UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Clinical and Experimental Sciences

THE RESPONSES OF CONVENTIONAL T CELLS AND MUCOSAL-ASSOCIATED INVARIANT T CELLS TO NONTYPEABLE *HAEMOPHILUS INFLUENZAE* INFECTION

by

Joshua Charles Wallington

Thesis for the degree of Doctor of Philosophy

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Abstract

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Nontypeable *Haemophilus influenzae* (NTHi) is a component of the normal lung microbiome, but is also highly associated with respiratory tract infections and exacerbations of major chronic respiratory diseases such as chronic obstructive pulmonary disease (COPD). It is not known how commensal bacteria can become pathogenic and cause inflammation in the lung, but is likely due to a breakdown in local immunity. T cell immunity may be key to controlling NTHi infection, although the responses of T cells to NTHi are not well understood. Mucosal-associated invariant T (MAIT) cells are a newly-discovered subset of innate-like T cells, which may play a role in controlling NTHi, as they recognise non-peptide antigens derived from the highly conserved vitamin B2 pathway. The role of MAIT cells in lung immunity to NTHi is also unclear.

The aim of this thesis was to study the cytokine and cytotoxic responses of MAIT cells to NTHi infection, comparing these responses to those of conventional T cells. The antigen presentation and co-stimulatory mechanisms that regulate MAIT cell activation have also been explored.

Conventional T cell and MAIT cell responses to NTHi were investigated using a human *ex vivo* lung tissue explant model and an autologous monocyte-derived macrophage (MDM)-T cell co-culture model. Cytokine and cytotoxic responses were measured by a combination of flow cytometry, ELISA and ELISpot. Blocking antibodies were used to determine the role of antigen presentation and cytokine signalling in conventional T cell and MAIT cell activation.

Lung MAIT cells significantly upregulated the cytokines; IFNγ, IL-17a and TNFα, and the cytotoxic markers; granzyme B and CD107a, following NTHi infection. A greater proportion of MAIT cells were active compared to conventional T cells. In the blood-derived MDM-T cell co-culture, IFNγ expression by MAIT cells was dependent on MR1 antigen presentation and IL-12 signalling in a time-dependent manner. Cytotoxic responses were regulated by MR1 but also by a novel mechanism where IL-12 and IL-7 signalling synergised to induce granzyme B expression by

upregulation of the IL-12 receptor. In contrast, conventional T cell responses predominantly relied on antigen presentation for activation. MAIT cell responses were also impaired by treatment with corticosteroids, which are commonly used to manage inflammation in chronic lung diseases, but are associated with a higher risk of developing pneumonia in COPD patients.

Overall, the data in this thesis provide evidence for a role for MAIT cells in controlling NTHi infection in the lung and also highlight key differences in the regulation of innate and adaptive T cells. A further understanding of the mechanism underpinning T cell responses to NTHi infection may yield new therapeutic opportunities and improve the outcome for patients with respiratory diseases.

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Publications

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Wallington JC, Williams AP, Staples KJ, Wilkinson TMA. (2015). Macrophages express MR1 and activate MAIT cells in response to NTHi. British Association for Lung Research Summer Conference, Bath, UK. – Poster Presentation

Wallington JC, Williams AP, Staples KJ, Wilkinson TMA. (2016). Immunomodulation of T cell responses to NTHi by corticosteroids: a mechanism for increased pneumonia risk in COPD? Wessex Immunology Group Annual Meeting, Southampton, UK. – Oral Presentation

Wallington JC, Williams AP, Staples KJ, Wilkinson TMA. (2016). Cytotoxic Responses of Mucosal-Associated Invariant T Cells to NTHi Infection: Implications for COPD. British Association for Lung Research Summer Conference, Sheffield, UK. – Oral Presentation (Student Investigator Award Finalist)

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Wallington JC, Williams AP, Staples KJ, Wilkinson TMA. (2017). Expression of PD1 and PD1 signalling in innate and adaptive T cell responses to NTHi. European Respiratory Society Lung Science Conference, Estoril, Portugal – Poster Presentation

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Declaration Of Authorship

I, Joshua Charles Wallington

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"The Responses Of Conventional T Cells And Mucosal-Associated Invariant T Cells To Nontypeable Haemophilus Influenzae Infection"

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

7. Parts of this work have been published as:

   Hinks TS*, Wallington JC*, Williams AP, Djukanović R, Staples KJ, Wilkinson TM. (2016). Steroid-induced Deficiency of Mucosal-associated Invariant T Cells in the COPD Lung: Implications for NTHi Infection. Am. J. Respir. Crit. Care Med. 187: rccm.201601-0002OC.
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Date: ……

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Abbreviations

APC	Antigen Presenting Cell
APM	Antigen Presenting Molecule
BAL	Bronchoalveolar Lavage
BCG	Bacillus Calmette-Guérin
BD	Budesonide
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CFU	Colony Forming Unit
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette smoke
CTLA4	Cytotoxic T-lymphocyte-associated Protein 4
DC	Dendritic Cell
DMSO	Dimethyl Sulfoxide
DN	Double Negative
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DP	Double Positive
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked ImmunoSpot
ER	Endoplasmic Reticulum
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FEV1	Forced Expiratory Volume in One Second
FP	Fluticasone Propionate
HiB	*Haemophilus influenzae* type b
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen

ICAM1	Intercellular Adhesion Molecule 1
ICOS	Inducible T-cell Costimulator
ICS	Inhaled Corticosteroids
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon Regulatory Factor
LDH	Lactate Dehydrogenase
Lin 1	Anti-Human Lineage Cocktail 1
LOS	Lipooligosaccharide
LPA	Lipid A
LPS	Lipopolysaccharide
MACS	Magnetic-Activated Cell Sorting
MAIT	Mucosal-associated Invariant T Cell
MDM	Monocyte-Derived Macrophages
MHC	Major Histocompatibility Complex
MLST	Multilocus Sequence Typing
MOI	Multiplicity Of Infection
MR1	Major Histocompatibility Complex, Class I-Related Protein
mRNA	Messenger RNA
Mtb	*Mycobacterium tuberculosis*
NAD	Nicotinamide Adenine Dinucleotide
NF-κB	Nuclear Factor kappa-light-chain-enhancer of Activated B Cells
NK cell	Natural Killer cell
NTHi	Nontypeable *Haemophilus influenzae*
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD1	Programmed Cell Death Protein 1
PDL1	Programmed Death-Ligand 1

PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-Myristate 13-Acetate
PRR	Pattern Recognition Receptor
qPCR	Quantitative PCR
RIG-I	Retinoic Acid-inducible Gene I
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal Ribonucleic Acid
RSV	Respiratory Syncytial Virus
SFC	Spot Forming Count
SMFI	Specific Mean Fluorescence Intensity
TCR	T cell Receptor
Th	T Helper
TIM3	T-cell Immunoglobulin and Mucin-domain containing-3
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
WHO	World Health Organization
β2M	Beta-2 Microglobulin

1. Introduction

The primary function of the human lungs is to facilitate gas exchange, which provides oxygen for all metabolic processes vital for life. However, the respiratory tract is a mucosal site with an incredibly large surface area which is continuously exposed to airborne pathogens and particles (Hasleton, 1972). In order to prevent infections, a number of key mechanisms are present which contribute to defence against invading pathogens (Martin and Frevert, 2005). These mechanisms present a complex challenge for the lung immune system, as it is required to balance inflammation aimed at clearing infection against minimising collateral damage to the lung architecture. This is particularly evident in severe respiratory viral infections, especially those associated with pandemics, where pneumonia occurs and lung pathology is induced by overproduction of pro-inflammatory cytokines; the so called the cytokine storm (Liu et al., 2016). Additionally, a dysfunctional immune response may lead to acute bacterial infections or chronic lung diseases, which are major causes of death worldwide (World Health Organization, 2017). Further complications may arise due to the complex interaction of the lung immune system with the lung microbiome. Understanding of these complex innate and adaptive immune responses of the human lung is limited. In particular, mechanistic studies giving insights into the immune processes of monitoring and responding to changes in the lung microbiome have not been performed in detail and are required to develop new strategies to improve clinical outcomes for respiratory infections.

1.1 The lung microbiome

Until recently, healthy human lungs were considered to be a sterile environment with bacteria only being detected during active infection or associated with chronic diseases (Dickson and Huffnagle, 2015). However, it is now known that normal, healthy lungs are in fact not sterile and are home to a wide variety of commensal bacterial species in the upper and lower airways and parenchymal tissue, which are not normally pathogenic (Sze et al., 2014)(Hilty et al., 2010)(Wang et al., 2016)(Dickson et al., 2014a). All of the microorganisms present in the human lung collectively make up the lung microbiome, which varies considerably between individuals (Shukla et al., 2017)(Costello et al., 2009). Infant airways are thought to be initially sterile but acquire a microbiome rapidly after birth, although the process for this is still not fully understood but does involves transfer of microbes from the mother (Dominguez-Bello et al., 2010)(Dickson et al., 2014b). Microbiome development may even occur *in utero*, as microbes have been detected from DNA obtained from the placenta, which have similarity to the oral microbiome (Aagaard et al., 2014).

The discoveries of the lung microbiome have been driven by advances in molecular detection methods, such as PCR, 16s rRNA sequencing and metatranscriptomics, that have identified species in the lung that were previously underappreciated due to lack of detection by traditional culture methods (Dickson et al., 2014a)(Shukla et al., 2017). Heterogeneity is observed throughout the airways; bacterial communities isolated from bronchoalveolar lavage (BAL) fluid or surgical tissue (Erb-Downward et al., 2011) differ from those found in the upper airways, such as the nasal cavity (Hilty et al., 2010), indicating that different bacterial species occupy their own ecological niche. The major phyla found in the lung include Proteobacteria, Firmicutes, and Bacteroidetes (Erb-Downward et al., 2011) although Proteobacteria appear to be associated with respiratory disease (Hilty et al., 2010). The source of the lung microbiome appears to be the oral cavity, not the nasal cavity or stomach, as many bacterial communities detected in the lung are also observed in the mouth (Bassis et al., 2015). However, microbiome analysis of the lower airway needs to be considered carefully as samples collected via bronchoscopy and BAL fluid can be subject to contamination. Indeed, one caveat is the issue of signal to noise ratio, which may confound the data, especially in the initial stages of disease where the microbial biomass is particularly low and contamination from the upper airways or external sources can occur (Twigg et al., 2013)(Wu and Segal, 2017).

Overall, much is still unknown about the role the microbiota play in lung health and disease, although immune mechanisms may be vital in control of the lung microbiome, preventing commensal species from becoming pathogenic.

1.1.1 Control of the lung microbiome by immune mechanisms

Mucociliary clearance, structural cells such as the airway epithelium, as well as innate and adaptive immune cells, including neutrophils, macrophages and T cells all play vital roles in airway defence and control of the lung microbiome (Whitsett and Alenghat, 2015)(Martin and Frevert, 2005). Innate immunity is classically defined as the arm of the immune system which provides a fast, germ-line encoded, generalised response to infection, but lacks immunological memory and typically recognises pathogens by expression of conserved pathogen-associated molecular patterns (PAMPs) (Martin and Frevert, 2005). Innate immune cells, such as macrophages and neutrophils attempt to contain and clear infection by rapidly phagocytosing and killing bacteria and viruses and releasing a multitude of cytokines to orchestrate the immune response. In contrast, adaptive immunity, which includes B cells (humoral/antibody-mediated immunity) and T cells (cell-mediated immunity), is slower to initiate during infection whilst antigen-receptor genes

undergo rearrangement but produces a highly antigen-specific response to pathogens and infected cells (Chiu and Openshaw, 2014). A key feature of the adaptive immune system is immunological memory, allowing enhanced responses upon subsequent infection with the same pathogen. However, this traditional paradigm is shifting with the discovery of innate-like T cells, such as mucosal-associated invariant T (MAIT) cells, which display features of both the innate and adaptive branches of the immune system and may be important in both host-defence against pathogens and in regulating the dynamics of the microbiome in the lung (Meierovics et al., 2013).

1.2 Dynamics of the lung microbiome

Three main factors dictate the composition of the airway microbiome: invasion of microbes into the lung, removal of microbes, and proliferation and spread of microbes within the lung, all of which are affected by innate and adaptive immune mechanisms (Dickson and Huffnagle, 2015)(O'Dwyer et al., 2016). Changes to any of these three factors can disrupt the lung microbiome which is associated with disease and pathology (Dickson et al., 2014b), as alterations to the microbiome may have considerable consequences. Commensal bacteria in the lung have a protective capacity against invading pathogens, including respiratory viruses, and also regulate local immunity (Ichinohe et al., 2011)(Ferreira et al., 2011)(Sze et al., 2014)(Hartwig et al., 2016). However, it is unknown how interactions of the microbiome with the lung immune system control the balance between a protective immune response and unwanted inflammation (Sze et al., 2014)(Hooper et al., 2012). The main questions that have been highlighted in microbiome research are; what actually is a resident, commensal species, what is transient and what is pathogenic? (Dickson et al., 2013). The processes needed to cause a commensal species to become pathogenic still remain to be determined, but are likely driven by impaired immune processes, and have implications for both acute infections and chronic lung diseases.

1.2.1 Acute dysbiosis in respiratory infections

Short-term microbial dysbiosis is observed in acute infections as microbial niches become dominated by certain species of bacteria (Dickson and Huffnagle, 2015), which normally reside in the respiratory tract as commensals (Pichichero and Almudevar, 2016). Three species that are commonly associated with a multitude of respiratory infections are nontypeable *Haemophilus influenzae* (NTHi), *Moraxella catarrhalis,* and *Streptococcus pneumoniae* (Pichichero and Almudevar, 2016)(Slack, 2017)(Cillóniz et al., 2016)(Faden, 2001). Of the three, NTHi is a major

cause of otitis media, sinusitis and pneumonia in children and adults (Van Eldere et al., 2014)(Slack, 2017)(Teele et al., 1989)(Murphy, 2003)(Behrouzi et al., 2017).

1.2.1.1 Nontypeable *Haemophilus influenzae* in the airway

Haemophilus influenzae is a gram-negative coccobacillus which is a member of the phylum Proteobacteria and typically resides in the nasopharynx of most healthy adults (King, 2012). However, NTHi can be found throughout both the upper and lower respiratory tract (Duell et al., 2016)(Clementi and Murphy, 2011).

H.influenzae can be divided into either encapsulated/typeable or non-encapsulated/nontypeable strains (NTHi) (Duell et al., 2016)(King, 2012). Encapsulated/typeable strains are further divided into six different serotypes (named A-F) depending on the polysaccharides present in their capsule and these polysaccharides constitute targets for vaccination, as is the case for the *H.influenzae* B (HiB) vaccine. NTHi on the other hand, which lacks a capsule, cannot be serotyped in this manner. NTHi is also an incredibly diverse bacterial species, as determined by multilocus sequence typing (MLST) (Schumacher et al., 2012). In culture, NTHi is catalase-positive and can be grown on chocolate agar at 37°C in 5% CO_2; aerobic growth requires hemin and nicotinamide adenine dinucleotide (NAD) (Thjötta and Avery, 1921)(King, 2012)(Kirkham et al., 2013)(Duell et al., 2016).

NTHi typically colonises the upper respiratory tract as a commensal bacteria beginning in infancy. Approximately 20% of children will have nasal cavity colonisation in their first year, rising to ~58% for children aged 5-6 years (Howard et al., 1988). By adulthood, the majority of people will have upper airway colonisation, with acquisition of new strains occurring transiently. It is thought that the nasopharynx acts as a reservoir and that NTHi becomes pathogenic by spreading into the lower airways (King, 2012). However, it remains unknown as to why NTHi spreads into the lower airways in the first place, although individuals with underlying comorbidities are more susceptible to lung colonisation (Van Eldere et al., 2014). NTHi is known to survive intracellularly, with both epithelial cells and macrophages appearing to be the primary targets (Morey et al., 2011)(Forsgren et al., 1994) (Clementi and Murphy, 2011). NTHi has been shown to survive in macrophages for up to 72 hours *in vitro* (Craig et al., 2001) and intracellular survival of NTHi is believed to be vital to its persistence and pathogenesis in the lung (Clementi and Murphy, 2011). Other survival mechanisms include the formation of biofilms, which protect NTHi from antibiotic treatment and the immune response (Swords, 2012)(Langereis and Hermans, 2013) and

expression of immunoglobulin (Ig)A proteases, which may impair mucosal humoral immunity to NTHi (Murphy et al., 2015).

1.2.1.2 NTHi as an infectious pathogen

NTHi is a leading cause of respiratory tract infections in both the young and old, although it can cause infections in any age group. Due to the introduction of the HiB vaccine, the majority of *H. influenzae* infections that now occur in children are caused by NTHi (Duell et al., 2016)(Giufrè et al., 2015)(Cerquetti and Giufrè, 2016). NTHi infection in childhood is associated with acute otitis media and will typically have affected 83% of children by the time they are 3 years old, with recurring infections occurring throughout childhood (Van Eldere et al., 2014)(Monasta et al., 2012)(Teele et al., 1989). Up to 20% of children will suffer with recurrent episodes of infection within a single year (Faden, 2001). As well as requiring antibiotics for treatment which can lead to antibiotic resistance, further complications include meningitis and hearing loss (Vergison et al., 2010)(Monasta et al., 2012). NTHi is also a cause of many other clinically important infections, including bacterial conjunctivitis, sinusitis, pneumonia and bacteraemia (Langereis and De Jonge, 2015)(Van Eldere et al., 2014)(Cillóniz et al., 2016)(Slack, 2017).

1.2.2 Chronic dysbiosis in lung disease

Whether an altered microbiome is a cause or consequence of chronic disease process is unclear, but dysbiosis of the microbiome is also observed in lung diseases such as cystic fibrosis, asthma, and chronic obstructive pulmonary disease (COPD) (Molyneaux et al., 2013)(Dickson et al., 2014b)(Hilty et al., 2010)(Wilkinson et al., 2017)(Sze et al., 2014)(Shukla et al., 2017)(O'Dwyer et al., 2016). Of these diseases, COPD is a major cause of morbidity and mortality, with disease progression often driven by exacerbations, and is currently the only chronic disease that is still increasing in incidence (World Health Organization, 2017)(Lopez et al., 2006)(Shukla et al., 2017)(Donaldson et al., 2002).

1.2.2.1 COPD: An overview

COPD is a progressive disease of the respiratory tract that is one of the leading causes of mortality worldwide (Mannino and Kiriz, 2006)(Cosio et al., 2009). According to the World Health Organisation (WHO), COPD affects over 64 million people worldwide, with an estimated 3 million

deaths in 2015 alone, with current projections indicating that COPD will become the 3rd biggest cause of death globally by 2020-2030 (World Health Organization, 2017)(Moghaddam et al., 2011)(Sheikh et al., 2016). The greatest risk factor for the development of COPD is a history of heavy tobacco smoking, although genetic factors, indoor use of biomass fuels, outdoor air pollution and exposure to dust and chemical irritants are considered to be additional risk factors for the development of COPD (Fairclough et al., 2008).

The common features of COPD are progressive irreversible airflow limitation and inflammation. COPD patients are susceptible to bacterial and viral infection, which typically result in recurrent exacerbations of disease. Exacerbations are defined as acute deterioration of symptoms leading to a sudden reduction in lung function, which does not recover to baseline, and potentially death. Overall, exacerbations promote disease progression, leading to structural remodelling of the lung architecture and reduced lung function, as measured by force expiratory volume in one second (FEV_1) (Finney et al., 2014)(Moghaddam et al., 2008)(Donaldson et al., 2005). Management of COPD usually requires regular medication over the life of the patient, including bronchodilators and inhaled corticosteroids (ICS) (Andersson et al., 2002). As exacerbations of COPD are thought to be driven by bacterial infection, COPD patients are usually treated with antibiotics, which increases the risk of antibiotic resistance and has an associated economic cost. Despite infiltration into the COPD lung, innate and adaptive immune cells seem unable to adequately clear bacterial infection.

1.2.2.2 Bacterial colonisation in COPD

A common hallmark of COPD is colonisation of the airways by potentially pathogenic bacteria (Sze et al., 2014). Current hypotheses for certain bacteria transitioning from being a commensal to pathogenic organism and colonisation of the lower airway in COPD involve smoke-induced changes to the oral microbiome and smoking-induced impairment of mucociliary function (Charlson et al., 2010). These may lead to a dysfunctional immune response against commensal bacteria and continuous inflammatory damage to the lung epithelium (Dye and Adler, 1994)(Sze et al., 2014). In contrast to what was previously thought, multiple studies have shown that there appears to be no difference in total bacterial load between non-smokers, smokers and COPD groups (Sze et al., 2012)(Huang et al., 2010). Therefore, the symptoms of COPD are not simply caused by an increase in the absolute amount of bacteria in the lungs. In this respect, COPD is different to other respiratory diseases, such as cystic fibrosis, where total bacterial load increases but diversity decreases (Sze et al., 2012). Rather, COPD patients have a microbial dysbiosis which

occurs with a reduction in microbial diversity alongside an overgrowth of certain bacterial species compared to non-COPD lungs (Sze et al., 2014). In particular, NTHi, *Moraxella catarrhalis,* and *Streptococcus pneumoniae* are commonly found in the airways of COPD patients. (Sethi et al., 2002)(Wilkinson et al., 2017)(Barker et al., 2015). Of the three, NTHi is the bacteria most commonly detected in the COPD airway during exacerbation but also induces inflammation in stable state (Hill et al. 2000)(Papi et al., 2006)(Wilkinson et al., 2017)(Sethi and Murphy, 2008).

1.2.2.3 NTHi and its role in COPD

It appears that acquisition of new strains of NTHi into the lung, as opposed to infection with new species of bacteria, increases the risk of COPD exacerbation, driving disease progression (Sethi et al., 2002). Of all the bacterial species found in the COPD lung, NTHi is the most commonly detected organism and is found in the lower respiratory tract in approximately 30% of COPD patients (Moghaddam et al., 2011). In addition to colonisation of the airway, NTHi is associated with up to 80% of exacerbations of COPD (King, 2012)(Van Eldere et al., 2014) and has been isolated in the sputum of COPD patients during exacerbation (Bandi et al., 2003)(Berenson et al., 2006a).

Current opinions regarding NTHi in COPD exacerbations suggest that it is not just the presence of NTHi in the airway that leads to exacerbations, but the changes in the frequencies of different NTHi strains and acquisition of new strains (Sethi et al., 2002)(Cerquetti and Giufrè, 2016). Interaction of bacterial species may also synergistically promote inflammation, such as combined infection with NTHi and *Streptococcus pneumoniae* (Lim et al., 2008). Viral infections such as rhinovirus, respiratory syncytial virus (RSV) and influenza are also major causes of exacerbation in COPD (Buss and Hurst, 2015)(Zwaans et al., 2014). Furthermore, there may be interactions between bacterial and viral infections in the lung which enhances immunopathology (Brundage, 2006), with exacerbations of COPD often being associated with co-infection (Papi et al., 2006), particularly *H. influenzae* strains and rhinovirus (Wilkinson et al., 2006)(Wilkinson et al., 2017).

In mouse models, exposure of NTHi lysate to the lungs has been shown to rapidly induce expression of NF-κB and proinflammatory cytokines, as well as recruitment of macrophages, neutrophils and $CD8^+$ T cells into the airways (Moghaddam et al., 2008) all of which are common features of COPD. Infection of NTHi in the presence of smoke is known to cause greater inflammation in mouse lungs compared to infection with NTHi without smoke (Gaschler et al., 2009). Mice exposed to both cigarette smoke (CS) and NTHi show symptoms similar to COPD; such as emphysema, lung inflammation and increased levels of mucin genes (Ganesan et al.,

2014). These dual exposed mice also displayed greater viral persistence when challenged with rhinovirus, compared to mice exposed to smoke or NTHi alone, indicating that dual exposure of the lungs to smoke and bacterial infection may worsen lung disease.

Overall, NTHi plays a major role in the pathogenesis of acute infections and in the progression of chronic diseases. Preventing infection and restraining inflammation induced by NTHi may be vital to controlling NTHi in the airway.

1.2.3 Treatments to prevent NTHi infection and lung inflammation

Two approaches commonly used to control the effects of microbial dysbiosis and inflammation are vaccination and corticosteroids. The former is aimed at preventing infection and the associated pathology by enhancing immunity, whereas the latter is aimed at reducing inflammation caused by dysfunctional or inappropriate immune responses. The relevance and consequences of these two approaches to treating NTHi infection and COPD exacerbation are discussed below.

1.2.3.1 Vaccination strategies to combat NTHi

As NTHi is an increasingly important pathogen the development of a vaccine for at-risk groups is desperately needed in order to combat infection and attempt to reduce acute exacerbations of COPD. However, in contrast to the HiB vaccine, the development of an effective vaccine for NTHi has been hampered by the fact that NTHi lacks a polysaccharide capsule, which may reduce the ability of the humoral response to target NTHi. NTHi is also incredibly heterogeneous, even within sample sites, and undergoes antigenic drift, so creating a vaccine which targets conserved antigens expressed by all strains of NTHi is difficult (Cerquetti and Giufrè, 2016)(Behrouzi et al., 2017)(King, 2012)(Duim et al., 1996). Oral vaccines involving inactivated NTHi have been trialled with some apparent success, causing some reduction in exacerbations of disease in COPD patients (Arandjus et al., 2006). However, further trials are needed in much larger cohorts before any conclusions can be made. Outer membrane proteins, lipooligosaccharide (LOS), *H. influenzae* protein D and adhesins have been proposed as potential vaccine candidates, but their efficacy remains to be seen (Cerquetti and Giufrè, 2016)(Murphy, 2015)(Behrouzi et al., 2017). Given that there is no vaccine currently available, the immune response to NTHi needs to be explored in order to understand the nature of lung immunity to NTHi and whether it can be modulated for therapeutic effect.

1.2.3.2 Immunomodulation of the immune response by steroids

Chronic respiratory diseases such as COPD and asthma are commonly treated with the use of ICS, such as fluticasone propionate and budesonide, which are anti-inflammatory drugs that have been shown to improve symptoms and reduce acute exacerbations (Burge et al., 2000)(Calverley et al., 2007)(Woods et al., 2014). Fluticasone has previously been shown to inhibit signalling of NF-κB, which may limit inflammation as NF-κB is required for interferon (IFN)γ expression (Escotte et al., 2003)(Blanco et al., 2006). However, current research is indicating that these drugs may not be as effective as first thought at improving mortality and controlling disease progression (Yang et al., 2012) and may only be effective in certain populations of patients (Celli et al., 2015). Chronic use of corticosteroids has also been implicated in the rising occurrence of community-acquired pneumonia amongst COPD patients (Festic and Scanlon, 2015)(Calverley et al., 2007)(Williams et al., 2017)(Calverley et al., 2011), although the issue may be confounded by trials involving different durations, potency and concentration of steroid used (Festic and Scanlon, 2015). As corticosteroids function by supressing immune activation and inflammation, but do not affect bacterial clearance (Euba et al., 2015), their use in treatment of chronic respiratory diseases may actually prevent an adequate immune response to respiratory pathogens, which may enable further bacterial colonisation of the airway. Steroids may also have direct effects on bacteria that favour persistence, such as by promoting biofilm formation (Earl et al., 2015).

Overall, understanding the complex interplay between the lung microbiome, species associated with dysbiosis and the host immune response may lead to better treatments to improve the pathogenesis of chronic lung diseases (O'Dwyer et al., 2016). Improving knowledge of the lung immune response to NTHi is therefore required in order to design appropriate therapeutics aimed at controlling the effects of microbial dysbiosis.

1.3 Lung immunity in NTHi infections and chronic respiratory diseases

Impairment in either innate and/or adaptive immunity (such as smoke-induced changes) may lead to bacterial infection and contribute to immune dysfunction, resulting in inappropriate, detrimental damage of the lung. In diseases such as COPD, abnormal inflammatory responses and impaired immune cells may be involved in the development and progression of disease. An overview of the immune cells involved in lung inflammation is shown in figure 1.1 and discussed below.

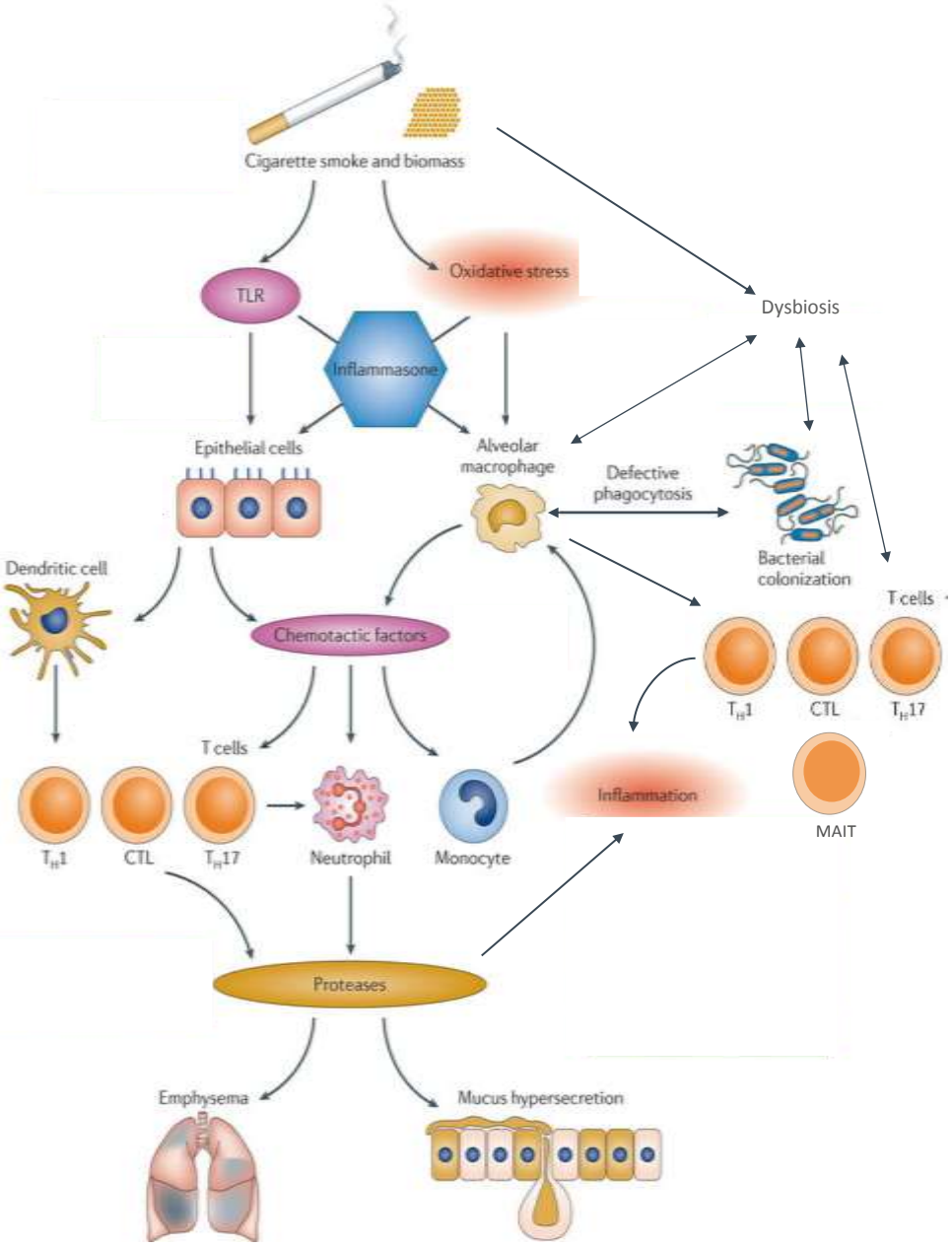


Figure 1.1 – Overview of immunity in the lung in chronic lung disease. Adapted from (Barnes, 2013). Inflammatory stimuli such as cigarette smoke may promote microbial dysbiosis either directly or indirectly by perturbing normal macrophage function and allowing bacterial colonisation. Impaired macrophage function may also result in dysfunctional T cells responses, furthering inflammation and microbial dysbiosis. Activated macrophages and epithelial cells can also release chemoattractants that recruit T cells and neutrophils, which subsequently release proteases and cause damage to the lung architecture.

1.3.1 Mechanical immunity

One of the first lines of defence against invading pathogens is mucus production and mucociliary clearance, where the mechanical beating of ciliated epithelial cells remove pathogens from the lung (Finney et al., 2014)(King, 2012)(King and Sharma, 2015). Smoking may impair this process leading to compromised first line defence against pathogens (Dye and Adler, 1994). NTHi can bind to mucus via its P5 membrane protein, which in cases of impaired mucociliary clearance, may allow NTHi to proliferate in stagnant mucus and lead to colonisation of the lung (Miyamoto and Bakaletz, 1996). NTHi may also actively subvert clearance by expression of cell wall LOS, which has been proposed to impair mucociliary activity, further promoting its persistence in the airway (Denny, 1974)(King and Sharma, 2015)(Pang et al., 2008).

1.3.2 Epithelial cells

Epithelial cells line the lung and provide an effective barrier by forming tight junctions. They also have innate immune function through expression of pattern recognition receptors (PRRs) (Martin and Frevert, 2005), release of antimicrobial peptides (McCormick and Weinberg, 2010) as well as cytokines and chemokines that modulate the immune system in response to pathogens (Khair et al., 1996). Epithelial cells are potent sources of proinflammatory cytokines and interferons, namely IFNβ, following viral challenge, which modulate the antiviral response (Gaajetaan et al., 2012)(Guillot et al., 2005). Culturing of human bronchial epithelial cells with NTHi has been shown to lead to activation of toll-like receptors (TLRs) (King and Sharma, 2015) and increased expression of proinflammatory cytokines, including interleukin (IL)-6, IL-8 and tumour necrosis factor (TNF)α, thus possibly contributing to recruitment of neutrophils as well as inflammatory damage in the COPD airway (Moghaddam et al., 2011).

1.3.3 Neutrophils

Neutrophils are present in the airways and are a vital part of the innate immune system (Finney et al., 2014). They are typically recruited to sites of infection by IL-8 and phagocytose bacteria and release cytokines to amplify the immune response. Increased numbers of neutrophils are detected in the COPD lung (Pesci et al., 1998) but these cells may have impaired phagocytosis of bacteria due to cigarette smoke exposure (Stringer et al., 2007). Elastase secreted by neutrophils enables them to clear infection but can cause considerable tissue damage in immune dysfunction and thus may contribute to lung inflammation in chronic diseases (Wilson, 1998).

1.3.4 Macrophages

Macrophages are mononuclear immune cells that are critical to host defence and display a high degree of plasticity by changing their functional phenotypes depending on the cytokine environment (Stout et al., 2005). In the lung, alveolar macrophages are key orchestrators of the immune response (Barnes, 2004) where they maintain lung homeostasis and protect the lung from pathogens. In the airways, macrophages are particularly abundant and are the predominant cell type recovered from the BAL fluid of COPD patients (Shapiro, 1999) and are increased in the COPD lung airway (Hogg et al., 2004).

Recognition of pathogens is typically driven by innate PRRs such as the TLRs (Janeway and Medzhitov, 2002). Macrophages may kill pathogens directly through release of reactive oxygen species (ROS) or recruit other immune cells by cytokine signalling to facilitate microbial killing (Barnes, 2004). NTHi infection of human alveolar macrophages results in production of ROS and proteases that may kill NTHi (King et al., 2015), but could lead to overproduction of ROS in chronic NTHi infection, which may result in lung oxidative stress and promote lung tissue damage.

Macrophages are also important in activating and co-ordinating the adaptive immune response (Unanue, 1984). Macrophages typically produce immune cytokines such as the pro-inflammatory TNFα, as well as a variety of interleukin cytokines, such as IL-1β, IL-6, IL-8 and IL-12 (Moghaddam et al., 2011). IL-8 and IL-12 are particularly important for neutrophil recruitment and T cell activation respectively, both of which result in the further release of cytokines and enhance inflammation. However, alveolar macrophages from COPD lungs have reduced production of TNFα, IL-1β and IL-8 to NTHi antigens (Berenson et al., 2006b), as well as impaired TLR signalling (Berenson et al., 2014). Cytokine production by macrophages in response to NTHi is reduced by exposure to cigarette smoke (Metcalfe et al., 2014), suggesting that impaired macrophage responses to bacteria may promote bacterial survival and colonisation. Airway macrophages from healthy people and normal smokers have shown inhibited release of IL-8 and TNFα when exposed to corticosteroids, however these drugs often appear ineffective in macrophages from COPD airways (Culpitt et al., 2003)(Barnes, 2004). A similar response is found in asthma, where macrophages from severe asthmatics appear to be corticosteroid insensitive (Bhavsar et al., 2008).

Another key function of macrophages is their ability to phagocytose pathogens to clear infection or cellular debris to prevent autoimmunity (Schaible, 2015). Following adhesion, NTHi is phagocytosed in a manner that is dependent on actin polymerisation and phosphatidylinositol 3-kinase (PI3K) (Martí-Lliteras et al., 2009). However, macrophages from COPD patients have

impaired phagocytosis of NTHi (Taylor et al., 2010)(Berenson et al., 2006a)(Berenson et al., 2013) which appears to be independent of surface expression of TLRs or receptors involved in phagocytosis (Taylor et al., 2010) but correlates with COPD severity (Berenson et al., 2013). Phagocytosis of NTHi also appears to be inhibited by cigarette smoke extract and this may be due to impaired PI3K signalling (Martí-Lliteras et al., 2009). Defective phagocytosis by pulmonary macrophages could explain the persistence of NTHi in the COPD airway, although intracellular killing appears unaffected by COPD status (Berenson et al., 2006a) and NTHi may survive within macrophages and allow it to persist in the airway (Craig et al., 2001).

Following phagocytosis, macrophages can process pathogens for antigen presentation to T cells. In the case of NTHi, intracellular processing is dependent on the phagolysosomal pathway (Martí-Lliteras et al., 2009). Expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules such as CD80 and CD86 allow macrophages to function as professional antigen presenting cells (APCs), capable of activating T cells (Pons et al., 2005)(Venet et al., 1985). Macrophages also express inhibitory proteins such as programmed death ligand 1 (PDL1) (Erickson and Gilchuk, 2012) (Staples et al., 2015), which signals via programmed death 1 (PD1) expressed on T cells and leads to inhibition of T cell proliferation and activation (Keir et al., 2007).

Despite much research regarding innate immune cells such as macrophages in COPD and in NTHi infections, less is known about the role of adaptive immune cells. Control of the lung microbiome by cell-mediated immunity is not well understood, neither is it known how T cells are involved in the response to NTHi infection.

1.4 T cells

T cells are a major component of the adaptive immune system and are vital in the control of intracellular and extracellular pathogens. One of the fundamental hallmarks of T cells is their ability to respond to pathogens in an antigen-specific manner whilst limiting responses against self-antigens to prevent autoimmunity. Conventional T cells specifically recognise antigens via their T cell receptors (TCRs) which bind to foreign antigens presented by MHC molecules; these antigens are typically short peptide sequences of approximately 8-20 amino acids in length (Rossjohn et al., 2014). Whilst the majority of T cells express TCRs which are comprised of alpha and beta subunits and typically recognise peptide antigens, a smaller population of T cells express TCRs with gamma and delta subunits, which are thought to recognise small phosphorylated antigens (Davis et al., 1998)(Attaf et al., 2015)(Singhal et al., 2013). T cells are defined by their

surface expression of co-receptors and as such, can be characterised into CD4+CD8- or CD4-CD8+ subsets.

1.4.1 T cell subsets

CD4+ cells are classically described as T helper (Th) cells, which produce cytokines and orchestrate the immune response (Luckheeram et al., 2012). Activation of CD4+ T cells is by antigen presentation via MHC-II, which is expressed on the surface of professional APCs, such as dendritic cells (DCs) and macrophages. Cytokine production by T helper cells is typically skewed and enables a more appropriate response depending on the type of pathogen (figure 1.2) (Luckheeram et al., 2012)(Murphy and Reiner, 2002)(Luckheeram et al., 2012). Th1 cells, driven by the transcription factor T-bet, produce IFNγ and are required for combating intracellular infection (Szabo et al., 2003)(Zhu and Paul, 2008)(Mosmann and Coffman, 1989). IFNγ is a potent antimicrobial cytokine which can activate macrophages and promote expression of nitric oxide and other reactive oxygen species, which kill bacteria (Held et al., 1999)(Gao et al., 1997)(Chua et al., 2012). IFNγ is also required for MHC-II upregulation (Steimle et al., 1994). Th2 and Th17 cells typically protect against extracellular bacteria and parasites and release IL-4, IL-13 and IL-5, and IL-17, IL-21 and IL-22 respectively (Szabo et al., 2003)(Murphy and Reiner, 2002)(Zhu et al., 2010)(Zhu and Paul, 2008). The main transcription factors driving Th2 and Th17 cells are GATA-3 and RORγt respectively (Zhu and Paul, 2008)(Zhu et al., 2010)(Ivanov et al., 2006).

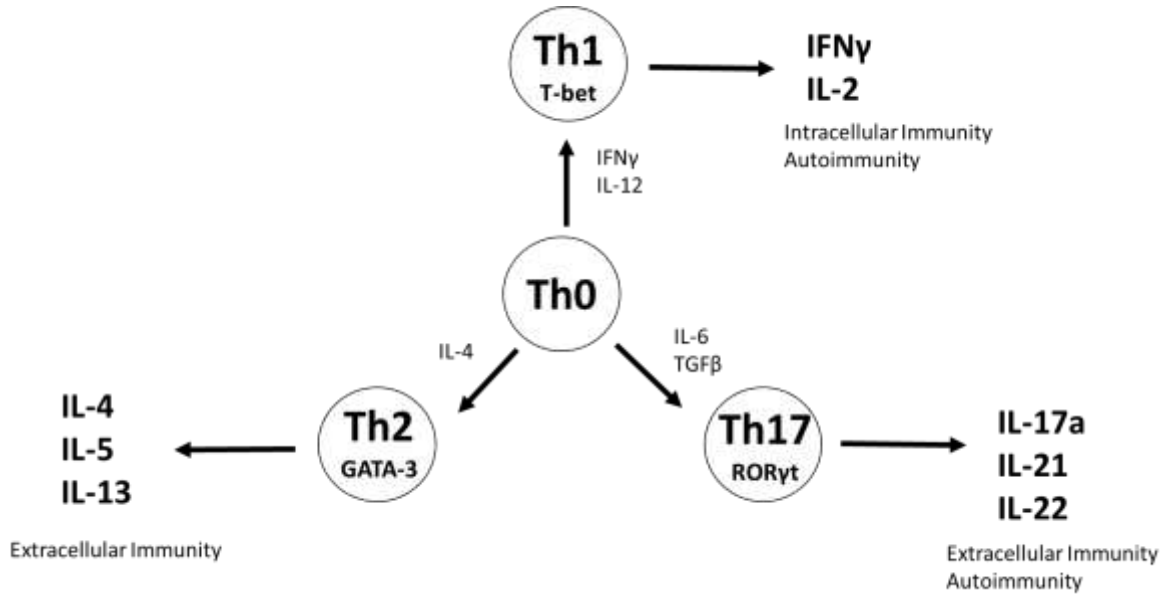


Figure 1.2 – Overview of T helper responses. Naïve T cells (Th0) can differentiate into multiple types of T helper cells based on cytokine stimulation and expression of transcription factors. Three subsets are shown here: Th1 cell, which express IFNγ and are required for immunity to intracellular pathogens; Th2 cell, which express IL-4,-5-13 and required for defence against extracellular immunity to bacteria and are involved in allergy; Th17 cells, which express IL-17a and are required for extracellular immunity, especially at mucosal sites.

CD8+ T cells are the canonical cytotoxic cell of adaptive immunity, which express cytotoxic mediators such as granzyme and perforin that make them capable of destroying bacterially or virally infected host cells by inducing apoptosis (Lewinsohn et al., 2011)(Lieberman, 2003)(Hodge et al., 2006). CD8+ recognise their targets cells through MHC-I antigen presentation. Research evidence suggests that defining CD8+ cells as cytotoxic may be inaccurate as CD4+ cells have also been shown to have cytotoxic capabilities (Wilkinson et al., 2012)(Marshall and Swain, 2011).

Both CD4-CD8- and CD4+CD8+ cells have also been identified although less is known about their function in health and disease (Martina et al., 2015)(Parel and Chizzolini, 2004).

T cells can also be categorised as either naïve, central memory or effector memory by expression of surface proteins such as CD45RA and -RO (Berard and Tough, 2002)(Sallusto et al., 2004). Antigen-inexperienced naïve T cells require MHC-TCR signalling and co-stimulation of molecules, such as CD28 and CD40L, and are slow to activate and differentiate. On the other hand, antigen-experienced memory T cells can quickly activate when they encounter their cognate antigen although co-stimulation by different receptors, such as CD27, OX40 and 4-1BB, is still required to ensure robust memory responses and T cell survival (Chen and Flies, 2013)(Dawicki et al., 2004)(Hendriks et al., 2003).

1.4.2 T cell development

Beginning as haematopoietic stem cells in the bone marrow, T cell precursors traffic through the blood to the thymus where they undergo multiple steps of development. When these T cell precursors initially enter the thymus, they lack expression of a TCR, CD4 and CD8 and are termed CD4-CD8- double negative (DN) thymocytes (Luckheeram et al., 2012)(Germain, 2002). DN thymocytes can be divided into four sub-stages, determined by their expression of CD25 and CD44: DN1 (CD25−CD44+); DN2 (CD25+CD44+); DN3 (CD25+CD44−) and DN4 (CD25−CD44−) (Godfrey et al., 1993). At the DN3 stage, TCR beta chain rearrangement occurs and is followed by expression of a pre-alpha TCR and CD3. Signalling though this pre-TCR causes the TCR beta chain to stop rearranging, activates expression of the CD4 and CD8 co-receptors, hence the thymocytes become double positive (DP). At this stage, TCR alpha chain recombination is initiated (Germain, 2002)(Krueger et al., 2017). Approximately 90% of DP thymocytes undergo death by neglect because their TCRs interact too weakly with self-peptide-MHCs on cortical epithelial cells, so do not receive sufficient survival signals. Thymocytes that bind too strongly to self-peptide-MHCs (approximately 5%) may cause autoimmunity and are deleted by apoptosis, a process called

negative selection. The remaining cells with TCRs that bind with an intermediate intensity are positively selected for, proliferate and undergo lineage-specific differentiation into either CD4+ or CD8+ T cells (Germain, 2002)(Morris and Allen, 2012)(Starr et al., 2003).

1.4.3 T cell activation

Following maturation, conventional T cells exit the thymus as naïve, antigen-inexperienced cells and enter blood and lymphatic circulation (Broere et al., 2011). In order for a T cell to become fully activated, they must receive three distinct signals or will otherwise become anergic or undergo apoptosis (Goral, 2011)(Smith-Garvin et al., 2009).

Lymph nodes are where DCs, which are the archetypal APC for activating naïve T cells, accumulate having sampled antigens and present these through the MHC class I or II molecules to the naïve T cell TCR (Broere et al., 2011)(Yin et al., 2012). Macrophages also express both MHC molecules and can present to T cells (Keskinen et al., 1997)(Pozzi et al., 2005)(Kopf et al., 2015). Presentation of antigen by MHC molecules to T cells is the first step (signal 1) in the process of T cell activation.

In the event that the TCR is specific for the antigen presented, T cells upregulate co-stimulatory molecules allowing further signalling from the APC (signal 2) (Smith-Garvin et al., 2009)(Jain and Pasare, 2017)(Schnare et al., 2001)(Pasare and Medzhitov, 2004). Important surface co-stimulatory molecules expressed on APCs include CD80 and CD86 (B7-1 and -2), which interact with CD28 on naïve T cells (Riley and June, 2005)(Goral, 2011). Inducible co-stimulator (ICOS) and CD40L also play substantial roles in co-stimulation of T cells (Smith-Garvin et al., 2009).

The third signal involved in activation of T cells is cytokine stimulation, typically derived from macrophages or DCs by TLR signalling, which is required to induce cytotoxic function in CD8+ T cells and proliferation and effector functions of CD4+ T cells (Goral, 2011)(Curtsinger and Mescher, 2010). IL-12, IL-1β and IFNβ have all been implicated as signal 3 co-stimulatory cytokines in T cell activation (Goral, 2011). Once instructed to activate, the T cells undergo clonal expansion and produce cytokines or perform other effector functions at the site of infection, as well as forming memory cells for subsequent responses (Broere et al., 2011)(Henrickson and von Andrian, 2007).

Overall, the three signal paradigm involves tightly regulated mechanisms aimed at preventing autoimmunity and ensuring correct activation of T cells in response to pathogens. Although alveolar macrophages are regarded as poor APCs, especially when compared to DCs, infection of macrophages does activate all three signals; processing of antigen and presentation via MHC,

upregulation of co-stimulatory molecules and release of cytokines, including IL-12 (Weinberg and Unanue, 1981)(Isler et al., 1999)(Magnan et al., 1998)(Ye et al., 2009)(Pons et al., 2005). Furthermore, alveolar macrophages are not the only macrophage population in the lung. Whilst alveolar macrophages primarily function by removing microbes from the alveolar space by phagocytosis, interstitial macrophages, which are present in the parenchymal space, may have a greater role in antigen presentation to T cells as they express greater levels of MHC-II compared to alveolar macrophages (Kopf et al., 2015)(Steinmüller et al., 2000).

1.4.4 T cells in COPD

CD8+ T cells are one of the main types of immune cells that increase in the airway and lung parenchyma in COPD, and may be a cause of airway inflammation (O'Shaughnessy et al., 1997)(Fairclough et al., 2008)(McKendry et al., 2016). This inflammatory response is thought to be driven in part by the recurrent infection and colonisation of the lower airways by bacteria (Moghaddam et al., 2011). In contrast, a decrease in proportion of CD4+ cells is found in the COPD lung compared to healthy lungs (McKendry et al., 2016)(Zhu et al., 2009) and COPD CD4+ T cells may have impaired IFNγ production (Freeman et al., 2014). T cells appear to be sensitive to the inhibitory actions of steroids, as glucocorticoids have been shown to induce apoptosis, inhibit T cell proliferation and inhibit cytokine and cytotoxic responses (Spahn et al., 1996)(Tuosto et al., 1994)(Shoeib et al., 2014)(Schleimer et al., 1984)(Herold et al., 2006). Steroid suppression of immune responses, particularly T cells, in COPD may be an important step in either the initial or further colonisation of the lower airway by bacterial pathogens such as NTHi.

T cell activation in COPD typically results in a Th-1 mediated immune response and the production of IFNγ (Fairclough et al., 2008). Both CD8+ cells in the lung and IFNγ levels are found to correlate with airflow limitation and severity of emphysema, indicating a direct role for these cells in immune-related pathogenesis of COPD (Cosio et al., 2009). There may also be a role for autoimmunity in COPD, as T cells specific for autoantigens derived from elastin (an extracellular matrix protein required for structural integrity) have also been found and may also play a role in lung destruction and disease progression (Lee et al., 2007)(Gadgil and Duncan, 2008).

Despite an apparent increase in the COPD lung, these CD8+ T cells appear to not grant increased immunity to bacterial and viral pathogens as shown by increased colonisation of the airway and exacerbations in COPD (Saetta et al., 1998) (Fairclough et al., 2008). However, Th1 and CD8+ T cells from COPD lung do produce IFNγ following activation by NTHi (King et al., 2013).

Furthermore, CD8+ T cells have been shown to be vital in controlling and clearing NTHi infection in a rat model (Foxwell et al., 2001) and CD8+ T cells may have dysfunctional cytotoxic functions in COPD (McKendry et al., 2016).

As well as the conventional, adaptive T cells, CD8 is also expressed on subsets of T cells that have innate-like properties and so may also be increased in the COPD lung. One such CD8+ innate-like T cell is the recently identified MAIT cell.

1.5 MAIT cells

In humans, MAIT cells are TCRαβ+ cell that are mainly CD8α+βlow/-, although CD4-CD8- (DN) and CD4+ populations also exist to lesser extents (Treiner et al., 2005)(Reantragoon et al., 2013). These innate T cells comprise up to 10% of peripheral blood (Le Bourhis et al., 2011) and airway (Hinks et al., 2016) CD3+ cells and locate to mucosal sites such as the gut and lungs (Gold et al., 2010). MAIT cells express both the natural killer (NK) cell marker, CD161, and the invariant T cell alpha chain region, Vα7.2, that combines with either a Jα12, Jα20 or Jα33 segment and pairs with a limited oligoclonal repertoire of Vβ chains, which may dictate the strength of antigen-specific responses (Dias et al., 2017)(Reantragoon et al., 2013)(Lepore et al., 2014)(Tilloy et al., 1999)(Martin et al., 2009). MAIT cells are therefore typically characterised as CD3+CD161+Vα7.2+ cells. However, MAIT cells are IL-18 receptor positive (Dusseaux et al., 2011)(Le Bourhis et al., 2010) and also express CD26 (Sharma et al., 2015), which have been shown to serve as an additional MAIT cell markers and may be incorporated into future MAIT definitions. MAIT cells appear to rapidly acquire an effector memory phenotype in the periphery following exit from the thymus, and typically express high levels of CD45RO, CD127 and CD95, but low levels of CD62L (Dusseaux et al., 2011)(Martin et al., 2009). Expansion and acquisition of a memory phenotype appear to be dependent on commensal bacteria, as humans rapidly acquire MAIT cells in the periphery after birth and germ free mice lack MAIT cells (Martin et al., 2009)(Treiner et al., 2003)(Le Bourhis et al., 2010)(Koay et al., 2016). Although first identified in the gut, MAIT cells have been detected in the lungs, liver, skin and female genital tract and are associated with a variety of diseases at these anatomical locations (Hinks et al., 2016)(Meierovics et al., 2013)(Gibbs et al., 2016)(Kurioka et al., 2016)(Reantragoon et al., 2013)(Jeffery et al., 2016)(Johnston and Gudjonsson, 2014).

1.5.1 MAIT cell development

Compared to conventional T cells, little is known about the development of MAIT cells. What is known is that MAIT cells develop in the thymus, but unlike conventional T cells, MAIT cells undergo selection following interaction with DP thymocytes expressing MR1 (Seach et al., 2013)(Treiner et al., 2003)(Tilloy et al., 1999). MAIT cell development is split into three distinct stages, based on expression of CD24 and CD44. Stage 1 MAIT cells are CD24+CD44−, stage 2 are CD24−CD44− and stage 3 are CD24−CD44+ (Koay et al., 2016). Stage 1 MAIT cells are characterised by low expression of a number of key surface receptors, including IL-2Rβ, IL-7Rα and IL-18R, as well as the transcription factor PLZF, compared to stage 3 MAIT cells (Koay et al., 2016). CD161 expression also occurs in the thymus and is lowly expressed at stage 1 but progressively increases by stage 3 (Dusseaux et al., 2011)(Koay et al., 2016). Following egress from the thymus between stage 2 and 3, naïve MAIT cells rapidly expand and acquire an effector memory phenotype in the periphery, in a manner dependent on MR1, commensal bacteria and B cells (Martin et al., 2009)(Treiner et al., 2003)(Le Bourhis et al., 2011). MAIT cells lacking commensal bacteria (i.e germ-free mice) have impaired development and maturation of MAIT cells (Koay et al., 2016). Whilst MAIT cells are capable of producing cytokines such as IFNγ and TNFα in the thymus by stage 3, it is not until maturation in the periphery that MAIT cells become fully functionally competent (Koay et al., 2016).

1.5.2 MAIT cell responses

MAIT cells have some similarity to conventional Th1 cells as they rapidly express both TNFα and IFNγ following activation, but also produce IL-17 in a similar manner to Th17 cells (Dusseaux et al., 2011) (Le Bourhis et al., 2010)(Chua et al., 2012)(Gibbs et al., 2016)(Dias et al., 2017)(Meierovics et al., 2013). As MAIT cells are early responders to infection (Meierovics et al., 2013)(Shaler et al., 2017), these cells may represent an early source of IFNγ which is required for antimicrobial killing (Chua et al., 2012) and to activate the adaptive immune system. Indeed, in bacterially-infected mice that lack MAIT cells, defects are observed in recruitment of conventional CD4+ and CD8+ into the lungs and in control of bacterial growth (Meierovics et al., 2013). MAIT cells also have cytotoxic function by expression of granzyme B and perforin (Kurioka et al., 2015) and have been shown to kill *Escherichia coli* infected cells (Kurioka et al., 2015) as well as inhibit bacterial growth (Chua et al., 2012). An overview of MAIT cell responses and functions is shown in figure 1.3.

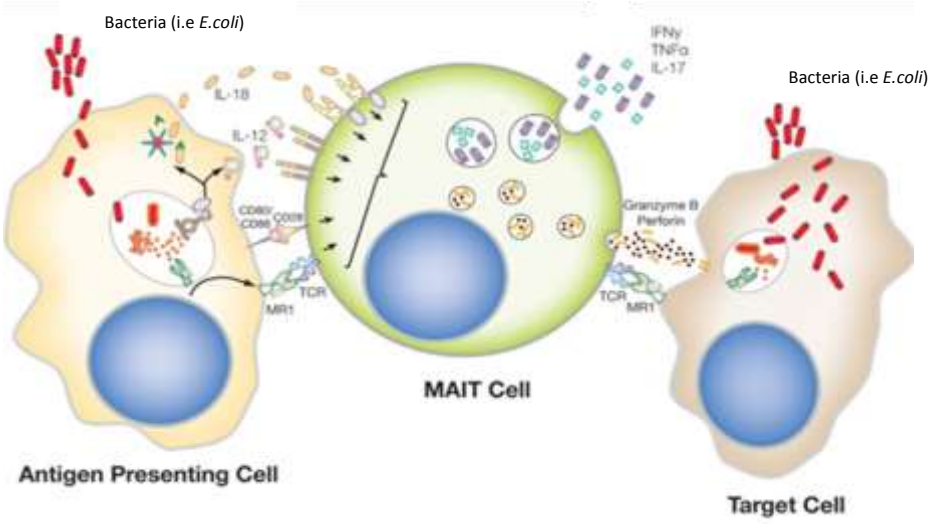


Figure 1.3 – Overview of MAIT activation and effector function. Adapted from (Ussher et al., 2014b). APCs such as macrophages internalise bacteria and become lysed within endosomes. Riboflavin antigens are then loaded into MR1 molecules which are then trafficked to the cell surface. Concurrently, co-stimulatory cytokines such as IL-12 and IL-18 are released by the activated APC. Combined MR1 and cytokine signalling activates MAIT cells, causing them to upregulate and release cytokine and cytotoxic mediators, which can then orchestrate further immune responses or lyse infected target cells.

1.5.3 MAIT antigens and MR1

It has recently been shown that, in contrast to recognising foreign peptides presented by MHC antigen presenting molecules, MAIT cells recognise highly conserved organic metabolites/by-products derived from bacterial vitamin B synthesis, which are presented to MAIT cells by the non-polymorphic MHC-related protein 1 (MR1) (Kjer-Nielsen et al., 2012). Of the vitamin B family, only vitamin B2 (riboflavin) and vitamin B9 (folic acid) pathways have been shown to produce MR1-presented antigens (Kjer-Nielsen et al., 2012). Of the two, only the vitamin B2 pathway has been shown to produce antigens capable of activating MAIT cells, whereas the vitamin B9 metabolites, 6-formyl pterin (6FP) and acetyl-6-formylpterin (Ac-6-FP), do not activate MAIT cells and act as an MR1 antagonist (Birkinshaw et al., 2014). The known antigens derived from the riboflavin pathway are: reduced 6-hydroxymethyl-8-D- ribityllumazine (rRL-6-CH2OH); 7-hydroxy-6-methyl- 8-D-ribityllumazine (RL-6-Me-7-OH); 6,7-dimethyl-8-D-ribityllumazine (RL-6,7-diMe); 5-(2-oxoethylidene-amino)-6-D-ribitylaminouracil (5-OE-RU); and 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (Kjer-Nielsen et al., 2012)(Corbett et al., 2014). In the cases of 5-OE-RU and 5-OP-RU, these are generated when 5-amino-6-D-ribitylaminouracil (5-A-RU) reacts with endogenous glyoxal or methylglyoxal groups respectively (Corbett et al., 2014). Whether there are other vitamin-derived ligands that MAIT cells respond to remains to be determined, but currently only bacteria that have been shown to have a functional vitamin B2 synthesis pathway can

activate MAIT cells, whereas bacteria lacking the B2 pathway cannot activate MAIT cells via MR1 (Le Bourhis et al., 2013). Recent evidence has also indicated that drug metabolites can bind to MR1, including metabolites of salicylates and diclofenac that have MAIT- antagonistic and -agonistic properties respectively (Keller et al., 2017). Figure 1.4 shows an overview of MR1 antigen presentation to MAIT cells and how this contrasts with MHC peptide antigen presentation to conventional T cells. The metabolic pathways which result in the riboflavin antigens is also shown.

MR1 is only found in mammals and the degree of sequence similarity is extremely high across species (Treiner et al., 2005). As vitamin B2 synthesis is restricted to only certain species of bacteria and yeast, the highly conserved MAIT-MR1 axis represents a unique mechanism of immunosurveillance (Reantragoon et al., 2013).

MR1 is ubiquitously found at the transcript level in the majority of cells, yet its expression at the cell surface under non-pathogenic conditions has been difficult to detect (Kjer-Nielsen et al., 2012)(Chua et al., 2011). A handful of studies have reported that MR1 expression increases at the cell surface of epithelial and B cells in response to infection with certain species of bacteria, such as *E.coli* and *Mycobacterium tuberculosis* (Mtb) (Gold et al., 2010)(Salerno-Goncalves et al., 2014) yet the exact mechanisms regulating MR1 expression are not known.

In contrast to other APMs, MR1 molecules do not bind and present endogenous self-ligands at baseline. MR1 is synthesised in the endoplasmic reticulum (ER) and remains there in an incompletely-folded state until riboflavin antigens become available. These antigens are processed independently of the MHC-related antigen processing protein TAP or the proteasome (Huang et al., 2008). Loading of these riboflavin antigens into the MR1 antigen binding site (or 'aromatic cradle') forms a Schiff base and triggers refolding of the MR1 protein, which allows the now completely-folded MR1 to traffic from the ER to the plasma membrane (McWilliam et al., 2016). Most MR1-antigen complexes will then remain at the plasma membrane until they are internalised and degraded. A small amount of MR1 traffics back from the cell surface through endosomal compartments, where it can sample the intracellular environment and acquire new antigens and recycle back to the cell surface (Huang et al., 2008)(Harriff et al., 2016).

The role of antigen presenting cells, such as macrophages, in activating MAIT cells is not fully understood (Gozalbo-López et al., 2009) (Chua et al., 2012), however MAIT cells have been shown to respond to monocytes infected with *E.coli* (Le Bourhis et al., 2010). Since macrophages play a key role in maintaining lung homeostasis, protection from pathogens and can activate

conventional T cells, lung macrophages may be important in activating MAIT cells in response to NTHi.

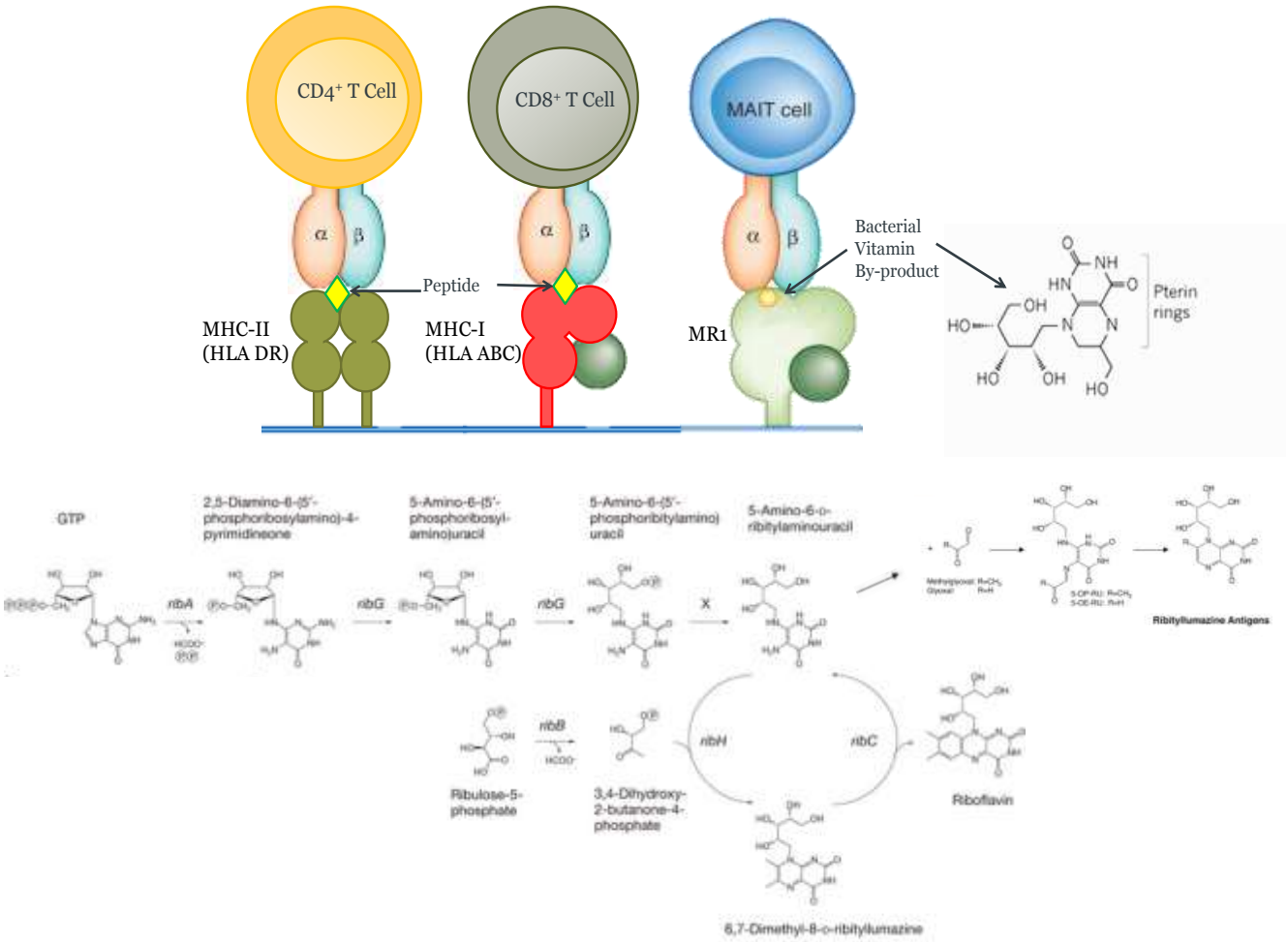


Figure 1.4 – CD4, CD8 and MAIT cell antigen recognition. Adapted from (Kronenberg and Zajonc, 2013). MR1/MAIT ligand produced as by-product of bacterial vitamin B2 synthesis. Adapted from (Chua and Hansen, 2012). Whereas CD4+ and CD8+ conventional T cells recognise peptide antigens presented by MHC-II and MHC-I respectively, MAIT cells recognise organic molecules produced as by-products of bacterial vitamin B2 synthesis which are presented by MR1. The metabolic pathway resulting in the MR1/MAIT agonist antigens is shown adapted from (Keller et al, 2017), and (Corbett et al, 2014).

1.5.4 MAIT cells in lung infections and chronic diseases

Most of what is known about MAIT cells concerns their role in the gut, whilst the function these cells play in the lungs is largely unknown. MAIT cells have been identified in the lungs of patients infected with Mtb, a pathogen that classically causes chronic pulmonary infection, and have been shown to produce IFNγ in response to Mtb-infected lung epithelial cells (Gold et al., 2010). In models of other pulmonary infections, MAIT cells appear to be vital in controlling *Francisella tularensis* infection and recruitment of conventional T cells into the lungs of mice (Meierovics et al., 2013). Since NTHi is a major pathogen that colonises the airways and exacerbates COPD, and

given that MAIT cells have been identified in the lungs in other respiratory infections, MAIT cells may play some role in controlling NTHi infection in COPD. Whilst it is currently unknown whether MAIT cells recognise and are activated by NTHi-infected cells, NTHi does possess a riboflavin synthesis pathway (Saeed-kothe et al., 2004)(Kanehisa and Goto, 2000) and therefore has the potential to activate MAIT cells.

Outside of pulmonary bacterial infections, MAIT cells have been explored in asthma, where they were found to be deficient in the airways of asthmatic patients receiving ICS therapy (Hinks et al., 2015). To date, only two studies have investigated MAIT cells in COPD. MAIT cells are decreased in the blood of COPD patients (Kwon et al., 2015b), which was expanded further by Hinks et al, who found that MAIT cells, but not conventional T cells, were deficient in the blood and airways of COPD patients receiving ICS (Hinks et al., 2016). It is currently not known whether steroids impair MAIT cell function.

1.6 Summary

Microbial dysbiosis is observed in acute respiratory infections and chronic airway diseases. NTHi is a dominant species found in both of these acute and chronic settings. Our current understanding of how the immune system surveys and controls the airway microbiome is inadequate. As NTHi lacks a capsule, expresses IgA proteases and is an intracellular pathogen, antibody-mediated immunity to NTHi may not be effective and therefore cell-mediated immunity may be vital in controlling NTHi infection in the lung.

However, NTHi is highly heterogeneous with a rich diversity of strains, and acquisition of new strains of NTHi commonly occurs, which may drive exacerbations of COPD. Therefore, conventional T cell immunity to specific peptide antigens may not elicit a long-term protective response to NTHi infection as T cells may not have a memory response to newly acquired strains. MAIT cells may therefore be vital in control of NTHi infection in the lung as they elicit both cytokine and cytotoxic response to bacterial infection and recognise highly conserved vitamin antigens, which are likely commonly expressed by all NTHi strains. Impairment or dysfunction of MAIT cells, such as by corticosteroid treatment, may impair MAIT cell function and leave the lung vulnerable to NTHi infection or further colonisation. Currently, it is not known whether MAIT cells can respond to and control NTHi infection and thus it is unclear if MAIT cells have a role in the immune response to NTHi in acute respiratory infections and in chronic lung diseases.

1.7 Hypothesis and aims

The overall hypothesis for this project is that human MAIT cells can demonstrate anti-bacterial immunity to NTHi infection through production of pro-inflammatory cytokines and cytotoxic effector molecules. Thus, MAIT cells may play a key role in lung mucosal immunity.

The specific aims for this project are to:

1. Characterise MAIT cells in the human lung and blood and confirm the effector function of lung MAIT cells by measuring cytokines such as IFNγ, IL-17a, and TNFα, and cytotoxic markers such as granzyme B.
2. Determine which cell types in the lung, such as macrophages, express the MAIT antigen presenting molecule, MR1, and may function as an APC for MAIT cells.
3. Measure MR1 expression on lung and monocyte-derived macrophages (MDM) following NTHi infection. MR1 expression on macrophages will be compared to the conventional antigen-presenting molecules, MHC-I (HLA-ABC) and MHC-II (HLA-DR).
4. Explore further how NTHi infection regulates cell surface expression of co-stimulatory molecules on macrophages.
5. Characterise MAIT cell cytokine responses to NTHi in a human lung explant model and in a blood-derived MDM-T cell co-culture model by measuring expression of the cytokines IFNγ, IL-17a, and TNFα. MAIT cell cytokine responses to NTHi will be compared to conventional CD4+ and CD8+ T cell responses
6. Characterise cytotoxic response of lung and blood-derived MAIT cells to NTHi infection by measuring CD107a, perforin and granzyme B in the lung explant and blood-derived co-culture models. MAIT cell cytotoxic responses to NTHi will be compared to conventional CD4+ and CD8+ T cell responses.
7. Determine whether activation of conventional T cells and MAIT cells is antigen specific by employing the use of MHC and MR1 blocking antibodies.
8. Explore other mechanisms controlling conventional T cells and MAIT activation, such as co-stimulatory molecules and cytokine signalling.
9. Explore the effects of corticosteroids on antigen presentation and conventional T cell and MAIT cell cytokine and cytotoxic responses.

To test these hypotheses and aims, macrophages and T cells will be explored in an *ex vivo* human lung tissue explant model and a peripheral-blood MDM-T cell co-culture model. Flow cytometry will be used to determine whether macrophages express MR1 and whether this is upregulated

during NTHi infection. Other antigen presenting molecules, such as HLA-ABC and HLA-DR (MHC-I and MHC-II respectively) will also be measured. Once MR1 expression and upregulation has been confirmed, the next stage will be to determine whether NTHi-infected macrophages can present to and activate total T cell populations, as measured by IFNγ ELISpot. Following on from this, MDM-mediated T cell activation will be measured by detection of pro-inflammatory cytokines and cytotoxic markers by flow cytometry and ELISA in order to determine if MAIT cells are specifically activated in response to NTHi. These responses will be compared to the responses of lung T cells from NTHi-infected lung tissue explants to ensure that responses seen in the blood model are equivalent to the lung. Blocking of antigen presentation will assess whether T cell activation is antigen-specific. Conventional T cell and MAIT cell numbers and responses will be analysed together with use of steroids, to determine what effect corticosteroid therapy in COPD patients may have on T cell activity.

2. Materials and Methods

2.1 Ethics and donor recruitment

The collection of blood for monocyte-derived macrophage (MDM) and T cell experiments was approved by Hampshire A Research Ethics Committee (13/SC/0416). Inclusion criteria: healthy volunteers were individuals between the ages of 18-65. Exclusion criteria: individuals who had not had a respiratory infection in the previous month and not taken antibacterial, antiviral or anti-inflammatory (including steroids) medication.

Human lung tissue for the explant model and lung macrophage infection was obtained from lung cancer patients undergoing lobectomy performed at Southampton General Hospital and approved by Southampton and South West Hampshire Research Ethics Committee (08/H0502/32). Patients were recruited under an existing, ethically approved collaboration with outpatient clinics and GP surgeries within Southampton. Inclusion criteria: patients over the age of 18; only surplus lung tissue was taken which was not required for diagnostic testing. Exclusion criteria: patients suffering from an infection, including human immunodeficiency virus (HIV) or hepatitis B/C. Patient demographics are displayed in table 2.1 and table 2.2.

Written informed consent was obtained from all participants.

Table 2.1 – Clinical characteristics of lung cancer patients undergoing lung resection surgery, where tissue was used for lung macrophage assays. Data are presented as median and IQR. Ex-smokers were defined as individuals who had stopped smoking for > 6 months. Data were collected by the Target Lung team. No other clinical data were available.

N	7
Age (years)	71.5 (68.75-78.75)
Gender (M/F)	3/3 (1 unknown)
Smoking status (current/former/never/unknown)	0/5/0/2
Pack Year	35 (14.5-37.5)
FEV1 (L)	1.86 (1.413-2.145)

Table 2.2 – Clinical characteristics of lung cancer patients undergoing lung resection surgery, where tissue was used for lung explant assays. Data are presented as median and IQR. Ex-smokers were defined as individuals who had stopped smoking for > 6 months. Data were collected by the Target Lung team. No other clinical data were available.

N	13
Age (years)	69 (57.5-74.5)
Gender (M/F)	8/5
Smoking status (current/former/never/unknown)	5/6/1/1
Pack Year	26.25 (17-45)
FEV1 (L)	2.04 (1.49-2.67)

2.2 Preparation of cells and tissue for infection

2.2.1 Blood monocyte isolation and maturation of MDM

Blood was obtained from healthy volunteers in BD vacutainer lithium heparin blood collection tubes (BD Biosciences, Oxford, UK). Blood was diluted 1:1 with Dulbecco's Phosphate-Buffered Saline (PBS) (Sigma-Aldrich, Gillingham, UK) and carefully layered onto approximately equal volumes of Ficoll Paque-Plus (GE Healthcare, Little Chalfont, UK) for density centrifugation at 800*g*, 20°C for 30 min. Peripheral blood mononuclear cells (PBMC) were harvested from the interface, washed in PBS and centrifuged at 400*g* for 10 min to remove excess platelets and to dilute excess Ficoll. PBMC were counted on a haemocytometer, washed in 50 ml PBS and centrifuged at 400*g* for 5 min, then resuspended in 80 µl sterile magnetic-activated cell sorting (MACS) buffer (2mM EDTA, 0.5% (v/v) Bovine Serum Albumin (BSA) in PBS) per 10^7 PBMC and 10 µl CD14+ magnetic microbeads (Miltenyi Biotec, Surrey, UK) per 10^7 PBMC. PBMC were incubated with the CD14+ beads on ice for 20 min, followed by washing in MACS buffer and centrifugation at 400*g*, 4°C for 5 min. PBMC were resuspended in 500 µl MACS buffer per 10^8 PBMC and allowed to run through a pre-washed LS MACS column (Miltenyi Biotec) connected to a magnetic stand. Any CD14+ PBMC, which are monocytes, are retained in the column whereas CD14- PBMC, which are lymphocytes, are collected as flow through in a collection tube. Once buffer had eluted, the column was washed 3 times with 3 ml MACS buffer, removed from the magnetic stand and 5 ml of MACS buffer was added to the column and plunged through to elute the CD14+ cells in the column into a separate collection tube. Isolation of monocytes by this method have been shown to yield >95% purity (Staples et al., 2012). Monocytes and lymphocytes were counted on a haemocytometer and the positively selected CD14+ monocytes were resuspended at 10^6 cells per ml in RPMI (Sigma-Aldrich) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS), 2 mg/ml L-glutamine, 0.05 U/ml penicillin, 50 µg/ml streptomycin, 0.5 µg/ml amphotericin B (all from Sigma-Aldrich) and 2 ng/ml GM-CSF (R&D Systems, Minneapolis, USA). Monocytes were then seeded at $5x10^5$ cells/well in 48-well culture plates, incubated at 37°C, 5% CO_2, and differentiated into macrophages (MDM) over 12 days, with media replaced every 48 h. The lymphocytes (CD14-depleted PBMC) were then either frozen at -80°C at a concentration of 10^7 cells/ml in 10% DMSO/ 90% FBS (v/v) or used immediately for T cell isolation.

2.2.2 Blood T cell Isolation

Autologous CD3+ T cells were positively selected from the lymphocyte (CD14- depleted PBMCs) flow through fraction using CD3+ microbeads (Miltenyi Biotec). The protocol for T cell isolation is almost identical to that of monocyte isolation, with the only difference being that 25 µl of CD3+ microbeads per 10^7 lymphocytes were used. Volumes of CD3+ beads were initially titrated and 25 µl gave an optimal yield of CD3+ cells. Following MACS separation, positively selected CD3+ T cells were analysed by flow cytometry and were found to have >99% CD3 purity, shown in figure 2.1. T cells were frozen at -80°C in 10% (v/v) DMSO in FBS until needed. CD3+ T cells were chosen, as opposed to sorting for CD4+ and CD8+ T cells, so the whole T cell population could be studied, so as not to exclude double negative (CD4-CD8-) T cells.

In some experiments, MAIT cells were isolated from whole lymphocytes by fluorescence-activated cell sorting (FACS). Briefly, lymphocytes were defrosted at 37°C for approximately 3 min, washed in pre-warmed RPMI and centrifuged at 400*g* for 5 min. Lymphocytes were then resuspended in 1 ml sterile FACS buffer (2mM EDTA, 0.5% (v/v) BSA in PBS) with 2 mg/ml sterile, purified IgG from human serum (Sigma-Aldrich) per FACS tube (BD Biosciences). Cells were then stained with CD3, Vα7.2 and CD161 antibodies (see table 2.3 for full details). After incubation on ice for 30 min, washed and then resuspended in 3 ml sterile FACS buffer. FACS was performed on a 9-colour FACSAria (BD Biosciences) and MAIT cells were sorted into 5 ml sterile RPMI. Median purity of FACS-sorted MAIT cells was greater than 93% (figure 2.2).

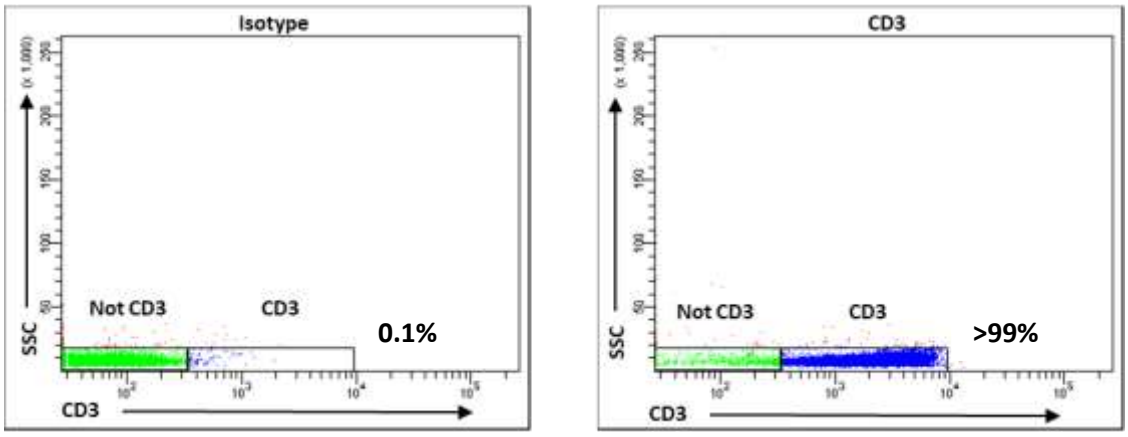


Figure 2.1 – Detection of T cells following CD3+ MACS separation. Lymphocytes were stained using an anti-CD3 antibody and compared to an isotype control (see table 2.3). T cells were detected using flow cytometry. The post-MACS purity was >99%.

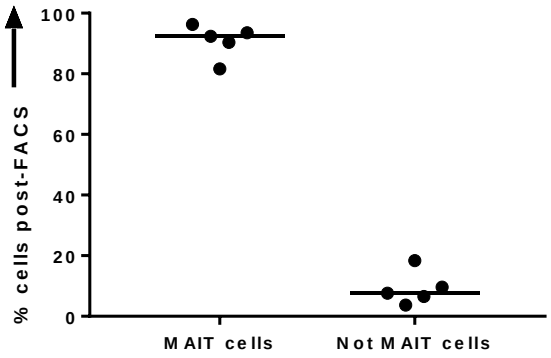


Figure 2.2 – Purity of MAIT cells following FACS. Lymphocytes were stained for CD3, Vα7.2 and CD161 (see table 2.3) and sorted on a 9-colour FACSAria (BD Biosciences). Post-sort flow cytometry revealed that MAIT cell purity was >93%.

2.2.3 Isolation of lung macrophages from tissue

Human lung tissue obtained from patients undergoing lobotomy for clinical reasons and was taken from sites distal to any tumours present, as judged by the surgical and pathology team. Lung tissue was transported in a sealed container containing sterile PBS and was mechanically disrupted using a sterile scalpel. Tissue fragments were then left in PBS for approximately 1 h to allow lung macrophages present in the tissue to egress into the PBS. PBS containing cells washed out from the lung tissue was then passed through 0.70 µm filters into multiple 50 ml collection tubes and centrifuged at 400*g* for 5 min. Cell pellets were resuspended in PBS, combined and made up to a total volume of 20 ml with PBS, before being carefully layered onto approximately equal volumes of Ficoll Paque-Plus (GE Healthcare) for density centrifugation at 800*g*, 20°C for 30 min. Cells at the interface were harvested and washed with 50 ml PBS, centrifuged at 400*g* for 10 min and resuspended in 5 ml PBS for counting on a haemocytometer in the presence of trypan blue to assess cell viability, which was found to be >95%. Cells from lung tissue were resuspended in RPMI (Sigma-Aldrich) supplemented with 2 mg/ml L-glutamine, 0.05 U/ml penicillin, 50 µg/ml streptomycin and 0.5 µg/ml amphotericin B (all from Sigma-Aldrich), seeded at $5x10^5$ cells/well in 48-well culture plates and incubated for 2 h at 37°C, 5% CO_2. Media was then replaced with the same supplemented RPMI as above but with an additional 10% (v/v) FBS and rested overnight at 37°C, 5% CO_2. Resting overnight in the presence of antibiotics and antifungals is important to remove any bacteria or fungi that may already be present in the tissue (Grivel and Margolis, 2009).

2.2.4 Preparation of whole lung explant

Lung tissue was obtained as described above and was cut into approximately $3mm^3$ cubes and washed extensively in RPMI before being placed in RPMI (Sigma-Aldrich, Poole, UK) supplemented with 2 mg/ml L-glutamine, 1% (v/v) gentamicin and 0.5 μg/ml amphotericin B (all from Sigma-Aldrich, Paisley, UK) and incubated overnight 37℃, 5% CO_2, prior to any further manipulation (McKendry et al., 2016).

2.3 Culture, storage and preparation of bacteria

Strains of NTHi were cultured from the sputum of Southampton COPD patients or healthy controls. The sequence types of the four strains of NTHi used were: ST201, ST1447, ST253 and ST14. NTHi were grown and frozen according to an established protocol (Kirkham et al., 2013). Briefly, single colonies of NTHi were isolated from chocolate agar plates that had been incubated overnight at 37℃ and used to inoculate BHI culture media supplemented with 44 mL/L glycerol, 30 mg/L hemin and 10 mg/L NAD. NTHi was grown to mid-log phase and heat-inactivated FCS was added to make a final concentration of 20% (v/v). 1 ml aliquots were then frozen at -80℃. Kirkham et al have shown that NTHi cultured and stored in this way retain viability and immunostimulatory properties (Kirkham et al., 2013). Viability of NTHi over time is shown in figure 2.3. NTHi was grown with the assistance of Denise Morris and Rebecca Anderson.

E.coli BL21 was kindly provided by Alastair Watson. Briefly, 5 ml of starter culture of *E.coli* BL21 was added to 500 ml of LB media and grown at 37℃ until absorbance reached λ=600 nm, typically 3 hours. *E.coli* BL21 was then frozen in LB media and 1 ml aliquots were frozen at -80℃.

Cell culture media used for the NTHi and *E.coli* infection assays consisted of RPMI with 0.1% (v/v) FBS and 2 mg/ml L-glutamine; this media is referred to as reduced serum RPMI (RS-RPMI) without antibiotics. Penicillin and streptomycin were not added as antibiotics would be detrimental to the bacteria. Amphotericin was also omitted in this media. Media used after the infection period is the same as above, but with 0.05 U/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml amphotericin B (all from Sigma-Aldrich); this media is referred to as RS-RPMI with antibiotics.

A single aliquot of NTHi or *E.coli* BL21 was defrosted at 37℃ for approximately 3 min. Following centrifugation at 800*g* for 5 min, NTHi was either resuspended in 1 ml RS-RPMI without antibiotics (for live NTHi and live *E.coli* BL21) and kept on ice until needed, or resuspended in 1 ml 2% (v/v)

paraformaldehyde (PFA) in PBS for 15 min at room temperature (PFA-NTHi). PFA-NTHi were then centrifuged 800*g* for 5 min and resuspended in 1 ml RS-RPMI without antibiotics as above.

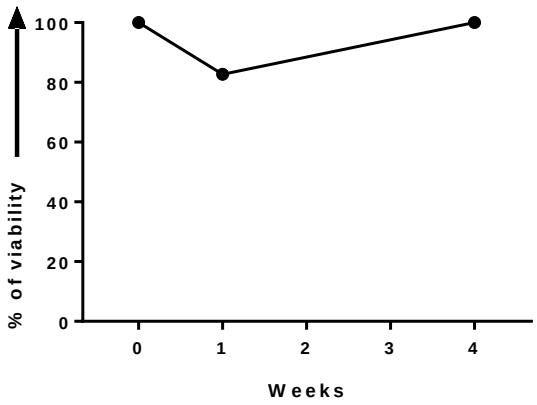


Figure 2.3 – Viability of NTHi following freezing. NTHi were frozen as described, then defrosted at the indicated timepoints and grown on chocolate agar plates. A decrease in NTHi viability was observed 1 week post-cryopreservation but this recovered by week 4, which was consistent with previous reports (Kirkham et al., 2013). N=2. Viability of NTHi was performed with the assistance of Zoe Pounce.

2.4 Bacterial infection and related assays

2.4.1 Bacterial infection of MDM and lung macrophages

MDM or lung macrophages were washed twice with 500 µl PBS in culture plates followed by addition of RS-RPMI without antibiotics followed by either live NTHi, PFA-NTHi or *E.coli* BL21 at multiplicity of infection (MOI) 1 or MOI10 as indicated. All wells were made up to a total volume of 500 µl with RS-RPMI without antibiotics. MDM and NTHi or *E.coli* were incubated for 2 h at 37°C, to allow sufficient time for bacterial uptake. After 2 h of infection, wells were washed twice with 500 µl PBS to remove extracellular bacteria, followed by addition of RS-RPMI with antibiotics and incubated at 37°C for a further 22 h.

In some experiments the effect of steroids on NTHi-induced responses was also investigated. In these experiments, NTHi infection was carried out in the presence of 100 nM fluticasone propionate (FP), 200 nM budesonide (BD), or dimethyl sulfoxide (DMSO) as a vehicle control. Double the amount of budesonide compared to fluticasone was used based on the difference in glucocorticoid receptor occupancy (Daley-Yates, 2015). Steroids were added at the beginning of the 2 h MDM NTHi infection and re-added for the 22 h incubation of MDM in RS-RPMI with antibiotics.

In experiments where IL-7 was measured intracellularly, the protein transport blocker monensin (eBioscience, Hatfield, UK), which prevents transport of proteins from the endoplasmic reticulum (ER) to the Golgi complex, was added for the final 5 h of culture to allow for accumulation of cytokines.

After incubation, supernatants were harvested and stored at -80°C. For gene expression analysis, cells were washed with PBS prior to lysis in 200 µl peqGold Trifast (Peqlab, Erlangen, Germany) reagent. TriFast was then harvested and stored at -80°C. For flow cytometry analysis, after washing with PBS, 200 µl of non-enzymatic cell-dissociation solution (Sigma) was added to each well and incubated at 37°C for 15 min. Cells were then removed from wells and added to polypropylene 5 ml FACS tubes (BD) ready for flow cytometry staining (see section 2.5). Cell viability was confirmed by trypan blue staining.

To measure NTHi phagocytosis, MDM were harvested after the 2 h infection and treated with 1% (v/v) gentamicin in RPMI for 90 min at 37°C to kill extracellular bacteria. MDM were then washed in PBS followed by 200 µl of sterile Perm/Wash (BD Biosciences) for 20 min at 37°C to lyse the MDM and release intracellular NTHi. The lysed solution was then plated out onto chocolate agar plate and cultured overnight to allow colonies to form.

2.4.2 Bacterial infection of MDM – T cell co-culture

For T cell activation, MDM were washed twice with 500 µl PBS in culture plates followed by addition of RS-RPMI without antibiotics and either live NTHi, PFA-NTHi, or *E.coli* at MOI1 or MOI 10. All wells were made up to a total volume of 500 µl with RS-RPMI without antibiotics. MDM and NTHi or *E.coli* were incubated for 2 h at 37°C. Autologous T cells were defrosted at 37°C for approximately 3 min, washed in pre-warmed RPMI and centrifuged at 400*g* for 5 min and resuspended in RS-RPMI with antibiotics. After 2 h of infection, MDM wells were washed twice with 500 µl PBS and 10^6 defrosted autologous T cells were added per well to MDM at an effector:target of 2:1. In some experiments, whole lymphocytes were used instead of CD3-sorted T cells and were prepared as described above. For cytokine readouts, T cells and MDM were incubated for 17 h to allow for presentation of antigens by MDM to T cells before exposure to monensin (eBioscience) for a further 5 h. For cytotoxic readouts, MDM and T cells were cultured for the full 22 h; no monensin was added. After 22 h total of co-culture T cells were then removed from wells, centrifuged at 400*g* for 5 min, and supernatant harvested and stored at -80°C. T cell pellets were resuspended in FACS buffer and added to polypropylene 5 ml FACS tubes (BD) and

stained for flow cytometry (see section 2.5). As a positive control for T cell cytokine production, T cell alone (without MDM) were stimulated with a commercially available phorbol 12-myristate 13-acetate (PMA; 40.5 uM) and ionomycin (670 uM) Cell Stimulation Cocktail (eBioscience), for 6 h, with monensin added after 1 h. Viability of all cells was confirmed by trypan blue staining. An overview of the total monocyte-T cell isolation process is shown in figure 2.4.

In some experiments, the effect of steroids on NTHi-induced T cell responses was also investigated. In these experiments, NTHi infection and T cell co-culture were carried out in the presence of 100 nM FP, 200 nM BD, or DMSO as a vehicle control. Steroids were added at the beginning of the 2 h MDM NTHi infection and re-added for the 22 h incubation of the MDM-T cell co-culture in RS-RPMI with antibiotics.

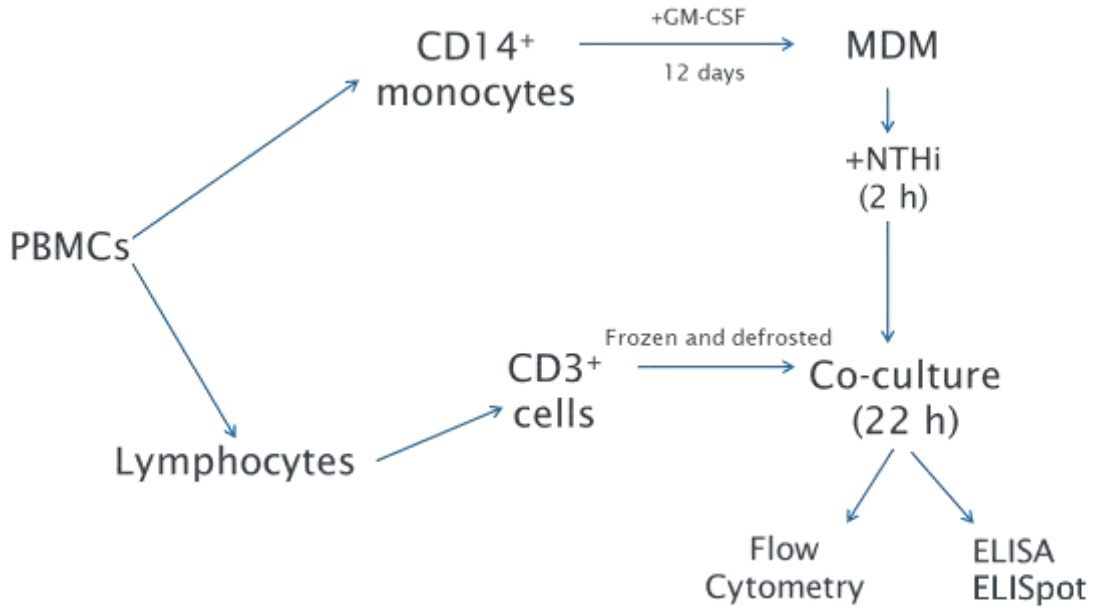


Figure 2.4 – Overview of NTHi-infected MDM-T cell co-culture. Monocytes and autologous lymphocytes were isolated from fresh PBMC from healthy donors as described. Monocytes were differentiated into monocytes-derived macrophages (MDM) by 2 ng/ml GM-CSF stimulation over 12 days. T cells (CD3+) were isolated from lymphocytes as described and frozen at -80°C until needed. After 12 days, MDM were infected with NTHi for 2 hours, washed and co-cultured with autologous T cells for 22 hours.

2.4.2.1 Investigating the role of antigen presentation, co-inhibition and cytokine signalling in the MDM-T cell co-culture

To investigate the role of antigen presentation, the NTHi-infected MDM-T cell co-culture was set up as described above, but with the addition of anti-HLADR (L243), -HLAABC (W6/32) (both at 10 µg/ml), -MR1 (26.5) (at 5 µg/ml) or IgG2a isotype control antibodies (all Biolegend San Diego, USA). Co-cultures were also adapted to include 0.4 µm Transwells® (Corning, New York, USA); MDM were present in the culture plate well, T cells were present in the transwell, as outlined in figure 2.5. To investigate the role of co-inhibitory pathways on T cell activation, the NTHi-infected MDM-T cell co-culture were repeated, but with the addition of anti-PDL1 (MIH1), -PD1 (EH12.2H7), -CTLA4 (L3D10 or IgG1 isotype control (all at 10 µg/ml). For the role of cytokines in T cell activation, the NTHi-infected MDM-T cell co-culture was set up as described, but with the addition of anti-IL-12p40 (C8.6), IL-7 (BVD10-40F6) (both at 10 µg/ml), or IgG1 isotype control antibodies (all Biolegend). All blocking antibodies were low-endotoxin, azide-free. Blocking antibodies were added at the same time as T cells.

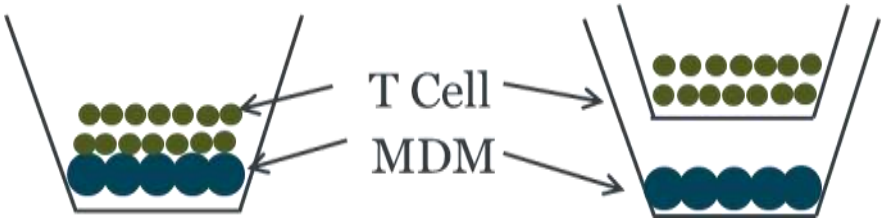


Figure 2.5 – Comparison of the standard co-culture set up compared to the co-culture using 0.4 µm transwells. In both cases, MDM were adherent to the bottom of the well. Transwells prevented physical contact of MDM and T cells. Transwell experiments were used to investigate the role of antigen presentation and cell-to-cell contact on conventional T cell and MAIT cell responses.

2.4.2.2 MDM-T cell co-culture and cytokine stimulation

For cytokine stimulations, the co-culture of uninfected MDM and T cells or just T cells alone (without MDM) were incubated with 10 ng/ml recombinant IL-12 or IL-7 as indicated (both Biolegend). Viability of cells was confirmed by trypan blue staining.

2.4.2.3 MAIT Killing Assay

To assess whether MAIT cells could kill NTHi-infected MDM, MDM were infected as before but this time were co-cultured with autologous MAIT cells. Following the 2 h infection of MDM, MAIT

cells and infected MDM were co-cultured at an effector:target of 2:1 for either 22 or 46 h, after which MAIT cells and MDM were separately harvested and transferred to FACS tubes and supernatants stored as described above. MDM were stained with a viability marker (Zombie violet fixable viability kit – Biolegend) to assess death of MDM by MAIT cells.

2.4.3 NTHi infection of the *ex vivo* human lung tissue explant

For infection of whole lung explant, lung tissue was washed extensively in RPMI following the overnight rest in antibiotics and cultured for a least 1 hour in RS-RPMI without antibiotics, to remove any remaining gentamicin or amphotericin. The lung tissue was then washed extensively again in RPMI and placed in RS-RPMI without antibiotics. Lung tissue explants were then infected with $5x10^6$ colony forming units (CFU) NTHi and cultured in 37 ℃, 5% CO_2 for 24 hours. For cytokine readouts, monensin was added after 1 h. After 24 h, cells were dispersed from tissue by collagenase digestion for 15 min at 37℃ using 0.5 mg/ml of pre-warmed collagenase type 1 (Sigma-Aldrich) dissolved in RPMI. A magnetic stirrer was also present to mechanically disrupt the tissue. The digested solution was filtered through a 70 µm pore straining cap into 5 ml polypropylene FACS tubes (BD Biosciences) and cells were then stained for flow cytometry. The lung tissue explant was also stimulated with the PMA and ionomycin Cell Stimulation Cocktail (eBioscience) for 6 h, with monensin added for after 1 h. Viability of cells was confirmed by trypan blue staining.

2.5 Flow Cytometry

2.5.1 Extracellular flow cytometry for surface expression of MDM, lung macrophages and cells dispersed from lung explant

For extracellular staining, $5x10^5$ – 10^6 cells were resuspended in 100 µl FACS buffer (2mM EDTA, 0.5% (w/v) BSA in PBS) with 2 mg/ml purified IgG from human serum (Sigma-Aldrich) per FACS tube (BD). Cells were then stained with appropriate antibodies or isotype controls (see table 2.3 for full details). After incubation on ice for 30 min, cells were washed and resuspended in FACSfix (2% (v/v) PFA in PBS), incubated on ice for 20 min, washed again and then resuspended in 350 µl FACS buffer. All staining was analysed by 9-colour FACSAria (BD Biosciences). Specific mean fluorescence intensity (SMFI) was determined by subtracting isotype fluorescence values from

each sample's MFI. An overview of the gating strategy for lung macrophage and cells dispersed from the explant is shown in figure 2.6.

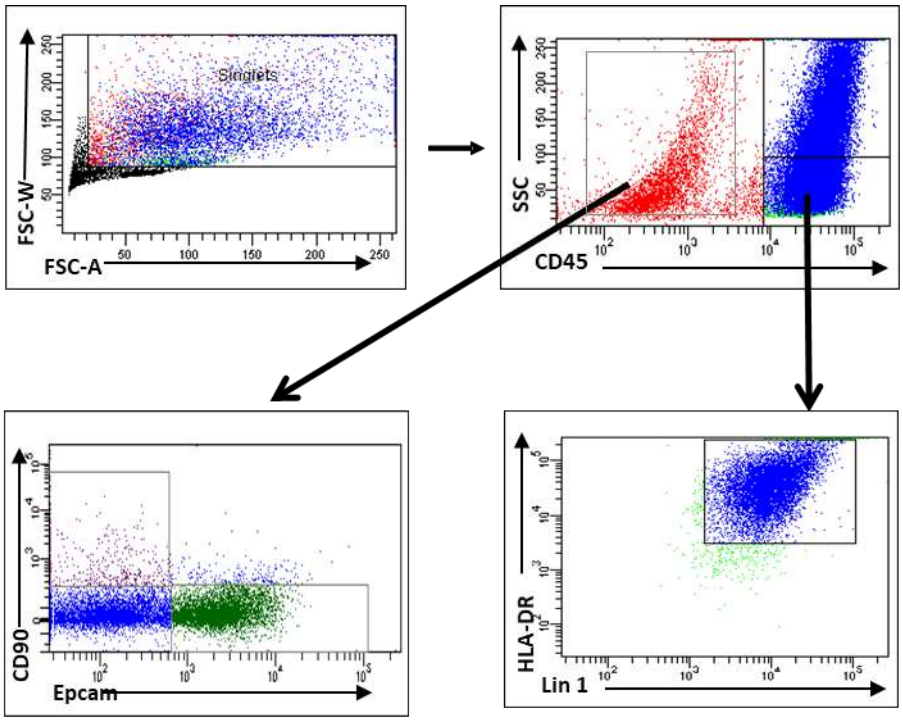


Figure 2.6 – Gating strategy for lung macrophage, epithelial cells and fibroblasts. Cells were first gated on size to remove lymphocyte sized cells. Cells were then gated on CD45 to select leukocytes. Macrophages were then defined as HLA-DR+ Lineage+ cells. HLA-DR+ Lineage- cells are defined as dendritic cells. CD45-CD90+ cells were fibroblast and CD45-Epcam+ cells were defined as epithelial cells. Gates were determined using isotype controls.

2.5.2 Intracellular flow cytometry for IL-7 expression of MDM

To stain for IL-7 expression of MDM, MDM were harvested from culture plates as described and resuspended in 200 µl Cytofix/Cytoperm (BD Biosciences). Following incubation for 20 min on ice, MDM were washed with 1xPerm/Wash (BD Biosciences), resuspended in 100 µl 1xPerm/Wash buffer and stained with 2 µl purified human anti-IL-7 (BVD10-40F6; Biolegend) for 30 min on ice. MDM were again washed with 1xPerm/Wash (BD Biosciences), resuspended in 100 µl 1xPerm/Wash buffer and stained with 1.25 µl anti-rat IgG1-PE (MRG1-58; Biolegend). After incubation on ice for 30 min, cells were washed again with 1xPerm/Wash and resuspended in 350 µl FACS buffer. All staining was analysed by 9-colour FACSAria (BD Biosciences). SMFI was determined by subtracting isotype fluorescence values from each sample's MFI.

2.5.3 Extracellular and intracellular flow cytometry for T cells from co-culture and lung explant

For staining of T cells, 10^6 T cells were resuspended in 100 µl FACS buffer with 2 mg/ml purified IgG from human serum per FACS tube. T cells were then stained appropriate antibodies or isotype controls (see table 2.3 for full details). After incubation on ice for 30 min, cells were washed, resuspended in 200 µl Cytofix/Cytoperm (BD Biosciences) and incubated for 20 min on ice before washing with 1xPerm/Wash (BD Biosciences) and resuspending in 100 µl 1xPerm/Wash buffer and stained with appropriate antibodies or isotype controls (see table 2.3 for full details). After incubation on ice for 40 min, cells were washed again with 1xPerm/Wash and resuspended in 350 µl FACS buffer. For lung T cells from the explant, or in cases where lymphocytes, not CD3+ MACS-sorted T cells, were stained CD3-PE-CY7 (BD-Biosciences) was also included in the panel. An overview of the T cell gating strategy for T cells from the lung explant (figure 2.7) and T cells from the co-culture (figure 2.8) is shown below. Representative gating strategies for extracellular and intracellular markers are also shown below; the extracellular marker 41BB, the intracellular cytokine IFNγ and intracellular marker granzyme B have been shown as examples (figure 2.9). All staining was analysed by 9-colour FACSAria (BD Biosciences).

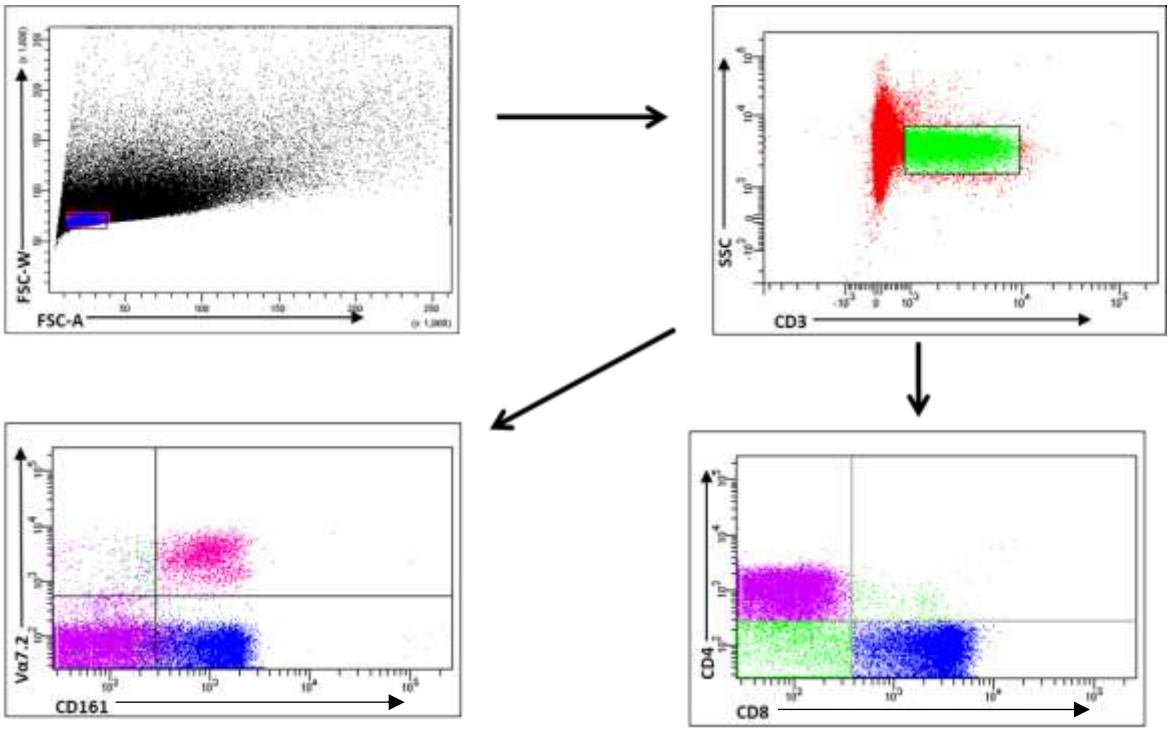


Figure 2.7 – Gating strategy for T cells from lung explant. Cells were first gated on lymphocytes by size. T cells were then identified by gating for CD3. T cells were then gated for CD4 or CD8 to identify conventional T cells, or gated for Vα7.2 and CD161 to detect MAIT cells. Gates were determined using isotype controls.

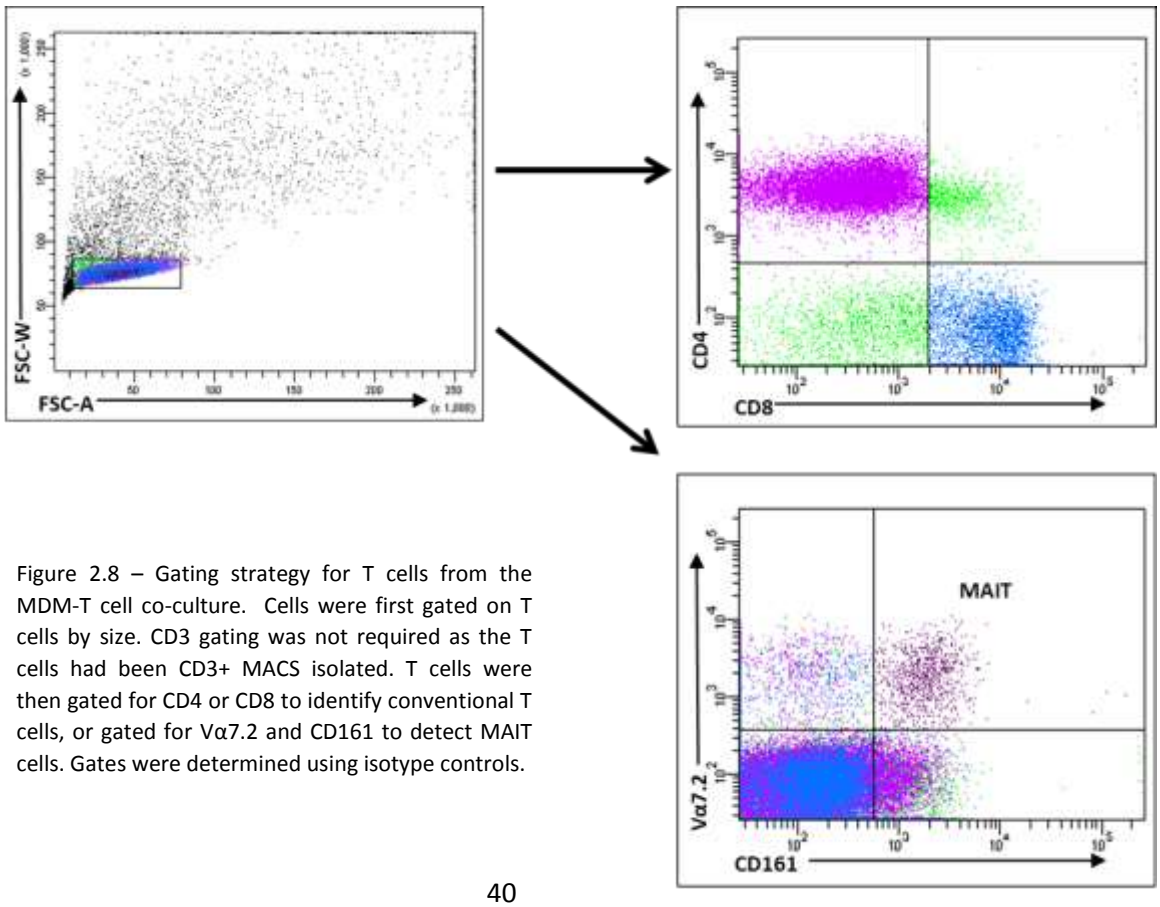


Figure 2.8 – Gating strategy for T cells from the MDM-T cell co-culture. Cells were first gated on T cells by size. CD3 gating was not required as the T cells had been CD3+ MACS isolated. T cells were then gated for CD4 or CD8 to identify conventional T cells, or gated for Vα7.2 and CD161 to detect MAIT cells. Gates were determined using isotype controls.

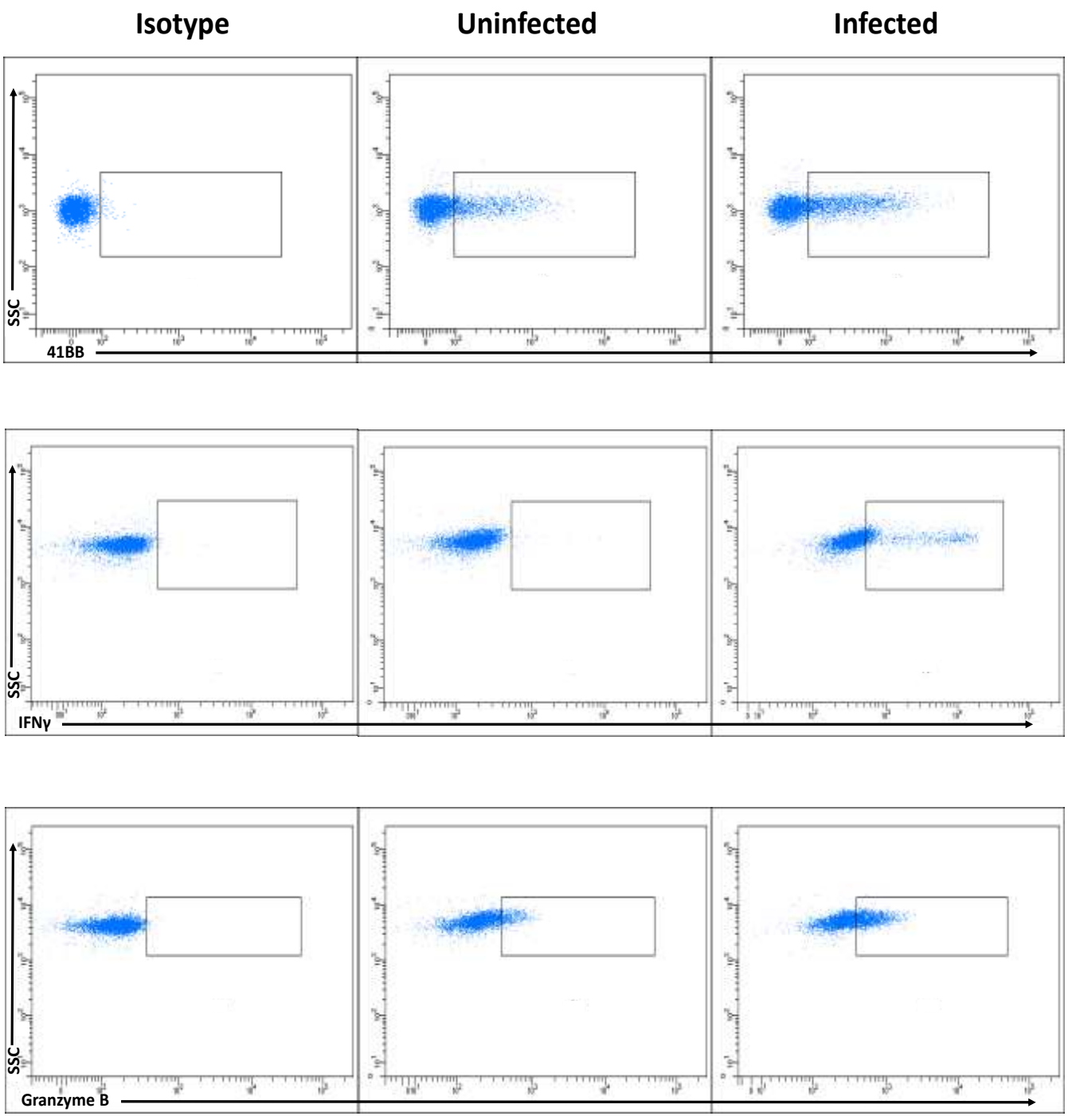


Figure 2.9 – Representative gating strategies for extracellular and intracellular T cell markers. Representative gating strategies for the extracellular marker 41BB, the intracellular cytokine IFNγ and intracellular marker granzyme B have been used as examples. Gates were determined using an isotype control as shown. Plots show expression of each T cell marker before and after NTHi infection.

Table 2.3 – Anti-human antibodies used for flow cytometry. All antibodies were raised in mice, unless specified otherwise

Marker	**Flurochrome**	**Isotype**	**Volume/test (µl)**	**Company**
CD3	PE-CY7	IgG1	2.5 or 10	BD Biosciences
CD4	PERCP-CY5.5	IgG1	5	BD Biosciences
CD8	APC-CY7	IgG1	5	BD Biosciences
CD161	APC	IgG1	20	BD Biosciences
Vα7.2	PE	IgG1	5	Biolegend
CD107a	BV510	IgG1	5	BD Biosciences
Granzyme B	FITC	IgG1	20	BD Biosciences
Perforin	FITC	IgG1	5	Biolegend
IFNγ	FITC	IgG1	5	Biolegend
IL17a	BV421	IgG1	5	Biolegend
TNFα	BV510	IgG1	5	Biolegend
CD28	FITC	IgG1	5	Biolegend
CD40L	BV510	IgG1	5	Biolegend
CTLA4	BV421	IgG2a	5	Biolegend
PD1	BV510	IgG1	5	Biolegend
41BB	BV421	IgG1	5	Biolegend
CD27	FITC	IgG1	5	Biolegend
ICOS	FITC	IgG (Hamster)	2	Biolegend
OX40	BV421	IgG1	5	Biolegend
IL-12Rβ1	FITC	IgG1	10	R&D SYSTEMS
IL-12Rβ2	FITC	IgG1	10	R&D SYSTEMS
IL-7Rα	BV510	IgG1	5	Biolegend
MR1	APC	IgG2a	2.5	Biolegend
MR1	PE	IgG2a	2.5	Biolegend
HLADR	APC-CY7	IgG2b	5	BD Biosciences
HLAABC	PE	IgG1	5	eBioscience
Lineage 1 (Lin1)	FITC	IgG1, IgG2b	10	BD Biosciences
EpCAM	PerCP-Cy5.5	IgG1	20	BD Biosciences
CD90	APC	IgG1	5	Biolegend
PDL1	PE	IgG1	20	BD Biosciences
PDL2	PerCP-Cy5.5	IgG1	5	BD Biosciences
CD45	BV510	IgG1	5	Biolegend

CD80	BV421	IgG1	5	Biolegend
CD86	APC	IgG2b	5	Biolegend
CD40	BV510	IgG1	5	Biolegend
CD70	FITC	IgG1	5	Biolegend
41BBL	APC	IgG1	5	Biolegend
ICOSL	PE-Cy7	IgG2B	5	Biolegend
OX40L	PE	IgG1	5	Biolegend
ICAM	FITC	IgG1	5	Biolegend
IL-7	None	IgG1 (Rat)	2	Biolegend
Anti-RAT IgG1	PE	IgG	1.25	Biolegend
ISOTYPE	FITC	IgG1	10	BD Biosciences
ISOTYPE	FITC	IgG (Hamster)	2	Biolegend
ISOTYPE	PE	IgG1	5	BD Biosciences
ISOTYPE	APC	IgG1	10	BD Biosciences
ISOTYPE	PE-CY7	IgG1	2.5	BD Biosciences
ISOTYPE	BV510	IgG1	5	BD Biosciences
ISOTYPE	PERCP-CY5.5	IgG1	2.5	eBioscience
ISOTYPE	APC-CY7	IgG2a	2.5	BD Biosciences
ISOTYPE	BV421	IgG2a	5	Biolegend
ISOTYPE	PE	IgG2a	2.5	eBioscience
ISOTYPE	APC	IgG2a	2.5	Biolegend
ISOTYPE	FITC	IgG2b	10	BD Biosciences
ISOTYPE	PE-Cy7	IgG2b	5	Biolegend

2.6 IFNγ ELISpot

On the day prior to MDM infection, ELISpot plates (Millipore, Billerica, USA) were coated with 50 µl of 15 µg/ml IFNγ capture antibody (Mabtech, Nacka-Strand, Sweden), incubated at 4°C overnight. On the day of infection, ELISpot wells were washed with PBS and blocked with RS-RPMI. MDM were washed twice with 500 µl PBS in culture plates followed by addition of RS-RPMI without antibiotics and either live or PFA-NTHi at MOI1. All wells were made up to a total volume of 500 µl with RS-RPMI without antibiotics. MDM and NTHi were incubated for 2 h at 37°C. After 2 h of NTHi infection, wells were washed twice with 500 µl PBS before addition of 200 µl non-enzymatic cell-dissociation solution and incubated for 15 min at 37°C. Cells were then removed from wells and $5x10^4$ infected MDM were added to ELISpot wells. Lymphocytes were defrosted at 37°C for approximately 3 min, washed with pre-warmed un-supplemented RPMI and 10^5

lymphocytes added to the infected MDM at an effector:target of 2:1. All ELISpot wells were made up to final volume of 200 µl with RS-RPMI with antibiotics and incubated at 37°C for a further 22h. After 24 h post-infection, wells were washed with PBS-0.05% (v/v) Tween followed by addition of 100 µl biotinylated IFNγ detection antibody (Mabtech) at 1 µg/ml in PBS-0.5% (v/v) FBS, incubated at 37°C for 90 min. Wells were then washed as before and 100 µl 1 µg/ml streptavidin (Mabtech) in PBS-0.5% (v/v) FBS was added, incubated at 37°C for 90 min. Following a final wash, 100 µl of BCIP/NBT substrate solution (Invitrogen) was added and removed by extensive washing once spots began to form. Plate was left to dry before spots were counted on an ELISpot Reader (AID, Strassberg, Germany).

2.7 RNA isolation, cDNA synthesis and Real-Time PCR

RNA from cells lysed in Trifast was isolated according to manufacturer's instructions (PeqLab). Briefly, Trifast samples were defrosted at room temperature, allowed to stand for 5 min before addition of 40 µl chloroform (Sigma), vortexed and left at room temperature for a further 10 min. Following centrifugation at 12,000*g*, 4°C for 10 min, RNA was isolated by removing the aqueous phase which was then mixed with 100 µl isopropanol (Sigma), left on ice for 5 min and centrifuged at 12,000*g*, 4°C for 10 min to pellet RNA. The pellet was washed twice with 500 µl 75% ethanol (Sigma), both times centrifuged at 12,000*g*, 4°C for 10 min before being resuspended in 15 µl RNase-free water (Sigma). Concentrations of RNA were determined by NanoDrop 1000 (Thermo Scientific, Wilmington, USA).

Reverse transcription was carried out in 20 µl reactions; 250 ng of RNA were added to 2 µl 10xRT buffer, 2 µl 10xRT random primers, 0.8 µl deoxyribonucleotide triphosphate (dNTP) mix, 1 µl MultiScribe Reverse Transcriptase and 1 µl RNase Inhibitor (all Applied Biosystems, Paisley, UK). Remaining volume was made up to 20 µl with RNAse-free water (Sigma). All samples were then briefly vortexed and centrifuged. The process of reverse transcription was carried out using a DNA Engine Thermal Cycler (MJ Research) with the following protocol:

- 25°C for 10 min
- 37°C for 2 h
- 85°C for 5 min

cDNA was then stored at 4°C until needed.

Following cDNA synthesis, quantitative PCR (qPCR) was carried out in 5 µl reactions. 2.5 µl TaqMan Universal Master Mix II (Applied Biosystems) was added to 1.25 µl RNAse-free water (Sigma) and 0.25 µl of appropriate TaqMan primers (see table 2.4 and table 2.5). This master mix cocktail was added to appropriate wells on a 384 well PCR plate before addition of 1 µl of sample cDNA. Gene expression was normalized to the housekeeping gene β_2 Microglobulin (β2M) and presented as either ΔCt or ΔΔCt to show fold induction. ΔΔCt was calculated as: 2^((*gene of interest Ct value from infected/stimulated sample minus β2M gene Ct value from infected/stimulated sample*) minus (*gene of interest Ct value from uninfected/unstimulated sample minus β2M gene Ct value from uninfected/unstimulated sample*)). The reactions were performed using a 7900HT Fast Real-Time PCR System with the following protocol:

- 95℃ for 10 min
- 40 cycles of 95℃ 15 sec, 60℃ for 1 min

Table 2.4 – TaqMan Primers used for qPCR (accession numbers). All primers with known accession numbers are displayed below.

Primer	Accession number (all Applied Biosystems)
β2M	Hs00984230_m1
IFN-beta	Hs01077958_s1
IRF-3	Hs01547288_m1
MR1	Hs00155420_m1
NF-κB (RELA)	Hs01042010_m1
RIG-I	Hs01061436_m1
TLR4	Hs00152939_m1
TLR7	Hs01933259_s1
IL-12p35	Hs00168405_m1
IL-12p40	Hs01011519_m1
IL-18	Hs010387877
IL-7	Hs00174202_m1

Table 2.5 – TaqMan Primers used for qPCR (sequences). All primers with known sequences are displayed below

Primer	Sequence
Hel (kindly provided by David Cleary)	hel-F: CCGGGTGCGGTAGAATTTAATAA hel-R: CTGATTTTTCAGTGCTGTCTTTGC hel-Pro: FAM-ACAGCCACAACGGTAAAGTGTTCTACG-TAMRA

2.8 ELISA

Supernatant was harvested from all assays described above and stored at -80℃ until needed. All ELISAs were performed according to the manufacturers' protocol: IL-12p70 ELISA MAX and IFNγ ELISA MAX (both Biolegend); IL-1β duoset ELISA and granzyme B duoset ELISA (both R&D Systems). Whilst all ELISA protocols had slight differences (see specific manufacturers' instructions), the following general protocol applied:

On the day prior to running the ELISA, Nunc™ MaxiSorp™ ELISA plates (Biolegend) were coated with 100 µl of appropriate capture antibody at working concentration and incubated either at room temperature or at 4℃, depending on the protocol. The following day, ELISA plates were washed in PBS-0.05% (v/v) Tween and blocked for 1 h at room temp using 200 µl 1% (v/v) BSA solution in PBS or equivalent blocking buffer, depending on the specific protocol. Following washing in PBS-0.05% (v/v) Tween, 100 µl supernatants (appropriately diluted) were added to the ELISA plates and incubated for 2 h at room temp. Following washing in PBS-0.05% (v/v) Tween, 100 µl appropriate detection antibody at working concentration was added to the ELISA plates and incubated at room temp for 2 hours. After further washing with PBS-0.05% (v/v) Tween, 100 µl streptavidin-HRP at working concentration was added to the ELISA plates and incubated at room temp for 30 min. Following a final wash in PBS-0.05% (v/v) Tween, 100 µl of TMB substrate solution was added for 20 min in the dark. The reaction was stopped by adding 50-100 µl stop solution/ 1M H_2SO_4. Plates were read on a microplate reader at 450 nm with 550 nm wavelength correction (Multiskan Ascent, Agilent Technologies, Wokingham, UK).

The limit of detection for each ELISA (according to the manufacturers' instructions) was as follows:

- IL-12p70 ELISA MAX (Biolegend): 4 pg/ml
- IFNγ ELISA MAX (Biolegend): 4 pg/ml
- IL-1β duoset ELISA (R&D Systems): 1 pg/mL
- Granzyme B duoset ELISA (R&D Systems): 2.4 pg/ml

2.9 Lactate Dehyrdrogenase (LDH) release assay

LDH was performed on supernatant from the MDM-MAIT co-culture to assess cell death, following the manufacturer's instructions (Promega, Madison, USA). Briefly, 50 µl of supernatant was added to wells of a 96-well plate, followed by 50 µl CytoTox 96® Reagent to each well and incubated at room temp for 30 min in the dark. Afterwards, 50 µl of stop solution was added to each well and absorbance were read at 490 nm on a microplate reader (Multiskan Ascent, Agilent Technologies).

2.10 Statistics

As the number of repeats were typically less than N=8, accurate testing for normality using the D'Agostino & Pearson normality test could not be performed. For this reason, the data were considered non-parametric. For the comparison of lung and blood T cells in chapter 3, the Mann Whitney test was used to determine statistical significance as the comparison was between different donors so was unpaired. For the remaining data in this thesis, due to the nature of the assays (e.g. uninfected vs infected cells from the same donor) all the data are paired. Statistical analyses were performed using Wilcoxon matched pairs signed-rank test for comparison of two groups or Friedman test with Dunn's multiple comparison testing as appropriate (GraphPad Prism version 6, GraphPad Software, San Diego, USA). All results were determined to be significant with a P value of at least $P<0.05$.

3. Characterisation of conventional T cells and MAIT cells and MR1 in the human lung and blood

3.1 Introduction

T cells are effector cells of the immune system which promote pro-inflammatory responses to pathogens and clear infection by secreting cytokines and releasing cytotoxic mediators. T cells are present in the human lung and CD8+ T cells are known to be increased in the airway in COPD, potentially acting as a major cause of airway inflammation (Fairclough et al., 2008)(McKendry et al., 2016). Less is known about the role of MAIT cells in the human lung.

Studying T cells at the site of infection (i.e. the lungs) is more appropriate than in peripheral blood as it is a more physiologically relevant to study tissue-resident cells. However, due to the nature of human tissue samples, the yields of cells derived from lung explants are often very limited. Blood-derived models are therefore often used in research as they overcome the low cell yields obtained from tissue. This thesis will investigate T cell responses to NTHi by first comparing both lung and blood T cells and then utilising blood T cells and blood-derived antigen presenting cells (APCs) to further explore the mechanisms controlling T cell responses. Human lung parenchyma tissue distal from tumour sites was obtained from patients who were undergoing lung resection. Human peripheral blood was obtained from healthy human volunteers.

Before measuring T cell responses to NTHi, T cell populations in the lung first needed to be identified, along with the T cell populations found in the blood. Whilst MAIT cells have been identified in the human airways before (Hinks et al., 2016), this was only in bronchial tissue. It is not currently known if MAIT cells are present in the lung parenchyma, so this needed to first be addressed. Furthermore it is not known whether the methods used to extract cells from tissue (namely collagenase digestion) affects expression of associated T cell markers, so this too will be investigated.

T cell responses to bacteria are varied and result in the production of a wide array of different effector molecules, including pro-inflammatory cytokines (Murphy and Reiner, 2002). The archetypal cytokine produced in response to intracellular pathogens is the Th1 cytokine, IFNγ (Das et al., 2001)(Zhu and Paul, 2008). However, T cells can have a variety of different responses to infection depending on the pathogen involved. IL-17+ T (Th17) cells have been identified in the lung, although their role in lung diseases has not been fully elucidated (Alcorn et al., 2010). Furthermore, MAIT cells have the capacity to produce IFNγ, IL-17a and TNFα, although production of these cytokines by lung MAIT cells is not well characterised (Le Bourhis et al., 2010)(Dusseaux et al., 2011).

In this chapter, flow cytometry will be used to first ensure detection of conventional T cells and MAIT cells in the lung and blood. Conventional T and MAIT cell populations will be analysed to determine which effector molecules these cells can produce. In addition, expression and location of the MAIT antigen presenting molecule, MR1, will also be evaluated.

3.2 Results

3.2.1 Identifying conventional T cell subsets in the blood and lung

As discussed previously, conventional T cells are CD3+ and are further defined by their expression of the co-receptor molecules CD4 and CD8 and the majority of T cells are characterised as either CD4+CD8- or CD4-CD8+. Double negative (CD4-CD8-) and double positive (CD4+CD8+) T cells have also been identified, although these subsets are typically thought to represent a small percentage of the total T cell population (Parel and Chizzolini, 2004)(Martina et al., 2015).

To first determine the proportion of T cell subsets present in the lung and how these compared to the blood, expression of CD4 and CD8 was measured by flow cytometry on both lung-derived and blood-derived T cells (see table 2.2 for patient demographics for lung tissue). As shown in figure 3.1, CD4+ T cells comprised the largest T cell subset within both the lung and blood compartments, with CD4+ T cell accounting for 44% and 55% of T cells respectively.

The second largest subset in both compartments was CD8+ T cells, which comprised 33% of lung T cells and 30% of blood T cells. Double positive populations were minor at 1% in both compartments; whereas double negative T cells were 19% of T cells in the lung and 13% in the blood.

Taken together, the data suggest that the CD4+ and CD8+ T cells represented the two largest T cell subsets in both the lung and blood. For the purpose of this thesis, only CD4+ and CD8+ conventional T cells were considered further and all mention of conventional T cells from here onwards refers only to either CD4+ or CD8+ T cells.

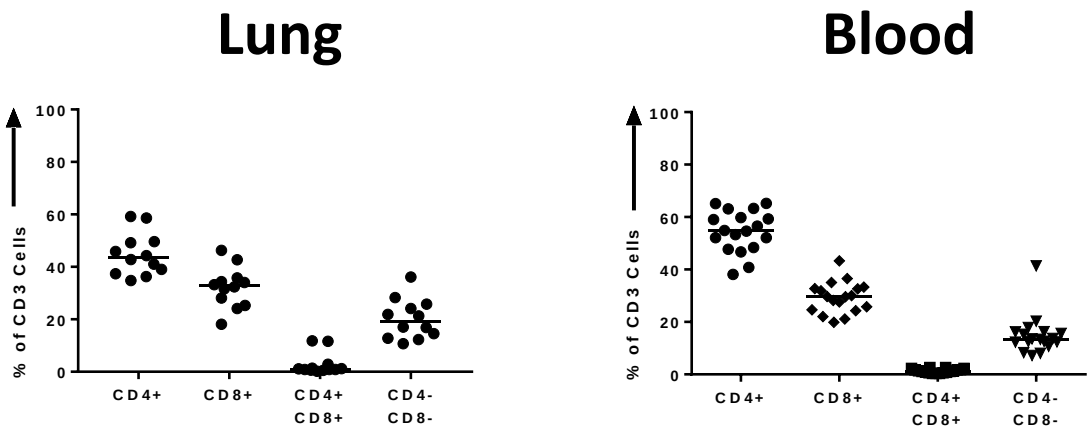


Figure 3.1 – Conventional T cell subpopulations in the lung and blood. Blood T cells and lung T cells were stained for CD4 and CD8 and numbers of CD4+, CD8+, CD4+CD8+ and CD4-CD8- T cell subpopulations were determined by flow cytometry. For all graphs, median values are shown.

3.2.2 Identifying MAIT cells in the blood and lung

One of the primary aims of this thesis is to investigate lung MAIT cell responses to NTHi. However, the presence of MAIT cells within the human lung has not been well characterised. To confirm that MAIT cells could be detected within the lung, T cells from human lung explant tissue were stained for CD161 and Vα7.2, in accordance with the MAIT cell literature as described previously (section 1.5). MAIT cells were found to comprise 1.5% of lung T cells, with a range of 0.3-5.6%, whereas in the peripheral blood, MAIT cells comprised approximately 3% of T cells (figure 3.2).

MAIT cells can be further defined by their expression of CD4 and CD8, with the majority of blood MAIT cells being reported in the literature as CD8+ (Treiner et al., 2003). Consistent with this, 61% of MAIT cells in the blood were CD8+, with the double negative population comprising 30% and both the CD4+ and the double positive populations making up only minor subsets (figure 3.2). In the lung, 60% of lung MAIT cells were also CD8+. However, a notable difference was that the CD4+ MAIT cell population in the lung was larger than in the blood compartment with both CD4+ and the double negative subsets comprising 13% of total lung MAIT cells (figure 3.2). Overall, MAIT cells are present in both the lung and blood, although more MAIT cells are found in the blood.

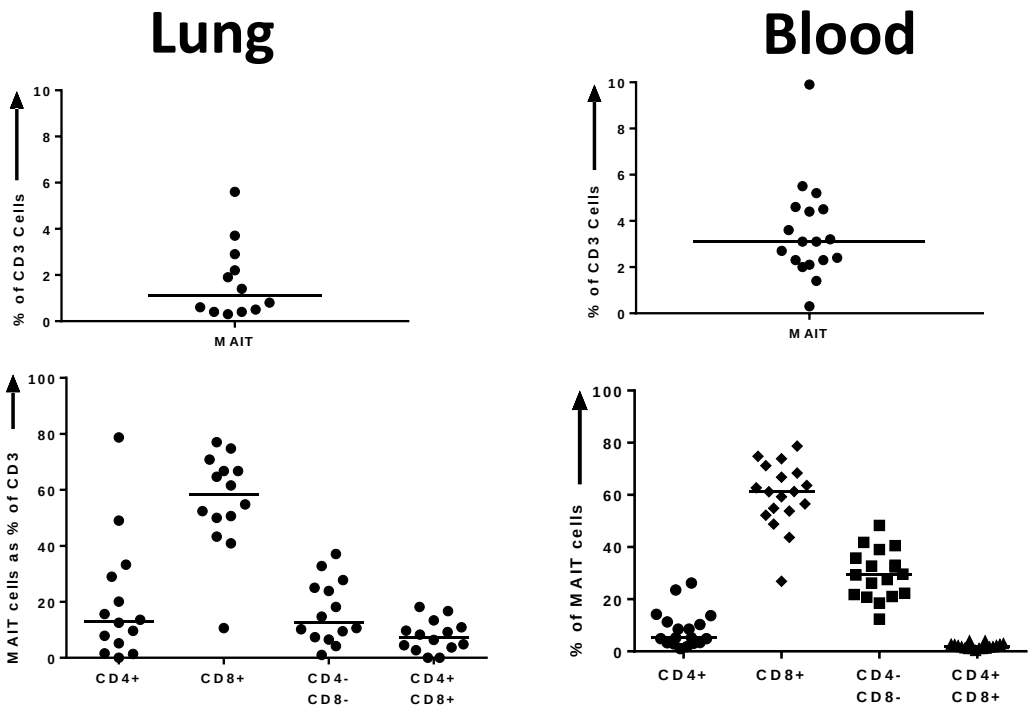


Figure 3.2 – MAIT cell populations in the lung and blood. Blood T cells and lung T cells were stained for Vα7.2 and CD161 and percentages of MAIT cells was determined by flow cytometry. Top graphs show MAIT cells as a % of the T cell population. Bottom graphs show CD4 and CD8 expression on MAIT cells. For all graphs, median values are shown.

3.2.3 Effect of collagenase on lung T cell subsets

In order to ensure maximal recovery of cells from tissue, collagenase is often used which causes degradation of collagen, releasing tissue-resident cells. However, one issue that arises is that collagenase has been shown to cause cleavage of certain surface markers, potentially impairing detection of certain cell types by flow cytometry (Autengruber et al., 2012)(Mulder et al., 1994). To ensure that detection of T cell populations in the lung was not affected by collagenase treatment, T cells from collagenase-treated (0.5mg/ml, 37°C, 15 minutes) lung explants were compared to T cell that had egressed from lung explants. No significant difference ($P>0.05$ for all paired comparisons) was observed in CD4+, CD8+ or MAIT cell populations from collagenase-treated explants compared to T cell that had egressed from lung tissue, indicating that collagenase does not impair detection of T cells (figure 3.3).

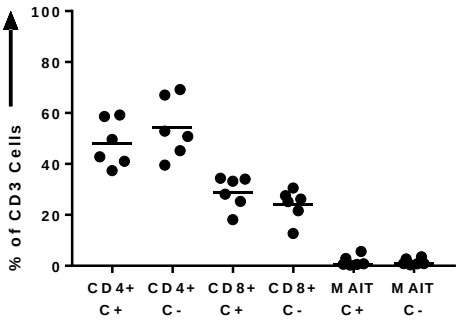


Figure 3.3 – Collagenase does not affect detection of T cell markers. T cells from collagenase-treated (C+) or non-collagenase-treated (C-) lung explants were stained for CD4+, CD8+ and MAIT cell markers. Median values are shown. Statistical significance was determined by Friedman test with Dunn's multiple comparison.

3.2.4 Cytokine production by lung and blood T cell subsets

Flow cytometry is a useful tool for detecting intracellular cytokines, which together with surface markers allow for the identification of cytokine-producing subpopulations of T cells. To this end, T cells from blood or lung explants were stimulated with the PMA and ionomycin Cell Stimulation Cocktail for 6 hours to induce non-specific cytokine production and T cells were analysed by flow cytometry.

Expression of IFNγ was detected in 24% and 45% of CD4+ and CD8+ lung T cells respectively following stimulation. However, the response of peripheral CD4+ and CD8+ T cells was smaller, with only 9% of CD4+ and 20% of CD8+ T cells positive for IFNγ (figure 3.4). Lung MAIT cells were also capable of producing IFNγ, which was comparable in amount to blood MAIT cells (lung 18% vs blood 20% IFNγ+).

IL-17a production was minimal in both lung and blood CD8+ T cells, whereas 2.9% of lung CD4+ cells were IL-17a+ and 1% of blood CD4+ T cells were IL-17a+. MAIT cells also produced IL-17a following stimulation, with 11% of lung MAIT cells IL-17a+ and just 0.7% of blood MAIT cells IL-17a+, indicating that MAIT cells could be a major source of IL-17 in the lung (figure 3.4).

Expression of TNFα was consistent across the three T cell subsets in the lung, with approximately 30% of each subset being TNFα+ following stimulation (figure 3.4). However, detection of TNFα was very low in all three blood T cell subsets, but this could be because TNFα production in blood T cells peaks earlier compared to the lung. No cytokines were detected in any T cell subset at unstimulated baseline. Overall, conventional T cells and MAIT cells in the lung are able to produce IFNγ, IL-17a and TNFα and as such, production of these cytokines in response to NTHi infection will be investigated in future chapters.

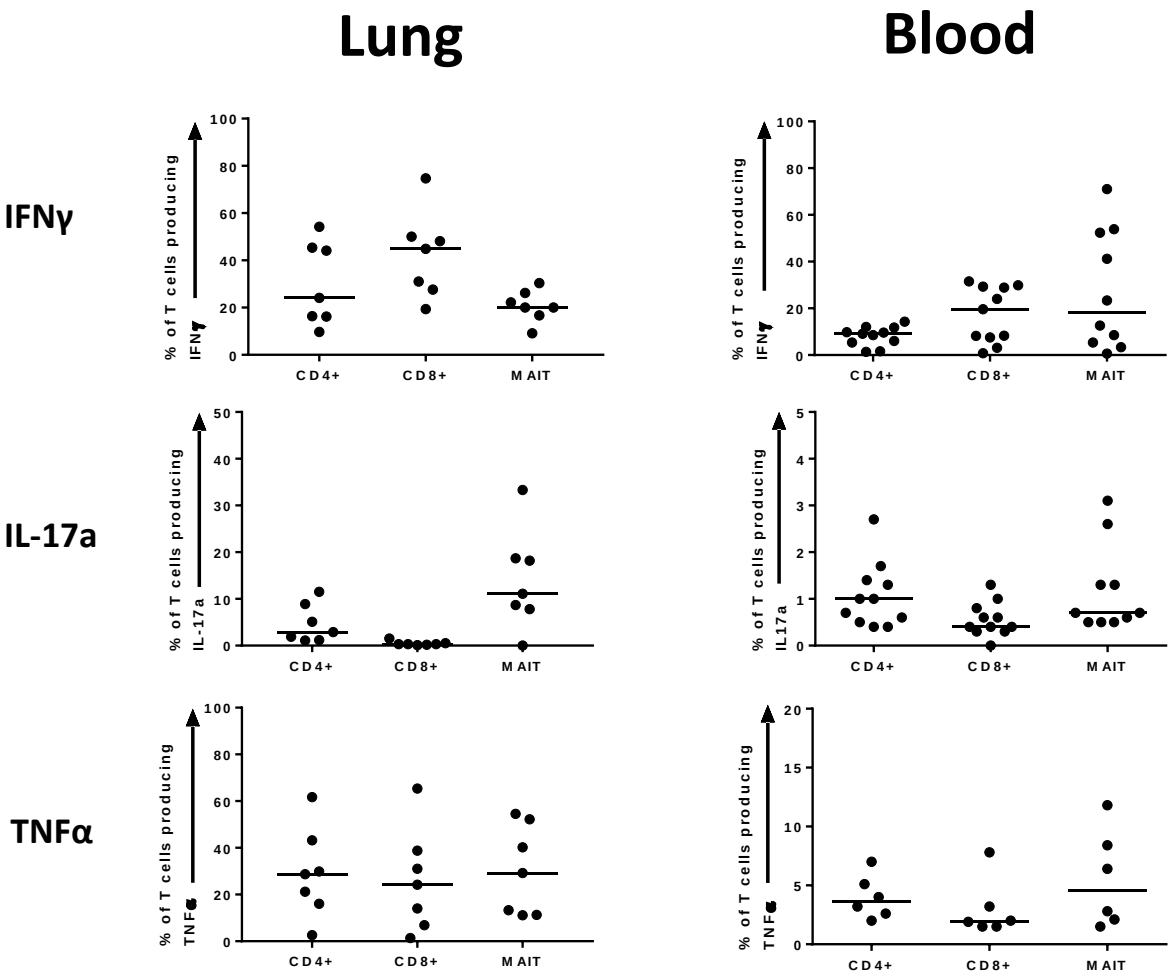


Figure 3.4 – Production of cytokines by T cell subpopulations in the lung and blood. Blood T cells and lung explants were stimulated with PMA/ionomycin for 6 hours. Blood T cells and lung T cells were stained for the IFNγ, IL-17a and TNFα and expression was determined by flow cytometry. No cytokines were detected in any T cell subset at baseline. For all graphs, median values are shown.

3.2.5 Cytotoxic T cell subsets in the lung and blood

Besides production of cytokines, another major feature of T cells is their cytotoxic ability, enabling them to destroy infected cells and clear infection. Whilst CD8+ T cells are classically thought of as cytotoxic, both CD4 and MAIT have been shown to have cytotoxic function (Wilkinson et al., 2012)(Marshall and Swain, 2011)(Le Bourhis et al., 2013). Lung and blood T cells were analysed by flow cytometry for their baseline expression of one of the primary cytotoxic enzymes, granzyme B.

Consistent with previously published reports, blood CD4+ and MAIT cells have no expression of granzyme B at baseline, whereas only 6% of blood CD8+ T cells were granzyme B+ (figure 3.5). However, in the lung, granzyme B was expressed in all three subsets, particularly in CD8+ T cells. The increased baseline expression of granzyme B indicates that lung T cells, including MAIT cells, may be better poised to induce cytotoxic responses to bacterial infection in the lung. Further work in subsequent chapters will explore how granzyme B expression of conventional T and MAIT cells is affected by NTHi infection.

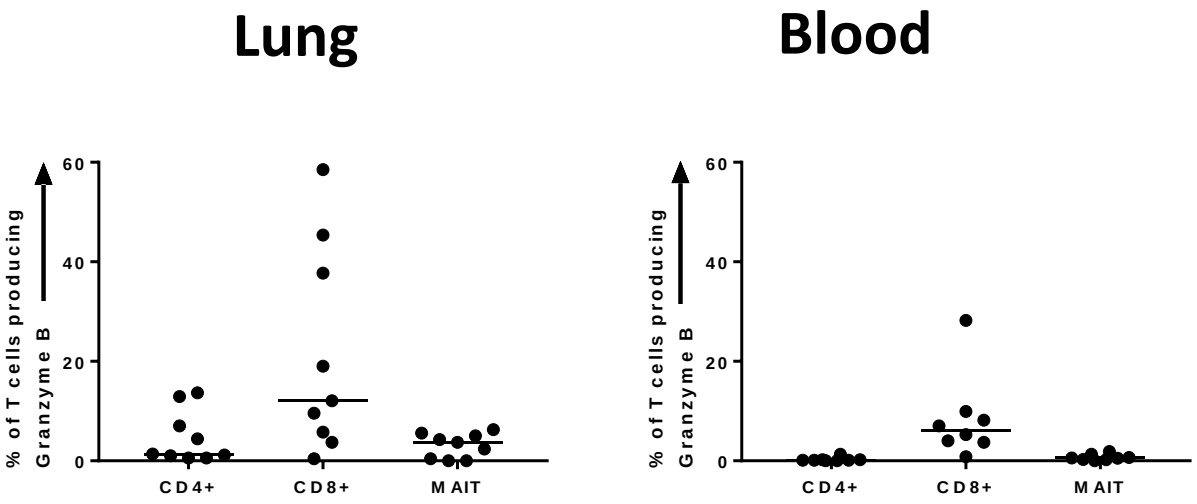


Figure 3.5 – Baseline expression of granzyme B by T cell subpopulations in the lung and blood. Blood T cells and lung T cells were stained for the granzyme B and expression was determined by flow cytometry. For all graphs, median values are shown.

3.2.6 MR1 expression in the lung

Having identified and characterised conventional T cells and MAIT cells in the lung and blood, the next step was to identify APCs in the lung which were likely to interact with and activate these T cell subsets. The ability of a professional APC to activate T cells is dependent on expression of antigen presenting molecules (APMs). Moreover, expression of APMs on lung macrophages, in particular HLA-DR (MHC-II), have been show to increase following viral infection (Staples et al., 2015)(Keskinen et al., 1997). Whilst HLA-DR and HLA-ABC (MHC-I) have been more extensively studied and are known to be expressed on macrophages in the lung, less is known about the expression of the MAIT antigen presenting molecule, MR1. Particularly, it is not known where MR1 is expressed in the lung nor to what extent it is expressed.

The three main non-lymphocyte cell types found within the lung are macrophages, epithelial cells and fibroblasts, so these cells were identified in lung explant tissue as described, stained for MR1 and analysed by flow cytometry. MR1 appeared to be expressed highest on macrophages, with a small expression of MR1 detected on epithelial cells; MR1 appeared to be absent on fibroblasts (figure 3.6A).

As macrophages were defined as CD45+ HLA-DR+ cells, it was possible that dendritic cells (DCs) were also being including in the macrophage gate. For this reason, lung cells were also stained with a lineage marker cocktail Lin1 (as described in the section 2.5), which positively identifies macrophages whereas its absence identifies DCs. Macrophages appeared to comprise >95% of CD45+ HLA-DR+ cells in the lung, confirming that macrophages, and not DCs, were the main cell type to express MR1 (figure 3.6B). As macrophages expressed the most MR1 and are the most abundant APC in the lung, macrophages were used as the APC for conventional T cell/MAIT cell activation in the subsequent chapters.

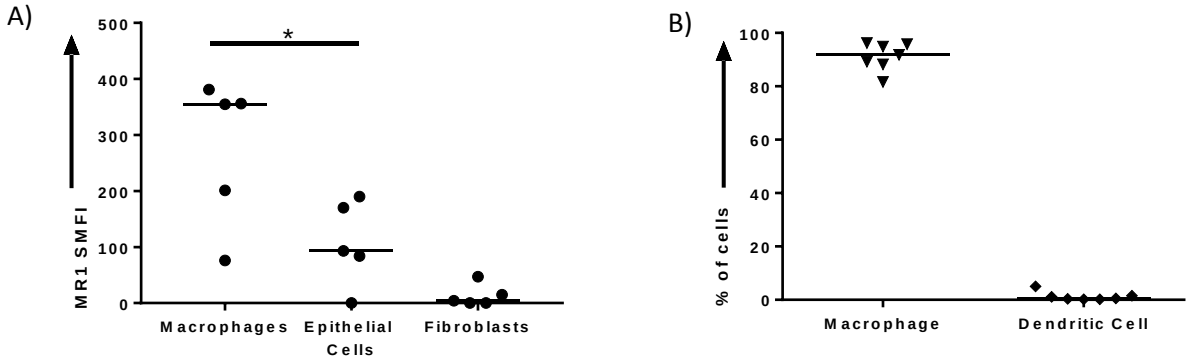


Figure 3.6 – Expression of MR1 in the lung. A) Lung cells were stained for CD45, HLA-DR, Epcam, CD90 and MR1 and expression of each marker was determined by flow cytometry. Macrophages were defined as CD45+ HLA-DR+; epithelial cell were defined as CD45- Epcam+; fibroblasts were defined as CD45- CD90+. B) Macrophages comprise >95% of CD45+ HLA-DR+ cells in the lung. Macrophages were defined as CD45+ HLA-DR+ Lin+, dendritic cells were defined as CD45+ HLA-DR+ Lin-. For all graphs, median values are shown. SMFI is where isotype control fluorescence values have been subtracted from marker MFI. Statistical significance was determined by Wilcoxon signed-rank test. * $P<0.05$.

3.3 Discussion and Conclusions

In this chapter, flow cytometry has been used to characterise the T cell populations present in the human lung, focusing on conventional CD4+ and CD8+ T cells and MAIT cells. Comparison of lung and blood showed that CD4+ were the most abundant conventional T cell subset present in both compartments, followed by CD8+ T cells, consistent with previous reports (Purwar et al., 2011). Double positive and double negative populations were much smaller in comparison, although as the double negative population was higher in the lung compared to the blood, it may represent an important lung population, which is worthy of further study. However, for the purpose of this thesis, only CD4+ and CD8+ were considered as conventional T cells. Although not investigated in this study, lung T cells have previously been shown to predominately be effector memory (CD45RO+ CCR7-) and central memory (CD45RO+ CCR7+) T cells, with few naive T cells (CD45RO-CCR7+) (Hutton et al., 2017). One caveat to consider is that the lung tissue was obtained from elderly patients (median age 69, see table 2.2), 11 of whom were either current or former smokers. Both age and smoking history may be factors which affect the number of T cell markers and the ability of T cells to respond to stimulation (Tollerud et al., 1989)(Lazuardi et al., 2005). This limitation should be considered in this chapter and all subsequent chapters where lung tissue has been used. Unfortunately, lung tissue was not available from younger, healthier donors as these individuals are not likely to be undergoing lung lobectomy.

MAIT cells are a recently discovered population of innate-like T cells which have not been well studied in the human lung. Here, MAIT cells were found to comprise approximately 1.5% of T cells in the lung parenchyma, consistent with observations made by Hinks et al, where MAIT cells from bronchial biopsies were a similar percentage of T cells (Hinks et al., 2016). Lung MAIT cells were also predominately CD8+, which was comparable to the data in blood reported here and consistent with the current literature regarding MAIT cells within PBMCs (Reantragoon et al., 2013). MAIT cells in the blood are also typically reported as $CD4^{low/-}$ (Reantragoon et al., 2013). However, CD4+ MAIT cells were found to be greater in the lung compared to the blood, which may represent a population of MAIT cells that are predominately cytokine producing, akin to conventional CD4+ T helper cells. Overall, given that MAIT cells are antibacterial T cells resident in the lung (Meierovics et al., 2013), it is likely that they are playing a role in host defence to lung bacterial pathogens, although the function of these cells in respiratory disease has been barely explored. Work in the subsequent chapters will explore the cytokine and cytotoxic functions of MAIT cells in response to bacterial infection.

In order to obtain cells from lung tissue, collagenase digestion was used to liberate T cells. This treatment seems to be suitable as collagenase did not affect expression of CD4, CD8 or MAIT cell markers compared to T cells obtained from lung tissue which was not collagenase treated.

Conventional T cells and MAIT cells have been further characterised by their ability to express three different cytokines: IFNγ, IL-17a and TNFα. These cytokines were chosen for two reasons. Firstly, in the MAIT cell literature, these three cytokines are typically investigated as MAIT cells are of a Th1/Th17 phenotype and have not been shown to greatly produce Th2 cytokines such as IL-4 or IL-13 (Dusseaux et al., 2011). Secondly, as the purpose of this thesis is to explore the responses of T cells to NTHi infection, IL-13 and IL-10 have not been studied as others have already shown that IL-13 and IL-10 are not the primary cytokines induced by NTHi (King et al., 2003)(Hutton et al., 2017). From the data presented in this chapter, conventional T cells in the lung are capable of producing IFNγ and TNFα, with IFNγ giving the strongest signal for these two cell types in the blood. MAIT cells, as well as expressing both IFNγ and TNFα, were also the most potent producers of IL-17a in the lung. Similar to conventional T cells, the strongest cytokine signal in blood MAIT cells was IFNγ. Taken together, conventional T cells and MAIT cells are sources of IFNγ, IL-17a and TNFα. In the subsequent chapters, the production of these cytokines in response to NTHi will be further explored.

In addition to cytokine production, T cells were also analysed for their expression of the cytotoxic effector, granzyme B. Cytotoxic T cells are vital in the control and clearance of intracellular bacterial pathogens, but little is known about the cytotoxic capabilities of MAIT cells. Lung CD8+ T cells and MAIT cells were granzyme B+ at baseline, indicating that these two cell types may have cytotoxic function in the lung. PMA/ionomycin stimulation was attempted to induce greater granzyme B expression, but this stimulus did not appear to be suitable for lung T cells. Chapter 6 will investigate T cell cytotoxicity further and the cytotoxic response of conventional T cells and MAIT cells to NTHi infection will be studied.

Expression of surface markers in combination with cytokine production allows for the identification of effector cell types. However, further approaches would have added to the definition of these T cell subsets. In particular, identification of Th1 and Th17 cells could have been confirmed by transcription factor staining for T-bet and RORγt (Broere et al., 2011). MAIT cells have also been shown to express both of these transcription factors, in addition to the transcription factor PLZF (Leeansyah et al., 2015)(Koay et al., 2016). Unfortunately, the available flow cytometry facilities only allowed for a maximum of 9 flurophores to be analysed, which meant that combining surface and cytokine staining with transcription factor staining was not

possible. As surface staining is the primary method used to identify T cell populations, the remainder of this thesis will just use surface antigen staining for CD4, CD8 and the MAIT markers Vα7.2 and CD161.

In order for T cells to become activated in response to invading pathogens, T cells require antigen presentation by APCs such as DCs and macrophages. To investigate T cell and MAIT cell responses to NTHi in the later chapters, the most appropriate APC needed to first be identified. In particular, expression of MR1 in the lung has not been characterised. In this chapter MR1 has been identified as being highly expressed on macrophages rather than DCs, with relatively little expression on epithelial cells, and no expression on fibroblasts. Together with previous reports that MHC-I and MHC-II, which present to CD8 and CD4 T cells respectively, are expressed on lung macrophages (Sprent, 1995)(Caulfield et al., 1999)(Martín-Orozco et al., 2001)(Kopf et al., 2015), the data here provide justification for using macrophages as the APC to study T cell responses to NTHi.

Overall, conventional T cells are present in the lung, with CD4+ T cells the most abundant conventional T cell subset present in the lung and in the blood. Furthermore, MAIT cells are also present in the lung and may be involved in immune defence against invading bacterial pathogens. T cells in the lung and the blood were investigated for their cytokine and cytotoxic potential; subsequent chapters will explore these immune mediators and their mechanisms following NTHi infection. As macrophages are abundant in the lung and express the necessary antigen presenting molecules, these phagocytes will be infected with NTHi and used as the APC to activate conventional and MAIT cells. Before T cell responses to NTHi can be characterised, the responses of macrophages to NTHi infection need to first be investigated. Particularly, it is currently unknown how NTHi affects macrophage expression of antigen molecules and other pathways involved in T cell activation. Therefore, the next chapter will study macrophage responses to NTHi.

4. Investigating macrophages as antigen presenting cells for conventional T cells and MAIT cells in NTHi infection

4.1 Introduction

The primary focus of this thesis is to explore the responses of T cells and MAIT cells to NTHi. However, T cells typically require antigen presentation in order to respond to invading pathogens (Smith-Garvin et al., 2009)(Broere et al., 2011). Having shown in the previous chapter that macrophages in the lung are abundant and have the highest expression of MR1, the next step was to explore how macrophages regulate their expression of antigen presenting molecules (APMs) following NTHi infection.

Macrophages are professional antigen presenting cells (APCs) and phagocytes, capable of internalising pathogens and presenting their antigens to T cells (Staples et al., 2015)(Weinberg and Unanue, 1981)(Pozzi et al., 2005), making macrophages a valid cell type to investigate T cell activation. In the lung, alveolar macrophages are orchestrators of the immune response (Barnes, 2004) and play a key role in maintaining lung homeostasis, protection from pathogens and activating conventional T cells. As opposed to naïve T cells which require antigen presentation by dendritic cells (DCs) in lymph nodes, lung T cells are mainly of a memory phenotype (Hutton et al., 2017) and may therefore be activated by lung macrophages. The role of lung macrophages in activating MAIT cells is unknown.

Expression of HLA-DR (MHC-II) and HLA-ABC (MHC-I) on macrophages has been characterised in response to viral infection (Staples et al., 2015), but the literature is limited on the response of these APMs to NTHi infection. To date, there are few studies of MR1 expression on macrophages and the effect NTHi infection has on MR1 expression has not been explored.

Whilst it is more appropriate to study infection in a physiologically relevant system, blood-derived models are often used as they overcome the issues of availability of lung tissue and low cell yields. Monocyte-derived macrophages (MDM) have been used as a model for lung macrophages, as these are easier to obtain in large quantities and have already been established in models of viral infection, showing similar responses to lung macrophages (Staples et al., 2015).

In this chapter, the expression of MR1, HLA-DR and HLA-ABC has been investigated on human MDM and human lung macrophages following NTHi infection. Further work has also explored the mechanisms regulating MR1 expression. In addition to APMs, the expression of MDM cell surface proteins involved in T cell co-stimulation and co-inhibition have also been investigated.

4.2 Results

4.2.1 NTHi is phagocytosed by MDM

To first confirm that macrophages could phagocytose NTHi, MDM were cultured with live NTHi for 2 hours in antibiotic-free media (as described in section 2.4) to allow for uptake of bacteria. MDM were then washed and treated with gentamicin for 90 minutes to kill extracellular bacteria, based on an established protocol (Edwards and Massey, 2011), after which the MDM were lysed to release internalised NTHi. The lysed solution was then plated out on chocolate agar plates and incubated overnight to facilitate growth of any NTHi present. No NTHi colonies were detected in uninfected MDM, whereas colonies were detected from MDM exposed to NTHi, demonstrating that NTHi is taken up by MDM (figure 4.1).

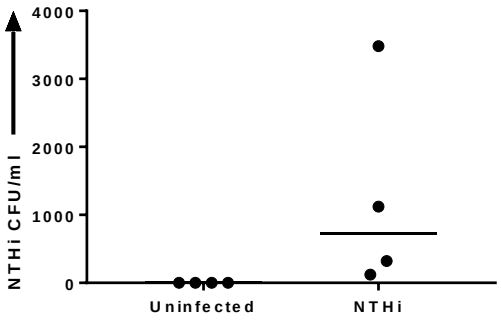


Figure 4.1 – NTHi is taken up by monocyte-derived macrophages (MDM). Viable counts of NTHi following a gentamicin protection assay. Data are from different donors and are paired; median values are shown. MDM were either uninfected or infected with NTHi at a MOI of 1.

4.2.2 NTHi is still detectable within MDM after 24 hours

As the purpose of this thesis is to ultimately co-culture T cells with MDM, the NTHi infection protocol was modified in line with previously established protocols for MDM-T cell co-cultures (Staples et al., 2015)(McKendry et al., 2016). MDM were exposed to NTHi for 2 hours as before, washed and this time cultured in media containing penicillin/streptomycin for a further 22 hours.

RNA harvested from MDM was analysed by qPCR for expression of the *hel* gene, which encodes lipoprotein e, an NTHi outer membrane protein (Yadav et al., 2003). Expression of *hel* was not detected in uninfected MDM, whereas the *hel* gene was present in MDM exposed to NTHi, indicating that NTHi products can still be detected within MDM 24 hours after first infection (figure 4.2). Detection of the *hel* gene doesn't prove that the NTHi is alive at this time point; indeed, given that the MDM have been exposed to penicillin/streptomycin for 22 hours, it would seem unlikely that the NTHi would be alive. However, for the purpose of this thesis, NTHi does not need to be viable at the endpoint of the experiment. The point of using MDM is for them to uptake the NTHi, process the NTHi antigens, and later present these antigens to T cells. Given that the MDM can phagocytose NTHi and that NTHi products are still detectable within MDM 24 hours later, MDM exposed to NTHi will herein be termed infected or referred to as NTHi-infected MDM.

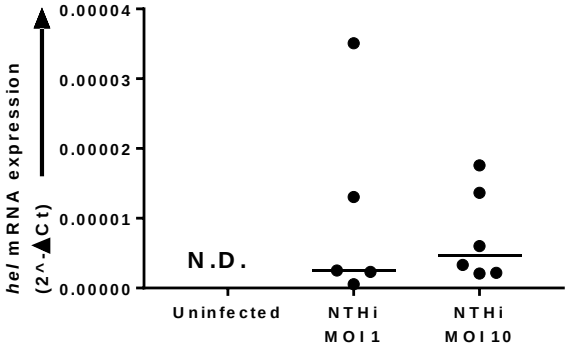


Figure 4.2 – Detection of NTHi within MDM. Expression of NTHi *hel* gene, as measured by qPCR. N.D. = not detected. For all graphs, data are from different donors and are paired; median values are shown. MDM were either uninfected or infected with NTHi at a MOI of 1 or 10.

4.2.3 Optimising the MOI of NTHi to detect antigen presenting molecules on lung macrophages and MDM

After confirming that NTHi infects MDM in this model, the next step was to determine which MOI of NTHi to use for further analysis. To this end, MDM were infected with NTHi at a MOI of 1 or 10 and surface expression of MR1, HLA-DR and HLA-ABC were investigated by flow cytometry.

MR1 was found to be expressed on uninfected MDM at low levels, with an MR1 SMFI of 302 which increased significantly ($P<0.05$) by approximately 3-fold on MDM following NTHi infection at both MOI 1 and 10. This is shown by an increase in fluorescence intensity after infection (figure 4.3). These data show that MDM upregulate MR1 in response to NTHi infection and may therefore be able to activate MAIT cells. Similar to MR1, HLA-DR surface expression also significantly ($P<0.05$) increased by approximately 2-fold on NTHi-infected MDM at both MOIs, as seen in figure 4.3. Upregulation of HLA-DR suggests that the macrophages have become activated and may present antigen to CD4+ T cells. In contrast to MR1 and HLA-DR, NTHi infection did not significantly affect HLA-ABC expression, despite a slight trend for an increase at MOI 10 (figure 4.3). However, as HLA-ABC has a high constitutive surface expression of approximately 30,000 SMFI, it may not be necessary for HLA-ABC to be upregulated in order to function and activate CD8+ T cells.

To ensure that the NTHi-induced upregulation of MR1 and HLA-DR were not an artefact of using blood-derived MDM, lung macrophages were obtained from human lung parenchymal tissue as described in chapter 2 and infected with NTHi at MOI 1 or 10. As seen in figure 4.3, NTHi at MOI 10 caused a small but significant ($P<0.01$) increase in surface MR1. An MOI of 1 however only produced a minor increase in MR1 expression. HLA-DR expression increased from SMFI 30406 to 46275 ($P<0.05$; 1.5-fold increase vs uninfected) for MOI 1 and further to 64241 ($P<0.01$; 2-fold increase vs uninfected) for MOI 10 (figure 4.3). However, HLA-ABC surface expression was unaffected by either MOI of NTHi (figure 4.3). Overall, NTHi infection caused lung macrophages to upregulate surface expression of HLA-DR and MR1.

Taken together, the MDM data show comparability with the lung macrophage data, as NTHi causes upregulation of MR1 and HLA-DR, providing a justification for using an MDM model to study macrophage responses to NTHi infection. The only obvious difference is that the MDM only required an MOI 1 for upregulation of MR1 and HLA-DR; in contrast to the lung macrophages, where only an MOI of 10 was sufficient to cause a greater increase in upregulation of both APMs. Due to the limited yields of lung macrophages obtained from tissue, the rest of the work in this

chapter utilises the NTHi-infected MDM model. As both MR1 and HLA-DR surface expression on MDM were not further elevated by increasing the amount of NTHi, the following work in the chapter uses NTHi at a MOI of 1.

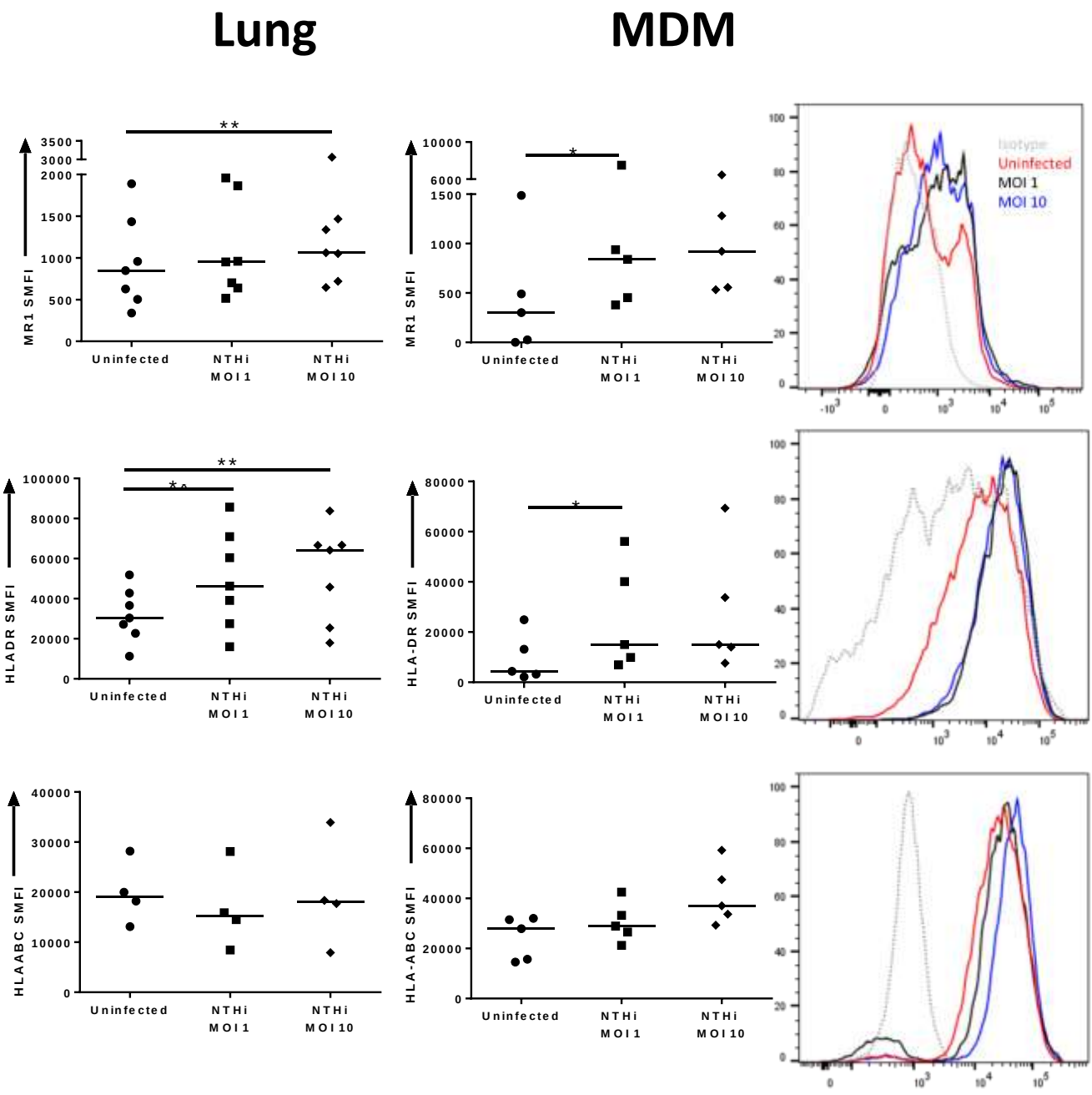


Figure 4.3 – Expression of antigen presenting molecules on NTHi-infected macrophages. Specific mean fluorescence intensity (SMFI) values of MR1, HLA-DR and HLA-ABC on lung macrophages and MDM either uninfected or infected with NTHi at a MOI of 1 or 10. For all graphs, data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Representative histograms of APM expression on MDM are shown adjacent to the relevant graph. Statistical significance was determined by Friedman test with Dunn's multiple comparison. *P<0.05 **P<0.01

4.2.4 Comparing the effects of bacterial strains on antigen presenting molecules on MDM

To next ensure that the observations described above were not due to bacterial strain dependent effects, MDM were infected at MO1 of 1 with one of four different clinical isolates of NTHi. Here, NTHi ST201 refers to the strain used previously in this chapter and the ST1447, ST253 and ST14 represent additional NTHi strains. Furthermore, MDM were also infected with a strain of *E.coli* as a positive control, as this bacteria is commonly used in the MR1/MAIT literature and has previously been shown to upregulate MR1 (Ussher et al., 2014a)(Dias et al., 2016)(Salerno-Goncalves et al., 2014). Both MR1 and HLA-DR were upregulated by all four strains of NTHi and no significant difference was observed between strains (measured by Friedman test with Dunn's multiple comparison), indicating that the effects of NTHi on MR1 and HLA-DR upregulation are not strain dependent (figure 4.4). Infection of MDM with *E.coli* also upregulated the two antigen presenting molecules to a similar level as with NTHi infection (figure 4.4). No significant differences in HLA-ABC expression were detected between strains (figure 4.4). Overall, no significant NTHi strain specific effects were observed. For this reason, the rest of this thesis will continue to use the ST201 strain of NTHi, unless otherwise stated.

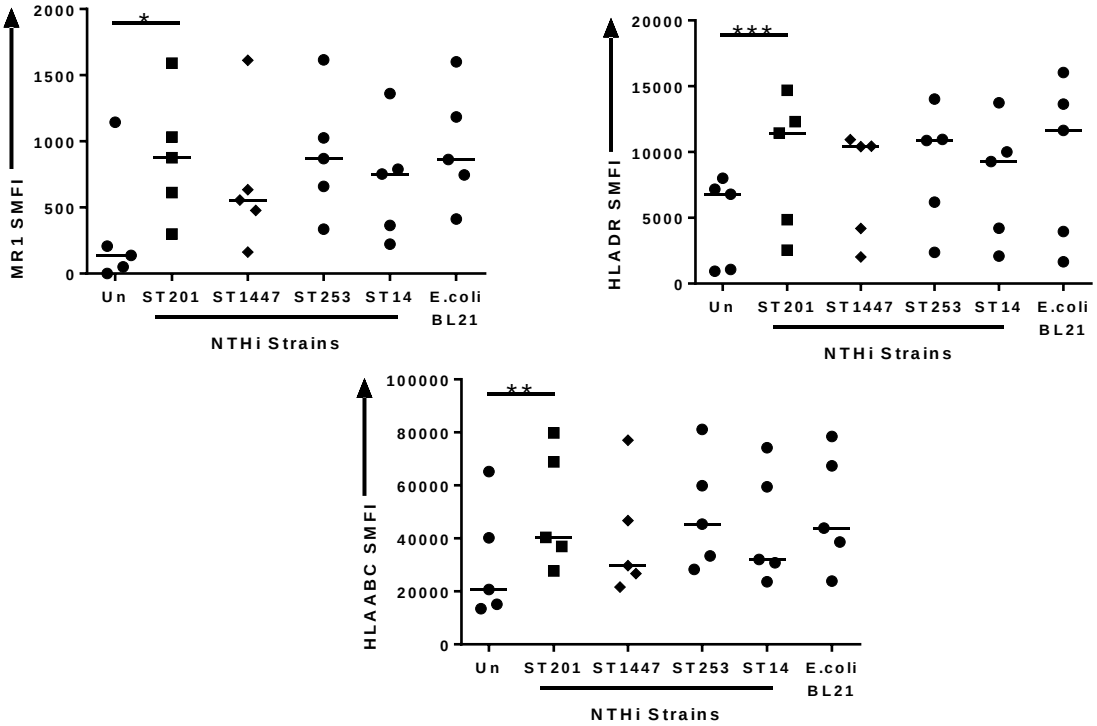


Figure 4.4 – Comparing different NTHi strains and expression of antigen presenting molecules. Specific mean fluorescence intensity (SMFI) values of MR1, HLA-DR and HLA-ABC on MDM either uninfected (Un) or infected with different strains of NTHi or *E.coli* at a MOI of 1. For all graphs, data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Friedman test with Dunn's multiple comparison. *P<0.05, **P<0.01, ***P<0.001

4.2.5 Expression of APMs on MDM is induced by live NTHi infection

To determine whether the observed upregulation of MR1 and HLA-DR were due to live bacterial infection or just due to activation of macrophages by innate recognition of PAMPs, MDM were additionally exposed to NTHi that had been killed by fixation in 2% (v/v) PFA (PFA-NTHi). Infection with inert bacteria could also indicate whether live bacteria are able to actively inhibit upregulation of antigen presentation molecules.

Similar to figure 4.3 and 4.4, expression of MR1 in these experiments increased significantly (P=0.002) by approximately 2-fold to 3992 SMFI on MDM following live NTHi infection. Live NTHi also caused a significantly (P=0.04) higher expression of MR1 on MDM compared to MDM infected with PFA-NTHi, indicating that live NTHi is required for optimal MR1 upregulation (figure 4.5). HLA-DR surface expression also significantly (P=0.006) increased by 4-fold in response to live NTHi, from SMFI of 1529 to 6329, as seen in figure 4.5. As with MR1, HLA-DR expression was significantly (P=0.01) higher with live NTHi compared to PFA-NTHi, again indicating that the upregulation observed requires infection with live NTHi. In agreement with the previous data, NTHi infection did not affect HLA-ABC expression and neither was it affected by infection with PFA-NTHi (figure 4.5). Overall, upregulation of MR1 and HLA-DR appear to be driven by live NTHi infection and are not strongly induced by killed bacteria.

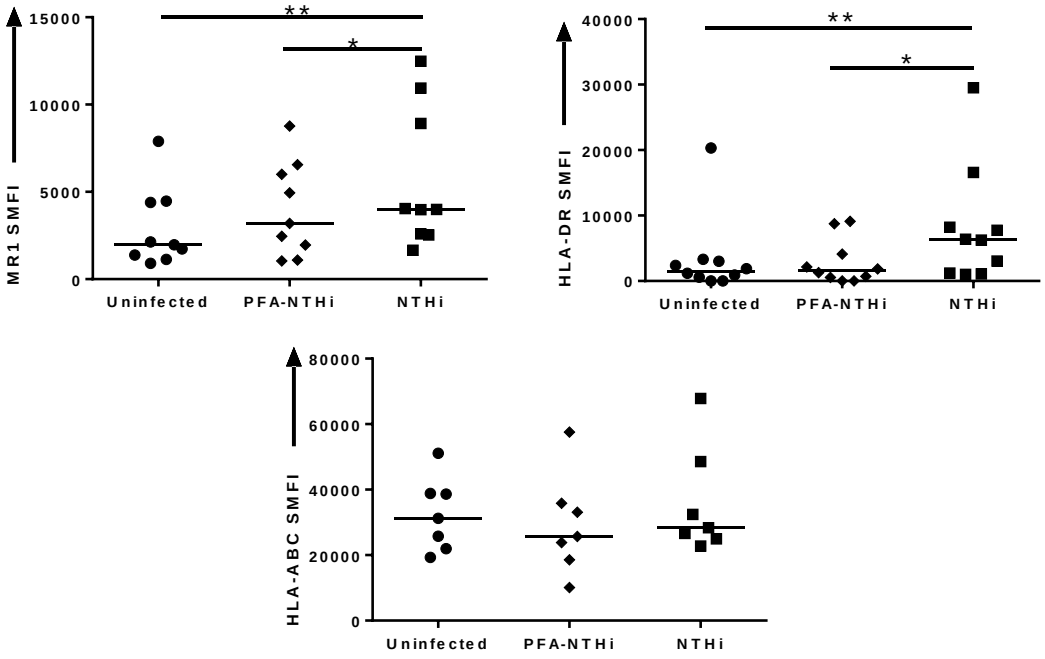


Figure 4.5 –Expression of antigen presenting molecules is affected by live NTHi infection. Specific mean fluorescence intensity (SMFI) values of MR1, HLA-DR and HLA-ABC on MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 1. For all graphs, data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05 **P<0.01

4.2.6 Upregulation of MR1 is specific to bacterial infection

Whilst HLA-DR and HLA-ABC have been extensively studied in the literature, less is known about the regulation of MR1. To further investigate the potential causes of MR1 upregulation, MDM were stimulated with pro-inflammatory cytokines produced by NTHi-infected macrophages; TNFα (Martí-Lliteras et al., 2009) and IL-6 (Punturieri et al., 2006) (both at 10 ng/ml). MDM were also stimulated with IFNγ (10 ng/ml), which is known to upregulate HLA-ABC and HLA-DR (Keskinen et al., 1997). All three cytokines produced only minor, inconsistent changes in surface expression of MR1, which were not statistically significant (figure 4.6A), indicating that MR1 was not regulated by autologous release of cytokines from infected MDM.

As NTHi is a gram-negative bacterial species, MDM were also stimulated with 100 ng/ml lipopolysaccharide (LPS) to determine whether TLR4 activation could account for upregulation of MR1. LPS however produced no significant change in MR1 expression, as shown in figure 4.6B. These data would suggest that the innate inflammatory response alone is not sufficient to affect MR1 protein expression.

Whilst MR1 is known to present vitamin B2 antigens derived from bacteria, to date viruses have not been shown to cause MR1 upregulation. To this end, MDM were infected with 500 pfu/ml of the respiratory virus influenza A (x31 strain). However, expression of MR1 was found to be unaffected by viral infection (figure 4.6C), despite the fact that x31 infects MDM and causes upregulation of HLA-DR (Staples et al., 2015).

Overall, these data suggest that NTHi-induced upregulation of MR1 is a specific response to NTHi bacterial infection and is not simply due to generic activation of the macrophage or innate inflammatory signalling. All MDM exposed to cytokines, LPS or virus underwent morphological changes, suggesting that these stimuli were biologically active and having some effect on the MDM.

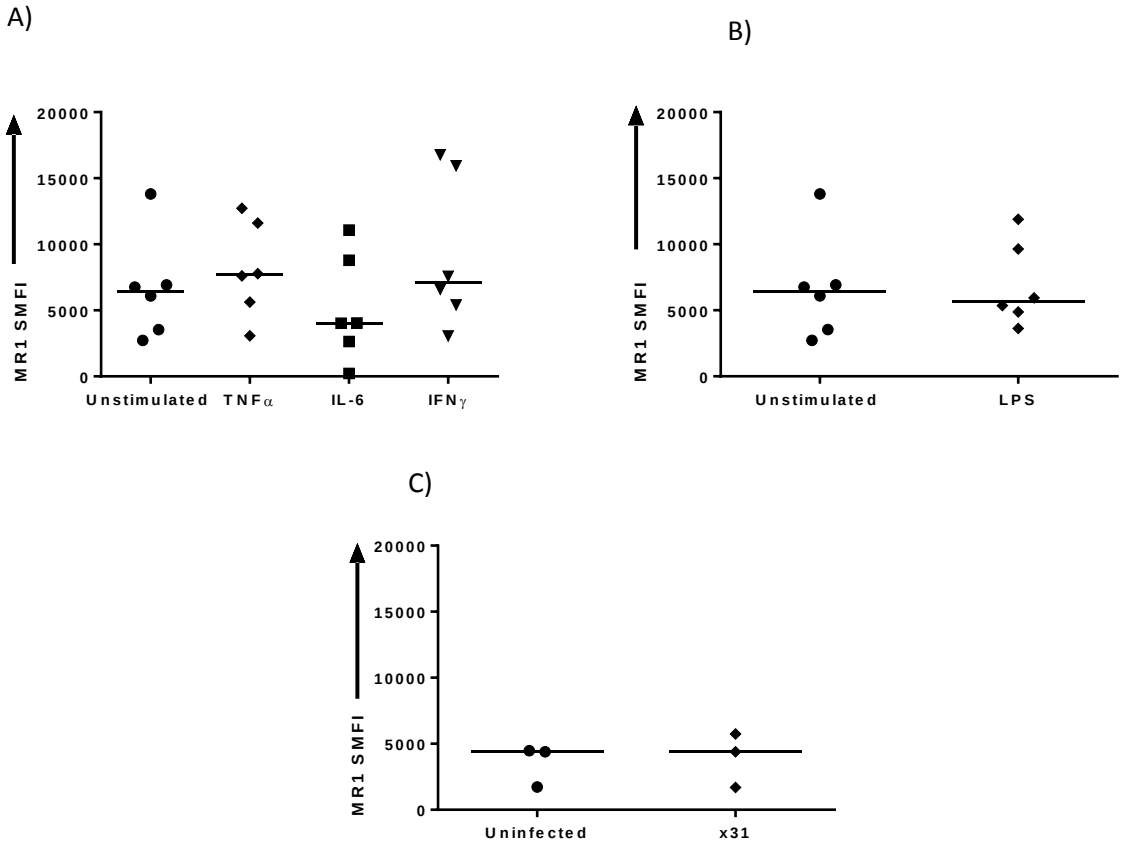


Figure 4.6 – MR1 expression is not induced by cytokines, TLR activation or viral infection. Specific mean fluorescence intensity (SMFI) values of MR1 expression on MDM either unstimulated or stimulated with A) TNFα, IL-6 & IFNγ, B) LPS or C) infected with influenza x31. For all graphs, data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test.

4.2.7 Upregulation of macrophage MR1 expression is not due to increased gene expression

Although upregulation of MR1 protein has been demonstrated, less is known about the regulation of MR1 at the mRNA level following infection, despite the fact that MR1 transcripts have been found in a variety of tissues and cell types (Riegert et al., 1998). To further uncover the mechanisms of MR1 upregulation, MR1 gene expression was analysed by qPCR following NTHi infection of MDM and compared by normalising to the house-keeping gene, β2M. As can be seen in figure 4.7A, NTHi infection did not increase MDM MR1 gene expression. MR1 cT values were well within the range of detection, with cT values of 26.6 for uninfected MDM compared to 26.4 for NTHi-infected MDM, confirming that the lack of upregulation of MR1 gene expression was not due to an impaired ability to detect MR1 mRNA (figure 4.7B). The cT values for β2M were also analysed to ensure that the normalisation to house-keeping gene was not impacting detection of MR1 gene expression, but β2M was also found to remain constant (figure 4.7C). Overall, the data presented here indicate that MR1 protein upregulation is not controlled by increases in MR1 gene expression and that MR1 transcripts may just be constitutively expressed.

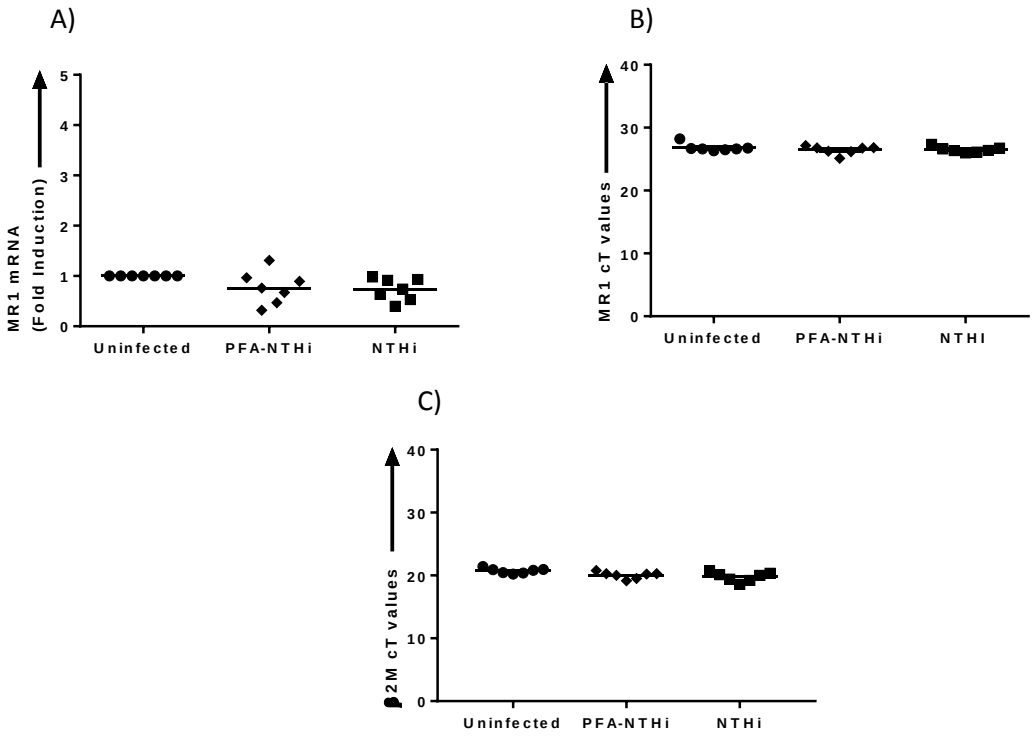


Figure 4.7 – MR1 gene expression in response to NTHi infection. A) MR1 gene expression of MDM either uninfected or infected with NTHi. β2M was used a housekeeping control gene to normalize the data. Data shown as 2^-ΔΔCt fold induction. cT values of B) MR1 and C) β2M from MDM either uninfected or infected with NTHi. For all graphs, data are from different donors and are paired; median values are shown.

4.2.8 NTHi infection causes *de novo* protein synthesis of MR1

As MR1 does not appear to be regulated at the gene level in MDM by NTHi infection, the upregulation on the surface of the NTHi-infected MDM could be due to increased trafficking of MR1 from an intracellular pool of endogenous protein to the cell surface. To investigate this, MR1 protein expression was analysed on both the cell surface alone and on the surface combined with intracellular expression (total expression) of NTHi-infected MDM. If MR1 surface upregulation was just due to increased trafficking to the cell surface, whilst surface expression would increase with infection, the total amount of MR1 present in the cell should remain unchanged.

As shown previously, surface upregulation of MR1 was observed following infection. However, when MDM were stained for combined surface and intracellular MR1 expression a clear increase ($P<0.05$) in total MR1 expression was also detected, indicating *de novo* MR1 protein production (figure 4.8).

Together with the data previously shown, infection of MDM with live NTHi results in new production of MR1 protein, which is then available to be trafficked to the MDM cell surface.

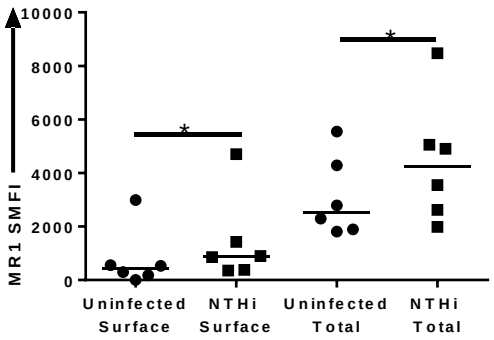


Figure 4.8 – Total MR1 protein increases in response to NTHi infection. Specific mean fluorescence intensity (SMFI) values of MR1 on either the surface alone or surface and intracellular combined (Total) of MDM either uninfected or infected with NTHi at a MOI of 1. Data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *$P<0.05$

4.2.9 Expression of macrophage co-stimulatory and co-inhibitory molecules

Although antigen presentation is one of the main factors responsible for driving T cell activation, by itself it is not sufficient to induce a robust T cell response to an antigen (Nurieva et al., 2009). Both naïve and memory T cells also require co-stimulatory signals from professional APCs in order to sufficiently activate, proliferate and survive following antigen-specific MHC-TCR engagement, or else they become anergic or undergo apoptosis (Chen and Flies, 2013)(Watts, 1999)(Smith-Garvin et al., 2009). In order to elucidate the role of MDM in activating T cells, the next step was to investigate the expression of co-stimulatory molecules on MDM in response to NTHi infection. Unfortunately, due to availability of cells, PFA-NTHi was not able to be tested.

One of the best characterised co-stimulation pathways is the CD80/86-CD28 pathway, where CD80 and CD86 on APCs bind to CD28 expressed on T cells to deliver a very potent activation signal. Here, CD80 and CD86 were expressed on MDM at baseline but expression increased significantly ($P<0.05$ for both) by 6-fold and 2-fold respectively following NTHi infection (figure 4.9). Similarly, another key co-stimulatory molecule, CD40, was potently upregulated ($P=0.03$) by 4-fold from 7382 SMFI uninfected to 29508 SMFI following infection (figure 4.9).

Additional co-stimulatory molecules investigated were CD70, 4-1BBL, ICOSL and OX40L, all of which were significantly (all $P<0.05$) upregulated on the surface of MDM following NTHi infection (figure 4.9). Successful signalling between APCs and T cells is furthered by stable cell-to-cell interactions. To this end, expression of the cell adhesion protein intracellular adhesion molecule 1 (ICAM1) was also investigated (figure 4.9). ICAM1 was found to be expressed on MDM at baseline and was significantly ($P=0.01$) upregulated by 3-fold following infection with NTHi, which may indicate a role for ICAM1 in MDM-T cell activation. Combined with the APM data presented earlier in this chapter, these data indicate that MDM express many of the proteins required for activating T cells in response to NTHi infection.

In addition to proteins that contribute to activate T cells by co-stimulation, APCs also express other molecules with co-inhibitory effects that dampen T cell responses. Once such protein is programmed death ligand 1 (PDL1) which is thought to be upregulated during infection and transmit an inhibitory signal to T cells via their PD1 receptor. PDL1 upregulation has been seen on macrophages following influenza infection (Staples et al., 2015) but whether PDL1 is increased as a result of NTHi infection has not been reported. Following exposure to NTHi, surface expression of PDL1 became upregulated by 8.5-fold on MDM from SMFI of 1238 to 10498 (figure 4.10) and

this was statistically significant (P=0.01). Expression of PDL2, the other ligand for PD1 also involved in T cell inhibition, was also examined. PDL2 was not expressed at baseline, but was upregulated following NTHi infection (P=0.01), albeit expression was considerably smaller compared to PDL1 (figure 4.10). Overall, the increase in PDL1 and PDL2 expression suggests that MDM may have the capacity to restrict T cell activation during infection, preventing over-activation and could contribute to T cell exhaustion. The functional effects of PDL1/2 are explored in the following chapters.

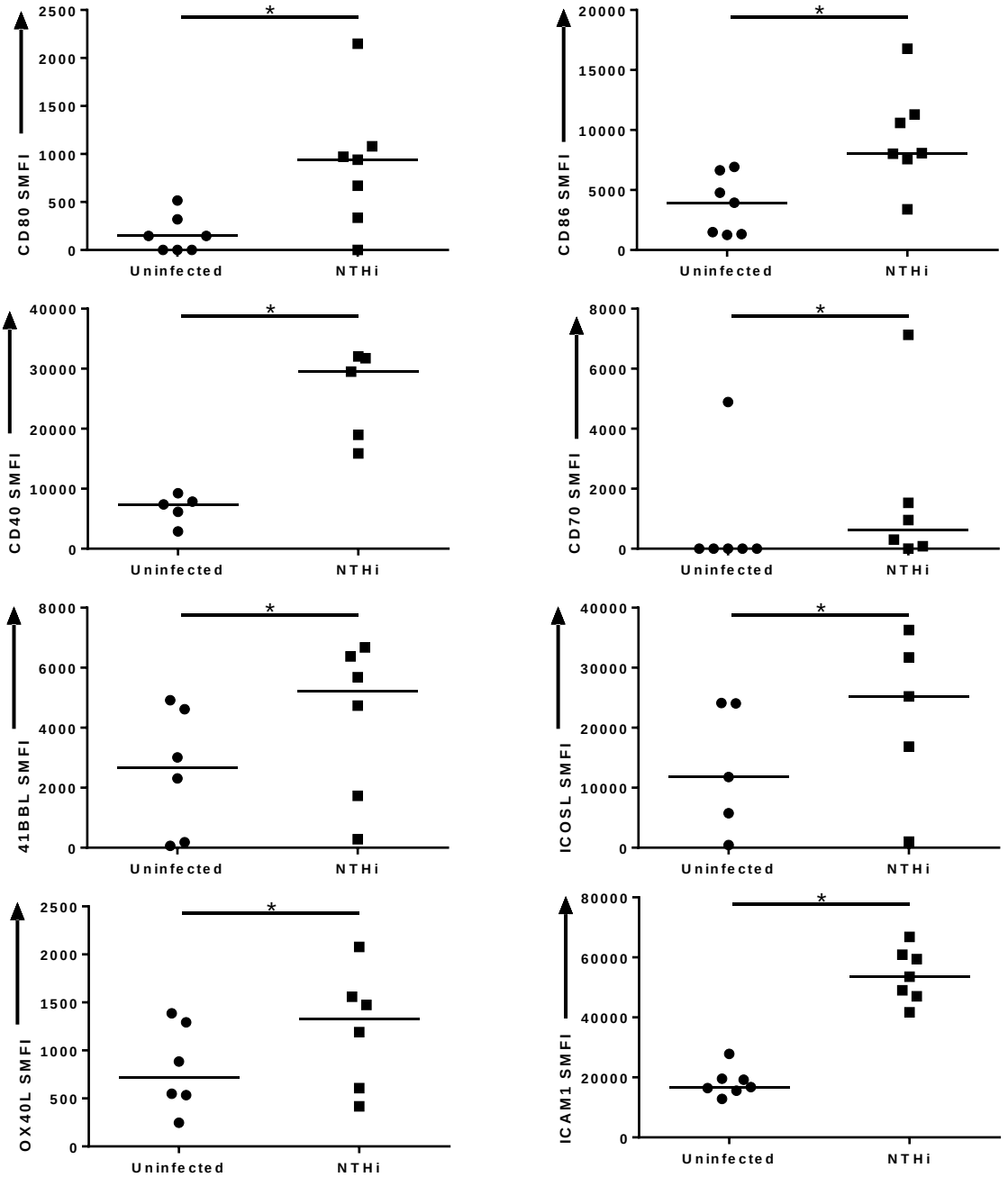


Figure 4.9 – Expression of co-stimulatory molecules on MDM following NTHi infection. Specific mean fluorescence intensity (SMFI) values of co-stimulatory molecules on the surface of MDM either uninfected or infected with NTHi at a MOI of 1. Data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

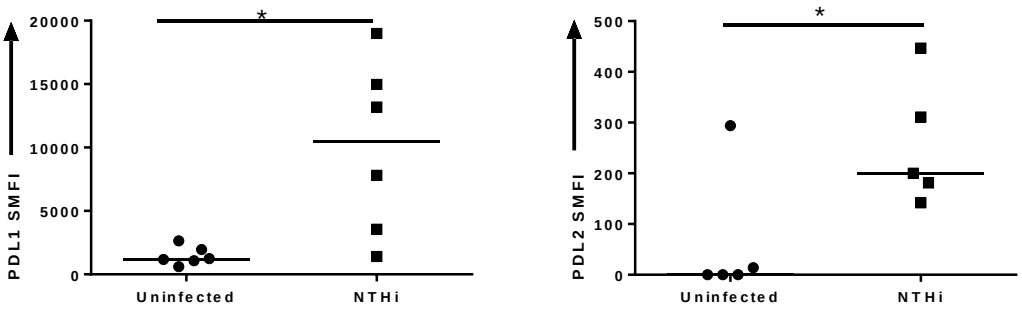


Figure 4.10 – Expression of co-inhibitory molecules following NTHi infection of MDM. Specific mean fluorescence intensity (SMFI) values of PDL1 and PDL2 on the surface of MDM either uninfected or infected with NTHi at a MOI of 1. Data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

4.2.10 Pattern recognition receptors and downstream signalling molecules are affected by NTHi infection

To further characterise the macrophage response to NTHi, RNA from infected MDM was analysed for expression of PRRs and their downstream signalling molecules, which are all involved in the detection of bacterial infection. TLR4 is a cell surface sensor of Gram-negative bacterial infection that detects LPS, which should therefore be involved in the response to NTHi. As NTHi is an intracellular pathogen (Craig et al., 2001)(Morey et al., 2011)(Clementi and Murphy, 2011) the expression of TLR7 and RIG-I, which are typically thought to recognise viral RNA, was also investigated as recent evidence suggests that they can recognise bacterial products as well (Schmolke et al., 2014)(Hagmann et al., 2013).

NTHi infection of MDM led to a significant (P=0.01) 65% reduction in gene expression of TLR4, whereas TLR7 expression increased by 3.8-fold (P=0.03) and RIG-I by 2-fold (P=0.03) (figure 4.11). To further assess the impact on innate immune signalling, gene expression of the inflammatory transcription factors NF-κB and IRF3, both of which are involved in signalling downstream of TLRs and RIG-I, was analysed.

Expression of NF-κB was not affected by NTHi infection whereas IRF3 gene expression significantly (P=0.01) decreased by 20%. As the purpose of IRF3 is to induce IFNβ, expression of IFNβ was measured and found to be significantly (P=0.03) reduced by 50%. Together these data suggest that NTHi infection may cause an impairment in innate immune signalling leading to reduction of certain cytokines.

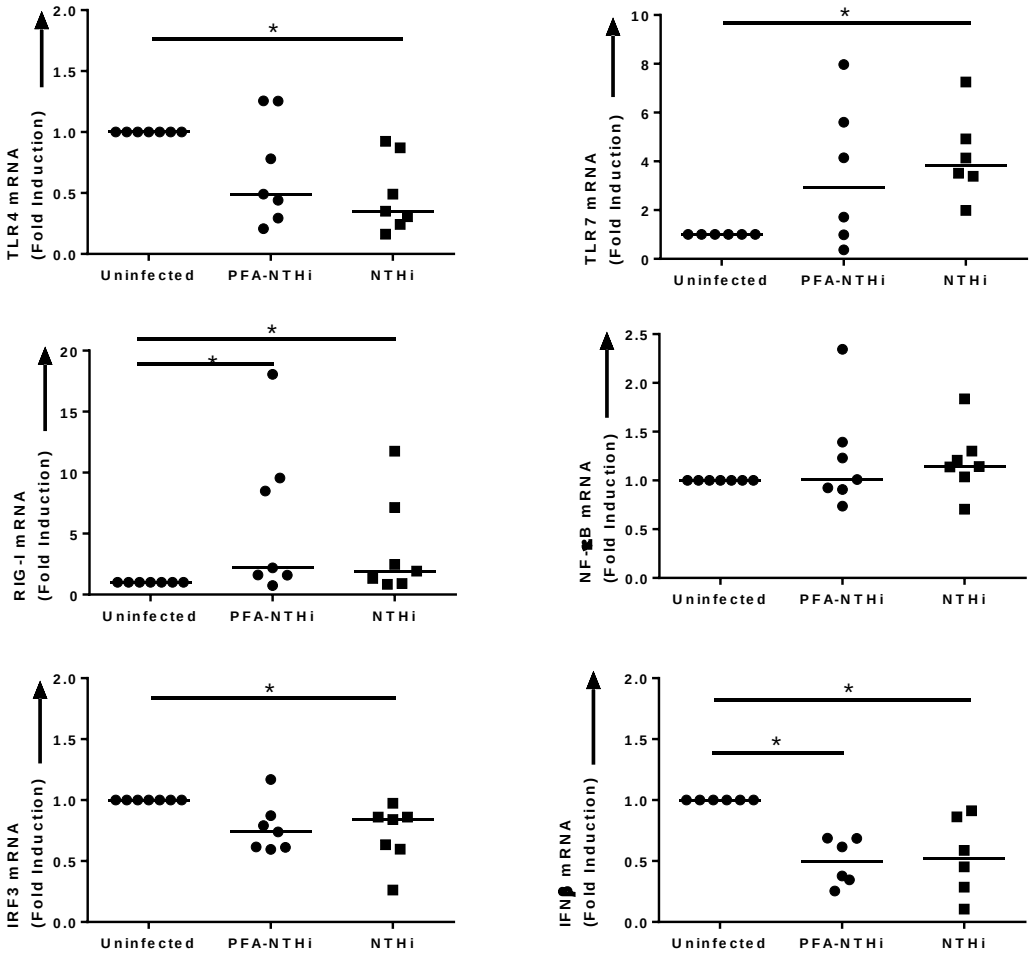


Figure 4.11 – Pattern recognition receptor and downstream signalling molecule gene expression in response to NTHi infection. TLR4, TLR7, RIGI, NFκB, IRF3 and IFNβ gene expression of MDM either uninfected or infected with NTHi. β2M was used a housekeeping control gene to normalize the data. Data shown as 2^-ΔΔCt fold induction. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

4.2.11 Macrophages release IL-1β in response to NTHi

As the qPCR data suggested that expression of cytokines by MDM could be impaired by NTHi infection, further analysis was performed to determine whether NTHi impaired production of other pro-inflammatory cytokines. Recently, high levels of IL-1β have been detected in the BAL of NTHi-positive COPD patients, compared to NTHi-negative COPD patients (Staples et al., 2016). To this end, IL-1β was measured in supernatant of NTHi-infected MDM. As seen in figure 4.12, no IL-1β protein was detected at baseline from uninfected MDM, but production rose to 412 pg/ml ($P<0.001$) following infection with NTHi. PFA-NTHi did not induce significant levels of IL-1β. In combination with the IFNβ qPCR data, whilst NTHi may impair expression of type I interferons, NTHi can induce other MDM pro-inflammatory cytokines. Further cytokine production by NTHi-infected MDM is explored in the following chapters.

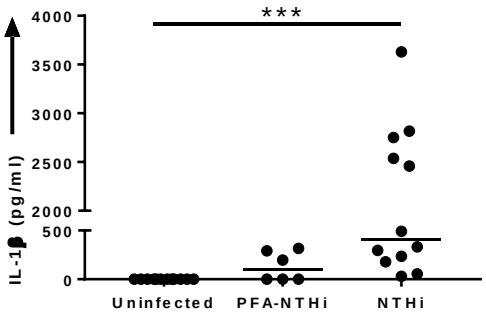


Figure 4.12 – Macrophages produce IL-1β in response to NTHi. IL-1β protein expression of MDM either uninfected or infected with NTHi, measured by ELISA. Data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. ***P<0.001

4.2.12 Corticosteroids reduce MDM responses to NTHi

Patients with COPD and other respiratory diseases are commonly treated with inhaled corticosteroids (ICS). However, current evidence suggests that these drugs may not be as effective as first thought at improving mortality and controlling disease progression (Yang et al., 2012). Whilst corticosteroids are effective during acute exacerbations (Woods et al., 2014), their use may cause an inadequate immune response to respiratory pathogens and therefore further bacterial colonisation and increase the risk of pneumonia (Festic and Scanlon, 2015). To determine whether corticosteroids have an impact on the immune response of macrophages to NTHi infection, MDM were infected with NTHi in the presence of either of the steroids fluticasone propionate (FP) or budesonide (BD).

As much of this chapter has focused on APMs, the effect of steroids on expression of MR1, HLA-DR and HLA-ABC was first explored to determine whether steroids impair expression of APMs. Both FP and BD completely prevented MR1 upregulation in response to NTHi ($P=0.0005$ and $P=0.01$ respectively; figure 4.13). To determine whether the impaired surface expression of MR1 was due to steroids inhibiting MR1 trafficking to the surface or inhibiting *de novo* protein, MR1 expression was analysed on both the surface and total (intracellular plus surface expression) of NTHi-infected MDM. Addition of fluticasone propionate significantly ($P=0.03$) impaired total MR1 expression back to baseline, suggesting that steroids impair production of new MR1 protein and do not just impair protein trafficking (figure 4.13). Steroid-induced inhibition of MR1 upregulation may have functional consequences and may lead to impairment of MAIT activation. The functional effects of steroids on MAIT activation are further explored in subsequent chapters.

In addition both steroids caused a significant ($P<0.05$) inhibition of HLA-DR upregulation, but in line with the lack of induction caused by NTHi, neither steroid had any effect on expression of HLA-ABC (Figure 4.14).

Fluticasone also significantly ($P<0.05$) inhibited release of IL-1β into the supernatant (figure 4.15). Overall, the data presented here indicate that steroids do not just affect expression of antigen presenting molecules but also impair the pro-inflammatory responses of macrophages to NTHi infection, which may have implications for effective immune responses to bacteria in the lungs of patients taking ICS. The effects of steroids on T cell responses to NTHi are explored in the following chapters.

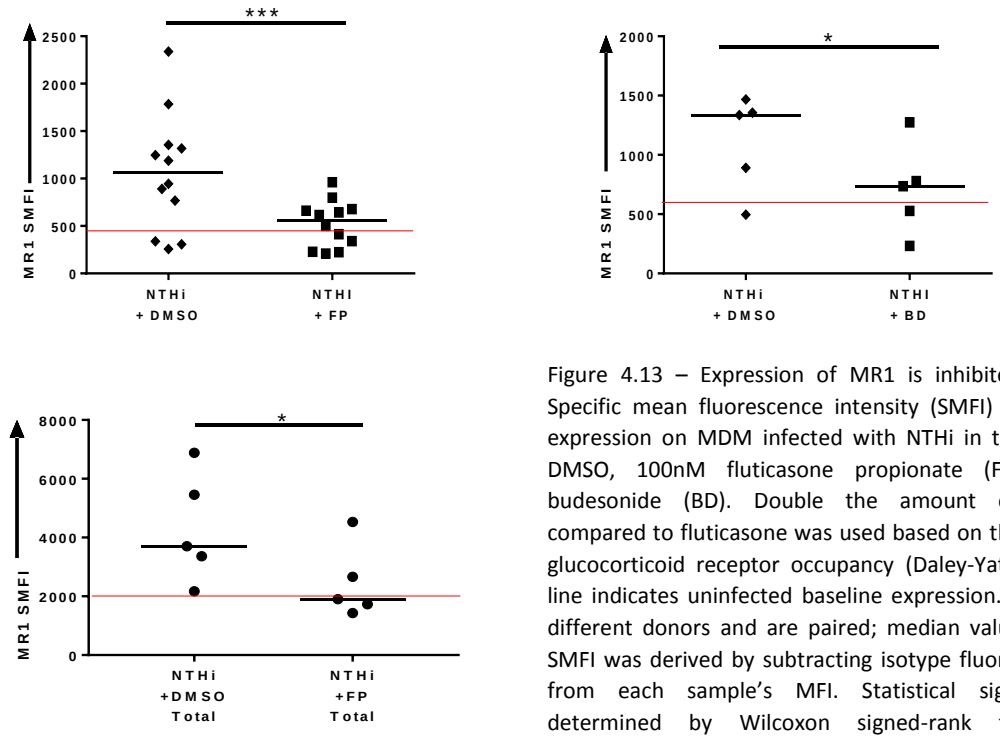


Figure 4.13 – Expression of MR1 is inhibited by steroids. Specific mean fluorescence intensity (SMFI) values of MR1 expression on MDM infected with NTHi in the presence of DMSO, 100nM fluticasone propionate (FP) or 200nM budesonide (BD). Double the amount of budesonide compared to fluticasone was used based on the difference in glucocorticoid receptor occupancy (Daley-Yates, 2015). Red line indicates uninfected baseline expression. Data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, ***P<0.001.

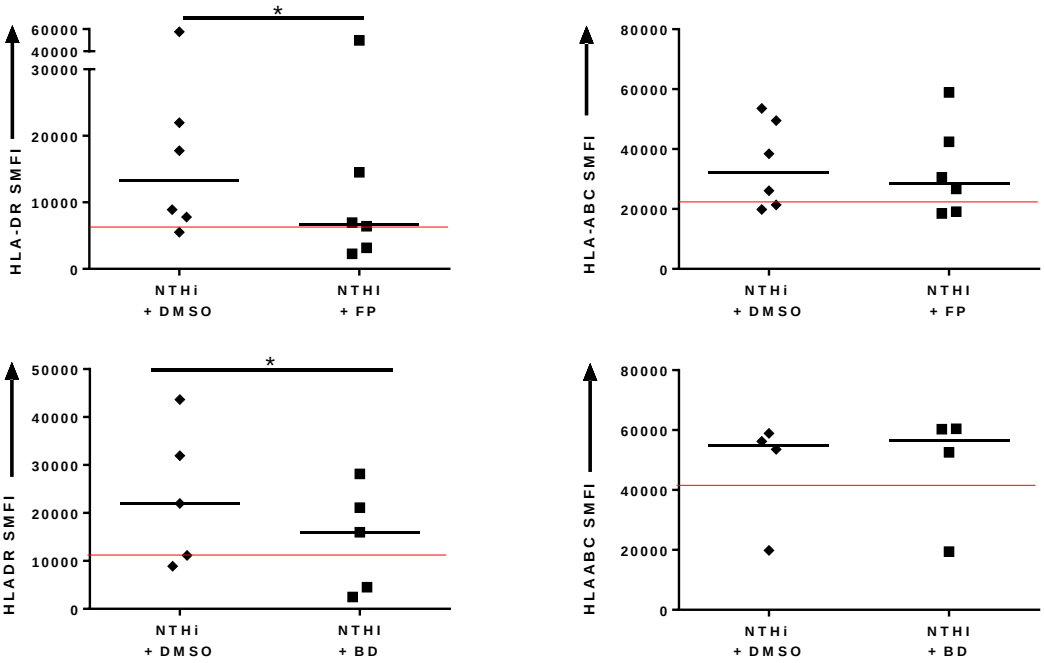


Figure 4.14 – Expression of HLA-DR but not HLA-ABC is inhibited by steroids. Specific mean fluorescence intensity (SMFI) values of MR1 expression on MDM infected with NTHi in the presence of DMSO, 100nM fluticasone propionate (FP) or 200nM budesonide (BD). Double the amount of budesonide compared to fluticasone was used based on the difference in glucocorticoid receptor occupancy (Daley-Yates, 2015). Red line indicates uninfected baseline expression. Data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

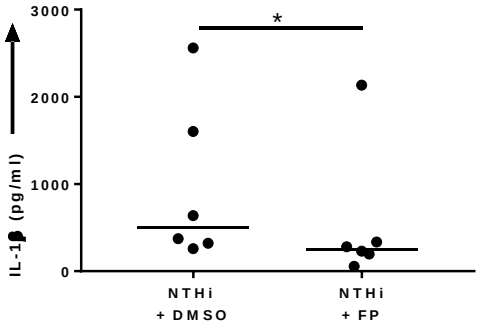


Figure 4.15 – Macrophages production of IL-1β is inhibited by steroids. IL-1β protein expression of MDM infected with NTHi in the presence of DMSO or 100nM fluticasone propionate (FP), measured by ELISA. Uninfected baseline was 0 pg/ml. Data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

4.3 Discussion and Conclusions

Macrophages are present in the lung and have a variety of functions in the response to bacterial infection. In this chapter, the main focus has been on the ability of macrophages to function as antigen presenting cells. Both lung and monocyte-derived macrophages express MR1 and this molecule becomes upregulated during infection with NTHi. The role of APCs, such as macrophages, in activating MAIT cells is not fully understood (Gozalbo-López et al., 2009)(Chua et al., 2012), however MAIT cells have been shown to respond to monocytes infected with *E.coli* (Le Bourhis et al., 2010). The standard model of naïve T cell activation is that DCs sample the airways and, following activation and trafficking to the local lymph nodes, present antigen to naive T cells. Whilst lung macrophages are not as potent at presenting antigen to naive T cells as DCs, lung macrophages do express high levels of both MHC I and II, express co-stimulatory molecules and present antigen to T cells (Staples et al., 2015)(Weinberg and Unanue, 1981)(Venet et al., 1985)(Lyons and Lipscomb, 1983)(Toews et al., 1984)(Pons et al., 2005). Furthermore, the data presented in the previous chapter indicated that the vast majority of HLA-DR+ cells (>95%) are CD45+/Lin+/HLA-DR+ macrophages rather than CD45+/Lin-/HLA-DR+ DCs. As macrophages express MR1 and HLA-DR and upregulate both these molecules in response to NTHi, using MDM to study T cell/MAIT cell activation appears an appropriate system to model what may happen in the airway. The data presented in this chapter indicate that macrophages are a plausible APC for MAIT cells in the lung.

Comparing MDM and lung macrophages, a clear observation is the difference in NTHi MOI required for MR1 upregulation, with MDM requiring the lower MOI of NTHi. The reasons for this could be many, but are likely due to the lung macrophages being exposed to a complex environment of cytokines as well as exposure to a complex microbiome and pollutants whilst *in situ* over the lifetime of the cell. Another consideration is that these lung macrophages were obtained from patients undergoing lung lobectomy surgery and thus likely have a history of smoking and also potentially COPD, both factors known to impair macrophage phagocytosis (Taylor et al., 2010). A higher MOI of NTHi may therefore be needed due to impaired phagocytosis, as suggested by Berenson et al (Berenson et al., 2013). That being said, HLA-DR expression was upregulated on lung macrophages with either MOI, suggesting that the macrophages were activated at least to some extent by NTHi infection with MOI 1. Levels of HLA-ABC protein expression remained unchanged on MDM and lung macrophages. Whilst effects of NTHi infection on HLA-ABC expression on macrophages has not been shown before, HLA-ABC expression remains unaltered on epithelial cells infected with NTHi (Frick et al., 2000) and

Mycobacterium tuberculosis (Mtb) (Gold et al., 2010). However, Choi et al found upregulation of HLA-ABC in response to lipooligosaccharide (LOS) and LPS derived from *Haemophilus influenzae* (Choi et al., 2014). Due to its high constitutive expression on MDM, HLA-ABC may not need to be further increased in order to function. In addition, comparison of different NTHi strains showed that the observed effects of MR1 and HLA-DR upregulation were strain independent. The NTHi isolates used in this chapter were all cultured from a combination of COPD patients and healthy volunteers. As such, the MDM response to NTHi is clinically relevant, not affected by COPD status and appears to be applicable to infection with many different strains of NTHi.

Previously, expression of MR1 has been difficult to detect and is thought to be typically expressed at low levels. One recent study measured MR1 surface expression on B cells during infection with a variety of pathogenic and commensal bacteria and found MR1 upregulation only in response to certain strains of *E. coli*, but not *Salmonella enterica* serovar Typhi (Salerno-Goncalves et al, 2014). Lung epithelial cells have also been shown to upregulate MR1 in response to Mtb (Gold et al., 2010). Expression of MR1 on lung macrophages has not be studied, neither has MR1 upregulation in response to NTHi infection in any other cell type. The data presented here demonstrate an NTHi-driven upregulation of MR1 on both MDM and lung macrophages. This is in contrast to a previous study which did not find expression of MR1 on MDM (Gozalbo-López et al., 2009). This difference may be explained by differences in the protocol used to derive macrophages. Differing protocols could give rise to higher expression of Fc receptors on the MDM, which could mask low level MR1 expression. A similar explanation has been postulated as to why detecting MR1 on blood-derived DCs can be difficult (Gold et al., 2010).

MAIT cells are known to recognise metabolites of vitamin B2 (riboflavin) synthesis presented by MR1 (Kjer-Nielsen et al., 2012), and only bacteria that possess this pathway can directly activate MAIT cells. A likely explanation for MR1 upregulation is that binding of these vitamin B ligands to MR1 facilitates its trafficking to the cell surface. MAIT cell activation may therefore be a tightly regulated mechanism, as MAIT cells may only be activated in response to bacteria that not only possess a ligand for MR1, but also cause MR1 to be upregulated in the first place. NTHi possesses the vitamin B2 pathway (Kanehisa and Goto, 2000) and one would expect that live NTHi produces more vitamin B2 than metabolically-inert PFA-NTHi, which could be why live NTHi caused the largest upregulation in MR1 expression on MDM. In other studies, C1R lymphoblastoid APCs expressing MR1 were found to upregulate surface MR1 in response to 5-amino-6-D-ribitylaminouracil, an MR1 antigen from the riboflavin synthesis pathway (Corbett et al., 2014). Another study has shown that, unlike other APMs, most MR1 molecules do not bind and present

endogenous self-ligands at baseline. Instead, newly synthesised MR1 appears to remain within the endoplasmic reticulum (ER) in an incompletely folded state which is receptive for riboflavin antigens. When these antigens are loaded into MR1 molecules, MR1 refolds, allowing MR1-antigen complex to then be released from the ER and traffic to the plasma membrane (McWilliam et al., 2016). MR1 can then be degraded or pick up new ligands via recycling and trafficking through endosomes, where MR1 can further sample the intracellular environment (Huang et al., 2008)(Harriff et al., 2016). It is important to consider that, in studies investigating MR1 expression and upregulation, processing of ligands for MR1 derived from phagocytised bacteria may differ from treatment of APCs with synthetic ligands (Harriff et al., 2016). However, it is known that processing of antigen for MR1 is independent of the antigen processing protein TAP and also independent of the proteasome (Huang et al., 2008). Overall, this would indicate that MR1 expression is not indirectly induced and that ligand availability is a limiting factor.

However, MR1 may not just be regulated by increased trafficking to the surface. The data presented in the chapter suggested that total MR1 protein may be increased following infection, as measured by comparing surface stained MDM to MDM which were stained on the surface and intracellularly combined. This is in contrast to a previous study, where MR1 in B cells was shown to only transiently shuttle to the cell surface from an endogenous MR1 protein source such as the ER, but total MR1 protein does not increase (Salerno-Goncalves et al., 2014), although this again may be due to differences in MR1 regulation in different cell types. The type of stimulus may also affect whether *de novo* MR1 protein production can be induced or not. One report has shown that whilst fixed *E.coli* or TLR9 stimulation could increase MR1 expression at the cell surface, total MR1 levels were again unaffected (Liu and Brutkiewicz, 2017), which could be because *de novo* protein synthesis of MR1 requires a stronger stimulation that only occurs with live bacterial infection. Further work using Western blotting would also be required to confirm that there was an actual increase in MR1 protein in the NTHi-infected MDM model. Changes in MR1 protein expression in the MDM model also appeared to be independent of an increase in MR1 gene expression, as NTHi infection did not change steady-state MR1 transcript levels despite increases in detectable surface protein expression. It is worth pointing out that the kinetics of MR1 gene expression are not known and thus the 24 h timepoint may not be optimal for detecting these mRNA transcript changes.

The exact signal initiating the upregulation of MR1 protein at the surface of the cell remains unclear. The data presented here suggest that this increased expression is independent of signalling by typical proinflammatory cytokines, LPS or viral infection. This would suggest that

MR1 upregulation is not merely just a generic inflammatory response to infection. Whilst cytokines, TLR stimulation and influenza infection do activate MDM and upregulate HLA-DR (Staples et al., 2015), the cytokines and LPS tested do not affect MR1 expression in this model. In each of these cases, live NTHi was used as the positive control suggesting that the lack of upregulation with cytokines, LPS or virus was not due to a problem with the cell cultures. Indeed, the MDM appeared morphologically healthy and were alive after infection/treatment, as determined by trypan blue staining. Other studies have found that Lipid A (LPA), the anchor component of LPS, failed to upregulate MR1 on LPA-stimulated B cells (Salerno-Goncalves et al., 2014). In this chapter, the lack of effect of both LPS and PFA-NTHi suggest that TLR4 stimulation may be dispensable for the upregulation of MR1 by macrophages. Thus, the stimulatory effect of live NTHi may be due to its metabolic activity rather than its ability to stimulate TLRs, in this MDM model. In contrast, TLR4 agonists have been shown to cause MR1 upregulation on THP-1 cells (Liu and Brutkiewicz, 2017). Furthermore, stimulation of B cells with a TLR9 agonist upregulated MR1, but MR1 was downregulated on TLR9-stimulated THP-1 cells (Liu and Brutkiewicz, 2017). Overall, regulation of MR1 appears to be different between primary cells and cell lines.

In addition to antigen presenting capabilities, the expression of co-stimulatory and co-inhibitory molecules in response to NTHi infection has also been studied in this chapter. The role of these molecules in activating MAIT cells is unclear. CD80/86 and CD40 are important for activation of naïve T cells, whereas the other co-stimulatory molecules may play a greater role in activation of memory T cells (Zhang et al., 2007)(Ochsenbein et al., 2004)(Gramaglia et al., 1998)(Rogers et al., 2001)(Bansal-Pakala et al., 2004)(London et al., 2000) (Salek-Ardakani et al., 2003). Taken together, the data presented in this chapter suggest that MDM may be capable of activating both naïve and memory T cells, although further work is required to address this. Unfortunately, due to limited lung tissue availability, expression of co-stimulatory and co-inhibitory molecules on lung macrophages were not able to be investigated as a comparison. In terms of co-inhibitory molecules, whilst the majority of research concerning the co-inhibition is in the field of cancer, recent studies have begun to explore the role of PDL1/PD1 in respiratory infection and disease. T cells from COPD patients were shown to have significantly higher levels of PD1 expression (Kalathil et al., 2014). MDM and lung macrophages were also shown to upregulate PDL1 in response to the respiratory viruses, influenza and respiratory syncytial virus (RSV) (Staples et al., 2015). The data presented in this chapter show upregulation of PDL1 and PDL2 on macrophages infected with NTHi, which could have consequences for NTHi-specific T cell activation. Whilst little is known about PDL/PD1 signalling in the context of MAIT cells, MAIT cells from patients with tuberculosis expressed elevated levels of PD1, which correlated with impaired immune responses

to bacillus Calmette-Guérin (BCG) and this impairment was rescued by treatment with PD1 blocking antibodies (Jiang et al., 2014). The PDL1/PD1 axis could therefore represent an important control mechanism for MAIT activation by NTHi. During chronic bacterial colonisation, upregulation of PDL1 by infected APCs and PD1 by chronically activated MAIT cells may impede further MAIT cell responses, allowing the airways to be overwhelmed by additional bacterial load.

In addition to the effects on APMs and co-stimulatory/co-inhibitory molecules, NTHi regulated MDM inflammatory pathways. NTHi infection caused a downregulation of TLR4 gene expression, suggesting that NTHi may be able to dampen immune activation by decreasing expression of PRRs. In contrast, TLR4 gene expression has been found to be significantly increased following LPS stimulation (Sarir et al., 2009). TLR7 and RIG-I transcripts were found to be upregulated following NTHi infection. Although both these PRRs are typically thought to respond to viral infection, RIG-I has been implicated in detection of intracellular bacteria (Schmolke et al., 2014)(Hagmann et al., 2013), which may therefore detect intracellular NTHi. Gene expression of IRF3 and IFNβ were significantly decreased after NTHi infection, despite other bacterial infections and LPS stimulation being shown to cause increases in IFNβ (Stockinger et al., 2004) (Reimer et al., 2008). As IRF3 leads to the production of IFNβ (Hiscott, 2007), the fact that both were downregulated could suggest that NTHi impairs IRF3, which then causes a decrease in IFNβ, as opposed to NTHi directly impairing expression of IFNβ. Decreased IFNβ production may impair downstream immune responses. This decrease in the interferon pathway could also indicate that NTHi can impair responses to subsequent viral infections. An alternative explanation is that due to the kinetics of IFNβ, 24 h may be an inappropriate time point to measure IFNβ transcript levels and shorter time courses may reveal different results. Analysis of MDM supernatant showed that IL-1β was increased in response to NTHi infection, indicating that NTHi infection was not impairing all cytokine responses of MDM.

COPD patients are commonly prescribed corticosteroids but data presented here suggest that use of steroids could in fact be detrimental, a finding also suggested in some clinical studies (Yang et al., 2012). Fluticasone prevented upregulation of MR1 and HLA-DR, and may therefore inhibit presentation of antigen by macrophages. One study has reported downregulation of HLA-DR on BAL macrophages from patients treated with fluticasone propionate (Bertorelli et al., 1998), which would also suggest impairment in the activation of CD4+ T cells. Fluticasone has been shown to exert anti-inflammatory effects by blocking signal transduction of NF-κB (Escotte et al. 2003), although it is unknown whether NF-κB is involved in *de novo* expression or trafficking of MR1 or HLA-DR. Furthermore, another study has suggested that steroids may impair uptake and/or processing of exogenous antigen but does not affect presentation of processed antigen

(Holt and Thomas, 1997). Although not investigated in this study, the downregulation of MR1 and HLA-DR could simply be due to reduced infection, as steroids may impair uptake of NTHi by MDM. However, steroid treatment of MDM from COPD patients has been shown to actually increase phagocytosis of *H. influenzae* (Taylor et al., 2010), indicating that the steroid-induced responses presented in this chapter may be independent of NTHi uptake. Overall, steroids reduced expression of MR1 and HLA-DR. The effects of fluticasone treatment on T cell/MAIT cell activation is described and discussed in chapters 5 and 6.

In summary, both MDM and lung macrophages upregulate HLA-DR and the MAIT antigen presenting molecule MR1, following NTHi infection, whilst expression of HLA-ABC is unaffected. Upregulation of MR1 appears to be specific to bacterial infection, as innate stimulation or viral infection did not affect MR1 expression. Treatment with corticosteroids caused a significant impairment in MR1 and HLA-DR. Now that the effects of NTHi infection on expression of MDM antigen presenting molecules has been established, the next chapters will explore T cell and MAIT cell responses to NTHi in an MDM-T cell co-culture model.

5. Investigating cytokine responses of T cells to NTHi infection

5.1 Introduction

One of the primary functions of T cells is to release various cytokines which co-ordinate the immune response. Classically, T cells are thought to require 3 sequential signals for full activation; presentation of antigen (signal 1), signalling of co-stimulatory molecules (signal 2), and cytokine stimulation (signal 3) (Goral, 2011)(Curtsinger and Mescher, 2010).

CD8+ T cells in particular are increased in the lung in patients with chronic lung diseases. Despite this, the lungs of these patients are commonly colonised with bacteria such as NTHi, which are associated with exacerbations and disease progression, suggesting an impairment in the ability of T cells to mount an effective immune response (King and Sharma, 2015)(Moghaddam et al., 2011)(Wang et al., 2016)(Van Eldere et al., 2014)(Cosio et al., 2009)(Fairclough et al., 2008). CD4+ and CD8+ T cells have been shown to produce pro-inflammatory cytokines in response to NTHi infection (King et al., 2008b), but nothing is known about the responses of MAIT cells to NTHi. IFNγ is the archetypal cytokine involved in the Th1 response; however T cells can have a variety of different responses to infection depending on the pathogen involved. In addition to a Th1 response, NTHi is known to induce IL-17a from conventional T cells (King et al., 2013). MAIT cells have previously been shown to produce IFNγ, IL-17a and TNFα, but it is not known if they can in response to NTHi. IFNγ is particularly important to investigate as it activates macrophages (Held et al., 1999) and MAIT cells have been shown to directly inhibit microbial growth and enhance production of nitric oxide by macrophages in a manner dependent on IFNγ (Chua et al., 2012). In the lung, IFNγ may contribute to lung destruction and COPD pathogenesis (Freeman et al., 2010), as evidence from mouse models has shown that over expression of IFNγ can result in lung inflammation and emphysema (Wang et al., 2000).

Utilising a human lung explant model to study host-pathogen responses is important, as lung T cells can be investigated in the context of respiratory bacterial infection. The nature of the explant means that T cell activation occurs within the 3D architecture of the lung, which is more representative of what occurs during infection *in vivo*.

In this chapter, expression of the cytokines IFNγ, IL-17a and TNFα have been measured from conventional CD4+ and CD8+ T cells and MAIT cells from NTHi-infected lung explants. These cytokine responses have then been modelled and studied further using peripheral T cells co-cultured with NTHi-infected MDM. To determine whether the observed T cell responses were antigen-specific or induced by other factors such as cytokines, T cells were also co-cultured with NTHi-infected MDM in the presence of blocking antibodies. Expression of co-stimulatory and co-

inhibitory T cell molecules and the effect that steroids have on T cell activation have also been explored.

5.2 Results

5.2.1 Lymphocytes produce IFNγ in response to NTHi

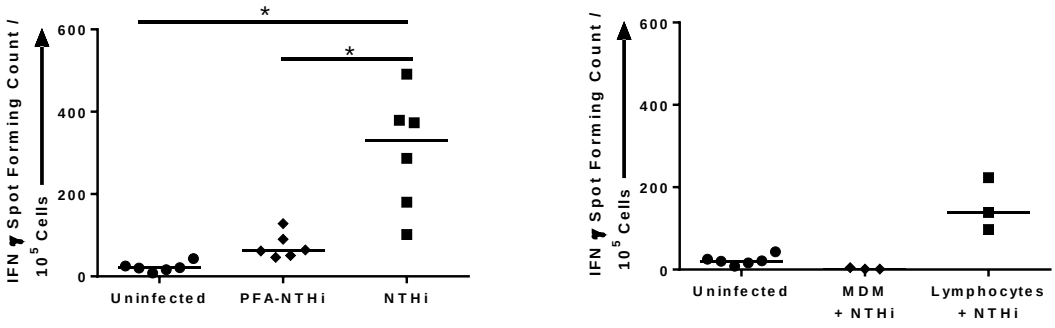
To first determine whether T cells can respond to NTHi antigens presented by macrophages, MDM were infected as before for 2 hours to allow bacterial uptake. MDM were then washed to remove excess bacteria and co-cultured with autologous lymphocytes for a further 22 hours to facilitate antigen presentation and T cell activation. Using the ELISpot assay, T cell activation was measured by IFNγ production in response to NTHi infection. Autologously matched cells were used to ensure that the IFNγ detected was due to NTHi-induced T cell activation, and not due to HLA mismatch.

Figure 5.1A shows a significant (P=0.01) increase in spot forming count (SFC) of IFNγ+ cells responding to NTHi-infected MDM, from 20 SFC to 330 SFC. MDM were also infected with PFA-NTHi to account for innate activation of MDM by PAMPs and subsequent T cell activation. PFA-NTHi was used as a control instead of heat-killed NTHi, because MAIT cells are not activated by heat-killed bacteria as the MR1/MAIT antigens are heat sensitive (Dias et al., 2016)(Le Bourhis et al., 2010). PFA-NTHi gave only a slight increase in SFC from 20 to 63.

Culturing of lymphocytes alone with live NTHi led to a spot forming count SFC of 140, showing that lymphocytes themselves can respond to NTHi in the absence of macrophages. This could be due to activation of T cells by bacterial PAMPs but also antigen presentation by B cells in the lymphocyte population to T cells. Culturing of infected MDM alone produced no increase in SFC compared to not infected, showing that macrophages themselves do not produce IFNγ as a result of NTHi infection.

By using whole lymphocytes in the ELISpot assay it is not possible to know whether T cells, B cells, or NK cells were responsible for the production of IFNγ. To analyse which lymphocyte populations were responding to NTHi, lymphocytes were co-cultured with NTHi-infected MDM and activation was measured by flow cytometry. Staining for CD3, a key phenotypical marker of T cells, and IFNγ allows the active T cell population to be identified. As can be clearly seen in figure 5.1B, both CD3+ (green) and CD3- (blue) populations produced IFNγ following NTHi infection, showing that T cells do become activated by NTHi-infected MDM, but so do CD3- cells, i.e NK cells or B cells.

A)



B)

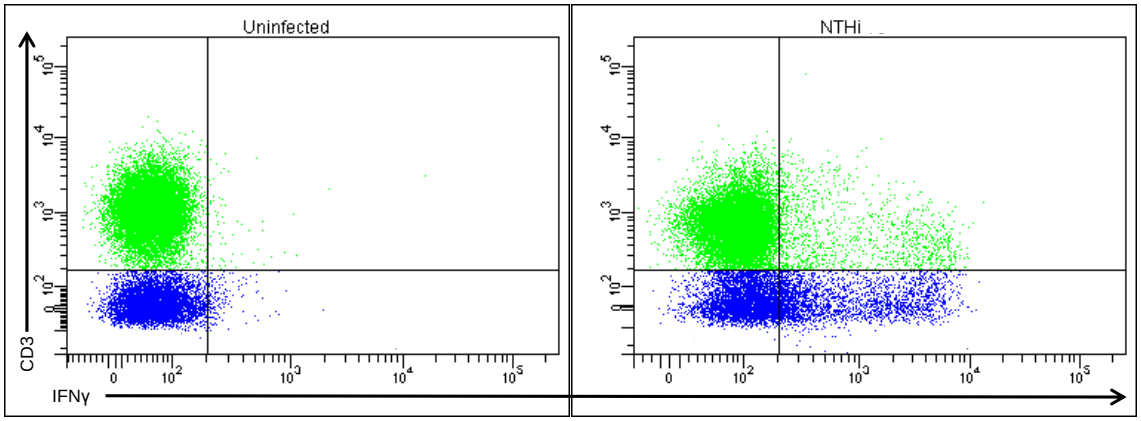


Figure 5.1 – Production of lymphocyte IFNγ following co-culture of lymphocytes with NTHi-infected MDM. A) IFNγ spot forming count (SFC) measured by ELISpot of lymphocytes co-cultured with autologous MDM either uninfected or infected with NTHi or PFA-NTHi at MOI 1. MDM were also incubated with NTHi alone, without lymphocytes present. Lymphocytes were also incubated with NTHi alone, without MDM present. B) FACS plots of CD3+ cells (green) and CD3- cells (blue) producing IFNγ following co-culture of lymphocytes with autologous MDM either uninfected or infected with NTHi at MOI 1. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.2.2 CD3 becomes downregulated following NTHi infection

One of the benefits of flow cytometry is being able to identify specific cell types within a heterogeneous population, such as T cells within lymphocytes. However, identification of specific cell types is dependent on phenotypic cell markers, such as CD3 on T cells. Issues arise when expression of these phenotypic markers become affected by activation (Bigby et al., 1990) (Leeansyah et al., 2013). Following flow cytometry of the lymphocytes from the MDM-lymphocyte co-culture, it became apparent that CD3 became significantly (P=0.02) downregulated following NTHi-induced activation (figure 5.2), measured by a 13% decrease in CD3+ cells. Overall, CD3 downregulation impairs detection of T cells by flow cytometry, as activated T cells lose expression of a marker that defines their identity.

To specifically measure the T cell response to NTHi and overcome the issue of activation-dependent CD3 downregulation, CD3+ MACS sorting was used to isolate pure T cells from lymphocytes as described in section 2.2.2. Setting up a pure MDM-T cell co-culture removes the issue of CD3 downregulation as the population is CD3+ to begin with. This also means that a CD3 antibody is not required for flow cytometry of these T cells. An additional benefit of using a pure T cell population is the CD3- cells from the lymphocyte population, NK cells and B cells, will be absent and will therefore not interfere with detection of cytokines or cytotoxic activity.

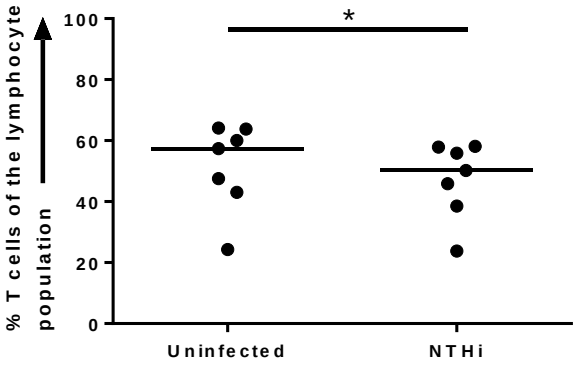


Figure 5.2 – Downregulation of surface CD3. Expression of CD3 following activation of T cells co-cultured with autologous MDM either uninfected or infected with NTHi at MOI 1. Data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

All the co-culture work that follows uses the pure MDM-T cell co-culture model; no NK or B cells were present, as shown in the methods chapter (section 2.2.2).

5.2.3 Titration of NTHi MOI for T cell activation

In the previous chapter, NTHi was used at an MOI of 1 as this was sufficient to induce upregulation of MDM APMs, which were not further expressed by increasing the MOI. However, it was not known whether an MOI of 1 would be enough to induce robust T cell activation. To address this, MDM were infected with NTHi at MOI 1 or 10 for 2 h, co-cultured with autologous T cells for a further 22 h and T cell activation was measured by IFNγ production (figure 5.3). CD4+ and CD8+ conventional T cells and MAIT cells all expressed IFNγ following co-culture with MDM infected with MOI of 1. In contrast to the APMs on MDM, IFNγ production by all T cell subsets was further increased in an MOI dependent manner, with MOI 10 giving a greater, statistically significant (all $p<0.05$) induction. Therefore, for the purpose of inducing T cell responses, an MOI of 10 was used in all subsequent T cell assays. Overall, whilst CD4 and CD8 T cells producing IFNγ in response to NTHi has been reported before (King et al., 2013), this is the first time that MAIT cells have been shown to respond to NTHi.

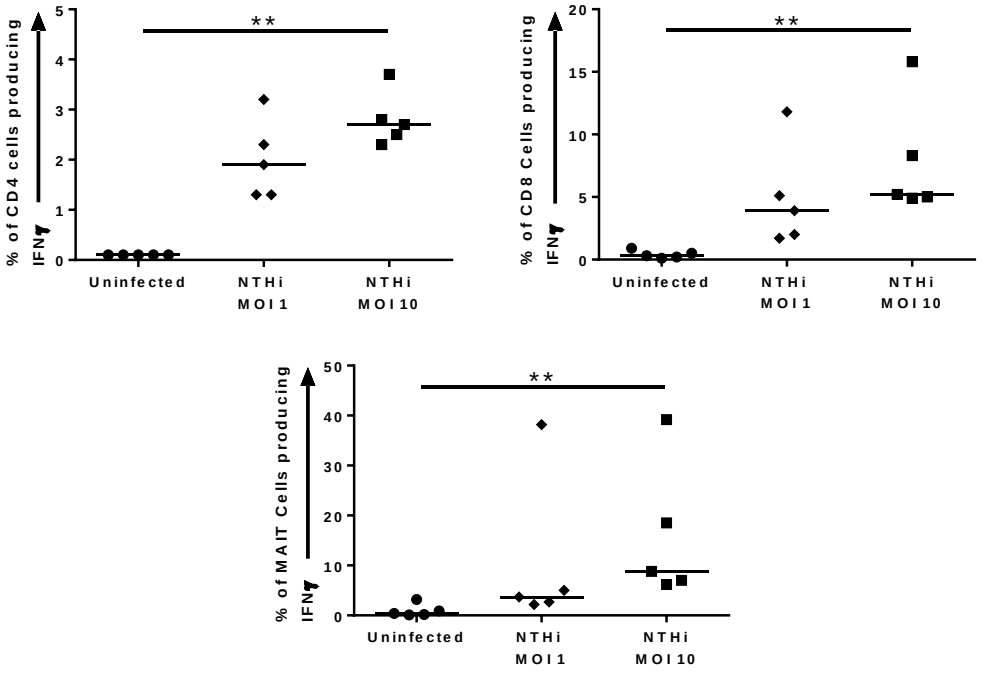


Figure.5.3 – MOI dependent expression of IFNγ. Expression of IFNγ by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 1 or 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Friedman test with Dunn's multiple comparison. **P<0.01

5.2.4 T cell responses to NTHi are not strain specific

The next step was to ensure that the IFNγ response of T cells to NTHi was representative of the whole species and not specific to the strain investigated. In line with the strain comparison data in the previous chapter, infection of MDM with one of the NTHi strains or with *E.coli*, followed by subsequent co-culture with T cells, induced IFNγ expression in all three T cell subsets (figure 5.4). However, there were no significant differences in IFNγ induction between strains, as measured by Friedman test with Dunn's multiple comparison. The rest of this thesis will therefore continue to use the NTHi ST201 strain at a MOI of 10 to measure T cell responses.

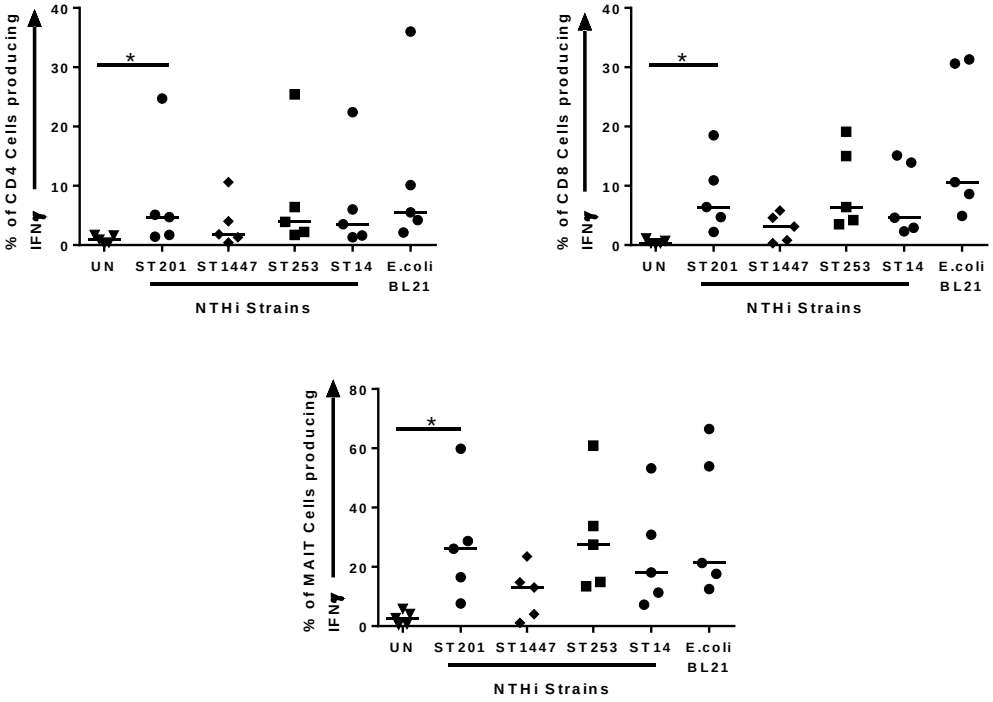


Figure 5.4 – Comparing different NTHi strains and expression of IFNγ. Expression of IFNγ by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM either uninfected or infected with different strains of NTHi or *E.coli* at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Friedman test with Dunn's multiple comparison. *P<0.05

5.2.5 Detection of conventional T cells and MAIT cell populations following NTHi infection

To confirm that this T cell-MDM model was suitable to investigate T cell responses to NTHi, expression of T cell markers were examined, as both CD4 and CD8 on conventional T cells have been shown to be downregulated following T cell activation (Bigby et al., 1990)(Xiao et al., 2007). Furthermore, on MAIT cells, which are defined as CD161+Vα7.2+ T cells, CD161 can become downregulated on MAIT cells following activation (Leeansyah et al., 2013). Downregulation of any of these markers could therefore impair detection of the T cell populations. As the co-culture model has been compared to the lung explant model at various stages throughout this thesis, expression of CD4, CD8 and MAIT markers was also examined on lung T cells from the explants infected with NTHi for 24 hours, to ensure that lung T cells could be adequately detected following NTHi infection.

In the co-culture model, neither CD4 nor CD8 expression were affected by activation of T cell by NTHi-infected MDM (figure 5.5), meaning that both of these T cell subpopulations could be accurately identified following NTHi infection. The same was true for expression of CD4 and CD8 on T cells from infected lung explants (figure 5.5).

In terms of MAIT cells however, it became apparent that there was a reduction in MAIT cell number in the co-culture model (figure 5.5), with a significant ($P<0.0001$) decrease from 2.2% of T cells to 1.5%. A minor downregulation of CD161 and slight increase in Vα7.2 were concurrently observed, suggesting that activation-induced changes in expression of these markers were affecting the ability to detect MAIT cells. In contrast, lung MAIT cell markers were unaffected by NTHi infection of lung explants, highlighting a minor difference between the co-culture and lung explant models (figure 5.5).

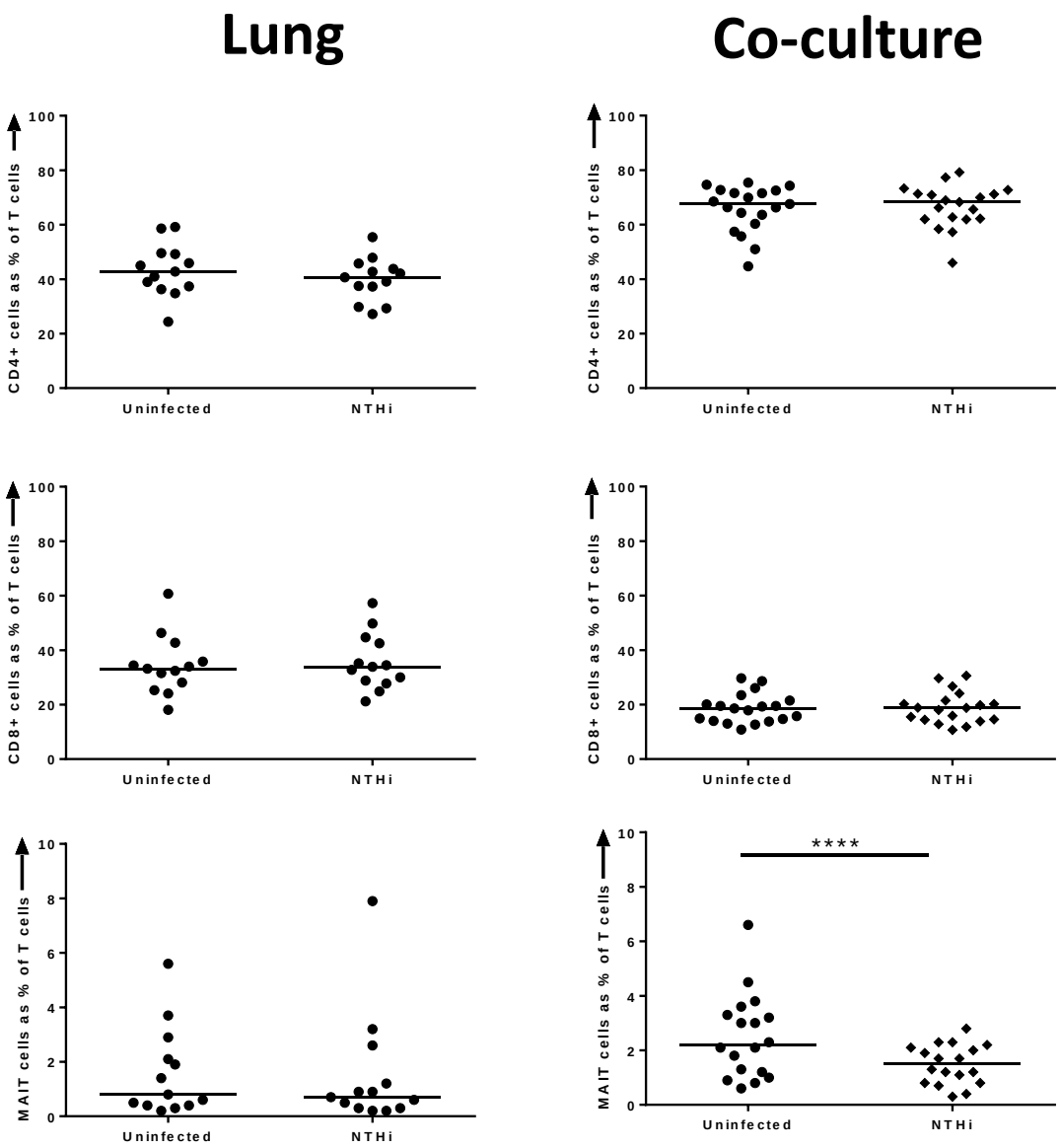


Figure 5.5 – Detection of T cell subsets before and after NTHi infection. Percentages of CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi or peripheral T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. ****P<0.0001

5.2.6 T cell production of cytokines in response to NTHi infection

Having shown that CD4+, CD8+, and most potently, MAIT cells all express IFNγ following co-culture with NTHi-infected MDM, the next step was to determine whether induction of IFNγ expression was due to live bacterial infection. In addition, the PMA/Ionomycin data presented in chapter 3 showed that T cells were also capable of producing IL-17a and TNFα, so the expression of these other pro-inflammatory cytokines in response to NTHi was investigated. Cytokine production by lung T cells from live NTHi-infected explants was also measured as a comparison; due to availability of tissue, a PFA-NTHi vs live NTHi comparison could not be conducted.

Analysis of lung T cells from infected tissue explants revealed that 3% of CD4+ T cells and 12% of MAIT cells significantly (both P=0.03) produced IFNγ in response to NTHi infection, but lung CD8+ T cells were unresponsive. In the blood-derived co-cultures, CD4, CD8 and MAIT cells all significantly (for all, P<0.0001) produced IFNγ in response to NTHi infection, with approximately 3%, 9% and 28% of T cell subsets responding respectively (Figure 5.6). In all three cases in the co-culture, PFA-NTHi infection caused only minor increases in IFNγ compared to uninfected controls. PFA-NTHi vs NTHi was significant in all three cases (all P<0.01), indicating that live NTHi infection is required for maximal IFNγ response. To confirm that cytokine expression also resulted in actual release of IFNγ, supernatants from lung explants and co-cultures were harvested and IFNγ was measured by ELISA. IFNγ was detected in both models, confirming release of cytokine, with infected explants releasing 255 pg/ml of IFNγ, compared to 170 ng/ml from co-cultures (figure 5.7).

The IL-17a response of lung CD4+ T cells was small (P<0.05), but the response of lung MAIT cells was drastically higher, rising from a high baseline of 3% to 17% with NTHi infection (P<0.05). CD4, CD8 and MAIT cells from co-cultures were stained for IL-17a, but IL-17a was only detected at a very minor level in CD4 and MAIT cells (figure 5.8). NTHi caused CD4+ cells to significantly (P=0.005) increase production of IL-17a, from 0.2% of CD4 cells uninfected to 0.4% with NTHi infection. PFA-NTHi caused a smaller, but still significant (P=0.003) increase to 0.3%. MAIT cells had a high production of IL-17a at baseline of 0.8%, which rose to 0.9% with NTHi infection, although this was not significant. PFA-NTHi however gave a significant (P=0.01) increase in IL-17a, to 1.1% of MAIT cells. Due to the small overall amounts of IL-17a detected, ELISA for IL-17a was not performed, as supernatant was a limited resource.

Finally, production of TNFα was measured in T cells and MAIT cells from both the lung explant and blood-derived co-culture following NTHi infection (figure 5.9). Induction of TNFα was minor in both lung and blood conventional T cells. MAIT cells on the other hand expressed significant (both

P<0.05) amount of TNFα in the lung and blood (19% and 8% respectively). Due to availability of cells, a PFA-NTHi vs live NTHi comparison was not performed in the co-culture. Again, due to the relatively minor amount of T cell TNFα detected, ELISA was not carried out. MDM have also been shown to release TNFα in response to NTHi, so any ELISA data would be confounded by MDM derived TNFα (Wajant et al., 2003). Overall, compared to conventional T cells, MAIT cells gave the strongest response in both the lung and co-culture for all three cytokines. As both IL-17a and TNFα responses were poor in the co-culture, further work studying the mechanisms controlling these cytokines could not be reliably performed. On the other hand, IFNγ was a strong readout in the co-culture model, so therefore all subsequent work in this chapter will just use IFNγ as a marker for cytokine production.

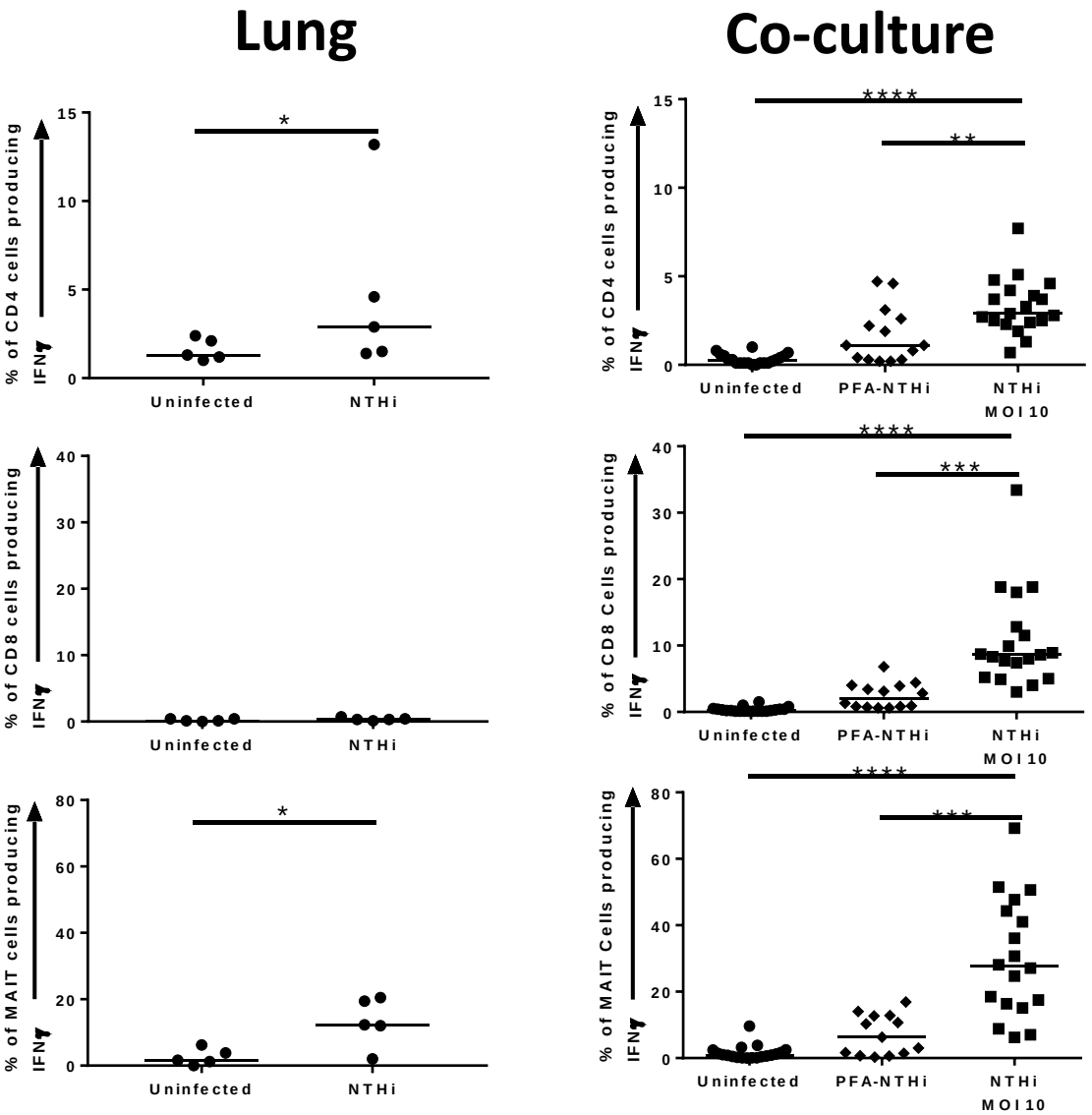


Figure 5.6 – Production of IFNγ by T cells after NTHi infection. Expression of IFNγ by CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

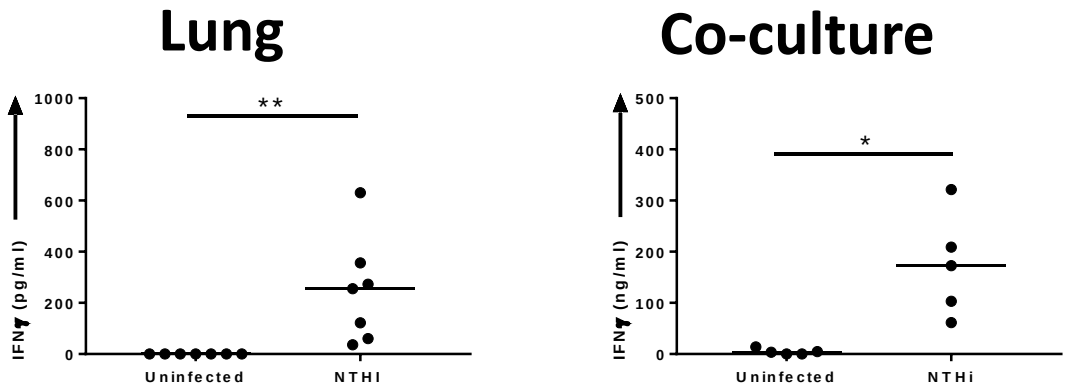


Figure 5.7 – Release of IFNγ after NTHi infection measured by ELISA. IFNγ production measured by ELISA of lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01

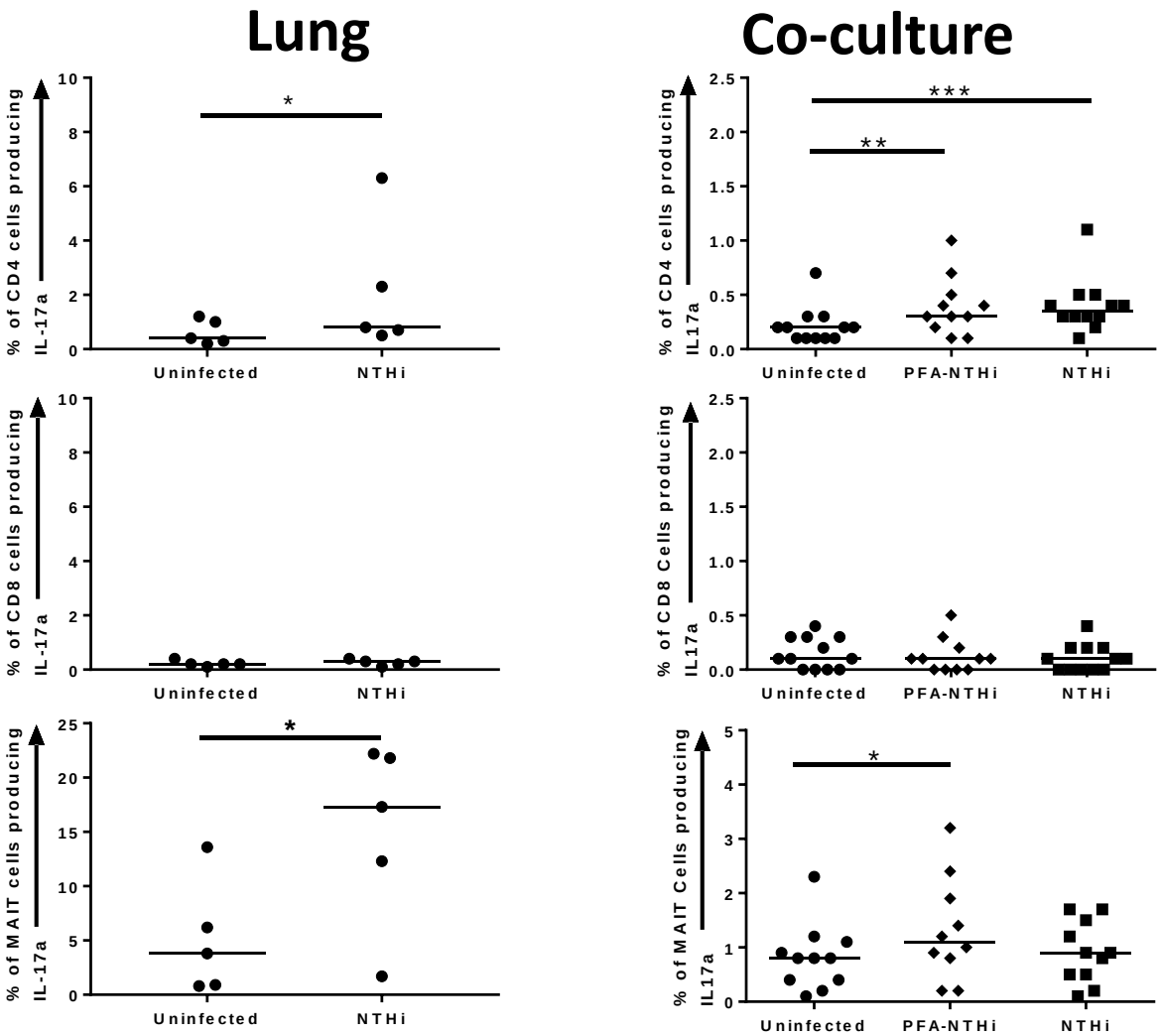


Figure 5.8 – Production of IL-17a by T cells after NTHi infection. Expression of IL-17a by CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01, ***P<0.001

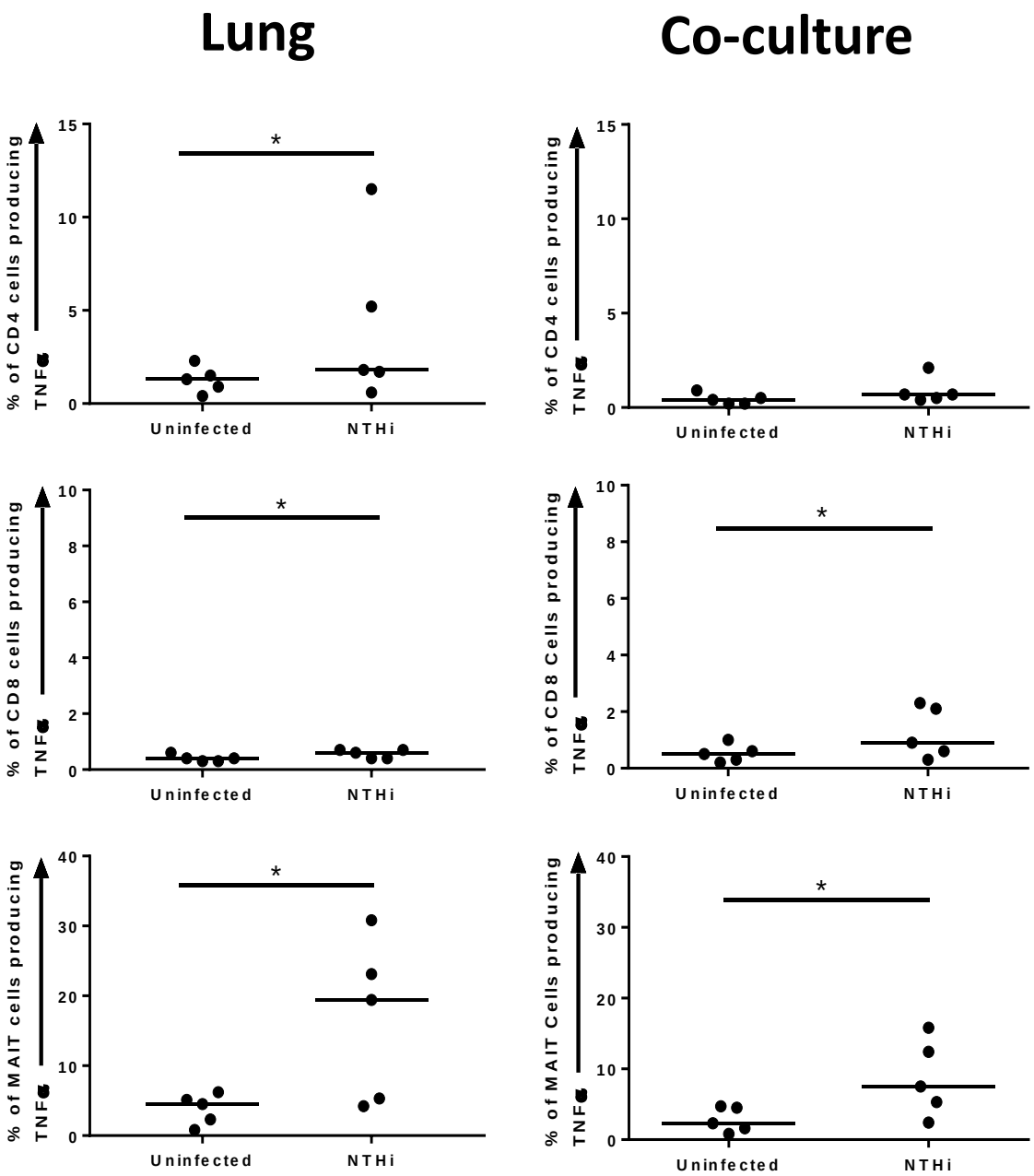


Figure 5.9 – Production of TNFα by T cells after NTHi infection. Expression of TNFα by CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.2.7 Cytokine responses of conventional T cells are antigen-specific

Activation of T cells by APCs can be caused by direct antigen presentation and signalling via the TCR pathway but also indirectly driven entirely by cytokine stimulation. To determine whether the CD4+ and CD8+ T cell response to NTHi infection were antigen specific, T cells were co-cultured with NTHi-infected MDM for 22 hours in the presence of either HLA-DR or HLA-ABC blocking antibodies respectively. In all cases, an isotype control antibody was used to account for the effect of just having an antibody present in the culture; all comparisons are blocking antibody vs isotype.

As seen in figure 5.10A, blocking of HLA-DR caused a significant (P=0.004) 42% reduction in IFNγ by CD4+ cells, from 3% of CD4+ cells producing IFNγ to 1.8%. Blocking of HLA-ABC inhibited IFNγ production by CD8+ cells, from 12% of CD8+ cells to 4.8% with blocking, a significant (P=0.003) reduction of 60%. Together, these data indicate that a large component of the CD4+ and CD8+ T cells response to NTHi is antigen specific. Blocking of HLA-DR and HLA-ABC also reduced IFNγ expression from CD8+ and CD4+ T cell respectively, suggesting that blocking the activation of one conventional T cell (i.e CD4+ cells) also impairs activation of the other (i.e CD8+ cells), which could indicate cross-talk between CD4+ and CD8+ T cells (figure 5.10B).

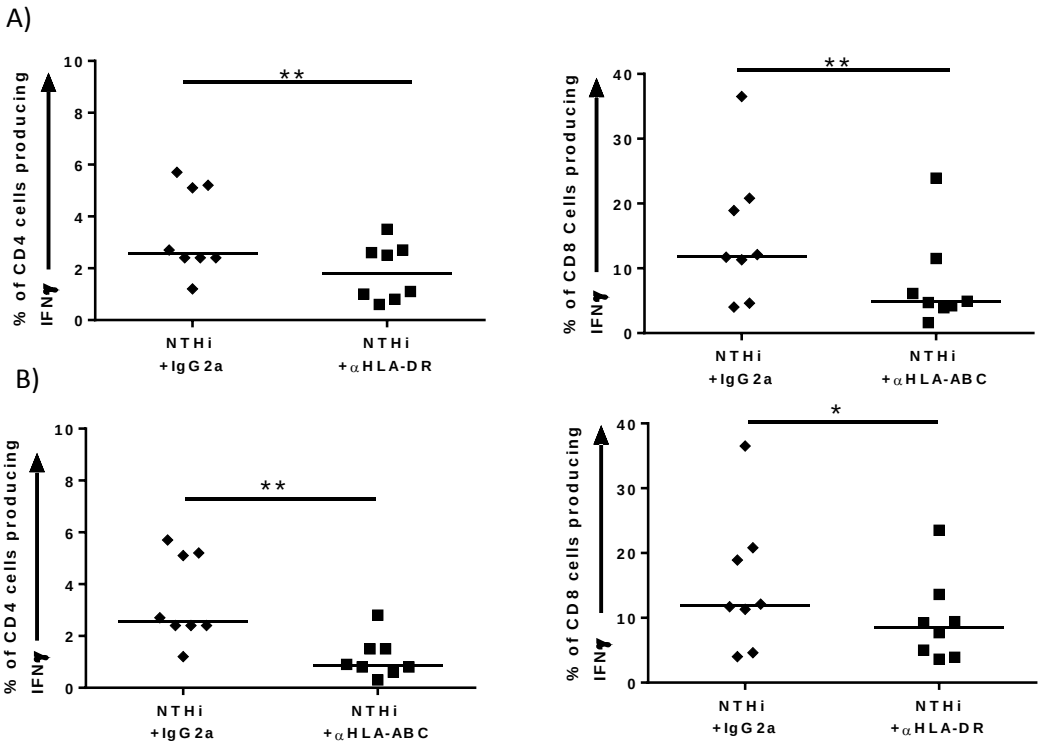


Figure 5.10 – Production of IFNγ by conventional T cells is impaired by blocking antigen presentation. A) and B) Expression of IFNγ by CD4+ and CD8+ cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-HLA-DR (L243), 10 µg/ml anti-HLA-ABC (W6/32) or IgG2a isotype control. For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01,

5.2.8 MR1 Is Required for MAIT cytokine responses in a time-dependent manner

T cells were next co-cultured with NTHi-infected MDM for 22 hours in the presence of an MR1 blocking antibody. However, blocking of MR1 did not affect expression of IFNγ at all by MAIT cells (figure 5.11), which would suggest that the observed MAIT cell response to NTHi was MR1-independent. However, it was plausible that the kinetics of MR1 antigen presentation were different to that of MHC and conventional T cells. Indeed, Ussher et al have previously shown that blocking of MR1 for just 5 hours strongly abrogated MAIT cell production of IFNγ in their model, but blocking of MR1 over a longer timecourse did not have such an impact (Ussher et al., 2014a). To investigate further, the MR1 blocking experiment was repeated over a shorter, 5 hour timecourse. IFNγ expression was significantly inhibited (P=0.03) by 70%, from 5.3% with isotype to 1.7% with the anti-MR1 antibody. A point of interest was that no IFNγ production by the conventional CD4+ or CD8+ T cell subsets was observed at this shorter timepoint, which suggests that MAIT cells can express IFNγ in response to infection quicker than conventional T cells. Overall, these data indicate that the MAIT IFNγ response to NTHi infection is antigen-specific initially, but could be driven by other factors later in the timecourse. These factors are investigated later in this chapter.

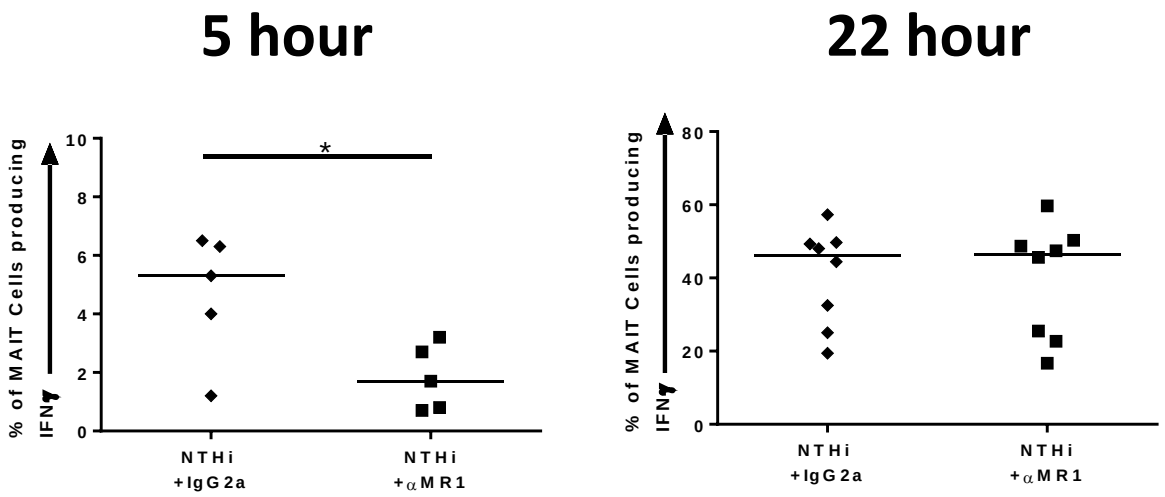


Figure 5.11 – Production of IFNγ by MAIT cells is impaired by blocking antigen presentation in a time dependent manner. Expression of IFNγ by MAIT cells from T cells co-cultured for 5 or 22 hours with autologous MDM infected with NTHi at a MOI of 10 in the presence of 5 μg/ml anti-MR1 (26.5) or IgG2a isotype control. For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.2.9 Expression of co-stimulatory and co-inhibitory molecules on lung and blood T cell subsets at baseline

Activation of T cells is not solely dependent on antigen presentation, but requires complex signals from additional co-stimulatory molecules, often called signal 2, in order to become fully activated (Nurieva et al., 2009)(Sharpe and Abbas, 2006)(Chen and Flies, 2013)(Smith-Garvin et al., 2009). In contrast, co-inhibitory signals help balance the immune response and prevent inappropriate over-activation. In order to understand the mechanism which lead to conventional T cell and MAIT cell responses to NTHi, expression of surface markers involved in co-stimulation or co-inhibition would need to be investigated following infection. Due to limited yields of cells from tissue, it was not possible to study the effect that NTHi infection had on lung T cell co-stimulatory and co-inhibitory molecules. Instead, expression of these molecules were first characterised at baseline on T cell populations present in the lung and compared to the T cells from the blood co-culture. The co-culture model was then used to investigate how NTHi infection affected expression of these co-stimulatory and co-inhibitory molecules.

One of the most well characterised co-stimulatory molecules expressed on T cells is CD28, which provides a critical co-stimulatory signal to naive T cell during activation, without which the T cell would become anergic (Nurieva et al., 2009)(Watts, 1999)(Smith-Garvin et al., 2009). CD28 was found to be highly expressed on almost all CD4+ lung T cells and 60% of CD8+ lung T cells, which was comparable to the same subsets in the blood-derived co-culture (figure 5.12). Expression of CD28 on both lung and co-culture MAIT cells was also very high at baseline, suggesting that CD28 may play a role in MAIT cell activation (figure 5.12). Another archetypal molecule involved in both naïve CD4+ and CD8+ T cell activation is CD40L, which binds to CD40 expressed on APCs such as dendritic cells (DCs) and macrophages or expressed by other T cells (Schoenberger et al., 1998). The expression and role of CD40L in MAIT cell activation is unknown. At baseline, CD40L was found to be lowly expressed on CD4+ and CD8+ T cell subsets from both lung and the co-culture (figure 5.12). However, MAIT cell expression of CD40L appeared slightly higher than expression on blood-derived co-culture MAIT cells, which may suggests a role for CD40L in MAIT cell activation in the lung.

In contrast to co-stimulatory molecules which enhance T cell activation, co-inhibitory molecules such as CTLA-4 and PD-1 function to negatively regulate T cells, leading to inhibition of T cell proliferation and cytokine release. At baseline, expression of CTLA-4 was low across all three T cell subsets in both the lung and the blood-derived co-culture (figure 5.12). However, expression of

PD-1 was approximately 20% which was consistent across the three T cell subsets in both compartments (figure 5.12).

In addition to the primary co-stimulatory/co-inhibitory molecules described above, other co-stimulatory pathways are known, which may enhance activation of memory T cells, promote survival and cytokine production, as well as contributing to different effector functions (Chen and Flies, 2013)(Hendriks et al., 2003)(Dawicki et al., 2004). For the purpose of this thesis, four of these additional molecules have also been investigated on conventional T cells and MAIT cells: 4-1BB, CD27, ICOS and OX40 (figure 5.13). 4-1BB was lowly expressed on CD4+ T cells and MAIT cells in both the lung and co-culture, whereas 4-1BB expression on blood-derived CD8+ T cells was 2-fold higher when compared to the lung. CD27 was consistently expressed at 65% across the three subsets on lung T cells but was expressed on almost all conventional T cells and the majority of MAIT cells in the blood co-culture.

In contrast to the previous co-stimulatory molecules mentioned above, expression of ICOS was drastically different between lung and co-culture derived T cells. Approximately 50% of CD4+ T cells and 20% of both CD8+ and MAIT cells expressed ICOS in the lung, whereas almost all conventional T cells and 55% of MAIT cells expressed ICOS in the co-culture. Notable differences were also observed with OX40 expression; OX40 was 2-fold higher on co-culture CD4+ T cells compared to lung CD4+ T cells, but was higher on lung CD8+ and MAIT cells compared to very low expression on blood-derived co-culture T cell counterparts. Taken together, these data indicate that expression of co-stimulatory/co-inhibitory molecules is not always similar and that differences in expression occur not just between T cell subsets, but also between the lung and co-culture compartments.

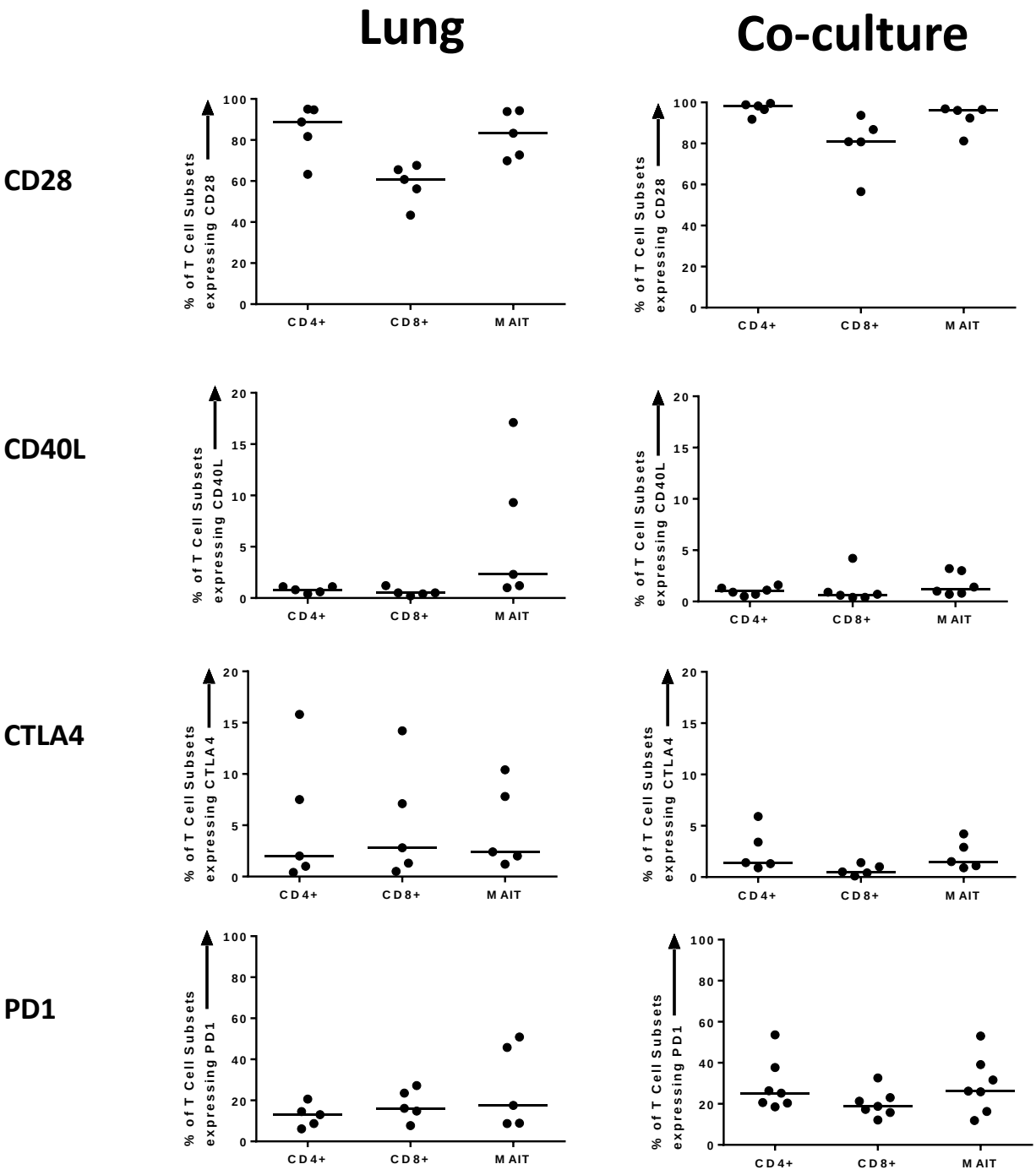


Figure 5.12 – Expression of co-stimulatory and co-inhibitory molecules on T cell subpopulations in the lung and blood. Blood T cells and lung T cells were stained for the indicated co-stimulatory/co-inhibitory markers and expression was determined by flow cytometry. For all graphs, median values are shown.

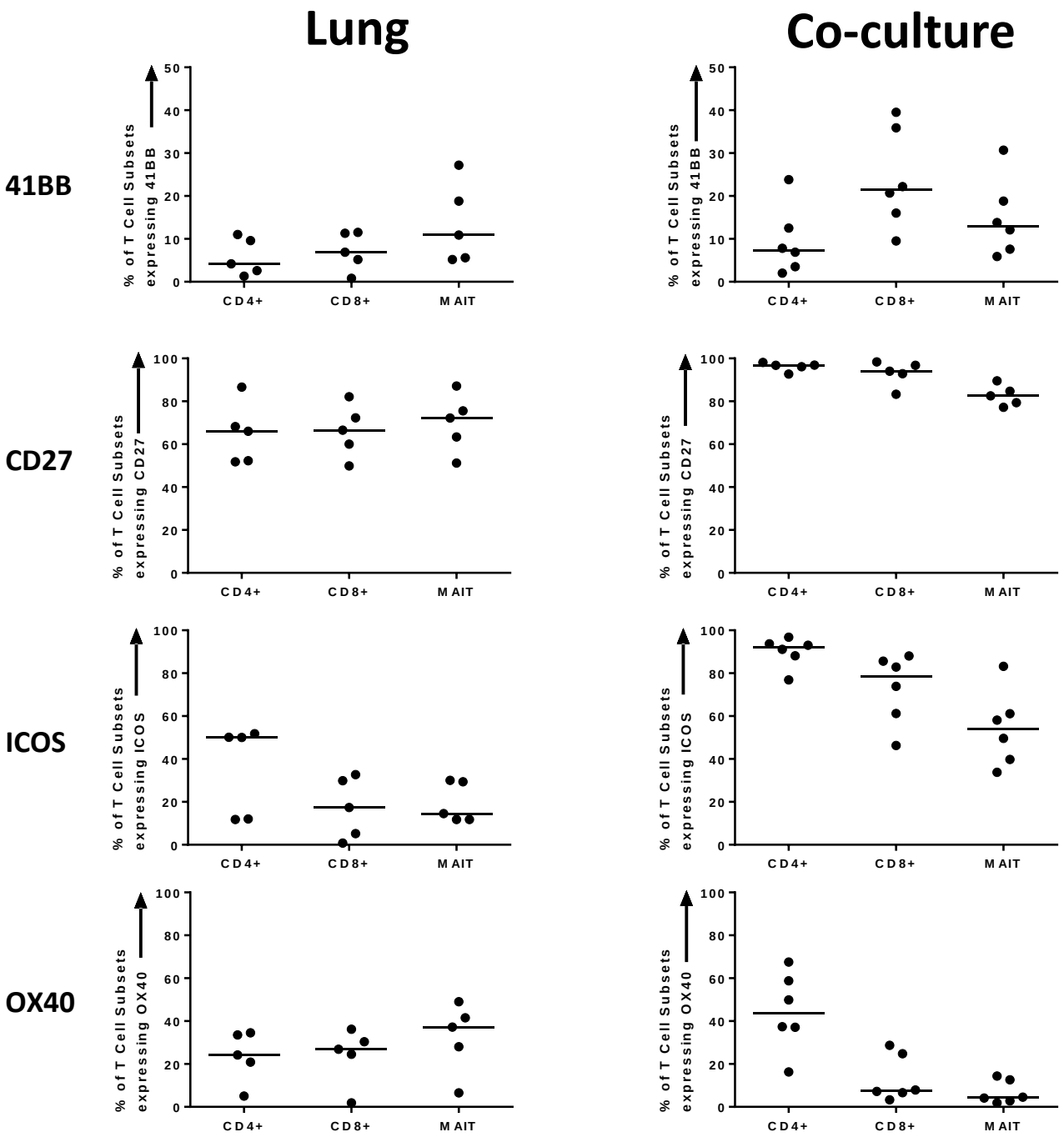


Figure 5.13 – Expression of other co-stimulatory molecules on T cell subpopulations in the lung and blood. Blood T cells and lung T cells were stained for the indicated co-stimulatory markers and expression was determined by flow cytometry. For all graphs, median values are shown.

5.2.10 Expression of co-stimulatory and co-inhibitory molecules on conventional T cell and MAIT cells following NTHi infection

The effect of NTHi infection on expression of the co-stimulatory and co-inhibitory molecules was next evaluated using the MDM-T cell co-culture model.

CD28 expression was not affected by NTHi infection on any T cell subset (figure 5.14), although it was very highly expressed at baseline on CD8+ T cells (80% CD28+) and was expressed on almost all CD4+ and MAIT cells in the first place. On the other hand, CD40L, which was lowly expressed at baseline, was significantly ($P<0.05$) upregulated on all three subsets, most potently on MAIT cells (figure 5.14).

PD1 expression was significantly ($P=0.03$) upregulated by almost 2-fold on MAIT cells following NTHi infection, but was unchanged on CD4+ and CD8+ conventional T cells (figure 5.14), suggesting that the PD1 pathway differentially regulates innate and adaptive T cells. Expression of the other co-inhibitory molecule, CTLA4, was also significantly (all $P<0.05$) upregulated on all three subsets, but the induction on conventional T cells was minor in comparison to the 3-fold induction on MAIT cells (figure 5.14). Together, these data suggest that MAIT cells have a higher expression of co-inhibitory molecules, which could indicate that these cells are under tighter control compared to conventional T cells.

Analysis of the other memory co-stimulatory pathways highlighted further differences between conventional T cell and MAIT cells. Expression of both OX40 and 41BB remained unchanged on conventional T cells following infection (figure 5.15). However, both markers were significantly elevated on MAIT cells; OX40 2-fold increase ($P=0.04$), 41BB 2.3-fold increase ($P=0.03$). ICOS expression was significantly (for both $P<0.05$) upregulated on CD8+ and MAIT cell, but the greatest induction was observed on MAIT cells, where expression rose by 1.5-fold (figure 5.15). In contrast, expression of CD27 remained highly expressed on all subsets (figure 5.15). Overall, here it has been shown for the first time that there are differences in the expression of the various co-stimulatory and co-inhibitory molecules between conventional T cells and MAIT cells following NTHi infection.

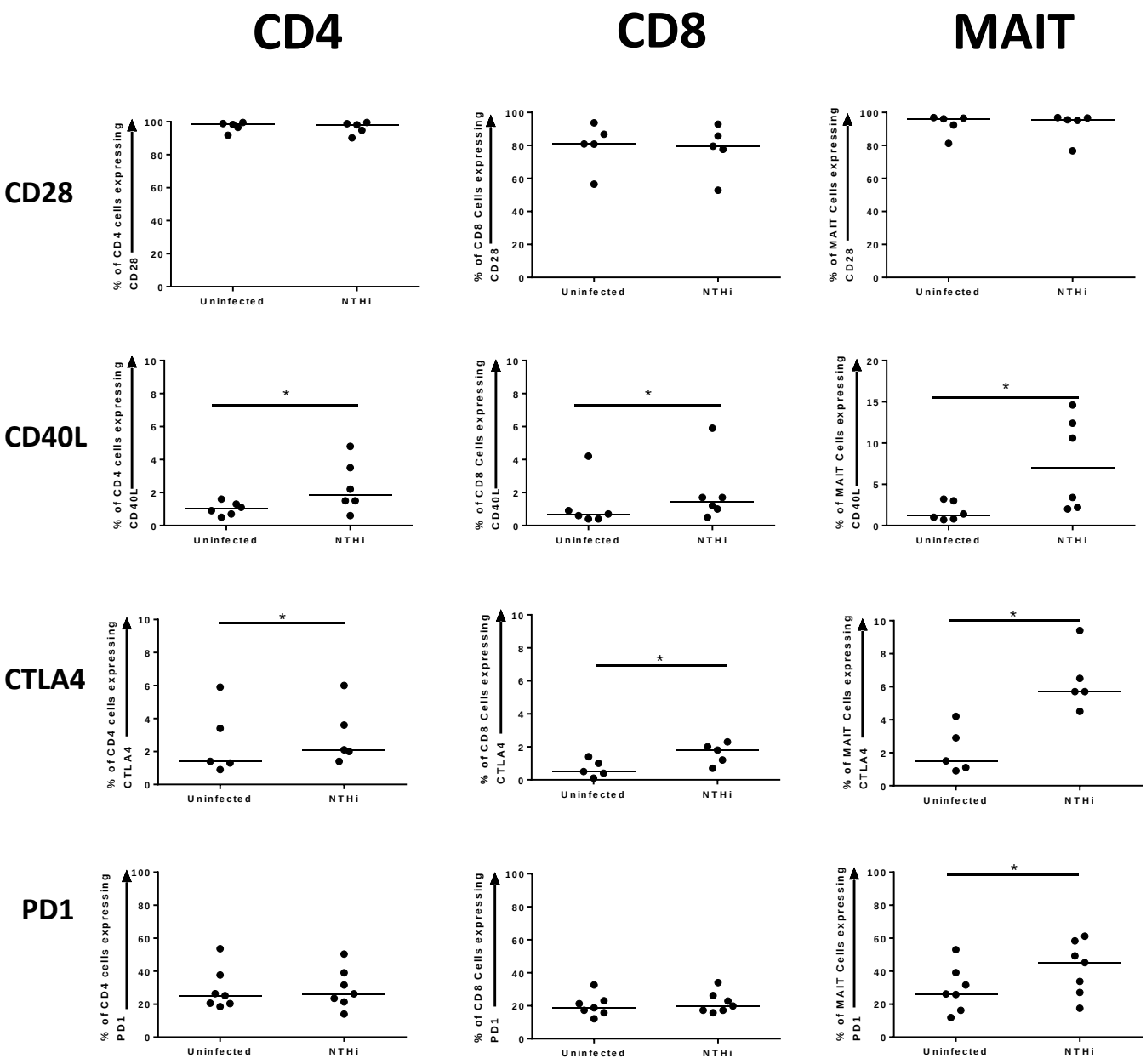


Figure 5.14 – Expression of co-stimulatory and co-inhibitory molecules on T cell following NTHi infection. Expression of CD28, CD40L, PD1 and CTLA4 by CD4+, CD8+ and MAIT cells from blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

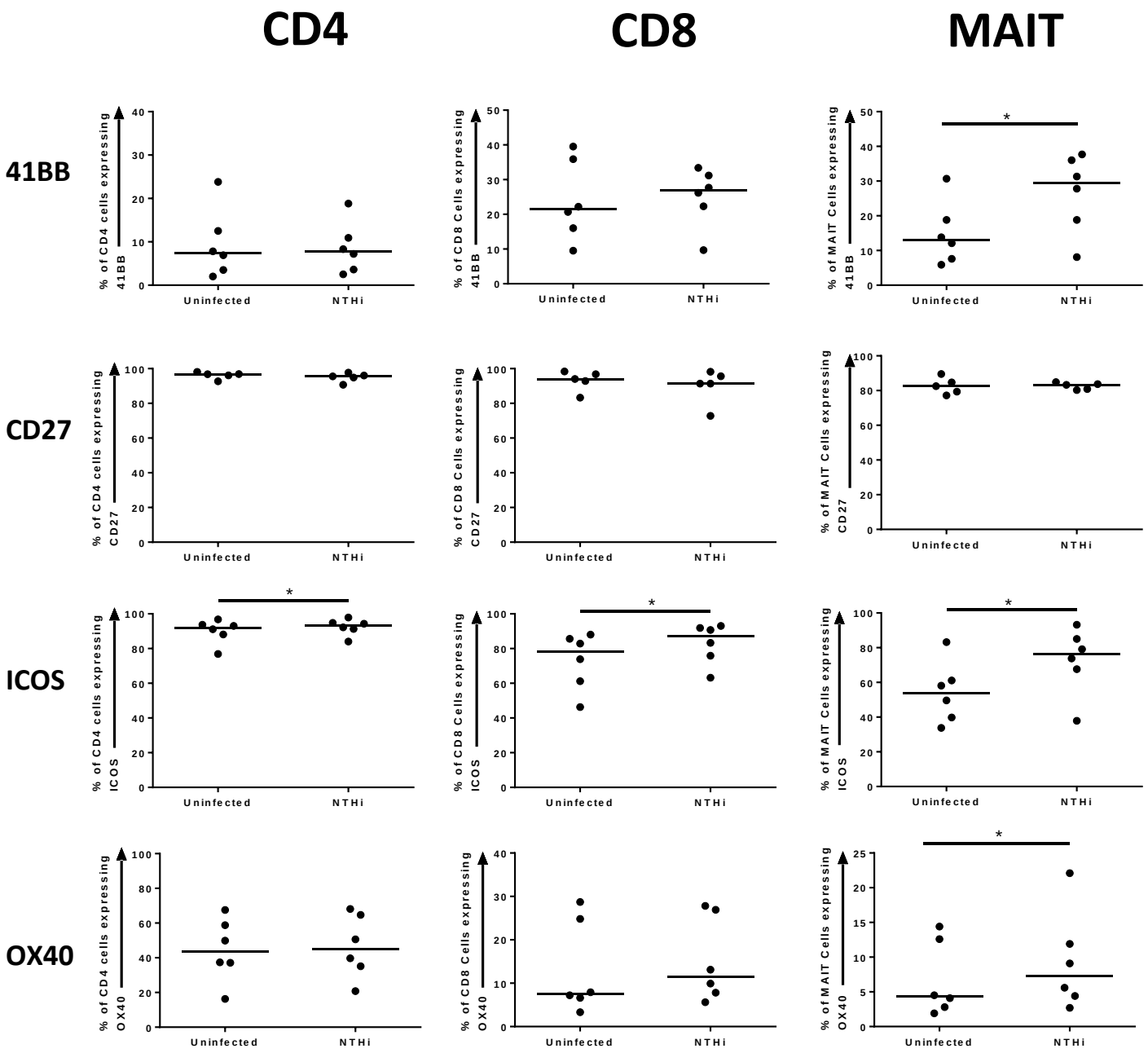


Figure 5.15 – Expression of other co-stimulatory molecules on T cell following NTHi infection. Expression of OX40, CD27, 41BB AND ICOS by CD4+, CD8+ and MAIT cells from blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.2.11 Co-inhibitory pathways do not regulate the IFNγ response of conventional T cell or MAIT cell following NTHi infection

In order to fully understand the role of the co-stimulatory and co-inhibitory pathways in regulating conventional and MAIT cells, blocking experiments would need to be performed. However, many of the blocking antibodies for the co-stimulatory molecules are either not suitable for human *in vitro* work or also had agonist properties, which would confound any results. Fortunately, blocking antibodies for the two co-inhibitory pathways were available, so the co-culture model was next used to investigate co-inhibition of conventional and MAIT cells.

As signalling of PDL1 on APCs to PD1 on T cells is known to inhibit T cell activation (Keir et al., 2007); blocking this inhibitory pathway should allow greater T cell responses during infection. Recently, blocking of PDL1 has been shown to enhance T cell activation in response to influenza-infected macrophages (Staples et al., 2015). In chapter 4 it was shown that PDL1 and PDL2 on MDM are both upregulated following NTHi infection, but the role the PDL/PD1 axis has on T cell responses to NTHi is unknown.

T cells were co-cultured with NTHi-infected MDM in the presence of a PDL1 blocking antibody and IFNγ expression was analysed on CD4, CD8 and MAIT cells by flow cytometry. In all three cases, blocking of PDL1 had no significant effect on cytokine production (figure 5.16). One alternative option was that it was PDL2, not PDL1, which was the main signalling molecule in this model; unfortunately, a PDL2 blocking antibody was not available. Instead, co-cultures were repeated with an anti-PD1 antibody, which would block signalling from both PDL1 and PDL2, but again, IFNγ production was not affected (figure 5.16). A second alternative was that flow cytometry may not be a suitable technique to observe the effects of the PD1 axis, as flow cytometry measures cytokine accumulation within the cell, not actual cytokine release. Instead supernatants were harvested from co-cultures and analysed for IFNγ protein by ELISA. An increase in release of IFNγ was detected from co-cultures with anti-PDL1 compared to co-cultures with isotype antibody, but this was not significant (figure 5.17). Overall, it would seem that PDL/PD1 does not increase IFNγ production in this model, as would be expected (Staples et al., 2015).

As the PDL/PD1 axis appeared to not be involved in regulating the T cell response to NTHi infection, the other co-inhibitory pathway, CTLA4, was explored to determine whether signalling via CTLA4 regulated the conventional T cell and MAIT cell IFNγ response to NTHi. Addition of an

anti-CTLA4 blocking antibody to the co-culture however did not affect IFNγ production by any T cell subset investigated (figure 5.18).

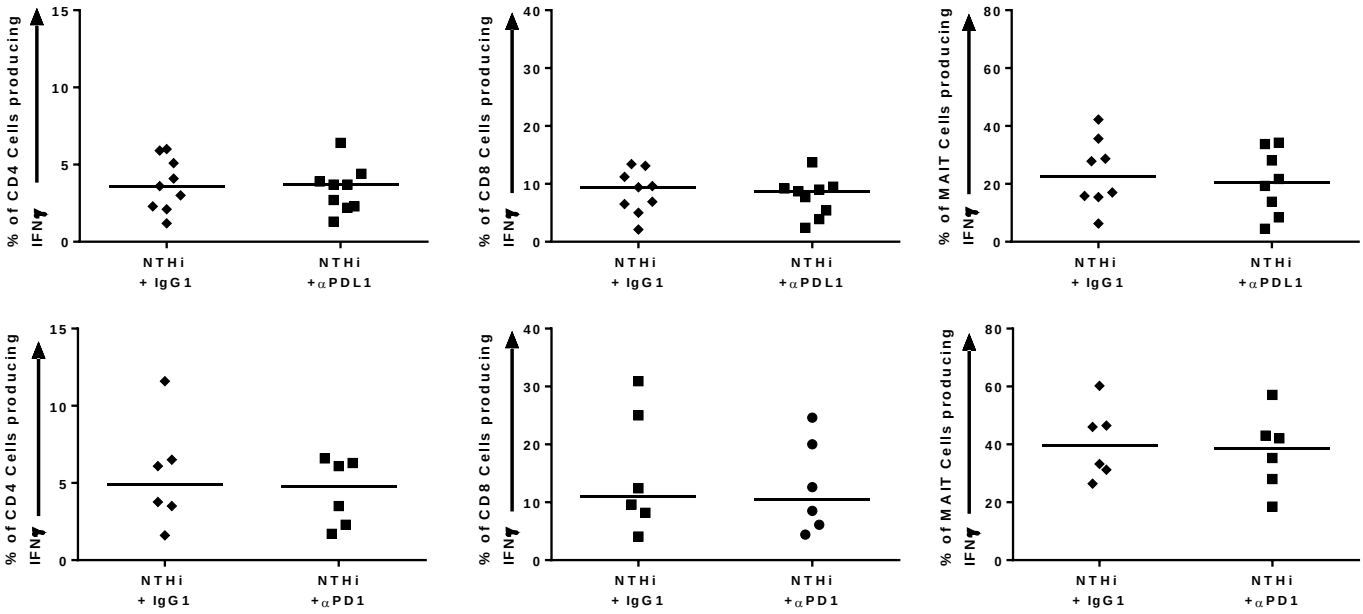


Figure 5.16 – Production of IFNγ is not affected by blocking PDL1 or PD1. Expression of IFNγ by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-PDL1 (MIH1), PD1 (EH12.2H7) or IgG1 isotype control. For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test.

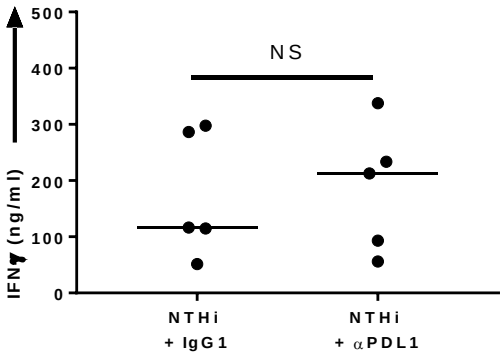


Figure 5.17 – Release of IFNγ is not affected by blocking PDL1. IFNγ production measured by ELISA from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-PDL1 (MIH1) or IgG1 isotype control. Baseline expression was 0 ng/ml; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. NS – Not Significant

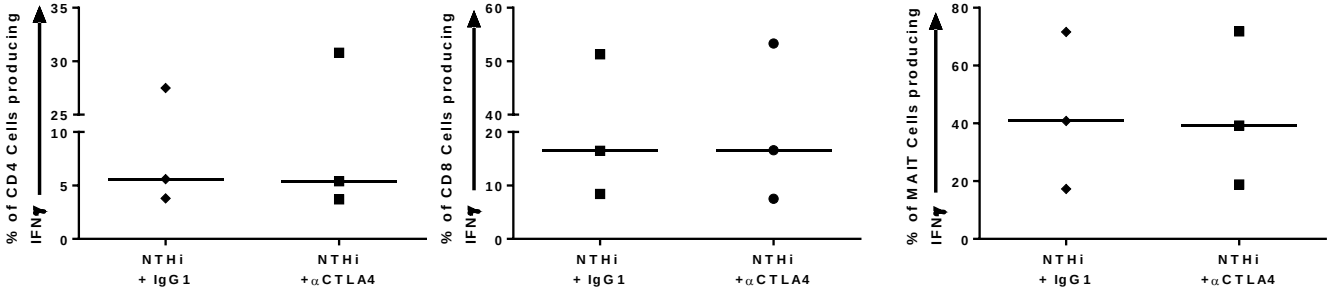


Figure 5.18 – Production of IFNγ is not affected by blocking CTLA4. Expression of IFNγ by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-CTLA4 (L3D10) or IgG1 isotype control. For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test.

5.2.12 Contact is required to induce conventional T cell but not MAIT cell cytokine response

Having explored antigen presentation and co-stimulation, the final step was to investigate the role of cytokine signalling (signal 3) in activation of the T cell subsets in the co-culture model. Cytokines are required for differentiation of naïve CD4+ cells into the different T helper subsets, such as IL-12 promoting a Th1 cells (Tominaga et al., 2000)(Murphy and Reiner, 2002)(Teng et al., 2015). Cytokine signalling can also result in non-specific activation of T cells (Lertmemongkolchai et al., 2001). In particular, MAIT cells have been shown to produce IFNγ in the absence of antigen presentation through IL-12 and IL-18 stimulation alone (Chua et al., 2012)(Ussher et al., 2014a). To see if stimulatory cytokines or other non-contact factors alone could activate conventional T cells and MAIT cells, the co-culture model was adapted to incorporate transwells (0.4 µm), preventing physical contact of T cells with the NTHi-infected MDM.

In the absence of contact, MAIT cells were able to significantly (P<0.05) produce IFNγ in response to NTHi infection, although this was substantially less than compared to MAIT cells in direct contact with NTHi-infected MDM (figure 5.19). CD4+ T cells were not activated in the absence of contact, whilst there was only a minor increase in CD8+ T cell producing IFNγ. Together, the data suggest that MAIT cells in particular are able to be activated in the absence of antigen presentation by non-contact factors, such as cytokines, which may suggest a role for these cells in bystander activation.

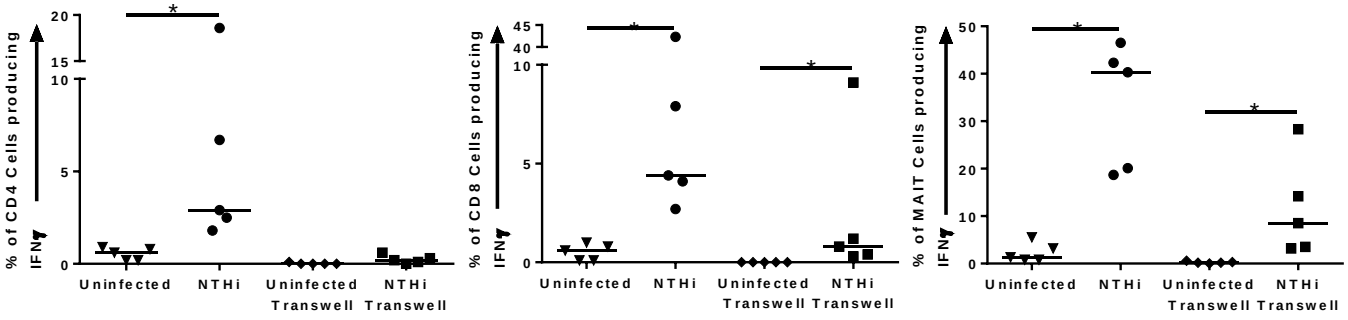


Figure 5.19 – Effect of contact-independent factors on IFNγ responses of T cells. Expression of IFNγ by CD4+, CD8+ and MAIT cells from T cells co-cultured in direct contact or in transwells with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.2.13 IL-12 is produced by MDM in response to NTHi infection and contributes to conventional T cell and MAIT cell IFNγ production

Previous reports in the MAIT cell literature have shown that IL-12 can activate MAIT cells independently of antigen presentation (Chua et al., 2012)(Ussher et al., 2014a), so the role of IL-12 in the NTHi-infected MDM-T cell co-culture model was next investigated. RNA and supernatants from uninfected and NTHi-infected MDM were harvested and IL-12 expression were measured by qPCR and ELISA.

IL-12 is comprised of two subunits, which are the p35 and p40 monomers that form the active heterodimer IL-12p70. NTHi infection caused a significant (P=0.01) 1200-fold induction in the IL-12p40 mRNA transcript but expression of IL-12p35 was not affected (figure 5.20). By ELISA, concentrations of the active IL-12p70 increased from 0 pg/ml at baseline to 290 pg/ml with NTHi infection. PFA-NTHi did not induce any detectable IL-12p70 protein, despite inducing a 120-fold induction in IL-12p40 transcript, again indicating that live bacteria are required to cause the greatest response. The production of IL-12p70 appeared time dependent, with very little protein detected at 5 hours post-infection but a significant increase detected at 22 hours. To determine whether IL-12 was relevant to the lung, IL-12p70 was also measured in supernatant from NTHi-infected lung explants and was found to be released ($p<0.01$) at low levels, but above the limit of detection (figure 5.21). Overall, IL-12 is induced by NTHi infection and may contribute to nonspecific activation of conventional T cells and MAIT cells.

Now that it had been established that IL-12 was released by NTHi-infected MDM, the co-cultures of NTHi-infected MDM and T cells were repeated with the addition of an anti-IL-12p40 capture antibody (figure 5.22). For all three T cell subsets, blocking of IL-12 significantly ($P<0.05$ for all) impaired expression of IFNγ to near-baseline, indicating that IL-12 was indeed involved in the IFNγ response of these T cells to NTHi infection.

To explore the role of IL-12 further, T cells were cultured alone (i.e. not with MDM) and stimulated with recombinant IL-12, but there was no induction of IFNγ, suggesting that IL-12 by itself was insufficient to activate these T cells (figure 5.23). However, when recombinant IL-12 was added to the MDM-T cell co-culture, a very minor induction of IFNγ was observed in conventional T cells, but a greater production of IFNγ was seen by MAIT cells, with 7.5% of MAIT cell expressing IFNγ (P=0.03). Clearly having MDM present in combination with IL-12 stimulation was having an effect, perhaps because IL-12 stimulated the MDM to release another cytokine which acted in

concert with the IL-12 to activate the MAIT cells. As described above, IL-12 and IL-18 can together induce non-specific activation of MAIT cells, so expression of IL-18 was analysed from IL-12 stimulated MDM. As seen in figure 5.24, IL-12 caused a 2-fold induction (P=0.03) in IL-18 gene expression. This could suggest that IL-12 can induce IL-18 protein, which together can cause non-specific MAIT cell activation.

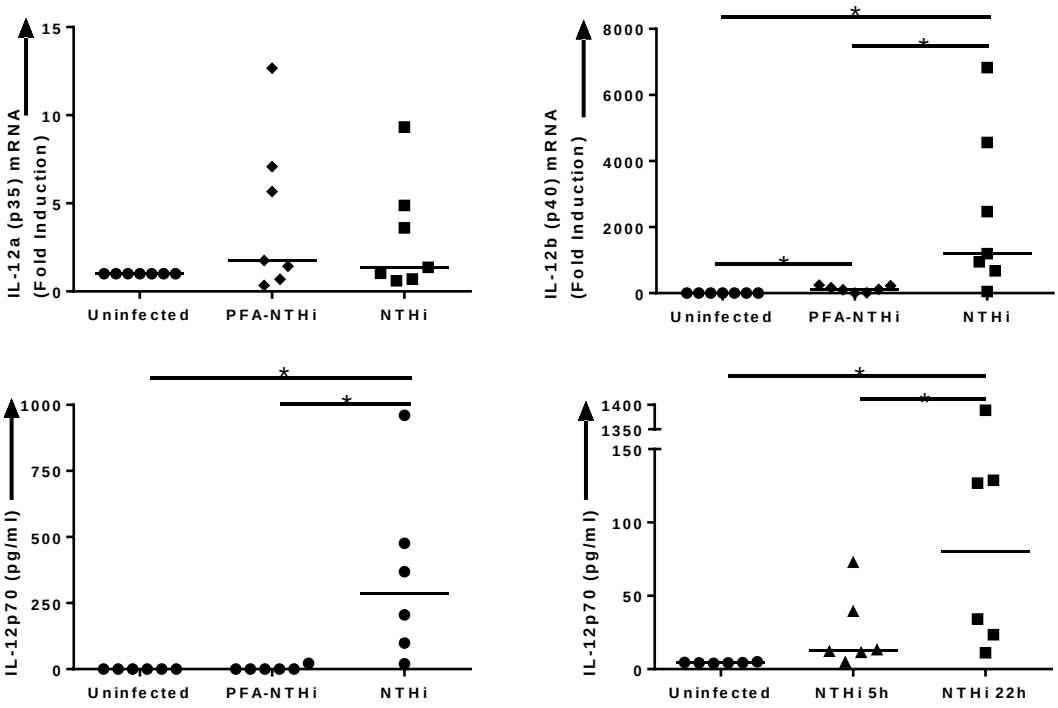


Figure 5.20 – Macrophages express IL-12 in response to NTHi. Top) Gene expression of *IL-12a (p35)* and *IL-12b (p40)* expressed as $\Delta\Delta C_t$ normalised to β_2M from autologous MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 10. Bottom) IL-12 p70 expression measured by ELISA from autologous MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 10, infected with NTHi at a MOI of 10 for 5 or 22 hours. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

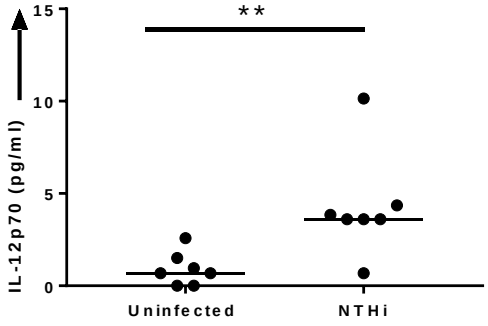


Figure 5.21 – Lung explants express IL-12 in response to NTHi. IL-12 p70 expression measured by ELISA from lung explants either uninfected or infected with NTHi at a MOI of 10, infected with NTHi at a MOI of 10. Data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. **P<0.01

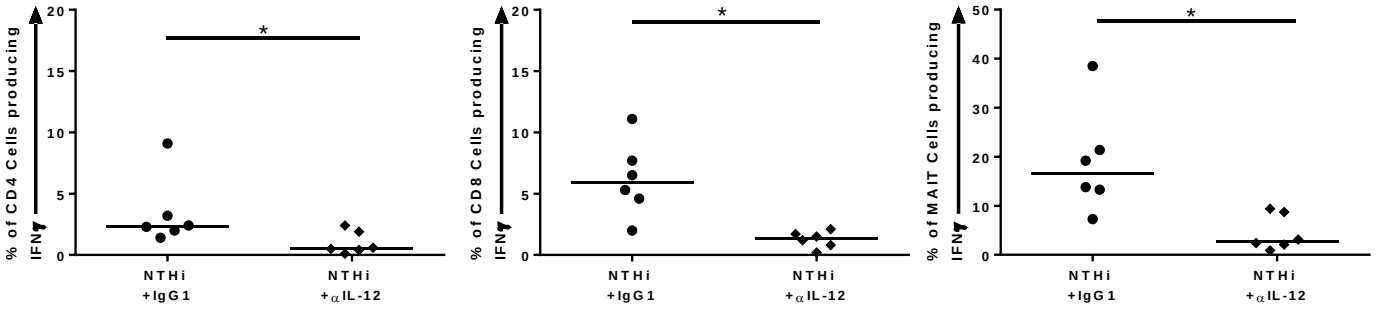


Figure 5.22 – Production of IFNγ by T cells is impaired by blocking IL-12. Expression of IFNγ by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-IL-12 (C8.6), IgG1 isotype control. For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

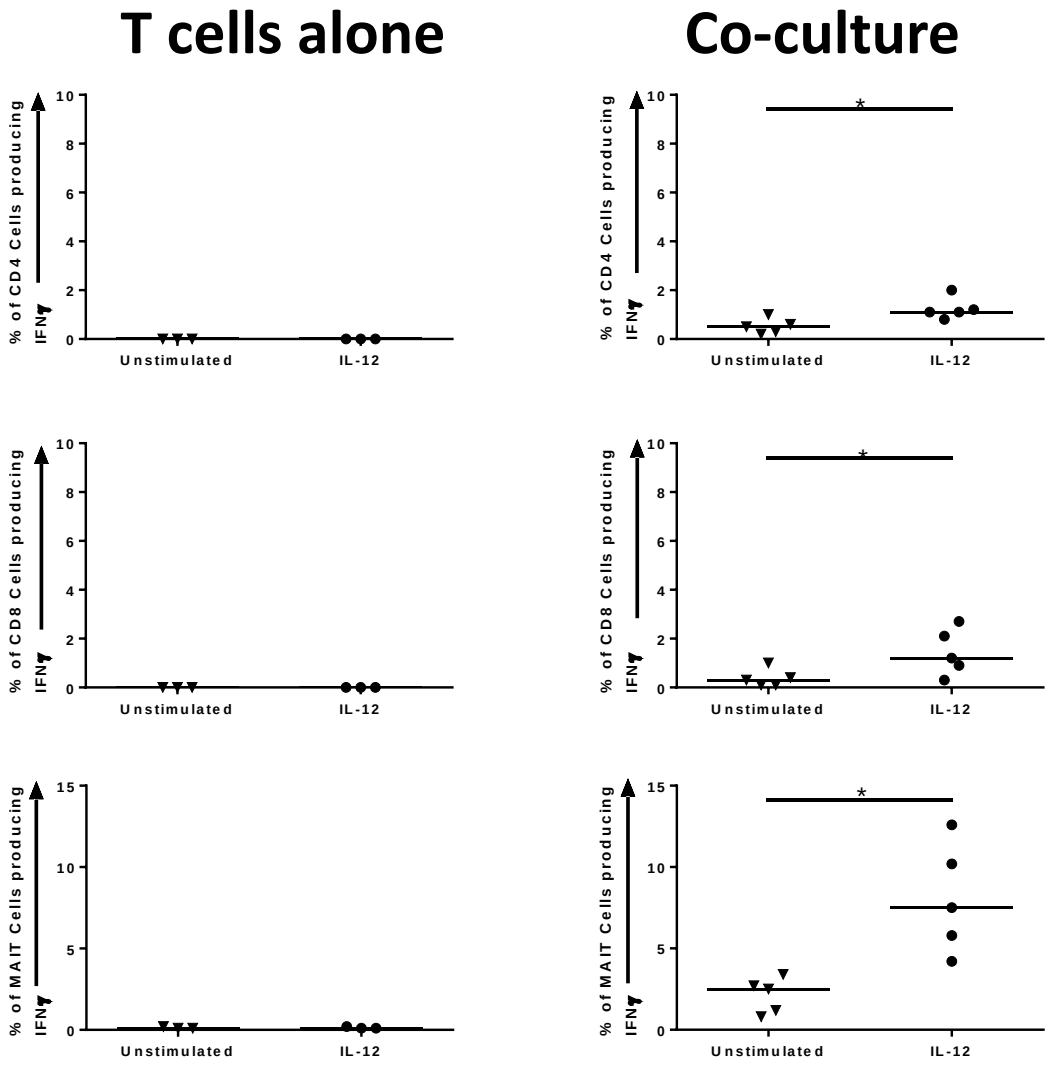


Figure 5.23 – Production of IFNγ by T cells is induced by IL-12. Expression of IFNγ by CD4+, CD8+ and MAIT cells from either T cells alone or co-cultures of T cells and autologous MDM stimulated with IL-12 (10 ng/ml). For all graphs; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

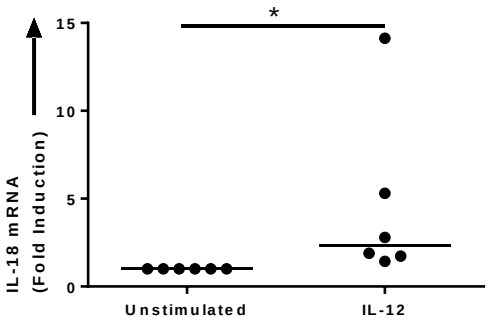


Figure 5.24 – Macrophages express IL-18 in response to IL-12 stimulation. Gene expression of *IL-18* expressed as $\Delta\Delta C_t$ normalised to β_2M from autologous MDM either unstimulated or stimulated with IL-12 (10 ng/ml). Data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.2.14 Steroids impair NTHi-induced T cell and MAIT cell activation

As discussed previously, the use of inhaled corticosteroids (ICS) to treat respiratory diseases such as COPD and asthma may potentially impair the immune response to respiratory pathogens and drive bacterial colonisation. In the previous chapter, it was found that fluticasone propionate and budesonide prevented upregulation of MR1 and HLA-DR on macrophages. The next step was to determine what effect these steroids have on T cell responses.

T cells were co-cultured with NTHi-infected MDM in the presence of either fluticasone (FP), budesonide (BD) or DMSO and analysed by flow cytometry (figure 5.25). For these experiments, steroids were present for the 2 hour infection of MDM, then following washing, added back to culture so they were present for the 22 hour co-culture with the T cells. CD4, CD8 and MAIT cells all had decreased IFNγ production in response to NTHi infection and FP, compared to just infection plus DMSO. Percentage of CD4+ cells producing IFNγ decreased significantly (P=0.003) from 2.8% to 0.75%, CD8+ cells IFNγ+ cells from 11% to 1.2% (P=0.003) and from 30% to 4.5% for MAIT cells (P=0.007). A similar effect was seen with BD; CD4+ 3.9% to 0.7%, CD8+ 7.2% to 0.9%, and MAIT 23.7% to 6.1% (all p<0.05).

Whilst steroids appear to inhibit IFNγ production, it was not clear whether the reduced T cell response is a due to impaired uptake of NTHi by the MDM or impaired T cell activation, as steroids were present for both the 2 hour NTHi infection stage of MDM and the 22 hour co-culture stage with T cells. To further explore this mechanism, the steroid experiments were

repeated with FP only present for just the 2 hour infection of MDM (figure 5.26), followed by 22h co-culture without steroids and production of IFNγ by all three T cell subsets was found to be significantly ($P<0.05$) impaired. However, when FP was not present for the 2 h infection but was only present for the 22 hour co-culture, IFNγ expression was also inhibited to the same extent. Overall, these data indicate that steroids impair both the MDM and the T cell response, as the inhibition of IFNγ was seen regardless of whether steroids were present for the 2 hour MDM infection stage or not. It would seem that steroid-induced inhibition of IFNγ may not be due to impaired phagocytosis of NTHi, in this model.

In terms of cell numbers, FP had no effect on the population percentages of CD4+ or CD8+ cells, but appeared to increase the percentage of MAIT cells from 1.4% of T cells with infection alone to 2.1% with infection and steroids ($P=0.03$). However, it seems likely that this apparent increase (figure 5.27) is due to fluticasone preventing activation-induced downregulation of CD161 and Vα7.2, returning MAIT cell numbers back to their uninfected level.

As the IL-12 pathway appears to be involved in the activation of conventional T cells and MAIT cells, the effect of steroids on production of IL-12 by MDM was next examined. As shown in figure 5.28, exposure of MDM to FP significantly impaired IL-12p70 production ($P=0.01$), reducing it to baseline. Together with the steroid data presented in the current and previous chapter, steroids appear to prevent T cell activation by inhibiting both the antigen-presenting and cytokine-signalling responses of MDM, which may have repercussions for lung immunity to NTHi.

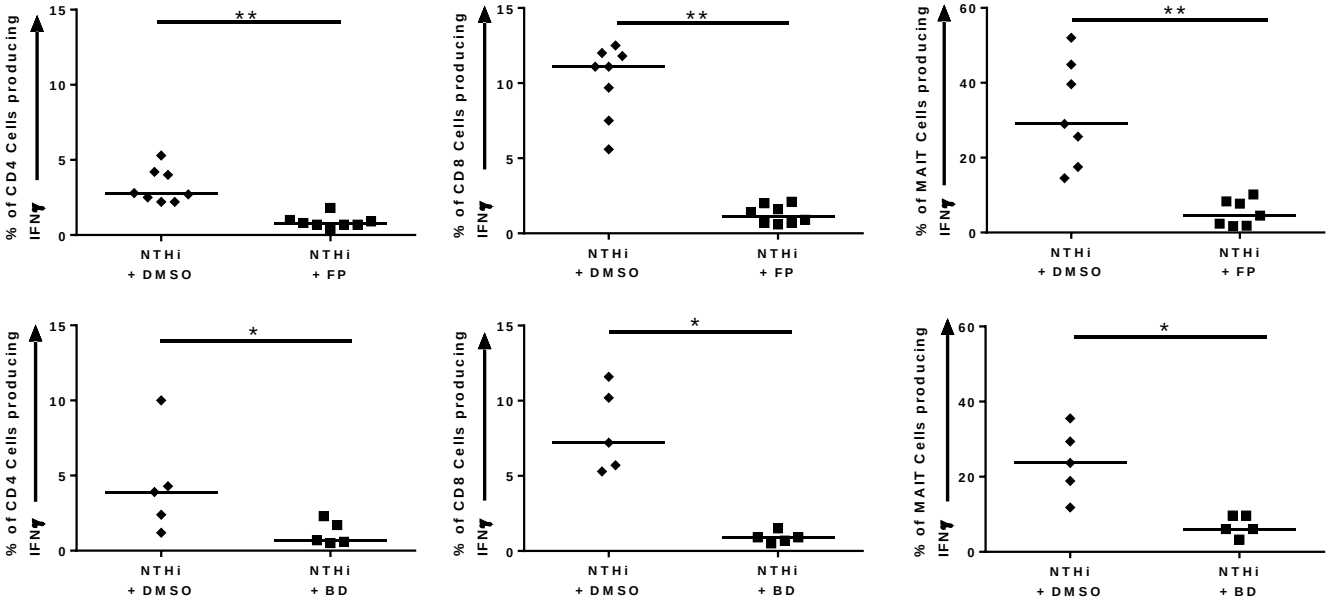


Figure 5.25 – Production of IFNγ is impaired by corticosteroids. Expression of IFNγ by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 100 nM fluticasone propionate (FP) or 200nM budesonide (BD) or DMSO. Double the amount of budesonide compared to fluticasone was used based on the difference in glucocorticoid receptor occupancy (Daley-Yates, 2015). For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **p<0.01

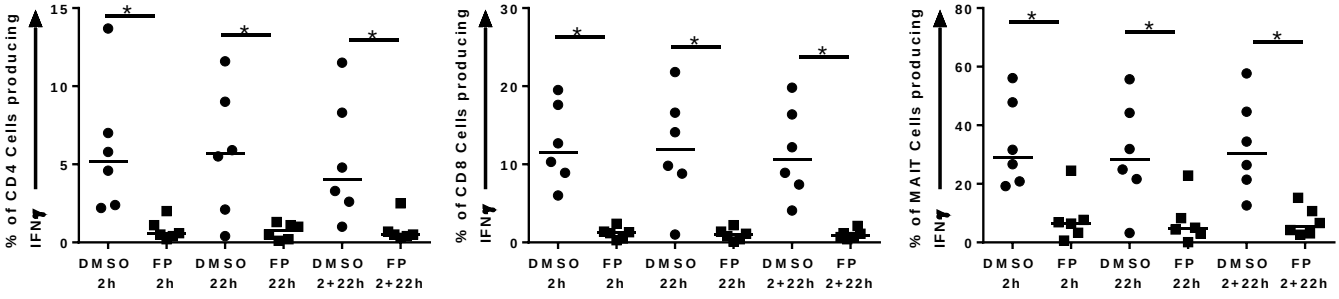


Figure 5.26 – Production of IFNγ is impaired by corticosteroids regardless of timepoint. Expression of IFNγ by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 100 nM fluticasone propionate (FP) or DMSO for different timepoints. For all graphs, baseline expression was 0%; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

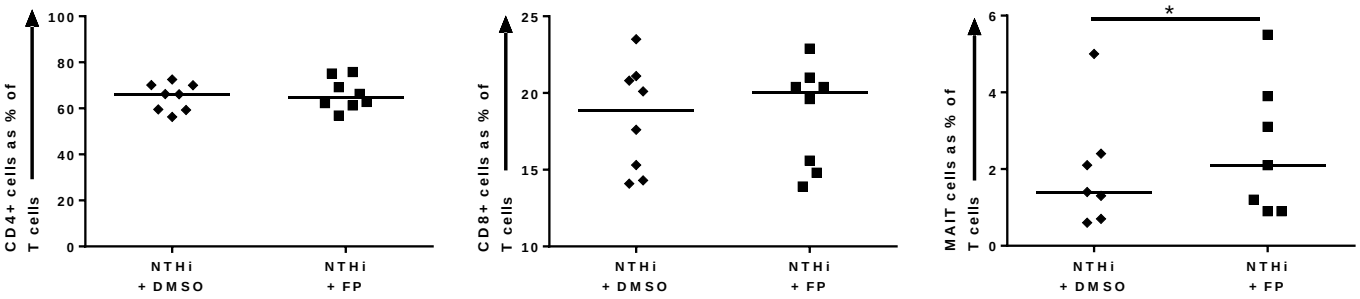


Figure 5.27 – Detection of T cell subsets after steroid treatment. Percentages of CD4+, CD8+ and MAIT cells from peripheral T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 100 nM fluticasone propionate (FP) or DMSO. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

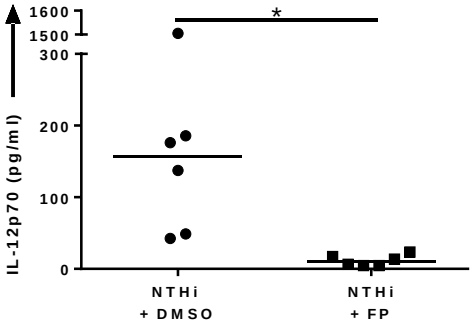


Figure 5.28 – Production of IL-12 is impaired by corticosteroids. IL-12 p70 expression measured by ELISA from autologous MDM infected with NTHi at a MOI of 10 in the presence of either 100 nM fluticasone propionate (FP) or DMSO. Baseline expression was 0 pg/ml; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

5.3 Discussion and Conclusions

NTHi is an important pathogen which colonises the human airway early in life but also causes infection such as otitis media and pneumonia. However, in respiratory diseases such as COPD and asthma, pathogens such as NTHi lead to exacerbations and further disease progression. As recurrent exacerbations cause a decline in lung function and increase mortality, the development of an efficient immune response may be vital for a better COPD prognosis (Lugade et al., 2014). The association of NTHi and exacerbations suggests an aberrant airway immune response which may be further compromised by immunomodulatory treatments such as steroids.

Given that MAIT cells are antibacterial T cells resident in the lung (Meierovics et al., 2013), it is likely that they are playing a role in host defence to lung bacterial pathogens, although the function of these cells in respiratory disease has been barely explored. This chapter has investigated the T cell response to NTHi and shown, for the first time, that MAIT cells are capable of being activated in response to NTHi. Lung MAIT cells upregulated significant levels of IFNγ, IL-17a and TNFα following NTHi infection, consistent with responses shown with other pathogens (Le Bourhis et al., 2010)(Chua et al., 2012), indicating that MAIT cells may be key responders to respiratory bacterial infections. IL-4, -5,-10 and -13 were not investigated as MAIT cells have not been shown to produce these cytokines (Dusseaux et al., 2011). In the blood co-culture, whilst IFNγ and TNFα were also observed, low levels of IL-17a were produced, indicating differences between the lung and blood models, although IL-17a is typically found at mucosal sites and so one would not expect to see much from peripheral blood-derived cells (Jin and Dong, 2013). As IL-17a production by MAIT cells from the co-culture appeared greater with killed PFA-NTHi than live NTHi, this may suggest that live NTHi can prevent the induction of an IL-17 response in this co-culture model although further work would be needed to confirm this. Other bacteria such as *E. coli* and *Mycobacterium bovis* have also been shown to induce IL-17a production by MAIT cells (Dusseaux et al., 2011)(Chua et al., 2012) although the extent to which IL-17a is induced appears to be dependent on the pathogen involved (Le Bourhis et al., 2010). One caveat is that it was not possible to ascertain whether individual MAIT cells were positive for more than one cytokine in response to NTHi, as separate flow cytometry panels were used. Therefore, it is not known whether the majority of MAIT cells are activated but only produce one cytokine each (i.e. some only produce IFNγ, some IL-17a, and some TNFα), or whether a small proportion of MAIT cells produce all three cytokines at once. Further work would be needed to address this.

Blocking of MR1 at the 22 hour timepoint however did not affect MAIT activation, which at first implied that MAIT activation in response to NTHi was MR1-TCR independent. This seemed strange

as NTHi is thought to produce riboflavin and therefore should be expressing the MAIT activating ligands (Kanehisa and Goto, 2000). Moreover, as discussed in chapter 4, MR1 upregulation on MDM by NTHi is also likely due to the presence of MR1 ligands. The timecourse of activation therefore seemed a likely key factor in determining whether the MR1 antigen presentation pathway was involved in MAIT activation. Ussher et al found that short incubations of infected APCs activated MAIT cells in an MR1-mediated manner, but longer incubations of 20 hours meant that MAIT activation was only partially dependent on MR1 (Ussher et al., 2014a). In agreement with this, shortening the length of NTHi-infected MDM-T cell co-culture with anti-MR1 antibody resulted in a significant reduction in IFNγ production by MAIT cells. Although IFNγ was the only readout for these blocking experiments as IL-17a and TNFα were not reliably detected in the co-culture, other reports have shown that IL-17a and TNFα production are impaired by MR1 blocking, indicating that these cytokines may also be mediated by MR1 (Gibbs et al., 2016)(Gracey et al., 2016).

Despite the fact that MR1 blocking has been shown to inhibit MAIT production of IFNγ (Gold et al., 2010)(Le Bourhis et al., 2013), other reports show that MAIT cell activation can be entirely MR1-independent and instead driven by IL-12 (Chua et al., 2012)(Ussher et al., 2014a). The type of pathogen, intracellular location of pathogen and/or type of infected cell may all be factors in determining whether MAIT activation is MR1-dependent or –independent. Chua et al found that macrophages infected with *Mycobacterium bovis* activated MAIT cells in a manner entirely dependent on IL-12, shown by IL-12 blocking antibody and $p40^{-/-}$ knockout (Chua et al., 2012). Here, in combination with the MR1 blocking data and transwell data, production of IFNγ by MAIT cells in response to NTHi is controlled by MR1 antigen presentation in a time dependent manner, and by IL-12 signalling, as summarised in figure 5.29. At 5 hours, MAIT activation appears entirely dependent on MR1 and production of IL-12 by MDM is minimal. However by 22 hours, MAIT activation has become MR1-independent but IL-12 dependent, as MDM produce high levels of IL-12 and IL-12 blocking abrogates MAIT activation. The fact that MAIT cells could still produce IFNγ even when contact and antigen presentation had been abolished, shows that cytokines are sufficient to induce IFNγ production by themselves in MAIT cells. The IL-12 pathway may also involve IL-18, as shown by others (Ussher et al., 2014a) and IL-12 was shown to induce IL-18 mRNA expression in MDM, but further work is needed to ascertain whether IL-18 has any role in this co-culture model. IL-18 is expressed by alveolar macrophages from COPD patients (Imaoka et al., 2008), and exposure of mice to IL-12 and IL-18 has been shown to induce lung inflammation (Okamoto et al., 2002). Overall, it would seem that MAIT cells utilise both the antigen presentation pathway and IL-12 signalling pathway independently of each other. If MAIT cells can

be activated purely by cytokine signalling, with no antigen-mediated activation, they may play an important role in bystander inflammation.

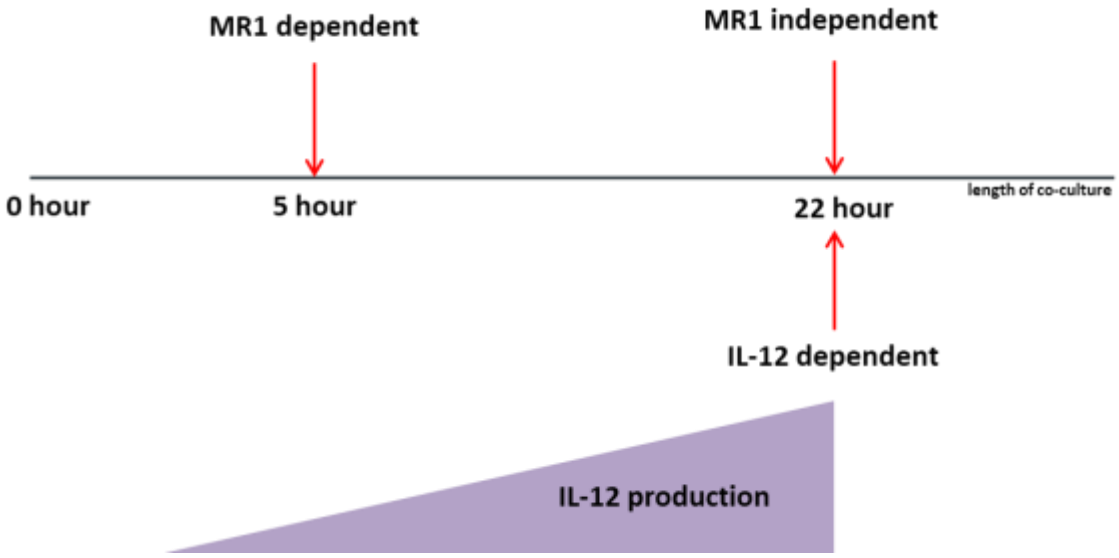


Figure 5.29 – Overview of MAIT cell activation as measured by IFNγ production. By 5 hours post co-culture of NTHi-infected MDM and T cells, the MAIT cell IFNγ response is small and is mediated by MR1 antigen presentation. IL-12 production is low at this time point. By 22 hours, IL-12 production has increased and now dominates control of the MAIT IFNγ response; MR1 antigen presentation is not involved.

In comparison to MAIT cells, lung CD4+ produced all three cytokines following NTHi infection, although lung CD8+ T cells did not, despite the fact that both blood CD4+ and CD8+ T cells also produced IFNγ in response to NTHi. The reasons for the differences in cytokine production by lung and blood CD8+ T cells are many, but could be due to differences in naïve and memory T cells in the lung and blood or different kinetics of activation between the two models. However as seen in chapter 3 (figure 3.4), lung CD8+ T cells did upregulate IFNγ and TNFα in response to PMA/Ionomycin stimulation, indicating they are capable of producing these cytokines. Lung CD4+ and CD8+ T cells have been previously shown to produce both IFNγ and IL-17a in response to NTHi (King et al., 2013). King et al also compared T cells from COPD and non-COPD patients and found a significant increase in IL-17a production in the diseased cohort, but no difference in production of IFNγ. Despite the fact that the proportion of CD8+ cells have been demonstrated to be increased in the airways during COPD (McKendry et al., 2016), they don't appear able to effectively control bacterial infection. Moreover, CD8+ cells correlate with the degree of airflow limitation, indicating that these cells play some role in the observed damage to lung tissue (Cosio et al., 2009). Lung T

cell responses to bacteria need to be investigated further to determine why they seem unable to control bacterial infection in the COPD airway.

The IFNγ response of the co-cultured CD4+ and CD8+ T cells was unexpected at first, as 3% and 9% of each population responding to a single pathogen is very high. However, it is perhaps not so surprising given the possible diversity of NTHi antigens that could be presented and how many of these antigens may be structurally-related to proteins shared with other bacteria, for example bacterial GAPDH (Madureira et al., 2011). Blocking of HLA-DR and HLA-ABC in the co-culture reduced expression of IFNγ by CD4+ and IFNγ by CD8+ cells respectively, indicating that these T cells were responding to NTHi in an antigen-specific manner. There also appears to be considerable cross-talk between CD4+ and CD8+ T cells, as blockade of one also reduced IFNγ production by the other. Many reports have indicated that the L243 antibody clone is specific for HLA-DR and the W6/32 antibody clone is specific for HLA-ABC (Anichini et al., 1985)(Santin et al., 1999)(Franssila et al., 2005)(Kalka-Moll et al., 2002)(Wang et al., 2011), so it seems likely that the antibodies used are only blocking their intended targets, i.e. the HLA-DR antibody is not blocking HLA-ABC. Instead, it would appear that CD4+ and CD8+ T cells in this model require help from each other in order to generate a maximal response. Whether this help is in the form of cytokine or physical stimulation from co-stimulatory molecules is unknown and would require further work. However, it is already established that CD4+ T cells help the generation of CD8+ T cell responses either directly or indirectly by production of IL-2 and IFNγ and ligation of the CD40-CD40L pathway, (Serre et al., 2006)(Zhang et al., 2009)(Bevan, 2004), all of which may be involved in the T cell responses in this co-culture model.

MHC blocking did not reduce IFNγ expression all the way to baseline and IL-12 blocking also impaired the conventional T cell response. The transwell data showed that, in the main, conventional T cell requires contact for IFNγ to be produced. Although a significant increase in IFNγ was seen with CD4+ and CD8+ T cells following IL-12 stimulation and an increase for CD8+ T cell IFNγ expression in the transwell, these increases were very minor in comparison to the induction seen in MAIT cells. This would suggest that antigen presentation, contact and IL-12 are all required together for the conventional T cell IFNγ response, unlike MAIT cells where antigen presentation and IL-12 signalling can function independently.

Conventional T cells and MAIT cells were further characterised by their expression of co-stimulatory or co-inhibitory markers, which are involved in ‘signal 2’ of T cell activation. Unfortunately, due to the limited numbers of T cells obtained from lung tissue, the potential effect that collagenase, which was discussed in chapter 3, has on expression of the various co-

stimulatory or co-inhibitory markers was not investigated. However, CD28, CD40L, CTLA4, PD1, 41BB and OX40 expression on T cells from collagenase-treated lung tissue appeared comparable to expression on T cells from blood, suggesting that collagenase may not have an affect these markers. On the other hand, due to the differences in expression of CD27 and ICOS between T cells from collagenase-treated lung tissue and blood, an effect of collagenase on these markers cannot be discounted.

CD28 and CD40L and are vital for activation of naïve T cells and work in tandem with the T cell receptor to support T cell activation (Chen and Flies, 2013). CD27, similar to CD28, is also highly expressed on T cells and promotes T cell survival by upregulating anti-apoptotic proteins (Hendriks et al., 2003)(Chen and Flies, 2013)(Croft, 2009). Both CD27 and CD28 were highly expressed and unaffected by NTHi infection, indicating that if they are involved in conventional and MAIT cell activation, upregulation is not required for them to function. Recently, CD27- MAIT cells have been shown to have greater production of IL-17a compared to CD27+ MAIT cells (Harms et al., 2015), which could explain the lack of IL-17a in the co-culture model, indicating that loss of CD27 is associated with polarisation towards a pro-inflammatory Th17 phenotype. CD40L on the other hand was upregulated following infection, with the greatest induction on MAIT cells. This may indicate that CD40L plays a greater role in MAIT cell activation. Alternatively, the upregulation of CD40L may have implications for other cell types such as causing MAIT cell-induced maturation of DCs in a CD40L-dependent manner (Salio et al., 2017) or allowing MAIT cells to exhibit other helper function, similar to a role proposed for CD40L+ CD8+ T cells (Frentsch et al., 2017). A possible paradigm therefore may be that MAIT cells are activated quickly in response to infection where they can induce DC maturation via mechanisms including CD40L signalling, allowing the DCs to then activate other conventional T cells. Thus MAIT cells may be important for the early phase of infection and be required to effectively orchestrate the immune response (Salio et al., 2017).

In contrast to CD28, OX40 is not thought to be expressed on naïve T cells, but is upregulated following antigen stimulation, where it is required to enhance T cell proliferation and survival (Gramaglia et al., 1998)(Rogers et al., 2001)(Bansal-Pakala et al., 2004). Memory T cells have been shown to be less dependent on CD28 and CD40L, but blocking of OX40 has been shown to supress activation of memory T cells, which indicates that memory T cells are still dependent on other co-stimulatory molecules (London et al., 2000) (Salek-Ardakani et al., 2003). OX40 upregulation was greatest on MAIT cells, again indicating that it may be required for antibacterial responses of MAIT cells. 4-1BB appears to be preferentially expressed on CD8+ T cells and may be important for enhancing IFNγ production and regulating cytotoxic T cells (Wen et al., 2002)(Shuford et al.,

1997) (Zhang et al., 2007). Given that there was a high upregulation of 4-1BB on MAIT cells following NTHi infection, the IFNγ response of MAIT cells may also utilises 4-1BB. ICOS is supposedly only expressed on activated T cells, so the high expression of ICOS here could be due to being in co-culture with the MDM, where the MDM are skewing the T cells to a more memory phenotype at baseline – further work would be needed to address this (Nurieva et al., 2009)(Watts, 1999)(Hutloff et al., 1999). Overall, just measuring upregulation of these molecules doesn't confirm whether they are actually involved in T cell activation. Unfortunately, this thesis was not able to investigate all of these interactions and further work with blocking antibodies would be needed to establish whether these co-stimulatory pathways regulate conventional or MAIT cell responses.

The expression of PD1 and CTLA4 were both upregulated on MAIT cells, again indicating that they could be involved in regulating the response to NTHi. Blocking of PDL1 has previously been shown to increase CD8+ T cell production of IFNγ in response to influenza viral infection (Staples et al., 2015). The other ligand for PD1, PDL2, (Keir et al., 2007) is known to be expressed on macrophages (Staples et al., 2015) and therefore could also be signalling to PD1 in the context of bacterial infection. Blocking of PD1 has been shown to cause increased T cell IFNγ production (Kalathil et al., 2014) and PD1 expression has also been demonstrated on MAIT cells and is upregulated in patients with active tuberculosis infection (Jiang et al., 2014). Blocking of PD1 in this case also caused increased production of IFNγ by MAIT cells. During chronic bacterial infection or colonisation, upregulation of a PD1 ligand by infected APCs and PD1 by chronically activated MAIT cells may impede further MAIT cell responses, allowing the airways to be overwhelmed by additional bacterial colonisation. However, the data presented here shows PDL1 and PD1 blocking had no effect on NTHi-induced activation of T cells, although increasing the number of repeats for the PDL1 ELISA could make the data significant. CTLA4 blocking also had no effect on the T cell response to NTHi. Together these data suggest that the PDL1/PD1 axis and CTLA4 pathway are not involved in regulating T cell cytokine responses to NTHi, in this model. However, these pathways may be involved in other T cell responses. In the next chapter, the potential role of PD1 and CTLA4 in regulating T cell cytotoxic responses will be investigated.

Steroids have long been known to impair T cell proliferation and activation (Tuosto et al., 1994), and in this study fluticasone and budesonide significantly impaired T cell IFNγ production in response to NTHi. Taken together with the effect of steroids on MR1 expression presented in chapter 4, use of steroids appears to impair the function of MAIT cells as well. The overall consequences of corticosteroids are unknown, as ICS treatment is thought to improve symptoms and reduce exacerbations of COPD (Woods et al., 2014) by dampening the immune response. On

the other hand, use of steroids in COPD and asthma has been associated with community acquired pneumonia (Festic and Scanlon, 2015). Steroid-induced suppression of T cells is likely detrimental, allowing initial colonisation of the lower airways by commensal bacteria from the upper airways, and therefore allowing NTHi to become pathogenic. Furthermore, MAIT cells, but not conventional T cells, are deficient in the airways and blood of COPD patients taking ICS (Hinks et al., 2016), suggesting that MAIT cells are both reduced in number and functionally impaired by steroids, further enforcing the fact that steroids may be detrimental long term.

Flow cytometry is only one way of detecting cytokines, but does not show whether these cytokines actually get released. Detection of IFNγ was also shown by ELISA and followed the same trend observed by flow cytometry, confirming that IFNγ was actually released and can therefore have functional effects. It is not known whether PFA-NTHi would induce release of IFNγ, but as the flow cytometry data showed that PFA-NTHi did not greatly upregulate IFNγ, very little if at all any IFNγ release would be expected. Unfortunately as the IL-17a response appeared very small, ELISA was not attempted. Even though 17% of MAIT cells were IL-17a+ in the lung, the fact that lung MAIT cells comprise 1.5% of lung T cells indicates that any total IL-17a release would be small. ELISA for TNFα was not performed as TNFα is produced by many cell types, most notably macrophages (Wajant et al., 2003).

T cell populations were measured following infection to ensure that infection-induced activation was not confounding the detection of T cells. Conventional T cells were unaffected by infection, but NTHi did cause a small deficiency in the percentage of MAIT cells in the co-culture. An alternative approach to identifying MAIT cells by CD161 and Vα7.2 would have been to use an MR1 tetramer, which would unequivocally identify MAIT cells through expression of their TCR, removing the issue of downregulation of phenotype markers (Leeansyah et al., 2013)(Reantragoon et al., 2013). However, MR1 tetramers were not commercially available during the majority of this project and therefore, for the purpose of this thesis, expression of CD161 and Vα7.2 had to be used to detect MAIT cells.

In summary, as well as conventional CD4+ and CD8+ T cells, MAIT cells also appear to have a role in the cytokine response to NTHi infection. This chapter has also demonstrated that macrophages do function as APCs for both conventional and MAIT cells and can present antigen from live pathogens. MAIT activation appears to be dependent on MR1 and IL-12 in a time-dependent manner, whereas conventional CD4+ and CD8+ T cells cytokine responses to NTHi infection have a greater reliance on MHC molecules and physical contact. Overall, this demonstrates that the abilities of conventional T cells and MAIT cells to activate in response to a pathogen may be

different. Corticosteroids significantly inhibited the activation of all T cell subsets explored in this study and this may have considerable clinical consequences. This chapter has been exclusively concerned with cytokine responses to NTHi infection. However, T cell mediated cytotoxicity is also required to clear bacterial infections and therefore the next chapter will explore whether conventional T and MAIT cells can mount cytotoxic responses to NTHi infection.

6. Investigating cytotoxic responses of T cells to NTHi infection

6.1 Introduction

A hallmark of T cell effector function is their cytotoxic ability, enabling them to destroy infected cells and clear infection. Whilst only CD8+ T cells are classically thought of as cytotoxic, both CD4+ T cells and MAIT have been shown to have cytotoxic function (Wilkinson et al., 2012)(Marshall and Swain, 2011)(Le Bourhis et al., 2013). Cell-mediated cytotoxicity may be key to controlling NTHi infection, but the cytotoxic responses of NTHi-specific T cell populations are not well understood. The mechanisms controlling MAIT cell cytotoxicity are not well characterised, neither is it known how these mechanisms and responses differ from those of conventional T cells.

One of the primary components of T cell cytotoxicity is the granule exocytosis pathway, where cytotoxic vesicles contained within the T cell are trafficked to the cell surface, fuse with the plasma membrane and the cytotoxic contents are released into the immunological synapse, leading to target cell apoptosis (Lieberman, 2003). As part of this process, CD107a, a molecule bound to the membrane of these cytotoxic vesicles, becomes expressed on the T cell surface and therefore CD107a expression typically serves as a marker of degranulation (Aktas et al., 2009)(Alter et al., 2004)(Wolint et al., 2004). Contained within the cytotoxic vesicles are the pore-forming molecule, perforin, and a family of serine proteases collectively known as granzymes, of which the apoptosis-inducing granzyme B has been intensively studied (Lewinsohn et al., 2011)(Lieberman, 2003)(Cullen et al., 2010)(Hodge et al., 2006)(Freeman et al., 2010).

In this chapter, expression of the degranulation marker CD107a and the effector molecules perforin and granzyme B have been characterised on conventional T cells and MAIT cells from both the NTHi-infected lung explants and the NTHi-infected MDM-T cell co-culture model. Similarly to the previous chapter, the co-culture model has been used to further explore whether the cytotoxic responses were antigen-specific or driven by cytokines such as IL-12. In addition, the impact of other factors such as co-inhibitory pathways and the effect of steroids on these cytotoxic responses have also been investigated.

6.2 Results

6.2.1 Conventional T cells and MAIT cells upregulate the degranulation marker CD107a in response to NTHi infection

To first determine whether NTHi infection could induce degranulation, surface expression of CD107a was measured on conventional T cells and MAIT cells from both the NTHi-infected lung explants and MDM-T cell co-culture. If CD4+, CD8+ or MAIT cells are able to mount a cytotoxic response to NTHi infection, an upregulation of CD107a on the surface of the cells would be expected. Again, due to limited availability of tissue, a PFA-NTHi vs live NTHi explant comparison could not be investigated.

Conventional lung CD4+ and CD8+ T cells had small inductions in CD107a expression following NTHi infection of the lung explant, with 5.9% of CD4+ cells CD107a+ at baseline increasing to 6.25% and 3.85% of CD8+ cells increasing to 4.05% CD107a+ with NTHi infection (figure 6.1). Despite the minor increase, the upregulation of CD107a on CD4+ cells was reported to be statistically significant (P=0.007), although this was not the case for CD8+ T cells. For lung MAIT cells on the other hand, NTHi infection significantly (P=0.004) upregulated CD107a expression from 5.7% CD107a+ at baseline to 17.3% with NTHi, indicating that MAIT cells were degranulating.

By comparison, in the MDM-T cell co-culture there was an increase in CD107a on CD4+, CD8+ and MAIT cells following NTHi infection (figure 6.1); for CD4+ 1.7% CD107a+ increasing to 2.5% (P=0.001), for CD8+ 2.8% CD107a+ increasing to 4.2% (P=0.002) and for MAIT cell 8.3% CD107a+ at baseline increasing to 19.8% (P=0.001). For all three subsets, PFA-NTHi did not significantly increase CD107a expression compared to uninfected and PFA-NTHi compared to NTHi was significant (all P=0.003), again indicating that live bacterial infection in this model is required for maximal responses. For all cases in the lung and co-culture, baseline expression of CD107a was not 0%, indicating that there is some level of degranulation at baseline. Overall, MAIT cells appear to have the strongest increase in degranulation following NTHi infection, indicating that they have cytotoxic function.

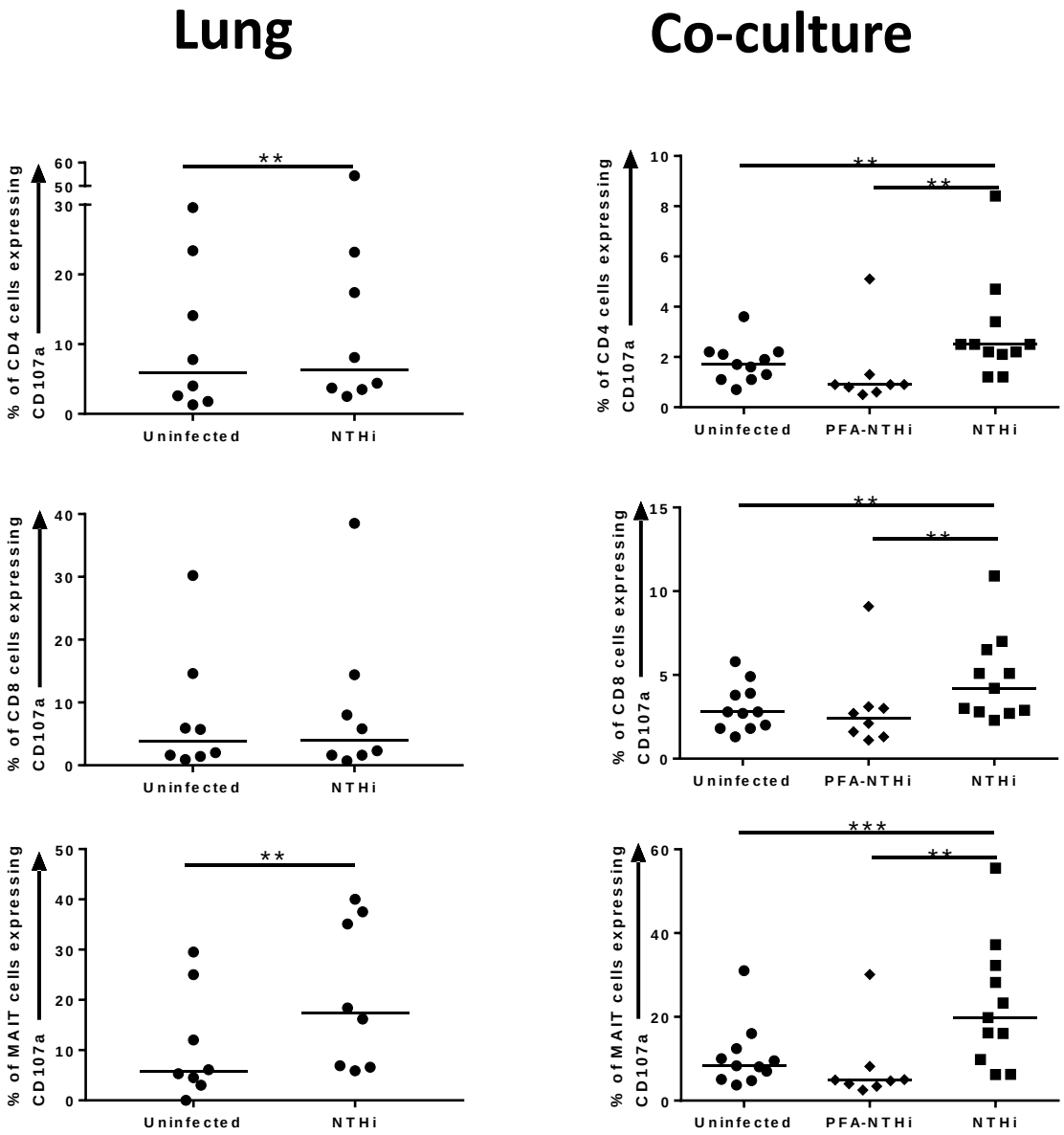


Figure 6.1 – Degranulation by T cells after NTHi infection as measured by CD107a expression. Expression of CD107a by CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. **P<0.01, ***P<0.001

6.2.2 The CD107a response of conventional T cells is antigen-specific

To determine whether the observed CD107a upregulation of CD4+ and CD8+ was the result of antigen presentation, co-cultures were repeated with either HLA-DR or HLA-ABC blocking antibodies respectively. All comparison are presented as isotype control antibody vs blocking antibody.

Blocking of HLA-DR significantly (P=0.01) reduced CD107a expression of CD4+ T cells from 2% CD107a+ to 1.4% (figure 6.2A), which brought CD107a below the uninfected baseline expression. Likewise, blocking of HLA-ABC impaired expression of CD107a in response to NTHi (figure 6.2A), reducing CD107a+ CD8+ T cells from 5% with isotype to 3% with the anti-HLA-ABC (P=0.008). Overall, these blocking data indicate that upregulation of CD107a on conventional T cells in response to NTHi infection is mainly driven by antigen presentation.

Blocking of HLA-ABC also reduced expression of CD107a on CD4+ T cells, which similar to the data presented in chapter 5, may indicate cross-talk between CD4+ and CD8+ T cells. Impairing antigen presentation by HLA-DR had no significant effect on CD8+ CD107a expression however (figure 6.2B).

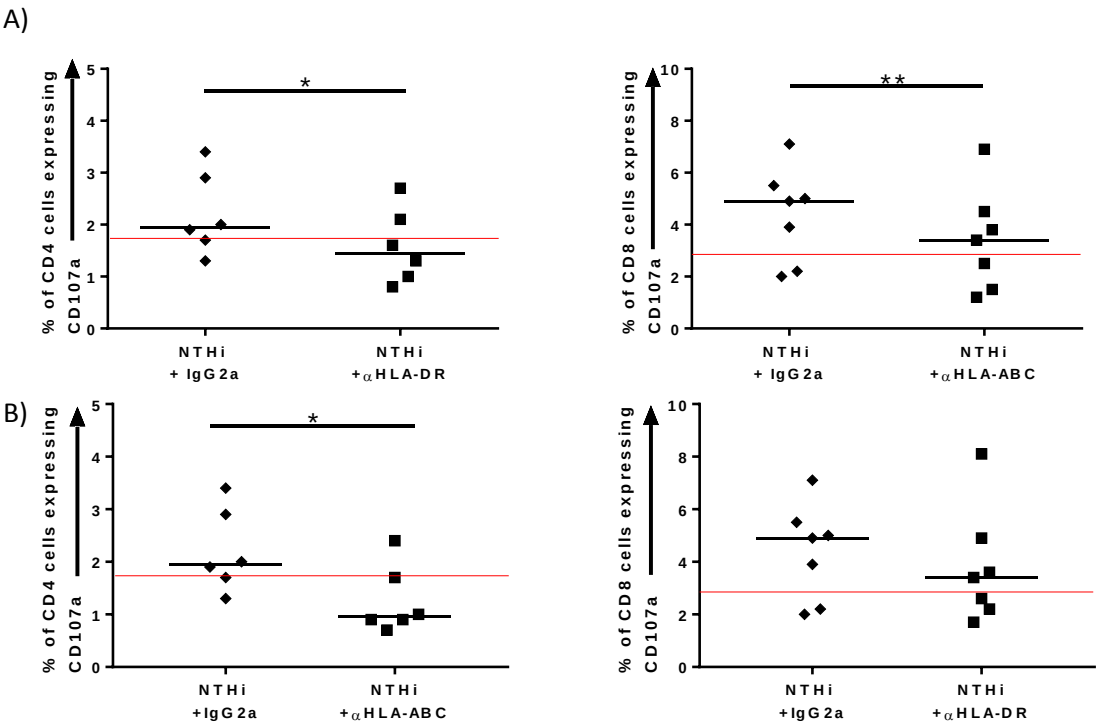


Figure 6.2 – Degranulation by conventional T cells is impaired by blocking antigen presentation. A) and B) Expression of CD107a by CD4+ and CD8+ cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-HLA-DR (L243), 10 µg/ml anti-HLA-ABC (W6/32) or IgG2a isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05 **P<0.01,

6.2.3 MAIT cell CD107a upregulation requires MR1 in a time-dependent manner

MDM-T cell co-cultures were next repeated in the presence of an MR1 blocking antibody to determine whether CD107a upregulation of MAIT cells required MR1 antigen presentation. In line with the results from chapter 5, co-cultures with the anti-MR1 antibody were performed for both 5 hours and 22 hours (figure 6.3).

In the shorter timecourse, blocking of MR1 significantly (P=0.03) reduced CD107a expression from 4.1% CD107a+ with the IgG2a isotype versus 2.6% CD107+ with anti-MR1, a reduction to near baseline. Blocking of MR1 for 22 hours on the other hand had no inhibitory effect, suggesting that at this later timepoint, CD107a expression was not regulated by antigen presentation and was driven by other factors. Similar to the IFNγ data from chapter 5, no significant increase in CD107a was seen in the CD4+ and CD8+ T cells at the 5 hour timepoint, again indicating that MAIT cells are activated in a faster manner.

Overall, in agreement with the previous IFNγ data, MAIT cell degranulation in response to NTHi infection appears to be antigen-specific in the first instance, but is driven by antigen-independent factors as the timecourse progresses.

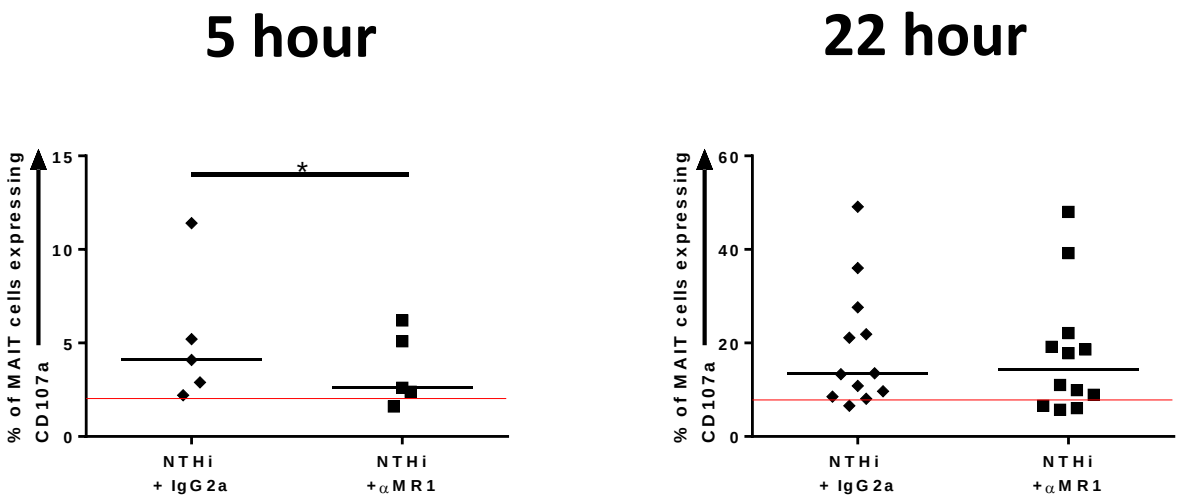


Figure 6.3 – Degranulation by MAIT cells is impaired by blocking antigen presentation in a time dependent manner. Expression of CD107a by MAIT cells from T cells co-cultured for 5 or 22 hours with autologous MDM infected with NTHi at a MOI of 10 in the presence of 5 μg/ml anti-MR1 (26.5) or IgG2a isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.4 Blocking of PDL1 and CTLA4 do not affect CD107a upregulation

Having investigated the effect of antigen presentation pathways on CD107a upregulation, the next step was to determine whether the co-inhibitory molecules PD1 or CTLA4 played any role in the regulation of CD107a. In the previous chapter, it was shown that CTLA4 was upregulated on all three T cell subsets following infection, whereas PD1 was only elevated on MAIT cells. Blocking of these pathways had no effect on IFNγ production however. If either of these pathways are involved in regulating degranulation, inhibiting them should result in a greater expression of CD107a.

Following co-culture of NTHi-infected MDM and T cells in the presence of the anti-PDL1 antibody, no significant increase in CD107a expression was observed in any T cell subset compared to IgG1 isotype (figure 6.4). Furthermore, to account for PDL2 signalling to PD1, blocking of PD1 was also performed but did not affect CD107a expression. This lack of inhibitory effect extended to CTLA4 as well, as CTLA4 blocking also had no effect on degranulation of any T cell subset (figure 6.5). Together, these data indicate that the PDL1/2-PD1 and CTLA4 pathways do not regulate CD107a expression in this model.

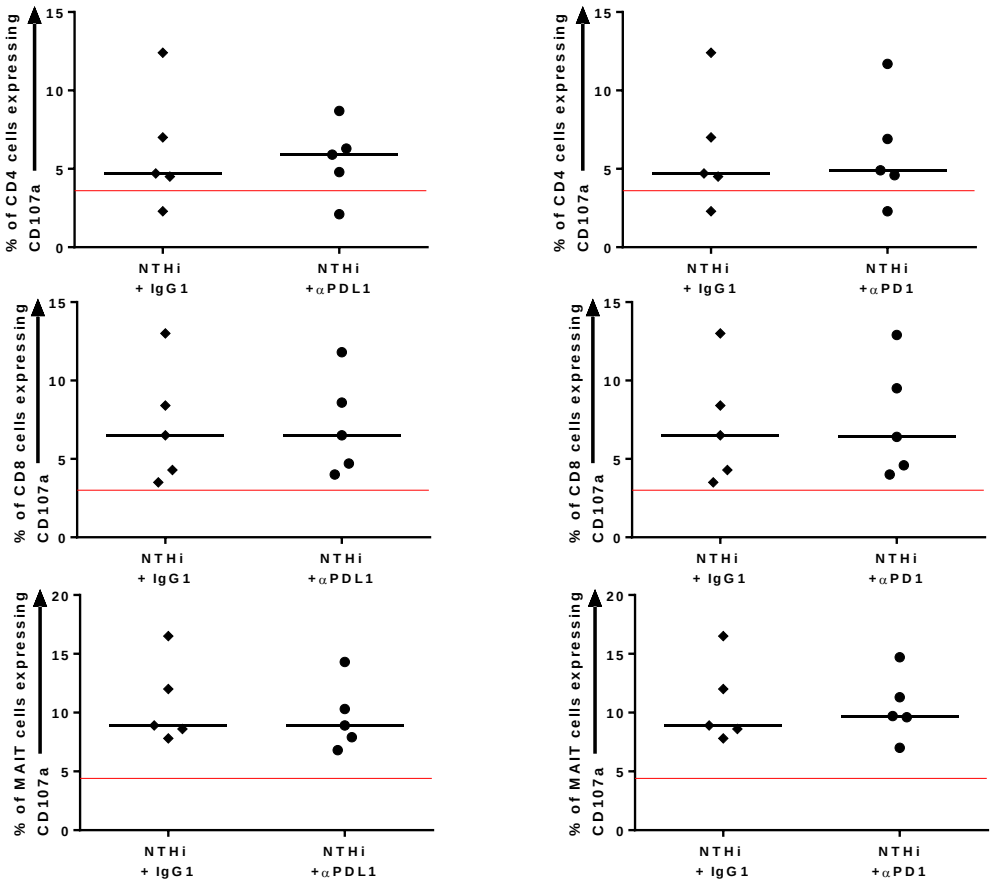


Figure 6.4 – Degranulation is not affected by blocking PDL1 or PD1. Expression of CD107a by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-PDL1 (MIH1), PD1 (EH12.2H7) or IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test.

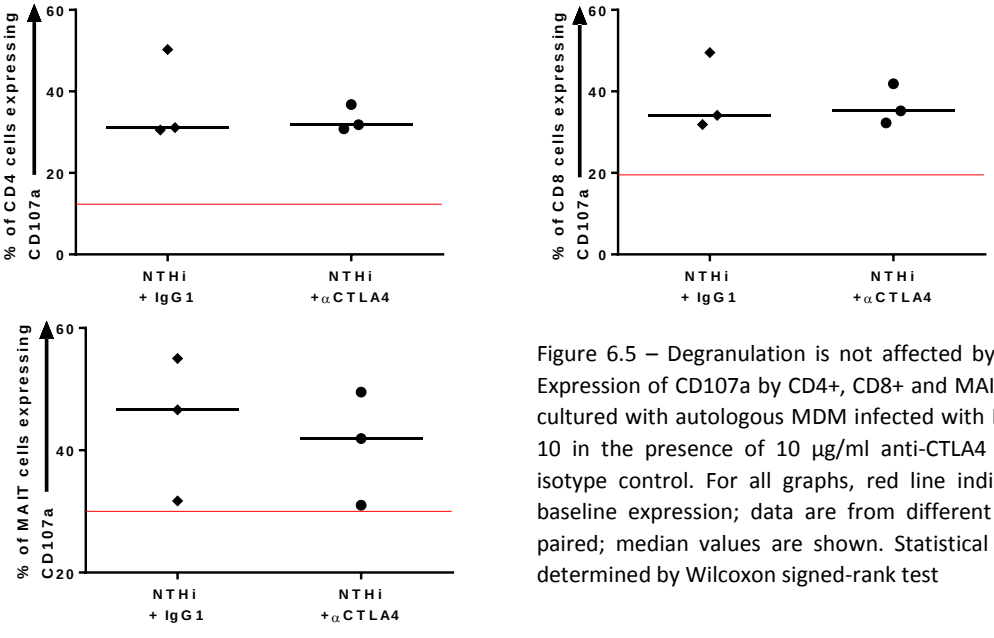


Figure 6.5 – Degranulation is not affected by blocking CTLA4. Expression of CD107a by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-CTLA4 (L3D10) or IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test

6.2.5 Cell contact is required for conventional T cell CD107a upregulation

To further investigate whether other factors independent of antigen presentation and cell contact could regulate degranulation, CD107a expression was measured on T cells in the 0.4 μm transwell model. Similar to the previous chapter, CD107a expression was not upregulated on conventional CD4+ T cells in the transwell setup. Likewise, no significant increase in CD107a was detected on CD8+ T cell either, indicating that degranulation of conventional T cells requires antigen presentation and physical contact between MDM and T cells.

However, for MAIT cells, a minor but significant (P=0.03) 2-fold increase in CD107a expression was detected in the transwell model, although this increase did not reach the maximal upregulation seen when NTHi-infected MDM and T cells were in direct contact (figure 6.6). Overall this would suggest that degranulation for both conventional and MAIT cells is predominately contact dependent, but MAIT cells have the capacity to undergo minor degranulation in response to non-contact factors.

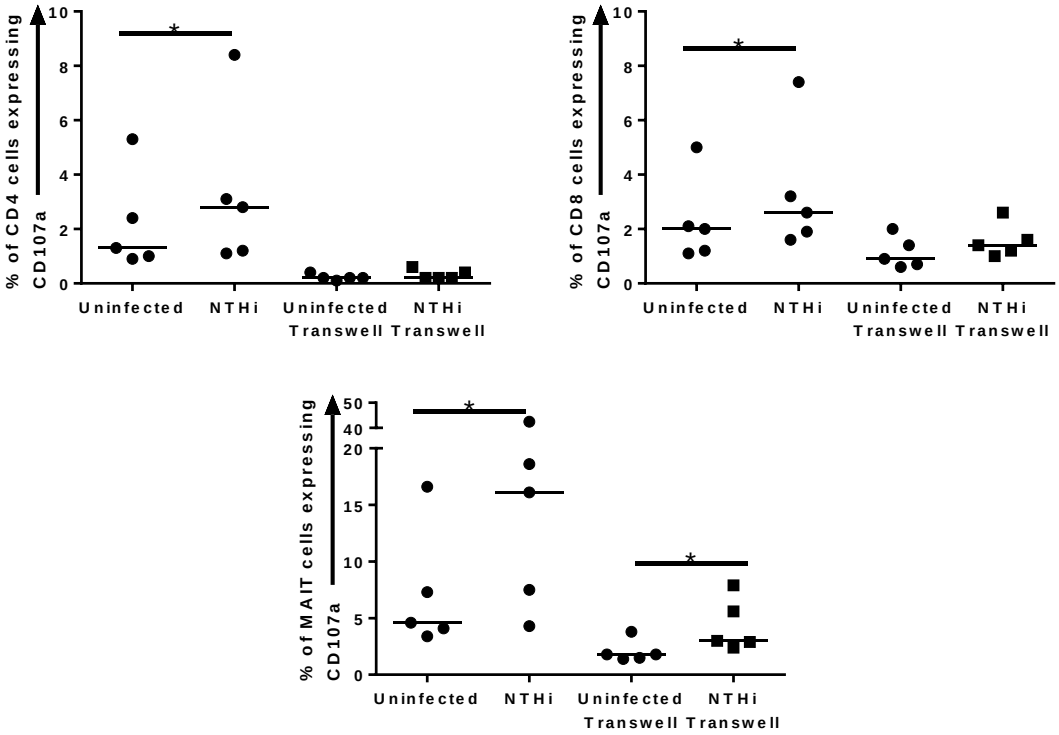


Figure 6.6 – Effect of contact-independent factors on degranulation. Expression of CD107a by CD4+, CD8+ and MAIT cells from T cells co-cultured in direct contact or in transwells with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.6 IL-12 does not regulate CD107a upregulation

As shown in chapter 5, NTHi-infected MDM produce active IL-12 protein which was required for IFNγ production in response to NTHi infection. To address whether IL-12 signalling controlled conventional T cell or MAIT cell degranulation, co-cultures were next repeated in the presence of the anti-IL-12p40 antibody. However, in all three cases, impairing IL-12 signalling did not impact NTHi-induced CD107a upregulation of conventional T cells or MAIT cells (figure 6.7). Furthermore, addition of IL-12 to either T cells alone or to the co-culture did not impact CD107a expression (figure 6.8), indicating that IL-12 plays no role in degranulation.

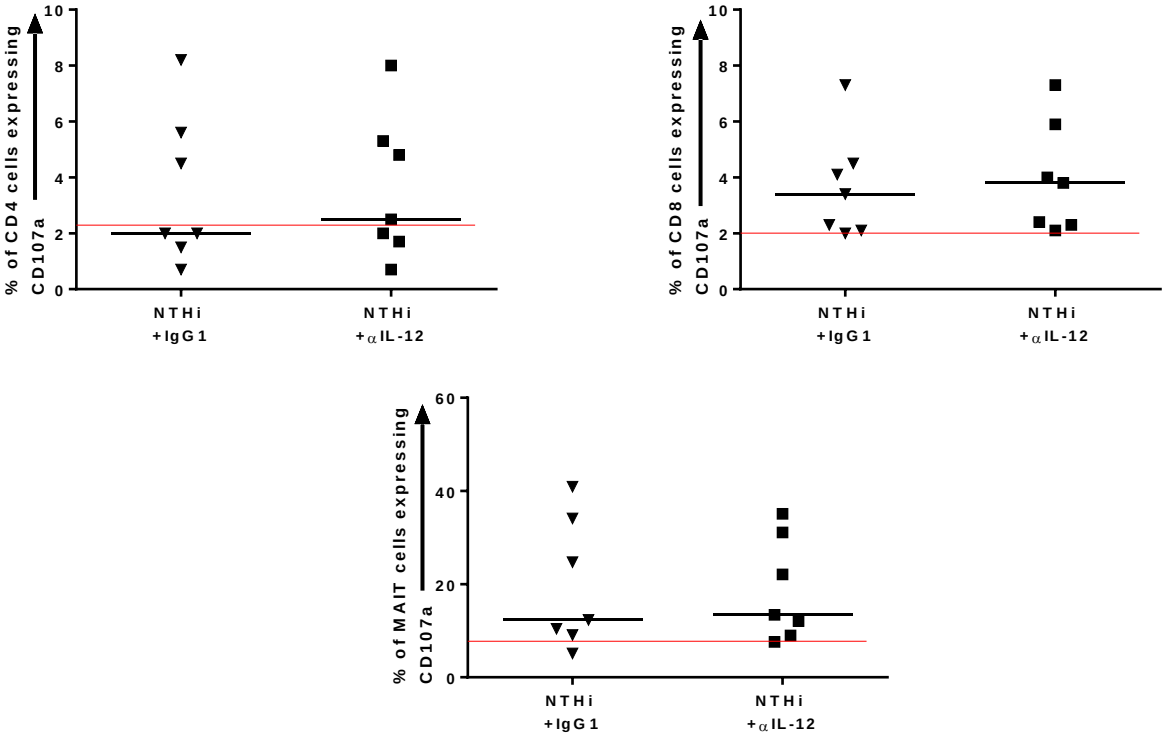


Figure 6.7 – Degranulation by T cells is not impaired by blocking IL-12. Expression of CD107a by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-IL-12 (C8.6), IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test.

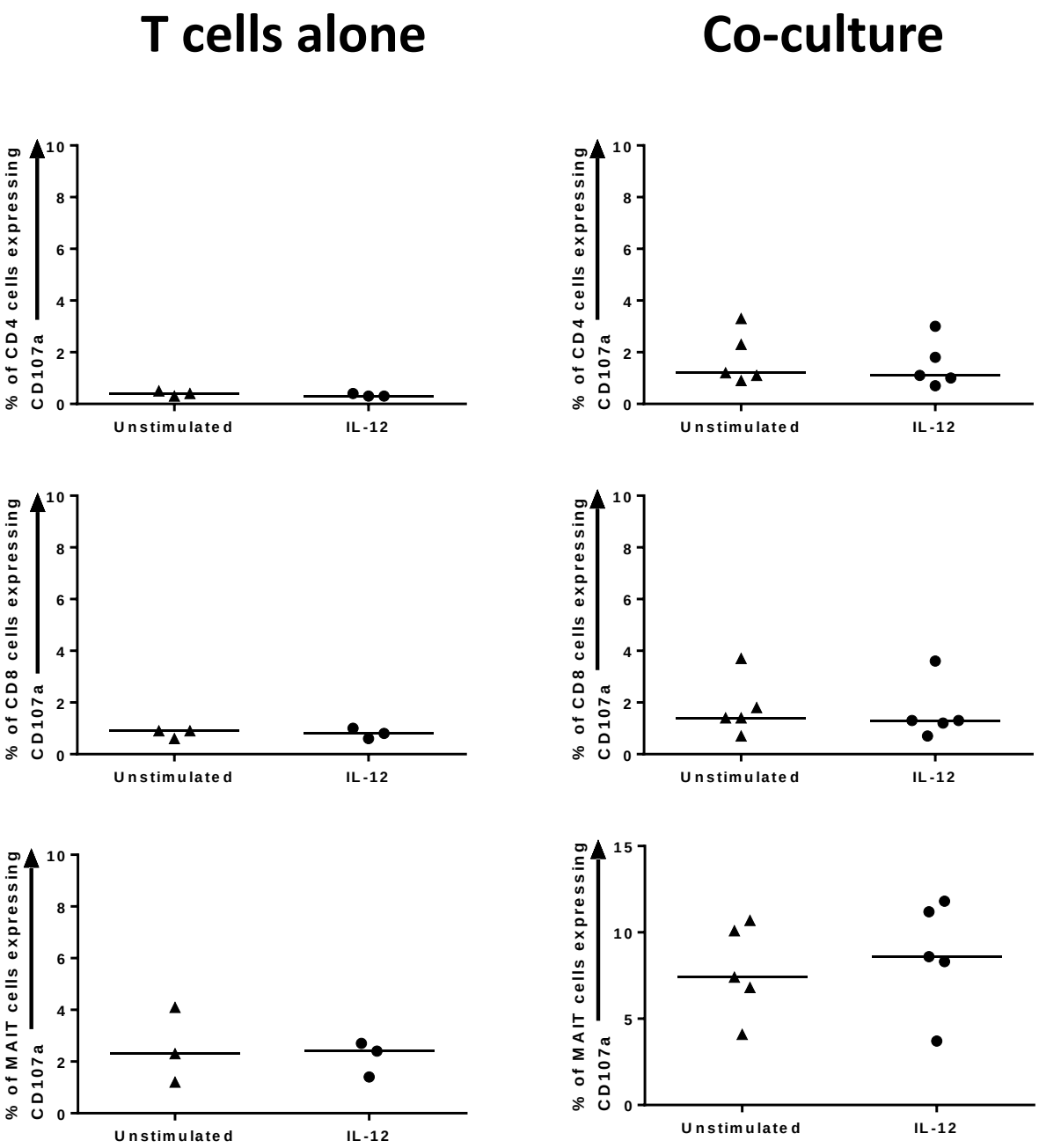


Figure 6.8 – Degranulation by T cells is not induced by IL-12. Expression of CD107a by CD4+, CD8+ and MAIT cells from either T cells alone or co-cultures of T cells and autologous MDM stimulated with IL-12 (10 ng/ml). For all graphs; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test.

6.2.7 Steroids inhibit upregulation of CD107a

To determine what effect corticosteroids had on conventional T cell and MAIT cell degranulation, NTHi-infected MDM-T cell co-cultures were repeated as before, with the addition of either 100 nM fluticasone propionate (FP) or DMSO to the co-culture, as described in chapter 5.

NTHi-induced degranulation of conventional T cells was significantly (both P=0.01) impaired by FP; CD4+ DMSO control 3.7% vs FP 2.2% CD107a+, CD8+ DMSO control 8.4% vs 4.8% CD107a+. Upregulation of CD107a by MAIT cells was also significantly (P=0.01) inhibited (DMSO control 13.2% vs FP 10.8% CD107a+) following NTHi-infection, almost reduced to baseline (figure 6.9). Taken together, corticosteroids appear to impair the ability of conventional T cells and MAIT cells to degranulate, which may impact the ability of these T cell to mount cytotoxic responses to NTHi infection.

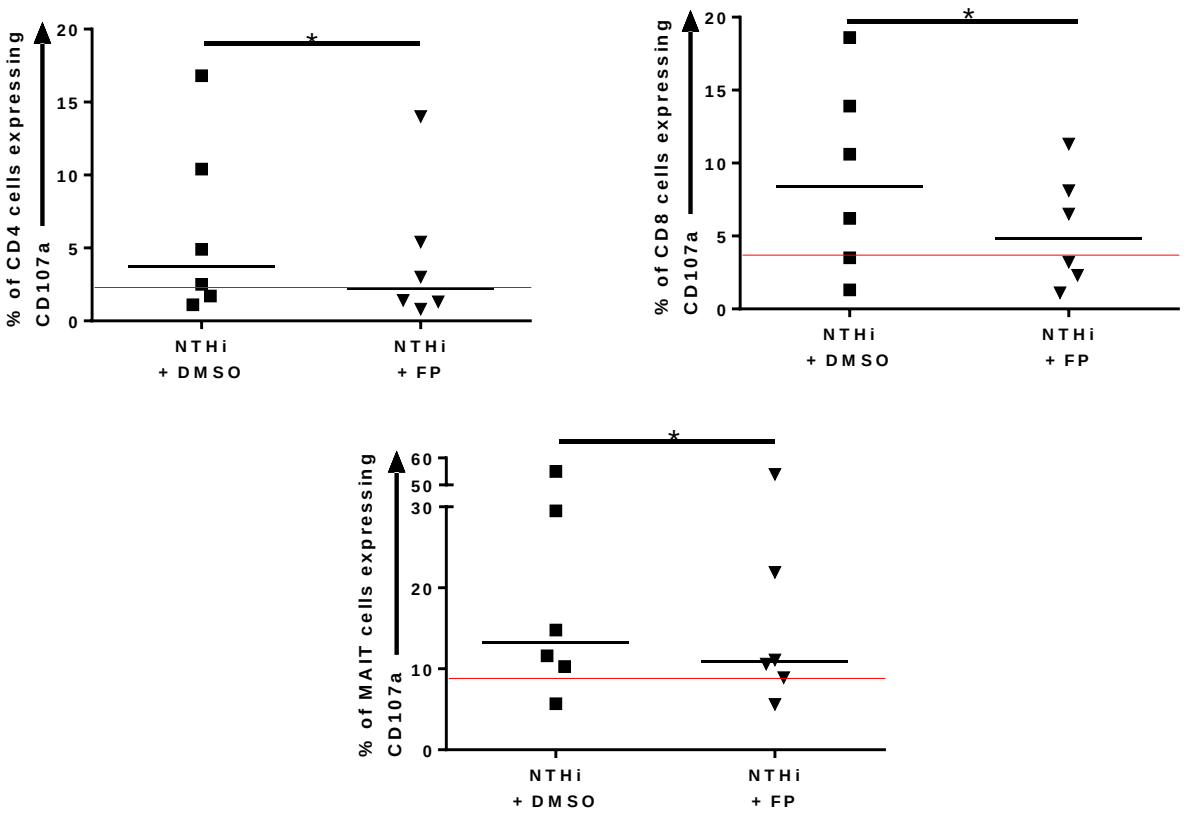


Figure 6.9 – Degranulation is impaired by corticosteroids. Expression of CD107a by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 100 nM fluticasone propionate (FP) or DMSO. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.8 Perforin is expressed by conventional T cells and MAIT cells

Having studied whether CD107a expression was affected by NTHi infection, the next step was to investigate whether cytotoxic effector molecules of conventional T cells and MAIT cells were regulated in response to NTHi. One of the key cytotoxic mediators released by degranulating cytotoxic cells is perforin, which is a membrane-perturbing protein that is thought to form pores in target cells, allowing delivery and trafficking of other cytotoxic proteins such as granzyme B (Lieberman, 2003)(Osińska et al., 2014)(Voskoboinik et al., 2015). Although perforin expression of T cells in response to NTHi is not known, T cells from blood and BAL T cells from COPD patients have been shown to have higher expression of perforin compared to healthy controls (Hodge et al., 2006). Expression of perforin was therefore analysed on conventional T cells or MAIT cells from NTHi-infected co-cultures.

As shown in figure 6.10, CD4+ T cells from the co-culture had very low levels of perforin at baseline, which were significantly (P=0.03) upregulated following NTHi infection; 0.5% perforin+ at baseline vs 2.1% following NTHi infection. Conventional CD8+ T cells from the co-culture had a higher baseline expression of perforin, with 23% of CD8+ T cell perforin+, which significantly (P=0.03) increased to 32% in response to NTHi infection. On the other hand, 70% of MAIT cells expressed perforin at baseline, (3-fold higher than CD8+ T cells) but this was not upregulated further with NTHi infection. As MAIT cells did not upregulate perforin, the role of antigen presentation, IL-12 signalling or steroids could not be investigated. For this reason, perforin was not considered any further.

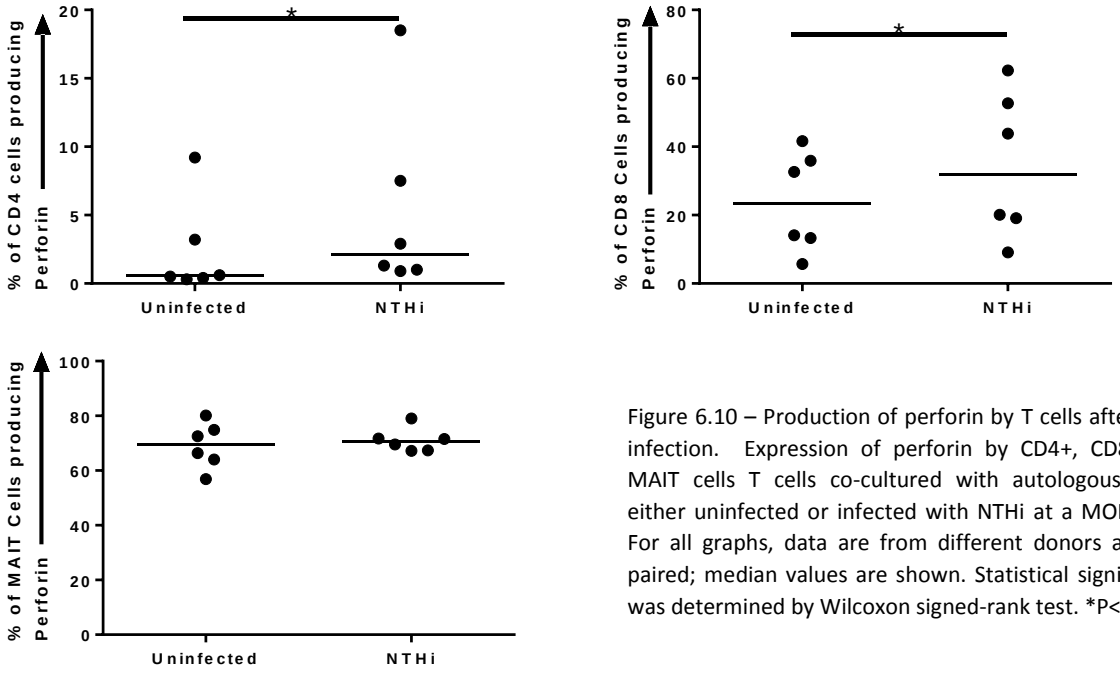


Figure 6.10 – Production of perforin by T cells after NTHi infection. Expression of perforin by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.9 Expression of granzyme B by conventional T cells and MAIT cells is upregulated following NTHi infection

Another key cytotoxic mediator contained within cytotoxic vesicles is granzyme B, which is one of a family of serine proteases that cause DNA damage, caspase activation and mitochondrial leakage, all of which result in apoptosis of the target cell (Cullen et al., 2010)(Lieberman, 2003)(Broere et al., 2011). Recent reports have shown that MAIT cells upregulate granzyme B following activation (Kurioka et al., 2015), but this has not been shown in the lung nor in response to respiratory pathogens. To further explore the cytotoxic response to of conventional T cells and MAIT cells to NTHi, expression of granzyme B was measured from lung explants or T cells co-cultured with NTHi-infected MDM.

Following NTHi infection, a statistically significant (P=0.009) increase in expression of granzyme B was detected in lung CD8+ T cells, although as this was very minor, it may not be biologically significant (figure 6.11). A 2-fold upregulation (P=0.003) of granzyme B was also detected in lung CD4+ T cells in response to NTHi infection, indicating that they too have cytotoxic function (figure 6.11). Furthermore, MAIT cells upregulated granzyme B expression by 3-fold (P=0.002), with an increase from 3.7% granzyme B+ to 11%. Overall, these data show that all three lung T cell subsets manifest a granzyme B response to NTHi, but MAIT cells displayed the greatest induction.

Similar responses were observed in the co-culture model, where upregulation of granzyme B occurred in all three subsets (figure 6.11). However, whilst the granzyme B expression of CD4+ T cells from the co-culture was upregulated again by 2-fold (P=0.0005), the magnitude of the CD8+ and MAIT cell granzyme B response was much greater in the co-culture compared to the lung explant. CD8+ T cells from the co-culture had a higher baseline of granzyme B expression compared to the lung, which was upregulated by 2-fold following NTHi infection; 22% vs 41% (P=0.0005). Significant upregulation of granzyme B (uninfected 27.3% vs NTHi 65.4%; P=0.0005) was also detected in MAIT cells in response to NTHi infection, a larger response compared to MAIT cells from the lung, albeit a similar fold induction. In all three cases, PFA-NTHi did not induce granzyme B expression, again highlighting that this model requires live bacterial infection for maximal responses. To confirm that upregulation of granzyme B detected by flow cytometry resulted in actual release of protein, lung explant supernatant and MDM-T cell co-culture supernatant were measured for granzyme B by ELISA. NTHi infection was found to cause a 2-fold (P=0.007) and 5-fold (P=0.01) induction of granzyme B release respectively (figure 6.12).

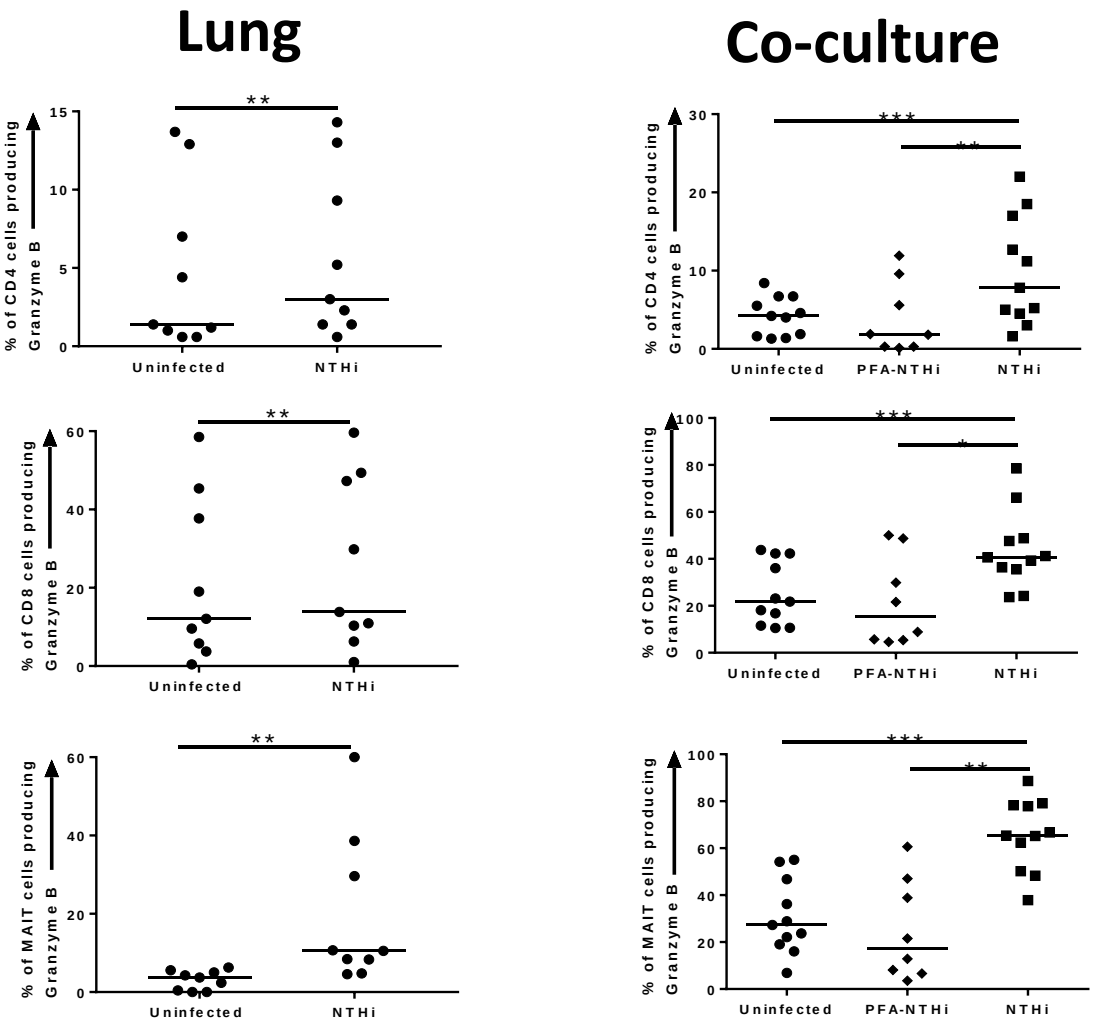


Figure 6.11 – Production of granzyme B by T cells after NTHi infection. Expression of granzyme B by CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi or PFA-NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01, ***P<0.001

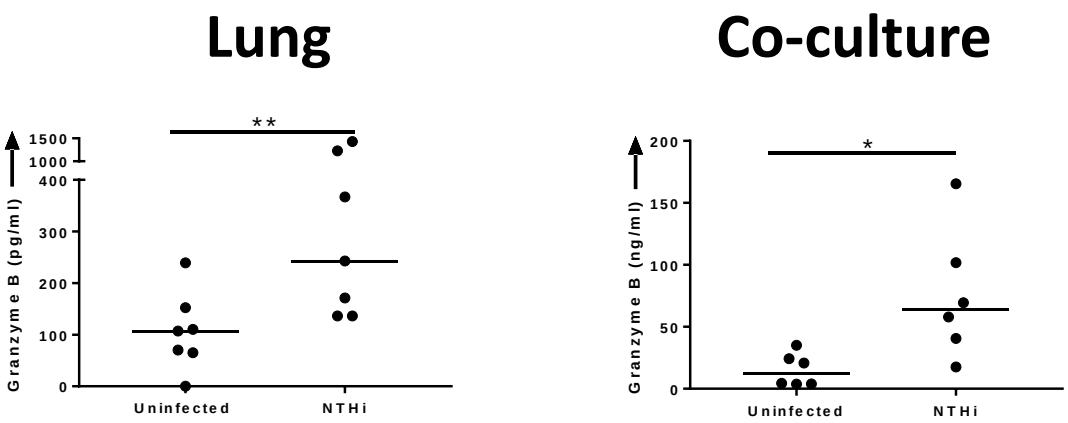


Figure 6.12 – Release of granzyme B after NTHi infection. Granzyme B production measured by ELISA of lung explants either uninfected or infected with $5x10^6$ CFU NTHi or blood T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05, **P<0.01

6.2.10 NTHi-induced upregulation of granzyme B by conventional T cells is inhibited by blocking antigen presentation

Blocking of HLA-DR and HLA-ABC were next performed to determine whether the NTHi-induced granzyme B responses of CD4+ and CD8+ T cells were antigen-dependent. All comparison are presented as isotype control antibody vs blocking antibody.

For both CD4+ and CD8+ T cells, addition of the anti-HLA-DR and anti-HLA-ABC antibodies respectively significantly reduced granzyme B expression back to baseline (both P=0.008, IgG2a isotype vs blocking antibody) (figures 6.13A). The decrease to baseline suggests that antigen presentation is the main factor driving the conventional T cell cytotoxic response. Furthermore, cross communication between conventional T cells appeared to be occurring, as blocking of HLA-ABC and HLA-DR also impacted granzyme B expression of CD4+ and CD8+ T cell respectively (figure 6.13B). This would suggest that if HLA-DR cannot present antigen to CD4+ T cells, not only do the CD4+ cells not upregulate cytotoxic markers as they are not activated, they also cannot provide co-stimulatory help to the CD8+ T cells. Whether that help is in the form of physical co-stimulation or cytokine stimulation is unknown and would require further work.

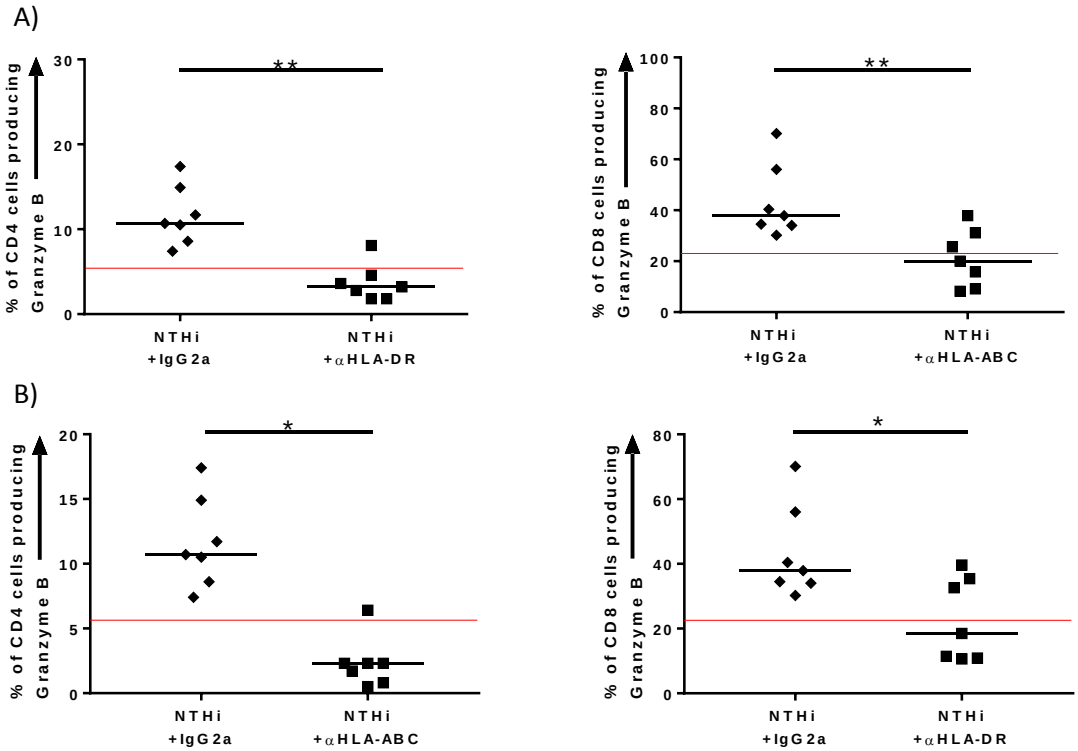


Figure 6.13 – Production of granzyme B by conventional T cells is impaired by blocking antigen presentation. A) and B) Expression of granzyme B by CD4+ and CD8+ cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-HLA-DR (L243), 10 µg/ml anti-HLA-ABC (W6/32) or IgG2a isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05 **P<0.01

6.2.11 MAIT cell granzyme B upregulation requires MR1 in a time-dependent manner

For the MAIT cell granzyme B response, MR1 blocking experiments were performed for both 5 and 22 hours as before to establish whether granzyme B was also regulated by MR1 antigen presentation in a time-dependent manner.

In contrast to the IFNγ and CD107a data, blocking of MR1 in the 5 hour co-culture with anti-MR1 antibody did not inhibit granzyme B upregulation, despite granzyme B increasing from 3% granzyme B+ in the uninfected to 17% with NTHi infection (figure 6.14). However, there was a statistically significant (P=0.01) inhibition in granzyme B upregulation in the 22 hour co-culture, from 74% granzyme B+ with the IgG2a isotype to 67% with the anti-MR1 antibody, although this reduction was only minor (so may not be biologically relevant) and was not back to baseline (figure 6.14). Similar to CD107a, no significant increase in granzyme B was seen in either CD4+ or CD8+ T cells in the 5 hour co-culture, in contrast to the MAIT cells, which again indicates that MAIT cells are early responders to bacterial infection.

Together, these data confirm the previous findings that MAIT cell activation requires MR1 and antigen presentation in a time-dependent manner. As MR1 blocking did not reduce granzyme B back to baseline at 22 hours of co-culture, this would suggest that other MR1-independent factors were also responsible for controlling the MAIT cell granzyme B response.

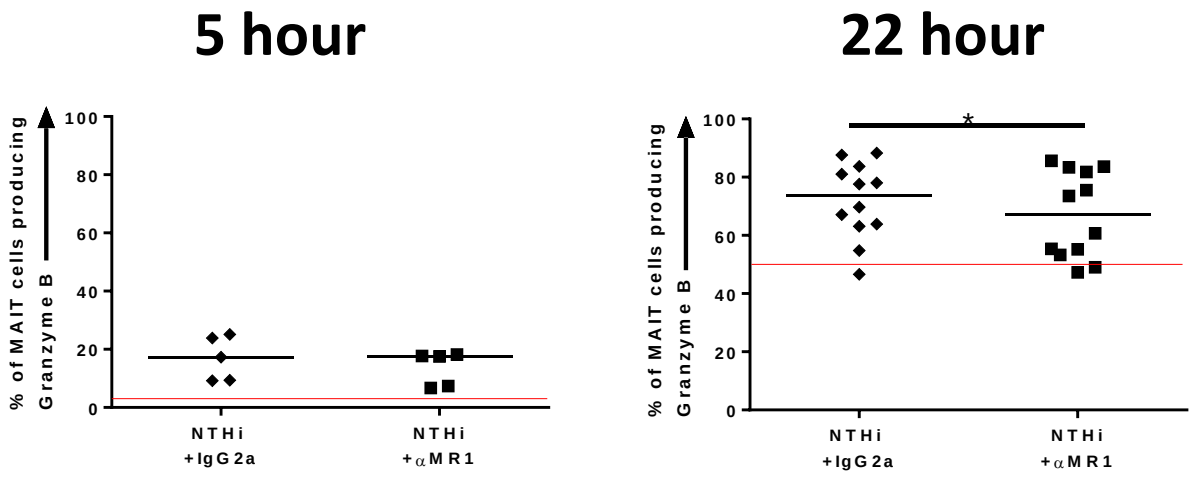


Figure 6.14 – Production of granzyme B by MAIT cells is impaired by blocking antigen presentation in a time dependent manner. Expression of granzyme B by MAIT cells from T cells co-cultured for 5 or 22 hours with autologous MDM infected with NTHi at a MOI of 10 in the presence of 5 μg/ml anti-MR1 (26.5) or IgG2a isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.12 Co-inhibitory molecules differentially regulate conventional T cell and MAIT cell granzyme B expression

In addition to the role of antigen presentation, the effects of blocking the co-inhibitory PDL/PD1 and CTLA4 pathways on granzyme B expression were next explored in the co-culture model. In contrast to the previous IFNγ and CD107a data, blocking of PDL1, which is expressed on MDM, significantly increased expression of granzyme B by both conventional CD4+ and CD8+ T cell. Expression of CD4+ granzyme B was increased by 1.7-fold (P=0.03) from 12.8% granzyme B+ with IgG1 isotype to 21.5% with anti-PDL1 antibody. For CD8+ T cells, granzyme B expression was significantly (P=0.03) upregulated from 53.2% to 65.2% with the anti-PDL1 antibody (figure 6.15). Blocking of PD1 on the other hand induced little to no increase in CD4+ and CD8+ T cell granzyme B, despite statistical significance being reported (figure 6.15). In complete contrast, blocking of either PDL1 or PD1 did not affect MAIT cell granzyme B expression. Together with the previous data, the PDL1/PD1 pathway not only differentially regulates cytokine and cytotoxic responses from conventional T cells, but also plays different roles with innate and adaptive T cells. Consistent with the previous IFNγ and CD107a data, blocking of CTLA4 had no effect on conventional T or MAIT cell granzyme B (figure 6.16).

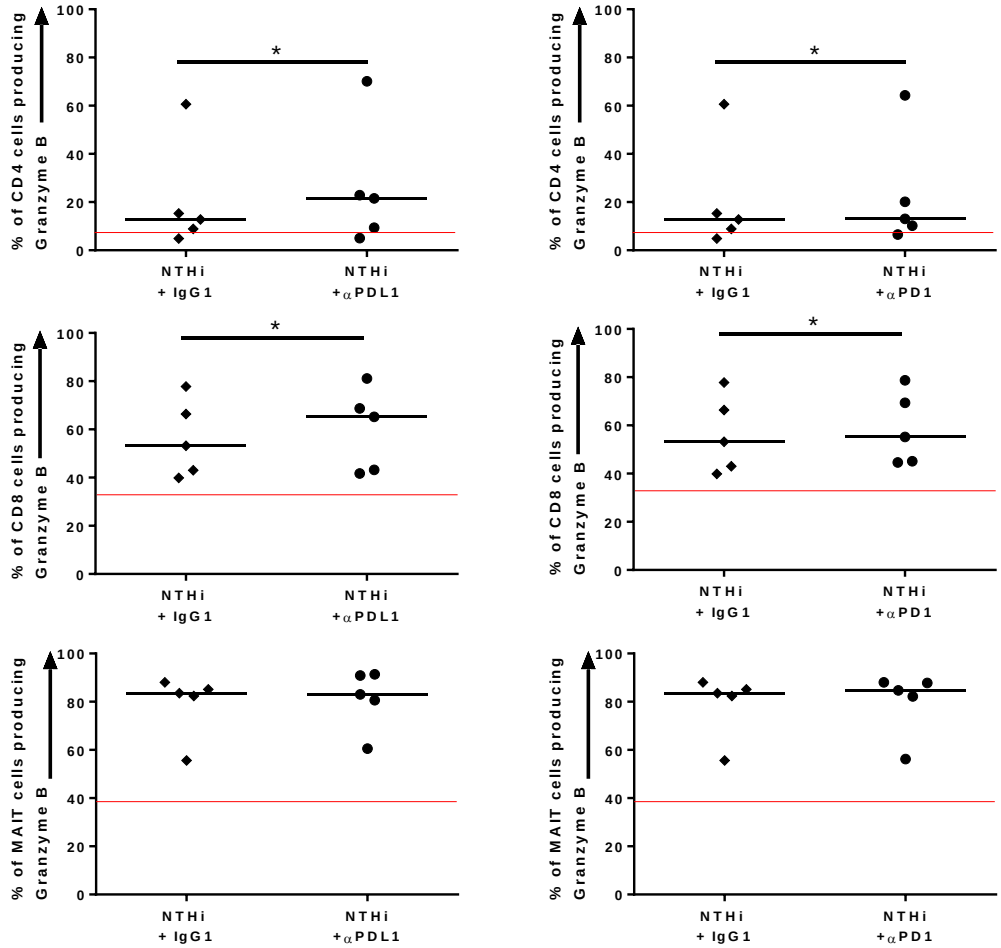


Figure 6.15 – Production of granzyme B is affected by blocking PDL1 or PD1. Expression of granzyme B by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-PDL1 (MIH1), PD1 (EH12.2H7) or IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

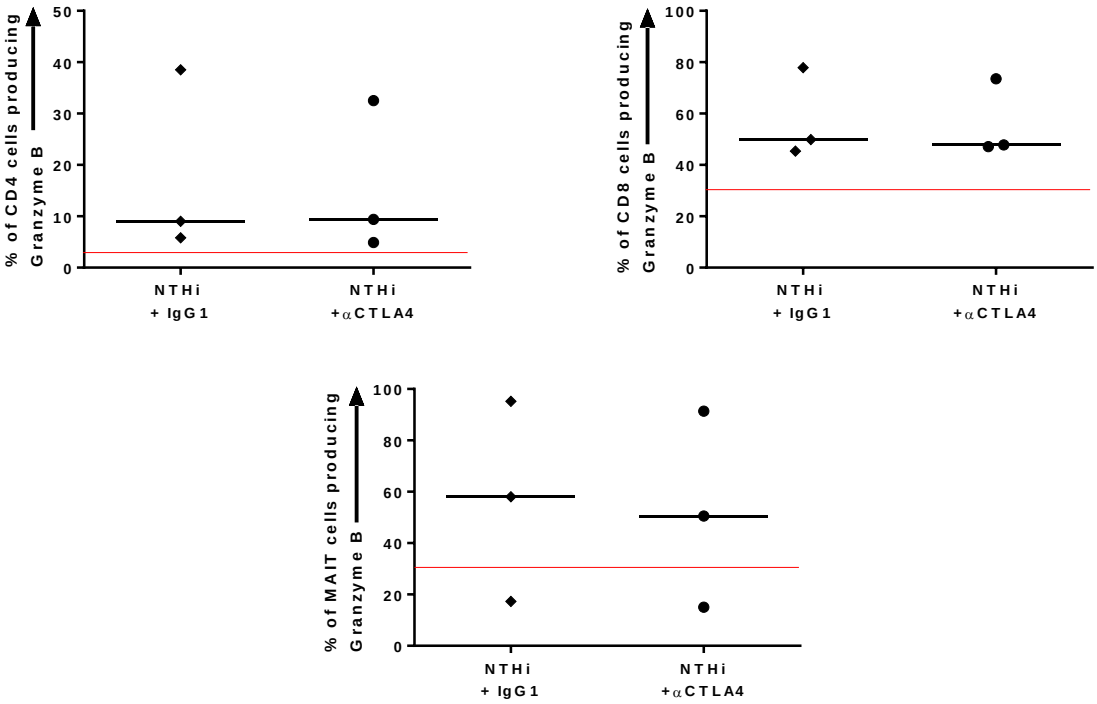


Figure 6.16 – Production of granzyme B is not affected by blocking CTLA4. Expression of granzyme B by CD4+, CD8+ and MAIT cells T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of 10 µg/ml anti-CTLA4 (L3D10) or IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test

6.2.13 Contact is required for conventional T cell but not MAIT cell granzyme B upregulation

Utilising the 0.4 µm transwell model to investigate the role of contact in driving granzyme B expression, a statistically significant upregulation (P=0.03) of granzyme B was observed in CD8+ T cells, although this upregulation was very minor and therefore may not be biologically relevant. No increase was observed with CD4+ T cells in the transwells (figure 6.17). Together this indicates that the granzyme B response of conventional T cells is largely dependent on antigen presentation and contact, similar to the response seen with CD107a.

MAIT cells in transwells still underwent a large upregulation of granzyme B (P=0.03; figure 6.17), although again this expression did not reach the maximal response as seen with direct contact. In combination with the MR1 blocking data, it is demonstrated here that MAIT cells can upregulate granzyme B in the absence of physical contact and antigen presentation, again indicating that soluble factors such as cytokines may play an important role in MAIT cell activation during infections.

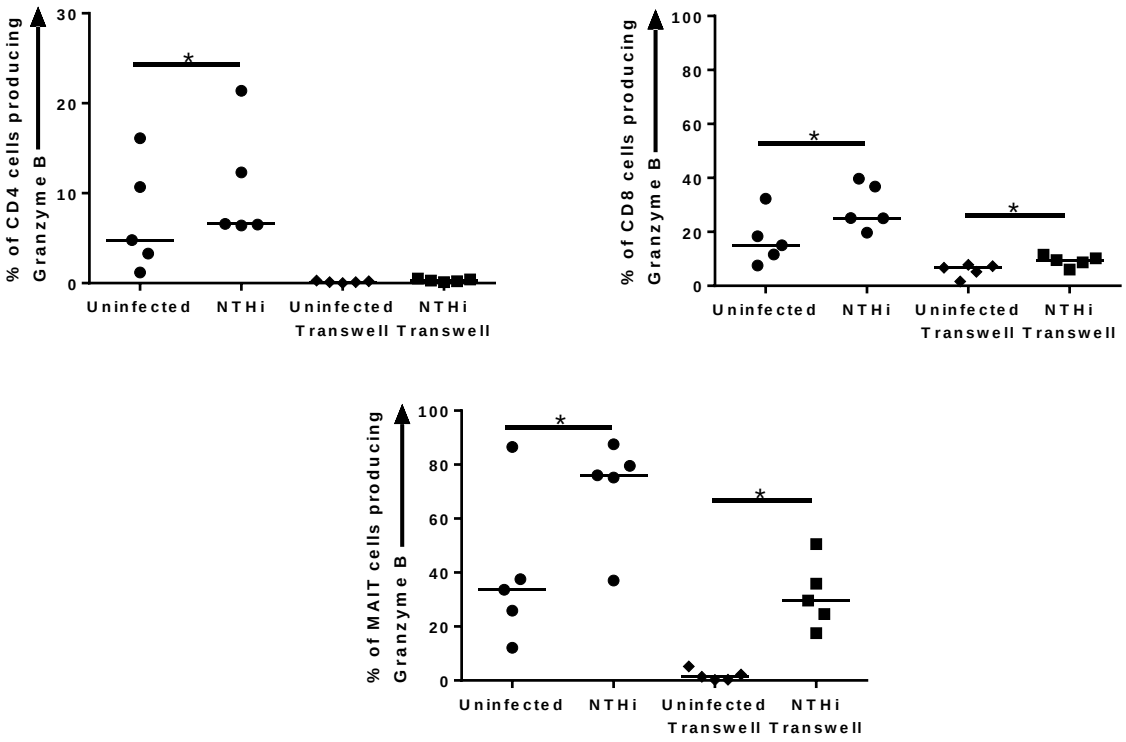


Figure 6.17 – Effect of contact-independent factors on granzyme B. Expression of granzyme B by CD4+, CD8+ and MAIT cells from T cells co-cultured in direct contact or in transwells with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.14 Blocking of IL-12 inhibits granzyme B expression

To next determine whether granzyme B expression requires IL-12 for induction in this model, NTHi-infected MDM-T cell co-cultures were repeated in the presence of the anti-IL-12p40 capture antibody. As seen in figure 6.18, impairing IL-12 signalling inhibited conventional T cell granzyme B expression (both P=0.01). However, the transwell data showed that in the absence of physical contact, CD4+ and CD8+ granzyme B upregulation was either non-existent or minimal respectively. In addition, blocking of MHC molecules reduced granzyme B expression to baseline for both CD4+ and CD8+ T cells. Taken together, it seems that whilst IL-12 signalling is involved in the conventional T cell granzyme B response, contact and antigen presentation are an absolute requirement.

Previous studies have shown that IL-12 played a role in MAIT cell granzyme B expression in response to *E.coli* infection (Kurioka et al., 2015), so it seemed likely that, given IL-12 was produced by NTHi-infected MDM, IL-12 would be also involved in the MAIT cell granzyme B response to NTHi. In agreement with this, the IL-12 blocking antibody caused a small but significant (P=0.01) impairment in granzyme B upregulation in response to NTHi infection (figure 6.18). As both MR1 and IL-12 blocking individually could not reduce MAIT cell granzyme B to baseline, it was possible that both of these pathways were independent of each another and combine together to induce a maximal granzyme B response in MAIT cells. Indeed, combined blocking of both MR1 and IL-12 together further impaired (P=0.03) MAIT cell granzyme B expression to baseline (figure 6.18). Overall, in combination with the MR1 blocking data, the mechanisms of MAIT granzyme B upregulation in response to NTHi are time dependent, with both MR1-dependent and MR1-independent, IL-12 dependent pathways. In contrast to the conventional T cell, the MAIT cell granzyme B response can still be activated in the absence of antigen presentation and cell contact, suggesting a potentially important role of MAIT cells in bystander activation driven by cytokines.

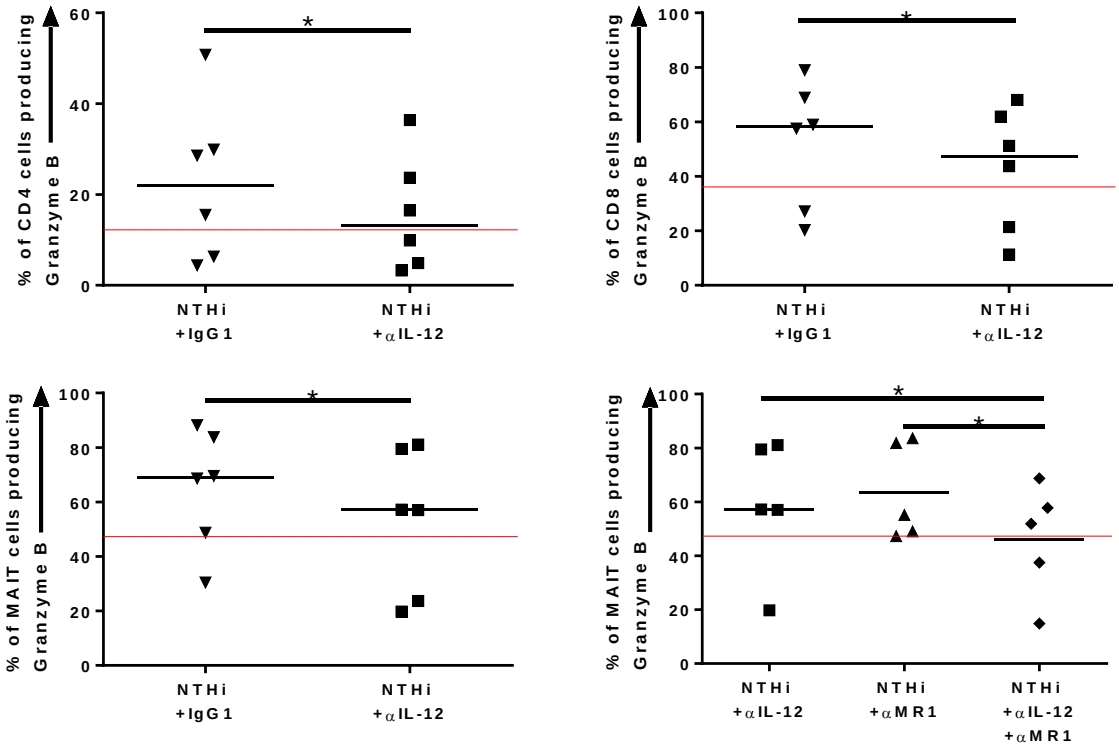


Figure 6.18 – Production of granzyme B by T cells is impaired by blocking IL-12. Expression of granzyme B by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-IL-12 (C8.6), IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.15 IL-12 stimulation can induce MAIT cell granzyme B expression in the absence of antigen presentation

To explore the role of IL-12 further, T cells by themselves (without MDM) or the co-culture of MDM and T cells were stimulated with IL-12 and granzyme B expression of conventional T cells and MAIT cells was analysed (figure 6.19). Stimulation of just T cells alone with IL-12 did not cause an increase in granzyme B expression of CD4+ or CD8+ T cells. When the co-culture of T cells and MDM together was stimulated with IL-12, a very minor increase in granzyme B was detected in conventional T cells (CD4+ 3.5% vs 4.6%, CD8+ 12.8% vs 14.8%), indicating that the presence of MDM was having some effect, although as these inductions were so minor they may not be biologically relevant, despite being statistically significant. In contrast to the conventional T cells, IL-12 stimulation of just T cells lead to a minor increase in MAIT cell granzyme B (1.3% vs 3.1%, P=0.004). However, a much larger increase (P=0.03) in MAIT cell granzyme B was observed in the IL-12 stimulated co-culture, approaching the same extent as with NTHi infection.

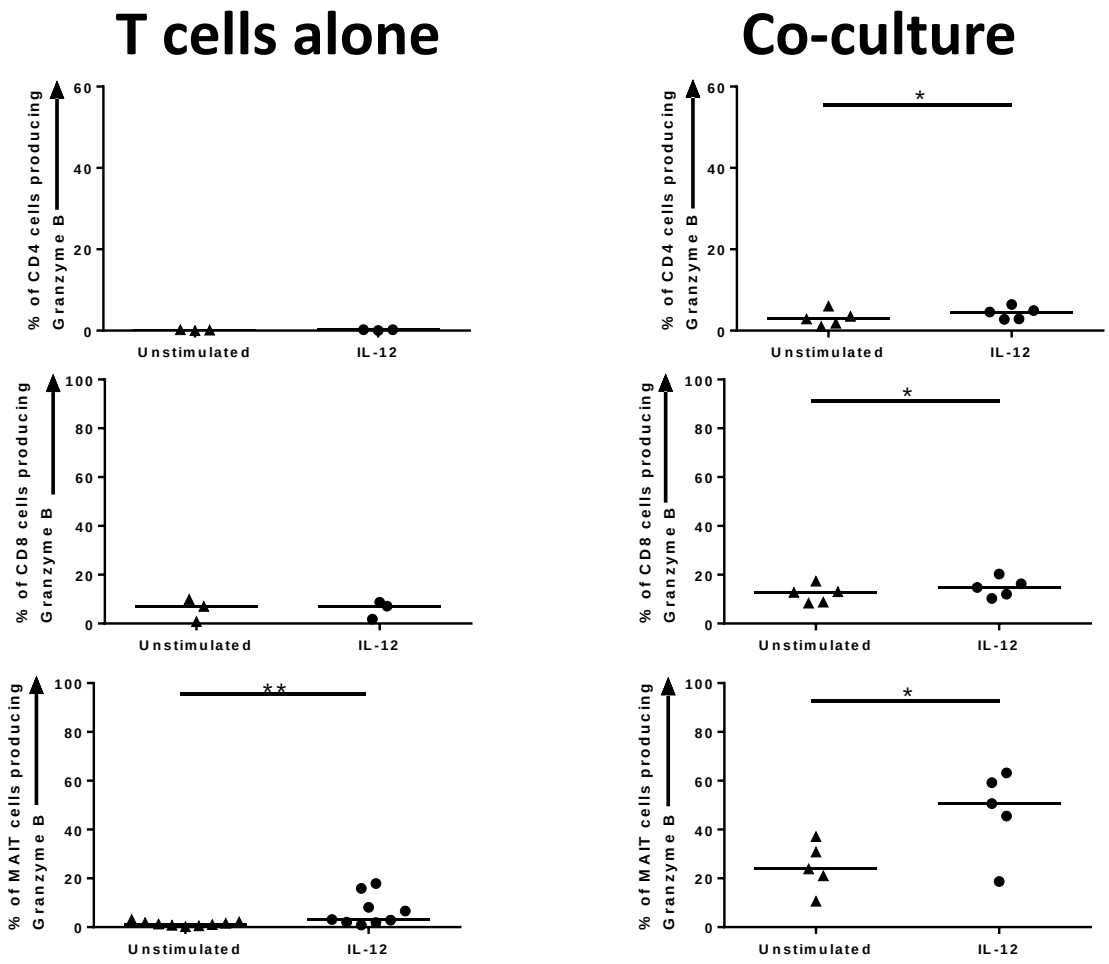


Figure 6.19 – Production of granzyme B by T cells is induced by IL-12. Expression of granzyme B by CD4+, CD8+ and MAIT cells from either T cells alone or co-cultures of T cells and autologous MDM stimulated with IL-12 (10 ng/ml). For all graphs; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05 **P<0.01

6.2.16 IL-7 is expressed by MDM and contributes towards granzyme B upregulation

In order to explain why the upregulation of granzyme B in response to IL-12 stimulation was greater in the presence of MDM, it seemed likely that the MDM may produce other cytokines which synergised with IL-12 to induce granzyme B expression. A recent study demonstrated that stimulation of MAIT cells with the pleiotropic cytokine, IL-7, induces upregulation of granzyme B (Leeansyah et al., 2015). Therefore, IL-7 was next explored to see if it was also involved in granzyme B upregulation in this model. qPCR revealed that MDM upregulated IL-7 mRNA expression in response to NTHi infection (figure 6.20).

However, IL-7 protein could not be observed by ELISA. Detection of IL-7 in supernatant may be difficult due to low expression of IL-7, rapid turnover of the protein or due to binding of IL-7 to IL-7 receptors. To confirm IL-7 expression at the protein level, MDM were stained for the presence of intracellular IL-7 protein by flow cytometry. IL-7 was expressed by MDM at baseline, which was upregulated further following NTHi infection (P=0.03; figure 6.20).

In line with the above data, use of an IL-7 capture antibody in the NTHi-infected co-culture model lead to a significant (P=0.03) 25% decrease in MAIT granzyme B expression, a small decrease in CD8+ granzyme B and did not affect CD4+ granzyme B expression (figure 6.21). Overall, it would seem that IL-7 plays a role in MAIT cell granzyme B expression.

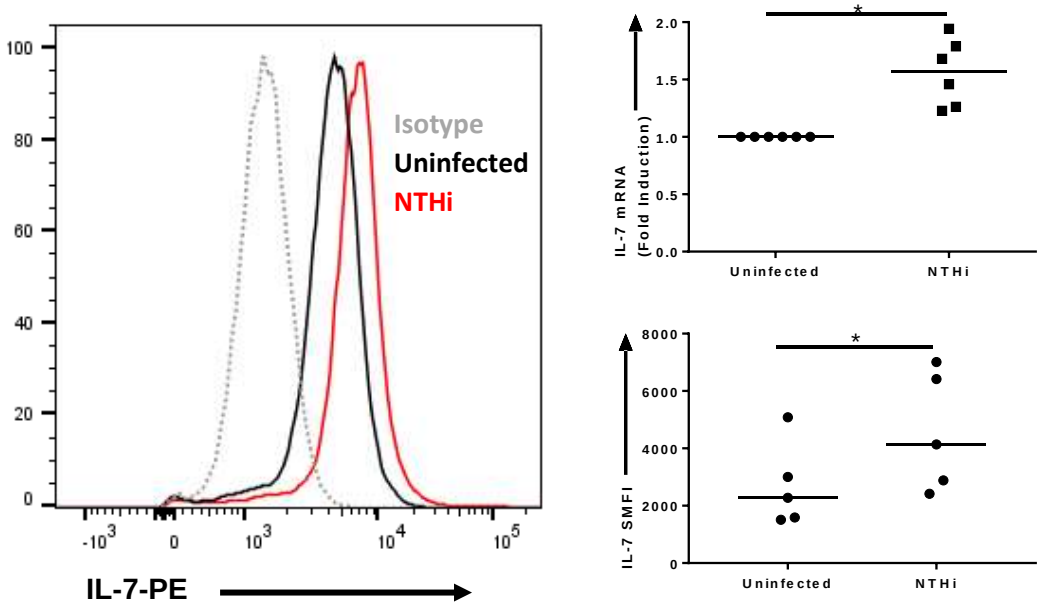


Figure 6.20 – Macrophages express IL-7 in response to NTHi. *IL-7* gene expression as ΔΔCt normalised to β_2M and IL-7 protein expression expressed as specific mean fluorescence intensity (SMFI) on MDM either uninfected or infected with NTHi at a MOI of 1. For all graphs, data are from different donors and are paired; median values are shown. SMFI was derived by subtracting isotype fluorescence values from each sample's MFI. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

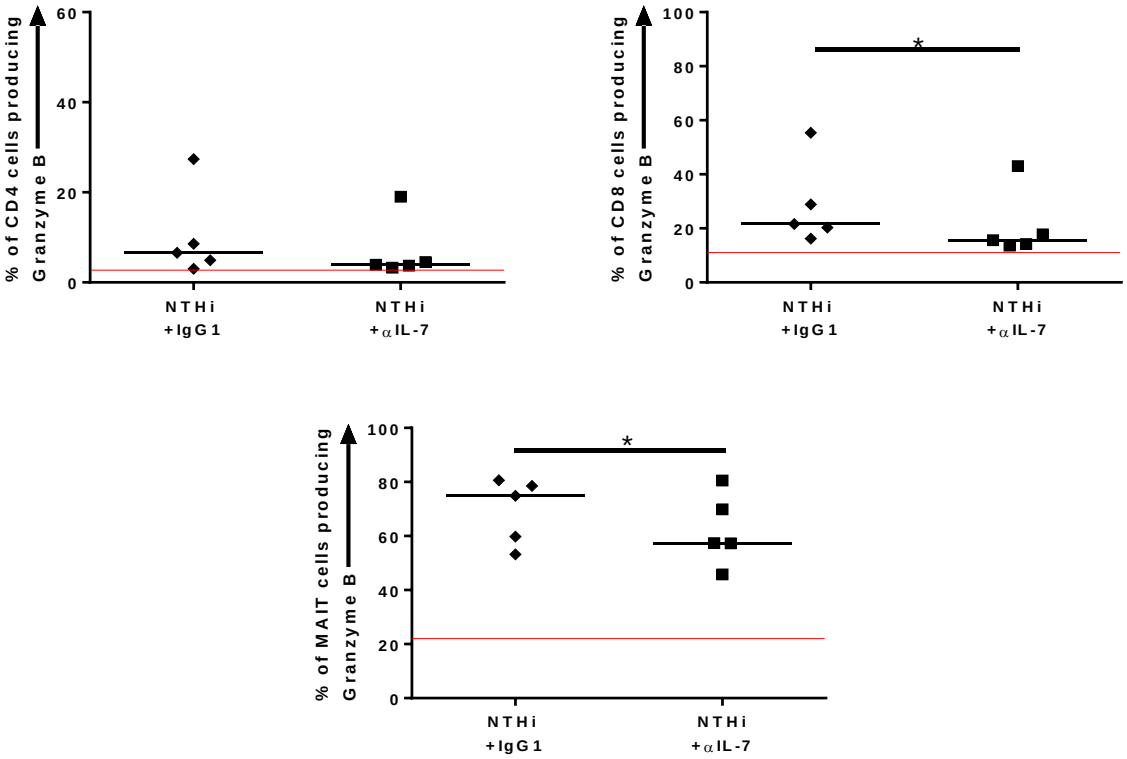


Figure 6.21 – Production of granzyme B by T cells is impaired by blocking IL-7. Expression of granzyme B by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 10 µg/ml anti-IL-7 (BVD10-40F6), IgG1 isotype control. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.17 IL-12 and IL-7 synergise to upregulate MAIT cell granzyme B expression

To next see if IL-12 and IL-7 worked together to induce granzyme B expression, T cells alone (without MDM) were stimulated with either IL-12 and/or IL-7; expression of granzyme B was measured by flow cytometry.

Combined IL-12 and IL-7 caused a very minor increase in granzyme B expression in both conventional CD4+ and CD8+ T cells. For MAIT cells, IL-7 and IL-12 individually caused a minor upregulation in MAIT cell granzyme B. However, when the IL-12 and IL-7 stimulation were combined together there was a synergistic upregulation of granzyme B expression (P=0.0002; figure 6.22), similar to the levels observed with NTHi infection. Furthermore, combined cytokine stimulation resulted in a significant (P=0.01) release in granzyme B measured by ELISA, from 0.3 ng/ml at baseline to 1.4 ng/ml with IL-12 and IL-7 stimulation (figure 6.22). In combination with the previous data in this chapter, the data show that granzyme B expression in MAIT cells can be activated by combined IL-12 and IL-7 signalling.

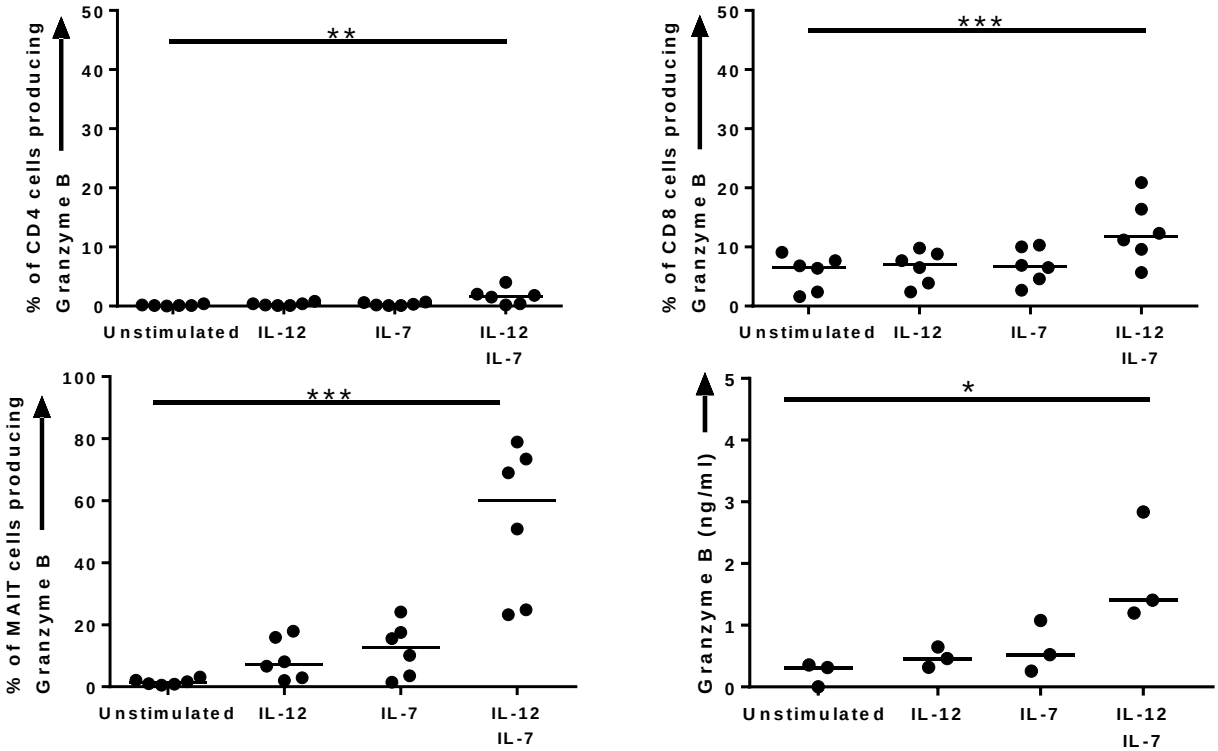


Figure 6.22 – Production of granzyme B by T cells is induced by IL-12 and IL-7. Expression or release of granzyme B by CD4+, CD8+ and MAIT cells from T cells alone stimulated with IL-12 or IL-7 (both 10 ng/ml). For all graphs, median values are shown; data are from different donors and are paired. Statistical significance was determined by Friedman test with Dunn's multiple comparison. *P<0.05 **P<0.01 ***P<0.001.

6.2.18 NTHi infection upregulates the IL-12 receptor on MAIT cells in a mechanism dependent on IL-12 and IL-7

It was possible that the synergistic effects of IL-12 and IL-7 on granzyme B were due to these cytokines affecting either the IL-12 or IL-7 receptor. Therefore, expression of both receptors were first measured on conventional T cells and MAIT cells following stimulation of T cells alone (without MDM) with IL-12 and/or IL-7. Expression of IL-12Rβ1, one of the two subunits that makes up the IL-12 receptor, was not affected by stimulation with either IL-12 or IL-7 in any of the T cell subsets. Expression of IL-12Rβ2 on the other hand was significantly (P=0.0024) and synergistically upregulated from 15% unstimulated to 28% in the presence of both cytokines on MAIT cells, but remained unaffected on conventional T cells (figure 6.23A). IL-7 stimulation caused a significant decreases in the IL-7Rα expression in all three subsets (all P<0.001; figure 6.23B).

Expression of both receptors was measured on T cells from the co-culture model, in order to confirm that the effects seen above with the IL-12 receptor were relevant to NTHi infection. NTHi infection caused a significant (both P<0.05) upregulation of both IL-12Rβ1 and IL-12Rβ2 on MAIT cells; IL-12Rβ1 4% vs 10%, IL-12Rβ2 16% vs 26% (figure 6.24A). Whilst the IL-12Rβ1 upregulation did not occur with the cytokine stimulation, the upregulation of IL-12Rβ2 in the co-culture was almost identical to the upregulation seen with IL-12 and IL-7 stimulation. A minor increase (5% vs 7%) in IL-12Rβ2 was also observed on CD4+ cells in response to infection, but both subunits remained unaffected on CD8+ T cells (figure 6.24A). For IL-7Rα expression, whilst expression remained largely unchanged on the conventional T cells, expression of IL-7Rα expression on MAIT cells was significantly (P=0.03) decreased by 2-fold following infection (figure 6.24B).

To finally ensure that the responses seen in the co-culture model were relevant to the lung, expression of the cytokine receptors was investigated on T cells from the NTHi-infected lung explant. IL-12Rβ2 was significantly (P=0.03) upregulated on lung MAIT cells from NTHi-infected explant to a similar extent as in the co-culture model, although infection did not affect expression of IL-12Rβ1 (figure 6.25A). Expression of IL-7Rα expression on MAIT cells was significantly (P=0.03) decreased by 30% following infection (figure 6.25B). Expression of either of the IL-12 receptor subunits or IL-7Rα were not altered on conventional lung T cells.

Together with the previous data in this chapter, IL-12 and IL-7 synergistically causes an upregulation of the MAIT cell IL-12Rβ2, which may allow greater IL-12 signalling and lead to an upregulation of MAIT cell granzyme B expression.

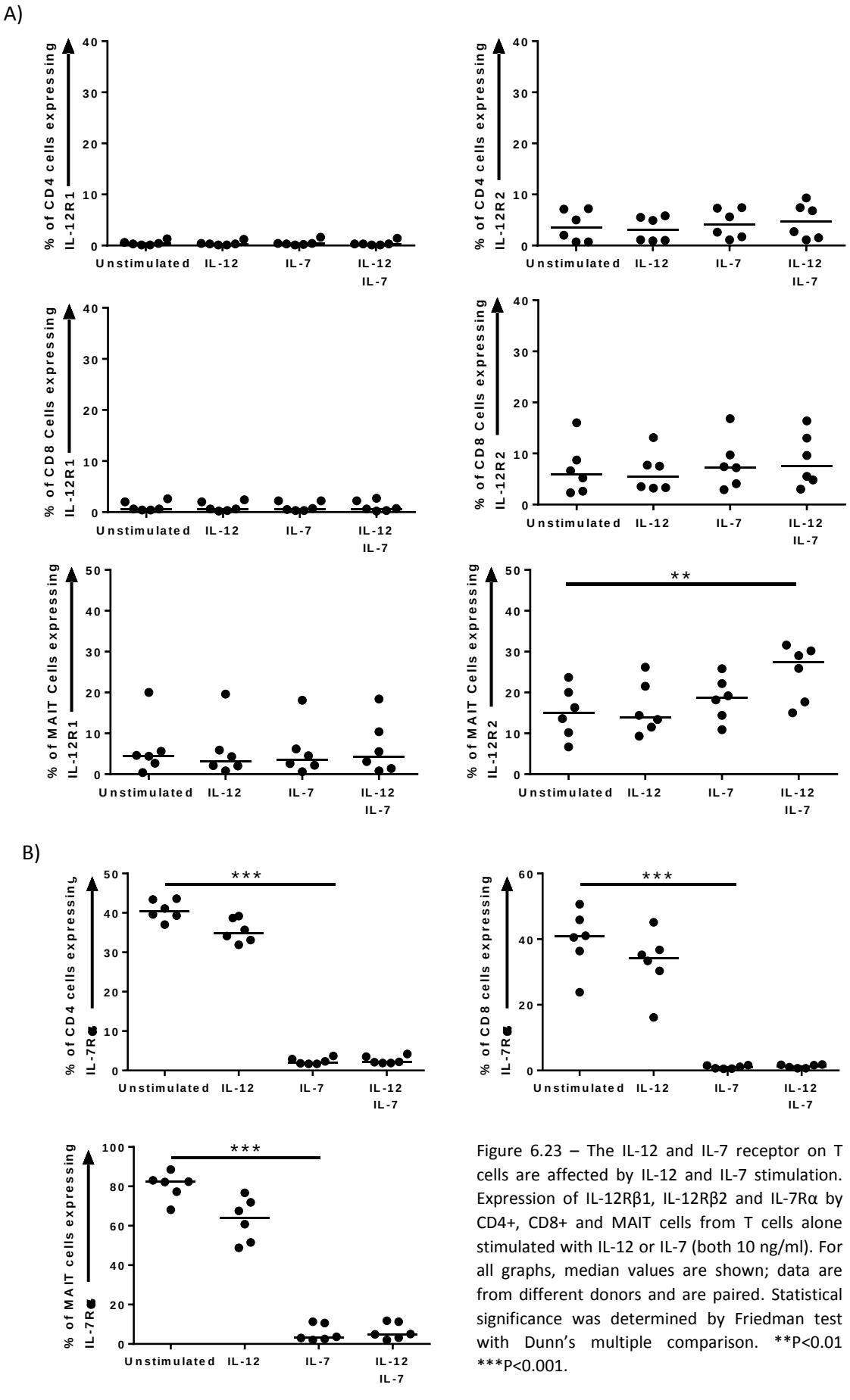
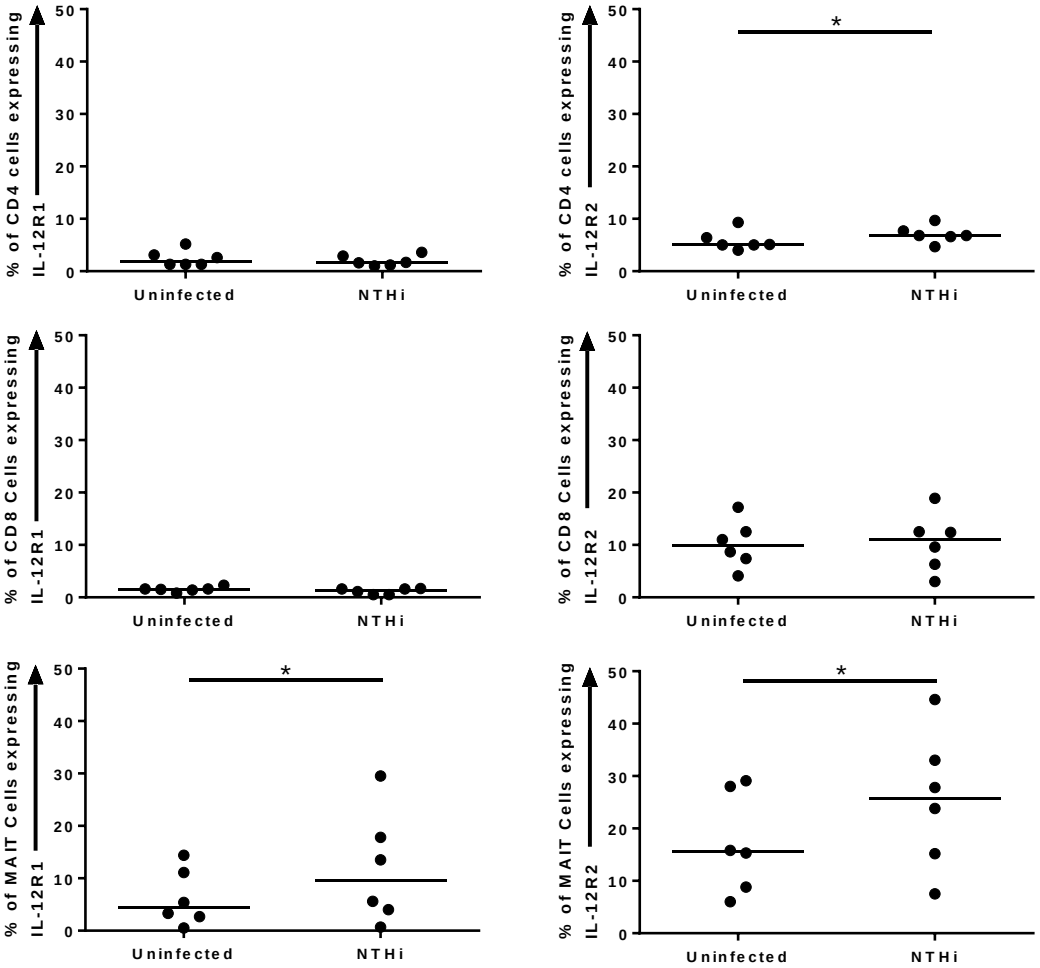


Figure 6.23 – The IL-12 and IL-7 receptor on T cells are affected by IL-12 and IL-7 stimulation. Expression of IL-12Rβ1, IL-12Rβ2 and IL-7Rα by CD4+, CD8+ and MAIT cells from T cells alone stimulated with IL-12 or IL-7 (both 10 ng/ml). For all graphs, median values are shown; data are from different donors and are paired. Statistical significance was determined by Friedman test with Dunn's multiple comparison. **P<0.01 ***P<0.001.

A)



B)

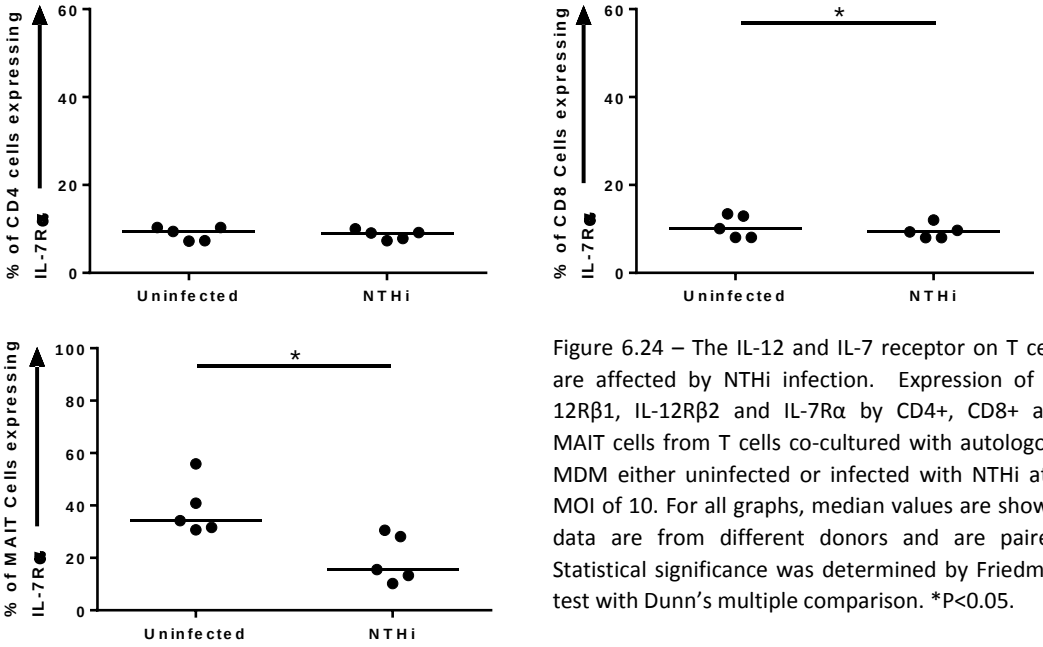


Figure 6.24 – The IL-12 and IL-7 receptor on T cells are affected by NTHi infection. Expression of IL-12Rβ1, IL-12Rβ2 and IL-7Rα by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM either uninfected or infected with NTHi at a MOI of 10. For all graphs, median values are shown; data are from different donors and are paired. Statistical significance was determined by Friedman test with Dunn's multiple comparison. *P<0.05.

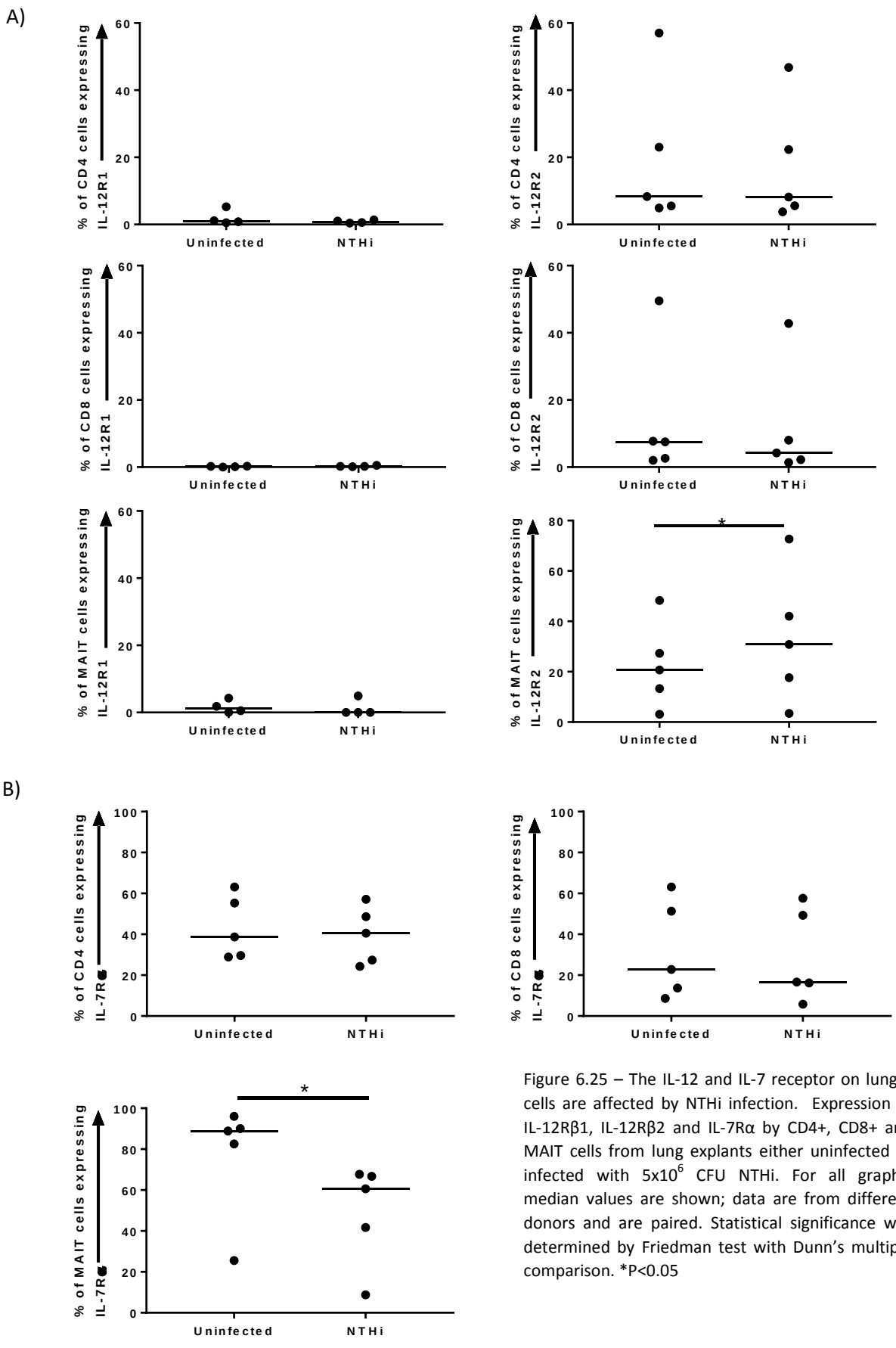


Figure 6.25 – The IL-12 and IL-7 receptor on lung T cells are affected by NTHi infection. Expression of IL-12Rβ1, IL-12Rβ2 and IL-7Rα by CD4+, CD8+ and MAIT cells from lung explants either uninfected or infected with $5x10^6$ CFU NTHi. For all graphs, median values are shown; data are from different donors and are paired. Statistical significance was determined by Friedman test with Dunn's multiple comparison. *P<0.05

6.2.19 Granzyme B expression is inhibited by steroids

To determine whether expression of granzyme B was affected by corticosteroids, NTHi-infected MDM-T cell co-cultures were repeated as before, with the addition of either 100 nM fluticasone propionate (FP) or DMSO to the co-culture.

As shown in figure 6.26, expression of granzyme B by both conventional CD4+ and CD8+ T cells following NTHi infection was significantly (both P<0.05) impaired by fluticasone, reducing expression to below the uninfected baseline; CD4+ DMSO 17% vs FP 4%, CD8+ DMSO 55% vs FP 26%. NTHi-induced upregulation of MAIT granzyme B production was also significantly (P=0.01) impaired to baseline; DMSO control 73% vs FP 42% (figure 6.26). Taken together with the CD107a data from this chapter, corticosteroids not only impair the ability of conventional T cells and MAIT cells to degranulate, but also impair their ability to produce cytotoxic effector molecules in response to infection. Overall, these data suggest that steroids impair the ability of both conventional T cells and MAIT cells to mount cytotoxic responses to bacterial infection.

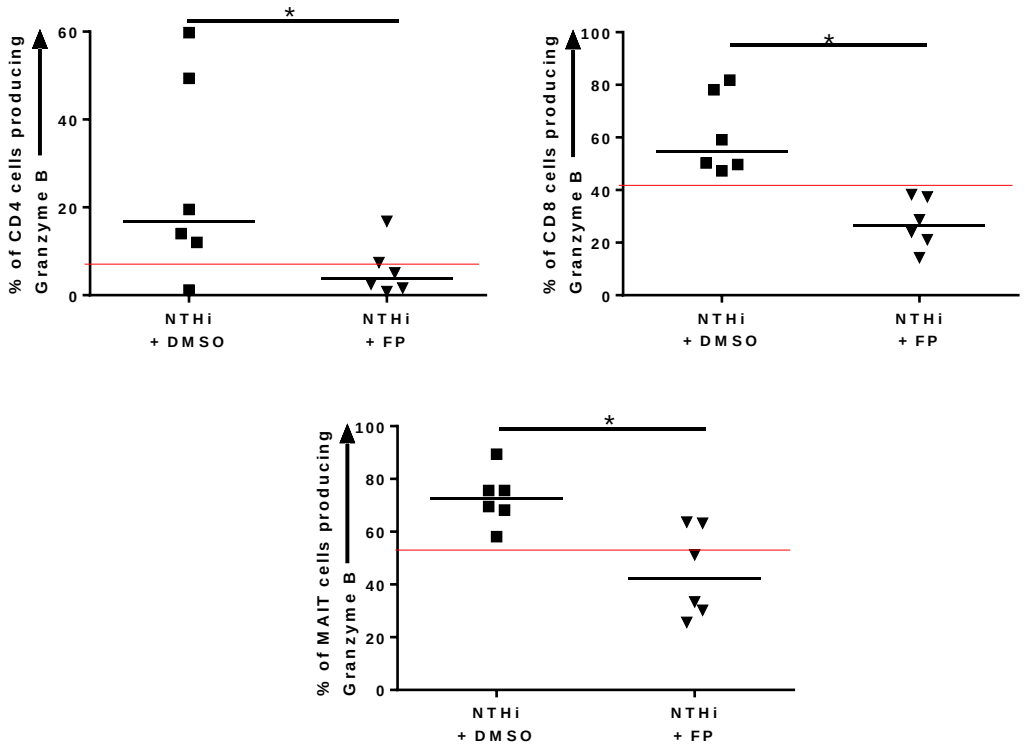


Figure 6.26 – Production of granzyme B is impaired by corticosteroids. Expression of granzyme B by CD4+, CD8+ and MAIT cells from T cells co-cultured with autologous MDM infected with NTHi at a MOI of 10 in the presence of either 100 nM fluticasone propionate (FP) or DMSO. For all graphs, red line indicates uninfected baseline expression; data are from different donors and are paired; median values are shown. Statistical significance was determined by Wilcoxon signed-rank test. *P<0.05

6.2.20 MAIT cells can kill NTHi-infected MDM

So far in this chapter, markers of cytotoxicity have been measured in response to NTHi infection. Although upregulation of CD107a is used as a surrogate marker for degranulation, it does not actually confirm that degranulation has occurred. To address this, expression of granzyme B was characterised by flow cytometry and was detected in the supernatant, thus confirming that granzyme B was actually being released in response to NTHi infection. However, none of these readouts directly demonstrate that the T cells are having a cytotoxic effect on the MDM. CD8+ T cells have previously been shown to kill target cells (Zaritskaya et al., 2010)(Noto et al., 2013), but whether MAIT cells are capable of killing target cells is not well understood.

In order to confirm that MAIT cells were able to kill NTHi-infected MDM, the co-culture had to be adapted so that MDM could be co-cultured with just MAIT cells. To do this, MAIT cells were FACS-sorted from lymphocytes to a median purity of >93% and co-cultured with autologous MDM either uninfected or infected with NTHi, as before. Uninfected and NTHi-infected MDM were also cultured alone (without MAIT cells present) as a control to account for death of MDM due to being in culture or infected with NTHi. Death of MDM was assessed by viability staining.

At 24 h of MDM-MAIT cell co-culture, no increase in death of MDM due to MAIT cells was detected so the co-culture was extended by another 24 h. At 48 h post infection, death of MDM cultured alone without MAIT cells was observed (figure 6.27). To determine whether MAIT cells were inducing MDM death in the MDM-MAIT co-culture, specific MAIT cell-induced death of MDM was measured by deducting the percentage cell death of the uninfected or NTHi-infected MDM cultured alone from the percentage cell death of uninfected or NTHi-infected MDM from the MDM-MAIT co-culture respectively.

A significant (P=0.03) increase was observed in MAIT cell-induced death of NTHi-infected MDM compared to uninfected MDM, indicating that MAIT cells can kill NTHi-infected macrophages (figure 6.27). Consistent with this, an increase in LDH release into the supernatant was detected in NTHi-infected MDM-MAIT co-cultures (figure 6.27). MAIT cell granzyme B was also significantly (P=0.03) upregulated in response to NTHi infection at 48 hours (figure 6.27). Overall, these data demonstrate that MAIT cells are cytotoxic cells that can respond to NTHi infection and can kill infected MDM.

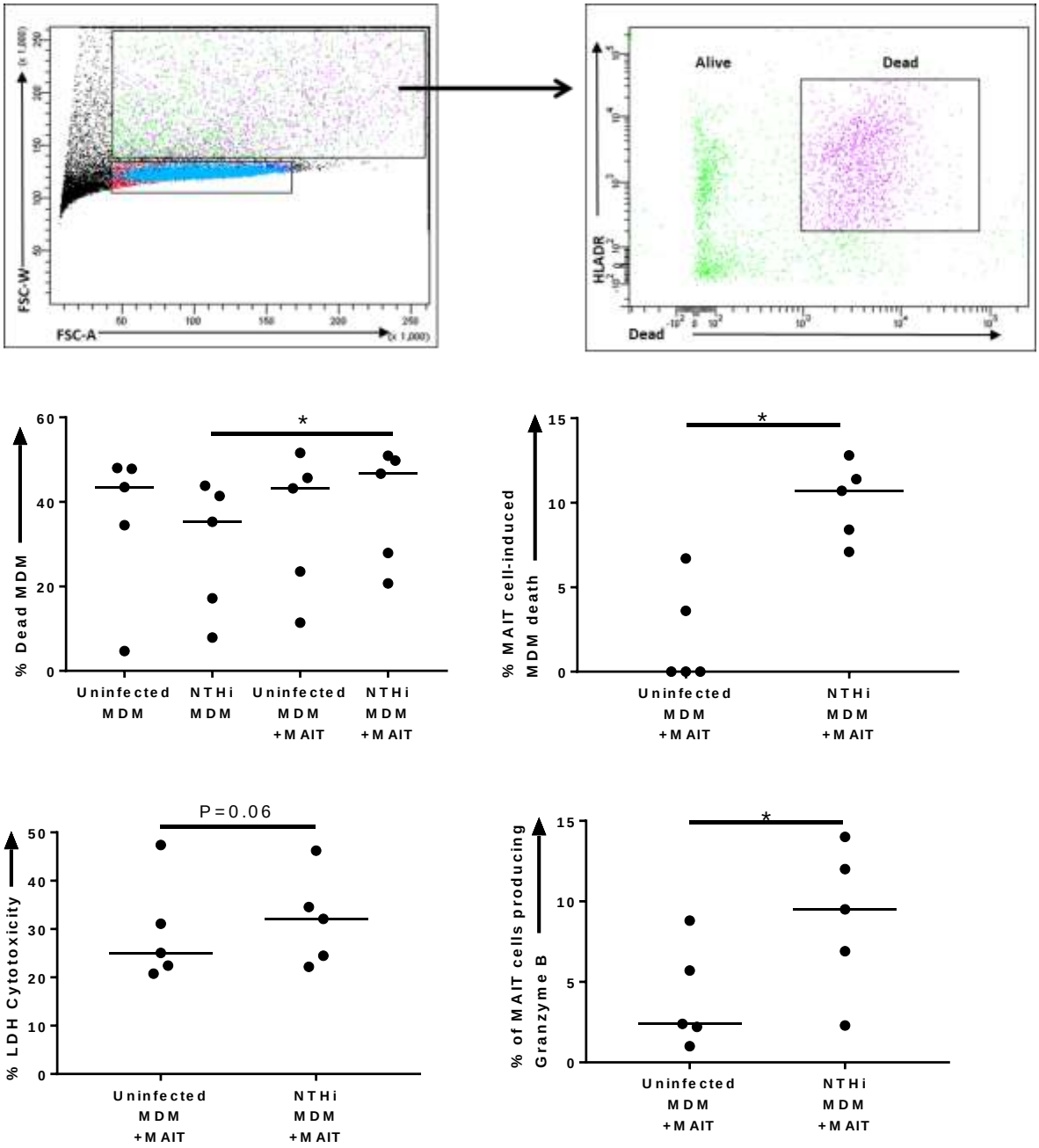


Figure 6.27 – Killing of NTHi-infected MDM by MAIT cells. Gating strategy, MDM cell death, LDH cytotoxicity and expression of granzyme B by MAIT cells from 48 hour co-cultures of FACS-sorted (>93% purity) MAIT cells and autologous MDM either uninfected or infected with NTHi at a MOI of 10. Killing of MDM by MAIT cells was assessed by viability staining. For all graphs, median values are shown; data are from different donors and are paired. Statistical significance was determined by Wilcoxon signed-rank test *P<0.05

6.3 Discussion and Conclusions

Cell-mediated immunity may be important in the control of NTHi infection in the lung and this has implications for both acute and chronic diseases associated with NTHi. Understanding T cell immune processes, especially the roles of novel cytotoxic T cells such as MAIT cells, may lead to the generation of effective treatments and better disease prognosis.

In this chapter, CD4+, CD8+, and MAIT cells all displayed cytotoxic responses to the respiratory pathogen, NTHi with the upregulation of CD107a and granzyme B. Furthermore, the data presented here have shown for the first time that human lung MAIT cells exhibit cytotoxic responses to NTHi infection. In combination with data from the previous chapter, these observations provide further evidence that NTHi is a target of lung MAIT cell immunity.

The cytotoxic responses of CD8+ T cells were expected as these cells have previously been shown to upregulate CD107a in response to NTHi infection (King et al., 2008b), consistent with the data presented here. Furthermore, lung CD8+ T cell granzyme B and perforin expression has been shown to inversely correlate with disease severity and FEV1 (% predicted) of COPD patients, suggesting an involvement of these cells in disease pathogenesis (Freeman et al., 2010). Although the archetypal conventional cytotoxic T cell is the CD8+ T cell, CD4+ T cells also have cytotoxic function, but less is known about the cytotoxic activity of these cells as they are typically regarded as just cytokine producing T helper cells. CD4+ T cells have been shown to express CD107a following viral challenge (Wilkinson et al., 2012) and also express granzyme B (Marshall and Swain, 2011), suggesting an important role of CD4+ cytotoxic activity in clearance of infection. These cytotoxic CD4+ T cells have not been investigated before in the context of NTHi infection but the data presented in this chapter suggest a role for cytotoxic CD4+ cells in responding to NTHi. Upregulation of CD107a, perforin and granzyme B indicate that CD4+ cells not only express cytotoxic mediators in response to infection but can also degranulate and release these mediators, suggesting an ability to kill infected cells.

Whilst NTHi infection did induce cytotoxic responses from the conventional T cells, these responses were minor in comparison to the proportion of cytotoxic responses of both lung and blood-derived MAIT cells, similar to the cytokine production from the previous chapter. Over recent years MAIT cells have rapidly gained recognition as key effector cells, with potential roles in a variety of different diseases. Cytotoxic activity of MAIT cells has been previously reported, with *Mycobacterium tuberculosis*-reactive MAIT cells producing granzyme (Gold et al., 2010) and upregulation of granzyme B and CD107a by MAIT cells in *E. coli* infection (Kurioka et al., 2015). The cytotoxic activity of these cells in response to NTHi was substantial, with large increases in

both degranulation and granzyme B production, which may indicate that MAIT cells are the primary cytotoxic cell responding to bacterial infection.

The mechanisms regulating MAIT cell cytotoxic responses are not fully understood, neither is it known how these pathways differ from those of the conventional T cells. Here blocking of HLA-DR and HLA-ABC reduced granzyme B upregulation of CD4+ and CD8+ T cells respectively in response to NTHi, suggesting that conventional T cell cytotoxic activity is dependent on antigen presentation. Contact also appeared to be required, again confirming that antigen presentation is essential. Although IL-12 blocking did impair granzyme B expression and is therefore involved in granzyme regulation, addition of IL-12 cytokine only induced a small granzyme B response. This would suggest that conventional T cell granzyme B responses requires both antigen presentation and cytokine signalling together and that impairing just one of these pathways prevents a granzyme B response. Antigen presentation therefore appears to be the primary driving factor, as cytokine stimulation in the absence of antigen presentation is insufficient for granzyme B upregulation. Conventional T cell responses also appear largely independent of IL-7.

MAIT cells on the other hand were regulated differently, as expression of granzyme B appeared to require a combination of both antigen presentation and IL-12 and IL-7 cytokine signalling pathways to result in maximum granzyme B expression. In contrast to conventional T cells, the antigen and cytokine pathways are independent of each other, as blocking of one pathway did not reduce granzyme B expression to baseline, suggesting the other pathway is still active. MAIT cell granzyme B was still upregulated in the absence of contact, again highlighting a role for non-contact factors such as cytokines. The transwell data presented in this chapter was unexpected, as another study using transwells suggested that MAIT cells require contact in order to activate, although the model in that study may have lacked IL-12 or IL-7 (Salerno-Goncalves et al., 2014). IL-12 and IL-7 have previously been reported to induce MR1-independent MAIT cell IFNγ and cytotoxic responses (Chua et al., 2012)(Ussher et al., 2014a)(Leeansyah et al., 2015). However, the data presented in this chapter show for the first time a novel mechanism that links these cytokines together, whereby both IL-12 and IL-7 synergistically upregulate the IL-12 receptor, which may allow increased IL-12 signalling (Zhu and Paul, 2008), leading to a greater expression of granzyme B (summarised in figure 6.28). This mechanism appears to be more important for MAIT cells, as cytokine stimulation of conventional T cells did not greatly affect IL-12 receptor expression and only resulted in minor granzyme upregulation. MAIT cells have a higher baseline expression of IL-12Rβ1 and IL-12Rβ2 to begin with, which confers them the ability to express granzyme B in response to IL-12 even in the absence of other stimulation. This response is not seen in conventional T cells due to lower expression of the IL-12 receptor.

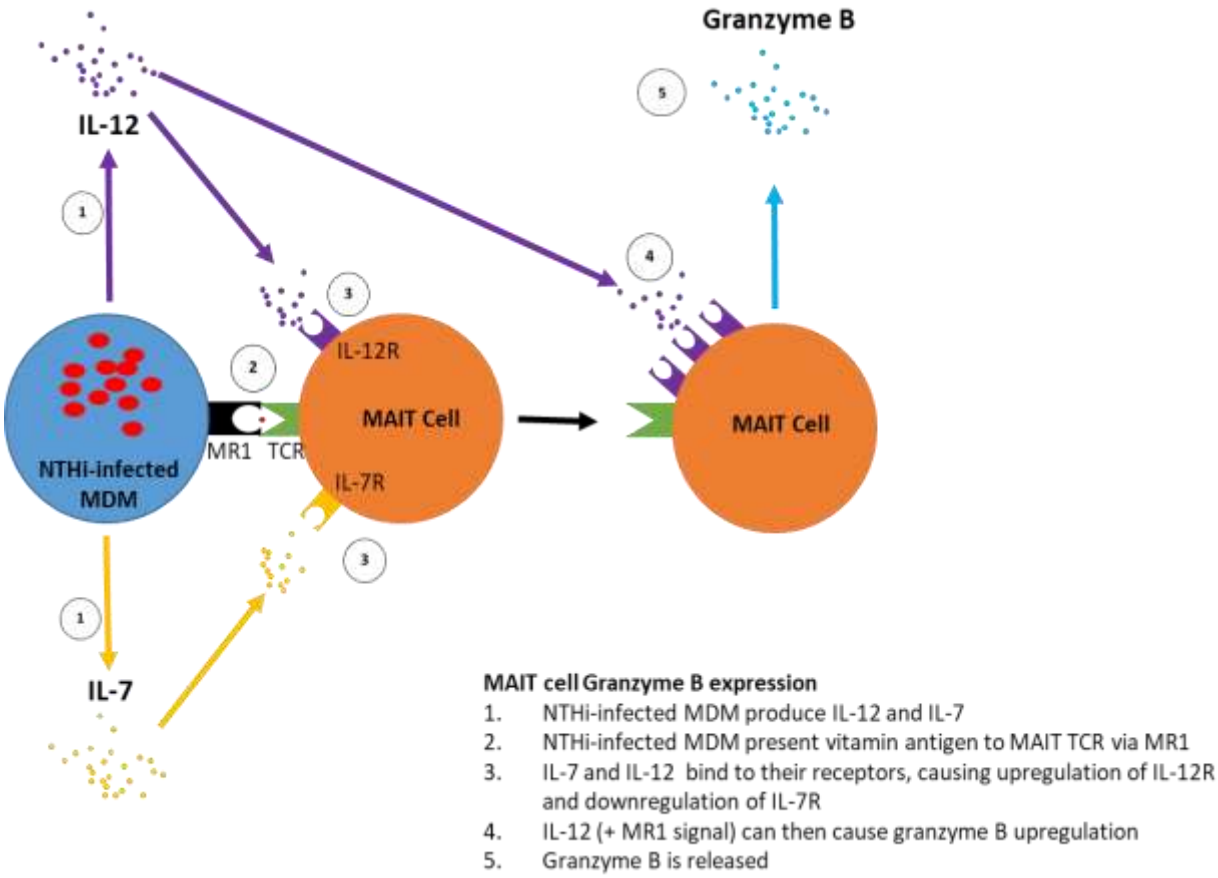


Figure 6.28 – IL-12 and IL-7 synergise to upregulate MAIT cell granzyme B via upregulation of the IL-12 receptor.

The blocking of MR1 only caused a small reduction in granzyme B expression, with IL-12 and IL-7 appearing to be largely responsible for granzyme induction. It is possible that a small antigen signal or minimal MR1-TCR engagement can be enhanced by cytokine signalling. Indeed, IL-7 has recently been shown to augment TCR signalling in MAIT cells by upregulating TCR-signalling genes as well as increased expression of Vα7.2 (Tang et al., 2013). This indicates another role for IL-7 in activating MAIT cells, besides its role in upregulating the IL-12 receptor. Alternatively, as the reduction in granzyme B following MR1 blocking was very minor, although a statistically significant result was found, this may actually not be biologically relevant.

In addition to IL-7 and IL-12, both IL-15 and IL-18 have been shown to activate MAIT cells (Sattler et al., 2015)(Wilgenburg et al., 2016). Further work will be needed in order to investigate the full interactions and combined effects of all of these cytokines on MAIT cell activation. With an ability to respond to cytokine stimulation in the absence of antigen presentation, the definition of MAIT cells as purely anti-bacterial cells has been expanded as they have a potential bystander role in

infections with non-riboflavin producing bacteria as well as viruses (Szabo et al., 2015)(Wilgenburg et al., 2016)(Loh et al., 2016). Furthermore, in diseases characterised by an influx of inflammatory cytokines, MAIT cells may undergo bystander activation which could be dangerous and induce further inflammation. Increased expression of IL-12 has been found in COPD patients which inversely correlated with lung function decline (Kalathil et al., 2014), although whether these increased IL-12 levels facilitate the decline in lung function by inducing MAIT cell cytotoxicity is a question that remains to be addressed. However, the anatomical location may be important in determining whether cytokine stimulation or MR1 are involved in MAIT cytotoxicity, as one study has recently shown that expression of granzyme B by MAIT cell from tuberculosis pleural effusions was not inhibited by IL-12 blocking (Jiang et al., 2016).

In contrast to the different mechanisms regulating granzyme B, CD107a expression for both conventional and MAIT cells appears to be mainly regulated independently of cytokines and driven by cell contact and antigen presentation. Particularly for MAIT cells, whilst they can upregulate cytotoxic mediators in response to IL-12 and IL-7, cytotoxic release is mainly controlled by antigen presentation and cell contact. This was further shown as IL-12 and IL-7 stimulation of T cells did result in a minor release of granzyme B into supernatant, but a far greater amount of granzyme B was detected in the NTHi-infected co-cultures. As degranulation required MR1 at the 5 hour timepoint but was MR1-independent at the later 22 hour timepoint, and as minor upregulation of CD107a was also seen in the transwell model, not just antigen presentation but other non-contact factors may also regulate degranulation. It would appear that MR1 antigen presentation initiates MAIT degranulation but MR1 is not required to induce granzyme B expression in the beginning stages of infection. Initial MR1-mediated degranulation may release other granzymes, such as granzyme A and granzyme K, whilst granzyme B release occurs later (Kurioka et al., 2015). Whether other factors are involved in MAIT cell cytotoxicity is currently unknown, but work by Le Bourhis et al indicated that secretion of granzyme B by MAIT cells is independent of CD161, whereas MAIT cytokine secretion is CD161-dependent (Le Bourhis et al., 2013).

One point of note is that in other studies, the CD107a flow cytometry antibody is often added during the beginning of the stimulation assay because of the fast kinetics of CD107a trafficking and recycling (Betts et al., 2003) (Alter et al., 2004) (Aktas et al., 2009). However, the fluorescently-labelled CD107a antibody available in this project could not be added during culture of NTHi-infected MDM and T cells as the antibody used was not produced low endotoxin or azide-free, and so this may account for the lower detection of CD107a compared to other studies.

In this model, a key difference between conventional T cells and MAIT cells is that MAIT cells upregulated CD107a and granzyme B expression faster than conventional T cells, as no substantial cytotoxic response of the conventional T cells was observed at the 5 h time point. The ability of MAIT cells to become activated in response to cytokine stimulation may allow them to become primed and rapidly respond to infection.

Many factors may determine whether MAIT cell activation requires MR1 presentation or not, such as the type of pathogen involved, the intracellular location of the pathogen, or the type of infected cell and its capacity to release cytokines. Another factor determining whether the MR1 pathway is required or not could be the length of activation. One recent report has shown that shorter co-cultures of infected APCs with MAIT cells causes upregulation of IFNγ in a MR1-mediated manner but longer time courses meant that MAIT activation was dependent on both MR1 and IL-12 (Ussher et al., 2014a).

The reasons why bacteria such as NTHi are able to change from a commensal to pathogenic organism and colonise the lower airways are not known, but could be caused by impaired T cell cytotoxic responses due to cigarette-smoke induced damage (Hernandez et al., 2013)(Kalra et al., 2000)(Feng et al., 2011). T cells may become either dysfunctional and respond to bacterial species that were previously regarded as commensals, or T cell responses become impaired, allowing unchecked commensal bacteria to advance to other niches, leading to inflammation. One possibility is that, as discussed previously in this thesis, T cells may become impaired in lung diseases such as COPD due to the use of steroids. Similarly to the IFNγ data in chapter 5, both CD107a and granzyme B expression for both conventional T cells and MAIT cells were impaired following treatment with the steroid fluticasone, again indicating that steroid use may have long term consequences for immune control. Indeed, granzyme B levels from sputum of asthmatics was lowered after ICS treatment (Shoeib et al., 2014). Another possibility is that aberrant regulation of co-inhibitory pathways represent a mechanism that allows T cells to contribute to excessive inflammation in response to respiratory pathogens. Previous research has shown that cytotoxic T cells are dysregulated in the COPD lung by increased expression of PD1 and diminished degranulation (McKendry et al. 2016). In this chapter, CD107a expression in response to NTHi was not affected by blocking the PDL/PD1 pathway, although as PD1 is only expressed on a small proportion of cells, it's possible that the T cells expressing PD1 are not the ones expressing CD107a, so PDL/PD1 blocking would not have an effect. However, a clear difference in regulation of granzyme B expression by PDL/PD1 was seen in conventional T cells vs MAIT cells. Very little is known about whether PD1 regulates MAIT cell cytotoxic responses, but the data here may indicate that the PDL/PD1 pathway is not involved, at least in response to NTHi. More research is

required to understand the mechanisms regulating T cell cytotoxic responses and how these mechanism are altered in diseases associated with chronic NTHi colonisation.

Cytotoxic function of MAIT cells was confirmed through the use of a cytotoxic killing assay, where MAIT cells induced death in NTHi-infected MDM. Due to the technical limitations of this model, it was not possible to show whether MR1 or the IL-12/IL-7 pathways were involved in the killing process. However, other studies have shown that MAIT cell killing of *E.coli*-infected target cells is impaired by blocking MR1 (Kurioka et al., 2015)(Le Bourhis et al., 2013) but augmented by IL-7 treatment due to upregulation of granzyme B (Leeansyah et al., 2015), so it would seem likely that killing of NTHi-infected MDM by MAIT cells is mediated by these pathways as well. MR1 blocking is reported to be more effective at inhibiting target cell killing by MAIT cells over short timecourses, but less effective in longer timecourses, which could be due to fast recycling of MR1 to/from the cell surface (Dias et al., 2016)(Chua et al., 2011). This observation may also explain why CD107a upregulation is blocked by MR1 at 5 hours, but not at 22 hours. The reason why no killing effect of infected MDM was seen at 22 hours of co-culture was surprising at first, but could be because macrophages, like other APCs such as dendritic cells and monocytes, express molecules called serpins (Shea-Donohue et al., 2014)(Hamerman et al., 2002)(Kaiserman and Bird, 2010)(Classen et al., 2006)(Hirst et al., 2003). These molecules are a family of proteinases, of which specifically serpin B9 (PI9) is an anti-granzyme proteinase (Hirst et al., 2003)(Classen et al., 2006)(Sun et al., 1996), which may confer MDM a resistance to granzyme B mediated apoptosis initially. However, as the co-culture progresses to 48 hours post-infection, granzyme B levels become too high for the MDM to cope with and they succumb to the apoptotic effects of granzyme B. Alternatively, MAIT cell production of granzyme B in the MDM-MAIT co-culture was less compared to MAIT cells from the whole T cell-MDM co-culture. This could be why a longer time point might be needed in the MDM-MAIT co-culture for killing, to allow sufficient levels of granzyme B to be induced. MAIT cell may also have a role in directly killing bacteria, as granzyme B is also delivered into bacteria and induces bacterial death by disrupting the electron transport chain and generating reactive oxygen species (Walch et al., 2014). Further work is required to fully investigate the role of MAIT cells in killing bacterially-infected target cells (and bacteria directly) and whether this process occurs *in vivo* in respiratory infections and diseases.

Perforin may also be involved in the chronic inflammation and pathogenesis of diseases such as COPD, as a high expression of perforin has been found in CD8+ T cells from COPD patients (Chrysofakis et al., 2004)(Hodge et al., 2006). Expression of perforin was also evaluated in this chapter, but whilst conventional T cells upregulated perforin in response to NTHi, MAIT cells did not. As such, it was not possible to perform MR1 or IL-12 blocking experiments as there was no

inducible response to inhibit. Previous studies have found that MAIT cells stimulated with anti-CD3/CD28 beads upregulated perforin (Le Bourhis et al., 2013), as do MAIT cells exposed to *E.coli*-infected THP1 cells (Kurioka et al., 2015), although in the latter study, perforin expression appeared independent of MR1 and IL-12 signalling. These observations would suggest that perforin expression in MAIT cells is regulated by other factors. However, just because perforin was not upregulated by MAIT cells does not mean it is not playing any role. Expression of MAIT cell perforin in the NTHi co-culture model was high at baseline, therefore further upregulation may not be required for function. Overall, the perforin data provide further evidence for differences between conventional and MAIT cells, although further work is needed to uncover the role of perforin in the cytotoxic response to NTHi.

This chapter has only explored the role of degranulation and the granule exocytosis pathway as measures of cytotoxicity. However, the alternative method cytotoxic T cells use to initiate target cell death is the FAS/FAS ligand pathway, which is a contact-dependent mechanism whereby signalling of FAS ligand on T cells to FAS expressed on target cells initiates apoptosis (Waring and Müllbacher, 1999)(Broere et al., 2011)(Lewinsohn et al., 2011). FAS ligand expression in response to NTHi infection has not been explored, although FAS ligand expression does not correlate with FEV1 in COPD patients, suggesting that cytotoxic immunopathology in COPD may be FAS/FAS ligand independent (Freeman et al., 2010). MAIT cells are reported to express very low levels of FAS ligand, but expression appears to be unaffected by CD3 stimulation or bacterial infection and does not contribute to MAIT cell-induced killing of target cells (Kurioka et al., 2015).

In summary, whilst conventional CD4+ and CD8+ T cells demonstrate cytotoxic responses to NTHi infection, a greater proportion of MAIT cells appear to have cytotoxic function, which further suggests that NTHi constitutes a target for MAIT cells in the lung. The mechanisms regulating these cytotoxic responses are different between innate and adaptive T cells and further work is required in order to understand how these mechanisms control the response to bacterial pathogens in respiratory diseases. As cytokine stimulation can activate MAIT cells cytotoxic responses, these innate cells could be an important cause of bystander-induced cytotoxic damage in many lung diseases and respiratory infections. The observations in this chapter that IL-12 and IL-7 upregulate granzyme B expression via the IL-12 receptor provide a new understanding of MAIT cell immune regulation but could also lead to possible therapeutics which aim to modulate the cytotoxic response of MAIT cells for the benefit of clinical outcome.

7. Discussion and Future Work

7.1 Introduction

The aim of this thesis was to explore the responses of T cells, with a particular focus on MAIT cells, to the lung pathogen NTHi, which is a major cause of respiratory tract infections and is a key pathogen involved in exacerbations of COPD (Van Eldere et al., 2014)(Murphy et al., 2004)(Bandi et al., 2003)(Sethi et al., 2002). Impaired immune responses have been reported in COPD (McKendry et al., 2016)(Bhat et al., 2015)(Schleimer, 2005)(Berenson et al., 2014), although it is not known why NTHi infection/colonisation may lead to exacerbations and immunopathology. The nature of the lung immune response to NTHi infection is not well understood and no effective vaccine is currently available (Behrouzi et al., 2017), although vaccines are in clinical trials and their use indicates a role for cell-mediated immunity (Leroux-Roels et al., 2016). As NTHi is an intracellular pathogen (Craig et al., 2001)(King et al. 2008)(Morey et al., 2011), cell-mediated T cell immunity may be important in controlling NTHi in the airway through expression of cytokines and degranulation of cytotoxic mediators (King et al. 2008)(King et al., 2013). What was not known was whether MAIT cells were involved in the response to NTHi infection.

By using an *ex vivo* lung tissue explant and an autologous, blood-derived MDM-T cell co-culture model, the data generated and presented in this thesis supports the hypothesis that MAIT cells have pro-inflammatory cytokine and cytotoxic responses to NTHi infection. These responses may be vital in not only controlling respiratory bacterial infections but also in modulating the airway microbiome. In this chapter, the overall findings from this thesis will be discussed in the wider context of lung immunity to infections and the potential clinical implications. Key areas of interest where the work can be developed further will also be addressed.

7.2 NTHi infection causes macrophages to upregulate MR1

One of the key hypotheses for this project was that macrophages would not only express MR1, but also present antigen to MAIT cells and thus function as an APC for MAIT cells. Previously, studies of MR1 on human lung macrophages and of human primary macrophages interacting with MAIT cells has been limited. MR1 was expressed on macrophages from lung tissue which became upregulated on lung macrophages following NTHi infection. A similar response occurred with MDM, suggesting that MR1 upregulation can lead to MAIT cell activation. Indeed, the surface expression of MR1 may represent one stage of control that contributes to MAIT cell regulation, as MR1 is typically lowly expressed at baseline but becomes upregulated in the presence of the bacterial vitamin antigens (Lamichhane and Ussher, 2017)(McWilliam et al., 2016). Whether

macrophages are the main APC for MAIT cells in the lung remains to be determined, but seem likely given that they express the highest level of MR1. Macrophages from COPD patients have reduced phagocytosis of *Haemophilus influenzae* compared to non-COPD macrophages (Taylor et al., 2010), which may be due to exposure to cigarette smoke (Martí-Lliteras et al., 2009) and this may reduce the ability of macrophages in the COPD lung to activate MAIT cells. Nonetheless, COPD macrophages can still take up some bacteria, which may be sufficient for antigen presentation to occur, as it is not known what threshold of vitamin antigens are required to activate MAIT cells. It is also not known whether macrophages can endocytose the bacterial vitamin antigens, independent of whole bacteria, and whether this process is impaired in COPD. Endocytosis of the vitamin antigens would be akin to the exogenous antigen pathway utilised by MHC-II (Watts, 2004). Indeed, C1R lymphoblastoid cells and epithelial cells have been shown to internalise and process exogenous vitamin antigens and present to MAIT cells, although MAIT cell activation likely requires live intracellular infection of APCs for maximal effect (Corbett et al., 2014)(Harriff et al., 2016). This is further implied as metabolically-inert PFA-NTHi did not elicit a strong MDM-MR1 upregulation or MAIT cell activation, compared to infection with live, metabolically active NTHi. Overall, macrophages express MR1 in the lung and MDM can present antigen to and activate MAIT cells.

7.3 MAIT cells are present in the human lung and respond to NTHi infection

In line with previous literature (Hinks et al., 2016)(Gold et al., 2010), MAIT cells have been identified in the human lung and, for the first time, have been shown to upregulate expression of the cytokines; IFNγ, IL-17a and TNFα, and the cytotoxic markers; granzyme B and CD107a in response to NTHi infection. The implications for these responses are many and indicate that MAIT cells may serve a protective role in the lung. Previous work has shown that Th1 responses are important in NTHi infection, as IFNγ activates macrophages to kill intracellular NTHi (King et al., 2003)(King et al. 2008). Furthermore, CD40L was also upregulated on MAIT cells to a greater extent compared to conventional T cells, and ligation of CD40 also initiates killing of NTHi by monocytes/macrophages (King et al., 2003)(King et al. 2008) and matures dendritic cells which can orchestrate adaptive immunity (Salio et al., 2017). IFNγ production and CD40L upregulation by MAIT cells may therefore contribute to the defence against intracellular pathogens. MAIT cells were also a source of IL-17 in the lung although its role in pathogen defence in humans in unclear.

Neutralisation of IL-17 in mice challenged with NTHi resulted in greater NTHi colonisation compared to mice without IL-17 neutralisation (Noda et al., 2011), which may indicate that MAIT cells, as a source of IL-17, are involved in mucosal immunity to NTHi in the respiratory tract. In addition to cytokine responses, MAIT cells upregulated granzyme B and killed NTHi-infected macrophages, which may indicate a cytolytic role for MAIT cells in the lung as another mechanism of controlling NTHi infection.

As NTHi is a target of MAIT cell immunity, it seems probable that MAIT cells play a role in surveillance of the lung microbiome and contribute to host defence against invading pathogens. The bacterial vitamin antigens that MAIT cells recognise are probably expressed by many different types of commensal lung bacteria (Kanehisa and Goto, 2000). MAIT cells may therefore be exposed to these antigens continuously as bacteria release them into their environment (Kjer-Nielsen et al., 2012). What is not understood is what prevents MAIT cells from activating in response to these commensal-derived antigens in health and whether these control mechanisms break down in disease settings. Whether MAIT cells play a major role in lung diseases has not been determined. It is not known whether MAIT cells contribute to immunopathology by overreacting to commensal bacteria, or have impaired responses to invading pathogens, allowing bacteria to further colonise the lung and cause inflammation. Data presented in this thesis support both paradigms. MAIT cells have the capacity to respond to IL-12 and IL-7 stimulation, which causes upregulation of IFNγ and granzyme B in a non-specific manner and may trigger potent responses when the MAIT cells encounter antigen, leading to unwarranted inflammation. In contrast, steroid treatment appeared to impair MAIT cell number (Hinks et al., 2016) and responses, which may allow otherwise commensal bacteria to become unrestricted and colonise other areas of the respiratory tract, causing inflammation or tissue damage. Further investigation is required to elucidate the full role of MAIT cells in the lung.

Although MAIT cell cytokine and cytotoxic responses have been explored, only the total MAIT population as a whole has been considered. It is possible that the different CD8+, CD4-CD8- double negative (DN) and CD4+ subsets of MAIT cells all have different functions and thus responses. Data in this thesis have shown that human MAIT cells are predominately CD8+ or DN, with few CD4+ T cells. A recent report has shown that whilst CD8+ and DN MAIT subsets appear to have similar responses, CD4+ MAIT cells are skewed to a Th2 phenotype and express IL-4 and IL-13 (Kurioka et al., 2017). Further work exploring the differences between these subsets may unveil roles for MAIT cell subpopulations in response to infection.

7.4 Potential role for MAIT cells in diseases independent of riboflavin-antigen presentation

MAIT cells are regarded as antibacterial T cells that respond to infection with riboflavin/vitamin B2-producing bacteria. However, this definition may change in the coming years, due to the capacity of MAIT cells to be involved in infections independent of vitamin B2 antigen presentation. Although, an initial study reported that MAIT cells could not respond to bacteria that lacked the vitamin B2 pathway (Le Bourhis et al., 2010), recently a new type of MR1-restricted T cell has been reported. Unlike typical MAIT cells that express Vα7.2 (also called TRAV1-2), the newly discovered MAIT cell subset is TRAV1-2 negative but expresses the atypical MAIT T cell receptor TRAV12-2. This receptor is MR1-restricted and has been shown to uniquely detect antigens from *Streptococcus pyogenes*, which lack the riboflavin pathway (Meermeier et al., 2016). This observation provides evidence for a new type of non-riboflavin MR1 antigen and indicates greater diversity in MR1/MAIT antigens than previously thought. As *S. pyogenes* can cause pneumonia and other respiratory infections (Tamayo et al., 2016)(Elmer et al., 2016), further work exploring the interactions of MAIT cells and *S. pyogenes* is necessary.

In addition to bacterial infection, a role for MAIT cells in viral infections is also plausible, given the fact that the data in this thesis have shown that MAIT cells can activate in response to just cytokine signalling. An antiviral role for MAIT cells in influenza infection has been previously shown, where IFNγ and granzyme B were quickly and robustly upregulated by MAIT cells in response to influenza-infected epithelial cells in a manner dependent on IL-18 derived from monocytes (Loh et al., 2016). In that study, the MAIT cell response was significantly greater than the conventional T cell response to influenza infection, similar to the data presented in this thesis with T cell responses and NTHi infection. Older reports have suggested that viruses could not activate MAIT cells, although this could be due to a lack of IL-18 (or other cytokines) in those systems (Le Bourhis et al., 2010)(Gold et al., 2010). The potential role for MAIT cells in viral infections is very interesting and may have implications in respiratory disease. A recent study has suggested that the increased risk of exacerbation in COPD may be driven by interaction of NTHi and rhinovirus co-infection in the airway (Wilkinson et al., 2017). It is therefore worth investigating whether MAIT cells are involved in viral infections on a chronic NTHi background and may therefore contribute to COPD exacerbations.

Outside of a role in infections, MAIT cells may have further implications for disease as MR1 can bind and present therapeutic drug-derived molecules such as salicylates and the nonsteroidal

anti-inflammatory drug, diclofenac, which have been shown to have either inhibitory or agonistic properties for MAIT cells (Keller et al., 2017). Treatment of patients with certain drugs may therefore inadvertently activate MAIT cells if these drugs produce agonist MR1 ligands, leading to immunopathology or sensitivity associated with these therapeutic drugs (Skypala et al., 2015). Alternatively, any drugs whose metabolites create inhibitory MAIT cell ligands may prevent MAIT cells from becoming activated and responding to infection, thus impairing the immune response.

7.5 Activation mechanisms and responses of conventional T cells and MAIT cells to NTHi are different

Few studies have previously explored conventional T cell responses to NTHi infection. The COPD lung is characterised by an increased CD8+/CD4+ T cell ratio, which may indicate a switch to a greater cytotoxic phenotype (Saetta et al., 1998)(McKendry et al., 2016). However, conventional T cells from COPD patients have been shown to express more IL-13 and IL-17 in response to NTHi infection compared to healthy controls (King et al., 2013), which may indicate an inappropriate response to NTHi in lung disease.

When comparing conventional T cells to MAIT cells, it was apparent that clear distinctions exist between these different T cell subsets. MAIT cells upregulate cytokines and cytotoxic markers more rapidly than conventional T cells, which may suggest that their role is to combat invading pathogens first whilst the adaptive arm of the immune system takes time to activate. In fact, cytokines or co-stimulation provided by MAIT cells may be required to activate conventional T cells either directly via cytokine stimulation or indirectly, such as MAIT cell maturation of DCs by CD40-CD40L signalling, which then allow the DCs to activate conventional T cells (Salio et al., 2017). MR1 knockout mice which lack MAIT cells display dysfunctional responses to *Francisella tularensis* infection, including impaired control of bacterial growth and reduced recruitment of conventional T cells into the lung (Meierovics et al., 2013), indicating that MAIT cells are important for inducing adaptive immune responses.

Data from this thesis have shown that the magnitude of responses between conventional T cells and MAIT cells is also different, which may be expected. Conventional T cells recognise a single specific peptide antigen from a highly diverse pool of antigens. Therefore only a minor increase in cytokine or cytotoxic markers by these T cells would be expected, as it is likely that only a very small percentage of the total conventional T cell population is specific for a given NTHi-peptide antigen. MAIT cells however recognise a limited repertoire of conserved antigens and therefore a

larger percentage of the total MAIT population can be activated, and this may explain why MAIT cells had the largest response to NTHi infection.

The antigen presentation blocking experiments showed that whilst interaction with MHC is vital for conventional T cell activation, MAIT cells appear to only rely on antigen presentation at certain timepoints (table 7.1). Although MR1 is structurally similar to MHC-I, its regulation on MDM in response to NTHi infection was more similar to MHC-II and upregulation of MR1 in infection may be a key control mechanisms in MAIT cell regulation. Furthermore, MAIT cell activation appears to have a greater reliance on cytokine stimulation, in particular IL-12 and IL-7 signalling, in line with previous reports (Chua et al., 2012)(Ussher et al., 2014a)(Leeansyah et al., 2015)(Tang et al., 2013). MAIT cells are also reported to have higher expression of the TLR4 co-receptor CD14 compared to conventional T cells (Singhania et al., 2017), which may represent a further non-antigen specific pathway that allows direct activation of MAIT cells by gram-negative bacteria. Overall, MAIT cells have the capacity to mount a large response to pathogens quickly, compared to the slower responses and smaller proportion of active conventional T cells. With the ability to greatly respond to cytokine stimulation in the absence of antigen, MAIT cells, but not conventional T cells, may be a source of bystander inflammation that could have detrimental consequences in the lung.

Table 7.1 – Overview of how MR1 antigen presentation and cytokine pathways control MAIT cell activation in the NTHi-infected MDM-T cell co-culture model. IFNγ production and CD107a upregulation by MAIT cells are only dependent on MR1 signalling early on in the timecourse, whereas granzyme B production by MAIT cells only requires MR1 signalling later in the timecourse. The IL-12 pathway contributes to IFNγ and granzyme B production by MAIT cells, but not CD107a upregulation. The IL-7 pathway appears to only contribute to MAIT cell granzyme B production.

	Activation Pathway			
Response	**MR1 (5 hours)**	**MR1 (22 hours)**	**IL-12**	**IL-7**
IFNγ	✓	✘	✓	**N/A**
Granzyme B	✘	✓	✓	✓
CD107a	✓	✘	✘	✘

7.6 Exhaustion pathways may differentially regulate innate and adaptive T cell responses

As discussed previously, one way T cells are regulated is by accessory molecules such as the exhaustion molecule PD1. Signalling via PD1 typically causes inhibition of T cell proliferation and cytokine release (Keir et al., 2007) and occurs with prolonged antigen presentation and T cell activation. PD1 may be involved in chronic respiratory diseases as PD1 expression is increased on T cells from COPD patients suggesting an involvement in COPD pathogenesis (Kalathil et al., 2014)(McKendry et al., 2016). Expression of PD1 and the role of the PDL/PD1 axis was explored in this thesis and revealed that this pathway may regulate the granzyme B response of conventional T cells, but not MAIT cells. This is despite the confusing observation that PD1 expression is elevated on MAIT cells, but not elevated on conventional T cells, in response to NTHi. CD107a and IFNγ expression measured by flow cytometry were not significantly affected by PDL/PD1 blocking in any T cell subset, although ELISA for IFNγ release into supernatant following PDL1 blocking suggested a trend for an increase in IFNγ production, which may be significant with more repeats. The differences between PD1 regulation of conventional T cells and MAIT cells may have implications for diseases treated with anti-PD1 therapy, as conventional T cell function may be restored by treatment but MAIT cell function is not. Other exhaustion markers were not investigated in this study, such as T-cell immunoglobulin and mucin domain (TIM)-3, as it is not expressed on lung T cells, whereas PD1 is (McKendry et al., 2016).

The reasons why PD1 does not seem to affect MAIT cell responses but does increase conventional T cell granzyme B in this model are not known. It is possible that as MAIT cell granzyme B expression is very high in response to NTHi infection, it cannot be upregulated further following blocking of PD1 signalling. The disease state may also determine whether PD1 is involved or not. Blocking of PD1 has been shown to increase production of IFNγ from BCG-stimulated MAIT cells from patients with active TB, but PD1 blocking has no effect on BCG-stimulated MAIT cells from healthy controls (Jiang et al., 2014). This could explain why blocking the PD1 pathway had no significant effect on IFNγ expression in response to NTHi shown in chapter 5, as the T cells utilised were from healthy donors. It is also possible that as cytokine signalling plays a greater role in MAIT cell activation compared to conventional T cells, activation by pro-inflammatory cytokines may interfere with PD1 signalling. In a recent study, high expression of PD1 was shown on MAIT cells in chronic hepatitis infection, which did not respond well to *E.coli* antigens compared to MAIT cells from healthy controls, but had the same level of response as healthy controls following IL-12 and IL-18 stimulation (Hengst et al., 2016). It could be that in chronic exhaustion, MAIT cells

cannot respond well to antigen stimulation (due to elevated PD1) but activation due to cytokine signalling is unaffected, as the cytokine stimulation overrides the MR1 antigen presentation pathway. Whereas for conventional T cells, which largely require antigen presentation, exhaustion affects them to a greater degree.

7.7 Steroids may have detrimental consequences for long-term lung T cell immunity to bacteria

The effects of corticosteroids have been investigated in this study as their use in treating symptoms and reducing severity of exacerbations in COPD is potentially offset by increasing the risk of developing community-acquired pneumonia (Burge et al., 2000)(Calverley et al., 2007)(Festic and Scanlon, 2015)(Calverley et al., 2011)(Anzueto et al., 2009)(Woodhead, 2007)(Welte, 2009). However, the reasons why steroids may have this effect remain unknown.

In this study, corticosteroids inhibited the ability of both conventional T cells and MAIT cells to mount cytokine and cytotoxic responses to NTHi. This inhibitory effect of cytokine was not just restricted to T cell responses, as steroids also impaired NTH-infected MDM upregulation of MR1 and HLADR, and reduced production of IL-12. Whether the inhibitory effects of steroids is purely due to impaired macrophage function, which then would prevent activation of T cells, or due to steroids having direct inhibitory effects on both the T cells and MDM is unclear. Furthermore, MAIT cells are particularly sensitive to ICS as they cause a deficiency of MAIT cells, but not conventional T cells, in COPD patients, which could be due to apoptosis associated with elevated expression of steroid-induced caspases (Hinks et al., 2016)(Gérart et al., 2013)(Distelhorst, 2002). The consequences of steroid-induced suppression of both MAIT cell number and function are unknown, but as MAIT cells do respond to NTHi, it is possible that impairing MAIT cells may contribute to NTHi colonising the lower respiratory tract. Once in this niche, NTHi may either become pathogenic or act in synergy with other bacterial or viral infections to induce inflammation and immunopathology, which has implications for many respiratory diseases or in chronic infections associated with biofilms (Swords, 2012). Indeed, a recent study has shown that ICS use is associated with an increase in *H. influenzae* in the sputum of COPD patients compared to patients not receiving ICS (Contoli et al., 2017) and so it is possible that this overgrowth of *H.influenzae* may be due to steroid-induced impairment of MAIT cell function. Another study has also recently found that ICS use increases the risk of nontuberculous mycobacterial pulmonary disease (Brode et al., 2017)(Chalmers and Keir, 2017) which could involve MAIT cells, as they are

deficient in patients with mycobacterial infection and have impaired IFNγ production following bacterial stimulation (Kwon et al., 2015a).

7.8 Using appropriate models to study lung immunity

A strength of this thesis is that it has utilised human lung tissue to specifically study T cell responses to a respiratory pathogen, which should more accurately reflect what occurs in the lungs *in vivo*. Due to the nature of human tissue work, it is not always possible to perform large scale experiments due to insufficient yields of cells from lung tissue. Another issue with human lung tissue work is the large variability in the data. All of the lung tissue was obtained from patients who were undergoing lung resection for clinical reasons and therefore these patients may be on different treatments or have other underlying conditions, which may affect their expression of the cytokine or cytotoxic markers, or make their T cell less responsive to stimulation. The heterogeneity in the data may also be due to the fact that each individual will have a different microbiome or different history of NTHi exposure previously in their lifetime, all of which may impact the immune response observed in the NTHi challenge experiments. However, because the lung data are paired for each donor between uninfected and infected, any upregulation of cytokines or cytotoxic markers in the lung model indicates that there is an NTHi-inducible response, however small. It is also difficult to perform mechanistic investigations in the explant model, such as using blocking antibodies, due to the physical limitation of blocking antibodies not penetrating into the tissue. Overall this means that although it is preferable to use lung derived T cells to study lung immunity, these types of models have limitations. To overcome all of these issues, the blood-derived MDM-T cell co-culture was established and compared to the lung explant model to ensure that similar responses were observed. The co-culture was then used further to explore the T cell responses and utilised blocking antibodies to interrogate the involvement of antigen presentation and cytokine signalling.

Pathogens such as NTHi and diseases such as COPD and asthma are exclusively human and therefore studies utilising human models are essential. However, models using human cells and tissues can only provide so much information and animal models often allow further investigations which are not possible in human models. That being said, T cell studies may be hampered by confounding data from animal models (Beura et al., 2016). Although MAIT cells are present in mammals (Martin et al., 2009)(Tilloy et al., 1999)(Treiner et al., 2005), differences between human and mouse MAIT cells have been observed. Whilst human MAIT cells express Vα7.2 as part of their TCR, mouse MAIT cells express a different TCR, Vα19, and the implications

of this are not known (Reantragoon et al., 2013). Another key issue is that as many mice used in animal models are kept in germ-free/sterile conditions, these mice lack a microbiome and therefore have very few if at all any MAIT cells to begin with, as MAIT cell development is dependent on the presence of commensal gut flora (Tilloy et al., 1999)(Treiner et al., 2003)(Rahimpour et al., 2015). To combat this, many murine MAIT cell models use over expressing transgenic lines to enrich MAIT cell numbers (Reantragoon et al., 2013)(Chua et al., 2012)(Le Bourhis et al., 2010)(Martin et al., 2009), which may alter the MAIT cell phenotype and therefore confound the data. Indeed, in some of these mouse models, a higher percentage of MAIT cells have been reported to be CD4+, whereas in humans CD4+ MAIT cells appear to be relatively rare (Chapter 3)(Reantragoon et al., 2013)(Rahimpour et al., 2015). CD4+ MAIT cells are reported to have different functions compared to CD8+ or DN MAIT cells (Kurioka et al., 2017), and appear to be skewed to producing Th2 cytokines, which could result in different outputs seen in mouse MAIT cells versus human MAIT cells following stimulation. It has also been reported that IL-17a is abundantly produced by MAIT cells from some strains of mice with relatively little IFNγ production (Rahimpour et al., 2015), which is in complete contrast to the data reported in this thesis using human MAIT cells. Ultimately, studies exploring the role of human infectious diseases should utilise human models first which can then be explored further in animal models. Whilst animal modelling is important and very useful for the study of many diseases, MAIT cell experiments using mouse models should be interpreted carefully.

7.9 Limitations of the data

One of the limitations of the work presented in this thesis is that the lung explant data are limited to a small number of repeats, as the yield of cells obtained from tissue was low. This is one of the reasons why the mechanistic work with blocking antibodies utilises the blood-derived MDM-T cell co-culture model. However, one caveat of the blood model is that the T cells are obviously not lung-resident cells, which is an important consideration when interpreting the results. The role of antigen presentation and cytokine signalling for lung T cells may be different to that of the blood-derived T cells. A further limiting factor is that due to small samples numbers, the lung tissue data were not able to be categorised into COPD versus non-COPD groups, therefore the role of T cells and MAIT cells in the process of chronic lung disease cannot be conclusively determined.

7.10 Future Work

MAIT cells are only now becoming more widely acknowledged as an important arm of the immune response to pathogens, although they are still largely unstudied in the clinical setting, even though they may have therapeutic applications (Schäfer et al., 2017). Further investigation into the role of MAIT cells in diseases is warranted.

Further work using *ex vivo* lung tissue is required where possible to confirm that the observations from the blood co-culture accurately reflect what occurs in the lung. Increasing the number of repeats would improve the clarity of the lung explant data. Once a sufficient number of repeats has been obtained, the data can be split by COPD status and steroid use to determine whether steroids actually have a functional effect on T cells within the lung. Although blocking experiments are not possible in the lung tissue, lung T cells and APCs could be dispersed from lung tissue and a lung T cell-APC co-culture set up, in a similar fashion to a recently published model (Hutton et al., 2017), which would allow further mechanistic investigation. Expression of co-stimulatory markers on lung T cells was only measured at baseline due to low cell numbers. However, baseline expression does not show whether infection can affect these molecules, so further work should address this. Immunohistochemistry should also be performed to confirm that macrophages and MAIT cells directly interact in the lung.

As IL-12 and IL-7 have been shown to promote MAIT cell IFNγ and granzyme B expression, the effect of other cytokines on MAIT cell activation should also be investigated. IL-1β has been shown to promote IL-17a production by MAIT cells (Turtle et al., 2011). However, other factors must be involved in the MDM-T cell co-culture, as large amounts of IL-1β were detected in response to NTHi infection but IL-17a expression by MAIT cells was minimal in the co-culture. Given that the MAIT cell IL-17a signal was greater in the lung, it seem likely that other lung-derived cytokines are also required. Future work is needed, such as IL-1β blocking antibodies, to determine what role IL-1β plays.

In terms of other responses, the killing assay could be expanded as it has only shown that MAIT cells are able to kill NTHi-infected MDM. It is not known whether this killing function is mediated by MR1, IL-12 and/or IL-7 in this model. A comparison of the cytotoxicity of conventional T cells to NTHi-infected MDM would also be interesting and provide further data on the similarities and differences to MAIT cells. Investigation of other pathways MAIT cells may use to kill infected cells is also needed, such as the FAS/FAS ligand pathway or whether MAIT cells can release molecules that have direct antibacterial effects, indicated by a recent report where granzymes and granulysin exerted killing effects directly on bacteria (Walch et al., 2014).

As the PDL/PD1 pathway appears to regulate conventional T cell but not MAIT cell granzyme B response, further investigation into the role of PD1 and MAIT cells is required. If MAIT cells do not respond to PD1 signalling but are affected by other inhibitory pathways, therapies utilising PD1 blocking may not rescue MAIT cell function and therefore an arm of the immune response may remain dysfunctional, impairing an adequate immune response. Other inhibitory or exhaustion markers could be explored, such as LAG-3 or TIGIT (Anderson et al., 2016). The PDL1 blocking data revealed an increase in IFNγ measured by ELISA, which was not significant; increasing the number of repeats may reveal a significant result. T cell populations may also need to be cultured separately, as CD4+ T cells may not be affected by PDL1 block, but CD8+ T cells could be.

MAIT cells are deficient in COPD patients receiving ICS therapy (Hinks et al., 2016) but in the co-culture model, steroids did not reduce MAIT cell numbers, although this was only a 22 hour co-culture which may be too acute to see any effects of steroids on MAIT cell apoptosis or proliferation. Extending the co-cultures past 24 hours may reveal whether *in vitro* steroid treatment causes MAIT cell reduction.

Expanding the co-culture model to involve other pathogens will be of particular interest, especially involving co-infection with NTHi and rhinovirus as these two pathogens may interact to promote COPD exacerbations (Wilkinson et al., 2006)(Wilkinson et al., 2017). As MAIT cells can respond to viral infections as well as bacterial (Loh et al., 2016), they may play a role in airway diseases associated with co-infections. Macrophages may also be involved in this process, as NTHi appeared to reduce IFNβ gene expression (although corresponding protein levels would need be confirmed), which may be responsible for the diminished interferon response to viral infection observed in COPD BAL (Mallia et al., 2011). Therefore, NTHi could predispose macrophages in the lung to have an impaired response to viral infection. Indeed, NTHi infection has already been shown to increase sensitivity to human rhinovirus and create an exaggerated inflammatory response to RSV (Gulraiz et al., 2014).

With many inflammatory diseases, a combination of multiple cell types and inflammatory pathways all work together to induce immunopathology. As well as macrophages, epithelial cells play an important role in airway defence, are also targets of NTHi (Morey et al., 2011)(Frick et al., 2000)(Goyal et al., 2014) and did express a small level of MR1. Adapting the co-culture model to investigate the role of epithelial cells as targets for MAIT cells in NTHi infection is required. Other cytokines released by activated MAIT cells may have a role in regulating barrier function and epithelial immunity. IL-22 is expressed by MAIT cells (Leeansyah et al., 2014)(Serriari et al., 2014)(Gibbs et al., 2016) and may contribute to barrier function and induce innate immunity of

epithelial cells in host defence against bacterial pathogens, which could be a pathway by which the lung microbiome is innately controlled (Wolk et al., 2004)(Zheng et al., 2008)(Sakamoto et al., 2017). IL-22 production by NTHi-activated MAIT cells needs to be investigated. Another mechanism by which epithelial cells contribute to antimicrobial immunity is by transporting IgA across the bronchial epithelium, but this process becomes impaired in COPD (Polosukhin et al., 2011). IgA is a mucosal antibody which is effective against NTHi, and may limit NTHi colonisation (Faden, 2001). Therefore MAIT cell induced-activation of epithelial cells may also be important in facilitating mucosal antibody immunity to NTHi and should be explored. Although as mentioned previously, NTHi secrete IgA proteases, which may allow certain strains to persist in the respiratory tract and induce exacerbations (Cerquetti and Giufrè, 2016)(Murphy et al., 2015). Overall, the role of MAIT cells and epithelial cells in response to NTHi infection should be studied further.

Overall it seems likely that the role of the macrophage is to prime/activate MAIT cells via antigen presentation, as well as co-stimulation and secretion of cytokines such as IL-7, IL-12 and IL-18. Activated MAIT cells can then release cytokines and recruit other immune cells or recognise other target cells, such as infected epithelial cells in the airways, and induce cell death (Ussher et al., 2014b). If the ability of macrophages to activate MAIT cells, or even conventional T cells, is impaired, this may contribute to T cell dysfunction and therefore the role of macrophages in T cell/MAIT cell activation in respiratory disease also requires further understanding.

The main question yet to be answered is whether MAIT cells are important in respiratory disease. It is not known whether MAIT cells have diminished effector function that lead to bacterial overgrowth and inflammation, or whether MAIT cells become hyperactive and induce inflammation. If it is the former, restoring MAIT cell function could be an important therapeutic strategy. Appropriate use of ICS in patients may be required to ensure that patients are not receiving steroids unnecessarily which could be impairing long-term MAIT cell function. Augmenting the MAIT cell response to bacteria could also be utilised. Emerging evidence indicates that MAIT cell responses to bacteria can be enhanced by pre-stimulation with synthetic MAIT cell antigens and TLR stimulation (Hinks et al., 2017), which means that MAIT cells may exhibit a recall memory response and could be utilised in vaccinations. A role for innate T cells in NTHi vaccinations has been previously shown, as mice immunised with NTHi P6 protein and the NKT cell antigen α-galactosylceramide demonstrated protective immunity of NKT cells to NTHi following infection (Noda et al., 2010)(Noda et al., 2011). If MAIT cells are impaired and cannot control NTHi infection, manipulation of MAIT cells by inhaled vitamin antigens to improve effector function could be a potential therapeutic strategy. However, if MAIT cells are overactive in

chronic diseases and induce excessive amounts of inflammation by inappropriate responses, therapies aimed at reducing MAIT cell activation, such as by blocking MR1 antigen presentation could be employed. It is here that animal models could be utilised to study therapeutic manipulation of MAIT cell in disease, although as discussed above, using mouse models to study MAIT cells needs to be considered carefully.

7.11 Summary

In summary, MAIT cells have potent cytokine and cytotoxic responses to NTHi infection, and MAIT activation appears to be driven by both MR1 antigen presentation and IL-12 and IL-7 signalling. Conventional CD4+ and CD8+ T cells also respond to NTHi infection, although with smaller responses compared to MAIT cells, and their activation is dependent on MHC molecules. Corticosteroids significantly inhibited the activation of all T cell subsets explored in this study and this may have considerable clinical consequences (figure 7.1).

This thesis has not investigated whether MAIT cells (or conventional T cells) are directly involved in COPD pathogenesis or whether they predispose some individuals to developing COPD. Immune interactions may keep the microbiome in check, preventing commensal bacteria from becoming pathogenic; a process which may become impaired in a variety of respiratory diseases.

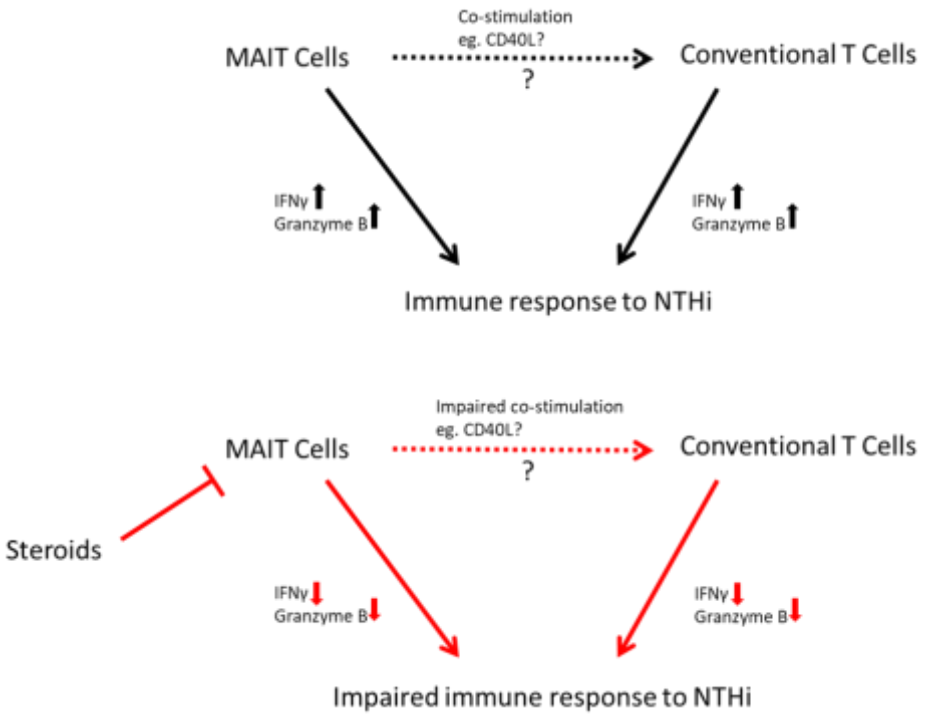


Figure 7.1 – Overview of main findings. MAIT cells and conventional T cells produce IFNγ and granzyme B in response to NTHi infection. MAIT cells may also promote conventional T cell activation. The presence of steroids impairs both MAIT cell and conventional T cell responses to NTHi, which may have detrimental clinical outcomes.

The question remains, are T cells and MAIT cells helpful or harmful in COPD? Is the paradigm that they are overactive and start responding to commensal bacteria inappropriately, contributing to unnecessary inflammation? Does the COPD inflammatory environment, such as smoking-induced cytokine production by macrophages skew/prime the T cells into an activated state, which causes them to induce inflammation and respond to bacteria they previously ignored? Or are they impaired, either by cigarette smoke exposure or by steroids, and are unable to protect the airways from further bacterial infections, leading to bacterial overgrowth? Most likely it is a combination of the two, which occurs at different stages of disease. As inflammation leads to damage of the lung architecture and exacerbations of COPD result in a decline in lung function and increase mortality, the development of an efficient immune response may be vital for a better prognosis. Understanding the mechanism behind innate and adaptive T cell responses to the airway microbiome may determine how T cells contribute to respiratory diseases and aid the development of potential treatments such as vaccines for NTHi and other respiratory infections.

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