

Modulation of responses in allergic airways disease by
***Haemophilus influenzae* infection**

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Synopsis

Asthma is a common chronic inflammatory disease of the airways that affects over 2.2 million people in Australia. Asthma is a heterogeneous inflammatory disease typically characterised by T helper lymphocyte type 2 (Th2)-mediated eosinophilic inflammation, exaggerated responses to innocuous stimuli, mucus hypersecretion leading to airways obstruction and airway remodelling. These physiological changes result in wheezing, chest tightness, and breathing difficulties. However, it has been established that eosinophilic inflammation is only present in 50% of asthmatic patients. Around 30% of non-eosinophilic asthmatics have neutrophilic rather than eosinophilic inflammation, which is a key feature of neutrophilic asthma.

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative bacterium that is commonly found in the upper respiratory tract of about 75% of healthy individuals. It is normally asymptotically carried in people, however it may cause otitis media and is a common cause of community-acquired pneumonia. NTHi has also been linked to a number of chronic airway diseases. It has been detected in patients with bronchiectasis, chronic bronchitis and is commonly associated with chronic obstructive pulmonary disease (COPD) exacerbations. It has also recently been associated with neutrophilic asthma, however, the role of NTHi in neutrophilic asthma has not been investigated.

Using murine models of NTHi infection and allergic airways disease (AAD), we investigated the relationship between infection and AAD. We showed that NTHi infection induced features of neutrophilic asthma; reduced Th2-mediated eosinophilic inflammation, reduced airways hyper-responsiveness (AHR) compared to eosinophilic AAD, and importantly, significantly increased Th17 responses and neutrophilic inflammation. In the first study it was demonstrated that the combination of infection and AAD reduced the

expression of MHC II and CD86 on dendritic cells (DCs), suggesting that infection induced changes in presentation of antigen to naïve T-cells and subsequent adaptive responses. Infection also induced Interleukin (IL)-17 production from innate cells and Th17 cells. Critically, we show that inhibiting IL-17 significantly reduced neutrophilic inflammation in the airways. This highlights the crucial role of IL-17 in infection-induced neutrophilic AAD.

The second study showed that the induction of AAD during infection delayed bacterial clearance from the lungs compared to infection alone controls. In contrast to Th2-mediated eosinophilic inflammation, this model of infection-induced neutrophilic AAD was resistant to dexamethasone treatment. All features of infection-induced neutrophilic AAD, including eosinophil and neutrophil influx, antigen-specific IL-5, IL-13 and Interferon (IFN)- γ , NTHi-specific IL-17, and AHR were unchanged with steroid treatment. This study also demonstrated that neutrophil and macrophage activation and function was inhibited in neutrophilic AAD. This lack of innate immune response may enable chronic bacterial infection.

The final study investigated clarithromycin, a macrolide, and combination therapy with dexamethasone, as possible treatment strategies for neutrophilic asthmatics. This study demonstrated that clarithromycin alone significantly reduced neutrophil influx and IL-17 responses, but increased Th2-mediated eosinophilic inflammation. However, the combination of clarithromycin and dexamethasone suppressed all key features of AAD, including eosinophilic and neutrophilic inflammation, ovalbumin (OVA)-specific IL-5, IL-13, and IFN- γ , NTHi-induced IL-17, and AHR.

These novel findings further the understanding of the potential role of NTHi in the development of neutrophilic asthma. We have identified some mechanisms of how infection

may lead to features observed in neutrophilic asthma, and importantly, possible treatment strategies for neutrophilic asthmatics, and perhaps, other neutrophilic airway diseases with evidence of infection.

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Abbreviations

AAD	Allergic airways disease	MHC	Major Histocompatibility Complex
AHR	Airways hyper-responsiveness	MLN	Mediastinal lymph node
APC	Antigen presenting cell	MMP-9	Matrix metalloproteinase-9
BALF	Bronchoalveolar lavage fluid	MSC	Mucus secreting cell
COPD	Chronic obstructive pulmonary disease	MyD88	Myeloid Differentiation factor 88
DC	Dendritic cell	NE	Neutrophil elastase
ECP	Eosinophil cationic protein	NF-κB	Nuclear Factor κ B
FCS	Foetal calf serum	NK	Natural Killer
FEV₁	Forced expiratory volume	NTHi	Non-typeable <i>Haemophilus influenzae</i>
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor	OVA	Ovalbumin
HBSS	Hanks buffered salt solution	PAMP	Pathogen-associated molecular patterns
IFN	Interferon	PBS	Phosphate buffered saline
Ig	Immunoglobulin	pDC	Plasmacytoid dendritic cell
IL	Interleukin	PMN	Polymorphonuclear cell
IN	Intranasal	PRR	Pattern recognition receptor
IP	Intraperitoneal	RBC	Red blood cell
KC	Keratinocyte chemokine	SEM	Standard error of the mean
LPS	Lipopolysaccharide	TCR	T-cell receptor
mDC	Myeloid dendritic cell		

TGF Transforming growth factor
Th T helper lymphocyte
Th1 Type 1 helper T lymphocyte
Th2 Type 2 helper T lymphocyte
Th17 Type 17 helper T lymphocyte
TLR Toll-like receptor
TNF Tumour necrosis factor
Treg T regulatory cell

Chapter 1:

Introduction

This chapter reviews the literature on the pathophysiology and immune responses associated with *Haemophilus influenzae* infection and asthma, and the link between infection and neutrophilic asthma.

1.1 Asthma

Australia has one of the highest rates of asthma prevalence in the world, with 10-12% adults and 10-15% of children affected. It is the only respiratory disease that is identified as a National Priority Health Area, and in 2000-2001 the Australian government spent approximately \$693 million on asthma (1). Despite advances and improvements in asthma diagnosis and therapy, it is estimated that worldwide there are about 300 million people with asthma. Studies indicate that the incidence of asthma has increased as communities have become more urbanised, and embraced western lifestyles (2).

Asthma is a chronic disease of the airways that is characterised by airway hyperresponsiveness (AHR), reversible airway obstruction, mucus overproduction, and airway remodelling, all of which lead to the clinical symptoms of wheeze, cough, breathlessness, and chest tightness (3-6).

1.1.1 Asthma pathophysiology

AHR is an exaggerated response of the airways to exposure to a non-specific stimulus, such as an allergen, exercise, cold air, pollution or an infection, that causes spasmodic bronchoconstriction (7-9). AHR is also an important risk factor for asthma, where asthmatics with more severe AHR have more symptoms and a greater decline in FEV₁ or lung function (8). Several mechanisms that underpin the development of AHR have been proposed, such as decreases in lung elasticity, changes in airway smooth muscle (8, 10) and increased eosinophilic inflammation of the airways. Interleukin (IL)-5 is a pivotal cytokine that is required for eosinophilic inflammation. Animal models have been used to demonstrate that when exposed to allergens, IL-5^{-/-} mice did not develop AHR (11), and

adoptive transfer of eosinophils to the lungs of these mice induced the development of AHR (12).

Mucus hypersecretion by mucus secreting cells (MSCs) in the airways plays an important role in the development of airway obstruction. Mucus production is an innate defence mechanism that facilitates the removal of inhaled foreign particles. However hypertrophy of submucosal glands, MSC hyperplasia, and increases in plasma exudation from the bronchial microvasculature lead to the excessive production of mucus. These factors lead to airway obstruction, and changes in airway responsiveness (13, 14). The three components of the mucociliary apparatus; cilia, a protective mucus layer, and the airway surface liquid (ASL) layer, also work together to protect the lungs from inhaled particles. Mechanisms for the dysfunction of this system are still not fully understood, however studies suggest that defects in cilia structure/function, and ASL dehydration may contribute to the dysfunction (15). A combination of reduced mucociliary clearance and mucus hypersecretion leads to mucostasis and the formation of mucus layers around the airway that may cause plugging and obstruction (16). IL-4 and IL-13, both of which are increased in asthmatic airways and signal through the IL-4R, have been implicated in mucus hypersecretion. Studies in mice have demonstrated that IL-4 administration up-regulates mucin gene expression (17), and IL-13 deficient mice have reduced mucus cell hyperplasia after allergen sensitisation (18).

Airway remodelling is characterised by subepithelial fibrosis and epithelial hypertrophy. It is defined as a change in mass, size or complexity of tissue, and is a normal transient phenomenon that occurs during growth and development of the airways and in response to injury or inflammation. However, in asthma it may become chronic, leading to collagen deposition, abnormal airways function, and MSC hyperplasia (19).

Airway remodelling is induced by chronic inflammation, however, the mechanisms that underpin the persistence of inflammation that causes abnormal remodelling responses are not yet known. It has been suggested that repeated allergen exposure, infection, a defective repair system, or a genetically abnormal inflammatory response may be responsible (20-22).

1.2 The inflammatory response in asthma

In healthy airways, exposure to inhaled allergens does not result in an inflammatory response. The respiratory tract has various protective features, such as the mucus layer, and intercellular tight junctions to limit antigen access to the immune system. Antigens that are able to penetrate these barriers are processed by mucosal DCs that initiate the differentiation of T regulatory cells (Tregs), that suppress the differentiation of effector T-cells and induce immune tolerance (9). However in asthma, the damaged airway epithelium allows allergens to constantly activate DCs that are involved in Th2 differentiation. This promotes pro-inflammatory mediator release which causes more epithelial damage and persistent inflammation (Figure 1.1) (9).

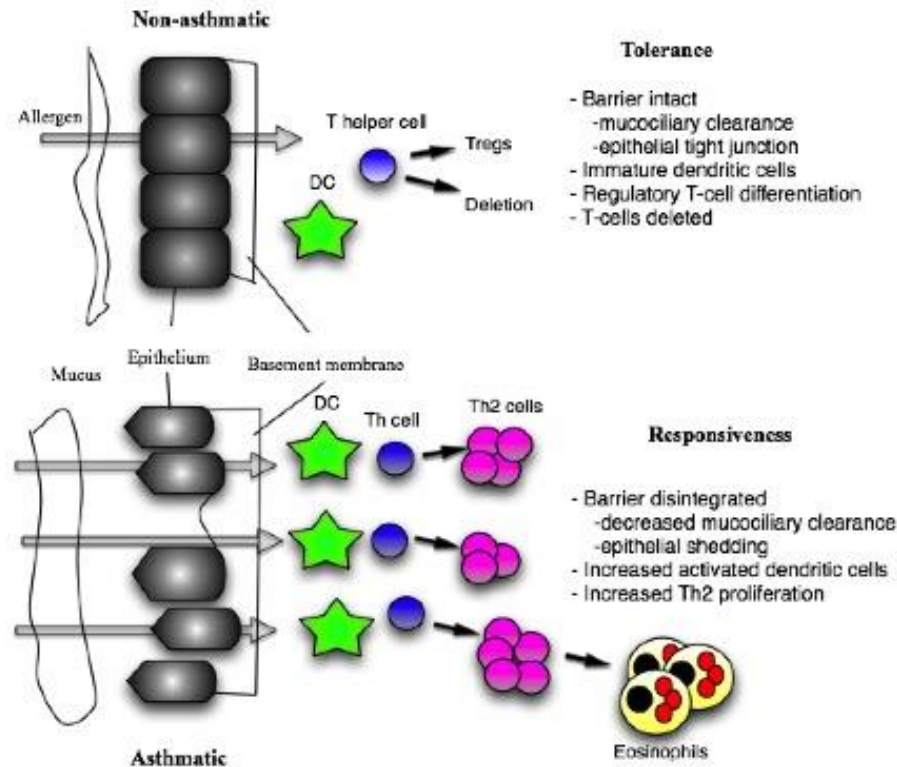


Figure 1.1: Schematic of the inflammatory response in asthma. In non-asthmatic lungs, antigens in the airway are detected by DCs that engulf, process, and present them to naïve T-cells. This presentation induces Treg development and subsequent tolerance. In the asthmatic lung, the damaged epithelium continuously allows allergens through, where they are processed by mature and activated DCs, which induce potent Th2 responses that recruit eosinophils to the airways. (Adapted from Cohn *et. al.*, 2004) (9).

1.2.1 Immunity

The immune system is divided into two arms; innate immunity, which is the first to detect the presence of foreign antigens, and adaptive immunity, which develops a memory response to antigen exposure, and is initiated 3-5 days after innate immune activation. Both work synergistically to protect against invading pathogens and ensure the survival and well-being of the host (23). Innate immune defences are initiated by germ line-encoded receptors that recognise common microbial components. Microorganisms have highly conserved cell wall motifs and membrane proteins termed pathogen-associated molecular patterns (PAMPs). Although distinct in structure, PAMPs have similarities, are often important in the survival of the microbe, and are involved in pathogenicity (23, 24).

PAMPs are recognised by cellular receptors expressed by the host, termed pattern recognition receptors (PRRs). Toll-like receptors (TLRs), which are a group of transmembrane receptors, are one such group of PRRs (25). TLRs play an important role in signalling the presence of infection, and constitute a family of type I transmembrane receptors. They have an extracellular leucine-rich repeat (LRR) domain, which mediates ligand-binding specificity, and an intracellular Toll/interleukin IL-1 receptor (TIR) domain that initiates intracellular signalling pathways (26). Thus far, 10 TLRs have been identified in humans and they recognise a range of PAMPs from bacteria, viruses, protozoa and fungi, however TLRs 2 and 4 are the most important in recognising bacterial components. TLRs are expressed on numerous immune cells including epithelial cells, macrophages, neutrophils, natural killer (NK) cells and DCs (27, 28).

1.2.2 Th cell response

T lymphocytes or T-cells originate from hematopoietic stem cells in the bone marrow that migrate to the thymus where they divide into immature thymocytes and eventually mature into naive T-cells. To mount a normal immune response, T-cells require two signals to be activated. The first is the binding of the T-cell receptor to a complex of antigen-peptide bound to a major histocompatibility-complex (MHC) molecule that is presented by antigen presenting cells (APCs). The second is a co-stimulatory signal, which often originates from CD80 and CD86 molecules located on the surface of APCs. The expression of CD80 and CD86 is induced by PAMP-activated PRRs, and once induced, CD80/CD86 bind to CTLA-4 and CD28 on the T-cell, resulting in complete activation. Self antigens do not induce the expression of CD80 or CD86 as they are not recognised by innate immune receptors and this provides a mechanism of T-cell activation only in the presence of infection-related antigens (23).

T-cells are divided into subclasses based on function and phenotype. CD4⁺ T-cells or Th cells recognise antigen-MHC II complexes, and are usually involved in the clearance of extracellular pathogens. CD4⁺ T-cells produce cytokines, including IL-4, IL-6, IL-9, IL-10, IL-17 and transforming growth factor (TGF)- β that direct antibody production in B cells (29). CD8⁺ T cells, or cytotoxic T cells, recognise antigens linked to MHC I, and are involved in the clearance of intracellular viral infections. CD8⁺ T-cells release cytokines such as IFN- γ and tumour necrosis factor (TNF)- α which have anti-viral properties (30).

Th cells and their cytokines are a crucial part of the immune response in asthma. Th cells may differentiate into either Th1, Th2, Th17 or Treg cells. There are a number of determinants for the differentiation of these Th cell types including the nature of antigenic

stimulus, genetic background, specific co-stimulatory signals during T-cell activation and importantly, the local cytokine environment (Figure 1.2).

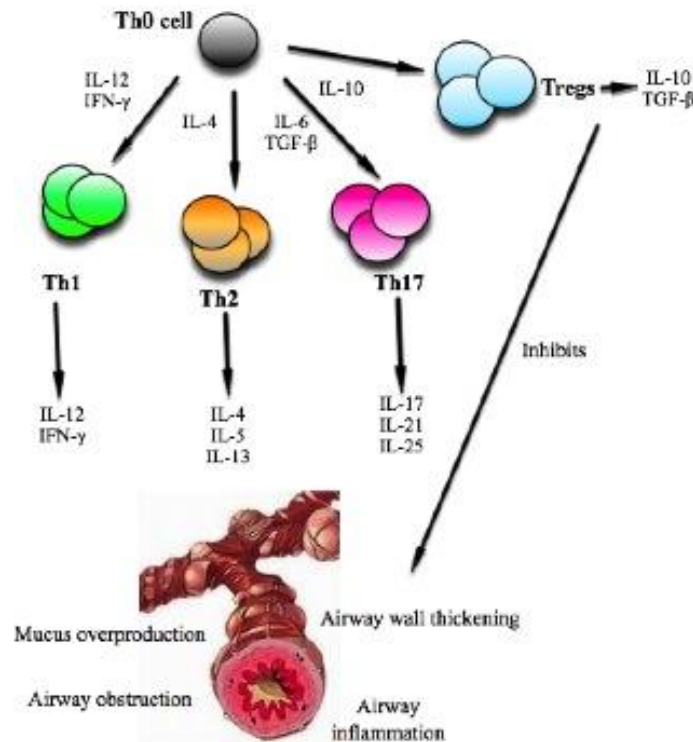


Figure 1.2: T-cell differentiation. The local cytokine environment determines the fate of T helper precursor cells (Th0). The development of Th1, Th2 and Th17 cells results in the release of cytokines that are involved in the development of the hallmark features of asthma. Th1 and Th2 cells are also able to regulate each other by releasing IFN- γ (suppresses Th2 cells) or IL-4 (suppresses Th1 cells). The development of Tregs results in immune tolerance and the suppression of features of asthma.

1.2.2.1 Th1 cells

In the presence of IL-12, naïve T-cells differentiate into CD4⁺ Th type 1 (Th1) cells, which release characteristic Th1 cytokines (31, 32). The role of Th1 cytokines in asthma is the subject of intense debate and is not yet completely understood. Of particular interest are IFN- γ , IL-12 and IL-18. Several studies have shown that IFN- γ inhibits AHR, mucus production and Th2-induced eosinophilia (33-35). However, in contrast, other studies have shown that IFN- γ and Th1 cells augment AHR and airway inflammatory responses (36-38). IL-12 is produced by APCs, and has an essential role in the differentiation of Th1 cells. Animal models have been used to demonstrate that administration of IL-12 suppresses Th2 development, antigen-induced AHR, and inflammation (39, 40). IL-18, which is secreted by macrophages, has been described as an IFN- γ releasing factor. More recently it has been shown that IL-18 collaborates with IL-12 to induce the release of IFN- γ and inhibit immunoglobulin (Ig) E synthesis as well as allergen-induced AHR (41).

1.2.2.2 Th2 cells

It is well established that CD4⁺ Th2 cells and the cytokines that they release promote immune responses that lead to asthma. Bronchoalveolar lavage (BAL) fluid and airway biopsies from asthmatic lungs have increased IL-4 and IL-5 mRNA (42), and IL-13 levels (43). More recently, there has been evidence to show a role for IL-33 and IL-25 in promoting IL-4 independent Th2-mediated responses in the asthmatic lung (44).

Interleukin-4 is involved in the differentiation of naïve T-cells to Th2 cells, and is required to induce a Th2 response (45, 46). IL-4 is also involved in the stimulation of mucus-producing cells and fibroblasts, linking it to remodelling of the airways, while inhalation of recombinant IL-4 induces airway eosinophilia, and AHR in asthmatics (47).

In allergic animal models, IL-4 is involved in eosinophil influx into the lungs and increases AHR (48). More recently, IL-33, a member of the IL-1 cytokine family, has been shown to be important in the induction of Th2 responses, and to be elevated in asthmatic patients (27, 65). IL-33, produced mainly by epithelial and endothelial cells, when cultured with naïve T-cells and dendritic cells, enhances the induction of an IL-5⁺ CD4⁺ T-cell/Th2 cell population (49). IL-33 also binds to other cells such as mast cells and basophils, and induces these cells to release chemoattractants for Th2 cells (49).

IL-25, a member of the IL-17 cytokine family, is also involved in the initiation of Th2 responses. Eosinophils and basophils are the main producers of IL-25, but it is also released by Th2 cells, mast cells and keratinocytes upon activation (50). Like IL-33, IL-25 expression is increased in asthmatic airways (51). Animal models have shown that IL-25 signals in a STAT6 (a crucial Th2 transcription factor) and CD4⁺ T-cell dependent-manner to enhance eosinophil recruitment and antigen-induced Th2 cytokine production (52). Studies have also shown that IL-25 enhances Th2 polarisation and expansion and is involved in the maintenance of Th2 activation (50).

IL-5 mediates eosinophil development, differentiation and activation and is one of the primary eosinophil chemoattractants to the airways (53). IL-5 is primarily produced by T-cells, but is also produced by basophils, mast cells and eosinophils (53). Eosinophils have been shown to be an integral factor in allergic inflammation. IL-5 deficient mice have reduced levels of circulating eosinophils, and during infection, these mice are unable to mount a normal eosinophilic response (54). Eotaxin functions as a potent chemoattractant, in concert with IL-5, to regulate the recruitment of tissue eosinophils (55, 56). Eotaxin is part of the CC chemokine subgroup, and this group targets cells such as eosinophils, macrophages, basophils and monocytes (57). Eotaxin regulates the

migration of progenitor and mature eosinophils from the bone marrow, whereas IL-5 is involved in the efflux of differentiated cells, and the induction of peripheral eosinophilia (58). Following the recruitment of eosinophils, eotaxins are also involved in their degranulation and the release of mediators such as major basic protein (MBP), which has cytotoxic effects on the airway epithelium and acts on smooth muscle, potentially contributing to the development of AHR (59).

IL-13 is up-regulated in the asthmatic airway, and induces mucus hypersecretion, by regulating MSC metaplasia and the release of mucin from storage granules in MSCs (60). IL-13 is predominantly produced by CD4⁺ and CD8⁺ T cells, and is also secreted by mast cells, basophils and eosinophils (60). It promotes IgE synthesis, attracts eosinophils, regulates airway smooth muscle (ASM) function by increasing histamine and acetylcholine-induced calcium influxes into the ASM, and induces AHR (47). IL-13 is also involved in regulating inflammation of the airways. Animal studies have demonstrated that the administration of rIL-13 causes inflammatory cell infiltration (including macrophages, eosinophils and lymphocytes) to the airways (61), by promoting the expression of genes that induce the recruitment, homing and activation of these cells (60).

1.2.2.3 Th17 cells

Th17 cells are a more recently discovered cell type and differentiate from naïve T-cells in the presence of IL-6 and TGF- β (62-64). Th17 cells secrete cytokines in the IL-17 family (IL-17A-F), and IL-21 (65-67). IL-17 is increased in the sputum of asthmatics, and correlates with increased IL-8 and neutrophils in these patients (68). IL-17 also induces the maturation, migration and function of neutrophils (67), but is also important in

inflammatory responses to bacterial infection (69, 70), arthritis (71), tumourigenicity of cervical tumours (72), and organ allograft rejection (73). Th17 cells are also the major producers of IL-22, which is also released by monocytes, DCs (74, 75), Th1 and Th2 cells (76). IL-22 induces pro-inflammatory responses by eliciting the production of chemokines and cytokines from different cells. It also promotes the secretion of antimicrobial peptides such as β -defensins to aid in host defence against infection, and induces tissue repair (76).

1.2.2.4 T regulatory cells

Tregs are a subset of $CD4^+$ T-cells that are critically involved in the control of the immune response, tolerance to self-antigens, and maintenance of homeostasis (77, 78). They can be divided into two groups, natural and induced Tregs. Naturally occurring Tregs are self antigen-specific and are central to peripheral tolerance (79). Peripheral depletion of these cells results in multiorgan-specific autoimmunity, and increased immunity to pathogens and allergens (80). Induced Tregs can be further subdivided into type 1 Tregs (Tr1), and Th3 cells. Tr1 cells are induced by chronic stimulation by infection and allergens, and produce TGF- β , IL-5 and high levels of IL-10 that are involved in immunoregulation (81). Th3 cells are induced by oral antigen administration, and exert their function through the release of high levels of TGF- β (82, 83). TGF- β suppresses the release of IL-1 and IL-2, thereby inhibiting the differentiation and proliferation of T-cells (84), while IL-10 down-regulates co-stimulatory properties of APCs, the expression of effector T-cell cytokines, and suppresses antigen presentation (85). Studies have demonstrated that Tregs from atopic individuals are less effective at suppressing T-cells and cytokine release compared to Tregs from healthy individuals (86,

87), and that allergic patients have fewer blood and sputum Tregs compared to healthy controls, and even fewer during asthma exacerbations (88, 89).

1.2.3 APCs in asthma

Professional airway APCs, mainly DCs and macrophages, are crucial in inducing the differentiation of naïve and memory T-cells, and are critical in determining the phenotype of the immune response to an allergen.

1.2.3.1 Dendritic cells

DCs are in constant contact with the airway epithelium. They sample the luminal environment, process antigens, and migrate to the draining lymph nodes where they present these antigens to naïve T lymphocytes, inducing their polarisation (90, 91). DCs are the principal cells involved in the bridging of the innate and adaptive immune responses. They can be broadly separated into two types, myeloid DCs (mDCs), and plasmacytoid DCs (pDCs), both of which are increased in the airways after allergen challenge (92, 93).

Studies have demonstrated that in naïve mice that received mDCs, Th2 responses were preferentially induced in the lymph nodes, which was accompanied by airway eosinophilic inflammation and the development of AHR, demonstrating mDC involvement in the induction of Th2-mediated eosinophilic inflammation and inhibition of tolerance (94, 95). mDCs are also increased in the lung after allergen sensitisation and challenge, and their depletion reduces airway inflammation, AHR, and mucus production (96).

Plasmacytoid DCs have an immunoregulatory role in the lung. Murine models have been employed to show that adoptive transfer of pDCs prior to the induction of asthma prevented inflammation of the airways (97). pDCs are involved in antigenic tolerance and are protective against asthma. This suggests that aberrant pDC function may contribute to allergic sensitisation (97, 98), and there are studies that indicate that under certain conditions, pDCs are able to switch from a suppressive phenotype, to one that increases allergic responses. *In vitro* studies have shown that pDCs can elicit both Th1 and allergen-specific Th2 memory responses (99).

DCs are important in the primary, but also the secondary response to an allergen, that may ultimately lead to the development of chronic inflammation. Lambrecht *et al.*, showed that the depletion of DCs during a secondary exposure to allergen in sensitised animals, significantly reduced T-cell and B cell numbers, and eosinophilic airway inflammation (100).

1.2.3.2 Macrophages

Alveolar macrophages play an important role in airway inflammation, however studies have demonstrated contradictory data on precisely how they are involved. Some studies show that alveolar macrophages increase the ability of T-cells to produce allergen-specific IL-5 in atopic versus non-atopic asthmatics (101), and increase IL-4 and IL-5 in asthmatic compared to non-asthmatic individuals (102). These results suggest a role for macrophages in promoting Th2 responses. In contrast, there is also evidence that macrophages have a protective role in asthma. One study showed that the administration of allergen-pulsed alveolar macrophages induced the production of IFN- γ , a Th1 cytokine, and the depletion of alveolar macrophages resulted in increases in the Th2

cytokines IL-4 and IL-5, as well as increased eosinophilic inflammation and AHR (103). Together these data suggest that alveolar macrophages have the ability to induce both Th1 and Th2 immune responses.

1.2.4 Eosinophils in asthma

Eosinophil recruitment into the lungs is a hallmark feature of asthma, with increased levels of activated eosinophils found in asthmatic lung tissue compared to healthy controls. Increases in the number of activated eosinophils in the lung also directly correlates with AHR and asthma severity (3, 20, 104). Eosinophils are bone marrow-derived leukocytes that are generated during haematopoiesis, then migrate from the blood to the lung tissue or other local tissues in response to allergen exposure and cytokine/chemokine release (104). Chemoattractants produced by airway epithelial cells, lymphocytes, and macrophages, including eotaxin-1 and -2, Th2 cytokines IL-5, IL-9, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), and RANTES, are all involved in the trafficking of eosinophils to the airways (18, 105-107).

Once eosinophils reach the lung tissue, they are primed, by IL-5 and IL-13, and become activated (53, 108). Upon activation eosinophils undergo cellular degranulation and release pro-inflammatory mediators; such as MBP, which increases smooth muscle activity, eosinophil cationic protein (ECP), leukotrienes, and eosinophil peroxidase (EPO), that cause epithelial damage, bronchospasms, and increased vascular permeability. These factors also induce shedding of airway epithelial cells, and AHR (109, 110). Eosinophils release IL-4, IL-5 and eotaxin which recruit more eosinophils and amplify the inflammatory response (109, 111). Eosinophils also contribute to chronic

asthma by secreting growth factors and metalloproteases that stimulate fibroblasts, and promote airway remodelling (3).

1.2.5 Neutrophils in asthma

Neutrophils are present in larger numbers than other inflammatory cells in lung tissues and blood, and are one of the first cells to respond to injury or allergen exposure in the airways. Increased neutrophil recruitment to the airways is associated with asthma severity (112, 113). Neutrophil influx usually occurs in response to an agent such as endotoxin or a pathogen, and neutrophils are the first cells to be recruited during the innate response to bacterial or viral infection. Respiratory infections are often associated with infection-induced asthma exacerbations (114, 115).

Neutrophil influx into the lungs is controlled by chemokines, such as CXCL8 (IL-8), which is released by the lung epithelium and neutrophils themselves, and cytokines such as IL-17 (116, 117). IL-8 is crucial for neutrophil recruitment and activation. It primes neutrophils for phagocytosis, induces the production of leukotriene B₄, oxygen radicals; and is involved in the secretion of the granular enzymes myeloperoxidase, gelatinase and β -glucuronidase (112). Neutrophils release several other mediators, such as neutrophil elastase (NE), an important secretagogue for goblet cells and inducer of mucus hypersecretion, matrix metalloproteinase (MMP)-9, which is implicated in smooth muscle hyperplasia (112, 118), and TGF- β , a pro-fibrotic factor that contributes to abnormal tissue repair and airway remodelling (112, 119).

Recently neutrophils have been recognised for their role as regulatory cells in asthma. Activated neutrophils release macrophage inflammatory protein (MIP)-1 α and MIP-1 β , which attract immature DCs, T-cells, monocytes and macrophages. These

immature DCs release IL-8, which allows co-localisation of DCs with neutrophils at inflammatory sites and subsequent cross-talk between them (120-122). Studies have shown that neutrophils express MHC II and T-cell receptors, and can acquire antigen-presenting properties which allow them to directly activate T-cells, and directly transfer antigens to DCs, subsequently also indirectly activating T cells (123, 124).

1.3 Asthma phenotypes

Asthma has long been thought to be a disease that is mediated by eosinophils and Th2 responses. While this is true for a large number of asthmatics it has recently been identified that there are in fact a number of different subtypes of asthma. Analysis of airway inflammation in induced sputum samples has been used to classify asthmatics based on different inflammatory profiles. Several subtypes of asthma are now recognised, however, all subtypes present with the same clinical symptoms (Figure 1.3) (125).

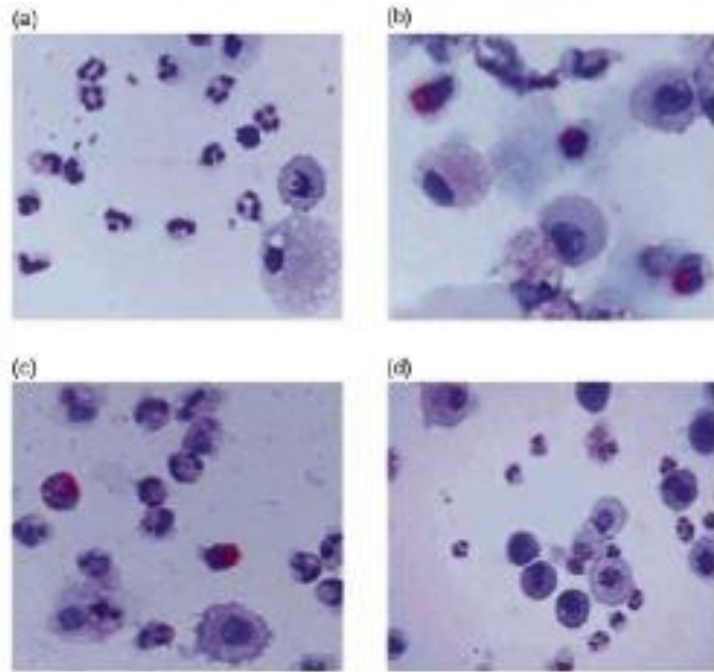


Figure 1.3: Sputum samples of asthma phenotypes. Sputum samples were induced from asthmatic patients and inflammatory cells isolated, stained and analysed. (a) Neutrophilic asthmatics have increased airway neutrophils, (b) while eosinophilic asthmatics have increased airway eosinophils. (c) Mixed granulocytic asthmatics have increased levels of both neutrophils and eosinophils, and finally (d) paucigranulocytic asthmatics have normal levels of neutrophils and eosinophils (125).

1.3.1 Non-eosinophilic asthma

Eosinophils are not the sole effector cells that are involved in the progression of asthma and in many individuals eosinophils may not play a dominant role. Studies have revealed that eosinophilic inflammation is present in the airways of only 50% of asthmatics, using cut off levels of 2-4% eosinophils to distinguish between eosinophilic and non-eosinophilic asthma (126). Non-eosinophilic asthma has been demonstrated in a number of disease states, including stable, persistent, severe, and steroid-naïve asthmatics (127-130).

1.3.2 Neutrophilic asthma

Several studies have investigated the possibility that neutrophilic inflammation is the mechanism that leads to non-eosinophilic asthma (127, 129, 131-133). Patients with sputum containing greater than 61% neutrophils are categorised as neutrophilic asthmatics (125).

Approximately one fifth of non-eosinophilic asthmatics have severe neutrophilic inflammation of the airways (125, 128). Although these asthmatics have similar clinical symptoms to eosinophilic asthmatics, there are some crucial differences that distinguish them. It has been shown that the intense neutrophilic bronchitis in neutrophilic patients, is a result of increased sputum IL-8 levels compared to eosinophilic asthmatics and healthy controls (127). Compared to eosinophilic asthma, neutrophilic asthmatics have reduced eosinophilic inflammation and AHR, and importantly, neutrophilic asthmatics are resistant to corticosteroid treatment (125, 128, 134-136).

1.3.2.1 Bacterial Colonisation in neutrophilic asthma

Assessment of bacterial factors and colonisation across different groups has revealed that sputum from neutrophilic asthmatics has 6-8 fold higher endotoxin levels compared to other asthma groups (137). Neutrophilic asthmatics also had evidence of increased bacterial colonisation compared to eosinophilic and paucigranulocytic asthmatics. In addition, 43% of neutrophilic asthmatics were colonised by bacteria, and *Haemophilus influenzae* (*H. influenzae*) was commonly isolated from these subjects (137). A more recent study showed that neutrophilic asthmatics have a significant load of potentially pathogenic bacteria, with bacteria detected in 41% of patients and *H. influenzae* detected in 60% of these individuals (138). These studies suggest that persistent bacterial colonisation may induce the influx of neutrophils and be a source of persistent innate immune stimulation.

1.3.2.2 Innate Immune factors in neutrophilic asthma

As mentioned above, neutrophils are key components of innate immunity and the innate immune response is the host's first response to antigen exposure. It is hypothesised that activation of innate immunity, either by infection, endotoxin, ozone or particulates, may be a possible trigger for neutrophilic inflammation in neutrophilic asthma.

A study by Simpson and colleagues, was the first to investigate the innate immune response in the airways of neutrophilic asthmatics and bronchiectasis patients, which is another airway disease characterised by neutrophilic bronchitis (137). By assessment of mRNA and protein levels in sputum samples, they demonstrated that neutrophilic asthma was associated with the up-regulation of innate immune receptors TLR-2, TLR-4 (both of which bind bacterial lipopolysaccharide [LPS]) and CD14, compared to eosinophilic and

paucigranulocytic asthmatics. Although both TLR-2 and -4 bind LPS, TLR-4 is ~100 fold more effective at binding LPS than TLR-2. TLR-2 binds mostly Gram-positive bacterial components such as peptidoglycan and lipoteichoic acid (139, 140). The authors also found increases in the innate cytokines IL-8, IL-1 β and TNF- α , which have been shown to up-regulate epithelial cell TLR-2 expression, subsequently further inducing innate immune responses (132).

Innate immune responses to substances such as endotoxin, may play roles in both the development of and protection against asthma. As mentioned above, increased exposure to innate immune activators increases the risk of the development of neutrophilic asthma. However, studies have shown that early exposure to PAMPs may protect against asthma by suppressing Th2 responses (141), although long term exposure may be required to maintain protection. Also of importance is the dose of PAMPs, in particular endotoxin. Low to moderate endotoxin exposure levels are protective against asthma, while high doses may induce exaggerated innate immune responses that result in the development of neutrophilic asthma (142).

1.3.2.3 Proteolytic Enzyme activity in neutrophilic asthma

Proteolytic enzymes, such as MMP-9 and NE are increased in asthma and are important in tissue repair and airway remodelling.

MMP-9 is produced by neutrophils during their differentiation, stored in granules and released upon activation. MMP-9 is also released by epithelial cells, eosinophils, macrophages and fibroblasts (143). It is released as a pro-enzyme, which is activated by reactive oxygen species, bacterial proteases and neutrophil proteins such as lipocalin. It is also involved in the degradation of extracellular matrices, and modulates other proteases

and cytokine activity (144-146). Importantly, *in vitro* studies show that MMP-9 release from asthmatic neutrophils is poorly suppressed by glucocorticosteroids (143).

NE, a protease that is also released by neutrophils, stimulates mucin gene expression and secretion, leading to mucus hypersecretion. It also breaks down a number of proteins, such as lung elastin, and studies have shown that an excess of NE can lead to lung degradation (147-150). A study of the activity of these enzymes in asthma phenotypes, demonstrated that high levels of active MMP-9 were present in the airways of eosinophilic asthmatics, while neutrophilic asthmatics had high levels of NE (151). The authors propose a feedback loop in neutrophilic asthmatics that involves IL-8, NE and MMP-9 and induces chronic neutrophilic inflammation. IL-8 promotes neutrophil recruitment and activation, which increases MMP-9 release from these neutrophils, and this MMP-9 cleaves IL-8 to a more potent form. This potent IL-8 recruits and activates more neutrophils, which release NE that is also able to induce IL-8 production. This feedback loop results in chronic inflammation (151).

1.4 Asthma therapy

The mainstay of asthma treatment is inhaled corticosteroids, however, an increasing number of studies have identified a significant proportion of asthmatics who do not respond to this medication. Most of these patients either have severe asthma, or are neutrophilic asthmatics (134, 136, 152, 153). As a result, other medications that target specifically neutrophilic inflammation are being investigated as treatments for these patients.

1.4.1 Corticosteroids

Corticosteroid hormones can be divided into two groups, glucocorticoids (also referred to as steroids), which have potent anti-inflammatory actions used to treat mild, moderate and severe asthma, and mineralocorticoids which control electrolyte and water levels (154). Glucocorticoids bind to an intracellular receptor (glucocorticoid receptor; GR), and once bound, this GR complex translocates to the nucleus where it binds to DNA sequences, known as glucocorticoid-inducible elements, that regulate gene transcription (155, 156). The GR is able to regulate transcription in one of two ways. Primary regulation involves the direct binding of the GR to regulatory regions of its primary target to enhance or inhibit transcription, and secondary regulation involves protein products from genes regulated at the primary level modulating the transcription of other genes (157). More recently it has been shown that primary regulation can also occur by the GR binding to other regulatory proteins that are bound to DNA. Both positive and negative regulation of transcription occurs as a result of different GR surface configurations, which allow it to interact with different proteins and therefore have opposing effects on transcription. Thus, variations such as post-translational modifications, ligand binding, and protein:protein interactions allow for the binding of co-factors that determine the outcome of transcription (158). The binding of a co-activator or co-repressor to the activated GR complex facilitates changes in chromatin structure at the target gene promoter site, resulting in transcriptional activation or suppression (159-161).

Inhaled corticosteroids (ICS) are highly effective in suppressing airway inflammation. They increase the rate of eosinophil apoptosis, subsequently reducing airway eosinophil numbers, and also decrease cytokine production from epithelial cells

and T-cells. These steroids also attenuate mucus hypersecretion and airway remodelling (136, 162, 163).

As previously mentioned, severe and neutrophilic asthmatics are largely resistant to steroid treatment, and a number of mechanisms have been proposed to explain this phenomenon. (1) reduced GR binding affinity in T lymphocytes, (2) a reduction in the number of GRs per cell, and (3) attenuated translocation and nuclear localisation of GR in response to high steroid doses. These changes may be the result of increased p38 MAP kinase activation, which phosphorylates the GR thereby reducing binding affinity. In addition, (4) CD4⁺ T cells may produce less of the anti-inflammatory cytokine IL-10 in response to steroids, and (5) increased expression of the GR- β variant which binds DNA and not glucocorticoid, and reduced expression of the active GR- α variant may also be involved (164-167). All of these alterations reduce the anti-inflammatory properties of steroids.

Importantly, steroid treatment also increases neutrophil survival by inhibiting neutrophil apoptosis and there has been much debate about whether the high levels of neutrophils observed in asthmatic patients taking high doses of ICS is a result of steroid treatment or if it is a primary pathological process (168, 169). However, it has recently been demonstrated that the neutrophilic phenotype is found in steroid-naïve asthmatics, suggesting that this is a stable and distinct disease phenotype (130).

These data and the fact that although neutrophilic asthmatics make up between 20-30% of asthmatics, they account for half of the health care costs for asthma, demonstrate that there is a desperate need for effective treatments for these patients (134).

1.4.2 Macrolides

Macrolide antibiotics are potent antimicrobial agents, and are first-line treatments for bacterial infections such as those that cause community-acquired pneumonia. Studies have shown that they function by inhibiting bacterial adherence, virulence and biofilm formation (170, 171). In addition, some macrolides also have anti-inflammatory properties, which are determined by their structure. The 14-, and 15-member macrolides (e.g. clarithromycin, roxithromycin, and azithromycin) have immunomodulatory effects, however, 16-member macrolides, such as josamycin, do not (172).

Clinical trials have shown that macrolides have beneficial effects in diseases such as cystic fibrosis, bronchiectasis, and COPD, but only a handful of studies have examined the effectiveness of macrolide therapy in asthma. Studies have demonstrated that clarithromycin treatment decreased inflammatory cell accumulation in the airways, inhibited airway hyperresponsiveness, and may potentiate glucocorticoid responsiveness in asthma (173-175). However, although these studies used the same macrolide they observed varying results, suggesting that more work is needed to elucidate the potential role of macrolide therapy in asthma.

Of interest, a recent study demonstrated that clarithromycin treatment targets neutrophilic inflammation in refractory asthma (176). The authors showed that after 8 weeks of treatment, clarithromycin reduced airway neutrophil numbers, and sputum IL-8, NE and MMP-9 levels. This indicates that there is potential for macrolide therapy in neutrophilic asthmatics. Potentially, neutrophilic asthmatics with evidence of bacterial colonisation would gain even more benefit from this treatment.

1.5 *Haemophilus influenzae* infection

Bacterial infections play an important role in both predisposition to, and suppression of asthma. Atypical bacteria, such as *Chlamydomphila pneumoniae*, and *Mycoplasma pneumoniae*, are associated with the development and exacerbation of asthma and *C. pneumoniae* infection has recently been implicated in the development of neutrophilic AAD (177, 178). In contrast, other bacteria such as *Streptococcus pneumoniae* and *Mycobacteria* may have suppressive effects in asthma (179-181).

H. influenzae is a non-motile Gram-negative bacterium that is asymptotically carried as normal flora in the nasopharynx of 75% of healthy children and adults (182). *H. influenzae* causes upper and lower respiratory tract infections, and can be capsulated (typeable) or non-capsulated (non-typeable). Typeable *H. influenzae* is classified into serotypes a to f according to the type of polysaccharide capsule. Serotype b is most commonly associated with pathogenic infections. A different classification system is used for the non-capsulated strains, which are grouped according to their outer membrane protein, molecular weight, and the antigenic heterogeneity of lipooligosaccharide (183). The occurrence of invasive *H. influenzae* disease has decreased by over 95% with the introduction of the Hib (*H. influenzae* serotype b) vaccine. Disease caused by NTHi is now the most prevalent, and accounts for a large percentage of community-acquired pneumonia.

NTHi is normally considered an extracellular bacterium, but studies have shown that it is also able to live intracellularly, especially in macrophages (184-186). Much research is now being performed to attempt to develop effective vaccines against NTHi.

1.5.1 Pathology of NTHi infection

NTHi causes lower tract infections that are responsible for substantial levels of morbidity and mortality in infants and children, and may predispose them to other serious diseases. NTHi is able to cause infection due to its ability to rapidly to evolve and evade the host defence system by variation of its antigenic proteins, and its multiple mechanisms of attachment during the infectious process.

NTHi commonly causes meningitis, otitis media (which occurs in 62% of children in developed countries), epiglottitis and pneumonia (187-189). *H. influenzae* becomes pathogenic, when it colonises areas where it is not normally located, and changes from normal flora, to a pathogenic parasite. Also, the presence of different *H. influenzae* subtypes encourages the exchange of genetic material, introducing new strains into the local environment.

NTHi is an opportunistic pathogen, and does not cause disease in healthy individuals with normal pulmonary function. However, those who have compromised airways, such as young children and the elderly, or individuals who suffer from chronic airway diseases and have lost efficient mucociliary clearance mechanisms, are susceptible to pathogenic NTHi infection (190-192).

H. influenzae is able to colonize and multiply in the airways as a result of its ability to adhere to structurally damaged respiratory epithelial cells, or epithelial cells that lack cilia (193). Bacterial cell wall components, such as lipooligosaccharide (LOS), and the release of heat-stable factors, initiate the recruitment of activated leukocytes, particularly neutrophils, to the airway, further impairing ciliary function and causing sloughing of epithelial cells. This facilitates the translocation of the bacteria across epithelial and endothelial cells and entry to other areas of the body (191, 193).

1.5.2 The inflammatory response to NTHi

The virulence of NTHi is determined by the extent of inflammatory responses of the host. NTHi does not release toxins, so disease is caused by the host response to bacterial components, specifically LOS. LOS structure, which differs between strains, is the major determinant of bacteria-host interactions. The first step in this interaction is bacterial adherence to epithelial cells, which initiates the formation of epithelial microvilli, and subsequent engulfment of NTHi (194).

TLR-2, TLR-4 and platelet-activating factor receptor (PAFr), are the main ligands for NTHi binding. NTHi outer membrane protein- (OMP) 2 and 6 bind to TLR-2 while LOS binds to TLR-4 and PAFr (189). Once bound, TLR signalling pathways are activated and induce signalling through the mitogen activated kinase cascade that results in the activation of the transcription factor nuclear factor- κ B. This then controls the transcription of pro-inflammatory cytokines such as IL-1 β and IL-6. Importantly, *H. influenzae* infection stimulates airway epithelial cells to express IL-8 and TNF- α , which promote neutrophil recruitment and activation (189, 195).

NTHi has developed a number of strategies for evading host immune responses. IgA is an innate antibody found at all mucosal sites that binds non-specifically to bacteria and induces bacterial cell lysis. IgA1 is the main IgA subtype in the upper respiratory tract. NTHi has 3 types of IgA1 proteases that are able to cleave IgA at several different sites, rendering it non-functional. Furthermore, under immunological stress, NTHi is able to change its IgA1 proteases to cleave at different sites (196, 197). During persistent infection, OMP P1, P2 and P5 have been shown to undergo antigenic drift, which causes small changes in protein sequences that allow persistence of infection, despite the induction of immune responses (198). NTHi can also invade endothelial cells, and survive

in membrane-bound vacuoles for long periods of time without eliciting an immune response (199).

A study examining responses to NTHi infection, found that healthy subjects had a predominantly protective Th1 response, releasing IFN γ and IL-2, however, patients with bronchiectasis released the Th2 cytokines IL-4 and IL-10, which may allow persistent infection. The authors suggest that this may have a significant role in the pathogenesis of bronchiectasis, as sufferers are prone to chronic bacterial infection (200, 201).

1.5.3 NTHi and chronic airway diseases

The lower respiratory tract is normally a sterile environment, however bacterial colonisation of this site often occurs in people with airway diseases such as COPD (202, 203), bronchiectasis (204), chronic bronchitis (205), and cystic fibrosis (206). During colonisation, patients may have no clinical symptoms, however the worsening of symptoms during exacerbations of disease may result from infection, alterations in the bacterial strain involved or bacterial load (203).

NTHi has been detected in the airways of patients with a number of chronic airways diseases. It is the bacteria most commonly isolated during exacerbations of COPD and a change in the site of bacterial infection in the airway, as well as NTHi-mediated inflammation are thought to be the main causes of these exacerbations (203, 207, 208). Recent *in vivo* studies indicate that the NTHi strains isolated during COPD exacerbations cause more neutrophil recruitment to the airways than colonising strains. These studies also demonstrated that strains isolated during exacerbations induced higher levels of IL-8 and were significantly more adherent to epithelial cells than colonising strains (209).

NTHi is also found in patients with bronchiectasis. Bronchiectasis, like neutrophilic asthma is a chronic airway disease that involves airway neutrophilic inflammation, and is associated with chronic cough, and recurrent chest infections (137, 210). A study demonstrated that 64% of clinically stable patients tested were positive for pathogenic bacteria, and 55% of these patients had *H. influenzae* (204). In addition, it has been shown that the higher the bacterial load in these patients, the more intense the airway inflammation (211).

These data validate the need for better understanding of the pathogenesis and contribution of infection, particularly those of NTHi to airway diseases. There is the potential that treatment strategies of infection may be a novel therapeutic strategy for neutrophilic asthma and may significantly improve disease outcomes.

1.6 Study rationale

The specific mechanisms that induce inflammation in the airways of neutrophilic asthmatics and how particular inflammatory cells are preferentially recruited remain unclear. *H. influenzae* infection of the airways induces the chemoattraction and activation of neutrophils, and is often involved in chronic disease, but the mechanisms of this association are not understood. It is unknown whether *H. influenzae* causes neutrophilic inflammation in asthma, or if neutrophilic asthmatics are predisposed to *H. influenzae* infection. The studies described in this thesis were designed to investigate these questions.

Chapter 2:

***Haemophilus influenzae* infection drives IL-17-mediated neutrophilic allergic airways disease**

I show that *Haemophilus influenzae* infection may be involved in the development of neutrophilic asthma-like disease through an IL-17-mediated mechanism in a mouse model. This may provide novel insights into the mechanisms that underpin infection-induced neutrophilic asthma.

A revised version of this chapter has been accepted for publication in *PLoS Pathogens*, September 2011.

2.1 Abstract

A subset of patients with stable asthma has prominent neutrophilic and reduced eosinophilic inflammation, which is associated with attenuated AHR. *Haemophilus influenzae* has been isolated from the airways of neutrophilic asthmatics, however the nature of the association between infection and the development of neutrophilic asthma is not understood. My aim was to investigate the effects of *H. influenzae* respiratory infection on the development of hallmark features of asthma in a mouse model of AAD. BALB/c mice were intraperitoneally sensitised to OVA and intranasally challenged with OVA 12-15 days later to induce AAD. Mice were infected with non-typeable *H. influenzae* during or 10 days after sensitisation, and the effects of infection on the development of key features of AAD were assessed on day 16. T helper 17 cells were enumerated by flow cytometry and IL-17 blocked with anti-IL-17 neutralising antibody. We show that infection significantly reduced eosinophilic inflammation, OVA-induced IL-5, IL-13 and IFN- γ responses and AHR during AAD, however, infection increased airway neutrophil influx in response to OVA challenge. Augmented neutrophilic inflammation correlated with increased IL-17 responses and IL-17 expressing macrophages (innate response) and T lymphocytes (adaptive response) in the lung. Significantly, depletion of IL-17 completely abrogated infection-induced neutrophilic inflammation during AAD. In conclusion, *H. influenzae* infections induce Th17 immune responses that drive the development of neutrophilic and suppress eosinophilic inflammation during AAD. This results in a phenotype that is similar to neutrophilic asthma. Infection-induced neutrophilic inflammation in AAD is mediated by IL-17 responses.

2.2 Introduction

Asthma is a complex disease of the airways that is generally characterised by symptoms of wheeze, cough, breathlessness and airway inflammation. While eosinophilic inflammation has been considered to be the hallmark of airway inflammation in asthma (5, 6), it is present in only 50% of asthmatics (126). Non-eosinophilic asthma has now been described in persistent (127, 128) and severe asthma, (129) as well as in steroid naïve asthma (130). Further investigation of the non-eosinophilic subtype has identified a subgroup with an intense neutrophilic bronchitis (125, 128) with increased IL-8 (127). Neutrophilic asthmatics are resistant to corticosteroid treatment, which results in a significant proportion of asthma-related health care costs (128, 134-136). IL-17 is also elevated in asthma and other obstructive airway diseases that are characterised by increased neutrophils (68, 212, 213).

IL-8 and IL-17 are important mediators of neutrophilic inflammation during infection and in disease states (127, 137, 212, 214, 215) and their elevated expression in neutrophilic asthma correlates with increased levels of neutrophils in sputum (68). IL-8 is a potent neutrophil chemoattractant, produced by macrophages, lymphocytes, epithelial cells and neutrophils (116, 216). IL-17 is produced by several cells including Th17 cells (117, 217, 218), $\gamma\delta$ T cells (70, 219), neutrophils (220), and macrophages (221, 222). IL-17 has critical roles in host defence against bacterial infections (223-226), suggesting a potential role in the pathogenesis of bacterial-induced neutrophilic asthma.

Chronic bacterial colonisation is evident in the airways of patients with neutrophilic asthma (137) and also associated with an intense neutrophilic bronchitis in asthma (138). *H. influenzae* is a common bacterium of the respiratory tract, is one of the bacteria most frequently isolated from the airways of neutrophilic asthmatics (137, 138), and often causes recurrent respiratory disease in those with compromised airways (189, 192, 203). *H.*

influenzae infection is also associated with the exacerbation of several chronic airways diseases, including COPD and bronchiectasis. Nevertheless, the nature of the association between *H. influenzae* infection and the development of neutrophilic asthma is yet to be determined. Specifically, whether infection promotes the pathogenesis of neutrophilic asthma, or if neutrophilic asthmatics are predisposed to infection is unknown.

In this study I used murine models of *H. influenzae* infection and OVA-induced allergic airways disease, which mimics hallmark features of human asthma, to elucidate the potential association between infection and the development of neutrophilic asthma.

2.3 Methods

2.3.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the NSW Animal Research Regulation 2005, and the Australian Code of Practice for the care and use of animals for scientific purposes (National Health and Medical Research Council). All protocols were approved by the Animal Care and Ethics Committee of the University of Newcastle (permit number 987/0111). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts made to minimize pain and suffering.

2.3.2 AAD model

Six to eight week old female BALB/c mice were used. Mice were sensitised by intraperitoneal (i.p.) injection, with OVA (50µg, Sigma-Aldrich, Castle Hill, Australia) with the Th2-inducing adjuvant Rehydrogel (1mg, in 200µl sterile saline, Reheis, Berkeley Heights, USA). On days 12 to 15 mice were challenged intranasally (i.n.) with OVA

(10µg/50µl saline) and AAD was assessed on day 16. Non-allergic control groups were sensitised with saline and adjuvant i.p. (227).

2.3.3 NTHi infection model

NTHi (NTHi-289, kindly supplied by M. Dunkley, Hunter Immunology Ltd) glycerol stocks were plated onto a chocolate agar plate, grown overnight (Oxoid, SA, Australia, 37°C, 5% CO₂), then washed off the plate and suspended in sterile phosphate buffered saline (PBS). To determine the effects of infection, mice were inoculated intratracheally (i.t.) with 5x10⁵ CFU non-typeable *H. influenzae* (in 30µl PBS, under alfaxane anaesthesia) during (Day 0) or after (Day 10) OVA sensitisation. Controls were infected but not exposed to OVA. In preliminary studies I determined that this inoculum induced an infection from which the mice recovered and could be used to study the effects of infection on AAD.

2.3.4 Cellular inflammation

Bronchoalveolar lavage fluid (BALF) was collected and processed as previously described (181). Briefly, the left lung was tied off and the right lung was washed twice with Hank's buffered salt solution (700µl, HBSS; Trace Scientific, Noble Park, Vic, Australia), and the right lung used for bacterial recovery. Cells in the BALF were pelleted and resuspended in 1ml red blood cell lysis buffer (RBC, on ice for 5 mins), washed and resuspended in HBSS, then cytocentrifuged (300g, 5 min, ThermoFisher Scientific, Waltham, MA) onto microscope slides. Blood smears were prepared from a drop of whole blood after a cardiac puncture. BALF and blood cells were stained with May-Grunwald-Giemsa according to manufacturer's instructions, and differential leukocytes were enumerated using light microscopy. A total of 250 cells were counted in a blinded fashion (181).

2.3.5 Bacterial recovery

Right lobes of lungs were aseptically removed and homogenised in 1ml of sterile PBS. Serial 1:10 dilutions of BALF and lung homogenates were each prepared in sterile PBS, plated onto chocolate agar plates and incubated overnight (37°C, 5% CO₂). Colonies were enumerated and bacterial numbers per right lung calculated.

2.3.6 Lung function

AHR was measured in response to increasing doses of aerosolised methacholine, by whole body invasive plethysmography as previously described (178). Briefly, mice were anaesthetised (ketamine/xylazine [90mg/kg and 10mg/kg respectively; Troy Laboratories, Smithfield, Australia) and tracheas were cannulated and attached to a ventilator set at 150 breaths/min. Peak dynamic compliance and transpulmonary resistance were assessed by analysis of pressure and flow waveforms following challenge with increasing doses of aerosolised methacholine (Sigma-Aldrich, NSW).

2.3.7 T-cell cytokines

Lung draining mediastinal lymph nodes (MLN) were collected and pushed through a 70µm sieve to form a single cell suspension (containing mostly T-cells). Cells were centrifuged and resuspended in supplemented RPMI media (10 % FCS, 20 mM HEPES, 10 µg/ml penicillin/streptomycin, 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate). Cells were subsequently restimulated with OVA (200µg/ml) or ethanol-killed NTHi (2x10⁷ CFU/ml) and cultured for six days (5% CO₂, 37°C, 1x10⁶ cells per well). After six days culture supernatants were recovered and analysed for IL-5, IFN-γ, (BD Biosciences,

North Ride, NSW), IL-17A and IL-13 (R&D Systems, Minneapolis, MN, USA) by ELISA, according to manufacturers instructions (228).

2.3.8 Cytokine expression in lungs

RNA was TRIZOL extracted from whole lung homogenates according to manufacturer's instructions (Invitrogen, Mount Waverly, Victoria). 1ug mRNA was reverse transcribed to cDNA then analysed by real-time PCR. Thermal cycling conditions included a denature step at 95°C, and annealing temperatures between 55 and 65°C depending on the optimal conditions for each primer. Target gene expression was determined relative to the reference gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) (228). Primers used were IL-17, Fwd 5'-aaacatgagtccaggagagcttt-3', Rev 5'-actgagcttcccagatcacagagg-3'; ROR- γ t, Fwd 5'-ccgctgagagggcttcac-3', Rev 5'- tgcaggagtaggccacattaca-3'; MIP2, Fwd 5'-ctagctgcctgcctcattctac-3', Rev 5'- caacagtgtacyyacgcagacg-3'; KC Fwd 5'-cttggggacaccttttagca-3', Rev 5'- gctgggattcacctcaagaa-3'; TGF- β Fwd 5'-cccgaagcggactactatgctaaa-3', Rev 5'- ggtaacgccaggaattgttgctat-3'; and HPRT Fwd 5'-aggccagactttgttgatttgaa-3', Rev 5'- caacttgcgctcatcttaggcttt-3'.

2.3.9 Flow cytometry

Single cell suspensions of MLNs and collagenase-D digested lungs were prepared. IL-17 producing cells were identified by stimulation with phorbol 12-myristate 13-acetate (PMA, 0.1 μ g/ml) and ionomycin (1 μ g/ml, Sigma-Aldrich, NSW) in the presence of Brefeldin A (8 μ g/ml, Sigma-Aldrich) for 4 hours at 37°C, 5% CO₂. Cells were incubated with Fc block for 15mins on ice, then stained for surface markers CD4 (FITC), CD3 (PE), CD11b (PerCP),

Gr-1 (APC; BD Bioscience), or F4/80 (PE; eBioscience, San Diego, CA, USA) for 30 minutes on ice, fixed with 4% paraformaldehyde (PFA), permeabilised with 0.1% saponin for 1 hour on ice, and stained for intracellular IL-17 (APC; or isotype control rat IgG_{2a}, eBioscience). Tregs were identified using surface markers CD4 (FITC), CD25 (PerCP) and a Treg staining kit for intracellular FoxP3 (PE; or IgG_{2a} isotype control) according to manufacturers instructions (eBioscience); pDCs were characterised as CD11c^{low}(PE)CD11b⁻(PerCP)B220⁺(FITC), and mDCs characterised as CD11c⁺CD11b⁺B220⁻ (BD Bioscience); using MHCII (FITC) and CD86 (APC; R&D Systems) for activation and co-stimulation status. All cells were analysed using a FACS Canto (BD Biosciences). 50 000 events were recorded and analysed (178).

2.3.10 Depletion of IL-17 during infection-induced neutrophilic AAD

Anti-IL-17A neutralising antibody (clone 50104, rat IgG_{2a}) was administered by i.p. injection (100µg/mouse, eBioscience) on days 11 and 13, and features of AAD were assessed on day 16. Control groups were un-infected and treated with anti-IL-17 or treated with IgG_{2a} isotype control antibody (178).

2.3.11 Statistics

Results are presented as mean $\pm$ standard error of the mean (SEM) from 6-8 mice, in duplicate. Significance was determined by one-way ANOVA or Student *t*-test (GraphPad Software, CA, USA).

2.4 Results

2.4.1 Non-typeable *Haemophilus influenzae* infection

In order to investigate the association between *H. influenzae* lung infection and asthma a murine model of NTHi lung infection was first established and characterised. Inoculation (i.t) with 5×10^5 CFU of NTHi resulted in a mild respiratory infection that induced inflammatory responses but did not significantly affect lung function (Figure 2.1).

Bacterial numbers in BALF and lung homogenates peaked at 5 days and were undetectable 16 days after inoculation (Figure 2.1A). NTHi infection induced airway inflammation. Neutrophil influx into the airways peaked at 24 hours post-infection while lymphocytes and eosinophils were significantly increased at 5 days. Neutrophil numbers returned to baseline after 5 days, while lymphocyte and eosinophil numbers returned to baseline after 10 days post-infection (Figure 2.1B).). Infection also induced significant but low level increases in NTHi-induced IL-5 and IL-13, and higher levels of IFN- γ release from MLN cultures after 5 days, which returned to baseline levels after 26 days (Figure 2.1C). Infection did not affect lung function, with no changes in dynamic compliance or airways resistance compared to sham infected (Saline) controls 5, 16 and 26 days after inoculation (Figure 2.1D-E).

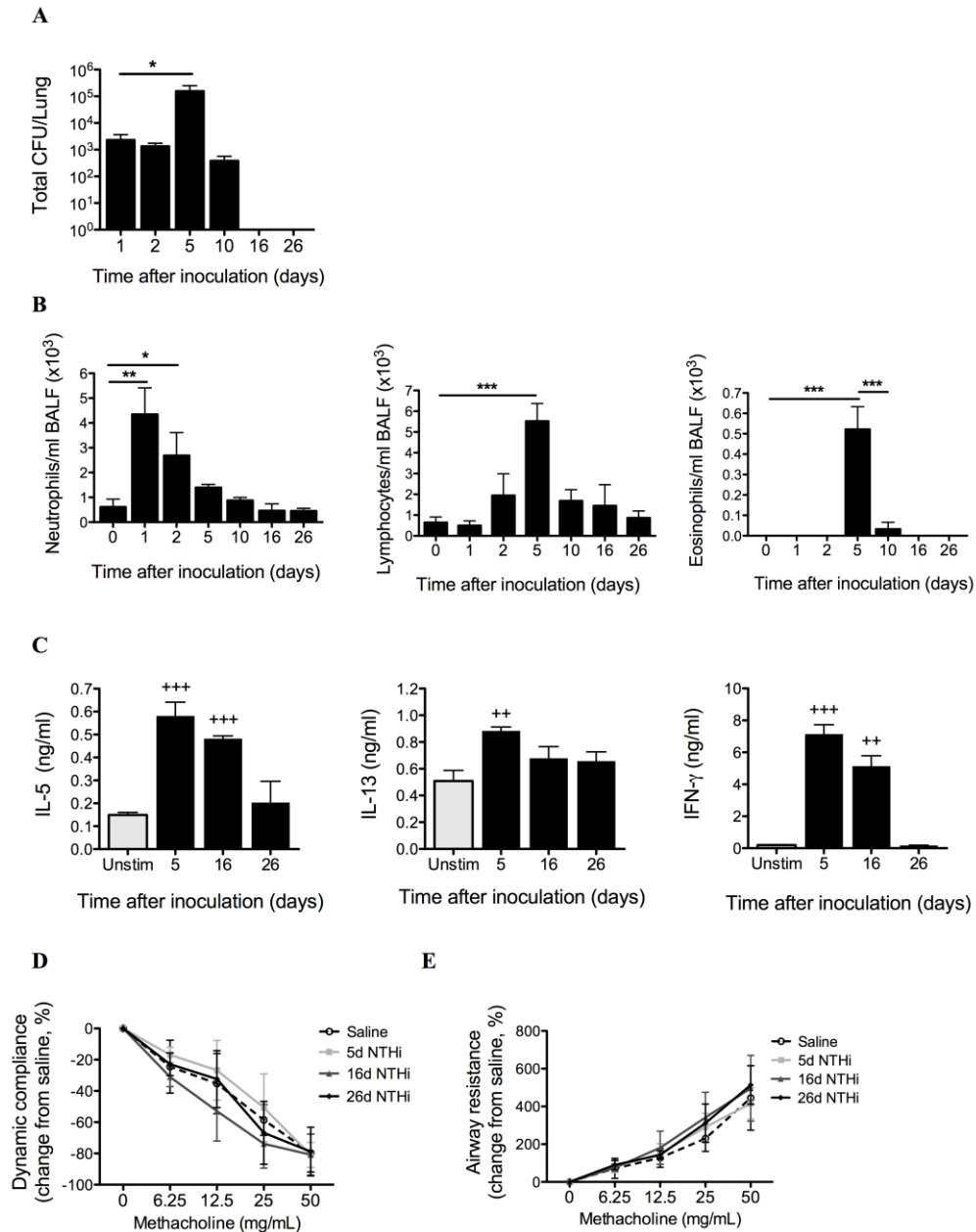


Figure 2.1: Characterisation of NTHi infection. The profile of infection was assessed in mice that only received NTHi (i.e. not OVA), by performing a time-course of bacterial recovery from BALF and lung homogenates (A), and airway inflammation represented by BALF neutrophils, lymphocytes and eosinophils (B). IL-5, IL-13 and IFN-γ (C) release from MLN T-cells stimulated with killed NTHi was also determined. Lung function in terms of dynamic compliance (D) and airways resistance (E) in response to increasing doses of methacholine was assessed 5, 16 and 26 days after inoculation. N.B. P values for compliance and resistance were determined for the entire dose response curve, **** p<0.001, ** p<0.01, * p<0.05.

2.4.2 NTHi infection suppresses key features of Th2-driven eosinophilic AAD

To investigate the effect of infection on AAD in sensitised animals, groups were infected during (d0 NTHi+OVA) or 10 days after (d10 NTHi+OVA) OVA sensitisation (Figure 2.2A). Infection suppressed OVA-induced T-cell cytokine responses, inflammatory cell influx and AHR in AAD (Figure 2.2).

The development of AAD (OVA groups) resulted in increased OVA-induced release of IL-5, IL-13 and IFN- γ from MLN and splenic T-cells, eosinophilic inflammation and AHR (decreased compliance and increased resistance), compared to uninfected, nonallergic (Saline) controls (Figure 2.2B-F and Figure 2.3). Infection during (d0 NTHi+OVA) or after (d10 NTHi+OVA) sensitisation resulted in significant reductions in OVA-induced IL-5, IL-13 and IFN- γ release from MLN T-cells (Figure 2.2B), compared to uninfected, allergic (OVA) controls. Infection also significantly reduced the numbers of total cells and eosinophils in the airways and blood (Figure 2.2C-D). This reduction in eosinophils correlated with the reduced release of IL-5 from MLN T-cells. Infection significantly suppressed, but did not abolish AHR in AAD; infection during sensitisation (d0 NTHi+OVA) had no effect on compliance, but significantly reduced resistance. However, infection after sensitisation (d10 NTHi+OVA), significantly suppressed both compliance and resistance (Figure 2.2E-F). Notably, compliance remained decreased in infected, allergic (NTHi+OVA) groups compared to uninfected, nonallergic (Saline) controls.

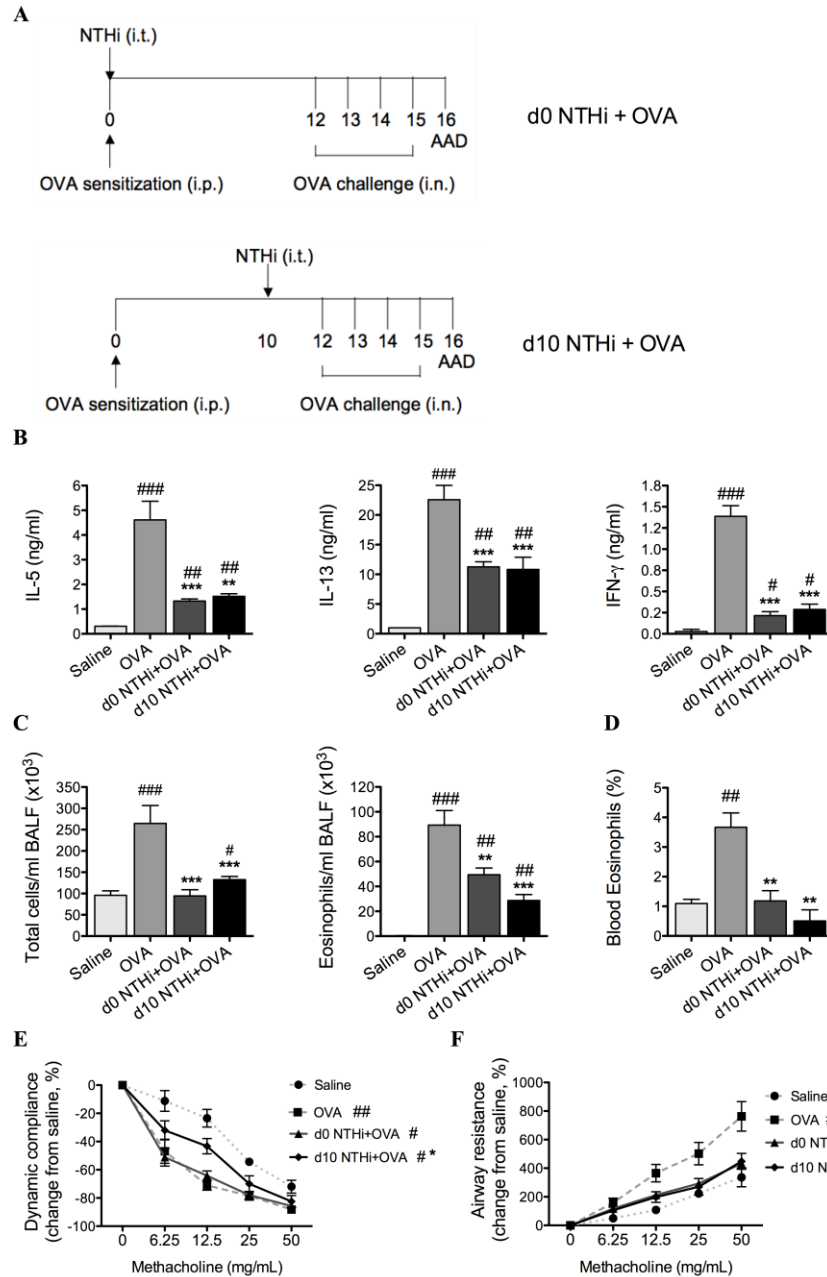


Figure 2.2. NTHi infection suppressed key features of Th2-driven eosinophilic AAD. Groups of mice were infected during (d0 NTHi+OVA) or 10 days after (d10 NTHi+OVA) OVA sensitisation (A), and AAD was analysed (day 16). The effects of infection on IL-5, IL-13, and IFN- γ (B) release from MLN T-cells stimulated with OVA, BALF total cell and eosinophil counts (C) and the percentage of blood eosinophils (D) were assessed. AHR in terms of dynamic compliance (E) and airways resistance (F) was also determined. N.B. P values for compliance and resistance were determined for the entire dose response curve, ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls.

2.4.3 NTHi infection suppresses systemic responses in AAD

Infection during (d0 NTHi+OVA) or after (d10 NTHi+OVA) sensitisation had no effect on IL-5 (Figure 2.3A) but significantly reduced systemic IL-13 and IFN- γ release from splenocytes (Figure 2.3B-C), compared to uninfected, allergic (OVA) controls.

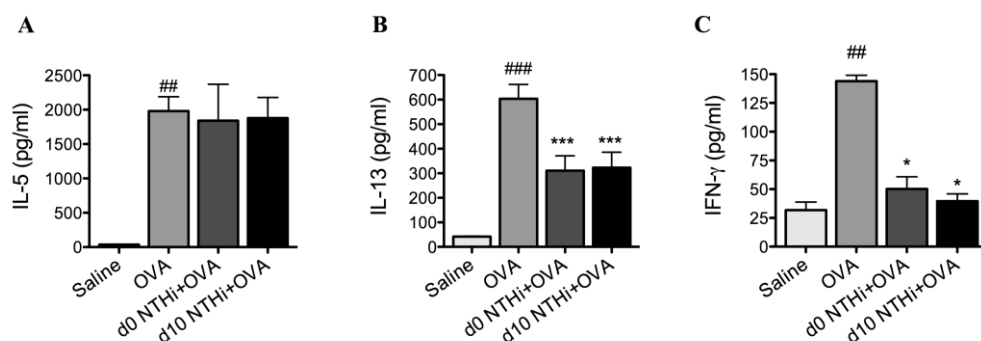


Figure 2.3. NTHi infection suppressed systemic IL-13 and IFN- γ responses in AAD. Groups of mice were infected during (d0 NTHi+OVA) or 10 days after (d10 NTHi+OVA) OVA sensitisation, and AAD was analysed (on day 16). The effect of infection on IL-5 (A), IL-13 (B), and IFN- γ (C) release from splenocytes stimulated with OVA was assessed. ### p<0.001, ## p<0.01 compared to Saline controls, *** p<0.001, * p<0.05 compared to OVA controls

2.4.4 NTHi infection does not alter T regulatory cells

To determine if Tregs were involved in the suppression of AAD, Tregs and TGF- β mRNA expression were quantified on day 16 of the model. TGF- β is a critical immunosuppressive factor that is produced by Tregs. NTHi infection during and after sensitisation did not alter the numbers of Tregs in the lung (Figure 2.4A), compared to uninfected allergic controls. Notably, infection decreased the expression of TGF- β mRNA in lung tissue (Figure 2.4B) in infected, allergic groups compared to uninfected, allergic controls.

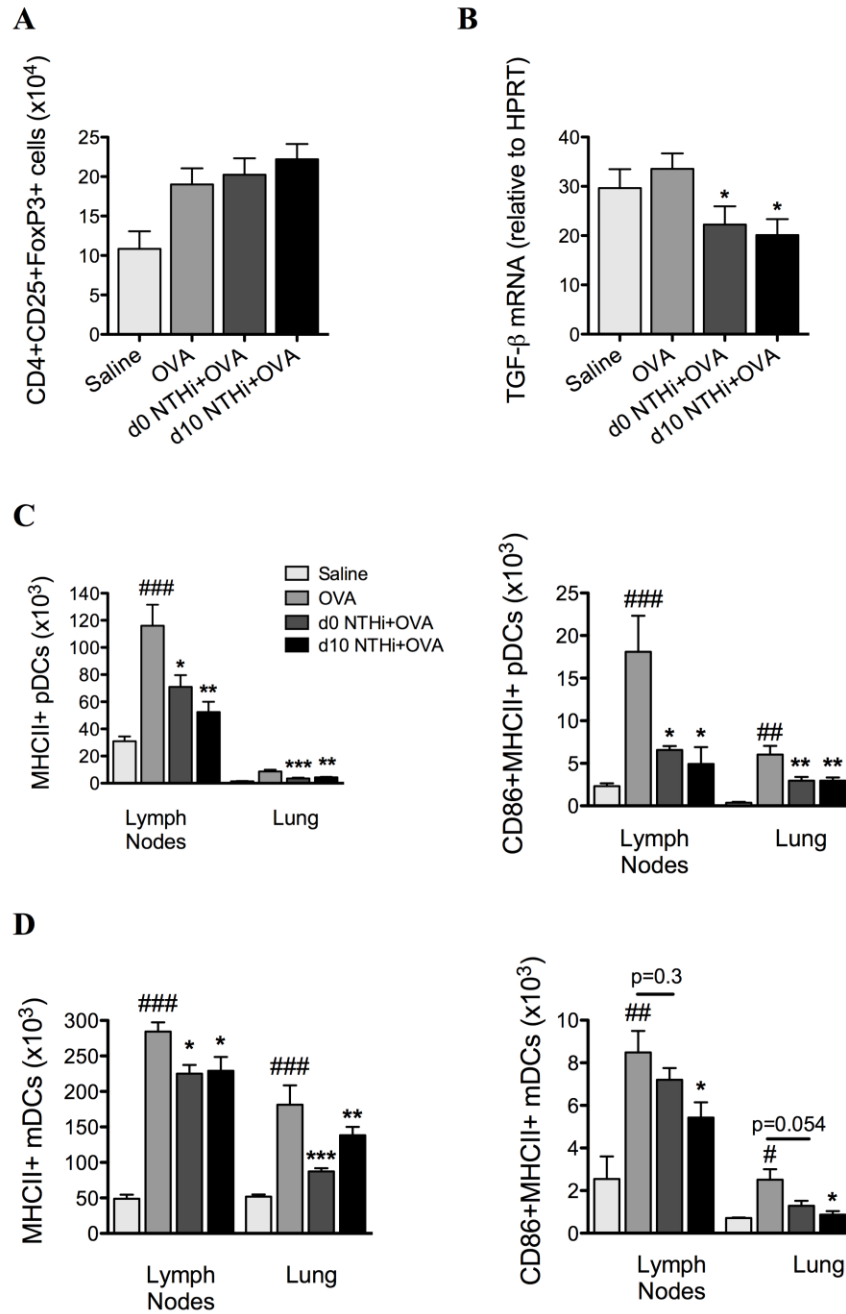


Figure 2.4. NTHi infection does not alter T regulatory cells but reduces markers of antigen presentation and activation in the suppression of AAD. Treg numbers in the lung (A), and TGF- β mRNA expression in lung tissue (B) were assessed (on day 16). The expression of MHCII and CD86 on pDCs (C) and mDCs (D) in MLNs and lungs was also determined. ###p<0.001, ## p<0.01, # p<0.05 compared to Saline controls, ***p<0.001, ** p<0.01, * p<0.05 compared to OVA controls.

2.4.5 Infection reduced markers of antigen presentation and co-stimulation in the suppression of AAD

To determine if alterations in APCs were involved in the suppression of AAD, the effect of infection on MHCII and CD86 expressing DCs was also investigated (on day 16). The development of AAD resulted in increases in the number of MHCII expressing pDCs and mDCs; and CD86 expressing MHCII⁺ pDCs and mDCs in MLNs and lungs (Figure 2.4D-E), compared to uninfected, nonallergic controls. Infection during or after sensitisation resulted in significant decreases in MHCII and CD86 expression of pDCs and mDCs compared to uninfected, allergic controls (Figure 2.4D-E).

2.4.6 Infection enhances neutrophilic inflammation in AAD

The effects of infection on other features of AAD (on day 16) were subsequently assessed. Significantly, whilst eosinophilic inflammatory responses were suppressed by infection during AAD, NTHi infection induced AAD with an enhanced neutrophilic inflammatory profile.

The development of AAD resulted in an increase in neutrophil influx into the airways (Figure 2.5A). Infection during or after sensitisation resulted in a two-fold increase in neutrophil recruitment into BALF compared to uninfected, allergic controls. Moreover, infected allergic groups had a four-fold increase in neutrophil recruitment compared to groups with infection alone (i.e. infected, nonallergic groups) at the same timepoint after infection (i.e. 16d and 5d after infection, Figure 2.5B). These results demonstrate that the combination of infection and AAD results in enhanced neutrophilic inflammation. Collectively, these results show that NTHi infections may induce a phenotype of neutrophilic AAD that resembles neutrophilic asthma in humans.

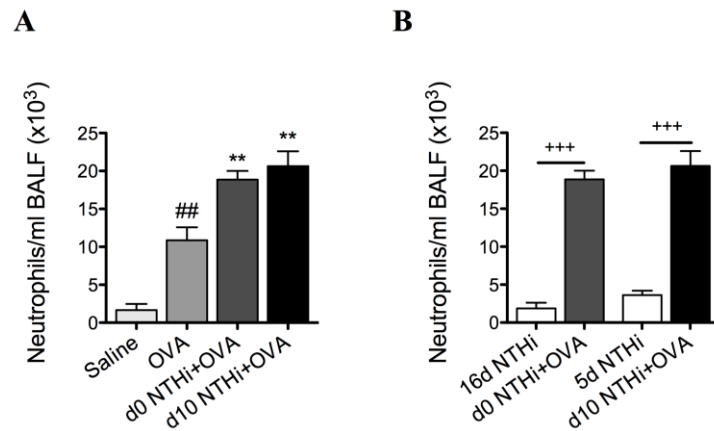


Figure 2.5. NTHi infection induces neutrophilic inflammation in AAD. The effect of NTHi infection on BALF neutrophil numbers in AAD was determined (day 16) and compared to groups with AAD without infection (A) or infection alone without AAD at the same timepoint after infection (i.e. 16d and 5d) (B). ## $p < 0.01$ compared to Saline controls, ** $p < 0.01$ compared to OVA controls, +++ $p < 0.001$ compared to infection alone controls.

2.4.7 Infection-induced neutrophilic inflammation is associated with increases in IL-17 responses.

Neutrophilic inflammation in asthma has been linked with increased IL-17 expression and IL-17 has been shown to be involved in neutrophil recruitment in response to bacterial infection. Therefore, the effects of infection on IL-17 responses during infection-induced neutrophilic AAD were further investigated. The experiments described hereafter were performed with infection during OVA sensitisation (d0 NTHi+OVA).

The profile of neutrophil influx into the airways and IL-17 production was determined in various tissues during the development of infection-induced neutrophilic AAD (1, 5, 12 and 16d, Figure 2.6A). The development of AAD resulted in increases in neutrophilic influx into the airways on days 12 and 16, and had minimal effects on IL-17 responses. Significantly greater numbers of neutrophils were recruited to the airways during infection and OVA sensitisation (1d) and after OVA challenge (16d) in infected, allergic compared to uninfected, allergic groups (Figure 2.6B).

Importantly, increases in the infection-induced neutrophil influx were accompanied by significant increases in IL-17 responses in pulmonary tissue and MLN cells. The expression of IL-17 mRNA in lung tissue was significantly elevated 1 day after infection in infected, allergic groups (Figure 2.6C) and returned to baseline levels by day 12, immediately prior to OVA challenge. Expression again significantly increased on day 16, after OVA challenges.

NTHi-induced IL-17 release from MLN T-cells was also increased from days 5 to 16 in infected, allergic compared to uninfected, allergic groups (Figure 2.6D). Interestingly, infection did not affect OVA-induced IL-17 release (Figure 2.6E). Taken together, these data

demonstrate that infection induces increased IL-17 responses in lung tissues and MLNs that correlate with elevated airway neutrophil numbers in infection-induced neutrophilic AAD.

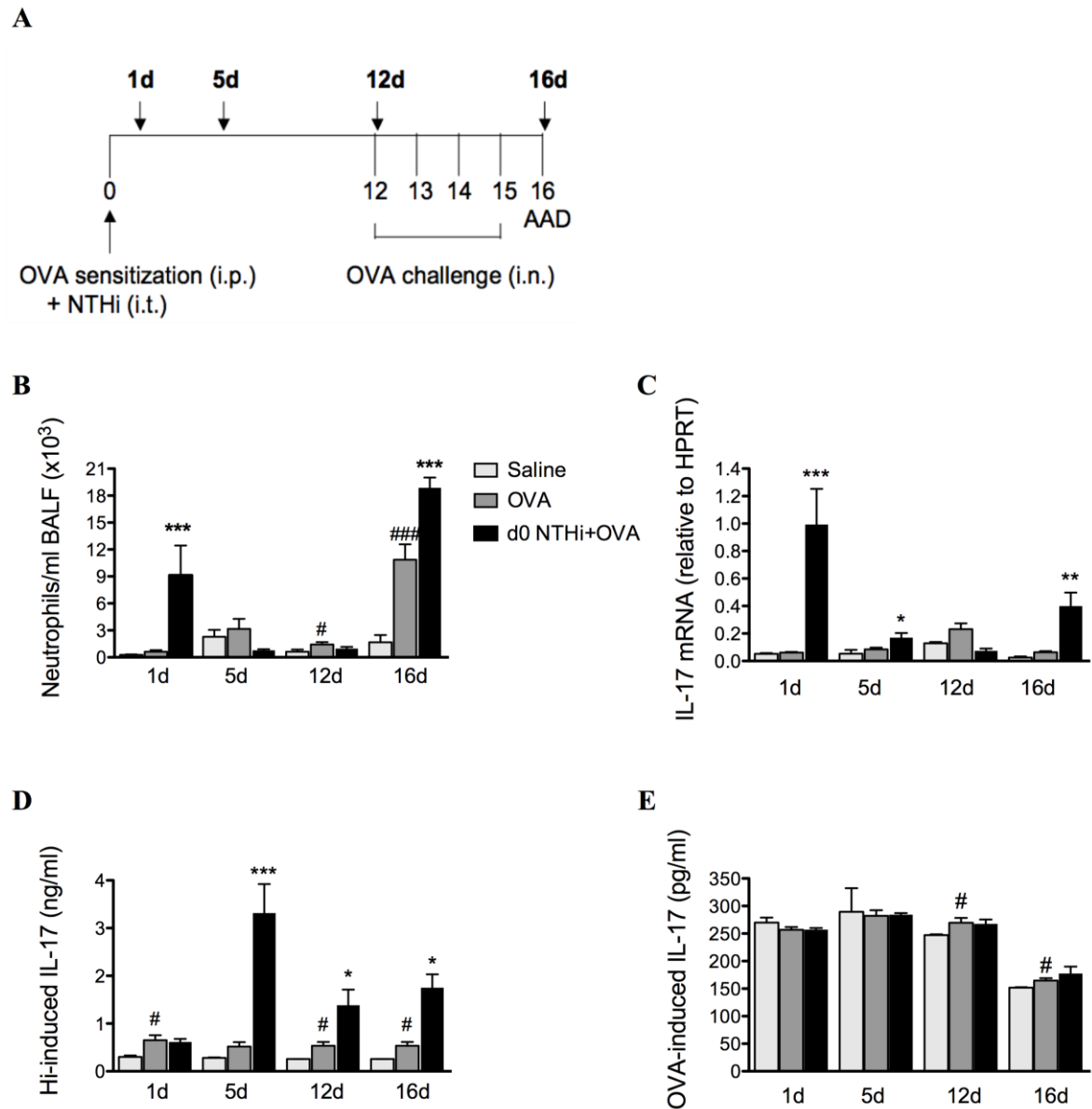


Figure 2.6. NTHi infection increases IL-17 responses that correlate with neutrophil influx in neutrophilic AAD. To investigate the association between neutrophil influx and IL-17 responses, a time-course (A), of neutrophils in BALF (B), IL-17 mRNA expression in lung tissue (C), and IL-17 release from MLN T-cells stimulated with killed NTHi (D), and OVA (E) was assessed. #### $p < 0.001$, # $p < 0.05$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls.

2.4.8 Infection-induced early influx of neutrophils is associated with early increases in neutrophil chemokine responses.

IL-17 can induce neutrophilic inflammation by enhancing the expression of the chemotactic factor IL-8. Therefore, the levels of MIP2 and KC mRNA expression, the mouse orthologs of IL-8, in lung tissues were also investigated. Both MIP2 and KC were elevated 1 day after infection and OVA sensitisation in the lungs of infected, allergic, compared to uninfected, allergic groups, (Figure 2.7). There were no differences between groups at later time-points. Therefore, the early induction of neutrophil influx into the lung during the development of infection-induced neutrophilic AAD is associated with early increases in neutrophil chemokine responses during infection.

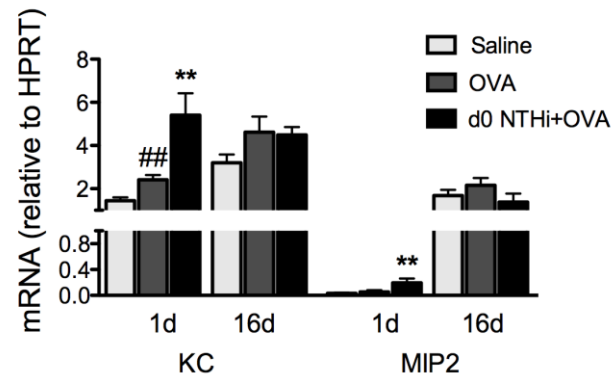


Figure 2.7. Early neutrophil influx is associated with enhanced neutrophil chemokine expression. To investigate the association between early neutrophil influx and neutrophil chemokine responses, KC and MIP2 mRNA expression in lung tissue was assessed. ## $p < 0.01$ compared to Saline controls, ** $p < 0.01$ compared to OVA controls

2.4.9 Infection induces Th17 cell differentiation and IL-17 production from Th17 cells, macrophages and neutrophils.

To investigate the mechanisms that underpin infection-induced neutrophilic AAD, the potential cellular sources of IL-17 and the role of adaptive and innate immune cells were investigated.

The development of AAD resulted in modest increases in IL-17 factors and responses (Figure 2.8A-E). ROR- γ t, the Th17 differentiation factor was assessed, and expression was significantly elevated after 12 and 16 days in the lungs of infected, allergic, compared to uninfected, allergic groups (Figure 2.8A), suggesting that there was enhanced Th17 polarisation. The numbers of Th17 cells in lung tissue and MLNs were then determined by flow cytometry. CD3⁺CD4⁺IL-17⁺ (Th17) cells were significantly increased in the lungs after 12 and 16 days in infected, allergic groups (Figure 2.8B), but in the MLNs were increased only at day 5 (Figure 2.8C). These results indicate that Th17 cells in the lungs and MLNs may be the potential adaptive immune source of IL-17 after day 5. I then assessed which cells were the early, innate source of IL-17 on day 1. Pulmonary macrophages and to a lesser extent neutrophils produced increased amounts of IL-17 at early but not other time-points (Figure 2.8D-E). Taken together these results demonstrate that infection induces early IL-17 responses from lung macrophages and neutrophils and later responses from Th17 cells in lungs and MLNs that are associated with neutrophil influx into the airways.

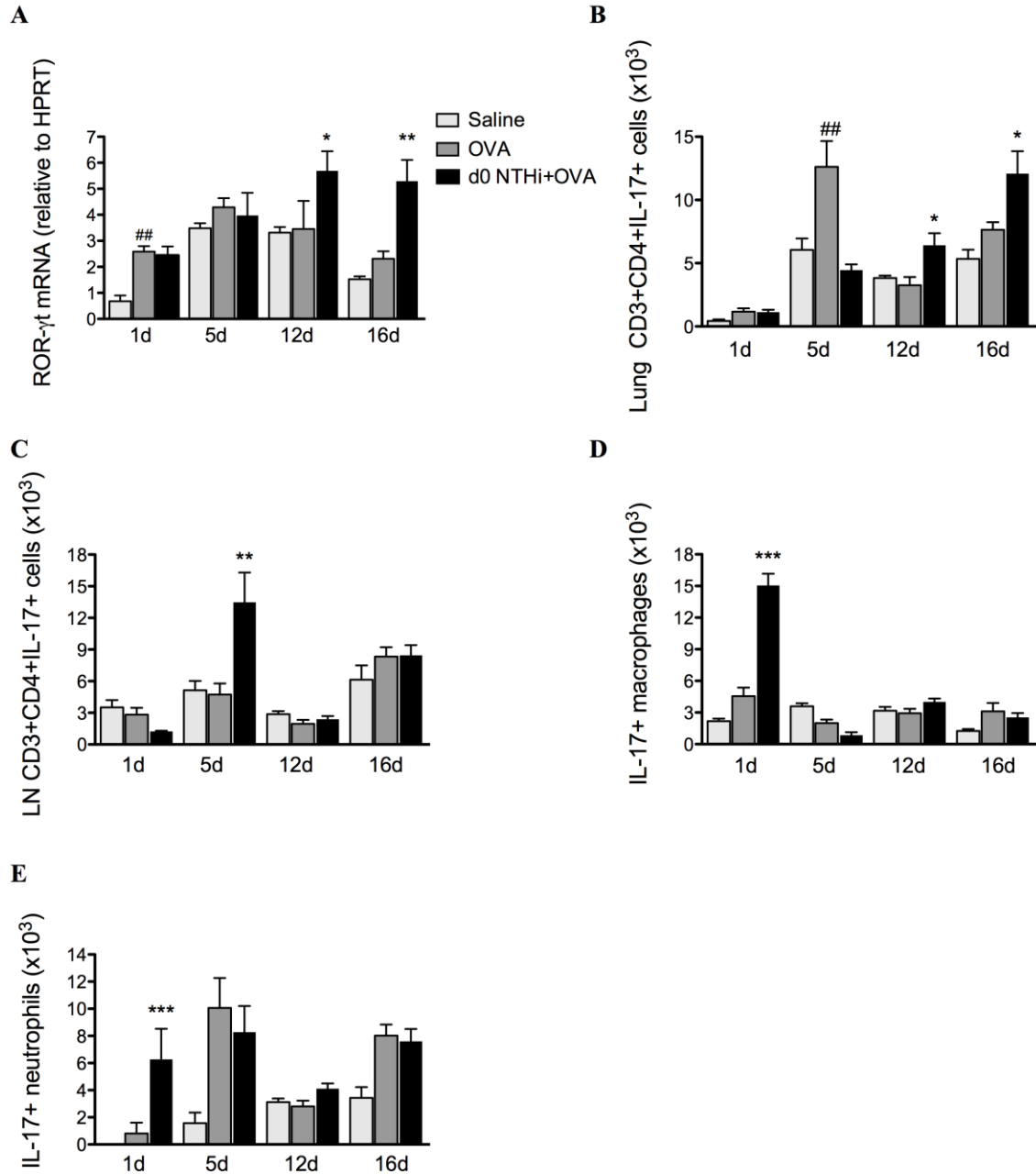


Figure 2.8. NTHi infection induces Th17 cell differentiation and IL-17 production from Th17 cells, macrophages and neutrophils in neutrophilic AAD. Th17 cell differentiation in lung tissues was examined by determination of the expression of the transcription factor ROR- γ t (A). Th17 cell numbers were quantified in lungs (B) and lymph nodes (C). IL-17 producing macrophage (D) and neutrophil numbers (E) in lungs were also assessed. ^{##} $p < 0.01$ compared to Saline controls, ^{***} $p < 0.001$, ^{**} $p < 0.01$, ^{*} $p < 0.05$ compared to OVA controls.

2.4.10 NTHi-induced neutrophilic AAD is dependent on IL-17.

Here I show that neutrophilic inflammation in infection-induced neutrophilic AAD correlates with increased expression of IL-17 during OVA challenge. To determine whether infection-induced neutrophilic inflammation is mediated by IL-17, IL-17 was depleted in infected, allergic groups during AAD, by administration of anti-IL-17 monoclonal antibody during OVA challenge on days 11 and 13 (Figure 2.9A). AAD was assessed on day 16. This approach has previously been shown to deplete IL-17 *in vivo* (117).

Importantly, IL-17 depletion significantly reduced the numbers of neutrophils in BALF compared to isotype treated infected, allergic groups (Figure 2.9B). Significantly, neutrophil numbers were not different to those observed in uninfected, allergic groups, which were unaffected by treatment. IL-17 depletion also significantly reduced KC mRNA expression levels in the lung, but had no effect on MIP2 mRNA (Figure 2.9C). Anti-IL-17 treatment of infected, allergic groups also partially restored IL-5 (3.198 ± 0.679 ng/ml in anti-IL-17- compared to 1.576 ± 0.238 ng/ml in isotype-treated infected allergic groups; $p < 0.01$) and IL-13 (18.240 ± 1.533 ng/ml in anti-IL-17 compared to 11.988 ± 0.938 ng/ml in isotype-treated infected allergic groups $p < 0.01$) responses, but had no effect on IFN- γ release. These results demonstrate that infection-induced IL-17 release is responsible for neutrophil influx into the airways and the induction of neutrophilic AAD.

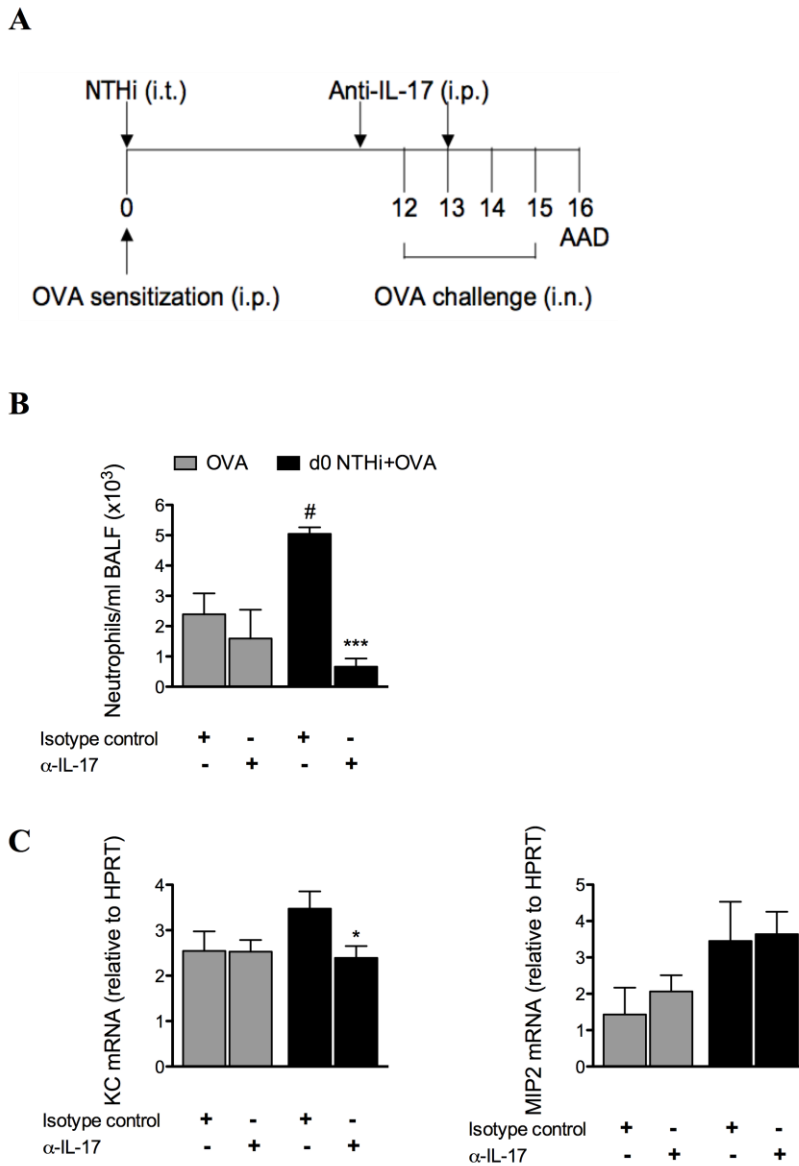


Figure 2.9. NTHi infection-induced IL-17 is required for the induction of neutrophilic AAD and is partially responsible for the effects of infection on T-cell responses. To confirm the role of IL-17, anti-IL-17 monoclonal antibody was administered i.p. on days 11 and 13 of infection-induced neutrophilic AAD, and AAD was analysed (day 16) (A). The effects on BALF neutrophil influx (B), and KC and MIP2 mRNA expression in lung tissue (C) were assessed. *** $p < 0.001$, * $p < 0.05$ compared to isotype controls.

2.5 Discussion

It has been demonstrated for the first time in this study that *H. influenzae* respiratory infection drives IL-17-mediated development of neutrophilic AAD. NTHi infection suppressed pulmonary and systemic eosinophilic inflammation and reduced Th2 cytokine responses and AHR in AAD. However, infection induced neutrophilic inflammation during AAD, by promoting early (innate) and late (adaptive) IL-17 responses from pulmonary macrophages and Th17 cells, respectively. This indicates that *H. influenzae* infection may modulate immune responses in asthmatics that promote the development of neutrophilic asthma.

NTHi is commonly isolated from the nasopharynx of healthy individuals, but is also associated with chronic airway diseases such as bronchiectasis (204), COPD (229), and chronic bronchitis (205). NTHi is the bacterium most commonly isolated during COPD exacerbations, and neutrophils isolated during these exacerbations induce higher levels of IL-8, and subsequent neutrophil recruitment to the airways, than colonising strains (209). *Simpson* and colleagues have recently demonstrated that a large proportion of neutrophilic asthmatics are colonised with *H. influenzae*, have increased innate immune activation, and 6-8 fold higher endotoxin levels compared to other asthma subtypes and healthy controls (137). A more recent study has demonstrated that 41% of neutrophilic asthmatics studied had a significant load of potentially pathogenic bacteria, and *H. influenzae* was identified in 60% of patients that tested positive for these bacteria (138). I have extended these studies to show that *H. influenzae* may promote neutrophilic asthma by suppressing Th2-mediated responses that are associated with alterations in APCs, and by inducing potent neutrophilic inflammation that is driven by Th17 responses.

These results show that infection during and after sensitisation inhibits characteristic features of eosinophilic asthma. Irrespective of the time of inoculation, infection significantly reduced both local and systemic allergen-induced cytokine release from MLNs and splenocytes, as well as airway and blood eosinophil recruitment. All of these effects may lead to the suppression of AHR. Tregs are an important cell involved in the suppression of inflammation and immune tolerance (230). We show that Treg numbers were not changed by infection, and TGF- β which is involved in the suppression of inflammation by Tregs, were reduced by infection. These results suggest that Tregs are not involved in the suppression of MLN cytokines or cellular inflammation. The role of infection on DC function was investigated as DCs play an integral role in the uptake and presentation of antigen to naïve T-cells, and as a result direct immune responses (231). Infection significantly decreased markers associated with antigen presentation and co-stimulation of DCs. Therefore, infection is able to alter the phenotype of APCs, which may alter the interaction between APCs and T-cells, and result in attenuated adaptive responses to allergen.

By contrast, NTHi infection induced potent neutrophilic inflammation in the airways. Persistent airway neutrophilia is also a feature common to chronic airway diseases, such as COPD (229), chronic bronchitis (205) and bronchiectasis (210), where recurrent infection is known to play an important role in pathogenesis. Neutrophilic inflammation is often associated with acute asthma exacerbations, and in particular infection-mediated exacerbations. Indeed, several studies have shown increased neutrophilic inflammation in both viral and bacterial infection-induced exacerbations (114, 115, 232). Here I show that NTHi induces strong neutrophilic inflammatory responses in a murine model of acute allergic asthma, and that NTHi may be involved in the development of neutrophilic asthma through the induction of neutrophilic inflammation. It has been demonstrated that immune

responses that lead to the development of NTHi-induced neutrophilic AAD occur in two phases. The first involves innate immune activation during infection that is likely to result in neutrophil chemoattraction to the airways. NTHi infection during OVA sensitisation resulted in a significant neutrophil influx to the airways 1 day after infection, a two-fold increase compared to NTHi infection alone. This correlated with the early expression of MIP2, KC, and IL-17 mRNA in the lungs. These neutrophils and to a greater extent macrophages were able to produce significantly more IL-17 than those from uninfected, allergic controls. Therefore these cells, particularly macrophages, may be sources of early (innate) IL-17 release. This observation may have important implications for other diseases where the innate source of IL-17 has not yet been identified.

The second phase involves adaptive immune responses during allergen challenge resulting in increased infection-mediated Th17 responses. During the challenge phase, days 12-15, there was a significant upregulation of ROR- γ t and IL-17 mRNA in the lungs of infected, allergic groups compared to uninfected, allergic controls. These results directly correlated with increases in Th17 cells in the lungs of infected, allergic groups. The increased production of IL-17 from T-cells in conjunction with increases in ROR- γ t expression suggest that infection drives Th17 responses that preferentially induce IL-17 production and neutrophilic inflammation during subsequent allergen challenge.

Collectively, our data suggest that infection induces early responses, involving neutrophils, IL-17, MIP2 and KC expression that may prime the host for enhanced Th17-mediated neutrophilic responses upon later allergen challenge, which subsequently induces neutrophilic AAD. Our findings are consistent with data from a recent study by Bullens *et al.*, which showed that increased IL-17 responses in asthmatics correlate with increased neutrophil numbers in sputum (68). Hellings *et al.*, (117) demonstrated that IL-17 is

important in lung neutrophil recruitment in response to an allergen, while Ye *et al.*, (223) showed that IL-17 responses and signalling through the IL-17R is vital for neutrophil recruitment and host defence against *Klebsiella pneumoniae* infection. Here these findings are extended by demonstrating that *H. influenzae* infection-induced IL-17 responses in AAD may play a role in driving neutrophilic inflammation in asthma. My colleagues have recently demonstrated that chlamydial respiratory infection is also able to drive neutrophilic asthma (178). Chlamydial infection also suppressed Th2-mediated eosinophilic inflammation and promoted neutrophilic inflammation.

Neutrophilic asthmatics are resistant to corticosteroid treatment, which is the mainstay of asthma therapy (135), and with evidence that asthmatics with infection are more resistant to steroids than asthmatics with no infection (233), alternative therapies are needed for infection-induced neutrophilic asthma.

Importantly I have shown that NTHi infection-induced neutrophilic AAD and the suppression of Th2 responses are dependent upon IL-17. Depletion of IL-17 with anti-IL-17 mAb during AAD prevented the induction of infection-induced neutrophilic AAD. This suggests that IL-17 is critical in the recruitment of neutrophils and may suppress Th2 responses in infection-induced neutrophilic inflammation and neutrophilic asthma. A study by Wakashin *et al.*, demonstrated that adoptive transfer of antigen-specific Th17 cells induced airway neutrophil recruitment, which supports our data (234). Little is known about how Th17 and Th2 cells interact with or regulate each other. These results demonstrate that infection inhibits cytokine release compared to uninfected allergic controls, and that anti-IL-17 treatment partially restored these effects, while having no effect on eosinophil recruitment (data not shown). This suggests that other mechanisms are also involved in the suppression of Th2 responses by NTHi infection, which requires further investigation. Several recent

studies have investigated the relationship between Th17 and Th2 cells. A study by Schnyder-Candrian *et al.*, showed that the administration of rIL-17 in a murine model of AAD significantly reduced allergen-induced eotaxin, thymus and activation-regulated chemokine (TARC) and IL-5, thereby reducing eosinophilic inflammation (235), while another study showed that inhibiting IL-17 in AAD also reduced airway eosinophils, neutrophils, AHR, and Th2 cytokines (236). These data suggest that IL-17 may suppress or promote eosinophilic inflammation, but the mechanisms that drive these different effects remain unknown. Our data is in agreement with Schnyder-Candrian *et al.*, who suggest that IL-17 interferes with DC activation and antigen uptake, which prevents T-cell activation and reduced IL-4, -5 and -13 production, leading to suppressed allergic responses. Interestingly, a recent study has shown a CD4⁺ T-cell subtype that expresses both Th17 and Th2 cytokines, including IL-17, IL-22, IL-4, IL-5 and IL-13, and this subset is increased in asthmatics compared to healthy controls (237). However, these cells have thus far only been found in the periphery, and confirmation of their presence is needed in BAL, sputum and/or bronchial biopsies.

In conclusion, I show that *H. influenzae* infection may be involved in the development of neutrophilic asthma. Infection suppressed features of Th2-mediated eosinophilic AAD, while inducing features of neutrophilic asthma that are mediated by infection-induced IL-17. Therefore, infection-induced IL-17 responses may play a major role in the pathogenesis of neutrophilic asthma. Our studies indicate the important role of infection in driving neutrophilic asthma-like disease, and identify new areas of investigation that may enhance the understanding of disease progression. Developing new treatments targeting infection may lead to better management of individuals with this disease phenotype.

Chapter 3:

Combined *Haemophilus influenzae* respiratory infection and allergic airways disease drives chronic infection and features of neutrophilic asthma

I show that the induction of AAD during *H. influenzae* infection impairs innate cell activation and function, which may result in delayed clearance of *H. influenzae* and the development of neutrophilic airways disease. This highlights the therapeutic potential of targeting infection as a novel treatment for neutrophilic asthma.

A revised version of this chapter has been accepted for publication in *Thorax*, December 2011.

3.1 Abstract

Neutrophilic asthmatics make up 20-30% of all asthmatics and are characterised by predominantly neutrophilic inflammation, and steroid insensitivity. These patients often have chronic airway colonisation with bacteria and *Haemophilus influenzae* is one of the most commonly isolated. The relationship between chronic airway bacterial colonisation and the development of steroid-resistant neutrophilic asthma is not understood. My aim was to investigate the relationship between *H. influenzae* respiratory infection and neutrophilic asthma using mouse models of infection and OVA-induced AAD. BALB/c mice were intratracheally infected with *H. influenzae* (day -10), then intraperitoneally sensitised (day 0) and intranasally challenged (day12-15) with OVA. Some groups were treated with intranasal dexamethasone during OVA challenge. Infection, key features of AAD, steroid sensitivity and innate immune responses were assessed after the final OVA challenge (day 16). The combination of *H. influenzae* infection and AAD resulted in chronic infection in the lung, and significant reduction in eosinophilic inflammation, OVA-specific IL-5, -13 and IFN- γ T-cell responses and airways hyperresponsiveness. By contrast, infection in AAD increased neutrophilic inflammation, Th17 cell development and IL-17 responses. Neutrophilic AAD in infected groups was steroid resistant. Innate immune cell activation was significantly reduced and the numbers of phagocytosing neutrophils and macrophages in the airways were significantly suppressed. In conclusion, the combination of infection and AAD promotes bacterial persistence, leading to the development of a disease phenotype that is similar to steroid-resistant neutrophilic asthma and may result from dysfunction in innate immune cells. This indicates that targeting bacterial infection in steroid-resistant asthma may have therapeutic benefit.

3.2 Introduction

Asthma is a chronic inflammatory condition of the airways. It is characterised by abnormal immune responses to environmental antigens, leading to recurrent episodes of cough, wheezing and breathlessness (5, 6, 238). Neutrophilic asthmatics make up between 20-30% of asthmatics, and are characterised by substantial increases in airway neutrophils and poor responsiveness to corticosteroids (125, 135). They are therefore difficult to treat and take up >50% of healthcare costs for asthma (134). Neutrophilic airway inflammation is common to many obstructive airway diseases including neutrophilic asthma (125), COPD (148), bronchiectasis (137), and allergic bronchopulmonary aspergillosis (239).

The cellular innate immune system of the respirator tract includes epithelial cells, neutrophils, macrophages, dendritic cells and lymphocytes (240). It is considered the first-line of immune defence, mounting an immediate non-specific response, and promoting antigen-specific responses by the adaptive component (240). Defects in innate immunity may be important in the development of steroid-resistant neutrophilic asthma, where increased sputum neutrophil, and IL-8 levels and increased expression of TLRs have been demonstrated (137).

Neutrophils and macrophages have critical roles in innate immunity, and are important contributors to airway inflammation, therefore any dysfunction in these cells may lead to the development or worsening of neutrophilic asthma. Airway neutrophils from asthmatics are less activated compared to healthy controls, which may lead to impaired local defence, and increased susceptibility to infections (241). Indeed, in COPD, which is also underpinned by neutrophilic bronchitis, airway neutrophils have impaired chemotaxis (242), and alveolar macrophages have reduced TLR-2 expression (243).

Infections, by both viruses and bacteria, have pathogenic roles in asthma and induce asthma exacerbations (244, 245). Recently we have shown that early-life infections play a role in the development of more severe AAD in later life (228). Chronic bacterial infection occurs frequently in airway diseases such as neutrophilic asthma (137, 138), COPD (203), and bronchiectasis (137). In neutrophilic asthma *H. influenzae* is one of the most commonly isolated bacteria (137, 138). However, the relationship between chronic *H. influenzae* infection and the development of steroid-resistant neutrophilic asthma is not understood.

In this study I used murine models of *H. influenzae* infection and OVA-induced AAD to investigate if and how the combination of infection and AAD may interact to promote chronic infection and the development of steroid-resistant neutrophilic asthma.

3.3 Methods

3.3.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the NSW Animal Research Regulation 2005, and the Australian Code of Practice for the care and use of animals for scientific purposes (National Health and Medical Research Council). All protocols were approved by the Animal Care and Ethics Committee of the University of Newcastle (permit number 987/0112). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts made to minimize pain and suffering.

3.3.2 Experimental protocols

Six to eight week old female BALB/c mice were supplied by the animal services unit. Mice received an i.t. inoculation of 5×10^5 CFU non-typeable *H. influenzae* (NTHi-289, in 30 μ l PBS), and AAD was induced 10 days later. Mice were sensitised to OVA (50 μ g, i.p., Sigma-

Aldrich, Sydney, NSW, Australia), with the adjuvant Rehydrogel (1mg, Reheis, Berkeley Heights, USA), and subsequently challenged intranasally with OVA (10µg/50µl saline) on days 12-15 to induce AAD. Saline groups were sham sensitised to saline with adjuvant and OVA challenges on d12-15, or infected and not exposed to OVA. Infection and hallmark features of AAD were assessed on day 16.

3.3.3 Bacterial recovery

Whole lungs were aseptically removed and homogenised in 1ml of sterile PBS. Serial dilutions of lung homogenates and BALF were plated onto chocolate agar plates (Oxoid, SA, Australia) and incubated overnight (37°C, 5% CO₂). Colonies were enumerated and total bacterial numbers in lungs were determined.

3.3.4 Cellular inflammation

Pentobarbitone overdose was used to euthanise mice. BALF was collected by washing the lungs twice with 1ml HBSS/wash. Cells were centrifuged (7 mins, 1400rpm), resuspended in RBC lysis buffer resuspended in sterile HBSS. Cytospins were prepared as previously described (181). Whole blood was collected by cardiac puncture and smears prepared on microscope slides. BALF cytopins and blood slides were stained with May-Grunwald and Giemsa, and differential leukocyte counts obtained using light microscopy, with a total of 200 cells counted in a blinded fashion (181).

3.3.5 T-cell cytokines

MLN T-cell single cell suspensions were prepared in media (RPMI supplemented with 10 % FCS, 20 mM HEPES, 10 µg/ml penicillin/streptomycin, 2 mM L-glutamine, 50 µM 2-

mercaptoethanol, 1 mM sodium pyruvate. This suspension was re-stimulated with either 200µg/ml OVA or 2×10^7 cfu/ml ethanol-killed NTHi for 6 days (5% CO₂, 37°C, 1×10^6 cells per well). After culture, supernatants were collected and analysed for IL-5, IFN-γ (BD Biosciences, North Ride, NSW), IL-17 and IL-13 (R&D Systems, Minneapolis, MN, USA) release by ELISA according to the manufacturer's instructions (178).

3.3.6 Lung function

AHR was measured as a change in dynamic compliance and transpulmonary resistance in response to increasing doses of methacholine, by whole body invasive plethysmography as previously described (180). Briefly, mice were anaesthetised, cannulated and connected to an inline ventilator and aerosol system. After dosing of increasing concentrations of aerosolised methacholine, peak pressure and flow waveforms were used to determine resistance and dynamic compliance.

3.3.7 Th17 cells

Single cell preparations of collagenase-D digested lungs were prepared. Briefly, MLN were pushed through a 70µm sieve, resuspended in RBC lysis buffer, washed and resuspended in 2% fetal calf serum/PBS. Lung tissues were processed using a gentleMacs according to manufacturer's instructions (Miltenyi). Cells were plated at 1×10^6 cells/well for staining. Th17 cells were identified by stimulation with PMA, (0.1µg/ml, Sigma-Aldrich) and ionomycin (1µg/ml, Sigma-Aldrich) in the presence of Brefeldin A (8µg/ml, Sigma-Aldrich) for 4 hours at 37°C, 5%CO₂. Cells were stained for surface markers CD3 (PerCP), CD4 (FITC; BD Bioscience) for 30 minutes on ice, fixed with 4% PFA, permeabilised with 0.1%

saponin for 1 hour at 4°C, and stained for intracellular IL-17 (APC: eBioscience San Diego, CA, USA). 50 000 events were recorded and cells were analysed using a FACS Canto and FACS Diva software (version 5.1, BD Biosciences).

3.3.8 Dexamethasone treatment

Dexamethasone (Sigma-Aldrich) was prepared in sterile saline (1mg/kg) and administered by i.n. droplets (50µl, days 13-15, Figure 3.4A).

3.3.9 Mucus secreting cells

The left lung was excised, immersed in formalin, then imbedded in paraffin. These blocks were sectioned (4-6µm), and stained with periodic acid Schiff. MSC numbers within the airway lumen (10 x 100µm fields) were enumerated using light microscopy, and the mean calculated (178).

3.3.10 Ciliary beat frequency

Tracheas were excised, thin transverse slices prepared, placed in a T-dish and cover-slipped. The tracheal slices were examined and the ciliary beat frequency measured using transmitted light photometry. Ciliary beating was measured from three epithelial region sections per mouse and was assessed on an inverted microscope connected to a receiver. Cilia beat frequency was assessed for one minute per section and mean frequency (Hz) was calculated.

3.3.11 Phenotyping of immune cells

Macrophages (CD11b⁺(PerCP)F4/80⁺(PE), eBioscience, San Diego, CA, USA) and neutrophils (CD11b^{hi}Gr-1^{hi}[APC], eBioscience) were stained and enumerated in

collagenase-D treated lungs, following manufacturer's instructions.. Cells were also stained for the activation marker CD62L(PE), and surface TLR-2(PerCP) and TLR-4 (FITC; eBioscience). 50 000 events were recorded and analysed using flow cytometry. Total cell numbers expressing the markers assessed were quantified by multiplying the total percentage of positive staining events by the total number of cells obtained.

3.3.12 Phagocytosis

NTHi was heat inactivated (60°C) for 1 hour and labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, 5µM) for 30 minutes, then opsonised by incubating with 50% pooled serum for 15 minutes (246). BALF cells were then incubated with CFSE-conjugated NTHi at a ratio of 1:20 (cell: bacteria) for 30 minutes (37°C, 5% CO₂). Excess NTHi was washed off, and cells were stained for CD11b(PerCP), F4/80(PE) and Gr-1(APC) and fixed with 4% PFA. Phagocytosis of CFSE-labeled NTHi was assessed in fixed cells using flow cytometry. 50 000 events were recorded and analysed.

3.3.13 Oxidative burst

A single cell suspension of lung cells were incubated with RBC lysis buffer, washed in HBSS and resuspended in RPMI. This suspension was pre-incubated with PMA (100ng/ml) for 15 minutes, washed and incubated with dihydroethidium (DHE, Sigma-Aldrich) for 15 minutes (37°C, 5% CO₂) (246). Reactive oxygen species oxidise DHE (colourless) to a red fluorescent compound. Reactions were terminated by placing on ice, and cells were washed and fixed with 4% PFA. Oxidative burst was assessed in fixed cells using flow cytometry.

3.3.14 Statistical analyses

Experiments were performed using 6-8 mice in duplicate and results presented as mean $\pm$ standard error of the mean (SEM). Statistical significance for multiple comparisons was determined by one-way ANOVA with the Bonferroni post-test. One-way repeated measures ANOVA with Bonferroni post-test was used to analyse AHR data for the entire dose-response curve (GraphPad Prism Software, La Jolla, USA).

3.4 Results

3.4.1 AAD during infection leads to chronic infection

To investigate the relationship between bacterial infection and AAD, I first assessed the effect of AAD on *H. influenzae* infection. In other studies I have shown that after inoculation with 5×10^5 CFU, *H. influenzae* infection in the absence of allergic airways disease peaks after 5d ($1.56 \times 10^6 \pm 9 \times 10^3$ CFU), declines after 10d ($4 \times 10^2 \pm 1 \times 10^2$ CFU) and is undetectable by 16d (data not shown).

Next, mice were inoculated with *H. influenzae* and 10 days later, after the majority of *H. influenzae* had been cleared, AAD was induced (Figure 3.1A). The induction of AAD promoted the development of chronic infection with substantial levels of *H. influenzae* recovered 26 days after inoculation of infected allergic (Hi/OVA) groups compared to infected non-allergic (Hi) groups (i.e. not OVA sensitised) (Figure 3.1B). Uninfected allergic controls were also assessed and neither Hi nor commensal bacteria were detected (data not shown), ruling out the possibility that AAD induces substantial alterations in commensal species.

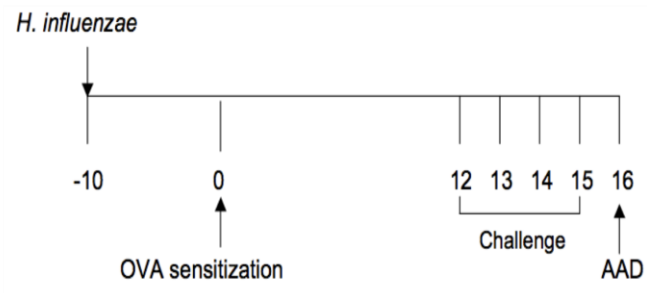
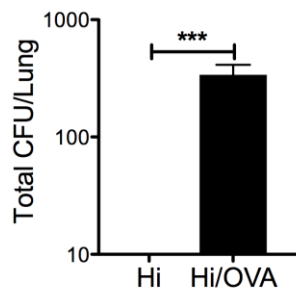
A**B**

Figure 3.1: AAD during *H. influenzae* infection promotes chronicity of infection. Mice were inoculated intratracheally with 5×10^5 CFU of NTHi, then 10 days later sensitised intraperitoneally (day 0) and challenged intranasally (days 12-15) with OVA. Allergic airways disease and infection were assessed (day 16) and compared to controls (A). The effects of AAD on bacterial recovery from lungs (CFU/lung) were characterised (B). *** $p < 0.001$.

3.4.2 Chronic *H. influenzae* in AAD suppresses eosinophilic inflammation

To investigate the effects of chronic *H. influenzae* infection on AAD, mice were infected, AAD induced (Figure 3.1A), and key features of AAD were assessed.

The induction of AAD (OVA groups) resulted in eosinophilic inflammation, OVA-induced IL-5, IL-13 and IFN- γ release from MLN T-cells and AHR, (decreased dynamic compliance and increased transpulmonary resistance) compared to uninfected non-allergic (Saline) controls (Figure 3.2A-G). Infection alone had no effects on these features. Infection during AAD significantly reduced the numbers of total cells and eosinophils in BALF compared to uninfected allergic (OVA) controls (Figure 3.2A-B). The suppression of airway responses were accompanied by reductions in OVA-induced IL-5, IL-13 and IFN- γ , and airways resistance (Figure 3.2A-G).

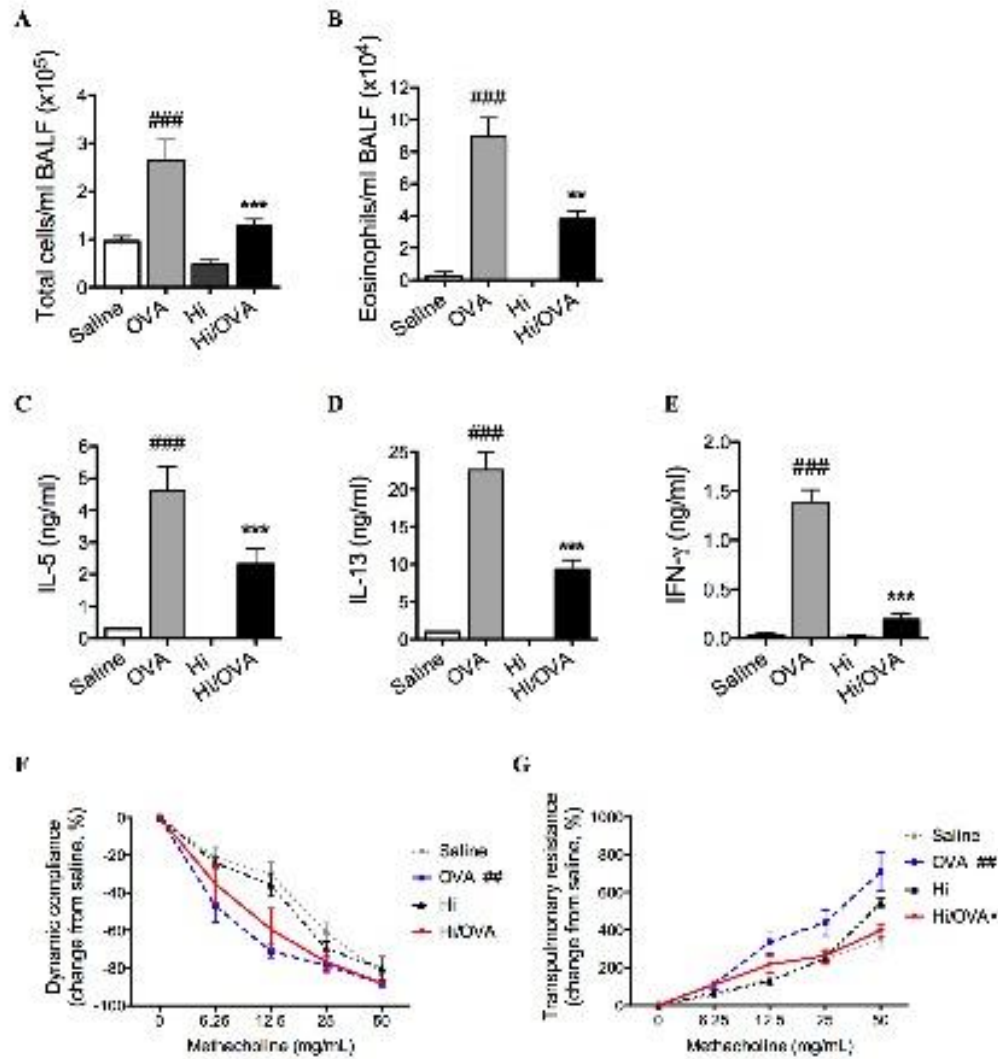


Figure 3.2: Chronic infection suppresses hallmark features of eosinophilic AAD. The effects of chronic *H. influenzae* infection in AAD on the numbers of total cells (A) and eosinophils (B) in BALF, and OVA-induced IL-5 (C), IL-13 (D) and IFN- γ (E) release from MLN T-cells were assessed. AHR, measured as dynamic compliance (F) and transpulmonary resistance (G) in response to increasing doses of methacholine was determined. P values for compliance and resistance were determined for the entire dose response curve. ### p<0.001, ## p<0.01 compared to Saline controls, *** p<0.001, ** p<0.01, * p<0.05 compared to OVA controls.

3.4.3 Chronic *H. influenzae* in AAD induces neutrophilic inflammation, Th17 cell and IL-17 responses

H. influenzae has frequently been isolated from neutrophilic asthmatics, and increased neutrophilic inflammation has also been observed in sputum from these patients. Therefore, it was investigated if chronic *H. influenzae* infection in AAD induced enhanced neutrophilic inflammation.

The induction of AAD was associated with mild neutrophilic inflammation (Figure 3.3A-B). The combination of chronic infection and AAD significantly increased the influx of neutrophils into the airway and blood, compared to un-infected allergic groups (Figure 3.3A-B).

Asthmatics have increased levels of IL-17 mRNA in sputum, which stimulates the recruitment of neutrophils. Therefore, the numbers and levels of Th17 cells and IL-17 in infection and AAD were assessed. The induction of AAD resulted in increased numbers of Th17 cells in lung tissue and IL-17 release from MLN T-cells stimulated with NTHi (Figure 3.3C-D). Infection in AAD also significantly increased the numbers of Th17 cells in the lung, and IL-17 release from MLN T-cells (Figure 3.3C-D). There were no differences in OVA-induced IL-17 production (data not shown).

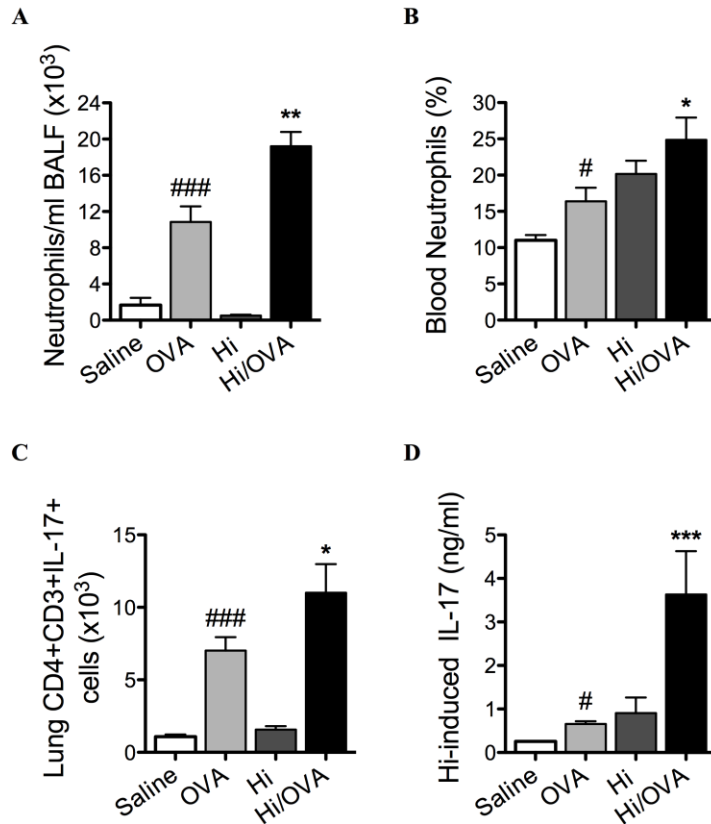


Figure 3.3: Chronic infection during AAD induces neutrophilic inflammation and Th17 responses. The effects of chronic *H. influenzae* infection in AAD on the levels of neutrophils in the airways (A) and blood (B), Th17 cell numbers in the lung (C) and NTHi-induced IL-17 release from MLN T-cells (D) were assessed. ^{###} $p < 0.001$, [#] $p < 0.05$ compared to Saline controls, ^{***} $p < 0.001$, ^{**} $p < 0.01$, ^{*} $p < 0.05$ compared to OVA controls.

3.4.4 Chronic *H. influenzae* infection in AAD induces steroid resistance

Many neutrophilic asthmatics are resistant to steroid therapy. Therefore, I investigated the sensitivity of infection and neutrophilic AAD to dexamethasone treatment.

Allergic and infected allergic groups were administered dexamethasone (1mg/kg) intranasally during OVA challenge (days 13-15, Figure 3.4A), and infection and key features of AAD were assessed. Steroid treatment had no effect on bacterial clearance (Figure 3.4B). Dexamethasone treatment of uninfected, allergic (OVA/Dex) groups resulted in a significant reduction in all of the key features of AAD (Figure 3.4C-J). In stark contrast, treatment of infected allergic groups had no effect on eosinophil or neutrophil recruitment to the airways, Hi-induced IL-17, or OVA-induced IL-5, IL-13 or IFN- γ release from MLN T-cells, or AHR, compared to untreated infected, allergic groups (Figure 3.4C-J).

Collectively, these data demonstrate that the induction of AAD promotes chronic infection, despite the presence of increased numbers of neutrophils, and leads to the development of neutrophilic AAD that is steroid resistant.

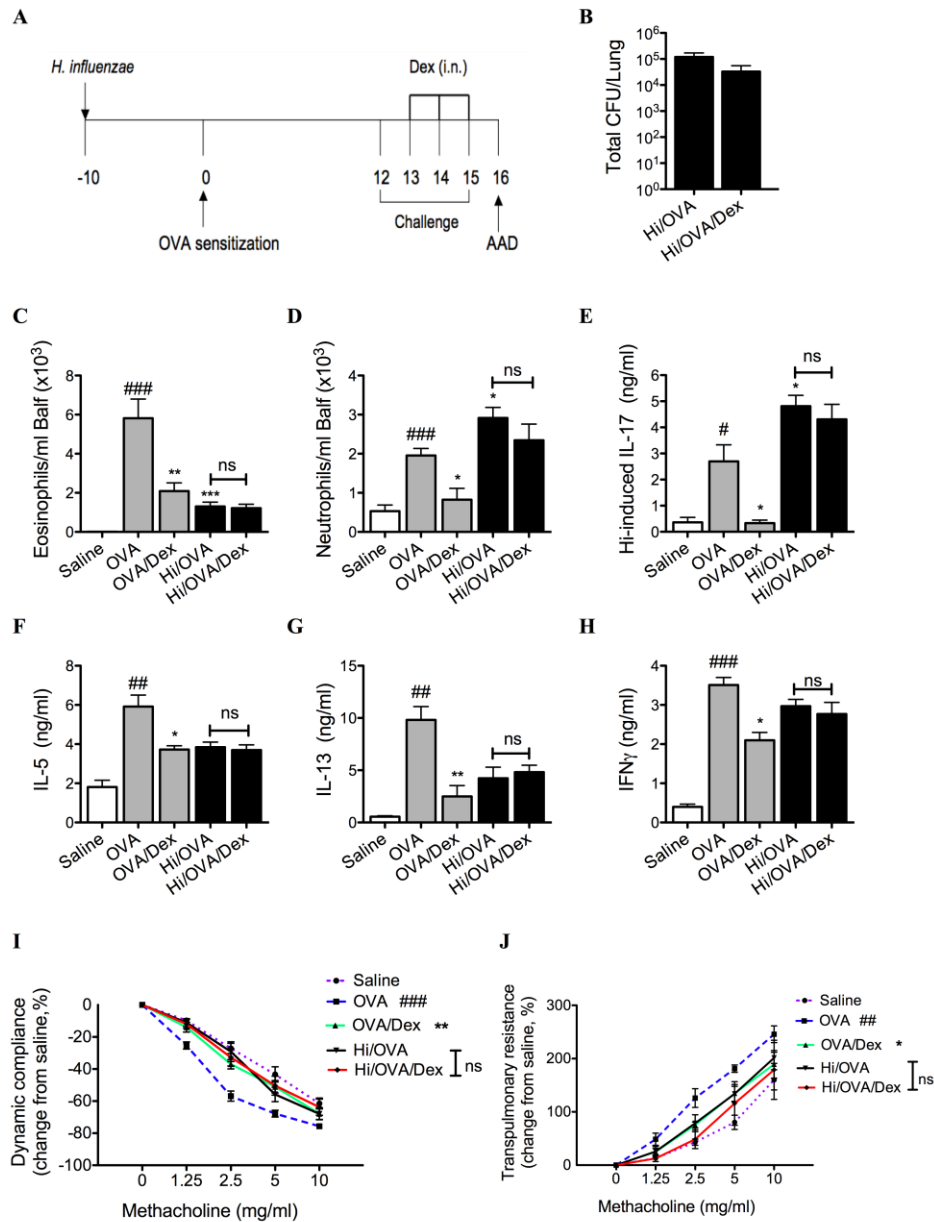


Figure 3.4: Chronic infection during AAD induces steroid resistance. The effects of chronic *H. influenzae* infection in AAD on responsiveness to steroid treatment were investigated. Infected allergic groups were treated intranasally with dexamethasone during OVA challenge (days 13-15) and AAD was assessed (day 16) and compared to controls (A). To determine the effect of treatment on infection, bacterial recovery (B) was assessed. To determine the effect of treatment on neutrophilic AAD numbers of eosinophils (C) and neutrophils (D) in BALF, Hi-induced IL-17 (E), OVA-induced IL-5 (F), IL-13 (G), IFN- γ (H), and dynamic compliance (I) and transpulmonary resistance (J) were determined. #### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls.

3.4.5 Chronic *H. influenzae* infection in AAD does not affect mucus secreting cell numbers or ciliary beat frequency

The mechanisms involved in promoting chronic infection were subsequently investigated. Reduction of both cilia function and mucus production in the airways may compromise bacterial clearance. Therefore, the effect of infection in AAD on MSC numbers and ciliary beat frequency in airway epithelia was assessed.

The induction of AAD significantly increased MSC numbers, but had no effect on ciliary beat frequency (Figure 3.5A-B). Infection in AAD had no effect on either MSCs or cilia beat frequency compared to uninfected allergic controls (Figure 3.5A-B).

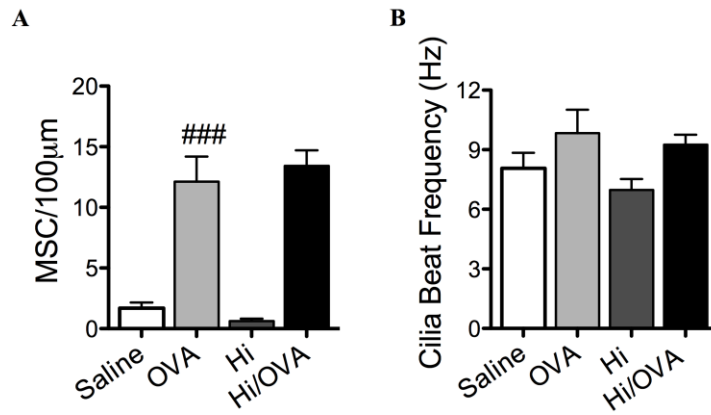


Figure 3.5: Chronic *H. influenzae* infection in AAD does not affect mucus secreting cell numbers or cilia beat frequency. The effect of chronic *H. influenzae* infection in AAD on tissue MSCs (A) and ciliary beat frequency (B) were assessed. ### $p < 0.001$ compared to Saline controls.

3.4.6 Chronic *H. influenzae* infection in AAD inhibits innate immune cell activation and function

Innate immune responses are impaired in neutrophilic asthma. Therefore, I examined if chronic infection and neutrophilic AAD were associated with alterations in phagocyte function by assessing TLR expression, activation and function of neutrophils and macrophages.

TLR-2 and -4 expression is indicative of increased activation of neutrophils and macrophages. The induction of AAD increased TLR-4 expression on macrophages but had no effects on the expression of TLR-2 or TLR-4 on neutrophils or TLR-2 on macrophages. Infection in AAD resulted in small but significantly increased expression of TLR-2 on macrophages but had no effects on the expression of TLR-2 or TLR-4 on neutrophils or TLR-4 on macrophages compared to uninfected allergic controls (Figure 3.6A-B).

Increased CD62L expression is a marker of reduced activation of innate immune cells. The induction of AAD had no effect on the expression of CD62L positive neutrophils and macrophages. However, infection in AAD resulted in a significant increase in CD62L positive neutrophils and macrophages, indicating reduced activation, compared to uninfected allergic controls (Figure 3.6C-D).

The function of neutrophils and macrophages in terms of phagocytosis and oxidative burst was then investigated. The induction of AAD increased the numbers of phagocytosing lung neutrophils and macrophages, but not oxidative burst in lung tissue. Critically, infection in AAD completely suppressed the numbers of phagocytosing neutrophils and macrophages in the airways (Figure 3.6E) but increased oxidative burst compared to uninfected allergic groups (Figure 3.6F). Indeed the numbers of phagocytosing neutrophils and macrophages was reduced to the same levels as in uninfected non-allergic (Saline) controls.

Collectively these results suggest that decreased activation and function of innate immune cells may contribute to the establishment of chronic infection in steroid-resistant neutrophilic AAD.

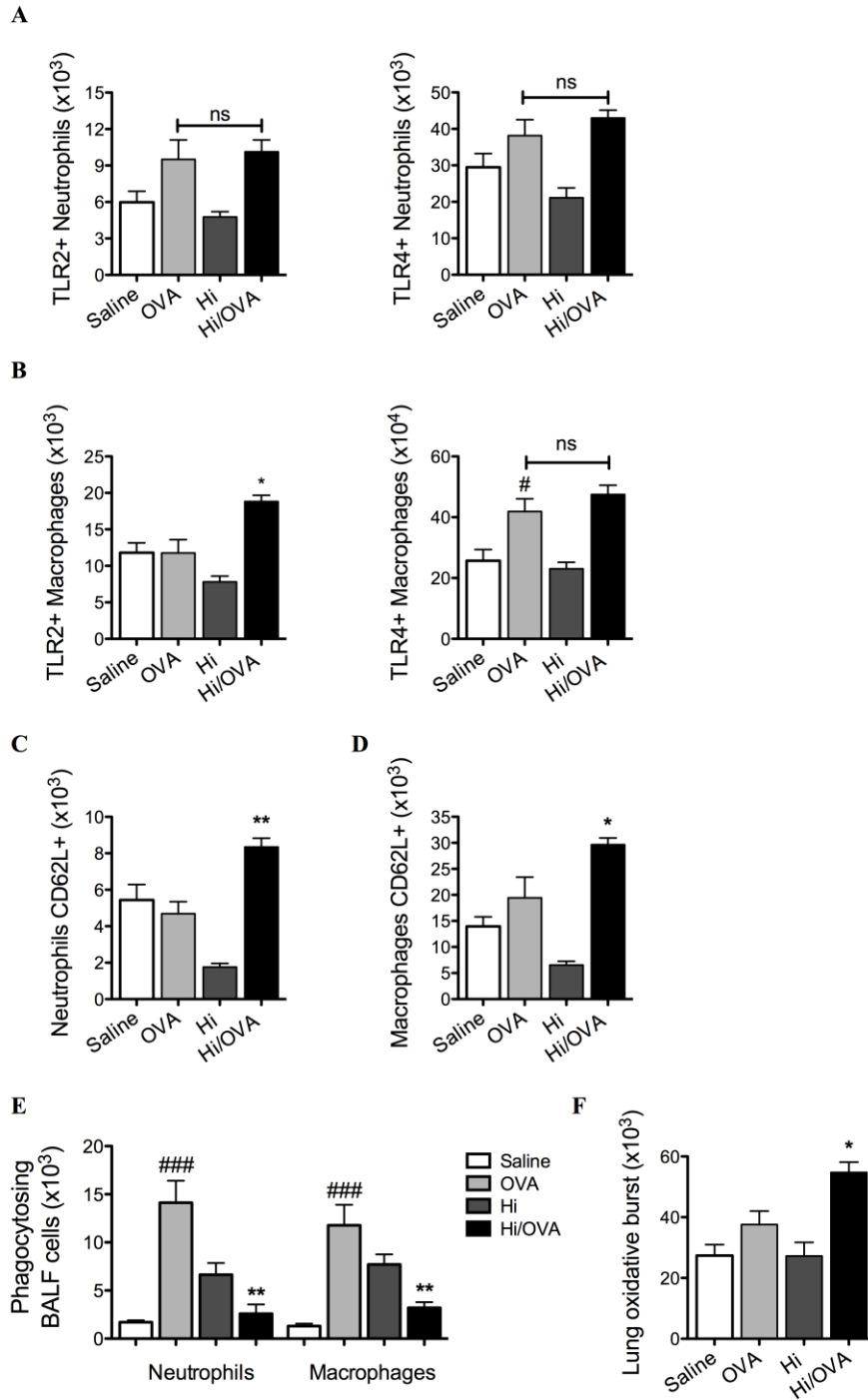


Figure 3.6: Chronic *H. influenzae* infection in AAD inhibits innate immune cell activation and function. The effect of chronic infection in AAD on neutrophil and macrophage TLR-2 and TLR-4 expression (A, B), and activation status (C, D) was examined. The effects of infection on neutrophil and macrophage phagocytosis (E) and oxidative burst (F) was also assessed. ### $p < 0.001$, # $p < 0.05$ compared to Saline controls, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls.

3.5 Discussion

This study demonstrates that the combination of *H. influenzae* infection and AAD results in chronic infection that drives the development of steroid-resistant neutrophilic AAD. I have also shown that chronic infection and AAD impairs the activation and function of airway neutrophils and macrophages, which may promote bacterial persistence.

Chronic infection is commonly associated with many chronic airway diseases that are characterised by neutrophilic inflammation such as neutrophilic asthma (137, 138), COPD (203, 247), bronchiectasis (204) and chronic bronchitis (205). A recent study showed that of the 41% of neutrophilic asthmatics that had a significant load of potentially pathogenic bacteria, *H. influenzae* was detected in the airway secretions of 60% of these patients (138). My data add to this observation by demonstrating that an allergic environment during *H. influenzae* infection results in the suppression of innate immune cell activation and function, which may lead to chronic infection.

These results show that NTHi infection in AAD suppressed features of eosinophilic asthma, including eosinophilic inflammation and allergen-induced Th2 cytokines. Infection in AAD simultaneously induced features of neutrophilic asthma, including intense airway neutrophilic inflammation and AHR that was reduced compared to eosinophilic AAD but still elevated above non-allergic controls (125, 127, 128, 135).

The role of infection in the development of neutrophilic asthma is a potentially important one. Our group has recently shown that respiratory infection with *Chlamydia* also drives the development of neutrophilic AAD, and that the suppression of neutrophils during infection prevented the development of this phenotype (178). In the current study I show that the expansion of neutrophils is accompanied by increases in both Th17 cells and IL-17 protein production. IL-17 has been detected in asthmatic airways (68), is important in

promoting neutrophilic inflammation (212, 226, 248), and has recently been identified in COPD patients (213). This provides further evidence of the potentially important contribution that Th17 cells and IL-17 have to infection-induced neutrophilic asthma.

An important feature of neutrophilic asthmatics is that they are unresponsive to corticosteroids, which are the mainstay of asthma therapy. This study demonstrates that all of the key features of infection-induced neutrophilic AAD, including infection, airway eosinophils and neutrophils, Th1, Th2 and Th17 cytokine responses and AHR are resistant to dexamethasone treatment. Several molecular mechanisms have been suggested to explain this steroid resistance: Abnormalities in glucocorticoid receptors, whereby increases in GR- β bind to DNA and not steroids, and increased activation of activator-protein-1, which counteracts the anti-inflammatory properties of steroids (249, 250). Interestingly, it has recently been shown that *in vitro*, IL-17 is able to induce the expression of GR- β mRNA in asthmatic epithelial cells (251). Another study showed that SCID mice reconstituted with Th17 cells were resistant to treatment with dexamethasone, while treatment in mice reconstituted with Th2 cells significantly reduced inflammation and AHR. However, the issue that has received the most attention is that *in vitro*, compared to eosinophils and T-cells, neutrophil apoptosis is resistant to steroid action, therefore steroids prolong neutrophil survival (252-254). Taken together these studies suggest that a number of factors may be involved in steroid resistance in neutrophilic asthmatics and that infection-induced increases in neutrophilic inflammation, Th17 cells and IL-17 production may be important in promoting steroid resistance.

The mechanisms involved in inducing chronic infection were then investigated. Chronic infection developed despite the increase in airway neutrophils in infected allergic groups compared to allergic controls. Firstly, I aimed to determine if there was a reduction in

mucus secretion that may inhibit the removal of bacteria. I found that mucus secreting cell numbers were not different in infected compared to uninfected allergic groups and probably only occurs as a result of the induction of AAD. It is possible that the increase in mucus production in AAD may actually inhibit the clearance of bacteria. In the healthy airway, normal mucociliary clearance of inhaled particles and bacteria requires the interaction of normally beating respiratory cilia and the overlying mucus blanket. Reduction of both ciliary function and mucus production in the airways may compromise bacterial clearance. Infection and inflammatory cells have been reported to impair both ciliary beat frequency and mucociliary clearance (255). The preservation of ciliary beat frequency suggests that the impairment of bacterial clearance was not a direct result of ciliotoxicity.

The cellular components of innate immunity consist mainly of neutrophils and macrophages, which when activated mount an immediate non-specific response and clear bacteria by phagocytosis. I therefore investigated the combined effects of infection and AAD on the activation and function of airway neutrophils and macrophages.

TLRs are pattern recognition receptors on innate immune cells and play an important role in resistance to infection (23). Although TLR-2 has an important role, TLR-4 is crucial for host defence and clearance of pulmonary NTHi infection (256). TLRs are expressed on neutrophils and macrophages and increases in their expression activation and signalling is involved in the activation, chemokine receptor expression and function of these phagocytic cells (257). The results here show that in infected allergic groups TLR-2 and TLR-4 expression was unchanged compared to allergic controls. The only exception was increased TLR-2 expression on macrophages. This demonstrates that infection in AAD does not substantially alter TLR expression levels on innate immune cells. TLR expression is significantly increased on neutrophils and macrophages from infected allergic compared to

infected only groups. This indicates that a lack of TLR stimulation is not responsible for chronic infection.

Neutrophil and macrophage activation was investigated. Activation of neutrophils and macrophages occurs in stages and includes a range of phenotypic and functional changes. Low levels of activation lead to the modulation of surface receptors and priming to subsequent stimuli (258), while higher activation levels enhance function (259). CD62L (L-selectin), is a surface receptor that is highly expressed on resting neutrophils and circulating monocytes, and mediates their migration to the site of inflammation (260, 261). During activation, migration and maturation, CD62L expression is down-regulated by shedding and its expression is therefore a marker of reduced activation and maturity (258, 260, 262, 263). This study demonstrates that chronic *H. influenzae* infection during AAD results in increases in the numbers of neutrophils and macrophages expressing CD62L, indicating that NTHi suppresses the activation of these cells.

Subsequently, the function of neutrophils and macrophages was examined. Once activated, neutrophils and macrophages gain microbicidal functions, the most important of which is phagocytosis and elimination of invading pathogens (264, 265). Critically I show that infection in AAD completely inhibited the influx and/or development of phagocytosing neutrophils and macrophages in the airways compared to allergic controls. The complete abrogation of phagocytosing cells may be the direct result of a lack of activation of these cells and is the likely reason for the development of chronic infection in neutrophilic AAD. Since the only resident cells in the airways are alveolar macrophages, it is likely that the inhibition of phagocytosing cells results from reduced influx into the airways, however, a reduction in the development of phagocytosing alveolar macrophages cannot be ruled out at this stage.

My results show that lung cells from infected allergic groups generated more oxidative burst than allergic controls. Oxidative burst increases oxidative stress and inflammation and has significant effects on the pathophysiology of asthma, such as increasing airway smooth muscle contractility (266). A recent study demonstrated increased oxidative stress in asthmatics who had increased bacterial load compared to healthy controls. The authors suggested that the oxidative stress was due to the increased bacterial load rather than increased neutrophilic inflammation (138).

Taken together these results may explain the association of infection with neutrophilic asthma. AAD promotes persistent infection and induces the influx of defective innate immune cells that fail to clear the bacteria and may increase oxidative stress.

In conclusion, I show that the induction of AAD during *H. influenzae* infection leads to chronic infection. Chronic infection together with infection-induced increases in Th17 cells and IL-17 production may lead to the development neutrophilic AAD. I also demonstrate that this phenotype is steroid resistant, which may be the result of a combination of increased neutrophils and Th17 cells. Chronic infection is likely due to the suppression of innate cell activation and function.

Further studies of the mechanisms of neutrophilic asthma are required to better understand disease progression. Such studies would enable the development of more effective treatments for patients with this disease phenotype, which may potentially target the infection as well as asthmatic responses.

Chapter 4:

Combination macrolide and steroid treatment: a potential therapy for neutrophilic asthma

I demonstrate that antibiotic treatment inhibited neutrophil and IL-17 responses in infection-induced neutrophilic AAD but did not reduce Th2-mediated eosinophilic responses. However, antibiotic and steroid combination treatment significantly reduced all features of neutrophilic airways disease, suggesting this combination therapy may be effective in neutrophilic asthmatics with evidence of infection.

This chapter will be submitted to the Journal of Immunology for publication in December 2011.

4.1 Abstract

Neutrophilic asthmatics are often resistant to steroid treatment and the airways of these patients are commonly chronically colonised with bacteria. There is an urgent need for effective therapies for neutrophilic asthma. Macrolides have potent antibiotic and anti-inflammatory functions, and may be effective in chronic inflammatory airway diseases such as asthma. My aim was to investigate the potential of macrolides alone or in combination with steroids as a therapy for neutrophilic asthma, using mouse models of *Haemophilus influenzae*-induced neutrophilic AAD. AAD was induced by intraperitoneal sensitisation with OVA, (day 0), followed by OVA intranasal challenges (days 12-13 and 33-34 or days 12-15). Mice were intratracheally inoculated with *H. influenzae* (day 14 or day -10). Mice were treated with 3 daily doses of clarithromycin, 2 days after infection, and in some groups were administered intranasal dexamethasone during OVA challenge (days 13-15). The effect of treatment on key features of AAD was assessed after the final OVA challenge (day 35 or 16 respectively). Infection in AAD reduced Th2 responses and eosinophilic inflammation, but increased airway neutrophil influx and IL-17 responses. Clarithromycin treatment reversed these effects and re-instated Th2 mediated responses and eosinophilic inflammation, but significantly reduced neutrophils and IL-17 responses. Clarithromycin combined with dexamethasone abrogated all key features of neutrophilic and eosinophilic AAD. In conclusion, macrolide treatment inhibited neutrophil and IL-17 responses in infection-induced neutrophilic AAD, which led to enhanced Th2-mediated eosinophilic responses. Combination macrolide and steroid therapy significantly reduced all features of eosinophilic and neutrophilic AAD, suggesting this combination therapy may be effective in patients with neutrophilic asthma with evidence of infection.

4.2 Introduction

Steroids are the mainstay of asthma treatment but around 10-20% of asthmatics respond poorly to steroids. Steroid resistance is defined as a lack of improvement in lung function of >15% after standard treatment, which is usually a daily single dose of 30-40mg of oral prednisone for 2 weeks (153, 165). Steroid-resistant asthma is difficult to treat and accounts for more than 50% of asthma-related healthcare costs (134, 165). Alternative treatments are urgently required to reduce disease morbidity and burden. Many patients that are steroid-resistant have predominantly neutrophilic inflammation of the airways and have persistent bacterial colonisation. Emerging evidence suggests that targeting infection in neutrophilic, steroid-resistant asthma may be an effective therapeutic approach, however this possibility has been little studied.

Glucocorticoids are widely used in steroid therapy and function by binding to an intracellular receptor (GR). This receptor consists of a DNA and steroid binding subunit and two heat-shock proteins. Binding to the receptor dissociates the heat-shock proteins, exposing the DNA-binding site. This GR complex translocates to the nucleus and binds to glucocorticoid response elements on DNA sites, and subsequently regulates transcription (155, 156). Regulation of transcription occurs either as a result of direct binding of the activated GR to regulatory DNA sequences, or proteins from primary-regulated genes subsequently modifying other genes. Regulation may also occur by the recruitment of co-activator and/or co-repressor proteins to the receptor complex that lead to activation or suppression of transcription (158, 159). Many possible mechanisms of steroid resistance in asthmatics have been proposed (267), including abnormally reduced GR binding affinity on T lymphocytes, or a normal binding affinity but a reduction in the number of GRs per cell (164). Other mechanisms include increased activation of AP-1 which directly interacts with

the GR, reducing the anti-inflammatory effects of glucocorticoids (268). Finally, steroid-resistant asthmatics have increased expression of the GR- β splice variant which binds DNA and not glucocorticoid, resulting in non-responsiveness to treatment (166). Asthma is poorly controlled in steroid-resistant patients, who are typically prescribed high-dose steroid treatment which have limited efficacy and side-effects. These side-effects include decreased bone density that doubles the risk of fractures (269), and adrenal insufficiency (270).

Macrolides are potent antimicrobials, and inhibit bacterial adherence, virulence and biofilm formation (170). Macrolides also have anti-inflammatory properties and decrease inflammatory cell accumulation in the airways, inhibit airway hyperresponsiveness, and may potentiate glucocorticoid responsiveness (173-175). Positive effects of macrolide treatment have been demonstrated in panbronchiolitis (271), chronic sinusitis (272), and COPD (273) and may be an alternative therapy for neutrophilic steroid-resistant asthma.

I have recently demonstrated that infection in mouse models of OVA-induced AAD leads to neutrophilic AAD that is steroid resistant, which agrees with other studies (178, 274). In this study murine models of NTHi-induced neutrophilic AAD were used to investigate the effectiveness of macrolides as a potential alternative or combination therapy with steroids, for neutrophilic AAD.

4.3 Methods

4.3.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the NSW Animal Research Regulation 2005, and the Australian Code of Practice for the care and use of animals for scientific purposes (National Health and Medical Research Council). All protocols were approved by the Animal Care and Ethics Committee of the University

of Newcastle (permit number 987/0112). All surgery was performed under sodium pentobarbital anaesthesia, and all efforts made to minimize pain and suffering.

4.3.2 Experimental protocols

AAD was induced by sensitisation to OVA (50µg, i.p., Sigma-Aldrich, Sydney, NSW, Australia), with the adjuvant Rehydrogel (1mg, Reheis, Berkeley Heights, USA), and subsequently challenged intranasally with OVA (10µg/50µl saline), on days 12-13 and 33-34; or on days 12-15. Infected groups received an i.t inoculation of 5×10^5 CFU NTHi-289 (in 30µl PBS). Control groups were sham sensitised to saline with adjuvant (Saline). Features of AAD were assessed 24 hours after the final challenge (day 35 or 16).

4.3.3 Cellular inflammation

Mice were euthanased with an overdose of pentobarbitone, and BALF was collected and processed. Cytospin slides were prepared, while whole blood was collected by cardiac puncture and blood smears prepared as previously described (178, 275). BALF and blood slides were stained with May-Grunwald and Giemsa, and differential cell counts were obtained using light microscopy in a blinded fashion (178).

4.3.4 T-cell cytokines

MLN single cell suspensions were restimulated with either 200µg/ml OVA or 2×10^7 cfu/ml ethanol-killed NTHi, and cultured for 6 days (5% CO₂, 37°C, 1×10^6 cells per well). Culture supernatants were analysed for IL-5, IFN-γ (BD Biosciences, North Ride, NSW), IL-17 and IL-13 (R&D Systems, Minneapolis, MN, USA) release by ELISA according to the manufacturer's instructions (228).

4.3.5 Lung function

Dynamic compliance and transpulmonary resistance in response to increasing doses of methacholine were investigated by whole body invasive plethysmography as a measure of AHR. This has been previously described (227).

4.3.6 Macrolide and dexamethasone administration

Clarithromycin (Sigma-Aldrich) was prepared in sterile PBS (500mg/kg). Mice were lightly anaesthetised with isoflurane (Abbott Australia), and clarithromycin was administered by gavage (200µl daily, 3 doses). Dexamethasone (Sigma-Aldrich) was prepared in sterile saline (1mg/kg) and delivered i.n., (50µl).

4.3.7 Bacterial recovery

Whole lungs were aseptically removed and homogenised in sterile PBS (1ml). Serial dilutions of lung homogenates and BALF were plated onto chocolate agar plates (Oxoid, SA, Australia) and incubated overnight (37°C, 5% CO₂). Colonies were enumerated and total bacterial numbers in lungs were determined by combining BALF and lung tissue counts.

4.3.8 Statistical analysis

Experiments were performed using 6-8 mice in duplicate and results presented as mean ± standard error of the mean (SEM). Statistical significance was determined by one-way ANOVA, or Student's t-test (GraphPad Software, CA) and accepted if $p < 0.05$.

4.4 Results

4.4.1 NTHi infection in established AAD suppresses Th2 mediated eosinophilic responses

I have previously shown that *H. influenzae* infection before, during or after allergic sensitisation suppresses eosinophilic and induces neutrophilic inflammation. However, the effects of infection in established AAD remains unknown. To examine the effect of infection on established AAD, mice were infected with NTHi after the induction of AAD and then re-challenged with allergen (OVA) 21 days later (Hi, Figure 4.1A). This models infection and allergen exposure in established disease. In all groups the key features of AAD were analysed on day 35.

The induction of AAD (OVA) induced eosinophilic inflammation, OVA-induced IL-5, IL-13 and IFN- γ production from MLN T-cells, and AHR (decreased dynamic compliance and increased transpulmonary resistance; Figure 4.1B-F).

Infection after AAD (Hi), significantly reduced features of AAD, including numbers of total cells and eosinophils in the airways, and OVA-induced IL-5, IL-13 and IFN- γ production compared to uninfected allergic (OVA) controls (Figure 4.1B-D). Although infection increased dynamic compliance and decreased transpulmonary resistance (i.e. reduced AHR) compared to allergic controls, this was not statistically significant (Figure 4.1E-F). There were no significant changes in the number of BALF macrophages or lymphocytes (data not shown).

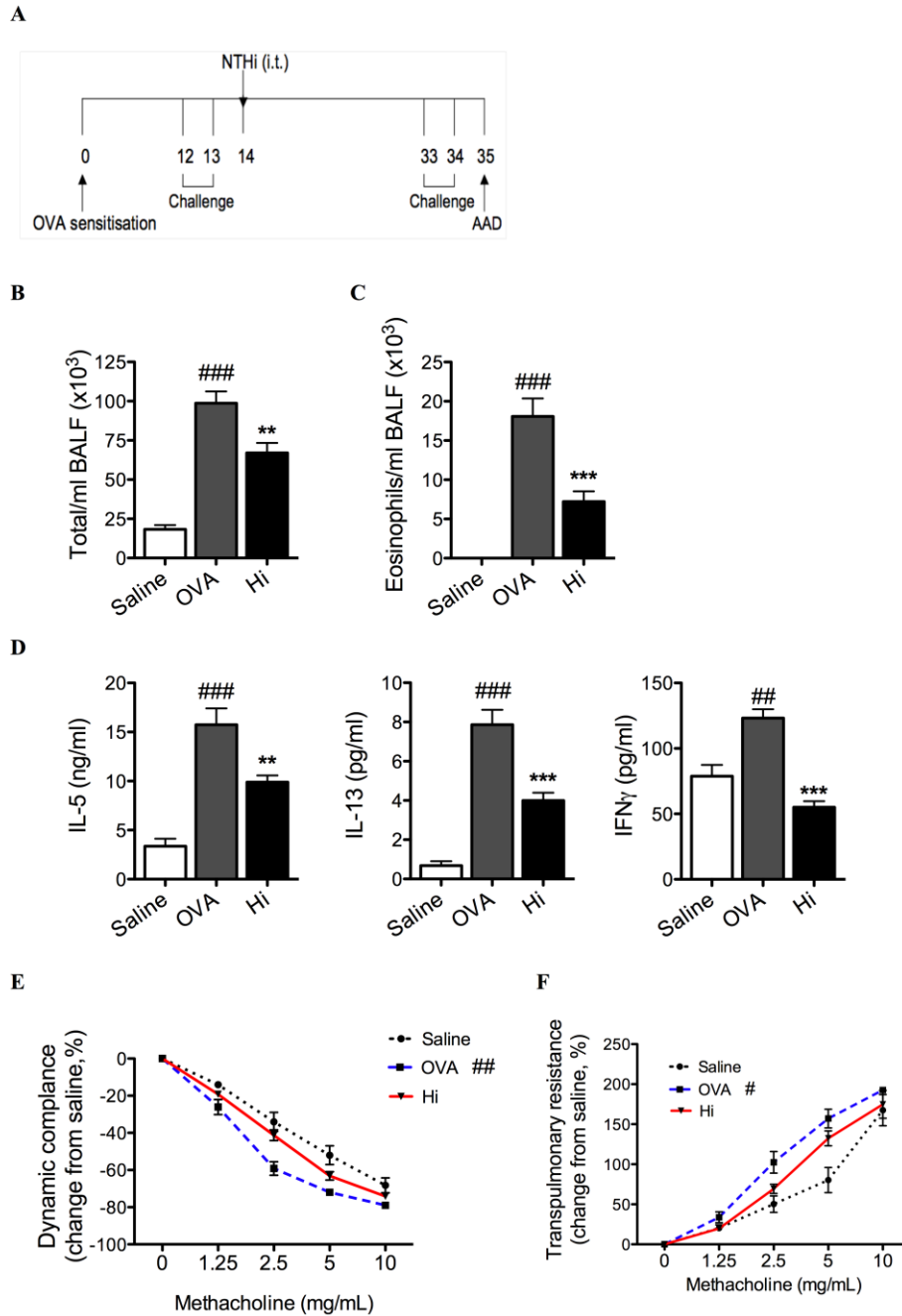


Figure 4.1: NTHi infection in established AAD decreases Th2-mediated eosinophilic responses. Mice were sensitised (day 0) and intranasally challenged (days 12-13 and 33-34) with OVA and inoculated with 5×10^5 CFU NTHi (day 14). AAD was assessed (day 35, A). The effects of infection on the numbers of total cells (B), and eosinophils (C) in BALF, OVA-induced IL-5, IL-13, and IFN- γ (D) production from MLN T-cells and dynamic compliance (E) and transpulmonary resistance (F) in AAD were assessed. ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$ compared to OVA controls.

4.4.2 NTHi infection in established AAD increases neutrophilic inflammation and IL-17 responses

I then determined if infection in established AAD induced neutrophilic inflammatory responses in the airways.

The induction of AAD (OVA) resulted in a mild neutrophilic influx into BALF and NTHi-induced IL-17 release from MLN T-cells (Figure 4.2A-B).

Infection after AAD significantly increased neutrophil influx and IL-17 production compared to allergic controls (Figure 4.2A-B).

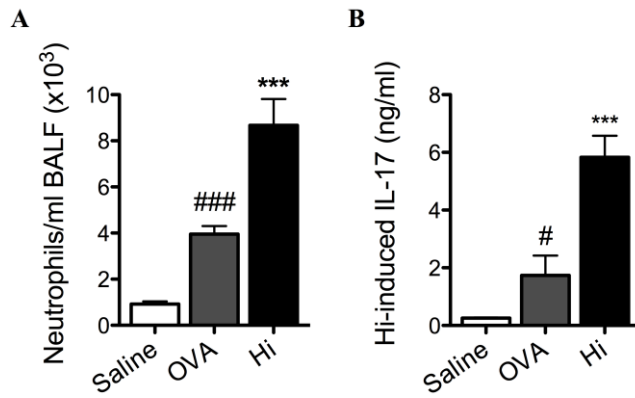


Figure 4.2: NTHi infection in established AAD increases neutrophilic inflammation and IL-17 responses. The effects of infection on neutrophil numbers in BALF (A), and NTHi-induced IL-17 release from MLN T-cells (B) were assessed. ### $p < 0.001$, # $p < 0.05$ compared to Saline controls, *** $p < 0.001$, compared to OVA controls.

4.4.3 Macrolide treatment of NTHi infection in established AAD reduces neutrophilic inflammation but restores Th2 mediated eosinophilic responses

Macrolides have both antibiotic and anti-inflammatory properties. Treatment with macrolides may be a possible therapy in asthmatics with neutrophilic inflammation, therefore, I investigated if clarithromycin reduced features of neutrophilic AAD. Mice were treated with clarithromycin for three days commencing two days after infection (days 16-18, Figure 4.3A), and the effects of treatment were assessed on day 35.

Clarithromycin treatment of uninfected allergic controls (OVA/Clar) significantly reduced eosinophilic inflammation and Th2 cytokines IL-5 and IL-13 compared to untreated allergic (OVA) controls (Figure 4.3B-C). However, treatment had no effect on airway neutrophils or IL-17 responses (Figure 4.3D-E).

By contrast, treatment of infected allergic groups (Hi/Clar) increased airway eosinophils and IL-13, but had no effect on IL-5 compared to untreated infected allergic groups (Figure 4.3B-C). Importantly, treatment of infected allergic groups reduced airway neutrophils and IL-17 levels (Figure 4.3D-E).

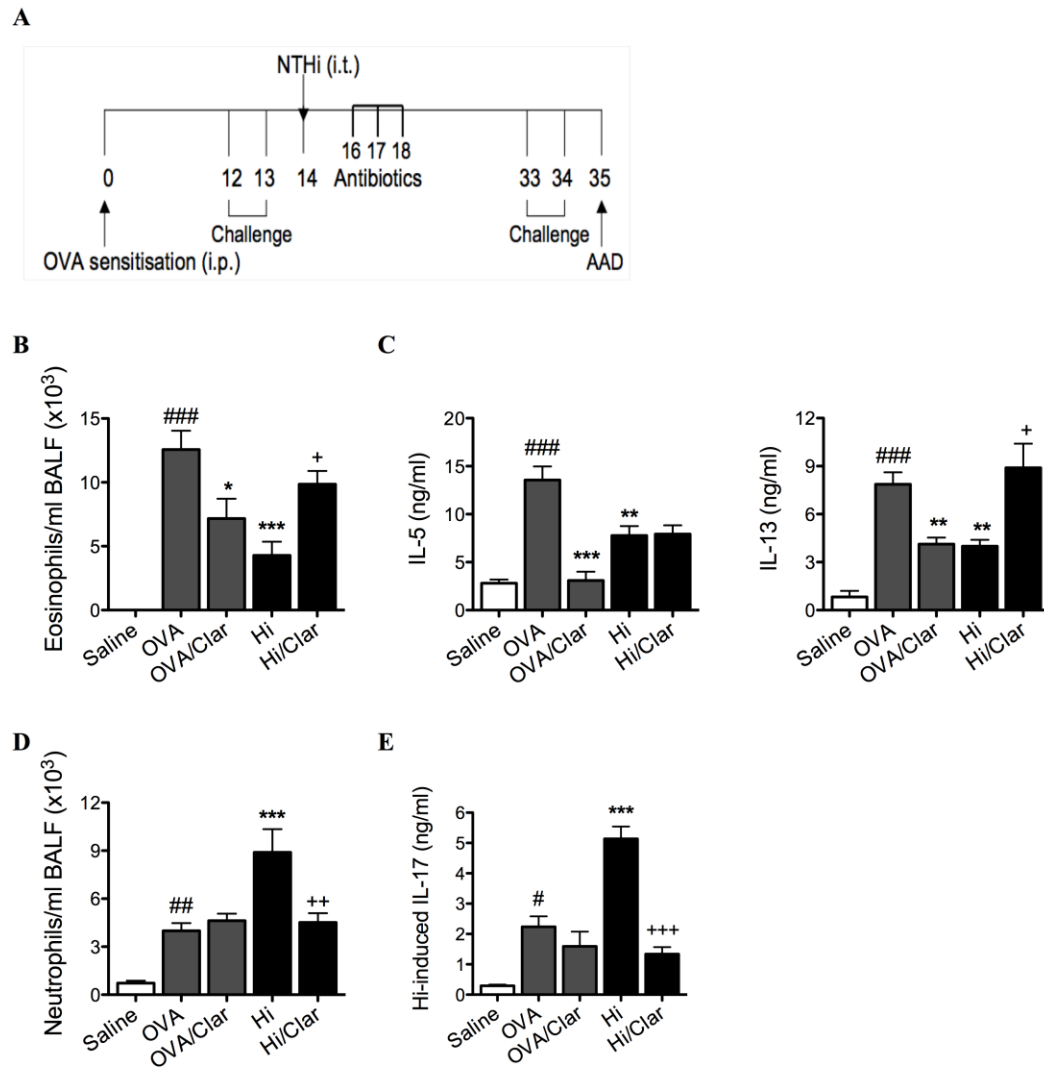


Figure 4.3: Macrolide treatment of NTHi infection in established AAD reduces neutrophilic inflammation but restores Th2 mediated eosinophilic responses. Mice were administered clarithromycin 2 days after infection (days 16-18, A). The effects of treatment on eosinophil numbers in BALF (B), OVA-induced IL-5 and IL-13 release from MLN cells (C), neutrophil numbers in BALF (D) and NTHi-induced IL-17 release from MLN T-cells (E) in neutrophilic AAD were assessed. ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls, +++ $p < 0.001$, ++ $p < 0.01$, + $p < 0.05$ compared to Hi/OVA untreated controls.

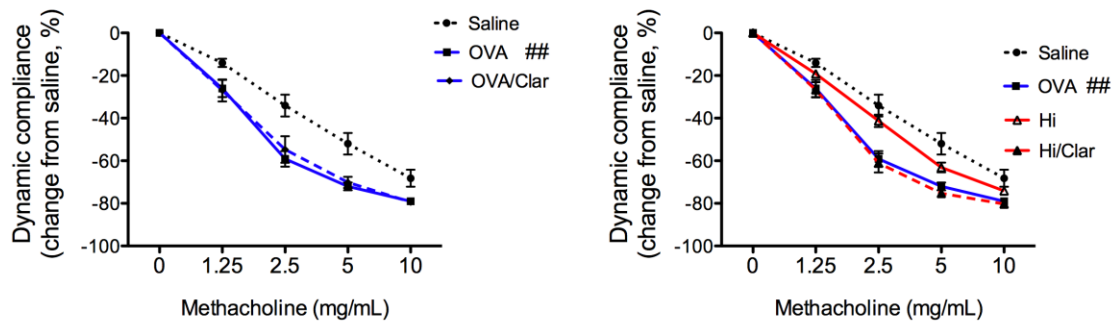
4.4.4 Macrolide treatment of NTHi infection in established AAD does not affect lung function

The effect of macrolide treatment on lung function was also examined.

Treatment of uninfected allergic controls (OVA/Clar) had no effect on dynamic compliance or transpulmonary resistance (Figure 4.4A-B). Treatment of infected allergic groups (Hi/Clar) did not significantly affect AHR, but there was a trend towards decreased dynamic compliance and increased resistance compared to untreated controls (Figure 4.4A-B).

Together these data show that the suppressive effect of NTHi infection on eosinophil inflammation and Th2 responses is reversed with clarithromycin treatment, and that treatment is effective in reducing neutrophilic inflammation and IL-17 responses. This suggests that treatment may reduce the severity of disease.

A



B

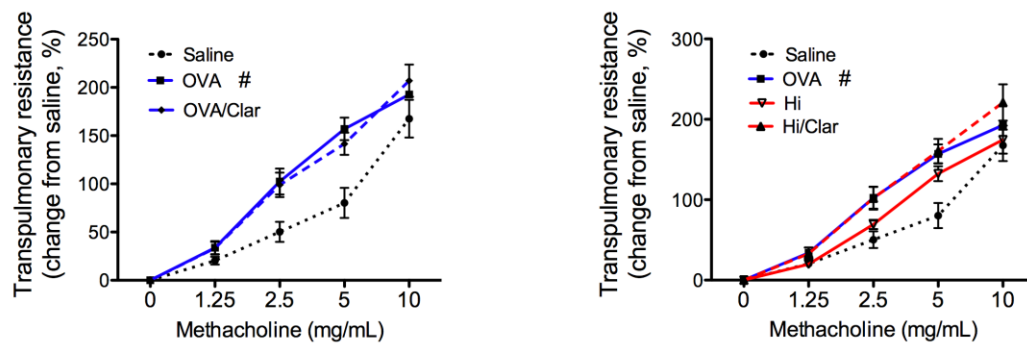


Figure 4.4: Macrolide treatment of NTHi infection in established AAD does not affect AHR.

The effects of clarithromycin treatment of infection in established AAD on dynamic compliance (A), and transpulmonary resistance (B) in neutrophilic AAD were assessed. ## $p < 0.01$, # $p < 0.05$ compared to Saline controls.

4.4.5 Combined macrolide and steroid treatment of chronic infection and neutrophilic AAD suppresses inflammation and cytokine responses

The effect of macrolide antibiotic treatment in a different model of chronic NTHi infection-induced neutrophilic AAD was also investigated. This model involves NTHi infection 10 days prior to the induction of AAD. This protocol induces chronic infection and neutrophilic AAD. Again, clarithromycin was administered for three days commencing 2 days after infection. In order to investigate the effect of steroid treatment on eosinophilic inflammation, dexamethasone was administered during the challenge phase (days 13-15, Figure 4.5A). The effect of clarithromycin alone and the combination of clarithromycin and dexamethasone treatment was assessed on day 16.

Clarithromycin treatment of uninfected allergic controls, either alone or combined with dexamethasone, significantly reduced BALF eosinophils and neutrophils, OVA-induced IL-13 and NTHi-induced IL-17 release from MLN T-cells compared to untreated controls (Figure 4.5B-E).

Similar to the previous model of treatment of infection in established disease, clarithromycin alone treatment of infected allergic groups increased eosinophil numbers in BALF and OVA-induced IL-5 and IL-13 release from MLN T-cells compared to untreated infected allergic controls (Figure 4.5B-C). However, treatment significantly reduced airway neutrophil influx and NTHi-induced IL-17 release from MLN T-cells compared to untreated controls (Figure 4.5D-E).

Importantly, combination clarithromycin and dexamethasone treatment of infected allergic groups, reduced BALF eosinophils, MLN IL-5 and IL-13 production, BALF neutrophils and IL-17 levels compared to untreated controls (Figure 4.5B-E).

These results show that crucially, combination therapy returned all parameters, with the exception of IL-17 release, to baseline levels.

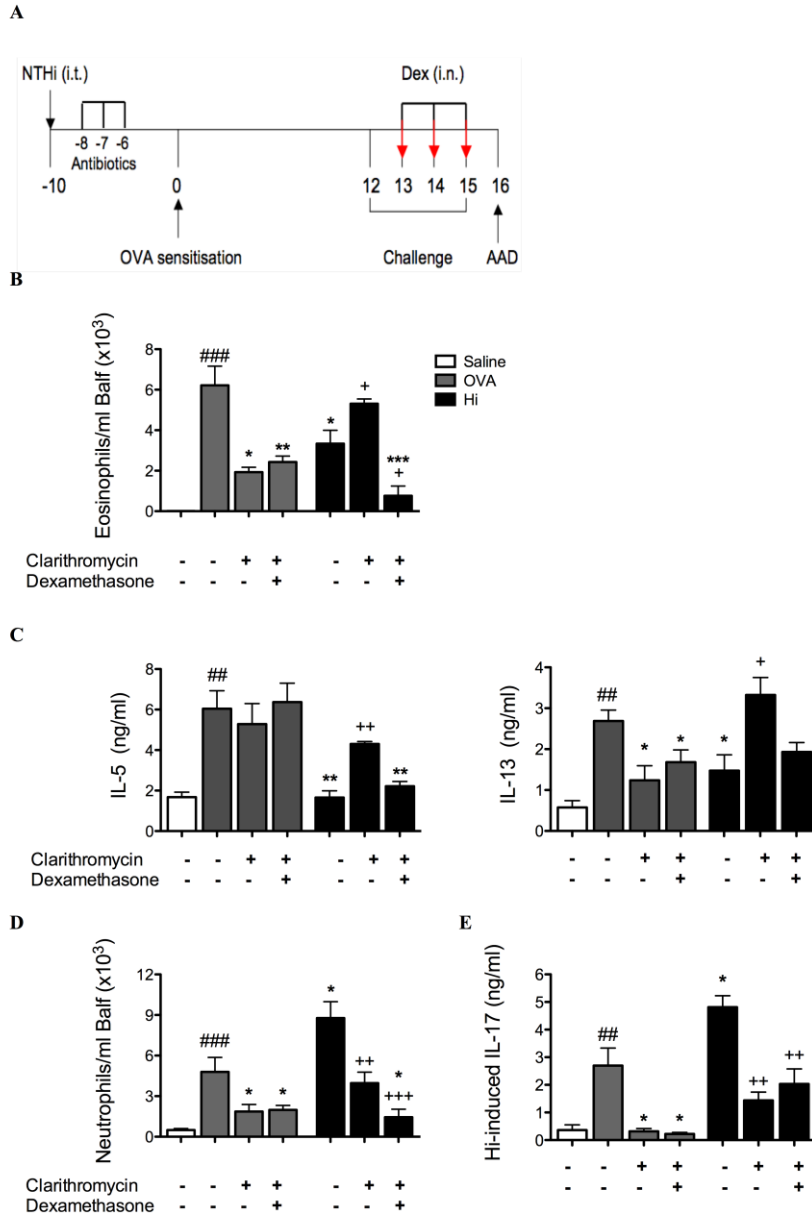


Figure 4.5: Combination macrolide and steroid treatment of chronic infection and neutrophilic AAD suppresses eosinophilic and neutrophilic AAD. Mice were inoculated with NTHi 10 days prior to OVA sensitisation, and challenged (days 12-15). Clarithromycin was administered 2 days after infection (days -8 to -6) and dexamethasone administered during challenge (days 13-15, A). The effects of treatment on eosinophil numbers in BALF (B), OVA-induced IL-5, IL-13 release from MLN cells (C), neutrophils numbers in BALF (D) and NTHi-induced IL-17 release from MLN T-cells (E) in neutrophilic AAD were assessed. ### $p < 0.001$, ## $p < 0.01$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls, +++ $p < 0.001$, ++ $p < 0.01$, + $p < 0.05$ compared to Hi untreated controls.

4.4.6 Combined macrolide and steroid treatment of chronic infection and neutrophilic AAD suppresses AHR

The effect of combined treatment on lung function was also examined.

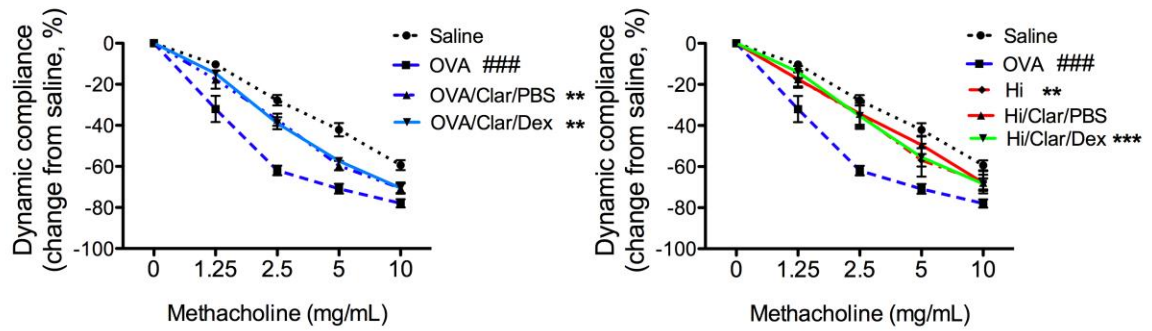
Treatment of uninfected allergic controls, with both clarithromycin alone (OVA/Clar/PBS) or in combination with dexamethasone (OVA/Clar/Dex), significantly reduced AHR compared to untreated uninfected allergic (OVA) controls (Figure 4.6A-B).

Treatment of infected allergic groups, with both clarithromycin alone (Hi/Clar/PBS) or in combination with dexamethasone (Hi/Clar/Dex), had no effect on dynamic compliance or transpulmonary resistance compared to untreated infected allergic (Hi) controls, but did suppress these parameters compared to untreated uninfected allergic (OVA) controls (Figure 4.6A-B).

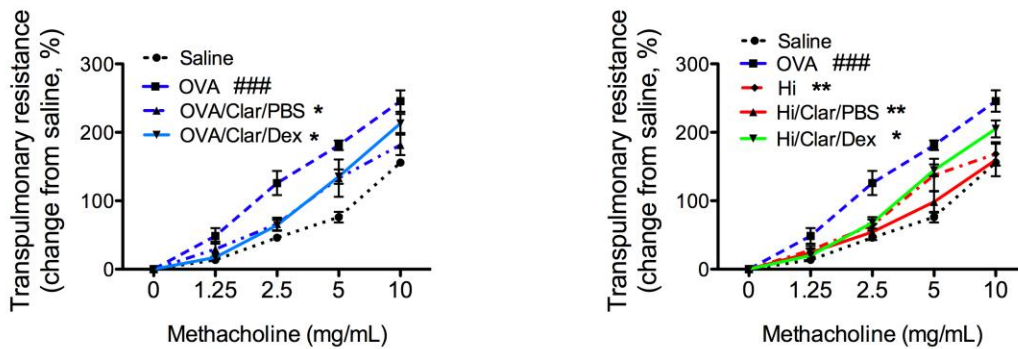
There were no significant changes in bacterial numbers in the lung with single or combined treatment compared to untreated groups (Figure 4.6C).

Together, these results show that antibiotic treatment in combination with corticosteroids suppresses eosinophilic and neutrophilic AAD and may, therefore, be a more effective therapy than macrolide treatment alone. These results also show the beneficial effects result from the anti-inflammatory rather than the antibiotic properties of clarithromycin since treatment did not affect the progression of infection.

A



B



C

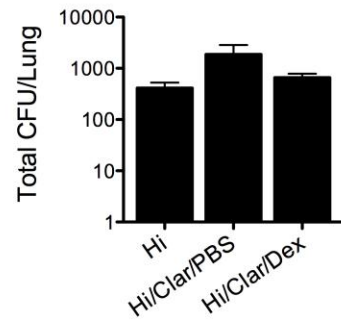


Figure 4.6: Combination macrolide and steroid treatment of chronic infection and neutrophilic AAD suppresses AHR. The effects of treatment of infection and neutrophilic AAD on dynamic compliance (A), and transpulmonary resistance (B) were assessed. The effect of treatment on bacterial numbers was also examined (C). ### $p < 0.001$ compared to Saline controls, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to OVA controls.

4.5 Discussion

Corticosteroids are the mainstay of asthma therapy and are effective in eosinophilic asthmatics. However, many neutrophilic asthmatics are steroid resistant, and effective therapies are urgently required. Here I have used two models of infection-induced neutrophilic AAD to show that macrolide treatment with clarithromycin alone reduced neutrophilic inflammation, but enhanced Th2-mediated eosinophilic responses and AHR. However, importantly, the combination of macrolide and steroid treatment abolished all key features of eosinophilic and neutrophilic AAD and may be an effective combination therapy for neutrophilic asthma.

Our previous studies have shown that NTHi infection during the induction of AAD induces features of neutrophilic asthma. These studies demonstrated that NTHi infection suppressed Th2-mediated eosinophilic inflammation, and induced innate and adaptive IL-17 responses that were crucial to neutrophilic inflammation. Here I extend these studies by investigating the role of NTHi infection in established disease. A model of established asthma was developed that involved a systemic sensitisation and two OVA challenges, which induced hallmark features of AAD, including increased airway eosinophils and lymphocytes, OVA-specific IL-5 and AHR. Twenty days later this was followed by a re-challenge to re-capitulate the features of AAD. This model was used to determine the effects of NTHi infection on established disease. Our results show that NTHi infection in established AAD also induces features of neutrophilic asthma, including reduced influx of eosinophil and Th2 responses but enhanced AHR, neutrophilic inflammation and IL-17 production, an important neutrophil chemoattractant (125, 126, 133, 135).

As evidence of distinct asthma subtypes has expanded, so has the interest in other treatment options for steroid-insensitive populations, particularly neutrophilic asthmatics. The use of macrolides has been investigated in different obstructive airways diseases including COPD (273, 276), bronchiectasis (277), panbronchiolitis (271), and sinusitis (272), and is now being assessed in asthma (176, 278). However, there are still only a small number of blinded, randomised, controlled trials, which have had mixed results.

Macrolides are potent antimicrobials that inhibit 50S ribosomes in bacteria, and block either protein translation initiation, or translocation of peptidyl tRNAs which prevents the elongation of peptide chains (279). Macrolides also have anti-inflammatory properties and inhibit inflammatory chemokine and cytokine production (280-282), adhesion molecule expression (283, 284), and airway mucus hypersecretion (285, 286).

A recent study showed that potentially pathogenic bacteria can be cultured from the sputum of approximately 41% of neutrophilic asthmatics (138), therefore it was hypothesised that treating infection with macrolides may abolish key bacterial and inflammatory features of infection-induced neutrophilic AAD. The administration of clarithromycin alone in infected groups with established AAD increased airway eosinophil influx, OVA-induced IL-5 and -13 release from MLN T-cells compared to untreated controls. This may be attributed to the fact that NTHi infection suppresses these features of eosinophilic AAD, and macrolide treatment reverses the effects of infection. I also show that clarithromycin treatment alone significantly reduced neutrophilic inflammation and IL-17 production in infected allergic groups, which corroborates several clinical studies that show macrolide therapy suppresses neutrophils and IL-8 production (277, 287, 288).

This study also shows that clarithromycin alone significantly reduced Th2 responses and eosinophilic inflammation but did not reduce neutrophil and IL-17 responses in uninfected allergic controls. This is similar to a study by Amayasu *et al.*, who demonstrated that clarithromycin treatment significantly reduced blood and sputum eosinophil numbers, as well as sputum eosinophil cationic protein levels, however the authors did not examine the effect of treatment on neutrophilic inflammation (173).

Treatment of infected allergic groups decreased dynamic compliance and increased transpulmonary resistance, but, this was not statistically significant. However, this result may have been different if AHR had been investigated at the peak of infection, i.e. on day 19. Nonetheless, these data are also similar to recent data from Simpson *et al.*, who showed a decrease in neutrophils and IL-8, but no change in AHR after 8 weeks of clarithromycin treatment (176).

Few studies have investigated the relationship between macrolide treatment and infection in asthma. Chronic infection with atypical bacteria is known to contribute to asthma pathogenesis and severity, and increased levels of *C. pneumoniae* are detected in asthmatics compared to healthy subjects (289, 290). Studies from our laboratory have shown that chlamydial respiratory infection promotes neutrophilic AAD in mice (178). A study by Black *et al.*, demonstrated that roxithromycin treatment of asthmatic patients with evidence of *C. pneumoniae*, resulted in significant improvement in lung function, but not in asthma symptoms (291). These differences in treatment outcomes may be due to the differences in how *H. influenzae* and *C. pneumoniae* induce features of disease. Another study showed that clarithromycin treatment in asthmatics, 56% of which were positive for either *C. pneumoniae*, or *Mycoplasma pneumoniae*, improved lung function in patients with evidence of infection, but no changes were observed in patients without

infection. These results were attributed to the antimicrobial effects of clarithromycin (292). These studies, although small, show the potential efficacy of macrolide use in asthmatics with infection.

Together the above results showed that treatment reduced Th2-mediated eosinophilic responses but not neutrophilic inflammation or IL-17-responses in uninfected allergic groups. This suggests that macrolide treatment may not be completely effective in uninfected asthmatics. Macrolide treatment of NTHi infection in established AAD was effective in substantially reducing neutrophilic inflammation and IL-17 responses, although Th2-mediated responses were enhanced. It is possible that a higher dose of clarithromycin is needed to inhibit these Th2-mediated responses. These results also suggest that other treatments, in combination with clarithromycin, may be necessary. Time restraints prevented us from investigating the effect of combination macrolide and dexamethasone treatment in this model.

Using a previously developed model of chronic infection and neutrophilic AAD, the combination of clarithromycin and dexamethasone treatment was investigated. The combination of treatments significantly reduced all key features of AAD, including airway eosinophil and neutrophil influx, and increases in OVA-induced IL-5 and IL-13, NTHi-induced IL-17 release from MLN T-cells and AHR. Combined macrolide and steroid treatment did not alter the bacterial load in the lungs. This corroborates studies that have shown that NTHi can persist in the lower respiratory tract for months during and after antimicrobial treatment (293, 294). Studies have also shown that the same NTHi strains with different OMP can colonise the respiratory tract at the same time, also leading to the persistence of bacteria after antibiotic treatment (198). Persistence of infection may be due to the fact that NTHi is able to survive in macrophages (185), which

may allow the bacteria to survive in the lung long after treatment. This also suggests that the beneficial effects observed with clarithromycin are due to its anti-inflammatory and not its antibiotic properties.

There are currently no studies that have examined the combination of macrolide and steroid therapies in asthmatics. Some studies have used combination macrolide and dexamethasone therapy for *M. pneumoniae* infection alone and have shown that this regime is more effective in treating infection-induced inflammation than treatment with macrolide or dexamethasone alone (295). Of interest, an *in vitro* pilot study demonstrated that PBMCs from asthmatic patients treated with clarithromycin were more able to suppress lymphocyte proliferation in the presence of dexamethasone compared to PBMCs from untreated patients. These data suggest that macrolides may potentiate asthmatic responsiveness to steroids (175).

In conclusion, I show that in models of infection-induced neutrophilic AAD, clarithromycin treatment suppresses airway neutrophils, and IL-17 responses, but enhances Th2-mediated eosinophilic responses. Importantly, the combination of clarithromycin with dexamethasone effectively reduced all features of eosinophilic and neutrophilic AAD. These data suggest that combination macrolide and steroid treatment may be effective in neutrophilic asthma, particularly in patients with persistent bacterial colonisation of the airways.

Chapter 5:

Discussion and conclusions

This chapter discusses the results from the investigation of the role of *H. influenzae* infection in inducing neutrophilic allergic airways disease, and the potential of macrolide treatment in neutrophilic airway diseases.

5.1 Significance of research

Infection plays an important role in the development and exacerbation of airways disease, and despite being investigated extensively, the role of infection in these diseases is still not fully understood. *H. influenzae* infection has been widely linked with chronic airway diseases such as COPD and bronchiectasis, both in stable and exacerbation states, and recently, it has been detected in the airways of a large proportion of neutrophilic asthmatics. Neutrophilic asthma has been identified, defined and characterised in the last 5 years, however, the role of infection in neutrophilic asthma, in particular *H. influenzae*, remains unknown. Using murine models of NTHi infection and OVA-induced AAD, the potential role of NTHi in the development of neutrophilic AAD was elucidated. I show that (1) NTHi infection reduces eosinophilic inflammation and preferentially promotes IL-17-mediated neutrophilic AAD, (2) the induction of AAD in infection promotes a dysfunctional innate immune response that promotes chronic infection and neutrophilic AAD, and (3) combination macrolide and steroid treatment may be an effective therapy for neutrophilic asthma. These studies further our understanding of the role of infection in the development of neutrophilic disease, and indicate possible treatment strategies for these patients.

5.2 NTHi infection in AAD induces features of neutrophilic asthma

The studies described here have shown that irrespective of timing, NTHi infection induces features of neutrophilic asthma by reducing eosinophilic inflammation and inducing intense neutrophilic inflammation in the airways, which concurs with published data (125, 127, 133, 135, 136). Our studies show that NTHi infection, whether it occurs before, during or after the induction of AAD, reduces both Th2 and Th1 cytokine responses, including local and

systemic IL-5, IL-13 and IFN- γ release. IL-5 is critically involved in eosinophil differentiation and activation, and both IL-5 and IL-13 are eosinophil chemoattractants (42, 43, 47, 53). The reduction in these factors correlates with the suppression of eosinophilic inflammation. Eosinophils numbers have been linked with asthma severity and AHR (3), subsequently, the suppression of eosinophils may also contribute to the reduction in AHR.

AHR, the result of a combination of excessive narrowing and increased sensitivity of the airway smooth muscle, is an important measure of disease severity, however in humans there is some variability in AHR intensity within similar patient groups. AHR, however, can be roughly divided into two types: variable and persistent (296-298). Variable AHR is thought to be the result of inflammatory processes induced by allergens, infections, or treatment; while persistent AHR is largely due to structural changes in the airway (e.g. smooth muscle hypertrophy, matrix deposition, or basement membrane thickening (298)). I show here that infection decreases lung transpulmonary resistance in all models of infection-induced neutrophilic AAD (except in the established asthma model, Chapter 4), and reduces dynamic compliance in one model. These data suggest that while neutrophilic asthmatics with infection have less severe AHR compared to eosinophilic asthmatics, they still have difficulty breathing compared to healthy individuals. So while I have not specifically shown how infection changes these features, these data suggest that infection may alter the variable component of AHR, (i.e. induces less eosinophilic and more neutrophilic inflammation), but perhaps also affects other processes (e.g. hyper-responsiveness of airway smooth muscle) that may dampen the severity of AHR. Further investigation is needed to determine the mechanisms of how NTHi infection reduces AHR.

The mechanism by which NTHi suppresses Th1 and Th2 responses is not yet fully understood, but our data suggest that infection alters antigen presentation to T-cells, which may suppress Th2 cytokine release and eosinophil development and mobilisation.

Our studies also show that NTHi infection at all time-points relative to AAD, induces significant neutrophilic inflammation and Th17 responses. Th17 cells release a number of cytokines, the most important of which are the IL-17 family cytokines, IL-17A-F. The function of all of these cytokines is not yet fully described, but extensive studies show that IL-17A is a chemoattractant for neutrophils (117, 218, 220, 299-301). The increases in Th17 cells and IL-17 protein observed, correlates with the significant increases in neutrophil numbers in the airways.

5.2.1 NTHi alters DC phenotype

I demonstrated, (Chapter 2), that NTHi infection in AAD significantly reduced the activation and co-stimulation of DCs in the lung and MLNs. DCs drive the innate and adaptive immune response to infection and allergens. DCs located in the airway mucosa and interstitia are usually in an immature state, and mature upon encountering and processing of antigens, at which point they migrate to the lymph nodes to present antigens to naïve T-cells (90, 98, 231). MHC II surface expression on DCs is an activation marker, and has been shown to increase DC antigen presentation, T-cell activation and subsequent immune responses (302-304); while the activity of co-stimulatory molecules, particularly CD80 and CD86, enhance the ability of DCs to induce T-cell responses (305, 306).

Infections are able to change the number, activation and phenotype of DCs (228). A study by Bilenki *et. al.* showed that adoptive transfer of DCs from mycobacteria-infected mice to sensitised mice decreased established Th2 responses compared to DCs from naïve

mice, and authors report that this may have been due to increased expression of the co-stimulatory molecules CD80 and CD86 (307). I show here that NTHi infection decreased expression of CD86 and MHC II, and this may alter the interaction between DCs and T-cells, resulting in the suppression of Th2 responses.

5.2.2 NTHi infection induces IL-17 responses

One of the most important findings from my studies was that IL-17 was implicated in responses to NTHi infection in AAD and played a critical role in NTHi infection-induced neutrophilic AAD. I have shown that NTHi infection induces both innate and adaptive IL-17 responses. NTHi infection during sensitisation induced the early production of IL-17 from neutrophils and macrophages at 24 hours, indicating that these innate cells may be the source of IL-17 in the initial stages of NTHi infection in AAD. This novel observation may be important in other diseases where the innate source of IL-17 is not known.

NTHi infection also induced adaptive IL-17 responses with increases in IL-17 production from Th17 cells in the lung. This immune response represents a bi-phasic response involving innate responses from lung macrophages and neutrophils, and adaptive responses from lung Th17 cells. This data suggests that early IL-17 production and neutrophilic inflammation may prime the host for enhanced Th17 responses later in disease, which subsequently induces neutrophilic AAD.

A study by Infante-Duarte *et al.* showed microbial lipopeptides are potent inducers of IL-17 production from T-cells (308), and indeed many other studies have demonstrated the important role of IL-17 responses in the host response to and clearance of bacterial infections (223, 226, 309, 310). My data add to this body of work, and are the first to show that NTHi induces Th17 responses in AAD.

5.2.2.1 Neutrophilic inflammation is mediated by IL-17

I showed that increases in neutrophils occurred concomitant with increases in IL-17 production, so to further investigate the role of IL-17 in neutrophilic inflammation, IL-17 was depleted during OVA challenge. Neutralising IL-17 prevented the induction of neutrophilic inflammation and reversed infection-mediated suppression of Th2 cytokines in infection-induced neutrophilic AAD. These data demonstrate the critical role of IL-17 in neutrophilic recruitment, and suggests that Th17 and Th2 cells cross-regulate each other. This is in agreement with a study by Schnyder-Candrian *et al.*, who showed that the administration of rIL-17 in a murine AAD model reduced allergen-induced eotaxin and IL-5, subsequently reducing eosinophilic inflammation. The authors suggest that this was the result of IL-17 reducing DC activation and antigen uptake, and chemokine synthesis (235). These studies emphasise the important role of IL-17, not only as a host defence mechanism against infection, but also its function in regulating allergic Th2 responses.

5.3 Induction of AAD during infection inhibits host responses

It was demonstrated, (chapter 3), that AAD during an ongoing NTHi infection adversely affects host immune responses to infection, which may lead to chronic infection.

5.3.1 Induction of AAD during infection promotes delayed bacterial clearance

The timing of infection relative to OVA sensitisation differentially affects the progression of infection. There was no change in bacterial clearance from the lungs in NTHi infection during or after OVA sensitisation compared to infection alone controls, however, in this study I showed that infection before OVA sensitisation and challenge inhibited the clearance

of bacteria compared to infection alone groups. My data adds to clinical studies that have shown that 41% of neutrophilic asthmatics had a significant load of potentially pathogenic bacteria in their sputum, 60% of these patients had *H. influenzae*, demonstrating the presence of chronic infection in a significant proportion of neutrophilic asthmatics (138).

Chronic infection is also commonly found in other chronic airway diseases characterised by neutrophilic inflammation such as COPD (203, 247), bronchiectasis (204) and chronic bronchitis (205). Several studies have focused on NTHi in COPD, due to the critical role of NTHi infection in the development, progression and exacerbation of disease. COPD is characterised by a progressive development of airflow limitation that is not fully reversible, and chronic inflammation of the small airways (which is quite often neutrophilic), resulting in narrowing of the small airways, and lung parenchymal destruction by NE and MMPs (311, 312). NTHi is commonly associated with recurrent and persistent lower respiratory infections in COPD, and is one of the leading causes of exacerbations in COPD (203, 313, 314). A study has shown that NTHi was detected extracellularly in 25-50% of stable COPD patients and 7% of acutely ill patients and NTHi was also demonstrated intracellularly in 33% of stable patients and 87% of acutely ill patients. Interestingly, typing of these NTHi strains revealed colonisation of multiple strains simultaneously (315). Another study showed that 83% of stable COPD patients were colonised with bacteria, some strains potentially pathogenic, and others not (207). These studies emphasise the significant role and widespread occurrence of chronic colonisation of *H. influenzae* in airway disease, prompting further investigation and understanding of the mechanisms of persistent infection.

5.3.2 Induction of AAD during infection induces steroid resistance

In this study I show that all the key features of infection-induced neutrophilic AAD were unresponsive to dexamethasone treatment, including airway eosinophils and neutrophils, Th1, Th2, and Th17 cytokine responses, AHR and bacterial numbers. This was in stark contrast to the effects of treatment on Th2-mediated eosinophilic AAD, where all features were significantly reduced by dexamethasone.

Neutrophilic asthmatics respond poorly to steroid therapy (135). Many studies have suggested different mechanisms of resistance, such as the increased activation of the transcription factor AP-1 which directly interacts with the GR, resulting in the reduction of the anti-inflammatory action of glucocorticoids (249, 268), or abnormal acetylation of histone-4 which is involved in the activation of steroid-responsive genes by corticosteroids, resulting in genes critical to the anti-inflammatory action of corticosteroids not being activated (250). Studies have also shown that steroid-resistant asthmatics have reduced GR- α expression, which is the active variant that binds corticosteroids, and instead have increased GR- β expression which binds DNA (164, 166). Interestingly, a recent study has shown that IL-17 can induce GR- β mRNA in asthmatic epithelial cells (251), and it is well established that steroids prolong neutrophil survival by inhibiting their apoptosis (252, 253). Taken together, these data suggest that infection-induced increases in neutrophilic inflammation and IL-17 production may play an important role in promoting steroid resistance in neutrophilic asthma.

5.3.3 Induction of AAD during infection inhibits innate immune cell activation and function

Another significant finding made in this study was that lung macrophages and neutrophils were dysfunctional in groups with the induction of AAD during infection. I demonstrated

that two crucial components of the innate immune system, macrophages and neutrophils, were less activated in infection-induced neutrophilic groups compared to allergic controls. In addition, these cells were significantly less able to phagocytose bacteria compared to allergic controls. The functional inability of these cells may be a direct result of them being inactivated, as studies report that once activated, macrophages and neutrophils carry out microbicidal functions such as phagocytosis and elimination of pathogens (264, 265). It is likely that the chronic infection described in infection-induced neutrophilic AAD, may be the result of a dysfunctional host immune response to infection.

Alveolar macrophages are the resident phagocytes in the lung, and therefore, are crucial to host defence against inhaled pathogens (316, 317). If a strong pro-inflammatory response by these macrophages is not induced during infection, pathogen clearance may be impaired. Impaired macrophage responses have also been shown in COPD. A study by Berenson and colleagues demonstrated that alveolar macrophages from COPD donors had impaired phagocytosis of NTHi compared to donors without COPD (318). The same authors also showed in a different study that alveolar macrophages from COPD patients stimulated with NTHi LOS induced less TNF- α , IL-1 β and IL-12 release compared to non-smokers. Authors suggested that this impaired responsiveness may impair bacterial clearance, and provide a constant source of inflammation (247).

There is also data to suggest that NTHi infection affects the function of neutrophils in COPD. A study has shown that neutrophils from COPD patients that had recently had a *H. influenzae* infection were able to phagocytose NTHi, but were being killed during phagocytosis instead of the bacteria being killed. The authors showed that NTHi was inducing necrosis in these neutrophils, resulting in the release of potentially harmful mediators that could damage surrounding cells, and importantly, bacteria being able to

persist in the lung (319). Together these data suggest that impairment of components of host innate immunity has detrimental effects on the ability of the host to clear infection.

5.4 The potential of macrolide antibiotics as a treatment strategy in asthma

Steroid-resistant asthma may occur in a small cohort of asthmatics, but is nonetheless a substantial problem, as it is responsible for about 50% of the total health care cost for asthma. Mechanisms involved in steroid resistance are continually being investigated, and potential treatments to reverse resistance to treatment are being explored. Macrolides are one such alternative therapy. Macrolides have potent anti-bacterial and anti-inflammatory effects, and new clinical studies are showing the potential use of macrolides in diseases such as bronchiectasis, COPD and post-transplant obliterative bronchiolitis (171).

5.4.1 Clarithromycin suppresses neutrophilic but not eosinophilic inflammation in infection-induced AAD

In the final study (chapter 4), it was demonstrated that clarithromycin treatment suppressed IL-17 production from MLN T-cells, and neutrophil influx in the airways compared to untreated controls, but increased Th2-mediated airway eosinophilic responses, with no effect on AHR. This may be due to the fact that NTHi infection reduces Th2-mediated inflammation, so clarithromycin targets this infection, reversing the suppressive effects of infection on features of AAD. These data agree with a recent study that showed that clarithromycin treatment reduced neutrophils, IL-8 and NE in patients with refractory asthma (176). A few other clinical studies have shown that macrolide treatment for 6-8

weeks decreases the patient's symptom score, and increases quality of life, however, in all the studies FEV₁ remained unchanged, and AHR largely unaffected (171). These studies demonstrate that while macrolide treatment has some benefits, alone it is not completely effective. Therefore, I investigated combination therapy.

5.4.2 Combined macrolide and dexamethasone treatment suppresses all features of neutrophilic and eosinophilic inflammation in AAD

The data in this study showed that the combination of clarithromycin and dexamethasone abolished all key features of neutrophilic and eosinophilic AAD, including airway eosinophils and neutrophils, antigen-specific IL-5, IL-13 and IFN- γ , NTHi-specific IL-17, and AHR. There is little clinical data on the interactions between macrolides and steroids in asthmatics, therefore further investigation is needed to elucidate the mechanisms of treatment outcomes, but these novel findings show the potential of macrolides in neutrophilic diseases, specifically patients with evidence of infection.

Bacterial numbers in the lung was the only feature of disease that was not ameliorated by treatment. Bacterial persistence despite antibiotic treatment has been previously demonstrated, and it is thought that this may be the result of NTHi being able to survive in macrophages, and not only escape detection by the immune system, but also, avoid the effects of antibiotics (185, 293). This would suggest that targeting intracellular NTHi may be necessary to completely eradicate bacteria, however further investigation into optimal doses and treatment regimes may show reveal better results in terms of infection.

5.5 Future directions

5.5.1 Further investigations into infection-induced IL-17 responses

I have shown in this thesis that NTHi infection induces Th17 responses, while reducing Th2 responses in all our models of infection-induced neutrophilic AAD. It is not known how infection induces IL-17 signalling, therefore I will investigate the roles of TLR-2 and TLR-4 in IL-17 responses during NTHi infection. TLR-2 and -4 are the main receptors that NTHi binds to, therefore infection using TLR knock-out mice would provide important information about signalling pathways that may be involved in IL-17 responses.

Importantly, the relationship between Th17 and Th2 responses involved in eosinophilic inflammation, specifically if these cells are able to counter-regulate each other needs to be investigated. Studies have shown that IL-17 may suppress or promote eosinophilic inflammation, but the mechanisms of this are not known. However, it has been shown that IL-17 affects DC function, therefore experiments that investigate this interaction, specifically looking at how infection-induced IL-17 from innate immune cells affect DC activation, and expression of co-stimulatory molecules will be conducted. Further to this, how these DCs then interact with naïve T-cells, establishing if T-cell proliferation is affected, as well as the cytokine profile of these T-cells will be investigated. This would help elucidate how NTHi infection is involved in the switch from Th2-mediated eosinophilic responses to Th17-mediated neutrophilic responses.

5.5.2 Further investigation into innate immune function in infection-induced neutrophilic AAD

The dysfunction of innate immunity plays a vital role in COPD progression, and I show a similar pattern here, that innate immune dysfunction may be the key to chronic infection. It therefore needs to be investigated how NTHi infection leads to the lack of shedding of CD62L and other activation markers on lung neutrophils and macrophages and subsequent

inactivation of these cells at the site of inflammation. To determine this, *in vitro* studies that co-culture lung neutrophils and macrophages with NTHi to elucidate the expression of activation markers on these cells will be conducted. Importantly, gene arrays to determine if genes involved in cell activation and function are altered by infection will be completed.

5.5.3 Further investigations into the mechanisms of action of macrolides

I have made some important novel observations that combination therapy with clarithromycin and dexamethasone effectively treats all features of neutrophilic and eosinophilic AAD. The next step is to investigate how macrolides achieve this effect. My studies here suggest that the suppression of inflammation in my infection-induced neutrophilic models may be due to the anti-inflammatory rather than the anti-microbial effects of macrolides, as I show that infection levels are not reduced with treatment. This may also suggest that further investigation is needed to determine optimal doses, it may be that a higher dose of clarithromycin is able to improve bacterial recovery and still have the same anti-inflammatory results. Therefore, the timing of treatment will also have to be optimised. Here clarithromycin was administered up to the peak of infection, however, treatment given during the challenge or exacerbation phase of disease may be effective, therefore potentially reducing the need for steroids all together.

5.5.4 NTHi vaccinations as a treatment strategy

Ultimately, the best form of treatment is prevention. Macrolide antibiotics may be effective in treating infection and infection-induced inflammation, but there is a risk of resistance to treatment. A study in our group has recently demonstrated that the Pneumococcal conjugate vaccine suppresses all key features of AAD, including Th2-mediated eosinophilic responses,

mucus hypersecretion, and AHR (320), therefore it is plausible that a NTHi vaccine may have the same effect in neutrophilic asthma.

Currently, there are no human NTHi vaccines, despite the prevalence of NTHi infections. This is due to some challenges in developing a vaccine. As NTHi has no polysaccharide capsule, OMPs have been investigated as potential vaccine targets. To be a suitable target, the OMP must be conserved between different NTHi strains and be immunogenic, however, not all NTHi strains cause disease, and NTHi strains display significant heterogeneity in their OMP molecules due to highly variable regions, leading to elimination of many proteins as vaccine targets. Given that one NTHi strain is able to cause recurrent infections, this suggests that finding a cross-strain protective epitope may be difficult (321). Nonetheless, efforts are still being made to develop a NTHi vaccine and there is potential that a vaccine would significantly benefit not only neutrophilic asthmatics, but also other chronic neutrophilic airway diseases such as COPD and bronchiectasis.

Publications

Refereed publications (accepted publications)

Essilfie, A-T., Simpson, J.L., Dunkley, M.L., Horvat, J.C., Gibson, P.G., Foster, P.S., Hansbro, P.M. (2011). “*Haemophilus influenzae* infection induces IL-17 mediated features of neutrophilic allergic airways disease.” *PLoS Pathogens*

Essilfie, A-T., Simpson, J.L., Dunkley, M.L., Morgan, L.C., Oliver, B.G., Gibson, P.G., Foster, P.S., Hansbro, P.M. (2011). “Combined *Haemophilus influenzae* respiratory infection and allergic airways disease drives chronic infection and features of neutrophilic asthma.” *Thorax*

Preston, J.A., **Essilfie, A-T.**, Horvat, J.C., Wade, M.A., Beagley, K.W., Gibson, P.G., Foster, P.S. & Hansbro, P.M. (2007). “Suppression of allergic airways disease by immunization with whole killed *Streptococcus pneumoniae*.” *Vaccine*

First author conference presentations/publications

Essilfie, A-T., Simpson, J.L., Dunkley, M.L., Morgan, L.C., Oliver, B.G., Gibson, P.G., Foster, P.S., Hansbro, P.M., “Combined *Haemophilus influenzae* respiratory infection and allergic airways disease drives chronic infection and features of neutrophilic asthma” *Newcastle Asthma meeting (NAME, 2011)*

Essilfie, A-T., Simpson, J.L., Dunkley, M.L., Horvat, J.C., Gibson, P.G., Foster, P.S., Hansbro, P.M., “*Haemophilus influenzae* infection induces IL-17 mediated features of neutrophilic allergic airways disease” *Proceedings of American Thoracic Society (ATS, 2010)*

Essilfie, A-T., Simpson, J.L., Dunkley, M.L., Horvat, J.C., Gibson, P.G., Foster, P.S., Hansbro, P.M. “*Haemophilus influenzae* infection induces features of neutrophilic asthma” *Thoracic Society of Australia and New Zealand, Darwin, Australia (TSANZ, 2009)*

Essilfie, A-T., Simpson, J.L., Dunkley, M.L., Horvat, J.C., Gibson, P.G., Foster, P.S., Hansbro, P.M “*Haemophilus influenzae* infection in asthma may drive the development of neutrophilic asthma” *Proceedings of American Thoracic Society* (ATS, 2008).

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