The role of microRNA in regulating antibacterial responses in innate immune cells

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Doctor of Philosophy (Immunology and Microbiology)

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Discipline of Immunology and Microbiology,
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Sep, 2013
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I hereby certify that the work embodied in this thesis has been done in collaboration with other researchers. I have included as part of the thesis a statement clearly outlining the extent of collaboration, with whom and under what auspices.

*Exposure of the mice to cigarette smoke was performed in collaboration with Professor Philip Hansbro.*
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHR</td>
<td>airway hyper-responsiveness</td>
</tr>
<tr>
<td>AM</td>
<td>alveolar macrophage</td>
</tr>
<tr>
<td>Ant</td>
<td>antagomir</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>AGO</td>
<td>argonaute protein</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinechinonic acid</td>
</tr>
<tr>
<td>BCG</td>
<td><em>Mycobacterium bovis</em> bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BCL</td>
<td>B-cell leukemia/lymphoma</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>carboxyfluorescein succinimidy ester</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestine alkaline phosphatase</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CXCL</td>
<td>chemokine ligand</td>
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<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-Phenyldlindole, dihydrochloride</td>
</tr>
<tr>
<td>DEX</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DGCR</td>
<td>DiGeorge syndrome chromosome region</td>
</tr>
<tr>
<td>DHE</td>
<td>dihydroethidium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double stranded ribonucleic acid</td>
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</table>
eIF  eukaryotic initiation factor
ELISA  enzyme-linked immunosorbent assay
ENaC  epithelial sodium channel
ERK  extracellular signal-related kinase
FCS  fetal calf serum
FEV  forced expiratory volume
FVC  forced vital capacity
GATA  GATA-binding protein
G-CSF  granulocyte colony-stimulating factor
GW182  glycine/tryptophan repeat protein, 182-KD
HAND  heart and neural crest derivatives
HBSS  hanks buffered saline solution
Hi  Haemophilus Influenzae
HIV  human immunodeficiency virus
HMW  high molecular weight proteins
HPRT  hypoxanthine phosphoribosyltransferase
ICAM  intercellular adhesion molecule
IFN  interferon
Ig  immunoglobulin
IL  interleukin
IN  intranasal
IT  intratracheal
IKK  inhibitory kappa B kinase
IRAK  interleukin receptor-associated kinase
I-κB  nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>JNK</td>
<td>C-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>LNA</td>
<td>locked nucleic acid</td>
</tr>
<tr>
<td>LOS</td>
<td>lipooligosaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>macrophage chemotactic protein</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>miRISC</td>
<td>miRNA-induced silencing complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases-12</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MRE</td>
<td>miRNA response element</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MTPN</td>
<td>myotrophin</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>NTHi</td>
<td>non-typeable <em>haemophilus influenzae</em></td>
</tr>
<tr>
<td>OMP</td>
<td>outer membrane protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
</tbody>
</table>
PCR  polymerase chain reaction
PMN  polymorphonuclear neutrophils
PRR  pattern-recognition receptor
PVDF polyvinylidene difluoride
PFV  primate foamy virus
RBC  red blood cell
RISC RNA-induced silencing complex
RNA  ribonucleic acid
ROS  reactive oxygen species
Scr  scrambled antagomir
SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM  standard errors of the means
siRNA small interfering RNA
Spn  *streptococcus pneumoniae*
ssRNA single stranded ribonucleic acid
TAK  transforming growth factor activating kinase
TIR  Toll/interleukin-1 receptor
TGF  transforming growth factor
Th   T-helper
TLR  Toll-like receptor
TNF  tumour necrosis factor
TRAF tumour necrosis factor receptor-associated factor
TRAM TRIF-related adaptor molecule
TRIF TIR-domain-containing adaptor-inducing interferon
UTR  untranslated region
<table>
<thead>
<tr>
<th>WHO</th>
<th>world health organization</th>
</tr>
</thead>
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<tr>
<td>α</td>
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</tr>
<tr>
<td>β</td>
<td>beta</td>
</tr>
<tr>
<td>γ</td>
<td>gamma</td>
</tr>
<tr>
<td>κ</td>
<td>kappa</td>
</tr>
</tbody>
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Abstracts

Respiratory infections are one of the major burdens to public health due to the ease of transmission and affect people of all ages. Respiratory infections are common and normal host defence mechanisms in the lung and are often adequate in clearing the invading pathogen. Infections, however, can be persistent or life threatening if patients have an underlying lung diseases such as chronic obstructive pulmonary disease (COPD) or if they are immunocompromised. Understanding the mechanisms and pathways responsible for regulating the host defence response to lung infections is central to developing new and novel therapeutic strategies. While the role of molecules such as pattern recognition receptors (PRRs) and the downstream signalling events they initiate to activate host defence has been extensively studied, the post-transcriptional regulation of molecules that are involved in immune regulation are just beginning to be explored. MicroRNAs are small non-coding RNA that bind to multiple target mRNA and regulate gene expression post-transcriptionally by repressing protein production. The role of miRNAs in initiating and controlling inflammation through cytokine production and toll-like receptor regulation in response to pathogens has received much attention. However, the role of specific miRNAs during bacterial lung infection, pathogen killing and phagocytosis remain unknown. Using a well-characterised model of respiratory infection with non-typeable *Haemophilus Influenzae* (NTHi), we show that miRNA-328 (miR-328) is downregulated in the lungs during NTHi infection. Further studies showed that pharmacological inhibition of miR-328 *in vitro* increased phagocytosis of bacteria by macrophages and neutrophils. Similarly, inhibiting miR-328 during infection of the lung improved bacterial clearance under normal conditions, and during heightened susceptibility to infection promoted by immune-suppression, and smoking-induced emphysema. Our studies uncover a novel role for
miRNA in the regulation of phagocytosis and indicate the potential for the inhibition of miR-328 as a therapy for antibiotic resistant bacterial infections in the lung.
Chapter 1: Introduction

1.1 Infectious diseases of the lung

Respiratory infections are a common and persistent health problem, having a major impact on both first and third world countries. Infection caused by respiratory pathogens results in a greater burden in all socio-economic populations compared to any other type of infectious disease (Mizgerd, 2006). Respiratory pathogens are normally spread through water droplets in the air, resulting in rapid transmission in densely populated areas. The severity of infection is dependent on the number of infectious particles introduced, the virulence factors expressed by the pathogen, as well as the host immune response. A low concentration of pathogens in the respiratory tract can usually be cleared by local innate host defences, such as airway surface fluids, antimicrobial proteins, alveolar macrophages, neutrophils and the mucociliary escalator (Mizgerd, 2008). However, infection by a highly virulent pathogen will stimulate an active inflammatory response, which is required for successful clearance of the invading pathogen. While the primary purpose of the inflammatory response is to clear pathogens from the lungs, it can also act as a “double-edged sword” if severe of persistent leading to abnormal pulmonary function and lung injury.

1.2 Bacterial infections of the lung

1.2.1 NTHi infection and disease prevalence

NTHi is a gram-negative bacteria commonly found in the upper respiratory tract of adults (Turk, 1984). Infection with NTHi results in the development of pneumonia, sinusitis, and chronic bronchitis (Moxon and Wilson, 1991, St Geme, 1997), and is the most common cause of otitis media in children (Murphy et al., 1987). Infection by NTHi also leads to
exacerbations of existing disease such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Groeneveld et al., 1990, Bilton et al., 1995).

1.2.2 NTHi and pathogenesis

NTHi infects cells lining the respiratory epithelium through bacterial-expressed cell surface structures, secreted proteins and lipooligosaccharides (LOSs), with preferential binding and infection of non-ciliated or structurally damaged epithelial cells. This preference for damaged cells may suggest why NTHi is commonly found in the lungs of COPD, cystic fibrosis, and to lesser extent asthma patients. The course of NTHi-induced disease is directed by bacterial-expressed antigens, factors that facilitate bacterial adherence to host tissue or aid in evasion of the host's defences.

LOSs are major surface antigens produced by NTHi, expressed in both secreted and cell-bound forms. Secretion of LOS results in increased host mucin production and lung damage, through activation of an inflammatory response (Zwahlen et al., 1983). Damage to the lungs and loss of cilia, caused by inflammation, in turn reduces the rate of bacterial clearance by epithelial cells and facilitates adherence of bacteria to the respiratory epithelium (Johnson and Inzana, 1986, Read et al., 1991).

After breaking down the first line of defence, the outer membrane protein (OMP) of NTHi, (P2 and P5), binds to mucin to initiate the infection process (Reddy et al., 1996). Other antigens involved in adhesion such as high-molecular-weight proteins (HMW)-1 and -2 bind to heparin sulphate on the epithelial cell surface, enabling colonisation of damaged lung tissue and subsequently the subepithelial tissue (Noel et al., 1994).
However, the presence and binding of NTHi to the host tissue alone is not sufficient for infection to occur, therefore the bacteria have also developed strategies necessary to evade the host’s immune defence mechanisms. One of these strategies is secretion of IgA1 proteases, which cleave the heavy chain of IgA antibodies, which causes the Fc portion to lose its function as an enhancer of bacterial phagocytosis by macrophages and polymorphonuclear cells (Kilian and Poulsen, 1992).

1.3 Innate immune responses and pulmonary host defence

1.3.1 Host defence systems of the lung

To investigate potential immunomodulatory therapies, it is critical to understand how key components of the immune system within the airways respond to challenge by respiratory pathogens. From birth the lungs are constantly exposed to stimuli such as airborne particles and invading microorganisms from the outside world during respiration. For this reason, complex defence mechanisms involving both innate (non-specific) and adaptive (specific) immune responses are essential to protect the host against these repeated exposures. The components of these immune responses are briefly summarized in (Table 1). During inhalation, particles greater than 5 µm are removed in the upper airway by first line defence, which includes mechanical, molecular, cellular processes (Zhang et al., 2000). The mechanistic events that are used to clear airborne particles include filtration by nasal hairs, coughing and mucociliary clearance (Taussig et al., 2008). Smaller particles such as bacteria can escape from these mechanical host defence mechanisms and enter the lower respiratory tract. Therefore, additional defence mechanisms are required to maintain a healthy lung environment. This is partly achieved by the secretion of antimicrobial peptides by epithelial cells and type II pneumocytes. Antimicrobial peptides such as defensins, lactoferrin, cathelicidin are directly bactericidal, while collectins and
complement proteins enhance phagocytosis and killing of bacteria by phagocytes (Schnapp and Harris, 1998, Caccavo et al., 2002, Zanetti et al., 1995, Crouch, 1998, Frank and Fries, 1991). During an acute infection, phagocytic defences by alveolar macrophages and neutrophils are required for effective clearance of pathogen. If innate immunity fails to overcome the infection, a greater role of adaptive immunity is required.

For the purpose of this thesis, we will be focusing on the innate arms of the immune system in particular on the role of macrophages and neutrophils in bacterial clearance. These will be discussed in more detail in the following section.

Table 1. Host defence systems of the lung (Taussig et al., 2008)

<table>
<thead>
<tr>
<th></th>
<th>Innate immunity</th>
<th>Adaptive immunity</th>
</tr>
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<tbody>
<tr>
<td><strong>Pathogen</strong></td>
<td>Extracellular bacteria</td>
<td>Virus, intracellular bacteria</td>
</tr>
<tr>
<td><strong>Response time</strong></td>
<td>Immediate</td>
<td>Lag time</td>
</tr>
<tr>
<td><strong>Immunologic component</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mechanical</strong></td>
<td>Filtration by nasal hair, coughing, mucociliary clearance</td>
<td></td>
</tr>
<tr>
<td><strong>Molecular</strong></td>
<td>Antimicrobial peptides: Complement, Defensin, Cathelicidin, Complement, Lactoferrin, Collectins</td>
<td>Antibody : Immunoglobulin (A and G), Granzymes, Perforin</td>
</tr>
<tr>
<td><strong>Cellular</strong></td>
<td>Macrophages, neutrophils</td>
<td>B-lymphocytes, T-lymphocytes</td>
</tr>
<tr>
<td><strong>Protection against re-exposure</strong></td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><strong>Target specificity</strong></td>
<td>Target both Infected and non-infected cells</td>
<td>Target infected cells only</td>
</tr>
</tbody>
</table>

1.3.2 Innate immunity and pulmonary host defence

During respiratory infection, one of the most important mechanisms in innate host defence is phagocytosis. Phases of phagocytosis are illustrated in Figure. 1. Initiation of
phagocytosis requires contact or binding of the pathogen to phagocytic receptors. Receptors that are known to be involved in phagocytosis are listed in Table 2.

Figure 1. Phases of phagocytosis. Attachment of bacteria to a phagocyte results in bacterial uptake into a membrane vesicle named phagosomes. Bacterial killing or digestion takes place when lysosomes fuse with phagosomes to form phagolysosomes.

Receptors such as the Fc receptor and complement receptor require antigens to be opsonized with antibody or complement before they are bound and engulfed by the phagocytes. Other receptors (e.g. MARCO, Scavenger receptor A, Dectin 1, and CD44) can also bind directly to the surface or a component of the pathogen for phagocytosis. Following phagocytosis, pathogens are contained in phagosomes. To kill or digest the
engulfed bacteria, phagosomes will fuse with lysosomes, forming phagolysosomes, leading to the release of high concentration of bactericidal compounds from lysosomes into the phagolysosome. This entire killing process occurs only in phagolysosomes, thus protecting the cytoplasm of the phagocyte and surrounding tissues from this toxic activity. The killing mechanism used by phagocytes can be organized into two processes: oxygen-dependent and oxygen-independent mechanisms. Oxygen-dependent mechanisms involve the generation of high levels of reactive oxygen species (ROS) that damage a wide variety of biomolecules present in the pathogen leading to their death (Slauch, 2011). Oxygen-independent killing can be achieved by a low pH environment (pH 4.0) in the phagolysosomes, which inhibits growth of many microorganisms. In addition to this, low pH increases the activity of many degradative enzymes including phospholipases, lysozyme, proteases, glycosylases, and nucleases, which can damage bacterial cell membranes or induce bacterial cell death (Blander and Medzhitov, 2006).

*Neutrophils and macrophages are the key phagocytes involved in pathogen clearance from the airways, which will be the primary focus of our studies.*
### Table 2. Receptors used for phagocytosis

<table>
<thead>
<tr>
<th>Types of phagocytosis</th>
<th>Receptors</th>
<th>Ligands</th>
<th>Reference</th>
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</thead>
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<tr>
<td>Opsonic phagocytosis</td>
<td>Fc receptor</td>
<td>Antibody-opsonized targets</td>
<td>(Ravetch and Bolland, 2001)</td>
</tr>
<tr>
<td></td>
<td>Complement receptors</td>
<td>Complement-opsonized targets</td>
<td>(van Lookeren Campagne et al., 2007)</td>
</tr>
<tr>
<td>Non-opsonic phagocytosis</td>
<td>MARCO</td>
<td>Surface proteins from bacteria</td>
<td>(Pluddemann et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Scavenger receptor A</td>
<td>LPS, LTA, CpG DNA</td>
<td>(Bowdish and Gordon, 2009)</td>
</tr>
<tr>
<td></td>
<td>Dectin 1</td>
<td>β-glucan</td>
<td>(Herre et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>TLR</td>
<td>Various TLR ligands</td>
<td>(West et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>CD44</td>
<td>Hyaluronan</td>
<td>(Vachon et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>αVβ5 integrin</td>
<td>Apoptotic cells</td>
<td>(Dupuy and Caron, 2008)</td>
</tr>
</tbody>
</table>

### 1.3.2.1 Macrophages

Alveolar macrophages (AMs) are resident within the lung and are the first phagocytes to encounter invading bacteria, once the microbes have successfully bypassed the host’s mechanical defences and entered the lower respiratory tract (Sibille and Reynolds, 1990). AMs have important microbicidal, phagocytic, and secretory functions to directly target bacteria, as well as playing a major role in amplifying anti-bacterial immune responses through secretion of chemokines and pro-inflammatory cytokines (Nelson and Summer, 1998). Upon recognition of bacteria, macrophages recruit neutrophils into the airways through secretion of neutrophil chemoattractants (e.g. macrophage inflammatory protein (MIP)-2, interleukin (IL)-8 and leukotriene B4) (Reynolds, 1983). AMs also secrete immune modulatory cytokines including IL-12, IL-10, interferon (IFN)-γ, granulocyte colony-stimulating factor (G-CSF), and tumour necrosis factor (TNF)-α (Zhang et al., 2000). TNF-α production by AMs is crucial in the recruitment of neutrophils into the lung and
neutralisation studies have shown that this process is ablated in the absence of TNF-\(\alpha\) (Lukacs et al., 1995). While the production of TNF-\(\alpha\) does not directly affect neutrophil chemotaxis, it can induce the production of neutrophil chemoattractants (such as IL-8) by AMs and airway epithelial cells (Kunkel et al., 1991). The pro-inflammatory cytokine IL-12 is also important in pulmonary defence as it enhances cell-mediated immunity against viruses, fungi, mycobacteria, parasites, and bacteria (Hogan et al., 1998, Qureshi et al., 1999, Wakeham et al., 1998). Neutralising IL-12 results in reduced TNF-\(\alpha\) production and an increase in bacterial growth within the lungs (Brieland et al., 1998). AMs also produce IL-10 when challenged with both gram-positive (van der Poll et al., 1996) and gram negative bacteria (Greenberger et al., 1995). IL-10 down-regulates pro-inflammatory cytokine secretion and suppresses neutrophil function, as a mechanism to dampen prolonged inflammation and limit host tissue damage during infection. IL-10 suppresses neutrophil function by reducing neutrophil superoxide production, phagocytosis, and CD11b expression (CD11b acts as a phagocytic receptor) (Laichalk et al., 1996). AM-produced G-CSF stimulates the maturation and proliferation of myeloid progenitor cells into neutrophils (Trneny and Klener, 1992). Contrary to the role of IL-10, G-CSF can enhance the microbicidal function of neutrophils by increasing chemotaxis, phagocytosis, adhesion molecule expression, and oxygen metabolism (Roilides et al., 1991, Sullivan et al., 1993).

### 1.3.2.2 Neutrophils

Neutrophils are one of the major cell types involved in innate immunity against bacterial infection. Neutrophils circulate in the blood and are recruited to the airways in response to chemokines such as MIP-2, IL-8, and leukotriene B4 (Zhang et al., 2000). In a normal healthy lung, few neutrophils are present, however in response to bacterial challenge
neutrophils are rapidly recruited within 3 hours, and total neutrophil numbers can make up to 80% of the total cells present in the airways at the peak of infiltration (Sibille and Marchandise, 1993). Following recruitment to the lung, neutrophils are activated by pro-inflammatory cytokines, including TNF-α, G-CSF, MIP-2, and IL-8. Activation by pro-inflammatory cytokines enhances the functional activities of neutrophils by increasing chemotaxis, adhesion molecule expression and adhesion, phagocytosis of bacteria, oxygen metabolism and killing of intracellular bacteria (Roiilides et al., 1991, Zhang et al., 1998, Zhang et al., 1997, Yong and Linch, 1992). Neutrophils kill extracellular pathogens through phagocytosis, by engulfing pathogens, followed by fusion of the resultant phagosomes with azurophil granules. Azurophil granules contain bactericidal enzymes including bacterial permeability protein, defensins, and lysozyme (Borregaard and Cowland, 1997). Following bacterial clearance, tissue neutrophils undergo programmed cell death, and are subsequently phagocytosed by resident macrophages to clear the cellular debris within the lung and prevent chronic inflammation (Dale et al., 2008).

1.3.3 Pattern recognition receptors

The activation of innate immune cells is dependent on the detection of pathogen-associated molecular patterns (PAMPs) by the host PRRs. One major family of PRRs is the Toll-like receptors (TLRs).

1.3.3.1 Toll-like receptors (TLRs)

A number of different cell types within the airways express TLRs, including epithelial (Adamo et al., 2004, Armstrong et al., 2004, Greene et al., 2005, Guillot et al., 2005, Guillot et al., 2004, Hertz et al., 2003) and endothelial cells (Zhang et al., 1999, Andonegui et al., 2003), fibroblasts (Rudd et al., 2005) and airway smooth muscle cells (Morris et al.,
2005, Sukkar et al., 2006). TLRs that are predominantly expressed on the cell surface (TLR-1, -2, -4, -5, and -6) play a role in recognising a range of bacterial components. For example, lipoteichoic acid (LTA) and lipoproteins present in the cell wall of gram-positive bacteria e.g. Streptococcus pneumonia (Spn) can activate TLR-2 signalling either alone or in conjunction with TLR-1 and TLR-6 (Knapp et al., 2004). TLR-4 is important for recognition of lipopolysaccharides (LPS) that are present on gram-negative bacteria (e.g. NTHi) and TLR-5 detects flagellin, which is expressed by motile bacteria (Hayashi et al., 2001, Smith et al., 2003). Intracellular TLRs such as TLR-3, -7, -8, and -9 generally recognise double-stranded RNA (Alexopoulou et al., 2001), single-stranded RNA (Diebold et al., 2004) and DNA that are found in bacteria and viruses (Krug et al., 2004).

Sensing of microbial PAMPs by TLRs leads to the initiation of inflammation (Figure 2). Binding of ligands to the receptors can induce either myeloid differentiation factor 88 (MyD88)-dependent or MyD88-independent signalling pathways, or both in the case of TLR-4 signalling (Figure 2). Apart from TLR-3, which signals through a TRIF dependent pathway only, all other TLRs utilize the MyD88 pathway for the activation of the innate immune response.

Following PAMP-recognition by TLRs, MyD88 is recruited and interacts with Toll / interleukin-1 receptor (TIR) domains on the TLR. This recruitment initiates a signalling cascade which involves activation of IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1, TNF receptor-associated factor (TRAF)-6, transforming growth factor (TGF)-β-activating kinase (TAK)-1, inhibitory kappa B kinase (IKK)-β, and IKK-α (Akira, 2006), leading to the degradation of inhibitor of I-κB. Degradation of I-κB allows the nuclear translocation of transcription factor nuclear factor-kappa B (NF-κB), which initiates the expression of pro-inflammatory cytokines (such as IL-1β, IL-8, IL-6, TNF-α and intercellular adhesion
molecule (ICAM)-1 (Baeuerle and Henkel, 1994). The increase in ICAM-1, IL-8, and TNF-α in turn recruits neutrophils to the site of infection (Folkesson et al., 1995, Windsor et al., 1993). The production of pro-inflammatory cytokines can also occur through the transcription factor, activator protein (AP)-1, which requires the activation of mitogen-activated protein kinases (MAPKs) and C-Jun N-terminal kinases (JNK) (Akira, 2006).

The sensing of PAMPs by TLR-3 and -4 can induce a type I IFN response through an MyD88-independent pathway (Figure 2). This pathway requires interaction of TIR-domain-containing adapter-inducing interferon-β (TRIF) with the TIR domain. During TLR-4 signalling, the recruitment of TRIF-related adaptor molecule (TRAM) is also required to activate the TRIF-dependent signalling pathway to induce type I IFN production (Akira and Takeda, 2004). It was recently shown that type I IFN signalling can enhance the macrophage response to Spn, indicating that TLR-4 signalling is important for resistance against Spn infection (Mancuso et al., 2007).

Tight regulation of these signalling pathways is important in maintaining a balance between efficient pathogen clearances and minimising damage to the host tissue resulting from inflammation. Further to this, activation of the TLR signalling pathway is also known to be important in linking the innate and adaptive arms of the immune system.
Figure 2. Toll like receptor (TLR) signalling pathways. Binding of respective ligands to TLRs initiates signalling pathways and activates transcription in the nucleus leading to activation of immune system and production of chemokines, pro-inflammatory cytokines, and antimicrobial mediators.

1.4 Bacterial infections and exacerbation in lung diseases

In many cases, severe respiratory infection may reflect deficiencies in host defence mechanisms (Reynolds, 1985).
1.4.1 Chronic obstructive pulmonary disease (COPD)

COPD is a heterogeneous disease that includes emphysema and chronic bronchitis (Barnes et al., 2008). Patients that are diagnosed with COPD have lung function levels of FEV1/FVC<70% with airway obstruction that is not fully reversible (Mannino and Buist, 2007, Lopez and Murray, 1998). COPD is the third leading cause of death in the United States of America (Minino et al., 2010) and the WHO has estimated that the burden of disease globally will increase from 12th in year 1990 to 5th by year 2020 (Pauwels et al., 2001).

Some of the risk factors that are associated with development of COPD include exposure to cigarette smoke, air pollutants, occupational exposure to dusts and gases, and repeated lower respiratory-tract infections (Tuder and Petrache, 2012, Salvi and Barnes, 2009). Although cigarette smoking is one of the major risk factors for COPD, it is estimated that only 20-25% of smokers will develop emphysema. Studies show that other factors, such as genetic susceptibility could increase the risk or progression of COPD. For example, α1-antitrypsin, a proteinase inhibitor, can provide protection against neutrophil elastase, which is central to alveolar injury and development of emphysema (Tuder and Petrache, 2012). Clinically, severe α1-antitrypsin-deficient patients often exhibit a poorer health status compared to COPD patients without the deficiency (Needham and Stockley, 2005, Travis and Salvesen, 1983, DeMeo and Silverman, 2004). Another proteolytic enzyme, matrix metalloproteinases-12 (MMP12), has also been linked to disease severity in emphysema patients (Mukhopadhyay et al., 2010). Interestingly, studies using MMP12-deficient mice in a mouse model of smoke-induced COPD demonstrate reduced macrophage accumulation in the lungs, as well as protection from the development of emphysema, after 6 months of smoke exposure (Hautamaki et al., 1997).
In addition to differences in genetic susceptibility, bacterial and viral infections can also lead to exacerbation and worsening of COPD disease (Monso et al., 1995). In healthy individuals, the lower respiratory tract is normally pathogen-free, however in patients with COPD the lower respiratory tract is often colonised with bacteria. The most common bacteria isolated in the lower airways of COPD patients include *Haemophilus influenzae*, *Streptococcus pneumonia*, and *Moraxella catarrhalis*. Further, bacterial loads are higher in patients with exacerbated COPD compared to stable COPD (Monso et al., 1995). The association between bacterial infections and COPD exacerbation suggests that bacteria could be responsible, and that bacterial clearance may be impaired during these events. This may further increase the risk of bacterial infection forming a vicious cycle and exacerbate disease progression. Notably, patients treated prophylactically with the antibiotics amoxicillin and clavulanate showed significant disease improvement with a longer times between exacerbations, compared with placebo-treated patients (Llor et al., 2012). This suggests that the presence of bacteria contributes to the exacerbation of disease.

Studies suggest that patients with COPD are at an increased risk of bacterial infection due to a weakened innate immune system (Schleimer, 2005). Human alveolar macrophagess isolated from COPD patients show an impaired ability to phagocytose NTHi and Spn (Berenson et al., 2006, Taylor et al., 2010, Donnelly and Barnes, 2012). Further, mucous obstruction in the airways can increase the likelihood of bacterial colonisation (Fahy and Dickey, 2010). Excess mucous production occurs in both early and advanced cases of chronic bronchitis and is associated with disease severity (Reid, 1954, Aikawa et al., 1989, Rogers, 2001). Studies also indicate that mucociliary clearance may be impaired in COPD patients. One of the mechanisms is through inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) function leading to dehydration and decreased mucous
transport (Clunes et al., 2012, Randell et al., 2006). Studies using beta-epithelial sodium channel (βENaC) mice (transgenic mice with increased airway Na⁺ absorption and decreased mucus clearance) suggest that depletion of airway surface liquid leads to increased mucous concentration, delayed mucous transport and mucous obstruction. In these mice, reduced mucous transport in the airway contributes to reduced bacterial clearance following infection by *H. influenzae* and *Pseudomonas aeruginosa* (Mall et al., 2004). In turn, failure to clear bacteria by the innate immune system may lead to bacterial colonisation of the lower airways, which results in increased frequencies of disease exacerbation.

### 1.4.2 Immunosuppression and pneumonia

The long-term use of steroids as a mainstay therapy for asthma and COPD can result in immunosuppression and an increased risk of hospitalisation (Singh and Loke, 2010). In diabetes mellitus, the use of drugs such as thiazolidinedione can increase the risk of pneumonia and lower respiratory tract infections (Singh et al., 2011), possibly due to an impaired innate immune response as a result of steroid treatment. Murine studies have shown that administration of the corticosteroid dexamethasone can suppress phagocytosis by macrophages. Similarly, human studies have found the use of glucocorticoid steroids (e.g. Budesonide) inhibits phagocytosis of *Escherichia coli* (*E. coli*) by alveolar macrophages (Becker and Grasso, 1985, Zetterlund et al., 1998). In addition, dexamethasone suppresses ROS generation from mononuclear cells and polymorphonuclear leukocytes (Dandona et al., 1999). Oxidative burst is one of the major mechanisms used in bacterial killing following phagocytosis. O₂ and H₂O₂ generated during oxidative burst are cytotoxic to bacteria as they promote DNA damage (Slauch, 2011, Imlay, 2008, Fang, 2011).
These studies indicate that usage of steroid could impair innate immunity by decreasing both phagocytosis of pathogen and microbicidal activity by phagocytes. Therefore, developing mechanism to boost innate immunity in setting of these diseases is urgently required.

1.5 MicroRNAs (miRNAs) as novel regulators of the immune response

1.5.1 Biogenesis of miRNAs

miRNAs are single-stranded non-coding RNAs that regulate gene expression at the post-transcriptional level. They are generally phylogenetically conserved and form imperfect base pairing with target sites in the 3’-untranslated region (UTRs) of targeted messenger RNAs (mRNAs) (Shivdasani, 2006). miRNAs are first transcribed as primary miRNA (pri-miRNA) before being processed into pre-miRNA (70-nucleotide stem-loop structures). This action is performed by the microprocessor complex consisting of the nuclease Drosha and the dsRNA binding protein Pasha (DGCR8) (Figure 3). Pre-miRNAs are then transported into the cytoplasm by exportin-5, where they are trimmed by the cytoplasmic RNaseIII endonuclease, Dicer, into duplex RNAs. The functional strand (mature miRNA) of each RNA duplex will then be incorporated into the miRNA-induced silencing complex (miRISC). The miRISC consists of a combination of glycine/tryptophan repeat protein, 182-KD (GW182), the argonaute (AGO) RNA binding protein and the functional miRNA strand (Jakymiw et al., 2005).
For copyright reason, this image was removed.

Image was sourced from:

**Micromanagement of the immune system by microRNAs.**

Lodish et al., 2008. Nat Rev Immunol, 8, 120-30. Figure 2

**Figure 3. Biogenesis of miRNA (Lodish et al., 2008).** MiRNAs are transcribed to primary miRNAs in the cell nucleus, further processed to pre-miRNAs, and then exported into the cytoplasm by Exportin-5, where they are processed into mature miRNAs. Mature miRNAs are then integrated into the miRISC and exert their repressive function on target mRNA transcripts.
1.5.2 Mechanism of action of miRNA

MiRNAs are known to play a major role in regulating gene expression in many biological processes. It has been shown that plant miRNAs bind perfectly and induce cleavage and degradation of the target mRNA through RNA interference machinery. However, mammalian miRNAs bind imperfectly to their target mRNA, and generally do not cause degradation of their target mRNA. The exact mechanisms by which miRNAs downregulate target gene expression remains unclear, however several possibilities have been proposed including: inhibition of the formation of initiation complexes, deadenylation of mRNA, direct ribosome drop-off (inhibiting protein translation), or protein degradation. Studies have shown that AGO-2 and eukaryotic initiation factor (eIF) -4E have the same structural features, thus binding of miRISC to mRNA can directly block the translation process (Kiriakidou et al., 2007), while studies using Caenorhabditis elegans showed that lin-4 miRNA actually suppressed lin-14 and lin-28 mRNA production after protein translation had occurred (Olsen and Ambros, 1999). A study by Nottrott et al.2006 using human cells, showed that miRNAs decrease protein production by either increasing ribosomal drop-off or inhibiting ribosome movement along their target mRNAs (Nottrott et al., 2006). Contrary to these observations, later studies (in vivo and in vitro) have shown that target mRNA concentration can be decreased by the action of miRNAs (Lim et al., 2005, Jing et al., 2005, Bagga et al., 2005, Wu and Belasco, 2005). Reduction in target mRNA expression in mammalian cells appears to be through a different mechanism from plants (where degradation is mediated by endonucleolytic cleavage), with miRNA-mediated decreases in transcripts resulting from deadenylation of target mRNAs (Behm-Ansmant et al., 2006). A better understanding on how miRNAs function to inhibit target gene expression is essential to the development of strategies aimed at modulating miRNA function.
1.5.3 The role of miRNAs in human diseases

MiRNAs are dysregulated in a number of human diseases such as heart, neural, metabolic, cancer, and viral diseases (Table 3). In heart disease, miR-1 and miR-133 are found to target heart and neural crest derivatives-expressed (HAND)-2 transcription factors and GATA-4 binding proteins, which are important in cardiomyogenesis (Zhao et al., 2005). In neural diseases, it was shown that 15 out of 16 miRNAs are up-regulated in the prefrontal cortex of schizophrenic patients when compared to healthy controls and this was not due to treatment with anti-psychotic drugs. The importance of miRNAs in neural disease is further demonstrated in Alzheimer’s patients as the expression of miR-9, -125b, and -128 are increased in the hippocampus (Lukiw, 2007). In addition, miR-375 expression was found to be up regulated in diabetes mellitus patients. One of the validated targets for miR-375 is myotrophin (MTPN), an important regulator of insulin secretion; thus by reducing miR-375 levels, MTPN expression could be restored making it a possible target for treating diabetes (Poy et al., 2004). In obesity, miR-143 is up-regulated in differentiating adipocytes. By reducing miR-143 levels, production of extracellular signal-related kinase (ERK)-5 proteins can be restored thereby preventing adipocyte differentiation (Esau et al., 2004). In some cancers, miRNAs can act as oncogenes or tumour suppressors. For example, CD5+ B-cells from patients with chronic lymphocytic leukaemia (CLL) expressed high levels of B-cell leukaemia/lymphoma-2 (BCL-2). BCL-2 is an anti-apoptotic protein and is known to be a target for miR-15a and miR-16, both of which are absent in CLL patients (Cimmino et al., 2005). In a mouse B-cell lymphoma model, up-regulation of miR-17 and miR-92 leads to a reduction in the rate of apoptosis, hence increasing tumour development (Ozcan, 2009). Interestingly, in viral disease it was found that miR-32 can interrupt the replication of retrovirus primate foamy virus type 1 (PFV-1), as the expression of miR-32 leads to down regulation of viral protein production.
(Lecellier et al., 2005). Together these observations suggest the potential of miRNAs as important regulators in the pathogenesis of several human diseases.
Table 3. MiRNAs in human diseases.

<table>
<thead>
<tr>
<th>Disease</th>
<th>miRNA</th>
<th>Function</th>
<th>Validated immune targets</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cancer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic Lymphocytic Leukemia</td>
<td>miR-15a and miR-16</td>
<td>Act as tumour suppressors as they suppress anti-apoptotic genes.</td>
<td>BCL-2</td>
</tr>
<tr>
<td></td>
<td>miR-17-miR92</td>
<td>Act as oncogenes by reducing the rate of apoptosis.</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Metabolic Disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Obesity</td>
<td>miR-143</td>
<td>Play a role in adipocyte differentiation as increased levels of miR-143 increases adipocyte differentiation.</td>
<td>ERK-5</td>
</tr>
<tr>
<td><strong>Diabetes Mellitus</strong></td>
<td>miR-375</td>
<td>Repress insulin secretion.</td>
<td>MTPN</td>
</tr>
<tr>
<td><strong>Heart Disease</strong></td>
<td>miR-1</td>
<td>Important for cardiac cell differentiation</td>
<td>GATA-4, HAND-2</td>
</tr>
<tr>
<td><strong>Neural Disease</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>miR-26b, miR-30b, miR-29b, miR-195, miR-92, miR-30a-5p, miR-30d, miR-20b, miR-29c, miR-29a, miR-212, miR-106b, miR-7, miR-24, miR-30e, miR-9-3p</td>
<td>Not determined. Expressed in schizophrenic patients while showing low levels of expression in healthy controls.</td>
<td>Not determined</td>
</tr>
<tr>
<td><strong>Alzheimer Disease</strong></td>
<td>miR-9, miR-128, miR125b</td>
<td>Not determined. Show elevated expression in the brain of Alzheimer’s sufferers.</td>
<td>Not determined</td>
</tr>
</tbody>
</table>
1.5.4 miRNAs and their role in immune function and inflammation

While investigations into the potential role of miRNAs in health and disease are still in their infancy, an understanding of how miRNAs interact with the immune system is essential for the development of novel therapeutic strategies to combat infectious diseases and inflammatory disorders. A recent study has shown that miR-155 plays an important role in normal immune function. Mice that are deficient in miR-155/bic are immunocompromised and develop features of airway remodelling (Rodriguez et al., 2007). Furthermore, mice deficient in miR-155 did not develop immunity against *Salmonella typhimurium* (*S.typhimurium*), even after vaccination with a live attenuated *S. typhimurium* vaccine (Rodriguez et al., 2007). Compared to wild type mice, vaccinated mice showed lower levels of IgM, IL-2 and IFN-γ (Rodriguez et al., 2007). In addition, *in vitro* studies have demonstrated that miR-155 deficient mice have increased T-helper 2 (Th2) cytokine and chemokine production compared to wild type controls (Rodriguez et al., 2007). This observation was confirmed by Thai *et al.*, (2007) who showed that T cells from miR-155 deficient mice tend to develop a Th2 phenotype compared to wild type mice after antigen challenge (Thai *et al.*, 2007), as demonstrated by the ability of the miR-155 target, transcription factor c-Maf, to induce IL-4, IL-5, and IL-10 production from Th2 cells.

A recent study conducted in our laboratory (Mattes *et al.*, 2009) demonstrated that miRNAs are also important in the regulation of allergic airways disease. Expression of miR-16, -21, and -126 were up-regulated in the lungs of mice following allergen challenge, in a house dust-mite induced model of allergic airways disease. This correlated with the development of hallmark features of allergic asthma such as an increase in IL-5 and IL-13 production, recruitment of eosinophils to the airways, and an increase in airway hyper-responsiveness (AHR) (Mattes *et al.*, 2009). MiR-126 in particular was found to target the transcription factor PU.1, an important regulator of
Th2 cell function. The function of miR-126 was demonstrated through intervention with a miRNA inhibitor, antagonomir (Ant), designed to specifically target miR-126. Antagonomir inhibition of miR-126 resulted in attenuation of the asthmatic phenotype through suppression of Th2 responses (Mattes et al., 2009).

MiR-146, a recently identified miRNA, targets TRAF-6 and IRAK-1, two important molecules involved in TLR signalling (Figure 2) (Taganov et al., 2006). A recent in vitro study has shown that increasing doses of LPS (a TLR-4 ligand) leads to a reduction in IRAK-1 protein expression (Li et al., 2000). This reduction, however, was not due to a decrease in mRNA expression, suggesting that miR-146 suppresses the translation of IRAK-1 protein rather than its transcription (Taganov et al., 2006). An in vivo mouse model designed to study the effect of LPS challenge on the expression of miRNAs found a peak in the levels of KC (the mouse equivalent of IL-8), MIP-2, and TNF-α in bronchoalveolar lavage (BAL) fluid 1 hour after exposure. Cellular infiltrates in the airways, however, took 3 hours to peak, coinciding with an increase in the expression of 46 miRNAs (Moschos et al., 2007). As such, the authors propose that these miRNAs are involved in the resolution of inflammation, rather than initiation of the inflammatory response. However, miR-146, previously identified in an in vitro study by Taganov et al., (2006) did not increase in this model. This is possibly due to differences in the dosage of LPS administered between the in vivo and in vitro study, or that miR-146 expression is being repressed by other miRNAs or regulators. However, together these studies demonstrate miRNAs play an important role in immune regulation.

The majority of early studies on miRNA expression in immune cells are based on responses to LPS challenge, however the role of miRNAs in broader infection and immunity remains largely unclear. It is known that pathogens can stimulate more than one TLR during a natural infection resulting in the activation of several signalling pathways, which in turn may enhance or suppress other pathways.
1.5.5 miRNAs and microbial infections

MiRNA expression patterns have been shown to be altered during bacterial infection. One study demonstrated that miR-155 could be induced by infection with *Helicobacter pylori* (*H. pylori*) in both gastric epithelial cells and mucosal tissues (Xiao et al., 2009). Over-expression of miR-155 in these cells resulted in decreases in IL-8 and CXCL1 production suggesting that *H. pylori* induces this miRNA to suppress inflammation. However, it is still unclear if miR-155 would be protective or detrimental to the progression of *H. pylori*-related diseases such as gastric cancers and peptic ulcers.

In another study, infection with *S.typhimurium* increased miR-21, miR-146a, miR-155 and decreased let-7a expression in a macrophage (RAW264.7) cell line. Let-7a was shown to target the mRNA transcripts of IL-6 and IL-10. This suggests that *S.typhimurium* infection can activate the production of these cytokines through down-regulation of let-7a expression (Schulte et al., 2011). Similar observations have been made following infection by *Listeria monocytogenes* (*L. monocytogenes*) of Caco2 epithelial cells (Izar et al., 2012). MiR-16, miR-146b, and miR-155 were up-regulated, while again let-7a was down-regulated. Further, it was demonstrated that mutant *L. monocytogenes* that lacked internalin virulence factors failed to upregulate miR-155 following infection, indicating that the uptake of bacteria into the cells is important for the induction of this miRNA. These studies demonstrate that bacterial infection alters miRNA expression, and suggest that induction of specific miRNAs may be dependent on the localisation of bacteria within cells.

Infection with *Francisella novicida* (*F.novicida*) in humans induced miR-155 expression in peripheral blood monocytes, while in mice, miR-155 expression was up-regulated in the lung, liver and spleen (Cremer et al., 2009). In contrast to *L. monocytogenes* however, induction of miRNA-155 by *F. novicida* did not require phagocytosis or internalisation of the bacteria, but instead was downstream of TLR2 and MyD88.
signalling. In this context, increased miR-155 expression promoted the production of TNF-α and IL-6 by monocytes. Interestingly, infection with highly virulent *Francisella tularensis* (*F. tularensis*), resulted in minimal increases in miR-155 and reduced pro-inflammatory cytokine production, suggesting that virulent bacteria have increased pathogenicity through the suppression of host immune responses. In support of this concept, an *in vivo* study by Ma *et al.*, 2011 showed that infection with intracellular pathogens such as *L. monocytogenes* or *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) suppressed miR-29 expression and IFN-γ production in NK cells, and CD4+ and CD8+ T cells (Ma *et al.*, 2011). Using bioinformatics approaches they found that the seed sequences of the miR-29 were complimentary to the 3' UTR of IFN-γ mRNA. Targeting of IFN-γ by miR-29 was further validated experimentally using a miR-29 mimic and inhibitor. The authors further demonstrated (using transgenic mice expressing a “sponge” target sequence to compete with miRNA binding of its target), that inhibition of miR-29 function in NK cells and T cells results in increased IFN-γ production and more effectively clearance of intracellular pathogens (Ma *et al.*, 2011).

In another study, biopsies obtained from the lesions of individuals with progressive leprosy had significant increases in miR-21 expression (Liu *et al.*, 2012). *In vitro* studies demonstrated that the up regulation of miR-21 in primary human monocytes correlated with increasing loads of the causative bacteria *Mycobacterium leprae*. Over-expression of this miRNA in macrophages showed that it could induce IL-10 and -6 while inhibiting CYP27B1 (an enzyme regulating the levels of biologically active Vitamin D), IL-1β, and the expression of transcripts of antimicrobial peptides such as CAMP and DEFB4A in a TLR-2 dependent pathway. Using a 3'UTR reporter assay, miR-21 was shown to bind directly to the 3'UTR of both CYP27B1 and IL-1β transcripts, but not the downstream antimicrobial peptide genes. Inhibition of miR-21 in primary human monocytes with specific antagonomers showed increased expression of these antimicrobial peptides.
1.5.6 Inhibition / over-expression of miRNAs as novel therapies

The fact that miRNAs have an important role in the pathogenesis of human disease suggests that the ability to inhibit or over-express miRNAs may provide novel therapeutic strategies. In 2005, Krutzfeldt et al. described ‘antagomirs’ which are chemically engineered ssRNAs that bind complementary to their corresponding mature target miRNAs to inhibit their function. This initial study demonstrated that antagomir-122 could suppress the expression of miR-122, in the liver for up to 23 days (Krutzfeldt et al., 2005).

Locked nucleic acids (LNAs) can also suppress the function of miRNAs in cell culture (Grunweller and Hartmann, 2007). LNAs are RNA oligonucleotides that are modified with the addition of a methylene bridge on the ribose moiety to connect the 2’ and 4’ carbons. This chemical modification improves affinity and stability of the RNA backbone, while also improving mismatch discriminations (Grunweller and Hartmann, 2007). LNAs can also be used as probes for in situ detection of miRNA expression (Obernosterer et al., 2007).

Another approach to suppress miRNA expression is through inhibition of miRISC binding to its target mRNA by introducing RNA binding proteins to the target 3’ UTRs of mRNAs. For example, Dnd-1 can be used to block the miRNA binding sites preventing attachment of miRNAs to their target (Kedde et al., 2007).

Enforced miRNA over-expression can also be useful in situations where expression of the miRNA of interest is deficient. For example, the absence of miR-15a and miR-16 in CLL patients results in high levels of the anti-apoptotic protein BCL-2 (Sampath et al., 2012). In this situation, miR-15a and miR-16 expression has been restored using gene-therapy or through administration of synthetic miRNAs (Garzon et al., 2010). A study by Doench et al., 2003 has also shown that introduction of short-interfering (siRNA) also
function as miRNAs. The ability of siRNA to translationally repress a target protein is dependent on the dose of siRNA and the number of miRNA binding sites (Doench et al., 2003).

Lentiviral vectors can also be used to increase miRNA expression. Lentiviral vectors are genetically engineered non-replicative viruses that are adapted to infect dividing, non-dividing, and differentiated cells in order to safely deliver transgenes of interest to target cells. Administration of miRNA mimics using a lentiviral vector has been shown to suppress the expression of Bcr-Abl oncoprotein both in vitro and in vivo, preventing the regrowth of leukemic cells (McLaughlin et al., 2007).

With increasing evidence indicating that miRNAs may be dysregulated during disease progression, various companies are developing miRNA related therapies by either inhibition or activation of miRNAs to treat hepatitis C, cancer, heart failure, fibrosis, and atherosclerosis (Table 4).

**Table 4. Companies developing miRNA related therapies**

<table>
<thead>
<tr>
<th>Company</th>
<th>Therapeutic</th>
<th>Target miRNAs</th>
<th>Product pipeline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Santaris Pharma A/S</td>
<td>Hepatitis C</td>
<td>miR-122</td>
<td>Phase II</td>
</tr>
<tr>
<td>MiRNA Therapeutics</td>
<td>Oncology</td>
<td>miR-34</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td>Oncology</td>
<td>Let-7</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Oncology</td>
<td>miR-16</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>miRagen Therapeutics</td>
<td>Heart Failure</td>
<td>miR-208</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Post-Myocardial Infarction</td>
<td></td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Remodeling</td>
<td>miR-15/195</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Vascular Disease</td>
<td>miR-145</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Fibrosis</td>
<td>miR-29</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Cardiometabolic</td>
<td>miR-208</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Regulus Therapeutics</td>
<td>Hepatitis C</td>
<td>miR-122</td>
<td>Phase I</td>
</tr>
<tr>
<td></td>
<td>Glioblastoma</td>
<td>miR-10b</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Hepatocellular Carcinoma</td>
<td>miR-21</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Kidney fibrosis</td>
<td>miR-22</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td></td>
<td>Atherosclerosis</td>
<td>miR-33</td>
<td>Pre-clinical</td>
</tr>
</tbody>
</table>
1.5.7 Targeting approach

The biggest challenge in miRNA research is identification of the target genes for each miRNA of interest, as there are various possibilities in the binding of miRNA-target mRNA as illustrated in Figure 4. Traditionally, binding of miRNA to the target mRNA was thought to occur in 3’UTR and required perfect complementary binding between the seed sequences (classical seed pairing) (Wightman et al., 1993, Lewis et al., 2003, Mayr et al., 2007). Seed sequences are 2nd-8th nucleotides that are located in 5’ region of a miRNA (as shown in red on Figure 4). It was later discovered that imperfect/seedless binding could also occur. This imperfect but complementary binding of seed sequence is compensated by binding of 3’ region of the miRNA to enhance target recognition (Bartel, 2009). Recently, a unique class of miRNA target site, centered pairing site, was discovered. These ‘centered sites’ pairing of miRNA-mRNA lacks both seed pairing and 3’ compensatory pairing (Shin et al., 2010, Lee and Shin, 2012). As an alternative perfect complementary binding between miRNA (nucleotides 4-15) for pairing with target mRNA is employed. It was noted that Mg$^{2+}$ concentration can directly influence both the efficacy and specificity of these miRNA to their target cleavage sites (Shin et al., 2010). A new miRNA-mRNA interaction in mouse brain was also identified using a biochemical approach to isolate AGO-bound mRNA followed by high-throughput sequencing of RNAs (Chi et al., 2009). By using MEME suite, a motif-based sequence analysis tool, the investigators identified a 5’-UGGCCUU-3’ sequence as the most significant motif in the AGO-bound mRNA. This motif was found to be complementary to miR-124 seed sequence if a G(nucleotide)-bulge in the mRNA was hypothesized. Further experiments demonstrate this specific “G-bulge binding” by miR-124 also leads to repression of its target Mink1 transcript (Chi et al., 2012). Although miRNA-target mRNA are conventionally known to take place in 3’UTR sites of the target mRNA, several recent studies indicate this is not entirely the case. For example, miR-148 binding to target DNMT3B occurs in a coding region of DNMT3B, which is
highly conserved (Duursma et al., 2008). In another study, binding of miR-296, -470 and -134 to their respective targets Nanog, Oct4, and Sox2 (in coding regions) are identified by combining both computational prediction programs (explained in next section) with experimental validation (Tay et al., 2008).

**Classical seed pairing - let-7 and Hmga2 (Mayr et al., 2007)**

5’ ...GCCAAGCUUUCUACUCA... 3’ mRNA (HMGA2)

3’ - UUGAUAGUUGGAUGAGA - 5’ miRNA (Let-7a)

**Imperfect/seedless binding - miR-24 and E2F2 (Lal et al., 2009)**

5’ ...GUGGGUGCU - CUGGGCUAGAACCA... 3’ mRNA (E2F2)

3’ -GACA - AGGACGACUUGACUGGU - 5’ miRNA (miR-24)

**Centered pairing – miR-21 and GSTM3 (Shin et al., 2010)**

5’ ...AGUUUUCAGUCUGAUAACUAUG3’ mRNA (GSTM3)

3’ -AGUUGUACAGCAUUAUCGA - 5’ miRNA (miR-21)

**G-bulge binding – miR-124 and MINK1 (Chi et al., 2012)**

5’ ...CUCUCCAUGUAGUGGCCUUGG... 3’ mRNA (MINK1)

3’ -CCUAAUGUGGCCGCACGGAAU - 5’ miRNA (miR-124)

**Outside of the 3’UTR – miR-148 and DNMT3B (Duursma et al., 2008)**

5’ ...UGGCAAGAGAGUUUUUCUUGUGCACUGAG... 3’ mRNA (DNMT3B)

3’ - UGUAUGAAUCACUGACU - 5’ miRNA (miR-148)
Figure 4. Examples of miRNA-target mRNA binding. MiRNA and target mRNA interaction takes place in 3’ untranslated regions (UTRs) of target mRNA. Seed sequence of miRNA is illustrated in red. Watson-Crick base pairing of miRNA and target mRNA are indicated by solid lines; G:U wobble represented by colon.

1.5.7.1 Bioinformatics approach

To assist in miRNA target prediction, several target prediction programs have been developed. These miRNA target prediction programs and their algorithms used for target prediction are listed in Table 5.

<table>
<thead>
<tr>
<th>Program</th>
<th>Prediction algorithm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Considering binding sites on 3’UTR only</td>
<td></td>
</tr>
<tr>
<td>TargetScan</td>
<td><a href="http://www.targetscan.org/">http://www.targetscan.org/</a></td>
</tr>
<tr>
<td>PicTar</td>
<td><a href="http://pictar.mdc-berlin.de/">http://pictar.mdc-berlin.de/</a></td>
</tr>
<tr>
<td>microcosm</td>
<td><a href="http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/">http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/</a></td>
</tr>
<tr>
<td>PITA</td>
<td><a href="http://genie.weizmann.ac.il/pubs/mir07/mir07_data.htmlrna22">http://genie.weizmann.ac.il/pubs/mir07/mir07_data.htmlrna22</a></td>
</tr>
</tbody>
</table>

These computer algorithms predict miRNA targets based on (i) complementarity of the miRNA seed region; (ii) conservation of the miRNA response element (MRE) and (iii) predicted free energy of the miRNA–mRNA hetero-duplex (Witkos et al., 2011). TargetScan and PicTar predict binding through perfect binding of the seed sequence to the 3’UTR of target mRNAs. Prediction is further improved by taking into account evolutionary conservation of targets and multiple predicted MREs (which are more likely to be the true targets) (Lewis et al., 2003, Krek et al., 2005). While these
prediction algorithms produce less false negative predictions, they do not account for other binding patterns, other than seed sequence pairing. To account for imperfect/seedless binding, microcosm, which uses miRanda algorithms, allows seed G:U wobbles and mismatches (John et al., 2004). However, the scoring given to the targets is higher when they have a seed sequence binding (Betel et al., 2008). Most of these algorithms only predict potential binding sites that are located in the 3'UTR of mRNAs. In some studies, however, it has been found that miRNAs bind not only to the 3'UTR region but also to the 5'UTR region or in the coding DNA sequence of mRNAs (Moretti et al., 2010, Forman and Coller, 2010). To address this, PITA and rna22 both predict MREs outside of the 3'UTR. PITA uses the difference between the free energy gained during miRNA-mRNA duplex formation and the free energy cost to unfold the mRNA secondary structure to determine the accessibility of miRNA to target sites (Kertesz et al., 2007). Rna22 searches for reverse complement sites of patterns within the target mRNA and determines the sites that have multiple patterns aligned. Predictions of the miRNAs to target are selected based on the lowest heteroduplex free energy cost. This prediction method allows seed mismatches and is not limited to 3'UTR binding (Miranda et al., 2006).

1.5.7.2 Experimental approach

Although miRNA target prediction algorithms are a great step forward in predicting miRNA targets, these assessments typically predict hundreds to thousands of target genes for any given miRNA. Most of these predictions are not in fact bona fide targets and also may not be transcribed in a given physiological setting. Therefore, the true targets of any individual miRNA, and their relevance in any given cell type or disease model, can only be identified using experimental approaches.

Binding of miRISC to an mRNA target inhibits the translation of that mRNA into protein followed by deadenylation and degradation of the target mRNA (Djuranovic et al.,
In order to determine which mRNAs are targeted by a given miRNA, gene expression correlation studies are commonly used following over-expression/inhibition of miRNAs (Andachi, 2008). However, while this experimental approach can identify mRNA changes resulting from altered miRNA expression, it cannot differentiate between direct and indirect targets. In addition, some studies report that miRNAs can regulate protein translation post-transcriptionally, without affecting mRNA abundance which complicates the identification of true targets using gene expression (Pillai et al., 2005, Humphreys et al., 2005).

Therefore proteomics analysis is sometimes used to identify potential targets. Differential protein expression can be analysed using 2D-SDS PAGE followed by mass spectrometry. In this technique, proteins extracted from miRNA over-expressed/inhibited cells are labelled with fluorescent dyes (e.g Cy3), while mock-transfected cells are labelled with a different dye (e.g Cy5). 2D-SDS PAGE is then performed to separate proteins based on isoelectric focusing and molecular size. Differential expression of proteins are identified by comparing Cy3/Cy5 ratios and excised from the gel, trypsinized and identified with mass spectrometry (Akbari Moqadam et al., 2013).

Although the above correlation studies would give us some ideas on the potential target, it is difficult to determine the direct miRNAs target from the indirect one as the changes in genes or proteins expression could also be regulated indirectly by the “true target”. In addition, studies using miRNA mimics to assess the effects of miRNA over-expression in these experiments can lead to artifacts, as the transfection of miRNA mimics can limit availability of miRISC to other endogenous miRNA, hence increasing the endogenous miRNA target leading to false positive results (Khan et al., 2009).

To overcome this deficiency, biochemical approaches can be used to increase the specificity of target discovery and directly identify miRNA-mRNA target interactions.
First, miRNAs of interest are overexpressed in cells. Using co-immunoprecipitation technique, miRNA-mRNA complexes can be isolated through pull-down assays, using specific monoclonal antibodies recognizing the AGO protein (mentioned in section 1.5.1). This precipitated fraction contains overexpressed miRNA that are associated with AGO protein and miRNA bound target mRNAs. Captured mRNA sequences are then analysed with gene expression arrays or deep sequencing to identify the gene name (Beitzinger et al., 2007, Karginov et al., 2007).

1.6 Hypothesis

The expression of miRNAs is often altered during disease pathogenesis. Previous studies in our laboratory have shown that this occurs during inflammation in response to allergic airways disease induced by allergens. However, the functional role and altered expression of miRNAs in response to bacterial infection of the lung has not been characterised. Very recent studies suggest an important role for miRNA in regulating both innate host defence responses and acquired immunity. In particular, miRNA expression is closely linked to activation of recognition pathways (Figure 5) that could lead to elimination of pathogens.

We hypothesise that:

1) NTHi infection stimulates the expression of miRNAs through the activation of common or divergent immune pathways during infection

2) The expression of specific miRNAs plays an important role in regulating innate immune cells function in response to NTHi infection.

3) Targeting by inhibiting or overexpressing specific miRNAs in innate immune cells may enhance bacterial clearance in lung.
Figure 5. Innate host defence and miRNA regulation. During respiratory bacterial infection, activation of PRR by bacterial ligands leads to production of pro-inflammatory cytokines and chemokines, leading to recruitment of inflammatory cells to the airways for the clearance of bacteria. MiRNAs can target both the PRR signalling pathway and inflammatory cytokines production. Role of miRNAs in regulating anti-bacterial responses in lung remain largely unknown.

1.7 Aims

1. To characterise the inflammatory response following pulmonary bacterial infection using a mouse model of NTHi infection.

2. To profile miRNA expression within the airways of adult mice following pulmonary infection with NTHi.

2. To identify the role of specific miRNAs following NTHi infection by using antagonimirs to inhibit their function.
3. To determine the therapeutic value of miRNA targeting for the clearance of bacterial infections in models of steroid-induced immunosuppression and COPD.
Chapter 2 Materials and methods

2.1 Mice

Specific pathogen-free male BALB/c mice (6-8 weeks old) were used in all experiments. Mice were obtained from the University of Newcastle Central Animal House (Newcastle, New South Wales) and were acclimatised for one week prior to experimentation. Mice were housed in individually ventilated cages and provided with autoclaved water, food and bedding. The animal protocols used in this study were approved by and used in accordance with guidelines by the Animal Care and Ethics Committee (ACEC, The University of Newcastle).

2.2 Bacterial respiratory infection

2.2.1 NTHi 289

NTHi biotype II (NTHi 289) was kindly provided by A/Prof Margaret Dunkley (Hunter Immunology Ltd, Newcastle, Australia). All bacterial work was performed in a biohazard hood under sterile conditions. Bacteria were prepared prior to infection by growing cultures overnight on chocolate agar plates (Oxoid, Australia) at 37 °C in an atmosphere of 5% CO₂. The following day, the bacteria were harvested, washed and resuspended in 1ml of phosphate buffered saline (PBS). Absorbance measurements were performed at 405 nm to determine bacterial concentration, by reference to a standardised regression curve previously established in our laboratory. This regression curve was generated by plotting the number of colony forming units (CFU) versus absorbance value at 405 nm in serial dilutions. The bacterial suspension was then diluted in sterile PBS to obtain $5 \times 10^5$ CFU / 30μl for infection.
2.2.2 NTHi 289 infection

Mice were anaesthetised (12.5 mg/kg Alfaxan, Jurox, NSW, Australia) intravenously via the tail vein, suspended vertically and infected intratracheally (i.t) through a catheter (Terumo, Sureflo Hospital Supplies of Australia) with $5 \times 10^5$ CFU of live NTHi 289 in 30 µl of sterile PBS. Control mice were sham infected with 30 µl of sterile PBS. Mice were left suspended for 1 minute to allow equal distribution of bacteria through the lungs before being returned to their cage. Mice were continually monitored until they made a full recovery from anaesthesia.

2.2.3 Bacterial Recovery

Both BAL fluid and right lung homogenates (homogenised in 1 ml sterile PBS (Gibco, Invitrogen, New Zealand) by using a tissue tearer) were used to determine bacterial load. Serial dilutions (neat, 1:10, and 1:100; 20 µl) were loaded onto chocolate agar plates (Oxoid, Australia) and incubated overnight at 37 °C in an atmosphere of 5% CO$_2$ to facilitate bacterial growth. After 16 hours, bacterial colonies for NTHi were counted. The total number of bacterial colonies recovered was obtained by combining the CFU in BAL fluid and lung homogenates.

2.3 Collection and analysis of BAL fluid

2.3.1 Cytospin preparation of BAL fluid

The left lobe of the lung was tied off using thread to block the bronchus (and kept for RNA extraction), leaving the right lung open for BAL fluid collection (tissue was also kept for bacterial recovery in NTHi infected mice). The right lung was washed with 2 x 700 µl of Hanks Buffered Saline Solution (HBSS) (Gibco, Invitrogen) through a cannula inserted into the trachea. BAL fluid from the same mice was pooled into 1.5 ml microcentrifuge tubes. 40 µl of BAL fluid was reserved for bacterial recovery (section
2.2.4) and the remainder was used for cytopsin preparations and subsequent differential cell counts. BAL samples were then centrifuged at 3000 rpm for 10 minutes at 4 °C to pellet the cells. The remaining pellet was resuspended in 400 µl of red blood cell (RBC) lysis buffer (155 mM NH₄Cl, 12 mM NaHCO₃, 0.1 mM EDTA, pH 7.35) for 5 minutes at 4 °C to lyse the erythrocytes before being centrifuged at 3000 rpm for 10 minutes at 4 °C. The pellet was then resuspended in 100 µl of HBSS. 10 µl of the cell suspension was mixed with 10 µl of Trypan Blue and the numbers of live cells per sample were determined using a Neubauer haematocytometer (Germany). A total of 100,000-200,000 cells were loaded into each cytofunnel and samples were spun onto histology slides at 300 rpm for 10 minutes in a cytopsin (Shandon-4). Slides were left to dry overnight before staining.

2.3.2 BAL fluid differential cell counts

Cytospin slide preparations were stained with May-Grunwald and Giemsa stain (Giemsa was diluted 1:25 with distilled water) (ACR, Australian Scientific). Slides were immersed in May-Grunwald stain for 5 minutes to stain the nuclei. Excess stain was removed by washing in distilled water for 1 minute before slides were immersed in Giemsa stain for 20 minutes to stain eosinophilic granulocytes. Excess staining was again removed by washing the slides in distilled water twice for 5 minutes. Slides were left to dry overnight before being cover-slipped using Normount mounting medium (Fronine, Australia) to prevent the stain from fading. Slides were analysed using light microscopy and differential cell counts were performed by counting a minimum of 300 cells per slide at 60 X magnification. Standard morphological criteria were used to determine the numbers of polymorphonuclear neutrophils (PMN), lymphocytes, macrophages and eosinophils in each sample. The percentage of each cell type was calculated by dividing the number of each cell type by the total number of cells counted.
per slide, while total cell numbers for each population were calculated based on multiplying the differential percentages by the total BAL cell count.

2.4 RNA extraction and miRNA analysis

2.4.1 Blunt dissection of airways

The left lobe of the lung was used for preparation of RNA for miRNA profiling. The airways were separated from the parenchyma by blunt dissection. Airway and parenchymal cells were immediately placed in RNA later (Ambion, Austin, TX) to inactivate any RNA nucleases present in the tissue. Samples were stored at -80 °C for further analysis.

2.4.2 Total RNA extraction

Total RNA was extracted using a mirVana™ miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer’s protocol. Airway that had been stored at -80 °C in RNA later was dissociated with scissors and ground with RNase free pestles in 1.5 ml microcentrifuge tubes containing 100 µl of ice cold Lysis/Binding buffer, to avoid possible degradation by RNA nucleases. Once homogenised, 200 µl of Lysis/Binding buffer was added to lyse the cells of the airway before 30 µl of miRNA homogenate additive was added to the lysate and incubated on ice for 10 minutes. Acid phenol chloroform was then added (300 µl/tube), samples vortexed for 30 seconds, and centrifuged for 5 minutes at 13,000 rpm to remove most of the cellular components, leaving a semi-pure RNA sample.

2.4.3 Small RNA extraction

RNA was further purified by transferring the aqueous layer into a fresh tube and adding 1.25 x 100% ethanol to precipitate the RNA. The mixture was transferred to a filter
cartridge fitted on a supplied collection tube and centrifuged for 30 seconds at 10,000 rpm. The filtrate was discarded and 700 µl of wash solution 1 (supplied in the kit) was added. The sample was centrifuged for 30 seconds at 10,000 rpm. This step was repeated twice by using 500 µl of wash solution 2 (supplied in the kit). For the final wash, samples were centrifuged for 90 seconds to remove excess ethanol in the filter cartridge. The cartridge was then transferred into a new collection tube. 100 µl of elution solution (nuclease-free 0.1 mM EDTA) pre-heated to 95 °C was added and samples spun immediately for 60 seconds at 10,000 rpm to elute the RNA from the filter. The eluate containing the RNA sample was analysed using a spectrophotometer (ND-1000, Thermo Scientific) to determine the RNA concentration and purity. Samples were stored at -80 °C until further analysis.

2.4.4 RNA quality analysis

The quality of each isolated RNA sample was analysed using a Bioanalyzer (Agilent 2100) and an RNA 6000 Nano Chip Kit (Agilent). Prior to sample analysis, the bioanalyzer was decontaminated with an RNAseZap Electrode Cleaner chip (filled with 350 µL of RNAseZap) for 1 minute followed by RNAse-free deionised (DI) water Electrode Cleaner chip (filled with 350 µL of RNAse-free DI water) for 10 seconds. RNA 6000 Nano gel matrix (supplied) was filtered through a column and centrifuged at 4,000 rpm for 10 min. 1 µL of dye concentrate was added to 65 µl of filtered gel, then 9 µl of the mix was added to one well of the bioanalyzer chip. The plunger on the chip priming station (Agilent) was depressed and held for 30 seconds to load the gel-dye mix into the chip. Another 9 µl of the gel dye mix was added to a second well while 5 µl of 6000 Nano Marker was added to 12 sample wells and the ladder well. In order to minimize the formation of secondary structures, RNA samples to be analysed as well as the RNA 6000 ladder (Ambion) were denatured at 70 °C for 2 minutes prior to loading. Equal volumes of sample were added to each sample well, while 1 µL of ladder was
added to the ladder well. The chip was vortexed at 240 rpm for 1 minute before being placed in the Bioanalyzer. Samples with a RNA integrity number (RIN) below 7.5 (indicating poor RNA quality) were not used for further miRNA profiling.

2.4.5 miRNA expression profiling

Steps for miRNA expression profiling are summarized in Figure 6. Briefly, 3 µl of Calf Intestine Alkaline Phosphatase (CIP) Master Mix (containing 0.7 µl 10 X CIP buffer, 1.6 µl RNase-Free Water, 0.7 µl GEHealthcare CIP (16 U/µl)) was added to 100 ng/4µl of RNA sample. RNA was dephosphorylated by heating at 37 °C for 30 minutes to avoid ligation of miRNA with other RNA fragments (which could interfere with the labelling process). Before labelling, RNA was added to 5 µl of 100 % DMSO and heated to 100 °C for 5 minutes to denature the RNA. 8 µl of Ligation Master Mix (containing 2 µl of 10 X T4 RNA Ligase Buffer, 2 µl of 0.1 % BSA, 3 µl of pCp-Cy3 dye, and 1 µl of diluted T4 RNA Ligase (15 U/µl)) was added to the sample and incubated at 16 °C for 2 hours to label the miRNA at the 3’ end. The sample was then added to 30 µl of RNase free water, purified using a Micro Bio-Spin 6 column and eluted by spinning at 1,000 g for 4 minutes. Purified, labelled filtrate was dried at 45 °C for 1 hour with a speed-vac. Dried sample was resuspended in 18 µl of RNase free water before adding 4.5 µl of 10 X Gene Expression (GE) Blocking Agent and 22.5 µl of Hi-RPM hybridization buffer was added. The mixture was heated to 100 °C for 5 minutes, then cooled immediately on ice for 5 minutes. The hybridized sample was then loaded onto miRNA microarray slides (Agilent) and samples allowed to hybridize in Agilent SureHyb chamber at 55 °C for 20 hours. Microarray slides were washed with GE Wash Buffer 1 and 2 for 5 minutes each to remove unbound RNA. The slides were scanned with an Agilent scanner and the data analysed using GeneSpring GX version 9 software.
Figure 6 miRNA microarray. Workflow for miRNA profiling adapted from Agilent miRNA microarray system online protocol.
2.5 Quantifying miRNA and mRNA expression using quantitative polymerase chain reaction (qPCR)

2.5.1 miRNA reverse transcription (RT)

cDNA was generated from RNA by adding 10 µl of RT master mix (containing 0.15 µl of 100mM dNTPs, 1.00 µl of MultiScribe™ Reverse Transcriptase, 1.50 µl of 10X Reverse Transcription Buffer, 0.19 µl RNase Inhibitor 20U/µl, 3 µl of Taqman primer, 4.16 µl of Nuclease-free water per sample) to 10 ng/ 5µl of purified RNA. The mixture was incubated at 16 °C for 30 minutes followed by 42 °C for 30 minutes to allow reverse transcription to occur. Reverse transcription was inactivated by heating to 85 °C for 5 minutes. The mixture was spun down and 30 µl of nuclease-free water added to dilute the cDNA. cDNA was stored at -20 °C for further analysis.

2.5.2 miRNA qPCR

1.33 µl of RT product from the RT reaction was added to 17.67 µl of qPCR mastermix (containing 10.00 µl of TaqMan 2X Universal PCR Master Mix No AmpErase UNG, and 7.67 µl of Nuclease-free water). The mixture was incubated at 95 °C for 10 minutes followed by 40 PCR cycles (denaturing step at 95 °C for 15 seconds and annealing step at 60 °C for 60 seconds). Sno-202 was used as a control for the baseline level of cDNA expressed in all samples. MiRNA taqman primers used were listed in Table 6.
Table 6 Taqman primer for miRNA qPCR

<table>
<thead>
<tr>
<th>miRNA name</th>
<th>Sequence (5' to 3')</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-223</td>
<td>UGUCAGUUUGUCAAAAUACCCCA</td>
<td>002295</td>
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<tr>
<td>mmu-miR-146b</td>
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<td>001097</td>
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<td>002498</td>
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<tr>
<td>mmu-miR-376c</td>
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<td>002450</td>
</tr>
<tr>
<td>mmu-miR-328</td>
<td>CUGGCCCUCUCUGCUCUUCCGU</td>
<td>000543</td>
</tr>
</tbody>
</table>

2.5.3 mRNA RT

cDNA was generated from RNA by adding 2 µl of primer/dNTP mix (containing 1 µl of 100 ng/µl Random Primers and 1 µl of 10 mM dNTPs per sample) to 1000 ng/11 µl of RNA. The mixture was incubated at 65 °C for 5 minutes to remove secondary mRNA structures, before being cooled on ice to allow the random primers to anneal to the mRNA. 7 µl of Superscript III master mix (containing 4 µl of 5 x 1st strand buffer (Invitrogen, Australia), 1 µl of 0.1 M DTT (Invitrogen, Canada), 1 µl of sterile Nuclease free water (Ambion, Austin, TX), and 1 µl of Superscript III reverse transcriptase (Invitrogen, Australia) was added per sample, the mixture incubated at 25 °C for 5 minutes and then heated at 50 °C for 60 minutes to allow reverse transcription to occur. Reverse transcription was inactivated by heating the sample to 70 °C for 15 minutes. The mixture was spun down and 30 µl of T10E0.1 buffer was added to inactivate DNA nucleases. cDNA was stored at -20 °C until further analysis.
2.5.4 mRNA qPCR

5 µl of cDNA was added to 20 µl of qPCR mastermix (containing 12.5 µl of PCR Supermix-UDG with ROX, 1 µl of 5 µM forward primers, 1 µl of 5 µM reverse primer, and 5.5 µl of nuclease free water). The sequence for the Muc5ac primers were: forward 5'-CGGCCGGAGAAAGTTGGTCCC -3', reverse 5'-GCACACCCGCCTGGATGTCC -3' (Invitrogen). The housekeeping gene mHPRT was used as a control for the baseline level of cDNA expressed in all samples. The sequence for the mHPRT primers were: forward 5'-AGGCCAGACTTTTGTGGATTTGAA-3', reverse 5'-CAACTTGCCTCATCTTTAGGGATT-3' (Sigma-Genosys).

2.6 Mouse primary cell isolation

2.6.1 Primary lung macrophage isolation

Lung tissue from naïve mice was forced through a 70 µM cell strainer, before being layered on Histopaque 1083 (Sigma) and centrifuged (3,000 rpm, 30 minutes, 20 °C). The interface layer containing mononuclear cells was collected and allowed to adhere to a culture plate for 3 hours at a concentration of 1 x 10^6 cells, following which non-adherent cells were removed. 300 µL of fresh culture media (made up with RPMI1640 supplemented with 0.1mM sodium pyruvate, 10%FCS, 2mM L-glutamine, 20mM HEPES) was added for cell culture.

2.6.2 Primary neutrophil isolation

Neutrophils were extracted from the bone marrow of naïve mice using a 3 layer Percoll gradient and magnetic bead separation. Briefly, bone marrow cells were isolated from femurs and tibias from both hind legs by flushing bones with HBSS-EDTA solution. Erythrocytes were lysed with lysis buffer. Cells were then layered on a three-layer gradient of 78%, 69%, and 52% Percoll and centrifuged (1,500 g, 20 minutes, 20 °C) at
the slowest deceleration. Cells from the 69%/78% interface were collected and
neutrophils further purified by negative immunomagnetic separation using labelling with
anti-CD4, -CD8, -CD11c, -CD49b, -CD117, -B220, and -F4/80 purified antibodies (BD
Biosciences, USA). The labelled cells were then depleted through magnetic separation
by first incubating with BD IMAG streptavidin particles plus-DM (BD Biosciences)
according to manufacturer’s protocol. The enriched fraction containing > 96%
neutrophils was utilised for further experiments.

2.7 Human primary cell isolation

2.7.1 Human monocyte-derived macrophages

Whole blood was obtained from healthy human adult volunteers, with written informed
consent. PBMCs were isolated by Ficoll centrifugation (GE Healthcare) and cells were
adhered to culture plates for 3 hours at 5 x 10^6 cells/ml, after which, non-adherent cells
were gently removed. Adherent cells were cultured in 50 ng/ml recombinant human M-
CSF (Peprotech) to induce macrophage differentiation, and fresh media supplemented
with M-CSF was replaced on day 3 and day 6 of culture. Monocyte-derived
macrophages were used on day 7.

2.7.2 Peripheral blood neutrophils

For neutrophil isolation, the remaining Ficoll layer was removed without disturbing the
layer of neutrophils/RBCs at the bottom. The thin white cell layer containing neutrophils
(above the RBC pellet) was collected and resuspended in an equal volume of HBSS
and dextran/saline solution (5% dextran T500 in 0.9% NaCl at room temperature),
before being incubated in an upright position for 20 minutes at room temperature. The
layer of neutrophils above the sedimented RBC layer was aspirated and remaining
RBCs lysed with lysis buffer. Cells were resuspended in culture media ready for
assays.
2.8 Inhibition of miRNA function using antagomirs

2.8.1 Synthesis of antagomir oligonucleotides

Complementary antagomir strands were generated using miRbase and were synthesised by Sigma-Aldrich. MiRNAs of interest were inhibited using the antagomirs outlined in table 1.

<table>
<thead>
<tr>
<th>Antagomir</th>
<th>Sequence</th>
</tr>
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<tr>
<td>Scrambled antagomir</td>
<td>5’mU.<em>.mC.</em>.mA.mC.mA.mA.mC.mC.mU.mC.mC.mU.mA.mG.mA.mA.mA.mG.mA.<em>.mU.</em>.mA.*.3’-Chol</td>
</tr>
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"m" represents 2’-OMe-modified phosphoramidites

"*" represents phosphorothioate linkages
“-Chol” was hydroxyprolinol-linked cholesterol to allow permeation of cell membranes

2.8.2 Antagomir treatment

Mice were treated in vivo with 50 µg of antagomir intratracheally. For in vitro cell assays, macrophages and neutrophils were treated with 30 µg/ml of antagomir 12 hours before the end points. Scrambled antagomirs (Scr) were used as both in vivo and in vitro controls.

2.9 In vitro assays following inhibition of miR-328

2.9.1 NTHi infection in vitro

In vitro macrophages were infected with a MOI of 100 NTHi for 8 hours while neutrophils were infected with a MOI of 10 NTHi for 1 hour. To measure extracellular bacteria, the supernatants were first removed from the macrophage or neutrophil cultures. To measure intracellular bacteria, macrophages or neutrophils were incubated with gentamicin (400 µg/mL) for 1 hour at 37 °C to eliminate extracellular bacteria. Cells were then washed three times with PBS before being lysed with 0.25% saponin solution to release intracellular NTHi. Extracellular and intracellular bacteria were plated onto chocolate agar plates and incubated overnight at 37 °C in an atmosphere of 5% CO₂. After 16 hours, bacterial colonies were counted.

2.9.2 Measurement of respiratory burst

Macrophages were detached from culture dishes in citric saline (0.135 M KCL, 0.015 M Na citrate) while neutrophils were removed by rinsing. Cells were spun down and resuspended in FACS buffer (2% FCS in PBS) before incubation with 10 µM dihydroethidium (DHE) for 30 minutes at 37 °C. Fluorescence intensity was measured by flow cytometry (BD FACSCanto™ II) with 488nm excitation and 610nm emission.
2.9.3 Measuring uptake of heat-killed NTHi

Both flow cytometry and immunofluorescence microscopy were used to measure uptake of CFSE-labelled, heat-killed NTHi. NTHi was labelled with 100 µM CFSE (Molecular Probes) (before heat-killing) for 10 minutes at 37 °C. Staining was quenched with fetal calf serum (FCS) for 1 minute, and bacteria washed, resuspended in PBS, and incubated with primary macrophages or neutrophils for 1 hour. Cells were then harvested and assessed to quantify cells positive for CFSE and mean fluorescence intensity by flow cytometry. To visualise the uptake of heat-killed NTHi by microscopy, macrophages or neutrophils were initially adhered to coverslips. Cells were incubated with rhodamine and DAPI for 5 minutes each to stain both cytoplasm and nucleus, respectively. Images were visualised using an Olympus BX51 microscope using a 40x objective and images were captured using an Olympus DP70 camera.

2.9.4 Immunofluorescence staining

Immunofluorescence assays were performed as described (Li et al., 2010). Macrophages were labelled with 5 µM of CFSE for 10 minutes at 37 °C. Staining was quenched with fetal calf serum (FCS) for 1 minute, and cells were washed and resuspended in PBS. Cells were fixed in 1% (w/v) paraformaldehyde in PBS buffer for 10 minutes at room temperature (RT), blocked and permeabilised with 0.2% (v/v) Triton X-100 and 5% normal goat serum for 1 hour at RT. Cathepsin D expression was detected by staining with rabbit monoclonal Ab to CTSD (Abcam) overnight at 4 °C. Cells were then incubated with Cy3-conjugated goat anti-rabbit IgG (GE Healthcare) for 45 minutes at RT and the nuclei counter-stained with DAPI. Images were analysed using Olympus FluoView™ FV1000 confocal microscope.
2.9.5 Cytokine analysis

The concentrations of TNF-α and IL-6 was measured in the supernatant of BAL, lung homogenate, and in vitro cell free culture supernatants using ELISA kits (ebioscience) according to the manufacturer's instructions.

2.9.6 Inhibition of intracellular signaling pathways

Macrophages were pre-treated with 5 µM doramapimod (LC lab) for 30 minutes to inhibit p38 MAPK activation prior to NTHi infection for 8 hours. DMSO was used as a vehicle control. MiR-328 expression was quantified using Taqman qPCR as described in section 2.5.1 and 2.5.2.

2.9.7 Western Blot

Cells were lysed and total protein levels were determined using a standard BCA protein assay (Thermo Scientific). Protein samples (20 µg) were resolved on SDS electrophoresis gels, transferred onto PVDF membrane by standard procedures and blotted using an antibody to phosphorylated p38 (Abcam). Samples were normalised to blots stained for β-actin expression, to control for loading variability. Bound antibodies were detected using chemiluminescent substrate (Bio-rad) and imaged using ChemiDoc XRS (Bio-Rad)

2.10 Inhibition of miR-328 function in vivo

2.10.1 Adoptive transfer of macrophages

Macrophages were isolated as described in section 2.6.1 and pre-treated with antagonir-328 (Ant-328) or Scr for 12 hours (refer to section 2.8.2). Cell were harvested and labelled with 5 µM of CFSE (Molecular Probes) for 10 minutes at 37 °C. Staining was quenched with FCS for 1 minute, wash and resuspended in PBS. $5 \times 10^5$
CFSE labelled macrophages were transferred into the lung of naïve mice intratracheally in 40 µL PBS. 24 hours after the macrophages instillation mice were challenged with 5 x 10^6 CFU of NTHi in 30 µl of sterile PBS as described in section 2.2.2. 12 hours post infection, end point was performed to measure miR-328 knockdown, bacterial load, cellular infiltration, and inflammatory cytokines in the lung.

### 2.10.2 Adoptive transfer of neutrophils

Neutrophils were isolated as described in section 2.6.2 and pre-treated with Ant-328 or Scr for 12 hours (refer to section 2.8.2). Cell were harvested and labelled with 5 µM of CFSE (Molecular Probes) for 10 minutes at 37 °C. Staining was quenched with FCS for 1 minute, wash and resuspended in PBS. 5 x 10^5 CFSE labelled neutrophils were transferred into the lung of naïve mice intratracheally in 40 µL PBS. 2 hours after neutrophils instillation, mice were challenged with 5 x 10^6 CFU of NTHi in 30 µl of sterile PBS as described in section 2.2.2. 6 hours post infection, end point was performed to measure miR-328 knockdown, bacterial load, cellular infiltrations, and inflammatory cytokines in lung.

### 2.10.3 Administration of Ant-328 as therapeutic agent

BALB/c mice were first infected with 5 x 10^6 CFU of NTHi for 6 hours as described in section 2.2.2. 6 hours post infection, Ant-328 was administered (refer to section 2.8.2) as treatment while Scr was used as control. 18 hours post infection, end point was performed to measure miR-328 knockdown, bacterial load, cellular infiltration, and inflammatory cytokines in lung.

### 2.10.4 Cigarette smoke exposure model

Mice were exposed to cigarette smoke (from 12 cigarettes) in a closed chamber twice a day, 5 days per week for a duration of 8 weeks, as previously published (Beckett et al.,
2013). 3 days following the end of cigarette smoke exposure, mice were challenged with 5 x 10^5 CFU of NTHi as described in section 2.2.2. 6 hours post infection, Ant-328 was administered (refer to section 2.8.2) as treatment while Scr was used as control. 18 hours post infection, end point was performed to measure miR-328 knockdown, lung function, bacterial load, cellular infiltration, and inflammatory cytokines in lung.

2.10.5 Steroid-induced immunosuppression model

Mice were injected intraperitoneally (i.p.) with 3 mg/kg of dexamethasone (DEX) (Sigma-Aldrich) for 3 consecutive days before they were inoculated with 5 x 10^5 CFU NTHi / mouse as described in section 2.2.2. 18 hours post infection, end point was performed to measure miR-328 knockdown, lung function, bacterial load, cellular infiltration, and inflammatory cytokines in lung.

2.10.6 Measuring lung function using forced oscillation technique (Flexivent)

Lung function assessment was performed as reported previously (Li et al., 2010). Mice were anaestheised (i.p.) with 50 µL/10g mixture containing xylazine (2 mg/mL; Troy laboratories, Smithfield, New South Wales, Australia) and ketamine (40 mg/mL; Parnell, Alexandria, New South Wales, Australia). Tracheostomy was performed to insert a cannula into the trachea. Mice were ventilated with a tidal volume of 8 mL/kg at a frequency of 450 breaths/minute and a positive end-expiratory pressure of 2 cm H$_2$O. FlexiVent perturbations were performed. Deep inflation, single compartment (snapshot), pressure-volume loops with stepwise or increasing volumes (PVs-V), or pressures (PVs-P), constant phase model (Primewave-8) perturbations were performed. Primewave-8 perturbation was used for tissue elastance and snapshot perturbations were used to measure compliance. Measurements were excluded if the coefficient of determination was lower than 95%.
2.11 Statistical analysis

All experimental results are presented as mean ± standard error of the mean (SEM). Results were analysed by one-way ANOVA, followed by a Bonferroni post-test for statistical significance or two-tailed Student's unpaired t-test. One-way ANOVA was used when comparing mean difference in a graph that has more than two treatment groups. Student's unpaired t-test was used where there are only two treatment groups in a graph. Analysis was performed with Prism v5.0 (GraphPad Software, CA. USA). p-values < 0.05 were considered statistically significant.
Chapter 3 Results

3.1 miRNA expression in the lung following NTHi infection

3.1.1 Kinetics of NTHi infection

In order to investigate the role of miRNA in the lungs during bacterial infection we first analysed the kinetics of NTHi clearance and cellular infiltration in mice. BALB/c mice were infected intratracheally (i.t) with low dose (5 x 10^5 CFU) NTHi. NTHi grew rapidly in lungs from 3-6 hours, reached peak bacterial loads between 6-12 hours after inoculation and was cleared rapidly thereafter to the point where it was only slightly above baseline levels by 24 hours (Fig. 7).

![Figure 7. Kinetics of NTHi clearance in lungs. Mice were infected (i.t) with 5 x 10^5 CFU of NTHi / 30 uL of PBS. Bacterial load in lungs at various time points were determined by colony counts. Mock infected mice are treated with 30uL of PBS. Results are expressed as mean ± SEM. n = 5-7 mice per group, *** p<0.001 compared to mock infected group.](image)
To characterize the inflammatory response following NTHi infection, BAL fluid was used to enumerate inflammatory cells present in the airways. Total cells (Fig. 8A) in the BAL fluid increased from 6 hours post NTHi infection and remained similar until 24 hours post NTHi infection. At 48 hours post NTHi infection, total cells increased further (2-fold increased). Cell numbers in the BAL fluids started to decrease on day 5 following infection.

To determine cell types present in lungs, cells stained with May-Grunwald and Giemsa stain were differentially counted based on cell morphology. Macrophages (Fig. 8B) level in lungs of infected mice was similar with mock infected group up to 24 hours post infection. At 48 hours following infection, macrophage numbers increased by 3-fold and continue to increase on day 5 post infection. Although low number of neutrophils (Fig. 8C) was observed as early as 3 hours post-infection, neutrophils were significantly increased at 6 hours and remained in similar numbers until 48 hours post-infection. Neutrophils inflammations were mostly resolved 5 days after infection. There was no eosinophils and lymphocytes observed in the BAL cells.
Figure 8. NTHi infection leads to recruitment of inflammatory cells. BAL fluid was collected at various time points to determine inflammatory cell numbers and types in the Airways following NTHi infection. (A) Total cells following NTHi challenge were counted using Trypan Blue staining. (B,C) Cellular infiltrates obtained from BAL fluid were cytopun and stations were stained with May-Grünwald. (B) Macrophage and (C) neutrophil numbers were determined by differential cell counts based on morphology of cells. Mock infected mice are treated with 30 uL of PBS. Results are expressed as mean ± SEM. n = 5-7 mice per group, * p<0.05, ** p<0.01, *** p<0.001 compared to mock infected group.

3.1.2 miRNAs expression changes following infection

To determine the expression and regulation of miRNA during respiratory bacterial infection, we performed miRNA microarray analysis on the total RNA isolated from the Airways of NTHi-infected versus mock-infected control mice at a time point at which cells were directed at bacterial clearance rather than resolution of inflammation (24 hours post-infection). At this time point, 72 out of 627 mouse miRNAs were altered in expression. Of these 27.7% of the differentially expressed miRNAs were up-regulated while 72.3% were downregulated > 2.5 fold (Fig. 9).

Although miRNA microarray analysis is a great tool for initial target discovery, microarrays results often vary from laboratory to laboratory, user to user and platform to platform. Moreover microarray results could be affected by non-specific and cross hybridization partly due to the small size of miRNA and high degree of similarity among miRNA sequences (Zhao et al., 2011, Chuaqui et al., 2002). Thus, we validated the miRNA microarray data for a number of miRNAs of interest using Taqman PCR (Fig. 10). These miRNAs were selected as they are highly conserved between species suggesting they could be essential for normal cellular function and regulation of
infection. We found that 4 out of 6 miRNAs (miR-146b, -21a-3p, -223, -328) validated with Taqman qPCR were correlated to the changes found in microarray data.

Figure 9. MiRNAs expression altered during bacterial infection. Mice were infected (i.t.) with NTHi for 24 hours. Total RNA from the airways of NTHi infected mice (n = 4...
mice) was extracted for miRNA microarray analysis. Differential expression of miRNAs was compared to mock infected control group (PBS) \( (n = 4 \text{ mice}) \). Relative miRNAs expression levels were shown with high expression miRNAs represented by yellow and low expression represented by blue.

**Figure 10. Validation of miRNA microarray expression.** Total RNA from the airways of NTHi infected mice was extracted. MiRNA microarray results were validated using TaqMan qPCR normalized against sno-202 and expressed as fold change compared to mock infected control group (PBS). Results are expressed as mean ± SEM. \( n = 8 \text{ mice per group}, ** p<0.01, *** p<0.001 \) compared to mock infected group.
3.2 miRNA regulating macrophages and neutrophils function \textit{in vitro}

3.2.1 miR-328 regulates killing of bacteria \textit{in vitro}

Among the miRNAs that were differentially expressed, we selected miR-328, specifically the 3p strand, as a candidate miRNA to regulate the host response to infection. At baseline, the expression of miR-328 was among the highest of the miRNAs identified, and uniquely it was also down-regulated rather than up-regulated or unchanged following infection. Additionally, miR-328 is highly conserved across species and the role of miR-328 in the immune and inflammatory response to microbial infection was unknown.

To examine the function of miR-328 in anti-bacterial responses in the lung, we isolated two major immune cell types found in the lung after NTHi infection, that is, macrophages and neutrophils. Macrophages were purified from lungs while neutrophils were isolated from bone marrow of naïve mice. They were pre-treated with an inhibitor with perfect complementarity to miR-328 (Ant-328) to block its function before the cells were infected with NTHi \textit{in vitro}. Scr sequence was used as a negative control. Knockdown of miR-328 expression in macrophages (Fig. 11A) and neutrophils (Fig. 11D) by Ant-328 was confirmed using Taqman qPCR.

Interestingly, macrophages treated with Ant-328 had a significantly reduced bacterial load in the culture supernatant (Fig. 11B) and increased bacterial uptake intracellularly (Fig. 11C) compared to Scr treated group. Similarly, the same result was also obtained with neutrophils. Knockdown of miR-328 in neutrophils using Ant-328 resulted in up to a 3-fold decrease in bacterial load in the culture supernatant (Fig. 11E) as early as 1 hour post infection and a dramatic increase in bacterial uptake intracellularly (Fig. 11F) compared to Scr treatment.
Figure 11. Inhibition of miR-328 function increases bacterial clearance in vitro.

(A-C) Primary lung macrophages were isolated from naïve mouse lungs and pre-treated with Ant-328 for 12 hours before infection with NTHi MOI 100 for 8 hours. (D-F) Primary neutrophils were isolated from bone marrow and pre-treated with Ant-328 for 12 hours before infection with NTHi MOI 100 for 1 hour. Scr was used as control. (A, D) MiR-328 knockdown by Ant-328 was validated using Taqman PCR, normalized against sno-202 and expressed as fold change compared to Scr. (B, E) Numbers of extracellular bacteria in the supernatant were measured by bacterial colony count. (C, F) Intracellular bacteria count was performed using gentamicin exclusion assay to kill
extracellular bacteria before cells were lysed to release intracellular bacteria. Results are expressed as mean ± SEM. n = 3-4 samples per group, * p<0.05, ** p<0.01, *** p<0.001 compared to Scr control or as indicated in graph.

Next we sought to determine if the function of miR-328 was specific to this miRNA. We inhibited the function of several miRNA whose expression on the array was either increased or decreased in the lungs of mice following bacterial infection. For this experiment, neutrophils were chosen over macrophages as greater number of neutrophils could be isolated from bone marrow, which allow us to do this extensive testing. Unlike inhibition of miR-328, we found that inhibition of miR-21 (control for no change in expression following bacterial infection), miR-21-3p, miR-146, miR-223 (increased expression following bacterial infection) and miR-376c (decreased expression following bacterial infection) in neutrophils using antagomirs did not produce observable effects on bacterial loads in culture supernatants (Fig. 12) compared to the Scr control group.
Figure 12. Inhibition of various miRNAs on bacterial clearance in vitro. Primary neutrophils isolated from bone marrow were pre-treated with antagomir for 12 hours before infection with NTHi MOI 100 for an hour. Scr was used as control. Number of extracellular bacteria in the supernatant was measured 1 hour post-infection by bacterial colony count. Results are expressed as mean ± SEM. n = 3 samples per group

3.2.2 Inhibition of miR-328 increased phagocytosis of bacteria and antimicrobial activity

To determine if the observed effects of miR-328 inhibition were due to a role of this miRNA in regulating phagocytosis of bacteria, or whether this was due to increased permissibility of phagocytes to active NTHi infection we conducted a phagocytosis assay. Heat-killed NTHi were labelled with CFSE and plated on macrophages or neutrophils in vitro for 1 hour, and uptake assessed both by flow cytometry and fluorescence microscopy. Inhibition of miR-328 function with Ant-328 in macrophages (Fig. 13A) and neutrophils (Fig. 13B) results in an increased CFSE intensity in cells. This indicates that blocking miR-328 function could enhance phagocytosis of heat-killed NTHi as early as 1 hour. This observation was also confirmed using fluorescence microscopy. Ant-328 treatment significantly increased green fluorescence dye (CFSE) in macrophages (Fig. 13C) compared to Scr control suggesting an increased uptake of heat-killed NTHi into the macrophages.
Figure 13. Phagocytosis assay using heat-killed NTHi. Bacteria were labeled with CFSE and heat-killed before being added to macrophages and neutrophils for 1 hour. Intracellular CFSE expression in (A) macrophages and (B) neutrophils was analysed using flow cytometry. (C) Uptake of heat-killed bacteria by macrophages imaged using fluorescence microscopy. DAPI (blue) represents cellular DNA, Rhodamine (red) cell protein, and CFSE (green) heat-killed NTHi. n = 3 samples per group.
Activation of the respiratory oxidative burst is one of the crucial events involved in bacterial killing by the lysosome. To determine whether miR-328 also played a role in activating the killing pathways of the phagocytes, ROS production was assessed in macrophages and neutrophils. Following NTHi infection, macrophages that were pre-treated with Ant-328 showed a significant increase in both the number of cells producing ROS and the intensity of ROS production on a per cell basis when compared to the Scr controls (Fig. 14A). Importantly, ROS was increased by Ant-328 treatment even in the absence of NTHi, although to a much lower extent, suggesting that a proportion of this effect on bacterial killing pathways was independent of phagocytosis. Inhibition of miR-328 in neutrophils produced the same result (Fig. 14B).

To extend our previous observation, we looked at some important events that occurred following phagocytosis. Using confocal microscopy, we showed that pre-treatment of macrophages with Ant-328 increased expression of lysosomal enzyme, Cathepsin D (increased red fluorescence in cytoplasm), following NTHi infection compared to Scr treated macrophages (Fig. 15).

Interestingly, these increased killing activities did not encompass general cell activation as the major pro-inflammatory cytokines, TNF-α (Fig. 16A) and IL-6 (Fig. 16B), were not altered by Ant-328 treatment of macrophages.
Figure 14. Reactive oxygen species production. Primary lung macrophages and neutrophils were pre-treated with Ant-328 for 12 hours before infection with NTHi MOI 100 for 8 hours in macrophages and 1 hour in neutrophils. Dihydroethidium (DHE) and flow cytometry was used to monitor superoxide production in (A) macrophages and (B) neutrophils following NTHi infection. Results are expressed as mean ± SEM. n = 3 samples per group, ** p<0.01, *** p<0.001 compared to Scr control.
Figure 15. Increased expression of cathepsin D in Ant-328 treated macrophages.
Confocal microscopy of cathepsin D expression 8 hours post NTHi infection. DAPI (blue) represents cellular DNA, CFSE (green) represents cell cytoplasm, CTSD (red) represent cathepsin D protein, merged represents overlay of all three images. n = 3 samples per group.
Figure 16. Proinflammatory cytokines production *in vitro*. Primary lung macrophages were pre-treated with Ant-328 for 12 hours before infection with NTHi MOI 100 for 8 hours. Culture supernatant was collected and protein levels of (A) TNF-α and (B) IL-6 was determined by ELISA. Results are expressed as mean ± SEM. n = 3 samples per group, no significance difference in mean value compared to NTHi group.

3.2.3 miR-328 expression was induced by p38 MAPK signalling

To investigate the pathway involved in the regulation of miR-328 expression, we used specific inhibitors to block the activation of a multitude of signalling pathways followed by NTHi treatment. NTHi infection or pre-treatment of macrophages with DMSO vehicle plus NTHi infection led to a significant 2-fold reduction in miR-328 expression (Fig. 17A). Pre-treatment of macrophages with doramapimod (a p38 inhibitor) prior to NTHi infection completely blocked this down-regulation of miR-328 expression (Fig. 17A). Western blot analysis of these cells confirmed that as early as 1 hour after exposure to NTHi the p38 pathway was activated in macrophages as p38 became rapidly phosphorylated (Fig. 17B). Overall this data suggests that NTHi activates the p38 signalling pathway, which regulates miR-328 expression and this in turn regulates bacterial phagocytosis and killing in macrophages and neutrophils *in vitro*.
Figure 17. MiR-328 expression is regulated by p38 MAPK. (A) Primary lung macrophages were pre-treated with 5 µM doramapimod for 30 minutes to inhibit p38 activation prior to NTHi infection for 8 hours. MiR-328 expression was measured using taqman PCR normalized against sno-202 and expressed as fold change compared to PBS control. (B) Western blot analysis of p-38 phosphorylation 1 hour following NTHi infection in lung macrophages, β-actin expression was used as a loading control. Results are expressed as mean ± SEM. n = 3-4 samples per group, ** p < 0.01 as indicated on graphs.

3.2.4 miR-328 regulates phagocytosis by human macrophages and neutrophils

MiR-328 is species conserved and has an identical sequence in mice and humans. Therefore, we investigated if miR-328 plays a similar role in human macrophages and neutrophils infected with NTHi in vitro. Neutrophils were purified from healthy adult blood and macrophages were differentiated in culture from monocytes derived from the
PBMC fraction. Similar to the results observed with murine cells, inhibition of miR-328 significantly increased bacterial uptake by human macrophages (Fig. 18A) and neutrophils (Fig. 18B) *in vitro*.

**Figure 18. Silencing miR-328 increases bacterial uptake in human monocyte-derived macrophages and neutrophils.** Human monocytes and neutrophils were isolated from healthy adult blood and macrophages were differentiated *in vitro*. Cells were pre-treated with Ant-328 or a Scr control. (A) Human monocyte-derived macrophages were infected with NTHi for 8 hours at MOI 100. (B) Human neutrophils were infected with NTHi for 1 h at MOI 10. Intracellular bacteria colony counts were determined using the gentamicin exclusion assay. Results are expressed as mean ± SEM. n= 6 human subjects per group, *** p<0.001 compared to Scr + NTHi.
3.3 Targeting miR-328 function in bacterial clearance in various *in vivo* models

3.3.1 MiR-328 regulates bacterial clearance by macrophages and neutrophils *in vivo*

To assess the role of miR-328 in macrophage- and neutrophil-mediated bacterial clearance in an *in vivo* setting we conducted a series of adoptive transfer experiments. A model scheme for the macrophage transfer experiment is illustrated in Fig. 19A while that for neutrophils is illustrated in Fig. 20A. To perform these studies, we isolated macrophages or neutrophils from naïve mice. Cells were treated *ex vivo* with Ant-328 to inhibit miR-328 function or Scr control for 12 hour before being transferred back into naïve BALB/c mice intratracheally. Mice were then infected with NTHi. To monitor effectiveness of the adoptive transfer, cells were stained with CFSE prior to transfer. It was evident that both CFSE-labelled macrophages (Fig. 19B) and neutrophils (Fig. 20B) successfully entered the lungs and remained there for a period of at least 12 hours. There was no difference in the numbers of these cells between Ant-328 treatments versus Scr control. In these experiments, total bacterial load from both BAL fluid and lung homogenate was measured by bacterial colony counts.

Importantly, mice that received Ant-328 treated macrophages showed significantly improved clearance of NTHi from the lungs compared to Scr control treatment (Fig. 19C). Total cell counts obtained from BAL fluid showed no difference in number (Fig. 19D). Differential cell counts indicate macrophage numbers (Fig. 19E) remained unchanged with neutrophil numbers (Fig. 19F) in BAL fluid being 2-fold lower following NTHi infection.

Similar to the results observed with the macrophages adoptive transfer experiment, mice that received Ant-328 treated neutrophils also had improved bacterial clearance
(Fig. 20C). Total cell counts obtained from BAL fluid showed no difference in numbers (Fig. 20D). Differential cell counts showed that both macrophage (Fig. 20E) and neutrophil (Fig. 20F) numbers remained similar compared to Scr control.

Concentration of pro-inflammatory cytokines, TNF-α and IL-6, in the BAL fluid were equivalent between mice receiving Ant-328 treated cells and Scr control treated cells (macrophages, Fig. 19G and H; neutrophils, Fig. 20G and H).
Figure 19. Inhibition of miR-328 in macrophages ex vivo improves bacterial clearance in the lung after adoptive transfer. (A) Primary macrophages were isolated and miR-328 expression was inhibited with Ant-328 for 12 hours ex vivo.
Control group macrophages were treated with Scr. Macrophages were then adoptively transferred to naïve recipient mice (i.t.) for 24 hours before infection with NTHi (i.t.) for 12 hours. (B) Graph shows the presence of CFSE labelled macrophages in the recipients' lungs following cell transfer compared to the non-treated mice (Naive). (C) Bacterial load in lungs measured by bacterial colony counts. (D) Total cells infiltration following NTHi challenge were counted using Trypan Blue staining of BAL fluid. (E, F) Cellular infiltrates obtained from BAL fluids were cytospun and slides were stained with May-Grunwald. (E) Macrophage and (F) neutrophil numbers were determined by differential cell counts base on morphology of cells. Lung homogenates were tested for (G) TNF-α and (H) IL-6 using ELISA. Results are expressed as mean ± SEM. n = 8 mice per group, ** p<0.01 compared to Scr control group.
A

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B

Naive

Scr

Ant-328

C

**Total CFU / Lung**

Scr + NTHi  Ant-328 + NTHi

D

**Total cells / mL BAL fluid**

Scr + NTHi  Ant-328 + NTHi

E

**Macrophages / mL BAL fluid**

Scr + NTHi  Ant-328 + NTHi

F

**Neutrophils / mL BAL fluid**

Scr + NTHi  Ant-328 + NTHi
Figure 20. Inhibition of miR-328 in neutrophils ex vivo improves bacterial clearance in the lung after adoptive transfer. (A) Primary neutrophils were isolated and miR-328 expression was inhibited with Ant-328 for 12 hours ex vivo. Control group neutrophils were treated with Scr. Neutrophils were then adoptively transferred to naïve recipient mice (i.t.) for 2 hours before infection with NTHi (i.t.) for 6 hours. (B) Graph shows the presence of CFSE labelled neutrophils in the recipients’ lungs following cell transfer compared to the non-treated mice (Naïve). (C) Bacterial load in lungs measured by bacterial colony counts. (D) Total cells infiltration following NTHi challenge were counted using Trypan Blue staining of BAL fluid. (E, F) Cellular infiltrates obtained from BAL fluids were cytopspun and slides were stained with May-Grunwald. (E) Macrophage and (F) neutrophil numbers were determined by differential cell counts base on morphology of cells. Lung homogenates were tested for (G) TNF-α and (H) IL-6 using ELISA. Results are expressed as mean ± SEM. n = 8 mice per group, ** p<0.01 compared to Scr control group.
3.3.2 Inhibition of miR-328 in the lung promotes bacterial clearance

We next sought to determine if inhibition of miR-328 in the lung by application of Ant-328 would be capable of regulating NTHi clearance. Furthermore, this question addresses whether the use of Ant-328 would be a viable option as a treatment to improve bacterial clearance during infection. Naïve BALB/c mice were infected with NTHi, and Ant-328 or Scr control was administered i.t. 6 hours post infection (Fig. 21A). Inhibition of miR-328 function in the lung by Ant-328 was confirmed during NTHi infection using Taqman qPCR (Fig. 21B). Bacterial recovery clearly demonstrated significantly enhanced (4-fold) NTHi clearance in the lung following Ant-328 treatment compared to Scr control (Fig. 21C). Once again total cellular infiltration in the BAL (Fig. 21D) was not significantly altered between Ant-328 and Scr control treatment following NTHi infection. Differential cell counts showed that macrophage numbers were increase by 1.8 fold while no changes observed for neutrophils (Fig. 21E and F). Consistent with previous results Ant-328 treatment did not alter levels of pro-inflammatory cytokines, TNF-α (Fig. 21G) and IL-6 (Fig. 21H) following infection.
Figure 21. Silencing miR-328 improves NTHi clearance in the lung in vivo. (A) Mice were first infected with NTHi for 6 hours before Ant-328 or Scr control were administered to the lung i.t. After 12 hours (B) miR-328 expression was determined using Taqman PCR, normalized against sno-202 and expressed as fold change compared to the Scr control, (C) bacterial load in the lung was measured by bacterial colony counts, (D) Total cells infiltration following NTHi challenge were counted using Trypan Blue staining on BAL fluid. (E, F) Cellular infiltrates obtained from BAL fluids were cytospun and slides were stained with May-Grunwald. (E) Macrophage and (F) neutrophil numbers were determined by differential cell counts base on morphology of cells. Lung homogenates were tested for (G) TNF-α and (H) IL-6 using ELISA. Results are expressed as mean ± SEM. n = 6 mice per group, * p < 0.05, *** p < 0.001 compared to control group
3.3.3 Inhibition of miR-328 overcomes corticosteroid-induced immune suppression to clear bacteria

The mainstay treatment of many respiratory diseases involves the prolonged use of inhaled or oral corticosteroids; however these drugs lead to local immunosuppression and an increased risk of hospitalization for pneumonia, especially in COPD patients (Ernst et al., 2007). Furthermore, treatment with corticosteroids, amongst numerous other effects, suppresses phagocytosis of various bacterial infections in both rodents and humans (Nakamura et al., 1996).

It was next important to determine whether miR-328 production was involved in the dexamethasone-mediated immune-suppression pathway or whether inhibition of miR-328 could overcome dexamethasone suppression of bacterial clearance. Mice were given dexamethasone via the intraperitoneal route each day for 3 days before they were infected with NTHi (Fig. 22A). Dexamethasone treatment does not alter miR-328 expression, suggesting this miRNA was not directly involved in the intracellular signalling pathway regulated by corticosteroid (Fig. 22B). As expected, dexamethasone pre-treated mice infected with NTHi had a substantially increased bacterial load in their lung post-infection (Fig. 22C). Interestingly, the pre-treatment with dexamethasone also increased the inflammatory cell infiltration into the lung, and this is likely due to a compensatory mechanism for the decrease in bacterial clearance following the increased bacterial load and hence recruitment of more inflammatory cells (Fig. 22D).
Figure 22. Systemic dexamethasone treatment impairs bacterial clearance in lung. (A) Mice were treated with dexamethasone (Dex) or vehicle (Veh) control intraperitoneally for 3 consecutive days before they were challenged with NTHi (i.t) for 18 hours. (B) MiR-328 expression in lung was determined using Taqman PCR, normalized against sno-202 and expressed as fold change compared to the vehicle group. (C) Bacterial load in the lung was measured by bacterial colony counts and (D) BAL fluid was collected to enumerate the total cellular infiltrate. Results are expressed as mean ± SEM. n = 7-12 mice per group. ** P < 0.01 compared to vehicle control.
Ant-328 treatment achieved a similar knockdown efficiency of miR-328 level in both dexamethasone and vehicle-treated mice infected with NTHi (Fig. 23A). Inhibition of miR-328 in dexamethasone pre-treated mice reversed the increased bacteria load present in these immune-suppressed mice treated with a Scr control (Fig. 23B). Notably, inhibition of miR-328 in dexamethasone-treated mice restored bacterial levels to below that in non-immune-suppressed mice (vehicle + Scr control) and at equivalent levels to non-immune suppressed mice treated with Ant-328 (vehicle + Ant-328). This suggests that inhibition of miR-328 is just as effective at clearing bacteria in an immune suppressed environment (Fig. 23B). Once again the number of infiltrating inflammatory cells in the BAL was only increased by dexamethasone treatment (similar to Fig. 22D) and not significantly affected by Ant-328 treatment (Fig. 23C). Differential cell counts show no difference in recruitment of macrophages (Fig. 23D) and neutrophils (Fig. 23E) into the airways following Ant-328 treatment.
Figure 23. Effect of miR-328 on bacterial clearance in dexamethasome immune suppressed mice. Mice were treated with dexamethasone (Dex) or vehicle (Veh) control intraperitoneally for 3 consecutive days before they were challenged with NTHi (i.t) for 18 hours. Mice were treated with Ant-328 or Scr control (Scr) 6 hours post NTHi challenge. (A) MiR-328 expression in lung was determined using Taqman PCR, normalized against sno-202 and expressed as fold change compared to the Scr control. (B) Bacterial load in the lung measured by bacterial colony counts and (C) BAL fluid was collected to enumerate the total cellular infiltrate. (D) Macrophage and (E) neutrophil numbers were determined by differential cell counts base on morphology
of cells. Results are expressed as mean ± SEM. n = 7-12 mice per group. ** P < 0.01 compared to vehicle control.

3.3.4 Inhibition of miR-328 promotes bacterial clearance in a model of cigarette smoke-induced emphysema

NTHi is one of the common bacteria isolated from airways of both stable and exacerbated COPD patients (Monso et al., 1995), we next sought to investigate if miR-328 can improve bacterial clearance in a COPD model. Mice that were exposed to cigarette smoke for 8 weeks were infected with NTHi followed by antagomir treatment (Fig. 24A). Knockdown of miR-328 expression using Ant-328 was validated in this model (Fig. 24B). Similar to the results observed before, treatment with Ant-328 during NTHi infection enhances NTHi clearance in a cigarette exposed mice (Fig. 24C). The total cellular infiltrates remain the same between Ant-328 and Scr (Fig. 24D). Differential cell counts indicate no difference in macrophage (Fig. 24E) or neutrophil (Fig. 24F) numbers following Ant-328 treatment.

In the same model, pulmonary function was measured using the flexivent system. We found that inhibition of miR-328 results in a decrease in pulmonary compliance (Fig. 24G) and an increase in pulmonary elastance (Fig. 24H). Inhibition of miR-328 also produced a near complete ablation of the increase in muc-5ac expression induced by smoking in combination with NTHi infection (Fig. 24I).
Figure 24. MiR-328 promotes bacterial clearance in cigarette smoke-exposed mice. (A) BALB/c mice were exposed to cigarette smoke (CS) daily for a period of 8 weeks. At the completion of the model, mice were challenged with NTHi for 6 hours before they were treated with Ant-328 or Scr control. (B) MiR-328 expression in lungs was determined using Taqman PCR, normalised to sno-202 and expressed as fold change compared to air control. (C) Total bacteria in lung 18 hours post infection was measured by colony count. (D) BAL fluid was collected to measure total cell numbers. (E) Macrophage and (F) neutrophil numbers were determined by differential cell counts base on morphology of cells. (G) Compliance and (H) elastance were measured using the flexivent system. (I) Expression of muc-5ac in lung tissue by quantitative PCR, normalised using HPRT as a housekeeping control and expressed as fold change over the NTHi + vehicle control. Results are expressed as mean ± SEM (* p<0.05, **p<0.01 compared to smoking + NTHi + Scr).
Chapter 4 Discussion

4.1 General discussion

miRNAs are known to be involved in regulating innate immune responses. They are induced by various stimuli such as TLR pathways (Taganov et al., 2006, Liu et al., 2009) or pro-inflammatory cytokines (Tili et al., 2007, O'Connell et al., 2007) and they can act as both positive and negative feedback signals to control these pathways. Furthermore, host pathogen interactions and activation of the immune system has been shown to alter, and be altered by, miRNA expression in various infection models (Liu et al., 2012, Schulte et al., 2011, Xiao et al., 2009, Izar et al., 2012, Cremer et al., 2009). However, it remains unknown how miRNA may regulate respiratory bacterial infections or the direct phagocytosis and killing of microbes. In this study we examine the role of miRNA in the host defence response to NTHi infection. We show that within 24 hours of NTHi infection the levels of miRNA in the lung are rapidly altered with expression of individual miRNAs being both increased and decreased. Alterations in the level of expression are strongly associated with bacterial clearance and recruitment of innate immune cells into the lung. Notably, miR-328 was significantly down-regulated by infection and had the highest level of basal expression of all down-regulated miRNA suggesting a key role in the host response to NTHi infection.

miR-328 is involved in cancer (Arora et al., 2011, Pan et al., 2009, Eiring et al., 2010), autoimmunity (Padgett et al., 2009), and neuronal disease (Boissonneault et al., 2009). Here we demonstrate a new role for miR-328 in regulating innate immune cell function, where inhibition of miR-328 function improves bacterial clearance in the lung. Inhibition of this miRNA in either macrophages or neutrophils increases uptake (of live or heat-killed NTHi) and decreases survival of bacteria, showing that reducing miR-328 expression improves phagocytosis of bacteria. Bacterial clearance is dependent on ROS production (Rada et al., 2004). In chronic granulomatous disease phagocytic
activity is normal, however, there is an inability of phagocytes to form ROS after phagocytosis that leads to increased bacterial survival (Quie et al., 1967) suggesting that phagocytosis alone is not sufficient for bacterial killing. Here we show increased production of ROS in Ant-328 treated phagocytes, indicating that bacterial killing mechanisms have also been enhanced. Oxygen-independent bacterial killing mechanisms (such as Cathepsin D) also operate following phagocytosis. Cathepsin D is a lysosomal cationic protease enzyme that is not only bactericidal, most importantly they also increase susceptibility of gram-negative bacteria to lysis by lysozyme (Thorne et al., 1976). Knocking down of this enzyme has results in delay clearance of pulmonary bacterial infection both in vivo and in vitro (Bewley et al., 2011, del Cerro-Vadillo et al., 2006). We observed increased Cathepsin D expression in Ant-328 treated macrophages suggesting that bacterial killing was also enhanced using an oxygen independent pathway. Thus miR-328 plays a critical role in regulating antimicrobial function of innate immune cells by augmenting phagocytosis, the production of ROS and microbicidal activity.

In cancer, miR-328 expression is regulated through the ERK1/2 pathway (Eiring et al., 2010). Here we found that p38 MAPK is activated by NTHi infection, and inhibition of p38 activation prior to bacterial infection, prevents the down-regulation of miR-328. This suggests that NTHi suppresses miR-328 expression through p38 MAPK-dependent pathways. These experiments were performed in vitro with macrophages. Thus, it is possible that miR-328 could be regulated by other pathways in a more complex in vivo environment.

Adoptive transfer of miR-328-deficient macrophages or neutrophils increased bacterial clearance in the lung, further supporting our in vitro observations and directly demonstrating that inhibition of miR-328 in these cells amplifies their ability to clear respiratory infections. In mice that received miR-328 deficient macrophages, neutrophil
counts in BAL were reduced. A study using Gram-negative bacteria showed that depleting alveolar macrophages increased bacteria in lung and neutrophils recruitment (Broug-Holub et al., 1997). This study demonstrated important roles of macrophages in bacterial clearance and failure of this process results in increased neutrophil recruitment to the lung (Broug-Holub et al., 1997). In our study, it is likely that bacteria have been cleared effectively by transfer of miR-328 deficient macrophages, hence less neutrophils are recruited for further clearance. We next explored the potential of Ant-328 as a therapeutic treatment for bacterial lung infection by directly administering the inhibitor to the lung after NTHi inoculation. Inhibition of miR-328 with the Ant-328 substantially increased bacterial clearance suggesting that targeting this miRNA could potentially be used as a new approach to anti-microbial therapy. Although we detected increased ROS production in vitro following Ant-328 treatment of infection, which is often linked to lung injury and activation of pro-inflammatory pathways, we did not observe any increase in inflammation as measured by cellular infiltration and pro-inflammatory cytokines production in the lung. One potential explanation is that the increased ROS production that occurs with more rapid bacterial clearance following Ant-328 treatment does not reach a threshold level or sustained level of production which is required to elicit deleterious inflammatory changes in the lung. As long as the ROS scavenging system is not impaired, ROS in the lung will be neutralised by intrinsic antioxidants when the infection is cleared (Roos et al., 1980). Thus, importantly, treatment with Ant-328 in vivo enhanced bacterial clearance without a consequent negative impact on lung inflammation (cellular recruitment or proinflammatory mediator production) or pathology.

In chronic lung disease, corticosteroid treatment can lead to immunosuppression and increase the risk of pneumonia (Ernst et al., 2007). In our current study, as anticipated, pre-treatment with dexamethasone prior to NTHi infection significantly impaired bacterial clearance despite an increase in cellular infiltration in the lung. Previous
studies have shown that dexamethasone inhibits macrophage phagocytosis in vitro while treatment in vivo suppresses clearance of bacteria (Becker and Grasso, 1985, Nakamura et al., 1996). Importantly, dexamethasone administration did not alter miR-328 expression despite the increased bacterial load in the lung. Notably, Ant-328 treatment of dexamethasone immune-suppressed mice brought about a dramatic reduction in bacterial load, to levels below that seen in immune competent mice. This data demonstrates that although dexamethasone treatment inhibits phagocytosis along with other inflammatory anti-bacterial pathways, this effect is overcome by treatment with Ant-328. These results suggest that it may be possible to use Ant-328 in conjunction with dexamethasone in the treatments of chronic lung diseases in order to better control bacterial infection. This would be of particular interest in the case of antibiotic resistant bacterial strains where conventional therapies tend to fail and in patients receiving steroid therapy where infections are difficult to control.

Bacterial colonisation and infection are commonly associated with COPD and exacerbation of disease, and NTHi is one of the most frequently isolated bacteria (Monso et al., 1995). The reason underlying this may be that, alveolar macrophages from COPD patients exposed to cigarette smoke extract are less efficient at phagocytosing NTHi (Berenson et al., 2006, Marti-Lliteras et al., 2009). Using a cigarette smoked-induced mice model of experimental COPD (Beckett et al., 2013), we demonstrated that inhibiting miR-328 brought about a 3-fold increase in bacterial clearance. A definitive characteristic of COPD is the loss of elastic recoil in the lung and increased lower airway remodelling including mucous cell hyperplasia (Baldi et al., 2001). Following infection with NTHi, treatment with Ant-328 significantly improved both elastic recoil in the lung and suppressed muc-5ac expression in the lung of chronically cigarette exposed mice. The exact mechanism of how this occurs remains to be elucidated but it may involve altered surface tension in lung by impairing mucus production (Ingenito et al., 2005). In a clinical study on acute exacerbation of COPD,
half of the sputum obtained from the patients was tested positive for bacterial growth. Approximately 50% of those strains isolated were *H. influenzae* and *Moraxella catarrhalis* and up to 90% of all isolates were resistant to penicillin (Larsen et al., 2009). Our study identifies a potential alternative treatment for bacterial exacerbations of chronic lung disease. The use of Ant-328 to enhance bacterial killing is further supported by the observation that it occurs without any additional inflammation that could lead to lung injury.

MiR-328 has a highly conserved sequence between mice and human. Inhibition of miR-328 function in human macrophages and neutrophils using Ant-328 again produced increased bacterial phagocytosis, indicating that our studies are potentially translatable into anti-microbial innate host defence pathways in human cells.

Our study identifies a potential alternative approach to the treatment of pathogenic microbial infections and bacterial induced exacerbation of chronic lung disease. Importantly, Ant-328 enhances bacterial killing by specifically augmenting bacterial clearance pathways without further promoting a proinflammatory environment that may be deleterious to lung tissue. Targeting miRNA would be of particular interest to enhance therapeutic outcomes in patients suffering from disorders such as COPD, cystic fibrosis and asthma where innate host defences may be compromised due to steroid therapy or underlying disease mechanisms. Innate immune defects also occur in HIV and transplantation patients. In HIV patients, phagocytosis and respiratory burst are both reduced in monocytes and neutrophils (Michailidis et al., 2012). After solid organ transplantation ~50% deaths are due to infection and during the first four months following bone marrow transplantation, phagocytosis by alveolar macrophage is impaired (Corensek et al., 1988, Winston et al., 1982). Although speculative, targeting miR-328 may be more broadly applicable and lead to improvement of innate immune cell function in these immune-deficient patients.
In summary, we have identified that miR-328 is important in modulating innate immune cell function. Inhibition of miR-328 enhanced bacterial clearance through enhanced phagocytosis and bacterial killing in macrophages and neutrophils. This study is the first to identify such role for miRNAs in regulating bacterial infection in the lung and provides proof-of-principle data that targeting specific host miRNAs could lead to future therapeutics to combat multi-drug resistant bacteria and infection in chronic lung diseases and in immune-compromised patients.
4.2 Future direction

Despite the interesting role of miR-328 in regulating anti-bacterial responses as discussed previously in the thesis there are a number of questions that remain unanswered.

1. **Is there any other miRNAs that are having a similar function to miR-328?**

In our studies we have identified at least 72 miRNAs (from miRNA microarray) that were either up or down regulated during NTHi infection. Some of these miRNAs such as miR-146, miR-21, miR-223, or let-7 family are known to regulate inflammatory responses by either targeting inflammatory cytokines or TLR signalling pathways (Taganov et al., 2006, Sheedy et al., 2010, Johnnidis et al., 2008, Schulte et al., 2011). The functional roles of other miRNAs, however, remain largely uncharacterised. MiRNAs are known to bind to their mRNA targets through several mechanisms (as discussed in section 1.5.7), therefore it would be possible that a few miRNAs are acting in concert to suppress protein translation of the same targets. In cancers, Wu et al., 2010 has experimentally validated a set of miRNAs that can inhibit p21Cip1/Waf1 expression post-transcriptionally to promote oncogenesis (Wu et al., 2010). Conversely, miRNAs could suppress several different targets, which operate in the same functional pathway. Mestdagh et al., 2010 showed in cancer diseases, miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a promote oncogenesis by inhibiting multiple key effectors of TGF-β signalling, resulting in increased proliferation and decreased cell adhesion (Mestdagh et al., 2010). Therefore apart from miR-328, it would be interesting to determine if other differentially expressed miRNAs in our models could also play a role in regulating bacterial clearance.
2. What are the targets for miR-328?

Finding the targets for miR-328 will allow us to answer 2 important questions:

(i) Which genes in the phagocytic pathway are involved in the increased uptake of bacteria caused by Ant-328?

(ii) Would there be any off-target or detrimental effect for inhibiting miR-328?

Several factors could contribute to the increased phagocytosis by Ant-328 including increased in: phagocytic receptors expression, opsonin production, or actin cytoskeleton rearrangement (Flannagan et al., 2012). There are hundred or more molecules that could be involved in these processes, making it difficult to identify the targets. Similarly it would be hard to determine off-target effects without first identifying the targets miR-328 acts upon. Therefore, the next important steps in this study are to identify targets for miR-328 by using biochemical approaches in combination with computational methods discussed in section 1.5.7.1 and 1.5.7.2. This targeting study would help to identify specific miRNA target involved in anti-bacterial responses and also determine potential off-target effects.

3. Does miR-328 expression change in human diseases for instance in COPD or immunocompromised patients?

Several human diseases have been associated with increased bacterial infection as a result of impaired immunity. For example, it was noticed that human alveolar macrophages isolated from COPD patients have reduced phagocytic activity (Berenson et al., 2006). Likewise glucocorticoid treatment suppresses phagocytosis of bacteria in human alveolar macrophages (Zetterlund et al., 1998). To date little is known about the miRNAs expression in such diseases. More importantly, it is unclear if miRNAs could have a role in regulating the immune response in such diseases. It would be interesting
to determine if the level of miR-328 expression in alveolar macrophages or neutrophils isolated from COPD patients or those of steroid therapy was dysregulated to further understand the mechanism involved in pathogenesis of these diseases.

4. Potential off-target effect of ant-328?

In our current studies, we have not determined the off-target effect of ant-328. The complementary sequence used to target mature miR-328 sequence could be incorporated into the miRISC followed by binding to its target mRNA. This possible off-target effect of ant-328 could also regulate gene expression through an alternative mechanism that could be independent of blocking miR-328 function. Alternatively, in a less likely scenario, could the ant-328 sequence be taken up by bacteria itself and has an inhibitory effect on the bacteria growth itself. More studies would be required to fully understand the exact mechanism on how ant-328 function.
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