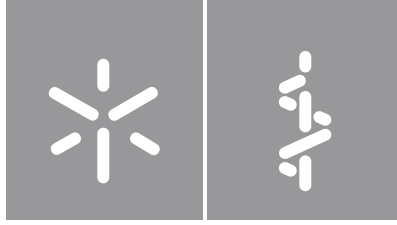Carolina Sousa Silva

The immune response to mycobacterial glycolipids in tuberculosis

outubro de 2022

Universidade do Minho
Escola de Medicina

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The immune response to mycobacterial glycolipids in tuberculosis

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Professor Doutor Christopher Sundling
e do
Professor Doutor João Carlos Sousa

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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TÍTULO: A RESPOSTA IMUNE A GLICOLÍPIDOS DE MICOBACTÉRIAS NA TUBERCULOSE

RESUMO

A tuberculose (TB) é uma doença infecciosa que continua sendo um importante problema de saúde pública, apesar de todos os esforços para eliminá-la, sendo a principal causa de morte por um único agente infeccioso após a COVID-19. Após exposição à bacteria *Mycobacterium tuberculosis* (Mtb), o sistema imunológico do hospedeiro pode eliminar a bactéria, controlar seu crescimento levando a tuberculose latente (TBL) ou não conter a infecção resultando em tuberculose ativa (TBA). Dado que apenas cerca de 5-10% dos indivíduos infectados latentemente irão progredir para TBA, a latência pode ser vista como um fenótipo na sua maioria protetor. Identificar os indivíduos com TBL que estão em risco de evoluir para ATB é de extrema importância na prevenção da TB. Os glicolipídios micobacterianos fosfatidilinositol manosídeo (PIM) e lipoarabinomanano (LAM) são abundantes na parede celular de Mtb e sabe-se que têm propriedades imunomoduladoras. No entanto, a resposta imune aos glicolipídios de Mtb em diferentes estágios da infecção por Mtb não tem sido muito explorada. Para resolver este problema, realizámos uma análise detalhada da resposta *in vitro* de células mononucleares do sangue periférico (PBMCs) de indivíduos com TBL e TBA para glicolipídios Mtb. Além disso, caracterizámos o perfil das PBMCs e avaliámos a função das células NK em indivíduos infectados com Mtb e comparámos com controles saudáveis.

Identificámos vários *clusters* de células mieloides, NK, B e T que responderam aos glicolipídios de Mtb produzindo diferentes padrões de citocinas. A resposta foi caracterizada por menor número de células mieloides produtoras de citocinas em indivíduos infectados por Mtb, mais proeminente em indivíduos com TBL em comparação com controlos saudáveis. Em células não estimuladas, as frequências de subpopulações de células NK foram semelhantes entre os grupos, mas uma investigação mais aprofundada da função destas células mostrou que indivíduos com TBL têm a função destas células comprometida, no que diz respeito à produção de citocinas, em comparação com indivíduos com TBA e/ou controlos saudáveis.

Estes resultados apontam para uma hiporresponsividade das células inatas em termos de produção de citocinas em indivíduos infectados latentemente com Mtb em resposta aos glicolipídios de Mtb. Uma investigação mais aprofundada dos mecanismos subjacentes a esta resposta pode fornecer informações sobre marcadores imunológicos da proteção natural contra a infecção por Mtb. A identificação de tais marcadores pode ajudar a melhorar testes de diagnóstico, estratégias de intervenção, terapia preventiva e investigação de novas vacinas.

Palavras-chave: glicolípidos de *Mycobacterium tuberculosis*; resposta imune; tuberculose (TB); tuberculose activa (TBA); tuberculose latente (TBL).

TITLE: THE IMMUNE RESPONSE TO MYCOBACTERIAL GLYCOLIPIDS IN TUBERCULOSIS

ABSTRACT

Tuberculosis (TB) is an infectious disease that remains a significant public health problem, despite all the efforts to eliminate it, being the leading cause of death from a single infectious agent after COVID-19. Upon exposure to *Mycobacterium tuberculosis* (Mtb) the host immune system might clear the bacteria, control its growth leading to latent tuberculosis (LTB), or fail to contain the infection resulting in active tuberculosis (ATB). Given that only about 5-10% of latently infected individuals will progress to ATB, latency can potentially be viewed as a generally protective phenotype. Identifying the individuals with LTB that are at risk of progressing to ATB is of great importance in TB prevention. The mycobacterial glycolipids phosphatidylinositol mannoside (PIM) and lipoarabinomannan (LAM) are abundant in the Mtb cell wall and are known to have immunomodulatory properties. However, the immune response to Mtb glycolipids in different stages of Mtb infection is poorly understood. To address this issue, we performed an in-depth analysis of the *in vitro* response of peripheral blood mononuclear cells (PBMCs) from individuals with LTB and ATB to Mtb glycolipids. Moreover, we profiled PBMCs and assessed NK cell function in individuals with LTB, and ATB and compared with healthy controls.

We identified various clusters of myeloid, NK, B, and T cells that responded to Mtb glycolipids by producing different patterns of cytokines. The response was characterised by lower numbers of cytokine-producing myeloid cells in Mtb-infected individuals, more prominent in individuals with LTB compared with healthy controls. In non-stimulated cells, frequencies of NK subsets were similar between groups, but further investigation of NK function showed that individuals with LTB had diminished NK cell function in terms of cytokine production, compared to individuals with ATB and/or healthy controls.

Our results point towards an innate immune hyporesponsiveness in terms of cytokine production in individuals latently infected with Mtb in response to Mtb glycolipids. Further investigation of the mechanisms underlying this response may provide insights into the immunological features of natural protection against infection by Mtb. Eventually, identifying markers of such protection may help improve diagnostic tests, intervention strategies, preventive therapy, and vaccine research.

Keywords: active tuberculosis (ATB); immune response; latent tuberculosis (LTB); *Mycobacterium tuberculosis* glycolipids; tuberculosis (TB).

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ABBREVIATIONS LIST

Greek letters/Units

α	Alpha
β	Beta
γ	Gamma
°C	Degree Celsius
%	Percentage
g/L	Grams per liter
ng	Nanograms
mL	Milliliter
mM	Millimolar
U	Units of penicillin
μg	Microgram
μL	Microliter
μM	Micromolar

A

Ac	Acylated
AGP	Arabinogalactan-peptidoglycan
ANOVA	Analysis of variance
APC	Antigen-presenting cell
AraLAM	Arabinose-capped lipoarabinomannan
Ara*p*	Arabinofuranose
ASC	Antibody-secreting cell
ATB	Active tuberculosis

B

B10	IL-10-producing B cells
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette–Guérin
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cell
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin

C

CD	Cluster of differentiation
CFP-10	Culture filtrate protein 10
cGAS	Cyclic gmp-amp synthetase
CLR	C-type lectin receptors
CM	Central memory
CMV	Cytomegalovirus
CRD	Carbohydrate-recognition domains
CXCL	Chemokine (C-X-C motif) ligand 1
CyTOF	Cytometry by time-of-flight

D

DC   Dendritic cell
DCAR   Dendritic cell immunoactivating receptor
DGS   *Direcção-geral da saúde* / Directorate-general of health
DMSO   Dimethyl sulfoxide
DN   Double negative
DNA   Deoxyribonucleic acid
DTH   Delayed-type hypersensitivity

E

ECDC   European centre for disease prevention and control
EDTA   Ethylenediaminetetraacetic acid
EM   Effector memory
ESAT-6   Early secreted antigenic target 6

F

FACS   Fluorescence-activated cell sorting
FBS   Fetal bovine serum
FCS   Flow cytometry standard
FSC   Forward scatter

G

GalCer   Galactosylceramide
GAMP   Guanosine monophosphate–adenosine monophosphate
GM-CSF   Granulocyte-macrophage colony-stimulating factor
GrzB   Granzyme B

H

HC   Healthy controls
HEPES   4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV   Human immunodeficiency virus

I

IFN   Interferon
Ig   Immunoglobulin
IGRA   Interferon-γ release assay
IL   Interleukin
iNKT   Invariant natural killer T

L

LAM   Lipoarabinomannan
LF-LM   Lateral flow urine lipoarabinomannan
LM   Lipomannan
LPS   Lipopolysaccharide
LTB   Latent tuberculosis

M

Mϕs   Macrophages

MAIT	Mucosal-associated invariant T
ManLAM	Mannose-capped lipoarabinomannan
Man*p*	Mannopyranose
MDR	Multi-drug resistant
MHC	Major histocompatibility complex
MR	Mannose receptor
MR1	MHC class I-related molecule
Mtb	*Mycobacterium tuberculosis*
MTC	*Mycobacterium tuberculosis* complex

N

NA	Not applicable
NAAT	Nucleic acid amplification test
NF	Nuclear transcription factor
NK	Natural killer
NKT	Natural killer T
NLR	NOD-like receptor
NOD	Nucleotide oligomerization domain
nSM	Non-switched memory
NTM	Non-tuberculous mycobacteria

P

PAMP	Pathogen-associated molecular patter
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PEA	Proximity extension assay
PHA	Phytohemagglutinin
PI	Phosphatidyl-*myo*-inositol
PIM	Phosphatidylinositol mannoside
PMA	Phorbol myristate acetate
PPD	Purified protein derivative
PRR	Pattern recognition receptor
P/S	Penicillin/streptomycin

S

SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SIGN	Specific intercellular adhesion molecule-3 grabbing nonintegrin
SM	Switched memory
SSC	Side scatter
STING	Stimulator of interferon genes

T

TB	Tuberculosis
TCR	T-cell receptor

TDM	Trehalose-dimycolate
TEMRA	Effector memory T cells re-expressing CD45RA
T$_H$	T helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TST	Tuberculin skin test
tSNE	t-stochastic neighbour embedding

W

| WHO | World health organization |

Z

| ZN | Ziehl-Neelsen |

FIGURES & TABLES LIST

CHAPTER I. INTRODUCTION

CHAPTER II. HIGH DIMENSIONAL IMMUNE PROFILING REVEALS DIFFERENT RESPONSE PATTERNS IN ACTIVE AND LATENT TUBERCULOSIS FOLLOWING STIMULATION WITH MYCOBACTERIAL GLYCOLIPIDS

CHAPTER III. IMMUNOPHENOTYPING AND FUNCTIONAL ANALYSIS OF PERIPHERAL IMMUNE CELL SUBSETS IN LATENT AND ACTIVE TUBERCULOSIS

THESIS LAYOUT

This thesis is organised in four chapters, the first one covering the state-of-the-art, two chapters containing the results and their discussion, and a fourth chapter addressing a general discussion, strengths and limitations, and future perspectives, as described below:

Chapter I reviews the state-of-the-art on tuberculosis and *Mycobacterium tuberculosis* (Mtb) glycolipids as well as the aims of this thesis. It provides an overview of the immune response to Mtb, and to Mtb glycolipids in particularly.

Chapter II presents a comprehensive characterisation of peripheral blood mononuclear cells of individuals infected with Mtb in response to the *in vitro* stimulation with mycobacterial PIM and LAM. The results presented in this chapter are part of an original research paper published in Frontiers in Immunology in 2021 (Silva, Carolina S. et al. 2021).

Chapter III focuses on the immunophenotyping and functional analysis of peripheral blood cells in latent and active TB.

Chapter IV covers a general discussion, giving an overview of the main findings in the state-of-the-art context. The strengths and limitations of this work as well as future perspectives are also briefly contemplated.

CHAPTER I

INTRODUCTION

1. TUBERCULOSIS – AN ANCIENT DISEASE IN THE 21ST CENTURY

Tuberculosis (TB), once known as the White Plague or the Consumption, is one of the oldest and deadliest diseases of humankind [Sabin et al., 2020; Zumla et al., 2009] and remains a significant public health problem in the twenty-first century, being the leading cause of death from a single infectious agent after COVID-19 [WHO, 2021a].

Since the declaration of TB as a global emergency by the World Health Organization (WHO) in 1993 [WHO, 1994], crucial efforts and progress have been made to control the disease. However, the target of the "End TB strategy" proposed in 2014, which aims to reduce TB incidence rate up to 90% and death up to 95% by 2035, is far from being achieved [WHO, 2014].

Since the end of 2019, the COVID-19 pandemic has reversed years of progress in reducing the global TB burden, leading to an estimated setback of 4 to 9 years [WHO, 2021a]. According to the WHO Global TB report 2021, in 2020 there was an 18% decline in TB case notifications compared to 2019, and for the first time in more than a decade, TB mortality has increased [WHO, 2021a]. Moreover, the TB incidence rate rose by 3.6% between 2020 and 2021, reversing declines of about 2% per year for most of the past two decades [WHO, 2022a]

Although TB can affect different organs, the most prevalent form of the disease affects the lungs [Sharma et al., 2021] and for that reason this work focus only on pulmonary TB. Thus, in the context of this thesis, only pulmonary TB will be addressed unless otherwise mentioned.

1.1. AETIOLOGY

On 24 March 1882, Robert Koch announced the discovery of the tubercle bacillus, *Mycobacterium tuberculosis* (Mtb), as the causal pathogen of TB, proving that the plague that had been devastating Europe was caused by an infectious agent [Koch, 1982]. Such revolutionary discovery earned him the title of father of the scientific study of TB and the Nobel Prize in Physiology or Medicine in 1905 [Sakula, 1983]. Although the primary aetiological agent of TB in humans is Mtb, there are other bacteria from the Mtb complex (MTC) that can cause human disease, such as *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium canettii*, and *Mycobacterium microti* [Kanabalan et al., 2021].

One of the key features behind Mtb success as a pathogen is the dynamic and complex cell envelope, which is roughly composed of three main layers (Figure 1): (i) the cytoplasmatic membrane, (ii) a cell wall consisting of the arabinogalactan-peptidoglycan (AGP) complex and the mycomembrane, and (iii) the capsule [Dulberger et al., 2020; Kalscheuer et al., 2019; Queiroz and Riley, 2017; Stokas et al., 2020].

The structure of the Mtb cell envelope, with emphasis on mycobacterial glycolipids, will be further reviewed in section 2 of this Chapter.
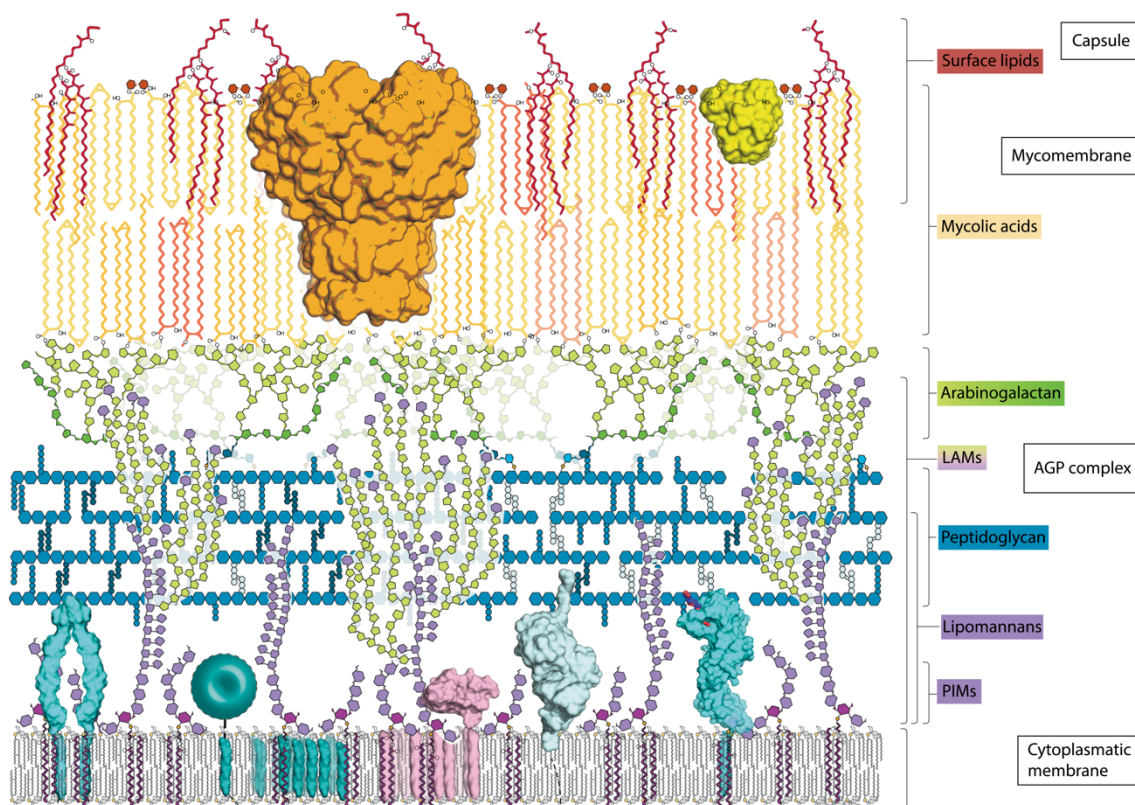
Figure 1. Model of the mycobacterial cell envelope. The cell envelope is composed of three main layers: (i) the cytoplasmatic membrane, (ii) a cell wall consisting of the arabinogalactan-peptidoglycan complex (AGP) and the mycomembrane, and (iii) the capsule. The cell envelope is populated by protein, glycolipids, and peptidoglycan that build and modulate the cell envelope. Adapted from [Dulberger et al., 2020].

1.2. EPIDEMIOLOGY

Worldwide

Tuberculosis is a communicable disease that with the proper treatment is almost always curable [Floyd et al., 2018]. Nevertheless, according to the latest WHO Global TB report, TB remains one of the leading causes of death worldwide and is the second cause of death by a single infectious agent [WHO, 2022a]. Furthermore, it is estimated that about one-quarter of the global population is or has been infected with Mtb [Houben and Dodd, 2016].

The COVID-19 pandemic, declared by the WHO on March 11, 2020, had devastating effects beyond the deaths caused directly by the virus SARS-CoV-2 (WHO, 2021b). Several TB essential health services have been disrupted worldwide, resulting in an unprecedented setback in global notification and mortality rates [WHO, 2021a]. In 2020, 5.8 million newly diagnosed TB cases were reported (a decrease of 18%

compared to 2019) falling far short of the estimated 10.6 million people who fell ill in 2020 [WHO, 2021a, 2022a]. Although in 2021 there was a partially recovery to 6.4 million [WHO, 2022a], the reduction in the reported number of people diagnosed with TB in these two years will likely result in an increased transmission of infection and number of deaths.

Another devastating consequence of the reduced access to health care services due to the ongoing pandemic was an increase in the TB mortality rate (1.3 million people died from TB in 2020, and 1.6 million in 2021), returning to the levels of 2017 [WHO, 2021a, 2022a]. It is the first time since 2005 that TB mortality rate increases.

Portugal

In Portugal, both TB notification and incidence rates have decreased considerably since 2000; in 2015, Portugal reached the threshold of less than 20 cases per 100 000 individuals per year, thus being considered a low incidence country [DGS, 2021]. The latest report by the Portuguese Directorate-General of Health shows that both notification and incidence decreased in 2020, compared to the previous year. The number of TB notifications decreased from 1848 to 1465, corresponding to a notification rate of 14.2 cases per 100 000 individuals, while the incidence rate went from 18 to 13.2 per 100 000 individuals [DGS, 2021]. However, despite the decrease in the incidence rate, Portugal is still the Western European country with the highest TB incidence rate [ECDC/WHO Regional Office for Europe, 2022].

The TB mortality rate increased by about 1% per year between 2011 and 2020, with 2.4 cases per 100 000 individuals in 2020 [ECDC/WHO Regional Office for Europe, 2022]. Moreover, in 2020 there was a median delay in diagnosis of 80 days, an increase of about 24% compared to 2008 (61 days). This will likely result in higher transmission, and, as expected by WHO, both notification and incidence rates will likely increase in subsequent years [DGS, 2021; WHO, 2021a].

Sweden

Also in Sweden both notification and incidence rates have been decreasing in the last decades [Public Health Agency of Sweden, 2021]. In 2020, the incidence rate was 3.6 per 100 000 individuals and 361 TB cases were notified, corresponding to a notification rate of 3.1 per 100 000 individuals [ECDC/WHO Regional Office for Europe, 2022]. However, in 2021, 365 TB cases were notified (incidence rate of 3.5 per 100 000 individuals) [Public Health Agency of Sweden, 2021], corresponding to a slight increase compared to the previous year. This is in line with the WHO forecasting of increase in TB cases due to underreported notification during 2020 [WHO, 2021a]. Regarding TB mortality rate, Sweden registered a

8.7% decrease between 2011 and 2020, with 0.2 deaths per 100 000 individuals in 2020 [ECDC/WHO Regional Office for Europe, 2022].

1.3. THE SPECTRUM OF TUBERCULOSIS

Tuberculosis is an airborne infectious disease transmitted when Mtb-containing aerosols expelled by an infected individual are inhaled by another individual [Dheda et al., 2016]. For convenience in clinical and public health practice, the infection by Mtb is commonly referred to as either active TB (ATB) or latent TB (LTB) based on the presence or absence of clinical symptoms, respectively [Lenzini et al., 1977; D. B. Young et al., 2009]. However, it is recognised and accepted by many researchers that Mtb infection can lead to diverse outcomes and thus could be better viewed as a dynamic spectrum, ranging from pathogen elimination to clinical manifestation of the disease (Figure 2) [Furin et al., 2019]. The outcome of infection depends essentially on the ability of the host's innate and adaptive immune systems to clear the bacterium or control Mtb replication and prevent disease establishment [Walzl et al., 2011].
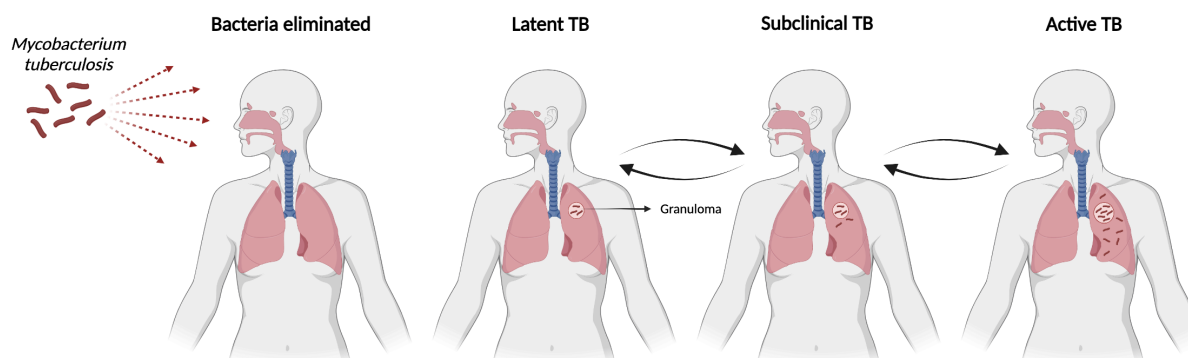
Figure 2. Tuberculosis spectrum. Upon exposure to *Mycobacterium tuberculosis* (Mtb), individuals might eliminate the bacteria, either through innate immune responses or acquire immune responses without T cell priming. Some individuals will eliminate the bacteria or control their growth and develop and retain T cell memory to Mtb antigens (latent TB). If the immune system fails in controlling bacterial growth, some individuals will be culture-positive but without clinical manifestations (subclinical TB), and others will develop symptoms such as cough, fever, and weight loss thus evolving to active TB (ATB). ATB diagnosis must be confirmed by sputum smear, culture and/or molecular tests. Created with BioRender.com

Bacteria elimination

Not all individuals who are exposed to Mtb (e.g., close contacts of individuals with ATB) develop the disease or show evidence of an acquired response to Mtb antigens [Anibarro et al., 2011; Morrison et al., 2008; Zellweger et al., 2015]. This suggests that some individuals may eliminate the bacteria through the innate immune system without T cell priming [Walzl et al., 2011]. If the innate immune response is

not sufficient, the pathogen may be eliminated with the help of the adaptive immune system and the individuals will develop immunological memory to Mtb antigens [Behr et al., 2019].

Latent tuberculosis

When both the innate and adaptive immune systems fail to clear the bacteria, it can persist in a dormant or quiescent state [Dutta and Karakousis, 2014], and these individuals will also show T cell-dependent responses to Mtb antigens [Furin et al., 2019; O'Garra et al., 2013]. According to WHO, LTB is defined as "a state of persistent immune response to stimulation by Mtb antigens with no evidence of clinically manifested active TB" [WHO, 2018]. Thus, the current definition of LTB infection includes both individuals who have eliminated the infection and those who harbour viable bacteria. This heterogeneity within the latently infected individuals happens because the existing diagnostic tests (further discussed in the following section) only allow to detect the host's adaptive immune memory to Mtb antigens, regardless of whether the bacteria have been eliminated or not [Ho et al., 2022; Walzl et al., 2018]

Subclinical and active TB disease

If both the innate and adaptive immune system fail in eliminating the bacteria, individuals will develop TB [Pai et al., 2016]. It is estimated that about 10% of the individuals with acquired immune memory to Mtb antigens will progress to disease [Behr et al., 2019]. Some of these individuals might not develop symptoms and remain in a subclinical TB status. In this case, viable bacteria can be detected by culture-based methods or may cause abnormalities observable by chest radiography [Drain et al., 2018]. Active TB disease implies the development of clinical symptoms including cough, fever, fatigue, night sweats and weight loss [Pai et al., 2016]. The diagnosis of TB disease, either subclinical or active, must always be confirmed by microbiologic detection of the bacilli [WHO, 2021a].

1.4. DIAGNOSIS

The diagnosis of TB is based on clinical and epidemiological data combined with laboratory techniques. The choice of the method/combination of methods depends on whether there is suspicion of ATB due to TB-related symptoms or if a person has had contact with someone infected, but does not develop symptoms [Pai et al., 2016]. As mentioned before, TB diagnosis must be confirmed by microbiological methods, namely, culture-based techniques, sputum smear microscopy, or nucleic acid amplification tests (NAATs) [WHO, 2021a]. The gold standard method to detect the presence of Mtb continues to be

the bacteriological culture of sputum specimens [ECDC, 2018]. The main advantages of this method are its high sensitivity and specificity [ECDC, 2018]. However, culture-based techniques can take up to 8 weeks, delaying the initiation of the treatment [ECDC, 2018]. Conventional sputum smear microscopy is a rapid and cheap method and the most widely used diagnostic method in high-burden and low/middle-income countries [Dheda et al., 2016; Pai et al., 2016]. It relies on the visualisation of the bacilli following Ziehl-Neelsen (ZN) staining, a technique used to detect acid-fast bacteria [Lawn and Zumla, 2011]. Thus, this method does not differentiate Mtb from other acid-fast bacteria. Moreover, the detection of infection with this technique requires a much higher concentration of bacilli (at least 5 000 bacilli/mL of sputum) compared to culture-based techniques [ECDC, 2018], and presents a limited and very variable sensitivity [Steingart et al., 2006]. In addition to conventional microscopy, Mtb can also be visualised through fluorescence microscopy, using fluorochrome staining (auramine or auramine-rhodamine) [ECDC, 2018]. The only rapid diagnostic tests with high sensitivity and specificity are NAATs (either based on polymerase chain reaction or loop-mediated isothermal amplification) [ECDC, 2018]. Diagnostic tests based on NAAT are used to detect a particular nucleic acid sequence of Mtb and additionally allow the detection of mutations associated with resistance to anti-TB drugs [ECDC, 2018]. Currently, the NAATs endorsed by WHO allow the detection of resistance to first and second-line antibiotics, contributing to ATB diagnosed people starting the most effective treatment regimen as early as possible [WHO, 2021c]. WHO recommends the use of a range of NAATs as the first-line diagnostic test in all people with suspected ATB [WHO, 2021c], but the use of these tests remains far too low, and it was only used in 33% of the people diagnosed with ATB in 2020 [WHO, 2021a]. This percentage is considerably lower in low- and middle-income countries [WHO, 2021a], which happens, in part, due to the costs, human resources, and infrastructure requirements for NAAT implementation [S. Brown et al., 2021; Nalugwa et al., 2020]. In 2015, WHO endorsed a lateral flow urine lipoarabinomannan assay to assist in ATB diagnosis for HIV-positive individuals [WHO, 2015]. Contrary to the previously described techniques that directly detect the bacteria or bacterial DNA, this method relies on the detection of lipoarabinomannan (LAM), a major component of the Mtb cell wall, in the urine of infected individuals. Detection of LAM in urine is a desirable diagnostic method as it is fast, can be used as a point-of-care test, and does not require expensive laboratory equipment or trained staff [Flores et al., 2021; WHO, 2015]. However, the method has very low sensitivity (44%) and is only recommended to be used in people with HIV infection with very low CD4^{+} T cell counts (less than or equal to 100 cells/μL), irrespective of signs and symptoms of TB, or people who are symptomatic or seriously ill (regardless of their CD4^{+} T cell counts) [WHO, 2019].

Contrary to ATB, there is no gold standard diagnostic test for LTB, and WHO guidelines on LTB management consider the risk group, the epidemiology and burden of TB, the availability of resources and the probability of a broad public health impact [WHO, 2018]. The methods to diagnose LTB rely on indirect approaches by evaluating the host acquired immune response to Mtb [Ho et al., 2022; Walzl et al., 2018]. For nearly one century, the only available method to diagnose LTB was the tuberculin skin test (TST), in which tuberculin or purified protein derivative (PPD) is injected intradermally, and the delayed-type hypersensitivity (DTH) response is evaluated 48-72 h post-injection by measuring the diameter of the induration at the site of injection [Yang et al., 2012]. PPD is obtained from Mtb culture filtrate and consists of an extract of Mtb proteins (>90%) and other molecules (polysaccharides and nucleic acids) [Seibert and Glenn, 1941]. However, the TST may lead to false-positive results in those who have received BCG vaccination, especially if BCG was administrated less than 10 years before testing, or in those that have been exposed to non-tuberculous mycobacteria (NTM) [Farhat et al., 2006]. Moreover, TST also has poor sensitivity in immunocompromised individuals [Pai et al., 2014]. In the early 2000s, a new test to detect the host acquired immune response to Mtb was developed, the interferon-γ (IFN-γ) release assay (IGRA). IGRAs are blood tests that quantify IFN-γ produced by T cells *in vitro* in response to the MTC antigens, early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) [Pai et al., 2014]. The IGRAs have higher specificity than TST as the two antigens used are not encoded by BCG vaccine strains nor most NTM species [Andersen et al., 2000]. However, they require a laboratory setting and are more costly than TST [WHO, 2022c]. Although both TST and IGRAs have been crucial in the detection and management of LTB [WHO, 2018], neither of them allows the discrimination of individuals that have successfully eliminated the bacteria but are still immunoreactive [Esmail et al., 2014]. Additionally, neither TST nor IGRAs provide a high predictive (prognostic) value for ATB [Auguste et al., 2017; Rangaka et al., 2012; Zhou et al., 2020].

To address these problems, new Mtb antigen-based skin tests (TBST) have been developed. Like TST, TBST consist of an intradermal injection of antigens and DTH evaluation, but instead of PPD, the tests rely on Mtb-specific antigens i.e., ESAT-6, CFP-10, and DPPD, the latter being encoded by a gene that is unique to the MTC and is absent in other members of the *Mycobacterium* genus [Badaro et al., 2020; Campos-Neto et al., 2001; F. Li et al., 2016; Ruhwald et al., 2017]. These new tests have a similar specificity to that of the IGRAs, but with the simplicity of the TST. Thus, they might be a valuable alternative in populations with a high BCG vaccination coverage and in settings where TSTs are commonly used [Krutikov et al., 2022; Ruhwald et al., 2017; WHO, 2022b]. Currently, the methods used to identify Mtb-reactive individuals (including individuals with LTB) have several limitations and do not allow prediction

of those who will progress to active disease [Auguste et al., 2017; Rangaka et al., 2012; Zhou et al., 2020], and that would benefit from preventive therapy [Shah and Dorman, 2021].

1.5. THE HOST IMMUNE RESPONSE TO *Mycobacterium tuberculosis*

When Mtb enters the lungs via inhalation, it is first recognised and/or phagocytised by cells of the innate immune system, comprising neutrophils, monocytes/macrophages, dendritic cells (DCs), natural killer (NK), and innate-like T cells [Ravesloot-Chavez et al., 2021]. Innate cells sense bacteria through pattern recognition receptors (PRRs), which trigger intracellular signalling cascades that ultimately lead to the expression and secretion of diverse pro-inflammatory molecules such as TNF, IL-6, IL-12, IL-1α and IL-1β. These pro-inflammatory cytokines have crucial roles in controlling the infection progression, such as the recruitment of other immune cells to the site of infection [Guilliams et al., 2013; Torrado and Cooper, 2013], and contribute to subsequent activation and shaping of the adaptive immune response [O'Garra et al., 2013]. Additionally, it has been described that memory-like innate immune responses, either trained immunity or tolerance, may have an important role in TB [Divangahi, 2018].

If the innate line of defence fails, Mtb bacilli invade the lung interstitial tissue, either by direct infection of epithelial cells or by migration of infected alveolar macrophages to the lung parenchyma [Pai et al., 2016]. Once the primary infection is established, both infected DCs and monocytes migrate to pulmonary lymph nodes where they promote T cell activation. This results in the recruitment of T and B cells to the site of infection, which might lead to the elimination of Mtb-infected cells and consequent resolution of the infection or to the formation of a granuloma [Philips and Ernst, 2012].

The granulomas developed in primary Mtb infection have a core of infected neutrophils and foamy macrophages surrounded by lymphocytes (mainly T cells) and associated tertiary lymphoid structures, which serve as sites of local antibody production and facilitate antigen presentation to T cells [Cohen et al., 2022]. If the granuloma does not contain the infection, the bacteria will eventually disseminate to the surrounding tissues, giving origin to other granulomas, to the bloodstream, and to other organs or re-enter the respiratory tract to be released into the environment via aerosols [Lin et al., 2013; Pai et al., 2016].

Despite the irrefutable role of innate immunity in the initial control and in the progress of the infection, the hallmark of protection in TB has been attributed to the adaptive branch of the immune system, namely, the production of IFN-γ and TNF by CD4+ T cells [Cooper, 2009]. However, it is clear that TB protective immunity goes much beyond CD4+ T cell-mediated responses [Nunes-Alves et al., 2014].

Although less studied compared to T cells, there is evidence that B cells and antibodies also take part and contribute to a protective immune response against Mtb [Rijnink et al., 2021].

1.5.1. *Mycobacterium tuberculosis* RECOGNITION

The innate immune cells are activated when PRRs recognize pathogen-associated molecular patterns (PAMPs), molecules conserved among a broad group of microorganisms. Mtb is recognised by several families of PPRs, such as toll-like receptors (TLR), nucleotide oligomerisation domain-like receptors (NLR), C-type lectin receptors (CLR), and cyclic GAMP synthase (cGAS)/stimulator of interferon genes (STING) [Ishikawa et al., 2017; Kleinnijenhuis et al., 2011].

TLRs are a family of 12 receptors that localise either to the membrane of cells or of intracellular organelles of mainly immune cells and recognise distinct or overlapping PAMPs such as lipids, proteins and nucleic acids [Mogensen, 2009]. Engagement of TLRs by mycobacterial PAMPs triggers, among others, the activation of a MyD88-dependent signalling cascade that ultimately leads to the activation of the nuclear transcription factor (NF)-κB, resulting in the expression of pro-inflammatory cytokines, such as IL-12, TNF, and IFN-γ [Kleinnijenhuis et al., 2011], that play a fundamental role in bacterial control [Torrado and Cooper, 2013]. TLR2 forms heterodimers with either TLR1 or TLR6 and binds to lipoproteins and cell envelope glycolipids, including lipomannan (LM), LAM, and phosphatidylinositol mannoside (PIM) [Kleinnijenhuis et al., 2011]; TLR4 recognises tri and tetra-acylated LMs [Doz et al., 2007]; TLR9 recognises unmethylated CpG motifs from Mtb DNA [Bafica et al., 2005]. Experimental studies using mice have identified TLR2, TLR4 and TLR9 as the more relevant TLRs for recognition of Mtb and control of the infection [Kleinnijenhuis et al., 2011]. TLR2 has been shown to have an important role in IL-1β production upon infection with Mtb [Kleinnijenhuis et al., 2009]. In contrast, the abrogation of TLR4 expression did not result in increased susceptibility to Mtb infection [Reiling et al., 2002]. However, TLR4 seems important to mount a protective response to Mtb by contributing to macrophage recruitment and production of pro-inflammatory cytokines [Abel et al., 2002]. The abolition of the expression of TLR9 leads to impaired IL-12 and IFN-γ responses upon infection with Mtb and contributes to host resistance to the bacteria [Bafica et al., 2005]. Mice lacking MyD88 have impaired IL-12 and IFN-γ responses leading to increased susceptibility to mycobacterial infection when compared to both TLR2 and TLR4 knockout mice [Feng et al., 2003], showing that resistance to infection is regulated by several MyD88-dependent signalling pathways. In humans, the importance of TLR signalling has been supported by different studies

showing that polymorphisms in various TLRs are associated with both susceptibility and protection in TB [Varshney et al., 2022].

NOD-like receptors are cytosolic PRRs [Zhong et al., 2013] that recognise bacterial molecules derived from the synthesis and degradation of peptidoglycan [Mogensen, 2009]. NLRPs are a subfamily of NLRs containing a pyrin domain that recruit the inflammatory caspases and the adaptor protein ASC, forming a protein complex known as the inflammasome [Zhong et al., 2013]. *In vitro* studies using murine macrophage cell lines have shown that Mtb infection inhibits inflammasome activation and subsequent IL-1β production [Master et al., 2008], a crucial cytokine for Mtb infection control [Torrado and Cooper, 2013]. Others observed that Mtb activates the NLRP3 inflammasome in bone marrow-derived macrophages (BMDMs) and that NLRP3 knockout mice control the infection through NLRP3-independent production of IL-1β [Dorhoi et al., 2012]. NOD-2 also belongs to the NLR family and contributes to IL-1β production *in vitro* [Kleinnijenhuis et al., 2009]. NOD-2 deficient mice have impaired inflammatory responses but show variable susceptibility to infection with Mtb [Divangahi et al., 2008; Gandotra et al., 2007]. In humans, the NOD-2 Arg702Trp polymorphism is associated with decreased risk of TB [Wang et al., 2014].

CLRs are a family of PRRs characterised by the presence of one or more carbohydrate-recognition domains (CRDs) that bind to carbohydrate molecules in a Ca^{2+}-dependent manner. Just like TLR signalling, CLR activation leads to pro-inflammatory cytokine expression mainly via NF-κB [Goyal et al., 2016]. The CLR family includes DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN), DC immunoactivating receptor (DCAR), dectin-1, dectin-2, macrophage-inducible C-type lectin (mincle) and mannose receptor (MR) [Ishikawa et al., 2017]. DC-SIGN is mainly expressed on DCs and recognises LAMs [Tallieux et al., 2003] and PIMs [Driessen et al., 2009; Torrelles et al., 2006], but the deletion of the DC-SIGN homolog gene SIGNR1 in mice does not affect the course of Mtb infection compared to wildtype mice [Court et al., 2010]. DCAR is predominantly expressed in peritoneal macrophages and monocyte-derived inflammatory cells in the lungs and spleen and recognises tri- and tetra-acylated PIMs [Toyonaga et al., 2016]. Dectin-1 is expressed on monocytes/macrophages and neutrophils [Taylor et al., 2002]. The receptor recognises β-glucans and is mainly involved in innate immune responses to fungal pathogens [G. D. Brown, 2005]. *In vitro* studies showed that dectin-1 signalling is involved in pro-inflammatory responses following Mtb infection [Dorhoi et al., 2010; Marakalala et al., 2011], but its role *in vivo* remains unclear as it does not seem to affect host resistance to infection [Marakalala et al., 2011].

Dectin-2 is present on DCs and macrophages and it was shown to recognise LAM *in vitro* [Ishikawa et al., 2017], leading to the production of anti and pro-inflammatory cytokines by bone marrow-derived DCs (BMDCs) and amplified antigen-specific T cell responses. Contrary to dectin-1, mice lacking the dectin-2 gene *Clec4n* have increased lung pathology following mycobacterial infection [Yonekawa et al., 2014]. Mincle is a receptor for the mycobacterial cell wall glycolipid trehalose-dimycolate (TDM) [Matsunaga and Moody, 2009]. In mice, purified TDM induces an inflammatory response and granuloma formation that is prevented in mincle knockout mice [Ishikawa et al., 2009]. However, the same was not observed when mincle-deficient mice were infected with Mtb H37Rv [Heitmann et al., 2013]. Thus, mincle abolition seems to be compensated by other PRRs and does not impact the infection outcome. The MR is expressed in some populations of macrophages and DCs that bind sulfated and mannosylated carbohydrates, such as LAM [Martinez-Pomares, 2012]. In mice, deletion of the expression of MR receptor does not impair resistance to Mtb infection [Court et al., 2010]. In monocyte-derived human macrophages, binding of the MR by LAM limits phagosome-lysosome fusion, thus enhancing macrophage survival [Kang et al., 2005], and a higher concentration of soluble MR (sCD206) in serum is associated with reduced survival [Suzuki et al., 2018].

cGAS is a cytosolic DNA sensor that recognises Mtb DNA and activates the STING pathway, resulting in type I IFN production [Watson et al., 2015], a family of cytokines that correlate with disease severity [Berry et al., 2010; Moreira-Teixeira et al., 2018]. However, others showed that cyclic-di-adenosine monophosphate, produced by the bacteria, activates STING in a cGAS-independent manner [Dey et al., 2015].

Thus, various PRRs belonging to diverse families recognise different components of the Mtb cell wall, triggering distinct signalling pathways. While some PRRs, such as TLRs, are crucial in mounting a protective immune response to Mtb infection that results in host survival, others seem to have redundant functions [Ravesloot-Chavez et al., 2021].

1.5.2. RECOGNITION OF *Mycobacterium tuberculosis*-INFECTED CELLS

Although T lymphocytes can recognise soluble antigens through TLRs engagement, T cell activation is mainly mediated by the T cell receptor (TCR) [Hwang et al., 2020; M. H. Young et al., 2016]. Once Mtb is phagocyted by antigen-presenting cells (e.g., macrophages and DCs) in the lung, these cells migrate to the lymph nodes, and present Mtb antigens to T cells [Philips and Ernst, 2012]. Classical T cells i.e.,

T cells that express αβ TCR, recognise peptide antigens that are presented by classical major histocompatibility complex (MHC) molecules, either MHC class I or MHC class II [Goadsby et al., 2016]. Apart from the classical peptide presentation, unconventional T cells can be primed by lipid antigens through the MHC class I-like molecules, such as CD1 and MHC class I-related molecule (MR1) glycoproteins [Huang, 2016]. CD1 family comprises different isoforms that can be classified into three groups: group 1 comprises CD1a, CD1b and CD1c; group 2 comprises CD1d; and group 3 comprises CD1e [Barral and Brenner, 2007].

The unconventional T cells include CD8$^+$ mucosal-associated invariant T (MAIT) cells that are MR1-restricted, invariant natural killer T (iNKT) cells [Huang, 2016] that recognise glycolipids through group 2 CD1 and have an invariant Vα24Jα18/Vβ11 TCR [Brennan et al., 2013], and γδ T cells that recognise CD1b, CD1c, CD1d and MR1 [Van Rhijn and Le Nours, 2021].

1.5.3. A VIEW FROM THE BLOOD

Due to the difficulty in accessing and obtaining biological samples from the site of infection (e.g., lung), most of the studies to understand the immune response to Mtb in humans have mainly resorted to blood and, to a lesser extent, bronchoalveolar lavage (BAL) fluid samples. Blood samples are easily obtained and contains millions of immune cells and molecules circulating to and from the infection site, providing valuable information to understand the host response to Mtb and the different clinical outcomes, as well as to predict the risk of progression to ATB [Walzl et al., 2018]. Here I review the main recent findings on the characterisation of the main peripheral immune cell subsets altered in individuals infected with Mtb, with a particular emphasis on the differences between active and latent TB.

Neutrophils are the most abundant immune cells in the blood and they rapidly enter tissues upon injury or infection [de Oliveira et al., 2016]. A study assessing the potential role of neutrophils in host resistance/tolerance to Mtb showed that the risk of development of ATB in contacts of newly diagnosed individuals was inversely and independently associated with peripheral neutrophil counts. In addition, in neutrophil-depleted whole blood, the release of microbial peptides in response to Mtb stimulation is reduced, as well as the capacity to limit Mtb growth *in vitro* [Martineau et al., 2007]. This suggests that neutrophils produce molecules that inhibit Mtb survival and are important in protection from disease. Berry et al. analysed the transcriptional profile of whole blood from individuals with ATB and found a distinct signature characterised by neutrophil-dependent IFN-γ and type I IFN signalling that distinguishes between ATB and other inflammatory diseases [Berry et al., 2010].

Monocytes play a dual role in the innate immune responses. On one hand, they are recruited to the lung and participate in the control of the infection, and on the other hand they have the ability to differentiate into either macrophages or DCs, contributing to bridge innate and adaptive immunity [Sampath et al., 2018]. Studies of the monocyte subsets in the peripheral blood showed that patients with ATB had increased frequencies of intermediate (CD14+CD16+) and non-classical (CD14+CD16++) monocytes [Castaño et al., 2011; Sánchez et al., 2006] that normalised after completing anti-TB treatment [Sánchez et al., 2006]. However, this was only partially confirmed by Dirix et al., that observed higher proportions of classical (CD14+CD16-) and intermediate monocytes in the blood of ATB patients compared to LTB and healthy controls (HC) [Dirix et al., 2018]. In contrast, another study reported that TST+ individuals have higher intermediate monocytes frequencies than both ATB and TST- individuals [Barcelos et al., 2008]. The differences between studies hinders the interpretation of the results and thus further studies are needed.

Dendritic cells play an essential role in Mtb antigen presentation to T cells, bridging innate and adaptive immunity [Ravesloot-Chavez et al., 2021]. In mice, depletion of CD11c+ DCs leads to a delayed T cell response, resulting in a higher Mtb burden in the lung [Tian et al., 2005]. In humans, several studies point to lower frequencies of peripheral myeloid DCs and plasmacytoid DCs in individuals with ATB compared to the ones with LTB and HC [Dirix et al., 2018; Y. Bin Lu et al., 2017; Uehira et al., 2002]. However, the same was not observed by others. Liu et al. reported higher frequencies of CD1c+CD11b+ DCs in patients with ATB, a subset that promoted T$_H$17 cell polarisation *in vitro* [Liu et al., 2018], while Mendelson et al. and Gupta et al. did not observe differences between Mtb-infected and non-infected individuals [Gupta et al., 2010; Mendelson et al., 2006].

Several studies report alterations in peripheral NK cell levels in individuals with ATB and LTB. Individuals recently diagnosed with ATB have lower peripheral NK frequencies than HC, with impaired function associated with decreased expression of the activation markers NKp30 and NKp46 [Bozzano et al., 2009]. A multi-cohort study showed that LTB is associated with higher levels of circulating NK cells with enhanced antibody-dependent cell-mediated cytotoxicity and higher higher target cell lysis. Moreover, individuals with LTB that progressed to ATB showed a gradual decrease in NK cells frequencies in the 180 days before TB diagnosis, while non-progressors did not show alterations in NK percentages [Roy Chowdhury et al., 2018]. Others did not observe changes in NK frequencies between active disease and latent infection. However, NK cells from treated individuals with ATB have increased IFN-γ expression and

degranulation compared to pre-treatment. In contrast, CD57 expression, a marker of NK cells maturation and cytotoxicity [Lopez-Vergè et al., 2010], was significantly lower in cases after treatment compared to before treatment [Garand et al., 2018]. Single-cell RNA-sequencing data revealed a gradual depletion of granzyme B (GrzB)$^+$ NK cells between healthy controls to Mtb-infected individuals. This observation was confirmed by flow cytometry, and it was also observed that the frequencies of this subset of NK cells increase after treatment of ATB [Cai et al., 2020].

A protective immune response in TB has long been associated with a T$_H$1-polarised response, characterised by CD4$^+$ T cell production of IFN-γ [Cooper, 2009]. For that reason, this subset of cells has been in the spotlight of TB research for decades. The most used strategy to study Mtb-specific T cells is to stimulate either whole blood, peripheral blood mononuclear cells (PBMCs), or sorted T cells *in vitro* with Mtb-derived antigens such as Mtb lysate, PPD, or ESAT-6 and CFP-10 peptide pools, and use IFN-γ secretion as a readout; but CD4$^+$ T cells can be further characterised based on the expression of differentiation/activation markers, Th polarisation, and polyfunctionality (the ability to produce multiple cytokines) [Morgan et al., 2021].

In TB, polyfunctional CD4$^+$ T cells are characterised by simultaneous production of two or three of the cytokines: IL-2, IFN-γ, and TNF [Wilkinson and Wilkinson, 2010]. These cells have been extensively studied in TB, and they are commonly used as a surrogate of protection in TB vaccine development trials [Zeng et al., 2018]. However, the protective role of these cells remains unclear. Some studies have reported increased frequencies of polyfunctional T cells (either triple or double producers) in TB patients [Caccamo et al., 2010; Petruccioli et al., 2013; Sutherland et al., 2009; J. M. Young et al., 2010], that reduced after treatment [J. M. Young et al., 2010]. Others observed that triple producer CD4$^+$ T cells are decreased in ATB [Day et al., 2011; Qiu et al., 2012] and increased following antibiotic treatment [Day et al., 2011]. A study including a cohort from Switzerland and another from South Africa points towards a protective role of polyfunctional CD4$^+$ T cells in TB, as these cells expanded more in LTB compared to ATB [Harari et al., 2011]. Furthermore, single-positive TNF-producing CD4$^+$ T cells proliferate more following stimulation in ATB, a finding also observed by Sutherland et al. [Sutherland et al., 2009], and then normalised after anti-TB treatment [Harari et al., 2011].

In addition to T$_H$1 lymphocytes, T$_H$17 lymphocytes, a CD4$^+$ T cell subset producing IL-17, have also been implicated in TB pathology [Lyadova and Panteleev, 2015; Torrado and Cooper, 2010]. Different studies using human blood samples from infected individuals reported amplified T$_H$17 responses in individuals with ATB, compared to LTB and/or HC [Jurado et al., 2011; Marín et al., 2012; Pollara et al., 2021],

that reverted after anti-TB treatment [Pollara et al., 2021]. Others have associated this subset with TB protection as individuals with LTB have higher frequencies of peripheral T_H17 cells compared to ATB or HC [Coulter et al., 2017; Scriba et al., 2008]. However, these findings were not observed by Babu et al., that reported lower frequencies of T_H17 cells in TST+ individuals compared to TST- [Babu et al., 2010]. Regulatory T cells (Tregs), a subset of T cells responsible for maintaining immune homeostasis and peripheral tolerance, have also been investigated as they may play an important role in disease resistance/tolerance [Cardona and Cardona, 2019]. Overall, individuals with ATB display higher percentages of peripheral Treg populations compared to LTB and/or HC [Agrawal et al., 2018; Chiacchio et al., 2009; Díaz et al., 2015; Guyot-Revol et al., 2006; Kim et al., 2014]. Some studies have reported that individuals with LTB have higher Treg percentages than HC but lower than ATB [Burl et al., 2007; Zewdie et al., 2016], while others did not observe differences between LTB and ATB groups [Wergeland et al., 2011]. However, it is important to highlight that Tregs can be phenotypically characterised by different combinations of markers, namely CD3, CD4, CD25, CD127, FoxP3, and CD39, and that not all studies used the same markers and/or reported frequencies within the same population [Cardona and Cardona, 2019].

As mentioned before, apart from the classical T cells, unconventional T cells can also recognise Mtb-infected cells. Although less studied than other T cell subsets, animal models of TB suggest that these cells may contribute to Mtb protection [Huang, 2016; James and Seshadri, 2020]. Human studies on peripheral MAIT cells revealed reduced frequencies of these cells in the blood of patients with ATB [Gold et al., 2010; Malka-Ruimy et al., 2019]. In addition to this, MAIT cells from individuals with ATB have an impaired immune response compared to LTB and HC, evaluated by *in vitro* secretion of IFN-γ and IL-15 upon stimulation with Mtb lysate [Jiang et al., 2016]. iNKT cells have also been found in lower frequencies in the blood of individuals with ATB compared to LTB and HC. The iNKT cells showed less proliferative capacity when stimulated with α-Galactosylceramide (GalCer), associated with an increased expression of the inhibitory programmed death-1 (PD-1) receptor [Kee et al., 2012]. Panquin-Proulx et al. reported higher percentages of both MAIT and NKT cells in individuals with LTB compared to HC and ATB, and no alterations in the expression of PD-1 or in the production of IFN-γ upon α-GalCer stimulation [Paquin-Proulx et al., 2018].

Human studies evaluating B cell frequencies during Mtb infection have contradictory results. While some have reported lower peripheral B cells frequencies in ATB [Hernandez et al., 2010; Joosten et al., 2016]

or LTB [Roy Chowdhury et al., 2018], others observed the opposite [Y. E. Wu et al., 2009]. However, some observations were not coherent across cohorts, highlighting the heterogeneity of TB in different settings [Roy Chowdhury et al., 2018]. Joosten et al. explored the phenotype and function of B cells in LTB, ATB, and in individuals successfully treated for ATB. Both active and treated TB patients had an increased proportion of Mtb-reactive antibodies. Moreover, active and latently infected individuals showed elevated circulating atypical (CD21$^-$CD27$^-$ or IgD$^-$CD27$^-$) and switched B cells (IgD$^-$CD27$^+$). Atypical B cells displayed impaired proliferation and immunoglobulin and cytokine production that was restored after treatment. Furthermore, improved T cell activity in treated individuals (compared to individuals with ATB and LTB) was dependent on the presence of functionally competent B cells [Joosten et al., 2016]. Mtb-specific antibodies produced by B cells may also contribute to TB control, either by direct neutralization of bacteria or by enhancing phagocytosis, inflammasome activation, cytotoxic NK cells activity or phagolysosome formation [H. Li and Javid, 2018]. A study with Mtb infected individuals showed that LTB is associated with a unique functional profile of antibody constant region (Fc) and glycosylation patterns and selective binding to FcγRIII (CD16), leading to enhanced phagolysosomal maturation, inflammasome activation and macrophage-dependent Mtb killing. [L. L. Lu et al., 2016]. Recently the authors have shown that Fc glycosylation patterns combined with PPD IgG titres provide high accuracy in discrimination between latent and active TB [L. L. Lu et al., 2020]. Li et al. investigated healthcare workers who are exposed to infectious doses of Mtb but remain healthy and found that 3 out of 48 participants that were IGRA$^-$ had protective antibodies against Mtb that depend on immune complexes (FcγRIII and FcγRIIa binding) and CD4$^+$ T cells for efficacy [H. Li et al., 2017]. Moreover, IGRA$^-$ household contacts of ATB patients have IgM, class-switched IgG antibody responses and IFN-γ-independent T cell responses to Mtb-specific peptides [L. L. Lu et al., 2019].

1.5.4. INNATE IMMUNE MEMORY IN TUBERCULOSIS

A very interesting recent finding in immunology was that the innate immune cells can exhibit features of immunological memory after the first encounter with a pathogen [Netea et al., 2011]. This phenomenon was initially termed "trained immunity" and defined as "a heightened response to a secondary infection that can be exerted both toward the same microorganism and a different one" [Netea et al., 2011]. Recently, it has been proposed that memory-like innate immune responses depend on the magnitude (low versus high dose) and duration (short versus long) of the first stimulation and that it should be classified as differentiation, priming, tolerance, and training [Divangahi et al., 2021]. Regardless of the functional outcome, innate memory responses depend on epigenetic and metabolic reprogramming of

the innate immune cells [Netea et al., 2020]. In the context of this thesis, only tolerance and training will be addressed.

One of the first indications that trained immunity is induced in vertebrates comes from mouse studies showing that BCG vaccination induces adaptive immunity-independent protection against secondary infections with *Candida albicans* mediated by macrophages [Wout et al., 1992]. Moreover, BCG vaccination is also associated with TB-independent improved survival in childhood, suggesting that BCG exerts non-specific protection in humans [Garly et al., 2003]. Recently, both mice and human studies have shown that BCG vaccination induces trained immunity in monocytes and NK cells [Arts et al., 2018; Kleinnijenhuis et al., 2014]. More importantly, BCG educates hematopoietic stem cells to generate trained monocytes/macrophages that protect against Mtb infection [Kaufmann et al., 2018]. *In vitro* studies showed that PBMCs from individuals recently exposed to Mtb, but not from remote LTB or BCG vaccinated individuals, control BCG outgrowth as a result of trained immunity mediated by CXCL10-producing nonclassical CD14$_{dim}$ monocytes [Joosten et al., 2018], suggesting that in addition to BCG, Mtb can also induce trained immunity.

It has also been shown that NK cells mediate memory-like responses to different pathogens, including Mtb [Brillantes and Beaulieu, 2020], regulated by transcription factors and epigenetic remodelling [Beaulieu, 2021]. Individuals with ATB have an accumulation of CD45RO$^+$ NK cells (a marker of T cell memory) in pleural fluid, and *in vitro* these cells produce more IFN-γ and have a more significant and faster cytotoxic capacity upon IL-12 stimulation [Fu et al., 2011]. An *in vitro* study using human PBMCs from individuals with LTB showed that a subset of memory-like NK cells expressing CD27, NKp46, and KLRG1 expanded in response to Mtb stimulation, compared to cells of individuals with ATB [Venkatasubramanian et al., 2017]. NK cells expressing CD27 appear to be more reactive regarding cytotoxicity, proliferation, and cytokine production [Inngjerdingen et al., 2011]. In mice, this subset expands following BCG vaccination in an IL-21-dependent manner and protects against Mtb challenge [Venkatasubramanian et al., 2017]. A non-human primate model study showed that CD27$^+$ NK cells accumulate in the lung of macaques with LTB [Esaulova et al., 2021]. Interestingly, PBMCs from individuals with LTB have higher percentages of CD27$^+$ NK cells upon *in vitro* stimulation with Mtb cell wall proteins [Esaulova et al., 2021], indicating a direct interaction between Mtb proteins and NK cells, although which parts of the cell wall remains unclear. Choreño-Parra et al. evaluated the levels of circulating NK cells expressing CXCR6, a chemokine crucial in generating long-lasting memory-like NK cells in mice [Paust et al., 2010], in Mtb-infected individuals and healthy control (HC) individuals. They observed a slight increase of different NK subsets expressing CXCR6 in individuals with ATB, but when

stimulated with Mtb, ATB memory-like NK cells expanded less as compared to LTB [Choreño-Parra et al., 2020]. Another activation and memory marker in NK cells, NKG2C, was found augmented in LTB compared to ATB [Garand et al., 2018].

In contrast to training, tolerance is a state where cells are unable to activate gene transcription and instead become hyporesponsive upon restimulation [Divangahi et al., 2021; Ifrim et al., 2014]. This phenomenon was first observed when macrophages exposed to a high dose of lipopolysaccharide (LPS) secreted lower TNF levels on a second stimulation with LPS [Mathison et al., 1990; Matic and Simon, 1991; Virca et al., 1989]. This was thought to be the result of receptor desensitization [Fujihara et al., 2003]. Later it was shown that pre-stimulation of monocytes and monocyte-derived DCs with PIMs and LAMs also induces a tolerance state, attenuating the cytokine production after subsequent stimulation (either with PIM, LAM or LPS) [Chávez-Galán et al., 2015; Knutson et al., 1998; Nigou et al., 2002, 2001]. Moreover, DCs differentiated in the presence of LAM suppresses cytokine secretion after LPS stimulation [Johansson et al., 2001].

Host defence strategies against infectious diseases includes both resistance and tolerance to the pathogen. Resistance is defined as the host's ability to prevent invasion or eliminate the pathogen, while tolerance mechanisms limit tissue damage caused by the pathogen and/or the immune response [Divangahi et al., 2018]. As mentioned before, about 90% of the individuals infected with Mtb do not develop symptoms [Behr et al., 2019; Divangahi et al., 2018]. In fact, different studies suggest that the immune profile in latently infected individuals is more protective than the immune profile in those who progress to ATB [L. L. Lu et al., 2016; Roy Chowdhury et al., 2018]. Thus, this protective phenotype may be a combination of resistance mediated by memory cells, both innate (trained immunity) and adaptive [Busch et al., 2016; Roy Chowdhury et al., 2018], with monocyte/macrophage tolerance protecting the host from excessive production of pro-inflammatory cytokines and ensuing tissue damage [Divangahi et al., 2018; Olive and Sassetti, 2018].

2. MYCOBACTERIAL CELL ENVELOPE AND ITS GLYCOLIPIDS

The Mtb cell envelope is a dynamic and complex structure that has low permeability and intrinsic resistance to many hydrophobic antibiotics and resistance to harsh environments [Garcia-Vilanova et al., 2019]. As mentioned in section 1.1 of this chapter, the Mtb cell envelope is constituted by three main layers: (i) the cytoplasmatic membrane, (ii) a cell wall consisting of the AGP and the mycomembrane,

and (iii) the capsule [Dulberger et al., 2020; Kalscheuer et al., 2019; Stokas et al., 2020]. The cytoplasmatic membrane is a standard phospholipid bilayer that also contains the glycolipids PIMs (mainly di- or hexa-mannosylated – PIM_2 and PIM_6, respectively) and their derivatives LMs and LAMs [Bansal-Mutalik and Nikaido, 2014]. The cell wall comprises the AGP complex, which maintains cell shape and supports the cytoplasmatic membrane against osmotic pressure of the cytoplasm, and the mycomembrane, which is rich in mycolic acids, but also lipids, glycolipids and secreted proteins [Dulberger et al., 2020]. The peptidoglycan is composed of linear strands of alternating saccharides, N-acetylglucosamine and N-acetylmuramic, cross-linked by short peptide chains into a layered structure [Meroueh et al., 2006] that are covalently attached to arabinogalactan polymers [Alderwick et al., 2015]. Finally, the capsule consists of polysaccharides, proteins and lipids that are weakly bound to the cell wall [Kalscheuer et al., 2019].

Mycobacterial glycolipids can be divided into three different families according to the carbohydrate residue that is attached to the lipid moiety: trehalose, mannose or 6-deoxy-pyranose-containing glycolipids [Garcia-Vilanova et al., 2019]. Trehalose containing glycolipids include di, tri, and penta-acyltrehaloses, mono and di-mycolyl-trehaloses, and tetra-acyl-trehalose sulfoglycolipids [Garcia-Vilanova et al., 2019]. This class of glycolipids is mainly located on the outer leaflet of the mycomembrane, covalently attached to the mycolic acids [Stokas et al., 2020]. The mannose-containing glycolipid family comprises PIMs and the mannosyl-β-1-phosphomycoketides (Garcia-Vilanova et al, 2019), and they are found in and anchored to the cytoplasmatic membrane but also exposed to the surface [Pitarquea et al., 2012], being critical for the cell envelope integrity [Fukuda et al., 2013]. Lastly, the 6-deoxy-pyranose-containing phenolic glycolipids, which are only present in some MTC strains [Reed et al., 2004].

2.1. PIM AND LAM, THE MOST ABUNDANT GLYCOLIPIDS OF *Mycobacterium tuberculosis*

PIMs and their multiglycosylated counterparts, LAMs, are the most abundant glycolipids found in the cell wall of the MTC [Garcia-Vilanova et al., 2019]. These mycobacterial glycoconjugates share a conserved phosphatidyl-*myo*-inositol (PI) anchor responsible for the non-covalent attachment to the cytoplasmatic membrane [Hunter and Brennan, 1990] and they are present in the cell wall as a mixture of molecules differing in the number and composition of the mannose and acyl groups [Garcia-Vilanova et al., 2019]. It is thought that PIMs are predominant during the rapid growth phase of the bacteria and downregulated in stasis, while LAMs seem to be abundant in the stationary growth phase [Chiaradia et al., 2017; Dulberger et al., 2020].
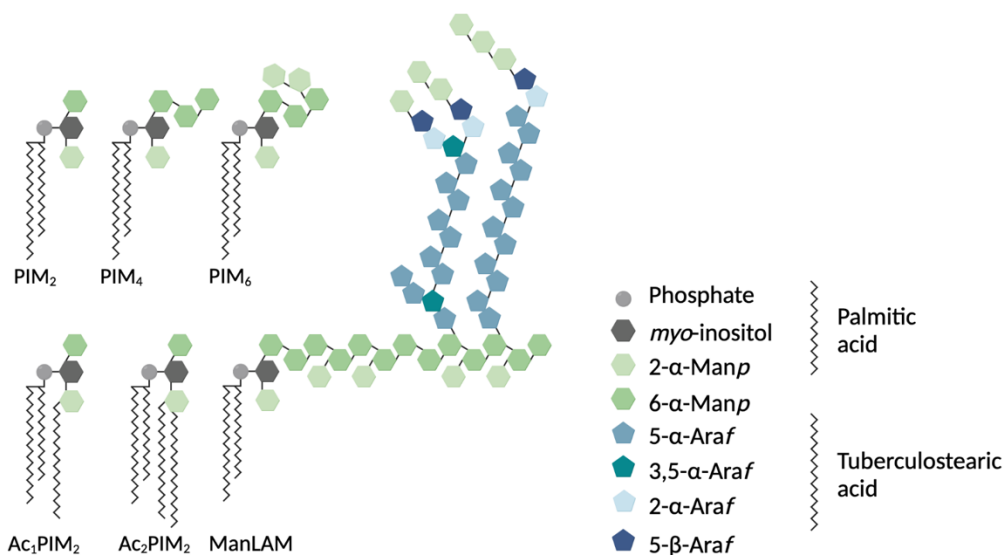
Figure 3. Schematic representation of the structures of PIMs and manLAM. PIMs, and manLAM share a common phosphatidyl-*myo*-inositol anchor. The structure of PIM$_2$ consists of two Man*p* units linked to the positions C-2 and C-6 of the *myo*-inositol ring. The Man*p* in C-6 can be further elongated with additional Man*p* to form higher PIM species (PIM$_{3-6}$). There are four possible acylation sites in the PIM core: the C-1 and C-2 positions of the glycerol moiety, the C-6 position of the Man*p* linked to the C-2 position of the *myo*-inositol (Ac$_1$PIM$_x$), and the C-3 position of the *myo*-inositol ring (Ac$_2$PIM$_x$). This acylation can occur with palmitic and/or tuberculostearic acids. ManLAM consists of a PI anchor, a polysaccharide backbone containing either Man*p* or Ara*f* residues, and mono-, di-, and trimannoside capping motifs. ManLAM also has four possible acylation sites (not represented). Created with BioRender.com

The basic structure PIMs consists of a mannopyranose (Man*p*) unit attached to the position C-2 of the *myo*-inositol of the PI anchor (PIM$_1$). PIM$_2$ is the result of the binding of a Man*p* to the position C-6 of the *myo*-inositol ring and can be further elongated with additional Man*p* to form higher PIM species (PIM$_{3-6}$); In PIM$_3$ and PIM$_4$, the elongation of the Man*p* occurs through a 1,2 glycosidic bond, whereas in PIM$_5$ and PIM$_6$ the binding of the fourth and fifth Man*p* occurs by a 1,6 glycosidic bond [Guerin et al., 2010]. In addition to this, PIMs occur in different acylated forms (AcPIMs), both in number and in nature. Up to four fatty acids chains can be linked to the PIM core: palmitic (C16) and/or tuberculostearic (C19) acids can bind on C-1 and C-2 positions of the glycerol moiety, on the C-6 position of the Man*p* linked to the C-2 position of the *myo*-inositol, and on the C-3 position of the *myo*-inositol ring [Gilleron et al., 2003]. The tri- and tetraacylated forms of PIM$_2$ and PIM$_6$ (Ac$_1$PIM$_2$/Ac$_2$PIM$_2$ and Ac$_1$PIM$_6$/Ac$_2$PIM$_6$) are the most abundant in Mtb [Guerin et al., 2010].

Such structural heterogeneity has an impact on PRRs binding [Garcia-Vilanova et al., 2019]. In fact, the recognition of mycobacterial glycolipids by CLRs depends on both the nature of the terminal carbohydrates and the degree of acylation. Higher-order PIMs and LAMs bind to MR, while PIM$_2$ does not. Moreover, the association of PIMs with MR is dependent on the degree of acylation. In contrast, both

higher-order PIMs and PIM$_2$, as well as LAMs, are recognised by DC-SIGN, regardless of their acylation degree [Torrelles et al., 2006], but it may be species-dependent [Driessen et al., 2009].

PIMs are the precursors of LAMs, and both families derive from the same biosynthetic pathway, although they can be differentially regulated [Crellin et al., 2008]. Both PIM$_5$ and PIM$_6$ have a terminal $\alpha(1\rightarrow2)$-Man$p$, a structure that is not found in the LAM mannan core. Thus, PIM$_4$ is the intermediate for subsequent biosynthesis of LAMs [Nigou, 2003].

LAMs are composed of three domains, the common PI anchor, a polysaccharide backbone, and capping motifs, occurring as a heterogeneous mixture of molecules that differ in the extent of glycosylation and acylation (Figure 3) [Nigou, 2003]. As in PIMs, there are four potential sites of acylation on the anchor, and LAMs are predominantly acylated by palmitic and tuberculostearic acids, and to a lesser extent, by stearic acids [Nigou, 2003]. Tetra-acylated LAM forms are also described as the most common molecular form of Mtb [Turner and Torrelles, 2018]. The polysaccharide backbone is composed of two polysaccharides with variable lengths containing either Man$p$ or arabinofuranose (Ara$f$) residues; the mannan core has an average of 30–35 Man$p$ residues, and the arabinan domain is composed of around 60 Ara$f$ units [Nigou, 2003]. The mannan core consists of an $\alpha(1\rightarrow6)$-Man$p$ backbone highly branched at the position C-2 by a single α-Man$p$ residue, whereas the arabinan structure is based on a linear $\alpha(1\rightarrow5)$Ara$f$ backbone branched by an $\alpha(1\rightarrow3)$ linked Ara$f$, that can occur as two different arrangements, linear tetra-arabinofuranosides (Ara4) and branched hexa-arabinofuranosides (Ara6). The link between mannan and arabinan polymers has not been determined yet [Nigou, 2003; Turner and Torrelles, 2018]. In MTC strains, LAMs may be capped with mono-, di-, and tri-Man$p$ units (manLAM) and are present on both Ara4 and Ara6 side chains [Chatterjee et al., 1992]. However, not all motifs are mannose-capped (in this case LAM is referred as araLAM), and the number of Man$p$ units per cap, as well as the degree of capping, varies according to the strain [Nigou, 2003].

2.2. PIMs AND LAMs AS IMMUNOMODULATORS

The mycobacterial glycolipids PIM and LAM are abundant in the Mtb cell wall and are key ligands of different PRRs, being extremely important for the host immune response. It is known that Mtb cell wall glycolipids are involved in the establishment and maintenance of the infection and in the progression to ATB, but the their exactly role is not well defined yet [Briken et al., 2004; Garcia-Vilanova et al., 2019]. So far, most studies of risk stratification and infection prognosis have focused on IGRA and/or TST tests,

that is, on the adaptive immune response to antigenic peptides [Correia-Neves et al., 2019]. However, these tests have been shown to have a limited predictive value of disease [Auguste et al., 2017; Rangaka et al., 2012; Zhou et al., 2020]. Thus, it is of fundamental interest to find alternatives to the antigens (ESAT-6 and CFP-10) and readout (released IFN-γ by T cells) used to date. Identifying an immune signature or specific markers of cell activation induced by Mtb glycolipids may represent an interesting alternative to the IGRA tests, not only as a potential diagnostic tool but, more importantly, as a way to better understand the LTB spectrum and to predict progression to ATB [Correia-Neves et al., 2019].

Most of the research on the immune response to mycobacterial glycolipids has been done using professional antigen presenting cells (APCs), namely, monocytes/macrophages and DCs. The first studies of the immunomodulatory effects of mycobacterial glycolipids showed that manLAM leads to a potent TNF production by human and murine monocytes/macrophages [Moreno et al., 1989]. However, subsequent mouse studies on BM-derived monocytes and peritoneal macrophages reported a limited effect on manLAM-induced TNF production by these cells [Bradbury and Moreno, 1993; T. I. A. Roach et al., 1995; T. I. Roach et al., 1993]. More recent studies using human DCs have reported that not only manLAM induces TNF production but also the secretion of other pro- and anti-inflammatory cytokines, namely IL-6, IL-10, and IL-12 [Dulphy et al., 2007; Mazurek et al., 2012; Nigou et al., 1997; T. Wu et al., 2011]. In some cases, manLAM boosted the production of IL-6, IL-10, IL-12 and TNF by LPS-primed DCs [Geijtenbeek et al., 2003; Gringhuis et al., 2009; Mazurek et al., 2012]. Dahl et al. studied the effect of mycobacterial glycolipids on human monocytes and observed that araLAM and, to a less extent, manLAM also lead to the secretion of pro-inflammatory cytokines in monocytes. However, the secretion of cytokines induced by the mycobacterial glycolipids was lower than that of LPS. Moreover, in LPS-stimulated monocytes, the cytokine production was enhanced by both glycolipids [Dahl et al., 1996]. In contrast, Nigou et al. reported that manLAM suppresses the LPS-induced IL-12 production by human DCs. This inhibitory effect was abolished when the mannose caps or the PI anchor were removed; but mannan, a ligand for MR, and antibodies targeting the MR, also inhibited IL-12 production, suggesting that MR engagement interferes with the LPS-induced response [Nigou et al., 2001].

The effect of manLAM on human DCs maturation is also contradictory. Dulphy et al. and Mazurek et al. observed that manLAM induced DCs maturation, similar to that induced by LPS in terms of cytokine production and expression of co-stimulatory molecules (CD80 and CD86) [Dulphy et al., 2007; Mazurek et al., 2012]. Moreover, manLAM augmented the LPS-induced DC maturation and pro-inflammatory cytokine production, while PIM supressed this effect [Mazurek et al., 2012]. However, others observed

that manLAM impaired mycobacteria or LPS-induced maturation of DCs [Geijtenbeek et al., 2003; T. Wu et al., 2011].

PIMs have been studied to a lesser extent compared to LAMs, but so far, several researchers have reported that these glycolipids induce an anti-inflammatory phenotype both *in vitro* and *in vivo*. Different PIMs, namely PIM$_1$, PIM$_2$ and PIM$_6$, have been shown to inhibit the secretion of TNF, IL-6, IL-10, and IL-12 by LPS-stimulated murine macrophages [Court et al., 2011; Doz et al., 2009], in a TLR-2 independent manner [Doz et al., 2009]. The same effect was observed by Mazurek et al. in human DCs. The presence of PIM inhibited pro-inflammatory cytokine production of DCs stimulated with LPS and LPS together with manLAM, compared to manLAM and/or LPS alone [Mazurek et al., 2012].

As mentioned before, in humans, the mycobacterial glycolipids PIMs and LAMs are presented to T cells by the CD1b isoform [Sieling et al., 1995], although it was shown that the cytosolic soluble CD1e isoform is crucial for PIM$_6$ processing and presentation [Cala-De Paepe et al., 2012; Salle et al., 2005; Tourne et al., 2008]. Due to their structural heterogeneity, the effect of these glycolipids on CD1b-restricted T cells may differ [Torrelles et al., 2004, 2012]. Torrelles et al. reported a relationship between the overall LAM charge and its capacity to stimulate CD1b-restricted T cells, suggesting that higher acylation degrees combined with lower LAM sizes favour its activation [Torrelles et al., 2012]. Moreover, MR seems to play an important role in this process. In an *in vitro* study using APCs derived from PBMCs it was reported that LAM is taken up and delivered by the MR to endosomes for CD1b presentation to T cells [Prigozy et al., 1997]. CD1b-restricted T cell activation is usually evaluated by IFN-γ production, but Busch et al. have shown that LAM induces a polycytotoxic phenotype in T cells (mostly CD8$^+$ T cells) characterised by the expression of IFN-γ, perforin, granulysin, and granzyme B; they also observed that the frequency of polycytotoxic LAM-responsive T cells correlates to LTB and post-treatment ATB. Moreover, TST$^+$ individuals react to several mycobacterial glycolipids, namely PIM and LAM, and LAM-responsive T cells reduce Mtb growth *in vitro* [Busch et al., 2016]. In contrast, murine studies have shown that manLAM induces CD4$^+$ T cell anergy by direct association with T cells membrane [Sande et al., 2016], possibly by interfering with TCR signalling [Mahon et al., 2012a]. Others showed that the suppressive effects on conventional T cells go beyond TCR inhibition and that manLAM stimulation leads to a global proteomic change, affecting Akt-mTOR signalling and resulting in broad functional impairment of CD4$^+$ T-cell activation [Karim et al., 2017]. ManLAM has also been shown to favour T$_H$2 cytokine production by human T$_H$ cells *in vitro*, to the detriment of T$_H$1 cytokines [Shabaana et al., 2005]. On the other hand, manLAM is indirectly involved in

the expansion of CD4$^+$CD25$^+$FoxP3$^+$ Tregs by stimulating prostaglandin E2 production in monocytes [Garg et al., 2010].

In addition to CD1b, mycobacterial glycolipids can be presented through the CD1d isoform to iNKT cells, a subset of T cells that simultaneously express a restricted TCR repertoire and several NK-associated receptors [Arora et al., 2013]. Consistently with this, Fischer et al. have shown that PIM$_4$ is presented to iNKT cells through CD1d, triggering IFN-γ and cell-mediated cytotoxicity [Fischer et al., 2004]. Moreover, crystal structure experiments revealed that PIM$_2$ also binds to CD1d molecules [Zajonc et al., 2006]. Notably, NKT cells may have a protective function in Mtb infection [Sada-Ovalle et al., 2008; Venkataswamy et al., 2009].

The effect of Mtb glycolipids on B cells has been less explored compared to myeloid and T cells, but recently Yuan et al. showed that manLAM leads to the expansion of IL-10-producing B cells (B10) in mice. B10 cells are increased in individuals with ATB, where they inhibit CD4$^+$ T$_H$1 polarization, resulting in increased Mtb susceptibility in mice. Moreover, the authors also observed that manLAM binds both TLR2 and MR, but the IL-10 production is dependent on TLR2 engagement [Yuan et al., 2019]. LAM-specific antibodies have been shown to be protective against Mtb infection in mice [De Vallière et al., 2005; Hamasur et al., 2004]. In humans, a low titre of anti-LAM IgG is associated with disseminated TB in children, suggesting that LAM-specific antibodies may also have a protective role in humans [Moreno et al., 1992]. Another study showed that serum containing anti-manLAM antibodies enhances Mtb uptake and killing by autologous macrophages [Kumar et al., 2015].

In conclusion, PIMs and LAMs are a family of mannose-containing glycolipids with different levels of complexity depending on the degree and nature of acylation, the length of the mannan and arabinan cores, as well as on the number and length of the mannose caps. Overall, the published studies show that PIMs and LAMs modulate the immune response mediated by both innate and adaptive immune cells. However, the findings are not coherent between different studies. This may be due to the heterogeneous nature of the glycolipids, which is species-specific but may also be derived from differences in the protocols used for PIMs and LAMs isolation. Moreover, the *in vitro* experiments setup, including the choice of immune cell types and procedures to generate them, may partly explain the discrepancy between studies [Källenius et al., 2016].

3. THESIS AIMS

Despite being one of the oldest known infections, TB is still one of the deadliest diseases and responsible for high morbidity and mortality worldwide. Some of the challenges in TB elimination highlighted by the WHO in the END-TB strategy is the prompt diagnosis and the management of LTB. Identifying latently infected individuals and those who are at risk of progress to ATB is of the utmost importance to improve TB control. Given this, our main goal is to contribute to a better understanding of the peripheral immune profile of individuals with latent and active TB. To achieve this, the following aims were established:

Aim 1. To study the immune response of PBMCs from Mtb-infected individuals to the mycobacterial glycolipids PIM and LAM. The immune response to mycobacterial glycolipids PIM and LAM is poorly understood. Such response might be useful as an alternative to the existing diagnostic methods, but also provide information about the immune status of those individuals.

Aim 2. To perform a comprehensive characterisation of the immune profiles of individuals in different stages of Mtb infection. Despite the vast research regarding the peripheral cell composition of individuals with TB, most of the studies focus on the T lymphocytes. Thus, there is a need for a comprehensive and broader characterisation of the peripheral immune profile of these individuals.

REFERENCES

Abel, B., Thieblemont, N., Quesniaux, V. J. F., Brown, N., Mpagi, J., Miyake, K., Bihl, F., and Ryffel, B. [2002]. Toll-like receptor 4 expression is required to control chronic Mycobacterium tuberculosis infection in mice. *Journal of Immunology (Baltimore, Md. : 1950)*, *169*(6), 3155–3162. https://doi.org/10.4049/JIMMUNOL.169.6.3155

Agrawal, S., Parkash, O., Palaniappan, A. N., Bhatia, A. K., Kumar, S., Chauhan, D. S., and Madhan Kumar, M. [2018]. Efficacy of T Regulatory Cells, Th17 Cells and the Associated Markers in Monitoring Tuberculosis Treatment Response. *Frontiers in Immunology*, *9*(157), 1–16. https://doi.org/10.3389/fimmu.2018.00157

Alderwick, L. J., Harrison, J., Lloyd, G. S., and Birch, H. L. [2015]. The Mycobacterial Cell Wall—Peptidoglycan and Arabinogalactan. *Cold Spring Harbor Perspectives in Medicine*, *5*(a021113), 1–15. https://doi.org/10.1101/cshperspect.a021113

Andersen, P., Munk, M., Pollock, J., and Doherty, T. [2000]. Specific immune-based diagnosis of tuberculosis. *The Lancet*, *356*(9235), 1099–1104. https://doi.org/10.1016/S0140-6736(00)02742-2

Anibarro, L., Trigo, M., Villaverde, C., Pena, A., Cortizo, S., Sande, D., Pazos, R. A., and Gonzalez-Fernandez, A. [2011]. Interferon- release assays in tuberculosis contacts: is there a window period? *European Respiratory Journal*, *37*(1), 215–217. https://doi.org/10.1183/09031936.00030610

Arora, P., Foster, E. L., and Porcelli, S. A. [2013]. CD1d and Natural Killer T Cells in Immunity to Mycobacterium tuberculosis. In *Advances in Experimental Medicine and Biology* (Vol. 23, pp. 199–223). https://doi.org/10.1007/978-1-4614-6111-1_11

Arts, R. J. W., Moorlag, S. J. C. F. M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., … Netea, M. G. [2018]. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host & Microbe*, *23*(1), 89-100.e5. https://doi.org/10.1016/j.chom.2017.12.010

Auguste, P., Tsertsvadze, A., Pink, J., Court, R., McCarthy, N., Sutcliffe, P., and Clarke, A. [2017]. Comparing interferon-gamma release assays with tuberculin skin test for identifying latent tuberculosis infection that progresses to active tuberculosis: systematic review and meta-analysis. *BMC Infectious Diseases*, *17*(200), 1–13. https://doi.org/10.1186/s12879-017-2301-4

Babu, S., Bhat, S. Q., Kumar, N. P., Kumaraswami, V., and Nutman, T. B. [2010]. Regulatory T Cells Modulate Th17 Responses in Patients with Positive Tuberculin Skin Test Results. *The Journal of Infectious Diseases*, *201*(1), 20–31. https://doi.org/10.1086/648735

Badaro, R., Machado, B. A. S., Duthie, M. S., Araujo-Neto, C. A., Pedral-Sampaio, D., Nakatani, M., and Reed, S. G. [2020]. The single recombinant M. tuberculosis protein DPPD provides enhanced performance of skin testing among HIV-infected tuberculosis patients. *AMB Express*, *10*(133), 1–8. https://doi.org/10.1186/s13568-020-01068-6

Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A., and Sher, A. [2005]. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. *Journal of Experimental Medicine*, *202*(12), 1715–1724. https://doi.org/10.1084/jem.20051782

Bansal-Mutalik, R., and Nikaido, H. [2014]. Mycobacterial outer membrane is a lipid bilayer and the inner membrane is unusually rich in diacyl phosphatidylinositol dimannosides. *Proceedings of the National Academy of Sciences*, *111*(13), 4958–4963. https://doi.org/10.1073/PNAS.1403078111/

Barcelos, W., Sathler-Avelar, R., Martins-Filho, O. A., Carvalho, B. N., Guimarães, T. M. P. D., Miranda, S. S., Andrade, H. M., Oliveira, M. H. P., and Toledo, V. P. C. P. [2008]. Natural Killer Cell

Subpopulations in Putative Resistant Individuals and Patients with Active Mycobacterium tuberculosis Infection. *Scandinavian Journal of Immunology*, *68*(1), 92–102. https://doi.org/10.1111/j.1365-3083.2008.02116.x

Barral, D. C., and Brenner, M. B. [2007]. CD1 antigen presentation: how it works. *Nature Reviews Immunology*, *7*(12), 929–941. https://doi.org/10.1038/nri2191

Beaulieu, A. M. [2021]. Transcriptional and epigenetic regulation of memory NK cell responses. *Immunological Reviews*, *300*(1), 125–133. https://doi.org/10.1111/imr.12947

Behr, M. A., Edelstein, P. H., and Ramakrishnan, L. [2019]. Is Mycobacterium tuberculosis infection life long? *BMJ*, *367*(l5770), 1–7. https://doi.org/10.1136/bmj.l5770

Berry, M. P. R. R., Graham, C. M., McNab, F. W., Xu, Z., Bloch, S. A. A. A., Oni, T., ... O'Garra, A. [2010]. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. *Nature*, *466*, 973–977. https://doi.org/10.1038/nature09247

Bozzano, F., Costa, P., Passalacqua, G., Dodi, F., Ravera, S., Pagano, G., Canonica, G. W., Moretta, L., and De Maria, A. [2009]. Functionally relevant decreases in activatory receptor expression on NK cells are associated with pulmonary tuberculosis in vivo and persist after successful treatment. *International Immunology*, *21*(7), 779–791. https://doi.org/10.1093/intimm/dxp046

Bradbury, M. G., and Moreno, C. [1993]. Effect of lipoarabinomannan and mycobacterium on tumour necrosis factor production by different populations of murine macrophages. *Clinical and Experimental Immunology*, *94*(1), 57–63. https://doi.org/10.1111/j.1365-2249.1993.tb05977.x

Brennan, P. J., Brigl, M., and Brenner, M. B. [2013]. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nature Reviews Immunology*, *13*(2), 101–117. https://doi.org/10.1038/nri3369

Briken, V., Porcelli, S. A., Besra, G. S., and Kremer, L. [2004]. Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response. *Molecular Microbiology*, *53*(2), 391–403. https://doi.org/10.1111/j.1365-2958.2004.04183.x

Brillantes, M., and Beaulieu, A. M. [2020]. Memory and Memory-Like NK Cell Responses to Microbial Pathogens. *Frontiers in Cellular and Infection Microbiology*, *10*(102), 1–10. https://doi.org/10.3389/fcimb.2020.00102

Brown, G. D. [2005]. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nature Reviews Immunology*, *6*(1), 33–43. https://doi.org/10.1038/nri1745

Brown, S., Leavy, J. E., and Jancey, J. [2021]. Implementation of GeneXpert for TB Testing in Low- and Middle-Income Countries: A Systematic Review. *Global Health: Science and Practice*, *9*(3), 698–710. https://doi.org/10.9745/GHSP-D-21-00121

Burl, S., Hill, P. C., Jeffries, D. J., Holland, M. J., Fox, A., Lugos, M. D., ... Brookes, R. H. [2007]. *FOXP3* gene expression in a tuberculosis case contact study. *Clinical and Experimental Immunology*, *149*(1), 117–122. https://doi.org/10.1111/j.1365-2249.2007.03399.x

Busch, M., Herzmann, C., Kallert, S., Zimmermann, A., Höfer, C., Mayer, D., ... Stenger, S. [2016]. Lipoarabinomannan-Responsive Polycytotoxic T Cells Are Associated with Protection in Human Tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, *194*(3), 345–355. https://doi.org/10.1164/rccm.201509-1746OC

Caccamo, N., Guggino, G., Joosten, S. A., Gelsomino, G., Di Carlo, P., Titone, L., ... Dieli, F. [2010]. Multifunctional CD4+ T cells correlate with active Mycobacterium tuberculosis infection. *European Journal of Immunology*, *40*(8), 2211–2220. https://doi.org/10.1002/eji.201040455

Cai, Y., Dai, Y., Wang, Y., Yang, Q., Guo, J., Wei, C., ... Chen, X. [2020]. Single-cell transcriptomics of blood reveals a natural killer cell subset depletion in tuberculosis. *EBioMedicine*, *53*(102686), 1–11. https://doi.org/10.1016/j.ebiom.2020.102686

Cala-De Paepe, D., Layre, E., Giacometti, G., Garcia-Alles, L. F., Mori, L., Hanau, D., ... Gilleron, M. [2012]. Deciphering the role of CD1e protein in mycobacterial phosphatidyl-myo- inositol

mannosides (PIM) processing for presentation by CD1b to T lymphocytes. *Journal of Biological Chemistry*, *287*(37), 31494–31502. https://doi.org/10.1074/jbc.M112.386300

Campos-Neto, A., Rodrigues-Júnior, V., Pedral-Sampaio, D. B., Netto, E. M., Ovendale, P. J., Coler, R. N., Skeiky, Y. A. W., Badaró, R., and Reed, S. G. [2001]. Evaluation of DPPD, a single recombinant Mycobacterium tuberculosis protein as an alternative antigen for the Mantoux test. *Tuberculosis*, *81*(5/6), 353–358. https://doi.org/10.1054/tube.2001.0311

Cardona, P., and Cardona, P.-J. [2019]. Regulatory T Cells in Mycobacterium tuberculosis Infection. *Frontiers in Immunology*, *10*(2139), 1–11. https://doi.org/10.3389/fimmu.2019.02139

Castaño, D., García, L. F., and Rojas, M. [2011]. Increased frequency and cell death of CD16+ monocytes with Mycobacterium tuberculosis infection. *Tuberculosis*, *91*(5), 348–360. https://doi.org/10.1016/j.tube.2011.04.002

Chatterjee, D., Lowell, K., Rivoire, B., McNeil, M. R., and Brennan, P. J. [1992]. Lipoarabinomannan of Mycobacterium tuberculosis. Capping with mannosyl residues in some strains. *Journal of Biological Chemistry*, *267*(9), 6234–6239. https://doi.org/10.1016/s0021-9258(18)42686-5

Chávez-Galán, L., Ocaña-Guzmán, R., Torre-Bouscoulet, L., García-De-Alba, C., and Sada-Ovalle, I. [2015]. Exposure of monocytes to lipoarabinomannan promotes their differentiation into functionally and phenotypically immature macrophages. *Journal of Immunology Research*, *2015*, 984973. https://doi.org/10.1155/2015/984973

Chiacchio, T., Casetti, R., Butera, O., Vanini, V., Carrara, S., Girardi, E., ... Goletti, D. [2009]. Characterization of regulatory T cells identified as CD4 +CD25 highCD39 + in patients with active tuberculosis. *Clinical and Experimental Immunology*, *156*(3), 463–470. https://doi.org/10.1111/j.1365-2249.2009.03908.x

Chiaradia, L., Lefebvre, C., Parra, J., Marcoux, J., Burlet-Schiltz, O., Etienne, G., Tropis, M., and Daffé, M. [2017]. Dissecting the mycobacterial cell envelope and defining the composition of the native mycomembrane. *Scientific Reports*, *7*(12807), 1–12. https://doi.org/10.1038/s41598-017-12718-4

Choreño-Parra, J. A., Jiménez-Álvarez, L. A., Muñoz-Torrico, M., Ramírez-Martínez, G., Jiménez-Zamudio, L. A., Salinas-Lara, C., García-Latorre, E. A., and Zúñiga, J. [2020]. Antigens of Mycobacterium tuberculosis Stimulate CXCR6+ Natural Killer Cells. *Frontiers in Immunology*, *11*(582414), 1–13. https://doi.org/10.3389/FIMMU.2020.582414

Cohen, S. B., Gern, B. H., and Urdahl, K. B. [2022]. The Tuberculous Granuloma and Preexisting Immunity. *Annual Review of Immunology*, *40*(1), 589–614. https://doi.org/10.1146/annurev-immunol-093019-125148

Cooper, A. M. [2009]. Cell mediated immune responses in Tuberculosis. *Annual Review of Immunology*, *27*, 393–422. https://doi.org/10.1146/ANNUREV.IMMUNOL.021908.132703

Correia-Neves, M., Fröberg, G., Korshun, L., Viegas, S., Vaz, P., Ramanlal, N., ... Källenius, G. [2019]. Biomarkers for tuberculosis: the case for lipoarabinomannan. *ERJ Open Research*, *5*(1), 1–14. https://doi.org/10.1183/23120541.00115-2018

Coulter, F., Parrish, A., Manning, D., Kampmann, B., Mendy, J., Garand, M., Lewinsohn, D. M., Riley, E. M., and Sutherland, J. S. [2017]. IL-17 Production from T Helper 17, Mucosal-Associated Invariant T, and γδ Cells in Tuberculosis Infection and Disease. *Frontiers in Immunology*, *8*(1252), 1–10. https://doi.org/10.3389/fimmu.2017.01252

Court, N., Rose, S., Bourigault, M.-L., Front, S., Martin, O. R., Dowling, J. K., ... Quesniaux, V. F. J. [2011]. Mycobacterial PIMs Inhibit Host Inflammatory Responses through CD14-Dependent and CD14-Independent Mechanisms. *PLoS ONE*, *6*(9), 1–12. https://doi.org/10.1371/journal.pone.0024631

Court, N., Vasseur, V., Vacher, R., Frémond, C., Shebzukhov, Y., Yeremeev, V. V., ... Quesniaux, V. F. J. [2010]. Partial Redundancy of the Pattern Recognition Receptors, Scavenger Receptors, and C-Type

Lectins for the Long-Term Control of Mycobacterium tuberculosis Infection. *The Journal of Immunology*, *184*(12), 7057–7070. https://doi.org/10.4049/jimmunol.1000164

Crellin, P. K., Kovacevic, S., Martin, K. L., Brammananth, R., Morita, Y. S., Billman-Jacobe, H., McConville, M. J., and Coppel, R. L. [2008]. Mutations in pimE restore lipoarabinomannan synthesis and growth in a Mycobacterium smegmatis lpqW mutant. *Journal of Bacteriology*, *190*(10), 3690–3699. https://doi.org/10.1128/JB.00200-08

Dahl, K. E., Shiratsuchi, H., Hamilton, B. D., Ellner, J. J., and Toossi, Z. [1996]. Selective induction of transforming growth factor β in human monocytes by lipoarabinomannan of Mycobacterium tuberculosis. *Infection and Immunity*, *64*(2), 399–405. https://doi.org/10.1128/iai.64.2.399-405.1996

Day, C. L., Abrahams, D. A., Lerumo, L., Janse van Rensburg, E., Stone, L., O'rie, T., ... Hanekom, W. A. [2011]. Functional Capacity of Mycobacterium tuberculosis -Specific T Cell Responses in Humans Is Associated with Mycobacterial load. *The Journal of Immunology*, *187*(5), 2222–2232. https://doi.org/10.4049/jimmunol.1101122

de Oliveira, S., Rosowski, E. E., and Huttenlocher, A. [2016]. Neutrophil migration in infection and wound repair: going forward in reverse. *Nature Reviews Immunology*, *16*(6), 378–391. https://doi.org/10.1038/nri.2016.49

De Vallière, S., Abate, G., Blazevic, A., Heuertz, R. M., and Hoft, D. F. [2005]. Enhancement of innate and cell-mediated immunity by antimycobacterial antibodies. *Infection and Immunity*, *73*(10), 6711–6720. https://doi.org/10.1128/IAI.73.10.6711-6720.2005

Dey, B., Dey, R. J., Cheung, L. S., Pokkali, S., Guo, H., Lee, J. H., and Bishai, W. R. [2015]. A bacterial cyclic dinucleotide activates the cytosolic surveillance pathway and mediates innate resistance to tuberculosis. *Nature Medicine*, *21*(LETTERS), 401–408. https://doi.org/10.1038/nm.3813

DGS. [2021]. *Relatório de vigilância e monitorização da Tuberculose em Portugal*. Lisboa.

Dheda, K., Barry, C. E., and Maartens, G. [2016]. Tuberculosis. *The Lancet*, *387*(Seminar), 1211–1226. https://doi.org/10.1016/S0140-6736(15)00151-8

Díaz, A., Santucci, N., Bongiovanni, B., D'Attilio, L., Massoni, C., Lioi, S., ... Bay, M. L. [2015]. Increased Frequency of CD4+ CD25+ FoxP3+ T Regulatory Cells in Pulmonary Tuberculosis Patients Undergoing Specific Treatment and Its Relationship with Their Immune-Endocrine Profile. *Journal of Immunology Research*, *2015*(985302), 1–8. https://doi.org/10.1155/2015/985302

Dirix, V., Corbière, V., Wyndham-Thomas, C., Selis, E., Allard, S., Hites, M., Aerts, L., Giese, T., and Mascart, F. [2018]. Blood tolerogenic monocytes and low proportions of dendritic cell subpopulations are hallmarks of human tuberculosis. *Journal of Leukocyte Biology*, *103*(5), 945–954. https://doi.org/10.1002/JLB.4A1117-448R

Divangahi, M. [2018]. Are tolerance and training required to end TB? *Nature Reviews Immunology*, *18*(Comment), 661–663. https://doi.org/10.1038/s41577-018-0070-y

Divangahi, M., Aaby, P., Khader, S. A., Barreiro, L. B., Bekkering, S., Chavakis, T., ... Netea, M. G. [2021]. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. *Nature Immunology*, *22*(1), 2–6. https://doi.org/10.1038/s41590-020-00845-6

Divangahi, M., Khan, N., and Kaufmann, E. [2018]. Beyond Killing Mycobacterium tuberculosis: Disease Tolerance. *Frontiers in Immunology*, *9*, 2976. https://doi.org/10.3389/fimmu.2018.02976

Divangahi, M., Mostowy, S., Coulombe, F., Kozak, R., Guillot, L., Veyrier, F., ... Behr, M. A. [2008]. NOD2-Deficient Mice Have Impaired Resistance to *Mycobacterium tuberculosis* Infection through Defective Innate and Adaptive Immunity. *The Journal of Immunology*, *181*(10), 7157–7165. https://doi.org/10.4049/jimmunol.181.10.7157

Dorhoi, A., Desel, C., Yeremeev, V., Pradl, L., Brinkmann, V., Mollenkopf, H. J., ... Kaufmann, S. H. E. [2010]. The adaptor molecule CARD9 is essential for tuberculosis control. *Journal of Experimental Medicine*, *207*(4), 777–792. https://doi.org/10.1084/JEM.20090067

Dorhoi, A., Nouailles, G., Jörg, S., Hagens, K., Heinemann, E., Pradl, L., ... Kaufmann, S. H. E. [2012]. Activation of the NLRP3 inflammasome by Mycobacterium tuberculosis is uncoupled from susceptibility to active tuberculosis. *European Journal of Immunology*, *42*(2), 374–384. https://doi.org/10.1002/EJI.201141548

Doz, E., Rose, S., Court, N., Front, S., Vasseur, V., Charron, S., ... Quesniaux, V. F. J. [2009]. Mycobacterial Phosphatidylinositol Mannosides Negatively Regulate Host Toll-like Receptor 4, MyD88-dependent Proinflammatory Cytokines, and TRIF-dependent Co-stimulatory Molecule Expression. *Journal of Biological Chemistry*, *284*(35), 23187–23196. https://doi.org/10.1074/jbc.M109.037846

Doz, E., Rose, S., Nigou, J., Gilleron, M., Puzo, G., Erard, F., Ryffel, B., and Quesniaux, V. F. J. [2007]. Acylation Determines the Toll-like receptor (TLR)-dependent Positive Versus TLR2-, Mannose Receptor-, and SIGNR1-independent Negative Regulation of Pro-inflammatory Cytokines by Mycobacterial Lipomannan *. *Journal of Biological Chemistry*, *282*(36), 26014–26025. https://doi.org/10.1074/JBC.M702690200

Drain, P. K., Bajema, K. L., Dowdy, D., Dheda, K., Naidoo, K., Schumacher, S. G., ... Sherman, D. R. [2018]. Incipient and Subclinical Tuberculosis: a Clinical Review of Early Stages and Progression of Infection. *Clinical Microbiology Reviews*, *31*(4), 1–24. https://doi.org/10.1128/CMR.00021-18

Driessen, N. N., Ummels, R., Maaskant, J. J., Gurcha, S. S., Besra, G. S., Ainge, G. D., ... Appelmelk, B. J. [2009]. Role of Phosphatidylinositol Mannosides in the Interaction between Mycobacteria and DC-SIGN. *Infection and Immunity*, *77*(10), 4538–4547. https://doi.org/10.1128/IAI.01256-08

Dulberger, C. L., Rubin, E. J., and Boutte, C. C. [2020]. The mycobacterial cell envelope — a moving target. *Nature Reviews Microbiology*, *18*(1), 47–59. https://doi.org/10.1038/s41579-019-0273-7

Dulphy, N., Herrmann, J.-L., Nigou, J., Réa, D., Boissel, N., Puzo, G., Charron, D., Lagrange, P. H., and Toubert, A. [2007]. Intermediate maturation of Mycobacterium tuberculosis LAM-activated human dendritic cells. *Cellular Microbiology*, *9*(6), 1412–1425. https://doi.org/10.1111/j.1462-5822.2006.00881.x

Dutta, N. K., and Karakousis, P. C. [2014]. Latent Tuberculosis Infection: Myths, Models, and Molecular Mechanisms. *Microbiology and Molecular Biology Reviews*, *78*(3), 343–371. https://doi.org/10.1128/MMBR.00010-14

ECDC/WHO Regional Office for Europe. [2022]. *Tuberculosis surveillance and monitoring in Europe 2022 - 2020 data*. https://doi.org/10.2900/488579

ECDC. [2018]. *Handbook on tuberculosis laboratory diagnostic methods in the European Union - Update 2018*. https://doi.org/10.2900/914169

Esaulova, E., Das, S., Singh, D. K., Choreño-Parra, J. A., Swain, A., Arthur, L., ... Khader, S. A. [2021]. The immune landscape in tuberculosis reveals populations linked to disease and latency. *Cell Host & Microbe*, *29*(2), 165–178. https://doi.org/10.1016/J.CHOM.2020.11.013

Esmail, H., Barry, C. E., Young, D. B., and Wilkinson, R. J. [2014]. The ongoing challenge of latent tuberculosis. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *369*(1645), 1–14. https://doi.org/10.1098/RSTB.2013.0437

Farhat, M., Greenaway, C., Pai, M., and Menzies, D. [2006]. False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? *INT J TUBERC LUNG DIS*, *10*(11), 1192–1204.

Feng, C. G., Scanga, C. A., Collazo-Custodio, C. M., Cheever, A. W., Hieny, S., Caspar, P., and Sher, A. [2003]. Mice Lacking Myeloid Differentiation Factor 88 Display Profound Defects in Host Resistance and Immune Responses to Mycobacterium avium Infection Not Exhibited by Toll-Like Receptor 2 (TLR2)- and TLR4-Deficient Animals. *The Journal of Immunology*, *171*(9), 4758–4764. https://doi.org/10.4049/jimmunol.171.9.4758

Fischer, K., Scotet, E., Niemeyer, M., Koebernick, H., Zerrahn, J., Maillet, S., ... Schaible, U. E. [2004].

Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(29), 10685. https://doi.org/10.1073/PNAS.0403787101

Flores, J., Cancino, J. C., and Chavez-Galan, L. [2021]. Lipoarabinomannan as a Point-of-Care Assay for Diagnosis of Tuberculosis: How Far Are We to Use It? *Frontiers in Microbiology*, *12*(638047), 1–16. https://doi.org/10.3389/FMICB.2021.638047

Floyd, K., Glaziou, P., Zumla, A., and Raviglione, M. [2018]. The global tuberculosis epidemic and progress in care, prevention, and research: an overview in year 3 of the End TB era. *The Lancet Respiratory Medicine*, *6*(4), 299–314. https://doi.org/10.1016/S2213-2600(18)30057-2

Fu, X., Liu, Y., Li, L., Li, Q., Qiao, D., Wang, H., Lao, S., Fan, Y., and Wu, C. [2011]. Human natural killer cells expressing the memory-associated marker CD45RO from tuberculous pleurisy respond more strongly and rapidly than CD45RO− natural killer cells following stimulation with interleukin-12. *Immunology*, *134*(1), 41. https://doi.org/10.1111/J.1365-2567.2011.03464.X

Fujihara, M., Muroi, M., Tanamoto, K. I., Suzuki, T., Azuma, H., and Ikeda, H. [2003]. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: Roles of the receptor complex. *Pharmacology and Therapeutics*, *100*(2), 171–194. https://doi.org/10.1016/j.pharmthera.2003.08.003

Fukuda, T., Matsumura, T., Ato, M., Hamasaki, M., Nishiuchi, Y., Murakami, Y., ... Morita, Y. S. [2013]. Critical Roles for Lipomannan and Lipoarabinomannan in Cell Wall Integrity of Mycobacteria and Pathogenesis of Tuberculosis. *MBio*, *4*(1). https://doi.org/10.1128/MBIO.00472-12

Furin, J., Cox, H., and Pai, M. [2019]. Tuberculosis. *The Lancet*, *393*(10181), 1642–1656. https://doi.org/10.1016/S0140-6736(19)30308-3

Gandotra, S., Jang, S., Murray, P. J., Salgame, P., and Ehrt, S. [2007]. Nucleotide-Binding Oligomerization Domain Protein 2-Deficient Mice Control Infection with Mycobacterium tuberculosis. *Infection and Immunity*, *75*(11), 5127–5134. https://doi.org/10.1128/IAI.00458-07

Garand, M., Goodier, M., Owolabi, O., Donkor, S., Kampmann, B., and Sutherland, J. S. [2018]. Functional and Phenotypic Changes of Natural Killer Cells in Whole Blood during Mycobacterium tuberculosis Infection and Disease. *Frontiers in Immunology*, *9*(257), 1–18. https://doi.org/10.3389/fimmu.2018.00257

Garcia-Vilanova, A., Chan, J., and Torrelles, J. B. [2019]. Underestimated Manipulative Roles of Mycobacterium tuberculosis Cell Envelope Glycolipids During Infection. *Frontiers in Immunology*, *10*(2909). https://doi.org/10.3389/fimmu.2019.02909

Garg, A., Barnes, P. F., Roy, S., Quiroga, M. F., Wu, S., García, V. E., Krutzik, S. R., and Weis, S. E. [2010]. PROSTAGLANDIN E2-DEPENDENT EXPANSION OF REGULATORY T CELLS IN HUMAN Mycobacterium tuberculosis. *October*, *38*(2), 459–469. https://doi.org/10.1002/eji.200737268.MANNOSE-CAPPED

Garly, M. L., Martins, C. L., Balé, C., Baldé, M. A., Hedegaard, K. L., Gustafson, P., Lisse, I. M., Whittle, H. C., and Aaby, P. [2003]. BCG scar and positive tuberculin reaction associated with reduced child mortality in West Africa: A non-specific beneficial effect of BCG? *Vaccine*, *21*(21–22), 2782–2790. https://doi.org/10.1016/S0264-410X(03)00181-6

Geijtenbeek, T. B. H., van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M. J. E., Appelmelk, B., and van Kooyk, Y. [2003]. Mycobacteria Target DC-SIGN to Suppress Dendritic Cell Function. *Journal of Experimental Medicine*, *197*(1), 7–17. https://doi.org/10.1084/jem.20021229

Gilleron, M., Quesniaux, V. F. J. V. F. J., and Puzo, G. [2003]. Acylation State of the Phosphatidylinositol Hexamannosides from Mycobacterium bovis Bacillus Calmette Guérin and Mycobacterium tuberculosis H37Rv and Its Implication in Toll-like Receptor Response *. *Journal of Biological Chemistry*, *278*(32), 29880–29889. https://doi.org/10.1074/JBC.M303446200

Goadsby, P. J., Kurth, T., and Pressman, A. [2016]. Present Yourself! By MHC Class I and MHC Class II Molecules. *Trends in Immunology*, *35*(14), 1252–1260. https://doi.org/10.1016/j.it.2016.08.010.Present

Gold, M. C., Cerri, S., Smyk-Pearson, S., Cansler, M. E., Vogt, T. M., Delepine, J., ... Lewinsohn, D. M. [2010]. Human mucosal associated invariant T cells detect bacterially infected cells. *PLoS Biology*, *8*(6), 1–14. https://doi.org/10.1371/journal.pbio.1000407

Goyal, S., Klassert, T. E., and Slevogt, H. [2016]. C-type lectin receptors in tuberculosis: what we know. *Medical Microbiology and Immunology*, *205*(6), 513–535. https://doi.org/10.1007/S00430-016-0470-1

Gringhuis, S. I., den Dunnen, J., Litjens, M., van der Vlist, M., and Geijtenbeek, T. B. H. [2009]. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nature Immunology*, *10*(10), 1081–1088. https://doi.org/10.1038/ni.1778

Guerin, M. E., Korduláková, J., Alzari, P. M., Brennan, P. J., and Jackson, M. [2010]. Molecular Basis of Phosphatidyl-myo-inositol Mannoside Biosynthesis and Regulation in Mycobacteria. *Journal of Biological Chemistry*, *285*(44), 33577–33583. https://doi.org/10.1074/jbc.R110.168328

Guilliams, M., Lambrecht, B. N., and Hammad, H. [2013]. Division of labor between lung dendritic cells and macrophages in the defense against pulmonary infections. *Mucosal Immunology*, *6*(3), 464–473. https://doi.org/10.1038/mi.2013.14

Gupta, V., Jaiswal, A., Behera, D., and Prasad, H. K. [2010]. Disparity in circulating peripheral blood dendritic cell subsets and cytokine profile of pulmonary tuberculosis patients compared with healthy family contacts. *Human Immunology*, *71*(7), 682–691. https://doi.org/10.1016/j.humimm.2010.03.010

Guyot-Revol, V., Innes, J. A., Hackforth, S., Hinks, T., and Lalvani, A. [2006]. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, *173*(7), 803–810. https://doi.org/10.1164/rccm.200508-1294OC

Hamasur, B., Haile, M., Pawlowski, A., Schroder, U., Kallenius, G., and Svenson, S. B. [2004]. A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab') fragment prolong survival of mice infected with Mycobacterium tuberculosis. *Clinical and Experimental Immunology*, *138*(1), 30–38. https://doi.org/10.1111/j.1365-2249.2004.02593.x

Harari, A., Rozot, V., Enders, F. B., Perreau, M., Stalder, M., Nicod, L. P., ... Hanekom, W. A. [2011]. Dominant TNF-α+ Mycobacterium tuberculosis–specific CD4+ T cell responses discriminate between latent infection and active disease. *Nature Medicine*, *17*(3), 372–376. https://doi.org/10.1038/nm.2299.Dominant

Heitmann, L., Schoenen, H., Ehlers, S., Lang, R., and Hölscher, C. [2013]. Mincle is not essential for controlling Mycobacterium tuberculosis infection. *Immunobiology*, *218*(4), 506–516. https://doi.org/10.1016/j.imbio.2012.06.005

Hernandez, J., Velazquez, C., Valenzuela, O., Robles-Zepeda, R., Ruiz-Bustos, E., Navarro, M., and Garibay-Escobar, A. [2010]. Low Number of Peripheral Blood B Lymphocytes in Patients with Pulmonary Tuberculosis. *Immunological Investigations*, *39*(3), 197–205. https://doi.org/10.3109/08820130903586346

Ho, C. S., Feng, P. J. I., Narita, M., Stout, J. E., Chen, M., Pascopella, L., ... Miller, T. [2022]. Comparison of three tests for latent tuberculosis infection in high-risk people in the USA: an observational cohort study. *The Lancet Infectious Diseases*, *22*(1), 85–96. https://doi.org/10.1016/S1473-3099(21)00145-6

Houben, R. M. G. J., and Dodd, P. J. [2016]. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. *PLOS Medicine*, *13*(10), 1–13. https://doi.org/10.1371/journal.pmed.1002152

Huang, S. [2016]. Targeting innate-like T cells in tuberculosis. *Frontiers in Immunology*, *7*(594), 1–19. https://doi.org/10.3389/FIMMU.2016.00594/BIBTEX

Hunter, S. W., and Brennan, P. J. [1990]. Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of Mycobacterium tuberculosis. *Journal of Biological Chemistry*, *265*(16), 9272–9279. https://doi.org/10.1016/s0021-9258(19)38843-x

Hwang, J. R., Byeon, Y., Kim, D., and Park, S. G. [2020]. Recent insights of T cell receptor-mediated signaling pathways for T cell activation and development. *Experimental & Molecular Medicine 2020 52:5*, *52*(5), 750–761. https://doi.org/10.1038/s12276-020-0435-8

Ifrim, D. C., Quintin, J., Joosten, L. A. B., Jacobs, C., Jansen, T., Jacobs, L., ... Netea, M. G. [2014]. Trained Immunity or Tolerance: Opposing Functional Programs Induced in Human Monocytes after Engagement of Various Pattern Recognition Receptors. *Clinical and Vaccine Immunology*, *21*(4), 534–545. https://doi.org/10.1128/CVI.00688-13

Inngjerdingen, M., Kveberg, L., Naper, C., and Vaage, J. T. [2011]. Natural killer cell subsets in man and rodents. *Tissue Antigens*, *78*(2), 81–88. https://doi.org/10.1111/j.1399-0039.2011.01714.x

Ishikawa, E., Ishikawa, T., Morita, Y. S., Toyonaga, K., Yamada, H., Takeuchi, O., ... Yamasaki, S. [2009]. Direct recognition of the mycobacterial glycolipid, trehalose dimycolate, by C-type lectin Mincle. *The Journal of Experimental Medicine*, *206*(13), 2879. https://doi.org/10.1084/JEM.20091750

Ishikawa, E., Mori, D., and Yamasaki, S. [2017]. Recognition of Mycobacterial Lipids by Immune Receptors. *Trends in Immunology*, *38*(1), 66–76. https://doi.org/10.1016/J.IT.2016.10.009

James, C. A., and Seshadri, C. [2020]. T Cell Responses to Mycobacterial Glycolipids: On the Spectrum of "Innateness". *Frontiers in Immunology*, *11*, 170. https://doi.org/10.3389/FIMMU.2020.00170/BIBTEX

Jiang, J., Yang, B., An, H., Wang, X., Liu, Y., Cao, Z., ... Cheng, X. [2016]. Mucosal-associated invariant T cells from patients with tuberculosis exhibit impaired immune response. *Journal of Infection*, *72*(3), 338–352. https://doi.org/10.1016/j.jinf.2015.11.010

Johansson, U., Ivanyi, J., and Londei, M. [2001]. Inhibition of IL-12 production in human dendritic cells matured in the presence of Bacillus Calmette-Guerin or lipoarabinomannan. *Immunology Letters*, *77*(1), 63–66. https://doi.org/10.1016/S0165-2478(01)00190-0

Joosten, S. A., Van Meijgaarden, K. E., Arend, S. M., Prins, C., Oftung, F., Korsvold, G. E., ... Ottenhoff, T. H. M. [2018]. Mycobacterial growth inhibition is associated with trained innate immunity. *Journal of Clinical Investigation*, *128*(5), 1837–1851. https://doi.org/10.1172/JCI97508

Joosten, S. A., van Meijgaarden, K. E., del Nonno, F., Baiocchini, A., Petrone, L., Vanini, V., ... Ottenhoff, T. H. M. [2016]. Patients with Tuberculosis Have a Dysfunctional Circulating B-Cell Compartment, Which Normalizes following Successful Treatment. *PLOS Pathogens*, *12*(6), e1005687. https://doi.org/10.1371/journal.ppat.1005687

Jurado, J. O., Pasquinelli, V., Alvarez, I. B., Peña, D., Rovetta, A. I., Tateosian, N. L., ... García, V. E. [2011]. IL-17 and IFN-expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. *J. Leukoc. Biol*, *91*, 991–1002. https://doi.org/10.1189/jlb.1211619

Källenius, G., Correia-Neves, M., Buteme, H., Hamasur, B., and Svenson, S. B. [2016]. Lipoarabinomannan, and its related glycolipids, induce divergent and opposing immune responses to Mycobacterium tuberculosis depending on structural diversity and experimental variations. *Tuberculosis*, *96*, 120–130. https://doi.org/10.1016/j.tube.2015.09.005

Kalscheuer, R., Palacios, A., Anso, I., Cifuente, J., Anguita, J., Jacobs, W. R., Guerin, M. E., and Prados-Rosales, R. [2019]. The Mycobacterium tuberculosis capsule: a cell structure with key implications in pathogenesis. *Biochemical Journal*, *476*(14), 1995–2016. https://doi.org/10.1042/BCJ20190324

Kanabalan, R. D., Lee, L. J., Lee, T. Y., Chong, P. P., Hassan, L., Ismail, R., and Chin, V. K. [2021].

Human tuberculosis and Mycobacterium tuberculosis complex: A review on genetic diversity, pathogenesis and omics approaches in host biomarkers discovery. *Microbiological Research*, *246*(126674), 1–18. https://doi.org/10.1016/j.micres.2020.126674

Kang, P. B., Azad, A. K., Torrelles, J. B., Kaufman, T. M., Beharka, A., Tibesar, E., DesJardin, L. E., and Schlesinger, L. S. [2005]. The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis. *The Journal of Experimental Medicine*, *202*(7), 987. https://doi.org/10.1084/JEM.20051239

Karim, A. F., Sande, O. J., Tomechko, S. E., Ding, X., Li, M., Maxwell, S., ... Boom, W. H. [2017]. Proteomics and Network Analyses Reveal Inhibition of Akt-mTOR Signaling in CD4+ T Cells by Mycobacterium tuberculosis Mannose-Capped Lipoarabinomannan. *Proteomics*, *17*(22), 1–13. https://doi.org/10.1002/pmic.201700233

Kaufmann, E., Sanz, J., Dunn, J. L., Khan, N., Mendonça, L. E., Pacis, A., ... Divangahi, M. [2018]. BCG Educates Hematopoietic Stem Cells to Generate Protective Innate Immunity against Tuberculosis. *Cell*, *172*(1–2), 176–190. https://doi.org/10.1016/j.cell.2017.12.031

Kee, S. J., Kwon, Y. S., Park, Y. W., Cho, Y. N., Lee, S. J., Kim, T. J., ... Ryang, D. W. [2012]. Dysfunction of natural killer T cells in patients with active Mycobacterium tuberculosis infection. *Infection and Immunity*, *80*(6), 2100–2108. https://doi.org/10.1128/IAI.06018-11

Kim, K., Perera, R., Tan, D. B. A., Fernandez, S., Seddiki, N., Waring, J., and French, M. A. [2014]. Circulating mycobacterial-reactive CD4+ T cells with an immunosuppressive phenotype are higher in active tuberculosis than latent tuberculosis infection. *Tuberculosis*, *94*(5), 494–501. https://doi.org/10.1016/j.tube.2014.07.002

Kleinnijenhuis, J., Joosten, L. A. B., van de Veerdonk, F. L., Savage, N., van Crevel, R., Kullberg, B. J., ... Netea, M. G. [2009]. Transcriptional and inflammasome-mediated pathways for the induction of IL-1β production by Mycobacterium tuberculosis. *European Journal of Immunology*, *39*(7), 1914–1922. https://doi.org/10.1002/EJI.200839115

Kleinnijenhuis, J., Oosting, M., Joosten, L. A. B., Netea, M. G., and Van Crevel, R. [2011]. Innate Immune Recognition of Mycobacterium tuberculosis. *Clinical and Developmental Immunology*, *2011*(405310), 1–12. https://doi.org/10.1155/2011/405310

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A. B., Jacobs, C., Xavier, R. J., van der Meer, J. W. M., van Crevel, R., and Netea, M. G. [2014]. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clinical Immunology*, *155*(2), 213–219. https://doi.org/10.1016/j.clim.2014.10.005

Knutson, K. L., Hmama, Z., Herrera-Velit, P., Rochford, R., and Reiner, N. E. [1998]. Lipoarabinomannan of Mycobacterium tuberculosis promotes protein tyrosine dephosphorylation and inhibition of mitogen-activated protein kinase in human mononuclear phagocytes. *Journal of Biological Chemistry*, *273*(1), 645–652. https://doi.org/10.1074/jbc.273.1.645

Koch, R. [1982]. The Etiology of Tuberculosis. *Clinical Infectious Diseases*, *4*(6), 1270–1274. https://doi.org/10.1093/clinids/4.6.1270

Krutikov, M., Faust, L., Nikolayevskyy, V., Hamada, Y., Gupta, R. K., Cirillo, D., ... Rangaka, M. X. [2022]. The diagnostic performance of novel skin-based in-vivo tests for tuberculosis infection compared with purified protein derivative tuberculin skin tests and blood-based in vitro interferon-γ release assays: a systematic review and meta-analysis. *The Lancet Infectious Diseases*, *22*(2), 250–264. https://doi.org/10.1016/S1473-3099(21)00261-9

Kumar, S. K., Singh, P., and Sinha, S. [2015]. Naturally produced opsonizing antibodies restrict the survival of Mycobacterium tuberculosis in human macrophages by augmenting phagosome maturation. *Open Biology*, *5*(12). https://doi.org/10.1098/RSOB.150171

Lawn, S. D., and Zumla, A. I. [2011]. Tuberculosis. *The Lancet*, *378*(9785), 57–72. https://doi.org/10.1016/S0140-6736(10)62173-3

Lenzini, L., Rottoli, P., and Rottoli, L. [1977]. The spectrum of human tuberculosis. *Clinical and Experimental Immunology, 27*(2), 230–237.

Li, F., Xu, M., Qin, C., Xia, L., Xiong, Y., Xi, X., ... Wang, G. [2016]. Recombinant fusion ESAT6-CFP10 immunogen as a skin test reagent for tuberculosis diagnosis: an open-label, randomized, two-centre phase 2a clinical trial. *Clinical Microbiology and Infection, 22*(10), 889.e9-889.e16. https://doi.org/10.1016/j.cmi.2016.07.015

Li, H., and Javid, B. [2018]. Antibodies and tuberculosis: finally coming of age? *Nature Reviews Immunology, 18*(9), 591–596. https://doi.org/10.1038/s41577-018-0028-0

Li, H., Wang, X. X., Wang, B., Fu, L., Liu, G., Lu, Y., Cao, M., Huang, H., and Javid, B. [2017]. Latently and uninfected healthcare workers exposed to TB make protective antibodies against Mycobacterium tuberculosis. *Proceedings of the National Academy of Sciences, 114*(19), 5023–5028. https://doi.org/10.1073/PNAS.1611776114/-/DCSUPPLEMENTAL

Lin, P. L., Ford, C. B., Coleman, M. T., Myers, A. J., Gawande, R., Ioerger, T., Sacchettini, J., Fortune, S. M., and Flynn, J. L. [2013]. Sterilization of granulomas is common in active and latent tuberculosis despite within-host variability in bacterial killing. *Nature Medicine 2013 20:1, 20*(1), 75–79. https://doi.org/10.1038/nm.3412

Liu, Y., Wang, R., Jiang, J., Cao, Z., Zhai, F., Sun, W., and Cheng, X. [2018]. A subset of CD1c+ dendritic cells is increased in patients with tuberculosis and promotes Th17 cell polarization. *Tuberculosis, 113*, 189–199. https://doi.org/10.1016/j.tube.2018.10.007

Lopez-Vergè, S., Milush, J. M., Pandey, S., York, V. A., Arakawa-Hoyt, J., Pircher, H., Norris, P. J., Nixon, D. F., and Lanier, L. L. [2010]. CD57 defines a functionally distinct population of mature NK cells in the human CD56dimCD16+ NK-cell subset. *Blood, 116*(19), 3865–3874. https://doi.org/10.1182/blood-2010-04-282301

Lu, Y. Bin, Xiao, D. Q., Liang, K. Di, Zhang, J. A., Wang, W. D., Yu, S. Y., ... Xu, J. F. [2017]. Profiling dendritic cell subsets in the patients with active pulmonary tuberculosis. *Molecular Immunology, 91*(1), 86–96. https://doi.org/10.1016/j.molimm.2017.08.007

Lu, L. L., Chung, A. W., Rosebrock, T. R., Ghebremichael, M., Yu, W. H., Grace, P. S., ... Alter, G. [2016]. A Functional Role for Antibodies in Tuberculosis. *Cell, 167*(2), 433-443.e14. https://doi.org/10.1016/j.cell.2016.08.072

Lu, L. L., Das, J., Grace, P. S., Fortune, S. M., Restrepo, B. I., and Alter, G. [2020]. Antibody Fc Glycosylation Discriminates Between Latent and Active Tuberculosis. *The Journal of Infectious Diseases, 222*(12), 2093–2102. https://doi.org/10.1093/infdis/jiz643

Lu, L. L., Smith, M. T., Yu, K. K. Q., Luedemann, C., Suscovich, T. J., Grace, P. S., ... Alter, G. [2019]. IFN-γ-independent immune markers of Mycobacterium tuberculosis exposure. *Nature Medicine, 25*(6), 977–987. https://doi.org/10.1038/s41591-019-0441-3

Lyadova, I. V., and Panteleev, A. V. [2015]. Th1 and Th17 Cells in Tuberculosis: Protection, Pathology, and Biomarkers. *Mediators of Inflammation, 2015*. https://doi.org/10.1155/2015/854507

Mahon, R. N., Sande, O. J., Rojas, R. E., Levine, A. D., Harding, C. V., and Henry Boom, W. [2012]. Mycobacterium tuberculosis ManLAM inhibits T-cell-receptor signaling by interference with ZAP-70, Lck and LAT phosphorylation. *Cellular Immunology, 275*(1–2), 98–105. https://doi.org/10.1016/j.cellimm.2012.02.009

Malka-Ruimy, C., Youssef, G. Ben, Lambert, M., Tourret, M., Ghazarian, L., Faye, A., Caillat-Zucman, S., and Houdouin, V. [2019]. Mucosal-associated invariant T cell levels are reduced in the peripheral blood and lungs of children with active pulmonary tuberculosis. *Frontiers in Immunology, 10*(FEB), 1–7. https://doi.org/10.3389/fimmu.2019.00206

Marakalala, M. J., Guler, R., Matika, L., Murray, G., Jacobs, M., Brombacher, F., Rothfuchs, A. G., Sher, A., and Brown, G. D. [2011]. The Syk/CARD9-coupled receptor Dectin-1 is not required for host resistance to Mycobacterium tuberculosis in mice. *Microbes and Infection / Institut Pasteur, 13*(2),

198–201. https://doi.org/10.1016/J.MICINF.2010.10.013

Marín, N. D., París, S. C., Rojas, M., and García, L. F. [2012]. Reduced frequency of memory T cells and increased Th17 responses in patients with active tuberculosis. *Clinical and Vaccine Immunology*, *19*(10), 1667–1676. https://doi.org/10.1128/CVI.00390-12

Martineau, A. R., Newton, S. M., Wilkinson, K. A., Kampmann, B., Hall, B. M., Nawroly, N., … Wilkinson, R. J. [2007]. Neutrophil-mediated innate immune resistance to mycobacteria. *Journal of Clinical Investigation*, *117*(7), 1988–1994. https://doi.org/10.1172/JCI31097

Martinez-Pomares, L. [2012]. The mannose receptor. *Journal of Leukocyte Biology*, *92*(6), 1177–1186. https://doi.org/10.1189/JLB.0512231

Master, S. S., Davis, A. S., Rampini, S. K., Keller, C., Ehlers, S., Springer, B., Sander, P., and Deretic, V. [2008]. Mycobacterium tuberculosis Prevents Inflammasome Activation. *Cell Host and Microbe*, *3*(4), 224–232. https://doi.org/10.1016/j.chom.2008.03.003

Mathison, J. C., Virca, G. D., Wolfson, E., Tobias, P. S., Glaser, K., and Ulevith, R. J. [1990]. Adaptation to bacterial lipopolysaccharide controls lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages. *Journal of Clinical Investigation*, *85*(4), 1108–1118. https://doi.org/10.1172/JCI114542

Matic, M., and Simon, S. R. [1991]. Tumor necrosis factor release from lipopolysaccharide-stimulated human monocytes: Lipopolysaccharide tolerance in vitro. *Cytokine*, *3*(6), 576–583.

Matsunaga, I., and Moody, D. B. [2009]. Mincle is a long sought receptor for mycobacterial cord factor. *The Journal of Experimental Medicine*, *206*(13), 2865–2868. https://doi.org/10.1084/JEM.20092533

Mazurek, J., Ignatowicz, L., Kallenius, G., Svenson, S. B., Pawlowski, A., and Hamasur, B. [2012]. Divergent Effects of Mycobacterial Cell Wall Glycolipids on Maturation and Function of Human Monocyte-Derived Dendritic Cells. *PLoS ONE*, *7*(8), e42515. https://doi.org/10.1371/journal.pone.0042515

Mendelson, M., Hanekom, W. A., Ntutela, S., Vogt, M., Steyn, L., Maartens, G., and Kaplan, G. [2006]. Quantitative and functional differences between peripheral blood myeloid dendritic cells from patients with pleural and parenchymal lung tuberculosis. *Clinical and Vaccine Immunology*, *13*(12), 1299–1306. https://doi.org/10.1128/CVI.00132-06

Meroueh, S. O., Bencze, K. Z., Hesek, D., Lee, M., Fisher, J. F., Stemmler, T. L., and Mobashery, S. [2006]. Three-dimensional structure of the bacterial cell wall peptidoglycan. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(12), 4404–4409. https://doi.org/10.1073/pnas.0510182103

Mogensen, T. H. [2009]. Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical Microbiology Reviews*, *22*(2), 240. https://doi.org/10.1128/CMR.00046-08

Moreira-Teixeira, L., Mayer-Barber, K., Sher, A., and O'Garra, A. [2018]. Type I interferons in tuberculosis: Foe and occasionally friend. *Journal of Experimental Medicine*, *215*(5), 1273–1285. https://doi.org/10.1084/jem.20180325

Moreno, C., Narayan, V., Akbar, M. S., Ahmed, S., Costello, A. M. L., Abou-Zeid, C., Rook, G. A. W., Stanford, J., and Stanford, J. [1992]. Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis? *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *86*(6), 686–692. https://doi.org/10.1016/0035-9203(92)90192-F

Moreno, C., Taverne, J., Mehlert, A., Bate, C. A. W., Brealey, R. J., Meager, A., Rook, G. A. W., and Playfair, J. H. L. [1989]. Lipoarabinomannan from Mycobacterium tuberculosis induces the production of tumor necrosis factor from human and murine macrophages. *Clinical and Experimental Immunology*, *76*(2), 240–245.

Morgan, J., Muskat, K., Tippalagama, R., Sette, A., Burel, J., and Lindestam Arlehamn, C. S. [2021].

Classical CD4 T cells as the cornerstone of antimycobacterial immunity. *Immunological Reviews*, *301*(1), 10–29. https://doi.org/10.1111/imr.12963

Morrison, J., Pai, M., and Hopewell, P. C. [2008]. Tuberculosis and latent tuberculosis infection in close contacts of people with pulmonary tuberculosis in low-income and middle-income countries: a systematic review and meta-analysis. *The Lancet Infectious Diseases*, *8*(6), 359–368. https://doi.org/10.1016/S1473-3099(08)70071-9

Nalugwa, T., Shete, P. B., Nantale, M., Farr, K., Ojok, C., Ochom, E., ... Katamba, A. [2020]. Challenges with scale-up of GeneXpert MTB/RIF® in Uganda: a health systems perspective. *BMC Health Services Research*, *20*(162), 1–7. https://doi.org/10.1186/s12913-020-4997-x

Netea, M. G., Domínguez-Andrés, J., Barreiro, L. B., Chavakis, T., Divangahi, M., Fuchs, E., ... Latz, E. [2020]. Defining trained immunity and its role in health and disease. *Nature Reviews Immunology*, 1–14. https://doi.org/10.1038/s41577-020-0285-6

Netea, M. G., Quintin, J., and Van Der Meer, J. W. M. [2011]. Trained immunity: A memory for innate host defense. *Cell Host and Microbe*, *9*(5), 355–361. https://doi.org/10.1016/j.chom.2011.04.006

Nigou, J. [2003]. Lipoarabinomannans: from structure to biosynthesis. *Biochimie*, *85*(1–2), 153–166. https://doi.org/10.1016/S0300-9084(03)00048-8

Nigou, J., Gilleron, M., Cahuzac, B., Bounéry, J. D., Herold, M., Thurnher, M., and Puzo, G. [1997]. The phosphatidyl-myo-inositol anchor of the lipoarabinomannans from Mycobacterium bovis bacillus Calmette Guerin: Heterogeneity, structure, and role in the regulation of cytokine secretion. *Journal of Biological Chemistry*, *272*(37), 23094–23103. https://doi.org/10.1074/jbc.272.37.23094

Nigou, J., Gilleron, M., Rojas, M., García, L. F., Thurnher, M., and Puzo, G. [2002]. Mycobacterial lipoarabinomannans: Modulators of dendritic cell function and the apoptotic response. *Microbes and Infection*, *4*(9), 945–953. https://doi.org/10.1016/S1286-4579(02)01621-0

Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M., and Puzo, G. [2001]. Mannosylated Lipoarabinomannans Inhibit IL-12 Production by Human Dendritic Cells: Evidence for a Negative Signal Delivered Through the Mannose Receptor. *The Journal of Immunology*, *166*(12), 7477–7485. https://doi.org/10.4049/jimmunol.166.12.7477

Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Jayaraman, P., Rothchild, A. C., and Behar, S. M. [2014]. In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, *12*(4), 289–299. https://doi.org/10.1038/nrmicro3230.In

O'Garra, A., Redford, P. S., McNab, F. W., Bloom, C. I., Wilkinson, R. J., and Berry, M. P. R. [2013]. The Immune Response in Tuberculosis. *Annual Review of Immunology*, *31*(1), 475–527. https://doi.org/10.1146/annurev-immunol-032712-095939

Olive, A. J., and Sassetti, C. M. [2018]. Tolerating the Unwelcome Guest; How the Host Withstands Persistent Mycobacterium tuberculosis. *Frontiers in Immunology*, *9*(2094), 1–8. https://doi.org/10.3389/fimmu.2018.02094

Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., ... Raviglione, M. [2016]. Tuberculosis. *Nature Reviews Disease Primers*, *2*(1), 16076. https://doi.org/10.1038/nrdp.2016.76

Pai, M., Denkinger, C. M., Kik, S. V., Rangaka, M. X., Zwerling, A., Oxlade, O., ... Banaei, N. [2014]. Gamma Interferon Release Assays for Detection of Mycobacterium tuberculosis Infection. *Clinical Microbiology Reviews*, *27*(1), 1–20. https://doi.org/10.1128/CMR.00034-13

Pai, M., Kasaeva, T., and Swaminathan, S. [2022]. Covid-19's Devastating Effect on Tuberculosis Care- A Path to Recovery. *The New England Journal of Medicine*. https://doi.org/10.1056/NEJMp2118145

Paquin-Proulx, D., Costa, P. R., Silveira, C. G. T., Marmorato, M. P., Cerqueira, N. B., Sutton, M. S., ... Kallas, E. G. [2018]. Latent Mycobacterium tuberculosis infection is associated with a higher

frequency of mucosal-associated invariant T and invariant natural killer T cells. *Frontiers in Immunology*, *9*(JUN). https://doi.org/10.3389/fimmu.2018.01394

Paust, S., Gill, H. S., Wang, B. Z., Flynn, M. P., Moseman, E. A., Senman, B., ... Von Andrian, U. H. [2010]. Critical role for the chemokine receptor CXCR6 in NK cell–mediated antigen-specific memory of haptens and viruses. *Nature Immunology*, *11*(12), 1127–1135. https://doi.org/10.1038/ni.1953

Petruccioli, E., Petrone, L., Vanini, V., Sampaolesi, A., Gualano, G., Girardi, E., Palmieri, F., and Goletti, D. [2013]. IFNγ/TNFα specific-cells and effector memory phenotype associate with active tuberculosis. *Journal of Infection*, *66*(6), 475–486. https://doi.org/10.1016/j.jinf.2013.02.004

Philips, J. A., and Ernst, J. D. [2012]. Tuberculosis Pathogenesis and Immunity. *Annual Review of Pathology: Mechanisms of Disease*, *7*(1), 353–384. https://doi.org/10.1146/annurev-pathol-011811-132458

Pitarquea, S., Larrouy-Maumusa, G., Payréb, B., Jacksonc, M., Puzoa, G., and Nigou, J. [2012]. The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface. *Tuberculosis*, *78*(2), 711–716. https://doi.org/10.1016/j.tube.2008.04.002.The

Pollara, G., Turner, C. T., Rosenheim, J., Chandran, A., Bell, L. C. K., Khan, A., ... Noursadeghi, M. [2021]. Exaggerated IL-17A activity in human in vivo recall responses discriminates active tuberculosis from latent infection and cured disease. *Science Translational Medicine*, *13*(592). https://doi.org/10.1126/scitranslmed.abg7673

Prigozy, T. I., Sieling, P. A., Clemens, D., Stewart, P. L., Behar, S. M., Porcelli, S. A., Brenner, M. B., Modlin, R. L., and Kronenberg, M. [1997]. The mannose receptor delivers lipoglycan antigens to endosomes for presentation to T cells by CD1b molecules. *Immunity*, *6*(2), 187–197. https://doi.org/10.1016/S1074-7613(00)80425-2

Public Health Agency of Sweden. [2021]. Tuberculosis - disease statistics. Retrieved 17 August 2022, from https://www.folkhalsomyndigheten.se/folkhalsorapportering-statistik/statistik-a-o/sjukdomsstatistik/tuberkulos/

Qiu, Z., Zhang, M., Zhu, Y., Zheng, F., Lu, P., Liu, H., Graner, M. W., Zhou, B., and Chen, X. [2012]. Multifunctional CD4 T cell responses in patients with active tuberculosis. *Scientific Reports*, *2*, 2–7. https://doi.org/10.1038/srep00216

Queiroz, A., and Riley, L. W. [2017]. Bacterial immunostat: Mycobacterium tuberculosis lipids and their role in the host immune response. *Revista Da Sociedade Brasileira de Medicina Tropical*, *50*(1), 9–18. https://doi.org/10.1590/0037-8682-0230-2016

Rangaka, M. X., Wilkinson, K. A., Glynn, J. R., Ling, D., Menzies, D., Mwansa-Kambafwile, J., Fielding, K., Wilkinson, R. J., and Pai, M. [2012]. Predictive value of interferon-γ release assays for incident active tuberculosis: a systematic review and meta-analysis. *Lancet Infectious Diseases*, *12*(1), 45–55. https://doi.org/10.1016/S1473-3099(11)70210-9

Ravesloot-Chavez, M. M., Van Dis, E., and Stanley, S. A. [2021]. The Innate Immune Response to Mycobacterium tuberculosis Infection. *Annual Review of Immunology*, *39*, 611–637. https://doi.org/10.1146/ANNUREV-IMMUNOL-093019-010426

Reed, M. B., Domenech, P., Manca, C., Su, H., Barczak, A. K., Kreiswirth, B. N., and Kaplan, G. [2004]. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. *Nature*, *431*(7004), 84–87. https://doi.org/10.1038/nature02837

Reiling, N., Hölscher, C., Fehrenbach, A., Kröger, S., Kirschning, C. J., Goyert, S., and Ehlers, S. [2002]. Cutting Edge: Toll-Like Receptor (TLR)2- and TLR4-Mediated Pathogen Recognition in Resistance to Airborne Infection with Mycobacterium tuberculosis. *The Journal of Immunology*, *169*(7), 3480–3484. https://doi.org/10.4049/JIMMUNOL.169.7.3480

Rijnink, W. F., Ottenhoff, T. H. M., and Joosten, S. A. [2021]. B-Cells and Antibodies as Contributors to

Effector Immune Responses in Tuberculosis. *Frontiers in Immunology*, *12*(640168), 1–19. https://doi.org/10.3389/fimmu.2021.640168

Roach, T. I. A., Barton, C. H., Chatterjee, D., Liew, F. Y., and Blackwell, J. M. [1995]. Opposing effects of interferon-γ on iNOS and interleukin-10 expression in lipopolysaccharide- and mycobacterial lipoarabinomannan-stimulated macrophages. *Immunology*, *85*(1), 106–113.

Roach, T. I., Barton, C. H., Chatterjee, D., and Blackwell, J. M. [1993]. Macrophage activation: lipoarabinomannan from avirulent and virulent strains of Mycobacterium tuberculosis differentially induces the early genes c-fos, KC, JE, and tumor necrosis factor-alpha. *Journal of Immunology*, *150*(5), 1886–1896. https://doi.org/10.1091/MBC.E03-05-0307

Roy Chowdhury, R., Vallania, F., Yang, Q., Lopez Angel, C. J., Darboe, F., Penn-Nicholson, A., ... Chien, Y. [2018]. A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. *Nature*, *560*(7720), 644–648. https://doi.org/10.1038/s41586-018-0439-x

Ruhwald, M., Aggerbeck, H., Gallardo, R. V., Hoff, S. T., Villate, J. I., Borregaard, B., ... Caylá, J. A. [2017]. Safety and efficacy of the C-Tb skin test to diagnose Mycobacterium tuberculosis infection, compared with an interferon γ release assay and the tuberculin skin test: a phase 3, double-blind, randomised, controlled trial. *The Lancet Respiratory Medicine*, *5*(4), 259–268. https://doi.org/10.1016/S2213-2600(16)30436-2

Sabin, S., Herbig, A., Vågene, Å. J., Ahlström, T., Bozovic, G., Arcini, C., Kühnert, D., and Bos, K. I. [2020]. A seventeenth-century Mycobacterium tuberculosis genome supports a Neolithic emergence of the Mycobacterium tuberculosis complex. *Genome Biology*, *21*(201), 1–24. https://doi.org/10.1186/S13059-020-02112-1/FIGURES/5

Sada-Ovalle, I., Chiba, A., Gonzales, A., Brenner, M. B., and Behar, S. M. [2008]. Innate invariant NKT cells recognize Mycobacterium tuberculosis-infected macrophages, produce interferon-γ, and kill intracellular bacteria. *PLoS Pathogens*, *4*(12). https://doi.org/10.1371/journal.ppat.1000239

Sakula, A. [1983]. Robert Koch: Centenary of the Discovery of the Tubercle Bacillus, 1882. *The Canadian Veterinary Journal*, *24*(4), 127.

Salle, H. de la, Mariotti, S., Angenieux, C., Gilleron, M., Garcia-alles, F., Malm, D., ... Libero, G. de. [2005]. Assistance of microbial glycolipid antigen processing by CD1e. *Science*, *310*(5752), 1321–1324.

Sampath, P., Moideen, K., Ranganathan, U. D., and Bethunaickan, R. [2018]. Monocyte Subsets: Phenotypes and Function in Tuberculosis Infection. *Frontiers in Immunology*, *9*(1726), 1–8. https://doi.org/10.3389/fimmu.2018.01726

Sánchez, M. D., García, Y., Montes, C., París, S. C., Rojas, M., Barrera, L. F., Arias, M. A., and García, L. F. [2006]. Functional and phenotypic changes in monocytes from patients with tuberculosis are reversed with treatment. *Microbes and Infection*, *8*(9–10), 2492–2500. https://doi.org/10.1016/j.micinf.2006.06.005

Sande, O. J., Karim, A. F., Li, Q., Ding, X., Harding, C. V., Rojas, R. E., and Boom, W. H. [2016]. Mannose-Capped Lipoarabinomannan from Mycobacterium tuberculosis Induces CD4 + T Cell Anergy via GRAIL. *The Journal of Immunology*, *196*(2), 691–702. https://doi.org/10.4049/jimmunol.1500710

Scriba, T. J., Kalsdorf, B., Abrahams, D.-A., Isaacs, F., Hofmeister, J., Black, G., ... Hanekom, W. A. [2008]. Distinct, Specific IL-17- and IL-22-Producing CD4 + T Cell Subsets Contribute to the Human Anti-Mycobacterial Immune Response . *The Journal of Immunology*, *180*(3), 1962–1970. https://doi.org/10.4049/jimmunol.180.3.1962

Seibert, F. B., and Glenn, J. T. [1941]. Tuberculin Purified Protein Derivative - Preparation and analyses of a large quantity for standard. *American Review of Tuberculosis*, *44*(1), 9–25.

Shabaana, A. K., Kulangara, K., Semac, I., Parel, Y., Ilangumaran, S., Dharmalingam, K., Chizzolini, C., and Hoessli, D. C. [2005]. Mycobacterial lipoarabinomannans modulate cytokine production in human T helper cells by interfering with raft/microdomain signalling. *CMLS Cellular and Molecular*

Life Sciences, *62*(2), 179–187. https://doi.org/10.1007/s00018-004-4404-5

Shah, M., and Dorman, S. E. [2021]. Latent Tuberculosis Infection. *New England Journal of Medicine*, *385*(24), 2271–2280. https://doi.org/10.1056/NEJMcp2108501

Sharma, S. K., Mohan, A., and Kohli, M. [2021]. Extrapulmonary tuberculosis. *Expert Review of Respiratory Medicine*, *15*(7), 931–948. https://doi.org/10.1080/17476348.2021.1927718

Sieling, P. A., Chatterjee, D., Porcelli, S. A., Prigozy, T. I., Mazzaccaro, R. J., Soriano, T., ... Modlin, R. L. [1995]. CD1-restricted T cell recognition of microbial lipoglycan antigens. *Science*, *269*(5221), 227–230. https://doi.org/10.1126/science.7542404

Steingart, K. R., Henry, M., Ng, V., Hopewell, P. C., Ramsay, A., Cunningham, J., ... Pai, M. [2006]. Fluorescence versus conventional sputum smear microscopy for tuberculosis: a systematic review. *The Lancet Infectious Diseases*, *6*(9), 570–581. https://doi.org/10.1016/S1473-3099(06)70578-3

Stokas, H., Rhodes, H. L., and Purdy, G. E. [2020]. Modulation of the M. tuberculosis cell envelope between replicating and non-replicating persistent bacteria. *Tuberculosis*, *125*, 102007. https://doi.org/10.1016/J.TUBE.2020.102007

Sutherland, J. S., Adetifa, I. M., Hill, P. C., Adegbola, R. A., and Ota, M. O. C. [2009]. Pattern and diversity of cytokine production differentiates between Mycobacterium tuberculosis infection and disease. *European Journal of Immunology*, *39*(3), 723–729. https://doi.org/10.1002/eji.200838693

Suzuki, Y., Shirai, M., Asada, K., Yasui, H., Karayama, M., Hozumi, H., ... Suda, T. [2018]. Macrophage mannose receptor, CD206, predict prognosis in patients with pulmonary tuberculosis. *Scientific Reports*, *8*(13129). https://doi.org/10.1038/s41598-018-31565-5

Tallieux, L., Schwartz, O., Herrmann, J. L., Pivert, E., Jackson, M., Amara, A., ... Neyrolles, O. [2003]. DC-SIGN Is the Major Mycobacterium tuberculosis Receptor on Human Dendritic Cells. *The Journal of Experimental Medicine*, *197*(1), 121. https://doi.org/10.1084/JEM.20021468

Taylor, P. R., Brown, G. D., Reid, D. M., Willment, J. A., Martinez-Pomares, L., Gordon, S., and Wong, S. Y. C. [2002]. The β-Glucan Receptor, Dectin-1, Is Predominantly Expressed on the Surface of Cells of the Monocyte/Macrophage and Neutrophil Lineages. *The Journal of Immunology*, *169*(7), 3876–3882. https://doi.org/10.4049/JIMMUNOL.169.7.3876

Tian, T., Woodworth, J., Sköld, M., and Behar, S. M. [2005]. In Vivo Depletion of CD11c + Cells Delays the CD4 + T Cell Response to Mycobacterium tuberculosis and Exacerbates the Outcome of Infection . *The Journal of Immunology*, *175*(5), 3268–3272. https://doi.org/10.4049/jimmunol.175.5.3268

Torrado, E., and Cooper, A. M. [2010]. IL-17 and Th17 cells in tuberculosis. *Cytokine & Growth Factor Reviews*, *21*(6), 455. https://doi.org/10.1016/J.CYTOGFR.2010.10.004

Torrado, E., and Cooper, A. M. [2013]. Cytokines in the balance of protection and pathology during mycobacterial infections. *Advances in Experimental Medicine and Biology*, *783*, 121–140. https://doi.org/10.1007/978-1-4614-6111-1_7

Torrelles, J. B., Azad, A. K., and Schlesinger, L. S. [2006]. Fine Discrimination in the Recognition of Individual Species of Phosphatidyl- myo -Inositol Mannosides from Mycobacterium tuberculosis by C-Type Lectin Pattern Recognition Receptors. *The Journal of Immunology*, *177*(3), 1805–1816. https://doi.org/10.4049/jimmunol.177.3.1805

Torrelles, J. B., Khoo, K. H., Sieling, P. A., Modlin, R. L., Zhang, N., Marques, A. M., ... Chatterjee, D. [2004]. Truncated structural variants of lipoarabinomannan in Mycobacterium leprae and an ethambutol-resistant strain of Mycobacterium tuberculosis. *Journal of Biological Chemistry*, *279*(39), 41227–41239. https://doi.org/10.1074/jbc.M405180200

Torrelles, J. B., Sieling, P. A., Zhang, N., Keen, M. A., McNeil, M. R., Belisle, J. T., Modlin, R. L., Brennan, P. J., and Chatterjee, D. [2012]. Isolation of a distinct Mycobacterium tuberculosis mannose-capped lipoarabinomannan isoform responsible for recognition by CD1b-restricted T cells. *Glycobiology*,

22(8), 1118–1127. https://doi.org/10.1093/glycob/cws078

Tourne, S., Maitre, B., Collmann, A., Layre, E., Mariotti, S., Signorino-Gelo, F., ... de la Salle, H. [2008]. Cutting Edge: A Naturally Occurring Mutation in CD1e Impairs Lipid Antigen Presentation. *The Journal of Immunology*, *180*(6), 3642–3646. https://doi.org/10.4049/jimmunol.180.6.3642

Toyonaga, K., Torigoe, S., Motomura, Y., Kamichi, T., Hayashi, J. M., Morita, Y. S., ... Yamasaki, S. [2016]. C-Type Lectin Receptor DCAR Recognizes Mycobacterial Phosphatidyl-Inositol Mannosides to Promote a Th1 Response during Infection. *Immunity*, *45*(6), 1245–1257. https://doi.org/10.1016/j.immuni.2016.10.012

Turner, J., and Torrelles, J. B. [2018]. Mannose-capped lipoarabinomannan in Mycobacterium tuberculosis pathogenesis. *Pathogens and Disease*, *76*(4), 1–15. https://doi.org/10.1093/femspd/fty026

Uehira, K., Amakawa, R., Ito, T., Tajima, K., Naitoh, S., Ozaki, Y., ... Fukuhara, S. [2002]. Dendritic cells are decreased in blood and accumulated in granuloma in tuberculosis. *Clinical Immunology*, *105*(3), 296–303. https://doi.org/10.1006/clim.2002.5287

Van Rhijn, I., and Le Nours, J. [2021]. CD1 and MR1 recognition by human γδ T cells. *Molecular Immunology*, *133*, 95. https://doi.org/10.1016/J.MOLIMM.2020.12.008

Varshney, D., Singh, S., Sinha, E., Mohanty, K. K., Kumar, S., Kumar Barik, S., Patil, S. A., and Katara, P. [2022]. Systematic review and meta-analysis of human Toll-like receptors genetic polymorphisms for susceptibility to tuberculosis infection. *Cytokine*, *152*(155791). https://doi.org/10.1016/J.CYTO.2021.155791

Venkatasubramanian, S., Cheekatla, S., Paidipally, P., Tripathi, D., Welch, E., Tvinnereim, A. R., Nurieva, R., and Vankayalapati, R. [2017]. IL-21-dependent expansion of memory-like NK cells enhances protective immune responses against Mycobacterium tuberculosis. *Mucosal Immunology*, *10*(4), 1031–1042. https://doi.org/10.1038/mi.2016.105

Venkataswamy, M. M., Baena, A., Goldberg, M. F., Bricard, G., Im, J. S., Chan, J., ... Porcelli, S. A. [2009]. Incorporation of NKT cell activating glycolipids enhances immunogenicity and vaccine efficacy of Mycobacterium bovis BCG. *Journal of Immunology*, *183*(3), 1644–1656. https://doi.org/10.4049/jimmunol.0900858.Incorporation

Virca, G. D., Kim, S. Y., Glaser, K. B., and Ulevitch, R. J. [1989]. Lipopolysaccharide induces hyporesponsiveness to its own action in RAW 264.7 cells. *Journal of Biological Chemistry*, *264*(36), 21951–21956. https://doi.org/10.1016/s0021-9258(20)88277-5

Walzl, G., McNerney, R., du Plessis, N., Bates, M., McHugh, T. D., Chegou, N. N., and Zumla, A. [2018]. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. *The Lancet Infectious Diseases*, *18*(7), e199–e210. https://doi.org/10.1016/S1473-3099(18)30111-7

Walzl, G., Ronacher, K., Hanekom, W., Scriba, T. J., and Zumla, A. [2011]. Immunological biomarkers of tuberculosis. *Nature Reviews Immunology*, *11*(5), 343–354. https://doi.org/10.1038/nri2960

Wang, C., Chen, Z.-L., Pan, Z.-F., Wei, L.-L., Xu, D.-D., Jiang, T.-T., ... Li, J.-C. [2014]. NOD2 Polymorphisms and Pulmonary Tuberculosis Susceptibility: A Systematic Review and Meta-Analysis. *International Journal of Biological Sciences*, *10*(1), 103–108. https://doi.org/10.7150/ijbs.7585

Watson, R. O., Bell, S. L., MacDuff, D. A., Kimmey, J. M., Diner, E. J., Olivas, J., ... Cox, J. S. [2015]. The Cytosolic Sensor cGAS Detects Mycobacterium tuberculosis DNA to Induce Type I Interferons and Activate Autophagy. *Cell Host & Microbe*, *17*(6), 811–819. https://doi.org/10.1016/J.CHOM.2015.05.004

Wergeland, I., Amus, J., and Dyrhol-Riise, A. M. [2011]. T Regulatory Cells and Immune Activation in Mycobacterium tuberculosis Infection and the Effect of Preventive Therapy. *Scandinavian Journal of Immunology*, *73*(3), 234–242. https://doi.org/10.1111/j.1365-3083.2010.02496.x

WHO. [1994]. *TB: a global emergency, WHO report on the TB epidemic*. Geneva.

WHO. [2014]. *The end TB strategy - global strategy and targets for tuberculosis prevention, care and control after 2015*. Geneva.

WHO. [2015]. *The use of lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis and screening of active tuberculosis in people living with HIV*.

WHO. [2018]. *Latent tuberculosis infection: Updated and consolidated guidelines for programmatic management*. Geneva.

WHO. [2019]. *Lateral flow urine lipoarabinomannan assay (LF-LAM) for the diagnosis of active tuberculosis in people living with HIV: Policy update*. Geneva.

WHO. [2021a]. *Global Tuberculosis Report*. Geneva.

WHO. [2021b]. *Impact of the COVID-19 pandemic on TB detection and mortality in 2020*.

WHO. [2021c]. *WHO consolidated guidelines on tuberculosis. Module 3: Diagnosis - Rapid diagnostics for tuberculosis detection*. Geneva.

WHO. [2022a]. *Global Tuberculosis Report*. Geneva.

WHO. [2022b]. *Rapid communication: TB antigen-based skin tests for the diagnosis of TB infection*. Geneva.

WHO. [2022c]. *Use of alternative interferon- gamma release assays for the diagnosis of TB infection: Policy Statement*. Geneva.

Wilkinson, K. A., and Wilkinson, R. J. [2010]. Polyfunctional T cells in human tuberculosis. *European Journal of Immunology*, *40*(8), 2139–2142. https://doi.org/10.1002/eji.201040731

Wout, J. W., Poell, R., and Furth, R. [1992]. The Role of BCG/PPD-Activated Macrophages in Resistance against Systemic Candidiasis in Mice. *Scandinavian Journal of Immunology*, *36*(5), 713–720. https://doi.org/10.1111/j.1365-3083.1992.tb03132.x

Wu, T., Guo, S., Wang, J., Li, L., Xu, L., Liu, P., ... Luo, Y. [2011]. Interaction between mannosylated lipoarabinomannan and dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin influences dendritic cells maturation and T cell immunity. *Cellular Immunology*, *272*(1), 94–101. https://doi.org/10.1016/j.cellimm.2011.09.001

Wu, Y. E., Zhang, S. W., Peng, W. G., Li, K. S., Li, K., Jiang, J. K., Lin, J. H., and Cai, Y. [2009]. Changes in lymphocyte subsets in the peripheral blood of patients with active pulmonary tuberculosis. *Journal of International Medical Research*, *37*(6), 1742–1749. https://doi.org/10.1177/147323000903700610

Yang, H., Kruh-Garcia, N. A., and Dobos, K. M. [2012]. Purified Protein Derivatives of Tuberculin - Past, Present, and Future. *FEMS Immunology and Medical Microbiology*, *66*(3), 273. https://doi.org/10.1111/J.1574-695X.2012.01002.X

Yonekawa, A., Saijo, S., Hoshino, Y., Miyake, Y., Ishikawa, E., Suzukawa, M., ... Yamasaki, S. [2014]. Dectin-2 is a direct receptor for mannose-capped lipoarabinomannan of mycobacteria. *Immunity*, *41*(3), 402–413. https://doi.org/10.1016/J.IMMUNI.2014.08.005/ATTACHMENT/F44D1ED6-547C-47B9-BAF0-D76C930F361D/MMC1.PDF

Young, D. B., Gideon, H. P., and Wilkinson, R. J. [2009]. Eliminating latent tuberculosis. *Trends in Microbiology*, *17*(5), 183–188. https://doi.org/10.1016/j.tim.2009.02.005

Young, J. M., Adetifa, I. M. O., Ota, M. O. C., and Sutherland, J. S. [2010]. Expanded polyfunctional T cell response to mycobacterial antigens in TB disease and contraction post-treatment. *PLoS ONE*, *5*(6). https://doi.org/10.1371/journal.pone.0011237

Young, M. H., Navarro, G., Askovich, P., and Aderem, A. [2016]. T cell receptor signaling and Toll-like receptor signaling converge to amplify T cell responses. *The Journal of Immunology*, *196*(1 Supplement).

Yuan, C., Qu, Z.-L., Tang, X.-L., Liu, Q., Luo, W., Huang, C., Pan, Q., and Zhang, X.-L. [2019]. Mycobacterium tuberculosis Mannose-Capped Lipoarabinomannan Induces IL-10-Producing B Cells and Hinders CD4+Th1 Immunity. *IScience*, *11*, 13–30.

https://doi.org/10.1016/J.ISCI.2018.11.039

Zajonc, D. M., Ainge, G. D., Painter, G. F., Severn, W. B., and Wilson, I. A. [2006]. Structural Characterization of Mycobacterial Phosphatidylinositol Mannoside Binding to Mouse CD1d. *The Journal of Immunology*, *177*(7), 4577–4583. https://doi.org/10.4049/jimmunol.177.7.4577

Zellweger, J. P., Sotgiu, G., Block, M., Dore, S., Altet, N., Blunschi, R., ... Lange, C. [2015]. Risk Assessment of Tuberculosis in Contacts by IFN-γ Release Assays. A Tuberculosis Network European Trials Group Study. *American Journal of Respiratory and Critical Care Medicine*, *191*(10), 1176–1184. https://doi.org/10.1164/RCCM.201502-0232OC/SUPPL_FILE/DISCLOSURES.PDF

Zeng, G., Zhang, G., and Chen, X. [2018]. Th1 cytokines, true functional signatures for protective immunity against TB? *Cellular and Molecular Immunology*, *15*(3), 206–215. https://doi.org/10.1038/cmi.2017.113

Zewdie, M., Howe, R., Hoff, S. T., Doherty, T. M., Getachew, N., Tarekegne, A., ... Abebe, M. [2016]. Ex-vivo characterization of regulatory T cells in pulmonary tuberculosis patients, latently infected persons, and healthy endemic controls. *Tuberculosis*, *100*(5), 61–68. https://doi.org/10.1016/j.tube.2016.06.007

Zhong, Y., Kinio, A., and Saleh, M. [2013]. Functions of NOD-Like Receptors in Human Diseases. *Frontiers in Immunology*, *4*(333), 1–18. https://doi.org/10.3389/FIMMU.2013.00333

Zhou, G., Luo, Q., Luo, S., Teng, Z., Ji, Z., Yang, J., ... Bao, F. [2020]. Interferon-γ release assays or tuberculin skin test for detection and management of latent tuberculosis infection: a systematic review and meta-analysis. *The Lancet Infectious Diseases*, *20*(12), 1457–1469. https://doi.org/10.1016/S1473-3099(20)30276-0

Zumla, A., Mwaba, P., Huggett, J., Kapata, N., Chanda, D., and Grange, J. [2009]. Reflections on the white plague. *The Lancet Infectious Diseases*, *9*(3), 197–202. https://doi.org/10.1016/S1473-3099(09)70045-3

CHAPTER II

HIGH DIMENSIONAL IMMUNE PROFILING REVEALS DIFFERENT RESPONSE PATTERNS IN ACTIVE AND LATENT TUBERCULOSIS FOLLOWING STIMULATION WITH MYCOBACTERIAL GLYCOLIPIDS

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High Dimensional Immune Profiling Reveals Different Response Patterns in Active and Latent Tuberculosis Following Stimulation With Mycobacterial Glycolipids

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Upon infection with *Mycobacterium tuberculosis* (Mtb) the host immune response might clear the bacteria, control its growth leading to latent tuberculosis (LTB), or fail to control its growth resulting in active TB (ATB). There is however no clear understanding of the features underlying a more or less effective response. Mtb glycolipids are abundant in the bacterial cell envelope and modulate the immune response to Mtb, but the patterns of response to glycolipids are still underexplored. To identify the CD45^{+} leukocyte activation landscape induced by Mtb glycolipids in peripheral blood of ATB and LTB, we performed a detailed assessment of the immune response of PBMCs to the Mtb glycolipids lipoarabinomannan (LAM) and its biosynthetic precursor phosphatidyl-inositol mannoside (PIM), and purified-protein derivate (PPD). At 24 h of stimulation, cell profiling and secretome analysis was done using mass cytometry and high-multiplex immunoassay. PIM induced a diverse cytokine response, mainly affecting antigen-presenting cells to produce both pro-inflammatory and anti-inflammatory cytokines, but not IFN-γ, contrasting with PPD that was a strong inducer of IFN-γ. The effect of PIM on the antigen-presenting cells was partly TLR2-dependent. Expansion of monocyte subsets in response to PIM or LAM was reduced primarily in LTB as compared to healthy controls, suggesting a hyporesponsive/tolerance pattern derived from Mtb infection.

Keywords: tuberculosis, mycobacterial glycolipids, active tuberculosis (ATB), latent tuberculosis (LTB), hyporesponsiveness, lipoarabinomannan (LAM), phosphatidylinositol mannoside (PIM)

INTRODUCTION

It is estimated that approximately 25% of the world population is latently infected with *Mycobacterium tuberculosis* (Mtb) (1). However, only about 10% of individuals with latent TB (LTB) are estimated to develop active TB (ATB) (2). It is clear therefore that in most cases Mtb infection is well controlled, but our understanding of what makes an effective immune response that controls and/or clears Mtb is limited.

Research on the host response to Mtb has so far mainly focused on protein-based antigens. However, the immune response to Mtb is initiated mainly through the interaction of Mtb cell envelope components, mostly glycolipids, with distinct cells of the innate immune system (3), which trigger activating or repressive responses in terms of cytokine production (4, 5). The ability of Mtb lipids to traffic outside infected cells (6–8) renders the direct contact of Mtb cell envelope glycolipids with distinct immune cells an important aspect of the immune response (9). Lipoarabinomannan (LAM) is a major glycolipid of the Mtb cell wall and has been studied quite extensively for its immunomodulatory properties (10, 11), compared to its biosynthetic precursors, the phosphatidyl-inositol mannosides (PIM_2 and PIM_6). Many host cell receptors take part in the initial interaction between mycobacteria and innate immune cells (12, 13). TLRs and C-type lectins are involved in this process, resulting in activation of several antimicrobial mechanisms by macrophages (Mφs) and dendritic cells (DCs) (14–17).

In addition to the extensive interaction with innate immune cells, PIM and LAM are also both recognized by CD1b-restricted T cells (9, 18–21). In fact, it was observed that purified-protein derivate (PPD) positive individuals respond through CD1-restricted T cells to several mycobacterial lipids, including PIM and LAM (18) and that this response may vary between individuals with ATB and LTB. Mtb whole lipid extract was shown to induce proliferation of CD1-restricted $CD4^+$ and, to a smaller extent, $CD8^+$ T cells in LTB. Interestingly, the same was observed for ATB patients only after the first two weeks of anti-TB treatment (22). A subset of LAM reactive CD1-restricted T cells co-expressing perforin, granulysin, and granzyme B (GrzB), mostly $CD8^+$, are more frequent in LTB than in individuals who developed ATB (evaluated after TB treatment) (22). Similarly, glycerol monomycolate-specific T cells are more frequent in LTB than ATB patients (18) and the response of these cells may vary between ATB and LTB individuals.

B cell-mediated immunity in Mtb infection has been less explored compared to monocyte- and T cell-mediated responses, although recent data strengthen the relevance of these cells in the immune response to Mtb. Recently it was shown that Mtb LAM induces IL-10 production by B cells and that these cells (B10) inhibit $CD4^+$ T_H1 polarization leading to increased Mtb susceptibility in mice (23). The response of B cells to LAM was shown to occur in a TLR2-dependent manner (23).

In the present study, we performed a detailed assessment and simultaneous comparison of the immune response to PIM, LAM and PPD from Mtb in peripheral blood mononuclear cells (PBMCs) from individuals with ATB or LTB and compared with healthy controls (HC). We performed immune profiling by secretome analysis and mass cytometry measuring simultaneously 37 cellular markers at the single-cell level to allow high-resolution of the cellular composition and secretion. We identified distinct subsets within memory T cells, NK cells, B cells and monocytes/DCs that were altered by PPD, PIM and LAM stimulation and further evaluated the role of TLR2 in this process.

MATERIALS AND METHODS

Study Participants

Participants were recruited in 2018 within an ongoing prospective cohort of adult (≥18 years) TB patients and contacts attending the TB Centre, Dept of Infectious Diseases Karolinska University Hospital Stockholm (**Supplemental Table 1**). ATB cases were defined upon microbiological (PCR and/or culture) verification. LTB participants were defined as asymptomatic, IGRA positive, close contacts to ATB cases. Healthy controls (HC) were defined as IGRA negative students and hospital staff without known previous Mtb exposure. Exclusion criteria were pregnancy, autoimmune diseases and HIV co-infection or other immunodeficiencies. ATB and LTB participants were screened with standard biochemical set-up and radiology.

Antigens

Tuberculin PPD (RT 50) was obtained from Statens Serum Institute, and PHA from *In vivo*gen. LAM and PIMs were prepared as previously described in detail (5). Briefly, heat-killed bacteria were frozen and thawed several times, sonicated and extracted in 40% hot phenol for 1 h at 70°C. ManLAM and PIM were obtained from the water and phenol phases respectively. The dialyzed water phase was submitted to affinity chromatography on Concanavalin A-Sepharose. After elution, bound material was subjected to hydrophobic interaction chromatography on Phenyl-Sepharose (Amersham, Sweden). Bound ManLAM was eluted and further separated from other glycolipids by gel filtration on Sephacryl S-100 (Amersham, Sweden). The phenol phase obtained above was washed 3 times with PBS and extracted with an equal volume of 2% SDS in PBS overnight at room temperature. The resulting water phase was precipitated with ice-cold ethanol. PIMs contained in the precipitate were purified to homogeneity by gel filtration on Sephacryl S-100. PIM contains both PIM_2 and PIM_6 isoforms, differing in number of fatty acyl constituents (5).

PBMC Isolation

Venous blood from each participant was collected into EDTA tubes and PBMCs were purified through density gradient centrifugation using Lymphoprep™ (Stemcell) according to the manufacturer's instruction, with some modifications. The cell isolation primarily removes granulocytes and red blood cells. Briefly, white blood cells were counted using a HemoCue instrument and the blood was diluted to a maximum of

240×10^6 cells per 22.5 ml that were then layered onto 10 ml Lymphoprep. The cells were centrifuged at $400g$ for 30 min without any break. The mononuclear cell layer was collected into a new 50 ml tube and resuspended to 45 ml with PBS. The cells were spun at 300g for 10 min with break after which the cells were resuspended into 1-5 ml PBS and filtered using a 100 μm poor size cell strainer and counted on a Countess (ThermoFisher Scientific). The cells were centrifuged at $400g$ for 10 min with break and resuspended with freeze media (90% FBS supplemented with 10% DMSO) and placed in a CoolCell freezing container (Sigma) before moving to –80°C overnight followed by long-term storage in liquid nitrogen.

PBMC Stimulation

PBMCs from 5 patients with ATB, 5 with LTB and five HCs were thawed at 37°C followed by addition of 1 mL RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin (P/S) and 250 U/mL Benzonase (all from ThermoFisher). The cells were washed twice (300g for 5 min) in media followed by resuspension in RPMI-1640 culture media supplemented with 10% FBS, 1% P/S, 0.3 g/L L-Glutamine and 25 mM HEPES and counted. The cells were then plated in 24-well plates at 2×10^6 PBMCs/mL in culture media containing either 5 μg/mL PHA, 10 μg/mL PPD, or 25 μg/mL LAM or PIM, or left untreated (PBS), for 24 h in a 37°C 5% CO_2 incubator. 4 h before collection, 5 μg/mL of brefeldin A and 2 μM Monensin (both ThermoFisher) were added to each well. PHA was used as positive control for PBMCs responsiveness (**Supplemental Figures 1**, **2**). The choice of concentration of LAM and PIM was based on titrations with cytokine secretion into supernatants as read-out (data not shown). The 24 h stimulation did not alter cell numbers between the conditions (**Supplemental Figure 3**).

Mass Cytometry Staining and Acquisition

After 24 h, cells were collected by centrifugation after a 15 min incubation with 2 mM EDTA. Supernatants were stored at –80°C and cells were fixed using the PBMCs fix kit (Cytodelics AB) and barcoded using Cell-ID[TM] 20-Plex Pd Barcoding Kit (Fluidigm Inc.), according to the manufacturer's recommendations. Samples were washed with CyFACS buffer (PBS with 0.1% BSA, 0.05% sodium azide and 2mM EDTA) and Fc receptors were blocked with 200 μL of blocking buffer (Cytodelics AB) for 10 min at RT. Cells were incubated with 200 μL of antibody cocktail (**Supplemental Table 2**) for 30 min at 4°C, washed with CyFACS buffer, and fixed with 1% formaldehyde. For intracellular staining, cells were permeabilized using an intracellular fixation and permeabilization kit (eBiosciences Inc.) according to the manufacturer's instructions. Subsequently, 200 μl of intracellular antibody cocktail (**Supplemental Table 3**) was added and incubated for 45 min at RT. Cells were washed, fixed in 4% formaldehyde at 4°C overnight, and stained with DNA intercalator (0.125 μM MaxPar[®] Intercalator-Ir, Fluidigm Inc.) on the following day. After that, cells were washed with CyFACS buffer, PBS and MiliQ water, counted and adjusted to 750,000 cells/mL. Samples were acquired in a CyTOF2 (Fluidigm) mass cytometer at a rate of

250-400 events/s using CyTOF software version 6.0.626 with noise reduction, a lower convolution threshold of 200, event length limits of 10-150 pushes, a sigma value of 3, and flow rate of 0.045 ml/min.

Analysis of Mass Cytometry Data

The mass cytometry FCS data files were gated for different cell subsets: $CD45^+$ leucocytes, $CD45^+CD3^+CD20^-$ T cells, $CD45^+CD3^-CD7^+$ NK cells, $CD45^+CD3^-$HLA-DR$^+$ antigen-presenting cells (APCs), and $CD45^+$ leukocytes producing IL-2, IL-4, IL-5, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, GrzB, and GM-CSF using FlowJo[TM] v10.6.1. The gated populations were exported to new FCS files that were then analyzed using the R-package Cytofkit v1.12.0, which includes an integrated pipeline for mass cytometry analysis (24). Cytofkit was run in R-studio version 1.1.463 and R version 3.6.1. For analysis of total leukocytes, 5000 cells were used per sample. For analysis of gated T cells, NK cells, and APCs, 10000 cells were used per sample. For analysis of cytokine$^+$ cells, a ceiling of 5000 cells were included per sample. Dimensionality was reduced using Barnes-Hut tSNE with a perplexity of 30 with a maximum of 1000 iterations. Clustering was then performed using density-based machine learning with ClusterX (24) and cell subsets were identified by visual inspection of marker expression for each cluster. The Cytoftkit analysis was performed using PBS, PPD, PIM, and LAM FCS files together, whereas PHA stimulated cells were evaluated independently, using only PBS and PHA FCS files.

Secretome Analysis of Culture Supernatants

Cell culture supernatants (n=75) were randomized in a 96-well plate and analyzed with a multiplex proximity extension assay (PEA) (25), enabling simultaneous quantification of 92 inflammatory markers from the Olink inflammation panel (**Supplemental Table 4**). Markers where all samples were below the limit of detection of the assay were removed from subsequent analysis. The samples were run by the Translational Plasma Profile Facility at SciLifeLab, Stockholm, Sweden.

TLR2-Dependence of PBMCs Activation

To investigate TLR2-dependent PBMCs activation by the Mtb glycolipids PIM and LAM, frozen PBMCs from HC (n=5) were thawed in a 37°C water bath, washed 2 times in complete media (RPMI-1640 culture media supplemented with 10% FBS, 1% P/S, 1 mM sodium pyruvate and 10 mM HEPES) and plated as described for mass cytometry. Prior to stimulation, the cells were pre-incubated for 30 min at 37°C with 5 μg/mL of anti-TLR2 monoclonal antibody (clone T2.5, *In vivo*Gen) or with an isotype control (mIgG1, eBiosciences).

Flow Cytometry

Cells stimulated in the presence or absence of anti-TLR2 antibody for 24 h were collected after an additional 15 min incubation with 2 mM EDTA. The cells were then washed with FACS buffer (PBS with 0.3% BSA and 2 mM EDTA) and Fc receptors were blocked with 20 μL of blocking buffer Fc Receptor

Binding Inhibitor (eBiosciences) for 10 min at 4°C. The cells were incubated with 50 µL of antibody cocktail (**Supplemental Table 5**) for 30 min at RT, washed with PBS and incubated with Fixable Viability Dye eFluor™ 450 (eBiosciences) for 30 min at 4°C. For intracellular staining, the cells were permeabilized using the FoxP3 intracellular fixation and permeabilization kit (eBiosciences) according to the manufacturer's instructions. Subsequently, 50 µl of intracellular Ab cocktail (**Supplemental Table 5**) was added and incubated for 30 min at 4°C. Finally, the cells were washed, resuspended in PBS and kept at 4°C until acquisition on the next day. The cells were acquired on a 12-color LSRII flow cytometer using FACSDiva software (Becton Dickinson, Franklin Lakes, NJ); data analysis was performed using FlowJo™ v10.6.1. Gating strategies are represented in **Supplemental Figure 4**.

Statistical Analysis

Comparisons of a single variable for paired data for >2 groups were evaluated by Friedman's test followed by Dunnet's *post-hoc* test. Comparisons of a single variable for unpaired data for >2 groups were evaluated by using a Kruskal-Wallis test followed by Dunn's post-test. Comparisons of >1 variable for paired data were evaluated using repeated measures 2-way ANOVA followed by Dunnett's *post-hoc* test. Differences were considered significant when p<0.05. Statistical analyzes were performed using Prism9 (GraphPad Software, USA).

Study Approval

Written informed consent was received from all participants before inclusion in the study, whereby they were pseudoanonymized. The study was approved by the Regional Ethical Review Board at the Karolinska Institute in Stockholm (approval numbers 2013/1347-31/2 and 2013/2243-31/4) and by the Ethics Committee for Research in Life and Health Sciences of the University of Minho, Portugal (approval number SECVS 014/2015) and it is in accordance with the Declaration of Helsinki.

RESULTS

Effect of Stimulants on Cell Types and Cytokine Production

To investigate the effect of the Mtb glycolipids LAM and PIM on the immune response, PBMCs from individuals with ATB or LTB, and HC (**Supplemental Table 1**) were thawed and stimulated for 24 h; PPD was used as a control for responses to Mtb proteins, while mock stimulation (PBS) or phytohemagglutinin (PHA) were used as negative and positive culture controls, respectively. Proteins released into the culture supernatant were analyzed using the Olink proximity-extension assay (PEA), that allows for simultaneous measurement of 92 inflammatory markers. Cells were analyzed using mass cytometry for changes in the expression of 27 surface and 10 intracellular markers (**Figure 1** and **Supplemental Tables 2**, **3**).

To assess the effect of LAM, PIM, and PPD on secretion of cytokines and chemokines from stimulated PBMCs, we assessed the culture supernatant for relative levels of 92 different soluble inflammatory markers (**Figure 2**). Of these, we observed changes in protein levels for 58 proteins. LAM and PIM stimulation produced very similar marker profiles, with a slightly stronger effect from PIM, suggesting a similar mechanism of action. PPD induced a markedly different response, with considerably higher IFN-γ levels, but also several other inflammatory proteins, such as IL1α and CCL8, compared to LAM and PIM, suggesting a different mechanism of action (**Figure 2A**).

There were also some indications of different levels of secretion between the groups (ATB/LTB/HC), primarily with a greater effect observed for HC compared with ATB or LTB (**Figure 2B**). IL-1α, IL-10, IL-18, and VEGF were detected at higher levels in HC compared with LTB or ATB while CCL8 was significantly decreased in HC compared with ATB and/or LTB. CCL8 functions as a strong monocyte chemoattractant but has also been associated with multiple other effects on leukocyte behavior, suggesting that its lower levels could be due to its

FIGURE 1 | Schematic of the study outline. **(A)** PBMCs from three groups, including active TB (ATB, red), latent TB (LTB, blue) and healthy controls (HC, black) were **(B)** stimulated for 24 h with the the mycobacterial glycolipids LAM or PIM. Control stimulations included PPD, or phytohemagglutinin (PHA), or mock stimulation (PBS). **(C)** Culture supernatants were analyzed by proximity extension assay (PEA), while **(D)** cells were analyzed by mass cytometry. Cells were either pre-gated for cytokine-secreting cells or different cell subsets prior to dimensionality reduction and cluster analysis. *p<0.05, ***p<0.001, ****p<0.0001.

FIGURE 2 | Secretion of inflammatory proteins after stimulation with LAM, PIM, and PPD. Culture supernatants were assessed for 92 protein markers using the Olink inflammation panel after 24 h of culture. Only markers above background levels were included (n=58). The graphs show normalized protein expression (ΔNPX) for stimulated (LAM, PIM, or PPD) minus unstimulated (PBS) PBMCs (**A**, n=15/group) and separated into individuals with active TB (ATB; red), latent TB (LTB, blue), and healthy controls (HC, black) (**B**, n=5/group). The lines indicate the mean and error bars indicate SEM. The protein markers were grouped into different functional groups (yellow highlight), where GF correspond to growth factors. Statistics was evaluated using two-way ANOVA followed by Dunnet's posthoc test with significant differences between ATB or LTB against HC indicated by #, corresponding to a p-value < 0.05.

increased uptake from the culture supernatant by activated monocytes (26). Another possibility is that it is produced to a larger extent in PPD stimulated cultures, potentially *via* synergistic effects from IFN-γ and IL-1 as has been proposed previously (27).

Intracellular Cytokine Production in Response to Stimulation

To evaluate the effect of each stimulus on intracellular production of cytokines and GrzB, regardless of the experimental group, the cumulative frequency of cytokine$^+$ cells among CD45$^+$ leukocytes was compared to that of unstimulated cells (PBS; **Figures 3A, B**). As expected, PPD stimulation resulted in an increase in IFN-γ-producing cells, but also led to higher levels of IL-2, IL-6, IL-10, IL-17A, TNF-α and GrzB-producing cells. Unlike PPD, PIM did not stimulate production of IFN-γ but instead stimulated early

production of IL-4 and GM-CSF. In addition, PIM stimulation led to increased levels of IL-2$^+$, IL-6$^+$, IL-10$^+$, IL17A$^+$ and TNF-α$^+$ cells (**Figure 3B**). To better understand if the cytokines were produced one their own or in combinations, we assessed polyfunctionality of the stimulated cells using the R-package COMPASS (28). Since GrzB is functionally distinct from the cytokines, it was excluded from the analysis. In total, 512 different combinations of cytokines, as defined by a Boolean gating strategy in FlowJo were included in the analysis. We observed that all stimulations resulted in polyfunctional cytokine production, although responses to LAM were significantly lower than to PIM and PPD (**Supplemental Figure 5**).

To get an overview of which cell types that were responsible for the cytokine production, we identified the cell subsets producing each cytokine, regardless of the group and stimuli. We observed that myeloid cells (identified through the

FIGURE 3 | Stimulation-induced cytokine production. **(A)** Representative gates for identification of cytokine producing CD45$^+$ cells *via* intracellular staining. **(B)** The frequency of cytokine positive cells out of CD45$^+$ cells for each stimulation at 24h. All donors merged (n=15/stimulation). Statistics was evaluated by Friedman's test with Dunnet's posttest where every group was compared with unstimulated cells (PBS). *p<0.05, **p<0.01, ****p<0.0001. **(C)** For each cytokine, the pie-charts indicate the frequency of cell type responsible for its production: B cells (blue), myeloid cells (red), NK cells (green), T cells (yellow), and others (grey).

expression of CD33) contributed strongly to the early production of IL-2, IL-6, IL-10, TNF-α, and GM-CSF. This is consistent with myeloid cells being the main source of pro-inflammatory cytokines such as IL-6 and TNF-α (29) (**Figure 3C**). This pattern largely overlapped with the cytokines stimulated by PIM, indicating that myeloid cells could be the main effector cells stimulated by Mtb glycolipids. T cells were the main cytokine producers of IFN-γ, IL-4, IL-5, IL-17A, and GrzB, while NK cells primarily produced IFN-γ, IL-2, IL-6, IL-17A, TNF-α, and GrzB. We also identified B cells, producing primarily IL-2, IL-4, IL-5, IL-17A, and TNF-α, although to a smaller extent compared with the other cell subsets (**Figure 3C**).

In summary, stimulation with Mtb glycolipids and PPD led to a polyfunctional cytokine response associated with production from multiple cell subsets.

Reduced Cytokine Production in Individuals With Active- or Latent TB

To investigate the overall cytokine response profile of the main cell populations in individuals with ATB, LTB and HC, we pooled all the cytokine-producing cells of each cell population after subtracting the number in unstimulated conditions for each donor and compared the cumulative production of cytokines within the different groups (**Figure 4**). For T cells, we observed a reduced cytokine production in individuals with ATB and LTB to PIM stimulation (**Figure 4A**). There was no overall significant effect on cytokine$^+$ NK cells associated with Mtb-infection (**Figure 4B**). For B cells, the overall cytokine production was reduced in individuals with ATB upon LAM and PIM stimulation compared with HC, primarily due to a reduced production of IL-5 (**Figure 4C**). For myeloid cells, a similar reduction of cytokine$^+$ cells was observed in individuals with

LTB to LAM, PIM, and PPD stimulations. This effect was mainly attributed to a reduced production of IL-10 and IL-6. For ATB this effect was only observed in response to LAM (**Figure 4D**).

In summary, individuals with ATB or LTB responded with less cytokine production by especially myeloid cells and somewhat by B and T cells upon stimulation with Mtb antigens.

To further analyze the effect of LAM, PIM, and PPD on cytokine production by individual cell subsets between the three groups (ATB, LTB, and HC), we proceeded with dimensionality reduction using t-stochastic neighbour embedding (t-SNE) and cluster analysis. This was performed by pooling all PBS, PPD, PIM, and LAM mass cytometry data files together followed by analysis using Cytofkit (24). To allow a high level of resolution in the analysis, cytokine producing CD45$^+$ cells were gated for the individual cytokines (see gates in **Figure 3A**) which were then analyzed separately (**Supplemental Figure 6**, **7**).

Qualitatively Different T Cell Responses to PIM and PPD

Stimulation with PPD resulted in an increased number of T cells (identified as CD3$^+$) producing IFN-γ, IL-2, IL-5, IL-6, IL-17A, TNF-α, and GrzB compared with LAM and/or PIM. Stimulation with PIM contributed to higher numbers of IL-2$^+$, TNF-α$^+$, and GM-CSF$^+$ T cells compared with LAM stimulated cells (**Figure 5A**). Of all cells producing IFN-γ at 24 h, T cells represented 39%, comprising 11 different clusters (clusters 2, 3, 5, 8, 10, 11, 12, 14 15, 16, and 24) (**Figure 5B**). Four of these clusters (clusters 5, 11, 12, and 15) were significantly elevated by PPD stimulation compared to PIM and/or LAM (**Figure 5C**). These clusters corresponded to different CD4$^+$ and CD8$^+$ T cells subsets, including central memory (CD4$^+$CD45RA$^-$CD27$^+$, cluster 5), effector memory (CD45RA$^-$CD27$^-$, clusters 11 and

FIGURE 4 | Overall effect of stimulation on cytokine production from individuals with active- and latent TB and healthy controls. Average number of cytokine+ cells following 24 h of stimulation with LAM, PIM, or PPD of PBMCs from individuals with active TB (ATB), latent TB (LTB), or healthy controls (HC) among **(A)** CD3+ T cells, **(B)** CD3−CD7+ NK cells, **(C)** CD20+ B cells, and **(D)** CD33+ myeloid cells. The groups average number of cytokine-producing cells were compared using the Friedman test followed by Dunn's posttest. *p<0.05, **p<0.01, ***p<0.001.

15), and effector memory T cells re-expressing CD45RA (TEMRA - CD8+CD45RA+CD27−, cluster 12; **Figure 5D**).

Approximately 23% of all IL-2 producing cells were identified as T cells (**Figure 5E**). These cells comprise six clusters, of which two (clusters 6 and 8) were significantly higher following PPD stimulation compared with LAM and/or PIM (**Figure 5F**). Cluster 6 corresponded to polyfunctional CD4+ T cells, co-producing IFN-γ and TNF-α, while cluster 8 was composed of cells producing only IL-2 (**Figure 5G**).

Although the regulatory effect of IL-6 on T cells is well known (30), the literature on IL-6 producing T cells is limited. We identified one cluster of IL-6+ T cells (cluster 5) corresponding to 5.3% of total IL-6+ leukocytes after 24 h of stimulation (**Figure 5H**). This cluster was significantly elevated by PPD stimulation compared to LAM and was mainly attributed to ATB and HC, but not LTB individuals (**Figure 5I**). Cluster 5 was a mixed cluster consisting of cells expressing CD8+, CD4+, and double negative (DN) T cells (data not shown) with 36% of the cells co-producing GrzB (**Figure 5J**).

Approximately 14% of the GM-CSF+ cells were T cells, represented by four different clusters (**Figure 5K**). Cluster 13 was significantly increased upon PIM stimulation compared to LAM and PPD (**Figure 5L**). The effect was more prominent in HC individuals compared to LTB. This cluster corresponded mostly to naïve (CD45RA+CD27+) CD8+ T cells (**Figure 5M**).

T cells represented 62% of the IL-17A+ cells (**Figure 5N**). Four out of 12 clusters (clusters 5, 14, 15, and 16) were increased by PPD compared with PIM and/or LAM stimulations, while clusters 1 and 2 were increased by PIM compared with PPD (**Figure 5O** left). In addition, the analysis of individual clusters

showed that stimulation with LAM reduced cluster 1 in ATB, compared with HC individuals. Also, cluster 16 was higher in LTB compared with HC upon PIM stimulation (**Figure 5O** right). Three of these clusters corresponded to polyfunctional T cells (clusters 5, 14, and 15), with clusters 5 and 15 co-producing IFN-γ, and cluster 14 co-producing IFN-γ and TNF-α (**Figure 5P**).

In summary, T cell responses were mainly observed upon stimulation with PPD. The T cells producing IFN-γ, IL-2, IL-6, and IL-17A, some of those with a polyfunctional phenotype, were significantly increased with PPD compared with LAM and/or PIM. Interestingly, however, PIM stimulation led to an increase in GM-CSF-producing T cells, particularly in HC individuals, potentially indicating a different mechanism for GM-CSF induction also associated with disease status.

NK Cells Are Primarily Stimulated by PPD

As for T cells, the NK cells (identified as CD3−CD7+) showed minor responses to PIM and LAM, and were mainly affected by PPD stimulation, which resulted in significantly higher numbers of NK cells producing IFN-γ, IL-2, IL-6, IL-17A, and GM-CSF, compared to PIM and LAM (**Figure 6A**). Most of IFN-γ producing cells at 24 h of stimulation were NK cells. They represented 51% of all IFN-γ-producing cells and could be further divided into 9 clusters (clusters 1, 6, 7, 13, 19, 21, 22, 23, and 25) (**Figure 6B**). Of these, four clusters were significantly increased following PPD stimulation, compared with LAM and PIM (**Figure 6C**). All of these clusters were CD57− but expressed different levels of CD56 suggesting that they belonged to different NK subsets. Moreover, all clusters expressed GrzB while cluster 13 also expressed IL-17A (**Figure 6D**).
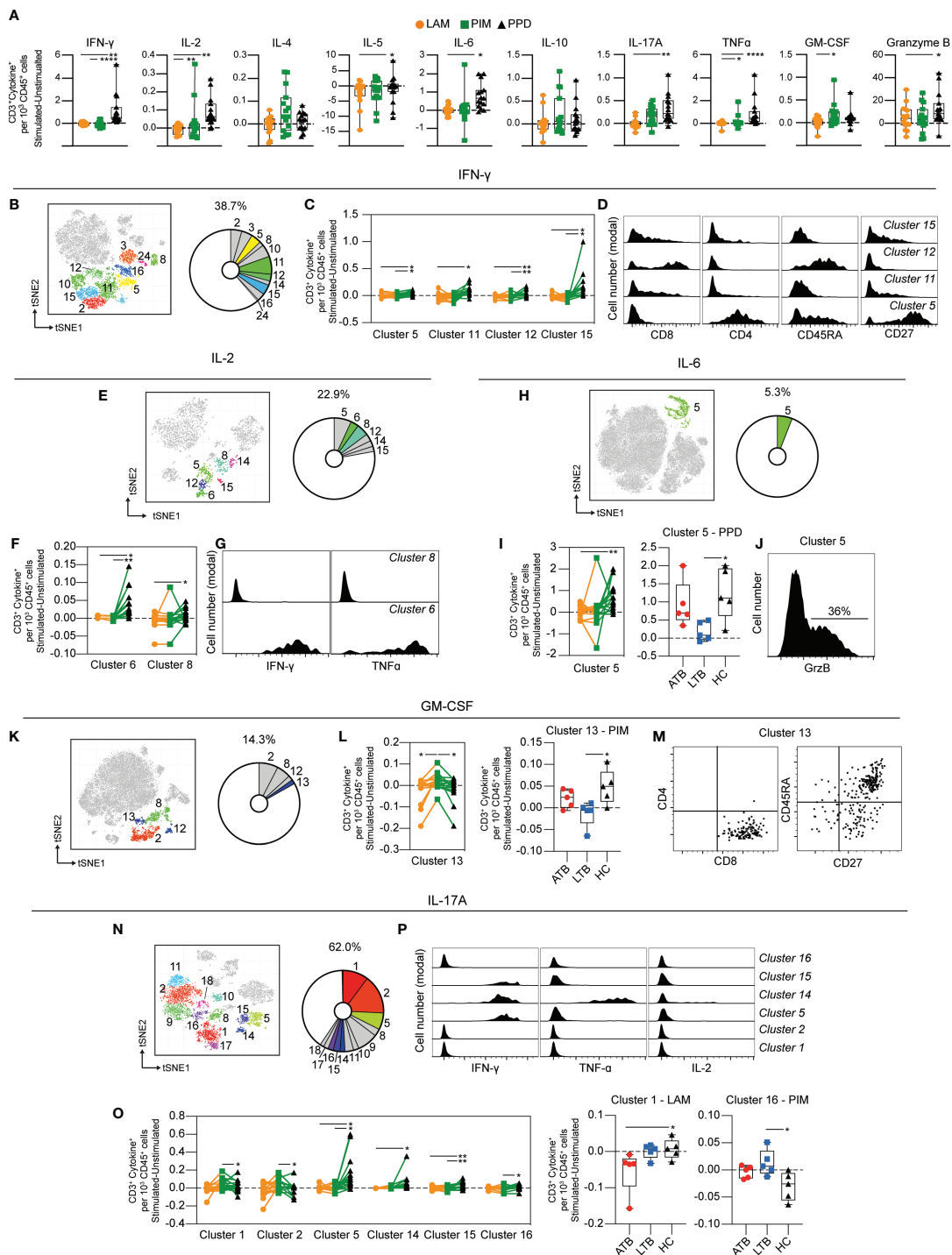
FIGURE 5 | Continued

FIGURE 5 | Cytokine production by stimulated T cells. **(A)** The number of cytokine-producing T cells per 1000 total CD45^{+} cells for each stimulation at 24 h with the background (unstimulated) cytokine-production removed. **(B)** Cluster analysis of IFN-γ secreting cells with clusters 2, 3, 5, 8, 10, 11, 12, 14, 15, 16, and 24 corresponding to T cells. **(C)** Clusters significantly affected by stimulation with **(D)** cluster histograms indicating CD4, CD8, CD45RA, and CD27. **(E)** Cluster analysis of IL-2 secreting cells with clusters 5, 6, 8, 12, 14, and 15 corresponding to T cells. **(F)** Clusters significantly affected by stimulation with **(G)** cluster histograms indicating IFN-γ and TNF-α secretion. **(H)** Cluster analysis of IL-6 secreting cells with cluster 5 corresponding to T cells. **(I)** Cluster 5 is significantly affected by stimulation (left) and comparison of PPD stimulation on donors with active TB (ATB), latent TB (LTB) and healthy controls (HC) in cluster 5 (right). **(J)** Cluster histogram indicating GrzB secretion. **(K)** Cluster analysis of GM-CSF secreting cells with cluster 2, 8, 12, and 13 corresponding to T cells. **(L)** Cluster 13 is significantly affected by stimulation (left) and comparison of PIM stimulation on donors with ATB, LTB and HC in cluster 13 (right). **(M)** Cluster dot plots indicating CD4, CD8, CD45RA, and CD27 expression. **(N)** Cluster analysis of IL-17A secreting cells with cluster 1, 2, 5, 8, 9, 10, 11, 14, 15, 16, 17, and 18 corresponding to T cells. **(O)** Clusters significantly affected by stimulation (left) and comparison of PIM and LAM stimulation on donors with active ATB, LTB and HC in clusters 1 and 16, respectively (right) **(P)** Cluster histograms indicating IFN-γ, TNF-α, and IL-2. Statistical differences between stimulations in **(A, I, L)** were evaluated by Friedman's test with Dunnet's posttest, while comparisons within multiple clusters (**C, F, O** left panels) were evaluated by a matched-pair two-way ANOVA with Geissner-Greenhouse correction followed by Tukey's posttest (n=15/stimulation). Groups (ATB/LTB/HC) (**I, L, O** right panels) were compared using Kruskal-Wallis with Dunn's posttest (n=5/group) [*]p<0.05, [**]p<0.01, [***]p<0.001, [****]p<0.0001.

IL-2-producing NK cells constituted 24% of all IL-2^{+} cells and represented two clusters (cluster 1 and 9) (**Figure 6E**). Cluster 1 was significantly increased by PPD, compared with LAM stimulation (**Figure 6F**). Both clusters were CD57^{−} while cluster 1 expressed intermediate levels of CD56 and no CD27 while cluster 9 expressed high levels of CD56 and CD27 (**Figure 6G**). Both clusters co-produced IL-6 (**Figure 6G**). NK cells represent approximately 8% of all GM-CSF-producing cells at 24 h of stimulation (**Figure 6H**). The cytokine was produced by two clusters (4 and 16), one of which (cluster 16) was significantly higher in response to PPD compared with LAM and PIM stimulation (**Figure 6I**). Similar to PPD-mediated IL-2 secreting NK cells, GM-CSF was primarily produced by CD57^{−} NK cells where >50% expressed intermediate CD56 levels while almost no cells expressed CD27 (**Figure 6J**). Cluster 16 cells were also co-producing IFN-γ (**Figure 6J**). Approximately 25% of all IL-6-producing cells at 24 h were identified as NK cells (**Figure 6K**), and two out of the six clusters (3 and 12) were significantly increased in numbers by PPD stimulation compared to LAM (**Figure 6L**). These two clusters belonged to different NK subsets with cluster 3 corresponding to CD56^{high}CD57^{−} NK cells, of which 48% also expressed CD27, while cluster 12 was composed of CD56^{int}CD57^{+}CD27^{−} NK cells (**Figure 6M**). The IL-17A producing NK cells were composed of two clusters at 24 h. However, they were not significantly different between the different stimulations (data not shown).

Thus, similar to T cells, NK cells were primarily induced to secrete cytokines through stimulation with PPD compared with the Mtb glycolipids LAM and PIM. The stimulation led to cytokine production by CD56^{int} and CD56^{bright} NK cells, independent on the expression of CD57. In summary, these results show that stimulation with PPD leads to rapid activation of different NK cell subsets with production of primarily pro-inflammatory cytokines.

Atypical B Cells Are a Major Source of Polyfunctional Cytokine Responses Following PIM Stimulation

Compared with T cells and myeloid cells, B cells (defined as CD3^{−}HLA-DR^{+}CD20^{+}) were minor producers of the measured cytokines (**Figure 3**). There was however a primarily PIM-derived effect leadin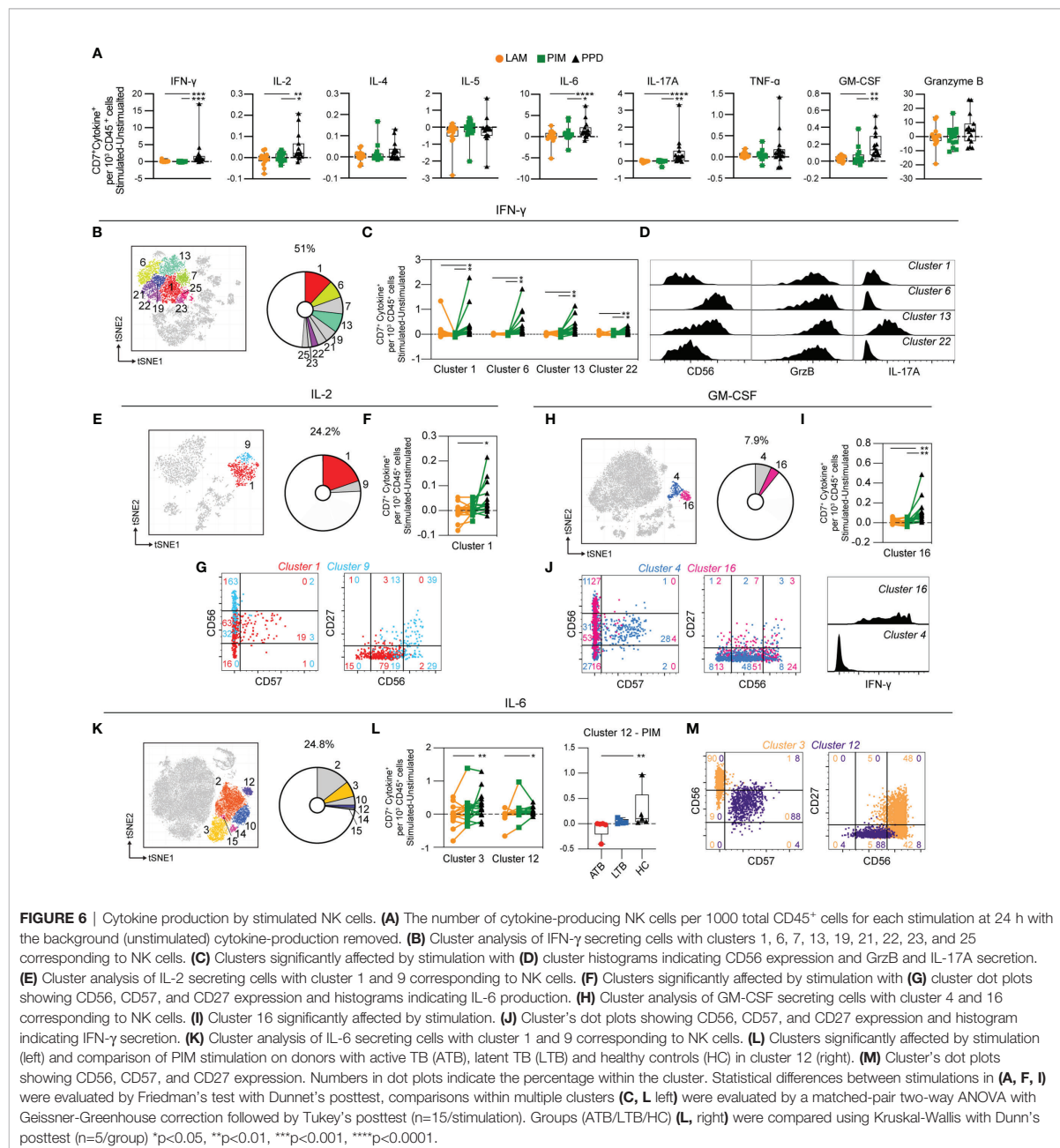g to significantly increased numbers of IL-4, IL-10, and GM-CSF producing B cells in comparison to LAM and/or PPD stimulation (**Figure 7A**). There were two B cell clusters producing IL-4 (cluster 6 and 12) (**Figure 7B**), but only cluster 6 was significantly increased by PIM stimulation, with PPD leading to the lowest numbers of cells in this cluster (**Figure 7C**). Cluster 6 was enriched for switched memory (CD27^{+}IgD^{−}) and double negative (DN; CD27^{−}IgD^{−}) B cells, while cluster 12 was enriched for naïve B cells (CD27^{−}IgD^{+}) (**Figure 7D**). Cluster 6 was further enriched for CD11c^{+} B cells, which are associated with recent B cell activation and formation of atypical B cells during infection or inflammatory conditions (31).

B cells producing IL-10 and GM-CSF were also significantly expanded by PIM stimulation (**Figures 7E–J**). As B cells responding to PIM stimulation presented a highly homogenous phenotype, we further evaluated the cells for co-expression of the three cytokines (**Figure 7K**). We found that 42% of GM-CSF-producing B cells also produced IL-4 and IL-10. Compared with total B cell populations, the phenotype of the polyfunctional cells was highly enriched for double negative (DN - IgD^{−}CD27^{−}) B cells but also for unswitched and switched memory B cells (CD27^{+}) compared with total B cell populations (**Figure 7L**). The polyfunctional B cells were also approximately 10-fold enriched for CD11c^{+} B cells compared with total B cells, suggesting that atypical B cells can respond to PIM stimulation (**Figure 7L**). We also quantified the levels of HLA-DR on the cell surface of the polyfunctional B cells and compared with the levels on total B cells and found an increased expression of HLA-DR on cells from cluster 7 (**Figure 7M**), consistent with previous reports on atypical B cells in mice and humans (32, 33).

Rapid Polyfunctional Response of Myeloid Cells to PIM Stimulation

The production of cytokines by CD33^{+} myeloid cells was compared for each stimulation (**Figure 8A**). PIM stimulation led to a robust increase of cells producing IL-2, IL-4, IL-6, IL-10, TNF-α, compared to PPD, and of IL-6, IL-17A, TNF-α and GM-CSF in comparison to LAM (**Figure 8A**). Interestingly, IL-10 producing cells were induced by both PIM and PPD (**Figure 8A**), contrasting with the other cytokines that were primarily induced by PIM.
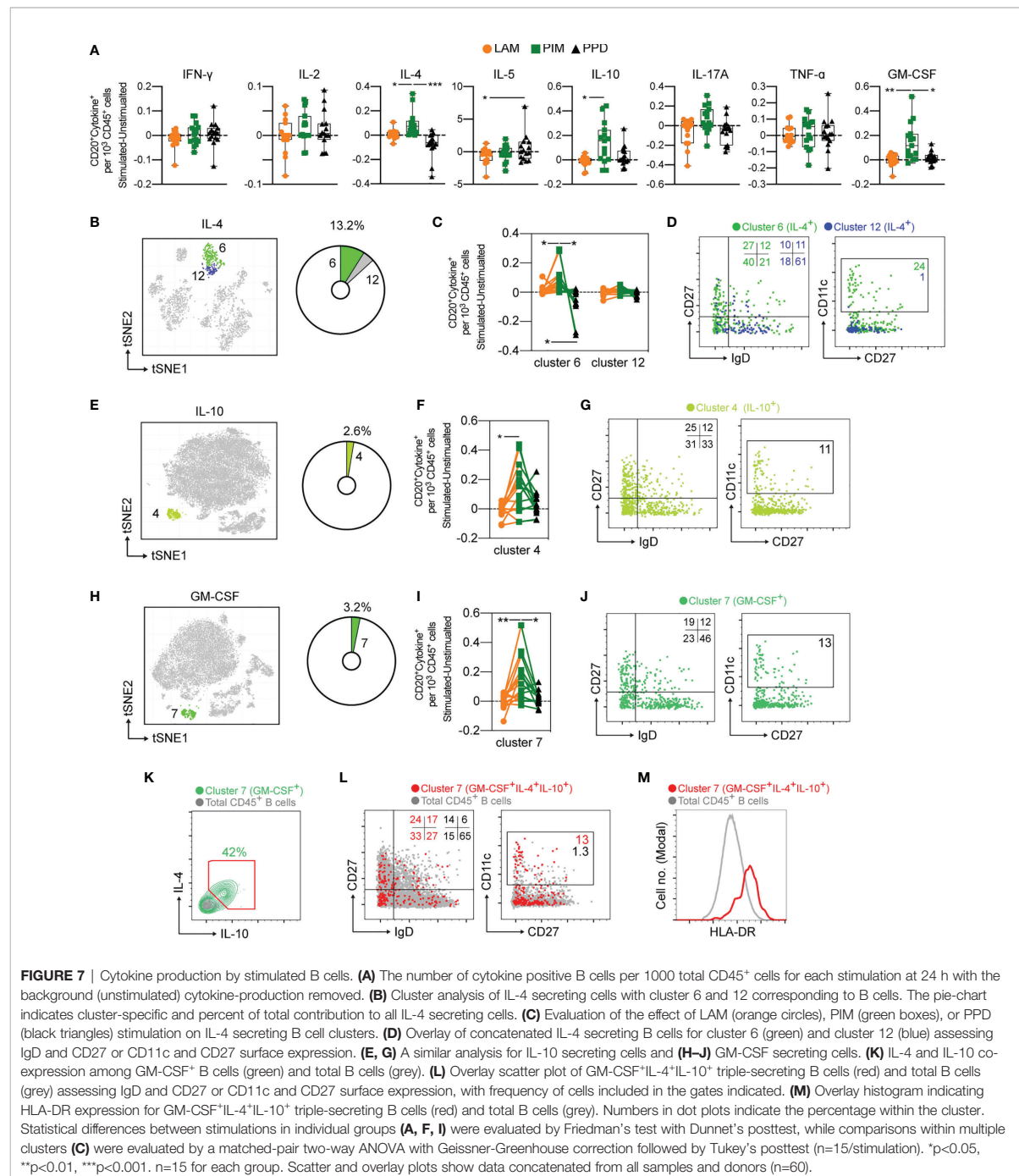
To understand if the effect of stimulation was associated with specific myeloid subsets, we further investigated the impact of

FIGURE 6 | Cytokine production by stimulated NK cells. **(A)** The number of cytokine-producing NK cells per 1000 total CD45+ cells for each stimulation at 24 h with the background (unstimulated) cytokine-production removed. **(B)** Cluster analysis of IFN-γ secreting cells with clusters 1, 6, 7, 13, 19, 21, 22, 23, and 25 corresponding to NK cells. **(C)** Clusters significantly affected by stimulation with **(D)** cluster histograms indicating CD56 expression and GrzB and IL-17A secretion. **(E)** Cluster analysis of IL-2 secreting cells with cluster 1 and 9 corresponding to NK cells. **(F)** Clusters significantly affected by stimulation with **(G)** cluster dot plots showing CD56, CD57, and CD27 expression and histograms indicating IL-6 production. **(H)** Cluster analysis of GM-CSF secreting cells with cluster 4 and 16 corresponding to NK cells. **(I)** Cluster 16 significantly affected by stimulation. **(J)** Cluster's dot plots showing CD56, CD57, and CD27 expression and histogram indicating IFN-γ secretion. **(K)** Cluster analysis of IL-6 secreting cells with cluster 1 and 9 corresponding to NK cells. **(L)** Clusters significantly affected by stimulation (left) and comparison of PIM stimulation on donors with active TB (ATB), latent TB (LTB) and healthy controls (HC) in cluster 12 (right). **(M)** Cluster's dot plots showing CD56, CD57, and CD27 expression. Numbers in dot plots indicate the percentage within the cluster. Statistical differences between stimulations in **(A, F, I)** were evaluated by Friedman's test with Dunnet's posttest, comparisons within multiple clusters **(C, L** left**)** were evaluated by a matched-pair two-way ANOVA with Geissner-Greenhouse correction followed by Tukey's posttest (n=15/stimulation). Groups (ATB/LTB/HC) **(L,** right**)** were compared using Kruskal-Wallis with Dunn's posttest (n=5/group) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

stimulation on individual cell clusters. The IL-2 producing myeloid cells constituted 43.6% of all IL2-producing cells and were composed of four different clusters (cluster 3, 4, 7, and 13), of which three were differently affected by the stimuli (**Figure 8B**). For cluster 3 and 13, PIM stimulation led to significantly more IL-2+ cells compared with PPD and/or LAM, while cluster 7 was higher in LAM compared to PPD (**Figure 8C**). Cluster 7 expressed CD14, while clusters 3 and 13 were mostly negative

for CD14 (**Supplemental Figure 7**). Cluster 3 was associated with the co-production of IL-6 (**Figure 8C**).

Approximately 29% of all IL-4 producing cells after 24 h of stimulation expressed CD33. (**Figure 8D**). These cells were further distributed into three clusters (3, 7, and 11), of which cluster 7 and 11 were significantly higher in number following PIM stimulation compared with LAM and PPD stimulation. LAM stimulation also led to more IL-4 producing cells compared

FIGURE 7 | Cytokine production by stimulated B cells. **(A)** The number of cytokine positive B cells per 1000 total CD45+ cells for each stimulation at 24 h with the background (unstimulated) cytokine-production removed. **(B)** Cluster analysis of IL-4 secreting cells with cluster 6 and 12 corresponding to B cells. The pie-chart indicates cluster-specific and percent of total contribution to all IL-4 secreting cells. **(C)** Evaluation of the effect of LAM (orange circles), PIM (green boxes), or PPD (black triangles) stimulation on IL-4 secreting B cell clusters. **(D)** Overlay of concatenated IL-4 secreting B cells for cluster 6 (green) and cluster 12 (blue) assessing IgD and CD27 or CD11c and CD27 surface expression. **(E, G)** A similar analysis for IL-10 secreting cells and **(H–J)** GM-CSF secreting cells. **(K)** IL-4 and IL-10 co-expression among GM-CSF+ B cells (green) and total B cells (grey). **(L)** Overlay scatter plot of GM-CSF+IL-4+IL-10+ triple-secreting B cells (red) and total B cells (grey) assessing IgD and CD27 or CD11c and CD27 surface expression, with frequency of cells included in the gates indicated. **(M)** Overlay histogram indicating HLA-DR expression for GM-CSF+IL-4+IL-10+ triple-secreting B cells (red) and total B cells (grey). Numbers in dot plots indicate the percentage within the cluster. Statistical differences between stimulations in individual groups **(A, F, I)** were evaluated by Friedman's test with Dunnet's posttest, while comparisons within multiple clusters **(C)** were evaluated by a matched-pair two-way ANOVA with Geissner-Greenhouse correction followed by Tukey's posttest (n=15/stimulation). *p<0.05, **p<0.01, ***p<0.001. n=15 for each group. Scatter and overlay plots show data concatenated from all samples and donors (n=60).

to PPD (**Figure 8E**). Both cluster 7 and 11 produced several other cytokines in addition to IL-4, with cluster 7 also producing IL-10, and cluster 11 producing IL-6, and IL-10 (**Figure 8E**). Interestingly, this effect of multiple cytokine production, was

significantly reduced in individuals with LTB compared with ATB and HC (**Figure 8F**).

IL-6 was the most frequent cytokine produced following PIM stimulation (**Figure 8A**). Approximately 70% of all IL-6 secreting
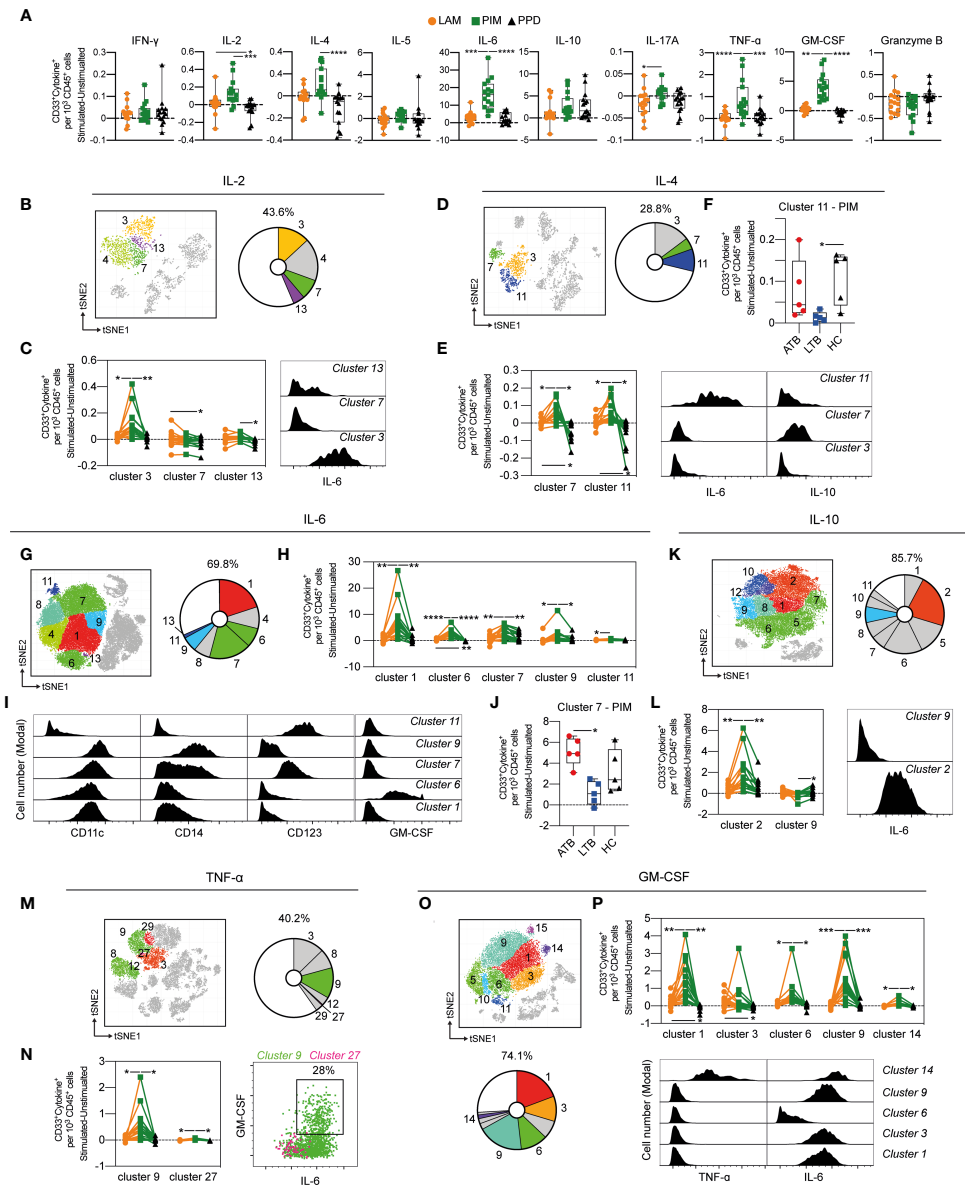
FIGURE 8 | Cytokine production by stimulated CD33+ myeloid cells. **(A)** The number of cytokine positive CD33+ myeloid cells per 1000 total CD45+ cells for each stimulation at 24 h with the background (unstimulated) cytokine-production removed. **(B)** Cluster analysis of IL-2 secreting cells with cluster 3, 4, 7 and 13 corresponding to myeloid cells. **(C)** Clusters significantly affected by stimulation (left) with cluster histograms indicating co-secretion of IL-6. **(D)** Cluster analysis of IL-4 secreting with cluster 3, 7, and 11 corresponding to myeloid cells. **(E)** Clusters significantly affected by stimulation (left) and co-expression with IL-6 and IL-10 (right). **(F)** Comparison of PIM stimulation on donors with active TB (ATB), latent TB (LTB) and healthy controls (HC) in cluster 11. **(G)** Myeloid clusters secreting IL-6 **(H)** significantly affected by stimulation. **(I)** Cell surface phenotype of indicated cluster. **(J)** Differential effect of PIM stimulation on cluster 7 cells in ATB, LTB, and HC. **(K)** Myeloid clusters secreting IL-10 with **(L)** clusters significantly affected by stimulation (left panel) and histograms indicating IL-10 co-expression with IL-6. **(M)** Myeloid clusters secreting TNF-α **(N)** significantly affected by stimulation (left) with co-expression of GM-CSF and IL-6 (right). **(O)** Myeloid clusters secreting GM-CSF. **(P)** Clusters differently affected by stimulation (left) with co-expression of TNF-α and IL-6 (right). Statistical differences between stimulations in **(A)** were evaluated by Friedman's test with Dunnet's posttest, while comparisons within multiple clusters **(C, E, H, L, N, P)** were evaluated by a matched-pair two-way ANOVA with Geissner-Greenhouse correction followed by Tukey's posttest (n=15/stimulation). Groups (ATB/LTB/HC) **(F, J)** were compared using Kruskal-Wallis with Dunn's posttest (n=5/group) *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

cells at 24 h were myeloid cells (**Figure 8G**) with 5 out of 7 clusters showing a significant increase following PIM stimulation compared with PPD and/or LAM (**Figure 8H**). Several subsets of myeloid cells responded with IL-6 production, including CD11c$^+$CD14$^-$CD123$^-$ DCs (cluster 1 and 6), intermediate/non-classical monocytes (CD11c$^+$CD14$^{int/-}$CD123$^+$, cluster 7), and classical monocytes (CD11c$^+$CD14$^+$CD123$^-$, cluster 9). Among these, the cluster 6 DCs also produced GM-CSF, in addition to IL-6 (**Figure 8I**). Similar to the IL-4$^+$IL-6$^+$ co-producing cluster 11 (**Figure 8F**), the intermediate/non-classical monocyte cluster 7 contracted in individuals with LTB, compared with those with ATB and HC (**Figure 8J**).

Myeloid cells were the main cell subset identified within IL-10, TNF-α and GM-CSF-producing cells, especially following stimulation with PIM (**Figures 8K, M, O**). One IL-10 cluster, two TNF-α clusters and five GM-CSF clusters were significantly increased compared with LAM and PPD (**Figures 8L, N, P**). Of these, parts of TNF-α cluster 9 and GM-CSF cluster 14 likely corresponded to the same polyfunctional cells as both clusters secreted TNF-α, GM-CSF, and IL-6 (**Figures 8L, N**). From the two IL-10 clusters that were affected by PIM, one only produced IL-10 while the other co-produced IL-6. GM-CSF cluster 1, 3,

and 9 also co-produced IL-6, but not TNF-α, while cluster 6 only produced GM-CSF.

In summary, several myeloid cell subsets rapidly responded to stimulation by producing cytokines. The response was primarily induced by PIM and included phenotypes of cells producing both single and multiple cytokines. Among the most polyfunctional responses were cells producing IL-4, IL-6, and IL-10, or TNF-α, GM-CSF and IL-6.

Stimulation of Myeloid Cells With PIM Is Partially TLR2 Dependent

PIM and LAM stimulation induced a robust immune response in myeloid cells (**Figure 8A**). To investigate the mechanism responsible for this effect, and in particular the dependence on interaction with TLR2, PBMCs from HC were treated with anti-TLR2 blocking antibody before stimulation with PIM, LAM and PHA. Blocking TLR2 led to a reduction in the percentage of CD33$^+$IL-6$^+$ myeloid cells upon PIM stimulation, but not with LAM or PHA (**Figure 9**). We did not observe any significant effect of blocking TLR2 on IL-6 production from T cells, NK cells, or B cells (data not shown), although the frequency of IL-6$^+$ cells was very low on those cell subsets.
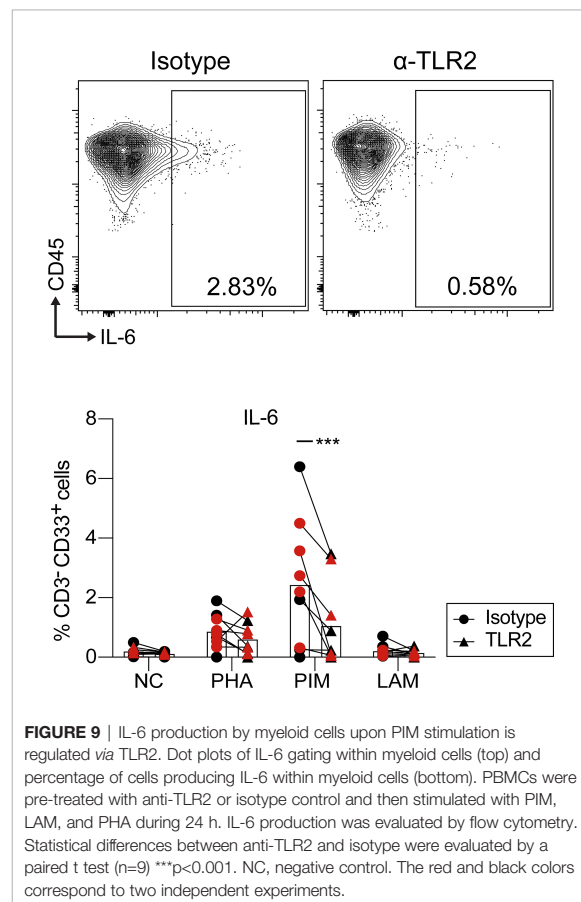
DISCUSSION

In the present study, we show that LAM and PIM induce responses in PBMCs from Mtb-infected individuals that can be distinguished from those obtained from HC. In addition, we show that the responses to these glycolipids are clearly different from those elicited by PPD. The responses involve both expansion and contraction of particular cell subsets and production and secretion of distinct patterns of cytokines and chemokines.

When analyzing intracellular cytokine production, we found that PIM mainly induced antigen-presenting cells to produce a defined set of pro-inflammatory cytokines consisting of IL-2, IL-6, IL-17A, TNF-α and GM-CSF, the anti-inflammatory IL-10 as well as IL-4, but not IFN-γ. LAM triggered responses that tended to be similar to the ones generated by PIM, but weaker in most instances. Classical and intermediate monocytes are known to secrete high levels of pro-inflammatory cytokines in response to microbial products (34). In addition, compared to non-classical monocytes, they were previously shown to exhibit a greater polyfunctional pro-inflammatory response (IL1-α, IL1-β, IL-6, IL-8, IL-10, and TNF-α) to lipomannan from *Mycobacterium smegmatis*, a TLR-2 agonist (34). Here we show that PIM induced multifunctional monocytes producing cytokines in a combination of either pro-inflammatory IL-2, IL-6, GM-CSF and TNF-α, or IL-4 and the anti-inflammatory IL-10. In particular GM-CSF, which is increasingly recognized for its potential role in innate resistance to TB (35), was in our study mainly produced by myeloid cells upon PIM stimulation.

This response contrasted with the quite well-known immune response triggered by PPD, which was dominated primarily by T and NK cells. They produced predominantly the pro-inflammatory cytokines IFN-γ, IL-2, IL-6, IL-17A, TNF-α, and

FIGURE 9 | IL-6 production by myeloid cells upon PIM stimulation is regulated *via* TLR2. Dot plots of IL-6 gating within myeloid cells (top) and percentage of cells producing IL-6 within myeloid cells (bottom). PBMCs were pre-treated with anti-TLR2 or isotype control and then stimulated with PIM, LAM, and PHA during 24 h. IL-6 production was evaluated by flow cytometry. Statistical differences between anti-TLR2 and isotype were evaluated by a paired t test (n=9) ***p<0.001. NC, negative control. The red and black colors correspond to two independent experiments.

GrzB, but also IL-10, although no IL-4. While T cells simultaneously producing combinations of cytokines have been extensively investigated in the context of the immune response in TB (36–38), we extended these findings to several other cell types. Our results reveal that multiple subsets of myeloid cells, NK, B and T cells respond to glycolipids and/or to PPD, with the production of different combinations of cytokines such as classical functional T cells producing IFN-γ, IL-2 and TNF-α, but also other combinations, such as IL6 and GrzB or IL-17A and TNF-α with or without IFN-γ.

B cells producing IL-10 and GM-CSF are known to be present at relatively low frequencies in human peripheral blood (39, 40). This is in agreement with our finding that B cells were minor producers of IL-10 and GM-CSF, even after stimulation. We did however identify subsets of polyfunctional B cells that produced a combination of GM-CSF, IL-4 and IL-10. These cells were enriched among DN (CD27⁻IgD⁻) B cells and unswitched and switched memory B cells (CD27⁺). The polyfunctional B cells were also approximately 10-fold enriched among B cells expressing CD11c, which was recently associated with B cell activation and formation of atypical B cells (31), also known to expand during ATB (41). Human GM-CSF–expressing B cells are notable for being among the highest producers of both TNF-α and IL-6, and most *in vitro*-induced human IL-10⁺ B cells are also reported to secrete TNF-α and/or IL-6 (42). However, human B cell subsets have been reported to show a near-mutually exclusive expression of GM-CSF and IL-10 (39). By contrast, in our study, B cells stimulated by PIM did not co-produce GM-CSF with TNF-α or IL-6, but rather with IL-4 and IL-10, indicating a different pathway of stimulation.

PIM and LAM did not trigger detectable polyfunctional T cells, although we identified several polyfunctional T cell subsets producing combinations of IFN-γ/IL-2/IL-6/TNF-α/IL-17A that were expanded by PPD stimulation, which is in agreement with previous reports of polyfunctional Mtb-specific T cells producing IFN-γ in combination with IL-2 and TNF-α (36, 37, 43).

One important observation in this study was that upon stimulation with PIM and LAM, cells and supernatants from individuals with ATB or LTB produced less cytokines than the cells from HC. This is obvious for myeloid cells and to a lesser extent for B and T cells. The hyporesponsive state in monocytes in response to PIM and LAM is compatible with trained immunity leading to a tolerogenic cellular response. Trained immunity is defined as a long-term adaptation of innate immune cells leading either to an enhanced responsiveness or a tolerance state to a subsequent challenge (44, 45). Chronic or repeated stimulation through TLRs can render immune cells unresponsive to subsequent challenges with the same or different TLR ligands (46–48) or other bacterial components (49). Our results support the hypothesis that continuous stimulation with LAM and PIM, in ATB and LTB individuals lead to a reduced response to these molecules compared with HC.

The response of myeloid cells to PPD was weaker in LTB compared to HC, indicating hyporesponsiveness also to PPD. This is in line with earlier observations of depression of PPD-induced proliferative responses by monocytes from TB patients (50, 51), where direct stimulation of monocytes primed during

Mtb infection appear to be responsible for *in vitro* suppression of PPD responses (50). Interestingly we also found that T cells were somewhat hyporesponsive to PIM. The overall cytokine production was reduced in individuals with ATB upon PIM stimulation. These results are in agreement with the systematic review by Li et al. that found lower levels of IL-17 and IFN-γ in ATB when compared to LTB (52). An additional interesting observation in our study was that PIM expanded a cluster of GMCSF⁺ CD8⁺ T cells in HC but not in LTB patients. This hyporesponsiveness to PIM might be caused by T cell exhaustion or tolerance in Mtb infected individuals. Exhaustion of T cells represents a state of functional hyporesponsiveness due to persistent antigen exposure and inflammation reported for TB and other chronic infections (53–55). This effect can also be induced by repeated exposure to mycobacterial antigens (56), including direct exposure of T cells to LAM (57).

Antigen-specific CD4⁺ T-cell activation can be directly inhibited by LAM (58–61) and PIM (59). By interfering with very early events in TCR signaling, LAM and PIM may drive cells to a state of anergy (59, 61), which could provide another explanation of the poor response of cells from ATB and LTB individuals to Mtb glycolipids. Alternatively, the hyporesponsiveness could be indirect, through upstream effects of hyporesponsive myeloid cells, since PIM and LAM also induce proliferation of specific T cells upon presentation by CD1 molecules on myeloid cells.

IL-6 is known to be strongly induced in monocytes and DCs upon TLR2 ligation (62). We observed that PIM stimulation induced IL-6 production mainly in myeloid cells (DCs and classical/nonclassical monocytes). Moreover, treatment with an anti-TLR2 antibody led to partial inhibition of PIM-induced IL-6 production in myeloid cells, suggesting that PIM induces IL-6 production through TLR2. This is in line with other studies where it was observed that PIMs and ManLAM from Mtb induce pro-inflammatory cytokine production in human and mouse Mφs *via* recognition by TLR2 (63–65). However, IL-6 production was not completely abolished suggesting that other mechanisms of PIM stimulation likely remain, or residual IL-6 production may be due to incomplete blocking rather than additional signaling pathways.

LAM and PIM had in general very similar effects, although LAM induced a weaker response than PIM. Presuming that LAM and PIM act through the same TLR2 pathway the different responses are potentially associated with structural differences, where a common active site may be partly masked in LAM compared to PIM. Nigou et al. showed that LAM induces a weaker signal through TLR2 compared to PIM₆, suggesting that the bulky arabinan domain may mask the mannan chain in such a way that they behave like molecules with a mannan restricted to a single mannosyl unit (65). This is also in line with observations by Shukla et al. that PIM₆ induces TLR2-mediated extracellular-signal-regulated kinase (ERK) activation and TNF-α secretion in Mφs, while LAM was not an effective functional activator of TLR2 signaling (66). The weaker effect of LAM compared to PIM may also in part depend on the fact that the LAM that was used in the present study has a higher molecular weight compared to PIM resulting in a lower molar concentration.

In contrast to the glycolipids, PPD displayed a markedly different response, mainly by inducing IFN-γ. PPD contains a complex mixture of proteins, including the antigens ESAT-6 and CFP10 that are the antigens used in the Mtb specific IFN-γ release assays. We did not identify which antigens in PPD that were responsible for the immune responses presented in this study. However, since PPD is still widely used in clinical testing, the high level of details presented here may be useful to better understand how individual immune cell subsets react with Mtb proteins.

The hyporesponsive state of monocytes observed in ATB and LTB in response to PIM was more prominent in LTB. The immune profile in LTB is thought to represent a more protective pattern than in ATB (67, 68). It is possible that during LTB a continuous level of stimulation maintains a pool of protective memory cells (18), while at the same time inducing tolerance in monocytes, which could indicate protection of the host from excessive production of pro-inflammatory cytokines and control of lung tissue damage (69).

In conclusion, the detailed high dimensional overview of the cellular source of cytokines produced in response to stimulation with the various antigens, suggesting several novel sources of important cytokines (NK cell and B-cell in particular), will provide a hypothesis- generating resource for future work.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved by the Regional Ethical Review Board at the Karolinska Institute in Stockholm (approval numbers 2013/1347-31/2 and 2013/2243-31/4) and by the Ethics Committee for Research in Life and Health Sciences of the University of Minho, Portugal (approval number SECVS 014/2015). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

GK, MC-N, and CS designed the study. CSS, CS, CN, JC-G, and TL performed experiments and/or analysis. EF, GF, and JB included patients and provided clinical data. CSS, CS, and EF generated the figures and tables. BC and PB provided key resources. CSS, CS, EF, JN, MC-N, and GK wrote the first draft and all authors contributed to manuscript revision. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.727300/full#supplementary-material

REFERENCES

1. Houben RM, Dodd PJ. The Global Burden of Latent Tuberculosis Infection: A Re-Estimation Using Mathematical Modelling. *PLoS Med* (2016) 13(10): e1002152. doi: 10.1371/journal.pmed.1002152
2. Getahun H, Matteelli A, Chaisson RE, Raviglione M. Latent Mycobacterium Tuberculosis Infection. *N Engl J Med* (2015) 372(22):2127–35. doi: 10.1056/NEJMra1405427
3. Garcia-Vilanova A, Chan J, Torrelles JB. Underestimated Manipulative Roles of Mycobacterium Tuberculosis Cell Envelope Glycolipids During Infection. *Front Immunol* (2019) 10:2909. doi: 10.3389/fimmu.2019.02909

4. Kallenius G, Correia-Neves M, Buteme H, Hamasur B, Svenson SB. Lipoarabinomannan, and Its Related Glycolipids, Induce Divergent and Opposing Immune Responses to Mycobacterium Tuberculosis Depending on Structural Diversity and Experimental Variations. *Tuberc (Edinb)* (2016) 96:120–30. doi: 10.1016/j.tube.2015.09.005
5. Mazurek J, Ignatowicz L, Kallenius G, Svenson SB, Pawlowski A, Hamasur B. Divergent Effects of Mycobacterial Cell Wall Glycolipids on Maturation and Function of Human Monocyte-Derived Dendritic Cells. *PLoS One* (2012) 7 (8):e42515. doi: 10.1371/journal.pone.0042515
6. Beatty WL, Rhoades ER, Ullrich HJ, Chatterjee D, Heuser JE, Russell DG. Trafficking and Release of Mycobacterial Lipids From Infected

Macrophages. *Traffic* (2000) 1(3):235–47. doi: 10.1034/j.1600-0854.2000.
010306.x

7. Brock M, Hanlon D, Zhao M, Pollock NR. Detection of Mycobacterial Lipoarabinomannan in Serum for Diagnosis of Active Tuberculosis. *Diagn Microbiol Infect Dis* (2019) 96(2):114937. doi: 10.1016/j.diagmicrobio.2019. 114937

8. Sakamuri RM, Price DN, Lee M, Cho SN, Barry CE3rd, Via LE, et al. Association of Lipoarabinomannan With High Density Lipoprotein in Blood: Implications for Diagnostics. *Tuberc (Edinb)* (2013) 93(3):301–7. doi: 10.1016/j.tube.2013.02.015

9. Rodriguez ME, Loyd CM, Ding X, Karim AF, McDonald DJ, Canaday DH, et al. Mycobacterial Phosphatidylinositol Mannoside 6 (PIM6) Up-Regulates TCR-Triggered HIV-1 Replication in CD4+ T Cells. *PLoS One* (2013) 8(11): e80938. doi: 10.1371/journal.pone.0080938

10. Turner J, Torrelles JB. Mannose-Capped Lipoarabinomannan in Mycobacterium Tuberculosis Pathogenesis. *Pathog Dis* (2018) 76(4). doi: 10.1093/femspd/fty026

11. Vergne I, Gilleron M, Nigou J. Manipulation of the Endocytic Pathway and Phagocyte Functions by Mycobacterium Tuberculosis Lipoarabinomannan. *Front Cell Infect Microbiol* (2014) 4:187. doi: 10.3389/fcimb.2014.00187

12. Ernst JD. Macrophage Receptors for Mycobacterium Tuberculosis. *Infect Immun* (1998) 66(4):1277–81. doi: 10.1128/IAI.66.4.1277-1281.1998

13. Liu CH, Liu H, Ge B. Innate Immunity in Tuberculosis: Host Defense vs Pathogen Evasion. *Cell Mol Immunol* (2017) 14(12):963–75. doi: 10.1038/ cmi.2017.88

14. Drummond RA, Brown GD. Signalling C-Type Lectins in Antimicrobial Immunity. *PLoS Pathog* (2013) 9(7):e1003417. doi: 10.1371/journal. ppat.1003417

15. Macauley MS, Crocker PR, Paulson JC. Siglec-Mediated Regulation of Immune Cell Function in Disease. *Nat Rev Immunol* (2014) 14(10):653–66. doi: 10.1038/nri3737

16. Drickamer K, Taylor ME. Recent Insights Into Structures and Functions of C-Type Lectins in the Immune System. *Curr Opin Struct Biol* (2015) 34:26–34. doi: 10.1016/j.sbi.2015.06.003

17. Toyonaga K, Torigoe S, Motomura Y, Kamichi T, Hayashi JM, Morita YS, et al. C-Type Lectin Receptor DCAR Recognizes Mycobacterial Phosphatidyl-Inositol Mannosides to Promote a Th1 Response During Infection. *Immunity* (2016) 45(6):1245–57. doi: 10.1016/j.immuni.2016.10.012

18. Busch M, Herzmann C, Kallert S, Zimmermann A, Hofer C, Mayer D, et al. Lipoarabinomannan-Responsive Polycytotoxic T Cells Are Associated With Protection in Human Tuberculosis. *Am J Respir Crit Care Med* (2016) 194 (3):345–55. doi: 10.1164/rccm.201509-1746OC

19. Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, et al. CD1-Restricted T Cell Recognition of Microbial Lipoglycan Antigens. *Science* (1995) 269(5221):227–30. doi: 10.1126/science.7542404

20. Torrelles JB, Sieling PA, Zhang N, Keen MA, McNeil MR, Belisle JT, et al. Isolation of a Distinct Mycobacterium Tuberculosis Mannose-Capped Lipoarabinomannan Isoform Responsible for Recognition by CD1b-Restricted T Cells. *Glycobiology* (2012) 22(8):1118–27. doi: 10.1093/glycob/ cws078

21. Fischer K, Scotet E, Niemeyer M, Koebernick H, Zerrahn J, Maillet S, et al. Mycobacterial Phosphatidylinositol Mannoside Is a Natural Antigen for CD1d-Restricted T Cells. *Proc Natl Acad Sci USA* (2004) 101(29):10685–90. doi: 10.1073/pnas.0403787101

22. Ulrichs T, Moody DB, Grant E, Kaufmann SH, Porcelli SA. T-Cell Responses to CD1-Presented Lipid Antigens in Humans With Mycobacterium Tuberculosis Infection. *Infect Immun* (2003) 71(6):3076–87. doi: 10.1128/ IAI.71.6.3076-3087.2003

23. Yuan C, Qu ZL, Tang XL, Liu Q, Luo W, Huang C, et al. Mycobacterium Tuberculosis Mannose-Capped Lipoarabinomannan Induces IL-10-Producing B Cells and Hinders CD4(+)Th1 Immunity. *iScience* (2019) 11:13–30. doi: 10.1016/j.isci.2018.11.039

24. Chen H, Lau MC, Wong MT, Newell EW, Poidinger M, Chen J. Cytofkit: A Bioconductor Package for an Integrated Mass Cytometry Data Analysis Pipeline. *PLoS Comput Biol* (2016) 12(9):e1005112. doi: 10.1371/ journal.pcbi.1005112

25. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. Homogeneous Antibody-Based Proximity Extension Assays Provide Sensitive and Specific

Detection of Low-Abundant Proteins in Human Blood. *Nucleic Acids Res* (2011) 39(15):e102. doi: 10.1093/nar/gkr424

26. Gschwandtner M, Derler R, Midwood KS. More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis. *Front Immunol* (2019) 10:2759. doi: 10.3389/fimmu.2019.02759

27. Struyf S, Proost P, Vandercappellen J, Dempe S, Noyens B, Nelissen S, et al. Synergistic Up-Regulation of MCP-2/CCL8 Activity Is Counteracted by Chemokine Cleavage, Limiting Its Inflammatory and Anti-Tumoral Effects. *Eur J Immunol* (2009) 39(3):843–57. doi: 10.1002/eji.200838660

28. Lin L, Finak G, Ushey K, Seshadri C, Hawn TR, Frahm N, et al. COMPASS Identifies T-Cell Subsets Correlated With Clinical Outcomes. *Nat Biotechnol* (2015) 33(6):610–6. doi: 10.1038/nbt.3187

29. Arango Duque G, Descoteaux A. Macrophage Cytokines: Involvement in Immunity and Infectious Diseases. *Front Immunol* (2014) 5:491. doi: 10.3389/ fimmu.2014.00491

30. Li B, Jones LL, Geiger TL. IL-6 Promotes T Cell Proliferation and Expansion Under Inflammatory Conditions in Association With Low-Level RORgammat Expression. *J Immunol* (2018) 201(10):2934–46. doi: 10.4049/jimmunol. 1800016

31. Karnell JL, Kumar V, Wang J, Wang S, Voynova E, Ettinger R. Role of CD11c (+) T-Bet(+) B Cells in Human Health and Disease. *Cell Immunol* (2017) 321:40–5. doi: 10.1016/j.cellimm.2017.05.008

32. Rubtsov AV, Rubtsova K, Kappler JW, Jacobelli J, Friedman RS, Marrack P. CD11c-Expressing B Cells Are Located at the T Cell/B Cell Border in Spleen and Are Potent APCs. *J Immunol* (2015) 195(1):71–9. doi: 10.4049/jimmunol.1500055

33. Reincke ME, Payne KJ, Harder I, Strohmeier V, Voll RE, Warnatz K, et al. The Antigen Presenting Potential of CD21(low) B Cells. *Front Immunol* (2020) 11:535784. doi: 10.3389/fimmu.2020.535784

34. de Pablo-Bernal RS, Canizares J, Rosado I, Galva MI, Alvarez-Rios AI, Carrillo-Vico A, et al. Monocyte Phenotype and Polyfunctionality Are Associated With Elevated Soluble Inflammatory Markers, Cytomegalovirus Infection, and Functional and Cognitive Decline in Elderly Adults. *J Gerontol A Biol Sci Med Sci* (2016) 71(5):610–8. doi: 10.1093/gerona/glv121

35. Mishra A, Singh VK, Actor JK, Hunter RL, Jagannath C, Subbian S, et al. GM-CSF Dependent Differential Control of Mycobacterium Tuberculosis Infection in Human and Mouse Macrophages: Is Macrophage Source of GM-CSF Critical to Tuberculosis Immunity? *Front Immunol* (2020) 11:1599. doi: 10.3389/fimmu.2020.01599

36. Beveridge NE, Price DA, Casazza JP, Pathan AA, Sander CR, Asher TE, et al. Immunisation With BCG and Recombinant MVA85A Induces Long-Lasting, Polyfunctional Mycobacterium Tuberculosis-Specific CD4+ Memory T Lymphocyte Populations. *Eur J Immunol* (2007) 37(11):3089–100. doi: 10.1002/eji.200737504

37. Scriba TJ, Tameris M, Mansoor N, Smit E, van der Merwe L, Isaacs F, et al. Modified Vaccinia Ankara-Expressing Ag85A, a Novel Tuberculosis Vaccine, Is Safe in Adolescents and Children, and Induces Polyfunctional CD4+ T Cells. *Eur J Immunol* (2010) 40(1):279–90. doi: 10.1002/eji.200939754

38. Wilkinson KA, Wilkinson RJ. Polyfunctional T Cells in Human Tuberculosis. *Eur J Immunol* (2010) 40(8):2139–42. doi: 10.1002/eji.201040731

39. Li R, Rezk A, Miyazaki Y, Hilgenberg E, Touil H, Shen P, et al. Proinflammatory GM-CSF-Producing B Cells in Multiple Sclerosis and B Cell Depletion Therapy. *Sci Transl Med* (2015) 7(310):310ra166. doi: 10.1126/ scitranslmed.aab4176

40. Rezk A, Li R, Bar-Or A. Multiplexed Detection and Isolation of Viable Low-Frequency Cytokine-Secreting Human B Cells Using Cytokine Secretion Assay and Flow Cytometry (CSA-Flow). *Sci Rep* (2020) 10(1):14823. doi: 10.1038/s41598-020-71750-z

41. Joosten SA, van Meijgaarden KE, Del Nonno F, Baiocchini A, Petrone L, Vanini V, et al. Patients With Tuberculosis Have a Dysfunctional Circulating B-Cell Compartment, Which Normalizes Following Successful Treatment. *PLoS Pathog* (2016) 12(6):e1005687. doi: 10.1371/journal.ppat.1005687

42. Lighaam LC, Unger PA, Vredevoogd DW, Verhoeven D, Vermeulen E, Turksma AW, et al. *In Vitro*-Induced Human IL-10(+) B Cells Do Not Show a Subset-Defining Marker Signature and Plastically Co-Express IL-10 With Pro-Inflammatory Cytokines. *Front Immunol* (2018) 9:1913. doi: 10.3389/fimmu.2018.01913

43. Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ, et al. Multifunctional TH1 Cells Define a Correlate of Vaccine-Mediated

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Protection Against Leishmania Major. *Nat Med* (2007) 13(7):843–50. doi: 10.1038/nm1592

44. Netea MG, Dominguez-Andres J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining Trained Immunity and Its Role in Health and Disease. *Nat Rev Immunol* (2020) 20(6):375–88. doi: 10.1038/s41577-020-0285-6

45. Divangahi M, Aaby P, Khader SA, Barreiro LB, Bekkering S, Chavakis T, et al. Trained Immunity, Tolerance, Priming and Differentiation: Distinct Immunological Processes. *Nat Immunol* (2021) 22(1):2–6. doi: 10.1038/s41590-020-00845-6

46. Cavaillon JM, Adib-Conquy M. Bench-To-Bedside Review: Endotoxin Tolerance as a Model of Leukocyte Reprogramming in Sepsis. *Crit Care* (2006) 10(5):233. doi: 10.1186/cc5055

47. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, et al. Cutting Edge: Endotoxin Tolerance in Mouse Peritoneal Macrophages Correlates With Down-Regulation of Surface Toll-Like Receptor 4 Expression. *J Immunol* (2000) 164(7):3476–9. doi: 10.4049/jimmunol.164.7.3476

48. Poovassery JS, Vanden Bush TJ, Bishop GA. Antigen Receptor Signals Rescue B Cells From TLR Tolerance. *J Immunol* (2009) 183(5):2974–83. doi: 10.4049/jimmunol.0900495

49. Ifrim DC, Quintin J, Joosten LA, Jacobs C, Jansen T, Jacobs L, et al. Trained Immunity or Tolerance: Opposing Functional Programs Induced in Human Monocytes After Engagement of Various Pattern Recognition Receptors. *Clin Vaccine Immunol* (2014) 21(4):534–45. doi: 10.1128/CVI.00688-13

50. Ellner JJ. Suppressor Adherent Cells in Human Tuberculosis. *J Immunol* (1978) 121(6):2573–9.

51. Ellner JJ. Regulation of the Human Cellular Immune Response to Mycobacterium Tuberculosis. The Mechanism of Selective Depression of the Response to PPD. *Bull Int Union Tuberc Lung Dis* (1991) 66(2-3):129–32.

52. Li Q, Li J, Tian J, Zhu B, Zhang Y, Yang K, et al. IL-17 and IFN-Gamma Production in Peripheral Blood Following BCG Vaccination and Mycobacterium Tuberculosis Infection in Human. *Eur Rev Med Pharmacol Sci* (2012) 16(14):2029–36.

53. Schietinger A, Greenberg PD. Tolerance and Exhaustion: Defining Mechanisms of T Cell Dysfunction. *Trends Immunol* (2014) 35(2):51–60. doi: 10.1016/j.it.2013.10.001

54. Blank CU, Haining WN, Held W, Hogan PG, Kallies A, Lugli E, et al. Defining 'T Cell Exhaustion'. *Nat Rev Immunol* (2019) 19(11):665–74. doi: 10.1038/s41577-019-0221-9

55. Khan N, Vidyarthi A, Amir M, Mushtaq K, Agrewala JN. T-Cell Exhaustion in Tuberculosis: Pitfalls and Prospects. *Crit Rev Microbiol* (2017) 43(2):133–41. doi: 10.1080/1040841X.2016.1185603

56. Liu X, Li F, Niu H, Ma L, Chen J, Zhang Y, et al. IL-2 Restores T-Cell Dysfunction Induced by Persistent Mycobacterium Tuberculosis Antigen Stimulation. *Front Immunol* (2019) 10:2350. doi: 10.3389/fimmu.2019.02350

57. Sande OJ, Karim AF, Li Q, Ding X, Harding CV, Rojas RE, et al. Mannose-Capped Lipoarabinomannan From Mycobacterium Tuberculosis Induces CD4+ T Cell Anergy *via* GRAIL. *J Immunol* (2016) 196(2):691–702. doi: 10.4049/jimmunol.1500710

58. Karim AF, Sande OJ, Tomechko SE, Ding X, Li M, Maxwell S, et al. Proteomics and Network Analyses Reveal Inhibition of Akt-mTOR Signaling in CD4(+) T Cells by Mycobacterium Tuberculosis Mannose-Capped Lipoarabinomannan. *Proteomics* (2017) 17(22). doi: 10.1002/pmic.201700233

59. Mahon RN, Rojas RE, Fulton SA, Franko JL, Harding CV, Boom WH. Mycobacterium Tuberculosis Cell Wall Glycolipids Directly Inhibit CD4+ T-Cell Activation by Interfering With Proximal T-Cell-Receptor Signaling. *Infect Immun* (2009) 77(10):4574–83. doi: 10.1128/IAI.00222-09

60. Athman JJ, Sande OJ, Groft SG, Reba SM, Nagy N, Wearsch PA, et al. Mycobacterium Tuberculosis Membrane Vesicles Inhibit T Cell Activation. *J Immunol* (2017) 198(5):2028–37. doi: 10.4049/jimmunol.1601199

61. Mahon RN, Sande OJ, Rojas RE, Levine AD, Harding CV, Boom WH. Mycobacterium Tuberculosis ManLAM Inhibits T-Cell-Receptor Signaling by Interference With ZAP-70, Lck and LAT Phosphorylation. *Cell Immunol* (2012) 275(1-2):98–105. doi: 10.1016/j.cellimm.2012.02.009

62. Flynn CM, Garbers Y, Lokau J, Wesch D, Schulte DM, Laudes M, et al. Activation of Toll-Like Receptor 2 (TLR2) Induces Interleukin-6 Trans-Signaling. *Sci Rep* (2019) 9(1):7306. doi: 10.1038/s41598-019-43617-5

63. Gilleron M, Quesniaux VF, Puzo G. Acylation State of the Phosphatidylinositol Hexamannosides From Mycobacterium Bovis Bacillus Calmette Guerin and Mycobacterium Tuberculosis H37Rv and Its Implication in Toll-Like Receptor Response. *J Biol Chem* (2003) 278(32):29880–9. doi: 10.1074/jbc.M303446200

64. Riedel DD, Kaufmann SH. Differential Tolerance Induction by Lipoarabinomannan and Lipopolysaccharide in Human Macrophages. *Microbes Infect* (2000) 2(5):463–71. doi: 10.1016/S1286-4579(00)00319-1

65. Nigou J, Vasselon T, Ray A, Constant P, Gilleron M, Besra GS, et al. Mannan Chain Length Controls Lipoglycans Signaling *via* and Binding to TLR2. *J Immunol* (2008) 180(10):6696–702. doi: 10.4049/jimmunol.180.10.6696

66. Shukla S, Richardson ET, Drage MG, Boom WH, Harding CV. Mycobacterium Tuberculosis Lipoprotein and Lipoglycan Binding to Toll-Like Receptor 2 Correlates With Agonist Activity and Functional Outcomes. *Infect Immun* (2018) 86(10):e00450-18. doi: 10.1128/IAI.00450-18

67. Roy Chowdhury R, Vallania F, Yang Q, Lopez Angel CJ, Darboe F, Penn-Nicholson A, et al. A Multi-Cohort Study of the Immune Factors Associated With M. Tuberculosis Infection Outcomes. *Nature* (2018) 560(7720):644–8. doi: 10.1038/s41586-018-0439-x

68. Lu LL, Chung AW, Rosebrock TR, Ghebremichael M, Yu WH, Grace PS, et al. A Functional Role for Antibodies in Tuberculosis. *Cell* (2016) 167(2):433–43.e14. doi: 10.1016/j.cell.2016.08.072

69. Divangahi M, Khan N, Kaufmann E. Beyond Killing Mycobacterium Tuberculosis: Disease Tolerance. *Front Immunol* (2018) 9:2976. doi: 10.3389/fimmu.2018.02976

6. SUPPLEMENTARY MATERIAL

Supplemental Table 1. Study individuals.

	ATB (n=5)	LTBI (n=5)	HC (n=5)
Male sex	5	2	4
Age (median, range)	39 (24-50)	39 (25-72)	25 (22-37)
Comorbidity	2*	0	0
Born in TB high endemic country	4	3	0
Years since immigration to Sweden	8 (0.5-20)	11 (3-16)	NA
BCG vaccination	unknown	3	1
Symptoms		NA	NA
- cough	4		
- fever/night sweats/weight loss	3		
IGRA			
- positive		4	0
- conversion (after 2 months)	NA	1	0
- negative		0	5
Chest X-Ray/Computed Tomography			
- infiltrates	5	2**	
- cavitaries	2	0	NA
- pleural effusion	3	0	
Mycobacteriology			
- microscopy positive	1	0	
- PCR positive	1	0	NA
- culture positive	5	0	

*Well-controlled diabetes mellitus type 2, chronic kidney disease stage 2; **Normal on repeat radiology

NA: not applicable; ATB: Active TB; LTB: latent TB; HC: Healthy controls.

Supplemental Table 2. Broad staining panel for mass cytometry.

Marker	Clone	Tag	Company	Dilution
CD45	HI30	89Y	Fluidigm	1:200
CD57	HCD57	115In	BioLegend	1:100
CD19	HIB19	142Nd	BioLegend	1:100
CD5	UCHT2	143Nd	BioLegend	1:200
CD16	3G8	144Nd	BioLegend	1:100
CD4	RPA-T4	145Nd	BioLegend	1:100
CD11c	Bu15	147Sm	Fluidigm	1:100
CD123	6H6	151Eu	BioLegend	1:100
CD3e	UCHT1	154Sm	Fluidigm	1:200
CD14	M5E2	160Gd	BioLegend	1:100
CD161	HP-3G10	161Dy	BioLegend	1:100
CD127	A019D5	165Ho	Fluidigm	1:100
CD38	HIT2	168Er	BioLegend	1:100
CD45RA	HI100	169Tm	Fluidigm	1:100
CD20	2H7	170Er	BioLegend	1:100
IgD	IA6-2	172Yb	BioLegend	1:100
Cell-ID™ Intercalator-Ir	DNAIr-191	-	Fluidigm	1:1000
Cell-ID™ Intercalator-Ir	DNAIr-193	-	Fluidigm	1:1000
CD31	WM59	148Nd	BioLegend	1:200
HLA-DR	L243	163Dy	BioLegend	1:200
CD44	BJ18	174Yb	BioLegend	1:100
CD8a	SK1	146Nd	BioLegend	1:300
CD11b (MAC1)	Mac-1	209Bi	Fluidigm	1:200
CD56	NCAM16.2	173Yb	BD	1:200
CD7	CD7-687	155Gd	BioLegend	1:200
CD27	L128	167Er	Fluidigm	1:200
Siglec-8	7C9	164Dy	Fluidigm	1:100
CD33	WM53	157Gd	BioLegend	1:100
TCRγδ	5A6.E9	152Sm	Fisher	1:100

Supplemental Table 3. Intracellular staining panel for mass cytometry.

Marker	Clone	Tag	Company	Dilution
IL-2	MQ1-17H12	158Gd	Fluidigm	1:200
IL-4	MP4-25D2	149Sm	BioLegend	1:100
IL-5	TRFK5	153Eu	BioLegend	1:100
IL-6	MQ2-13AS	156Gd	Fluidigm	1:100
IL-10	JES3-9D7	159Tb	BioLegend	1:100
IL-17A	N49-653	166Er	BioLegend	1:100
IFN-γ	B27	150Nd	BioLegend	1:125
TNF	MAb11	175Lu	BioLegend	1:75
Granzyme B	GB11	171Yb	Fluidigm	1:100
GM-CSF	BVD2-21C11	176Yb	BioLegend	1:75

**Supplemental Table 4. Olink inflammation panel.**

Protein	UniProt ID	Protein	UniProt ID
Adenosine Deaminase (ADA)	P00813	Fractalkine (CX3CL1)	P78423
Artemin (ARTN)	Q5T4W7	Glial cell line-derived neurotrophic factor (GDNF)	P39905
Axin-1 (AXIN1)	O15169	Hepatocyte growth factor (HGF)	P14210
Beta-nerve growth factor (Beta-NGF)	P01138	Interferon gamma (IFN-gamma)	P01579
Caspase-8 (CASP-8)	Q14790	Interleukin-1 alpha (IL-1 alpha)	P01583
C-C motif chemokine 3 (CCL3)	P10147	Interleukin-2 (IL-2)	P60568
C-C motif chemokine 4 (CCL4)	P13236	Interleukin-2 receptor subunit beta (IL-2RB)	P14784
C-C motif chemokine 19 (CCL19)	Q99731	Interleukin-4 (IL-4)	P05112
C-C motif chemokine 20 (CCL20)	P78556	Interleukin-5 (IL5)	P05113
C-C motif chemokine 23 (CCL23)	P55773	Interleukin-6 (IL6)	P05231
C-C motif chemokine 25 (CCL25)	O15444	Interleukin-7 (IL-7)	P13232
C-C motif chemokine 28 (CCL28)	Q9NRJ3	Interleukin-8 (IL-8)	P10145
CD40L receptor (CD40)	P25942	Interleukin-10 (IL10)	P22301
CUB domain-containing protein 1 (CDCP1)	Q9H5V8	Interleukin-10 receptor subunit alpha (IL-10RA)	Q13651
C-X-C motif chemokine 1 (CXCL1)	P09341	Interleukin-10 receptor subunit beta (IL-10RB)	Q08334
C-X-C motif chemokine 5 (CXCL5)	P42830	Interleukin-12 subunit beta (IL-12B)	P29460
C-X-C motif chemokine 6 (CXCL6)	P80162	Interleukin-13 (IL-13)	P35225
C-X-C motif chemokine 9 (CXCL9)	Q07325	Interleukin-15 receptor subunit alpha (IL-15RA)	Q13261
C-X-C motif chemokine 10 (CXCL10)	P02778	Interleukin-17A (IL-17A)	Q16552
C-X-C motif chemokine 11 (CXCL11)	O14625	Interleukin-17C (IL-17C)	Q9P0M4
Cystatin D (CST5)	P28325	Interleukin-18 (IL-18)	Q14116
Delta and Notch-like epidermal growth factor-related receptor (DNER)	Q8NFT8	Interleukin-18 receptor 1 (IL-18R1)	Q13478
Eotaxin (CCL11)	P51671	Interleukin-20 (IL-20)	Q9NYY1
Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1)	Q13541	Interleukin-20 receptor subunit alpha (IL-20RA)	Q9UHF4
Fibroblast growth factor 21 (FGF-21)	Q9NSA1	Interleukin-22 receptor subunit alpha-1 (IL-22 RA1)	Q8N6P7
Fibroblast growth factor 23 (FGF-23)	Q9GZV9	Interleukin-24 (IL-24)	Q13007
Fibroblast growth factor 5 (FGF-5)	P12034	Interleukin-33 (IL-33)	O95760
Fibroblast growth factor 19 (FGF-19)	O95750	Latency-associated peptide transforming growth factor beta-1 (LAP TGF-beta-1)	P01137
Fms-related tyrosine kinase 3 ligand (Flt3L)	P49771	Leukemia inhibitory factor (LIF)	P15018
Leukemia inhibitory factor receptor (LIF-R)	P42702	STAM-binding protein (STAMBP)	O95630
Macrophage colony-stimulating factor 1 (CSF-1)	P09603	Stem cell factor (SCF)	P21583
Matrix metalloproteinase-1 (MMP-1)	P03956	Sulfotransferase 1A1 (ST1A1)	P50225
Matrix metalloproteinase-10 (MMP-10)	P09238	T cell surface glycoprotein CD6 isoform (CD6)	P30203

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Monocyte chemotactic protein 1 (MCP-1)	P13500	T-cell surface glycoprotein CD5 (CD5)	P06127
Monocyte chemotactic protein 2 (MCP-2)	P80075	T-cell surface glycoprotein CD8 alpha chain (CD8A)	P01732
Monocyte chemotactic protein 3 (MCP-3)	P80098	Thymic stromal lymphopoietin (TSLP)	Q969D9
Monocyte chemotactic protein 4 (MCP-4)	Q99616	TNF-beta (TNFB)	P01374
Natural killer cell receptor 2B4 (CD244)	Q9BZW8	TNF-related activation-induced cytokine (TRANCE)	O14788
Neurotrophin-3 (NT-3)	P20783	TNF-related apoptosis-inducing ligand (TRAIL)	P50591
Neurturin (NRTN)	Q99748	Transforming growth factor alpha (TGF-alpha)	P01135
Oncostatin-M (OSM)	P13725	Tumor necrosis factor (Ligand) superfamily, member 12 (TWEAK)	O43508
Osteoprotegerin (OPG)	O00300	Tumor necrosis factor (TNF)	P01375
Programmed cell death 1 ligand 1 (PD-L1)	Q9NZQ7	Tumor necrosis factor ligand superfamily member 14 (TNFSF14)	O43557
Protein S100-A12 (EN-RAGE)	P80511	Tumor necrosis factor receptor superfamily member 9 (TNFRSF9)	Q07011
Signaling lymphocytic activation molecule (SLAMF1)	Q13291	Urokinase-type plasminogen activator (uPA)	P00749
SIR2-like protein 2 (SIRT2)	Q8IXJ6	Vascular endothelial growth factor A (VEGF-A)	P15692

**Supplemental Table 5. Membrane and intracellular staining panel for flow cytometry.**

Marker	Clone	Fluorochrome	Company	Dilution
Surface staining				
CD3	UCHT1	V500	BD Horizon	1:10
CD4	HI30	APC-Cy7	BioLegend	1:80
CD8	SK1	BV650	BioLegend	1:40
CD19	HIB19	BV711	BioLegend	1:40
CD33	WM53	PE-Cy7	BioLegend	1:40
CD45	HI30	BV605	BioLegend	1:10
CD56	NCAM16.2	BV786	BD Horizon	1:10
Viability staining				
Fixable viability dye	-	eFluor™ 450	eBiosciences	1:1000
Intracellular staining				
IL-2	MQ1-17H12	PerCP-Cy5.5	BioLegend	1:20
IL-6	MQ2-13AS	FITC	Fluidigm	1:7
TNF	MAb11	PE	BioLegend	1:20
GM-CSF	BVD2-21C11	APC	BioLegend	1:10
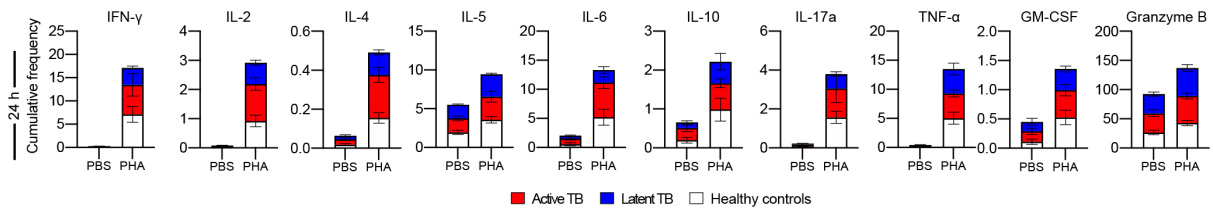
Supplemental Figure 1. Intracellular cytokine staining after PHA stimulation. Frequency of cytokine-producing leukocytes with PHA stimulation after 24 hours. All donors merged (n=15/condition).
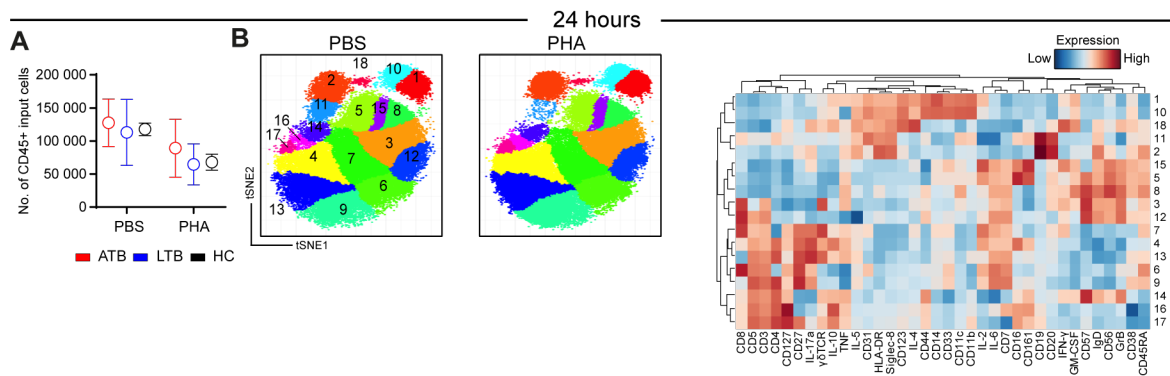
Supplemental Figure 2. Clustering analysis of total leukocytes after 24 hours of stimulation. (A) Numbers of CD45^{+} input cells in unstimulated (PBS) and PHA-stimulated PBMCS in ATB, LTB, and HC, after 24 hours of stimulation. **(B)** Cluster identification and median heatmap expression of each marker.
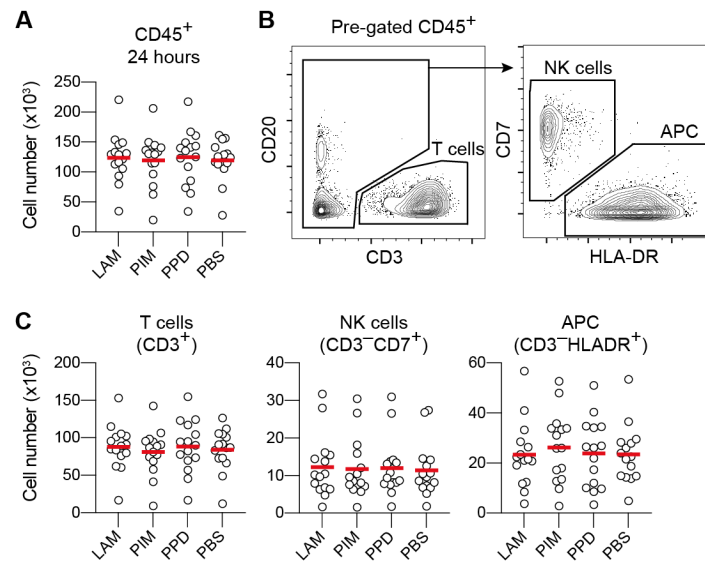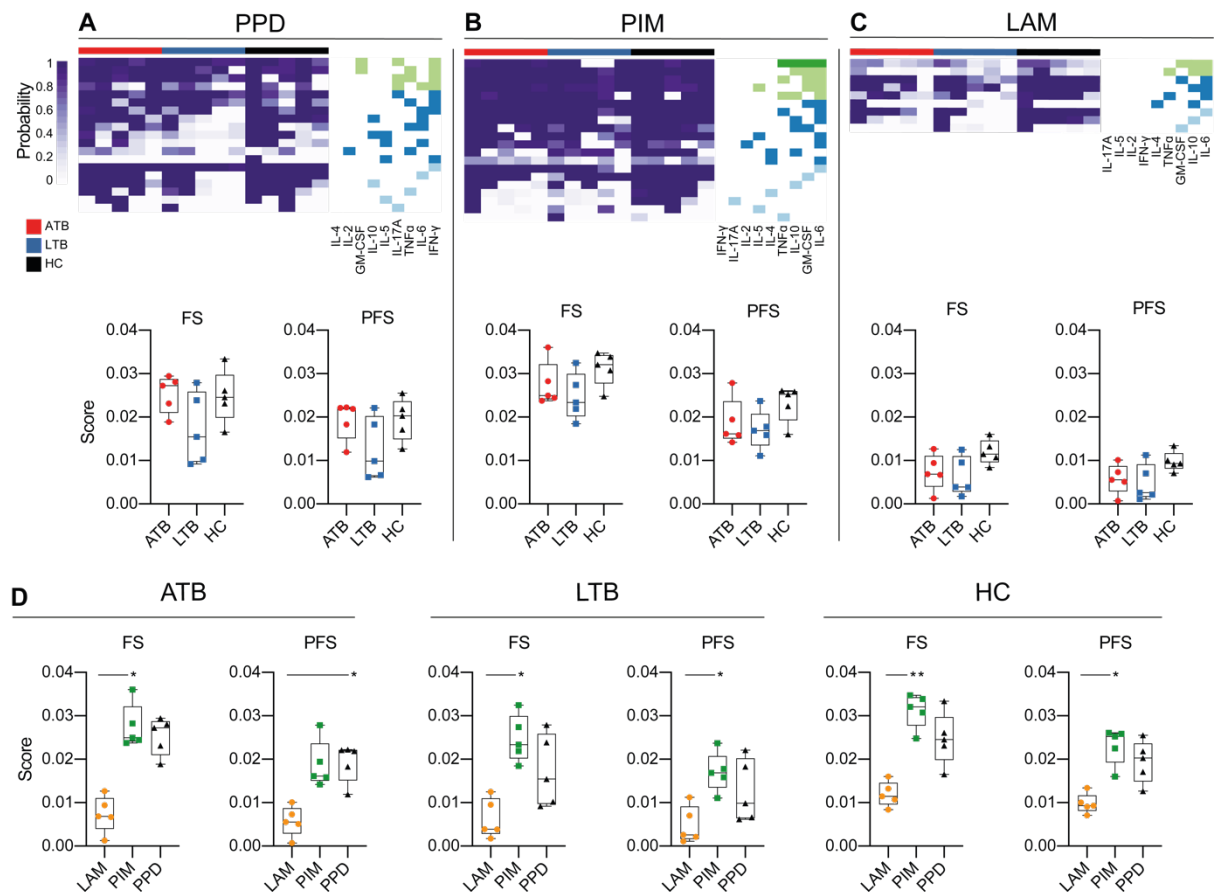
Supplemental Figure 3. Effect of stimulation with LAM, PIM, and PPD on culture cell numbers. (A) Number of total CD45+ cells for each condition at 24 hours of stimulation. **(B)** Representative gating strategy to further subdivide CD45+ cells into CD3+ T cells, CD3-CD7+ NK cells, and CD3-CD7-HLA-DR+ antigen-presenting cells (APC). **(C)** Number of T cells, NK cells and APCs after 24 h. Statistics was evaluated by Friedmans test with Dunn's posttest where every group was compared with unstimulated cells (PBS). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
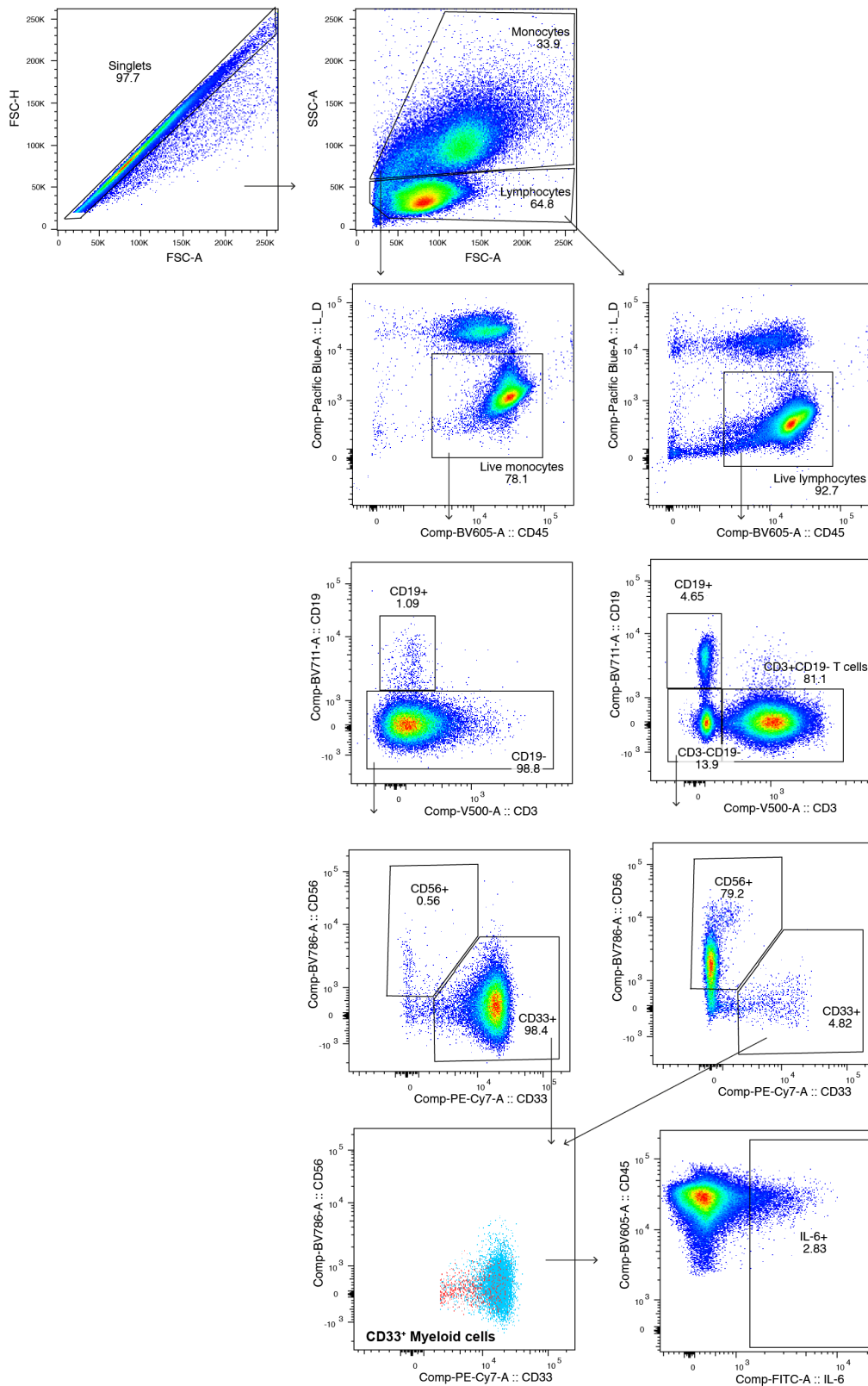
Supplemental Figure 4. Flow cytometry gating strategy for the identification of IL-6-producing-myeloid cells.
Total lymphocytes and monocytes were gated from singlets (based on FSC-H/FSC-A) based on SSC-S/FSC-A. Live lymphocytes and live monocytes were gated from total lymphocytes and total monocytes, respectively. Myeloid cells (CD3-CD33-) were gated on CD3-CD19- both in lymphocytes and monocytes gate and merged using a Boolean gate. IL-6 producing cells were gated on myeloid cells.

Supplemental Figure 5. Polyfunctionality analysis of CD45+ cells in response to PPD, PIM, and LAM stimulations. Boolean gates were created from the nine individual cytokines in FlowJo to divide responding cells into 512 distinct subsets corresponding to all possible combinations of these functions, and data was analyzed using COMPASS. Only subsets having over five cells in more than two subjects were included in the analysis. Heatmaps of COMPASS posterior probability for the **(A)** PPD (top), **(B)** PIM (top), and **(C)** LAM (top) stimulations. Rows correspond to the different cell subsets modeled by COMPASS, each color represent the cytokines expressed (white = "off", shaded = "on", color = "degree of functionality") and are ordered by degree of functionality from one function (bottom) to four functions (top). Columns correspond to the individuals. Each cell of the heatmap shows the probability that a given cell-subset (row) has an antigen-specific response in the corresponding subject (column), where the probability is color-coded from white (zero) to purple (one). Box plots of FS and PFS stratified by (**A**, **B**, and **C** - bottom) groups and **(D)** stimulations. The FS is defined as the proportion of antigen-specific subsets detected among all possible ones, while PFS weighs the different subsets by their degree of functionality. Statistical differences between groups in (A, B, and C) were evaluated by Kruskal-Wallis, while comparisons between stimulations in (D) were evaluated by Friedman's test with Dunns's posttest *p<0.05, **p<0.01.
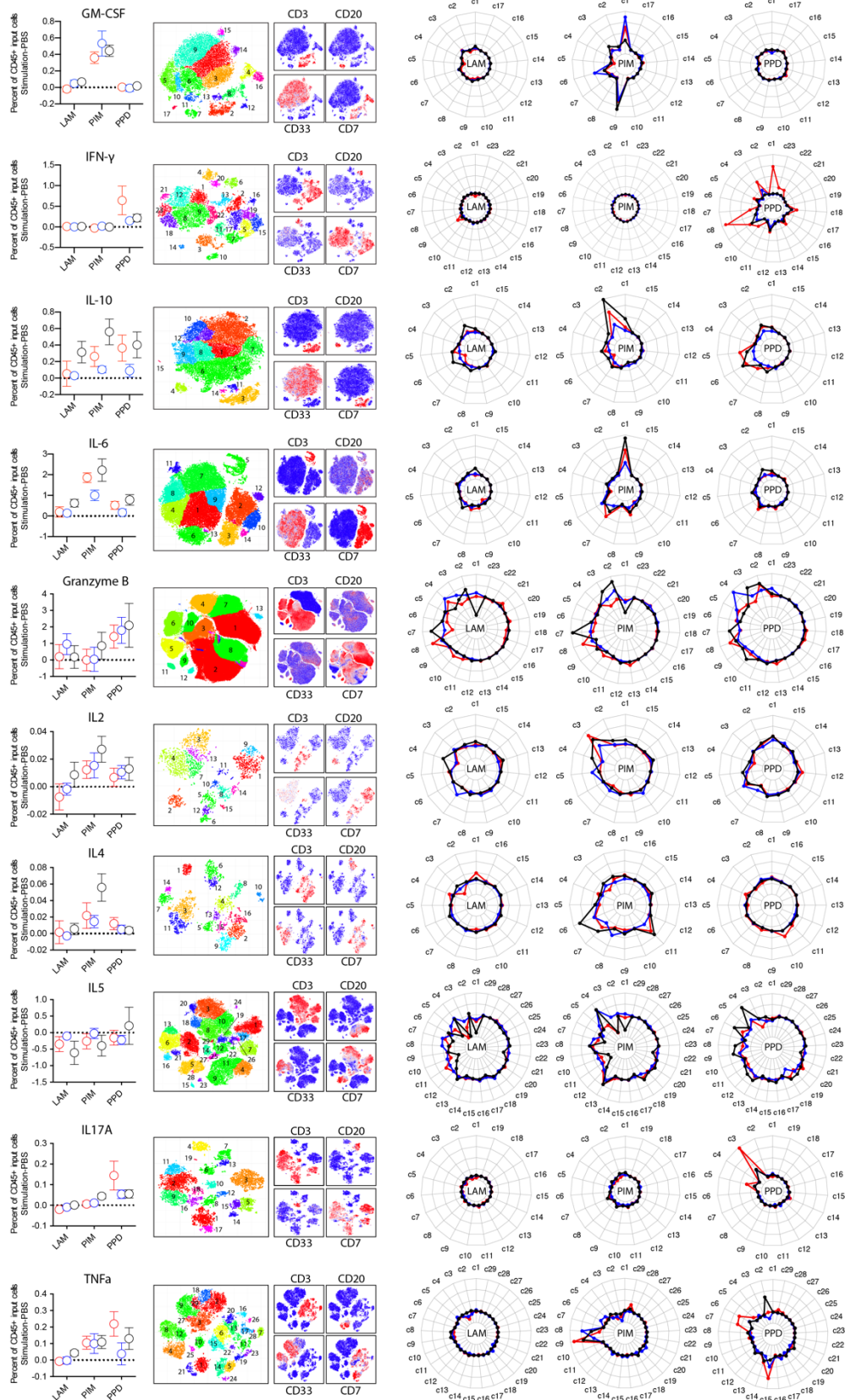
Supplemental Figure 6. Group and cluster analysis of cytokine+ cells after 24 hours of stimulation. Cytokine+ cells were exported after 24 h of stimulation as gated in figure 3 and analyzed by tSNE dimensionality reduction and clustering using cytofkit with ClusterX. To provide some guidance on the type of cells that compose the different clusters the expression of CD3 (T cells), CD20 (B cells), CD33 (myeloid cells), and CD7 (NK cells if negative for CD3). In tSNE plots, red indicates

high expression and blue indicates low expression. The frequency of cytokine+ cells for each stimulation minus background signal (PBS) grouped (left graphs, mean +/– SEM) and from each cluster (spider plots, mean) out of total CD45+ cells, were compared between individuals with active TB (red), latent TB (blue), and healthy controls (black).
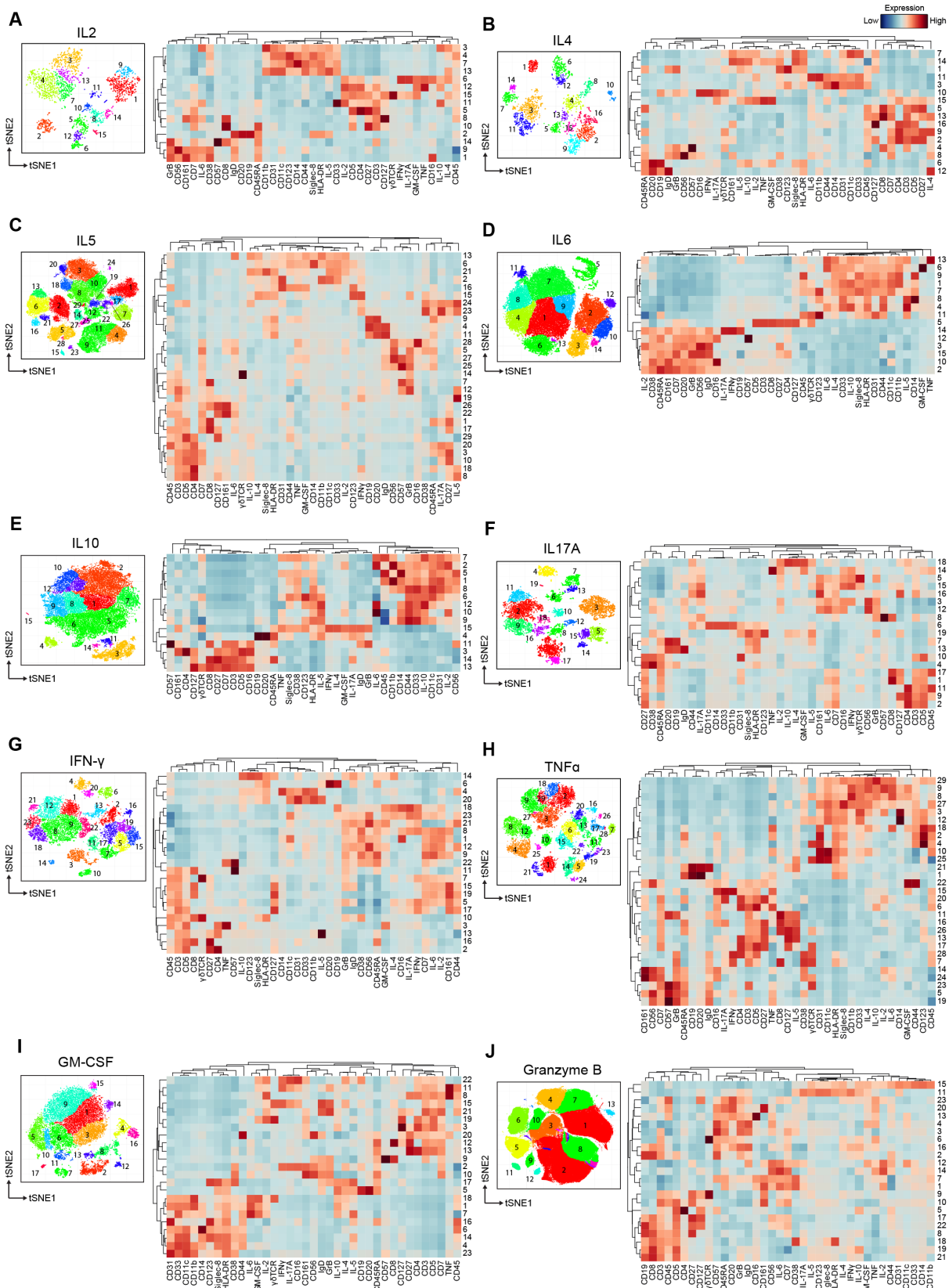
Supplemental Figure 7. Marker expression of cytokine+ cells after 24 hours of stimulation. Cluster identification (Y-axis) and median heatmap expression of each marker (X-axis) in (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-6, (E) IL-10, (F) IL-17A, (G) IFN-γ, (H) TNF-α, (I) GM-CSF, and (J) granzyme B-producing cells. The expression is normalized within the observed range for each marker.

CHAPTER III

IMMUNOPHENOTYPING AND FUNCTIONAL ANALYSIS OF PERIPHERAL
IMMUNE CELL SUBSETS IN LATENT AND ACTIVE TUBERCULOSIS

IMMUNOPHENOTYPING AND FUNCTIONAL ANALYSIS OF PERIPHERAL IMMUNE CELL SUBSETS IN LATENT AND ACTIVE TUBERCULOSIS

ABSTRACT

Tuberculosis remains a significant public health problem in the twenty-first century, being the leading cause of death from a single infectious agent after COVID-19. Upon infection with *Mycobacterium tuberculosis* (Mtb) the immune system might clear the bacteria, control its growth leading to latent tuberculosis (LTB), or fail to control Mtb growth resulting in active tuberculosis (ATB). About 10% of the individuals with LTB will progress to ATB throughout their life, but the features underlying this progression are poorly understood. Thus, to better understand the immune cell populations underlying the immune response in various stages of Mtb infection, we performed a comprehensive immune profiling and comparison of peripheral blood mononuclear cells from individuals with LTB, with ATB and healthy controls. Moreover, we evaluated the function of NK cells in terms of degranulation, cytotoxicity activity, and cytokine production. We identified distinct immune profiles among the different status of Mtb infection. Individuals with ATB have higher percentages of naïve B cells. Moreover, individuals with LTB present higher percentages of IgM^{bright} B cells, while individuals with ATB have higher percentages of IgM^{dim} B cells. Although NK subsets were similar between groups, further investigation of NK functions revealed that individuals with LTB showed decreased NK cell function in terms of cytokine production, compared to individuals with ATB and healthy controls.

Keywords: tuberculosis (TB); active tuberculosis (ATB); latent tuberculosis (LTB); immunophenotyping; functional assays; NK cells; B cells.

CHAPTER III

1. INTRODUCTION

Despite considerable efforts, tuberculosis (TB) is still one of the deadliest infectious diseases worldwide [WHO, 2022]. Infection with *Mycobacterium tuberculosis* (Mtb) is commonly divided into active TB (ATB) or latent TB (LTB), depending on whether the individuals develop clinical symptoms (ATB) or not (LTB) [Pai et al., 2016]. However, inhalation of droplets containing Mtb results in different clinical outcomes that essentially depend on the host immune response to the pathogen [Pai et al., 2016]. This ranges from bacteria elimination, either through the innate or the adaptive immune system, to the development of disease symptoms. Most of the individuals who have had contact with the bacterium retain immunological memory of Mtb antigens but do not progress to an active form of the disease [Behr et al., 2019]. This suggests that these individuals can naturally control Mtb growth. However, the immune factors underlying the control or progression to TB disease are not clearly defined.

T cell-dependent adaptive immunity was thought to be the hallmark of protection in TB, with the production of IFN-γ by CD4+ T cells being a key mediator [Cooper, 2009]. These cells have been extensively investigated while other immune cell populations have been less explored. However, a protective immune response to Mtb is orchestrated by multiple immune cells and the cytokines secreted by these cells [Nunes-Alves et al., 2014].

NK cells are innate immune cells that target infected host cells via antibody-mediated cytotoxic mechanisms and secretion of proinflammatory cytokines [Mujal et al., 2021]. The Mtb bacilli survive in intracellular niches in infected macrophages, potentially indicating an important role of NK cells in controlling the infection. This is further supported by observations of a unique profile of antibody constant region (Fc) in individuals with LTB associated with enhanced NK cell-mediated Fc effector function [Lu et al., 2016], suggesting a potential interplay between antibodies and NK cells in TB [Li and Javid, 2018].

Thus, to better understand the immune cell populations underlying the immune response in various stages of Mtb infection, we performed comprehensive immune profiling and comparison of peripheral blood mononuclear cells (PBMCs) from individuals infected with Mtb who control bacterial growth (LTB) or progress to disease (ATB) and healthy individuals. Moreover, we evaluated the function of NK cells in terms of degranulation and cytokine production. We identified distinct immune profiles among the different status of Mtb infection. Individuals with ATB have higher percentages of naïve B cells. Furthermore, individuals with LTB present higher percentages of IgM^bright B cells, while individuals with ATB have higher percentages of IgM^dim B cells. We observed that frequencies of NK subsets were similar between groups, but further investigation of NK functions revealed that individuals with LTB have a diminished NK cell function in terms of cytokine production, compared to individuals with ATB and healthy controls.

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2. METHODS

Study participants

Participants were recruited between 2018 and 2022 within an ongoing prospective cohort of adult (≥18 years) TB patients, contacts to active cases, and IGRA⁺ migrants attending the TB Centre, Dept of Infectious Diseases Karolinska University Hospital Stockholm (Supplemental Table 1). Active TB cases were defined upon microbiological (PCR and/or culture) verification; Latent TB participants were defined as asymptomatic and IGRA⁺. Healthy controls (HC) were defined as IGRA negative students and hospital staff without known previous TB exposure. Exclusion criteria were pregnancy, autoimmune diseases, and HIV co-infection or other immunodeficiencies. Participants with ATB and LTB were screened with standard biochemical set-up and radiology.

PBMCs isolation

Venous blood from each participant was collected into EDTA tubes and PBMCs were purified through density gradient centrifugation using Ficoll (Cytiva) and SepMate tubes (StemCell) according to the manufacturer's instructions. Briefly, whole blood was diluted with an equal volume of PBS supplemented with 2% of heat-inactivated FBS (2% FBS-PBS) and then layered onto 15 mL Ficoll. The cells were centrifuged at $1200g$ for 10 min with the brake on. The mononuclear cell layer was collected into a new 50 ml tube and resuspended to 45 ml with 2% FBS-PBS. The cells were spun at $300g$ for 10 min, after which the cells were resuspended into 1-5 mL FBS and counted on a Countess (ThermoFisher Scientific) with trypan blue to indicate dead cells. The cells were centrifuged at $250g$ for 10 min and resuspended with freeze media (90% FBS supplemented with 10% DMSO) and placed in a Mr.Frosty freezing container (Sigma) before moving to –80°C overnight followed by long-term storage in liquid nitrogen.

Mass cytometry phenotyping (staining and acquisition)

Mass cytometry data was repurposed from the study in CHAPTER II where PBMCs from five patients with ATB, five with LTB and five HCs, were assessed for phenotype and cytokine production after 24 h of culture.

Analysis of mass cytometry data

The mass cytometry FCS data files were gated for different cell subsets: CD45⁺ leucocytes, CD45⁺CD3⁺CD20⁻ T cells, CD45⁺CD3⁻CD7⁺ NK cells, CD45⁺CD3⁻HLA-DR⁺ antigen-presenting cells (APCs),

using FlowJo™ v10.6.1. The gated populations were exported to new FCS files that were then analyzed using the R-package Cytofkit v1.12.0, which includes an integrated pipeline for mass cytometry analysis [Chen et al., 2016]. Cytofkit was ran in R-studio version 1.1.463 and R version 3.6.1. For analysis of total leukocytes, 5000 cells were used per sample. For analysis of gated T cells, NK cells, and APCs, 10000 cells were used per sample. Dimensionality was reduced using Barnes-Hut tSNE with a perplexity of 30 with a maximum of 1000 iterations. Clustering was then performed using density-based machine learning with ClusterX [Chen et al., 2016] and cell subsets were identified by visual inspection of marker expression for each cluster.

Functional assays

PBMCs were thawed, washed, resuspended in complete RPMI media supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 % heat-inactivated FBS (all from Thermo Fisher Scientific) and then incubated at 37°C over-night. The cells were then plated at 2.5×10^6 PBMCs/mL and stimulated with either eBioscience™ Cell Stimulation Cocktail (0.081 μM PMA and 1.34 μM ionomycin, Thermo Fisher Scientific), or anti-CD16 (1μg/mL, clone 3G8, Biolegend), IL-12 (10 ng/mL, Biolegend), and IL-18 (100ng/mL, Biolegend) in the presence of brefeldin A and monensin (Thermo Fisher Scientific) for 6 hours. To evaluate cell degranulation, 1μL of anti-CD107a PE-CF594 (BD Biosciences) was added to each well. Stimulated cells were subsequently stained with antibodies targeting surface markers (Supplemental Table 2), according to manufacturers' instruction. Cells were then fixed and permeabilized using the eBioscience™ FoxP3 / Transcription Factor Staining Buffer (Thermo Fisher Scientific) and stained with intracellular antibodies for TNF, IFN-γ, Ki-67 and granzyme B. For the exclusion of dead cells, LIVE/DEAD aqua fixable viability staining kit (Invitrogen) was used. Cells were acquired using an Aurora spectral flow cytometer (Cytek Biosciences, Fremont, California, USA) and the data analysed using FlowJo version 10.8.1, with the gating strategy shown in Supplemental figure 2.

Flow cytometry phenotyping

PBMCs were thawed, washed, and resuspended in complete RPMI media supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 % heat-inactivated FBS (all from Thermo Fisher Scientific) and then incubated at 37°C over-night. Cells were then stained and acquired as described in the section 2.5 of this Chapter. The antibodies used are listed in the Supplemental Table 3.

Statistical analysis

The histogram and measures of asymmetry and kurtosis were evaluated, and the D'Agostino and Pearson tests performed to assess the normality assumption for parametric tests; for data not following a normal distribution, non-parametric tests were used. For quantitative data, depending on the underlying distributions, comparisons between groups were performed through independent 2-way ANOVA or Kruskal-Wallis test followed by Dunn's post-test. Differences were considered significant when $p<0.05$. Statistical analyses were performed using Prism9 (GraphPad Software, USA).

Study approval

Written informed consent was received from all participants before inclusion in the study, whereby they were pseudo-anonymised. The study was approved by the Regional Ethical Review Board at the Karolinska Institutet in Stockholm (approval numbers 2013/1347-31/2 and 2013/2243-31/4), and it is in accordance with the Declaration of Helsinki.

3. RESULTS

Investigating the landscape of peripheral blood mononuclear cells in active and latent TB at the single cell level

We performed a comprehensive profiling of the immune cell populations of individuals infected with Mtb, to characterise the immunological status in latent and active TB. We included a total of 19 individuals with ATB, 44 individuals with LTB, and 19 age- and sex-matched HC (Supplemental table 1). Different numbers of individuals were included in the analyses below and are indicated in the caption of each figure.

To get an overview of the blood immune profile at the single-cell level, we first performed a mass cytometry analysis of isolated PBMCs from individuals with ATB (n=5), LTB (n=5) and HC (n=5) (Figure 1). Frozen PBMCs were thawed and left to rest for 24 h, and then a similar number of cells were analysed by mass cytometry (Figure 1A). First, we performed a dimension reduction using t-stochastic neighbour embedding (t-SNE) and clustering analysis of total pre-gated live CD45+ cells, and we identified four main cell types based on cell expression of immune cell markers: T cells (CD3), B cells (CD20), NK cells (CD7), and myeloid cells (CD33) (Figure 1B-C). T cells were the most abundant cell subset, representing on average 70% of the cells, followed by NK, myeloid cells, and B cells (Figure 1C). The relative frequencies of the identified subsets were similar between the groups (Figure 1D).
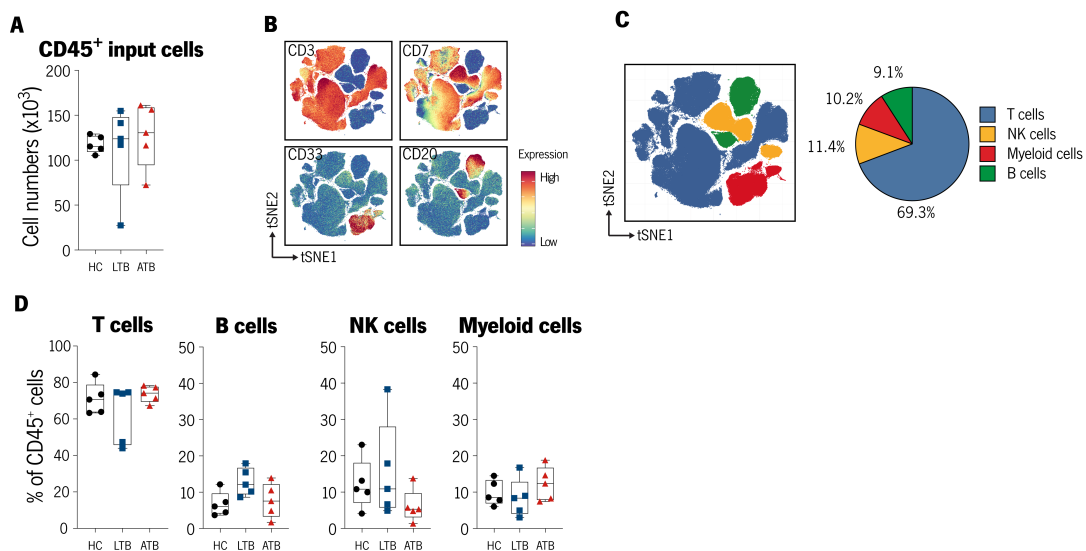
Figure 1. Characterisation of the immune landscape of PBMCs from individuals with ATB and LTB, and HC by mass cytometry. (A) Comparison of total CD45+ cells input cells between groups. **(B)** tSNE plots with the expression markers used to characterise the main lymphoid populations. **(C)** tSNE plot of the clustering of CD45+ lymphocytes from all samples together coloured according to cell types (left) and the mean proportions of each cell type (right). **(D)** Comparison of the percentage of T, B, NK, and myeloid cells within total lymphocytes between groups. Statistical differences between groups were analysed using Kruskal-Wallis with Dunn's post-test (n=5/group).

Following the exploratory analysis, we extended the analysis to include more individuals. Although in the mass cytometry data there were no differences between groups for B cell frequencies, there was a tendency for higher percentages in individuals with LTB compared with ATB and HC. For that reason, we performed further B cell phenotyping by flow cytometry using antibodies targeting IgD, IgM, CD27, CD21, CD38, CD11c, and T-bet allowing the identification of seven different B cell subsets (Figure 2 and Supplemental figure 1). We observed that individuals with ATB had higher percentages of naïve B cells and plasmablasts compared to individuals with LTB, but not double negative (DN) or atypical B cells, as was shown before (Figure 2A-C) [Joosten et al., 2016]. Moreover, both switched memory and non-switched memory B cells were present in lower percentages in individuals with ATB, compared with LTB and HC, respectively. Consistent with having more naïve B cells, individuals with ATB showed higher percentages of IgD$^+$IgM^{dim} B cells (Figure 2D or E), while the percentage of IgD$^-$IgM^{bright} B cells was decreased in individuals with ATB compared to the other two groups. We further evaluated the effect of sex and age on these variables using a standard least squares linear mixed effects model with emphasis on effect screening but could not find any significant association (data not shown).

In summary, naïve B cells and plasmablasts as well as IgD$^+$IgM^{dim} B cells were more frequent in ATB compared to LTB, while both switched memory and non-switched memory B cells as well as IgD$^-$IgM^{bright} B cells were less frequent in individuals with ATB compared to LTB and in some cases in HC.
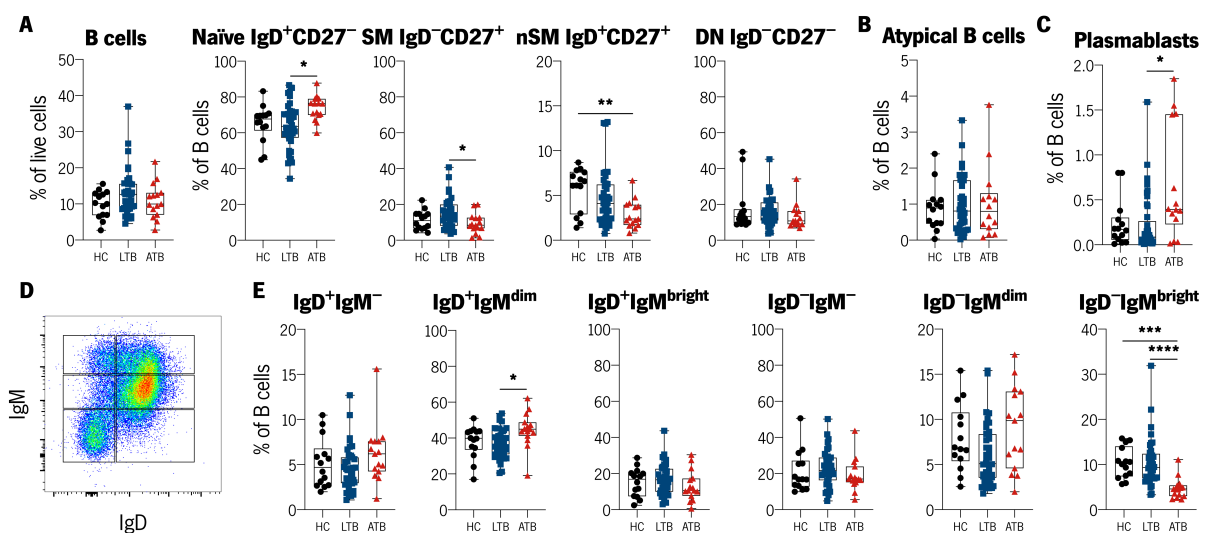

Figure 2. Percentage of B cells and B cell populations evaluated by flow cytometry. (A) Total B cell frequency of live PBMCs followed by the frequency of naïve (IgD$^+$CD27$^-$), switched memory (IgD$^-$CD27$^+$), non-switched memory (IgD$^+$CD27$^+$) and double negative (IgD$^-$CD27$^-$) cells out of total B cells. **(B)** Frequency of atypical B cells (CD11c$^+$T-bet$^+$) out of total B cells. **(C)** Frequency of plasmablasts (CD27$^+$CD38$^+$CD20^{low}) out of total B cells. **(D)** Gating of total B cells into populations based on the expression of IgD and IgM. **(E)** Frequency of B cells based on the expression of IgD and IgM out of total B cells. Each dot represents one individual. Statistical differences between groups were analysed using one-way ANOVA or Kruskal-Wallis with Dunn's post-test according to data distribution. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (HC=14, LTB=40, ATB=15)

**Individuals with LTB and ATB have similar percentages of NK cell subsets**

To further dissect the immune profile of the leukocytes from the mass cytometry dataset, we analysed the different cell subsets individually. Cells were gated based on CD3, CD7, and HLA-DR expression, and we performed a clustering analysis of each cell subset separately. We identified several subsets among T and APCs, but no differences were found between groups (Supplemental Figures 2 and 3).

Within NK cells (CD3⁻CD7⁺), three subsets were identified based on the different expression levels of CD56, CD57, and granzyme B (GrzB) (Figure 3 A-B). Although CD56 in combination with CD16 are commonly used as markers to identify NK cell subsets, CD16 is rapidly downregulated in culture, thus not providing a reliable identification. For that reason, CD56 was used in combination with CD57. A subset of $CD56^{dim}CD57^{+}$ NK cells, expressing higher levels of GrzB, was found to be increased in individuals with LTB compared to individuals with ATB (Figure 3C).
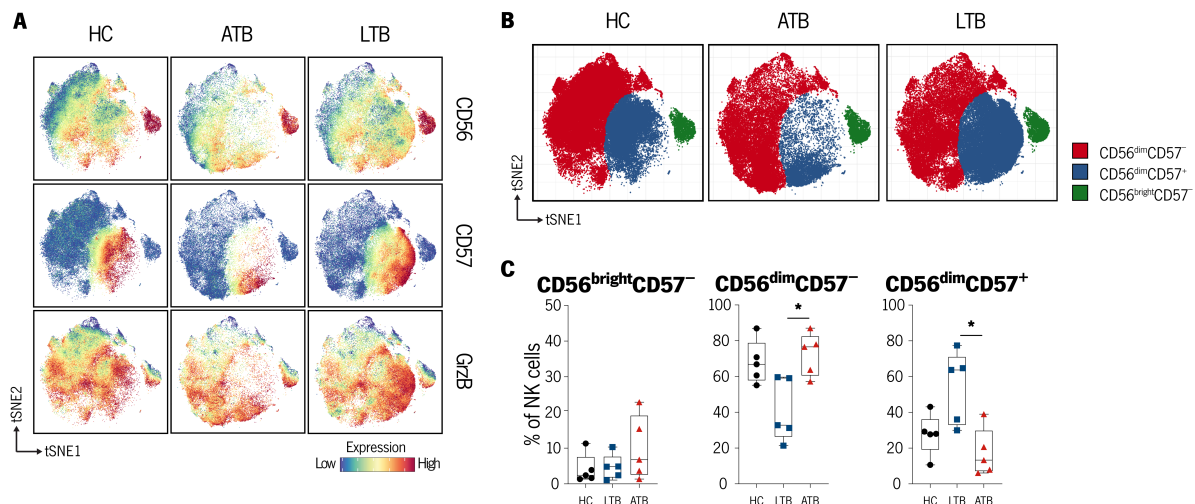
Figure 3. Mass cytometry profiling of NK cells in individuals infected with Mtb shows higher percentages of $CD56^{dim}CD57^{+}$ NK cells in LTB. (A) tSNE plots of NK cells with the expression of CD56, CD57, and GrzB in the different groups. **(B)** tSNE plots of the clustering of NK cells across the groups, coloured according to the three main populations identified. **(C)** Percentage of the NK subpopulations within total NK cells from the ATB, LTB, and HC groups. Each dot represents one individual. Statistical differences between groups were analysed using Kruskal-Wallis with Dunn's post-test (n=5/group). *p<0.05.

We then sought to validate the results with a larger number of individuals in each group using flow cytometry. The NK cells were subset based on expression levels of CD56 and CD57, similar to the mass cytometry data and we also analysed canonical (CD57⁺NKG2C⁻) and adaptive (CD57⁺NKG2C⁺) NK cell subsets. Contrary to the observations in the exploratory mass cytometry analysis, no differences in cell frequencies were observed between the groups among the NK cell subset (Figure 4A-C).
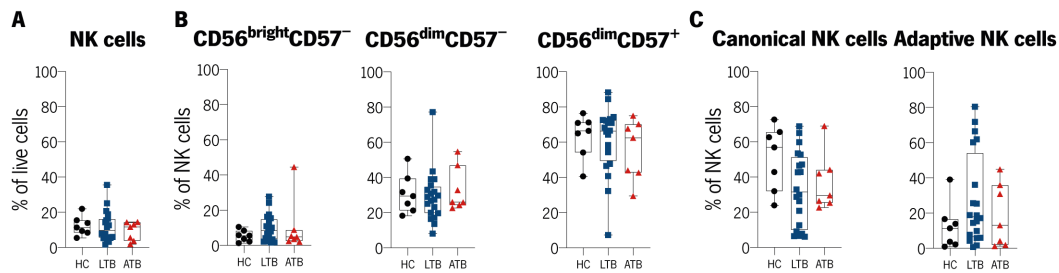
Figure 4. Percentage of NK cells and NK subpopulations evaluated by flow cytometry. (A) Frequency of total NK cells (CD3⁻CD56⁺) out of Live PBMCs. **(B)** Frequency of NK cell subpopulations out of total NK cells based on expression of CD56 and CD57. **(C)** Frequency of canonical (CD57⁻NKG2C⁻) and adaptive (CD57⁺NKG2C⁺) NK cell subsets out of total NK cells. Each dot represents one individual. Statistical differences between groups were analysed using one-way ANOVA or Kruskal-Wallis with Dunn's post-test according to data distribution. (HC=7, LTB=20, ATB=7).

Individuals with LTB have impaired NK cell function

We investigated the function of NK cells by evaluating cell degranulation, cytokine production, and proliferation (Supplemental figure 4). Degranulation was assessed by the expression of surface CD107a and intracellular GrzB levels, and proliferation by Ki-67 staining. Cytokines IL-12 and IL-18 are commonly used to stimulate NK cells [Sarhan and Miller, 2019]. However, adaptive NK cells are less responsive to IL-12 and IL-18, likely due to low expression of the receptor for these cytokines, but are more sensitive to stimulation via CD16 [Schlums et al., 2015]. Thus, to stimulate all NK cells equally, we used anti-CD16 in combination with IL-12 and IL-18. Moreover, cells were also stimulated with PMA and ionomycin (PMA/ion) to asess general cell functionality. Total PBMCs were seeded and stimulated for 6 h prior to analysis.

We first observed that the function of unstimulated cells was similar between groups (Supplemental Figure 5). We then evaluated the effect of the stimulations, regardless of the group (Supplemental Figure 6). Both stimulations (PMA/Ion and anti-CD16/IL-12/IL-18) resulted in degranulation by NK cells as shown by expression of CD107a on the cell surface and lower expression of GrzB intracellularly [Alter et al., 2004; Prager and Watzl, 2019]. Moreover, we observed an increased frequency of cells producing IFN-γ, TNF, and proliferating, as shown by the expression of Ki-67.

Stimulation with PMA/ion resulted in differences in NK cell function between groups (Figure 5). Individuals with LTB showed impaired total NK cell function in terms of cytokine production, compared to individuals with ATB and HC. To better understand if this difference was specific to a particular NK cell subset, we also evaluated the cell function of five main NK cell subsets (Figure 5). In all subsets, individuals with LTB had less cells producing IFN-γ and TNF in the two CD57⁻ subsets and the canonical and adaptive NK cells compared to individuals with ATB and/or HC. Moreover, individuals with LTB had less CD56ᵇʳⁱᵍʰᵗCD57⁻

and adaptive NK cells degranulating compared to HC, as showed by lower percentages of cells expressing CD107a.
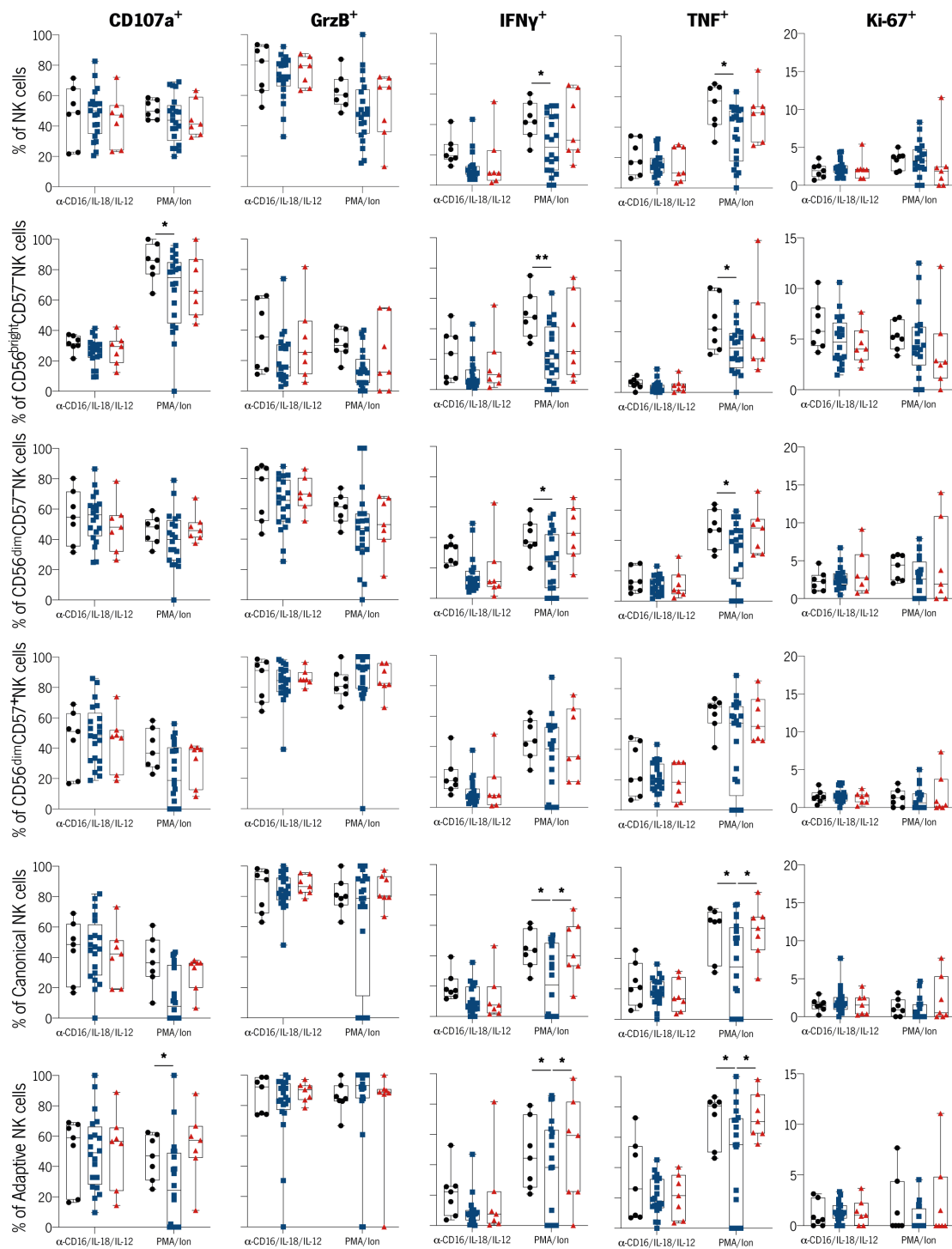
Figure 5. Functional analysis of NK cells and NK subpopulations evaluated by flow cytometry. PBMCs isolated from healthy controls or individuals with ATB, or LTB were stimulated with α-CD16/IL-18/IL-12 or PMA/ionomycin and NK cells function was determined by evaluating degranulation (CD107a and GrzB), cytokine production (IFN-γ and TNF) and proliferation (Ki-67). Each dot represents one individual. Statistical differences between groups were compared using one-way ANOVA or Kruskal-Wallis with Dunn's post-test according to data distribution. *p<0.05, **p<0.01. (HC=7, LTB=20, ATB=7)

4. DISCUSSION

In this study we found that in Mtb infection different B cell compartments were altered and that individuals with LTB had impaired NK cell functions. While we did not observe any changes in circulating total B cell frequencies in TB patients, in line with a study by Barcelos et al. [Barcelos et al., 2006], others found an increase [Wu et al., 2009] or decrease [Corominas et al., 2004; Hernandez et al., 2010; Joosten et al., 2016; Veenstra et al., 2006] compared to healthy controls. Further analysis of our data for different B cell subsets demonstrated that individuals with ATB had higher frequencies of peripheral naïve B cells and plasmablasts and lower frequencies of switched and non-switched memory B cells. This is in line with a study by Sebina et al. [Sebina et al., 2014] where percentages of memory B cells were markedly higher in individuals with LTB compared to active TB patients, while the frequency of plasmablasts was higher in active TB cases.

Likewise, in a study on multi-drug resistant (MDR)-TB, decreased frequencies of non-switched memory (IgD$^+$CD27$^+$) B cells were observed in individuals with MDR-TB [Joosten et al., 2016]. However, in contrast, Joosten et al. found that individuals with ATB had lower percentages of naïve B cells and higher switched-memory B cells [Joosten et al., 2016]. They also observed an increased number of atypical B cells in individuals with ATB. Expanding atypical B cell numbers are associated with inflammatory responses [Courey-Ghaouzi et al., 2022], potentially indicating that the ATB donors in their study were either more severely ill, or had presented with symptoms for a longer time, compared with the ATB donors in the current study. Either way, this data suggest that diverse B cell subsets are affected during Mtb infection, but the mechanisms of this effect remains unclear. We also observed that individuals with ATB had lower frequencies of IgD$^-$IgM^{bright} B cells and higher frequencies of IgD$^+$IgM^{dim} B cells. A recent study showed that different levels of IgM and IgD expression define two subpopulations with a distinct phenotype and function [Bautista et al., 2020]. IgD$^-$IgM^{bright} B cells are similar to marginal zone B cells that can enter germinal centres (IgM only and switched memory B cells), while IgD$^+$IgM^{dim} B cells are less differentiated and phenotypically close to naïve B cells [Bautista et al., 2020], consistent with our phenotyping data based on IgD and CD27. Stimulation of IgD$^-$IgM^{bright} B cells with differentiates the cells into antibody-secreting cells (ASC). Plasmablasts are antibody-secreting cells and precursors of short- and long-lived plasma cells that can be detected in the periphery shortly after viral infections and vaccination [Ellebedy et al., 2016]. We and others detected higher percentages of these cells in individuals with ATB at diagnosis that then normalised after treatment [Sebina et al., 2014]. Thus, reduced frequencies of

peripheral IgD⁻IgM^bright B cells might be due to differentiation into plasmablasts during ongoing Mtb infection.

There is increasing evidence that NK cells play an important role in the immune response to Mtb [Choreño Parra et al., 2017]. In a comprehensive multi-cohort study, Chowdhury et al. found that the levels of NK cells decreased during progression from latency to active disease and were restored after anti-TB treatment, suggesting a protective role of NK cells against Mtb [Roy Chowdhury et al., 2018]. In the exploratory mass cytometry analysis, our study individuals with LTB had higher percentages of CD56^{dim}CD57$^+$ NK cells. However, when we evaluated the level of these cells in a larger group of patients, we did not repeat the observation. Instead, the NK subsets analysed were present in similar frequencies between the groups, consistent with a study by Garand et al. who found no difference in NK cell frequencies between pre- and post-treatment TB cases, and tuberculin skin test (TST)$^+$ and TST$^-$ household contacts [Garand et al., 2018].

NK cells can directly kill infected cells through their cytotoxic activity, but they can also produce cytokines that enhance the immune response to intracellular infections, such as Mtb. Roy Chowdhury et al. showed that individuals with LTB had higher levels of circulating cytotoxic NK cells compared with healthy controls [Roy Chowdhury et al., 2018]. These changes in NK cells inversely correlated with the lung inflammatory state of individuals with ATB. Garand et al. observed a significant decrease in IFN-γ expression and degranulation in NK cells from TB cases pre-treatment compared to post-treatment [Garand et al., 2018]. Here we evaluated NK cell function and observed that individuals with LTB had an impaired NK cell function in terms of cytokine production and degranulation. This is contrary to the observations where individuals with LTB have a stronger NK cytotoxic response. However, it is important to note that the experimental setup of the above-mentioned studies differs from ours. Roy Chowdhury et al. evaluated NK cell cytotoxicity by calcein-release assay using target K562 cells and Garand et al. used whole blood instead of isolated PBMCs.

The diagnosis of LTB is based on the detection of an adaptive immune response to Mtb antigens, which can be done using different tests (either TST or IFN-γ release assays [IGRAs]) and different commercial brands of the same type of test. Moreover, there are discrepancies between different tests/brands in the diagnosis of LTB [Young et al., 2005]. The fact that different studies use different methods to diagnose LTB, may lead to differences in the experimental groups. We observed a large variability between individuals within the LTB group, which is in line with a study by Garand et al. who observed a large inter-

individual variation in the TST[+] (latent TB) group and concluded that this likely made the analysis difficult [Garand et al., 2018]. In addition to the inclusion of more participants in the study, a better definition of the LTB group may contribute to a more robust analysis. It is known that only about 5-10% of people diagnosed with LTB will progress to disease in the first years after the contact with an index case [Behr et al., 2019]. Some of these individuals may clear the bacteria, suggesting that Mtb control is different in Mtb-immunoreactive individuals. Thus, it would be valuable to better stratify individuals with LTB into more well-defined groups, such as recent versus remote infection, as well as to include longitudinal samples.

Cytokines are important players during Mtb infection [Domingo-gonzalez et al., 2016]. In particularly, IFN-γ is critical in the protection against Mtb infection. However, excessive production of pro-inflammatory cytokines leads to tissue damage [Divangahi et al., 2018]. Thus, the control of the infection requires a balance between activation and hyporesponsiveness. A weakened cytokine production in latency was also observed by us in myeloid cells in response to mycobacterial glycolipids [Silva et al., 2021]. This phenomenon is known as innate tolerance and has mainly been explored for myeloid cells but is also described for NK cells in the context of viral infection and cancer [Frutoso and Mortier, 2019]. Since most of the individuals with LTB do not develop the disease, their immune status could be considered as associated with protection against Mtb. Thus, in latency, chronic stimulation of immune cells may potentially lead to a hyporesponsiveness that protects the host from tissue damage and ensures survival. Romera-Cárdenas et al. have established an experimental setup to study NK cell hyporesponsiveness using cells from healthy donors and they conducted a transcriptomic analysis but did not find any correlation with the impaired NK response [Romera-Cárdenas et al., 2016]. Moreover, they did not observe changes in activating or inhibiting NK cell receptors.

These contrary observations might be due to heterogeneity of the groups but also due to the lack of control of variables that may affect peripheral NK cell proportions. Chronic viral infections have an impact on the frequency of different NK cell subsets. Adaptive NKG2C^{+}CD57^{+} NK cells are expanded in healthy individuals infected with cytomegalovirus (CMV) compared to those not infected [Lopez-Vergès et al., 2011]. It is estimated that the worldwide seroprevalence of CMV is 83% in the general population [Zuhair et al., 2019]. However, this variable was not considered in any of the studies.

In conclusion, our results provide new knowledge on the immune profile and NK cell function in ATB and LTB that may contribute to a better understanding of Mtb control. However, the discrepancies between our results and the literature also highlight the heterogeneity across different groups of patients analysed and the importance of further studies.

5.   REFERENCES

Alter, G., Malenfant, J. M., and Altfeld, M. [2004]. CD107a as a functional marker for the identification of natural killer cell activity. *Journal of Immunological Methods*, *294*(1–2), 15–22. https://doi.org/10.1016/J.JIM.2004.08.008

Barcelos, W., Martins-Filho, O. A., Guimarães, T. M. P. D., Oliveira, M. H. P., Spíndola-de-Miranda, S., Carvalho, B. N., and de Toledo, V. de P. C. P. [2006]. Peripheral Blood Mononuclear Cells Immunophenotyping in Pulmonary Tuberculosis Patients before and after Treatment. *Microbiology and Immunology*, *50*(8), 597–605. https://doi.org/10.1111/j.1348-0421.2006.tb03834.x

Bautista, D., Vásquez, C., Ayala-Ramírez, P., Téllez-Sosa, J., Godoy-Lozano, E., Martínez-Barnetche, J., Franco, M., and Angel, J. [2020]. Differential Expression of IgM and IgD Discriminates Two Subpopulations of Human Circulating IgM+IgD+CD27+ B Cells That Differ Phenotypically, Functionally, and Genetically. *Frontiers in Immunology*, *11*, 736. https://doi.org/10.3389/FIMMU.2020.00736/BIBTEX

Behr, M. A., Edelstein, P. H., and Ramakrishnan, L. [2019]. Is Mycobacterium tuberculosis infection life long? *BMJ*, *367*(l5770), 1–7. https://doi.org/10.1136/bmj.l5770

Chen, H., Lau, M. C., Wong, M. T., Newell, E. W., Poidinger, M., and Chen, J. [2016]. Cytofkit: A Bioconductor Package for an Integrated Mass Cytometry Data Analysis Pipeline. *PLoS Computational Biology*, *12*(9), 1–17. https://doi.org/10.1371/journal.pcbi.1005112

Choreño Parra, J. A., Martínez Zúñiga, N., Jiménez Zamudio, L. A., Jiménez Álvarez, L. A., Salinas Lara, C., and Zúñiga, J. [2017]. Memory of Natural Killer Cells: A New Chance against Mycobacterium tuberculosis? *Frontiers in Immunology*, *8*, 967. https://doi.org/10.3389/fimmu.2017.00967

Cooper, A. M. [2009]. Cell mediated immune responses in Tuberculosis. *Annual Review of Immunology*, *27*, 393–422. https://doi.org/10.1146/ANNUREV.IMMUNOL.021908.132703

Corominas, M., Cardona, V., Gonzalez, L., Caylà, J. A., Rufi, G., Mestre, M., and Buendia, E. [2004]. B-lymphocytes and co-stimulatory molecules in Mycobacterium tuberculosis infection. *The International Journal of Tuberculosis and Lung Disease : The Official Journal of the International Union against Tuberculosis and Lung Disease*, *8*(1), 98–105.

Courey-Ghaouzi, A. D., Kleberg, L., and Sundling, C. [2022]. Alternative B Cell Differentiation During Infection and Inflammation. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/FIMMU.2022.908034/PDF

Divangahi, M., Khan, N., and Kaufmann, E. [2018]. Beyond Killing Mycobacterium tuberculosis: Disease Tolerance. *Frontiers in Immunology*, *9*, 2976. https://doi.org/10.3389/fimmu.2018.02976

Domingo-gonzalez, R., Prince, O., Cooper, A., and Khader, S. [2016]. Cytokines and chemokines in Mycobacterium tuberculosis infection. *Miicrobiology Spectr*, *4*(5), 1–58. https://doi.org/10.1128/microbiolspec.TBTB2-0018-2016.Cytokines

Ellebedy, A. H., Jackson, K. J. L., Kissick, H. T., Nakaya, H. I., Davis, C. W., Roskin, K. M., ... Ahmed, R. [2016]. Defining antigen-specific plasmablast and memory B cell subsets in human blood after viral infection or vaccination. *Nature Immunology*, *17*(10), 1226–1234. https://doi.org/10.1038/ni.3533

Frutoso, M., and Mortier, E. [2019]. NK Cell Hyporesponsiveness: More Is Not Always Better. *International Journal of Molecular Sciences*, *20*(18), 4514. https://doi.org/10.3390/ijms20184514

Garand, M., Goodier, M., Owolabi, O., Donkor, S., Kampmann, B., and Sutherland, J. S. [2018]. Functional and Phenotypic Changes of Natural Killer Cells in Whole Blood during Mycobacterium tuberculosis Infection and Disease. *Frontiers in Immunology*, *9*(257), 1–18. https://doi.org/10.3389/fimmu.2018.00257

Hernandez, J., Velazquez, C., Valenzuela, O., Robles-Zepeda, R., Ruiz-Bustos, E., Navarro, M., and

Garibay-Escobar, A. [2010]. Low Number of Peripheral Blood B Lymphocytes in Patients with Pulmonary Tuberculosis. *Immunological Investigations*, *39*(3), 197–205. https://doi.org/10.3109/08820130903586346

Joosten, S. A., van Meijgaarden, K. E., del Nonno, F., Baiocchini, A., Petrone, L., Vanini, V., ... Ottenhoff, T. H. M. [2016]. Patients with Tuberculosis Have a Dysfunctional Circulating B-Cell Compartment, Which Normalizes following Successful Treatment. *PLOS Pathogens*, *12*(6), e1005687. https://doi.org/10.1371/journal.ppat.1005687

Li, H., and Javid, B. [2018]. Antibodies and tuberculosis: finally coming of age? *Nature Reviews Immunology*, *18*(9), 591–596. https://doi.org/10.1038/s41577-018-0028-0

Lopez-Vergès, S., Milush, J. M., Schwartz, B. S., Pando, M. J., Jarjoura, J., York, V. A., ... Lanier, L. L. [2011]. Expansion of a unique CD57 + NKG2C hi natural killer cell subset during acute human cytomegalovirus infection. *Proceedings of the National Academy of Sciences*, *108*(36), 14725–14732. https://doi.org/10.1073/pnas.1110900108

Lu, L. L., Chung, A. W., Rosebrock, T. R., Ghebremichael, M., Yu, W. H., Grace, P. S., ... Alter, G. [2016]. A Functional Role for Antibodies in Tuberculosis. *Cell*, *167*(2), 433-443.e14. https://doi.org/10.1016/j.cell.2016.08.072

Mujal, A. M., Delconte, R. B., and Sun, J. C. [2021]. Natural Killer Cells: From Innate to Adaptive Features. *Annual Review of Immunology*, *39*(1), 417–447. https://doi.org/10.1146/annurev-immunol-101819-074948

Nunes-Alves, C., Booty, M. G., Carpenter, S. M., Jayaraman, P., Rothchild, A. C., and Behar, S. M. [2014]. In search of a new paradigm for protective immunity to TB. *Nature Reviews Microbiology*, *12*(4), 289–299. https://doi.org/10.1038/nrmicro3230.In

Pai, M., Behr, M. A., Dowdy, D., Dheda, K., Divangahi, M., Boehme, C. C., ... Raviglione, M. [2016]. Tuberculosis. *Nature Reviews Disease Primers*, *2*(1), 16076. https://doi.org/10.1038/nrdp.2016.76

Prager, I., and Watzl, C. [2019]. Mechanisms of natural killer cell-mediated cellular cytotoxicity. *Journal of Leukocyte Biology*, *105*(6), 1319–1329. https://doi.org/10.1002/JLB.MR0718-269R

Romera-Cárdenas, G., Thomas, L. M., Lopez-Cobo, S., García-Cuesta, E. M., Long, E. O., and Reyburn, H. T. [2016]. Ionomycin Treatment Renders NK Cells Hyporesponsive. *PLOS ONE*, *11*(3), e0150998. https://doi.org/10.1371/journal.pone.0150998

Roy Chowdhury, R., Vallania, F., Yang, Q., Lopez Angel, C. J., Darboe, F., Penn-Nicholson, A., ... Chien, Y. [2018]. A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. *Nature*, *560*(7720), 644–648. https://doi.org/10.1038/s41586-018-0439-x

Sarhan, D., and Miller, J. S. [2019]. Assessing Canonical and Adaptive Natural Killer Cell Function in Suppression Assays In Vitro. In *Methods in Molecular Biology* (Vol. 1913, pp. 153–166). https://doi.org/10.1007/978-1-4939-8979-9_11

Schlums, H., Cichocki, F., Tesi, B., Theorell, J., Beziat, V., Holmes, T. D., ... Bryceson, Y. T. [2015]. Cytomegalovirus Infection Drives Adaptive Epigenetic Diversification of NK Cells with Altered Signaling and Effector Function. *Immunity*, *42*(3), 443–456. https://doi.org/10.1016/J.IMMUNI.2015.02.008

Sebina, I., Biraro, I. A., Dockrell, H. M., Elliott, A. M., and Cose, S. [2014]. Circulating B-Lymphocytes as Potential Biomarkers of Tuberculosis Infection Activity. *PLoS ONE*, *9*(9), e106796. https://doi.org/10.1371/journal.pone.0106796

Silva, C. S., Sundling, C., Folkesson, E., Fröberg, G., Nobrega, C., Canto-Gomes, J., ... Källenius, G. [2021]. High Dimensional Immune Profiling Reveals Different Response Patterns in Active and Latent Tuberculosis Following Stimulation With Mycobacterial Glycolipids. *Frontiers in Immunology*, *12*(November), 1–17. https://doi.org/10.3389/fimmu.2021.727300

Veenstra, H., Baumann, R., Carroll, N. M., Lukey, P. T., Kidd, M., Beyers, N., Bolliger, C. T., Van Helden,

P. D., and Walzl, G. [2006]. Changes in leucocyte and lymphocyte subsets during tuberculosis treatment; prominence of CD3dimCD56+ natural killer T cells in fast treatment responders. *Clinical and Experimental Immunology*, *145*(2), 252. https://doi.org/10.1111/J.1365-2249.2006.03144.X

WHO. [2022]. *Global Tuberculosis Report*. Geneva.

Wu, Y. E., Zhang, S. W., Peng, W. G., Li, K. S., Li, K., Jiang, J. K., Lin, J. H., and Cai, Y. [2009]. Changes in lymphocyte subsets in the peripheral blood of patients with active pulmonary tuberculosis. *The Journal of International Medical Research*, *37*(6), 1742–1749. https://doi.org/10.1177/147323000903700610

Young, A. K., Hye, W. L., Ho, I. Y., Cho, B. L., Sung, K. H., Shim, Y. S., and Yim, J. J. [2005]. Discrepancy Between the Tuberculin Skin Test and the Whole-Blood Interferon γ Assay for the Diagnosis of Latent Tuberculosis Infection in an Intermediate Tuberculosis-Burden Country. *JAMA*, *293*(22), 2756–2761. https://doi.org/10.1001/JAMA.293.22.2756

Zuhair, M., Smit, G. S. A., Wallis, G., Jabbar, F., Smith, C., Devleesschauwer, B., and Griffiths, P. [2019]. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Reviews in Medical Virology*, *29*(3), e2034. https://doi.org/10.1002/rmv.2034

6. SUPPLEMENTARY MATERIAL

Supplemental Table 1. Study individuals.

	HC (n=19)	LTB (n=44)	ATB (n=19)
Female sex (n)	5	20	9
Age (median, range)	27.5 (21-66)	39 (18-66)	38 (18-70)
Comorbidity (n)	0	7	6
BCG vaccination	2	16	unknown

HC: Healthy controls; LTB: Latent TB; ATB: Active TB.

Supplemental Table 2. Membrane and intracellular B cell staining panel for flow cytometry.

Marker	Clone	Fluorochrome	Company	Dilution
Surface staining				
IgD	IA6-2	PerCP-eFluor710	Invitrogen	1:160
CD38	HIT2	PerCPCy5.5	BD Biosciences	1:40
IgM	G20-127	BB515	BD Horizon	1:20
CD19	HIB19	PE-Cy7	BD Biosciences	1:100
CD27	L128	BV786	BD Horizon	1:160
CD11c	B-ly6	BV650	BD Horizon	1:80
CD20	2H7	BV570	BD Biosciences	1:40
CD57	NK-1	eFluor450	BD Horizon	1:40
CD21	B-ly4	BV421	BD Horizon	1:40
Viability staining				
LIVE/DEAD fixable dye	-	Aqua	Invitrogen	1:600
Intracellular staining				
T-bet	O4-46	AlexaFluor647	BD Biosciences	1:80

Supplemental Table 3. Membrane and intracellular NK cell staining panel for flow cytometry.

Marker	Clone	Fluorochrome	Company	Dilution
Surface staining				
CD107a*	H4A3	PE-CF594	BD Biosciences	1 µL/well
NKG2C	S19005E	PE	BioLegend	1:80
CD3	SK7	APC-Fire810	BioLegend	1:40
CD19	SJ25C1	BV510	BD Horizon	1:20
CD14	MϕP9	BV510	BD Horizon	1:40
CD56	NCAM16.2	BV786	BD Horizon	1:160
CD57	TB01	eFluor450	Invitrogen	1:40
Viability staining				
LIVE/DEAD fixable dye	-	Aqua	Invitrogen	1:600
Intracellular staining				
Ki-67	SolA15	AlexaFluor532	Invitrogen	1:40
GrzB	QA16A02	APC	BioLegend	1:160
IFN-γ	4S.B3	BV711	BD Horizon	1:40
TNF	Mab11	BV650	BD Horizon	1:40

*CD107a was added before stimulation as described in the section 2 of this chapter.
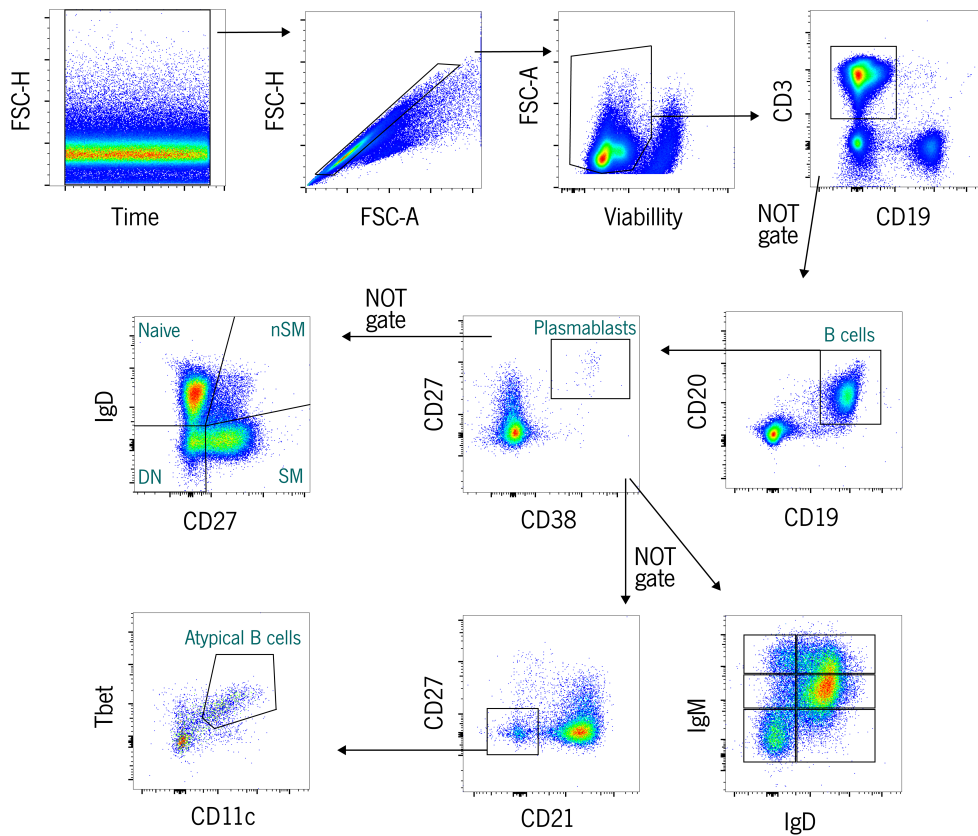
Supplemental Figure 1. Flow cytometry gating strategy for the immunophenotyping of B cells. Cells acquired during a stable flow were gated based on time of acquisition. Live cells were gated from singlets (based on FSC-H/FSC-A) based on FSC-A/Viability. B cells (CD19$^+$CD20$^+$) were gated on CD3$^-$ cells. Plasmablasts were identified within B cells as CD27$^+$CD38$^+$ and excluded from further gating (not gate). Atypical B cells were defined as CD21$^-$CD27$^-$CD11c$^+$T-bet$^+$. Different B cell subsets were identified using IgD in combination with CD27, and IgD in combination with IgM.
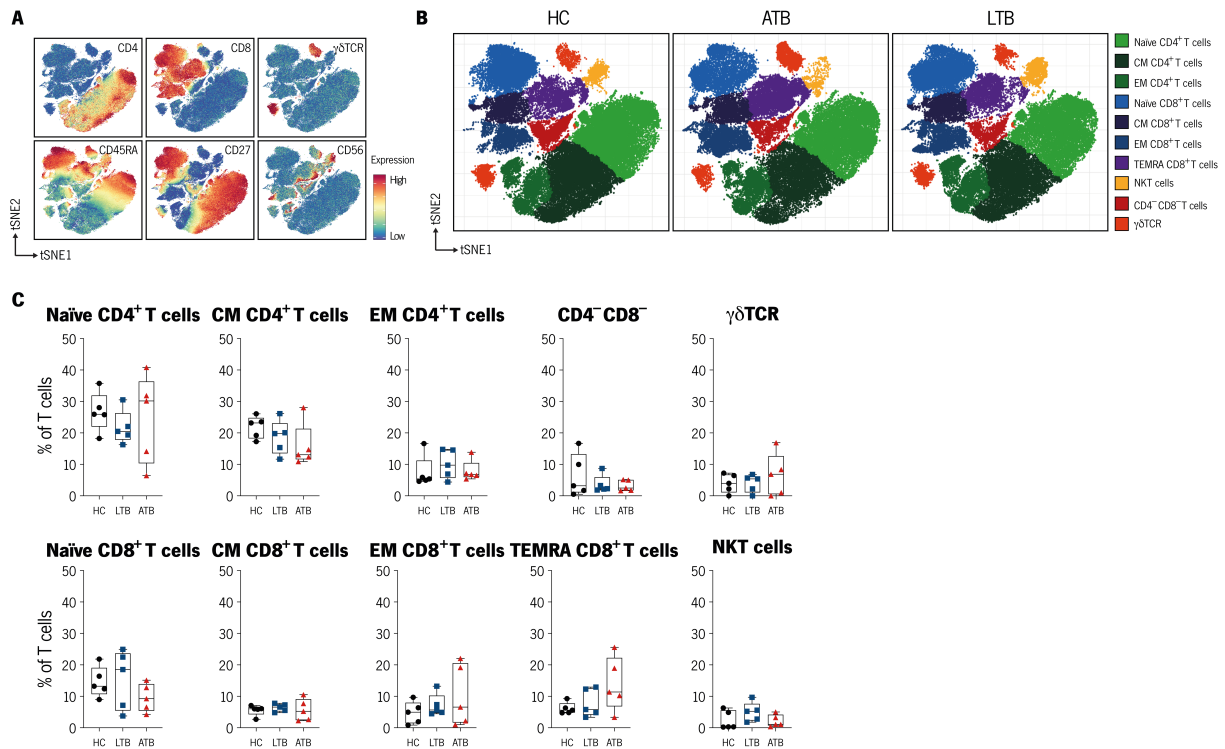
Supplemental Figure 2. Mass cytometry profiling of T cells in individuals with ATB and LTB, and healthy controls. (A) tSNE plots with the expression markers used to characterise the main T cell subsets. **(B)** tSNE plots of the clustering of T cells across the groups, coloured according to the main populations identified. **(C)** Percentage of the T cell subpopulations out of total T cells from the ATB, LTB, and HC groups. Each dot represents one individual. Statistical differences between groups were analysed using Kruskal-Wallis with Dunn's post-test (n=5/group).
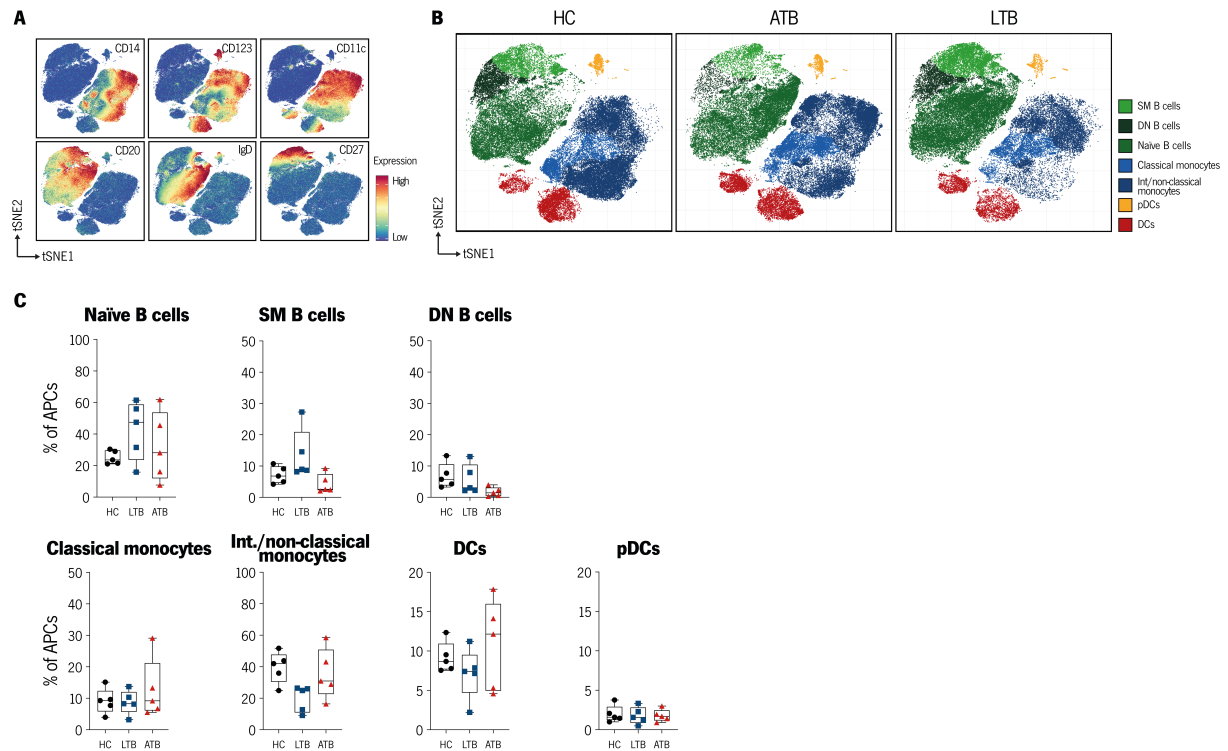
Supplemental Figure 3. Mass cytometry profiling of APCs in individuals with ATB and LTB, and healthy controls. (A) tSNE plots with the expression markers used to characterise the main APC subpopulations. **(B)** tSNE plots of the clustering of APCs across the groups, coloured according to the main populations identified. **(C)** Percentage of B cell and monocyte subsets, DCs, and pDCs out of total APCs from the ATB, LTB, and HC groups. Each dot represents one individual. Statistical differences between groups were analysed using Kruskal-Wallis with Dunn's post-test (n=5/group).
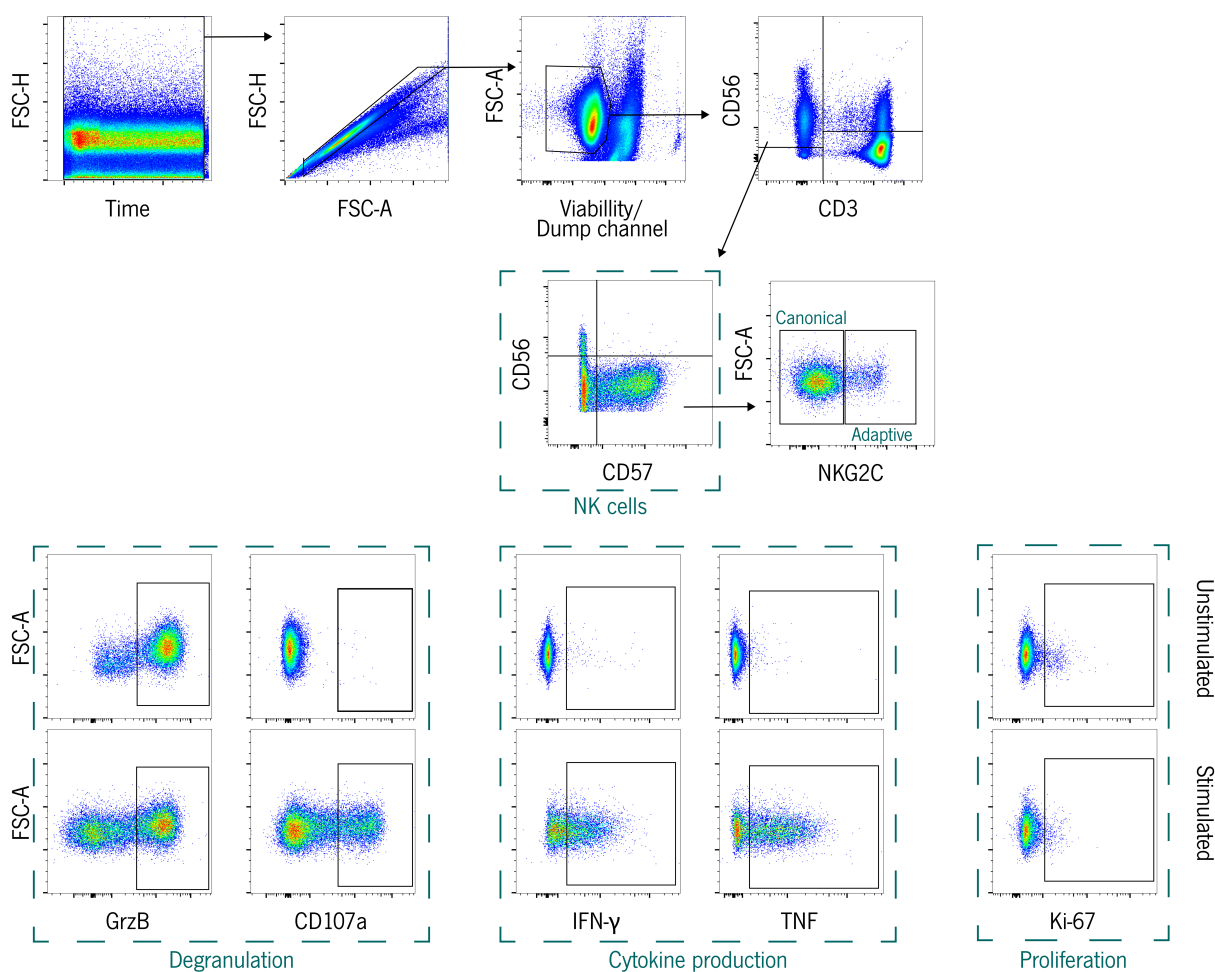
Supplemental Figure 4. Flow cytometry gating strategy for the evaluation of NK cells' function. Cells acquired during a stable flow were gated based on time of acquisition. Live cells were gated from singlets (based on FSC-H/FSC-A) based on FSC-A/Viability+Dump channel (CD14 and CD19). Different NK cell (CD3⁻CD56⁺) subsets were identified using CD56 in combination with CD57. Canonical and adaptive NK cells were identified within $CD56^{dim}CD57^+$ based on the expression of NKG2C. Cell function was then evaluated in terms of degranulation, cytokine production and proliferation.
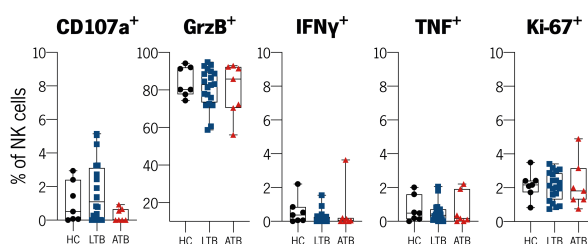
Supplemental Figure 5. Functional analysis of NK cells and NK subpopulations evaluated by flow cytometry in unstimulated (negative control) cells. Cell function was determined by evaluating degranulation (CD107a and GrzB), cytokine production (IFN-γ and TNF) and proliferation (Ki-67). Each dot represents one individual. Statistical differences between groups were compared using one-way ANOVA or Kruskal-Wallis with Dunn's post-test according to data distribution. (HC=7, LTB=20, ATB=7)

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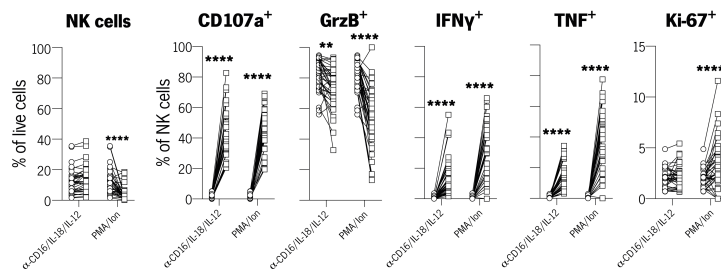
Supplemental Figure 6. Effect of stimulation on NK cells regardless of the experimental group. PBMCs isolated from individuals with ATB, LTB, and HC were stimulated with α-CD16/IL-18/IL-12 or PMA/ionomycin and cell function was determined by evaluating degranulation (CD107a and GrzB), cytokine production (IFN-γ and TNF) and proliferation (Ki-67). Each dot represents one individual. Statistical differences between groups were compared using paired t-test or Wilcoxon matched pairs signed rank test according to data distribution. (n=37/group)

CHAPTER IV

GENERAL DISCUSSION, LIMITATIONS, AND FUTURE PERSPECTIVES

Diagnosis of latently infected individuals and, more importantly, identification of those that are at risk to progress to ATB is crucial to achieving the "END TB strategy goals" [WHO, 2014]. Thus, it is of the utmost importance to better understand the immune status of these individuals. The work presented in this thesis gives some insights into the immune response of individuals with LTB and ATB and contributes to expanding the knowledge behind Mtb control. In CHAPTER II we present a characterisation of the *in vitro* immune response to the mycobacterial glycolipids LAM and PIM in ATB and LTB and found that myeloid cells of these individuals have a dampened immune response compared to HC. In the work in CHAPTER III, we assessed the NK cell function of Mtb-infected patients and observed that individuals with LTB have a diminished NK cell response in terms of degranulation and cytokine production compared to healthy controls. In the present chapter, I critically discuss the data generated, the strengths and limitations of this work, and propose how our findings can be further explored.

The mycobacterial glycolipids LAM and PIM are part of the Mtb cell wall and are recognised by cells of the immune system [Garcia-Vilanova et al., 2019]. Although it is known that Mtb glycolipids have immunomodulatory properties, there is controversy about the specific patterns of response induced by these molecules [Källenius et al., 2016]. Moreover, studying how immune cells from Mtb-infected individuals respond *in vitro* to stimulation by mycobacterial glycolipids might help us to understand the features behind a more or less protective immune response. In CHAPTER II we performed a comprehensive characterisation of the immune response to LAM and PIM in individuals with ATB and LTB and compared it with HC. We identified diverse subsets of myeloid, NK, B, and T cells that respond to Mtb glycolipids by producing and secreting distinct patterns of cytokines and chemokines. Particularly in myeloid cells, stimulation with PIM resulted in the expansion of cells producing anti- and pro-inflammatory cytokines such as IL-6, IL-10, TNF, and GM-CSF, compared to stimulations with LAM or PPD. When comparing the overall cytokine response between the three groups we observed that PBMCs from individuals with ATB and LTB responded differently from those of HC upon stimulation with LAM and PIM. The response was characterised by lower numbers of cytokine-producing myeloid cells in Mtb-infected individuals, more prominent in individuals with LTB. The dampened immune response by myeloid cells in these individuals resembles the *in vitro*-induced hyporesponsiveness by Mtb glycolipids observed in several studies, where LAM and PIM have been shown to downmodulate the production of various cytokines induced by LPS in monocytes and DCs [Blanc et al., 2017; Mazurek et al., 2012; Nigou

et al., 2001]. This was observed upon simultaneous stimulation with LPS or in a sequential stimulation where cells were first primed with LAM and with LPS added later. Contrary to this, others observed that LAM boosted the LPS-induced production of pro-inflammatory cytokines [Geijtenbeek et al., 2003; Gringhuis et al., 2009, 2007].

Hyporesponsiveness in myeloid cells was first observed when LPS-stimulated macrophages were subjected to a second challenge with LPS, resulting in lower levels of secreted TNF [Mathison et al., 1990; Matic and Simon, 1991; Virca et al., 1989]. On the other hand, BCG vaccination results in stronger responses against secondary infections mediated by innate immune cells [Arts et al., 2018; Kleinnijenhuis et al., 2014; Wout et al., 1992]. These two concepts of dampened (tolerance) and enhanced (trained) responses were recently proposed as being memory-like innate immune responses, resulting from epigenetic, transcriptional, and metabolic reprogramming of innate immune cells [Netea et al., 2020, 2011]. Whether the first stimulation augments or weakens the immune response to a second challenge depends on the magnitude and duration of the stimulation [Divangahi et al., 2021]. The mechanisms behind cells' reprogramming are poorly understood but include histone modifications and DNA methylation [Netea et al., 2016] which are associated with alterations in gene transcription and signalling pathways [Kong et al., 2021; Novakovic et al., 2016]. Innate immune tolerance has been mostly studied in the context of TLR4 signalling but our results suggest that other PRRs may be involved in this process. Thus, it would be interesting to develop an *in vitro* model of tolerance induction in monocytes by Mtb glycolipids and investigate the role of PRRs in this process. For instance, a two-step stimulation of isolated monocytes from non-infected individuals with LAM and PIM in the presence or absence of blocking antibodies for PRRs such as TLR2, Desctin-2, or DC-SIGN could help us understand which PPRs drive tolerance in monocytes and which signalling pathways are modulated. It also remains unclear if alterations in signalling pathways lead to epigenetic changes or vice-versa. Single-cell technologies such as single-cell-RNA sequencing and single-cell-chromatin accessibility analysis complemented with DNA methylation profile would allow a more detailed characterisation of Mtb glycolipid-induced tolerance.

Innate immune cells trigger and modulate adaptive immune responses by presenting antigens to T cells and by secreting cytokines and chemokines [Kleinnijenhuis et al., 2011]. So far, it is not known how innate tolerance impacts T cell activity. In CHAPTER II we also observed a hyporesponsiveness of some T cells clusters in individuals with ATB and LTB in response to PIM and LAM. Whether this is a direct effect of Mtb glycolipids on T cells, or a consequence of innate immune tolerance needs to be further

elucidated. To explore this, it would be pertinent to isolate T cells and stimulate them with LAM and PIM, and in parallel co-culture non-stimulated T cells with autologous tolerised monocytes, and assess T cell function in terms of polarisation, proliferation, cytokine production, and degranulation. T cells can recognise Mtb glycolipids directly via lipid rafts [Mahon et al., 2012] or indirectly through CD1b presentation [Barral and Brenner, 2007]. In fact, it was shown that blocking CD1b *in vitro* results in weaker T cell responses [Busch et al., 2016; Gong et al., 1998]. Thus, it would be interesting to study the role of CD1b in this process by performing the co-culture experiment described above in presence of CD1b-blocking antibody.

Most of the individuals latently infected with Mtb do not progress to ATB. In fact, it is thought that some eliminate the bacteria but retain an Mtb-specific T cell memory [Behr et al., 2021]. This is supported by evidence that after prophylactic treatment individuals still show Mtb-immunoreactivity, and by the fact that most of the immunosuppressed IGRA+ individuals (either due to HIV co-infection or after organ transplantation) do not progress to ATB [Behr et al., 2018, 2019]. Moreover, in several highly endemic countries, many IGRA+ individuals do not have access to prophylactic treatment and still do not progress to ATB [Pai and Behr, 2016]. Thus, latency can potentially be viewed as a largely protective phenotype, where most individuals either have cleared Mtb or are controlling Mtb growth. Together with our observations, this suggests that the hyporesponsiveness of myeloid cells to Mtb glycolipids can be a relevant part of a protective immune response. This tolerant state of myeloid cells might be a consequence of Mtb infection, where Mtb glycolipids can potentially play a role in innate cell reprogramming. A weaker immune response might protect from excessive production of pro-inflammatory cytokines that lead to tissue damage [Divangahi et al., 2018] and help control Mtb growth. Thus, insights into the immune mechanisms of these individuals might help us to understand infection control and identify markers of protection. This could be relevant not only for the implementation of LTB management strategies, but also for the development of vaccines against TB. In CHAPTER II we evaluated cytokine production and secretion in 15 individuals (5 per group) at a single-cell level and identified interesting patterns of hyporesponsiveness. It would be valuable to expand this analysis to more individuals and to complement this study by investigating the transcriptomic and epigenomic profiles. Moreover, we do not know the duration of this innate immune tolerance or if it fades upon bacteria clearance. This could be addressed in a longitudinal study by collecting samples of recently diagnosed LTB individuals who have initiated chemoprophylactic treatment. To enable such studies, it would be necessary to monitor and sample longitudinally Mtb-exposed individuals (such as house-hold contacts to active TB cases) who do

not initiate anti-TB treatment, to investigate if they progress ATB or not. Comparison of individuals that develop ATB with the ones that do not, would be important to not only identify indicators of protection, but also markers of progression to ATB. In Europe, upon diagnosis of LTB, most individuals initiate treatment and thus this type of studies would be very difficult to implement. Such studies are however possible in settings where prophylactic treatment is not widely implemented or by recruiting individuals who choose not to be treated.

Another curious observation was that, overall, LAM resulted in weaker cytokine responses compared to PIM. Studies investigating the role of TLR2 in Mtb glycolipids stimulation have also showed that LAM induces weaker responses compared to PIM [Nigou et al., 2008]. Although LAM and PIM share a common PI anchor, the bulky arabinan domain of LAM may cover the TLR2 engagement site [Nigou et al., 2008]. However, others observed that LAM induces higher cytokine production compared to PIM [Mazurek et al., 2012]. As reviewed in CHAPTER I, these Mtb glycolipids are a complex and heterogeneous family of molecules. Moreover, a given glycolipid can be recognised by several PRRs, resulting in activation and regulation of different signalling pathways. Structural differences of the glycolipids might potentially lead to different combinations of activated PRRs. In addition to the heterogeneity of Mtb glycolipids, differences in the experimental setup such as the type of immune cells used (i.e., PBMCs, isolated monocytes/DCs, monocyte-derived macrophages/DCs), the incubation time or the simultaneous or sequential stimulation with other stimuli might lead to differences among studies.

In CHAPTER III we investigated the function of NK, T, and NKT cells in Mtb-infected individuals and saw that individuals with LTB have a dampened NK cell response in terms of cytokine production compared to healthy controls. Our observations are contrary to others that showed that individuals with ATB, but not LTB, have an impaired IFN-γ production by NK cells [Garand et al., 2018]. These differences between studies may be due to the lack of control of variables that affect NK cell responses. For instance, vaccination with BCG induces trained immunity in NK cells, resulting in stronger responses to a secondary infection [Kleinnijenhuis et al., 2014]; on the other hand, viral infections such as cytomegalovirus lead to the expansion of adaptive NKG2C+CD57+ NK cells [Lopez-Vergès et al., 2011]. A multi-cohort and comprehensive study to identify stage-specific host responses to Mtb infection including individuals of different ages, genetic backgrounds, geographical locations, and infection stages also emphasises the

discrepancy in results across cohorts [Roy Chowdhury et al., 2018]. As mentioned before, individuals with LTB might have developed different immune responses against Mtb: some clear the bacteria, others are likely to control Mtb growth and about 5-10% will progress to ATB [Behr et al., 2019]. This heterogeneity of the immune response in latency highlights the importance of a comprehensive characterisation of the LTB group. For instance, in our study it would have been important to have information regarding the time since exposure to an index case or history of previous TB disease and possible treatment. Most of the individuals enrolled in this study are migrants and, in some cases, it is difficult to obtain information about previous medical history, such as records of BCG vaccination.

Several aspects regarding our experimental setup deserve some consideration. Flow cytometry is the golden standard tool for single-cell phenotypic and functional analysis of PBMCs. Despite all the advantages, conventional cytometers detect a limited number of parameters (up to 28) due to overlapping emission spectra of the fluorochromes. In the last 10 years, technologies such as spectral flow cytometry and mass cytometry have emerged and became important tools in the analysis of immune cells as they allow analysing a higher number of parameters. Mass cytometry is a high dimensional single-cell analysis tool that allows comprehensive immunophenotyping with up to 50 markers [Iyer et al., 2022]. For that reason, we have chosen to first use mass cytometry as an exploratory tool, and we decided to include a low number of samples (five per group) and instead investigate several different stimulations and time-points. This allowed us to obtain a detailed overview of the immune response to stimulation in Mtb-infected individuals and we were able to identify diverse populations differentially present in the study groups. Thus, despite mass cytometry being a powerful hypothesis-generating tool, the low number of individuals included in the study is a limitation, and the observed findings require investigation in a larger number of samples. For instance, in CHAPTER III we observed by mass cytometry changes in the percentage of CD57+ NK cells which disappeared when more individuals were included in the analysis by conventional flow cytometry. This highlights the value of mass cytometry as an exploratory tool, but also the need to be critical when drawing conclusions. Another important aspect to keep in mind is that our study was conducted in PBMCs isolated from peripheral blood samples and not in samples from the site of infection (e.g., lung). Although peripheral blood samples are easily accessible and provide valuable information one should be careful when interpreting the results. It would be valuable to collect biological samples of the site of the infection and correlate that data with observations from peripheral blood. However, tissues biopsies are not routinely performed in Mtb-infected individuals, making it difficult to

access such samples. Alternatively, in the case of pulmonary TB, BAL fluid samples could provide a valuable source of data. Nevertheless, blood samples contain immune cells and molecules circulating to and from the infection site that reflect the immune status of the individuals and might help us understand Mtb infection. It should also be noted that we used total PBMCs instead of isolated cell subsets. It can be relevant to study PBMCs as interactions between different cells cannot be neglected and this may better reflect what happens *in vivo.* However, when investigating the exact mechanisms of Mtb glycolipid-induced tolerance in monocytes it is crucial to resort to isolated cell subsets.

Taken together, the results presented in this thesis point towards an hyporesponsiveness on innate immune cells in terms of cytokine production in individuals latently infected with Mtb, namely in response to Mtb glycolipids. This work contributes to a better knowledge to the immune state of LTB and raises several questions, both concerning the mechanisms underlying Mtb glycolipids-induced myeloid tolerance, such as: how do Mtb glycolipids induce tolerance in myeloid cells? How does innate immunity tolerance affect T cell responses? But also regarding the implication of these findings in latency: What are the epigenetic and transcriptomic profiles behind innate immune cell tolerance to Mtb glycolipids in latency? Do all patients with LTB (e.g., recent contacts, remote contacts or treated individuals) share the same immune profile? Can we use the immune response to Mtb glycolipids to predict the risk to progress to ATB? Answering these questions will provide insights into the immunological features of natural protection to Mtb infection. Ultimately, identifying markers of such protection may help improve diagnostic tests, intervention strategies, preventive therapy, and vaccine research.

REFERENCES

Arts, R. J. W., Moorlag, S. J. C. F. M., Novakovic, B., Li, Y., Wang, S.-Y., Oosting, M., ... Netea, M. G. [2018]. BCG Vaccination Protects against Experimental Viral Infection in Humans through the Induction of Cytokines Associated with Trained Immunity. *Cell Host & Microbe*, *23*(1), 89-100.e5. https://doi.org/10.1016/j.chom.2017.12.010

Barral, D. C., and Brenner, M. B. [2007]. CD1 antigen presentation: how it works. *Nature Reviews Immunology*, *7*(12), 929–941. https://doi.org/10.1038/nri2191

Behr, M. A., Edelstein, P. H., and Ramakrishnan, L. [2018]. Revisiting the timetable of tuberculosis. *BMJ*, *362*(k2738), k2738. https://doi.org/10.1136/bmj.k2738

Behr, M. A., Edelstein, P. H., and Ramakrishnan, L. [2019]. Is Mycobacterium tuberculosis infection life long? *BMJ*, *367*(l5770), 1–7. https://doi.org/10.1136/bmj.l5770

Behr, M. A., Kaufmann, E., Duffin, J., Edelstein, P. H., and Ramakrishnan, L. [2021]. Latent Tuberculosis: Two Centuries of Confusion. *American Journal of Respiratory and Critical Care Medicine*, *204*(2), 142–148. https://doi.org/10.1164/rccm.202011-4239PP

Blanc, L., Gilleron, M., Prandi, J., Song, O. ryul, Jang, M. S., Gicquel, B., ... Nigou, J. [2017]. Mycobacterium tuberculosis inhibits human innate immune responses via the production of TLR2 antagonist glycolipids. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(42), 11205–11210. https://doi.org/10.1073/pnas.1707840114

Busch, M., Herzmann, C., Kallert, S., Zimmermann, A., Höfer, C., Mayer, D., ... Stenger, S. [2016]. Lipoarabinomannan-Responsive Polycytotoxic T Cells Are Associated with Protection in Human Tuberculosis. *American Journal of Respiratory and Critical Care Medicine*, *194*(3), 345–355. https://doi.org/10.1164/rccm.201509-1746OC

Divangahi, M., Aaby, P., Khader, S. A., Barreiro, L. B., Bekkering, S., Chavakis, T., ... Netea, M. G. [2021]. Trained immunity, tolerance, priming and differentiation: distinct immunological processes. *Nature Immunology*, *22*(1), 2–6. https://doi.org/10.1038/s41590-020-00845-6

Divangahi, M., Khan, N., and Kaufmann, E. [2018]. Beyond Killing Mycobacterium tuberculosis: Disease Tolerance. *Frontiers in Immunology*, *9*, 2976. https://doi.org/10.3389/fimmu.2018.02976

Garand, M., Goodier, M., Owolabi, O., Donkor, S., Kampmann, B., and Sutherland, J. S. [2018]. Functional and Phenotypic Changes of Natural Killer Cells in Whole Blood during Mycobacterium tuberculosis Infection and Disease. *Frontiers in Immunology*, *9*(257), 1–18. https://doi.org/10.3389/fimmu.2018.00257

Garcia-Vilanova, A., Chan, J., and Torrelles, J. B. [2019]. Underestimated Manipulative Roles of Mycobacterium tuberculosis Cell Envelope Glycolipids During Infection. *Frontiers in Immunology*, *10*(2909). https://doi.org/10.3389/fimmu.2019.02909

Geijtenbeek, T. B. H., van Vliet, S. J., Koppel, E. A., Sanchez-Hernandez, M., Vandenbroucke-Grauls, C. M. J. E., Appelmelk, B., and van Kooyk, Y. [2003]. Mycobacteria Target DC-SIGN to Suppress Dendritic Cell Function. *Journal of Experimental Medicine*, *197*(1), 7–17. https://doi.org/10.1084/jem.20021229

Gong, J., Stenger, S., Zack, J. A., Jones, B. E., Bristol, G. C., Modlin, R. L., Morrissey, P. J., and Barnes, P. F. [1998]. Isolation of Mycobacterium-reactive CD1-restricted T Cells from Patients with Human Immunodeficiency Virus Infection. *J. Clin. Invest*, *101*(2), 383–389. https://doi.org/0021-9738/98/01/0383/07

Gringhuis, S. I., den Dunnen, J., Litjens, M., van der Vlist, M., and Geijtenbeek, T. B. H. [2009]. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to Mycobacterium tuberculosis, HIV-1 and Helicobacter pylori. *Nature Immunology*, *10*(10), 1081–1088. https://doi.org/10.1038/ni.1778

Gringhuis, S. I., den Dunnen, J., Litjens, M., van het Hof, B., van Kooyk, Y., and Geijtenbeek, T. B. H. [2007]. C-Type Lectin DC-SIGN Modulates Toll-like Receptor Signaling via Raf-1 Kinase-Dependent Acetylation of Transcription Factor NF-κB. *Immunity*, *26*(5), 605–616. https://doi.org/10.1016/j.immuni.2007.03.012

Iyer, A., Hamers, A. A. J., and Pillai, A. B. [2022]. CyTOF® for the Masses. *Frontiers in Immunology*, *13*, 1636. https://doi.org/10.3389/FIMMU.2022.815828/BIBTEX

Källenius, G., Correia-Neves, M., Buteme, H., Hamasur, B., and Svenson, S. B. [2016]. Lipoarabinomannan, and its related glycolipids, induce divergent and opposing immune responses to Mycobacterium tuberculosis depending on structural diversity and experimental variations. *Tuberculosis*, *96*, 120–130. https://doi.org/10.1016/j.tube.2015.09.005

Kleinnijenhuis, J., Oosting, M., Joosten, L. A. B., Netea, M. G., and Van Crevel, R. [2011]. Innate Immune Recognition of Mycobacterium tuberculosis. *Clinical and Developmental Immunology*, *2011*(405310), 1–12. https://doi.org/10.1155/2011/405310

Kleinnijenhuis, J., Quintin, J., Preijers, F., Joosten, L. A. B., Jacobs, C., Xavier, R. J., van der Meer, J. W. M., van Crevel, R., and Netea, M. G. [2014]. BCG-induced trained immunity in NK cells: Role for non-specific protection to infection. *Clinical Immunology*, *155*(2), 213–219. https://doi.org/10.1016/j.clim.2014.10.005

Kong, L., Moorlag, S. J. C. F. M., Lefkovith, A., Li, B., Matzaraki, V., van Emst, L., … Xavier, R. J. [2021]. Single-cell transcriptomic profiles reveal changes associated with BCG-induced trained immunity and protective effects in circulating monocytes. *Cell Reports*, *37*(7). https://doi.org/10.1016/j.celrep.2021.110028

Lopez-Vergès, S., Milush, J. M., Schwartz, B. S., Pando, M. J., Jarjoura, J., York, V. A., … Lanier, L. L. [2011]. Expansion of a unique CD57 + NKG2C hi natural killer cell subset during acute human cytomegalovirus infection. *Proceedings of the National Academy of Sciences*, *108*(36), 14725–14732. https://doi.org/10.1073/pnas.1110900108

Mahon, R. N., Sande, O. J., Rojas, R. E., Levine, A. D., Harding, C. V, and Henry Boom, W. [2012]. Mycobacterium tuberculosis ManLAM inhibits T-cell-receptor signaling by interference with ZAP-70, Lck and LAT phosphorylation. *Cellular Immunology*, *275*(1–2), 98–105. https://doi.org/10.1016/j.cellimm.2012.02.009

Mathison, J. C., Virca, G. D., Wolfson, E., Tobias, P. S., Glaser, K., and Ulevith, R. J. [1990]. Adaptation to bacterial lipopolysaccharide controls lipopolysaccharide-induced tumor necrosis factor production in rabbit macrophages. *Journal of Clinical Investigation*, *85*(4), 1108–1118. https://doi.org/10.1172/JCI114542

Matic, M., and Simon, S. R. [1991]. Tumor necrosis factor release from lipopolysaccharide-stimulated human monocytes: Lipopolysaccharide tolerance in vitro. *Cytokine*, *3*(6), 576–583.

Mazurek, J., Ignatowicz, L., Kallenius, G., Svenson, S. B., Pawlowski, A., and Hamasur, B. [2012]. Divergent Effects of Mycobacterial Cell Wall Glycolipids on Maturation and Function of Human Monocyte-Derived Dendritic Cells. *PLoS ONE*, *7*(8), e42515. https://doi.org/10.1371/journal.pone.0042515

Netea, M. G., Domínguez-Andrés, J., Barreiro, L. B., Chavakis, T., Divangahi, M., Fuchs, E., … Latz, E. [2020]. Defining trained immunity and its role in health and disease. *Nature Reviews Immunology*, 1–14. https://doi.org/10.1038/s41577-020-0285-6

Netea, M. G., Joosten, L. A. B., Latz, E., Mills, K. H. G., Natoli, G., Stunnenberg, H. G., O'neill, L. A. J., and Xavier, R. J. [2016]. Trained immunity: a program of innate immune memory in health and disease. *Science*, *352*(6284), 1–23. https://doi.org/10.1126/science.aaf1098

Netea, M. G., Quintin, J., and Van Der Meer, J. W. M. [2011]. Trained immunity: A memory for innate host defense. *Cell Host and Microbe*, *9*(5), 355–361. https://doi.org/10.1016/j.chom.2011.04.006

Nigou, J., Vasselon, T., Ray, A., Constant, P., Gilleron, M., Besra, G. S., Sutcliffe, I., Tiraby, G., and Puzo, G. [2008]. Mannan Chain Length Controls Lipoglycans Signaling via and Binding to TLR2. *The Journal of Immunology*, *180*(10), 6696–6702. https://doi.org/10.4049/JIMMUNOL.180.10.6696

Nigou, J., Zelle-Rieser, C., Gilleron, M., Thurnher, M., and Puzo, G. [2001]. Mannosylated Lipoarabinomannans Inhibit IL-12 Production by Human Dendritic Cells: Evidence for a Negative Signal Delivered Through the Mannose Receptor. *The Journal of Immunology*, *166*(12), 7477–7485. https://doi.org/10.4049/jimmunol.166.12.7477

Novakovic, B., Habibi, E., Wang, S. Y., Arts, R. J. W., Davar, R., Megchelenbrink, W., ... Stunnenberg, H. G. [2016]. β-Glucan Reverses the Epigenetic State of LPS-Induced Immunological Tolerance. *Cell*, *167*(5), 1354-1368.e14. https://doi.org/10.1016/j.cell.2016.09.034

Pai, M., and Behr, M. [2016]. Latent Mycobacterium tuberculosis Infection and Interferon-Gamma Release Assays. *Microbiology Spectrum*, *4*(5), 379–388. https://doi.org/10.1128/microbiolspec.TBTB2-0023-2016

Roy Chowdhury, R., Vallania, F., Yang, Q., Lopez Angel, C. J., Darboe, F., Penn-Nicholson, A., ... Chien, Y. [2018]. A multi-cohort study of the immune factors associated with M. tuberculosis infection outcomes. *Nature*, *560*(7720), 644–648. https://doi.org/10.1038/s41586-018-0439-x

Virca, G. D., Kim, S. Y., Glaser, K. B., and Ulevitch, R. J. [1989]. Lipopolysaccharide induces hyporesponsiveness to its own action in RAW 264.7 cells. *Journal of Biological Chemistry*, *264*(36), 21951–21956. https://doi.org/10.1016/s0021-9258(20)88277-5

WHO. [2014]. *The end TB strategy - global strategy and targets for tuberculosis prevention, care and control after 2015*. Geneva.

Wout, J. W., Poell, R., and Furth, R. [1992]. The Role of BCG/PPD-Activated Macrophages in Resistance against Systemic Candidiasis in Mice. *Scandinavian Journal of Immunology*, *36*(5), 713–720. https://doi.org/10.1111/j.1365-3083.1992.tb03132.x