Asthma induced exacerbation of bacterial pneumonia.

A thesis submitted for the Degree of Doctor of Philosophy in the National Heart and Lung Institute, Imperial College London

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Statement of Originality

All experimental work submitted in this thesis is entirely the result of the candidate’s own investigations. All experiments were carried out at the National Heart and Lung Institute, Imperial College London.

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Abstract

Asthma and bacterial pneumonias are major causes of human mortality and morbidity throughout the world. To date many studies have investigated the possibility that bacteria exacerbate asthma but only a handful consider that asthma may cause bacterial infections. Recent clinical evidence suggests that bacterial infections cause serious complications in patients with asthma and that asthmatics show a 2-fold increased risk of invasive pneumococcal disease. This thesis examines the molecular mechanisms causing susceptibility of house dust mite (HDM) exposed lungs to bacterial infection.

The main finding of this thesis is that HDM-induced allergic airways disease increases susceptibility to *Streptococcus pneumoniae* infection. Furthermore, the molecular pathways leading to the production of neutrophil chemoattractants in the lung are compromised and that despite the complexity of anti-bacterial pathways that are disrupted, the re-introduction of a single chemokine to the lungs with allergic airway disease enables clearance of *S. pneumoniae* that would otherwise prove fatal. However, a reduction in HDM-induced eosinophilia seen in ST2 deficient mice does not restore anti-bacterial immunity.

This deficit in anti-bacterial immunity in HDM exposed lungs is associated with a change in resident alveolar macrophages into an alternatively activated phenotype, characterised by high mRNA and protein levels of RELMα, Ym1 and Arg1. These altered alveolar macrophages produce considerably less TNFα in response to Toll-Like Receptor (TLR) stimulation that is not a result of reduced TLR mRNA levels but due to an upregulation of TLR negative regulators particularly A20. A20 targets TRAF-6 that is found upstream of NF-κB activation. To prove a causal link naïve alveolar macrophages were transferred into allergic lungs prior to bacterial infection; such lungs handled the infection better confirming that alveolar macrophages are important in initiating the anti-bacterial response. Overall our findings highlight a change in specific innate immune pathways in the allergic lung that participate in susceptibility to bacterial pneumonia.
## Contents

Title page 1  
Statement of originality 2  
Acknowledgements 3  
Abstract 4  
Contents page 5  
Abbreviations 10  
List of figures 14  
List of tables 19

### Chapter 1 - Introduction 20

1.1 Asthma 21  
1.1.1 Burden of asthma and pneumonia 21  
1.1.2 Risk of infection in asthma aetiology 22  
1.1.3 Genetic susceptibility to asthma 26  
1.1.4 Allergic sensitisation 28  
1.1.5 House dust mite and its pathogenesis 29  
1.1.6 Asthma pathogenesis 31  
1.1.7 Asthma therapies 33  
1.1.8 Mouse models of asthma 34  
1.2 Respiratory bacteria: *Streptococcus pneumoniae* 34  
1.2.1 Epidemiology 34  
1.2.2 Virulence factors 35  
1.2.3 Host response to pneumococcal infection 37  
1.2.4 Vaccines and therapeutics 40  
1.2.5 Mouse models of *S. pneumoniae* infection 40  
1.3 Cells of the innate immune response in allergic airway disease and bacterial infection 41  
1.3.1 Airway epithelial cells 41
Chapter 2- Materials and Methods

1.3.2 Macrophages
1.3.3 Neutrophils
1.3.4 Eosinophils
1.4 Pathogen recognition by Toll-like receptors
1.5 Aims and hypothesis

Chapter 2- Materials and Methods

2.1 Laboratory animals

2.2 Streptococcus pneumoniae (D39) stock

2.3 Animal allergy and infection models

2.3.1 Murine allergic airway disease induction

2.3.2 Respiratory bacterial challenge

2.3.3 Recombinant murine chemokine administration

2.3.4 Intranasal PKH26-PCL dye administration

2.4 Lung Histology

2.4.1 H&E and PAS staining of lung tissue

2.4.2 Immunohistochemistry

2.5 Sample recovery and cell preparation of BAL and lung tissue

2.5.1 Analysis of bone marrow mobilisation of neutrophils

2.6 Determination of bacterial load in blood, BAL and lung

2.7 Flow cytometric analysis

2.7.1 Extracellular antigen analysis

2.7.2 Intracellular cytokine expression

2.8 Cell Sorting by flow cytometry

2.8.1 Sorting of airway cells dyed with PKH26-PCL red fluorescent cell linker

2.9 Cytokine detection using ELISA

2.10 Isolation of mRNA and real-time PCR
Chapter 3 - Defective anti-pneumococcal immunity in the allergic lung.

3.1 Introduction
3.1.1 Asthma and S. pneumonia
3.1.2 Prior lung inflammation alters susceptibility to pneumonia
3.1.3 Hypothesis and Aims

3.2 Results
3.2.1 HDM exposure induces allergic airway disease
3.2.2 HDM exposure prior to S. pneumoniae infection reduced bacterial clearance
3.2.3 Blunted cell recruitment in HDM exposed mice after bacterial infection compared to PBS controls
3.2.4 HDM exposure reduces recruitment of neutrophils to the airways and lung
3.2.5 Instillation of CXCL1 or CXCL2 to the HDM-exposed lung restores anti-bacterial immunity
3.2.6 Reduced anti-bacterial cytokine production in the allergic airways
3.2.7 Reduced pro-inflammatory receptor expression on key anti-bacterial immune cells
3.2.8 Exacerbation of bacterial infection wanes without re-exposure to allergen

3.3 Discussion
3.3.1 Allergen exposure increases susceptibility to respiratory bacterial infections
3.3.2 Reduced PRR expression on innate immune cells

Chapter 4 - Probing macrophage dysfunction in the allergic lung.

4.1 Introduction
### 4.1.1 Macrophages in allergic airways disease

- Reduced airway neutrophil levels after TLR stimulation *in vivo*
- HDM exposure changes resident alveolar macrophage
- Restoration of naïve alveolar macrophages restores anti-bacterial immunity in the allergic airway
- Responsiveness of alveolar macrophages to TLR agonists *ex vivo*
- Regulation of alveolar macrophages by regulators of TLR responses
- Addition of macrophage chemoattractant protein, CCL2 restores anti-bacterial immunity in the allergic airway

### 4.2 Results

- Hypothesis and Aims

### 4.3 Discussion

### 5.1 Introduction

- Hypothesis and Aims

### 5.2 Results

- Bacterial susceptibility in WT and ST2-/- mice
- Increased neutrophil recruitment in the airways of ST2-/-+HDM compared to WT+HDM
- Th2 cytokine levels in WT and ST2-/- lungs
- Reduced AAM markers in ST2-/- lungs and alveolar macrophages
- ST2-/- alveolar macrophage features

### 5.3 Discussion

### 6.1 Final Discussion
6.2 Specific innate immune pathways are modulated in the allergic lung that precipitates life threatening bacterial pneumonia and sepsis 163

6.3 Bacterial pneumonia in a murine model of reduced house dust mite induced airway inflammation 166

6.4 Asthma therapeutics and bacterial exacerbation 167

6.5 Conclusion of HDM induced innate mechanisms increasing bacterial susceptibility in the allergic lung and areas suitable for therapeutic manipulation 167

6.6 Future work 170

Reference List 171

Publications 195

Appendices 196
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAMs</td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td>AHR</td>
<td>Airway Hyper-reactivity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>Arg1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCL2</td>
<td>Chemokine ligand 2</td>
</tr>
<tr>
<td>C. pneumoniae</td>
<td><em>Chlamydia pneumoniae</em></td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CD200R</td>
<td>CD200 receptor</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine phosphate deoxiribose Guanine</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>D39</td>
<td><em>Streptococcus pneumoniae</em> strain D39</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3‘-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>Der p</td>
<td><em>Dermatophagoides pteronyssinus</em></td>
</tr>
<tr>
<td>DNA</td>
<td>DeoxyriboNucleic Acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>Fragment Crystallisable</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-colony stimulating factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony-Stimulating Factor</td>
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Abbreviations

H&E Haematoxylin and Eosin
HBSS Hank’s buffered salt solution
HDM House Dust Mite
HIV Human Immunodeficiency Virus
HRP Horseradish peroxidase
i.n Intranasal
i.t Intratracheal
ICAM-1 Intercellular Adhesion Molecule 1
IFN Interferon
Ig Immunoglobulin
IL Interleukin
IMS Industrial Methylated Spirit
IPD Invasive Pneumococcal Disease
IRAK-1 Interleukin-1 receptor associated kinase 1
ITAM Immunoreceptor tyrosine-based activation motif
LPXTG Carboxy-terminal sortase motif
LTB4 Leukotriene B4
KC CXCL1
KO Knock out
LPS Lipopolysaccharide
LTA Lipoteichoic acid
LytA Autolysin
µg Microgram
M. pneumoniae Mycoplasma pneumoniae
MARCO Macrophage Receptor with Collagenous structure
MIP-2 Macrophage Inflammatory Protein-2 / CXCL2
miRNA Micro RNA
MMP Matrix metalloproteinase
mRNA Messenger RNA
**Abbreviations**

Abbreviation | Definition
--- | ---
MyD88 | Myeloid differentiation factor 88
NET | Neutrophil Extracellular Traps
NF-κB | Nuclear factor–kappa-light-chain-enhancer of activated B cells
NKT | Natural killer T cells
OD | Optical density
OVA | Ovalbumin
P/S | Penicillin/streptomycin
PAMPS | Pathogen associated molecular patterns
PAS | Periodic acid-Schiff stain
PBS | Phosphate buffered saline
PCR | Polymerase chain reaction
PCV | Pneumococcal Conjugate Vaccine
PE | Phycoerythrin
PerCP | Peridinin chlorophyll protein
Pg | Picogram
PMA | Phorbol-12-myristate-13-acetate
PRR | Pattern recognition receptor
PspA | Pneumococcal Surface Protein A
PspC | Pneumococcal Surface Protein C
R | Receptor
Relmα | Resistin-like molecule-α
RNA | Ribonucleic Acid
ROS | Reactive oxygen species
RPMI | Roswell Park Memerial Institute
RSV | Respiratory Syncytial Virus
RT | Room Temperature
RV | Rhinovirus
SD | Standard deviation
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>S.pn</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>SIGNR1</td>
<td>SIGN-related 1</td>
</tr>
<tr>
<td>SIT</td>
<td>Allergen-Specific Immunotherapy</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>SP</td>
<td>Surfactant protein</td>
</tr>
<tr>
<td>SPLUNC1</td>
<td>Short palate, lung and nasal epithelium clone 1</td>
</tr>
<tr>
<td>sST2</td>
<td>Soluble ST2</td>
</tr>
<tr>
<td>ST2</td>
<td>Suppression of tumorigenicity 2</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper type 2</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TOLLIP</td>
<td>Toll interacting protein</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF-receptor associated factor 6</td>
</tr>
<tr>
<td>T reg</td>
<td>T regulatory</td>
</tr>
<tr>
<td>TREM</td>
<td>Triggering Receptor Expressed on Myeloid Cells</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoietin</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Wild-type</td>
<td>WT</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula Occludens-1</td>
</tr>
</tbody>
</table>
List of Figures

Chapter 1

Figure 1.1: Involvement of inflammatory cells in asthma.

Figure 1.2: Route for Streptococcus pneumoniae infection.

Figure 1.3: A summary of pneumococcal virulence factors.

Figure 1.4: Early immune response to bacterial infection in the airway.

Figure 1.5: Macrophage phenotypes.

Figure 1.6: Macrophage interaction with T cell.

Figure 1.7: Antibacterial properties of neutrophils.

Figure 1.8: Toll-like receptor (TLR) signalling pathway.

Chapter 3

Figure 3.1: Induction of allergic airway inflammation after repeated intranasal HDM administrations into the airways.

Figure 3.2: Allergic airway inflammation induces exacerbation of lung Streptococcus pneumoniae (S.pn) infection.

Figure 3.3: Enhanced mucus production in HDM-sensitised mice is exacerbated following bacterial infection.

Figure 3.4: Total airway and lung cell counts in PBS or HDM exposed group after Streptococcus pneumonia (S.pn) infection.

Figure 3.5: Airway and lung eosinophils and CD4 T cells of HDM- exposed and control mice after Streptococcus pneumoniae (S.pn) challenge.

Figure 3.6: Reduced neutrophil numbers HDM- exposed group following bacterial challenge.

Figure 3.7: Equivalent numbers of neutrophils are recruited into the airways and lungs of control (PBS) and HDM- exposed group after recombinant CXCL1 or CXCL2 instillation.

Figure 3.8: CXCL2 administration to HDM- exposed group reduces blood neutrophil levels.
**Figure 3.9:** Airway cytokine and chemokines levels after *Streptococcus pneumoniae* infection.

**Figure 3.10:** Elevated IL-17A producing CD4+ and γδ TCR expressing T cells in the HDM-exposed airways compared to PBS controls.

**Figure 3.11:** IL-17A levels in the airways of HDM-exposed airways compared to PBS controls.

**Figure 3.12:** Reduced expression of pathogen recognition receptors (PRRs) on macrophages and neutrophils in allergic mice compared to PBS controls.

**Figure 3.13:** Increased expression of regulatory receptors on macrophages (CD11c+ cells) in allergic mice compared to PBS controls.

**Figure 3.14:** Expression of TLR negative regulators in whole lung of allergic mice were compared to PBS controls.

**Figure 3.15:** Antimicrobial substances in HDM-sensitised group after bacterial infection.

**Figure 3.16:** The percentage of lungs with bacteria reduces and the numbers of neutrophils in the airways of HDM/*Streptococcus pneumoniae* challenged group increases as HDM exposure wanes.

**Figure 3.17:** TLR2 signalling pathway leading to activation of NF-κB associated cytokine genes.

**Figure 3.18:** The attenuated innate immune response to infection in allergic-HDM exposed mice explored in this chapter.

---

**Chapter 4**

**Figure 4.1:** Neutrophil recruitment in response to *in vivo* stimulation of TLR2, TLR4 and TLR5 ligands.

**Figure 4.2:** Proportion of neutrophils and eosinophils in response to *in vivo* stimulation of TLR2, TLR4 and TLR5 ligands.

**Figure 4.3:** CXCL1 levels in response to *in vivo* stimulation of TLR2, TLR4 and TLR5 ligands in the airways.

**Figure 4.4:** *In vivo* labelling of resident alveolar macrophages.
**Figure 4.5:** Total dye positive resident alveolar macrophages.

**Figure 4.6:** Regulatory receptors T1/ST2 and TREM2 expression on CD11c+Dye+ and CD11c+Dye- airway cells.

**Figure 4.7:** Analysis of alternatively activated markers RELMα, YM1 and Arg1 in CD11c+dye+ airway cells.

**Figure 4.8:** Transfer of naïve CD11c+CD11b- alveolar macrophages restores bacterial clearance in HDM exposed mice.

**Figure 4.9:** TLR stimulation of sorted dye+ airway cells *ex vivo*.

**Figure 4.10:** TNF-α levels released by alveolar macrophages *in vitro*.

**Figure 4.11:** TNF-α production of naïve alveolar macrophages in response to TLR stimulation after HDM, IL-4 or IL-33 incubation.

**Figure 4.12:** TLR and TLR negative regulators analysis of dye+ alveolar macrophages by real-time PCR.

**Figure 4.13:** MicroRNA-146b and its targets TRAF-6 and IRAK-1.

**Figure 4.14:** Airway cellular influx peaks 6 hours after administration of macrophage chemoattractant protein, CCL2.

**Figure 4.15:** Addition of CCL2 reduces total cell numbers in the airways and lung of allergic mice.

**Figure 4.16:** Addition of CCL2 reduces anti-inflammatory receptors ST2L and TREM2 on alveolar macrophages the airways.

**Figure 4.17:** Addition of CCL2 reduces inflammatory airway cells.

**Figure 4.18:** Addition of CCL2 improves bacterial clearance from the airways and lung.

**Figure 4.19:** Addition of CCL2 restores anti-bacterial immunity.

**Figure 4.20:** Anti-microbial substances after replenishment of macrophages in the allergic airways.

**Figure 4.21:** An illustration of the effects of HDM exposure on airway macrophages.

**Figure 4.22:** CCL2 addition improves the proportion of alveolar macrophages in the airways that are uneducated by the allergic response.
Chapter 5

Figure 5.1: ST2 knock out and wild-type control (BALB/c) genotyping.

Figure 5.2: Reduced airway cellular infiltrate and eosinophils in ST2-/- compared to WT controls mice after HDM exposure.

Figure 5.3: Susceptibility of WT and ST2-/- to Streptococcus pneumonia after house dust mite exposure.

Figure 5.4: Reduced infiltrated cell recruitment in ST2-/- after Streptococcus pneumonia infection compared to WT PBS group.

Figure 5.5: Mucus production in the lungs of WT and ST2-/- before and 24 hours after infection.

Figure 5.6: Increased percentage of airway myeloid cells that are neutrophils in ST2-/- mice compared to WT controls.

Figure 5.7: Neutrophils and eosinophils in the lungs of WT and ST2-/- 24 hours after Streptococcus pneumonia infection.

Figure 5.8: Lung T cells numbers after bacterial infection in WT and ST2-/- with or without HDM exposure.

Figure 5.9: Levels of IL-33 and IL-13 levels cytokines in the lungs.

Figure 5.10: TNFα levels in the airways and lung of WT and ST2-/-.

Figure 5.11: Reduced mRNA expression of alternatively activated macrophage markers RELMα, Arg1 and YM1 in ST2-/- alveolar macrophages compared with WT after exposure to HDM.

Figure 5.12: Reduced mRNA expression of alternatively activated macrophage markers RELMα, Arg1 and YM1 in ST2-/- lungs compared with wild type after exposure to HDM.

Figure 5.13: TLR2 and TREM2 expression on CD11c+ airway cells.

Figure 5.14: TNFα release by CD11c+CD11b- airway cells in response to LPS and LTA stimulation.
Chapter 6

Figure 6.1: Possible factors involved in bacterial exacerbation in the allergic lung.
List of Tables

**Chapter 1**

*Table 1.1*: Top 5 causes of death in the world as on the World Health Organisation website accessed on 31 Jan 2012.

*Table 1.2*: Bacterial species involved in exacerbations of lung inflammatory disorders.

*Table 1.3*: A list of susceptibility genes for asthma.

*Table 1.4*: Important HDM allergens and their biochemical properties.

*Table 1.5*: Examples of Mouse Models of *Streptococcus pneumoniae*.

*Table 1.6*: Biomarkers for three macrophage populations: classically activated macrophages, alternatively activated macrophages and regulatory macrophages.

*Table 1.7*: Toll-like receptors and their ligands.

**Chapter 2**

*Table 2.1*: List of antibodies used in experimental work in this thesis.
CHAPTER 1

Introduction
1.1 Asthma

1.1.1 Burden of asthma and pneumonia

Respiratory diseases are responsible for a significant proportion of morbidity and mortality globally. Asthma is a major respiratory public health problem affecting 300 million people worldwide\(^1\). Although the incidence of asthma is variable between populations, the prevalence has risen over the past few decades\(^3\). In the UK, 3.4 million people have asthma\(^2\). Asthma has a high economic burden costing the UK’s National Health Service £750 million per year\(^4\) and is an important cause of incapacity in terms of absence from school and work\(^5\). The World Health Organisation (WHO) ranks lower respiratory diseases as the 3\(^{rd}\) leading cause of death worldwide\(^6\) (Table 1.1) and *Streptococcus pneumoniae* (*S. pneumoniae*), also known as the pneumococcus is a major cause of respiratory bacterial infections worldwide. In 2000, there were 14.5 million global pneumococcal cases\(^7\). The main forms of severe *S. pneumonia* disease are pneumonia, meningitis and sepsis\(^8\). The WHO estimates 1.6 million annual deaths due to pneumococcal diseases\(^9\). Therefore, asthma and bacterial infection independently affect millions of people worldwide causing significant mortality and an associated high economic burden.

<table>
<thead>
<tr>
<th></th>
<th>Death in millions</th>
<th>% Death</th>
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<tbody>
<tr>
<td>1</td>
<td>Ischaemic heart disease</td>
<td>7.25</td>
</tr>
<tr>
<td>2</td>
<td>Stroke and other cerebrovascular disease</td>
<td>6.15</td>
</tr>
<tr>
<td>3</td>
<td>Lower respiratory infections</td>
<td>3.46</td>
</tr>
<tr>
<td>4</td>
<td>Chronic obstructive pulmonary disease</td>
<td>3.28</td>
</tr>
<tr>
<td>5</td>
<td>Diarrhoeal diseases</td>
<td>2.46</td>
</tr>
</tbody>
</table>

Table 1.1: Top 5 causes of death in the world as on the World Health Organisation website accessed on 31 Jan 2012 (factsheet No. 310 updated June 2011)\(^6\).
1.1.2 Risk of infection in asthma aetiology

Lower respiratory tract infections are associated with the initiation of asthma, but more commonly cause asthma exacerbations. There is increasing evidence to indicate that early childhood infections may affect the subsequent sensitisation to allergens and development of asthma. For example, there is an inverse relationship between measles in childhood and allergen sensitisation\textsuperscript{10}. Similarly, children who exhibited strong positive tuberculin skin tests, indicating natural exposure to tuberculosis, have reduced serum immunoglobulin (Ig)-E, T helper type (Th)-2 cytokines and fewer symptoms of asthma\textsuperscript{10}. However, not all infections are protective, wheezing respiratory syncytial virus (RSV) infected infants are more likely to develop asthma by their third birthday\textsuperscript{11}. These results suggest that early childhood infections modulate immune responses that can initiate or inhibit allergic inflammatory responses later in life.

There is a large body of evidence that viral infections are a potent trigger of asthma symptoms. It is reported that 80% of childhood asthma exacerbations and 50% of all adult asthma attacks are associated with viral infections\textsuperscript{12}. Most commonly identified viruses linked to wheezing under the age of 2 years are RSV (50-60%) and rhinovirus (RV; 30%), while RV account for 60% of viruses in children over 2 years of age\textsuperscript{13}. These viruses were mainly detected by PCR assays. Viruses cause increased airway inflammation affecting airway function. However, the association between respiratory viral infections and asthma exacerbations is not completely understood.

The most common cause of viral exacerbations of asthma is RV. In one study, a respiratory virus was associated in 78% of acute exacerbations of asthma and of these, 83% were RV\textsuperscript{14}. RV predominately infect the conducting airways by interacting with intercellular adhesion molecule 1 (ICAM-1) or low-density lipoproteins receptors on epithelium that internalise the virus. The virus initiates a cascade of molecular pathways resulting in activation of NF-κB and the release of a variety of cytokines and chemokines i.e. interleukin (IL)-17F, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-8\textsuperscript{15}. The asthmatic epithelial cells also facilitate virus penetration into the airways and then into the circulation-this is linked to abnormal bronchial epithelial cell responses to RV infection as a result of a major defect in interferon (IFN)-β secretion and delayed apoptosis of
bronchial epithelial cells\textsuperscript{15}. In addition, \textit{in vitro}, studies show that Th2-associated cytokines in the asthmatic lung increase ICAM-1 that is the major RV receptor. This would facilitate viral attachment to epithelial cells and entry.

Of the pathogens studied, viruses have received the most attention. However, retrospective studies in humans establish a strong relationship between asthma and \textit{S. pneumonia} infections. In 2005, Talbot \textit{et al} showed asthmatics to have a 2-fold increased risk of invasive pneumococcal disease (IPD)\textsuperscript{16} and a number of other studies published since adds support to this finding. A retrospective study on 1282 patients with IPD in Finland show a 5\% disease burden attributed to asthma\textsuperscript{17}. In a recent study, children born to asthmatic mothers were investigated for neonatal airway colonisation and pneumonia until three years of age. 21\% of the children were colonised with \textit{S. pneumonia}, \textit{Haemophilus influenza} and/or \textit{Moraxella catarrhalis} at four weeks of age and colonisation with at least one of these bacteria was significantly associated with increased incidence of pneumonia\textsuperscript{18}.

\textit{Streptococcus pneumoniae}, \textit{Haemophilus influenzae} and \textit{Staphylococcus aureus} complications are often reported in lung inflammatory diseases including asthma, chronic obstructive pulmonary disease (COPD) and influenza (Table 1.2). The similarity in the bacterial complications that occur following these conditions could be driven by a mechanism common to all for example the causative agents of these disorders alter respiratory epithelial barrier. Although, the type of infiltrating cells are different in asthma and COPD; eosinophils, mast cells and Th2 cells dominate in asthma, whereas neutrophils and Th1 cells are prominent in COPD. However, there are subphenotypes of these diseases that are similar, for example some COPD patients present with high sputum eosinophil counts similar to asthma, while some asthmatics have high neutrophil numbers. Eosinophilic airway inflammation plays a role in exacerbations of both asthma and COPD and treatments to lower eosinophilia significantly reduce the frequency of exacerbations\textsuperscript{19}. In a study of 82 patients with moderate-severe COPD who received therapy to minimise eosinophilic inflammation (\textgreater{}3\% of non-squamous sputum cells) experienced fewer severe exacerbations (defined by score of symptoms, quality of life and lung function)\textsuperscript{19}. Furthermore, a study in asthmatics with high sputum eosinophil counts after discontinuation of inhaled corticosteroids augmented asthma exacerbations\textsuperscript{20}. Sputum eosinophilia is associated with corticosteroid responsiveness\textsuperscript{21}, whereas an exacerbation
characterised by high bacterial load will have a favourable response to antibiotics. In a randomised control trial, treatment targeted to sputum eosinophil counts significantly reduced asthma exacerbations in patients with severe asthma with eosinophilic phenotype\textsuperscript{22}. Neutrophilic inflammation also predomnates in some patients with severe asthma\textsuperscript{23,24}. Asthmatics with viral infection have an increase in sputum neutrophils and neutrophil elastase\textsuperscript{25}. COPD exacerbations are associated with impaired lung function and increased sputum neutrophilia as a result of viral (48.4\%) and bacterial (54.7\%) infections\textsuperscript{26}. Furthermore, airway neutrophilia correlated with exacerbation severity regardless of the cause of infection\textsuperscript{26}.

Inhaled corticosteroids have broad anti-inflammatory effects and are widely used in the treatment of asthma and COPD. Inhaled corticosteroids do not alter disease progression but reduce AHR, exacerbations and mortality in all age groups\textsuperscript{27}. A study in Lothian, UK involving 490 COPD patients admitted with community-acquired pneumonia of which 76.7\% were inhaled corticosteroid users show no difference on outcome and in pneumonia severity\textsuperscript{28}. Whereas, another study showed the probability of having pneumonia was higher among subjects receiving medications containing inhaled corticosteroids compared with the placebo group\textsuperscript{29}. However, in this latter study, the lack of effect of inhaled corticosteroids on mortality and disease progression may reflect resistance to the anti-inflammatory effects of corticosteroids in COPD patients. Another retrospective study also show an improved survival of COPD patients with inhaled corticosteroids from all causes\textsuperscript{30}.

<p>| Table 1.2: Bacterial species involved in exacerbations of lung inflammatory disorders. |
|-----------------------------------------------------|-----------------|----------------|---------------|----------------|
|                                                   | Influenza       | RSV            | COPD          | Asthma         |
| Human studies                                     |
| Typical Bacteria                                  |
| Streptococcus pneumoniae                          | ✓ 31,32         | ✓ 33           | ✓ 34,35       | ✓ 16,36-38     |
| Haemophilus influenzae                            | ✓ 39            | ✓ 40           | ✓ 34,35       | ✓ 36,37,31     |
| Moraxella catarrhalis                             | ✓ 42            | ✓ 43           | ✓ 35          | ✓ 36,37/41     |
| Staphylococcus aureus                             | ✓ 39,44         | ✓ 45           | ✓ 45          | ✓ 46           |
| Atypical bacteria                                 |
| Chlamydia pneumoniae                              | ✓ 47            |               | ✓ 48,49       |</p>
<table>
<thead>
<tr>
<th>Mycoplasma pneumoniae</th>
<th>✓ 45,49</th>
</tr>
</thead>
</table>

### Animal studies

#### Typical Bacteria

<table>
<thead>
<tr>
<th>Streptococcus pneumoniae</th>
<th>✓ 51-53</th>
<th>✓ 54</th>
<th>✓ 55</th>
<th>✓ 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemophilus influenzae</td>
<td>✓ 57</td>
<td>✓ 58</td>
<td>✓ 59</td>
<td></td>
</tr>
<tr>
<td>Moraxella catarrhalis</td>
<td>✓ 60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>✓ 61,62</td>
<td></td>
<td>✓ 63,64</td>
<td></td>
</tr>
</tbody>
</table>

#### Atypical bacteria

<table>
<thead>
<tr>
<th>Chlamydia pneumoniae</th>
<th>✓ 55</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>✓ 66,67</td>
</tr>
</tbody>
</table>

All references restricted to examples. RSV-Respiratory syncytial virus; COPD-Chronic obstructive disease.
Blank boxes: To our knowledge no reported complication of the bacteria in the associated lung condition and this table was originally made for a review in future microbiology submitted in 2012.

There is growing evidence for the role of atypical bacteria such as *Mycoplasma pneumoniae* (*M. pneumoniae*) and *Chlamydia pneumoniae* (*C. pneumoniae*) in asthma exacerbations. For example, one study found 56% of asthmatics positive for *C. pneumoniae*, *M. pneumoniae* or both compared to 9% of controls. One group associate the increased *M. pneumoniae* susceptibility to downregulated toll-like receptor (TLR)-2 and IL-6 production during *M. pneumoniae* infection in ovalbumin (OVA)-induced allergic inflammation. Low-level *M. pneumoniae* infection enhances Th2 responses (IL-4 and exotaxin), whereas a high-level exposure reduced lung eosinophilia in OVA-induced allergic airway disease. *Chlamydia muridarum* infection can subvert dendritic cells from a protective Th1 response to a non-protective Th2 response, which promote asthma. Furthermore, IL-13 increases susceptibility to *Chlamydia muridarum* infection as the cytokine reduces uptake of bacteria by macrophages. The role of bacteria in asthma exacerbation is strengthened by studies using anti-atypical bacteria antibiotics (macrolides and ketolides) where improvement in asthma symptoms is noted. However, some antibiotics possess anti-inflammatory properties making the role of atypical bacteria controversial in asthma exacerbations. It should be noted that in most studies to date...
the role of outgrowth of specific bacterial commensals have been ignored. This is mostly due to their detection by culture in the laboratory. More recently, Hilty et al have shown the bronchial tree to contain a characteristic microbiota by molecular analysis of the polymorphic bacterial 16S ribosomal RNA gene, and suggest that this microbiota is altered; *Haemophilus, Moraxella* and *Neisseria* species being more frequent in bronchi of asthmatics. This study also shows that the airways are not sterile.

1.1.3 Genetic susceptibility to asthma

Data suggests 40-60% of the risk of developing asthma can be linked to genetic factors. A number of alleles, chromosomes and polymorphisms are associated with asthma susceptibility (Table 1.3).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome</th>
<th>Function and pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1</td>
<td>1p13.3</td>
<td>Environmental and oxidative stress-detoxification</td>
</tr>
<tr>
<td>FLG</td>
<td>1q21.3</td>
<td>Epithelial barrier integrity</td>
</tr>
<tr>
<td>IL10</td>
<td>1q31-q32</td>
<td>Immunoregulation</td>
</tr>
<tr>
<td>CTLA4</td>
<td>2q33</td>
<td>T cell response inhibition and immunoregulation</td>
</tr>
<tr>
<td>IL13</td>
<td>5q31</td>
<td>Th2 effector functions</td>
</tr>
<tr>
<td>IL4</td>
<td>5q31.1</td>
<td>Th2 differentiation and IgE induction</td>
</tr>
<tr>
<td>CD14</td>
<td>5q31.1</td>
<td>Innate immunity-microbial recognition</td>
</tr>
<tr>
<td>SPINK5</td>
<td>5q32</td>
<td>Epithelial serine protease inhibitor</td>
</tr>
<tr>
<td>ADRB2</td>
<td>5q31-q32</td>
<td>Bronchial smooth muscle relaxation</td>
</tr>
<tr>
<td>HAVCR1</td>
<td>5q33.2</td>
<td>T cell response regulation-HAV receptor</td>
</tr>
<tr>
<td>LTC4S</td>
<td>5q35</td>
<td>Cysteinyi leukotriene biosynthesis-inflammation</td>
</tr>
<tr>
<td>Gene</td>
<td>Chromosome</td>
<td>Function</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>LTA</td>
<td>6p21.3</td>
<td>Inflammation</td>
</tr>
<tr>
<td>TNF</td>
<td>6p21.3</td>
<td>Inflammation</td>
</tr>
<tr>
<td>HLA-DRB1</td>
<td>6p21</td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>HLA-DQB1</td>
<td>6p21</td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>HLA-DPB1</td>
<td>6p21</td>
<td>Antigen presentation</td>
</tr>
<tr>
<td>GPRA</td>
<td>7p14.3</td>
<td>Regulation of cell growth and neural mechanisms</td>
</tr>
<tr>
<td>NAT2</td>
<td>8p22</td>
<td>Detoxification of drugs and carcinogens</td>
</tr>
<tr>
<td>FCERIB</td>
<td>11q13</td>
<td>High affinity Fc receptor for IgE</td>
</tr>
<tr>
<td>CC16</td>
<td>11q12.3-q13.1</td>
<td>Epithelium derived anti-inflammatory protein</td>
</tr>
<tr>
<td>GSTP1</td>
<td>11q13</td>
<td>Environmental and oxidative stress-detoxification</td>
</tr>
<tr>
<td>IL18</td>
<td>11q22.2-q22.3</td>
<td>Induction of IFNγ and TNF</td>
</tr>
<tr>
<td>STAT6</td>
<td>12q13</td>
<td>IL-4 and IL-13 signalling</td>
</tr>
<tr>
<td>NOS1</td>
<td>12q24.2-q24.31</td>
<td>Nitric oxide synthesis- cell-cell communication</td>
</tr>
<tr>
<td>CMA1</td>
<td>14q11.2</td>
<td>Mast-cell chymotryptic serine protease</td>
</tr>
<tr>
<td>IL4R</td>
<td>16p12.1-p12.2</td>
<td>α-chain of the IL-4 and IL-13 receptors</td>
</tr>
<tr>
<td>CCL11</td>
<td>17q21.1-p21.2</td>
<td>Epithelium derived eosinophil chemoattractant</td>
</tr>
<tr>
<td>CCL5</td>
<td>17q11.2-q12</td>
<td>Monocyte, T cell and eosinophil chemoattractant</td>
</tr>
<tr>
<td>ACE</td>
<td>17q23.3</td>
<td>Inactivation of inflammatory mediators</td>
</tr>
<tr>
<td>TBXA2R</td>
<td>19p13.3</td>
<td>Smooth-muscle contraction, inflammation</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>19q13.1</td>
<td>Immunoregulation, cell proliferation</td>
</tr>
<tr>
<td>Gene</td>
<td>Chromosome</td>
<td>Function</td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>ADAM33</td>
<td>20p13</td>
<td>Cell-cell and cell-matrix interactions</td>
</tr>
<tr>
<td>GSTT1</td>
<td>22q11.23</td>
<td>Environmental and oxidative stress-detoxification</td>
</tr>
<tr>
<td>ORMDL3</td>
<td>17q21</td>
<td>Negative regulator of sphingolipid synthesis</td>
</tr>
<tr>
<td>IL33</td>
<td>9</td>
<td>Inducing Th2 associated cytokines</td>
</tr>
<tr>
<td>TSLP</td>
<td>13q31.1</td>
<td>Eosinophilic esophagitis</td>
</tr>
<tr>
<td>IL1RL1</td>
<td>2</td>
<td>IL-33 receptor (ST2)</td>
</tr>
<tr>
<td>SMAD3</td>
<td>15</td>
<td>Transcriptional modulator</td>
</tr>
</tbody>
</table>

Table 1.3: A list of susceptibility genes for asthma. Adapted from 76–78

ACE, angiotensin I converting enzyme 1 (also known as peptidyl-dipeptidase A); ADAM33, a disintegrin and metalloproteinase domain 33; ADRB2, β2 adrenergic receptor; CC16, Clara cell-specific 16 kD protein (also known as SCGB1A1); CCL11, CC-chemokine ligand 11 (also known as eotaxin1); CCL5, CC-chemokine ligand 5 (also known as RANTES); CD14, monocyte differentiation antigen 14; CMA1, chymase 1, mast cell; CTLA4, cytotoxic T lymphocyte antigen 4; FCERIB, high-affinity Fc receptor for IgE β-chain; FLG, filaggrin; GPRA, G-protein-coupled receptor for asthma susceptibility (also known as NPSR1, and GPRA154); GSTM1, glutathione S transferase M1; GSTP1, glutathione S transferase P1; GSTT1: glutathione S transferase T1; HAVCR1, hepatitis A virus cellular receptor 1 (also known as TIM1); IL, interleukin; IL4R, interleukin-4 receptor (α-chain); LTA, lymphotoxina (also known as TNFβ); LTC4S, leukotriene C4 synthase; NAT2, N acetyltransferase 2; NOS1, nitric oxide synthase 1 (neuronal); SPINK5, serine protease inhibitor, Kazal-type, 5; STAT6, signal transducer and activator of transcription 6; TBX2A2R, thromboxane A2 receptor; TGFB1, transforming growth factor-β1; TNF, tumour necrosis factor; SMAD 3, mothers against decapentaplegic homologue); IL1RL1 (ST2) and IL1 receptor accessory protein activate IL-33; TSLP, thymic stromal lymphopoietin.

1.1.4 Allergic sensitisation

Asthma is a multifactorial disease but there is growing evidence for the role of local immune events in development of allergic airway inflammation. Asthma is characterised by reversible airway obstruction, mucus hyper-secretion, airway
remodelling and infiltration of eosinophils and Th2 cells. Asthma sub-phenotypes (allergic, non-allergic, occupational) have differing pathology, clinical expression and response to treatment. Asthma occurs not purely a result of allergen exposure; nonetheless allergic asthma is the most common form. Common allergens include HDM, pollen, cat dander and fungi. It is reported that 60-100% of asthmatics are sensitised to HDM. In Europe, the most predominating species of HDM is *Dermatophagoides pteronyssinus (Der p)*. Allergens of *Der p* have different biochemical properties but most are digestive enzymes that are secreted in their faeces. For example, *Der p 1* has cysteine and serine protease activities.

### 1.1.5 House dust mite and its pathogenesis

10-15% of individuals in the western world have asthma with 15% of asthmatics, 85% are HDM allergic. HDM’s environment contains bacteria, lipopolysaccharide (LPS), β-glucans and fungi. HDM extract consists of whole mites, faecal pellets, nymphs, eggs and culture media. HDM has a lifetime of 10 weeks and produces 2000 fecal particles. The major HDM species are *Der p* and *Dermatophagoides farinae (Der f)*, however, in the UK *Der p* predominates. *Der p* has 21 allergens (Table 1.4) and absolute IgE binding measurement shows 50% binding associated with *Der p 1* and 2 and 30% was equally contributed by *Der p 4, 5* and *7*. The level of *Der p 1* in house dust is 100-10,000 ng/g of dust.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Biochemical property</th>
<th><em>Dermatophagoides</em> Allergenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Cysteine protease</td>
<td>Dominant</td>
</tr>
<tr>
<td>Group 2</td>
<td>ML domain lipid-binding protein</td>
<td>Dominant</td>
</tr>
<tr>
<td>Group 4</td>
<td>A-amylase</td>
<td>Midpotency</td>
</tr>
<tr>
<td>Group 5</td>
<td>Unknown</td>
<td>Midpotency</td>
</tr>
<tr>
<td>Group 7</td>
<td>Lipid-binding protein</td>
<td>Midpotency</td>
</tr>
<tr>
<td>Group 21</td>
<td>Unknown</td>
<td>Midpotency</td>
</tr>
</tbody>
</table>

**Table 1.4:** Important HDM allergens and their biochemical properties. More than 20 HDM allergens groups have been defined based on sequence and functional homologies. Adapted from. ML=MD-2 related lipid-recognition.
The host immune response to HDM comprises both innate and antigen-specific adaptive immunity. HDM in the airway first comes in contact with airway epithelial cells. HDM can act through a number of cell surface receptors on airway epithelial cells and HDM-induced TLR4 signalling through epithelial cells leads to secretion of TSLP, GM-CSF, IL-25 and IL-33 cytokines associated with induction of allergic inflammation. These cytokines can induce allergic airway disease without the help of Th2-derived cytokines. Over-expression of TSLP and intranasal administration of IL-25 result in AHR. IL-33 is member of the IL-1 family and signals through T1/ST2 receptor (ST2L, IL-33 receptor) on Th2 cells, nuocytes and macrophages. Exogenous administration of IL-33 causes eosinophilia, induction of Th2 cytokines and mucus hypersecretion, whereas blocking the receptor abolishes many features associated with Th2 responses. ST2 is present in two forms, ST2L and soluble ST2. The extracellular domain is common to ST2 and ST2L; however, sST2 lacks the transmembrane and intracellular Toll-interleukin 1 receptor domains. ST2L is expressed on many cell types including macrophages, eosinophils, vascular endothelial and mast cells but is preferentially expressed in murine and human Th2 cells. A newly described subset of cells, innate lymphoid cells also express ST2L even though they are lineage negative. These lineage negative innate lymphoid cells produce IL-5 and IL-13 in response to in vivo HDM exposure and are thought to be involved in allergic responses in vivo. Although anti-ST2 antibody treatment attenuates Th2 responses, ST2 deficient mice developed normal Th2 responses after helminth parasite infection.

The cysteine proteases (Der p 1) in HDM can cleave epithelial tight junction proteins occludin and zonula occludens-1 (ZO-1), impairing an essential structural barrier. Furthermore, Der p 1 can stimulate protease dependent release of TSLP and IL-25 from airway epithelial cells. In addition, the proteases can inactivate lung surfactant proteins A and D (SP-A and SP-D), which are known to have important roles in host defense against bacteria. Proteinase-activated receptor is activated by serine proteases in HDM resulting in an inflammatory response. Therefore, HDM allergy is not only dependent on allergen-specific Th2 responses but also on the activation of innate immune cells. Although, non-allergic individuals are also subject to the same level of HDM exposure, they do not develop
inappropriate immune responses. The explanation for this could be genetic susceptibility and/or the intensity of innate immune activation in allergic people.\textsuperscript{106}

1.1.6 Asthma pathogenesis

Exposure to an allergen in susceptible individuals leads to allergen-specific IgE cross-linking high-affinity Fc receptors for IgE (FcεRs) on the surface of mast cells and basophils resulting in immediate release of leukotrienes, prostaglandins and histamine that can constrict airway smooth muscle and induce mucus secretion, while local chemokines recruit a number of other immune cells such as eosinophils, T cells and neutrophils.\textsuperscript{107} Release of toxic granules from eosinophils can damage the epithelium. Also, some allergens with proteolytic activity such as HDM can damage epithelium directly by cleaving epithelial tight junction molecules.\textsuperscript{108} The inflammatory cells also release Th2-associated cytokines such as IL-4, IL-5, IL-9 and IL-13 to enhance the allergic inflammation of the airways and lung (Fig. 1.1). Other than Th2 cells, T regulatory (T reg), Th17 and Th9 cells are associated with allergic responses to inhaled allergens.\textsuperscript{79}

Some allergens including HDM are reported to induce an allergic response through activation of innate immune receptors such as TLRs. The complex interactions of allergens, inflammatory cells and cytokines result in airway hyper-responsiveness and remodelling. Airway hyper-responsiveness caused by a stimulus like HDM leads to airway hyper-reactivity causing bronchoconstriction and inflammatory cell infiltration. Chronic exposure of the lungs to a stimulus can result in lung tissue remodelling- that involves changes in composition and structural organisation of the tissue.\textsuperscript{109}
Figure 1.1: Involvement of inflammatory cells in asthma. Early phase (occurs within minutes) mainly involve secretion of mediators by mast cells. However, in the late phase response (hours after allergen challenge) slow release of cytokines and chemokines occur by engagement of antigen presenting cells e.g. dendritic cells (DCs) with T helper (Th) type 2 cells recruit other inflammatory immune cells i.e. eosinophils, T cells and B cells. Together the early and late phase products influence airway and lung endothelial, epithelial, fibroblasts and smooth muscle cells leading to wheezing, nasal blockage and mucus production. IL-Interleukin, TSLP-Thymic Stromal Lymphopoietin, TGF-β-Transforming Growth Factor, GM-CSF-Granulocyte Macrophage Colony Stimulating Factor.
1.1.7 Asthma therapies

There is currently no cure for asthma and compliance with medication is not frequently maintained. Medication used in the clinic such as relieving bronchoconstriction with inhaled bronchodilators or oral cysteinyl leukotriene receptor 1 antagonists or corticosteroids is primarily to reduce symptoms.

More specific therapies have been developed, for example, anti-IgE antibodies. Cross-linking of these receptors by FcɛRI IgE release preformed mediators such as histamine, heparin and tryptase within 5 minutes and newly generated arachidonic acid such as leukotriene-D₄, prostaglandin-D₂ and cytokines such as tumour necrosis factor-α (TNFα) and IL-4. The importance of IgE is seen during treatment of allergic disease with blocking recombinant humanised monoclonal antibodies to IgE, Omalizumab. Omalizumab reduces the expression of high affinity IgE receptors on mast cells, basophils and dendritic cells by blocking IgE binding to high and low affinity IgE receptors.

Allergen-specific immunotherapy (SIT) involves repeated administration of an allergen and modifies cellular and humoral responses to allergens such as increasing T regs, IL-10 and transforming growth factor (TGF)-β production. Anti-Th2 cytokine treatments have also been developed with limited clinical success. Combination therapies such as SIT with anti-cytokine antibodies may have a greater potential for success than single therapies. There is growing evidence that asthma treatments needs to be patient specific. Thus, there is still a clear need for research and development of more asthma therapies.

Cytokines elevated in asthmatics such as IL-5, IL-13 and IL-4 are targeted for their therapeutic potential. High numbers of eosinophils are present in sputum and airways of asthmatics. IL-5 (eosinophil recruiting cytokine) deficient mice have no eosinophilia in response to Th2 cell inducing stimuli, while baseline eosinophil counts remain normal. Therefore, a number of human and mouse studies suggest that targeting eosinophil recruitment should provide therapeutic benefit for the treatment of asthma. However, blocking IL-5 with monoclonal antibodies (reslizumab) turned out to be effective (reduction in eosinophils and marked improvement in clinical symptoms) in only a specific group of asthmatics defined as poorly controlled, steroid-resistant eosinophilic asthma. Lebrikizumab, an anti-IL13 monoclonal
antibody reduced the late asthmatic response in mild asthmatics by reducing markers of Th2 inflammation\textsuperscript{118}. However, monoclonal antibodies directed against IL-4 lack clinical efficacy while a variant of IL-4 that acts as an antagonist of IL-4R\(\alpha\) and IL-13R\(\alpha\)\(1\) is more effective in reducing the frequency of asthma exacerbations in patients with single nucleotide polymorphisms in IL-4R\(\alpha\)\textsuperscript{119}.

1.1.8 Mouse models of asthma

A variety of allergens, allergen doses and application routes have been used to induce allergic airways disease in mice to resemble common features of human asthma. The most common mouse strain used is BALB/c mouse that show a genetically determined tendency to develop allergic immune responses\textsuperscript{120,121}. However, asthma features can be induced in C57BL/6 mice that is less susceptible to allergic airway disease\textsuperscript{122}. Airway hyper-reactivity (AHR) can be assessed in murine models after exposure to metacholine, a bronchoconstrictory agent\textsuperscript{123}. Although, chicken egg ovalbumin lacks clinical relevance, the OVA model is widely used as it provides the opportunity to study Th2 immune responses\textsuperscript{123}.

1.2 Respiratory bacteria: \textit{Streptococcus pneumoniae}

1.2.1 Epidemiology

\textit{S. pneumoniae} is a gram-positive \(\alpha\)-haemolytic facultative anaerobic organism\textsuperscript{124}. It commonly inhabits the upper respiratory tract especially in the nasopharyngeal as a commensal\textsuperscript{125}. Asymptomatic carriers can transmit the organisms by coughing. Over 90 serotypes have been recognised that colonise for weeks in adults or months in children\textsuperscript{126}. However, if the colonisation spreads it can progress to a number of inflammatory diseases including pneumonia or systemic disease (Fig.1.2). The risk factors for nasopharyngeal carriage of \textit{S. pneumoniae} are cigarette smoking, asthma, upper respiratory infections and immunocompromised health\textsuperscript{127}. The incidence of IPD is also higher in children (<2 years), the elderly (>65 years) or in crowded environments such as day care centres and hospitals\textsuperscript{128}.
Figure 1.2: Route for *Streptococcus pneumoniae* infection. Adapted from^{129}. *Streptococcus pneumoniae* is an airborne pathogen and can reside in the upper respiratory tract as a commensal. If it spreads, however, it can cause a number of inflammatory diseases such as pneumonia.

1.2.2 Virulence factors

*S. pneumoniae* has two main survival strategies- the invasive strains induce disease rapidly and spread efficiently, whereas the less invasive strains use surface adhesins and immune evasion strategies to allow long-term colonisation^{126}. The bacterium has a number of virulence factors important in facilitating the disease process (Fig. 1.3). The capsule surrounding the bacterium is a critical virulence factor. 99% of IPDs are caused by encapsulated *S. pneumoniae*^{128}. 91 different capsular polysaccharide types that are structurally and antigenically different have been identified, but the distribution of serotypes differs among geographic regions^{127}. The capsule protects the bacteria from opsonophagocytosis by reducing the access of opsonins to the bacterial surface molecules and prevents mucosal clearance^{129}. Below the capsule is the cell wall that consists of polysaccharides and lipoteichoic acid (LTA). The bacterium produces a 53-kDa pore forming toxin called pneumolysin during the logarithmic phase of growth^{130}. This toxin binds to membrane cholesterol to form large pores^{130}. Some activities attributed to pneumolysin are inhibition of ciliary...
beating, inhibition of the phagocyte respiratory burst and induction of cytokine synthesis\textsuperscript{131}.

Three major groups of \textit{S. pneumoniae} cell surface proteins identified are choline binding proteins, lipoproteins and proteins that are covalently linked to the bacterial cell wall by a carboxy-terminal sortase motif (LPXTG). \textit{S. pneumoniae} also has a number of LPXTG-anchored proteins such as hyaluronidase (breaks down the hyaluronic acid component of extracellular matrix) and neuraminidase (cleaves N-acetylneuraminic acid from glycolipids, lipoproteins and oligosaccharides)\textsuperscript{132}. Choline binding proteins are anchored to the cell surface of \textit{S. pneumoniae}. For example, autolysin (LytA) is an amidase that releases pneumolysin from the cytoplasm, pneumococcal surface protein A (PspA) that binds to lactoferrin to protect from bactericidal activity of apolactoferrin and interferes with the complement-mediated opsonisation and PspC that binds to immunoglobulin receptors and complement regulatory protein factor H to resist complement\textsuperscript{133}. \textit{S. pneumoniae} also has divalent metal ion binding lipoproteins such as pneumococcal surface antigen A, pneumococcal iron acquisition A and pneumococcal iron uptake A that are important in pneumococcal virulence\textsuperscript{131,132}. \textit{S. pneumoniae} depletion or mutations in all the virulence factors described above reduce virulence in animal models of pneumonia and bacteraemia.
Figure 1.3: A summary of pneumococcal virulence factors. *Streptococcus pneumoniae* synthesises several factors such as polysaccharide capsule, the cell wall and protein factors (pneumolysin, neuraminidase and hyaluronidase). Immunoglobulin (Ig), Complement protein (C3), Neuraminidase (nanA, B & C), Autolysin (LytA), Pneumococcal surface antigen A (PsaA), Pneumococcal adhesion and virulence A (PavA) and Enolase (Eno). Adapted from 131.

1.2.3 Host response to pneumococcal infection

*S. pneumoniae* clearance requires both cellular and humoral immune responses as well as non-immune factors e.g. anatomical barriers and mucus. Intact respiratory epithelium is essential for bacterial clearance by providing mucociliary clearance and releasing soluble anti-microbial mediators. The early cellular host response to the bacterium is mediated by alveolar macrophages followed by neutrophils 134 (Fig. 1.4). Interaction of *S. pneumoniae* LTA and pneumolysin with TLR2 135 and TLR4 136, respectively on airway epithelium or alveolar macrophages triggers anti-bacterial
cytokine production including CXCL1 (KC, murine homology of IL-8), IL-6, TNFα and IL-1β and co-operate to facilitate chemotaxis of neutrophils\textsuperscript{137,138}. TNFα has an essential role in initial defense because its inhibition greatly enhances bacterial susceptibility\textsuperscript{139,140}. Similarly, IL-8\textsuperscript{138} and IL-6\textsuperscript{141} deficiency are detrimental. The importance of neutrophils in pneumococcal elimination is seen in neutrophil depletion studies and in neutropenic patients who present increased infection and mortality\textsuperscript{142}.

TLR2 deficient mice produce significantly less neutrophil attracting chemokines (CXCL1 and CXCL2) and therefore show an increased susceptibility to bacterial infection\textsuperscript{143}. More importantly, TLR2 deficient alveolar macrophages do not respond to LTA or \textit{S. pneumoniae}\textsuperscript{143}. Interestingly, TLR9 (associated with the recognition of bacterial DNA, CpG) deficient mice are highly susceptible to lethal infection\textsuperscript{144}. CD4\textsuperscript{+} T cells also express TLR2 and TLR4 are likely to contribute to the adaptive immune response to pneumococcus\textsuperscript{145}. Furthermore, patients with human immunodeficiency virus (HIV) are more susceptible to pneumococcal infection.

Recognition of \textit{S. pneumonia} by TLRs is not the only form of innate mediated recognition of \textit{S. pneumonia}. Opsonins such as complement proteins, acute phase proteins (C-reactive protein (CRP) and surfactant proteins) and antibodies directed against the capsule of the pneumococcus are essential for opsonophagocytosis\textsuperscript{146} and activation of classical complement pathway via C1q.

Mice deficient in other receptors, such as receptor for platelet-activating factor, SIGNR1 (C-type lectin), macrophage receptor with collagenous structure (MARCO, class A scavenger receptor), are highly susceptible to pneumococcal infection\textsuperscript{126}.

Antibodies to the capsule are important to protect against invasive pneumococcal disease. Pneumococcal vaccines aim to enhance antibody IgG to capsular polysaccharides and improve antibody/antibody-complement dependent phagocytosis. However, antibodies may not be primarily responsible for resistance to colonisation because COPD patients colonised with \textit{S. pneumonia} did not have a lower concentration of antibodies to pneumococcal antigens\textsuperscript{147}. The reduction in CD4\textsuperscript{+} T cell counts in human immunodeficiency virus infected individuals correlate with increased \textit{S. pneumonia} infection\textsuperscript{148}. CCL5 deficiency reduces IFN-γ producing CD4\textsuperscript{+} T cells and IgG2a and IgG1 antibodies in serum and these changes correspond with the transition from pneumococcal carriage to severe pneumonia\textsuperscript{149}. 


In addition, human pneumococcal carriage increases the proportion of IL-17A producing T cells and this cytokine enhances opsonisation of bacteria by alveolar macrophages\textsuperscript{150}. Interestingly, patients with hyper-IgE syndrome that have mutations in STAT3 gene undergo recurrent \textit{S. pneumoniae} infections as a result of defective Th17 cell production\textsuperscript{151}. However, some experiments have shown CD4 T cell numbers may adversely impact the cytokine network and survival during \textit{S. pneumoniae} induced pneumonia\textsuperscript{152}. In contrast, CD8 T cells play an important role in improving survival from pneumococcal pneumonia\textsuperscript{153}.

\textbf{Figure 1.4:} Early immune response to bacterial infection in the airway. \textit{A}) Bacterial components are recognised by alveolar macrophages and epithelial cells. \textit{B}) These cells in turn secrete cytokines and chemokines to recruit \textit{C}) neutrophils to site of infection. \textit{D}) Neutrophil granule release promotes monocyte recruitment to help combat infection quicker. \textit{E}) Macrophages also clear apoptotic neutrophils to promote resolution of infection.
1.2.4 Vaccines and therapeutics

Antimicrobial resistance by *S. pneumonia* has considerably increased worldwide particularly with the overuse of penicillin and erythromycin\(^{154}\). Resistance to macrolides, fluoroquinolones, and vancomycin are also reported\(^{126}\). The increased resistance to antibiotics highlights the importance of safe and effective vaccines.

Introduction of 7-valent pneumococcal conjugate vaccine (PCV7) in 2000 helped reduced the incidence of IPD, though the incidence of invasive disease caused by serotypes not included in the vaccine has arisen\(^{154}\). This led to the introduction of vaccines containing more pneumococcal serotypes e.g. PCV23. The PCV7 is part of the routine infant immunisation schedule and PCV23 is recommended for the elderly\(^{9}\).

1.2.5 Mouse models of *S. pneumoniae* infection

Animal models using *S. pneumoniae* isolates from human experimental colonisation studies have been used to define the host-pathogens factors that contribute to bacterial colonisation and spread\(^{131}\). Pneumonia due to *S. pneumoniae* has been studied largely by use of mouse models (Table 1.5).

<table>
<thead>
<tr>
<th>Route of infection</th>
<th>Mouse Strain</th>
<th>Pneumococcal serotype/strain</th>
<th>Utility</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>i.n</td>
<td>BALB/c</td>
<td>Serotype 14</td>
<td>Therapy in neutropenic mice</td>
<td>155</td>
</tr>
<tr>
<td>i.n</td>
<td>C57BL/6</td>
<td>Strain D39</td>
<td>Model of secondary bacterial pneumonia</td>
<td>51</td>
</tr>
<tr>
<td>i.t</td>
<td>C57BL/6</td>
<td>Serotype 3</td>
<td>Drug efficacy and pharmacokinetics</td>
<td>156</td>
</tr>
<tr>
<td>Aerosol nebuliser</td>
<td>CBA/J</td>
<td>Serotype 28</td>
<td>Model of chronic obstructive pneumonia</td>
<td>157</td>
</tr>
</tbody>
</table>

Table 1.5: Examples of Mouse Models of *Streptococcus pneumonia*. Adapted from\(^{138}\). i.n-intranasal; i.t-Intratracheal.
1.3 Cells of the innate immune response in allergic airway disease and bacterial infection

1.3.1 Airway epithelial cells

Airway epithelial cells not only form a physical barrier to environmental antigens, but also secrete a number of first line defence molecules to eliminate bacteria such as mucins (bind infectious agents), surfactant protein A and D (opsonises pathogen), antimicrobial peptides (defensins, cathelicidins and PLUNC that induce direct antimicrobial action) and complement products to promote phagocytosis. Epithelial cells express a range of receptors that enable them to respond to inhaled microorganisms and amplify host responses through the secretion of cytokines and chemokines.

Antimicrobial peptides are polypeptides of 15-45 amino acids with a net positive charge. In the airways, antimicrobial peptides are mainly released by epithelial cells to opsonise and eliminate pathogens. Defensins, cathelicidins and short palate, lung and nasal epithelial clone-1 (SPLUNC1) are major antimicrobial peptide families. Deficiencies of these peptides impair antibacterial immunity. For example, deficiency in murine cathelicidin (cathelicidin-related antimicrobial peptide, CRAMP) increases gram-negative Klebsiella pneumoniae dissemination. Mice deficient in β-defensin-1 reveal a delay in Haemophilus influenzae clearance from the lungs. SPLUNC1 belongs to bacterial/permeability increasing protein family and regulates airway surface liquid volume. SPLUNC1 deficient mice have increased mortality after Pseudomonas aeruginosa infection possibly as a result of increased neutrophil mediated immunopathology. However, SPLUNC1 can be suppressed easily by direct insult of respiratory epithelial cells by PAMPs and IFN-γ. In OVA models of allergic airway disease, SPLUNC1 decreases and negatively correlated with numbers of eosinophils in the lung. Furthermore, IL-13 inhibits SPLUNC1 expression and M. pneumonia clearance. Overall, antimicrobial peptides are important components of the innate immune system and allergic airway inflammation can suppress their activity.

In the healthy airways, 5% of airway epithelial cells are goblet cells; this increases to 20-25% in asthma. Goblet cells produce mucus that forms a semi-permeable barrier that enables the exchange of nutrients, water and gases and allow removal of
Chapter 1: Introduction

pathogens trapped in the mucus through the mucociliary escalator. However, overproduction of mucus can obstruct the airway lumen.

HDM components and airway epithelial cell receptors such as Dectin-1 (binds beta-glucan), TLR2 (beta-glucan), TLR4 (LPS) and platelet activated receptor-2 (proteinases) interact to mediate allergic sensitisation, inflammation and remodelling. HDM allergen, Derp 1 cleaves epithelial tight junction molecules such as occludin and increases penetration of allergens and pathogens into the lungs. In response to an allergen, epithelial cells secrete IL-33, IL-25 and TSLP. These epithelial derived cytokines induce activation of innate lymphoid cells and alternatively activate macrophages to produce IL-13 and IL-4 that in turn, polarise CD4+ T cells into Th2 phenotype. Moreover, murine allergic inflammation and AHR can be inhibited by antibodies directed against IL-33 receptor and IL-25.

1.3.2 Macrophages

Macrophages are found in most tissues and assigned different names depending on which tissue they reside in: alveolar macrophages (airway/lung), kupffer cells (liver), microglia (nervous system) and langerhans cells (epidermis). In the lungs, alveolar macrophages and interstitial macrophages represent two major populations of macrophages based on their anatomical location. Alveolar macrophages line the surface of alveoli and are better phagocytes than interstitial macrophages. Interstitial macrophages reside in the space between alveolar epithelium and vascular endothelium and are better antigen presenting cells than alveolar macrophages.

Most lung resident macrophages are derived from bone marrow-derived monocytes. A study on murine macrophages shows that alveolar macrophages do not originate directly from blood monocytes, but require a lung macrophage intermediate. However, this concept has been challenged recently and genetic fate mapping data defined a lineage of tissue macrophages that derive from the yolk sac. Furthermore, in a recent paper, Guilliams et al found fetal monocyte transferred to the lung of neonatal mice acquired an alveolar macrophage phenotype dependent on GM-CSF and persisted for three months. Taken together these data suggest at least two lineages of macrophages in mice; one derived from the yolk sac.
(F4/80+CD11b-, most likely to constitute the majority of alveolar macrophages) and a second from the bone marrow (F4/80-CD11b+ macrophages).

In a naïve mouse, 90-95% of airway cells are alveolar macrophages\textsuperscript{178} that express Ig receptors, complement receptors, mannose receptor and scavenger receptors to allow phagocytosis of pathogens and debris\textsuperscript{179}. Phagocytosis can occur with or without opsonisation with complement, and subsequently release pro-inflammatory mediators such as IL-12 (activates natural killer cells), IL-8/CXCL1/CXCL2/TNF (recruits neutrophils), TNF/IL-1 (stimulates T cells) and lactoferrin/lysozyme (antimicrobial products)\textsuperscript{179}.

Macrophages become heterogenous and display various phenotypes depending on their microenvironment created by cytokines released by immune and lung structural cells. The diversity of macrophage functions has led to classification of macrophages into three groups: classically activated, alternatively activated (AAMs) or immune regulatory macrophages (\textbf{Fig. 1.5})\textsuperscript{180}.

Classically activated macrophages, activated by IFN-\gamma and TNF or TLR ligands, have enhanced microbicidal activity. Cytokines (IL-6, IL-1 and IL-23) produced by these cells are an important part of host defense\textsuperscript{68}. These macrophages will interact with T cells to give rise to Th17 cells that produce IL-17, a cytokine that is associated with increased neutrophil and Th1 cells recruitment (\textbf{Fig. 1.6}). In contrast to classically activated macrophages, AAMs have reduced anti-microbial capacity. The presence of IL-4 and IL-13 converts macrophages into AAMs to promote wound healing\textsuperscript{181} and this process is amplified by IL-33 and IL-25 produced by epithelial cells, macrophages and Th2 cells\textsuperscript{182}. Innate immune cells, basophils and mast cells also produce IL-4. These macrophages produce less pro-inflammatory cytokines, fail to present antigens and are less efficient at killing intracellular pathogens such as \textit{Cryptococcus neoformans} and \textit{Mycobacterium tuberculosis}\textsuperscript{180,183}. Also, treatment of influenza-infected mice with IL-4 significantly delays viral clearance\textsuperscript{184}, although, AAMs are essential in helminth and nematode clearance.

AAMs have some signature markers: Ym1 is a member of chitinase-like molecule family, induced by Th2 cytokines on macrophages\textsuperscript{182}. Another marker, resistin-like molecule-\alpha (Relm\alpha also known as Fizz1) is induced by IL-13 in macrophages, epithelium and eosinophils\textsuperscript{182}. Arginase 1 (Arg1) is induced by Th2 cytokines in
Figure 1.5: Macrophage phenotypes. A. Classically activated macrophages generate in response to T helper cell type 1 (Th1) cytokine, interferon-γ (IFN-γ) or tumour-necrosis factor (TNF) produced by antigen presenting cells (APCs). B. Exposure of macrophages to T helper type 2 (Th2) cytokines such as interleukin-4 (IL-4) produced by granulocytes or Th2 cells. C. Regulatory macrophages arise in response to many stimuli and produce high levels of IL-10 to suppress immune responses. Adapted from\textsuperscript{180}. 

Adapted from 180.
macrophages and ultimately, results in fibroblast proliferation and collagen production\textsuperscript{185}. In chronic murine schistosomiasis, arginase is induced in Th2 granuloma associated macrophages important in enhancing granuloma size and fibrosis\textsuperscript{186}. Therefore, activation of AAMs increases fibronectin/matrix associated proteins, promotes cell growth/tissue repair, increases parasite killing and enhances allergic immunity\textsuperscript{173}.

In summary, classically activated macrophages are induced by IFN-\(\gamma\), expression of AAMs is induced by IL-4 and TLRs plus other stimuli e.g. immune complexes induce regulatory macrophages. The different macrophage populations express separate biomarkers, summarised in \textbf{table 1.6}.

Alveolar macrophages are vital in host airway defence but their activation must be tightly controlled to prevent host-tissue damage. Alveolar macrophages express regulatory receptors to control their activation and pro-inflammatory cytokine secretion. IL-10 and TGF-\(\beta\) released by epithelial cells induce alveolar macrophages to express high levels of the myeloid inhibitory receptor, CD200R. This receptor interacts with CD200 expressed on the luminal side of airway epithelium. After the resolution of influenza, the intensity of CD200R expression is even higher than at homeostasis\textsuperscript{52}. Inhibition of CD200R prevents \textit{S. pneumoniae} induced sepsis in
influenza-infected mice by reducing early viral titres and subsequent damage of the lung. Another macrophage inhibitory receptor is Triggering Receptors Expressed on Myeloid cells (TREM)-2. TREM2 attenuates macrophage activation and reduces cytokine production in response to TLR agonists. TREM2 reduces macrophage TLR responses via DAP12 and inhibits pro-inflammatory cytokine production in response to TLR ligands. IL-4 induced AAMs express TREM2 with reduced pro-inflammatory cytokine production but enhanced phagocytosis of bacteria. Furthermore, IL-4 can induce TREM2 on resident peritoneal macrophages.

Similar to murine respiratory macrophages, humans have alveolar, bronchial and interstitial macrophages. However, humans also have pulmonary intravascular macrophages. As with murine monocytes, human monocytes are recruited to sites of inflammation by CCL2 and growth factors like GM-CSF are involved in proliferation of both murine and human macrophages. Human bone marrow derived macrophages constitutively express ST2. Both murine and human alveolar macrophages suppress T cells in a similar manner e.g. inhibiting T cell response to IL-2. Murine AAMs and regulatory macrophages produce greater amounts of IL-10 than classically activated macrophages. Alveolar macrophages from asthmatics release more IL-10 compared to non-asthmatic patients and corticosteroid treatment can further enhance IL-10 production. Moreover, human IL-10 producing monocytes can differentiate into AAMs. Bronchial macrophages from mild asthmatics have impaired phagocytosis and downregulation of phagocytic receptors CD16 and CD64. A recent study has found that sputum macrophages from asthmatics did not express all the AAM markers such as Arg1, IL-10, IL-13, CD206 and CLEC10A, however, one AAM marker CCL17 was upregulated in mild steroid-naïve and moderate steroid-treated patients compared to non-atopic controls.
### Table 1.6: Biomarkers for three macrophage populations: classically activated macrophages, alternatively activated macrophages and regulatory macrophages. Adapted from\(^{180}\).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Classically activated macrophages</th>
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<tbody>
<tr>
<td></td>
<td>IL-12</td>
</tr>
<tr>
<td></td>
<td>iNOS</td>
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<tr>
<td></td>
<td>CCL15</td>
</tr>
<tr>
<td></td>
<td>CCL20</td>
</tr>
<tr>
<td></td>
<td>CXCL9</td>
</tr>
<tr>
<td></td>
<td>CXCL10, CXCL11</td>
</tr>
<tr>
<td></td>
<td>TREM1</td>
</tr>
<tr>
<td></td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td></td>
<td>CCL18</td>
</tr>
<tr>
<td></td>
<td>YM1</td>
</tr>
<tr>
<td></td>
<td>RELMα</td>
</tr>
<tr>
<td></td>
<td>CCL17</td>
</tr>
<tr>
<td></td>
<td>IL-27Rα</td>
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<tr>
<td></td>
<td>IGF1</td>
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<tr>
<td></td>
<td>CCL22</td>
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<tr>
<td></td>
<td>TREM2</td>
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<td></td>
<td>Regulatory macrophages</td>
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<tr>
<td></td>
<td>IL-10</td>
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<tr>
<td></td>
<td>LIGHT</td>
</tr>
<tr>
<td></td>
<td>CCL1</td>
</tr>
<tr>
<td></td>
<td>SPHK1</td>
</tr>
</tbody>
</table>

### 1.3.3 Neutrophils

Neutrophils (also known as polymorphonuclear cells or granulocytes) are essential effector cells in the innate immune system that kill microorganisms by phagocytosis, release of granules and neutrophil extracellular traps (NETs)\(^{197}\). They derive and mature in the bone marrow and, when terminally differentiated, they are released into the blood\(^{197}\). However, during an infection neutrophils readily migrate to inflammatory sites to clear the pathogens. Important mouse neutrophil
Chemoattractants are KC/CXCL1, MIP-2/CXCL2 and IL-17, as well as other pro-inflammatory cytokines such as TNF-α and IL-1β. Apoptotic neutrophils are removed by macrophages to prevent tissue damage. Neutrophil granules contain antimicrobial peptides, as shown in figure 1.7. NETs are extracellular structures composed of chromatin with antibacterial proteins from neutrophil granules and cytoplasm attached\(^\text{197,198}\), although, \textit{S. pneumoniae} capsule reduces binding to NETs\(^\text{199}\). Individuals with marked neutrophil dysfunction, known as chronic granulomatous disease, suffer from recurrent infections including \textit{S. pneumoniae} and \textit{Haemophilus influenzae}\(^\text{200}\). Recently, it was shown that local cell death initiates interstitial neutrophil recruitment and clustering with leukotriene B4 (LTB4) playing a critical role\(^\text{201}\).

Neutrophils are involved in the pathogenesis of asthma. Severe asthmatics in particular have greater sputum neutrophil counts\(^\text{202}\) and their airway smooth muscle cells upregulate neutrophil chemoattractant, IL-8\(^\text{203}\). Furthermore, another neutrophil recruiting cytokine IL-17 is increased in the airways of severe asthmatics\(^\text{204}\).

**Neutrophil**

1. **Primary granules (peroxidase positive)**
   - Elastase
   - Cathepsin G
   - Protease 3
   - Myeloperoxidase

2. **Secondary and tertiary granules**
   - lactoferrin-iron binding proteins
   - Lipocalin inhibits bacterial siderophores that deliver Iron to bacteria
   - Lysozyme
   - MMP8/9/25

Reactive oxygen intermediates (ROIs)

Bactericidal phospholipases

**Figure 1.7:** Antibacterial properties of neutrophils. Neutrophils have three main groups of granules: 1. Primary granules develop first and contain myeloperoxidase, 2. Secondary granules lack peroxidase and lactoferrin 3. Tertiary granules are formed last.
1.3.4 Eosinophils

Immune functions of eosinophils involve release of cationic proteins, antibody and complement mediated cytotoxicity, phagocytosis and recognition of pathogens and endogenous danger signals through TLRs and other pathogen recognition receptors (PRRs) to produce an efficient antibacterial response\textsuperscript{205}. However, the quick decline in eosinophil numbers after bacterial infection suggests a very limited role.

Resembling neutrophils, eosinophils have granules composed of cytotoxic cationic proteins and also store cytokines, chemokines and growth factors, available for rapid release\textsuperscript{206}. Eosinophil derived TGF\(\beta\), matrix metalloproteinases (MMPs) and Th2 cytokines effect tissue repair and remodelling\textsuperscript{206}. Siglec F is member of the sialic acid binding Ig-like lectins and possibly the most specific marker for murine eosinophils\textsuperscript{207}. In OVA-induced mice, anti-Siglec F antibodies reduce eosinophilic inflammation and AHR\textsuperscript{208}. SiglecF (Siglec8 in humans), IL-5 receptor \(\alpha\) and CCR3 are the main receptors that define eosinophils\textsuperscript{209}.

Eosinophils are a common feature in allergic inflammation. Eosinophils are granulocytes that develop in the bone marrow with a half-life of 18 hours in the peripheral blood and recruited into tissues in response to appropriate stimuli e.g. IL-5 and eotaxin\textsuperscript{209}. In addition, molecules such as YM1 released by alternatively activated macrophages can recruit eosinophils. Eosinophils also secrete CCL17 and CCL22 to recruit Th2 cells to augment allergic airways disease\textsuperscript{210,211}.
1.4 Pathogen recognition by Toll-like receptors

The airways are constantly exposed to inhaled pathogens. Therefore, it is vital that the host detects pathogens efficiently and mounts a rapid anti-bacterial immune response. To do this innate immune cells express a number of PRRs that recognise pathogen-associated molecular patterns.

The TLRs have evolved to detect conserved patterns on pathogens. So far twelve TLRs (TLR1-TRL9 and TLR11-TRL13) in mice and ten TLRs (TLR1-TRL10) in humans have been identified (Table 1.7). Ligands have been identified for all TLRs except for human TLR10, mouse TLR12 and mouse TLR13. All TLRs are found on the plasma membrane except TLR3, TLR7, TLR8 and TLR9 that are found intracellularly, in the endosome. The TLR signalling cascade is shown in figure 1.8.

TLR activation can be regulated in a number of ways including release of anti-inflammatory cytokines (IL-10 and TGF-β), reduced messenger RNA (mRNA) of TLRs, induction of cell apoptosis, increased micro RNA (miRNA), internalization of TLRs, cleavage and release of soluble TLR receptors or increased expression of intracellular TLR negative regulators \(^{212,213}\).

The triggering receptor expressed on myeloid cells (TREM) family consists of four myeloid transmembrane glycoprotein receptors and modulate TLR responses. TREM1 is expressed by monocytes/macrophages and neutrophils, associates with the immunoreceptor tyrosine-based activation motif (ITAM) containing signalling molecule DAP12 and stimulation of TREM1 induces cytokine production- IL-8 and MIP-1\(^{214}\). TREM1 is assigned the role of an amplifier of inflammation\(^{215}\).
### Toll-like receptors and their ligands

<table>
<thead>
<tr>
<th>Toll-like receptor</th>
<th>Toll-like receptor ligands</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Human</td>
</tr>
<tr>
<td>1:2</td>
<td>Bacterial lipoproteins</td>
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</tr>
<tr>
<td>2:6</td>
<td>Diacylated lipopeptide</td>
<td>✓</td>
</tr>
<tr>
<td>2</td>
<td>Lipotechoic acid (LTA)</td>
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</tr>
<tr>
<td>3</td>
<td>Viral dsRNA</td>
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</tr>
<tr>
<td>4</td>
<td>Lipopolysaccharide (LPS)</td>
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</tr>
<tr>
<td>5</td>
<td>Flagellin</td>
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<tr>
<td>7</td>
<td>Viral ssRNA</td>
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<tr>
<td>9</td>
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<tr>
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</tr>
<tr>
<td>10</td>
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</tr>
<tr>
<td>13</td>
<td>Bacterial RNA</td>
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</tbody>
</table>

**Table 1.7:** Toll-like receptors and their ligands. 13 Toll-like receptors have been identified that recognise various microbial components. Adapted from\(^\text{216}\).
Figure 1.8: Toll-like receptor (TLR) signalling pathway. Activation of TLRs by their ligands initiates a cascade of intracellular signalling molecules that result in activation of NF-κB and transcription of antimicrobial products. Marked in red are Intracellular negative regulators of TLR signalling molecules.
1.5 Aims and hypothesis

In this thesis chronic HDM exposure will be used to induce murine allergic airways disease. In particular, the three week HDM exposure protocol that induces AHR, mucus hypersecretion, eosinophils, Th2 cells, upregulation of epithelial derived cytokines such as IL-25 and IL-33 and airway remodelling 217. This model reflects HDM-induced allergic asthma, which is the most common cause of mild to moderate asthma; furthermore 40% of severe asthmatics are HDM allergic. Therefore, the murine model represents a clinically relevant model of human asthma.

The hypothesis for this thesis is that the allergic lung mounts a defective anti-bacterial response to S. pneumoniae and is therefore more susceptible to bacterial infection. This thesis addresses the possibility that asthma exacerbates bacterial infection rather than bacteria exacerbating underlying asthma.

The aims are:

1. To assess the susceptibility of the allergic lung to bacterial infection.
2. To analyse the anti-bacterial immune response in mice with prior HDM induced allergic inflammation.
3. To investigate features of alveolar macrophages after three weeks of HDM exposure.
4. To evaluate bacterial susceptibility in a model where there is reduced allergen induced eosinophilic pathology (immune response).
CHAPTER 2

Materials and Methods
2.0 Materials and Methods

2.1 Laboratory animals

Seventeen to nineteen gram or 6-8 weeks old female BALB/c mice were purchased from Harlan Olac Ltd, Bicester UK. ST2 deficient mice were originally generated and kindly provided by Professor Andrew McKenzie (University of Cambridge). All mice were maintained in specific pathogen free conditions at Bio Safety Level 2 and kept in accordance with institutional and UK Home Office guidelines.

2.2 Streptococcus pneumoniae (D39) stock

Wild type (WT) S. pneumoniae (serotype 2) strain D39 (NCTC 7466, National Collection of Type Cultures, London, UK). Bacteria were cultured at 37°C in 5% CO2 on blood agar plates or in Todd-Hewitt broth (Sigma) supplemented with 0.5% yeast extract (OXOID) (THY broth) to an OD₆₀₀ of 0.2 (approximately 2x10^8 CFU/ml) and stored at -80°C in 10% glycerol as single-use aliquots. Exact inoculum colony forming units (CFU) used in each experiment and the recovered tissue CFU were determined by manually counting colonies of serial tenfold dilutions plated on Columbia agar supplemented with 5% defibrinated horse blood.

2.3 Animal allergy and infection models

2.3.1 Murine allergic airway disease induction

BALB/c mice were anaesthetised with isoflurane and intranasally (i.n.) inoculated with 15µg HDM (Chapters 3 & 4: Greer, Lenoir, N.C, USA, batch number XPB82D3A2.5, lot: 151176- Derp 1 149.02 µ/vial, Protein 4.29 mg/vial, 125 Endotoxin EU/vial and Chapter 5: Greer, Lenoir, N.C, USA, batch number XPB70D3A2.5, lot: 213051- Derp 1 145.56mcg/vial, Protein 2.87 mg/vial, endotoxin 31.25 EU/vial) extract dissolved in phosphate buffered saline (PBS) sterile or PBS alone for controls for 3 times/week for 3 weeks.

2.3.2 Respiratory bacterial challenge

S. pneumoniae (serotype 2) strain D39 was diluted in endotoxin free sterile PBS and administered i.n in a volume of 50µl to mice held in an upright position. Groups were
followed daily for illness. Mice were harvested at predetermined time points post infection by injection of 3 mg pentobarbitone and exsanguinated via the femoral vessels.

2.3.3 Recombinant murine chemokine administration
To restore anti-bacterial immunity 1µg of recombinant murine CXCL1 (KC, Peprotech; Cat. No: 250-11), recombinant murine CXCL2 (MIP-2, Peprotech; Cat. No: 250-15) or recombinant murine CCL2 (MCP-1, Peprotech: Cat. No: 250-10) was given i.n per mouse reconstituted in 20µl sterile PBS.

2.3.4 Intranasal PKH26-PCL dye administration
Mice were given intranasal administration of 50µl of 10µM PKH26 red fluorescent cell linker kit for phagocytic cell labeling (PKH26-PCL) from Sigma that is ingested by phagocytic cells in the airways\(^2\)\(^1\). PKH26-PCL (Sigma) labels cells with phagocytic capabilities through ingestion of dye micro-aggregates. We administered dye to BALB/c mice 72 hours before the first HDM exposure and then exposed the lungs to three weeks of 15µl PBS or 15µg HDM (3 times a week).

2.4 Lung Histology

2.4.1 H&E and PAS staining of lung tissue
One right lung lobe of BALB/c mouse was placed in formalin for 24 hours then transferred into 70% ethanol or industrial methylated spirit (IMS) and 4 micron sections were stained with Haematoxylin and Eosin (H&E) or Periodic acid-Schiff stain (PAS). The H&E and PAS stainings were performed by Lorraine Lawrence, NHLI, Imperial College, UK.

Mucus scoring: mucus-containing cells were counted on PAS stained sections using an arbitrary scoring system. Goblet cells in the airway epithelium were quantified according to the following scoring system; 0 score = <0.5%, 1 score = 5-25%, 3 = 25-50%, 3 = 50-75% and 4 = >75% of positive purple stained cells. The total scores for each section were divided by the number of airways examined and expressed as mucus index in arbitrary units.

2.4.2 Immunohistochemistry

A. CXCL1: Paraffin-embedded lung sections were incubated with goat anti-mouse CXCL1 IgG (15µg/ml, R&D systems Cat: AF-453-NA) with incubation for 1 hour at room temperature. An avidin/biotin horseradish peroxidase cell staining kit from R&D
systems (CTS008) was used with diaminobenzidine to give a brown-colored product at the site of CXCL1 presence. Subsequently, sections were counter-stained in haematoxylin, mounted and photographed using a light microscope.

B. RELMα and YM1: Paraffin-embedded sections lung sections were incubated with goat anti-mouse RELMα (1µg/ml, R&D systems, Cat no: AF1523) or YM1 (1µg/ml, R&D systems, Cat no: AF2446) and detected with anti-goat horseradish peroxidase (HRP)- 3,3’-diaminobenzidine tetrahydrochloride (DAB) cell staining kit (R&D systems, CTS008). Sections were counter stained with haematoxylin, mounted with DPX and photographed using a light microscope.

2.5 Sample recovery and cell preparation of BAL and lung tissue

Bronchoalveolar lavage (BAL), lung tissue and blood were harvested. Blood clotting was prevented by heparin (1:10 dilution).

1. Chapter 3: BAL was obtained by inflating the lungs of each mouse 3 times with 1.5ml 1mM ethylenediaminetetraacetic acid (EDTA) in Hank’s buffered salt solution (HBSS) and placed in sterile tubes on ice. BAL was centrifuged for 5 minutes at 1200 RPM and the supernatant removed and stored at -80°C for analysis of cytokines by ELISA.

2. Chapter 4 & 5: BAL was obtained by inflating the lungs of each mouse 3 times with 1.2ml 1mM EDTA in HBSS and placed in sterile tubes on ice. This was repeated one more time, however, supernatant was only stored from the first wash. Cells from both washes were used for counting and flow cytometry staining. BAL was centrifuged for 5 minutes at 1200 RPM and the supernatant removed and stored at -80°C for analysis of cytokines by ELISA. The number of BAL washes was increased in chapters 4 and 5 to increase the total cell numbers recovered from the airways for multiple flow cytometry staining panels.

The pellet was re-suspended in RPMI-1640 supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (P/S) at a final concentration of 1x10^6 cells/ml for fluorescence-activated cell sorting (FACS) analysis.

Lung tissue was finely minced and digested with dispase (0.4 Wunsch units/ml Roche) and DNase (50µg/ml of 100mg DNase I 10104159001 Roche 1:2000) with shaking for 30 minutes at 37°C. This digested preparation or whole lung tissue was disrupted to a single cell suspension by passage through a 70µM sieve (BD
labware/falcon, USA Cat. No: 352350). The cell suspension was subsequently spun for 5 minutes at 1200 RPM and red blood cells lysed by re-suspending pellets in ACK buffer (0.15M ammonium chloride, 1M potassium hydrogen carbonate and 0.01mM EDTA, pH7.2) for 3 minutes at room temperature (RT) before centrifugation for 5 minutes at 1200 RPM and washing in RPMI. Cell viability in all tissues was assessed using Trypan Blue exclusion and subsequently re-suspended in RPMI+FCS+P/S to a final concentration of 1x10^6 cells/ml.

2.5.1 Analysis of bone marrow mobilisation of neutrophils
Femurs were isolated from BALB/c mice and the bone marrow was flushed with HBSS (1x), HEPES 30mM, EDTA 15mM using a 23G needle. Any clumps were resuspended using a 21G needle and spun at 1200rpm for 5 minutes. Red blood cells were lysed using 3ml 0.2% NaCl followed immediately by 3ml 1.6% NaCl and made up to 20ml with RPMI and 0.1% BSA. Cell viability was assessed using Trypan Blue exclusion and subsequently re-suspended in RPMI+0.1%BSA. Cells were FACS stained for neutrophils as described in section 2.7.1.

2.6 Determination of bacterial load in blood, BAL and lung
Airway, lung and blood bacterial titres were determined by serial dilution of 20µl aliquots from single cell suspensions of each tissue in sterile PBS. Serial dilutions were plated on Columbia blood agar with 5% supplemented with 5% defibrinated horse blood (TCS Bioscience HB034) and incubated overnight at 37°C in 5% CO2. The total CFU per tissue was determined manually by counting bacterial colonies on the plates (number of colonies x dilution factor x original cell suspension volume).

2.7 Flow cytometric analysis

2.7.1 Extracellular antigen analysis
Cells were stained for surface markers and analysed by flow cytometry. A minimum of 2x10^5 or a maximum of 1x10^6 BAL or lung derived cells were stained using various combinations of FITC, PE, PerCP-Cy5.5, PE-Cy7 and APC antibodies. All antibodies (Table 2.1) were diluted in PBS containing 1% Bovine serum albumin (BSA) with 0.5% sodium azide [PBA]. Cells were stained for 30 minutes on ice, washed with PBA and spun for 2 minutes at 2000 RPM. After washing, cells were fixed for 20 minutes at RT with 2% formaldehyde/PBS. Cells were then washed in
PBS and re-suspended in a final volume of 200µl PBA. Data acquired on a BD FACS CANTOII (BD) or Fortessa II (BD) and analysed on FlowJo software. Forward scatter and side scatter gates were used to exclude debris on organ homogenates.

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Table 2.1: List of antibodies used in experimental work in this thesis.
In most experiments (all experiments in chapter 4 and 5), dead cells were excluded using a fixable near-IR dead cell stain kit for 633 or 635nm excitation (Invitrogen; L10119).

Fluorescence minus one (FMO) control was included for each fluorescent marker, the expression of a particular marker was calculated by subtracting FMO fluorescence values from fluorescent antibody levels.

2.7.2 Intracellular cytokine expression

To detect intracellular cytokines, 1x10^6 cells/ml were incubated with 50ng/ml Phorbol-12myristate-13-acetate (PMA), 500ng/ml ionomycin (Calbiochem, Nottingham, UK) and 10µg/ml brefeldin A for 3 hours at 37°C. Cells were then stained for extracellular antigens such as anti-CD4-PerCP-Cy5.5, anti-γδ T cell receptor (TCR)-FITC and anti-CD3-PE-Cy7 (all eBioscience) and fixed as described in 2.7.1. After permeabilisation with PBS containing 1% saponin/ 1% BSA/ 0.05% azide (saponin buffer) for 10 minutes:

A. Cytokine staining: Cells were stained with anti-IL-17-APC (eBioscience) diluted 1:50 in saponin buffer for 30 minutes. Cells were then washed once in saponin buffer and once in PBA.

B. Intracellular protein staining (IRAK and TRAF-6): After permeabilisation in saponin buffer for 15 minutes, cells were incubated with primary antibodies rabbit polyclonal anti-IRAK (1:200 dilution, Abcam, ab238) or rabbit monoclonal anti-TRAF-6 (1:200 dilution, Abcam, ab33915) for 16h at 4°C followed by 2 washes and then incubation with secondary antibody chicken anti-rabbit-FITC (1:500 dilution, Abcam, ab6825) for one hour.

Data was then acquired as described in section 2.7.1.

2.8 Cell Sorting by flow cytometry

BAL was obtained by inflating the lungs of each mouse multiple times with 1.2ml 1mM EDTA in HBSS and placed in sterile tubes on ice. The cell suspension was subsequently spun for 5 minutes at 1200 RPM and red blood cells lysed by resuspending pellets in ACK buffer (0.15M ammonium chloride, 1M potassium hydrogen carbonate and 0.01mM EDTA, pH7.2) for 3 minutes at room temperature.
(RT) before centrifugation for 5 minutes at 1200 RPM and washing in RPMI. Cells were then stained with 1µl in 1ml of fixable near-IR dead cell stain kit for 633 or 635nm excitation (Invitrogen; L10119) in PBS incubated for 20-30min at 4°C. After washing the cells in PBS:

1. Dye positive cells were resuspended in RPMI (no FCS+P/S) and ready to run for sorting.

2. Cells were stained with CD11b and CD11c in RPMI for 30 minutes at 4°C and then washed in RMPI before cell sorting.

2.8.1 Sorting of airway cells dyed with PKH26-PCL red fluorescent cell linker

The PKH26 red fluorescent cell linker kit for phagocytic cell labelling (PKH26-PCL) from Sigma was used to label airway macrophages.

An example of cell sorting profile of airways that received no dye (unstained)
An example of cell sorting profile of PBS exposed airways that received dye
An example of cell sorting profile of HDM exposed airways that received dye
2.9 Cytokine detection using ELISA
TNF (BD or eBioscience), CXCL1 (KC, R&D systems), CXCL2 (MIP-2, R&D systems), CRP (R&D systems), SP-D (Uscn Life Science Inc. SEB039MU), IL-17A (eBioscience), IL-33 (eBioscience) and IL-6 (eBioscience) in BAL fluid were quantified using Duoset ELISA kits and following manufacturer’s instructions. Microtiter plates (Nunc, Roskilde, Denmark) were coated with 100µl of capture antibody diluted in the recommended buffer and incubated according to the manufacturer’s protocol. After 3-5 washes with PBS containing 0.5% Tween 20, plates were blocked with 200µl of recommended reagent diluent and left for 1 hours at RT. Samples and standards (diluted in reagent diluent) were then incubated for a further 2 hours at RT. After 3-5 washes, bound cytokine was detected using biotinylated antibodies with avidin-HRP followed by tetramethylbenzidine (TMB). Optical densities were read at 450nm using 570nm as a reference. The mean optical density of blank wells (no cytokine) was subtracted from the results obtained for samples and standards. A standard curve was used to calculate the concentration of cytokine in each sample using Excel or GraphPad Prism 5 software.

2.10 Isolation of mRNA and real-time PCR
A. mRNA: Total lung RNA was extracted using RNeasy Mini kit (QIAI GEN, UK), reverse transcribed into complementary (c) DNA (SuperScript III Reverse transcriptase, Invitrogen, Cat. No: 18080), and then amplified using specific primers for the TLR negative regulators tested. All values were normalised against the expression of 18S and then to a control sample (PBS) using ABI7900HT (Applied Biosystems, CA) sequence detection system and software. The primers for the genes of interest were purchased from Applied Biosystem (all TaqMan probes): Toll interacting protein (Tollip) (Mm00445841), Irakm (Mm00518541), A20 (Mm00437121), Triad3A (Mm012005634), Ym1 (Mm00657889), Relmα (Mm00445109), Arg1 (Mm00475988), Tlr2 (Mm00442346), Tlr3 (Mm01207404), Tlr4 (Mm00445273), Tlr9 (Mm00446193), Irak1 (Mm01193538) and Traf6 (Mm00493836).

B. miRNA: miRNA was extracted and detected using the TaqMan MicroRNA Cells-to-CT kit (Ambion, Life technologies, 4391848) manufacturer’s instructions. TaqMan MicroRNA assays were used to detect mmmu-miR-146b-5p (Applied Biosystems,
001097). All values were normalised against the expression of snoRNA202 (001232) and then to a control sample (PBS) using ABI7900HT (Applied Biosystems, CA) sequence detection system and software.

2.11 Western blot
SPLUNC1 was detected by western blotting. Briefly, all samples were electrophoresed by reducing sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were blocked in PBS containing 5% milk and 0.1% Tween-20 for 2h at RT and incubated with sleep anti-mouse PLUNC (SPLUNC1; 1µg/ml, R&D systems) diluted in 5% milk/PBS/0.1% Tween-20, overnight at 4°C. After three washes of 15 min in PBS/0.1% Tween-20, membranes were incubated with Donkey anti-sheep IgG-HRP (1:1000 dilution, R&D systems) and detected using ECL chemiluminescent kits (Pierce, Rockford, IL).

2.12 Genotyping
BALB/c wild-type controls and ST2 deficient mice were genotyped using REDExtract-N-Amp Tissue PCR kit (Sigma). Primers used for genotyping were Primer 1 854 TGT TGA AGC CAA GAG CTT ACC and Primer 2 TTG GCT TCT TTT AAT AGG CCC. 2% agarose gel was used with hyperladder 100bp Plus (Bioline Bio-33071) and SYBR Safe DNA gel stain was used for visualization of DNA. Wild-type band were 300bp band, whereas the homozygous ST2 knock out displayed no band on the gel.

2.13 Statistics
GraphPad Prism software was used for all statistical calculations. Statistical test used was Mann-Whitney compared to PBS controls (or as indicated in the figure). Data presented as box and whisker plots show median and range of data. ELISA data is shown as mean ± standard deviation (SD) and unpaired two-tailed t test was used. p values < 0.05 were considered significant (* p<0.05, ** p<0.01, ***p<0.001).
CHAPTER 3

Defective anti-pneumococcal immunity in the allergic lung.
3.1 Introduction

3.1.1 Asthma and *S. pneumonia*

There is no life long immunity against IPD through natural infection or vaccination. Moreover, individuals with immunocompromised lungs are more likely to suffer from *S. pneumoniae* induced pneumonia. As discussed earlier, clinical data show that asthmatics are more susceptible to bacterial infections and present defective antibacterial immune responses. For example, HDM-sensitised children have reduced IgG1 antibody levels for PspC, which is a surface protein on *S. pneumoniae*\(^\text{219}\).

In contrast, murine models of allergic airways disease using the atypical allergen OVA for sensitisation and challenge show no defect in bacterial clearance\(^\text{220-222}\). Kang *et al* assessed development of pneumococcal pneumonia in OVA sensitised mice using bioluminescent *S. pneumoniae* strain (A 66.1 serotype 3)\(^\text{220}\) and concluded that allergic airways disease actually reduced susceptibility to pneumococcal pneumonia. However, the experimental protocol used by Kang *et al* did not significantly increase Th2 associated cytokines IL-4, IL-13 and IL-5 (a common feature of allergic response) in the OVA sensitised mice compared to the control group. In addition, the authors did not present data on cellular infiltrate after OVA to validate their allergic airways disease protocol. Another study using OVA and *S. pneumonia* (serotype and dose unknown) show bacterial infection after OVA sensitisation but before OVA challenge suppresses allergic airways disease possibly due to an increase in Foxp3+ T regulatory cells. Similarly, treatment with ethanol killed *S. pneumonia* or administering the bacteria before OVA sensitisation and challenge suppresses allergic airway disease\(^\text{221}\).

The main difference between these studies and the allergic and bacterial protocols to be used in our studies is the use of a clinically relevant allergen, HDM. OVA sensitisation requires intraperitoneal priming while HDM will be administrated intranasal (mimicking the natural route of exposure) and does not require peritoneal sensitisation with aluminum hydroxide (Th2 skewing adjuvant). Therefore, allergic inflammation will result after mucosal sensitisation within the lungs. Furthermore,
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.

HDM replicates the environment created by common allergens better than OVA, which contains no proteolytic activity or TLR ligands.

3.1.2 Prior lung inflammation alters susceptibility to pneumonia

It is not simply asthma that increases susceptibility to S. pneumonia 16. Clearance is also hampered following influenza infection 51,52,223 and chronic COPD224. Many reasons for enhanced susceptibility of influenza-infected mice to bacterial pneumonia have been identified. For example, influenza virus neuraminidase removes sialic acid from the lung exposing receptors for bacterial adherence225. Influenza enhances the anti-inflammatory cytokine IL-10226,227, upregulates platelet-activating factor receptor228, reduces tracheal mucociliary rate229, type I interferon mediated impaired production of CXCL1 and CXCL2 after bacterial infection230 and induces alternative activation of lung macrophages231. Clearly, a prior lung infection can alter innate immune responses to a subsequent pathogen and the effect may last for several months53. In this chapter the impact of HDM on the ability to clear S. pneumoniae and the mechanisms responsible for any modifications are elucidated based on our knowledge of influenza infection.

3.1.3 Hypothesis and Aims

To date many studies have investigated the possibility that bacteria exacerbate asthma, but only a handful considers that asthma may cause a bacterial exacerbation. Our hypothesis is to study how asthma affects immune responses to inhaled bacteria. Therefore, the aim is to elucidate the innate anti-bacterial immune mechanisms that are modified following HDM exposure and that subsequently impact on control of respiratory bacteria. The specific aims were:

1. To examine the response of the allergen exposed lung to S. pneumoniae.
2. To assess the molecular and functional alterations that occur in anti-pneumococcal immunity in the allergic lung.
3. To examine alteration of immune potentiators and negative regulators of innate immunity.
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.

Data from this chapter was published in the journal of Mucosal Immunology$^{232}$.

3.2 Results

3.2.1 HDM exposure induces allergic airway disease

The ability of HDM allergen to induce experimental asthma in BALB/c mice was first examined. 50-85% of asthmatics are HDM allergic and it is therefore a clinically relevant allergen to use that unlike OVA, does not require intraperitoneal priming$^{82}$. HDM naturally contains LPS. The Greer HDM used in this thesis has a low LPS content (0.1-1ng) per 25µg dose, which is far lower than the dose of LPS (100ng) required to promote Th2 responses in OVA models$^{233}$. We first ascertained that the standard hallmarks of airway disease were present by sampling prior to bacterial challenge. Intranasal administration of HDM three times a week for three weeks (Fig. 3.1A) resulted in increased mucus production and increased airway and lung cellularity and eosinophils as described before$^{234}$.

HDM-induced airway inflammation was monitored by histology and BAL & lung eosinophilia. HDM exposure resulted in significantly increased cellularity in the airways and lungs compared to PBS controls in H&E stained lung sections and by enumerating total viable cell counts (Fig. 3.1B & C). Similarly, only the HDM exposed group produced mucus as seen in PAS stained lung sections (Fig. 3.1B) with significantly increased eosinophil numbers in BAL (p=0.0079) and lung (p=0.0079) (Fig. 3.1D). Furthermore, three weeks of HDM exposure significantly increased airway and lung CD4$^+$ T cells (Fig. 3.1E).
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.
Figure 3.1: Induction of allergic airway inflammation after repeated intranasal HDM administrations into the airways. (A) Experimental design: BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract three times a week for 3 weeks and subsequently harvested on day 0 (72 hours post final HDM exposure). As a control we included BALB/c mice intranasal exposed to phosphate-buffered saline (PBS) instead of HDM. (B) Histologic sections of lungs were stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) to determine lung cellularity and the degree of mucus production and goblet cell hyperplasia and analysed by light microscopy (original magnification x10). Pictures show representative samples of five per group from one experiment and are representative of two independent experiments. (C) After 3 weeks of HDM challenge, the total viable bronchoalveolar lavage fluid (BALF/airway) & lung cell count determined using trypan blue exclusion. (D) The number of CD11b⁺SiglecF⁺ eosinophils and (E) CD4⁺ T cells were discriminated in airway and lung by flow cytometry. Data presented as box and whisker plots showing the median and minimum to maximum range. Data representative of five per group and are representative of three independent experiments. *P<0.05; ** P<0.01.
3.2.2 HDM exposure prior to *S. pneumoniae* infection reduced bacterial clearance

At 72 hours after the final allergen challenge, *S. pneumonia* was administered intranasal (Fig. 3.2A) and the lung and BAL sampled after 4, 48 and 96 hours. 100% of HDM exposed group had bacteria in the BAL and lung compared to 0% of PBS controls 48h post infection (Fig. 3.2B). The increased bacterial titres in the HDM group correlated with the increased numbers developing bacteraemia. The interval between 48 and 96 hours post infection seemed to be the most crucial in determining whether the groups could recover from the infection or develop bacteraemia. The bacterial titres at the 96 hours post infection is not shown as some had to be culled in the HDM/S. pneumonia group and so the results were biased towards survivors. 40% of HDM exposed mice were sacrificed at 96 hours after infection (n=5-6 of two independent experiments).

Allergen exposure typically induces mucus by increased secretion of mucins by goblet epithelial cells. Despite, cessation of HDM exposure mucus production remained. Strikingly, following *S. pneumonia* infection, mucus production actually increased as reflected in histology (Fig. 3.3A) and mucus scores (Fig. 3.3B). This was not observed in PBS infected group and is therefore not a feature of bacterial infection alone.
Figure 3.2: Allergic airway inflammation induces exacerbation of lung *Streptococcus pneumoniae* (S.pn) infection. (A) Experimental setup of house dust mite (HDM)-induced allergic airway inflammation and bacterial (*Streptococcus pneumoniae*) infection in BALB/c mice. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and subsequently infected with 1x10^6 CFU of *Streptococcus pneumoniae* (D39 strain) harvested on day 0 (72 hours post final HDM exposure). (B) Colony forming units (CFUs) were calculated using serial dilutions of single cell suspensions of each sample and plated on blood agar plates. Bacterial titres were measured at 0, 4 and 48 hours post infection in bronchoalveolar lavage fluid (BALF/airway), lung & blood (ND= not detected). Data representative of five per group and are representative of 2 independent experiments at 4 hours and 4 independent experiments at 48 hours.
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.
Figure 3.3: Enhanced mucus production in HDM-sensitised mice is exacerbated following bacterial infection. (A) Histologic sections of lungs were stained with periodic acid Schiff (PAS) to determine the degree of mucus production (arrows) and goblet cell hyperplasia analysed by light microscopy (original magnification x10). Pictures show representative samples of five per group from one experiment and are representative of two independent experiments. (B) Mucus-containing cells were counted on PAS stained sections using an arbitrary scoring system. Goblet cells in the airway epithelium were quantified according to the following scoring system; 0 score = <0.5%, 1 score = 5-25%, 3 = 25-50%, 3 = 50-75% and 4 = >75% of positive purple stained cells. The total scores for each section were divided by the number of airways examined and expressed as mucus index in arbitrary units. S.pn=S. pneumonia
3.2.3 Blunted cell recruitment in HDM exposed mice after bacterial infection compared to PBS controls

The inflammatory response to bacteria in allergic lungs was measured next. Total cellularity measured 72 hours after final HDM exposure but prior to bacterial infection, was significantly increased in the airways and lung of HDM exposed group (0 hour after infection) compared to PBS controls (Fig. 3.4). In contrast, 48 & 96 hours after infection, the total viable cell numbers decreased in the airways of HDM exposed mice (Fig. 3.4A). This is depicted more clearly as percentage of baseline-total cell count at time of infection (0 hour) (Fig. 3.4B). Total cellularity in the lungs of PBS exposed mice increased significantly 48 hours (p=0.0079) post infection; while there were no changes in the HDM exposed mice that already possessed high cell numbers (Fig. 3.4C & D). Since the numbers of cells in the HDM groups are similar in the lung yet the number recruited in the airspace is blunted in the allergen exposed groups 48 hours post infection this may suggest impaired recruitment of cells from the lung to the airways or increased apoptosis of cells in the airway.

Flow cytometric analysis was used to determine the cellular phenotype in the airways and lung in response to HDM exposure. Prior to bacterial infection the airway and lung was dominated by eosinophils (Fig. 3.5A & B) and CD4⁺ T cells (0 hour post infection) in HDM exposed mice (Fig. 3.5C & D). Eosinophils were absent in the PBS group prior to bacterial infection and CD4⁺ T cells absent or low, following bacterial infection the number of both populations decreased. Therefore, the reduced cellularity from baseline in the airspaces can be explained by a reduction in eosinophils and CD4+ T cells in both the airway and lung.
Figure 3.4: Total airway and lung cell counts in PBS or HDM exposed group after *Streptococcus pneumonia* (S.pn) infection. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and subsequently infected with 1x10^6 CFU of *Streptococcus pneumoniae* (D39 strain) harvested on day 0 (72 hours post final HDM exposure), 48 and 96 hours after bacterial infection. (A) Airway and (C) lung total viable cell numbers. Data presented as box and whisker with the median of 5 per group representative of 3 independent experiments' *P<0.05; ** P<0.01. (B) Airway and (D) lung cell counts presented as percentage of baseline (0 hour after infection).
Figure 3.5: Airway and lung eosinophils and CD4 T cells of HDM- exposed and control mice after Streptococcus pneumoniae (S.pn) challenge. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and subsequently infected with 1x10^6 CFU of Streptococcus pneumoniae (D39 strain). Samples were harvested on day 0 (72 hours post final HDM exposure), 48 and 96 hours after bacterial infection. The numbers of (A, B) CD11b^+SiglecF^+ eosinophils and (C, D) CD3^+CD4^+ T cells in the allergic airways and lungs were determined by flow cytometry. Data presented as box and whisker with the median of 5 per group representative of 3 independent experiments. *P<0.05; ** P<0.01.
3.2.4 HDM exposure reduces recruitment of neutrophils to the airways and lung

Neutrophils are an important cell type in anti-bacterial immunity. Next, it was examined whether their recruitment was affected by prior HDM exposure. Neutrophil numbers were significantly higher in the lungs of HDM (p=0.0317) exposed mice prior to bacterial infection unlike the airways (0h) (Fig. 3.6A & B). This lung neutrophil infiltrate to HDM has been described before\(^\text{120}\). There was a substantial increase in neutrophils in PBS treated mice in response to \textit{S. pneumoniae} at 48 hours. However, this increase was blunted in HDM exposed mice in both airways and lung (Fig. 3.6). On comparison to PBS, neutrophil numbers in the allergic group were significantly reduced 48 hours after infection in BAL (p=0.0079) and lung. At 96 hours post infection there is no difference in neutrophil numbers between the two groups and they had essentially returned to pre-infection levels.

The impairment of neutrophil recruitment in HDM exposed mice may be a result of structural changes in the lung or reduced chemotactic signals (Fig. 3.7A). Therefore, after 3 weeks of HDM or PBS exposure, mice were given recombinant CXCL1 (1µg CXCL1/mouse), which is an important mouse neutrophil chemoattractant. HDM exposed mice (without infection) were able to recruit as many neutrophils as PBS controls after CXCL1 exposure (Fig. 3.7B) into the airways and lung (Fig. 3.7C). This suggests that chemotactic signals for neutrophils may be reduced in the asthmatic lung and that perhaps their restoration could prevent subsequent bacterial infection.

3.2.5 Instillation of CXCL1 or CXCL2 to the HDM-exposed lung restores anti-bacterial immunity

The HDM-exposed lung is clearly deficient in multiple factors required for clearance of \textit{S. pneumoniae} and this deficit likely occurs at the level of innate recognition and responsiveness of TLRs. We therefore attempted to bypass this deficiency by restoring the neutrophil chemoattractants, CXCL1 or CXCL2 (Fig. 3.7D). Intranasal administration of CXCL1 or CXCL2 at the time of infection (72 hours following cessation of HDM) restored Ly6G\(^+\) neutrophil numbers in the airway and lung and
Figure 3.6: Reduced neutrophil numbers in HDM- exposed mice following bacterial challenge. (A) Airway and (B) lung neutrophil (identified by flow cytometry as CD11b+ Ly6G+) recruitment in HDM- or PBS- exposed group were compared after intranasal administration of Streptococcus pneumoniae (S.pn). Data presented as box and whisker plots with the median. *P<0.05; ** P<0.01. Data show representative samples of five per group from one experiment and are representative of three independent experiments.
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.
Figure 3.7: Equivalent numbers of neutrophils are recruited into the airways and lungs of control (PBS) and HDM- exposed mice after recombinant CXCL1 or CXCL2 instillation. (A) An illustration of the CXCL1 administration protocol. (B) Airway and (C) lung neutrophil (identified as CD11b+Ly6G+ by flow cytometry) recruitment in HDM- or PBS- exposed mice was analysed 6 and 24 hours after intranasal administration of 1µg of recombinant murine CXCL1 per mouse on day 0. (D) An illustration of CXCL1 or CXCL2 with or without S. pneumonia administration protocol. Airway and lung bacterial titres were determined 6 hours after infection +/- (E) CXCL1 or (F) CXCL2. Administration of 1µg recombinant murine (G) CXCL1 or (H) CXCL2 per mouse overcomes the inability of HDM exposed group to recruit neutrophils 6 hours after bacterial infection. Box and whisker plots of 5 per group representative of 2 experiments. *P<0.05; ** P<0.01.
the neutrophil numbers even exceeded those in the PBS control group 6 hours after infection (Fig. 3.7G & H). Bacterial CFU in the airway and lung were significantly reduced in HDM-sensitised group where CXCL1 (Fig. 3.7E) or CXCL2 (Fig. 3.7F) had been restored. Furthermore, there was a significant reduction in the percentage of neutrophils in the blood after i.n CXCL2 administration suggesting an improved migration of neutrophils from blood to the airways and lungs (Fig. 3.8A), whereas there was no change in the percentage of neutrophils in the bone marrow between the groups that received CXCL2 and those that did not (Fig. 3.8B).

3.2.6 Reduced anti-bacterial cytokine production in the allergic airways

To evaluate further the cause of the impaired neutrophil recruitment, cytokines and chemokines essential for neutrophil recruitment to sites of infection were assessed. The trend for all cytokines and chemokines (TNF, IL-6, CXCL1 and CXCL2) measured was a significant reduction in HDM exposed mice compared to PBS controls at early hours after infection (6 hours) (Fig. 3.9A-E). Both CXCL1 and CXCL2 act on the chemokine receptor CXCR2 on neutrophils. TNF levels were significantly reduced at 4 (p=0.0225) and 48 hours (p=<0.0001) after S. pneumoniae infection compared to PBS (Fig. 3.9A). MMP-9 airway levels were studied as it cleaves CXCL1 into a 10-fold more potent form\textsuperscript{235}.

CXCL1 is an important neutrophil chemoattractant secreted mainly by epithelial and macrophages in response to infection. Therefore, we next sought to identify CXCL1 production by airway epithelial cells. Immunohistochemistry of lung sections showed that CXCL1 was strongly expressed in PBS, while levels comparable to control (no primary antibody added) were visible in HDM exposed mouse lung sections, 4 hours after infection (Fig. 3.9F).

IL-17A is an important stimulus for granulocyte-colony stimulating factor (G-CSF) secretion that is essential for production of neutrophils. Furthermore, endothelial cells activated by IL-17A can release CXCL1 and CXCL2 that activate and recruit neutrophils. Major IL-17A producing cells are γδ T cells, CD4\textsuperscript{+} Th17 and natural killer T (NKT) cells\textsuperscript{236}. Therefore, the number of IL-17 producing cells in the lungs were examined. There were significantly (p=0.0079) elevated numbers of Th17 cells 72
Figure 3.8: CXCL2 administration to HDM- exposed group reduces blood neutrophil levels. The frequency of (A) blood and (B) bone marrow neutrophils were determined by flow cytometry. Data presented as box and whisker plots showing the median of n=5 per group in 2 independent experiments. *P<0.05; ** P<0.01.
Figure 3.9: Airway cytokine and chemokines levels after *Streptococcus pneumoniae* infection. Quantification of (A) TNF, (B) IL-6, (C) CXCL1 (D) CXCL2 and (E) MMP-9 in the bronchoalveolar lavage (BAL) fluid by ELISA. Data presented are the means ± SD  *P*<0.05;  **P**<0.01;  ***P***<0.001. (F) Immunohistology of formalin fixed lung tissue for CXCL1. Brown coloured stain shows CXCL1 on the lung sections detected by HRP-DAB and examined by light microscopy (original magnification x 40) and is representative of the lungs from 5 mice per group.
hours after the final HDM exposure compared to PBS controls that wanes down to PBS levels 120 hours after the final exposure (Fig. 3.10A). However, after *S. pneumoniae* infection there was no difference in Th17 cells in the lungs between the two groups. IL-17 producing γδ T cells were similarly high in HDM exposed mice (72h after final exposure) and reduced to PBS levels by 120 hours in the absence of infection or allergen (Fig. 3.10B). Similar to Th17 cells, there was no difference in IL-17+ γδ T cells 48 hours after infection. This suggests that impairment in IL-17 production from CD4+ T cells or γδ T cells is not responsible for the impaired neutrophil recruitment and subsequent bacterial complications in HDM exposed group. There was no difference in total IL-17 secretion examined by ELISA between PBS and HDM exposed mice (Fig. 3.11).

### 3.2.7 Reduced pro-inflammatory receptor expression on key anti-bacterial immune cells

The expression of TLRs on key innate immune cells such as macrophages (airway CD11c+TLR2+) and neutrophils (airway CD11b+Ly6G+TLR2+) were investigated. In particular, we analysed TLR2, as it is an important PRR in gram-positive bacterial infections. HDM exposure significantly reduced TLR2 expression measured as frequency of CD11c+ macrophages and CD11b+Ly6G+ neutrophils in the airways when analysed 72 hours after final HDM exposure (Fig. 3.12A-F). Even following *S. pneumoniae* infection (48 hours), HDM exposure significantly reduced TLR2 expression compared to PBS controls. The percentage of airway neutrophils (p=0.0079) and alveolar macrophages (p=0.0317) expressing TLR2 were also significantly reduced in the HDM exposed group compared to PBS controls 48 hours after infection. However, at 96 hours, TLR2 expression was similar between the PBS and HDM groups and had returned to levels prior to infection (Fig. 3.12A & B).

Another important innate immune receptor on neutrophils is TREM1 that modulates the innate response by amplifying TLR-induced cytokine secretion for better anti-microbial immunity(215). HDM exposed group had a significantly reduced percentage (Fig. 3.12G) and total number (Fig. 3.12H) of TREM1+ neutrophils in the airways at 78 (p=0.0079) and 96 (p=0.0159) hours after the final HDM challenge.
Figure 3.10: Elevated IL-17A producing CD4+ and γδ TCR expressing T cells in the HDM-exposed airways compared to PBS controls. 48 hours after *Streptococcus pneumoniae* infection the number of IL-17A producing (A) CD4+ and (B) γδ TCR expressing T cells were determined in lung by flow cytometry. n=5 per group. *P<0.05; **P<0.01.
Figure 3.11: IL-17A levels in the airways of HDM-exposed airways compared to PBS controls. HDM-exposed and PBS control mice were infected with *S. pneumoniae* and at the time points shown BAL performed. IL-17A (pg/ml) levels in BAL supernatant were measured by ELISA after *S. pneumonia* infection.
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.
Figure 3.12: Reduced expression of pathogen recognition receptors (PRRs) on macrophages and neutrophils in allergic mice compared to PBS controls. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and harvested on 72 hours post final HDM exposure. Expression of surface TLR2 involved in bacterial recognition on (A, B) airway neutrophils and (C, D) CD11c+ airway macrophages is shown as percentage of cells and representative histograms and was determined by flow cytometry. Total cell counts of TLR2 positive (E) neutrophils and (F) CD11c+ macrophages in the airways were calculated from total viable cells. (G) Percentage of total airway cells that are Ly6G+TREM1+ and (H) total cells number of Ly6G+TREM1+ airway cells. Data presented as box and whisker plots with the median of n = 5 in 2 independent experiments. *P<0.05; ** P<0.01.
Dead cells were excluded using a live/dead marker. In contrast, TREM2, mainly expressed on macrophages, inhibits TLR-induced cytokine production. The percentage of airway macrophages identified as CD11c+ cells that expressed TREM2 was significantly higher in HDM exposed airways than PBS controls measured three days after the final HDM exposure ($p=0.0079$) (Fig. 3.13A & B).

Inhibition of TLR responsiveness can also occur by the up-regulation of receptors that recruit similar adapter molecules to initiate signalling. In this respect it is interesting that the IL-33 receptor, ST2L (associated with development of allergic airway disease) that recruits the TLR signalling adapter myeloid differentiation factor 88 (MyD88) is raised on CD11c+ airway macrophages (Fig. 3.13C) together with the negative regulator CD200R (Fig. 3.13D) in the HDM-sensitised lung at the onset of bacterial infection.

Additionally, analysis of intracellular TLR negative regulators (A20, Tollip, IRAK-M and Triad3A) by real time PCR revealed a trend of increased mRNA transcripts of these inhibitors in HDM exposed lungs compared to PBS controls (Fig. 3.14). Therefore, the TLR responsiveness of airway macrophages and neutrophils is inhibited on many levels.

Furthermore, anti-microbial peptides were comparable in airway lavage of PBS and HDM exposed mice. A recently identified antimicrobial protein SPLUNC1 produced by epithelial cells has an important role in M. pneumoniae clearance, but is reduced in mouse models of allergic inflammation. However, reduced levels of SPLUNC1 were not detected in the HDM exposed airway fluid (in fact there seems to be more in HDM airway fluid, Fig. 3.15A) and only a partial decrease in CRP that opsonises S. pneumoniae for opsonophagocytosis in HDM-sensitised mice (Fig. 3.15B).
Figure 3.13: Increased expression of regulatory receptors on macrophages (CD11c+ cells) in allergic mice compared to PBS controls. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and harvested on 72 hours post final HDM exposure. (A) Percentage and (B) total cell count of TREM2 positive alveolar macrophages (CD11c+) 78 hours after final HDM exposure. Percent of CD11c+ macrophages expressing (C) ST2L or (D) CD200R was determined by flow cytometry. Data presented as box and whisker plots with the median of n=5 in 2 independent experiments. *P<0.05, ** P<0.01.
Figure 3.14: Expression of TLR negative regulators in whole lung of allergic mice were compared to PBS controls. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and harvested on 72 hours post final HDM exposure. The level of different RNA transcripts, as indicated, was assessed at this time point by real-time PCR. Results are expressed as the ratio between the gene of interest and a PBS control sample.
Figure 3.15: Antimicrobial substances in HDM-sensitised group after bacterial infection. HDM-exposed and control mice were infected with *S. pneumoniae* and (A) short palate, lung and nasal epithelium clone 1 (SPLUNC1) 6 hours after infection detected by western blotting. Data representative of 2 independent experiments of n=2-3 per group. (B) C-reactive protein (CRP) levels were measured by ELISA. Data is presented as box and whisker plots showing the median of n=5 per group in 2 independent experiments. *P<0.05.
3.2.8 Exacerbation of bacterial infection wanes without re-exposure to allergen

In order to determine whether the timing of bacterial exposure is critical, HDM was given on alternate days for 3 weeks and 3, 7, 10 or 14 days after the last HDM exposure infected with *S. pneumoniae* (Fig. 3.16A). Invasion of lung tissue with bacteria was lost after an absence of HDM for 7 days. The proportion of lungs harbouring bacteria at 48 hours after *S. pneumoniae* infection also reduced after day 3 of an absence of allergen, but never reached the low level shown in PBS exposed mice (Fig. 3.16B). The improvement with increasing time post allergen exposure was associated with a return in the ability to recruit neutrophils to the airspaces (Fig. 3.16C) and a decrease in eosinophils (Fig. 3.16D). This suggests that allergen exposure causes increased susceptibility to invasive bacterial infection within a specific time window but that some remain susceptible for prolonged periods of time, and that allergen load may be a factor in determining the seriousness of concomitant bacterial infection.
Chapter 3: Defective anti-pneumococcal immunity in the allergic lung.

A

Allergen exposure

Week 1 | Week 2 | Week 3 | Day 3 | Day 7 | Day 10 | Day 14

15 µg: HDM | 72h | Tissue harvest and analysis 48h after infection

S. pn Infection 1x10^6 CFU

B

% of mice with bacteria

Days post final HDM exposure

3 | 7 | 10 | 14

CFU

BAL | Lung

C

Ly6G^+

% of airway CD11b^+ cells

Days post final HDM exposure

D

SiglecF^+

% of airway CD11b^+ cells

Days post final HDM exposure

*
Figure 3.16: The percentage of lungs with bacteria reduces and the numbers of neutrophils in the airways of HDM/Streptococcus pneumoniae challenged group increases as HDM exposure wanes. (A) Experimental design: Five BALB/c mice per group were inoculated intranasal with 15µg of house dust mite (HDM) three times a week for 3 weeks and subsequently infected on days 3, 7, 10 and 14 after final house dust mite (HDM) administration and harvested 48 hours after infection. (B) The percentage of with bacteria reduces as HDM exposure wanes. (C) The frequency of CD11b\(^+\)Ly6G\(^+\) neutrophils and (D) CD11b\(^+\)SiglecF\(^+\) in the airways were determined by flow cytometry. The dotted line illustrates PBS/S. pneumonia infected group harvested 48 hours after infection (baseline). Data presented as box and whisker plots with the median of n=5 per group in two independent experiments. *P<0.05.
3.3 Discussion

In this chapter we focused on susceptibility of the allergic lung to *S. pneumonia* infection. We show for the first time that HDM exposed mice have increased susceptibility to *S. pneumonia*, that the molecular pathways leading to the production of neutrophil chemoattractants in the lung are compromised and that despite the complexity of anti-bacterial pathways that are disrupted, the re-introduction of neutrophils by i.n administration of neutrophil chemoattractants to lungs with allergic airway disease enables clearance of *S. pneumoniae* that would otherwise prove fatal.

3.3.1 Allergen exposure increases susceptibility to respiratory bacterial infections

In our study the effect of HDM exposure prior to bacterial infection was analysed. Intranasal inoculation of HDM exposed mice with *S. pneumonia* resulted in significantly increased bacterial titres in BAL and lung compared to PBS exposed controls, suggesting increased replication of *S. pneumonia* or a reduced clearance of the bacteria from the allergic lung. Furthermore, the severity of disease was so high in the HDM exposed mice that some mice were sacrificed 3 days after infection.

Neutrophils are essential to eliminate *S. pneumonia*. However, the findings in this chapter show that HDM exposed lungs are unable to recruit as many neutrophils as the PBS controls after bacterial infection. Rolling and attachment of neutrophils to sites of infection is determined by adhesion molecule (selectins and integrins) expression on endothelial cells that increases in response to TNF-α, IL-1β and IL-17. The effect of TNF-α is essential during early stages of infection in recruiting neutrophils and enhancing anti-bacterial cytokine secretion. A number of studies have shown blocking/neutralising TNF-α to be detrimental in *S. pneumonia* infection. Analysis of most of these chemokines and cytokines in the airways revealed significantly reduced levels after bacterial infection in HDM exposed compared to controls. Therefore, the signals needed to attract neutrophils are impaired in allergic airways. To support this Zhao *et al* show reduced production of IL-17 in OVA sensitised/challenged lungs compared to controls after *S. pneumoniae*
serotype-3 infection\textsuperscript{242}. When the impaired chemotactic signal was restored by administering intranasal CXCL1 or CXCL2, the impaired neutrophil recruitment diminished and the bacterial clearance improved considerably.

Most MMPs degrade proteins of the extracellular matrix (ECM). However, MMP-9 can also break down a variety of non-ECM proteins such as chemokines and myelin basic protein\textsuperscript{243}. Furthermore, MMP-9 cleaves IL-8 into a form that is 10-fold more potent\textsuperscript{243,244} and activates TNFα. Therefore the reduced neutrophil recruitment may not only be due to reduced CXCL1, but also a reduction in this more active form due to alterations in MMP-9 activity. MMP-9 deficiency reduces systemic bacterial clearance\textsuperscript{245}. The role of MMP-9 in asthma is controversial with airway and sputum levels noted as elevated in asthmatic patients\textsuperscript{246} that is mimicked in vivo models where MMP-9 inhibition caused reduced airway hyperresponsiveness\textsuperscript{247}. However, MMP-9/-/- show enhanced allergen-induced airway inflammation\textsuperscript{248}. Moreover, the role of MMP-9 has not been studied in HDM model of allergic airway disease. Therefore, the proteolytic cleavage of CXCL1 (murine homologue of human IL-8) to a more biologically active one may be defective in the HDM exposed lung as a result of reduced MMP-9 (and/or other chemokine endopeptidases).

The airway epithelium and alveolar macrophages are the first line of host defense against environmental antigens. Airway epithelial cells recognise invading microbes such as \textit{S. pneumonia} and subsequently produce a range of mediators such as mucins to trap the pathogens for mucociliary clearance, cytokines and antimicrobial peptides (β-defensins and cathelicidin) and proteins (lactoferrin). A recently identified antimicrobial protein SPLUNC1 produced by epithelial cells has an important role in \textit{M. pneumonias}e clearance, but is reduced in mouse models of allergic inflammation\textsuperscript{166,237}. However, there was no reduction in anti-microbial peptide (SPLUNC1) in airway lavage and only a partial decrease in CRP in HDM-sensitised mice. In future experiments, CRAMP and defensins should be analysed in the airway fluid after HDM exposure with or without bacterial infection. We identify that airway macrophages seem to have a suppressed phenotype with increased expression of regulatory receptors such as the myeloid negative regulator CD200R and ST2L (IL33 receptor) that inhibits the MAL component of the TLR signalling pathway (further analysed in Chapters 4 and 5).
3.3.2 Reduced PRR expression on innate immune cells

PRRs provide early recognition and initiate the inflammatory response to infection. Several TLRs are implicated in the host response to *S. pneumonia* but, most importantly, TLR2 that recognises LTA on gram-positive bacteria amongst others. In models of pneumococcal meningitis, TLR2-/- show increased disease severity and higher bacterial titres compared to wild-type controls. Similarly, in a nasopharyngeal carriage model, TLR2-/- show impaired clearance of bacteria. Pneumococcal pneumolysin is detected by TLR4. Overall, TLR4 may play a limited role in defense against *S. pneumoniae* as some studies show no difference between WT and TLR4-/-. MyD88 is an important signalling adaptor protein during TLR signalling and MyD88 deficiency increase susceptibility to *S. pneumonia*.

Heightened susceptibility of aged mice (19-20 months) compared to young mice (4-5 months) is attributed to reduced TLR 1, 2 and 4 protein levels in the lungs together with reduced NF-κB activation and reduced pro-inflammatory cytokines, TNF-α and IL-6, production. Furthermore, IL-6 production is predominately dependent on TLR2. Alveolar macrophages have a significant reduction in TLR2 expression in HDM exposed mice compared to controls, as well as an increased gene expression of TLR negative regulators- Tollip, A20, IRAKM and TRIAD3A (an E3 ubiquitin-protein ligase that enhances ubiquitination and proteolytic degradation of some TLRs though it has not been shown to act on TLR2), in whole lung homogenate (Fig. 3.17). Collectively, TLR signalling cascades in the allergic lung are impaired in response to infection compared to non-allergic lungs.
Figure 3.17: TLR2 signalling pathway leading to activation of NF-κB associated cytokine genes. TLR negative regulators (A20, Tollip, IRAK-M and A20) can inhibit this pathway at multiple sites.

Not only was TLR2 downregulated but also TREM-1 (Triggering receptor expressed on myeloid cells-1) that is considered to be a potent amplifier of the inflammatory response to invading microbes. TREM-1 has a vital role in accelerating the induction of the early immune response to S. pneumoniae resulting in augmented bacterial clearance and improved survival\(^{260}\).

To control excessive inflammation our immune system as a number of receptors that act as ‘off’ switches such as TREM2 expressed on macrophages, which we show is
increased. *In vitro* studies show increased TLR responsiveness and cytokine production in TREM2/- or bone marrow derived macrophages silenced for TREM2 using short hairpin RNAi\(^{261}\). Therefore, TREM1 is a positive regulator of cytokine synthesis and TLR activation, while TREM2 is a negative regulator. The allergic lung may cause TLR desensitisation as it has high levels of IL-4 that increases TREM2 expression on airway macrophages\(^{187}\).

Our results show that the allergen-exposed lung is defective in its ability to respond to bacteria (Fig. 3.18). This culminates in an inability to recruit neutrophils and ultimately results in bacterial invasion and bacteraemia. Furthermore, the reprogramming of the lung innate immunity after allergen exposure persists for prolonged periods of time.

**Figure 3.18:** The attenuated innate immune response to infection in allergic-HDM exposed lungs explored in this chapter. In our studies so far we have shown that HDM exposed lungs produce reduced levels of neutrophil chemoattractants such as KC/CXCL1, IL-6 and TNF (mainly produced by alveolar macrophages and airway epithelial cells) to recruit neutrophils. Furthermore, there is reduced expression of pathogen recognition receptors on neutrophils and alveolar macrophages. Collectively, they reduce the threshold of pathogen detection in the airways and lungs.
CHAPTER 4

Probing macrophage dysfunction in the allergic lung.
CHAPTER 4

4.1 Introduction

In the airways, epithelial cells together with alveolar macrophages are the first cells to interact with allergens and respiratory pathogens. Macrophages play a crucial role in innate and adaptive immunity in response to microorganisms and are major mediators of the inflammatory response. Alveolar macrophages maintain lung tissue homeostasis and scavenge pathogens and debris. The last chapter showed that bacterial infection was exacerbated in the allergic lung and further analysis implied a substantial affect of airway macrophages.

4.1.1 Macrophages in allergic airways disease

Allergic airways disease is more robust in the absence of alveolar macrophages-depletion of alveolar macrophages in OVA-sensitised lungs before OVA challenge results in increased AHR and pulmonary eosinophilia suggesting they play an anti-allergic role in this specific model. Furthermore, allergen-sensitised alveolar macrophages show a reduced capacity for phagocytosis and, consequently, diminished production of anti-inflammatory mediators such as TGF-β and prostaglandin E2 (normally released when alveolar macrophages phagocytose apoptotic cells).

In response to the microenvironment macrophages can assume a number of different phenotypes and roles. In a Th2 rich cytokine (IL-4 and IL-13) environment macrophages become alternatively activated, however, IL-4Rα-induced AAMs do not play a role in protection against allergic airway disease. HDM challenge induces higher numbers of AAMs which correlates with higher eosinophil numbers. In addition HDM exposure of the rat alveolar macrophage cell line (NR8383) liberates pro-inflammatory mediators such as nitric oxide. This pro-inflammatory phenotype is also observed on Dermatophagoides farinae exposure that causes NF-κB activation in alveolar macrophages and upregulation of IL-6, TNFα and nitric oxide. In a recent paper, resident alveolar macrophages are shown to gain pro-inflammatory function after OVA sensitisation and challenge but have reduced...
phagocytic capacity and increased production of cytokines and chemokines in response to TLR3 (poly I:C), TLR4 (LPS) and TLR7 (imiquimod) ligands\textsuperscript{268}. This reduced phagocytic activity in inflammation is interesting since influenza infection also desensitises alveolar macrophages to TLR ligands\textsuperscript{53}. This suggests that generic processes, rather than antigen specific influences may be responsible. Since bacterial phagocytosis, phagolysosomal destruction and/or recruit inflammatory cells to the site of infection using chemokines and acute phase proteins\textsuperscript{269} are critical for anti-bacterial immunity, subsequently alveolar macrophages were examined in more detail.

4.1.2 Regulation of immune cells by Micro RNAs

A number of studies show miRNAs can regulate TLR signalling\textsuperscript{270}. miRNAs are short double-stranded RNA molecules approximately 22 nucleotides long and repress the translation of target mRNAs\textsuperscript{270,271}. The RNA silencing depends on sequence specific interaction between the target RNA molecule and a small RNA incorporated into the RNA-induced silencing complex\textsuperscript{272}. The binding of this complex inhibits the expression of mRNA either through degradation (removal of the polyA tail of target mRNA) or blockade of translation. Many miRNAs target cell-signalling pathways.

4.1.3 Hypothesis and Aims

Data presented in the earlier chapter show that neutrophil recruitment is defective in the airways of \textit{S. pneumonia} infected allergic mice compared to non-allergic controls possibly due to reduced neutrophil recruiting cytokines and chemokines. As one of the first cells to contact airborne bacteria, alveolar macrophages are essential in initiating an effective anti-bacterial response including neutrophil recruitment. Therefore, the next aim was to determine the anti-bacterial capacity of resident alveolar macrophages in the HDM exposed airways. The specific aims are:

1. To define the phenotype of resident alveolar macrophages.
2. To examine the response of alveolar macrophages to bacterial components (TLR agonists).
4.2 Results

4.2.1 Reduced airway neutrophil levels after TLR stimulation in vivo

The ability of the allergic airway to support neutrophil migration in response to specific TLR stimulation was assessed. Administration of TLR2 (LTA), 4 (LPS) and 5 (flagellin) ligands (Fig. 4.1A) induced significantly increased cellular infiltrate into the airways (Fig. 4.1B) and lung (Fig. 4.1C) of mice exposed to PBS and especially HDM. However, the percentage of infiltrating (CD11b+) cells that were Ly6G+ neutrophils was reduced in the airways and lung of allergen exposed mice compared with PBS controls (Fig. 4.1D & E). The ratio of neutrophils to eosinophils was similar after flagellin (Fig. 4.2B) challenge between the PBS and HDM exposed groups. However, after LPS and LTA (Fig. 4.2C & D) challenge, the ratio of neutrophils was considerably higher than eosinophils. Cytokine and chemokine levels in the airspaces of HDM-exposed mice were compared to PBS controls following TLR challenge. Cytokines, IL-6 and TNFα were not detectable at 16 hours after TLR agonist challenge (data not shown). The neutrophil chemoattractant CXCL2 was also undetectable at the time point tested (the chemokine levels may have been detectable at earlier timepoints after TLR stimulation); while, CXCL1 was detected mainly in the HDM exposed airways (Fig. 4.3). Within the PBS groups, CXCL1 was significantly elevated in response to LPS compared to no agonist PBS group. These data together with our previous work on reduced neutrophil recruitment following *Streptococcus pneumoniae* infection in the allergic airways show reduced response to bacterial pathogen recognition receptors.

4.2.2 HDM exposure changes resident alveolar macrophage

Alveolar macrophages can initiate the recruitment of neutrophils via secretion of relevant chemokines. Therefore, alveolar macrophages were examined in more detail. Alveolar macrophages express high levels of the integrin CD11c (that is not expressed by other macrophages)\(^{273}\) and low levels of CD11b. The longevity of alveolar macrophages was assessed by i.n. administration of a dye PKH26-PCL (Sigma) that is ingested by phagocytic cells in the airways (Fig. 4.4A). The dye was
Figure 4.1: Neutrophil recruitment in response to in vivo stimulation of TLR2, TLR4 and TLR5 ligands. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and 72 hours post final HDM exposure 10µg LPS, 50µg LTA and 2µg Flagellin was administered. (A) Illustration of HDM and TLR agonist administration protocol. Total (B) airway and (C) lung cells were enumerated before and after TLR agonist addition. Percentage of Ly6G+ CD11b+ CD11c- neutrophils was assessed by flow cytometry in the (D) airway and (E) lungs. Data presented as box and whisker plots with the median of n=5 per group in two independent experiments. *P<0.05.
Figure 4.2: Proportion of neutrophils and eosinophils in response to *in vivo* stimulation of TLR2, TLR4 and TLR5 ligands. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and 72 hours post final HDM exposure 10µg LPS, 50µg LTA and 2µg Flagellin. (A-D) shows the proportion of neutrophils and eosinophils between PBS and HDM groups in the airways. Data presented as mean±SEM of n=5 per group. *P<0.05.
Figure 4.3: CXCL1 levels in response to in vivo stimulation of TLR2, TLR4 and TLR5 ligands in the airways. BALB/c mice were inoculated intranasal with 15µg of house dust mite (HDM) extract in 15µl phosphate-buffered saline (PBS) or 15µl PBS alone three times a week for 3 weeks and 72 hours post final HDM exposure 10µg LPS, 50µg LTA and 2µg Flagellin. CXCL1 levels in the airways. Data presented as box and whisker plots with the median of n=5 per group. Significance compared to PBS group with TLR agonist. *P<0.05, ** P<0.01.
Chapter 4: Probing macrophage dysfunction in the allergic lung.

A. Allergen exposure

Week 1  Week 2  Week 3
72h  15 μg: HDM  72h
15 μl: PBS

B. 50 μl of 10 μM PKH26 PCL Sigma Dye
Tissue harvest and analysis

Dye+ (PKH26PCL)

C. PBS airway

HDM airway

D. Dye+CD11c+ airway cells

Control  PBS  HDM

F4/80+
**Figure 4.4: In vivo labelling of resident alveolar macrophages.** PKH26-PCL (Sigma) labels cells with phagocytic capabilities through ingestion of dye micro-aggregates. (A) We administered dye to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then exposed the mice to three weeks of 15µg HDM or 15µl PBS (3 times a week). (B) Flow cytometry plots show uptake of dye at the time of the first HDM exposure (72 hours after dye inoculation). (C) The gating strategy used to analyse PKH26-PCL dye+ airway cells and the majority of these cells are (D) F4/80+. Data presented as representative flow cytometry plots.
given 72 hours before the start of HDM exposure. PKH26-PCL administration allowed us to track the phenotype of this starting population following subsequent exposure to allergen (Fig. 4.4A). 75% of CD11c+ airway cells were dye+ at the start of HDM exposure (Fig. 4.4B). A comparable percentage of dye+ macrophages were evident following three weeks of HDM exposure (Fig. 4.4C) that were also F4/80+ (Fig. 4.4D). The majority of dye+ CD11c+ cells (98%) were CD11b- (Fig. 4.5A). About 20% of airway CD11c+ macrophages did not take up dye at the start of the experiment and a comparable percentage was present after three weeks of HDM exposure. These dye-CD11c+ airway cells showed a similar phenotype as the dye+ cells (upregulated regulatory receptors and alternatively activated markers). This data suggests that airway macrophages are long lived and are either non-migratory or return to the airspaces rapidly. Therefore, macrophages that ingested the PKH26-PCL dye before the start of HDM exposure (in a naïve airway) were the resident alveolar macrophages (PKH26-PCL dye+ and CD11c+). Both PBS and HDM exposed mice had comparable numbers of dye+ alveolar macrophages (Fig. 4.5B). Although, the percentage of airway myeloid cells that were CD11c+Dye+ reduced after HDM exposure as a result of increased cellular infiltrate following allergen exposure. HDM exposure increased the percentage (Fig. 4.6 left), geomean (Fig. 4.6 middle) and total numbers (Fig. 4.6 right) of ST2L (IL-33R or T1/ST2) and Triggering Receptor Expressed on Myeloid Cells (TREM2) on dye positive macrophages that are associated with decreased TLR responses. Therefore, an increase in these regulatory receptors indicates an altered phenotype of macrophages after HDM exposure. Messenger RNA analysis of the sorted dye+CD11c+ alveolar macrophage after 3 weeks of HDM exposure also highlighted increased levels of arginase I, resistin-like molecule alpha (RELMα) and YM1, expression of which are characteristic of AAM phenotype (Fig. 4.7A). In addition, RELMα and YM1 proteins were only present in the allergic lung when assessed by immunohistochemistry (Fig. 4.7B). Thus, alveolar macrophages in the HDM-exposed lung are altered in phenotype towards an alternatively activated or wound healing phenotype.
Figure 4.5: Total dye positive resident alveolar macrophages. PKH26-PCL (Sigma) dye micro-aggregates were intranasal inoculated to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then mice were exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). (A) Flow cytometry plot to show dye+CD11c+ airway macrophages are CD11b-.

(B) Percentage of dye+ airway myeloid cells and total airway cells was determined by flow cytometry. Data presented as box and whisker plots with the median of n=5 per group in two independent experiments. *P<0.05.
Figure 4.6: Regulatory receptors T1/ST2 and TREM2 expression on CD11c+Dye+ and CD11c+Dye- airway cells. PKH26-PCL (Sigma) dye was given (intranasal) to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then exposed mice to three weeks of 15µg HDM or 15µl PBS (3 times a week). Flow cytometry was used to analyse T1/ST2 and TREM2 expression on dye positive and negative alveolar macrophages. Data presented as box and whisker plots with the median of n=5 per group in two independent experiments. *P<0.05.
Figure 4.7: Analysis of alternatively activated markers RELMα, YM1 and Arg1 in CD11c+dye+ airway cells. PKH26-PCL (Sigma) dye that stains only phagocytic cells was intranasal inoculated to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and mice then exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). The alternatively activated markers were measured by (A) real-time PCR and (B) immunohistochemistry. Data presented as box and whisker plots with the median of n=5 per group in two independent experiments. *P<0.05, ** P<0.01.
4.2.3 Restoration of naïve alveolar macrophages restores anti-bacterial immunity in the allergic airway

The next step was to assess whether administering naïve alveolar macrophages to the allergic lung would restore the anti-bacterial immune response. Alveolar macrophages express high levels of CD11c and low expression of CD11b compared to other lung macrophages\textsuperscript{273}. \(1 \times 10^6\) CD11c+CD11b- alveolar macrophages from naïve or HDM exposed lungs were purified by flow sorting and transferred intranasal to allergic mice 48h after the final HDM exposure and 18h before \textit{Streptococcus pneumonia} infection (Fig. 4.8A). The allergic lungs that received alveolar macrophage from age matched naïve mice performed better in removing bacteria from the airways and lung compared to allergic lungs that received HDM-exposed allergic alveolar macrophages or allergic lungs without cell transfer (Fig. 4.8B-C). Furthermore, allergic mice receiving naïve alveolar macrophages had more airway neutrophils (Fig. 4.8D & E) following \textit{S. pneumoniae} challenge. A similar trend was seen when neutrophil recruitment was assessed 6 hours after infection, although the bacterial titres at this time point was not significantly different between the groups.
Figure 4.8: Transfer of naïve CD11c+CD11b- alveolar macrophages restores bacterial clearance in HDM exposed mice. (A) BALB/c mice were exposed to 15µg of HDM three times a week for three weeks. 48 hours after the final HDM exposure, allergen exposed mice were given intranasal 1x10^5 FACS sorted CD11c+CD11b- airway macrophages either from naïve or allergen exposed lungs. 18 hours after the cell transfer mice were infected with 1x10^6 CFUs of *Streptococcus pneumoniae* strain D39 (B) Bacterial titres in the airways, lung and blood of the infected groups were determined by plating out respective issues. (C) Percentage of mice with bacteria in the different organs. Airway neutrophils (CD11b^+^CD11c^+^Ly6G^-^SiglecF^-) as (D) percentage of live airway cells and (E) total airway cell numbers. Figure includes data from two independent experiments.
4.2.4 Responsiveness of alveolar macrophages to TLR agonists ex vivo

The next aim was to examine whether the reduced responsiveness of airway macrophages persisted ex vivo. Isolation of HDM exposed CD11c+CD11b- airway macrophages stimulated with LTA or LPS in vitro released significantly less TNF-α compared to controls (Fig. 4.9A). In addition, isolation of live dye+CD11c+ alveolar macrophages after three weeks of HDM exposure and ex vivo incubation with a range of TLR agonists, showed a similar reduction in TNFα to CD11c+DC11b- (Fig. 4.9B), most significantly in response to flagellin (TLR5) and LPS (TLR4) stimulation. Similarly, lower levels of neutrophil chemoattractant protein, CXCL2 were released (Fig. 4.9C). CXCL1 was only decreased in response to bacterial flagellin (TLR5) (Fig. 4.9D).

We next assessed in vitro whether it was the HDM or the cytokine milieu in the airway lavage that causes the changes in airway macrophages. To test this 0.5x10^6 naïve airway cells (>90% are alveolar macrophages) were exposed to 200µl of 5µg/ml HDM, 100µl media + 100µl BAL fluid from lungs exposed to PBS for 3 weeks, 100µl media + 100µl BAL fluid from lungs exposed to HDM for 3 weeks or media alone overnight. Responses to TLR agonists 1µg/ml LTA (TLR2), 1µg/ml flagellin (TLR5) or 0.1µg/ml LPS (TLR4) were then assessed.

Incubating naïve alveolar macrophages with HDM in vitro increased the production of TNFα after TLR 2, 4 and 5 stimulation compared to no HDM addition (Fig. 4.10). However, addition of BAL fluid from lungs exposed to PBS or HDM for 3 weeks, inhibited TNFα production (Fig. 4.10). This could be a result of the airways washed in HBSS with EDTA. As Hanks’ Salts are mainly used in media exposed to atmospheric condition as opposed to CO₂ incubation, incubating cells media with HBSS may exceed Hanks’ Salts in the buffer in a CO₂ environment and may result in cell death. To avoid these issues the experiment was repeated with the airways washed with PBS with no HBSS and EDTA (EDTA can detach adherent cells). Data from this experiment shows no difference in TNF response between cells exposed to airway fluid from PBS or HDM exposed mice after LPS or LTA stimulation (Fig. 4.11).
Figure 4.9: TLR stimulation of sorted dye+ airway cells *ex vivo*. PKH26-PCL (Sigma) dye that stains only phagocytic cells was given (intranasal) to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then exposed the lungs to three weeks of 15µg HDM or 15µl PBS (3 times a week). (A) FACS sorted 0.42x10^5 CD11c+CD11b- cells were stimulated with 1µg/ml LTA (TLR2) or LPS (TLR4) and TNFα was measured in the supernatant. (B) TNFα, (C) CXCL2 and (D) CXCL1 were measured in response to 0.3x10^5 dye+ cells that were stimulated with 1µg/ml LPS (TLR4), LTA (TLR2), FLAG (flagellin, TLR5) or Pam3CSK4 (TLR1/2) agonists for 16-18 hours. White bars=PBS and Grey bars=HDM exposed alveolar macrophages *in vivo*. Data presented as mean+/−SD representative of two independent experiments. *P<0.05, ** P<0.01.
Figure 4.10: TNF-α levels released by alveolar macrophages in vitro. Airways of naïve lungs were washed multiple times and red blood cell lysed. 0.5x10^6 cells were plated on a 96-well plate. Cells were incubated for 2 hours in 5% CO₂ at 37°C (for alveolar macrophages to stick to the plate). 2 hours later supernatants were removed and fresh media, media+5µg/ml house dust mite, media+bronchoalveolar lavage (BAL) washed in HBSS+EDTA from lungs exposed to PBS for 3 weeks or media+BAL from lungs exposed to HDM for 3 weeks were added to wells and incubated O/N. Subsequently, supernatants were removed and fresh media with or without TLR agonists (1µg/ml LTA (TLR2), 1µg/ml Flagellin (TLR5) or 0.1µg/ml LPS (TLR4)) were added and incubated for 4 hours. TNF-α was measured by ELISA. Data presented as mean with SD.
Figure 4.11: TNF-α production of naïve alveolar macrophages in response to TLR stimulation after HDM, IL-4 or IL-33 incubation. Airways of naïve lungs were washed multiple times and red blood cell lysed. 0.5x10^6 cells were plated on a 96-well plate. Cells were incubated for 2 hours (for alveolar macrophages to stick to the plate) in 5% CO₂ at 37°C. 2 hours later supernatants were removed and fresh media alone, 5µg/ml house dust mite, 20ng/ml recombinant IL-4, 20ng/ml recombinant IL-33, 100µl media+100µl bronchoalveolar lavage (BAL) washed in PBS from lungs exposed to PBS for 3 weeks or media+BAL from lungs exposed to HDM for 3 weeks were added to cells and incubated O/N. Subsequently, supernatants were removed and fresh media with or without TLR agonists: (A) 0.1µg/ml LPS (TLR4) or (B) 1µg/ml LTA (TLR2) were added and incubated for 4 hours. TNF-α levels in the supernatant were measured by ELISA. Data presented as Mean with SD. *P<0.05, ** P<0.01, *** P<0.001 using unpaired Student’s t test. All significances are to media only control (plus TLR stimulation).
Cytokines IL-4 and IL-33 are indicative of a Th2 response in the lungs\textsuperscript{120}. Therefore, the next investigation was to examine whether addition of these cytokines to naïve alveolar macrophages induced changes that would result in lower production of TNFα to TLR stimulation. Overnight incubation of HDM or IL-4 induced significantly increased release of TNFα when TLR2 was stimulated by addition of LTA compared to media alone control or IL-33 incubation (Fig. 4.11B). No difference was seen between IL-33 and media alone control after LTA addition (Fig. 4.11B). Adding LPS (Fig. 4.11A) to cells incubated with IL-4 had considerably higher TNFα levels compared to media alone, HDM or IL-33 groups. IL-33 stimulated cells produced similar levels of TNFα as media alone control after both LPS and LTA. TNFα levels after overnight HDM and cytokine stimulation were undetectable without TLR stimulation. Therefore, the conclusion was that cytokines dominant in HDM-induced allergic airway disease do not individually account for the altered airway macrophage phenotype.

4.2.5 Regulation of alveolar macrophages by regulators of TLR responses

An increase in TLR negative regulator mRNA transcripts were observed in macrophages taken from the HDM exposed airways relative to PBS controls (tested in FACS sorted resident alveolar macrophages) comparable to increased TLR negative regulators seen in the whole lung after HDM exposure (chapter 3) (Fig. 4.12). In particular, the TLR negative regulator A20 that targets TNF-receptor associated factor 6 (TRAF-6)\textsuperscript{274} was significantly increased in alveolar macrophages sorted by flow cytometry (Fig. 4.12A). TRAF-6 is an important intracellular component of TLR signalling pathway. A reduced response to TLR agonists was not explained by a reduction in mRNA transcript levels for TLR 2, 3 and 4 in HDM-exposed alveolar macrophage; TLR9 was in fact significantly upregulated (Fig. 4.12B).

TRAF-6 and Interleukin-1 receptor associated kinase 1 (IRAK-1) are regulated by the microRNA, mmu-miR-146b-5p\textsuperscript{187} that was up-regulated in dye+ alveolar macrophages exposed to HDM compared to PBS when normalised to snoRNA 202 and a PBS control sample (Fig. 4.13A). However, there was no difference in the
Figure 4.12: TLR and TLR negative regulators analysis of dye+ alveolar macrophages by real-time PCR. PKH26-PCL (Sigma) dye that stains only phagocytic cells was given (intranasal) to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then exposed the mice to three weeks of 15µg HDM or 15µl PBS (3 times a week). (A) TLR negative regulators Irak-M, A20, Tollip and Triad-3a and (B) TLR-2, 3, 4 and 9 mRNA levels are expressed relative to 18S and a PBS control sample. n=5 per group and 2 independent experiments performed. *P<0.05, **P<0.01.
Figure 4.13: MicroRNA-146b and its targets TRAF-6 and IRAK-1. PKH26-PCL (Sigma) dye that stains only phagocytic cells was intranasal inoculated to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then exposed the lungs exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). (A) miR-146b levels are expressed relative to snoRNA 202 and a PBS control sample and were determined by RT-PCR. (B) mRNA for TRAF-6 and IRAK-1 are expressed relative to 18S and a PBS control sample. (C-D) TRAF-6 and (E-F) IRAK protein expression was determined by flow cytometry in live CD11c+Dye+. *P<0.05.
mRNA levels of IRAK-1 and TRAF-6 in dye+ airway macrophages (Fig. 4.13B). Consequently, intracellular protein expression of TRAF-6 and IRAK were determined by flow cytometry. The percentage of resident alveolar macrophages that express TRAF-6 (Fig. 4.13C-D) and IRAK (Fig. 4.13E-F) were significantly reduced in the airways of HDM exposed group compared to PBS controls. Therefore, the adaptor molecules of the TLR signalling pathway were altered by HDM exposure and the mRNA levels of their regulators are increased.

4.2.6 Addition of macrophage chemoattractant protein, CCL2 restores anti-bacterial immunity in the allergic airway

Addition of alveolar macrophages benefited the allergic lung in clearing bacteria. Although, lungs that received naïve macrophages performed the best in bacterial elimination, in general addition of more macrophages reduced bacterial numbers in the airways and lung. A macrophage chemokine CCL2 (chemokine ligand-2) was next introduced in order to promote in vivo recruitment of macrophages and analysed bacterial response.

Intranasal administration of 1µg of CCL2 in naïve mice resulted in an increase in airway cellular recruitment that peaked at 6 hours (Fig. 4.14A) but no significant change was observed in the lungs (Fig. 14B). Increase in airway cell recruitment correlated with an increase in airway myeloid cell numbers (Fig. 4.14C). At all timepoints tested the majority of the airway cells were CD11c+CD11b- (resident alveolar macrophages) (Fig. 4.14D). Recruited macrophages numbers (CD11c-CD11b+) (Fig. 4.14D) and neutrophils (Fig. 4.14E & F) also increased.

In HDM-sensitised mice however, total airway and lung cell numbers reduced after addition of 1µg of CCL2 (Fig. 4.15A). This reduction was reflected in the reduced percent of live airway and lung myeloid cells that were CD11b+CD11c-, markers of recruited cells (Fig. 4.15 B & C) and eosinophils in the airways and lung (Fig. 4.15 D & E).
Chapter 4: Probing macrophage dysfunction in the allergic lung.
Figure 4.14: Airway cellular influx peaks 6 hours after administration of macrophage chemoattractant protein, CCL2. 1µg of CCL2 was intranasal inoculated per mouse and 0, 2, 6 and 24 hours later analysis was performed. Total cell numbers in the (A) airways and (B) lungs. (C) Total numbers of live airway myeloid cells. (D) Percentage and numbers of resident alveolar macrophages (CD11c+CD11b-) and recruited airways cells (CD11c-CD11b+). (E) CD11c+CD11c-Ly6G+ neutrophils as percentage of live myeloid airway cells and (F) total numbers in the airways by trypan blue exclusion. n=5 per group.
Figure 4.15: Addition of CCL2 reduces total cell numbers in the airways and lung of allergic mice. 1µg of CCL2 was i.n 48 hours after the final HDM exposure and lungs were analysed 24 hours later. (A) Total cell numbers in airways and lungs was enumerated by trypan blue exclusion. Percentages of live (dead cells excluded by fixable live/dead marker) myeloid cells that were CD11b+CD11c- (B) airway and (C) lung and the percentage of live myeloid cells that were eosinophils (D-E) was determined by flow cytometry. n=4 per group. *P<0.05.
The majority of cells in the airways of allergic mice were recruited CD11b+, whereas in PBS exposed airways the main cells were CD11c+ (Fig. 4.15B & 4.16B). Addition of CCL2 restored the dominance of CD11c+ macrophages in the airspaces of HDM exposed mice. More importantly CCL2 administration reduced the proportion of CD11c+ macrophages expressing ST2L (Fig. 4.16 C & D) and TREM2 (Fig. 4.16 E & F) that inhibit TLR responses and elevated the expression of TLR2 (Fig. 4.16G).

Similarly, total numbers of CD11b+CD11c-, eosinophils, CD11c+ST2L+ and CD11c+TREM2+ were elevated in the allergic airways, but significantly reduced with CCL2 addition except CD11c+ST2L+ airway cells (Fig. 4.17A-D).

When bacteria were introduced (Fig. 4.18A), the HDM exposed group that received CCL2 cleared bacteria better than the control HDM group (Fig. 4.18B). 33% of the mice in the HDM without CCL2 had bacteraemia, compared to none in the HDM with CCL2 group (Fig. 4.18C). There was no difference in airway or lung total cells numbers 48 hours after infection (Fig. 4.18D), though HDM+CCL2 mice had more cells than HDM group without CCL2 (Fig. 4.18E).

As expected the majority (~80%) of airway myeloid cells after infection of PBS naïve mice were neutrophils; which dropped to ~30% in the HDM exposed airways (Fig. 4.19A). However, CCL2 administration in HDM exposed airways improved neutrophil recruitment to over 40% (Fig. 4.19A). CCL2 i.n. before infection reduced the percentage of airway myeloid cells that were eosinophils (identified by FSC and SSC plots) (Fig. 4.19B). This environment created by CCL2 administration seemed to be better in neutrophil recruitment and bacterial clearance.

Anti-microbial peptides that opsonise bacteria and enhance the uptake of bacteria by immune cells, were analysed after the introduction of new macrophages by either intranasal transfer or addition of CCL2. Although, the data showed no difference in SP-D and CRP levels with or without new macrophage recruitment in the airways 48 hours after bacterial infection (Fig. 4.20).
Figure 4.16: Addition of CCL2 reduces anti-inflammatory receptors ST2L and TREM2 on alveolar macrophages the airways. PKH26-PCL (Sigma) dye that stains only phagocytic cells was i.n to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then exposed the lungs to three weeks of 15µg HDM or 15µl PBS (3 times a week). 1µg of CCL2 was i.n 48 hours after final HDM exposure and 24 hours before infection. Percentages of live (dead cells excluded by fixable live/dead marker) airway...
myeloid cells that were (A) CD11c+Dye+ or (B) CD11c+ was determined by flow cytometry. The percentage of (C) CD11c+Dye+ and (D) CD11c+ cells that are ST2L+ and the percentage of (E) CD11c+Dye+ and (F) CD11c+ cells that are TREM2+ airway. (G) Percentage of CD11c+ cells that are TLR2+. n=4 per group. *P<0.05.

Figure 4.17: Addition of CCL2 reduces inflammatory airway cells. PKH26-PCL (Sigma) dye that stains only phagocytic cells was i.n to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then the mice exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). 1µg of CCL2 was i.n 48 hours after final HDM exposure and 24 hours before infection. Total airway (A) CD11b+CD11c-, (B) eosinophils, (C) CD11c+ST2L+ and (D) CD11c+TREM2+ was determined by flow cytometry *P<0.05.
Figure 4.18: Addition of CCL2 improves bacterial clearance from the airways and lung. PKH26-PCL (Sigma) dye that stains only phagocytic cells was i.n to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then mice exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). 1µg of CCL2 was i.n 48 hours after final HDM exposure and 24 hours before infection. Mice were then infected with 1x10^6 CFU of Streptococcus pneumoniae (D39) and analysed 48h after infection. (A) Experimental plan for CCL2 administration and bacterial infection. (B) Bacterial titres in the airways and lungs determined by plating on agar. Data representative of 4-5 mice per group and are representative of 3 independent experiments. (C) Percentage of mice with bacteria. Total (D) airway and (E) lung cells determined by trypan blue exclusion. *P<0.05.
Figure 4.19: Addition of CCL2 restores anti-bacterial immunity. PKH26-PCL (Sigma) dye that stains only phagocytic cells was i.n to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then mice exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). 1µg of CCL2 was i.n 48 hours after final HDM exposure and 24 hours before infection. Mice were then infected with 1x10^6 CFU of *Streptococcus pneumoniae* (D39) and analysed 48h after infection. Percentage of live airway myeloid cells that are (A) neutrophils (CD11b+CD11c-Ly6G+SiglecF-) and (B) Eosinophils gated from FSC and SSC plots were determined by flow cytometry. Data representative of 4-6 mice per group and are representative of 2-3 independent experiments. *P<0.05.
Chapter 4: Probing macrophage dysfunction in the allergic lung.

Figure 4.20: Anti-microbial substances after replenishment of macrophages in the allergic airways. (A-B) BALB/c mice were exposed to 15µg of HDM three times a week for three weeks. 48 hours after the final HDM exposure, allergen exposed mice were given intranasal FACS sorted CD11c+CD11b- airway macrophages either from naive or allergen exposed lungs. 18 hours after the cell transfer mice were infected with 1x10^6 CFUs of *Streptococcus pneumoniae* (S.pn) strain D39. Surfactant protein-D (SP-D) and C – reactive protein (CRP) levels in the airways were measured by ELISA. (C-D) PKH26-PCL (Sigma) dye that stains only phagocytic cells was i.n to BALB/c mice 72 hours before the first house dust mite (HDM) exposure and then mice exposed to three weeks of 15µg HDM or 15µl PBS (3 times a week). 1µg of CCL2 was i.n 48 hours after final HDM exposure and 24 hours before infection. Mice were then infected with 1x10^6 CFU of *S. pneumonia* (D39) and analysed 48h after infection. SP-D and CRP levels in the airways were measured by ELISA. Data presented as box and whisker diagrams.
4.3 Discussion

The main finding of this chapter is that three weeks of HDM exposure changes resident alveolar macrophages into an alternatively activated phenotype that produces considerably less TNFα in response to TLR ligand stimulation. During the early stages of bacterial infection, the early cytokines TNFα and IL-1 are released. Many studies have shown TNFα to have a key role in *S. pneumonia* elimination. Anti-TNFα antibody administered against endogenous TNFα reduces survival by reducing neutrophil numbers and increasing bacteria\(^{240}\). TNF and IL-1 signalling receptor deficiency impairs *S. pneumonia* clearance and reduces neutrophil recruitment into the airspaces\(^{239}\). TNF is also important in enhancing the release of other cytokines and chemokines from macrophages and other immune cells.

In this chapter it was observed that alveolar macrophages present at the start of HDM exposure are also present 3 weeks later (dye+). Such alveolar macrophages defined as dye positive had increased mRNA and protein levels of RELMα, Ym1 and Arg1, which may signify alternative activation that may be driven by an environment rich in IL-4 and IL-13. Our data also strongly supports other studies showing enhanced IL-10 secretion\(^{275}\), expression of a number of anti-inflammatory receptors such as TREM2\(^{187}\), ST2L and IL-27 receptor\(^{276}\) and inhibition of expression of pro-inflammatory cytokines IL-1, TNFα, IL-6 and IL-12\(^{277}\). Other cytokines that enhance AAMs are IL-33, IL-25 and IL-21\(^{182}\) but were not studied directly here. Unfortunately such alternatively activated or regulatory macrophages are not conducive to antibacterial immunity. For example, AAMs are linked to secondary bacterial pneumonia\(^{231}\) and have impaired phagocytosis\(^{278,279}\). Transfer of naïve airway macrophages restores anti-bacterial immunity in the HDM-allergic lung not only suggests a therapeutic strategy might be to recruit replacements or cause turnover of ones already there but also that macrophages are critical for immunity in the airway.

Bone marrow derived immune cells, such as neutrophils and inflammatory monocytes appear early at the site of infection to promote innate immune responses and CCL2 is essential for the mobilisation of monocytes\(^{280}\). CCL2 is also called monocyte chemoattractant protein-1 (MCP-1). Deletion of ccl2 reduces monocyte recruitment by 50% in *Listeria* infection\(^{281}\). CCL2 deficient mice infected with
Escherichia coli have reduced bacterial clearance with reduced neutrophil and LTB4 levels, whilst, addition of exogenous CCL2 in CCL2-/ improved bacterial elimination and neutrophil influx. Furthermore, the addition of recombinant CCL2 to mice with allergic Airways disease reduces total airway and lung cells and this correlated with a reduction in recruited CD11b+ airway cells compared to CD11c+ alveolar macrophages. Moreover, pre-treatment of mice with CCL2 reduces eosinophils. A study shows eotaxin-3 (eosinophil chemoattractant) binds to CCR2 (the CCL2 receptor) and inhibits CCL2 mediated responses on human monocytes. Although, mice do not have eotaxin 3, there is a possibility that CCL2 competes with endogenous mouse eotaxin to reduce eosinophil recruitment. The addition of CCL2 also had an impact of CD11c+ alveolar macrophages by reducing their expression of ST2L and TREM2 while increasing expression of TLR2. Furthermore, addition of CCL2 did not allow bacteraemia to develop.

CCL2 administration may have the potential to be used in therapy in man. CCR2 (receptor for CCL2) deficiency shows enhanced allergic airway inflammation to Aspergillus fumigatus and OVA challenge/sensitisation. As evidenced by data in this chapter administration of CCL2 after HDM but before bacterial infection is beneficial in combating S. pneumonia. Of course, further experiments are needed to elucidate and confirm the benefits of CCL2. For example, can a dose lower than 1µg per mouse of CCL2 exert the same effects? What are the consequences of giving CCL2 during HDM exposure rather than after the cessation of exposure? What are the effects of CCL2 on resolution after HDM exposure with/without bacterial infection and the side effects of treatment?

TREM2 is expressed on AAMs and induced by IL-4 on peritoneal macrophages. TREM2 deficient bone marrow derived dendritic cells produces more TLR-induced cytokines than WT dendritic cells. TREM2 on bone marrow derived macrophages inhibits through DAP12. Therefore, TREM2 expressed on alveolar macrophages could induce anti-inflammatory properties in alveolar macrophages. It is therefore pertinent that CCL2 reduces TREM-2 expression, which may be via CCR2 signalling on myeloid cells or be recruitment of fresh macrophages that become CD11c+ in the lung microenvironment but not TREM-2+ because of the reduced allergic environment. The same explanation could be used for the reduction of the IL-33.
receptor ST2L (that impairs macrophage activation via TLRs by sequestering MyD88) in CCL2 treated mice.

The restoration of TLR responses is unlikely to be due to a down-regulation of surface TLRs themselves, as there were no significant changes at mRNA level. We did however observe an upregulation of TLR negative regulators particularly A20. A20 targets TRAF-6 that is found upstream of NF-κB activation. Although the mRNA levels of TRAF-6 and IRAK-1 were not affected, the percentage of resident alveolar macrophages that express TRAF-6 and IRAK at the protein level are significantly less in the HDM exposed airways (<20%) than PBS controls (>40%). TRAF-6 and IRAK-1 could also be targeted by other inhibitors e.g. miR146 [287]. Therefore, alveolar macrophages were analysed for this miRNA and HDM exposure was found to induce miRNA146. It would be important to ascertain whether CCL2 administrations cause the recruitment of new macrophages where such TLR negative regulators are reduced.

Overall, HDM exposure or the HDM induced immune response causes a number of changes (Fig. 4.21) in alveolar macrophages that could reduce their ability to respond to bacterial components that signal through TLRs. However, administration of macrophage chemoattractant protein significantly improves bacterial clearance possibly as a result of recruitment of new macrophages that are uninfluenced by the allergic response in the airways.
Figure 4.21: An illustration of the effects of HDM exposure on airway macrophages. Data in this chapter illustrates that after house dust mite exposure: alternatively activated macrophage markers YM1 and RELMα are highly expressed, there is increased messenger RNA levels of the TLR negative regulator A20 and reduced levels of TLR adaptor molecules TRAF6 and IRAK1. All these changes could play roles in reducing TNF cytokine production in response to TLR stimulation and may be driven by the up-regulation of microRNA species.

Figure 4.22: CCL2 addition improves the proportion of alveolar macrophages in the airways that are uneducated by the allergic response. CCL2 administration reduces regulatory receptors on resident alveolar macrophages. These changes could enhance the antibacterial capacity of alveolar macrophages.
CHAPTER 5

Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.
5.1 Introduction

T1/ST2 (the IL-33 receptor) is upregulated on airway macrophages from HDM-exposed mice (chapter 4). IL-33, the ligand for ST2L\textsuperscript{288}, is produced in a precursor form that is cleaved by caspase-1\textsuperscript{93}. IL-33-ST2L signalling leads to NF-κB and MAP kinases activation\textsuperscript{93}. IL-33 increases eosinophil survival and ST2 blocking antibodies inhibit eosinophil responses to IL-33\textsuperscript{289}. Furthermore, intranasal administration of IL-33 induces airway hyper-reactivity and mucus production even in T cell deficient mice (RAG2\textsuperscript{-/-})\textsuperscript{290}, therefore, IL-33 responsive innate immune cells have a critical role. Soluble human ST2 in sera correlates with asthma exacerbations\textsuperscript{291}. IL-33 signalling via ST2L plays an important role in allergic responses. For example, the levels of Th2 cytokines are decreased in allergic lungs by administration of ST2L blocking antibody\textsuperscript{101}. ST2 deficiency causes mice to develop attenuated OVA-induced airway inflammation. In response to OVA sensitisation and challenge, ST2 knock outs have significantly less cellular infiltrate, macrophages and eosinophils than WT OVA exposed lungs\textsuperscript{292}, this is in contrast to another study showing normal Th2 responses after OVA induced airway inflammation\textsuperscript{293}. ST2\textsuperscript{-/-} macrophages are not alternatively activated during airway inflammation induced by OVA sensitisation and challenge\textsuperscript{94}. Therefore, IL-33-ST2 signalling is important in AAMs in response to OVA.

IL-33 signals through ST2L and IL-1 receptor associated protein (IL-1RAcP) heterodimer and recruits MyD88 to ST2L, which activates downstream NF-κB and MAP kinase pathways\textsuperscript{288}. sST2 is shown to act as a decoy receptor and inhibit LPS induced signalling\textsuperscript{294}. IL-33 and ST2L signalling protects from parasitic infection. ST2\textsuperscript{-/-} mice infected with *Toxoplasma gondii* has more severe pathology\textsuperscript{295}. Lung innate lymphoid cells expressing ST2 are important in restoring epithelial integrity after influenza infection\textsuperscript{296}. Furthermore, ST2L inhibition by monoclonal anti-ST2L treatment during RSV infection prevents Th2 pathology\textsuperscript{297}. In contrast, ST2L\textsuperscript{-/-} mice have a normal antibacterial response against respiratory infection with *Mycobacterium tuberculosis*\textsuperscript{298}.
5.1.1 Hypothesis and Aims

Since ST2 and its ligand, IL-33 are current targets in the treatment of allergic conditions we decided to elucidate this interaction further and its effect on anti-bacterial immunity in the HDM-sensitised lung. It is not currently known whether the immune response to, or the allergen itself, is responsible for increased susceptibility to bacterial pneumonia and sepsis. The hypothesis for this chapter is that the allergic immune response plays a dominant role in susceptibility and that the initial effects of IL-33 is reduced in mice lacking functional ST2 given HDM, this restores anti-bacterial immunity. The specific aims were:

1. To assess the response to HDM in mice lacking functional ST2 and assess the allergic airway inflammation.

2. To assess bacterial clearance in WT and ST2/-/- mice following prior exposure to HDM.

3. To study the cellular recruitment profile in the airways of ST2/-/- with or without bacterial infection.

4. To assess ST2/-/- macrophages response to bacterial components.
5.2 Results

5.2.1 Bacterial susceptibility in WT and ST2-/- mice

ST2-/- mice (confirmed by genotyping Fig. 5.1) exposed to HDM had reduced cellular infiltrate and reduced total eosinophil numbers in the airways (Fig. 5.2). Both WT and ST2-/- mice exposed to PBS or HDM had bacteria in the airways 24 hours after S. pneumonia infection. However, 48 hours after infection only WT HDM and ST2-/-+HDM airways had bacteria (Fig. 5.3A & B). All groups had bacteria in the lungs except WT PBS 48 hours after infection. Therefore, ST2-/- PBS exposed mice (33%) had bacteria in the lungs while all the control (WT PBS) mice had eliminated the bacteria. Bacterial dissemination was present in both WT and ST2-/- HDM exposed groups 48 hours after infection (Fig. 5.3). However, 50% of the mice in WT HDM group developed bacteraemia whereas, only 28.5% of ST2-/- mice had bacterial dissemination (Fig. 5.3B). ST2-/- mice are thus still susceptible to bacterial complications but at a lower level than WT HDM exposed mice. Therefore, the cellular infiltrate to HDM in the presence or absence of ST2 was examined.

There was reduced infiltration of leukocytes into the airways of ST2-/- mice compared to WT controls 48 hours after S. pneumonia infection (Fig. 5.4). WT+PBS had elevated numbers of cells in the airways after infection compared to numbers present at baseline (0h after infection), however, this increase in cell recruitment after infection was absent in WT+HDM exposed group (Fig. 5.4) as shown in the chapter 3. Similarly, ST2-/-+PBS had more cellular recruitment than ST2-/-+HDM compared to baseline (0 hour) levels (Fig. 5.4B). Thus ST2-/- HDM-exposed mice still display reduced cell recruitment.

HDM exposure induces mucus production. At the time of infection, mucus staining was higher in the WT+HDM group compared to ST2-/-+HDM group (Fig. 5.5). However, 24 hours after bacterial infection both WT and ST2-/- HDM groups had similar levels of mucus production (Fig. 5.5).
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.1: ST2 knockout and wild-type control (BALB/c) genotyping. PCR image to confirm animals used in this chapter were ST2-/− homozygous knockouts. Representative three wild-type (WT) controls and four ST2-/− mice were ear punched and DNA extracted and PCR performed using REDExtract-N-Amp Tissue PCR kit (Sigma). The expected band for ST2 is at 300 bp in wild-type mice (lanes 1-3).
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.2: Reduced airway cellular infiltrate and eosinophils in ST2-/- compared to WT controls mice after HDM exposure. Wild-type BALB/c and ST2 deficient mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure BAL was performed and analysed for (A) Total airway cells by trypan blue exclusion, (B) total eosinophil numbers by flow cytometry and (C) lung IL-33 levels by ELISA. Data shows the mean with SEM of n=10 mice per group. *P<0.05, **P<0.01, ***P<0.001.
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.3: Susceptibility of WT and ST2-/- to Streptococcus pneumoniae after house dust mite exposure. Wild-type BALB/c and ST2 deficient mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure mice were infected with 1x10^6 CFU Streptococcus pneumoniae and bacterial titres were analysed 24 and 48 hours after infection by plating out airway lavage (BAL), lung and blood. n=5 at 24h and n=8-9 at 48h. Data shows the mean with SEM.
Figure 5.4: Reduced infiltrated cell recruitment in ST2/- after *Streptococcus pneumonia* infection compared to WT PBS group. Wild-type BALB/c and ST2/- mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure mice were infected with 1x10^6 CFU *Streptococcus pneumonia*. Total airway cells by trypan blue exclusion are shown 24 hours after infection (A) numbers and (B) % of baseline (baseline = total cells at time of infection and 72hrs after final HDM exposure). Data presented as mean with SEM. *P<0.05, ** P<0.01, *** P<0.001.
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.5: Mucus production in the lungs of WT and ST2-/- before and 24 hours after infection. Wild-type BALB/c and ST2-/- mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure mice were infected with 1x10^6 CFU *Streptococcus pneumoniae*. Histology shows Periodic Acid-Schiff (PAS) staining of lung tissue that was fixed in 10% formalin for 24 hours. Only HDM exposure induces mucus production in WT and ST2-/- groups. Magnification x40.
5.2.2 Increased neutrophil recruitment in the airways of ST2-/–+HDM compared to WT+HDM

The percentage of neutrophils (CD11b+CD11c-Ly6G+SiglecF–) in the airways of ST2-/–+HDM group was higher than WT+HDM mice 24 hours after infection (Fig. 5.6A). After infection, there were fewer neutrophils in HDM groups (WT and ST2-/–) compared to PBS controls 48 hours after S. pneumoniae infection (Fig. 5.6C). The percentage of eosinophils (CD11b+CD11c-Ly6G-SiglecF+) was significantly reduced in ST2-/–+HDM group compared to both PBS (WT and ST2-/–) controls in the airways after infection eosinophils as percentage of total airway cells (Fig. 5.6B) and in total numbers compared to WT+HDM group (Fig. 5.6D). Thus despite a reduction in eosinophils and an increase in neutrophils, bacterial infection was still a problem in the HDM-exposed airway.

Total lung cells were similar in all groups (Fig. 5.7A). The percentage of recruited CD11b+ lung cells that were Ly6G+SiglecF– (neutrophils) was significantly reduced in WT+HDM compared to WT+PBS; however, the difference in WT+PBS and ST2-/–+HDM was not significant 24 hours after infection (Fig. 5.7B). The percentage of CD11b+ that were Ly6G-SiglecF+ (eosinophils) was significantly higher in HDM exposed groups compared to PBS controls (Fig. 5.7C). Lung neutrophil numbers were highest in WT+PBS group compared to the rest of the groups that had similar numbers (Fig. 5.7D). However, eosinophil numbers in ST2-/–+HDM were significantly reduced in comparison to WT+HDM (Fig. 5.7E). There was no difference in CD4+ and CD8+ T cell numbers in the lungs (Fig. 5.8). It is interesting to note that cell recruitment in ST2-/– mice to HDM or bacteria is blunted in general compared to WT mice suggesting a more generalised defect in these animals.
Figure 5.6: Increased percentage of airway myeloid cells that are neutrophils in ST2/- mice compared to WT controls. Wild-type BALB/c and ST2/- mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure mice were infected with 1x10^6 CFU Streptococcus pneumonia. (A) CD11b+Ly6G+SiglecF- cells and (B) CD11b+Ly6G-SiglecF+ as percentage of airway myeloid cells and total airway (C) neutrophil and (D) eosinophil numbers 48 hours after infection were determined by flow cytometry. n=5 at 24 hours and n=12 at 48 hours per group. *P<0.05, ** P<0.01, *** P<0.001.
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.7: Neutrophils and eosinophils in the lungs of WT and ST2-/− mice 24 hours after *Streptococcus pneumonia* infection. Wild-type BALB/c and ST2-/− mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure mice were infected with 1x10^6 CFU *Streptococcus pneumonia* and harvested 24 after infection. (A) Total number of lung cells (by trypan blue exclusion), percentage of CD11b+ lung cells that are (B) Ly6G+SiglecF− or (C) SiglecF+Ly6G− and number of lung (D) neutrophils and (E) eosinophils was determined by flow cytometry. Data presented as box and whisker diagrams. *P<0.05, **P<0.01, ***P<0.001.
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.8: Lung T cells numbers after bacterial infection in WT and ST2-/- with or without HDM exposure. Wild-type BALB/c and ST2-/- mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM exposure mice were infected with 1x10^6 CFU Streptococcus pneumoniae and harvested 24 after infection. T lymphocytes in lung homogenate were identified as (A) CD3+CD4+CD8- or (B) CD3+CD8+CD4- T cells by flow cytometry. n=5 per group. *P<0.05.
5.2.3 Th2 cytokine levels in WT and ST2-/- lungs

After bacterial infection the Th2 cytokine IL-13 was undetectable in the lungs (Fig. 5.9). 0 hour after infection, the lungs of WT+HDM and ST2-/-+HDM group had detectable levels of IL-13 compared to PBS controls (Fig. 5.9B). Similarly, IL-33 levels were significantly higher in lungs after HDM exposure before infection (Fig. 5.9A). However, after infection the levels of IL-33 significantly increased in all groups and at this stage there was no difference in IL-33 levels between the groups (Fig. 5.9A). WT+HDM, ST2-/-+PBS and ST2-/-+HDM had similar levels of TNFα in the airways 24 hours after infection and this level was significantly reduced compared to WT+PBS (Fig. 5.10).

5.2.4 Reduced AAM markers in ST2-/- lungs and alveolar macrophages

The data in the previous chapter showed an AAM skew of alveolar macrophages after HDM exposure. In ST2-/- alveolar macrophages, although the levels of Arg1, RELMα and YM1 RNA expression were significantly lower after HDM exposure compared to WT HDM, they did not reach baseline levels seen in PBS controls (Fig. 5.11). This is also shown by whole lung mRNA analysis (Fig. 5.12). Thus, alveolar macrophages in the WT+HDM-exposed lung are altered strongly in phenotype towards alternatively activated or wound healing and much less so when the allergic immune response is attenuated e.g. in ST2-/-+HDM group.
Chapter 5: Reduced HDM-induced eosinophilic pathology does not restore anti-bacterial immunity.

Figure 5.9: Levels of IL-33 and IL-13 levels cytokines in the lungs. Wild-type and ST2−/− BALB/c mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM challenge, mice were infected with 1x10^6 CFU Streptococcus pneumonia and harvested 24 hours after infection. Levels of cytokines; (A) IL-33 and (B) IL-13 were analysed by ELISA. *P<0.05, **P<0.01, ***P<0.001.
Figure 5.10: TNFα levels in the airways and lung of WT and ST2-/-. Wild-type BALB/c and ST2-/ mice were exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM challenge, mice were infected with 1x10^6 CFU *Streptococcus pneumonia* and TNF levels in the (A) airways and (B) lungs were analysed 24 hours after infection. n=5 per group. *P<0.05, ** P<0.01, *** P<0.001.
Figure 5.11: Reduced mRNA expression of alternatively activated macrophage markers RELMα, Arg1 and YM1 in ST2-/− alveolar macrophages compared with WT after exposure to HDM. BALB/c mice exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM challenge live CD11c+CD11b− alveolar macrophages were sorted by flow cytometry and stored in RNA lysis buffer+β-ME. TaqMan probes were used to detect the mRNAs. mRNA levels are expressed relative to 18S and a WT PBS control sample. Data presented as mean+/SEM representative of two independent experiments. n=4-5 per group of 2 independent experiments. *P<0.05, ** P<0.01, *** P<0.001.
Figure 5.12: Reduced mRNA expression of alternatively activated macrophage markers RELMα, Arg1 and YM1 in ST2/- lungs compared with wild type after exposure to HDM. BALB/c mice exposed to 15µg of HDM or 15µl PBS 3 times a week for 3 weeks. 72 hours after the final HDM challenge whole lung was stored in RNA lysis buffer+β-ME. TaqMan probes were used to detect the mRNAs. mRNA levels are expressed relative to 18S and a WT PBS control sample. Data presented as mean+/−SEM representative of two independent experiments. n=4-5 per group of 2 independent experiments. *P<0.05.
5.2.5 ST2/- alveolar macrophage features

CD11c+ alveolar macrophages expressed significantly lower levels of TLR2 and higher levels of TREM2 after HDM exposure in both WT and ST2/- groups (Fig. 5.13A & C). However, the percentage of airways myeloid cells that were CD11c+TLR2+ were reduced in WT+HDM compared to ST2/-+HDM (Fig. 5.13B). In contrast, the percentage of airway myeloid cells that were CD11c+TREM2+ was significantly higher in ST2/-+HDM compared to WT+HDM (Fig. 5.13D).

In the last chapter, it was found that alveolar macrophages from the HDM exposed airways produced less TNFα in response to TLR stimulation compared to PBS exposed alveolar macrophages. Here, the response of CD11c+CD11b- (alveolar macrophages) airway cells was assessed from WT and ST2/- to overnight TLR stimulation. Overall, WT+HDM exposed alveolar macrophages produced less TNFα compared to WT+PBS (Fig. 5.14) as shown in chapter 4. WT+PBS cells produced less TNFα compared to ST2/-+PBS group after TLR stimulation (Fig. 5.14). Alveolar macrophages from ST2/-+HDM produced lower levels of TNFα compared to ST2/-+PBS in response to TLR4 and TLR1/2 stimulation (Fig. 5.14).
Figure 5.13: TLR2 and TREM2 expression on CD11c+ airway cells. Wild-type BALB/c and ST2-/ mice were exposed to HDM three times a week for 3 weeks. TLR2 and triggering receptor expressed on myeloid cells (TREM2) were analysed 72 hours after final HDM exposure. Data is shown (A) TLR2 and (C) TREM2 expression as percentage of airway CD11c+ cells and (B) CD11c+TLR2+ and (D) CD11c+TREM2+ as percentage of airway myeloid cells. n=5 per group of 2 independent experiments. *P<0.05, ** P<0.01.
Figure 5.14: TNFα release by CD11c+CD11b- airway cells in response to LPS and LTA stimulation. Wild type and ST2−/− BALB/c mice were exposed to PBS or HDM 3 times a week for 3 weeks. 72 hours after the final HDM exposure, CD11c+CD11b- airway cells were sorted by flow cytometry. 3x10^4 cells per well were plated out and stimulated with 1µg/ml TLR4 ligand LPS and 1µg/ml TLR2 ligand LTA. TNFα release by the cells was measured by ELISA. Data presented as mean with SD. Representative of 2 experiments. *P<0.05, ** P<0.01, *** P<0.001.
5.3 Discussion

The effect of HDM exposure in ST2 deficient mice was less marked than WT controls. A significant reduction in HDM induced cellular recruitment and in particular eosinophils was present in ST2 deficient mice. Therefore, it might be expected that lacking a receptor that binds IL-33 that in turn drives allergic disease would be of benefit in subsequent bacterial infection. In HDM-exposure of the lung followed by *Streptococcus pneumonia* infection, this appears not to be the case. In this chapter, fewer ST2-/- mice exposed to HDM have bacteraemia 48 hours after bacterial infection, whereas, 50% of WT HDM controls had bacteria in the blood. Both WT and ST2-/- HDM exposed groups had bacteria in the airways and lung 48 hours after infection.

ST2 deficiency does protect from secondary lung infection during sepsis in a murine model of cecal ligation and puncture induced sepsis followed by a secondary respiratory infection with *Pseudomonas aeruginosa*\(^\text{299}\). This study showed that cecal ligation and puncture reduces IFN-γ and TNFα production from T cells, whereas this was absent in ST2 deficient mice \(^\text{299}\). In contrast, another study shows ST2 deficiency increases susceptibility to polymicrobial sepsis as a result of impaired phagosome maturation and reactive oxygen species production in ST2-/- phagocytes \(^\text{300}\). This may explain the heightened bacterial numbers in ST2-/- PBS exposed mice in the lungs 48 hours after *S. pneumonia* infection compared to WT PBS controls. Furthermore, ST2 deficient mice infected with influenza A virus have a comparable recovery pattern to WT controls but display higher bacterial titres after subsequent pneumococcal pneumonia associated with increased lung IFN-γ levels\(^\text{301}\). A recent study show increased cytokine secretion by the ST2 deficient cells using whole blood leukocytes from WT and ST2-/- mice stimulated *ex vivo* with *S. pneumonia*\(^\text{302}\). Taken together these studies highlight the importance of the possible protective effect of IL-33/ST2 signalling after bacterial infection. Although, ST2L signalling does not seem to be critical after lung *Mycobacterium tuberculosis* infection, ST2 deficient mice display a normal host immune response\(^\text{298}\). These data exemplify the complexity of innate immunity receptors.

LPS-induced shock *in vivo* is dramatically reduced after administration of soluble ST2 that would presumably mop up IL-33; whereas, administration of anti-ST2
antibody (that binds, but it is unclear if it inhibits or signals through ST2) exacerbates LPS-induced inflammation. Increased soluble ST2 in serum is seen in patients with sepsis. Administration of ST2 ligand, IL-33 reduces sepsis by increasing neutrophil influx for better bacterial clearance, this occurs as a result of IL-33 preventing downregulation of CXCR2. In our model, the levels of IL-33 may not have a major role, as after bacterial infection all groups had similar levels of IL-33 in lung homogenate.

ST2L expression is induced on macrophages by IL-13 and promotes differentiation into AAMs. In vivo, HDM exposed ST2-/- alveolar macrophages expressed less RELMα, YM1 and Arg1 mRNA levels compared to WT HDM controls. This may explain the improved response of ST2-/-+HDM exposed alveolar macrophages to TLR2 and 4 agonists, compared to WT+HDM. This is supported by observations that peritoneal macrophages from ST2-/- mice cultured with IL-1α, IL-1β or LPS produce more pro-inflammatory cytokines (IL-6, IL-12 and TNFα) than WT controls. ST2-/- macrophages could produce more TNFα than WT controls due to less sequestering of MyD88 and MAL, adaptor molecules involved in the TLR signalling pathway. MyD88 is required for NF-κB activation by TLR2, TLR4 and TLR9. By affecting MyD88 and MAL, ST2L will prevent signalling through IRAKs, TRAF6 and NF-κB and inflammatory gene expression. By contrast addition of IL-33 to peritoneal macrophages enhances LPS and LTA induced TNFα release. Whether ST2 promotes or inhibits TLR responses is currently confusing and seems to depend on whether it is the receptor or ligand that is blocked.

In summary, ST2 signalling is important in eosinophil recruitment in response to allergen exposure since deficiency in this receptor impairs eosinophil recruitment into the airways. However, despite a reduction in HDM induced allergic inflammation the impact of ST2 deficiency on protection against bacterial pneumonia is limited. Therefore, this pathway does not appear to be critical hindrance to anti-bacterial immunity in the lungs of HDM exposed mice. Nonetheless the effect of ST2 seems to be important in protection against S. pneumonia infection shown by increased bacterial susceptibility of lungs without functional ST2.
CHAPTER 6

Final Discussion
6.1 Final Discussion

Bacteria are associated with asthma exacerbations, but the mechanisms responsible are unknown. Our data shows delayed clearance and increased invasiveness of *S. pneumonia* in the murine lung undergoing inflammation induced by chronic exposure to HDM. This “asthma exacerbation of bacterial infection” rather turns the concept of microbial exacerbation of asthma on its head. There is also clinical support for this scenario in that individuals with asthma have a greater than 2-fold increased risk of invasive pneumococcal disease\(^{16}\). This study identified *S. pneumoniae* as the most prevalent bacterium. There is currently a discrepancy with other models of allergic airway inflammation. The murine OVA model of sensitisation shows reduced susceptibility to *S. pneumonia* \(^{220,222}\). In addition, *S. pneumonia* before OVA challenge induces T regulatory cells in lymph nodes and suppresses allergic airway disease\(^{308}\). However, OVA is not a clinically relevant allergen, requires intraperitoneal priming and does not contain the complex mix of allergen, enzymatic activity and TLR agonists common in most clinically relevant allergens like HDM. Therefore, HDM allows us to study a clinically relevant allergen that affects lung mucosal immune cells.

The main findings of this thesis are that HDM exposed lungs have increased susceptibility to *S. pneumonia* and that the molecular pathways leading to the production of neutrophil chemoattractants in the lung are compromised. Despite the complexity of anti-bacterial pathways that are disrupted, the re-introduction of neutrophils by i.n administration of neutrophil chemoattractants to airways with allergic airway disease enables clearance of *S. pneumoniae* that would otherwise prove fatal.

6.2 Specific innate immune pathways are modulated in the allergic lung that precipitates life threatening bacterial pneumonia and sepsis

HDM exposure modifies anti-bacterial pathways in the allergic airway disease that impact on subsequent control of respiratory bacteria. The HDM exposed lung recruits considerably fewer neutrophils than PBS controls. Blockade or depletion of
neutrophils is fatal in bacterial pneumonia \(^{142}\). The inability to recruit neutrophils could be a result of reduced neutrophil recruiting cytokines and chemokines such as TNF and CXCL2 present in the HDM exposed airways after bacterial infection. The lack of involvement of IL-17A in our study was surprising since this cytokine is involved in neutrophil recruitment in another lung allergic/infection models\(^{309}\). This latter study, however examines *Haemophilus influenzae* administered during OVA peritoneal sensitisation, but before allergen challenge and does not examine the effect of established allergy on bacterial clearance.

Alveolar macrophage alterations in the allergic lungs may also contribute to the reduced neutrophil recruitment following bacterial infection as they secrete neutrophil recruiting cytokines. Alveolar macrophages are long lived cells with a slow turnover \(^{177}\). The reduced responsiveness to TLR ligands after HDM exposure will therefore impact on antibacterial immunity for significant periods of time. Furthermore, even with turnover, their replacements may be conditioned by the allergic lung to be equally affected.

HDM exposure results in prolonged phenotypic and functional changes in murine alveolar macrophages that reduce their ability to respond to PAMPs. Alveolar macrophages (CD11c+ airway cells) have reduced levels of TLR2 after three weeks of HDM exposure compared with PBS controls. Surprisingly, neutrophils also have reduced TLR2 levels during bacterial challenge of HDM-exposed mice compared to non-allergic controls. This may suggest that it is the altered environment that affects immune cells and not on inherent deficit in the cells themselves. Future studies should address whether the altered TLR responsiveness is restricted to TLR2 or extends to others that use different signaling pathways.

In addition alveolar macrophages from the allergic lung express higher levels of regulatory receptors CD200R, ST2L and TREM2 that limit myeloid cell activity and reduce anti-bacterial cytokine secretion. Blockade of these receptors in the allergic lung and analysis of bacterial susceptibility should be tested in the future as it may be possible to re-educate alveolar macrophage responsiveness. Moreover, alveolar macrophages gain an alternatively activated phenotype in the allergic lung determined by increased mRNA and protein levels of RELMα, Arg1 and YM1.
Functionally these macrophages are not able to produce as much TNFα in response to TLR agonists as PBS exposed alveolar macrophages. An increase in these TLR regulatory proteins will impact the level of activation of NF-κB (and other transcription factors) and therefore, anti-bacterial cytokine and chemokine responses. *In vitro*, direct exposure of naïve alveolar macrophages to HDM overnight did not reduce their capacity to produce TNFα after TLR 2 and 4 stimulation. This again points to effects of an altered or more complex microenvironment of the lungs after HDM exposure than direct impact of HDM on alveolar macrophages.

There is a possibility that the reduced responsiveness to TLR ligands is a result of TLR cross-tolerance, which is a process whereby TLR-activated cells are, for a short period of time, refractory to subsequent TLR stimulation\(^{310}\). Der p2 and Der p7 have structural and functional homology with MD-2; the LPS binding component of the TLR4 signaling complex \(^{311,312}\) and HDM extracts contain low levels of LPS and so it is possible that there is a similar refractory effect via TLRs alone. However, chronic pulmonary LPS (1µg) exposure for 4 consecutive days and then challenge with 10µg of LPS 24 hours later results in only a selective immunosuppression without affecting the neutrophilic response\(^{313}\). Additionally, LPS tolerance does not cause tolerance to TLR2 ligands such as Pam3Cys\(^{313}\).

If macrophage responsiveness is the problem, then we should be able to rectify the situation by replacing them. Transfer of naïve alveolar macrophages prior to bacterial challenge in HDM-exposed mice reduced bacterial titres compared to mice receiving HDM-exposed alveolar macrophages. However, the increase in alveolar macrophages seemed to be beneficial whether naïve or allergic. From this we can conclude that fresh macrophages, or more allergic ones (albeit producing less chemokines) is beneficial in combating bacterial growth and spread. Another way of increasing airway macrophages is to increase monocyte release from the bone marrow by CCL2\(^ {280}\). Indeed, CCL2 administration before bacterial infection prevented bacteraemia 48 hours after infection. The defect in the allergic lung can therefore be overcome by manipulating macrophage numbers in the airspaces. This does not mean they are the only defect, but they are sufficient to overcome the problems that exist.
Future studies should address whether airway macrophage turnover or recruitment can be improved in models of allergy. Despite our studies showing that anti-bacterial immunity improves the longer the gap between the last allergen exposure, in reality, in patients the problem is likely to persist as allergen may be ubiquitous.

6.3 Bacterial pneumonia in a murine model of reduced house dust mite induced airway inflammation

In chapter 5, we addressed whether it is the Th2 environment that causes bacterial complications or the HDM itself. Previous studies show that administration of monoclonal ST2 antibody reduces the severity of RSV in allergic mice. Furthermore, IL-33 treatment in mice infected with influenza virus resulted in considerably reduced lung inflammation (Hussell et al, unpublished observations). In addition, administration of neutralising antibody against ST2 in an OVA model of airway inflammation attenuates eosinophilic inflammation and mucus hypersecretion and reduces Th2 cytokine production. However, when the OVA model of allergic inflammation was used in ST2 knockout mice, allergic inflammation was unaffected. In contrast, another OVA study in ST2-/- mice shows attenuated inflammation. The differences between these two studies could be a result of different OVA sensitisation and challenge protocols. In this thesis we show that HDM induced eosinophilic inflammation is lower in ST2-/- mice compared to wild type controls. Though both wild type and ST2-/- mice exposed to HDM developed bacterial pneumonia in the lungs 48 hours after infection, fewer ST2-/- HDM mice had bacteria in the blood compared to PBS controls. An absence of the IL-33R, ST2L, therefore reduces but does not fully eliminate bacterial complications after HDM exposure.

ST2 knockout mice had reduced TLR2 expression on alveolar macrophage, but not as profound as in WT mice. Similarly, mRNA levels of AAM markers Arg1, YM1 and RELMα are significantly lower in ST2-/-+HDM compared to WT+HDM alveolar macrophages. In response to TLR2 and TLR4 agonists, alveolar macrophages from ST2-/- group produced more TNF than WT controls. This suggests that alveolar macrophages are less affected in the reduced allergic airway inflammation in ST2-/- mice, which improves handling of bacteria. However, a lack of ST2 does not
completely remove the problem. Future studies should address macrophage production of neutrophil chemoattractants in this model.

6.4 Asthma therapeutics and bacterial exacerbation

Asthma is a complex disease encompassing a number of different subtypes. Despite better understanding of asthma pathogenesis, the main therapy remains inhaled corticosteroids, beta-2 agonists and long-acting bronchodilators. These reduce symptoms and the frequency of exacerbations, but many patients remain highly symptomatic and these therapies have little impact on the decline in lung function. Many studies in mice show a reduction in allergic inflammation by manipulating cytokines such as IL-4, IL-5 and IL-13, but the underlying dysfunction in airway hyper-responsiveness, neurons and smooth muscle cells is either not studied or unaltered. Neutralising these cytokines that dominate in overt disease have proved successful in specific groups of patients. For example, in patients with severe, steroid-resistant, eosinophilic asthma, anti-IL-5 (Mepolizumab) significantly reduced asthma exacerbations; this was in contrast to the same treatment in milder asthmatics. Similarly, anti-IgE monoclonal antibody (omalizumab) is only effective in a small patient population of difficult-to-treat asthma. Such findings reinforce the importance of employing patient specific treatments. One deficit in the literature regarding cytokine blockade in patients is whether subsequent bacterial complications are reduced, which requires longitudinal follow up studies. In other words, the underlying asthma may not have changed in clinical trials of severity but downstream complications, such as bacterial exacerbation may have improved.

6.5 Conclusion of HDM-induced innate mechanisms increasing bacterial susceptibility in the allergic lung and areas suitable for therapeutic manipulation.

If Streptococcus pneumonia gains entry into the lungs undergoing an allergic immune response, there are a number of factors that could increase the threshold of initiation of an effective antibacterial response (Fig. 6.1). Many allergens have protease activity and can cleave the epithelial tight junctions molecules such as claudin increasing penetration of the allergen and subsequently other pathogens into the lungs (Fig. 6.1A). HDM is often associated with TLR agonists such LPS. TLR (or other, PRRs) signalling on epithelial cells and macrophages induces NF-κB
activation and subsequent cytokine release. Epithelial derived cytokines IL-25, IL-33 and TSLP stimulate innate lymphoid cells (ILC)\textsuperscript{316} and alternatively activate macrophages leading to secretion of IL-13 (Fig. 6.1B). All of these reduce the antibacterial capacity of alveolar macrophages. Our data shows alveolar macrophages from HDM exposed lungs have lower TLR2 expression, therefore their detection of bacterial component LTA maybe impaired and also they express more alternatively activated proteins Arg1 and YM1 (Fig. 6.1C). Dendritic cells sample the airway lumen by forming dendritic extensions between epithelial cells. TSLP also conditions dendritic cells to release CCL17 and CCL22 to recruit T helper 2 (Th2) cells\textsuperscript{317}. Th2 cells have limited antibacterial effects compared to Th1 cells (Fig. 6.1D). Th2-associated cytokines IL-4, IL-5, IL-9 and IL-13 have an important role in the pathogenesis of allergic airways disease. These cytokines are involved in prolonging the survival of eosinophils, activation of mast cells and induction of goblet cell hyperplasia. Eosinophils and mast are critical in helminth infections but they have limited role in anti-bacterial immunity\textsuperscript{318} (Fig. 6.1E). In resolution of allergic airway response, resident macrophages uptake and clear apoptotic cells from the lung while releasing TGF-\(\beta\), IL-10 and prostaglandin E\(_2\) involved in lung structural changes\textsuperscript{319}. However, these mediators are anti-inflammatory and can dampen antibacterial mechanisms. Disrupted epithelial tight junctions allow basolateral epithelial growth factor to reach its receptor (EGFR) on the apical side. EGFR signalling in epithelial cells releases TGF-\(\beta\), which activates fibroblasts to differentiate into myofibroblasts (sensitises more extracellular matrix components)\textsuperscript{320}. Damaged epithelial cells (and innate lymphoid cells) also secrete amphiregulin that can bind EGFR and promote airway remodelling \textsuperscript{296} (Fig. 6.1F & G). Remodelled airways cause structural changes that will impact on the immune response against respiratory bacteria.
Figure 6.1: Possible factors involved in bacterial exacerbation in the allergic lung. If Streptococcus pneumoniae gains entry into the lungs undergoing an allergic immune response, there are a number of factors that could increase the threshold of initiation of an effective antibacterial response—highlighted below. A. Many allergens can cleave the epithelial tight junctions molecules and subsequently other pathogens into the lungs. B. TLR signalling on epithelial cells induces NF-κB activation and subsequent cytokine release. IL-25, IL-33 and Thymic stromal lymphopoietin (TSLP) stimulate innate lymphoid cells and C. alternatively activate macrophages leading to secretion of IL-13, reducing the antibacterial capacity of alveolar macrophages. D. TSLP also conditions dendritic cells to release CCL17 and CCL22 to recruit T helper 2 (Th2) cells. Th2 cells have limited antibacterial effects compared to Th1 cells. E. Th2-associated cytokines IL-4, IL-5, IL-9 and IL-13 are involved in prolonging the survival of eosinophils, activation of mast cells and induction of goblet cell hyperplasia. Eosinophils and mast are critical in helminth infections, they have limited role in bacterial infections. F. In resolution, resident macrophages uptake and clear apoptotic cells from the lung while releasing transforming growth factor (TGF)-β, IL-10 and prostaglandin E2.
(PGE2) involved in lung structural changes. G. Damaged epithelial cells (and innate lymphoid cells) also secrete amphiregulin that can bind EGFR and promote airway remodelling. Remodelled airways involve structural changes that will impact on the immune response against respiratory bacteria.

6.6 Future work

The next set of experiments that can carry forward the theme of this thesis is assessing S. pneumonia susceptibility in mice deficient in key allergic airways disease associated cytokines such as IL-33 and IL-13. Furthermore, HDM has a substantial effect on alveolar macrophages and their role can further be assessed by macrophage depletion studies.

We were unable to assess lung function in this thesis because we did not have the equipment in category II facility. However, it will be useful to measure lung function as a functional correlate to cellular and structural changes in the airways.

The role of airway epithelial cells in initiating an effective bacterial response should be characterised including the response of airway epithelial cells to S. pneumonia and TLR ligands in vitro and in vivo by assessing cytokine/chemokine (TNF and CXCL2) and anti-microbial peptide (CRAMP and β-defensins) release and also the impact of HDM proteases and S. pneumonia virulence factors on epithelial tight junctions.

In chapter 5, ST2 deficient mice were used to assess the impact of attenuated allergic airway inflammation on susceptibility to bacterial infection. We could take this work further and assess the impact of an allergen with (HDM) and without (OVA) protease activity on bacterial susceptibility.
Reference List


Publications


Abstract presentations

- Poster Presentation at Imperial College Graduate School Research Symposium (July 2013) – Highly Commended Poster Prize Award
- Poster presentation at FOCIS 2012, Vancouver (June 2012)
- Poster presentation at BSI, Liverpool (Dec 2011) – Best Poster Prize for ‘Innate Immunity’ Section
- Oral presentation at CRI symposium, London (Mar 2011)
- Oral presentation at MRC-Asthma UK Science Day, London (Mar 2011)
- Poster presentation at EAACI Winter Allergy School, Davos, Switzerland (Feb 2011)
- Poster presentation at BSI, Liverpool (Dec 2010)
- Oral presentation at MRC-Asthma UK Away Day, Brighton (Sept 2010)
Appendices


Email replies from editors of Mucosal Immunology.

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Dear Maryam,

As long as you appropriately cite the original published manuscript in MI as the source then you may certainly use your data and figures in your thesis.

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Denise

Denise Gibson
Managing Editor
Mucosal Immunology

From: maryam.habibzay09@imperial.ac.uk
To: c.lobello@us.nature.com; mi@nature.com
Subject: FW: Mucosal Immunology, DOI: MI.2012.28, Online Publication Notification
Date: Thu, 18 Jul 2013 12:52:15 +0000

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Regards,

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From: Habibzay, Maryam [mailto:maryam.habibzay09@imperial.ac.uk]
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To: Lobello, Carol; Mucosal Immunology
Subject: FW: Mucosal Immunology, DOI: MI.2012.28, Online Publication Notification

Dear MI editors

We published our research last year in your journal. The title of the article is 'Altered regulation of toll-like receptor responses impairs anti-bacterial immunity in the allergic lung' (available free in pubmed).

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