College of Public Health, Medicine and Veterinary Science

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DECONSTRUCTING THE IMMUNOPATHOGENESIS OF LUNG INFECTIONS

Thesis submitted for the degree of
Doctor of Philosophy

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Abstract

Lung disease caused by non-tuberculous mycobacteria (NTM) are growing increasingly common globally. *Mycobacterium avium* complex (MAC) and *Mycobactereoides abscessus* complex (MABS) are the leading causative species groups, world-wide. These infections are highly antibiotic resistant, even more so than the more virulent *M. tuberculosis*. Intensive therapy with multiple antibiotics for 12 to 18 months is required, often accompanied by serious drug side effects, poor compliance, and increased morbidity in patients. Despite treatment, relapse or persistence of infection is a common clinical problem. Escalating health care costs associated either directly with the expensive treatment regimens, or indirectly due to frequent admissions to tertiary care centres and high all-cause mortality have resulted in this disease becoming a rising research priority. Despite years of research into NTM infection, particularly MAC infection in the immunocompromised, little is known about how and why these infections occur in apparently immune competent populations. Whether the diversity of risk groups indicates a diversity of immune susceptibility or whether the disease is the same in all patients is not known. T cell immunity is essential but how it is perturbed in infection and whether the perturbation is the same in all infections is not known. The objective of this study was to answer some of these fundamental questions.

The study was conducted in two stages. The first stage was a pilot study of MABS infection in patients with cystic fibrosis (CF), a young patient group with structural lung compromise, as well as elderly patients with no known immune compromise. The second stage was an in-depth study of MAC and MABS infection in elderly patients during active infection, disease remission and persistent infection. Peripheral blood mononuclear cells (PBMCs) from patients and controls, both within-disease controls and healthy controls were compared by phenotype and function.

Key findings of this study are that MAC and MABS induce characteristic T cell exhaustion marker phenotypes with MAC increasing TIM3 expression on predominantly $CD8^+$ T cells while MABS increased CTLA4 expression on predominantly $CD4^+$ T cells. Quantification of $CD4^+$ and $CD8^+$ T cell gene expression revealed signatures that could distinguish active infection from controls as well as differentiate infecting species group based on peripheral blood analysis. Functional interrogation of cells showed no broad underlying deficits that could predispose elderly patients to infection but revealed that CF patients predisposed to MABS

infection had reduced TNF secretion capacity. Antigen specific immune response assessment proved more informative in elderly patients with MAC infection, showing antigen specific proliferating $IFN\gamma^+$ TNF^+ $CD4^+$ T cells were the hallmark of disease remission. In vitro testing of the α-PD1 checkpoint inhibitor Nivolumab resulted in significant increase in IFNγ levels in all patient groups except patients with persistent infection, who had PBMCs that were apparently refractory to checkpoint modulation.

MABS lung disease in patients with CF and the elderly are immunologically different entities. MAC and MABS infection in elderly patients are immunologically different entities. These differences can be exploited to develop much needed diagnostic and screening tests that will make clinic level risk-group screening practical rather than cumbersome and costly. The immune signatures identified, both phenotypic and genotypic, show new, druggable pathways that could be used as adjunctive treatment. With appropriate immune manipulation, reduction in duration and intensity of these toxic antibiotic regimes are a real possibility. Persistent infection however, seems better managed by prevention rather than cure. The finding that persistent infection is a turning point that takes place after onset of treatment makes further work to identify the immune correlates of this decisive moment necessary. Collectively, these results offer new avenues of diagnosis and treatment for this emerging infectious disease.

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List of Abbreviations

APC	Antigen presenting cell
BAL	Broncho-alveolar lavage
BCG	Bacillus Calmette Guerin
BMI	body mass index
BTLA	B and T lymphocyte attenuator
CBA	Cytometric bead array
CD40L	CD40 Ligand
CF	Cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator gene
CLR	C-type lectin receptor
CM	central memory
COPD	chronic obstructive pulmonary disease
CPS	Capture probe set
CTLA4	Cytotoxic T lymphocyte associated protein 4
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
ELISA	enzyme linked immune-sorbent assay
EM	effector memory
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
GORD	Gastro-oesophageal reflux disease
HTS	High Throughput System
ICS	Intra cellular staining
IFN?	Interferon gamma
IL-	Interleukin
iNOS	inducible nitric oxide synthase
JAK1	Janus Kinase 1
KLF2	Kruppel Like Factor 2
LCMV	lymphocytic choriomeningitis virus
LOOCV	Leave one out classifier
LTBI	Latent tuberculosis infection

MABS	*Mycobacterium/ Mycobactereoides abscessus* complex
MAC	*Mycobacterium avium* complex
MFI	Median fluorescence intensity
MoDCs	Monocyte derived macrophages
MSMD	Mendelian susceptibility to Mycobacterial Disease
MTB	*M. tuberculosis*
NEMO	NFκβ essential modulator
NK	Natural Killer cells
NKT	Natural Killer T cells
NLRP3	NOD- LRR- and pyrin domain containing 3
NOD2	Nucleotide binding oligomerization domain protein
NTM	Non tuberculous mycobacteria
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCA	Principle component analysis
PD1	Programmed cell death protein -1
PD-L1	Programmed Death Ligand -1
PGE2	Prostaglandin E2 receptor
PID	primary immune deficiency
PMA	phorbol-12-myristate-13-acetate
PNTM	Pulmonary non tuberculous mycobacteriosis
PPD	Purified protein derivative
PTGER2	prostaglandin E2 receptor subtype 2
QLD	Queensland
QMRL	Queensland Mycobacterial Reference Laboratory
R10	RPMI +10% Foetal calf serum
RCS	Reported code set
RDA	Redundancy analysis
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
S1PR1	Sphingosine - 1- phosphate receptor 1
SCID	severe combined immunodeficiency
SPICE	Simple Presentation of Incredibly Complex cytometry data

STAT5A	Signal transducer and activator of transcription 5A
TB	Tuberculosis
TCR	T cell receptor
TEMRA	Terminally differentiated effector memory cell with RA phenotype
Tfh	T follicular helper cells
Th	T helper
TIM3	T cell Ig and mucin domain 3
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	T regulatory cell
t-SNE	t distributed stochastic neighbour embedding
ViSNE	Visual stochastic neighbour embedding

Chapter 1: Introduction

1.1 Non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) are ubiquitous, free living, environmental saprophytic organisms known to inhabit water systems, soil and vegetation. Belonging to the genus *Mycobacterium* (like the better-known *M. tuberculosis* and *M. leprae*), there are over 150 identified species of NTM with that number increasing each year (1-3). NTM are microaerobic organisms which grow in 6-12% oxygen and have lipid-rich cell walls and metabolic characteristics that result in a slow doubling time for the bacteria (approximately 20-24 hours) (2). These organisms can withstand a wide range of environmental temperatures, do not readily grow in standard bacterial culture media, and are antibiotic and disinfectant resistant. Given these characteristics, NTM are found worldwide and cause infections that are easily missed, difficult to diagnose, and difficult to treat.

First described in the late 19th century (soon after Robert Koch's seminal description of *M. tuberculosis* as the causative agent of tuberculosis in 1882), decades passed before human infection with NTM was identified (4). Since then over 90 species have been identified from human samples with several more remaining either unclassified or unidentified (5). NTM are conventionally classified as 'slow' or 'rapid' growers and this classification remains in use as it provides a practical, easy way to narrow down possible species in the diagnostic setting, particularly when molecular methods are not available. Species classification based on biochemical characteristics, however, are gradually being replaced by 16S rRNA sequencing which has revealed a great deal of complexity within the genus (3). Human infection is most commonly caused by the slow growing *Mycobacterium avium* Complex (MAC) which now includes *M. avium* subspecies *avium*, subspecies *silvaticum*, subspecies *hominissuis*, and subspecies *paratuberculosis*, *M. intracellulare*, *M. arosiense*, *M. chimaera*, *M. columbiense*, *M. marseillense*, *M. timonense*, *M. bouchedurhonense* and *M. ituriense* (2). Other common NTM isolated in relation to human disease include *M. xenopi, M. fortuitum* complex, *M. kansasii* and the rapidly growing *M. abscessus* group (MABS) which were recently grouped as a separate clade named *Mycobacteriodes abscessus* based on phylogenetic characteristics (6). This group includes subspecies *abscessus sensu stricto*, subspecies *massiliense* and subspecies *bolletii* (5, 7). Collectively, these species comprise 80% of NTM isolated globally from clinical specimens (5).

The natural habitats for NTM range from natural brackish and marshy waters to municipal water distribution systems and household plumbing including shower heads (8). NTM are also found in potting soil and other peat rich soils. This overlap of bacterial habitat with human habitation provides an ideal opportunity for human infection. The lipid-rich hydrophobic cell walls of these organisms are ideal for biofilm formation which allows long-term persistence of bacterial colonies that are effectively resistant to disinfectants and aerosolization, particularly from shower heads, as well as soil dusts which facilitate inhalation and pulmonary infection (9, 10). Organism density in shower aerosols is significantly higher than in the main water stream and is thought to be the most likely source for pulmonary infection (2, 11). More disturbingly, NTM have also been identified in hospital ice machines and water-cooling systems and haemodialysis unit water supplies. Exposure to these organisms is therefore likely to occur widespread from home to healthcare (2, 4, 12, 13). Recent evidence of person-to-person transmission of MABS has revealed another source of infection that is discussed in section 1.12 (14).

1.2 Pulmonary NTM infection

NTM disease presents with a wide variety of clinical syndromes, from aseptic meningitis to sterile pyuria and lymphadenopathy (commonly cervical lymph nodes) as these organisms can infect every system of the body. Infection of the lung is one of the most common clinical manifestations. Termed pulmonary NTM disease (PNTM), this manifestation is an evolving and complex pathology with many questions including the mode of transmission, the range of incubation periods and the true disease burden remaining unresolved. Three forms of PNTM are described based on distinct pathology. The three forms comprise fibro-cavitary disease, nodular bronchiectatic disease and hypersensitivity pneumonitis. Given the generally low virulence of these organisms together with their slow growth rate, onset of disease symptoms is often insidious. Incubation periods can vary from months to years making diagnosis difficult and tracing the source of infection nearly impossible. A rise in the number of NTM infections documented globally has led to NTM being officially recognized as emerging pathogens that cause significant morbidity and mortality in both immune competent and immune compromised populations (1, 15, 16). *Mycobacterium avium* complex (MAC) and *Mycobacterioides abscessus* complex (MABS) are the most common organism groups causing PNTM worldwide (17-19)

1.3 Risk groups

NTM are considered opportunistic pathogens to humans. Exposure to these organisms in day-to-day life is common, through shower aerosols and hot tubs, but infection and clinical disease occur only in a small percentage of those exposed (10). Over the last decades it has become evident that several groups of patients are especially prone to NTM lung disease. These include patients with structural lung diseases such as cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), non-CF bronchiectasis, alpha-1 antitrypsin deficiency, allergic bronchopulmonary aspergillosis, previous pulmonary tuberculosis and lung cancer (20-22). Patients with immune suppression due to primary immune deficiency syndromes (PIDs) such as Mendelian Susceptibility to Mycobacterial Disease (MSMD) associated with IL12-p40 and IL12 and IFNγ receptor abnormalities – *IFNγR1, IFNγR2, IL12RB1, IL12B, STAT1, IKBKG, CYBB, ISG15, IRF8, GATA2* gene deformities), severe combined immunodeficiency (SCID), or autoantibodies to IFNγ are susceptible to NTM infection (23-25). In addition, patients with acquired immunodeficiency syndromes including AIDS, haematological malignancies, those on immunosuppressive drugs including inhaled and oral corticosteroids and cytotoxic medication such as those given to stem-cell and solid organ transplant recipients have been identified as susceptible to NTM infection (26, 27). These patients however, usually develop disseminated NTM infection rather than isolated pulmonary disease and are considered a separate risk group.

The increase in scientific research into the epidemiology, diagnostics and treatment of this once obscure group of diseases stems from the increasing numbers of cases being identified from populations with previously unknown and currently unidentified risk factors (1). Advances in therapeutics in all fields of medicine have seen unexpected NTM disease susceptibilities emerge which pose a challenge in terms of patient care, but which also provide insight into disease pathology. The susceptibility of patients with rheumatoid arthritis started on anti-TNF therapy (infliximab, adalimumab, golimumab and certolizumab) to NTM infections is a prime example of unexpected NTM susceptibility (28-32).

A fourth disease susceptibility group include elderly white post-menopausal females who present classically with NTM infection of the middle or lingular lobe of the lung. Described as “Lady Windermere syndrome” these patients often have a distinct physical phenotype of slender build, pectus excavatum or scoliosis and mitral valve prolapse, though notably they have no known immune dysfunction (20, 25, 33, 34). Recently identified genetic defects that

could contribute to susceptibility in these 'Lady Windemere' patients include cystic fibrosis transmembrane conductance regulator gene (CFTR) related mutations, ciliary function and other connective tissue related genetic defects as well as the DNA damage response protein TTK defects (26, 35-37). Finally, gastro-oesophageal reflux disease (GORD), vitamin D deficiency, rheumatoid arthritis and low body mass index (BMI) are known NTM lung disease associated comorbidities (32, 38).

1.4 Global disease burden

Studies from the North America, Europe and Asia have all shown increasing NTM disease incidence over the last two decades. Estimated NTM disease prevalence rose from 2.4 cases/100.000 in the early 1980s to 15.2 cases/100,000 in 2013 in the US (39). The prevalence in the elderly (>65 years) population more than doubled from 20 cases/100,000 to 47cases /100,000 population between 1997 and 2007 (40). A study on clinical laboratory reports in five states in the US showed NTM positive culture rates increased from 8.2 per 100,000 persons in 1994 to 16 per 100,000 persons in 2014 (41). Similar figures are recorded in a Canadian study published in 2017 with NTM pulmonary disease prevalence increasing from 4.65 cases/ 100,000 in 1998 to 9.08 cases/ 100,000 in 2010, while laboratory isolation rate increased from 11.4 isolates/ 100,000 in 1998 to 22.22 isolates / 100,000 population in 2010 (42). The prevalence of NTM disease in non-cystic fibrosis (NCF) bronchiectasis in the US is estimated as 37% with the most common isolate being MAC (40). Laboratory isolation of NTM is now more common than *M. tuberculosis* in the US and Canada with an increase of 8.4% annually being documented between 1997 and 2003 (21).

A study from the UK shows similar trends with the NTM infection rates more than tripling from 0.9 cases / 100,000 in 1995 to 2.9 cases/ 100,000 population in 2006 primarily due to increase in PNTM disease in the elderly (43). Similar rates have been documented in Denmark (44) and Germany (45).

Studies in South Korea showed a 62% increase in NTM lung disease from 2002 to 2008 with a marked increase in MABS infection (46). This is in contrast to European studies that show a predominance of MAC infection (47, 48) Data from Japan has shown a marked increase in both NTM infection and mortality from 1994 to 2010 (49) while a population-based Chinese study showed an increase in NTM isolation rate from 3% to 8.5% from 2008 to 2012 (50). As NTM disease is not a 'notifiable' disease in most countries, accurate epidemiological data is

limited, particularly in countries with low development indices'. Nonetheless, an increasing number of NTM cases have been recorded in Brazil, Taiwan and the Middle East (51-55).

Globally, the most common NTM pathogens are the MAC organisms though prevalence varies greatly with geographic region, gender and age (52). MABS is a significant problem particularly because of very high levels of antibiotic resistance and the disease is a growing problem in East Asian countries including Japan, Korea and Taiwan (56). NTM are also a particularly difficult problem in patients with cystic fibrosis who are highly prone to MABS infection (43).

1.5 Local disease burden

NTM pathology has been a notifiable disease in Queensland (QLD) since the inception of the tuberculosis (TB) control programme in the 1960s and is currently notifiable under the *Queensland Public Health Act of 2005* (57, 58) . The increase in disease incidence in QLD over the last several decades has been clearly documented. Clinical cases of MAC disease were reported as 0.63 cases/100,000 population in 1985, 1.21 cases/100,000 population in 1994 and 2.2 cases/100,000 population in 1999 (57). Significant NTM species isolation rate rose from 9.1 cases/100,000 to 13.6 cases/100,000 from 1999 to 2005. 1171 isolates were reported in 2016 (up to 27th November), which is almost double the 672 isolates reported for the same period in 2012 (58). An increase in MABS isolates was also seen during this period. A change in the gender distribution from male predominance in 1999 to female predominance in 2005, particularly in the elderly population was observed (57). Overall, a pattern of increasing non-cavitary disease in elderly females at a rate of 2.2 cases/100,000 to 3.2 cases/100,000 population per year has emerged. Subsequent investigation showed MAC and MABS are present in household and municipal water sources and shower aerosols in QLD homes (59-61). An increase in NTM disease was also documented in the Northern Territory (NT) from 1989 to 1997 though the data are not as extensive or comprehensive as the QLD data (62).

The availability of data in QLD highlights the importance of surveillance in recognizing emerging disease threats. Improved diagnostics and greater awareness probably accounts for part of global increase in NTM lung disease prevalence. However, by the same argument, this would also mean that in areas where notification is poor, awareness is low and diagnostics and

rudimentary the disease still goes largely unrecognized and what we currently see is still the tip of an indolently progressing ice-berg.

1.6 Cystic fibrosis and NTM lung disease

CF is an autosomal recessive disorder caused by mutation in the CFTR1 gene leading to viscid mucus accumulation in airways. Disease progression is characterized by early onset airway inflammation due to bacterial colonization and infection, generally followed by airway remodelling and bronchiectasis in later life with chronic infection (63). CF is the most common life-limiting autosomal recessive disorder in people of European descent (64). 1 in 2,500-3,500 children of northern European heritage have CF with 1 in 25 being carriers (65, 66). In Australia, 3,235 CF patients were registered in the Australian Cystic Fibrosis data registry in 2013, with approximately 50% being adults. QLD recorded the 2nd highest number of registrants with 796, of whom 50% were male (67). Chronic infection is the most common complication of CF and NTM infection is a well-documented cause or morbidity and mortality in these patients (68, 69). Similar to trends seen in the general population, an increase in NTM infection in CF patients has been documented from studies performed in the UK, France, Scandinavia and the US (48, 70-75).

Cultures from CF patients have an approximately 10,000-fold higher NTM prevalence compared with the general population (76). NTM isolation rates in CF vary from 3% to 17% with an increase in median prevalence from 9% to 13% seen in pre- and post-millennial studies (77). Increased prevalence of NTM positive cultures is seen with increasing age (78). Prevalence rates in the Australian adult CF population was 4.1% in a 2001-2014 retrospective study carried out in Queensland (79). Though not as common as other bacterial pathogens, NTM infection was recognized as an important clinical entity in these patients as it was associated with significant deterioration in lung function (80). Chronic persistent infection is seen as a relative contra-indication for lung transplantation. Transplant is the last resort available to these patients as lung function deteriorates (81-84). A geographical variance is seen in NTM species prevalent in the CF population, though MABS and MAC infection remain the most common in most regions (77). In the QLD CF population, MABS infection is the most common organism isolated which has caused concern among clinicians and patients alike as antibiotic resistance rates are very high in this patient cohort (85).

1.7 Treatment, complications and economic burden

In general, PNTM treatment requires prolonged (>12 to 18 months) multi-drug therapy (86). Disease remission rates vary depending on infecting species, patient age and comorbidities (40, 87). Recurrence is common with rates of 30-50% being recorded in MAC infection (88). MABS infection is more likely to result in treatment failure and recurrence. Many patients develop persistent chronic infection despite treatment while others succumb to the disease (7, 87). Side effects of antibiotics are numerous, and regimes are difficult to tolerate. High costs (estimated at USD $14,730 for MAC infection and USD $47,240 for MABS infection) (87) associated with long term treatment with multiple antibiotics, increasing resistance to the antibiotics available and increasing prevalence of disease, with some evidence of person-to-person transmission, make MAC and MABS infection a serious and highly expensive health care problem (87). A multicentre study of MAC infection across Canada, France, Germany and the UK conducted in 2018 showed average direct medical costs per person year ranged from $US12,200 in Canada to $US25,500 in France (89). In addition to direct disease related costs, these patients were also shown to have almost six times higher secondary care utilization events for disease-related and -unrelated illnesses. (22)

Adjuvant therapies have been tested with little success. Preliminary trials of adjunctive IFNγ therapy were abandoned due to lack of response (90-92) although early case studies performed in patients with refractory disease showed promise (93, 94). IFNγ therapy (by intramuscular injection, as opposed to the original trials done with nebulized IFNγ) showed promise in a recent Cuban study (95) but no other studies have supported this data in recent times (38). Other immune modulatory agents tested include recombinant IL12 in mice (96, 97) and GM-CSF in HIV infected patients as well as recent case reports of successful use in two CF patients with MABS infection (98, 99). A phase 2 open labelled drug trial is currently underway to test the efficacy of inhaled GM-CSF in persistent NTM infection (ClinicalTrials.gov Identifier: NCT03421743)

1.8 The host-bacterial interaction

NTM are not primary pathogens, rather they are environmental saprophytic organisms that make use of the new living opportunity presented when human habitation and bacterial

habitation overlap. What percentage of a given population are exposed, how infection occurs and what host and bacterial factors determine whether the organism is cleared completely, establishes itself as a colonizer without causing tissue invasion, causes latent infection or progresses to a symptomatic active infection are yet to be determined. All that is currently known is that specific groups of patients are at risk, some with known immune dysfunction and others with specific characteristics that define them as a group, but it is not yet understood why these individuals are susceptible to infection. To understand NTM pathogenesis and pathomechanisms, similarities to what is known about the immune response to tuberculosis are likely to be further investigated.

1.9 Immune response in tuberculosis

The immunopathogenesis of tuberculosis (TB) has been well studied for decades. Though much light has been shed on the immunological progression of this disease in the human lung, the exact mechanisms that underlie formation and propagation of the characteristic tubercle, immune correlates of sterilizing immunity and long term protection are yet to be deconstructed (100). The story uncovered so far is one of a complex, multi-pronged interactome between the infecting bacilli and the innate and adaptive cells of the hosts' immune system. A complex equipoise that shifts one way and then another depending on a multitude of factors not yet fully understood. Current understanding is that initial infection occurs via bacterial aerosols inhaled into the lung alveoli. *M. tuberculosis* (MTB) primarily target alveolar macrophages for infection while intra epithelial dendritic cells (DCs) and other antigen presenting cells (APCs), lung epithelial cells and neutrophils can also be infected (101).

The innate immune response

Toll-like receptors (TLRs) 2, 4 and 9 (pattern recognition receptors- PRRs- on the surface of antigen presenting cells) recognize mycobacterial lipoproteins (TLR2) and mycobacterial DNA (TLR9) and signal via the TLR adaptor molecule Myeloid differentiation primary response 88 (MYD88 - which is also key in IL1β and IL1α signalling via IL1R1) resulting in activation of macrophages (102). Macrophage activation is a key step in the mobilisation of the innate immune response. Currently defined pathways include activation of NFκβ pathways, inducible nitric oxide synthase (iNOS) and secretion of TNF, IL1β and IL1α, IL12p40 and IFNγ (101, 103, 104). C-type lectin receptor molecules (CLR) including dectin 1 and mincle

also recognize mycobacterial antigens and mediate activation through signalling via adaptor molecule CARD9 (105). Cytosolic pattern recognition receptors nucleotide binding oligomerization domain protein 2 (NOD2) and NOD- LRR- and pyrin domain containing 3 (NLRP3) also recognize substances secreted by the bacterium. Together these PRRs activate macrophages and stimulate intracellular bacterial killing as well as IL12p40 dependent migration of antigen laden dendritic cells (DCs) to draining lymph nodes for antigen presentation (105, 106). T cell Ig and mucin domain 3 (TIM3), which is an exhaustion marker and regulatory molecule expressed on T cells as well as monocytes, macrophages and DCs, has been shown to decrease in monocytes in active TB patients (107).

Bacilli infect macrophages while simultaneously inhibiting macrophage activation and phagosomal maturation pathways resulting in bacilli escaping lysosomal killing (105). Bacilli also inhibit apoptosis of infected cells which allows prolonged bacterial survival and release of large numbers of viable bacteria into tissue (105). Studies have also shown that bacilli are able to reduce innate recognition via TLRs thereby reducing the number of activated macrophages recruited to the site of infection. Other immune evasion mechanisms include modulation of CCL2 and CCR2 that drive the recruitment of susceptible macrophages thereby increasing the number of cells available for infection, while simultaneously limiting the innate response (100, 108). This recruitment of 'permissive macrophages' is seen as an important step during early infection. Innate immune cells including neutrophils, invariant NKT (iNKT) cells, γδ Tcells and mucosal associated invariant T (MAIT) cells are activated (109) and thought to play a role in limiting early infection (100).

As tissue macrophages (both activated and permissive) and DCs are recruited to the site of infection, the characteristic 'tubercle' or granuloma begins to form. The granuloma is the hallmark of chronic inflammation, characterized by a ring of macrophages, epitheloid giant cells and lymphocytes that surround a centre of caseous necrosis. It is hypothesized that this dynamic immune structure serves to 'wall-off' the infection, however studies have shown that there may be a constant influx and efflux of immune cells to and from the granuloma that may facilitate bacterial infection to other organs. During this early phase of infection, adoptive transfer of antigen-specific T cells has been shown to have no impact on the outcome of infection. Rather, it is the response of the innate immune cells, including the early neutrophil response that has been shown to determine whether infection is established or cleared before the adaptive immune response comes online. Genetic factors of the host and other variables such as lung damage due to smoking, diabetes, malignancy and malnutrition of the host also

add to the mix of variables. This sets the stage for a rather wide spectrum of potential outcomes from complete clearance to established latent infection to active pulmonary TB (105).

The adaptive response

The onset of the adaptive immune response is unusually slow in TB. IL12p40 dependant DC migration to lymph nodes is delayed up to 8-10 days in mice following aerosol infection, possibly by inhibition of migration response to CCR7, though exact mechanisms are still unclear (105). Both MHC class-I and class-II restricted peptide antigen presentation and activation of both CD4+ and CD8+ (αβ) T cell subsets occur. In addition, CD1-restricted T cells, activated by mycobacterial lipid antigens, and MR1-restricted MAIT cells are mobilised (105, 110-112). A predominantly T helper 1 (Th1) type response is seen in protective immunity to TB. Both Th1 and Th17 type T cells develop and the balance of these cells plays an important role in the outcome of the infection (113). Once antigen-specific T cells proliferate and differentiate, they then traffic to the lung. Recent evidence from mouse studies has shown that antigen-specific T effector cells expressing CX3CR1, KLRGI and T-bet can be found in peripheral blood, however they have no protective action as they are unable to traffic to the lung. CXCR3+ CCR6+ T memory cells have been shown to be lung trafficking cells with multifunctional transcription profiles that mediate protection in latent infection. CXCL13 and CXCR5 expression has been shown to be necessary for T cell migration from the lung perivasculature to the granuloma (100, 108). A cuff of lymphocytes around the activated macrophages and epithelioid giant cells of the granuloma, represents the mature fully formed ‘tubercle’, which is the characteristic pathological feature of mycobacterial infection.

Currently, activated $CD4^+$ and $CD8^+$ T cells in the lung parenchyma are considered the main source of IFNγ. Other sources of IFNγ 'backups' include iNKT cells, γδT cells and NK cells. IFNγ is crucial to immunity against mycobacterial infection and acts in both an activating and regulatory capacity. Activation of macrophages for increased intracellular killing of mycobacteria is a vital function of IFNγ. In addition, IFNγ acts to counteract IL17 action on neutrophils which has been shown to be detrimental in late stages of infection (103, 114).

Activated macrophages secrete TNF which is important from early stages in phagocyte activation, chemokine expression, cell influx and formation of granuloma. Experimental work has shown that both secreted and membrane bound TNF and lymphotoxin (LT – previously TNFβ) are important in protective immunity, though levels depend on multiple factors related to the infecting strain (103, 115, 116). Type I IFNs (α/β) have been shown to enhance

susceptibility to infection in mice and a study of the gene expression signature in human whole blood has shown an increase in type I IFN induced genes in neutrophils in patients with active TB, as well as 10% of patients with latent TB, indicating that these cytokines are likely negative regulators of protective immunity (103). Other cytokines associated with active disease and poor outcome include IL10 and IL27 which are associated with regulation of inflammation and T regulatory cell (Treg) activity. Increased number of Tregs have also been associated with increased susceptibility to infection (100, 105). In contrast, activated lymphocytes (both innate and adaptive) secreting perforins, granulysin, granzymes, and NOS2 have been shown to mediate protection (103, 105). In addition to immune cells, chemokines, cytokines and their receptors, lipid mediators of inflammation like lipoxins and leukotrienes, such as lipoxin A4 and prostaglandins like PGE2, have been shown to play an important role in protection (117). This stage of infection where the complex balance of the hosts innate and adaptive immune cells, chemokines, cytokines and lipid mediators can both hinder or help the organism in infection has been described as 'immunological equilibrium' (105). It is hypothesized that latent TB, once thought to be a state where the bacterium lies dormant in the body, may in fact be a spectrum of disease where there is a dynamic equilibrium between host immunity and bacterial growth.

Reactivation

An important question is why and how latent TB becomes active TB. Known risk factors discussed above are found in patients who have deficiencies in the IFNγ/IL12 axis, TNF deficiency (or inhibition), quantitative or qualitative deficiency in $CD4^+$ T cell surveillance (HIV/AIDS) which directly affect the immune equilibrium that keeps the disease in check. In the majority of cases however, the cause of the immune failure is unclear. Patients with diabetes mellitus, malignant disease, steroid treatment, malnutrition and thin body habitus and those of advanced age are known to be at risk but the true immune correlate/s of susceptibility in each of these groups are unknown.

One of the theories behind immune failure is immune exhaustion and/or loss of T cell polyfunctionality. Initial data supporting this theory have been provided by HIV studies where polyfunctional T cells were seen in patients with latent TB (tri-function with IFNγ, TNF and IL2) while active TB patients had mono- or bi-functional T cells, particularly IFNγ and IFNγ + TNF secreting cells (118-121). However later studies showed contradictory findings with elevated tri-functional cells seen in active infection compared to latent infection or post TB

treatment (113). Investigations into the role of Programmed cell death protein-1 (PD1) molecule in patients with active TB showed that the checkpoint molecule was up-regulated with antigen stimulation and IFNγ production. Likewise, blocking PD1 and its ligand was associated with increased $CD8^+$ degranulation and pathogen-specific IFNγ production (122). Interestingly, investigation of PD1 expression in mice infected with TB showed that PD1 expressing cells were actually 'more' polyfunctional than those without PD1, and blocking PD1 resulted in worsening of disease (105)

Many questions remain to be answered with regards to the immune response to mycobacteria, particularly in humans. Given the heterogeneity in disease it has been hypothesized that investigating the individual "inflammatory tendency" may be useful in determining TB outcome (100). This provides a framework on which to base the immune response to NTM infection. Although NTM are opportunistic pathogens and have not evolved the same virulence mechanisms of TB, the aerosol infection route, infection of APCs (antigen presenting cells) in the lung, particularly the alveolar macrophages, formation of granulomas and the clinical manifestation of both active and latent infection are strikingly similar across both diseases. Many of the risk groups are also common for both TB and NTM infection and indicate common pathways in pathogenesis.

1.10 Immune response in pulmonary NTM infection

The immune responses seen in human NTM infection have shown similarities to TB. However, no consistent phenotype of immune protection or immune susceptibility has been described. Immune compromise caused by genetic mutations MSMD, acquired defects due to infection (HIV), iatrogenic causes, (inhaled corticosteroids, anti-TNF treatment, chemotherapeutic and systemic immune suppressive agents) and defects in lung structural and functional integrity (primary ciliary dyskinesia and other mutations leading to ciliary dysfunction, CFTR mutations, bronchiectasis, COPD, pulmonary alveolar proteinosis), and anti-cytokine antibodies are known predispositions (22, 26, 123). Previous or concomitant TB infection and Aspergillosis independently increase risk of NTM lung disease (124).

These predispositions tell a story of both local and systemic innate and adaptive immunity being required to quell NTM infection. Innate defence mechanisms like efficient respiratory

epithelial ciliary function are likely required to keep colonizing NTM bacterial counts under control. When airway muco-ciliary clearance is impaired and/ or when more virulent strains of bacteria can locally invade tissue, cellular defence mechanisms are activated. The sequence of events that follows, from macrophage activation, local recruitment of innate cells including neutrophils, iNKTs and NK cells to control early infection, migration to lymph nodes, antigen presentation and activation of antigen specific T cells follows a similar pathway as described for TB. An excellent, though rarely cited review by Tomioka (2004) describes the cytokines and other factors involved in macrophage activation as well as the key players involved in moving Th0 naïve cells to either Th1 type or Th2 type response during mycobacterial infection (125).

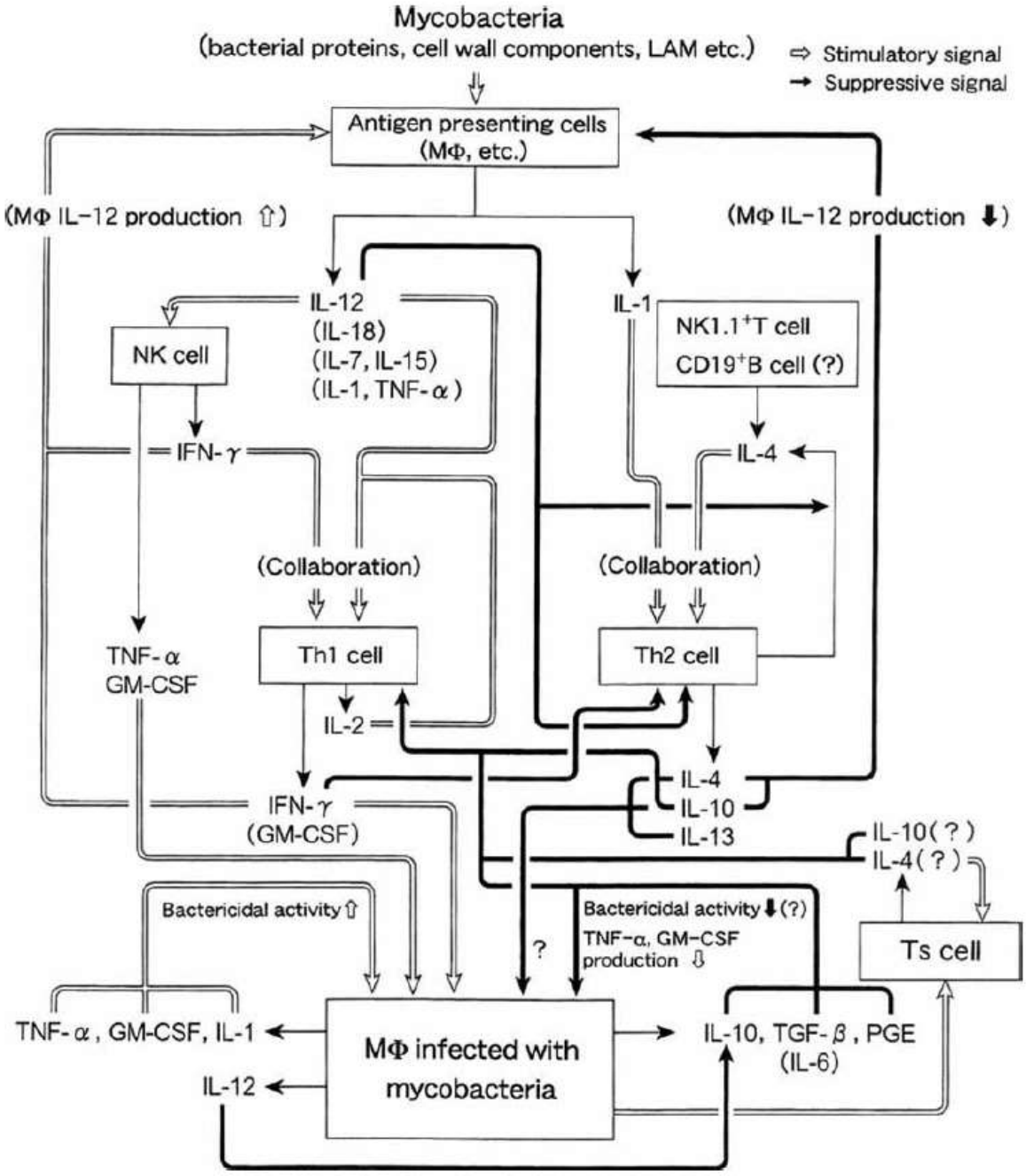


Figure 1.1 Cytokine networks in hosts with mycobacterial infection. Reproduced from Tamioka et al. Current Pharmaceutical Design. 2004, 10;26: 3300.

Briefly, the schematic above shows the complex cytokine networks and cells that are activated during mycobacterial infection. Macrophage (Figure 1). Macrophage and NK cell IL12/ IFNγ push responsive T cells towards a Th1 type phenotype. Th1 IFNγ and IL2 then promote intracellular killing of mycobacteria. The exact triggers for a Th2 type response are not known, but should a Th2 type response predominate, type 2 cytokines IL4, IL10 and IL13 promote suppressive pathways with increased Treg cells.

1.10.1 Experimental studies – of mice

Mouse studies have shown evidence that RORγt induced Th17/IL17 responses are mobilised in MAC infection however the importance of this not clear (126, 127). Studies showing the importance of CCL2, CCL5 and TLR signalling via MAPK, MyD88 and NFκβ are similar to TB in mouse models of NTM infection (128-130). The protective effects of membrane bound TNF has also been shown in mouse models of mycobacterial infection using MTB and Bacillus Calmette Guerin strains (BCG) (116, 131), though NTM data and corresponding human data is lacking. However the difficulty in causing and maintaining infection and measuring immune responses particularly for the mouse model of MABS is highly challenging (132).

1.10.2 Experimental work on MAC and MABS immune responses – human studies

Laboratory and clinical studies of NTM immunity have partially shed light on some aspects of why these opportunistic infections occur. Most studies have either measured cytokine levels directly in serum or cell culture supernatants where cell preparations have been stimulated with antigens or other nonspecific stimulant such as lipopolysaccharide (LPS), which activates macrophages through TLR signalling, or phytohaemaglutinin (PHA) which stimulates T cells.

A comparison of MAC infected patients with no evidence of compromised immunity and *M. avium* sensitin skin test positive healthy controls, showed that infected patient peripheral blood mononuclear cells (PBMCs) stimulated with mycobacterial antigens produced higher levels of IL10 but lower levels of IFNγ, IL12 and TNF. Other studies have shown similar results for IFNγ and IL10, but not for the other cytokines (133-137). A study of serum cytokine levels comparing newly diagnosed MAC patients to healthy controls showed a significant reduction in IL6, IL8, IL23 and IFNγ (127). CD40L was also significantly reduced. Longitudinal assessment of Th1 and Th17 cytokines in these patients after 1 year of antibiotic therapy showed that while low Th1 cytokine levels could accelerate infection, Th17 cytokine levels at diagnosis (IL17, IL23) could act as indicators of treatment outcome (sputum conversion vs failure). A comparison of immune responses in MAC and MABS infection showed that MABS stimulated PBMC produced higher levels of TNF, IFNγ, IL1β and MIP1α than MAC stimulated PBMC (138). A study that compared IFNγ, IL12 and IL10 production in response to mitogen-stimulated PBMCs in patients with MAC, MABS and healthy controls showed a reduction in IL10 production in patients compared to healthy controls. Cytokine assays were

performed using ELISA techniques (139). A more comprehensive study, that used multiplexed bead-based assays to evaluate 22 cytokines in 24 MABS patients, showed reduced IFNγ, IL12, IL4 and IL13, with higher levels of IL17 and IL23 in patients compared to healthy controls. This finding is similar to other studies in MAC infection (127, 135). The authors also noted higher levels of monokine induced by IFNγ (MIG) and (FNγ induced protein (IP-10) in patients who were classified as treatment failures in comparison to those who had successful treatment (140). A recent small study on cytokine levels in three CF patients with MABS infection compared to three patients with non-CF PNTM infection and healthy controls showed no difference in TNF and IL1β levels between CF and non-CF patients, however the non-CF patients showed higher TNF and IL1β production following LPS stimulation (141).

Cell subset specific functional data, particularly on T cell subsets, was lacking in all these studies. Some preliminary evidence that T cell defects may play a role in MAC infection was shown in a study where T cells from healthy control subjects exhibited superior MAC growth inhibition in monocytes compared with patients (142). A recent study by Shu *et al*. (143) showed higher PD1 expression in T cells in patients with MAC lung disease compared to controls. This study also showed reduced IFNγ and TNF production in MAC patients which was partially corrected after 2 months of antibiotic treatment and could also be increased by blocking PD1. However, this study also performed cytokine assays on supernatants and T cell function was not directly evaluated. The control subjects included in this study were also significantly younger than the MAC patients.

A study using monocyte derived macrophages (MoDCs) showed no difference in MoDC cytokine responses between patients and controls (144) while a more recent study showed that Keap1 (an oxidative stress sensor) negatively regulated inflammatory signalling from primary macrophages in MAC infection (145). Other studies of TLR and dectin-based signalling in MAC and MABS infection showed TLR signalling to be crucial (138, 144-149). In addition, MAPK signalling, ERK1/2 and p38 have been shown to be down regulated in patients with MABS infection with subsequent reduction in TNF, IL6 and IL10 (150).

Similar to studies in TB, different strains of NTM have been shown to elicit different immune responses in both human cells and murine models showing the importance of pathogen genetics on host response (151, 152).

Studies on human cells have varied in the specimen used (PBMC vs broncho-alveolar lavage (BAL) fluid vs whole blood), the stimulants used (PHA, LPS, killed bacteria) and the patient groups (age, infecting species and stage of treatment) making both cross-study comparisons and interpretation challenging. Very little is known on this pathophysiology in CF patients. As CF patients present a unique group who have their own inherent immune dysregulation, the data from current studies may not reflect the overarching immune deficiencies in these patients. In addition, patient age ranges often vary widely, include multiple risk groups and other confounders.

Indirect evidence of the immune response in NTM infection has been theorised from the susceptibilities through immune dysfunction. Mutations known to cause susceptibility include those affecting IL12β, IL12Rβ1, IFNγR1, IFNγR2 and transcription factor STAT1 and RORC (153). Deficiency in NFκβ essential modulator (NEMO) and other primary immunodeficiency syndromes like GATA-2 deficiency and SCID and isolated $CD4^+$ T cell deficiency have also been implicated in NTM susceptibility (76, 154). A recent study in an Australian cohort of patients showed association between TNFA-1031 and IL10-1082 alleles and NTM infection (155). HIV infection increases the risk of NTM disease when $CD4^+$ T cell counts drops below 50/mm^3. Broadly immunosuppressed patients with haematological malignancies, organ transplants and stem cell transplants are at high risk. The timing of this increased risk does not coincide with the neutropenic phase of these diseases, highlighting the probable lack of importance of neutrophils in NTM immunity (76). Current available data supports the increased risk of NTM in patients being treated with anti-TNF therapy (28, 156). There is also evidence for increased risk in patients on the anti-IL6 agent tocilizumab while other agents including IL12/IL23 inhibitor ustekinumab (associated with TB reactivation), and the JAK pathway inhibitors tofacitinib and ruxolitinib (interferes with IFN signalling) pose a theoretical risk, as solid data is not yet available (31, 76).

Early markers or predictors for patients that are likely to develop active NTM disease (vs airway colonization which is commonly seen in chronic lung diseases like CF, COPD and bronchiectasis) are of high clinical value. Likewise, the identification of patients likely to recover, and patients likely to develop serious life-threatening infection would be of enormous benefit to clinicians to advise the therapeutic decision-making process. Data from mouse models of MAC infection is available, though less so for MABS. Human data is limited to small studies of generally <10 patients (132). Data is still lacking about the immune profiles of

CF patients with MAC and MABS disease in comparison to non-CF patients with disease. Longitudinal follow-up data of the changes seen in the immune profile of these patients during treatment is also not available. In-depth analysis of the immune function and dysfunction seen in these groups of patients will provide much needed insight into disease pathophysiology and ultimately therapeutics (immunotherapeutics etc) that could be developed and/or repurposed to enhance immune responses to these life-threatening infections.

1.11 Immunity in the cystic fibrotic lung

The CFTR gene is defective in CF resulting in abnormal ion transport in the mucosal membranes, particularly in the respiratory mucosa, resulting in viscid mucus and reduced mucociliary clearance (157). This causes a cascade of events that eventually leads to a state of chronic inflammation and permanent remodelling of the airways. Chronic inflammation is characterized by persistent neutrophilia and accumulation of neutrophil proteases and elastases that cause the disease to progress by reducing lung tissue integrity and immune impairment by acting on immune receptors. Impaired neutrophil mediated gram-negative bacterial killing related to TIM3 expression in CF has recently been shown (158). Other mechanisms that dysregulate immune pathways have also been characterized in CF; activation of NFκβ resulting in increased TNF, IL1β, IL6 and IL8 and subsequently increased numbers of lung neutrophils, abnormal fatty acid metabolism that changes lipoxin and leukotriene generation with potential consequences on the inflammatory response, as well as reduced IFNγ signalling, have been described (157). Impaired lung macrophage function has also been described in CF with increased inflammatory responses and reduced bacterial killing efficiency. A reduction in the number of MHC class-II receptors on the surface of DCs in CF patients has also been reported, as well as cleavage of CD86 by neutrophil elastase that may contribute to impaired DC maturation, function and impaired antigen presentation. Increased Th2 and Th17 Reponses have also been shown in the CF lung (157).

As mentioned in section 1.6, only a small percentage of CF patients develop NTM disease. Therefore, although the immune dysfunction described above are seen in all CF patients, they are likely not the only factors present in NTM susceptible patients. It is likely that a subset of patients have compounding immune predispositions that favour NTM pathology. Certainly, the presence of an abnormally enhanced neutrophil response with impaired macrophage and IFNγ action seem to be the most likely immune environment for NTM infection to occur. However,

the ultimate mechanism/s are likely far from simple and require the study of patients both with and without NTM infection in the context of CF, robustly performed experiments using high dimensional analysis and excellent controls.

1.12 Recent developments and research priorities

Recent findings in NTM research are cause for global concern. Firstly, the recent emergence of evidence of person-to-person transmission of MABS across CF patient cohorts (14). Regardless of whether true person-to-person transmission is occurring, or whether long lived environmental aerosol and fomite contamination has led to these clusters of infection, isolation of patients in patient waiting areas, and other infection control practices have been put in place in the US, UK and Australia (38, 78, 159).

Secondly, there is evidence suggesting an increasing incidence of childhood NTM infection. A nationwide, population-based study from Finland published in 2018 showed a significant increase in childhood NTM infection following a change in national policy on BCG vaccination from 'universal' to 'selective'(160). This study suggests that while BCG may provide some degree of protection to children from NTM infection, unvaccinated children and other populations with respiratory compromise like CF could be a susceptible to this disease. Other studies that document similar trends, particularly in relation to extra-pulmonary NTM infection in children, support this theory (161)

Thirdly, it has been postulated that that MAC infection and associated inflammatory responses could lead to an increased risk of breast and lung cancer as tumour associated gene expression is increased during infection (162). Other studies that have associated NTM infection with diseases such as Sjogren's syndrome in Taiwan (163) and Sweets syndrome in Japan (164), though few, highlight the possibility that this infection leads to non-infective sequalae that add to morbidity.

Fourthly, there are disturbingly high death rates in patients following diagnosis with NTM lung infection. A 2018 systematic review showed five year mortality was 27% in Europe, 35% mortality in the US, and 33% mortality in Asia. (165). Predictors of high mortality included male gender, presence of comorbidities and fibro cavitary disease. These findings have been replicated in other studies that showed that male patients, with fibro cavitary disease, low BMI

and malignancy were prognostic indicators of poor outcome (44, 166, 167). In addition, with persistent infection (those who remain culture positive despite 12 months of treatment) have higher rates of death attributable to NTM infection than those who manage to clear the sputum (38). Significantly higher numbers of hospitalizations due to illness, leading to increasing health care costs compound this issue (45).

Research priorities recommended in the US and UK include rapid diagnostic tools especially rapid identification of the infecting species, (38) and screening methods to identify patients at risk (124, 159). These are considered high impact research targets that would alert clinicians to at-risk patients enabling better screening and faster initiation of appropriate treatment.

1.13 Immune checkpoints: Infectious disease and immune exhaustion

Immune checkpoints blocking therapeutic agents were initially developed to rectify T cell dysfunction during malignant disease. These checkpoint receptors act to suppress immune cell function, and in their natural state are essential in limiting autoimmunity and immune pathology associated with unchecked activation of the immune system. Checkpoint therapies have been highly successful in the cancer therapeutics space, revolutionizing melanoma therapy and are now the 4th pillar of cancer treatment (168). A logical next step is to explore the re-tasking of these novel therapeutics to treat other chronic diseases, particularly refractory infectious diseases where new antimicrobial treatment options are few and far between in the development pipeline (169).

Investigation into the influence of immune checkpoints, particularly PD1 and Cytotoxic T lymphocyte associated protein 4 (CTLA4), in infectious diseases has provided several intriguing findings. Tuberculosis patients were shown to have high PD1 expression on antigen specific T cells; the level of expression correlated to IFNγ production. Blocking PD1 or PD1 and ligands PD-L1, PD-L2 increased $CD8^+$ T cell degranulation as well as IFNγ production by antigen specific cells (122). PD1 and TIM3 were both elevated, primarily on antigen specific $CD8^+$ T cells in chronic lymphocytic choriomeningitis virus (LCMV) infection which correlated to reduced functionality and proliferation capacity. These functions were returned by blocking antibodies against PD1 and TIM3, showing the reversible nature of this immune exhaustion (170). Investigation of hepatitis B and C virus infections, HIV and cryptococcal infection has yielded similar results, showing elevated PD1/CTLA4 levels with reversible

dysfunction (171, 172). Other chronic infections that are being investigated include malaria, toxoplasma and leishmania infection though these studies are all performed in mouse models (173).

Data on immune checkpoint profiles in NTM infection is minimal. One study showing elevated PD1 expression on lymphocytes from patients with MAC lung disease was published in 2017 (143). However, the control group used for comparison in this study were younger than the patient group which likely affected results (174). Data on other exhaustion markers is not available in published literature. Given the chronic nature of the disease, the high rates of antibiotic treatment and the lack of current adjuvant therapies, checkpoint inhibitors are an intriguing avenue worthy of investigation.

1.14 Gene signatures as diagnostic tools

Gene expression signatures are currently being explored for their value as diagnostic and prognostic tools in cancer and other disease settings. Laboratory methods for quantification of gene expression have also undergone major change in recent years. One such technology is the Nanostring nCounter platform (https://www.nanostring.com) that combines fluorescent barcoding technology with molecular imaging to provide rapid quantification of gene expression without the inherent bias introduced by amplification. This has proven to give robust and sensitive data suitable for utilization at clinic-level (175). Both end-user customized chips as well as commercial kits containing common genes are available, making this a versatile technique suitable for both research and routine clinical use. Gene signature based diagnostics based on the Nanostring nCounter system include 'Prosignia' (www.nanostring.com/diagnostics/prosigna-uk) which is a breast cancer risk prediction tool already approved and in-use in both the US and the UK (176). Other gene signatures under investigation, based on this technology include a B cell lymphoma classification tool (177, 178)

Nanostring gene expression has also been used in the study of infectious diseases, though commonly for pathogen mRNA based diagnostics (179). Pathogenesis and treatment response investigations have also used Nanostring based signatures to better understand mechanisms that underly disease and response to therapy. A recent study on C57BL/6 BCG infected mice used this platform to show that resistance was associated with rapid upregulation of *Camp*

Cathelicidin (180). An investigation of the innate immune response during anti HCV therapy showed dynamic immune signatures with therapy (181).

1.15 Rationale for the study

Lung diseases caused by MAC and MABS species are increasing in prevalence worldwide. An increasing population of susceptible individuals is postulated to be one of the reasons for this increase. Immune susceptibility to these infections is described for many though not all identified risk groups. MAC and MABS lung disease cause significant morbidity and mortality in affected patients with escalating treatment costs, frequent recurrence of infection, and antibiotic resistance, which are complicating care.

Much of the laboratory research performed in relation to NTM infection has been carried out in mice. Studies in human patients have often focused on monocyte/macrophage systems and thus far failed to uncover a robust model of immune susceptibility in the patient groups of interest. T cells, particularly $CD4^+$ T cells, are known to be vital in the defence against NTM, as demonstrated by the increased susceptibility of HIV patients to infection but have been poorly studied in the context of NTM. I therefore focused my doctoral investigation on T cell phenotypes and dysfunction in the greater sphere of pulmonary NTM associated disease.

No studies published to date have comprehensively dissected the immune response in MAC and MABS infection in defined patient populations. Most past studies treated NTM infection as a single entity, though some stratification by species has been attempted in more recent literature. No studies to date have compared the response of a specific infection in two different patient groups, or the response to two different infecting species in a single patient group. Immune phenotyping in different stages of infection, which includes both phenotyping and functional data has not yet been attempted. Whether species-specific, disease stage-specific, patient group-specific immune signatures exist that could be translated into clinical diagnostic tools and therapeutic monitoring tools is not yet known. These are the knowledge gaps that I set out to address with this project.

Patients with CF present a group of young patients with a genetic disorder resulting in structural lung compromise. All CF patients are prone to respiratory infections. Some CF patients are susceptible to NTM infection, particularly MABS infection which can prove life threatening.

I therefore investigated CF patients with known susceptibility to MABS infection to characterize immune cell phenotypes associated with this infection. I also compared MABS infection in CF patients to MABS infection in middle aged/elderly patients, a second at-risk group susceptible to NTM lung disease. This comparison demonstrates whether the susceptibility to infection is specific to the patient group or specific to the infecting species, or whether a combination of signatures exists.

Middle aged / elderly patients with MAC and MABS lung disease comprise a large cohort of patients in whom neither pulmonary nor specific immune susceptibility to infection has been identified thus far. I next investigated this group of patients studying the immune phenotypes of disease with different pathogen groups and at different stages of disease. Identification of potential immune risk factors, biomarkers of active disease and treatment response as well as an immunological basis for persistence of infection was sought. I additionally trialled αPD1 (nivolumab) *in vitro* in these patient groups to evaluate if there was potential for redeploying checkpoint blockade inhibitors for this difficult to treat infection.

1.16 Hypotheses

The following hypotheses were tested.

1. Patients with (i) cystic fibrosis and (ii) middle aged/elderly patients with MAC/MABS lung disease have blood-specific signature immune profiles that are:
    a. Specific to each patient group
    b. Specific to each species group
2. These immune signatures can be developed into prognostic biomarkers and screening/diagnostic point-of-care tools
3. T cell immune function in MAC infection can be improved with use of checkpoint inhibitors such as anti-PD1.

1.17 Objectives

1. Compare T cell immune signatures in CF patients and elderly patients with MABS to identify:

1.1 patient phenotype-specific responses to MABS infection

1.2 immune susceptibility features of CF patients with MABS infection

2. Characterise immune phenotyping of peripheral blood cells from middle aged/elderly patients with MAC/MABS infection disease states (i) during active disease, (ii) post treatment disease remission and (iii) persistent infection, and to compare these immune phenotypes to those of control subjects to identify:
    2.1 the immune cell composition of peripheral blood T cells
    2.2 the immune exhaustion marker fingerprint of peripheral blood T cells
    2.3 polyfunctionality profiles of peripheral blood T cells
3. Perform T cell gene expression profiling of $CD4^+$ and $CD8^+$ peripheral blood T cells in patients and control subjects to identify:
    3.1 gene signatures associated with active infection of both MAC and MABS (common signatures)
    3.2 gene signatures associated with specific-species infections (MAC vs MABS)
    3.3 gene signatures associated with NTM persistence
4. Perform antigen stimulation tests evaluating:
    4.1 antigen-specific proliferation and cytokine responses associated with active infection, disease remission and disease persistence
    4.2 whether antigen-specific cytokine IFNγ response can be improved by modulation with anti-PD1 antibodies.

1.18 Thesis outline

This thesis is structured as follows.

Chapter No.	Title	Details
2	Materials and Methods	Contains all materials and methods relevant to this thesis. Two of the flow cytometric panels developed by the student (CR) are currently being prepared for publication as OMIPs (optimized multi-colour immunophenotyping panels)
3	Results Chapter-1 Published as Lutzky VP**, Ratnatunga CN**, Smith DJ, Kupz A, Doolan DL, Reid DW, et al. Anomalies in T Cell Function Are Associated With Individuals at Risk of *Mycobacterium abscessus* Complex Infection. Front Immunol. 2018;9.	SB and JM conceptualized the study. VL, CR, DS, AK, DD, DR, RT, and JM performed the experiments and analyzed the data. CR, VL, and JM wrote the manuscript with input from all authors. DR, RT, SB, and JM supervised the study. **VL and CR are co first authors of this paper. Contains results and discussion pertaining to objective 1.
4	Results Chapter-2 Immune phenotyping of elderly patients with MAC and MABS lung disease	Contains results and discussion pertaining to objective 2.*
5	Results Chapter-3 Nanostring nCounter gene expression quantification	Contains results and discussion pertaining to objective 3.*
6	Results Chapter-4 Antigen specific recall response	Contains results and discussion pertaining to objective 4.* *Chapters 4-6 are to be published as a single manuscript which is currently being drafted. CR will be first author of this manuscript.
7	Discussion	Contains an overall summary of results, general discussion including the implications of the study as well as study limitations and future directions to be explored.

Chapter 2: Materials and Methods

2.1 Introduction and overview of methods

This project was conducted in two stages. The first stage was a pilot study designed for descriptive analysis of MABS infection with a proof-of-concept aim of identifying immune signatures from a single infection across two different patient groups. My objective was to characterize MABS infection in CF and elderly patients to ascertain if patient group-specific susceptibilities lead to MABS infection. The second stage was designed as an in-depth immune study of MAC and MABS lung disease in middle aged/ elderly patients at three different stages of infection/recovery. The overarching aim was to characterize the immune phenotype and functionality of the elderly patient population and uncover immune signatures underlying species-specific active disease, disease remission and disease persistence. High-depth phenotyping by multi-colour flow cytometry was also performed for biomarker discovery and mechanics of disease research. I then went on to evaluate antigen-specific immune responses and the utility of novel immune modulatory agents as new biologics in refractory NTM patients.

An overview of the patient groups and methods used in stage I and stage II are shown in figure 2. 1 and 2. 2

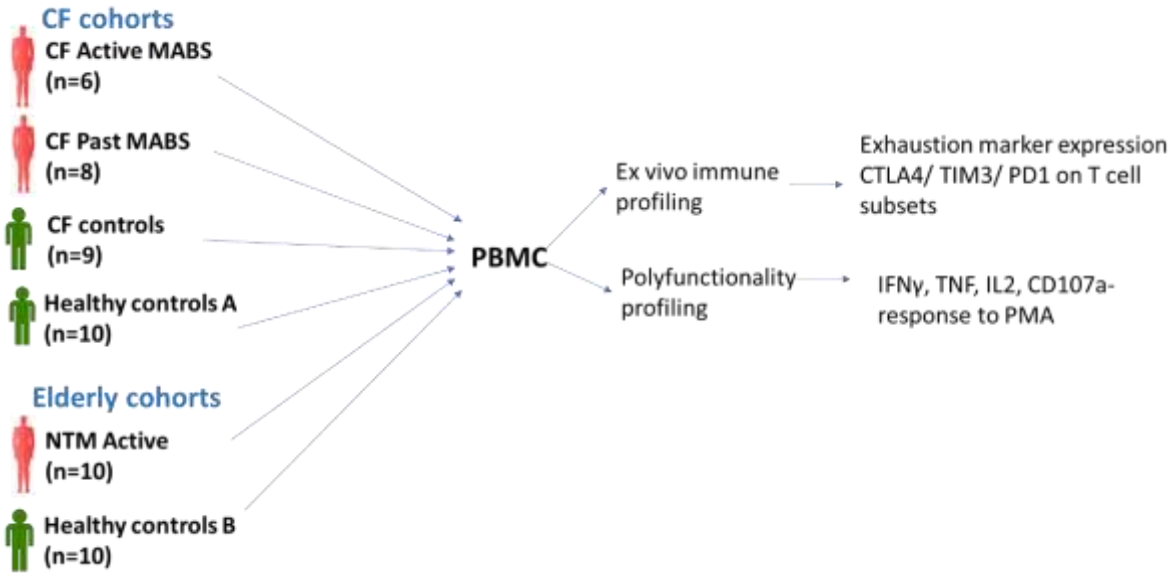


Figure 2.1 Patient cohorts and laboratory methods used in stage I.

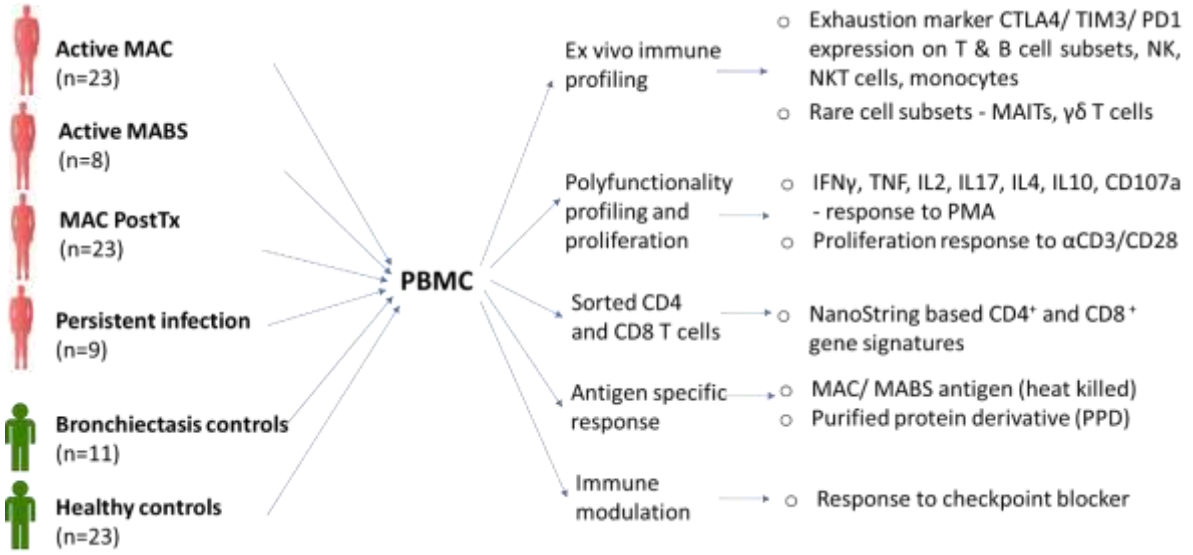


Figure 2.2 Overview of patient groups and laboratory methods used in stage II.
Tx- Treatment. Persistent infection – included patients with persistent MAC or MABS infection.

2.2 Patient recruitment and ethics

All samples were obtained with written consent and all protocols were approved by the Human Research Ethics Committees of the University of Queensland (Approval 2013001411) QIMR Berghofer Medical Research Institute (HREC P2045), The Prince Charles Hospital (EC2718) and Greenslopes Private Hospital (Protocol 12/12 and 14/14), Australia. Patients were recruited from Greenslopes Private Hospital, Brisbane QLD, The Prince Charles Hospital, Brisbane, QLD, and The Princess Alexandra Hospital, Brisbane QLD, Australia. Clinical details and patient categorizations were obtained from the collaborating physicians. All patient personal details were deidentified and coded for scientists while full details were available only to the principle clinical investigators.

2.3 Patient cohorts for stage I- pilot study on MABS infection in CF and elderly patients

Two patient cohorts were studied (see Figure 2.1). The CF patient cohort (n=24) included three groups of patients: (i) CF^{Act}: CF patients with active pulmonary MABS infection (at least one positive MABS culture within a 12 month period; n=6) and one with MAC infection; (ii) CF^{Past}:

patients who had a previous diagnosis of MABS infection who had undergone treatment and were now in remission (at least six negative sputum samples over a one year period; n=8) and; (iii) $CF^{Control}$: patients with chronic *Pseudomonas aeruginosa* (Pa) infection defined by the Leeds criteria (31), but with no history of past or current NTM infection included as a 'within-disease' control group (n=9). Five of the six patients with active MABS infection had chronic Pa infection and one had intermittently positive sputum cultures (≤50% of serial sputum cultures positive) for Pa. Sex, age and matched healthy controls were recruited from unrelated adult volunteers (HC^{A} n=10). The second patient cohort were elderly patients diagnosed with active NTM (NTM^{Act}; n=10) all of whom had confirmed MABS infection. Healthy controls (HC^{B}) were gender and age matched to the NTM^{Act} patients and recruited from healthy adult volunteers (n=10).

2.4 Patient cohorts for stage II- comprehensive study on MAC and MABS infection in elderly patients

A total of 96 subjects belonging to four patient cohorts and two control cohorts were studied (see Figure 2.2).

2.4.1 Patient cohorts

Elderly patients with MAC lung disease and those with MABS lung disease were studied at three disease stages. Disease stages were defined based on the American Thoracic Society 2007 guidelines for diagnosis of pulmonary NTM infection (1). Patients with active infection were defined as symptomatic individuals with microbiological and radiographic (high resolution CT) evidence of pulmonary infection according to the ATS/IDSA criteria (1). These patients were recruited prior to starting antibiotic therapy. Patients with active MAC infection (Active MAC n=23) and patients with active MABS infection (Active MABS n=8) who had no known immune compromise and were not on immune suppressive medication were recruited. HIV infection was excluded in most patients. Patients who were in remission were defined as those who had completed antibiotic therapy and remained sputum culture negative for 12 consecutive months. Due to time constraints, only MAC patients who were in remission (PostTx MAC) were recruited. An adequate number of MABS post treatment patients could not be recruited during the study period. Patients with persistent infection were defined as those who remained

sputum culture positive despite treatment. The cohort of patients with persistent infection (Persist Inf n=9) included patients who had either MAC or MABS infection.

2.4.2 Control groups

Two control cohorts were recruited. Healthy individuals, age and gender matched to the patient cohorts, were recruited from volunteers (HC; n=23). A group of patients who were diagnosed with bronchiectasis but had no history of NTM lung disease were recruited as a 'within disease' control group (BronchC; n=11). Including individuals with a background of respiratory disease who had no history of mycobacterial infection has been used methodologically in other studies and provides a valuable baseline adjustment for the effect of underlying similar lung disease (182).

2.5 Blood Samples and Processing and cryopreservation

PBMCs were separated from venous blood by Ficoll-Paque PLUS (GE Health) density gradient method and were cryopreserved in R10 medium (RPMI-1640- (Gibco 21870-076), containing 10% heat inactivated Foetal Bovine Serum (FBS)) containing 100U/ml Penicillin and 100µg/ml Streptomycin (Gibco) supplemented with 10% DMSO (Sigma-Aldrich).

2.6 Flow Cytometric Analysis

2.6.1 Stage I- pilot study; MABS infection in CF and elderly patients

Thawed cells were rested overnight in R10 medium at 37^0C. Cells were then stained for viability, counted and aliquoted into three 96-well plates. Cells in plate one were resuspended in staining buffer (PBS with 2% FBS) and stained *ex vivo* for flow cytometric analysis. Cells in plate two and three were split into two aliquots. One aliquot was activated with phorbol-12-myristate-13-acetate + ionomycin (PMA/I) (Ebioscience) at 1x final concentration in the presence of Brefeldin A 1µg/ml (Ebioscience) and Monensin 0. 1µg/ul (Ebioscience) for 6 hrs at 37°C. The other aliquot was incubated in R10 without PMA/I as an *ex vivo* baseline control.

The cells in plate one were stained for surface markers delineating major immune cell lineages and evaluated for Tim-3 expression. Panel one included surface markers αCD4-FITC (BD), αCD8-Percp-Cy5. 5 (Biolegend), αCD16-PECY7 (BD), αCD19-BV421 (BD), αCD14-APC

(BD) and αTIM-3-PE (R&D Systems). Cells in plate two were stained with panel 2 which included surface markers αCD4-FITC (BD), αCD8-Percp Cy5.5 (Biolegend), activation marker αCD25-PE (BD) and exhaustion marker αPD-1-BV605 (BD). Staining for intracellular exhaustion marker αCLTA-4-BV421 (BD) and nuclear transcription factor αFOXP3-APC was performed after fixation and permeabilization on ice with Foxp3 Permeabilization kit (Ebioscience) according to manufacturers' instructions. Plate three cells were activated with PMA/I and incubated with αCD107a-FITC (BD) during activation. Cells were then washed and resuspended in staining buffer and surface staining was performed with panel 3; αCD3-PECY7 (BD), αCD4-BV711 (BD) and αCD8-Percp-Cy5. 5 (BD) markers. Intracellular cytokines were stained with αINFγ-AlexaFlour700 (BD), αTNF-APC (BD) and αIL2-PE (BD) after fixation and permeabilization with Fix/Perm buffer kit (BD) for intracellular staining. Stained samples were run on a BD LSR Fortessa 4 laser cytometer (BD).

2.6.2 Stage II- Comprehensive study on MAC and MABS infection in the elderly

Three multicolour immunophenotyping panels were optimized for this study. Panel 1 and panel 2 were direct *ex vivo* staining panels for phenotyping most major immune cell lineages and their subsets, while panel 3 was optimized for intracellular cytokine staining after *in vitro* stimulation of PBMCs.

2.6.3 Panel 1 and 2

A 16-colour panel (panel 1) which discriminated T cells, B cells, NKT cells, NK cells, monocytes and their subsets was developed. This panel evaluated the expression of the immune checkpoints/exhaustion markers CTLA4, TIM3 and PD1 on immune cells. The optimized panel is shown in table 2.1.

A second 12-colour panel (panel 2) was optimized at the Curtin school of medicine Flow facility for discrimination of rare cell subsets including mucosa associated invariant T cells (MAIT) cells, γδ T cells and Vδ 1 subset of γδ T cells. This panel also evaluated the activation marker HLA-DR on T cell subsets. Details of panel 2 are shown in Table 2.2.

2.6.4 Staining method for panel 1 and 2

Thawed cells were suspended in R10 media containing DNAse (Deoxyribonuclease I from bovine pancreas, Sigma Aldrich 10µg/ml) and allowed to rest for 1hr. Viable cells were then counted using Trypan blue (Gibco 0.4%) viability stain and $1x10^6$ cells were stained per panel.

Table 2.1 Multicolour phenotyping panel 1

Fluorochrome	Specificity	Stain	Purpose	Clone	volume	Manufacturer	Cat no
BUV395	CD4	Surface	Lineage	SK3	1. 5	BD Biosciences	563790
BUV737	PD1	Surface	Exhaustion	EH 12. 1	0. 6	BD Biosciences	565299
BV421	CTLA4	ICS	Exhaustion	BN13	2	BD Biosciences	565931
BV510	CD56	Surface	Lineage	B159	1. 5	BD Biosciences	740171
BV605	CD16	Surface	Subset	3G8	0. 6	BD Biosciences	563172
BV650	CD27	Surface	Subset	L128	1. 5	BD Biosciences	653228
BV786	CCR7	Surface	Subset	3D12	2. 5	BD Biosciences	563710
FITC	CD3	Surface	Lineage	UCHT1	1. 5	BD Biosciences	555916
PerCP-Cy5. 5	CD8	Surface	Lineage	HI100	0. 3	Biolegend	344710
PE	TIM3	Surface	Exhaustion	344823	1	R&D Systems	FAB2365P
FV620	Viability	Amine	Viability	-	0. 1	BD Biosciences	564996
PE-CY5	CD19	Surface	Lineage	HIB19	0. 3	BD Biosciences	555414
PE-Cy7	CD25	Surface	Subset	BC96	2	Biolegend	302611
AF647	FOXP3	ICS	Subset	259D/CD	5	BD Biosciences	560046
AF700	CD45RA	Surface	Subset	HI100	0. 6	BD Biosciences	560673
APC-H7	CD14	Surface	Lineage	M5E2	2	BD Biosciences	561384

Table 2.2 Multicolour phenotyping panel 2

Fluorochrome	Marker	Stain	Purpose	Clone	Volume	Manufacturer	Cat no
BUV395	CD4	Surface	Lineage	SK3	1. 5	BD Biosciences	563550
BUV737	CD27	Surface	Subset	L128	1. 2	BD Biosciences	564301
BV421	Vα7. 2	Surface	Lineage	3C10	1	Biolegend	351716
BV510	NK-Tcell	Surface	Lineage	6B11	5	BD Biosciences	563267
BV650	HLA-DR	Surface	Activation	G46	1. 25	BD Biosciences	564231
BV786	CD8	Surface	Lineage	RPA-T8	1	BD Biosciences	563823
FITC	Vδ1	Surface	Lineage	REA173	2	Miltenyi Bio	130-100-532
PE	CD161	Surface	Lineage	191B8	1	Miltenyi Bio	130-092-677
PE-Cy7	γδ TCR	Surface	Lineage	11F2	2. 5	BD Biosciences	655410
APC	CD3	Surface	Lineage	UCHT1	1. 5	BD Biosciences	555335
AF700	Viability	Amine	Viability	-	0. 1	BD Biosciences	564997
APC-H7	CD45RA	Surface	Subset	HI100	1. 2	BD Biosciences	560674

Cells were first stained with fixable viability dye for 20mins at room temperature in phosphate buffered saline (PBS). Excess stain was then washed with PBS, and cells were resuspended in PBS with 2% FCS (FACS buffer) for surface staining. CCR7 BV786 stain was added to panel 1 cells in 25µl FACS buffer and incubated at room temperature for 30mins. Subsequently, all other stains were added as a master mix (to a final volume of 50µl) to panel 1 as well as panel 2 cells and incubated on ice for a further 30mins. Cells stained with panel 2 were then washed and resuspended in PBS for flow cytometric analysis. Cells stained with panel 1 were washed and resuspended in eBioscience Foxp3 fixation/permeabilization solution (cat no 00-5521) and incubated on ice for 1hr. Cells were then washed in permeabilization buffer and stained with the intracellular stains for CLTA4 and Foxp3 in permeabilization buffer for 45mins on ice. Panel 1 cells were then washed and resuspended in PBS for flow cytometric analysis.

2.6.5 Panel 3

A 15-colour panel (panel 3) which included seven cytokine/functional markers was optimised to evaluate the functional capabilities of PBMCs in patients and controls. These included Th1, Th2, Th17 cytokines and degranulation markers encompassing a broad range of immune function. Details of panel 3 are shown in table 2.3.

Table 2.3 Multicolour functional panel 3

Fluorochrome	Marker	Stain	Purpose	Clone	Volume	Manufacturer	Cat no
BUV395	CD4	Surface	Lineage	SK3	1. 5	BD Biosciences	563550
BUV737	IL2	ICS	Function	MQ1-17H1	0. 5	BD Biosciences	564446
BV421	IL17	ICS	Function	N49-653	2. 5	BD Biosciences	562933
BV510	CD56	Surface	Lineage	B159	1. 5	BD Biosciences	740171
BV605	CD45RA	Surface	Subset	HI100	0. 5	BD Biosciences	562886
BV650	CD3	Surface	Lineage	UCHT1	0. 5	BD Biosciences	563851
BV786	CCR7	Surface	Subset	3D12	2. 5	BD Biosciences	563710
FITC	CD107a	Surface	Function	H4A3	7	BD Biosciences	555800
PerCP-Cy5. 5	CD8	Surface	Lineage	HI100	0. 3	Biolegend	344710
PE	IL10	ICS	Function	ES3-19F1	1. 2	BD Biosciences	554706
FV620	Viability	amine	Viability	-	0. 1	BD Biosciences	564996
PE-Cy7	IL4	ICS	Function	8D4-8	2. 5	BD Biosciences	560672
APC	TNF	ICS	Function	MAb11	0. 3	BD Biosciences	554514
AF700	IFNγ	ICS	Function	B27	0. 3	BD Biosciences	557995
APC-H7	CD14	Surface	Lineage	M5E2	2	BD Biosciences	561384

2.6.6 Staining method panel 3.

The functional capability of cells to produce cytokines was tested by stimulating PBMCs *in vitro* with mitogen. Thawed counted cells were aliquoted into 96-well -plates. Invitrogen eBioscience Cell Stimulation Cocktail (500X) 50 ng/ml of PMA and 1 µg/ml of ionomycin was used in a final concentration of 1x in to $1x10^6$ cells in R10 media. Cytokine secretion was blocked by using eBioscience Protein Transport Inhibitor Cocktail (500X brefeldin and monensin) at a final concentration of 1x. Baseline level of cytokine secretion was evaluated by an unstimulated control well. Cells were incubated for 6 hrs at 37^0C in 5% CO2 humidified incubator.

Selected stains were added to cell culture media during incubation per optimized protocol. These included lineage markers CD3, CD4, CD8, CD56 and CD14 as well as degranulation marker CD107a as surface expression of CD107a is transient. Plates were covered in foil to prevent fluorescent dye breakdown during incubation.

Following incubation, cells were washed and resuspended in PBS and stained with viability dye on ice for 20mins. Cells were then washed and stained with CCR7 and incubated at room temperature for 20mins. CD45RA stain was then added and cells were incubated for a further 20mins on ice. Cells were then washed and resuspended in BD cytofix/cytoperm permeabilization solution and incubated on ice for 20mins. After washing with permeabilization buffer, a master mix containing all intracellular cytokine stains was added and cells were incubated for a further 30mins on ice. Cells were then washed again and resuspended in PBS for flow cytometric analysis.

2.6.7 Gene expression validation panels

Gene expression patterns in $CD4^+$ and $CD8^+$ T cells were analysed by the Nanostring nCounter gene expression quantification platform (see methods section 2.10 below). Identified genes of interest were analysed to ascertain if protein expression levels followed observed gene expression. This was done by flow cytometric analysis of protein markers. Two 14-colour panels were optimized evaluating the expression of 8 markers of interest on $CD4^+$ and $CD8^+$ T cell subsets, as well as NK and NKT cells. Markers included in panel 1 and 2 (PEX1 and PEX2) included the transcription factors STAT5A, JAK1, and KLF, surface receptors IFNγR1, IL4R,

OX40, S1PR1and CXCR3, and cytokine CXCL8 (IL8) as shown in tables 2.4 and 2.5. Previously un-thawed cells were used to minimize freeze-thaw related changes. As above, cells were thawed, rested for 1hr in DNAse, counted, and $0.5x10^6$ cells per sample were stained per panel.

Table 2.4 PEX1 staining panel for validation of gene expression

Fluorochrome	Marker	Stain	Purpose	Clone	Volume	Manufacturer	Cat no
BUV395	CD4	Surface	Lineage	SK3	1. 5	BD Biosciences	563550
BUV496	CD3	Surface	Lineage	UCHT1	1. 25	BD Biosciences	564809
BV421	CXCL8/IL8	ICS	Marker	G265-8	5	BD Biosciences	563310
BV510	CD56	Surface	Lineage	B159	1. 5	BD Biosciences	740171
BV605	CD16	Surface	Subset	3G8	0. 6	BD Biosciences	563172
BV786	CCR7	Surface	Subset	3D12	2. 5	BD Biosciences	563710
FITC	STAT5A	ICS	Marker	REA549	2. 5	Miltenyi Biotec	130-108-901
PE	JAK1	ICS	Marker	REA700	0. 6	Miltenyi Biotec	130-110-549
FV620	Viability	amine	Viability	-	0. 1	BD Biosciences	564996
PE-Cy7	CD19	Surface	Lineage	HIB19	0. 3	BD Biosciences	555414
APC	KLF2	ICS	Marker	REA766	1. 2	Miltenyi Biotec	130-111-039
APCR700	CD8	Surface	Lineage	RPA-T8	0. 3	BD Biosciences	565165
APC-H7	CD45RA	Surface	Subset	H100	1. 25	BD Biosciences	560674

Table 2.5 PEX2 Staining panel for validation of gene expression

Fluorochrome	Marker	Stain	Purpose	Clone	Volume	Manufacturer	Cat no
BUV395	CD4	Surface	Lineage	SK3	1. 5	BD Biosciences	563550
BUV496	CD3	Surface	Lineage	UCHT1	1. 25	BD Biosciences	564809
BV421	CXCR3	Surface	Marker	1C6	2. 5	BD Biosciences	562558
BV510	CD56	Surface	Lineage	B159	1. 5	BD Biosciences	740171
BV605	CD16	Surface	Subset	3G8	0. 6	BD Biosciences	563172
BV711	OX40	Surface	Marker	(ACT35	5	Biolegend	350030
BV786	CCR7	Surface	Subset	3D12	2. 5	BD Biosciences	563710
PE	IFNγR1	Surface	Marker	GIR-94	1. 25	Biolegend	308704
FV620	Viability	amine	Viability	-	0. 1	BD Biosciences	564996
PE-CY5	CD19	Surface	Lineage	HIB19	0. 3	BD Biosciences	555414
PE-Cy7	IL4R	Surface	Marker	G077F6	2. 5	Biolegend	355004
eFlour 660	S1PR1	Surface	Marker	SW4GYPP	1. 25	eBioscience	50-3639-42
APCR700	CD8	Surface	Lineage	RPA-T8	0. 3	BD Biosciences	565165
APC-H7	CD45RA	Surface	Subset	H100	1. 25	BD Biosciences	560674

Cells were first stained for viability in PBS and subsequently stained for surface markers. Samples stained with PEX1 were then fixed and permeabilised with eBioscience Foxp3 permeabilization kit and stained for intracellular cytokines and intranuclear transcription factors. As fresh samples were not available from all samples at this stage of experiments, only 46 samples that had no previous freeze-thaw cycles were analysed which included Active MAC n=7, PostTx MAC n=9, Active MABS n=4, Persist Infection n=7, BronchC n=9 and HC n=10.

2.6.8 Antigen specific recall response

Antigen specific cytokine response in patient and control PBMCs was tested as described in section '2.12 Antigen stimulation and immunomodulation'. Recall response after overnight re-stimulation with antigen was evaluated by flow cytometry and intracellular staining for cytokines.

Specific immunogenic epitopes for NTM have not been identified. Peptide – MHC class I/II multimers specific to responding T cells are not yet available for this infectious disease. Therefore, surrogate markers were used to differentiate antigen-specific T cell responses from nonspecific responses and bystander responses.

CD40L (CD154) is considered a marker of activated $CD4^+$ T cells and has been successfully used as a surrogate marker of antigen-specific T cell activation in experimental settings in both mouse cells and human PBMCs (183-185). CD40L has more recently been used in TB antigen response investigation in humans (186) and mice (187) with similar success. This panel was designed to assess Th1 (IFNγ, TNF, IL2), Th2 (IL4), and Th17 (IL17) responses in CD4+ and $CD8^+$ T cells. CD40L was used to define antigen specific $CD4^+$ T cells. In addition, a proliferation dye was included to identify proliferating cells. Therefore, proliferating $CD40L^+$ $CD4^+$ T cells and proliferating $CD8^+$ T cells (as CD40L is not highly expressed on $CD8^+$ T cells) were quantified as antigen-specific T cells.

2.6.9 Antigen-specific response panel staining method

Cells were stained with Violet Proliferation Dye 450 (VPD450 BD) as described in section 2.11. After 7 days incubation with antigen, cells were re-stimulated overnight (with PPD or crude MAC antigen as described below in section 2.12). Protein transport inhibitor (eBioscience PTI cocktail 500x) was added after 4 hrs of incubation with antigen at a final concentration of 1x. On the following day, cells were transferred to a -96-well -plate and

resuspended in PBS. Cells were then stained for viability and with surface stains including αCD3, αCD4, and αCD8 as well as dump stains αCD56 and αCD19 (to exclude NK, NKT and B cells). Cells were then permeabilized with BD cytofix/cytoperm buffer kit as described in section 2.6.6 and stained for intracellular αCD40L (185) and intracellular cytokines mentioned in 2.6.8 above. Cells were then washed and suspended in PBS for flow cytometric analysis. Staining panel is shown in table 2.6.

Table 2.6 Multicolour staining panel for antigen specific cytokine response and proliferation

Fluorochrome	Marker	Stain	Purpose	Clone	Volume	Manufacturer	Cat no
BUV395	CD4	Surface	Lineage	SK3	2	BD Biosciences	563550
VPD450*	Cytosol	Cytosol	Proliferation	NA	**	BD Biosciences	562158
BV650	CD3	Surface	Lineage	UCHT1	1	BD Biosciences	563851
BV711	IL2	ICS	Function	SK7	3	BD Biosciences	563946
FITC	CD8	Surface	Lineage	HIT8a	2	BD Biosciences	555634
PerCP-Cy5. 5	IL17	ICS	Function	N49-653	2. 5	BD Biosciences	560799
PE	CD40L	ICS	Antign sp[$]	89-76	12	BD Biosciences	340477
FV620	Viability	amine	Viability	-	0. 2	BD Biosciences	564996
PE-CY5	$CD19^{+}CD56$	Surface	Dump	HIB19	0. 3	BD Biosciences	555414
PE-Cy7	IL4	ICS	Function	8D4-8	2. 5	BD Biosciences	560672
APC	TNF	ICS	Function	MAb11	0. 3	BD Biosciences	554514
AF700	IFNγ	ICS	Function	B27	0. 3	BD Biosciences	557995

***** VPD450- Violet proliferation dye 450

[$]Antigen specificity

2.7 Flow cytometric data acquisition and analysis

Samples for panel 1, 2 and 3 described above were acquired on LSR-II 5 laser, 18 parameter Fortessa (BD) at QIMR Berghofer Medical Research Institute Flow Cytometry Facility, QLD. Samples from PEX1, PEX2 and the antigen-specific response panel were acquired on a LSR-II 5 laser, 20 parameter Fortessa (BD) at the Australian Institute of Tropical Health and Medicine (AITHM) flow cytometry facility, James Cook University, Cairns, QLD. Samples were acquired on FACSDiva software (BD). Automated compensation using single stained compensation beads, experiment templates and application settings were used to maintain as much consistency between samples run on different days as possible. As many events as

possible, up to a maximum of 750 000 live cells were acquired from each sample. Acquisition threshold was set to 7000 to reduce noise generated by subcellular events.

2.7.1 Quality control measures

All flowcytometric data was analysed on Flowjo V10. 1 (Treestar inc.). FSC 3.0 files were imported and checked for sample quality. The gating strategy included an initial cell gate to exclude subcellular events, singlet gate, time gate to exclude flowrate related anomalies, dead cell exclusion gate, and a dump gate where relevant. Fluorescence minus one (FMO) controls were used as gating controls for all panels. A technical replicate sample was run with each set of samples to assess run-to-run variability. Gating was performed blind to the patient/control status of the sample. Once all gating had been finalized and rechecked, an annotation with the sample group was added. The live cell count in each sample was assessed in each sample in all panels. Samples with low numbers of viable cells were excluded from downstream analysis.

2.7.2 Gating strategies

Gating strategies used for each panel are shown in Appendix 2. Both rectangle gates for discrimination of a single marker as well as quadrant gates for biaxial gating of two markers were used. The proliferation analysis tool available in Flowjo v10 was used for cell proliferation analysis. Cell proliferation indexes were automatically calculated based on the proliferation model applied to each sample.

2.8 Fluorescence activated cell sorting

$CD4^+$ and $CD8^+$ T cells were sorted using fluorescence activated cell sorting (FACS) for gene expression experiments described below in section 2.10. Previously un-thawed samples were rested for 1hr in DNAase as described above. 1-1. 5 $x10^6$ viable cells were then counted and stained with a 5-colour panel for viability, dump stains to exclude $CD14^+$ monocytes, $CD19^+$ B cells and $CD56^+$ NK and NKT cells, and T cell markers CD3, CD4 and CD8. Stained cells were then resuspended in 200µl of 2% FCS in PBS (FACS buffer) and filtered through a cell strainer (Falcon™ Cell strainer 70µm nylon) to get a single cell suspension into a sterile 5ml polypropylene FACS tube with lid.

Cell suspensions were transported on ice to a sorting facility, where $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells were sorted using a BD Aria 4-laser cell sorter, 2-way sort. Sorts were performed under safety precautions (Class II safety cabinet) by flow cytometry core facility staff. Approximately 50 000- 100 000 cells were collected into 400µl of R10 media in 1.5ml Eppendorf tubes and stored on ice until RNA extraction was performed as described below in section 2.9.

2.9 RNA extraction and quantification

Procedures were carried out in an RNA extraction dedicated biosafety hood. Sorted $CD4^+$ and $CD8^+$ T cells were centrifuged at 250g for 7mins. As much media as possible was removed by pipetting and 400µl of cold RNAzol-RT (Sigma-Aldrich R4533) was added. 160µl (0.4x volume) of RNAse free water (Sigma Aldrich) was added and samples were vigorously shaken by hand for 15sec creating an emulsion. Samples were then incubated for 15mins at room temperature and then centrifuged at 16,000g for 15mins. DNA, proteins and polysaccharides form a semisolid pellet at the bottom of the tube while RNA remained in the supernatant. Approximately 500µl of supernatant was aspirated (without disturbing the DNA pellet) and added to a new tube that contained 4µl RNAse free water and 1µl molecular grade glycogen (20 µg/µl Sigma Aldrich). Tubes were vortexed and incubated at RT for 10mins after which they were centrifuged at full speed (21,000g) for 15mins to pellet RNA. Supernatant was carefully aspirated until approximately 50µl volume remained and 750µl 75% ethanol (ultra-pure molecular grade, Sigma Aldrich) was added. Tubes were inverted to mix and centrifuged at full speed for 2mins. Supernatant was aspirated using progressively smaller pipette tips until the now visible RNA pellet remained. The ethanol wash was repeated a second time. After aspiration of supernatant, the pellet was left to dry and then dissolved in 10µl of ultra-pure molecular biology grade water. Extracted RNA was then quantified on a NanoDrop ND-1000 spectrophotometer. Samples were then stored at -80^0C until further analysis.

2.10 Gene expression quantification by Nanostring nCounter platform

Gene expression quantification of 131 selected T cell genes was performed using the Nanostring nCounter® platform (Nanostring Technologies, USA). A custom set of 131 T cell

genes (previously optimized from other projects), as well as five housekeeping genes for normalization were included in the probe-set. This technology uses a fluorescent camera to directly count barcoded target RNA molecules, giving quantified results without an amplification step. We chose the Nanostring system, given it is the first fully digital platform for gene quantification (with sensitivity down to femtomole range). At the time, Nanostring was also an order of magnitude cheaper than RNAseq per sample. Finally, promising Nanostring signature codesets can be fast-tracked through regulatory bodies for clinical use given the system already has approval as a diagnostic by the FDA and TGA.

2.10.1 Nanostring setup method

Sample hybridization was set up according to manufacturer's instructions. Briefly, capture probe set (CPS) which contains the probes specific to each RNA strand of each gene of interest, and reporter code set (RCS), which contains the barcoded fluorescent reporter molecules specific for each capture probe/ target RNA sequence, were thawed on ice. 70µl of hybridization buffer was added to the RCS. 8µl of this mix along with 150ng of sample RNA were added to 200µl tubes sets provided. Tubes were then spun in a picofuge to mix sample RNA and RCS. 2µl of CPS was added after inversion of the tube to mix. Tubes containing sample and probe-sets were inverted to mix reagents, briefly spun down and immediately placed in a thermocycler set at 65^0C for 16 hrs.

Hybridized samples were then setup on the Nanostring Prepstation according to manufacturer's instructions. Briefly, an nCounter cartridge, two reagent plates, required tip sheaths and tubes were setup in the prep station along with the hybridized samples. Following 3hrs of automated preparation, loaded cartridges were then read on the nCounter reader station using maximum fields of view. Downloaded raw data (as RCC files) was then inputted into nSolver (software dedicated for Nanostring data annotation).

2.11 αCD3/αCD28 stimulated cellular proliferation

The proliferative capacity of T cells under nonspecific stimulation was tested to evaluate if T cell proliferation was defective in patients with active or persistent lung infection with MAC. αCD3/αCD28 antibodies were used to stimulate T cell proliferation *in vitro*. Fluorescent proliferation dye was used to stain cells prior to stimulation. Proliferation was then measured by flow cytometry.

Thawed cells were suspended in PBS at $4x10^6$ cells/ml in 50ml falcon tubes. Reconstituted violet proliferation dye (VPD450 BD) was added at 1µl/ml and tubes were incubated at 37^0C for 15mins while shaking the tube every 5mins. Tubes were then filled up to 50ml with PBS and centrifuged. Supernatant was discarded, and cells were resuspended in R10 media containing GlutaMAX-1 (100X Gibco, USA) 1x at $4x10^6$ cells/ ml. 500 µl of cell suspension was added to a 48 well plate.

Purified NA/LE (no azide/ low endotoxin) mouse anti-human CD3 (clone HIT3a; 1.0mg/ml) was added to a final concentration of 1µg/ml (0.5µl volume) to each well along with purified NA/LE mouse anti-human CD28 (clone CD28. 2; 1. 0mg/ml) to a final concentration of 5µg/ml (2.5µl volume) as per optimized protocol.

Culture plates were incubated at 37^0C in a humidified 5% CO2 incubator for 8 days. Plates were observed daily for cell growth, media colour or evidence of microbial contamination. On day 4, an additional 250µl of R10 media supplemented with GlutaMax-1 was added to all wells. No other cytokines or growth supplements were added. On day 7 of incubation, cells were re-stimulated with αCD3/αCD28 antibodies and incubated overnight with protein transport inhibitor and analysed by flow cytometry.

2.12 Antigen stimulation and immunomodulation

The phenotypic characteristics, gene expression profiles, functional and proliferative capacity of T cells were investigated in the assays described above. I then went on to perform antigen-specific stimulation assays to determine T cell immune dysfunction present in patients with MAC infection. Antigen-specific responses were measured by flow cytometry as described in section 2.6.9 above. In addition, culture supernatants were quantified for multiple cytokines by cytometric bead array (CBA) (described in section 2.12.3) as this method provides a more sensitive quantification of cytokine levels in cell culture supernatants.

This assay was combined with testing the immune checkpoint inhibitor Nivolumab (clinical grade), in *in vitro* cell culture to assess whether immune function of cells could be improved with this biologic. Nivolumab (anti-PD1 10µg/ml) was graciously provided by the Khanna lab, QIMR Berghofer, Brisbane, Australia.

2.12.1 Antigen preparation

Antigen-specific responses were assessed by stimulating PBMCs with two forms of antigen. The first was crude antigen from heat killed cultures of clinical MAC isolates. Five heat killed clinical isolate cultures were obtained from the Queensland Mycobacterial Reference Laboratory (QMRL). These crude antigen extracts were pooled, and protein content measured by Bicinchoninic (BCA) colorimetric assay (ThermoFisher, USA). As this crude antigen extract contained lipids, lipopolysaccharide (LPS), nucleic acid etc in addition to proteins and peptides, a high background level of non-specific cytokine production was seen during optimization experiments. Therefore, a purified peptide antigen was needed to evaluate antigen specific response by T cells. As purifiable crude antigen for any NTM species was unavailable, Purified Protein Derivative (PPD) was substituted.

PPD is made from the filtered proteins of a standard *M. bovis* strain and is used to perform the *in vivo* tuberculin skin test (TST) in patients with suspected latent tuberculosis. One of the drawbacks of the test is that many of the antigens of *M. bovis*, and indeed all species of the *M. tuberculosis* complex are shared with NTM so false positive results in patients exposed to MAC and other NTM species is commonly seen (188, 189). However, this cross reactivity can be exploited in experimental studies where PPD is used in NTM research as a surrogate antigen (135).

Tuberculin PPD (Tubersol, 0.1ml Sanofi Aventis, Australia) was quantified for protein content by the BCA method. Both crude antigen and PPD concentrations were titrated, with and without IL2 stimulation and the final protocol was optimized over several months.

2.12.2 Cell culture, antigen stimulation, immune modulation

As shown in Figure 2.3, $2x10^6$ cells per well were stained with VPD450 as per protocol described in section 2.11 and plated in a 48 well cell culture plates (Corning, USA). One well was stimulated with crude antigen at a final protein concentration of 10µg/ml as per the optimized assay. Further increases in concentration were not possible as increased cell death was observed in positive correlation. A second well was stimulated with PPD at a final concentration of 40µg/ml. Immune modulation with αPD1 was tested with PPD stimulation as this antigen gave clearer responses with lower background levels in optimization assays. One well was treated with αPD1 at a final concentration of 10µg/ml while a second well was treated with the antibody isotype control (Ultra-LEAF™ Purified Human IgG4 Isotype Control

Recombinant Antibody, Biolegend) at a similar final concentration. A final control well, with no antigen was setup to establish baseline levels of cytokine secretion for each sample.

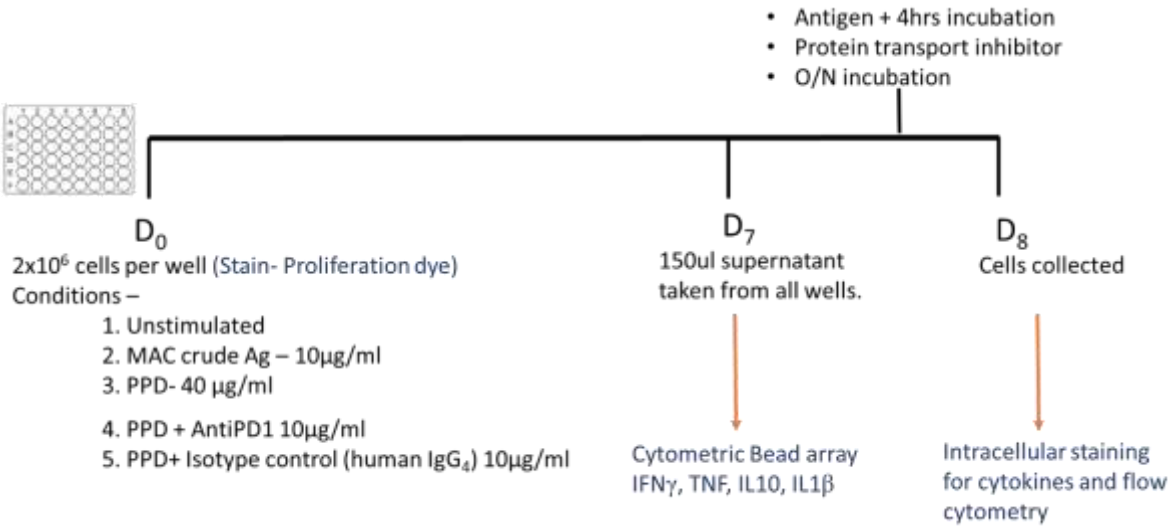


Figure 2.3 Experimental design of antigen stimulation and immune modulation assay

Culture plates were then incubated in humidified 5% CO2 incubator at 37^0C for 7 days with daily observation. On day 7 of incubation, 150µl of culture supernatant was taken from all wells and stored at -80^0C for CBA analysis.

PPD and MAC antigen was then added at the same concentration as mentioned above to relevant wells and culture plates were incubated for 4hrs to allow antigen uptake. Protein transport inhibitor was then added, and cells were incubated overnight. Antigen recall response was measured by intracellular staining and flow cytometry.

2. 12. 3 Cytometric Bead Array

A multiplexed CBA (BD) was performed on day 7 cell culture supernatants to quantify six cytokines, ie. IFNγ, TNF, IL-8, IL10, IL2 and βIL-1β. Assay standards were reconstituted according to manufacturer instructions. Serial dilution of assay standards was prepared. Culture supernatants were thawed and centrifuged to precipitate any debris. 20µl of supernatant was diluted 1:1 in assay diluent for a final 1:2 dilution in 96 well plates. A capture bead master mix was prepared and 10µl of capture bead mix and 10µl of diluted samples/standards were mixed

for 5mins in a plate shaker at RT. Plates were then incubated for 1hr. A PE detection bead mix was prepared and 10µl was added to wells and mixed in a plate shaker for 5mins. After incubation at RT for 2 hrs, 100µl of filtered wash buffer was used to wash all wells by centrifugation. Supernatant was discarded, and beads were resuspended in 80µl of wash buffer for acquisition by high throughput system (HTS) on the LSR Fortessa II flow cytometer.

Sample acquisition was performed on a CBA template on FACSDiva software according to manufacturer's instructions. HTS settings were set to pre-optimized values. Acquired data was exported as FCS 2.0 files to FCAP Array 3.0 (BD) software and analysed. Standard curves were calculated for each analyte and analyte concentrations were reported based on these. If standard curves failed to provide a good R^2 and fit, median fluorescence intensity of the analyte was reported.

2. 13 Statistical analysis

Statistical analysis performed with each data type is described below.

2. 13. 1 Flow cytometric data

Gating of flowcytometric data was performed as previously described in section 2.7. Statistics on percentage cells and median fluorescent intensity (MFI) of specific markers of interest on cell subsets were exported. Samples that had low counts of live cells (<500-1000 gated events) were excluded from subsequent analysis.

Data was analysed using SPSS 25 (IBM SPSS Statistics v25) and GraphPad Prism v7. Comparison of cell percentage or MFI across multiple groups was performed using the Kruskal Wallis (KW) test with Dunns post hoc comparison of means if the KW test was significant. Comparison of two groups was performed with the Mann-Whitney U test. Significance level of $p \leq 0.05$ were reported.

Boolean gating was applied to single marker positive gates to create combination gates i.e. subgates for all possible combinations of marker expression in cells. These data tables were formatted for use with Pestle (available from http://drmr.com/pestle.zip) and subsequently analysed for patterns of marker expression using SPICE (Simple Presentation of Incredibly Complex Evaluations- available from http://exon.niaid.nih.gov/spice) (190). SPICE enables rapid representation of phenotype patterns (fingerprints) using pie charts, comparison of

phenotypes using pie chart comparison statistics in-built to the programme, and comparison of cell subset percentages between groups using multiple Wilcoxon tests (where each chosen group was compared to another against chosen group). Data were analysed to compare overall phenotype and polyfunctionality patterns per cell subset between groups, as well as specific combinations of markers, in specific cell subsets between patient group and healthy controls. For example, $TIM3^+$ CTLA4- $PD1^+$, $CD8^+$ central memory T cells were compared between patients and relevant healthy controls groups.

FCS files were then uploaded into the web-based analytics platform Cytobank (Cytobank. org-Cytobank Premium v). After basic gating and sample categorization were performed, viSNE (visual stochastic neighbour embedding) analysis, utilizing the t-SNE algorithm (t distributed stochastic neighbour embedding) was performed. The process was repeated with different analysis parameters (iterations, theta and perplexity) until a robust level of visual separation of cell subsets was obtained. FCS files that contained the additional new t-SNE1 and 2 axes were then used to setup Citrus analyses, which uses automated gating algorithms based on specified channels to find subpopulations that differ between groups (191). Citrus analyses for differences in cell abundance as well as marker intensity for markers of interest were run according to recommendations with assistance of the Cytobank team. Stable predictive models (validated by multiple citrus runs) were then analysed and reported.

In the antigen stimulation experiments, a different approach to flow cytometric and CBA data analysis was necessary. All gating was performed as mentioned above, however, statistical analysis was performed on fold change values. All cell percentages were divided by the value of the unstimulated control well of that sample. When the value in the control well was zero, the mean value of the unstimulated wells of that patient/control group was substituted. Therefore, all estimates of fold change in cell populations analysed were semiquantitative.

2.13.2 Gene expression data

Data was put through the inbuilt quality control pipeline in nSolver and samples that failed QC in any of the observed parameters were omitted from analysis. Samples that passed QC were then normalized by positive controls and housekeeping genes. Any samples where the normalization factors fell outside the specified limits were excluded from analysis. Mean + 2 standard deviations (SD) of the negative control counts of each sample were subtracted from all genes so only expression 2 SD above background was considered. Log2 of these normalized

counts were then exported and analysed using GMine (http://cgenome.net/GMine/) online analysis platform (192) and the R statistical package.

Multivariate principle component analysis (PCA) and redundancy analysis (RDA) were used to identify potential underlying patterns (latent variables) that showed broad differences between patient and control groups in gene expression. Univariate analysis was then performed. Biomarkers that differentiated groups of interest were identified by unpaired t tests with p values adjusted for multiple comparisons at false discovery rate (FDR) of $p<0.05$. Multiple groups were compared by ANOVA tests again with FDR adjustment. Post hoc analysis of significant genes for mean separation was performed using Tukeys test.

Identification of gene expression signatures of active infection in $CD4^+$ and $CD8^+$ T cells was performed by Dr. Carla Proietti, AITHM, James Cook University. Briefly, the data set was randomly split the into a training set (randomly selected 70 % of all data) and validation set (randomly selected 30% of the remaining data). Random forest models were used on the training set to rank each gene based on importance in differentiating samples into the defined groups (active infection of MAC or MABS vs. control BronchC or HC). The model was then tested on the independent validation data set. This process was repeated five times with each iteration considered a 'run'. Variable importance (mean decrease in model accuracy) for each gene was used as a measure for its predictive importance in discriminating active from control. Within each iteration, models based on different top ranked genes were tested for their performance on the validation dataset by calculating sensitivity, specificity and accuracy of model. Assuming genes that appeared in potential models more frequently were more important as classifiers, a final model was built. This model was then tested on the whole data set using the Leave One Out Classifier (LOOC) method and model accuracy was calculated.

Gene expression/protein expression correlation was evaluated by Spearmans correlation. Log2 counts and MFI of marker were compared in each $CD4^+$ and $CD8^+$ cell subset as well as total cells. In addition, MFI and positive cell percentages were compared between groups to test whether they had the same pattern of differences seen in gene expression.

Sample size required for a validation study was calculated based on the following parameters. Power of test was set to 0.8, effect size was estimated the smallest effect size seen in the top 10 differentially expressed genes, and significance level (α error) was set to 0.0005 which would allow correction for multiple testing.

2.13.3 CBA data

FCS files from CBA data were analyzed using FCAP array 3.0 (BD Biosciences). Experiment layouts were annotated, and concentration curves based on serially diluted standard samples were calculated. Concentration of cytokines was calculated as pg/ml. If the standard curve calculation failed, due to errors in fluorescence, the MFI of samples was used.

Analysis was performed in a similar manner to flowcytometric data with fold change of cytokine level in each condition over the level of the unstimulated well being analyzed. For samples where the unstimulated well MFI was negative or 0.0xxx decimals (<1.0), MFI was converted to 1. Negative and decimal values in other conditions were also set to zero to aid log conversion calculations (where negative values around zero are due to random error in measuring extremely low or no fluorescence). Ratio of MFI condition/MFI NA was considered for each condition. Log2 of these ratios were plotted and analyzed.

Chapter 3: *Mycobacterium abscessus* infection in cystic fibrosis and elderly patients

3.1 Introduction

The first hypothesis tested was that MABS infection in different patient groups (CF patients and elderly patients) had unique peripheral blood immune signatures specific to each patient group. This hypothesis was tested in the pilot study described in Materials and Methods section 2.3 and 2.6.1. The results shown here characterize the functional and phenotypic peripheral blood immune signatures in the patient cohorts described. These findings were published in the article "Anomalies In T Cell Function Are Associated With Individuals At Risk Of *Mycobacterium abscessus* Complex Infection" published in 'Frontiers in Immunology' doi: 10. 3389/fimmu. 2018. 01319 (193). Excerpts of this paper have been included here as results and discussion sections.

3.2 Distinct T Cell Phenotype in CF Patients Susceptible to MABS Infection

We first investigated the phenotypic and functional immune profiles of PBMCs in CF patients to probe for functional deficiencies that could underlie predisposition to NTM infection. Cohorts were categorised as CF with active NTM infection (CF^{Act}), CF with a history of NTM infection (CF^{Past}), CF with chronic *Pseudomonas aeruginosa* (Pa) infection and no history of NTM infection ($CF^{Control}$) and healthy controls (HC^{A}), all matched in both age (ANOVA P = 0. 350) and gender distribution (chi sq P = 0. 445). Demographic and clinical characteristics of patient groups are shown in Table 3.1. All patients had either active or past MABS infection with the exception of one patient who had active MAC infection.

Flow cytometric analysis of PBMCs revealed no significant differences in the percentage of B cells ($CD19^{+}$), total $CD4^{+}$ T cells ($CD3^{+}CD4^{+}$), total $CD8^{+}$ T cells ($CD3^{+}CD8^{+}$) or in TIM3 expression levels on cell subsets between the CF patients and healthy controls (data not shown). Comparison of Tregs ($CD4^{+}$ $CD25^{+}$ $FOXP3^{+}$) between cohorts showed higher percentages in CF^{Act} and CF^{Past} groups compared to the $CF^{Control}$ group (Figure 3.1(A)). Analysis of the individual expression of activation marker CD25 and exhaustion marker CTLA4 on $CD4^{+}$ T cells revealed significantly higher expression of CD25 and CTLA4 in CF^{Act} group compared to the $CF^{Control}$ group (Figure 3.1(A)). Higher co-expression of CD25 and CTLA4 was seen on $CD4^{+}$ T cells in CF^{Act} group compared to the $CF^{Control}$ group and higher percentages of CD25 and CTLA4 double negative $CD4^{+}$ T cells were observed in the $CF^{Control}$ group and HC^{A} group (Figure 3.1(A)).

We next compared the overall pattern of immune marker expression of the $CD4^{+}$ and $CD8^{+}$ T cell compartments in terms of surface 'phenotypic fingerprint'. Analysis of triple, double, single or nil expression of markers CD25, CTLA4 and PD1 on $CD4^{+}$ T cells revealed a common fingerprint in CF^{Act} and CF^{Past} groups which was distinct from the $CF^{Control}$ group (Figures 3.1(B) and (C)). A higher number of CLTA4 single-positive cells was seen in patients with either active or past NTM infection as compared with the healthy control subjects (Figures 3.1(B) and (C)). The T cell fingerprint following PMA/I activation was not significantly different to *ex vivo* T cells (Figures 3.1 (B) and (C)). The T cell fingerprint on $CD8^{+}$ T cells was similar between groups with significance found in $CD8^{+}$ CD25 single positive T cells in the *ex vivo* CF^{Act} group and in the PMA/I activated CF^{Past} group (Figure 3.2 (A)). These data reveal a difference in systemic T cell phenotypes in CF patients with active or past NTM

disease, particularly in $CD4^+$ T cells, compared to CF patients with more common chronic Pa infection. There was no difference between CF patients with active or past NTM disease and healthy controls in terms of T cell fingerprint.

Table 3.1 Demographic and clinical characteristics of CF and elderly patients

	CF^{Act}	**CF^{Past}**	**$CF^{Controls}$**	**NTM^{Act}**
Mean age (SD)	32. 6 (13. 6)	34. 9 (10. 4)	33. 1 (8. 0)	75. 6 (9. 23)
Male : Female	06:01	06:02	06:03	03:07
NTM Infection at time of sample	MABS (6) MAC (1)[a]	None	None	MABS (10)
History of NTM Infection				
MABS	0	8	0	0
MAC	3[b]	0	0	3[c]
Other infections				
Pseudomonas aeruginosa	5	8	9	4
Aspergillus spp	0	0	0	1
Burkholderia spp[d]	0	1	1	0
Lung function				
>70% FEV1	4	1	3	8
30-70% FEV1	3	6	5	2
<30% FEV1	0	1	1	0
Radiographic features				
Bronchiectasis	7	8	9	10

CF^{Act} -CF patients with active NTM infection. **CF^{Past}** -CF patients with past NTM infection. Both groups of patients had a history of *P. aeruginosa* infection. **$CF^{Controls}$** -control CF patients with chronic *P. aeruginosa* infection. **NTM^{Act}** -elderly patients with active NTM infection. N/A not applicable. MABS– *Mycobacterium abscessus* complex*,* MAC -*Mycobacterium avium* complex.

[a]One patient in the **CF^{Act}** group had active MAC infection. This patient was excluded from ANOVA and biomarker analysis but included in general profiling analysis. Data point is shown as a blue circle in scatter plots for ANOVA analysis in Figures 1A and 2A.

[b]One patient in **CF^{Act}** group had prior history of MAC infection while two subsequently developed MAC infection

[c]In **NTM^{Act}** elderly patient group, one patient had a history of MAC infection prior to MABS infection while two others developed MAC infection after treatment of current episode of MABS infection.

[d]*Burkholderia cepecia* complex

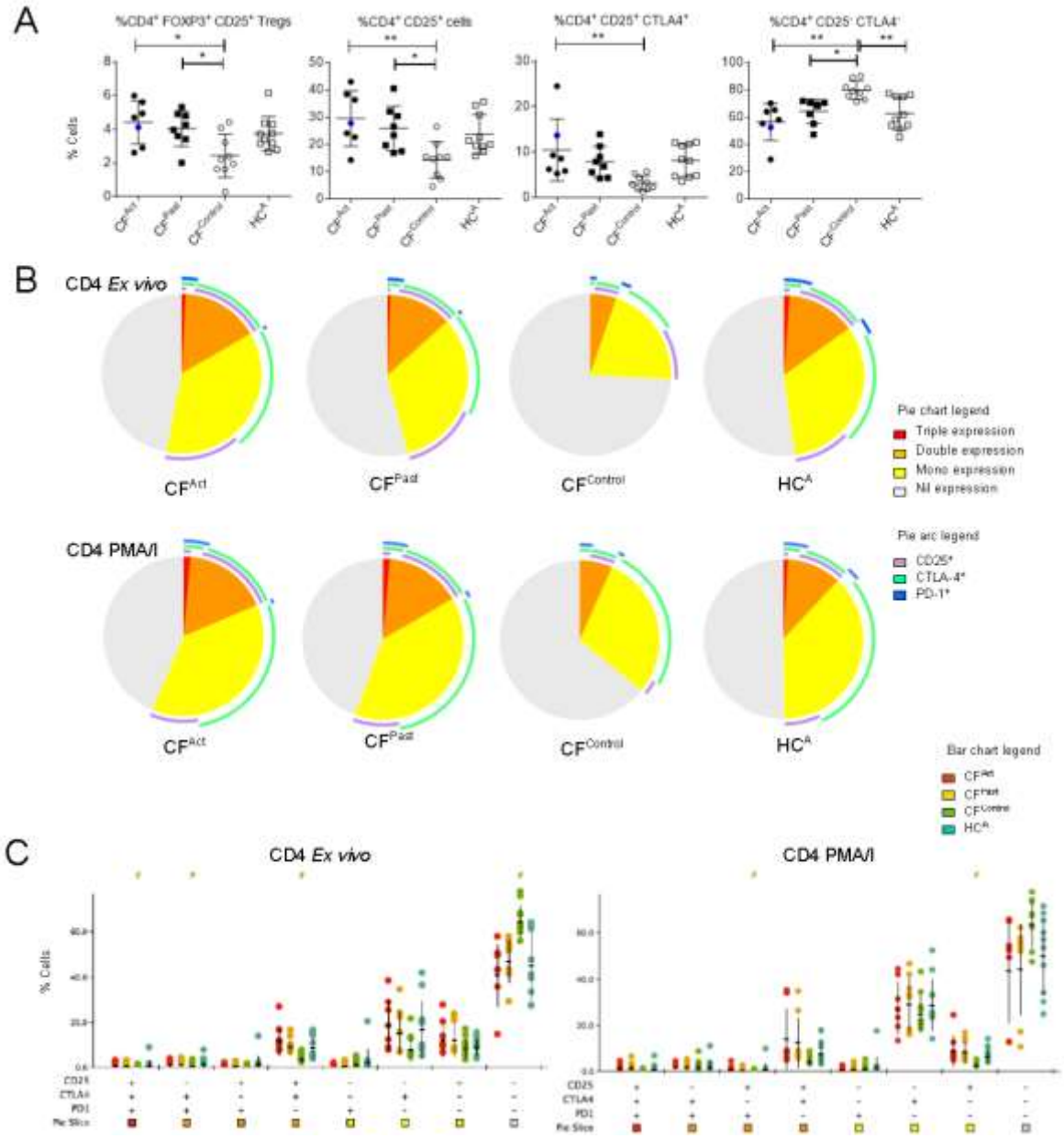


Figure 3.1 Divergent T cell activation and exhaustion profiles in CF patients based on NTM infection status.

(**A**) Flow cytometric analysis of ex vivo $CD4^+$ T cells show significant differences in Treg percentages and marker expression between patient groups. Significantly more Tregs were seen in both CF^{Act} and CF^{Past} groups compared to $CF^{Control}$ (one-way ANOVA with post hoc testing $P = 0.013$ and $P = 0.042$, respectively). Significantly higher $CD25^+$ $CD4^+$ T cells were observed in both CF^{Act} and CF^{Past} groups compared to $CF^{Control}$ group ($P = 0.0056$ and $P = 0.037$, respectively). CD25 CTLA-4 double-positive T cells were significantly higher in CF^{Act} than in $CF^{Control}$ ($P = 0.019$). A reciprocal reduction in CD25 CTLA-4 double-negative $CD4^+$ T cells were seen in CF^{Act}, CF^{Past} and HC^A groups compared to $CF^{Control}$ ($P = 0.001$, $P = 0.027$ and P

= 0.008, respectively). CF^{Act} patient with active MAC infection is shown as a blue circle in scatter plots. This data point was not included in the ANOVA analysis but is shown here to demonstrate activation and exhaustion profile of a patient with an active NTM infection that is not MABS (**B**) Immune marker profiling of $CD4^+$ T cells by SPICE showed differences in ex vivo phenotype in the $CF^{Control}$ group compared to CF^{Act} (p=0.0002), CF^{Past} (p=0.0002), and HC^A (p=0.005). PMA/I stimulation resulted in minor changes in maker profile with the $CF^{Control}$ profile still being significantly different to CF^{Act} (P = 0.025) and CF^{Past} (P = 0.018) though the difference with HC^A was reduced (P = 0.057). (**C**) SPICE dot plots show expression levels of all combinations of markers CD25, CTLA-4 and PD1 in $CD4^+$ T cells both *ex vivo* and after PMA/I stimulation in CF patient and control groups. Groups with significantly different expression compared to HC^A (Wilcoxon rank test $P < 0.05$) are indicated with # symbol. Symbol colour indicates significantly different group.

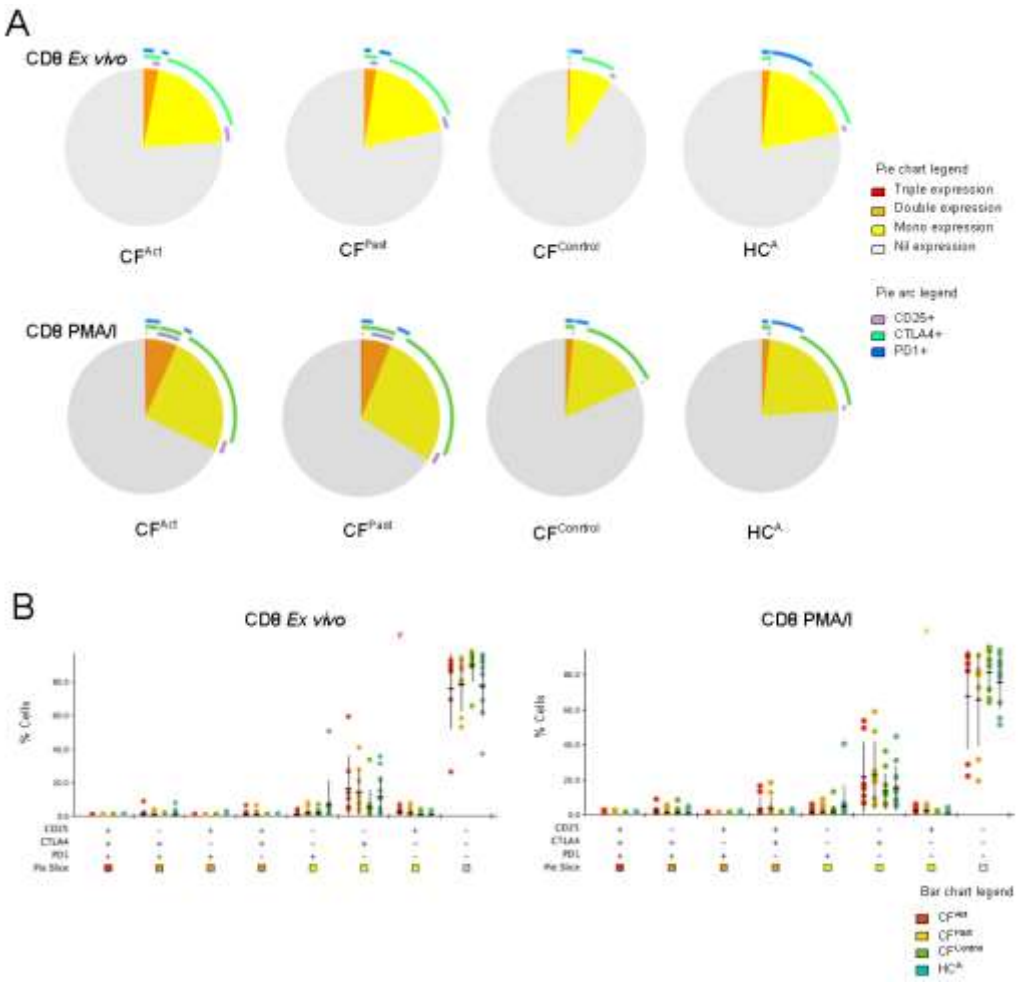


Figure 3.2 Immune activation and exhaustion marker profile in $CD8^+$ T cells in CF patient and control cohorts.

Flow cytometric phenotype profiling of $CD8^+$ T cells by SPICE showed no significant differences between CF^{Act}, CF^{Past}, $CF^{Control}$ and HC^A groups directly *ex vivo* and post PMA/I stimulation. (**A**) Pie charts showing marker fingerprint on $CD8^+$ T cells before and after PMA/I stimulation. (**B**) Dot plots are shown showing all combinations of marker expression on $CD8^+$ T cells before and after PMA/I stimulation. Groups with significantly different expression compared to HC^A (Wilcoxon rank test $p<0.05$) are indicated by a # symbol. Increased expression of CD25 on $CD8^+$ T cells was seen in NTM infection.

3.3 Distinct T Cell Function in CF Patients Susceptible to MABS Infection

Given the differences in surface T cell phenotypes between cohorts we next analysed cytokine production post mitogen stimulation. T cell cytokine production after PMA/I stimulation revealed a specific signature associated with NTM disease. TNF producing $CD4^+$ T cells were significantly lower in both CF^{Act} and CF^{Past} groups compared to the $CF^{Control}$ group (Figure 3.3 (A)). TNF production in $CD4^+$ T cells was also markedly lower in both CF^{Act} and CF^{Past} compared to HC^A group, though this difference did not reach statistical significance (Bonferroni post hoc test). $IFN\gamma^+$ $CD4^+$ T cells in the CF^{Act} group were higher than the $CF^{Control}$ group (Figure 3.3 (A)). In the $CD8^+$ subset, $IFN\gamma^+$ T cells from the CF^{Act} and CF^{Past} group were higher than the $CF^{Control}$ group (Figure 3.3 (A)).

Polyfunctionality in $CD4^+$ and $CD8^+$ T cells was next examined. As CD107a represents degranulation and cytolytic activity, the expression of this marker was included in the polyfunctionality profile in addition to TNF, IFNγ and IL2. Both CF^{Act} and CF^{Past} groups showed a unique polyfunctionality profile compared to both $CF^{Controls}$ and HC^A groups (Figure 3.3 (B)). Both $CD4^+$ TNF producing single positive T cells (mono-functional) and $CD4^+$ TNF^+ $IL2^+$ double positive cells (dual-functional) were seen to be significantly reduced in both CF^{Act} and CF^{Past} groups compared to both control groups (Figure 3.3 (B) and (C)). Significantly higher IFNγ mono-functional T cells were seen in the CF^{Past} group compared to the $CF^{Control}$ group though there was no difference compared to the HC^A group. There was also no difference in the number of triple- and quadruple-functional T cells between groups. When $CD8^+$ T cell polyfunctionality was compared, significantly higher numbers of IFNγ producing mono-functional cells were seen in both CF^{Act} and CF^{Past} groups compared to both control groups though there was no significant difference in terms of overall polyfunctionality profile (Figure

3.4 (A)). Total TNF producing $CD8^+$ T cells were significantly higher in the HC^A and $CF^{Control}$ groups compared with CF^{Act} and CF^{Past} groups.

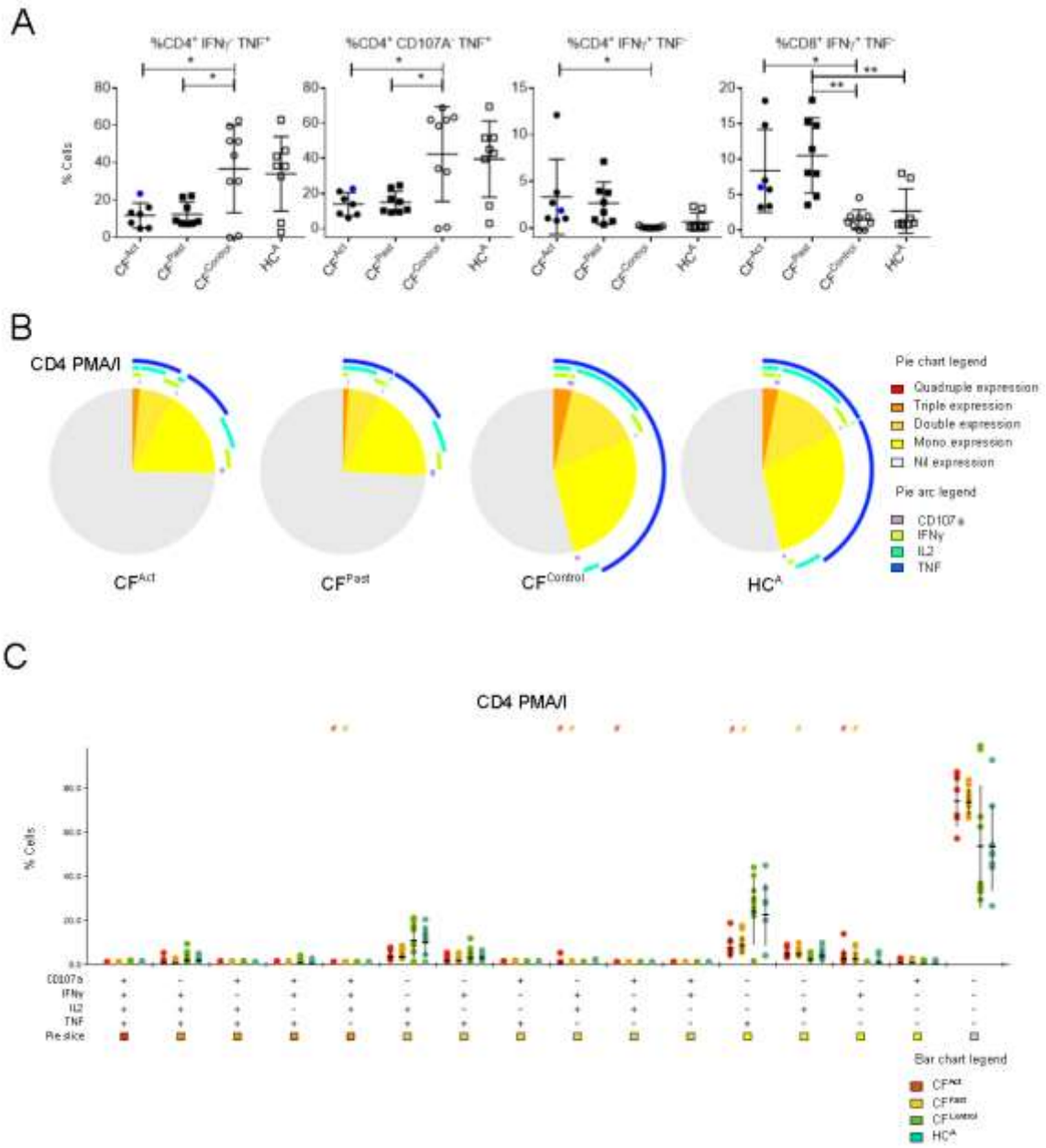


Figure 3.3. Divergent T cell cytokine profiles in CF patients based on NTM infection status.

(**A**) Flow cytometric analysis of *ex vivo* activated $CD4^+$ T cells showed significantly lower IFNγ- TNF^+ ($P = 0.026$ and $P = 0.030$) and CD107a- TNF^+ ($P =$ 0 026 and $P = 0.027$) T cells in CF^{Act} and CF^{Past} groups, respectively compared to the $CF^{Control}$ group. Differences were not significant when comparing the HC^A group with ANOVA post-hoc testing. A similar pattern of increased TNF^+ $CD4^+$ T cells was seen in the HC^A group. Significantly more $IFN\gamma^+$ TNF-

$CD4^+$ T cells were seen in CF^{Act} patients compared to $CF^{Control}$ patients (P = 0.0315) and significantly more $IFN\gamma^+$ TNF- $CD8^+$ T cells were seen in both CF^{Act} and CF^{Past} groups (P = 0.014 and P = 0.0047, respectively) compared to $CF^{Control}$ group. CF^{Past} had significantly more $IFN\gamma^+$ TNF- $CD8^+$ T cells compared to HC^A (P = 0.005). $CF^{Act\ patient}$ with active MAC infection is shown as a blue circle in scatter plots. This data point was not included in the ANOVA analysis but is shown here to demonstrate cytokine profile of a patient with an active NTM infection that is not MABS. (**B**) Polyfunctionality profiling of $CD4^+$ T cells by SPICE showed differences in *ex vivo* functions in CF^{Act} and CF^{Past} groups compared to $CF^{Control}$ (P = 0.056 and P = 0.041, respectively) and HC^A groups (P = 0.022 and P = 0.012, respectively). TNF mono-expressing $CD4^+$ T cells (blue arc) were significantly lower in the two NTM patient groups compared to the $CF^{Control}$ and HC^A groups. (**C**) SPICE dot plots show polyfunctionality profile of all combinations of cytokine expression in $CD4^+$ T cells after PMA/I stimulation. Groups with significantly different expression compared to $CF^{Control}$ (Wilcoxon rank test P < 0. 05) are indicated with # symbol. Symbol colour indicates significantly different group.

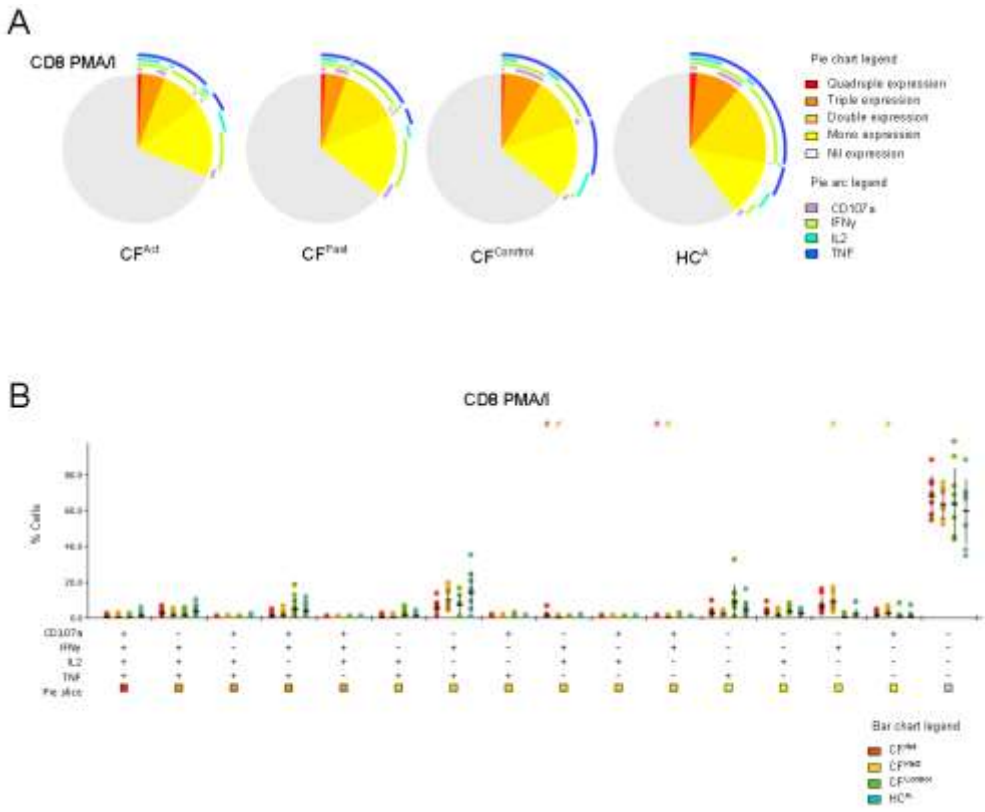


Figure 3. 4 Polyfunctionality profile of $CD8^+$ T cells post mitogen stimulation.

Flow cytometric phenotype profiling of $CD8^+$ T cells post PMA/I stimulation by SPICE showed no significant differences between CF^{Act}, CF^{Past}, $CF^{Control}$ and HC^A groups overall. (**A**)

Pie charts showing cytokine polyfunctionality fingerprint of $CD8^+$ T cells. (**B**) Dot plots show cytokine secretion profile in $CD8^+$ T cells after PMA/I stimulation. Groups significantly different to HC^A are indicated with an # symbol. Colour indicates significantly different group. Significantly higher IFNγ single positive cells are seen, corresponding to Figure 3.3 (A).

Hierarchical clustering analysis of cytokine production and CD107a expression data showed a grouping of CF^{Act} and CF^{Past} groups while the $CF^{Control}$ and HC^A groups clustered together (Figure 3.5 (A)). Based on global cytokine and CD107a expression profiles, patients with NTM disease (either past or present) could be grouped together. There was no clear separation of the active and past NTM infection groups indicating that based on all clustering variables, no global differences were seen between these two groups. The same pattern was observed in the chronic Pa infection group $CF^{Control}$ and the HC^A group where both groups clustered together.

Given this common hierarchical clustering result between CF^{Act} and CF^{Past} cohorts, we next redefined the cohorts for subsequent data analysis. Patients who had either active or past NTM infection (CF^{Act} and CF^{Past}) were defined as the 'NTM disease' cohort and CF patients with chronic Pa infection and healthy controls ($CF^{Control}$ and HC^A) were defined as the 'control' cohort (ie. persons immune to NTM pathology). These two variables were then used as outcomes to analyse data for predictive biomarkers using GMine multivariate analysis software (194). CCA analysis showed significant clustering between these groups (Figure 3.5 (B)). Biomarker analysis identified 13 significant predictors of 'NTM disease' after correction for multiple comparisons (FDR) (Figure 3.6(A)). Unsurprisingly, significant predictors included combinations of TNF and IFNγ production. The stepwise regression model (area under the curve AUC 100%) identified percentage $CD8^+$ $IL2^+$ TNF- T cells and percentage $CD8^+$ $IFN\gamma^+$ TNF- T cells as the best predictors of NTM disease (Figure 3.6 (B)).

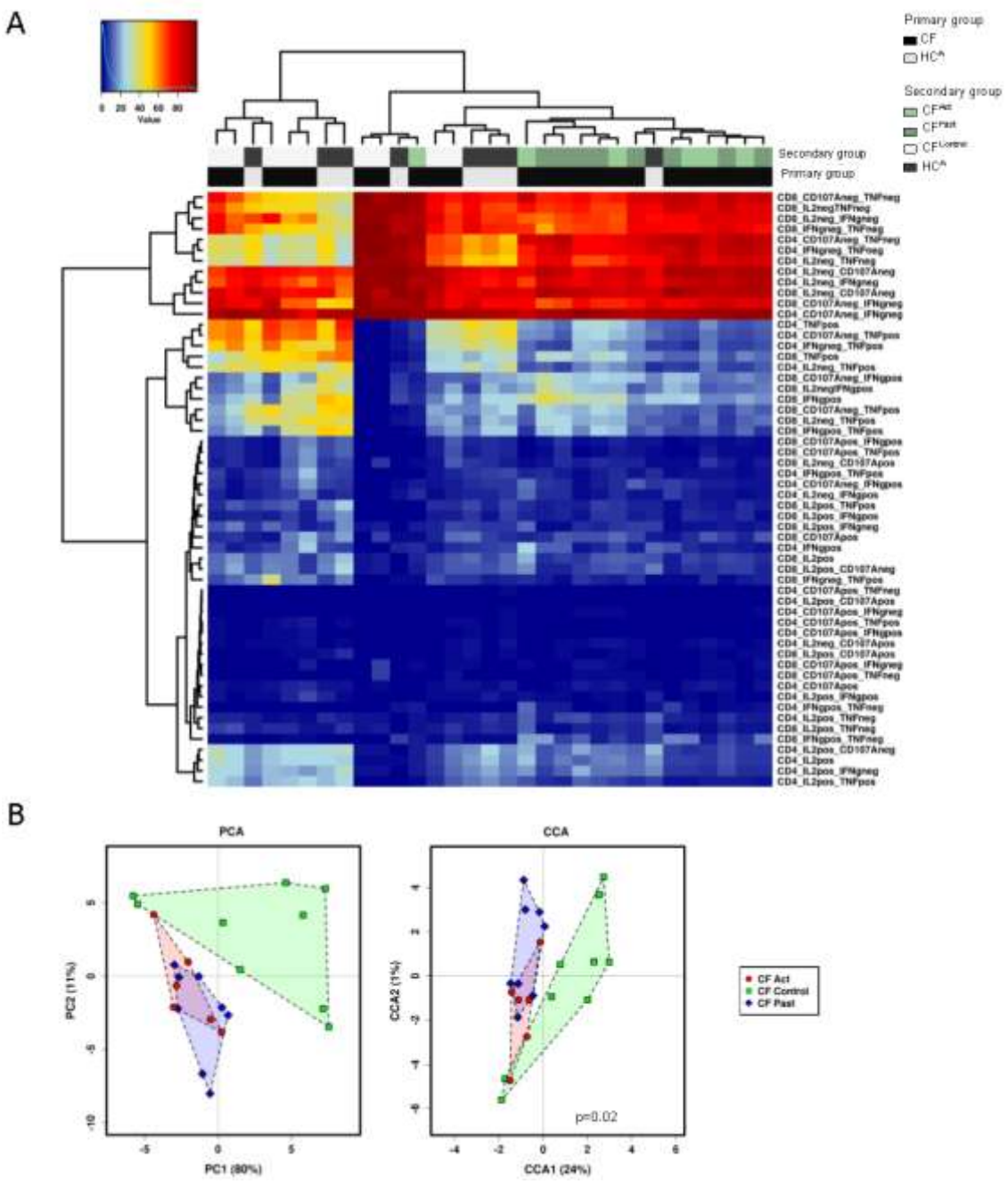


Figure 3.5. Multivariate T cell analysis of mitogen stimulated cytokine secretion profiles. (**A**) Unsupervised hierarchical clustering of mitogen stimulated $CD4^+$ and $CD8^+$ T cell cytokine secretion profile show divergence based on NTM infection status**.** Each column represents an individual (patient/control) and each row represents a clustering variable (cytokine secretion pattern). Clustering patterns shown according to primary group (patient with CF black, or healthy control grey) and secondary group (CF^{Act}-light green, CF^{Past}-dark green, $CF^{Control}$-white, and HC^A-black). Clustering of CF^{Ac} and CF^{Past} patients with one exception and clustering of $CF^{Control}$ and HC^A with one exception. (**B**) Principle component analysis (PCA) and canonical covariate analysis (CCA) show significant clustering ($P = 0.02$) of subjects by patient

group. Marked overlap between CF^{Act} and CF^{Past} groups is seen in terms of global cytokine secretion profile indicating an overall similarity in secretion profile. $CF^{Control}$ group diverges further from CF^{Act} and CF^{Past} groups.

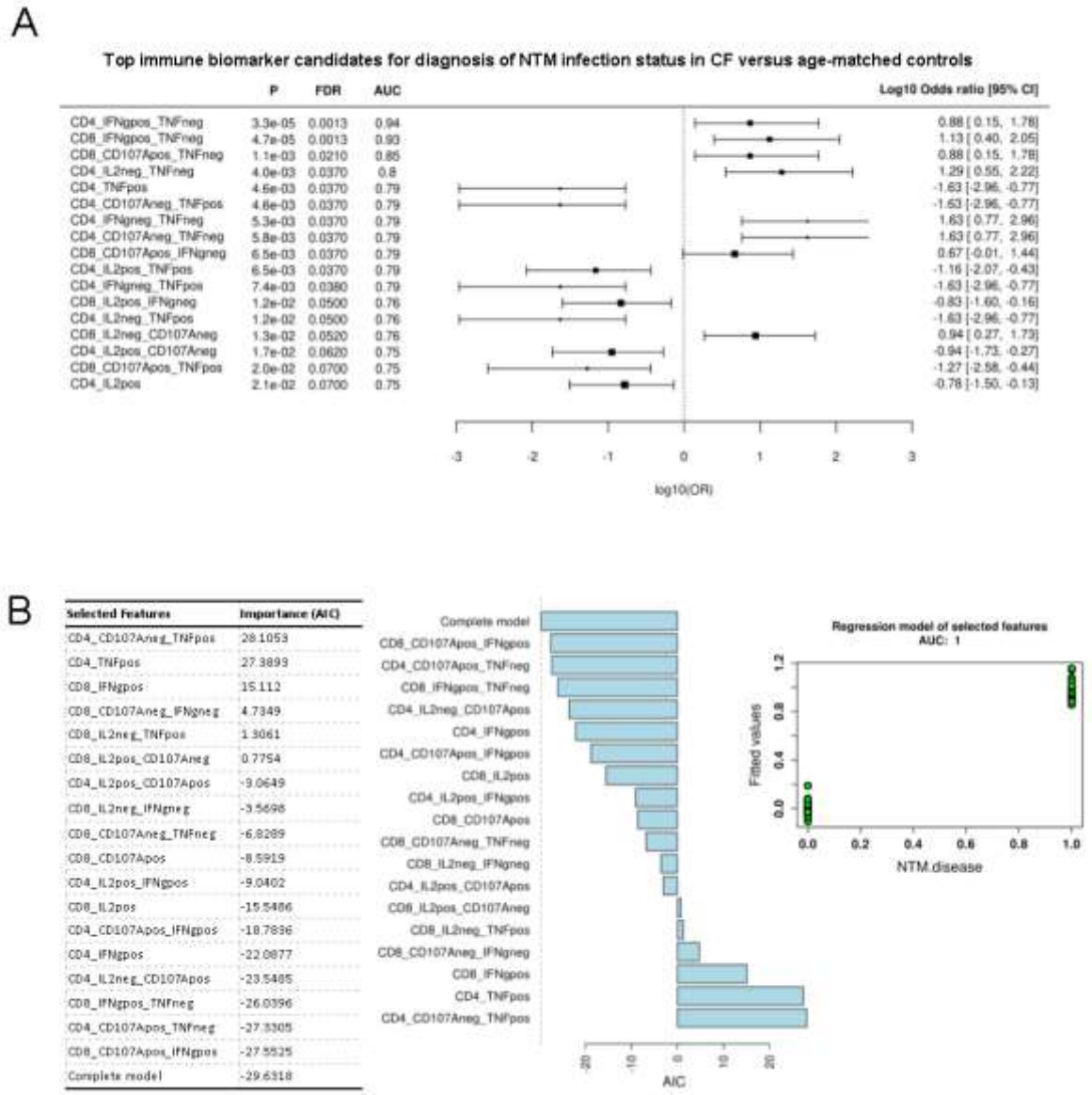


Figure 3.6. Top immune biomarker candidates for diagnosis of NTM infection status in CF cohorts.

(**A**) Flow cytometric biomarkers for NTM disease state from cytokine secretion profile data. (Wilcoxon test) Graph shows log odds ratio (log OR) for each biomarker candidate for diagnosis of NTM disease state (negative versus exposed state) with 95% CI. (P- Probability, FDR- false discover rate, AUC- area under the curve). (**B**) Stepwise regression model (forward selection) for NTM infection status. Fitted model contains 18 variables shown in table with a

final AIC of –30 and an AUC 1. Plots show contribution of each variable to model and fitted value of for each patient/control (green dots) when model is applied showing an AUC =1

3.4 Distinct T Cell Function in Elderly Patients with Active MABS Infection

To determine whether this immune profile would also be found in other independent disease cohorts, we next investigated elderly patients with active NTM infection (NTM^{Act}). In Australia, the rate of notified NTM cases per 100,000 population has increased by approximately 17% per year between 2012 and 2015 (58). The reasons for this increase is unknown, and there is as yet no predictor to identify at-risk individuals. To determine if underlying immune dysfunction may be a predictive factor for NTM infection we compared NTM^{Act} patients with elderly healthy controls (HC^{B}). We found that Tregs were increased in the peripheral blood of elderly NTM^{Act} patients compared to elderly HC^{B} (Figure 3.7 (A)). Elevated CD25 and CTLA4 expression was also seen on $CD4^{+}$ T cells in the NTM^{Act} group alongside increased PD1 expression on $CD4^{+}$ $CD25^{+}$ T cells. Analysis of the phenotypic fingerprint of CD25, CTLA4 and PD1 expression in $CD4^{+}$ T cells showed a specific signature in NTM^{Act} patients with elevated CD25 and CTLA4 double-positive T cells as well as elevated CD25 single-positive T cells the in NTM^{Act} group (Figure 3.7(A) and (B)). T cells negative for all three markers (triple-negative) were significantly higher in the HC^{B} group compared to NTM^{Act} patients. Four significant phenotypic differences *ex vivo* and during mitogen stimulation were observed between the NTM^{Act} group and HC^{B} group (Figure 3.7 (C)). *Ex vivo* $CD8^{+}$ T cell fingerprint were identical between HC^{B} and NTM^{Act} cohorts (Figure 3.9 (C) and (D)).

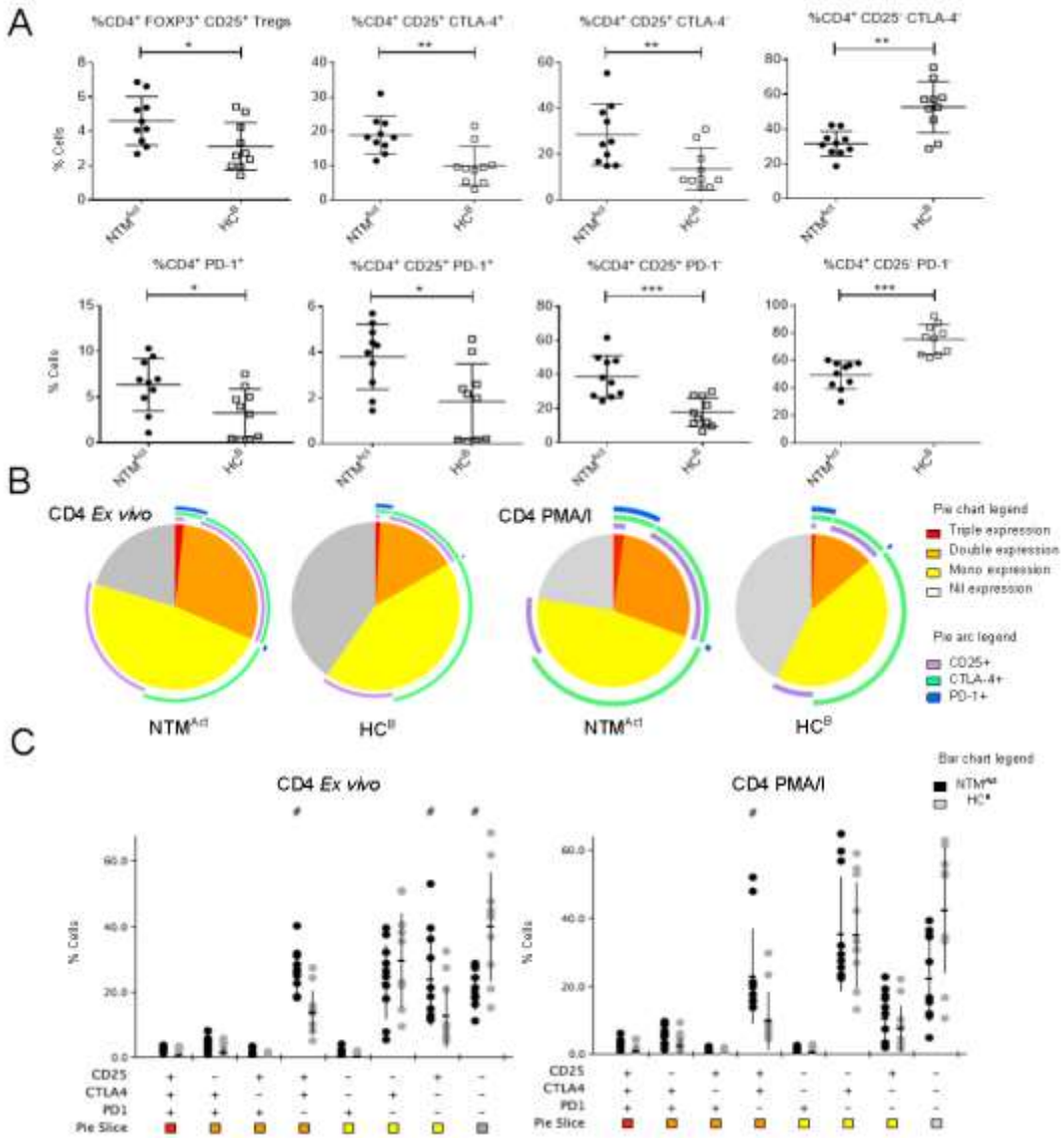


Figure 3.7. Evidence of CD4$^+$ T cell activation and exhaustion in immunocompetent individuals with active NTM infection.

(**A**) Flow cytometric analysis of *ex vivo* CD4$^+$ T cells showed significantly more Tregs in NTM^{Act} group compared to HC^B group (p=0.028). Significantly more CD25$^+$ CTLA4$^+$ and CD25$^+$ CTLA4- CD4$^+$ T cells were seen in NTM^{Act} group (p=0.002 and p=0.009, respectively) compared to HC^B. A reciprocal increase in CD25- CTLA4- CD4$^+$ T cells was seen in the HC^B group (p=0.001) compared with disease group. Higher numbers of PD1$^+$ CD4$^+$ T cells (p=0.021) and CD25$^+$ PD1$^+$ CD4$^+$ T cells (p=0 011) were seen in the NTM^{Act} group. CD25$^+$ PD1- T cells were significantly higher in NTM^{Act} group (p<0.001) and CD25- and PD1- CD4$^+$ T cells were significantly higher in HC^B group (p<0.001). (**B**) Phenotyping and polyfunctionality profiling of CD4$^+$ T cells by SPICE showed differences in NTM^{Act} and HC^B

groups directly *ex vivo* and post PMA/I stimulation. Phenotype profiles were significantly different between groups both *ex vivo* ($p=0.0013$) and post stimulation ($p=0.022$). Significantly more $CD25^+$ $CTLA4^+$ T cells were observed in NTM^{Act} groups compared HC^B. (**C**) SPICE dot plots show a phenotype profile of all combinations of markers CD25, CTLA4 and PD1 in $CD4^+$ T cells both *ex vivo* and post PMA/I stimulation between NTM^{Act} and HC^B groups. Significantly different expression compared to HC^B (Wilcoxon rank test $p<0.05$) is indicated with a # symbol.

Mitogen stimulation and polyfunctionality analysis in the elderly cohorts revealed a very different profile to that seen in CF patients. In the NTM^{Act} group, significantly higher TNF production by $CD8^+$ T cells was observed (Figure 3.8 (A) and (B)) while there was no difference in the number of TNF producing $CD4^+$ T cells between groups (Figure 3.9 (A)). IFNγ single-positive $CD8^+$ T cells were similar in patients and controls as was the overall polyfunctionality profile in $CD4^+$ T cells (Figure 3.9 (A) and (B)).

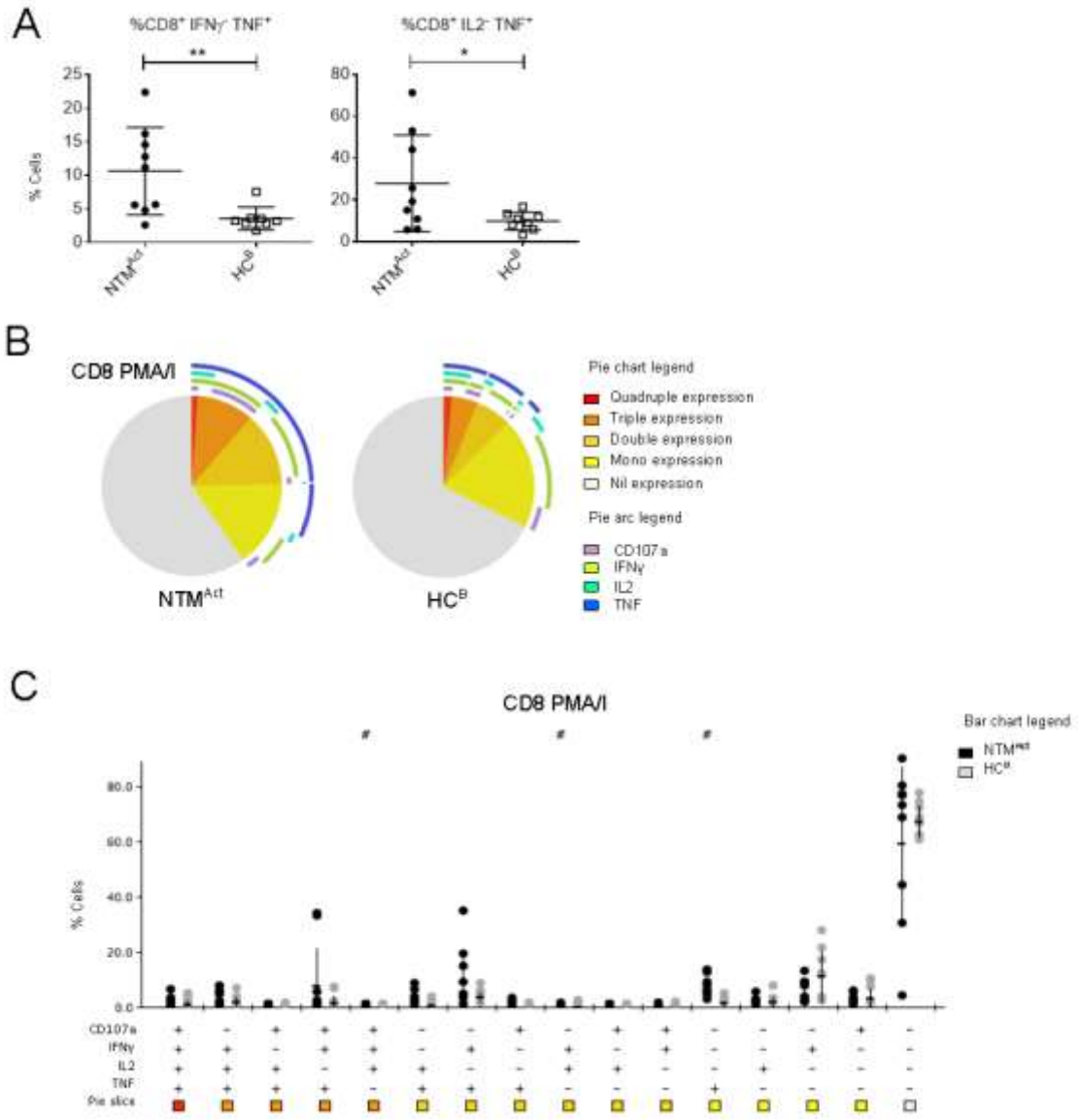


Figure 3.8. Divergence of CD8$^+$ T cell cytokine profiles in immunocompetent individuals with active NTM infection.

(A) Flow cytometric analysis of *ex vivo* CD8$^+$ T cells showed significantly more IFNγ- TNF$^+$ cells (p=0.01) and IL2- TNF$^+$ cells (p=0.048) in NTM^{Act} compared to HC^B. **(B)** Polyfunctionality profiling of CD8$^+$ T cells shown in pie charts were not significantly different between the two groups. However, significantly higher TNF mono-functional CD8$^+$ T cells were observed in NTM patient group (p=0.002). **(C)** SPICE dot plots show polyfunctionality profile of all combinations of cytokine expression in CD8$^+$ T cells after PMA/I stimulation between NTM^{Act} and HC^B groups. Significantly different expression compared to HC^B (Wilcoxon rank test p<0.05) is indicated with a # symbol.

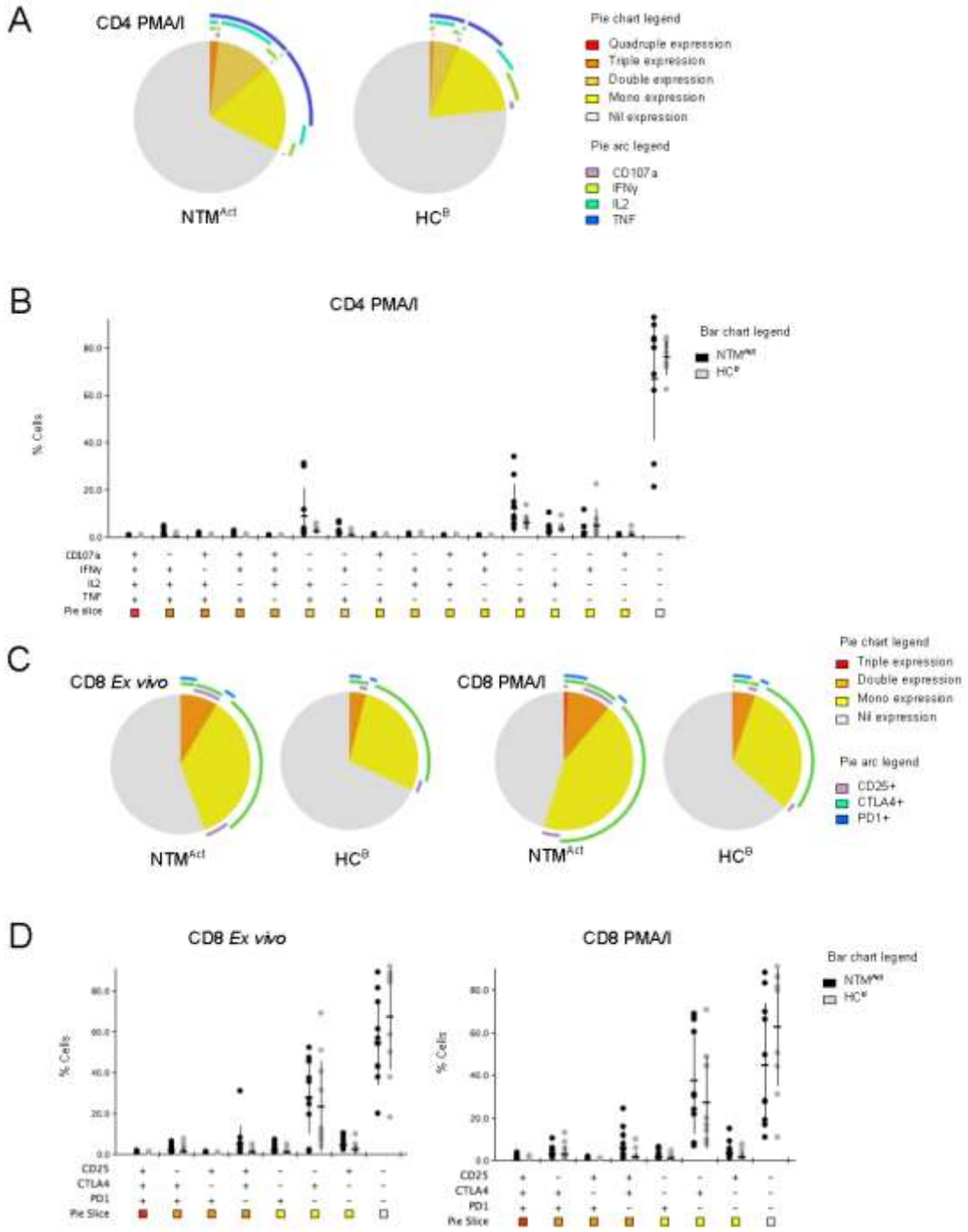


Figure 3.9. Phenotype and polyfunctionality profiles of T cells in immunocompetent individuals with active NTM infection.

(**A**) Flow cytometric polyfunctionality profiling of PMA/I stimulated $CD4^+$ T cells showed no significant differences between NTM^{Act} and HC^B groups. (**B**) SPICE dot plots showed no significant differences between NTM^{Act} and HC^B groups. (**C**) Flow cytometric phenotyping of *ex vivo* PMA/I stimulated $CD8^+$ T cells showed no significant differences between NTM^{Act} and HC^B groups. (**D**) SPICE dot plots showed no significant differences between NTM^{Act} and HC^B groups.

3.5 Discussion

The increased incidence and prevalence of NTM disease in recent years, necessitates a more comprehensive understanding of mechanisms of action and failures in immunity for susceptible individuals (52). Due to increasing antibiotic resistance of NTM strains and poor patient-outcomes, in particular MABS, immunomodulatory strategies may emerge as critical adjuvants to combine with conventional antimycobacterial therapy in NTM disease. Here, I dissect faults in immunity in two cohorts of patients (CF and elderly) with NTM lung infection and matched controls to identify the underlying blood immune signatures of each disease. Blood was examined given: (i) T cells are known to traffic between the blood and lung (195); (ii) NTM-specific T cells have been observed in the blood (196); and (iii) blood is easy to access for diagnostic tests. The first cohort included CF patients with active NTM disease, CF patients with past NTM disease who had been successfully treated and were now in disease remission, CF patients with chronic Pa infection who had no history of NTM infection and a group of matched healthy controls. The second cohort included elderly patients with NTM infection and matched healthy controls. All control individuals in both cohorts had active or past MABS infection with the exception of one patient who had MAC infection.

The frequency of Tregs was significantly increased in CF patients with active and past NTM infection compared to CF patients with chronic Pa infection. These data contrast with the elderly cohort where Tregs were significantly higher in elderly NTM patients compared to healthy controls. This was similarly observed by Hector et al, where lower number of Tregs were found in both the airways and peripheral blood of CF patients compared to healthy controls and a further reduction in Tregs was seen in patients with chronic Pa infection (197). The lack of difference in Treg percentages between CF NTM patients and healthy controls could be an indication of an increase in Tregs following NTM infection. This rationale would align with our findings of elevated Tregs in the elderly immunocompetent patients with active NTM infection. Of note, increased Treg numbers have been observed in the peripheral blood of TB patients (198-200) indicating overlap between TB and NTM immunopathology.

When examining the T cell fingerprint, CF patients with active and past MABS infection were different to control patients with chronic Pa infection and, interestingly, very similar to the healthy control group. This finding suggests differences in T cell immunity between CF patients susceptible to MABS and CF patients who have no history of NTM infection. These data indicate a new disease subclass within the umbrella of CF.

Distinct activation and exhaustion profiles were seen in both patient cohorts with NTM infection compared to corresponding controls. Higher numbers of $CD4^+$ T cells co-expressing the activation marker CD25 and the exhaustion marker CTLA4 were seen in CF patients with both active and past NTM infection. In the elderly cohort, a similar pattern of increased CD25 and CTLA4 co expression was observed on $CD4^+$ T cells. PD1 expression was also increased on $CD4^+$ T cells, suggesting an exhausted immune phenotype. High PD1 expression on T cells associates with increased TB disease burden (122). Increased PD1 expression on T cells, B cells, NK cells and monocytes has also been reported in patients with MAC infection (143). In mice, PD1 gene knockout can enhance TB resistance by preventing over-production of IFNγ (201). However, another study showed PD1 gene knockout mice can be more susceptible to NTM infection (202). To date, the immune checkpoint CTLA4 has not been studied in the context of NTM. Our data presents the first finding of elevated CTLA4 on T cells in NTM infection. The significance of this data as well as elevated PD1 expression on T cells indicates a degree of immune suppression in NTM infection. No PD1 (or CTLA4) antibody blockade therapy has been examined *in vivo* on TB or NTM patients. Future research should focus on the prospect of treating NTM patients using PD1 and/or CTLA4 antibody blockade.

TNF directly activates macrophages to restrict mycobacterial growth and induces apoptosis of infected macrophages leading to bacterial killing (203-205). TNF is also essential for granuloma formation and disease restriction during mycobacterial pathogenesis highlighting its importance for *in vivo* control of the pathogen (206). Animals deficient in TNF are highly susceptible to disseminated forms of TB (207). The cytokine polyfunctionality profiles seen in CF cohorts revealed underlying global TNF deficiencies that could explain susceptibility to NTM infection in CF patients. Low TNF secretion (mono-functional TNF secreting $CD4^+$ T cells) were seen in CF patients with both active and past NTM infection while increased TNF secreting mono-functional $CD8^+$ T cells were seen in elderly patients with NTM infection. Given that CF patients with both active and past infection exhibit this TNF deficiency in response to mitogen, it is more likely that this phenotype is an underlying predisposition to disease rather than a direct effect of the disease. The contrasting pattern of TNF secretion in the two disease scenarios is significant in the larger context of mycobacterial pathobiology. Studies of NTM immunity are conflicting and show both low and high levels of TNF production in supernatants of stimulated PBMCs (73, 135, 138, 139, 143). However, these studies did not perform in depth mapping of cell subsets. TNF production has an important role in host resistance as treatment with anti-TNF therapies are associated with increased

susceptibility to active TB and reactivation of latent TB infection (208) and is correlated with NTM activation in autoimmune diseases such as rheumatoid arthritis, Crohn's disease, ankylosing spondylitis and psoriasis (209). Here, we have shown that TNF profiles can correlates with specific disease settings. Thus, it is important to introduce personalised care when interpreting these findings. Moreover, we have also shown that TNF secretion levels vary according to cell subset and it may be specific deficiencies in specific cell subsets that predispose disease. It is possible that low dose TNF replacement therapy, via aerosol for example, may aid standard of care in NTM treatment in CF. Indeed, other cytokine replacement therapies such as IFNγ, IFNα, IL2, GM-CSF and IL12 have shown promise against TB, MDR-TB, MAC and MABS (reviewed (208)). Systemically administered IFNγ has shown the most promise for clinical use (210).

The importance of IFNγ in anti-mycobacterial immunity is widely accepted and extensively studied in TB (211). Reduced IFNγ production in NTM infection has been shown in several studies (133, 139, 212) though contradictory results have also been reported (137). Akin to TNF, we show that IFNγ secretion varies with cell subset and disease scenario. In the CF NTM cohort, increased IFNγ secretion was seen in both $CD4^+$ and $CD8^+$ T cells compared to healthy controls, whereas in the elderly NTM cohort, there was no significant increase in IFNγ secreting cells in both $CD4^+$ or $CD8^+$ T cells. This could indicate an inadequate protective response rather than a deficiency of cytokine. Comparison of MABS infection in these two disease cohorts once again highlights the importance of the clinical context when searching for risk factors. However, one limitation of the study is the profiling of circulating lymphocytes which may differ to those at the site of disease. Studies have shown that blood /tissue T cell trafficking occurs extensively (213) though to what extent the phenotype of blood vs lung T cells overlap is still unknown.

The search for environmental and behavioural risk factors for NTM infection in CF patients has found increased acquisition in the tropics and decreased acquisition with macrolide treatment (85). Here we show that it is also possible to define immune parameters in the circulatory blood that help identify at-risk individuals. Specifically, we showed that CF patients can be stratified into two groups based on Treg frequencies, $CD4^+$ T cell surface phenotype (CD25 and CTLA4) and cytokine production by $CD4^+$ and $CD8^+$ T cells (IFNγ, TNF, CD107a and IL2), with at-risk patients exhibiting a distinct deficiency in TNF production. We suggest that this immune signature could be further refined and validated in independent cohort studies, and ultimately developed as a diagnostic tool to identify individuals who are at high risk of

developing NTM infection. Targeted behavioural interventions for at-risk patients may subsequently decrease the risk of NTM acquisition from the environment or infected patients.

The role of $CD8^+$ T cells in NTM infection in humans remains unclear. Research in TB in primate and mouse models have shown that $CD8^+$ T cells are important in controlling experimental infection. However, the precise mechanism by which they contribute to protection is unknown (214). Regulatory $CD8^+$ T cells have been identified in human TB, though their role in protection is also not clear (214).

This study specifically focused on MABS infection. It is important to highlight that the CF patient with active MAC infection (shown as a blue circle in Figure 3.1(A) and Figure 3.3(A)) exhibited a T cell fingerprint and T cell functional profile very similar to patients with MABS infection (note; this patient was not included in statistical analysis). Additionally, it is also important to note the two-of-six CF MABS patients and three-of-ten elderly MABS patients either had a previous history of MAC infection or subsequently developed MAC infection. This tentatively suggests that the susceptibility to NTM infection may occur through a common immune dysfunction pathway, although further mechanistic studies and antigen-specific immune studies are required to validate this hypothesis. If NTM-specific immune dysfunction is common in the individuals, targeted immunotherapies may help in correcting this deficit.

Limitations of this study include the small sample size due to restrictions in patient recruitment within a confined study duration. An additional, larger patient cohort will be essential to validate our predictive model, ideally as a longitudinal follow-up study. Limitations in sample volumes also restricted the analysis to *ex vivo* phenotyping and mitogen activation signatures. Thus, antigen-specific immune responses were not conducted in this study. However, further research is warranted given the identification of significant global immune anomalies in this exploratory study. Anomalies in mitogen-triggered activation signatures in the global T cell compartment suggest an underlying immune deficiency in the patients that would likely translate to NTM-specific responses as seen in TB (215, 216). Cross sectional design limits result interpretation to association of TNF and checkpoint molecules with NTM infection in these two patient cohorts. Mechanistic studies, identifying correctable T cell immune dysfunction by checkpoint blockade are required to pave the way for potential adjunctive therapies. A combination of longitudinal data with immune modulation showing susceptibility and reversal of susceptibility would be necessary to show causation of infection.

In summary, our study presents the first data on immune checkpoint expression on T cell subsets in human MABS infection as well as the first comparison of T cell polyfunctionality between CF and non-CF patient groups with MABS infection in response to mitogen stimulation. We show that MABS infection in two different patient groups' exhibit specific immune phenotypes and show dysregulation in type 1 cytokine production and a global decrease in T cell 'quality'. In CF patients, TNF-mediated immunity may hold the key to understanding the increased risk of MABS infection and guide future therapeutic interventions. In elderly individuals, interventions with checkpoint molecules (PD1 and CTLA4) may be included in frontline therapies. Collectively, the study has revealed many potential biomarkers that could allow early detection and diagnosis of patients with MABS disease as well as targetable pathways for immunotherapy.

Chapter 4: Immune phenotyping of elderly patients with MAC and MABS lung disease.

4.1 Introduction

Based on the findings of the pilot study described in Chapter 3, a second larger study was designed, focusing on the middle aged/elderly patient group (aged 50-84yrs). The objectives of this study were to: (i) perform a comprehensive analysis of the peripheral blood immune cell compartment; (ii) identify species-specific and disease stage-specific immune signatures that could be used as screening and prognostic tools and; (iii) to evaluate the responsiveness of PBMCs in patients with regulatory approved immune modulators originally designed for the cancer space.

The experimental design is described in Chapter 2: Materials and Methods, section 2.4. Briefly, elderly patients with MAC lung disease were recruited before (Active MAC) and after successful treatment (PostTx MAC) along with patients who had active MABS lung infection (active MABS) as well as patients with persistent infection (either MAC or MABS- Persist Inf). These patients were compared to two control groups: 1) healthy controls (HC) and 2) patients with bronchiectasis who had no history of NTM infection (BronchC). PBMCs from these patients and control cohorts were then evaluated for immune cell composition, exhaustion marker expression, cytokine production and proliferation capacity. Subsequently, antigen specific cytokine responses and immune modulatory agents were evaluated *in vitro*. This chapter presents the results of peripheral blood phenotyping assays T cell functional assays in response to non-specific stimulation

4.2 Patient demographics and clinical measures

A total of 96 middle-aged to elderly subjects were included in the study distributed amongst the four patient cohorts and two control cohorts. Age and gender distribution within each cohort are shown in Table 4. 1. A Dunnetts T3 post hoc test showed no significant difference in mean age between groups (p values for all comparisons >0.05). Gender distribution could not be tested accurately as some cells in the cross tabulation had expected counts of <5. Female

predominance was seen in all patient groups with no male patients with persistent infection being recruited during this time.

Body mass index (BMI) data was available for a subset of patients and healthy controls. Comparison revealed a significantly lower BMI in the PostTx MAC group (mean BMI =22.2kg/m2) and Persist Infection group (mean BMI 20.8kg/m2) compared to the HC group (mean BMI 26. 7kg/m2; unpaired *t* test, p = 0.022 and p=0.047, respectively) while Active MAC patients showed a similar trend (mean BMI 22.83kg/m2; unpaired t test p=0.094). *M. intracellulare* was the most common causative species in the MAC patient groups, while the nodular bronchiectatic pathological type of disease was diagnosed in most patients across all groups.

Outcome of treatment in the Active MAC patient group as per patient records showed thirteen patients in the cohort went into disease remission at the end of treatment. Two patients were untreated as they had stable disease and did not require antibiotic management at that time while one patient was lost to follow up. Two patients developed persistent infection due to identified risk factors. A similar distribution of outcome was seen in the Active MABS group with three patients going into disease remission with treatment, two patients progressing to persistent infection and one patient being lost to follow up. Five patients in the Active MAC group and two patients in the Active MABS group had a history of recurrent infection, with two or more episodes of NTM infection (different species) though each episode was successfully treated. Three patients in the PostTx MAC group also had a history of, or subsequently developed another episode of NTM lung infection. In total, 10/53 (18%) of patients with active or past infection had recurring infections in this study cohort.

Sample collection in PostTx MAC group occurred from one month post treatment to over five years post treatment. A breakdown of patient numbers by time to sampling is shown in Table 4. 1. Although some patients had a history of recurrence of infection or subsequently developed a recurrence as mentioned above, at the time of sampling all patients were in disease remission.

Table 4. 1 Clinical and demographic characteristics of patients included in study stage II

	Active MAC infection- Pre Treatment (Active MAC)	MAC infection—Post treatment (PostTx MAC)	Active MABS infection—Pre treatment (Active MABS)	Persistent infection (Persist Infection)	Bronchiectasis control (BronchC)	Healthy control (HC)
Sample n	23	22	8	9	11	23
Mean age (SD)	67.17 (6.43)	66.91 (9.99)	75.0 (8.19)	67.11 (8.95)	74.81 (8.91)	64.40 (8.84)
Female : Male	15:8	21:1	6:2	9:0	8:3	15:7
BMI (kg/m^2) (n)	22.83 (8)	22.1 (13)	19.7 (2)	20.8 (5)		26.7 (11)
Species	M.a*–4	M.a–3		M.a–2		
	M.i **–19	M.i –15		M.i –4		
	Other MAC$^{\$}$ spp.–0	Other MAC spp–4		M. t^{+}–1		
				MABS$^{\$\$}$-2		
Disease type-						
NB	18	20	8	6		
NB + Cavity	3	2	0	2		
Cavitatory	2		0	1		
Treatment outcome	Remission –18		Remission-5			
	Persistence –2		Persistence-2			
	Other[1] -3		Other[1] -1			
Post treatment sampling		<1year—8				
		1-2years—5				
		2-5years—5				
		>5years—4				
Comorbidity						
Malignancy	6	4	0	0		1
GORD	2	2	1	2		3

* *Mycobacterium avium*

** *Mycobacterium intracellulare*

$ *Mycobacterium abscessus* subspecies *masseliense*

$$ *Mycobacterium absessus* subspecies *bollettii*

+ *Mycobacterium triplex*

[1] Other– untreated, still on treatment or lost to follow up.

NB– Nodular bronchiectatic

COPD– Chronic Obstructive Pulmonary disease

GORD– Gastro oesophageal reflux disease

4.3 Immune cell composition of peripheral blood

I performed a comprehensive analysis of the immune cell composition of peripheral blood mononuclear cells utilizing two multicolour flow cytometric panels as described in the Materials and Methods section 2.6.3, utilizing viSNE (217) and SPICE (190).

Panel 1 characterized both lymphocyte and monocyte cell populations utilizing twelve lineage and subset markers. The major cell subsets characterized are shown in the viSNE plot (Figure 4. 1). Total $CD4^+$ T cells, and $CD4^+$ T cell subsets including T regulatory cells (Tregs) ($CD4^+$, $CD25^+$ $FOXP3^+$), naïve ($CD45RA^+$ $CCR7^+$), central memory ($CD45RA^-$ $CCR7^+$), transitional effector memory ($CD45RA^-$ $CCR7^-$ $CD27^+$), effector memory ($CD45RA^-$ $CCR7^-$ $CD27^-$), and terminally differentiated effector cells (TEMRA $^-$ $CD45RA^+$ $CCR7^-$) were characterized. $CD4^+$ T cell subsets showed similar percentage distribution across all patient and control groups. The same pattern was seen in total $CD8^+$ T cells as well as $CD8^+$ T cell subsets, naïve ($CD45RA^+$ $CCR7^+$), central memory ($CD45RA^-$ $CCR7^+$), transitional effector memory ($CD45RA^-$ $CCR7^-$ $CD27^+$), effector memory ($CD45RA^-$ $CCR7^-$ $CD27^-$), and terminally differentiated effector cells ($CD45RA^+$ $CCR7^-$), with no difference in percentage of cells subsets across groups. In addition, NKT cells ($CD3^+$ $CD56^+$), NK cells ($CD3^-$ $CD56^+$) including subsets $CD56^{bright}$ $CD16^{dim}$, $CD56^{dim}$ $CD16^{bright}$, $CD56^{dim}$ $CD16^{dim}$ and B cells ($CD3^-$ $CD19^+$), including subsets naïve ($CD19^+$ $CD27^-$) and memory ($CD19^+$ $CD27^+$) B cells, all comprised similar percentages in peripheral blood across patients and controls. Monocytes subsets analysed included $CD14^+$ $CD16^-$ classical monocytes, $CD14^+$ $CD16^+$ intermediate monocytes and $CD14^-$ $CD16^+$ non-classical monocytes. No significant difference in monocyte composition was observed between groups.

ViSNE analysis enabled a top down view of the lymphocyte lineages. Negative cells (ie. $CD3^-$ $CD4^-$ $CD8^-$ $CD56^-$ $CD19^-$ $CD14^-$) made up a significant portion of the peripheral blood. These cells comprised approximately 1 to 12% of lymphocyte gate in patients and controls with no significant difference between groups. These cells are likely to represent innate lymphoid cells (ILCs) other than NK cells.

Panel 2 was used to characterize mucosa associated invariant T cells ($CD3^+$ $CD161^+$ $V\alpha7.2^+$) (MAIT cells) and γδ T cells ($CD3^+$ γδ TCR^+) which comprised 0-16% and 0-40% of PBMCs in all patient and control cohorts, respectively. There was no significant difference in the percentage of these cells in peripheral blood between groups.

Overall, no significant differences in peripheral blood immune cell makeup were seen between patients and controls or between patient groups. This analysis included both innate cells like NK cells and monocytes as well as the sub-lineages of adaptive T cell and B cell subsets.

4.4 Exhaustion marker expression patterns on peripheral blood cell immune subsets

A main aim of this study was to characterize the expression of the immune checkpoint markers CTLA4, TIM3 and PD1 on peripheral blood immune cells. An initial overview of the percentage of cells that were positive for these exhaustion markers (gating based on FMO controls - see Methods section 2.7) and level of expression (as quantified by MFI) was done utilizing the viSNE map (Figure 4.1A). An increase in CLTA4 on multiple cell subsets, particularly $CD4^+$ effector cells was seen in both MABS and MAC infection. A more pronounce increase occurred in MABS infection. In contrast TIM3 was increased in MAC infections, particularly on NK cells. Detailed analysis was then performed on cell subsets by conventional biaxial gating and SPICE analysis (190).

SPICE analysis (see Methods section 2.13.1) generated pie charts that characterized the exhaustion marker 'fingerprint' in each cell subset. Based on the expression pattern (ie. triple expression, all three exhaustion markers; double expression, any two of the markers; mono expression, any one of the markers; nil expression, no markers), $CD4^+$ $CD25^+$ $FOXP3^+$ T regulatory cells and $CD4^+$ TEMRA cells showed an exhaustion fingerprint that was unique to patients with MABS infection when compared to healthy controls (Figure 4.1B). There was a notable increase in mono expressing (yellow pie slice), $CLTA4^+$ cells (red pie arc), with a lower increase in $TIM3^+$ (blue pie arc) cells in $CD4^+$ TEMRA cells in Active MABS compared to HCs. A similar evaluation of other cells, including $CD8^+$ T cell subsets, NKT and NK cells, showed no difference in overall marker fingerprint between groups.

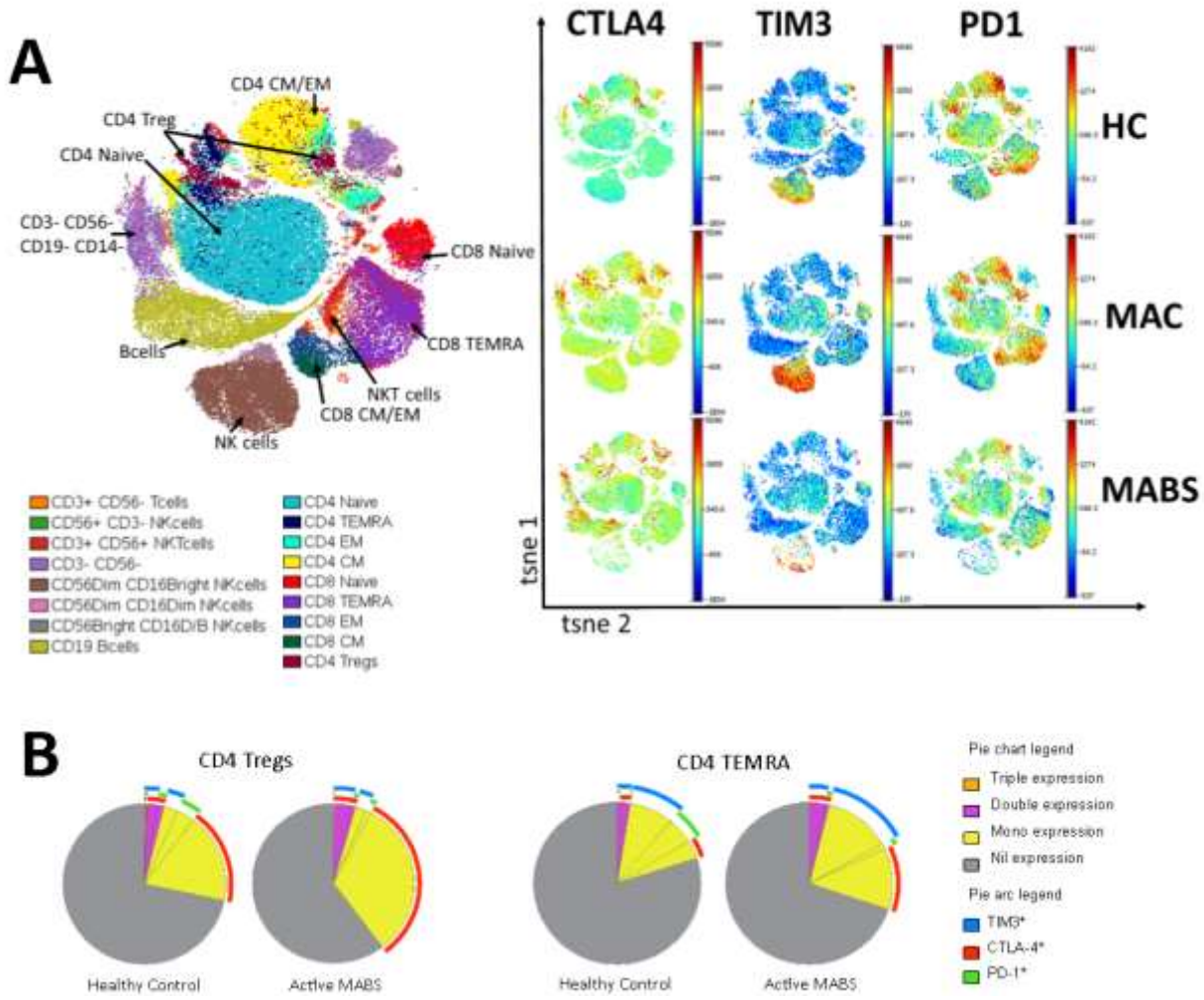


Figure 4.1 Peripheral blood phenotyping analysis showing increased Tim3 and CTLA4 in patients with Active MAC and MABS infection.

(A) ViSNE plot showing all cell subsets that were analysed in the lymphocyte gate. Representative viSNE plots from a healthy control (HC), patient with active MAC infection (MAC), and patient with active MABS infection (MABS) are shown, coloured by level of expression of CTLA4, TIM3 and PD1. Multiple cell subsets with increased CTLA4 and TIM3 expression in active MAC and MABS infection are seen. **(B)** SPICE analysis showing exhaustion marker fingerprint of $CD4^+$ $FOXP3^+$ $CD25^+$ Treg cells and $CD4^+$ $CD45RA^+$ CCR7- TEMRA cells. Pie slices denote number of positive exhaustion markers. Pie arcs denote each individual marker as shown in the legend. Significant difference in exhaustion marker fingerprint of T reg cells (p=0.0144) and in $CD4^+$ TEMRA cells (p=0.0128) were observed between Active MABS and HCs.

A complete breakdown of cell subsets by exhaustion marker fingerprinting is shown in Table 4.2. Major cell lineages and cell subsets are shown with the patterns of CTLA4, TIM3 and PD1 expression where significant differences between groups were identified. Also shown is the respective p value along with either increase (+) or decrease (-) of that cell population in the given patient group as compared to HCs.

The pattern that emerges here is consistent with elevated $CD4^+$ subsets including $CTLA4^+$ $CD4^+$ central memory T cells, effector memory T cells, TEMRA and Treg cells in active MABS infection compared to healthy controls. $TIM3^+$ $CD4^+$ effector memory cells were increased in active MAC infection. The increase of TIM3 expression in active MAC infection was more prominent in $CD8^+$ T cells with a transitional effector memory, effector memory and TEMRA cells all showing this same signature. NK cells showed a similar increase in TIM3 in active MAC infection. Classical monocytes showed a reduction in $TIM3^+$ cell percentage while non-classical monocytes showed an increase in TIM3 positivity in active MAC infection. Post treatment MAC patients showed fewer differences compared to healthy controls than patients with active infection. $CD8^+$ transitional and effector memory T cells retained the increased $TIM3^+$ signature seen in active infection while $CD4^+$ T cells had returned to baseline states. The exhaustion marker signature in persistent infection was also remarkably like healthy controls with only $CD4^+$ TEMRA cells showing an increase in $CLTA^+$ $TIM3^+$ cells, while $CD8^+$ transitional effector memory cells showed a reduction in $TIM3^+$ $PD1^+$ cells. BronchC differed from HCs only with a comparative increase in $CTLA4^+$ $CD4^+$ TEMRA cells and Treg cells. In all other subsets, BronchC and HCs showed no difference in exhaustion marker expression.

Table 4. 2 SPICE analysis-based exhaustion marker fingerprint on immune cell subsets in elderly patient cohorts compared to healthy controls

Cell type	Subset	CTLA4	TIM3	PD1	Active MAC p value	PostTx MAC p value	Active MABS p value	Persist infection p value	Bronch control p value
$CD3^+$ $CD4^+$ T cells	Central memory	+	-	-	(+) 0.039		(+) 0.001		
	Transitional Effector memory	-	-	+			(-) 0.029		
	Effector memory	-	+	-	(+) 0.009				
		-	-	+			(-) 0.033		
		+	-	-			(+) 0.002		
	TEMRA	+	+	-			(+) 0.018	(+) 0.014	(+) 0.002
		+	-	-			(+) 0.002		(+) 0.047
	Tregs	+	-	-			(+) 0.018		(+) 0.031
$CD3^+$ $CD8^+$ T cells	Central memory	+	-	-			(+) 0.019		
	Transitional Effector memory	-	+	+		(+) 0.016		(-) 0.036	
		-	+	-	(+) 0.024				
	Effector memory	-	+	-	(+) 0.002	(+) 0.016			
		-	-	+	(-) 0.033				
	TEMRA	-	+	-	(+) 0.005				
		-	-	+			(-) 0.013		
$CD3^+$ $CD4^-$ $CD8^-$ T cells		-	+	-	(+) 0.001	(+) 0.002	(+) 0.018		
$CD3^-$ $CD56^+$ NK cells	$CD56^{bright}$ $CD16^{dim/bright}$	-	+	-	(+) 0.006		(+) 0.026		
		-	-	-	(-) 0.007		(-) 0.035		
	$CD56^{dim}$ $CD16^{bright}$	-	+	-	(+) 0.021				
		-	-	-	(-) 0.020				
	$CD56^{dim}$ $CD1^{6dim}$	+	+	-				(+) 0.029	
		-	+	-	(+) 0.035				
		-	-	-	(-) 0.023				
$CD3^+$ $CD56^+$ NKT cells		-	-	+			(-) 0.024		
Monocytes	$CD14^+$ $CD16^-$ classical	-	+	-	(-) 0.010				
		-	-	-	(+) 0.012				
	$CD14^{dim}$ $CD16^+$ non classical	-	+	-	(+) 0.024				
		-	-	-	(-) 0.020				

(+) Significant increase in cell population compared to healthy controls.

(-) Significant decrease in cell population compared to healthy controls

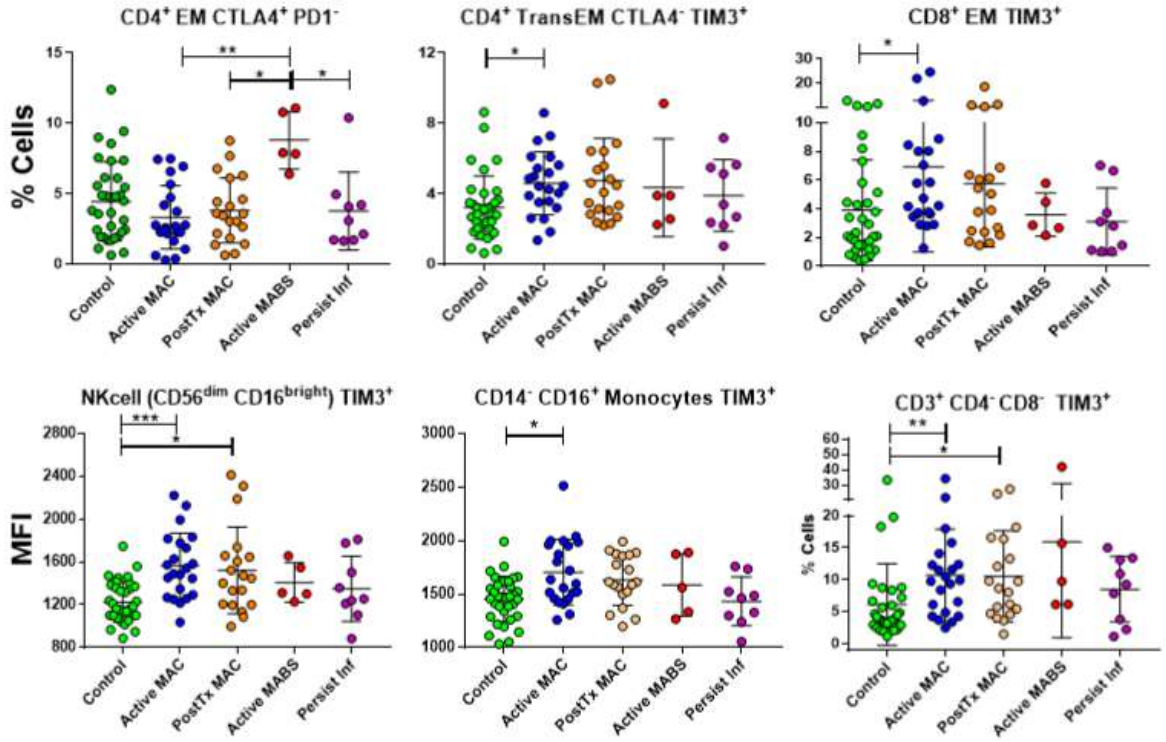


Figure 4.2 Tim3 and CTLA4 expression on $CD4^+$, $CD8^+$, $CD4^-CD8^-$ T cells, NK cells and non-classical monocytes

Conventional biaxial gating shows: i) significant increase in $CTLA4^+$ $PD1^-$ $CD4^+$ effector memory (EM) cells in Active MABS infection compared to active MAC (p=0.0061), PostTx MAC (p=0.0261) and Persist Inf (p=0.0401) patient groups; ii) significant increase in $CTLA4^-$ $TIM3^+$ $CD4^+$ transitional effector memory (TransEM) cells in Active MAC compared to controls (p=0.0355) with a similar increase in PostTx MAC patients though this did not reach statistical significance (p=0.140). iii) Increased $TIM3^+$ $CD8^+$ effector memory (EM) cells in Active MAC compared to Controls (p=0.043); iv) increased TIM3 expression (MFI) in $CD56^{dim}$ $CD16^{bright}$ NK cells in both Active MAC (p=0.0002) and PostTx MAC (p=0.0301) patients compared to controls ; v) increased TIM3 expression in $CD14^-$ $CD16^+$ non-classical monocytes in Active MAC compared to controls (p=0.03); and vi) $TIM3^+$ $CD3^+$ $CD4^-$ $CD8^-$ cells in Active MAC compared to controls (p=0.009). In this double negative subset PostTx MAC patients also significantly higher $TIM3^+$ cells compared to controls (p=0.03).

Conventional biaxial gating confirmed the $CD4^+$ T cell fingerprint, giving a more precise description of the specific immune cell subsets with the CTLA4 and TIM3 signatures (Figure

4. 2). HCs and BronchC samples were pooled into a common control group for this analysis as it advanced downstream data interpretation. A significantly higher percentage of $CTLA4^{+}$ $PD1^{-}$ $CD4^{+}$ effector memory T cells were seen in Active MABS patients compared to Active MAC and PostTx MAC groups as well as the Persist Inf group (Kruskal- Wallis test p=0.0133, multiple comparisons of mean rank p=0.0061, p=0.0261 and p=0.040, respectively). A similar trend was seen compared to the control group though this comparison did not reach statistical significance (p=0.062). A higher percentage of $TIM3^{+}$ $CTLA4^{-}$ $CD4^{+}$ transitional effector memory T cells was seen in Active MAC patients compared to the control group (p=0.012). In addition, $TIM3^{+}$ $CD8^{+}$ effector memory T cells were increased in Active MAC patients compared to controls (p=0.043).

The $CD56^{dim}$ $CD16^{bright}$ subset of NK cells as well as non-classical monocytes had higher expression of TIM3, as measured by MFI, in Active MAC patients compared to controls (p=0.0002 and p=0.03 respectively), which corresponded to what was seen in the viSNE plot.

Collectively, these results show that MAC and MABS infection results in increased TIM3 and CTLA4 expression across multiple immune cell subsets, a finding confirmed by multiple analytical methods. Both the adaptive and innate immune compartments were affected by these changes. PostTx MAC patients showed what could be residual changes with persistence in TIM3 elevation in NK cells and non-classical monocytes. Interestingly, of all patient groups, patients with persistent infection showed the least difference in exhaustion marker signature compared to healthy controls. Bronchiectasis control subjects showed some elevation of CTLA4 on $CD4^{+}$ TEMRA cells but showed no other difference in exhaustion marker fingerprints as compared to HCs. Combining these two groups into a single control group that represented a “non-infection related baseline” immune signature showed that the elevation of TIM3 and CTLA4 in active MAC and MABS infection was likely the result of NTM infection.

4.5 Differentiation of active infection from controls by flow cytometry

The second major aim of this study was to identify unique cell clusters or markers that could differentiate active infection from background controls i.e. healthy individuals as well as patients with bronchiectatic lung disease but no NTM infection. The results described above

which showed by multiple analytical methods that species-specific exhaustion marker signatures existed during active infection. The Citrus algorithm available in Cytobank (Cytobank. org) was then leveraged to identify cell clusters which best differentiated active infection from controls, to elucidate mechanism of action and to provide predictive models of disease. Figure 4.3(A) shows the marker output plot generated by Citrus (191) showing CD3 expression as a heatmap. All cells in the lymphocyte gate are sequentially subdivided into two daughter nodes based on expression of markers of interest i.e. lineage markers. The plot represents all cells in the lymphocyte gate broken down into nodes of similarity. Figure 4.3(B) are feature plots generated by Citrus. These show the selected nodes (dark brown) identified as having differential expression of TIM3 or PD1 (as MFI) between patients with active infection (either MAC or MABS) compared to controls (BC or HC). NK cells, NKT cells, $CD3^+$ $CD4^-$ $CD8^-$ T cells and $CD8^+$ T cell nodes showed differential expression of TIM3 as shown. Several nodes had differential expression of TIM3 while only one node had differential expression of PD1. CTLA4 was not differentially expressed between groups in any of the nodes based on this analysis. Probable reasons for this include the lower number of MABS patients in the active infection group compared to MAC patients, resulting in a stronger TIM3 signal which was detected by the algorithm and the staining for intracellular CTLA4 being more variable between subjects.

A deep dive analysis of selected nodes with differential TIM3 expression highlighted by coloured circles in Figure 4.3(B) was performed to cross-validate data. Dot plots in Figure 4.3(C) show TIM3 MFI of each of the nodes indicated by the colour coded rings overlaid on features plots in section (B). Comparison of TIM3 MFI between patients with active infection and controls shows significantly higher TIM3 expression in active infection in all nodes identified by the algorithm. The most significant differences were seen in nodes from $CD8^+$ T cells (black circle) and NK cells (orange circle). These data correspond with the findings of the SPICE analysis. The PAMR (nearest shrunken centroid) prediction model generated from this analysis had a cross validation error rate of 25% which is not deemed an adequate predictive model.

Citrus analysis identified the $CD3^+$ $CD4^-$ $CD8^-$ subset of cells as having differential expression of TIM3 in active disease compared to controls. This subset of cells was not previously analysed by biaxial gating. Reanalysis and applying conventional biaxial gates to this subset of cells on FlowJo followed by SPICE analysis revealed that this finding was indeed accurate. The increased expression of TIM3 on these cells is shown in Figure 4.1(C) and in Table 4.2.

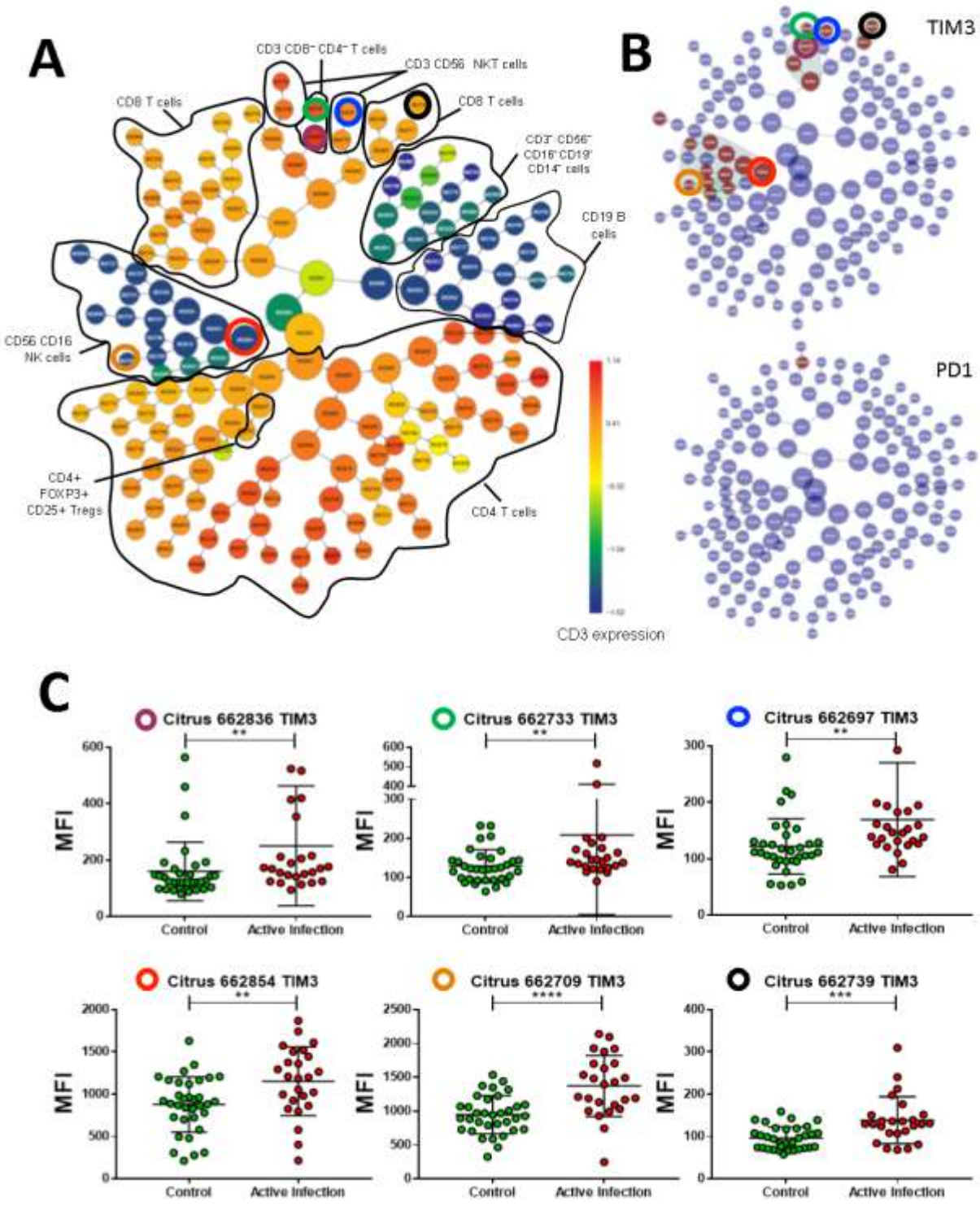


Figure 4.3 Citrus analysis showing cell nodes that differentially express TIM3 in active infection compared to controls.

All cells of lymphocyte gate in patients with Active infection (Active MAC + Active MABS) and Controls (BronchC + HC) were analysed with Citrus (Cytobank. org). **(A)** Marker plot shows all cells (central node) sequentially divided into daughter nodes based on dissimilarity

of expression of lineage markers specified in the analysis setup. Marker plot is coloured by expression of CD3 as a heatmap. Black lines show cell subsets identified based on other marker plots not shown here. Total lymphocyte gate is represented in this plot**. (B)** The same marker plot as shown in **A**. is coloured by cell nodes (brown) that have significantly different expression of i) TIM3 and ii) PD1. Nodes that were analysed further are highlighted with coloured circles. Corresponding circles in marker plot **A**. show which cell subset each node belongs to. **(C)** Nodes identified for further analysis are identified by the coloured circle and by the node number assigned by the citrus algorithm. MFI of TIM3 for the cells assigned to the given node for each sample are plotted. Comparison between groups done by Mann-Whitney test. Maroon circle (node 662836) $CD3^+$ $CD4^-$ $CD8^-$ T cell parent node ($p=0.0039$); Green circle (node 662733) subset of $CD3^+$ $CD4^-$ $CD8^-$ T cells ($p=0.0071$); blue circle (node 662697) $CD3^+$ $CD56^+$ NKT cells ($p=0.0014$); red circle (node 662854) $CD3^-$ $CD56^+$ $CD16^+$ NK cell parent node ($p=0.0041$); orange circle (node 662709) $CD3^-$ $CD56^+$ $CD16^+$ NK cell daughter node ($p<0.0001$); black circle (node 662739) $CD3^+$ $CD8^+$ T cells ($p=0.0005$). All nodes show increased TIM3 expression in active infection.

4.6 Functional and proliferative capacity of peripheral blood immune cells in response to non-specific stimulus.

Having found phenotypic evidence of immune dysfunction associated with active NTM infection, functional profiling was next carried out assessing cytokine secretion capacity and the proliferation variabilities of T cells across cohorts.

4.6.1 Polyfunctionality response to mitogen stimulation

Type 1 (Th1) cytokine production is the corner stone of anti-mycobacterial immunity. The functional response generated by T cells, NK cells, and NKT cells to a 6-hour PMA (nonspecific mitogen) stimulation was tested, quantifying Th1, Th2, Th17 responses by intracellular staining. Cells were evaluated for overall polyfunctionality (the number of cytokines they produced) as well as the specific cytokine production pattern and quantity (as MFI and percentage of positive cells).

The overall polyfunctionality signatures (as SPICE generated pie charts) are shown in Figure 4.4. There was no significant difference in polyfunctionality between patient or control groups at a $p<0.05$ level of significance. Patients had no significant change in polyfunctional T cells,

NK cells or NKT cells compared to controls. $CD4^+$ T cells in all groups showed similar levels of IFNγ, TNF, IL2, IL17, IL4 and IL10 secretion while $CD8^+$ T cells, NKT cells and NK cells in all groups showed similar levels of IFNγ, TNF, IL2, IL4, IL10 and CD107a secretion/expression following PMA stimulation.

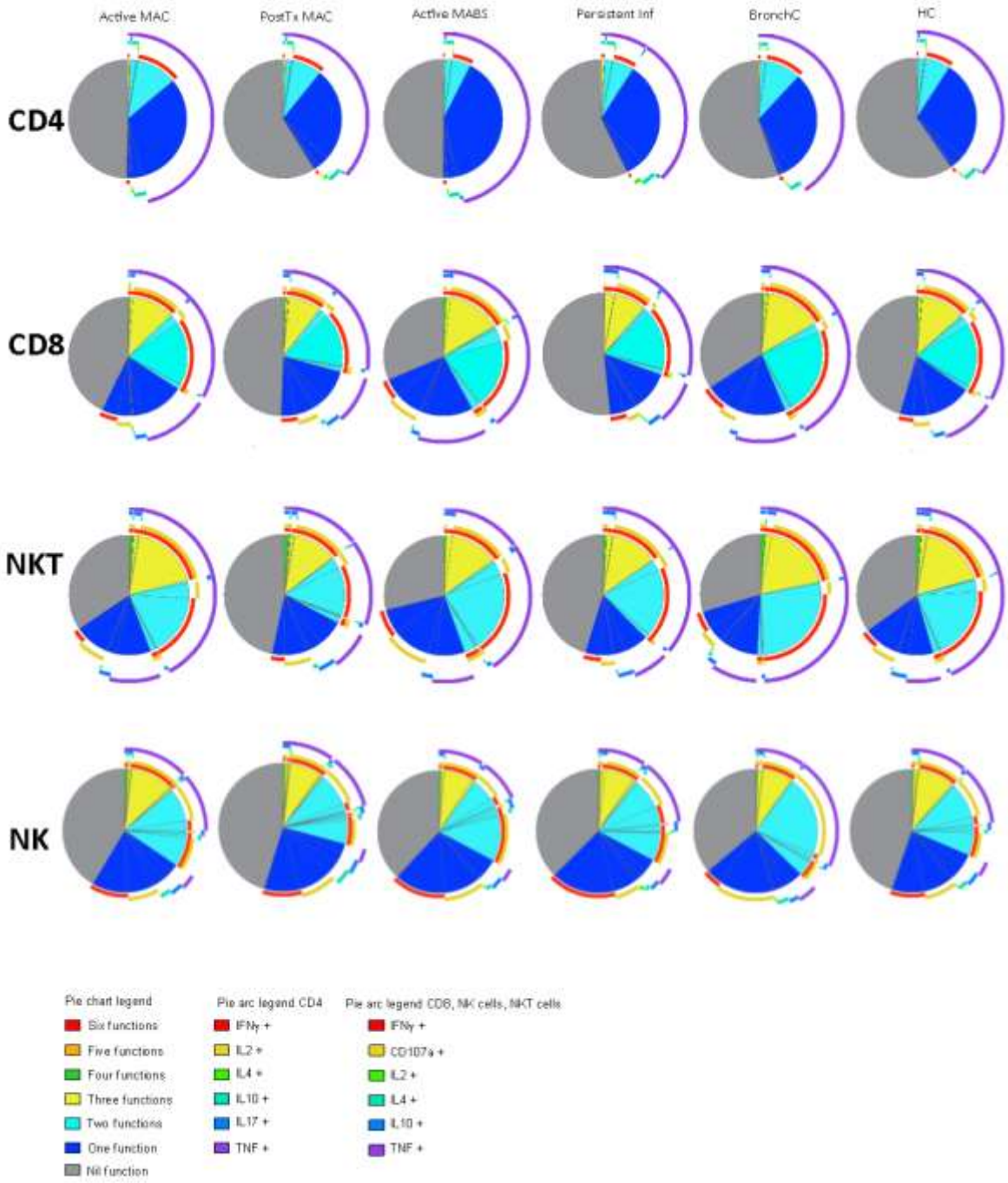


Figure 4.4 Polyfunctionality signatures of $CD4^+$ and $CD8^+$ T cells, NKT cells and NK cells in response to 6-hour PMA stimulation.

Cytokines were evaluated by intracellular staining and flow cytometry. Pie charts generated by Boolean gating of cytokine positive populations and SPICE analysis. Pie slice represents

percentage of cells that have a given number of functions colour coded in the legend. Pie arcs indicate the specific cytokines produced in each functionality set, shown by colour coded legends separately for $CD4^+$ T cells and $CD8^+$ T cells, NKT and NK cells. No significant difference in polyfunctionality was observed between any of the groups for any of the cell subsets tested.

Dot plots in Figure 4.5 show that unstimulated cells in Active MAC patients had higher $IFN\gamma^+$ $IL17^-$ $CD4^+$ T cells compared to both controls and patients with Active MABS infection (KW test, Dunns multiple comparisons p=0.03 and p=0.03, respectively). This indicates elevated IFNγ production in peripheral blood during active MAC infection. After PMA stimulation, $IFN\gamma^+$ TNF^+ $CD4^+$ T cells were significantly increased in Active MAC patients compared to controls (p=0.042), though there was no difference in total $IFN\gamma^+$ $CD4^+$ T cells or total TNF^+ $CD4^+$ T cells between groups. This result was confirmed with SPICE analysis which showed $IFN\gamma^+$ TNF^+ double positive $CD4^+$ T cells were increased in patients with active MAC infection compared to healthy controls (Wilcoxon test p=0.038). $IL17^+$ $IFN\gamma^-$ CD4+ T cells and $IL17^+$ TNF^- CD4+ T cells were significantly elevated in PostTx MAC patients compared to Active MAC patients (p=0.03 and p=0.01, respectively) indicating an elevation of IL17 production in patients who responded to treatment. $IL17^+$ TNF^- CD4 T cells were also increased in Active MABS infection compared to Active MAC infection (p=0.03). These findings could not be confirmed by SPICE analysis as statistical comparisons were limited to comparing each group to healthy controls only.

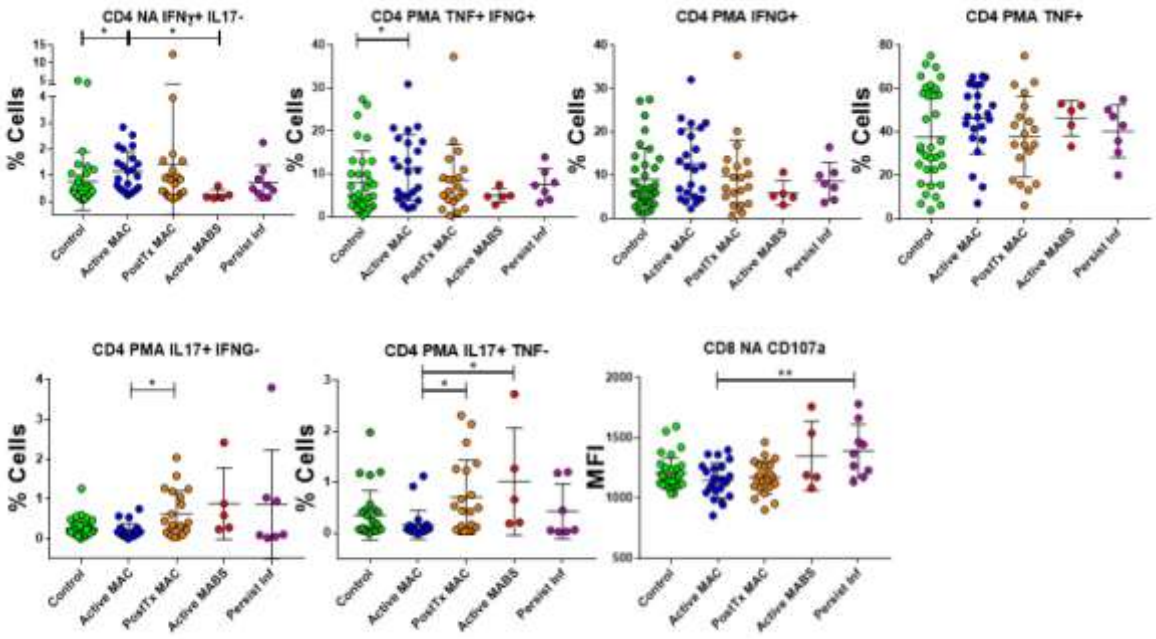


Figure 4.5 $CD4^+$ and $CD8^+$ T cell cytokine profiles in patients with MAC and MABS infection

Biaxial gating of $CD4^+$ and $CD8^+$ T cells shows cytokine signatures in both non-activated (NA) and activated (PMA) conditions. Significantly higher $IFN\gamma^+$ $IL17^-$ non-activated $CD4^+$ T cells are seen in Active MAC infection compared to controls (p=0.03) as well as Active MABS patients (p=0.03). Post PMA activation $IFN\gamma^+$ TNF^+ $CD4^+$ T cells are seen in Active MAC infection compared to controls (p=0. 042) though total $IFN\gamma^+$ and TNF^+ $CD4^+$ T cells are similar across all groups. An increase in $IL17^+$ $CD4^+$ T cells (both IFNγ- and TNF) is seen in PostTx MAC patients compared to Active MAC patients (p=0.03 and p=0.01). Persistent infection shows increased $CD8^+$ $CD107a^+$ cells under NA conditions (p=0.03).

An increase in CD107a expression (MFI) in resting $CD8^+$ T cells in patients with persistent infection compared to patients with active MAC infection was the only differential cytokine signature seen in persistent infection. This signature was not seen in PMA activated cells. Detailed analysis of cytokine secretion characteristics by both conventional biaxial gating, as well as the more complex SPICE and Citrus methods confirmed that there were no other underlying immune signatures associated with infecting species or disease stage.

Overall, no underlying deficit in Th1 cytokines IFNγ and TNF production, Th2 type IL4 and IL10 or Th17 IL17 production capacity in response to mitogen stimulation was found. Patients

with active as well as persistent MAC infection had T cell, NKT cell and NK cell compartments with comparable immune functionality to both patients who were post treatment in remission as well as to control subjects. An increase in IFNγ and TNF double positive activated $CD4^+$ T cells in active MAC infection and an increase in IL17 producing cells $CD4^+$ T cells in PostTx MAC patients were the identified signatures.

4.6.2 Proliferation response to αCD3/αCD28 antibody stimulation

T cell proliferation to αCD3/αCD28 antibody stimulation was measured after 8 days of *in vitro* culture. Proliferation modelling was done using the dedicated tool available on FlowJo v10. Proliferation indexes of CD4+ and CD8+ T cells were compared across groups using one-way ANOVA tests.

Proliferation indexes tested included: (1) division index (average number of cell divisions that a cell including the original population has undergone); (2) proliferation index (average number of cell divisions that a cell in the proliferating population has undergone); (3) expansion index (ratio of final cell count to starting cell count); (4) replication index (fold expansion over the culture time for proliferating cells), and; (5) percent divided for both $CD4^+$ and $CD8^+$ T cells (218).

Due to limitations in cell availability in some samples, proliferation assay was performed on n=11 Active MAC, n=11 PostTx MAC, n=6 Persist infection, n=9 BronchCs and n=10 HC samples.

There was no significant difference between groups in any of the proliferation indexes evaluated as shown in Figure 4.6. On average 40-50% of $CD4^+$ and $CD8^+$ T cells proliferated (Figure 4.6 (A)). Cytokine production was also evaluated by testing culture supernatants by CBA. Levels are expressed as fold change above the control well for each sample (Figure 4.6 (B)). Proliferating cells produced high levels of IFNγ, IL10 and IL1β compared to the non-stimulated control cells, whereas by day 7, TNF levels were highest in the control wells (Figure 4.6 (B)). There was no difference in the levels of IFNγ, TNF, IL10 or IL1β in culture supernatants between patient and control groups (one-way ANOVA).

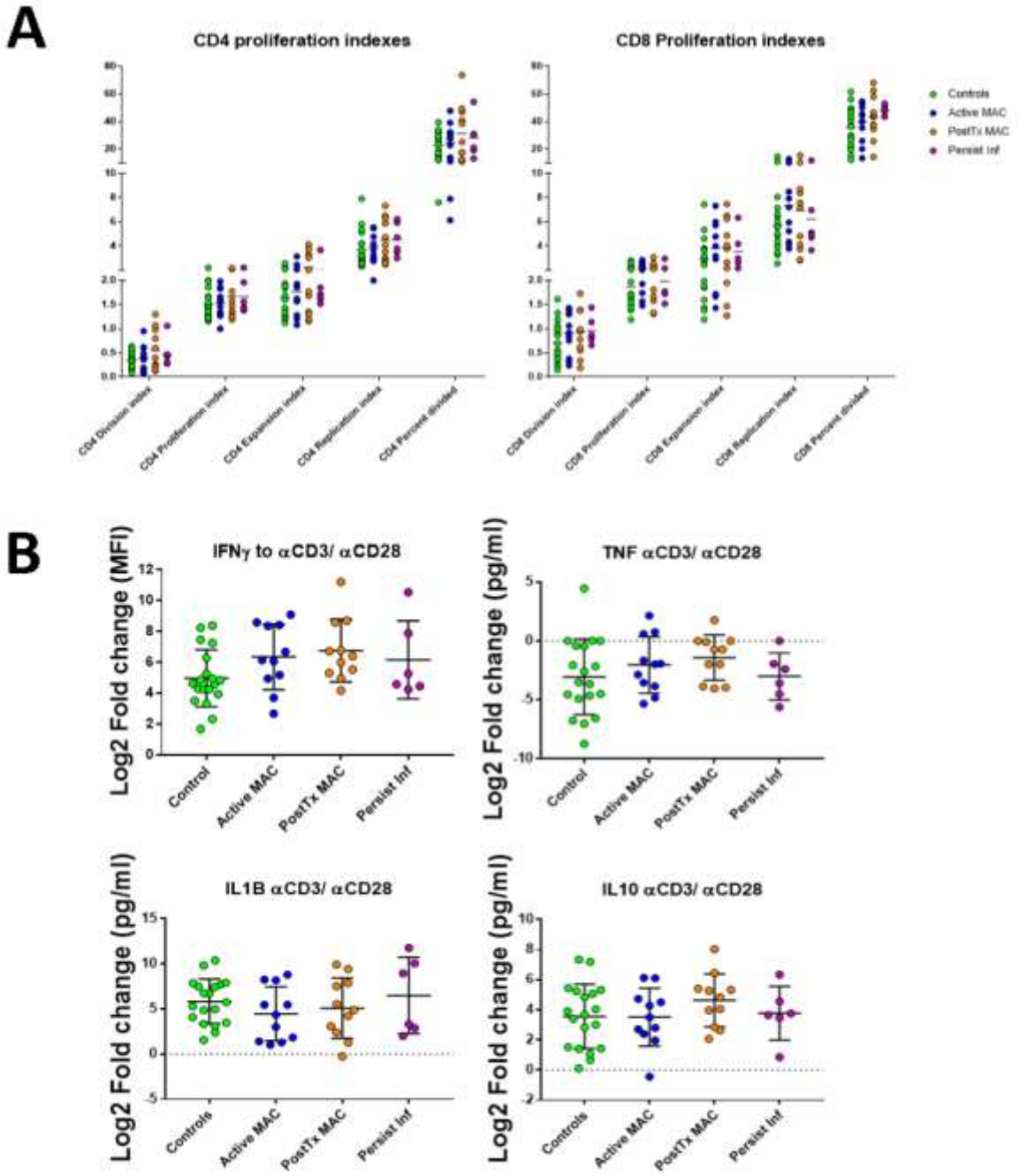


Figure 4. 6 Proliferation indexes and cytokine secretion levels after 7 days of stimulation with αCD3/CD28 antibodies.

(A) Proliferation indexes shown include division index, proliferation index, expansion index, replication index and percent divided cells. No significant differences seen between groups. **(B)** cytokine levels in αCD3/CD28 stimulated wells presented as log2 fold change over control wells show increased IFNγ, IL1β and IL10 upon stimulation.

4.7 Discussion

Increasing prevalence of MAC and MABS infection in the middle-aged to elderly population poses a significant burden on healthcare globally (22, 89). Patient morbidity due to disease related complications as well as a six-fold increase in all-cause mortality for these diseases pose a significant cause for concern. Despite the wealth of information made possible by in-depth immunophenotyping, data generated by such technologies in these patients has been lacking. In particular, immune cell compartment composition, cellular exhaustion marker phenotypes or 'fingerprints' and cellular level functional data has not been available from human studies. As such, the immune landscape of MAC and MABS lung disease remains sparsely researched. Considering the fact that immune cells are well known to circulate through the lungs and blood and the fact that blood draws are cheap and non-invasive, I analysed the peripheral blood immune cell compartment in middle aged/ elderly patients with MAC and MABS infection using high dimensional immune phenotyping methods. Combining these methods with several multiparametric data analytic pipelines gave a comprehensive picture of the cellular composition, exhaustion marker fingerprint and functional capacity of T cell, NKT cell and NK cell subsets in the peripheral blood of NTM patients.

Four patient groups at three different stages of disease were studied. Patients with active MAC infection or active MABS infection prior to treatment were included to identify immune signatures associated with active infection as well as species-specific immune perturbations. Patients who had successfully undergone treatment and were now in disease remission (PostTx MAC) were included to ascertain the immune signatures associated with a desirable treatment outcome. Patients with persistent infection, who remained sputum culture positive despite intensive treatment were recruited to ascertain what, if any immune predisposition they may have to be unable to clear the infection. These four patient groups were compared to age and gender matched healthy control subjects as well as patients who had bronchiectasis (i.e. structural lung disease) but no history of NTM infection and therefore presumably, did not have other underlying susceptibilities to this infection.

Patients susceptible to NTM infection (i.e. patients with Active MAC, PostTx MAC, and Persistent infection) had lower BMI compared to healthy controls. This thin body habitus is commonly seen in patients with NTM lung infection and recent studies have identified gene variants associated with ciliary function, *CFTR* mutations and connective tissue in this particular risk group (35). With an average age of 64 -75 years, a female predominance and a

recurrence rate of 18% in patients with active infection (in prospective follow-up), this patient cohort is representative in many important respects of the typical risk cohort for NTM infection described worldwide (26).

A total of 23 immune cell subsets in the peripheral blood of patients and controls were characterized using multi-colour immunophenotyping. Three independent analysis strategies were used to mine this high dimensional data, including an automated gating algorithm. Only findings that were confirmed by two or more methods after stringent sample and gating quality control measures are presented here.

Percentages of peripheral blood immune cells in these elderly patient cohorts corresponded to published values (219-221). The reduction in naïve $CD4^+$ and $CD8^+$ T cell populations with age were also seen in this middle aged/ elderly cohort. The lineage marker negative subset of cells accounted for 1-12% of lymphocytes in this study. These cells are probably type 2 innate lymphoid cells (ILCs), though this cannot be confirmed without using additional surface markers. Few studies reported the frequencies of ILCs in peripheral blood, though ranges given are usually <1% of mononuclear cells (222) Whether these cells are more frequent in the elderly or what, if any, role they play in NTM infection is not yet known. Overall, the immune cell composition of peripheral blood was not significantly changed from control levels during MAC or MABS infection in this study.

Treg cells represent a vital immune regulatory mechanism in the body. Increased Tregs have been associated with active TB where they perform immune suppressive functions (198, 199). In this study, there was no difference in the percentage of Tregs between patients and controls. Results of the pilot study which looked at MABS infection in CF and elderly patients, found increased Tregs in elderly patients compared to healthy controls, while in CF patients with MABS infection, either past or current, Tregs were not different to healthy controls. The findings of stage II of the study are different to the pilot study in this respect. However, the mean percentage of Tregs in Active MABS group was highest of all groups (7.09% vs <5.8% for all other groups). Higher variance of values seen in this larger study cohort accounts for this difference in result.

Conventional biaxial gating of multiparametric data is cumbersome and time consuming. Due to the extremely high number of permutations in any given dataset, most studies focus on only common cell subsets, thus not achieving the true depth and detail of immune profiling that is possible. ViSNE converts high-dimensional data to a two-dimensional plot, making data

exploration highly efficient and unbiased. With this approach, global signatures that would otherwise be lost were clearly visible across whole data sets. Two important insights were gained from the viSNE analysis. First, the highly variable distribution of exhaustion markers, CTLA4, TIM3 and PD1 across immune cells in healthy controls was consistent with that reported in the literature (171). CTLA4 expression was high in Treg cells, PD1 was expressed primarily in T effector cell populations, and TIM3 was highly expressed in NK cells. These findings established that the phenotyping panel was specific and sensitive, and extrapolations on these findings would be robust. Second, the viSNE maps highlighted many lineage negative populations which would otherwise have been completely omitted and gated out. Further characterization of these cells was not possible with the current staining panel but these cells are likely to be ILC2 cells which as associated with Th2 type responses in asthma and allergy. The significance of these cells in NTM infection is yet to be established. This avenue of investigation was not pursued in the current study due to lack of sample material and time for further analysis.

The most significant finding of this Chapter is the increased expression of exhaustion marker TIM3 in active MAC infection and CTLA4 in active MABS infection. The increase of CLTA4 in active MABS infection in Tregs supports the findings of the pilot study. The additional definition of $CD4^+$ and $CD8^+$ T cell subsets allowed a more precise quantification of CLTA4. This uncovered additional signatures with $CD4^+$ memory cells also showing CTLA4 elevation. TIM3 elevation in MAC infection was seen predominantly in $CD8^+$ T cells and NK cells. Though the significant increase was seen in active infection compared to controls, patients who were in disease remission also had comparatively high levels of TIM3 expression.

The implications of these findings are: (i) that each species group triggers different immune response pathways resulting in upregulation of unique immune suppressor molecules; although the immune susceptibility may be common, the pathogen-specific immune response is not bulked together under a single NTM genus. MABS showed a CTLA4 dominant picture in mainly $CD4^+$ effector T cell subsets and Tregs, while MAC infection showed a predominantly TIM3 signature in effector $CD8^+$ T cells and NK cell subsets; (ii) that immune perturbation is seen in the peripheral blood, indicating that systemic immunity is compromised as a result of infection, which could lead to increased susceptibility to other infections. This could also lead to increased risk of malignancy and could be a reason for the recent findings of increased expression of breast cancer associated genes in these patients (162) and; iii) that the immune system is compromised across multiple cell subsets including Tregs, $CD4^+$ and $CD8^+$ effector

cells, $CD3^+$ CD4/8 double negative cells, NK cell subsets and monocyte subsets, particularly in active MAC infection.

TIM3 is an immune checkpoint molecule with functions that include induction of peripheral tolerance to antigens, prevention of autoimmunity and inducing cell death (223). Constitutively expressed in high levels on NK cells, dendritic cells and blood monocytes, TIM3 is associated with both enhanced cross-presentation, effector function as well as regulation of innate immune responses. In $CD4^+$ and $CD8^+$ T cells, TIM3 is considered a marker of exhaustion and apoptosis. It is generally expressed in low levels on circulating Tregs but is expressed at high levels in Tregs at the site of inflammation, where its function is still not clear (224). Most studies to date have focused on TIM3 as an exhaustion marker of $CD8^+$ T cells in tumour immunology. TIM3 elevation in tumour antigen specific tumour infiltrating lymphocytes indicates T cell exhaustion in melanoma and non-small cell lung cancer (223). TIM3 has been shown to increase in peripheral blood of several other cancers, including renal cell carcinoma, hepatocellular carcinoma and cervical and ovarian cancer, leading to current development of anti-TIM3 antibodies as cancer immunotherapeutic at various stages for phase trials.

No studies published to date have reported TIM3 expression in NTM infection. It has, however, been studied in chronic viral infections HIV, HBV and HCV. In these diseases, TIM3 expression increases on antigen-specific $CD8^+$ T cells which are dysfunctional and correlates with viral load. Vial load negatively correlates with TIM3 expression during treatment and resolution of infection (223). A study on HIV patients showed patients with chronic HIV had elevated TIM3 levels in both $CD4^+$ and $CD8^+$ T cells. In this same study, *ex vivo* experiments showed that TIM3 blockade enhanced mycobacterial control by improving macrophage function (225). More relevant to NTM infection, studies of TIM3 in TB are consistent in that they show elevated TIM3 levels in $CD4^+$ and CD8 T^+ cells (226, 227) and a positive correlation with disease severity (228). However, evaluation of effector functions showed divergent results, with one study on lung T cells from infected mice showing exhaustion in $TIM3^+$ antigen-specific cells (227), while another study of peripheral blood in human patients showed increased effector functions in of $TIM3^+$ T cells in response to antigen (229).

NK cells that are actively degranulating and secreting IFNγ are known to express high levels of TIM3. However, elevated NK cell TIM3 expression has also been shown to mark dysfunctional cells and expression levels correlated with prognosis in metastatic melanoma (223, 230). Elevated TIM3 levels on NK cells have recently been shown in the peripheral blood

of patients with active TB (231). This study also showed that TIM3 expression correlated with disease severity, was associated with impaired IFNγ production and degranulation and TIM3 blocking antibodies could rescue function. The importance of NK cells in TB was highlighted in a recent publication by Chowdhury et. al. (232) where increased circulating NK cells were associated with latent infection. Circulating NK cell levels were shown to correlate to bacterial burden in the lung. This study did not evaluate levels of immune checkpoints on these cells. These findings are supported by other studies that show NK cell IFNγ response in TB is diminished in active infection (233).

Taken together, these findings indicate that TIM3 is elevated on multiple immune cell types and acts to suppress function in many instances. The results of this Chapter showing increased TIM3 on $CD8^+$ effector T cells and NK cells in active MAC infection correspond well with findings in TB and chronic viral infections. The role of NK cells in NTM infection is not yet clear. Mouse models show that NK cell derived IFNγ is necessary for both innate and adaptive immune responses in *M. kansasii* infection (234). Human data however is lacking. I found no difference in the total percentage of circulating NK cells or NK cell subsets in any stage of the disease, unlike TB. Functional capacity of the $TIM3^+$ NK and $CD8^+$ T cell subsets could not be evaluated as panel limitations precluded combining phenotyping and functionality data in this lineage.

CTLA4 and PD1 were the first immune checkpoints identified and researched in the context of tumour immunity and subsequently in infectious disease immunity (171). Human studies in HIV, HBV and HCV have shown elevated levels of PD1 and/or CLTA4 on $CD4^+$ and $CD8^+$ T cells (173). There is very limited human data on CTLA4 in other bacterial and parasitic diseases. This is possibly due to the high adverse event rate seen with anti-CTLA4 therapy in cancer patients. Most studies focus on PD1 and PD-L1 which have therapeutic blocking agents with minimal side effects. The data in Chapter 3 and this Chapter represent the first data on CLTA4 expression in human MAC and MABS infection. The consistent elevation of CTLA4 in Treg cells across two patient groups and performed with different panels and different control groups points to the robustness of this result. CTLA4 is a potent suppressor of T cell function that is constitutively expressed on Treg cells and plays an essential role in peripheral tolerance (235). The increased CTLA4 in both Treg cells as well as effector $CD4^+$ T cell populations in active MABS infection is consistent with asuppressive immune environment in the peripheral blood. Therapeutic targeting of this molecule is a possibility worth consideration in refractory disease.

PD1 expression in MAC infection was investigated by Shu *et. al* who showed an increase in PD1 and PD-L1 in MAC infection compared to controls (143). That study, however, compared patients to significantly younger controls, in the absence of essential flow cytometric gating controls which would have affected the outcome of analysis. The results of this Chapter do not corroborate these findings. Patients with active MABS infection had reduced $PD1^{+}$ effector and transitional effector memory $CD4^{+}$ T cells. These results do not align with the results of the pilot study where $CD4^{+}$ T cell PD1 expression was shown to be increased in elderly patients with active MABS infection. The increased variance of the larger patient and control groups analysed during this second stage as well as staining panel variability could account for this inconsistency. The increase of PD1 in TB is however well documented. It is possible that PD1 is the signature exhaustion marker of TB infection.

Other significant results include that reduced number of exhaustion marker negative ($CLTA4^{-}$ $TIM3^{-}$ $PD1^{-}$) NK cells of all subsets were seen in active MAC infection, while $CD56^{bright}$ $CD16^{dim/bright}$ cells were also reduced in MABS infection. A significant inhibition of NK cell mediated immunity is indicated by these results. In addition, the similarity of exhaustion marker fingerprint between PostTx MAC patients, Persistent infection patients and BronchC patients to the healthy controls needs to be highlighted. This indicates that peripheral blood immune exhaustion is reversible post treatment, with only minimal residual TIM3 elevation in $CD8^{+}$ effector T cells. In addition, patients with persistent infection show minimal differences from healthy controls, specifically, only in exhaustion marker double positive cell subsets which are relatively few in number. The direction of change is also not consistent. Collectively, this indicates that patients with persistent infection have no significant difference in exhaustion marker fingerprint compared to healthy controls. The significance of this finding is that exhaustion marker fingerprint cannot be used for prognostic follow-up of patients, as both patients who go in to remission as well as those who go into persistence show the same changes in peripheral blood. Bronchiectasis controls were different to healthy controls only in the increased expression of CTLA4 in $CD4^{+}$ TEMRA and Treg cells, which is remarkably similar to the Active MABS signature. It is possible that part of the signature seen in MABS infection is due to underlying bronchiectasis, though this is not seen in patients with active MAC infection.

The results presented in this Chapter lead to several questions. Firstly, the increase of TIM3 in $CD8^{+}$ T cells and NK cells raises the issue of the role of these cytotoxic cells in MAC infection. If an exhaustion marker fingerprint is indicative of cells that are/were activated, then MABS

infection would appear to elicit the expected $CD4^+$ dominant responses while MAC infection may be tilted towards a $CD8^+$ cytotoxic responses. Whether this is due to MHC-1 restricted antigens or cross-presentation cannot be identified until immunogenic peptides specific to each species group are mapped.

Secondly, this Chapter outlines whether the species-specific signature of active infection can be used to diagnose infection by blood tests. Due to the low number of patients with MABS infection, the analysis was limited to differentiation of active infection from controls, by combining both patients with active MAC and MABS infection into one group. Citrus based predictive modelling aimed at finding cell nodes with differential abundance was unsuccessful. The algorithm identified cell nodes that had differential expression of TIM3, but the model predictive accuracy was low. The observation that CTLA4 was not a significant differentiating marker is likely due to: (i) the low number of MABS patients in the group and; (ii) the elevated CLTA4 in BronchC which obscures the separation. Overall, this method did not provide a useful signature that could be further developed. The analysis did, however, identify a previously ignored subset of cells, $CD3^+$ $CD4^-$ $CD8^-$ cells which were confirmed to have a similar signature to other T cells. This study is one of the first that leverages the high dimensional analytical capability of Citrus for immune-monitoring (191)

Functional analysis of PBMCs showed no significant underlying deficits in $CD4^+$ or $CD8^+$ T cells, NK cell or NKT cell function as measured by response to PMA and proliferation. Importantly, no deficit in type 1 cytokines IFNγ and TNF or any excess of Th2 / regulatory cytokines IL4 and IL10 was found. It is difficult to compare these results to others in the literature as most studies to date performed total cytokine level evaluation on culture supernatants. These studies have shown varying results with both increased and decreased IFNγ, TNF, IL4, IL10 and other cytokine levels (127, 133-138). Cell subset level functional data is available from the pilot study where patients with elderly patients with active MABS infection had higher TNF production in $CD8^+$ T cells compared to controls. This finding was not replicated in the second stage, again due to the increased variability seen in the larger group of controls that were included.

Biaxial gating showed some subtle functional differences between groups. Patients with active MAC infection had higher $IFN\gamma^+$ $IL17^-$ $CD4^+$ T cells in *ex vivo* staining compared to controls as well as patients with active MABS infection. An increase in $IFN\gamma^+$ TNF^+ $CD4^+$ T cells after PMA stimulation in patients with active MAC infection compared to controls was also

identified. These two results show that a lack of IFNγ production capacity in $CD4^+$ T cells is unlikely to be the predisposing factor to MAC infection. An antigen-specific deficit cannot be excluded by these results, however and is an avenue that will be investigated further in Chapter 6. Proliferation response to αCD3/CD28 stimulation in both $CD4^+$ and $CD8^+$ T cells showed no evidence of global defects.

Collectively, the results of this Chapter show that in active infection with MAC and MABS, peripheral blood $CD4^+$ and $CD8^+$ T cell, NK T cell and NK cells compartments show TIM3 and CTLA4 exhaustion phenotypes. These signatures are similar to that described in chronic viral infections and TB. This signature reverts to near normal with successful treatment. Persistent infection is also marked by a lack of signature rather than critical exhaustion pointing toward the underlying pathomechanisms which are responsible for the failure to ultimately clear NTM infection. In this study, the functional capacity of these cell compartments to mitogen stimulation was not affected in individuals susceptible to NTM infection, indicating any major deficiency in response is likely associated with antigen-specific responses rather than an underlying global abnormality. Species group-specific disease signatures identified in these studies will help delineating the pathomechanisms of NTM disease but may be too early for preliminary predictive models and diagnostic tools. Thus, I moved on to high dimensional, highly sensitive gene expression profiling in T cells to identify useful biomarkers for disease diagnosis and disease outcome.

Chapter 5: Nanostring nCounter gene expression quantification

5.1 Introduction

Phenotyping experiments identified species-specific exhaustion marker signatures with CTLA4 and TIM3 elevation in MABS and MAC infection, respectively. Citrus-based analysis revealed the presence of specific cell clusters with differential exhaustion marker expression between active infection (either MAC or MABS) and controls (both HC and BronchC).

The recent technology of Nanostring based gene expression quantification presents a new, highly sensitive, highly reproducible method to measure RNA transcripts without amplification. This digital technology is also TGA/FDA approved as a clinical diagnostic platform for breast cancer prognosis (Prosignia https://www.nanostring.com/diagnostics/prosigna-uk) and is currently being tested for diffuse large B cell lymphoma classification with the 'Lymph2CX' test (177, 178). Being a highly multiplexable assay where hundreds of target molecules can be evaluated irrespective of sample input type (biopsy, blood, body fluids, FFPE, frozen samples etc) is an added advantage. I applied this Nanostring technology to investigate the T cell gene expression signatures in each of my patient and control cohorts. As the platform is already in clinical use for diagnostic tests, it has the advantage of rapid clinical translatability should clinically useful disease signatures emerge.

Since flow cytometric data showed that $CD4^+$ and $CD8^+$ T cells were the most likely cells to show gene expression alterations, I focused my study on these cell subsets. $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells were sorted from freshly thawed aliquots of sample PBMCs utilizing FACS. RNA extracted from sorted cells was then quantified for expression levels (copy numbers) of a custom set of 131 T cell genes using the Nanostring nCounter platform. Normalized and standardized data that passed inbuilt quality control measures were analyzed as log2 counts. Multivariate and univariate analysis was performed using GMine online analysis platform, SPSS v23 and GraphPad Prism V7. A total of 95 samples were analysed. All $CD4^+$ samples and 93 $CD8^+$ samples passed quality control measures and were used for subsequent analysis.

5.2 Healthy controls and Bronchiectasis controls

Bivariate comparison of the two controls groups included in this study showed no significant differences in gene expression between healthy individuals and patients with bronchiectasis in either $CD4^+$ or $CD8^+$ T cells. (Wilcoxon test, significance at $p<0.05$ adjusted for false discovery rate [FDR]). Interferon regulatory factor 4 (IRF4), aryl hydrocarbon receptor (AHR) and interleukin 32 (IL32) were the top three markers that were differentially expressed with IRF4 and AHR being reduced in bronchiectasis while IL32 was increased compared to healthy individuals in $CD4^+$ T cells. Galectin 1 (LGALS1), IL32 and granzyme H (GZMH) had higher expression in $CD8^+$ T cells of patients with bronchiectasis. These differences were not significant. Multivariate PCA and CCA showed no significant latent variables that defined to these two groups. Therefore, for subsequent analysis, these two groups were combined and analyzed as a single 'Control' group.

5.3 Global comparison of patient group gene signatures to control group

Univariate comparison (independent t tests with FDR correction) of all 131 genes between each patient group i.e., Active MAC, PostTx MAC, Active MABS, and persistent infection group vs the control group revealed patients with active MAC and MABS infection had the most divergent gene signature in both $CD4^+$ and $CD8^+$ T cells. Figure 5.1A shows heatmaps of p values where red indicates upregulated genes compared to controls and blue indicates down regulated genes compared to controls. Intensity of colour represents the magnitude of the p value as shown in the figure legend. Figure 5.1B shows genes that are differentially expressed between MAC and MABS infection in $CD4^+$ and $CD8^+$ T cells.

Significantly upregulated genes in Active MAC infection included prostaglandin E2 receptor subtype 2 (PTGER2), early growth response 2 (EGR2, a transcription factor associated with T cell activation), TNF, and CD27 (T cell activation marker) in $CD4^+$ T cells. $CD8^+$ T cells also upregulated CD27 and prostaglandin E receptor 2 (PTGER2), in addition to which CD272 (B and T lymphocyte attenuator, BTLA) which is a co-inhibitory receptor on activated T cells (236), CD226 (DNAX accessory protein-1/ DNAM- a co inhibitory receptor) and cell signalling/adhesion molecules CD38 and SELL (L-selectin/ CD62L) were upregulated. The

general signature was one of activation with some increase in inhibitory signalling pathways in $CD8^+$ T cells.

Significantly down regulated genes included the IL4 receptor, transcription factors STAT5A and JAK1, and cell trafficking receptor S1PR1 (sphingosine 1 phosphate receptor) in $CD4^+$ T cells. $CD8^+$ T cells had a remarkably similar pattern of down regulated genes in addition to which the interferon γ receptor 1 (IFNγR1) was also highly down regulated. As STAT5A and JAK1 together with the IFNγR1 are involved in the IFNγ signalling pathway, together these results indicate a suppression of downstream IFNγ function, particularly in $CD8^+$ T cells.

Active MABS infection had a different gene signature compared to controls. In both $CD4^+$ and $CD8^+$ T cells, in addition to JAK1, STAT5A, and S1PR1, transcription factors JUN, Kruppel Like Factor 2 (KLF2 which is involved with S1PR1 in regulating lymphocyte trafficking) and interleukin-2 inducible T cell kinase (ITK) were also down regulated. Highly significant upregulation of CXCL8 (IL8) was seen in both $CD4^+$ and $CD8^+$ T cells. PostTx MAC patients and persistent infection patients had few differentially expressed genes compared to control levels in both $CD4^+$ and $CD8^+$ T cells. Figure 5.1B shows the gene signature of patients with active MABS infection when compared to patients with active MAC infection. Active MABS infection showed significant upregulation of CXCL8, CD134 (OX40, a TNF receptor superfamily molecule) and IL21 receptor when compared to active MAC infection while KLF2 and JUN were significantly down regulated in active MABS.

These results closely followed phenotypic data of Chapter 4, indicating that active infection had both a gene signature that differed from controls and a species-specific signature. Detailed analysis with a view to eventual clinical translation were then performed to identify gene signatures that could be useful in diagnosis and/ or screening for active MAC/MABS lung infection, and differentiation of infecting species.

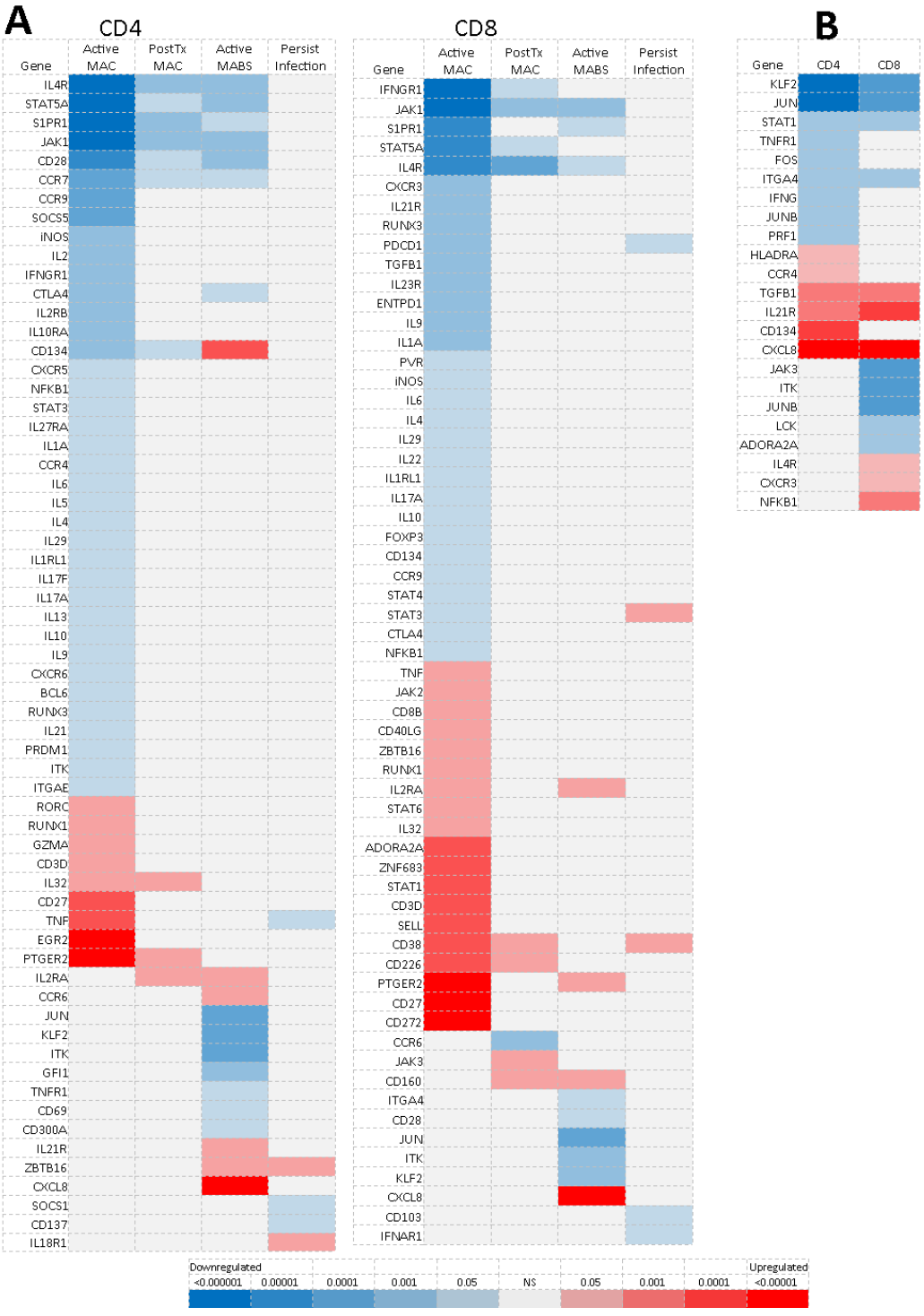


Figure 5.1 Heat map of Nanostring gene expression levels in CD4$^+$ and CD8$^+$ T cells in patients with MAC and MABS infection.

(A) Heatmaps showing up and down regulated genes in patients with active MAC infection (MAC), treated MAC infection (PostTx MAC), active MABS infection (MABS) or persistent MAC or MABS infection compared to control group. Independent t-tests were performed using GMine. **(B)** Heatmap of up- and downregulated genes in patients with active MABS infection compared to patients with active MAC infection. Colour code based on significance p value of comparison as shown in legend.

5.4 Gene expression profiling for gene signature of active infection and causative species group

A main objective of this study was identification of biosignatures that could be developed into diagnostic/ screening tools and prognostic tools. Therefore, the following comparisons were targeted. (i) Comparison of patients with active infection (either MAC or MABS) to Controls; (ii) comparison of patients with Active MAC to patients with Active MABS infection for identification of species- specific signature in active infection; (iii) comparison of patients with active infection to patients with persistent infection to identify early markers of treatment failure.

Pooled MAC and Active MABS patients were defined as having 'active infection'. Markers that differentiated these patients from controls were analysed. The PostTx MAC group and Persist Infection group were also included in the ANOVA with Tukeys posthoc analysis at this stage to look for disease stage-specific signatures. Figure 5.2A shows the log2 counts of the main markers differentially expressed between patients with active infection and controls in $CD4^+$ and $CD8^+$ T cells. Significant suppression of surface receptors IFNγR1 ($CD4^+$ p=0.0363; $CD8^+$ p=0.0001) and S1PR1 ($CD4^+$ p<0.0001; $CD8^+$ p<0.0001) as well as transcription factors STAT5A ($CD4^+$ p<0.0001; $CD8^+$ p=0.0004) and JAK1 ($CD4^+$ p<0.0001; $CD8^+$ p<0.0001) were seen. Two observations of interest include: (i) the commonality between the $CD4^+$ and $CD8^+$ T cell signatures; and (ii) evidence that these markers show a suppression in active disease that appears to revert to control levels during both post treatment disease remission and persistent infection. Mean expression levels in persistent infection were higher than levels in disease remission. S1PR1 expression was significantly increased in persistent infection compared to active infection in both $CD4^+$ (p=0.043) and $CD8^+$ (P=0.0036) T cells, as were STAT5A levels ($CD4^+$ p=0.0006; CD8 p=0. 0023) JAK1 levels ($CD4^+$ p=0.0011; $CD8^+$ p<0.0001) and $CD8^+$ IFNγR1 levels (p=0.0217).

PTGER2 was significantly upregulated in both $CD4^+$ (p=0.0039) and $CD8^+$ (p=0.0086) T cells, as were $CD4^+$ TNF expression (p=0.0364) and CD4+ EGR2 expression (p=0.0069). These markers also showed a trend towards patients with persistent infection having lower i.e. control levels of expression. In particular, $CD4^+$ TNF expression was significantly lower in persistent infection compared to active infection (p=0.0058) as was $CD4^+$ EGR2 (p=0.020). $CD8^+$ T cells had a significant suppression of lung trafficking marker CXCR3 expression (p=0.0034) and STAT3 expression (p<0.0001) in active infection compared to controls.

PostTx MAC patients generally had gene expression levels between that of patients with active infection and controls, and this mean expression level often fell between that of patients with active infection and patients with persistent infection. Collectively these results indicate that the inflammatory profile of active disease slowly reverts towards normal levels, but even when patients are in disease remission, some systemic effects remain. As patients' blood samples were collected up to 5 years post treatment, it would appear that MAC infection may have a rather long-standing global impact on the systemic immunity.

Patients with persistent infection gave no significant signature across all 131 T cell gene analysed. Combined with the results mentioned above, it seems persistent infection presents a state of immune ignorance in the adaptive immune arm. T cell signatures most similar to control levels were seen at this disease stage in both $CD4^+$ and $CD8^+$ T cells.

Next, genes that were expressed differentially between Active MAC and Active MABS patients were investigated, to inform a potential species-specific gene signature. Six genes that were significantly different between the two groups were identified (Figure 5.2B). Active MABS infection showed a significant increase in IL8 expression ($CD4^+$ $p=0.001$; $CD8^+$ $p=0.007$) as well as IL21R expression ($CD4^+$ $p=0.0152$; $CD8^+$ $p=0.010$) compared to MAC infection. IL8 expression was significantly higher in Active MABS infection compared to all other patient and control groups in $CD4^+$ T cells (vs Controls $p=0.004$, vs PostTx MAC $p=0.003$, vs Persist infection $p=0.003$) and $CD8^+$ T cells (vs Controls $p=0.05$, vs PostTx MAC $p=0.01$, vs Persist infection $p=0.04$) indicating a strong association with MABS species infection. In contrast, IL21R levels were comparatively high in MABS infection due to a suppression in MAC infection rather than an increase associated with MABS. In addition, there was a highly significant increase in $CD4^+$ CD134 (OX40) expression in MABS infection compared to all other patient and control groups (vs Controls $p<0.0001$, PostTx $p<0.001$, Persist infection $p<0.001$). $CD4^+$ T cell KLF2 was the only marker that was suppressed in MABS infection compared to both MAC patients ($p=0.03$) and controls (0.02).

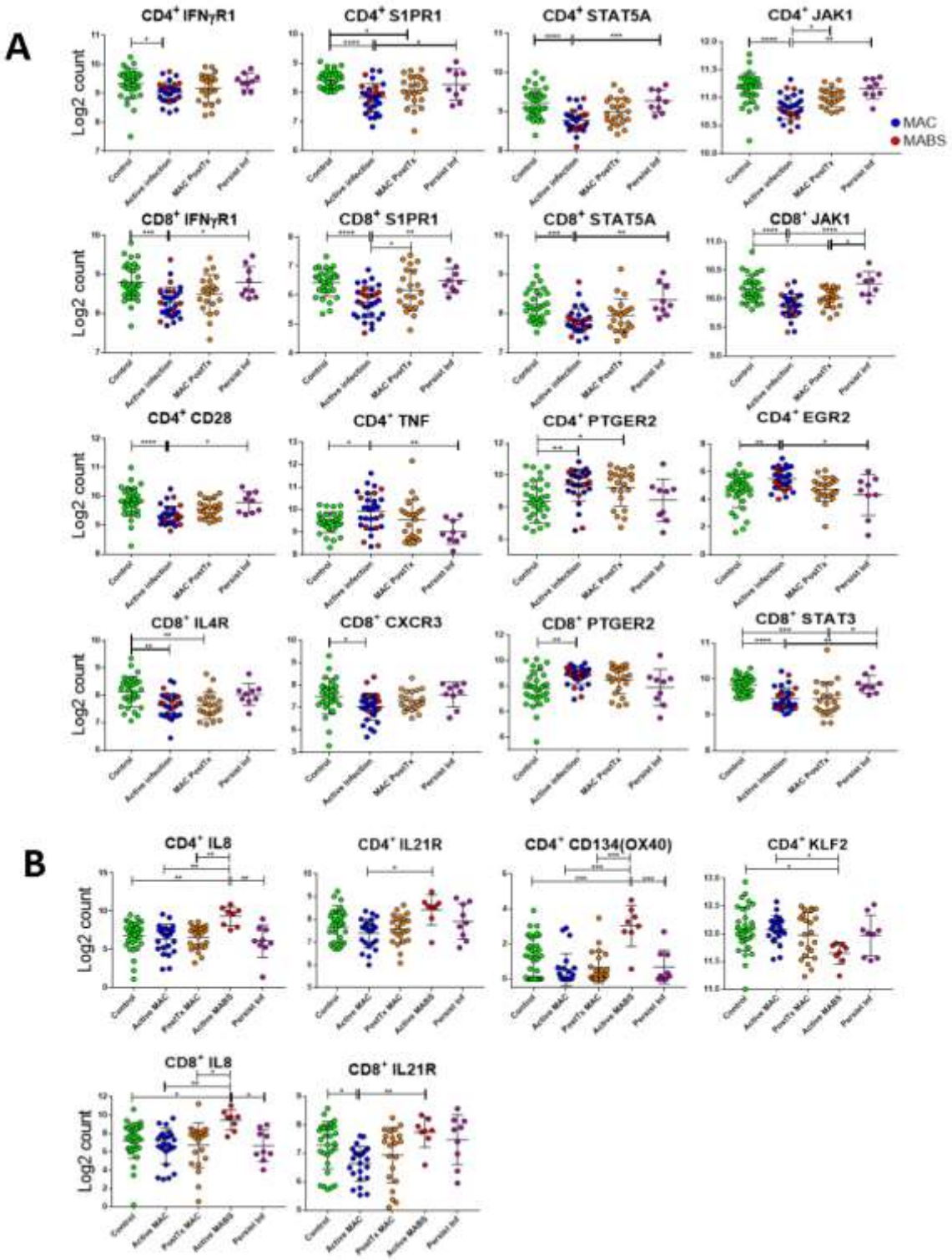


Figure 5.2 Relative expression of individual immune genes in $CD4^+$ and $CD8^+$ T cells that differentiate (A) active MAC/ MABS infection from controls and (B) differentiate infecting species group in active infection.

Shown are log2 counts of RNA copy numbers in patient and control groups. Healthy controls and bronchiectasis controls were combined as a single 'Control' group (green circle). **(A)** Dot

plots show $CD4^+$ and $CD8^+$ T cell genes that had significant differential expression between controls and patients with active infection. Active MAC infection is shown in blue circles while active MABS infection is shown in red circles. PostTx MAC patients are shown in orange circles and persistent infection patients are shown in purple circles. **(B)** Dot plots show genes that are differentially expressed between active MABS infection and active MAC infection. Comparisons between groups by ANOVA with Tukeys post hoc test. P values adjusted for multiple comparisons ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Forest plots showing the top biomarkers for diagnosis of active infection in $CD4^+$ and $CD8^+$ T cells are shown in Figure 5.3A and B, respectively. In $CD4^+$ T cells markers with the best log odds ratios were, S1PR1 (p= 0.000026), IL4R (p=0.000026), STAT5A (p=0.000026), JAK1 (p=0.00028), CD28 (p=0.00064), CCR7 (p=0.00071), CTLA4 (p=0.012), PTGER2 (p=0.012), SOCS5 (p=0.015) and EGR2 (p=0.015). $CD8^+$ T cells showed a similar set of markers which included JAK1 (P=0.000096), S1PR1 (p=0.00012), IFNΓR1 (p=0.0003), STAT5A (p=0.0015), IL4R (p=0. 0031), PTGER2 (p=0.014), and CD272/BLTA (p=0.014). (FDR adjusted p value, biomarker identification by t test). Based on these results, a multigene signature that could be developed as a potential screening tool for active infection was investigated.

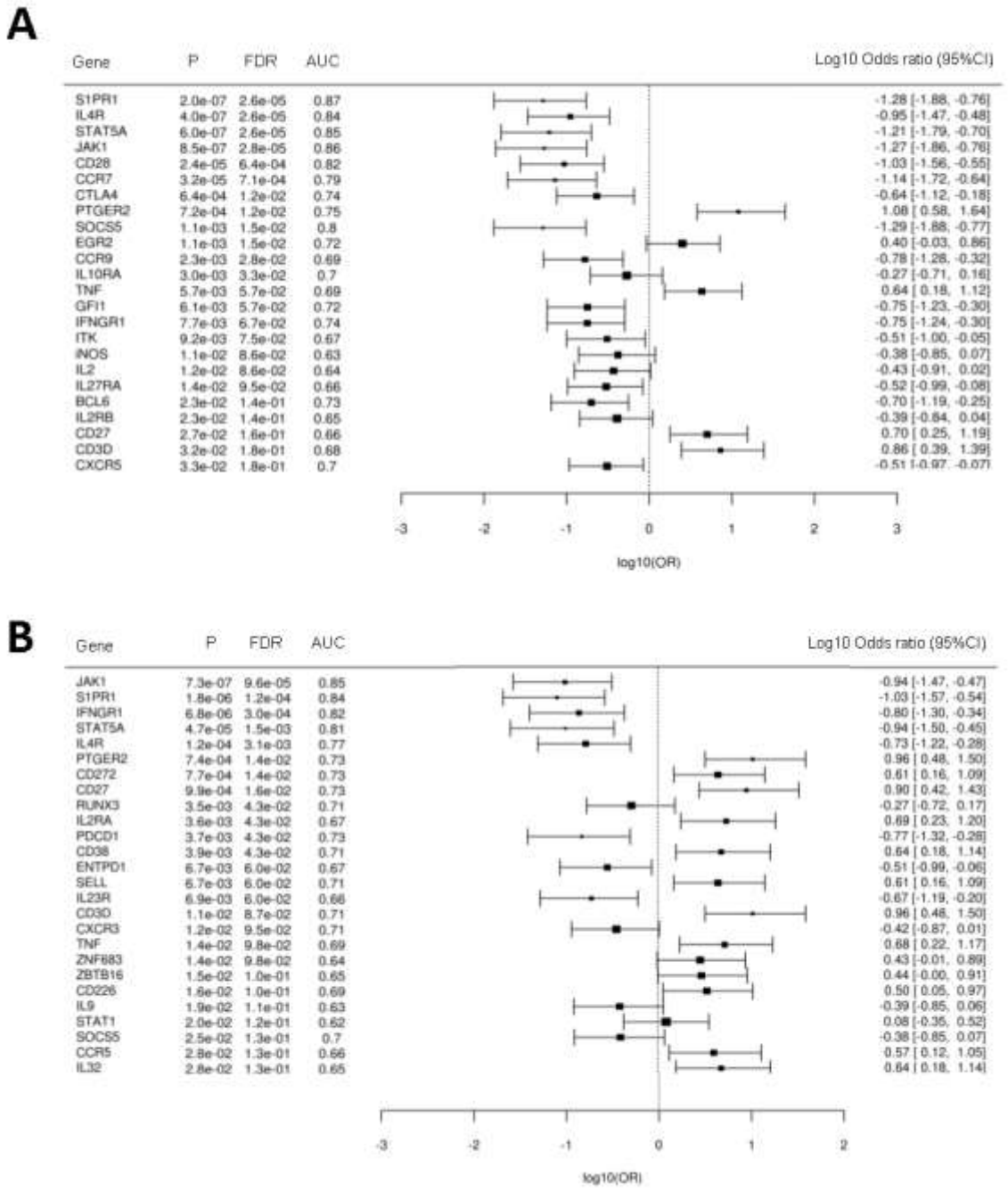


Figure 5.3 Forest plots of top biomarkers differentiating active infection from controls with log odds ratio.

Forest plots of top biomarkers (t test with FDR correction for multiple comparisons) that differentiate active infection from controls in **(A)** $CD4^+$ T cells and **(B)** $CD8^+$ T cells. P value of t test and FDR corrected t test are shown. Area under the curve (AUC) values for separation of active infection from controls as well as log odds ratio (95%CI) also shown.

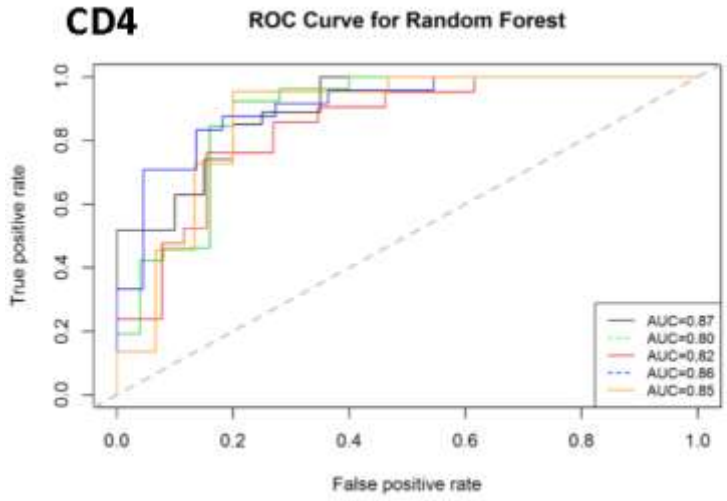
5.5 Diagnostic gene signature for active MAC/MABS lung infection: test and internal validation

A blood-based gene signature screening tool that could be rapidly deployed to investigate at-risk populations for active MAC/MABS lung infection would be an asset to current clinical practice. Current investigative methods for NTM lung disease involve cumber some and time-consuming tests such as high-resolution CT coupled with sputum for mycobacterial culture. Results presented in section 5.4 indicated that peripheral blood T cell gene signatures could be potentially used to build such a screening tool, since multiple genes were differentially expressed between active infection and controls. Although no single gene showed a perfect differentiating capacity as presented in the forest plots in Figure 5.3, a combination of genes could be developed as a predictive model that would give reasonable sensitivity and specificity for diagnosis of active NTM lung infection.

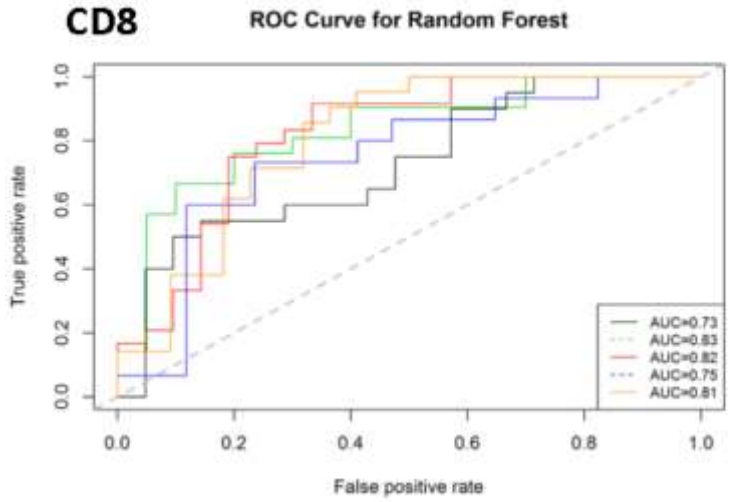
Model development and validation method used are described in Methods section 2.13.2. Briefly, the data set was randomly split into 70% test and 30% validation sets. Predictive accuracy of models developed on test data was tested on validation data. The process was repeated five times. Receiver operated characteristic (ROC) curves for each iteration are shown in Figure 5.4 with the accuracy, sensitivity and specificity of the each predictive model. Classification performance of the five models on the five random validation data sets returned an accuracy that ranged from 0.75 to 0.95. The sensitivity of models ranged from 0.67 to 1.0 and specificity ranged from 0.78 to 1.0 in $CD4^+$ T cell data. Corresponding values for $CD8^+$ T cell data were: model accuracy ranging from 0.73 to 0.91, sensitivity ranging from 0.70 to 1. 0 and specificity from 0.66 to 1.0.

Genes that were most frequently included in the models were assumed to give the best classification performance. Based on this, a 9-gene signature, comprising all genes that were in the top ranked genes for at least 3 of the 5 iterations, was identified for CD4 T cells. These genes were S1PR1, IL4R, STAT5A, JAK1, CD28, SOCS5, IL10RA, GFI1 and BCL6 all of which were down regulated in active infection. For $CD8^+$ T cells, a 7-gene signature was identified which included JAK1, S1PR1, IFNγR1, STAT5A, PTGER2, CD27 and CXCR3 of which PTGER2 and CD27 were upregulated in active infection. These models were then tested for classification accuracy on the total data set using LOOCV method. The 9-gene signature for $CD4^+$ T cells correctly classified active infection with an accuracy of 0.88, sensitivity of

0.81 and specificity of 0.93. The 7-gene signature for $CD8^+$ cells correctly classified active infection with an accuracy of 0.84, sensitivity of 0.81 and specificity of 0.86.



	Accuracy	Sensitivity	Specificity
run1	0.85	0.89	0.82
run2	0.83	0.88	0.80
run3	0.92	0.81	1
run4	0.75	0.67	0.78
run5	0.95	1	0.9



	Accuracy	Sensitivity	Specificity
run1	0.91	0.80	1
run2	0.73	0.73	0.73
run3	0.82	0.70	0.75
run4	0.75	0.76	0.71
run5	0.81	1	0.66

Figure 5.4 Classification performance of predictive models on validation data sets for diagnosis of active infection.

Figure shows the receiver operated characteristic (ROC) curves for predictive model accuracy. Random forest models developed on 70% test data sets were validated on 30% validation datasets (Methods section 2.13.2). Process was repeated 5 times. Model performance of each iteration is shown in tables. AUC- Area under Curve.

5.6 Gene trends associated with clinical outcome

Having identified gene signatures associated with active infection, I then investigated whether the $CD4^+$ and $CD8^+$ T cells gene signatures seen in active infection (Active MAC and Active MABS cohorts together) prior to treatment had any association with the final treatment outcome. Outcome of treatment as obtained from clinical records of patients at the end of treatment. Patients were categorized as 'Remission' for patients who went into disease remission and remained healthy, or 'Remission-relapse' for patients who went into disease remission but subsequently developed or had a history of recurring relapses. Of 31 patients with active infection (both MAC and MABS active infection), 16 were categorized as 'Remission' while 7 were categorized as 'Remission-relapse' patients. Four patients were either lost to follow-up or were untreated, while four patients had persistent infection due to identified factors (i.e. inadequate treatment regime at onset, persistent heavy smoking) and were excluded from this analysis.

There were no differentially expressed genes between 'Remission' and 'Relapse-remission' patients in either $CD4^+$ or $CD8^+$ T cells after correction for multiple comparisons. This indicated that at diagnosis there was no identifiable T cell signature that could predict outcome of treatment in these patients. Further analysis to test if either patient group had a T cell signature similar to those of patients in persistent infection (Persist Inf group) was then performed. A multivariate comparison of $CD4^+$ gene profiles of these two groups to patients with persistent infection (Persist Infection group) revealed that 'Remission' patients had gene expression profiles most similar to patients with persistent infection whereas 'Remission-relapse' patients were significantly different from persistent infection (Redundancy analysis - Persist infection vs Remission p=0.089; Persist infection vs Remission-relapse p=0. 37) as shown in Figure 5.5(A). Univariate analysis showed that 'Remission' patients had significantly higher TNF gene expression (FDR, p=0.026) and significantly lower JAK1 expression (FDR p=0.026) compared to Persist infection patients. In contrast, 'Remission-relapse' patients had significantly lower IFNγR1 (FDR, p=0.017), STAT5A (FDR, p=0.017) and JAK1 (FDR, p=0.04) expression compared to patients with persistent infection.

Similar analysis of $CD8^+$ T cell profiles showed the opposite pattern of differences in multivariate analysis (Redundancy analysis - Persist infection vs Remission p=0.031; Persist infection vs Remission-relapse p=0.132) with 'Remission-relapse' patients being most like patients with persistent infection. Univariate analysis of genes showed no significant

differences in gene expression between 'Remission' patients and persistent infection, while IFNγR1 (FDR, p=0.049,) JAK1 (FDR, p=0.049,) and STAT5A (FDR, p=0.049,) were all significantly reduced in patients with Remission-relapse compared to persistent infection. Overall these results indicate that during active infection, before treatment, it is difficult to predict what the outcome of treatment will be based on peripheral blood T cell gene signatures.

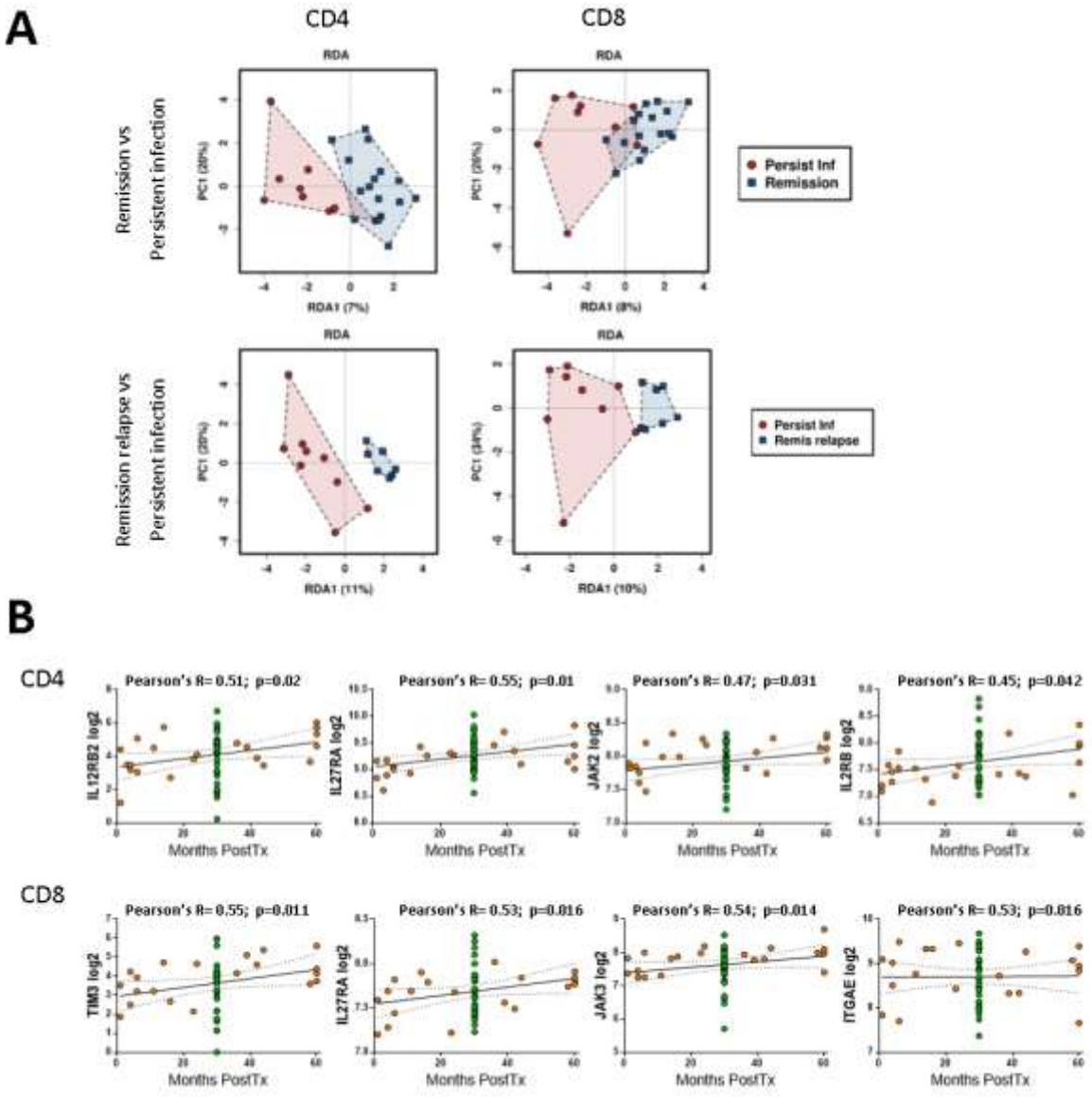


Figure 5.5 Gene expression association with treatment outcome and correlation with time of sampling post treatment.

(A) redundancy analysis (RDA) plots comparing global $CD4^+$ and $CD8^+$ T cell gene expression profiles between patients with active infection (with MAC or MABS) who at follow-up went into 'Remission' and remained healthy, or went into remission but had a history of or

subsequently developed relapses 'Remission-relapse' as compared to patients who currently had persistent infection. $CD4^+$ RDA Persist inf vs Remission p=0.089; Persist inf vs Remission-relapse p=0.037. $CD8^+$ RDA Persist inf vs Remission p=0.031; Persist inf vs Remission-relapse p=0.132. **(B)** Pearson's correlation between gene expression log2 count and time post treatment (months post stopping treatment) in PostTx MAC patients (orange circles) are shown for $CD4^+$ and $CD8^+$ T cells. Gene expression level in Controls (green circles) are overlaid on each graph to show range of normal values.

The PostTx MAC patient group had blood samples taken at various time after completion of treatment ranging from 1 month to over 5 years post treatment. The data was analysed to identify genes that correlated with time of sampling (in months post treatment). A strong positive correlation with IL27RA (IL27 receptor alpha subunit), IL12RB2 and IL12RB as well as JAK2 was seen in $CD4^+$ T cells (figure 5.5(B)). $CD8^+$ T cells strong positive correlations were seen with HAVRC2/TIM3, IL27RA, JAK3 and ITGAE (CD103). However, when control sample values were overlaid, the wide range of gene expression levels in the normal population is seen.

5. 7 Correlation of gene expression with protein expression in selected markers

Protein expression levels of the genes of interest identified by Nanostring were evaluated by flow cytometric analysis. Transcription factors STAT5A and JAK1 as well as IL8 were evaluated by intracellular staining. $CD4^+$ TEMRA STAT5A levels correlated with gene expression counts (Spearman's r =0.324, p=0.039 – Figure 5. 6 (A)) though there was no correlation with total $CD4^+$ T cell STAT5A expression. IL8 expression pattern matched the gene expression signature with patients with active MABS infection having significantly higher $IL8^+$ $CD4^+$ (vs PostTx MAC p=0.04) and $CD8^+$ (vs PostTx MAC p=0.03) T cells (Kruskal Wallis test with Dunns multiple comparisons) (Figure 5.5B).

Surface marker interrogation included IFNγR1, CXCR3, S1PR1 and OX40 (CD134) expression. $CD8^+$ IFNγR1 expression pattern matched gene expression with significant reduction in surface expression of IFNγR1 in active MAC infection compared to controls (p=0.046). S1PR1 expression in $CD8^+$ central memory T cells showed significant increase (0.017) in persistent infection compared to active MAC patients which was similar to gene

expression. CXCR3 MFI in $CD4^+$ T cells correlated well (Spearmans r=0.051, p<0.01) with gene expression log 2 counts. Of the markers tested in $CD4^+$ and $CD8^+$ T cells, approximately 32% correlated with protein expression. The overall pattern of expression matched that of gene expression in IL8, IFNγR1, and S1PR1.

In some instances, however, protein abundance was negatively correlated with RNA abundance. For example, IFNγR1 expression positively correlated with gene expression (Spearmans r =0.0319, p=0.042) in naïve CD4 T cells but was negatively correlated in effector memory $CD4^+$ T cells (Spearmans r =-0.0415, p=0.007). Similarly, S1PR1 expression in $CD4^+$ T cells did not correlate to log2 gene expression in total $CD4^+$ T cells and was negatively correlated in naïve $CD4^+$ T cells (Spearmans r=-0.0384, p=0.013) and effector memory $CD4^+$ T cells (Spearmans r=-0.0367, p=0.018). OX40 expression in total $CD8^+$ T cells was negatively correlated (Spearmans r=-0.332, p=0. 042) with gene expression.

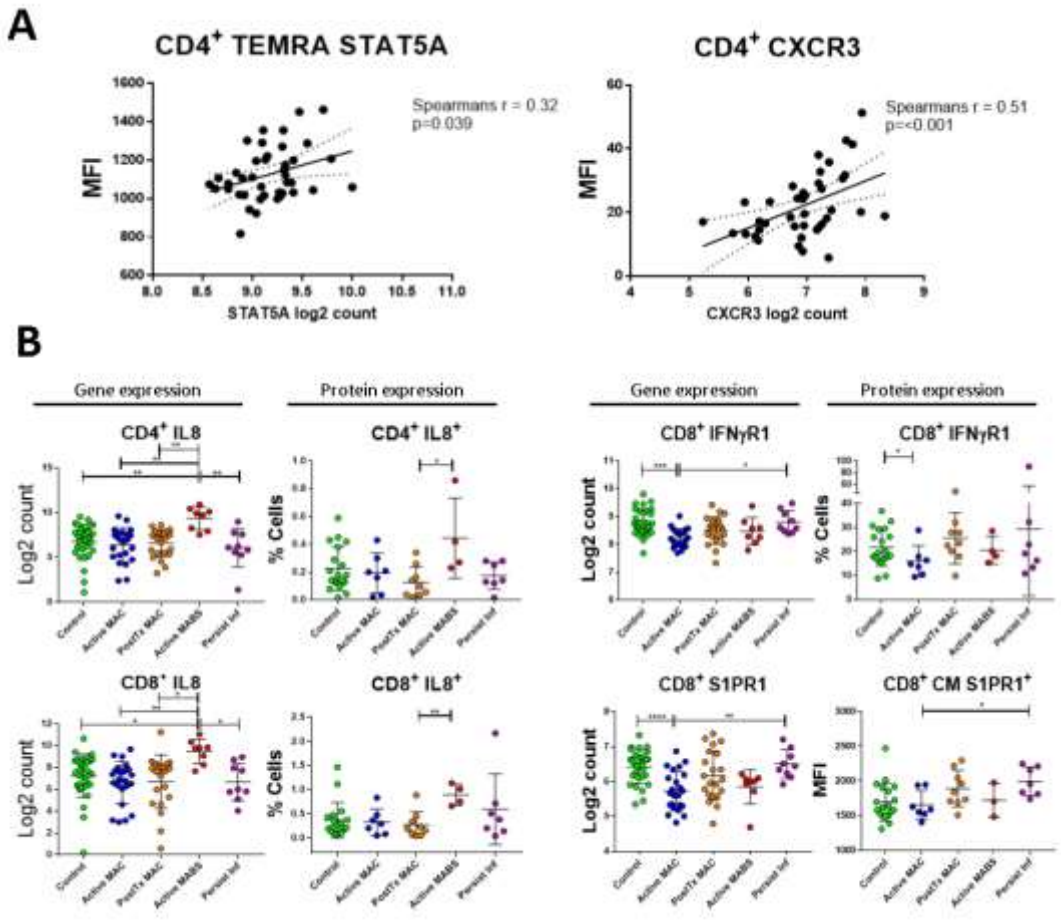


Figure 5. 6 Correlation of gene expression and protein expression.

(A) Correlation between gene expression log2 counts and protein expression as evaluated by flowcytometry MFI in markers of interest: $CD4^+$ terminally differentiated effector memory

cells (TEMRA) STAT5A. and $CD4^+$ CXCR3 count. **(B)** Gene expression log2 counts tested by ANOVA with Tukeys post hoc test. Protein expression cell percentage and median fluorescence intensity (MFI) tested by Mann Whitney test of predetermined comparisons. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

5. 8 Discussion

Gene expression quantification presents a rapidly expanding field where the complex nature of biological processes can be explored in detail from small quantities of sample. Assays can be low or high dimensional depending on the investigator's purpose. New platforms such as the Nanostring nCounter and Nanopore present user-friendly options that provide rapid, robust results of tissue gene expression profiling, which are in-turn more easily transferrable from the research laboratory to the hospital bedside (175). With the objective of identifying gene signatures that could be of clinical use for diagnosis and prognostication of active NTM infection, I analysed $CD4^+$ and $CD8^+$ T cell RNA using a custom probe set that quantified expression of 131 T cell genes. The Nanostring nCounter system proved a rapid, highly automated method with an efficient work flow. A total of 95 samples of $CD4^+$ T cell and 93 samples of $CD8^+$ T cell RNA that passed stringent quality control parameters were analysed.

There were no significant differences in T cell gene expression in either $CD4^+$ or $CD8^+$ T cells between bronchiectasis controls and healthy controls. The similarity in gene expression resembles the likeness of immune phenotype between the two groups described in Chapter 4. This is consistent with bronchiectatic inflammation during clinically stable disease remaining largely confined to the airways, with minimal systemic spill. NTM infection, on the other hand, activates systemic immune signatures which appear to be unique to the infection. It is not simply a matter of increasing bronchiectatic inflammatory response in infection. IL32 expression was increased in patients with bronchiectasis though not significantly. This finding is consistent with other data (unpublished) where I have shown IL32 gene expression was increased in T cells in patients with COPD (chronic obstructive pulmonary disease). IL32 has been implicated in airway inflammation and lung pathology of COPD (237) and likely represents some systemic evidence of airway inflammation in this study as well. A study of *in vitro* MAC and MABS infection of respiratory epithelial cells showed upregulation of IL32 gene expression, corroborating this theory (238).

Global comparison of each patient group with the controls (healthy controls plus bronch controls) showed that patients with active infection, either MAC or MABS, had the most divergent gene signature in both $CD4^+$ and $CD8^+$ T cells. Again, this closely follows the phenotypic changes described in Chapter 4. Upregulated genes in $CD4^+$ and $CD8^+$ T cells in Active MAC infection included PTGER2 and CD27, CD272 (BTLA) and CD226 (DNAM). Down regulated genes in $CD4^+$ and $CD8^+$ T cells in Active MAC infection included IFNγR1, JAK1, STAT5A and S1PR1.

Mouse models of TB have demonstrated that prostaglandin E2 (PGE2) directs macrophages towards apoptosis (103). The complex effects of PGE2 on T cells is a current field of research. The effects appear to be concentration dependant, target cell type and signalling receptor specific (239). Recent studies have shown that T cell secrete picomolar levels of PGE2 during $CD4^+$ T cell activation which have autocrine action. In addition, PGE2 signalling through the EP2 receptor (coded by *ptger2* gene) is required for Th1 differentiation and IFNγ secretion in $CD4^+$ T cells (240). In contrast, another study showed that EP2 receptor was upregulated on virus specific $CD8^+$ T cells in chronic LCMV infection. This study also showed that combined blocking of PD1 and PGE2 increased the number of functional virus-specific $CD8^+$ T cells which lead to improved viral control (241). BTLA is a protein molecule expressed on the surface of activated T cells that interacts with the TNFSR14 (HVEM) to inhibit T cell function (242). It has been shown to inhibit tumour specific $CD8^+$ T cells (236), and suppress early host responses to bacteria (242). DNAM or CD226 is a co-stimulatory ligand of PVR (CD155/ Polio virus receptor) down regulated in exhausted $CD8^+$ T cells in chronic HIV infection (243). Taken together, these findings point towards an activated T cell response with elevated inhibitory signalling molecules. Whether this inhibitory signalling takes the form of normal regulation or pathological suppression is difficult to tease out at present. It is possible that there is PGE2 and BTLA mediated inhibition of Th1 and $CD8^+$ cytotoxic responses, which would indicate a broad suppressive state induced by the infecting organism. As PGE2 is also known to inhibit myeloid cells, the PGE2 signalling pathway may represent an avenue for immune therapeutic modulation in mycobacterial disease which warrants further investigation.

IFNγR1 deficiency is classified in the family of primary immunodeficiencies termed Mendelian Susceptibility to Mycobacterial Disease (MSMD) known to predispose those affected to NTM disease (24, 153). Partial or complete loss of IFNγR1 mediated downstream signalling results in susceptibility. The downregulation of IFNγR1 gene expression in both $CD4^+$ and $CD8^+$ T cells of patients with active infection is indicative of a similar trend. This

suppression was seen in both MAC and MABS patients. Flow cytometric analysis confirmed that IFNγR1 protein was expressed at lower levels on patients with active infection on $CD8^+$ T cells. Together these findings indicate that IFNγR1 expression on $CD8^+$ T cells may be suppressed during active infection which could be a cause of pathology in these patients. As the signalling deficiency is localized to a subset of immune cells, these patients would not be susceptible to disseminated infection as patients with MSMD are. Reversal of the suppression seen in post treatment patients could restore $CD8^+$ T cell IFNγ mediated function. This observation raises the following possibilities: (i) it is a possible explanation for why there does not seem to be any consistent evidence for IFNγ deficiency in elderly patients with MAC infection and why trials of inhaled IFNγ did not show consistent results, since pathology may be mediated by down regulated signalling and; (ii) when correlated with the exhaustion marker fingerprint of TIM3 elevation in $CD8^+$ T cells, the role of cytotoxic T cells in MAC infection is significant. Antigen-specific MHC-I restricted conventional $CD8^+$ T cells are known to be required for protective immunity in mouse models of TB. Unlike HIV and $CD4^+$ T cell deficiency, a human disease model for $CD8^+$ T cell deficiency does not exist. However, BCG vaccination and TB infection have been shown to produce antigen-specific CTLs (214). $CD8^+$ T cells can kill mycobacterium infected cells as well as kill organisms directly via secreted granulysins. It is possible that $CD8^+$ T cell mediated immunity has an even greater role to play in MAC infection. Investigation is hampered by the lack of peptide antigens and MHC-peptide multimers. As such, identifying the MHC-I and MHC-II restricted antigenic epitopes of MAC and MABS organisms needs to be a priority for further work. Downregulation of the IFNγR1 due to ligand mediated autoregulatory feedback is also possible. However, given that IFNγR1 downregulation on macrophages is described as a virulence factor in both TB (244) and Listeriosis (245), the possibility that such a mechanism is utilized by pathogenic strains of MAC and MABS and could act on T cells as wells does not seem farfetched.

The JAK-STAT signalling pathway represents a major transmembrane receptor-to-nucleus signalling mechanisms that regulates gene expression. JAK1 and STAT5A, both of which had suppressed expression in both $CD4^+$ and $CD8^+$ T cells, are required in multiple cytokine signal transduction including IFNγ, IL2, IL7 and GM-CSF (246). Suppression of this signalling pathway is known to lead to susceptibility to infection. However, as protein expression pattern did not correlate to gene expression for JAK1 and STAT5A ($CD8^+$ T cells and $CD4^+$ total T cells) it is difficult to hypothesize on whether this finding is of true biological significance. The importance of the JAK-STAT signalling pathway in NTM immunity will likely be better

understood when the new generation of JAK-STAT blocking/enhancing therapeutics come into clinical use. This pathway, together with IFNγR1 modulation present another potential avenue for therapeutic intervention.

S1PR1 is a surface receptor expressed on both innate and adaptive immune cells. It is involved in immune cell trafficking and increases both B and T lymphocyte egress from lymph nodes. S1PR1 has been shown to increase differentiation of T cells as well, though *ex vivo* studies of the effect on effector responses has shown both inhibitory and stimulatory action. Suppression of S1PR1 in mouse models has shown deficient late stage of T cell development and lymphocyte egress (247, 248). S1PR1 gene expression was significantly suppressed in both $CD4^+$ and $CD8^+$ T cells in patients with MAC infection. This was measured in PBMCs so presumably, some cells with even lower expression were retained in lymph nodes. $CD8^+$ central memory T cells showed a significant reduction of S1PR1 protein expression in active MAC infection compared to controls. Other cell subsets and total $CD4^+$ and $CD8^+$ T cells did not display this suppression. Although the evidence for S1PR1 mediated suppressed trafficking is not substantial in this case, an investigation into peripheral blood vs lymph node resident antigen specific cell S1PR1 expression could prove valuable. The importance of this receptor in infectious disease as a whole is still poorly documented and remains restricted to murine research.

IL8 (CXCL8) is produced by macrophages as well as respiratory epithelial cells and has been shown to play a role in neutrophil recruitment, macrophage mediated killing as well as T cell recruitment in TB (249). In MABS infection, rough variants of MABS induce innate immune responses by TLR2 mediated IL8 secretion (250). The significant increase in IL8 gene expression in both $CD4^+$ and $CD8^+$ T cells together with increased *ex vivo* IL8 protein expression in both cell types indicates that T cells are also a significant source of IL8 in MABS infection. On-going IL8 secretion would explain the strong neutrophilic response seen in MABS infection. This species-specific signature was seen despite a small sample number in the Active MABS patient group. Therefore, T cell IL8 levels has potential for development into a rapid screening test. Longitudinal follow-up studies may show whether T cell, or indeed serum IL8 levels, correlate to disease remission or progression. Given the close relationship IL8 has with the neutrophil rich pathology of MABS infection, IL8 presents an excellent target molecule both for diagnostics as well as therapeutic intervention.

KLF2 is a transcription factor that inhibits T follicular helper cell (Tfh) generation and has also been shown to increase expression of S1PR1. S1PR1 suppression is required for Tfh production (251). In our study, both KLF and S1PR1 were suppressed in MABS infection, indicating an environment that promotes Tfh cell development. KLF2 is also down regulated during TCR mediated T cell activation and regulate both T cell quiescence and activation as well as monocyte inhibition during sepsis (252). Little is known of its role in specific infection settings. Whether protein expression correlates with gene expression in our study patients with MABS infection could not be ascertained as limited sample availability constrained *ex vivo* testing.

Other genes with differential expression in active infection included CD28, CD27, and OX40 a member of the TNF superfamily of receptors, all of which are markers of T cell activation. OX40 upregulation in MABS infection, indicates a special role for TNF in MABS infection compared to MAC infection. OX40 expression on T cells has been associated with increased risk of latent TB reactivation (253). BCG vaccination of mice with OX40 ligand fusion protein has shown increased protective responses (254). Together these findings indicate that OX40 upregulation in T cells marks an active immune response to disease. Findings of the present study indicate that OX40 mediated T cell activation could be more pronounced in MABS infection compared to MAC infection. As protein expression levels did not correlate to gene expression however, we currently have no evidence to show that OX40 mediated signalling is upregulated in MABS infection. However, that MABS infection was shown to cause significantly higher inflammatory gene expression in respiratory epithelial cells (in vitro infection) than MAC infection (238), which supports the T cell gene signature seen here with significant upregulation of inflammatory cytokine IL8.

The importance of TNF in antimycobacterial immunity is well documented (206). TNF gene expression was significantly increased in patients with active infection compared to controls as well as significantly decreased in patients with persistent infection compared to those with active infection. In post treatment MAC patients, TNF gene expression remained relatively high. Results shown in Chapter 4 showed there was no difference in cytokine production capacity between any of the patient or control groups with mitogen stimulation. These findings together indicate that in persistent infection, the lack of immune response appears to be due to immune ignorance, where the T cells, though capable of responding to strong stimulus, are not activated by NTM and so do not orchestrate a robust antimycobacterial response. Indeed, the gene expression pattern in almost all genes evaluated showed that patients with persistent infection had very little T cell activation, and displayed a phenotype closely resembling that of

control subjects. The data from this study represents the first comparative evaluation of the immune phenotype of patients with persistent MAC and MABS infection.

Diagnosing latent TB infection (LTBI) and what immunological factors differentiate it from active TB have been troublesome research questions for decades. Gene expression signature analysis is the most recent tool deployed in the quest for these elusive answers (255). A recent study identified a 74-gene signature that could identify $CD4^+$ T cells from healthy LTBI donors (asymptomatic persons who have positive interferon gamma release assay results) from healthy controls (256). Genes of interest with high differentiating capacity included the Th1 genes ABCB1, c-KIT, and GPA33. A whole blood gene expression analysis of patients with pulmonary NTM infection showed a differential expression of over 200 genes compared to healthy controls. Many of the genes identified were associated with cell mediated immune responses of which the most striking finding was a 1.38-fold suppression of IFNγ transcripts (182). While several other T cell genes were identified as differentially expressed, none of them overlap with the signature identified in the present study which included S1PR1, STAT5A and JAK1.

The major finding of this chapter is the T cell gene signature associated with active infection. The 9-gene signature identified in $CD4^+$ T cells (S1PR1, IL4R, STAT5A, JAK1, CD28, SOCS5, IL10RA, GFI1 and BCL6) and 7-gene signature identified in $CD8^+$ T cells (JAK1, S1PR1, IFNγR1, STAT5A, PTGER2, CD27 and CXCR3) proved to have good discriminatory capacity in the validation data sets tested. These signatures were able to identify active infection against controls who included patients with bronchiectasis as well. This is advantageous as in a clinical setting many of the patients who would be screened for active NTM infection would have underlying airway inflammatory diseases like bronchiectasis and COPD. Internal validation done here is a preliminary step. Further analysis for a combined CD4/CD8 T cell signature is planned though, as no validation cohort is available at present, this is of purely academic interest. Further validation of both the genes involved and the accuracy of prediction in independent cohorts is necessary. The current data provides basic parameters on which approximate sample size for a validation cohort can be estimated. At a power of 0.8, significance level of 0.0005 which would allow for multiple comparisons, to find significance at the lowest effect size of the top 10 genes in each group, approximately 50 subjects per group (Active vs Control) would be required. In addition, to validate the species group specific signature, a minimum of 15 subjects in each species group is necessary.

Clinical records of patients with active infection (MAC and MABS) were reviewed at the end of the study, by which time all recruited patients had completed their treatment course. Outcome of treatment as well as medical history were used to classify patients as 'remission' and 'remission-relapse' based on whether they went into disease remission and remained healthy or went into remission but developed one or more relapses of infection respectively. Gene expression of these two groups were compared to patients who were in persistent infection to ascertain if a signature similar to that seen in persistence was seen at the beginning of infection in patients who tended to relapse. No such similarity was found. There were no markers that could predict treatment outcome at the onset of infection either. As this analysis was limited to 131 T cell genes, the existence of such markers cannot be excluded. However, it is also possible that the outcome of treatment was not already determined at diagnosis and all/most patients have the potential to recover fully from the infection. Longitudinal follow-up of patients during treatment is necessary to determine if and when the turning point of treatment occurs that push patients into remission or persistence.

PostTx MAC patients' blood sampling was done at post treatment timepoints that ranged from 1 month to over 5 years. Gene expression patterns over time were analysed. IL27RA gene expression showed a significant positive correlation with time post treatment in both $CD4^+$ and $CD8^+$ T cells. IL27 has been associated with IL10 secretion and increased Treg responses in cancer and infectious diseases including TB (257) and HIV (258). It is possible that an increasingly tolerogenic/suppressive T cell environment develops with time after treatment which could potentially lead to relapse. Increase in HAVCR2/TIM3 expression in $CD8^+$ T cells with time is also supportive of this theory. There are currently no data on what changes occur in the immune compartment with time after disease remission. As the samples used in this study were not paired samples, collected from the same patient over a period of time, biological significance is difficult to comment on. In addition, the wide range of values seen in the control population indicates that these could well be spurious findings because of small sample size.

Correlation of gene expression with protein expression was seen at 32% which is approximately what is mentioned in the literature (259).Other factors likely to have affected the results include the smaller sample size (approximately half the sample number) tested for protein expression due to limitations in sample availability and the greater sensitivity of Nanostring gene expression quantification compared to flow cytometric analysis.

The current results are limited to T cell genes. While differing signatures for active infection with different species were identified, signatures of disease remission and persistent infection were not apparent. Patients in clinical remission had gene signatures that were often in-between those of active infection and controls indicating that although the immune response to infection was regressing, it had not returned to normal levels. It is possible that once infection is established, true sterilizing immunity does not occur often or quickly. This could explain why some patients tend to get recurrent infections, though these infections tend to be from different infecting species rather than relapses of the same species. The search for markers of disease persistence will likely have to be extended to other immune cell types. Whole blood-based screening tools would be the ideal method for this as they would encompass all blood-based immune cell subsets as well as provide an easy sample to use for clinical tests. While the JAK-STAT signalling pathway was affected at mRNA level, correlation at the level of protein expression level was not apparent. In addition, protein expression as well as activated phosphorylated-protein levels are required to assess whether signalling pathways are affected. The current study did not address these issues and leaves areas for further study. Particularly as JAK/STAT pathway modifying drugs are now becoming available, these molecules could be viable therapeutic targets for MAC/ MABS lung infection.

Collectively the results of this chapter show that disease stage-specific and species genus-specific T cell gene signatures are seen between MAC and MABS lung disease in the blood. These signatures have excellent potential for development into: (i) Point of Care (PoC) screening assays that can be deployed at clinic level for rapid screening of patients presenting with symptoms and; (ii) prognostic assays for prediction of treatment outcome which can assist therapeutic decision making at early stages. Based on current results, a longitudinal follow-up study of approximately 50 patients with active infection, consisting of 20 patients with MABS infection, and a control cohort of 50 healthy + bronchiectasis controls would be the logical next step for this research. Plans for such a study are currently being drawn up.

The results of Chapter 4 and 5 show that patients with active infection have a T cell compartment with features of both activation and suppression mediated by immune checkpoint molecules. Further experiments were then planned to test if immune modulation using checkpoint inhibition could improve the antigen specific cytokine response in PBMCs, as there was no apparent underlying functional defect that would prevent improved function in these patients.

Chapter 6: Antigen-specific recall response

6.1 Introduction

Data presented in Chapters 4 and 5 showed that while phenotypic and gene expression related T cell signatures associated with active infection could be identified, cytokine response capacity for the cytokines tested with mitogen stimulation, appeared to be the same across all patient and control groups. To evaluate if antigen-specific cytokine responses associated with disease stage could be identified, antigen stimulation assays were carried out using two antigens. Crude heat-killed mycobacterial extract from clinical isolates (MAC antigen) was used as this simulates the *in vivo* antigen milieu during infection. Additionally, tuberculin purified protein derivative (PPD), a purified protein fraction of *Mycobacterium tuberculosis*, was used to provide a clearer picture of pure T cell responses without interference from the background innate response.

Responses were measured by two methods. At day 7 after incubation with antigens, culture supernatants were harvested and quantified using a multiplexed cytometric bead array (CBA) for levels of cytokines IFNγ, TNF, IL2, IL10, IL1β and IL8. Cytokine levels are reported as log2 of the fold change of cytokine level/MFI over the negative control well of each sample. Additionally, cultured PBMCs were re-stimulated overnight with antigen and the antigen-specific recall response was quantified by intracellular staining and flow cytometry. Percentage of positive cells are reported as fold change over levels of corresponding cell population in the negative control well of each sample.

6.2 MAC antigen vs PPD antigen response- measured by CBA

Cytokine responses to heat-killed MAC antigen and PPD followed similar patterns though differed in magnitude. A more pronounced response was noted with MAC antigen compared to PPD (Figure 6.1). Control samples (bronchiectasis controls and healthy controls) also showed cytokine responses above baseline levels when stimulated with MAC antigen for all cytokines measured, albeit at lower levels than the disease groups, whereas with PPD stimulation cytokine response was predominantly seen in patient samples; these data indicate that the crude antigen was eliciting an innate type response in addition to the desired antigen-

specific response. Log2 of the fold change of cytokine concentration above unstimulated control well cytokine concentration was used for statistical analysis.

IFNγ levels in response to MAC antigen was significantly higher in patients with active MAC infection and post treatment patients compared to control samples (ANOVA with Dunnetts post hoc p=0.033 and p=0.036 respectively). Patients with persistent infection had a slightly higher IFNγ response compared to controls though this difference was not significant (Figure 6.1). Patients with active infection and post treatment patients showed no significant difference in IFNγ production in response to MAC antigen indicating that during active infection, antigen specific peripheral blood T cell IFNγ responses are in-tact.

The same pattern was seen with PPD stimulation, where IFNγ production in patients with active disease and post treatment were higher than baseline levels. Patients with persistent infection had an IFNγ response that was lower than that of the other patient groups, indicating a lower response to PPD. However, none of these differences were statistically significant. IFNγ response to PPD in active infection and post treatment patients were similar.

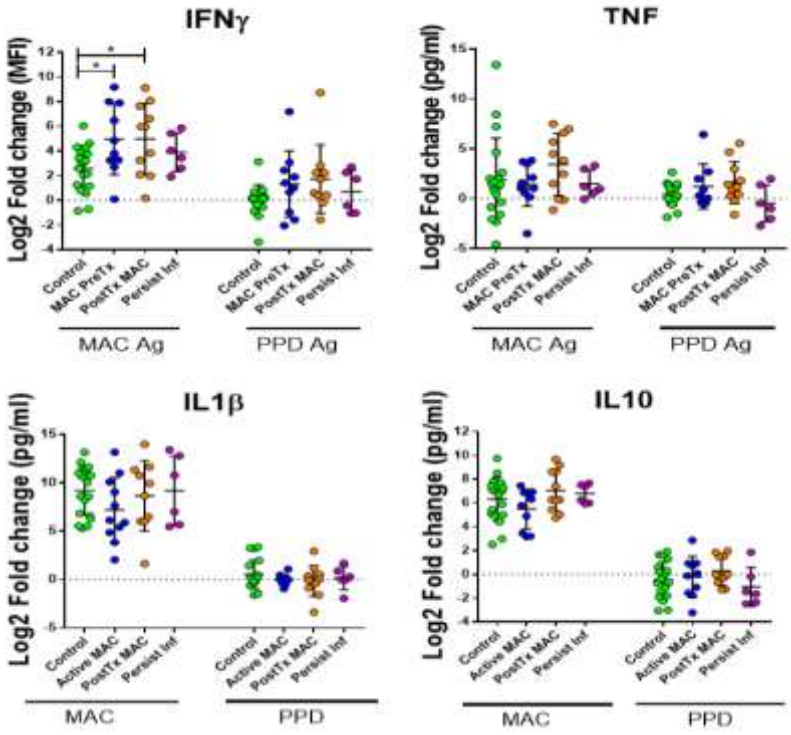


Figure 6.1 Cytokine responses to MAC antigen and PPD Ag stimulation measured by CBA on day 7 of stimulation.

IFNγ, TNF, IL1β and IL10 cytokine levels after antigen stimulation are presented as log2 of fold change over unstimulated control. Antigens used for stimulation were heat killed organism

crude extract (MAC antigen) and purified protein derivative (PPD). Significantly higher IFNγ responses are seen in patients with active MAC infection and PostTx MAC patients compared to controls with MAC antigen stimulation. Comparisons by ANOVA with Tukeys post hoc comparisons. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

TNF response to MAC or PPD (Figure 6.1) antigen was not significantly different between groups. The observed trend in both stimulations was that PostTx patients had a higher TNF response than the other patient or control groups. IL10 and IL1β levels were similar across all groups (Figure 6.1). Higher levels of IL1β and IL10 were seen with MAC antigen stimulation than with PPD, where these cytokines remained at baselines levels in all patient and control groups.

6. 3 MAC antigen and PPD antigen recall response: measured by ICS and flow cytometry after overnight re-stimulation

Antigen recall responses were measured by ICS and flow cytometry to more comprehensively evaluate the cellular immune response. Analysis focused on $CD4^+$ proliferating cells that were $CD40L^{+.}$ In the absence of peptide-MHC multimers to specifically stain antigen-specific cells, proliferation and CD40L upregulation were used as surrogate markers of antigen-specific activation. Significant findings are shown in Figure 6.2

$CD4^+$ proliferating $CD40L^+$ $IFN\gamma^+$ TNF^+ cells were significantly higher in PostTx MAC patients compared to the controls in both MAC antigen stimulation (KW test with Dunns multiple comparisons p=0.006) and PPD Ag stimulation (p=0.007). The cytokine response to MAC antigen was more pronounced than to PPD, similar to what was seen with the CBA analysis.

$CD4^+$ proliferating $CD40L^+$ $IFN\gamma^+$ $IL2^+$ were significantly higher in PostTx MAC patients compared to both the controls (p=0.005) as well as patients with active infection (p=0.02) with MAC antigen stimulation. With PPD stimulation, both PostTx patients and active infection patients had increased levels of these cells, though this increase was not statistically significant. $IFN\gamma^+$ IL2- cells were significantly increased in Post Tx patients compared to controls (p= 0.01) with PPD stimulation, while this pattern was not seen with MAC antigen stimulation.

TNF^{+} IL4^{+} cells as well as TNF^{+} IL2- cells were increased in PostTx patients compared to controls with MAC antigen stimulation (p=0.006 and p=0.04, respectively). This trend was not seen in PPD stimulated cells.

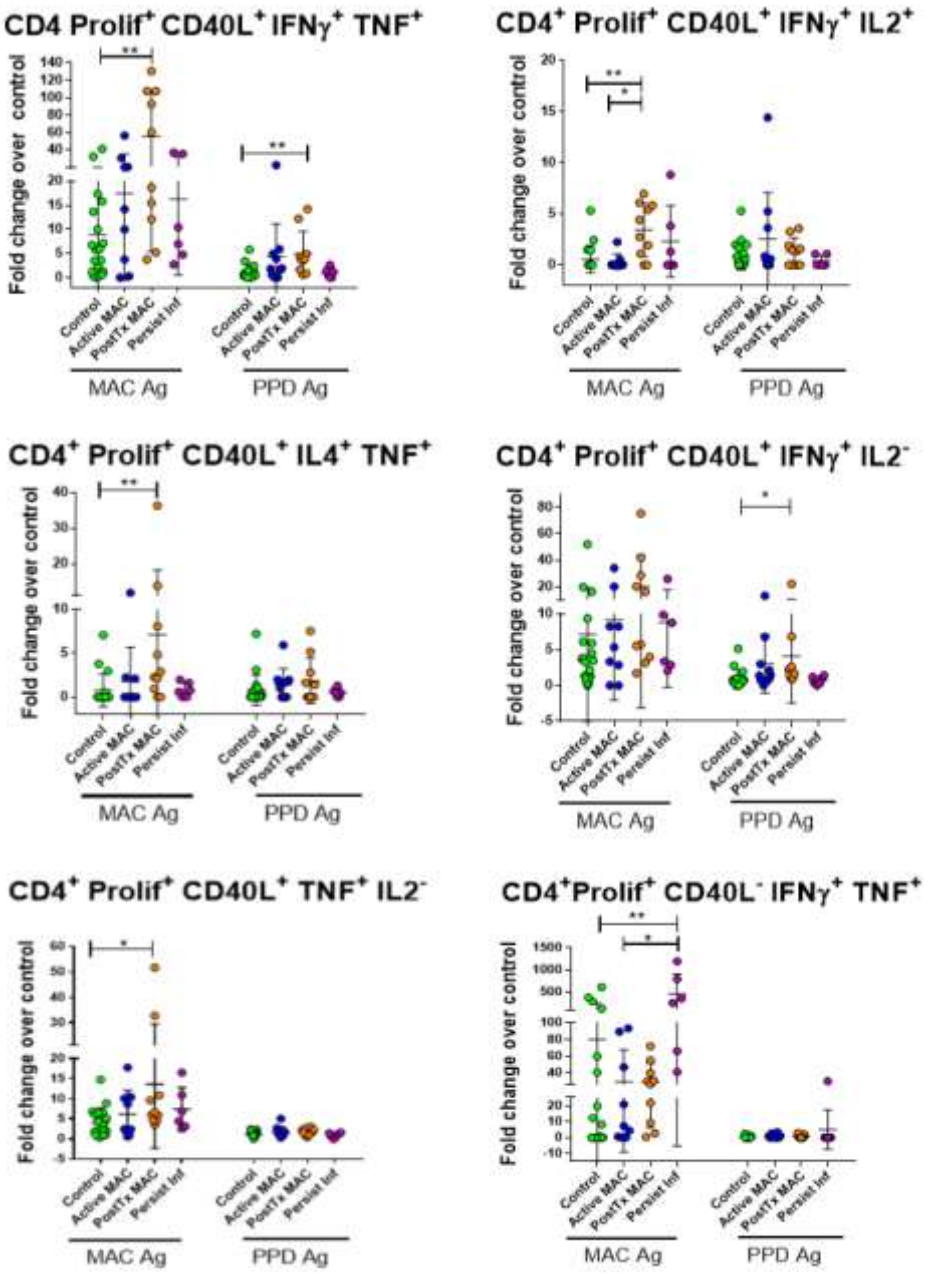


Figure 6.2 Antigen-specific immune responses to MAC and PPD antigens after overnight restimulation.

Figure shows antigen specific cytokine responses measured by proliferating CD40L^{+}ve CD4^{+} T cells when stimulated with heat killed MAC antigen (MAC Ag) or purified protein derivative (PPD). Percentage of positive cells are shown as fold change over unstimulated control levels.

Comparisons Kruskall Walliis test with Dunns post hoc comparisons. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

In addition to the proliferating $CD40L^+$ cells, $CD40L^-$, $CD4^+$ proliferating cells were also analysed. Interestingly, in these cells, amongst these groups, $IFN\gamma^+$ TNF^+ cells were present in significantly high numbers in patients with persistent infection. This increase was compared to both control subjects (p=0.006) and patients with active infection (p=0.05). A significantly higher IL4 secretion in $CD40L^-$ proliferating $CD4^+$ T cells was seen in controls compared to patients with persistent infection (Kruscal Wallis test with Dunns post hoc p=0.05). There were no other differences between groups in this cell subset. There was no difference in the total percentage of $CD4^+$ proliferating cells or percentage of $CD40L^+$ cells, either proliferating or non-proliferating, between groups.

There were no statistically significant differences in $IL17^+$ T cells, either as monofunctional cells, or as polyfunctional cells in combination with other tested cytokines between groups with either MAC antigen or PPD stimulation. IL10 production could not be evaluated due to limitations in panel size.

$CD8^+$ T cell responses were analysed although antigen-specific responses could not be accurately distinguished as CD40L is not greatly increased on activated $CD8^+$ T cells. $CD8^+$ T cells showed no difference between groups in proliferation or in cytokine profile with MAC antigen stimulation. However, PPD stimulation produced very minimal cytokine or proliferation response in $CD8^+$ T cells.

Interestingly, MAC antigen stimulation produced a significant proliferation of NKT cells in all patient and control groups, likely due to the high concentration of LPS in the antigen extract. Mean percent of proliferating NKT cells unstimulated and PPD stimulated wells (all groups) were 7. 3% and 6. 8% respectively. With MAC antigen stimulation, 46.1% of NKT cells were proliferating. (KW test p<0.0001). There was no significant difference in NKT cell proliferation between patient and control groups in response to PPD or MAC antigen.

The above results indicated that the assay detected antigen-specific responses. Stimulation with MAC antigen, though producing a stronger immune response, was also stimulating high levels of non-specific responses from innate type cells. PPD stimulation, on the other hand, gave a similar pattern of IFNγ response to MAC antigen stimulation and appeared to activate $CD4^+$ T

cells specifically without background innate cell activation. Therefore, immune modulation was tested using PPD as the stimulating antigen.

6.4 Immune modulation with αPD1 immune checkpoint inhibitor Nivolumab

The regulatory approved immune checkpoint inhibitor Nivolumab that blocks the exhaustion marker PD1 was used to assess the effect of immune modulating therapeutics on antigen specific responses. αPD1 effect was evaluated based on day 7 assessing IFNγ levels in cell culture supernatant by CBA. Human IgG4 antibodies were used as an isotype control to match the same isotype as Nivolumab.

IFNγ levels (as log2 of fold change over unstimulated control well level) with PPD antigen alone and with PPD $^{+}$ αPD1 are shown in Figure 6.3. A significant increase in IFNγ production was seen in patients with active MAC infection in the presence of αPD1 (paired t test $p=0.043$, $R^2 =0.38$). PostTx MAC patients and control subjects showed a more consistent increase in IFNγ response to αPD1 modulation ($p=0.0025$, $R^2 = 0.612$ and $p<0.0001$, $R^2 = 0.589$) with the R^2 values indicating the unit increase in IFNγ due to the treatment. In contrast, patients with persistent infection showed no significant change in IFNγ levels in response to αPD1 treatment. This finding is important as in all experiments performed thus far, this was the only test parameter that showed a difference between persistent infection and controls. The isotype control IgG4 had no significant effect on IFNγ levels in any of the patient or control groups indicating that the effect seen was due to αPD1 action.

αPD1 treatment had no effect on the levels of TNF and IL1β with PPD stimulation. IL10 levels were, however, significantly reduced in all patient and control groups with both αPD1 and isotype control treatment.

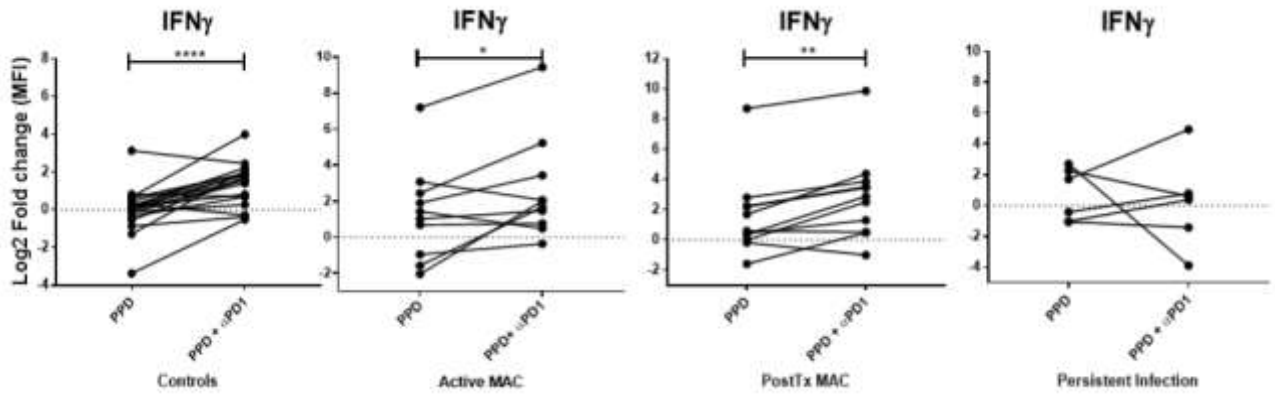


Figure 6.3 IFNγ response to immune modulation by checkpoint inhibitor αPD1.
Dot plots show IFNγ response (measured by cytometric bead array) to PPD in patients and controls with and without αPD1 treatment. Difference in IFNγ tested by Wilcoxon signed rank test. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Collectively, these findings present an intriguing picture of T cell responsiveness in NTM infection. A protective $CD4^+$ T cell response was associated with $IFN\gamma^+$ TNF^+ proliferating antigen-specific cells which are increased in PostTx MAC patients. Increased IFNγ alone is not indicative of disease remission. TNF^+ signatures were also identified as associated with PostTx patients, but it is the $IFN\gamma^+$ TNF^+ signature that was consistent for both MAC antigen and PPD Ag testing. In persistent infection, an increase of $CD4^+$ proliferating $IFN\gamma^+$ TNF^+ cells is seen, but these cells are CD40L negative and may not be able to mount an effective response to antigens. Importantly, patients with persistent infection do not respond to αPD1 treatment, while patients with active MAC and PostTx MAC show a significant increase in $IFN\gamma^+$ secretion in response to this immune modulator. Controls also showed an excellent $IFN\gamma^+$ response to αPD1 treatment showing that the lack of response and lack of immune signature seen in persistent infection is quite different to that seen in control subjects. These results also suggest that healthy controls may have been exposed to NTM in the past.

6.5 Discussion

Quantifying antigen-specific immune responses is a cornerstone of immunology. The complexity of signals that determine the immune response to a given antigenic epitope *in vivo* makes this quantification difficult to replicate in *in vitro* models. This chapter focuses on

antigen-specific T cell responses to two antigens; heat killed mycobacteria was used as a crude antigen mix (MAC antigen) while PPD was used as a purer form of protein antigen. Crude MAC antigen consisted of complex proteins, lipids, nucleic acids and other structural components of mycobacteria presenting a broad antigen range that could stimulate both innate and adaptive responses. As total protein concentration was relatively low in the crude antigen, a large volume had to be used to get an adequate protein concentration in each cell culture well. Strong IL1β cytokine responses in supernatant and high proliferative responses in NKT cells irrespective of patient group illustrate the strong innate response stimulated by this antigen, likely due to high concentrations of LPS. In this milieu it is difficult to deconvolute the peptide-specific response of T cells. It does however represent a more accurate picture of the response that occurs *in vivo* where the antigenic complexity of the organism and a multi-pronged cellular response all play a role in immune pathology and protection. Use of heat killed MAC antigen in patients compared to controls has shown varying results for TNF and IFNγ depending on concentration used and time of incubation (143). As such, it is difficult to compare results of other published research with the present study or indeed, between other studies.

PPD presents a commonly used antigen for testing of antigen specific responses in mycobacterial infections. Significant overlap between *M. bovis* antigens and NTM antigens results in NTM infection giving positive PPD test results (189). While useful for defining peptide specific T cell responses *in vitro* as described here, the lack of innate cell, particularly macrophage activation with co-stimulation signals and other immune cell responses, raises the question of real-world translation of these responses. The difference observed in response to these two antigens, measured by both total cytokine quantification as well as cell specific response, show how variable responses can be, even when all other conditions are controlled. Variability introduced by each experimental condition including antigen dose, duration of cell culture, culture conditions etc are unavoidable errors that need to be kept in mind when interpreting results. Method of quantification also affects the outcome as cytokine measurement in supernatants measures total cytokine levels while ICS measures cell specific cytokine. This variability also accounts for the diversity in immune signatures of MAC and MABS disease published from antigen stimulation studies (133, 134, 137, 139, 164, 260).

The MCH-peptide multimer is a combination of multimerized MHC molecules displaying their cognate peptide fragment. In combination with a fluorescent marker, this molecule is used for identifying peptide antigen-specific T cells. Defining antigen-specific T cells without MHC-peptide multimers is a challenge in most diseases. In NTM infection even antigenic epitopes

remain undefined. The search thus far has proved difficult with studies showing patients often have better responses to PPD than to NTM derived peptide pools (261). Use of CD40L (CD154) as a surrogate marker of antigen specific $CD4^+$ T cells has proven useful in studying many diseases including TB (186, 187). This technique has the added advantage of expanding the analysed cell repertoire beyond a single peptide responsive clone to all antigen responsive $CD4^+$ cells. For these reasons, antigen-specific response testing was performed defining responsive $CD4^+$ T cells as $CD40L^+$ proliferating cells. There was no difference in the percentage of $CD4^+$ proliferating cells or percentage of $CD40L^+$ cells, either proliferating or non-proliferating, between groups. This indicates that proliferation and CD40L upregulation could be occurring as bystander effects in a highly stimulatory, antigen and cytokine rich environments. It was cytokine secretion profiles that differed between patient groups, further indicating that while T cell activation may occur, T cell function is more specific for antigen stimulation. A combination of activation, as measured by proliferation and CD40L upregulation as well as functional profile, as measured by cytokine secretion appears the more suitable method of T cell evaluation, particularly in the absence of MHC-peptide multimers.

Antigen-specific $CD4^+$ T cells that produce both IFNγ and TNF were hallmarks of patients in disease remission (PostTx MAC). This signature was consistent with both MAC antigen and PPD stimulation. This is the first report of in-depth antigen-specific T cell polyfunctionality in MAC lung disease patients. Antigen-specific $CD4^+$ T cells that were $IFN\gamma^+$ $IL2^+$ were significantly higher in disease remission PostTx MAC patients compared to both controls and patients with active infection when MAC antigen was used for stimulation. Antigen specific $CD4^+$ T cells that were $IFN\gamma^+$ IL2- were significantly higher in PostTx MAC patients compared to controls when PPD was used for stimulation. Together these findings indicate that polyfunctional antigen-specific $CD4^+$ T cells that can produce combinations of IFNγ, with other Th1 cytokines TNF and IL2 are seen in patients with protective immune responses. These findings match what is known of the pathogenesis of mycobacterial infection, where a Th1 response involving IFNγ, IL2 and TNF signalling is required for control of mycobacterial infection (132). The significant increase in TNF^+ $IL4^+$ antigen-specific $CD4^+$ T cells with MAC antigen stimulation in PostTx MAC patients compared to controls indicate that, despite the predominant Th1 type response seen, there is some level of Th1/Th2 balance in the memory response generated.

It is interesting to note that the Th1 type cells increased in persistent infection lacked CD40L. CD04L acts as a co-receptor which is activated by elevated CD40 on activated antigen

presenting cells and leads to T helper cell activation. It is possible that these T cells, though stimulated by antigen, are unable to fully respond to the threat due to lack of co-signalling. There may be a similarity with the X linked form of Hyper IgM syndrome, which is known to predispose to NTM infection. In these patients, CD40L gene mutation leads to lack of CD40L expression. CD40L mediated downstream signalling in monocyte derived cells was shown to be required for effective immunity to mycobacteria (23). Investigation of macrophage killing function in patients with persistent infection, as well as modulation with CD40L agonist/antagonist agents would provide useful insight into this pathway in NTM infection. It is possible that this pathway could be amenable to therapeutic intervention in these patients.

The lack of an identified marker that delineates antigen-specific $CD8^+$ T cell responses in my disease setting is a significant drawback. The results thus far point toward $CD8^+$ T cell mediated immunity playing a significant role in MAC infection. However, as the $CD8^+$ T cells proliferation and cytokine response profiles in both patients and controls were similar in response to MAC antigen as well as PPD, a specific cell population could not be defined as being antigen specific. Using the simple definition of $IFN\gamma^+$ $CD8^+$ T cells would lead to both erroneous inclusion and exclusion of cells as $CD8^+$ T cells perform many more functions. As mentioned in Chapter 3, the role of $CD8^+$ T cells in immunity against NTM is not yet known. The importance of $CD8^+$ immunity in TB is, however, now better elucidated. A recent study by Lewinsohn et. al. described the first HLA allele-independent $CD8^+$ antigen discovery study which found and validated 16 previously known as well as several new CD8 antigens to which immune responses are mounted in patients with TB infection (262). Importantly this study further highlighted that the anti-TB vaccine trial of Ag85a did not elicit $CD8^+$ T cell responses, which may have led to failure of the Phase 2b vaccine trial (263). Antigen specific $CD8^+$ T cells are generated during BCG vaccination and are known to contribute to immunity though the exact mechanisms are still unknown (264). Very recent findings show that BCG vaccination provides cross protective T cells that increase MABS and MAC intra-cellular killing (265) indicating it is likely that cross-reactive $CD8^+$ T cell epitopes play a significant role in heterologous immunity to NTM infection; however this remains an unexplored field.

Having ascertained that the assay used in my work could detect antigen-specific responses, I then proceeded to test if immune modulation with checkpoint inhibitors could increase the desired Th1 cytokine response. αPD1 and αCTLA4 therapeutic agents are available. Given the

MAC and MABS species specific signature identified (Chapter 4) TIM3 or CTLA4 blockade therapy testing was considered. Given that PD1 was not reduced compared to controls in either infection, αPD1 testing was also an option. However, as αTIM3 therapy is still in trial stages and not available by MTA (266) and αCTLA4 therapy, though effective, is prone to give high levels of immune side effects including pneumonitis the safer and more clinically suitable drug αPD1 was chosen for testing (267). As PD1 levels were not significantly reduced in patient T cells, it was reasoned that there would be no significant loss of effectivity of αPD1 agent Nivolumab.

The most significant finding of this Chapter is that αPD1 treatment increased IFNγ secretion in all patient and control groups except patients with persistent infection. The increase in IFNγ secretion corresponds to the findings of Shu et. al. who showed an increase in IFNγ levels when MAC heat killed antigen stimulated cells from patients with active infection and controls were treated with PD1 and PD-L1 blocking antibodies (143). The consistent increase in IFNγ levels in both patients with active MAC as well as PostTx MAC show that during both active disease as well as successful treatment, $CD4^+$ and $CD8^+$ T cells, and possibly other αPD1 responsive cells like NKT cells remain in a functional state similar to that of T cells of controls. However, in persistent infection, T cells become refractory to αPD1 indicating other mechanisms beyond checkpoint inhibition are at play. In a recent paper Splitt et. al. tested αPD1, αCTLA4 and αTIM3 for efficacy in reversing T cell exhaustion in lethal re-infection of *Toxoplasma gondii* in mice and found no effect of therapy, despite all three markers being increased on T cells (268). The resistance to αPD1 could be due to senescence of T cells, where they are no longer in a reversibly exhausted state. Level of senescence marker CD57 was not tested (269). However, other markers such as KLRG-1 were not elevated in the gene profile of these T cells making this hypothesis unlikely. Lack of response to αPD1 is also the most significant difference between persistent infection and control PBMCs, which up to now were remarkably similar in phenotype, gene expression and functional profile. This finding suggests that mechanisms other than immune ignorance to infection are at play in persistent NTM infection.

It is possible that αTIM3 or αCTLA4 agents would exert a far higher stimulatory response in patients with MAC and MABS infection respectively, given the phenotypic signature of T cells described in Chapter 4. Indeed TIM3 blocking has been shown to be beneficial in control of TB in mouse models of infection (173). Whether such a response would be beneficial or detrimental due to its magnitude is also a question that needs to be answered. The increase of PD1 on $CD4^+$ and $CD8^+$ T cells in patients with TB has documented (270, 271). Blocking

experiments in mouse models have shown an increased susceptibility to infection due to exacerbated immune responses (202). Together, these findings highlight the time sensitive nature of checkpoint inhibitor therapy. Treatment that is too early could be detrimental to the host while treatment that is too late could see T cells unresponsive to treatment.

Treatment options for patients with persistent infection are a high priority need. Currently, GM-CSF inhaled therapy is being trialled (ClinicalTrials.gov Identifier: NCT03421743) in these patients. As GM-CSF would act directly on macrophages, by-passing T cell signalling, there is a good chance that this therapy will prove useful in these patients with poorly responsive T cells. If the mechanism of T cell resistance to activation is elucidated, GM-CSF therapy could be combined with T cell stimulants that would perhaps offer better immune responsiveness in these chronic infections.

The complexity of deconstructing the immune response to a chronic intracellular infection that has both localized and disseminated forms and requires both the innate and adaptive immune system to work in unison over an extended period of time is described by Sakai et. al. (201). This study looked at the contribution of $CD4^+$ derived IFNγ to resistance to TB in the lungs and spleen of infected mice and showed that it played a minimal role in the lung while playing a larger role in the spleen. The advantage of mouse models remains the access to site-of-lesion samples, and the highly controlled nature of the experiments, where single variables can be tested. The disadvantages are that in-bred mouse immune systems in laboratory environments are not representative of the natural diversity of human immune responses. Mouse infection models are by nature short term, whereas infections like NTM have incubation periods of months to years. This difference in models is exceptionally important when evaluating therapeutic responses in chronic infections as demonstrated by the LCMV model, where early αPD1 blockage exacerbated pathology, whereas during the chronic phase of the infection it increased viral control. Similar results were seen in TB models, with PD1-KO mice showing worsened immunopathology compared to controls with early use of αPD1 (202) while use in the chronic phase showed no change in either bacterial control or pathology (201). The differences in IFNγ production between lung parenchyma homing T cells ($CXCR3^+$ KLRG1- CX3CR1- $CD4^+$) compared to T cells that home to the lung vasculature ($KLRG1^+$ $CX3CR1^+$ $CD4^+$) shows the subtlety of the immune response and highlights the difficulty of broad interpretation of low definition results (272). In human studies, access to samples from site of pathology is often limited and so data is extrapolated from accessible samples like peripheral

blood. Evidence that lymphocytes circulate between the blood and lungs (195) is published and the fact that antigen specific T cells are isolated from PBMCs shows that this occurs. However, increasing evidence of tissue specific T cell populations (273) clearly shows that for specific understanding of disease pathology, tissue samples would be necessary. For this reason, while I will speculate on the possible immunopathological implications of the results of this study, it is possible that in patients, checkpoint blockade could mediate very different systemic effects. A notable similarity between the above experimental conditions and the clinical syndrome of Immune Reconstitution Inflammatory Syndrome (IRIS) does exist. HIV patients on antiretroviral therapy who have occult NTM infection, develop IRIS and T cell function (in this case, $CD4^+$ T cell number) is suddenly restored in the presence of established infection. This circumstance is immunologically similar to the experimental setting of acute infection with robust T cell response, further heightened by checkpoint blockade. If this parallel holds true, it would indicate despite the differences between experimental mouse infection and chronic human infection, the well-timed use of checkpoint inhibitors in addition to antibiotics could afford increased bacterial control in the chronic phase of NTM infection.

Consistent suppression of IL10 in all patient and control groups by both αPD1 Nivolumab as well as the human IgG4 isotype control presents an interesting effect, probably mediated by the Fc segment of the antibody. Other studies have reported IL10 gene upregulation in macrophages with αPD1 and isotype controls mediated by FcγI receptors (274). An increase in serum IL10 in ovarian cancer associated with αPD1 treatment has also been documented (275). While my findings are the opposite of what is shown in these studies, there is clear interaction between the IgG4 antibody Fc segment and IL10 production. The effect of IgG4 antibodies in PBMCs may ultimately result in elevated IL10.

Overall, these results suggest that protective polyfunctional Th1 type responses are seen in patients in MAC disease remission while patients with persistent infection produced a Th1 type response in the absence of co-signalling molecule CD40L. αPD1 agent Nivolumab treatment of PBMCs significantly increased IFNγ levels in all patient and control groups except for patients with persistent infection, where it is likely that suppressive mechanisms other than checkpoint pathways are active. Further study into the time dependant nature of checkpoint inhibitor action as well as the utility of other checkpoint inhibitors in this clinical scenario need to be tested.

Chapter 7: General discussion

7.1 Overview and summary of results

Lung disease caused by NTM is a global public health problem that is steadily increasing in prevalence and associated economic burden. NTM presents a diagnostic and treatment dilemma for clinicians. Current guidelines require microbiological, radiological and clinical criteria for diagnosis. No screening tests are available other than sputum microbiology when clinically suspected. Treatment courses involve multiple antibiotics over 18 months or more. Many of these can be associated with critical side effects, represent morbidity to the patient and incur substantial health care costs (26). Outcome of treatment varies from complete remission to persistent infection that is refractory to further antibiotic treatment often over periods of years. Together with increased all-cause mortality, increased tertiary health care visits, recent evidence of person-to-person transmission (for some species of NTM), as well as increased incidence in children, NTM lung disease presents an area of research importance.

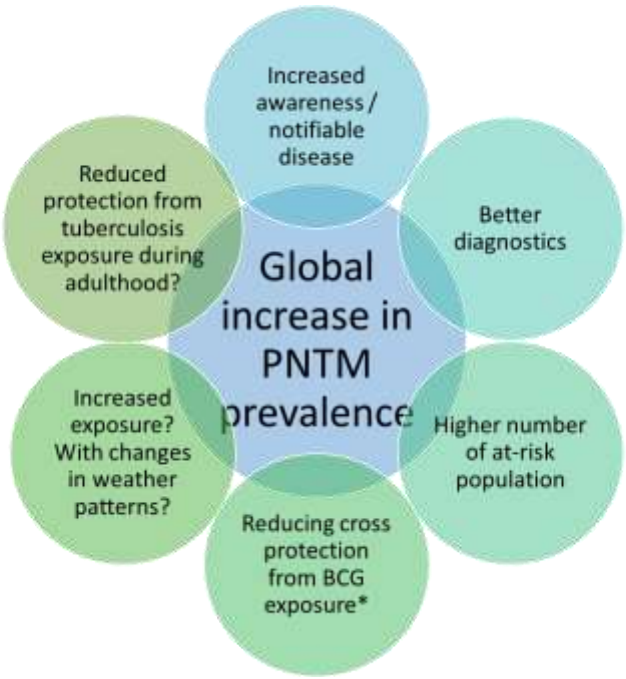


Figure 7.1 Potential reasons for increasing global prevalence of pulmonary non-tuberculous mycobacterial infection.

Increasing awareness of the magnitude of the problem has led to NTM infection now being a notifiable disease in many countries, resulting in better statistics on global disease burden. Improving diagnostics and molecular identification assays to differentiate NTM from TB as

well as identify NTM infection at the species level have helped. With progress in modern medical practice, there are increasing numbers of patients in known risk groups. These are the result of either increased iatrogenic risk, introduction of new immune suppressive therapies, or increased life span of patients with disease as medical treatments and health outcomes improve in general. The increase in cervical lymphadenitis and NTM infection in children after routine BCG vaccination of the general population ceased that was described in Finland (160) together with evidence of cross protective immunity generated by BCG vaccination (265) suggest that the decline in BCG coverage of populations could be a cause of increased NTM infection. The corollary of this is the possibility that as TB infection has become less common in developed nations and exposure to TB has become rare during adult life, together with reduced community BCG immunity, there is an increase in susceptibility to NTM species in the elderly through decreased immunity. In addition, as weather patterns change, and extreme weather conditions become more common, it is possible that the organism density in water systems and the natural environment (soil) increases, leading to greater potential for human exposure and spread of infection. NTM density has been shown to be affected by temperature and pH with higher organism density associated with areas of high evapotranspiration (9, 276) supporting this theory.

M. avium complex and *M. abscessus* complex are the most common species groups causing human lung disease worldwide. They are organisms that are classified as opportunistic pathogens, causing disease only when host immunity is compromised. The nature of immune compromise is clear in some susceptible populations, such as individuals with primary immune deficiencies (MSMD), HIV/AIDS or haematological malignancy. In other risk groups, such as cystic fibrosis, structural lung compromise is present. However, whether any additional immune compromise contributes to the increased risk of NTM in some of these patients is unknown. In middle aged and elderly patients who have specific morphological characteristics that prompted the name ‘Lady Windemere syndrome’, the immune defect is unknown. Previously regarded as one uniform entity, emerging evidence that MABS and MAC result in unique immunopathological responses has led to a more focused approach in some studies of human disease. However, studies that compare these infections between risk groups, dissect the immune compartment at different stages of this chronic disease, and perform in-depth genotypic, phenotypic and functional characterization are not available.

These doctoral projects were designed to address these important questions. The first stage was a pilot study of MABS infection in CF patients and elderly patients. CF patients with active MABS infection and those with a history of MABS infection were compared to CF patients who had no history of MABS or other NTM infection as well as matched healthy controls. In addition, elderly patients with no known immune compromise who had active MABS infection were recruited along with a second set of matched healthy controls. Increased expression of the immune checkpoint CTLA4 in $CD4^+$ T cells was seen in both CF and middle aged/ elderly patients with active infection. CF patients who were predisposed to MABS infection (had current or past infection) showed significant reduction in TNF production in response to mitogen compared to both CF and healthy controls while the opposite was seen with the elderly patients. Together, these results suggested that risk group specific immune dysfunction related to T cell quality, and immune exhaustion signatures related to infecting species, are present.

The second stage of the project studied MAC and MABS lung disease in middle aged and elderly patients who had no known immune compromise. A detailed study of peripheral blood immune cells during active disease, remission and persistent infection showed the following. First, that active MAC infection is characterized by TIM3 elevation predominantly on $CD8^+$ effector T cells and NK cells, while active MABS infection is characterized by CTLA4 elevation predominantly on $CD4^+$ effector T cells and Treg cells. The TIM3 levels reverted to near normal levels in PostTx MAC patients, while persistent infection showed no discernible exhaustion fingerprint. Second, the functional capacity both in terms of cytokine production and proliferation of $CD4^+$, $CD8^+$, NK and NKT cells in peripheral blood showed no dysfunction in response to strong mitogens. Patient cells were as responsive as healthy control cells. Third, gene expression in $CD4^+$ and $CD8^+$ T cells showed increases in both activation and suppression markers in active MAC as well as active MABS infection. Intriguing evidence that the IFNγR1-JAK-STAT pathway may be suppressed in active infection posed new possibilities for immune susceptibility as well as therapeutic intervention in these patients. Based on collective data of all patients with active infection (MAC + MABS), a 9-gene $CD4^+$ T cell signature and 7-gene $CD8^+$ T cell signature for diagnosis of active infection was identified and validated using internal validation cohorts. These signatures had high accuracy (0.73 to 0.95), sensitivity (0.67 to 1.0) and specificity (0.66 to 1.0). Species group differentiation based on IL8 levels, either serum or gene expression is a possibility that needs further exploration. Fourth, T cell gene signatures in individuals with persistent infection and control subjects were remarkably similar, providing new insight into the pathology of persistence and immune 'ignorance'. Finally,

antigen-specific immune response testing showed that while T cells from patients with active infection and those in disease remission were responsive to immune modulation by αPD1 checkpoint blockade, T cells from patients with persistent infection were refractory to such therapeutic intervention.

7. 2 A unified model of MAC and MABS lung disease

The greatest insights into NTM disease immune pathology have come from detailed studies of susceptible populations and the disease mechanisms that increase risk. Equally important in this context are the diseases that do not increase risk. A unified model of NTM colonization, infection, immune response and immune failure can be built based on these facts.

Aerosol inhalation of NTM leads to colonization of the respiratory tract. Virulence of the organism and infectious dose/duration of exposure to aerosols play a role during initial establishment of infection. Respiratory innate immune barriers are necessary for keeping colonizing bacterial numbers under control. This requires intact mucosal barriers, efficient ciliary function, and clearance of mucus. Structural lung diseases where ciliary function is compromised and mucus clearance in impaired therefore result in increased organism retention. Such diseases would include bronchiectasis, COPD and CF. The impact of smoking on ciliary function would explain the common incidence of recurrent infection in smokers. Patients with skeletal abnormalities like pectus excavatum and scoliosis, tall, thin body habitus have recently been shown to have connective tissue and ciliary function related gene defects. It is possible that these patients are all susceptible due to innate defence breakdown leading to colonization of the airways and subsequent invasion of tissue. Patients with gastro oesophageal reflux disease (GORD) are also prone to develop NTM infection. The association of GORD with other chronic cough syndromes such as COPD and asthma indicate that the predisposition lies in impaired airway immunity.

It is evident that some degree of tissue invasion occurs even with low levels of colonization when airway defences are intact. Patients who have healthy airways but have systemic immune defects like HIV/AIDS are susceptible to infection, indicating that barriers alone do not keep the organisms within the lung, but rather keep numbers low enough that a functioning immune system can deal with infection. Initial infection results in activation of a broad spectrum of innate PRRs including TLRs, C-type lectins and NLRs. Downstream signalling through the

NFκB pathway and NLRP3 inflammasome activation are necessary for intracellular killing of phagocytosed bacteria as well as secretion of pro-inflammatory cytokines IFNγ, IL12p40 and TNF. Dysfunction of these pathways as seen in the PIDs with NFκB pathway related NEMO deficiency leads to increased susceptibility to infections. Nramp (natural resistance associated macrophage protein) expressed on the phagosomes acts to sequester iron and enhance phago-lysosome fusion. It has been proposed that the natural resistance of African Americans to NTM infection has been associated with Nramp expression (277) while predisposition has been associated with Nramp allelic variants in Korean studies.

Activation of TLRs results in upregulation of vitamin D receptor VDR on the surface of macrophages. Vitamin D2 and the active form vitamin D3 is known to potentiate macrophage function by increasing expression of antimicrobial peptides, such as Cathelicidin, and increasing reactive oxygen species (ROS) among several other mechanisms. Vitamin D deficiency has been associated with reduced macrophage induced killing in patients with diabetes mellitus, which was correctable with vitamin D supplementation (106). Studies showing both the presence and absence of vitamin D deficiency in NTM infection are published (278, 279). It is possible that vitamin D deficiency does play a role in NTM susceptibility through impaired macrophage mediated intracellular killing, though probably not in all patients which could explain these divergent findings. Deficiency in ROS is also seen in patients with chronic granulomatous disease who have NADPH mutations. These patients are also susceptible to mycobacterial infections (23) supporting the theory that causes of poor macrophage mediated killing predisposes to infection.

Postmenopausal females with thin body habitus (‘Lady Windemeres’) are a risk group. These patients have low estrogen levels and altered adiponectin/leptin balance (280). A recent review published by Holt et. al. (2019) describes the endocrine changes seen in these patients with possible mechanisms of susceptibility. The common thread that relates these hormones, vitamin D levels and bone mineral density, is fat mass. It is possible that body metabolic balance predisposes to infection which is an area of ongoing research. In future studies by my colleagues which will build on my doctoral research findings, and metabolic and endocrine data from this patient cohort will be incorporated with the current immune data for comprehensive analysis. We anticipate this will shed further light on the interrelationships between hormones and immune response in NTM infection.

During the initial stage of infection, neutrophils and innate type NK cells are recruited to the site of infection. This response is probably not essential in controlling infection as patients with PIDs of neutrophil deficiency/dysfunction do not show increased predisposition to NTM infection. Neither do patients with NK cell and cytolytic pathway deficiencies (perforins) nor do patients with complement deficiencies. Possibly, these cells and proteins perform redundant functions (281).

Antigen presentation to T cells in a proinflammatory IFNγ, IL12 environment polarizes the T helper cell response towards the desirable Th1 type. Whether antigen presentation is predominantly class II restricted or class I restricted, or both is not known. The role of lipid antigens and responding MAIT cells, γδ cells and NKT is not known. IL12/IL23 (with its common IL12p40 subunit) and signalling mediated via IL12Rβ1 and IFNγ signalling via the IFNγR1 and IFNγR2 is essential at this stage. Deficiency in IL12Rβ1 pathway confers moderate susceptibility to NTM infection while IFNγR deficiency confers a more severe form of susceptibility to the host. This syndrome is named Mendelian Susceptibility to Mycobacterial Disease. The importance of IFNγ/IL12 and intact signalling pathways in developing a Th1 response is highlighted here (281). Interestingly patients with HLA-I or HLA-II deficiencies in APCs and patients with T cell dysfunction related to complex immune deficiencies like Wiskott-Aldrich syndrome, do no show increased susceptibility.

Aspergillosis is a common co-infection seen in patients with NTM lung infection. Immunity to *Aspergillus* also requires a robust Th1 type response as $CD4^+$ T cell IFNγ is necessary to mediate efficient fungal clearance. A Th2 type response is associated with poor clearance and allergic bronchopulmonary aspergillosis (ABPA) (282). The association between these two diseases could be due to a common tendency to develop Th2 type responses which would be detrimental in both cases.

Factors that lead to impaired $CD4^+$ Th1 proliferative and cytokine response include iatrogenic causes like systemic steroids, cytotoxic drugs and other classes of immune suppressants which are known to predispose to NTM infection. In addition, data from the current study shows that patients with active infection, particularly MABS infection, generate Treg cells that express multiple exhaustion markers and are highly suppressive. The exhaustion marker increase is seen on many immune cell subsets and likely leads to a global impairment of immunity. The consequences of this could include increased susceptibility to other infections, as well as malignancy. Metrics in PBMCs during active infection show characteristic T cell gene

signatures. Results from this study also show that T cells at this stage are responsive to immune checkpoint blockade. As disease progresses, T cell signatures change and patients who have persistent disease show modulation refractory T cells. When and how this change occurs is a question that needs to be answered.

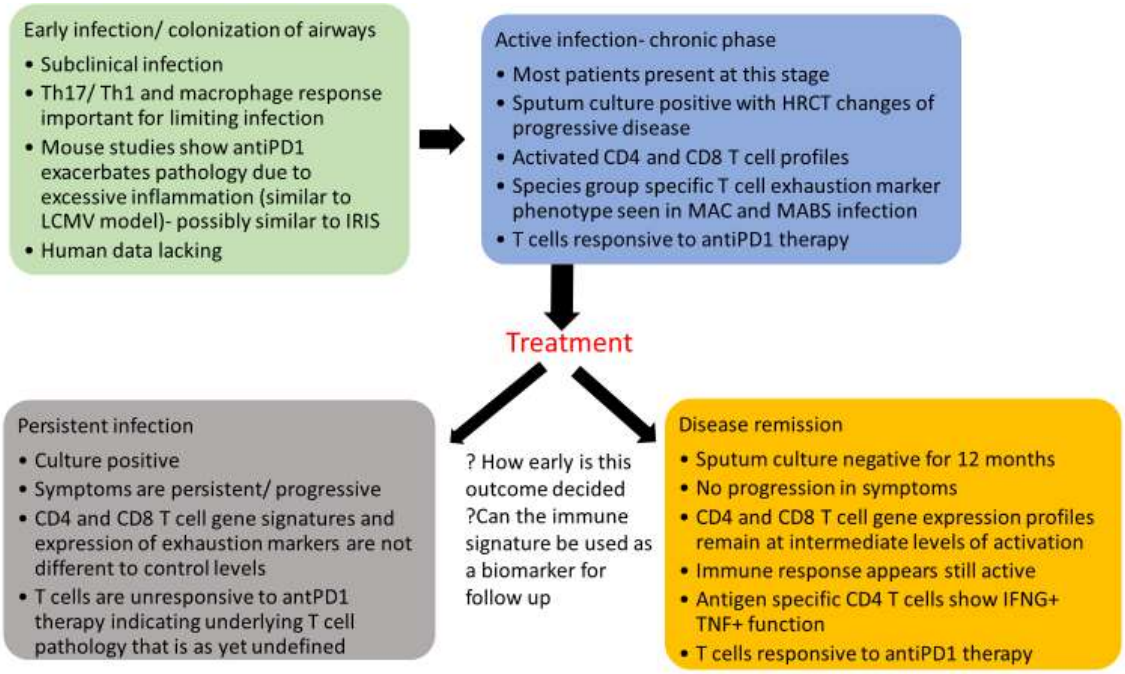


Figure 7.2 Disease model of chronic MAC infection.

Figure shows an outline of possible sequence of events in MAC infection in humans. Major findings of this study as well as important areas that need further work are identified in association with the course of infection. *IRIS- immune reconstitution inflammatory syndrome.

T cell activation includes CD40L upregulation. Patients with X-linked hyper IgM syndrome who have deficient CD40L have been shown to be at risk of NTM infection in one study (23). These patients had deficiency in T cell stimulated IL12 production which along with other deficiencies lead to susceptibility. Though the evidence is not overwhelming, it is possible that patients with persistent infection have a subtle deficiency in CD40L upregulation on antigen specific cells which leads to IL12 deficiency related persistence of infection. The role of CD40L in NTM immunity is certainly worth investigating. Another source of data will likely be the new generation of immune therapeutics include CD40L blocking drugs (283). These, together with the JAK/STAT modulating drug family, will likely uncover new predispositions to NTM infection.

Th1 cell trafficking to the lung with secretion of type 1 cytokines results in increased macrophage activation for intracellular killing. MABS has been shown to induce IL17 type responses from T cells which would increase neutrophil influx which is characteristic of MABS infection. Data from this study has shown IL8 is increased in MABS infection which would also contribute to neutrophil migration. TNF secreted from T cells and macrophages is essential for granuloma formation and limiting spread of infection. Blocking of TNF action by anti-TNF drugs would result in numerous downstream effects including poor granuloma formation (284).

Both TNF and IFNγ are described as double-edged swords in mycobacterial immunity. They are essential for a protective immune response but too much results in excessive inflammation, tissue damage and overwhelming immune pathology. This is what is seen in M-IRIS (mycobacterial immune reconstitution inflammatory syndrome) in HIV/AIDS patients started on anti-retroviral therapy. Initial immune suppression allows opportunistic infection to get established. However, as $CD4^+$ T cell response is minimal, there is little inflammatory response and no clinical features of illness. Once anti-retroviral therapy starts to increase $CD4^+$ T cell counts, an intense immune response is mounted against the now abundant infection leading to extensive tissue injury (285). The regulation of T cell response is therefore essential for limiting immune pathology. However, excessive suppression mediated by highly suppressive Tregs may lead to poor control of infection. Lung levels of Tregs, and regulatory cytokines IL10 and TGFβ levels during active infection and persistence would give valuable insight into the suppressive state prevalent at site of infection.

7.3 Strengths and limitations

This doctoral study presents the first detailed deconstruction of the immune landscape of MAC and MABS infection comparing risk groups, disease stage, and infecting organism. The focus on T cell biology yielded biosignatures of active infection, species-specific signatures, and functional insight into the pathobiology of acute, recurrent and persistent infection. The patient cohorts included in the study are representative of risk populations in age and gender distribution. CF patients with MABS infection were compared to young healthy controls as well as other CF patients who had chronic *Pseudomonas* infection highlighting the impact of MABS in CF compared to other well-known chronic infections. Similarly, the inclusion of patients with bronchiectasis as a ‘within disease’ control group in the second phase, highlighted

the significant changes that occur in the immune compartment due to NTM infection, rather than underlying bronchiectasis, which has comparatively little impact on systemic T cells. The study of T cell, NK cell and NKT cell subsets showed the broad-spectrum effects NTM infection has on immune cells. This is not a disease restricted to macrophages or $CD4^+$ T cells alone. Utilizing new data analytical pipelines proved useful for identification of cell subsets that would be missed by biaxial gating as well as detailed analysis of functional data. Two T cell gene signatures associated with active infection were identified using the Nanostring nCounter platform. Further validation and development of the identified signature as a potential clinical screening tool would be facilitated by the validated, licenced platform used for performing this test. Inclusion of patients with persistent infection provided interesting insights into mechanisms of persistence. Antigen specific immunomodulation showed comparative T cell unresponsiveness in these patients which could provide the first clues as to mechanisms of persistence though much work remains to be done.

Limitations of the study include limited sample size, particularly in the active MABS patient group. A group of post-treatment MABS patients were not included in the elderly cohorts, so post-treatment disease remission data is limited to MAC infection. Patients with persistent infection were also comparatively few. Limitations in sample availability prevented all assays from being performed on all samples. Gene signature confirmation assays and antigen specific stimulation/immune modulation was performed only on approximately half of the samples. Gene signature validation required PBMC samples that had not gone through previous freeze-thaw cycles, and antigen stimulation assays required large cell numbers; therefore, only samples that met these criteria could be included. This reduced the power of analysis and impacts the robustness of the results. Antigens used included crude heat killed bacteria and PPD tuberculin. Purified protein derived from *M. avium* 'sensitin' may have presented a clearer picture of MAC specific T cell responses. Both the pilot study and second phase study focused on T cell biology. Although additional data on NK cells and NKT cells were gathered during the second phase, monocyte/macrophage data is limited. Gene expression analysis was limited to 131 genes and $CD4^+$ and $CD8^+$ T cells. A more comprehensive RNAseq analysis may have presented a more complete picture of immune changes in active, remissive and persistent disease but was beyond our budget. Longitudinal follow-up samples were not available. Although serial sampling was attempted, this was not feasible for most patients during the study period. As such, we do not have data on changes that occur with treatment and we cannot

assess at what stage of treatment patients start to progress towards remission vs persistence. One of the biggest limitations of the study is that all experiments were done on PBMCs. Whether T cells in the lung mirror these changes is not known.

7.4 Conclusions and future directions

The objectives of these studies within this thesis aimed to characterize immune signature of MAC and MABS infection in two disease risk groups aiming to identify immune susceptibilities associated with infection. Based on the results presented in Chapters 3 to 6, the following conclusions can be made. First, NTM lung infection is not one uniform entity. Risk group specific and species-specific immune signatures are present that indicate this disease has unique immunological characteristics in each clinical situation. There is a species-specific systemic immune dysfunction with each pathogen. These changes could potentially be developed into a much-needed PoC screening test for active infection, which could probably incorporate species identification. Patients with active infection have immune responses that can be modulated by checkpoint inhibitor αPD1 which could be used as an adjunctive therapy to antibiotics. However, timing of this intervention is of paramount importance as patients who develop persistent infection appear to be refractory to this treatment.

The field of NTM infection and immunology is still in its infancy. There are many gaps to be filled. Key areas include: (i) T cell and B cell epitope identification; (ii) lung vs blood immune profile comparison; (iii) quantitative measurements of macrophage-T cell interactions ; (iv) RNAseq, proteomic and phosphor-proteomic approaches to decipher pan immune signatures and networks; (v) the development of humanized mouse models that can closely mimic the chronic phase of infection; (vi) multi-cohort studies with patients carefully stratified by ethnicity, risk factors, infecting organism, etc. and (vii) longitudinal follow-up studies with serial sampling.

In terms of T cell responses, immunogenic epitopes specific to each species need to be identified. The HLA restriction of these peptides must also be determined as it seems likely the $CD4^+$ T cells and $CD8^+$ T cells play different roles depending on the infection. Using surrogate markers for antigen-specific stimulation assumes that: (i) these markers are upregulated with all antigens; (ii) there is no abnormality in antigen-specific markers in any given individual, and (iii) they are upregulated for no other reason/condition that could occur during stimulation.

In the current study, it is possible that the CD40L-ve $CD4^+$ proliferating cells that were TNF^+ $IFN\gamma^+$ were the antigen-specific responsive cells in persistent infection, and the lack of CD40L is part of the pathology we are trying to decipher. Without mapped epitopes and MHC-peptide multimers, there is likely a great deal of data loss as limitations in the data included. The importance of $CD8^+$ T cell mediated immunity in NTM infection is almost certainly underestimated. Given the findings of this study of $CD8^+$ T cell exhaustion in MAC infection and $CD8^+$ T cell gene signature in active infection together with mounting evidence that $CD8^+$ immunity is likely to be essential for protection in TB, this area requires further work.

Comparison of lung vs blood immune profiles, cell functional capacity and antigen-specific cell repertoires is vital. Although there is documented evidence of trafficking of cells from the lung to blood and vice versa, the causes for a cell to leave the site of infection, or the reason a blood lymphocyte will home to the site of pathology, are not yet fully elucidated. Previous research has shown there is trafficking of lymphocytes from lung to blood. However, it is not known to what degree the lymphocyte phenotypic and functional signatures of lung vs blood overlap. This is an area of current research interest (273). The immune signature in the lung will inform on immunopathogenesis while the blood signature will be a better tool for development of diagnostic and prognostic tests. Research in both areas is necessary. Paired peripheral blood and bronchoalveolar lavage fluid samples taken at the same time would enable comparison of both lung macrophages and blood monocytes as well as lung and blood T cells. This is the first step to identifying which paths of investigation would be most appropriate for a given objective.

It is evident from current results and previous work that both macrophage mediated immunity and T cell responses are required for control of NTM infection. Once established, both innate and adaptive immunity are required for clearance of infection. Combined assays that evaluate macrophage mediated killing as well as killing when activated by antigen specific T cells would provide deeper insight into where dysfunction occurs. In patients with persistent infection , this would be a good place to start to evaluate immune function.

The role of other cell types, including NK cells, NKT cells, other ILCs, γδ T cells, macrophage subsets and dendritic cells need to be evaluated for a holistic picture of the immune landscape to be drawn. Omics approaches, including RNAseq and proteomic analysis of these cells after FACS sorting would provide these answers. The advantage of proteomics would be that given the low correlation between gene expression and protein expression, directly measuring

proteins would provide a more accurate molecular picture of disease. However, for diagnostics development, the Nanostring approach, particularly on whole blood samples would be of greater clinical utility.

Mouse models are a useful tool in deconstructing immunopathogenesis as a range of experimental tools can be used (i.e gene knock-in, knock-out mice etc). However, such results are limited by the fact that the mouse model of most diseases, including mycobacterial disease, does not reflect the chronic human disease. Pathology changes based on route of administration of pathogen, dose of pathogen etc. The chronic phase of infection cannot be replicated in mouse models as yet. Until a reliable mouse model that mimics human pathology is developed, mouse experiments tend to be of academic interest with little clinical translation.

Patient and control cohorts would need to be better stratified based on ethnicity, comorbidities and other risk factors that could affect the immune susceptibility to infection. A detailed multi cohort multi-centre study would be ideal to compare differences due to geography, ethnicity etc. Multi cohort studies would also enable external validation of findings such as the $CD4^+$ and $CD8^+$ T cell signatures identified in this study which is an essential next step. Longitudinal studies with serial sampling from patients are also necessary. Identifying the point at which active infection progresses to remission or deteriorates to persistence is of vital importance. If biomarkers of this transition can be identified, more aggressive treatments could be started earlier during patient management.

The ideal study would therefore be a multi-centre prospective cohort study where patients being investigated for NTM infection but found to be negative for disease (like patients with bronchiectasis and COPD) were prospectively recruited. Stratification into risk groups by history and clinical examination as well as genotyping would enable identification of patients in whom there is truly no known predisposition. Blood and BAL samples prior to treatment of patients with NTM-PD, and then follow-up samples during and after treatment would be beneficial. Samples would have to be stored in aliquots. The sequence of experiments would be determined by an initial broad RNAseq/ proteomic approach applied to sorted cells subsets, with focus on both T cells and macrophages. Using both live organisms to mimic real life infection, as well as purified peptide pools/ MHC-peptide multimers would be beneficial as the first shows what happens in the inflammatory milieu of infection, while the second shows specifically what antigen specific memory T cells are able to do. Based on current findings, T

cell gene expression can be used as a follow up lab test on serial samples to identify when disease pathology changes.

In conclusion, these studies have elucidated characteristics of NTM lung infection which showed signature immune checkpoint elevation in active disease. Evidence of a putative T cell gene signature for screening risk populations is presented and further work to validate this is currently being planned. Exploration of the potential of immune checkpoint modulation as an adjuvant to antibiotic therapy revealed the disease stage specific milieu of the cytokine response. This provides an excellent foundation for further work on deciphering immune dysfunction in specific stages of MAC and MABS infection in elderly and CF patients.

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Appendixes

Appendix 1- Publication from results chapter 3.

frontiers in Immunology

ORIGINAL RESEARCH
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Anomalies in T Cell Function Are Associated With Individuals at Risk of *Mycobacterium abscessus* Complex Infection

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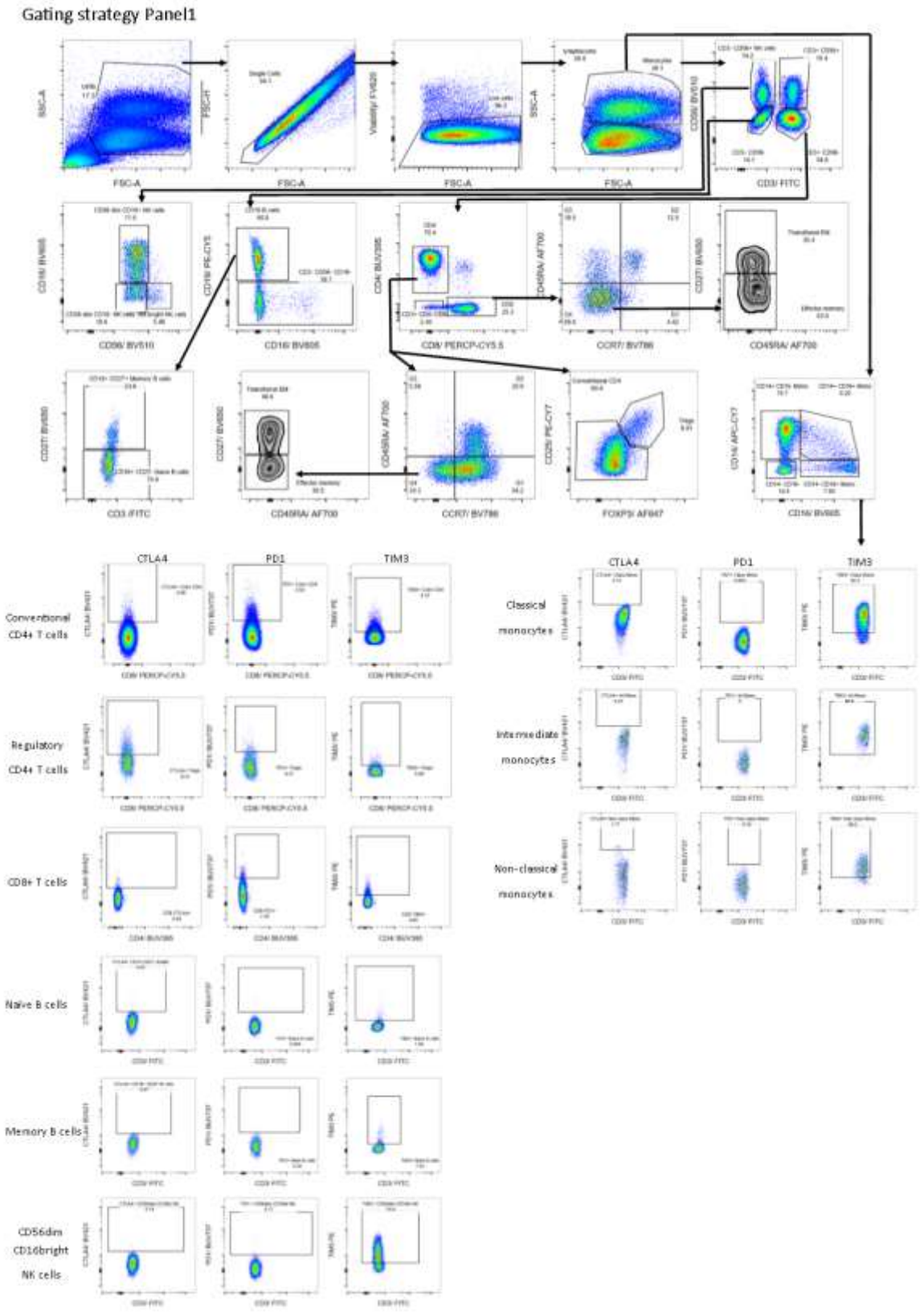
The increasing global incidence and prevalence of non-tuberculous mycobacteria (NTM) infection is of growing concern. New evidence of person-to-person transmission of multidrug-resistant NTM adds to the global concern. The reason why certain individuals are at risk of NTM infections is unknown. Using high definition flow cytometry, we studied the immune profiles of two groups that are at risk of *Mycobacterium abscessus* complex infection and matched controls. The first group was cystic fibrosis (CF) patients and the second group was elderly individuals. CF individuals with active *M. abscessus* complex infection or a history of *M. abscessus* complex infection exhibited a unique surface T cell phenotype with a marked global deficiency in TNFα production during mitogen stimulation. Importantly, immune-based signatures were identified that appeared to predict at baseline the subset of CF individuals who were at risk of *M. abscessus* complex infection. In contrast, elderly individuals with *M. abscessus* complex infection exhibited a separate T cell phenotype underlined by the presence of exhaustion markers and dysregulation in type 1 cytokine release during mitogen stimulation. Collectively, these data suggest an association between T cell signatures and individuals at risk of *M. abscessus* complex infection, however, validation of these immune anomalies as robust biomarkers will require analysis on larger patient cohorts.

Keywords: non-tuberculous mycobacteria, cystic fibrosis, immunoprofiling, pulmonary non-tuberculous mycobacteria infection, T cells

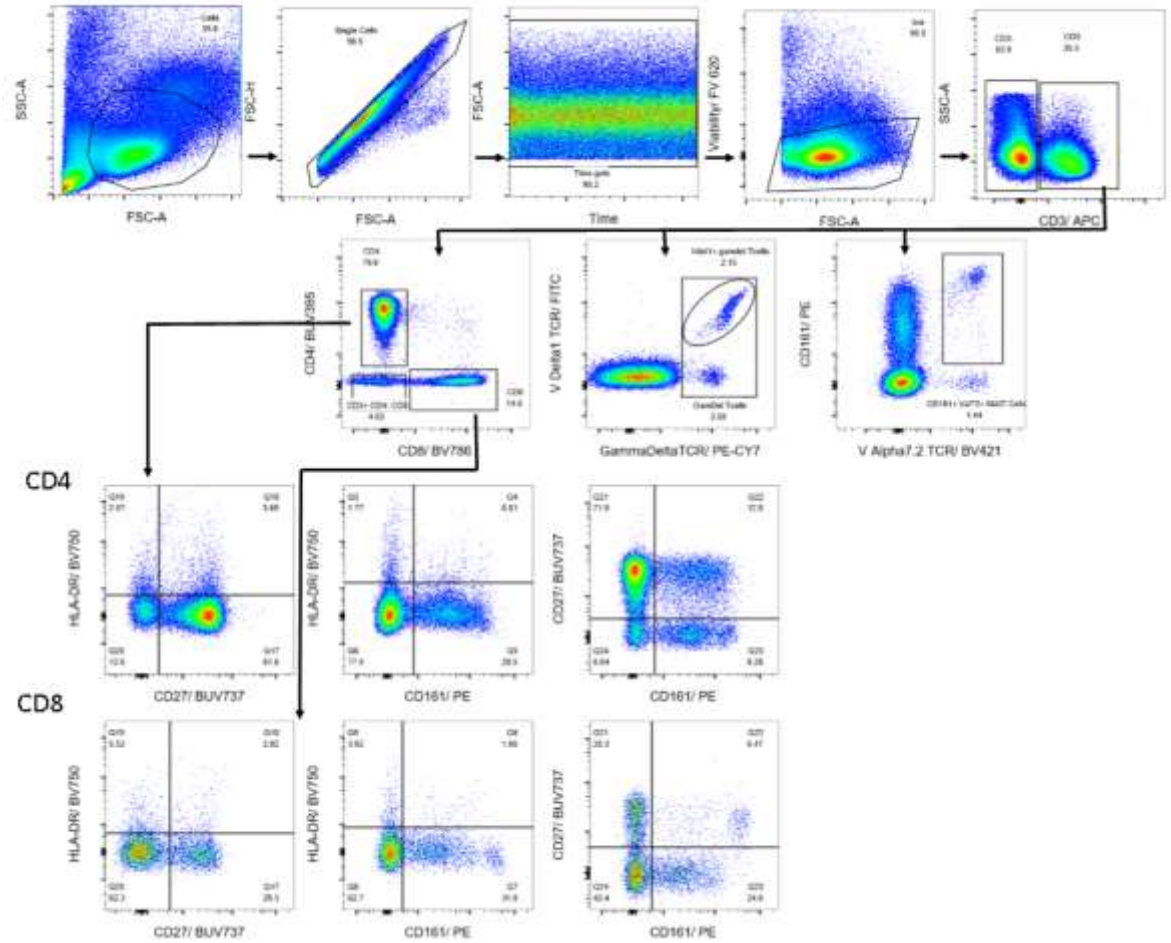
INTRODUCTION

Pulmonary infection caused by non-tuberculous mycobacteria (NTM) is an emerging threat with serious public health consequences. Mortality rates of 10–40% due to lung disease caused by these lesser known "cousins" of *Mycobacterium tuberculosis* (TB) have been increasingly reported in the developed world (1–4). The prolonged treatment regimens lasting months to years and increasing antibiotic resistance to front-line antibiotics make these pathogens difficult and expensive infections to treat. Over 180 species of NTM are known to cause disease in humans of which the *Mycobacterium*

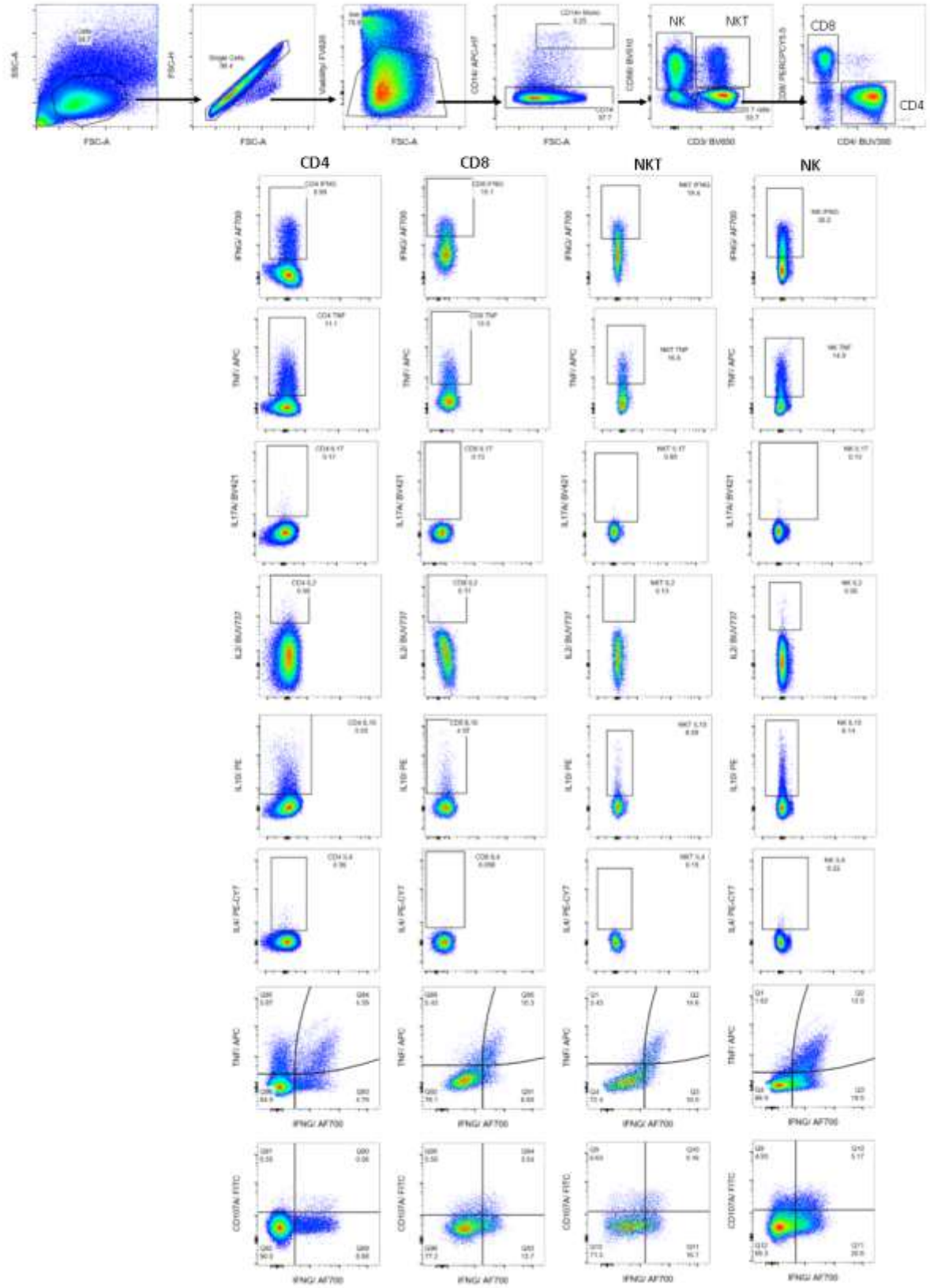
Appendix 2- Gating strategies for panel 1,2,3, PEx1 and 2, and stimulation panel



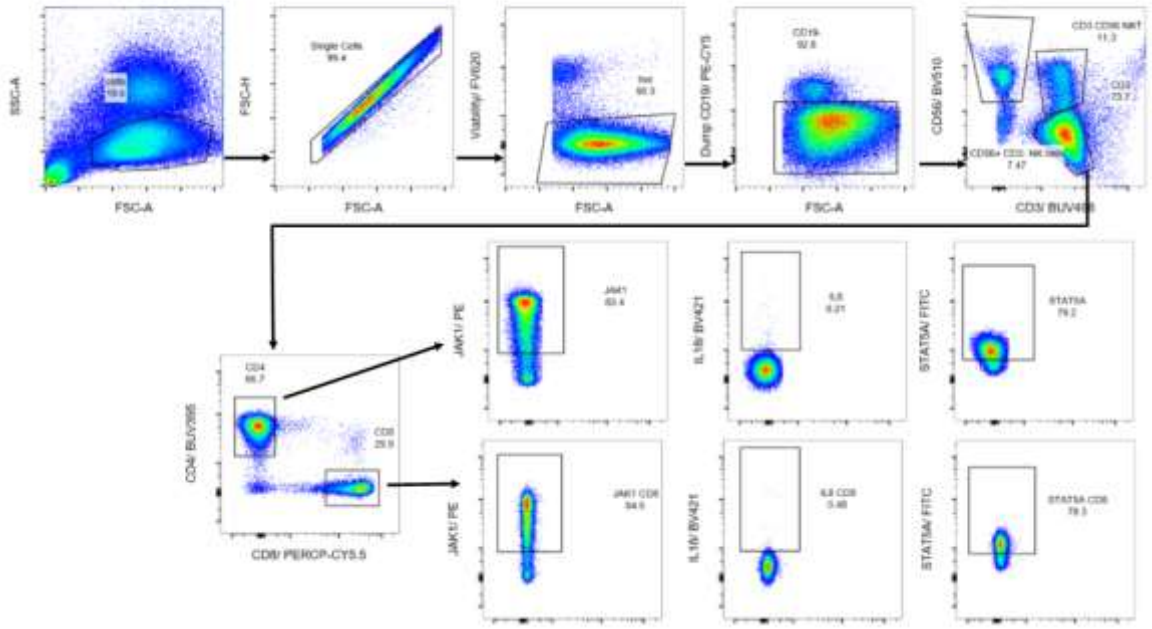
Gating strategy Panel2



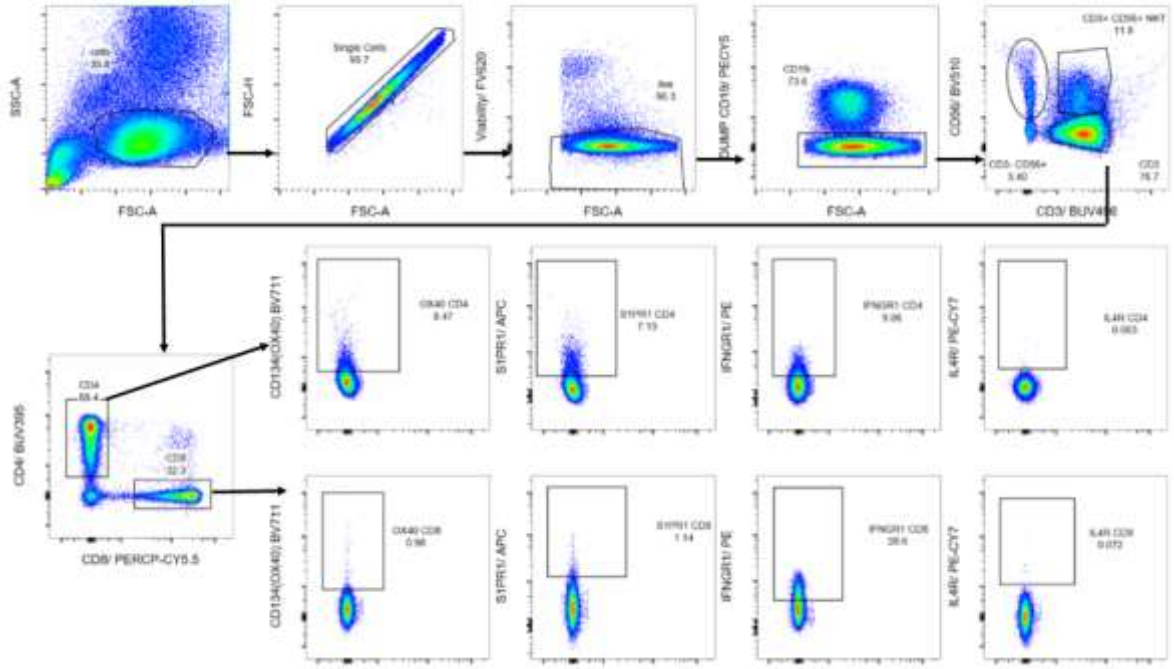
Gating strategy Panel3



Gating strategy PEX1



Gating strategy PEX2



Gating strategy Antigen stimulation panel