The Epithelial Cell in Host-Pathogen Interactions in Airways Disease

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I am also grateful for the support and encouragement of my partner James, and for my dog Anja, and my horse Izzy.

Abstract

Non-typeable *Haemophilus influenzae* (NTHi) is the most common lower respiratory tract bacterium, where it causes persistent infections and is associated with neutrophilic airway inflammation and increased risk of exacerbations in patients with asthma and chronic obstructive pulmonary disease (COPD). This work aimed to investigate the persistence of NTHi within the epithelium, the role of bacterial internalisation in the epithelial immune response, and evaluate the differential immune signalling in the epithelium of upper compared with lower airway epithelium.

In an *in vitro* model of NTHi infection using cell lines and primary cells, differing internalisation rates between NTHi strains did not correlate with induction of proinflammatory cytokines. Treatment with cytochalasin D to block invasion diminished IL-1b production, but did not affect other cytokines. Imaging demonstrated that intracellular NTHi localised primarily within the late endosome of differentiated epithelial cells. Viable bacilli did not persist beyond 48 h in cell lines in submerged culture, or 24 h in primary cells grown at ALI, suggesting the NTHi does not persist in epithelial cells alone.

Although NTHi causes inflammation in the lower airways, it is considered a commensal organism in the nasopharynx. Comparison of primary cells cultured at air-liquid interface inoculated with NTHi showed substantially more upregulation of TLR signalling, NOD signalling, and of ICAM1, Caspase1 and IL-8 in nasal epithelial cells than bronchial cells. This suggests that the increased pathogenicity of NTHi in the lower airways compared with the nasopharynx is not due to increased innate immune responsiveness of bronchial epithelial cells.

These data demonstrate that NTHi potently induces an innate response in the bronchial epithelium independent of internalisation rate, with limited NLR inflammasome dependence, implicating surface immune sensing via TLR2 and 4. These data also demonstrate differential immune signalling in nasopharyngeal compared with lower airway epithelium. Overall, these data suggest that NTHi is internalised transiently but has the capacity to drive inflammation in airway epithelial cells.

Covid Impact Statement

My research has been affected by COVID beginning in late March 2020 when the first lockdown was announced in the UK. Our lab was fully closed for non-coronavirus related work through late July 2020, and as someone with a chronic respiratory condition I was advised to shield as well. I was able to begin some lab work at the end of July, however, it was primarily limited to work related to the ATOMIC2 clinical trial. In August I began to be able to start some non-Covid related work again, but on a limited basis as our lab was operating on a rota system to maintain social distancing, and we were only allocated a couple hours a day in the lab, curtailing time course experiments. In January our lab moved to a booking system, which allowed me to resume my experiments on a mostly normal schedule and load. However, disruption in the supply chain continued to affect some lab work, with shortages of PCR kits, pipette tips and other plasticware, and a backlog of experiments which has delayed the return of our RNA sequencing data by almost a year while it was in a queue to be processed. Overall, I have had 4 months where I was unable to do any lab work and an additional 5 months of limited lab work, as well as the ongoing supply chain disruptions.

During the initial lockdown, I began writing my thesis introduction and have co-written a review article. In addition, I wrote up my methods, and the results I had so far. During the period of limited lab access we were able to recruit a higher number of patients for our biobank than usual, so I have been able to use frozen samples from them once the lab reopened fully. I have also compressed my research schedule and have attempted to expedite experiments by combining some experiments and using more time-efficient although more expensive techniques, such as MSD rather than ELISA, and a Fluidigm PCR array rather than individual PCRs.

Contributions

Thanks are due to my fellow members of the Respiratory Medicine Unit for their collaboration and advice throughout my DPhil. In particular, I would like to thank Dr. Yuqing Long for her microbiology expertise and assistance with the generation of azithromycin-resistant NTHi strains, and collaboration on the RNA-sequencing experiment. In addition, I would like to thank Wojciech Lason, who has kindly supported us in analysing the RNA sequencing data.

I would also like to thank Katie Horton for designing and performing the initial optimisation work to develop a flow cytometry panel to evaluate NTHi infection in different types of epithelial cells. In addition, I would like to acknowledge the expertise of Dr. Helen Ferry at the flow cytometry facility, who performed the flow sorting experiments included in this thesis.

I would also like to thank Dr. Derek Hood at MRC Harwell for sharing his expertise and selection of NTHi strains with me, without which this work would not have been possible.

List of Abbreviations

ATCC	American type culture collection
ALPK1	Alpha kinase-1
BAL fluid	Broncho-alveolar lavage fluid
BGA	Bermuda grass allergen
BEAS-2B	Human bronchial epithelial cell line
BHI	Brain-heart infusion
CC16	Club cell secretory protein-16
CCL2 (MCP1)	C-C motif chemokine ligand
CD107a (LAMP-1)	Cluster of differentiation107a
CF	Cystic fibrosis
CFU	Colony-forming unit
COPD	Chronic Obstructive Pulmonary Disease
COX-2	Cyclooxygenase 2
CRNN	Cornulin
CXCL9	Chemokine CXC motif ligand 9
DAMP	Damage associated molecular pattern
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco’s phosphate-buffered saline
DSG-1	Desmoglein-1
ECM	Extracellular Matrix
ELISA	Enzyme-linked immunosorbent assay
EMTU	Epithelial-mesenchymal trophic unit
ET-1	Endothelin-1
FBS	Foetal bovine serum
FISH	Fluorescence in situ hybridisation

HBSS	Hank’s balanced salt solution
HDMA	House dust mite allergen
Hib	*Haemophilus influenzae* type B
HRV	Human rhinovirus
ICAM-1 (CD54)	Intercellular adhesion molecule 1(Cluster of differentiation 54)
ICS	Inhaled corticosteroids
IgaA	Human immunoglobulin A type A protease
IgaB	Human immunoglobulin A type B protease
IL-1	Interleukin-1
IL-4	Interleukin-4
IL-8 (CXCL8)	Interleukin-8
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-37	Interleukin-37
IRF	Interferon regulatory factor
KRT1	Keratin 1
LABA	Long-acting β agonist
LAMP-1 (CD107a)	Lysosomal-associated membrane protein-1
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP1 (CCL2)	(Monocyte chemoattractant protein 1)
MDM	Monocyte-derived macrophage
MOMP	Major outer membrane protein
MSC	Microbiological safety cabinet
NAD	Nicotinamide adenine dinucleotide

NEB	Neuroepithelial bodies
NEC	Nasal epithelial cell
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor-κB
NLR	Nod-like receptor
NOD	Nucleotide-binding oligomerization domain
NTHi	Non-typeable Haemophilus influenzae
OD	Optical density
OM	Otitis Media
OVA	Ovalbumin
PAMP	Pathogen associated molecular pattern
PBB	Protracted bacterial bronchitis
PBECs	Primary bronchial epithelial cells
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PRR	Pattern recognition receptor
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RelA	NF-kB p65 subunit
RIG-I	Retinoic acid-inducible gene I
RSV	Respiratory syncytial virus
SABA	Short-acting β agonist
SPINK5	Serine peptidase inhibitor kazal 5
TC	Tissue culture
TEER	Transepithelial electrical resistance
TEM	Transmission electron microscopy

TJ	Tight Junction
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factor
ZO-1	Zona occludens-1

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Chapter 1 Introduction

1.1 Chronic Airways Diseases

1.1.1 Asthma Overview

Asthma is a chronic inflammatory disease of the pulmonary airways affecting 235-360 million people worldwide (Possa *et al.*, 2013; Dunican and Fahy, 2015; Soriano *et al.*, 2017). It is characterized by variable airway obstruction, elevated mucus production, bronchial hyperresponsiveness and airway remodelling (Saetta and Turato, 2001; Brannan, 2010; Peter, 2013; Fehrenbach, Wagner and Wegmann, 2017). People who suffer from asthma typically experience coughing, wheezing and shortness of breath affecting their daily lives and often affecting their ability to exercise and work, as well as acute episodes of exacerbation with more severe symptoms. Asthma patients experience lower quality of life measures, particularly during asthma-related crisis events (Crossman-Barnes *et al.*, 2019). The true societal cost of a lifelong condition like asthma is difficult to estimate, encompassing direct expenses of healthcare, indirect costs of lost work, and intangible effects of reduction in quality of life associated with chronic disease burden (Nunes, Pereira and Morais-Almeida, 2017). Asthma is estimated to cost at least £1.1 bn annually in the UK in healthcare costs, disability claims and hospital care (Mukherjee *et al.*, 2016). These costs are disproportionately associated with patients with severe refractory asthma, highlighting the need for new treatments for these patients for whom existing therapies are not effective (Godard *et al.*, 2002; O'Neill *et al.*, 2015). Children with asthma miss more days of school than their healthy peers, and absenteeism at school was related to poor disease control, mould in the home, and cost barriers to asthma treatment (Hsu *et al.*, 2016). In a multinational study of 1,598 patients with symptomatic asthma despite following treatment regimens, nearly three-quarters of patients reported that asthma affected their workplace productivity (Gruffydd-Jones *et al.*, 2019). Participants reported loss of productivity both due to missed days due to asthma symptoms, as well as the symptoms they experience at work, notably shortness of breath, tiredness and mental strain (*ibid*). Asthma-related deaths have been declining for the past few decades following the introduction of corticosteroids as a treatment for asthma, but the

WHO estimates that 250,000 people die prematurely every year due to asthma (D'Amato *et al.*, 2016; Chang *et al.*, 2020).

Asthma is a disease of the pulmonary airways, where the bronchial epithelium acts as a barrier between the lung and the external environment, and damage to the epithelium is a key mediator of the airway remodelling and inflammatory signalling seen in asthma (Davies, 2009). Allergic asthma is considered to be initiated by damage to the airway epithelium leading to release of pro-inflammatory alarmins, and by penetration of the airway epithelium by aeroallergens, where they are sensed by dendritic cells and the processed antigen is presented to T cells in the lymph node (Nabe, 2013). B cells then produce antigen-specific IgE, which binds to Fcε receptors on a variety of immune cells, notably mast cells and basophils (*ibid*). When the sensitised individual is later exposed to that allergen, it binds to specific IgE and leads to activation of mast cells and basophils, which in turn release histamine, arachidonic acid metabolites and proteases into the airway, resulting in an early asthmatic response, occurring within 30 minutes of allergen exposure (*ibid*). However, in addition to this classical asthma pathway, typically leading to allergic eosinophilic inflammation in patients with persistent asthma, there are also patients with eosinophilia who are sensitised to non-allergic stimuli, as well as patients with paucigranulocytic inflammation (Busse, 2019a). In addition, a portion of asthma patients develop neutrophilic inflammation, which is often severe and associated with non-typeable *Haemophilus influenzae* (NTHi) infection and resistant to treatment with corticosteroids (Busse, 2019a, 2019b).

Figure 1. Epithelial cell signalling in asthma and health

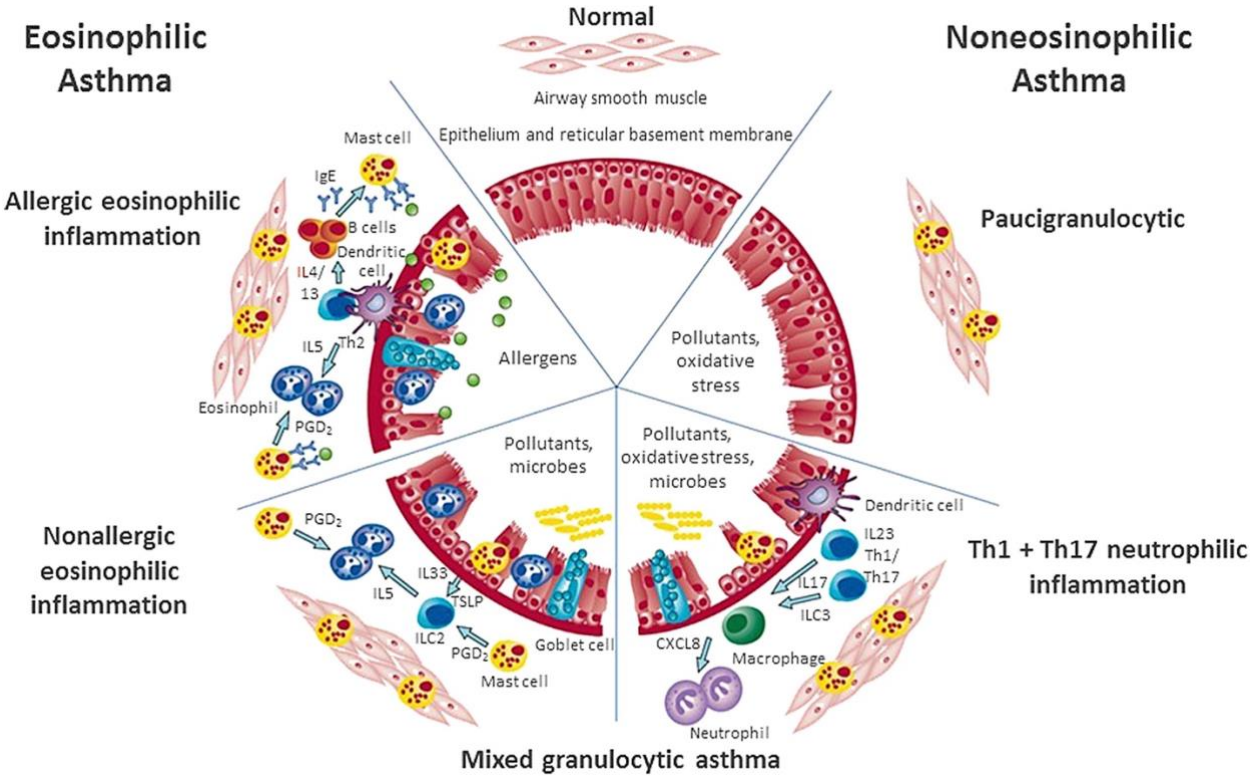


Figure 1. Diagram showing a cross-sectional view of the airway, depicting the cytokine milieu and immune cells associated with asthma. Normal tissue, allergic eosinophilic inflammation, non-allergic eosinophilic inflammation, Th1+ Th17 neutrophilic inflammation and paucigranulocytic asthma phenotypes are included, demonstrating the differential triggers, immune cell infiltration, and epithelial damage associated with each type. Figure reproduced from (Busse, 2019a).

1.1.2 Prevalence and geographical distribution of asthma

Rates of asthma are increasing worldwide, but the reasons for this are poorly understood (Cookson, 1987; Beasley *et al.*, 2000; Lai *et al.*, 2009; Dharmage, Perret and Custovic, 2019). Cases have more than doubled in the US between 1980 and 2002 (Redd, 2002), and rates continue to increase in the 21st century, affecting 1 in 12 Americans in 2009 versus 1 in 14 in 2001 (Dunican and Fahy, 2015). The cause of this increase in prevalence is still not understood, although a number of hypotheses have been put forward, including the hygiene hypothesis, increased obesity rates, and exposure to pollutants from traffic and industry. Other data has suggested that obesity predisposes people to the development of asthma, so increasing rates of obesity may also contribute to rising rates of asthma (Figueroa-Muñoz, Chinn and Rona, 2001;

Redd, 2002; Sin and Sutherland, 2008; Kim, Sutherland and Gelfand, 2014; Mohanan *et al.*, 2014). In addition, environmental exposure to various home and workplace allergens is associated with development of asthma (Baxi and Phipatanakul, 2010; Peden and Reed, 2010; Sousa-Silva *et al.*, 2021). Pollutants from traffic and industry, as well as from cigarette smoke, have also been associated with a predisposition to asthma (Sunyer, 2009; Guarnieri and Balmes, 2014; Hernandez *et al.*, 2018; Keet, Keller and Peng, 2018; Holst *et al.*, 2020).

Asthma prevalence is highest in Western countries, but rates are increasing in developing nations as they become more westernised (Beasley *et al.*, 2000; Al-Hajjaj, 2008; Sinharoy, Mitra and Mondal, 2018). Despite the higher rates of asthma in developed countries, asthma related mortality (ARM) is higher in developing countries, likely due to limited access to healthcare, poor health education, and high costs of effective drugs (Al-Hajjaj, 2008; Sinharoy, Mitra and Mondal, 2018). There are highly variable rates of asthma throughout the world; in 2000 – 2003, a questionnaire of 388,811 children in 91 countries age 6-7 found rates of current wheeze ranging from 2.4% of children in Jodhpur, India to 37.6% in Costa Rica (Lai *et al.*, 2009). A related study in older children found similarly disparate rates; 13-14 year olds over the same time period across 97 countries had rates of wheeze varying from, 0.8% in Tibet to 32.6% in Wellington, New Zealand (Lai *et al.*, 2009). There are several hypotheses about the increasing prevalence of asthma, including the hygiene hypothesis, association with higher levels of pollution, and increasing rates of obesity (Figueroa-Muñoz, Chinn and Rona, 2001; Redd, 2002; Brooks, Pearce and Douwes, 2013; Guarnieri and Balmes, 2014; Peters, Dixon and Forno, 2018).

One possible explanation for the increasing prevalence of asthma in the developed world is the hygiene hypothesis (Redd, 2002; Brooks, Pearce and Douwes, 2013; Liu, 2015; Weber *et al.*, 2015; Scudellari, 2017; van Tilburg Bernardes and Arrieta, 2017). This theory was put forward in 1989, based on the observation that while rates of asthma were increasing, incidence of childhood infections such as measles and mumps were decreasing, and suggesting that this decrease has resulted in altered immune system development. A more modern interpretation is that, while a decrease in exposure to pathogens is desirable, reduced exposure to a varied beneficial microbial

population in the environment as homes in developed countries become increasingly sanitised may contribute to the development of autoimmune conditions, including asthma, allergies, multiple sclerosis and Crohn's disease (Scudellari, 2017). Children with older siblings and those who have attended day-care are less likely to develop asthma than their peers who have not (Redd, 2002; Brooks, Pearce and Douwes, 2013; Liu, 2015; Weber *et al.*, 2015; Scudellari, 2017; van Tilburg Bernardes and Arrieta, 2017). The hygiene hypothesis may partly explain why the prevalence of asthma is higher in developed countries, where the increasing sanitization of environments and use of antibiotics has coincided with increasing rates of asthma.

Many studies have shown an association between obesity and both the probability of developing asthma and the severity of disease (Figueroa-Muñoz, Chinn and Rona, 2001; Redd, 2002; Sin and Sutherland, 2008; Kim, Sutherland and Gelfand, 2014; Mohanan *et al.*, 2014). In the US, almost 60% of adults with severe asthma are obese (Peters, Dixon and Forno, 2018). Obesity is associated with poorer disease control, a higher probability of hospitalisation and poor response to standard treatments such as ICS and combination ICS-long acting β agonists (LABA) (Peters, Dixon and Forno, 2018). In a cross-sectional study of 14,908 children aged 4-11 years, obesity as measured by BMI was associated with increased incidence of asthma attacks and wheeze (Figueroa-Muñoz, Chinn and Rona, 2001). As in adults, this correlation was stronger in girls than boys (Figueroa-Muñoz, Chinn and Rona, 2001; Eneli, Skybo and Camargo, 2008). However, it is unclear whether there is a causative effect where obesity causes a predisposition to development of asthma, or merely a correlation because obesity and asthma share inflammatory mechanisms (Kim, Sutherland and Gelfand, 2014). There is some evidence that obesity is a risk factor in the development of asthma, notably a dose-response relationship and correct temporal order, where obesity develops prior to asthma (Eneli, Skybo and Camargo, 2008). Despite the lack of conclusive evidence that obesity causes asthma, there is evidence that weight loss aids the control of asthma (Mohanan *et al.*, 2014; Peters, Dixon and Forno, 2018). A prospective, randomised study of 33 adults with severe asthma and moderate obesity showed that weight reduction was associated with significant improvement in disease control, without altering markers of lung inflammation, suggesting this improvement was not related to lung inflammation (Dias-Júnior *et al.*, 2014). Likewise, a systematic

review showed that weight loss resulted in 48-100% remission of asthma symptoms, again without a reduction in markers of eosinophilic inflammation (Juel *et al.*, 2012). These data suggest that obesity may contribute to the severity of asthma, although it is unclear how it contributes to the development of asthma.

Pollution from traffic and industry are the primary sources of urban air pollution, and are associated with increased development of asthma, as well as exacerbation of existing asthma (Sunyer, 2009; Guarnieri and Balmes, 2014; Hernandez *et al.*, 2018; Keet, Keller and Peng, 2018; Holst *et al.*, 2020). The number of disability-adjusted life years lost related to exposure to pollution was markedly higher in 2010 than in 2000 (Guarnieri and Balmes, 2014). A study including all Danish children born between 1997 and 2015 from the age of 1-15 years found that exposure to particulate matter PM $\leq$ 2.5 μm and $\leq$10 μm was associated with higher risk of asthma and recurrent wheeze, with a hazard ratio of 1.05 for $PM_{2.5}$ and 1.04 for PM_{10} per 5 $\mu g/m^3$ increase in pollution (Holst *et al.*, 2020). In the USA, a study of 7,810,025 children aged between 5 and 20 found that increased exposure to course $PM_{2.5\text{-}10}$ was associated with increased rates of asthma diagnosis (rate ratio 1.006, 95% CI) and increased hospitalisations (rate ratio 1.023, 95% CI) per 1 $\mu g/m^3$ increase in pollutant exposure (Keet, Keller and Peng, 2018). These data suggest that increased exposure to pollutants may contribute to elevated rates of asthma.

Exposure to airborne allergens contributes to both the development and severity of asthma (Baxi and Phipatanakul, 2010; Peden and Reed, 2010; Sousa-Silva *et al.*, 2021). Indoor allergens at home and in schools and offices are a significant source of exposure, such as house dust mite allergen, moulds, and allergens from pets and arthropods such as cockroaches (Peden and Reed, 2010). Exposure to unusual allergens in workplaces are also important, causing up to 5% of asthma cases in adults in the USA (Peden and Reed, 2010). Outdoor allergens include moulds and pollens (Peden and Reed, 2010; Sousa-Silva *et al.*, 2021). An increasing proportion of the population in western countries now live in urban areas where they are exposed to a relatively narrow spectrum of tree species, increasing their risk of developing allergies to those pollens (Sousa-Silva *et al.*, 2021). Utilising data about the allergenicity of different tree species and cultivars would allow city planners to plant a more diverse

population of less allergenic trees to help reduce the burden of highly allergenic pollens in urban areas (Sousa-Silva *et al.*, 2021). In addition to trees, grasses and weeds are also significant sources of allergenic pollen throughout the summer (Baxi and Phipatanakul, 2010). Climate change is also likely to alter distribution and growth of plants, which may contribute to increased exposure to allergenic pollen (Peden and Reed, 2010). For instance, one study showed that ragweed plants, which produce highly allergenic pollen, grew larger and produced more pollen in warmer urban areas compared to cooler rural areas (Shea *et al.*, 2008). These data suggest that increasing urbanisation, which exposes people to a higher concentration of a few pollens, together with climate change, may contribute to the increasing numbers of asthma cases.

Figure 2. Map showing global asthma prevalence in 2017

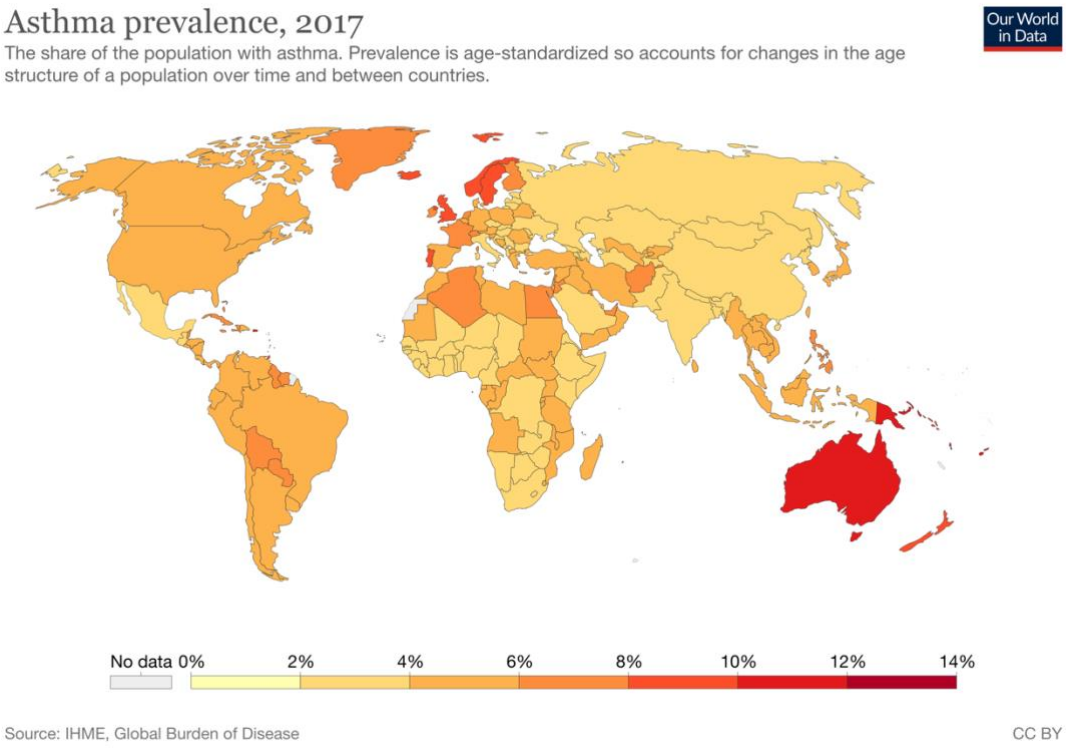


Figure 2. Diagram showing the global burden of asthma. The share of the population affected by asthma is highest in western countries, with particularly high rates in Australia, the UK and Scandinavia. In addition, rates in developing countries have begun to rise as they become increasingly developed. Figure reproduced from the Global Burden of Disease Study 2017 (Global Burden of Disease Collaborative Network, 2018).

1.1.3 Heterogeneity of asthma

Over the last two decades asthma has been divided into type-2 high asthma (frequently associated with an eosinophilic phenotype) and type-2 low asthma (often a non-eosinophilic phenotype) (Wenzel, 2012; Mccann *et al.*, 2016). Type-2 high asthma is characterized by a T_H2 response with production of interleukin (IL)-4, IL-5 and IL-13. Type-2 high disease is the most common form of asthma, both in childhood and adult life (Wenzel, 2012; Hinks, Levine and Brusselle, 2021) but is typically responsive to inhaled corticosteroids, which have constituted the mainstay of preventative therapy since the 1970s (Gelfand, 1951; Brockbank and Pengelly, 1958; Helm and Heyworth, 1958). By contrast, a subset of type-2 low, non-eosinophilic, asthma seen in a significant proportion of patients is characterized by elevated levels of neutrophils in the airway lumen, and is often severe and non-responsive to steroid treatment (Fahy *et al.*, 1995; Simpson *et al.*, 2006; McGrath *et al.*, 2012; Hinks, Levine and Brusselle, 2021). Elevated levels of neutrophil chemoattractants chemokine CXC motif ligand 8 (CXCL8, IL-8) and IL-17 are common features of neutrophilic asthma (Simpson *et al.*, 2007; Hynes and Hinks, 2020). Production of IL-8 and the pro-inflammatory activation associated with this may contribute to the pathogenesis of neutrophilic disease, although the pathogenesis and mechanisms underlying neutrophilic asthma currently remain poorly understood.

Figure 3. Th2 vs non-Th2 signalling in asthma

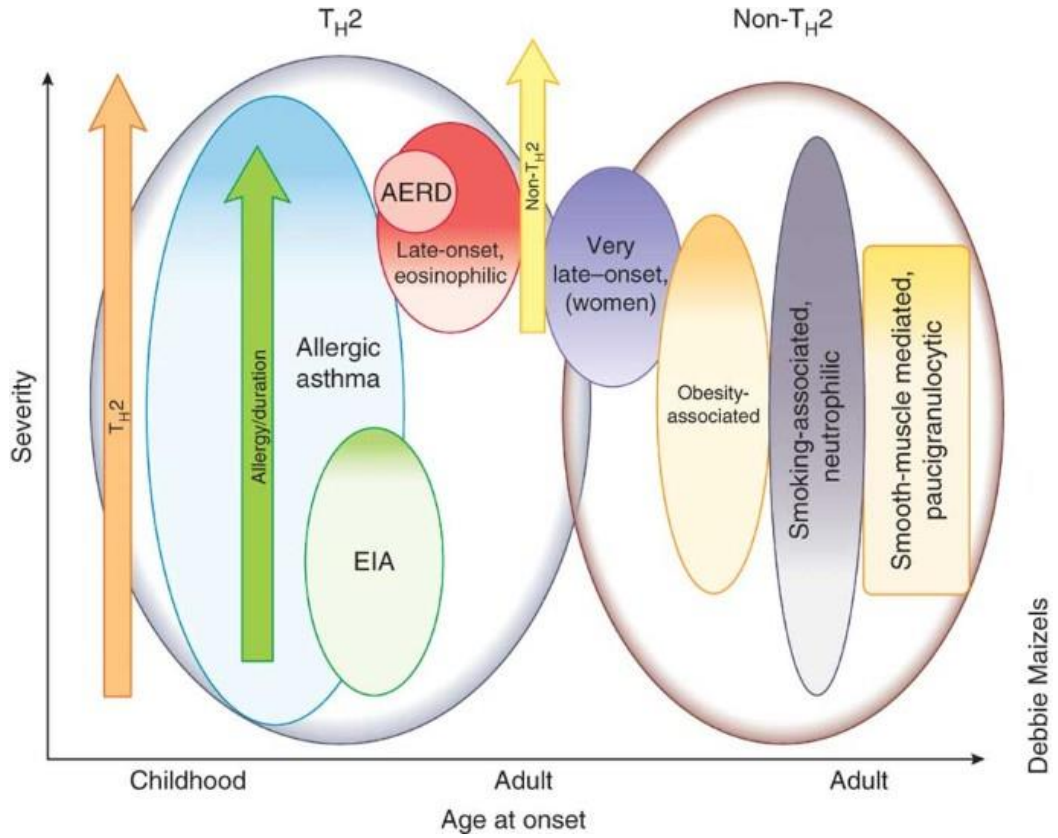


Figure 3. Inflammatory signalling associated with type-2 high and type-2 low asthma. Asthma has been divided into two types, type-2 high asthma, often associated with eosinophilia, and type-2 low asthma, which is typically associated with a non-eosinophilic phenotype. Type-2 high asthma is characterized by a T_H2 response with production of interleukin (IL)-4, IL-5 and IL-13. Type-2 high disease is the most common form of asthma, often with its onset in childhood, and in mild and moderate cases is typically responsive to inhaled corticosteroids. Patients with severe cases of type-2 high asthma are more likely to experience poor responsiveness to corticosteroids, and it is these patients which current biologics aim to treat. By contrast, type-2 low asthma is often adult-onset and associated with obesity, smoking, bacterial infection and paucigranulocytic inflammation. A subset of type-2 low asthma seen in a significant proportion of patients is characterized by elevated levels of neutrophils in the airway lumen, likely triggered by persistent bacterial infection, and is often severe and non-responsive to steroid treatment. Figure reproduced from Wenzel (Wenzel, 2012).

1.1.3.1 Neutrophilic Inflammation in asthma

Neutrophilic inflammation in asthma is associated with a phenotype characterized by severe symptoms which are refractory to treatment with steroids (Ray and Kolls, 2017). Neutrophil “high” asthma is defined varyingly by ≥61 to 73% sputum

neutrophils on an induced sputum cytospin (McGrath *et al.*, 2012; da Silva *et al.*, 2017; Hinks, Levine and Brusselle, 2021). Definitions are not well-defined for differentials based on bronchoalveolar lavage (BAL), as this remains a research tool, but one study has used a definition of 5% or greater BAL neutrophils (Grunwell *et al.*, 2019). Bronchoalveolar lavage involves the instillation of isotonic saline solution via flexible bronchoscope into a region of the lung and its subsequent aspiration to harvest airway cells and secretions (Meyer and Raghu, 2011). Neutrophilic asthma is associated with increased production of a variety of cytokines including IL-1β, IL-6, IL-8, IL-12, IL-17A and TNF (Hinks *et al.*, 2016; Yang *et al.*, 2018; Busse, 2019b). IL-17 is the signature cytokine produced by Th17 cells, and has a role in host defence against pathogens, but also has a role in inflammatory pathology of airways disease (Griffin *et al.*, 2012). IL-17 is associated with asthma, however, its role is unclear, or rather context-dependent at times appearing to be protective, whilst in other instances appearing pathogenic (Hynes and Hinks, 2020).

IL-1β is a pleiotropic cytokine mediated by the NLRP3 inflammasome (Whelan *et al.*, 2004; Kim *et al.*, 2017). It is part of the IL-1 axis, which exerts both pro- and anti-inflammatory effects, and is involved in the changes to airway smooth muscle (ASM) found in asthma (Whelan *et al.*, 2004). IL-1β upregulation is associated with chronic obstructive pulmonary disease (COPD), airway infections and neutrophilic asthma (Kim *et al.*, 2017). A mouse model of virus-induced asthma exacerbation demonstrated that IL-1β signalling was necessary for expression of neutrophil chemotactic factors, Th2 mediated IL-33, and Muc5ac in response to viral infection, suggesting it may have a role in both neutrophilic and type-2 inflammation in virus-induced exacerbations (Mahmutovic Persson *et al.*, 2018).

IL-6 has long been considered an inflammatory cytokine in asthma, although it may also have a regulatory role (Dimitrova *et al.*, 2017; Schmit *et al.*, 2020). IL-6 is significantly higher in patients with asthma compared with healthy controls, but also significantly higher in asthma patients who have had an exacerbation in the past year compared with those who have not (Dimitrova *et al.*, 2017). In addition, elevated sputum IL-6 is associated with impaired lung function, giving it potential as a biomarker for severity of asthma (Poynter and Irvin, 2016; Lipworth, Chan and Kuo,

2020). Conversely, a mouse model of allergic asthma demonstrated that IL-6 deficiency resulted in increased airway inflammation, eosinophil recruitment and tissue pathology, as well as disruption of the epithelial tight junctions (Schmit *et al.*, 2020). In addition, it abrogated protection provided by the host immune response to infection with Streptococcus pneumoniae (*ibid*). These results suggest that IL-6 may have a protective role in some cases, but appears to be associated with increased severity of asthma.

IL-8 (CXCL8) is a potent neutrophil chemoattractant produced by airway epithelial cells in response to bacteria, viruses and allergens and is a hallmark of innate immunity (Pease and Sabroe, 2002; Reynolds *et al.*, 2018). In a study of uncontrolled asthmatics, elevated IL-8 serum levels were associated with higher neutrophil counts, and IL-8 levels were higher in patients requiring systemic corticosteroids compared with those on inhaled corticosteroids (Zhang and Bai, 2017). Both IL-8 and neutrophil numbers were significantly higher in tracheal aspirates from patients intubated with severe acute asthma exacerbations compared with healthy controls (Ordoñez *et al.*, 2000). Airway neutrophilia is associated with higher bacterial burden and poor responsiveness to corticosteroids (Crisford *et al.*, 2021). In all, IL-8 signalling is strongly associated with airway neutrophilia, and may be a useful biomarker for identifying patients with poorly controlled, steroid-resistant asthma (Pease and Sabroe, 2002; Zhang and Bai, 2017).

IL-12 is a macrophage-derived cytokine which modulates T-lymphocytes and allergic inflammation (Bryan *et al.*, 2000; Meyts *et al.*, 2006). Some evidence suggests that IL-12 contributes to allergic inflammation in the airways of asthma patients (Meyts *et al.*, 2006). However, another study showed that recombinant IL-12 was able to significantly reduce sputum and peripheral blood eosinophils, with a trend towards lower airway hyper-responsiveness to histamine (Bryan *et al.*, 2000). It did not have an effect of hyperresponsiveness following inhaled allergen challenge (*ibid*). Another study found no significant difference between levels of peripheral blood IL-12 in children during asthma exacerbations compared with baseline (Soferman, Rosenzwig and Fireman, 2007). In addition, elevated IL-12 has been observed in sputum from patients with neutrophilic asthma compared with sputum from patients with non-neutrophilic asthma, where it was significantly positively correlated with neutrophil

percentage in sputum (Yang *et al.*, 2018). In addition to IL-12, a milieu of other cytokines were elevated in patients with neutrophilic asthma, which may together contribute to the disordered bacterial community observed in these patients (*ibid*). These data suggest that IL-12 may have varying roles in controlling airway inflammation during asthma.

TNF, formerly called TNF-α, is a proinflammatory cytokine produced by macrophages and mast cells involved in many aspects of airway pathology in asthma (Brightling, Berry and Amrani, 2008; Nabe, 2013; Grimstad, 2016). Elevated TNF levels have been observed in bronchial biopsies from patients with severe asthma, and it is thought to play a role in severe steroid-resistant asthma (Brightling, Berry and Amrani, 2008; Holgate *et al.*, 2011). There is some evidence that TNF may have a pleiotropic, anti-inflammatory effect by mediating expansion of immunosuppressive Tregs, tolerogenic dendritic cells, and MDSCs (Ahmad *et al.*, 2018). This effect may be through differential activation of TNFR2, which is preferentially expressed on immunosuppressive cells, whilst activation via TNFR1 is thought to be responsible for pro-inflammatory effects (*ibid*). In some patients with steroid-refractory asthma, anti-TNF therapy has shown improvement in asthma-related quality of life, lung function and reduction in airway hyper-responsiveness and frequency of exacerbations (Brightling, Berry and Amrani, 2008). However, this seems to be effective only in a small subset of steroid-resistant asthma patients suggesting there are several mechanisms at work (Brightling, Berry and Amrani, 2008; Holgate *et al.*, 2011).

Neutrophilic asthma is also associated with increased production of myeloperoxidase and elastin, two factors which are key components of neutrophil granules and are necessary for the formation of neutrophil extracellular traps (NETs) (Okubo *et al.*, 2016; Busse, 2019b). However, lactoferrin, which is an inhibitor of NET formation, was not upregulated in neutrophilic asthma, suggesting not only an increase in the number of neutrophils but also greater neutrophilic activation (Okubo *et al.*, 2016; Busse, 2019b). This suggests that airway neutrophils are active participants in the progression of disease.

One specific phenotype of neutrophilic asthma is early preschool wheeze. In a study of 138 preschool-age children with asthma, airway neutrophilia was associated with

recurrent wheezing, whereas, in 104 school-age children, wheezing was associated with elevated eosinophil numbers (Guiddir, 2017). In sixty-seven children with severe neutrophilic asthma refractory to high dose inhaled corticosteroids, neutrophils showed increased markers of degranulation and activation, including elevated release of elastase, lactoferrin and myeloperoxidase protein in BAL fluid, compared to neutrophils from children with moderate, steroid-responsive asthma (Grunwell *et al.*, 2019). In addition, primary neutrophils from healthy donors that were exposed to BAL fluid from these children showed enhanced phagocytic ability, elevated elastase production and increased formation of NETs, which suggested that the cytokine milieu present in the lungs of these children contributes to a pro-inflammatory neutrophil phenotype. Given the majority of asthma exacerbations in childhood are driven by viruses (Xepapadaki and Papadopoulos, 2010; Chowdhury and Chakraborty, 2017; Jartti *et al.*, 2020) which typically induce neutrophilic inflammation (McDougall and Helms, 2006; Deschildre *et al.*, 2017; Ray and Kolls, 2017) it is likely neutrophilic asthma in children with pre-school wheeze is a consequence of recurrent viral infections.

1.1.3.2 Bacterial Infection in Airway Neutrophilia

In adults, neutrophilic asthma is associated with increased bacterial burden in the airways (Yang *et al.*, 2018). A 2018 study by Yang *et al* using 16S rRNA copy number and phylogenetic analysis showed that there was both a higher bacterial burden and greater relative abundance of *Haemophilus* spp and *Moraxella* spp in neutrophilic asthma patients. In addition, Yang found less diversity in bacterial species and lower relative abundance of bacteria from phyla firmicutes, actinobacteria and saccharibacteria. Induced sputum from 28 severe steroid-resistant asthmatics at a stable state showed that 17/28 patients were colonised with *Haemophilus*, *Moraxella catarrhalis* or *Streptococcus* (Green *et al.*, 2014). This colonisation was associated with higher asthma disease duration, higher sputum neutrophil differential cell counts and worse post-bronchodilator percent predicted FEV_1. Higher bacterial burdens were correlated with increased IL-8 levels in sputum and higher neutrophil counts. Taken together, these data suggest that patients with neutrophilic asthma have a disordered bacterial community composition and a distinct microbial constitution, and specifically that a few potentially pathogenic species become dominant within the

microbiome. This may contribute to the inflammatory cascade associated with neutrophilic asthma.

In a recent investigation in a mouse model of chronic obstructive pulmonary disease (COPD), chronic exposure to NTHi induced airway neutrophilia and recruitment of lymphocytes (Ritzmann *et al.,* 2018). In addition, this inflammation activated PD-1 checkpoint signalling, and inhibition of this checkpoint ameliorated recruitment of neutrophils. Children that have had early nasopharyngeal colonization with NTHi were more likely to develop asthma later during childhood, and monocytes from these children showed aberrant inflammatory signalling in response to bacterial antigens (Bisgaard *et al.*, 2007; Mccann *et al.*, 2016). In a study of 321 infants born to mothers with asthma, 21% of neonates were colonised at 1 month with *Haemophilus influenzae*, *Streptococcus pneumoniae, Moraxella catarrhalis*, *Staphylococcus aureus* or a combination (Bisgaard *et al.*, 2007). Colonisation with one or more of these organisms, aside from S. aureus, was significantly associated with increased prevalence of persistent wheeze, severe acute exacerbation of wheeze and hospitalisation for wheezing. In addition, neonatal colonization was associated with elevated blood eosinophils at 4 years of age, and higher prevalence of asthma at 5 years. When this effect was modelled in mice, juvenile mice inoculated with intranasal NTHi at 3 days had exacerbated airway disease in an OVA allergic asthma model (Mccann *et al.*, 2016). In addition, the mice had increased infiltration with both neutrophils and eosinophils, as well as upregulated IL-5 and IL-13 signalling and increased mucus production. The authors suggested that early-life colonization with NTHi is partly responsible for immune system reprogramming that contributes to the development of asthma. However, mouse models of NTHi infection are imperfect, because mice are able to rapidly eradicate the infection from their lungs, unlike the chronic infection seen in humans.

1.1.4 Rhinovirus infection in asthma exacerbations

Rhinovirus infection is one of the most common triggers of asthma exacerbations in both children and adults (Hammond, Kurten and Kennedy, 2015; Holgate *et al.*, 2015). A 13-month longitudinal community based study which included 108 children aged 9-11 used peak expiratory flow measurements and a diary card of respiratory

symptoms to monitor episodes of wheeze and upper respiratory symptoms (Johnston *et al.*, 1995). Viruses were isolated from patients during 80% of episodes of reduced peak flow or wheeze, and from 85% of episodes with upper respiratory tract symptoms, wheeze, cough and decreased peak expiratory flow. These were primarily rhinoviruses, although coronaviruses, respiratory syncytial virus (RSV), influenza and parainfluenza viruses were also found. A longitudinal study of 138 adults found that 80% of episodes of wheeze and chest tightness or shortness of breath occurred in conjunction with cold symptoms, while 89% of colds were associated with asthma symptoms as measured by reduced peak expiratory flow rate (Nicholson, Kent and Ireland, 1993). Epithelial cells are a key target in RHV mediated exacerbations

1.1.5 COPD Overview

Chronic obstructive pulmonary disease (COPD) is a diverse group of disease syndromes which are characterised by persistent limitation in expiratory airflow (Devine, 2016). COPD is defined by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) as airflow limitation which is not fully reversible with pharmacological bronchodilation. While airflow limitation and inflammation are also characteristics of asthma, asthma is distinguished from COPD by bronchial hyperresponsiveness to stimuli and functional deficits in airflow which are reversible. COPD is a poorly reversible condition, and in 2016 was the fourth most common cause of death in the USA, and is a significant cause of morbidity and mortality worldwide (Devine, 2016; Rabe and Watz, 2017). Smoking is a major cause of COPD, so smoking cessation is an important step in stopping disease progression in individuals, and preventing the development of disease in the population as a whole. However, 50% of COPD globally, particularly in developing countries, is not related to tobacco use, but is due to exposure to biomass cooking fumes (Liu *et al.*, 2007; Sana *et al.*, 2018; Mazumder *et al.*, 2019; Pathak, Gupta and Suri, 2020). Rates of COPD are growing more quickly in women, likely due to their increased exposure to biomass fumes during household activities (Sana *et al.*, 2018). A study compared lung function of 200 women who used either biomass cooking fuels or liquid petroleum gas (LPG) (Mazumder *et al.*, 2019). Although participants were often exposed to multiple sources of pollution and had poor overall lung function, use of biomass cooking fuels was associated with lower FEV_1/FVC and increased lung impairment with age compared

to women who used LPG. There are currently no treatments available which stop the progression of disease, but use of inhaled bronchodilators are central to COPD management presently. Short-acting bronchodilators are commonly prescribed for immediate relief in mild COPD, progressing to the use of long-acting bronchodilators as disease becomes more severe. A subset of COPD patients have significant airway eosinophilia which contributes to exacerbations (Bafadhel *et al.*, 2016; Bafadhel, Pavord and Russell, 2017; David *et al.*, 2021). These patients are responsive to inhaled and systemic corticosteroids, and severe acute eosinophilic exacerbation is associated with shorter duration of hospitalisation compared to non-eosinophilic exacerbation (Bafadhel *et al.*, 2016).

NTHi colonisation in COPD patients contributes to both acute exacerbations and promotes disease progression (Short *et al.*, 2021). Although COPD is triggered by inhaled irritants, chronic colonisation with NTHi is thought to contribute to disease progression by causing chronic inflammation and increasing the frequency of exacerbations (Moghaddam *et al.*, 2011; Finney *et al.*, 2014). NTHi is present in many COPD patients even during stable state; a 2004 study has demonstrated that some COPD patients are persistently colonised with NTHi for months at a time despite periods of negative sputum culture (Murphy *et al.*, 2004).

Infection with NTHi is a common trigger in COPD exacerbations (Short *et al.*, 2021). Acute exacerbations cause lasting respiratory, physical, social and emotional damage even after the acute stage has resolved (Anzueto, 2010). In addition, frequent exacerbations accelerate disease progression and are associated with higher mortality (*ibid*).

1.1.6 Bronchiectasis Overview

Bronchiectasis is a chronic condition characterised by persistent abnormal widening of the airways and incomplete clearance of mucus (Chalmers *et al.*, 2018). This build-up of mucus leads to greater susceptibility to infections, which are often recurrent. It is often caused by lung infections, such as pneumonia or whooping cough, or a wide range of defects of immune regulation or mucociliary clearance, such as primary ciliary dyskinesia (Rubbo and Lucas, 2017). Infants and young children can have

congenital bronchiectasis, due to foetal malformation of the airways (Chalmers *et al.*, 2018). As the precise aetiology in many cases is unknown, treatments aim to reduce exacerbations and disease progression. In some instances, specific therapies are now available such as intravenous immunoglobulin replacement for immunoglobulin deficiencies, and cystic fibrosis transmembrane conductance regulator modulators in many forms of cystic fibrosis. However, in general no specific treatments are available, and antibiotics and mucolytic agents are currently employed. It is an increasingly prevalent condition that can occur in any age group.

1.1.7 Pharmacotherapy of asthma

1.1.7.1 Bronchodilators

Bronchodilators are used for patients with suboptimal airflow in the lungs, targeting the smooth muscle in bronchioles resulting in dilatation (Almadhoun and Sharma, 2021). They are used in both asthma and COPD patients (*ibid*). The currently available bronchodilators are short acting β agonists used for immediate asthma relief, long-acting β agonist used for long-term control in conjunction with ICS, and long-acting antimuscarinic agents (LAMA) which can be used interchangeably with LABAs in some patients (Cazzola, Segreti and Matera, 2010; Almadhoun and Sharma, 2021).

β agonists act on the β_2 adrenoceptors on airway smooth muscle cells, resulting in activation of the receptor and subsequent smooth muscle relaxation and dilatation of the airways (Billington, Penn and Hall, 2017). However, consistent use of a β_2 agonist results in gradual downregulation of the β_2 adrenoceptors in the airways, leading to the need for increasing dosages of β agonists to achieve the same effect (Almadhoun and Sharma, 2021). Short acting β agonists (SABA) are used for rapid relief for acute asthma symptoms, typically taking effect within a few minutes and lasting 4-6 hours (Sockrider and George, 2019). SABAs are presently the standard treatment recommended by GINA and BTS guidelines for an acute asthma episode, administered on an as needed basis by the patient (BTS, 2019b; Sockrider and George, 2019; Almadhoun and Sharma, 2021). Long acting β agonists (LABA) are used only in conjunction with ICS due to evidence that they may increase risk of mortality alone, but are an important tool in the treatment of moderate-severe asthma in combination with ICS (Beasley *et al.*, 2018). Ultra-long acting β agonists have now been developed

in the hopes of simplifying the treatment regimen with once-daily dosing (Cazzola, Segreti and Matera, 2010). Adverse effects from β agonists are due to activation of the sympathetic nervous system, most commonly tremor, nervousness, heart palpitations and muscle cramps, but more severe side effects including sudden constriction of the bronchial airways, paradoxical bronchospasm, hypokalaemia and, rarely, myocardial infarction (Almadhoun and Sharma, 2021).

Long acting muscarinic antagonists (LAMA) have long been used in the management of COPD, but have more recently been shown to be effective add-on therapies in asthma in both adults and children (Hamelmann, 2018; Papi *et al.*, 2021). Acetylcholine receptor signalling increases bronchoconstriction, mucus production and airway remodelling, as well as increasing airway hyper-responsiveness (Papi *et al.*, 2021). LAMAs are thought to ameliorate this effect by blocking acetylcholine signalling, thus reducing smooth muscle contractility associated with increased cholinergic tone (*ibid*). Long acting muscarinic antagonists were considered less effective than β_2 adrenoceptor agonists, but recent trials have shown LAMAs to be a useful add-on therapy for patients with poorly controlled asthma on ICS or LABA-ICS treatments (Papi *et al.*, 2021). Tiotropium is the first LAMA approved as an add-on treatment in adults and children over 6 (Hamelmann, 2018; Papi *et al.*, 2021). GINA guidelines now recommend addition of LAMA therapy for patients with severe asthma regardless of phenotype, particularly as a step-up option prior to the use of systemic corticosteroids (*ibid*).

1.1.7.2 Inhaled corticosteroids

Inhaled corticosteroids (ICS) are a mainstay of asthma treatment, and are effective at treating asthma symptoms for a majority of patients with mild-moderate asthma and reduce the risk of exacerbations (Keeley, 2019). ICS treatment revolutionised the treatment of asthma and has for decades been widely used (Barnes, 1998). Corticosteroids are effective at supressing eosinophilic inflammation, and reduce airway inflammation in several ways, inhibiting recruitment of eosinophils, macrophages, T-lymphocytes and dendritic cells (*ibid*). In addition, corticosteroids reduce expression of pro-inflammatory cytokines associated with airway inflammation in asthma, notably IL-4 and IL-5, as well as eosinophil chemoattractants (*ibid*). Use of

ICS reduces the need for LABA use, and is recommended as the first step in asthma treatment by GINA and BTS guidelines (BTS, 2019a; Keeley, 2019). Whilst low-dose ICS poses minimal risk, higher doses present more side-effects, so once asthma control has been achieved with ICS, dosage should be stepped down if possible (NICE, 2018; Keeley, 2019). Due to the adverse effects of high-dose ICS, addition of a leukotriene inhibitor or long-acting bronchodilator may be preferable to increasing ICS dose (Barnes, 1998; NICE, 2018). However, there is some diversity of opinion on this point, with some suggesting LABA use is indicative of poor control of underlying conditions (Keeley, 2019).

However, a significant number of patients have symptoms that are refractory to steroid treatment, often patients with severe asthma (Ramsahai and Wark, 2018; Kalchiem-Dekel, Yao and Levine, 2020). Some of these patients exhibit persistent type-2 inflammation despite high-dose steroids, but a significant proportion of these patients have neutrophilic inflammation, which is not responsive to corticosteroid treatment (Kalchiem-Dekel, Yao and Levine, 2020).

1.1.7.3 Combination fixed dose LABA-ICS

A combination treatment of ICS and long-acting β agonists (LABA) is a mainstay of asthma treatment for moderate and severe asthma, and is recommended in GINA guidelines for steps 3, 4 and 5 (Beasley *et al.*, 2018). β agonists act on the β_2 adrenoceptors on airway smooth muscle cells, resulting in smooth muscle relaxation and dilatation of the airways (Billington, Penn and Hall, 2017). LABA are only suitable as a combination therapy with ICS in a single inhaler, as LABA monotherapy is associated with increased risk of mortality whilst its use in combination with ICS does not appear to present this risk (*ibid*). The most common regimen involves LABA-ICS as maintenance, often fluticasone propionate and salmeterol, with a short-acting β agonist rescue inhaler (Beasley *et al.*, 2018; Tashkin *et al.*, 2021). Alternatively, LABA-ICS can be used as both maintenance and reliever therapy (the MART regimen), which may be more effective than the standard LABA-ICS with as needed short-acting β agonist (Beasley *et al.*, 2018). A 2021 Cochrane review has found that there is now good evidence that fixed-dose LABA-ICS used as-required is the most effective form of treatment for mild asthma (Crossingham *et al.*, 2021).

1.1.7.4 Systemic corticosteroids

Systemic treatment with corticosteroids is an effective treatment for severe type-2 high/eosinophilic asthma, targeting several aspects of the type-2 inflammatory pathway and rapidly reducing airway eosinophilia, as well as more slowly reducing airway hyper-responsiveness (Ramsahai and Wark, 2018; Gaga and Zervas, 2019). Oral corticosteroids are the default treatment for acute asthma exacerbations and are important tools for long-term treatment of severe asthma (Ramsahai and Wark, 2018). However, systemic corticosteroids, particularly chronic use, is associated with a long list of side effects, including osteoporosis, weight gain, mood disturbance, gastro-oesophageal reflux, hypertension, hyperglycaemia, skin atrophy and glaucoma (Gaga and Zervas, 2019). In addition to the side effects, a portion of patients with severe type-2 high asthma are insensitive or resistant to corticosteroids, as well as well-known steroid insensitivity in type-2 low asthmatic patients (Busse, 2019a; Kalchiem-Dekel, Yao and Levine, 2020). Thus, although systemic corticosteroids are important tools in asthma management, they are not a panacea.

1.1.7.5 Other non-biological treatments

Leukotriene receptor agonists (LTRA) work by reducing immune cell recruitment to lung tissue (Paggiaro and Bacci, 2011). The first available leukotriene receptor antagonist was zafirlukast, which was discontinued by AstraZeneca in March 2018 (McCarthy, 1997). Montelukast is the safest and most effective of the LTRAs, and is commonly used as a monotherapy and in conjunction with ICS (Paggiaro and Bacci, 2011). Early *in vivo* studies demonstrated that montelukast can reduce activation and recruitment of airway eosinophils, and *in vitro* data has shown that at high doses montelukast decreases cytokine release (*ibid*). In addition, *in vivo* studies of asthmatic patients have shown that montelukast is capable of reducing sputum eosinophilia, exhaled nitric oxide concentrations and bronchial hyperresponsiveness (*ibid*). Montelukast is recommended by GINA as the go-to alternative to low-dose ICS monotherapy, as well as in conjunction with ICS or with an ICS plus LABA combination treatment, and is particularly useful in younger patients with mild exercise-induced asthma or asthma with allergic rhinitis (*ibid*).

Aminophylline is a theophylline class of bronchodilator which is used for acute asthma exacerbations, working by relaxing the smooth muscle of the bronchial airways and pulmonary blood vessels and reducing responsiveness to histamine, methacholine and adenosine (Aralihond *et al.*, 2020). It is also licensed for use in reversible airflow restriction caused by chronic bronchitis and emphysema, as well as to prevent apnoea in preterm infants (Zafar and Zulfigar, 2021). However, there are varying clinical opinions on the efficacy and safety of aminophylline; BTS guidelines support its use while GINA does not, citing poor efficacy and potentially fatal side-effects (Aralihond *et al.*, 2020).

1.1.7.6 Biologic Therapies

A significant proportion of patients with severe asthma exhibit steroid resistance (Pavord *et al.*, 2018). In addition, high dose systemic steroids have significant side-effects. Recently, therapies have been directed at modulating specific aspects of inflammatory signalling associated with asthma. However, so far these therapies have been focussed on treating allergic and eosinophilic asthma. Type-2 low asthma with neutrophilic inflammation is often severe and responds poorly to corticosteroids, representing a significant unmet clinical need (Kalchiem-Dekel, Yao and Levine, 2020).

Omalizumab is a monoclonal antibody therapy for severe allergic asthma targeting IgE in the bloodstream (NICE, 2013; Henriksen *et al.*, 2020). A recent meta-analysis found that omalizumab reduced the rate of exacerbations in children and adults, and allowed oral corticosteroid doses to be reduced in adult patients whilst maintaining asthma control (Henriksen *et al.*, 2020).

Benralizumab, Reslizumab, and Mepolizumab are monoclonal antibody treatments for severe eosinophilic asthma. They target the type-2 cytokine IL-5, and have been shown to improve lung function and quality of life, as well as reducing airway hyperresponsiveness. Benralizumab is a humanised IgG1 monoclonal antibody which targets the IL-5 receptor on eosinophils and basophils, reducing eosinophilia by promoting apoptosis and preventing maturation of these cells (‘Benralizumab for Asthma’, 2018; The National Institute for Health and Care Excellence (NICE), 2019; Padilla-Galo *et al.*, 2020). A cross-sectional multicentre study of patients with severe

eosinophilic asthma demonstrated that treatment with benralizumab improved asthma control and lung function, and reduced the number of hospitalisations and steroid cycles per patient (Padilla-Galo *et al.*, 2020). Likewise, mepolizumab also selectively targets IL-5, and clinical trials have demonstrated a significant reduction in the number of exacerbations, systemic corticosteroid use, and blood eosinophil counts (Pavord *et al.*, 2012; Taillé *et al.*, 2020; The National Institute for Health and Care Excellence (NICE), 2021). Anti-IL-5 monoclonal antibody therapy reslizumab was also effective in reducing the number of exacerbations and improving lung function in patients with severe treatment-refractory asthma, although trials of low-dose reslizumab yielded poor results (Bernstein *et al.*, 2020; Christian Virchow *et al.*, 2020).

Anti-TNF therapies show promise in treating a subset of patients with severe refractory asthma, improving asthma-related quality of life, lung function and a reduction in airway hyper-responsiveness and the number of exacerbations patients experienced (Brightling, Berry and Amrani, 2008). However, a 12 week randomised, double-blind, placebo-controlled phase 2 clinical trial of anti-TNF therapy etanercept found no difference in any endpoint measure compared to placebo, including measures of forced expiratory volume in 1 second (FEV_1)% predicted, asthma control, quality of life and number of exacerbations (Holgate *et al.*, 2011). These results suggest that anti-TNF therapy may only be suitable for a limited subset of severe asthma patients.

Tezepelumab is a potential first in class therapy which targets the epithelial-derived alarmin thymic stromal lymphopoietin (TSLP) (Menzies-Gow *et al.*, 2021). It is presently in phase III clinical trials, demonstrating efficacy in improving severe asthma regardless of eosinophil count at the beginning of treatment (*ibid*). 1061 patients with severe uncontrolled asthma aged 12-80 years were randomly assigned to receive tezepumab or placebo. Tezepelumab reduced the annualised rate of asthma exacerbations in all patients to 0.93 (95% CI, 0.8 – 1.07) compared to 2.10 (95% CI, 1.84 – 2.39) in the control. In patients with initial blood eosinophil counts of <300 cells/μl, the annualised exacerbation rate with Tezepelumab was 1.02 (95% CI, 0.84 – 1.23) compared with 1.73 (95% CI, 1.46 – 2.05) with placebo, representing the first biologic treatment effective in patients without high eosinophil counts, although the effect was more modest than in patients with eosinophilia. Treatment with

Tezepelumab also improved lung function as measured by pre-bronchodilator FEV_1, as well as quality of life scores and asthma control.

These therapies show great promise for patients with severe type-2 high asthma that is uncontrolled despite high-dose corticosteroids. However, the currently available biologics focus on type-2 cytokines, making them suitable only for patients with type-2 high asthma. Tezepelumab is a notable exception to this, providing a useful addition to the armoury of treatments available to treat type-2 low asthma. However, type-2 low neutrophilic asthma is often associated with NTHi infection, with evidence pointing to NTHi as a treatable trait in the development of neutrophilic disease. Neutrophilic asthma is also often severe and notably non-responsive to steroid treatment. There is therefore an unmet need for effective treatments for neutrophilic asthma which address the trigger of airway neutrophilia at the source, by eliminating airway infections.

Figure 4. BTS Sign 158 management of asthma guidelines

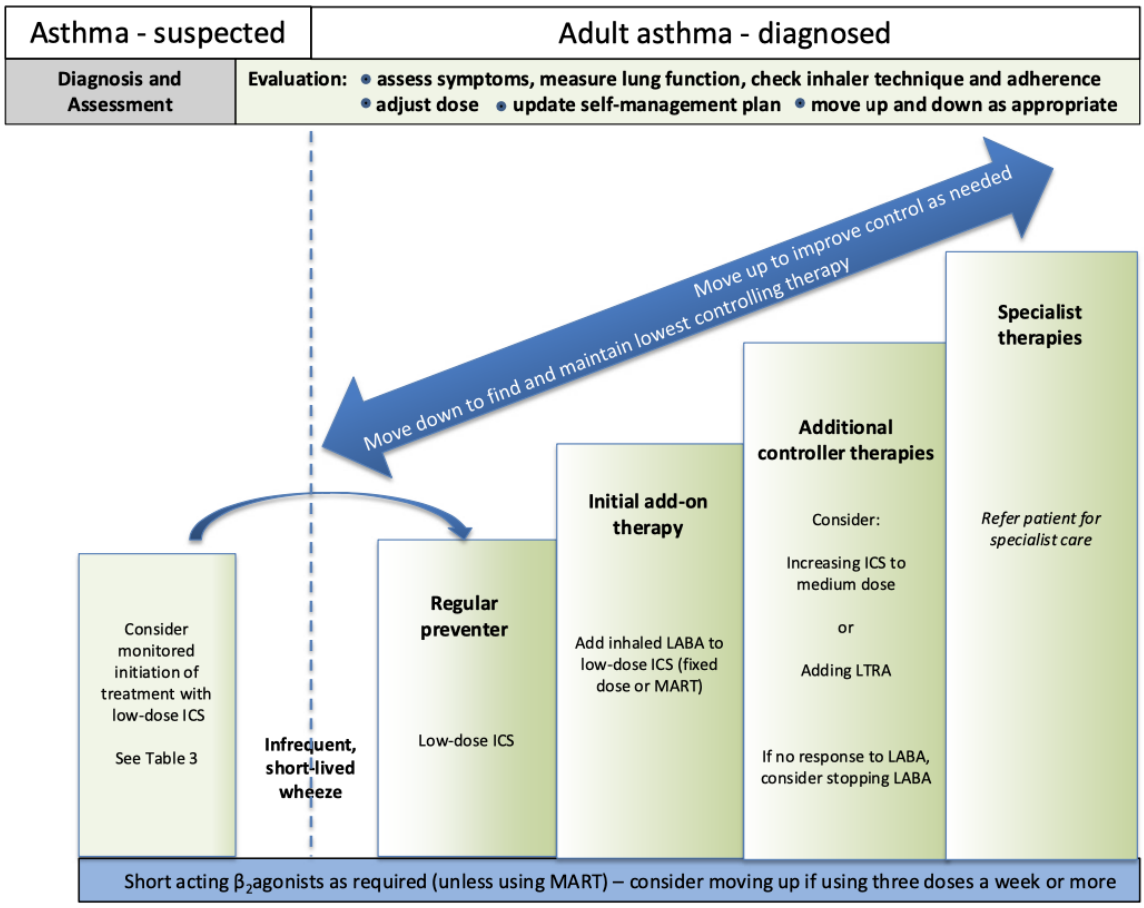


Figure 4. Diagram depicting the BTS guidelines for asthma management. The guidelines recommend the use of low-dose ICS as a first step in the control of mild disease, followed by add-on LABA if needed to improve asthma control. The next step is the addition of other controller therapies, such as increasing ICS dose or adding a LTRA. Patients who are not responsive to this level of treatment should then be referred to specialist therapies, such as mepolizumab or Tezepelumab. Across all steps, short-acting β agonists may be used as-needed as a reliever inhaler, unless the MART regimen is being followed, which uses LABA-ICS as both controller and reliever. Figure reproduced from the BTS Sign 158 British guideline on the management of asthma (BTS, 2019b).

1.2 Experimental models of asthma

1.2.1 Animal models of asthma

Disease-relevant animal models are lacking in asthma, as it is a uniquely human disease (Holmes, Solari and Holgate, 2011). Mice are the most commonly used model,

as they are well-characterised, relatively easily managed and a wide variety of transgenic knockout models are available (Nials and Uddin, 2008; Aun *et al.*, 2017; Adner *et al.*, 2020). Mice do not spontaneously develop asthma, so most mouse models of asthma rely on acute exposure to allergens. However, in humans, asthma is a chronic disease developing through repeated, intermittent exposure to allergens or pollutants, so chronic exposure models have also been developed (Nials and Uddin, 2008). A study compared airway inflammation, IgE levels and mucus production in different routes of OVA challenge in an OVA-induced asthma model (Kim, Song and Lee, 2019). Both routes exhibited a typical asthma phenotype, including airway inflammation, elevated IgE levels and airway hyper-responsiveness. However, the inhaled route showed elevated inflammation of pulmonary vessels, alveolar ducts and alveoli compared with the intranasal route. In addition, both Th2 and epithelial inflammatory cytokines, especially IL-25, were more strongly induced by the inhaled challenge route. Models that recapitulate specific disease phenotypes are particularly important in developing treatments for severe steroid-resistant asthma, the treatment of which is an unmet clinical need (Gubernatorova *et al.*, 2019). A study compared models mimicking eosinophilic and neutrophilic asthma (Yu and Chen, 2018). Eosinophilic asthma was induced by intraperitoneal sensitisation with OVA on days 0 and 7, followed by inhaled OVA challenge on days 14-17. Neutrophilic asthma was modelled by adding high-dose LPS intratracheal instillation on days 15 and 17 to the eosinophilic OVA challenge model. Similarly, mixed-granulocytic asthma was induced using the OVA challenge model with low-dose LPS on day 15. Compared with healthy controls, the number of eosinophils and BALF Th2 cytokines were elevated in the eosinophilic asthma model. In the neutrophilic model, elevated levels of neutrophils and Th1 and Th17 cytokines in BALF were seen. The mixed-granulocytic model produced enhanced numbers of both neutrophils and eosinophils, as well as Th1, Th2 and Th17 cytokines in BALF. This study has demonstrated the ability to recapitulate these distinct clinical phenotypes in mice (Yu and Chen, 2018). A study on the effects of steroid and macrolide treatment in a mouse model of neutrophilic asthma sensitised with OVA and LPS showed that clarithromycin with or without dexamethasone reduced airway inflammation, BALF neutrophils, IL-17, and TNF, suggesting that macrolides may be useful as a steroid-sparing treatment in

neutrophilic asthma (An *et al.*, 2018). Another study has demonstrated steroid-resistance in mice with acutely induced neutrophilic asthma, recapitulating a common characteristic of neutrophilic asthma in humans (Ito *et al.*, 2008). These data show promise in effectively using mice to mimic human disease. However, there are also limitations of mouse models, particularly differences in the innate and adaptive immune system which may lead to poor translation of treatments from mice to humans (Mestas and Hughes, 2004). For instance, ILC2 cells are important in mice, but are much less abundant in humans and their importance remains unclear (Mazzurana *et al.*, 2018; Cavagnero and Doherty, 2020) In addition, mice lack direct homologues of many human cytokines and chemokines and their receptors, exhibit differences in Th1/Th2 cell differentiation and have altered TLR and defensin function compared to humans (Mestas and Hughes, 2004; Ito *et al.*, 2018). Humanisation of the mouse genome by strategically replacing parts of the mouse genome with orthologous human genes is becoming increasingly possible and may help to develop a mouse model that more readily translates to humans in the future (Ito *et al.*, 2018; Zhu *et al.*, 2019).

Although mice are the most common model of asthma, guinea pigs have more similar airway physiology and anatomy to that of humans, and more consistently predict therapeutic targets that are effective in humans (Ricciardolo *et al.*, 2008; Adner *et al.*, 2020). Like mice, OVA sensitization is a common pulmonary inflammation model in guinea pigs, via both acute and chronic exposures (Evans *et al.*, 2012). Use of guinea pig asthma models has been stymied by a lack of specific molecular tools and reagents, but going forward it may be a promising tool in providing more reliable translational data than mice.

Equine asthma, often termed ‘heaves’, presents some significant similarities to human disease, featuring reversible airflow obstruction, airway remodelling, and airway neutrophilia (Bullone and Lavoie, 2015). However, in one study of equine asthma, BAL fluid neutrophilia of >20% was associated with lower airway mucostasis and milder peripheral pulmonary lesions during exacerbation (Bullone *et al.*, 2018). However, this study suggested that BAL fluid neutrophils of >5% in otherwise healthy horses is associated with bronchiolitis. This suggests the relationship between airway neutrophilia and remodelling remains poorly understood in equine asthma. Another

more recent study used proteomic analysis of horses during exacerbations versus healthy control herd-mates, and found a correlation between differential expression of proteins that promote neutrophilic inflammation in asthmatic horses (increased neutrophilic infiltration, chemotaxis, cell spreading and transmigration), compared to high representation of proteins that reduce neutrophil lifespan in healthy control horses (Dittmar, Burgess and Swiderski, 2019). Thus, equine asthma is an interesting example of a naturally-occurring animal model, which may be of interest for translational research once it is more thoroughly understood.

Other novel animal models have been developed using cats, dogs, pigs, calves, sheep and non-human primates (Kirschvink and Reinhold, 2008). Large-animal models offer potential for lung function studies using techniques similar to those used in humans, although they are expensive and lacking in specific immunologic and molecular tools (Kirschvink and Reinhold, 2008). Feline models are of particular interest, as cats commonly develop spontaneous airways disease with many of the hallmarks of asthma (Norris Reinero *et al.*, 2004; Cohn *et al.*, 2010). Airway allergen sensitization with house dust mite (HDM) and Bermuda grass (BMA) allergens led to allergen-specific IgE, IgG and IgA production, airway hyper-reactivity, eosinophilia and airway remodelling (Norris Reinero *et al.*, 2004). However, there are few current studies using a model of feline asthma, and more investigation is necessary to develop it as a practical model.

Overall, although many animal models are available, none are able to recapitulate all the features of asthma. In addition, although mice are widely used, there is a low rate of success in translating therapeutics tested in mice into humans, usually due to safety or lack of efficacy in humans (Holmes, Solari and Holgate, 2011).

1.2.2 Animal models of NTHi infection

Mice do not naturally harbour NTHi infection, which is usually rapidly cleared from both inner ear and respiratory tract. Models of transient NTHi infection recapitulate only the infectious process, rather than the inflammatory response and remodelling associated with chronic infection (Saliu *et al.*, 2021). Mutant Junbo mice, which spontaneously develop inner ear inflammation in a pathogen-free environment, have

been used to develop a model of acute otitis media, with NTHi persisting in most ears until 21-35 days post infection (Hood *et al.*, 2016). However, Junbo mice are not permissive to chronic NTHi infection in the lung. An alternative approach might use intact or immunocompromised A/J mice pre-treated with hydrocortisone and cyclophosphamide inoculated intranasally with NTHi, which harbour substantial bacterial burdens in BALF and lung 24 hours post infection, with 50-fold higher CFU counts in immunocompromised mice compared with healthy (Kimura *et al.*, 2020).

Work by Phil Hansbro has used an OVA challenge model of allergic asthma inoculated with NTHi either concomitantly with OVA or 10 days after, which maintained significant NTHi infection up to 10 days post-infection (Essilfie *et al.*, 2011). Results from this model have suggested that inflammatory signalling from NTHi infection and allergic asthma synergistically induced neutrophil infiltration of the airways, as well as reducing airway eosinophilia, in an IL-17 dependent manner (*ibid*).

Despite the limitations of mouse models of NTHi, they have been widely used. A mouse model of COPD suggested that cigarette smoke exposure increases immune response to NTHi challenge, as well as increasing titres of antibodies which promote bacterial clearance (Gaschler *et al.*, 2010). Another mouse model of COPD/emphysema has also demonstrated reduced ICAM-1 expression and impaired bacterial clearance from the lung when challenged with NTHi (Pang *et al.*, 2008). Early-life colonisation of an OVA-induced allergy model of asthma with NTHi demonstrated that bacterial colonisation increased severity of airways disease (Mccann *et al.*, 2016). However, there are limitations to mouse models of NTHi, due to the short duration of NTHi colonisation achievable in the lung of mice.

A new model of NTHi infection in the lung has recently been developed by inoculating C57BL6/NCrl mice via intratracheal injection of agar beads containing NTHi (Saliu *et al.*, 2021). 80% of mice had detectable bacterial loads at 14 days post infection. Comparison of mice at 2- and 14-days post infection showed that the immune response at the early stage of infection was characterised primarily by macrophage and neutrophil recruitment, while at 14 days post infection an adaptive immune response had developed. In addition, keratinocyte derived chemokine (KC or CXCL1) and macrophage inflammatory protein (MIP), functional homologues of human IL-8, as

well as IL-6, IL-17A, IL-17F and G-CSF were elevated in response to NTHi infection compared to sham-infected controls (Saliu *et al.*, 2021). This model shows promise in developing a model of chronic NTHi infection in the lung.

In a recently-described ferret model of COPD NTHi infection in animals exposed to cigarette smoke induced lung inflammation and neutrophilia (Hunt *et al.*, 2020). Furthermore NTHi colonised the lungs of animals exposed to cigarette smoke more readily than controls, and NTHi was able to persist within the lungs by forming biofilms (Hunt *et al.*, 2020).

Chinchillas are a preferred model of otitis media (OM) infection, capable of modelling nasopharyngeal infection and ascent to the eustachian tubes to establish OM as seen in humans (Yang *et al.*, 1998; Mason, Munson and Bakaletz, 2003; Davidoss, Varsak and Santa Maria, 2018). However, despite this success using chinchillas as a model for OM, a model of NTHi infection in the lung has not been established, and reagents and facilities for this species are limited.

Overall, although much has been learned from animal models, the difficulties in translation from animals to humans limits the usefulness of animal models for asthma research, which is a uniquely human disease. Therefore, I have chosen to use *in vitro* culture techniques in my thesis work.

1.2.3 *In vitro* models of epithelium

In vitro epithelial cell cultures are indispensable in investigating epithelial diseases, allowing researchers to precisely evaluate questions involving cell signalling, bacteria-host interactions, as well as physical characteristics such as epithelial tight junctions (Hiemstra *et al.*, 2018; Aydin *et al.*, 2021). *In vitro* models lack the complexity of an *in vivo* model, allowing researchers to isolate one element of the system, but also limiting information from interactions between cell types. Traditional submerged cultures are powerful tools, but they do not completely recapitulate the architecture of lung epithelium, with limited polarisation and undifferentiated cells. Air liquid interface (ALI) culture of primary cells provides a useful model for studying asthma at the level of the epithelium (Stewart *et al.*, 2012a; Ehlers *et al.*, 2017).

Figure 5. Diagram of air-liquid interface model

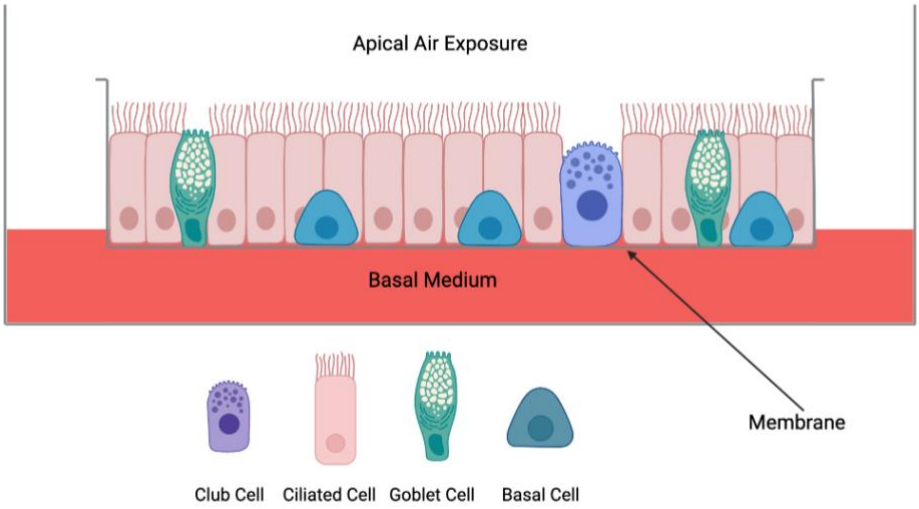


Figure 5. Diagram showing differentiated epithelium cultured at air-liquid interface including ciliated, basal, and secretory epithelial cell types. The air-liquid interface involves culturing epithelial cells on transwells, exposed to media on the basolateral side and air on the apical side, which encourages polarisation of cells, the development of tight junctions and high transepithelial electrical resistance, and differentiation into distinct cell types. Created with BioRender.com

ALI culture is able to model differentiated epithelium, containing differentiated ciliated, basal and goblet cells, unlike submerged culture. The main drawback of submerged culture is the loss of polarisation and ciliation. Primary bronchial epithelial cells cultured at ALI express differentiation markers, form stable, high transepithelial electrical resistance (TEER) readings, and express tight junction proteins (Stewart *et al.*, 2012a). In addition, culture at ALI results in altered protein expression that more accurately recapitulates that found in native epithelium (Wang *et al.*, 2018). ALI Cultures have been used successfully for a wide range of asthma, drug transport, and air pollution toxicity studies (Stewart *et al.*, 2012a; Upadhyay and Palmberg, 2018; He *et al.*, 2021). It is possible to co-culture epithelial cells with many types of immune cells with or without direct contact (Yonker *et al.*, 2017; He *et al.*, 2021). More recently, 3D culture and organ-on-a-chip systems have been developed which allow differentiation and interaction with co-cultured immune cells, while also introducing

mechanical stimuli to mimic lung movement (Chandiramohan *et al.*, 2021; de Dios-Figueroa *et al.*, 2021).

In vitro epithelial cultures can be either use immortalised cells, which are transformed either because they are derived from tumour tissues or because they have been transformed *in vitro* using a viral vector (eg SV40), or primary cells from normal tissues can be cultured. Cell lines are readily available, generally fast-growing and are uniform, giving consistency across experiments. However, they do have some drawbacks, with some varying in key signalling pathways and others differing in growth habit. For instance, A549 cells have abnormal TLR signalling, whilst BEAS-2B cells fail to form tight junctions at ALI (Stewart *et al.*, 2012b). It is therefore important to select an appropriate cell line for the work being performed. However, cell lines have been used to great effect in innumerable key experiments. Important cell lines in airways disease studies include BEAS-2B, NHBE, A549, and Calu-3.

Taken together, cell culture models are an important tool for asthma research, which are readily available, versatile, and are able to accurately recapitulate native epithelium using human cells. I have therefore chosen to focus on cell lines for my work, using a combination of cell lines for preliminary work with validation performed in primary cells at ALI whenever possible.

1.3 Macrolides as a Treatment for Asthma

It has been repeatedly demonstrated that long-term treatment with azithromycin reduces the number of exacerbations patients with severe non-eosinophilic asthma (Seemungal *et al.*, 2008; Brusselle *et al.*, 2013; Gibson *et al.*, 2017). The mechanism is unclear; it may simply work as an antibiotic, but 14- and 15-membered macrolide rings also have well-documented anti-inflammatory properties which may be at play (Wales and Woodhead, 1999; Brusselle *et al.*, 2013). Long-term azithromycin treatment does not appear to lower numbers of many common airway bacteria, but there is emerging evidence that the therapy is most effective in patients with a high initial NTHi burden (Taylor *et al.*, 2019, 2020). This suggests that inflammation caused by NTHi may be an important factor driving inflammation in severe non-

eosinophilic asthma, and eliminating this chronic infection presently is a likely mechanism by which azithromycin reduces exacerbations.

1.3.1 Azithromycin

Azithromycin is a second-generation macrolide antibiotic commonly used to treat a wide range of skin, urogenital and respiratory infections, including airways infections in patients with COPD (Albert *et al.*, 2012) and asthma (Brusselle *et al.*, 2013; Gibson *et al.*, 2017). It is a broad-spectrum, bacteriostatic antibiotic effective against intracellular bacteria. Conversely, gentamicin, the aminoglycoside used in the classical gentamicin exclusion assay, is effective only against extracellular bacteria, making it a useful tool to eliminate extracellular bacteria while leaving intracellular bacteria intact (Sharma and Puhar, 2019). Azithromycin is a 15-membered lactone ring azalide, developed in the 1980's as a semi-synthetic derivative of erythromycin (Parnham *et al.*, 2014). Like other macrolides, azithromycin interferes with bacterial protein synthesis by binding to the large 50S ribosomal subunit, where it disrupts the 50S subunit assembly and the growing polypeptide chain (Parnham *et al.*, 2014). A study evaluating the pharmacokinetics of long-term azithromycin treatment of cystic fibrosis (CF) patients with 500 mg per day showed that azithromycin accumulated in polymorphonuclear neutrophils (PMNs) at a concentration up to 2100 times the concentration found in blood plasma (Wilms, Touw and Heijerman, 2006). This promotes concentration of azithromycin within tissues at sites of infection. Sputum concentrations of azithromycin were higher than that in blood or plasma, and were still detectable up to 10 days after the last dose. In addition, an *in vitro* study of *Pseudomonas aeruginosa* infection demonstrated that azithromycin inhibited quorum sensing-regulated virulence factors and production of o-acetylated alginate, a key factor in biofilm formation (Hoffmann *et al.*, 2007). Biofilms allow bacteria to evade antibiotics and the innate immune system, thus inhibition of biofilm formation by azithromycin may aid in eradicating infections.

1.3.1.1 Antibiotic Role in Asthma

There is evidence from clinical trials that long-term macrolide treatment reduces the risk of exacerbations in patients with severe, refractory asthma, both those with eosinophilic and non-eosinophilic inflammation.

The AZISAST study, a double-blind, placebo-controlled study of 109 patients with severe, exacerbation prone asthma, examined the efficacy of azithromycin treatment as an add-on therapy in addition to combination ICS-LABA treatment for 6 months (Brusselle *et al.*, 2013). Overall, there was not a significant difference in the number of exacerbations or lower respiratory tract infections requiring antibiotics in the azithromycin versus placebo groups, with rates of primary endpoint incidents of 0.75 vs 0.81. However, in patients with non-eosinophilic inflammation, analysis showed that azithromycin treatment resulted in significantly lower numbers of exacerbations and lower respiratory tract infections (0.44 vs 1.03). In addition, azithromycin treatment significantly improved scores on the asthma quality of life questionnaire, but did not affect the asthma control score. These data suggested that azithromycin treatment may be beneficial for patients with non-eosinophilic asthma.

The AMAZES study, a multi-centre, double-blind placebo controlled long-term clinical trial, was performed to evaluate the efficacy and safety of long-term treatment with azithromycin in patients with persistent asthma despite treatment with medium to high-dose inhaled corticosteroids plus long-acting bronchodilators (Gibson *et al.*, 2017). 420 adults with frequently exacerbating asthma were randomized to 500 mg thrice weekly oral azithromycin or placebo for 48 weeks. The AMAZES trial showed that azithromycin reduced the number of exacerbations experienced by patients with severe asthma, reducing the number of exacerbations per patient year to 1.07 from 1.86 in the placebo group (Gibson *et al.*, 2017). 61% of patients in the placebo group experienced at least one exacerbation during the study, reduced to 44% in the group which received azithromycin, whilst asthma related quality of life was significantly improved by azithromycin treatment (adjusted mean difference 0.36). Patients with severe asthma were identified from AMAZES, using definitions of severe asthma as: Global Initiative for Asthma (GINA) step 4 with poor asthma control, the International Severe Asthma Registry definition, and the American Thoracic Society and European Respiratory Society task force definition (Gibson *et al.*, 2017, 2019). Analysis of patients meeting the ATS/ERS definition of severe asthma showed a significant reduction in exacerbation frequency, declining from 2.01 exacerbations per patient year in the placebo group to 1.2 per patient year with azithromycin treatment. Patients with asthma defined as severe by the GINA step 4 and International Severe Asthma

Registry (ISAR) definitions saw similar benefits of azithromycin treatment. In addition, asthma related quality of life improved in patients with severe asthma treated with azithromycin, with mean scored improving from 5.45 to 5.61. Similar benefits are seen in both patients with eosinophilic and non-eosinophilic asthma. In patients with non-eosinophilic severe asthma defined by ISAR and GINA step 4, exacerbations were significantly reduced. Exacerbations in GINA step 4 severe non-eosinophilic severe asthma patients were reduced from 1.90 per patient year to 1.19 per patient year, while for ISAR, exacerbation frequency declined from 1.89 to 1.21. There was not a significant reduction in exacerbations in non-eosinophilic severe asthmatics defined by ATS/ERS, likely due to small sample size, but there was a similar trend. Likewise, exacerbations in patients with severe eosinophilic asthma defined by GINA step 4 declined from 2.04 to 1.13 exacerbations per patient year in the azithromycin group, reduced from 2.03 to 1.10 in severe asthma defined by IFAR, and from 2.49 to 1.36 based on the ATS/ERS definition. 16S RNA sequencing and qPCR quantification of sputum bacteria was performed pre- and post-treatment for 61 patients, revealing that overall bacterial burden was not significantly changed by azithromycin treatment (Taylor *et al.*, 2019). Bacterial diversity was decreased, and 5 macrolide and 2 tetracycline resistance genes were increased, of 89 genes that were examined. *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Moraxella catarrhalis* levels were not significantly affected by azithromycin treatment. However, azithromycin treatment did significantly reduce sputum NTHi numbers ($P<0.0001$). This suggested that azithromycin may reduce the exacerbations by treating chronic NTHi infections. In addition, further analysis demonstrated that azithromycin treatment was most likely to be effective in patients with high initial NTHi burden (Taylor *et al.*, 2020). Sputum NTHi was quantified by qPCR before treatment, and NTHi counts were similar in the azithromycin and placebo groups. High NTHi burden pre-treatment was associated with a higher frequency of exacerbations in the placebo group, but lower frequency of exacerbations in the azithromycin group. These data suggest that azithromycin treatment is most effective in patients with high baseline NTHi abundance, likely by eliminating chronic NTHi burden. One limitation of this study is that there is not a comparison with another antibiotic treatment which would eliminate chronic NTHi infection, such as doxycycline, making it difficult to

definitively dissect the antibiotic role of azithromycin from its anti-inflammatory properties. However, the ability of high initial NTHi abundance to predict responsiveness to azithromycin treatment, coupled with the decline in NTHi levels, suggest that the primary mechanism is by eliminating NTHi infection and the accompanying inflammation. Taken together, the AMAZES trial provides robust evidence that azithromycin is useful for reducing the rate of exacerbations in patients with asthma which is severe and poorly controlled despite ICS-LABA therapy.

The BTS guidelines for clinical practice recommend using macrolides to reduce the frequency of exacerbations in patients with persistent asthma despite good adherence to ICS treatment who have had at least one exacerbation requiring systemic corticosteroids in the past year (Smith, 2020). However, they do not recommend using it as a means to reduce oral steroid dose. Azithromycin therapy is generally well-tolerated, with the most common side-effect being gastrointestinal (GI) symptoms. In patients with a prolonged QTc interval, treatment with azithromycin is contraindicated, and liver function should be monitored 1 month after starting treatment and every 6 months thereafter.

1.3.1.2 Non-Antibiotic Role of Azithromycin

Some studies have reported improvements in asthma symptoms with azithromycin treatment despite the bacterial population being resistant to azithromycin. This suggests that azithromycin may also have a non-antibiotic role in reducing asthma exacerbations. Azithromycin has known anti-inflammatory and anti-viral effects, which may be responsible for its ability to reduce exacerbations in patients with asthma or COPD (Oliver and Hinks, 2021).

Infections with rhinovirus (HRV) are a major cause of exacerbations of asthma and COPD (Menzel *et al.*, 2016). Azithromycin has anti-viral effects, which may contribute to its ability to reduce exacerbations of airways disease. Azithromycin pre-treatment of bronchial epithelial cells increases viral-induced interferon (IFN) and reduces release of viral particles (Gielen, Johnston and Edwards, 2010; Porter *et al.*, 2016). Likewise in primary bronchial epithelial cells from patients with CF azithromycin pre-treatment increased gene expression of IFN and downstream factors

of IFN, as well as reducing replication of HRV (Schögler *et al.*, 2015). An *in vitro* study of COPD primary bronchial epithelial cells showed that pre-treatment with azithromycin reduced viral load of HRV16, and increased expression of IFN and RIG-I like helicases in both uninfected and infected cells from COPD patients, but not in healthy control cells (Menzel *et al.*, 2016). Taken together, these data suggest that azithromycin may ameliorate rhinovirus infection via its antiviral effects, likely by upregulating HRV-induced IFN signalling.

The 14 and 15-membered ringed members of the macrolide family are widely credited with immunomodulatory effects (Wales and Woodhead, 1999). They have been credited with reduction of endothelin-1 (ET-1), reduction in neutrophil oxidant burst and neutrophil chemotaxis, as well as reduced IL-6, IL-8, IL-1β, sICAM-1 and TNF (Wales and Woodhead, 1999). Primary bronchial epithelial cells isolated from asthma patients treated with clarithromycin or erythromycin showed a reduction in expression and production of ET-1, a potent bronchoconstrictor (Takizawa *et al.*, 1998). Dexamethasone also reduced ET-1, but theophylline, salbutamol and immunosuppressor FK506 had no effect (Sano *et al.*, 1995; Takizawa *et al.*, 1998). A randomised controlled trial of patients with influenza compared treatment with the neuraminidase inhibitor oseltamivir to oseltamivir plus azithromycin, and observed a more rapid reduction in pro-inflammatory cytokines IL-6, IL-17 and CXCL9 with oseltamivir plus azithromycin treatment (Lee *et al.*, 2017). However, the mechanisms underlying the well-known anti-inflammatory and immunomodulatory effects of azithromycin are not well understood (Zimmermann *et al.*, 2018).

Azithromycin treatment may have clinical benefits in a non-antibiotic role by improving barrier function, which is often impaired in airways diseases such as asthma and COPD (Holgate, 2008; Schleimer and Berdinikovs, 2017). Treatment with azithromycin improved lung function in cystic fibrosis patients without reducing the bacterial count (Halldorsson *et al.*, 2010). However, the mechanism remains unclear, potentially suggesting an azithromycin-induced increase in epithelial barrier function may be responsible. Initial experiments *in vitro* using A549 cells showed that azithromycin treatment increased expression of tight junction (TJ) proteins (Halldorsson *et al.*, 2010). However, these experiments showed that azithromycin

treatment resulted in increased production of intracellular proteins, rather than increased proteins localised to the tight junction, so it is unclear whether this represented a functional improvement in tight junction barrier function. Another *in vitro* study using air-liquid interface of bronchial VA10 epithelial cell lines showed that treatment with azithromycin for up to 22 days improved epithelial barrier function as measured by increased transepithelial electrical resistance (TEER) (Arason *et al.*, 2019). Bioinformatic analysis showed that long-term azithromycin treatment resulted in upregulation of epidermal barrier differentiation markers KRT1, CRNN, SPINK5 and DSG1, which was confirmed by immunostaining. In addition, azithromycin treatment induced the formation of multivesicular and lamellar bodies in which azithromycin accumulated intracellularly. This activation of epidermal characteristics and alteration of lipid trafficking may be part of the mechanism by which azithromycin improves barrier function.

1.3.2 Macrolides in diseases other than asthma

How effective might macrolides be in airways diseases? Apart from their potential utility in asthma, macrolides have shown promising results in the control of other airways diseases, notably non-CF bronchiectasis, CF and COPD.

Several clinical trials evaluating macrolides in non-CF bronchiectasis have demonstrated some efficacy of macrolides in reducing the frequency of exacerbations. BLESS, a randomised, double-blind controlled study of 107 patients found that low-dose erythromycin treatment modestly reduced the rate of pulmonary exacerbations overall and in the subgroup of patients with baseline *Pseudomonas aeruginosa* infection (Serisier *et al.*, 2013). Further analysis suggested that exacerbation rate was reduced significantly only in patients with *Pseudomonas* as the dominant microbe (Rogers *et al.*, 2014). Erythromycin treatment reduced 24-hour sputum production and ameliorated decline in lung function (Serisier *et al.*, 2013). However, long term treatment did increase the incidence of macrolide-resistant oropharyngeal streptococci. Further analysis of sputum bacterial composition using 16S rRNA genetic sequencing showed that in patients with prominent *Pseudomonas aeruginosa* colonisation, there was not a significant change in microbiome, while in patients without *Pseudomonas*

there was a shift away from *Haemophilus influenzae* towards more macrolide-tolerant bacteria in the microbiome (Rogers *et al.*, 2014).

Another trial, BAT, demonstrated that long term azithromycin treatment in 83 patients with non-CF bronchiectasis resulted in significantly fewer infectious exacerbations per patient, positively effecting quality of life for these patients (Altenburg *et al.*, 2013). Gastrointestinal side effects were seen in 40% of azithromycin-treated patients compared with 5% of placebo, but no patients discontinued the study due to these side effects. Increased macrolide resistance increased in azithromycin-treated patients, with an average resistance rate of 88% compared with 26% of placebo, which is a concern for ongoing use of long-term macrolide therapy. An earlier study of 141 patients with non-CF bronchiectasis demonstrated a reduction in exacerbations in patients receiving azithromycin daily for 6 months, with an event-based rate of exacerbations of 0.59 in patients treated with azithromycin compared with 1.57 in the placebo (Wong *et al.*, 2012). Taken together, these data suggest that long term macrolide antibiotic treatment, particularly with azithromycin, may be a valuable tool in reducing exacerbations in patients with non-CF bronchiectasis.

A prospective, randomised, double-blind, placebo controlled study of 60 patients with CF evaluated azithromycin treatment on lung function, weight and quality of life over three months of treatment (Wolter *et al.*, 2002). Chronic pulmonary inflammation is a key feature of CF which contributes to morbidity and mortality, and macrolide treatment was selected for its known immunomodulatory effect (Peckham, 2002; Wolter *et al.*, 2002). Treatment with azithromycin maintained lung function measured by forced expiratory volume in 1 second (FEV_1%) and forced vital capacity (FVC%) predicted, whereas lung function in the placebo group declined over the three month period (Wolter *et al.*, 2002). Average C reactive protein levels declined in the azithromycin group, while they remained constant in the control. In addition, patients treated with azithromycin required fewer courses of intravenous antibiotics, and quality of life improved. More recently, the OPTIMIZE (Optimising Treatment for Early *Pseudomonas aeruginosa* Infection in Cystic Fibrosis) study demonstrated that azithromycin treatment for up to 18 months reduced the risk of exacerbations, which

are important factors in declining lung function, by 44% (Southern *et al.*, 2012; Schultz and Sly, 2018).

Erythromycin is also effective in treating diffuse panbronchiolitis. an inflammatory condition prevalent in east Asia, which has high morbidity and mortality (Nagai *et al.*, 1991; Lin *et al.*, 2015). In 1982, Dr. Kudoh began using erythromycin to treat diffuse panbronchiolitis, which significantly improved cough, shortness of breath and mortality rates (Kudoh *et al.*, 1998). Long-term low-dose erythromycin treatment is now the standard of care for these patients (*ibid*).

A randomised, placebo-controlled, double-blind study of 109 patients with COPD demonstrated that treatment with twice-daily 250 mg doses of erythromycin reduced the number and duration of exacerbations compared with the placebo (Seemungal *et al.*, 2008). However, there was not a significant difference in stable FEV_1, sputum IL-6, IL-8, myeloperoxidase, or change in sputum bacterial flora. Nonetheless, these data provide further evidence that long-term macrolide treatment may be useful in reducing exacerbations in patients with chronic airways diseases.

1.4 Non-typeable Haemophilus Influenzae

A likely predominant mechanism of the efficacy of azithromycin in severe asthma is its antibiotic effect against common airway bacterial pathogens, of which the most commonly isolated in the human airways is non-typeable *Haemophilus influenzae* (NTHi). NTHi is a gram-negative, non-encapsulated bacterium (King and Sharma, 2015). The *Haemophilus influenzae* family is divided into typeable strains, which have a polysaccharide capsule, and non-typeable strains which do not have a capsule. Following development of the *Haemophilus influenzae* type b (Hib) vaccine, NTHi is now the primary cause of clinical disease within the family. It is a commensal organism in the human nasopharynx, and also a common cause of otitis media in children, as well as being capable of causing invasive diseases such as pneumonia, bronchitis, sinusitis and conjunctivitis. It causes persistent lower airway infections and is associated with neutrophilic airway inflammation, impaired lung function and increased exacerbations in patients with asthma or chronic obstructive pulmonary disease (COPD). Severe invasive diseases caused by NTHi are most common in young

children and the elderly (Laupland *et al.*, 2011). A study characterizing the lung microbiome using 16S rRNA bacterial sequencing showed that *Haemophilus* spp are more commonly isolated from the airways of patients with asthma than from healthy airways (Hilty *et al.*, 2010). In addition, several studies have suggested that NTHi infections also play a role in the progression of respiratory disease, likely by contributing to chronic inflammation and increasing the frequency of exacerbations (Martí-Lliteras *et al.*, 2011; Finney *et al.*, 2014).

1.4.1 Infection in Epithelial Cells

NTHi was traditionally thought to be primarily an extracellular pathogen, however, it has consistently been observed clinically within middle ear epithelium and airway epithelial cells (Hardison *et al.*, 2018). It uses a variety of techniques to promote adhesion and internalisation into the epithelium.

NTHi utilises several adhesion molecules to interact with the epithelium, which are often highly immunogenic (Duell, Su and Riesbeck, 2016; Atack *et al.*, 2018). It uses the host ICAM-1 receptor expressed by epithelial cells, and blockade of ICAM-1 has been shown to reduce adhesion by 37% in A549 cells and 69% in Chinese hamster ovary cells transfected with human ICAM-1 (Avadhanula *et al.*, 2006). In addition, infection with NTHi increases expression of ICAM-1 (Avadhanula *et al.*, 2006; Sajjan *et al.*, 2006). Most NTHi strains expressed the phase variable HMW1 and HMW2 adhesins, which have different lectin activities, allowing NTHi to occupy distinct niches depending on phase variable gene expression (Atack *et al.*, 2018). In addition, ubiquitously expressed Protein E (PE) and *Haemophilus* adhesin protein (Hap) mediate adhesion by binding to laminin, a basement-membrane glycoprotein (Ronander *et al.*, 2009; Hallström *et al.*, 2011).

Following adhesion, NTHi is internalized in several ways, including clathrin-mediated endocytosis, actin remodelling, lipid rafts and macropinocytosis. Once internalized, NTHi is typically trafficked by the endolysosomal pathway and degraded in the lysosome (Hardison *et al.*, 2018). However, NTHi has evolved a variety of ways to evade detection by the immune system and clearance from the airways. In patients with COPD, NTHi can colonise the airways for months at a time (Murphy *et al.*, 2004).

There is some evidence that NTHi may preferentially infect ciliated epithelial cells (Ren *et al.*, 2012; Kuek and Lee, 2020). As a key part of the ciliary escalator, cilia are often the first point of contact between inhaled pathogens and the host (Kuek and Lee, 2020). A differentiated *in vitro* model using primary human epithelial cells suggested that NTHi may preferentially infect ciliated epithelial cells (Ren *et al.*, 2012), whilst another *in vitro* experiment showed using confocal imaging that NTHi appears to primarily interact with ciliated epithelial cells as the first step in colonisation (Baddal *et al.*, 2015a). Patients with primary cilia dyskinesia also show increased susceptibility to NTHi infection (Walker *et al.*, 2017). In addition, there is evidence that NTHi infection supresses ciliary beating in vitro (Bailey *et al.*, 2012). However, the role of respiratory cilia in NTHi infection remains poorly understood and requires further study (Kuek and Lee, 2020).

1.4.2 Persistence in Epithelial Cells

There is evidence that patients with COPD are colonised by the same strain of NTHi for long periods of time, suggesting a capability for long-term evasion of host barrier and immune defences (Murphy *et al.*, 2004). To promote its persistence, NTHi is capable of forming biofilm-like structures, displaying antigenic variation of surface molecules, and expressing multiple adhesins (Ahearn, Gallo and Murphy, 2017). In addition, invasion of the host cells may allow it to evade the host immune response by creating a reservoir of NTHi infection (Clementi and Murphy, 2011).

In a 7-year prospective study of COPD spanning 345 patient-months, 122 episodes of negative sputum cultures lasting 1 month or more were identified, followed by recolonization with apparently the same strain of NTHi (Murphy *et al.*, 2004). In further analysis of 17 episodes of negative sputum culture lasting a minimum of 6 months, molecular typing confirmed that the re-emerging strain was identical to the infecting strain prior to the period of negative culture (Murphy *et al.*, 2004). The authors suggested that it is unlikely that NTHi survives as a reservoir in mucus, as that would likely be detectable in sputum samples. Instead, it is suggested that NTHi are capable of persisting intracellularly in airway epithelial cells. The authors do note, however, that strain-specific NTHi deoxyribonucleic acid (DNA) is sometimes detectable in sputum samples despite being negative on culture, suggesting that sputum

cultures may underestimate rates of infection. In addition, it is possible that, during periods of negative sputum culture, the infection is eradicated from the lungs while remaining as a commensal organism in the nasopharynx, later opportunistically re-colonising the lungs. Concurrent sampling from the nasopharynx for sequencing could confirm whether the nasopharynx is colonised during periods of negative sputum culture and is responsible for subsequent re-infection. Another longitudinal study found that the same NTHi strain persisted in COPD patients for up to 1422 days (Pettigrew *et al.*, 2018). Sequencing from 269 patients revealed that strains vary key virulence factors during persistence in the airways primarily by slipped-strand mispairing, which alters adherence, nutrient uptake and modification of surface molecules.

There is evidence in human adenoid tissue that *H. influenzae* is able to replicate intracellularly (Forsgren *et al.*, 1994). In 10 clinically uninfected children whose adenoids were removed due to nasal obstruction, *in situ* hybridisation showed *H. influenzae* in all 10 patients, though only 6 were positive on sputum culture. The *H. influenzae* were localised within large macrophage-like mono-nuclear cells, primarily in the subepithelial layer, but also in the reticular crypt epithelium. Electron micrograph images show dividing *H. influenzae* within these macrophage-like cells. It is presently unclear whether NTHi is similarly able to replicate intracellularly in the lung. There is some evidence that adenoid tissue may be a reservoir for NTHi biofilms (Nistico *et al.*, 2011).

Adherence and invasion of epithelial cells are important persistence mechanisms for NTHi (Clementi, Håkansson and Murphy, 2014; Duell, Su and Riesbeck, 2016; Atack *et al.*, 2018). A recent study analysed the genomes of NTHi strains isolated from COPD patients and identified novel virulence factor NTHI1441, which is conserved among strains (Ahearn *et al.*, 2019). Isogenic knockout mutation of the NTHI1441 open reading frame reduced invasion of primary bronchial and alveolar epithelial cells by 77 +/- 6%, although decreased invasion appeared to be independent of intracellular persistence or adherence. Adults with COPD had increased IgG serum against NTHI1441 following exacerbations with NTHi. In addition, enzyme-linked immunosorbent assay (ELISA) and flow-cytometry data suggested that NTHI1441 has

surface-expressed epitopes, which make it a potential target for preventative and therapeutic treatments for disease caused by NTHi.

A 2014 study (Clementi, Håkansson and Murphy, 2014) showed *in vitro* that IgA1 protease production by NTHi is necessary for optimal invasion, adherence and persistence. The IgA1 protease cleaves peptides within the hinge region sequence of the human immunoglobulin A1 gene. The igaA protease is ubiquitous in NTHi, while some strains also express igaB, which is associated with greater pathogenicity and is homologous to that found in pathogenic Neisseria spp. The authors compared NTHi strain 11P6H, a COPD isolate, with versions which lack igaA, igaB, or both. *In vitro* infections with these strains in NCI-H292 cells, a human bronchial mucoepidermoid carcinoma cell line, showed that igaA is necessary for efficient invasion. In addition, although igaB did not affect internalisation, it promoted intracellular survival by cleaving lysosome-associated protein-1 (LAMP1). However, IgaB did not prevent lysosomal acidification. The authors suggest that cleavage of LAMP1 may take place at the plasma membrane rather than within the lysosome, as igaB is more effective at a higher pH.

NTHi infections are often able to recur following treatment with common antibiotics which would eradicate extracellular bacterial populations, which provides further evidence that they may evade antibiotics by sheltering intracellularly. An ability to internalise may also offer a mechanism by which NTHi elude clearance by the immune system. A 1999 study by van Alphen *et al* showed *in vitro* that NTHi are able to persist within the epithelial cell layer despite treatment with gentamicin, an antibiotic effective against extracellular bacteria, but which is unable to cross the cell membrane (Van Schilfgaarde *et al.*, 1999). In addition, NTHi located within the epithelial layer was able to resist clearance by a specific antibody to major outer membrane protein (MOMP) P2 using the same *in vitro* co-culture model, suggesting that internalisation may also provide a mechanism by which NTHi evades clearance by the immune system. Garmendia *et al* go went farther towards describing NTHi localisation in the epithelial layer, showing in submerged culture of A549 cells that NTHi was located in an acidified endosome-like subcellular compartment (Morey *et al.*, 2011). They also confirmed the finding that intracellular NTHi were able to survive gentamicin

treatment up to 28 hours post-treatment. Additionally, it was demonstrated using fluorescence in situ hybridization (FISH), a fluorescence technique that illuminates metabolically active bacteria, that the intracellular bacteria were viable and metabolically active, though the gradually diminishing numbers suggested that they were not replicating (Morey *et al.*, 2011).

1.4.3 Can NTHi persist paracellularly?

It is presently unclear whether NTHi persists solely intracellularly, or whether it is able to shelter in the paracellular space within the epithelial layer. A 2012 study using normal human primary bronchial epithelial cells (NHBE/PBEC) cells at ALI showed NTHi survival within the paracellular space up to 10 days post infection following gentamicin treatment to eliminate extracellular NTHi (Ren *et al.*, 2012). Conversely, bacteria found within vacuoles were degraded (Ren *et al.*, 2012). Deletion of the Sap transporter reduced adhesion of NTHi to the epithelial cell, but increased the invasion rate (Raffel *et al.*, 2013). Sap expression also altered trafficking within the cell, with Sap deficient NTHi appearing free within the cytoplasm, whereas NTHi with intact Sap confined to the endosomes (Raffel *et al.*, 2013). In addition, Sap mutant strains elicited a weaker cytokine response *in vitro* and less severe disease in a chinchilla model of OM. These results suggested that host inflammatory signalling in response to NTHi may be influenced by intracellular processing, and may promote disease. A study performed in A549 cells in submerged culture examined the kinetics of intracellular localisation, showing that NTHi have disappeared in the early endosome by 120 minutes post infection, whereas they persist in the late endosome up to 24 hours post infection (Morey *et al.*, 2011). These data suggest that in addition to survival within the epithelial cell, NTHi may be capable of persisting in the paracellular space as well. However, replication to confirm reproducibility is an important step. In addition, it is still unclear whether different inflammatory signalling is elicited by intracellular compared to paracellular bacteria.

1.4.4 NTHi Biofilm Formation

Classical biofilms are multidimensional microbial communities existing within self-derived extracellular matrices (Magana *et al.*, 2018). Bacteria are typically excluded or rapidly cleared from deep tissues, but at mucosal surfaces bacteria can persist in

biofilms (*ibid*). Biofilms are responsible for up to 80% of chronic human diseases, and contribute to upper and lower respiratory tract infections, persistent otitis media, urinary tract infections and periodontitis amongst others. Biofilms can also form on abiotic surfaces, notably medical devices such as artificial cardiac valves, coronary stents and urinary catheters. The stages of biofilm formation are broadly conserved among species, progressing from the planktonic phase, irreversible attachment of live bacteria to a surface, growth of the adherent bacteria and formation of a mature biofilm containing live and dead bacteria, as well as often host cells. Mature biofilms serve as a bacterial reservoir and disperse bacteria. Extracellular matrices are typically composed of polysaccharides, proteins and DNA, but the composition varies between bacterial species, conditions during development of the biofilm, and expression of various bacterial factors. Existing within these communities allows the bacterial community to evade environmental threats such as treatment with antimicrobials as well as host defence mechanisms (Langereis and Hermans, 2013; Magana *et al.*, 2018).

NTHi may persist in the airways through its ability to form biofilms. NTHi is capable of switching between planktonic and biofilm-like growth modalities in response to changes in its microenvironment (Langereis and Hermans, 2013). NTHi biofilms *in vivo* contained both significant quantities of ds-DNA, likely providing structural stability, along with pilin IV protein aggregates (Jurcisek and Bakaletz, 2007). Classic biofilm formation requires the ability of the bacteria to produce extracellular polysaccharides. However, despite their inability to produce extracellular polysaccharides, NTHi appear to be capable of forming a biofilm-like structure (Langereis and Hermans, 2013), although, due to the lack of extracellular polysaccharide production, the ability of NTHi to form traditional biofilms is still disputed, though arguably this is ultimately a question of semantics (Moxon *et al.*, 2008).

Several studies suggest that NTHi induces formation of neutrophil extracellular traps (NETs) (Juneau *et al.*, 2011; Langereis and Hermans, 2013). Neutrophils extrude DNA to form neutrophil extracellular traps (NETs), which allows them to ensnare bacteria and plays a role in the clearance of infections, although it is unclear whether they are capable of direct killing (Langereis and Hermans, 2013). However, NETs are

ineffective at clearing NTHi infection (Juneau *et al.*, 2011). Instead, NTHi are able to establish a biomass within them, forming a *de facto* biofilm despite not being adherent to the mucosa. Juneau *et al* show that NTHi within NETs are protected from both extracellular killing mechanisms as well as from phagocytic killing by neutrophils joining the NET structure (Juneau *et al.*, 2011).

NTHi is often found in multispecies biofilms, notably with *S. pneumoniae* (Tikhomirova and Kidd, 2013). However, it is uncertain whether their relationship is competitive or cooperative, although there is evidence that airway infection with one bacterial species may facilitate subsequent colonisation by additional species (Van Eldere *et al.*, 2014; Whiley *et al.*, 2015; Aziz *et al.*, 2019). Existence in this biofilm structure has been postulated as a reason for the often chronic nature of NTHi and *S. pneumoniae* infections (Tikhomirova and Kidd, 2013). A study of recurrent otorrhea in chronic suppurative OM showed that multispecies biofilms dominated by NTHi, *S. aureus*, and anaerobic bacteria were a common feature in patients with active disease (Jensen *et al.*, 2017). In addition, colonisation with NTHi and recurrent episodes of protracted bacterial bronchitis in children was a significant risk factor in the development of future bronchiectasis (Wurzel *et al.*, 2016). Interactions between *S. pneumoniae* and NTHi altered expression of virulence factors, promoting expression of pilin IV in NTHi (Cope *et al.*, 2011). This relationship was also evident in chronically infected tissue from patients with chronic rhinosinusitis (Cope *et al.*, 2011). These data suggest that colonisation with multiple species is a common feature of biofilms and may contribute to the recurrence of infection.

1.4.5 Immune response to NTHi

The immune response to NTHi infection remains incompletely understood, but given the diverse roles NTHi plays as a commensal organism in the nasopharynx to persistent invasive pathogen in the lung, understanding the immune response to NTHi infection may be important in understanding the role it plays in driving mucosal inflammation in airways diseases.

1.4.5.1 Innate Immune response to NTHi

The innate immune response is the first line of defence against pathogens, consisting of several physical, chemical and cellular defences. These highly conserved

mechanisms unite to stem the prevent the spread and movement of pathogens throughout the body.

1.4.5.1.1 Inflammasomes

Inflammasomes are cytosolic multiprotein complexes which are important in the detection and response to both viral and bacterial exposure, and are able to respond to both pathogen-associated molecular patterns (PAMPs) as well as damage-associated molecular patterns (DAMPS) (Dagenais, Skeldon and Saleh, 2012; Broz and Dixit, 2016). They are intracellular protein complexes composed of a sentinel protein which informs the type of inflammasome – notably NLRP1, NLRP3, NLRC4 or AIM2. Inflammasome activation leads to cleavage of procaspase-1 and the processing of IL-1 family members into their active forms. The NLRP3 inflammasome is upregulated during NTHi infection in *ex vivo* human lung tissue (Rotta detto Loria *et al.*, 2013). The NLRP3 inflammasome is a member of the Nod-like receptor (NLR) family, which responds to a wide variety of stimuli including viruses and bacteria via pattern recognition receptors (PRRS). NLRP3 inflammasome upregulation results in activation of IL-1β and IL-18 signalling in a caspase-1 dependent manner by processing them into their active forms.

Figure 6. NLRP3 Inflammasome activation

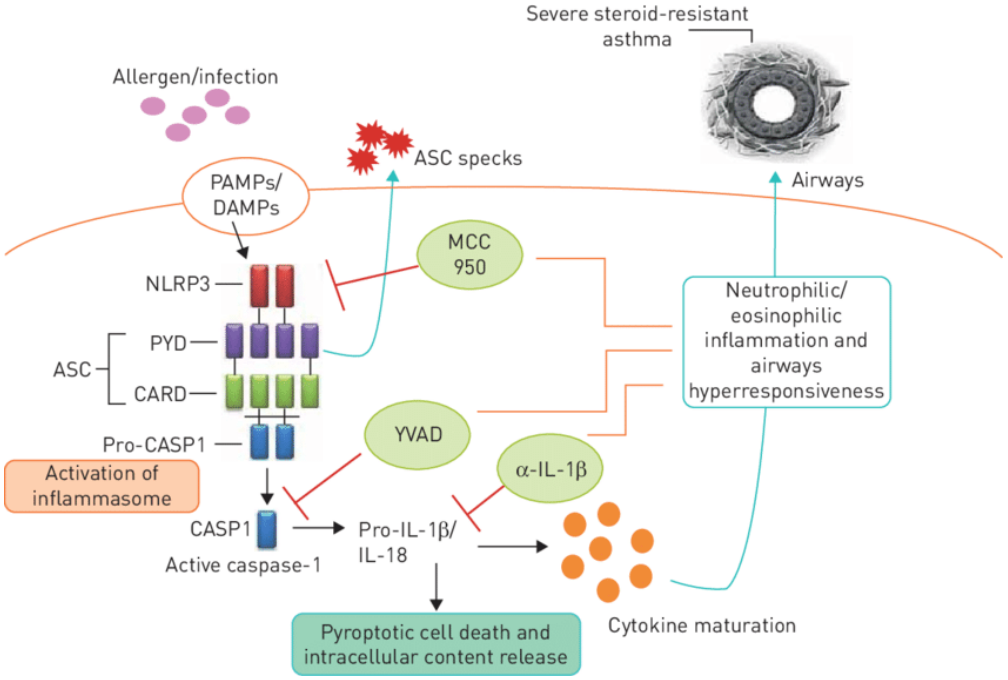


Figure 6. Diagram demonstrating the activation of the NLRP3 inflammasome and its contribution to airway inflammation. Exposure to allergic or infectious stimuli activate NLRP3 via DAMPs and PAMPs, resulting in production of Caspase-1 and downstream induction of IL-18 and pro-IL-1β. Maturation of these cytokines leads to elevated neutrophilic and eosinophilic airway inflammation and increased airway hyperresponsiveness. Reproduced from (Wadhwa *et al.*, 2019)

During NTHi infection in children with protracted bacterial bronchitis (PBB), multiple inflammasomes were involved in response to NTHi in macrophages isolated from bronchoalveolar lavage fluid and in PBMCs (Chen *et al.*, 2018). IL-1β upregulation is a common feature of PBB. NTHi stimulation of PBMCs from patients with PBB induced IL-1β, but this effect was blocked when treated with NLRP3 or caspase-1 inhibitors, suggesting that IL-1β in PBB is mediated by inflammasomes. In addition, in macrophages from BAL fluid, stimulation with NTHi resulted in activation of both NLRP3 and AIM2 inflammasome complexes. Taken together, these results suggest that both the NLRP3 and AIM2 inflammasomes play a role in regulating the inflammatory response to NTHi. One limitation of this study is the use of only one clinical isolate of NTHi throughout, particularly given that NTHi strains are highly heterogeneous.

1.4.5.1.2 Toll like receptors

Toll-like receptors are a family of proteins which mediate inflammatory responses to damage and infection via adaptive and innate immune signalling (Arora *et al.*, 2019). TLRs are conserved innate immune sensors capable of recognising a range of endogenous and microbial molecules, as well as allergens (Zakeri and Russo, 2018). Cellular innate immunity is part of the first line of defence against infection (King, 2012). Macrophages and neutrophils are the primary players in cellular immunity mediating NTHi infection, predominantly by sensing bacterial pathogens via TLRs (King, 2012; King and Sharma, 2015). TLRs are differentially expressed on both hematopoietic and non-hematopoietic structural and stromal lung cells which can then be activated by TLR agonists and modulate immune signalling (Zakeri and Russo, 2018). TLR signalling plays a diverse role in asthma, varyingly contributing to the development of disease and to decreasing disease progression, possibly dependent on the cell type that has been activated (hematopoietic vs non-hematopoietic), type of allergen, and route of exposure (Zakeri and Russo, 2018). TLRs, particularly TLRs 2 and 4, are also thought to be important in the development of COPD, and is capable of sensing particulate pollutants and cigarette smoke as well bacteria (Hansbro *et al.*, 2017; Sidletskaya, Vitkina and Denisenko, 2020). In addition to its role in infectious disease, TLR signalling has also been implicated in lung cancer, where TLRs on cancer cells may activate downstream factors that promote tumorigenesis (Gu *et al.*, 2018).

NTHi lipoprotein P6 elicited a strong adaptive immune response via TLR2 signalling and increased lung inflammation in one mouse model of NTHi exposure (Lugade *et al.*, 2011). Another study in a mouse model of NTHi infection demonstrated that an intact MyD88-dependent pathway of TLR4 is necessary for effective bacterial clearance and immune response (Wieland *et al.*, 2005). In contrast to the prior study which suggests that TLR2 signalling contributes to lung inflammation, this study suggested that TLR2 KO mice infected with NTHi had unchanged inflammation and slightly elevated NTHi CFUs compared to controls.

1.4.5.1.3 Complement System

The complement system is the first line of defence against bacteria, labelling bacteria for phagocytosis and directly killing them via pore formation (Hovingh, van den Broek

and Jongerius, 2016). The components of the complement system normally circulate in the blood and tissue fluids in an inactive state, which can be activated sequentially as part of an enzymatic cascade upon the recognition of microorganism components (Gani, 2008). There are approximately 20 proteins that make up the complement system, and activation of one leads to the enzymatic cleavage and activation of the next. In addition, there is signalling between the complement system and the TLR response, as well as activating the adaptive immune system by stimulating B-cells. Intracellular complement activation in T cells also mediates adaptive immunity in response to infection. There are three branches to the complement system, the classical pathway (CP), alternative pathway (AP) and lectin pathway (LP). However, effective pathogens have developed a variety of mechanisms to circumvent the complement system (Hallström and Riesbeck, 2010; Riesbeck, 2020). One important method of complement evasion is by recruiting complement regulatory proteins, which are ordinarily present on host cells to avoid being targeted, to the bacterial surface instead (Hovingh, van den Broek and Jongerius, 2016). NTHi uses a wide range of outer membrane proteins (OMPs) to bind host complement regulators C4b binding protein and factor H, preventing both the classical and alternative complement system (Riesbeck, 2020). Lipo-oligosaccharide, a highly variable close relative of LPS produced by NTHi, also has been shown to promote resistance to complement killing (Hallström and Riesbeck, 2010).

1.4.5.2 Adaptive Immune Response to NTHi

In a cohort of 15 patients with idiopathic bronchiectasis and chronic severe infections with NTHi and 24 healthy control participants, both healthy control participants and patients with bronchiectasis had detectable antibody responses to NTHi, suggesting that most healthy people have an adaptive immune response to NTHi (King *et al.*, 2003). Further characterisation showed that healthy participants had a Th1-mediated response to NTHi with distinct CD40 ligand production, whilst in contrast bronchiectasis patients had a primarily Th2 cytokine response to NTHi, decreased CD40 ligand production, and different IgG production. These results suggest that chronic infection with NTHi in bronchiectasis patients was associated with altered adaptive immune responses, which may be important in the pathogenesis of persistent infections.

1.4.5.3 NTHi Vaccines

There is high demand for an effective vaccine against NTHi infection, which is now the primary cause of disease within the *Haemophilus* family since the Hib vaccine has become widely available. Developing an NTHi vaccine presents several challenges due to the heterogeneity between different strains and antigenic variation within strains (Poolman *et al.*, 2000). Early vaccines used killed NTHi as an oral vaccine, while more recent vaccines have been consisted of recombinant versions of major and minor outer membrane proteins and adhesins. However, currently no vaccine is available that provides broad protection against NTHi infection and the associated exacerbations in airways disease.

A 1985 trial using killed NTHi as an oral vaccine in patients with COPD saw a 10-fold reduction in the incidence of infections over a three-month winter period (Clancy *et al.*, 1985). However, without receiving a booster dose, the vaccine did not provide significant protection from infection over the following winter period. Another study of sixty-four patients with chronic bronchitis evaluating the efficacy of an oral killed vaccine found that patients given the vaccine developed fewer acute infective episodes and required fewer antibiotic prescriptions in total, although a similar number patients required at least one course of antibiotics (Tandon and Gebski, 1991).

A phase II, observer-blind, controlled clinical trial of a protein vaccine against NTHi was carried out in 145 COPD patients aged 40-80 with a history of exacerbations (Wilkinson *et al.*, 2019). The patients received two doses 60 days apart of either placebo or vaccine containing NTHi surface proteins recombinant protein D (PD) and combined protein E and Pilin A (PE-PilA). Specific CD4+ T cells increased following two doses of vaccine, and antibodies against PD, PE and PilA increased up to 30 days post inoculation and then decreased, remaining higher than baseline concentrations for up to 13 months. Patients who received the vaccine had a statistically non-significant decrease in yearly moderate or severe exacerbations, with a vaccine efficacy rate of 13.3%.

NTHi and *Moraxella catarrhalis* are frequently found in bacterial exacerbations of COPD, thus a combined vaccine containing NTHi antigens PD and PE-PilA and *M. catarrhalis* antigen UspA2 was administered in 2 doses 60 days apart in participants

with ≥10 pack-years smoking history to mirror the background of COPD patients (Van Damme *et al.*, 2019). In 90 smokers/ex-smokers aged 50-70 years, participants who received an adjuvanted formulation containing 10 μg of each NTHi antigen and 3.3 μg of UspA2 developed the best humoral immune response against NTHi, while the *M. catarrhalis* response was similar across varieties, waning after 30 days from the second dose. Overall, the NTHi-*M. catarrhalis* vaccine had good immunogenicity in older adults with a history of smoking.

Efforts at producing an effective vaccine have focussed on generating a robust humoral immune response. However, a study in mice inoculated with a killed NTHi strain showed that while immune antibodies protected only from a homologous strain, transfer of Th cells provide broad immunity against heterogeneous strains (Li *et al.*, 2018). Further characterisation showed a strong Th17 response which recognised conserved cytosolic and membrane associated proteins, whereas immune antibodies responded to surface antigens in a highly strain-specific way. Mice were immunised with a two highly conserved proteins which are recognised by Th17 cells, which provided protection against challenge with various strains of NTHi as well as against an encapsulated Haemophilus strain. These results suggest that Th17 cells are important in generating broad protection against NTHi infection, and that incorporating Th17 antigens in vaccines may be important in creating a vaccine which protects against a wide range of NTHi strains.

1.4.6 Immune response in commensal NTHi nasopharyngeal colonisation

There is increasing interest in the human microbiome in various niches and its role in disease progression and immunity (Dimitri-Pinheiro, Soares and Barata, 2020). The nasal cavity is a reservoir for a multiplicity of organisms that cause opportunistic infections in the respiratory tract. NTHi is a commensal organism in the nasopharynx (NP), colonising the nasopharynx of even most healthy people (Miyamoto and Bakaletz, 1997; Pichichero and Almudevar, 2016). NTHi does not cause disease in the NP of most people, although colonisation of the NP is a pre-requisite for development of OM and infections of the respiratory tract (Yang *et al.*, 1998).

The development of otitis media (OM) involves the ascension of NTHi up the eustachian tubes from the nasopharynx to the middle ear (Miyamoto and Bakaletz, 1997; Yang *et al.*, 1998). An adenovirus model of OM in chinchillas showed using fluorescent and electron microscopy that NTHi preferentially bound to mucus rather than epithelial cells during the ascent to the middle ear (Miyamoto and Bakaletz, 1997). Clinical onset of OM and microscopy show that NTHi reach the middle ear 7-10 days following nasal inoculation. These data suggest that growth of NTHi in mucus within the eustachian tubes may be important in the ascension of NTHi from the nasopharynx and the development of acute OM.

Another model of OM in chinchillas utilised antibiotic-resistant NTHi strains, eliminating the need for a viral co-infection (Yang *et al.*, 1998). This model showed that although young chinchillas and adults are susceptible to nasopharyngeal colonisation, young animals were more likely to develop OM. In addition, animals were immune to subsequent infections with the same strain after recovery, and inoculation with inactivated whole-cell NTHi preparations provided protection against homologous and incompletely against heterologous strains.

A study of 85 healthy children from 6-36 months of age analysed serum samples and nasal cultures to evaluate NP colonisation with *S. pneumoniae*, NTHi, and *M. catarrhalis* and serum levels of sICAM-1, IL-10 and S100A12, a marker of inflammation (Meijer, Gearry and Day, 2012; Pichichero and Almudevar, 2016). Serum levels of these markers were elevated by asymptomatic NP colonisation by *S. pneumoniae*, NTHi or *M. catarrhalis*, and a statistical model using serum cytokine levels was highly predictive of positive NP culture (Pichichero and Almudevar, 2016). These data could suggest that even asymptomatic infection with NTHi could alter immune signalling.

1.4.7 Antibiotic susceptibility

NTHi is increasingly the prevalent serotype of *Haemophilus influenzae* following the development of the Hib vaccine, and is a priority pathogen. Resistance to beta-lactam antibiotics, such as ampicillin, is increasing via production of beta-lactamase or by decreasing affinity of beta-lactams via alteration of the penicillin-binding protein

(PBP) (Dabernat and Delmas, 2012; Li *et al.*, 2020). A study evaluating antibiotic resistance in Haemophilus influenzae isolates from blood, cerebrospinal fluid, bronchial secretions, middle ear fluid, nasopharynx or conjunctiva of children in France between 2001-2008 showed that 27.5% of isolates produced beta-lactamase, while a further 16.9% were beta-lactamase-negative ampicillin-resistant and 6.4% were beta-lactamase-negative amoxicillin-clavulanate resistant (Dabernat and Delmas, 2012). Analysis of microbiological data from the AMAZES study have shown that prolonged treatment with azithromycin effectively eliminated NTHi strains, evidently without inducing azithromycin resistance, despite substantial increases in resistance in other species (Taylor *et al.*, 2019). Because of the increased resistance to macrolides in other bacterial species, caution must be used when using long-term macrolide therapy, which should be restricted to patients who will benefit most from it (Serisier, 2013).

1.4.8 Strain Variation

NTHi is a heterogeneous organism, with wide variation between strains observed in many virulence factors, such as adhesins, outer membrane proteins, nutrient uptake and internalisation capacity (Foxwell, Kyd and Cripps, 1998; Ackland *et al.*, 2020). For instance, NTHi is able to use both pilus and non-pilus adhesins to facilitate adhesion and colonisation, but while 80% of strains produce the most common non-pilus adhesins HMW1 and HMW2, another 20% produce the adhesin Hia only (Buscher *et al.*, 2004). There is also significant variation in the highly immunogenic outer membrane proteins between strains (Foxwell, Kyd and Cripps, 1998). In addition, NTHi strains are able to vary characteristics during persistent infection to better adapt to their biological niche (Buscher *et al.*, 2004; Pettigrew *et al.*, 2018). Recent data has also suggested monocyte-derived macrophages (MDMs) produce different inflammatory responses to different clinical strains of NTHi (Ackland *et al.*, 2019). 8 diverse clinical isolates elicited unique responses in MDMs *in vitro*, inducing varying levels of RIG-I, IFN-β, IL-1β, IL-6 and IL-8. However, induction of TLR4 and 7 were not different between strains. These data highlight the heterogeneity of NTHi strains and the importance of acknowledging strain differences both experimentally and clinically.

1.4.9 Phase Variation in NTHi

Phase variation is an important aspect of NTHi biology which it utilises to evade the host immune system, and has material effects on the reproducibility of results and the importance of multiple replicates (Poole *et al.*, 2013; Davis, Marino, *et al.*, 2014; Atack *et al.*, 2018, 2019; Zachary N. Phillips *et al.*, 2019; Elango and Schulz, 2020; Kadowaki *et al.*, 2021). Phase variation is the random, reversible switching of bacterial genes, typically encoding surface structures such as adhesins, pili, and lipooligosaccharides (Zachary N. Phillips *et al.*, 2019). Phase variation allows a bacterial population to produce a variety of phenotypes, some of which may be more fit than others, giving the bacteria a better chance of survival (*ibid*). In addition, many of the regions which are phase-variable are highly immunogenic, so phase variation may promote immune evasion (*ibid*). One study has evaluated On/Off switching of LOS biosynthetic genes, demonstrating that the combination of *oaf*A ON and *lic2*A OFF promotes invasion, suggesting that phase variation of LOS genes may contribute to NTHi invasive disease (Zachary N Phillips *et al.*, 2019). Some phase variable methyltransferase *mod*A alleles are over-represented in NTHi isolated from the children with middle ear infections, whilst COPD isolates preferentially express different *mod*A alleles, suggesting that different alleles confer advantages in the lung compared to middle ear niches (Atack *et al.*, 2019). These data demonstrate that phase variable regions in the NTHi genome allow it to adapt to different environments and modulate host-cell interactions such as adhesion and invasiveness.

1.4.10 Human rhinovirus co-infection with NTHi

Infection with human rhinovirus (HRV) infection is a common cause of exacerbations in people with asthma (Nicholson, Kent and Ireland, 1993; Holgate, 2006). In addition, immune responses induced by airway bacteria modulate viral interactions in the airways. For instance, NTHi infection predisposes people with asthma to further infections with HRV, possibly via upregulation of ICAM-1 (Sajjan *et al.*, 2006; Gulraiz, Bellinghausen, Cathrien A Bruggeman, *et al.*, 2015).

Rhinovirus infections are a major cause of exacerbations in COPD (Sajjan *et al.*, 2006; Kelly and Busse, 2008; Gulraiz, Bellinghausen, Cathrien A. Bruggeman, *et al.*, 2015). NTHi is a common cause of chronic bacterial infections in COPD (Ahearn, Gallo and

Murphy, 2017; Barbara A. Maciejewski *et al.*, 2017). Sajjan *et al* sought to examine the effect of NTHi infections on co-infection with rhinoviruses (Sajjan *et al.*, 2006). In an *in vitro* model using human bronchial epithelial cell line 16HEBo- and primary bronchial epithelial cells (PBECs), pre-incubation with NTHi followed by infection with HRV39 resulted in upregulation of IL-8, epithelial-derived neutrophil chemoattractant-78, and growth-related oncogene-alpha, compared with cells that were not exposed to NTHi. In addition, exposure to NTHi upregulated expression of ICAM-1, a receptor used by major-type rhinoviruses. Binding of major-type rhinovirus HRV39 was increased in epithelial cells exposed to NTHi. Thus, upregulation of ICAM-1 may be a mechanism by which NTHi infection increases infectivity of rhinoviruses. Expanding on this study, a 2015 paper by Gulraiz *et al* corroborated the finding that exposure to heat-inactivated NTHi upregulated ICAM-1. Using an *in vitro* model of bronchial epithelial cell line (BEAS-2B cells) and PBECs, major-type rhinovirus HRV16 were compared with minor-type rhinovirus HRV1B and respiratory syncytial virus (RSV). Pre-treatment with NTHi increased release of HRV16 viral particles, which uses ICAM-1, but not of HRV1B or RSV. Similarly, copies of HRV16 RNA increased in response to NTHi exposure, though copies of HRV1B and RSV also increased. Taken together, these data suggest that NTHi-mediated upregulation of ICAM-1 may have a role in increasing susceptibility to viral infections, particularly for viruses which bind via ICAM-1.

Other mechanisms may also contribute to interactions between NTHi and viruses. A mouse model of RHV-NTHi co-infection demonstrated that co-infection with RHV increased persistence of NTHi by reducing NTHi-induced IL-8 via IRAK-1 degradation (Unger *et al.*, 2012). Conversely, another study demonstrated in primary human bronchial epithelial cells and BEAS-2B cells that HRV co-infection with NTHi or *Pseudomonas aeruginosa* synergistically induced CXCL8 (IL-8) and CCL20 compared to either stimulus alone (Barbara A Maciejewski *et al.*, 2017). These data provide interesting insights into the interactions between bacteria, HRV and epithelium in the presence or absence of immune cell interactions. A recent study showed that type A rhinovirus (HRV16) infection resulted in defective phagocytosis of NTHi by monocyte-derived macrophages (MDMs) and alveolar macrophages (AMs) (Sabroe, Ho and Dockrell, 2019). Similarly, HRV16 infection caused defective

phagocytosis of *S. pneumoniae* in MDMs, but not in alveolar macrophages. In addition, infection with HRV16 reduced proinflammatory signalling in response to NTHi in MDMs derived from COPD patients (*ibid*). Furthermore, in the absence of HRV challenge, both MDMs and AMs from COPD patients had lower phagocytosis of both NTHi and *S. pneumoniae* compared to macrophages from healthy controls (*ibid*). These results suggested that airway macrophages from patients with airways diseases may have defective phagocytosis, and that this may have been exacerbated by rhinovirus infection.

1.4.11 NTHi coinfection with other respiratory viruses

In contrast to its role in HRV susceptibility, NTHi appears to be protective against RSV infection in an *in vitro* infection model (Hartwig *et al.*, 2016). RSV is a common opportunistic pathogen, commonly causing sometimes severe respiratory infections in children. Interestingly, like NTHi, RSV infection induces upregulation of ICAM-1 surface expression, although it does not utilize the ICAM-1 receptor itself (Arnold, Neumann and König, 2007). Pre-incubation of transformed human bronchial epithelial cells (16HBE14o- cells) with live NTHi significantly reduced expression of RSV RNA. Exposure to HT-NTHi or NTHi 2019delta licD, which lacks phosphorylcholine and hence is unable to enter epithelial cells, was much less protective against subsequent RSV infection, suggesting that invasion is important for initiating protection. The authors suggested that NTHi may directly bind to RSV particles and thus prevent entry, or may inhibit RSV binding to its receptor, nucleolin. This protective effect of NTHi was not observed with influenza A challenge. Taken together, these data suggest that NTHi infection may have a dichotomous role in mediating secondary viral infection, being in some instances protective and in others increasing infectivity, likely related to the target cell type being infected.

1.4.12 NTHi coinfection with bacteria

Haemophilus haemolyticus is an upper respiratory tract bacterium which is closely related to NTHi. It is often a commensal, although it is sometimes capable of causing invasive disease. Historically, NTHi and *H. haemolyticus* were often confused because they are difficult to differentiate by culture. Both NTHi and *H. haemolyticus* require both haemin and nicotinamide adenine dinucleotide (NAD). Traditionally, *H.*

haemolyticus is lytic, but a significant proportion of strains are not, as they can lose their lytic phenotype when passaged or lack it altogether, and these strains were often mistaken for NTHi (Pickering, Richmond and Kirkham, 2014). An *in vitro* model of infection in nasopharyngeal and bronchoalveolar epithelial cell lines (D562 and A549 cells, respectively) showed that NTHi and *H. haemolyticus* elicit different immune responses (Pickering *et al.*, 2016). *H. haemolyticus* strains are generally less invasive, but induce stronger IL-6 and IL-8 signaling in nasopharyngeal epithelial cells. However, in PBMCs, both *H. haemolyticus* and NTHi induced potent IL-6, IL-8, IL-10, IL-1β and TNF signaling, but only NTHi induced IFNγ. It is hypothesized that NTHi and *H. haemolyticus* may exist in competition, given their relatedness. Data showed that pre-incubation with *H. haemolyticus* significantly reduced the ability of NTHi to associate with epithelial cells (Pickering *et al.*, 2016). Taken together, these results suggest that *H. haemolyticus* and NTHi may compete, and that further investigation of their interactions may yield a potential target for ameliorating NTHi infections.

1.5 Epithelial Cell Biology

Epithelial cells line cavities and structures throughout the body, from the skin to the gut, glands, and respiratory tract (U. S. National Institutes of Health, 2021). The epithelium provides the first line of defence against stimuli from either outside the body or from, for example, within the lumen of the gut. The epithelium is the first organised multicellular tissue found in evolution and is highly conserved, found in all metazoans (Miller *et al.*, 2013). Epithelial tissue can be derived from either ectoderm or endoderm, depending on the site. Epithelium derived from the ectoderm include the epidermis, all hollow organs which have cavities open to a surface covered by epidermis, as well as the lining of the nose (U. S. National Institutes of Health, 2021). Epithelium of endodermal origin includes the lining of the digestive tract, and the lining of all hollow structures formed as outpockets of the digestive tract, including the pharynx and respiratory tract, with the exception of the nose (*ibid*). The epithelial layer can either be simple, a monolayer of cells, or stratified, with multiple layers of cells. There are three basic shapes of epithelial cell, squamous cells, which are flat, cuboidal epithelial cells which are roughly square, or columnar cells, which are taller

than they are wide. As a rule, epithelium derived from the ectoderm is squamous, whilst that derived from endoderm is glandular. The bronchial airway epithelium is made up of pseudostratified columnar epithelial cells, which is a monolayer composed of cells of varying heights giving the illusion of stratification (Crystal *et al.*, 2008; Maynard and Downes, 2019).

1.5.1 Airway Structure

The airways are divided into the conducting and respiratory airways (Wittekindt, 2017). The conducting airways are from the trachea to the 16th generation of terminal bronchioles. The partitions of the airway structure are denoted by generations of dichotomous branching, where the trachea is designated generation 0 and each subsequent branching is a higher generation, from generation 0 to the last order of the terminal bronchioles at generation 23. Conducting airways are further subdivided into the cartilaginous region, up to the 10th generation bronchial tree, and the non-cartilaginous region from the 11th generation bronchial tree to the 16th generation terminal bronchioles (Hyde, Hamid and Irvin, 2009). The respiratory airways are non-cartilaginous and comprise the 17th-23rd generation of terminal bronchioles, terminating in the alveoli, where gas-exchange takes place.

The airway epithelium is an important barrier and the first line of defence protecting the host from inhaled particulates and pathogens (Kojima *et al.*, 2013; Carsin *et al.*, 2016). It is pseudostratified columnar epithelium, the composition of which alters throughout the airways (Vareille *et al.*, 2011). The epithelium of the conducting airways is composed of ciliated epithelial cells, goblet cells, brush cells, basal cells and small granule cells, whilst the bronchioles, which are at the intersection between the conducting and respiratory airways, are primarily composed of ciliated epithelial cells with a minority of microvillar cells, small granule cells and club cells (Rokicki *et al.*, 2016). The pulmonary alveolar epithelium is composed of type I, II and III pneumocytes as well as numerous macrophages.

Figure 7. Diagram of epithelial makeup throughout the airways

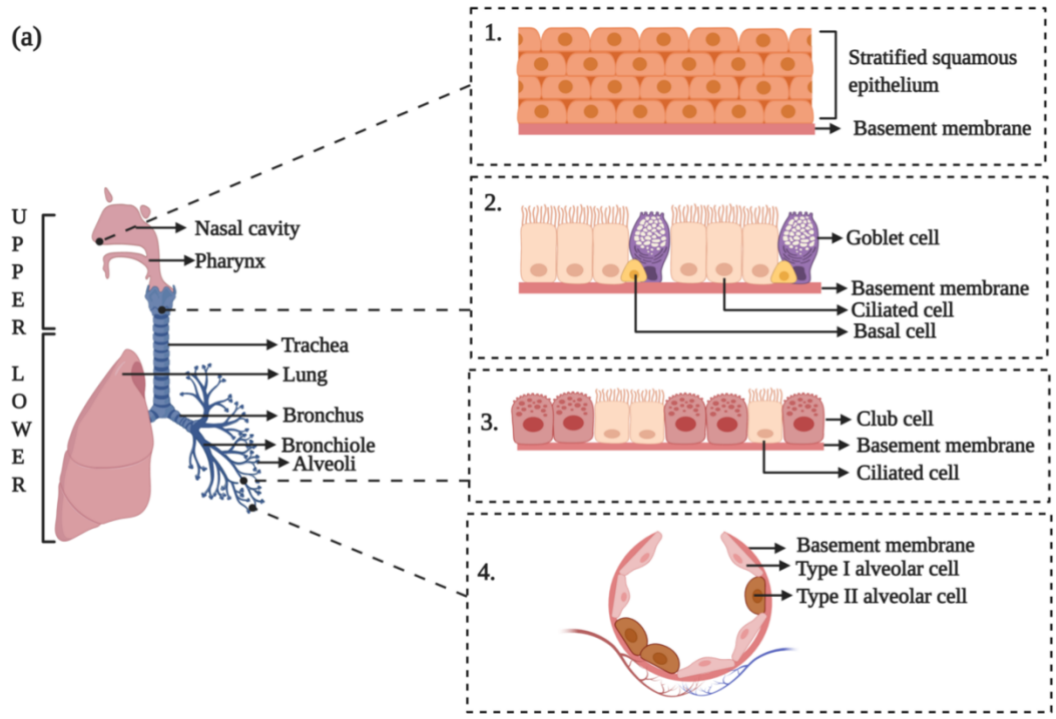


Figure 7. Diagram showing the types of epithelia present throughout the airways. The nasal cavity is composed of stratified squamous epithelium, while the epithelium of the conducting airways is composed of ciliated epithelial cells, goblet cells, brush cells, basal cells and small granule cells, whilst the bronchioles, which are at the intersection between the conducting and respiratory airways, are primarily composed of ciliated epithelial cells with a minority of microvillar cells, small granule cells and club cells. At the terminus of the lower airways, the alveoli are lined by type I and type II alveolar epithelial cells. Reproduced from (Adivitiya *et al.*, 2021).

1.5.1.1 Ciliated Epithelial Cells

Ciliated epithelial cells remove particulates and inflammatory stimuli from the airways (Vareille *et al.*, 2011). Cilia are specialized hair-like projections which beat in concert to form a key part of the mucociliary transport apparatus (Yaghi and Dolovich, 2016). They are responsible for transporting mucus and debris up the respiratory escalator to prevent irritation of the airways. Both endogenous and exogenous factors can affect this movement. In addition, the production of excess or abnormally thick mucus can also impede the ability of ciliated cells to clear the airways.

1.5.1.2 Club cells

Club cells are secretory cells found primarily in the terminal bronchioles, making up about 9% of the total number of epithelial cells (Rokicki *et al.*, 2016). They are cubical cells which are non-ciliated and do not produce mucus, and are sometimes referred to as non-ciliated non-mucous secretory cells. They were previously referred to as Clara cells. Their main product is club cell protein 16 (CC16), also known as uteroglobin, among other names. A mouse model of CC16 deficiency suggested that loss of expression led to greater susceptibility to viral infection and oxidative stress (Broeckaert and Bernard, 2000). Production of CC16 is influenced by expression of cytokines, notably IFN-γ. In addition to their secretory role, they also act as progenitor cells in the lung. They are also present in the uterus, kidneys, and prostate.

1.5.1.3 Pulmonary Brush Cells

The pulmonary brush cell, also known as the tuft, multivesicular, caveolated or fibrillovesicular cells, are found in both lung and gastrointestinal epithelium (Reid *et al.*, 2005). They have short, blunt microvilli that protrude from the apical surface between ciliated cells. The purpose of brush cells in not well-understood, although it is speculated that it may be involved in regulating the passage of fluid and solutes across the membrane.

1.5.1.4 Small Granule Cells

Small granule, or neuroendocrine-like epithelial cells, are found in both foetal and adult mammals across multiple species (DiAugustine and Sonstegard, 1984). They can occur as single cells, which are found throughout the airways, or as organoids, called neuroepithelial bodies (NEBs), which are found only in the intrapulmonary airways. The purpose of small granule cells is poorly understood, but studies have suggested that they function as intrapulmonary sensors of change in gas composition, such as hypoxia.

1.5.1.5 Basal Cells

Basal cells provide an anchor for the other types of epithelial cells, particularly for the ciliated epithelial cells. In addition, they are multi-potent progenitor cells in many tissues, including the lung (Hong *et al.*, 2004; Morrisey, 2018). Airway basal cells are capable of differentiating into ciliated and goblet cells. Basal cells more susceptible to

infection by major-type RHV than ciliated epithelial cells, possibly due to higher expression of ICAM-1 (Jakiela *et al.*, 2008). In their role as progenitor cells, airway basal cells are important for both maintenance of the epithelium in adulthood, as well as epithelial regeneration following acute injury such as that induced by influenza infections (Hong *et al.*, 2004). They are present in both large and small airways.

1.5.1.6 Goblet Cells

Goblet cells are an important source of mucus production in the conducting airways (Rogers, 1994). They line the mucosal surface, often protruding from the apical surface into the lung lumen. Goblet cells secrete a variety of mucins, high molecular-weight mucus glycoproteins, which are necessary for the efficient entrapment of foreign particles and microbes, and their subsequent removal by the mucociliary apparatus.

1.5.2 Immune Cells in the Lung

1.5.2.1 Eosinophils

Eosinophils are bone marrow-produced granulocytes which circulate in the blood at low levels, where they can be recruited to areas of immunological or inflammatory response (Possa *et al.*, 2013). Eosinophils have historically been considered cytotoxic effector cells, due to their ability to release toxic granule proteins, lipid mediators, cytokines and reactive oxygen species. However, eosinophils are capable of storing pre-formed chemokines and cytokines, enabling them to rapidly respond in various ways, with roles in initiating and maintaining inflammation, maintaining epithelial barrier function, mediating tissue remodelling, and providing interactions between the innate and adaptive immune system. Due to the low numbers of eosinophils in the blood (1-3% of peripheral blood leukocytes during health), eosinophils have been under-appreciated until recently, and their precise role remains poorly understood due to their varied roles (Possa *et al.*, 2013; Varricchi *et al.*, 2017). Despite the low numbers of circulating eosinophils in the blood, they are able to adhere to activated endothelial cells and accumulate in tissue in areas of allergic inflammation, such as the lungs of patients with eosinophilic asthma (Varricchi *et al.*, 2017). Eosinophilia is present in a significant proportion of patients with severe asthma, and is associated with more severe disease, increased exacerbations, decreased lung function and poor

pharmacological control (Possa *et al.*, 2013; Varricchi *et al.*, 2017; Pavord *et al.*, 2021).

1.5.2.2 Neutrophils

Neutrophils are bone marrow-derived white blood cells which mature in response to cytokine signalling, followed by carefully controlled emigration into the bloodstream to maintain a steady population, forming the majority of circulating peripheral white blood cells (Lacy, 2006). Neutrophils are densely packed with toxic secretory granules which they can release at sites of infection, and they are usually short-lived and tightly controlled to prevent uncontrolled inflammation (Lacy, 2006; Aulakh, 2018; Snelgrove *et al.*, 2018). Circulating neutrophils are divided into circulating and marginated pools, with lungs being the largest reservoir for marginated neutrophils (Lacy, 2006; Aulakh, 2018). Neutrophils are important for controlling infection in the lung via degranulation and release of pro-inflammatory membrane-bound secretory molecules, as well as by releasing a variety of antimicrobial peptides and cytokines which recruit additional leukocytes (Lacy, 2006). However, excessive numbers of neutrophils in patients with asthma contribute to inflammation and are associated with more severe asthma and poor responsiveness to corticosteroids (Snelgrove *et al.*, 2018).

1.5.2.3 Macrophages

Pulmonary macrophages are one of the most abundant leukocytes in the lung, forming a key part of the mononuclear phagocyte system, which also includes monocytes and dendritic cells (Lumeng, 2016). Macrophages were first described in 1892 by Metchnikoff as phagocytic cells, however it is now known that macrophages are responsible for many roles including maintaining pulmonary homeostasis, removing cellular debris, immune surveillance and response to infection, microbial clearance and resolution of inflammation (Byrne *et al.*, 2015). The pulmonary macrophage population is heterogeneous and shows remarkable plasticity to allow them to fulfil many roles (*ibid*). Macrophages have been underappreciated in the pathogenesis of asthma, partly because of their diversity of roles (Lumeng, 2016; Draijer and Peters-Golden, 2017). Changes in macrophages polarization associated with asthma have diverse effects, including defective phagocytosis, efferocytosis, changes to

inflammatory signalling and induction of the inflammasome (van der Veen, de Groot and Melgert, 2020). Macrophages are generally divided into two subsets based on their function and cytokine expression: pro-inflammatory M1 macrophages which respond to intracellular pathogens, and anti-inflammatory M2 macrophages which mediate apoptosis and phagocytosis of foreign pathogens (Balhara and Gounni, 2012). In addition, macrophages in the lung are subdivided by location into bronchial, interstitial and alveolar macrophages (*ibid*).

1.5.2.4 T cells

Asthma has until relatively recently been considered an atopic disease driven by allergen exposure and sensitisation and a Th2 CD4+ lymphocyte response, leading to IL-5 release and eosinophilic inflammation (Douwes *et al.*, 2002). While Th2-driven inflammation is an important contributor to inflammation in many asthma patients, only about 50% of asthma patients have Th2-high eosinophilic asthma, suggesting other T-cell types also have a role in asthmatic inflammation (Robinson *et al.*, 1992; Lloyd and Hessel, 2010). In general, Th2 cell activation results in IL-4, IL-5, IL-9 and IL-13 production, IgE production, eosinophil recruitment, mast cell activation and mucus production in response to parasites or as an inappropriate response to allergens (Herrick and Bottomly, 2003). Meanwhile, Th1 cells result in IFN-γ and TNF-β production and subsequent IgG2a production and macrophage activation in response to intracellular pathogens or autoantigens (Herrick and Bottomly, 2003). However, more recently additional types of T cells have been implicated in asthma, such as Th17 cells, CD8+ Tc2 cells and regulatory T cells. Th17 cells, one of the Th2-low cell types, produces IL-17, which is associated with airway neutrophilia and airway remodelling (Zhou *et al.*, 2017). Human CD8+ Tc2 cells are an underappreciated cell type which secretes type-2 cytokines and is enriched in patients with eosinophilic asthma, whereas Th2 cells are not (Hilvering *et al.*, 2019; Hinks, Hoyle and Gelfand, 2019). In healthy lung, regulatory T cells supress inflammation driven by Th2 cells in response to harmless particles, maintaining tolerance and preventing unchecked inflammation (Lloyd and Hawrylowicz, 2012).

1.5.3 Differences in nasopharynx compared to lower airways

Upper and lower airway diseases often mirror one another, with asthma patients often also experiencing rhinitis, as well as continuity in microbial communities between the nasopharynx and lower airways (De Boeck *et al.*, 2017; Samitas *et al.*, 2018). These similarities have led to the term 'one airway' or 'united airways'.

There is continuity in microbial communities between the lower airways and nasopharynx, with common bacterial species occupying both (De Boeck *et al.*, 2017). As seen in the lower airway, patients with positive nasopharyngeal cultures for Haemophilus influenzae and Streptococcus pneumoniae had less microbial diversity (Chonmaitree *et al.*, 2017). In addition, co-infection with airway pathogens and episodes of acute otitis media alter composition of the nasopharyngeal microbiome in infants (Chonmaitree *et al.*, 2017). The nasopharyngeal microbiome composition may alter host susceptibility to respiratory infections, chronic rhinosinusitis, asthma, pneumonia and otitis media (Clark, 2020; Dimitri-Pinheiro, Soares and Barata, 2020). The kinetics of NTHi accension from the nasopharynx to the Eustachian tubes and development of lower airway infection with the same bacteria suggests that bacteria are mobile within the airways. However, although the same bacteria may occupy both the upper and lower airways, the immune response mounted by each may be distinct.

Nasopharynx-associated lymphoid tissue (NALT) can be considered the primary inductive site of the mucosal immune system as the initial site of contact for many viruses and bacteria, whilst the lung and blood are effector sites (Vintiñi and Medina, 2014; Gallo *et al.*, 2021). NALT is a highly conserved, ancient immune compartment located in the most cranial pharyngeal mucosa in humans (*ibid*). It plays a key role in the induction of Th1- and Th2-polarised lymphocytes, as well as IgA-committed B cells (Gallo *et al.*, 2021). Together with nasal epithelial cells, goblet cells and dendritic cells, the nasopharynx is a key site for mediating the immune response in the respiratory tract and systemically (Gallo *et al.*, 2021). Analysis of the humoral and cellular immune responses in the nasopharynx in response to live or heat-killed *Lactobacillus casei* associated with pneumococcal antigen (P-Ag) demonstrated that both live and heat-killed vaccines induced specific IgG and IgA both in the upper and lower respiratory tract and systemically (Vintiñi and Medina, 2014).

The majority (up to 80%) of allergic rhinitis is driven by Th2 inflammation, however, the upper airway appears to be more adept at resisting injury than the lower airways, possibly because the immune response is milder, or because the nose is routinely exposed to environmental insults and has robust repair mechanisms (Samitas *et al.*, 2018). A methods paper used nasal curettes to collect cells from the inferior turbinate and absorptive matrices to collect nasal lining fluid (Jochems *et al.*, 2017). The immune cell proportions and cytokine profile using each technique were evaluated and compared to blood, showing that both T cells and granulocytes in nasal tissue were more highly activated than those in the blood (Jochems *et al.*, 2017). T cells were absent from the nasal lumen, and this was reflected in low levels of T cell cytokines IL-10, -17, -4, -5, -2, IFN-gamma and TNF in fluid from nasal washes (*ibid*). Few macrophages were found in nasal epithelial tissue from healthy patients, whilst exposure to influenza A virus resulted in macrophage recruitment (ibid). Research into the nasopharyngeal immune response has been primarily focussed on NALT and T-cell responses, while less is known about the role of epithelial cells.

1.6 Epithelial Barrier and Ciliary Function in the Lung

1.6.1 Barrier function in health

Epithelial cells produce mucus, remove particulate foreign material by ciliary beating, and produce antimicrobial peptides. Epithelial cells are the first line of defence in resisting infection by viruses (Vareille *et al.*, 2011).

The ciliated epithelium covers the surface of airways, providing a barrier to the external environment (Thomas *et al.*, 2010). It is also the first contact with the mucosal immune system for inhaled pathogens, antigens and particulate materials. The ciliated epithelium is lined by a layer of airway surface liquid and a mucus layer. The airway liquid layer covers the cilia and provides an optimal environment for the coordinated ciliary beating that occurs in healthy airways and clears the mucus, which is essential in pulmonary defence.

Mucus acts as a physical barrier between the respiratory tract and external environment (Zanin *et al.*, 2016). The primary role of mucus is to maintain hydration of the

respiratory tract and to trap foreign particles, including pathogens. These particles can then be swept from the respiratory tract by mucociliary clearance. This involves the rhythmic beating of cilia to form the 'mucociliary escalator'. It also interacts with the host, as well as being the first barrier to pathogens. Damage to the respiratory epithelium, from insults such as tobacco smoking, inhaled foreign bodies or infections, resulting in defective mucociliary clearance leads to increased susceptibility to infection and inflammation (Thomas *et al.*, 2010).

1.6.2 Defective barrier function in asthma

The epithelial barrier is controlled by the apical junctional complex, which is composed of the tight junctions and adherens junctions (Gon and Hashimoto, 2018). In allergic diseases such as asthma, epithelial homeostasis is shifted towards loss of differentiation, reduced junctional integrity and impaired innate defence (Schleimer and Berdinikovs, 2017). These abnormalities may pre-date atopy and rather contribute to the development of allergic disease. Exposure to external stimuli such as proteases, allergens and injury also increase mucosal permeability.

Decreased airway epithelial barrier function allows increased transcytosis of bacteria and particulates and is a common feature in the airways of patients with asthma and COPD (Gon and Hashimoto, 2018). Previous work has shown that NTHi infection in alveolar epithelium decreases expression of E-Cadherin, which is integral to maintenance of epithelial barrier function (Kaufhold *et al.*, 2017). Azithromycin treatment improves lung function in patients with cystic fibrosis (CF) without reducing bacterial populations, although the mechanisms remain unclear (Halldorsson *et al.*, 2010). Halldorsson *et al* (Halldorsson *et al.*, 2010) suggest that an azithromycin-induced increase in epithelial barrier function may be responsible. Their initial *in vitro* experiments suggested that azithromycin treatment increases expression of tight junction proteins in uninfected epithelial cells.

A 2008 review by Holgate postulates that the airway epithelium of patients with asthma is fundamentally abnormal and has undergone structural changes which profoundly alter the function of the airway wall (Holgate, 2008). Further data suggest that airway epithelial abnormalities are present in asthma patients from a young age

(Carsin *et al.*, 2016) and loss of lung function occur early in childhood (Covar *et al.*, 2004). The epithelial abnormalities were hypothesised to be via activation of the epithelial-mesenchymal trophic unit (EMTU) (Holgate, 2008). Mammalian lung development is a tightly regulated process which involves concerted activities of transcription factors, signalling molecules, and interactions between the epithelium and mesenchyme (Knight, Lane and Stick, 2004). The pulmonary mesenchyme is thought to influence the fate of the epithelium by producing a variety of growth factors, and was able to re-program the epithelial cells in a study where lung epithelium was engrafted on tracheal mesenchyme or vice versa (*ibid*). However, several groups have hypothesised that the EMTU may be activated inappropriately in patients with asthma, leading to structural changes to the epithelium (Knight, Lane and Stick, 2004; Holgate, 2008; Osei, Booth and Hackett, 2020). This activation is associated with impaired barrier function caused by abnormal formation of tight junctions, leading to increased susceptibility to injury as well as impeding epithelial repair. This concept of epithelial damage being the primary driver in asthma pathology suggests that future treatments for asthma should be directed to the epithelium and focus on protecting vulnerable airways from environmental insults, rather than on supressing inflammation and immune responses. Indeed this is consistent with promising data emerging from biological studies targeting epithelial 'alarmins', with phase 2 clinical trial from thymic stromal lymphopoietin (TSLP) inhibitor Tezepelumab reducing the frequency of clinically significant exacerbations in patients with moderate-to-severe asthma (Corren *et al.*, 2017).

1.6.3 Compromised barrier function in infection

Infection with NTHi reduced expression of the tight-junction protein E-cadherin in an in-vitro model (Kaufhold *et al.*, 2017). TGF-β is a key mediator of E-cadherin expression, directly repressing E-cadherin by upregulating Snail, Twist and Slug. In an *in vitro* model using alveolar epithelial cell type II (AECII) and A549 cells, a transformed alveolar epithelial cell line, E-cadherin mRNA expression and protein detection by western blot analysis was significantly reduced in cells infected with NTHi. In addition, the mTOR downstream target p70S6kinase was increased in NTHi infection, and blockade of mTOR by rapamycin ameliorated the reduction in E-cadherin induced by NTHi, suggesting that NTHi infection upregulates the mTOR

signalling cascade. In addition, expression of Slug is increased in response to NTHi, possibly implicating TGF-β signaling in the downregulation of E-cadherin and thus reduction of epithelial barrier integrity.

Basal cells also mediate the airway epithelial response to injury by producing the antimicrobial protein RNase 7 (Amatngalim *et al.*, 2015). In a model using PBEC culture composed primarily of basal cells, exposure to NTHi resulted in an increase in Rnase 7. In addition, in a model of air-liquid interface differentiated PBEC culture, exposure to cigarette smoke extract induced EGF-receptor mediated expression of Rnase 7 in basal cells visualized by microscopy. These data suggest that basal cells may have a role in epithelial injury as a second line of defence.

1.6.4 Abnormalities in Ciliary Function in Asthma

Ciliary and ultrastructural abnormalities in the airway epithelium are a feature of asthma, and are related to disease severity (Thomas *et al.*, 2010). Observations using digital high-speed video imaging revealed that the frequency of ciliary beating is decreased in bronchial epithelial strips in moderate and severe asthma, in addition to increased ciliary dyskinesia and higher immotility indices associated with disease severity. Transmission electron microscopy (TEM) examination revealed structural abnormalities in severe asthma, namely a decrease in ciliated cells and an increase in dead cells and ciliary disorientation compared to mild, moderate or healthy controls. Additionally, compared to mild asthma and healthy control epithelium, the epithelium of severe asthmatics displayed increased ciliary depletion, microtubular defects, mitochondrial damage and cytoplasmic blebbing. These results suggest that defects in ciliary beating are present in even moderate asthma, while only the airways of severe asthmatics have significant ultrastructural abnormalities. Deficiencies in ciliary beating reduce the airway's ability to clear mucus and pathogens, possibly contributing to the prevalence of NTHi infection in patients with asthma, although it is presently unclear whether the abnormalities in ciliary function contribute to the progression of disease or are merely symptomatic of airway damage.

1.7 Project Aims

Aim 1:

There is evidence both from patient-derived tissue biopsies and *in vitro* models that NTHi is found within the epithelial monolayer (Morey *et al.*, 2011). However, it is not clear whether NTHi actually replicates within the epithelial monolayer or if the bacteria replicate elsewhere, such as a biofilm or within macrophages. In addition, there is some evidence that NTHi preferentially infects ciliated epithelial cells (Ren *et al.*, 2012; Baddal *et al.*, 2015b).

My first aim was to examine interactions between NTHi and the airway epithelium.

Specifically, I addressed the following objectives:

- Does invasion rate differ by strain?
- Does invasion differ between cell types?
- Are some strains more adherent than others?
- Is NTHi capable of selectively replicating within a differentiated airway epithelial monolayer?
- Is NTHi found intracellularly within a particular type of epithelial cell in a differentiated epithelium?
- Is intracellular NTHi found within the early or late endosome?

I evaluated preferential infection in differentiated epithelial cells using flow cytometry and microscopy. In addition, I performed experiments to evaluate whether a portion of NTHi associated with the monolayer are paracellular rather than intracellular, possibly due to damaged tight junctions between the epithelial cells. Adhesion, paracellular and intracellular internalisation rates of several NTHi strains were also evaluated. Further microscopy experiments were directed at understanding the intracellular lifestyle of NTHi and its localisation within early versus late endosomes.

Aim 2:

Bacterial internalisation may contribute to persistence of NTHi and act as a trigger for immune activation. I initially hypothesised that highly invasive strains of NTHi

would elicit a greater immune response than less-invasive or non-invasive NTHi. In addition, I hypothesised that greater adhesion would lead to more robust immune activation.

My second aim was to evaluate the importance of adhesion and internalisation of NTHi in the lung epithelium for immune activation or evasion.

Specifically, I addressed the following objectives:

- Does increased invasion correlate with increased immune activation?
- Do more adherent strains provoke a stronger immune response?
- Transcriptomic analysis of signalling associated with NTHi infection and treatment with azithromycin.

Using an *in vitro* model of NTHi infection, I evaluated the immune activation in response to NTHi strains with varying rates of internalisation and adhesion. In collaboration with other members of the lab, I also performed a transcriptomic analysis to evaluate gene expression in epithelial cells in response to NTHi infection and azithromycin treatment.

Aim 3:

NTHi is a common commensal organism in the nose, where it does not cause significant disease. Conversely, NTHi infection in the lung is pathogenic and likely contributes to the inflammation in steroid-resistant asthma. I hypothesised this is due to differing immune responses mounted by different tissue types

The third aim of this project was to determine whether human-NTHi host-pathogen interactions differ between the upper and lower respiratory tract.

Specifically, I addressed these objectives:

- Are invasion rates different in nasal versus lower airway epithelium?
- Does immune activation differ between NTHi infection in lower airway and nasal cells?
- Is this differential immune response specific to NTHi?

I initially evaluated invasion rates of several NTHi strains in nasal and lung epithelium *in vitro*. Following this, I evaluated a wide range of immune markers by qPCR and PCR array to compare the inflammatory cascade triggered by NTHi infection in nasal versus bronchial epithelium.

Chapter 2 Materials and Methods

2.1 Cell culture

2.1.1.1 BEAS-2B Cell Subculture

BEAS-2B cells are an immortalised bronchial epithelial cell line widely used for asthma studies (Han *et al.*, 2020). Although immortalized, BEAS-2B cells share many characteristics with primary bronchial epithelial cells (PBECs), and they are similarly susceptible to bacterial and viral infection (Sajjan *et al.*, 2006). In addition, I have cultured BEAS-2B cells in defined serum-free media, which is thought to promote a more epithelial phenotype than culture in FBS-supplemented media (Malm, Amouzougan and Klimecki, 2018). However, BEAS-2B cells do not form robust tight junctions or develop a high TER, so they are not comparable to primary cells for barrier function studies (Stewart *et al.*, 2012b). Cell lines have formed an important part of my thesis investigations due to their availability, fast growth, and consistency from one experiment to the next. However, due to their limitations in recapitulating the features of native tissue, I have attempted to replicate key experimental findings from cell lines using primary cells whenever possible. BEAS-2B cells (ATCC) were maintained in collagen-coated flasks in serum-free airway epithelial cell media (PromoCell) supplemented with 100 μg/mL Penicillin/Streptomycin (Gibco). To encourage adherence of BEAS-2B cells, flasks were collagen coated by incubating at RT for 2 hours, or overnight at 4°C with 100μg/mL PureCol (Advanced BioMatrix, 97% type I atelocollagen, 3% type III collagen) diluted in H_2O. Collagen is a common extracellular matrix protein which is widely used in cell culture applications, encouraging cell attachment, proliferation and appropriate differentiation to recapitulate normal tissue physiology. PureCol solution was then removed, the flasks washed with Dulbecco's phosphate buffered saline (DPBS) (Sigma), and allowed to dry. Cells were seeded for maintenance at 3000-5000 cells/cm^2 and sub-cultured upon reaching 70-80% confluence. To sub-culture, trypsin – ethylenediaminetetraacetic acid (EDTA) (Sigma, 0.1mM Trypsin, 0.9mM EDTA) was supplemented with 0.5% polyvinylpyrrolidone (PVP), an antioxidant. Soybean trypsin inhibitor (0.5mg/mL in DPBS, Gibco) was used to inactivate the trypsin at a 1:1 ratio. BEAS-2B cells were

sub-cultured at 70% confluency and media were renewed every 48 hours. Cells were cryopreserved in 45% conditioned media, 45% new media, and 10% DMSO (Sigma) at $1x10^6$ cells/mL.

2.1.2 Calu-3 Cell Culture

Calu-3 cells are epithelial cells derived from lung adenocarcinoma and are widely used both as a model for bacterial activation as well as immune activation (Sajjan *et al.*, 2008; Hirakata *et al.*, 2010; Stewart *et al.*, 2012b). They exhibit TLR1, TLR2, TLR3, TLR4 and TLR6 signalling, as well as expressing downstream factors MyD88, MD2 and CD14 (Melkamu and O'Grady, 2007; Ragupathy *et al.*, 2014). In addition, they are capable of forming tight junctions and are suitable for studies evaluating barrier function (Stewart *et al.*, 2012b). Calu-3 cells were cultured at 37°C in a 5% CO_2 incubator in Eagle's minimum essential media (EMEM) with non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 1500 mg/mL sodium bicarbonate supplemented with 10% foetal bovine serum (FBS). Cultures were maintained in media with 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco). Cells were subcultured upon reaching 80% confluence and seeded at a 1:3 to 1:6 ratio. Media were renewed every 48 hours. Cells were cryopreserved in complete media supplemented with 5% DMSO.

2.1.3 RPMI 2650 Cell Culture

The RPMI 2650 cell line is an epithelial cell line derived from nasal septum metastatic pleural effusion. It is widely used as a model of nasal epithelium (Salib, Lau and Howarth, 2005; Kreft, Lasi and Kristan, 2015). I have used it in conjunction with primary nasal epithelial cells for comparison between infection in commensal nasal colonisation with NTHi and pathogenic lower airways infection in bronchial epithelial cells. RPMI 2650 cells were cultured at 37°C in a 5% CO_2 incubator in Eagle's minimum essential media (EMEM) with non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate and 1500 mg/mL sodium bicarbonate supplemented with 10% foetal bovine serum (FBS). Cultures were maintained in media with 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco). Cells were subcultured upon reaching 70% confluence and seeded at a 1:2 to 1:4 ratio. RPMI2650 cells grow in clumps and pile on top of one another, so they may never reach 100%

confluence. Media were renewed every 48 hours. Cells were cryopreserved in complete medium supplemented with 5% DMSO.

2.1.4 Primary Bronchial Epithelial Cell (PBEC) Culture

Primary bronchial epithelial cells were cultured from bronchial brushings taken from 3rd to 5th order bronchi from healthy controls after obtaining written informed consent. All PBECs used in this thesis were isolated from healthy control donors. The study was reviewed by Oxford Research Ethics Committee B (18/SC/0361) and Leicestershire, Nottinghamshire and Rutland Ethics Committee, UK (08/H0406/189). In addition, all samples were processed and stored in accordance with the Human Tissue Act. Bronchoscopies were performed using Pentax video colour CCD flexible bronchoscope (Pentax UK, Slough, UK) in an endoscopy suite with the assistance of at least 2 nurses and a laboratory technician and in accordance with the BTS guidelines (2014) and with established research protocols (Jarjour *et al.*, 1998; British Thoracic Society, 2014). Briefly, subjects who had been starved for at least 4 hours underwent routine physical examination and routine measurement of vital signs. An intravenous cannula was inserted and the procedure performed under light sedation with fentanyl 0-100 mcg and / or midazolam 0-10 mg with continuous pulse oximetry. The analgesia and suppression of gag and cough reflexes was achieved with 6-8 sprays (60-80 mg) of 10% lidocaine orally, 2-5 ml of Instillagel (CliniMed, High Wycombe, UK) nasally, 6 ml of 2% lidocaine to the vocal cords and 5-10 ml of 2% lidocaine to the bronchial tree. Bronchial brushings were obtained from right and left lower and middle lobe 3rd to 5th order bronchi by gentle brushing using sterile 2 mm disposable cytology brushes (BC-202D-2010, Olympus UK, Southend-on-Sea, UK) and samples gently agitated in 5ml of ice-cold PBS. Brushings were taken prior to other samples to minimise contamination with blood which would decrease the cellular purity of samples and can impair growth of primary bronchial epithelial cells *in vitro*. After the procedure subjects were observed for at least 60 minutes, before having their swallow tested.

The brushes were collected in 15 mL Falcon tubes containing 4 mL serum-free airway epithelial cell media (PromoCell) supplemented with 200 μg/mL gentamicin and 0.515 μg/mL amphotericin B (Lonza), 100 IU/mL penicillin and 100 μg/mL streptomycin

(Gibco) and transported on ice. The cells were centrifuged at 200 g for 10 minutes and resuspended in 1 mL of medium. If blood was visible in the sample, a red blood cell lysis step was performed by resuspending the cells in low-endotoxin tissue-culture grade dH2O (Gibco) for 30 seconds before adding 10X Hank's balanced salt solution (HBSS) (Gibco) and topping up with DPBS prior to centrifugation and resuspension in media. The cells were counted and plated in the same media at 5000–8000 cells/cm^2 in 6-well plates pre-coated with PureCol (Advanced BioMatrix). The cells were incubated for 4 hours before the supernatants were removed and cultured in new plates, and the media replaced with airway epithelial cell media supplemented with 30 μg/mL gentamicin and 15 ng/mL amphotericin, and 100 IU/mL penicillin and 100 μg/mL streptomycin. Media were renewed every 48 hours and subcultured at 70% confluency until reaching passage 2, at which time cells were either plated for bacterial infection experiments or cryopreserved for later use. Cells were cryopreserved in 45% conditioned media, 45% new media, and 10% dimethyl sulfoxide (DMSO) (Sigma) at $1x10^6$ cells/mL.

2.1.5 Nasal Epithelial Cell (NEC) Culture

Nasal epithelial cells are used as a model of airway epithelial cells due to the scarcity and difficulties in culturing PBECs (Comer, Elborn and Ennis, 2012; Kreft, Lasi and Kristan, 2015), compared with the ready availability of NEC which can be obtained via a minimally invasive procedure after obtaining informed consent. The study was reviewed by Oxford Research Ethics Committee B (18/SC/0361) and Leicestershire, Nottinghamshire and Rutland Ethics Committee, UK (08/H0406/189). In addition, all samples were processed and stored in accordance with the Human Tissue Act. There are differing opinions regarding the suitability of NECs as an alternative experimental substitute for PBECs, particularly in infection experiments given that some bacteria such as NTHi readily colonise the nasopharynx, while generally being cleared from the lungs more rapidly (Comer, Elborn and Ennis, 2012). However, other groups do use nasal epithelial cultures as a model of airway epithelium, though like any tissue culture model it does have limitations (Broadbent *et al.*, 2020). I have therefore used NECs for preliminary and optimization experiments in conjunction with cell lines, because of the presumed similarity in innate immune responses in these two tissue

types, but critical experiments have been performed using PBECs wherever possible. Nasal epithelial cells were cultured from nasal brushings collected from healthy volunteers. The brushes were collected using 2 cm cytology brushes (19H19, Medical Wire & Equipment, Corsham, UK) in 15mL Falcon tubes containing 4 mL serum-free airway epithelial cell media (PromoCell) supplemented with 200 μg/mL gentamicin and 0.515 μg/mL amphotericin B (Lonza), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco) and transported on ice. The cells were passed through a cell strainer to remove debris, and washed with 20 mL DPBS. The sample was then centrifuged at 200 g for 10 minutes and resuspended in 1mL of media. The cells were counted and plated in the same media at 5000–8000 cells/cm^2 in 6-well plates pre-coated with PureCol (Advanced BioMatrix). The cells were incubated for 4 hours before the supernatants were removed and cultured in new plates, and the media replaced with airway epithelial cell media supplemented with 30μg/mL gentamicin and 15ng/mL amphotericin, and 100 IU/mL penicillin and 100μg/mL streptomycin. Media were renewed every 48 hours and sub-cultured at 70% confluency until reaching passage 2, at which time cells were either plated for bacterial experiments or cryopreserved for later use. Cells were cryopreserved in 45% conditioned media, 45% new media, and 10% DMSO (Sigma) at 1x10^6 cells/mL.

2.1.6 Air-liquid Interface (ALI) Culture

Air liquid interface (ALI) culture of primary cells provides a useful model for studying asthma at the level of the epithelium (Ehlers *et al.*, 2017). ALI culture is able to model differentiated epithelium, containing differentiated ciliated, basal and goblet cells, unlike submerged culture. In addition, culture at ALI results in different protein expression that more accurately recapitulates that found in native epithelium (Wang *et al.*, 2018). Nasal epithelial cells or primary bronchial epithelial cells were cultured on 0.4 μm pore polyester membrane permeable inserts (Corning, catalogue number 3801) coated with collagen in ALI media (StemCell Pneumacult). They were maintained in submerged culture until reaching confluence, approximately two days. Upon reaching confluence, the cultures were brought to ALI by removing the media from the upper well. They were maintained at ALI for four weeks to allow the cells to form a

differentiated epithelial monolayer. Media in the lower well was exchanged by 50% every two days in cultures at ALI.

Figure 1. Air-liquid interface culture

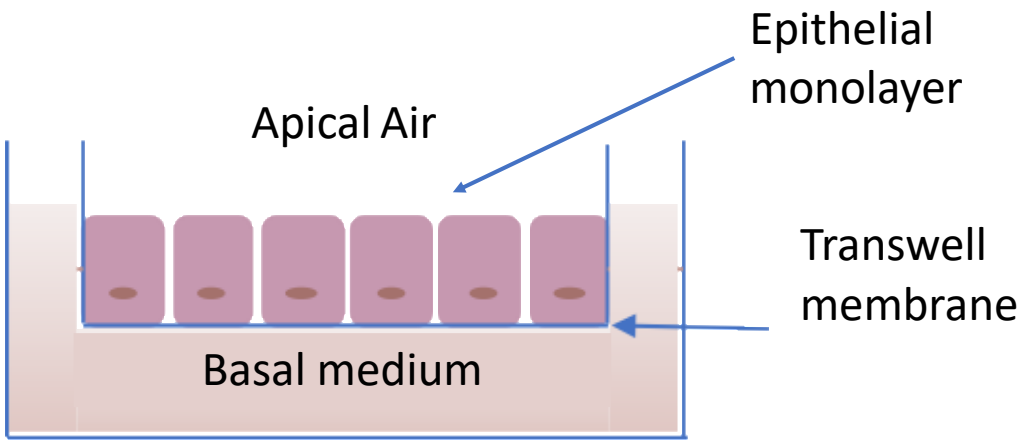


Figure 1. Diagram showing a cross-sectional view of the air-liquid interface culture technique. The air-liquid interface involves culturing epithelial cells on transwells, exposed to media on the basolateral side and air on the apical side, which encourages polarisation of cells, the development of tight junctions and high transepithelial electrical resistance, and differentiation into distinct cell types.

2.1.7 Transepithelial Electrical Resistance (TER) Measurements

Transepithelial electrical resistance (TER) is used as a measure of epithelial barrier function by measuring electrical resistance across the membrane from apical to basal compartment (Shuler and Hickman, 2016). An EVOM (World Precision Instruments) voltmeter is used with a probe to take measurements while the transwell inserts remain in the plate. The probe has two prongs, the shorter one will be submerged in the media in the top well, while the other will reach the bottom well, enabling measurement across the membrane. In ALI culture, where there is no medium in the top well, PBS is added while the measurements are being taken. All measurements were taken in Ohms and normalised using the area of the insert by multiplying the measurement by the area of the insert, resulting in a value expressed in Ohms.cm^2. For example, a

12mm diameter insert has a surface area of 1.12 cm^2, thus if the measurement was 500 Ohms, the normalised value would be expressed as 560 Ohms.cm^2.

2.2 Immunofluorescence Imaging

Immunofluorescence (IF) imaging visualises target molecules using fluorescence-tagged antibodies. IF provides information not only about the relative intensity of expression, but also of the localisation. It is widely used for infection and characterisation studies, as it visualises localisation of bacteria within different cell types within a heterogeneous population. Cell cultures grown at ALI on permeable polyester transwell inserts are amenable to immunofluorescence imaging. PBEC cultures were grown at ALI for a minimum of 28 days to ensure differentiation prior to infection. NTHi strain 398 were labelled using Carboxyfluorescein succinimidyl esther (CFSE) (Invitrogen CellTrace, C34554) according to the manufacturer's instructions, incubating bacteria in the presence of CFSE dye for 20 minutes at room temperature prior to washing to remove dye (Table 1). CFSE covalently binds to intracellular amines within the bacteria, producing a stable fluorescent dye which can be preserved using aldehyde fixation methods. Cell cultures were then infected with the stained bacteria according to the standard protocol. Following experimental infection, the transwells were fixed at room temperature in 4% paraformaldehyde (PFA) for 15 minutes. The transwells were then washed 3x with PBS for 5 minutes, followed by incubation with blocking buffer for 1 hour at room temperature (Cell Signalling Technology Immunofluorescence Blocking buffer, 12411). Primary antibodies (Table 1) were diluted using antibody diluent containing 1% BSA and 0.3% Triton-X100 in PBS, applied to transwells and incubated for overnight at 4°C. The antibody solution was then removed and the transwells were washed 3x 5 minutes. The secondary antibodies were then applied and incubated at room temperature for 1-2 hours. The transwells were then washed 3 x 5 minutes with PBS. The transwell membranes were excised using a scalpel and mounted face down onto glass coverslips using hard-setting mounting media (ThermoFisher, ProLong Glass). The coverslips were then applied to glass slides, positioned so that the transwell membrane was sandwiched between the slide and coverslip, with the apical side facing upward through the coverslip. The cover slips were allowed to cure protected from light for 24

hours prior to imaging. Imaging was then performed on a Zeiss 880 confocal microscope using various oil objectives.

Table 1. Immunofluorescence Antibody Information

Target	**Marker**	**Species**	**Dilution**	**Source and catalogue #**	**2º antibody**	**Source and catalogue #**	**2º Antibody dilution**	**Conjugate**
Epithelial Cell Panel								
Integrin subunit alpha 6 (CD49f)	Basal cells	Rat	1:50 (Oki *et al.*, 2012)	R&D Systems, MAB13501	Goat anti Rat IgG H&L	Abcam, ab150167	1ug/ml	Alexa Fluor 647
Acetylated alpha-tubulin	Ciliated cells	Rabbit	1:500	Abcam, ab179484	Goat anti Rabbit IgG H&L	Abcam, ab175652	2ug/ml	Alexa Fluor 405
MUC5AC	Goblet cells	Mouse	1:100	Abcam, ab3649	Goat anti mouse IgG H&L	Abcam, ab150118	5ug/ml	Alexa Fluor 555
NTHi strain 398 stained with CFSE								CFSE (488 nm)
Endosome Marker Panel								

EEA1	Early endosomes	Goat	5ug/ml	Abcam, ab206860	Donkey anti goat IgG H&L	Abcam, ab150135	2ug/ml	Alexa Fluor 647
LAMP1 (H4A3)	Late endosomes	Mouse	1:50	Santa Cruz, sc-20011	Goat anti mouse IgG H&L	Abcam, ab150118	5ug/ml	Alexa Fluor 555
Nuclear dye								NucBlue (358 nm)
NTHi strain 398 stained with CFSE								CFSE (488nm)

2.3 Growth of NTHi

NTHi strains were grown overnight on chocolate Columbian horse blood agar (ThermoFisher) at 37°C in a 5% CO_2 incubator. A loop of plated bacteria was taken with from an area of dense bacterial growth and added to 15 mL sBHI (Brain-Heart Infusion (BHI, Sigma) supplemented with 2 μg/mL nicotinamide adenine dinucleotide (NAD) and 10 μg/mL bovine hemin) in a 50 mL Falcon tube. It was then incubated at 37 °C and 5% CO_2 in a shaking incubator at 200 g until reaching an optical density at 600 nm (OD_{600}) of 0.4 to 0.8, as determined by growth curve analysis of each strain (Figure 2). Upon reaching the exponential growth stage, the number of colony forming units (CFU) per mL of bacterial suspension was estimated by performing a CFU count assay. Bacterial suspension was diluted $1:10^4$, $1:10^5$, $1:10^6$ or $1:10^7$ and 10 μl of each dilution applied in triplicate to chocolate agar plates and cultured overnight at 37°C and 5% CO_2. The number of colonies per 10 μl dot was counted in triplicate for 1 dilution per sample and CFU/mL calculated. 500 μl aliquots of bacterial suspension were supplemented with 125 μl 80% glycerol (final concentration 16%) as a cryoprotectant to reduce formation of ice crystals for cryopreservation at -80°C. Alternatively, NTHi to be used for experiments requiring heat-inactivation were heat-treated for 30 minutes at 65°C and plated on chocolate agar to confirm inactivation. All strains were a kind gift from Dr. Derek Hood at MRC Harwell.

Figure 2. Growth curve analysis of NTHi strains

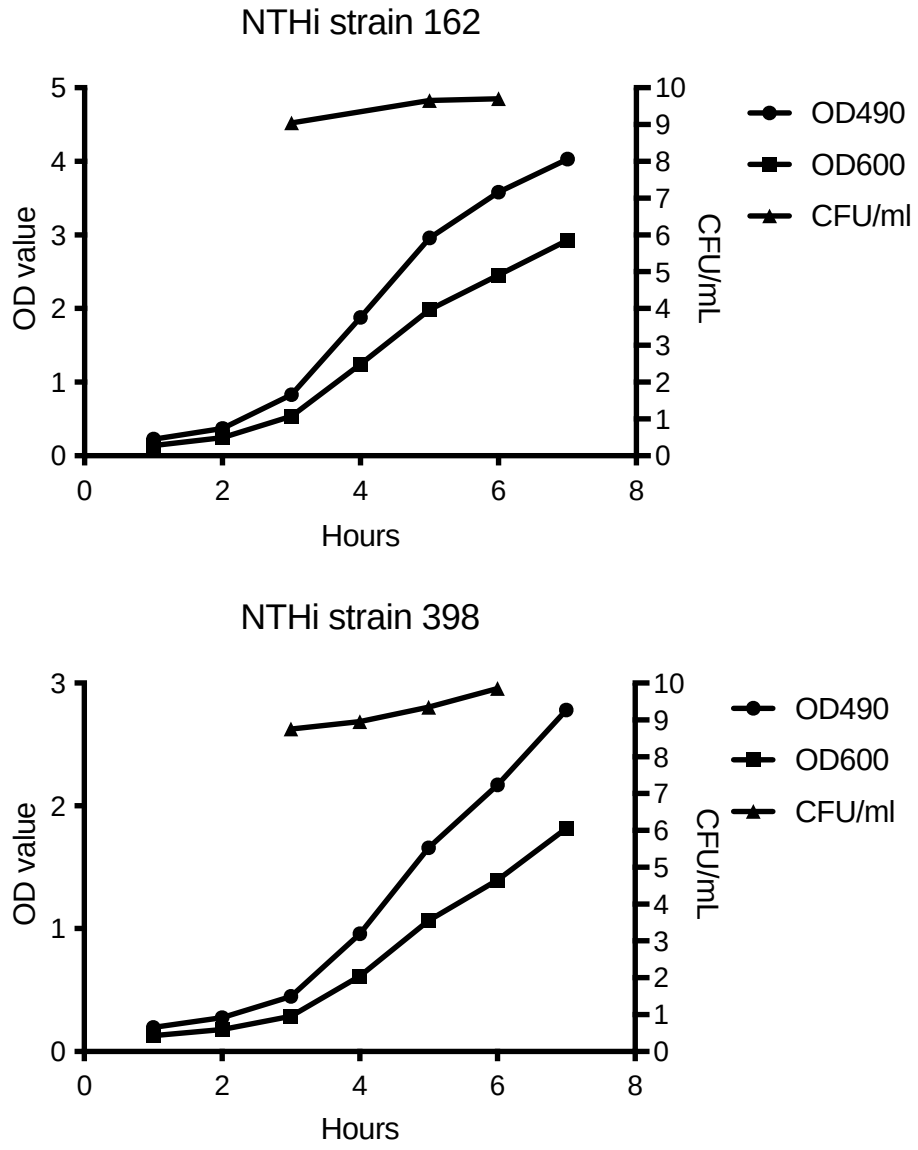


Figure 2. Growth curve analysis comparing OD readings with numbers of viable NTHi was performed to allow accurate estimation of bacterial numbers in culture. Several NTHi colonies were used to inoculate BHI broth culture and incubated 37 °C and 5% CO_2 in a shaking incubator at 200 g, and OD measurements were taken at hourly intervals to determine when the exponential phase of growth was reached. Once the exponential phase had begun, a series of CFU assays were performed to allow estimation of the CFU/mL present at a given OD. Given the heterogeneity of NTHi, several strains were evaluated to ensure accurate estimation, with strains 162 and 398 shown here with similar results. Both OD values from 490 nm and 600 nm measurements are used in the literature, and I have performed measurements at both wavelengths with consistent results.

Table 2. NTHi Strains Selected for Investigation

Strain	Clinical Presentation	Literature Reference
398sr[1]	COPD	(Martí-Lliteras *et al.*, 2011)
1566sr	COPD	(Martí-Lliteras *et al.*, 2011)
1553	COPD	(Martí-Lliteras *et al.*, 2011)
1500	COPD	(Martí-Lliteras *et al.*, 2011)
1560	COPD	(Martí-Lliteras *et al.*, 2011)
1607	COPD	(Martí-Lliteras *et al.*, 2011)
162sr	Otitis Media	(Fox *et al.*, 2008)
375sr	Otitis Media	(Fox *et al.*, 2008)
176sr	Otitis Media	(Fox *et al.*, 2008)
Finland 51sr	Otitis Media	
Finland 54sr	Otitis Media	
285sr	Otitis Media	(Fox *et al.*, 2008)
1124sr	Otitis Media	(Fox *et al.*, 2008)
1158sr	Otitis Media	(Fox *et al.*, 2008)
723sr	Otitis Media	(Fox *et al.*, 2008)

2.3.1 Bacterial Internalisation Assay

Bacterial internalisation assays are a well-established method to determine rates of internalisation into epithelial cells *in vitro* (Virji *et al.*, 1992). In brief, epithelial monolayers are inoculated with bacteria and are then treated with gentamicin to eliminate extracellular bacteria prior to lysis of the epithelial cells and estimation of

[1] sr indicates that the strain is resistant to streptomycin.

the number of intracellular bacteria. Gentamicin is an aminoglycoside antibiotic effective only against extracellular bacteria, leaving the intracellular bacteria intact. Although widely used, the rates of internalisation are notably variable in literature. To ameliorate this, I have optimised this assay and established a strict procedure to reduce variability. Epithelial cells were cultured overnight in 24 or 48 well plates pre-coated with PureCol. BEAS-2B cells were cultured at $5.3x10^4$ cells/cm^2 in 24 or 48 well plates to achieve approximately 70% confluence after overnight culture. NTHi stocks were thawed and resuspended in 0.5 mL antibiotic-free airway epithelial cell culture media (PromoCell). The bacterial suspension was then diluted in antibiotic-free airway epithelial cell media using previous estimates of stock concentration, and CFU/mL concentration confirmed by plating 10-fold dilutions on chocolate agar plates and calculating CFU/mL. The epithelial cells were incubated with bacteria at MOIs of approximately 5-25 for 4 hours at 37°C in a humidified CO_2 incubator and then washed twice with sterile DPBS. Medium containing 200 mg/mL gentamicin (Gibco) was then added, and they were incubated for a further 1.5 hours to eliminate extracellular bacteria. Cells were then washed twice with DPBS. Epithelial cells were then lysed to release the intracellular bacteria. Early experiments utilised 1% Saponin as a lysis agent, incubating on ice for 45 min – 1hr. Later experiments used 1% Triton-X 100 diluted in PBS for lysis, incubated for 15 minutes at room temperature. An optimisation experiment was performed to ensure that Triton-X did not damage bacterial viability and to determine incubation time (Figure 3). Triton-X more completely lysed cells, yielding more consistent results than Saponin.

Figure 3. NTHi viability with Triton-X

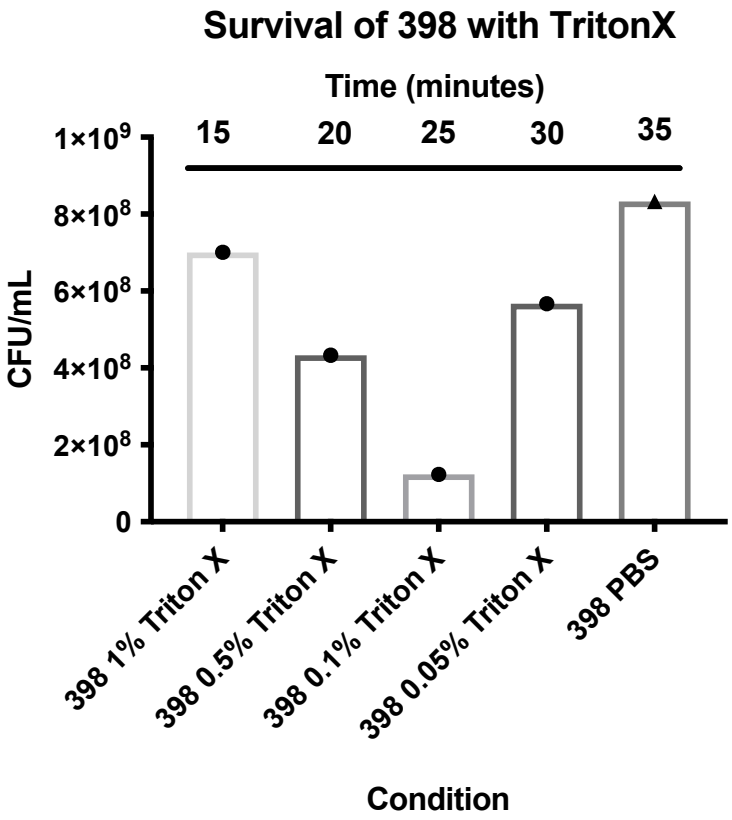


Figure 3. NTHi strain 398 was incubated in the presence of Triton-X at different concentrations. Incubation times varied from 15 minutes at 1% Triton-X increasing in 5 minutes increments to 30 minutes at 0.5% Triton-X, to reflect the time it takes for the diminishing concentrations to lyse epithelial cells. Based on this data, going forward I have used 1% Triton-X for 15 minutes.

Suspension containing lysed cells was then plated on chocolate agar plates either neat or at 10-fold dilutions to calculate CFU/mL concentrations and estimate numbers of intracellular bacteria.

Percent internalisation is calculated by dividing the total number of colony forming units (CFUs) released from the epithelial cells when lysed by the number of colony forming units that were added to the epithelial cells, and the result multiplied by 100.

Eg. If the CFU count shows that 1000 bacteria were released from the epithelial cells after incubation, and 100,000 bacteria had been initially added to the well then % internalisation = 1,000/100,000 x 100 = 1% internalisation

Figure 4. Diagram of Bacterial Internalisation Assay

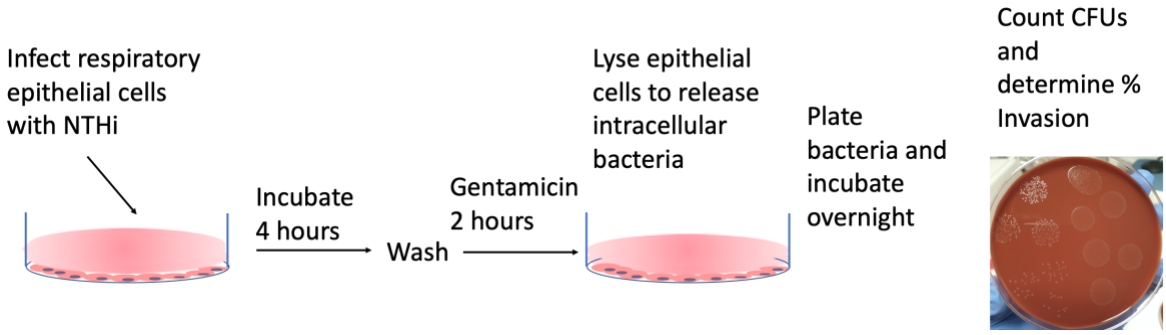


Figure 4. Bacterial internalisation, or CFU assays, were performed to evaluate the number of bacteria present within the monolayer of epithelial cells following inoculation with NTHi. Epithelial cells were inoculated with bacteria for a minimum of 4 hours, washed with PBS, and then treated with gentamicin for 2 hours to eliminate extracellular NTHi. Epithelial cells were then lysed using either a saponin or Triton-x protocol described above, and serial dilutions were performed prior to cell lysates being dotted on chocolate agar and incubated overnight. The CFUs on each plate were then counted, and calculations performed to determine the number of internalised bacteria, and compared with the number of bacteria in the inoculum to determine internalisation rate.

2.3.2 Enumeration of adherent bacteria

To enumerate the number of bacteria that have adhered to the epithelial monolayer, cell cultures infected with NTHi were washed to remove the non-adherent bacteria, and were then lysed and plated to determine numbers of viable CFUs. To account for the intracellular bacteria that would be included in the CFU count, matched wells which were treated with gentamicin to kill extracellular bacteria were lysed and the number of recovered intracellular CFUs subtracted from the number of adherent and internal bacteria to leave the number of adherent, non-internalised bacteria. This technique was performed on both submerged and ALI cultures.

2.3.3 Comparison of paracellular and intracellular bacterial numbers

An adaptation of the internalisation CFU experiment was developed to determine the numbers of bacteria sheltering from antibiotics in the paracellular space rather than intracellularly. Submerged or ALI cultures were infected with NTHi as above,

however, prior to gentamicin treatment the cells were trypsinised for 15 minutes to separate the epithelial cells so that paracellular bacteria would be exposed to gentamicin. The cell suspension was treated with gentamicin for 1.5 hours at 37°C in a 5% CO_2 incubator gently shaking at 80 RPM to prevent cells from clumping. The suspension was then washed with PBS prior to lysing epithelial cells and plating bacteria for the CFU count assay as above. These CFU counts were subtracted from the CFU counts of the standard internalisation assay to provide estimates of paracellular and intracellular bacteria individually.

2.3.4 Isolation of azithromycin-resistance plasmids

Azithromycin resistance plasmids in shuttle vector pLS88 in *Haemophilus influenzae* Rd were a kind gift from Dr. Christopher Atkinson at the University of Tasmania (Atkinson, Kunde and Tristram, 2017). The resistant *H. influenzae* Rd were grown overnight on chocolate agar plates at 37°C in a 5% CO_2 incubator. A loopful of bacteria from an area of dense growth was used to inoculate 10 mL sBHI with 10 μg/mL kanamycin and grown at 37°C in a 5% CO_2 incubator shaking at 200 rpm until reaching an OD_{600} of 0.4 to 0.8. The cells were then centrifuged at 13000 rpm and the plasmid DNA isolated using a Qiagen Miniprep Plasmid isolation kit according to the manufacturer's instructions. The plasmid DNA was eluted in 10 mM Tris-Cl pH 8.5 (Qiagen EB Buffer) as recommended and stored at -20°C.

Figure 5. pLS88 vector map

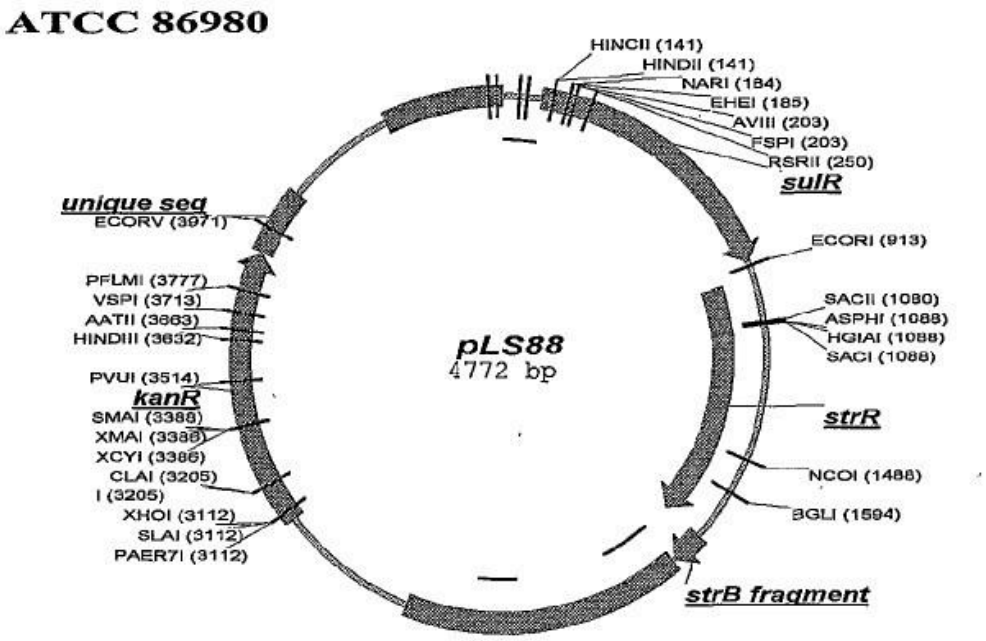


Figure 5. Diagram showing the vector map for shuttle vector pLS88. Azithromycin resistance plasmids inserted into the pLS88 shuttle vector were used to generate azithromycin-resistant NTHi for use in transcriptomics studies.

2.3.5 Generation of Azithromycin-resistant NTHi

NTHi stocks were thawed and cultured overnight at 37°C and 5% CO_2 on chocolate horse blood agar plates. A loopful of bacteria was taken from a dense area of growth and used to inoculate 20 mL of sBHI and cultured for approximately 4 hours at 37°C in a 5% CO_2 incubator until reaching an OD_{650} of 0.2 to 0.4. Cells were then centrifuged at 13,000 rpm for 5 minutes. To induce competence, the cell pellet was washed in 20 mL MIV starvation media, resuspended in 5 mL MIV and incubated for exactly 100 minutes in a shaking incubator at 90 rpm. MIV non-growth medium contained 80 mM NaCl, 10 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KH_2PO_4, and per liter: 1.0 g fumarate, 0.00g g citrulline, 4.0 g aspartate, 0.2 g glutamate, 0.004 g cysteine, 0.01 g tyrosine, 0.02 g phenylalanine, 0.03 g serine, 0.02 g alanine, 0.5 g Difco-free casamino acids and 0.02% Tween-80, with a pH adjusted to 7.4 (Herriott, Meyer and Vogt, 1970; Macfadyen *et al.*, 2001). 3-5 μg of previously isolated plasmid DNA was

added to 250 μl competent bacteria and incubated for 30 minutes prior to addition of 1 mL sBHI and incubation for a further 30 minutes. The suspension was then centrifuged and resuspended in 200 μl sBHI. Suspension was plated either neat or at 1:10 or 1:100 dilutions on BHI plates supplemented with hemin, NAD and 10 μg/mL kanamycin and incubated overnight. BHI supplemented with hemin, NAD and 64 μg/mL azithromycin was inoculated with a loopful of bacteria to evaluate azithromycin resistance. This protocol has produced bacteria that are resistant to kanamycin, but has not yet successfully produced azithromycin-resistant NTHi.

Alternatively, in collaboration with my colleague Yuqing Long, the protocol was modified to include an electroporation step as suggested in the literature (Atkinson, Kunde and Tristram, 2017). This technique used repeated washes with SG solution (1X SG solution contained 15% glycerol and 272 mM sucrose). NTHi stocks were thawed and cultured overnight at 37°C and 5% CO_2 on chocolate horse blood agar plates. A loopful of bacteria was taken from a dense area of growth and used to inoculate 20 mL of sBHI and cultured for approximately 4 hours at 37°C in a 5% CO_2 incubator until reaching an OD_{650} of 0.16. Competence was induced by chilling cells on ice for 30 minutes, followed by 4 washes with 0.5X SG solution on ice. Following washes, bacteria were resuspended in 1X SG solution and 50 μl NTHi suspension was then electroporated using a BioRad Electropulser with 3-5 μg plasmid DNA. 1 mL warm supplemented BHI broth was then added to the electroporated NTHi, which was then incubated at 37°C and 5% CO_2 for 2 hours. Suspension was then plated neat on BHI plates supplemented with hemin, NAD and 10 μg/mL azithromycin, and incubated overnight.

2.3.6 Heat-inactivation of NTHi

NTHi strains were grown in liquid culture to a specified OD. They were then heat-inactivated for 30 minutes at 65°C in a waterbath, as previously described in the literature (Hartwig *et al.*, 2016). Heat-inactivation was verified by plating on chocolate agar plates.

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

Enzyme-linked Immunosorbent Assays (ELISA)s are a sensitive quantitative assay which measures protein using an antibody capture technique (Alhajj and Farhana, 2020). It is advantageous because it directly detects protein production, unlike PCR, which detects RNA, which may undergo post-transcriptional modifications or degradation. CXCL8 (IL-8) ELISAs were performed on cellular supernatants collected from PBEC, BEAS-2B, or NEC cultures to evaluate the effect of infection with strains of NTHi. Throughout, a minimum of biological duplicates were used, with technical duplicate wells used for each biological replicate. ELISAs were performed according to manufacturer instructions (R&D Systems DuoSet ELISA). Assay range is 31.2 – 2000 pg/mL. Supernatants were applied either undiluted or diluted 1:1 with reagent diluent as suggested in the protocol.

2.5 qPCR Analysis

Quantitative polymerase chain reaction (qPCR) is a sensitive tool used to evaluate relative gene expression (Pabinger *et al.*, 2014). PCR uses complementary primers flanking the gene of interest, resulting in the gene being amplified exponentially. Quantitative, or real-time, PCR uses a fluorescence molecule to tag the product, which is detected by the thermocycler as the reaction proceeds. There are several ways to analyse qPCR results. I have used the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method, which normalises against a housekeeping gene such as the commonly-used GAPDH or beta-actin. GeNORM data provided by my colleague Gareth Hynes compared variability of gene expression in asthmatic and healthy human epithelial tissue and identified RPL13A and ATP5B as the most stably expressed reference gene in this tissue. Experiments have used both RPL13A and GAPDH for comparison, giving comparable results. Assays were performed using technical duplicate wells throughout. Whole-cell RNA was extracted using the Qiagen RNEasy kit following the standard protocol recommended by the manufacturer. Reverse-transcription was performed using the Applied Biosystems High-Capacity cDNA reverse transcription kit. qPCR analysis was done using the Qiagen QuantiFAST SYBR Green system. The SYBR green method measures luminescence generated by double-stranded DNA as the target is amplified, allowing relative quantification of the target gene. However, because all double-stranded DNA produces fluorescence, including primer-dimers and non-target DNA, the primers must be carefully designed to

minimize this. Despite these drawbacks, SYBR green is relatively inexpensive and allowed me to screen a large number of genes easily. Primers were designed using the NCBI PrimerBLAST tool. Wherever possible, primers were intron-spanning.

Table 3. PCR Primer Information

GOI	Forward Primer	Reverse Primer
Claudin 8	GTGGATGAATTGCGTGAGGC	TAGGTCCGGAGAAAGAGCCA
E-Cadherin	GCTGGACCGAGAGAGTTTCC	CAAAATCCAAGCCCGTGGTG
Occludin	TGCGGCGAGCGGATTG	TGGACTTTCAAGAGGCCTGG
Zona Occludens 1	AGAGGAAGCTGTGGGTAACG	AGGGTTTTCCTTGGCTGACA
Claudin 7	TCCAGTTAGGAGCCTTGATGC	ACACACAAATCGACCCCTCC
Claudin 1	TGAGGATGGCTGTCATTGGG	GACCAAATTCGTACCTGGCA
RPL13A	GTGGTCGTACGCTGTGAAGG	GGGCAGCATACCTCGCAC
ATP5F1B	CCTGCTACTACGTTTGCCCA	TGGAGGGATTTGTAGTCCTGC
GAPDH	AATGGGCAGCCGTTAGGAAA	GCCCAATACGACCAAATCAGAG
ICAM-1	CCTCACCGTGTACTGGACTC	GGGTAAGGTTCTTGCCCACT
COX-2	AACTGCTCAACACCGGAATTT	TAGTGCACTGTGTTTGGAGTG
TLR2	GAGTTCTCCCAGTGTTTGGTGT	ACCCTGTCTTCCTGCCTTCAC
TLR4	AACCCAGAGCTTTCAGACTCC	ATTAGGAACCACCTCCGTGA
IL-1b	TTCGAGGCACAAGGCACAA	TGGCTGCTTCAGACACTTGAG
Alpha-Defensin 6	CAAGTCTTAGAGCTTTGGGCTC	AGTGCAGGTCCCATAGGAAT
Lysozyme	GGGCAAGGTCTTTGAAAGGTG	GGCCAAACACATCCAGTTTGC
NF-kB	GCTTAGGAGGGAGAGCCCA	CTGCCATTCTGAAGCCGGG
CCL2 (MCP1)	CATGAAAGTCTCTGCCGCCC	GGGCATTGATTGCATCTGGCT
IL-8 (CXCL8)	AGTTTTTGAAGAGGGCTGAGA	TGCTTGAAGTTTCACTGGCA

IL-6	CCCACCGGGAACGAAAGAG	GGACCGAAGGCGCTTGT
TNF	GCCCATGTTGTAGCAAACCC	TATCTCTCAGCTCCACGCCA
IL-37	CTTAGAAGACCCGGCTGGAAG	CTTCACCTTTGGACTTGTGTGA

2.6 Meso-Scale Discovery Experiments

Meso-Scale Discovery (MSD) is a technique which measures protein quantitatively using a capture antibody system similar to an ELISA. However, it utilises multiplexing so that several targets can be measured in the same well. MSD uses Sulfo-Tag labelling, which emit light when electrically stimulated. Only labels bound near the electrode surface at the bottom of the well are activated, so any unbound labels are not amplified, eliminating the need for repeated washing steps and improving specificity. MSD analysis was performed according to the standard protocol, with duplicate wells, using supernatants from apical and basal compartments of PBECs and NECs at ALI inoculated with NTHi strain 398, 162, 11931, 1158, 375, or TLR2 (Heat-killed *Listeria monocytogenes*) or TLR4 (LPS) agonist for 24 hours, or supernatants from uninfected controls. Supernatants were diluted 1:1 prior to addition to the plate. Assay range was as follows: IL-8: 0.07 – 375 pg/mL, IL-6: 0.06 – 488 pg/mL, IL-1β: 0.05 – 375 pg/mL, and TNF: 0.04 – 248 pg/mL.

2.7 Fluidigm PCR Array

Fluidigm DeltaGene Assays are a PCR gene expression array technique which uses an integrated fluidic circuit in a microfluidic chip format to analyse multiple genes simultaneously. It is based on the same paradigm as qPCR, but uses a bespoke Fluidigm thermocycler for amplification and detection. This assay uses EvaGreen dye which binds to double-stranded DNA much like SYBR green, producing fluorescein-like emission spectra. However, it has minimised PCR inhibition compared to traditional SYBR green dyes. Custom targets were selected for this assay to compliment the conventional PCRs I have performed. This experiment used RNA from PBECs and NECs at ALI inoculated with NTHi strain 398, 162, 11931, 1158, 375, TLR2 (Heat-killed *Listeria monocytogenes*) or TLR4 (LPS) agonist for 24 hours, or RNA from uninfected controls, isolated using the Qiagen RNEasy micro kit. cDNA

conversion was performed using the proprietary Fluidigm cDNA conversion kit. The PCR array was then performed according to the standard protocol and analysed using Biomark software. The Fluidigm software performs an initial quality control analysis to identify values which are outside the conventional range of detection. Analysis of gene expression was performed using the delta-delta Ct ($2^{-\Delta\Delta Ct}$) method, which normalises against a housekeeping gene such as the commonly-used GAPDH. GeNORM data provided by my colleague Gareth Hynes compared variability of gene expression in asthmatic and healthy human epithelial tissue and identified RPL13A and ATP5B as the most stably expressed reference gene in this tissue. Experiments have been analysed using both RPL13A and GAPDH and yield comparable results.

Table 4. Fluidigm Target Information

Gene of interest	Target
AGER (RAGE)	Upstream of IL-33, associated with neutrophilic asthma
ALPK1	PRR recognising ADP-heptose
ATP5B	Housekeeping gene
CAMP (Cathelicidin)	Antimicrobial peptide, TLR signalling
CASP1(ICE)	Inflammasome marker
CCL2 (MCP1)	Pro-inflammatory cytokine, downstream of NF-κB
CXCL8 (IL-8)	Cytokine
GAPDH	Housekeeping gene
ICAM-1	Host receptor for NTHi adhesion
IL-1b	Cytokine

IL-25	Cytokine
IL-33	Cytokine
IL-37	Antimicrobial peptide
IL-6	Cytokine
IRAK1	TLR marker
IRF5	TLR marker
IRF7	TLR marker
Lysozyme	Antimicrobial peptide
MAPK3 (ERK1)	MAPK family
MAPK8	MAPK family
MYD88	TLR marker
NLRC4	Inflammasome marker
NLRP1	Inflammasome marker
NLRP3	Inflammasome marker
NOD1	NF-κB pathway (upstream)
NOD2	NF-κB pathway, MAPK family (upstream)
RelA	NF-κB marker
RPL13A	Housekeeping gene
TLR2	TLR marker
TLR4	TLR marker

TNF	Proinflammatory marker
TRAF6	TLR marker
TSLP	Cytokine

2.8 Flow Cytometry Sorting

ALI cultures of NECs and PBECs which were infected with a CellTrace Far Red-labelled NTHi strain isolated from a ciliary dyskinesia patient were sorted using flow cytometry to evaluate prevalence of bacteria in ciliary, basal and secretory cells, and to compare upper with lower respiratory epithelium. PCDNTHi4 was cultured overnight in supplemented BHI broth, centrifuged at 3500 x g for 5 minutes and resuspended in PBS. CellTrace Far Red staining was performed according to manufacturer's instructions, added to bacterial suspension in PBS at 1:1000 and incubated for 20 minutes at 37 C. Bacteria were then pelleted again, washed with PBS, and resuspended in PBS. ALI transwells cultured in antibiotic-free medium were inoculated with NTHi at a MOI of 50 and incubated for one hour. The bacterial suspension was then removed, the transwells washed with PBS, and media containing gentamicin (200 μg/ml, Gibco) added for 1 hour. The gentamicin-containing media was then removed and the cells rinsed 3 times with PBS. Staining with a tubulin tracker as a marker for ciliated cells was performed while monolayers were intact by adding the dye to the apical compartment and incubating for 20 minutes. Following this, the cells were washed with PBS and trypsinised. The trypsinised cells were then centrifuged and resuspended in ALI medium, and the single-cell suspension was then stained with CD49f as a marker for basal cells and CD66C for secretory cells at the dilutions listed below. In addition, a live/dead control was performed using Zombie Near IR staining. Sorting was then performed on an Aria III to sort populations of infected or uninfected control ciliary, basal and secretory cells from NECs and PBECs. A portion of these cells were then lysed and CFU/cell calculated to determine the prevalence of bacteria within each cell type.

Table 5. Flow Cytometry Staining Information

Target	Dilution	Catalogue number	Fluorophore
CellTrace (Bacterial dye)	1:1000	Invitrogen, C34572	Far Red
CD49f (Basal Cells)	1:100	BD Biosciences, 562474	PE CF594
CD66c	1:100	Invitrogen, KOR-SA3544	PE
Tubulin tracker (Ciliated cells)	1:100	Invitrogen, T34078	FITC
Zombie (live/dead)	1:500	BioLegend, 423105	Near IR

Gating Strategy:

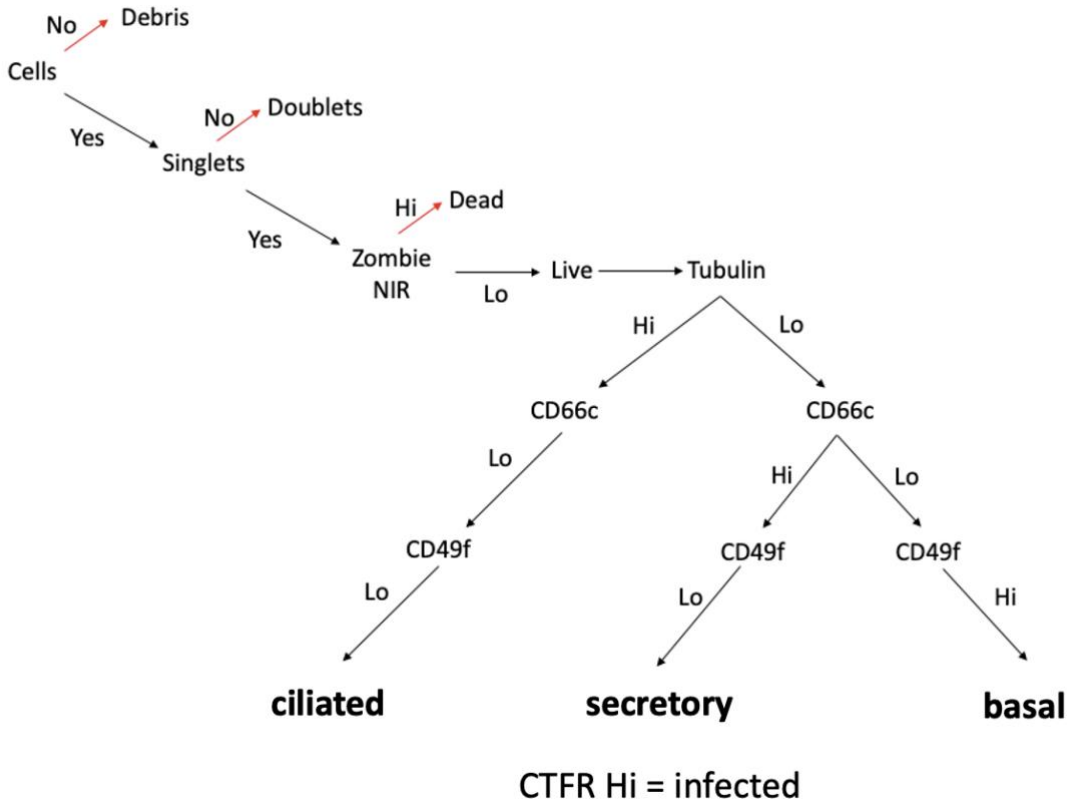


Table 6. Reagent List

Application	Reagent	Source
NTHi culture	Brain heart infusion 500g	Sigma, DM095
CXCL8 (IL-8) ELISA	DuoSet CXCL8(IL-8) ELISA Kit	Bio-Techne (R&D Systems), DY208-05
Whole-cell RNA isolation for qPCRs	RNEasy Plus RNA isolation kit	Qiagen, 74134
qPCR	QuantiFAST SYBR Green system	Qiagen, 204056
cDNA reverse transcription	High-capacity cDNA transcription kit	Applied Biosystems, 4374966
Induction of competence in NTHi	MIV	
NTHi electroporation	Bio-Rad GenePulser/MicroPulser Cuvettes, 0.2cm gap	Bio-Rad, 1652086
qPCR primers	qPCR primers	Merck Life Sciences Ltd, VC00027N
Tissue culture media – expansion – primary cells	Pneumacult-Ex Plus Basal media	StemCell, cat. No 05040
Tissue culture media – maintenance – primary and BEAS-2B cells	PromoCell Airway epithelial cell growth medium kit	PromoCell, 21160
ALI tissue culture media	Pneumacult-ALI media	StemCell, cat. No. 05001

Tissue culture media – maintenance and expansion – RPMI2650 and Calu-3 cells	EMEM with Earle's balanced salt solution, non-essential amino acids, 2mM L-glutamine, 1mM sodium pyruvate and 1500 mg/L sodium bicarbonate	LGC/ATCC, 30-2003
Transwells for ALI	24-well Thincert, 0.4 um pore size, transparent (PET)	Greiner Bio-One, 662641
Azithromycin dihydrate		Abcam, ab141064-50mg
Gentamicin sulfate		Gibco, 15710064
Blockade of bacterial internalisation	Cytochalasin D	Santa Cruz Biotechnology, sc-201442
Nasal brushes	Cytotak Transwab cytology brushes	VWR, 720-0136
NTHi Staining	CFSE CellTrace Cell proliferation kit	Life Technologies, C34554
Immunofluorescence blocking buffer		Cell Signaling Technology, 12411
Mounting medium	ThermoFisher ProLong Glass Antifade mounting medium	ThermoFisher, P36982
Mounting medium with nuclear dye	ThermoFisher ProLong Glass Antifade mounting medium with NucBlue dye	ThermoFisher, P36983

2.9 Data Management and Statistics

Clinical data associated with collection of primary bronchial and nasal epithelial cells were recorded by hand and stored securely in the Oxford Airways Studies (OAS) database using Microsoft Access. Records of cryopreserved PBECs and NECs were recorded on the Respiratory Medicine Unit shared drive after being anonymised and labelled with OAS number. Following subculture to passage 2 or higher, primary nasal and bronchial cells no longer contain original material from donors, and are not subject to HTA storage requirements.

Experimental data from non-clinical sources were stored on the Hinks Lab storage drive and my personal computer backed up to my iCloud account. Data from experiments is stored with information detailing the date, replicate number, and OAS numbers of any primary cells used both electronically and in a physical lab notebook to ensure it is easily accessible.

Data were processed for downstream analyses using Microsoft Excel, and statistics were performed using Prism (GraphPad Software, 2021). Statistical analyses used a combination of parametric tests, which assume data is normally distributed, and non-parametric tests, which do not make this assumption.

Log-normality was assessed by visually inspecting the data to assess distribution. The literature suggests that formal normality tests lack power when used on small samples, so I have not used them. Log-normally distributed data was analysed in the log-space. If data contained zero values, a small value was added to all values before transformation to avoid losing any data.

To compare two groups of parametric data, a Student t-test was used, which evaluates the ratio between group mean difference over within-group differences and sample size to generate a *t*-statistic and p-value. Unpaired t-tests were used to evaluate the differences between two unrelated or independent groups, whilst paired tests were used to compare the means of dependent groups under different circumstances.

Comparison of more than two groups was performed using analysis of variance (ANOVA). ANOVA involves the evaluation of ratios of mean differences over

variability within groups and sample size to generate an F-statistic and p-value. One-way ANOVAs compare the differences in instances where there is one independent variable. Two-way ANOVA is a more powerful test which compares multiple groups where there are two independent variables. 2-way ANOVA analysis has been followed by a Sidak's multiple comparison test to evaluate the difference between all groups.

P-values are the probability that a similar result would occur in randomly generated data with the same spread and mean. Throughout, p-values are indicated as follows: *≤0.05, **≤0.01, ***≤0.001, and ****≤0.0001.

Chapter 3 Does NTHi selectively replicate within the epithelial monolayer?

NTHi has traditionally been considered primarily an extracellular pathogen, but there is evidence that NTHi can be internalised in epithelial cells, and is found intracellularly and in the paracellular space in cells isolated from the lower airways of patients with NTHi infections (Clementi, Håkansson and Murphy, 2014). However, it is still unknown whether NTHi replicates within the epithelial cells, or whether internalisation is merely a transient immune-escape mechanism. There is some evidence *in vitro* that NTHi is metabolically active within epithelial cells, but that it does not replicate intracellularly (Morey *et al.*, 2011). However, these data were generated primarily in undifferentiated A549 cells in submerged culture, which limits the appropriateness of their generalisation to humans. Preliminary data generated by my supervisor's group suggested that while NTHi does not replicate within submerged epithelium, it may be capable of replicating within differentiated primary bronchial epithelial cell (PBEC) cultures polarised at ALI for up to 72 hours post infection (hpi). One aim of this chapter was to resolve this disparity by evaluating the ability of NTHi to replicate within the epithelial monolayer in ALI cultures which more accurately mimic *in vivo* conditions.

In addition, I sought to investigate whether NTHi preferentially infects a particular epithelial cell type using immunofluorescence imaging and flow cytometry. There is some evidence that NTHi preferentially infects ciliated epithelial cells (Morey *et al.*, 2011; Baddal *et al.*, 2015a). However, there are also data which suggest that ciliary function is important for mediating host-pathogen interactions (Walker *et al.*, 2017; Kuek and Lee, 2020). In addition, mucus production also mediates host-pathogen interactions in conjunction with ciliary function (Fahy and Dickey, 2014; Zanin *et al.*, 2016). I have also sought to evaluate the relationship between mucus production and internalisation and adhesion rates.

Further experiments were directed at evaluating physical interactions between NTHi and the host epithelial cells, examining adhesion and evaluating whether internalised bacteria were present intracellularly or within the paracellular spaces in the epithelial monolayer. I then sought to investigate the intracellular lifestyle of NTHi. There is a

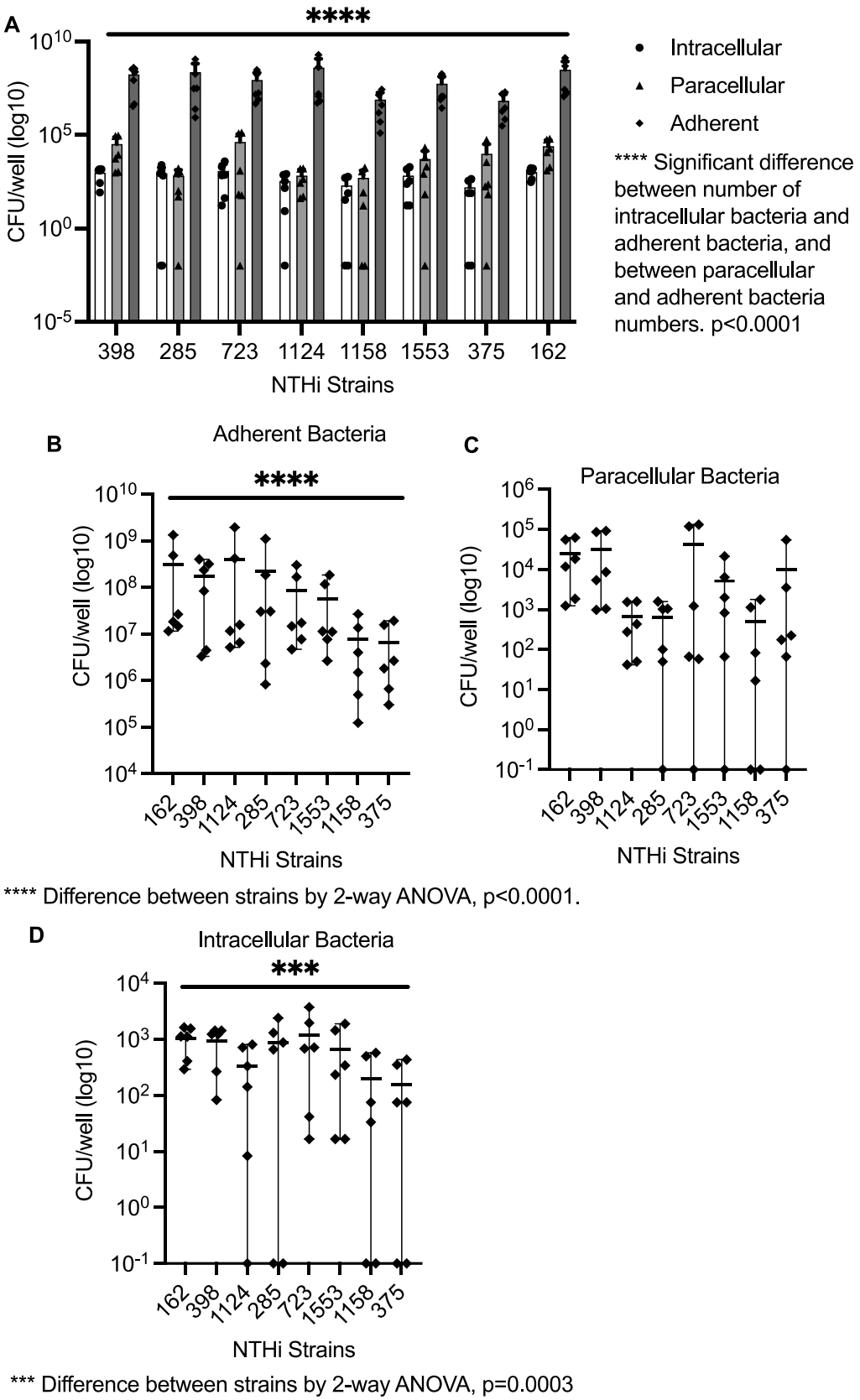
diversity of opinion regarding the intracellular localisation of NTHi within the early and late endosomes. One such study using BEAS-2b cells inoculated with NTHi for 2 hours identified NTHi only within vesicles characterised by early endosome marker EEA1 (Ikeda *et al.*, 2015). Conversely, another study found NTHi primarily within vesicles with late-endosome like characteristics (Morey *et al.*, 2011). To further investigate this disparity, I evaluated trafficking of NTHi within the early and late endosome in a PBEC ALI model by conducting microscopy experiments to visualise the localisation of NTHi within the epithelial cell.

3.1 Is NTHi internalised paracellularly or intracellularly?

Localisation of NTHi within the epithelial monolayer was evaluated by using a modified gentamicin exclusion assay. Confluent epithelial cells were infected with NTHi for 4 hours, followed either by washing to leave adherent bacteria, gentamicin only as normal to eliminate extracellular bacteria only, or trypsin and gentamicin to eliminate paracellular as well as extracellular bacteria. The number of remaining bacteria was then estimated by lysing the epithelial cells to release the internalised bacteria and performing a colony forming unit (CFU) count assay, involving plating the recovered bacteria on agar at various dilutions and counting the colonies. Calculations were then performed to yield numbers of adherent, paracellular or intracellular bacteria.

In Calu-3 cells grown to confluence in submerged culture, adherent bacteria account for over 99% of all bacteria interacting with the epithelial cells ($p=0.018$) (Figure 1A). There was a tendency towards a higher number of paracellular compared with intracellular bacteria across strains, but this was not significant. The number of adherent bacteria varied significantly between strains, with strain 1158 and 375 being markedly lower (Figure 1B). Likewise, intracellular bacteria similarly varied between strains, with lowest numbers of 1158 and 375 (Figure 1C). Numbers of paracellular bacteria varied, but were not significantly different between strains (Figure 1D). Overall, strains 398 and 162 tended towards higher rates of adhesion and internalisation, whilst strain 1158 and 375 had lower rates of adhesion and internalisation into the intracellular space. Future experiments used these strains to compare lower and higher adhesion and internalisation.

Figure 1. NTHi adhesion and internalisation in Calu-3 cells.



**** Difference between strains by 2-way ANOVA, p<0.0001.

*** Difference between strains by 2-way ANOVA, p=0.0003

Figure 1. Enumeration of bacterial adhesion and internalisation into Calu-3 cells and the paracellular space following incubation with NTHi strains for 4 hours. A. Adherent bacteria accounted for over 99% of bacteria interacting with epithelial cells. B. There was significant variation between the proportion of adherent bacteria between strains, with lowest adhesion by strains 1158 and 375. C. There is variation in numbers of paracellular bacteria between strains, but this was not significant. D. Rates of intracellular internalisation varied significantly between strains, with a trend towards reduced numbers of strain 1158 and 375 compared with 398 and 162. Data were evaluated using a 2-way ANOVA on log-transformed data to correct for the log-normal distribution of the data. 3 independent replicates were performed with technical duplicate wells for each experiment.

These experiments were recapitulated in PBECs grown at ALI (Figure 2). Overall numbers of adherent and internalised bacteria were significantly lower in ALI cultures than in PBECs in submerged culture (2A). Numbers of adherent bacteria accounted for the majority of bacteria associated with the epithelium, although this is not as pronounced in ALI cells as in submerged cultures (2A). Numbers of adherent strain 162 and 398 bacteria tended towards higher levels than strain 1158 (2B). There was not significant variation between numbers of intracellular and paracellular bacteria between strains (Figure 2, C and D). ALI experiments were performed in 3 biological replicates from different healthy donors in duplicate wells in each experiment.

Figure 2. NTHi is internalised at lower rates in PBEC ALI cultures.

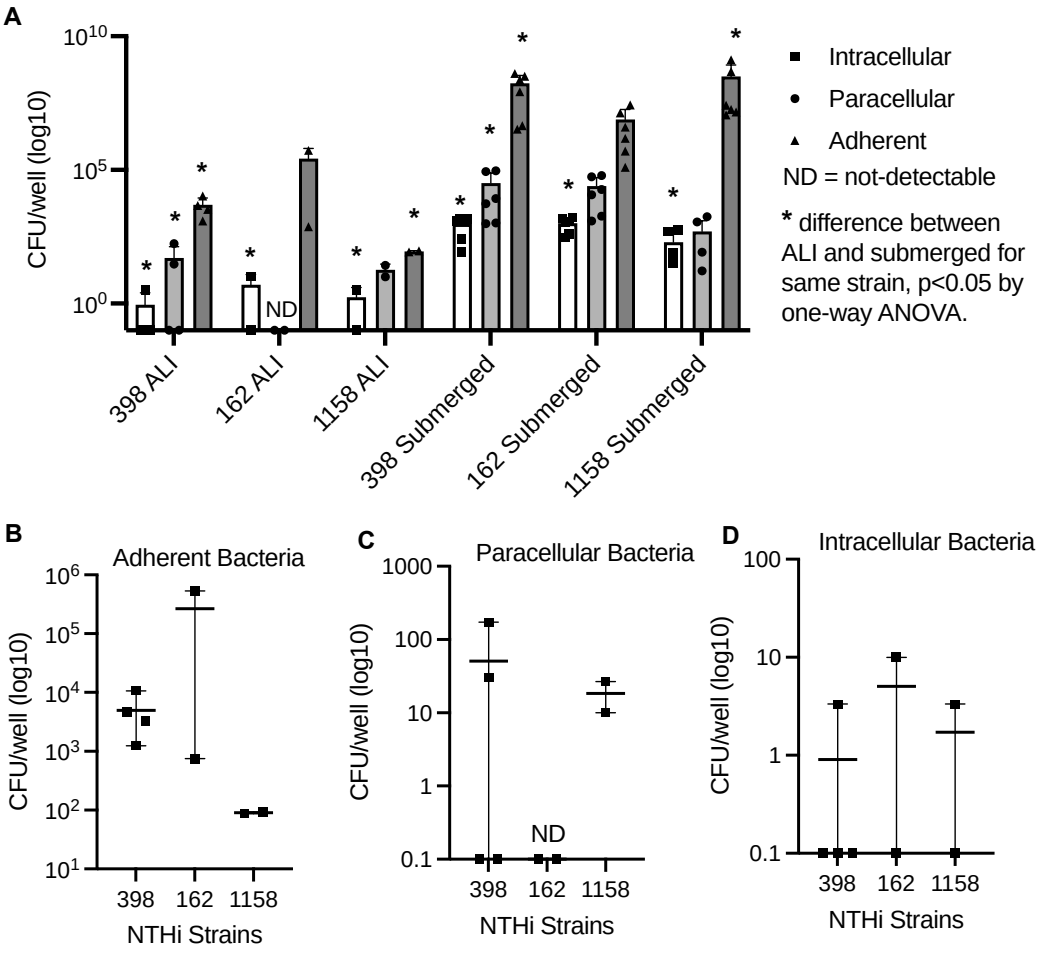


Figure 2. Enumeration of adherent, intracellular and paracellular bacteria in PBECs grown in submerged or ALI cultures following incubation with NTHi strains for 4 hours. Strains are arranged from most to least internalised. A. Adherent bacteria account for a high proportion of bacteria associated with the epithelium in both ALI and submerged cultures. Numbers of intracellular, paracellular and adherent bacteria are higher in submerged compared with ALI cultures. The number of internalised bacteria of all strains were significantly higher in submerged compared with ALI cultures, while paracellular bacteria were significantly higher in submerged cultures for strain 398, and numbers of adherent bacteria were higher in strains 398 and 1158. Significance was evaluated using one-way ANOVA with multiple comparisons to evaluate difference between ALI and submerged for each strain using log-transformed data. B. Numbers of adherent bacteria were numerically lower in strain 1158 compared with 398 and 162, but this is not significant. C. Numbers of viable paracellular bacteria vary between strains, with no detectable bacteria strain 162 in the paracellular space. D. Intracellular bacteria numbers vary insignificantly between strains 398, 162 and 1158. 2-way ANOVA was performed to evaluate significance of differences between strains in B, C and D. Experiments were performed on a minimum of two biological replicates from two healthy control donors.

I postulated that the reduction in internalised and adherent bacteria in ALI cultures compared with submerged culture may be due to the presence of mucus acting as a physical barrier (Fahy and Dickey, 2014; Zanin *et al.*, 2016), or the development of strong tight-junctions and polarisation of the cells, preventing bacteria accessing intracellular or basal surfaces. Mucus production was evaluated by performing ELISA analysis on supernatants from the apical surfaces of control and infected submerged Calu-3 cells and PBECs grown at ALI to measure Mucin-5AC (MUC-5AC), a structural component of mucus often used as a marker for mucus production (Figure 3). This showed that MUC-5AC expression was significantly higher in Calu-3 cells compared with PBECs at ALI. NTHi is capable of binding to mucins (Clementi and Murphy, 2011), so these results may suggest that the increased production of MUC-5AC in Calu-3 cells enables more efficient NTHi adhesion. Alternatively, the thickness of mucus found on the surface of ALI cultures may have made it difficult to sample effectively, so it is possible that this is an experimental artifact. There was not a significant difference in MUC-5AC between infected and uninfected samples of the same cell type (Figure 3).

Figure 3. MUC-5AC production in ALI PBECs and NECs compared with submerged Calu-3 cells

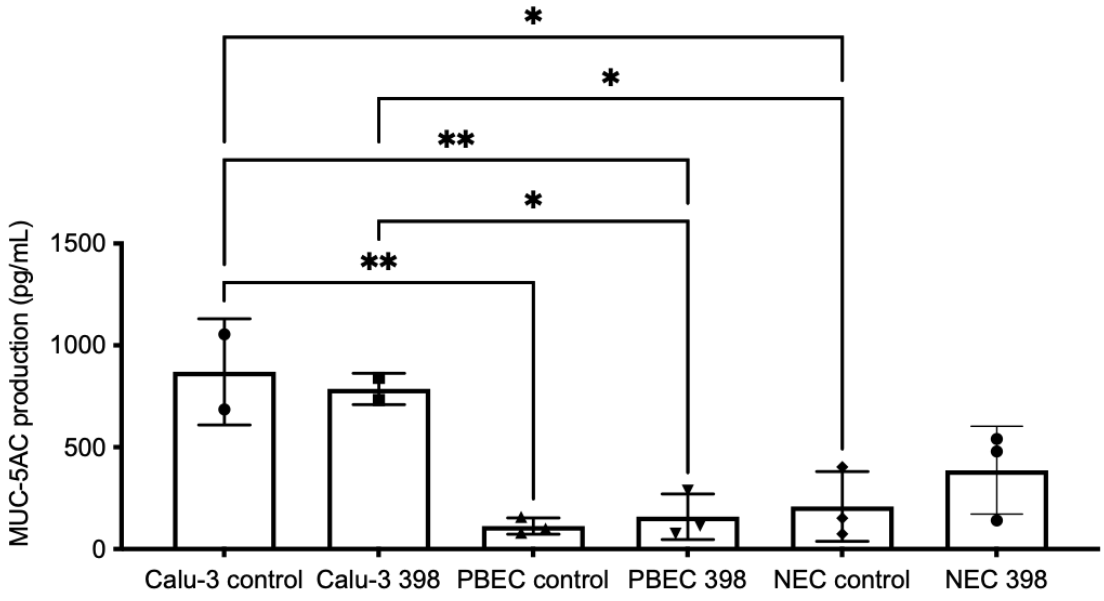


Figure 3. Evaluation of MUC-5AC production after 24 hours as a measure of mucous production in ALI PBECs and NECs compared with Calu-3 cells grown in submerged culture. An ELISA performed on supernatants from the apical compartment of submerged Calu-3 cells or PBECs and NECs grown at ALI showed that Calu-3 cells produced significantly higher levels of MUC-5AC than PBECs or NECs. Infection with NTHi strain 398 for 24 hours did not affect mucin production. n=3 biological replicates with PBECs and NECs from 3 different healthy donors. Significance in difference between cell types was evaluated by ordinary one-way ANOVA with Tukey's multiple comparisons post-hoc test.

3.2 Is NTHi persisting within the epithelial monolayer?

NTHi is capable of maintaining a persistent infection *in vivo*, and is found within the epithelial monolayer both *in vivo* and *in vitro*. However, it is unclear whether NTHi persists and replicates within the epithelium. To explore this, I have used a gentamicin exclusion assay, where confluent epithelium is infected with NTHi for 4 hours, followed by incubation in the presence of gentamicin for up to 48 hours to ensure that any recovered bacteria have been continuously harboured within the monolayer. In submerged cultures of BEAS-2B cells, NTHi strains 398 and 162 were internalised in significant numbers at 4 hours post-infection, but intracellular bacteria were eliminated rapidly in the absence of extracellular bacteria, with no detectable viable bacteria within the monolayer by 48 hours post-infection (Figure 4, A-D). Likewise, in PBECs grown in submerged culture, internalised bacteria were eliminated by 24 hours post-infection in cells treated with gentamicin to eliminate extracellular bacteria (Figure 4E). Throughout, submerged cultures were visually assessed to ensure that the epithelial monolayers remained intact. However, future experiments would benefit from including viability measures at each timepoint to ensure that the diminishing number of bacteria is not due to the demise of the epithelial cells. Optimisation experiments performed by another member of our lab demonstrated that the TERs of PBECs cultured at ALI were not affected by inoculation with NTHi at low MOIs, such as those used in these studies (MOIs of 10-30), whilst at MOIs of 100 or more, the epithelial monolayers exhibited both diminished TERs and morphological abnormalities (data not shown). These data suggest that the diminished number of bacteria isolated from the epithelium is likely not due to mortality of the epithelial

monolayer, but future studies would benefit from the inclusion of TER measurements at each time point.

Figure 4. NTHi does not persist within the epithelial monolayer in the absence of extracellular bacteria in submerged cultures.

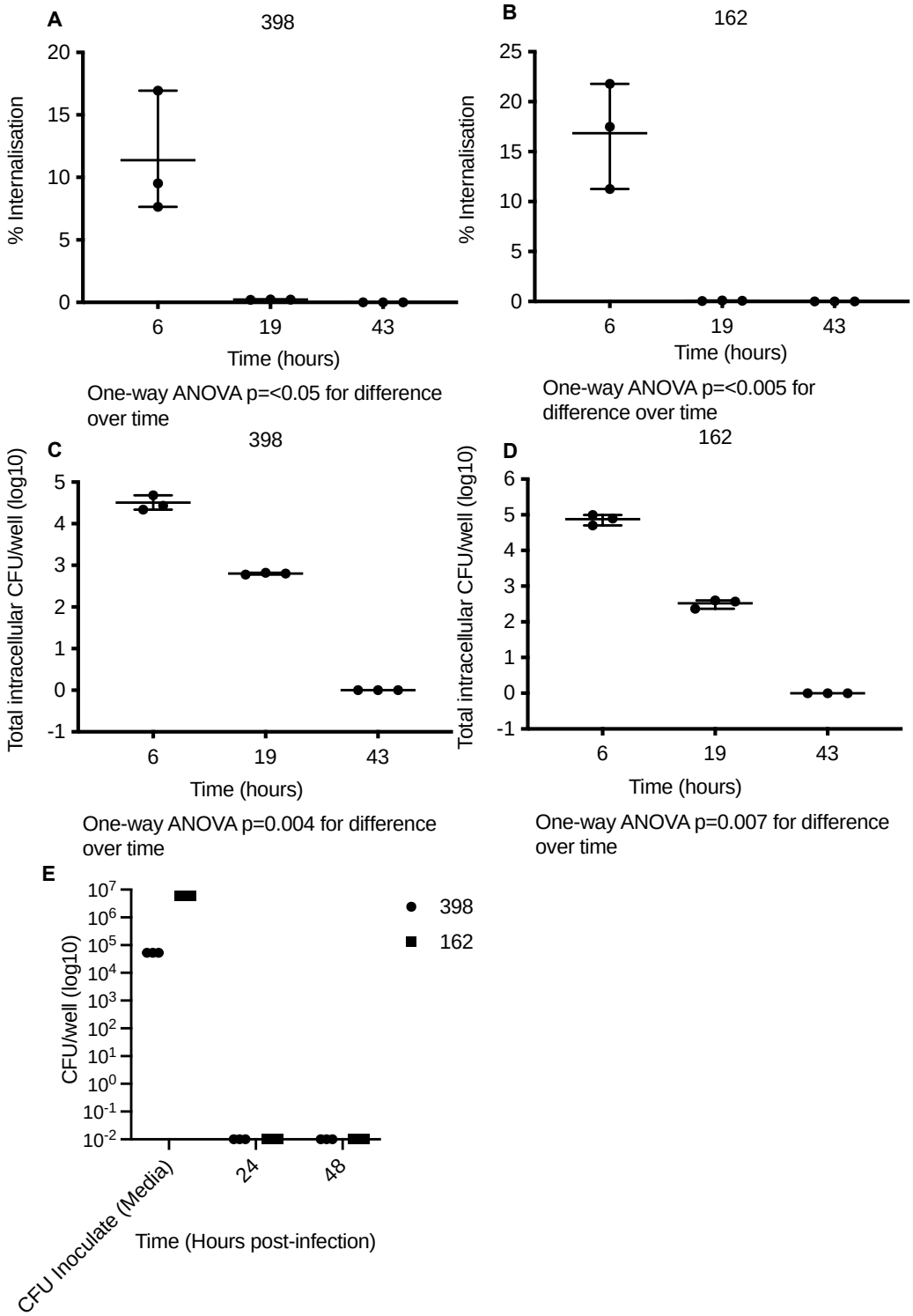


Figure 4. NTHi does not persist within BEAS-2B cells or PBECs grown in submerged culture in the absence of continuous exposure to extracellular bacteria. BEAS-2B cells or PBECs were inoculated with NTHi, treated with gentamicin 4 hours post-infection to kill extracellular bacteria, and incubated for up to 43 hours in the presence of gentamicin to inhibit extracellular growth of NTHi. A, C. Strain 398 is internalised by BEAS-2B cells, but no live intracellular bacteria remain at 43 hours post-infection. B, D. This result is replicated by infection with strain 162, which was likewise being cleared by 43 hours, suggesting that this is not a strain-specific effect. E. Both strain 398 and 162 are rapidly cleared from PBECs grown in submerged culture. n=2 biological replicates from healthy control donors. One-way ANOVA yielded $p<0.05$ for variance over time for both strains when expressed as percent internalisation or absolute numbers.

To ensure that this was not a cell-line specific effect, or an artifact of the submerged culture system, this experiment was recapitulated in mature PBEC ALI cultures (Figure 5). NTHi strains 398 and 162 were internalised at 6 hours post-infection, but did not persist within PBECs at ALI in the absence of extracellular bacteria, with internalised bacteria disappearing by 24 hours post-infection (Figure 5, A and B). These results suggest that NTHi does not persist within epithelial cells alone.

Figure 5. NTHi does not persist in the absence of extracellular bacteria in PBECs cultured at ALI.

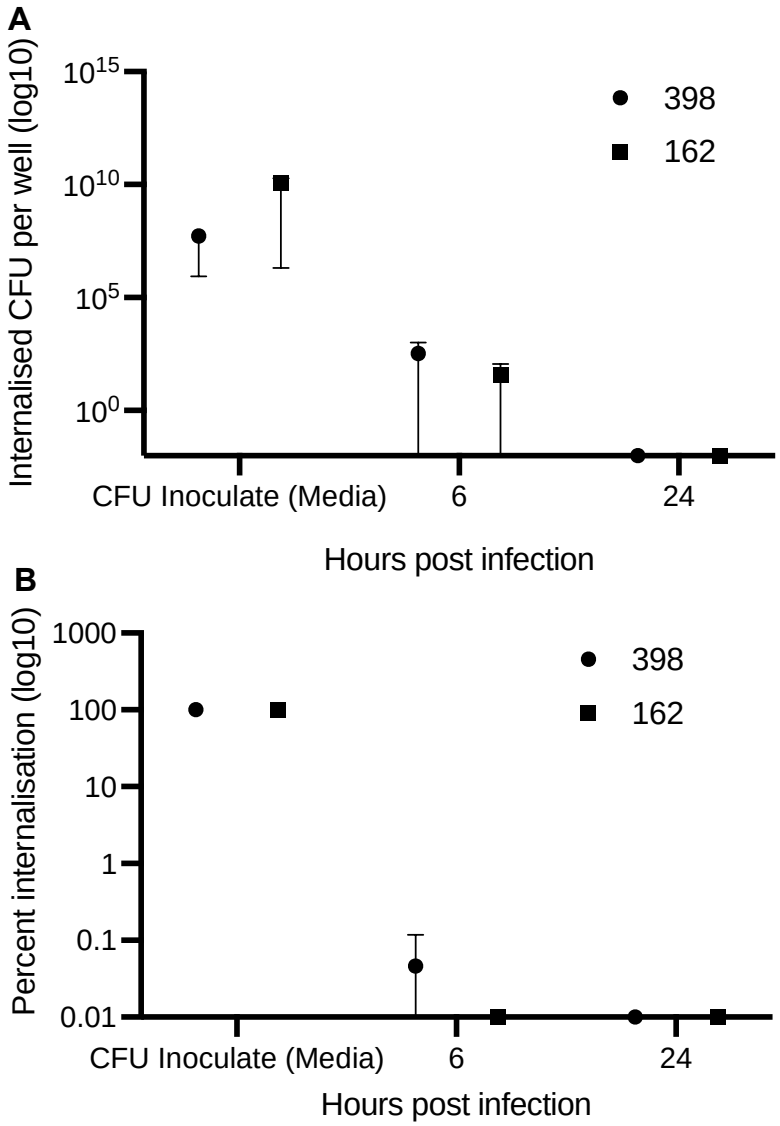


Figure 5. NTHi does not persist within PBECs cultured at ALI in the absence of extracellular bacteria. PBECs cultured at ALI for a minimum of 28 days were inoculated with two highly invasive strains, 398 and 162. They were incubated with the bacteria for 4 hours, followed by treatment with gentamicin to kill extracellular bacteria, and incubated for up to 72 hours in the presence of gentamicin to inhibit extracellular growth of NTHi. A, B. Strain 398 and 162 are internalised at low levels by PBEC ALI cultures, but no live intracellular bacteria remain by 24 hours post-infection. n=3 biological replicates using different healthy control donors.

In a similar assay, continuous internalisation was possible when extracellular NTHi were present. In BEAS-2b cells in submerged culture, numbers of NTHi strains 398 and 1566 were elevated within the media compared with baseline (Figure 6, A and B). Both strains were detectable within the epithelial monolayer up to 48 hours post infection, but numbers of internalised bacteria significantly decline over time ($p<0.05$ for difference over time) (Figure 6, C and D). In PBECs in submerged culture, NTHi numbers were maintained in the media, and intracellular bacteria were detectable up to 168 hours post-infection (Figure 6, E and F). However, despite the increasing numbers of bacteria in the media, viable internalised bacteria decreased significantly over time, suggesting the acquisition of resistance to internalisation ($p=0.04$ for difference over time) (Figure 6F).

Figure 6. Continuous invasion of the epithelium is possible in the presence of extracellular NTHi.

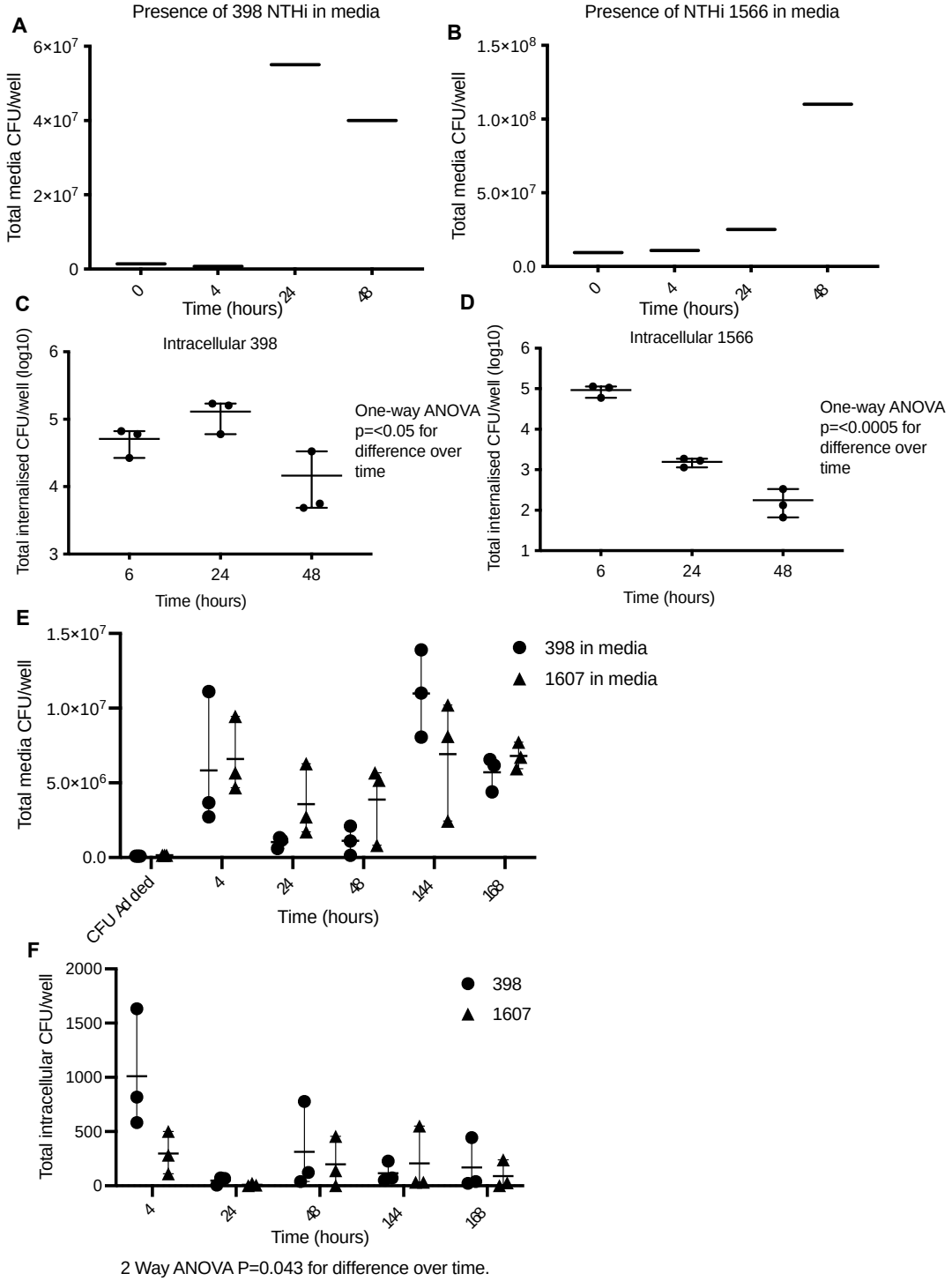


Figure 6. NTHi is able to replicate in media with BEAS-2B cells or PBECs in submerged culture, and some strains have the ability to be continually internalised for up to 7 days. BEAS-2B cells were inoculated with either NTHi strain 398 or strain 1566 and incubated for up to 48 hours post-infection. A, B. A CFU count assay was performed to determine the number of extracellular NTHi CFUs present in the media over the 48-hour time course. Both strain 398 and 1566 proliferate in serum-free airway epithelial cell media when co-cultured with BEAS-2B cells. C, D. Gentamicin was applied to cultures 2 hours prior to lysing to eliminate extracellular bacteria, and the number of internal bacteria calculated using a CFU count assay. This shows that intracellular numbers of strain 398 are maintained at higher levels than strain 1566, although both decline intracellularly over time despite high numbers of bacteria in the media. (3 technical replicates from 1 representative experiment). E. In PBECs, a CFU assay showed that NTHi strains 398 and 1607 proliferate in serum-free media in the presence of PBECs. F. Live intracellular NTHi are detectable by CFU assay up to 7 days post-infection in PBECs when extracellular bacteria are present, though there is a decline in intracellular numbers over time. Strain 398 invades more rapidly, but the difference in invasion rate is reduced over time. This experiment was performed in biological triplicates with PBECs (5.3e4 cells/cm^2) from three healthy donors. Each biological replicate was performed with three wells per condition.

These data suggest that in persistent infections seen clinically, NTHi is likely to be replicating in other cell types or in biofilms, rather than replicating and persisting within the epithelial monolayer as has been previously hypothesised. However, these data do confirm that NTHi is internalised into the epithelial monolayer, although they suggest that this internalisation is transient.

3.3 Evaluation of NTHi prevalence in different epithelial cell types within an epithelial monolayer

The airway epithelium comprises a variety of distinct cell types. To determine which cell types are particularly susceptible to NTHi invasion, and hence a potential reservoir of persistent airways infection *in vivo*, the prevalence of NTHi infection within different types of epithelial cell was characterised in an ALI model of differentiated epithelium using immunofluorescence imaging and flow cytometry (Figure 7).

PBECs grown at ALI were infected with NTHi stained with CFSE for 4 hours, fixed using PFA and stained for markers for ciliated cells (acetylated α-tubulin), basal cells (Integrin subunit α6, ITGA6 or CD49f), and goblet cells (MUC5ac) (Bonser *et al.*,

2021). Imaging was performed using confocal microscopy us a 63x oil objective. Panels 7A, B and C show staining for basal cells, secretory cells and ciliated cells alone, whilst panel D shows stained NTHi. Merged images of staining of cells and NTHi suggested that there was limited NTHi co-localised with basal cells (7E). There was modest overlap between staining for secretory cells and NTHi (7F). NTHi was substantially co-localised with staining for ciliated cells (7G). These data showed that NTHi infects both secretory and ciliated cells, but suggested it may preferentially infect ciliated cells within the epithelium.

Figure 7. Imaging showing co-localisation of bacteria with particular cell types

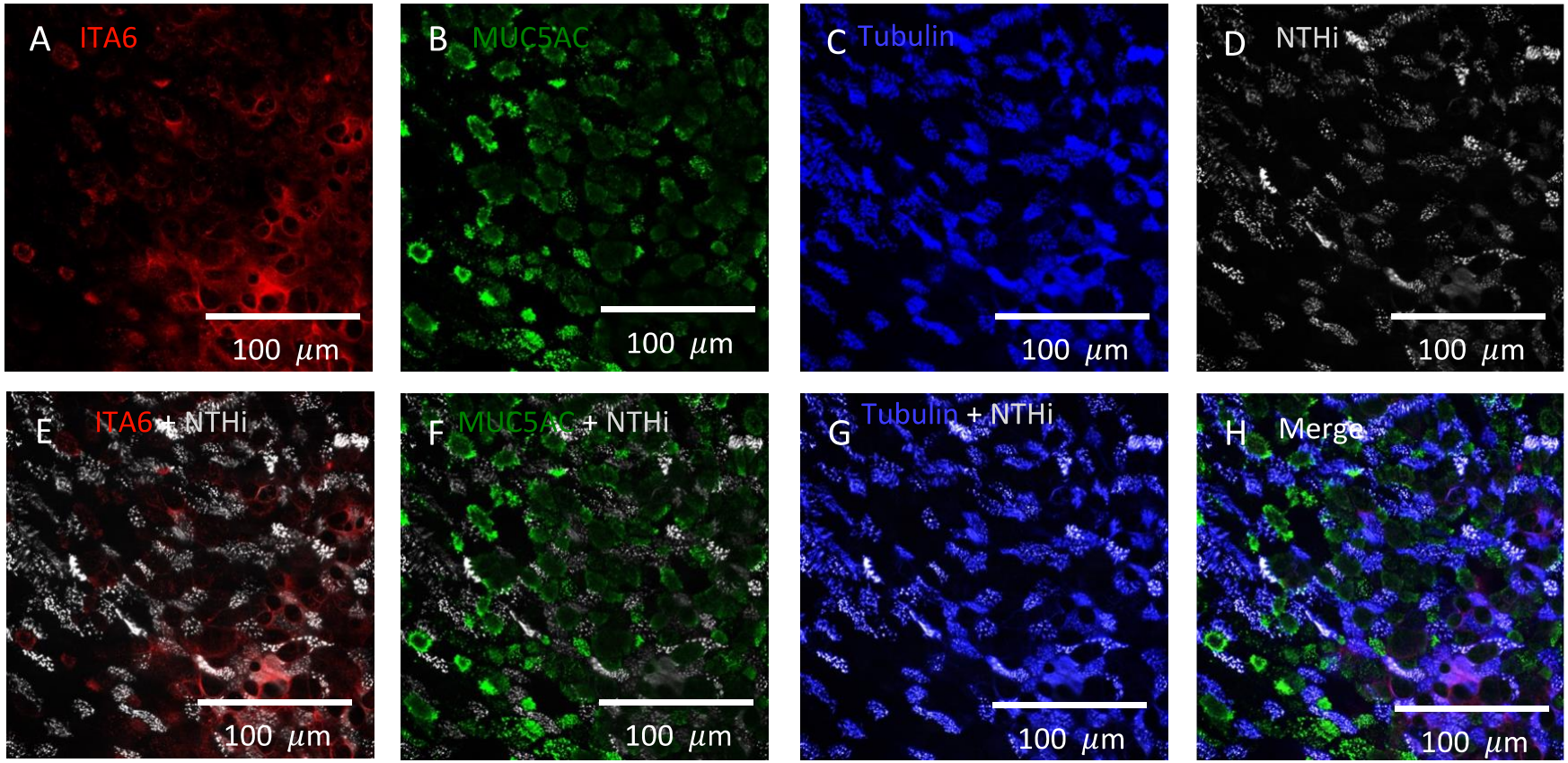


Figure 7. PBECs cultured at ALI were infected with NTHi CFSE-stained strain 398 for 4 hours, fixed in 4% PFA and then stained for ciliated, basal and goblet cell markers. A. Basal cell marker Integrin subunit alpha-6 (ITA6). B. MUC5AC marks secretory cells in green. C. Acetylated alpha-tubulin indicates ciliated cells. D. NTHi are labelled in grey to indicate CFSE-stained bacteria. E. Merging of NTHi and basal cell staining show limited co-localisation. F. There is minimal co-localisation of NTHi with MUC5AC staining. G. There is pronounced co-localisation between NTHi and ciliated epithelial cells. H. Merge of all staining.

A flow cytometry panel was developed in conjunction with Katie Horton (University of Southampton) to isolate different epithelial cell types within a model of PBECs at ALI infected with NTHi and evaluate the number of each cell type that is infected with NTHi (Figures 8 and 9). As with imaging, tubulin was used as a marker for ciliated cells and ITGA6 (CD49f) for basal cells. To avoid permeabilising the cells, CEACAM6 (CD66c) was chosen as a marker for secretory cells. Mature PBEC ALI cultures were infected with NTHi strain 398 at an MOI of 50 for 1 hour and then treated with gentamicin for 1 hour to eliminate extracellular bacteria. Cells were then stained and prepared for flow cytometric sorting. Populations were sorted into infected and uninfected populations of CD49f+ basal cells, tubulin+ ciliated cells and CD66c+ secretory cells. Figure 8 shows the gating strategy for this experiment. This experiment was performed in 3 healthy control donors.

The greatest number of infected cells was highest in the secretory cell population, which was significantly higher than the number of infected basal cells (Figure 9A). There were numerically more ciliated cells than basal cells, but this did not reach statistical significance (Figure 9A). The total number of infected and uninfected cells was also calculated, with the highest number of secretory cells followed by ciliated and then basal cells (9B). The proportion of infected to total cells of each type showed that the highest percent of basal cells were infected, followed by secretory cells and ciliated cells (9C). However, there was considerable variation between donors and these differences were not significant. These data showed that all cell types are permissive to NTHi infection, and while the highest total number of infected cells were secretory, given the low number of basal cells present they were most highly infected.

Figure 8. Gating strategy for flow sorting of infected secretory, basal and ciliated epithelial cells

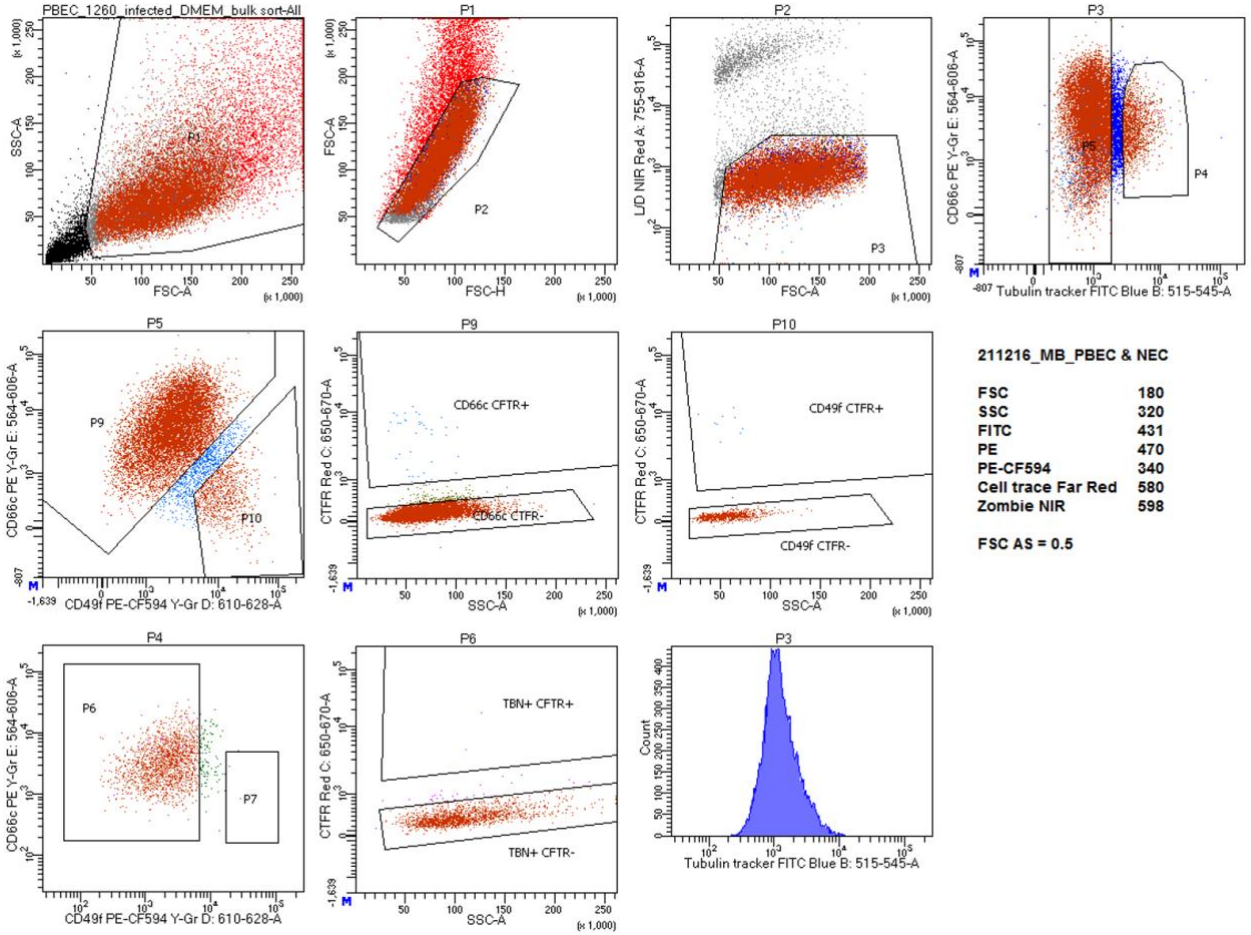


Figure 8. PBECs cultured at ALI were infected with COPD strain 398 NTHi stained with CellTrace FarRed (CTFR) for 1 hour, treated with gentamicin for 1 hour to eliminate extracellular bacteria, and then stained for ciliated (tubulin), basal (CD49f) and goblet cell (CD66c) markers. Cells were flow-sorted to determine proportion of each cell type infected by NTHi. This gating strategy allowed us to isolate infected (CTFR+) and uninfected cells of each cell type. Flow plot shown from one representative experiment.

Figure 9. Flow cytometry data showing proportion of each cell type that is infected in PBECs grown at ALI

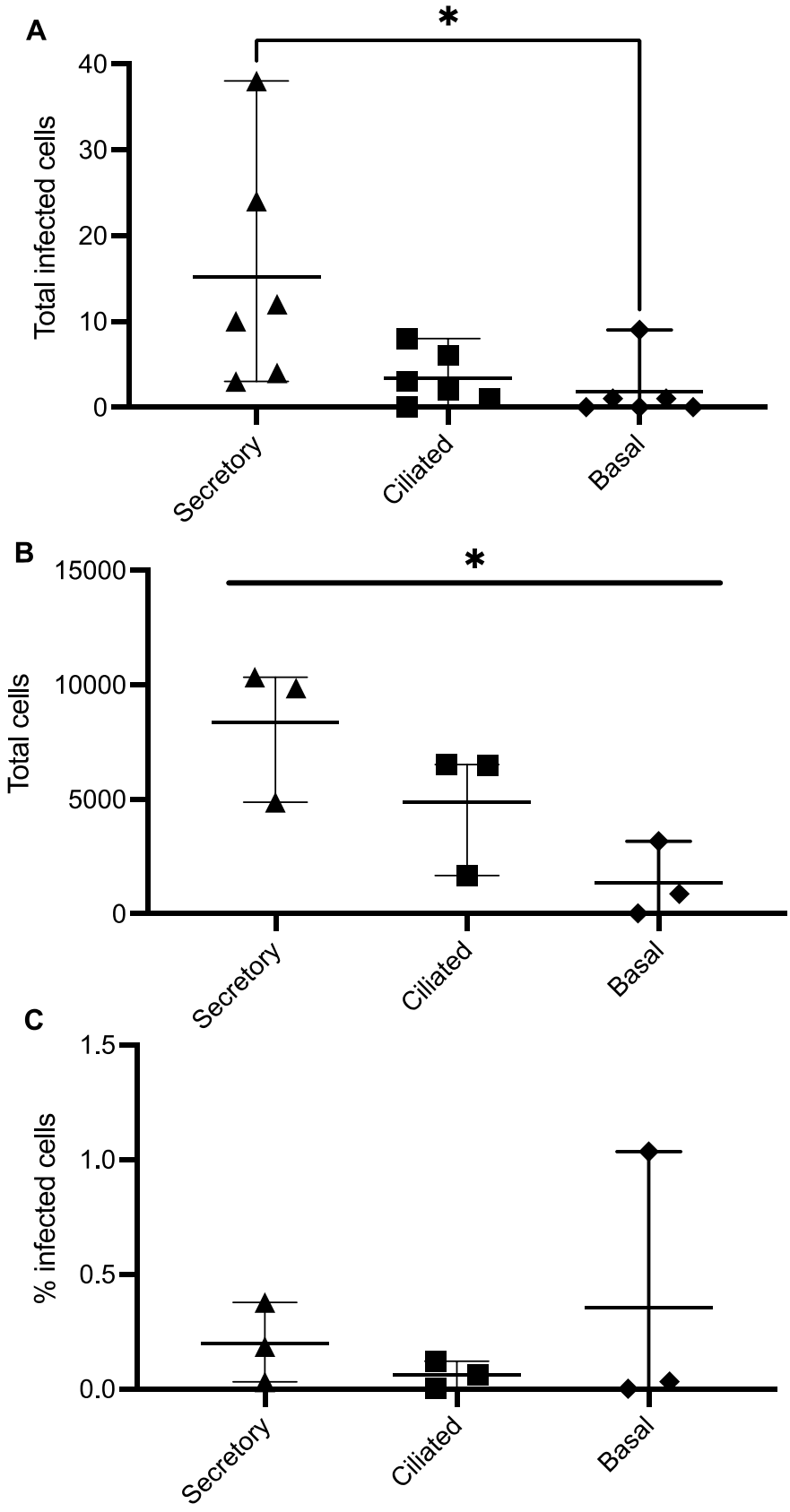


Figure 9. PBECs cultured at ALI were infected with COPD strain 398 NTHi stained with CellTrace FarRed for 1 hour, treated with gentamicin for 1 hour to eliminate extracellular bacteria, and then stained for ciliated (tubulin), basal (CD49f) and goblet cell (CD66c) markers. Cells were flow-sorted to determine proportion of each cell type infected by NTHi. A. The total number of infected cells in each sample was highest for secretory cells, while modest numbers of ciliated and basal cells were infected. Mean and range are indicated and dots represent technical replicates from 3 biological replicate experiments. B. The total number of each cell type in each sample mirrors the proportions of infected cells, with significant variation between the number of each cell type. Mean and range are indicated and dots represent the mean of replicates from each of 3 biological replicates. C. The percent of infected cells of each cell type was calculated. The cell type with the highest percentage of infected cells was basal cells, followed by secretory cells, with the lowest proportion of infected ciliated cells. Mean and range are indicated and dots represent the mean of replicates from each of 3 biological replicates.

3.4 Intracellular localisation of NTHi

NTHi has been identified varyingly within intracellular vesicles with early and late endosome-like characteristics. In addition, it is currently unclear whether NTHi is trafficked from early to late endosome and eventually merged with the lysosome resulting in the elimination of the bacteria, or whether NTHi is able to subvert the process. To evaluate whether NTHi is located in early/sorting endosomal or late endosomal vesicles, the intracellular lifestyle of NTHi was investigated by using confocal microscopy. PBECs cultured at ALI for a minimum of 28 days were infected for 4 hours with NTHi strain 398 stained with CFSE CellTrace dye, fixed with 4% PFA and stained for early or late endosome markers EEA1 and LAMP,1 respectively (Figure 10). Panels A, B, C and D show nuclear staining, early endosome staining, late endosome staining and stained NTHi. Merged images of NTHi and EEA1 showed limited co-localisation, as indicated by arrows (Figure 10F). There appeared to be greater co-localisation of NTHi with late endosome marker LAMP1, also marked with arrows (Figure 10G). These data suggest that although NTHi may initially be contained in vesicles with early endosome characteristics, it is rapidly moved to the late endosome during intracellular processing.

Figure 10. Localisation of NTHi in early vs late endosome

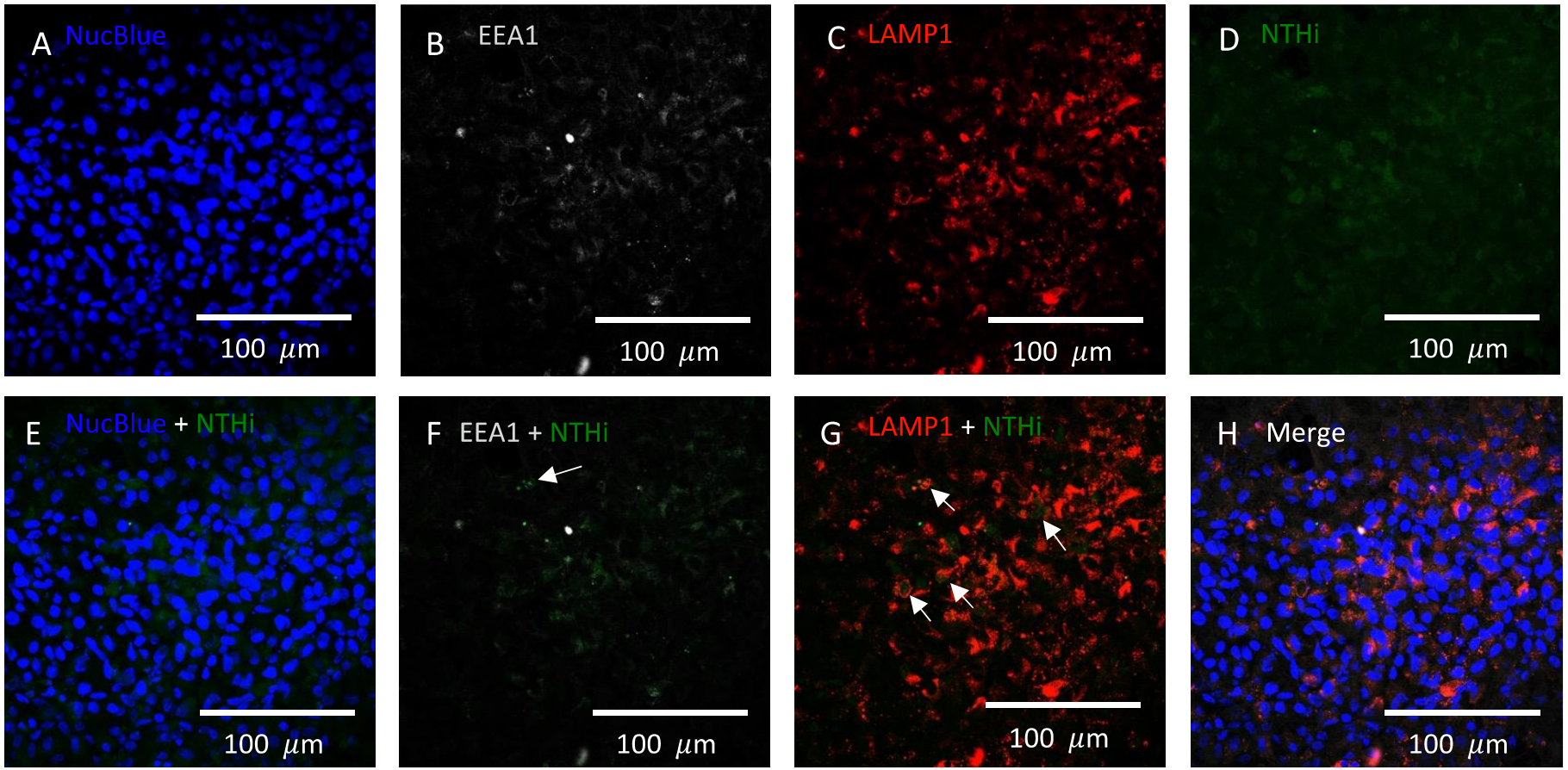


Figure 10. PBECs cultured at ALI were infected with NTHi CFSE-stained strain 398 for 4 hours, fixed in 4% PFA and then stained for early and late endosome markers to track localisation of bacteria through the endosome. A. NucBlue staining of nuclei. B. Staining for EEA1, an early endosome marker. C. Staining for the late endosome marker, LAMP1. D. CFSE-staining of NTHi. E. Merge of NTHi and nuclei. F. Merged images of NTHi and EEA1 show limited co-localisation. Arrow indicates NTHi-EEA1 co-localization. G. Co-localisation is more pronounced in NTHi-LAMP1 imaging, as indicated by arrows. H. Merge of all image layers.

3.5 Conclusions

This chapter has investigated the physical interactions between epithelial cells and NTHi, and attempted to characterise the lifestyle of NTHi within the epithelial monolayer. Initial experiments evaluating NTHi-epithelial cell interactions in Calu-3 cells have demonstrated that a high proportion of NTHi associated with the epithelium are adherent to the cell surface, while a much smaller number are internalised into the paracellular and intracellular spaces. There was significant heterogeneity in adhesion and internalisation between NTHi strains, likely due to heterogeneous expression of cell surface molecules. These trends are also seen in PBEC ALI cultures. However, the total number of adherent and internalised bacteria interacting with ALI cultures is significantly lower compared with submerged culture.

Mucus is an important physical barrier and first line of defence against infection in the lower airways (Fahy and Dickey, 2014; Zanin *et al.*, 2016). Therefore, I hypothesised that the lower adhesion and internalisation in ALI cultures may be due to greater production of mucus by ALI cultures acting as a physical barrier. However, analysis of supernatants from PBECs at ALI and Calu-3 cells in submerged culture showed significantly higher levels of MUC5AC, a structural component of mucus, in submerged cells. NTHi is capable of binding to mucins (Clementi and Murphy, 2011), so these results could suggest that increased Muc5ac production, rather than acting as a protective barrier, enables more efficient NTHi adhesion. Alternatively, the thickness of mucus found on the surface of ALI cultures may have made it difficult to effectively sample, so it is possible that this is an experimental artifact. In addition, differences in the cell makeup and media composition between ALI and submerged cultures may have affected mucus production and sampling, making it difficult to draw concrete conclusions from these data. Future studies could benefit from imaging techniques to more accurately assess mucus interactions with NTHi.

NTHi does not persist within epithelial cells in submerged culture, nor does it appear to persist solely intracellularly in cultures at ALI. This suggests that the bacteria may be replicating in another niche in persistent infections seen *in vivo*, and being only

transiently internalised into the epithelial layer. This view is supported by previous work which demonstrated that in NTHi within the epithelial monolayer was metabolically active but not replicating (Morey *et al.*, 2011). Further studies including measures of viability would be beneficial to ensure that the lack of viable NTHi is not due to the demise of the epithelial monolayer. NTHi has several other potential persistence mechanisms which would explain its ability to persistently infect human airways. NTHi is capable of forming biofilm-like structures which incorporate neutrophil NETs, allowing NTHi to subvert both antibiotic and cell-mediated killing. Another possibility is that NTHi may be internalised and persist within macrophages *in vivo*, which are not present in the current cell culture model (Craig *et al.*, 2001). Gentamicin exclusion assays are valuable for evaluating intracellular bacterial numbers, but due to the use of a lysing agent, it is possible that they affect bacterial viability and underestimate the number of viable bacteria (Baddal *et al.*, 2015a). Future experiments utilising imaging or flow cytometry techniques to evaluate the presence of viable NTHi would be beneficial to ensure that the viability is not affected during the lysis step.

The epithelium is composed of several cell types with distinct properties. To investigate the permissiveness of each of these to NTHi infection, I then attempted to evaluate whether NTHi preferentially infects one type of epithelial cell. There is some evidence that NTHi preferentially infects ciliated epithelial cells compared with other cell types (Morey *et al.*, 2011; Baddal *et al.*, 2015a), whilst other data suggest that ciliary function is important for mediating host-pathogen interactions and reduces bacterial binding (Walker *et al.*, 2017; Kuek and Lee, 2020). Using a model of differentiated epithelial cells infected with NTHi, imaging data showed substantial co-localisation between NTHi and ciliated cells, while fewer basal and secretory cells were localised with bacteria. In infected epithelial cells analysed by flow cytometry, secretory cells were the most highly infected, followed by ciliated and then basal cells. However, because the relative numbers of each cell type were different, when corrected for this, the proportion of infected cells of each type was not significantly different. This suggests that all epithelial cell types are permissive to NTHi infection. However, there was significant variation in internalisation between donors, complicating analysis of results using primary cell cultures, limiting the

relevance of these findings. Inclusion of a larger number of donors would ensure that these findings are representative of the population more broadly.

The intracellular lifestyle of NTHi was then investigated using confocal microscopy to evaluate trafficking of NTHi to the early/sorting endosomal or late endosomal vesicles.

It is presently unclear whether NTHi is trafficked from early to late endosomal vesicles and then killed during lysosomal acidification, or whether some NTHi strains are able to subvert this process, although there is evidence for both (Morey *et al.*, 2011; Clementi, Håkansson and Murphy, 2014; Ikeda *et al.*, 2015). Intracellular NTHi is contained within vesicles, but there is some data suggesting that NTHi is found only within early endosome-like vesicles (Ikeda *et al.*, 2015), while other studies found NTHi primarily within vesicles with late-endosome like characteristics (Morey *et al.*, 2011). However, many of these studies were conducted in cell lines, which limits the generalisability of these results. To more accurately represent lower airway epithelium, confocal imaging experiments using PBECs cultured at ALI were performed, demonstrating limited co-localisation of NTHi with early endosomes but substantial co-localisation with late endosomes. This suggests that NTHi is successfully trafficked from early to late endosome in ALI cells. It would be of interest to conduct time-course experiments to determine whether NTHi is subsequently trafficked to the lysosome, or whether NTHi is capable of subverting lysosomal acidification to promote persistence.

Overall, the experiments in this chapter have demonstrated that NTHi is internalised in submerged and ALI culture models in significant numbers, but does not persist in the absence of extracellular bacteria. The number of adherent and internalised bacteria vary significantly between strains. NTHi is internalised into all types of epithelial cells in a model of differentiated PBECs, but infects a particularly high proportion of basal cells. NTHi within epithelial cells is contained within primarily within vesicles with late endosome-like properties, but is also present in early endosomes, suggesting that it is trafficked from early to late endosome, although it may subvert the lysosomal acidification process. Having investigated how NTHi

interacts physically with the host cell, the next steps were to investigate induction of immune response due to NTHi.

Chapter 4 Epithelial immune response to NTHi infection

NTHi has been identified repeatedly intracellularly within the epithelial cells and paracellular spaces in the lower airways of patients with NTHi infections (Clementi, Håkansson and Murphy, 2014). Exposure to NTHi elicits an inflammatory immune response in epithelial cells of the lower airways (King and Sharma, 2015). NTHi mediates immune responses using several pathways, such as signalling via TLRs, NLRP3 and AIM2 inflammasomes, and NOD1 and 2 (Chen *et al.*, 2018). Activation of these pathways results in induction of cytokines such as neutrophil chemoattractant IL-8, TNF, IFN-γ and IL-1β, which are thought to drive neutrophilic inflammation. The role of invasion in the progression of NTHi disease remains poorly understood, but it has been suggested that there may be a correlation between invasion and symptomatic infection (Clementi and Murphy, 2011). Invasion and adhesion are also thought to be mechanisms for NTHi persistence in the lower respiratory tract (Ahearn *et al.*, 2019). In addition, NTHi expresses a large number of outer membrane proteins which are thought to mediate adhesion and invasion, and which are highly immunogenic (Ronander *et al.*, 2009; Hallström *et al.*, 2011; Duell, Su and Riesbeck, 2016; Atack *et al.*, 2018). However, NTHi has several strategies by which it evades the immune response and promotes persistent infection. NTHi is capable of undergoing genome evolution and phase variation to facilitate persistent infection in the hostile airway environment, varying expression of outer membrane proteins, membrane lipooligosaccharide and adherence and nutrient uptake mechanisms (Pettigrew *et al.*, 2018).

There is marked heterogeneity within NTHi, with variations in adhesion and internalisation rates, as well as known variations in specific outer membrane proteins and adhesins (LaCross *et al.*, 2008; Schumacher *et al.*, 2012). In addition, differences in phase variable regulons, a mechanism of bacterial gene regulation, have been identified between otitis media and COPD isolates (Atack *et al.*, 2019). I initially hypothesised that strains isolated from lower airway infections in patients with COPD would exhibit higher internalisation rates than those isolated from the middle ear during otitis media infections.

Several studies have investigated the role of various adhesion proteins in bacterial interactions with epithelial cells, however, few have evaluated the consequences differing adhesion and internalisation rates have for immune response in the epithelium. In this aim I have also evaluated the significance of invasion rate on strength of immune response in epithelial cells, initially hypothesising that increased numbers of internalised bacteria would lead to higher immune activation. In addition, I have hypothesised that increased adhesion would correspond with a more robust immune response in epithelial cells infected with NTHi strains with high and low adhesion rates.

4.1 Internalisation rates differ between strains

To determine whether strains differed in invasiveness depending on their clinical source, initial experiments characterised the invasiveness of NTHi strains isolated from either COPD patients or otitis media (OM) infections. Percent internalisation is a calculation of the invasiveness of strains by comparing the number of internalised bacteria with the number of bacteria in the initial inoculum. The number of internalised bacteria was estimated by lysing the epithelial cells to release the internalised bacteria after applying gentamicin to eliminate extracellular bacteria, and performing a colony forming unit (CFU) count assay, involving plating the recovered bacteria on agar at various dilutions and counting the colonies. BEAS-2B cells in submerged culture were infected with five COPD and five OM strains for 4 hours prior to treatment with gentamicin to remove extracellular bacteria, and cell lysis at 6 hours post-infection (hpi) (Figure 1). Internalisation rates differed between NTHi strains in terms of both total numbers of internalised bacteria and the proportion of bacteria which have been internalised (Figure 1, A and B). However, this variation did not correlate with the disease from which the strain was isolated.

Figure 1. Internalisation differs between NTHi strains.

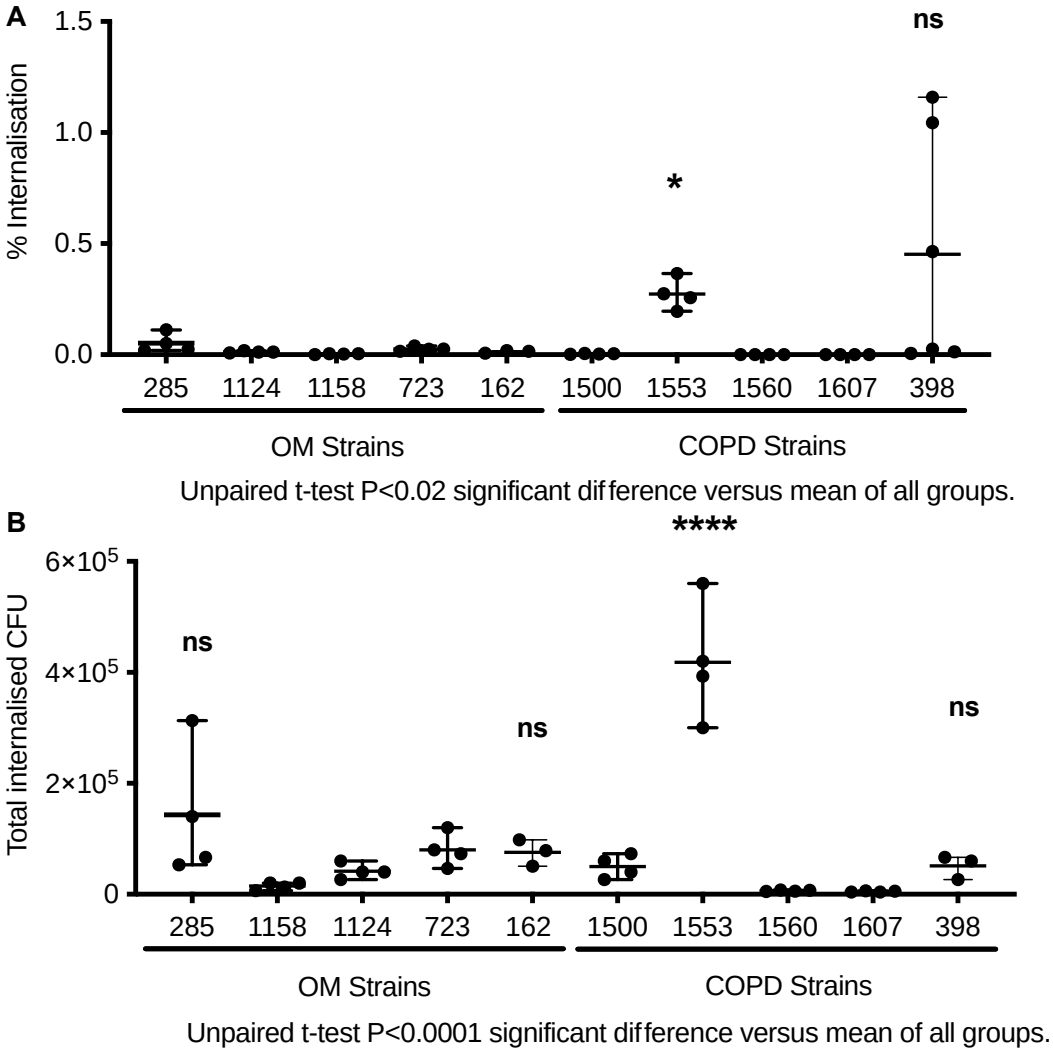


Figure 1. Internalisation by epithelial cells varies between NTHi strains. BEAS-2B cells (5.3e4 cells/cm$^{2)}$ were infected with a selection of NTHi strains and the number of internalised bacteria evaluated by lysing the cells 6 hours post-infection and performing a colony forming units (CFU) assay. A. Percent invasion was calculated by comparing the number of internalised bacteria calculated from the CFU assay with the number of bacteria the cells were inoculated with. Percent internalisation differs substantially between NTHi strains in BEAS-2B cells, although only strain 1553 differs statistically when compared to the mean of all other groups. B. Absolute numbers of NTHi CFUs recovered from infected BEAS-2B cells shows substantial variation in internalisation between NTHi strains, although again only strain 1553 is significantly different. n=4 independent replicates. Significance was evaluated using paired t-tests on log-transformed data to compare each strain with the mean of all other strains. Means and range are indicated on plots and dots represent technical replicates.

4.2 Does invasion rate correlate with immune activation?

I initially hypothesised that bacterial internalisation is necessary for robust immune activation, as this would be likely to activate intracellular pathogen associated molecular pattern (PAMP) receptors including inflammasome-associated NOD-like receptors (NLRs) NLRP3 and AIM2 and NOD1 and 2. To evaluate this, experiments were performed to compare immune activation in cells infected with NTHi strains with higher and lower internalisation rates.

Initial experiments established that the proportional internalisation of NTHi strain 398 is significantly higher than NTHi lab strain 11931 in Calu-3 cells grown in submerged culture, in terms of both percent internalisation and absolute numbers ($p<0.008$ by unpaired t-test) (Figure 2, A and B).

Figure 2. NTHi strain 11931 is internalised to a significantly lower extent than NTHi 398.

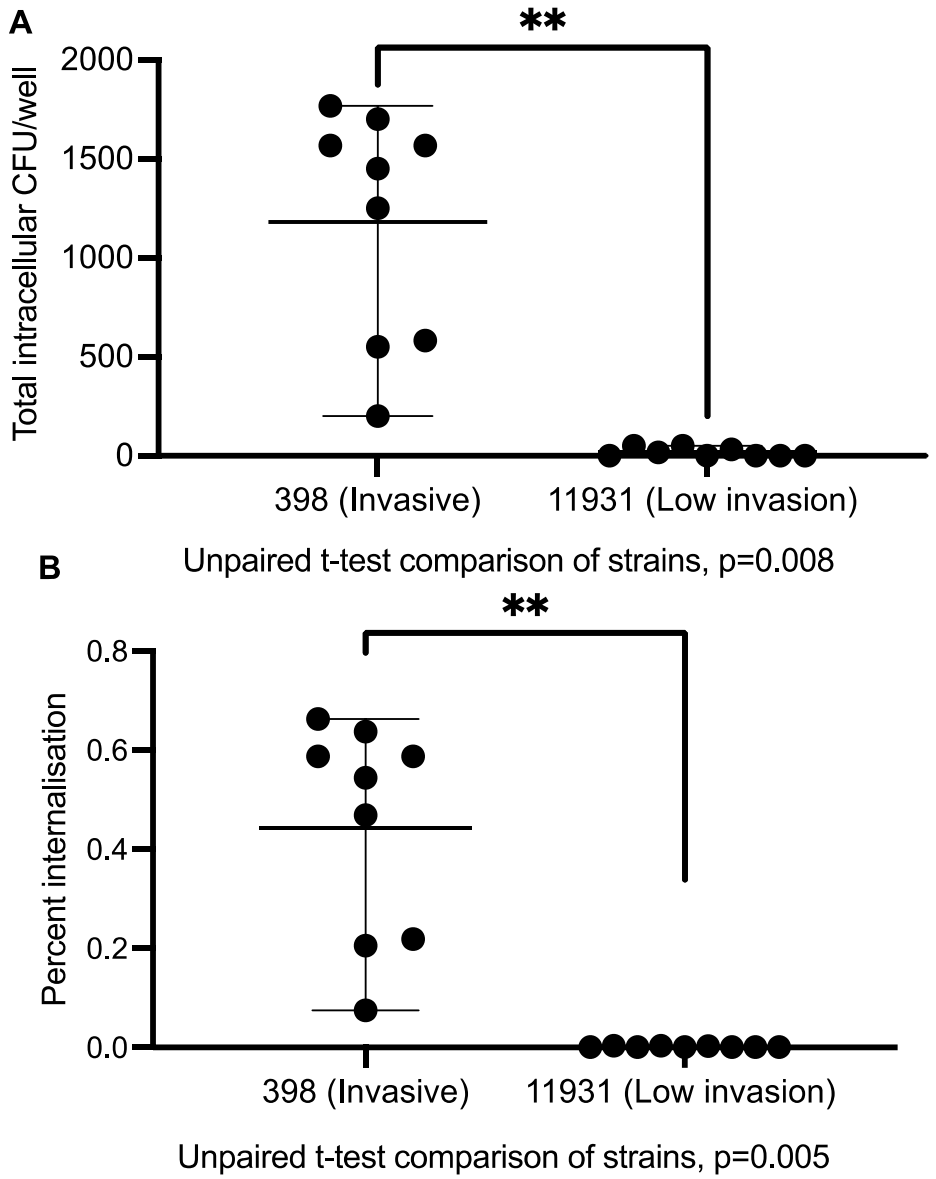


Figure 2. Internalisation of NTHi strain 398 was significantly higher than NTHi lab strain 11931 at 24 hours post-infection. Confluent Calu-3 cells in submerged culture were incubated in the presence of bacteria for 24 hours. A CFU assay was then performed to enumerate internalised bacteria. Both total numbers of internalised bacteria (A) and the proportion of bacteria that were internalised compared to the total inoculum (B) were significantly higher for strain 398 compared with strain 11931. Unpaired t-tests were performed on log-transformed data to evaluate the difference between strains. Mean and range are indicated on plots. n=3 biological replicates with 3 technical replicates each. Dots represent technical replicates.

Analysis by qPCR showed that immune response to highly internalised NTHi strain 398 was comparable with that of non-invasive lab strain 11931 in Calu-3 cells exposed to bacteria for 24 hours (Figure 3). Expression of pro-inflammatory markers IL-1β, IL-8, CCL2, IL-6 and TNF was significantly upregulated in response to NTHi. However, there was not a substantial difference between gene expression in response to NTHi strain 398 compared with 11931 for any target (Figure 3, A-E).

Figure 3. qPCR analysis has demonstrated that NTHi strain 11931 and strain 398 induce inflammatory cytokine mRNA expression at comparable levels.

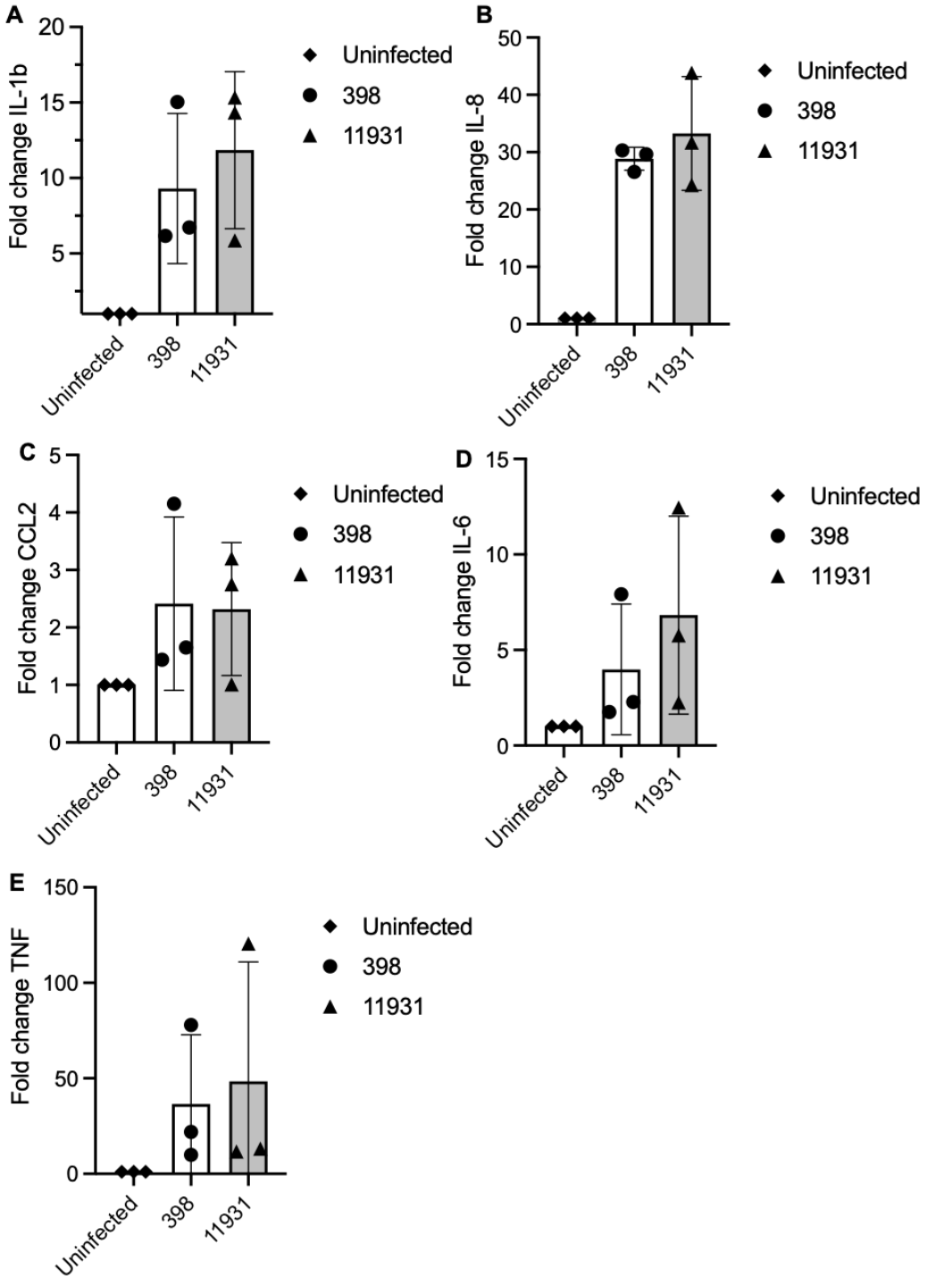


Figure 3. Infection with a more highly internalised NTHi strain did not correlate with more robust immune activation in Calu-3 cells inoculated with NTHi strains 398 (high internalisation) compared with NTHi lab strain 11931 (low internalisation). A-E. Each marker was significantly increased in cells treated with NTHi, but there was no significant difference in induction between strains. Significance was evaluated using 2-way ANOVAs to evaluated the difference between strains. n=3 biological replicates with 2 technical replicates each. Dots represent mean of technical replicates.

To confirm that the results comparing strain 11931 and 398 were not strain-dependent, but were specifically related to the extent of internalisation, a model was established using cytochalasin D treatment to limit NTHi internalisation. Cytochalasin D binds to the barbed end of actin filaments and prevents polymerisation of actin monomers, which functionally blocks phagocytosis of bacteria into the cell. Initial results confirmed that treatment with cytochalasin D at low concentrations did not affect bacterial viability (Figure 4).

Figure 4. Cytochalasin D does not reduce bacterial viability at low concentrations.

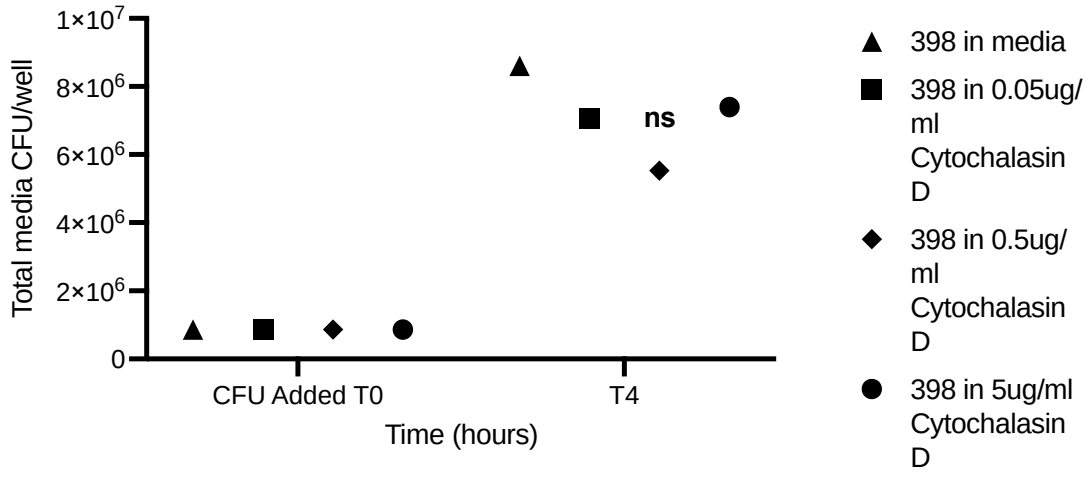


Figure 4. In Calu-3 cell cultures inoculated with NTHi strain 398, treatment with cytochalasin D at concentrations up to 5 μg/ml did not affect bacterial viability for up to 4 hours of exposure. Dots represent mean of 3 technical replicates.

Pre-treatment with cytochalasin D significantly reduced internalisation of NTHi strain 398 in a concentration-dependent manner at 4 hours post infection (Figure 5, A and B). However, due to the ability of NTHi to be internalised by several mechanisms,

blockade of phagocytosis by cytochalasin D does not completely eliminate internalisation.

Figure 5. Treatment with cytochalasin D partially inhibited internalisation of NTHi into Calu-3 cells.

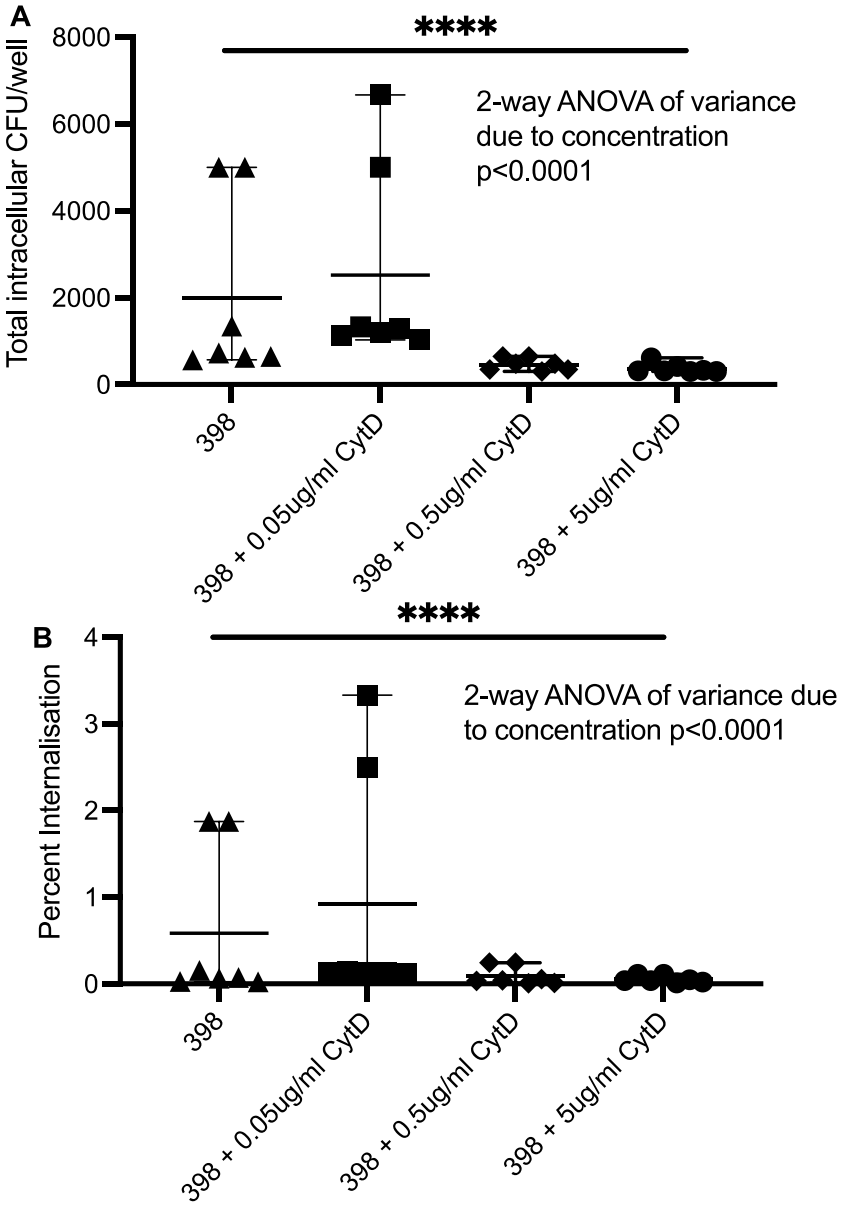


Figure 5. Cytochalasin D reduces internalisation rate of 398 in Calu-3 cells in submerged culture in a dose-dependent manner. Internalisation by NTHi strain 398 is significantly reduced in Calu-3 cells pre-treated with cytochalasin D in increasing concentrations, in terms of both total numbers of internalised bacteria (A) and as a percent of total bacteria (B). Significance was evaluated using a 2-way ANOVA on log-transformed data. Means and ranges are indicated on plots and dots represent technical replicates from 3 biological replicates.

The reduction in NTHi internalisation in cells treated with cytochalasin D did not result in altered immune activation (Figure 6). PCR analysis was performed to evaluate gene expression of IL-8, IL-6, TNF and IL-1β in response to NTHi strain 398 with or without cytochalasin D pre-treatment. IL-8, IL-6 and TNF expression were increased in response to NTHi, but this was not altered by diminished NTHi internalisation in cells treated with cytochalasin D (Figure 6, A-C). There was a trend towards reduced expression of IL-1β in a dose-dependent manner in cells treated with cytochalasin D (Figure 6D). IL-8, IL-6 and TNF expression were comparable between untreated samples and those treated with cytochalasin D alone (Figure 6, A-D).

Figure 6. Reduction in bacterial internalisation did not result in lower immune activation.

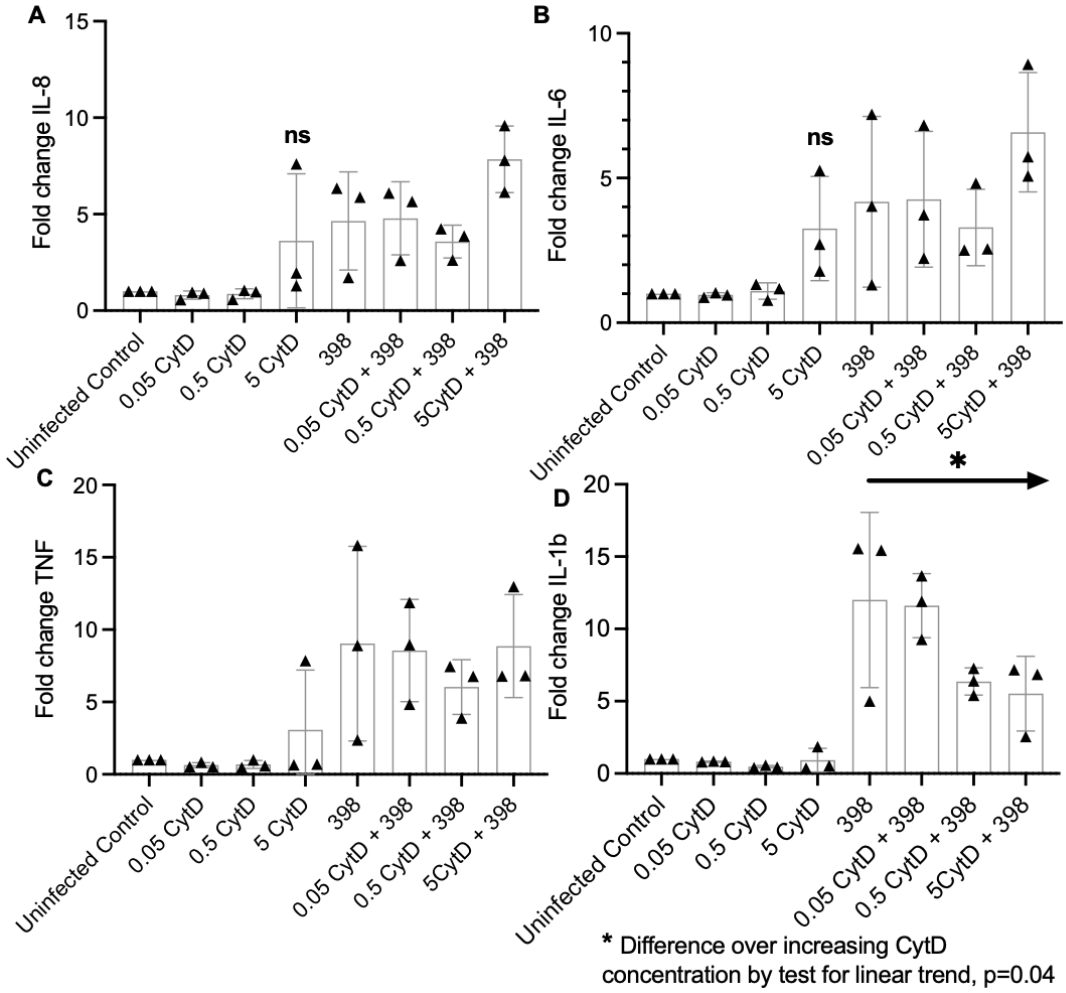
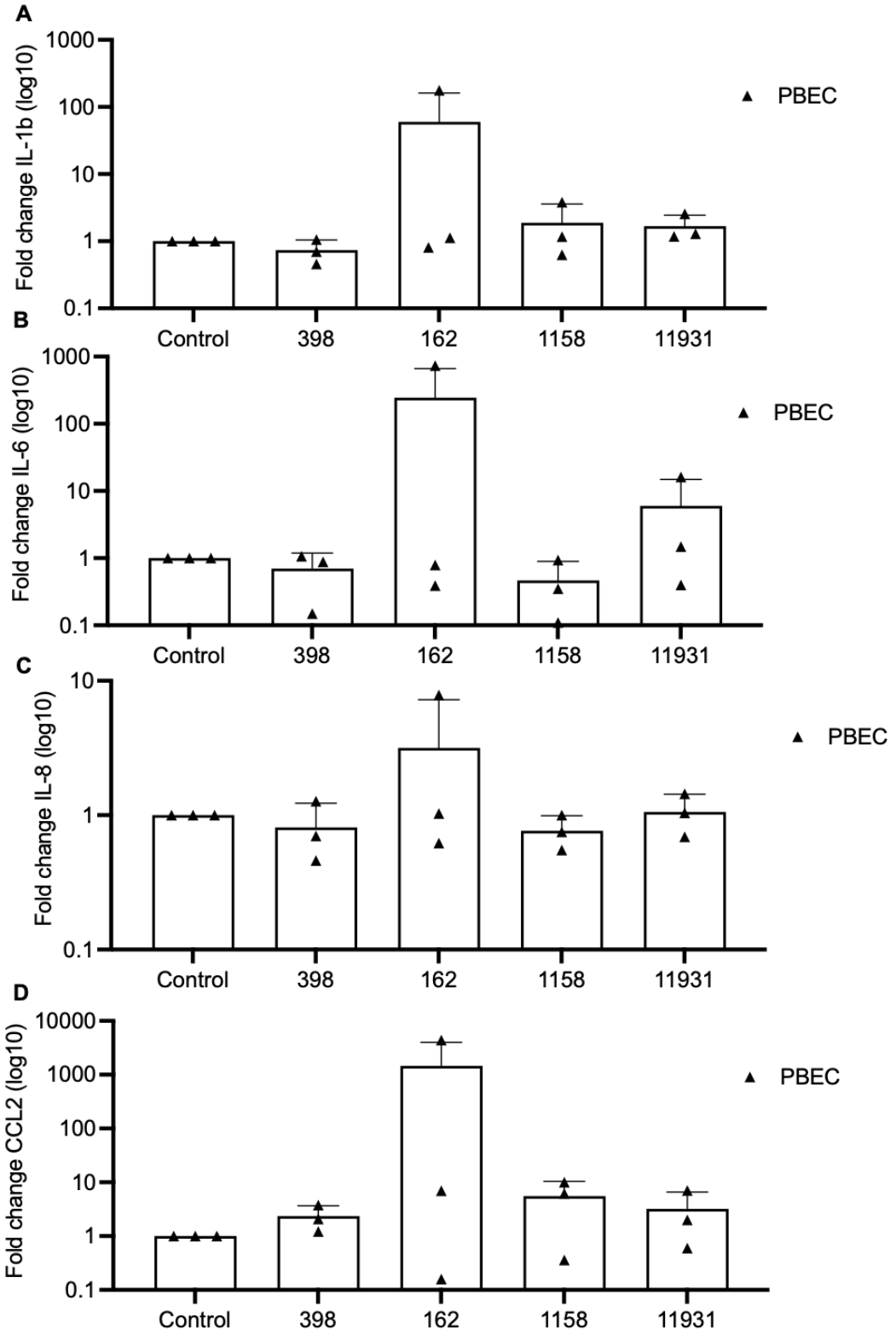


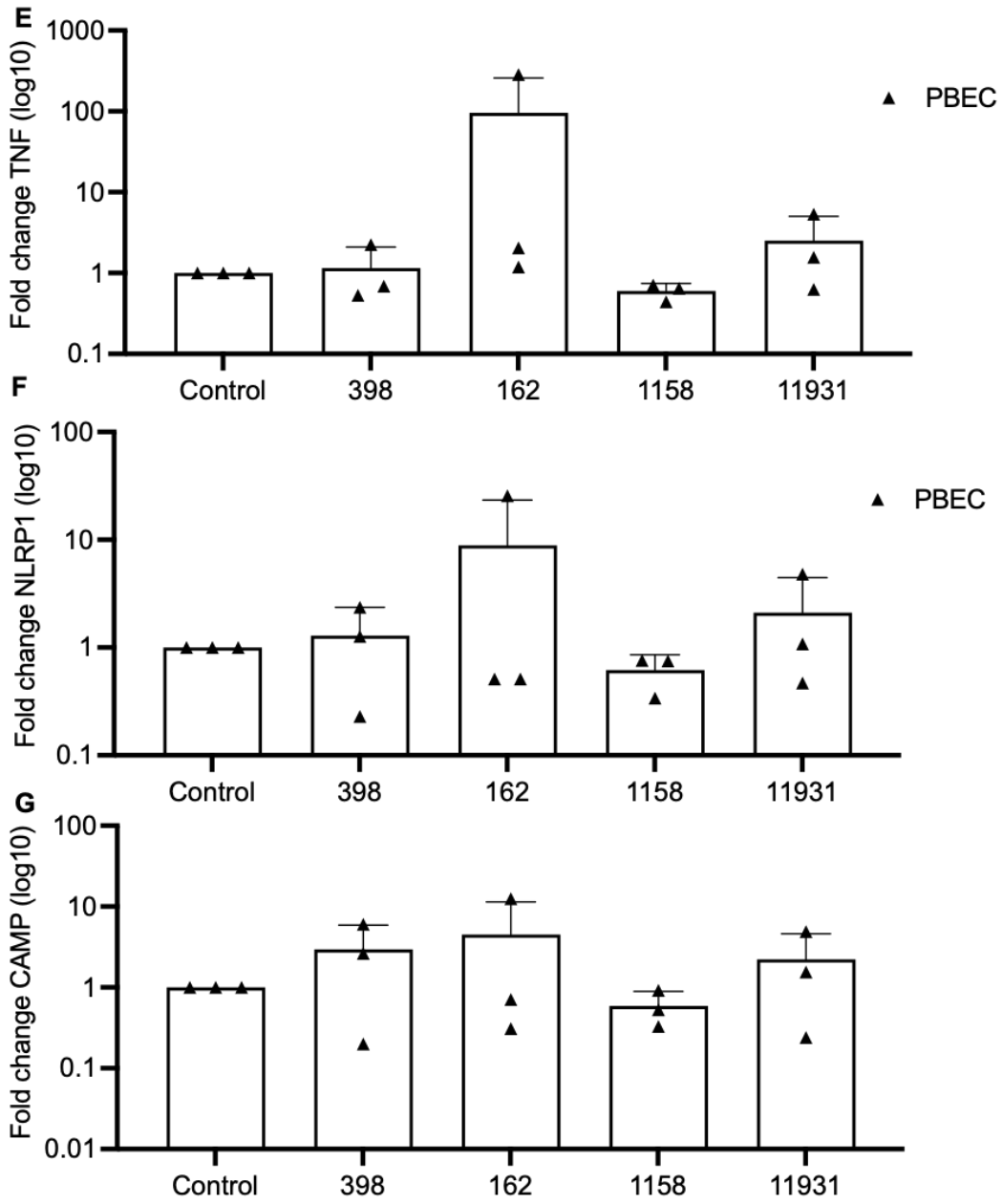
Figure 6. In confluent Calu-3 cells inoculated with NTHi strain 398 and treated with increasing concentrations of cytochalasin D, reduction in NTHi strain 398 internalisation in cells treated with cytochalasin D did not result in significantly reduced immune activation. Treatment with 5 μg/ml cytochalasin D trended towards increasing expression of IL-8, IL-6 and TNF, but this was not significant for any gene (A, B, C). Expression of IL-8, IL-6 and TNF was not altered due to decreased bacterial internalisation (A, B, C). IL-1β expression trended towards a concentration-dependent decrease in response to diminishing NTHi internalisation (D). The significance of this finding was confirmed using a test for linear trend across groups treated with increasing concentrations of cytochalasin D. n=3 independent experiments performed in technical duplicates.

To verify these findings in a primary cell model, PBECs cultured at ALI were exposed to NTHi strains with high and low proportional internalisation for 24 hours, and RNA and supernatants were collected for analysis using a Fluidigm PCR array and Meso-Scale Discovery (MSD) assay.

A Fluidigm PCR array experiment was performed to evaluate gene expression in response to NTHi strains with varying degrees of internalisation and adhesion. Expression of cytokines IL-6, IL-8, TNF, inflammasome marker IL-1β and CCL2 (MCP1) were evaluated. In addition, NLRP1 and antimicrobial peptide cathelicidin (CAMP) were investigated. Overall, there was only modest induction of gene expression in response to NTHi. Comparison of strains with high and low internalisation rate in PBECs cultured at ALI demonstrated that lower internalisation did not affect expression of cytokines (Figure 7). IL-1β was induced modestly by infection with strains 162, 1158 and 11931 compared to uninfected control cells, but this was not related to internalisation rate (7A). IL-6 was induced by infection with strains 162 and 1158, but this did not reach statistical significance and there was significant variation in response between donors (7B). IL-8 was not significantly induced by treatment with NTHi (7C). All NTHi strains modestly induced expression of CCL2, although this was not statistically significant (7D). Both TNF and NLRP1 were numerically elevated in cells infected with 162 and 1158, but this was not significant and there was substantial variation between donors (Figure 7, E and F). Expression of Cathelicidin (CAMP) was slightly increased by infection with 398, 162 and 11931 (7G). There was variation in induction of gene expression between strains, but this was not related to internalisation.

Figure 7. Lower internalisation rates did not affect pro-inflammatory gene expression in PBECs.




E
Fold change TNF (log10)
1000
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0.1
Control
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162
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PBEC
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Fold change NLRP1 (log10)
100
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1
0.1
Control
398
162
1158
11931
PBEC
G
Fold change CAMP (log10)
100
10
1
0.1
0.01
Control
398
162
1158
11931

Figure 7. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi internalised to different extents in PBECs cultured at ALI. Mature ALI cultures were inoculated with a selection of NTHi strains, 2 highly internalised (398 and 162) and two less highly internalised strains (1158 and 11931) and incubated in the presence of extracellular bacteria for 24 hours prior to isolation of RNA. Degree of internalisation did not correlate with induction of any signalling target. A. Infection with strain 162, 1158 and 11931 modestly induced IL-1β expression compared to uninfected control cells, but this was not related to internalisation rate. B. IL-6 was induced by strain 162 and 1158, but this was not significant, and there was significant variation between response in donors. C. IL-8 was not significantly induced by treatment with NTHi. D. CCL2 was modestly induced by all NTHi strains, although this was not statistically significant. E, F. Both TNF and NLRP1 were numerically elevated in cells infected with 162 and 1158, but this was not significant and there was substantial variation between donors. G. Cathelicidin (CAMP) was slightly induced by infection with 398, 162 and 11931. Overall, there was no correlation between degree of internalisation and gene expression. These experiments were performed in 3 biological replicates from healthy control donors. qPCRs were performed in duplicate wells. Significance was evaluated for all targets using 2-way ANOVA with Sidak's multiple comparison tests on log-transformed data.

To address potential for disparity between gene expression and actual production of cytokine proteins, ELISA and MSD assays were performed to measure levels of supernatant cytokines. PBECs from 3 healthy control donors were grown in submerged culture and infected with highly internalised NTHi strain 398 and less internalised strain 1607 and incubated for up to 168 hours (7 days) (Figure 8). NTHi significantly increased concentrations of IL-8 in cell supernatants by 24 hpi. IL-8 is constitutively expressed at low levels in uninfected PBECs (Marshall *et al.*, 2001). At 168 hpi, IL-8 concentration in uninfected PBECs was equal to that of infected cells at 48 hpi, likely due to the accumulation of constitutively expressed IL-8. Media were not exchanged throughout the timecourse, promoting the accumulation of IL-8 in the media. There was not a significant difference between IL-8 production in cells infected with different strains at any timepoint.

Figure 8. Inoculation of PBECs grown in submerged cultures with NTHi results in robust production of IL-8 regardless of internalisation rate.

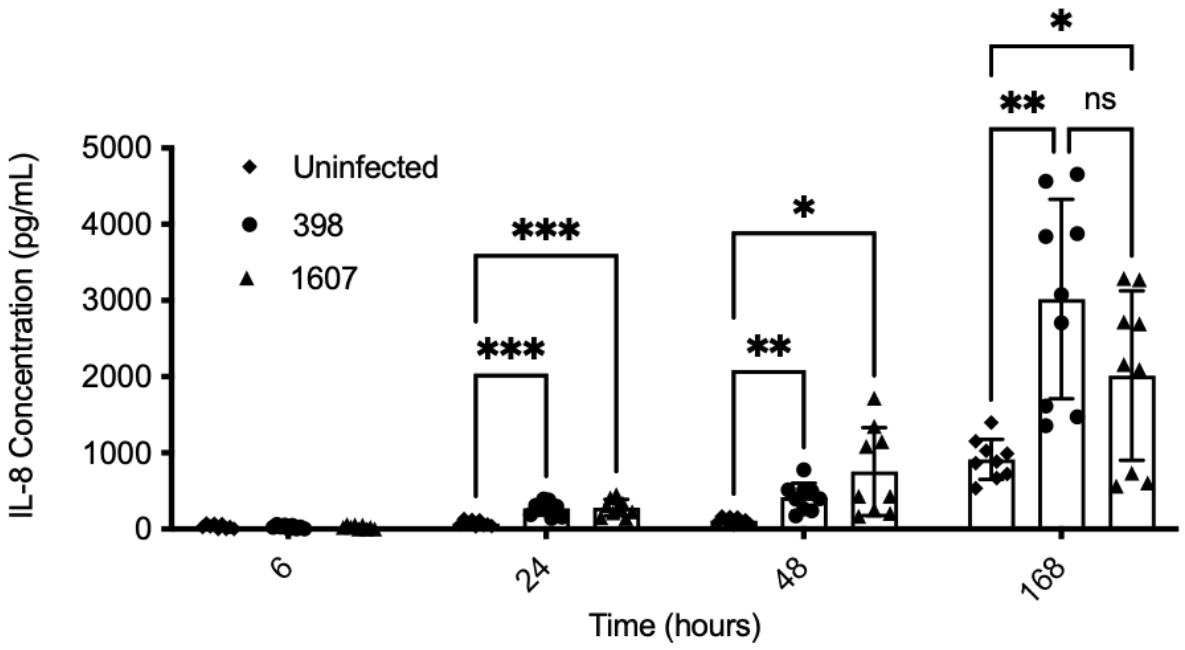


Figure 8. PBECs in submerged culture were inoculated with a highly internalised strain (398) and less internalised strain (1607). Supernatants were collected at various timepoints and levels of IL-8 were evaluated using ELISA. IL-8 production was significantly induced in cells treated with either strain of NTHi from 24 hours post-infection. IL-8 production did not differ significantly between strains at any time point. Significance was evaluated using 2-way ANOVA with Sidak's multiple comparison tests to evaluate significance between each treatment and timepoint. n=3 biological replicates assayed in technical triplicates. Mean and standard deviation represented on plots. Dots represent technical replicates.

Protein levels of IL-6, IL-1β and TNF were evaluated in pooled apical and basal supernatants from PBECs cultured at ALI in the presence of NTHi for 24 hours using MSD analysis (Figure 9). Strains are shown from least to most highly internalised. IL-1β was not induced by any NTHi strain (Figure 9A). There was modest induction of IL-6 by strains 1158, 375 and 398, but this was not significant (Figure 9B). There was not induction of TNF by any strain compared to uninfected cells (Figure 9C). Overall, NTHi only slightly induced signalling in PBECs at ALI.

Figure 9. Production of pro-inflammatory cytokines did not depend on internalisation rate

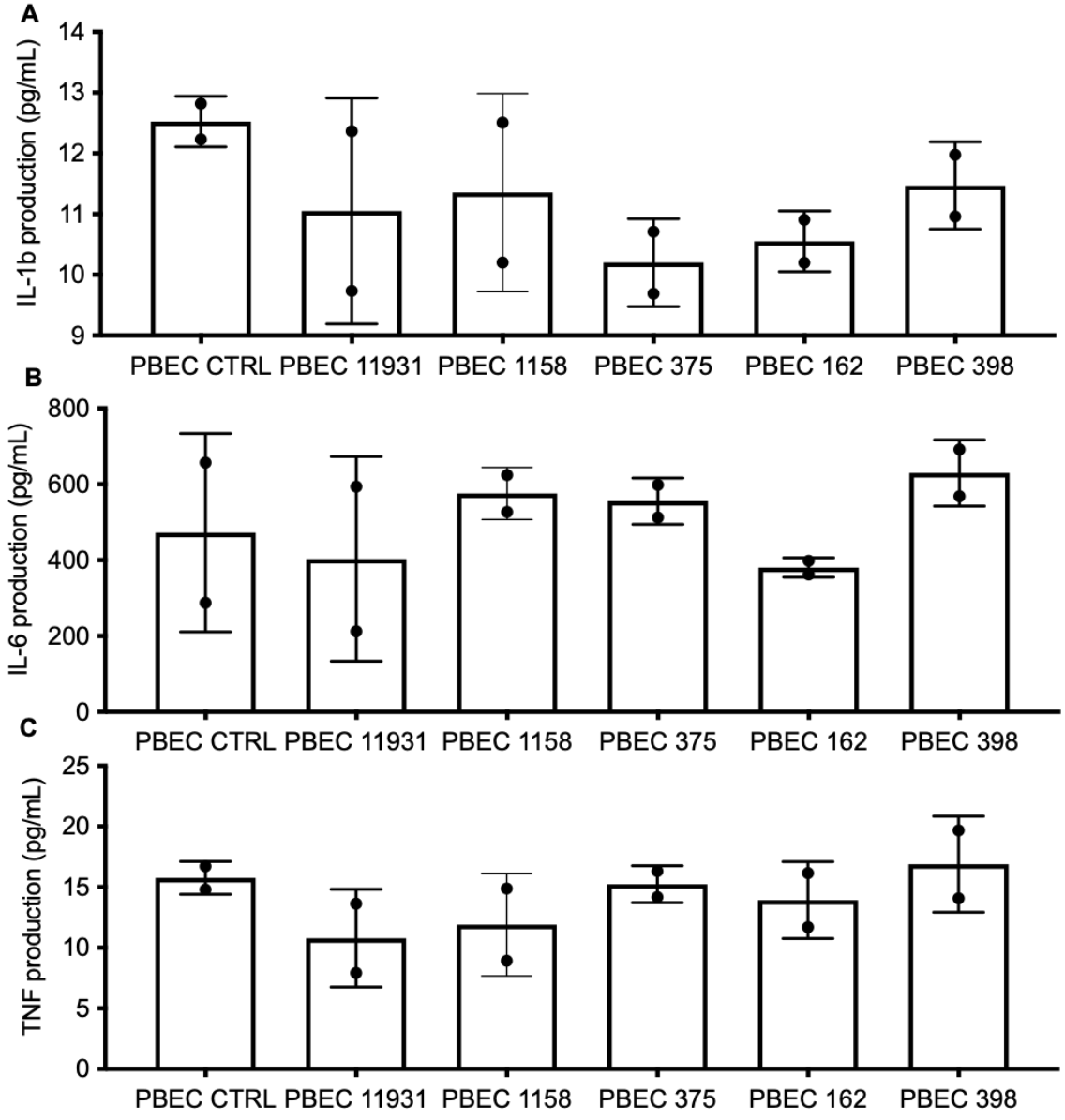


Figure 9. PBECs grown at ALI and inoculated with a selection of NTHi strains were incubated in the presence of extracellular bacteria for 24 hours prior to sampling of supernatants. Pooled apical and basal supernatants were evaluated for levels of IL-1β, IL-6, and TNF using a meso-scale design (MSD) assay. Strains are shown from least to most highly internalised. A. IL-1β was not induced by any NTHi strain. B. IL-6 was modestly induced by 1158, 375 and 398, but this was not significant. C. TNF was not induced by any strain compared to control. Significance for each target was evaluated by 2-way ANOVA with Sidak's multiple comparison tests on log-transformed data. These experiments were performed in 3 biological replicates from healthy control donors. MSD assays were performed on duplicate wells.

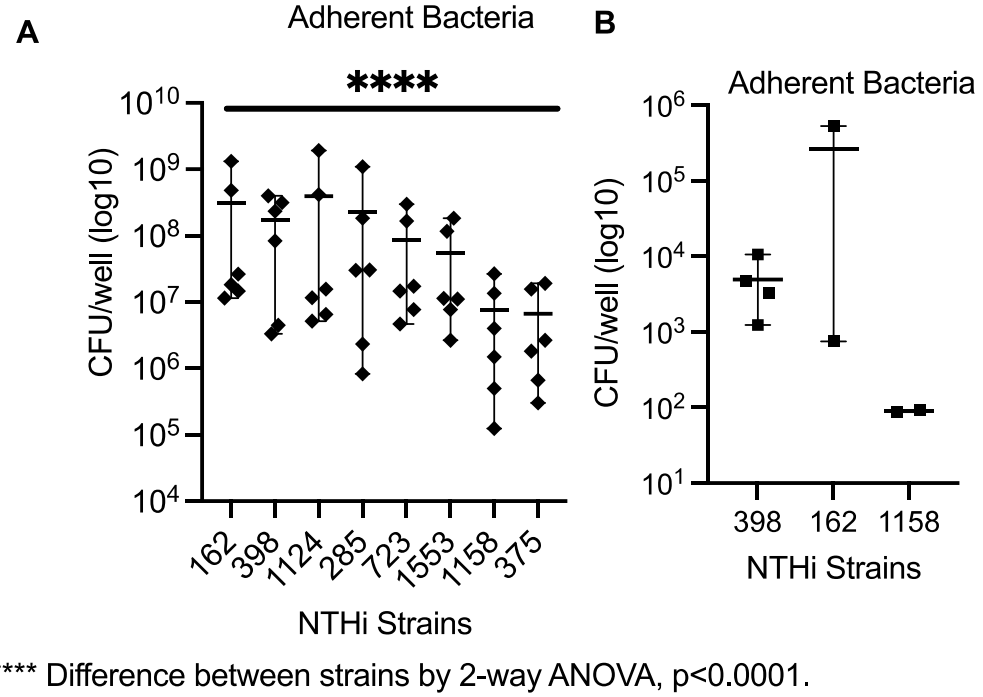
Taken together, these data show that NTHi potently induces a cytokine response in cell lines and PBECs in submerged culture, which may suggest that NTHi-induced epithelial immune responses are a driver of inflammation in the lung. This induction varies between strains, but does not appear to depend on the internalisation capacity of NTHi strains. Induction of cytokine signalling in response to NTHi was substantially diminished in PBECs at ALI compared with results from submerged cultures, perhaps due to lower overall interaction between bacteria and host cells, or differing cell phenotypes. Analysis of data from a PBEC ALI model is further complicated by the high levels of heterogeneity in responses from cells isolated from different donors.

4.3 Does adhesion differ between strains?

Adhesion rate is an often-cited factor in persistence and pathogenicity of NTHi, and adhesion molecules are often highly immunogenic (Davis, Patel, *et al.*, 2014). I therefore hypothesised that more adherent strains of NTHi may induce more robust immune responses.

Calu-3 cells in submerged culture were incubated with NTHi for 4 hours and washed with PBS to remove non-adherent bacteria. The total number of adherent and internalised bacteria were then enumerated using a CFU assay. A gentamicin exclusion assay was also performed to determine the number of internalised bacteria, and this number was subtracted from the total number of bacteria to yield the number of adherent bacteria for each strain. There was significant variation in numbers of adherent bacteria between strains, with NTHi strains 162 and 398 being substantially more adherent than strains 375 and 1158 (Figure 10A).

Figure 10. NTHi adhesion rates vary between strains in Calu-3 and ALI PBECs.



**** Difference between strains by 2-way ANOVA, p<0.0001.

Figure 10. Enumeration of adherent bacteria associated with Calu-3 cells in submerged culture (A) and PBECs at ALI (B) following incubation with NTHi strains for 4 hours. A. There is significant variation in adhesion between strains, with a tendency towards lower levels of adhesion in strains 1158 and 375. Means and ranges are indicated on plots and dots represent technical replicates. B. Similarly, in PBECs at ALI, numbers of adherent bacteria have a tendency towards reduced adhesion in strain 1158 compared to 398 and 162, but this is not significant (p=0.25 by t-test between each strain and the mean of all strains). Means and ranges are indicated on plots and dots represent biological replicates.

These data were recapitulated in PBECs cultured at ALI. Strain 1158 was less adherent numerically than 398 and 162, but this was not statistically significant (Figure 10B). Overall numbers of adherent and internalised bacteria were lower in ALI cells (10B). These data suggest that there is variation in the capacity for adhesion of different NTHi strains, and that this is consistent across cells in submerged and ALI cultures.

4.4 Does increased adhesion correlate to higher immune activation?

Initial experiments to evaluate differences in immune response in more and less adherent NTHi strains were performed in Calu-3 cells. Calu-3 cells in submerged culture were exposed to NTHi for 24 hours and PCRs were performed to evaluate gene expression of IL-8, IL-6, IL-1β, CCL2 and TNF (Figure 11). IL-8, IL-1β and TNF expression were robustly induced infection with any NTHi infection strain (Figure 11, A, B and D). NTHi exposure induced modest expression of IL-6 and CCL2 (Figure 11, C and E). However, there were no significant differences between induction of any target by strains with higher and lower adhesion. These results suggest that adhesion ability of NTHi may not be related to the degree of immune activation it induces.

Figure 11. Adhesion rate does not significantly relate to immune activation in Calu-3 cells

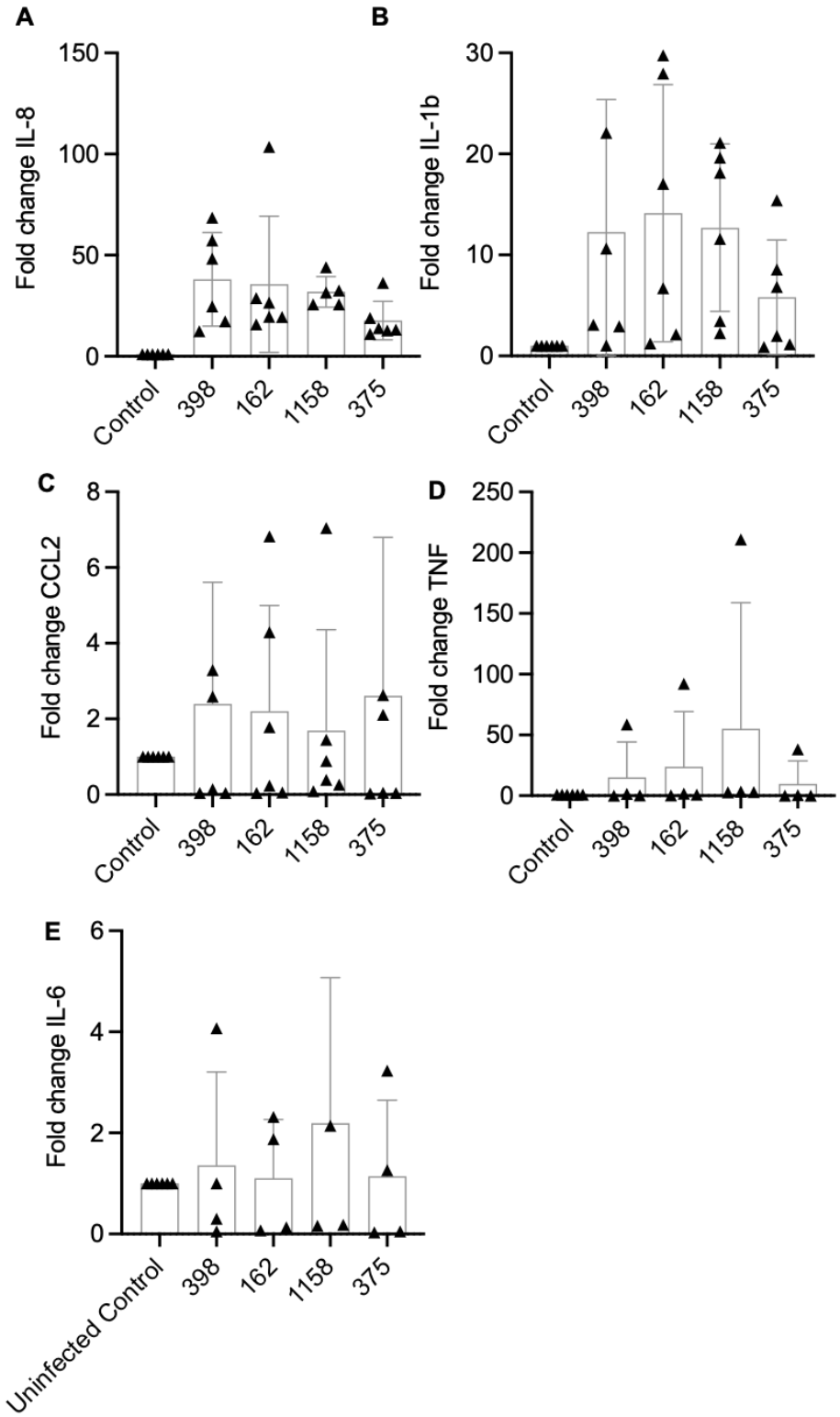
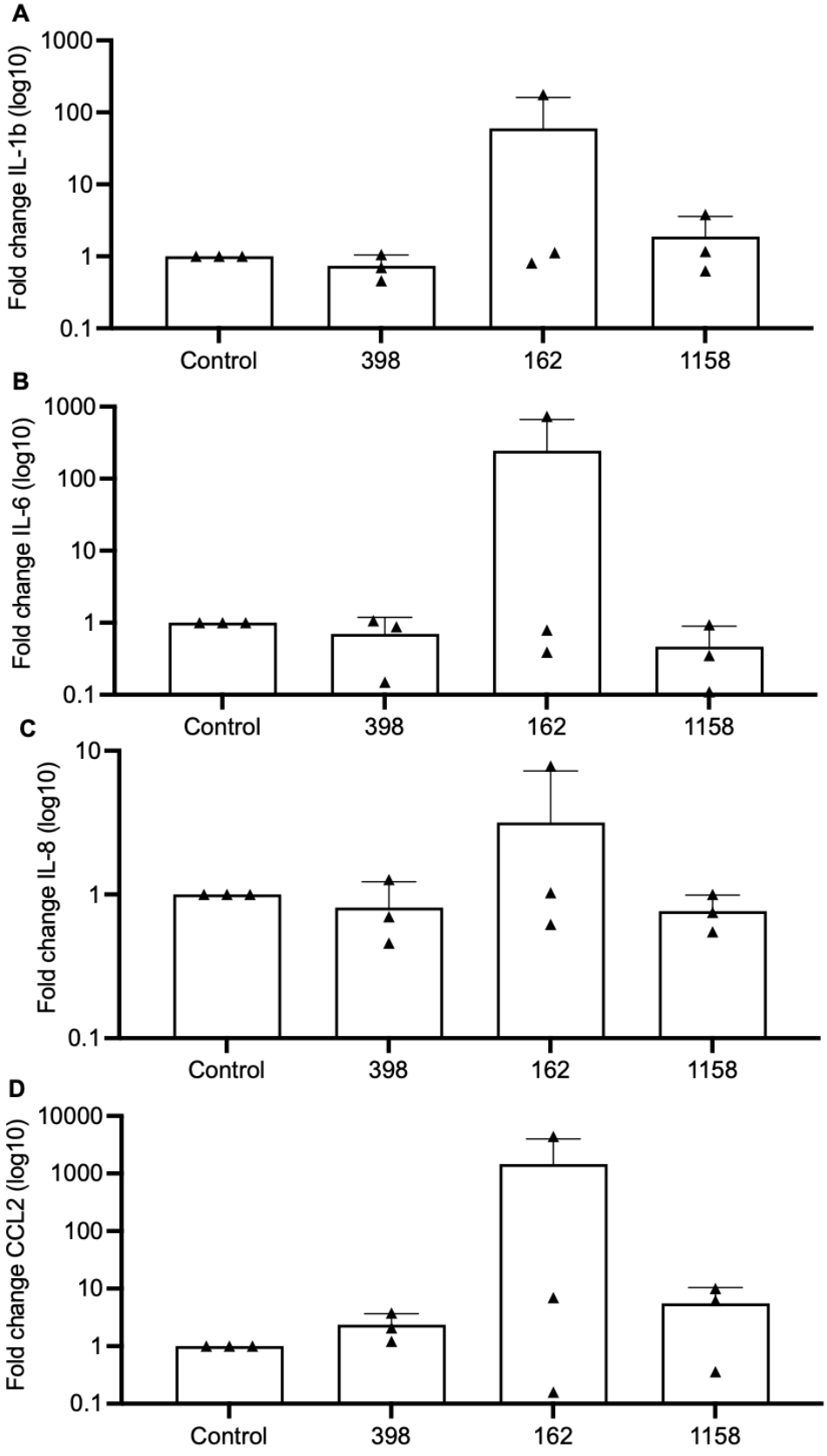


Figure 11. Differences in degree of adhesion did not affect immune activation in Calu-3 cells inoculated with NTHi strains and incubated in the presence of NTHi for 24 hours. Strains are ordered from highest to lowest adhesion. A. IL-8 expression is induced by NTHi, but there is no trend towards lower activation in strains with lower adhesion rates. B. IL-1β is induced by all NTHi strains at equivalent levels. C. CCL2 is induced at a low level by all strains of NTHi at comparable levels. D. TNF is induced at low levels by all strains of NTHi at similar rates. E. IL-6 is induced at low levels by exposure to NTHi, but there is no trend towards lower expression in less adherent strains. Significance was evaluated using 2-way ANOVA. This experiment was performed in 3 independent replicates with duplicate wells within each experiment.

To ensure that this was not a cell line specific effect, this experiment was repeated in PBECs cultured at ALI. Immune responses in PBECs cultured at ALI and inoculated with highly adherent NTHi strains 398 and 162, and less adherent strain 1158, for 24 hours were evaluated for their relationship with adhesion rate. Gene expression of IL-8, IL-6, IL-1β, CCL2, TNF, NLRP1 and cathelicidin was evaluated using a Fluidigm PCR array (Figure 12). IL-1β and IL-6 were modestly induced by strains 162 and 1158 (Figure 12, A and B). No strain significantly induced IL-8 (Figure 12C). CCL2 was modestly induced by all NTHi strains, although this was not statistically significant. Strains 162 and 1158 resulted in numerically elevated TNF and NLRP1, but this was not significant and there was substantial variation between donors (Figure 12, E and F). Cathelicidin (CAMP) was slightly induced by infection with 398 and 162 (Figure 12G). These data suggest that induction of cytokines in PBECs at ALI is modest, and is not related to the proportional adhesion of each NTHi strain.

Figure 12. Adhesion rate does not influence immune activation in response to NTHi.



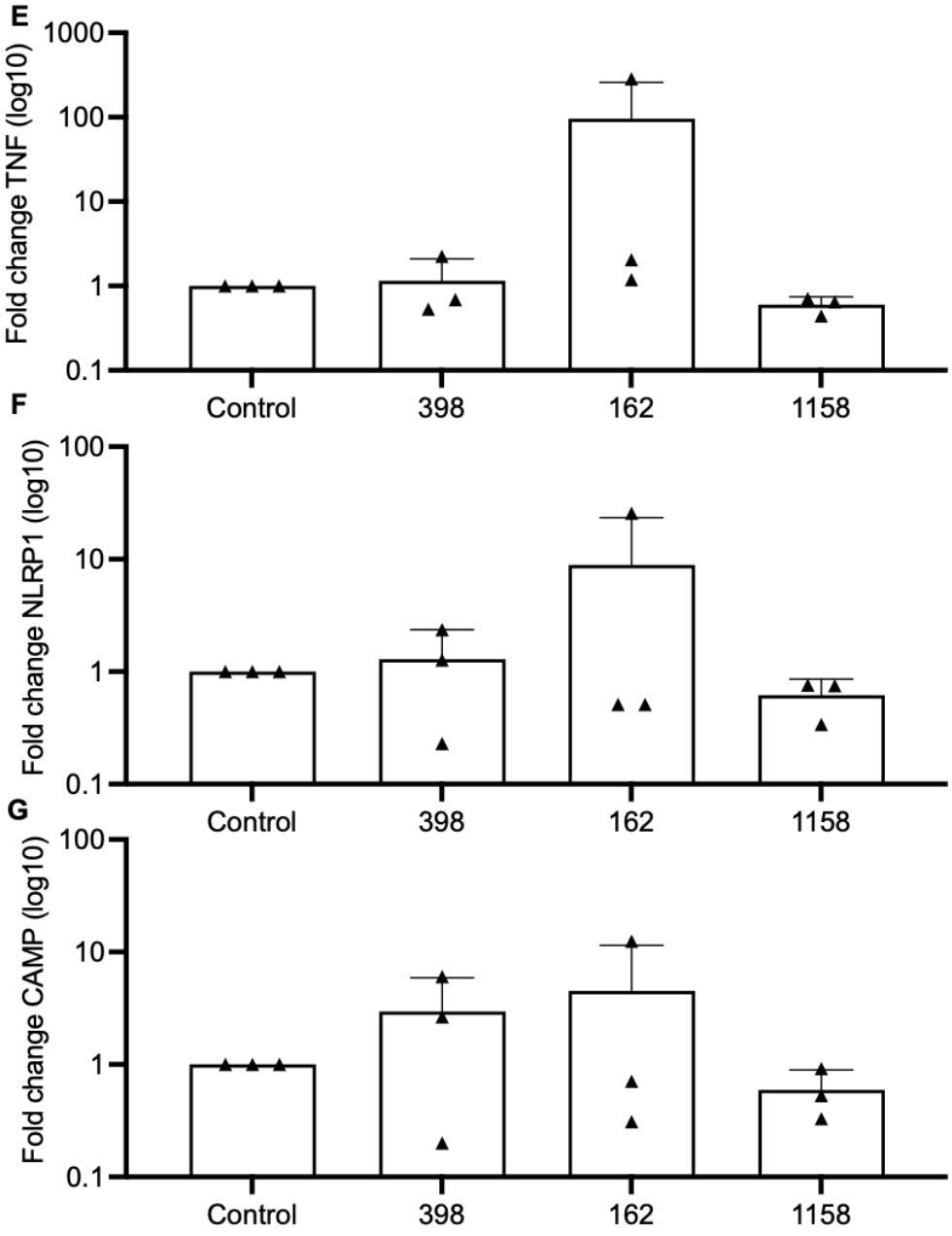

E
Fold change TNF (log10)
1000
100
10
1
0.1
Control
398
162
1158
F
Fold change NLRP1 (log10)
100
10
1
0.1
Control
398
162
1158
G
Fold change CAMP (log10)
100
10
1
0.1
Control
398
162
1158

Figure 12. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi internalised to different extents in PBECs cultured at ALI. Mature ALI cultures were inoculated with a selection of NTHi strains, 2 highly adherent strains (398 and 162) and the less adherent strain 1158, and incubated in the presence of extracellular bacteria for 24 hours prior to isolation of RNA. Number of adherent bacteria did not correlate with induction of any signalling target. A. Infection with strain 162 and 1158 modestly induced IL-1β expression compared to uninfected control cells, but this was not related to internalisation rate. B. IL-6 was induced by strain 162 and 1158, but this was not significant, and there was significant variation between response in donors. C. IL-8 was not significantly induced by treatment with NTHi. D. CCL2 was modestly induced by all NTHi strains, although this was not statistically significant. E, F. Both TNF and NLRP1 were numerically elevated in cells infected with 162 and 1158, but this was not significant and there was substantial variation between donors. G. Cathelicidin (CAMP) was slightly induced by infection with 398 and 162. Overall, there was no correlation between degree of adhesion and gene expression. These experiments were performed in 3 biological replicates from healthy control donors. qPCRs were performed in duplicate wells. Significance was evaluated for all targets using 2-way ANOVA with Sidak's multiple comparison tests on log-transformed data.

Production of IL-6, IL-1β and TNF were evaluated in pooled apical and basal supernatants from PBECs cultured at ALI infected with NTHi strains with high and low adhesion rates (Figure 13). NTHi exposure did not significantly increase supernatant concentrations of IL-1β or TNF (Figure 13, A and C). Strains 1158, 375 and 398 modestly induced production of IL-6, but this was not significant. Like the results from the gene expression experiment, response from PBECs cultured at ALI was modest compared to that seen in cell lines and PBECs grown in a submerged culture system. In addition, there was significant variation in host response in cells from different donors. These highlight the challenges of working with primary cells, but also underline the differences between results obtained from work with cell lines.

Figure 13. Supernatant immune markers were expressed at comparable levels in PBECs infected with strain with low and high adhesion rates.

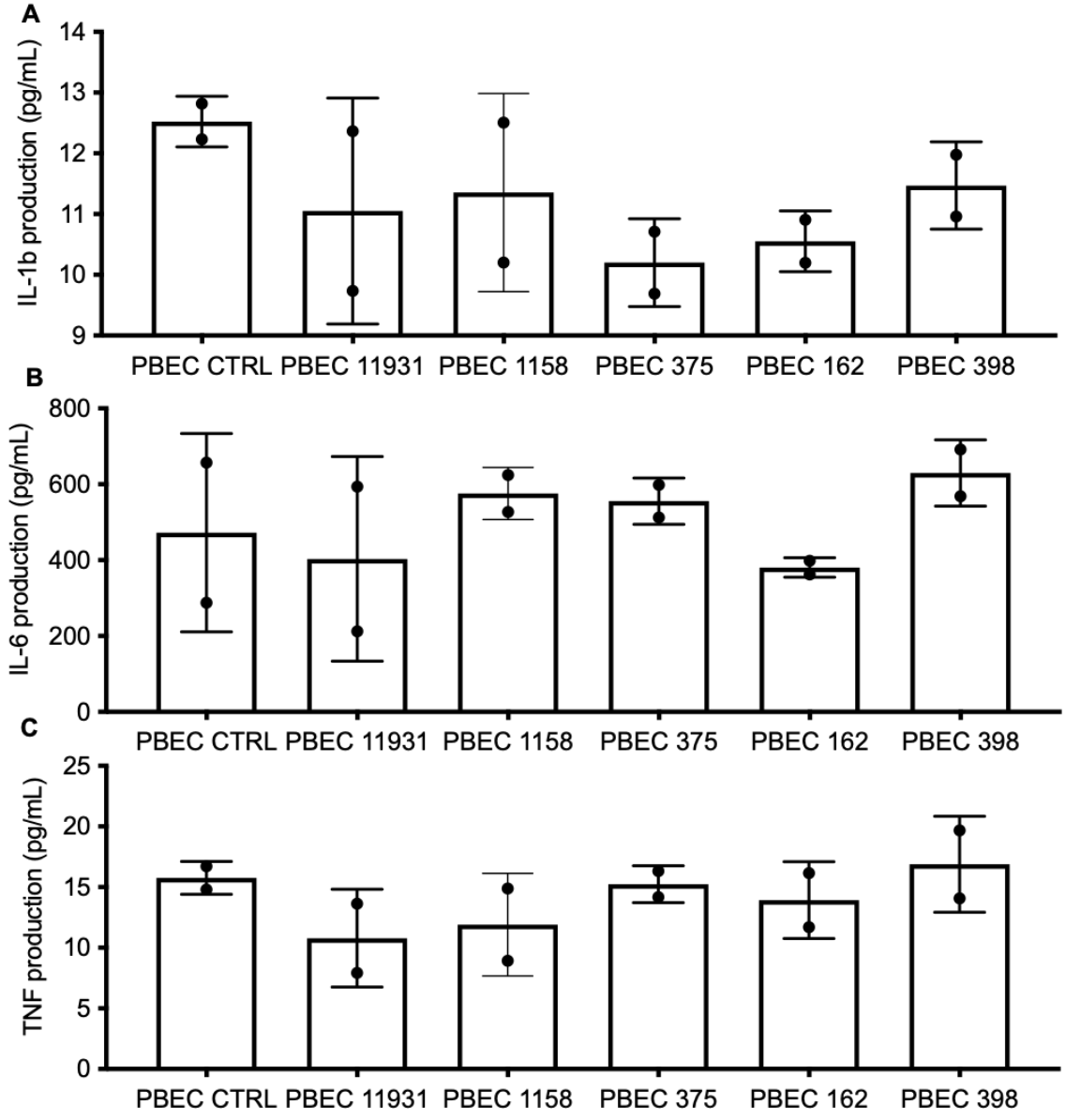


Figure 13. PBECs grown at ALI and inoculated with a selection of NTHi strains were incubated in the presence of extracellular bacteria for 24 hours prior to sampling of supernatants. Pooled apical and basal supernatants were evaluated for levels of IL-1β, IL-6, and TNF using a meso-scale design (MSD) assay. Strains are shown from least to most adherent. A. IL-1β was not induced by any NTHi strain. B. IL-6 was modestly induced by 1158, 375 and 398, but this was not significant. C. TNF was not induced by any strain compared to control. Significance for each target was evaluated by 2-way ANOVA with Sidak's multiple comparison tests on log-transformed data. These experiments were performed in 2 biological replicates from healthy control donors. MSD assays were performed on duplicate wells.

4.5 Does Azithromycin alter immune activation in response to NTHi infection?

NTHi is thought to be an important driver of inflammatory signalling in the epithelium. A bulk RNA sequencing experiment sought to provide additional information on differential gene expression in the epithelium in response to NTHi infection. In addition, gene expression was evaluated in PBECs infected with azithromycin-resistant NTHi infection followed by treatment with azithromycin to examine the non-antibiotic effects of azithromycin on the immune response to NTHi. Azithromycin has widely-reported anti-inflammatory effects, so there has been speculation that this may be partly responsible for its efficacy in reducing the number of exacerbations seen in severe asthma, as discovered in the AMAZES trial (Gibson *et al.*, 2017).

Figure 14. RNA sequencing experiment on PBECs from asthma patients cultured at ALI exposed to NTHi

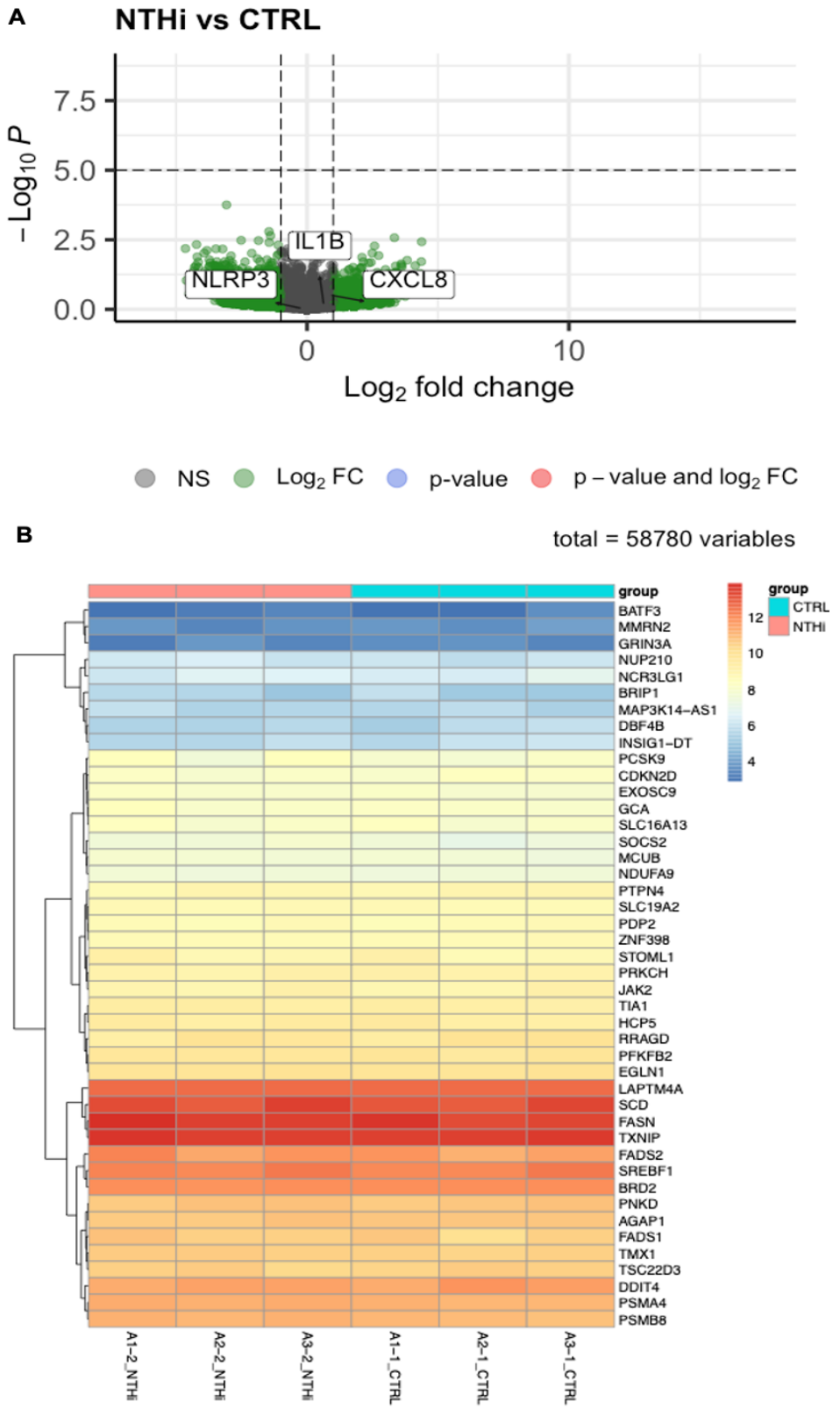


Figure 14. Bulk RNA sequencing was performed on PBECs from asthma patients inoculated with NTHi strain 398 for 1 hour prior to treatment with gentamicin and incubation overnight. A. A volcano plot shows little change in gene expression between uninfected and infected cells, including no significant change in IL-1β, NLRP3, and IL-8. B. There are no significant changes between infected and control cells in the top 50 differentially expressed genes.

To evaluate differential gene expression in response to NTHi treatment, PBECs from patients with asthma were cultured at ALI for a minimum of 28 days prior to infection with azithromycin-resistant NTHi strain 398 at an MOI of 10 for 1 hour, followed by incubation with gentamicin for 24 hours post infection. Strain 398 was chosen for its robust ability to adhere to and be internalised into epithelial cells. RNA was then isolated from these samples and analysed by bulk RNA sequencing. However, analysis has yielded no significantly differentially expressed genes comparing uninfected control samples to those infected with NTHi (Figure 14). IL-1β, NLRP3 and CXCL8 were not altered in response to NTHi in this experiment (Figure 14A). A heatmap of the top 50 differentially expressed genes show no significant differences between conditions (Figure 14B).

PBECs from donors with asthma were cultured for a minimum of 28 days at ALI and inoculated with azithromycin-resistant NTHi strain 398, or NTHi plus azithromycin or azithromycin alone for 1 hour prior to gentamicin treatment and incubation for a further 24 hours in the presence of azithromycin (Figure 15). RNA was then isolated and sent for bulk sequencing. In response to azithromycin treatment, several genes had significantly altered gene expression compared to untreated controls (Figure 15A). However, these were primarily genes related to cholesterol metabolism and fatty acid synthesis, rather than those related to inflammatory signalling. Similarly, in PBECs exposed to azithromycin-resistant NTHi compared with azithromycin alone, only SREBF1 was differentially expressed, which is a gene controlling fatty acid biosynthesis and cholesterol metabolism (Figure 15B). There is some evidence in viral infections that fatty acid biosynthesis is altered in infection, which may suggest that these are early changes in host cell metabolism in response to NTHi (Yu, Maguire and Alwine, 2012; Lo *et al.*, 2018; Yuan *et al.*, 2019).

Figure 15. RNA sequencing comparing gene expression in PBECs at ALI exposed to azithromycin or NTHi with azithromycin treatment.

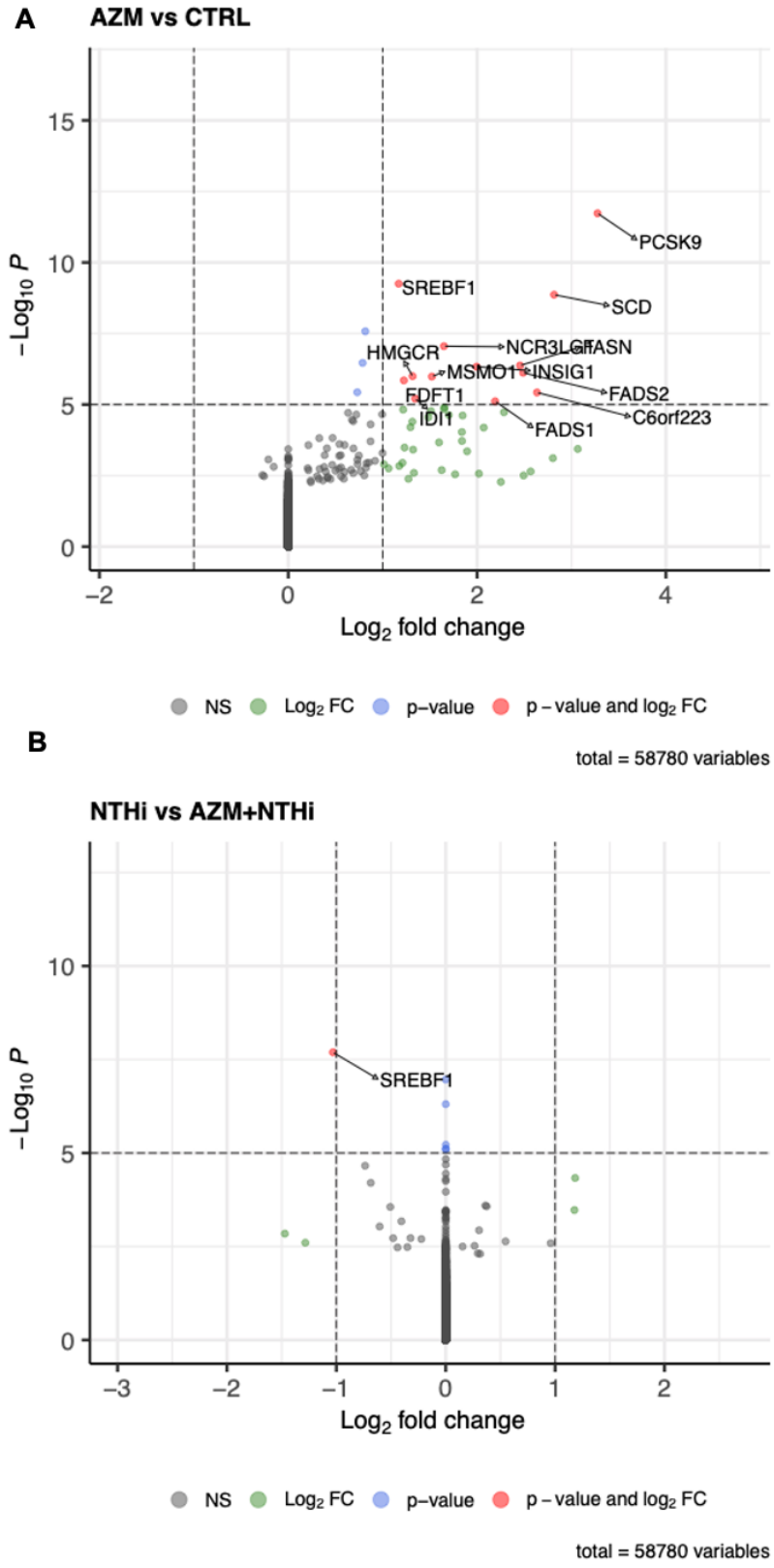


Figure 15. Bulk RNA sequencing was performed on PBECs from asthma patients treated with azithromycin compared with control cells or PBECs inoculated with NTHi strain 398 for 1 hour prior to treatment with gentamicin and incubation overnight. A. Several genes were upregulated in response to treatment with azithromycin, primarily genes related to metabolism and fatty acid synthesis. B. In PBECs treated with azithromycin compared with those infected with azithromycin-resistant NTHi strain 398 in addition to azithromycin treatment, SREBF1 is differentially expressed, but no cytokines are upregulated in response to infection.

There are several possible reasons these experiments have produced so few differentially expressed genes. The epithelial cells may have been exposed to these stimuli for an insufficient period or at low concentrations, which may explain the lack of differentially expressed genes. The cells were infected at an MOI of 10 for only 1 hour prior to gentamicin treatment. As PBEC at ALI are much more resistant to infection than epithelial cells grown in submerged culture, this may have led to a very low number of internalised bacteria remaining during the subsequent incubation period. Future experiments may benefit from a higher MOI as well as an extended incubation period. Alternatively, there is some evidence that PBECs at higher passage numbers may have altered or less robust expression of some genes (Reeves *et al.*, 2018; Rayner *et al.*, 2019). These PBECs were purchased from Lonza, whilst those used in other experiments were collected from donors by our lab, so they may also have been processed differently early on. Due to our receipt of these cells at passage 2, they were used at passage 4 rather than earlier passages used for other experiments. Taken together, I am unable to draw conclusions regarding the differential gene expression in response to NTHi and azithromycin from these data.

4.6 Conclusions

The results of this chapter confirm that NTHi exposure induces a robust pro-inflammatory immune response in submerged cultures. This induction of neutrophil chemoattractant signalling such as IL-6 and IL-8 may explain the association seen between NTHi infection and neutrophilic inflammation in patients with asthma. In addition, activation of the NLRP3 inflammasome and IL-1β production are also drivers of neutrophilic inflammation and airway hyperreactivity. These data suggest

that inflammatory signalling from the epithelium in response to NTHi may contribute to the inflammation observed in asthma and COPD patients with NTHi infections.

Although NTHi exposure is a potent activator of cytokines, invasion does not appear to correlate with increased immune activation of pro-inflammatory cytokines. I show that there are significant differences in the rates of internalisation between NTHi strains. However, this does not correlate with the disease from which the strain was isolated, with strains isolated from otitis media being no less invasive than those isolated from COPD. Further, in models of lower airway epithelium, internalisation does not appear to be critical for induction of IL-8, IL-6, CCL2 or TNF. However, IL-1β was significantly reduced by decreasing internalisation of NTHi in a model of Calu-3 cells in submerged culture, suggesting that, unlike other signalling pathways using surface or cytosolic receptors, bacterial internalisation may be necessary for induction of inflammasomes. One limitation of these experiments is their use of cell lines, which limits their ability to recapitulate the epithelial architecture present *in vivo*.

Further experiments comparing immune activation in strains that adhere more efficiently to the surface of the epithelium suggests that increased bacterial adhesion does not lead to more robust immune activation. This may be due to a 'threshold effect', in which further adhesion does not induce higher induction of cytokines as long as a minimum number of bacteria have already been bound. Future studies utilising NTHi strains with mutated adhesion molecules which inhibit adhesion to investigate its role in immune induction would allow more precise study of this concept. In addition, investigation of NTHi's ability to induce cytokines without physical contact, for instance through shedding of membrane molecules or small molecule metabolites, would be of interest.

In collaboration with other lab members, RNA sequencing was performed on PBECs isolated from asthma patients cultured at ALI infected with azithromycin-resistant NTHi with or without treatment with NTHi. This experiment yielded limited results, with few genes meeting the threshold for statistical significance.

Future RNA sequencing experiments may benefit from longer incubation periods with NTHi to allow greater immune activation. A similar experiment performed by Baddal

et al investigating the induction of immune signalling in response to NTHi infection at 24 hpi showed only significant upregulation of IL-8, while at 72 hpi, IL-8, IL-1β and TNF were significantly upregulated in infected compared to control PBECs (Baddal *et al.*, 2015b). It also showed significant reduction in IL-33 and TSLP during infection. These results suggest that a longer incubation period in the presence of NTHi may be necessary to appreciate the inflammatory signalling associated with NTHi infection.

In addition, there is some evidence that PBECs at higher passages have altered gene expression (Reeves *et al.*, 2018; Rayner *et al.*, 2019). The literature recommends using PBECs at a maximum passage of 3 for the most consistent results in cells from both asthma and healthy control donors. The ALI cultures used in our RNA sequencing experiment were from passage 4, which may have contributed to the limited induction of differential gene expression. Overall, these experiments have shown that infection with NTHi induces a robust immune response in submerged epithelial cell cultures in a manner which does not depend on bacterial internalisation, but these responses are substantially muted in primary cultures at ALI.

Chapter 5 Pathogenic versus commensal NTHi-host cell interactions

NTHi is a common commensal organism colonising the nasopharynx without causing disease (Pichichero and Almudevar, 2016). However, infection by NTHi in the lung is capable of causing exacerbations in asthma and COPD patients and contributing to the progression of disease (Finney *et al.*, 2014; Taylor *et al.*, 2019). The mechanisms underlying the differential response of upper and lower airways remains poorly understood. My aim in this chapter was to examine the host-pathogen interactions and immune responses which differ between infection in the nasopharyngeal epithelium compared with epithelium in the lower respiratory tract.

There are several bacterial species which are commensals in the nasopharynx but pathogens in the lung (Verduin *et al.*, 2002). For instance, *Moraxella catarrhalis* is a commensal organism in the nasopharynx, but it has more recently been recognised as a cause of otitis media in children, and a trigger for COPD exacerbations when it infects the lower airways (Verduin *et al.*, 2002; Murphy and Parameswaran, 2009; Aebi, 2011). However, even asymptomatic carriage of bacteria can induce inflammatory cytokines in the nasopharynx (Pichichero and Almudevar, 2016), suggesting that, although different signalling pathways may be activated, the nasopharyngeal epithelium is not unresponsive.

There is a lack of data comparing immune signalling in the upper and lower respiratory tract in response to bacterial stimuli. In this chapter, I have hypothesised that immune responses to NTHi are different in the epithelium in the nasopharynx compared with the lower respiratory tract, and that this may partly explain the role of NTHi as a commensal in one tissue and a pathogen the other. I have also sought to evaluate whether this is an NTHi-specific difference, or whether this is a broader difference in signalling in the two tissues.

I hypothesised that the commensal nature of NTHi infection in the nasopharynx may be due to lower rates of adhesion and internalisation. Initial experiments have evaluated the numbers of bacteria which are adherent to or are internalised into the monolayer of upper respiratory epithelial cells compared with the lower respiratory

tract using a modified gentamicin exclusion assay. In addition, flow cytometry experiments have been performed in a differentiated ALI culture to evaluate the proportion of each cell type which have been infected with NTHi in PBECs and NECs cultured at ALI

5.1 Is there a difference in invasion in upper versus lower airway epithelial cells?

Initial experiments were directed at evaluating differences in physical interactions between NTHi and the lower and upper respiratory tract epithelium. The RPMI2650 cell line was used as a model of nasal epithelium and compared with Calu-3 cells, a lower respiratory epithelial cell line. In submerged cultures infected with several strains of NTHi for 24 hours in the presence of extracellular bacteria, internalisation rates varied between strains in both RPMI2650 and Calu-3 cells (Figure 1A). At 24 hours post-infection (hpi), every tested strain was internalised in significantly higher numbers in RPMI2650 cells compared with Calu-3 cells. However, at 120 hpi viable NTHi were still present in Calu-3 cells, but not in RPMI2650 cells, despite the presence of extracellular bacteria (1B). Numbers of viable bacteria in the media at 120 hpi had declined when cultured in the presence of RPMI2650 cells compared with Calu-3 cells, which may contribute to the elimination of intracellular bacteria (Figure 1, C and D). One possibility is that RPMI2650 cells mount an immune response that result in the elimination of NTHi both intracellularly and in the media. Alternatively, the viability of RPMI2650 cells may decline more when infected with NTHi than Calu-3 cells, resulting in fewer host cells the bacteria are able to interact with. I have hypothesised that the rapid elimination of internalised bacteria in RPMI2650 cells compared with Calu-3 cells may be due to differential immune responses in each cell type.

Figure 1. NTHi internalisation rates in RPMI2650 (upper airway) and Calu-3 (lower airway) cells.

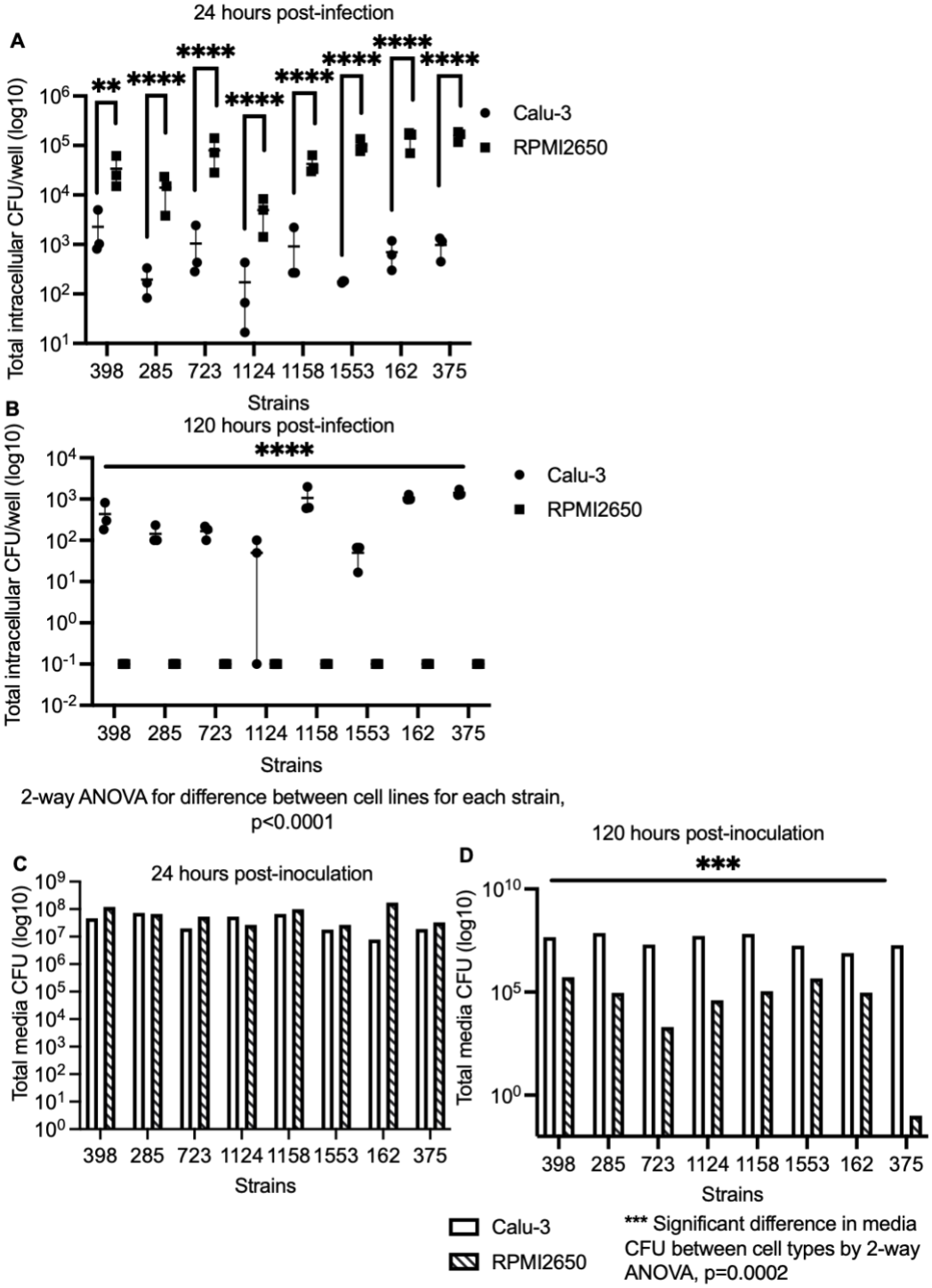


Figure 1. Confluent RPMI2650 (upper airway) or Calu-3 (lower airway) cells grown in submerged culture were inoculated with NTHi strains and incubated for 24 or 120 hours in the presence of bacteria. A. Using a CFU assay as previously described to enumerate internalised bacteria, NTHi strains were internalised in significantly higher numbers in RPMI2650 cells compared with Calu-3 cells. Mean and range are indicated and dots represent mean of each biological replicate. B. Following 120 hours of culture in the presence of NTHi, viable internalised bacteria had been eliminated from RPMI2650 cells, while a significant number of viable bacteria remained within the monolayer of Calu-3 cells. Mean and range are indicated and dots represent mean of each biological replicate. C. CFU numbers in media cultured with Calu-3 or RPMI2650 cells are comparable at 24 hours. D. Media CFU numbers in the media are significantly lower when cultured in the presence of RPMI2650 compared with Calu-3 cells at 120 hours post-inoculation. n=3 independent replicates with 2 replicate wells in each experiment. 2-way ANOVA with Sidak's multiple comparisons was performed on data which have been log-transformed.

To further evaluate bacteria-epithelial interaction differences in nasal and lower respiratory epithelium, I have used a modified gentamicin exclusion assay to enumerate the number of adherent, intracellular and paracellular bacteria (Figure 2). The numbers of adherent bacteria were comparable between RPMI2650 and Calu-3 cells (Figure 2A). Numbers of intracellular bacteria were comparable in both cell types for most strains, while numbers of highly internalised strain 162 and 398 were considerably higher in RPMI2650 cells (2B). Bacteria recovered from the paracellular space had a tendency towards being lower in all strains in Calu-3 cells, but this was not significant ($p=0.056$) (2C). These data suggest that NTHi interacts with nasal epithelial cells at comparable or higher levels than lower airway epithelium.

Figure 2. Adhesion, intracellular, paracellular bacteria numbers in RPMI2650 vs Calu-3 cells

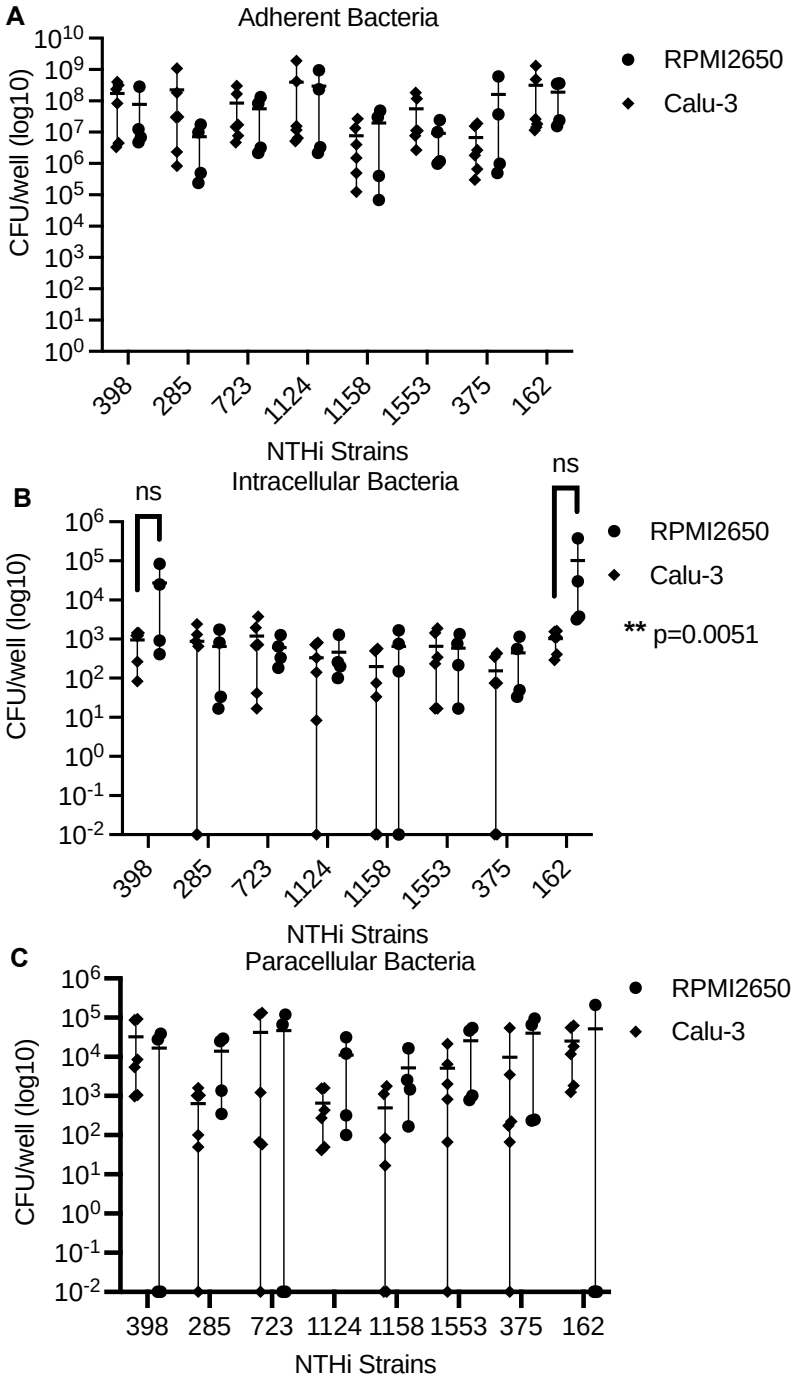


Figure 2. Adherence and internalisation were compared in Calu-3 and RPMI2650 cells grown in submerged culture and infected with NTHi strains for 4 hours. A. Numbers of adherent bacteria were not significantly different between Calu-3 and RPMI2650 cells. B. Intracellular bacterial numbers after 4 hours of infection were comparable for most strains, but strains 162 and 398 were numerically substantially higher in RPMI2650 compared with Calu-3. C. Paracellular bacteria tended towards lower values in Calu-3 cells compared with RPMI2650 cells, but this difference was not significant (p=0.056). n=3 independent experiments with duplicate wells for each condition. Significance was assessed using 2-way ANOVA with Sidak's multiple comparisons on log-transformed data.

Figure 3. Comparison of internalised NTHi numbers in submerged NEC vs PBEC culture

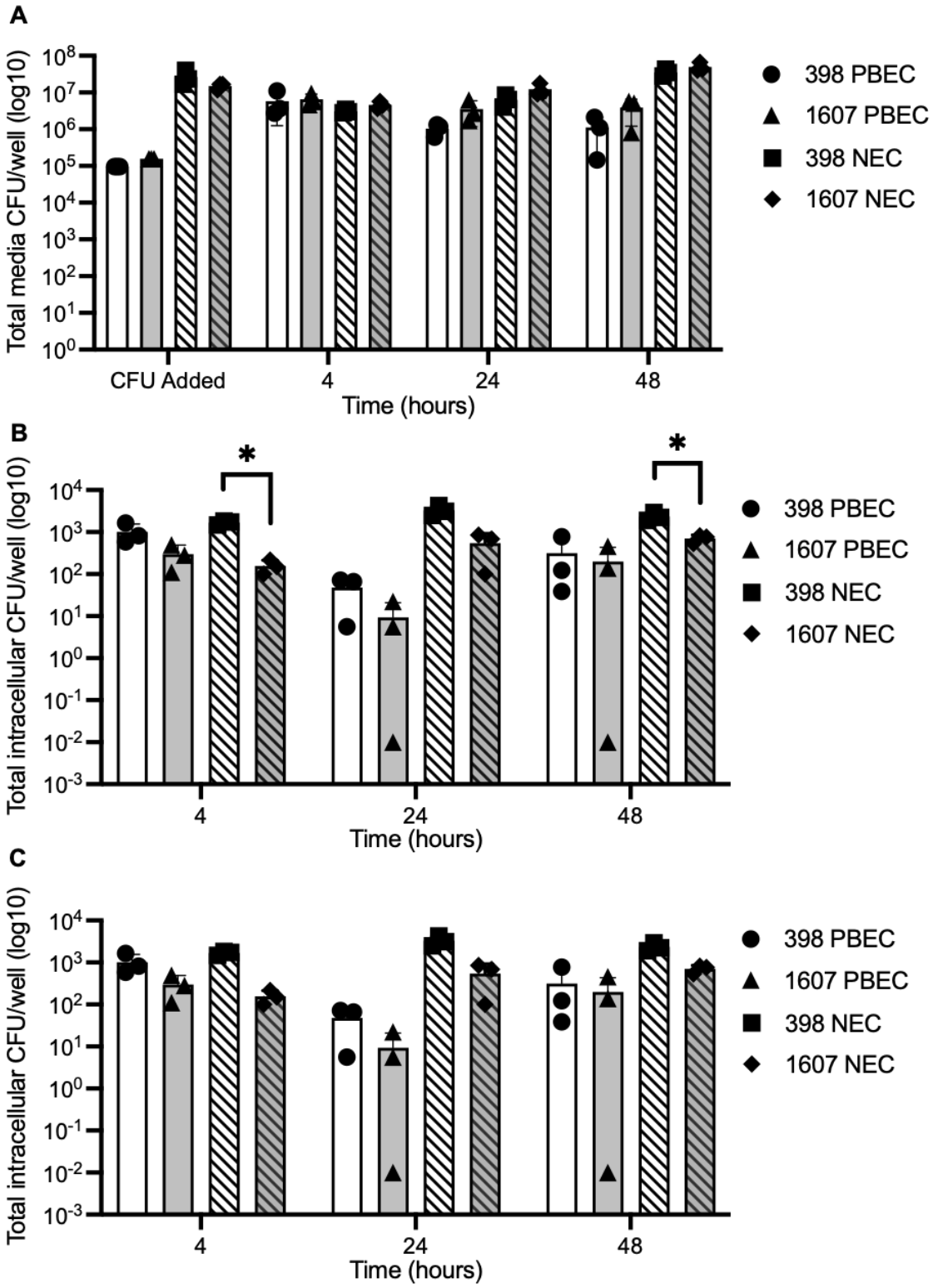


Figure 3. NECs cultured in submerged culture were inoculated with a highly internalised strain (398) and a less internalised strain (1607). A. A CFU assay shows that NTHi strains 398 and 1607 proliferate in serum- free media in the presence of NECs and PBECs. There was not significant variability between bacterial numbers in the media based on strain or cell type. B. Live intracellular NTHi are detectable by CFU assay up to 2 days post-infection in NECs and PBECs when extracellular bacteria are present. Strain 398 was internalised in higher numbers than strain 1607 in NECs at all time points, reaching statistical significance at 4 and 48 hours post-infection. In PBECs there was a tendency towards higher internalisation of 398 compared with 1607 at all time points, but this was not significant. C. There was a tendency towards lower numbers of internalised bacteria in PBECs compared with NECs at 24 and 48 hpi. This experiment was performed on NECs from one healthy donor in triplicate wells, and in PBECs from 3 healthy donors in 3 independent experiments with triplicate wells. Significance was evaluated on log-transformed data using 2- way ANOVA with Sidak's multiple comparison tests.

These findings were confirmed in primary cells, comparing numbers of bacteria in the media and internalised bacteria in NECs and PBECs grown in submerged culture (Figure 3). Numbers of bacteria in the media of NECs and PBECs was comparable across all time points (Figure 3A). In the presence of extracellular bacteria, viable internalised bacteria were detectable up to 2 days post-infection in both NECs and PBECs (3B). In NECs, strain 398 was internalised in higher numbers than strain 1607 at all time points, whilst in PBECs there was also a tendency towards higher internalisation of 398 compared with 1607 at all time points, but this was not significant (3B). There were numerically lower numbers of internalised bacteria in PBECs compared with NECs at 24 and 48 hpi, but this difference was not statistically significant (3C). Overall, these experiments suggest that strain-dependent trends in internalisation and adhesion are consistent between cell types, but that the absolute number of bacteria interacting with the epithelial cells is higher in NECs than PBECs. These data are consistent with those found in RPMI2650 and Calu-3 cells, suggesting that they are not cell-line specific effects.

To further describe the interactions between NTHi and the epithelium, a flow cytometry panel was developed in conjunction with Katie Horton (University of Southampton) to isolate different epithelial cell types infected with NTHi in a model of NECs and PBECs at ALI, enabling us to evaluate the proportion of each cell type that is infected (Figures 4 and 5). As with imaging, tubulin was used as a marker for ciliated cells, whilst ITGA6 (CD49f) was used to mark basal cells. To avoid permeabilising the cells, CEACAM6 (CD66c) was chosen as a marker for secretory cells. Mature NEC and PBEC ALI cultures were infected with NTHi strain 398 at an

MOI of 50 for 1 hour and then treated with gentamicin for 1 hour to eliminate extracellular bacteria. Cells were then stained and prepared for flow cytometric sorting. Populations were sorted into infected and uninfected populations of CD49f+ basal cells, tubulin+ ciliated cells and CD66c+ secretory cells. The gating strategy for these experiments is shown in figure 4.

In NECs, the highest total number of uninfected and infected cells in the differentiated epithelium is ciliated cells, followed by secretory and then basal cells (Figure 5A). Secretory cells were the most highly infected cell type, followed closely by ciliated cells, in terms of both absolute numbers and percent of total cells infected (Figure 5, B and C). Only a very small number of basal cells were infected. However, differences between cell types were not significant. These data show that all types of epithelial cells are permissive to infection, but suggests that secretory cells may be preferentially infected.

Figure 4. Gating strategy for flow sorting infected NECs

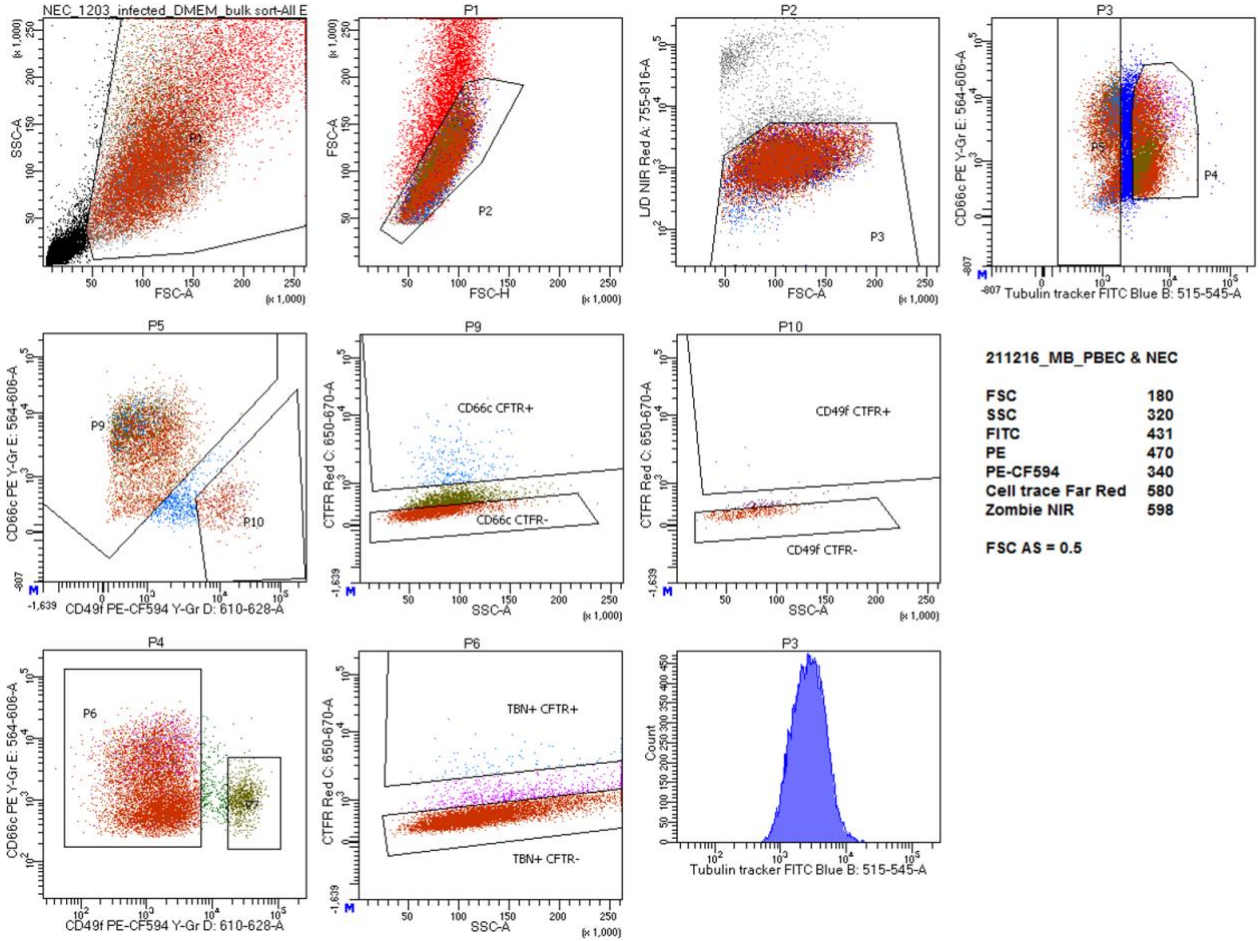


Figure 4. NECs cultured at ALI for a minimum of 28 days were infected with COPD strain 398 NTHi stained with CellTrace FarRed (CTFR) for 1 hour, treated with gentamicin for 1 hour to eliminate extracellular bacteria, and then stained for ciliated (tubulin), basal (CD49f) and goblet cell (CD66c) markers. Cells were flow-sorted to determine proportion of each cell type infected by NTHi. This gating strategy allowed us to isolate infected (CTFR+) and uninfected cells of each cell type. Flow plot shown from one representative experiment.

Figure 5. Flow sorting showed secretory cells were the most highly infected cell type in NECs cultured at ALI

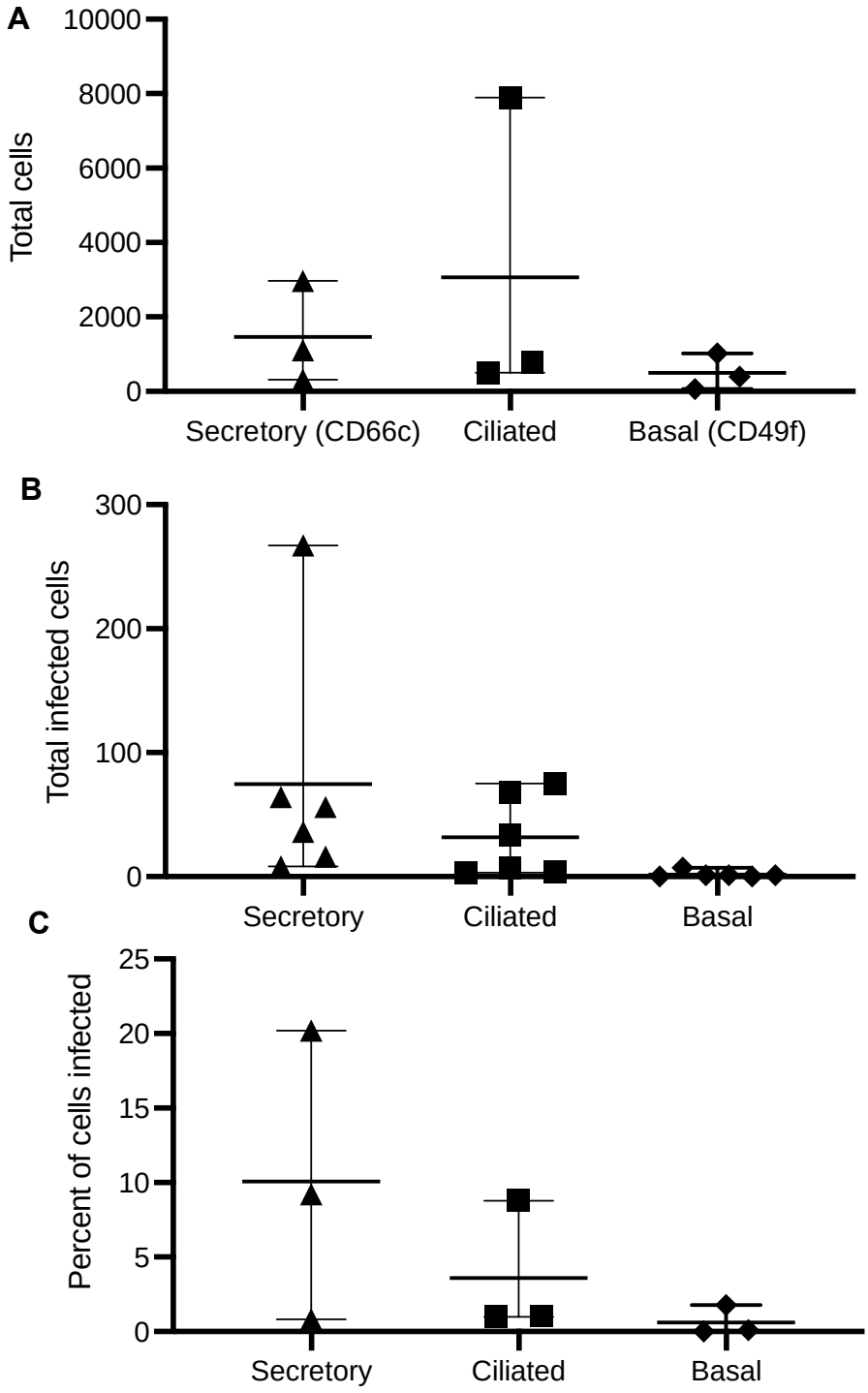


Figure 5. NECs cultured at ALI for a minimum of 28 days were inoculated with NTHi strain 398 for 1 hour, treated with gentamicin to eliminate extracellular bacteria, and stained for ciliated, basal and secretory cell markers. The total numbers of each cell type and the proportion of infected cells in each cell type was determined. A. The sum of infected and uninfected cells of each cell type was calculated. B, C. In NECs, secretory cells were the most highly infected cell type, closely followed by ciliated cells, with basal cells being the least infected, in terms of both absolute numbers and percent of total cells infected. However, differences between cell types were not significant. Significance was evaluated using a 1-way ANOVA with Tukey multiple comparison tests on log-transformed data.

Comparison between the number of each epithelial cell type infected with NTHi in NECs and PBECs showed significantly higher numbers of infected NECs (Figure 6, A and B). There were greater numbers of infected secretory and basal cells in NEC cultures compared with PBECs, whilst the numbers of infected basal cells were comparable in both populations. This was true both in terms of the total number of cells, and in the proportion of cells infected out of the total number of each cell type.

Figure 6. A higher proportion of NECs compared with PBECs are infected by NTHi

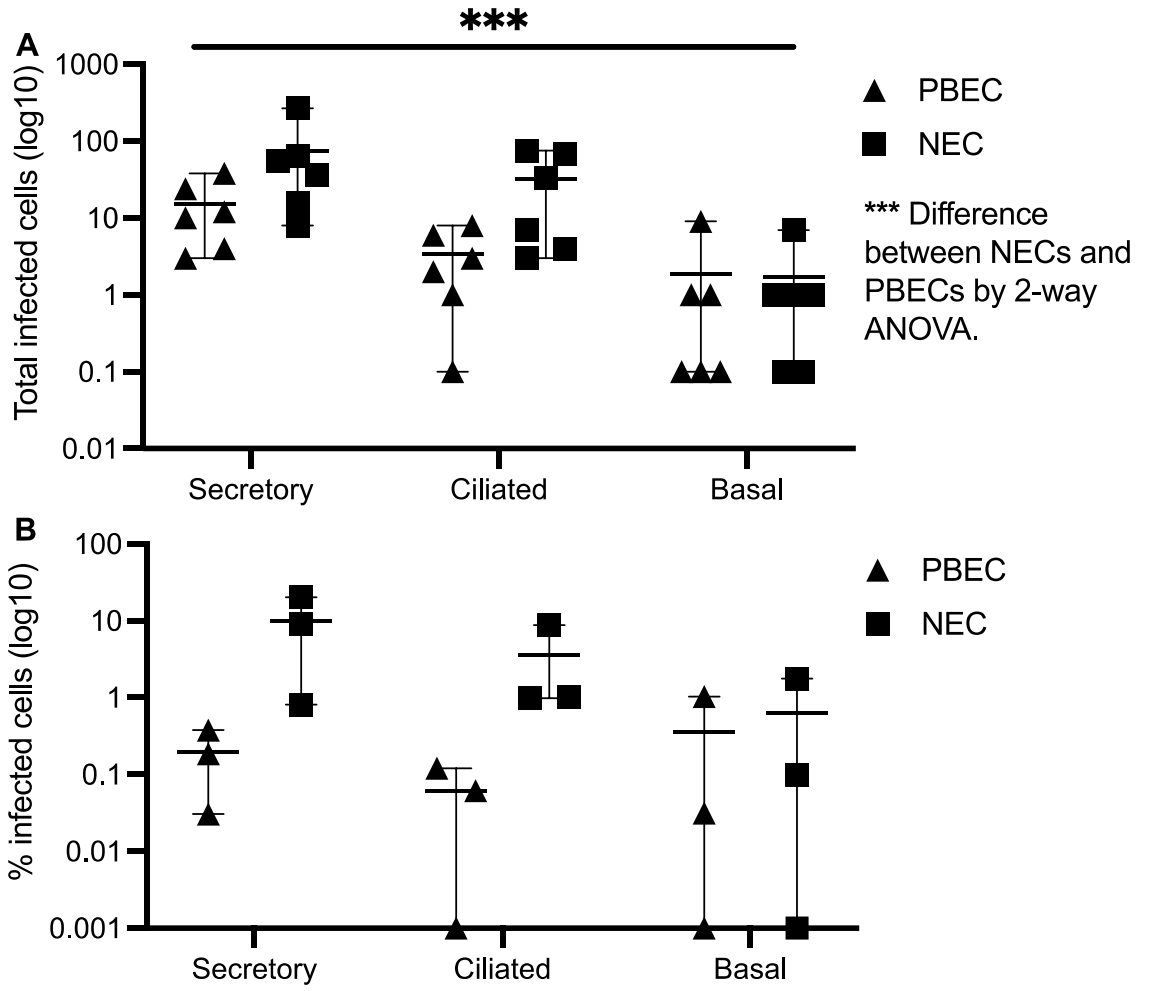


Figure 6. NECs and PBECs cultured at ALI were infected with COPD strain 398 NTHi stained with CellTrace FarRed for 1 hour, treated with gentamicin for 1 hour to eliminate extracellular bacteria, and then stained for ciliated, basal and goblet cell markers. Cells were flow-sorted to determine proportion of each cell type infected by NTHi. A. The total number of cells harbouring intracellular NTHi was significantly higher in NECs than in PBECs in secretory and ciliated cells, but there was no difference in basal cells. Mean and range are indicated on plots and dots represent technical replicates from 3 biological replicates. B. The percentage of infected cells was also higher in NECs than PBECs in all cell types. Means and ranges are indicated on plots and dots represent means of each biological replicate. Throughout experiment, n=3 biological replicates from different healthy donors. 2-way ANOVA was performed on log-transformed data to evaluate significance.

Overall, the data in this section show that NTHi interacts with upper airway epithelial cells in equal or higher rates than lower airway cells, showing that NECs are permissive to infection by NTHi. This suggests that the differential immune response leading to commensal colonisation of the nasopharynx is not due to lower bacterial internalisation or adherence.

5.2 Is the pro-inflammatory immune response different in the upper versus lower airway epithelium?

This section aimed to evaluate the inflammatory signalling induced by NTHi in nasopharyngeal compared to lower respiratory epithelial cells. To examine the differential immune responses in upper compared with lower respiratory tract epithelium, immune activation of common inflammatory signalling molecules IL-8 (CXCL8), IL-6, TNF and IL-1b, a marker of NLRP3 inflammasome activation, was evaluated in response to NTHi exposure. Initial experiments compared induction of these markers in Calu-3 compared with RPMI2650 cell lines inoculated with a selection of NTHi strains (Figure 7) using qPCR. Expression of IL-8 was robustly induced by NTHi infection in Calu-3 cells, while induction in RPMI2650 cells was significantly lower (Figure 7A). IL-1b expression was induced by NTHi exposure at comparable levels in both Calu-3 and RPMI2650 cells (Figure 7B). CCL2 was not strongly induced by NTHi in either cell type (Figure 7C). Unlike IL-8 and IL-1b, IL-6 were induced at significantly higher levels in RPMI2650 cells compared with Calu-3 cells, although induction was modest in both (Figure 7D). TNF expression was induced more highly in Calu-3 cells than RPMI2650 cells across all NTHi strains, although this varied widely between strains (Figure 7E). These results suggest that NTHi induction may induce different responses in the upper versus lower respiratory tract epithelium, with a stronger IL-8 and TNF signalling response in the lower respiratory tract.

Figure 7. Differences in NTHi-induced inflammatory gene expression between RPMI and Calu-3 cells

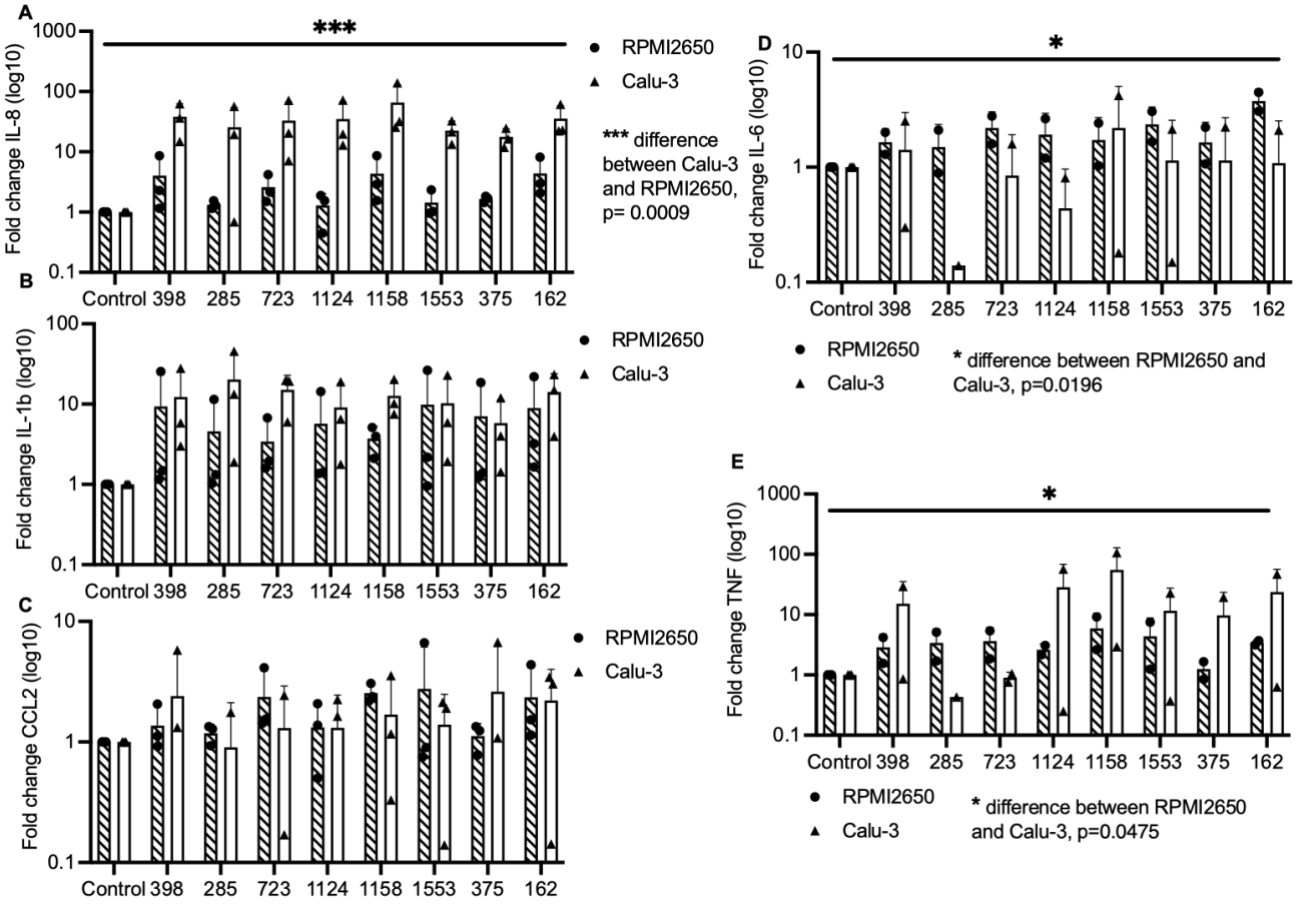


Figure 7. Confluent RPMI2650 and Calu-3 cells grown in submerged culture were inoculated with NTHi strains for 24 hours. RNA was isolated from these cells and qPCR analysis was performed to evaluate pro-inflammatory immune responses. A. IL-8 expression was significantly higher in Calu-3 cells compared with RPMI2650 cells (paired t-test, p=0.0009). B. Expression of NLRP3 inflammasome marker IL-1b was upregulated by NTHi treatment, but did not differ between RMPI2650 and Calu-3 cells. C. CCL2 was modestly upregulated following NTHi infection, but this was not different between cell types. D. Induction of IL-6 was significantly higher in RPMI2650 compared with Calu-3 cells (paired t-test, p=0.019). E. TNF expression was significantly higher in Calu-3 compared with RPMI2650 cells (paired t-test, p=0.047). Three independent replicates were performed with duplicate wells in each experiment.

To evaluate immune signalling using a primary cell model, a PCR array was designed using Fluidigm microfluidic technology to evaluate gene expression in NECs and PBECs cultured at ALI. Mature ALI cultures were inoculated with NTHi strains and incubated in the presence of extracellular bacteria for 24 hours. RNA was isolated for PCR array experiments (Figures 9-16) and supernatants were collected to evaluate levels of secreted cytokines (Figures 17 and 18).

Based on literature review, I have postulated that TLR2 and 4, NOD1 and 2, and inflammasome-associated NLRs (NLRP1, NLRP3, NLRC4, AIM2) may be involved in immune sensing and activation in response to NTHi (Figure 8). I have investigated induction of inflammatory signalling via these three routes. One route is via TLR2 and 4 signalling and downstream IRF5 and 7 and the MYD88-IRAK1-TRAF6 signalling transduction pathway. Another is via NOD1 and NOD2 and downstream activation of MAPK signalling and NF-kB. Inflammasome-associated NLRs have also been implicated in NTHi-induced inflammatory signalling in a caspase-1-dependent manner resulting in cleavage of pro-IL-1b to its active form. NF-kB signalling is downstream from several of these mechanisms, in addition to possible activation via ALPK1. Infection with NTHi is thought to induce transcriptional upregulation of cytokines CCL2(MCP1), TNF, IL-1b, IL-6 and IL-8 and alarmins (TSLP, IL-33 and IL-25). In addition, cell surface receptor ICAM-1 may also be upregulated in epithelial cells infected with NTHi. I have also sought to investigate whether antimicrobial peptides lysozyme and cathelicidin (CAMP) are affected by NTHi infection. Figure 8 shows the proposed signalling pathways.

Figure 8. Proposed immune activation pathways.

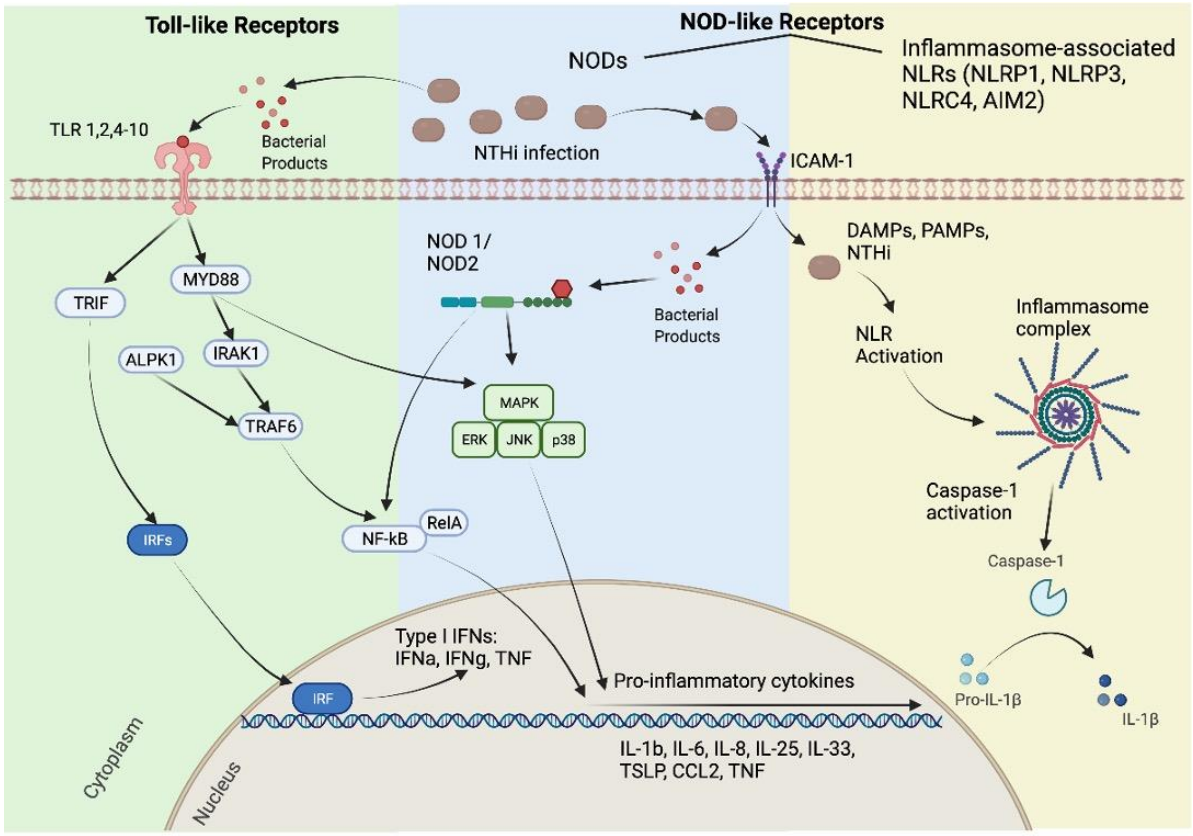


Figure 8. Based on literature review, I have proposed that the following pathways may be involved in immune sensing and activation in response to NTHi. I have investigated three potential pathways by which NTHi may mediate immune activation. One route is via TLR2 and 4 signalling and downstream IRF5 and 7 and the MYD88-IRAK1-TRAF6 signalling transduction pathway. Another is via NOD1 and NOD2 and downstream activation of MAPK signalling and NF-kB. Inflammasome-associated NLRs (NLRP1, NLRP3, NLRC4, AIM2) have also been implicated in NTHi-induced inflammatory signalling in a caspase-1 dependent manner resulting in cleavage of pro-IL-1b to its active form. NF-kB signalling appears to be activated by several of these mechanisms, in addition to possible activation via ALPK1. Transcriptional upregulation of cytokines CCL2(MCP1), TNF, IL-1b, IL-6 and IL-8 and alarmins TSLP, IL-33 and IL-25 is thought to be induced by NTHi. In addition, cell surface receptor ICAM-1 is thought to be upregulated in epithelial cells infected with NTHi. I have also sought to investigate whether antimicrobial peptides lysozyme and cathelicidin (CAMP) are affected by NTHi infection.

This experiment aimed to evaluate several questions. One of these was whether signalling induced by NTHi is different in upper airway compared to lower epithelium. It also evaluated whether NF-kB signalling was induced by NTHi, and whether this occurred partially via ALPK1 activation. The experiment also evaluated NTHi-induced expression of AGER (RAGE), which has been implicated in the development of inflammation associated with airways disease, in particular neutrophilic asthma.

Figure 9. Fluidigm analysis of TLR and IRF signalling pathways in NECs and PBECs in response to NTHi

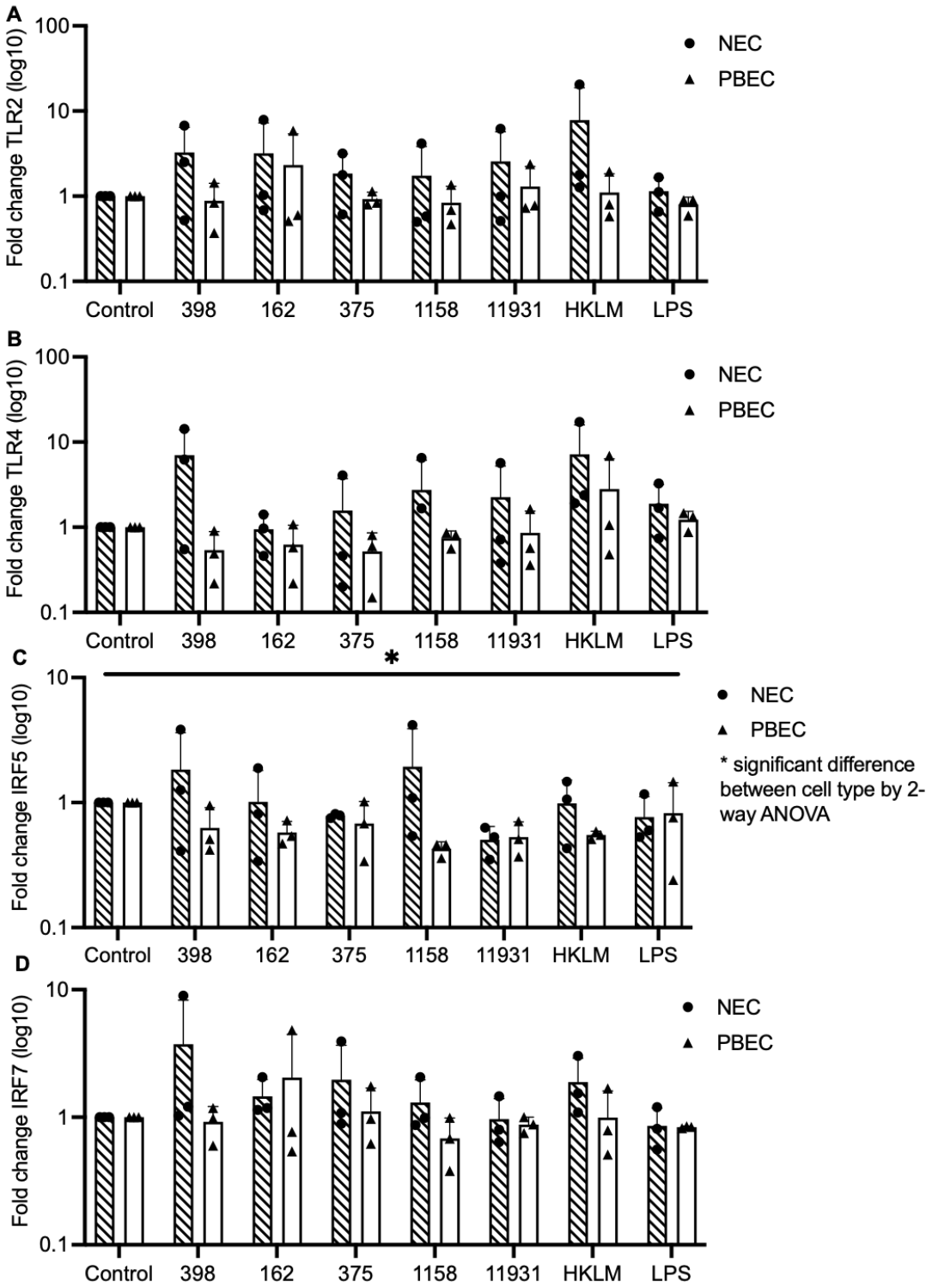


Figure 9. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. Mature ALI cultures were inoculated with a selection of NTHi strains, ordered from highest to lowest internalisation, and incubated in the presence of extracellular bacteria for 24 hours prior to isolation of RNA. Treatment with TLR2 and TLR4 agonists HKLM and LPS was included to evaluate differential TLR signalling in NECs compared with PBECs. A, B. TLR2 and TLR4 were modestly upregulated in NECs in response to NTHi, while they were unaffected in PBECs. C. IRF5, a factor downstream from the TLRs, was significantly higher in NECs compared with PBECs. D. There was a tendency towards higher expression of IRF7 in NECs than PBECs, but this was not significant. Overall, NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs, suggesting that they are capable of responding to TLR agonists. Significance for all results was evaluated by 2-way ANOVA and Sidak's multiple comparisons test performed on log-transformed data.

Results from the Fluidigm BioMark PCR array experiment showed TLR2 and TLR4 were modestly upregulated in NECs in response to NTHi, while they were unaffected in PBECs, although these findings were not statistically significant (Figure 9, A and B). The interferon regulatory factor (IRF) family are transcriptional regulators of IFN, downstream of PRRs such as TLRs, NOD2 and RIG-I, which mediate IFN production (Jefferies, 2019). Bacterial sensing via NOD2 has been implicated in activation of IRF5, leading to enhanced IFN production (*ibid*). It has also been implicated in production of IL-6, IL-12 and TNF in a MYD88-dependent manner (*ibid*). IRF7 is constitutively expressed at very low levels except in pDC, but is inducible by LPS, viral infection, TNF and type-I IFNs (Ning, Pagano and Barber, 2011). The coordinated activation of IRFs in response to pathogen sensing is involved in the downstream induction of IFNs and the pattern of cytokine expression induced by infection (Jefferies, 2019). Expression of IRF5 was significantly higher in NECs inoculated with NTHi compared with PBECs (Figure 9C). There was a tendency towards higher expression of IRF7 in NECs than PBECs, but this was not significant (Figure 9D). Treatments with TLR2 and TLR4 agonists HKLM and LPS were included to evaluate differential TLR signalling in NECs compared with PBECs, but NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs, suggesting that they are capable of responding to TLR agonists.

I next evaluated expression MYD88, IRAK1 and TRAF6, factors downstream from TLR2 and 4 (Figure 10). MYD88 and IRAK1 higher expression were numerically higher in NECs compared with PBECs, although this difference was not statistically significant, whilst TRAF6 expression was significantly higher in NECs compared with PBECs.

Figure 10. Fluidigm MYD88, IRAK1, TRAF6

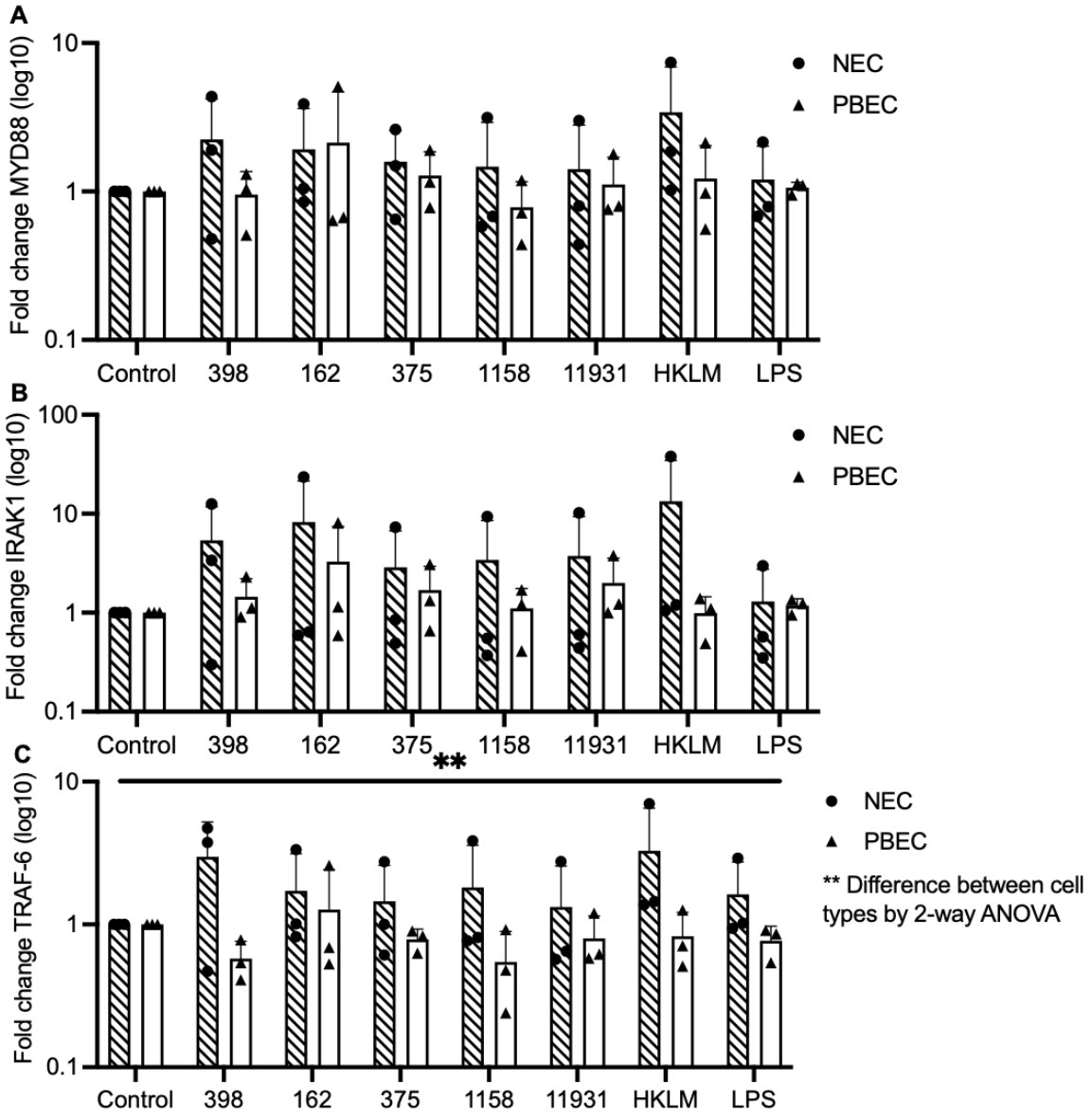


Figure 10. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. Mature ALI cultures were inoculated with a selection of NTHi strains, ordered from highest to lowest internalisation, and incubated in the presence of extracellular bacteria for 24 hours prior to isolation of RNA. Treatment with TLR2 and TLR4 agonists HKLM and LPS was included to evaluate differential TLR signalling in NECs compared with PBECs. A, B. MYD88 and IRAK1 tended towards higher expression in NECs compared with PBECs, but this was not significant. C. TRAF6 was significantly higher in NECs compared with PBECs. Overall, NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs. Significance for all results was evaluated by 2-way ANOVA and Sidak's multiple comparisons test performed on log-transformed data.

Evaluation of NF-kB subunit protein, RelA, which is involved in the translocation of NF-kB into the nucleus, demonstrated that expression was significantly higher in NECs compared with PBECs (Figure 11A). Expression of ALPK1, which activates NF-kB via TRAF6, was also significantly higher in NECs compared with PBECs exposed to NTHi (Figure 11B).

Figure 11. NF-kB pathway components RelA and ALPK1 were higher in NECs than PBECs

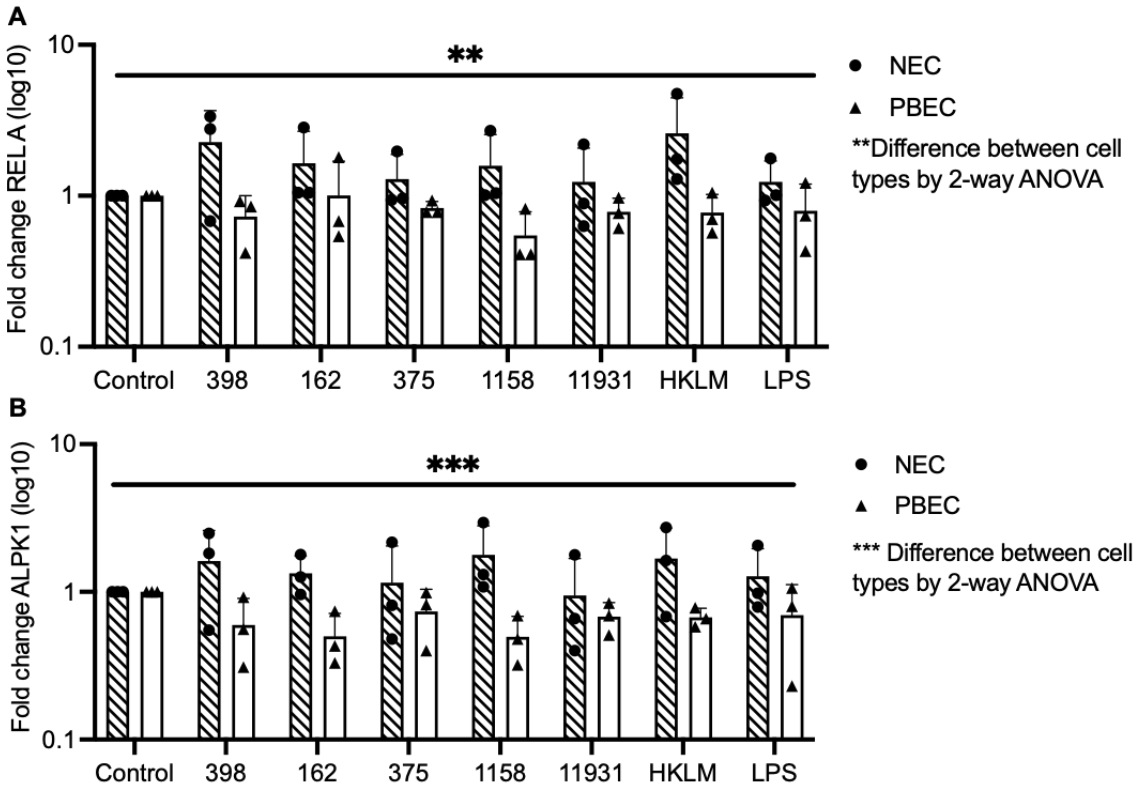


Figure 11. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. A. NF-kB subunit protein, RelA, which is involved in the translocation of NF-kB into the nucleus, was significantly higher in NECs compared to PBECs. B. ALPK1, which activates NF-kB via TRAF6, was also significantly higher in NECs compared with PBECs exposed to NTHi. Overall, NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs. Significance for all results was evaluated by 2-way ANOVA performed on log-transformed data.

Figure 12. Fluidigm NLRs

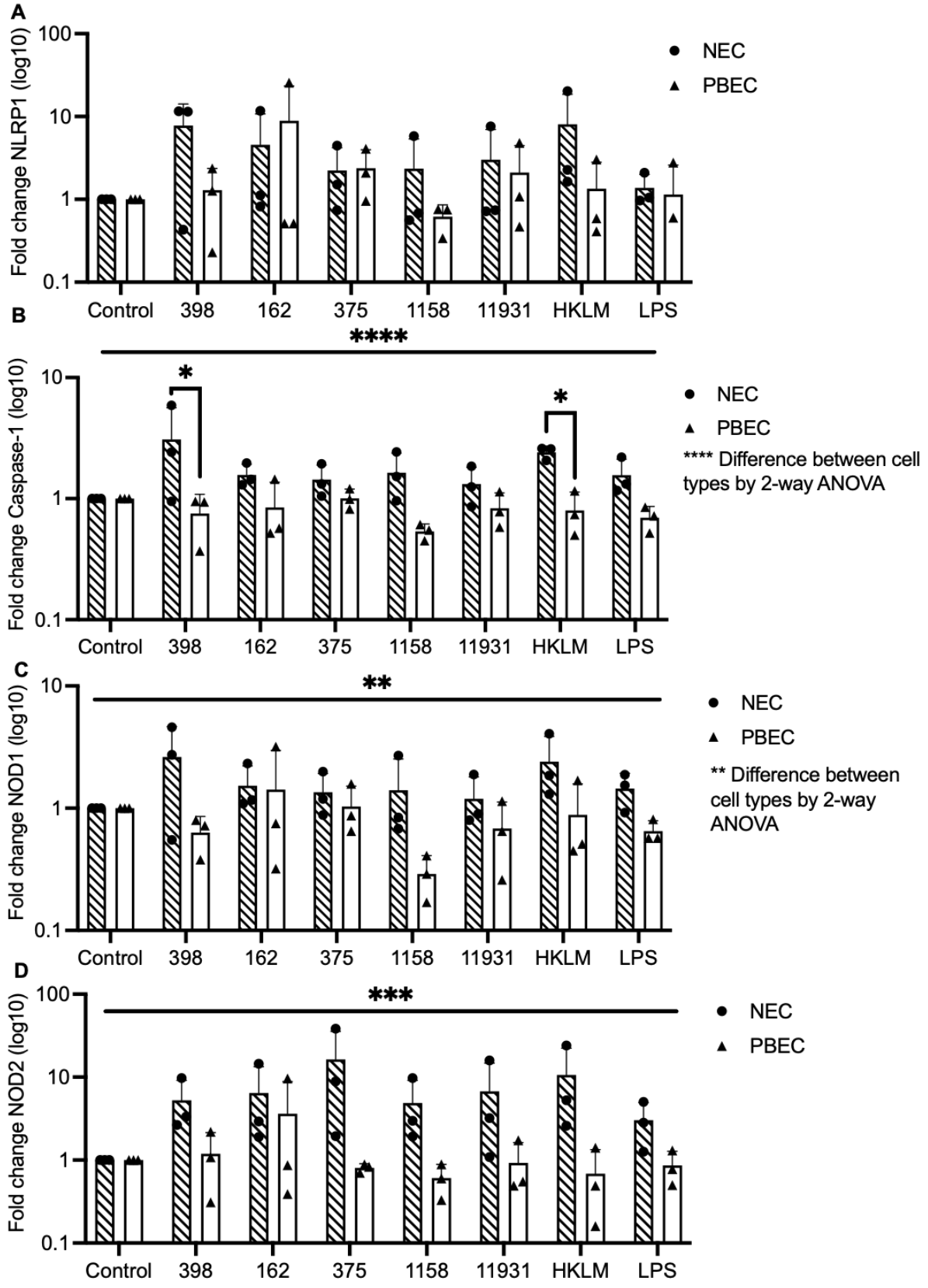


Figure 12. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. A. NLRP1 was modestly upregulated in both NECs and PBECs when exposed to NTHi, with no significant difference between cell types. B. Caspase-1, a factor downstream from NLR-associated inflammasomes, was significantly more highly expressed in NECs compared with PBECs. C, D. Both NOD1 and NOD2 were more highly expressed in NECs than PBECs. Significance for all results was evaluated by 2-way ANOVA performed on log-transformed.

Activation of the NLRP1 inflammasome was then evaluated (Figure 12, A and B). NLRP1 was modestly upregulated by NTHi in both NECs and PBECs, but there was not a significant difference between cell types. A downstream factor from NLR-associated inflammasomes, Caspase-1, was significantly more highly expressed in NECs compared with PBECs. Evaluating another branch of the signalling pathway, intracellular receptors NOD1 and NOD2 were both significantly more highly expressed in NECs than PBECs (Figure 12, C and D).

To examine the next step of the pathway, I evaluated expression of components of the MAPK pathway (Figure 13). MAPK signalling is downstream from both TLR signalling via MYD88, and NOD receptor signalling. Stimulation with NTHi only modestly induced MAPK3 (ERK1) in NECs, but expression was significantly higher than in PBECs (Figure 13A). Similarly, MAPK8 (JNK1) expression was also modestly induced in response to NTHi and was significantly higher in NECs compared with PBECs (Figure 13B).

Figure 13. Activation of MAPKs is higher in NECs than PBECs

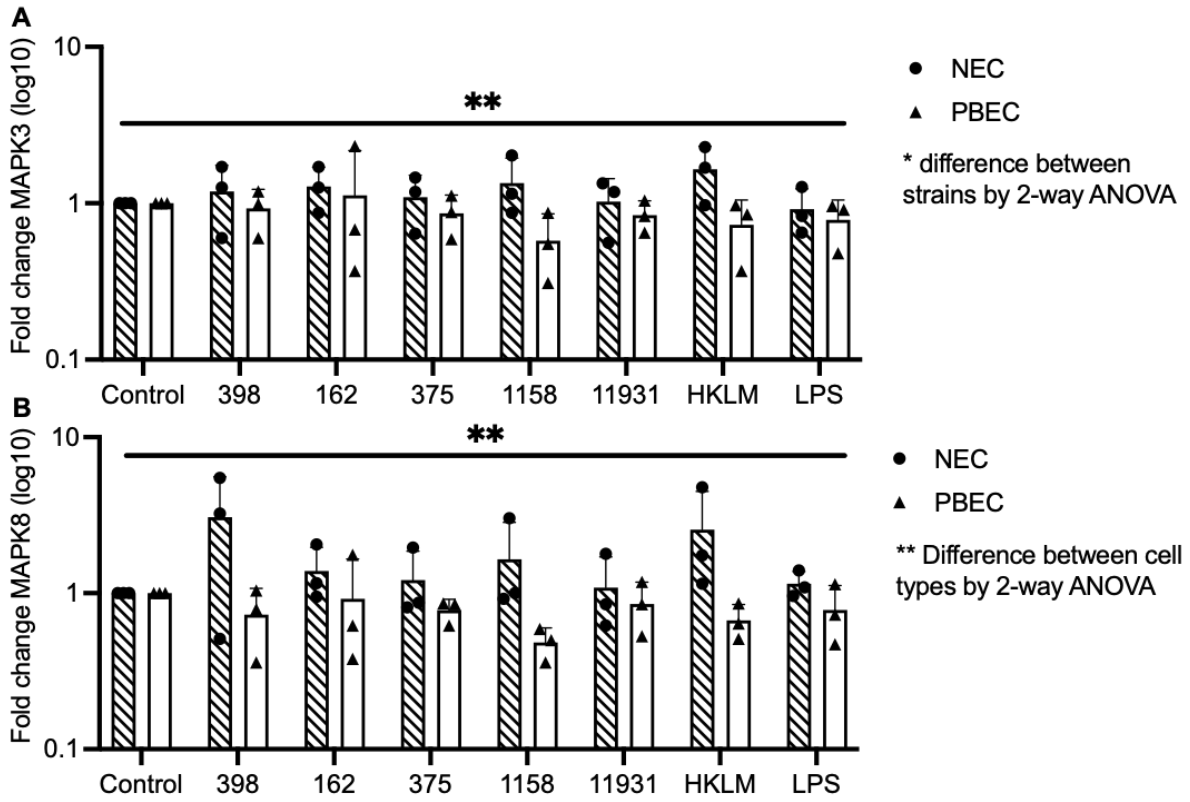


Figure 13. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. A. MAPK3 (ERK1) was only slightly induced in NECs, but was significantly higher than in PBECs. B. Similarly, MAPK8 (JNK1) was also modestly induced in response to NTHi, but was significantly higher in NECs. NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs. Significance for all results was evaluated by 2-way ANOVA performed on log-transformed data.

Expression of downstream pro-inflammatory cytokines was then evaluated (Figure 14). IL-1b, a cytokine driven by inflammasome activation, was substantially induced in NECs and only slightly induced in PBECs (Figure 14A). However, the differences between cell types were not significant. Both NECs and PBECs showed moderately upregulated IL-6 expression in response to NTHi (Figure 14B). Expression of IL-8 expression was modestly upregulated in NECs in response to NTHi and was significantly higher than in PBECs (Figure 14C). NTHi induced modestly elevated expression of TNF was induced at comparable levels in both cell types (Figure 14D).

Figure 14. Fluidigm Cytokines

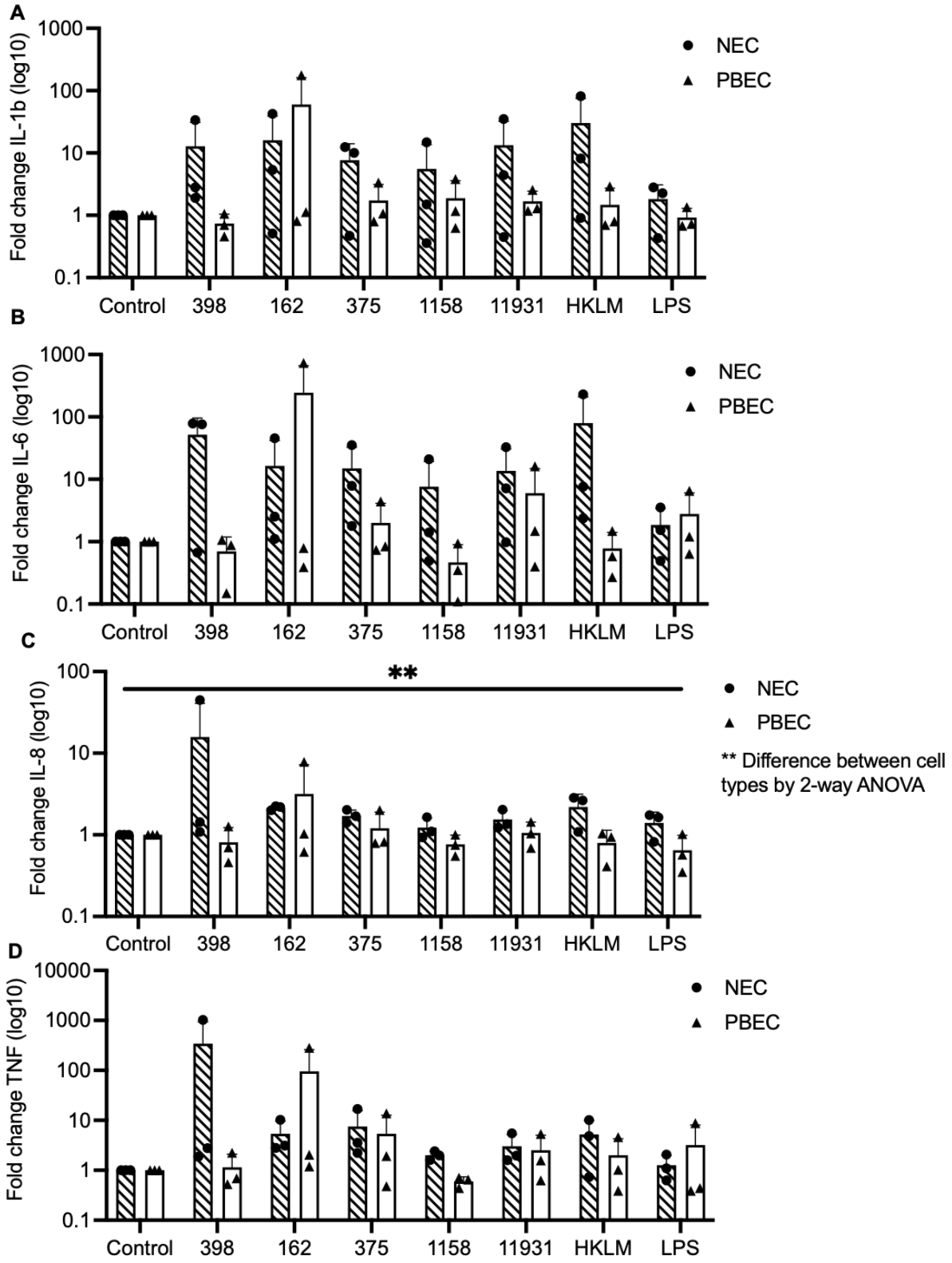


Figure 14. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. Mature ALI cultures were inoculated with a selection of NTHi strains, ordered from highest to lowest internalisation, and incubated in the presence of extracellular bacteria for 24 hours prior to isolation of RNA. Treatment with TLR2 and TLR4 agonists HKLM and LPS was included to evaluate differential TLR signalling in NECs compared with PBECs. A. IL-1b was substantially induced in NECs and only slightly induced in PBECs, but the differences between cell types was not significant. B. IL-6 was moderately induced in both NECs and PBECs in response to NTHi. C. IL-8 expression was significantly higher in NECs than PBECs. D. TNF was induced at comparable levels in both cell types. NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs. Significance for all results was evaluated by 2-way ANOVA performed on log- transformed data.

Analysis of further cytokines showed similar trends (Figure 15). NTHi induced CCL2 at comparable levels in both cell types (15A). IL-33, IL-25 and TSLP belong to a group of cytokines collectively referred to as alarmins, which are induced by a variety of infectious agents and allergic stimuli, notably house dust mites. IL-33 was substantially induced by NTHi in NECs, but unaffected in PBECs. However, the differences between cell types were not significant, and there was significant variation between donors (15B). TSLP expression was not substantially altered in either cell type (15C). Altered expression of AGER (RAGE) is associated with allergic inflammation in airways diseases and neutrophilic asthma. However, neither NECs nor PBECs expressed significantly elevated AGER (RAGE) in response to any stimuli (15D).

Figure 15. Fluidigm Alarmins

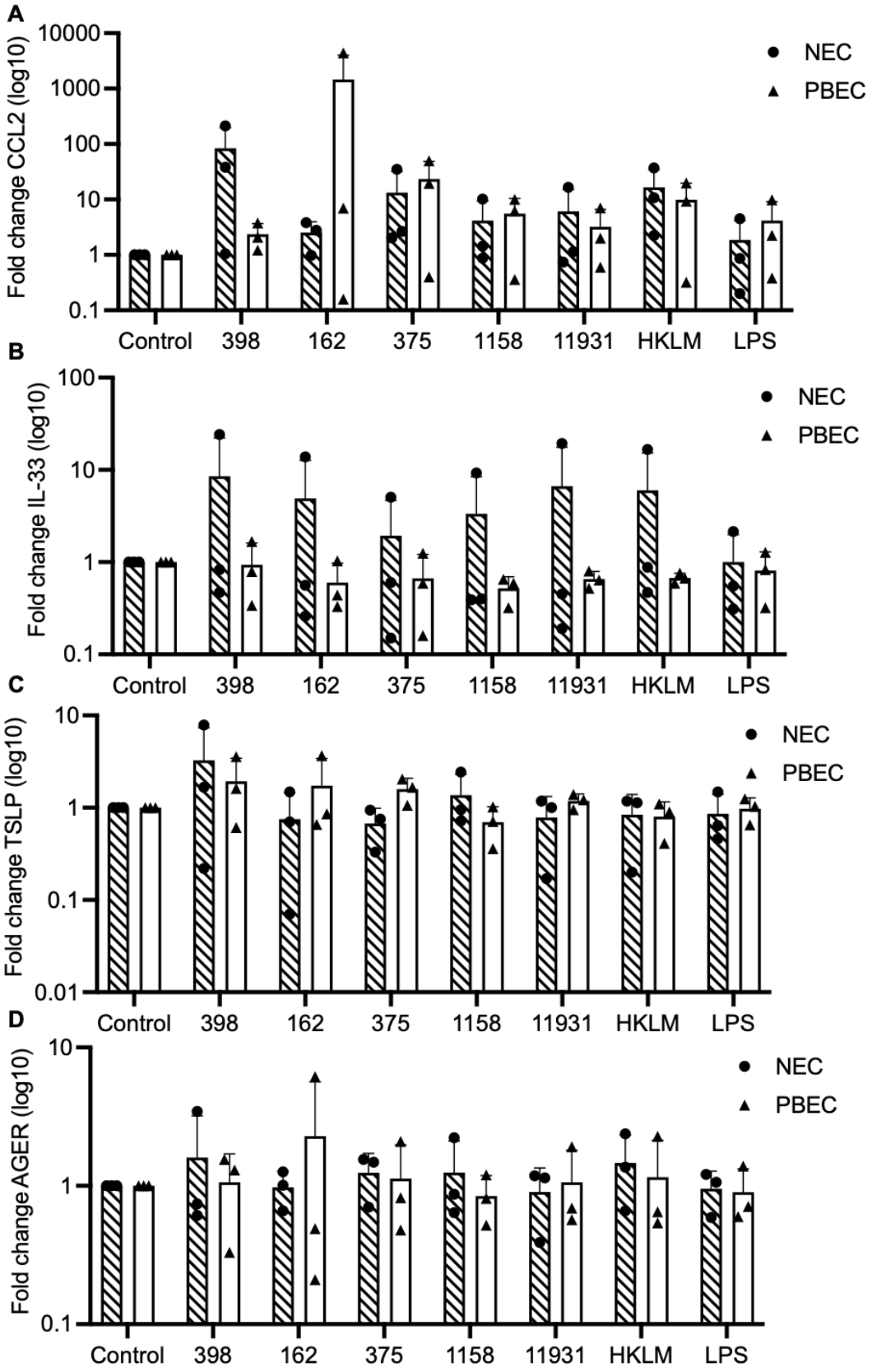


Figure 15. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. A. CCL2 was induced at comparable levels in both cell types. B. IL-33 was substantially induced in NECs and unaffected in PBECs, but the differences between cell types was not significant and there was significant variation between donors. C. TSLP was not induced in either cell type. D. Neither NECs nor PBECs expressed significantly elevated AGER (RAGE) in response to any stimuli. NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs. Significance for all results was evaluated by 2-way ANOVA performed on log-transformed data.

Figure 16. Surface binding and antimicrobial peptides

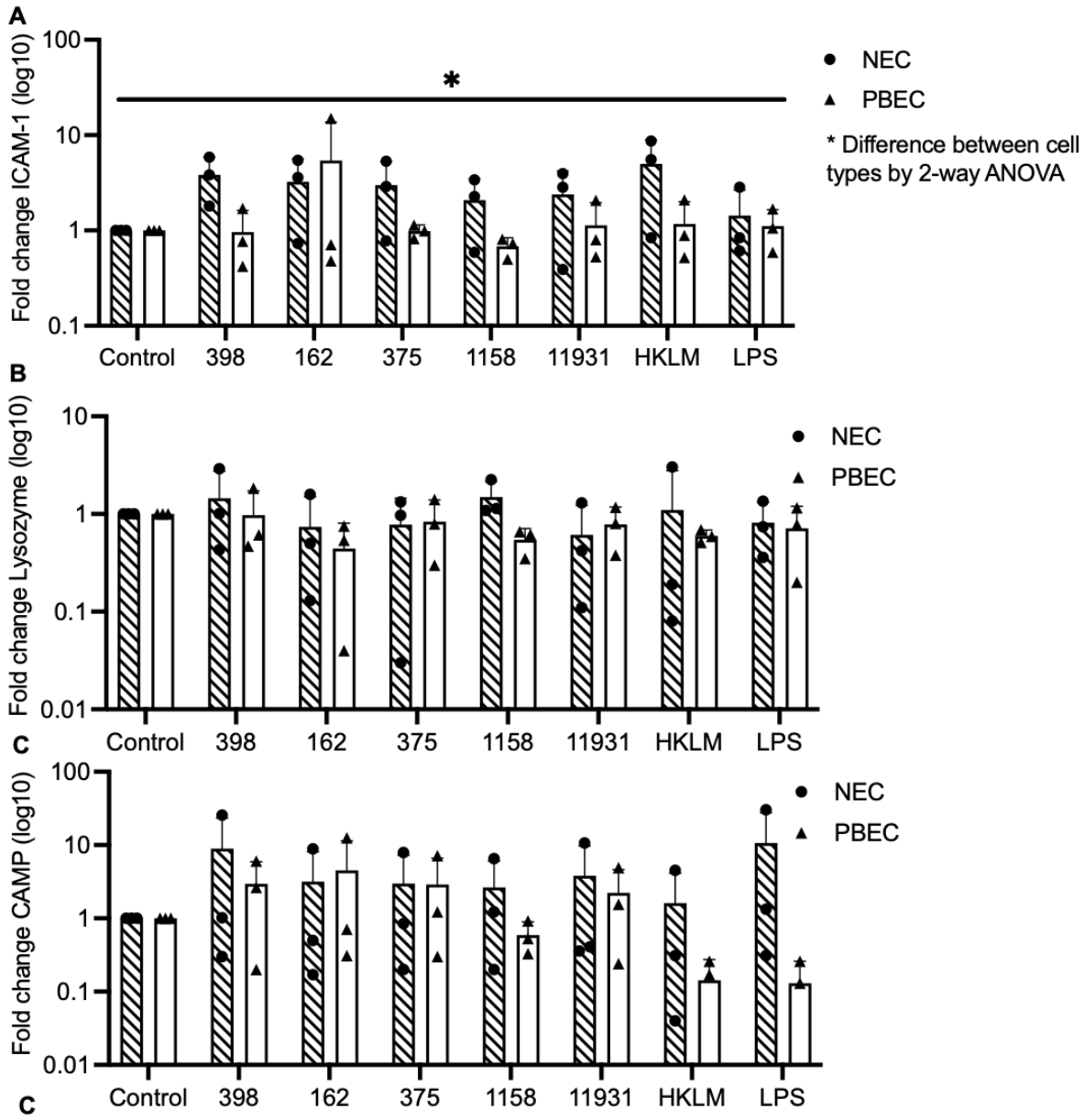


Figure 16. A Fluidigm BioMark PCR array experiment was performed to evaluate gene expression induced by NTHi in PBECs compared with NECs cultured at ALI. Mature ALI cultures were inoculated with a selection of NTHi strains, ordered from highest to lowest internalisation, and incubated in the presence of extracellular bacteria for 24 hours prior to isolation of RNA. Treatment with TLR2 and TLR4 agonists HKLM and LPS was included to evaluate differential TLR signalling in NECs compared with PBECs. A. Cell surface molecule ICAM-1 was significantly higher in NECs than PBECs. B. Lysozyme was not significantly upregulated in either cell type. C. Cathelicidin (CAMP) was modestly induced by NTHi in both cell types at comparable levels. NECs responded to stimulation with HKLM and LPS at comparable or higher levels than PBECs. Significance for all results was evaluated by 2-way ANOVA performed on log- transformed data.

ICAM-1 is a cell surface molecule which used as a receptor by NTHi and major type rhinoviruses, and which may be upregulated by NTHi. In this model, ICAM-1 expression was moderately increased in NECs in response to NTHi, and was significantly higher than in PBECs (Figure 16A). Evaluation of antimicrobial peptides showed that expression of lysozyme was not significantly upregulated in either cell type (16B), whilst cathelicidin (CAMP) was modestly induced by NTHi at comparable levels in both NECs and PBECs (16C).

To evaluate the protein production in epithelial cells in response to NTHi, analysis was performed on supernatants from NECs and PBECs inoculated with NTHi for 24 hours using a Meso-Scale Design (MSD) kit (Figure 17). Production of cytokines IL-1b, IL-6 and TNF were evaluated in pooled apical and basal supernatants from NECs and PBECs grown at ALI. Exposure to NTHi did not induce significantly elevated production of IL-1b in either NECs or PBECs (17A). NTHi did not induce IL-6 production in NECs, while in PBECs, IL-6 was modestly induced by infection with strains 1158, 375 and 398 (17B). NTHi did not alter production of TNF in either cell type (17C).

Figure 17. Cytokine production by NECs and PBECs in response to NTHi.

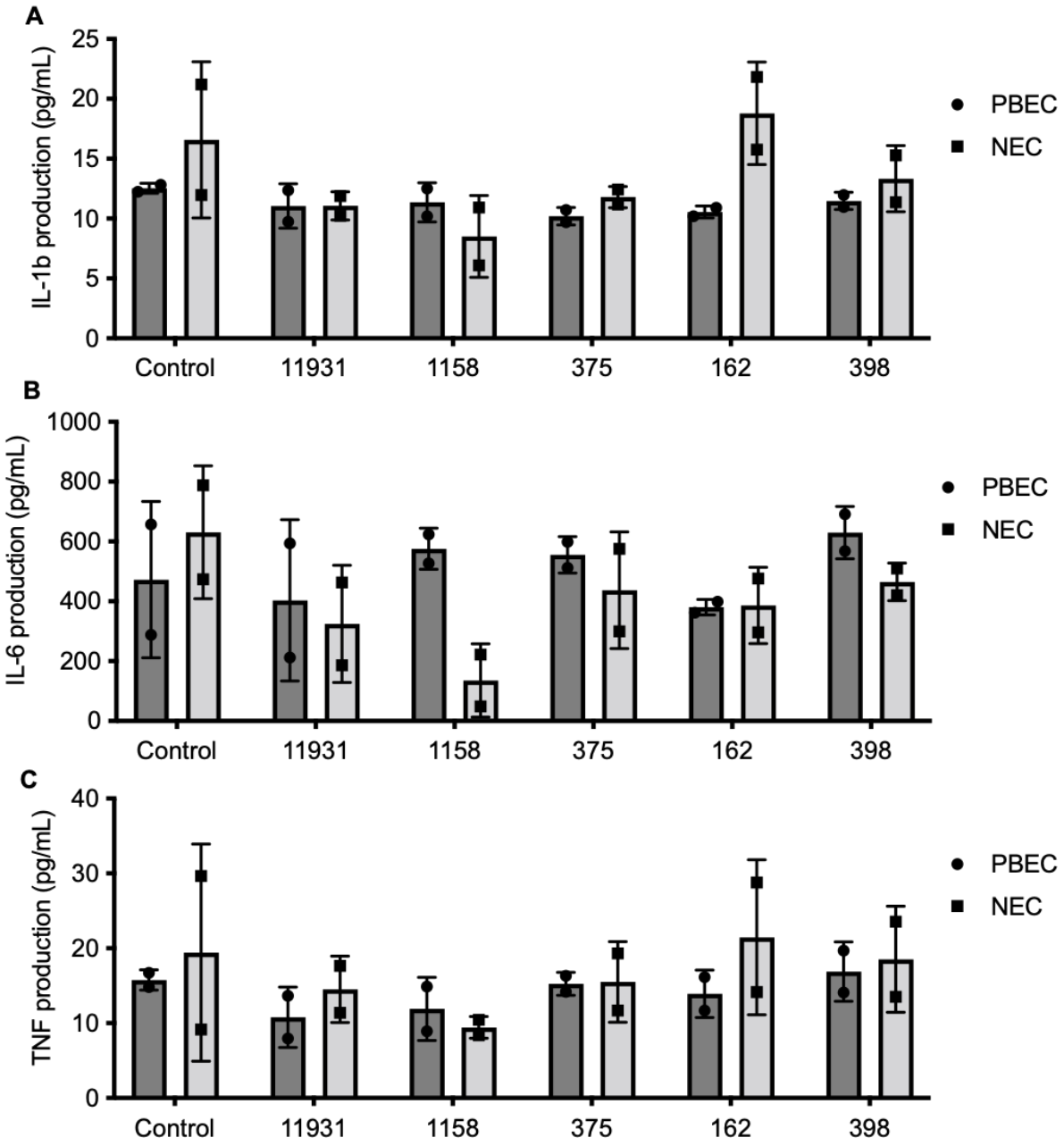


Figure 17. NECs and PBECs from 2 healthy control donors were cultured at ALI for a minimum of 28 days prior to inoculation with NTHi strains for 24 hours. Strains are arranged from lowest to highest internalisation. Supernatants pooled from the apical and basolateral compartments were analysed using an MSD analysis. A. IL-1b was not significantly induced by NTHi inoculation in either NECs or PBECs. B. IL-6 was expressed at baseline or lower levels in NECs infected with NTHi, while in PBECs IL-6 was modestly induced by infection with strains 1158, 375 and 398. C. TNF production was not significantly induced in either cell type. Significance was evaluated using 2-way ANOVA.

Taken together, the results from this series of experiments suggests that NECs are more immunologically sensitive to NTHi exposure compared with PBECs and express significantly more pro-inflammatory signalling molecules at various steps in the signalling cascades. These experiments have also demonstrated that NTHi activates inflammatory signalling via several pathways, including via TLR signalling, NOD1 and NOD2 sensing, and by upregulation of inflammasome-associated NLRs.

5.3 Is differential immune activation specific to NTHi?

To evaluate whether differential immune responses are an intrinsic property of the different cell types, or an NTHi-specific effect, induction of inflammatory cytokines following stimulation with TLR2 and TLR4 agonists was evaluated (Figure 18). NECs and PBECs cultured at ALI were exposed to NTHi or TLR2 and TLR4 agonists, HKLM and LPS respectively, for 24 hours. Cytokine production was evaluated in pooled apical and basal supernatants using an MSD assay. IL-1b production was not substantially elevated by treatment with HKLM or LPS, and it was comparable in NECs and PBECs (18A). Although IL-6 was numerically lower in NECs compared with PBECs treated with HKLM or NTHi strain 398, this difference was not significant and there were not substantial differences between cell types treated with LPS (Figure 18B). TNF production was not substantially upregulated, but levels were comparable in NECs and PBECs for all treatments (Figure 18C). Overall, production of cytokines in response to TLR2 and TLR4 agonists were comparable between NECs and PBECs. In qPCR data, the response to TLR agonists in NECs was consistently higher than in PBECs, as was seen in response to NTHi, suggesting that differential signalling between cell types is not NTHi-specific (Figures 9-16).

Figure 18. MSD analysis of TLR 2 and TLR4 agonists in NECs and PBECs

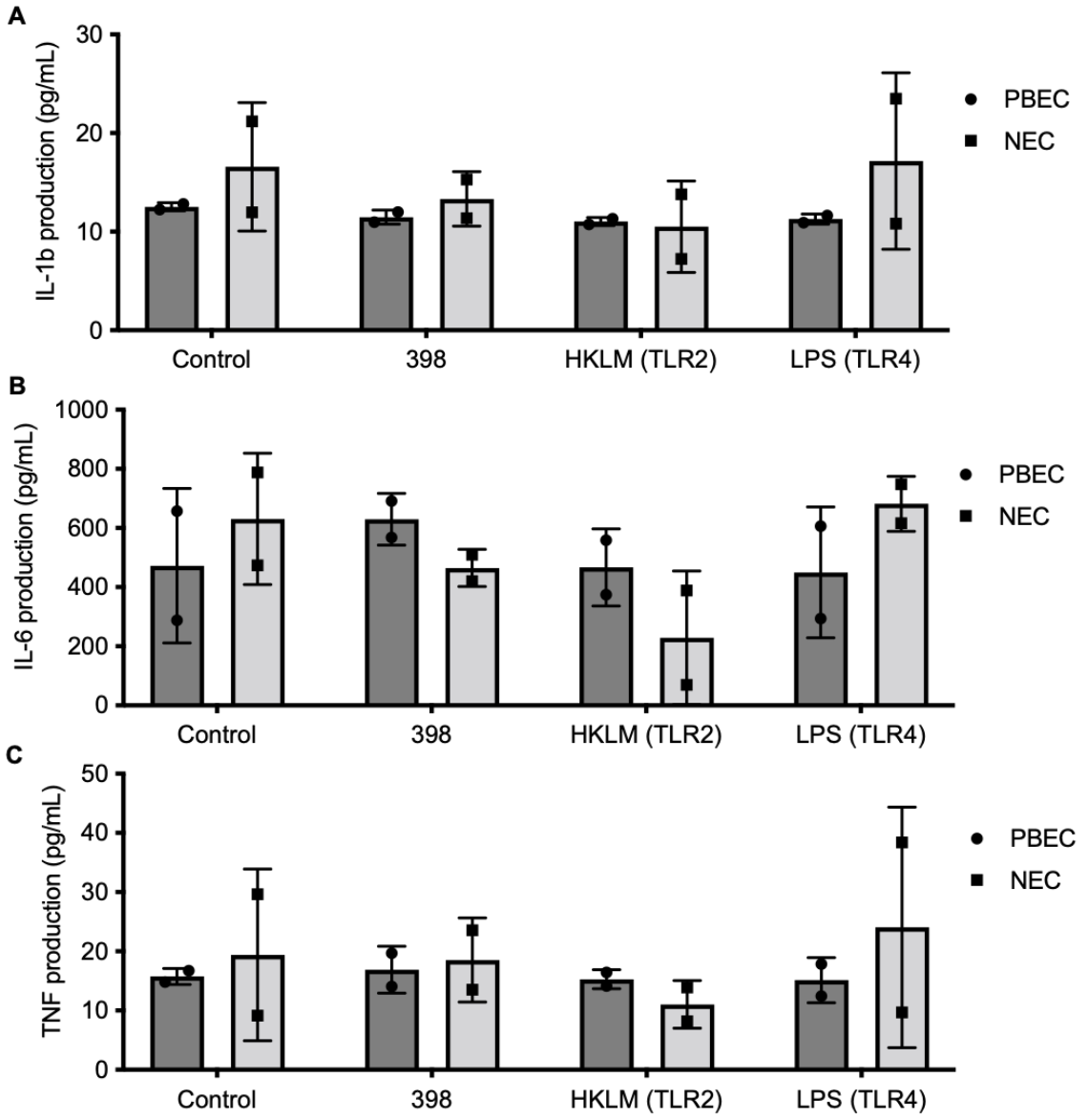


Figure 18. NECs and PBECs from 2 healthy control donors were cultured at ALI for a minimum of 28 days prior to inoculation with NTHi strains for 24 hours or treatment with TLR2 agonist HKLM or TLR4 agonist LPS. Supernatants pooled from the apical and basolateral compartments were analysed using an MSD analysis. Comparison of cytokine induction in NECs compared with PBECs demonstrated that there was not a significant difference between signalling induced by TLR agonists in NECs compared with PBECs. A. IL-1b production was comparable in NECs and PBECs, although it was not substantially elevated by treatment HKLM or LPS. B. IL-6 was numerically lower in NECs compared with PBECs treated with HKLM, but this was not significant and there were not substantial differences between cell types treated with 398 or LPS. C. TNF production was comparable in NECs and PBECs for all treatments. Significance was evaluated by 2-way ANOVA.

5.4 Conclusions

This chapter has sought to examine the differences in infection by, and the innate epithelial cell immune response to, NTHi infection in the upper airway epithelium compared with the lower airways. Clinically, NTHi is a commensal organism in the upper airway, whilst it causes clinically significant, persistent infections in the lungs, often of patients with asthma and COPD, and contributes to inflammation and disease exacerbations. Several other bacteria are also commensals in the nasopharynx but cause invasive disease in the lung, notably *Moraxella catarrhalis*, suggesting that there may be differential signalling induced by bacterial infection in upper compared with lower airway epithelium.

Initial experiments were directed at examining differences in physical interactions between upper and lower airway epithelium, demonstrating that nasal epithelial cells are significantly more highly infected than bronchial epithelial cells in both submerged and ALI cultures. In a cell line model, adherent and intracellular bacterial numbers were modestly elevated in nasal epithelium, whilst differences in paracellular numbers were more marked, perhaps suggesting that the difference in internalisation may be related to differences in tight junction function. Further investigation using primary cell cultures would be beneficial to ensure that these are not cell line-specific effects.

Flow cytometry analysis of NECs cultured at ALI infected with NTHi demonstrated that secretory cells were the most highly infected cell type, followed closely by ciliated cells, whilst only a small number of basal cells were infected. Comparison of NECs and PBECs examined the number of infected secretory, ciliated and basal cells, and showed that NECs were more highly infected in all cell types. One limitation is that these experiments were performed on cell cultures that had been infected for only a short time. Initial data in cell lines suggested that upper airway epithelium were able to eliminate NTHi infection more rapidly than lower airway cells, so repeating these experiments at ALI with an extended timecourse would be of interest to determine whether primary cells behave similarly.

The second half of this chapter focussed on evaluating the differences in immune activation in response to NTHi in upper compared with lower airway epithelium.

Initial experiments used cell lines infected with a selection of NTHi strains, and showed that Calu-3 cells, a model of lower airway epithelium, expressed higher levels of IL-8, IL-6, and TNF in response to NTHi compared with nasal epithelial cell line RPMI2650 cells. Conversely, CCL2 was more robustly induced in RPMI2650 cells than Calu-3 cells, suggesting that NTHi may induce differential activation of immune responses in each cell type. Due to the lower expression of cytokines, I hypothesised that the nasal epithelial cells may be deficient in TLR2 and TLR4 signalling.

Further examination of the signalling pathways involved was performed using a PCR array experiment to evaluate activation of signalling via TLR-2 and 4, NOD1 and 2, and inflammasome-associated NLRs in NECs and PBECs grown at ALI infected with NTHi. Overall, NTHi induced more robust activation of inflammatory signalling in NECs compared with PBECs across all pathways (Figure 19). TLR2 and 4, and NOD1 and 2 signalling pathways were activated in NECs, whilst NLRs, which require intracellular sensing of bacterial components, were activated in both NECs and PBECs. In addition, expression of most cytokines was elevated in NECs in response to NTHi, whilst CCL2 and TNF were induced by NTHi in both NECs and PBECs. CCL2 is a chemokine that mediates migration and infiltration of monocytes and macrophages, suggesting that PBECs may focus on recruiting immune cells. Unlike the other cytokines that were evaluated, TNF is a type I interferon, perhaps suggesting that PBECs prioritise induction of type I signalling. Induction of signalling by TLR2 agonist HKLM and TLR4 agonist LPS were equivalent or higher in NECs compared with PBECs in both qPCR and MSD experiments measuring protein levels of cytokines, suggesting that TLR signalling is intact in upper airway cells. The trend towards greater activation in NECs is discordant with results from cell line data, underlining the importance of performing confirmatory experiments in primary cells. One limitation of these experiments is the limited availability of primary cells, precluding their use for preliminary studies, which limits the value of initial work due to cell line-specific effects which do not recapitulate the conditions *in vivo*. However, there was also substantial variation in responses between donors, which is a well-known challenge associated with primary cell work. Further experiments using

samples from a greater number of donors would be beneficial to confirm that these results are relevant to the wider population.

Figure 19. Signalling pathways activated by NTHi in NECs and PBECs

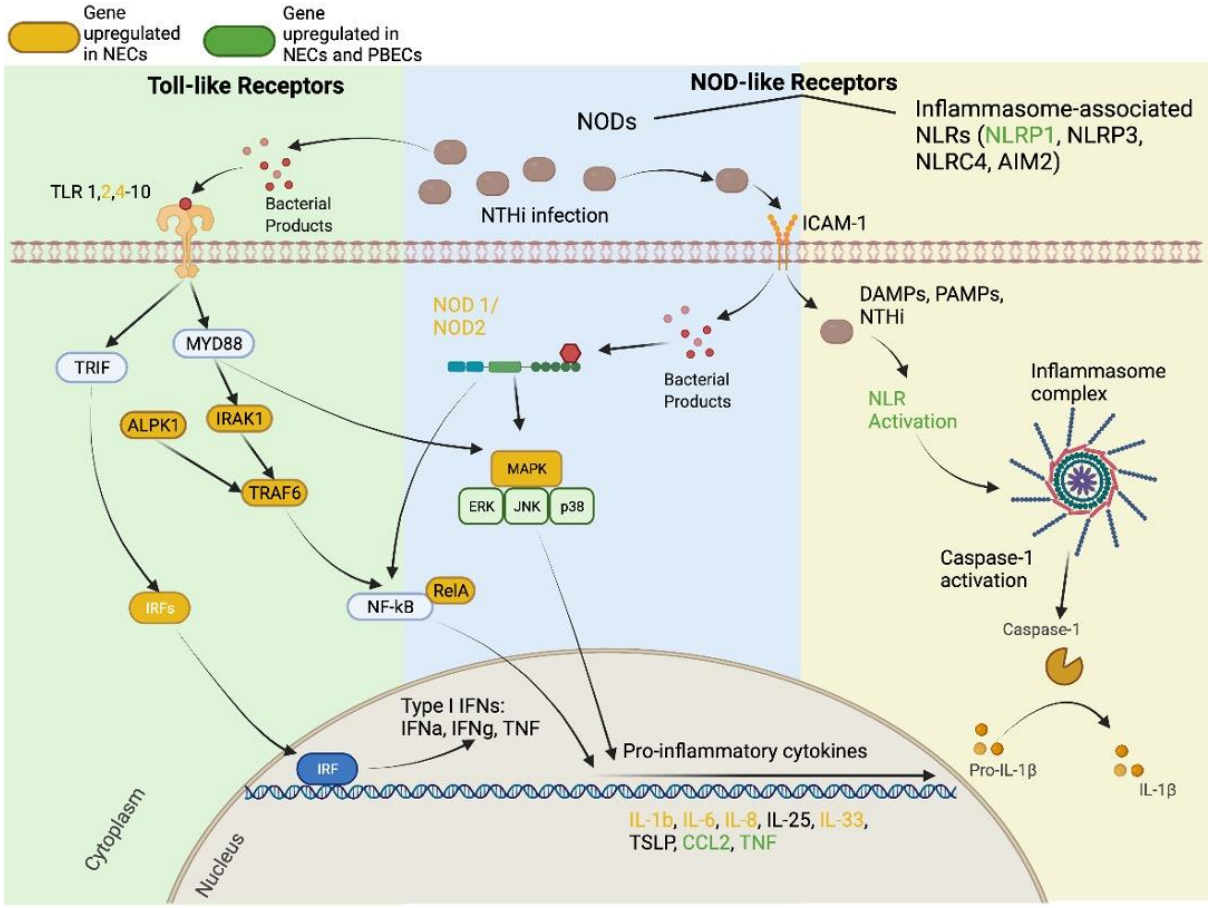


Figure 19. Based on literature review, I have investigated three potential pathways by which NTHi may mediate immune activation. One route is via TLR2 and 4 signalling and downstream IRF5 and 7 and the MYD88-IRAK1-TRAF6 signalling transduction pathway. Another is via NOD1 and NOD2 and downstream activation of MAPK signalling and NF-kB. Inflammasome-associated NLRs (NLRP1, NLRP3, NLRC4, AIM2) have also been implicated in NTHi-induced inflammatory signalling in a caspase-1 dependent manner resulting in cleavage of pro-IL-1b to its active form. NF-kB signalling appears to be activated by several of these mechanisms, in addition to possible activation via ALPK1. Transcriptional upregulation of cytokines CCL2(MCP1), TNF, IL-1b, IL-6 and IL-8 and alarmins TSLP, IL-33 and IL-25 is thought to be induced by NTHi. In addition, cell surface receptor ICAM-1 is thought to be upregulated in epithelial cells infected with NTHi. I have also investigated whether antimicrobial peptides lysozyme and cathelicidin (CAMP) are affected by NTHi infection. Targets which have been upregulated in NECs compared with PBECs are marked in yellow, while those upregulated in both cell types are coloured green.

These data suggest that there is differential activation between NECs and PBECs in response to NTHi infection, with NECs being more sensitive to infection overall. Due

to the pro-inflammatory nature of NTHi infection in the lower airways, and commensal nature in the nasopharynx, these findings were unexpected. Further work is necessary to unravel the signalling involved, but I postulate that these differences may be due to differences in immune cells in the lung and nasopharynx, and that perhaps lower levels of signalling in the lower airway epithelium are amplified by greater numbers of macrophages in the lung.

Chapter 6 Discussion

Despite major advances in the treatment of type II airways inflammation, chronic airways infection remains a major cause of morbidity and mortality in airways diseases including asthma, COPD and bronchiectasis (Moghaddam *et al.*, 2011; Chalmers *et al.*, 2018; Saliu *et al.*, 2021). A significant proportion of patients with severe asthma have steroid-resistant disease, with an estimated 10% of patients experiencing steroid-resistant disease (Chung *et al.*, 2014; Pavord *et al.*, 2018). Biologic treatments such as Benralizumab, Mepolizumab, and Reslizumab, which target the type-2 cytokine IL-5 or its receptor, improve lung function and quality of life, reduce airway eosinophilia and improve airway hyperresponsiveness (Pavord *et al.*, 2012; Padilla-Galo *et al.*, 2020; Taillé *et al.*, 2020). Currently in phase III clinical trials, Tezepelumab targets TSLP and has shown promising results in reducing the number of annual exacerbations and improving lung function and quality of life in patients regardless of their initial eosinophil counts (Menzies-Gow *et al.*, 2021). These therapies are promising for patients with severe steroid-resistant eosinophilic disease, but as they focus on type-2 cytokines, there is still an unmet need for treatments for patients with neutrophilic, type-2 low disease. These patients often have underlying bacterial infections which are driving inflammation, notably chronic NTHi infection (Zhang *et al.*, 2020).

Epithelial cells are critical players in mediating airways infections as the most numerous cell type in the airways, constituting the predominant physical barrier to invading pathogens (Holgate, 2006, 2008). More broadly, epithelial cells are central to the aetiopathogenesis of asthma and other inflammatory diseases of the airways and are drivers of inflammatory signalling in response to airway pathogens including NTHi infection (Holgate, 2008). In addition, prolonged inflammatory signalling may induce differentiation of epithelial cells into mucus hypersecretory cells, contributing to the pathophysiology of asthma and increasing susceptibility to bacterial infection (Evans *et al.*, 2009).

I chose to investigate the interaction of NTHi with the human airway epithelial cell because of its role in the pathogenesis of neutrophilic asthma and the emerging role of the epithelium as a mediator of infection. NTHi is associated with the development of

neutrophilic, steroid-resistant asthma (Zhang *et al.*, 2020). It is also the most commonly isolated airway pathogen in metagenomic studies of airways diseases (Simpson *et al.*, 2016; Abdel-Aziz *et al.*, 2019). One study examining the pathogen burden in patients with uncontrolled asthma isolated NTHi from 76% of patients (Simpson *et al.*, 2016). NTHi was previously considered a primarily extracellular pathogen, but it has now been shown that it is capable of being internalised in airway epithelium (Clementi, Håkansson and Murphy, 2014). NTHi can be isolated within epithelial cells isolated from patients, suggesting that the epithelium may act as a reservoir for NTHi facilitating persistent infection. Overall, the results of this thesis suggest that, although NTHi is internalised by all types of epithelial cells in both submerged and ALI cultures, it does not persist solely within the epithelium. This is supported by another study which has shown that viable NTHi is present within the epithelium in submerged culture, but that it does not replicate within epithelial cells (Morey *et al.*, 2011). Together these results suggest that the persistent infections seen in patients with airways diseases require additional or alternative reservoirs for survival of viable bacteria. These may be via persistence of NTHi within biofilms formed in conjunction with NETs or due to impaired phagocytosis by alveolar macrophages associated with airways disease.

Despite its inability to produce extracellular polysaccharides, a requirement for traditional biofilms, NTHi is able to form biofilm-like structures, often in conjunction with other bacterial species (Juneau *et al.*, 2011; Langereis and Hermans, 2013; Tikhomirova and Kidd, 2013). Bacteria within biofilms are protected from killing by both antimicrobial peptides and phagocytosis (Magana *et al.*, 2018). Some evidence has suggested that infection with NTHi can induce production of NETs, and that, whilst NETs are able to kill many types of bacteria, they are not able to efficiently eliminate NTHi, allowing them to form a *de facto* biofilm shielding them from immune clearance (Juneau *et al.*, 2011). NET formation has been identified as a key marker of disease severity and responsiveness to azithromycin treatment in patients with bronchiectasis (Keir *et al.*, 2021). This is one potential mechanism by which NTHi may be able to chronically infect the lower airways.

Despite their classical role for immune surveillance and pathogen clearance of the lung, there is evidence that NTHi is able to persist for extended periods within airway macrophages (Ackland *et al.*, 2019, 2020). One study demonstrated using mouse macrophage-like cell line J774 that a virulent strain of NTHi was able to be recovered from macrophages up to 72 hours after phagocytosis (Craig *et al.*, 2001). A dual-RNAseq study has shown that, despite enrichment of immune response genes in macrophages, NTHi underwent transcriptomic adaptation and persisted intracellularly (Ackland *et al.*, 2020). In addition, alveolar macrophages in patients with airways diseases have impaired phagocytosis of NTHi (Berenson *et al.*, 2006), and exposure to cigarette smoke also impairs bacterial clearance by alveolar macrophages (Martí-Lliteras *et al.*, 2009). In addition, alveolar macrophages from exacerbation-prone COPD patients exhibited reduced sensitivity to TLR2 and TLR4 ligands from NTHi and lower activation of NF-kB (Berenson *et al.*, 2014). These data suggest that the function of alveolar macrophages is fundamentally impaired in patients with airways disease, and their reduced capacity to phagocytose and kill NTHi may provide an immunologic explanation for the chronic NTHi infection common in these patients (Berenson *et al.*, 2006).

After describing the physical interactions between NTHi and the epithelium, I investigated the induction of innate immunity in the epithelium by NTHi infection. NTHi is capable of provoking a strong immune response in the epithelium via TLR2, TLR4, NOD and inflammasome signalling (Wieland *et al.*, 2005; Lee *et al.*, 2008, 2019; Chen *et al.*, 2018). In submerged culture of lower airway epithelial cell lines and primary bronchial epithelial cells, NTHi robustly induces expression of several common cytokines. The neutrophil chemoattractant IL-8, IL-6, TNF, CCL2 and NLR inflammasome marker IL-1β were all markedly induced by inoculation with NTHi. Given the immunogenicity of adhesion molecules and the evidence of intracellular signalling activated by NTHi, I hypothesised that NTHi strains that were more adherent and more highly internalised would trigger a more robust immune response. Although there is marked variation between strains with regard to the proportion of bacteria which become adherent or are internalised into the intracellular or paracellular spaces, the number of internalised or adherent bacteria does not appear to be critical

for the induction of IL-8, IL-6, TNF and CCL2. This suggests that induction of these cytokines is driven primarily by NTHi components interacting with receptors on the cell surface, such as TLR2 and 4. However, there is a trend towards reduced induction of IL-1β expression in cells which have been treated with cytochalasin D to block bacterial internalisation, suggesting that intracellular bacteria are necessary for activation via intracellular inflammasome-associated NLRs. These data suggest that extracellular sensing of NTHi, perhaps by TLRs, dominate the induction of cytokines in the epithelium. Alternatively, there may be a 'threshold effect' of intracellular bacterial sensing, so a dose-response may not be seen in response to additional bacteria when a minimum number of bacteria have already been internalised.

NTHi is a commensal organism in the nasopharynx, but is capable of causing persistent invasive infection in the lower airways and contributing to exacerbations. I initially hypothesised that the lack of pathogenicity in the nasopharynx may be due to either reduced internalisation or lack of cytokine induction in the epithelium of the nasopharynx compared with the lower airway epithelium. However, experiments using primary nasal and bronchial epithelial cells cultured at ALI have demonstrated that NTHi is internalised in significantly higher numbers in nasal compared with bronchial epithelium across all types of epithelial cells. This was unexpected, given the commensal nature of NTHi infection in the nasopharynx. Initial experiments using cell lines also demonstrated higher internalisation rates in a nasal epithelial model, but those experiments suggested that immune activation was higher in lower airway epithelial cells. However, a more extensive PCR array experiment performed on primary nasal and bronchial epithelial cells grown at ALI showed overall significantly higher induction of a proinflammatory immune markers in NECs compared with PBECs. This experiment investigated activation of immune signalling via TLR2 and 4, NOD1 and 2, and inflammasome-associated NLRs. Expression of TLRs, NODs, and their downstream factors was significantly higher in NECs compared with PBECs in cells inoculated with a variety of NTHi strains. This effect was consistent across NTHi strains regardless of internalisation and adhesion rate, suggesting that it is not a strain-specific effect. In addition, similar The NLRP1 inflammasome was numerically upregulated in both NECs and PBECs in response to NTHi, but this difference was not

statistically significant. In addition, expression of IL-8, IL-6, IL-1β and IL-33 was significantly higher in NECs than PBECs when inoculated with NTHi, whilst only expression of CCL2 and TNF was numerically increased in both NECs and PBECs when exposed to NTHi. Due to the more pro-inflammatory nature of NTHi infections in the lower airways compared with the nasopharynx *in vivo*, these findings were unexpected.

My original hypothesis postulated that PBECs would be more sensitive to NTHi infection and have a more robust activation of cytokine responses via a range of pathways, given the inflammatory signalling seen in asthma patients who have chronic NTHi infection compared with the commensal existence of NTHi in the nasopharynx. I now postulate that these unexpected findings may be due to the variations in the immune cell populations in the different tissues. In the nose and upper airways, dendritic cells form a tight network, draining pathogens to the lymph nodes to generate an adaptive immune response, whilst alveolar macrophages are the dominant immune cell type in the lower airways (Nicod, 2005). Dendritic cells are more efficient antigen-presenting cells, but alveolar macrophages are more effective phagocytes and may constitute an important reservoir of persistent NTHi infection in the lower airways (Gordon and Read, 2002). In addition, alveolar macrophages are capable of recruiting a large influx of neutrophils to the lower airways in more severe infections by generating neutrophil-recruiting chemokines such as IL-8 and leukotriene B_4 (Nicod, 2005). An *in vitro* model of NTHi infection in alveolar macrophages from COPD patients or healthy controls demonstrated that infection with NTHi induced steroid-resistant production of IL-8, suggesting that NTHi-induced inflammatory signalling in macrophages may contribute to steroid insensitivity in COPD and asthma patients (Khalaf *et al.*, 2017). These data suggest that the modest signalling seen in the epithelial cells of the lower airways may be amplified by immune cells such as alveolar macrophages and neutrophils which are present in greater numbers in the lower airways compared with the nasopharynx (Nicod, 2005).

The inability to examine these interactions between epithelial and immune cells is one disadvantage of the *in vitro* cell culture model, as it is necessarily reductionist.

However, it does lend valuable insights into the interactions of infection in a single cell type. Air-liquid interface culture of primary epithelial cells takes a step towards more accurate physiological recapitulation of the epithelial architecture (Ehlers *et al.*, 2017), and yields substantially different results even from primary cells grown in submerged culture in my experiments. Most data available in the literature comparing ALI and submerged cultures focus on the physical characteristics of the differentiated epithelium and development of cilia, mucin-producing cells, and tight junctions (Rayner *et al.,* 2019; Aydin *et al.,* 2021). However, one study examining H5N1 and H1N1 influenza infections in differentiated and undifferentiated PBECs demonstrated that viral replication and IFN responses were divergent in undifferentiated compared with differentiated cells for each viral strain (Chan *et al.*, 2010).

Further experiments using co-culture models of epithelial cells and macrophages would be of interest to examine cell-cell interactions along with host-pathogen interactions. The presence of immune cells in the culture system may be critical in unravelling bacterial interactions with the host (de Waal *et al.*, 2022). For instance, examining cytokine induction in co-culture systems stimulated with NTHi could confirm whether macrophages and epithelial cell interactions result in synergistic induction of pro-inflammatory signalling. Additional examination of interactions between NECs and macrophages would also yield interesting insight into the differences in signalling between the lung and nasopharynx in response to bacteria.

The use of primary cells comes with several well-known challenges. Notably, there are limitations to the number of patients it is possible to access, primary cells have much slower growth rates in culture, and there is high variability between donors, likely due to the mixed populations of cells retrieved during each bronchoscopy (Hodge *et al.*, 2004). Bronchoscopy brushes also contain substantial numbers of bacteria and fungi, leading to low rates of survival of cultures to maturity despite the use of antibiotics and antifungal agents in the media. Cell lines offer greater homogeneity, but results are likely to be of less clinical relevance. They are often derived from cancerous tissue, or are transformed *in vitro*, which may alter key metabolic functions, or induce states of enhanced antimicrobial resistance, and is likely to also affect how the cells respond to stimuli. In addition, being derived from one

individual, a cell line is unable to represent the variability in patients across the population.

Overall, data from *in vivo* models of NTHi infections are still important for understanding interactions between bacteria and individual cell types. NTHi is a uniquely human pathogen, so mouse models are thus far unable to recapitulate all the features of NTHi infection in humans, particularly the chronic nature of the condition (Nials and Uddin, 2008; Holmes, Solari and Holgate, 2011). However, fundamental differences in the immune makeup of mice compared with humans, such as the relative abundance of ILC2 cells and of invariant natural killer T cells, with relative deficiency of mucosal associated invariant T cells, or the lack even of a murine homolog of CXCL8 (IL-8), mean findings from mouse models often do not translate directly to humans (Mestas and Hughes, 2004; Holmes, Solari and Holgate, 2011). This underlines the importance of studying samples from patients with known NTHi infections. These studies would require bronchoscopies in infected individuals, ideally performed before and after an intervention such as long-term treatment with an antibiotic such as azithromycin. Performing transcriptomic analysis such as RNA sequencing on epithelial and immune cells from these patients could yield valuable insights into the immune activation induced by NTHi infection. Flow cytometric analysis of these cells to evaluate proportions of epithelial, macrophage and neutrophil populations, followed by RNA sequencing of individual populations would allow us to tease apart the activation of each cell type in response to NTHi. Proinflammatory cytokines, particularly those related to neutrophil recruitment, are likely to be elevated in patients infected with NTHi. Results from my ALI experiments suggest this may be largely in macrophage populations, as epithelial cells at ALI have low levels of cytokine production, although I postulate that the crosstalk between cell types may result in synergistic activation of proinflammatory signalling. Dual RNA-sequencing to evaluate the changes in bacterial gene expression in response to cytokine production and increased immune cell recruitment would provide insights into bacterial modifications that contribute to persistence.

1.1 Proposed future work

Further studies are necessary to expand these findings and to explore the consequences of my key results. One of my next steps would be to perform further transcriptomics studies using primary cells of a lower passage number to ensure they have not been exhausted, and infect them with NTHi for a longer period of time. I postulate that PBECs at ALI may respond more slowly to NTHi stimulation than those in submerged cultures, so infecting ALI cultures with NTHi for 48 or 72 hours in the presence of extracellular bacteria may induce more robust cytokine signalling than infection for 24 hours without extracellular NTHi. Alternatively, a lack of strong response to NTHi in PBECs at ALI would confirm the current data set which suggests that PBECs may not produce a robust cytokine response to NTHi, potentially as a consequence of various immune evasion strategies employed by NTHi which allow it to survive in the human airways.

The inclusion of NECs, which were not part of the original RNA sequencing data set, would also be of interest, as it would allow expansion of the data from the Fluidigm PCR array experiment. I postulate that, like the results of the Fluidigm experiment, RNA sequencing of NECs will result in more robust differential gene expression of TLRs and NOD-related genes, whilst NLR-associated inflammasomes are upregulated in both tissues. *In vitro* results in submerged cultures suggested that NTHi is more rapidly cleared from upper airway epithelial cells compared to those from the lower airways. This may suggest that proinflammatory signalling in NECs may be resolved earlier, whilst that in PBECs may increase over a more sustained time course. Earlier clearance of NTHi and faster resolution of inflammation in NECs compared with PBECs may be one reason NTHi is a commensal in the nasopharynx but a pro-inflammatory pathogen in the lower airways.

In addition to these *in vitro* ALI experiments, which are limited in their scope as they do not include immune cells, RNA sequencing on immune and epithelial cells isolated from asthma patients with known NTHi infections would be of interest, as I have mentioned above. As cross-sectional studies are by nature only correlative, and therefore unable to distinguish cause and effect, well designed studies would involve

experimental manipulation of the system, such as studies performed before and after therapeutic interventions.

The lack of an adequate *in vivo* model which can recapitulate persistent airways infection with NTHi remains an important challenge for this field of research. As my data point away from the epithelium as the primary site of persistent infection, it is likely that obtaining persistent infection within the macrophage will prove critical in developing such a model, and that the differences between the behaviour of NTHi in mouse and humans is related to differences between the phagocytic or intracellular cytolytic capacity of macrophages. Therefore, an important first step in the rationale design or selection of an *in vivo* murine model would be the comparison of human and murine macrophages in their interactions *in vitro* with NTHi. This could initially be done by directly comparing phagocytosis assays and intracellular killing assays in murine bone marrow derived macrophages with human mononuclear derived macrophages. Positive findings would then be investigated in murine and human alveolar macrophages. The potential would be to identify a murine strain with deficiency of a specific macrophage function, which could then be used with a mouse-adapted strain of NTHi.

Additional investigation of the intracellular lifestyle of NTHi would also be of interest. There is a lack of consensus on whether NTHi is trafficked from early to late endosomal vesicles and then killed during lysosomal acidification, or whether some NTHi strains are able to subvert this process, although there is evidence for both (Morey *et al.*, 2011; Clementi, Håkansson and Murphy, 2014; Ikeda *et al.*, 2015). My data have shown that NTHi is able to be trafficked from the early to late endosome. However, further time course experiments to evaluate whether NTHi is trafficked to and killed in the lysosome, or whether it is able to subvert this process, would be beneficial. There are some technical challenges in evaluating the presence of viable bacteria within the lysosome, as it is impossible to determine whether the NTHi were not trafficked to the lysosome, or if they have been killed. However, staining of NTHi which does not depend on viability in addition to immunofluorescence imaging to monitor phagolysosome fusion and acidification could provide some of these answers.

1.2 Conclusions

The most important clinical implications from these experiments are that NTHi is capable of internalisation into the epithelial monolayer, despite its inability to persist subsequently within the epithelium in the absence of other immune cells. This may partly explain how this otherwise relatively-innocuous pathogen is able to establish a persistent niche within the human airways. Nonetheless, the short-term nature of the persistence I have observed *in vitro* implies that other factors too may be at play to drive persistent NTHi infection, such as defective phagocytosis, subversion of neutrophil extracellular traps, or evasion of intracellular killing. It is likely that similar internalisation may also occur in professional phagocytes, such as neutrophils or macrophages. Given the ability of these cells to kill phagocytosed bacteria, it would be expected that NTHi would then deploy additional immune evasion strategies, such as inhibition of phagolysosome fusion or acidification as employed by mycobacteria (Craig *et al.*, 2001; Jamwal *et al.*, 2016; Zhai *et al.*, 2019). There is evidence both that alveolar macrophages in patients with airways diseases have defective phagocytosis of NTHi, and that some virulent strains of NTHi are able to subvert killing within macrophages (Craig *et al.*, 2001; Ackland *et al.*, 2019, 2020). There is also evidence that NTHi is capable of sheltering in NETs to escape clearance by both other neutrophils and other immune mechanisms, as well as protecting it from antibiotic clearance (Juneau *et al.*, 2011; Langereis and Hermans, 2013; Tikhomirova and Kidd, 2013; Magana *et al.*, 2018). Taken together, this evidence suggests that NTHi is skilled at exploiting host mechanisms to establish a persistent niche.

Once that niche has been established, NTHi is capable of driving an inflammatory response, and therefore in individuals with persistent bacterial colonisation of the lower airways, is likely to be contributing substantially to inflammatory signalling and type-2 low airway inflammation associated with asthma. These findings are in line with the identification of NTHi infection as a strong predictor of the success of long-term azithromycin treatment in reducing the frequency of exacerbations in patients with severe asthma (Taylor *et al.*, 2020). This elimination of NTHi was also associated with reduced concentrations of sputum IL-6 and IL-1β (Shukla *et al.*, 2021). One of the differences between submerged and ALI cultures is the development of tight

junctions in differentiated culture, suggesting that the reduction in inflammatory signalling in ALI cultures in response to NTHi may be due to enhanced barrier function. Patients with asthma and COPD often have defective barrier function, which may make their lungs more permissive to NTHi infection (Gon and Hashimoto, 2018). In addition, exacerbations following viral infections may be due to barrier function damage caused by the virus allowing NTHi to establish infection in the already compromised epithelium, thus triggering further inflammation.

NTHi is a potent driver of inflammation in the airways, and a clinically-important potential treatable trait in patients with severe neutrophilic asthma, suggesting that long-term antibiotic treatment to eliminate persistent NTHi infection may be an important component in the arsenal of therapies used to treat severe steroid-refractory asthma. Long-term azithromycin treatment is a mainstay of long-term antibiotic therapy, and has shown efficacy in the treatment of persistent infection in patients with COPD, asthma and bronchiectasis (Calverley *et al.*, 2011; Serisier, 2013; Gibson *et al.*, 2017). However, azithromycin is not appropriate for all patients. Some people treated with azithromycin experience side-effects such as diarrhoea, tinnitus, allergic reactions, drug interactions and QTc prolongation (Hancox *et al.*, 2013; McMullan and Mostaghim, 2015). In addition, there are concerns surrounding the long-term use of antibiotics, with reports of elevated rates of antibiotic resistance in common respiratory pathogens such as *Moraxella* and *Pseudomonas* in patients treated with long-term azithromycin therapy (Taylor *et al.*, 2019). Potential alternative treatments may include other macrolides, such as erythromycin, alternative classes of antibiotics which target intracellular bacteria – although positive clinical trial data are currently lacking – or therapeutics focussed on reducing recruitment of neutrophils and eliminating the reservoir of NTHi present in NETs, or able to reduce the activation of neutrophil serine proteases, such as Brensocatib, an orally acting dipeptidyl peptidase 1 inhibitor (Chalmers *et al.*, 2020). However, more research is needed to identify effective treatments which are better tolerated by patients and which are less susceptible to rising antibiotic resistance levels. In addition, several questions about how NTHi interacts with the host still remain. One of these is determining the precise role the epithelium plays in the inflammatory milieu, with my data suggesting that

immune cells are necessary for inflammatory signalling in the epithelium. Another is resolving the differences in immune induction in the epithelial cells in the nasopharynx compared with the lower airways, given the unexpected evidence that induction of TLR and NOD signalling is more strongly induced in the nasopharynx in response to NTHi. Finally, the interplay between epithelial and immune cells in response to NTHi infection remains poorly understood and may yield valuable insights into the clinical implications of infection. In all, given the significant unmet clinical need associated with NTHi infections and with these questions remaining unanswered, the role of NTHi as a driver of airway inflammation remains an exciting area for research.

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