

Ackland Jodie (Orcid ID: 0000-0003-3120-3620)
Barber Clair (Orcid ID: 0000-0001-5335-5129)
Azim Adnan (Orcid ID: 0000-0001-5960-1159)

NTHi infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma

Jodie Ackland^a, Clair Barber^{a,c}, Ashley Heinson^a, Adnan Azim^{a,c}, David W. Cleary^{a,c}, Myron Christodoulides^a, Ramesh J. Kurukulaaratchy^{a,c}, Peter Howarth^{a,c}, Tom M.A. Wilkinson^{a,b,c}, Karl J. Staples^{a,b,c}# On behalf of the WATCH study investigators.

^aClinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, UK

^bWessex Investigational Sciences Hub, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, UK

^cNIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton, UK

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#Address correspondence to Karl J. Staples, K.Staples@soton.ac.uk

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Abstract

Background: Nontypeable *Haemophilus influenzae* (NTHi) is a respiratory tract pathobiont that chronically colonises the airways of asthma patients and is associated with severe, neutrophilic disease phenotypes. The mechanism of NTHi airway persistence is not well understood, but accumulating evidence suggests NTHi can persist within host airway immune cells such as macrophages. We hypothesised that NTHi infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma.

Methods: Bronchoalveolar lavage (BAL) samples from 25 severe asthma patients were assessed by fluorescence *in situ* hybridisation to quantify NTHi presence. Weighted gene correlation network analysis (WGCNA) was performed on RNASeq data from NTHi-infected monocyte-derived macrophages to identify transcriptomic networks associated with NTHi infection.

Results: NTHi was detected in 56% of BAL samples (NTHi+) and was associated with longer asthma duration (34 vs 22.5 years, $p=0.0436$) and higher sputum neutrophil proportion (67% vs 25%, $p=0.0462$). WGCNA identified a transcriptomic network of immune-related macrophage genes significantly associated with NTHi infection, including upregulation of T17 inflammatory mediators and neutrophil chemoattractants *IL1B*, *IL8*, *IL23* and *CCL20* (all $p<0.05$). Macrophage network genes *SGPP2* ($p=0.0221$), *IL1B* ($p=0.0014$) and *GBP1* ($p=0.0477$) were more highly expressed in NTHi+ BAL and moderately correlated with asthma duration (*IL1B*; $\rho=0.41$, $p=0.041$) and lower pre-bronchodilator FEV1/FVC% (*GBP1*; $\rho=-0.43$, $p=0.046$ and *IL1B*; $\rho=-0.42$, $p=0.055$).

Conclusions: NTHi persistence with pulmonary macrophages may contribute to chronic airway inflammation and T17 responses in severe asthma, which can lead to decreased lung function and reduced steroid-responsiveness. Identifying therapeutic strategies to reduce the burden of NTHi in asthma could improve patient outcomes.

Introduction

Asthma is a complex, heterogeneous disease of the airways characterised by episodic and reversible airway obstruction, hyper-responsiveness and inflammation^{1,2}. Asthma has long been characterised by an excessive type 2 (T2) inflammatory response and eosinophilic infiltration, mediated by IL-4, IL-5 and IL-13³⁻⁵. As such, asthma is broadly classified into T2-high and T2-low endotypes based on the presence or absence of eosinophils and associated T2 markers.

Due to the heterogeneous nature of asthma, novel approaches to inflammatory endotyping based on sputum inflammatory profiles including eosinophilic, neutrophilic, mixed granulocytic and paucigranulocytic inflammatory phenotypes have been developed^{6,7}. Advances in stratification methodologies utilising biomarkers to develop sophisticated classification algorithms, as well as increased availability and analysis of longitudinal clinical data and patient history has demonstrated the predominance of eosinophilia in severe asthma^{8,9}. As such, current asthma therapies including corticosteroids and monoclonal antibodies aim to reduce eosinophilic and T2 inflammation, with eosinophil levels determined to be a clinical marker of steroid-responsiveness^{10,11}.

However, certain subsets of severe asthma patients do not respond well to conventional steroid treatment¹². As individuals with severe asthma account for 50-80% of the total health care burden of asthma, further studies aimed at uncovering the mechanisms underlying airway inflammation are needed to better understand the multifactorial nature of asthma¹³. The neutrophilic asthma phenotype accounts for up to 20% of adult asthma cases and is associated with increased steroid-resistance, airflow obstruction, risk of hospitalisation and lung microbial dysbiosis^{6,14-21}. In particular, increased abundance of potentially pathogenic Proteobacteria, such as nontypeable *Haemophilus influenzae* (NTHi) is associated with more severe, neutrophilic phenotypes of asthma²²⁻²⁴. NTHi airway colonisation in chronic respiratory disease is suggested to vary from days to years²⁵⁻²⁸ and is detectable during both stable and exacerbated periods of asthma^{22,29,30}.

Despite an increasing number of studies indicating associations between chronic NTHi airway colonisation and asthma, it is not clear how NTHi persists in the airway and whether this persistence modulates the airway immune response. Although NTHi was previously regarded as an extracellular pathogen, accumulating evidence suggests that NTHi can invade and persist within host immune cells to facilitate airway survival³¹. Numerous *in vitro* studies have demonstrated the ability of NTHi to invade and persist within macrophages^{32–35} with *in vivo* evidence of NTHi intracellular invasion within macrophage-like cells from adenoid tissue also apparent^{36,37}. Despite this, the ability of NTHi to persist within macrophages in the asthmatic airway is less clear.

Although fundamental to the airway immune response, macrophages have an altered phenotype in asthma³⁸ and are functionally impaired, which worsens with disease severity³⁹. A role for macrophages in shaping the T17 response in asthmatics and promoting neutrophilic inflammation has also been suggested, with increased presence of IL-17+ macrophages detected in the bronchoalveolar lavage (BAL) of asthmatic patients⁴⁰. Furthermore, we have recently shown that NTHi-infected macrophages upregulated genes involved in the IL-17 pathway and increased release of neutrophil-related mediators including IL-8 and IL-17C⁴¹. It is unclear whether NTHi presence in the airway modulates macrophage activity and subsequently contributes to the complex chronic inflammatory environment characteristic of severe asthma.

We hypothesised that NTHi infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma. As such, the aim of our study was to investigate NTHi-macrophage interactions in severe asthma. We first assessed the presence of NTHi in severe asthma BAL samples. We then utilised RNASeq data (GSE180166) from an *in vitro* NTHi-monocyte-derived macrophage (MDM) intracellular persistence model to investigate macrophage transcriptomic networks and identify the central NTHi-response genes. Finally, we determined whether NTHi-modulation of these identified genes were detectable in severe asthma and correlated with patient clinical characteristics and airway inflammation.

Results

NTHi association with airway macrophages can be detected in severe asthma

To first assess bacterial presence in severe asthma, fluorescence *in situ* hybridisation (FISH) was performed on BAL cytopins generated from 25 severe asthma patients undergoing bronchoscopy as part of the WATCH study⁴². BAL cytopin slides were stained with DAPI (Figure 1A), a universal bacterial (EUB338A) FITC probe (Figure 1B) and a NTHi-specific bacterial 16s rRNA (HAIN16S1251) CY3 probe (Figure 1C), to visualise the presence of host cells, bacteria and NTHi, respectively. The NTHi-specific CY3 and pan-bacteria FITC signal overlapped, indicating NTHi presence only (Figure 1D). Furthermore, overlaying the individual host and NTHi fluorescent signals demonstrated that the NTHi signal closely associated with host BAL cell single-lobed nuclei (Figure 1E). Differential cell counting determined that macrophages were the predominant BAL cell type (median 91.6%, Figure S1), indicating NTHi association with pulmonary macrophages. In total, 56% of patient BAL samples (14/25) demonstrated a detectable NTHi-specific CY3 signal (herein referred to as NTHi+, Figure 1F).

To determine the impact of NTHi presence on airway inflammation and clinical characteristics, NTHi+ and NTHi- patient groups were compared (Table 1 & S1). NTHi+ individuals exhibited increased sputum neutrophil proportion ($p=0.0462$) which corresponded to a decrease in macrophage sputum proportion ($p=0.0221$). Stratification of patients into neutrophilic or non-neutrophilic sputum inflammatory phenotypes found a higher number (12/14; 86%) of NTHi+ individuals were neutrophilic, compared to only 2/14 (14%) NTHi- individuals (Figure S2). However, this difference did not reach statistical significance ($p=0.081$), which could be due to the overall neutrophilic nature of this cohort (17/25, 68%; $p=0.0227$), with 5/11 NTHi- patients also categorised as neutrophilic (45.5%). NTHi presence was also associated with longer asthma duration (34 years), compared to NTHi- individuals (22.5 years, $p=0.0436$).

WGCNA identifies modules significantly correlated to the macrophage response to NTHi

As NTHi presence in BAL was visualised to be associated with pulmonary macrophages, we next sought to determine the impact of NTHi presence on macrophage gene expression. We have previously modelled intracellular NTHi within human MDM and identified numerous macrophage differentially expressed genes (DEGs) using RNASeq⁴¹. However, this previous work did not consider the importance of gene co-expression or identify which hub genes are the crucial components within a transcriptomic network. In this current study, we further interrogated the RNASeq data (GSE180166) using WGCNA⁴³ to identify the key biological networks and macrophage hub genes which could then be compared between the NTHi+ and NTHi- groups within our severe asthma cohort by qPCR.

Network topology analysis assigned 15048 MDM genes to 15 modules (Figure 2A&B), with 10 modules significantly correlated with NTHi infection (Figure 2C, all $p < 0.05$). To determine the significance between the genes assigned to each module and NTHi infection, correlation of module membership (MM) and gene significance (GS) was performed. High correlation between GS and MM indicates that genes which are significantly correlated with the trait of interest (GS) are also important components of the module (MM). The blue module showed the strongest correlation, indicating that genes within this module are both significantly associated with the infection trait and are important components of the blue module (Figure S3A; 0.93, $p < 0.001$).

Clustering of modules with the infection trait further emphasised the significant relationship of the blue module with NTHi infection (Figure 2D). Four distinct clusters of modules were visible and gene list enrichment analysis identified enrichment of divergent processes for each cluster (Figure S4). Cluster I, which included the blue module and infection trait, was significantly enriched in immune processes, with significant overlap of terms shared between the blue module individually and Cluster I overall, suggesting the genes assigned to the blue module were driving the enrichment of terms in cluster I (Figure S5A&B), which were significantly

enriched in immune processes (Figure 3A&B). The expression profile of the 2333 MDM genes assigned to the blue module clustered depending on infection status (Figure S5C), indicating these genes were associated with NTHi infection. As a result, the blue module was chosen to explore further.

Identification of the most highly connected genes in the blue module gene network

To further explore the importance of the macrophage genes assigned to the blue module, a network of the 62 most highly connected macrophage genes was constructed in Cytoscape (Figure 3C). All 62 genes were more highly expressed in response to NTHi compared to uninfected controls (Figure S6A). To determine the key genes in this network, genes were ranked by the Maximal Clique Centrality (MCC) scoring method using the Cytoscape cytohubba plugin. *CASP4*, *PNRC1* and *SGPP2* were ranked as the top three genes in the network (Table S2).

Enrichment analysis of the 62 genes present in this network identified significant enrichment of Biological Processes involved in immune responses (Figure S6B). KEGG pathway analysis further confirmed enrichment of macrophage immune responses, including asthma-related inflammatory pathways 'IL-17 signalling pathway' and 'Th17 cell differentiation' (Figure S6C). To assess macrophage release of T17 mediators, levels of IL-1 β , CXCL8, IL-23 and CCL20 were measured in cell culture supernatants. All T17 mediators were significantly upregulated at both time points during NTHi infection compared to uninfected controls (Figure S7A-D, all $p < 0.05$).

We also validated the expression of the 3 top ranked *in vitro* NTHi response genes (*CASP4*, *SGPP2* and *PNRC1*) in our NTHi-MDM model by qPCR. We selected 3 other immune-related genes from the network (*IL1B*, *GBP1* and *LAMP3*) which have previously shown to be important for macrophage immune responses to intracellular pathogens⁴⁴⁻⁴⁶. Increased expression of all 6 genes was measured in response to NTHi compared to uninfected controls (Figure S8A-F, all $p < 0.05$).

Expression of macrophage genes in severe asthma samples

Next, it was important to determine whether this transcriptomic signal of NTHi modulation of macrophage immune responses could be detected *in vivo*. To do this, 25 BAL cell pellets matched to the 25 BAL cell cytopins were used to investigate expression of the selected 6 macrophage blue module network genes using qPCR (Figure 4). Whilst expression of *CASP4*, *PNRC1* and *LAMP3* did not significantly differ between groups ($p>0.05$), *SGPP2* ($p=0.0221$), *IL1B* ($p=0.0014$) and *GBP1* ($p=0.0477$) expression was higher in NTHi+ compared to NTHi- samples (Figure 4C, D & E respectively). We also assessed BAL supernatants for CXCL8 and found significantly higher levels of CXCL8 in NTHi+ samples (Figure S7E, $p=0.0217$).

Correlation of NTHi-related macrophage gene expression with clinical characteristics

Correlation analysis for all 25 patients found levels of co-expression between the 6 chosen genes in BAL (Figure 5), in line with the *in vitro* WGCNA MDM data. In particular, the two genes both more highly expressed in NTHi+ patients, *IL1B* and *GBP1*, were the most strongly positively correlated genes in BAL samples ($\rho=0.67$, $p=0.00031$). Furthermore, these two genes correlated with clinical characteristics. A weak to moderate, but significant, positive correlation between *IL1B* expression and asthma duration was identified ($\rho=0.41$, $p=0.041$), with both *IL1B* ($\rho=-0.42$; $p=0.055$) and *GBP1* ($\rho=-0.43$, $p=0.046$) negatively correlated with pre-BD FEV₁/FVC%. Therefore, NTHi modulation of macrophage gene expression could be detected in the severe asthmatic airway and importantly, was significantly associated with decreased lung function.

Discussion

Our study suggests that NTHi airway presence is facilitated by interactions with airway macrophages and is associated with sputum neutrophilic inflammation in severe asthma. We demonstrate NTHi association with BAL macrophages obtained from individuals with severe asthma during a stable period of disease. We investigated these interactions by performing WGCNA on RNASeq data from an NTHi-MDM intracellular persistence model⁴¹, which identified a transcriptomic gene network enriched in immune responses, including T17 responses. Confirmation of MDM release of T17 mediators in cell culture supernatants further demonstrated the contribution of NTHi-infected macrophages to T17 inflammation. NTHi presence and modulation of *IL1B* and *GBP1* gene expression *in vivo* correlated with clinical characteristics including increased sputum neutrophilic inflammation and lower lung function measures. These findings have important implications for understanding the drivers of chronic airway inflammation and disease progression in severe asthma.

The mechanisms underlying NTHi airway persistence remain unclear. Although NTHi has the capacity to form biofilms *in vitro*, NTHi biofilm formation in the human lung is less understood⁴⁷. We did not observe any evidence of NTHi biofilm formation in our samples, but rather saw the presence of cell-associated NTHi in 56% of individuals during a stable period of disease. A limitation of our fluorescence microscopy is that it did not allow for ascertainment of whether the BAL macrophage-associated NTHi was intracellularly or extracellularly localised within our samples. Nonetheless, our findings are corroborated by Dickson and colleagues, who found that significant components of the lung microbiota are in fact cell-associated⁴⁸. Indeed, our recent work reported the ability of multiple clinical NTHi strains to invade macrophages and persist intracellularly⁴¹.

In this current study we performed WGCNA on macrophage RNASeq data to identify biologically significant transcriptomic networks and the hub genes that were important during NTHi-macrophage interactions. *CASP4* was the top ranked gene in the blue module gene network, suggesting a role of the noncanonical inflammasome pathway in the macrophage

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response to NTHi. Elevated expression of inflammasome components in severe asthma including NLRP3, caspase-1 and caspase-4 have previously suggested a role for the inflammasome pathway in asthma⁴⁹. However, we found no difference in *CASP4* expression between NTHi+ and NTHi- patients. The lack of *CASP4* expression between patients stratified by NTHi presence may be due to the underlying inflammatory phenotype-dependent differences. Simpson and colleagues detected higher *CASP4* gene expression in neutrophilic asthmatics compared to eosinophilic and paucigranulocytic asthmatics⁴⁹. As such, *CASP4* expression may already be elevated compared to healthy individuals or other asthma inflammatory phenotypes, independently of NTHi presence.

Our results indicate an important role for NTHi-modulation of IL-1 β pathways; we found significant upregulation of *IL1B* gene expression and protein release by NTHi-infected MDM and elevated *IL1B* gene expression in NTHi+ BAL samples, with this signal absent in uninfected MDM or NTHi- BAL. Elevated BAL *IL1B* gene expression is in agreement with previously published studies identifying elevated airway IL-1 β gene and protein expression in asthma^{49–54}. Moreover, a recent sub-study of the AMAZES trial demonstrated a strong correlation between *H. influenzae* load and IL-1 β in those with non-eosinophilic asthma following add-on azithromycin, further emphasising the role of NTHi in driving airway inflammation in asthma⁵⁵.

Macrophages have recently been shown to display an altered transcriptomic profile in neutrophilic asthma⁵⁶, thus could be an alternative cell type, other than the neutrophil, to therapeutically target in neutrophilic asthma. Here, we show co-expression of *IL1B* and *GBP1* in BAL, suggesting modulation of *IL1B* by macrophage intracellular immune pathways involving GBPs, which could offer insights into how to target IL-1-mediated inflammatory pathways. As our work suggests that NTHi-infected macrophages may be the source of IL-1 β -induced inflammation in the asthmatic airway, stratifying patients based on NTHi presence may result in better therapeutic outcomes. IL-1 pathway interventions have yet to be shown to

be successful in asthma, and may only be effective in certain patient cohorts, such as those colonized with NTHi⁵⁷.

NTHi presence in our severe asthma sub-cohort was associated with increased sputum neutrophil proportion, supporting previous work demonstrating that individuals possessing a *Haemophilus*-dominant microbiome were more likely to be neutrophilic^{23,24}. It is not clear why NTHi is associated with neutrophilic asthma, however we found NTHi presence was associated with increased asthma duration. Long term exposure of mice to *H. influenzae* resulted in airway remodeling following a bacteria-induced switch from a T2, eosinophilic inflammatory phenotype to T17, neutrophilic inflammation⁵⁸. Furthermore, murine studies have demonstrated that the combination of NTHi infection and allergic airways disease promote a steroid-resistant, neutrophilic inflammatory phenotype^{59,60}.

We have previously shown NTHi-infected macrophages upregulate IL-17C release, providing further evidence of NTHi-macrophage interactions contributing to T17 responses⁴¹. In this current work, we demonstrate enrichment of immune pathways involved in T17 inflammation and confirmed MDM release of T17 mediators in response to NTHi intracellular persistence, as well as elevated CXCL8 levels in NTHi+ BAL samples. Given previous studies reporting independent associations of NTHi and macrophage contribution to T17 inflammation^{40,60,61}, our work links these observations and proposes that NTHi association with pulmonary macrophages may contribute to and propagate the development of the T17-driven neutrophilic phenotype in asthma.

Therefore, investigating the role of NTHi persistence in the airway of asthmatics prior to severe disease development could elucidate the exact role of NTHi interactions with macrophages on asthma progression. Although the main aim of our study was to specifically investigate NTHi modulation of the severe asthmatic airway, we acknowledge the small cohort size and severe nature of asthma experienced by our cohort is a limitation. Furthermore, WGCNA identified clusters of genes which were significantly associated with NTHi infection using an *in vitro* MDM NTHi persistence model, which demonstrated significant enrichment of innate

immune responses. However, the gene signature from this analysis may be too acute to be recapitulated in the chronically colonised airway, with NTHi known to persist from days to years^{25–28}. Thus, future transcriptomic work using a larger cohort of BAL samples longitudinally obtained from both health and asthma will better allow for determining global transcriptomic alterations associated with NTHi persistence *in vivo*. Such studies will also allow determination of whether potential confounding factors, such as steroid usage or antibiotic treatment, impact the expression of the genes identified in this current analysis. A further potential confounding factor is the heterogenous nature of our BAL cell population; although 91.6% of cells were macrophages, other cell types are present and may influence gene expression results. Although we only observed NTHi association with BAL macrophages within our samples, both *in vitro* and *in vivo* work has demonstrated the ability of NTHi to infect epithelial cells and induce inflammatory responses⁶², so these cells could also be an important source of airway inflammation. Use of single cell methodologies would provide further resolution of cell-specific gene expression within the asthmatic airway.

Finally, although GM-CSF differentiated MDM are commonly used and are phenotypically comparable to alveolar macrophages, including similar phagocytic capacity⁶³, cytokine production⁶⁴ and expression of cell surface markers⁶⁵, they may not fully recapitulate *in vivo* NTHi-macrophage interactions, especially given the known functional impairments of macrophages in asthma^{38,39}. Future modelling work would benefit from using macrophages isolated from both healthy and asthmatic airways, as well as using a culture system which attempts to retain the 3D lung architecture and complex cellular milieu, to allow for a better understanding of NTHi-macrophage interactions *in vivo*. Due to the complex, multifactorial nature of asthma, it is likely that additional factors contribute to disease progression, rather than a single transitional event. Unpicking the complex relationship between NTHi persistence, the host immune response and severe asthma pathogenesis over an extended period using a longitudinal cohort could reveal the opportune moment to therapeutically intervene to prevent disease progression.

In conclusion, NTHi infection of pulmonary macrophages may drive components of complex chronic airway inflammation, including inflammasome activation and T17 responses, leading to the development of a neutrophilic, severe asthma phenotype. Our work highlights a potential benefit of stratifying patients based on NTHi presence to improve treatment efficacy. Although antibiotic therapy has shown to be successful in reducing *H. influenzae* load, not all patients experienced depletion of *H. influenzae* and the treatment was associated with increased antibiotic resistance gene carriage⁶⁶. Therefore, uncovering novel, non-antibiotic therapeutic targets to reduce the burden of NTHi in asthma may alleviate chronic airway inflammation, prevent the progression to more severe disease and improve patient outcomes.

Materials and Methods

WATCH patient recruitment

Bronchoalveolar lavage (BAL) samples were obtained from a sub-cohort of patients undergoing bronchoscopy as part of the Wessex Asthma Cohort of Difficult Asthma (WATCH) study in accordance with the protocol approved by the West Midlands – Solihull Research Ethics Committee (REC:14/WM/1226), as per Azim *et al*⁴². All patients included did not have a clinical diagnosis of COPD. All subjects who provided samples gave informed consent. Further information about lung sample processing for generation of cytopsin slides and RNA extraction is available in the supplementary material.

Fluorescence *in situ* hybridisation (FISH)

FISH was performed on stored BAL cytopsin slides as previously described⁶⁷, using a NTHi specific Cy3-labelled probe (HAIN16S1251) and a pan-bacteria FITC-labelled probe (EUB338A). Slides were visualised using a Zeiss Axioskop2 Mot fluorescence microscope using x 40 magnification immersion oil objective. Images were acquired using Micro-Manager and analysed in ImageJ/FIJI (National Institutes of Health, USA, version 1.53c). Further details are supplied in the supplementary material.

MDM-NTHi intracellular infection model

MDM culture and infection was performed as previously described⁴¹. Briefly, monocytes were isolated from the blood of healthy volunteers (in accordance with Hampshire A Research Ethics Committee, 13/SC/0416) by magnetic associated cell sorting (MACS) and differentiated into monocyte-derived macrophages (MDM) in the presence of 2 ng/mL GM-CSF over 12 days as previously described⁶⁸. NTHi ST14 was grown and stored in aliquots at -80°C until required in accordance with the protocol described by Kirkham and colleagues⁶⁹. NTHi infection of MDM was performed at multiplicity of infection (MOI) 100 for 6 h in antibiotic-free RPMI media. After 6 h, MDM were incubated in RPMI media supplemented with 500 µg/ml gentamicin for

90 min to remove and kill extracellular NTHi. Gentamicin-containing media was removed and cells incubated in antibiotic-free media until 24 h.

Weighted gene correlation network analysis (WGCNA)

We utilised RNA Sequencing data from an NTHi-MDM intracellular persistence model previously reported by Ackland et al.⁴¹ and deposited at <http://www.ncbi.nlm.nih.gov/geo/> under GEO accession number GSE180166. Normalised and transformed data was analysed using the WGCNA R package⁴³. Further information is available in the supplementary methods.

RNA isolation and qPCR

RNA was extracted from BAL cell pellets or MDM and reverse transcribed to cDNA as previously described⁴¹. Expression of macrophage genes was assessed by quantitative PCR (qPCR) using TaqMan universal PCR master mix (Applied Biosystems), with all primers obtained from Applied Biosystems (Table S3). The qPCR reactions were performed at 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min using a 7900HT Fast Real-Time PCR System. The expression of target genes was normalised to *B2M* expression and expressed as $\Delta\Delta C_t$.

Mediator release assays (ELISA and Luminex)

IL-1 β and CXCL8 release into cell culture or BAL supernatants was assessed by DuoSet ELISA kits, which was carried out according to manufacturer's instructions (R&D Systems). IL-23 and CCL20 release into cell culture supernatants was assessed by a customised Luminex Human Magnetic Assay according to manufacturer's instructions (R&D Systems). Further information is supplied in the supplementary methods.

Statistics

Statistical analysis was performed using GraphPad Prism (version 8 GraphPad Software, San Diego, USA) and statistical significance was determined as $p < 0.05$. Wilcoxon signed rank test

between paired data or Mann Whitney U test between unpaired data was performed. Correlation analyses were performed using Spearman rank correlation coefficient. Fisher's exact test was used for analysis of categorical data.

Conflicts of interest

DC reports that he was a post-doctoral researcher on projects funded by Pfizer and GSK between April 2014 and October 2017. RJK received a non-promotional grant (£35,000) from Novartis to assist funding initial database support for the WATCH study. PH reports that he is an employee of GSK. TW reports grants and personal fees from AstraZeneca, personal fees and other from MMH, grants and personal fees from GSK, personal fees from BI, and grants and personal fees from Synairgen, outside the submitted work. KS reports grants from AstraZeneca, outside the submitted work. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

JA contributed to study design, data collection, analysis and drafted the initial manuscript. CB, AA, RJK contributed to study design, undertook longitudinal data collection and contributed to manuscript preparation. AH contributed to data analysis and manuscript preparation. DWC, MC, PH and TMAW contributed to study design, and manuscript preparation. KJS contributed to study design, drafted the initial manuscript and acts as guarantor for the paper.

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Table

Table 1. Comparison of demographics and clinical characteristics between NTHi+ and NTHi- samples.

	Total (n=25)	NTHi + (n=14)	NTHi - (n=11)	p-value
General demographics				
Age (yr)	56 [47 – 66.5]	59 [54.25 – 67.25]	50 [41 – 68]	0.2167
Gender (M/F)	15/10	10/4	5/6	0.240
Smoking status (Ex/Never)	11/14	7/7	4/7	0.6887
Pack years	0 [0 – 4.9]	0 [0 – 6.625]	0 [0 – 2]	0.6805
Asthma-related characteristics				
Asthma duration	21 [9.5 – 42.5]	34 [18.5 – 47.75]	22.5 [8 – 27]	0.0436
ACQ Score	2.3 [1.5 – 2.9]	2.25 [1.3 – 3.1]	2.3 [1.5 – 2.8]	0.6959
Exacerbation (last 12 months)	1 [1-2.5]	1 [1 – 1.5]	2[0 – 4]	0.4918
Pulmonary function				
BD FEV ₁ % [#]	83.5% [71% - 92.25%]	81.5 [47 – 90.75]	83.5 [90 – 93.5]	0.3207
Pre BD FEV ₁ /FVC % [#]	69.5% [64.42% - 76.01%]	67.2 [54.9 – 75.3]	73.68 [67.41 – 76.88]	0.1802
Asthma management				
Inhaled corticosteroids	18/25 (72%)	10/14 (71.4%)	8/11 (72.7%)	>0.99
BDP equivalent dose(µg)	2000 [2000-2000]	2000 [1000 - 2000]	2000 [2000 – 2000]	0.2279
Maintenance oral steroids	6/25 (24%)	4/14 (29%)	2/11 (18%)	0.6609
Long term antibiotics	4/25 (13%)	2/14 (14%)	2/11 (18%)	>0.99
Sputum cell count				
Macrophage (%)	35.6 [16.6 – 53.5]	19.1 [12.9 – 46.4]	51.3 [51.3 – 63.6]	0.022
Neutrophil (%)	57 [24.8 – 73.7]	67 [49.2 – 80.7]	24.9 [16.3 – 62]	0.046
Eosinophil (%)	0.8 [0.2 – 5.4]	0.9 [0.3 – 3.2]	0.8 [0.1 – 9.6]	0.43
Lymphocyte (%)	0 [0 - 0]	0 [0 - 0.1]	0 [0 - 0]	0.08

The 25 severe asthma patients were split based on the outcome of the FISH analysis into NTHi+ (14) and NTHi- (11) groups. Values reported are medians [IQR] or (%). # indicates missing patient data; pre-bronchodilator spirometry measures and asthma duration history was not available for 2 patients. Statistical analysis was performed between NTHi+ and NTHi- groups. Continuous data were analysed by Mann-Whitney U test, categorical data were analysed by Fisher's exact test. **Bold** indicates p-values determined as statistically significant ($p < 0.05$). ACQ = asthma control questionnaire, BD = bronchodilator, BDP = beclometasone dipropionate, BMI = body mass index, FEV₁ = forced expiratory volume in one second, FVC = forced vital capacity.

Figure legends

Figure 1. Detection of NTHi colonisation of asthmatic BAL cells by FISH. Images show representative cytopsin slide of severe asthma BAL cells colonised with NTHi. Slides were stained with **(A)** DAPI, **(B)** a pan-bacteria FITC probe (EUB338A) and **(C)** an NTHi-specific CY3 probe (HAIN16S1251). **(D)** Merged colour composite of all three fluorescent channels. **(E)** Enlarged subfield view of **(D)**, indicated by the white arrow. Images were acquired using a x 40 magnification immersion oil objective. Scale bar shows 10 μm . **(F)** Quantification of NTHi-colonisation of asthmatic BAL cells for all 25 patients within the study cohort. The total DAPI (host cell nucleus) and CY3 (NTHi) signal detected in 25 asthma BAL cytopsin slides was quantified in Image J. Each individual dot indicates the total DAPI count and CY3 count for each patient cytopsin. **(G)** The percentage of host cells colonised with NTHi per field of view for each cytopsin slide was determined by the number of CY3 counts over the DAPI count for each individual patient. The median percentage of colonised cells per field of view was 24.2%. Only samples that returned a CY3 count >1 , indicating NTHi presence were included. Line on graphs indicates median.

Figure 2. Construction of macrophage transcriptomic gene network using WGCNA identifies significant modules associated with NTHi infection. **(A)** Clustering dendrogram of all 15048 genes with dissimilarity based on topological overlap with assigned module colours below. The grey module was categorised as the 'bin' module containing genes not assigned to any module. **(B)** Barplot depicting the number of genes assigned to each module. **(C)** Association between modules and sample trait data. Each row corresponds to a module eigengene and each column to a trait. Each cell contains the correlation score (top) and p-value (bottom) assigned to each relationship. **(D)** Module membership (MM) and gene significance (GS) correlation scores for infection. The correlation score for each module is present above each bar and modules are ordered based on significance (most significant to least significant, left to right) for the correlation (all $p < 0.05$). **(E)** Visualisation of the eigengene network as a dendrogram and heatmap showing the relationships between individual modules

and the NTHi infection. Four distinct clusters of modules were visible in the dendrogram; cluster I (yellow, blue, pink and salmon), cluster II (turquoise), cluster III (magenta, brown, green and red) and cluster IV (tan, cyan, greenyellow, black and purple). Each row and column in the heatmap corresponds to one module eigengene (labelled by colour) or NTHi infection. In the heatmap, purple colour represents low adjacency, while orange represents high adjacency.

Figure 3. Blue module gene expression profile and gene ontology analysis indicates enrichment of macrophage immune processes in response to intracellular NTHi. (A)

Enrichment of biological processes indicates activation of MDM immune responses in response to NTHi, with **(B)** KEGG pathway analysis indicating enrichment of immune responses involved in the response to an intracellular pathogen. Pathway/category IDs are ordered by enrichment significance (FDR) which is indicated by the colour of each dot, with the size of the dot representing the number of genes assigned to each pathway/category. **(C)**

To identify the top connected genes within this blue module, visualisation of the blue module network was generated in Cytoscape using a topological overlap threshold of 0.34. Gene ranking performed by Maximal Clique Centrality (MCC) using the cytoHubba plugin in Cytoscape. Nodes are coloured blue, with darker shades indicating high network importance, lighter shades indicate decreasing network importance and solid lines show connecting edges/interactions between nodes

Figure 4. Expression of macrophage genes was detectable in BAL from severe asthma

The expression of six MDM genes identified in the blue module network deemed to be significantly associated with NTHi infection were investigated in BAL samples by qPCR. Patients were split into NTHi+ or NTHi- groups based on the outcome of the FISH analysis identifying NTHi presence in matched BAL cell cytopins. The top three ranked genes, **(A)** *CASP4*, **(B)** *PNRC1* and **(C)** *SGPP2* and three other genes present in the blue module network

and associated with an intracellular immune response **(D)** *IL1B*, **(E)** *GBP1* and **(F)** *LAMP3* were measured. Gene expression was normalised to *B2M*. Graphs show medians, n=25. Data were analysed by Mann Whitney U test, *p<0.05, **p<0.01.

Figure 5. Correlation of expression of genes from the blue module network with asthma clinical characteristics in severe asthma BAL samples.

Spearman's correlation was used to determine correlations between the level of gene expression and patient characteristics or demographics. Larger sized dots indicate more significant correlations, with the correlation coefficient (ρ) represented by the coloured bar (right), with blue representing positive correlations and red representing negative correlations.

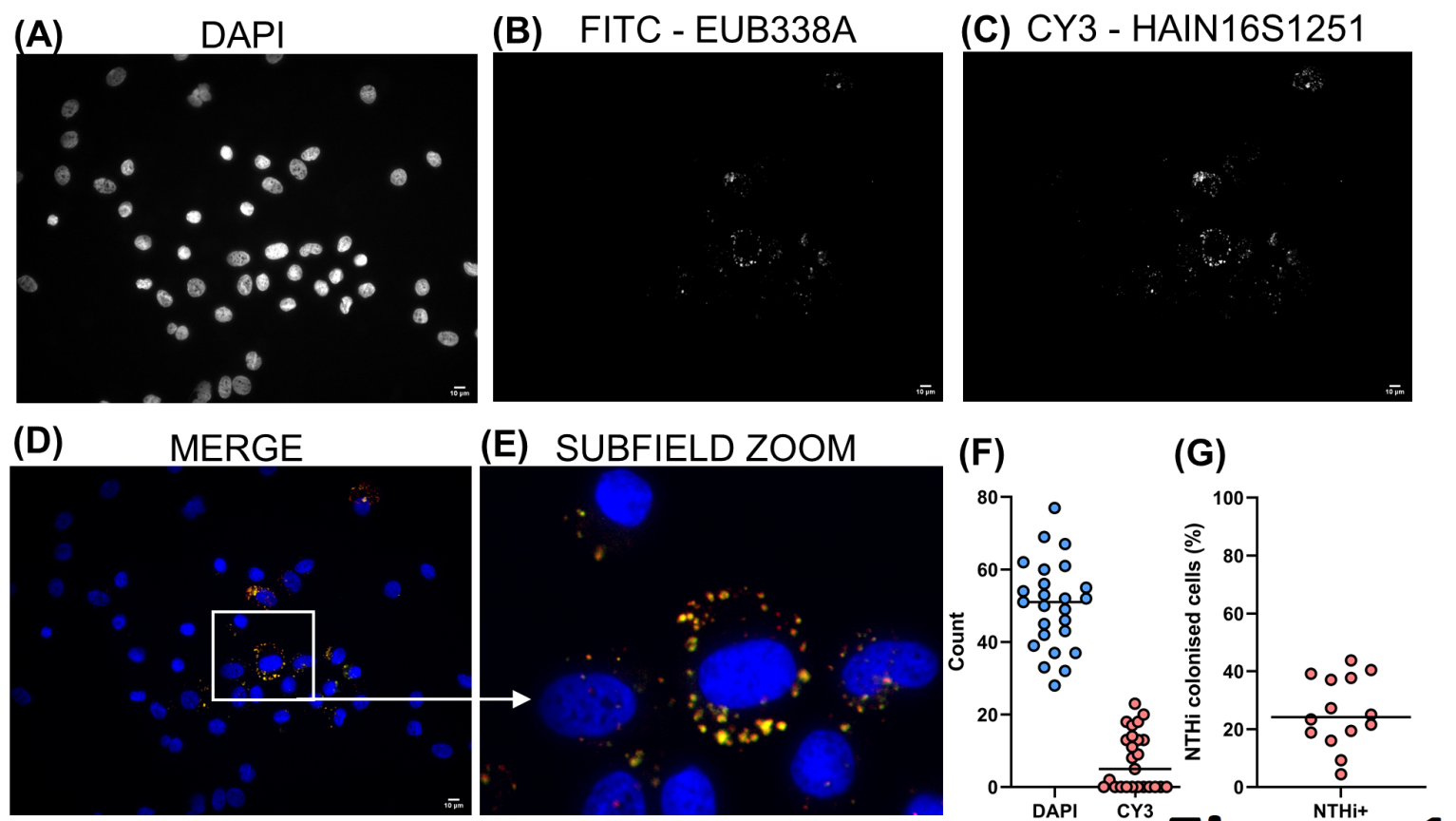


Figure 1

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Cluster Dendrogram

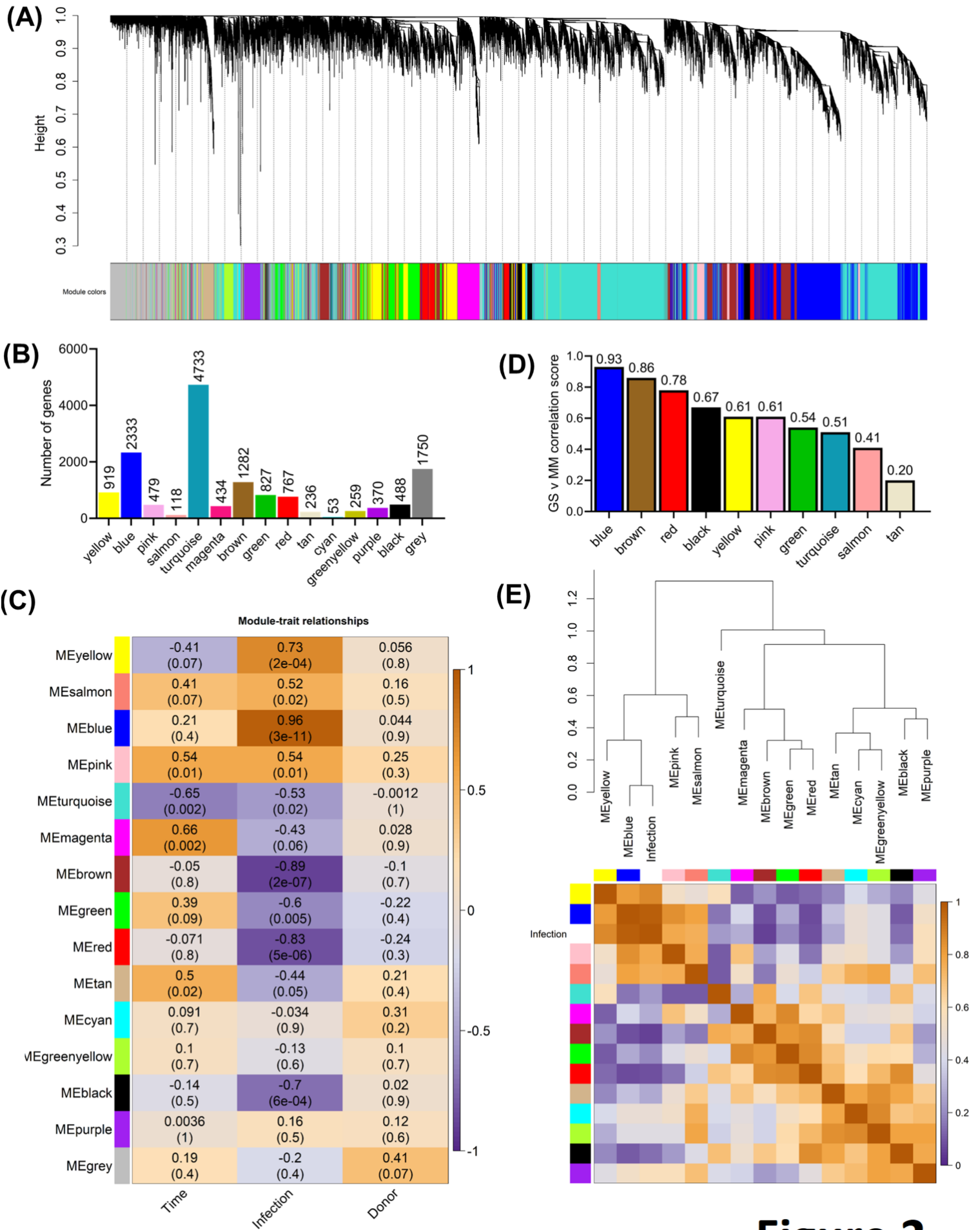
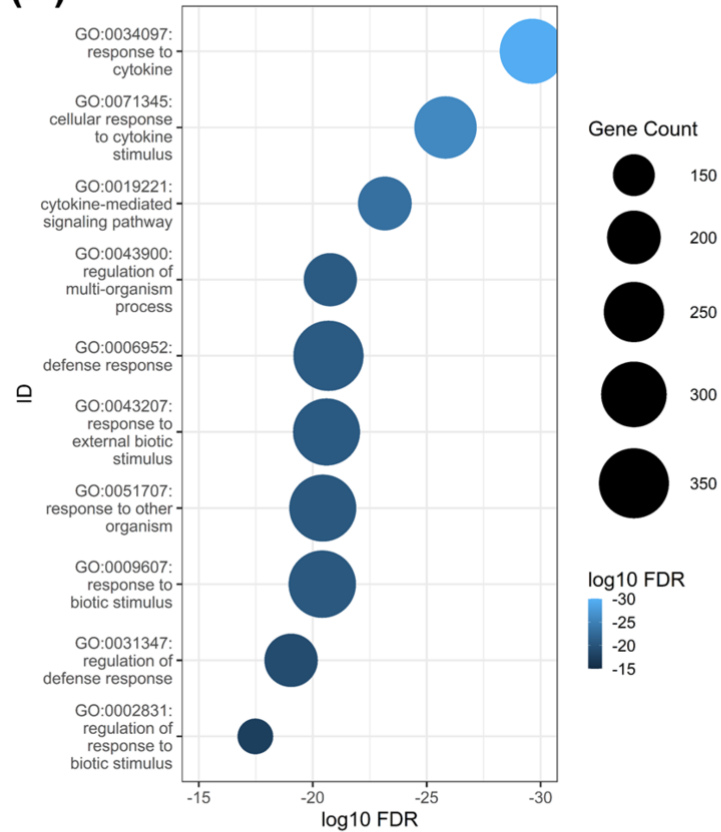
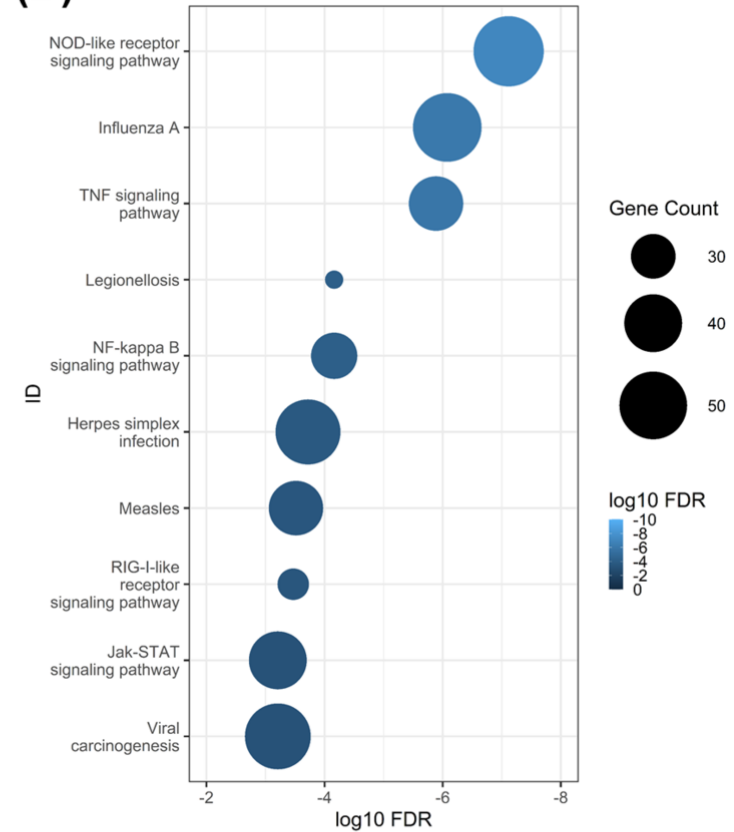


Figure 2

(A) Top 10 Biological Processes Blue module



(B) Top 10 KEGG Blue module



(C)

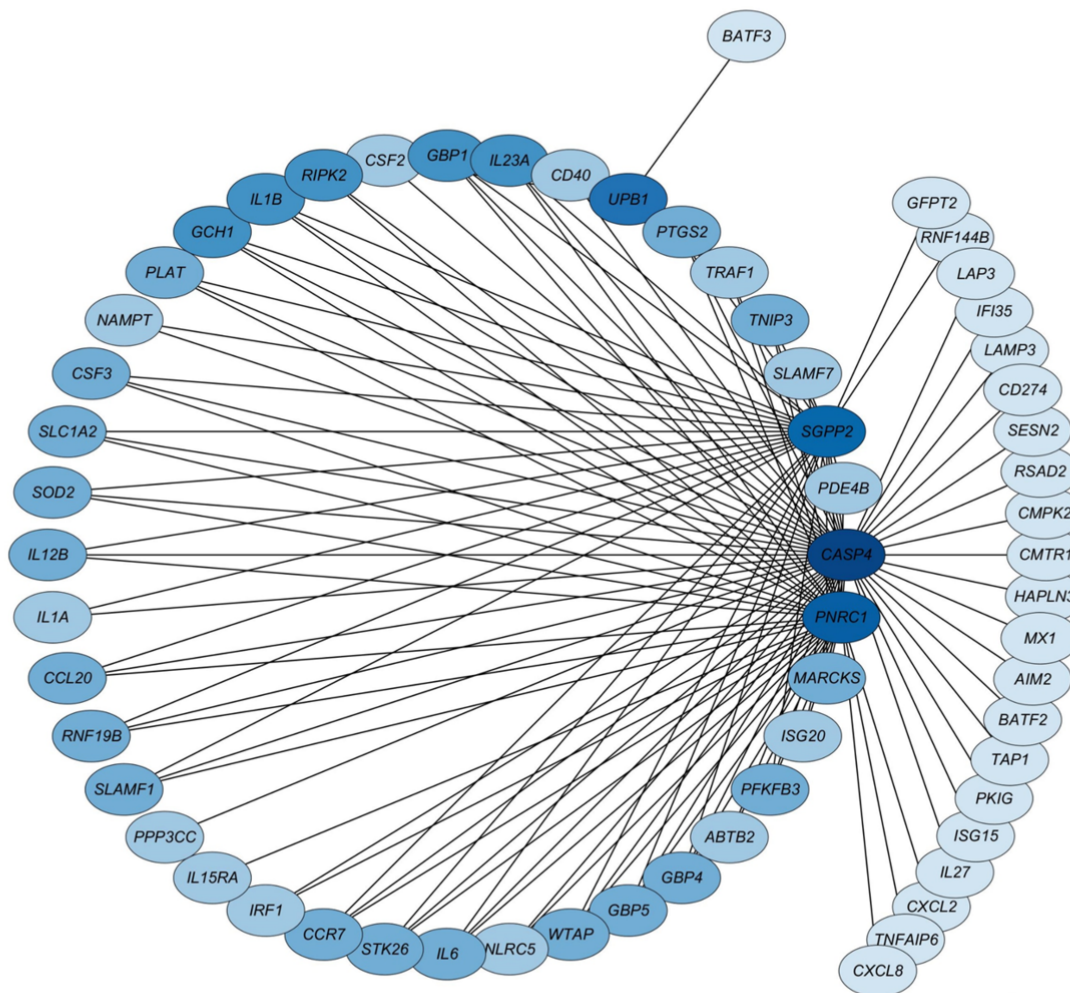


Figure 3

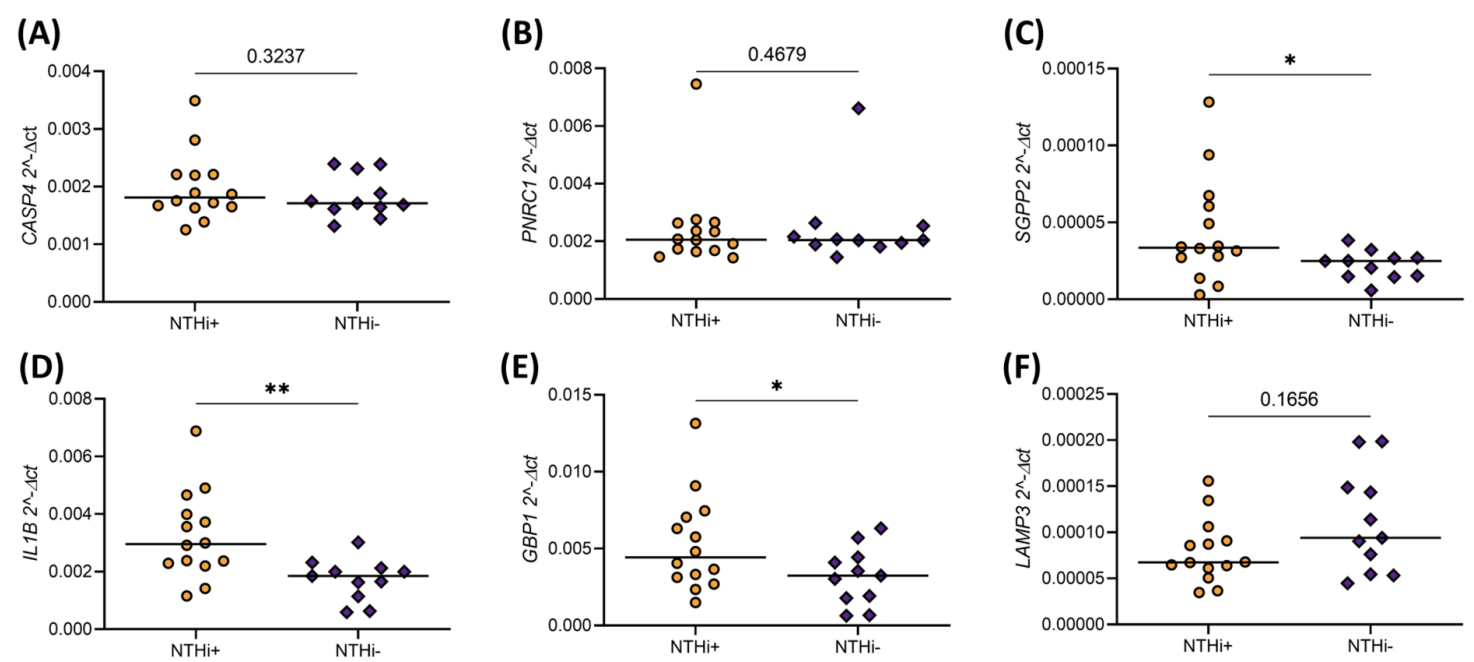


Figure 4

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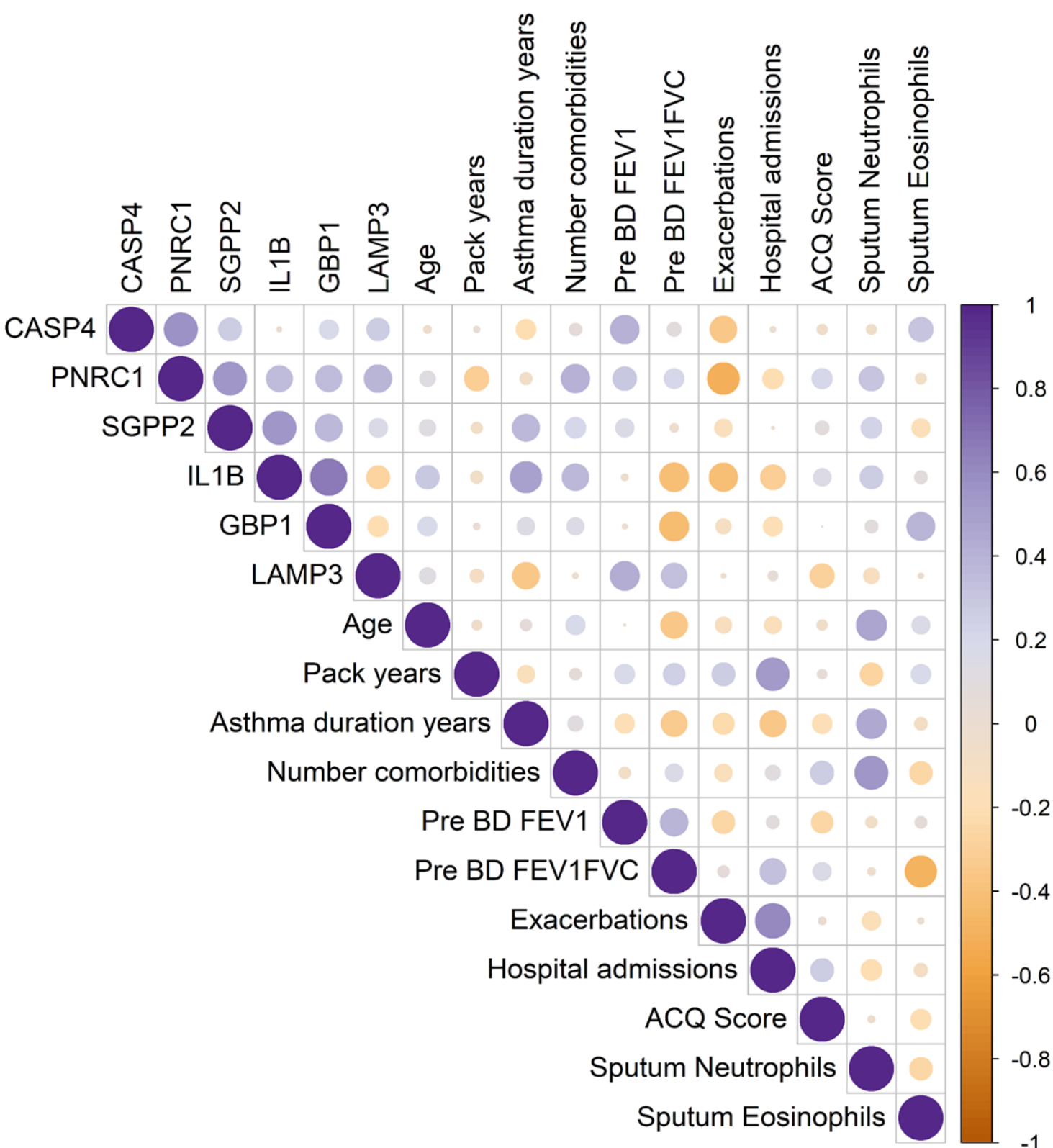


Figure 5

Online Supplement for

NTHi infection of pulmonary macrophages drives neutrophilic inflammation in severe asthma

Jodie Ackland^a, Clair Barber^{a,c}, Ashley Heinson^a, Adnan Azim^{a,c}, David W. Cleary^{a,c}, Myron Christodoulides^a, Ramesh J. Kurukulaarachy^{a,c}, Peter Howarth^{a,c}, Tom M.A. Wilkinson^{a,b,c}, Karl J. Staples^{a,b,c} # On behalf of the WATCH study investigators.

^aClinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, UK

^bWessex Investigational Sciences Hub, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, UK

^cNIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust, Southampton, UK

Short Title: NTHi drives neutrophilic inflammation in asthma

#Address correspondence to Karl J. Staples, K.Staples@soton.ac.uk

Supplementary Material and Methods

Lung sample processing from WATCH study

BAL was collected from patients and stored on ice until processing by members of the WATCH study team. Briefly, samples were filtered using a 100 µm cell strainer and centrifuged at 790 *g* for 10 min at 4°C. The cell pellet was resuspended in PBS and a cell count using the trypan blue exclusion method was performed to assess cell viability, which identified a median BAL cell viability of 77%. Subsequently, the cell suspension was adjusted to 5 x 10⁵ cells per ml in PBS. Cell cytospins were generated, with 75 µl of the cell suspension added to a cytospin funnel and subjected to centrifugation using a Shandon Cytospin® centrifuge at 450 rpm for 6 min. Slides were left to air dry at room temperature for at least 2 h before use or stored at -20°C until required for Fluorescence *in situ* hybridization (FISH, as described in 2.24). The remaining cell suspension was resuspended to allow for a minimum of 5 x 10⁵ cells per ml and centrifuged at 790 *g* for 10 min at 4°C. The sample was resuspended in 500 µl QIAzol and vortexed for 30 s. Samples were stored at -80°C until RNA isolation was performed.

Sputum was also collected and processed for each patient, and differential sputum cell counts were performed in order to determine patient inflammatory phenotypes. Briefly, sputum was obtained by members of the WATCH study team and was stored on ice until processing. Sputum was weighed and diluted with 2 volumes of 0.2% (w/v) dithiothreitol (DTT) to processed sputum weight (g) and vortexed for 15 s. Sample was placed on a bench roller in ice for 30 min. The sample was then passed through a 100 µm cell strainer and centrifuged at 790 *g* for 10 min at 4°C. The cell pellet was resuspended and a cell count using the trypan blue exclusion method was performed to assess cell viability, which indicated a median sputum cell viability of 70%. Cells were resuspended to a concentration of 5 x 10⁵ cells per ml to generate cytospin slides, with 75 µl of the cell suspension added to a cytospin funnel and subjected to centrifugation using a Shandon Cytospin® centrifuge at 450 rpm for 6 min. Slides were left to air dry at room temperature for at least 2 h before use or stored at -20°C until required. Once dry, slides were manually counted under a light microscope, with a total of 400

cells manually counted to determine the proportion of macrophages, neutrophils, eosinophils, lymphocytes and epithelial cells present in each sample. Sputum cell counts were used to determine the inflammatory phenotype of patients using published cut off values^{50,68,69}: neutrophilic = $\geq 40\%$ neutrophils, eosinophilic = $\geq 3\%$ eosinophils, mixed granulocytic = $\geq 40\%$ neutrophils and $\geq 3\%$ eosinophils and paucigranulocytic $< 40\%$ neutrophils and $< 3\%$ eosinophils. No data regarding bacteria or viral culture were available for analysis.

Fluorescence *in situ* hybridisation (FISH)

Stored BAL cytospin slides were thawed and allowed to come to room temperature. Slides were washed in PBS before being fixed in a paraformaldehyde-acetic acid fixative solution (36.5M paraformaldehyde and 5% acetic acid in PBS) for 15 min. Slides were subsequently washed twice in PBS for 5 min, before two final quick washes in water. Slides were dehydrated using an ethanol gradient of 70% ethanol for 5 min, 90% ethanol for 5 min and 100% ethanol for 5 min before being left to completely air dry. The NTHi specific probe HAIN16S1251 and the pan-bacteria probe EUB338A were diluted to a concentration of 50 $\mu\text{g}/\mu\text{l}$ in hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 0.01% SDS, and 30% formamide pH 7.5) and 20 μl added to each slide. Hybridization was performed for 2 h at 46°C using a ThermoBrite StatSpin humid chamber before being washed for 15 min in wash buffer (80 mM NaCl, 20 mM Tris- HCl pH 8, 5 mM EDTA, and 0.01% SDS) at 48°C. Slides were again washed in PBS before addition of 150 μl of Vector® TrueVIEW® Autofluorescence Quenching reagent (Vectorlabs) for 4 min. Slides were washed in PBS before glass coverslips were added using Vectashield mounting solution containing 4',6-Diamidino-2-Phenylindole (DAPI) nuclear stain (Vectorlabs). Slides were stored at 4°C until visualisation.

Quantification of NTHi colonisation in BAL samples

Quantification of the presence of NTHi was performed in ImageJ/FIJI. The number of macrophages in each image, represented by DAPI staining of single cell nucleus, was quantified using the Analyse Particles in-built ImageJ tool. This was achieved by setting the threshold for each image and running the 'Convert to Mask' and 'Erode' functions before the 'Analyse Particles' function to count the individual nuclei. The CY3 NTHi-specific signal was next quantified using the Find Maxima in-built ImageJ tool. Firstly, each image was adjusted using the 'Enhance Contrast', 'Smooth' and 'Gaussian Blur' in-built ImageJ functions. The 'Find Maxima' function was then used to identify individual CY3 signal per host cell which passed a set 'prominence' level, which was set to reduce potential background or autofluorescence being counted as NTHi. The output of DAPI and CY3 signal quantification was used to determine the number of DAPI-stained cells associated with the CY3-NTHi signal, which was expressed as a percentage of cells colonised with NTHi.

Weighted gene correlation network analysis (WGCNA)

Weighted gene correlation network analysis (WGCNA) was performed using the WGCNA R package⁴² on the normalised, log transformed CPM MDM data set. Construction of the gene network was performed using the WGCNA automatic network construction method. A soft thresholding power of 18 was chosen based on the scale-free topology fit output of the *pickSoftThreshold* function using default values provided by the WGCNA package, with a power of 18 the lowest power to completely intersect the high value red line ($R^2 = 0.9$) on the scale independence plot (Figure S9).

Identification of modules and genes of interest

Correlation analyses were performed using Pearson correlation as described in the WGCNA package. Correlation analysis between module eigengene and sample trait information was performed to identify which modules were significantly association with NTHi infection. The

significant modules were assessed for correlations between gene significance (GS, representing the correlation between each gene and the trait of interest) and module membership (MM, representing the significance of gene assignment to the module) to determine module-trait relationships. To visualise the gene network within the significant blue module, the network file was exported to Cytoscape. Due to the large size of the module (2333 genes), a topological overlap threshold of 0.34 was used to extract a meaningful network of highly interconnected genes for further exploration. The cytoHubba plugin in Cytoscape was used to rank genes present in the blue module gene network using the Maximal Clique Centrality (MCC) scoring method⁷⁰.

Gene list enrichment analysis

Gene list enrichment analysis and Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis was performed using ToppFunn, which is part of the online ToppGene Suite, using default parameter settings (FDR multiple correction method and enrichment significance cut off level 0.05).

Quantification of MDM mediator release by Luminex

Analysis of MDM cell culture supernatants for quantification of mediator release also performed using a customised 14-plex Luminex Human Magnetic Assay according to the manufacturer's instructions (R&D Systems). Briefly, 50 µl of the premixed microparticle cocktail was added to each well of a 96 well plate and incubated with 50 µl of neat MDM culture supernatant or the pre-prepared standard at 4°C overnight. The plate was then washed with 100 µl Wash Buffer (R&D Systems) whilst attached to a Handheld Magnetic Washer, which held the microparticles and bound sample analytes to the bottom of the plate to prevent them washing away. The biotin antibody cocktail provided was diluted with the provided diluent concentrate (R&D Systems) and 50 µl was added to each well and incubated for 1 h on a plate

shaker. The plate was again washed as described above, before 50 µl of Streptavidin-PE was added and again incubated for 30 min on a plate shaker. The plate was read using a Bio-Plex 200 system (Bio-Rad), with the concentration of each analyte calculated from the concentration of the 1 in 3 diluted standard curve for each analyte. The lower and upper limit of quantitation (LLOQ and ULOQ) for each analyte is indicated in Table S4.

Supplementary Tables

Table S1. Additional clinical characteristics and demographic data for the 25 severe asthma patients used in this stud

	Total (n=25)	NTHi + (n=14)	NTHi – (n=11)	p-value
Total IgE [#]	147.6 [32.7 – 366.1]	213.2 [21.2-399.1]	139.7 [44.3 – 239.3]	0.8718
Atopy [#]	10 (40%)	6 (42.4%)	4 (36.4%)	0.6084
Blood eosinophils	0.2 [0.1 – 0.4]	0.15 [0.1 – 0.3]	0.2 [0.1 – 0.4]	0.4963
Comorbidities (Y/N)	16/9	9/5	7/4	>0.9999

The 25 severe asthma patients were split based on the outcome of the FISH analysis into NTHi+ (14) and NTHi- (11) groups. Values reported are medians [IQR] or (%). # indicates missing patient data; Total IgE was not available for 1 patient, and atopy was determined as a positive skin prick test to at least one common aeroallergen, however this was not performed or data were missing for 10 patients. Comorbidities indicate the number of patients reporting at least 1 or more comorbidities (Y) or no comorbidities (N). Statistical analysis was performed between NTHi+ and NTHi- groups. Continuous data were analysed by Mann-Whitney U test. **Bold** indicates p-values determined as statistically significant (p<0.05).

Table S2. Ranking scores of the 62 blue module network genes.

Rank	Gene	Score
1	CASP4	28
2	PNRC1	21
3	SGPP2	9
4	GBP5, GBP4	3
6	ABTB2, UPB1, GBP1 , RIPK2, GCH1, PLAT, NAMPT, SLC1A2, CCL20, CCR7, STK26, IL6, TRAF1	2
19	CXCL8 , PTGS2, PFKFB3, LAMP3 , CXCL2, MARCKS, IL23A , MX1, IL1B , ISG15, SOD2, IL12B, IL1A, SLAMF1, PPP3CC, IL15RA, SESN2, GFPT2, RSAD2, CMPK2	1
39	IFI35, ISG20, PDE4B, CD40, BATF3, CSF2, AIM2, BATF2, TAP1, PKIG, CSF3, IL27 , RNF19B, CD274, TNFAIP6, IRF1, CMTR1, RNF144B, LAP3, HAPLN3, SLAMF7, NLRC5, WTAP, TNIP3	0

Genes were ranked using Maximal Clique Centrality (MCC) method using the cytoHubba plugin in Cytoscape. *CASP4*, *SGPP2* and *PNRC1* were the three highest ranked genes in the blue module. **Bold** indicates genes chosen for qPCR and/or ELISA/Luminex validation in MDM or BAL samples.

Table S3. List of TaqMan gene expression assays (all from Thermo Fisher) used for validation of macrophage gene expression by qPCR.

Gene	Protein	Assay ID
<i>B2M</i>	Beta-2-microglobulin	Hs00984230_m1
<i>CASP4</i>	Caspase-4	Hs01031951_m1
<i>GBP1</i>	Guanylate Binding Protein 1	Hs00977005_m1
<i>IL1B</i>	Interleukin 1 Beta	Hs01555410_m1
<i>LAMP3</i>	Lysosomal Associated Membrane Protein 3	Hs01111316_m1
<i>PNRC1</i>	Proline Rich Nuclear Receptor Coactivator 1	Hs00199095_m1
<i>SGPP2</i>	Sphingosine-1-Phosphate Phosphatase 2	Hs00544786_m1

Table S4. The lower and upper limit of quantitation (LLOQ and ULOQ) for each analyte (pg/ml) measured by a customised Luminex Human Magnetic Assay (R&D).

Analyte	Lower Limit of Quantification	Upper Limit of Quantification
IL-23	45.919	34817.527
CCL20	8.558	2169.997

Supplementary Figures

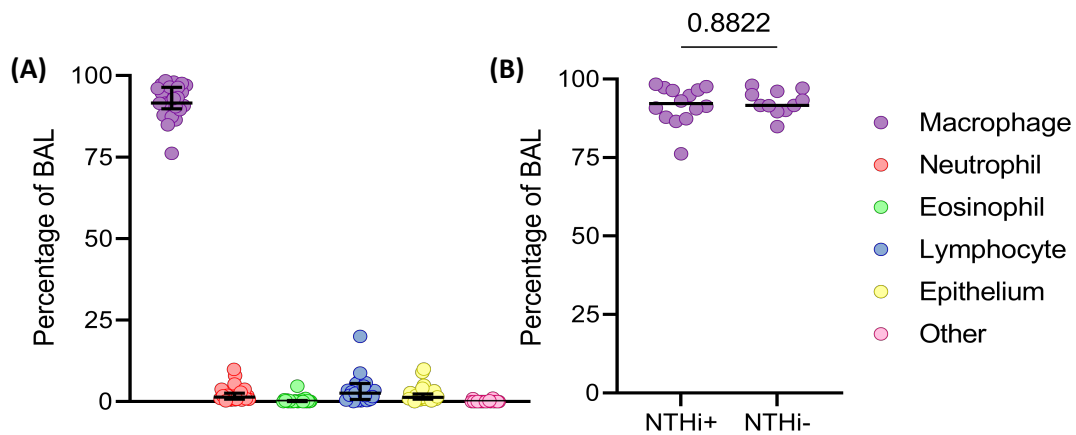


Figure S1. Proportion of BAL cell types isolated from the airways of severe asthma patients. (A) The cells recovered from BAL were predominantly macrophages (91.6%). **(B)** No differences in BAL macrophage proportions were detected between NTHi+ (92.2%) and NTHi- (91.6%) groups. Data were analysed by Mann Whitney U test. Lines indicate median values. Key shows cell types included for differential cell counting.

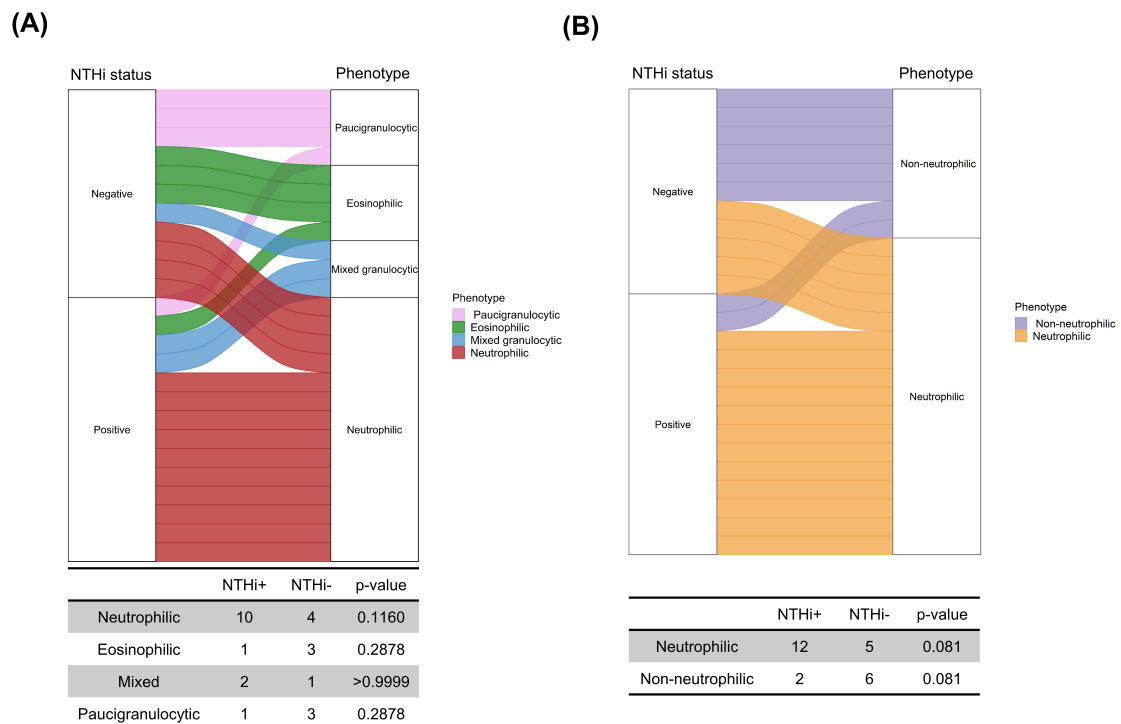


Figure S2. Stratification of the 25 severe asthma BAL samples by NTHi colonisation and inflammatory phenotype status. Patients were stratified into neutrophilic ($\geq 40\%$ neutrophils, $< 3\%$ eosinophils), eosinophilic ($< 40\%$ neutrophils, $\geq 3\%$ eosinophils), mixed granulocytic ($\geq 40\%$ neutrophils, $\geq 3\%$ eosinophils) or paucigranulocytic ($< 40\%$ neutrophils, $< 3\%$ neutrophils) inflammatory phenotypes. Accompanying table (bottom) indicates the significance between NTHi+ and NTHi- groups as performed by Fisher's exact test.

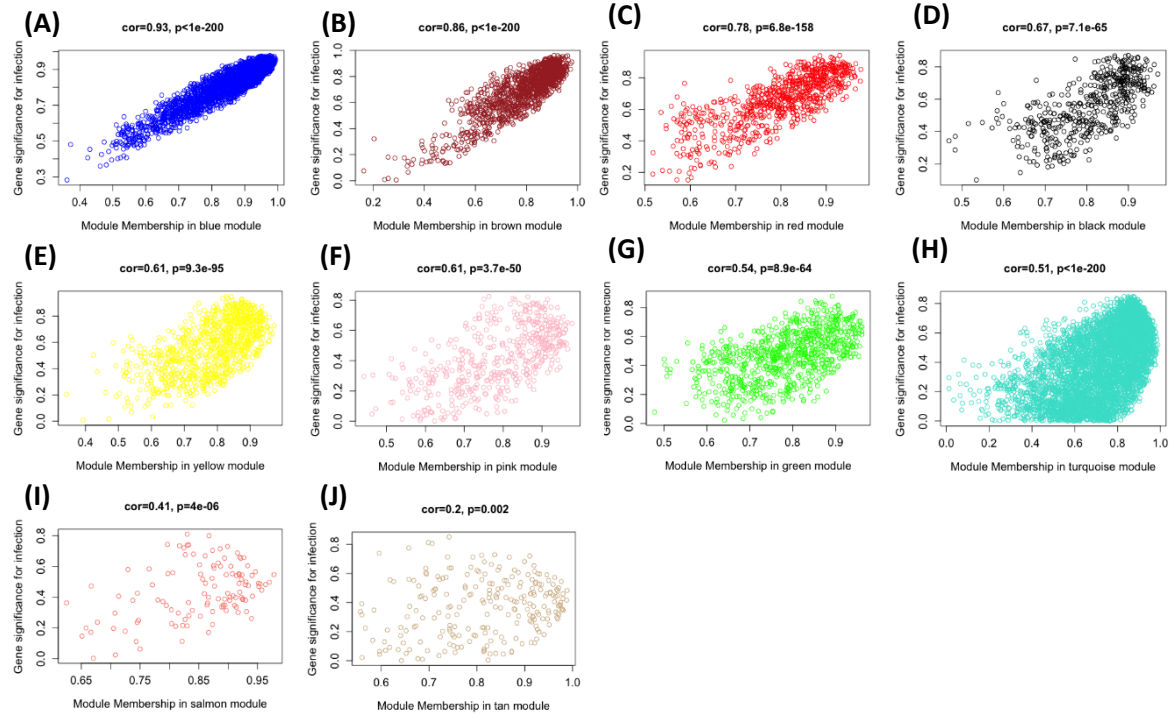


Figure S3. Individual module membership and gene significance correlation plots for the ten modules significantly associated with NTHi infection. Identification of gene significance in the ten modules (A) blue, (B) brown, (C) red, (D) black, (E) yellow, (F) pink, (G) green, (H) turquoise, (I) salmon and (J) tan found to be significantly correlated with the NTHi infection. Module membership indicates the correlation of each individual gene to the specific module eigengene. The correlation and corresponding p-value for each comparison is present above each scatterplot and modules are ordered based on level of correlation (high to low).

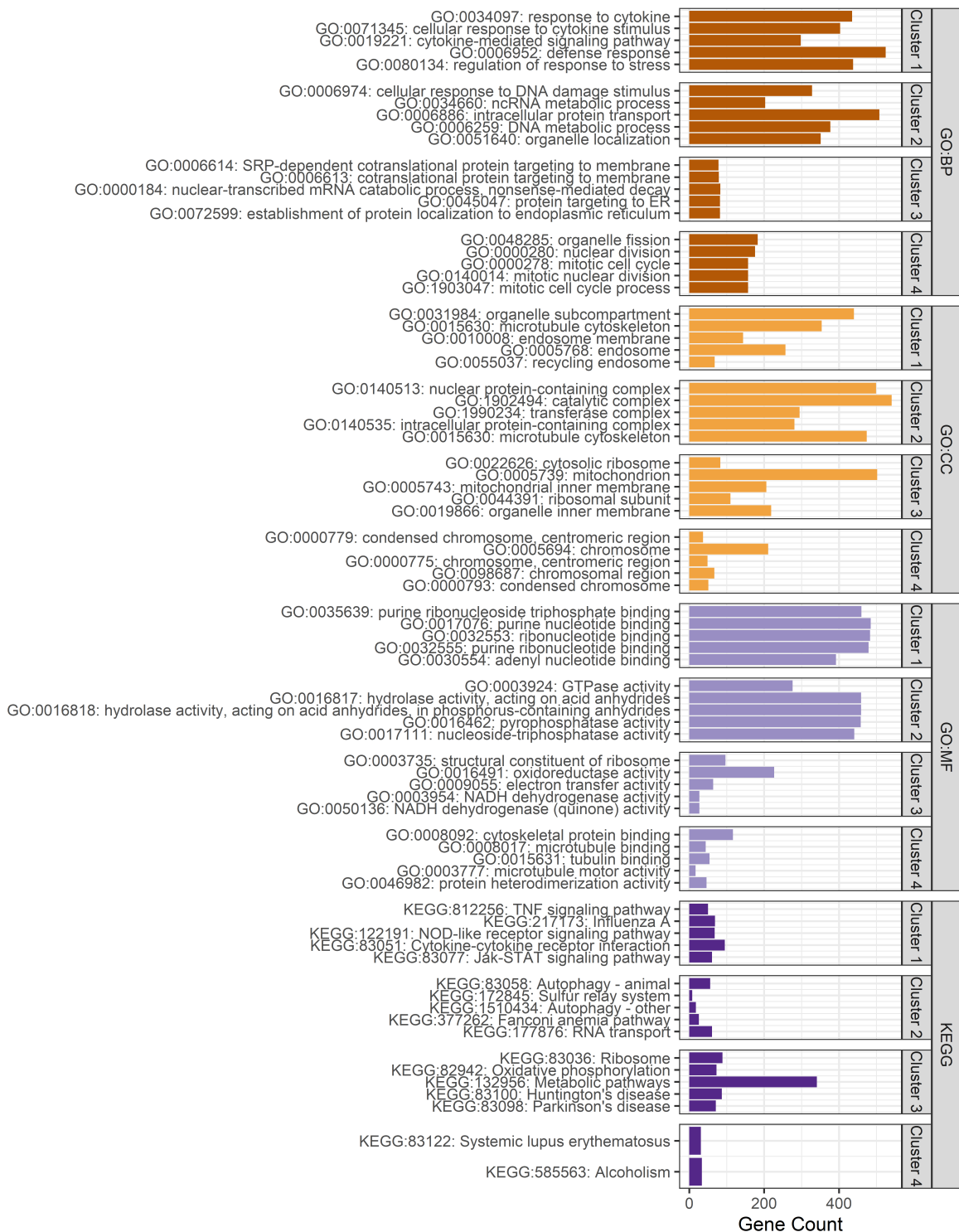


Figure S4. Gene ontology and KEGG pathway analysis indicates intracellular immune response pathways are modulated in the cluster associated with infection. The top 5 significantly enriched terms for Biological Processes (BP), Cellular Component (CC), Molecular Function (MF) and KEGG pathway are shown for each cluster, with Pathway/category IDs ordered by enrichment significance (FDR), of which all categories were deemed as statistically significantly functionally enriched (FDR $p < 0.05$). **(A)** Cluster 1 indicating enrichment of immune response pathways, **(B)** Cluster 2 showing enrichment of metabolic and cell activation processes, **(C)** Cluster 3 is enriched in gene regulation and metabolic processes, **(D)** Cluster 4 is enriched in cell cycle processes, however no significant KEGG pathways were identified for this cluster. Gene ontology performed using ToppGene.

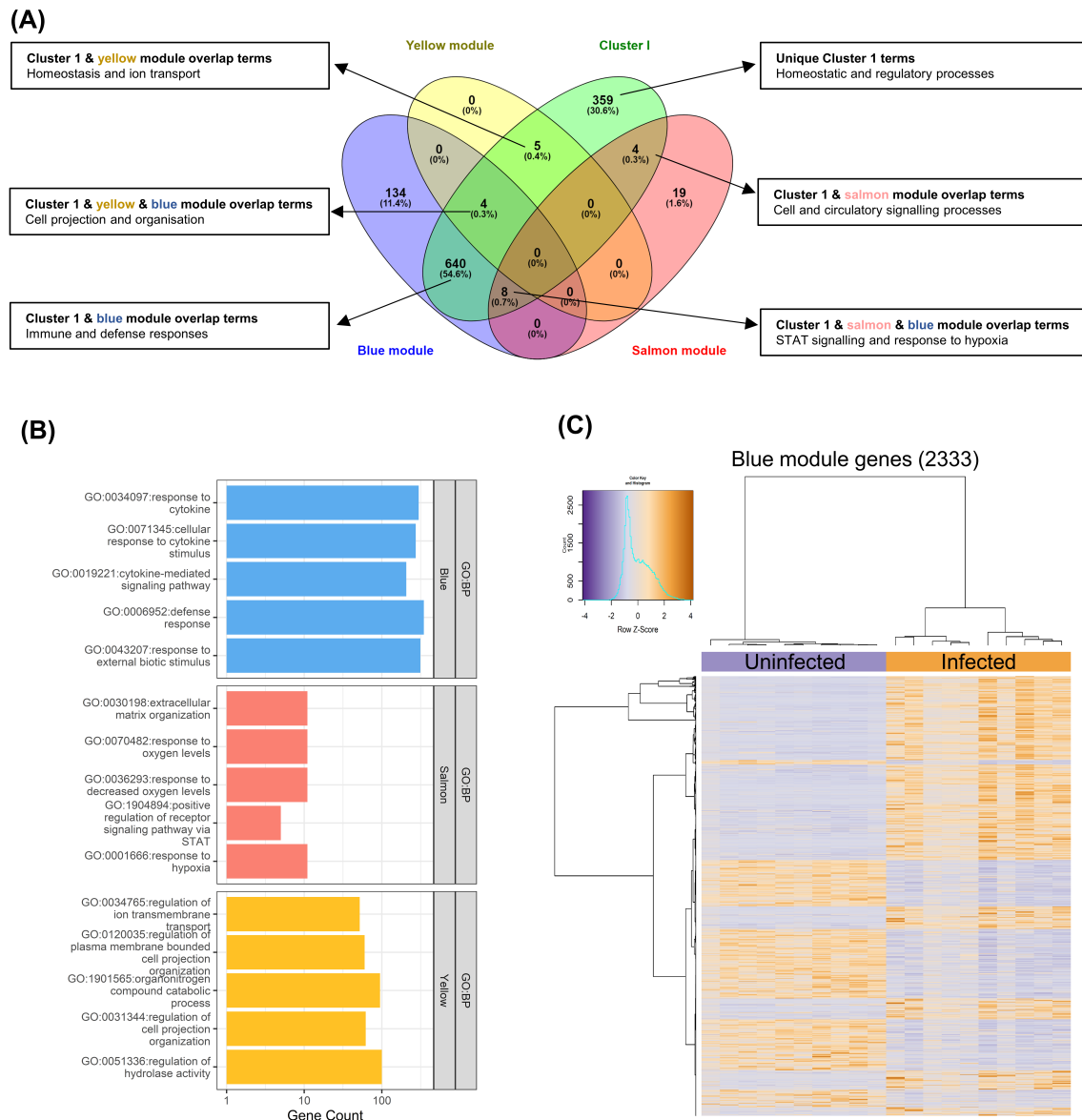


Figure S5. The blue module drives the enrichment of immune responses in cluster I and is associated with NTHi infection. **(A)** Enrichment analysis performed on each of the modules assigned to cluster I individually showed enrichment of different process for each module. The total number of enriched Biological Process terms for each category were: Cluster I: 1022, Blue module: 786, Yellow module: 9 and Salmon module: 31. No enriched terms for the pink module were found. Venn diagram shows the overlapping terms between each of the modules and cluster I, with the blue module and cluster I sharing the highest proportion of terms, indicating the contribution of the genes within this module to the enrichment of terms within Cluster I. **(B)** Barplot shows the top 5 most significantly enriched (adjusted p-val < 0.05) Biological Process terms for the blue module, salmon module and yellow module respectively, further highlighting the immune processes specific to the blue module. **(C)** Heatmap of the 2333 genes present in the blue module constructed using Euclidean distance and Ward linkage. Samples cluster according to infection status (uninfected or infected).

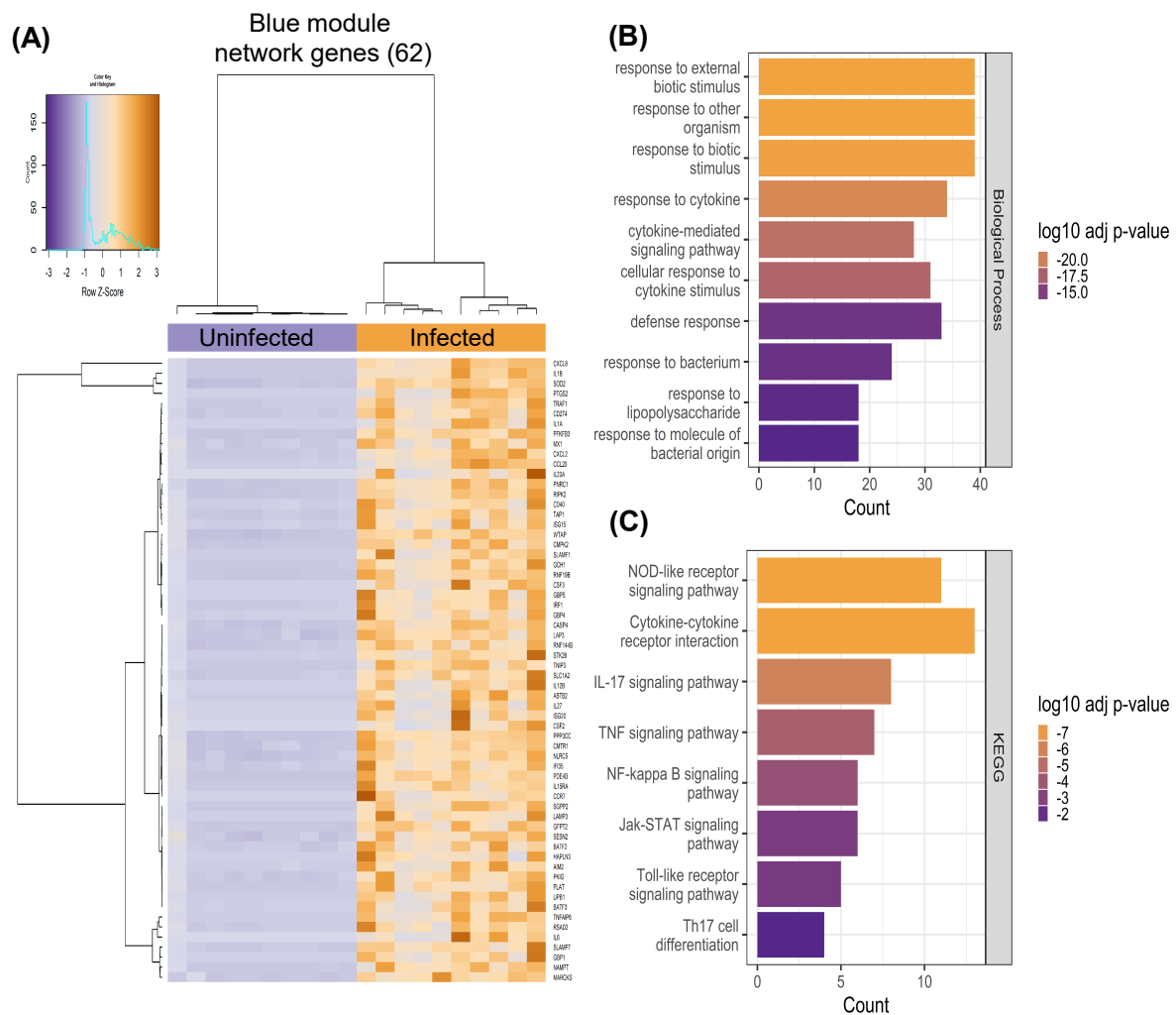


Figure S6. The most highly interconnected blue module genes are more highly expressed in response to NTHi infection and are enriched in immune responses. (A) Heatmap visualising the expression profile of the 62 most highly connected blue module network genes. All genes were more highly expressed in response to NTHi infection compared to uninfected controls. Clustering was performed using Euclidean distance and Ward linkage methods. Gene enrichment analysis performed on the 62 genes using ToppGene demonstrated significant enrichment of **(B)** Biological Processes and **(C)** KEGG pathways (adjusted p-value <0.05 for all).

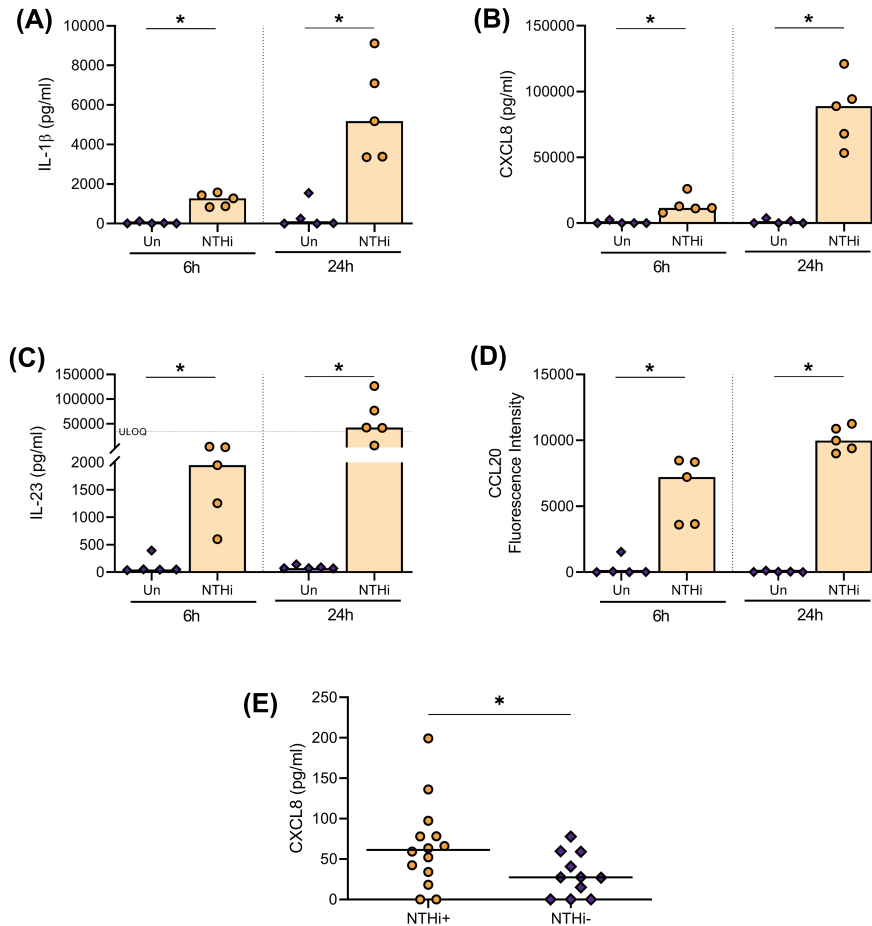


Figure S7. Validation of inflammatory mediator release. To validate the upregulation of genes for inflammatory mediators present in the blue module gene network resulted in increased protein level release, macrophage release of T17 mediators **(A)** IL-1 β , **(B)** IL-8, **(C)** IL-23 and **(D)** CCL20 into cell culture supernatants at 6 h and 24 h in response to NTHi infection of MDM (N=5) was measured by Luminex (IL-23 and CCL20) or ELISA (CXCL8 and IL-1 β). Horizontal dotted lines on graphs show the upper limit of quantification (ULOQ) or lower limit of quantification (LLOQ) of the Luminex assay where required. CCL20 was released into cell culture supernatants at such high amounts that the concentration of this chemokine was unable to be accurately quantified, so is represented here as fluorescence intensity. **(E)** Levels of CXCL8 in severe asthma BAL supernatants were assessed by ELISA (n=25). Graphs A-D show paired data and data were analysed by Wilcoxon signed rank test. Graph E shows unpaired data and data were analysed by Mann Whitney U test. *p<0.05. Lines indicate medians.

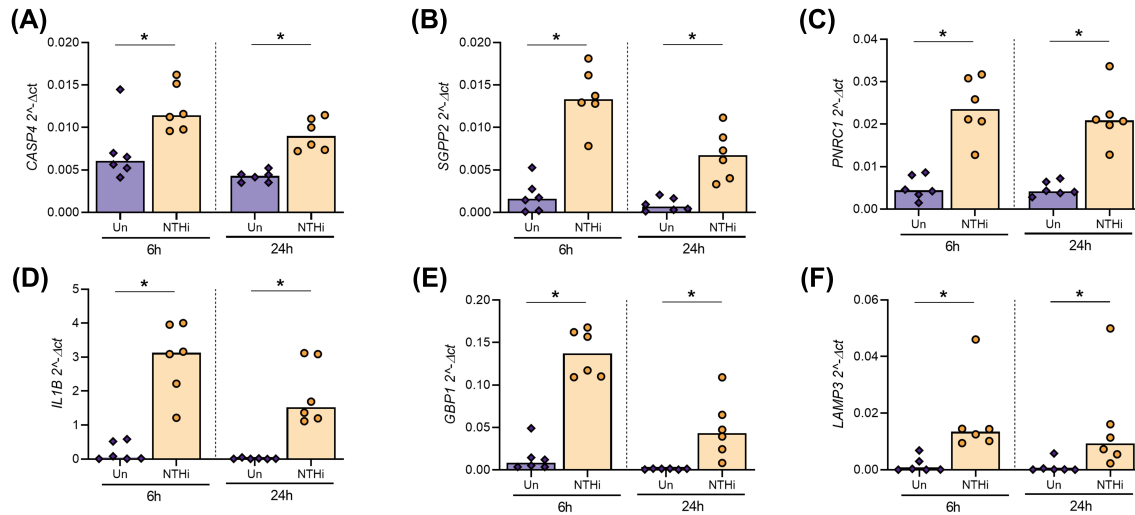


Figure S8. Validation of macrophage gene expression in the MDM model. To validate the expression of the genes in the blue module network, the top three ranked genes **(A) CASP4**, **(B) PNRC1** and **(C) SGPP2** and three other immune related genes in the blue module gene network **(D) IL1B**, **(E) GBP1** and **(F) LAMP3** were validated by qPCR using the MDM model (N=6). Gene expression was normalised to *B2M* and is expressed as 2^{-ΔΔCt}. All genes were more highly upregulated in response to NTHi infection compared to uninfected controls. Data were analysed by Wilcoxon signed rank test, *p<0.05. All graphs show paired data and bars indicate median values. Data were analysed by Wilcoxon signed rank test, *p<0.05.

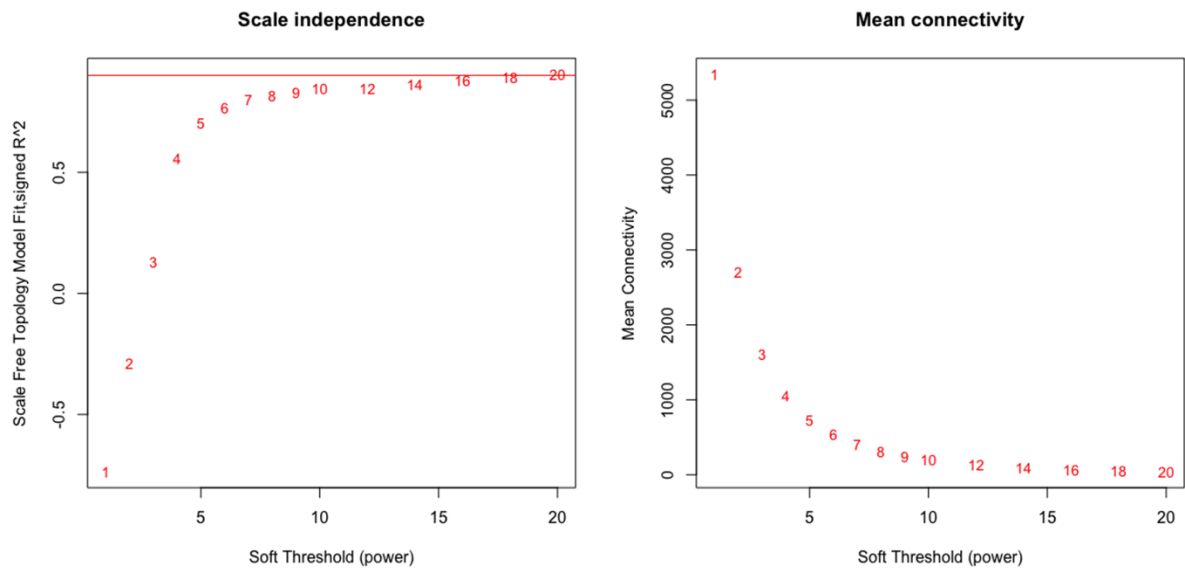


Figure S9. Network topology analysis for determining soft-threshold analysis power to be used for automatic network construction. (A) scale-free fit index (y axis) as a function of the soft-thresholding power (x axis). **(B)** mean connectivity degree (y axis) as a function of the soft thresholding power (x axis).