

**The immunopathology of chronic suppurative lung
disease in Northern Territory children**

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DECLARATION

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is the result of my own investigations, and all references to the ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

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ABSTRACT

Northern Territory Indigenous children experience a high burden of chronic suppurative lung disease (CSLD) associated with recurrent lower respiratory infection. There are currently limited data to guide effective long-term management strategies for CSLD.

This thesis investigates the role of the immune response, particularly in relation to non-typeable *Haemophilus influenzae* (NTHi), in the pathogenesis of chronic suppurative lung disease in Northern Territory children. The findings from this thesis provide biological evidence in support of novel management strategies to improve the clinical outcome for Northern Territory children with CSLD.

Principal findings:

1. A prospective evaluation study showed that flexible bronchoscopy with lavage substantially contributes to the clinical management of children with CSLD by identifying airway eosinophilia and respiratory pathogens not addressed by current empiric therapies.
2. In vitro studies of innate, and adaptive immune responses to NTHi showed that:
 - a. Children with CSLD generate a functionally distinct cell-mediated immune phenotype in response to NTHi compared with respiratory-healthy children.
 - b. The NTHi-driven cytokine profile in children with CSLD is associated with mechanisms regulating inflammation in the lungs.

3. A study of children vaccinated with a Pneumococcal *H. influenzae* Protein D conjugate vaccine provided proof-of-concept that the functional immune profile can be modified in children with CSLD.

The findings from this thesis contribute to the development of novel management strategies for CSLD in Northern Territory children by identifying factors of the immune response, likely contributing to the pathogenesis of CSLD, that may be targeted by novel therapeutic interventions. These data lay the foundation for studies to evaluate the effect of novel interventions on the clinical outcomes of Northern Territory children with CSLD.

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1. Hare KM, Marsh RL, Binks MJ, Grimwood K, Pizzutto SJ, Leach AJ, Chang AB, Smith-Vaughan HC. Quantitative PCR confirms culture as the gold standard for detection of lower airway infection by nontypeable *Haemophilus influenzae* in Australian Indigenous children with bronchiectasis. *Journal of Microbiological Methods*. 2013;92(3):270-2.
2. Smith-Vaughan HC, Binks MJ, Marsh RL, Kaestli M, Ward L, Hare KM, Pizzutto SJ, Thornton RB, Morris PS, Leach AJ. Dominance of *Haemophilus influenzae* in ear discharge from Indigenous Australian children with acute otitis media with tympanic membrane perforation. *BMC Ear, Nose, and Throat Disorders*. 2013;13(1):12.
3. Marsh RL, Thornton RB, Smith-Vaughan HC, Richmond P, Pizzutto SJ, Chang AB. Detection of biofilm in bronchoalveolar lavage from children with non-cystic fibrosis bronchiectasis. *Pediatric Pulmonology*. 2015; 50:284-292.

CONFERENCE PRESENTATIONS

This sections lists the conference presentations made that are relevant to this thesis.

1. Pizzutto SJ, Schutz KL, Upham JW, Yerkovich ST, Morris PS, Chang AB Airway cellularity in children with non-cystic fibrosis bronchiectasis: implications for clinical management. Thoracic Society of Australia and New Zealand, Darwin, April 2009.

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5. Pizzutto SJ, Yerkovich ST, Hales BJ, Thomas WR, Upham JW, Chang AB. Children with chronic suppurative lung disease have a Th2 polarised immune response to *H. influenzae*. European Respiratory Society Lung Science Conference, Estoril, Portugal, March 2013.

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LIST OF ABBREVIATIONS

BAL	bronchoalveolar lavage
BE	bronchiectasis
CFU	colony forming units
cHRCT	chest high resolution computed tomography
CI	confidence interval
COPD	chronic obstructive pulmonary disease
COX-2	cyclooxygenase-2
CSLD	chronic suppurative lung disease
CXCL10	chemokine (C-X-C motif) ligand 10
DELFA	dissociation-enhanced lanthanide fluorescent immunoassay
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FB	flexible bronchoscopy
FBS	fetal bovine serum
HC	healthy control
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	interferon gamma-inducing protein 10

IQR	interquartile range
LPS	lipopolysaccharide
LTA	lipotechoic acid
MHC	major histocompatibility complex
ng	nanograms
NSAIDs	non-steroidal anti-inflammatory drugs
NTHi	non-typeable <i>Haemophilus influenzae</i>
OMP	outer membrane protein
PBB	protracted bacterial bronchitis
PBMC	peripheral blood mononuclear cell
pg	picograms
PGE	prostaglandin E
PHA	phytohaemagglutinin
PHiD-CV	pneumococcal <i>H. influenzae</i> -protein D conjugate vaccine
Poly(I:C)	polyinosinic:polycytidylic acid
RDH	Royal Darwin Hospital
rHu	recombinant Human
RNA	ribonucleic acid
SD	standard deviation
TGF	transforming growth factor
Th	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor

LIST OF FIGURES

This section lists the figures presented in this thesis (chapter 3) that are not included in published journal articles. Figures that have been published in journal articles (chapters 2, 4, 5, 6 and 7) are not listed in this section.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Chapter 1 INTRODUCTION AND LITERATURE REVIEW

1.1 Chapter overview

This chapter reviews the current understanding of the respiratory illness, chronic suppurative lung disease (CSLD). It outlines the definition of CSLD in relation to this thesis and provides evidence for the relevance of this thesis globally and more specifically to our local region, the Northern Territory of Australia. This literature review briefly describes the clinical and microbiologic aspects of CSLD before discussing the role of host immune responses in the pathogenesis of CSLD. The review concludes with the aim of this thesis and the research hypotheses generated from the current literature. Further literature, specific to the research hypotheses, is incorporated into the respective chapters.

1.2 The definition of chronic suppurative lung disease

Chronic suppurative lung disease is a clinical syndrome in children that describes symptoms characteristic of persistent and recurrent endobronchial infection. The defining symptom common to all children with CSLD is a recurrent and persistent wet cough (2 or more episodes greater than 4 weeks duration per year) [1]. The phenotype of CSLD overlaps with bronchiectasis, and in circumstances when diagnosis of

bronchiectasis is difficult, the term CSLD is used interchangeably with bronchiectasis [2].

Bronchiectasis is traditionally defined as destruction of the structure of the airway architecture resulting in dilation of the bronchi. It is diagnosed by high-resolution computerised tomography of the chest (cHRCT). In adults, a diagnosis of bronchiectasis is made according to well-defined criteria, including a bronchial diameter 1 to 1.5 times greater than that of the accompanying artery and the irreversibility of this dilation [3]. However, these criteria are under debate in the paediatric setting where lung morphology differs considerably from that of the adult lung [1, 4]. The bronchoarterial ratio in the normal lung varies according to age [5] and is likely to be lower in children than adults [4]. In children, dilation of the bronchi may occur without exceeding the diameter of the adjacent artery, thus using the adult criteria may result in an under-diagnosis of bronchiectasis. Furthermore, whilst repeated cHRCT are not recommended in children due to radiation exposure, multiple studies have described a resolution of bronchial dilation over time in some children previously diagnosed with bronchiectasis [6, 7]. This raises questions regarding the point at which bronchiectasis becomes permanent [8].

Evidence linking the progression of CSLD to bronchiectasis is anecdotal as there are no longitudinal studies documenting the physiological changes to the airways resulting in bronchiectasis. However, there is a large clinical overlap between CSLD and bronchiectasis. Children and adults with bronchiectasis often have a preceding clinical history of persistent or recurrent respiratory symptoms and in adults this is often traced back to early childhood [9].

Thus, due to the above-mentioned difficulties in distinguishing CSLD from bronchiectasis and the substantial clinical overlap, the term CSLD is used in this thesis in the broad sense of the clinical spectrum, that is chronic suppurative lung disease with and without HRCT-confirmed bronchiectasis.

1.3 The burden of CSLD

1.3.1 The global burden

There are few epidemiologic studies of CSLD, thus the true extent of the global burden of CSLD is unknown. Furthermore, current studies focus primarily on bronchiectasis, the severe end of the disease spectrum. Not all children with CSLD have bronchiectasis, thus the global burden of CSLD is likely much higher than that depicted in the literature. A downturn in prevalence of bronchiectasis in affluent countries from the mid 1900's is attributed to improvements in antibiotics and childhood immunisation strategies. Today, bronchiectasis is considered more common in populations where access to health care is poor [10, 11]. However, the prevalence of bronchiectasis is also high in Indigenous populations of affluent countries, including those in Australia, New Zealand and the United States of America [2, 12-14]. Furthermore, the past few decades have seen an increase in the diagnosis of bronchiectasis across Europe [15-17] and North and South America [18, 19]. The reason for the increased prevalence in bronchiectasis is unknown. A heightened clinical awareness and the use of cHRCT likely contribute to an increase in the number of diagnosed cases. However, it cannot be ruled out that changes in antibiotic use may also contribute to an increase in the prevalence of CSLD and hence

bronchiectasis. Long-term use of antibiotics may lead to persistent infections through the emergence of antibiotic resistant bacteria [20]. Alternatively, the recent reduction in use of antibiotics during viral infections may have unwittingly increased the number of secondary bacterial infections [21].

In the region where my thesis has been undertaken (Northern Territory, Australia), the burden of CSLD is reflected by the burden of respiratory infections (refer to section 1.3.2 below regarding the relationship between acute respiratory infections and CSLD). The burden of respiratory disease is disproportionately high in Australian Indigenous children compared with non-Indigenous children [22]. In the Northern Territory, 1 in 5 Indigenous children are hospitalised for an acute lower respiratory infection within the first 12 months of life and 7% admitted at least twice [23].

1.3.2 The burden of CSLD in the Northern Territory of Australia

Hospitalisation for respiratory infection early in life is a risk factor for CSLD [13] and respiratory illness is the leading cause of hospitalisation in Northern Territory (NT) children less than 5 years of age [23]. However, there are very few data on the prevalence or incidence of CSLD in Australia. Much of the data regarding CSLD in Australia, including the Northern Territory, are derived from reviews of hospitalisation records for respiratory admissions or studies of bronchiectasis [23, 24]. Both factors represent severe disease, thus the true burden of CSLD in the Northern Territory is likely underestimated in the literature. Characterising the burden of CSLD in the Northern Territory is further complicated by region-specific studies offset by differences in the

risk of respiratory illness between regions. Even within the Northern Territory, the risk of lower respiratory illness in the Central Australian region is almost twice that of the Top End region [23]. The reasons for this discrepancy are unknown. Despite these limitations in the literature, the estimated prevalence of CSLD with bronchiectasis in Central Australian Indigenous children (14.7 per 1000 children) is one of the highest in the world [24] and more than 1 in 1000 Indigenous children will be diagnosed with bronchiectasis within the first 12 months of life [23].

In Australia, the hospitalisation rate for bronchiectasis (adults and children) has risen more than 60% in the past 15 years and is more than 6 times higher for Indigenous Australians than non-Indigenous Australians [25]. At least half of Indigenous adults with bronchiectasis were first diagnosed in childhood [26]. These bronchiectasis data are indicative of the broader importance of CSLD in Northern Territory children.

1.4 The aetiology of CSLD

CSLD describes a syndrome of symptoms rather than a specific disease entity and thus has multiple possible aetiologies. On a global scale and using studies of bronchiectasis as a guide, severe lower respiratory infection during childhood accounts for the greatest number of CSLD cases [27]. Infection followed by primary immune deficiency, defects in mucociliary clearance mechanisms (primary ciliary dyskinesia, congenital malformations) and aspiration of a foreign body account for approximately 85% of known causes [27]. Region-specific studies suggest that geographic locality and socioeconomic environment play a large role in determining the likely aetiology of

CSLD [13, 28]. In affluent countries, an underlying primary immune deficiency disorder, or a physiologic defect in mucus-clearing mechanisms (such as primary ciliary dyskinesia and congenital defects) are the most common known causes of CSLD [27, 28]. However, in under-privileged populations, including populations within affluent societies (such as Indigenous populations of Australia, New Zealand and Alaska), severe lower respiratory infection is the most likely cause [13, 28, 29]. It is estimated that severe bacterial or viral pneumonia accounts for approximately 60% of the cases of post-infection paediatric bronchiectasis, whilst measles and tuberculosis combined account for 25% [27].

There is often considerable delay between the first onset of symptoms and clinical diagnosis of CSLD [11, 28, 30]. Even in countries such as Australia, England and Italy where access to health services is good, children may have symptoms for up to 7 years prior to diagnosis [6, 17, 31]. A delay in diagnosis and incomplete clinical history can make it difficult to determine the aetiology of CSLD. Thus, up to half of CSLD diagnoses have no apparent underlying cause and are regarded as idiopathic [27]. Where aetiology has been determined, a severe lower respiratory infection early in life accounts for approximately 50-90% of CSLD cases in Indigenous populations of Australia, New Zealand and Alaska [12, 13, 29], compared with 5-37% in socially privileged populations (including those in Australia) [28].

Bacterial and viral pneumonia is a significant risk factor for CSLD and is reportedly responsible for more than half of post-infectious cases in the Northern Territory and globally. However, only a proportion of children develop CSLD following an episode of

pneumonia [23]. The mechanisms involved in progressing from acute lower respiratory infection to persistent infection and chronic illness are poorly understood. It is likely that establishment of chronic infection with consequent inflammation is a combination of pathogen virulence factors and impaired host immune responses. The focus of my thesis is the role of the host immune response.

1.4.1 Understanding the aetiology CSLD: importance and clinical investigations

Determining the aetiology of CSLD is important for directing clinical management [32]. Early diagnosis followed up with appropriate therapy can stabilise CSLD and potentially resolve current damage to the airways [6, 7, 31]. Advances in immunotherapy, mucus-clearing techniques and surgical interventions have improved the clinical outcomes of children where CSLD has an underlying cause such as primary immune disorders and structural abnormalities. Hence, it is standard management that children with CSLD undergo certain investigations as outlined in Thoracic Society of Australia and New Zealand guidelines [3]. These investigations include full blood counts and levels of the major immunoglobulin classes IgG (including subclasses), IgM and IgA, a sweat test (to exclude cystic fibrosis) and collection of a lower airway specimen to assess the microbiology of the lower airway. Optimal clinical practice is best achieved by individualising therapy to the specific microbiologic and pathophysiologic lung environment [20, 33, 34].

As young children cannot usually expectorate, flexible bronchoscopy with bronchoalveolar lavage (BAL) is used to investigate the lower airways. Bronchoscopy with BAL is an invasive procedure usually performed under anaesthesia, requires resourcing in a hospital setting and is not without risk. At the time of this thesis there were no studies that had evaluated the benefit of bronchoscopy with BAL in guiding therapy for children with CSLD. Bronchoscopy with BAL can be used to identify respiratory pathogens requiring antibiotics that differ from those used empirically. There is also increasing evidence to support the use of airway cellularity in guiding clinical management of children with chronic respiratory disorders [35-37]. However, the value of bronchoscopy in guiding clinical therapy for children with cystic fibrosis has recently been questioned [38]. The lack of data regarding the value of bronchoscopy for children with CSLD was identified as a clinical gap and is addressed as the first aim of my thesis in chapter 2.

1.5 Pathophysiology of CSLD

Whilst CSLD is recognised globally as an important respiratory condition, little is known about the mechanisms contributing to the persistent airway inflammation characteristic of the disease. Over recent years, considerable progress has been made in understanding airway inflammation in other suppurative lung disorders such as cystic fibrosis and chronic obstructive pulmonary disease (COPD). Some parallels exist between CSLD and these chronic respiratory disorders in relation to airway inflammation, and thus inflammatory processes involved in cystic fibrosis and COPD are sometimes used in an attempt to understand the pathophysiology of CSLD in

children and bronchiectasis in adults [39]. However, cystic fibrosis and COPD have distinctly different aetiologies and extrapolating data to CSLD, and indeed adult bronchiectasis, should be done with caution.

Nevertheless, similarities between CSLD and other chronic respiratory diseases suggest that CSLD arises from exaggerated inflammation in response to challenge from a respiratory pathogen. Thus, the vicious cycle hypothesis of self-perpetuating infection and inflammation proposed by Cole in 1997 to explain the pathogenesis of bronchiectasis [40] remains the most likely explanation for the pathogenesis of CSLD.

Suppurative airway diseases are typically characterised by neutrophilic airways and persistent or recurrent bacterial infection. Neutrophilic airways are characteristic of chronic bronchitis and bronchiectasis in adults. Neutrophil numbers as well as inflammatory cytokines including IL-1 β , IL-6, IL-8 and TNF α are elevated during periods of infective exacerbation [41, 42] and these key markers of inflammation continue to persist during periods of clinical stability [41, 43].

As described earlier, geographic locality and socioeconomic environment play a large role in determining the likely aetiology of CSLD [13, 28]. However, consistent with bronchiectasis in adults, studies of CSLD in children from various environments consistently show neutrophilic inflammation, in the presence and absence of respiratory pathogens, as well as elevated levels of proinflammatory cytokines IL-8 and TNF- α [44-46]. These paediatric studies, although limited by their cross-sectional and/or

retrospective design, are important to our understanding of the pathogenesis of CSLD as well as established bronchiectasis in adults. Inflammation in the absence of bacterial infection in children with relatively new disease may indicate that neutrophilic inflammation is not just a flow on effect of chronic infection, rather an indication of abnormal immune regulation.

It is likely that a combination of bacterial virulence mechanisms and impaired host immune responses contribute to the environment of persistent and recurrent infection [47]. Respiratory pathogens employ a variety of strategies to avoid clearance by host defence mechanisms. These strategies contribute to an environment supportive of chronic infection. Biofilm has been reported in the lower airways of children with CSLD [48] and can impede the action of antibiotics [49]. Secreted proteases can damage the structure of the bronchial wall including cilia, further hampering sputum clearance from the lungs and promoting inflammatory processes. Neutrophils degranulate in response to bacterial challenge and deploy potent anti-microbial proteases. Inadvertently it is thought that these host proteases are in part responsible for degrading the structural matrix of the airway walls, causing dilation of the airways and leading to established bronchiectasis [50].

Early diagnosis and intensive treatment protocols can stabilise or even improve the clinical prognosis of children with CSLD [31, 51]. However, understanding the host immunologic mechanisms that contribute to recurrent infection and prolonged inflammation would contribute substantially to effective prevention strategies for children at risk of CSLD [47, 52]. This thesis, and thus the remainder of this literature

review, will focus on the current understanding of the role of the host immune response to a common respiratory pathogen in the pathogenesis of CSLD.

1.6 Pathogens associated with CSLD

It is widely accepted that bacterial infection plays an important role in the pathogenesis of CSLD. Limited but important research on airway microbiology has identified three species of bacteria that are commonly associated with CSLD in children. Non-typeable *Haemophilus influenzae* (NTHi) is most common followed by *Streptococcus pneumoniae* and *Moraxella catarrhalis* [20, 45, 46, 53]. These three species are also commonly associated with acute exacerbations in adults with bronchiectasis [54, 55]. *Pseudomonas aeruginosa* is rarely reported in children with CSLD, although relatively common in adults with established bronchiectasis [56, 57]. Lower respiratory infection with *P. aeruginosa* is a predictor of accelerating lung function decline [58, 59] and is therefore likely associated with long-standing or severe disease. The dominance of *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* in CSLD forms the basis of empiric antibiotic therapy to treat acute exacerbations in children [3]. However, this evidence is based primarily on cross-sectional studies of children who are clinically stable or from retrospective chart reviews. Detailed data that relate microbiologic findings to inflammation and clinical severity are lacking.

A recent study by Hare and colleagues [60] reported a high level of concordance in bacterial strains between the nasopharynx and the lungs in children with CSLD, leading to the conclusion that colonisation of the upper respiratory tract may be a source of

lower respiratory infection in children with CSLD. The upper respiratory tract of Australian Indigenous children is densely colonised by respiratory pathogens early in life [61, 62]. Furthermore, this burden is likely higher than in non-Indigenous children [63]. It is probable that the high and early burden of upper respiratory pathogens in the nasopharynx contributes to the high burden of lower respiratory infections experienced by Indigenous children. However, there are challenges associated with identifying the pathogen responsible for a respiratory exacerbation. Spontaneous or induced sputum is a reliable and accessible source of specimen routinely used for microbiologic analysis in adults. However, collecting sputum from young children is problematic as they find it difficult to expectorate. Thus, the organism responsible for an exacerbation is rarely identified and physicians generally rely on empiric evidence to treat respiratory exacerbations in children with CSLD.

Identifying the pathogen responsible for a respiratory exacerbation is further complicated by the limitations in laboratory methods. Only a small proportion of bacteria are culturable using routine laboratory methods and identification of respiratory pathogens from sputum or BAL alone does not prove causality of infection. Furthermore, improvements in culture-independent molecular technologies show that the bronchial tree is host to a diverse microbiota, including respiratory pathogens, in people without respiratory illness [64]. Thus, it is difficult to distinguishing pathogenic colonisation from commensal or opportunistic colonisation in an individual patient.

Respiratory pathogens employ a variety of strategies to avoid clearance by host defence mechanisms. Some of the primary strategies employed by common respiratory

pathogens include formation of protective structures such as biofilm (*H. influenzae*, *P. aeruginosa*) [48, 65, 66], secretion of immune-blocking agents such as IgA proteases (*H. influenzae*,) [67] and the secretion of toxins which damage mucus-clearing structures (including cilia) of the epithelium [68]. Some pathogens associated with chronic respiratory infections, including *H. influenzae* and *Mycobacterium tuberculosis*, also avoid the host humoral response by manipulating the host's own phagocytic cells, hijacking antimicrobial mechanisms [69] and establishing an intracellular niche. It is beyond the scope of this literature review to detail virulence factors that likely contribute to CSLD and instead the reader is referred to the following reviews [70-72].

There are no data characterising viral infections in Northern Territory children with CSLD. However, data from two single-centre studies published in 2014 indicate that attention is warranted. In a study of 69 children with bronchiectasis (Queensland, Australia), respiratory viruses were associated with 48% of respiratory exacerbations [73]. In a study of bronchiectasis in 58 adults experiencing exacerbations (Guangdong, China), respiratory viruses were detected during 49% of 100 exacerbations [74]. In the paediatric study, rhinovirus was the most commonly detected virus, whilst in the adult study coronavirus was most common, followed by rhinovirus and influenza. In both studies, the presence of virus was associated with more severe symptoms. The similar findings from two distinctly different populations suggest that viral infections may be an under-recognised factor contributing to acute exacerbations and the pathogenesis of CSLD. Whilst rhinovirus is commonly carried in healthy adults, respiratory viruses are an important cause of exacerbation in other chronic respiratory illnesses including asthma and COPD [75, 76]. It has been postulated that viruses may alter immune

responses and promote respiratory exacerbations from bacterial infection [77]. Furthermore, bacteria/virus co-infections reportedly result in more severe symptoms [77, 78]. Australian Indigenous children carry a high burden of respiratory bacteria from a very young age [61, 62] and it is plausible that viral infection could be a potential initiator of bacterial exacerbation. Furthermore, viruses may contribute to the persistent inflammation reported in the absence of bacterial infection. The potential contribution of respiratory viruses to the airway pathophysiology of children with CSLD was investigated in this thesis and the resulting data is presented within chapter 5.

1.6.1 The importance of non-typeable *Haemophilus influenzae* in CSLD

Non-typeable *H. influenzae* (NTHi) are Gram-negative bacteria that are commensals of the upper airways and sometimes considered commensals of the lower respiratory tract [64, 79, 80]. However, NTHi is also the leading known cause of respiratory exacerbation in bronchiectasis, COPD and suppurative otitis media [43, 54, 81, 82] Although *S. pneumoniae* and *M. catarrhalis* are common, NTHi is more frequently isolated from the airways of children with CSLD [45, 53]. Thus, it is likely that NTHi contributes substantially to recurrent respiratory infections in children with CSLD.

A notable feature of NTHi carriage in children is the high and regular turnover of strains as well as the multiplicity of strains carried at any one time [83]. Similar carriage characteristics have been reported in adults with chronic respiratory conditions [54, 84]. Transition from a commensal to a pathogenic role for NTHi likely involves a complex

combination of immune avoidance strategies and ineffective immune responses on behalf of the host. There are a number of virulence factors employed by NTHi that provide an environment conducive to chronic colonisation. Some of these, including production of biofilm and secretion of proteases have been mentioned previously in this review (section 1.5). Biofilm has recently been described in children with CSLD and recent evidence from in vitro studies suggest that NTHi may manipulate activated neutrophils into facilitating the production of biofilm [85]. NTHi also produces human IgA proteases that, in vitro, contribute to invasion of and survival within human respiratory epithelial cells [86].

In contrast to the literature regarding virulence factors of NTHi, there are very few studies investigating the contribution of the host immune response to recurrent or persistent lower respiratory infection. A series of recent studies in adults with bronchiectasis and COPD indicate that impaired cell-mediated immune responses contribute to chronic infection with NTHi [87-90]. However, it is unknown if similar mechanisms are important in children with CSLD. Prior to the studies undertaken in this thesis (chapters 4, 5 and 7) there were no data from children. The remainder of this review summarises the current literature on the role of the host immune response in the pathogenesis of CSLD, with a particular focus on NTHi. Given the lack of data relevant to children in our population of interest, this question forms the second aim of my thesis: to identify features of the immune response in children that contribute to an increased susceptibility to lower respiratory infection with NTHi.

1.7 Inflammation and CSLD

The inflammatory response is one of the rapid response units of the immune system. When pathogens penetrate the physiological barriers, such as the epithelium, a series of coordinated cellular and non-cellular events function to rapidly contain the infection. In addition to its role as a first response unit, the inflammatory response also orchestrates the initiation of the adaptive response. The whole process involves a highly regulated system of cellular recruitment, phagocytosis, antigen processing and both intracellular and extracellular killing mechanisms. When functioning optimally, the inflammatory process resolves as rapidly as it begins, culminating in maintenance of tissue homeostasis.

1.7.1 Airway inflammation

Suppurative airway diseases are typically characterised by chronic airway inflammation associated with persistent bacterial colonisation. However, difficulties associated with obtaining lower airway specimens from young children have precluded comprehensive study of the inflammatory mechanisms contributing to CSLD.

As a result, much of our current knowledge of airway inflammation in children with CSLD is derived from small, cross-sectional studies and retrospective chart reviews. Macrophages are one of the most abundant cells found in the healthy lung, typically comprising 85-95% of the cellular profile of BAL fluid [91]. Indications of airway inflammation include changes in the cellular profile and elevation of proinflammatory cytokines. Current evidence indicates that CSLD in children is associated with

neutrophilic inflammation of the airways [12, 46, 92], and elevated levels of proinflammatory cytokines such as IL-8, TNF α and IL-1 β [45, 93].

Airway neutrophilia is also characteristically present in many chronic respiratory diseases in adults, including chronic obstructive disease, chronic bronchitis and bronchiectasis. However, elevated levels of macrophages, B-cells and CD8 $^{+}$ T-cells are also reported [94]. Neutrophil numbers and the associated inflammatory cytokines, IL-1 β , IL-6 and IL-8, are highest during periods of infective exacerbation [41, 95]. However, prolonged airway inflammation following infection, is recognised as a key pathological mechanism of these chronic lung diseases [40].

The underlying mechanisms resulting in persistent inflammation have not been determined but it is proposed that an exaggerated inflammatory response and impaired regulation are involved [47]. IL-1 β is produced by a variety of pulmonary cells, including macrophages and neutrophils, in response to a microbial challenge. IL-1 β drives the inflammatory cascade by localising neutrophils and promoting the production of inflammatory modulators such as IL-6 and IFN- γ -inducible protein 10 (IP-10; CXCL10). IL-6 plays a complex role in the inflammatory response, from promoting inflammation to wound healing. Dysregulation of IL-6 pathways is associated with chronic inflammation [96]. In addition to its inflammatory modulating properties, IL-6 is integral to initiating the adaptive response and in directing its primary phenotype. In the lung, IL-6 polarises the adaptive immune response in favour of the humoral response by

inhibiting IL-12 production [97, 98] and by promoting the differentiation of B-cells into antibody-producing plasma cells [99].

Several studies in children and adults indicate that increased levels of inflammatory markers in the lungs, including IL-8, TNF- α and neutrophil counts are associated with more severe respiratory symptoms including poorer lung function [45, 100-102].

Furthermore, in adults with bronchiectasis, neutrophil counts correlate positively with the anatomical extent of bronchiectasis [101].

1.7.2 Systemic inflammation

CSLD is traditionally recognised as an inflammatory disease of the airways. However, emerging data from studies of adults with bronchiectasis suggest that local inflammation may have systemic consequences. Elevated levels of circulating inflammatory cells (neutrophils and total white cell count) as well as soluble serum mediators (including transforming growth factor (TGF)- β , C-reactive protein (CRP), fibrinogen, and soluble adhesion molecules) have been described in adults with bronchiectasis [41, 103-106].

Furthermore, a high level of systemic inflammation is associated with an accelerated decline in lung function [59]. Whether systemic inflammation is actively involved in bronchiectasis or rather a symptom of ‘spill over’ from excessive lung inflammation (as suggested in COPD [107]) is unknown. There are currently no data regarding the role of systemic inflammation in the pathogenesis of CSLD in children.

1.7.3 The role of neutrophils in CSLD

Neutrophilic inflammation of the airways is often present in CSLD. In the healthy lung, the neutrophil is one of the key cells involved in driving the inflammatory response against invading pathogens. Neutrophils are recruited from peripheral circulation by local populations of macrophages and neutrophils. Circulating neutrophils rapidly migrate to the airways in response to pro-inflammatory mediators including IL-1 β , IL-8 and TNF α . The primary role of the neutrophil is to eliminate the invading pathogen, first by phagocytosis, followed by release of an arsenal of antimicrobial products within the phagosome. When significantly stimulated, neutrophils degranulate and deploy potent proteases (including neutrophil elastase, cathepsin and myeloperoxidase). Inadvertently, the release of these proteases can also degrade matrix proteins of the airway walls and promote further inflammation. In the healthy lung, this process is tightly regulated to prevent excessive damage to host tissue.

Impaired neutrophil function has been described in some studies in adults with bronchiectasis [108] but not in others [109]. Several studies have demonstrated that an impaired capacity for phagocytosis and a reduced capacity for oxidative burst by neutrophils may be associated with severe disease [108, 110]. King and colleagues [111] recently demonstrated a high prevalence of impaired bacterial-specific oxidative burst function by circulating neutrophils in a large group of adults with bronchiectasis. Furthermore, patients with a low capacity for neutrophil-generated oxidative burst also demonstrated a reduced capacity for intracellular bactericidal activity by the neutrophils. These data however, were not reflected in two small studies of adult bronchiectasis

where no impairment in oxidative burst by circulating neutrophils was found [109, 110], although impairment of airway neutrophils was observed [110]. The discrepancies between these data may be attributed to the methods used to induce oxidative burst (bacterial phagocytosis versus synthetic peptide) or the clinical characteristics of the study cohorts (age and severity of disease). In King et al.'s study, phagocytosis and intracellular killing were investigated using a bacterial challenge, which may represent an alternate and more accurate pathway of activation compared with using peptide as the challenge. Despite the conflicting data, King et al. and Chalmers et al. both found evidence of impaired microbicidal activity, albeit in different populations of neutrophils, suggesting that functional phagocytosis accompanied by impaired intracellular killing may be one strategy employed by NTHi to establish an intracellular niche and avoid host clearance mechanisms. There are currently no data regarding neutrophil function in children with less severe disease. The possibility that impaired phagocytosis and intracellular killing mechanisms correlate with disease severity highlights the need to investigate the role of neutrophil function in the progression of CSLD in children who are in the early stage of chronic disease.

1.7.4 The role of macrophages in CSLD

Macrophages are phagocytic cells with multiple phenotypes. They are the most abundant cell in the normal lung, located in and around interstitial tissue (interstitial macrophages) and within the surfactant fluid lining the alveoli (alveolar macrophages). Alveolar macrophages hold a unique position at the interface between the outside environment and the lung tissue and are therefore at the forefront of pathogen surveillance. The

phagocytic capabilities of macrophages afford multiple distinct functions including intraphagosomal killing of bacterial pathogens, clearance of apoptotic neutrophils and epithelial cells (known as efferocytosis), antigen processing and subsequent instigation of inflammatory responses.

Macrophage numbers are increased in the airways of adults with bronchiectasis [112]; however, there are few published data regarding the role of the macrophage in the pathogenesis of the disease. One possible mechanism that has been demonstrated in adults with chronic obstructive pulmonary disease and asthma is an impaired capacity for efferocytosis (phagocytosis of apoptotic cells) [113, 114]. When neutrophils apoptose, the potential exists for a myriad of toxic factors to be released into the lung microenvironment. Efficient efferocytosis is paramount to preventing secondary necrosis and damage to lung tissue. Defective efferocytosis has been described in asthma and COPD and may contribute to airway inflammation in these disorders [115]. Whilst increased numbers of apoptotic cells have been reported in adults with bronchiectasis, there are currently no data regarding the role of efferocytosis in the pathogenesis of bronchiectasis in adults or CSLD in children [116]. Whilst it is plausible that impaired efferocytosis may contribute to excessive inflammation and thus the pathogenesis of CSLD, it was not the focus of my PhD and investigations of efferocytosis and related mechanisms were beyond the scope of this thesis.

1.8 Adaptive immune responses in CSLD

1.8.1 Overview

Despite the dual existence of NTHi as commensal organism and important respiratory pathogen, little is known about the development of natural immunity to NTHi. It is well recognised that both the lungs and the immune system continue to mature and develop throughout childhood [117, 118]. Regular antigen exposure drives immune maturation from a predominantly Th2 type response to a balance of Th1 and Th2 responses. Environmental, lung and immune pressures influence the maturation process [119-121]. A recent study of Papua New Guinean infants demonstrated that high and early upper respiratory carriage of respiratory pathogens might be associated with delayed or impaired maturation of antigen-specific Th1 responses [122]. Similar to Papua New Guinean infants, the upper respiratory tract of Northern Territory Indigenous children is densely colonised by respiratory pathogens early in life [62]. Furthermore, a recent study by Hare and colleagues [53] reported a high level of concordance between bacterial strains in the nasopharynx and the lungs of children with CSLD, leading to the conclusion that colonisation of the upper respiratory tract may be a source of lower respiratory infection in children with CSLD [53]. Thus, it is possible that the high and early burden of respiratory pathogens in the nasopharynx of Northern Territory Indigenous infants influences the developing immune response and predisposes young children to exaggerated inflammatory responses, impaired immune regulation and the perpetuation of chronic endobronchial infection.

1.8.2 Dendritic cells

Airway dendritic cells are potent antigen presenting cells and reside throughout the lung architecture. They are key instigators of the adaptive immune response, activating and directing the differentiation of naïve T-cells. The mechanisms controlling the transition between innate and adaptive responses are slowly being elucidated. However, there are difficulties associated with isolating dendritic cells from the airways, particularly in children. Thus, much of the basic knowledge regarding dendritic cells in paediatric lower airways has been derived from animal studies or cohorts of children with allergic disease such as asthma or upper respiratory infections such as otitis media [123]. There are at least two types of dendritic cells, plasmacytoid and myeloid. Both are derived from progenitor cells produced in the bone marrow and each has distinctive functional properties. Myeloid dendritic cells are primarily responsible for the antigen-specific activation and phenotypic polarisation of naïve T-cells in the lung lymph nodes [124], whilst plasmacytoid dendritic cells are important in T-cell responses to viral infections [125, 126] and in regulating inflammation [97].

There are no data regarding dendritic cell function in children susceptible to lower respiratory infections with NTHi. However, dysfunctional dendritic cells have been implicated in the pathogenesis of other respiratory illness including recurrent otitis media and chronic infection with *M. tuberculosis* [69]. Preliminary data from a small group of children (n=12) suggest that dendritic cell immaturity, as determined by low MHC II expression, contributes to recurrent otitis media in children [127].

1.8.3 Cell-mediated immune responses to NTHi

The NTHi-specific cell mediated immune response in adults with bronchiectasis is phenotypically distinct from that of adults without respiratory disease [88, 89]. In a landmark study by King and colleagues [88], circulating CD4⁺ T-helper cells from healthy adults responded to an in vitro NTHi challenge with increased expression of IL-2 and IFN- γ , in a predominantly classic Th1 manner. In contrast, the cytokine response from adults with bronchiectasis and chronic NTHi infection was polarised in favour of IL-4 and IL-10 and complemented by low expression of IL-2 and IFN- γ . These data suggest that Th1 polarised cell-mediated responses contribute to protective immunity against NTHi.

Several factors may contribute to the differences in cytokine profiles including a reduction in the size of the pool of CD4⁺ memory T-cell. However, King et al [89] showed that the polarisation of cytokine profiles were in direct relation to a skewing of CD4⁺ T- cell phenotype, rather than an overall reduction in the absolute number of CD4⁺ T cells. In addition to CD4⁺ T-helper cells, CD8⁺ cytotoxic T-cells have been implicated in chronic infection with NTHi [89]. CD8⁺ T-cells have the capacity to switch between IFN- γ and IL-4 polarised responses [128]. CD8⁺ phenotype switching has not been investigated with respect to chronic NTHi infection, however CD8⁺ T - cells from adults with bronchiectasis demonstrated a non-specific capacity to produce IFN- γ that was not realised in response to a specific challenge with NTHi [89].

Phenotype switching is one mechanism that may explain this. These data suggest that adults with bronchiectasis have the necessary cell-mediated immune architecture to

respond to NTHi. Both T-helper and cytotoxic T cells in adults with chronic NTHi infection had a capacity to produce IFN- γ that was similar to healthy adults, but failed to do so in response to NTHi. Whilst the mechanism causing the impaired IFN- γ response is yet to be determined, these data suggest that, at least in vitro, the cell-mediated immune response is modified, rather than delayed and this modified response contributes to chronic infection with NTHi in adults.

These adult studies provide important data on the pathogenesis of NTHi infections, however extrapolation of this data to the paediatric setting may not be appropriate. It is well regarded that immune responses of children differ from those of adults [123, 129, 130]. Furthermore, accumulating evidence indicate that the immune response may be influenced by chronic inflammation, airway remodelling and exposure to pollutants such as cigarette smoke [131]. It remains unclear whether the altered immune response described in adults with established disease is involved in the initiation of their disease, or rather, develops as a consequence of chronic inflammation and longstanding tissue damage. This represents a knowledge gap addressed by this thesis, which aims to determine if NTHi-specific cell-mediated immune responses are impaired in children with considerable milder disease (chapter 4).

1.8.4 Humoral immune responses to NTHi

The role of the humoral response in the pathogenesis of CSLD is unclear. There are considerable published data showing chronic colonisation and active infection of the lower airways with NTHi despite the presence of an active humoral immune response

[84, 90, 132, 133]. Adults with chronic respiratory diseases including bronchiectasis, chronic bronchitis and COPD exhibit a comprehensive humoral immune response against NTHi, with ample amounts of specific total IgG, IgG subclasses, IgA and IgM present in circulation [88, 90] [134]. These antibodies are functional against NTHi in vitro and when combined with serum complement form a potent NTHi-clearance mechanism. Although the amount of circulating immunoglobulin is age dependent, children also appear to be proficient at producing NTHi-specific IgG [135, 136]. It is thought that this strong, universal humoral response is one of the main reasons why NTHi rarely causes systemic infection. However, high systemic antibody levels do not appear to correlate with protection from respiratory infections [90].

Secretory IgA is the main immunoglobulin associated with mucosal immunology. Whilst data on NTHi-specific secretory IgA are lacking, a small study of 25 adult patients indicated that deficient secretory IgA was not associated with bronchiectasis or chronic bronchitis [134]. In contrast, in vitro studies have shown that NTHi produce human IgA proteases that may facilitate the internalisation and persistent survival within lung epithelial cells [86]. It is plausible that impaired antibody function rather than deficient antibody may contribute to recurrent infection with NTHi in CSLD. Alternatively, the presence of bacterial biofilm or host bronchial secretions may impede the activity of antibodies. However, there are no data to confirm or refute this in children or adults with suppurative lung disease.

Collectively, these data indicate that chronic infection with NTHi is not significantly attributed to an overall deficiency in antibody production. However, NTHi is a

heterogeneous species and can induce the production of strain-specific antibodies [84, 137]. Thus, it has been suggested that antibody specificity may explain the inability of a previous infection to protect against a future infection with NTHi. Contrary to this hypothesis, a considerable degree of functional antibody cross-reactivity has been demonstrated between different NTHi strains [90]. Furthermore, there is a high turnover of strain carriage and multiple strains are often carried concurrently [80, 83]; hence, it would be expected that a high level of antibody diversity is circulating at any one time which should significantly restrict the number of infections regardless of strain specificity. These data do not preclude the role of humoral immunity in protection against respiratory infections with NTHi as humoral immunity likely plays a significant role in protection from systemic and airway disease. However, high prevalence of recurrent infection in the presence of high levels of antibody supports the argument that an increased susceptibility to infection with NTHi is more closely linked to the cell-mediated immune response than the humoral immune response. It is important to note that the data described here were derived from studies involving quite distinct disease pathologies and it is possible that the specific immunopathology associated with different respiratory diseases, such as exposure to cigarette smoke and airway remodelling, influence the effectiveness of the humoral response and its role in disease progression. Thus, extrapolation of data obtained from adults with severe disease may not be applicable to children with CSLD where the disease is of more recent onset.

In light of the knowledge gap regarding NTHi-specific humoral responses in children with CSLD, one of the aims of this thesis is to determine if the production of IgG

subclass antibodies to NTHi-specific proteins is impaired in children with CSLD. This aim is addressed in chapter 4.

1.8.5 Gaps in methodology

In this thesis immune responses to NTHi were investigated using blood mononuclear cells. NTHi is a pathogen of the respiratory tract and it may be suggested that immune responses differ between the local and peripheral environments. Lymphocytes are a minority population in BAL and this limits investigation of local (lung) immune responses in children with CSLD. T-lymphocytes derived from lung biopsies have recently been used in a novel study of NTHi-specific immune responses in adults with COPD and lung cancer [138]. However, obtaining lung tissue from children for research purposes was not appropriate in the setting of my research. Furthermore, when studying chronic respiratory disease in children, peripheral blood mononuclear cells may be more indicative of an antigen-specific recall response as the lymphocytes are not under the influence of inflammatory mediators associated with chronic inflammation. In vivo, blood mononuclear cells provide a reservoir of cells that are mobilised to the lung when needed.

One of the gaps in the literature concerns the lack of data regarding protective immune responses against NTHi in children. Blood is a reliable and convenient source of lymphocytes for immunologic studies and has been used successfully in recent large cohort studies of other respiratory illnesses including asthma [117, 139] as well as in efficacy trials of vaccines against respiratory pathogens [140, 141]. In this thesis, blood

mononuclear cells were used to compare the systemic immune response in children with CSLD and healthy control children, as presented in chapters 4 and 7.

Other knowledge gaps in currently used methods relate to the heterogeneity of NTHi as over 100 different strains have been proposed. It has been suggested that immune responses towards NTHi may be strain-specific [142]. When my PhD commenced, there were limited data regarding strain-specific cell-mediated immune responses towards NTHi [87, 89]. Challenges associated with studying immune responses against a heterogeneous species include standardising antigen type and challenge dose such that in vitro assays are reproducible. These challenges have been addressed in chapter 3.

1.9 Clinical management of CSLD

1.9.1 Overview of management strategies

It is increasingly recognised that with early diagnosis and appropriate therapy the clinical progression of CSLD in children can be prevented [1, 143, 144]. It is also widely accepted that bacterial infection plays an important role in the pathogenesis of CSLD. Thus, current clinical management strategies focus on antibiotic therapies combined with physiotherapy to clear the airways of mucus [3, 145]. Whilst antibiotic therapy is generally used short-term for acute exacerbations there have been several studies showing that extended (maintenance) antibiotic therapy can reduce the number of acute exacerbations and sputum production, and thereby improve quality of life [20, 146]. Yet despite the best clinical management efforts, CSLD can progress and other innovative approaches should be considered to prevent or minimise disease progression.

A number of innovative therapeutics have been proposed to break the cycle of inflammation and infection. The combined antimicrobial and anti-inflammatory properties of macrolides have received recent attention for use in children with CSLD, and were the focus of a recent multicentre clinical trial [20].

Controlling inflammation through inhibitors of neutrophil elastase and TNF- α has also been proposed [147, 148] but trials of these potential therapeutics are in their infancy. It is beyond the scope of my thesis to present a comprehensive review of novel therapeutic strategies proposed for children with CSLD, rather the reader is referred to several recent reviews [52, 149, 150]. Presented here instead is an overview of therapeutics related to my PhD research.

1.9.2 Anti-inflammatories

As described in section 1.5, CSLD is a chronic illness often characterised by intense neutrophilic inflammation that can persist in the absence of bacterial exacerbation. One proposed management strategy involves the use of inhaled anti-inflammatories drugs such as corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) to reduce the persistent neutrophilic inflammation. Kapur et al. recently conducted a systematic review on the use of inhaled corticosteroids for adults and children with bronchiectasis, concluding that no recommendations could be made for their use. At the time my PhD study commenced, there were no reviews regarding the use of NSAIDs for CSLD or bronchiectasis. Thus, as part of my PhD studies I undertook a systematic review to

investigate the efficacy of inhaled NSAIDs in the management of bronchiectasis. The results from this are presented in chapter 6.

1.9.3 Vaccination against NTHi

Immunisation against respiratory pathogens is another possible strategy to prevent or reduce infections with NTHi. Trials of oral vaccines targeting NTHi have shown considerable short-term success in reducing the number of acute exacerbations in adults with chronic bronchitis, bronchiectasis and COPD [151-153]. However, none have been trialled in children and there remains no vaccine licenced specifically for NTHi.

The 10-valent Pneumococcal *H. influenzae* protein D conjugate vaccine (PHiD-CV) [154] is the sole licensed vaccine that contains a component of *H. influenzae* and therefore may be effective against lower respiratory infections with NTHi. PHiD-CV is licensed for use in children up to 2 years of age, to prevent infection with *S. pneumoniae*. It comprises 10 antigens specific to *S. pneumoniae* serotypes conjugated to protein D, a highly conserved, outer membrane lipoprotein common to both typeable and non-typeable strains of *H. influenzae*. In its natural form, protein D is a virulence factor of NTHi. It impairs the ciliary function of epithelial cells and facilitates intracellular colonisation of phagocytic and epithelial cells [155]. Protein D is also highly immunogenic [156, 157] and protein D-specific antibodies have been shown to offer protection against experimentally induced otitis media in animal models [158]. However, to date there are no published human studies that have examined the efficacy of vaccination with PHiD-CV on lower respiratory infections with NTHi.

Studies of adults with bronchiectasis and children with otitis media indicate that the cell-mediated immune response is important for protective immunity against NTHi. Thus, a vaccine that improves the cell-mediated immune response to NTHi may be beneficial for children with, or at risk of, CSLD. As PHiD-CV contains an antigenic lipoprotein of NTHi and is currently licenced for use in children (albeit for *S. pneumoniae*), the final aim of my thesis (chapter 7) was to investigate the potential of vaccination with PHiD-CV to improve the NTHi-specific cell-mediated immune response in children.

1.10 Summary of chapter 1 and the aim of this thesis

1.10.1 Relevance

CSLD is a respiratory illness that has considerable impact on Australian Indigenous children, their families and an overburdened health system. The factors contributing to the increased susceptibility of children with CSLD to lower respiratory infection with NTHi have not been defined. Published data regarding the pathogenesis of lower respiratory infections with NTHi are limited and primarily restricted to adults with long-standing and severe respiratory disease or extrapolated from studies of paediatric illness with distinctly different aetiologies such as cystic fibrosis and asthma. Furthermore, evidence-based clinical management strategies for children with CSLD are restricted to limiting acute exacerbations of CSLD rather than in the prevention of CSLD in at-risk children. Understanding the pathogenesis of CSLD in children is important for guiding effective clinical management strategies for a condition that should be largely preventable in Australia.

1.10.2 Overall aim

The overall aim of my PhD was to identify factors of the host immune response contributing to the pathogenesis of CSLD that may be targeted in future management strategies.

The specific objectives of my thesis are to:

1. Evaluate the benefit of flexible bronchoscopy with BAL to the clinical management of children with CSLD (Chapter 2).
2. Identify aspects of systemic innate, cell-mediated and humoral immune responses which may increase susceptibility to respiratory infections with NTHi (Chapters 3 and 4).
3. Determine if systemic or lung inflammation is associated with a modified NTHi-specific cell-mediated immune response (Chapter 5).
4. Review the clinical evidence of the efficacy of inhaled NSAIDs in the management of bronchiectasis (Chapter 6).
5. Determine if receipt of a conjugate vaccine containing protein D from *H. influenzae* is associated with improvement in NTHi-specific cytokine responses in children with chronic suppurative lung disease (Chapter 7).

The main hypotheses associated with the specific objectives are that:

1. CSLD is heterogeneous condition that cannot be addressed by empiric antibiotic therapy alone.

2. Children with CSLD have an impaired immune response to NTHi that likely contributes to an increased susceptibility to lower respiratory infections.
3. Systemic NTHi-specific immune responses are associated with airway inflammation.
4. A systematic review on the efficacy of inhaled NSAIDs in the management of children and adults with bronchiectasis will help guide clinical practice.
5. Impaired immune responses to NTHi may be improved through vaccination.

1.11 Thesis design

Studies were designed to address the specific thesis hypotheses and in doing so, fulfil the primary aim of this thesis.

Chapter 2 addresses the first hypothesis that CSLD is a heterogeneous condition that cannot be addressed by empiric antibiotic therapy alone. Flexible bronchoscopy and BAL data were collected prospectively in 56 children during initial investigations for bronchiectasis. It was determined *a priori* that investigations contributed to a change in empiric therapy if any of the following were identified:

- a) airway obstruction requiring additional intervention
- b) airway inflammation other than neutrophilic
- c) culture of a respiratory pathogen requiring change from usual empiric antibiotics.

Chapter 3 addresses the methodology gaps that have been identified in the literature and describes the methods used to conduct the studies comprising this thesis. Included are

ethics clearance number, specimen collection, immunology assays and statistical analysis.

Chapter 4 addresses the second hypothesis that impaired NTHi specific immune responses contribute to an increased susceptibility to infection with NTHi. Inflammatory and cell-mediated immune responses to NTHi in vitro and NTHi-specific antibodies in plasma were investigated in 80 children with CSLD and 51 healthy control children.

Chapter 5 addresses the third hypothesis that an impaired cell-mediated immune response and airway inflammation are inter-related in children with CSLD. A panel of cytokines and antimicrobial compounds involved in the generation and control of inflammation were measured in the BAL and plasma of 70 children with CSLD. Multivariate regression analysis was used to determine the relationship between airway inflammation and the capacity to generate an appropriate systemic cell-mediated immune response to NTHi.

Chapter 6 addresses the fourth hypothesis that a systematic review of the literature of clinical trials pertaining to the efficacy of inhaled NSAIDs in the management of children and adults with bronchiectasis will help guide clinical practice.

Chapter 7 addresses the final hypothesis and ultimate aim of this thesis; to provide proof-of-concept that impaired immune responses may be improved in children with CSLD. This study took advantage of the introduction of the 10 valent Pneumococcal *H. influenzae* Protein D conjugate vaccine (PHiD-CV) into the Northern Territory

childhood immunisation schedule to investigate the potential of an NTHi-vaccine to improve NTHi-specific cell-mediated immune responses in 107 young children with CSLD and 32 healthy control children.

CHAPTER 2

The value of bronchoscopy with bronchoalveolar lavage to the clinical management of children with chronic suppurative lung disease

Chapter 2 THE VALUE OF BRONCHOSCOPY WITH BRONCHOALVEOLAR LAVAGE TO THE CLINICAL MANAGEMENT OF CHILDREN WITH CHRONIC SUPPURATIVE LUNG DISEASE

2.1 Chapter Overview

The overall aim of this chapter is to evaluate the benefit of flexible bronchoscopy with bronchoalveolar lavage (BAL) to the clinical management of Northern Territory children with CSLD.

As data regarding the pathogenesis of CSLD are limited, management strategies for children in the Northern Territory are primarily based on empiric evidence or extrapolated from clinical management strategies of other chronic respiratory illnesses such as cystic fibrosis. Bronchoscopy with BAL is one strategy increasingly used to investigate chronic respiratory disease in children.

Bronchoscopy is a valuable procedure used to visually inspect the lower airways for lesions and structural abnormalities that may be causing chronic respiratory symptoms. In conjunction with bronchoscopy, BAL is often performed to collect a lower airway specimen for microbiologic and cellular analysis. However in children, bronchoscopy

with BAL is an invasive procedure performed under general anaesthetic and is therefore not without risk.

As described in Chapter 1 bacterial infection is the most common identified cause of CSLD in Northern Territory children. Non-typeable *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* are the respiratory pathogens most frequently associated with CSLD in children from this region and form the basis of empiric antibiotic therapy. Given that CSLD is closely associated with three common respiratory pathogens targeted by empiric therapy, the question arose as to whether bronchoscopy with BAL was of clinical benefit to children with CSLD. There are currently no data regarding the benefit of bronchoscopy with BAL in children with CSLD, especially with respect to empiric antibiotic therapy. Furthermore, the role of bronchoscopy with BAL in the clinical management of children with cystic fibrosis has recently been questioned [38].

Thus the first aim of my PhD is to prospectively evaluate the benefit of bronchoscopy with BAL to the clinical management of children with CSLD beyond empiric antibiotic therapy. The results from this study are presented in section 2.2 as a published article. The main findings are summarised in section 2.3.

2.2 Journal article: Bronchoscopy contributes to the clinical management of Indigenous children newly diagnosed with bronchiectasis.

2.3 Chapter summary

The study described in this chapter is the first to evaluate the benefit of flexible bronchoscopy with BAL to the clinical management of children with CSLD and therefore addresses the first aim of this thesis. The main finding was that bronchoscopy with BAL substantially contributes to the clinical management of Northern Territory children with CSLD. Data readily obtained from bronchoscopy with BAL added to or resulted in a change from empiric antibiotic therapy in 41% of children with CSLD. BAL data regarding the inflammatory phenotype of the airways was the highest contributing factor (34% of children) followed by the identification of bacterial species requiring different antibiotic therapy from those used empirically (9% of children).

There was a high prevalence of airway eosinophilia with 34% of children identified with BAL eosinophils greater than 2.5%. Although data describing the nature of airway inflammation in children with CSLD are limited, airway eosinophilia has not previously been described in children with CSLD. Furthermore airway eosinophilia is rarely reported in adults with bronchiectasis and is thought to be associated with severe or end-stage disease. Thus the high prevalence of eosinophilia in this population of children with CSLD was an unexpected, novel finding. The role of airway eosinophilia in the pathogenesis of CSLD in children, as for bronchiectasis in adults, is unknown however prolonged systemic or airway inflammation is associated with end-organ damage and therefore requires investigation. Treatment of airway eosinophilia varies depending on the aetiology but differs from that for neutrophilia. Due to the cross sectional nature of

this study, limited investigations were conducted to identify the cause of the eosinophilia. These investigations are discussed further in Chapter 5.

The data presented in this chapter support the first hypothesis of my thesis; that CSLD is a heterogeneous condition that cannot be managed by empiric antibiotic therapy alone. Bronchoscopy with BAL added to the clinical management of children with CSLD by identifying unexpected airway eosinophilia, respiratory pathogens and a foreign body, all of which required intervention beyond empiric antibiotic therapy. These data do not advocate for the indiscriminate use of bronchoscopy with BAL for monitoring CSLD. However these data support the use of bronchoscopy to investigate persistent respiratory symptoms earlier rather than later to prevent the progression of CSLD.

CHAPTER 3

General methods

Chapter 3 GENERAL METHODS

3.1 Chapter overview

Chapter 3 describes the general methods used for the laboratory studies undertaken in chapters 4, 5 and 7. The laboratory studies were undertaken to achieve the primary aim of my thesis (to identify factors of the immune response contributing to the pathogenesis of CSLD that may be targeted in future management strategies), and address the following thesis objectives:

Objective 1: Evaluate the benefit of flexible bronchoscopy with BAL to the clinical management of children with CSLD (Chapter 2)

Objective 2: Identify aspects of systemic innate, cell-mediated and humoral immune responses that may increase susceptibility to respiratory infections with NTHi (Chapter 4).

Objective 3: Determine if systemic or lung inflammation is associated with a modified NTHi-specific cell-mediated immune response (Chapter 5)

Objective 5: Determine if receipt of a conjugate vaccine containing protein D from *H. influenzae* is associated with improvement in NTHi-specific cytokine responses in children with chronic suppurative lung disease (Chapter 7).

This chapter includes an overview of ethics approval, study participants, specimen collection and immunologic assays. Establishment of immunologic assays were necessary prior to undertaking the targeted studies as these methods were not previously done in our laboratory. These methods were:

- (a) Optimising assays to measure cytokine and inflammatory biomarker protein in culture supernatant, BAL and serum (section 3.4).
- (b) Preparation and standardising of the NTHi inoculum (section 3.5)
- (c) Optimisation of cell culture conditions for measuring cytokine protein post stimulation (section 3.6).

Each panel of cytokines, inflammatory biomarkers and *H. influenzae*-specific antibodies investigated in this thesis were study specific and are described in the respective chapters. Overall these included IFN- γ , IL-13, IL-5, IL-10, IL-1 β , IL-6, TNF- α , IP-10, LL-37 and IgG subclasses to *H. influenzae* Protein D and outer membrane proteins P4 and P6.

Here, I provide a full description of the methods I established in our Menzies laboratory. These methods are also described in the appropriate published journal articles of chapters 4, 5 and 7.

3.2 Ethics approval

The studies comprising this thesis were approved by the Human Research Ethics Committee (HREC) of the Northern Territory Department of Health and Menzies School

of Health Research (project number 07/63), including approval from the Aboriginal subcommittee. All children were enrolled following written informed consent from the parent/carer. Healthy adult volunteers provided written informed consent.

3.2.1 Study participants

Children with CSLD and healthy control children up to 10 years of age were recruited prospectively between the years 2008 and 2013 from Royal Darwin Hospital or at Menzies School of Health Research, Darwin, Northern Territory, Australia.

Children in the CSLD group were under the care of a paediatrician and currently undergoing routine investigations for their CSLD [3]. Briefly, these investigations included cHRCT scan, flexible bronchoscopy with BAL, blood analysis including total IgG and subclass IgG, IgA, IgM and IgE, sweat test (to exclude cystic fibrosis) and microbiologic analysis of BAL. Only children with CSLD not attributed to an underlying disorder (for example cystic fibrosis, primary immune deficiency, physiological defect) were included in the data analysis.

Children in the healthy control group were enrolled primarily through the elective surgery list of Royal Darwin Hospital. Healthy control was defined as an absence of clinical history of chronic respiratory or other non-respiratory illness. The healthy control group did not have the same clinical investigations as the CSLD group as these investigations were not indicated for their procedure. Thus blood was collected from the healthy controls but BAL was not collected.

All children were clinically stable at the time of sample collection. Clinical stability was defined as no known respiratory exacerbation or acute infection within the preceding 3 weeks.

Peripheral blood was also obtained from adult volunteers (laboratory and clinical staff). These samples and data were for establishing the assays in this chapter only. Data from these adult samples were not included in any of the subsequent research chapters.

3.3 Sample and data collection

Clinical and socio-demographic data were collected using standardised data collection forms. Routine clinical investigations were performed by the regional reference laboratory at Royal Darwin Hospital. Blood and BAL specimens for both clinical and research purposes were collected simultaneously at the time of intravenous access (at the start of general anaesthesia). BAL was not collected from healthy control children.

3.3.1 Bronchoalveolar lavage

BAL was obtained from the most abnormal lobe identified by bronchoscopy or cHRCT scan. Two aliquots of sterile normal saline (1 ml/kg, max 10ml for the first aliquot and 2ml/kg, maximum 20mL for the second aliquot) were instilled into the lobe and suctioned immediately into separate mucus traps. BAL in excess of clinical requirements was used for research. BAL for research were immediately placed on ice and sent to the Menzies laboratory for processing. The first aliquot was used for microbiologic analysis, the second for immunologic analysis.

3.3.2 BAL for microbiology and virology

BAL from pot one was used for microbiology and virology. NTHi, *S. pneumoniae* and *M. catarrhalis* culture and identification were performed by our research laboratory (Microbiology Laboratory, Child Health Division, Menzies School of Health Research) using established protocols [159]. As previously reported, a threshold greater than 10^4 cfu/ml was considered clinically important [53, 160]. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* culture and identification were performed by the diagnostic laboratory at the Royal Darwin Hospital.

Presence of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and respiratory viruses (rhinovirus, adenovirus, enterovirus, bocavirus, respiratory syncytial virus, Wu virus, Ki virus, coronavirus, parainfluenzae and metapneumovirus) were determined by PCR on undiluted BAL stored at -80°C , by the Queensland Paediatric Infectious Disease Laboratory (Brisbane, Queensland, Australia) as previously reported [73].

3.3.3 BAL for immunology studies

Total and differential cell counts were performed on unfiltered BAL from the 2nd aliquot. Total cell counts were determined using a haemocytometer. The remaining BAL was separated into cellular and acellular fractions by centrifugation (800 xg for 8 minutes).

For cell differential counts, cytospins were prepared with a Cytospin 4 (Thermo Scientific, USA), loading approximately 5×10^4 cells per slide, and stained with Quick Dip (Fronine, Australia). The macrophage, neutrophil, lymphocyte and eosinophil percentages were calculated from a total count of 300 cells. The differential cell counts

were performed by myself. The procedure was initially calibrated by comparing independently obtained differential cell counts from two operators (myself and Professor Anne Chang) and also by comparing repeat counts (myself). These data were used primarily to address thesis objectives 1 and 3. The acellular fraction was stored in single use aliquots (to avoid freeze-thaw cycles) at -80°C and used to address thesis objective 3.

3.3.4 Blood processing

Up to an additional 3ml of heparinised venous blood was collected for research. Blood was collected into preservative-free heparin and separated into peripheral blood mononuclear cells (PBMC) and plasma within 2 hours. PBMC were isolated by centrifugation over Ficoll-Paque™ Plus (GE Healthcare Bio-Sciences AB, Sweden) and cryopreserved in liquid nitrogen in 10%DMSO/ heat-inactivated fetal bovine serum (FBS; Gibco Life technologies, Australia). The separated plasma was stored at -80°C. These specimens were used to address objectives 2, 3 and 5.

3.4 Measurement of cytokines, circulating antibodies and antimicrobial proteins

Sandwich (non-competitive) Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIATM; Perkin Elmer, USA) was used to quantify cytokine protein from the culture supernatant, cytokine and antimicrobial proteins from BAL and plasma and NTHi-specific plasma antibodies. DELFIATM was chosen to measure protein levels for several reasons. Firstly DELFIATM has a large dynamic range (3-30 000 pg/ml) with low background and thus requires minimal handling of specimens. This was important in

order to maximise data from the limited volume of blood obtained from young children. Secondly, DELFIA™ measures the total concentration of individual cytokines in the culture supernatant and thus provides an overall view of the cytokine environment generated in response to NTHi.

Coating and detection antibodies used for the assay were first titrated to determine the optimal working concentration of each. Details of the recombinant proteins and coating and detection antibodies used for DELFIA™ are presented in Appendix 1. Quantitative standard curves were generated from serial dilutions of recombinant human (rHu) cytokine proteins and included on each assay plate. Representative standard curves are presented in Figure 3.1.

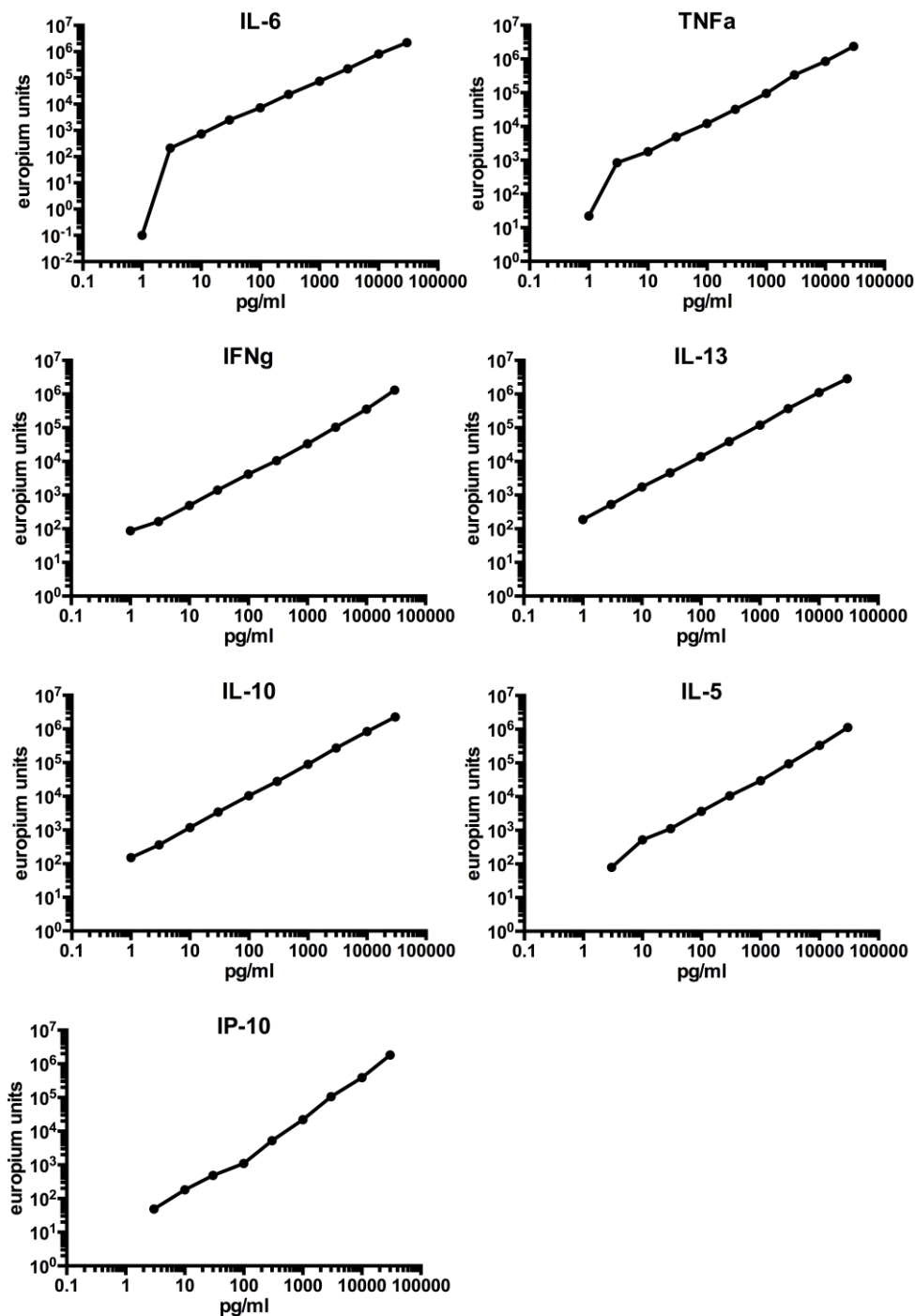


Figure 3.1 Representative standard curves used to determine cytokine and biomarker concentration by DELFIA™ time-resolved fluorescence assays. Each data point represents the mean europium fluorescence unit from duplicate wells.

3.5 Preparation of the non-typeable *H. influenzae* inoculum

The NTHi-driven cell-mediated immune response was investigated in blood mononuclear cells using a single strain of live NTHi. This strain was originally isolated from the sputum of an adult with pneumonia and confirmed as NTHi by the Phadebact Haemophilus Test (Bactus AB, Huddinge, Sweden) and by PCR [161]. A pilot study to determine the immunogenicity of 20 NTHi strains associated with lower respiratory infection shows that the strain used in this thesis was representative of an NTHi-directed cell-mediated immune response (Figure 3.2).

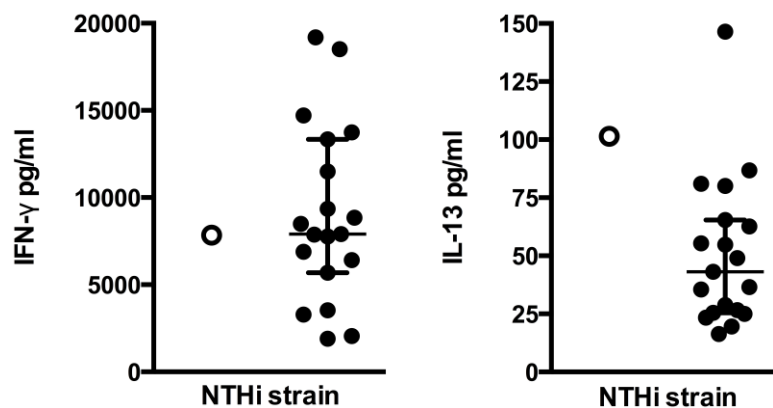


Figure 3.2 IFN- γ and IL-13 production by PBMC from one adult in response to an in vitro challenge with 20 clinical strains of NTHi. Each data point represents cytokine production by PBMC (combined duplicate wells) in response to a 72 hour challenge with an individual NTHi strain. The strain used in this thesis is represented by the open circle. Median with interquartile range is shown.

All studies were conducted using a single batch of live, cryopreserved NTHi prepared by a method adapted from the laboratory of Dr Lea-Ann Kirkham (The University of Western Australia, Western Australia). NTHi was grown on chocolate agar plates containing selective antibiotics (Oxoid, Australia) overnight at 37°C, 5% CO₂. Brain heart infusion broth supplemented with hemin (30 mg/L), nicotinamide adenine dinucleotide (30 mg/L) and glycerol (4%) was inoculated with isolated colonies and incubated with shaking at 37°C overnight. This starter culture was used to inoculate a large volume broth culture and grown to an optical density at 600 of 0.47 units. Single use aliquots were stored in 20% heat-inactivated fetal bovine serum (FBS) at -80°C. The same batch of NTHi was used in all experiments. Viability of the stock inoculum was quantified by plating serial dilutions on chocolate agar. Batch viability was routinely monitored and remained consistent over the course of this thesis.

3.6 Cell mediated immune responses

NTHi-driven adaptive and inflammatory cell-mediated immune responses were investigated in vitro by challenging cultured PBMC with NTHi and measuring representative cytokines in the culture supernatant by DELFIA™ as described in section 3.4. The establishment of this assay including preparation of the NTHi inoculum and optimisation of cell culture conditions are described in this section.

3.6.1 The effect of cryopreservation on PBMC function

Cell mediated immune responses were investigated in PBMC rather than fresh whole blood. As samples were collected over a 5 year period, this approach allows

cryopreservation and batch testing of PBMC samples thus minimising potential interassay variation. Batch testing of samples was also more logistically feasible as only 1-3 samples were available on any one day. Preliminary studies included investigations to determine the effect of cryopreservation on PBMC function. The effect of cryopreservation on early innate response was investigated by measuring IL-6 and TNF- α production by cryopreserved PBMC in response to Toll-like receptor (TLR) ligands and comparing with freshly prepared PBMC from the same donor. The TLR ligands used were the TLR-4 ligand, lipopolysaccharide (LPS; *E. coli* 055:B5; Alexis® Biochemicals, Enso Life Sciences, USA), the TLR-3 ligand, polyinosinic:polycytidylic acid (Poly(I:C); Sigma-Aldrich, MO, USA), and the TLR-2 ligand, lipoteichoic acid (LTA-SA; from *Staphylococcus aureus*; Invivogen, USA). In addition to early innate responses, the effect of cryopreservation on the NTHi-driven response was determined by measuring IFN- γ , IL-13 and IL-10 at 72 hours and IL-6 at 24 hours by PBMC in response to 4 different strains of NTHi. Comparing cytokine production by cryopreserved and freshly prepared PBMC (from the same adult) indicated that cryopreservation did not impair the ability of PBMC to produce the panel of cytokines investigated in this thesis. Selected data representative of this work are presented in Figure 3.3.

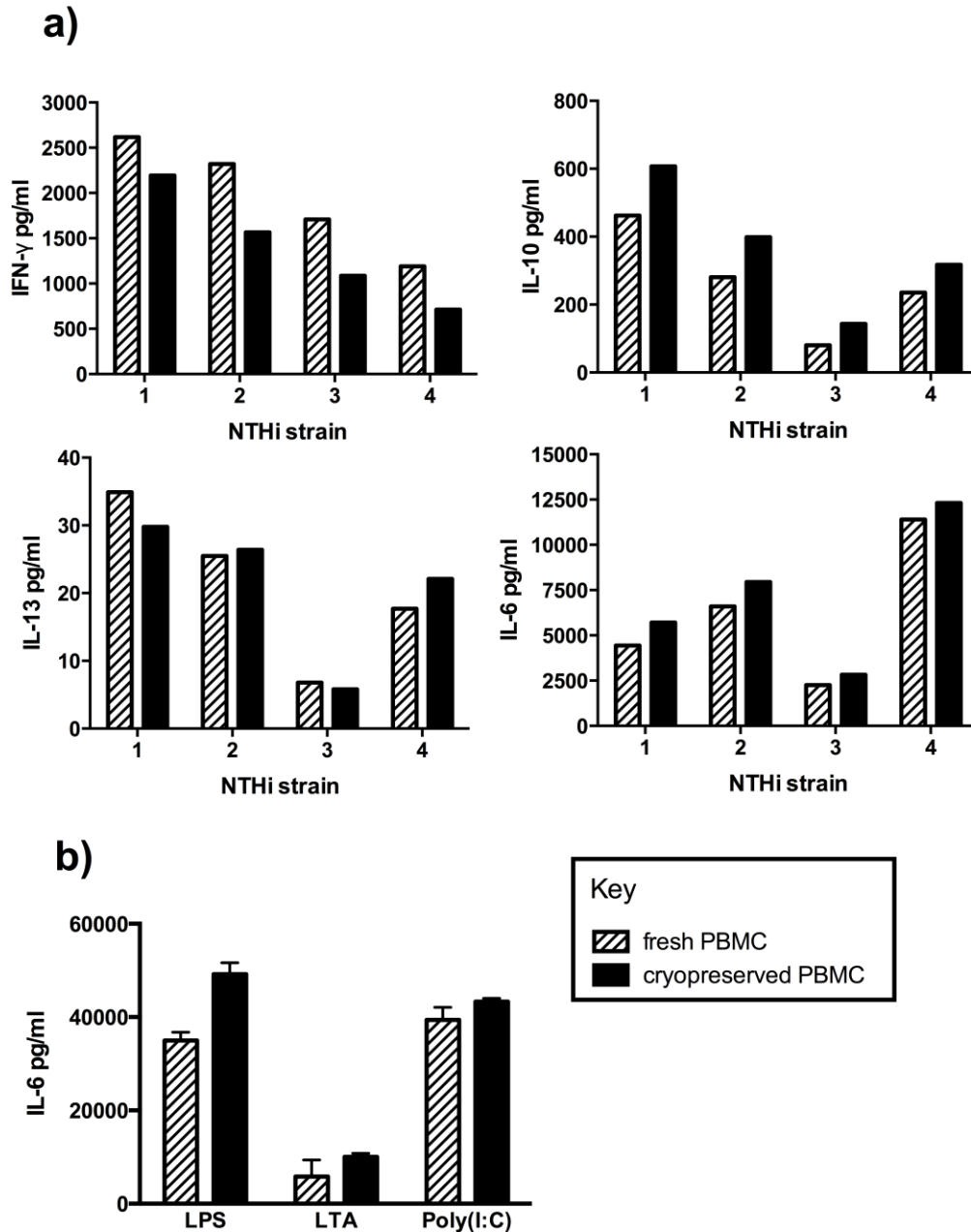


Figure 3.3 Cytokine production by cryopreserved (striped bar) or freshly prepared (solid bar) PBMC in response to four strains of NTHi a) or Toll-like receptor ligands b). Data is presented from combined duplicate wells a) or mean with standard deviation of duplicate well b).

3.6.2 Optimization of the NTHi/PBMC challenge assays

As the number of cells obtained from children was limited, the basic conditions for the challenge assays (including immunogenicity of the NTHi strain, NTHi challenge dose and challenge duration) were first determined in adult PBMC.

Duplicate wells of PBMC were challenged with log fold concentrations of NTHi for various duration. Cytokines representative of the early innate response (TNF- α , IL-6 and early IL-10) and the T-helper recall response (IFN- γ , IL-13, IL-10 and IL-5) were measured in the culture supernatant over 3 to 24 hours and 48 to 96 hours respectively. Cells cultured with 2 μ g/ml phytohaemagglutinin (PHA; Sigma Aldrich, USA) or media alone were included as positive and baseline controls. Selected data representative of these optimisation assays are presented in Figure 3.4. The culture conditions (dose and challenge duration) for the recall response were confirmed in a sub-group (n=9) of PBMC from the study cohort (Figure 3.5). As the number of PBMC from children was limited, the culture conditions used to investigate the early innate response to NTHi were extrapolated from adult data. Optimum conditions varied between individual cytokines thus the conditions that best represented each panel of cytokines as a group were used in the final assays.

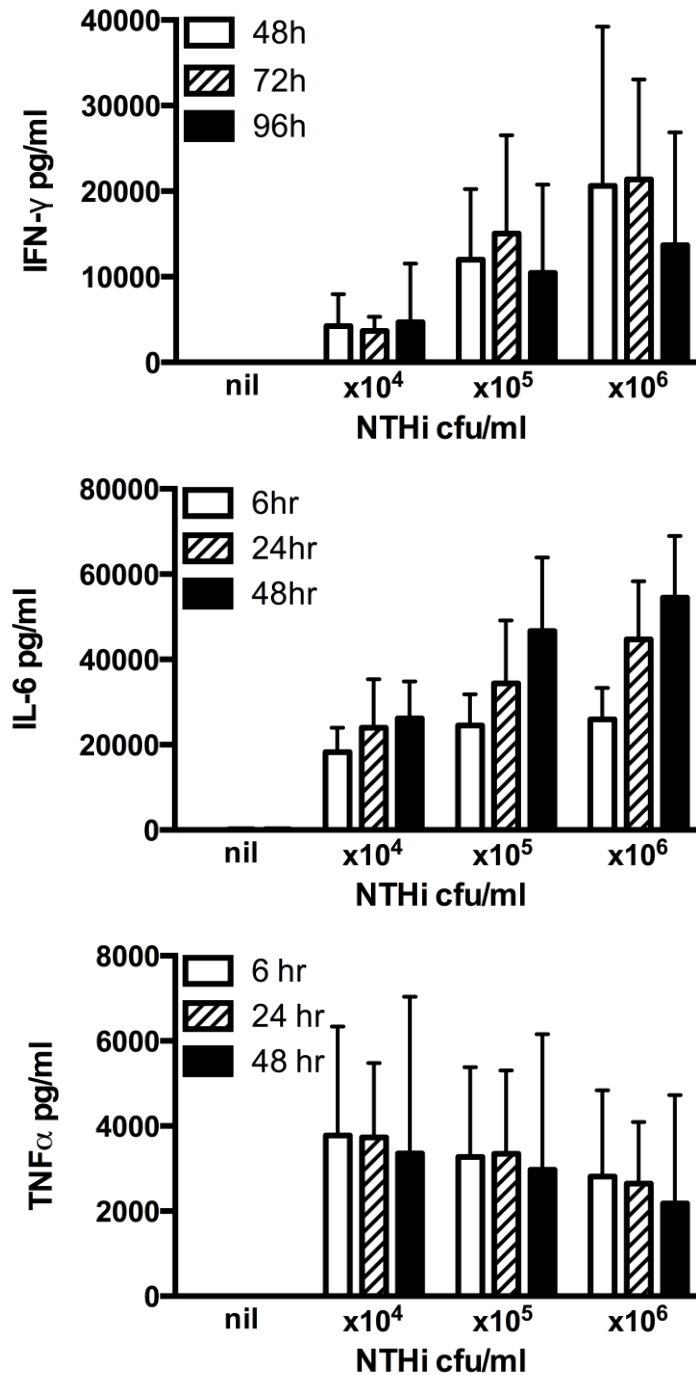


Figure 3.4 Optimisation of NTHi dose and culture duration. IFN- γ , IL-6 and TNF α production by PBMC following challenge with NTHi (study strain). Data from 5 adults are presented as mean with standard deviation.

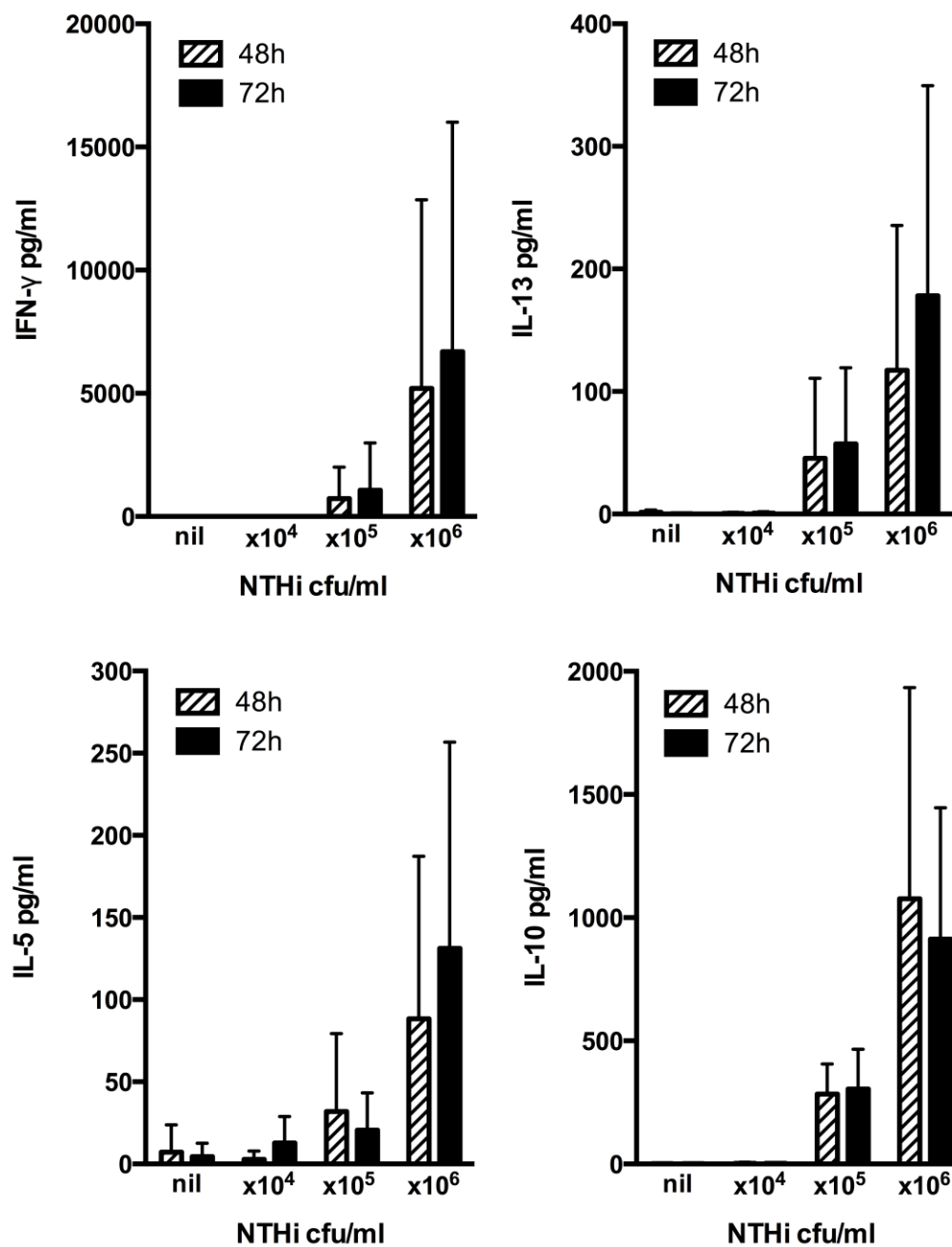


Figure 3.5 Confirmation of culture conditions in PBMC from children. Cytokine production by PBMC from 9 children (CSLD and HC) following a 48 hour or 72 hour challenge with log fold concentrations of NTHi. Data are presented as mean with standard deviation.

3.6.3 The optimised NTHi/PBMC challenge assay

To measure adaptive cell mediated immune function, PBMC were resuspended in AIM-V serum-free medium (Gibco Life Technologies, Australia) supplemented with 2-mercaptoethanol (0.04 mM final concentration), and cultured in duplicate at 2.5×10^5 cells per well in 96 well plates with either live NTHi (4×10^6 CFU/ml final concentration), PHA (2 µg/ml; Sigma, Missouri, USA) or medium alone for 72 hours at 37°C, 5% CO₂. To measure the innate immune response, PBMC were cultured in RPMI 1640 with 10% non-heat inactivated FCS and NTHi (4×10^6 CFU/ml final concentration) or medium alone for 24 hours at 37°C, 5% CO₂. Supernatants were collected and stored at 4°C for up to 7 days prior to quantification of cytokines in a Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIATM, Perkin Elmer, USA) using time-resolved fluorometry (section 3.4).

3.6.4 Reproducibility of NTHi inoculum

Although a single batch of NTHi inoculum was used in this thesis, reproducibility of the inoculum and robustness of the challenge assays was investigated by comparing IFN- γ production by PBMC in response to two separately prepared batches of NTHi (prepared in 2010 and 2012). Using the optimised methods described in this chapter, PBMC from four adult volunteers were challenged with both batches of NTHi at two concentrations, $\times 10^5$ and $\times 10^6$ cfu/ml. IFN- γ was measured in the culture supernatant of duplicate wells. The data in Figure 3.6 show that both batches, prepared 2 years apart, had a similar effect on IFN- γ production and demonstrates the robustness of this assay.

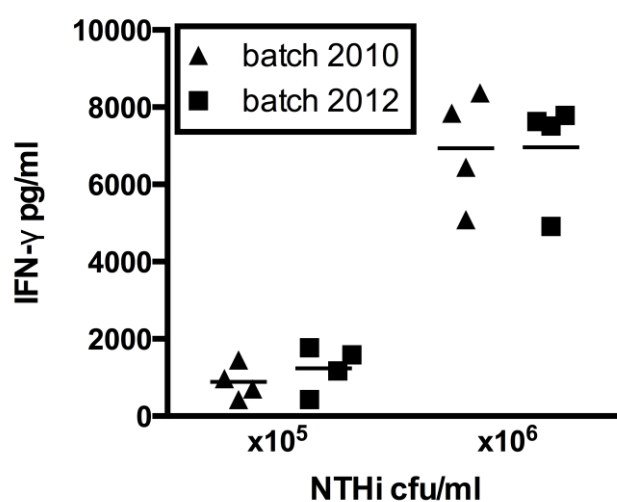


Figure 3.6 The effect of two separate batches of NTHi inoculum on IFN- γ production.

3.7 *H. influenzae*-specific Immunoglobulin levels

The IgG subgroups IgG₁ and IgG₄, specific for the recombinant outer-membrane proteins (OMP) P4 and P6 from *H. influenzae*, and the NTHi vaccine candidate Protein D were quantified from plasma by DELFIA™ as previously described by Hales et al [135, 162]. Standard curve and negative control plasma for each OMP/antibody combination were included on each plate. Representative standard curves are presented in Figure 3.7

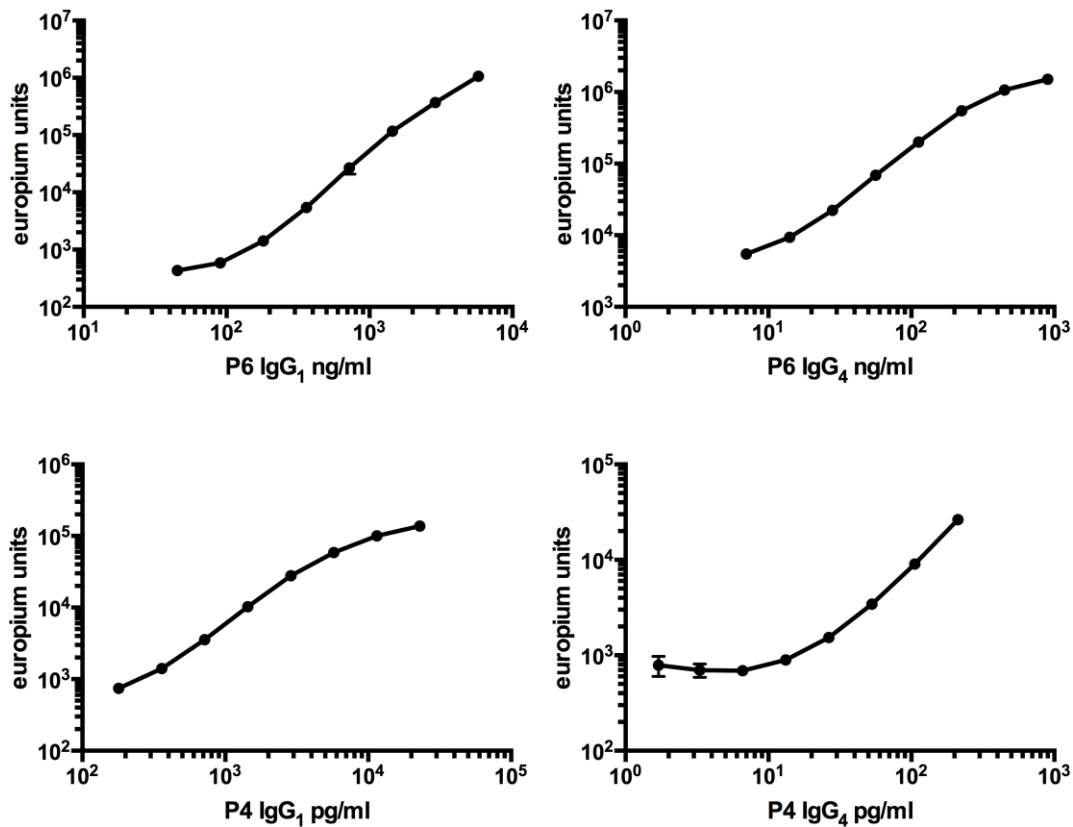


Figure 3.7 Representative standard curves for P4- and P6-specific IgG1 and IgG4 by DELFIA™ time-resolved fluorescence assays. Each data point represents the mean europium fluorescence units from duplicate plates.

3.8 Significance of the methods

There are few studies that have investigated cell-mediated immune responses to NTHi thus there are no universal methods currently available. Several studies have investigated responses to single recombinant or purified membrane proteins of NTHi [129, 163] and data from these studies contribute important information to vaccine development. However NTHi are a complex and heterogeneous species and immune responses to single antigens may not be representative of natural infection. The primary aim of this thesis is to identify factors of the immune response that contribute to the pathogenesis of CSLD through recurrent infection with NTHi. This is best achieved by using an antigen that best represents natural infection with NTHi. Thus a whole cell inoculum was used to challenge PBMC. King and colleagues pioneered this approach by using a heat-killed, sonicated, multi-strain inoculum to show that the cell-mediated immune response is important for protective immunity against NTHi [88]. They furthered these studies in cultured natural killer and cytotoxic T cells using freshly prepared individual live strains of NTHi [89].

The methods used in my thesis have contributed to the repertoire of laboratory techniques used to investigate host pathogen interactions particularly in regards to NTHi. In this thesis a single batch of live NTHi was prepared and cryopreserved for use in all PBMC challenge assays. Cytokine data obtained from two separate batches of NTHi indicate that the method presented in this thesis is robust and reliable. This method allows PBMC to be batch tested over a period of time with minimal interassay variation.

These methods contributed to achieving the aims of this thesis and will be important for future studies of host-NTHi interaction. Indeed this NTHi inoculum has recently been used successfully in a study of macrophage phagocytosis and efferocytosis in children with bronchiectasis [164].

CHAPTER 4

Immune responses to non-typeable *Haemophilus influenzae* in children with chronic suppurative lung disease

Chapter 4 IMMUNE RESPONSES TO NON-TYPEABLE

***HAEMOPHILUS INFLUENZAE* IN CHILDREN WITH CHRONIC**

SUPPURATIVE LUNG DISEASE.

4.1 Chapter overview

As discussed in section 1.6 NTHi is the most common respiratory pathogen identified in the BAL of children with CSLD. Thus it has been proposed that recurrent lower respiratory infection with NTHi contributes to the pathogenesis of CSLD in children. However the reasons for a heightened susceptibility in some children to infection with NTHi are unknown. The overall aim of this chapter is to identify aspects of the immune response that may contribute to an increased susceptibility to lower respiratory infections with NTHi in children.

In this chapter, cytokine representing T-helper and inflammatory responses produced by blood mononuclear cells in response to challenge with NTHi are described in 80 children with CSLD. These responses are compared with the responses from 51 healthy control children. The methods used in this study are described in Chapter 3. The results from this study are presented section 4.2 as a published article. Supportive data associated with the published article is presented in section 4.2.1. The main findings from this study are summarised in section 4.3.

4.2 Journal article: Children with chronic suppurative lung disease have a reduce capacity to synthesize interferon-gamma in vitro in response to non-typeable *Haemophilus influenzae*



Children with Chronic Suppurative Lung Disease Have a Reduced Capacity to Synthesize Interferon-Gamma *In Vitro* in Response to Non-Typeable *Haemophilus influenzae*

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Abstract

Chronic suppurative lung disease (CSLD) is characterized by the presence of a chronic wet or productive cough and recurrent lower respiratory infections. The aim of this study was to identify features of innate, cell-mediated and humoral immunity that may increase susceptibility to respiratory infections in children with CSLD. Because non-typeable *Haemophilus influenzae* (NTHi) is commonly isolated from the airways in CSLD, we examined immune responses to this organism in 80 age-stratified children with CSLD and compared their responses with 51 healthy control children. Cytokines involved in the generation and control of inflammation (IFN- γ , IL-13, IL-5, IL-10 at 72 hours and TNF α , IL-6, IL-10 at 24 hours) were measured in peripheral blood mononuclear cells challenged *in vitro* with live NTHi. We also measured circulating IgG subclass antibodies (IgG₁ and IgG₄) to two *H. influenzae* outer membrane proteins, P4 and P6. The most notable finding was that PBMC from children with CSLD produced significantly less IFN- γ in response to NTHi than healthy control children whereas mitogen-induced IFN- γ production was similar in both groups. Overall there were minor differences in innate and humoral immune responses between CSLD and control children. This study demonstrates that children with chronic suppurative lung disease have an altered systemic cell-mediated immune response to NTHi *in vitro*. This deficient IFN- γ response may contribute to increased susceptibility to NTHi infections and the pathogenesis of CSLD in children.

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Introduction

Bronchiectasis and chronic suppurative lung disease (henceforth collectively termed CSLD) describe a syndrome of persistent or recurrent respiratory symptoms predominantly characterized by chronic productive or wet cough. CSLD is increasingly recognized as an important chronic respiratory disorder affecting children [1–4] and adults [5] globally and may represent a precursor to bronchiectasis [1].

Non-typeable *Haemophilus influenzae* (NTHi) are Gram-negative bacteria commonly associated with chronic upper and lower respiratory disease. It is the dominant species isolated from the lower airways of children and adults with chronic respiratory symptoms [6–8]. However, NTHi is also a commensal organism in

healthy adults [9] and children [10] and as healthy adults and children both develop antibodies against NTHi [11,12], the relationship between host and bacterium and the transition from commensal organism to pathogen is likely influenced by a complex interaction of host and bacterial factors.

One such host factor identified as important in adults is the cell-mediated immune response. Altered NTHi-specific cytokine responses, including Th2-skewed cytokine profiles have been reported in adults (>50 years of age) with established bronchiectasis or COPD and impaired lung function [11,13]. However, it is unclear whether these alterations were involved in disease induction, or rather arose as a consequence of systemic inflammation in adults with chronic, severe disease [14,15]. A

study in children with milder disease of short duration may help elucidate some of these unresolved issues.

In the absence of published data to explain the susceptibility of some children to recurrent lower respiratory infections, we characterized systemic immune responses to NTHi in children with CSLD and healthy children. Our key outcome measures included NTHi-specific cytokine profiles (24 hour and 72 hour) *in vitro* and serum antibodies specific for the *H. influenzae* outer membrane proteins (OMP) P4 and P6. In this study we describe these profiles and identify key differences which may contribute to an increased susceptibility to lower respiratory infections in children.

Materials and Methods

Study population and sample collection

Eighty children (aged ≤ 10 years) undergoing chest computed tomography (CT) scan and flexible bronchoscopy for suspected CSLD (CSLD group) and 51 age-matched children without acute infection or clinical history of respiratory or chronic illness (healthy control, HC group) were prospectively recruited (2008–2011) from the Royal Darwin Hospital (RDH), Darwin, Northern Territory (NT), Australia.

All children in the study group were clinically stable (absence of respiratory exacerbation) at the time of sample collection. Blood and bronchoalveolar lavage (BAL) for clinical and research investigations were collected at the time of intravenous access (i.e. at the start of general anesthesia), prior to chest CT scan/bronchoscopy. Clinical and socio-demographic data were collected using standardized data collection forms. Routine clinical investigations [1] were performed using the regional reference laboratory (RDH) and subsequently two children were excluded from analysis following a diagnosis of primary immunodeficiency (final $n = 80$). Radiographic diagnosis of bronchiectasis was made by the pediatric respiratory physician (AC). *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* culture and identification were performed by our laboratory (diagnostic threshold $>10^4$ CFU/ml [6]). *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* culture and identification were performed by the diagnostic laboratory at the Royal Darwin Hospital.

Healthy controls (absence of a history of chronic respiratory, other non-respiratory illness and no acute illness within 4 weeks) were enrolled primarily through RDH elective surgery list. They did not have the same clinical work up as the CSLD group (including chest CT, bronchoscopy) as these investigations were not clinically indicated for their procedure. Blood was collected under the same conditions as described for the group with CSLD, prior to any procedure. BAL was not collected from the control children.

The Human Research Ethics Committee (Northern Territory Department of Health and Menzies School of Health Research) approved this study (#07/63). The children were enrolled following written informed consent from the parent/carer.

NTHi preparation

A single NTHi strain originally isolated from the sputum of an adult with pneumonia (confirmed by the Phadebact *Haemophilus* Test (Bactus AB, Huddinge, Sweden) and by PCR [16]) was used for this study. The immunogenicity of this strain and optimization of culture conditions were first determined in peripheral blood mononuclear cell (PBMC) cultures from healthy adults. A pilot study of 19 additional NTHi strains indicated that the strain used in this study was representative of NTHi-specific cytokine responses in a healthy adult.

NTHi was grown on chocolate agar plates overnight at 37°C, 5% CO₂. Brain heart infusion broth supplemented with hemin (30 mg/L), nicotinamide adenine dinucleotide (30 mg/L) and glycerol (4%) was inoculated with isolated colonies and incubated with shaking at 37°C overnight. This starter culture was used to inoculate a large volume broth culture and grown to an optical density at 600 of 0.47 units. Single use aliquots were stored in 20% heat-inactivated fetal bovine serum (FBS) at –80°C. The same batch of NTHi was used in all experiments. Viability was quantified by plating serial dilutions on chocolate agar and batch viability was routinely monitored.

Peripheral Blood Mononuclear Cell (PBMC) isolation

Venous blood was collected into preservative-free heparin and processed within 2 hours. PBMC were isolated by centrifugation over Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Sweden) and cryopreserved in liquid nitrogen in 10%DMSO/heat-inactivated FBS. These techniques have been successfully used in studies of immune function in large human cohorts [17,18]. Matched plasma for antibody analysis was stored at –80°C.

PBMC challenge assays

In order to measure adaptive, cell mediated immune function, PBMC were resuspended in AIM-V serum-free medium (Gibco Life Technologies, Australia) supplemented with 2-mercaptoethanol (0.04 mM final concentration), and cultured in duplicate at 2.5×10^5 cells per well in 96 well plates with either live NTHi (4×10^6 CFU/ml final concentration), PHA (2 µg/ml; Sigma, Missouri, USA) or medium alone for 72 hours at 37°C, 5% CO₂. IFN γ , IL-13, IL-5 and IL-10 were measured in supernatant collected at 72 hours. Where sufficient cells were available, innate immune responses (IL-6, TNF α and early IL-10) were also assessed. PBMC were cultured in RPMI 1640 with 10% non-heat inactivated FCS and NTHi (4×10^6 and 4×10^5 CFU/ml final concentration), or medium alone for 24 hours at 37°C, 5% CO₂. Supernatants were collected and stored at 4°C for up to 7 days prior to cytokine quantification.

Quantification of cytokines

Cytokines were measured from the 72 and 24 hour culture supernatants using dissociation-enhanced lanthanide fluorescent immunoassays (DELFA) as described previously [19]. Quantitative standard curves were generated from serial dilutions of recombinant human cytokine proteins and included on each plate. The limit of detection for all cytokines was 10 pg/ml; samples with concentrations below the limit of detection were assigned the value of 1 pg/ml. The data is presented as delta concentrations (amount of cytokine from NTHi challenge minus nil challenge). Children were identified as responders if cytokine production was at least two-fold greater than the nil treatment or two-fold above the assay limit of detection (whichever was greater).

Immunoglobulin levels

IgG₁ and IgG₄ specific for the recombinant outer-membrane proteins (OMP) P4 and P6 from *H. influenzae* were quantified from plasma by Dissociation Enhanced Lanthanide Fluorescent Immunoassay (DELFA; PerkinElmer) as previously described [20,21]. Negative control plasma for each OMP/antibody combination were included on each plate. Plasma IgE was measured using ELISA. Children were identified as responders if their antibody level was at least two-fold greater than the mean concentration of the negative control plasma.

Data analysis

For the primary analysis, the children were stratified by age (< 18, 18–36, 37–60 and >60 months) and the data analyzed according to respiratory diagnosis (healthy control or CSLD). As a secondary analysis, the age groups were combined and potential confounding factors of the data identified and assessed in the analysis. For the secondary analysis, only cytokines which maintained a statistical difference following correction for potential confounding factors are described.

Statistical analysis was performed using STATA 13 (StataCorp, USA) and a p-value <0.05 was considered statistically significant. Group differences (by age, diagnosis of CSLD and cultural background) were assessed by the Mann-Whitney U test and categorical data were assessed by Fisher's exact test. Cytokine and antibody data were non-parametric and are presented as median with interquartile range. Potential factors that might confound analysis of the immunologic data were identified (cultural background, clinical characteristics) and regression analysis used to investigate their effects.

Results

Study population

The demographic characteristics of the children are shown in table 1. All of the children in the current study came from a similar geographic region, northern Australia. High generational cultural diversity is a characteristic of the population in this region and studies to determine the influence of race on the immune response are not feasible. Despite this, we found that a high proportion of children in the CSLD group were identified by their parent as having Indigenous Australian ancestry, thus we have grouped children as either having known Indigenous Australian ancestry (described as Indigenous descent) or with no, or unknown, Indigenous Australian ancestry (non-Indigenous descent).

Children with CSLD have altered 72 hour NTHi-cytokine profiles

We detected IFN- γ , IL-13 and IL-10 in the NTHi-challenged PBMC culture supernatants from almost all children (figure 1a). Whilst the number of IL-5 responders was comparatively low, the CSLD group had almost 2-fold more responders than the HC group.

For the three older age groups, PBMC from children with CSLD produced significantly less IFN- γ compared with HC children but no significant difference between groups were present in the <18 month age group (figure 1a). When all age groups were combined, IFN- γ production was also significantly lower in the CSLD group compared to HC (median (IQR) 876 (145–2536) versus 7861 (1939–17840) pg/ml, age-adjusted $p < 0.001$; table S1).

In the HC group there was a dramatic upward trajectory in IFN γ production between the <18 month age group and the 18–36 month age groups (median 845 versus 8115 pg/ml; $p = 0.06$) and this level of production remained high in the older children. In contrast, IFN γ production in children with CSLD <18 months of age was similar to that seen in children with CSLD in the 18–36 month age group (median 928 versus 678 pg/ml; $p = 0.36$) and also similar to that seen in the older age groups (figure 1b).

Furthermore, children with CSLD aged <18 months produced higher amounts of the Th2 associated cytokine IL-13 than the age-matched HC children (median 63 versus 19 ng/ml; $p = 0.03$). In contrast, IL-13 production was similar in CSLD and HC children in each of the three older age groups. The production of IL-5 and

Table 1. Demographic characteristics of the population by age group.

	<18 months		18–36 months		37–60 months		>60 months	
	HC	CSLD	HC	CSLD	HC	CSLD	HC	CSLD
n	6	18	9	38	14	12	22	12
Age in months								
median	8	14	26	26	51	47	81	82
(IQR)	(5–13)	(10–15)	(24–35)	(21–29)	(46–56)	(40–54)	(66–107)	(71–106)
p value[#]	0.09		0.19		0.21		0.59	
Male	5 (83)	9 (50)	8 (89)	22 (58)	6 (43)	5 (42)	12 (55)	6 (50)
Indigenous descent	0	13 (72)	5 (56)	36 (95)	6 (43)	12 (100)	4 (18)	12 (100)

[#]analysis of age between HC and CSLD at each age group.
doi:10.1371/journal.pone.0104236.t001

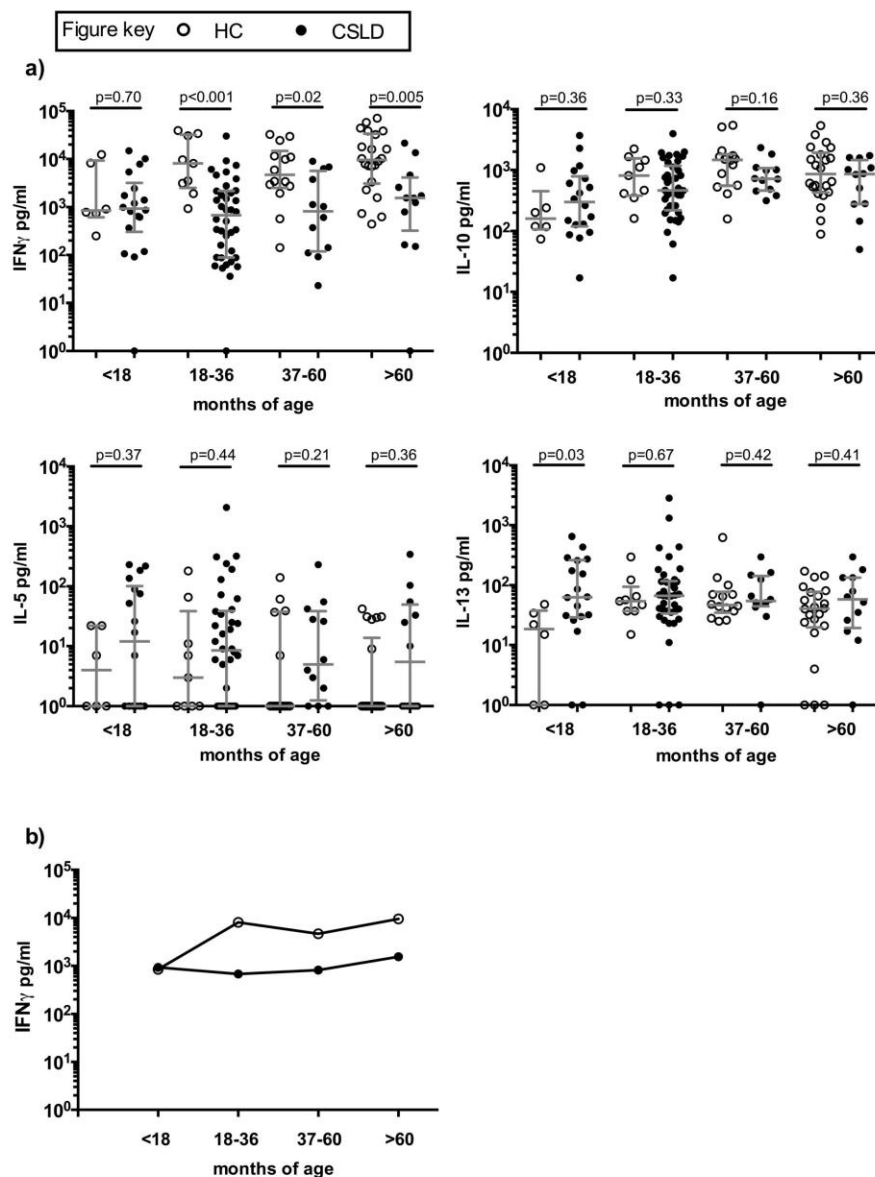


Figure 1. Adaptive (72 hour) cytokine production to NTHi. a) In vitro cytokine production by HC (open circles) and CSLD (filled circles) PBMC following 72 hour challenge with NTHi. Delta concentration (NTHi challenge minus nil challenge) with median and IQR. b) Trajectory of adaptive (72 hour) IFN- γ production to NTHi with age. In vitro cytokine production by HC (open circles) and CSLD (filled circles) PBMC following 72 hour challenge with NTHi. Median (delta) concentration, pg/ml.
doi:10.1371/journal.pone.0104236.g001

IL-10 did not differ between the CSLD and HC groups across any age group.

We used the non-specific mitogen PHA to test the global capacity for cytokine production and found minimal differences between CSLD and HC children (figure 2). These data indicate that the reduced capacity to synthesise IFN- γ observed in CSLD children aged >18 months is specific to NTHi and cannot be attributed to a generalised impairment in the capacity of T-cell and natural killer cells to produce these cytokines.

As CSLD was found to be prevalent in children of Indigenous descent, we investigated if this contributed to the differences in IFN- γ observed between children with CSLD and HC children. We found that Indigenous children with CSLD produced significantly less IFN- γ than age-matched Indigenous HC children (median 703 versus 3497 pg/ml; $p < 0.001$) and non-Indigenous HC children (median 703 versus 10017 pg/ml $p < 0.001$) (table 2). Finally, using regression analysis, Indigenous descent was not an independent predictor of IFN- γ production (table S1).

Innate (24 hour) cytokine responses to NTHi

Because innate immunity may modulate adaptive immunity, we examined the strength and nature of the innate immune response

to NTHi by measuring IL-6, TNF α and IL-10 production in 24 hour NTHi-stimulated PBMC (figure 3). Measurable quantities of the cytokines were produced by almost all children. Children with CSLD aged 18–36 months produced less IL-6 and TNF α than HC children, but these differences were not observed in the three other age groups. IL-10 production at 24 hours was similar across all groups. When all age groups were combined, the CSLD group produced less IL-6 than the HC group (median (IQR) 5527 (3131–8733) versus 11425 (4835–18458) pg/ml, age-adjusted $p = 0.007$; table S1).

As found with the 72 hour cytokine data, none of the 24 hour cytokine data varied in relation to Indigenous descent (table 2).

Serum antibodies specific for *H. influenzae* outer membrane proteins P4 and P6

Most children had measurable serum IgG₁ to both P4 and P6. The number producing detectable levels of IgG₄ was low. Only minor differences were observed between the children with CSLD and the HC children (figure 4).

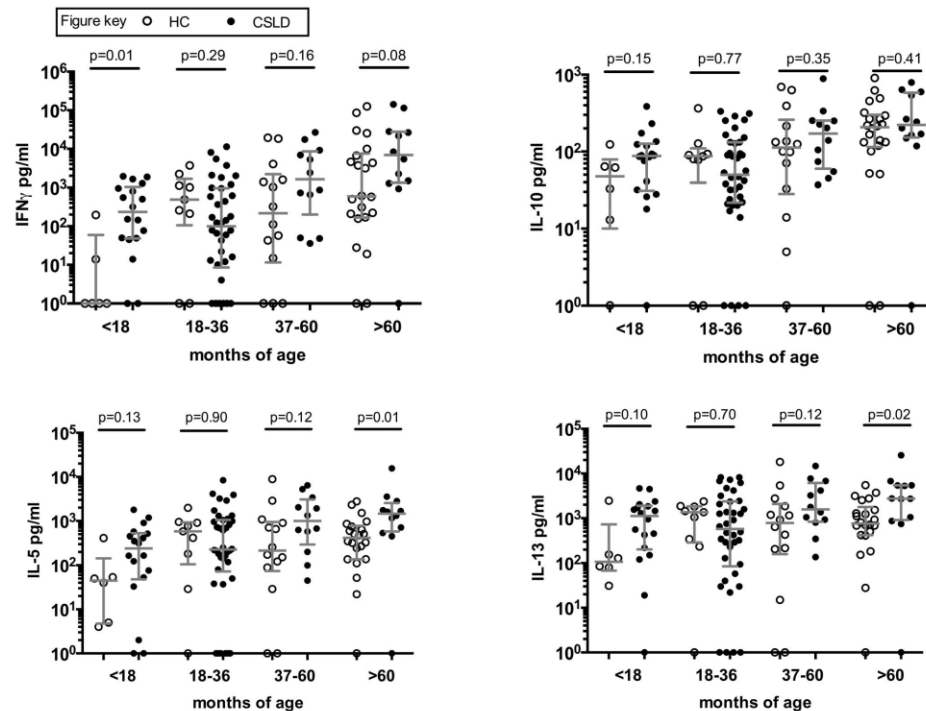


Figure 2. Adaptive (72 hour) cytokine production to PHA. In vitro cytokine production by HC (open symbols) and CSLD (filled symbols) PBMC following 72 hour challenge with the non-specific mitogen PHA. Delta concentration (NTHi challenge minus nil challenge) with median and IQR. doi:10.1371/journal.pone.0104236.g002

Table 2. Cytokine production (pg/ml; median (IQR)) from NTHI-challenged PBMC from children ≥ 18 months; HC Indigenous descent n = 15, HC non-Indigenous descent n = 30, CSLD Indigenous descent n = 60, CSLD non-Indigenous descent n = 2.

		Indigenous descent	non-Indigenous descent	p [#]
IFN- γ (72 hr)	HC	3497 (1939–33950)	10017 (3331–24437)	0.36
	CSLD	703 (102–2190)	557, 3858**	n/a [*]
	p ^{##}	<0.001	n/a [*]	
IL-13 (72 hr)	HC	44 (37–123)	46 (25–66)	0.36
	CSLD	65 (33–129)	50, 69**	n/a [*]
	p ^{##}	0.76	n/a [*]	
IL-5 (72 hr)	HC	0 (0–37)	0 (0–3)	0.07
	CSLD	7 (0–40)	0, 9**	n/a [*]
	p ^{##}	0.54	n/a [*]	
IL-10 (72 hr)	HC	562 (352–1454)	1336 (555–2230)	0.03
	CSLD	682 (279–1109)	391, 467**	n/a [*]
	p ^{##}	0.83	n/a [*]	
TNF (24 hr)	HC	656 (541–1094)	1556 (727–2086)	0.10
	CSLD	588 (226–1267)	181, 533**	n/a [*]
	p ^{##}	0.53	n/a [*]	
IL-6 (24 hr)	HC	7154 (4221–18458)	12819 (8966–17698)	0.18
	CSLD	6563 (3701–10969)	2304, 2736**	n/a [*]
	p ^{##}	0.38	n/a [*]	

#Analysis between Indigenous and non-Indigenous descent.

##analysis between HC and CSLD.

*p value not calculated due to small numbers.

**absolute values for the 2 participants.

doi:10.1371/journal.pone.0104236.t002

Effect of patient and clinical characteristics on cytokine response

Although all children were clinically stable at the time of sample collection, 45% were receiving maintenance antibiotics for CSLD or chronic suppurative otitis media, a common co-morbidity in this population. BAL culture showed lower airway infection (growth of $\geq 10^4$ colony-forming units (CFU)/mL of any pathogen using standard culture methods) in 21 (25.6%) children. NTHi was the most common pathogen identified (24% of children), followed by *Streptococcus pneumoniae* (5%) and *Moraxella catarrhalis* (5%), *Pseudomonas aeruginosa* (2.5%) and *Klebsiella pneumoniae* (1%). Serum total IgE was similar between healthy controls (HC) (median 449 ng/mL; IQR 73–1142 ng/mL) and the CSLD group (641 ng/mL; 149–1623 ng/mL) ($p = 0.07$). All but one child were up to date with their childhood vaccination schedule. Using regression analysis, Mann-Whitney U test or Spearman's rank order correlation, we investigated whether these characteristics of the children with CSLD at the time of sample collection could explain variations in NTHi-induced cytokine production. The observed NTHi-stimulated cytokine responses were not associated with antibiotic use (including azithromycin), current isolation of NTHi or other respiratory pathogens in the BAL, evidence of parasitic infection (strongyloides), serum IgE and inflammatory cell counts in BAL and blood (table S2).

Discussion

Despite advances in the prevention and management of pediatric respiratory infections, CSLD remains a problem in children and adults globally. This prospective, cross-sectional study is the first to compare NTHi-driven innate, cell-mediated

and humoral immune responses across a large group of children with CSLD ($n = 80$) and healthy control children ($n = 51$). The most notable finding was that, in children aged ≥ 18 months, PBMC from those with CSLD produced significantly less IFN- γ than PBMC from their healthy peers. In contrast, in children aged < 18 months, those with CSLD produced more IL-13 than the healthy control children. We found comparatively minor differences in humoral and innate immune responses. These data are consistent with our hypothesis that impaired cell mediated immune responses may contribute to the pathogenesis of CSLD in children.

Immune development begins in utero [22,23]. Various environmental, respiratory and immune pressures drive the maturation of the Th1 response from the Th2-dominated response of neonates [24–26]. By 18 months of age there is a substantial rise in IFN- γ function [27] and a delay in the development of the IFN- γ response may predict Th2-associated disorders in childhood [28]. IFN- γ , primarily secreted by T lymphocytes and natural killer cells, is integral to the orchestration of antiviral and antibacterial activity and is a dominant Th1 cytokine. Furthermore impaired IFN- γ responses have been implicated in the pathogenesis of rhinovirus-induced asthma exacerbations in adults [29]. Whilst our study was not designed to investigate the mechanistic process of immune maturation, our findings have highlighted a disparity between children with CSLD and HC children in age-related IFN- γ and IL-13 production. We found a log-fold increase in IFN- γ production between HC aged < 18 months compared to those > 18 months. The capacity to produce IFN- γ remained high in the older age groups. These data are consistent with normal maturation of the Th1 response through early childhood. This age-related increase in IFN- γ response observed in the HC

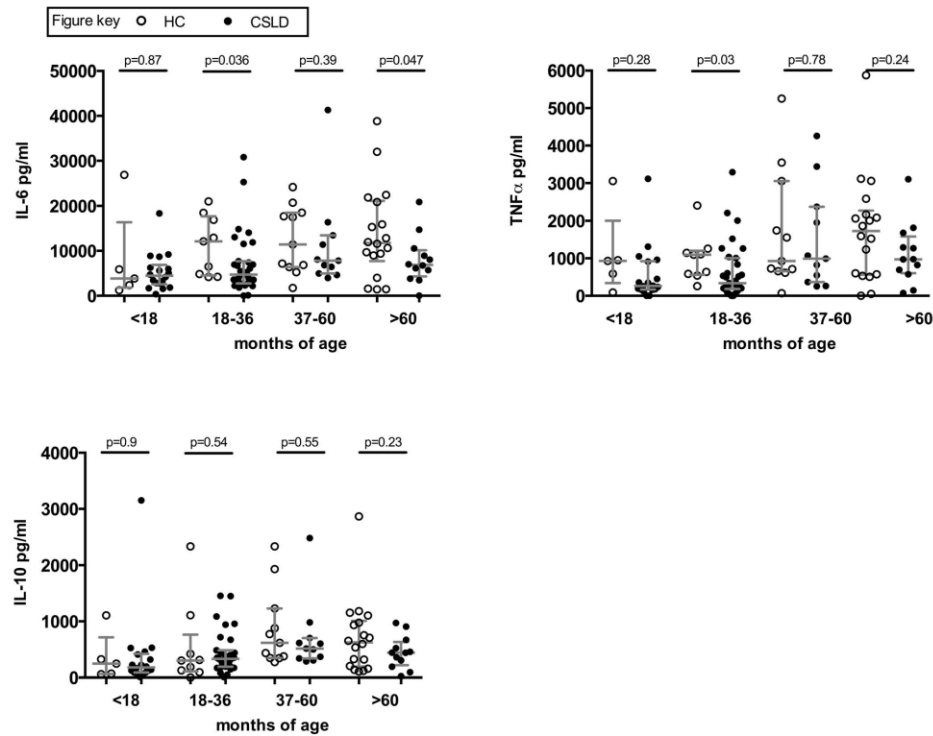


Figure 3. Innate (24 hour) cytokine production. In vitro cytokine production by HC (open circles) and CSLD (filled circles) PBMC following 24 hour challenge with NTHi. Delta concentration (NTHi challenge minus nil challenge) with median and IQR.
doi:10.1371/journal.pone.0104236.g003

children was not reflected in the children with CSLD. Children < 18 months of age with CSLD had a similar capacity for NTHi-specific IFN- γ production as their HC peers, however unlike the HC group, the CSLD group maintained a significantly lower capacity for IFN- γ production over subsequent increasing age groups. Conversely, IL-13 production was significantly higher in younger children with CSLD, with no significant difference between the two groups in older children. Whilst future longitudinal studies are required, these data support the notion that normal maturation of the cell mediated immune response is impaired in children who develop CSLD.

We also examined cytokines representative of innate immune responses in PBMC challenged for 24 hours with NTHi reasoning that altered innate immunity might provide a mechanism for altered adaptive immunity. No studies have previously addressed this issue in children with CSLD. IL-6 is an important cytokine in facilitating the transition from the innate to the adaptive response and is also important for the resolution of acute inflammation (reviewed in Jones [30]). The low IL-6 response to NTHi which we observed in children with CSLD as a group and in the 18–36 month age group is consistent with our data demonstrating an

impaired cell-mediated immune response. That TNF α production was also low in 18–36 month CSLD children may point to an immaturity or impaired function of innate immune cells such as monocytes or dendritic cells. Immaturity of such antigen presenting cells would also account for low IFN- γ responses to antigenic stimulation via impaired IL-12 or IL-27 pathways [31]. The possible contribution of IL-6 and other Th1-polarising cytokines, including IL-12 and IL-27, to the pathogenesis of NTHi infections and chronic inflammation requires exploration in future longitudinal studies.

P4 and P6 are highly conserved, immunogenic outer membrane proteins ubiquitously expressed on *H. influenzae*. Given the association of OMP antibodies with protection from NTHi infection in animal models [32] and the association of low P4 and P6 IgG₁ titres with asthma and atopy, in children [20], we investigated if children with CSLD were deficient in NTHi-specific antibodies, especially since clinical investigations found no deficiency in total IgG or any IgG subgroup overall. Consistent with published data [20,21], most children in our study produced IgG₁ to P4 and P6 whilst the number of children (CSLD and HC) producing IgG₄ to either antigen was low. In contrast to published

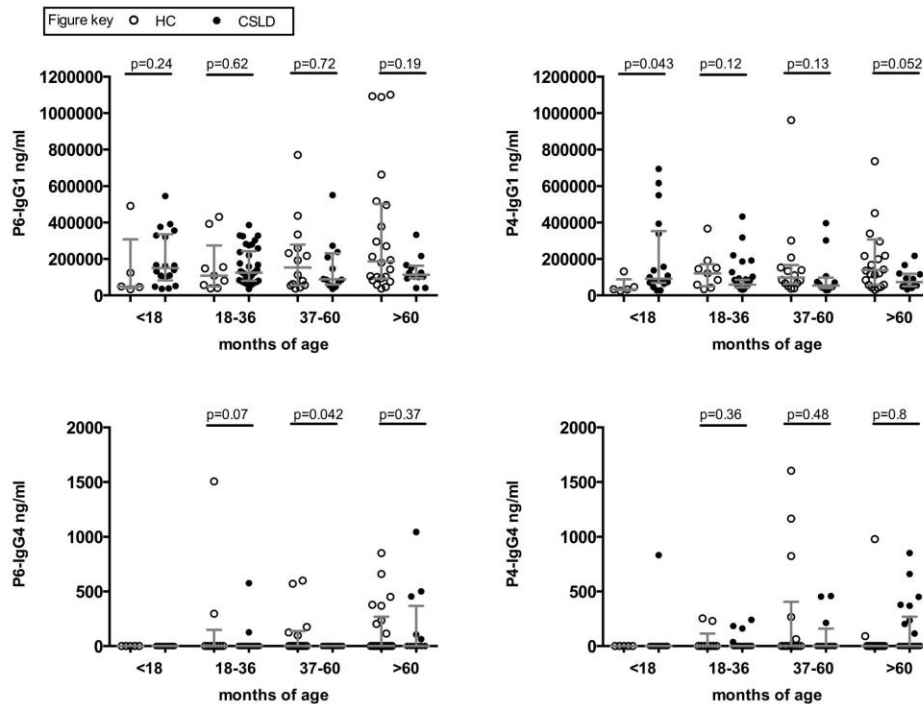


Figure 4. Serum IgG1 and IgG4 to outer membrane proteins P4 and P6. HC (open circles) and CSLD (filled circles). Concentration with median and inter-quartile range.
doi:10.1371/journal.pone.0104236.g004

data of children with Th2-associated disorders asthma and atopy [20] we found no significant difference in P4 and P6 IgG1 levels between children with CSLD and HC children. These results plus the comparable levels of early (24 hour) and later (72 hour) IL-10 responses support the hypothesis that non-protective immunity to NTHi in children with CSLD is more closely linked to aberrant cell-mediated immune function rather than the humoral arm of the immune response.

Our finding that children with CSLD have an impaired IFN- γ response to NTHi has some interesting parallels with that described in adults with the chronic respiratory conditions associated with infection (COPD and bronchiectasis). In these studies involving smaller cohorts, King and colleagues demonstrated that persistent or recurrent lower respiratory infection with NTHi in adults with COPD ($n = 39$) or bronchiectasis ($n = 16$) was associated with high IL-13 production in the airways and low systemic IFN- γ production [13,11]. These studies provide important data on the pathogenesis of NTHi infection in adults, however extrapolation of this data to the pediatric setting may not be appropriate. The strengths of our study, which also distinguish it from the adult studies, lie in our large sample size of young children with early onset chronic disease. 75% of our total cohort ($n = 80$) were aged <3.5 years and had comparatively mild disease

of recent onset. The effect of age, chronic inflammation, airway remodeling and pollutants on immune responses is well documented [33–36]. Thus studying CSLD in children with early stage disease may be more instructive to understanding the early pathogenesis of bronchiectasis. The differences in immune function described in the current study are more likely to be involved in disease initiation rather than an effect of chronic disease and long standing tissue damage.

It is now evident that the airways are colonized with microbial flora and that respiratory illness is frequently characterized by changes in the normal microbiota [9]. Furthermore emerging evidence suggests that normal immune profiles vary between populations; children from regions of high pathogen burden have functionally distinct immune profiles compared with children from regions of low burden [23,24]. Whilst all of the children in our study came from a similar geographic location, a large proportion of our CSLD group identified with an Indigenous Australian ancestry. Many factors beyond the scope of this study may contribute to this disparity including both biological and socio-economic determinants of health. We undertook several approaches to ensure that the differences in cytokine profiles were a result of the CSLD and not merely explained by the demographics of the population.

There are numerous host and environmental factors which may influence the immune response thus, whilst our current study has novel findings, we acknowledge some limitations. We chose to use NTHi because it is the most common bacterium in children with CSLD but it was beyond the scope of our study to examine if other pathogens also induce low IFN- γ responses. Secondly it is a cross-sectional study thus we cannot explain the cause or the effect of the observed impaired IFN- γ response in children with CSLD. Also, the association between systemic and local airway responses is unknown as limited cell numbers obtained from BAL precluded measurement of IFN- γ production by airway T-cells in our study. Nonetheless, our findings suggest that altered systemic immune responses are characteristic of CSLD, and we would argue that both local and systemic immunity are likely to be important in defending the lungs from respiratory pathogens.

In conclusion, this is the first study to compare NTHi-driven innate, cell-mediated and humoral immune responses between a large group of children with CSLD and HC children. Our key finding is that the ability to induce protective cell mediated immune responses against NTHi is compromised in children with CSLD. We found clear evidence of impaired IFN- γ responses by PBMC, *in vitro*, in children with CSLD and limited evidence of impaired innate and humoral responses. Furthermore, whilst our data on young infant responses is preliminary it suggests that this impairment begins at an early age. This study has important implications for research into management strategies for children at risk of CSLD. Effective management strategies require an understanding of host responses to respiratory pathogens and the contribution of these responses to the pathogenesis of chronic infection. Future intervention studies that can improve cell mediated immune responses along with longitudinal studies examining the relative contributions of delayed immune develop-

ment, and high microbial load to the pathophysiology of CSLD in children, are required.

Supporting Information

Table S1 a) Linear regression model of IFN- γ production at 72 hours. b) Linear regression model of IL-13 production at 72 hours. c) Linear regression model of IL-10 production at 72 hours. d) Linear regression model of IL-6 production at 24 hours. (DOC)

Table S2 Analysis of patient characteristics on NTHi-specific cytokine production. # p value from analysis by Mann-Whitney U test, ### p value from analysis by ANOVA, ### p value from analysis by Spearman's rank order correlation. (DOC)

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Author Contributions

Conceived and designed the experiments: SJP STY JWU BJH WRT ABC. Performed the experiments: SJP STY. Analyzed the data: SJP STY. Contributed reagents/materials/analysis tools: SJP STY JWU BJH WRT ABC. Contributed to the writing of the manuscript: SJP STY JWU BJH WRT ABC.

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4.2.1 Supporting information (published online)

Supporting Information Table S1

a) Linear regression model of IFN- γ production at 72 hours

Univariate analysis	β	p	95% CI for β
Age (months)	0.201	<0.001	0.01 – 0.03
Indigenous background	-1.498	<0.001	-2.15 – -0.84
CSLD	-1.963	<0.001	-2.55 – -1.38
Multivariate analysis	β	p	95% CI for β
CSLD	-1.758	<0.001	0.09
age	0.01	0.09	-0.001 – 0.02

b) Linear regression model of IL-13 production at 72 hours

Univariate analysis	β	p	95% CI for β
Indigenous background	0.38	0.055	-0.01 – 0.77
CSLD	0.38	0.046	0.006 – 0.76
Multivariate analysis ($r^2=0.28$)	β	p	95% CI for β
CSLD	0.25	0.31	-0.23 – 0.73
Indigenous background	0.22	0.38	-0.28 – 0.72

c) Linear regression model of IL-10 production at 72 hours

Univariate analysis	<i>B</i>	<i>p</i>	<i>95% CI for B</i>
Age	0.01	0.008	0.002 – 0.02
Indigenous background	-0.38	0.07	-0.79 – 0.03
CSLD	-0.45	0.02	-0.85 – -0.06
Multivariate analysis ($r^2=0.07$)	<i>B</i>	<i>p</i>	<i>95% CI for B</i>
CSLD	-0.21	0.43	-0.74 – 0.32
Age	0.01	0.04	<0.001 – 0.01
Indigenous background	-0.15	0.57	-0.68 – 0.37

d) Linear regression model of IL-6 production at 24 hours

Univariate analysis	<i>B</i>	<i>p</i>	<i>95% CI for B</i>
Age	0.01	0.007	0.002 – 0.01
CSLD	-0.50	0.006	-0.85 – -0.14
Multivariate analysis ($r^2=0.10$)	<i>B</i>	<i>p</i>	<i>95% CI for B</i>
CSLD	-0.38	0.04	<0.001 – 0.01
Age	0.001	0.052	<-0.001 – 0.01

Supporting Information, Table S2. Analysis of patient characteristics on NTHi-specific cytokine production. # p value from analysis by Mann-Whitney U test, ## p value from analysis by ANOVA, ### p value from analysis by Spearman's rank order correlation

	number of children (%)	p value			
		IFN γ	IL-13	IL-10	IL-5
Antibiotic use[#]					
Azithromycin	25 (31)	0.38	0.99	0.64	0.87
Other	11 (14)				
Lower airway infection[#]					
Any pathogen	21 (26)	0.52	0.63	0.77	0.87
non-typeable <i>H. influenzae</i>	16 (20)	0.28	0.77	>0.9	0.71
<i>Streptococcus pneumoniae</i>	4 (5)	-	-	-	-
<i>Moraxella catarrhalis</i>	4 (5)	-	-	-	-
<i>Pseudomonas aeruginosa</i>	2 (2.5)	-	-	-	-
<i>Klebsiella pneumoniae</i>	1 (1.3)	-	-	-	-
Strongyloides serology	7 (9.7)	0.64	0.71	0.70	0.45
positive[#]					
(n tested=72)					
Serum total IgE^{##}		0.41	0.74	0.74	0.83
Blood inflammatory cells^{###}					
(n tested=76)					
White cell count		0.26	0.84	0.84	0.85
neutrophils		0.80	0.47	0.78	0.73
Lymphocytes		0.72	0.53	0.96	0.39
Airway inflammatory cells^{###}					
neutrophils		0.76	0.90	0.94	0.80
eosinophils		0.18	0.88	0.50	0.33

4.3 Chapter summary

This is the first prospective study to investigate host-related factors that may contribute to an increased susceptibility to lower respiratory infections and hence the pathogenesis of CSLD in children. The data from this chapter demonstrate that children with chronic suppurative lung disease have an altered systemic cell-mediated immune response to NTHi in vitro.

The most notable finding of this study was that, compared with healthy control children, blood mononuclear cells from children with CSLD have a reduced capacity to produce IFN- γ in response to NTHi. Interestingly the capacity for NTHi-driven IFN- γ production was comparable between groups in children less than 18 months of age. The difference in NTHi-specific IFN- γ production became notable in children older than 18 months of age suggesting a possible developmental delay in IFN- γ associated immunity in children with CSLD.

These data support the second hypothesis of this thesis; that an increased susceptibility to lower respiratory infection in children with CSLD may be due to an impaired NTHi-specific immune response.

CHAPTER 5

Non-typeable H. influenzae-directed immune responses are associated with airway inflammation

Chapter 5 NON-TYPEABLE *H. INFLUENZAE*-DIRECTED

IMMUNE RESPONSES ARE ASSOCIATED WITH AIRWAY

INFLAMMATION

5.1 Chapter overview

There is a growing body of evidence that persistent airway or systemic inflammation may contribute to impaired T-cell responses. Having found that PBMC from children with CSLD had a diminished capacity for NTHi-specific IFN- γ production (Chapter 4), I looked for some possibilities why this was so. As described in Chapter 2 there is a high prevalence of neutrophilic and eosinophilic inflammation in the airways of children with CSLD. Furthermore airway eosinophils positively correlated with circulating eosinophils suggesting there may be a degree of systemic inflammation active in these children. Thus the aim of Chapter 5 is to determine if the modified IFN- γ response described in children with CSLD is associated with airway or systemic inflammation. This study addresses objective 3 of my thesis.

Indications of airway inflammation include changes in cellular profile and the presence of pro-inflammatory cytokines in the BAL or sputum. The methods described in Chapter 3 were used to measure a panel of soluble cytokines (IFN- γ , IL-1 β , IL-6, IL-8, IL-12 p70) and antimicrobial proteins (LL-37 and IP-10) involved in the generation and

control of inflammation in the BAL and plasma of 70 children with CSLD. These were compared to the capacity of blood mononuclear cell to produce IFN- γ in response to NTHi in vitro. The results from this study are presented as a submitted manuscript currently under review with PLoS ONE (manuscript number PONE-S-14-71838) in section 5.2. Supporting information submitted with this manuscript are presented in section 5.2.1. A summary of the main findings is presented in section 5.3.

5.2 Manuscript under review: High pulmonary expression of IL-6 and IL-1 β in children with chronic suppurative lung disease is associated with impaired recall responses to non-typeable *Haemophilus influenzae*

This section presents a submitted manuscript in the format currently under review with PLoS ONE. Manuscript number: PONE-S-14-71838

1 **High pulmonary expression of IL-6 and IL-1 β in children with chronic suppurative lung**
2 **disease is associated with impaired recall responses to non-typeable *Haemophilus***
3 ***influenzae***

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5

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13

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15 **Abstract**

16 Non-typeable *Haemophilus influenzae* (NTHi) is commonly associated with chronic suppurative lung
17 disease in children. We have previously shown that children with chronic suppurative lung disease
18 have a reduced capacity to produce IFN- γ in response to NTHi compared with healthy control
19 children. The aim of this study was to determine if deficient NTHi-specific IFN- γ production is
20 associated with heightened systemic or airway inflammation. We measured a panel of cytokines
21 (IFN- γ , IL-1 β , IL-6, IL-8, IL-12 p70), antimicrobial proteins (LL-37, IP-10) as well as cellular and clinical
22 factors associated with airway and systemic inflammation in 70 children with chronic suppurative
23 lung disease. IFN- γ was measured in peripheral blood mononuclear cells challenged *in vitro* with live
24 NTHi. Regression analysis was used to assess the association between the systemic and airway
25 inflammation and the capacity to produce IFN- γ . On multivariate regression, NTHi-specific IFN- γ
26 production was significantly negatively associated with BAL concentrations of the inflammatory
27 cytokines IL-6 (β =-0.316; 95%CI -0.49, -0.14; p =0.001) and IL-1 β (β =-0.023; 95%CI -0.04, -0.01;
28 p =0.001). This association was independent of bacterial or viral infection, BAL cellularity and the
29 severity of bronchiectasis (using modified Bhalla score on chest CT scans). We found limited evidence
30 of systemic inflammation in children with chronic suppurative lung disease. In summary, airway
31 inflammation involving elevated IL-6 and IL-1 β may impair the capacity of children with CSLD to
32 produce IFN- γ in response to NTHi.

33 **Introduction**

34 Non-typeable *Haemophilus influenzae* (NTHi) is a common and important pathogen associated with
35 suppurative lung diseases (including bronchiectasis) in children and adults [1-3]. Chronic suppurative
36 lung disease (CSLD) is a common childhood condition in respiratory clinics and is characterised by
37 persistent wet or productive cough and recurrent lower respiratory infections. CSLD shares many
38 clinical characteristics with and is thought to be a common antecedent of bronchiectasis [4]. Thus
39 studying CSLD in young children may provide important insight into the pathogenesis of
40 bronchiectasis.

41

42 Studies in adults with suppurative lung conditions (bronchiectasis) have indicated that a Th1
43 polarised cell-mediated immune response contributes to immunity against NTHi [5,6]. Similarly our
44 previous studies have shown that young children with CSLD have a reduced systemic capacity to
45 produce the Th1-associated cytokine IFN- γ , in response to NTHi [7,8]. Understanding the
46 mechanisms that contribute to impaired immune responses and the pathogenesis of CSLD are
47 important for the development of intervention therapies. However factors leading to the impaired
48 IFN- γ response described in children and adults have not been examined.

49

50 There is a growing body of evidence that persistent airway or systemic inflammation may contribute
51 to impaired T-cell responses. Severe acute inflammation and chronic infection can disrupt
52 macrophage and T-cell activation and subsequently induce an environment of immune tolerance or
53 exhaustion [9,10]. Furthermore, it is increasingly recognised that lung and systemic immune
54 responses may be inter-related [11,12]. To further our understanding of the role played by IFN- γ in
55 the pathogenesis of CSLD, we aimed to determine if an impaired systemic ability to produce IFN- γ in
56 response to NTHi was associated with lung or systemic inflammation in 70 children with CSLD. We
57 found that NTHi-specific IFN- γ production by blood mononuclear cells was associated with markers
58 of airway inflammation.

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56 response to NTHi was associated with lung or systemic inflammation in 70 children with CSLD. We
57 found that NTHi-specific IFN- γ production by blood mononuclear cells was associated with markers
58 of airway inflammation.

59 **Materials and Method**

60 ***Study participants***

61 Children ≤ 10 years of age with CSLD, undergoing chest high resolution computed tomography
62 (HRCT) and flexible bronchoscopy for suspected bronchiectasis, were prospectively recruited (2010-
63 2013) from the Royal Darwin Hospital, Northern Territory, Australia. All children were clinically stable
64 (defined as absence of recent exacerbation) and under the care of a specialist paediatrician at the
65 time of sample collection. Radiographic diagnosis of bronchiectasis was made by the respiratory
66 paediatrician (AC) and the severity of bronchiectasis scored using a modified Bhalla scale as
67 previously done [13]. The total score was the sum of the score for each lobe, including lingula, based
68 on the extent of bronchiectasis, bronchial wall thickness and dilation (maximum score 48). Routine
69 clinical investigations [14] were undertaken in all children evaluated for bronchiectasis. Clinical and
70 socio-demographic data were collected using standardised data collection forms. Blood and
71 bronchoalveolar lavage (BAL) for clinical and research investigations were collected immediately
72 prior to chest CT scan/bronchoscopy as previously described [8]. This study was approved by the
73 Human Research Ethics Committee (Northern Territory Department of Health and Menzies School of
74 Health Research; #07/63) and children enrolled following written informed consent from the
75 parent/carer.

76

77 ***Sample collection***

78 Peripheral blood mononuclear cells (PBMC) and plasma were collected and stored as previously
79 described [8]. Briefly, PBMC were isolated from heparinised blood using standard density-gradient
80 techniques and cryopreserved in liquid nitrogen until use. Matched plasma was stored at -80°C .
81
82 BAL was collected and processed as previously described [15]. Briefly, BAL was collected as two
83 separate aliquots from the most affected lobe and maintained on ice for up to 3 hours prior to
84 processing. The first aliquot was used for microbiologic/viral analysis, the second for cytology.

85

86 BAL used for bacterial culture was stored diluted 2 fold in skim milk tryptone glucose glycerine broth
87 at -80°C. BAL for viral or bacterial analysis by PCR was stored undiluted at -80°C. Samples were
88 maintained at -80°C prior to testing.

89

90 ***Microbiology/virology Investigations***

91 Semi-quantitative culture identification of *Haemophilus influenzae*, *Streptococcus pneumoniae* and
92 *Moraxella catarrhalis* from BAL were performed by our research laboratory and a threshold of $>10^4$
93 CFU/ml considered clinically important [2,16]. *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*
94 culture and identification were performed by the diagnostic laboratory at the Royal Darwin Hospital.
95 Presence of *Chlamydia pneumoniae*, *Mycobacteria pneumoniae* and the respiratory viruses
96 (rhinovirus, adenovirus, enterovirus, bocavirus, respiratory syncytial virus, Wu virus, Ki virus,
97 coronavirus, parainfluenzae and metapneumovirus) were determined by PCR by the Queensland
98 Paediatric Infectious Disease Laboratory as previously done [17].

99

100 ***PBMC investigations***

101 PBMC were challenged with a single strain of NTHi as previously described [8]. Briefly, PBMC were
102 cultured with live NTHi, PHA (positive control) or medium alone (base line control) for 72 hours, as a
103 reflection of memory T cell function, and IFN- γ measured in the harvested supernatant.

104

105 ***Quantification of systemic and airway Inflammatory markers***

106 Routine clinical investigations (including full blood count with white cell differential count, platelets,
107 C-reactive protein (CRP) and serum protein) [4] were performed using the regional reference
108 laboratory Royal Darwin Hospital.

109

110 IFN- γ , IL-1 β , IL-6, IL-8, IL-12 p70, LL-37 and IP-10 in plasma and BAL, and IFN- γ and in PBMC culture
111 supernatants were measured in our research laboratory using an in-house dissociation-enhanced
112 lanthanide fluorescent immunoassay (DELFIATM) as described previously [8,18]. Quantitative standard
113 curves were generated from serial dilutions of recombinant human proteins and included on each
114 plate. The limit of detection was 10 pg/ml. The cathelicidin LL-37 was measured in plasma and BAL
115 using a high sensitivity ELISA kit (Hycult Biotech, The Netherlands). The limit of detection was 0.14
116 ng/ml. For analysis purposes, a value of 1/10 the limit of detection was assigned if the marker
117 concentration was below the limit of detection.

118

119 ***Data analyses***

120 Data were analysed using the statistics package STATA 13 (StataCorp, USA). As the data did not
121 follow a normal distribution, group data was described as median with interquartile range (IQR).
122 Differences between groups were assessed using the Mann-Whitney U test. A two-tailed p-value $\leq$
123 0.05 was considered significant. Univariate regression analysis was used to assess the potential of
124 systemic and airway inflammatory markers and clinical factors to predict in vitro NTHi-specific IFN- γ
125 production. NTHi-specific IFN- γ concentration was natural log transformed prior to regression
126 analysis. Variables with a p value <0.1 were included in a multivariate regression model. Within this
127 multivariate model, a p value of <0.05 was considered an independent predictor of the capacity to
128 produce IFN- γ in vitro in response to NTHi.

129 **Results**

130 **Characteristics of the children in the cohort**

131 The majority of the 70 children in this study (table 1) were young, with 84% of the children aged <48
132 months. All but 3 children had radiographic evidence of bronchiectasis and of these, 68% had 3 or
133 more lobes affected. The aetiology of bronchiectasis was presumed to be post-infectious as more
134 than 80% of the children had been hospitalised at least once and 50% hospitalised at least twice for a
135 lower respiratory infection. None of the children had primary immune deficiencies.

136

137 Airway neutrophilia (>15%) was found in 41% of children. A clinically important level of bacterial
138 infection (>10⁴ cfu/ml BAL) was present in 20 children (28%) and one or more viral pathogens
139 detected in 30 (44%). Non-typeable *H. influenzae* (15.5%), *S. pneumoniae* (15.5%) and rhinovirus
140 (29.4%) were the most common pathogens identified (detailed microbiology is presented in
141 supporting information Table S1).

142

143 BAL neutrophil % was not significantly associated with the presence of any bacteria ($\beta=0.19$, 95%CI -
144 12.8-13.2; $p=0.98$), or virus ($\beta=11.44$, 95%CI -0.335-23.2; $p=0.057$). However, BAL eosinophil % was
145 significantly and positively associated with viral infection ($\beta=2.534$, 95%CI 0.414-4.654; $p=0.020$). BAL
146 IL-1 β , IL-6, IL-8 and IP-10 significantly correlated with BAL % neutrophils but not with BAL %
147 eosinophils (supporting information Table S2).

148

149 **Relationship of airway and systemic profiles with NTHi-specific IFN- γ production**

150 On univariate analysis for the BAL data (Table 2 and Figure 1), % neutrophils, IL-1 β , IL-6, IL-8, IP-10,
151 NTHi infection and respiratory viruses were inversely associated with NTHi-specific IFN- γ production
152 by blood mononuclear cells.

153

154 On univariate analysis for the systemic inflammation data (Table 3), blood platelet levels (although
155 within the normal clinical reference range) were inversely associated with NTHi-specific IFN- γ
156 production by blood mononuclear cells. In contrast, total white cell count, CRP and serum
157 inflammatory mediators were not associated with NTHi-specific IFN- γ production.

158

159 **Multivariate analyses**

160 Using a cut off of $p < 0.1$ from the univariate analyses described in tables 2 and 3 above and
161 considering confounding factors, we combined the inflammatory markers in the BAL and blood into a
162 multivariate linear regression model. We found that in vitro IFN- γ production by blood mononuclear
163 cells in response to NTHi was significantly and inversely associated with BAL IL-1 β ($\beta = -0.023$; 95%CI -
164 0.04, -0.01; $p = 0.001$) and IL-6 ($\beta = -0.316$; 95%CI -0.49, -0.14; $p = 0.001$). In contrast, BAL IL-8, IP-10,
165 neutrophil % and infection status, as well as blood platelets were no longer independent predictors
166 of the IFN- γ response to NTHi by blood mononuclear cells

349 **Tables**

350 Table 1 Demographic characteristics and respiratory history of the cohort

Variable	N=70
Age, months, median (IQR)	27 (19-40)
Male, n (%)	39 (55.7)
Indigenous, n (%)	65 (92.9)
Gestational age (n=66), weeks; median (IQR)	38 (35-40)
Bronchiectasis, number of children (%)	68 (95.8)
number of lobes, median (IQR)	3 (2-4)
[§] Severity score, median (IQR)	7 (5-10)
Chronic suppurative otitis media, n (%)	9 (12.9)
History of hospitalisation (respiratory), n (%)	59 (84.3)
Number of hospitalisations, median (IQR)	2 (1-3)
Age at first hospitalisation, months, median (IQR)	6 (3-9)
Respiratory vaccinations up to date, n (%)	70 (100)
Passive cigarette smoke exposure (n=52), n (%)	42 (80.8)
Family history asthma (n=48), n (%)	23 (47.9)

351 [§] scored according to a modified Bhalla scale as previously reported [13].

352 Table 2. Univariate regression analysis of NTHi-specific IFN- γ production by blood mononuclear cells
 353 and BAL inflammatory markers

BAL variable	median (IQR)	β	95% CI	p value
Total cell count $\times 10^6/\text{ml}$,	0.36 (0.22-0.50)	-0.071	-0.222, 0.079	0.347
Neutrophil %	11 (4.6-39)	-0.023	-0.041, -0.005	0.015
Lymphocyte %	0 (0-0.7)	-0.081	-0.310, 0.149	0.485
Eosinophil %	2 (0.3-4.0)	-0.016	-0.122, 0.089	0.80
# IFN- γ pg/ml	1.0 (1.0-16.9)	-0.228	-0.938; 0.481	0.52
# IL-1 β pg/ml	133.6 (44.6-1279.4)	-0.026	-0.039, -0.012	<0.001
# IL-6 pg/ml	47.2 (19.3-77.8)	-0.349	-0.539, -0.158	0.001
# IL-8 pg/ml	105.4 (30.5-547.7)	-0.067	-0.141, 0.008	0.078
# IL-12 p70 pg/ml	27.2 (1-63.6)	0.178	-0.401, 0.757	0.54
# IP-10 pg/ml	340.7 (52.7-604.6)	-0.044	-0.067, -0.020	<0.001
# LL-37 ng/ml	2.6 (1.9-4.2)	-0.275	-0.921, -0.371	0.40
Any bacterial pathogen	20 (28.1)	-0.2686	-1.298, 0.0761	0.60
<i>H. influenzae</i> (non-typeable)	11 (15.5)	-1.223	-2.4689, 0.022	0.054
Any viral pathogen (n=68)	30 (44.1)	-0.9149	-1.850, 0.020	0.055
Rhinovirus	20 (29.4)	-0.5244	-1.564, 0.516	0.318

354 # β and confidence intervals reported as 100x concentration.

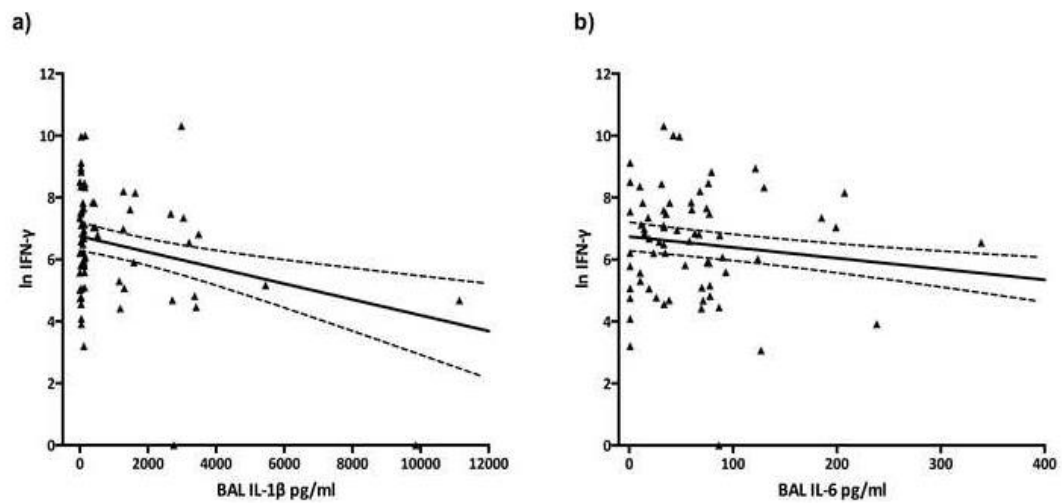
Table 3: Univariate regression analysis of NTHi-specific IFN- γ production by blood mononuclear cells and systemic inflammatory markers.

Blood variable	median (IQR)	β	95% CI	p value
White cell count $\times 10^9/L$	11.4 (9.1-14.3)	0.013	0.002, 0.029	0.087
Neutrophil	4.1 (2.85-5.55)	-0.064	-0.222, 0.095	0.42
Lymphocyte	4.9 (3.65-6.25)	-0.005	-0.143, 0.134	0.95
Eosinophil	0.9 (0.5-1.4)	0.311	-0.122, 0.744	0.16
CRP (n=36) mg/L	1 (1-3.3)	-0.013	-0.061, 0.035	0.59
Protein g/L	73 (70-77)	0.009	-0.058, 0.075	0.80
Platelets $\times 10^9/L$	337 (274.5-377.5)	-0.006	-0.010, -0.001	0.017
[#] IFN- γ pg/ml	26.6 (16.9-97.5)	0.074	-0.102, 0.251	0.40
[#] IL-1 β pg/ml	144.4 (16.7-349.2)	-0.012	-0.089, 0.066	0.77
[#] IL-6 pg/ml	5.7 (1.0-19.4)	1.015	-0.621, 2.651	0.22
[#] IL-8 pg/ml	11.6 (1.0-17.8)	-0.111	-4.099, 3.876	0.96
[#] IL-12 p70 pg/ml	40.1 (15.3-206.5)	0.037	-0.127, 0.201	0.65
[#] IP-10 pg/ml	756.8 (506.6-1079.7)	-0.030	-0.135, 0.074	0.56
[#] LL-37 ng/ml	28.9 (22.0-38.3)	0.033	-0.404, 0.470	0.88

[#] β and confidence intervals reported as 100x concentration.

Figure 1 Univariate regression model of NTHi-specific IFN- γ production by blood mononuclear cells and IL-1 β a) and IL-6 b) concentration in the bronchoalveolar lavage fluid.

Figure 1
[Click here to download high resolution image](#)



167 **Discussion**

168 This study is the first to investigate the association between the systemic cell-mediated immune
169 response and airway inflammation in children with CSLD. Our study of 70 children found that a
170 reduced capacity for systemic NTHi-specific IFN- γ production was significantly associated with
171 heightened airway inflammation as shown by high concentrations of IL-1 β and IL-6 in BAL fluid. We
172 found limited evidence of systemic inflammation in this cohort and neither markers of systemic
173 inflammation or clinical markers of CSLD severity (including radiological scores of bronchiectasis and
174 socio-demographic factors) predicted the capacity of the systemic NTHi-specific recall response.
175 These data support the hypothesis that systemic adaptive immune responses are linked to persistent
176 airway inflammation in children susceptible to lower respiratory infections.

177
178 Indications of airway inflammation include changes in cellular profile and the presence of pro-
179 inflammatory cytokines in the BAL or sputum. In adults and children with suppurative lung disease,
180 airway inflammation is characterised by elevated levels of neutrophils, IL-1 β , IL-6 and IL-8 [11,19,20].
181 Consistent with these studies, in our cohort of young children with CSLD, IL-1 β , IL-6, IL-8 as well as IP-
182 10 and LL-37 in the BAL were significantly correlated with BAL neutrophils. They were also all
183 significantly and negatively correlated with systemic NTHi-specific IFN- γ production on univariate
184 analysis. However, on multivariate regression, only IL-6 and IL-1 β were significantly and
185 independently associated with NTHi-specific IFN- γ production.

186
187 Whilst we found that the capacity for IFN- γ production was significantly inversely associated with
188 airway inflammation, the direction of this association, be that cause or effect, is unknown. The
189 mechanism driving this relationship is likely complex. However, one possible mechanism involves
190 regulation of the transition between the inflammatory and adaptive response, as these processes are
191 integral for homeostasis through rapid identification and elimination of the invading pathogen and
192 prompt resolution of inflammation.

193

194 IL-1 β , produced by a variety of pulmonary cells in response to a microbial challenge, drives the
195 inflammation cascade, localises neutrophils and promotes the production of inflammatory
196 modulators, such as IL-6 and IP-10 [21]. Consistent with this, we found a strong correlation between
197 levels of BAL IL-1 β , IL-6 and IP-10 in children with CSLD. IL-6 plays a complex role in the inflammatory
198 response, from promoting inflammation to wound healing. Dysregulation of IL-6 is associated with
199 chronic inflammation). In addition to its inflammatory modulating properties, IL-6 is integral to
200 initiating the adaptive response and in directing its primary phenotype. In the lung, IL-6 polarises the
201 adaptive immune response in favour of the humoral response. Animal and in vitro studies indicate
202 this is accomplished in two ways. Firstly, dendritic cell-derived IL-6 suppresses activation of the Th1
203 pathway by inhibiting IL-12 production [22,23]. Secondly IL-6, in synergy with macrophage-derived IP-
204 10, promotes the differentiation of B-cells into antibody-producing plasma cells [24]. Minimal
205 numbers of lymphocytes in our BAL samples precluded our ability to study airway cells directly,
206 however our data are consistent with the hypothesis that localised immune responses may not be
207 contained to the lungs. We found that IL-1 β , IL-6 and IP-10 were positively correlated with each
208 other. Furthermore BAL IL-1 β was positively associated with BAL IL-13. An environment high in these
209 cytokines is conducive to a polarised humoral immune response. Thus it is plausible that the low
210 systemic capacity for NTHi-specific IFN- γ production, also associated with BAL IL-1 β and IL-6, may be
211 a reflection of prolonged humoral responses in the lungs.

212

213 The mechanism responsible for translating this local response to peripheral T-cells has not been
214 investigated. However, it is well regarded that pulmonary dendritic cells preferentially activate naïve
215 T-cells in neighbouring lymph nodes rather than in the lung. Here they instigate an antigen-specific
216 polarisation of the mature T-helper phenotype. Furthermore dendritic cells are potent stimulators of
217 circulating T cells in vitro [22]. Thus it is conceivable that prolonged elevation of IL-6 and IL-1 β in the
218 chronically inflamed lung modifies the functional phenotype of the migrating dendritic cell such that

219 it continues to suppress Th1 activity in the lymph node. Future studies are required to characterise
220 the functional phenotype of dendritic cells from chronically inflamed lungs and their role in T-helper
221 activation.

222

223 An alternative explanation for the association between localised inflammation and the systemic
224 adaptive response is that insufficient IFN- γ may contribute to increased susceptibility to infections
225 with NTHi and subsequently a heightened state of local inflammation. There is increasing evidence
226 to indicate that a strong systemic IFN- γ response is associated with protective immunity against NTHi
227 [5,8] An impaired ability to produce sufficient IFN- γ in response to a challenge by NTHi may lead to
228 an increased susceptibility to respiratory infections and prolonged inflammation due to ineffective
229 clearing mechanisms. While the current cross-sectional study has found no relation between NTHi-
230 specific IFN- γ production and severity of bronchiectasis, resolving this issue will require prospective
231 studies examining the relationship between capacity for IFN- γ production and clinical outcomes.

232

233 In adults with bronchiectasis, King and colleagues have shown that a low NTHi-specific IFN- γ
234 response was due to polarisation of the T helper phenotype in favour of IL-4 production [5,6]. In the
235 current study, we measured secreted cytokine and therefore do not know the phenotype of the cells
236 responsible. However blood mononuclear cells from the children with NTHi infection produced
237 approximately half the amount of IFN- γ in response to an in vitro NTHi challenge than those without
238 NTHi infection, whilst showing equivalent IFN- γ responses to mitogen stimulation. This is consistent
239 with King et al's study, that the NTHi-specific recall response is predetermined and polarised away
240 from IFN- γ production rather than deficient in amplification of the T-cell response. Polarisation of the
241 immune response may result in delayed and inefficient clearance of NTHi and promote the
242 establishment of a niche within biofilm [25] or host cells [26], further stimulating and prolonging
243 local inflammatory responses.

244

245 Systemic inflammation has been reported in adults with bronchiectasis but appears to be transient
246 and associated with bacterial infection [11,27]. Consistent with this we found limited evidence of
247 systemic inflammation in our cohort of young children. There were isolated exceptions but overall,
248 clinical markers of systemic inflammation including CRP, white cell count, neutrophils and platelets
249 were within normal ranges. We found no significant association between eosinophils (circulating or
250 BAL) and the capacity for IFN- γ production or the panel of inflammatory markers investigated. There
251 was no correlation between plasma and BAL IL-1 β or IL-6 and no significant association between
252 markers of systemic inflammation and the capacity for NTHi-specific IFN- γ production by blood
253 mononuclear cells. Furthermore, clinical markers of CSLD severity (including radiological scores of
254 bronchiectasis and socio-demographic factors) did not predict systemic NTHi-specific IFN- γ
255 production. Thus, in children with CSLD, systemic NTHi-specific IFN- γ production is unlikely to be a
256 direct consequence of systemic inflammation.

257

258 The cross-sectional design of the current study meant we were unable to determine how resolution
259 of airway inflammation affects the systemic NTHi-specific IFN- γ response. Also, minimal numbers of
260 lymphocytes in our BAL samples precluded our ability to study airway cells directly. However our
261 data show a strong association between local inflammation and the systemic adaptive immune
262 response to NTHi in children susceptible to lower respiratory infections. Future studies to identify the
263 mechanisms driving this relationship between the adaptive IFN- γ response and airway inflammation
264 are required to better inform effective, long-term prevention strategies for children at risk of CSLD.
265 Understanding the nature of this association will help to determine if improving NTHi immunity
266 would be best achieved by directly boosting the adaptive IFN- γ response through vaccination or
267 immunotherapy strategies or if improved adaptive responses may be better realised by targeting IL-
268 1 β and IL-6 pathways in the lungs.

269

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268 1 β and IL-6 pathways in the lungs.

269

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5.2.1 Supporting information

Supporting information

Table S1: Bacterial and viral pathogens identified in the BAL.

Pathogen	Number of children (% of total N)
Infection type identified	
Any (n=70)	41 (58.6)
[§] bacteria only	9 (13.2)
[§] virus only	21 (30.9)
[§] bacterial/viral co-infection	9 (13.2)
Any bacterial pathogen	20 (28.1)
<i>H. influenzae</i> (non-typeable)	11 (15.5)
<i>S. pneumoniae</i>	11 (15.5)
<i>M. catharrhalis</i>	3 (4.2)
<i>K. pneumoniae</i>	1 (1.4)
<i>P. aeruginosa</i>	3 (4.2)
<i>C. pneumoniae</i>	1 (1.5)
<i>M. pneumoniae</i>	0 (0)
Any viral pathogen (n=68)	30 (44.1)
Rhinovirus	20 (29.4)
Adenovirus	6 (8.8)
Enterovirus (n=35)	3 (4.4)
Bocavirus	3 (4.4)
RSV	2 (2.9)
Coronavirus	2 (2.9)
Wu	2 (2.9)
Ki	1 (1.5)
Parainfluenzae	1 (1.5)
Metapneumovirus	0 (0)

[§] n=68; 2 children were not tested for BAL viruses.

Supporting information

Table S2: Spearman's rank correlation of bronchoalveolar lavage neutrophils and eosinophils from bronchoalveolar lavage, with bronchoalveolar lavage markers of inflammation and infection.

BAL	Neutrophils		Eosinophils	
	p value	r _s	p value	r _s
IL-1 β pg/ml	0.003	0.356	0.36	0.110
IL-6 pg/ml	<0.001	0.545	0.59	0.066
IL-8 pg/ml	<0.001	0.729	0.10	0.200
IP-10 pg/ml	<0.001	0.428	0.061	0.225
Eosinophils	0.059	0.227	-	-
Bacteria	0.51	0.081	0.31	0.124
Viruses	0.010	0.311	0.005	0.339

5.3 Chapter summary

This prospective study is the first to show an association between inflammation and the cell-mediated immune response in children with CSLD. The primary finding from this chapter was that a reduced capacity for NTHi-directed IFN- γ production by blood mononuclear cells was significantly associated with heightened airway inflammation. Furthermore BAL IL-1 β and IL-6 were independent predictors of NTHi-driven IFN- γ production, independent of BAL neutrophils, IL-8, IP-10, LL-37 and infection with respiratory pathogens. There was limited evidence of systemic inflammation.

Elevated levels of lung neutrophils, IL-1 β , IL-6 and IL-8 have been reported in adults with suppurative lung disease and correlate with respiratory pathogen load. The novelty of the current study is the linking of airway inflammation to the systemic cell-mediated immune response. In the current study, IL-1 β , IL-6, IL-8 as well as IP-10 and LL-37 in the BAL were significantly correlated with BAL neutrophils indicating active inflammation in the lungs. Each of these inflammatory markers was negatively associated with NTHi-driven IFN- γ production by blood mononuclear cells. However, IL-1 β and IL-6 alone were independent predictors of IFN- γ production.

IL-1 β and IL-6 are integral to the initiation and regulation of inflammation and the transition between inflammatory and adaptive immune responses. Thus the independent association between BAL IL-1 β and IL-6 and systemic NTHi-driven IFN- γ production

supports the third hypothesis of this thesis and may provide one mechanism contributing to recurrent lower respiratory infection in children and the pathogenesis of CSLD.

Understanding the nature of this association will help to determine if improving NTHi immunity would be best achieved by targeting IL-1 β and IL-6 inflammation pathways in the lungs or better realised by directly boosting the adaptive IFN- γ response through vaccination or immunotherapy strategies. The first of these strategies will be investigated in Chapter 6 and the second strategy will be investigated in Chapter 7.

CHAPTER 6

Inhaled non-steroidal anti-inflammatories for children and adults with bronchiectasis

Chapter 6 INHALED NON-STEROIDAL ANTI- INFLAMMATORIES FOR CHILDREN AND ADULTS WITH BRONCHIECTASIS

6.1 Chapter overview

As established in chapters 5, there is a significant association between airway inflammation (as measured by IL-1 β and IL-6) and the NTHi-driven memory recall response of blood mononuclear cells in children with CSLD. Although the factors driving this association between IL-1 β and IL-6 in the lungs and systemic NTHi-induced IFN- γ production are unknown it is plausible that targeting dysregulated IL-1 β and IL-6 pathways in the lungs may have systemic effects.

One of the roles of IL-1 β and IL-6 in airway inflammation is the synergistic activation of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) both directly and via an increased expression of prostaglandin E₂ (PGE₂) [165, 166]. One novel therapy that has been proposed to address airway inflammation in children with CSLD is the use of inhaled non-steroid anti-inflammatories (NSAIDs) [52]. Non-steroid anti-inflammatories (NSAIDs) block the COX-2 pathway by inhibiting the activity of IL-1 β on PGE₂ and COX-2 directly.

Chapter 6 presents a systemic review of the efficacy of inhaled NSAIDs in the management of bronchiectasis in children and adults and addresses objective 4 of my thesis. The aim of chapter 6 is two-fold:

1. To inform and guide the clinical management of CSLD in children
2. To direct future studies regarding the mechanisms driving the interaction between airway and systemic immune responses.

An evaluation of the current evidence regarding the efficacy of inhaled NSAIDs in the management of CSLD in children and bronchiectasis in adults is presented as a published article in section 6.2. A summary of the main findings and directions for future research are presented in section 6.3.

**6.2 Journal article: Inhaled non-steroid anti-inflammatories
for children and adults with bronchiectasis.**

Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis (Review)

Pizzutto SJ, Upham JW, Yerkovich ST, Chang AB



This is a reprint of a Cochrane review, prepared and maintained by The Cochrane Collaboration and published in *The Cochrane Library* 2010, Issue 4

<http://www.thecochranelibrary.com>



Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis (Review)
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[Intervention Review]

Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

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ABSTRACT

Background

Chronic neutrophilic inflammation, both in the presence and absence of infection, is a feature of bronchiectasis in adults and children. The anti-inflammatory properties of non-steroid anti-inflammatory drugs (NSAIDs) may be beneficial in reducing airway inflammation and thus potentially improve lung function and quality of life in patients with bronchiectasis.

Objectives

To evaluate the efficacy of inhaled NSAIDs in the management of non-cystic fibrosis bronchiectasis in children and adults.

Search strategy

We searched the Cochrane Airways Group Trials Register, the Cochrane Central Register of Controlled Trials (CENTRAL) (*The Cochrane Library* 2009, issue 3), MEDLINE, OLDMEDLINE and EMBASE databases. The latest searches were carried out in October 2009

Selection criteria

All randomised controlled trials comparing inhaled NSAIDs to a control group (placebo or usual treatment) in children or adults with bronchiectasis not related to cystic fibrosis.

Data collection and analysis

We reviewed the results of the searches against pre-determined criteria for inclusion.

Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis (Review)
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Main results

One small, short-term trial was eligible for inclusion. We included this study of 25 adults with chronic lung disease (including bronchiectasis) as the other conditions were linked to development of bronchiectasis and all had chronic sputum production.

The single trial in adults reported a significant reduction in sputum production over 14 days in the treatment group (inhaled indomethacin) compared to placebo (difference -75.00 g/day; 95% CI -134.61 to -15.39) and a significant improvement in a dyspnoea score (difference -1.90; 95% CI -3.15 to -0.65). There was no significant difference between groups in lung function or blood indices. No adverse events were reported.

Authors' conclusions

There is currently insufficient evidence to support or refute the use of inhaled NSAIDs in the management of bronchiectasis in adults or children. One small trial reported a reduction in sputum production and improved dyspnoea in adults with chronic lung disease who were treated with inhaled indomethacin, indicating that further studies on the efficacy of NSAIDs in treating patients with bronchiectasis are warranted.

PLAIN LANGUAGE SUMMARY

Inhaled non-steroid anti-inflammatories (NSAIDs) for children and adults with bronchiectasis

The airways of patients with bronchiectasis are characterised by chronic inflammation. The anti-inflammatory effects of inhaled non-steroid anti-inflammatory drugs (NSAIDs) may be beneficial in patients with bronchiectasis. However, the short and long-term benefits in both adults and children require investigation, in addition to the potential side effects of the long-term use of NSAIDs. For this review we found one small study that reported an improvement in sputum production and dyspnoea (shortness of breath) in adults with chronic lung disease (chronic bronchitis, bronchiectasis or diffuse panbronchiolitis) who received inhaled indomethacin compared to the placebo group. There was no significant improvement in lung function (forced expiratory volume in one second (FEV₁) and vital capacity (VC)). However, the small scale of this study and the collective analysis of data from the three disease states made it difficult to draw any solid conclusions on the benefit of using NSAIDs to treat adults with bronchiectasis. There were no studies identified on the use of NSAIDs in children with bronchiectasis.

BACKGROUND

Description of the condition

Bronchiectasis, previously termed an 'orphan disease', is increasingly recognised as a major cause of respiratory morbidity, especially in developing countries (Karadag 2005; Karakoc 2001) and in pockets of affluent countries (Chang 2008). The underlying aetiology of bronchiectasis varies; it may follow recurrent respiratory infections or be secondary to rare immune deficiencies. However, bronchiectasis is also a common pathway for a variety of diseases. Thus, the presence of bronchiectasis is also increasingly recognised in common (e.g. chronic obstructive pulmonary disease (COPD) (O'Brien 2000) and uncommon respiratory diseases (e.g. bronchiolitis obliterans and sarcoidosis (Lewis 2002)) as well as non-primary respiratory (e.g. autoimmune) diseases. When bronchiectasis is present with another underlying disorder, it increases the mor-

bidity and mortality of the underlying disease (Keistinen 1997; Lewis 2002). For example, in diseases such as COPD the presence of bronchiectasis has been reported in 29% to 50% (O'Brien 2000) of cohorts and when present increases the severity and frequency (Gursel 2006) of respiratory exacerbations.

The dominant symptoms and signs of bronchiectasis are productive or wet cough, dyspnoea on exertion and presence of other respiratory signs (clubbing, chest wall deformity, respiratory noises such as wheeze or crepitations on auscultation). In the long term pulmonary decline may occur (Keistinen 1997). Also, as in patients with COPD, children and adults with bronchiectasis also suffer from recurrent acute exacerbations, some of which require hospitalised treatment (Chang 2008). Effective management regimes for bronchiectasis improve quality of life (Courtney 2008; Martinez-Gracia 2005; Muthalithas 2008), and could reduce the frequency or severity of respiratory exacerbations (Cymbala 2005)

and/or the long-term pulmonary decline (Chang 2008). Thus, management of the symptoms and severity of bronchiectasis is important.

Description of the intervention

Non-steroid anti-inflammatory drugs (NSAIDs) are a class of medication that act as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes. Non-steroidal anti-inflammatories have analgesic, antipyretic and anti-inflammatory effects and reduce pain, fever and inflammation. NSAIDs are usually given orally but the inhaled formulation has been also used in people with bronchorrhoea, a feature present in many patients with bronchiectasis (Tamaoki 1992). A Cochrane Review of oral NSAIDs for people with bronchiectasis did not find any suitable randomised controlled trials (Kapur 2007).

How the intervention might work

Based on Cole's 'vicious circle hypothesis', microbial colonisation/infection is important in the pathophysiology of bronchiectasis as it leads to bronchial obstruction and an abnormal or exaggerated inflammatory response (Cole 1986). Anti-inflammatory drugs may reduce the inflammatory cascade and thus ameliorate symptoms and reduce long-term pulmonary decline.

As the airways of patients with bronchiectasis have intense neutrophilic inflammation (Cole 1986), the anti-inflammatory effect of NSAIDs may have a beneficial effect for patients with bronchiectasis. "Blockade of cyclooxygenase pathway with indomethacin could decrease respiratory tract fluid and mucus by inhibiting chloride secretion and glandular secretion and by enhancing Na absorption across airway mucosa" (Tamaoki 1992). Animal studies have shown that pre-treatment with inhaled indomethacin protects the airway from distilled water and ozone which increases lung resistance through swelling of airway epithelial cells (Mochizuki 2002).

Why it is important to do this review

Although NSAIDs may have potential benefits for those with bronchiectasis, oral NSAIDs are associated with a number of adverse events, particularly of the gastrointestinal tract (Behrman 2003). NSAIDs may be better tolerated when inhaled, however transient upper airway irritation has been reported (Ong 2004; Sestini 1999). It is therefore important to assess any additional side effects associated with the inhalation of NSAIDs.

In cystic fibrosis (CF), preliminary evidence suggests that NSAIDs may prevent pulmonary deterioration in people with mild lung disease due to CF (Lands 2007). However, extrapolation of treatment for CF to non-CF bronchiectases may be harmful (e.g. re-

combinant human DNase efficacious in CF causes harm in non-CF bronchiectasis (Crockett 2001)). Thus, a systematic review on the efficacy of inhaled NSAIDs in the management of children and adults with bronchiectasis would help guide clinical practice.

OBJECTIVES

To evaluate the efficacy of inhaled NSAIDs in children and adults with bronchiectasis:

- (a) during stable bronchiectasis;
and for reducing:
 - (b) the severity and frequency of acute respiratory exacerbations;
and
 - (c) long-term pulmonary decline.

METHODS

Criteria for considering studies for this review

Types of studies

All randomised controlled trials (RCTs) comparing inhaled NSAIDs to a control group (placebo or usual treatment) in patients with bronchiectasis

Types of participants

Children or adults with bronchiectasis (defined clinically or radiologically) not related to cystic fibrosis. We excluded participants with cystic fibrosis or with other diseases where bronchiectasis was not present.

Types of interventions

All types of inhaled NSAIDs.

Types of outcome measures

Primary outcomes

We planned to obtain data on at least one of the following outcome measures.

- (A) **For short-term effectiveness (12 months or less):** mean difference in bronchiectasis severity control (quality of life (QOL), cough scores).

(B) For medium to long-term outcomes (> 1 year): lung function data (forced expiratory volume in one second (FEV₁) % predicted).

Secondary outcomes

(A) For short-term effectiveness (12 months or less):

- a) total numbers of days with respiratory symptoms;
 - b) mean difference in lung function indices (spirometry, other lung volumes, airway hyper-responsiveness);
 - c) proportions of participants who had respiratory exacerbations and/or hospitalisations;
 - d) total number of hospitalised days;
 - e) mean difference in other objective indices (airway markers of inflammation, exhaled nitric oxide etc.);
 - f) proportions experiencing adverse effects of the intervention (e.g. gastritis, haematemesis, ecchymoses, etc.);
 - g) serious adverse events (e.g. haemoptysis, bronchospasm etc.).
- (B) For medium to long-term outcomes (> 1 year):
- h) radiology scores (high resolution computed tomography scans or chest radiograph);
 - i) clinical indices of bronchiectasis severity control (QOL, cough diary, Likert scale, visual analogue scale, level of interference of cough, etc.);
 - j) mortality;
 - k) proportions experiencing adverse effects of the intervention (e.g. gastric bleeding, gastritis, haematemesis, cardiac events, etc.);
 - l) serious adverse events (e.g. haemoptysis, bronchospasm etc.).

Search methods for identification of studies

Electronic searches

We used the following topic search strategy to identify the relevant randomised controlled trials listed in the electronic databases: ("bronchiectasis" OR "suppurative lung disease" as (textword) or (MeSH)) AND ("inhaled" OR "nebulise" OR "nebulised" as (textword) or (MeSH)) AND ("anti-inflammatory" OR "diclofenac" OR "etodolac" OR "ketorolac" OR "sulindac" OR "tolmetin" OR "diflunisal" OR "salsalate" OR "meloxicam" OR "piroxicam" OR "flurbiprofen" OR "ibuprofen" OR "ketoprofen" OR "naproxen" OR "oxaprozin" OR "indomethacin" OR "COX2 inhibitors" OR "celecoxib" OR "rofecoxib" OR "valdecoxib" as (textword) or (MeSH))

We identified trials from the following sources:

1. the Cochrane Airways Group Trials Register;
2. the Cochrane Central Register of Controlled Trials (CENTRAL) (*The Cochrane Library* 2009, issue 3);
3. MEDLINE (1966 to present);
4. OLDMEDLINE (1950 to 1965); and
5. EMBASE (1980 to present).

For MEDLINE, OLDMEDLINE and EMBASE we combined the topic search strategy with the RCT search filter as outlined in the Airways Group module.

Searching other resources

We also searched the references in relevant publications. We planned to communicate with the authors of trials included in the review, if necessary.

Data collection and analysis

Selection of studies

From the title, abstract or descriptors, two authors (SP, AC) independently reviewed the literature searches to identify potentially relevant trials for full review. We conducted searches of bibliographies and texts to identify additional studies. From the full text and using the specified criteria, the same two authors independently selected trials for inclusion. We planned to resolve any disagreement by third party adjudication (JU).

Data extraction and management

We reviewed trials that satisfied the inclusion criteria for the following information: study setting; year of study; source of funding; patient recruitment details (including number of eligible subjects); inclusion and exclusion criteria; other symptoms; randomisation and allocation concealment method; numbers of participants randomised; blinding (masking) of participants, care providers and outcome assessors; dose and type of intervention; duration of therapy; co-interventions; numbers of patients not followed up; reasons for withdrawals from study protocol (clinical, side effects, refusal and other); details on side effects of therapy; and whether intention-to-treat analyses were used where possible. We would have extracted data on the outcomes described previously. Where required we planned to obtain further information from the authors.

Assessment of risk of bias in included studies

In order to assess the risk of bias, two review authors (SP, AC) independently assessed the quality of the studies according to the criteria described by Juni ([Juni 2001](#)).

Allocation concealment

We assessed allocation concealment as follows.

1. Adequate: if the allocation of participants involved a central independent unit, on-site locked computer, identically appearing

numbered drug bottles or containers prepared by an independent pharmacist or investigator, or sealed opaque envelopes.

2. Unclear: if the method used to conceal the allocation was not described.

3. Inadequate: if the allocation sequence was known to the investigators who assigned participants or if the study was quasi-randomised.

Generation of the allocation sequence

Each study was to be graded for allocation concealment as follows.

1. Adequate: if methods of randomisation included using a random number table, computer-generated lists or similar methods.

2. Unclear: if the trial was described as randomised, but no description of the methods used to allocate participants to treatment group was described.

3. Inadequate: if methods of randomisation included alternation, the use of case record numbers, dates of birth or day of the week, and any procedure that was entirely transparent before allocation.

Blinding (or masking)

Each study was graded for blinding as follows.

1. Blinding of clinician (person delivering treatment) to treatment allocation.

2. Blinding of participant to treatment allocation.

3. Blinding of outcome assessor to treatment allocation.

Follow up

Each study was graded as to whether numbers of and reasons for drop-outs and withdrawals in all intervention groups were described, or if it was specified that there were no drop-outs or withdrawals.

Dealing with missing data

The authors planned to request further information from the primary investigators where required but as the only included study was published in 1992, we did not contact the authors (Tamaoki 1992).

Assessment of heterogeneity

We planned to describe any heterogeneity between the study results and test this to see if it reached statistical significance using the χ^2 test. We would have considered heterogeneity to be significant if the P value was less than 0.10 (Higgins 2008). We also planned to use the I^2 statistic, where heterogeneity is categorised such that a value of under 25% is considered low, around 50% is

considered moderate and over 75% is considered a high degree of heterogeneity (Higgins 2003).

Assessment of reporting biases

If meta-analysis had been possible, we would have assessed publication bias using a funnel plot. We intended to investigate and report on any selective reporting.

Data synthesis

For the dichotomous outcome variables of each individual study, we would have calculated the odds ratios (OR) using a modified intention-to-treat analysis. This analysis assumes that children not available for outcome assessment have not improved (and probably represents a conservative estimate of effect). An initial qualitative comparison of all the individually analysed studies examines whether pooling of results (meta-analysis) is reasonable. This would take into account differences in study populations, inclusion/exclusion criteria, interventions, outcome assessment and estimated effect size.

The results from studies that met the inclusion criteria and reported any of the outcomes of interest were to be included in the subsequent meta-analyses. We planned to calculate the summary weighted odds ratio and 95% confidence interval (CI) (fixed-effect model) (Cochrane statistical package, RevMan version 5 (RevMan 2008)). We would only have combined data from parallel studies. We planned to calculate numbers needed to treat (NNT) from the pooled OR and its 95% CI applied to a specified baseline risk using an online calculator (Cates 2003). If studies reported outcomes using different measurement scales, we planned to estimate the standardised mean difference (SMD). We would have described and explored any heterogeneity between the study results. We would have included the 95% CI estimated using a random-effects model whenever there were concerns about statistical heterogeneity.

Subgroup analysis and investigation of heterogeneity

The following a priori subgroup analyses were planned:

1. children (aged 18 years or less) and adults (> 18 years);
2. severity of bronchiectasis (based on FEV₁: > 80% classified as mild, 50% to 79% classified as moderate, 30% to 49% classified as severe, < 30% classified as very severe).

Sensitivity analysis

We also planned sensitivity analyses to assess the impact of the potentially important factors on the overall outcomes:

- variation in the inclusion criteria;
- differences in the medications used in the intervention and comparison groups;
- differences in outcome measures;

- analysis using random-effects model;
- analysis by treatment received; and
- analysis by intention-to-treat.

The method used for allocating treatment groups was not described.

Blinding

The patients and investigators responsible for disease follow up and data analysis were blinded. The doctor responsible for allocating treatment groups was not blinded but was not involved in follow up or data analysis.

Incomplete outcome data

Data were complete for all participants. However, data analysis did not distinguish bronchiectasis patients from the other respiratory groups, other than that for sputum production.

Selective reporting

We identified no selective reporting bias in the study.

Other potential sources of bias

We identified no other potential sources of bias.

RESULTS

Description of studies

See: [Characteristics of included studies](#); [Characteristics of excluded studies](#).

See the '[Characteristics of included studies](#)' and '[Characteristics of excluded studies](#)' tables.

Results of the search

The Airways Group specialised register/literature search performed in Oct 2008 and October 2009 yielded 173 (153 and 20 respectively) references. There were no RCTs which focused specifically on adults or children with bronchiectasis. We identified two publications which were considered for inclusion in this review. One study [Tamaoki 1992](#) was included; the second ([Llewellyn-Jones 1995](#)) did not meet the eligibility criteria and was excluded.

Included studies

There were no studies identified which focused solely on bronchiectasis in either adults or children. However a single, small study on adults with chronic lung disease, including bronchiectasis, was included in this review as the additional two chronic lung disease conditions in the study lead to bronchiectasis, and bronchorrhoea is a key clinical feature of bronchiectasis. The detail of this study is described in the '[Characteristics of included studies](#)' table. Tamaoki and colleagues ([Tamaoki 1992](#)) examined the short-term effect (14 days) of inhaled indomethacin on sputum and blood indices, dyspnoea scale and lung function in 25 adults with chronic lung disease (eight with bronchiectasis, 12 with chronic bronchitis and five with diffuse panbronchiolitis).

Excluded studies

We excluded one study ([Llewellyn-Jones 1995](#)) as it was not a randomised controlled trial.

Risk of bias in included studies

Allocation

Effects of interventions

The one study included in this review evaluated the effect of inhaled indomethacin on sputum production, quality of life and lung function in 25 patients with chronic lung disease, including eight patients with bronchiectasis.

Respiratory symptoms

The only clinical data reported were in the form of the Borg score, which showed a significant difference between groups (difference of -1.90; 95% CI -3.15 to -0.65) ([Analysis 1.1](#)). (The minimum clinically important difference (in COPD) is 1 unit ([Ries 2005](#))). Days with respiratory symptoms (our primary outcome measure) was not reported in the study.

Lung function

There was no significant difference between groups for FEV₁ % predicted (difference between groups of -2.90%; 95% CI -13.30 to 7.50) or for vital capacity (VC) % predicted (difference -2.90%; 95% CI -10.58 to 4.78) ([Analysis 2.1](#) and [Analysis 2.2](#)).

Other indices

For sputum indices (Analysis 3.2), a significant decrease in sputum production in the indomethacin group was found compared with the placebo group (difference -75.00 g/day; 95% CI -134.61 to -15.39) but there was no difference in the density of bacteria per gram of sputum (difference -0.30; 95% CI -1.71 to 1.11).

For blood indices (Analysis 3.3) no significant difference between groups was found for either erythrocyte sedimentation rate (ESR) (difference -2.00 mm/hr; 95% CI -13.42 to 9.42) or total white cell count (difference -400.00 cells/ml; 95% CI -1654.94 to 854.94).

Adverse events

No adverse events were reported in the study.

DISCUSSION

Summary of main results

Data from one small, short-term (14-day) study of 25 adults with chronic lung disease (12 with chronic bronchitis, eight with bronchiectasis and five with panbronchiolitis) suggest that inhaled indomethacin (a type of NSAID) was significantly beneficial in reducing sputum production and improving dyspnoea compared to placebo. The clinically important difference for the Borg scale in bronchiectasis is unknown but that for COPD is 1 unit (Ries 2005) and thus the difference between groups for dyspnoea (-1.90; 95% CI -3.15 to -0.65) is likely to be clinically important.

There was no difference between groups for lung function or blood indices.

Overall completeness and applicability of evidence

The small study and limited number of patients with bronchiectasis in the sole included study limits any definitive conclusion. We included this study on the same basis for inclusion as a study in the Cochrane Review of pneumococcal vaccination for bronchiectasis (Chang 2009).

No randomised controlled trials of inhaled NSAIDs in children with bronchiectasis were identified.

Quality of the evidence

The sole included study in this review (Tamaoki 1992) was a double-blind, randomised study but the sample size was small and allocation concealment remains unknown. Data from the three disease

states were described and analysed collectively, thus bronchiectasis-specific data are unknown. Data were expressed as means $\pm$ SEM (standard error of the mean). Two-way analysis of variance and Student's paired *t* test were used for normally distributed variables. The Newman-Keuls test was used for multiple comparisons. A *P* value of less than 0.05 was considered statistically significant.

Agreements and disagreements with other studies or reviews

The Cochrane Review of oral NSAIDs for cystic fibrosis concluded that NSAIDs are likely to slow the progression of lung disease (Lands 2007). Data on sputum production or dyspnoea were not reported in the review. The Cochrane Review of oral NSAIDs for bronchiectasis (Kapur 2007) did not find any relevant studies.

AUTHORS' CONCLUSIONS

Implications for practice

Although a single study has shown some benefit in the short-term use of inhaled indomethacin in adults with chronic lung disease (including bronchiectasis and those at risk of bronchiectasis), there is currently insufficient evidence to support or refute the use of inhaled NSAIDs in children or adults with bronchiectasis. NSAIDs may be beneficial in the immediate term in reducing sputum production and therefore improving quality of life in adults with chronic lung disease. However, there were too few bronchiectasis patients included in the study group and the duration of treatment was too short to provide adequate information on the beneficial or adverse effects of inhaled NSAIDs in adults with bronchiectasis. There is no data currently available on the effectiveness of inhaled NSAIDs in children with bronchiectasis.

Implications for research

The data presented in the one study included in this review indicate that a double-blind, randomised, placebo-controlled trial is warranted to investigate the short-term (< 12 months) and long-term (> 12 months) beneficial and adverse effects of inhaled NSAIDs in both adults and children with bronchiectasis. Randomised controlled trials should investigate children and adults separately and include data as highlighted in the 'Types of outcome measures' section of this review.

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* Indicates the major publication for the study

CHARACTERISTICS OF STUDIES

Characteristics of included studies [ordered by study ID]

Tamaoki 1992

Methods	Double-blind, randomised, placebo-controlled trial Pulmonary function was assessed by a change in vital capacity (VC) and FEV ₁ pre-treatment (day 0) and on day 14. Quality of life was assessed by Borg's ratio scale to questions related to breathlessness and dyspnoea Sputum was analysed for change in production (g/day), cyclooxygenase products (PGE ₂ , PGF _{2α} , 6-oxo-PGF _{1α} , TxB ₂) and microbiological culture Statistical analysis: data were expressed as means +/- SEM. Two-way analysis of variance and Student's paired <i>t</i> test were used for normally distributed variables. The Newman-Keuls test was used for multiple comparisons. A P value of less than 0.05 was considered statistically significant.	
Participants	25 adults (age 29 to 78 years) diagnosed with chronic lung disease (chronic bronchitis, diffuse pan-bronchiolitis or bronchiectasis) and bronchorrhoea of at least 4 weeks. Eight of the 25 participants had bronchiectasis but all had symptoms of bronchiectasis and 21 had chronic colonisation with respiratory pathogens present in adults with bronchiectasis - 17 had <i>Pseudomonas aeruginosa</i> , 3 with <i>Haemophilus influenzae</i> and one with <i>Staphylococcus aureus</i> . Of the 8 subjects with bronchiectasis, 4 were allocated to the indomethacin group and 4 to the placebo group. All had no history of respiratory allergy.	
Interventions	Treatment group 1: inhaled indomethacin, 2 ml aerosol preparation of 1.2 µg/ml in saline 3 times daily for 14 days Treatment group 2: inhaled placebo, 2 ml aerosolised saline alone 3 times daily for 14 days Method of delivery: nebuliser delivering aerosolised particles with a median particle diameter of 4.5 to 5 µm	
Outcomes	Data for all 3 disease states were analysed collectively. Outcomes were sputum indices (% solid composition, sputum bacterial density and inflammatory markers - prostaglandin E ₂ , PGF _{2α} , 6-oxo-PGF _{1α} , TxB ₂), Borg score ratio scale for breathlessness and dyspnoea, white cell count (WCC), erythrocyte sedimentation rate (ESR) and spirometry. The only outcome for which results were reported separately for bronchiectasis patients was effect on sputum production	
Notes	We elected to include all outcomes as although not all had the diagnosis of bronchiectasis, the additional 2 diseases (chronic bronchitis and panbronchiolitis) overlap with bronchiectasis and can eventually lead to bronchiectasis. Furthermore, the high number colonised with bacteria especially with <i>Pseudomonas</i> indicates that bronchiectasis would have been likely to be present if a multi-detector high resolution CT scan was performed in all subjects.	
<i>Risk of bias</i>		
Item	Authors' judgement	Description
Adequate sequence generation?	Unclear	Not described

Tamaoki 1992 (Continued)

Allocation concealment?	Unclear	The authors state that the study is a double-blind, randomised, placebo-controlled trial but do not describe the randomisation process, including allocation concealment
Blinding? Sputum volume	Yes	The patients and investigators responsible for disease follow up and data analysis were blinded. The doctor responsible for allocating treatment groups was not blinded but was not involved in follow up or data analysis.

Characteristics of excluded studies [ordered by study ID]

Llewellyn-Jones 1995	Study using oral indomethacin
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DATA AND ANALYSES

Comparison 1. Clinical symptoms

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Borg score	1		Mean Difference (IV, Fixed, 95% CI)	Subtotals only

Comparison 2. Lung function

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 FEV1 % predicted (end of study)	1		Mean Difference (IV, Fixed, 95% CI)	Subtotals only
2 VC % predicted (end of study)	1		Mean Difference (IV, Fixed, 95% CI)	Subtotals only

Comparison 3. Other indices

Outcome or subgroup title	No. of studies	No. of participants	Statistical method	Effect size
1 Wet weight of sputum at end of study (g/day)	1		Mean Difference (IV, Fixed, 95% CI)	Totals not selected
2 Bacterial load of sputum at end of study (Log10 cfu/g)	1		Mean Difference (IV, Fixed, 95% CI)	Totals not selected
3 White cell count at end of study (per mm ³)	1		Mean Difference (IV, Fixed, 95% CI)	Totals not selected
4 ESR at end of study (mm/h)	1		Mean Difference (IV, Fixed, 95% CI)	Totals not selected

Analysis 1.1. Comparison 1 Clinical symptoms, Outcome 1 Borg score.

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 1 Clinical symptoms

Outcome: 1 Borg score

Study or subgroup	Indomethacin		Placebo		Mean Difference IV,Fixed,95% CI	Mean Difference IV,Fixed,95% CI
	N	Mean(SD)	N	Mean(SD)		
Tamaoki 1992	13	4.5 (1.44)	12	6.4 (1.73)		-1.90 [-3.15, -0.65]

-4 -2 0 2 4
Favours indomethacin Favours placebo

Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis (Review)
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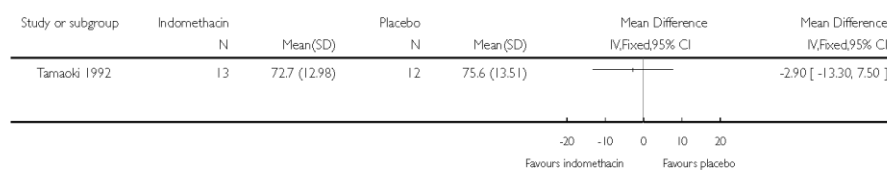
12

Analysis 2.1. Comparison 2 Lung function, Outcome 1 FEV1 % predicted (end of study).

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 2 Lung function

Outcome: 1 FEV1 % predicted (end of study)

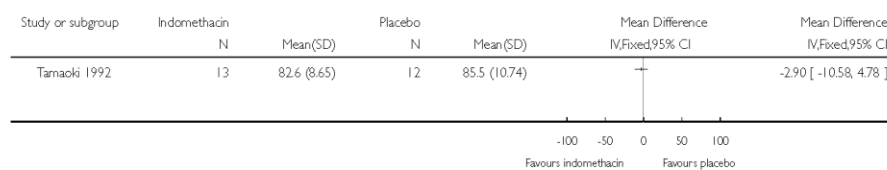


Analysis 2.2. Comparison 2 Lung function, Outcome 2 VC % predicted (end of study).

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 2 Lung function

Outcome: 2 VC % predicted (end of study)

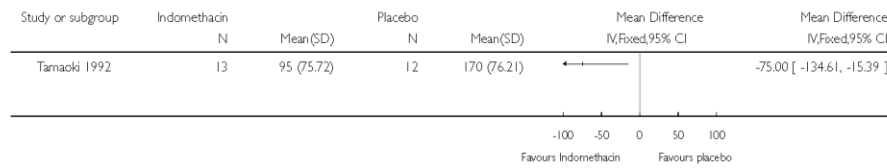


Analysis 3.1. Comparison 3 Other indices, Outcome 1 Wet weight of sputum at end of study (g/day).

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 3 Other indices

Outcome: 1 Wet weight of sputum at end of study (g/day)

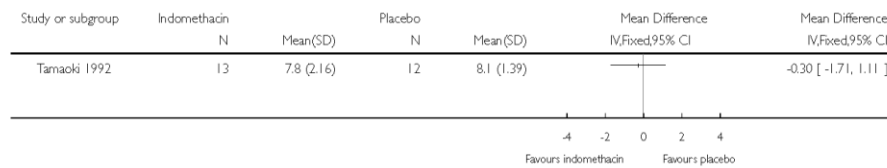


Analysis 3.2. Comparison 3 Other indices, Outcome 2 Bacterial load of sputum at end of study (Log10 cfu/g).

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 3 Other indices

Outcome: 2 Bacterial load of sputum at end of study (Log10 cfu/g)

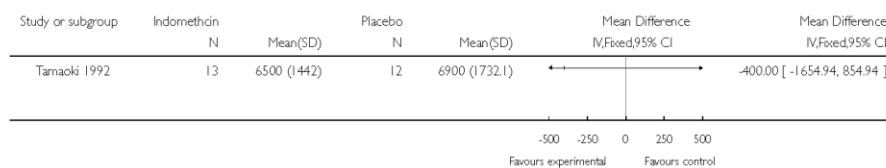


Analysis 3.3. Comparison 3 Other indices, Outcome 3 White cell count at end of study (per mm3).

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 3 Other indices

Outcome: 3 White cell count at end of study (per mm3)

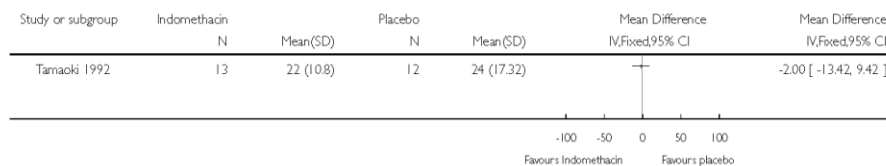


Analysis 3.4. Comparison 3 Other indices, Outcome 4 ESR at end of study (mm/h).

Review: Inhaled non-steroid anti-inflammatories for children and adults with bronchiectasis

Comparison: 3 Other indices

Outcome: 4 ESR at end of study (mm/h)



HISTORY

Protocol first published: Issue 1, 2009

Review first published: Issue 4, 2010

CONTRIBUTIONS OF AUTHORS

SP and AC wrote the protocol and review based on previous protocols and reviews. JU and ST contributed to writing the protocol and review.

DECLARATIONS OF INTEREST

None of the authors have any conflict of interest.

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INDEX TERMS

Medical Subject Headings (MeSH)

Administration, Inhalation; Anti-Inflammatory Agents, Non-Steroidal [*administration & dosage]; Bronchiectasis [*drug therapy]; Dyspnea [drug therapy]; Sputum [secretion]

MeSH check words

Adult; Child; Humans

6.3 Chapter summary

A comprehensive systematic review of randomised, controlled clinical trials investigating inhaled NSAIDs in children and adults with suppurative lung disease found no studies to guide the clinical management of children with CSLD. A single, small study in adults was identified. The adult study suggested that NSAIDs may reduce airway inflammation (measured by a reduction in sputum production) however the small number of patients and limited data collection restricted the conclusion that could be drawn. Thus this chapter does not support hypothesis 4 of this thesis, that a systematic review on the use of NSAIDs would guide clinical management of CSLD in children. Instead this chapter highlights a gap in the knowledge of therapeutics for children with CSLD. The data from this study indicate that a double blind, randomised, placebo controlled trial is warranted to investigate the short term (<12 months) and long term (>12 months) beneficial and adverse effects of inhaled NSAIDs in children with CSLD. In addition to clinical outcomes indicated in the methods of this study, outcome measures should include the effect of NSAID therapy on markers of airway inflammation (in particular IL-1 β , IL-6, COX-2 and PGE₂). Given the association between airway inflammation and the NTHi-driven systemic cell-mediated immune response described in chapter 4 of this thesis, secondary outcome measures should also include the long-term effects on memory recall responses to NTHi.

An updated search performed on 20-01-2015 did not identify any new studies since the review was published in 2010.

CHAPTER 7

The effect of vaccination with the pneumococcal *H. influenzae* protein D conjugate vaccine on NTHi-driven immune responses in children with chronic suppurative lung disease

Chapter 7 THE EFFECT OF VACCINATION WITH THE PNEUMOCOCCAL H. *INFLUENZAE* PROTEIN D CONJUGATE VACCINE ON NTHI-DRIVEN IMMUNE RESPONSES IN CHILDREN WITH CHRONIC SUPPURATIVE LUNG DISEASE

7.1 Chapter overview

As described in Chapter 4 children with CSLD have a reduced capacity to produce IFN- γ in response to NTHi. It is proposed that a deficient IFN- γ response may contribute to an increased susceptibility to colonisation by NTHi, persistent or exaggerated airway inflammation and the pathogenesis of CSLD (Chapter 5).

As discussed in section 1.8.1 innovative approaches or strategies should be considered to prevent and/or reduce CSLD progression in children. Chapter 7 explores if receipt of a currently licenced vaccine containing protein D from *H. influenzae* (Pneumococcal *H. influenzae* Protein D conjugate vaccine; PHiD-CV) modifies NTHi-driven cytokine responses in children with CSLD and promotes cytokine profiles similar to that of healthy control children. The results of this investigation are presented in section 7.2 as a published manuscript. A summary of the main findings is presented in section 7.3.

7.2 Journal Article: Improving immunity to *Haemophilus influenzae* in children with chronic suppurative lung disease

7.3 Chapter summary

The main finding from this study was that children with chronic suppurative lung disease who received ≥ 3 doses of PHiD-CV produced significantly more IFN- γ than children who received the alternative pneumococcal vaccines without protein D (median 939 versus 338 pg/ml; $p=0.007$). Importantly, the amount of IFN- γ produced by those vaccinated with the conjugate vaccine approached the levels observed from healthy children. Vaccination with PHiD-CV was also associated with small but significant increases in IL-13 and IL-5. Thus these data support the fifth hypothesis of this thesis that impaired cell-mediated immune responses to NTHi may be improved in children with CSLD.

The importance and novelty of this study is that it provides proof-of-concept that an NTHi vaccine may improve the cell-mediated immune response to NTHi in children with a particular vulnerability to lower respiratory infections. It is increasingly recognised that the cell-mediated immune response, particularly the IFN- γ response, is important for protective immunity against NTHi. Augmenting the NTHi-driven cell-mediated immune response in children with or at risk of CSLD may reduce the number of infective exacerbations and impede the progression of CSLD.

CHAPTER 8

Final Discussion

Chapter 8 FINAL DISCUSSION

8.1 Chapter overview

The overall aim of this thesis was to identify aspects of the immune response contributing to the pathogenesis of CSLD in Northern Territory children that may be targeted in future management strategies. Chapter 8 summarises the main findings of this thesis in relation to the overall aim and discusses these findings in relation to each other. The main limitations of my overall studies are then discussed. I have then presented further research questions that arose from my thesis and this is followed by a conclusion to my thesis.

8.2 Main study findings, discussion and significance

As outlined in section 1.3 Northern Territory Indigenous children have a high burden of CSLD. Prior to my PhD, there were no immunology-based studies that examined the reasons for this high burden. To address this, I embarked on a series of studies to address some of the knowledge gaps, raised in chapter 1, regarding the role of the host immune response to NTHi in the pathogenesis of CSLD in children.

Firstly, I needed to address whether flexible bronchoscopy (with its associated risks) should be performed in children suspected of having bronchiectasis. To do this, I looked at whether the data obtained from a bronchoscopy and lavage contributed to clinical

management. The results as described in chapter 2, showed that bronchoscopy with BAL contributed to the clinical management in 41% of children with CSLD. Surprisingly we found that 34% of children had clinically important eosinophilia, which has not been described previously in children with CSLD. This study addressed a gap in the knowledge regarding evidence based best clinical practice for children with CSLD. The findings from chapter 2 have contributed to clinical care by identifying airway eosinophilia as well as bacterial pathogens not addressed by current empiric therapies.

Having established that bronchoscopy with BAL should continue to be performed my subsequent chapters investigated the role of the immune response in the pathogenesis of CSLD and how this might be targeted in novel therapeutic interventions.

Despite current comprehensive immunisation and antibiotic strategies, children with CSLD have a propensity for lower respiratory infections. This may be explained in part by one of the key findings of this thesis presented in chapter 4. In vitro challenge assays of blood mononuclear cells demonstrated that children with CSLD have a reduced capacity to produce IFN- γ in response to NTHi suggesting that the protective cell-mediated immune response may be compromised in children with CSLD. Subsequent investigations into reasons for the modified cell-mediated immune response found a significant association between a low capacity for NTHi-driven IFN- γ production by blood mononuclear cells and elevated levels of IL-1 β and IL-6 in the lungs (chapter 5). The findings from these two studies have addressed important gaps in our knowledge regarding host-driven mechanisms that may contribute to the pathogenesis of CSLD,

first by showing that protective immunity to NTHi is likely compromised in children with CSLD and secondly this compromised adaptive immune response is linked to airway inflammation. Importantly this association was independent of bacterial infection and lung severity scores indicating that lung inflammation and adaptive immune responses to NTHi are linked rather than coincidental symptoms of CSLD. These findings are significant for the development of novel therapeutic strategies that target the mechanisms driving persistent lung inflammation including impaired cell-mediated immune responses. The strong association between low systemic IFN- γ production and elevated levels of IL-1 β and IL-6 in the lungs raised two plausible therapeutic strategies, one to target airway inflammation with inhaled anti-inflammatories and a second strategy targeting the cell-mediated immune response to NTHi.

Given the link between airway inflammation and systemic ability to mount a response to NTHi (Chapter 5), inhaled anti-inflammatories may be an effective management strategy in people to bronchiectasis. This was examined in Chapter 6. Although inhaled NSAIDs are sometimes used to treat airway inflammation in cystic fibrosis and have been proposed for the management of CSLD, a comprehensive systematic review of randomised, controlled clinical trials found no evidence to support their use in children with CSLD. The importance of this systematic review is that it emphasised a gap in the knowledge regarding novel therapeutics for children with CSLD. It also highlighted the need for clear data regarding the effect of inhaled NSAIDs on airway inflammation and the long-term effect of NSAIDs on recurrent infection in children with CSLD prior to introducing NSAIDs into the therapeutic regime.

An alternative strategy for improving clinical outcomes for children with CSLD that is supported by data from this thesis is to target the NTHi-driven cell-mediated immune response (chapter 7). We showed that NTHi-driven IFN- γ production by children with CSLD was highest in those who had been vaccinated with PHiD-CV. Furthermore, the levels of IFN- γ in PHiD-CV vaccinated children approach the levels produced by healthy children. The significance of this finding is that it provides proof-of-concept that an NTHi-specific vaccine has the potential to improve the NTHi-specific cell-mediated immune response in children with CSLD. These data suggest that the functional immune profile in children with CSLD may not be rigid, and that with appropriate therapy it may be possible to promote a more protective response to respiratory pathogens, particularly NTHi.

8.3 Limitations

The limitation of each study was discussed within their respective chapters. Here I discuss the overall limitations to my work.

The greatest limitation within my thesis is its cross-sectional nature. This restricted interpretation of the data and its implications for clinical outcomes. There are two main factors that confined this to a cross-sectional study. Firstly, samples for this study were collected opportunistically from children attending Royal Darwin Hospital for investigations of their respiratory symptoms (children with CSLD) or elective surgery (healthy control children) and follow-up of study participants for research was not

available. Secondly, bronchoscopy with BAL is an invasive procedure performed under anaesthesia and is therefore not without risk. While some researchers have undertaken bronchoscopies (including repeated bronchoscopies) in children purely for research purposes, my supervisors and I considered this approach unethical in our setting. Also, bronchoscopy with BAL is a specialist procedure and requires resourcing in a hospital setting. Prior to this thesis there was a lack of evidence to support the use of bronchoscopy in the clinical management of children with CSLD. Thus in the Northern Territory bronchoscopy is generally reserved to investigate the cause of deteriorating respiratory symptoms (primarily, investigations for bronchiectasis) rather than for monitoring purposes. Furthermore BAL is rarely available from respiratory-healthy children. Thus, reference values are estimated from studies where sputum has been used or extrapolated from studies of other respiratory disorders including asthma and cystic fibrosis. Longitudinal BAL and peripheral blood samples would be invaluable to understanding the pathogenesis of CSLD, to identify the factors driving excessive and persistent inflammation and for monitoring the effect of novel therapeutic interventions on adaptive and inflammatory immune responses. However, as mentioned above, this is not feasible or ethical in our setting.

Another limitation is the absence of data regarding the relationship between lower airway cellularity and microbiology profiles in young healthy Indigenous children. For the reasons noted above and in Chapter 1, lower respiratory samples from healthy children in our region are rare. Data regarding airway cellularity and inflammatory profiles in children without chronic respiratory disease in general are limited and primarily derived from small studies of children from large urban centres. However it is

becoming increasingly evident that children from regions of high pathogen burden have functionally distinct immune profiles compared with children from regions with low pathogen burden. Thus clinical thresholds derived from small studies in geographically distinct populations (particularly from Europe and the USA) to interpret the inflammatory profile of Northern Territory children may not be appropriate.

A further limitation came from the laboratory methods used to investigate immune responses to NTHi. Using DELFIA™ to measure cytokine protein we were able to determine the magnitude and profile of cytokine expression and thus provide an overall view of the functional immune phenotype of children with CSLD. This was important as there were previously no such data. However, it did limit our investigations into the cell subpopulations of T-lymphocytes responsible for the response, hence we could only speculate as to the mechanisms driving the low IFN- γ response to NTHi.

8.4 Future directions

Whilst my studies achieved the objectives outlined in section 1.10 and in doing so addressed a number of gaps in the literature, our understanding of the pathogenesis of CSLD in children is incomplete. The findings from my thesis raised a number of questions regarding the immunopathology of CSLD and immunologic mechanisms contributing to recurrent infection and persistent inflammation. Below I describe possible future work that may help elucidate a few of the important questions that remain.

8.4.1 Development of non-invasive methods to define and monitor airway inflammation

Data regarding airway cellularity and inflammatory profiles in children without chronic respiratory disease are limited. Obtaining lower airway specimens from children is difficult in general and as a result much of the published data are derived from small cross-sectional studies of children from large urban centres. However it is becoming increasingly evident that the normal immune phenotype may vary between populations particularly between children from regions with distinct immunological pressures. It will be important to characterise the lower airways of Northern Territory Indigenous children without respiratory illness in order to define diagnostic thresholds of inflammatory markers in this population and subsequently monitor therapeutic interventions and disease progression in children with CSLD. These data are currently unavailable. To achieve this goal novel, non-invasive approaches for monitoring lower airway

inflammation are required. Approaches that warrant investigation include the use of exhaled breath condensate [167] and upper airway mucosal secretions [168] to identify biomarkers reflective of lower airway inflammatory processes including the resolution of inflammation.

8.4.2 What is the clinical significance of elevated levels of airway and systemic eosinophils in children with CSLD?

Prior to the study presented in chapter 2 airway eosinophilia had not been reported in children with CSLD. Indeed, in adults with chronic airway diseases such as bronchiectasis and COPD, eosinophilia is believed to be associated with very severe disease. Thus finding airway eosinophilia in a third of this cohort of young children with CSLD is an intriguing and clinically important finding. The nature of my PhD limited clinical investigations in this cohort thus the aetiology of the eosinophilia and whether it was transient or sustained is unknown. As asthma was a rare diagnosis in this cohort of children, preliminary investigations in chapters 2 and 5 as to the possible cause of eosinophilia found limited associations with strongyloides and viral infections respectively. Further studies to help guide the clinical management of CSLD in Northern Territory children might include investigations regarding the role of infection with scabies and gut parasites (endemic to this region but often difficult to detect with routine clinical investigations) to the aetiology of airway and systemic eosinophilia, including an evaluation of the benefit, if any, of antiparasitic and antiviral therapies to clinical outcomes for children with CSLD.

8.4.3 The mechanisms responsible for low IFN- γ production in children with CSLD

One of the most notable findings from this thesis was that blood mononuclear cells from children with CSLD have a low capacity for IFN- γ production in response to NTHi. These data in children, together with adult studies by King et al [88, 89, 108] strongly suggest that a low IFN- γ response to NTHi increases susceptibility to lower respiratory infection. The mechanisms leading to the low IFN- γ response are unknown. However, as shown in children with CSLD who were vaccinated with PHiD-CV (Chapter 7), the capacity to produce IFN- γ in response to NTHi can be improved. Evidence presented in this thesis, particularly Chapters 4 and 5, support several plausible mechanisms for a diminished capacity to produce IFN- γ in response to NTHi that warrant further investigation and these are presented in this section.

What is driving the association between lung IL-6/IL-1 β and NTHi-driven IFN- γ ?

In chapter 5 of this thesis we demonstrated an association between the level of IL-6 and IL-1 β in the lung and the systemic memory recall response to NTHi (measured by IFN- γ production). This association was independent of classic inflammatory markers (eg neutrophils, IL-8, bacterial infection) and radiologic severity scores. These data suggest that IL-6 may be more than a marker of inflammation, but rather indicative of T-helper pathways that increase susceptibility to CSLD. IL-6 and IL-1 β inhibit the differentiation of Th1 effector cells by suppressing IL-12 production. Furthermore murine and human studies respectively show that IL-6 actively drives the differentiation of naïve T-cells into Th17 effector cells and also promotes the differentiation of B-cells into antibody-

producing plasma cells. Thus it is possible that the association between IL-6/IL-1 β and IFN- γ described in children with CSLD is indicative of Th2 or Th17 polarisation in the airways.

Th17 pathways have a protective role against acute respiratory infections with *K. pneumoniae* and *Mycoplasma pneumoniae*. However Th17 has also been implicated in the exacerbation of pathology associated with *B. pertussis* and human rhinovirus infections. Furthermore, emerging evidence from studies of chronic respiratory disease such as cystic fibrosis and fungal infections suggest that Th17 may be more pathologic than protective in chronic respiratory disease. Murine studies suggest a protective role of Th17 in NTHi immunity [169], however, how this translates to children with CSLD is unknown. The cultured cell pellets and BAL cell pellets from the studies presented in chapters 4, 5 and 7 are currently stored in RNA preservative and could be used to investigate the Th2 and Th17 profile in children with and without CSLD by measuring the transcription factors and cytokines responsible for driving the respective pathways; STAT4 and IL-12 (Th1), GATA3 and STAT6 (Th2) and STAT3, IL-23 and TGF β (Th17).

Is normal immune development impaired in children with CSLD?

It is possible that the maturation of the Th1 (hence IFN- γ) response, which naturally occurs during infancy, is delayed or modified in children with CSLD. It is well established that immune development begins in utero with both maternal health and environmental influences contributing to the process. Immune function during early

infancy is typically characterised by a suppressed IL-12-driven IFN- γ response to pathogens in favour of a classic Th2 response.

The capacity to produce IFN- γ increases substantially from approximately 12-18 months of age although adult-like responses may not develop until puberty. Delayed development of the IFN- γ response has been associated with childhood disorders of a Th2 phenotype, such as asthma. Emerging data indicate that antigenic type and load early in life may influence the development of the functional immune phenotype. Whilst regular antigen exposure is important for immune development, there are no data regarding the influence of high, early pathogen burden on the development of functional immunity in Northern Territory Indigenous children and the role this has in the pathogenesis of CSLD. A longitudinal birth cohort study to investigate the association between pathogenic and non-pathogenic antigen load (aspirate or nasopharyngeal swab) and functional immune phenotype (transcription factors and cytokines driving Th1 (such as STAT4, IL-12, IFN- γ), Th2 (GATA3, STAT6, IL-13, IL-5) and Th17 (STAT 3, IL-23, TGF β , IL-6)) at birth (cord blood) 18 months and 3 years of age and how this associates with recurrence and severity of respiratory illness, would fill this gap in our knowledge. Ideally these studies would include maternal prenatal microbiologic and immunologic data to determine the in utero factors that increase the risk for CSLD and may help guide prenatal care of at risk children.

Is the reduced capacity for NTHi-driven IFN- γ production a symptom of immune exhaustion?

There is a growing body of evidence that severe acute infection or chronic inflammation may contribute to impaired macrophage activation and T-cell responses, resulting in an environment of immune tolerance or exhaustion. Several studies have described a hierarchical loss in the ability of T-cells to produce IL-2, TNF- α and IFN- γ , particularly in response to chronic viral infection [170]. As shown in Chapter 5 there is a high prevalence of asymptomatic viral infection in Northern Territory Indigenous children with CSLD that was significantly and positively associated with eosinophilic inflammation of the airways. Furthermore the presence of respiratory virus was inversely associated with NTHi-driven IFN- γ production by blood mononuclear cells. As this was a cross-sectional study, the nature of the viral infection, be it transient or persistent, is unknown. However the high prevalence of respiratory viruses detected in this cohort suggests that viral infection contributes substantially to persistent airway inflammation. These data are consistent with the notion that the low capacity for IFN- γ production by blood mononuclear cells is indicative of immune exhaustion brought about by the high and early burden of bacterial respiratory pathogens accompanied by recurrent or persistent viral challenge. There are currently no studies that have investigated the concept of immune tolerance as a contributing factor to recurrent lower respiratory infections in children with CSLD. Studies to investigate the effect of viral, bacterial and parasite load on dendritic cell, T-cell and macrophage function in children with CSLD, and the possible contribution of immune exhaustion to recurrent respiratory

infection will fill important gaps in our knowledge regarding the pathogenesis of CSLD in Northern Territory children.

Does this immune deficit extend to other respiratory pathogens?

NTHi is the most common known pathogen identified in the lower airways of children with CSLD, however other respiratory pathogens, including viruses (as described in Chapter 5) are also common. Furthermore accumulating data indicate that the airways are host to a diverse microbiota, including respiratory pathogens, in people without respiratory disease. Thus distinguishing infection from opportunistic colonisation can be difficult. It has been proposed in the literature that respiratory disease may be better characterised by changes in the phenotypic profile of the microbiota [64]. Detailed data investigating the relative effect of changes in the lung microbiota on immune function in children are lacking. Such studies would provide important information regarding immune development and the pathogenesis of CSLD.

8.4.4 Defining the immune phenotype that correlates with protective immunity against NTHi in Northern Territory

Indigenous children

A finding of this thesis that has important implications for the clinical management of CSLD is that appropriate interventions have the potential to improve the functional immune profile of children with CSLD. It was shown in Chapter 7 of this thesis that children who received at least 3 doses of a vaccine (PHiD-CV) containing a single NTHi antigen produced a different cytokine profile in response to NTHi compared with

children who had received an equivalent vaccine without the NTHi component. This was a cross-sectional study and thus it is unknown if the amended cytokine profile resulted in increased protection from lower respiratory infection with NTHi. Whilst it appears that a strong IFN- γ response is important in immunity to NTHi the specific cytokine profile that correlates with protection is unknown. Indeed, whilst the IFN- γ levels produced by children with CSLD approached the levels obtained by healthy children, the children with CSLD also produced higher levels of Th2 associated cytokines (IL-13 and IL-5). As mixed Th1/Th2 responses are associated with suboptimal protection from pertussis [171] further investigations are required to ascertain the functional cell-mediated phenotype that best correlates with protection from infection with NTHi in Northern Territory Indigenous children. Such studies might include a longitudinal study to determine if the functional immune profile (as described in section 8.4.3) at 12-18 months of age can predict the prevalence and severity of lower respiratory infection at 3-4 years of age. Alternatively a cross-sectional study of the microbiologic and functional immune profile in siblings with and without CSLD may provide useful information regarding protective immune profiles of children exposed to similar environmental pressures. Regardless of approach, multicentre studies including children from Central Australia, Northern Australia and a large urban centre will be important in determining how protective immune profiles vary between children from different sociodemographic and geographic environments. These data will be important for effective vaccine development and immunisation strategies not only for Northern Territory Indigenous children but also for vulnerable children in other regions of high pathogen burden.

8.5 Overall conclusion

CSLD, an under-researched chronic illness, is particularly prevalent in Indigenous children worldwide including those living in the Northern Territory.

Many factors contribute to the high prevalence of CSLD in the Northern Territory including socioeconomic [13] and microbiologic [53, 60, 172] pressures. However prior to this thesis almost nothing was known regarding the intrinsic host factors that increase susceptibility to CSLD. The studies described in this thesis were directed at filling this knowledge gap. The overall aim of this thesis was to identify factors of the immune response contributing to the pathogenesis of CSLD in Northern Territory children that may be targeted in future management strategies.

The importance of investigating immunologic parameters associated with CSLD was highlighted by identifying a high prevalence of eosinophilic inflammation both systemically and in the airways of children with CSLD. Current management strategies depend primarily on antibiotic therapy for the treatment or prevention of acute bacterial infection and associated airway neutrophilia. However investigations of BAL showed that Northern Territory children with CSLD often have a mixed inflammatory phenotype, highlighting the need for novel therapeutic interventions and management strategies.

Thus this thesis contributes to the development of novel management strategies by identifying factors of the immune response contributing to the pathogenesis of CSLD in Northern Territory children that can be targeted by novel therapeutic interventions. The findings of this thesis suggest that dysregulated airway inflammation is linked to the

functional phenotype of the adaptive cell-mediated immune response to NTHi in children with CSLD. Importantly it has shown that with novel interventions it may be possible to improve immunity to NTHi and thus impede the progression of CSLD in Northern Territory Indigenous children.

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Appendix

APPENDIX A

Reagents for Dissociation Enhanced Lanthanide Fluorescent Immunoassay by time resolved fluorescence.

Reagent	Brand	Product	working conc.
IL-1 β	Biolegend Sapphire		0.5 μ g/ml
IL-1 β biotin	Biolegend Sapphire		0.5 μ g/ml
IL-1 β rHu protein	Biolegend Sapphire		-
IL-5 Rat IgG ₁ , k	BD Pharmingen TM	554393	2 μ g/ml
IL-5 biotin Rat IgG _{2a}	BD Pharmingen TM	554491	1 μ g/ml
IL-5 rHu protein	BD Pharmingen TM	554606	-
IL-6 Rat IgG ₁	BD Pharmingen TM	554543	4 μ g/ml
IL-6 biotin Rat IgG _{2a}	BD Pharmingen TM	554546	1 μ g/ml
IL-6 rHu protein	BD Pharmingen TM	550071	-
IL-10 Rat IgG _{2a}	BD Pharmingen TM	554705	2 μ g/ml
IL-10 biotin Rat IgG _{2a}	BD Pharmingen TM	554499	0.5 μ g/ml
IL-10 rHu protein	BD Pharmingen TM	554611	-
IL-13 Rat IgG ₁	BD Pharmingen TM	554570	2 μ g/ml
IL-13 Biotin Ms IgG ₁ , k	BD Pharmingen TM	555054	1 μ g/ml
IL-13 rHu protein	R&D Bioscientific	213IL005	-

Appendix A continued

Reagent	Brand	Product #	working concentration
IFN- γ Mouse IgG ₁ , k	BD Pharmingen TM	551221	2 μ g/ml
IFN- γ biotin Ms IgG ₁ , k	BD Pharmingen TM	554550	1 μ g/ml
IFN- γ rHu protein	BD Pharmingen TM	554616	-
TNF- α Mouse IgG ₁	BD Pharmingen TM	551220	3 μ g/ml
TNF- α biotin Ms IgG ₁ , k	BD Pharmingen TM	554511	1 μ g/ml
TNF- α rHu protein	BD Pharmingen TM	554618	-
IP-10 Mouse IgG _{2a} , k	BD Pharmingen TM	555046	3 μ g/ml
IP-10 biotin Mouse IgG _{2a} , k	BD Pharmingen TM	555048	1 μ g/ml
IP-10 rHu protein	BD Pharmingen TM	551130	-
LL-37 ELISA Kit	Hycult Biotech	HK321-02	-
IgG1 biotin Mouse IgG _{2b} , k	BD Pharmingen TM	555869	1/2000 dilution
IgG4 biotin Mouse IgG ₁ , k	BD Pharmingen TM	555882	1/2000 dilution
DELFIA® Eu-N1 Streptavidin	Perkin Elmer	1244-360	1/1000 dilution
DELFIA® Enhancement Solution	Perkin Elmer	1244-105	-

