Respiratory Innate Immune Factors Regulate Steroid-resistant Airway Hyperreactivity and Asthma

By

Jingjing Li

Master of Science

Research Center for Asthma and Respiratory Disease,

Discipline of Immunology and Microbiology,

School of Biomedical Sciences and Pharmacy,

Faculty of Health,

The University of Newcastle

NSW, Australia

A dissertation submitted as fulfillment of the requirements for the award of a PhD degree (Immunology and Microbiology).

January, 2014
LIST OF PUBLICATIONS

PUBLICATION #1

**Jing Jing Li**, Wan Wang, Katherine J. Baines, Nikola A. Bowden, Philip M. Hansbro, Peter G. Gibson, Rakesh K. Kumar, Paul S. Foster and Ming Yang. IL-27/IFN-γ Induce MyD88-Dependent Steroid-Resistant Airway Hyperresponsiveness by Inhibiting Glucocorticoid Signalling in Macrophages. *J. Immunol.* 2010; 185; 4401-4409

PUBLICATION #2


PUBLICATION #3

LIST OF ADDITIONAL PUBLICATIONS WITH RELEVANCE TO THIS THESIS


THESIS STATEMENTS

This thesis contain no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Jingjing Li
January 2014
ACKNOWLEDGEMENTS

Firstly I would like to thank my primary supervisor, Dr. Ming Yang, and co-supervisor, Prof. Paul Foster, whose encouragement, support and supervision made this work possible. Thank you also to Prof. Philip M. Hansbor, Dr. Nicole Hansbro, Dr. Steven Maltby, Ms Fiona Eyers and Dr. Jay Horvat. Your expertise, knowledge and advice have been proven invaluable during my studies. Special thanks to Prof. Paul D. Allen of Harvard Medical School and Prof. Rakesh K. Kumar of The University of New South Wales for their stimulating discussions and critical suggestions.

Thank you also to all the staff and students in the groups of Foster, Hansbro and Mattes, who have provided their kindly assistance and friendship throughout these years. Special thanks to Mr. Hock L. Tay, Mr. Maximilian Plank, Dr. Gerard Kaiko, Dr. Eric Lam, and Dr. Adeline Foo. I am very lucky to have a brother-sister-hood friendship with you guys.

I would very much like to thank my family, my parents and my brother, who inspired, encouraged and supported me throughout my study. Also thanks to my dear friends, Prof. Xudong Zhang, Dr. Nagaraj Gopisetty and Ms. Donna Meredith, who have provided their generous helps and guides.

At last I would like to thank University of Newcastle and Asthma CRC who provided scholarships to make this work possible.
# TABLE OF CONTENTS

- LIST OF FIGURES AND TABLES ................................................................. 11
- ABBREVIATIONS .................................................................................. 13
- ABSTRACT ............................................................................................ 16

Chapter 1: Introduction ........................................................................... 18

1.1 Overview of Asthma Disease ......................................................... 19
1.2 Immunology of Asthma ................................................................. 19
1.3 Asthma Management ..................................................................... 24
  1.3.1 Glucocorticoids ......................................................................... 25
  1.3.1.1 Molecular Basis of GC Function ........................................ 26
  1.3.1.1.1 GC Receptor Structure and Expression .......................... 26
  1.3.1.2 GR Nuclear Translocation ...................................... 28
  1.3.1.3 GRs and Gene Transcription ......................................... 29
1.4 Asthma and Steroid Resistance ..................................................... 31
  1.4.1 Steroid Resistant Asthma ........................................................ 31
  1.4.2 Molecular Basis of Steroid Resistance .................................... 33
  1.4.2.1 Defect of GRs Expression and Ligand Binding ............... 33
  1.4.2.2 Impaired GRs Nuclear Translocation and GR-GRE Binding ......................................................... 34
  1.4.2.3 Crosstalk with Other Transcription Factors ...................... 35
1.5 Asthma Phenotype .......................................................................... 36
  1.5.1 Non-eosinophilic Asthma ........................................................ 37
  1.5.2 Innate Immune Response in Steroid Resistant Asthma .......... 37
  1.5.3 Infection and Asthma .............................................................. 38
  1.5.3.1 Toll-like Receptors (TLRs) ........................................... 39
  1.5.4 Interferon-γ (IFN-γ) ............................................................... 42
1.5.5 Chemosensors and Asthma ........................................................................... 43
1.5.5.1 Olfactory Receptor (OR) ....................................................................... 44
1.5.6 Innate Immune Cells .................................................................................. 46
1.5.6.1 Macrophages ......................................................................................... 46
1.5.6.2 Alveolar Macrophages .......................................................................... 47
1.5.6.3 Macrophage Polarization ...................................................................... 48
1.5.6.4 Macrophage Recruitment and Chemotaxis .......................................... 49

1.6 MicroRNAs in Immune Response and Asthma ............................................... 50
1.6.1 Biogenesis of microRNA .......................................................................... 50
1.6.2 Modulation of miRNAs Expression .......................................................... 52
1.6.3 Emerging Role of microRNA in Asthma .................................................... 52
1.6.4 Therapeutic Potential of Targeting miRNAs as New Approach to Treat Asthma ................................................................................................................. 55

1.7 Study rational .................................................................................................. 55

Chapter 2: IL-27/IFN-γ Induce MyD88- Dependent Steroid-Resistant Airway Hyperresponsiveness by Inhibiting Glucocorticoid Signalling in Macrophage ................................................................................................................. 57

2.1 Abstract ........................................................................................................... 58

2.2 Introduction ...................................................................................................... 58

2.3 Materials and Methods .................................................................................. 62
2.3.1 Animals .................................................................................................... 62
2.3.2 Administration of IL-27, IFN-γ, and/or LPS, and Ab neutralization of IL-27 .............................................................................................................. 63
2.3.3 Analysis of induced sputum samples from human asthma patients .......... 63
2.3.4 Administration of dexamethasone ............................................................. 64
2.3.5 Measurement of lung function .................................................................. 65
2.3.6 Isolation of adherent cells, epithelial cells, and nonadherent cells
2.3.7 Analysis of cytokines by ELISA
2.3.8 Quantitative PCR
2.3.9 Inhibition of NF-κB, JNK, or p38 activation
2.3.10 Depletion of IFN-γ, CD4+, CD8+, and NK cells and pulmonary Macrophages
2.3.11 Flow cytometry
2.3.12 Transfer of isolated wild-type macrophages into MyD88-/- mice
2.3.13 Immunofluorescence detection of GRs
2.3.14 Data analysis

2.4 Results
2.4.1 IFN-γ/LPS increases activation and numbers of CD11b+ pulmonary macrophage
2.4.2 IFN-γ/LPS administration induces increased expression of IL-27 in pulmonary macrophages
2.4.3 Levels of IL-27 p28 and IFN-γ are concurrently increased in neutrophilic but not eosinophilic asthma
2.4.4 Cooperative interaction between IL-27 and IFN-γ contributes to the development of AHR
2.4.5 IL-27/IFN-γ–induced AHR is abolished by the depletion of pulmonary macrophages
2.4.6 IL-27/IFN-γ–induced AHR is not inhibited by the suppression of NF-κB, JNK, or p38
2.4.7 IL-27/IFN-γ–induced AHR is underpinned by the activation of MyD88 in pulmonary macrophages
2.4.8 IL-27/IFN-γ induces steroid-resistant AHR and suppresses the translocation of the GR to the nucleus of pulmonary macrophages
Chapter 3: Activation of Olfactory Receptors on Mouse Pulmonary Macrophages Promotes Monocyte Chemotactic Protein-1 Production

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.3.1 Mice

3.3.2 Administration of IFN-γ, LPS or γ/LPS

3.3.3 Gene chip microarray

3.3.4 Pulmonary macrophage isolation and treatment

3.3.5 Preparation of peritoneal macrophages and macrophage migration assay

3.3.6 Bone marrow derived macrophage (BMDM) culture and polarization

3.3.7 Quantitative polymerase chain reaction (q-PCR)

3.3.8 Immunofluorescence detection of OR622

3.3.9 ELISA

3.3.10 Macrophage phagocytosis of non-typeable Haemophilus influenzae (NTHi)

3.3.11 Data analysis

3.4 Results

3.4.1 Treatment with γ/LPS up-regulates OR expression levels in mouse airway tissue

3.4.2 γ/LPS synergistically enhance the expression levels of ORs in pulmonary macrophage

3.4.3 Olfactory agonists stimulate MCP-1 production by pulmonary macrophages
3.4.4 Octanal exposure promotes γ/LPS-induced MCP-1 macrophage migration

3.4.5 Octanal does not influence macrophage polarization

3.4.6 Octanal does not affect the phagocytosis of NTHi by pulmonary macrophages

3.5 Discussion

Chapter 4: MiR-9 Induction Contributes to Steroid-resistant Airway Hyperresponsiveness By Reducing PP2A Activity

4.1 Abstract

4.2 Introduction

4.3 Methods

4.3.1 Mice

4.3.2 Pulmonary macrophage isolation and stimulation

4.3.3 Collection of induced sputum samples from human asthma patients

4.3.4 In vivo administration of IFN-γ, LPS or γ/LPS or DEX or AAL(s)

4.3.5 Induction of allergic airway inflammation and prolonged airway hyperresponsiveness

4.3.6 AHR measurement

4.3.7 MiRNA target prediction and luciferase reporter assay

4.3.8 Quantitative assessment of miRNA expression

4.3.9 Quantitative assessment of mRNA expression

4.3.10 Western Blot

4.3.11 Immunofluorescent detection of GR localisation

4.3.12 PP2A activity assay

4.3.13 Data analysis

4.4 Results
4.4.1 MiR-9 expression in upregulated under conditions that induce steroid-resistant AHR.................................................................139
4.4.2 Inhibition of miR-9 restores steroid-sensitivity, reducing AHR induced by γ/LPS.................................................................................142
4.4.3 MiR-9 expression inhibits PP2A activity by targeting the regulatory subunits PPP2R5D and PPP2R2A.................................................................................145
4.4.4 MiR-9 reduces GR nuclear translocation.......................................................................................................................150
4.4.5 Altered PP2A activity effects induced AHR.................................................................152
4.4.6 Ant-9 treatment reduces AHR in LPS co-exposure and exacerbation models of OVA-induced allergic AHR.................................................................................................154

4.5 Discussion.................................................................................................................156

Chapter 5: Discussion.................................................................................................162

Chapter 6: Future Directions.......................................................................................169

REFERENCES.............................................................................................................174
LIST OF FIGURES AND TABLES

Fig.1.1: Immune system activation in the induction of asthma……………………………………23
Fig.1.2: Structure of the GR gene and proteins. .................................................................28
Fig.1.3: Mechanism of glucocorticoid action.................................................................31
Fig.1.4: Multiple patterns of agonist-ORs combination.................................................45
Fig.1.5: The biogenesis process and function of miRs.................................................51
Fig.2.1: Exposure of the airway to IFN-γ and LPS induces recruitment and activation of
CD11b+ macrophages, with increased production of IL-27 from macrophages…75
Fig.2.2: Synergism between IL-27 and IFN-γ contributes to the development of AHR
independently of neutrophilia.....................................................................................77
Fig.2.3: Macrophages are critical for IL-27/IFN-γ–induced AHR.........................................79
Fig.2.4: The development of IL-27/IFN-γ–induced AHR does not involve NF-κB, JNK, or
p38................................................................................................................................81
Fig.2.5: The induction of AHR by IL-27/IFN-γ is dependent on MyD88 signalling in
pulmonary macrophages. ............................................................................................83
Fig.2.6: IFN-γ/LPS and IL-27/IFN-γ administration leads to the development of
DEX-resistant AHR. ........................................................................................................85
Fig.2.7: IFN-γ/LPS and IL-27/IFN-γ administration impair steroid induced nuclear
translocation of GR in pulmonary macrophages..........................................................86
Fig.3.1: OR expression in the lung is induced by IFN-γ + LPS stimulation......................106
Fig.3.2: OR expression in different mouse tissues.........................................................107
Fig.3.3: ORs are expressed by mouse pulmonary macrophages and upregulated by
IFN-γ + LPS stimulation............................................................................................109
Fig.3.4: OR622 protein expression in mouse pulmonary macrophages...........................110
Fig.3.5: Effects of macrophage OR activation on the expression of proinflammatory
genes, chemokines and cytokines.............................................................................113
Fig.3.6: Octanal stimulation increases IFN-γ and LPS-induced MCP-1 expression in
mouse pulmonary macrophages..............................................................................114
Fig.3.7: OR agonists induce MCP-1 expression in mouse pulmonary macrophages.

Fig.3.8: Supernatants from macrophage cultures stimulated with octanal, IFN-γ and LPS induce macrophage migration.

Fig.3.9: OR activation has no effect on cultured pulmonary macrophage polarization.

Fig.3.10: OR activation has no effect on cultured bone marrow derived macrophage polarization.

Fig.3.11: Exposure to OR agonist has no effect on macrophage phagocytic capacity for NTHi.

Fig.4.1: miR-9 expression is induced by γ/LPS stimulation.

Fig.4.2: Inhibition of miR-9 restores DEX sensitivity attenuating AHR induction by γ/LPS.

Fig.4.3: Effect of ant-9 on airway cell infiltration following γ/IFN administration.

Fig.4.4: Targeting of PPP2R5D and PPP2R2A mRNA by miR-9.

Fig.4.5: Targeting miR-9 restores PPP2R5D expression and PP2A activity.

Fig.4.6: Effect of miR-9 on PPP2R2A expression.

Fig.4.7: Inhibition of miR-9 restores DEX-induced GR nuclear translocation.

Fig.4.8: Increasing PP2A activity restores steroid-sensitivity and GR nuclear translocation.

Fig.4.9: Inhibition of miR-9 restores steroid sensitivity, thus blocking the development of AHR in allergen-induced models of steroid-resistant AHR.

Fig.4.10: Schematic representation of miR-9 effects on glucocorticoid function in pulmonary macrophage following LPS+IFN-γ exposure.

Fig.5.1: Proposed mechanism of steroid-resistance induced by LPS and IFN-γ.

Fig.5.2: Proposed mechanism of interaction between OR pathway and IFN-γ/LPS pathway in inducing MCP-1 production.

Table 3.1: Primer sequences of ORs.

Table 3.2: Primer sequences of other genes.
ABBREVIATIONS

AAL(S) 2-amino-4-(4-heptyloxyphenol)-2-methylbutanol
AAVs adeno-associated viruses
Ant-9 antagonomir-9
AHR airway hyperresponsiveness
AP-1 activator protein 1
APCs antigen presenting cells
ARG1 arginase-1
BALF broncho alveolar lavage fluid
pCAF p300/CBP-associated factor
CBP cyclic AMP response element-binding protein
(CREB)-binding protein
CDK cyclin-dependent kinase
CSF-1 colony-stimulating factor
DAMPs damage associated molecular patterns
DC dendritic cell
DEX dexamethasone
DMEM Dulbecco's Modified Eagle Medium
EBI3 EBV-induced gene 3
FCS fetal calf serum
FEV1 forced expiratory volume in one second
FGR familial GC resistance
FIZZ1 resistin-like molecule-α
GCs glucocorticoids
GM-CS granulocyte macrophage colony-stimulating factor
GLIZ GC-induced leucine zipper protein
GP G protein-coupled receptor
GR Glucocorticoid receptor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>GREs</td>
<td>GC response elements</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HAT</td>
<td>histone acetyltransferase</td>
</tr>
<tr>
<td>HAD</td>
<td>CHAT activity and recruit histone deacetylase</td>
</tr>
<tr>
<td>HDM</td>
<td>house dust mite</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>Hsps</td>
<td>heat shock proteins</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of nuclear factor-κB</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>IFNγR</td>
<td>IFN-γ receptor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IRFs</td>
<td>interferon regulatory factors</td>
</tr>
<tr>
<td>i.t.</td>
<td>intratracheally</td>
</tr>
<tr>
<td>KC</td>
<td>keratinocyte-derived chemokine</td>
</tr>
<tr>
<td>LBD</td>
<td>ligand-binding domain</td>
</tr>
<tr>
<td>lipo-C12MDP</td>
<td>liposome-encapsulated clodronate</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>miR</td>
<td>microRNA</td>
</tr>
<tr>
<td>miRISC</td>
<td>miRNA-induced silencing complex</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>MKP</td>
<td>mitogen-activated protein kinase phosphatase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappaB</td>
</tr>
<tr>
<td>iNOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NTHi</td>
<td>non-typeable <em>Haemophilus influenzae</em></td>
</tr>
</tbody>
</table>
OR  olfactory receptor
OVA  ovalbumin
PAMPs  pathogen associated molecular patterns
PBMC  peripheral blood mononuclear cell
PMN  polymorphonuclear neutrophil
PP2A  protein phosphatase 2A
PPP2R5D  protein phosphatase 2, regulatory subunit B
PRRs  pattern recognition receptors
Raw  airway resistance
RLRs  RIG-I-like receptors
pri-miRNA  primary RNA
RSV  respiratory syncytial virus
RT  room temperature
SABAs  beta2-adrenoceptor agonists
SCFAs  short chain fatty acids
SEB  staphylococcus aureus enterotoxin B
SLPI  secretory leukoprotease inhibitor
SRC  steroid receptor co-activator
STATs  signal transducer and activator of transcriptions
Th  T helper cell
Ticam-1  Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1
TIR  Toll / interleukin-1 receptor-like domain
TLRs  Toll-like receptors
(TNF)-α  tumor necrosis factor-α
Tirap  TIR domain-containing adaptor protein
Trif  TIR-domain-containing adapter-inducing interferon
UTR  untranslated regions
ABSTRACT

Asthma is a chronic inflammatory disease of the airways and a combination of genetic and environmental factors underpin the pathogenesis. The clinical symptoms of asthmatics most mild to moderate, allergic asthma patients can be effectively managed by combination therapy with broad-spectrum anti-inflammatory agents and bronchodilators (typically inhaled glucocorticoids and long acting β-agonists). Indeed, glucocorticoids remain the forefront therapeutical approaches for the treatment of asthma. However, 5-10% of asthmatics who have severe asthma do not respond to treatment, and these patients account for almost 50% of asthma-related healthcare costs. Thus it is essential to understand the pathogenesis of steroid resistance in severe asthma for the development of more efficient therapies for those patients. With well-established animal models of steroid resistant airway hyper-responsiveness (AHR, a hallmark feature of asthma) and in vitro culture systems of pulmonary macrophages, the underlying mechanisms regulating steroid resistance and exacerbation of asthma have been thoroughly investigated, particularly on the causative roles of innate immune factors. This thesis consists of three publications. The first publication identifies changes in the expression of key innate immune molecules and their signalling pathways in a mouse model of steroid-resistant AHR and demonstrates the central role of pulmonary macrophages in the induction of steroid-resistant AHR. The second publication investigates the expression of olfactory receptors in the respiratory system and on immune cells in response to innate immune activation, and identifies a potential
role of olfactory receptors in regulating the function of pulmonary macrophages. The final publication discusses the modulation of small non-coding RNAs, microRNA, expression by innate immune activation in a steroid-resistant mouse model of asthma and evaluates the role of key microRNAs involved in the induction of steroid-resistant AHR by regulating the activity of a critical phosphatase, protein phosphatase-2A, which further affected the function of glucocorticoid.
Chapter 1.

Introduction
1.1 Overview of Asthma

Asthma is a worldwide public health problem affecting 235-300 million people in 2011, and approximately 250,000 people per year die from this disease (GINA, 2011). Asthma is also an important health problem in Australia. In 2011-2012, it is reported that 10.2% of Australians had asthma and about 8 people die from asthma every week in Australia, according to the Australian Bureau of Statistics. Although great advancement has been achieved in asthma diagnosis and treatment, it is estimated by the World Health Organization that by 2025 the number of people with asthma will grow by more than 100 million people worldwide (WHO, 2007).

Asthma is a chronic inflammatory disorder of the airways characterised by recurring clinical symptoms, reversible airflow obstruction, and bronchospasm, which causes substantial morbidity and mortality among its sufferers (NHLBI, 2007). Common clinical symptoms include chest tightness, wheezing, coughing, and breath shortness (Bateman et al., 2008). Airway hyper-responsiveness (AHR) to non-specific spasmogens is one of the hallmark features of asthma. Furthermore, airway inflammation, tissue remodelling and mucus hypersecretion may contribute to the development of AHR in asthma (Hershenson et al., 2008).

1.2 Immunopathogenesis of Asthma

Asthma is increasingly recognised as a heterogeneous inflammatory disorder, critically regulated by aberrant immune responses (including both innate and adaptive forms), and results in airway dysfunction (Fig.1.1). Thus, the chronic pathophysiology process
leads to that airway is more susceptible to a variety of environmental insults, such as bacteria, viruses, air pollutants, chemicals and aeroallergens, and generates various altered repair responses (Holt et al., 2010; Holgate, 2012; Holtzman, 2012). Different combination of these processes results in different asthma subphenotypes, so the understanding of immunological decision making in asthma natural history is fundamental. The perturbation of airway epithelium by allergen, infection and environmental pollutants generates initial danger signal and recruit immature dendritic cells (DCs) to the mucosal epithelium. Moreover, a certain class of microbes shares a certain conserved molecular motif, called pathogen-associated molecular pattern (PAMP), which can be recognised by DCs via pattern recognition receptors (PRRs) (Holgate, 2012). PAMPs can also be detected by other innate immune cells leading to the release of various endogenous tissue factors or damage associated molecular patterns (DAMPs) which then activate DCs (Holgate, 2012). Activated DCs process allergens and migrate to secondary lymphoid tissues where they regulate the differentiation and polarization of T helper (Th) cells. Conventionally, the adaptive immune system can be divided into Th1 and Th2 types of responses according to T cells polarization subsets (Bruce Alberts, 2002). A Th1 type response is characterised by the production of Th1 cytokines (e.g. IFN-γ and IL-12) which activate the bactericidal activities of macrophages, induces B cells to make opsonizing and complement-fixing antibodies, and leads to the activation of “cell-mediated immunity”, primarily mediating clearance of intracellular bacterial and viral infections (Adler, 2008). A Th2 type
response is characterised by the secretion of Th2 cytokines including (e.g. IL-4, IL-5 and IL-13) from Th2 cells (Adler, 2008). This results in the activation of B cells to make neutralizing non-cytolytic antibodies, and leads to “humoral immunity”, primarily mediating protection against extracellular bacteria and parasites (Adler, 2008). The pathogenesis of asthma is classically hypothesised as the bias toward an aberrant allergen-specific CD4+ Th2 response with an increase in Th2 cellular responses and a decrease in Th1 responses (Ying et al., 1995; Cohn et al., 1998). Several studies have suggested that a Th1 response counteracts a Th2 response, thus inhibiting Th2 cells-regulated inflammation and AHR. However, other studies showed that severe asthma, different from mild to moderate allergic asthma, is characterised by a mixed Th2/Th1 phenotype with Th1 cytokines, such as IFN-γ, induced neutrophils or a mixed granulocytic airway infiltration (Hansbro et al.; Magnan et al., 2000; Cho et al., 2005). This sub-population of asthmatics is refractory to steroid treatment and microbe infections are implicated in the induction and progression of disease (Litonjua et al., 2003; Hansbro et al., 2004).

Over the last few years, there are emerging studies suggesting that other T cell subsets, such as Th17 cells (characterised by producing IL-17, IL-22, TNF-α and IL-6) and regulatory T (Treg) cells (characterised by producing IL-10 and TGF-β), play important roles in the development of asthma (Alcorn et al., 2010; Krishnamoorthy et al., 2012; Lloyd et al., 2009; Cosmi et al., 2011)). A Th17 response mainly mediates protection against extracellular bacteria and fungi (Martinez et al., 2008). IL-17 levels
in serum and airway are increased in severe compared to mild asthma (Agache et al.; Bullens et al., 2006). IL-17 promotes neutrophiles influx into the airway and airway remodelling (Chakir et al., 2003). Th17 also has been shown responsible for steroid-resistance in murine models of asthma (McKinley et al., 2008). Treg cells suppresses Th1, Th2 and Th17 over-responses and maintain a homeostatic balance to avoid over activation of immune responses (Larche, 2007). In asthma patients, both the number and the suppressive function of Treg cells are significantly reduced in serum and/or sputum, and individuals with exacerbations their number drops further (Grindebacke et al., 2004; Ling et al., 2004; Lee et al., 2007; Mamessier et al., 2008). Studies with murine models of asthma have shown the requirement of Treg in controlling asthma, and that adoptive transfer of Tregs attenuates the development of airway inflammation and AHR (Kearley et al., 2005; Strickland et al., 2006; Kearley et al., 2008).

Moreover, increasing studies suggest that asthma is more than an allergic inflammation. In particular, the role of innate immune pathway in the exacerbation and the steroid resistance of asthma has drawn attention (Dombrowicz, 2005; Herbert et al., 2010; Yang et al., 2010; Yang et al., 2012). Innate immune cells (pulmonary macrophage) are at the front line of host defense against microbial infection, in turn affecting the development of asthma (Holgate, 2012). For example, human rhinorvirus causes wheezing in babies and young children, which is a major predictor of asthma at 6 years of age (Jackson et al., 2008; Gern, 2009). Moreover, human rhinorvirus is the
most frequent viral cause of adult asthma exacerbations (Wos et al., 2008). Recent findings suggest that allergens drive allergic responses in asthmatic patients by stimulating innate immune cells, called microbial mimics, and the protease activity of allergens increase their interactions with in immune system (Wills-Karp et al., 2010). Thus, it is critical to investigate the roles of innate immune cells in the development of asthma for exploring novel and more effective method to manage the disease.

![Immune system activation in the induction of asthma](image)

Fig.1.1 Immune system activation in the induction of asthma. Perturbation of airway epithelium with infection provides the initial danger signal and leads to the trafficking of DC to respond to danger signals via PRRs. Activated DC then presents antigen to naive T cells via TCR, MHC II and co-stimulatory molecules to drive differentiation of Th cells and further results in activation of two types of immunity--- cell-mediated
immunity and humoral immunity. Pathogens that accumulate inside macrophage and dendritic cells tend to stimulate the differentiation of Th1 cells, and extracellular antigens tend to stimulate the differentiation of Th2 cells. Th1 cells initiate cell-mediated immunity by activating microbicidal properties of macrophages and induction of B cells to make IgG antibodies to opsonizing extracellular pathogens for uptake by phagocytic cells. Th2 cells initiate humoral immunity by activating naïve antigen-specific B cells to produce IgM antibodies, and subsequently produce IgA, IgE and weakly opsonizing subtypes of IgG.

1.3 Asthma Management

As asthma is a chronic disease, appropriate management of the disease can control its development and improve the quality of patients’ life. Customized plans for asthma sufferers include reducing exposure to allergens, testing to assess the severity of symptoms, and medications (GINA, 2011).

Asthma medications are generally divided into two general classes: quick-relief medications used to treat acute symptoms; and long-term control medications used to prevent further exacerbation (NHLBI, 2007). Fast-acting medications include: β2-adrenoceptor agonists (SABAs), such as salbutamol, which are generally the first line treatment for asthma symptoms; anticholinergic medications, provide greater benefits of treatment when used in combination with SABAs or can be used alone if patients cannot tolerate SABAs; and adrenergic agonists, which have a similar efficacy
to SABAs (Rodrigo and Nannini, 2006). Glucocorticoids (GCs) are generally considered the most effective long-term treatment for asthma (NHLBI, 2007). GCs are a class of steroid hormones that bind to the glucocorticoid receptor (GR), which is present in almost every vertebrate animal cell (Kadmiel and Cidlowski, 2013). GCs are part of the feedback mechanism in the immune system that turns immune activity (inflammation) down. Other long-term medications, such as long-acting beta-adrenoceptor agonists (LABA) and leukotriene antagonists, can be used in addition to inhaled corticosteroids (Ducharme et al., 2010).

1.3.1 Glucocorticoids

GCs suppress overreactive immune responses, and is therefore used in clinic to treat inflammatory diseases, such as asthma (Szefler, 1991). GCs suppress both innate immunity and adaptive immunity. GCs inhibit the phagocytic and microbicidal function of phagocytes, (e.g. neutrophils and macrophages), and suppress the release of inflammatory cytokines (such as IL-1, TNF) (Smoak and Cidlowski, 2004). GCs also inhibit the survival of eosinophils and degranulation of mast cells (Druilhe et al., 2003; Zhou et al., 2008). GCs suppress mature DC but not immature DC due to differential expression of GR translational isoforms (Cao et al., 2013). GCs decrease the number of circulating T cells by inhibition of IL-2 and IL-2 signaling, impaire the migration of T cells from lymphoid tissues (Bianchi et al., 2000). GCs inhibit the generation of Th1 and Th2 cytokines, and inhibitory effect on Th1 cytokines is greater (Ashwell et al., 2000). GCs amplify IL-2-dependent expansion of functional T regulatory cells and
enhance their suppressive capacity (Chen et al., 2006). GCs mildly reduce the number of circulating B cells and low concentration of glucocorticoid could suppress mitogen activation of B cells, but physiological concentration or typical therapeutic-range doses exerted stimulatory effects on B cells antibody production (Kovacs, 2014). Dexamethasone is a potent synthetic member of the glucocorticoid class of steroid drugs that has anti-inflammatory and immunosuppressant properties (Le Jeunne, 2012).

1.3.1.1 Molecular Basis of Glucocorticoid Function

1.3.1.1.1 Glucocorticoid Receptor Structure and Expression

GCs exert their function by binding to the GC receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors. GRs contain several functional domains, including a ligand-binding domain (LBD), a DNA-binding domain and two domains with activation function; AF-1 and AF-2, which are involved in gene transactivation (Encio et al., 1991) (Fig.1.2). Alternative splicing of the human form of glucocorticoid receptor (GR) primary transcript generates two protein isoforms: GRα and GRβ. In physiological conditions, the default splicing pathway is the one leading to GRα (Pujols et al., 2002). While GRα binds only GCs and functions as a ligand-dependent transcription factor, GRβ is constitutively localised in the nucleus of cells and interacts with DNA but not with GCs, and GRβ does not activate glucocorticoid-responsive promoters (Hollenberg et al., 1985; Giguere et al., 1986). Increasing studies revealed GRβ is widely expressed and acts as a dominant negative inhibitor for GRα transcriptional regulation and correlated with glucocorticoid
resistance (Bamberger et al., 1995; Oakley et al., 1997). In addition to GRα and GRβ, several other GR splice variants have also been identified, and have been linked to glucocorticoid resistance in childhood acute lymphoblastic leukemia (Beger et al., 2003). Other splice variants have been reported in mouse cells: GRP retains the intron between exons 7 and 8 while missing the appropriate exons 8 and 9; GRA lacks the entire sequences of exons 5, 6 and 7 (de Lange et al., 2001). These alternatively spliced GR transcripts may also participate in the reduction of GC responsiveness in myeloma patients (Moalli et al., 1993; Krett et al., 1995). However, their clinical significance has been questioned due to the relatively low expression level (Pujols et al., 2002; Pedersen et al., 2003). As GRβ is not expressed in mouse cells and we used mouse model in our whole study, so this study will focus on the functional isoform GRα (all GR mentioned below is refer to mouse GRα).
Fig.1.2 Structure of the GR gene and proteins. Alternative processing of exon 9 of GR primary transcript generates multiple GR messages, and results in two protein isoforms: GR-α and GR-β, both contain several functional domains, including a ligand-binding domain (LBD), a DNA-binding domain (DBD) and two domains with activation function; AF-1 and AF-2, which are involved in gene transactivation.

1.3.1.1.2 Glucocorticoid Receptor Nuclear Translocation

In the absence of GC, GR resides in the cytosol and combines with a chaperone complex, including various heat shock proteins (Hsps) (such as hsp90 and hsp70), immunophilins (such as FKBP51, FKBP52, Cyp44 and PP5) and other factors to prevent degradation and to assist maturation (Pratt et al., 1997; Cheung et al., 2000) (Fig.1.3). Once GC diffuses through the cell membrane into the cytoplasm and binds to GR, Hsps dissociate allowing the nuclear localization of activated GR-GC complex and its binding to DNA (Adcock, 2003). Then GR subsequently form a dimer combines with
another GR at consensus DNA sites termed GC response elements (GREs), which are located in the regulating regions of corticosteroid responsive genes (Adcock, 2003). This combination allows the GR to interact with transcriptional co-activators, SRC-1 and cyclic AMP response element-binding protein (CREB)-binding protein (CBP), to regulate gene transcription (Adcock, 2003). Therefore, the combination of GC with the GR, and the number and position of GREs will determine the response to GCs.

1.3.1.1.3 Glucocorticoid Receptors and Gene Transcription

Upon binding with GCs, activated GRs mediate the transcription of target genes in the nucleus of responsive cells. The major anti-inflammatory effects of GCs are through switching off activated inflammatory genes coding for cytokines, chemokines, adhesion molecules, inflammatory enzymes and receptors. There is no negative GREs in most responsive inflammatory genes promoter regions, thus, GCs exert its inhibitory effect through interaction between activated GRs and between GR with other transcription factors, such as Nuclear Factor-KappaB (NF-κB) and activator protein 1 (AP-1) (Ismaili and Garabedian, 2004). Activated GRs can directly bind to coactivators to inhibit their histone acetyltransferase (HAT) activity and recruit histone deacetylase (HDAC) 2 to reverses histone acetylation and suppress inflammatory genes activation (Ito, Barnes et al. 2000). In addition, it has been reported that GCs may inhibit the action of mitogen-activated protein kinases (MAPKs) through inhibiting their phosphorylation or inducing specific inhibitors (Reichardt, Kaestner et al. 1998; Reichardt, Tuckermann et al. 2001).
GCs also can exert their anti-inflammatory effect by increasing transcription of small numbers of anti-inflammatory genes. GR homodimers bind to the promoter region of steroid-responsive genes, where they interact with co-activator molecules, such as CBP, steroid receptor co-activator (SRC)-2/300/CBP-associated factor (pCAF) (Ito, Barnes et al. 2000; Ito, Jazrawi et al. 2001). The complex have intrinsic histone acetyltransferase (HAT) activity, thus selectively activating lysine acetylation of histone H4 at residues 5 and 16 (Ito, Barnes et al. 2000; Ito, Jazrawi et al. 2001). These interactions then lead to the increased expression of anti-inflammatory genes, such as secretory leukoprotease inhibitor (SLPI), GC-induced leucine zipper protein (GILZ), inhibitor of nuclear factor-κB (IκB) and mitogen-activated protein kinase phosphatase (MKP)-1 (Mittelstadt and Ashwell 2001; Lasa, Abraham et al. 2002). In addition, GRs can form heterodimers with other transcription factors, such as signal transducer and activator of transcriptions (STATs), to bind to DNA and specific co-activator or co-repressor complexes (Barnes and Karin 1997; Barnes and Adcock 1998; Hart, Lim et al. 2000). In addition, some side effects of GCs, such as osteoporosis, growth retardation in children, cataracts, metabolic effects and skin fragility, are caused by gene activation (Belvisi, Wicks et al. 2001; Schacke, Schottelius et al. 2004).
Fig.1.3 Mechanism of glucocorticoid action. After passing the cell membrane by passive diffusion, glucocorticoids bind to GR, and associated chaperon proteins are released. The ligand-bound receptor translocates into nucleus, and further exerts transcription suppression of pro-inflammatory genes or activation of anti-inflammatory genes.

1.4 Asthma and Steroid Resistance

1.4.1 Steroid Resistant Asthma

Currently, GCs are the front-line therapy used for asthma management because they effectively inhibit the development of airway inflammation and greatly improve the
clinical symptoms. However, a subset of asthmatic patients (approximately 5% to 10%) responds poorly to GC treatment. These patients contribute to half of the total health care costs and spend a disproportionate amount of physician time and resources (Barnes et al., 1998; Chung et al., 1999). Steroid-resistant asthma is defined as a failure of the forced expiratory volume in one second (FEV1) to improve from a baseline value of 75% of the predicted value or less by 15% or more after 14 days of treatment with 40mg of prednisolone taken orally, despite demonstrating greater than 15% reversibility to inhaled β2-agonist (Barnes, 1995). Steroid-resistant asthma was first reported in 1968 in six patients who had no clinical response or reduction in blood eosinophilia after treatment with high doses of systemic GCs (Schwartz et al., 1968). Carmichael et al., reported a larger group of 58 patients with less improvement in lung function after treatment with oral prednisolone. These steroid-resistant patients showed a longer duration of symptoms, lower morning lung function, and a more significant AHR than those steroid-sensitive patients (Carmichael et al., 1981). The clinical symptoms of the majority of steroid-sensitive asthma patients are well controlled with GCs with no side effects. However, steroid-resistant patients are subjected to prolonged high-doses of GC therapy, which may result in adverse side effects including osteoporosis, cataracts, diabetes and growth suppression (Barnes, 2006). Steroid-resistant asthmatic patients have worse health and poorer quality of life compared to their steroid-sensitive counterparts (Wenzel, 2005). The specific mechanisms responsible for a poor response to GC treatment in steroid-resistant asthma remain unclear.
Hypothalamic-pituitary-adrenal (HPA) axis was demonstrated to be impaired in asthma (Adcock et al., 2008). The severity of HPA axis suppression is associated with duration, route, dose and time of glucocorticoid administration (Moghaddam et al., 2012). Pro-inflammatory factors stimulate glucocorticoid release from all three levels (Sternberg et al., 1989; Calogero et al., 1992; Silverman and Sternberg, 2012). Thus, local environmental factors change can induce glucocorticoid resistance, including chronic inflammation or exposure to infectious agents. Glucocorticoid effects are ultimately determined by GR---its expression, binding affinity to ligands, nuclear translocation, DNA binding or interaction with other transcription factors. Thus, even the circulating glucocorticoid concentration is normal or elevated, impaired glucocorticoid responses can still occur at the cellular or molecular level.

1.4.2 Molecular Basis of Steroid Resistance

The mechanisms that lead to steroid-resistance may vary between patients. A reduction in the response to GC treatment may be the result of defects in GR expression, altered combination of ligand to GRs, blockage of activated GR nuclear translocation, reduced binding of GRs to DNA, or an increase in expression of inflammatory transcription factors that competitively bind to DNA.

1.4.2.1 Defect of Glucocorticoid Receptor Expression and Ligand Binding

The inherited syndrome familial GC resistance (FGR) is a very rare genetic disease and caused by mutations of the GR. These patients have high circulating levels of cortisol but lack any signs or symptoms of Cushing’s syndrome, a condition resulting from
prolonged exposure to GCs (Carmichael et al., 1981; Lamberts, 2001). Several abnormalities in GR function have been indicated in patients with familial glucocorticoid resistance, including decreased binding for cortisol, reduced numbers, thermolability and an abnormality binding to DNA, all of which are due to mutations of GR. (Carmichael, Paterson et al. 1981; Lamberts 2001). Another proposed mechanism for steroid-resistant asthma is an increase in the expression of GRβ. GRβ weakly interacts with transcriptionally inactive heat shock proteins, and dose-dependently inhibits GR-mediated transactivation through competing with GRα in the nucleus for co-activators or forms inactive transcriptional heterodimers (Bamberger et al., 1995; Gougat et al., 2002). It is reported that increased levels of GRβ were observed in bronchoalveolar lavage (BAL) cells from steroid-resistant asthma patients, but without GRβ gene expression in monocytes of patients with GR-insensitive compared to GR-sensitive asthma (Hamid et al., 1999; Gagliardo et al., 2000).

1.4.2.2 Impaired GRs Nuclear Translocation and GR-GRE Binding

Steroid-resistant asthma patients exhibited a poor response to a high concentration of dexamethasone with impaired nuclear translocation of activated GRs (Adcock et al., 1995). GR-GRE binding analysis showed a reduced number of GRs available for DNA binding, however there was no change in binding affinity in steroid-resistant asthma patients (Matthews et al., 2004). This defect might be associated with GR phosphorylation and interaction with importin-α, increased c-Fos expression, and c-Jun N-terminal kinase (JNK) activity and over-activation of AP-1, in response to
inflammatory stimuli, such as tumor necrosis factor-alpha (TNF-α) (Ismaili et al., 2004; Matthews et al., 2004; Szatmary et al., 2004). In addition, another group of steroid-resistant asthma patients showed normal nuclear localisation of GRs, but there was a defect in acetylation of histone 4 (Matthews et al., 2004). This defect might suggest that in these patients GCs were not able to activate certain genes to initiate the desired anti-inflammatory effect.

GRs can be phosphorylated at different sites, such as Human Serine 203 (Rat Ser 224/ Mouse Ser 212), Human Serine 211 (Rat Ser 232/ Mouse Ser 220), Human Serine 226 (Rat Ser 246/ Mouse Ser 234) and Human Serine 404 (Rat Ser 424/ Mouse Ser 412), by cyclin-dependent kinases (CDKs), CDK2 and CDK5, ERK, p38, JNK and glycogen synthase kinase 3 (GSK3) (Krstic et al., 1997; Itoh et al., 2002; Wang et al., 2002; Miller et al., 2005; Blind et al., 2008). The phosphorylation of GRs resulted in reduced GR activity. The phosphorylation of GR on Ser203 results in a transcriptionally inactive form(Takabe et al., 2008). Phosphorylation of Ser226 site of GR leads to blunted signalling due to enhanced nuclear export of GRs (Chen et al., 2008). Phosphorylation of Ser404 site attenuates GC signalling by recruitment of altered co-factor and re-direction of transcriptional response of GR (Galliher-Beckley et al., 2008).

1.4.2.3 Crosstalk with Other Transcription Factors

One of the main inhibitory effects inhibitory effect of GCs is due to protein / protein interactions between activated GRs and transcription factors of pro-inflammatory genes, such as NF-κB and AP-1 (Barnes et al., 1997; Lane et al., 1998; Hart et al., 2000;
Shaulian et al., 2002). Previous studies have demonstrated direct protein / protein
between activated GR and AP-1, and between NF-κB p65 and STAT5, suggesting that
GCs mediate the binding or activation of these transcription factors and thus regulate
inflammatory gene expression (Goleva et al., 2002). It is reported that the basal AP-1
DNA binding levels increase in peripheral blood mononuclear cells (PBMCs) of
steroid-resistant asthma patients but without alteration in the sequences of AP-1mRNA
(Adcock et al., 1995). It is also observed that expression of c-Fos increased significantly
in bronchial biopsies and PBMCs of patients with steroid-resistant asthma (Lane et al.,
1998). The tuberculin mononuclear cell inflammation model revealed that resistance to
corticosteroids in asthmatic subjects may be caused, at least in part, by failure to
suppress JNK phosphorylation, leading to failure to suppress c-jun N-phosphorylation
(Sousa et al., 1999).

1.5 Asthma Phenotypes

A large number of asthmatics have been identified with different subtypes of asthma
based on different airway inflammatory profiles. These subtypes include eosinophilic and
non-eosinophilic asthma. The main characteristics of eosinophilic asthma include an
imbalance in the inflammatory cell profile towards Th2 cells, increased IgE production
and increased recruitment of eosinophils and mast cells. However, asthma may persist
in absence of eosinophilic inflammation, which subtype is termed non-eosinophilic
asthma (Douwes et al., 2002). While these subtypes are differentiated at a cellular level
they all may present with similar clinical symptoms (Wenzel, 2012).

1.5.1 Non-eosinophilic Asthma

Although eosinophilic inflammation is classically recognised as one of the hallmarks of asthma (Gould and Sutton, 2008), accumulated studies have demonstrated these granulocytes may not play a dominant role in some subtypes, particularly in severe asthma (Wenzel et al., 1999; Ordonez et al., 2000). Indeed, only about 50% of asthmatics are characterised with eosinophilic asthma according to the cut off level of eosinophils infiltrated in the airway (2~4%) (Douwes et al., 2002). Non-eosinophilic asthma involves aberrant innate immune response, with Th1 lymphocyte-mediated cytokines release and an influx of neutrophils (Douwes et al., 2002). In patients with severe, persistent asthma symptoms, increased number of lung neutrophils is quite often observed (Gibson et al., 2001; Simpson et al., 2006). More importantly, non-eosinophilic asthma patients respond poorly to inhaled GC (Berry et al., 2007; Boulet, 2010). Studies have shown the presence of airway neutrophilain non-eosinophilic exacerbations (Wark et al., 2002). Patients with persistent asthma symptoms experience more non-eosinophilic exacerbations than eosinophilic exacerbations and GC treatment is not effective as a preventative therapy (Pavord et al., 1999; Green et al., 2002). Thus, it is important to further explore the pathogenesis of non-eosinophilic asthma for the development of novel and more effective treatment strategies.

1.5.2 Innate Immune Response in Steroid Resistant Asthma
Airway inflammation underpins the pathogenesis of asthma. Viral and bacterial infections can cause lower respiratory tract inflammation, acute bronchiolitis and exacerbations of asthma and these infections may contribute to GC-resistance in asthma. Chronic inflammation induced by persistent infection has also been associated with the development of steroid resistance (Adcock et al., 2003; Weinberger, 2004). Innate immunity is immediate host defense system within a short time after exposure to microbes. The major functions of the human innate immune system include: production of cytokines to recruit more immune cells to the site of infection; removing dead cells, antibody complexes or foreign substances; and activation of the adaptive immune system (Janeway, 2001). However, little is known of the role of innate immune in the regulation of steroid resistance. Therefore, in this study we aim to investigate the innate immune factors that contribute to steroid resistance in a mouse model of asthma and provide insightful clues for clinical treatment.

1.5.3 Infection and Asthma

Microbial infections, particularly in early childhood, are related to the development of asthma (Maddox and Schwartz, 2002). It has previously been demonstrated that respiratory bacterial infections have a significant role in the pathogenesis of asthma (Saglani et al., 2005; Saglani et al., 2007). Bacterial infections were consistent with pathological changes in infants and young children with recurrent wheeze, which is the major predictor of later asthma development (Bisgaard et al., 2007; Keski-Nisula et al., 2009). Epidemiologic data firstly link Chlamydia and Mycoplasma species to asthma
exacerbations, and were later shown to play a role in asthma pathogenesis (Leaver et al., 1985; Kraft et al., 1998). There is also a great deal of data supporting a causal link between respiratory viral infections during infancy and childhood with the later development of asthma, particularly picornaviruses (rhinovirus/enterovirus), respiratory syncytial virus (RSV), and human metapneumovirus (MPV) (Lee et al., 2007). Rhinovirus is most commonly associated with exacerbations of asthma and clinical studies have shown early rhinovirus infections were related with wheezing (Jackson et al., 2008; Kelly et al., 2008). Although the direct causation remains unclear, many studies suggest a clear relationship between viral infection and asthma development.

1.5.3.1 Toll-like Receptors (TLRs)

The microbe-specific molecules that are called pathogen-associated molecular patterns (PAMPs), including bacterial carbohydrates (such as LPS or mannose), nucleic acids (such as bacterial or viral DNA or RNA), bacterial peptides (flagellin, ax21), peptidoglycans and lipoteichoic acids (from Gram positive bacteria), N-formylmethionine, lipoproteins and fungal glucans, are recognised by given receptors, pattern recognition receptors (PRRs), expressed by immune cells. PRRs include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs), and DNA sensors (Fritz et al., 2006; Ishii et al., 2008; Beutler, 2009; Opitz et al., 2009). These receptors are widely expressed in alveolar macrophages, lung epithelial cells and DCs, which recognise invading pathogens and subsequently recruit immune cells (Fritz et al., 2006; Ishii et al., 2008; Beutler, 2009; Opitz et al., 2009). The PRRs within the
respiratory tract play an indispensable role in both acute and chronic disorders affecting the lung (Opitz et al., 2010). PRRs activation induce the production of inflammatory cytokines, interferons (IFNs), and chemokines on transcriptional and post-translational levels, which further activate surrounding cells, for example, trigger recruitment of macrophages and neutrophils. PRRs can activate cell-autonomous defense mechanisms within macrophages or epithelial cells that fight intracellular pathogens (Radtke and O'Riordan, 2006), and the expression of inducible antimicrobial peptides that combat primarily extracellular microbes (Ganz, 2003). PRRs on DCs and macrophages further provide an obligatory signal for the induction and shaping of subsequent T cell responses (Schnare et al., 2001; Fritz et al., 2007).

TLRs are highly conserved from Drosophila to humans and share structural and functional similarities. TLRs are transmembrane proteins that recognise extracellular or endosomal pathogen-associated molecular patterns (Gay and Keith, 1991). The TLR family includes 13 members, and they recognise a range of pathogen-associated molecular patterns (PAMPs) from bacteria, viruses, protozoa and fungi (Maris et al., 2006).

TLR4 is one of the most critical receptors in recognising bacterial components. Particularly, TLR 4 can recognise LPS that is a critical component of the cell wall of gram-negative bacteria. LPS is ubiquitous in the environment and is often present in high concentrations in organic dusts, air pollution and household dust (Rylander et al., 1985; Michel et al., 1996; Bonner et al., 1998). There is convincing evidence that LPS
participates in the pathogenesis of asthma (Brass et al., 2003; Lambrecht and Hammad, 2003). Epidemiological studies also suggested that inhalation of LPS contaminated air is linked to the classical features of asthma, such as airway inflammation, reversible airflow obstruction, airway remodelling and AHR (Kennedy et al., 1987; Schwartz et al., 1995; Becker et al., 1996; Bonner et al., 1998). Human studies have also shown that LPS exacerbates airflow obstruction and airway inflammation in allergic asthma patients. LPS induces a more significant bronchoconstrictive effect in allergic asthmatics compared to non-asthmatics (Michel et al., 1989). Furthermore, mouse models have shown that inhalation of LPS exacerbates the late phase inflammatory response to ovalbumin (OVA), which potentiated the allergen-induced inflammatory cell influx into BALF and increased OVA-specific serum IgE levels (Tulic et al., 2000).

The TLR family is characterised by the presence of leucine-rich repeats (mediates ligand binding), and the Toll / interleukin-1 receptor-like domain (TIR) (mediates interaction with intracellular signalling proteins) (Taguchi et al., 1996). The different abilities of TLRs to activate transcription factors depends on differential engagement of the TIR domain-containing adapter molecules, Myeloid differentiation primary response gene 88 (MyD88), MyD88 adapter-like (Mal) / TIR domain-containing adaptor protein (Tirap), TIR-domain-containing adapter-inducing interferon (Trif) / Toll-interleukin 1 receptor domain (TIR)-containing adaptor molecule-1 (Ticam-1). MyD88 / TIR domain-containing adapter protein (TIRP), and MyD88, among which MyD88 is the pivotal adaptor (Wesche et al., 1997; Burns et al., 1998). All TLRs except TLR3 can
induce NF-κB activation via a MyD88-dependent signalling pathway (O'Neill, 2008). MyD88 deficient mice showed an impaired inflammatory gene expression profile, reduced neutrophil recruitment to the lung, reduced bacterial clearance and enhanced mortality after infection with *K. pneumonia* (Cai et al., 2009). MyD88 deficiencies predispose patients to invasive bacterial infection (von Bernuth et al., 2008; Picard et al., 2010; Picard et al., 2011). Emerging in vitro evidence also reveals a role of MyD88 participating IFN-γ signalling as an adaptor protein. MyD88 and IFN-γR formatted a signalling complex in macrophage stimulated with IFN-γ. This complex is termed signalosome, which functions as mediator of host defense responses, and IFN-γ induces proinflammatory molecules transcription is MyD88 dependent (Shi et al., 2003; Sun and Ding, 2006). Our previous study demonstrated that LPS+IFN-γ induced MyD88 dependent AHR and airway neutrophil influx (Yang et al., 2009).

### 1.5.4 Interferon-γ (IFN-γ)

Interferon (IFN) was identified during influenza-virus study (Isaacs and Lindenmann, 1957), and was designated because of its ability to interfere with virus growth (Isaacs and Lindenmann, 1957). Currently, interferons are generally classified as type I IFNs (IFNα/β and related molecules), type II IFN (IFN-γ) and type III IFN (IFN-λ). Type I IFNs are essential for immunity to viruses infection, and are produced in direct response to virus infection, whereas Type II IFN, rather than being induced directly by virus infection, is synthesized in response to the recognition of infected cell during virus and intracellular bacterial infections, and IFN-λ displays similar function as type I IFNs.
In this thesis, I focus on the Type II IFN, IFN-γ. IFN-γ, acting as the ‘macrophage activating factor’, is a critical factor for innate and adaptive immunity for its inhibition of viral replication, immunostimulatory and immunomodulatory effects, such as promotes Th1 differentiation and suppresses Th2 differentiation; increases antigen presentation and lysosome activity of macrophages; activates inducible nitric oxide synthase (iNOS); promotes NK cell activity and induces IgG2a and IgG3 secretion from activated plasma B cells (Schroder et al., 2004). IFN-γ exerts its action through IFN-γ receptor (IFN-γR) complex and activates JAK-STAT signalling pathway (Ozato et al., 2007; Schindler et al., 2007).

IFN-γ has been associated with worsen airway inflammation in clinical studies. Asthma has been associated with elevated production of IFN-γ by BALF cells (Krug et al., 1996). IFN-γ was expressed by greater frequencies of peripheral blood CD8+ T cells in asthmatic airways, which are correlated with asthma severity and bronchial hyperresponsiveness (Magnan et al., 2000). Increased IFN-γ level was identified in induced sputum form asthma patient (Kim et al., 2010). An increased percentage of IFN-γ-expressing cells is found in the airways of severe asthmatics and been linked to regulate AHR induction (Magnan et al., 2000; Shannon et al., 2008).

1.5.5 Chemosensors and Asthma

Chemosensation (smell and taste), to detect chemicals which exist in the external environment or are produced by internal metabolic systems, is essential for the survival of all animals (Hildebrand, 1987). This ancient sense enables animals to seek nutritious
foods and mating partners, and to avoid dangerous substances. The generation of chemosensation relies on a massive repertoire of receptors to recognise various chemicals. Olfactory receptors are dedicated to the sense of smell and taste receptors are implicated in bitter, sweet sour, salty and umami taste (Mombaerts, 2004).

1.5.5.1 Olfactory Receptors (ORs)

The detection of diverse sensory information from volatile odorants by the vertebrate olfactory system is accomplished by a large family of ORs (Buck and Axel, 1991). ORs are expressed on the primary sensory neurons in the olfactory epithelium and have been identified in many vertebrate species with an estimated 1000 receptors found in mice (Godfrey et al., 2004). ORs are members of the G protein-coupled receptor (GPCR) superfamily. GPCRs play key roles in a variety of cellular and physiological processes, particularly in regulating the inflammatory response through modulating macrophage chemotaxis, survival and activation (Lattin et al., 2007). ORs share sequence motifs but are exceptionally diverse in protein sequence. Each OR protein responds to a broad spectrum of ligands due to non-specific combination (Malnic et al., 1999; Abaffy et al., 2007; Triballeau et al., 2008) (Fig.1.4). Although ORs are mainly recognised to be expressed in olfactory tissues, ORs are also identified in non-olfactory tissues and display multiple functions. ORs in testis and germ cells are involved in sperm chemotaxis (Parmentier et al., 1992; Feldmesser et al., 2006). ORs (OR78) expressed in kidney, the renal juxtaglomerular apparatus and smooth muscle cells of small resistance vessels, were found to respond to short chain fatty acids (SCFAs), the end product of
fermentation by the gut microbiota and are absorbed into the circulation (Pluznick et al.).

The biological function of ectopic ORs in non-olfactory tissues is not fully understood.

A recent study demonstrated OR activation resulted in an anti-bacterial effect. The olfactory tissue produced increasing levels of inflammatory cytokines (IL-6, TNF-α and iNOS) in response to bacterial challenge (Herbert et al., 2012). In addition, a recent study showed that other chemosensor receptors, the bitter taste receptors T2Rs were found ectopically expressed in human respiratory epithelium. T2Rs could be activated by bacteria-secreted ligands and triggered nitric oxide (NO) production, resulting in stimulation of mucociliary clearance and direct antibacterial effects (Lee et al., 2012). T2Rs expressed on smooth muscle were related to airway smooth muscle contraction and bronchospasm (Deshpande et al., 2010). Due to the high similarity of these chemosensor receptors, these studies strongly suggest that ORs expressed in the airway are involved in the host defense response within the respiratory system as well as AHR, and opens up other avenues for investigating the development and progression of asthma.

Fig.1.4 Multiple patterns of agonist-ORs combination. The recognition of agonists is
non-specific. ORs are able to recognise different features of molecules, and one agonist may consist of a number of ‘epitopes’ that possess some of these features.

1.5.6 Innate Immune Cells

It is increasingly recognised that innate immune cells, such as macrophages, neutrophils, may play critical or even dominant roles in asthma development and correlated with disease severity and steroid sensitivity, especially in non-eosinophilic asthma (Wenzel et al., 1997; Goleva et al., 2008; Essilfie et al., 2012; Yang et al., 2012). In this study, we will focus on discussing the biological significance of macrophages.

1.5.6.1 Macrophages

Macrophages are the most abundant inflammatory immune cell found in the alveoli, distal airspaces and conducting airways, which suggests that they play an important role in the pulmonary system against invading pathogens. The mononuclear phagocyte system is generated from pluripotent progenitor cells in the bone marrow. Cells in the mononuclear phagocyte lineage progress through a series of morphologically-distinct stages to differentiate into committed cells. Myeloid progenitors give rise to monoblasts, pro-monocytes and then monocytes, which migrate into tissue (Hume, 2000). The differentiation of mononuclear phagocytes from progenitor cells is directed by colony-stimulating factors with lineage restriction and hierarchy, including macrophage colony-stimulating factor (CSF-1), granulocyte macrophage colony-stimulating factor (GM-CSF) and fms-like tyrosine kinase 3 ligand (Flt3L), which instruct progenitor cells
to differentiate to macrophage (Stanley, 2009).

1.5.6.2 Alveolar Macrophages

Monocytes generated from bone marrow are released into circulation and further migrate from circulation and extravastate through vessel endothelium, then differentiate into tissue-resident macrophages or DCs (Geissmann et al., 2010). Macrophages are divided into subpopulations based on their anatomical location and functional phenotypes (Gordon and Taylor, 2005). Specialised tissue-resident macrophages include alveolar macrophages (lung), osteoclasts (bone), histiocytes (interstitial connective tissue) and kupffer cells (liver) (Murray and Wynn, 2011). Alveolar macrophages play an important role in pulmonary innate immune responses, homeostasis, host defense, the response to foreign substances, and tissue remodelling (Twigg, 2004; Lambrecht, 2006). Alveolar macrophages are the major residential immune effector cells in alveolar spaces and airways, and are an important cellular source of mediators including cytokines, chemokines, proteases, and arachidonic metabolites, initiating and resolving pulmonary inflammation (Hocking et al., 1979; Peters-Golden, 2004). The function of macrophages may be dependent on their sensitisation status. Several studies suggested unsensitised alveolar macrophages protect against allergen specific immune response and airway inflammation and AHR (Thepen et al., 1991; Careau et al., 2010). And other studies showed sensitised alveolar macrophages promoted eosinophilic airway inflammation and asthma exacerbation by stimulating CD4+ T cells to secrete cytokines (Careau et al., 2006; Moon et al., 2007; Herbert et al., 2010).
1.5.6.3 Macrophage Polarisation

Cells from the monocyte-macrophage lineage are heterogeneous in response to exposures of versatile micro-environmental signals (Ma et al., 2003). Cytokines and microbial products affect the polarisation and function of macrophages (Mantovani et al., 2004). IFN-γ alone or together with LPS, activates macrophages, which are referred as M1 macrophages (Endlich et al., 2002). These cells are characterised by their enhanced antigen presenting capacity; enhanced IL-12 and IL-23 production; and increased production of toxic intermediates, such as NO, and characteristically produce chemokines, CXCL9 and CXCL10 (Mantovani et al., 2004; Verreck et al., 2004). Another distinct activation program was identified, which is induced by IL-4 and IL-13 and other Th2 cytokines, and referred to as ‘alternative activation’ (M2) (Gordon, 2003). M2 macrophages typically produce chemokines, such as CCL17, CCL22, and CCL24 (Stein et al., 1992; Martinez et al., 2006; Mantovani, 2008). Studies suggest that classically activated M1 macrophages participated in Th1 responses as potent effector cells to clear microorganisms and produce pro-inflammatory cytokines, while M2 macrophages participate in inflammatory responses and adaptive immunity, promoting tissue remodelling and repair (Stein et al., 1992; Verreck et al., 2004). Th1 responses inhibit Th2 responses through the production of cytokines such as IL-12 and IFN-γ. IL-12 plays important role in inhibiting inappropriate IgE synthesis and allergic inflammation as a result of allergen exposure (Gavett et al., 1995). These effects of IL-12 are mediated by IFN-γ (Bruselle et al., 1997). The production of IL-12 and IL-12
induced IFN-γ release is reduced in patients with allergic asthma compared to nonatopic controls (van der Pouw Kraan et al., 1997). A recent study has found an increase in the number of Th1 macrophages in the BALF of steroid-resistant asthma patients (Goleva et al., 2008). The inducers of M1 macrophages (LPS, IFN-γ and TNF-α) were found to be significantly higher in asthmatics, especially in patients with severe asthma (Berry et al., 2006; Kim et al., 2007; Shannon et al., 2008). Corticosteroid treatment resulted in increased IL-12 expression in steroid-sensitive asthmatics, but had no effect in steroid-resistant asthmatics (Naseer et al., 1997). The neutralization of Th1 cytokine, IL-12, could abolish the development of allergic airway inflammation and this effect is IFN-γ dependent (Meyts et al., 2006). Thus, the presence of M1 inducers (LPS, IFN-γ and TNF-α) and M1 cytokine, proinflammatory factor IL-12, all contribute to asthma development, which suggest the critical role in the development of severe steroid-resistant asthma.

1.5.6.4 Macrophage Recruitment and Chemotaxis

Macrophages are recruited into inflammatory sites in response to various stimuli to perform their functions. The migrating process of macrophages is known as chemotaxis; macrophages in peripheral blood adhere to the vessel wall, move cross the endothelial barrier, and migrate towards the inflammatory sites in response to pro-inflammatory stimuli (such as IL-1, TNF-α, LPS and viruses). Inflammatory stimuli induce the production of cell adhesion molecules such as P-selectin that make the vessel walls sticky and therefore more easily adhered to, as well as inducing the production of
chemokines which attract the movement of macrophages (Ono et al., 2003; Sallusto et al., 2008). Chemokines can be classified into four main subfamilies: CXC, CC, CX3C and XC, and exert their effects by binding to chemokine receptors, which are selectively found on the surfaces of different immune cells (Le et al., 2004). Typical inflammatory chemokines for macrophages migration include: CCL2 (MCP-1), CCL3 (MIP-1), CCL5 (RANTES), CCL7 (MCP-3), CCL8 (MCP-2), CCL13 (MCP-4), CCL17 (TARC) and CCL22 (MDC) (Ono et al., 2003; Laing et al., 2004).

1.6 MicroRNAs in Immune Response and Asthma

MicroRNAs (miRNAs) were first identified in C. elegans as RNA molecules of approximately 22 nucleotides (nt) that are complementary to the 3’ untranslated regions (UTR) of the target transcripts (Lee et al., 1993; Lau et al., 2001). Subsequently, these small RNA molecules have also been identified in a diverse range of organisms. Currently a large amount of studies have demonstrated that miRNAs play important roles in mediating gene expression involved in many biological processes (Lagos-Quintana et al., 2003; He and Hannon, 2004).

1.6.1 Biogenesis of microRNA

The biogenesis of miRNA in vertebrates consists of five steps: 1) primary RNA (pri-miRNA) is transcribed from the genome and mediated by RNA polymerase II (Pol-II) (Lin et al., 2003; Lee et al., 2004); 2) pri-miRNA is excised by Drosha-like RNase III endonucleases and/or spliceosomal components to form 60- to 70-nt pre-miRNA(Lee et al., 2003); 3) Ran-GTP and Exportin-5 exported pre-miRNA out of
the nucleus (Yi et al., 2003); 4) pre-miRNA is cleaved by Dicer-like endonucleases to form mature 18- to 25-nt miRNA in the cytoplasm; and 5) mature miRNA is incorporated into the miRNA-induced silencing complex (miRISC) and executes RNA interfering-related gene silencing (Khvorova et al., 2003; Schwarz et al., 2003) (Fig.1.5).

![Fig.1.5 The biogenesis process and function of miRs. Pri-miRNA is transcribed from genome and then sliced to form pre-miRNA, and pre-miRNA is further cleaved to form mature miRNA. Mature miRNA is incorporated into RISC and executes RNA interfering-related gene silencing.](image-url)
1.6.2 Modulation of miRNA Expression

Currently, there are several approaches for miRNA silencing including genetic knockouts, miRNA sponges and antisense oligonucleotides (termed antagomir) (Kluiver et al., 2012; Velu and Grimes, 2012; Han et al., 2013). There are now a number of individual miRNA knockout animals available (Park et al., 2010). The genetic knockout approach is also extensively used in miRNA function studies using C. elegans and Drosophila (Abbott et al., 2005; Sokol et al., 2005). MiRNA sponges are highly expressed transgenes harboring multiple miRNA target sites to sequester the expression of target miRNAs and are able to inhibit entire miRNA seed families or manipulate miRNA activity (Ebert and Sharp, 2010). Antagomirs are a widely employed approach in miRNA loss-of-function studies, as they compete with mature miRNAs and lead to functional inhibition of the miRNA and derepression of the target genes (Stenvang et al., 2012). In this study, I made use of antagomir to against the target miRNA.

The expression of miRNAs can be increased or re-initiated by using synthetic RNA duplexes designed to mimic the endogenous function of the target miRNA. Another method of increasing the expression level of miRNAs is by using of adeno-associated viruses (AAVs). The miRNAs of interest can be continually expressed, resulting in robust replacement expression of miRNAs down-regulated during disease development (van Rooij, 2011).

1.6.3 Emerging Role of microRNA in Asthma

Approximately 2000 miRNAs are expressed in human cells, and their expression
pattern varies depending on the different tissue and cell type examined (Sanchez-Jimenez et al., 2013). There are accumulating evidences to show that miRNAs are involved in diverse biological processes and aberrant miRNA expression has been associated with the pathogenesis of multiple diseases, such as heart, neural, cancer and multiple allergic inflammatory diseases, including asthma, eosinophilic esophagitis, allergic rhinitis, and atopic dermatitis (Zhao et al., 2005; Gee et al., 2008; Yoo et al., 2009; Lu and Rothenberg, 2013). MiRNAs mainly function to modulate and fine-tune expression levels of key regulatory proteins, however more studies have increasingly revealed that miRNAs are expressed in immune cells to target proteins involved in regulating inflammation and consequently control the magnitude of immune responses (O'Neill et al., 2011; O'Connell et al., 2012). For example, decrease of miR-16, miR-15a and miR-223 cooperatively suppresses the differentiation of monocytes to macrophages through controlling NF-κB activity (Li et al., 2010). Following exposure to LPS, miRNAs expression rapidly increases in the mouse lung, including miR-21, -25, -27b, -100, -140, -142-3p, -181c, -187, 194, -214, -223, and -224, and these increases are correlated with reduced expression of TNF-α, macrophage inflammatory protein (MIP)-2 and keratinocyte-derived chemokine (KC), and these changes suggested that miRNAs play potential roles in mediation of inflammatory cytokine production (Moschos et al., 2007). MiR-155, miR-146a and miR-21 are also crucial miRNAs in TLR signalling and activation. MiR-155 targets and down-regulates the negative regulators of cytokine signals, Src homology-2 domain-containing inositol 5-
phosphatase 1 (SHIP1) and suppressor of cytokine signalling 1 (SOCS1), and thus leads to increased activation of AKT and IFN response genes (Androulidaki et al., 2009; O'Connell et al., 2009). The negative regulatory role of miR-146a occurs through inhibition of TNF receptor associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1), which are involved in the transduction of TLR signalling and lead to NF-κB activation (Taganov et al., 2006; Boldin et al., 2011). Similarly, miR-21 is induced by NF-κB in a MyD88-dependent manner, and it targets programmed cell death 4 (PDCD4) to down-regulate NF-κB signalling (Sheedy et al., 2010).

MiRNAs may participate in the pathogenesis of asthma by regulating immune cells and structural cell responses. Analysis of airway miRNA expression in a mouse model of house dust mite (HDM)-induced allergic asthma showed that miRNA-16, -21 and -126 were significantly up-regulated in sensitised mice (Mattes et al., 2009). MiR-146a is widely involved in the responses of cell types that are associated with human asthma including bronchial epithelial cells, alveolar epithelia cells and bronchial smooth muscles cells (Holgate, 2008; Larner-Svensson et al., 2010). OVA-challenged, *Aspergillus fui-gatus*-induced IL-13 transgenic and IL-4 transgenic mice demonstrated increased miR-21 expression compared with control mice. IL-12p35 mRNA, a potential target gene of miR-21, was decreased in these models (Lu et al., 2009).

MiRNA have also been shown to potentially involve in the regulation of GC function by modulating GR expression. It is reported that miR-18 and miR-124a could regulate
GC sensitivity by suppressing the production of GR protein. Some miRNAs (such as miR-18a, -128b and -221) are involved in differentially regulating translation of GRs to contribute to GC resistance (Uchida et al., 2008; Kotani et al., 2009).

1.6.4 Therapeutic Potential of Targeting miRNAs as New Approach to Treat Asthma

Increasing studies have revealed the important role of miRNAs in regulating the immune system as well as their contribution to the development and severity of asthma (Wang et al., 2011; Greene and Gaughan, 2013). These studies have identified potential new targets for the therapeutic manipulation and treatment of asthma. Triggering stimuli, which are produced during inflammation and bacterial and viral infections, usually induce transcriptional changes of a group of miRNAs (Skalsky and Cullen, 2010; Staedel and Darfeuille, 2013). In this context, to identify the critical miRNAs, which play the dominant roles, and their targets, which propagate the development of disease, are vital for more specific interventions.

1.7 Study Rationale

Innate immune responses during microbial infections contribute to asthma exacerbations and steroid-resistance. In severe and difficult-to-control (steroid-resistant) asthma, the activation of innate immune pathways (for example by IFN-$\gamma$ and LPS) contributes to disease pathogenesis. The aim of this study is to identify the mechanisms contributing to innate immune activation mediated steroid-resistant AHR. I will
characterise the role of innate immune cells, specifically pulmonary macrophages, identify the key genes or molecules that manipulate the steroid-resistance and investigate the underlying mechanisms

I hypothesise that aberrantly expressed genes or miRNAs regulate the expression of pro-inflammatory cytokines, chemokines and transcription factors. In turn these factors affect the activity of innate immune system which further contributes to the development of steroid-resistant AHR.
Chapter 2.

IL-27/IFN-γ Induce MyD88- Dependent Steroid-Resistant Airway Hyperresponsiveness by Inhibiting Glucocorticoid Signalling in Macrophage
2.1 Abstract

Inflammation and airway hyperresponsiveness (AHR) are hallmark features of asthma and often correlate with the severity of clinical disease. Although these features of asthma can be effectively managed with glucocorticoid therapy, a subgroup of patients, typically with severe asthma, remains refractory to therapy. The mechanisms leading to steroid resistance in severe asthmatics are poorly understood but may be related to the activation of innate host defense pathways. Previously, we have shown that IFN-γ-producing cells and LPS, two factors that are associated with severe asthma, induce steroid-resistant AHR in a mouse model. We now demonstrate that cooperative signalling induced by IFN-γ and LPS results in the production of IL-27 by mouse pulmonary macrophages. IL-27 and IFN-γ uniquely cooperate to induce glucocorticoid-resistant AHR through a previously unknown MyD88-dependent mechanism in pulmonary macrophages. Importantly, integrated signalling by IL-27/IFN-γ inhibits glucocorticoid-induced translocation of the glucocorticoid receptor to the nucleus of macrophages. Furthermore, expression of both IL-27 and IFN-γ was increased in the induced sputum of steroid-refractory asthmatics. These results suggest that a potential mechanism for steroid resistance in asthma is the activation of MyD88-dependent pathways in macrophages that are triggered by IL-27 and IFN-γ, and that manipulation of these pathways may be a therapeutic target.

2.2 Introduction
Asthma is a chronic inflammatory disorder of the airways that is clinically characterised by recurrent airflow obstruction, wheezing and airway hyperresponsiveness (AHR) (Eder et al., 2006). AHR is an exaggerated narrowing of the airways in response to nonspecific spasmogenic stimuli (Sont et al., 1999; Cockcroft et al., 2006; Eder et al., 2006). Importantly, the degree of AHR often correlates with the severity of disease, and titration of therapy based on the control of AHR may produce superior outcomes in asthma (Sont et al., 1999). CD4+ Th2 lymphocytes have been identified as a central component of the allergic inflammatory response (Cohn et al., 2004). However, there is increasing evidence that asthma is a heterogeneous inflammatory condition in which the contribution of Th2- driven pathways is not always dominant. Indeed, responsiveness to inhaled corticosteroids was recently shown to correlate with the molecular phenotype, defined in terms of the degree of Th2 inflammation (Woodruff et al., 2009).

In severe and difficult-to-manage forms of the disease, where steroids may have limited efficacy, factors associated with activation of host defense pathways (such as IFN-γ and LPS) are likely to contribute to pathogenesis (Michel et al., 1996; Wenzel et al., 1997; ten Hacken et al., 1998; Simpson et al., 2007; Hansbro et al., 2008; Shannon et al., 2008; Al-Ramli et al., 2009; Barnes et al., 2009; Simpson et al., 2009; Wilson et al., 2009). Patients with refractory asthma are more susceptible to allergen- and infection-induced exacerbations, and they often have increased neutrophilic rather than eosinophilic infiltrates (Wenzel et al., 1997; Simpson et al., 2007; Hansbro et al., 2008). Although eosinophilic inflammation may still be important in severe asthma (Eder et al.,
the lack of control of inflammation and AHR by steroid therapy in these patients suggests that factors underpinning the expression of disease are either not controlled by glucocorticoid-responsive elements or are suppressors of this pathway.

Two factors that may be relevant in this context are the expression of IFN-$\gamma$ by inflammatory cells and the activation of inflammatory cells by LPS. An increased percentage of IFN-$\gamma$-expressing cells is found in the airways of severe asthmatics, and this cytokine has been linked to the mechanisms regulating AHR (Heaton et al., 2005; Shannon et al., 2008). LPS is a major component of the cell walls of Gram-negative bacteria and of allergens that trigger asthma. High levels of LPS have been detected in the bronchoalveolar lavage fluid (BALF) from steroid resistant asthmatics and are an important determinant of the severity of asthma (Michel et al., 1996; Goleva et al., 2008). We and others have shown that IFN-$\gamma$-producing cells and LPS contribute to the induction of airway inflammation and AHR in mouse models; furthermore, we have demonstrated that IFN-$\gamma$/LPS-induced responses are steroid resistant (Hayashi et al., 2007; Yang et al., 2009). Our investigations revealed a novel interaction between IFN-$\gamma$ and LPS that leads to the activation of pulmonary macrophages and the induction of steroid-resistant AHR (Yang et al., 2009). Specifically, we provided the first demonstration that LPS-triggered signalling through TLR4 and MyD88 pathways cooperated with signalling through the IFN-$\gamma$R to modulate airway inflammation and reactivity to spasmogens (Yang et al., 2009). These data were consistent with earlier in vitro observations suggesting that these pathways may be integrated and regulate
inflammation in macrophages (Sun et al., 2006) and block the anti-inflammatory effects of glucocorticoids in human monocytes (Szefer et al., 1989).

Nevertheless, the critical downstream molecules that regulate IFN-γ and LPS-TLR4/MyD88–mediated inflammation and steroid-resistant AHR remain unknown. Analysis of recent studies suggests that IL-27 may be an important mediator of IFN-γ and LPS induced steroid-resistant AHR, because of its potential role as a regulator of pulmonary macrophage function (Pflanz et al., 2004; Ruckerl et al., 2006; Liu et al., 2007). A recently discovered member of the IL-6/IL-12 family, IL-27 is a heterodimeric cytokine that has two subunits, an EBV-induced gene 3 (EBI3) and a p28 chain (Pflanz et al., 2002). It is produced by activated monocytes (or monocyte-derived dendritic cells (DCs)) and macrophages after microbial exposure (Pflanz et al., 2002; Liu et al., 2007) and its expression is critically dependent on signalling through MyD88 (Liu et al., 2007).

IL-27 acts on a wide range of immune cells, including CD4+ and CD8+ T lymphocytes, NK cells, monocytes/macrophages, and activated DCs (Yoshida et al., 2001; Holscher et al., 2005; Morishima et al., 2005; Ruckerl et al., 2006; Wang et al., 2007). The effect of IL-27 on monocytes/macrophages is complex, as it is able to induce or suppress the expression of surface activation molecules or cytokines by these cells (Holscher et al., 2005; Ruckerl et al., 2006; Feng et al., 2008; Imamichi et al., 2008; Kalliolias et al., 2008). The pleiotropic effects of IL-27 on monocytes/macrophages may depend on the timing and context of surrounding inflammatory signals and the nature of the local immune environment.
The contribution of IL-27 in different phenotypes of asthma remains poorly understood. However, IL-27 is known to promote IFN-γ production by Th1 cells, as well as innate host defense responses to infection (Pflanz et al., 2002; Pearl et al., 2004), both of which may contribute to severe forms of asthma and exacerbations. In this study, we extend our investigations of molecular and cellular mechanisms regulating steroid-resistant AHR. Specifically, we demonstrate a critical role for IL-27, integrated with IFN-γ, in the activation of pulmonary macrophages in asthma. IL-27- and IFN-γ-induced AHR was dependent on MyD88 expression in macrophages, thus identifying a novel interaction between IL-27R, IFN-γR, and MyD88 signalling pathways in this cell. Importantly, this interaction prevented steroid induced translocation of the glucocorticoid receptor (GR) to the nucleus of pulmonary macrophages. We also observed that the expression of IL-27 and IFN-γ was significantly greater in the sputum of patients with neutrophilic asthma, compared with those with eosinophilic asthma. Collectively, our observations suggest that a novel IL-27- and IFN-γ- driven pathway may be important in the regulation of AHR, and that the innate immune system may thus potentially contribute to the pathogenesis of steroid-resistant asthma.

2.3 Materials and Methods

2.3.1 Animals

Wild-type- and MyD88 knockout (MyD88−/−)-specific pathogen-free BALB/c mice
(6–8 wk) were obtained from the animal services unit of the University of Newcastle. MyD88−/− mice were backcrossed for 12 generations onto the BALB/c strain. All experiments were performed with approval from the animal ethics committee of the University of Newcastle.

2.3.2 Administration of IL-27, IFN-γ, and/or LPS, and Ab neutralization of IL-27

Mice were anesthetized (i.v. of 100 ml Saffan solution [1:4] diluted with PBS) and the trachea was intubated with a 22-gauge catheter needle. Optimized doses of murine rIL-27 (200 ng/mouse; R&D Systems, Minneapolis), rIFN-γ (1.5 mg/mouse; PeproTech, Rocky Hill, NJ) and/or LPS (50 ng/ mouse; Sigma-Aldrich, St Louis, MO), or control vehicle (0.1% BSA/PBS) were intratracheally (i.t.) instilled into the airways. Anti–IL-27 Ab (100 mg/ mouse; R&D Systems) or isotype control was delivered i.v.into some mice at the same time as treatment with IFN-γ/LPS. Endpoints were assessed 12 h after treatment with IL-27, IFN-γ, and/or LPS.

2.3.3 Analysis of induced sputum samples from human asthma patients

Patients with asthma, defined by clinical diagnosis with evidence of AHR to hypertonic saline and/or bronchodilator response (Simpson et al., 2008), were recruited and categorized via induced sputum inflammatory cell counts. Participants with a sputum eosinophil count of ≥3% in the absence of an increase in sputum neutrophil numbers were classified as eosinophilic asthma. Participants with a sputum neutrophil count of
≥63% and ≤3% eosinophils were classified as neutrophilic asthma. All participants gave written informed consent prior to their inclusion in the study, which was approved by the Hunter New England Area Health Service and the University of Newcastle Research Ethics Committees.

Induced sputum samples were obtained using nebulized hypertonic (4.5%) saline (Gibson et al., 1998). For the assessment of inflammatory cells, DTT was used to disperse cells from mucus. Total cell counts and cell viability (trypan blue exclusion) were performed with a hemocytometer, followed by preparation of cytospins for differential cell counts using May-Grünewald-Giemsa. Selected sputum plugs (100 ml) were stored for RNA analysis in RLT buffer (Qiagen, Valencia, CA) at 280°C.

RNA was extracted from induced sputum plugs using RNeasy Mini kits (Qiagen) and quantitated using the Quant-iT RiboGreen assay (Invitrogen, Carlsbad, CA). RNA (200 ng) was reverse-transcribed to cDNA using high capacity cDNA reverse transcription kits according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). TaqMan qPCR primer and probes for IFN-γ, IL-27 p28, and EBI3 genes were purchased in kit form (Applied Biosystems) and combined in duplex real-time PCRs using an ABI 7500 real-time PCR machine (Applied Biosystems). The levels of expression of IFN-γ and IL-27 p28 and EBI3 subunit genes were calculated using $2^{-\Delta\Delta C_t}$ relative to the reference gene eukaryotic 18S rRNA and an internal calibrator.

2.3.4 Administration of dexamethasone
Dexamethasone (DEX) (1 mg/kg; Sigma-Aldrich) was administered by daily i.p. injection for 3 consecutive days, commencing 3 d before IL-27/IFN-\(\gamma\) or IFN-\(\gamma\)/LPS treatment. Endpoints were assessed 12 h after treatment with IL-27/IFN-\(\gamma\) or IFN-\(\gamma\)/LPS.

2.3.5 Measurement of lung function

Airway responses to methacholine challenge were measured using Flexivent apparatus (Scireq; Montreal, Quebec, Canada) as previously reported (Yang et al., 2009). Briefly, mice were anesthetized (50 ml/10 g i.p.) with a mixture containing xylazine (2 mg/ml; Troy Laboratories, Smithfield, New South Wales, Australia) and ketamine (40 mg/ml; Parnell, Alexandria, New South Wales, Australia). A further dose (25 ml/10 g) of anesthetic was administered when the animal was attached to the ventilator and every 30 min thereafter. A tracheostomy was performed and a cannula (length, 1.0 cm; internal diameter, 0.0813 cm) inserted into the trachea. Animals were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min, with a positive end expiratory pressure of 2 cm H\(_2\)O. Mice were then challenged with aerosolized saline followed by increasing concentrations of b-methacholine (5, 10, 20, and 40 mg/ml; Sigma-Aldrich) for 10 s at each dose. Aerosols were delivered by an ultrasonic nebulizer (Scireq) installed in a bypass branch of the inspiratory tubing. The constant-phase model was used to describe the mechanical properties of airways and parenchyma (Hantos et al., 1992). Measurements were excluded if the coefficient of determination was < 95%. Raw represents the Newtonian resistance of the airways, which predominantly reflects airflow, and AHR was defined as a significantly increased change in Raw relative to
control mice. Changes in Raw were calculated as percentage increase over saline control.

2.3.6 Isolation of adherent cells, epithelial cells, and nonadherent cells

Pulmonary macrophages were isolated from mouse lungs according to previously described methods with some modifications (Loewen et al., 2005). Briefly, macrophages were mechanically extracted from minced mouse lung tissue, purified by gradient centrifugation (Histopaque-1083; Sigma-Aldrich) and plated at a concentration of \(1 \times 10^6\) cells/ml in RPMI 1640 containing 10% FCS. After 3 h, 95% of adherent cells were macrophages, which was confirmed by FACS. To obtain epithelial cells, lung pieces (after macrophages were mechanically extracted) were further incubated with 5 volumes of trypsin for 15 min at 37°C. Enzymatic digestion was repeated once. Cells released during the second digestion were cultured as described for airway epithelial cells (Loewen et al., 2005). More than 90% of adherent cells were of the epithelial cell population, which was determined by FACS with positive staining of anti-epithelial cell adhesion molecule (mouse epithelial cell marker; BioLegend, San Diego, CA). Nonadherent cells were collected from macrophage and epithelial cell isolations and cultured at a concentration of \(5 \times 10^6\) cells/ml in RPMI 1640 containing 10% FCS.

2.3.7 Analysis of cytokines by ELISA

IL-27 concentrations were determined in supernatants from IFN-\(\gamma\)- and/or LPS-stimulated and nonstimulated (control) macrophages, epithelial cells, and
nonadherent cells isolated from mouse lung by ELISA according to the manufacturer’s instructions (R&D Systems).

2.3.8 Quantitative PCR

Quantitative PCR was performed as previously described (Yang et al., 2006). Briefly, RNA was prepared from cells or tissue using the TRIzol RNA isolation buffer following the manufacturer’s instructions (Invitrogen). cDNA was synthesized using oligo(dT)-primed reverse transcriptase reactions using 0.5 mg RNA from each sample. Quantitative PCR was performed in an ABI PRISM 7000 sequence detection system (Applied Biosystems) using the following primers: murine IL-27 p28 (p28) (forward, 5'-CTGTTGCTGCTACCCTTGCTT-3'), and reverse, 5'-CACTCCTGGCAATCGAGATTC-3'); EBI3 (forward, 5'-GCAGCCTCCTAGCCTTTGTG-3', and reverse, 5'-GGAGTCGGTACTTGAGAGAGAAG-3'); and GAPDH (forward, 5'-CAGGTTGTCTCCTGCGACTT-3', and reverse, 5'-CCCTGTTGCTGTAGCGCTA-3'). SYBR Green was used to detect changes in amplicon levels with each sequential amplification cycle. The fluorescence intensity was normalised to the rhodamine derivative ROX as a passive reference label, which was present in the buffer solution. The levels of mRNA from treated groups were normalised to GAPDH.

2.3.9 Inhibition of NF-κB, JNK, or p38 activation

NF-κB and JNK were depleted by i.p. injection with BAY11-7082 (10 mg/kg;
Calbiochem, Darmstadt, Germany), or by i.v. injection with SP600125 (25 mg/kg; Sigma-Aldrich), or vehicle (2% DMSO/saline) 1 h before IFN-γ/ LPS or IL-27/IFN-γ treatment. Previous studies have shown that these doses of BAY11-7082 or SP600125 can efficiently inhibit the activation of NF-κB (Alvira et al., 2007) and JNK (Bennett et al., 2001). For inhibition of p38, mice were i.v. injected with SB203585 (10 mg/kg; LC Laboratories, Woburn, MA) or vehicle control 1 h before IFN-γ/LPS or IL-27/IFN-γ treatment. An additional dose was administered 1 h before the measurement of lung function. Previous studies have shown that these doses of SB203585 used can efficiently inhibit p38 activation (Liu et al., 2008).

2.3.10 Depletion of IFN-γ, CD4+, CD8+, and NK cells and pulmonary macrophages

CD4+ or CD8+ cells were depleted by i.p. injection with 500 mg anti-CD4 (GK1.5) (Hogan et al., 1998) or 500 mg anti-CD8 (YTS169.4) mAbs (Schwarze et al., 1999) or the corresponding isotype controls 3 d and 1 h before IL-27/IFN-γ or IFN-γ/LPS treatment. Depletion of CD4+ or CD8+ cells was confirmed by FACS. NK cells were depleted by i.v. injection with 50 ml anti-ASIALO GM1 Ab (Wako Chemicals, Osaka, Japan) or rabbit serum 2 d and 1 h before IL-27/IFN-γ instillation (Hansen et al., 2007). The efficiency of depletion of NK cells by anti-ASIALO GM1 Ab was determined as previously described and confirmed by FACS. Pulmonary macrophages were depleted using liposome-encapsulated clodronate (lipo-Cl2MDP), which was prepared as described previously (Van Rooijen, 1989). Briefly, phosphatidylcholine (86 mg;
Sigma-Aldrich) and cholesterol (8 mg; Sigma-Aldrich) were dissolved in chloroform (10 ml). A lipid film was generated by vacuum rotary evaporation and resuspended in either 4 ml PBS or 10 ml Cl2MDP (Sigma-Aldrich). The suspension was maintained at room temperature (RT) for 2 h, sonicated for 3 min, and again maintained for 2 h at RT. Lipo-Cl2MDP was diluted in 90 ml PBS and centrifuged at 100,000 3 g for 30 min. Pellets were washed with 10 ml PBS and resuspended in 4 ml PBS. Liposomes were either used immediately or stored under N2 gas at 4°C for up to 1 wk. Either lipo-Cl2MDP or liposome encapsulated PBS (100 ml/mouse) was i.t. instilled to deplete pulmonary macrophages 3 d before IL-27/IFN-γ or IFN-γ/LPS treatment. Cellular depletion was confirmed by examination of BALF and FACS.

2.3.11 Flow cytometry

Lungs were isolated from mice after treatment with IL-27, IFN-γ, and/or LPS. Samples were dissociated into single-cell suspensions and depleted of erythrocytes using 0.86% (w/v) ammonium chloride. Cells were then washed and immediately stained for surface marker expression using the following fluorescent mAbs: anti-F4/80, anti-CD11b, and anti-CD11c (BD Pharmingen, San Diego, CA). Subpopulations of pulmonary macrophages were categorized as previously described (Hall et al., 2008). Briefly, typical alveolar macrophages were defined as F4/80 high CD11b^var^CD11c^high^FS^high^SS^high^, CD11b^+^ macrophages as F4/80^high^CD11b^high^CD11c^var^FS^var^SS^var^, monocytes as F4/80^low^CD11b^mid^CD11c^low^FS^low^SS^low^, and DCs as F4/80^low^
CD11b\textsuperscript{var}CD11c\textsuperscript{high}FS\textsuperscript{var}SS\textsuperscript{mid}. Anti-CD3, anti-CD4, and anti-CD8 (Bio-Legend) were used to detect CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells. Anti-CD49b and anti- Fc\varepsilon RII (BioLegend) were used to detect NK cells (CD49b\textsuperscript{+}Fc\varepsilon RII2). All samples were analyzed using a FACS Canto flow cytometer (BD Biosciences, San Jose, CA).

2.3.12 Transfer of isolated wild-type macrophages into MyD88\/- mice

Pulmonary macrophages from wild-type (WT) mice were isolated, harvested, and resuspended in sterile PBS. MyD88\/- mice were i.v. injected with 3 \times 10^6 cells per mouse. Some cells were labeled with CFSE to confirm transfer efficiency. One hour after macrophage transfer, recipients were i.t. administered either IL-27/IFN-\gamma (200 ng and 1.5 mg, respectively) or vehicle.

2.3.13 Immunofluorescence detection of GRs

The detection of GRs was performed as described previously with minor modification (Goleva et al., 2002). Briefly, macrophages isolated from mouse lungs were seeded on cover slips at 3\times10^6 cells/ml and allowed to settle and adhere for 48 h before treatment at 37°C under 5% CO2. Cells were then stimulated with vehicle, LPS (50 ng/ml), IFN-\gamma (1.5 mg/ml), IL-27 (200 ng/ml), IFN-\gamma (1.5 mg/ml) plus LPS (50 ng/ml), IL-27 (200 ng/ml) plus IFN-\gamma (1.5 mg/ml), IFN-\gamma (1.5 mg/ml) plus LPS (50 ng/ml) plus anti–IL-27 Ab (50 mg/ml), or IFN-\gamma (1.5 mg/ml) plus LPS (50 ng/ml) plus control Ab (70 mg/ml) for 24 h and treated with 1 \mu M DEX or vehicle for 1 h. Pulmonary macrophages were washed with cold PBS, fixed in 4% (w/v) paraformaldehyde in PBS buffer for 20 min
on ice, permeabilized with 0.2% (v/v) Triton X-100 for 10 min at RT, and blocked with 5% BSA/PBS for 30 min at RT. Cells were then incubated with the polyclonal Ab to GRs (Santa Cruz Biotechnology, Santa Cruz, CA) or control (purified non-immune rabbit IgG [Santa Cruz Biotechnology]) diluted (1:50) in 1% BSA/PBS overnight at 4°C. Macrophages were washed with PBS for 15 min at RT and incubated with Cy3-conjugated goat anti-rabbit IgG (10 mg/ml; GE Healthcare, Buckinghamshire, U.K.) diluted in 1% BSA/PBS for 45 min in the dark at 37°C, and again washed with PBS for 15 min. The nucleus was stained with DAPI (Sigma-Aldrich) for 10 min at RT. Slides were washed with PBS and mounted. GRs in macrophages were visualised using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) with a 3100 objective lens; images were captured using a digital camera (DP70; Olympus) and analyzed using Image J 1.42 software (National Institutes of Health, Bethesda, MD). Sampling was performed on 8–10 different areas for 40–60 cells of each slide. The intensity of Cy3 staining per unit area in the nucleus and cytoplasm of each cell was quantified and the ratios of nucleus/cytoplasm were calculated.

2.3.14 Data analysis

An initial one-way ANOVA (or a Kruskal-Wallis test for nonparametric data) was followed by appropriate comparisons to test for differences between means of groups. Values are reported as the mean ± SEM for each experimental group. The number of mice in each group ranged from 8 to 10. Differences in means were considered significant if p<0.05.
2.4 Results

2.4.1 IFN-γ/LPS increases activation and numbers of CD11b⁺ pulmonary macrophages

We have previously shown that pulmonary macrophages have a critical role in the development of IFN-γ/LPS–induced AHR (Yang et al., 2009). This steroid-resistant AHR develops 12 h after exposure of the airways to these factors. To characterize the phenotype of these macrophages, we administered IFN-γ/LPS to the lung and 12 h later performed FACS analysis on the cells isolated from lung homogenates (see Materials and Methods for macrophage phenotypes). A greater frequency of CD11b⁺ macrophages (F4/80⁺CD11b⁺CD11c-var) was observed in lungs of mice treated with IFN-γ/LPS (34.2%) compared with treatment with IFN-γ (25.4%), LPS (20.6%), or vehicle alone (20.1%) (Fig.2.1A). A significant increase in the absolute numbers of CD11b⁺ macrophages in the lungs of IFN-γ/LPS–treated mice (2.26 ± 0.14 × 10⁵ cells/ml; data expressed as mean ± SEM, n = 6, p < 0.05) was also detected compared with controls treated with IFN-γ (1.35±0.23×10⁵ cells/ml), LPS (1.16 ± 0.17 × 10⁵ cells/ml), or vehicle (0.97 ± 0.12 × 10⁵ cells/ml). In contrast, a reduced frequency of typical alveolar macrophages (F4/80⁺CD11b⁻CD11c⁺) was noted in the lungs of mice treated with IFN-γ/LPS (18.3%) compared with treatment with IFN-γ (34%), LPS (44.3%), or vehicle (44.3%) alone (Fig.2.1A). There were no significant changes in numbers of monocytes or DCs (unpublished data). In clinical studies, the levels of
CD14 on pulmonary macrophages after allergen challenge correlates with airway sensitivity to methacholine (Viksman et al., 2002). To determine the effect of IFN-γ/LPS on the activation of pulmonary macrophages, we also examined the expression of CD14 on this cell population. Treatment with IFN-γ/LPS upregulated the expression of CD14 on CD11b+ macrophages compared with controls (Fig.2.1B). IFN-γ treatment alone also slightly increased CD14 expression on these cells compared with LPS or vehicle-treated controls. These data suggest that the development of IFN-γ/LPS–induced AHR is associated with activation and increased numbers of CD11b+ pulmonary macrophages.

2.4.2 IFN-γ/LPS administration induces increased expression of IL-27 in pulmonary macrophages

In preliminary experiments, we dissected airways from lung tissue of mice 12 h after IFN-γ/LPS treatment, performed gene array analysis on these airways, and identified that IL-27 p28 was upregulated compared with IFN-γ-, LPS-, or vehicle-treated controls (unpublished data). This result was confirmed by quantitative PCR (Fig.2.1C). The IL-27 EBI3 subunit was significantly increased in response to IFN-γ/LPS, IFN-γ, and LPS administration compared with vehicle-treated controls (Fig.2.1C). To analyze the cellular production of IL-27 p28 protein, we separated homogenized lung into fractions highly enriched for macrophages or epithelial cells, or containing other non-adherent cells. IFN-γ/LPS treatment induced the production of IL-27 p28 by pulmonary macrophages, which was significantly increased at 6 h and peaked at 12 h (Fig.2.1D).
Although IFN-γ had no effect, LPS caused a slight increase in production of IL-27 p28. We did not detect production of IL-27 p28 by epithelial cells or in the nonadherent cell population (containing CD4+ and CD8+ T lymphocytes and NK cells; confirmed by FACS; unpublished data). These data indicate that the development of IFN-γ/LPS-induced AHR is associated with the production of IL-27 by pulmonary macrophages.

2.4.3 Levels of IL-27 p28 and IFN-γ are concurrently increased in neutrophilic but not eosinophilic asthma

To determine the relevance of our observations to human disease, we measured the levels of IL-27 p28 and IFN-γ in induced sputum samples from patients with neutrophilic and eosinophilic asthma using quantitative PCR. Levels of IL-27 p28 and IFN-γ were significantly increased in neutrophilic asthma compared with eosinophilic asthma (Fig.2.1E). No difference was observed in the expression of the IL-27 EBI3 between the different groups (unpublished data). These results support the concept that interactions between IL-27 and IFN-γ may be involved in the pathogenesis of asthma, and they are particularly relevant to the difficult-to-manage neutrophilic form of the condition.
Fig. 2. Exposure of the airway to IFN-γ and LPS induces recruitment and activation of CD11b+ macrophages, with increased production of IL-27 from macrophages. Exposure of the airways to IFN-γ and LPS induces increases in (A) CD11b+ macrophages, (B) expression of CD14 on CD11b+ macrophages, (C) expression of IL-27 p28 mRNA in the airway tissues, and (D) production of IL-27 protein in cultured pulmonary macrophages (data are representative of four independent experiments). Levels of both (E) IL-27 p28 and IFN-γ were significantly and concurrently increased in induced sputum samples from neutrophilic (n = 9) compared with eosinophilic asthmatics (n = 11). *p < 0.05 compared with other groups; #p < 0.05 compared with groups treated with LPS, IFN-γ, or IFN-γ/LPS; • p < 0.05 compared with eosinophilic asthmatics.

2.4.4 Cooperative interaction between IL-27 and IFN-γ contributes to the development of AHR

To elucidate the role of IL-27 in the mechanism of steroid-resistant AHR, we treated mice with anti–IL-27–neutralizing Ab, exposed the airways to IFN-γ/LPS, and measured airway reactivity and inflammatory infiltrates 12 h later. Neutralization of IL-27 abolished IFN-γ/LPS-induced AHR, but not neutrophil influx, into the airways (Fig. 2.2A, 2B).
Next we treated mice with vehicle, IL-27, IL-27/IFN-γ, or IL-27/LPS and assessed AHR and airway inflammation 12 h later. Treatment with IL-27/IFN-γ resulted in significantly increased airway reactivity to methacholine compared with the other groups (Fig. 2.2C). Treatment with IL-27/LPS or IL-27 had no effect on baseline reactivity to methacholine. However, IL-27/IFN-γ did not induce neutrophilic inflammation; this was only triggered by treatment with IL-27/LPS (Fig. 2.2D). This finding indicates that development of IL-27/IFN-γ–induced AHR is not dependent on neutrophilic inflammation.
Fig. 2.2 Synergism between IL-27 and IFN-γ contributes to the development of AHR independently of neutrophilia. Neutralization of IL-27 during IFN-γ and LPS treatment abolished (A) the induction of AHR but did not affect (B) numbers of neutrophils in BALF. Cooperation between IL-27 and IFN-γ was required for (C) the induction of AHR. LPS but not IL-27 was required for (D) the increased number of neutrophils in BALF. *p < 0.05 compared with other groups.

2.4.5 IL-27/IFN-γ–induced AHR is abolished by the depletion of pulmonary macrophages

To examine if pulmonary macrophages are critical in IL-27/IFN-γ–induced AHR, we depleted these cells using lip-CI2MDP 3 d before IL-27/IFN-γ treatment. Depletion of pulmonary macrophages abolished IL-27/IFN-γ–induced AHR (Fig. 2.3A). Because IL-27Rs are also found on CD4+ and CD8+ T lymphocytes and NK cells, we also tested the effect of depleting these cells with mAbs to determine their roles in IL-27/IFN-γ–induced AHR. In contrast to the macrophage depletion studies, depletion of CD4+ or CD8+ T cells or NK cells did not affect IL-27/IFN-γ–induced AHR (Fig. 2.3B–D). These data identify the critical role of pulmonary macrophages in the development of IL-27/IFN-γ–induced AHR.
Fig. 2.3 Macrophages are critical for IL-27/IFN-γ–induced AHR. IL-27/IFN-γ–induced AHR was markedly attenuated by (A) depletion of pulmonary macrophages. In contrast, depletion of (B) CD4⁺ or (C) CD8⁺ T cells or (D) NK cells had no effect. Cl2MDP represents liposome-encapsulated clodronate. PBS represents liposome-encapsulated PBS. *p < 0.05 compared with other groups; #p < 0.05 respective controls or depletion groups compared with vehicle.
2.4.6 IL-27/IFN-\(\gamma\)-induced AHR is not inhibited by the suppression of NF-\(\kappa\)B, JNK, or p38

NF-\(\kappa\)B, JNK, and p38 are critical mediators of TLR-MyD88 signalling pathways in response to LPS (Beutler, 2004). Inhibition of NF-\(\kappa\)B by BAY11-7082 significantly suppressed IFN-\(\gamma\)/LPS-- but not IL-27/IFN-\(\gamma\)--induced AHR (Fig.2.4A, 4B). Activation of NF-\(\kappa\)B is important for production of IL-27 p28 in macrophages after IFN-\(\gamma\)/LPS stimulation (Liu et al., 2007). BAY11-7082 also suppressed IL-27 production from macrophages treated with IFN-\(\gamma\)/LPS compared with vehicle-treated controls (unpublished data). Therefore, NF-\(\kappa\)B appears to be important in IFN-\(\gamma\)/LPS--induced AHR because of its role in the production of IL-27, but it is not required for IL-27/IFN-\(\gamma\)--induced AHR. Blocking JNK (Fig.2.4C, 4D) or p38 (Fig.2.4E, 4F) did not affect either IFN-\(\gamma\)/LPS-- or IL-27/IFN-\(\gamma\)--induced AHR. Collectively, these data suggest that NF\(\kappa\)B, JNK, or p38 do not contribute to IL-27/IFN-\(\gamma\)--induced AHR.
Fig. 2.4 The development of IL-27/IFN-γ–induced AHR does not involve NF-κB, JNK, or p38. Inhibition of activation of NF-κB by administration of BAY11-7083 markedly suppressed (A) IFN-γ/LPS but did not affect (B) IL-27/IFN-γ–induced AHR. Blocking of JNK function by treatment with SP600125 did not affect (C) IFN-γ/LPS or (D)
IL-27/IFN-\(\gamma\)–induced AHR. Inhibition of p38 action by administration of SB203585 did not affect (E) IFN-\(\gamma\)/LPS– or (F) IL-27/IFN-\(\gamma\)–induced AHR. *\(p< 0.05\) compared with other groups; \#\(p < 0.05\) respective controls or treated groups compared with vehicle

2.4.7 IL-27/IFN-\(\gamma\)–induced AHR is underpinned by the activation of MyD88 in pulmonary macrophages

MyD88 has been shown in vitro to contribute to IFN-\(\gamma\)–regulated macrophage activation (Shi et al., 2003). To examine the role of MyD88 in IL-27/IFN-\(\gamma\)–induced AHR, we treated mice deficient in this molecule (MyD88-/-) with IL-27/IFN-\(\gamma\). MyD88-/- mice did not develop AHR after administration of IL-27/IFN-\(\gamma\) (Fig.2.5A), and pulmonary macrophage numbers in BALF were the same as in WT mice (unpublished data). Notably, baseline reactivity to methacholine was equivalent in MyD88-/- and WT mice (Fig.2.5A). To investigate the importance of MyD88 in macrophages in IL-27/IFN-\(\gamma\)–induced AHR, we adoptively transferred macrophages derived from the lungs of WT mice into MyD88-/- mice and then treated these animals with IL-27/IFN-\(\gamma\). Transfer of WT macrophages reconstituted the IL-27/IFN-\(\gamma\)–induced AHR in MyD88-/- mice (Fig.2.5B). Baseline reactivity to methacholine in these recipient mice was also equivalent to responses in WT mice. Therefore, the presence of MyD88 in macrophages is essential for the development of IL-27/IFN-\(\gamma\)–induced AHR, and macrophage-derived factors appear to have a central role in the induction of enhanced airway reactivity.
Fig. 2.5 The induction of AHR by IL-27/IFN-γ is dependent on MyD88 signalling in pulmonary macrophages. The instillation of IL-27/IFN-γ into the airways of (A) MyD88−/− mice did not induce AHR. However, (B) the development of IL-27/IFN-γ–induced AHR was reinstated in MyD88−/− mice by transfer of WT pulmonary macrophages. *p < 0.05 compared with other groups; #p < 0.05 compared with vehicle controls.
2.4.8 IL-27/IFN-γ induces steroid-resistant AHR and suppresses the translocation of the GR to the nucleus of pulmonary macrophages

Next we determined if IL-27/IFN-γ–induced AHR was affected by steroid treatment. AHR induced by Th2 cells and LPS significantly suppressed by DEX in our previous study (Yang et al., 2009). Treatment of mice with DEX did not suppress AHR induced by IL-27/IFN-γ (Fig.2.6), indicating that IL-27 was integral to the steroid-resistant mechanism induced downstream in IFN-γ/LPS signalling pathways. Glucocorticoids act by binding to GR, which leads to the translocation of the resulting complex to the nucleus and the inhibition of gene transcription (Barnes et al., 2009). To determine if IL-27/IFN-γ treatment altered the ability of DEX to promote the translocation of GR, we visualised its intracellular localization by immunocytochemistry in pulmonary macrophages exposed to DEX and stimulated with LPS, IFN-γ, IL-27, IFN-γ/LPS, or IL-27/IFN-γ. Exposure of macrophages to DEX promoted the translocation of the GR to nucleus, whereas the receptor largely remained in the cytoplasm in vehicle-treated controls (Fig.2.7). Exposure of pulmonary macrophages to LPS, IFN-γ, or IL-27 prior to treatment with DEX did not inhibit nuclear translocation of GR (GR staining nuclear/cytoplasm ratio, 2.74 ± 1.01, 2.17 ± 0.61, or 2.25 ± 0.51, respectively; n = 4) and translocation was similar to that observed after DEX treatment alone (GR staining nuclear/cytoplasm ratio, 2.90 ± 0.81; n = 4) (Fig.2.7). In contrast, IFN-γ/LPS or IL-27/IFN-γ exposure resulted in a significant reduction of GR nuclear translocation (GR staining nuclear/cytoplasm ratio, 0.76± 0.23 or 0.80 ± 0.32, respectively; n = 4; p
<0.05) compared with the cells treated with DEX only (as above), and levels of cytoplasmic GR were not significantly different from vehicle control in the absence of DEX treatment (GR staining nuclear/ cytoplasm ratio, 0.55 ± 0.29; n = 4) (Fig.2.7).

Furthermore, neutralization of anti–IL-27 inhibited IFN-γ/LPS–induced blockade of GR nuclear translocation (GR staining nuclear/cytoplasm ratio, IFN-γ/LPS plus anti–IL-27 of 2.66 ± 0.78 or IFN-γ/LPS plus control Ab of 0.56±0.14; n = 4; p<0.05) (Fig.2.7).

These data indicate that exposure of pulmonary macrophages to IFN-γ/LPS or IL-27/IFN-γ results in defective nuclear translocation of GR to the nucleus, which underpins the mechanism of steroid-resistant AHR.

Fig.2.6 IFN-γ/LPS and IL-27/IFN-γ administration leads to the development of DEX-resistant AHR. IFN-γ/LPS– or IL-27/IFN-γ–induced AHR was not inhibited by DEX. #p< 0.05 compared with vehicle controls.
Fig. 2.7 IFN-γ/LPS and IL-27/IFN-γ administration impair steroid induced nuclear translocation of GR in pulmonary macrophages. Treatment of IL-27/IFN-γ or IFN-γ/LPS led to deficient GR nuclear translocation in cultured pulmonary macrophages in response to DEX stimulation (original magnification 31000; blue,
DAPI nuclear staining; red, Cy3 GR). Furthermore, neutralization of IL-27 recovered GR nuclear translocation, which was inhibited by IFN-γ/LPS. Each panel is representative of four independent experiments. The ratio of GR staining for nucleus and cytoplasm was also defined (see text).

2.5 Discussion

The mechanisms predisposing to steroid insensitivity in asthma remain largely unknown. Because of the complexity of the inflammatory process in these patients, we have initiated studies to identify the potential contribution of key immune factors that are implicated in pathogenesis. Recently, we demonstrated the integrated signalling between IFN-γ and LPS predisposed to the induction of steroid resistant AHR in a mouse model; these two factors are associated with severe asthma and exacerbations (Yang et al., 2009). In this study, we extend these observations and show that IL-27 production in macrophages plays a critical role in this mechanism of steroid resistance, by signalling with IFN-γ. Importantly, the effects of IL-27/IFN-γ are mediated via a novel MyD88-dependent pathway that suppresses nuclear translocation of the GR in pulmonary macrophages.

Although pulmonary macrophages are known to be critical in host defense against respiratory infection and are activated in the airways of asthmatics (Gosset et al., 1991), their contribution to the disease process and the development of AHR is unclear. Exposure to Ags or increased level of LPS in the airways is associated with the activation of macrophages in asthma (Viksman et al., 2002; Goleva et al., 2008).
Interestingly, the levels of CD11b\(^+\) macrophages in the lung are almost doubled after allergen challenge in patients with asthma (Viksman et al., 2002). CD11b is a component of \(\alpha\)-chains of b2 integrins (Arnaout, 1990). Higher expression of CD11b reflects the activation of pulmonary macrophages (Lensmar et al., 1999; Viksman et al., 2002). Indeed, adoptive transfer of CD11b\(^+\) pulmonary macrophages induces exaggerated AHR and airway inflammation in a mouse model of asthma (Moon et al., 2007). This suggests that these cells may contribute to the pathogenesis of AHR and inflammation, particularly in relation to recurrent infections (Lensmar et al., 1999; Taylor et al., 2005; Kirby et al., 2006).

In our study, administration of IFN-\(\gamma\)/LPS to the airways resulted in the influx of increased numbers of activated CD11b\(^+\) pulmonary macrophages (a small percentage of these CD11b\(^+\) cells may also be DCs and LPS-induced recruitment of monocytes) into the airways and increased expression of IL-27 in the lung. Treatment of isolated pulmonary macrophages with IFN-\(\gamma\)/LPS substantially increased the expression of IL-27 p28 (but not the EBI3 subunit), and these cells were the primary source of the induced IL-27 production in the lung. This finding is analogous to the observation that CD11b\(^+\) microglia/macrophages are important sources of IL-27 during the development of inflammation associated with autoimmune encephalomyelitis (Li et al., 2005). We showed that neutralization of IL-27 completely inhibited the development of IFN-\(\gamma\)/LPS–induced AHR, indicating that this molecule played a central role downstream of these signals in the induction of steroid-resistant AHR. Although
IFN-γ/LPS regulated the production of IL-27 from macrophages, and this cytokine was required for the induction of AHR, it alone could not alter airway reactivity. Importantly, a second signal from IFN-γ was required in conjunction with IL-27 to induce AHR. We also found that IL-27/IFN-γ-mediated AHR was resistant to DEX therapy, further confirming that these molecules were integral to the mechanism of IFN-γ/LPS–induced steroid-resistant AHR. Thus, IFN-γ has two pivotal roles in the mechanism for the induction of AHR: the first in promoting LPS-induced production of IL-27 from macrophages, and the second in acting cooperatively with IL-27 to alter airway reactivity. Administration of IL-27/IFN-γ to the airways did not induce neutrophil or eosinophil influx into the lung, and NK cells and CD4+ and CD8+ T cells were not required for the induction of AHR. However, IL-27/IFN-γ–induced AHR was attenuated by the depletion of pulmonary macrophages. Collectively, these observations demonstrate that IL-27 and pulmonary macrophages play a central role in the regulation of steroid-resistant AHR.

We have previously shown that TLR4-MyD88 signalling pathways are critical in the development of steroid-resistant AHR induced by IFN-γ/LPS (Yang et al., 2009). In this study, we have established that MyD88 also has a central role in IL-27/IFN-γ–induced steroid resistant AHR. AHR induced by IL-27/IFN-γ was not suppressed in TLR4-deficient mice (unpublished data), and treatment with IL-27 and/or IFN-γ did not induce pulmonary neutrophilic influx, ruling out the possibility that the requirement for MyD88 was associated with low levels of endotoxin exposure during cytokine...
treatments. Moreover, adoptive transfer of WT macrophages to MyD88-deficient mice restored IL-27/IFN-γ–induced AHR. Taken together, these results suggest that MyD88, specifically expressed in macrophages, is critical for IL-27/IFN-γ signal transduction that leads to alterations in airway reactivity.

Although MyD88 was initially recognised as a key molecule in the transduction of TLR pathways, emerging in vitro evidence also reveals a role for this adaptor protein in IFN-γ signalling (Shi et al., 2003). IFN-γ stimulation of macrophages induces a novel physical association between IFN-γR and MyD88 and the formation of a signalling complex, termed a signalosome, without affecting IFN-γ–induced JAK-STAT signalling/phosphorylation (Sun et al., 2006). During this process, MyD88 directly interacts with the cytoplasmic domain of the IFN-γR (Sun et al., 2006). The signalosome may function to regulate specific aspects of host defense responses, as subsets of proinflammatory molecules are not transcribed in macrophages deficient in MyD88 when stimulated with IFN-γ (Sun et al., 2006). Importantly, the specific and critical role of MyD88 in IFN-γsignalling pathways was further supported in our studies by the lack of effect of the inhibition of NF-κB or other MAPK pathways (e.g., JNK and p38) on IL-27/IFN-γ–induced AHR. These molecules are important components in TLR-MyD88–linked signalling cascades. Collectively, our data indicate that the induction of IL-27/IFN-γ–induced AHR is dependent on an integrated cytokine signalling network in macrophages that involves the IL-27R, IFN-γR, and MyD88.

Glucocorticoids suppress inflammation and asthma by attenuating the expression of
inflammatory factors. This occurs through binding of the steroid to the GR, which results in the translocation of the GR to the nucleus and the inhibition of gene transcription (Barnes et al., 2009). We found that the GR remained in the cytoplasm of macrophages instead of translocating to the nucleus after DEX treatment in the presence of IFN-γ/LPS or IL-27/IFN-γ. Exposure to IFN-γ, LPS, or IL-27 alone did not affect DEX–induced translocation of the GR. Collectively, our results demonstrate that IL-27/IFN-γ induces steroid-resistant AHR possibly by suppressing steroid activation of the GR pathway. Furthermore, MyD88 has a critical role, directly or indirectly, in the regulation of GR signalling in macrophages in response to steroid activation, in addition to its well-described function as an adaptor molecule for TLRs. To our knowledge, this is the first demonstration of a collaborative role between IL-27R, IFN-γR, and MyD88 signalling network in the suppression of the GR pathway. The role of the endogenous glucocorticoid pathway in immune responses is to promote the expression of anti-inflammatory proteins, while downregulating the expression of proinflammatory proteins (Barnes et al., 2009). Although speculative, the IL-27R/IFN-γR/MyD88 signalling system may act physiologically to suppress the GR pathway and promote the proinflammatory function of macrophages. Furthermore, chronic respiratory infection and activation of this signalling system could lead to desensitisation and dysfunction of the GR pathway.

Our study indicates that the underlying inflammatory processes in patients are likely to have significant effects on the pattern of AHR and its responsiveness to steroid
treatment. Clinical and experimental studies suggest that Th2 cell/eosinophil–dominated asthma, which is dependent on signalling through STAT6, can be effectively managed by steroids (Evans et al., 1993; Pauwels et al., 1997; Mathur et al., 1999; Kumar et al., 2003). In contrast, pathways activated by host defense mechanisms (classically nonallergic) such as IL-27/IFN-γ and Th17 cells are more likely to be steroid-resistant (Szefler et al., 1989; McKinley et al., 2008; Al-Ramli et al., 2009; Yang et al., 2009). Thus, nonallergic pathways that lead to AHR are fundamentally different from those regulated by Ag-reactive CD4+ Th2 cells. Interestingly, nonallergic pathways may be able to cross-regulate allergic inflammatory mechanisms suppressing Th2-like disease.

While IL-27 and its receptor WSX-1 are important in the initial stages of CD4+ Th1 cell differentiation (Pflanz et al., 2002), they also negatively regulate Th2 cell development and activity (Miyazaki et al., 2005; Yoshimoto et al., 2007). WSX-1–deficient mice exhibit enhanced Th2 responses in models of allergic airways inflammation and gastrointestinal nematode Trichuris muris infection (Artis et al., 2004; Miyazaki et al., 2005). Intranasal administration of IL-27 during Ag challenge in a mouse model of allergic asthma inhibited IL-13 production from Th2 cells and the development of AHR (Yoshimoto et al., 2007).

Although preliminary, analysis of the expression of IL-27 and IFN-γ in the sputum from neutrophilic and eosinophilic asthmatics supports a potential role for these factors in disease pathogenesis. Of note, IL-27 and IFN-γ were expressed to a greater extent in the airway secretions of neutrophilic asthmatics, who show insensitivity to steroid
treatment. Neutrophilic airway inflammation is a recognised feature of severe and difficult-to-manage asthma, but the contribution of this cell type to the expression of disease remains unresolved. Interestingly, neutrophilic asthmatics are allergic individuals, as evidenced by positive atopic tests, yet they have substantially reduced eosinophil levels (~0.4%) in their sputum compared with eosinophilic asthmatics (~4.3%)(Simpson et al., 2005).

In summary, the pathogenesis of severe and steroid-resistant asthma is complex, and our lack of knowledge prevents the development of targeted therapeutics. By focusing on the role of innate host defense pathways, we have developed models that allow dissection of the mechanisms predisposing to the induction of nonallergic inflammation and steroid-resistant AHR. In this study, we demonstrate the importance of integrated signalling events between IL-27R, IFN-γR, and MyD88 pathways specifically in pulmonary macrophages for the induction of steroid-resistant AHR. This mechanism, which does not require eosinophilic or neutrophilic inflammation and occurs independently of T lymphocytes, may be clinically relevant. Importantly, the IL-27R–linked IFN-γR pathway operates by inhibiting steroid-induced GR trafficking into the nucleus. Understanding the contribution of this novel macrophage pathway to subtypes of asthma may provide new therapeutic approaches for difficult-to-treat asthma.
Chapter 3.

Activation of olfactory receptors on mouse pulmonary macrophages promotes monocyte chemotactic protein-1 production
3.1 Abstract

Emerging evidence suggests that non-olfactory tissues and cells can express olfactory receptors (ORs), however, the exact function of ectopic OR expression remains unknown. We have previously shown in mouse models that a unique cooperation between IFN-γ and LPS drives the activation of pulmonary macrophages and leads to the induction of pathogenetic responses in the respiratory tract. Further, through gene array studies, we have shown that activation of macrophages by these molecules results in the selective expression of a number of ORs. In this study, we validated the expression of these ORs in mouse airway and pulmonary macrophages in response to IFN-γ and LPS (γ/LPS) stimulation, and further explored the effect of odorant stimulation on macrophage function. OR expression in airway and pulmonary macrophages in response to IFN-γ, LPS or γ/LPS treatments was assessed by microarray and validated by q-PCR. OR expression (e.g. OR622) on macrophages was confirmed by visualisation in immunofluorescence assays. Functional responses to odorants were assessed by quantifying inflammatory cytokine and chemokine expression using q-PCR and cell migration was assessed by a modified Boyden chamber migration assay. Our results demonstrate that eight ORs are expressed at basal levels in both airway and pulmonary macrophages, and that γ/LPS stimulation cooperatively increased this expression. Pulmonary macrophages exposed to the combined treatment of γ/LPS+octanal (an odorant) exhibited a 3-fold increase in MCP-1 protein production, compared to cells treated with γ/LPS alone. Supernatants from γ/LPS+octanal exposed macrophages also
increased macrophage migration in vitro. Eight different ORs are expressed at basal levels in pulmonary macrophages and expression is upregulated by the synergistic action of γ/LPS. Octanal stimulation further increased MCP-1 production and the motility of macrophages. Our results suggest that ORs may mediate macrophage function by regulating MCP-1 production and cell migration.

3.2 Introduction

Olfactory receptors (ORs) are one of the most ancient sensory systems in animals and are crucial for animal survival, procreation and evolution (Firestein, 2001; Margolskee, 2002; Zhang et al., 2003; Mombaerts, 2004). In mammals, ORs on the nasal olfactory epithelium detect scents by binding to odorant molecules (Ressler et al., 1994; Buck, 2004). These ORs belong to the G-protein coupled receptor (GPCR) superfamily, the largest known superfamily of cell-surface receptors (Malnic et al., 1999; Godfrey et al., 2004). Notably, ORs (like immune recognition receptors), are responsible for distinguishing molecules of immense diversity (Firestein, 2001). Although primarily associated with the nasal sensory systems, recent studies have demonstrated OR expression across a range of other tissues (such as testis, kidney, heart, etc.) (Parmentier et al., 1992; Weber et al., 2002; Feldmesser et al., 2006; Pluznick et al., 2013). However, their function in non-olfactory tissues and cells remains largely unknown.

IFN-γ and LPS play central roles in the activation of innate host defence pathways during infection (Beutler, 2000; Shtrichman et al., 2001). Previously, we have reported a
unique signalling pathway that is activated cooperatively by IFN-γ and LPS. In a mouse model, we demonstrated that IFN-γ and LPS/Toll like receptor 4 (TLR4) pathways synergistically activate a novel steroid-resistant MyD88-dependent pathway in pulmonary macrophages (Yang et al., 2009; Li et al.). This leads to (i) production of the proinflammatory cytokine interleukin (IL)-27, (ii) glucocorticoid receptor (GR) dysfunction, and (iii) the development of airways hyperreactivity (AHR, enhanced airway smooth muscle contractility in response to spasmogenic stimuli) (Yang et al., 2009; Li et al., 2010). This pathway may have significant implications for our understanding of how infection and immune molecules inhibit the endogenous glucocorticoid pathway to promote inflammation. Furthermore, we have demonstrated that this pathway may also contribute to the pathogenesis of infection-induced, steroid-resistant asthma (Li et al., 2010). In an attempt to further explore how IFN-γ and LPS regulate pulmonary macrophage function, we performed transcriptome profiling of the airways 12 hours after exposure to IFN-γ and LPS (γ/LPS). Surprisingly, exposure of the airways to γ/LPS resulted in markedly increased expression of a number of ORs. Subsequent investigations demonstrated that this OR expression was present on pulmonary macrophages. Therefore, we speculated that the increased OR expression induced by IFN-γ and LPS may play a functional role in innate immune responses of pulmonary macrophages, and as such, may contribute to the host defence process.

Interestingly, the concept that sensory receptors (both taste and smell receptors) contribute to host defence responses is beginning to emerge. Although examples are
limited, both taste and smell receptors have been linked to the activation of resident cells in human and mouse airways that contribute to anti-microbial responses (Shah et al., 2009; Deshpande et al., 2010). For example, non-specific stimulation of ‘ectopically’ expressed taste receptors by flavonoids leads to increased ciliary beat frequency on airway epithelial cells and the relaxation of airway smooth muscle cells (Shah et al., 2009; Deshpande et al., 2010). Furthermore, activation of taste receptors in the human respiratory epithelium increases the production of nitric oxide (NO), resulting in enhanced mucociliary clearance and direct anti-microbial effects (Lee et al., 2012). Intranasal application of female odorants also stimulates leukocyte mobilization, in the lungs of male mice (Moshkin et al., 2013). Collectively, these investigations suggest that the host defence system (including pulmonary macrophages) may employ sense receptors to respond to invading microbes.

As with other innate immune cells, pulmonary macrophages express a wide range of receptors, which enable them to recognise a wide variety of endogenous and exogenous stimuli (Taylor et al., 2005). These receptors collectively regulate differentiation, activation, migration, phagocytosis and cytotoxicity of macrophages (Gordon, 2002; Taylor et al., 2005). Thus, we speculated that ORs could also function to regulate one or more of these critical functions of macrophages in response to odorants. Macrophage migration to the site of infection is critical for microbial clearance and is regulated by a range of chemokines (Haskill et al., 1992; Lauffenburger et al., 1996). Among these, monocyte chemotactic protein-1 (MCP-1) plays a central role (Yoshimura et al., 1989;
Rollins, 1997). MCP-1 is primarily secreted by activated macrophages during the host defence response suggesting autocrine and paracrine functions (Yoshimura et al., 1989; Yoshimura et al., 1991; Leonard et al., 1993).

In this study, we demonstrate OR expression in pulmonary macrophages and show that γ/LPS synergistically increase the expression of a number of ORs. Furthermore, we demonstrate that odorant-receptor stimulation greatly enhances both MCP-1 production by γ/LPS-activated pulmonary macrophages and cell migration. Thus, we propose that odorants produced by the metabolic activity of infecting microbes may activate ORs on macrophages leading to increased MCP-1 production and macrophage recruitment to the site of infection.

3.3 Materials and Methods

3.3.1 Mice

Wild type pathogen free BALB/c mice (6–8 wk) were obtained from the animal services unit of the University of Newcastle. All experiments were performed with approval from the animal ethics committee of the University of Newcastle (ethics approval number: A-2010-132).

3.3.2 Administration of IFN-γ, LPS or γ/LPS

Mice were anesthetized (100 μl Alfaxan solution [1:4] diluted with PBS i.v.) and the trachea was intubated with a 22-gauge catheter. Optimized doses of murine IFN-γ (1.5 μg/mouse; PeproTech, Rocky Hill, NJ), LPS (50 ng/mouse; Sigma-Aldrich, St Louis,
MO) or γ/LPS (IFN-γ 1.5 μg + LPS 50 ng/mouse) in 50 μl vehicle (saline) were instilled intratracheally (i.t.) into the airways. Airway samples were then collected at 12hr after treatment.

3.3.3 Gene chip microarray

Airway tissues were collected by blunt dissection, a procedure that permits effective separation of the airway tissue without significant contamination by parenchymal tissue (Yang et al., 2009; Li et al.). Airway tissues were disaggregated and homogenized and total RNA was extracted using the RNeasy Mini Kit (QIAGEN) as per the manufacturer’s instructions. For gene chip hybridization (Illumina microarray platform) and data analysis, RNA samples were stored in dry ice and shipped to the SRC Microarray Facility, The University of Queensland.

3.3.4 Pulmonary macrophage isolation and treatment

Pulmonary macrophages were isolated from mouse lungs according to previously described methods [29]. Briefly, mouse lung tissue was minced and single cell suspensions prepared. Macrophages were separated by density gradient centrifugation with Histopaque-1083 (Sigma-Aldrich, St Louis, MO) and plated at a concentration of $6 \times 10^6$ cells/ml in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal calf serum (FCS). After 3 hr, 95% of adherent cells were macrophages, as confirmed by flow cytometry. Macrophages were cultured overnight, and then stimulated with different OR agonists, including amyl acetate (5mM, Sigma-Aldrich, St Louis, MO), DL-α, ε-diaminopimelic acid (2.5μM, MP Biomedicals, Santa Ana, CA), octanal (10μM,
Sigma-Aldrich, St Louis, MO) or vehicle (PBS or DMSO 0.1% v/v) for 12 hr. In some experiments, as indicated, macrophages were pretreated with γ/LPS (IFN-γ 0.5 μg + LPS 50 ng/ml) for 12 hr before exposure to octanal.

3.3.5 Preparation of peritoneal macrophages and macrophage migration assay

Brewer’s thioglycolate (3 ml of 4% solution, BD Bacto, Franklin Lakes, NJ) was injected into the peritoneum of mice, and peritoneal-derived macrophages were isolated 4 days later, by washing the peritoneal cavity with 5 ml of ice-cold Hank's buffered salt solution (HBSS). Erythrocytes were then eliminated using red cell lysis buffer as previously described and peritoneal-derived macrophage plated at $5 \times 10^5$ cells/well in 6-well plates with complete Roswell Park Memorial Institute (RPMI) 1640 medium (Yang et al., 2009). After 3 hr, non-adherent cells were washed away with PBS, and adherent cells were plated in fresh complete RPMI 1640 medium. Over 99% of adherent cells were macrophages, as determined by flow cytometry. For migration assays, $1 \times 10^6$ peritoneal macrophages were seeded in the 24-well upper chamber of a cell culture insert with 8-μm pore-size membrane (BD Lifesciences, San Jose, CA). Macrophages were allowed to attached for 3hr, then 600 μl of cell culture media cell culture media with anti-MCP antibody or control IgG (Biolegend, San Diego, CA)or media containing MCP-1 (10 ng/ml) was added to the lower chamber and migrating macrophages were quantified in the lower chamber after 5 hr.

3.3.6 Bone marrow derived macrophage (BMDM) culture and
polarization

Mouse femurs were flushed with 5 ml ice cold HBSS through a 70µm cell strainer. After red blood cell lysis and washing with PBS, bone marrow cells were plated at 4 × 10^6 cells per 10-cm dish with 10 ml of macrophage complete medium (L929 conditioned DMEM/12 medium). After culturing for 3 days, 5 ml of fresh medium was added to the culture. On day 7, floating cells were discarded and adherent cells used for further polarization studies. BMDM were polarized to M1 phenotype with IFN-γ (100 ng/ml) and LPS (10 ng/ml) or M2 phenotype with IL-4 (20 ng/ml) in the absence or presence of octanal. Polarized BMDM were then further stimulated with octanal (10µM) or vehicle (DMSO, 0.1% v/v) for 12 hr.

3.3.7 Quantitative polymerase chain reaction (q-PCR)

Quantitative PCR was performed as described in detail elsewhere (Triballeau et al., 2008; Yang et al., 2009). Briefly, total RNA was isolated from treated pulmonary macrophages in TriReagent (Sigma-Aldrich, St Louis, MO) and reverse transcribed using Superscript III (Invitrogen, Carlsbad, CA). q-PCR was performed using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Carlsbad, CA) using SYBR green reagents and expression was normalised to the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primers sequences are shown in Tables 1 and 2.

3.3.8 Immunofluorescence detection of OR622

Immunofluorescence assays were performed as described previously [16]. Briefly,
macrophages isolated from mouse lungs were seeded on cover slips at $6 \times 10^6$ cells/ml in DMEM containing 20% FCS. Cells attached to the slips were washed with cold PBS, fixed in 1% (w/v) paraformaldehyde in PBS buffer for 10 min at room temperature (RT), and blocked with 5% bovine serum albumin (BSA)/PBS for 1hr at RT. Cells were then incubated with a polyclonal Ab to OR622 (antibodies-online, Atlanta, GA, USA) or isotype control (purified nonimmune rabbit IgG, Santa Cruz Biotechnology, Santa Cruz, CA) in 1% BSA/PBS overnight at 4°C. Cover slips were then washed with PBS and incubated with Cy3-conjugated goat anti-rabbit IgG (10 mg/ml; GE Healthcare, Buckinghamshire, U.K.) diluted in 1% BSA/PBS for 45 min in the dark at RT. Coverslips were then washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) define (Sigma-Aldrich, St Louis, MO) for 10 min at RT. Slips were fixed on slides and visualised using a fluorescence microscope (BX51; Olympus, Tokyo, Japan) using a 100× objective lens. Fluorescence images were captured using a digital camera (DP70; Olympus, Tokyo, Japan). Mean fluorescence intensity was calculated and expressed as mean ± SEM.

3.3.9 ELISA

Cell culture supernatants were collected after treatment as described and the MCP-1 ELISA kit (eBioscience, San Diego, CA) was used to detect MCP-1 protein levels according to manufacturer’s instructions.

3.3.10 Macrophage phagocytosis of non-typeable *Haemophilus influenzae* (NTHi)
Isolation of pulmonary macrophages was performed as described above. Cells were then infected in vitro with 100 MOI NTHi for 8 hr as previously described (Marti-Lliteras et al., 2009). To quantify intracellular bacteria load, macrophages were incubated with gentamicin (400 μg/mL) for 1 hr at 37°C to eliminate extracellular bacteria. After gentamicin treatment, cells were washed three times with PBS before being lysed with 0.25% saponin to release intracellular NTHi. To quantify extracellular bacteria numbers, supernatants were removed, plated onto chocolate agar plates and incubated overnight at 37 °C in 5% CO₂. Bacterial colonies were counted after incubation for 16 hr.

3.3.11 Data analysis
An initial one-way ANOVA was used to test for differences between groups. Values are presented as mean ± SEM for each experimental group. The number of mice ranged from 8 to 10 per group. Differences in means were considered significant if p was < 0.05.

3.4 Results
3.4.1 Treatment with γ/LPS up-regulates OR expression levels in mouse airway tissue.
We have previously shown that pulmonary macrophages play a critical role in the development of γ/LPS-induced steroid-resistant inflammation and AHR by inhibiting endogenous glucocorticoid pathways (Triballeau et al., 2008; Yang et al., 2009). This suggests that these molecules activate pathways that enhance inflammation and host
defence responses. Steroid-resistant inflammation and AHR develops 12 hr after exposure of the airways to these factors and is critically dependent on cooperative signalling (Li et al., 2010). To determine the pathways selectively activated by γ/LPS, we profiled mRNA expression in airway tissue by microarray 12 hr after exposure to LPS, IFN-γ and LPS + IFN-γ (γ/LPS). Interestingly, the data showed that a group of ORs (including of OR65, OR272, OR352, OR446, OR568, OR622, OR657 and OR1014) were significantly increased (Fig. 3.1). Further, the OR transcript levels following exposure to γ/LPS were profoundly elevated (~50 to 250 fold increase) compared to groups exposed to IFN-γ or LPS alone. To further characterize OR expression, we quantified these receptors in compartments of the airways and in different tissues using q-PCR (Fig.3.2). The same OR receptors were expressed in the trachea, bronchi and parenchyma of the lung, albeit at levels lower than those in nasal mucosa, where ORs are typically studied. While expression levels were very low in the liver, a number of ORs were also highly expressed in the testis.
Fig. 3.1. OR expression in the lung is induced by IFN-γ + LPS stimulation. Mice were intratracheally instilled with IFN-γ, LPS or γ/LPS, and airway samples were collected 12 hr later. Gene expression profiles were determined by microarray. Values are presented as mean ±SEM (n=4), *P<0.05 (vs. other groups).
Fig. 3.2 OR expression in different mouse tissues. Tissue samples from naïve BALB/c mice were collected. RNA was extracted and gene expression was determined with q-PCR. Values are presented as mean ± SEM (n=4 mice).
3.4.2 \( \gamma \)/LPS synergistically enhance the expression levels of ORs in pulmonary macrophages

Previously, we have shown that \( \gamma \)/LPS exposure activates pulmonary macrophages to induce steroid-resistant inflammation and AHR (Yang et al., 2009; Li et al.). We therefore examined whether OR expression occurred in pulmonary macrophages and whether levels could be synergistically and selectively upregulated by exposure to \( \gamma \)/LPS conditions in vitro. Pulmonary macrophages were isolated and treated with IFN-\( \gamma \), LPS or \( \gamma \)/LPS and expression levels of the eight candidate ORs quantified by PCR. IFN-\( \gamma \) or LPS exposure alone failed to induce the expression of any of the eight ORs, compared with vehicle treatment (Fig.3.3). However, combined treatment with \( \gamma \)/LPS induced a significant upregulation of all eight OR transcripts (Fig.3.3). To further confirm OR expression in macrophages, we used the only commercially available antibody to any of the identified ORs (OR622) for immunofluorescence staining. Staining demonstrated that OR622 was expressed at low levels at baseline in pulmonary macrophages, and that combined treatment with \( \gamma \)/LPS dramatically increased OR622 antibody fluorescence intensity (Fig.3.4 A and B).
Fig. 3.3 ORs are expressed by mouse pulmonary macrophages and upregulated by IFN-γ + LPS stimulation. Pulmonary macrophages were isolated by concentration gradient centrifugation and plated into 6-well plates. After 3 hr of attachment, adherent cells were treated with either IFN-γ, LPS or IFN-γ plus LPS (γ/LPS) for 12 hr. RNA was extracted and q-PCR was performed to assess OR gene expression. Values are presented as mean ± SEM (n=3 separate experiments). *P<0.05 (vs. vehicle control).
Fig.3.4 OR622 protein expression in mouse pulmonary macrophages. Pulmonary macrophages were isolated and attached to cover slides. After 3 hr of attachment, cells were treated with γ/LPS or vehicle control for 12 hr. OR622 protein expression was detected by immunofluorescence antibody staining. A) Representative cell images. Magnification ×1000; blue = DAPI nuclear staining; red = Cy3-conjugated secondary antibody (anti-OR622 antibody) staining. B) Quantification of red fluorescence intensity (in arbitrary units, a.u), values are presented as means ± SEM (n=40 cells), *P<0.05 (vs. vehicle control).
3.4.3 Olfactory agonists stimulate MCP-1 production by pulmonary macrophages

Octanal is a model agonist used to stimulate OR activation and the first odorant where a structure-function relationship has been established (Araneda et al., 2004). To determine whether macrophage-expressed ORs are functional, we exposed pulmonary macrophages to octanal and assessed changes in macrophage function. Resting pulmonary macrophages were stimulated with 10 μM octanal for up to 12 hr and the expression levels of macrophage-derived chemokines (macrophage inflammatory protein 1(MIP-1), monocyte chemotactic protein-1 (MCP-1), monocyte chemotactic protein-2 (MCP-2), monocyte chemotactic protein-3 (MCP-3), chemokine (C-X-C motif) ligand 1(KC)), cytokines (tumor necrosis factor-α (TNF)-α, IFN-γ), interleukin-12 (IL-12)), and other proinflammatory factors (nitric oxide synthase-2 (NOS2), resistin-like molecule-α (FIZZ1), chitinase-3-like protein 3 (YM1) and arginase-1 (ARG1)) were quantified by qPCR. Of the molecules assessed, MCP-1 mRNA expression was significantly increased following 12 hours of octanal exposure (Fig.3.6 A). There was no change in expression levels of other proinflammatory genes following octanal treatment (Fig.3.5). Next, we stimulated pulmonary macrophages with γ/LPS in combination with octanal for 12 hr to assess changes following γ/LPS-induced increases in OR expression. γ/LPS treatment alone resulted in ~65 and ~32 fold increases in the levels of MCP-1 mRNA transcripts and protein in culture supernatants, respectively (Fig.3.6 B and C), over vehicle exposure. The combined
treatment with γ/LPS and octanal resulted in a further increase in MCP-1 levels, with ~140 and ~114 fold increases in the levels of MCP-1 mRNA transcripts and protein in culture supernatants (Fig.3.6 B and C, respectively), as compared to vehicle.

Because ORs are G-protein coupled receptors, we also investigated the role of Ca²⁺ flux in OR signalling using diltiazem (a Ca²⁺ ion channel blocker). Diltiazem has been previously used to inhibit OR function (Restrepo et al., 1993; Restrepo et al., 1993). Here, we showed the addition of 50 μM diltiazem eliminated the octanal- but not the γ/LPS-induced expression of MCP-1 (Fig.3.6 D), demonstrating a role for OR activation in increasing MCP-1 expression.

To further demonstrate that macrophage-expressed ORs are functional, we employed two more odorants - amyl acetate and diaminopimelic acid- to stimulate pulmonary macrophages. Amyl acetate is commonly used to activate ORs experimentally (Cui and Evans, 1997). Diaminopimelic acid is a component of Gram-negative bacterial cell walls and an intermediate in the bacterial biosynthetic pathways for lysine and peptidoglycans, which has recently been identified to act as an OR agonist (Triballeau et al., 2008). Exposure of resting pulmonary macrophages to each of these two OR agonists for 12 hr also resulted in increased MCP-1 expression (Fig.3.7).
Fig.3.5 Effects of macrophage OR activation on the expression of proinflammatory genes, chemokines and cytokines. Pulmonary macrophages were isolated and plated into 6-well plates. After 3 hr of attachment, adherent cells were exposed to octanal for 12 hr. RNA was extracted and the mRNA expression of NOS2, ARG1, FIZZ1, YM1, IL-10, KC, MCP-2, MCP-3, MIP-1, TNF-α, IFN-γ and IL-12 was determined by q-PCR. Values are presented as mean±SEM (n=3 separate experiments).
Fig. 3.6 Octanal stimulation increases IFN-γ and LPS-induced MCP-1 expression in mouse pulmonary macrophages. Pulmonary macrophages were isolated and plated into 6-well plates. After 3 hr for attachment, adherent cells were stimulated with (A) octanal for 3 hr, 6 hr or 12 hr or (B/C) stimulated with octanal for 12 hr after exposure to γ/LPS (γ/LPS+Oct). MCP-1 mRNA expression was assessed by qPCR and protein levels were determined by ELISA. In addition, (D) macrophages were stimulated with γ/LPS or γ/LPS+Oct in the presence or absence of a diltiazem pre-treatment. Values are presented as mean ± SEM (n=3), *P<0.05, #P<0.05.
Fig.3.7 OR agonists induce MCP-1 expression in mouse pulmonary macrophages. Pulmonary macrophages were isolated and plated into 6-well plates. After 3 hr of attachment, adherent cells were stimulated with the OR agonists amyl acetate or diaminopimelic acid. Exposure to (A) amyl acetate or (B) diaminopimelic acid induced MCP-1 expression, as assessed by qPCR. Values are presented as mean ± SEM (n=3 separate experiments), *P<0.05.
3.4.4 Octanal exposure promotes \( \gamma \)/LPS-induced MCP-1 macrophage migration

To further characterize OR function on macrophages, we assessed whether \( \gamma \)/LPS-induced macrophage migration was enhanced by octanal-stimulated MCP-1 production using a chemotaxis chamber (Fig. 3.8). Supernatants derived from macrophages treated with \( \gamma \)/LPS and octanal resulted in significantly enhanced macrophage migration \((4.0 \pm 0.632 \times 10^4 \text{ cells/ml})\) compared to supernatants from \( \gamma \)/LPS treatment alone \((2.5 \pm 0.555 \times 10^4 \text{ cells/ml})\) (Fig. 3.8). We also compared migration rates to groups stimulated with recombinant MCP-1 \((10 \text{ ng/ml})\) at the concentration of MCP-1 found in \( \gamma \)/LPS and octanal stimulated cultures (Fig 3.8A). Results indicated that supernatant from octanal and \( \gamma \)/LPS exposed cultures could induce macrophage migration at levels comparable to those induced by recombinant MCP-1 alone \((10 \text{ ng/ml})\) (Fig.3.8A). We further confirmed the migration of macrophages was caused by the release of MCP-1 with MCP-1-neutralizing antibody (Fig.3.8B). Octanal-treated culture supernatants alone were unable to induce significant macrophage migration (Fig.3.8A). These data demonstrate that the OR agonist octanal enhances macrophage migration, potentially by driving increased MCP-1 production in \( \gamma \)/LPS-treated pulmonary macrophages.
Supernatants from macrophage cultures stimulated with octanal, IFN-\(\gamma\) and LPS induce macrophage migration. Pulmonary macrophages were isolated and stimulated with octanal, \(\gamma/LPS\) or \(\gamma/LPS\) plus octanal (\(\gamma/LPS+Oct\)), and cell culture supernatants were collected. Additional peritoneal macrophages were prepared and plated into modified Boyden chambers. Stimulated culture supernatants were added to the lower chamber and macrophage migration was assessed (A). MCP-1- neutralizing antibody or control IgG were added into the stimulated culture supernatants and macrophage migration was assessed (B). Medium spiked with MCP-1 (10ng/ml) was used as a positive control group. Values are presented as mean ±SEM (n= 3 separate experiments), P<0.05 was considered statistically significant.
3.4.5 Octanal does not influence macrophage polarization

Next we determined whether OR activation could effect the expression of markers that define the polarisation status of M1 or M2 macrophages or production of the anti-inflammatory cytokine IL-10. First, we assessed whether octanal altered the baseline expression of phenotypic markers of M1 and M2 cells in pulmonary macrophages. Exposure to octanal did not alter the expression NOS2 (M1 marker), or FIZZ1, YM1 or ARG1 (M2 markers) (Fig.3.9). γ/LPS exposure alone induced the expression of NOS2 in pulmonary macrophages, but not M2 cell markers, as previously described [29]. Octanal again had no effect on expression of these factors in the presence of γ/LPS exposure (Fig.3.9). We also generated BMDM (M0) and polarised them to the M1 or M2 phenotype (Fig.3.10). Again octanal had no effect on the expression of M1 or M2 markers. These data indicate that OR activation does not influence the polarization of macrophages.
Fig.3.9 OR activation has no effect on cultured pulmonary macrophage polarization. Pulmonary macrophages were isolated and plated into 6-well plates. After 3hr for attachment, adherent cells were exposed to either octanal alone or exposed to octanal after γ/LPS stimulation (γ/LPS+Oct). RNA was extracted and genes expression for NOS2, ARG1, FIZZ1 and YM1 were determined with q-PCR. Values are presented as mean ± SEM (n=3).
Fig 3.10 OR activation has no effect on cultured bone marrow derived macrophage polarization. Bone marrow derived macrophages were prepared and polarized toward M1 or M2 type macrophages, before being stimulated with octanal for 12 hr. RNA was extracted and gene expression for the macrophage polarization marker genes NOS2, ARG1, FIZZ1 and YM1 were determined by q-PCR. Values are presented as mean ± SEM (n=3).
3.4.6 Octanal does not affect the phagocytosis of NTHi by pulmonary macrophages

Finally, we assessed whether octanal exposure could alter the phagocytic capacity of pulmonary macrophages during bacterial infection. Macrophages were exposed to NTHi in the absence or presence of octanal and the rate of bacterial uptake was determined by colony formation assay. Exposure to octanal did not alter the number of bacteria taken-up by macrophages (nor affect bacterial replication in the culture medium). γ/LPS treatment of macrophages leads to rapid activation of these cells and exhaustion, which decreased their ability to clear bacteria from the supernatant. Octanal did not alter the effect of γ/LPS on bacterial clearance from the culture medium (Fig.11).

![Graph](image)

**Fig.3.11** Exposure to OR agonist has no effect on macrophage phagocytic capacity for NTHi. Pulmonary macrophages were prepared and exposed to NTHi in the absence or presence of octanal, and the rate of bacterial uptake was determined by colony formation assay. Colony forming units (CFU) of both intracellular (A) and
extracellular (B) bacteria were counted. Values are presented as mean ± SEM (n=3).

<table>
<thead>
<tr>
<th>OR</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>OR65</td>
<td>5′ AGGACCGCTGATCATCCACACAGCA3′</td>
<td>5′ AAGGAGGGTGCCGTTGCCAAGT3′</td>
</tr>
<tr>
<td>OR272</td>
<td>5′ ACCTGCTCTGCCCACCTGAGCT3′</td>
<td>5′ TGAGGGCCTCCATACGCTTTGA3′</td>
</tr>
<tr>
<td>OR352</td>
<td>5′ TTGCCCACCTCTCTTTCAGTGGGG3′</td>
<td>5′ TGAGAGCCACACGTTGACAAAGGC3′</td>
</tr>
<tr>
<td>OR446</td>
<td>5′ CTGGAGATGCTCCTGTGTTGCTGT3′</td>
<td>5′ TCCAGGCAGATGAGCCCAAGGATA3′</td>
</tr>
<tr>
<td>OR568</td>
<td>5′ GGCTGAGAGCAGCTAGGTCTG3′</td>
<td>5′ TGGCCAGCAAGACTCCGGATCC3′</td>
</tr>
<tr>
<td>OR622</td>
<td>5′ TCTGCAGCTCTGAAAGATGGCTTGCA3′</td>
<td>5′ GGGCAGGTGGCAAACCATGAGCACA3′</td>
</tr>
<tr>
<td>OR657</td>
<td>5′ GCTGGACGATTTGGCTCTCCCTACC3′</td>
<td>5′ GGCAAGGCCCAATCTCCACCACAGAG3′</td>
</tr>
<tr>
<td>OR1014</td>
<td>5′ ACCCTGTCA TGCAGCTGGTTCTGC3′</td>
<td>5′ GCCACACAGCCAGAAAGGAG ATGC3′</td>
</tr>
</tbody>
</table>

Table 3.1. Primer sequences of ORs.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>5’ AGGCCAGACCTTTGTTGGATTGAA 3’</td>
<td>5’ CAACTTGCCTCATCTTTAGCCTT 3’</td>
</tr>
<tr>
<td>IL-10</td>
<td>5’ CATTGAATTCCCTGGGTGAGAAG 3’</td>
<td>5’ GCCTTGTGACACCTTTGCTTGGG 3’</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5’ TCTTGAAAGACAATCAGGCCATCA 3’</td>
<td>5’ GAATCAGCAGCGACTCTTTTCC 3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’ GTCTACTGAACCTGCGGTATCG 3’</td>
<td>5’ AGCCTTTGCCCTTGAAGAAGAG 3’</td>
</tr>
<tr>
<td>IL-12</td>
<td>5’ CTGTGCGCTTGGGTCATCG 3’</td>
<td>5’ GCAGAGGCACCTCCATTGATT 3’</td>
</tr>
<tr>
<td>NOS2</td>
<td>5’ CTCGAGGATGTCACACTG 3’</td>
<td>5’ CAGAAACTTCGGAAGGAGCAAT 3’</td>
</tr>
<tr>
<td>ARG1</td>
<td>5’ GCTCCAAGGCAAAGTCCCTAGAGAT 3’</td>
<td>5’ AGGAGCTTGCACATTGGAAGACT 3’</td>
</tr>
<tr>
<td>MR</td>
<td>5’ GCCATGGGGCTTCTCTGCTTCTG 3’</td>
<td>5’ TGCCGTCCTGACACTGAGATGGGACT 3’</td>
</tr>
<tr>
<td>FIZZ1</td>
<td>5’ TACTTGCAACCTGCGCTTACT 3’</td>
<td>5’ TATCGAGGTGGTCTCCACTC 3’</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward</td>
<td>Reverse</td>
</tr>
<tr>
<td>--------</td>
<td>------------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>YMI</td>
<td>5′ GACAGGCCAATAGAAGGGAGTTTCA3′</td>
<td>5′ GACGGTTCTGAGGAGTAGAGACCAT3′</td>
</tr>
<tr>
<td>KC</td>
<td>5′ CAATGAGCTGCCTGTCAGTG3′</td>
<td>5′ CTTGGGGACACCTTTTAGCATC3′</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5′ AGAGCCAGACGGGAGGAAG3′</td>
<td>5′ CCAGCCTACTCAATTGGGATC3′</td>
</tr>
<tr>
<td>MCP-2</td>
<td>5′ GGGTGCTGAAAAAGCTACGAG3′</td>
<td>5′ TCCAGCTTTTGCTGCTCT3′</td>
</tr>
<tr>
<td>MCP-3</td>
<td>5′ AATGCAATCCACATGCTGCTA3′</td>
<td>5′ CTTTTGGAGTTGGGGTTTTCA3′</td>
</tr>
<tr>
<td>MIP-1</td>
<td>5′ CCTCTGACCTGCTCAACA3′</td>
<td>5′ GATGAATGGCGTGAAATC3′</td>
</tr>
</tbody>
</table>

Table 3.2. Primer sequences of other genes.
3.5 Discussion

ORs are classically regarded as smell sense receptors that are predominantly expressed in the olfactory epithelium within the nasal cavity (Firestein, 2001). However, various studies have reported that ORs may also be expressed in non-olfactory tissues, where the roles of these ectopically expressed receptors remain unclear (Feldmesser et al., 2006). In the present study, we demonstrate that a group of ORs (OR65, OR272, OR352, OR446, OR568, OR622, OR657 and OR1014) are expressed in mouse airway tissue and pulmonary macrophages (Fig. 3.1 and 3.2). We further demonstrate that IFN-\(\gamma\) and LPS stimulation act synergistically to enhance the expression of this group of ORs (Fig.3.3). We further demonstrate the presence of OR622 protein in pulmonary macrophages by immunofluorescence (Fig.3.4). Our data suggest that innate immune signals linked to the control of infection (IFN-\(\gamma\) and LPS) may regulate OR expression and suggest that these receptors may play a role in host defence responses, regulating macrophage function.

Although there are a limited number of studies on the role of ORs in immunity, a previous study demonstrated a role for odorants in activation of pulmonary immune response. Intranasal application of odorants derived from female mice stimulated IL-1\(\beta\)-independent activation of lung immunity and leukocyte mobilization in the lungs of male mice(Moshkin et al., 2013). In our study, stimulation of ORs with the odorant agonists, octanal, amyl acetate and diaminopimelic acid, increased MCP-1 production in pulmonary macrophages (Fig.3.6 and 3.7). The effect appears to be selective for MCP-1,
as no changes were observed in other major macrophage inflammatory mediators including NOS2, ARG1, FIZZ1, YM1, IL-10, MIP-1, MCP-2, MCP-3, KC, TNF-α, IFN-γ and IL-12 (Fig. 3.5) (Mantovani et al., 2004; Martinez et al., 2006; Benoit et al., 2008; Sica et al., 2012; Yang et al., 2012). Furthermore, supernatants from octanal-stimulated macrophages (with exposure to γ/LPS) significantly enhanced the migration of peritoneal-derived macrophages, at levels that were comparable to the levels observed by the addition of 10 ng/ml MCP-1 protein (Fig. 3.8). As MCP-1 predominantly acts on macrophages, these data collectively suggest that the activation of ORs in pulmonary macrophages leads to increased production of MCP-1, which further amplifies macrophage migration during immune responses (Carr et al., 1994; Xu et al., 1996).

Pulmonary macrophages are the most abundant innate immune cells in the lung and they are critical for maintaining pulmonary homeostasis, contributing to the clearance of foreign substances, elimination of infectious agents through phagocytosis and regulating aspects of innate and adaptive immune responses (Gordon, 2003; Gordon et al., 2005; Kaiko et al., 2008). Broadly, macrophages can be classified into M1 and M2 subtypes based on their expression of specific markers (Mantovani et al., 2004; Gordon et al., 2005; Murray et al., 2011). M1 macrophages are associated with T helper type 1 (Th1) immune responses and contribute to the clearance of intracellular pathogens, killing of tumour cells and removal of tissue debris (Mantovani et al., 2004; Gordon et al., 2005; Biswas et al., 2010). By contrast, M2 macrophages are associated with T helper type 2
(Th2) immune responses that underpin parasite eradication, suppression of inflammation and tissue remodelling (Mantovani et al., 2013). We investigated whether OR stimulation using octanal influences polarization of macrophages to specific phenotypes and phagocytosis of NTHi bacteria. Interestingly, octanal stimulation of bone marrow derived macrophages did not alter their M1/M2 polarisation phenotype (Fig. 3.9). Furthermore, exposure of pulmonary macrophages to octanal did not alter the rate of phagocytosis or clearance of NTHi by pulmonary macrophages (Fig. 3.10). These results imply that the roles of ORs expressed on macrophages are to facilitate the recruitment of these cells through the production of MCP-1, rather than directly altering their proinflammatory or innate host defence function.

Interestingly, many bacterial metabolites can act as OR agonists. For example, a recently identified odorant, diaminopimelic acid, is an intermediate product of the peptidoglycan and lysine biosynthetic pathways of bacteria (Triballeau et al., 2008). This in conjunction with our data suggests that pulmonary macrophages have the potential to detect bacteria in their surroundings by sensing bacterial metabolites. Similarly, this notion is supported by the findings that P. aeruginosa-generated acyl-homoserine lactones (quorum-sensing molecules) activate taste receptors in human upper respiratory epithelium (Lee et al.). This pathogenic bacterium is commonly found in the lungs of patients with cystic fibrosis (Lee et al.). Although it is not clear at present if these quorum-sensing molecules can stimulate ORs, studies in C. elegans have shown that nematodes can respond to acyl-homoserine lactones through ORs expressed on
their neuron cells (Shiner et al., 2005; Beale et al., 2006). These studies suggest that bacterial quorum-sensing molecules not only directly stimulate taste receptors but also have the potential to activate the ORs of mammalian cells. Highly volatile compounds such as acetic acid, ethanol and 4-methylphenol are well-known odorant molecules that have also been identified in the culture of *P. aeruginosa* (Zhu et al.). Interestingly, Fukuda and colleagues have recently shown that acetate (an acetic acid molecule lacking H+) released by *Bifidobacteria* protects the host from pathogenic infection through the activation of intestinal epithelial cells in a mouse model of *E. coli* O157 infection (Fukuda et al.).

Our findings demonstrate that stimulation of macrophages with the bacterial bio-product LPS in conjunction with the host defence cytokine IFN-γ synergistically up-regulates the expression of ORs. Activation of these ORs with the odorant octanal results in the increased expression and production of MCP-1 (but not other proinflammatory or host defence molecules). Although the fundamental roles of ORs on pulmonary macrophages needs to be further explored we speculate that the ectopic expression of ORs on macrophages may serve as another pathogen-recognition pathway for the sensing of odorants linked to the metabolic activity of infecting bacteria. Thus, OR activation may act to promote the migration and accumulation of macrophages at the site of bacterial infection, where they can be further activated by specific pathogen recognition pathways (e.g. Toll like receptors).
Chapter 4.

MiR-9 induction contributes to steroid-resistant airway hyperresponsiveness by reducing PP2A activity.
4.1 Abstract

We previously demonstrated that two factors, lipopolysaccharide (LPS) and interferon-γ (IFN-γ) drive induction of steroid-resistant airway hyperresponsiveness (AHR). Steroid-resistance is a major clinical problem in severe asthma patients and limited treatment options are available. MicroRNAs (miRs) are small non-coding RNAs that play an important regulatory role in innate immune activation; however their functional role in steroid-resistance remains unexplored. This study is to characterize the expression and functional role of miR-9 in macrophage responses to inflammatory signals in vitro and the induction of steroid-resistant AHR in vivo. MiRNA-9 expression was assessed by q-RT-PCR in mouse lung tissue and primary pulmonary macrophages following LPS + IFN-γ stimulation, as well as human asthmatic sputum samples. Potential miR-9 targets were determined using in silico prediction software and confirmed in luciferase reporter assays. Specific miR-9 blocking antagomirs were used to assess miR-9 function. Lung function was assessed in vivo and macrophage function was assessed using glucocorticoid receptor (GR) immunofluorescence and PP2A enzyme assays. LPS and IFN-γ synergistically induced miR-9 expression in lung tissues in vivo and pulmonary macrophages in vitro, resulting in decreased expression of the target gene PPP2R5D. MiR-9 expression was also increased in patients with neutrophilic asthma. Inhibition of miR-9, using specific antagomirs, increased both PP2A catalytic activity and GR nuclear translocation in macrophages and restored AHR steroid-sensitivity in multiple steroid-resistant AHR models. Increased miR-9
expression contributesto steroid-resistance, through decreased PP2A activity. Blocking miR-9 function restores steroid sensitivity and may serve as a novel approach for the treatment of steroid-resistant AHR.

4.2 Introduction

Asthma is a heterogeneous disorder divided into a number of distinct subsets (endotypes) based on airway inflammatory cell profiles (Wenzel et al., 1999; Simpson et al., 2006; Anderson, 2008; Gibson, 2009). Glucocorticoids are a front line anti-inflammatory treatment for disease management, however, some patients respond poorly to therapy even at high doses (Barnes and Adcock, 2009; Currie et al., 2009). Steroid-resistance occurs predominantly in severe and neutrophilic asthmatics where innate immune cells (e.g. neutrophils and macrophage rather than eosinophils) are activated and thought to contribute to pathogenesis (Simpson et al., 2005; Simpson et al., 2007; Li et al., 2010; Yang et al., 2012). An improved understanding of mechanisms underlying steroid-resistance and alternative approaches to overcome poor treatment-responsiveness are urgently required.

Numerous lines of evidence suggest a link between innate immune activation and steroid-resistant asthma. Activated macrophages and neutrophils are prominent features in the inflammation linked to steroid-resistant disease (Anderson, 2008; Yang et al., 2012). In patients, early-life infections are linked to increased asthma susceptibility, exacerbations and a decline in lung function (ten Hacken et al., 1998; Simpson et al.,
Recently, we demonstrated novel synergistic signalling between IFN-γ and LPS (two factors linked to the pathogenesis of steroid-resistant asthma), which induces macrophage-dependent steroid-resistant AHR in a mouse model (Yang et al., 2009; Li et al., 2010). We also demonstrated that macrophages and IFNγ induce persistent AHR in an allergen-induced model of steroid-resistant asthma (Yang et al., 2010). Furthermore, we demonstrated that microRNA (miRNA) play a pivotal role in allergic inflammation in mouse asthma models (Mattes et al., 2009; Collison et al., 2011), however, the role of miRNA in steroid-resistance remains unknown.

MiRNAs are short (19–25nt) non-coding RNAs that regulate gene expression, inhibiting translation or destabilizing target mRNAs (Lee et al., 1993; Lau et al., 2001). MiRNA bind target mRNA 3’-UTR s with partial complementarity, so a single miRNA can target multiple mRNAs. This allows miRNA to fine-tune specific target expression or act broadly on transcriptional pathways controlling immune cell function (Lodish et al., 2008; Hafner et al., 2011; Ma et al., 2011; O’Neill et al., 2011; Wang et al., 2011). As miRNA play important roles maintaining cellular function, dysregulation may predispose to disease (e.g. cancer and heart disease). However, the functional roles of miRNA in inflammatory and immune disorders are just beginning to be explored (Iborra et al., 2010; McCarthy, 2010; Oglesby et al., 2010; Kasinski and Slack, 2011; Pekow and Kwon, 2011; Foster et al., 2013; Plank et al., 2013). Already, a subset of miRNAs has been identified as potential therapeutic targets in asthma (Mattes et al., 2009;
MiR-126 and miR-21 have been implicated in mouse eosinophilic asthma models. Furthermore, we showed that targeted inhibition of miR-126 reduces clinical symptoms by inhibiting T helper type-2 inflammatory responses (Mattes et al., 2009). Other miRNAs (e.g., miR-18a, miR-128b and miR-221) have also been implicated in the regulation of glucocorticoid function, modulating glucocorticoid receptor (GR) expression (Uchida et al., 2008; Kotani et al., 2009). However, the role of miRNAs in the regulation of steroid-resistant asthma pathways remains unclear.

In the current study, we demonstrate that miR-9 expression is increased in pulmonary macrophages and lung samples in our IFN-γ/LPS-induced mouse model of steroid-resistant AHR and sputum samples from neutrophilic asthmatics. MiR-9 expression decreased protein phosphatase 2A (PP2A) activity and DEX-induced GR nuclear translocation, through targeting of protein phosphatase 2 regulatory subunit B (B56) δ isoform (PPP2R5D), a key regulatory subunit of PP2A. Specific inhibition of miR-9 enhanced PP2A activity and restored DEX-induced GR nuclear translocation. Furthermore, in vivo miR-9 inhibition, or PP2A activation (using 2-amino-4-(4-heptylophenol)-2-methylbutanol (AAL(S)), restored steroid efficacy, reducing AHR when administered with DEX. MiR-9 inhibition also restored steroid efficacy in mouse models of infection-induced AHR exacerbation, suggesting miR-9 as a novel target for treatment of steroid-resistant inflammation and AHR.
4.3 Methods

4.3.1 Mice

Wild type specific pathogen free BALB/c mice (6–8 wk) were obtained from the animal services unit of the University of Newcastle. All experiments were performed with approval from the animal ethics committee of the University of Newcastle (ethics approval number: A-2010-132).

4.3.2 Isolation and stimulation of pulmonary macrophages

Pulmonary macrophages were isolated from lungs as previously described (Loewen et al., 2005). Lung tissue was minced and cell suspensions prepared, separated by density gradient centrifugation (Histopaque-1083; Sigma-Aldrich) and plated at 6×10^6 cells/ml in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal calf serum (FCS). After 3 hr, 95% of adherent cells were macrophages, as confirmed by flow cytometry, which were cultured overnight, and stimulated as indicated.

4.3.3 Collection of induced sputum samples

Asthmatic patients (defined by clinical diagnosis with evidence of AHR to hypertonic saline and/or bronchodilator response) (Nair et al., 2009), were recruited and categorized by induced sputum inflammatory cell counts as eosinophilic (eosinophil count ≥2%, neutrophil <61%) or neutrophilic (neutrophil ≥61%, eosinophils<2%). Participants provided written informed consent, approved by the Firestone Institute for Respiratory Health and the McMaster University Research Ethics Committees.

Induced sputum samples were obtained using nebulized hypertonic (4.5%) saline
(Nair et al., 2009), treated with dithiothreitol and counts and viability (trypan blue exclusion) were determined by hemocytometer. Differential cell counts were performed on May-Grunwald-Giemsa-stained cytospin samples. Selected plugs were stored in Trizol (Life Technologies) for RNA analysis at -80°C.

4.3.4 Induction of IFN-γ/LPS-induced steroid-resistant AHR

Mice were anesthetized (100μl Alfaxan solution [1:4] in PBS i.v.) and intubated with a 22-gauge catheter (Yang et al., 2009). Optimized doses of murine IFN-γ (1.5μg/mouse; PeproTech), LPS (50ng/mouse; Sigma-Aldrich) or IFN-γ+LPS (γ/LPS; 1.5μg+50ng/mouse) in 50μl vehicle (saline) were instilled intratracheally (i.t.). After 12h (peak of steroid-resistant AHR) airway samples were collected. Where specified, mice were treated with DEX (1mg/kg, i.p., twice/week) and/or a non-phosphorylatable FTY720 analog, AAL(S) (10μg/mouse, i.n., daily).

4.3.5 Induction of allergic airway inflammation and persistent steroid-resistant airway hyperresponsiveness

Mice were sensitised with ovalbumin (OVA) (i.p., 50mg, fraction V, Sigma-Aldrich) and Alhydrogel (1mg, Reheis Inc.) in 200μL 0.9% sterile saline. Non-sensitised control mice were injected with Alhydrogel alone. On days 13-16 mice were aerosol-challenged with OVA (10mg/mL in 0.9% saline) for 20 min. Mice were also exposed to LPS (i.n., 50ng/mouse; Sigma-Aldrich (to mimic infection)) with or without DEX (i.p., 1mg/kg, Sigma-Aldrich). Antagomir-9 (ant-9, miR-9 sequence-specific antagonim) or scrambled antagonim control (scr) (50μg/mouse, Dharmacon) were administered (i.t.) 3 times as
indicated (see Fig. 7). On day 17 or 1 week after the last OVA challenge, AHR was assessed and inflammatory infiltrates quantified as previously described (Yang et al., 2009).

4.3.6 AHR measurement

Airway responsiveness was analysed using a FlexiVentsystem (Scireq) as previously described (Yang et al., 2009). Mice were anesthetized and ventilated by tracheal cannula, then sequentially challenged with increasing concentrations of β-methacholine in saline (Sigma-Aldrich). Airway reactivity was recorded and presented as percentage increase over baseline (saline).

4.3.7 MiRNA target prediction and luciferase reporter assay

MiR-9 mRNA targets were predicted using TargetScan(Lewis et al., 2005). Predicted targets were limited to genes with known involvement in glucocorticoid signalling pathways.

To assess miRNA/target interactions, the 3’-UTR of murine PPP2R5D and protein phosphatase 2 regulatory subunit B (B56) α isoform (PPP2R2A) were cloned from total mouse genomic DNA (Promega) into the PsiCheck2dual-luciferase vector (Promega) using the following primers: PPP2R5D forward (5’-3’,

GTTTAAACCCTTCCACAGGATTCCA) and reverse (5’-3’,

GCGGCCGCCCACCTTTATTCCAGTCTGATCATATG):PPP2R2A forward (5’-3’,

GTTTAAACGTGCTGCTGCTTATATCTG) and reverse (5’-3’,

GCGGCCGACAATAGGCAGTGGAATATATAAG). Resulting clones were
sequenced to verify proper sequence identity.

HEK293 cells (2×10^4 cells/well seeding in 96-well plate) were co-transfected with each dual-luciferase construct (200 ng/ml), with miR-9-mimic or control miR-mimic (Qiagen) using Fugene-HD transfection reagent (Promega) and lysed 48h later using Glo-Lysis buffer (Promega). Both firefly and renilla luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) and quantified on a SpectraMax M5 Multimode Microplate Readers (Molecular Devices). Firefly luciferase was normalised to renilla luciferase activity, presented as relative luciferase units.

4.3.8 Quantitative assessment of miRNA expression

Mouse RNA was isolated using the mirVana miRNA extraction kit (Ambion). Human sputum RNA was phenol-chloroform extracted and quantified on a Nanodrop spectrophotometer (Thermo-Scientific). MiRNA expression levels were measured by TaqMan MicroRNA Assay (Life Technologies) on an ABI Prism 7700 or an ABI Viia7 real-time PCR machine (Applied Biosystems). Mouse results were normalised to sno-202 and human results were normalised to RNU48.

4.3.9 Quantitative assessment of mRNA expression

Total RNA was isolated in TriReagent (Sigma-Aldrich) and reverse-transcribed using Superscript III (Invitrogen) (Bateman et al., 2008; Yang et al., 2009). Quantitative RT-PCR (q-PCR) was performed on an ABI Prism 7700 real-time PCR machine (Applied Biosystems) using SYBR green reagents and normalised to hypoxanthine-guanine phosphoribosyltransferase (HPRT). Primer sequences: PPP2R5D
forward: 5′CTTGCCAAAGGCACAGCCAA3′, reverse: 5′GTCGCTTGTTGGATGATGGT3′;
PPP2R2A-variant 1 forward: 5′TGTTCGTTGGAATGGGTCTG3′, reverse: 5′CCGTGCGAGGCTTTATTGTTC3′;
PPP2R2A-variant 2 forward: 5′ATTTCAGCATAGCGTGCA3′, reverse: 5′CTGTGCGAGGCTTTATTGTTC3′;
HPRT forward: 5′AGGCCAGACTTTGTTGGA TTTGAA3′, reverse: 5′CAACTTGCGCTCATCTTAGGCTTT3′.

4.3.10 Western blot

Pulmonary macrophages were harvested at the indicated time points, and 20 µg of cell lysate was separated on 12% SDS/PAGE gels and transferred to a nitrocellulose membrane. Levels of total JNK and phosphorylated JNK were determined with anti-mouse antibodies against JNK (D-2, Santa Cruz) and p-JNK (J1807, Santa Cruz) as previously reported (Rogatsky et al., 1998). Western blots were developed, using an ECL system (Amersham Biosciences).

4.3.11 Detection of GR localization by immunofluorescence

Immunofluorescence assays were performed as described previously (Goleva et al., 2002). Isolated lung macrophages were seeded on cover slips (6×10^6 cells/ml) in DMEM containing 20% FCS. Adherent cells were washed with cold PBS, fixed in 1% (w/v) paraformaldehyde in PBS for 10 min and blocked with 5% bovine serum albumin (BSA)/PBS for 1h. Cells were stained with a polyclonal anti-GR antibody (Santa Cruz Biotechnology) or isotype control (purified non-immune rabbit IgG, Santa Cruz
Biotechnology) in 1% BSA/PBS overnight at 4°C, washed with PBS and incubated with Cy3-conjugated goat anti-rabbit IgG (10mg/ml; GE Healthcare) in 1% BSA/PBS for 45 min, then washed with PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) for 10 min at RT. Slips were fixed on slides and visualised using a fluorescence microscope (BX51; Olympus) using a 100× objective lens and digital camera (DP70; Olympus).

4.3.12 PP2A activity assay

PP2A activity was assessed using the Active PP2A DuoSet IC Activity assay kit (R&D Systems), using manufacturer’s specifications.

4.3.13 Data analysis

Two-way ANOVA was used to identify differences in lung function, and Student t-tests were used for all other comparisons. Values are presented as mean ± SEM for 8 to 12 samples per group and differences were considered significant if p < 0.05.

4.4 Results

4.4.1 MiR-9 expression is increased under steroid-resistant AHR conditions

To explore roles for miRNA in steroid-resistant AHR, we performed microarrays on lung samples from γ/LPS-treated mice, and identified several miRNAs with altered expression (data not shown). Most notably, miR-9 levels were markedly increased following administration of γ/LPS, but not IFN-γ or LPS alone, as confirmed by q-PCR
Fig. 4.1A). This pattern linked miR-9 to steroid-resistant AHR, as the hyperreactivity was only induced by combined γ/LPS-treatment (14). Based on the critical role of macrophages in steroid-resistant AHR, we assessed miR-9 expression in pulmonary macrophages treated with LPS, IFN-γ or γ/LPS for 3h, 6h, 12h and 24h. Q-PCR demonstrated a modest increase in miR-9 expression following exposure to IFN-γ, but not LPS (Fig. 1B). Further, miR-9 expression was synergistically upregulated by γ/LPS treatment, peaking at 12h (Fig. 4.1B). Interestingly, miR-9 expression was also increased in sputum samples from neutrophilic asthmatics compared to eosinophilic patients (Fig. 4.1C). These findings demonstrate that miR-9 is induced in pulmonary macrophages and the lung under conditions that induce macrophage-dependent steroid-resistant AHR as well as in a subset of human asthmatic patients that are often resistant to steroid therapy.
Fig. 4.1. miR-9 expression is induced by γ/LPS stimulation. MiR-9 expression levels in A) lung samples from mice 12h after exposure to LPS, IFN-γ or γ/LPS, B) pulmonary macrophages stimulated with LPS, IFN-γ or γ/LPS for 3/6/12/24h and C) lung sputum.
samples from asthmatic patients (eosinophilic (EOS); neutrophilic (NEU)). Mouse samples normalised to sno-202, human samples normalised to RNU48. Values represented as mean ± SEM, *P<0.05 (vs. vehicle control).

4.4.2 Inhibition of miR-9 restores steroid-sensitivity reducing AHR induced by γ/LPS

To assess the functional role of miR-9 in steroid-resistant AHR, we inhibited miR-9 through i.t. administration of ant-9, prior to γ/LPS exposure. Ant-9 administration effectively blocked miR-9 induction in the lung (Fig. 4.2A), but alone had no effect on AHR induced by γ/LPS (Fig. 4.2B). As we have previously described, γ/LPS induces steroid-resistant AHR, which is not inhibited by DEX treatment (Fig. 4.2C) (Li et al., 2010). However, inhibition of miR-9 in conjunction with DEX attenuated AHR (Fig. 4.2C). While ant-9+DEX treatment inhibited AHR, ant-9 administration had no effect on inflammatory cell lung infiltration (Fig. 4.3). These findings indicate that ant-9 exposure alone has no effect on AHR despite reducing miR-9 levels; however ant-9 treatment restores steroid-sensitivity.
Fig.4.2 Inhibition of miR-9 restores DEX sensitivity attenuating AHR induction by γ/LPS. Mice administered ant-9 or scrambled antagonir (scr) and/or DEX 12h before γ/LPS. Lung function was assessed and lung tissues isolated 12h later. A) MiR-9 levels in lung normalised to sno-202, B) lung function measurements after exposure to γ/LPS and ant-9 or scr and C) mice pretreated with DEX and ant-9 or scr. Values represented as mean ± SEM, *P<0.05 (vs Veh), #P<0.05 (γ/LPS+DEX+scr vs γ/LPS+DEX+ant-9).
Fig. 4.3 Effect of ant-9 on airway cell infiltration following γ/LPS administration. Mice treated with ant-9 or scrambled control (scr) and/or DEX 12 h before γ/LPS. A) Total BALF cell numbers assessed using a hemocytometer and B) differential cell counting for neutrophils determined after May-Grunwald-Giemsa staining. Values represented as mean ± SEM, *P<0.05 (vs. vehicle control).
4.4.3 MiR-9 expression inhibits PP2A activity by targeting PPP2R5D

To clarify the mechanism underlying miR-9 inhibition of steroid-sensitivity, we performed *in silico* target prediction for molecules involved in GR signalling, which identified two PP2A regulatory subunits, PPP2R5D and PPP2R2A. Predicted miR-9 target sites are conserved within the 3’UTR of PPP2R5D and PPP2R2A, matching the miR-9 seed sequence at position 398-404 and position 1361-1367, respectively (Fig. 4.4A). To verify miR-9 targeting of these transcripts, we cloned the 3’UTR regions of each gene into dual-fluorescence luciferase reporter constructs. Co-transfection of luciferase constructs with miR-9 mimic resulted in a dose-dependent decrease in luciferase activity, compared to control mimic, demonstrating that miR-9 directly targets PPP2R5D and PPP2R2A transcripts (Fig. 4.4B).

To determine whether PPP2R5D or PPP2R2A are regulated by miR-9 in our model, we assessed mRNA expression in γ/LPS-stimulated macrophages with or without ant-9. Q-PCR confirmed efficient antagonir-mediated depletion of miR-9 (Fig. 4.5A). PPP2R5D expression was reduced following γ/LPS stimulation and restored by ant-9 treatment (Fig. 4.5B), indicating that PPP2R5D is downregulated by miR-9 in pulmonary macrophages. Interestingly, miR-9 inhibition had limited effects on PPP2R2A, with slight decreases in PPP2R2A-variant 1 expression following ant-9 treatment (Fig. 4.6).

As PPP2R5D levels affect PP2A catalytic activity (Ahn et al., 2007; Yu and Ahn, 2010; Ahn et al., 2011; Louis et al., 2011), we hypothesised that increased miR-9
expression may reduce overall PP2A catalytic activity. PP2A activity was reduced following γ/LPS treatment and ant-9 significantly recovered this activity, compared with scrambled control in macrophages (Fig.4.5C). Assessment of PP2A activity in γ/LPS-stimulated lung samples revealed similar findings, with increased miR-9 expression and reduced PP2A enzyme activity (Fig.4.5D). Western blots also demonstrated increased c-Jun N-terminal kinase (JNK) phosphorylation 12 hr after γ/LPS administration to pulmonary macrophages, which was inhibited by ant-9 pretreatment (Fig.4.5E/F). Taken together, these findings suggest that increased miR-9 expression reduced expression of its target, PPP2R5D, leading to reduced PP2A activity, increased JNK phosphorylation and thus suppression of the GR pathway and steroid efficacy.
Fig. 4.4 Targeting of PPP2R5D and PPP2R2A mRNA by miR-9.

A) Conserved miR-9-binding sites (highlighted) in the 3’UTR of PPP2R5D and PPP2R2A mRNAs. B) Luciferase activity in lysates of HEK293 cells transfected with constructs encoding the 3’UTR regions of PPP2R5D or PPP2R2A and miR-9 mimic or scrambled control at indicated concentrations. Values represented as mean ± SEM, *P<0.05, **P<0.005.
Fig. 4.5 Targeting miR-9 restores PPP2R5D expression and PP2A activity. Pulmonary macrophages pretreated with ant-9 or scrambled antagonir (scr) for 12h before γ/LPS. Samples were collected 12h later. A) MiR-9 and B) PPP2R5D transcript levels. PP2A catalytic activity normalised to untreated control, in C) pulmonary macrophages and D) lung. E) Western blot staining of pulmonary macrophages for phosphorylated (p-JNK) and total JNK and F) ratios of p-JNK/JNK, normalised to vehicle control. Values represented as mean ± SEM, *P<0.05.
Fig. 4.6 Effect of miR-9 on PPP2R2A expression. Pulmonary macrophages pretreated with ant-9 or scrambled antagonir control (scr) for 12h, then γ/LPS. 3h, 6h and 12h later RNA samples were collected. A) Expression levels of PPP2R2A variant-1 and B) variant-2. Values represented as mean ± SEM, *P<0.05. ns = not significant.
4.4.4 MiR-9 reduces GR nuclear translocation

As we demonstrated reduced PP2A activity concurrent with increased miR-9 expression, and PP2A activity has previously been implicated in regulating GR nuclear translocation (DeFranco et al., 1991; Kobayashi et al., 2011), we assessed the impact of ant-9 on GR localization. Previously we demonstrated (Li et al., 2010) that exposure of pulmonary macrophages to DEX alone results in nuclear GR translocation, which is blocked in the presence of γ/LPS (Fig. 4.7A/B). Pretreatment with ant-9 restored DEX-driven GR nuclear translocation (Fig. 4.7A/B). Collectively, our findings demonstrate that miR-9 blocks DEX-mediated GR nuclear translocation by modulating PP2A activity, promoting steroid-resistant AHR. Blocking miR-9 reverses this effect, restoring DEX-induced GR nuclear translocation.
Fig 4.7 Inhibition of miR-9 restores DEX-induced GR nuclear translocation. Pulmonary macrophages pretreated with ant-9 or scrambled control (scr) for 12h before γ/LPS for
11h, then DEX for 1h. GR cellular localization determined by immunofluorescence staining. B) Ratio of fluorescence intensity (nuclear/ cytoplasm). Values represented as mean ± SEM, *P<0.05. L=LPS, I=IFN-γ and D=DEX.

4.4.5 PP2A activity directly affects AHR

To directly determine the impact of altered PP2A activity on both steroid-resistant AHR and GR nuclear translocation, we administered AAL(s), a compound that upregulates PP2A activity. Intranasal AAL(s) administration significantly reduced airway resistance when administered with DEX (Fig.4.8A), comparable to the effect of anti-9. Further, AAL(s) administration to γ/LPS-stimulated pulmonary macrophages in vitro also restored GR nuclear translocation (Fig.4.8B/C). These findings demonstrate that increased PP2A activity is sufficient to restore DEX-induced nuclear GR translocation and render AHR sensitive to steroid.
Fig.4.8 Increasing PP2A activity restores steroid-sensitivity and GR nuclear translocation. Mice administered DEX and/or PP2A activator, AAL(s), following γLPS.
A) Lung function measured 12h later. B) Isolated pulmonary macrophages treated with DEX +/- AAL(s) and GR cellular localization determined by immunofluorescence staining and C) the ratio of fluorescence intensity (nuclear/cytoplasm). Values represented as mean ± SEM, *P<0.05 (vs. vehicle control). L=LPS, I=IFN-γ and D=DEX.

4.4.6 Ant-9 inhibits LPS-induced exacerbations of steroid-resistant AHR in allergen challenge models of asthma

Having identified a role for miR-9 in steroid sensitivity in our γ/LPS model, we extended our study to more complex models of steroid-resistant AHR. Mice were sensitised and challenged with OVA to induce asthmatic-like inflammation and exposed to LPS to mimic infection and activate macrophages. LPS was administered during OVA challenge (co-exposure model (allergen with infection)) (Fig. 4.9A) or after challenge (post-exposure model (infection after allergen exposure)) (Fig. 4.9C). In the LPS co-exposure model mice were pretreated with DEX in the presence or absence of ant-9 (Fig. 4.9A). Measurement of lung function (1 day after the last OVA exposure) identified a LPS-induced steroid-resistant exacerbation of AHR by comparison to the OVA/OVA group (Fig. 4.9B, left panel). Administration of ant-9+DEX significantly reduced AHR as compared to scr control (Fig. 4.9B, right panel). Similarly, post-exposure with LPS assessed 1 week after OVA (Fig. 4.9C) induced steroid-resistant AHR (Fig. 4.9 D, left panel). In this model, ant-9+DEX
completely reduced airway responsiveness to baseline levels (Fig. 4.9 D, right panel).

Collectively, these findings demonstrate that ant-9 is sufficient to restore steroid-sensitivity, allowing DEX to inhibit AHR in these models.

Fig. 7 Li JJ., et al
Fig.4.9 Inhibition of miR-9 restores steroid sensitivity, thus blocking the development of AHR in allergen-induced models of steroid-resistant AHR. Schematic representation of A) co-exposure and C) post-exposure models of LPS administration indicating timing of OVA sensitisation, challenges and DEX/ant-9 treatments. Lung function was assessed on B) day 17 post-sensitisation and D) day 24 post-sensitisation, respectively. Values represented as mean ± SEM, *P<0.05.

4.5 Discussion

MiRNAs are important modulators of gene expression and their expression levels are dramatically altered during immune responses (Yang et al., 2009; Li et al., 2010; Foster et al., 2013). However, the functional roles of miRNAs in the regulation of immune responses are only beginning to be identified. In the current study, we assessed miRNA expression within the lung in our model of steroid-resistant AHR. We observed a synergistic upregulation of miR-9 expression by IFN-γ and LPS in lung tissue and pulmonary macrophages, linking this miRNA to the mechanism of steroid resistance. Moreover, inhibition of miR-9 restored steroid sensitivity and inhibited AHR in multiple models of steroid-resistant AHR. Taken together, our findings provide support for a model where by activation of macrophages results in increased expression of miR-9, leading to decreased PPP2R5D expression and overall PP2A activity (Fig.4.10). Decreased PP2A activity results in increased JNK phosphorylation, increased GR phosphorylation and inhibited GR nuclear translocation. Blocking miR-9 with ant-9
reverses these effects, results in increased PPP2R5D expression and PP2A activity, decreased JNK phosphorylation and restoration of DEX-induced GR nuclear translocation (Fig. 4.7). Our findings establish a new link between miRNA and glucocorticoid sensitivity in macrophages, and highlight the potential of targeting miRNA pathways for the treatment of steroid-resistant disease.

MiR-9 was initially identified as a neuronal-specific miRNA and implicated in mammalian nerve development (Leucht et al., 2008; Shibata et al., 2008). Subsequently, miR-9 was identified in non-neural diseases, with a role regulating insulin cell secretory function (Plaisance et al., 2006). This miRNA has also been implicated in regulating cancer cell proliferation and metastasis, and has been associated with cardiotoxicity by regulating Platelet-derived Growth Factor Receptor β expression (Almeida et al., 2010; Wan et al., 2010; Zhang et al., 2011). Recently, miR-9 expression was shown to be induced by high dose (100ng/mL) LPS in primary human polymorphonuclear neutrophils and monocytes in a MyD88- and NF-κB-dependent manner (Bazzoni et al., 2009). Further, miR-9-dependent inhibition of NFκB1 transcription suggests that the rapid induction of miR-9 operates as a feedback control of NF-κB-dependent responses in these cells (Bazzoni et al., 2009). MiR-9 was also induced by LPS and Staphylococcus aureus enterotoxin B in bovine monocytes (Dilda et al., 2011). By contrast, in our study we demonstrate synergistic upregulation of miR-9 expression by γ/LPS in pulmonary macrophages. LPS stimulation alone (at 5ng/mL) was not sufficient to induce miR-9 expression, but enhanced the upregulation observed following
exposure to IFN-γ alone.

Glucocorticoids exert their anti-inflammatory effect through GR activation, directing downstream transcription and signal transduction. A number of possible mechanisms have been proposed to explain the development of steroid-resistance, including decreased histone deacetylase activity, overexpression of pro-inflammatory transcription factors and abnormal expression of GR (Adcock and Barnes, 2008; Barnes and Adcock, 2009). Post-transcriptional modifications of GR, such as phosphorylation, are critical for modulating GR function and may also underlie one mechanism of steroid resistance (Chen et al., 2008; Galliher-Beckley and Cidlowski, 2009). GR phosphorylation impairs nuclear translocation and modifies transcriptional activity. One phosphorylation site on the human GR (Ser 226) is mainly phosphorylated by JNK (Rogatsky et al., 1998). JNK is activated by inflammatory stimuli, including inflammatory cytokines and LPS, and its activation results in the phosphorylation of various transcription factors, promoting their transcriptional activity (Ip and Davis, 1998). In our observations, γ/LPS treatment results in increased JNK phosphorylation, which is reversed following ant-9 administration. This activation of JNK may in turn lead to the observed alteration in GR nuclear translocation, mediating steroid resistance in our model.

PP2A is ubiquitously expressed and functions as a serine/threonine phosphatase (Shanley et al., 2001). PP2A functions as a heterotrimeric enzyme composed of a catalytic C subunit, a structural subunit, and multiple regulatory B subunits, which regulate PP2A function by modulating substrate binding, enzyme activity, and
subcellular localization (Xu et al., 2006). Previous studies suggest PP2A may increase GR activity through dephosphorylation of JNK and also regulate GR translocation to the nucleus (DeFranco et al., 1991; Kobayashi et al., 2011). Further, in an animal model of viral-induced exacerbation of asthma, PP2A activity was significantly decreased, while forced PP2A activity resulted in reduced AHR (Collison et al., 2013).

PPP2R5D is a critical PP2A regulatory subunit and its expression and phosphorylation regulate the catalytic activity of PP2A (Ahn et al., 2007; Yu and Ahn, 2010; Ahn et al., 2011; Louis et al., 2011). To clarify the mechanism behind miR-9 function, we performed a dual luciferase reporter assay and demonstrated miR-9-mediated inhibition of PPP2R5D and PPP2R2A expression. Further investigations in pulmonary macrophages demonstrated that ant-9-mediated inhibition of miR-9 restored expression of PPP2R5D, but not PPP2R2A. APP2A activity assay confirmed that γ/LPS stimulation resulted in decreased catalytic PP2A activity, both in vitro and in vivo, while inhibition of miR-9 restored activity. These results suggested that miR-9 exerts its effect by regulating the activity of PP2A, through modulation of the regulatory PPP2R5D subunit.

PP2A activity is significantly reduced in multiple inflammatory diseases, and reactivation using various activators, such as AAL(s) or FTY-720, has been shown to abrogate the development of inflammation and AHR in mouse models of asthma (Sawicka et al., 2003; Idzko et al., 2006; Collison et al., 2013). Previous studies have also demonstrated a critical role for PP2A in the regulation of GR phosphorylation and
nuclear translocation (DeFranco et al., 1991; Kobayashi et al., 2011). In peripheral blood mononuclear cells from severe asthmatic patients, PP2A expression and activity were significantly reduced as compared to healthy volunteers, suggesting a potential mechanism for PP2A activity in regulating the development of steroid-resistance in these patients (Kobayashi et al., 2011). In our study, ant-9 treatment restored PP2A activity but also concomitantly reduced JNK phosphorylation, resulting in restored DEX-induced GR nuclear translocation.

Our findings also reveal that localized targeting of miR-9 within the lung, using ant-9, was sufficient to restore DEX sensitivity and reduce AHR in a number of models of steroid-resistant AHR. Interestingly, the observed reduction in AHR following ant-9 administration occurred only in the presence of DEX treatment, with no observed changes in immune cell infiltration to the lung. These results suggest that upregulation of miR-9 blocks the normal glucocorticoid-induced signalling pathway and targeting miR-9 could serve as a novel approach to restore glucocorticoid responsiveness, without altering the immune response.

In summary, our data suggests that increased miR-9 expression contributes to steroid-resistant AHR by decreasing PPP2R5D levels and subsequently PP2A activity. Blocking miR-9 function restores steroid sensitivity and may serve as a novel approach for the treatment of steroid-resistant AHR.
Fig. 4.10 Schematic representation of miR-9 effects on glucocorticoid function in pulmonary macrophage following LPS+IFNγ exposure. MiR-9 reduces PP2A activity by reducing PPP2R5D levels, thus down-regulating GR nuclear translocation and inhibiting glucocorticoid function. Ant-9 recovers PP2A activity, leading to improved glucocorticoid function. LPS = lipopolysaccharide, IFN-γ = interferon-γ, miR-9 = microRNA-9, JNK = c-Jun N-terminal kinase, PPP2R5D = protein phosphatase 2, regulatory subunit B δ, PP2A = protein phosphatase 2, GR = glucocorticoid receptor, ant-9 = antagonim-9, 🔺 = increased, 🔻 = phosphorylation, 🔻 = decreased, Red arrow=miR-9 effect, blue arrow = ant-9 effect.
Chapter 5.

Discussion
This thesis explores the innate immune factors involved in the pathogenesis of asthma and the development of steroid-resistance. It demonstrates the interaction between pathogen infection and innate immune cells as well as the molecular processes that occur as a result of these interactions in asthma development. This thesis attempts to delineate the underlying mechanisms of how the innate immune system may be activated by infection and how this process further contributes to the development of steroid-resistance.

The first publication within this thesis was based on our well-established murine model of LPS plus IFN-γ induced AHR. Here we demonstrate a potential role for pulmonary macrophages and key molecules in the development of steroid-resistant AHR. We found that LPS and IFN-γ synergistically induce IL-27 production in pulmonary macrophages, and IL-27 and IFN-γ uniquely co-operate to induce steroid-resistant AHR through a MyD88-dependent pathway. The implication of these observations is that a non-allergic host defense response in lung is important for the development of steroid-resistant AHR. This study also attempts to thoroughly explore the role of pulmonary macrophages, the most abundant innate immune cell in the airways, in the development of severe asthma. The role of macrophages as effector cells in severe asthma has only recently come of the attention of researchers in the field of airways disease (Moon et al., 2007; Bhavsar et al., 2008; Goleva et al., 2008; Yang et al., 2012). However the mechanisms of how they are activated, what phenotype they develop into, and how they are involved in the steroid-resistant asthma are not fully
understood. In this study, pulmonary macrophages are typically activated by two important innate immune factors involved in infection, LPS and IFN-γ, which have been widely linked to exacerbations of asthma as well as steroid-resistance.

LPS and IFN-γ co-stimulation resulted in the production of IL-27 in pulmonary macrophages. IL-27, a new member of the IL-12 family, has been shown to have a double sided effect in regulating the immune response (Villarino et al., 2005). The immunosuppressive effects of IL-27 depend on IL-2 suppression, inhibition of Th17 development, and induction of IL-10 production (Artis et al., 2004; Stumhofer et al., 2006; Stumhofer et al., 2007). On the other hand, IL-27 acts as an initiator of the immune response by driving the differentiation of Th1 cells. Mice deficient in the IL-27 receptor (WSX-1−/− mice) showed impaired IFN-γ production compared with wild-type mice. Accordingly, WSX-1−/− mice showed remarkable susceptibility to L. major, an intracellular pathogen, whose clearance mostly depends on a Th1 response (Yoshida et al., 2001). We revealed for the first time co-operative interaction between IL-27 and IFN-γ in the induction of steroid-resistant AHR, which occurs independently of neutrophils but is dependent on pulmonary macrophages. Further, this co-operative interaction modulates GR nuclear translocation. Further studies are required to determine the exact mechanisms as to how macrophages exert their function to regulate the induction of AHR, and how IL-27/IFN-γ regulates GR movement (Fig.5.1).

In the second publication, we reported for the first time the expression, regulation, and potential function of ORs on pulmonary macrophages. This study reveals that there
is significant expression of ORs on innate immune cells and suggests their very existence may contribute to the development of pulmonary diseases by regulating the activity of the cells they are expressed on, when exposed to LPS and IFN-\(\gamma\). These ectopically expressed chemosensors have only recently attracted the attention of researchers. Recent identification showed the expression of one member of chemosensor family, taste receptor, on immune cells has been linked to the pathogenesis of severe asthma (Orsmark-Pietras et al., 2013). With a similar mechanism of activation, the role of ORs in the immune response is only now just beginning to be revealed. Innate immune cell activation (via LPS and IFN-\(\gamma\) co-stimulation) resulted in increased OR expression on pulmonary macrophages. In addition, macrophage migration was significantly increased in response to stimulation by OR agonists, resulting in MCP-1 production, suggesting a new mechanism for innate immune cell activation in response to environmental stimuli. This investigation paves the way to explore the interaction between environmental exposure and the innate immune response and to further clarify the mechanisms underpinning the development of airway diseases such as asthma (Fig.5.2).

As ORs belong to the G-protein coupled receptor (GPCR) family, activation of the OR signalling pathway in macrophages may have crosstalk with other GPCR pathways (such as chemokine receptors, inflammatory lipid receptors, peptide hormone receptors) or non-GPCR pathways, (such as TLRs). Elucidating the signalling pathway used by ORs will provide important insight into macrophage biology and may lead to novel
approaches for targeting macrophage-mediated disease (Lattin et al., 2007).

In our final publication, we demonstrate the important role of miRNAs as a fine-tuner of the innate immune response and their contribution to the development of steroid-resistance. LPS and IFN-γ synergistically induced miR-9 upregulation in pulmonary macrophages, which targeted PPP2R5D and resulted in reduced PP2A activity. Interestingly, neutralization of miR-9 expression could recover PP2A activity and restore steroid sensitivity by restore GR nuclear translocation. Recent studies suggest that PP2A, which increases the fidelity of a signal transduction pathway by tightly regulating activation and deactivation processes, is reduced in response to pathogen or allergen exposure and its activity is related to AHR induction and steroid-resistance (DeFranco et al., 1991; Juntila et al., 2008; Budziszewska et al., 2010; Kobayashi et al., 2011; Collison et al., 2013). Our study establishes a new connection that regulates innate immune activation and steroid-sensitivity. Innate immune activation (by LPS and IFN-γ co-stimulation) triggered multiple signalling pathways, ultimately blocking GC function. In addition to the key molecule IL-27 that we identified in our first publication, miR-9 is also suggested to be an important regulatory factor in the genesis of steroid-resistance. The production of IL-27 and miR-9 is dependent on different down-stream signalling pathways. IL-27 production is MyD88-dependent, while miRNA-9 expression is both TLR-4 dependent and MyD88-independent (not shown in this publication), suggesting the complexity of the down-stream network arising from co-operative signalling between TLR4 and IFN-γR.
This study also demonstrated that production of IL-27 was partially regulated by miRNA-9 through manipulation of IL-27 transcriptional factor IRF-1 expression. The interaction between IL-27 and miRNA-9 in regulating GC activity needs to be further investigated. This study provides valuable insight into understanding the role of the innate immune response, in particular of pulmonary macrophages in the development of steroid-resistant asthma, and developing novel therapeutic approaches for this difficult to control disease.
**Fig 5.1** Proposed mechanism of steroid-resistance induced by LPS and IFN-γ. LPS activates MyD88-dependent and independent pathways, both of which can co-operate with IFN-γ-activated pathway and results in the synergistic production of IL-27 and miR-9.  
?? indicates an unknown aspect of the pathway.  
P indicates phosphorylation activation.  
X indicates pathway blockage.

**Fig 5.2** Proposed mechanism of interaction between OR pathway and IFN-γ/LPS pathway in inducing MCP-1 production.  
+ indicates enhansive effect to signalling activation.
Chapter 6.

Future directions
1. Further investigations into macrophage-induced steroid-resistant AHR

In this thesis, I have shown that pulmonary macrophages play a critical role in the induction of steroid-resistant AHR. It is still not known how changes to the phenotype or bio-activity of macrophages resulted in the altered airway hyper-responsiveness or how macrophages communicate with airway smooth muscle cells directly or indirectly to regulate their contraction and dilatation.

The interaction between macrophages and other pulmonary cells is also important for airway inflammation and disease development and these relationships need to be further investigated. Previous studies have shown the potential for IL-27 to amplify airway inflammation. In addition to the effects of IL-27 in regulating T cells differentiation, IL-27 and TNF-α synergistically induced CXCL-10 production in lung fibroblasts (Dong et al., 2013). Thus, IL-27 produced by pulmonary macrophages in response to LPS/IFN-γ may generate multiple regulatory effects as an intermediate factor. In future studies, it will be necessary to detect the expression of IL-27 in clinical samples from different subtypes of asthmatic patients, as well as to clarify the relationship between IL-27 expression levels and infection-induced exacerbations in asthmatic patients. Experiments will be conducted to investigate how infection-induced IL-27 from pulmonary macrophages affect the activation of other immune cells, such as T cells, and other lung structural cells, such as fibroblast and epithelial cells. The co-stimulatory molecules, cell phenotype changes and cytokine profiles will be investigated.
Elucidating these relationships will direct us to form a targeted strategy for asthma diagnosis and treatment.

2. Future investigations into the potential role of ectopically expressed ORs

This thesis reported the novel functional expression of ORs in pulmonary macrophages, which may trigger more studies into the diverse roles of ORs in immune responses and diseases development. To reveal the unknown regulatory role of ORs may also provide us theoretical background for identifying new clinical diagnostic markers and exploring new treatment strategies for asthma or other related diseases. Therefore, future studies will profile OR expression on other immune cells, regulation of their expression in response to immune system activation, and the effect of OR activation in regulating other immune cell activity. To reveal the role of ORs in asthma or other immune disease development will pave the way for exploring new treatment strategies with odorant products.

In this study, we also demonstrated that OR expression was regulated by innate immune activation. LPS and IFN-γ co-operatively activated a signalling pathway which contributed to synergistic production of various regulatory factors, such IL-27 and miR-9. The underlying mechanism of how LPS and IFN-γ produce this synergistic effect needs to be further investigated. Future studies may also include the detection of expression and activation levels of ORs in response to viral or bacterial infection in animal models.

3. Further investigation into miRNAs in regulating innate immune function and
identifying potential targets for asthma treatment

The fine-tuning of miRNA expression may exert extensive effects on innate immune responses and further contribute to disease development. In this thesis, it was shown that the aberrant expression of miR-9 in response to innate immune activation modulated the biological behavior of macrophages and may underpin the mechanism of steroid-resistance. MiR-9 regulated PP2A activity was dependent on the expression of PP2A regulatory subunit PPP2R5D instead of PPP2R2A, and further study is needed to fully reveal the mechanism. In this study, we confirmed previous report that GRs nuclear translocation is regulated by JNK phosphorylation, which is determined by PP2A activity, it is still not clear whether there are other underlying mechanisms regulating GRs nuclear translocation determined by PP2A activity. Thus, it is necessary to perform further study to fully establish the relationship between PP2A activity and GRs nuclear translocation. PP2A activity regulated by miR-9 plays various roles in inflammatory signalling pathways and disease development. Thus, to further examine the effect of this feedback loop within the innate immune system, future investigations into the expression and potential roles of miR-9 should include other infection-induced asthma models (i.e. viral- or bacterial-induced asthma), or in other pulmonary inflammation models (i.e. Chronic Obstructive Pulmonary Disease (COPD) or pulmonary fibrosis).

Due to the multiple targets of miRNAs, other miRNA-9 targeted genes may also be involved, such as NFKB1, which is suggested to be important in pro-inflammatory
signalling and cancer development. Thus to further reveal the role of this conserved small regulatory RNA, it is necessary to investigate its regulatory effects in a broad range of diseases.

Collectively, the work presented in this thesis may help to improve our understanding of the pathogenesis and development of asthma, the double-sided role of the innate immune system in maintaining homeostasis and disease exacerbation, and the gene-environment interaction important in disease development. Investigations into the key regulatory factors and underlying mechanisms will contribute to the design of more targeted molecular therapies aimed at modulating the innate immune response as a way to treat asthma patients.
REFERENCES:


airway CD4+ and CD8+ T cells in atopic asthma." Am J Respir Crit Care Med 171(3): 224-230.


