Human myeloid cell and innate lymphocyte responses to mycobacterial vaccination or infection

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Declaration

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1. **Murphy M** *et al.,* "Newborn BCG vaccination induces robust IFNγ-expressing NK cell responses to mycobacteria" (Submitted, under review).

2. Gela A, **Murphy M** *et al*., "Effects of BCG vaccination on donor unrestricted T cells in two prospective cohort studies", *eBioMedicine*. 2022 Jan Vol. 76.

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Summary

We investigated immune responses beyond conventional T cells in the context of BCG vaccination and tuberculosis disease. The first objective was to determine whether neonatal BCG vaccination modulates mycobacteria-reactive myeloid, NK, B, $\gamma\delta$ and MAIT cell responses. We analysed infants in whom BCG vaccination was either administered at birth or delayed to 5 or 9 weeks of age. Whole blood was stimulated with BCG and cell phenotype and functions measured by flow cytometry. Secreted pro- and anti-inflammatory mediators were quantified by bead array. The second objective was to determine which immune subsets are associated with outcomes of inflammation and infectiousness in adult tuberculosis patients. Immune subsets were enumerated by flow cytometry from ex-vivo whole blood from tuberculosis patients and healthy controls with and without HIV. Inflammation and lung pathology were measured by expression of selected gene transcripts in blood and radiology, respectively. Infectiousness was characterised as the amount of *Mycobacterium tuberculosis*-containing aerosols expelled by tuberculosis patients during normal breathing in a novel Respiratory Aerosol Sampling Chamber.

Frequencies of BCG-reactive IFNγ-producing NK, $\gamma\delta$ T and $CD8^{-}CD26^{hi}CD161^{hi}$ T cells were higher in BCG vaccinated compared to unvaccinated infants. NK cell responses in vaccinated infants were not associated with enhanced NK cell maturation, differentiation or cytokine receptor expression. No differences in cytokine-producing myeloid and B cells were detected. BCG-reactive NK cell responses correlated with levels of IL-2 and IFNγ and the innate pro-inflammatory cytokines IL-6, IL-1β and TNF in BCG vaccinated infants only. Overall, our results suggest that innate lymphocytes are modulated by BCG vaccination in infants.

In adults, frequencies of intermediate monocytes could differentiate tuberculosis patients from healthy controls, regardless of HIV status, a biomarker that may be useful for diagnosis of tuberculosis disease. *Mycobacterium tuberculosis*-containing aerosols were higher in patients with cavitary disease, compared to individuals with no cavitation, indicating that patients with severe lung pathology are more likely to expel infectious droplets. MAIT cell frequencies negatively correlated with

Mycobacterium tuberculosis-containing aerosols, suggesting that depletion of MAIT cells from peripheral blood is an indicator of infectiousness. In addition, transcriptomic tuberculosis risk signature scores were positively correlated with frequencies of monocyte subsets and negatively correlated with lymphocyte subsets.

Our results suggest that myeloid cells and innate lymphocytes are modulated by mycobacterial vaccination and disease. NK and $\gamma\delta$ T cells represent attractive targets for vaccination. In addition, intermediate monocyte frequencies may be used as a biomarker of TB disease and MAIT cell frequencies potentially used as an indicator of infectiousness.

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Abbreviations

%	Percent
°C	Degrees centigrade
Ab	Antibody
ADCC	Antibody-dependent cellular cytotoxicity
AM	Alveolar macrophage
APC	Antigen presenting cell
ART	Antiretroviral therapy
AUC	Area under the curve
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette Guérin
BDCA	Blood dendritic cell antigen
Be	Effector B cells
C.albicans	*Candida albicans*
CASS	Cough aerosol sampling system
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CFU	Colony forming unit
CO_2	Carbon dioxide
COVID-19	Coronavirus disease 2019
CRRS	Chest radiograph reading and recording system
CV	Coefficient of variation
CXCL	Chemokine (C-X-C motif) ligand
CXCR	C-X chemokine receptor
DC	Dendritic cell
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DURT	Donor unrestricted T cell
FDR	False discovery rate
Foxp3	Forkhead box p3
GLR	Granulocyte to lymphocyte ratio

H3K27Ac	Histone 3 lysine 27 acetylation
H3K4me1	Histone 3 at lysine 4 methylation
H3K4me3	Histone 3 lysine 4 trimethylation
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
i.d	intradermal
IFN	Interferon
IGRA	Interferon gamma release assay
IL	Interleukin
IL-1R	Interleukin 1 receptor
ILC	Innate lymphoid cell
INH	Isoniazid
i.v	Intravenous
KIR	Killer immunoglobulin-like receptors
LIR	Leukocyte immunoglobulin-like receptors
LN	Lymph node
LXA4	Lipoxin A4
MAIT	Mucosal associated invariant T cell
MHC	Major histocompatibility complex
ML	Millilitre
MLR	Monocyte to lymphocyte ratio
MMP	Matrix metalloproteinase
MR1	Major histocompatibility complex-related protein 1
M.tb	*Mycobacterium tuberculosis*
NHP	Non-human primate
NK	Natural killer
NKG	Natural killer group
NLR	Neutrophil to lymphocyte ratio
NTM	Non-tuberculosis mycobacteria
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
Ph	Potential of hydrogen

PLZF	Promyelocytic leukaemia zinc finger
PMA	Phorbol 12-myristate 13-acetate
PPD	purified protein derivative
PRR	Pathogen recognition receptor
RASC	Respiratory aerosol sampling chamber
RD	Region of difference
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROC	Receiver under the curve
S.Aureus	*Staphylococcus Aureus*
SA	South Africa
SATVI	South African Tuberculosis Vaccine Initiative
SNP	Single nucleotide polymorphism
TB	Tuberculosis
TCR	T cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAJ	T cell receptor alpha joining region
TRAV	T cell receptor alpha variable region
TST	Tuberculin skin test
UCT	University of Cape Town
UK	United Kingdom
VACI	Viable Anderson Cascade Impactor
WB-ICS	Whole blood intracellular cytokine staining
WT	Wild type
μg	Microgram
μL	Microlitres
μm	Micrometres

Chapter 1: Introduction and literature review

1.1. General introduction

Tuberculosis (TB) is caused by an infection with *Mycobacterium tuberculosis* (*M.tb),* first identified by Robert Koch in 1882 (Koch, 1882). More than 100 years later, TB is still a major public health problem with one-quarter of the world's population estimated to be sensitised to *M.tb* (WHO, 2021). It is estimated that 10 million people have had TB disease and 1.5 million people**,** including 214 000 people with human immunodeficiency virus (HIV) infection, have died from TB disease in 2020 alone (WHO, 2021). South Africa (SA) is a high TB burden country, with high rates of HIV-TB co-infection (WHO, 2021). TB is mainly thought of as a pulmonary disease but can develop anywhere in the body. Pulmonary TB can range from mild to cavitary disease, the latter characterised by destructive immunopathology and infectiousness (Rodrigo et al., 1997, Ehlers and Schaible, 2012). Miliary TB is defined as the dissemination of large numbers of *M.tb* throughout the body via the blood (Sharma and Mohan, 2017).

The Bacille Calmette Guérin (BCG) vaccine is the only licensed vaccine against TB and is usually administered to children at birth. It protects against miliary TB and TB meningitis, but confers variable efficacy against pulmonary TB (Trunz et al., 2006, Mangtani et al., 2014, Roy et al., 2014). The lack of an immune correlate of protection against TB has hampered the development of new TB vaccines. The importance of conventional cluster of differentiation (CD)4^+ and $CD8^+$ T cells in resistance against *M.tb* infection has been shown experimentally (Orme and Collins, 1983, Flory et al., 1992, Flynn et al., 1992, Lin et al., 2012). It is further supported by cases in which individuals with mutations in the T-helper (Th) 1 axis have increased susceptibility to mycobacterial disease (Casanova et al., 2012). It is likely that new vaccines that are developed will have to work in conjunction with BCG given at birth (or replace it with a safer and/or more efficacious candidate), due to its effectiveness against severe forms of TB**.** However, the MVA85A vaccine, designed to boost the Th1 response primed by BCG, induced durable Th1 and Th17 responses, but failed to enhance protection against TB disease in BCG-vaccinated infants (Tameris et al., 2013). This has led to a re-evaluation of putative protective responses in the TB vaccinology field and a shift

towards investigating immune responses beyond conventional T cells in the context of TB (Joosten et al., 2019). In this thesis, we investigated how BCG modulates the immune response beyond conventional $CD4^+$ and $CD8^+$ T cells in infants. In addition, we also explored the role that immune responses play in TB disease, immunopathology and potential infectiousness.

1.2. Key host immune leukocyte subsets in TB

In this section we provide a broad overview of host cell subsets involved in the immune response to TB. We reviewed in more detail the immune cell subsets investigated in the different chapters of this thesis. Their specific role in TB is further discussed in the introduction section of each chapter. Other cell types are only briefly reviewed for completeness.

1.2.1. Innate cells

Innate cells are the first line of defence against invading pathogens. They respond rapidly once activated and, until recently, were considered to be short-lived and without the ability to develop a memory response. In this thesis, we were interested in monocytes and natural killer (NK) cells due to the increasing literature on the memory-like features displayed by these cells in response to BCG (discussed in **section 1.5.4**). We focus particularly on NK cell responses here.

1.2.1.1. Myeloid cells

Myeloid cells are comprised of distinct subsets of innate cells, which include granulocytes, monocytes, macrophages and dendritic cells (DC)s. The major innate subsets present in blood, however, are granulocytes, monocytes and DCs and are discussed further below.

Granulocytes

In blood, granulocytes are made up of three major subsets: neutrophils, eosinophils and basophils. Neutrophils make up the majority of granulocytes and are also the most abundant white blood cell in blood. Neutrophils are professional phagocytes and are rapidly recruited to the site of infection, where they recognise, phagocytose and kill pathogens (Silva, 2010). Neutrophils also produce antimicrobial molecules, cytokines and chemokines but can also contribute to the pathogenesis of various infections, including TB (Silva, 2010, Zhang et al., 2021).

Monocytes

Monocytes are professional antigen presenting cells (APC)s and are able to carry out multiple functions including phagocytosis and the production of antimicrobial molecules, cytokines and chemokines (Ziegler-Heitbrock, 2007, Frankenberger et al., 1996, Belge et al., 2002). Monocytes are divided into 3 subsets based on the relative expression of CD14 and CD16. Classical monocytes express high levels of CD14 and do not express CD16 ($CD14^{++}CD16^{-}$), intermediate monocytes express high levels of CD14 and co-express low levels of CD16+ ($CD14^{++}CD16^{+}$) and the non-classical subset expresses low levels of CD14 and co-expresses high levels of CD16 ($CD14^{+}CD16^{++}$) (Ziegler-Heitbrock et al., 2010).

Classical monocytes are the most abundant subset and make up more than 80% while intermediate and non-classical monocytes make up 5-15% of total monocytes in blood. The $CD16^{+}$ monocyte subsets, however, increase in number in response to inflammation and infection and produce high levels of cytokines (also discussed in **section 6.1.4.**) (Ziegler-Heitbrock, 2007). Classical monocytes are considered to be highly phagocytic, intermediate monocytes are the antigen presenting subset while non-classical monocytes are believed to patrol the endothelium for inflammation and injury (Wong et al., 2012).

Dendritic cells

DCs make up less than 1% of white blood cells in peripheral blood. DCs are professional APCs and are critical in the initiation of the adaptive immune response. They also produce cytokines and chemokines during infection (Steinman and Hemmi, 2006). In the blood, DCs are divided into two major subsets, plasmacytoid DCs and myeloid DCs, which are defined by their expression of blood DC antigens (BDCA). The plasmacytoid DCs express BDCA-2 (CD303) while the myeloid DCs can be further divided based on their expression of BDCA-1 (CD1c) and BDCA-3 (CD141) (Ziegler-Heitbrock et al., 2010).

Myeloid cell effector functions

Altogether, the bactericidal mechanisms of myeloid cells include secretion of antimicrobial molecules, autophagy, apoptosis, secretion of cytokines and chemokines which activate and recruit other immune cells, and are collectively responsible for the induction of the adaptive immune response to *M.tb* (Lerner, Borel and Gutierrez, 2015).

1.2.1.2. Innate lymphoid cells

Innate lymphoid cells (ILC)s are lymphocytes that do not express diverse antigen receptors such as those expressed by T and B cells but are activated by cytokines. Their functions include contributing to a protective immune response against pathogens at mucosal sites, tissue remodelling and maintaining homeostasis (Panda and Colonna, 2019). ILCs are comprised of three groups: ILC1s produce the prototypical Th1 cytokine interferon (IFN)γ and respond to interleukin (IL)-12, IL-18 and IL-15. They have many characteristics in common with NK cells, including IFNγ as their principal cytokine; ILC2s produce IL-4, IL-5 and IL-13 and respond to IL-25, IL-33 and thymic stromal lymphopoietin; and ILC3s produce IL-17 and IL-22 and respond to IL-23 and IL-1β (Vivier et al., 2018). In recent years ILC1s and ILC3s have been linked to playing a role in the immune response to TB in animal and human studies (Steigler et al., 2018, Ardain et al., 2019).

1.2.1.3. NK cells

NK cells consist of two major subsets

NK cells are large granular lymphocytes and were considered to mainly form part of the host immune response against viruses and tumours (Trinchieri, 1989). In humans the two major NK subsets are $CD56^{bright}$ and $CD56^{dim}$ **[Figure 1]** (Lanier et al., 1986, Cooper et al., 2001a). However, NK cells can be further divided into different subsets based on the relative expression of CD56 and CD16 in the CD3 negative lymphocyte compartment (Poli et al., 2009). A consensus exists that $CD56^{bright}$ cells are precursors of $CD56^{dim}$ NK cells (Poli et al., 2009). This is supported by findings that $CD56^{bright}$ NK cells have longer telomeres than $CD56^{dim}$ NK cells and that isolated $CD56^{bright}CD16^{-}$ NK cells cultured with skin fibroblasts can differentiate into $CD56^{dim}$ cells (Chan et al., 2007). Approximately 90% of NK cells in peripheral blood are $CD56^{dim}$ and 10% are $CD56^{bright}$ cells, the latter subset is most represented in secondary lymphoid tissues (Cooper et al., 2001a).

NK cell effector functions

NK cells are known for their cytotoxic potential and are able to lyse (via perforin, granzymes and Fas), tumours and cells infected with intracellular pathogens, viruses in particular, without prior exposure. They are also able to produce cytokines and chemokines which can influence the adaptive immune response **[Figure 1]**. NK cells are capable of producing large amounts of IFNγ, which can activate infected macrophages to control intracellular infections (Trinchieri, 1989, Morandi et al., 2006, Vivier et al., 2008, Fauriat et al., 2010). *In vitro* studies have also shown that NK cells are able to play a role in DC maturation through the production of tumour necrosis factor (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) and lyse autologous immature DCs via NKp30 in certain circumstances. It is thought that the lysis of immature DCs might limit a hyperinflammatory immune response (Cooper et al., 2004). NK cell recognition of *M.tb*, in particular, and subsequent effector mechanisms are discussed in **section 4.1**.

Activation of NK cells

NK cells work through a complex network of germline-encoded activating and inhibiting receptors. NK cell recognition of self-ligands that are downregulated in transformed or infected cells ('missing self') prevents attack on the cell, while the recognition of ligands which are upregulated in transformed or infected cells ('induced self') can lead to cell lysis (Lanier, 2005). The inhibitory receptors include killer immunoglobulin-like receptors (KIR)s, leukocyte immunoglobulin-like receptors (LIR)s and NK group (NKG)2A which dimerises with CD94 to form the NKG2A-CD94 receptor. The KIR, LIR and NKG2A receptors recognise classical human leukocyte antigens (HLA-A, B and C), HLA-G and HLA-E respectively (Lanier, 2008, Bryceson et al., 2006). The activating receptors include natural cytotoxicity receptors which are comprised of NKp46, NKp30 and NKp44. Other activating receptors include CD16, NKG2D and CD244 (2B4) (Lanier, 2008, Bryceson et al., 2006). NK cells also possess pattern recognition receptors (PRR)s and therefore can directly recognise pathogen-associated molecular patterns (PAMP)s (Adib-Conquy et al., 2014).

NK cells can also be activated by cytokines such as type I IFNs, IL-2, IL-18 and IL-15 without contact with its activating receptor ligands (Lanier et al., 1985, Cooper et al., 2001c, Cooper et al., 2009, Romee et al., 2012). The $CD56^{bright}$ NK subset, which is the main cytokine-producing subset, usually requires IL-12 in combination with another activating cytokine or activating receptor-ligand interaction to secrete IFNγ (Cooper et al., 2001c, Caligiuri, 2008).

NK cell development and associated markers

The development of $CD56^{bright}$ into $CD56^{dim}$ involves expression of specific receptors which are related to distinct maturation states (Abel et al., 2018). Inhibitory and activating receptors can be variably expressed in different maturation states with high expression of CD16 and multiple KIRs associated with mature NK subsets, and CD57 indicating terminal differentiation **[Figure 1]** (Bjorkstrom et al., 2010, Lopez-Verges et al., 2010). Low expression of CD8, CD16, and CD57 and high expression of CD56 and the inhibitory receptor CD94-NKG2A are associated with NK cell immaturity **[Figure 1]** (Nagler et al., 1989). CD161 is an early marker expressed by NK cells

during maturation and is expressed by the majority of NK cells (Bennett et al., 1996). The expression of CD161 positively associates with the cytotoxicity of $CD16^+$ NK cells (Konjevic et al., 2009). In this thesis we used the CD57, CD158b (the inhibitory KIR receptor), CD161 and CD8 markers to characterise the phenotypic and/or differentiation state of the two major NK cell subsets in response to BCG vaccination.

Functional and phenotypic differences between $CD56^{bright}$ and $CD56^{dim}$ NK cells

The $CD56^{dim}$ subset is highly cytotoxic with low proliferative capacity in response to IL-2; the expression level of CD16 will determine the strength of antibody-dependent cellular cytotoxicity (ADCC), as this subset can play a role in killing through binding opsonised pathogens or cells (Lanier et al., 1986, Nagler et al., 1989, Baume et al., 1992). The $CD56^{bright}$ subset is considered to be the main IFNγ producer, is less cytotoxic and possesses high proliferative capacity in response to low doses of IL-2 **[Figure 1]** (Lanier et al., 1986, Nagler et al., 1989, Cooper et al., 2001c). Both NK cells express the heterodimeric IL-2 receptor (IL-2R$\beta\gamma$) which has intermediate affinity for IL-2. The $CD56^{bright}$ NK cell subset, however, also constitutively expresses the high affinity heterotrimeric IL-2R (IL-2R$\alpha\beta\gamma$) which gives $CD56^{bright}$ cells the ability to be activated by low doses of IL-2 **[Figure 1]** (Caligiuri et al., 1990, Nagler et al., 1990).

NK cells constitutively express receptors for monocyte-derived cytokines, including IL-15, IL-18, IL-12 and IL-1 (Carson et al., 1994, Kunikata et al., 1998, Wang et al., 2000, Cooper et al., 2001b). The level of expression of some of these receptors differ between the two major NK subsets; as higher expression of IL-18 receptor (IL-18R) and IL-1 receptor (IL-1R) has been shown in $CD56^{bright}$ compared to $CD56^{dim}$ NK cells (Kunikata et al., 1998, Cooper et al., 2001b). In addition, the $CD56^{bright}$ subset expresses high levels of the C-C chemokine receptor (CCR)7 (important for homing to secondary lymphoid tissues) and C-X chemokine receptor (CXCR)3 while the $CD56^{dim}$ subset expresses high levels of CXCR1 and CX_3CR1 **[Figure 1]** (Campbell et al., 2001).

NK cells exhibit memory-like properties

In recent years NK cells have garnered much interest as a TB vaccine target; this is due to memory-like properties exhibited by NK cells (Khader et al., 2019). In 2009, Sun et al. showed in the murine cytomegalovirus infection model, that the viral protein m157 expressed on the surface of infected cells was recognised by Ly49H, the NK activating receptor in mice, and that this led to the proliferation of NK cells and the generation of memory-like m157-responsive NK cells. It was shown that upon the transfer of these cells to naive mice, they responded robustly to subsequent murine cytomegalovirus infection (Sun et al., 2009). This study was able to show that NK cells can display an antigen-specific recall response. On the other hand, O'Leary et al. were able to show that haptens can induce memory-like NK cells during a delayed hypersensitivity response in RAG knockout mice and when these cells were transferred to naive mice, they were able to mount hapten-specific responses (O'Leary et al., 2006). It was also shown that these cells were able to mount viral antigen-specific memory-like responses (Paust et al., 2010). These cells were reported to be liver-restricted and dependent on CXCR6 (O'Leary et al., 2006, Paust et al., 2010). In addition, human and murine NK cells stimulated with IL-12 and IL-18 were shown to produce cytokine-induced memory-like NK cells which secreted increased levels of IFNγ and cytotoxic molecules upon restimulation with infected cells, transformed cells or cytokines (Cooper et al., 2009, Romee et al., 2012, Sun et al., 2012). Altogether, these studies show various contexts in which NK cells can become memory-like and in which they do not adhere to the traditional view on innate cells. NK cell adaptive properties in the context of TB, in particular, are discussed in **section 4.1**.

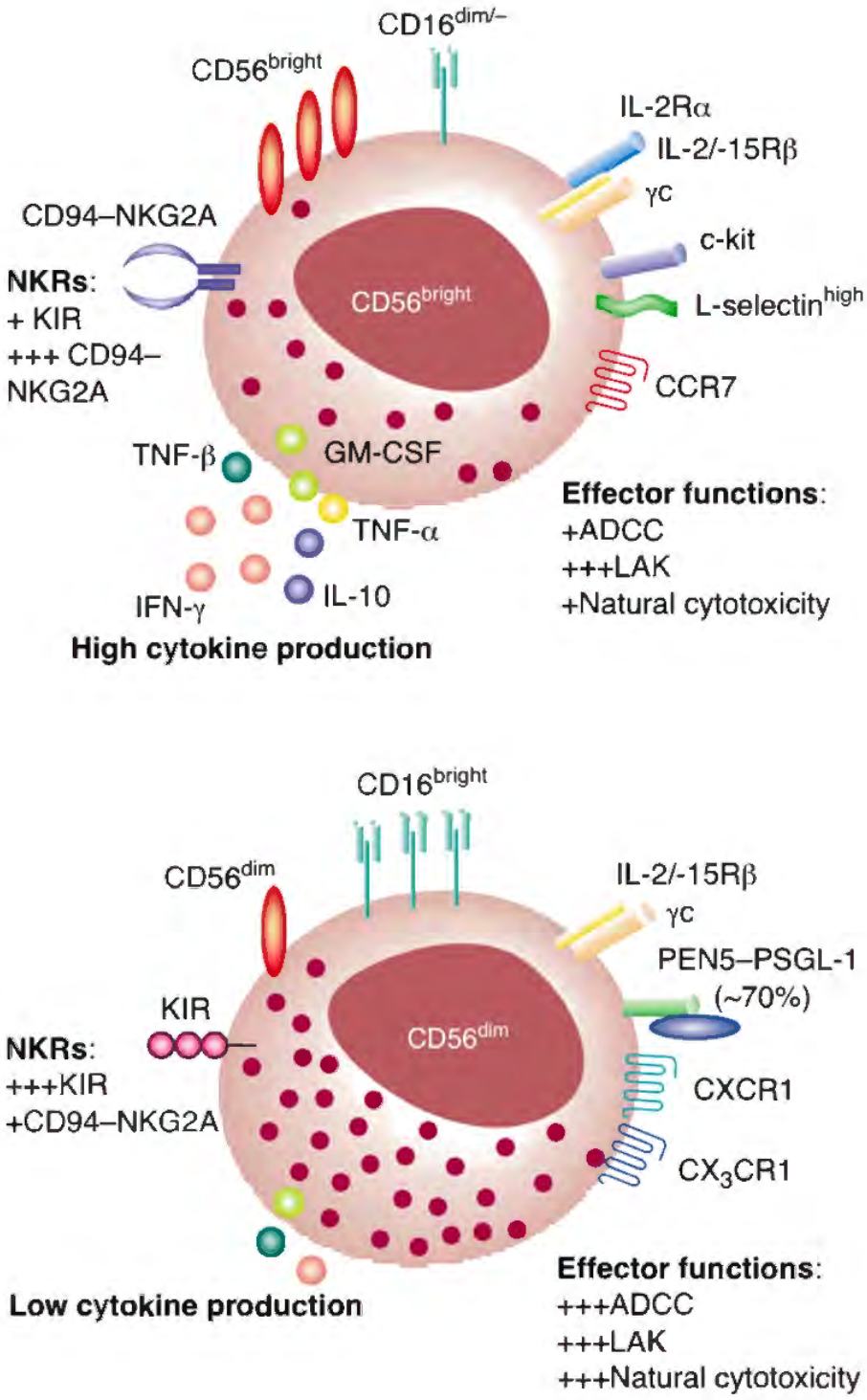


Figure 1. An illustration of the phenotype of two major NK cell subsets. ADCC, antibody-dependent cellular cytotoxicity; KIR, killer-immunoglobulin-like receptor; LAK, lymphokine-activated killer; NKR, natural killer receptor. Adapted from Cooper et al. (Cooper et al., 2001a).

1.2.2. Conventional adaptive lymphocytes

The hallmark of conventional adaptive immunity is the induction of specific long-lasting memory cells following primary infection and/or vaccination. The key concept of vaccination is to induce memory T and B lymphocytes, so that a rapid and enhanced immune response is elicited upon secondary exposure to cognate antigens. In this thesis we investigated $CD4^+$ T and B cell cytokine responses to BCG.

1.2.2.1. $CD4^+$ and $CD8^+$ T cells

Extracellular antigens, once phagocytosed by professional APCs, are collected in endosomes, loaded onto major histocompatibility complex (MHC) II molecules and presented to $CD4^+$ T cells. Intracellular antigens found in the cytosol of any nucleated cell are loaded onto MHC I molecules and are presented to $CD8^+$ T cells (Sallusto et al., 1999). Although intracellular bacteria such as *M.tb* are captured through phagocytosis and are not found in the cytosol (Schaible et al., 2003), $CD8^+$ T cells have been shown to play an important part in the immune defence against TB (Grotzke and Lewinsohn, 2005). This is thought to occur via cross-presentation: DCs take up extracellular antigens, such as apoptotic bodies containing antigen, and load them onto MHC I molecules, which then present to $CD8^+$ T cells (Schaible et al., 2003, Winau et al., 2004, Winau et al., 2006). The recognition of antigen in the context of MHC by T cells leads to activation, clonal expansion, differentiation, cytokine production and other effector functions. $CD8^+$ T cells are known for their ability to kill target cells directly or indirectly through the release of cytotoxic molecules, Fas-FasL interactions and the production of cytokines (Zhang and Bevan, 2011).

1.2.2.2. $CD4^+$ T cells differentiate into distinct T helper subsets

$CD4^+$ T cells differentiate into distinct Th subsets based on the cytokines in the milieu during priming by DCs in the lymph node (LN) and are classified according to their cytokine profiles (Zhu et al., 2010). Th1 cells, polarised by IL-12 and IFNγ, produce IL-2, TNF and IFNγ and play an important role against viruses and intracellular bacteria (Hsieh et al., 1993, Glimcher and Murphy, 2000). Th2 cells, polarised by IL-4 and IL-

2, express IL-4, IL-5 and IL-13 and are involved in immunity against extracellular pathogens, asthma and allergies (Zhu, 2015). Th17 cells, polarised by transforming growth factor (TGF)-β and IL-6, produce IL-17A, IL-17F and IL-22 and are important against fungi and bacteria (Bettelli et al., 2006, Zhou et al., 2007). Forkhead box p3 $(Foxp3)^+$ regulatory T cells, polarised by TGF-β and IL-2, express TGF-β and IL-10 and are involved in the suppression of autoreactive and effector T cells (Chen et al., 2003, Huber et al., 2004, Davidson et al., 2007). $CD4^+$ T cell immune responses in the context of *M.tb* infection and TB disease are discussed in **section 3.1**.

1.2.2.3. Classical T cell immunological memory

Following the expansion of antigen-specific T cells by infection or vaccination, contraction of effector T cells occurs, however, a minor subset of memory cells remains. These cells are antigen-specific, long-lasting and have a heightened ability to fight infection (Seder and Ahmed, 2003). Based on the expression of CCR7 and CD45RA, T cells can be divided into naive and memory subsets as following: $CD45RA^+CCR7^+$, naive cells; $CCR7^+CD45RA^-$, central memory (Tcm); $CCR7^-CD45RA^-$, effector memory (Tem) and; $CCR7^-CD45RA^+$, effector memory re-expressing RA (Temra) (Sallusto et al., 1999). Many studies have investigated and reported on $CD4^+$ and $CD8^+$ memory T cell phenotype and function, particularly in the field of TB vaccinology (Seder et al., 2008, Kirman et al., 2016), however, this is not the focus of this thesis and therefore is not covered in detail here.

1.2.2.4. B cells

B cells are important immune modulators

Traditionally, B cells and antibodies (Abs) were thought to be most effective against extracellular organisms and toxins. However, recent studies have shown that B cells and Abs may play an important role against intracellular pathogens such as *M.tb,* as further discussed in **section 3.1.** B cells have many functions including the production of Abs, secretion of cytokines and antigen presentation. They are also important in the formation of secondary and tertiary lymphoid tissue (Shen and Fillatreau, 2015, Chen

and Jensen, 2008). B cells are classified into different subsets based on their location, migratory capabilities and whether their activation is dependent on T cells (Allman and Pillai, 2008).

B cell effector functions

The most well-known function of B cells is the production of Abs, which are derived from antigen-specific plasma cells. The overall effector functions of Abs include complement activation, antigen neutralisation, ADCC and antibody-dependent cellular phagocytosis (Lu et al., 2018, Schroeder and Cavacini, 2010). There has been much interest in Ab responses to *M.tb* in recent years (Jacobs et al., 2016, Rijnink et al., 2021), however, we do not address Ab responses in this thesis. B cell cytokine production is also an important immune regulation mechanism. B cells can be divided into different subsets based on the cytokines they produce. Regulatory B cells produce IL-10 while effector B (Be) cells can produce IL-12, TNF and IFNγ (Be-1) or IL-6, IL-4 and TNF (Be-2) (Lund, 2008). As previously stated, in this thesis we investigated B cell cytokine responses to BCG.

1.2.3. Donor unrestricted T cells

Donor unrestricted T (DURT) cells have garnered much attention as potential targets for new vaccine candidates. They express an invariant T cell receptor (TCR), which is present in a relatively conserved manner in humans, and recognise antigens presented by non-polymorphic antigen presenting molecules. Activation of DURT cells involves antigen presentation by the non-polymorphic MHC-related protein 1 (MR1), butyrophilin 3A1, CD1 and HLA-E to mucosal-associated invariant T- cells (MAIT), $\gamma\delta$ T cells, CD1-restricted T cells and HLA-E-restricted T cells, respectively (Godfrey et al., 2015). Rare subsets of $\gamma\delta$ T cells (non-Vγ9Vδ2) are also able to recognise antigens presented by CD1 (Godfrey et al., 2015, Uldrich et al., 2013, Bai et al., 2012, Luoma et al., 2013). In addition, $\gamma\delta$ T cells, MAIT cells and certain CD1-restricted T cells can also be activated through bystander activation (Suliman et al., 2019, Ussher et al., 2014, Sada-Ovalle et al., 2008, Gutierrez-Arcelus et al., 2019). In this thesis we focus on MAIT and $\gamma\delta$ T cell responses to BCG.

1.2.3.1. MAIT cells

MAIT cells are abundant in blood and enriched in mucosal tissues

MAIT cells recognise metabolites from the riboflavin pathway (vitamin B2) presented by MR1 (Kjer-Nielsen et al., 2012, Corbett et al., 2014), which allows them to detect a wide variety of yeast and bacteria, including *M.tb* **[Figure 2]** (Gold et al., 2010, Le Bourhis et al., 2010). They are enriched in mucosal tissues, including the liver and gut (Dusseaux et al., 2011, Treiner et al., 2003), but also circulate in the blood from where they are able to traffic to the site of infection (Salou et al., 2017). MAIT cells make up 1-10% of T cells in peripheral blood (Martin et al., 2009, Le Bourhis et al., 2010). They express a semi-invariant TCR made up of TCR alpha variable region (TRAV)1-2 and TCR alpha joining region (TRAJ)33/12/20 alpha chain and TRBV6/20 beta chain (Salou et al., 2017). Interestingly, it was reported that a TRAV12-2 TCR clone was also MR1-restricted and recognised diverse microbial antigens, including those from *S. pyogenes* which is a riboflavin auxotroph (Meermeier et al., 2016). This suggests that there are other MR1-restricted T cells that may recognise different classes of microbial metabolites.

MAIT cell effector functions

MAIT cells are capable of rapid effector functions after leaving the thymus and are known to secrete cytokines such as IFNγ, TNF and cytotoxic molecules (Dusseaux et al., 2011, Le Bourhis et al., 2010). As mentioned above, MAIT cells are also activated by cytokines such as IL-12 and IL-18 in the milieu. These cytokines can act synergistically or in the absence of TCR engagement to activate MAIT cells. **[Figure 2]** (Suliman et al., 2019, Ussher et al., 2014). MAIT cell responses to mycobacteria, in particular, are discussed in **section 3.1**.

MAIT cell phenotype

MAIT cells were initially defined based on surface expression of CD161 and TRAV1-2 in the $CD8^+$ (which make up the majority of MAIT cells in adults) and $CD4^-CD8^-$ T cell compartments (Gold et al., 2010, Martin et al., 2009, Le Bourhis et al., 2010). More

recently the phenotypic definition has expanded to include CD26 (Sharma et al., 2015). In addition, $CD4^+$ MAIT cell subsets have also been reported (Gherardin et al., 2018, Reantragoon et al., 2013). MAIT cells express the tissue homing chemokine receptors CXCR6, CCR6, CCR5 and CCR9 (Dusseaux et al., 2011). They also express high levels of IL-18R (Dusseaux et al., 2011, Le Bourhis et al., 2010).

The development of MR1 tetramers has enabled a better understanding of MAIT cell biology. However, our routine whole blood intracellular cytokine staining (WB-ICS) assay (Hanekom et al., 2004, Kagina et al., 2015) which is used to assess the function of immune cells (direct stimulation of cells from whole blood followed by cell fixation prior to flow cytometry staining) is not compatible with MR1 tetramer staining of fixed cells. Stimulation of cells typically affects accuracy of tetramer staining due to TCR downregulation and addition of tetramers during stimulation can block the TCR-antigen interaction. Other *ex-vivo* assays that have been developed to assess tetramer-positive DURT function require a large number of cells and do not allow the simultaneous assessment of different cell types and/ or cytokine production (Seshadri et al., 2015, Swarbrick et al., 2020). Thus, phenotypic markers are still generally used to identify MAIT cells when assessing the function of these cells.

Recently it was proposed that the classification of MAIT cells be broadened to take into account the diversity of MAIT cell populations in humans. Classical MAIT cells would include MAIT cells which express TRAV1-2, TRAJ33/12/20 alpha chains and TRBV6/20 beta chains. The non-classical MAIT cell would possess more variable TRAV and/or TRAJ chains and a similar phenotype to classical MAIT cells. Finally, atypical MR1-restricted T cells would be different in phenotype and have variable antigen reactivity compared to classical MAIT cells. These cells also do not possess the innate-like transcription factor promyelocytic leukemia zinc finger (PLZF) typically expressed by MAIT and NKT cells (Godfrey et al., 2019).

MAIT cells differ in infants and adults

MAIT cells are more diverse in phenotype and TCR repertoire in infants and become adult-like (classical MAIT cells become the predominant subset) in the first few months of life (Swarbrick et al., 2020, Ben Youssef et al., 2018). A study by Gherardin et al.

also showed that MR1-restricted T cells, except $CD4^+$ cells, increased in frequency from infancy to 25 years of age, after which frequencies declined (Gherardin et al., 2018).

1.2.3.2. γδ T cells

γδ T cells are able to recognise diverse antigens

γδ T cells make up approximately 5% of total T cells in peripheral blood and are enriched in solid organs and mucosal tissues (Parker et al., 1990, Groh et al., 1989). The major γδ T cell subset in blood is Vγ9Vδ2, which is known to recognise phosphorylated metabolites (phospohoantigens) from microbes and eukaryotic cells presented by butyrophilin 3A1 **[Figure 2]** (Bonneville et al., 2010, Vavassori et al., 2013). They are also activated by cytokines such as type I IFNs, IL-12 and IL-18 in the absence of TCR engagement with its cognate antigen (Gutierrez-Arcelus et al., 2019). Other γδ T cells consist mainly of Vδ1 and Vδ3 paired with various Vγ TCR segments and are abundant in solid organs and mucosal tissues (Bonneville et al., 2010). These γδ T cells do not recognise phosphoantigens, and for some their cognate antigen has been identified (Vermijlen et al., 2018). In addition, non-Vγ9Vδ2 subsets have been shown to recognise lipids presented by CD1 molecules (Godfrey et al., 2015, Uldrich et al., 2013, Bai et al., 2012, Luoma et al., 2013). γδ T cells are also able to recognise stressed cells or infection using toll-like receptors (TLR)s and NK receptors such as NKG2D (Thedrez et al., 2007). Interestingly, a subset of γδ T cells has also been shown to recognise *M.tb* protein antigens (Ding et al., 2015). A plausible explanation for the diverse antigen classes recognised by γδ T cells are the structural studies showing that these cells bind their antigen in an immunoglobulin-like fashion (Adams et al., 2005).

γδ T cell effector functions

γδ T cells are thought to link the innate and adaptive immune response and are able to respond rapidly to infection and/or stressed cells before the induction of an adaptive immune response (Chien et al., 2014, Meraviglia et al., 2011). The frequency of these

cells increases substantially in the blood of individuals during infections (Balbi et al., 1993, De Maria et al., 1992). γδ T cells produce cytokines such as IFNγ, TNF and IL-17 in response to viruses and bacteria and cytokines such as IL-4, IL-13 and IL-5 in response to extracellular parasites (Ferrick et al., 1995, Chien et al., 2014). They are also known for their cytotoxic capabilities, producing perforin, granzymes and granulysin in addition to other antimicrobial molecules (Dieli et al., 2001, Spencer et al., 2013). Thus, they are able to kill infected and transformed cells. γδ T cell responses to mycobacteria, in particular, are discussed in **section 3.1**.

γδ T cells differ in infants and adults

γδ T cells in newborns express more diverse Vγ and Vδ TCR segments compared to adults (Morita et al., 1994). The predominant subset is Vγ9-Vδ1+ cells, differing to that of adults. The abundance of Vγ9+Vδ2+ cells in blood increases with age, presumably due to exposure to phosphoantigen-producing microbes (Parker et al., 1990, Morita et al., 1994). Also, it was reported that Vγ9Vδ2 responses to phosphoantigens were reduced in newborns and infants compared to adults (Papadopoulou et al., 2020, Engelmann et al., 2006).

1.2.3.3. CD1-restricted T cells

CD1 molecules are MHC class I-like antigen presentation molecules that present lipids and glycolipids (host and microbial) to T cells (Godfrey et al., 2004, Adams, 2014). In humans, they are comprised of group 1 (CD1a, CD1b and CD1c) and group 2 (CD1d) **[Figure 2]** (Calabi et al., 1989). Mice only express CD1d, which has hampered studies to better understand the group 1 CD1-restricted T cells antigen repertoire and function. Human studies, however, have shown that following recognition of mycobacterial lipid antigens by group 1 CD1 T cells, that they are able to proliferate and produce cytokines (Moody et al., 2000, Ulrichs et al., 2003, Montamat-Sicotte et al., 2011). It was shown *in vitro* that CD1-restricted T cells isolated from individuals were able to lyse *M.tb* infected macrophages. In addition, in animal studies, these cells have been implicated in a protective immune response against *M.tb* infection (Dascher et al., 2003, Zhao et al., 2015).

1.2.3.4. HLA-E-restricted T cells

HLA-E is a non-classical MHC class 1b molecule that presents self-peptides to NKG2A/CD94, thereby inhibiting NK cell cytolytic activity towards normal cells (Lee et al., 1998, Braud et al., 1997). HLA-E molecules are also able to present microbial peptides and glycopeptides to $CD8^+$ T cells (Heinzel et al., 2002, Joosten et al., 2010, McMurtrey et al., 2017). It was reported that HLA-E restricted T cells were able to inhibit *M.tb* growth of infected macrophages *ex-vivo* (van Meijgaarden et al., 2015).

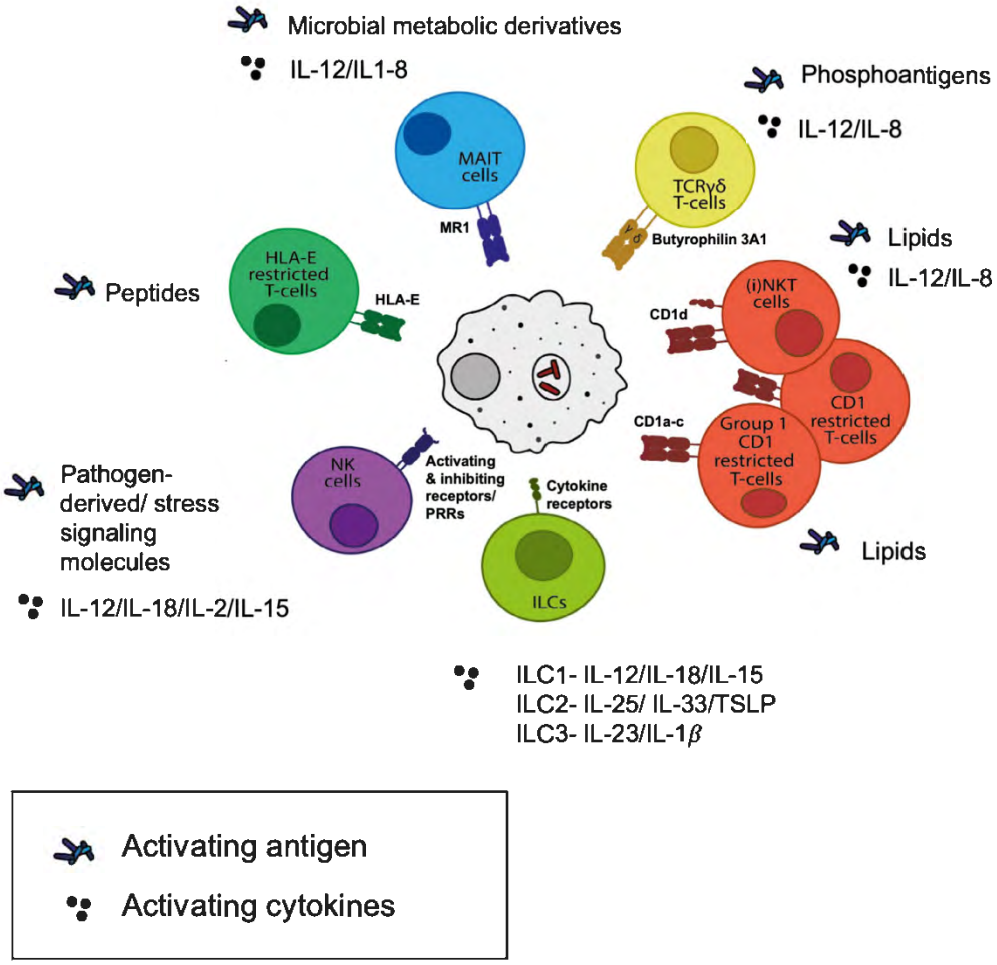


Figure 2. An illustration of DURTs, ILCs and NK cell activating antigens and cytokines in the context of TB. PAMPS, pathogen-associated molecular patterns; TSLP, thymic stromal lymphopoietin. Adapted from Ruibal et al. (Ruibal et al., 2021).

1.3. The immune response to M.tb

1.3.1. M.tb is spread through infectious droplets

M.tb spreads from person to person through the aerosolisation of infectious *M.tb*-containing particles (Riley et al., 1995)**.** The infectious aerosols are expelled from the lungs of patients with TB disease. The size of aerosols expelled by TB individuals range from 0.65 micrometres (μm) to more than 7 μm (Fennelly and Jones-López, 2015). An infectious droplet, however, is thought to be in the range of 1 μm to 5 μm, as they are small enough in size to allow their passage through the upper airways, bronchi and eventually the alveoli of the lung (Fennelly and Jones-López, 2015). We investigated host immune determinants of *M.tb* aerosolisation, which we propose are associated with infectiousness, in adults with TB disease in this thesis and more details are provided in chapter 6.

1.3.2. The different outcomes following M.tb exposure

There are multiple outcomes that can occur following exposure to *M.tb* (**Figure 3**) which include (i) no infection, either due to an insufficient infectious dose of *M.tb* or mucosal barriers preventing *M.tb* from entering the lung. It is also possible that the innate immune system clears the infection prior to the induction of a detectable adaptive immune response; (ii) the establishment of an infection leading to failed immune containment and progression to primary TB disease; (iii) the establishment of infection which is controlled by the immune system and does not lead to TB disease in the vast majority of individuals; and (iv) in approximately 10% of individuals with a controlled *M.tb* infection, reactivation occurs (months to years after primary infection) and the development of TB disease (O'Garra et al., 2013).

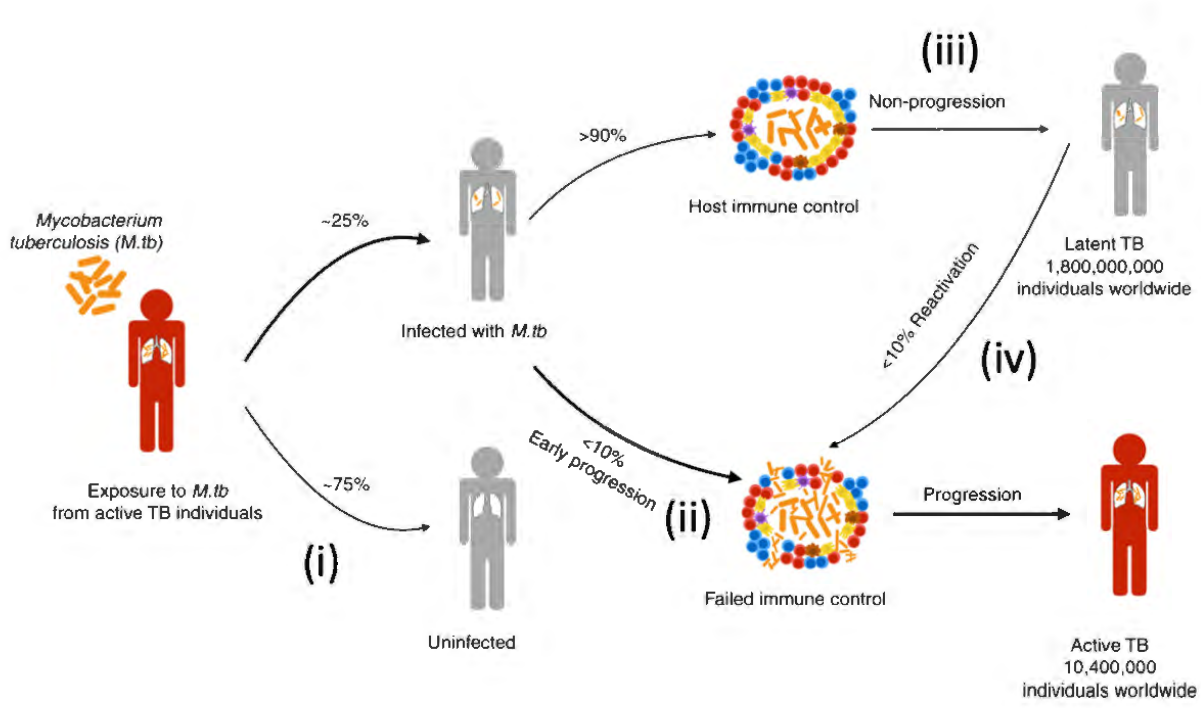


Figure 3. An illustration of the different clinical outcomes following *M.tb* exposure. Adapted from Lou et al. (Luo et al., 2019).

1.3.3. The initial immunological events following M.tb infection

In the alveoli space, the bacteria encounter alveolar macrophages (AMs), DCs, neutrophils and epithelial cells**.** *M.tb* is able to infect other cell types, but preferentially infects AMs (Bermudez and Goodman, 1996, Wolf et al., 2007). Innate cells express germline encoded PRRs such as TLRs, nod-like receptors, C-type lectin receptors, complement receptors and scavenger receptors that recognise conserved PAMPs which are present on the bacteria but are absent in the host (Killick et al., 2013). *M.tb* expresses a number of PAMPs, including lipoarabinomannan, mycolic acids and lipoproteins which are recognised by multiple different PRRs (Stamm et al., 2015). This receptor-ligand interaction triggers phagocytosis and activates a signalling cascade which includes the NF-κB and MAP kinase pathways (Kawai and Akira, 2008), resulting in the initiation of the innate arm of the immune response and the secretion of antimicrobial peptides (cathelicidin and α/βdefensins), cytokines (IL-12, IL-6, TNF and IL-1β) and chemokines [(C-C motif) ligand (CCL3) and CCL2 [**Figure 4**, (1a and 1b)] (Lerner et al., 2015). This process leads to the recruitment of other

cells from nearby locations in the lung and innate cells from the blood to the site of infection.

There are two major mechanisms by which phagocytes such as macrophages recognise *M.tb*. One mechanism occurs when PRRs on macrophages recognise their target directly on the bacteria. The other mechanism occurs when secreted proteins or molecules on the surface of *M.tb* activate complement proteins in the extracellular space, which are then recognised by complement receptors on macrophages (Schlesinger et al., 1990, Schlesinger, 1993, Kang and Schlesinger, 1998). Following the ingestion of *M.tb* through phagocytosis, bacteria are sequestered inside the phagosome. There are many mechanisms that phagocytes such as macrophages can use to eliminate *M.tb* once the bacillus is inside the phagosome: lowering of Ph levels occur, the bacilli are exposed to reactive oxygen intermediates, reactive nitrogen intermediates (RNI) and toxic metals, which creates an intolerable environment for the bacteria. Phagosomes are also able to fuse with lysosomes to form phagolysosomes which leads to the degradation of *M.tb* (van Crevel et al., 2002). Another important cellular mechanism is autophagy, this process involves the sequestering of organelles and intracellular pathogens in an autophagosome which is delivered to the lysosome for *M.tb* killing (Kundu and Thompson, 2008). The activation of autophagy can occur in response to IFNγ (produced by NK cells and T cells) which leads to phagosomal maturation, further decreases in Ph levels and enhanced *M.tb* killing (Gutierrez et al., 2004). However, *M.tb* is able to circumvent these processes, inhibit the phagosome-lysosome fusion and survive in the phagosome (discussed in **section 1.3.5**).

A balance between immune regulation and inflammation is essential and determines clinical outcomes following *M.tb* infection. A hyperinflammatory immune response can lead to an ineffective cellular response, lung pathology and cavitary disease. However, a supressed host immune response can lead to bacterial growth and a delay in the induction of innate and adaptive immune responses (Dorhoi and Kaufmann, 2014a). Neutrophils are the first immune cells to be recruited from the blood to the site of infection and are the predominant cell type infected with *M.tb* in the airways of active TB patients (Eum et al., 2010). Neutrophils are able to engulf bacilli and secrete antimicrobial enzymes which limit *M.tb* growth. They are also able to produce cytokines and chemokines, leading to the recruitment of more cells to the site of

infection (Segal, 2005, Appelberg, 2007). Cytokines secreted by immune cells act on infected macrophages to become more mycobactericidal and can also lead to apoptosis (discussed below) (Behar et al., 2011). Neutrophils can also undergo apoptosis once infected (Serhan and Savill, 2005). Although neutrophils have been reported to play an important role in *M.tb* control (Blomgran and Ernst, 2011, Martineau et al., 2007), they may also contribute to TB pathogenesis (Nandi and Behar, 2011, Lowe et al., 2013). Monocytes from the blood also migrate into the tissues in response to chemokines produced by infected cells and can differentiate into macrophages or DCs (Coillard and Segura, 2019). Other cell types such as NK cells and DURT cells are able to recognise *M.tb* antigens, are known for their rapid proinflammatory effector functions, and likely also play a role during the initial stages of *M.tb* infection (Kaipilyawar and Salgame, 2019).

Apoptosis is an important innate immune mechanism that contributes to early containment of infection and involves programmed cell death of the macrophage, which results in an intact plasma membrane and *M.tb* killing [**Figure 4**, (1a and 1b)] (Behar et al., 2011). However, infection of macrophages with *M.tb* can also lead to necrosis, which is a type of cell death that results in cell lysis and the release of cellular contents including reactive oxygen species and lysosomes into the extracellular space [**Figure 4**, (1a and 1b)] (Lee et al., 2011, Chen et al., 2006). This results in damage of the surrounding tissue and the spread of *M.tb*. Necrosis is more favourable for *M.tb* and it has been reported that virulent strains of *M.tb* are able to induce necrosis of macrophages (Chen et al., 2008, Divangahi et al., 2010, Behar et al., 2011). The type of cell death (apoptosis or necrosis) is regulated by the signalling lipids prostaglandin E2 (PGE2) and lipoxin A4 (LXA4), which induce apoptosis and necrosis respectively (Bafica et al., 2005, Chen et al., 2008). Engulfment of virulent *M.tb* strains by macrophages can lead to the bacilli blocking PGE2, inducing LXA4 and causing necrotic death of the macrophage (Chen et al., 2008). Efferocytosis is another important mechanism which leads to early containment of *M.tb* and involves the phagocytosis of apoptotic macrophages by uninfected macrophages [**Figure 4**] (Martin et al., 2012). This process allows the further containment of *M.tb* and delivers the bacillus to the lysosome for elimination.

The factors that determine whether early clearance of *M.tb* occurs or infection is established and possibly progression to TB disease takes place are complicated and influenced by both bacterial and host interactions that are still not fully understood (Kaipilyawar and Salgame, 2019). In cases where the innate immune system is unable to clear *M.tb*, the induction of the adaptive immune response is initiated.

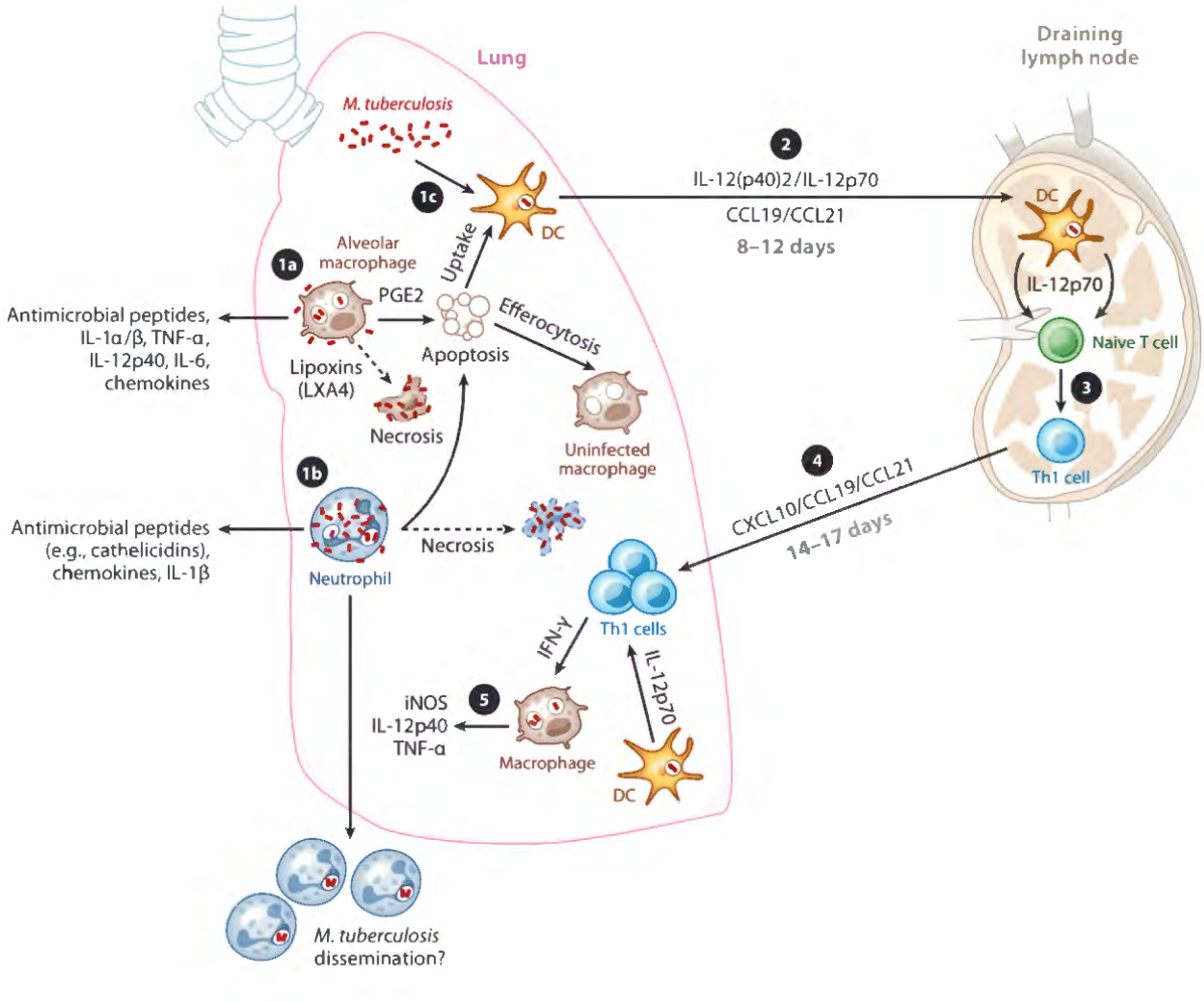


Figure 4. An illustration of the innate and adaptive immune response to M.tb infection. Adapted from O'Garra et al. (O'Garra et al., 2013).

1.3.4. The initiation of the adaptive immune response

Upon recognition of *M.tb*, DC maturation takes place, which involves the downregulation of phagocytic functions and the upregulation of chemokine receptors and costimulatory molecules which improve antigen presentation capabilities of the cell [**Figure 4**, (1c)] (Rescigno et al., 1997, Winzler et al., 1997). Subsequently, migration to the lymphatic vessels and then the lung draining LN occurs. Trafficking out of the tissues by DCs is dependent on CCR7, CCL19 and CCL21 [**Figure 4**, (2)] (Wolf et al., 2007). The secretion of IL-12p40 homodimers by myeloid cells is also required for trafficking out of the lung [**Figure 4**, (2)] (Khader et al., 2006). In order to elicit a *M.tb*-specific T cell response, bacilli must be transported to the draining LN (Wolf et al., 2008) where antigen presentation takes place: (i) DCs present *M.tb* antigens in the context of MHC I and MHC II to naive $CD8^+$ T and $CD4^+$ T cells respectively (referred to as priming); (ii) The MHC-antigen complex colocalises with co-stimulatory molecules such as CD40 and CD86 which bind to CD40 ligand and CD28 on T cells respectively; and (iii) high levels of cytokines such as IL-12 drive differentiation and clonal expansion of naive $CD4^+$ T cells to Th1 cells (IL-2-, TNF- and IFNγ- producing $CD4^+$ T cells) [**Figure 4**, (3)] (Huppa and Davis, 2003, Athie-Morales et al., 2004, Cooper and Khader, 2008). $CD4^+$ T cells must then migrate from the LN to the site of infection, directed by a chemokine gradient. The induction of the adaptive immune response is typically slow from the initial onset of infection [14-17 days, **Figure 4**, (4)]. It is thought that mechanisms carried out by *M.tb* to subvert the immune response contribute to the slow initiation of adaptive immunity. Macrophages and neutrophils infected with *M.tb,* and that have undergone apoptosis, are able to deliver *M.tb* antigens to DCs, leading to rapid migration of DCs to LNs (Blomgran and Ernst, 2011, Divangahi et al., 2010). However, virulent *M.tb* is able to block apoptosis leading to delayed priming of $CD4^+$ T cells in the LNs (Blomgran et al., 2012).

1.3.5. M.tb immune evasion

M.tb has evolved numerous mechanisms to evade and subvert the host immune response in order to ensure its survival and spread. The most well-known mechanism executed by *M.tb* is the disruption of phagosome maturation and phagosome-

lysosome fusion (Armstrong and Hart, 1971, Fratti et al., 2000). In mice, IFNγ-mediated production of RNIs is essential for killing of *M.tb* in infected macrophages (Flynn et al., 1993), however, this is not clear in humans (Nathan and Shiloh, 2000). The bacillus is also able to persist in the presence of RNI in the phagosome. It has been reported that *M.tb* is able to produce enzymes which convert RNI to a less toxic form, thus allowing the persistence of bacilli (St John et al., 2001, Bryk et al., 2002) and providing a mechanism for *M.tb* tolerance of reactive chemicals. *M.tb* is also able to interfere with antigen presentation by downregulating MHC II expression on APCs, which has implications for the initiation of innate and adaptive immune responses (Flynn and Chan, 2003). As discussed above, the bacillus is also able to block apoptosis of *M.tb* infected phagocytes and induce necrosis, delaying the onset of the adaptive immune response and also promoting lung damage and *M.tb* spread (Blomgran et al., 2012).

1.3.6. CD4$^+$ T cells are essential for M.tb control

The activation of *M.tb*-infected macrophages by IFNγ-producing CD4$^+$ T cells is an essential mechanism to establish immunological control [**Figure 4,** (5)] (Flynn and Chan, 2001). In mice, IFNγ can synergise with TNF and induce the production of nitric oxide and RNI in macrophages, which is catalysed by inducible nitric oxide synthase. This is a major mechanism to control *M.tb* growth in mice, however as stated above, this is not clear in humans (Nathan and Shiloh, 2000). Other immune subsets (NK, CD8$^+$ T and DURT cells) are also able to produce IFNγ in response to *M.tb* (Cooper et al., 2001a, Grotzke and Lewinsohn, 2005, Ruibal et al., 2021). The predominant role of CD4$^+$ T cells, however, is evident in studies showing that MHC II knockout mice or depletion of CD4$^+$ T cells leads to rapid loss of control of *M.tb* infection and death. Green et al. created a model in which RAG knockout mice were reconstituted with CD4$^+$ depleted splenocytes and either (wild-type) WT CD4$^+$ T cells or IFNγ knockout CD4$^+$ T cells. Mice that received IFNγ knockout CD4$^+$ T cells lost control of *M.tb* infection and died earlier than mice that received WT CD4$^+$ T cells (Green et al., 2013). This study further supports the thinking that IFNγ produced by CD4$^+$ T cells, in particular, is essential for protection against TB (Flory et al., 1992, Casanova et al., 2012, Green et al., 2013). In humans, HIV-infected individuals not on antiretroviral

therapy (ART) experience depletion and dysfunction of $CD4^+$ T cells and are at the greatest risk of developing TB disease (Kwan and Ernst, 2011). Although IFNγ and other proinflammatory cytokines produced by T cells are essential in the immune response against *M.tb*, excessive amounts can lead to TB pathogenesis and poor outcomes. The mechanisms to curb an over-exuberant proinflammatory response include the production of IL-10 by various innate and adaptive immune cells (Redford et al., 2011, Urdahl et al., 2011) as well as the induction of $Foxp3^+$ regulatory T cells (Scott-Browne et al., 2007). Recently, the role of other non-conventional T cell subsets in the immune response against *M.tb* has gathered much attention and is discussed in later chapters.

1.3.7. The lung granuloma and immunopathology in TB disease

After the initial events following *M.tb* infection, it is believed that, in most cases, myeloid cells are unable to kill *M.tb* due to virulence factors, such as those present in the region of difference (RD)1, and provide a permissible environment for the survival and spread of *M.tb* (Corleis et al., 2012). The *M.tb* virulence factor ESAT-6 has been shown to induce matrix metalloproteinase 9 (MMP-9) production by epithelial cells, which recruits macrophages and is thought to be advantageous for bacilli (Volkman et al., 2010). MMPs are a family of proteases which have been linked to lung damage and worse TB outcomes (Salgame, 2011, Elkington et al., 2011a, Elkington et al., 2011b). Immune cells such as macrophages, neutrophils and NK cells are recruited to the site of infection and form the early aggregates of infected and uninfected innate cells of the granuloma (**Figure 5**). Macrophages in the granuloma can also fuse to form giant cells or differentiate into foam cells which are made up of lipid bodies (**Figure 5**) (Russell et al., 2009, Helming and Gordon, 2007). Previous thinking has been that granuloma formation is dependent on an adaptive immune response, however, animal studies such as those using the zebrafish larvae model (in which adaptive immunity has not developed) have shown that granulomas can form in the absence of adaptive immunity (Davis et al., 2002). TNF was also considered to be critical in TB granuloma formation and maintenance (Kindler et al., 1989, Roach et al., 2002, Algood et al., 2005), however, studies in zebrafish larvae observed that in the absence of TNF, granuloma formation is accelerated, but an increase in bacterial

numbers and necrosis of infected cells leads to granuloma collapse (Clay et al., 2008). Thus, TNF is essential for granuloma maintenance through bacterial control.

The initiation of the adaptive immune response allows activation of infected myeloid cells by T cells, which increases mycobactericidal capabilities and microbial killing (North and Jung, 2004). T and B cells make up the outer cuff of the granuloma structure (**Figure 5**). At this stage, a dynamic interaction between the host and pathogen commences, with cell death (either due to apoptosis or necrosis), the recruitment of immune cells to the site of infection and tissue remodelling occurring (Ramakrishnan, 2012). This could lead to sterilisation of the granuloma, dissemination of infected cells to form new structures (thought to occur early in infection), or the bacteria could switch to a dormant state and remain in-check for years or a lifetime without reactivation (Lin et al., 2014, Martin et al., 2017, Feldman and Baggenstoss, 1939). Studies have shown that individuals who appeared healthy prior to a non-TB related death had lung pathology consistent with TB lesions (Feldman and Baggenstoss, 1939). In some cases, however, a loss of immune control takes place, characterised by inflammation, high bacterial numbers and lung pathology, driven by an uncontrolled $CD4^+$ T cell inflammatory response (Ehlers et al., 2001, Aly et al., 2009). This stage is characterised by a caesium in the centre of the granuloma structure, which is formed mainly by necrotised infected macrophages which may liquefy and form cavities near the airway, providing optimal conditions for bacterial growth and transmission of *M.tb* to the next host. However, a previous theory postulated that the caesium and subsequent cavitation develops from the necrosis of TB lipid pneumonia in the alveolar space; it's plausible that both scenarios could occur (Sarathy and Dartois, 2020). The link between TB lung pathology and the infectiousness of an individual is further discussed and investigated in chapter 6 of this thesis.

Studies in humans and non-human primates (NHP)s using ^{18}FDG positron emission tomography-computed tomography technology, which detects and visualises metabolically active sites in the lung, have shed light on the heterogeneity and spectrum of granulomas in an individual and in disease states. These studies have shown that granulomas develop independently within an individual host and that sterilised granulomas exist in individuals with *M.tb* infection and active TB cases (Lin

et al., 2013, Lin et al., 2014, Coleman et al., 2014). It is therefore likely that the sum of the outcomes of each granuloma in an individual determines clinical manifestations. This is also evident in the spectrum of *M.tb* infected individuals observed (discussed in **section 1.3.8**). In addition, Gideon et al. showed that a balance between pro- and anti-inflammatory cytokines produced by T cells within granulomas was associated with low bacterial burden and sterilised granulomas in NHPs infected with *M.tb* (Gideon et al., 2015). In another study, the authors showed that granulomas that were able to control bacterial growth developed later in *M.tb* infection and immune cells were more proinflammatory compared to those granulomas that lacked bacterial control (Gideon et al., 2021). Thus, these studies support previous work indicating the need for a balanced response to *M.tb* to achieve immune control.

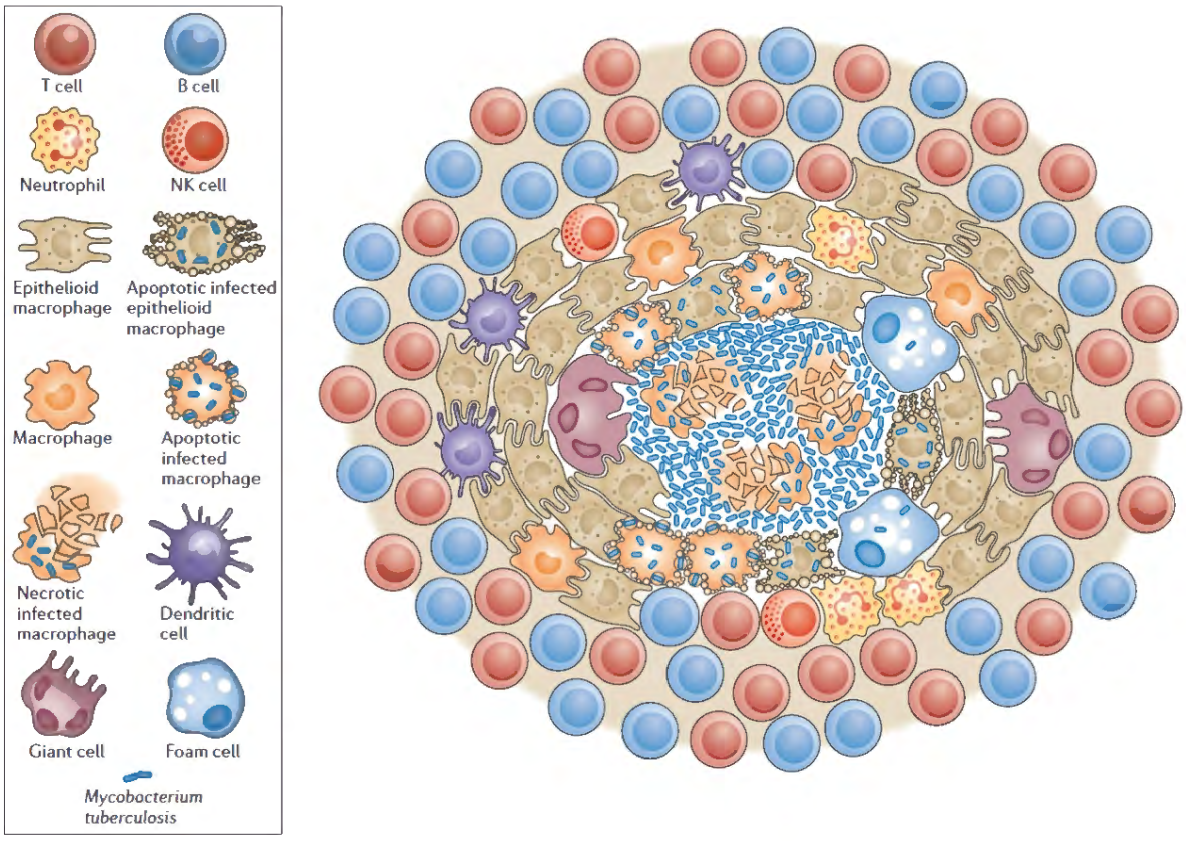


Figure 5. An illustration of innate and adaptive immune cells forming the structure of a classic TB lung granuloma. Adapted from Ramakrishnan et al. (Ramakrishnan, 2012).

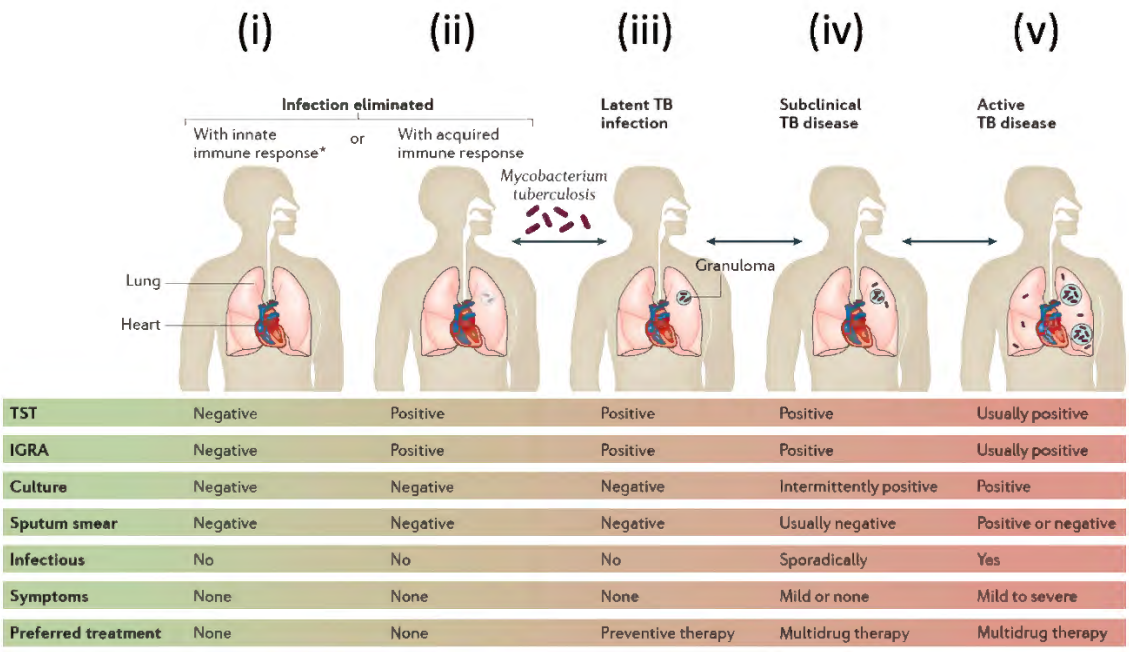
1.3.8. The TB spectrum

It is not possible to detect *M.tb* in infected healthy individuals using current diagnostics (microbiological culture and sputum smear). Thus, *M.tb* infection is indirectly diagnosed based on immunological sensitisation to *M.tb* antigens, measured by tuberculin skin test (TST), which detects $CD4^+$ T cell-driven delayed hypersensitivity skin reaction to mycobacterial antigens, or IFNγ release assay (IGRA), which quantifies $CD4^+$ T cell IFNγ secretion in response to *M.tb*-specific antigens (Pai and Sotgiu, 2016). In recent years, it has become clear that individuals who have been infected with *M.tb* may exhibit different phenotypes, which challenges the traditional binary viewpoint that individuals infected with *M.tb* either develop primary TB disease or have a life-long, latent *M.tb* infection (Barry et al., 2009, Esmail et al., 2014) (**Figure 6**).

Some individuals, termed 'resisters', do not show evidence of infection, measured by TST or IGRA, despite high exposure to *M.tb* [**Figure 6** (i)] (Simmons et al., 2018, Meermeier and Lewinsohn, 2018). These resisters could have cleared *M.tb* through innate mechanisms (including DURT cell functions), or some might be infected but are able to control infection through adaptive IFNγ-independent immune mechanisms, or have T cells that recognise antigens other than those tested in the IGRA and thus immune responses are not detected (Kaipilyawar and Salgame, 2019, Lu et al., 2019). It has also been shown that a proportion of *M.tb* culture positive individuals have no TB symptoms [**Figure 6** (iv)]. These individuals have been referred to as having subclinical TB disease and could be contributing to TB transmission despite being asymptomatic (Esmail et al., 2018, Frascella et al., 2021).

A blood transcriptional signature, composed of type I/II IFN-related genes, identified individuals at risk of developing TB disease up to 18 months prior to symptoms and TB diagnosis. These genes were highly upregulated in individuals closer to TB diagnosis and provide insight into the biological mechanisms at play prior to the onset of TB disease (Zak et al., 2016). In this thesis we used this blood transcriptional signature as an indicator of type I/II IFN driven inflammation (**Chapter 6**). Overall,

recent studies in the field suggests that *M.tb* infection is dynamic, and the early stages of disease can occur sometime before the onset of clinical presentation.



TST	Negative	Positive	Positive	Positive	Usually positive
IGRA	Negative	Positive	Positive	Positive	Usually positive
Culture	Negative	Negative	Negative	Intermittently positive	Positive
Sputum smear	Negative	Negative	Negative	Usually negative	Positive or negative
Infectious	No	No	No	Sporadically	Yes
Symptoms	None	None	None	Mild or none	Mild to severe
Preferred treatment	None	None	Preventive therapy	Multidrug therapy	Multidrug therapy

Figure 6. An illustration of the spectrum of TB. Adapted from Pai et al (Pai et al., 2016).

1.4. Key host immune mediators in TB

In this section we discuss key host immune mediators in TB and provide detail on host mediators that have been studied in the different chapters of this thesis.

1.4.1. IFNγ

As discussed above, IFNγ is considered one of the most important effector molecules in the elimination of *M.tb*. It is known to induce signalling through transcriptional pathways which improve immune cell function and migratory capacity in order to eliminate *M.tb* (Flynn and Chan, 2001). It also acts on cells by inducing cytokine production (promoting a Th1 response) and is also able to synergise with vitamin D to induce phagosomal maturation, autophagy and the production of antimicrobial molecules such as cathelicidin in *M.tb* infected macrophages (Fabri et al., 2011). IFNγ knockout mice were shown to be highly susceptible to *M.tb* infection, characterised by

uncontrolled bacilli growth, tissue necrosis and rapid death (Cooper et al., 1993, Flynn et al., 1993). Also, humans with mutations in IFNγ receptor show increased susceptibility to mycobacterial infection (Ottenhoff et al., 1998).

1.4.2. IL-12

IL-12 is a critical cytokine in the host defence against *M.tb*, and serves as a link between the innate and adaptive immune response. It is largely produced by phagocytes (Trinchieri, 1995, Ladel et al., 1997b) and is a potent inducer of IFNγ production by NK cells, conventional T cells and some DURT cell subsets, which is essential for a controlled immune response against *M.tb* infection (Flynn et al., 1993, Cooper et al., 1993, Chan et al., 1991, Ussher et al., 2014, Sada-Ovalle et al., 2008, Cooper et al., 2001c). Following *M.tb* infection, IL-12 is required for DC migration to the draining LN and for T cell priming and differentiation in the LN to initiate a Th1 adaptive immune response (Khader et al., 2006). IL-12 knockout mice are highly susceptible to mycobacterial infections (Wakeham et al., 1998, Cooper et al., 1997b). Also, humans with genetic mutations in the genes encoding IL-12 and IL-12R also have increased susceptibility to infections by mycobacteria (Altare et al., 1998a, Altare et al., 1998b). These individuals have a reduced ability to produce IFNγ (Ottenhoff et al., 1998).

1.4.3. IL-18

IL-18 is produced by a variety of cell types and induces the production of proinflammatory cytokines (Netea et al., 2000, Wawrocki et al., 2016). It is particularly important for the optimal induction of IFNγ (Sugawara et al., 1999, Kinjo et al., 2002). In addition, IL-18 in combination with IL-12 induces high levels of IFNγ by NK cells and some DURT cell subsets (Cooper et al., 2001c, Sada-Ovalle et al., 2008, Ussher et al., 2014, Suliman et al., 2019). IL-18 knockout mice have been shown to have higher *M.tb* colony forming units (CFU)s and lower levels of IFNγ (Sugawara et al., 1999, Kinjo et al., 2002, Schneider et al., 2010). The absence of IL-18 in mice was also shown to result in the accumulation of neutrophils and chemokine (C-X-C motif) ligand (CXCL)1 and CXCL2 (Schneider et al., 2010). Thus, these studies indicate that IL-18

is required for an optimal Th1 response following *M.tb* infection and prevents accumulation of permissive cells.

1.4.4. TNF

TNF is a critical proinflammatory cytokine that is produced mainly by macrophages, although it is secreted by other immune cells such as lymphocytes (Wajant et al., 2003). It plays a key role in immune processes, including chemokine expression (Roach et al., 2002, Algood et al., 2005), apoptosis of infected cells (Laster et al., 1988), macrophage activation (Dorhoi and Kaufmann, 2014b, Flesch and Kaufmann, 1990) and granuloma structure (Roach et al., 2002, Bean et al., 1999). The neutralisation of TNF or the mutation of the TNF receptor in mice infected with *M.tb* leads to bacterial growth and death (Flynn, 1995). In a study by Roach et al. it was shown that TNF knockout mice infected with *M.tb* resulted in an initial delay of chemokine induction and cellular recruitment to the granuloma. In addition, it was reported that mice failed to form tight granulomas and clear *M.tb* (Roach et al., 2002). The important role of TNF in TB is highlighted in cases of progression to disease in humans infected with *M.tb* on anti-TNF therapy (Wallis, 2008). Too much production of TNF, however, can lead to lung pathology and contribute to the spread of *M.tb* (Roca and Ramakrishnan, 2013).

1.4.5. IL-1β

IL-1β is produced by monocytes, macrophages and DCs and is critical in the immune response to *M.tb* (Fremond et al., 2007). It has been shown to induce antimicrobial molecules and proinflammatory cytokines such as TNF by macrophages, leading to the restriction of *M.tb* growth (Jayaraman et al., 2013). IL-1β also induces PGE2, resulting in the downregulation of type I IFNs (discussed below) and improved disease outcomes (Mayer-Barber et al., 2014). Also, mice deficient in IL-1β or IL-1R have been found to have increased disease severity after *M.tb* infection (Yamada et al., 2000, Fremond et al., 2007, Mayer-Barber et al., 2010). In addition, polymorphisms in the IL-1 and IL-1R antagonist genes were associated with differential susceptibility to TB disease in humans (Bellamy et al., 1998, Wilkinson et al., 1999).

1.4.6. IL-6

IL-6 is a pleiotropic cytokine, produced by many cell types, that can exert both proinflammatory and anti-inflammatory effects. It has been shown to be important for B cell and macrophage differentiation and T cell activation (Van Snick, 1990, Hunter and Jones, 2015). IL-6 has also been shown to be important for an optimal T cell response to mycobacterial vaccination and infection (Appelberg et al., 1994, Leal et al., 1999). On the other hand, it has also been shown to suppress proinflammatory responses such as IL-1β and TNF (Schindler et al., 1990), as well as macrophage responsiveness to IFNγ (Nagabhushanam et al., 2003). In a high dose intravenous infectious model, IL-6 knockout mice are particularly susceptible to *M.tb* infection compared to control mice (Ladel et al., 1997a). In a low dose aerosol infection model, however, IL-6 knockout mice displayed an early increase in bacterial load linked to a delay in IFNγ production before the onset of the adaptive immune response (Saunders et al., 2000). These results indicate that IL-6 may play an important role in the early stages of infection and can exert different effects depending on the context.

1.4.7. IL-17

IL-17 is thought to mainly be produced by T cells, however, other innate and adaptive immune cells are also able to produce it (Korn et al., 2009). IL-17 has been shown to play an important role in the sterilisation of granulomas (Gideon et al., 2015) and the induction of chemokines leading to the trafficking of neutrophils and Th1 cells to the site of infection to control *M.tb* infection (Khader et al., 2007). However, unrestrained IL-17 production can lead to neutrophil-rich granulomas and neutrophil-driven pathology (Redford et al., 2010, Cruz et al., 2010). IFNγ has also been shown to be able to dampen IL-23 and thereby limit IL-17 production (Nandi and Behar, 2011, Desvignes and Ernst, 2009). In mice, IL-17 has been shown to be protective against virulent strains of *M.tb* (Gopal et al., 2014), and contributes to an enhanced vaccine-induced immune response against other *M.tb* strains (Aguilo et al., 2016). Recent studies in NHPs, which have administered BCG to NHPs via the mucosal or intravenous routes, have shown that BCG-induced protection was associated with Th17 and Th1 responses (Dijkman et al., 2019, Darrah et al., 2020).

1.4.8. IL-10

IL-10 is an anti-inflammatory cytokine produced by many immune cells including myeloid and T cells (Saraiva and O'Garra, 2010). IL-10 is necessary to dampen an excessive proinflammatory immune response to pathogens such as *M.tb*, although too much IL-10 can lead to chronic infection (Redford et al., 2011). IL-10 is able to inhibit phagosome maturation of *M.tb*-infected macrophages (O'Leary et al., 2011) and antigen presentation by macrophages and DCs (Moore et al., 2001). It is also able to inhibit cytokines such as TNF and IL-12 leading to the inhibition of macrophage activation and the induction of a protective Th1 response (Demangel et al., 2002, Oswald et al., 1992, Moore et al., 2001). In some studies, it was shown that IL-10 knockout mice had an early decrease in bacterial burden compared to controls when infected with mycobacteria (Redford et al., 2010, Murray and Young, 1999), however, other studies showed no effect (North, 1998, Erb et al., 1998).

1.4.9. The type I IFN response

Type I IFNs are known for their antiviral activity but have been linked to poor outcomes in the context of TB disease (Trinchieri, 2010). IFNα receptor knockout mice, unresponsive to type I IFNs, infected with *M.tb* have a reduced bacterial burden and increased survival, indicating that type I IFNs have a detrimental role in TB disease (Manca et al., 2005, Ordway et al., 2007, Stanley et al., 2007, Antonelli et al., 2010). It has been shown that the type I IFN response is induced by virulent strains of *M.tb* which contain the ESAT-6 secretion system 1 (Stanley et al., 2007). The increased levels of type I IFNs lead to diminished proinflammatory cytokine production and increased bacterial burdens (Manca et al., 2001, Manca et al., 2005, Ordway et al., 2007). Excessive amounts of type I IFNs can also lead to the trafficking of phagocytes to the lung, that cannot be optimally activated and thereby provide a permissive environment for *M.tb* growth (Antonelli et al., 2010). Also, in a study by Berry et al. it was shown that a predominantly type I IFN-stimulated gene expression signature was present in the blood of active TB patients, correlated with extent of lung pathology measured by chest X-ray and resolved following successful TB treatment (Berry et al., 2010).

1.4.10. Cytotoxic molecules

A major killing mechanism carried out by lymphocytes is the release of the cytolytic molecule perforin, which forms pores in the membranes of cells and allows the entry of granzymes and granulysin into the cell, which may lead to direct killing of *M.tb* or apoptosis of the infected cell (Liu et al., 1996, Krensky and Clayberger, 2009). Another important killing mechanism is the induction of apoptosis of an infected cell through the ligation of CD95 (Fas) by CD95 ligand (Liu et al., 1996). Studies *in vitro* have shown that lymphocytes release perforin in conjunction with cytotoxic granules, leading to killing of *M.tb* directly and *M.tb*-infected cells (Lu et al., 2014, Stenger et al., 1998). However, other studies have reported that deficiencies in perforin, granzyme and Fas receptor in mice infected with *M.tb* did not affect the course of infection in comparison to controls (Laochumroonvorapong et al., 1997, Cooper et al., 1997a).

1.5. The BCG vaccine

1.5.1. The BCG vaccine elicits specific and heterologous effects

BCG is a live attenuated strain of *Mycobacterium bovis* and is considered to be one of the most widely used vaccines (Lancione et al., 2022). Yet, it confers variable protection against pulmonary TB, the predominant form of disease in adults (Mangtani et al., 2014). The reasons for this variability are not well understood, but it is known that the protection provided by BCG is greater at higher latitudes and diminished over time (Mangtani et al., 2014). In a recent study it was reported that household contacts (HHC)s of TB patients with persistent negative IGRA tests were more likely to be BCG vaccinated (as evidenced by a BCG scar) compared to those HHCs who were IGRA converters (Verrall et al., 2020a). It was also shown that these protective effects of BCG waned with increasing exposure to *M.tb* (Verrall et al., 2020a, Campbell et al., 2021). In a case-contact study it was shown that the risk of IGRA conversion was higher in individuals who were exposed to TB patients with the *M.tb* Beijing strain compared to other strains. In addition, BCG vaccination was associated with protection in individuals exposed to other strains, however, this was not the case in

those individuals exposed to the *M.tb* Beijing strain (Verrall et al., 2020b). BCG is also able to protect against related pathogens such as *Mycobacterium leprae* (Setia et al., 2006) and non-tuberculous mycobacteria (NTM) which can cause lymphadenitis and Buruli ulcer (Zimmermann et al., 2018). In addition, BCG given at birth has been shown to reduce all-cause neonatal and infant mortality due to non-TB diseases (Higgins et al., 2016); the underlying mechanism that has been proposed is "training" of innate immunity (Blok et al., 2015) (**Figure 7**) (discussed in detail below).

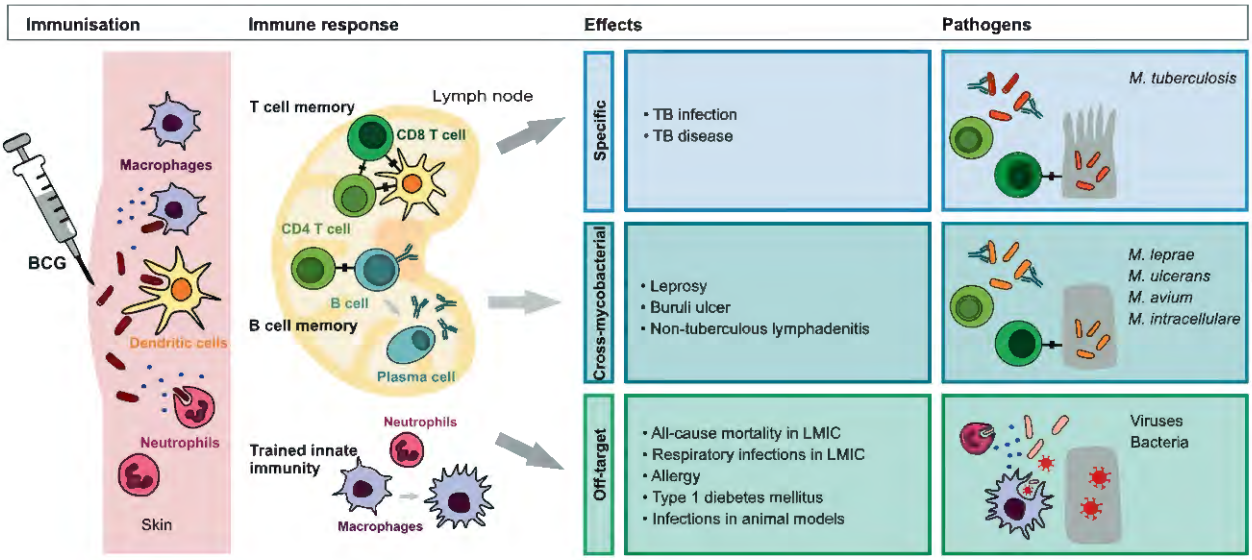


Figure 7. An illustration of the specific, cross-mycobacterial and off-target effects of BCG vaccination in children. Adapted from Fritschi et al. (Fritschi et al., 2020).

1.5.2. Immune responses elicited by BCG

BCG vaccination is routinely administered via the intradermal (i.d.) route (discussed below), therefore, it is taken up by APCs in the i.d. tissue and not by AMs, as is the case during natural infection with *M.tb*. BCG vaccination elicits a strong Th1 response in infants and adults; as previously discussed, the production of IFNγ by $CD4^+$ T cells is considered to be one of the main protective mechanisms against TB disease (Flynn and Chan, 2001). BCG is able to overcome the Th2 bias of the infant immune response so that infants are able to produce IFNγ comparably to adults in response to vaccination (Marchant et al., 1999). I.d. BCG elicits poor Th17 responses; this is thought to be because of the absence of the immunodominant ESAT-6 containing RD1 region in BCG, which is present in *M.tb* (Dockrell and Smith, 2017). Recently, studies

in NHPs in which BCG was administered via the mucosal or intravenous (i.v.) route, showed increased protection compared to i.d. BCG, which was associated with increased Th1/Th17 responses in the lung (Dijkman et al., 2019, Darrah et al., 2020).

It is still unclear which immune responses are associated with BCG-induced protection in infants. The heterogeneity of BCG-induced immune responses may confound identification of correlates of protection and risk (Fletcher et al., 2016a). In a previous study from our group, frequencies of BCG-specific Th1 and polyfunctional responses in 10-week-old infants who were vaccinated at birth did not differ between infants who progressed or did not progress to active TB disease (Kagina et al., 2010). In a cohort of SA infants, the expression of the activation marker HLA-DR on bulk $CD4^+$ T cells in response to BCG was associated with increased TB risk while the number of cells expressing IFNγ (the cellular source was not identified and could be cells other than conventional T cells) was associated with reduced risk of TB (Fletcher et al., 2016b). This association was not very strong and does not fully explain why BCG protects in some infants and not others. Thus, other immune responses, beyond conventional T cells, elicited by BCG vaccination such as NK cells, Abs, B cells and DURT cells have gathered much interest in recent years and are discussed in later chapters. A better understanding of the immune responses elicited by BCG vaccination is of critical importance. Many TB vaccine candidates in clinical development are boosters to be administered after BCG (Andersen and Scriba, 2019); it is important that these vaccines are able to enhance the different components of the BCG-induced immune response, or complement it by inducing responses to additional antigens (Woodworth et al., 2021). Also, new whole cell mycobacterial vaccines could improve on BCG-induced immune responses.

Studies investigating infant immune responses to BCG vaccination in TB endemic settings are challenging, thus longitudinal studies are not routinely undertaken. Also, many studies use cord blood as a surrogate for early infant immune responses, however, there is evidence that cord blood is not a good proxy for infant immune responses in peripheral blood (Olin et al., 2018). In this thesis we studied a cohort of infants in whom BCG was either administered at birth, as is routine, or delayed, for the purpose of this study. This allowed us to conduct cross-sectional comparisons between vaccinated and unvaccinated infants from the same community. Other

studies have investigated immune responses to mycobacteria in infants from TB endemic settings in whom BCG vaccination was delayed. These studies, however, have largely focused on conventional T cell responses and/ or host secreted immune mediators (Marchant et al., 1999, Hussey et al., 2002, Burl et al., 2010, Kagina et al., 2010, Lutwama et al., 2014, Blakney et al., 2015, Jensen et al., 2015, Tchakoute et al., 2015, Subbian et al., 2020). In this thesis, we studied the phenotype and function of diverse immune subsets from whole blood in infants (who have or have not received BCG) in response to mycobacterial stimulation.

1.5.3. Heterologous beneficial effects induced by BCG vaccination

i. BCG effects on all-cause mortality

Historical case control studies (Shann, 2013) have shown reductions in all-cause mortality in infants who received BCG compared to those who did not receive BCG. More recently, 3 randomised trials in Guinea-Bissau reported BCG-mediated protection against all-cause mortality in low-birth weight (LBW) infants. (Aaby et al., 2011, Biering-Sorensen et al., 2012, Biering-Sorensen et al., 2017). In a meta-analysis of all 3 trials, a reduction in mortality of 38% in the neonatal period and 16% in infancy was reported in infants who received BCG at birth compared to those who did not (Biering-Sorensen et al., 2017). It is thought that BCG vaccine heterologous effects may be altered or diminished by subsequent non-live vaccines given to infants. In addition, stronger effects were reported in males in the first week of life while in females this was the case at 2-4 weeks of age (Biering-Sorensen et al., 2018). The decrease in all-cause mortality was linked to the reduction of neonatal sepsis and respiratory infections (Biering-Sorensen et al., 2017), which are major causes of mortality in children younger than 5 years of age. However a recent study in India observed no differences in all-cause mortality between LBW infants who received BCG and those who did not (Jayaraman et al., 2019). The possible reasons for these conflicting results could be attributable to the fact that BCG-Denmark was used in the studies in Guinea-Bissau while BCG-Russia was used in the Indian study. Also, infants in India were <2000g and in an intensive care unit compared to the Guinea-Bissau studies, where the inclusion criteria was a birthweight of <2500g.

ii. BCG effects on non-related diseases

Studies from high- and low-income countries in which BCG vaccination is no longer administered, either due to lack of supply or discontinuation, observed increases in cases of respiratory infections (Stensballe et al., 2005, de Castro et al., 2015). In a large study using survey data from 33 countries (middle- and low- income) it was reported that BCG vaccination in children younger than 5 years of age was associated with a 17% to 37% reduced risk of developing suspected acute lower respiratory infections (Hollm-Delgado et al., 2014). Recently, a study in Uganda showed a reduction of infectious diseases other than TB in infants who received BCG compared to those who did not receive BCG at birth (Prentice et al., 2021). However, studies investigating heterologous effects of BCG on all-cause hospitalisation and hospitalisation for infections in infants from Denmark, a high income and latitude country, showed no differences in hospitalisations up to 15 months of age between those who received BCG and those who did not (Stensballe et al., 2017, Stensballe et al., 2019). Interestingly, in a subset of this cohort it was shown that infants born to mothers who were BCG-vaccinated had a 32% reduction in infections in the first 3 months of life compared to infants of mothers who did not receive BCG. (Kjaergaard et al., 2016). These results indicate that the protective effects of BCG vaccination may be more pronounced in low-income settings. In light of these studies, there are clinical trials currently underway all over the world to determine whether BCG protects against Coronavirus disease 2019 (COVID-19) (Gonzalez-Perez et al., 2021).

1.5.4. BCG and trained immunity

1.5.4.1. The molecular mechanism underpinning BCG-induced trained immunity

Trained immunity refers to the process whereby innate cells such as monocytes, macrophages and NK cells can be programmed by initial exposure to certain stimuli to retain the ability to respond with greater (or lesser) magnitude or function to subsequent related or heterologous exposures (Netea et al., 2016). Whole cell vaccines such as BCG and measles, including the molecule β-glucan have all been

implicated in the induction of trained immunity (Blok et al., 2015, Moorlag et al., 2020a). A pivotal study by Kleinnijenhuis et al. reported that peripheral blood mononuclear cells (PBMC)s from BCG-vaccinated adults produced increased levels of cytokines such as IL-6, IL-1β and TNF in response to stimulation with *M.tb* and unrelated organisms such as *Candida albicans* (*C.albicans)* and *Staphylococcus aureus* (*S.aureus)* up to 3 months post vaccination (Kleinnijenhuis et al., 2012). BCG-induced trained immunity effects appear short-lived and have been shown to last up to 1 year (Kleinnijenhuis et al., 2014a). The authors also measured increased occupancy of the active epigenetic mark, histone 3 lysine 4 trimethylation (H3K4me3), at the promoters of *tnf* and *il6* from sorted monocytes of these BCG vaccinated individuals (Kleinnijenhuis et al., 2012). This was the first study to link BCG-induced epigenetic reprogramming of innate cells to an enhanced immune response upon unrelated secondary immune challenge, thereby providing a plausible mechanism for the heterologous beneficial effects conferred by BCG vaccination (Blok et al., 2015). Interestingly, in a human infection model, BCG vaccinated individuals who were given the yellow fever vaccine showed reduced viremia compared to BCG unvaccinated controls (Arts et al., 2018)

Recent work has also uncovered the relationship between immunological signalling, metabolism and epigenetic reprogramming (**Figure 8**) thereby providing the molecular mechanism underpinning trained immunity. This work could potentially provide targets for interventions aimed to enhance or dampen trained immunity effects. The current framework of trained immunity is that after engagement of PRRs by a specific stimuli, a signalling cascade ensues. In the case of BCG, this process occurs in a NOD2 dependent manner (Kleinnijenhuis et al., 2012) causing a metabolic shift from oxidative phosphorylation to glycolysis, known as the Warburg Effect (Cheng et al., 2014). This leads to the accumulation of metabolites such as succinate and mevalonate which allow the retention of H3K4me3 marks by blocking demethylases and amplifies the signalling cascade respectively (Arts et al., 2016, Bekkering et al., 2018). Other metabolic pathways such as glutamine, cholesterol and glutathione metabolism have also been shown to contribute to trained immunity (Arts et al., 2016, Ferreira et al., 2021, Fanucchi et al., 2021). Sirtuine1, which is part of a family enzymes which deacetylate lysine residues from histone and non-histone substrates, has also been linked to modulatory effects on BCG-induced trained immunity (Mourits

et al., 2021). The promoter regions of innate immune genes are then loaded with ribonucleic acid (RNA) polymerase II with nearby histones possessing active marks such as H3K4me3 and histone 3 lysine 27 acetylation (H3K27Ac) at specific gene sites. This provides a permissive environment for transcription of innate genes to occur. These active histone marks are only partially lost after the eradication of the initial stimulus, with the retention of 'latent enhances' such as histone 3 at lysine 4 methylation (H3K4me1) resulting in an enhanced immune response to a secondary challenge (Netea et al., 2016). Studies have also shown that the depositing of histone marks is facilitated by a class of long non-coding RNAs (lncRNA)s called immune gene-priming lncRNAs (IPL)s, which engage with a histone modification complex, the COMPASS complex, which in turn conducts the trimethylation of H3K4me3 (Fanucchi et al., 2019, Gomez et al., 2013, Wang et al., 2011). This process occurs in topologically associated domains, which are folded regions of long stretches of chromatin, bringing together multiple complexes and allowing the depositing of histone marks at particular gene sites that would otherwise be dispersed across the genome (Li et al., 2012). This structural mechanism allows a rapid and coordinated immune response to stimuli.

The immune cells, such as monocytes and macrophages, that have been implicated in trained immunity, which can last up to a year, are short-lived. A study by Kaufmann et al. showed in a mouse model that BCG can reprogramme hematopoietic stem cells in the bone marrow towards myelopoiesis and subsequently generate a protective immune response upon *M.tb* infection [Figure 8] (Kaufmann et al., 2018). Mitroulis et al. showed that β-glucan administered to mice also induced an expansion of hematopoietic stem cells and myelopoiesis in the bone marrow which was associated with an increase in soluble mediators such as IL-1β (a key trained immunity cytokine) and elicited improved responses against subsequent challenge with systemic inflammation-inducing lipopolysaccharide (Mitroulis et al., 2018). It was also shown in healthy adults that BCG vaccination induced a myeloid-associated transcriptomic signature in the hematopoietic stem and progenitor cells of the bone marrow. In addition, epigenetic changes were also observed in monocytes from peripheral blood (Cirovic et al., 2020). These studies provide a plausible explanation for the long-lived trained immunity effects that have been observed in short-lived innate cells. However, this mechanism might not work as effectively in infants and could account for trained

immunity effects not being as pronounced in infants compared to adults (discussed in detail below). Interestingly, a recent study also identified bacterial species of the gut microbiome which was associated with trained immunity in BCG-vaccinated adults (Strazar et al., 2021). Therefore the composition of the gut microbiome (influenced by diet, location etc.) could also influence training of the innate immune response.

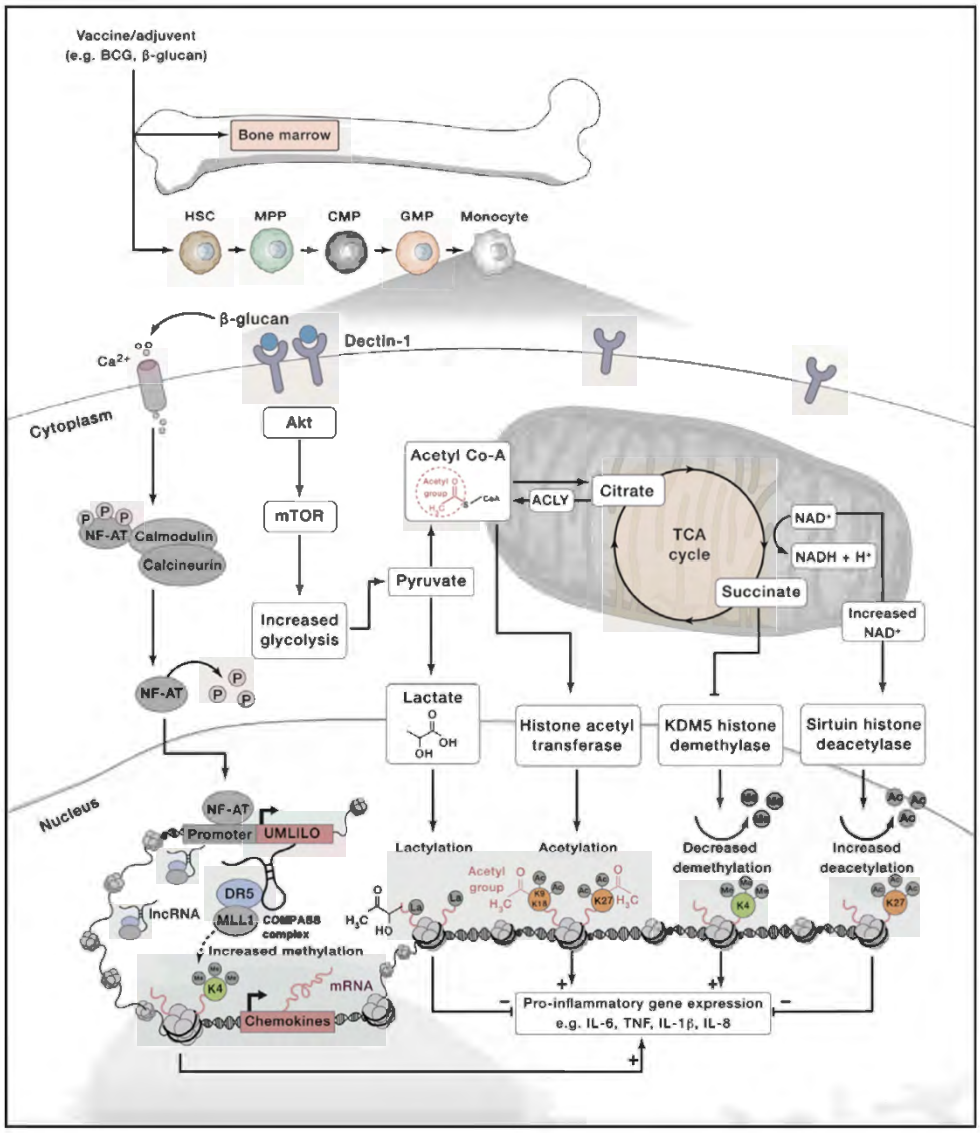


Figure 8. An illustration of the molecular mechanism underpinning trained immunity. Adapted from Fanucchi et al.(Fanucchi et al., 2021).

1.5.4.2. BCG-induced trained immunity in infants

Much of the studies investigating BCG-induced trained immunity have been conducted in adults from non-endemic TB settings (Kleinnijenhuis et al., 2012, Kleinnijenhuis et al., 2014b, Kleinnijenhuis et al., 2014a, Moorlag et al., 2020b). In a study in United Kingdom (UK) infants, no differences were observed in the activation phenotype of monocytes in response to non-*M.tb* related organisms between vaccinated and unvaccinated infants. Interestingly, higher expression of CD69 on NK cells was observed in response to Pam3Cys stimulation in infants who received BCG vaccination compared to those who did not receive BCG at birth. This result indicates that BCG-induced training of NK cells in infants can occur. However, increased levels of the secreted cytokines IL-6, EGF and PDGF-AB/BB from whole blood stimulated with non-*M.tb* related stimuli (Pam3Cys, *C.albicans* and *S.aureus*) were reported, differing from the cytokine pattern in adults (Smith et al., 2017). A study in LBW infants from Guinea-Bissau showed higher levels of IFNγ, TNF, IL-1β and IL-6 in response to Pam3Cys in infants vaccinated with BCG compared to those who were not, which is in line with previous studies in adults (Jensen et al., 2015). On the other hand, a study in Australian infants did not observe any differences in the commonly identified trained immunity cytokines in response to *M.tb*-unrelated stimuli in the two groups of infants based on BCG status (Freyne et al., 2020). As previously mentioned, a study in Ugandan infants showed a reduction in infectious diseases other than TB in infants who were BCG-vaccinated compared to those who were not. In this study there were no differences in cytokine levels to non-mycobacterial stimuli. The authors also measured the permissive H3K4me3 and the repressive histone 3 lysine 9 trimethylation (H3K9me3) histone marks. Contrary to previous studies, they showed no difference in the occupancy of these marks at the *il6*, *ilβ* and *tnf* genes of total PBMCs between BCG vaccinated and unvaccinated infants. However, lower levels of H3K9me3 occupancy at *tnf* in the BCG vaccinated compared to the BCG unvaccinated infants was shown, which has been described before (Prentice et al., 2021). Overall, these studies show that BCG training effects might differ in infants compared to adults. Therefore, standardised studies in infants from various TB-endemic settings are required to better understand training of innate cells in this vulnerable population.

1.5.5. BCG and heterologous adaptive immunity

Heterologous adaptive T cell responses have also been implicated in the heterologous beneficial effects observed after BCG vaccination (Kleinnijenhuis et al., 2014a, Blakney et al., 2015, Butkeviciute et al., 2018). It is plausible that after the waning of trained immunity, that heterologous T cells may take over and lead to the long-term heterologous beneficial effects that have been observed (de Castro et al., 2015). As such, there have been a number of underlying immunological mechanisms proposed for this phenomenon. BCG, which can persist after administration, may cause sustained activation of the innate immune response which in turn may produce cytokines such as IL-β and IL-6 that act on T cells with diverse specificities by enhancing maturation and function. In a study in healthy adults, increased levels of secreted IL-17 and IL-22 were measured in response to *C. albicans* and *S .aureus* in individuals up to 1 year post BCG vaccination (Kleinnijenhuis et al., 2014a). This would presumably lead to relatively short-lived heterologous effects. In a study in SA HIV-exposed uninfected infants, it was reported higher frequencies of IL-2 producing $CD8^+$ T cells in response to *Bordetella pertussis* in BCG vaccinated compared to unvaccinated infants at 8 weeks of age (Blakney et al., 2015). It has also been shown that subsets of $CD8^+$ T cells were able to secrete Th1 cytokines in response to IL-12 and IL-18 (produced by innate cells upon infection) in the absence of a cognate antigen (Berg et al., 2003). It is therefore likely that BCG may modulate $CD8^+$ T cell responses as well. Another possible explanation is the expression of common epitopes by the primary and secondary invading pathogen, however, this is unlikely in the case of BCG-induced heterologous T cell immunity to non-related organisms such as *C. albicans* and *S. aureus.*

1.5.6. Factors which may influence the variable efficacy of BCG vaccination

Factors which may influence the efficacy of BCG vaccine include: (i) maternal sensitisation to mycobacteria; (ii) prior exposure to mycobacteria; (iii) route of administration; (iv) BCG strains; and (v) host genetic factors.

i. Maternal sensitisation to mycobacteria

It has been hypothesised that the maternal *M.tb* infection status may influence infant immune responses to BCG vaccination and contribute to the variable efficacy of BCG. Exposure to mycobacterial antigens in utero and/ or the passive transfer of mycobacteria-specific Abs to the foetus, that later block BCG from eliciting protective immune responses, may occur. However, recent studies indicate that maternal *M.tb* status has minimal effect on infant immune responses to BCG (Jones et al., 2015, Mawa et al., 2015, Lubyayi et al., 2020, Mawa et al., 2021). Interestingly, studies have reported that infants born to mothers that are BCG-vaccinated have reduced morbidity and mortality, which is attributed to heterologous beneficial effects (Kjaergaard et al., 2016, Stensballe et al., 2019, Berendsen et al., 2020, Berendsen et al., 2021). A recent study in Guinea-Bissau reported that LBW infants born to mothers with a BCG scar had 60% reduction in mortality in the first 6-weeks of life compared to those born to mothers without a BCG scar. In addition, infants born to fathers with a BCG scar had 49% reduction in mortality compared to those of fathers with no BCG scar. However, these effects waned in the period of 6 weeks to 1 year of age (Berendsen et al., 2021). A recent study showed that infecting mice with *C.albicans* or zymosan (a PAMP) induced trained immunity effects that protected their offspring against heterologous infections (Katzmarski et al., 2021). However, another study in mice did not observe any differences in susceptibility to related and non-related infections in the offspring of mice that were BCG-vaccinated, infected with *C.albicans*, or β-glucan (Kaufmann et al., 2022).

ii. Prior exposure to mycobacteria

A systematic review reported that the efficacy of BCG was reduced in school age children previously sensitised to mycobacteria, confirmed by skin test positivity to purified protein derivative (PPD) (Mangtani et al., 2014). It was also reported that the efficacy of BCG was reduced in studies carried out closer to the equator (Mangtani et al., 2014). These results indicate that exposure to NTMs, which are particularly abundant in the tropics, might interfere with the protective effect of BCG (Andersen and Doherty, 2005). It is has been hypothesised that prior exposure to NTM can affect the efficacy of BCG vaccination by providing a level of protection against TB that BCG

cannot improve upon and thereby masking BCG effects (Palmer and Long, 1966), and/ or by inducing pre-existing immunity to mycobacterial antigens which could block the replication of live BCG and the induction of a protective immune response (Brandt et al., 2002). In TB endemic settings, however, infants are vaccinated shortly after birth and therefore are most likely immunologically naive when BCG is given. This is supported by the fact that BCG works relatively well in infants (Mangtani et al., 2014, Roy et al., 2014). In adults, the efficacy of BCG varies from 0-80%. This could be associated with the waning of protection offered by BCG, which has been shown to last 10-15 years (Abubakar et al., 2013), although other studies have shown longer protection (Aronson et al., 2004, Nguipdop-Djomo et al., 2016). In adults, infection of NTMs and/ or *M.tb* infection could be at play. This has implications for TB vaccine candidates: 1) vaccines designed to replace BCG will have to work as well as BCG against severe forms of TB and overcome any exposure to NTMs after birth in infants; 2) vaccines designed to be administered after BCG (and potentially given in the adolescent or adult population) should not be affected by previous exposure to mycobacteria (be it BCG, *M.tb* or NTMs) (Andersen and Doherty, 2005).

iii. Vaccine route of administration

As stated above, BCG is mostly commonly administered intradermally, which is the only vaccine route recommended by the WHO (WHO, 2018). BCG has also been given orally and percutaneously in the past. Previous studies have compared immune responses elicited when BCG is administered intradermally or percutaneously. However, different studies used different BCG strains and in the studies that used the same BCG strain, results were contradictory (Kemp et al., 1996, Davids et al., 2006, Hawkridge et al., 2008). Sharpe et al. reported that BCG delivered intravenously to Rhesus macaques was associated with reduced lung pathology following a high dose aerosol *M.tb* challenge compared to i.d. BCG or i.d. boosted with intratracheal BCG (Sharpe et al., 2016). Another study in Rhesus macaques showed that BCG i.v. provided 90 percent (9/10) protection from subsequent *M.tb* challenge compared to low dose i.d., high dose i.d., aerosol and the combination of aerosol and i.d. (Darrah et al., 2020). Unfortunately, correlates of immune protection could not be determined from this study because virtually all the animals in the i.v. group were protected. Pulmonary installation of BCG followed by a repeated limiting-dose *M.tb* challenge

was also associated with reduced lung pathology and improved protection compared to i.d. BCG in NHPs (Dijkman et al., 2019). In this study, enhanced Th1/Th17 responses, IgA Abs and IL-10 secretion in the lung were associated with protection from *M.tb* infection and TB disease (Dijkman et al., 2019). Recently, a study reporting on a low dose aerosol infection model in NHPs showed comparable protection between aerosol BCG and i.d. BCG vaccination, although pathology in the extra-pulmonary tissues was significantly reduced in NHPs that received aerosol BCG compared to i.d. BCG (White et al., 2020). Further studies are required to determine whether changing vaccination route in humans could improve upon BCG efficacy.

iv. BCG strains

The original BCG strain was first developed in 1921 by serial passage of *Mycobacterium bovis*. BCG was distributed all over the world, however, strains were passaged and maintained using different protocols, leading to daughter strains that differ genetically (Zhang et al., 2013). It is not known which BCG strain in use today is more efficacious. A systematic review showed no evidence that differences in protection against TB were related to the strain of BCG given (Mangtani et al., 2014). A study in guinea pigs, which compared the efficacy of 6 widely used BCG strains, reported no differences between strains upon subsequent *M.tb* challenge (Horwitz et al., 2009). Interestingly, the presence of a BCG scar in infants has been associated with improved survival (Shann, 2015, Benn et al., 2020). A study in Guinea-Bissau in normal birth weight infants showed higher scarring in infants who received BCG-Denmark compared to BCG-Russia, however, there were no differences in consultation rates (Frankel et al., 2016). BCG-Denmark was used to vaccinate infants in the studies showing BCG-mediated protection against all-cause mortality in LBW infants in Guinea-Bissau (discussed above) (Aaby et al., 2011, Biering-Sorensen et al., 2012, Biering-Sorensen et al., 2017). Conversely, in a recent study in India there was no reduction in all-cause mortality in LBW infants vaccinated with BCG-Russia (Jayaraman et al., 2019). It is possible that the heterologous beneficial effects induced by BCG may differ by strain and this requires further investigation.

v. Host genetic factors

Previous studies of mono- and dizygotic twins and individuals with Mendelian susceptibility to mycobacterial disease (MSDM) (affecting IFN-mediated responses) have shown that host genetics is a contributing factor to TB susceptibility (Kallmann and Reisner, 1943, van der Eijk et al., 2007, Cottle, 2011). Heritable susceptibility to TB disease, however, only partially accounts for the variability observed in populations; single nucleotide polymorphisms (SNPs) have also been identified that influence TB susceptibility and immune responses to *M.tb* (Messina et al., 2020). Interestingly, a study using a collaborative cross mouse model showed that *M.tb* susceptibility and BCG efficacy in mice were independently controlled by host genotype (Smith et al., 2016a). IFNγ responses following BCG vaccination are known to be variable (Finan et al., 2008) and it is possible that host genetic factors may contribute to this phenomenon (Randhawa et al., 2011). BCG is a live stimulant that contains ligands for multiple PRRs, and it is possible that the triggering of multiple PRRs and the strength of downstream signalling leads to the induction of trained immunity (Ramos et al., 2020). SNPs in genes important for BCG-mediated trained immunity may dampen heterologous effects (Arts et al., 2016, Arts et al., 2018) and should be further investigated.

1.6. Immune responses in infants differ to adults

Newborns and infants have distinct immune responses that markedly differ from those of adults, including lower proinflammatory innate responses, antimicrobial proteins, Th1-inducing cytokines and higher IL-10 production (Kollmann et al., 2009, Strunk et al., 2009, Corbett et al., 2010). The infant immune response is also known to be Th17 and Th2-biased (Kollmann et al., 2009, Barrios et al., 1996, Debock and Flamand, 2014). These unique features are thought to be necessary to protect young infants against excessive inflammation when transitioning from the intrauterine to the non-sterile environment after birth. However, it appears that this can also lead to insufficient immunity against pathogenic challenge (Levy, 2007). Thus, infants are highly susceptible to intracellular pathogens, including *M.tb,* and have a high risk of developing TB disease (Vanden Driessche et al., 2013). In a study in SA infants, it

was shown that proinflammatory cytokine responses by monocytes in response to mycobacterial stimulation increased with age (Shey et al., 2014). The unique features of the infant immune system could also have implications for infant responses to immune therapeutics and vaccinations.

1.7. Overall aim

The overall aim of this thesis was to investigate immune responses beyond conventional T cells in the context of BCG vaccination and TB disease. A better understanding of the immune responses elicited by BCG vaccination, a whole cell vaccine, in infants is required. Despite the fact that infants are very susceptible to TB, adults are the main drivers of *M.tb* transmission. A better understanding of the components of the immune response which contribute to or are affected by inflammation, lung pathology and resulting infectiousness of TB patients is also required. Thus, we also sought to identify host immunological determinants of *M.tb* infectiousness in adults.

There were two main objectives:

1. To determine whether neonatal BCG vaccination modulates mycobacteria-reactive innate cell (myeloid and NK cells), B cell and unconventional T cell ($\gamma\delta$ T and MAIT cell) responses.

   We hypothesised that functional responses to mycobacterial stimulation would be higher in BCG vaccinated compared to unvaccinated infants in the immune subsets that we investigated. We also hypothesised that there would be alterations in the phenotypic (memory and/ differentiation) profile of key immune subsets.

2. To identify immune responses that are associated with inflammation, TB disease severity and aerosolization of *M.tb* in adults with active TB disease.

We hypothesised that TB patients with excessive inflammation and pathology would expel higher levels of M.tb-containing aerosols (a surrogate for infectiousness) than TB patients with minimal inflammation and pathology. We also hypothesised that immune subsets would be altered in response to inflammatory processes and that levels of immune cell subsets in the blood could serve as an indicator of infectiousness of TB patients.

Chapter 2: Materials and methods

2.1. Recruitment of study participants

2.1.1. Infant participants

Study participants were recruited from Worcester in the Western Cape, SA. Blood was collected from infants in whom BCG vaccination was either administered at birth, as is routine, or delayed to after collection of blood samples at 6 or 10 weeks of age in different groups of participants. Analysis of samples collected prior to delayed BCG administration allowed a cross-sectional comparison between age-matched BCG-vaccinated and unvaccinated infants (**Figure 9**). In the birth-vaccination cohort, mothers were approached and invited to participate and provide consent at public and private vaccination clinics. In the delayed BCG-vaccination cohort, mothers were contacted and consented at hospitals prior to giving birth. Infants received i.d. BCG Danish strain 1331 from the Statens Serum Institut (SSI) at the standard dose of 1-4 x 10^5 CFUs. Blood was collected from infants in the birth or delayed BCG cohorts at 5 or 9 weeks of age. Blood samples were collected from infants vaccinated at birth for the period of September 2011 to August 2012. Blood samples were collected from infants in whom BCG vaccination was delayed from February 2012 until March 2013. The demographic characteristics of the infant cohorts are summarised in **Table 1**. The University of Cape Town (UCT) research ethics committee approved protocols for infants vaccinated at birth (REF: 126/2006) and infants who received delayed BCG vaccination (REF: 177/2011) studies.

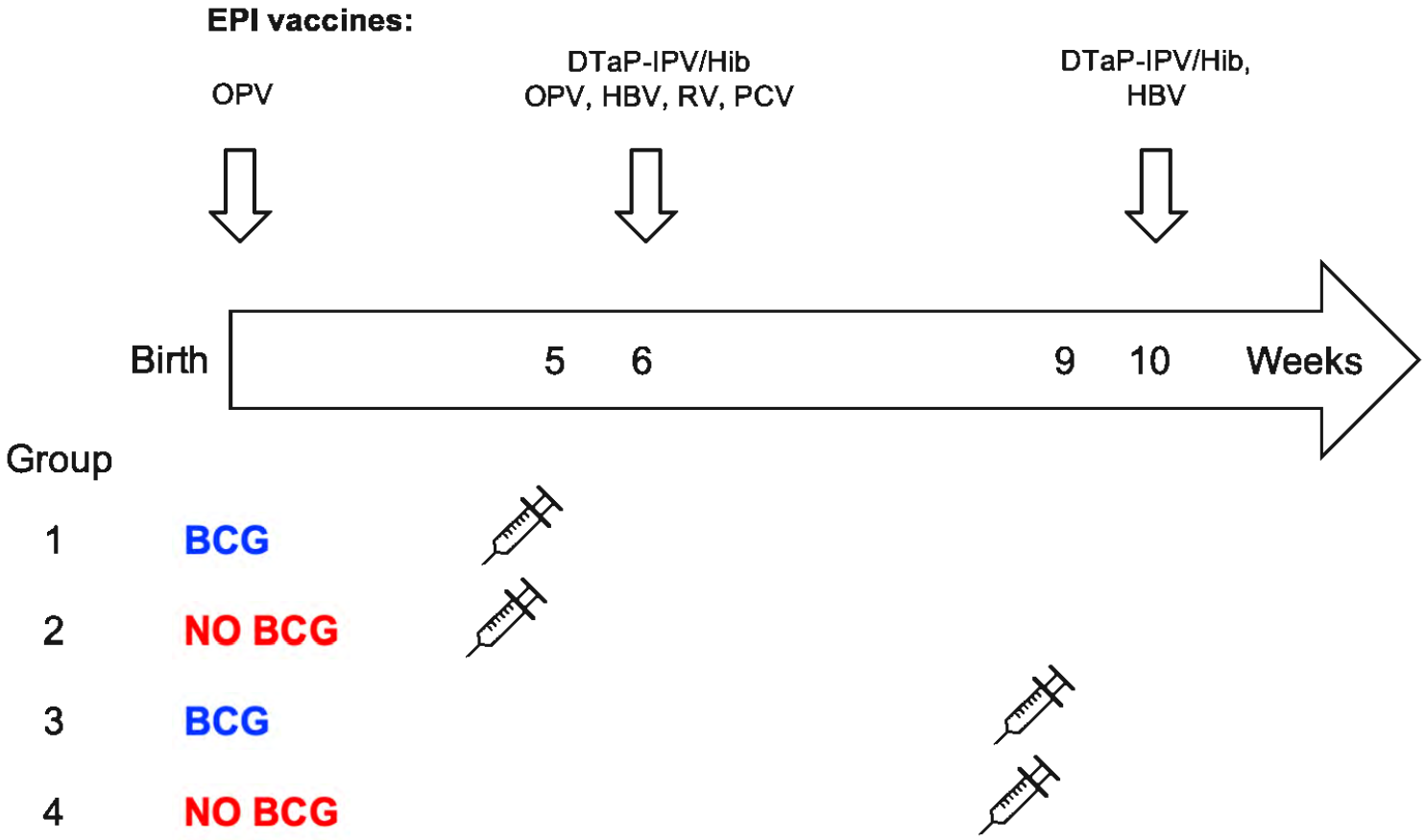


Figure 9. Delayed BCG Infant study design. Schematic representation of administered vaccines and blood draws in four cohorts of infants. Group 1 and 3 received BCG vaccination at birth. Group 2 and 4 received BCG vaccination at 6 weeks and 10 weeks of age respectively. Syringes indicate when blood draws were taken. The arrows indicate when the respective vaccines were administered. BCG, Bacille Calmette-Guérin vaccine; OPV, oral polio vaccine; DTaP-IPV//Hib, diphtheria, tetanus, acellular pertussis, inactivated polio vaccine, haemophilus influenzae type b; PCV, pneumococcal conjugate vaccine; RV, rotavirus vaccine; HBV, hepatitis B virus.

Table 1. Demographic characteristics of infant cohorts.

Variable	5-week-old (Group1)	5-week-old (Group 2)	9-week-old (Group 3)	9-week-old (Group 4)
BCG vaccination	Yes	No	Yes	No
Participants, n	26	30	50	24
Age, median (week, range)	5 (4-8)	5 (4-5)	9 (8-11)	9 (8-11)
Female, n (%)	10 (38)	15 (50)	25 (50)	11 (46)
Ethnicity, n (%)				
Black African	4 (15)	5 (17)	13 (26)	4 (17)
Mixed ancestry	22 (85)	25 (83)	37 (74)	20 (83)

2.1.2. Exclusion criteria (birth-vaccination cohort)

1. Infants who had not received BCG within 48 hours of birth, as is routine.
2. Infants who were enrolled in another experimental protocol.
3. Mothers who had known chronic infections or any acute infection during the last trimester of pregnancy.
4. Mothers who were HIV+ or who refused an HIV test.
5. Infants born through Caesarean section and those with delivery complications.
6. Infants born before 37 weeks of gestation and those with low birth weight (<2500g).
7. Infants who had congenital malformations and those with perinatal complications.
8. Infants who had a history of TB, or close contact with someone with TB disease.

9. Infants who had received isoniazid (INH) therapy during the first 5 or 9 weeks of life.
10. Infants with any chronic disease in the first 5 or 9 weeks of life, or any acute disease within 2 weeks of blood collection.
11. Infants who were on chronic or immune-modifying drugs.

2.1.3. Exclusion criteria (delayed-BCG cohort)

1. Infants who were enrolled in another experimental protocol.
2. Mothers who had significant pregnancy complications.
3. Mothers who had a high chance of relocating.
4. Mothers who were HIV+ or who refused an HIV test.
5. Mothers who had known chronic infections or any acute infection during the last trimester of pregnancy.
6. Mothers who had TB disease or contact with someone with TB disease.
7. Infants born through Caesarean section and those with delivery complications.
8. Infants born before 37 weeks of gestation and those with low birth weight (<2500g).
9. Infants who had congenital malformations and those with perinatal complications.
10. Infants who had a history of TB, or close contact with someone with TB disease.
11. Infants who had received INH therapy during the first 5 or 9 weeks of life.
12. Infants with any chronic disease in the first 5 or 9 weeks of life, or any acute disease within 2 weeks of blood collection.

2.2. Whole Blood stimulation experiments

2.2.1. Antigens

BCG Vaccine SSI (Biovac; Cape Town, SA) was prepared from the vaccine vial by reconstituting with Roswell Park Memorial Institute Medium (RPMI, Adcock Ingram), to a final concentration of 1.2 x 10^6 CFU per millilitre (mL) blood (Hanekom et al.,

2004). Phytohaemagglutinin (PHA, Sigma-Aldrich) was used as a positive control at 5 microgram per mL (μg/mL) and RPMI alone was used as a negative control.

2.2.2. WB-ICS assay

In both the delayed BCG and birth vaccination cohorts, 8 mL of blood was collected in sodium heparin. Blood was processed, within 75 minutes of blood collection, using a standardised 12-hour WB-ICS assay developed at the South African Tuberculosis Vaccine Initiative (SATVI) (Hanekom et al., 2004, Kagina et al., 2015). Blood was stimulated with BCG, PHA or left unstimulated. In all stimulation conditions, co-stimulants anti-CD28 and anti-CD49d (BD Biosciences; San Diego, USA) were added at 0.25 μg/mL to optimally activate conventional T cells. Blood was stimulated for 7 hours at 37°C, after which 100 microlitre (μL) of plasma was removed from samples and stored at -80°C. Plasma samples were later used to quantify soluble cytokines by multiplex bead array (discussed in detail below)**.** Brefeldin-A (Sigma-Aldrich) was added at a concentration of 10 μg/mL to blood for the remaining 5 hours of stimulation. At the end of the 12-hour stimulation, 2 mM of ethylenediaminetetraacetic acid (Sigma-Aldrich) was added to detach adherent cells, red blood were lysed using 1:10 FACS Lysing solution (BD Biosciences), washed, and white blood cells cryopreserved in cryosolution [50% RPMI (Adcock Ingram), 40% foetal calf serum (FCS, Adcock Ingram), 10% dimethyl sulfoxide (Merck)] in liquid nitrogen.

2.3. Flow cytometry experiments

2.3.1. Flow cytometry panel marker selection

Different flow cytometry panels were developed to address the key objectives (**Tables 2-4)**. Identical markers for NK cells (CD16 and CD56) and phenotypic MAIT cells (CD26 and CD161) were added to panel 1 and 2 to address the question of BCG vaccination effects in both 5-week-old and 9-week-old infants. NK differentiation markers (CD8, CD57, CD158b and CD161) were selected for panel 1 **(Table 2)** to explore the differences in NK cell differentiation between BCG vaccinated and unvaccinated infants. Cytokine receptors (CD212, CD218 and CD122) were selected

for panel 2 **(Table 3)** to investigate the responsiveness of immune subsets to cytokines produced by the innate and adaptive arms of the immune response. Myeloid (CD33), B cell (CD20, IgD, CD27) and $\gamma\delta$ T (TCR-$\gamma\delta$) cell markers were selected for panel 3 **(Table 4)** to explore immune responses to BCG beyond MAIT, NK, and conventional T cells. Conventional T cell markers (CD3, CD4 and CD8), however, were also included in the flow cytometry panels.

In addition, cytokine and cytotoxic markers were selected to investigate whether BCG vaccination modulates the effector functions of certain immune subsets (mentioned above) in infants. As discussed in chapter 1, TNF **(Table 2 and Table 4)** is a key proinflammatory cytokine primarily produced by macrophages but is also expressed by other myeloid cells and lymphocytes (Wajant et al., 2003). The importance of CD4 T cells producing IFNγ in the immune response against *M.tb* **(Tables 2-4)**, is well established (Flynn and Chan, 2001). Other cell types, which include non-CD4 T cell subsets and NK cells, also produce IFNγ when stimulated with mycobacterial antigens (Esin et al., 2004, Le Bourhis et al., 2010, Hoft et al., 1998). IL-1β **(Table 4)**, like TNF, is primarily produced by monocytes and macrophages but is also produced by B cells. (Cooper et al., 2011, du Plessis et al., 2016b). IL-6 **(Table 4)** is produced by many cell types including innate cells and lymphocytes (Van Snick, 1990, VanHeyningen et al., 1997). Perforin **(Table 2 and Table 3)** is released by NK and cytotoxic T cells, such as MAIT and $\gamma\delta$ T cells (Thiery et al., 2011). GrnB **(Table 4**) is also mainly produced by NK cells and cytotoxic T cells but other cell types such as B cells have also been shown to produce GrnB in other disease models (Chowdhury and Lieberman, 2008).

Due to limited sample availability, only a subset of 5-week-old infant samples were stained with panel 1, a subset of 9-week-old infant samples were stained with panel 2 and the entire 5-week-old and 9-week-old infant cohorts were stained with panel 3**.**

Fluorochrome conjugates were then allocated to markers based on a set of criteria: the instrument configuration, marker expression level, fluorochrome brightness and spectral overlap. Markers with low expression were preferably allocated to fluorochromes that were bright while markers that were expressed in high abundance were allocated to fluorochromes that were less bright. Antibody-fluorochrome

combinations that have been tested before in our lab as well as the commercial availability of Abs was also taken into account during panel development. Some antibody clones that were previously tested using PBMCs in our lab did not work optimally on the fixed, stimulated whole blood samples used in our experiments, the next available working antibody-clone-fluorochrome combination therefore had to be selected.

2.3.2. Antibodies

Abs used in all flow cytometry panels were titrated to determine their optimal concentration to achieve optimal resolution. Eight serial dilutions were carried out for each antibody in separate sample tubes. Antibody concentrations were chosen based on the lowest titre to achieve saturation and the highest signal-to-noise ratio. This was done to achieve the best separation of the positive and negative populations and to minimise background staining. Fluorescence minus one experiments were then carried out to identify any artefacts due to signal spread into the channel for which no antibody was added, in addition to autofluorescence, which may result in false positive signal. In a fluorescence minus one experiment, a sample tube is stained with all the Abs in a flow cytometry panel except one antibody; this procedure is repeated for each antibody. A sample tube was also stained with all Abs in the panel. All flow cytometry panels described in this thesis were optimised in this manner. The flow cytometry panels showing fluorochrome-antibody combinations are shown in **Tables 2-4**. Single-stained rat κ- chain was used for the IL-6-PE antibody while mouse κ- chain BD CompBeads were used to compensate all other parameters in order to accurately calculate the compensation matrix. To address key research questions, a gating strategy for each flow cytometry panel was developed in order to identify immune subsets and investigate their phenotype and function in the context of BCG vaccination **(Figures 10-12).**

Table 2. Markers and fluorochrome combinations for Panel 1. Optimal antibody titers and manufacture details are provided.

Fluorochrome	Antigen	Clone	Manufacturer	Volume (μL/50μL)
FITC	CD45	2D1	BD	0.5
Brilliant Violet 510	CD16	3G8	BioLegend	0.5
Brilliant Violet 711	CD56	HCD56	BioLegend	0.6
APC-H7	CD3	SK7	BD	0.4
Brilliant Violet 650	CD8α	RPA-T8	BD	0.6
APC	CD26	BA5b	BioLegend	0.3
PeCy5	CD161	DX12	BD	2.5
Brilliant Violet 605	CD57	NK-1	BioLegend	1
PE	CD158b	CH-L	BD	0.5
AlexaFlour700	IFNγ	B27	BD	0.6
PeCy7	TNF	Mab11	eBioscience	1
Brilliant Violet 421	Perforin	B-D48	BioLegend	1

Figure 10 shows the gating strategy that was used to identify NK, phenotypic MAIT and conventional T cells in 5-week-old infants.

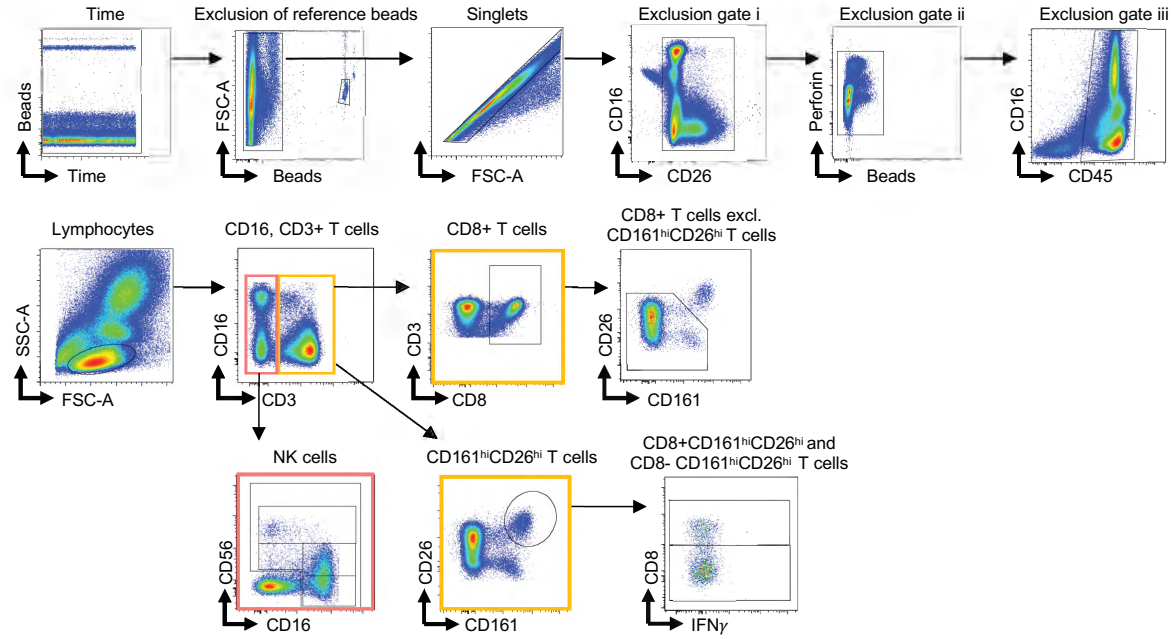


Figure 10. Gating strategy used for Panel 1. The first gate was set on time to ensure that uniform fluorescence signal was obtained during acquisition of the sample. Reference beads were added in order to quantify the number of cells per μL. The next gate excluded reference beads from cells. Singlets were then selected followed by three exclusion gates to remove artefacts due to antibody aggregates and autofluorescent cells. Using FSC-A and SSC-A lymphocytes were then selected. Next, T cells were selected by gating on CD3+ cells. CD16+CD3- cells were further gated to identify NK cells which were divided into subsets based on CD16 and CD56 expression. Two different immune subsets were further gated on from the CD3+ gate. Conventional CD8 T cells were gated using CD8 followed be the exclusion of CD161^{hi}CD26^{hi} T cells. CD161^{hi}CD26^{hi} T cells, also known as phenotypic MAIT cells, were then gated and further divided into CD8+ CD161^{hi}CD26^{hi} T cells and CD8- CD161^{hi}CD26^{hi} T cells.

Table 3. Markers and fluorochrome combinations for Panel 2. Optimal antibody titers and manufacture details are provided.

Fluorochrome	Antigen	Clone	Manufacturer	Volume (μL/50μL)
Brilliant Violet 510	CD16	3G8	BioLegend	0.5
Brilliant Violet 711	CD56	HCD56	BioLegend	0.6
APC-H7	CD3	SK7	BD	0.4
ECD	CD4	SFCI1274011	Beckman Coulter	0.5
Brilliant Violet 650	CD8α	RPA-T8	BD	0.6
APC	CD26	BA5b	BioLegend	0.3
PeCy5	CD161	DX12	BD	2.5
Brilliant Violet 605	TRAV1-2	3C10	BioLegend	1.5
BB515	CD212	2.4E6	BD	0.5
PeCy7	CD122	TU27	BioLegend	0.3
PE	CD218α	H44	BD	0.125
AlexaFlour700	IFNγ	B27	BD	0.5
Brilliant Violet 421	Perforin	B-D48	BioLegend	1

Figure 11 shows the gating strategy that was used to identify NK, phenotypic MAIT and conventional T cells in 9-week-old infants.

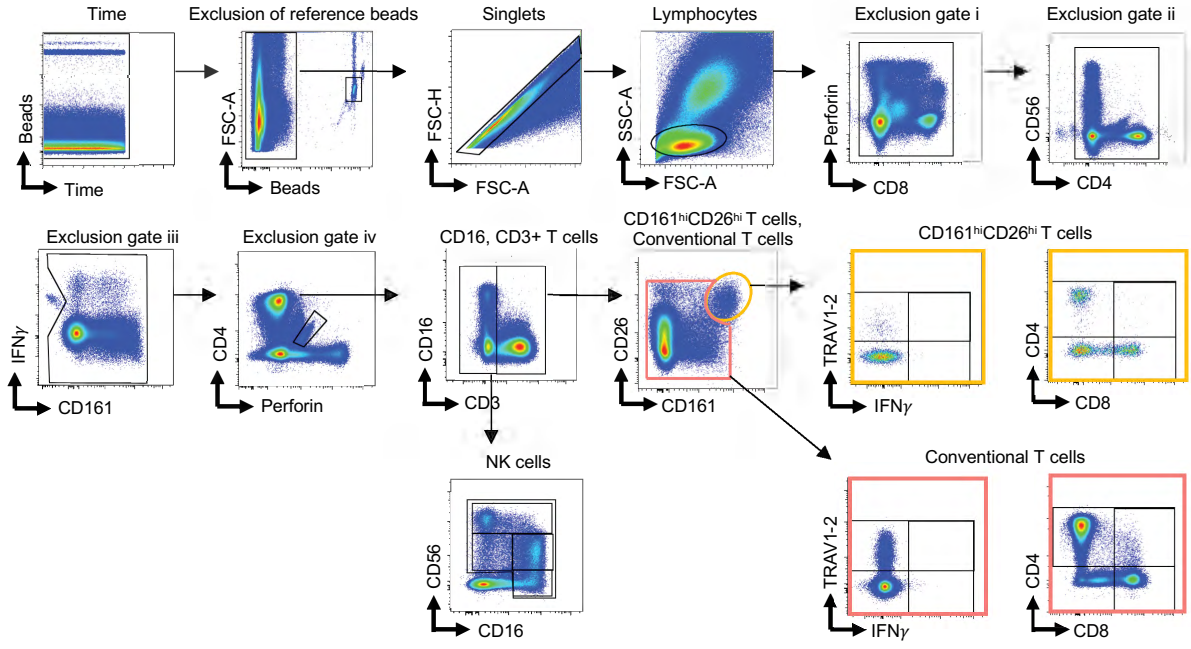


Figure 11. Gating strategy used for Panel 2. The first gate was set on time to ensure that uniform fluorescence signal was obtained during acquisition of the samples. Reference beads were added in order to quantify the number of cells per μL. The next gate excluded reference beads from cells. Singlets were then selected followed by a gate on lymphocytes. Next, four exclusion gates were used to remove artefacts due to antibody aggregates and autofluorescent cells. T cells were then selected by gating on CD3+ cells. CD16+CD3- cells were further gated to identify NK cells, which were divided into subsets based on CD16 and CD56 expression. Two immune subsets were further gated on from the CD3+ gate: conventional T cells and CD161^{hi}CD26^{hi} T cells, also known as phenotypic MAIT cells. Both of these subsets were further characterised based on TRAV1-2, IFNγ, CD4 and CD8 expression. Boolean analysis was carried out to obtain 16 combinations of these markers for each T cell subset.

Table 4. Markers and fluorochrome combinations for Panel 3. Optimal antibody titers and manufacture details are provided.

Fluorochrome	Antigen	Clone	Manufacturer	Volume (μL/50μL)
BV650	CD33	VM53	BD	0.16
PeCy5	CD16	3G8	BD	2
APC-H7	CD3	SK7	BD	0.4
Brilliant Violet 786	CD4	SK3	BD	1.5
Brilliant Violet 421	TCR-$\gamma\delta$	B1	BioLegend	1
Brilliant Violet 711	CD20	2H7	BioLegend	2
BB15	IgD	IA6-2	BD	0.6
PE-CF594	CD27	M-T271	BD	0.5
Brilliant Violet 605	HLADR	L243	BioLegend	1
Brilliant Violet 510	Granzyme B	GB11	BD	1
PE	IL-6	MQ2-13A5	BD	0.2
AlexaFlour647	IL-1β	JK1B-1	BioLegend	1
AlexaFlour700	IFNγ	B27	BD	0.5
PeCy7	TNF	Mab11	eBioscience	1

Figure 12 shows the gating strategy that was used to identify myeloid cells, B cells, $\gamma\delta$ T cells and conventional T cells in 5-week-old and 9-week-old infants.

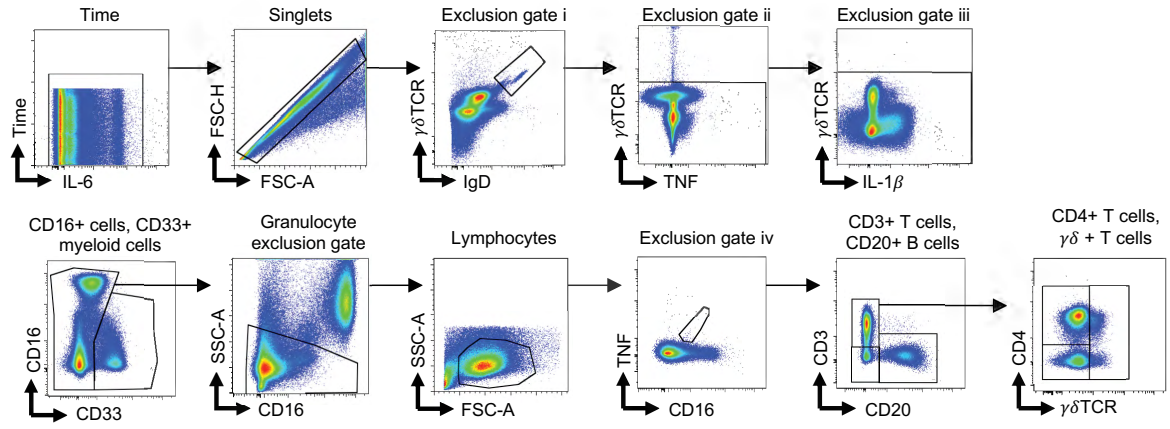


Figure 12. Gating strategy used for Panel 3. The first gate was set on time to ensure that uniform fluorescence signal was obtained during acquisition of the sample. Singlet cells were then selected followed by three exclusion gates to remove artefacts due to antibody aggregates and autofluorescent cells. CD33+ myeloid cells were gated in addition to CD16+ cells. In the next gate granulocytes were removed from the CD16+ cells. Lymphocytes were then selected based on FSC-A and SSC-A. An additional exclusion gate was added followed by the positive selection of CD20+ B cells and CD3+ T cells. CD4+ and $\gamma\delta$ T cells were then gated from CD3+ T cells.

2.3.3. Flow cytometry Panel 3 optimisation experiments

In the course of the optimisation experiments for panel 3, we discovered that certain immune markers and antibody clones did not work optimally, the reasons for this are discussed in detail below. Please note that we omitted discussing antibody markers that performed well during optimisation experiments.

i. B cell lineage staining

CD19 is expressed on B cells throughout B cell differentiation, although its expression is downregulated during the final stages of B cell differentiation to plasma cells, which are present in tissues and the bone marrow (Perez-Andres et al., 2010). Variable staining of CD19 was observed in infant samples during optimisation experiments for Panel 3. The frequencies of $CD19^+$ cells were low in some samples, while in other samples the frequencies of $CD19^+$ cells were in the expected range **(Figure 13A).** The staining was first optimised in adult samples, where the variability was seen to a lesser degree. Different vials of the same antibody, with the same lot numbers, were tested with the same result (not shown). To test whether fixation could influence the CD19 antibody binding, infant whole blood samples from the same donor were stained in parallel with the standard staining protocol (cell fixation followed by permeabilisation and staining) or an alternative staining protocol (staining of fresh cells followed by fixation). The results showed that fixation greatly affected the CD19 antibody staining of cells **(Figure 13B).** Because all whole blood samples in this study were stimulated and fixed prior to staining, an alternative B cell marker conjugated to the same fluorochrome was required. CD20 is similar to CD19 in that it is a B cell lineage marker expressed throughout B cell differentiation. However, CD20 is not expressed on plasmablasts and plasma cells whereas CD19 is expressed on plasmablasts but downregulated on plasma cells (Perez-Andres et al., 2010). We next compared CD20-BV711 and CD19-BV711 staining of B cells using our standard protocol. Higher frequencies of B cells were consistently observed using the CD20 antibody compared to the CD19 antibody (**Figure 13C**). We therefore used CD20 instead of CD19 in order to immunophenotype the majority of B cells present in peripheral blood.

ii. B cell memory

In order to investigate whether BCG vaccination changed the memory profile of B cells in infants, we included detection of IgD and CD27 in Panel 3 to delineate the following B cell memory subsets: $IgD^{+}CD27^{-}$ (naive B cells), $IgD^{+}CD27^{+}$ (non-switched memory B cells), $IgD^{-}CD27^{+}$ (classical memory B cells) and $IgD^{-}CD27^{-}$ (atypical memory B cells) (Lyashchenko et al., 2020). **Figure 14** shows that these memory subsets can be distinguished in adult samples but not in infant samples. This was the case for all infant samples regardless of whether B cells were defined by CD19 or CD20 staining. Other studies have shown that IgD and CD27 are able to identify different memory subsets in B cells in infants (Morbach et al., 2010, Duchamp et al., 2014), however we were unable to resolve these subsets sufficiently. It is likely that our WB-ICS assay does not allow optimal staining for these antigens. We therefore analysed median fluorescence intensity (MFI) values for the CD27 and IgD markers in infant samples, rather than gating on positive or negative populations.

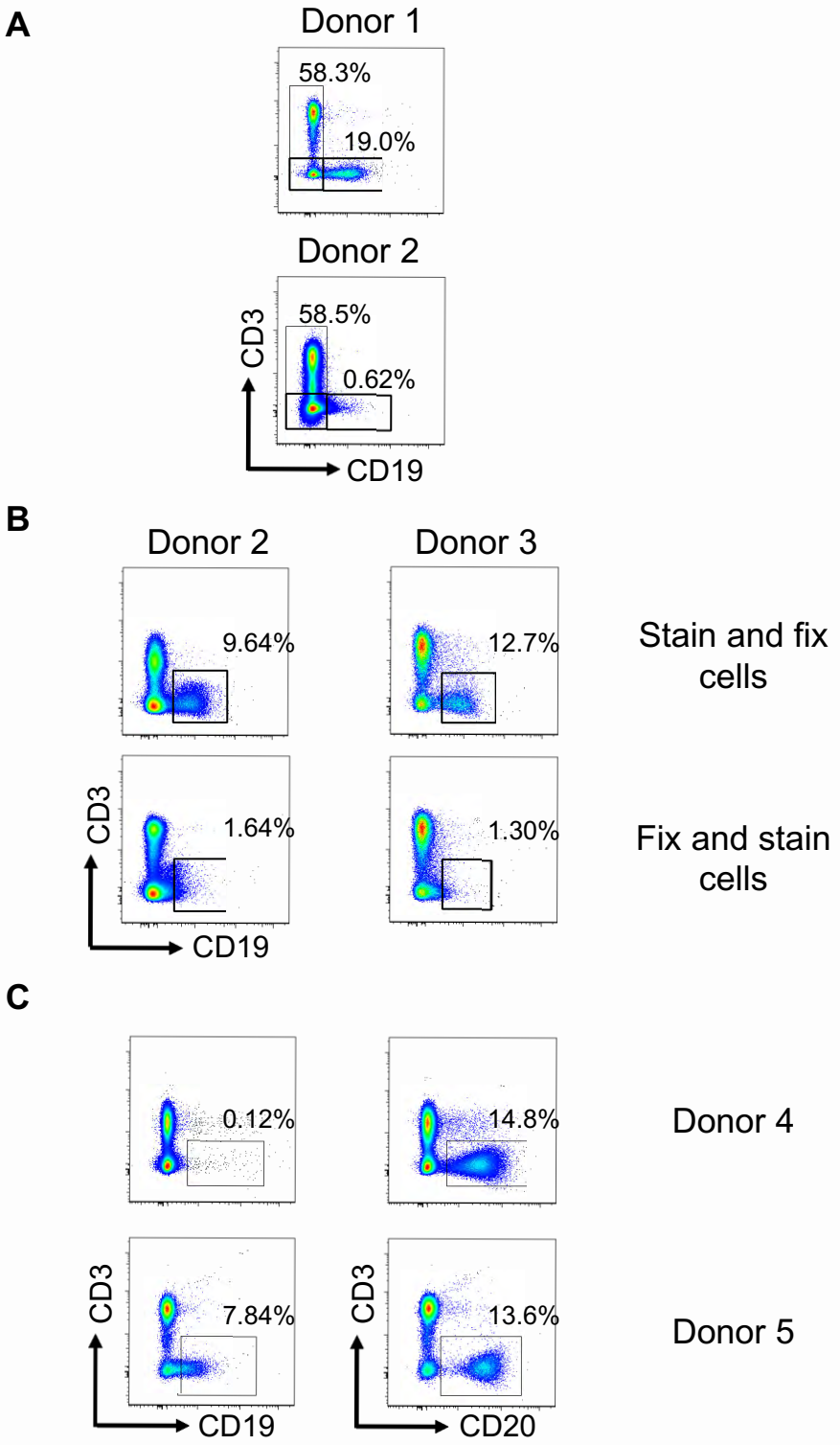


Figure 13. The effect of fixative on B cell marker staining in whole blood samples from infants. Representative flow cytometry plots showing **(A)** differing CD19+ staining in infant donors **(B)** comparison staining of CD19 staining under two different staining conditions in the same donor **(C)** comparison staining of CD19 and CD20 in the same donor.

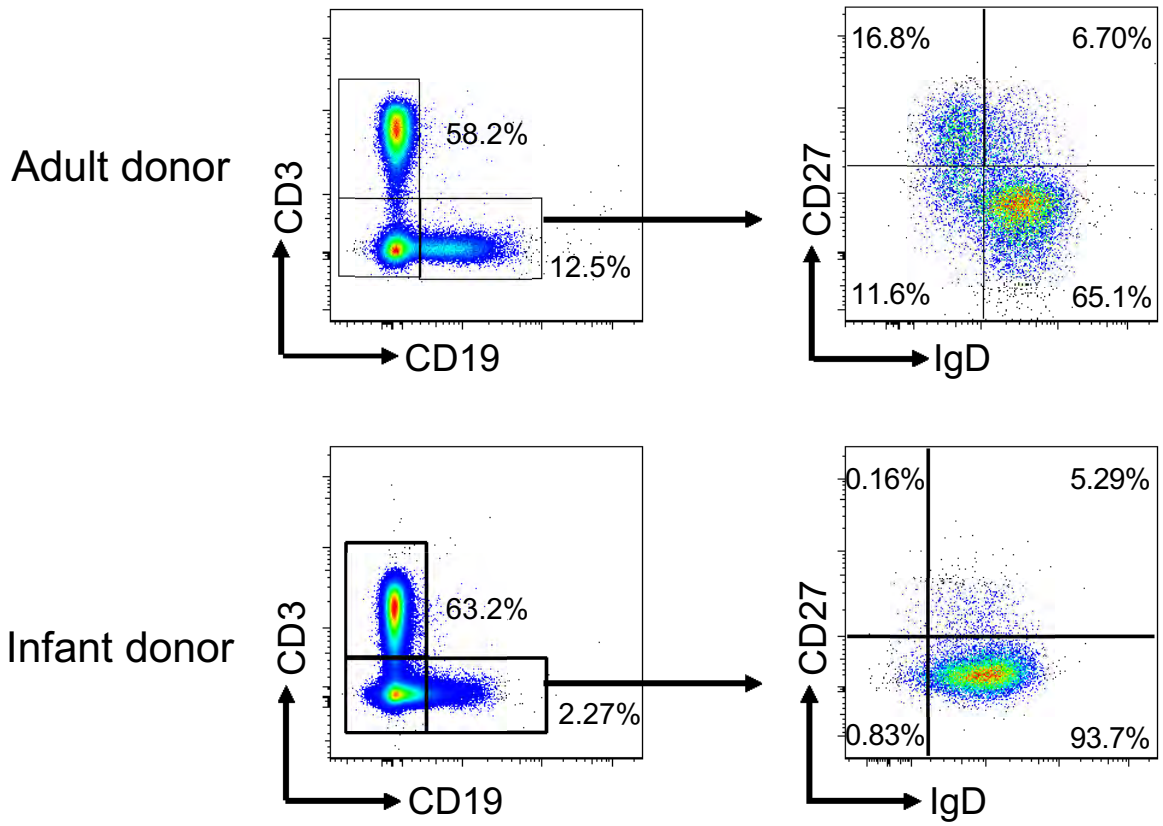


Figure 14. Staining of memory B cell markers differ in infants and adults. Representative flow cytometry plots comparing IgD and CD27 staining in adult and infant fixed whole blood samples.

iii. $\gamma\delta$ T cell staining

We compared staining of the $\gamma\delta$ TCR with two different clones: the B1 clone, which has been previously used in our laboratory but does not stain 100% of $\gamma\delta$ T cells (including some δ1 and δ2 subsets), and the 11F2 clone, which stains all $\gamma\delta$ T cell subsets (Wistuba-Hamprecht et al., 2014). **Figure 15A** shows that the 11F2 clone does not stain as well as the B1 clone in fixed whole blood samples from infants. We then tested whether fixation and the type of washing buffer might affect the antibody staining optimally. In **Figure 15B** we show that the 11F2 clone is indeed affected by fixation and washing buffer and its use is therefore not compatible with the sample type available for this study. We therefore used the $\gamma\delta$ B1 clone, despite the limitation that not all the $\gamma\delta$ T cells would be stained using this antibody. However, we were able to capture the $\gamma9^{+}\delta2^{+}$ subset which is of particular interest since this subset recognises mycobacterial antigens (Kabelitz et al., 1991).

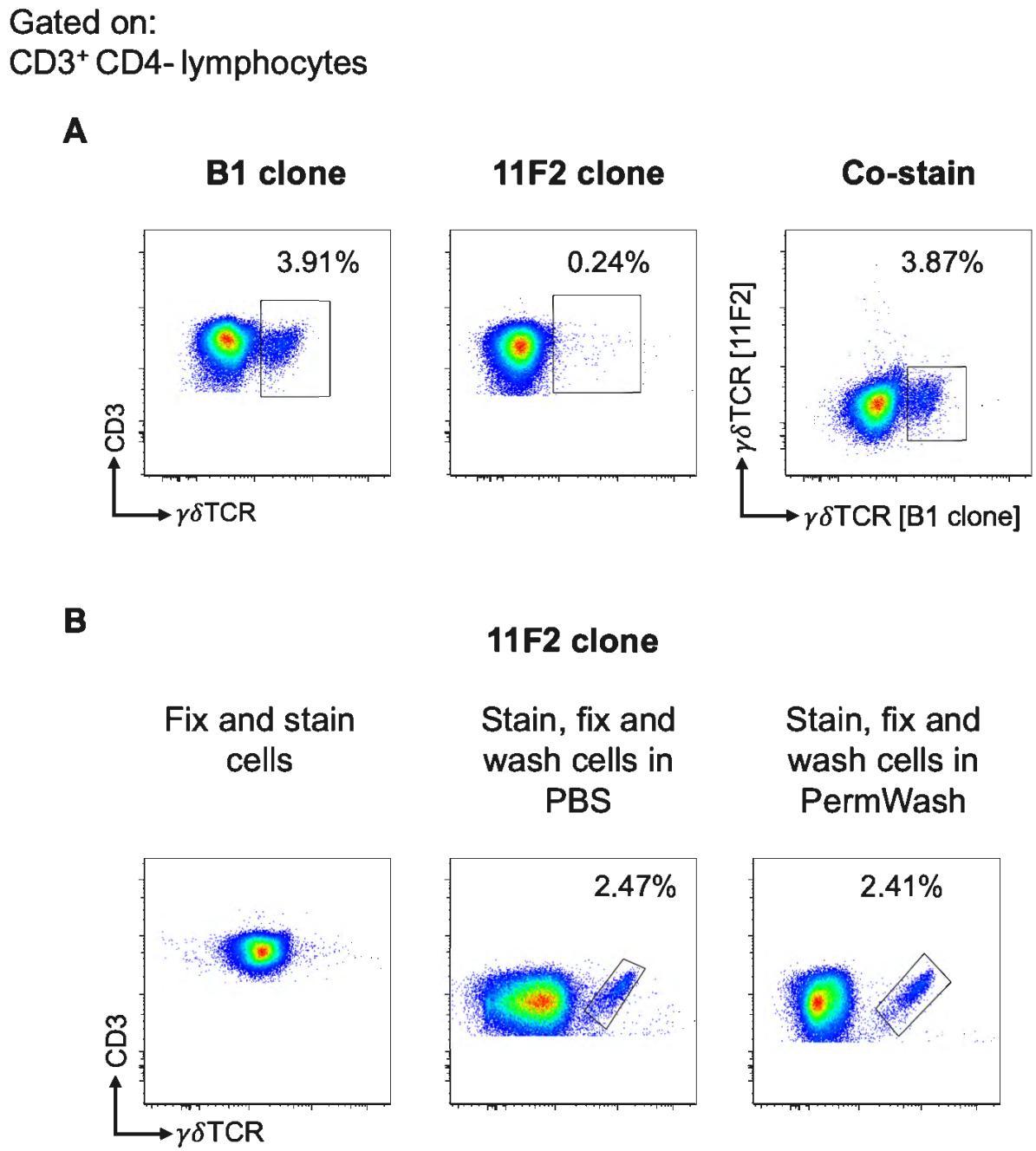


Figure 15. The effect of fixatives on $\gamma\delta$ T cell clones in whole blood samples from infants. Representative flow cytometry plots showing **(A)** staining of the B1 (left) and 11F2 (middle) $\gamma\delta$ T cell clone, and staining with both B1 and 11F2 in the same sample (right) in the same infant donor in fixed whole blood **(B)** staining of the 11F2 clone under different staining conditions (left) fixing cells first before staining, (middle) staining cells first, fixation followed by washing cells in PBS, (right) staining cells first, fixation followed by washing cells with PermWash.

2.4. Flow cytometer configuration

Samples were acquired on a BD LSRII flow cytometer which is equipped with 488 nm blue, 405 nm violet, 635 nm red and 532 green lasers. The mirror and filter configuration is shown in **Table 5.** Cytometer Setup and Tracking beads (BD Biosciences) were run daily to ensure that target MFI and signal coefficient of variation (CV)s were maintained (Perfetto et al., 2012). Photomultiplier tube voltages were calibrated daily using SPHERO Rainbow Fluorescent Particles (Spherotech; Lake Forest, USA) to ensure that consistent MFIs were maintained on the flow cytometer.

Table 5. BD LSRII flow cytometer configuration**.**

Laser	Detector	Band pass (nm)	Long pass (nm)	Flourochrome
Blue (488nm)	A	710/50	685	PerCP-Cy5.5
	B	525/20	505	FITC
	C	488/10		SSC
Violet (405nm)	A	785/60	770	BV785/800
	B	750/40	730	BV750
	C	710/40	685	BV711
	D	655/40	630	BV650
	E	605/40	585	BV605
	F	572/36	550	BV570
	G	515/20	505	BV510
	H	450/50		BV421
Red (635nm)	A	780/60	740	APC-H7
	B	710/50	680	Alexa Fluor 700
	C			APC
Green (532nm)	A	780/40	740	PE-Cy7
	B	695/40	690	PE-Cy5.5
	C	660/20	635	PE-Cy5
	D	610/20	595	PE-Texas Red
	E	575/25		PE-Green

2.5. Multiplex bead array experiments

2.5.1. Quantification of soluble cytokines by multiplex bead array

Thawed plasma samples from whole blood incubated with BCG or medium for 7 hours were used to quantify secreted cytokines. Concentrations of IFNγ, TNF, IL-12p70, IL-18, IL-1β, IL-6, IL-2, GM-CSF, IL-10, IL-13, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-4, IL-5 and IL-9 were measured using the Th1/ Th2/ Th9/ Th17 Cytokine 18-Plex ProcartaPlex™ Panel (Thermo Fisher Scientific; Waltham, USA) on the Bio Plex™ platform (Bio Rad laboratories; Berkeley, USA). This was done in accordance with the manufacturer's instructions (Thermo Fisher Scientific). The standard curve for all analytes ranged from 1.78-82500 picogram per mL.

2.5.2. Optimisation of multiplex bead array experiments

Pilot experiments were carried out to determine the optimal dilution of plasma samples to ensure that all analytes measured were detected within the range of the standard curve. Plasma samples from 5 healthy infants were used for the optimisation experiments. Unstimulated and BCG-stimulated samples from each infant were diluted 3-fold in assay buffer, with 4 serial dilutions carried out. Each sample was run in duplicate. Standard curve values were considered outliers and excluded if the ((observed value/expected value) *100) was outside the range of 100 $\pm$ 30. Only analytes that had CV values below 30% across replicates were analysed and samples with more than 9 analytes with CV values above 30% were excluded.

2.5.3. Results from multiplex bead array optimisation experiments

Concentration in range values were used for all multiplex bead array analyses. Samples that were below the limit of detection were assigned a zero value for the optimisation experiments only. Undiluted BCG-stimulated samples were used to investigate the frequency at which each analyte was detected in the pilot infant samples **(Figure 16A)**. Eight analytes were detected in all participant samples (IFNγ, IL-2, IL-18, IL-1β, IL-6, TNF, IL-10 and IL-21), 5 analytes were detected in 4

participants (IL-17A, IL-22, IL-27, IL-9 and GM-CSF), 1 analyte (IL-23) was detected in 3 participants and 4 analytes (IL-5, IL-4, IL-13 and IL-12p70) were detected in zero participants **(Figure 16A)**.

In order to address key objectives, seven analytes were prioritised, and the dilution factor was chosen based on the concentration in range values of these analytes (**Figure 16B).** IL-6, IL-1β and TNF are key cytokines linked to trained immunity (Kleinnijenhuis et al., 2012). IL-12 and IL-18 are cytokines that play an important role in bystander activation of NK and phenotypic MAIT cells (Cooper, 2009, Romee et al., 2012, Suliman et al., 2019) and may also be expressed at higher levels due to trained immunity. IL-2 and IFNγ are Th1 cytokines that are predominantly expressed by immune cells after BCG vaccination (Soares et al., 2008). A 1/3 dilution was selected for all samples, as most samples had optimal concentration in range values at this dilution factor.

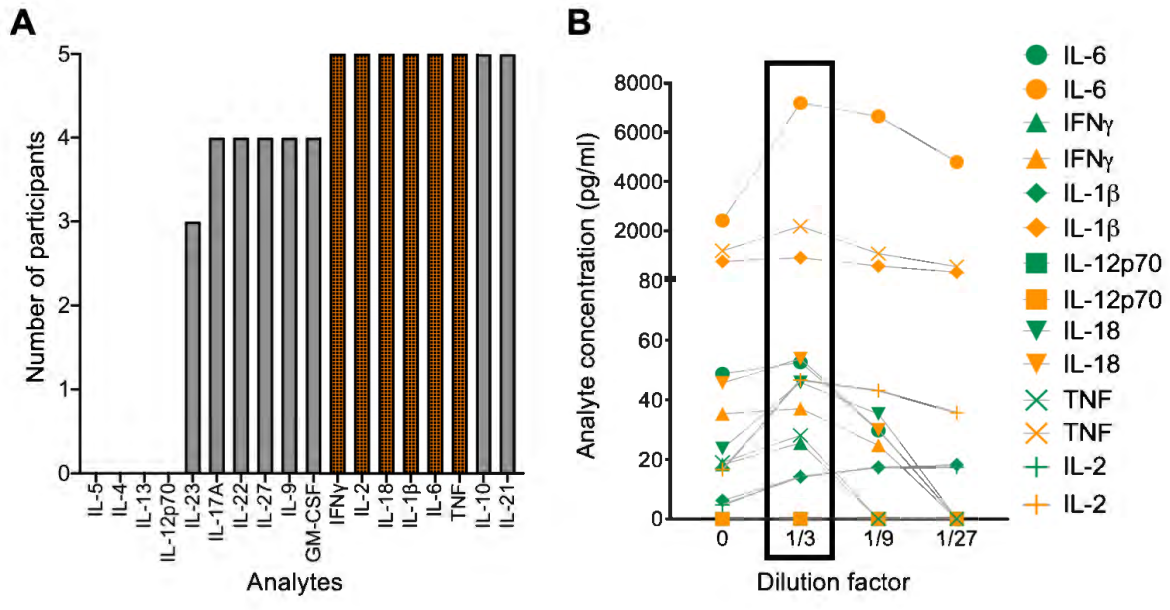


Figure 16. Detection of analytes in infant supernatants. (A) The number of participants in which analytes were detected (n=5). Samples highlighted in dark orange represent detected analytes of interest. **(B)** Concentration of six analytes of interest at different dilution factors. Green, unstimulated sample; orange, BCG-stimulated sample.

2.5.4. Multiplex bead array experiments on infant study samples

Based on the optimisation experiments, unstimulated and BCG-stimulated samples were diluted 1/3 in assay buffer. Each sample was run in duplicate. Analyte concentrations detected in unstimulated samples were subtracted from those measured after BCG stimulation (BCG-UNS). Values below the limit of detection and BCG-UNS values that were zero were assigned the value 0.89. This value was derived using the lowest standard value in the kit (1.78) divided by 2. This was done because the range of values for analytes amongst participants varied greatly and it was more appropriate to visualise the values on a logarithmic scale, where “zero” values cannot be plotted.

2.6. Data analysis

Flow cytometry files were analysed using FlowJo version 9.7-9.9.6 (Treestar; Ashland, OR). Background subtractions were performed in Pestle version 1.7- 2.0 and Boolean cytokine combinations were analysed in Spice version 5.3 – 6.0 (Roederer et al., 2011). Absolute cell numbers were calculated using the formula: [(cell event count/bead count) x #beads/ test/initial blood volume)] (Nemes et al., 2015). Acquisition and analysis for multiplex bead array experiments were performed using the Bio-Plex Manager Software. Statistical analyses were performed, and graphs were created using GraphPad Prism version 6-8 or R (http://www.r-project.org). Heatmaps were generated using the ComplexHeatmap package (Gu et al., 2016). The Mann-Whitney U test was applied to compare different groups. The Wilcoxon signed-rank test was used for comparisons between the same group. To measure the degree of association between two variables the Spearman rank correlation was applied. The p values were shown unadjusted; bolded values represent those comparisons that are significant after corrections for multiple comparisons. The p values were corrected for multiple comparisons using the false discovery rate (FDR) or the Bonferroni correction method where appropriate.

2.7. Contributions to this chapter

Multiplex bead array experiments were carried out by Melissa Murphy with the assistance of Candice I. Snyders, Ilana C. van Rensburg and Assoc. Prof Novel N. Chegou.

Flow cytometry experiments were carried out by Melissa Murphy.

All analyses were carried out by Melissa Murphy under the supervision of Dr. Sara Suliman, Assoc. Prof. Elisa Nemes and Prof. Thomas J. Scriba.

Chapter 3: BCG-induced T & B lymphocyte responses to mycobacteria in infants

3.1. Introduction

Although it is widely accepted that conventional T cells are critical in the defence against TB, it is likely that other immune subsets may play a greater role than previously thought. In recent years, interest in DURT cells has increased. The less polymorphic nature of antigen presentation and innate-like characteristics, such as immediate effector functions, make DURT cells an attractive target for new TB interventions (Joosten et al., 2019, Ruibal et al., 2021). There has also been a renewed interest in B cells and the humoral response against mycobacteria. In addition to antibody production, B cells are able to produce cytokines and are capable antigen presenting cells, which might be of significance and warrant further investigation (Rijnink et al., 2021). In this chapter we focused on $CD4^+$ T cells, B cells, MAIT cells and $\gamma\delta$ T cells.

Mouse models have provided the framework for our understanding of the initial conventional T cell response to *M.tb* infection. Studies in mice have shown that $CD4^+$ T cells are indispensable in the control of *M.tb* infection and the prevention of mortality (Flory et al., 1992, Orme and Collins, 1983, Caruso et al., 1999). An important function is the production of IFNγ by $CD4^+$ T cells, which is associated with the restriction of *M.tb* replication in infected macrophages in the lung (Flynn and Chan, 2001). Soares at al. measured the longitudinal $CD4^+$ T cell cytokine and cytotoxic response in infants following BCG vaccination up to 1 year of age. They showed that the BCG-specific $CD4^+$ T cell response peaked at 6-10 weeks of age, after which a gradual waning occurred. They also showed that the BCG-specific response was mainly comprised of IFNγ, TNF and/ or IL-2-expressing T cells (Soares et al., 2013). In addition to IFNγ production by T cells, it is thought that a IFNγ^+TNF$^+$IL-2$^+$ polyfunctional T cell response is required for a favourable outcome against several intra-cellular pathogens (Seder et al., 2008). Higher frequencies of single and dual cytokine-expressing $CD4^+$ T cells have been shown in active TB patients in comparison to healthy *M.tb* infected

individuals, who have higher frequencies of $CD4^+$ T cells co-expressing $IFN\gamma^+TNF^+IL\text{-}2^+$ (Day et al., 2011, Harari et al., 2011, Riou et al., 2014). In addition, in a study in BCG-vaccinated infants, polyfunctional T cells correlated with increased *in vitro* mycobacterial growth inhibition (Smith et al., 2016b). However, there are also contradicting studies reporting the opposite effect (Sutherland et al., 2009a, Caccamo et al., 2010, Mueller et al., 2008). As stated before, a study in SA infants showed that frequencies of BCG-specific Th1 and polyfunctional responses in 10-week-old infants who were vaccinated at birth did not differ between infants who progressed or did not progress to active TB disease. In a recent study, the administration of BCG intravenously to NHPs induced protection against subsequent *M.tb* challenge, which was associated with antigen-specific Th1/ Th17 cells in bronchoalveolar lavage (BAL) fluid. In the same study, transient increases of MAIT cells, $\gamma\delta$ T cells, IgG and IgA in BAL fluid was also reported (Darrah et al., 2020). Since intravenous BCG administration induced all these immune responses and virtually all animals were protected, it was not possible to determine which of these responses were essential for protection. However, these studies have paved the way for the assessment of other components of the immune response, which could complement conventional immune responses if modulated with new interventions such as new whole cell vaccines, adjuvants or host-directed therapies.

M.tb is a facultative intracellular pathogen and as such it has been thought that B cells and Abs, which are mostly effective against extracellular organisms and toxins, do not play a major role in the immune response against *M.tb*. Contradicting reports on whether B cells and Abs are important in the immune response against *M.tb* have provided little impetus for their investigation in the context of TB (Maglione et al., 2007, Turner et al., 2001, Vordermeier et al., 1996, Glatman-Freedman and Casadevall, 1998, Johnson et al., 1997). However, more recently, it has been shown that transfer of Abs from health care workers who were highly exposed to *M.tb* provided some protection against *M.tb* aerosol challenge in mice (Li et al., 2017). In South African BCG-vaccinated infants, higher levels of IgG Abs specific for the mycobacterial antigen Ag85A were associated with reduced risk of developing TB (Fletcher et al., 2016b). Also, NHP models in which BCG was administered mucosally or intravenously and induced protection, showed an increase in antibody levels compared to BCG administered intradermally (Darrah et al., 2020, Dijkman et al., 2019). It was also

reported that Abs from healthy *M.tb* infected individuals had distinctive Fc profiles and glycosylation patterns, including selective binding of FcγRIII, compared to active TB patients. In addition, these Abs were capable of driving enhanced macrophage killing of intracellular *M.tb* (Lu et al., 2016). A recent study also reported a transient induction of antibody-secreting plasmablasts and a durable memory B cell response following BCG vaccination in humans (Bitencourt et al., 2022).

Non-humoral B cell functions against *M.tb* include antigen presentation and cytokine production (Chan et al., 2014). B cells can produce cytokines in response to mycobacterial stimulation, including IL-1β, TNF, IL-17, IL-21 and IL-10 (du Plessis et al., 2016b, Joosten et al., 2016, Phuah et al., 2016), which could have a modulatory role on other effector cells. Studies in mice have indicated that B cells may also influence the production of IL-10 in the lungs during *M.tb* infection (Maglione et al., 2007, Junqueira-Kipnis et al., 2005). In *M.tb* infected NHPs, B cells were found in clusters in the lung granuloma, with some B cells expressing a proliferative and active phenotype. In addition, plasma cells in the granuloma produced *M.tb*-specific IgG (Phuah et al., 2012). These results indicate that B cells are actively involved in the immune response at the site of infection. Ectopic B cell follicle-like structures near granulomas in the lungs have also been reported in TB patients (Ulrichs et al., 2004); it is presumed that it is most likely a site of antigen presentation and B cell maturation (Maglione and Chan, 2009). $CD20^+$ B cell depletion in NHPs did not result in different disease severity and outcomes compared to controls with intact B cell compartment, however, in some granulomas in the lung there was a decrease in inflammation and increase in bacterial burden (Phuah et al., 2016). In addition, it was reported that B cell deficient mice had higher bacterial loads in the lung compared to controls (Maglione et al., 2007). In another study, however, a reduction in bacterial load was observed in BCG-vaccinated mice after subsequent *M.tb* challenge (Vordermeier et al., 1996). Other studies in mice have reported that B cells do not play a significant role during *M.tb* infection (Johnson et al., 1997, Turner et al., 2001, Bosio et al., 2000). In humans, the assessment of B cell frequencies in active TB compared to healthy controls have yielded conflicting results and in most of these studies, only total B cells were assessed (Joosten et al., 2016, Chowdhury et al., 2018, Hernandez et al., 2010, Wu et al., 2009). It has been shown that atypical and activated B cells are elevated, and naive B cells reduced, in LTBI and active TB patients compared to healthy controls

(Joosten et al., 2016). In another study, frequencies of memory B cells were higher at TB diagnosis compared to post treatment. The investigators also reported that marginal zone B cell frequencies could differentiate between start of treatment and end of treatment (du Plessis et al., 2016a). It is clear that B cells and Abs are diverse in phenotype and function. In-depth analyses are required in the context of *M.tb* studies and vaccine candidate assessments to establish the quality of the B cell and antibody response that should be targeted. In addition, studies undertaking a comprehensive characterisation of the infant B cell response to BCG vaccination are lacking. Therefore, in this chapter we sought to investigate whether BCG modulates the cytokine response and memory profile of B cells in infants. We were unable to investigate antibody responses in this study due to restraints in the availability of reagents and funding.

MAIT cells have been shown to be depleted in peripheral blood of active TB patients when compared with healthy controls (Sharma et al., 2015, Le Bourhis et al., 2010, Gold et al., 2010, Balfour et al., 2021, Jiang et al., 2014, Kwon et al., 2015) and it has been hypothesised that MAIT cells leave the periphery and traffic to the site of *M.tb* infection (Wong et al., 2019). This recent study showed a depletion of MAIT cells in peripheral blood but an enrichment in BAL fluid of active TB patients, compared to healthy controls. However, another recent study in a TB-endemic setting showed no differences in MAIT cell frequencies between TB patients, LTBI individuals and healthy controls (Suliman et al., 2020). It was reported that MR1 deficient mice had higher bacterial loads at earlier time points after aerosol BCG infection compared to wild-type (WT) mice (Chua et al., 2012). Other studies in mice showed that MAIT cells were able to inhibit BCG-infected macrophages and reduce the bacterial burden following *M.tb* infection (Chua et al., 2012, Sakala et al., 2015). In line with studies in mice, Green et al. observed an early and transient increase in MAIT cell activation in peripheral blood of NHPs following BCG vaccination, indicating a role for MAIT cells in the early stages of infection (Greene et al., 2017). Also, a transient increase in MAIT cell frequencies were observed in NHPs after the administration of intravenous BCG, which provided protection from *M.tb* infection (Darrah et al., 2020). However, recent studies in mice showed that vaccinating mice with 5-OP-RU (a MAIT cell activating metabolite) and a TLR agonist did not reduce bacterial growth after *M.tb* challenge (Sakai et al., 2021, Vorkas et al., 2020, Yu et al., 2020). Although MAIT cells have

been extensively studied in recent years and much has been uncovered, the role that MAIT cells play in the human protective immune response against *M.tb* infection and their utility as a vaccine or adjuvant target is still unknown. In this chapter we investigated the phenotype and function of phenotypic MAIT cells in infants in the context of BCG vaccination.

As discussed in chapter 1, $\gamma\delta$ T cells are activated by host and pathogen-derived phosphoantigens, which are present in BCG and *M.tb* (Vermijlen et al., 2018)**.** Indeed, BCG vaccination elicits $\gamma\delta$ T cell functional responses to mycobacterial antigens in both adults and infants (Suliman et al., 2016, Zufferey et al., 2013, Kagina et al., 2010). Vγ9Vδ2 is the predominant and *M.tb*-reactive $\gamma\delta$ subset in peripheral blood in human adults (Kabelitz et al., 1991). In 10-week-old infants, the Vγ9Vδ2 subset had higher cytotoxic and Th1 cytokine potential compared to conventional and other $\gamma\delta$ T cell subsets (Papadopoulou et al., 2020). When infected with BCG, mice that lack $\gamma\delta$ T had lower levels of IFNγ production compared to control mice (Dieli et al., 2003, Ladel et al., 1995). $\gamma\delta$ T cells are also able to kill *M.tb* directly by producing cytotoxic molecules (Dieli et al., 2001) and releasing granzyme A, which has been shown to induce monocytes to produce TNF and inhibit BCG growth (Spencer et al., 2013). The adoptive transfer of $\gamma\delta$ T cells into NHPs conferred protection against challenge with *M.tb* (Qaqish et al., 2017). In NHPs vaccinated with BCG or infected with *M.tb* a rapid expansion of $\gamma\delta$ T cells was reported (Shen, 2002, Lai et al., 2003, Shen et al., 2015). In addition, human adults vaccinated with BCG showed an expansion of $\gamma\delta$ T cells, compared to BCG-unvaccinated adults, in response to mycobacteria (Hoft et al., 1998), suggesting memory properties that could be exploited by vaccination. However, in a recent study in infants recruited from Worcester, Cape Town, it was shown that Vγ9Vδ2 T cells rapidly expands from birth to 10 weeks of age independently of BCG vaccination, as the frequencies of Vγ9Vδ2 T cells were the same between BCG-vaccinated and unvaccinated infants (Papadopoulou et al., 2020). Also, frequencies of BCG-reactive $\gamma\delta$ T cells in infants vaccinated with BCG at birth did not correlate with protection against TB (Kagina et al., 2010). As mentioned above, in a study in NHPs in which intravenously administered BCG provided protection upon challenge with *M.tb*, a transient increase of $\gamma\delta$ T cells was reported (Darrah et al., 2020), which is in line with other immunisation studies in NHPs showing an early increase in $\gamma\delta$ T

cells (Shen, 2002, Shen et al., 2015, Lai et al., 2003). Overall, these studies indicate that $\gamma\delta$ T cells may play a role in the early defence against *M.tb* infection and act as a bridge between innate and adaptive immunity. Studies exploring an array of cytokines expressed by $\gamma\delta$ T cells in response to whole cell mycobacteria in infants is lacking. Therefore, in this chapter, utilising our unique study design, we set out to investigate the $\gamma\delta$ T cytokine response elicited by BCG vaccination in infants.

3.2. Aims

In this chapter we sought to investigate whether BCG vaccination modifies infant $CD4^+$ T cell, $CD20^+$ B cell, MAIT cell and $\gamma\delta$ T cell responses to mycobacteria. We hypothesised that cytokine and cytotoxic molecule production in response to mycobacterial stimulation would be higher in BCG vaccinated compared to unvaccinated infants in all the immune subsets that we investigated. We also hypothesised that the memory profile in B cells would be altered in the BCG vaccinated compared to unvaccinated infants. The following aims were addressed:

1. To investigate infant $CD4^+$ and $\gamma\delta$ T cell effector functions induced by BCG vaccination in infants.
2. To determine whether BCG vaccination modulates infant $CD20^+$ B cell cytokine production and expression of B cell memory markers.
3. To investigate the phenotype of functional attributes of phenotypic MAIT cells in infants.

3.3. Materials and methods

3.3.1. Study participants

Infant samples used in this chapter were collected from the delayed-BCG and the birth-vaccination cohorts, as described in chapter 2.

3.3.2. WB-ICS assay

Heparinised whole blood for the standardised 12-hour WB-ICS assay was processed as described in chapter 2.

3.3.3. Flow cytometry

Cryopreserved whole blood samples were thawed, washed in PBS, permeabilised in Perm/Wash buffer (BD Biosciences) and stained with flow cytometry panels as described in **(Chapter 2, Tables 2-4)** for 45 minutes at 4℃. Data shown in this chapter are from infant samples stained with panel 1 **(Chapter 2, Table 2)**, panel 2 **(Chapter 2, Table 3)** and panel 3 **(Chapter 2, Table 4)** as described in **Table 6** below. All functional markers are reported after background subtraction in this chapter.

Table 6. The number of infant samples stained for each flow cytometry panel stratified according to infant cohort and age.

Panel no	Age (weeks)	Vaccination status	
		No BCG (n)	BCG (n)
1	5	15	14
2	9	10	28
3	5	30	26
	9	24	50

3.4. Results

3.4.1. BCG-specific CD4$^+$ T cells are induced after BCG vaccination

It has been shown longitudinally that BCG elicits a strong CD4 Th1 response in addition to cytotoxic CD4$^+$ T cells in infants (Soares et al., 2013). Thus, we measured frequencies of CD4$^+$ T cells expressing IFNγ, TNF and GrnB in response to BCG stimulation in 5-week-old and 9-week-old infants who did (BCG) or did not (no BCG) receive BCG vaccination at birth. Upon BCG-stimulation, IFNγ and TNF production by CD4$^+$ T cells were significantly higher in the BCG group compared to the no BCG group at both ages **(Figure 17A and 17B)**. However, frequencies of CD4$^+$ T cell producing GrnB were not different between the vaccination groups in 5-week-old and 9-week-old infants **(Figure 17C-17E).**

3.4.2. BCG-reactive IFNγ-expressing $\gamma\delta$ T cells are induced after BCG vaccination in 5-week-old infants only.

$\gamma\delta$ T cells are known to produce IFNγ and TNF when activated and are also highly cytotoxic (Boom, 1999, Bonneville et al., 2010). Therefore, we measured frequencies of $\gamma\delta$ T cells expressing IFNγ and TNF cytokines and GrnB in response to BCG stimulation in 5-week-old and 9-week-old in the BCG and no BCG group. Frequencies of IFNγ expressing $\gamma\delta$ T cells were higher in the BCG group compared to the no BCG group in 5-week-old infants only **(Figure 18A-18B)**. The frequencies of IFNγ-expressing $\gamma\delta$ T cells were not different between the two vaccination groups in 9-week-old infants **(Figure 18B)**. In addition, frequencies of $\gamma\delta$ T cell producing TNF and GrnB were not different between the two vaccination groups in 5-week-old and 9-week-old infants **(Figure 18A-18D)**.

3.4.3. CD20$^+$ B cell functional and memory marker expression are unchanged in response to mycobacterial stimulation after BCG vaccination.

B cells have been shown to produce proinflammatory cytokines such as IL-1β, IL-17 and TNF in humans (du Plessis et al., 2016b) and IL-6 in NHP (Phuah et al., 2012) in

response to mycobacterial antigens. In addition, GrnB production by B cells has been shown in experimental models and exerted important regulatory properties (Chesneau et al., 2020). To investigate whether BCG vaccination elicited changes in the $CD20^+$ B cell cytokine and cytotoxic production, we measured frequencies of $CD20^+$ B cells expressing IL-1β, IL-6, GrnB and TNF in response to BCG stimulation in 5-week-old and 9-week-old infants in the BCG and no BCG group. There were no differences in cytokine expression by $CD20^+$ B cells between the no BCG and BCG group in both age groups **(Figure 19)**.

We were also interested in determining whether BCG vaccination changed the frequencies of B cell memory subsets. We therefore measured IgD and CD27 memory marker expression on $CD20^+$ B cells. Using these markers, we would be able to detect IgD^+CD27^- (naïve B cells), IgD^+CD27^+ (non-switched memory B cells), IgD^-CD27^+ (classical memory B cells) and IgD^-CD27^- (atypical memory B cells). However, as discussed in chapter 2, we were unable to resolve these subsets sufficiently. We therefore analysed MFI values for the CD27 and IgD markers, rather than gating on positive or negative populations. There were no differences in expression of IgD and CD27 on $CD20^+$ B cells between the BCG and no BCG group in 5-week-old and 9-week-old infants **(Figure 20)**.

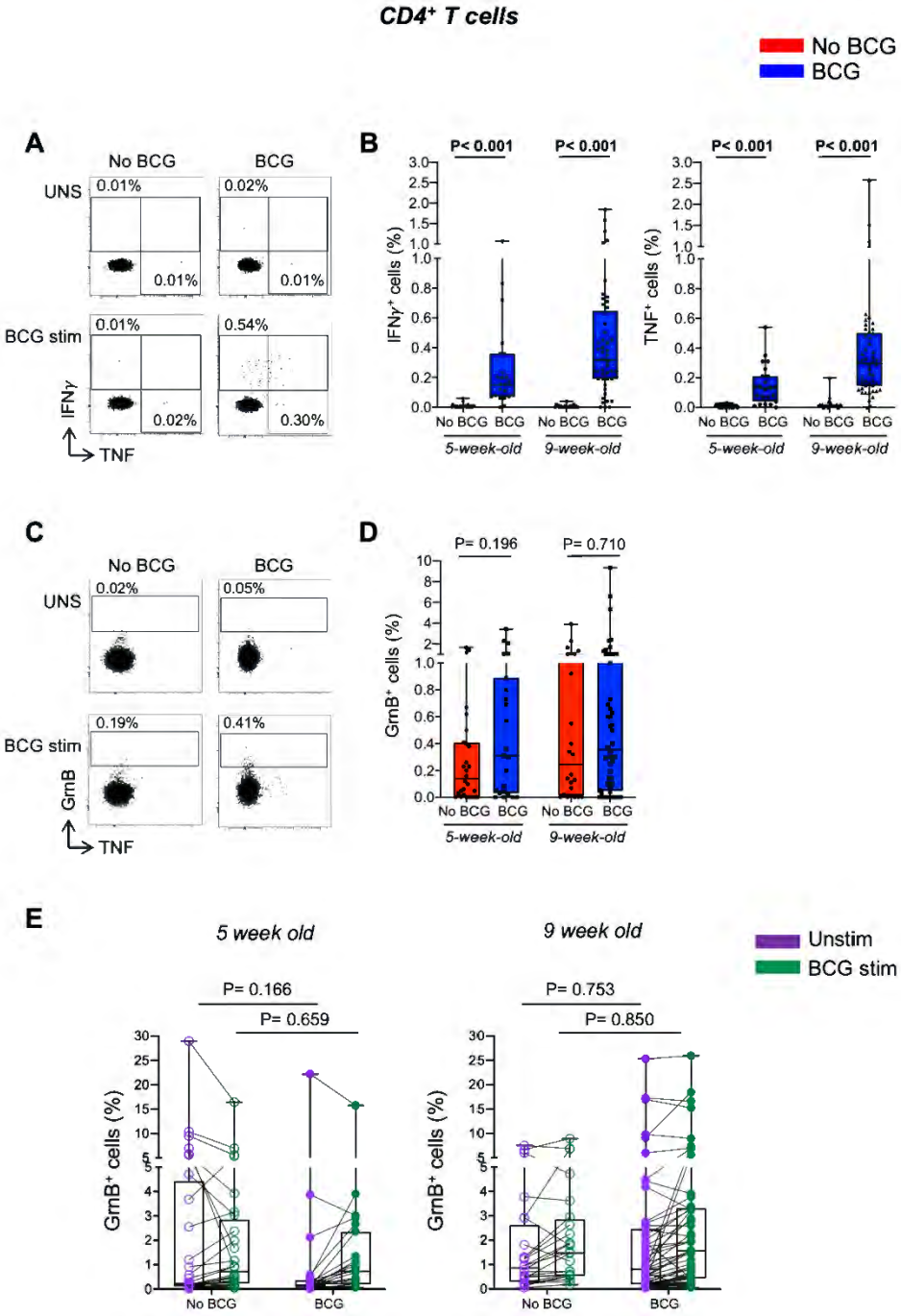


Figure 17. Frequencies of BCG-reactive CD4⁺ T cells in peripheral blood in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing IFNγ versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) infant and a vaccinated (BCG) 5-week-old infant. **(B)** Frequencies of IFNγ (left) and TNF (right) expression by CD4⁺ T cells in 5-week-old and 9-week-old infants. **(C)** Representative flow cytometry plots showing GrnB versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) infant and a vaccinated (BCG) 5-week-old infant. **(D)** Frequencies of GrnB expression by CD4⁺ T cells in 5-week-old and 9-week-old infants. **(E)** Frequencies of GrnB expression by CD4⁺ T cells in 5-week-old (left) and 9-week-old infants (right) in unstimulated (purple) and BCG stimulated (green) samples in the no BCG and BCG infant groups. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. The p values < 0.025 were considered statistically significant.

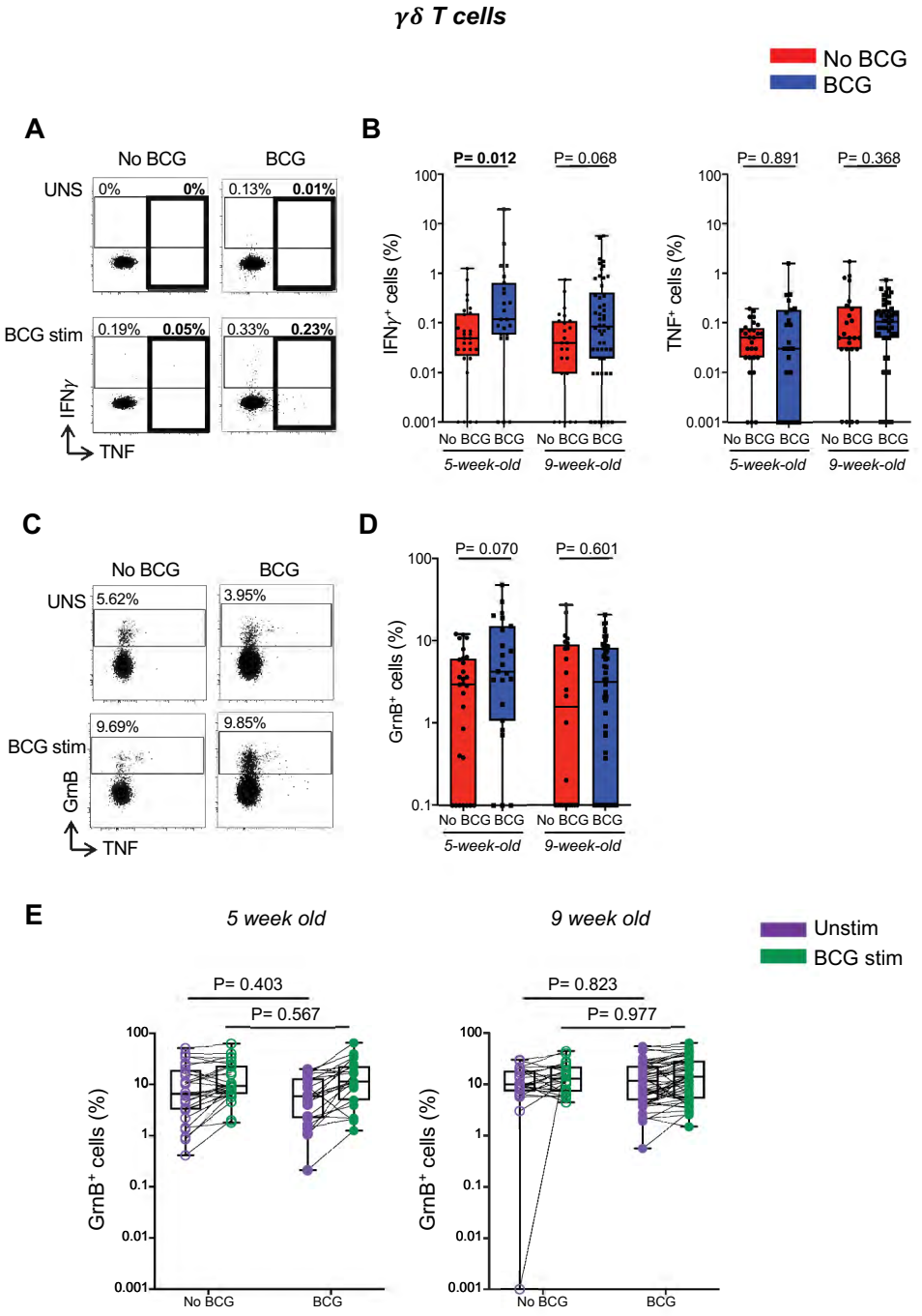


Figure 18. Frequencies of BCG-reactive $\gamma\delta$ T cells in peripheral blood in 5-week-old and 9-week-old infants. **(A)** Representative flow cytometry plots showing IFNγ versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(B)** Frequencies of IFNγ (left) and TNF (right) expression by $\gamma\delta$ T cells in 5-week-old and 9-week-old infants. **(C)** Representative flow cytometry plots showing GrnB versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(D)** Frequencies of GrnB expression by $\gamma\delta$ T cells in 5-week-old and 9-week-old infants. **(E)** Frequencies of GrnB expression by $\gamma\delta$ T cells in 5-week-old (left) and 9-week-old infants (right) in unstimulated (purple) and BCG stimulated (green) samples in the no BCG and BCG infant groups. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. The p values < 0.025 were considered statistically significant.

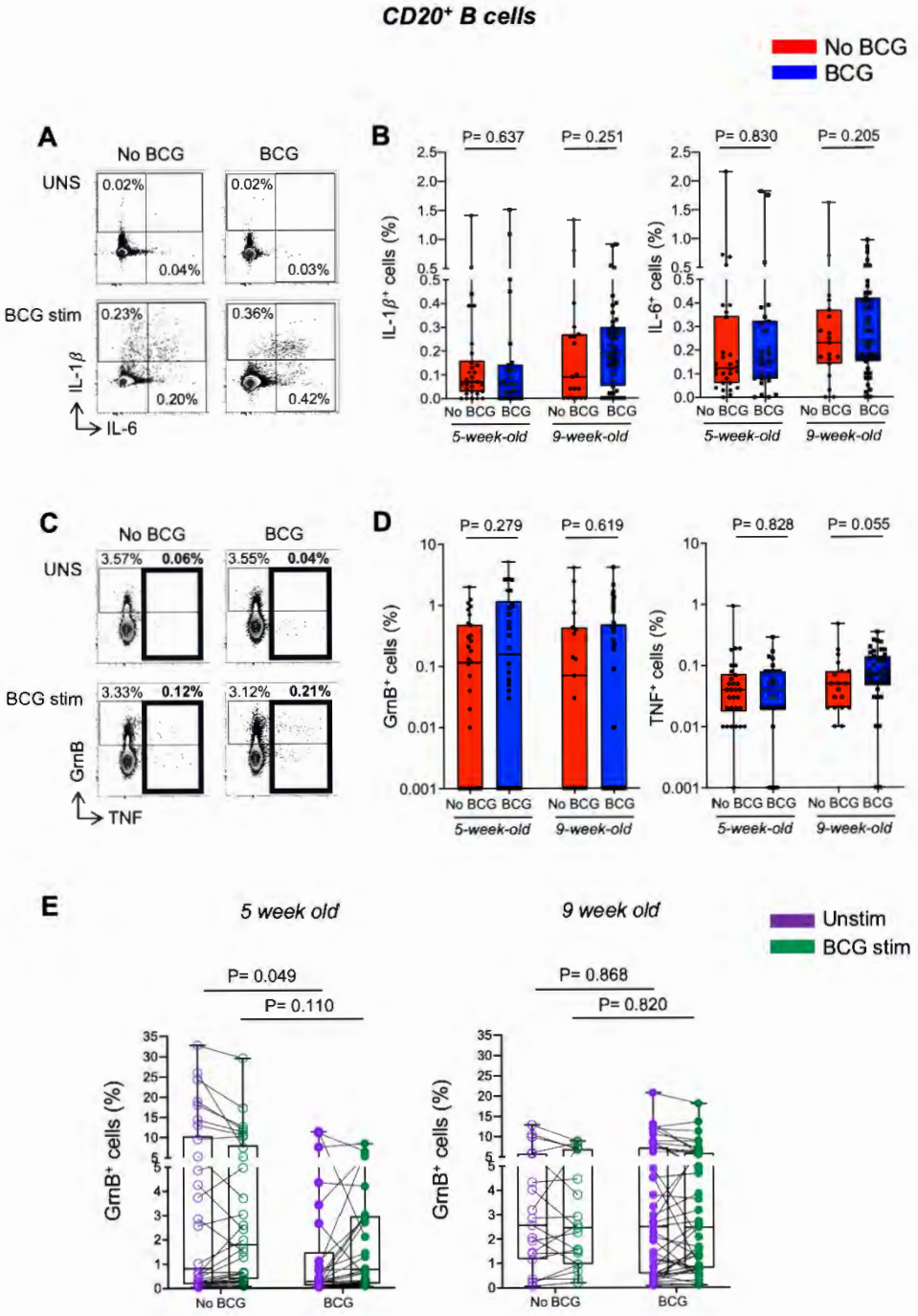


Figure 19. Frequencies of BCG-reactive CD20⁺ B cells in peripheral blood in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing IL-β versus IL-6 expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 9-week-old infant. **(B)** Frequencies of IL-1β (left) and IL-6 (right) expression by CD20⁺ B cells in 5-week-old and 9-week-old infants. **(C)** Representative flow cytometry plots showing GrnB versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 9-week-old infant. **(D)** Frequencies of GrnB (left) and TNF (right) expression by CD20⁺ B cells in 5-week-old and 9-week-old infants. **(E)** Frequencies of GrnB expression by CD20⁺ B cells in 5-week-old (left) and 9-week-old infants (right) in unstimulated (purple) and BCG stimulated (green) samples in the no BCG and BCG infant groups. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. The p values < 0.025 were considered statistically significant.

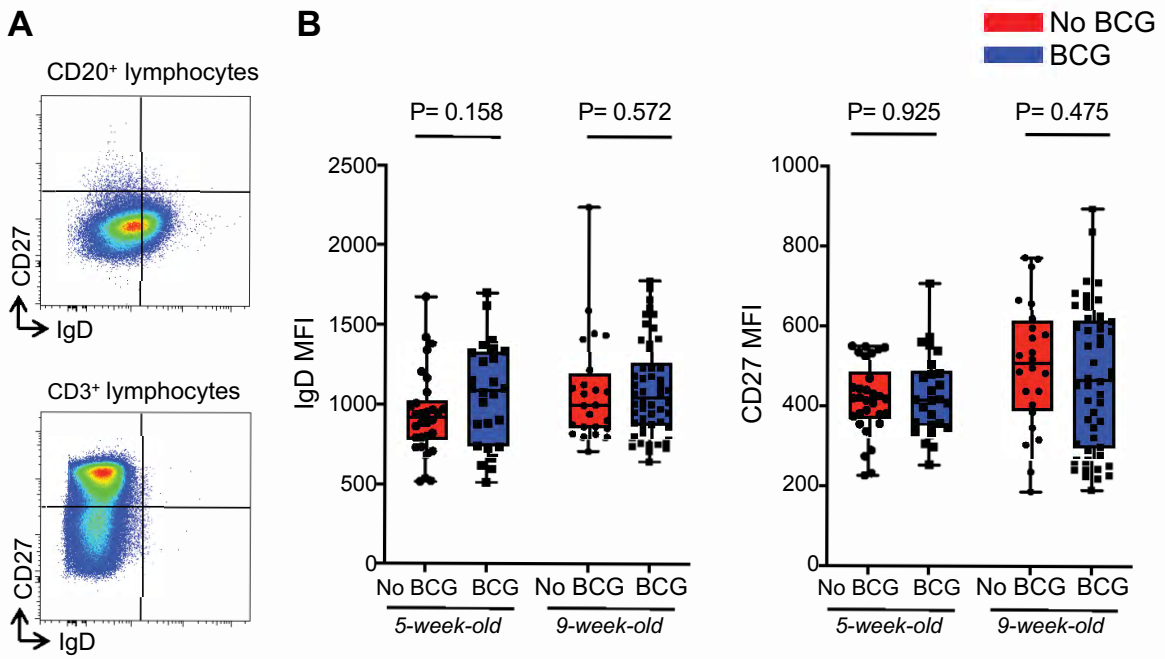


Figure 20. CD20⁺ B cell memory marker expression in peripheral blood in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing IgD versus CD27 expression in CD20⁺ lymphocytes (top) and CD3⁺ lymphocytes (bottom). **(B)** Median fluorescence intensity (MFI) of IgD (left) and CD27 (right) on CD20⁺ B cells in 5-week-old and 9-week-old infants. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. The p values < 0.025 were considered statistically significant (two comparisons).

3.4.4. IFNγ⁺CD3⁺ phenotypic MAIT cells are elicited after BCG vaccination in response to mycobacterial stimulation in 5-week-old and 9-week-old infants.

We defined phenotypic MAIT cells as $CD26^{hi}CD161^{hi}$ T cells in infants based on previous studies in adults [**Figure 21A**, (Sharma et al., 2015, Suliman et al., 2019)] using flow cytometry Panel 1 **(Chapter 2, Table 2)**. We first sought to determine the effect of BCG vaccination on the functional response of CD3⁺ MAIT cells. We measured the frequencies of IFNγ-expressing CD3⁺ phenotypic MAIT cells in response to BCG stimulation in the BCG and no BCG group in 5-week-old and 9-week-old infants. Frequencies of IFNγ-expressing CD3⁺ phenotypic MAIT cells were higher in the BCG group compared to the no BCG group in both age groups **(Figure 21B and Figure 21C)**. There were no differences in perforin expression on CD3⁺ phenotypic MAIT cells between the BCG and no BCG group in both age groups **(Figure 21D and Figure 21E)**.

Since functional MAIT cells had been described mainly among $CD8^+$ T cells at the time of these experiments, we did not include a CD4 marker in Panel 1 **(Chapter 2, Table 2)**, which we used to stain a subset of the 5-week-old infants. Therefore, we sought to determine if the functional IFNγ-producing $CD3^+$ phenotypic MAIT cells were in the $CD8^+$ or $CD8^-$ compartment in the infant cohort **(Figure 22A)**. Frequencies of $CD8^+$ phenotypic MAIT cells were not different between the two vaccination groups in 5-week-old and 9-week-old infants **(Figure 22B)**. Surprisingly, frequencies of $CD8^-$ phenotypic MAIT cells were higher in the BCG group compared to the no BCG group in both age groups **(Figure 22C)**.

3.4.5. BCG-reactive $CD26^{hi}CD161^{hi}$ T cells elicited after BCG vaccination are $IFN\gamma^+CD8^-CD4^+TRAV1\text{-}2^-$ and $IFN\gamma^+CD8^-CD4^-TRAV1\text{-}2^-$ T cells in 9-week-old infants.

We later embarked on an in-depth characterisation of phenotypic MAIT cells **(chapter 2, Table 3)**. CD4 was added to other MAIT cell markers into Panel 2 **(Chapter 2, Table 3),** which we used to stain a subset of the 9-week-old infants, in order to detect the expression of CD4 and TRAV1-2 on the functional phenotypic MAIT cell subset. BCG-reactive IFNγ^+CD26^{hi}CD161^{hi} T cells that were higher in frequency in the BCG compared to the no BCG group were $CD8^-CD4^+TRAV1\text{-}2^-$ and $CD8^-CD4^-TRAV1\text{-}2^-$ T in phenotype **(Figure 23A and Figure 23B)**. Next, we measured perforin expression on IFNγ-positive and IFNγ-negative cells in the two functional subsets. However, there were not enough IFNγ-positive cells in the no BCG group to include in this analysis. We observed no differences in perforin expression between the IFNγ-positive and IFNγ-negative cells in the BCG group in the $CD8^-CD4^+TRAV1\text{-}2^-$ phenotypic MAIT subset **(Figure 23C)**. In addition, there were no differences in perforin expression on IFNγ-negative cells between the BCG and no BCG group **(Figure 23C)**. Perforin expression was higher in the IFNγ-positive compared to the IFNγ-negative cells in the BCG group in the $CD8^-CD4^-TRAV1\text{-}2^-$ phenotypic MAIT subset **(Figure 23C)**. However, no differences in perforin expression on IFNγ-negative cells between the BCG and no BCG group were detected **(Figure 23C)**.

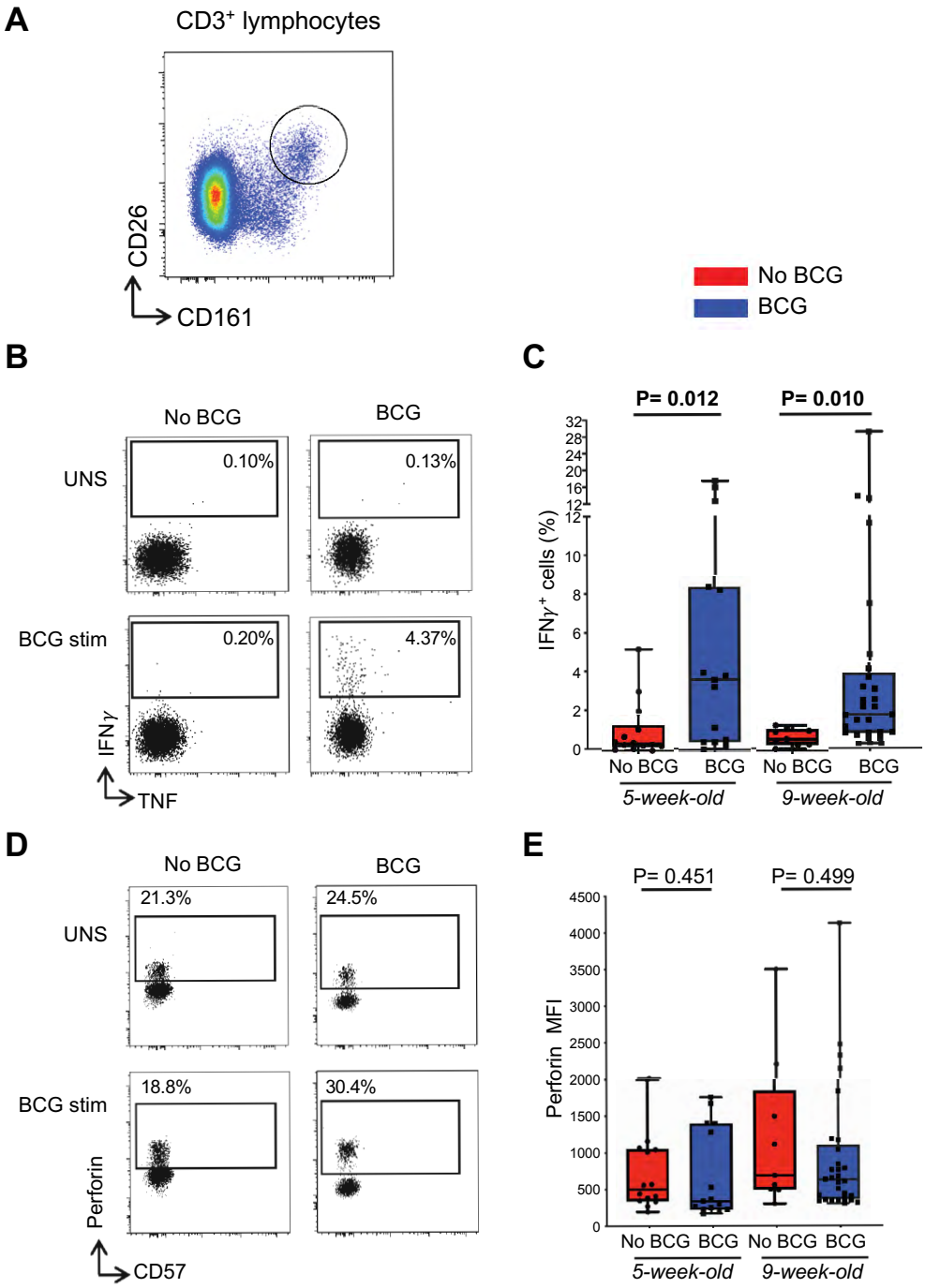


Figure 21. Frequencies of IFNγ^+CD3$^+$ phenotypic MAIT cells in 5-week-old and 9-week-old infants. (A) A representative flow cytometry depicting the gating of phenotypic MAIT cells (CD3$^+$CD26^{hi} CD161^{hi}). **(B)** Representative flow cytometry plots showing IFNγ versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(C)** Frequencies of IFNγ expression by CD3$^+$ phenotypic MAIT cells in 5-week-old and 9-week-old infants. **(D)** Representative flow cytometry plots showing perforin versus CD57 expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(E)** Perforin Median fluorescence intensity (MFI) of CD3$^+$ phenotypic MAIT cells in 5-week-old and 9-week-old infants. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. The p values < 0.025 were considered statistically significant (two comparisons).

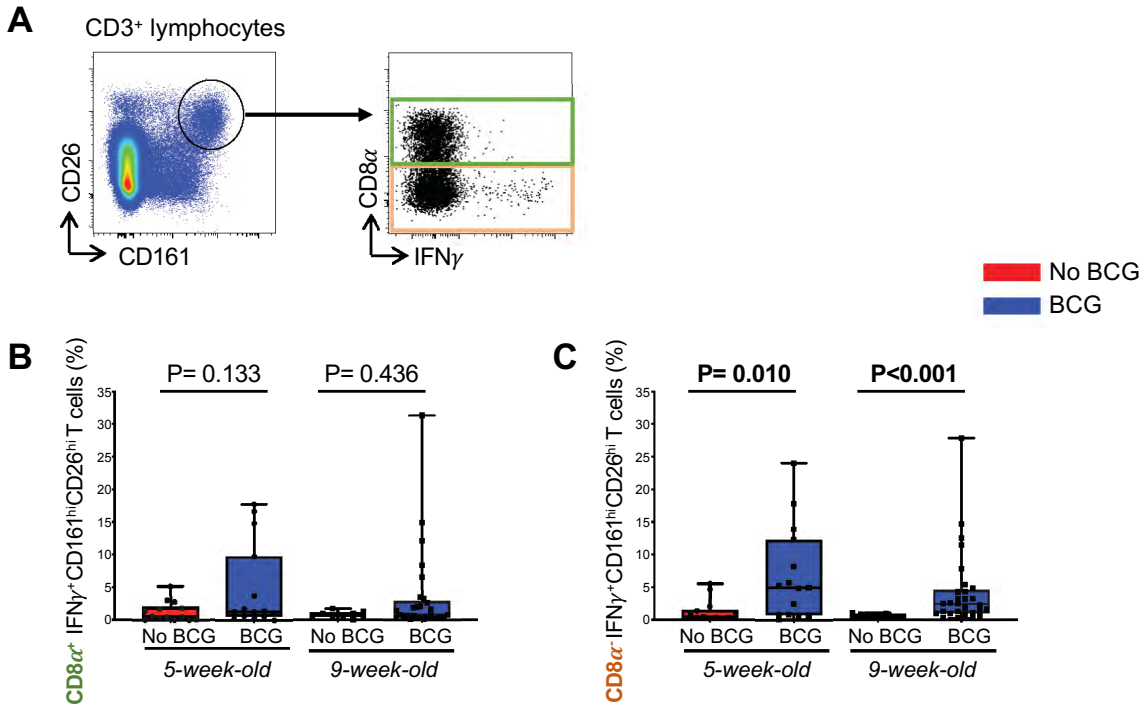


Figure 22. Frequencies of IFNγ^+CD8$^+$ and CD8$^-$ phenotypic MAIT cells in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots depicting the gating of CD8$^+$ and CD8$^-$ phenotypic MAIT cells. Frequencies of IFNγ expression by phenotypic CD8$^+$ **(B)** and CD8$^-$ **(C)** phenotypic MAIT cells in 5-week-old and 9-week-old infants. The p values are calculated with the Mann-Whitney U or Wilcoxon signed-rank test. Bolded p values are significant after correcting for multiple comparison testing using the Bonferroni method. The p values < 0.025 are considered statistically significant (two comparisons).

3.4.6. Expression of cytokine receptors on CD26^{hi}CD161^{hi} T cell subsets is not altered after BCG vaccination in 9-week-old infants.

Next, we sought to ascertain whether BCG vaccination changes cytokine receptor expression on the functional CD26^{hi}CD161^{hi} T cell subsets. Higher expression of cytokine receptors might indicate higher responsiveness of the cell to the corresponding cytokines in the milieu. We measured IL-2Rβ (CD122), IL-18Rα (CD218a) and IL-12Rβ1 (CD212) expression **(Figure 24A and Figure 24B)**. There was no difference in the levels of cytokine receptor expression on the functional IFNγ-positive phenotypic MAIT cells and IFNγ-positive CD3$^+$ T cells **(Figure 24C)**. Next, we analysed cytokine receptor expression on IFNγ-positive and IFNγ-negative cells on CD8$^-$CD4$^+$TRAV1-2$^-$ **(Figure 24D)** and CD8$^-$CD4$^-$TRAV1-2$^-$ **(Figure 24E)** phenotypic MAIT cell subsets. As stated above, there were not enough IFNγ-positive cells in the no BCG group to allow reliable measurement for this analysis. CD212 expression on

CD8$^-$ CD4$^+$TRAV1-2$^-$ T cells was higher on IFNγ-positive compared to IFNγ-negative cells in the BCG group **(Figure 24D)**. In addition, the expression of CD122 on CD8$^-$ CD4$^-$TRAV1-2$^-$ T cells was higher on IFNγ-positive cells compared to IFNγ-negative cells **(Figure 24E)**. However, there were no differences for the other markers between the groups **(Figure 24D and Figure 24E)**.

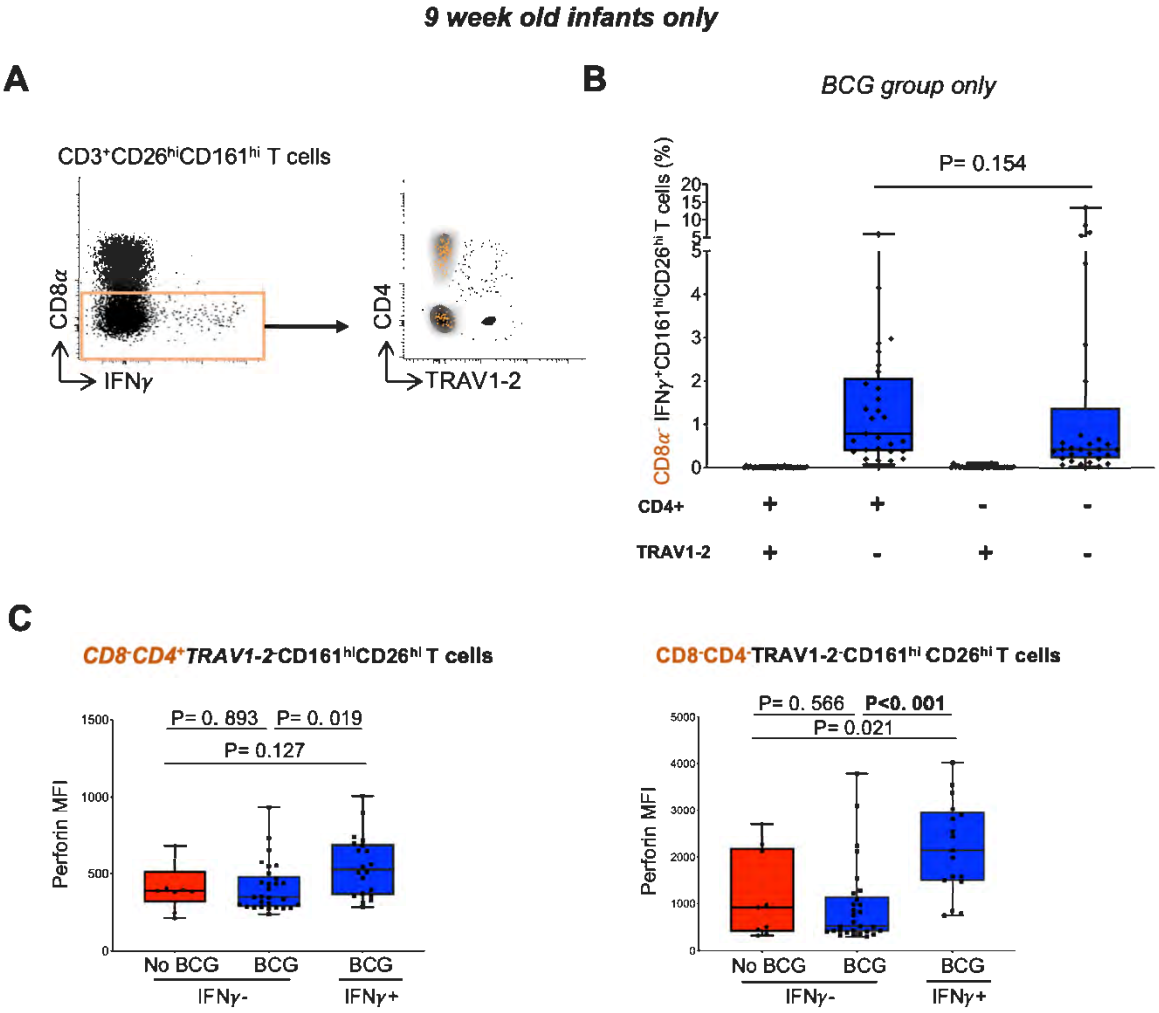


Figure 23. Characterisation of IFNγ^+CD8$^-$ phenotypic MAIT cells in 9-week-old infants. (A) Representative flow cytometry plots depicting the gating IFNγ^+CD8$^-$ (left) and CD4 +/- and TRAV1-2$^-$ (right) T cells in a 9-week-old. **(B)** Frequencies of CD8$^-$IFNγ^+CD161^{hi}CD26^{hi} T cells expressing combinations of CD4 and TRAV1-2. **(C)** Perforin MFI of CD8$^-$ CD4$^+$TRAV1-2$^-$ (left) and CD8$^-$CD4$^-$TRAV1-2$^-$ (right) phenotypic MAIT cells in IFNγ-positive and IFNγ-neagtive cells. The p values are calculated with the Mann-Whitney U or Wilcoxon signed-rank test. Bolded p values are significant after correcting for multiple comparison testing using the Bonferroni method. In **C** the p values < 0.017 are considered statistically significant (three comparisons).

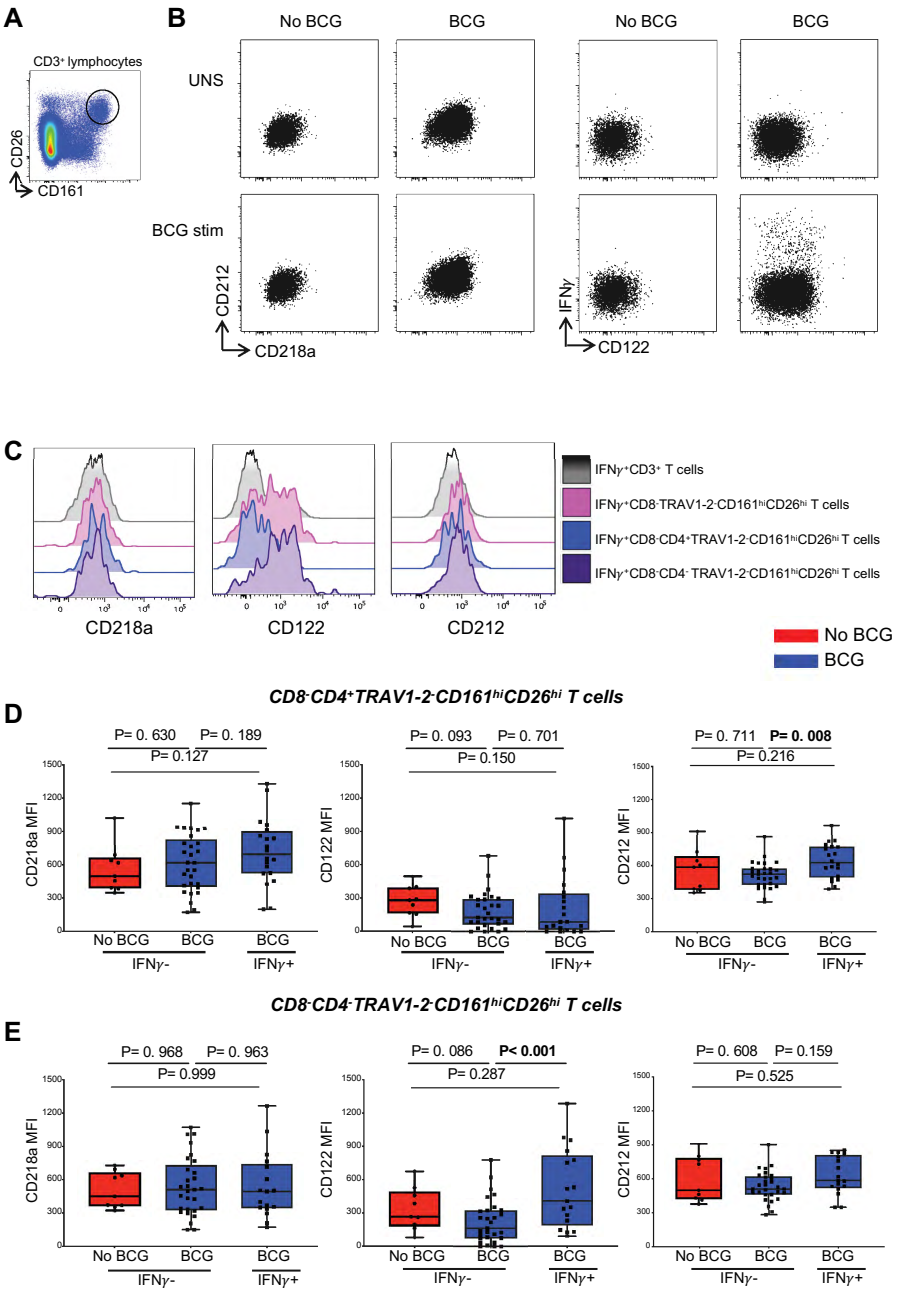


Figure 24. Cytokine receptor expression on functional phenotypic MAIT cells in 9-week-old infants. (A) A representative flow cytometry plot depicting the gating of $CD3^+$ phenotypic MAIT cells based on CD161 and CD26 expression. **(B)** Representative flow cytometry plots showing CD212 versus CD218 (left panel) and IFNγ versus CD122 (right) in $CD3^+$ phenotypic MAIT cells in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) infant. **(C)** Histograms showing relative expression of CD218a (left), CD122 (middle) and CD212 (right) in IFNγ-producing phenotypic MAIT subsets and $CD3^+$ T cells in a 9-week-old infant. **(D and E)** CD218a (left) CD122 (middle) and CD212 (right) median fluorescence intensity (MFI) in IFNγ-negative (No BCG and BCG cohorts) and IFNγ-positive (BCG cohort) in $CD8^-CD4^+TRAV1\text{-}2^-$ (top) and $CD8^-CD4^-TRAV1\text{-}2^-$ (bottom) phenotypic MAIT cells. The p values are calculated with the Mann-Whitney U or Wilcoxon signed-rank test. Bolded p values are significant after correcting for multiple comparison testing using the Bonferroni method. The p values < 0.017 are considered statistically significant (three comparisons).

3.5. Discussion

In this chapter we investigated T and B cell functional responses to mycobacteria in infants after BCG vaccination. In particular, we were interested in understanding immune responses beyond conventional T cells better. Important findings include: 1. As expected, IFNγ and TNF production by $CD4^+$ T cells were significantly higher in the BCG group compared to the no BCG group in 5-week-old and 9-week-old infants; 2. Frequencies of IFNγ-expressing $\gamma\delta$ T cells were higher in the BCG group compared to the no BCG group in 5-week-old infants only; 3. There were no differences in cytokine expression by $CD20^+$ B cells or in the expression of CD27 and IgD on $CD20^+$ B cells between the no BCG and BCG group at either age; 4. BCG-reactive phenotypic MAIT cells were higher in the BCG group compared to the no BCG group in 5-week-old and 9-week-old infants and; 5. We defined two novel T cell subsets of BCG-reactive $CD4^+CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ and $CD4^-CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ cell in 9 week old infants.

It is known that infants exhibit a low IFNγ response compared to adults, and it is believed that the infant immune response is skewed towards a more Th2 phenotype (Zaghouani et al., 2009, Kollmann et al., 2017). Previous studies have shown, however, that BCG vaccination of infants at birth elicits IFNγ production (Marchant et al., 1999, Vekemans et al., 2001, Soares et al., 2008) at levels comparable to adults (Marchant et al., 1999). Our result showing that frequencies of BCG-specific IFNγ and TNF producing $CD4^+$ T cells was higher in BCG vaccinated compared to unvaccinated infants is in line with prior studies from TB endemic settings in which BCG vaccination had been delayed (Kagina et al., 2009, Lutwama et al., 2014, Ritz et al., 2016). These data further indicate that BCG has a potent polarising effect that primes the infant immune response to a Th1 phenotype (Marchant et al., 1999, Vekemans et al., 2001, Soares et al., 2008).

Infants vaccinated with BCG show $\gamma\delta$ T cell expansion and cytokine production to mycobacterial antigens (Zufferey et al., 2013, Mazzola et al., 2007, Tastan et al., 2005, Kagina et al., 2010). In addition, $\gamma\delta$ T cells are known for their ability to produce

IFNγ and granzymes (Boom, 1999, Bonneville et al., 2010). In our study, we observed that expression of IFNγ was higher in the BCG group compared to the no BCG group in 5-week-old infants only. Despite the modest difference in IFNγ production at 5 weeks of age, GrnB production by $\gamma\delta$ T cells was not different. In addition, there was only a trend towards increased expression of IFNγ in the BCG group compared to the no BCG group in 9-week-olds. It is possible that with a larger sample size that we would have observed a statistically significant difference. It is also possible that the different vaccines given at 6 weeks of age could have interfered with BCG effects on $\gamma\delta$ T cells. Alternatively, the BCG effect on $\gamma\delta$ T cells may be only transient, regardless of the other vaccines given in the EPI schedule. Studies have shown robust IFNγ responses in 10-week-old infants vaccinated with BCG at birth, but in these studies there were no age-matched BCG-unvaccinated controls (Zufferey et al., 2013, Kagina et al., 2010). Other studies have only investigated frequencies of $\gamma\delta$ T cells following BCG in infants and adults (Mazzola et al., 2007, Tastan et al., 2005, Hoft et al., 1998). A recent study by Papadopoulou et al. reported robust IFNγ and TNF responses by the Vγ9Vδ2 subset in infants, but this was in response to phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation. Low IFNγ responses in 10-week-olds to (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate stimulation, which is present in BCG and *M.tb,* was also reported. However, the authors did not investigate intracellular cytokine production by $\gamma\delta$ T cells in infants vaccinated with BCG at birth to whole cell mycobacteria (Papadopoulou et al., 2020).

Recent studies have demonstrated renewed interest in investigating the role of B cells during *M.tb* infection (Rijnink et al., 2021). Our results show that there were no differences in B cell cytokine production (IL-6, IL-1β, TNF and GrnB) between the BCG and no BCG groups in 5-week-old and 9-week-old infants. To our knowledge this is the first study from a TB endemic setting that investigated an array of intracellular cytokines produced by infant B cells in response to mycobacterial stimulation using our unique study design. In a Danish infant study, in which BCG was delayed, there were no overall differences in B cell frequencies between BCG vaccinated and unvaccinated infants; cytokine measurements were not reported in this study (Birk et al., 2017). We also assessed memory properties of $CD20^+$ B cells and observed no differences in CD27 and IgD MFI. As discussed above, we were unable to resolve the

combinatorial expression of IgD and CD27 (IgD^+CD27^-, IgD^+CD27^+, IgD^-CD27^+ and IgD^-CD27^-) in fixed whole blood from infants, despite successful staining in adults during the optimisation of this panel. It is possible that modulation of distinct memory populations by BCG vaccination in infants might have occurred and these changes could not be detected by measuring total CD27 and IgD MFI. A study in adults showed higher PPD-specific memory B cells in BCG-vaccinated compared to unvaccinated adults (Sebina et al., 2012). Also, a recent study showed durable antibody responses by memory B cells in response to mycobacterial antigens following BCG vaccination in adults (Bitencourt et al., 2022). However, it is important to note that not only were these studies carried out in adults in a non-TB endemic setting, but B cell ELISPOT assays were used to determine memory B cell responses and not flow cytometry (which is the case in our study). Since we did not measure Abs induced by BCG vaccination in this study, our results cannot provide further information on the main function of B cells.

MAIT cells are abundant in the peripheral blood of humans, and they are capable of immediate effector functions upon activation by their cognate antigen, bystander cytokines or both (Martin et al., 2009, Le Bourhis et al., 2010, Dusseaux et al., 2011, Ussher et al., 2014, Suliman et al., 2019). Therefore, MAIT cells have gained much interest for their potential role in the immune response against *M.tb*. We first sought to determine whether BCG vaccination was able to modulate phenotypic MAIT cell ($CD3^+CD26^{hi}CD161^{hi}$ T cells) cytokine responses to mycobacterial stimulation in infants. We observed higher frequencies of IFNγ-producing total $CD3^+CD26^+CD161^+$ T cells in the BCG group compared to the no BCG group in 5-week-old infants. When we first embarked on this study, we expected that infant phenotypic MAIT cells would follow the same co-receptor phenotype as adults, the major subsets being $CD4^-CD8^-$ and $CD8^+$ T cells. We also assumed that the functional subset would express the CD8 co-receptor and therefore we did not include a CD4 marker in flow cytometry Panel 1. Interestingly, when we further characterised the co-receptor expression of the $CD3^+CD26^{hi}CD161^{hi}$ T cells, we discovered that the IFNγ-producing cells were in the $CD8^-$ compartment. In our next flow cytometry panel (Panel 2) we added CD4 and TRAV1-2 alongside CD8, CD26 and CD161 to further characterise this subset in the 9-week-old infant group. We observed that the IFNγ-producing T cells are in fact

$CD4^+CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ and $CD4^-CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ T cells. Since we did not include a $\gamma\delta$ T cell marker in Panel 2 we cannot exclude that this subset may make up a percentage of the $CD4^-CD8^-TRAV1\text{-}2^-$ T cells. It is likely that the $CD4^+CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ T cells are activated conventional T cells. However, a recent study showed that a subset of 5-OP-RU-MR1-tetramer positive MAIT cells in neonates and infants express a $TRAV1\text{-}2^-$ and $CD4^+$ phenotype (Swarbrick et al., 2020). Interestingly, a recent study by Nathan et al. reported that a Th17 $CD4^+CD26^+CD161^+CCR6^+$ memory T cell subset was reduced in TB-exposed individuals who developed TB disease compared to non-progressors from Peru (Nathan et al., 2020). It is possible that the two BCG-reactive subsets described in our study are 'non-classical' MAIT cells, which have been proposed as MR1-restricted T cells which have variable TCR alpha genes but are phenotypically similar to MAIT cells on the basis of markers such as CD26, CD161, and the innate-like transcription factor PLZF (Godfrey et al., 2019). It is also possible that this subset represents another DURT cell type. A study by Fergusson et al. described $TRAV1\text{-}2^-CD161^{hi}$ and $TRAV1\text{-}2^+CD161^{hi}$ T cells which were both highly positive for PLZF (Fergusson et al., 2014). Overall, this subset, which may comprise two CD4+ and CD4- subpopulations, represent an interesting phenotype in infants vaccinated at birth with BCG, which is not present in their unvaccinated counterparts and warrants further investigation.

We also investigated perforin expression and cytokine receptor expression on these two BCG-reactive subsets to give an indication about whether these cells were more cytotoxic or more receptive to cytokines (IL-2, IL-12, IL-18) in the milieu. However, we observed no differences in perforin and cytokine receptor expression between the no BCG and BCG groups on the two bulk (the total cells) subsets in 9-week-old infants (not shown). Interestingly, when we stratified based IFNγ positivity ($IFN\gamma^+$ and $IFN\gamma^-$), we observed higher perforin expression on the $IFN\gamma^+$ subset compared to the $IFN\gamma^-$ subset in BCG-vaccinated infants. However, after correction for multiple comparisons, perforin expression on the $CD4^+CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ T cell subset did not meet the significance threshold. Regardless, our observations indicate that those cells that are receptive to BCG vaccination are modified towards a proinflammatory and cytotoxic phenotype. We also observed a similar effect for CD122 expression on $CD4^-CD8^-TRAV1\text{-}2^-CD26^{hi}CD161^{hi}$ T cells and CD212 expression on $CD4^+CD8^-TRAV1\text{-}2^-$

$CD26^{hi}CD161^{hi}$ T cells. We speculate that the two T cell subsets are differentially activated by cytokines, leading to higher IFNγ production. In addition, the higher expression of the phenotypic and cytotoxic markers on the IFNγ^+ cells only in the BCG group, is most likely indicative of a vaccine-induced activated phenotype.

In conclusion, our data show that BCG vaccination induces increased frequencies of mycobacteria-reactive IFNγ and TNF expressing $CD4^+$ T cells in 5-week-old and 9-week-old infants and IFNγ-expressing $\gamma\delta$ T cells in 5-week-old infants. We also identified novel, BCG-reactive IFNγ-producing $CD4^+CD8^-$TRAV1-$2^-CD26^{hi}CD161^{hi}$ and $CD4^-CD8^-$TRAV1-$2^-CD26^{hi}CD161^{hi}$ T cells, which may be novel DURT subsets and warrant further investigation.

3.6. Contributions to this chapter

Flow cytometry experiments were carried out by Melissa Murphy.

All analyses were carried out by Melissa Murphy under the supervision of Dr. Sara Suliman, Assoc. Prof. Elisa Nemes and Prof. Thomas J. Scriba.

Chapter 4: Modulation of infant NK cell responses to mycobacteria after BCG vaccination

4.1. Introduction

BCG vaccination can induce immune responses beyond classical T cells in infants, including NK and unconventional T cells (Zufferey et al., 2013, Smith et al., 2017). NK cells constitute approximately 10 percent of peripheral lymphocytes (Lanier et al., 1986, Angelo et al., 2015) and were primarily considered to be part of the innate immune response against tumours and viruses (Trinchieri, 1989).

In response to mycobacterial stimulation, NK cells produce various cytokines including IFNγ, TNF, IL-22 and cytotoxic molecules which can modulate the immune response (Esin et al., 2004, Dhiman et al., 2009, Kleinnijenhuis et al., 2014b, Suliman et al., 2016). IFNγ is the prototypical cytokine produced by NK cells, this cytokine activates macrophages to kill and inhibit the spread of intracellular pathogens (Cooper et al., 2001a, Flynn and Chan, 2001). It has been shown that NK cell production of IL-22 can restrict *M.tb* growth by enhancing phagolysosome fusion (Dhiman et al., 2009). In C57BL/6 mice infected with *M.tb*, the depletion of NK cells had no effect on the bacterial burden (Junqueira-Kipnis et al., 2003). However, in RAG knockout (T & B cell deficient) mice, NK cells were shown to be the main source of IFNγ. In the same study it was shown that NK cells contribute to bacterial containment and the prevention of neutrophil-driven pathology (Feng et al., 2006). Isolated NK cells are also able to detect and respond directly to *M.tb* cell wall components *in vitro* through TLR2 and NKp44 (Esin et al., 2008, Esin et al., 2013, Marcenaro et al., 2008). They are able to lyse monocytes and macrophages infected with *M.tb* through the involvement of their NKp46 and NKG2D receptors (Vankayalapati et al., 2002, Vankayalapati et al., 2005). NK cells are also able to lyse extracellular mycobacteria through a contact-dependent release of perforin and granulysin (Lu et al., 2014). Interestingly, it was shown that NK cells from individuals were able to kill autologous *M.tb* infected monocytes *in vitro*, this killing was not associated with IFNγ or cytotoxicity but dependent on contact between NK cells and infected monocytes (Brill et al., 2001). NK cell and DC cross-talk *in vitro*

is well established (Cooper et al., 2004), it has also been shown that NK cells activated by dendritic cells infected with live BCG were able to lyse autologous immature DCs (Ferlazzo et al., 2003, Marcenaro et al., 2008). They also shape the adaptive response by enhancing $CD8^+$ T cell function and lysis capabilities against *M.tb* infected monocytes (Vankayalapati et al., 2004). It has also been reported that NK cells are able to induce $\gamma\delta$ T cell expansion through cell-to-cell contact or cytokine production (Zhang et al., 2006). NK cells are also able to lyse regulatory T cells expanded by *M.tb in vitro* (Roy et al., 2008). These data suggest that NK cells may play an important role early in *M.tb* infection through the production of cytokines and direct killing. In addition, NK cells also exhibit a regulatory role by enhancing and/ or curbing other immune cell responses against mycobacteria.

NK cells have traditionally been classified as innate cells, but evidence has emerged that they exhibit adaptive properties. As discussed in chapter 1, numerous studies have shown that, following stimulation with haptens, viruses or cytokines, NK cells can exhibit memory-like properties and enhanced recall responses (O'Leary et al., 2006, Cooper et al., 2009, Sun et al., 2009, Romee et al., 2012, Sun et al., 2012, Paust et al., 2010). NK cells have also been linked to trained immunity. Kleinnijenhuis et al. reported that NK cells from BCG-vaccinated adults had enhanced proinflammatory cytokine production in response to *M.tb* and other unrelated organisms compared to their pre-vaccination timepoint (Kleinnijenhuis et al., 2014b). In the same study in mice, it was shown that BCG conferred nonspecific protection against *C.albicans*, at least partially through NK cells (Kleinnijenhuis et al., 2012). It was also been shown in mice that following BCG vaccination, memory-like NK cells developed and provided protection following *M.tb* challenge (Venkatasubramanian et al., 2017).

Recent studies in humans have also highlighted the potential importance of NK cells in the immune response to TB. In a study by Fu et al. memory-like NK cells expressing CD45RO were isolated from the pleural fluid of TB patients and were shown to produce IL-22 in response to IL-15 or mycobacterial antigens (Fu et al., 2016). Chowdhury et al. reported higher frequencies of NK cells in healthy *M.tb*-infected individuals compared to uninfected controls. Interestingly, NK cell frequencies decreased during progression from latent infection to active TB disease and were re-established after successful treatment (Chowdhury et al., 2018). In a study by Suliman

et al. revaccination of *M.tb* infected adults with BCG elicited BCG-reactive IFNγ-expressing NK cells up to 1 year post vaccination; these responses correlated with IL-2 expressing $CD4^+$ T cells and were dependent on IL-12 and IL-18 (Suliman et al., 2016).

A comprehensive investigation of the modulation of infant NK cells by BCG vaccination has not been undertaken before. In this chapter we investigated proportions of different NK cell subsets, effector functions, differentiation markers, cytokine receptor expression and associations between BCG-reactive NK responsiveness and host adaptive and innate secreted cytokines in 5-week-old and 9-week-old infants.

4.2. Aims

To investigate whether neonatal BCG vaccination modulates NK cell responses to mycobacteria the following specific aims were addressed in BCG vaccinated compared to unvaccinated infants:

1. To investigate whether BCG vaccination changes proportions of NK cell subsets in 5-week-old and 9-week-old infants.

   We hypothesised that BCG vaccination would lead to maturation of NK cells, and we would detect lower numbers of $CD56^{bright}$ (immature NK cell subset) and higher numbers of the $CD16^{+}$ NK cell subsets (mature NK cell subset) following BCG vaccination.

2. To investigate infant NK cell effector functions induced by BCG vaccination in 5-week-old and 9-week-old infants.

   We hypothesised that NK cell functional responses to mycobacterial stimulation would be higher in BCG vaccinated compared to unvaccinated infants.

3. To determine NK cell differentiation induced by BCG vaccination in 5-week-old infants.

   We hypothesised that BCG vaccination would lead to increased differentiation of NK cells following BCG vaccination.

4. To quantify BCG-mediated modulation of cytokine receptor expression in 9-week-old infants.

   We hypothesised that cytokine receptor expression would be higher in BCG vaccinated compared to unvaccinated infants.

5. To quantify secreted cytokine levels in response to BCG stimulation and to investigate associations between BCG-reactive NK subsets and secreted cytokine levels in 5-week-old and 9-week-old infants.

   We hypothesised that levels of secreted cytokines would be higher in BCG vaccinated compared to unvaccinated infants. In addition, we hypothesised that secreted cytokine levels would associate with BCG-reactive NK subsets in the BCG vaccinated infants only.

4.3. Materials and methods

Study participants

Infant samples used in this chapter were obtained as described in chapter 2 from the delayed-BCG cohort and the birth-vaccination cohort.

WB-ICS assay

Heparinised whole blood for the standardised 12-hour WB-ICS assay was processed as described in chapter 2.

Flow cytometry

Cryopreserved whole blood samples were thawed, washed in PBS, permeabilised in Perm/Wash buffer (BD Biosciences) and stained with flow cytometry panels as previously described **(Chapter 2, Table 2 and Table 3)** for 45 minutes at 4℃. Data shown in this chapter are from infant samples stained with panel 1 **(Chapter 2, Table 2)** and panel 2 **(Chapter 2, Table 3)** as previously described **(Chapter 3, Table 6)**.

Secreted cytokine detection by multiplex bead array

Plasma samples from the WB-ICS were used to quantify secreted cytokines using the multiplex bead array assay as described in the methods section in chapter 2. Data shown in this chapter are from infant supernatant samples processed in multiplex bead array experiments as follows:

Twenty-eight supernatant samples from 5-week-old infants were processed in total. Thirteen samples from the delayed BCG cohort and 15 samples from the birth-vaccination cohort.

Thirty-eight supernatant samples from 9-week-old infants were processed in total. Ten samples from the delayed BCG cohort and 28 samples from the birth-vaccination cohort.

4.4. Results

4.4.1. Proportions of NK cell subsets are not modulated by BCG vaccination

We were able to define four distinct subsets based on CD56 and CD16 expression: $CD56^{+}CD16^{-}$, $CD56^{++}$, $CD56^{+}CD16^{+}$ and $CD56^{-}CD16^{+}$ in addition to total NK cells (**Figure 25A**). In order to investigate whether BCG vaccination modulates NK cell abundance, we compared proportions of each NK subset (among total NK) between infants who did not receive BCG vaccination (no BCG) and infants who did (BCG) at 5 and 9 weeks of age (**Figure 25B and Figure 25C**). There were no differences in NK cell subset proportions between the BCG and no BCG infant groups at 5-weeks of age **(Figure 25B and Figure 25C)**. The composition of NK cell subsets in 9-week-old infants was different by Permutation Test **(Figure 25B)**, however, the proportions of each individual subset was not significantly different between the infant groups **(Figure 25C)**. As expected, the $CD56^{+}CD16^{+}$ and $CD56^{-}CD16^{+}$ NK cells made up the majority while $CD56^{+}CD16^{-}$ and $CD56^{++}$ NK cells were in the minority in peripheral blood (**Figure 25B and Figure 25C**).

4.4.2. BCG-reactive IFNγ-expressing NK cells are induced after BCG vaccination

NK cells are a major source of IFNγ in response to mycobacterial stimulation in infants (Watkins et al., 2008, Zufferey et al., 2013). To address whether effector functions were modified by BCG vaccination in infants we measured frequencies of BCG-reactive IFNγ-expressing NK cells in the BCG and no BCG infant groups (**Figure 26A and Figure 26B**). Among 5-week-old infants, frequencies of $CD56^{++}$ and $CD56^{+}CD16^{+}$ IFNγ-expressing NK cells were higher in the BCG compared to the no BCG group (**Figure 26C**). There were no differences observed for the other NK subsets between the two groups of infants. Among 9-week-old infants, frequencies of all IFNγ-expressing NK cell subsets were significantly higher in the BCG compared to the no BCG group (**Figure 26B and Figure 26C**), although frequencies of total IFNγ-expressing NK cells did not maintain significance after adjustment for multiple testing.

NK cells also produce other proinflammatory cytokines in response to mycobacteria, such as TNF and IL-22 (Dhiman et al., 2009, Kleinnijenhuis et al., 2014b, Suliman et al., 2016). We therefore measured the frequencies of TNF-expressing NK cells in the no BCG and BCG groups in 5-week-old infants. However, TNF-expressing NK cell frequencies were very low overall and not different between the two infant groups (not shown).

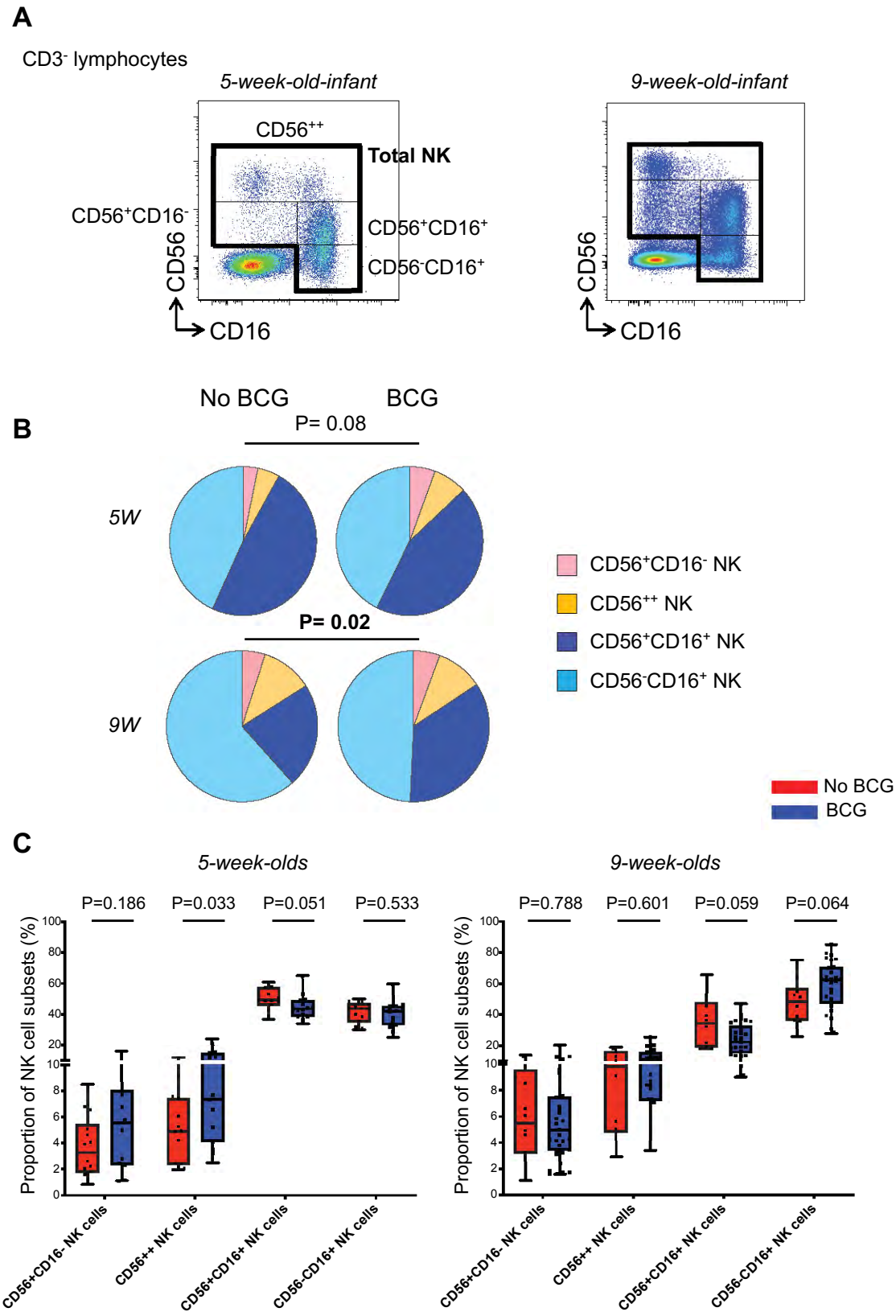


Figure 25. Proportions of NK subsets in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots depicting the gating of NK subsets in 5-week-old (left) and 9-week-old (right) infants based on CD56 and CD16 expression. **(B)** Pie charts showing proportions of NK subsets ($CD56^+CD16^-$, $CD56^{++}$, $CD56^+CD16^+$ and $CD56^-CD16^+$) among total NK cells. P-values were computed with a Permutation Test. **(C)** Box-and-whisker plots of proportions of NK cell subsets in 5-week-old (left) and 9-week-old (right) infants. Horizontal lines represent medians, boxes represent the interquartile range and whiskers represent the range. P-values were calculated with the Mann-Whitney U test. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.0125, four comparisons).

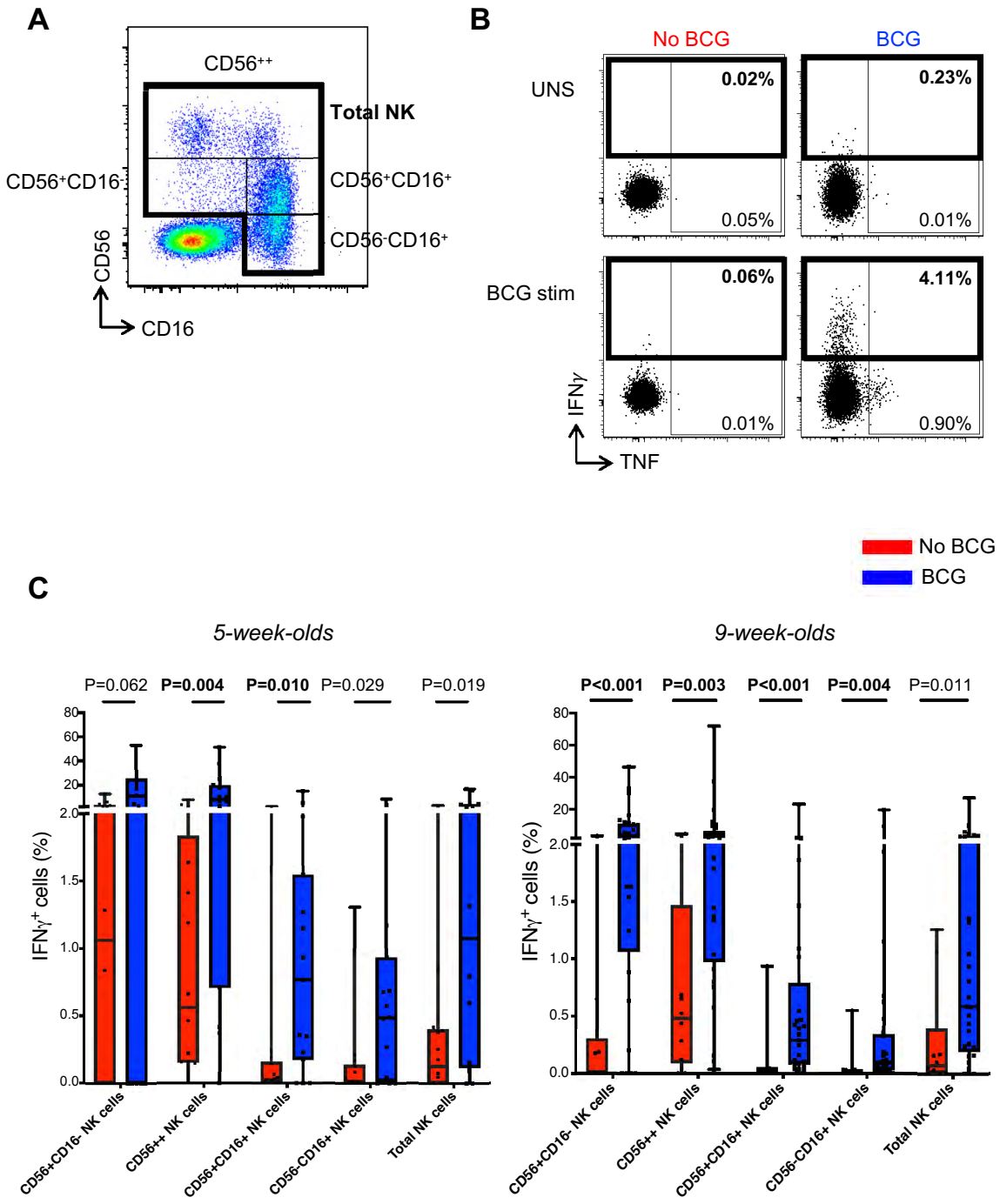


Figure 26. Frequencies of BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants. (A) A representative flow cytometry plot depicting the gating of NK subsets ($CD56^+CD16^-$, $CD56^{++}$, $CD56^+CD16^+$, $CD56^-CD16^+$ and total NK) in a 5-week-old infant. **(B)** Representative flow cytometry plots showing TNF versus IFNγ expression in the total NK subset in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(C)** Frequencies of IFNγ-expressing NK subsets in 5-week-old (left) and 9-week-old (right) infants. Horizontal lines represent medians, boxes represent the interquartile range and the whiskers represent the range. P values were calculated with the Mann-Whitney U test. Bolded p values were significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.010, five comparisons).

4.4.3. BCG modulation of NK cell cytokine responses cells is not sex-dependent

Previous studies have reported that non-specific effects of BCG vaccination is more prominent in male compared to female infants in early life (Biering-Sorensen et al., 2012, Biering-Sorensen et al., 2018, Prentice et al., 2021). To determine if a sex-associated effect was present in the NK response to BCG, we stratified the IFNγ response for all the NK cell subsets according to sex **(Figure 27)**. Overall, when we stratified according to age and sex, there were higher frequencies of IFNγ^+ NK cells in male infants in the BCG group compared to the no BCG group (**Figure 27A, Figure 27B and Figure 27D**). Whereas this was generally not the case for female infants except for the $CD56^+CD16^+$ NK subset in which females in the BCG group had higher IFNγ-expressing NK responses compared to the no BCG group (**Figure 27C**). When we combined the cytokine-expressing NK responses across the ages, both male and female infants had higher cytokine-positive cells in the BCG group compared to the no BCG group for the $CD56^{++}$ and $CD56^+CD16^+$ NK cell subsets **(Figure 27B and Figure 27C)**. Males had higher frequencies of cells that express IFNγ in the BCG group compared to the no BCG group for the $CD56^+CD16^-$ and total NK cell subsets (**Figure 27A and 27E**) and females had higher frequencies of IFNγ^+ cells in the BCG group compared to the no BCG group for the CD56-CD16+ NK cell subset (**Figure 27D**). In addition, there were no differences in the frequencies of IFNγ-expressing cells between males and females for any of the NK cell subsets (not shown).

4.4.4. Perforin expression by NK subsets is not modulated by BCG vaccination in 5-week-old and 9-week-old infants.

Cytotoxic activity is a major NK cell function and the $CD56^{bright}$ subset typically has less cytotoxic activity than the $CD56^{dim}$ subset (Lanier et al., 1986, Nagler et al., 1989). To determine if BCG vaccination influences the cytotoxic potential of NK cells, we measured perforin expression on NK cell subsets in the two infant vaccination groups at 5 and 9 weeks of age (**Figure 28**). The majority of total NK cells expressed high levels of perforin (median >80%) which differed to the expected low perforin expression by $CD3^+$ T cells (**Figure 28A**). However, there were no statistical differences in the proportions of NK cell subsets expressing perforin (not shown) nor

levels of perforin (measured as MFI) between no BCG and BCG groups in 5-week-old or 9-week-old infants (**Figure 28B**). As expected, the $CD56^{+}CD16^{+}$ and $CD56^{-}CD16^{+}$ NK cell subsets had higher perforin expression than the $CD56^{+}CD16^{-}$ and $CD56^{++}$ NK subsets (**Figure 28B**). Interestingly, when we compared perforin expression in the two age groups, irrespective of vaccination status, 9-week-old infants had higher perforin expression than 5-week-old infants in the $CD56^{++}$**,** $CD56^{+}CD16^{+}$ and $CD56^{-}CD16^{+}$ and total NK subsets (**Figure 28C**). Also, a sub-analysis in 9-week-olds revealed that expression of perforin was lower in IFNγ-expressing total NK cells when compared to bulk NK cells in both no BCG and BCG infant groups at 5 weeks of age were observed (**Figure 28D**).

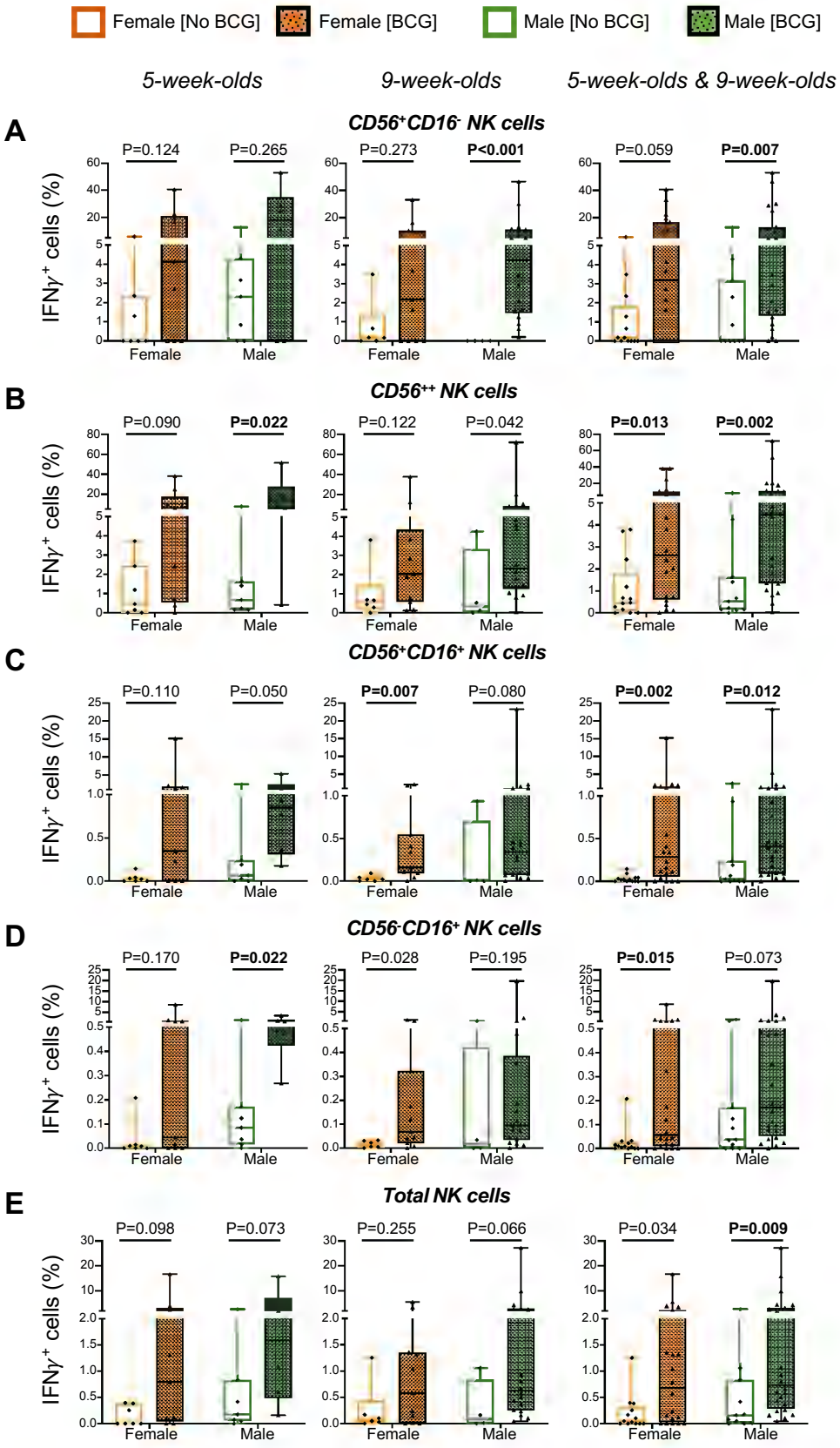


Figure 27. Sex-associated differences in BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants. Frequencies of IFNγ-expressing **(A)** $CD56^{+}CD16^{-}$ **(B)** $CD56^{++}$ **(C)** $CD56^{+}CD16^{+}$ **(D)** $CD56^{-}CD16^{+}$ **(E)** total NK subsets in 5-week-old (no BCG = 14: n= 7 female, n=7 male; BCG = 15: n=9 female, n= 6 male) (left), 9-week-old (no BCG = 10: n= 6 female, n=4 male; BCG = 28: n=11 female, n= 17 male) (middle) and both 5-week-old and 9-week-old infants (right) in female (orange) and male (green) infants from the BCG and no BCG groups. Horizontal lines represent medians, boxes represent the interquartile range and the whiskers represent the range. P values were calculated with the Mann-Whitney U test. Bolded p values were significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.025, two comparisons).

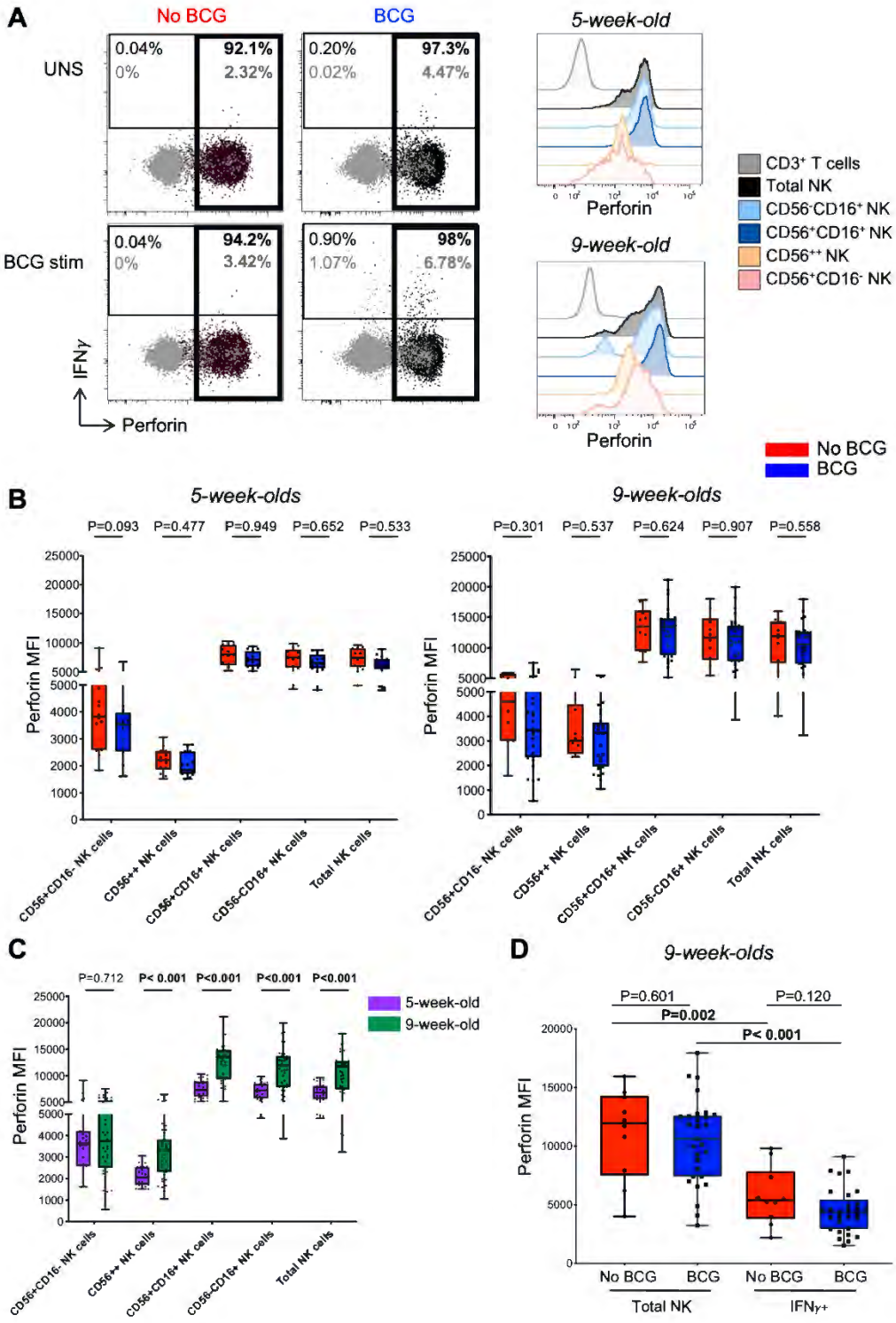


Figure 28. Perforin expression by NK subsets in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing perforin versus IFNγ expression in the total NK subset in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 9-week-infant (left) and histograms showing relative expression of perforin in NK subsets ($CD56^+CD16^-$, $CD56^{++}$, $CD56^+CD16^+$, $CD56^-CD16^+$ and total NK) and $CD3^+$ T cells in a 5-week-old (top right) and 9-week-old (bottom right) infant. **(B)** Perforin median fluorescence intensity (MFI) in NK subsets in 5-week-old (left) and 9-week-old (right) infants (BCG stimulated condition). **(C)** Perforin MFI in 5-week-old and 9-week-old infants irrespective of BCG vaccination status (BCG and no BCG groups combined) (BCG stimulated condition). **(D)** Perforin MFI in bulk NK and IFNγ^+ total NK cells in 9-week-old infants (BCG stimulated condition). Horizontal lines represent medians, boxes represent the interquartile range and whiskers represent the range. P-values were calculated with the Mann-Whitney U or Wilcoxon signed-rank tests. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.010 in **B** and **C**, five comparisons; p values < 0.0125 in **D**, four comparisons).

4.4.5. Expression of NK cell differentiation markers is not modulated after BCG vaccination in 5-week-old infants.

We next sought to determine if frequencies of IFNγ^+ NK cells were associated with NK phenotypic and/ or differentiation marker expression. We measured CD57, CD158b, CD161 and CD8α expression on bulk cells and IFNγ^+ CD56^{hi}CD16^{lo} and CD56^{dim}CD16$^+$ NK cells (**Figure 29**). The two major NK subsets were selected because they were sufficiently frequent to allow reliable combinatorial analysis (**Figure 29A and Figure 29B**). No differences in expression of CD8, CD57, CD158b and CD161 on bulk or IFNγ^+ CD56^{dim}CD16$^+$ and CD56^{hi}CD16^{lo} NK cells between the BCG and no BCG groups were observed (**Figure 29C**).

4.4.6. Expression of cytokine receptors on NK cells is not altered after BCG vaccination in 9-week-old infants.

Since a major mechanism for NK cell function is by activation through cytokine stimulation, we also determined whether BCG vaccination leads to changes in cytokine receptor expression on NK cell subsets, and thereby give an indication of the responsiveness of NK cells to cytokines in the milieu. Expression of IL-18Rα (CD218a), IL-2Rβ (CD122), and IL-12Rβ1 (CD212) were measured, which have been shown to be among the cytokines that NK cells are most responsive to, in the no BCG and BCG groups in 9-week-old infants (**Figure 30A and Figure 30B**). As expected, the expression of CD218a was higher in the CD56^{++} subset compared to the other NK subsets (**Figure 30C**). CD218a, CD122 and CD212 expression were not different between the no BCG and BCG groups for any of the NK subsets (**Figure 30D**). Interestingly, CD218a expression was higher in IFNγ-expressing total NK cells when compared to bulk total NK cells in both the no BCG and BCG infant groups (**Figure 30E**). CD122 expression was also higher in IFNγ-expressing total NK cells than bulk total NK cells, but only in the BCG infant group (**Figure 30E**). CD212 expression on NK subsets was low overall and indistinguishable between NK subsets (**Figure 30C and Figure 30D**) but was higher than total CD3$^+$ T cells (**Figure 30C**), confirming successful CD212 antibody staining.

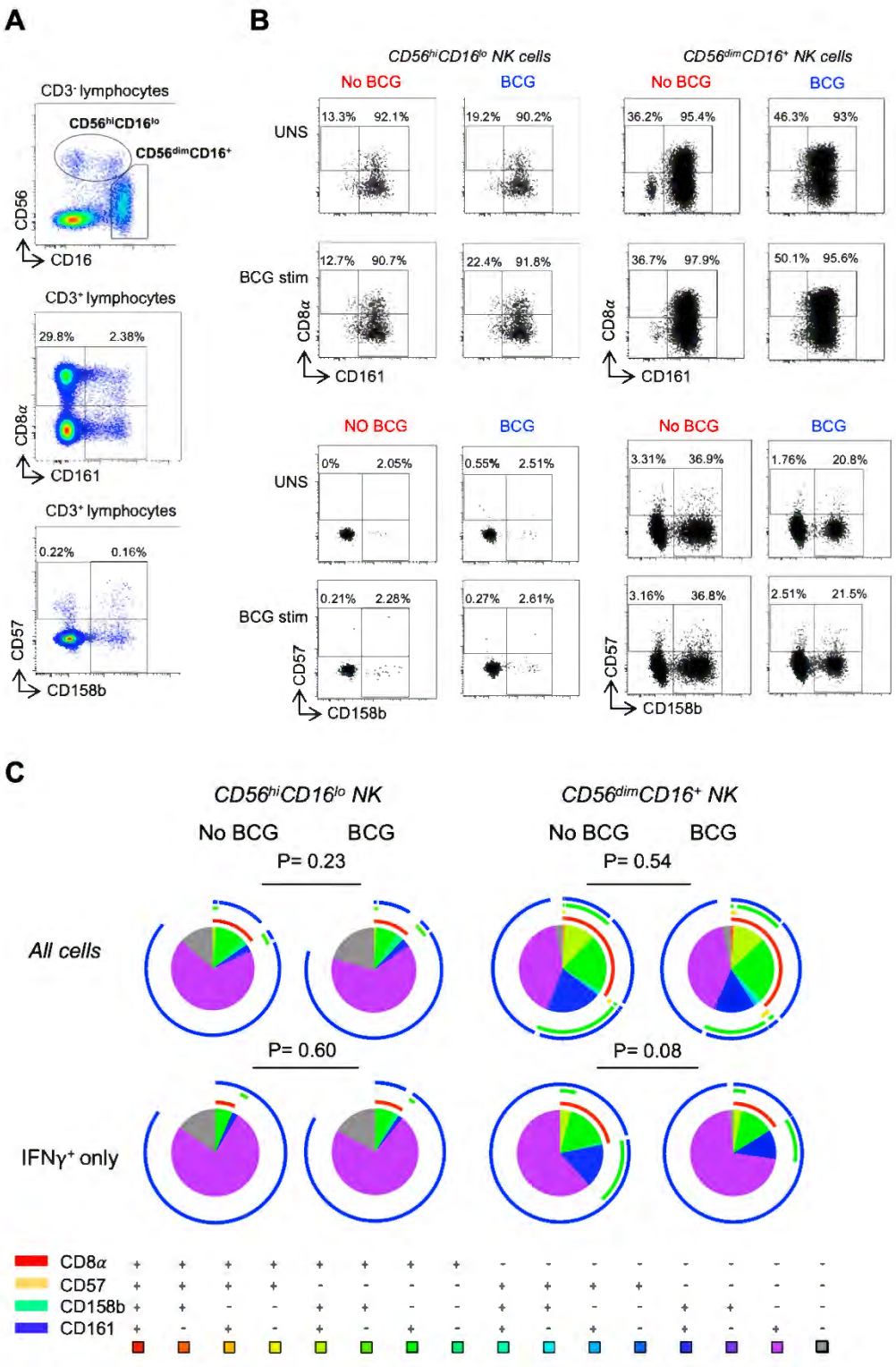


Figure 29. Expression of NK differentiation markers in 5-week-old infants. (A) Representative flow cytometry plots depicting the gating of NK subsets ($CD56^{hi}CD16^{lo}$ and $CD56^{dim}CD16^{+}$) based on CD56 and CD16 expression in a 5-week-old infant (top), CD8 versus CD161 in $CD3^{+}$ lymphocytes (middle) and CD57 versus CD158b in $CD3^{+}$ lymphocytes (bottom). **(B)** Representative flow cytometry plots showing CD8 versus CD161 expression in $CD56^{hi}CD16^{lo}$ NK (top left panel) and $CD56^{dim}CD16^{+}$ NK (top right panel) and CD158b versus CD57 in $CD56^{hi}CD16^{lo}$ NK (bottom left panel) and $CD56^{dim}CD16^{+}$ NK (bottom right panel) in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) infant. **(C)** Pie charts showing combinatorial expression of phenotypic markers (CD8, CD57, CD158b and CD161) as median proportions of $CD56^{hi}CD16^{lo}$ NK and $CD56^{dim}CD16^{+}$ NK cells (BCG stimulated condition). P-values < 0.05 were considered statistically significant.

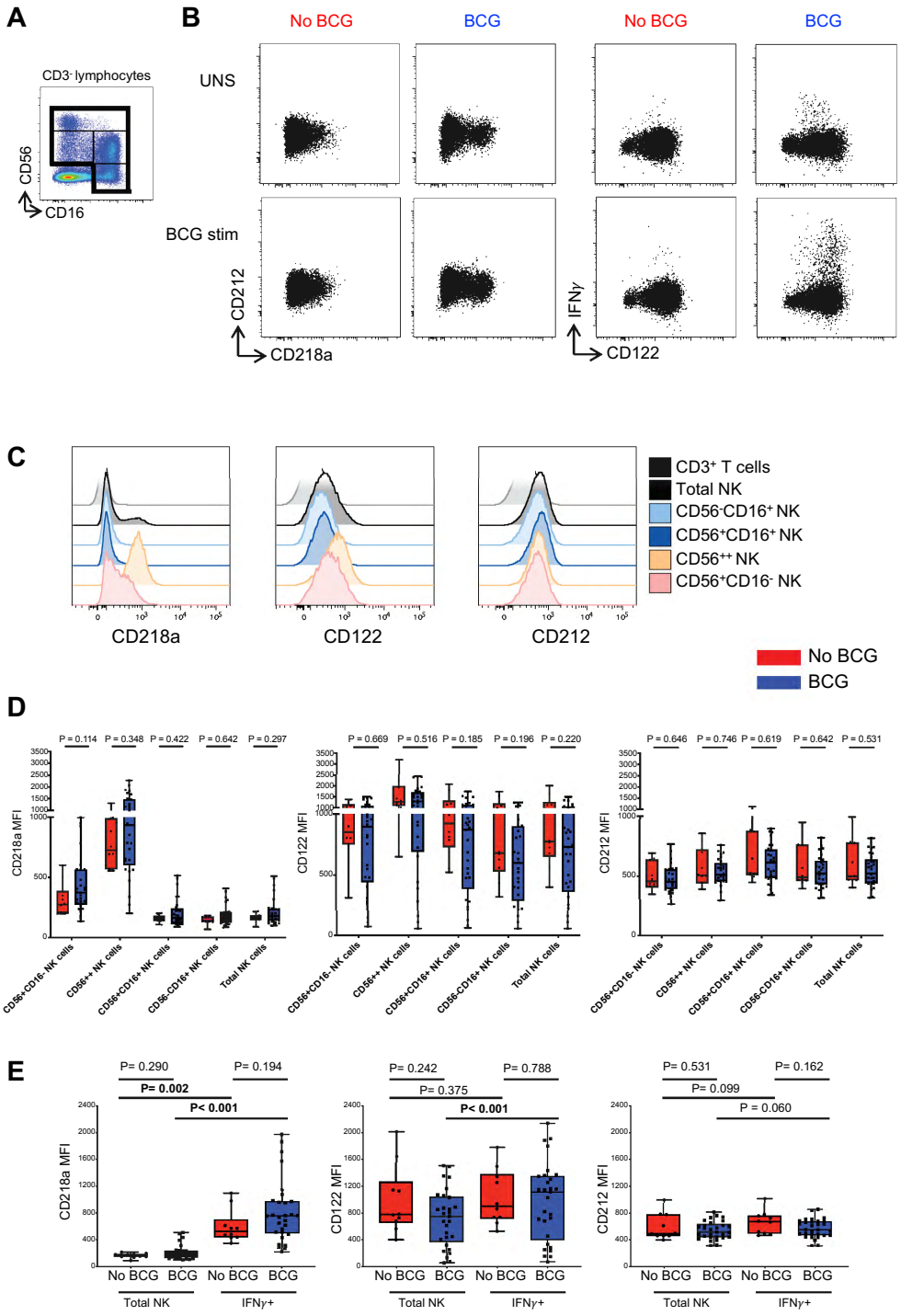


Figure 30. Cytokine receptor expression in 9-week-old infants. (A) Representative flow cytometry plot depicting the gating of NK subsets based on CD56 and CD16 expression. **(B)** Representative flow cytometry plots showing CD212 versus CD218 (left panel) and IFNγ versus CD122 in the total NK subset in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) infant. **(C)** Histograms showing relative expression of CD218a (left), CD122 (middle) and CD212 (right) in NK subsets and CD3$^+$ T cells in a 9-week-old infant (BCG stimulated condition). **(D)** CD218 (left) CD122 (middle) and CD212 (right) median fluorescence intensity (MFI) in infant NK subsets (BCG stimulated condition). **(E)** CD218a (left) CD122 (middle) and CD212 (right) MFI in bulk NK and IFNγ^+ total NK cells (BCG stimulated condition). Horizontal lines represent medians, boxes represent the interquartile range and whiskers represent the range. P-values were calculated with the Mann-Whitney U or Wilcoxon signed-rank tests. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.010 in **D**, five comparisons; p values < 0.0125 in **E**, four comparisons).

4.4.7. Host secreted Th1 cytokines are modulated by BCG vaccination in 5-week-old and 9-week-old infants.

Previous studies have shown that BCG vaccination can induce epigenetic changes in monocytes, which can lead to higher production of proinflammatory cytokines such as IL-1β, TNF and IL-6 in response to mycobacteria and unrelated organisms (Kleinnijenhuis et al., 2012). These studies have mostly been done in adults in TB non-endemic countries. We sought to detect similar changes, such as enhanced cytokine levels after BCG vaccination in infants from a TB endemic setting. In addition, we hypothesised that levels of monocyte-derived cytokines such as IL-12 and IL-18 would also be elevated after BCG vaccination. This could potentially explain the increased frequencies of BCG-reactive IFNγ-expressing NK cells after BCG vaccination (**Figure 26**) as these cytokines are potent activators of NK cells. We measured levels of 18 secreted cytokines in BCG-stimulated (above levels in unstimulated blood) in plasma samples from all study groups. Nine analytes were too low to be detected, including IL-12p70 (not shown). We initially analysed the 5-week-old and 9-week-old infants separately because the two groups not only differ in age but have received different types and numbers of childhood vaccinations as part of the Extended Programme for Immunisation, as previously shown (**Chapter 2, Figure 9**). Since there were no differences detected for each analyte in the two age groups, we combined the data for the secreted cytokines from 5- and 9-week-old infants in the no BCG and the BCG groups in order to maximise statistical power. IFNγ was higher in infants who received BCG vaccination compared to those who did not receive BCG (**Table 7**). There were no other differences between the no BCG and BCG infant groups for the other analytes measured (**Table 7**).

4.4.8. Host secreted cytokines positively correlate with BCG-reactive NK cells in the BCG vaccination group in 5-week-old and 9-week-old infants.

Next, we examined associations between BCG-reactive IFNγ^+ NK cells and levels of secreted cytokines. Interestingly, although there were no differences in secreted cytokine concentrations, except for IFNγ, between the infant groups (**Table 7 and Figure 31**), frequencies of BCG-reactive IFNγ^+ NK subsets correlated with secreted

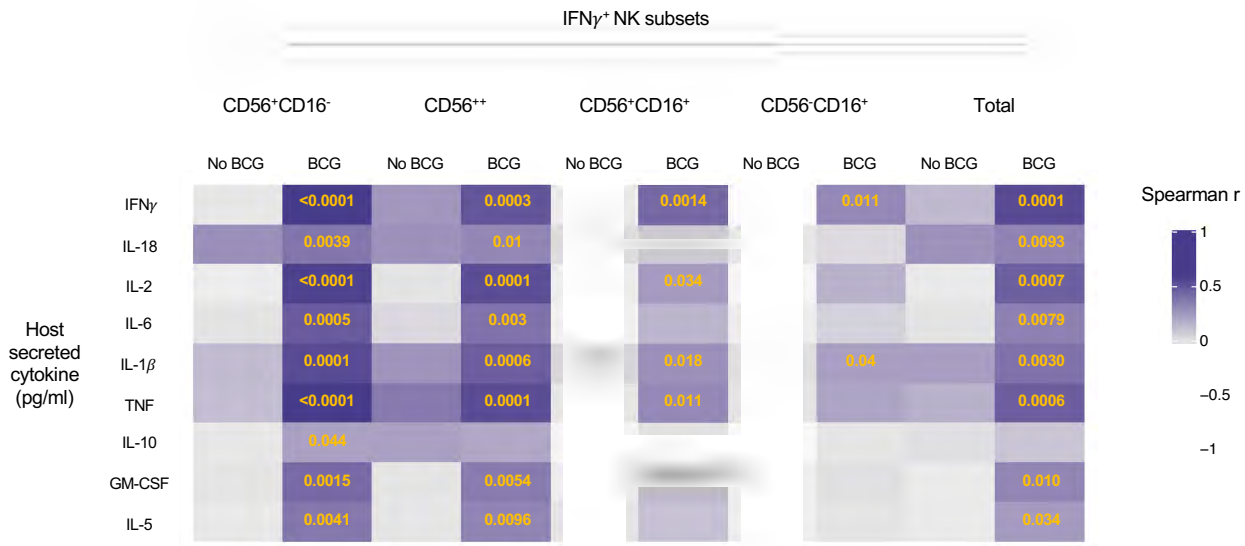
cytokines in the BCG group, but not in the no BCG group (**Table 8 and Appendix A**). In the BCG group only, IFNγ^+CD56$^+$CD16$^-$ NK cells positively correlated with all detectable analytes, (**Figure 31**, **Table 8 and Appendix A**), IFNγ^+CD56^{++} NK cells and IFNγ^+ total NK cells positively correlated with all detectable analytes except IL-10, while IFNγ^+CD56$^+$CD16$^+$ NK cells and IFNγ^+CD56$^-$CD16$^+$ NK cells positively correlated with only some of the cytokines (**Table 8 and Appendix A**). Defining the four distinct NK subsets based on their expression of CD56 and CD16 showed that CD56 expression by BCG-reactive NK cell subsets was an indicator of their responsiveness (association with) to soluble secreted cytokines in the BCG-vaccinated infant group (**Table 8 and Appendix A**).

Table 7. Host secreted cytokine concentration values in vaccinated (no BCG) and vaccinated (BCG) infants.

Host secreted cytokines (pg/ml)	No BCG	BCG	*p*	Bonferroni *p*
	n= 23	n=43		
Higher in BCG-vaccinated infants Median (LQ-UQ)				
IFNγ	0.89 (0.89-2.95)	26.78 (0.89-89.68)	< 0.001	0.011
No statistically significant difference Median (LQ-UQ)				
IL-18	7.74 (0.89-20.38)	13.39 (0.89-58.10)	0.18	1
IL-2	51.8 (41.75-66.38)	71.86 (47.63-171.05)	0.020	0.36
IL-6	8231.72 (3250.70-10741.68)	9272.25 (3250.70-10741.70)	0.38	1
IL-1β	605.51 (336.64-1961.32)	737.84 (341.23-2347.61)	0.72	1
TNF	1442.90 (607.74-2753.98)	1409.54 (648.66-4322.26)	0.52	1
IL-10	52.58 (14.22-123.15)	40.31 (24.02-102.12)	0.76	1
GM-CSF	63.45 (19.94-151.15)	61.48 (0.89-194.23)	0.86	1
IL-5	28.79 (22.04-80.92)	28.84 (10.58 – 109.15)	0.84	1

Host secreted cytokines were measured on thawed plasma samples from whole blood incubated with BCG or medium for 7 hours (no BCG= 23: n=13 5-week-olds, n= 10 9-week-olds; BCG= 43: n=15 5-week-olds, n=28 9-week-olds) using the Bio Plex™ platform. Values from unstimulated samples were subtracted from those values measured after BCG stimulation (BCG-UNS).
IL-12p70, IL-13, IL-17A, IL-21, IL-23, IL-4, IL-9, IL-22 and IL-27 were too low to be detected.
Cytokine values presented as median with interquartile range (IQR): median (IQR).
The p values were calculated with the Mann-Whitney U test and corrected for multiple comparisons with the Bonferroni method.

Table 8. Associations between host secreted cytokines and BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants.



Heat map showing Spearman correlation coefficients for host secreted cytokines and BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants (no BCG= 23: n=13 5-week-olds, n= 10 9-week-olds; BCG= 43: n=15 5-week-olds, n=28 9-week-olds). The coefficients with p values that were significant after correcting for multiple comparisons using the FDR method are shown.

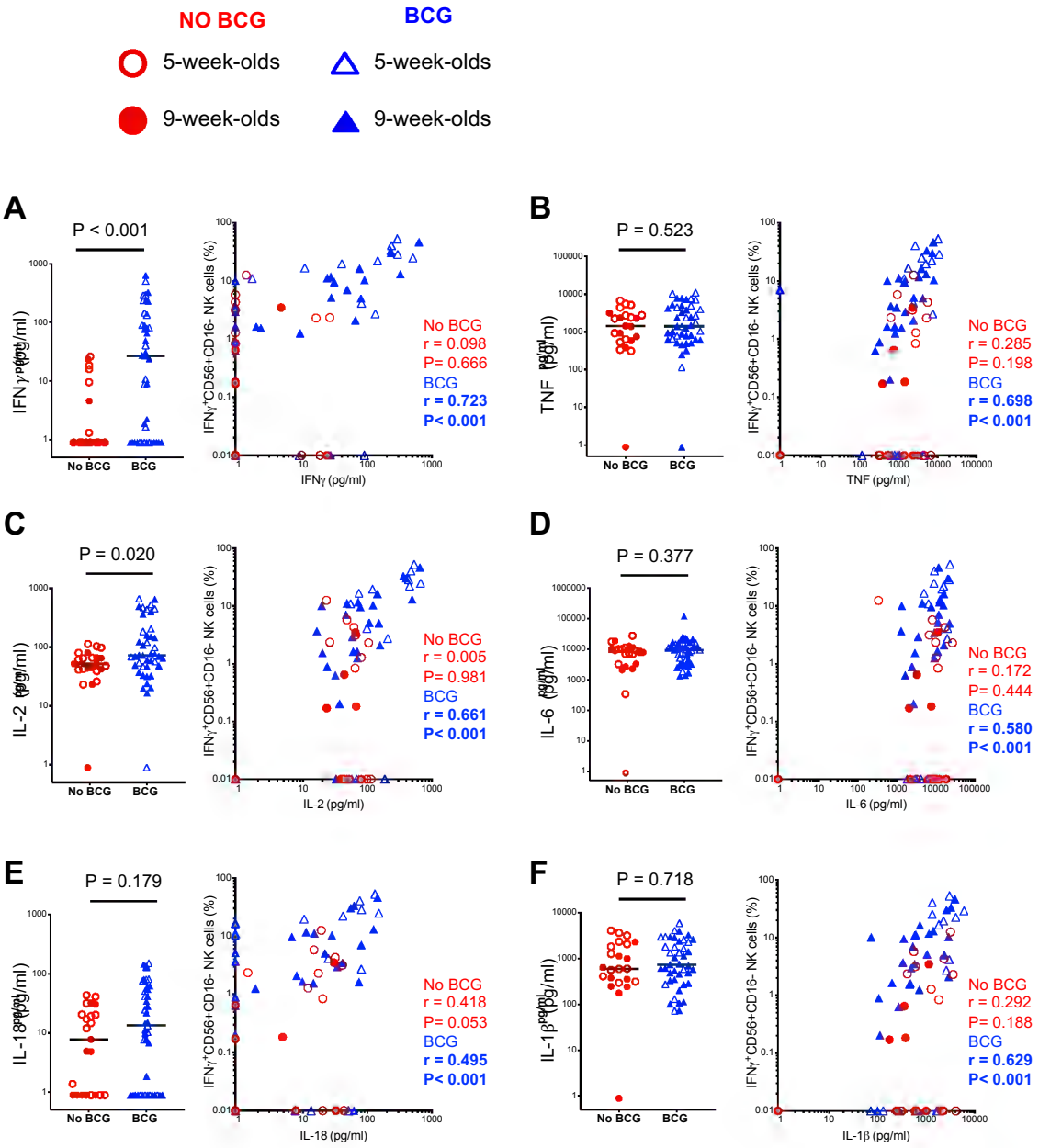


Figure 31. Host secreted cytokines correlated with CD56$^+$CD16$^-$ NK cells in 5-week-old and 9-week-old infants. (A) IFNγ **(B)** TNF **(C)** IL-2 **(D)** IL-6 **(E)** IL-18, **(F)** IL-1β levels (left) and correlations with BCG-reactive IFNγ+ CD56$^+$CD16$^-$ NK cells (right) in unvaccinated (no BCG, red) and vaccinated (BCG, blue) infants. Comparisons between the delayed (no BCG) and BCG vaccinated groups were done using the Mann-Whitney U test. Correlation coefficients and respective p-values were calculated using the nonparametric Spearman Correlation test. Unadjusted p-values are reported (see Supplementary Table 3 for FDR-adjusted p values).

4.5. Discussion

We investigated whether neonatal BCG vaccination modulates NK cell responses to mycobacteria in 5-week-old and 9-week-old infants. We observed the following: 1. Proportions of NK cell subsets in peripheral blood were not changed by BCG vaccination in 5-week-old and 9-week-old infants; 2. Frequencies of BCG-reactive IFNγ-expressing NK cells were higher in BCG-vaccinated compared to unvaccinated infants; 3. Perforin expression by NK subsets was not modified by BCG vaccination in infants; 4. Expression of NK cell differentiation markers were not modulated by BCG vaccination in 5-week-old infants; 5. Cytokine receptor expression on NK cells were not altered by BCG vaccination in 9-week-old infants; 6. Host secreted Th1 cytokines were increased after BCG vaccination in infants and; 7. Host proinflammatory secreted cytokines positively correlated with BCG-reactive NK cells in the BCG vaccination group only in infants.

The $CD56^{bright}$ NK subset is considered to be immature and through various maturation stages can become terminally differentiated $CD56^{dim}CD16^{+}CD57^{+}$ NK cells (Bjorkstrom et al., 2010, Lopez-Verges et al., 2010). We hypothesised that we would be able to detect lower numbers of $CD56^{bright}$ and higher numbers of the $CD16^{+}$ NK cell subsets following BCG vaccination because we reasoned that BCG would promote NK cell differentiation leading to maturation of NK cells. However, BCG vaccination did not modulate proportions of NK cell subsets in infants. In a previous study in adults, proportions of the two major NK cell subsets before and after BCG vaccination were also reported to be unchanged in PBMCs (Kleinnijenhuis et al., 2014b). It is important to note, however, that in our study we compared NK cells in whole blood in two separate groups of infants (BCG and no BCG). The results indicate that BCG vaccination does not alter the proportions of NK cell subsets.

Our observation that BCG-reactive IFNγ-expressing NK cells were increased by BCG vaccination in infants is consistent with the findings of a previous study of BCG revaccination in healthy, tuberculin skin test-positive South African adults (Suliman et al., 2016). In this study, BCG revaccination boosted frequencies of IFNγ-expressing BCG-reactive $CD56^{++}$ NK cells, which remained elevated above pre-vaccination levels

for at least one year. Other studies have reported increased frequencies of IFNγ-expressing NK cells after *in vitro* BCG stimulation (Watkins et al., 2008, Kativhu and Libraty, 2016, Portevin and Young, 2013, Esin et al., 2004, Batoni et al., 2005). In addition, NK cells from BCG vaccinated individuals had enhanced function following stimulation with mycobacterial and unrelated stimuli (Smith et al., 2017, Kleinnijenhuis et al., 2014b). Possible mechanisms underlying the induction of BCG-reactive IFNγ-expressing NK cells remain unknown, but may include: 1. vaccination-induced NK cell maturation, augmenting frequencies of IFNγ-expressing NK cells; 2. BCG-mediated upregulation of cytokine receptors on responsive NK cells, leading to elevated NK responsiveness to cytokines expressed by other cells; 3. Increased sensitivity of NK cells to bystander activation by BCG-specific IL-2-expressing $CD4^+$ T cells and/ or pro-inflammatory cytokines expressed by innate cells, such as IL-12 and IL-18; or 4. Direct training of NK cells through epigenetic modifications by BCG.

Previous studies have shown in LBW infants that BCG vaccination is able to protect against all-cause mortality and that this effect may be stronger in males early in life (Biering-Sorensen et al., 2018). We determined if BCG modulation of IFNγ-producing NK cells differed by sex and observed, overall, that cytokine responses were not more pronounced in males than females. At smaller sample sizes (when we separated the analysis by age) the differences, in general, were more easily detected in males. However, when we analysed both age groups together, we generally observed differences between the BCG and no BCG groups in both males and females.

NK cells are known for their cytotoxic potential and $CD16^+$ NK cells in particular are considered to be highly cytotoxic (Lanier et al., 1986). We used perforin as a measure of cytotoxic potential and hypothesised that we would detect higher expression of perforin in the BCG group in comparison to the no BCG group. However, perforin expression by NK cell subsets was not modified in infants in the BCG group compared to the no BCG group. The frequencies of perforin-positive NK cells were high overall regardless of vaccination status and it is likely that the cytotoxic potential could not be further increased by BCG vaccination. In a study comparing NK cell function between cord blood and adult PBMCs, similar frequencies of IFNγ-producing NK cells were reported, however, higher frequencies of perforin and granzyme B expressing NK cells

were present in cord blood, indicating that NK cells are highly cytotoxic at birth (Dalle et al., 2005). Interestingly, when we compared perforin MFI between 5-week-old and 9-week-old infants, regardless of vaccination status, there was significantly higher perforin expression in older infants for the $CD56^{++}$, $CD56^{+}CD16^{+}$ and $CD56^{-}CD16^{+}$ and total NK subsets. This result suggests that NK cells in 9-week-olds are more mature and therefore more cytotoxic than 5-week-old infants. In a sub-analysis in 9-week-olds, $IFN\gamma^{+}$ total NK had lower perforin expression than bulk NK cells in both the BCG and the no BCG groups. We postulate that this is a vaccine-independent effect and may be consistent with cytokine producing NK cells being less cytotoxic, or, alternatively that IFNγ-producing NK cells have degranulated perforin upon activation.

Consistent with the finding above, expression of NK cell differentiation markers was not modulated after BCG vaccination in 5-week-old infants. We hypothesised that the higher frequency of $IFN\gamma$-expressing NK cells in the BCG group was linked to changes in NK cell differentiation and/ or phenotype. Although there were no differences in differentiation marker expression between the BCG and no BCG groups, co-expression profiles were more diverse in $CD56^{dim}CD16^{hi}$ NK cells compared with $CD56^{hi}CD16^{lo}$ NK cells, which exhibited a predominantly $CD8^{-}CD57^{-}CD158b^{-}CD161^{+}$ phenotype, as shown before in adults (Suliman et al., 2016). The $CD56^{bright}$ subset typically has low expression of CD8, CD16 and CD57 (Nagler et al., 1989) and infants in particular express low levels of CD57 (Abo et al., 1984). It is not surprising that this subset is mostly CD161 single positive, with BCG having no effect on the markers measured, and CD161 being one of the first markers to be expressed during NK development (Montaldo et al., 2012).

We were also interested in exploring whether BCG vaccination changes the expression of key cytokine receptors (CD122, CD218a and CD212), which would give an indication of the potential responsiveness of NK cells to cytokines secreted by other immune cells upon BCG stimulation. NK cells are activated in response to cytokines such as IL-2, IL-18 and IL-12 and this leads to rapid proliferation and production of effector molecules (Lanier et al., 1985, Cooper et al., 2001c, Cooper et al., 2009, Romee et al., 2012). Individuals with a CD122 deficiency are more susceptible to severe BCG infections; a key function affected is IL-12-mediated induction of $IFN\gamma$

(Lichtenauer-Kaligis et al., 2003). We postulated that the increased expression of IFNγ by NK cells in the BCG vaccinated infants was linked to a BCG-mediated increase in cytokine receptor expression. However, cytokine receptors on NK cells were not altered between BCG vaccination groups in 9-week-old infants. A study in mice infected by aerosol with *M.tb* reported unchanged expression of CD122 on NK cells in the lung (Junqueira-Kipnis et al., 2003), indicating that perhaps mycobacterial infection does not alter total cytokine receptor expression on NK cells. The observation that CD218a expression was more highly expressed on IFNγ^+ total NK cells compared to bulk total NK cells could indicate that cells with higher levels of CD218a were preferentially activated and produced IFNγ (Romee et al., 2012, Lauwerys et al., 1999). Interestingly, higher expression of CD122 on IFNγ^+ NK cells compared to bulk NK cells was observed in the BCG group only, suggesting a vaccine-specific response.

The finding of higher levels of soluble IFNγ in the supernatant of BCG-stimulated whole blood is consistent with the increased T and NK cell responses observed in BCG vaccinated infants. However, the lack of difference in soluble IL-6, TNF and IL-1β in the BCG group compared to the no BCG group was unexpected, since other studies have reported enhanced production of these cytokines by BCG vaccination (Kleinnijenhuis et al., 2012, Kleinnijenhuis et al., 2014b). We also hypothesised that concentrations of IL-12 and IL-18, potent activators of NK cells (Cooper et al., 2001c, Cooper et al., 2009, Romee et al., 2012), would be increased in the BCG group, resulting in stronger by-stander activation of NK cells in vaccinated infants. This hypothesis is supported by studies showing that BCG vaccination induced trained immunity in monocytes, leading to enhanced functional responses including higher expression of cytokines, upon *in vitro* re-stimulation to mycobacteria and unrelated organisms (Kleinnijenhuis et al., 2012, Netea et al., 2016). However, it is important to note that these studies were mostly performed on purified monocytes and isolated from adults in TB non-endemic settings. It is probable that baseline responses in non-endemic settings are lower, thus the magnitude of change that BCG can induce is higher and more easily detectable. In a study in infants from the UK, levels of secreted IL-6 and TNF after 48-hour stimulation of whole blood with *M.tb* lysate was higher in BCG-vaccinated infants compared to unvaccinated infants, while no difference was

found for IL-1β (Smith et al., 2017). As previously mentioned, in a recent study conducted in Uganda (Prentice et al., 2021), where BCG vaccination was associated with lower incidence of infectious diseases other than TB, the authors attempted to link these effects to trained immunity. They measured histone modifications at promoters of inflammatory genes in total PBMCs, as well as secreted cytokines upon 24-hour stimulation of whole blood with mycobacteria and unrelated organisms. Surprisingly, they did not find any differences in the occupancy of H3K4me3 at the *il6* or ilβ promoters in vaccinated compared to unvaccinated infants, differing from previous results (Kleinnijenhuis et al., 2012, Arts et al., 2015, Arts et al., 2016). In addition, there were no differences in levels of secreted TNF, IL-6, IL-1β, IL-10 or IFNγ in response to unrelated organisms in the BCG compared to the no BCG groups, however, differences were found in response to *M.tb* PPD stimulation.

It is possible that the 7-hour stimulation time point at which supernatants were harvested in our WB-ICS assay protocol was suboptimal to detect differences in secreted cytokine levels. In other studies of delayed BCG vaccination and in which cells were stimulated for 7 hours, few analytes, mainly Th1 and Th2 cytokines, were measured, showing that levels of secreted TNF (Ritz et al., 2016) were higher in the BCG group while IL-10 (Lutwama et al., 2014) was higher in the no BCG group. Despite differences in study design and location, other studies in delayed BCG cohorts measuring heterologous effects in infants (with larger bead array panels and in which the duration of stimulations were 24 hours or longer) showed higher levels of some of the same innate and Th1 cytokines in vaccinated compared to unvaccinated infants after mycobacterial stimulation (Jensen et al., 2015, Smith et al., 2017, Freyne et al., 2020, Prentice et al., 2021). Although these technical differences may explain the discrepancies between our results and those published by others, we were mostly interested in myeloid-derived cytokine responses, which would peak at an earlier time-point than 24 or 48 hours.

A limitation of our study was that we did not include stimulations with other pathogens, which would allow assessment of innate training. The small volume of blood that can be safely collected from infants for immunological studies did not allow measurement of additional time points or stimuli. Despite these limitations, levels of IL-2 secreted

upon whole blood stimulation with BCG, most likely produced by T cells, were strongly correlated with frequencies of BCG-reactive IFNγ-expressing NK cell subsets in the BCG group, but not in unvaccinated infants. This is in line with other studies that have reported an association between IL-2 expressing $CD4^+$ T cells and NK cell functional responses after vaccination (Horowitz et al., 2010a, Horowitz et al., 2010b, Jost et al., 2014, Penn-Nicholson et al., 2015). We also found strong positive correlations in vaccinated infants between BCG-reactive IFNγ-expressing NK cells and IL-6, IL-1β and TNF, hallmark innate cytokines of trained immunity (Kleinnijenhuis et al., 2012). We speculate that, despite observing no differences between the BCG vaccinated and unvaccinated infants for these key cytokines, BCG vaccination does enhance crosstalk between innate and adaptive immune cells, resulting in strong correlations observed between BCG-reactive functional NK cells and the secreted cytokines. As previously mentioned, revaccination of *M.tb* infected adults with BCG is associated with increased frequencies of BCG-reactive IFNγ^+ NK cells up to 1 year post vaccination; these responses were dependent on IL-12 and IL-18 (Suliman et al., 2016). However, in the present infant analyses, correlation between IL-18 and IFNγ-expressing NK cells was weak, and soluble IL-12 levels were not detected with the kit we used. Another limitation is the cross-sectional design of our study, which precluded paired comparisons before and after BCG stimulation.

Overall, our data shows that BCG vaccination is associated with increased frequencies of BCG-reactive IFNγ-expressing NK cells in 5-week-old and 9-week-old infants. We observed that IFNγ-expressing NK cells were positively correlated with Th1 cytokines, IL-2 and IFNγ, and the innate proinflammatory cytokines IL-6, IL-1β and TNF in BCG vaccinated infants only. These associations could be linked to higher levels of IFNγ and IL-2 in BCG-stimulated supernatants, although IFNγ was the only cytokine that remained significant after correction for multiple comparisons. Interestingly, there were no changes in the levels of IL-6, IL-1β and TNF in BCG-stimulated supernatants. Also, the induction of BCG-reactive IFNγ-expressing NK cells after BCG vaccination could not be linked to NK cell maturation, differentiation or cytokine receptor expression changes; the underlying mechanism therefore requires further study.

4.6. Contributions to this chapter

Multiplex bead array experiments were carried out by Melissa Murphy with the assistance of Candice I. Snyders, Ilana C. van Rensburg and Assoc. Prof Novel N. Chegou.

Flow cytometry experiments were carried out by Melissa Murphy.

All analyses were carried out by Melissa Murphy under the supervision of Dr. Sara Suliman, Assoc. Prof. Elisa Nemes and Prof. Thomas J. Scriba.

Chapter 5: Infant myeloid cell functional responses induced by BCG vaccination to whole cell mycobacteria

5.1. Introduction

Studies investigating immune responses elicited by BCG in infants are limited and largely focused on T lymphocytes (Hussey et al., 2002, Murray et al., 2006, Soares et al., 2008, Soares et al., 2013, Kagina et al., 2010, Ritz et al., 2012). A previous study in BCG-vaccinated infants from Worcester, Cape Town, showed that the proinflammatory cytokine response by monocytes, when stimulated with mycobacteria, increased with age (Shey et al., 2014). In this chapter we investigated myeloid cell responses, we were particularly interested in monocyte responses, to BCG vaccination in 5-week-old and 9-week-old infants.

The host innate immune response plays an important role in preventing the early establishment of an *M.tb* infection (Sia et al., 2015). This is supported by case contact studies which show that approximately half of *M.tb* exposed individuals remain negative to TST (Morrison et al., 2008). In this instance, it is likely that when *M.tb* is inhaled it is contained and cleared before the onset of adaptive immunity. The binding of PRRs to PAMPs enables innate immune cells to recognise microbial molecular structures as non-self antigens and generate an immune response (Medzhitov and Janeway, 2002). This response has been classified as not being antigen-specific and not possessing immunological memory.

Previous thinking in the field was that the innate immune response is limited to providing the first line of defence against pathogens and the initiation of the adaptive immune response. Experimental evidence, however, suggests that innate cells can also elicit pathogen-specific responses and enhanced secondary responses through trained innate immunity (Netea et al., 2016, Moorlag et al., 2020b). As previously discussed, BCG vaccination can induce trained immunity through epigenetic changes in monocytes, which can lead to higher production of proinflammatory cytokines in response to mycobacteria and unrelated organisms (Kleinnijenhuis et al., 2012). NK

cells have also been shown to exhibit adaptive properties (discussed in detail in chapter 1). A recent study by Moorlag et al. reported that BCG vaccination of adults induced increased neutrophil expression of activation markers and enhanced antimicrobial function, which were associated with epigenetic modifications (Moorlag et al., 2020b). Interestingly, Cirovic et al. showed that BCG vaccination elicited a myeloid-associated transcriptomic signature 90 days after BCG vaccination in the hematopoietic stem and progenitor cell compartments in the bone marrow of adults. In the same study, a randomised controlled trial in infants showed an increased number of neutrophils in peripheral blood of the infant group that received BCG shortly after birth compared to BCG-unvaccinated infants (Cirovic et al., 2020).

As discussed in chapter 1, recognition of *M.tb* by myeloid cells leads to cell activation and the production of cytokines, amongst other functions, which play an important role in the outcome of mycobacterial infections (Cooper and Khader, 2008). Cell surface CD33 expression can be used as a defining marker to identify myeloid cells, which is expressed at high levels on myeloid progenitor cells. Its expression is sustained on monocytes, including during their differentiation into macrophages in the tissues. CD33 is also continuously expressed on myeloid DCs, although downregulation is observed on granulocytes with maturation although they continue to express CD33 at low levels (Freeman et al., 1995, Kelm et al., 1996).

BCG is a potent mediator of monocyte training, which has been extensively studied (Kleinnijenhuis et al., 2012, Arts et al., 2016, Arts et al., 2015, Kaufmann et al., 2018). Thus, we were interested in investigating BCG vaccination effects on monocytes, in particular, in infants in a TB endemic setting. We used the CD33 myeloid marker instead of CD14 to delineate monocytes because of technical difficulties in our flow cytometry staining assay (discussed in detail below).

5.2. Aims

In this chapter we investigated $CD33^+$ myeloid cell functional responses to mycobacterial stimulation in BCG vaccinated compared to unvaccinated infants. The following aims were addressed:

1. To investigate infant myeloid effector functions induced by BCG vaccination in 5-week-old and 9-week-old infants.

We hypothesised that myeloid cell expression of proinflammatory cytokines would be higher in BCG-vaccinated compared to unvaccinated infants.

2. To determine whether BCG vaccination modulates the cross-talk between $CD33^+$ myeloid and adaptive immune cell subsets.

We hypothesised that BCG vaccination would enhance the cross-talk between CD33+ myeloid cells and adaptive immune cells in the BCG vaccinated infants only,

5.3. Materials and methods

Study participants

Infant samples used in this chapter were collected from the delayed-BCG and the birth-vaccination cohorts, as described in chapter 2.

WB-ICS assay

Heparinised whole blood for the standardised 12-hour WB-ICS assay was processed as described in chapter 2.

Flow cytometry

Cryopreserved whole blood samples were thawed, washed in PBS, permeabilised in Perm/Wash buffer (BD Biosciences) and stained with the flow cytometry panels as previously described in (**Chapter 2, Tables 2-4**) for 45 minutes at 4℃.

Data shown in this chapter are from infant samples stained with panel 1 (**Chapter 2, Table 2**), panel 2 (**Chapter 2, Table 3**) and panel 3 (**Chapter 2, Table 4**) as described in the materials and methods section in chapter 3.

5.4. Results

5.4.1. Frequencies of BCG-reactive CD33$^+$ myeloid cells are not altered by BCG vaccination in infants.

As stated above, we were interested in investigating the CD14$^+$ monocyte functional response to BCG vaccination in infants. We were unable to use the CD14$^+$ marker to stain monocytes during flow cytometry experiments because of massive downregulation of CD14 expression on BCG-stimulated cells from our 12-hour WB-ICS. We, therefore, used the CD33 marker and based on immune cell characteristics, were able to use our flow cytometry gating strategy to exclude non-CD33$^+$ immune cells and granulocytes (which are large in size and express high levels of CD16) (**Figure 32A**). The cell population of interest in the CD33$^+$ gate would primarily consist of CD14$^+$ monocytes and a very small population of myeloid DCs. We felt confident that this population was representative of CD14$^+$ monocytes in peripheral blood.

To determine the modulatory effects of BCG vaccination on myeloid cell function, we measured frequencies of myeloid cells expressing IL-6, IL-1β and/ or TNF in response to BCG stimulation in 5-week-old and 9-week-old infants who did (BCG) or did not (no BCG) receive BCG vaccination at birth. We hypothesised that higher frequencies of myeloid cells would express the cytokines measured in the BCG compared to the no BCG groups. Upon BCG re-stimulation, IL-6, IL-1β and TNF expression was highly upregulated compared to unstimulated myeloid cells (**Figure 32B and Figure 32C**). However, there were no differences in BCG-induced cytokine expression (IL-1β, IL-6 and TNF) by myeloid cells between the no BCG and BCG groups in 5-week-old and 9-week-old infants (**Figure 32D and Figure 32E**). There were also no differences in cytokine MFI on myeloid cells between the groups (**Figure 32F and Figure 32G**).

When we compared frequencies of cytokine-expressing myeloid cells in the two age groups, irrespective of vaccination status, 9-week-old infants had higher TNF expression than 5-week-old infants (**Figure 32H**). There were no differences between the two age groups for the other cytokines (IL-6 and IL-1β) measured (**Figure 32H**).

We also compared cytokine co-expression (IL-6, IL-1β and/ or TNF) by myeloid cells between the infant vaccination groups in 5-week-old and 9-week-old infants (**Figure 33**). No differences were detected in the myeloid polyfunctional cytokine response in the infant groups (**Figure 33**).

5.4.2. Crosstalk between myeloid and adaptive lymphoid cells is enhanced by BCG vaccination.

It is well established that the innate immune response is responsible for the induction of the adaptive immune response and influences the activation and function of surrounding immune cells. To determine if BCG vaccination modulates the crosstalk between innate and adaptive immune subsets, we correlated BCG-reactive myeloid cells with functional BCG-reactive $CD4^+$ T cells, $\gamma\delta$ T cells, $CD20^+$ B cells, NK cells and $CD3^+CD26^{hi}CD161^{hi}$ T cells in the BCG and no BCG group in 5-week-old and 9-week-old infants (**Figure 34**). Strong associations were evident between frequencies of cytokine-expressing myeloid cells and the conventional adaptive cells in the BCG group only but not for the other immune subsets (**Figure 34**). Positive correlations were observed for TNF^+CD33^+ myeloid and $IFN\gamma^+CD4^+$ T cells, TNF^+CD33^+ myeloid and TNF^+CD20^+ B cells, $IL\text{-}6^+CD33^+$ myeloid and $IL\text{-}6^+CD4^+$ T cells, $IL\text{-}6^+CD33^+$ myeloid and $IL\text{-}6^+CD20^+$ B cells, $IL\text{-}6^+CD33^+$ myeloid and TNF^+CD20^+ B cells, including $IL\text{-}1\beta^+CD33^+$ and $IL\text{-}1\beta^+CD20^+$ B cells in the BCG group only (**Figure 34**).

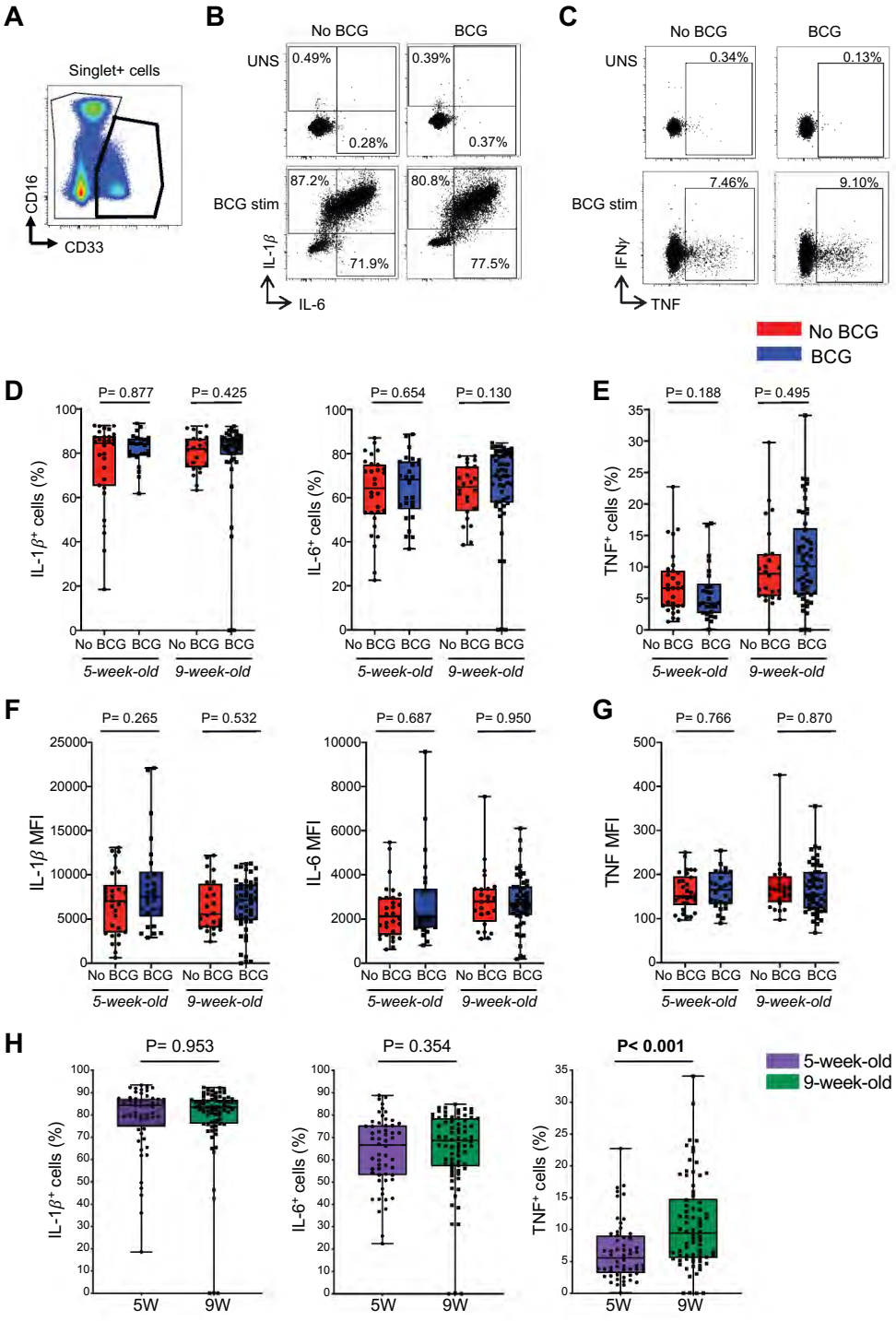


Figure 32. Frequencies of BCG-reactive CD33$^+$ myeloid cells in peripheral blood in 5-week-old and 9-week-old infants. Representative flow cytometry plots showing (**A**) the gating of CD33+ myeloid cells (**B**) IL-1β versus IL-6 and (**C**) IFNγ versus TNF expression in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. Frequencies of (**D**) IL-1β (left), IL-6 (right) and (**E**) TNF expression by CD33$^+$ myeloid cells in 5-week-old and 9-week-old infants. **(F)** IL-1β (left), IL-6 (right) and (**G**) TNF MFI expression by CD33$^+$ myeloid cells in 5-week-old and 9-week-old infants. (**H**) **)** IL-1β (left), IL-6 (middle) and TNF (right) expression by CD33$^+$ myeloid cells in 5-week-old and 9-week-old infants irrespective of BCG vaccination status (BCG and no BCG groups combined). Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.0167 in **G**, three comparisons).

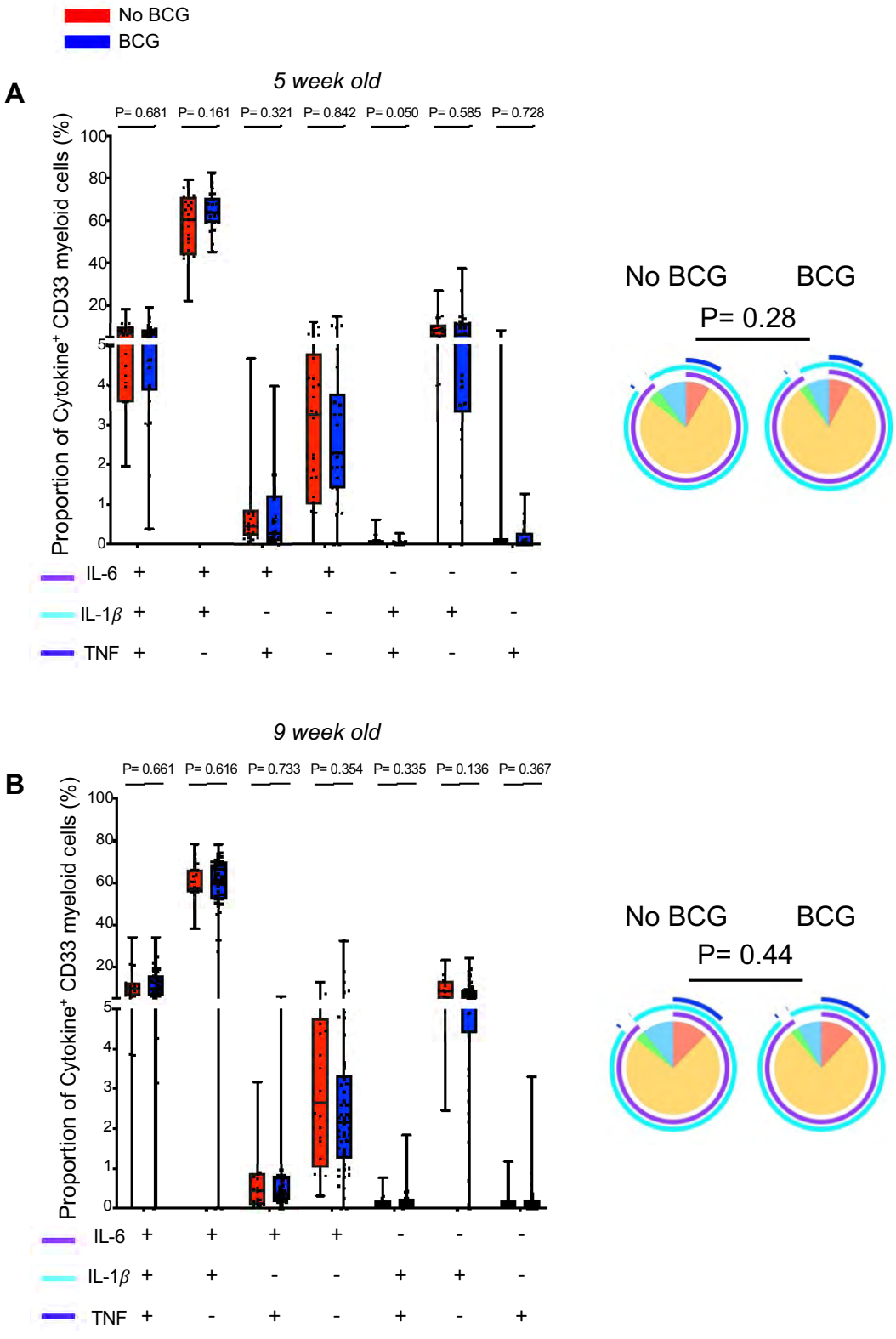


Figure 33. Frequencies of cytokine co-expressing CD33$^+$ myeloid cells in response to BCG stimulation in peripheral blood in 5-week-old and 9-week-old infants. (left) Frequencies of myeloid cells co-expressing proinflammatory cytokines (IL-6, IL-1β and/ or TNF) in response to BCG stimulation and (right) pie charts showing combinatorial expression of proinflammatory cytokines (IL-6, IL-1β and/ or TNF) as median proportions of myeloid cells in (**A**) 5-week-old and (**B**) 9-week-old infants. P-values < 0.05 were considered statistically significant.

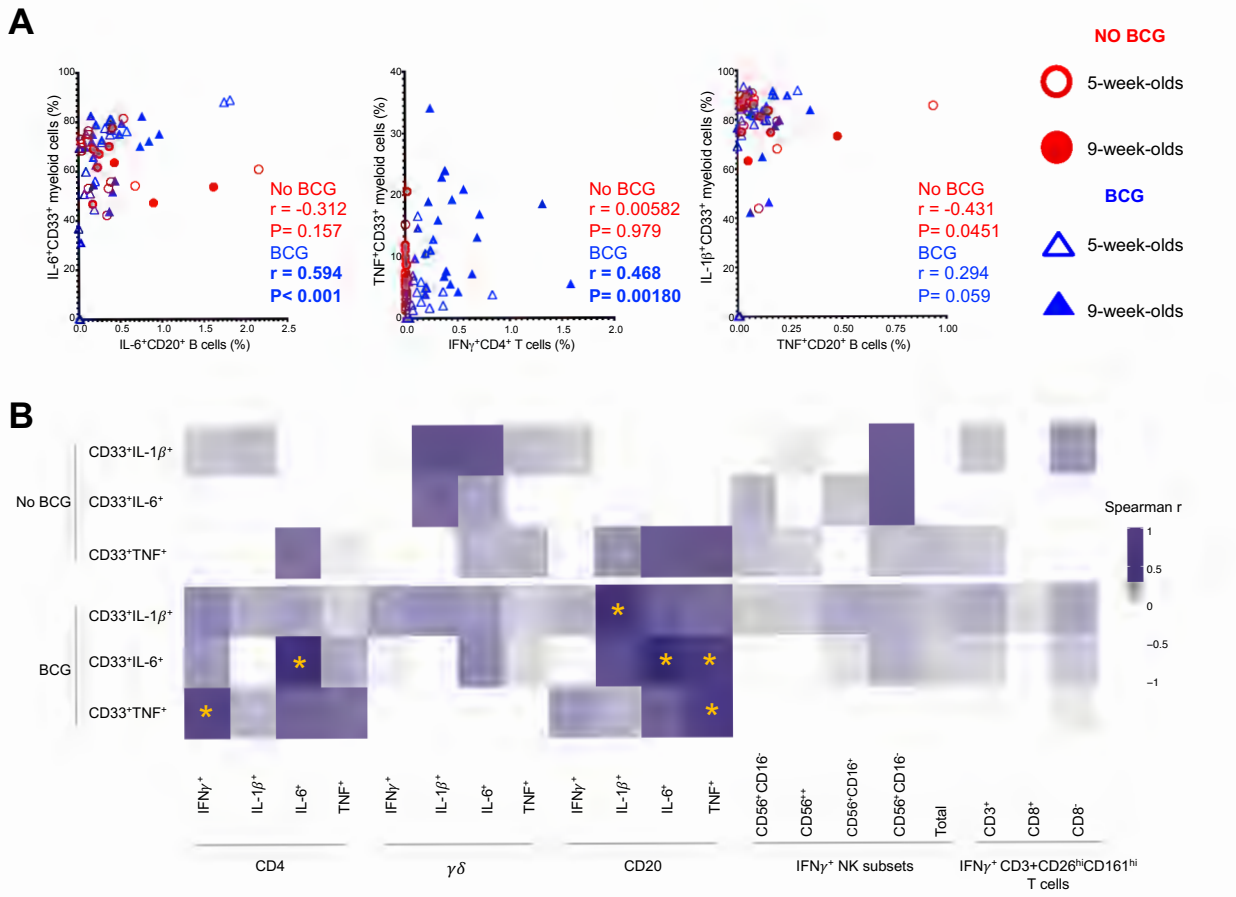


Figure 34. Myeloid cell functional responses associate strongly with functional adaptive immune cells. (**A**) Correlations between frequencies of (left; strong correlation) IL-6$^+$CD20$^+$ B cells and IL-6$^+$CD33$^+$ myeloid cells (middle; intermediate correlation) IFNγ^+CD4$^+$ T cells and (right; weak correlation) TNF$^+$CD20$^+$ B cells and IL-1β^+CD33$^+$ myeloid cells. (**B**) Heatmap showing spearman correlations shown for BCG-reactive immune subsets in 5-week-old and 9-week-old infants. Spearman coefficients with corresponding p values that are significant after correcting for multiple comparisons using the FDR method are shown with asterisks. Correlation coefficients and respective p-values were calculated using the nonparametric Spearman Correlation test. Bolded p-values were considered significant after correcting for multiple comparison testing using the FDR method.

5.5. Discussion

Comprehensive immune studies investigating BCG-mediated modulation of immune cell types beyond conventional T cells in infants are lacking. We sought to better understand the myeloid immune response, with a particular interest in monocyte responses, to BCG vaccination in infants. In this chapter, we showed that $CD33^{+}$ myeloid cell functional responses are not different between BCG-vaccinated and unvaccinated 5-week-old and 9-week-old infants in response to mycobacterial stimulation. We also reported that myeloid and adaptive cell functional responses strongly associate with one another in BCG-vaccinated infants only.

Other studies which have also investigated immune responses to mycobacteria in infants in a TB endemic setting in which BCG vaccination has been delayed have largely focused on T cell responses and/ or host secreted cytokine levels (Marchant et al., 1999, Hussey et al., 2002, Burl et al., 2010, Kagina et al., 2010, Lutwama et al., 2014, Blakney et al., 2015, Jensen et al., 2015, Tchakoute et al., 2015, Subbian et al., 2020). In a study by Smith et al., infants from the UK displayed higher expression of CD11b and CD206 by monocytes in BCG-vaccinated infants compared to the unvaccinated arm in response to *M.tb* lysate, however, intracellular cytokine staining of monocytes was not assessed (Smith et al., 2017). To our knowledge, this is the first study to investigate myeloid cell intracellular cytokine expression in response to whole cell mycobacteria in BCG vaccinated and unvaccinated infants from a TB endemic setting.

Our results showing that there were no differences in frequencies of myeloid cytokine (IL-6, TNF and/ or IL-1β) expression, co-expression or MFI between the BCG and no BCG groups were unexpected. The cytokines measured have been linked to BCG-mediated trained immunity, thus our hypothesis was that higher levels of IL-6, TNF and IL-1β expression would be observed in the BCG group compared to the no BCG group. However, these results correspond with our previous results (**Chapter 4, Table 7**) showing that secreted levels of IL-6, TNF and IL-1β, in response to BCG stimulation, were also not different in the two vaccination groups in 5-week-old and 9-week-old infants. It is known that particular innate functions in newborns are reduced in

comparison to adults (Vanden Driessche et al., 2013) which could account for this surprising result in infants. It is plausible that these reduced functions could affect infant myeloid immune responses to BCG vaccination and result in changes too small to detect. In addition, it is possible that our study sample size was too small and therefore underpowered to detect these small differences in cytokine production in the two groups of infants. Also, the high multiplicity of infection of BCG used in the assay, which is a potent stimulus, could mask subtle differences that may have been detectable at sub-saturating doses of BCG. It is also possible that by broadly measuring myeloid cell responses, which are composed of granulocytes, monocytes, macrophages and dendritic cells, small and distinct differences between the BCG and no BCG infant groups in discrete immune subsets would not be detected. However, we excluded granulocytes in our flow cytometry gating strategy of immune subsets for panel 3 (**Chapter 2, Figure 12**), therefore monocytes and DCs would be the two subsets detected in peripheral blood, of which monocytes would be the predominant subset. Previous studies have shown that BCG vaccination can induce epigenetic changes in monocytes, which can lead to higher production of IL-6, TNF and IL-1β (Kleinnijenhuis et al., 2012). These studies have mainly been done in adults; it is possible that BCG-training is not as efficient in the first few hours of life which could account for the similar cytokine production by myeloid cells in the BCG and no BCG group in our infant cohort. Previous trained immunity studies have been carried out on isolated monocytes, whereas we measured $CD33^+$ myeloid cells. On the other hand, in this chapter we show cytokines on a per cell level and not secreted cytokines measured by enzyme-linked immunosorbent assay or multiplex bead array, which is what was done for these studies. As discussed in chapter 4, a limitation in our study is that we did not include a stimulation condition containing an organism unrelated to mycobacteria, which is the typical experimental approach to show non-specific enhanced function by innate cells after BCG vaccination. It is also possible that the stimulation time of our 12-hour WB-ICS assay was suboptimal, likely too long, to detect differences in myeloid cells between the two groups of infants and that a shorter stimulation time was needed. Indeed, in previous studies conducted in our lab, a 6-hour stimulation was optimal for intracellular detection of cytokines produced by myeloid cells (Shey et al., 2012). As previously discussed in chapter 4, studies in infants are difficult to carry out and yield low blood volumes, we therefore had limited sample availability to carry out different stimulations and stimulation times for the

different study endpoints. Since we intended to measure cytokine production by different cell types ($CD33^+$ myeloid, NK, $CD4^+$ T, phenotypic MAIT, $\gamma\delta$ T and $CD20^+$ B cells), we decided to process whole blood collected using our standard 12-hour WB-ICS assay with three stimulation conditions.

Of interest is our data which revealed that myeloid cell and adaptive cell functional responses strongly associated with one another in the BCG-vaccinated infant group alone. Interestingly, functions between myeloid cells correlated with $CD4^+$ T and $CD20^+$ B cells but not NK cells and the other T cell subset measured. This might be indicative of BCG-mediated enhanced cross-talk between myeloid and conventional adaptive cells. It is also known that BCG vaccination induces Th1 cytokine responses in infants that are comparable to adults (Marchant et al., 1999). Th1 cytokines act on myeloid cells which in turn leads to cell activation and killing of intracellular pathogens (Kaufmann, 2002). It is also likely that our data is suggestive of this vaccine-specific response to mycobacterial stimulation.

Studies investigating the innate immune responses in infants have largely focused on responses to purified TLR ligands (Kollmann et al., 2009, Corbett et al., 2010, Nguyen et al., 2010, Burl et al., 2011, Reikie et al., 2012). These studies show differing responses based on population, age and stimulation condition. In addition, many studies use cord blood samples as a proxy for the young infant immune response. A study by Olin et al. reported that major changes occur in plasma protein levels, immune cell proportions and immune cell phenotypes during the first few days of life that cannot be predicted from cord blood measurements (Olin et al., 2018). This study suggests that cord blood samples should not be used as a proxy for immune responses in young infants. Therefore, it is important that more comprehensive studies be carried out in infants in the first few weeks of life. Shey et al. investigated maturation of monocyte and myeloid DC responses to whole cell mycobacteria in infants over the first year of life. Monocyte expression of TNF, IL-6 and IL-12p40 increased with age in response to BCG stimulation. Increased expression of CD40 with age was also shown in myeloid DCs and monocytes (Shey et al., 2014). This is in line with our observation that frequencies of myeloid cells expressing TNF was higher in 9-week-old infants than 5-week-old infants regardless of vaccination status. However, there were no differences in IL-6 and IL-1β expressing myeloid cells between the two age

groups. Altogether these studies show that the newborn and infant immune response are distinct and further studies are required to better understand the postnatal immune response to mycobacteria.

Our data shows that there are no differences in myeloid cell production of IL-6, IL-1β and TNF in response to mycobacterial stimulation between BCG vaccinated and unvaccinated infants in 5-week-old and 9-week-old infants. Our data suggests that BCG effects (potentially including trained immunity) on myeloid cells may be subdued in infants due to their distinct immune response in early life. Mores studies are needed in infants to understand these differences, so that reduced innate immune functions may be overcome with future TB interventions.

5.6. Contributions to this chapter

Multiplex bead array experiments were carried out by Melissa Murphy with the assistance of Candice I. Snyders, Ilana C. van Rensburg and Assoc. Prof. Novel N. Chegou.

Flow cytometry experiments were carried out by Melissa Murphy.

All analyses were carried out by Melissa Murphy under the supervision of Dr. Sara Suliman, Assoc. Prof. Elisa Nemes and Prof. Thomas J. Scriba.

Chapter 6: Host immune cell subsets associated with inflammation, disease severity and aerosolised *M.tb* in TB patients

6.1. Introduction

The previous chapters addressed immune responses induced by BCG vaccination in healthy infants. In this chapter we investigated immune responses that are associated with inflammation, TB disease severity and aerosolisation of *M.tb* in adults with active TB disease. A better understanding of the immunogenicity of BCG in infants is imperative as infants are very susceptible to TB. However, adults are the main drivers of transmission in the TB epidemic, thus this part of the thesis focuses on host determinants of *M.tb* transmission.

6.1.1. Overview of M.tb transmission

There are multiple steps involved in the successful transmission of *M.tb* from an infectious individual to a new host **(Figure 35)** (Churchyard et al., 2017). If host and pathogen interactions result in unsuccessful containment of *M.tb* infection, growth of *M.tb* in infected lung tissue or draining lymph nodes occurs. Progression through these events, from early infection to TB disease, can take months to years and is characterised by inflammation and lung immunopathology driven by the host immune response itself (BoseDasgupta and Pieters, 2014, Ehlers and Schaible, 2012). In certain cases, granulomas in the lung may become caseous and cavitary (discussed in chapter 1), leading to favourable environmental conditions for the bacilli, such as oxygen availability, which allows for the permissive growth of *M.tb* (Kaplan et al., 2003). Individuals with cavitary disease, especially where cavities communicate with the airways, are thought to be highly infectious (characterised by a high sputum bacillary load) and more likely to exhibit symptoms such as cough (Jones-Lopez et al., 2013, Turner et al., 2018), which is considered the main mechanism whereby the bacilli is spread (Esmail et al., 2018). However, recent studies show that tidal breathing may be a major contributor to *M.tb* transmission as well (Dinkele et al., 2021a). Once

the bacilli are expelled from the lung of an individual, they must survive in the air until inhaled by a new host. Inhaled bacteria must then circumvent physical barriers, evade immune mechanisms, and seed themselves in the alveoli of the lung to establish a new infection and disease in a susceptible host (Turner et al., 2017). At each step there are potential interventions that could be put in place to halt the cycle of transmission. In this chapter we investigate host immune factors associated with infectiousness, disease severity and inflammation in individuals with active TB, which could shed light on targets for host-directed interventions.

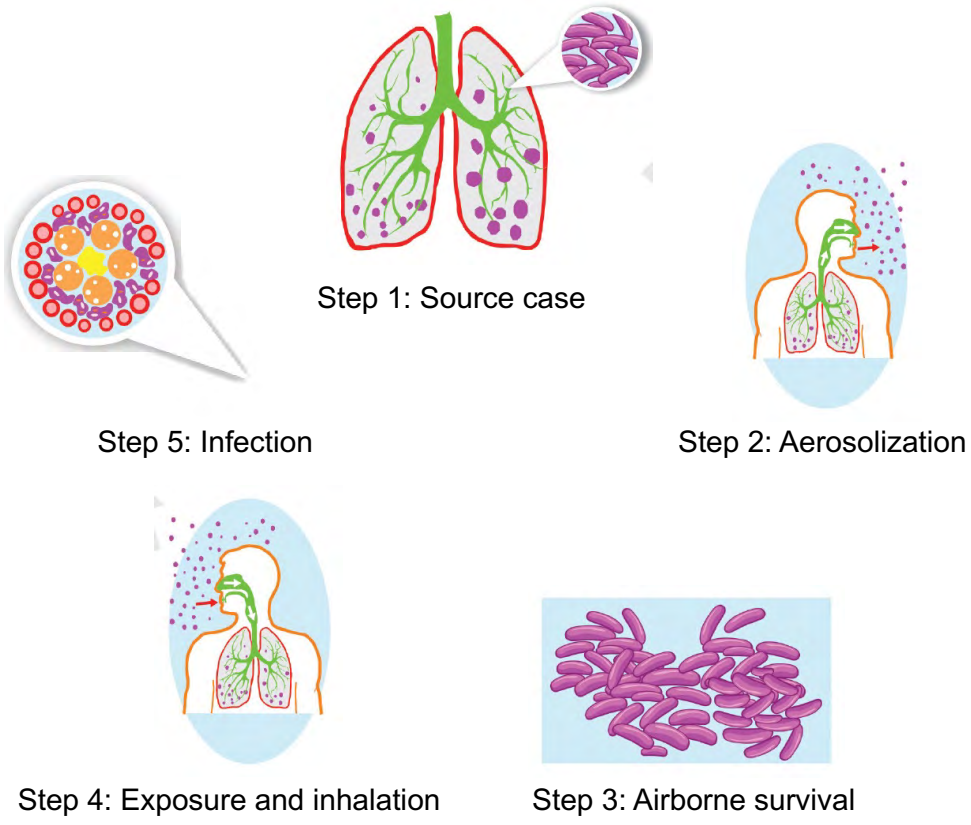


Figure 35. An illustration of the steps involved in *M.tb* transmission (discussed in detail below). Adapted from Churchyard et al., 2017.

6.1.2. Drivers of TB transmission

i. The source case

A major risk factor for *M.tb* transmission is the infectiousness of the source case. Individuals with more severe TB disease and cavitation are thought to expel more infectious droplets (Rodrigo et al., 1997, Kaplan et al., 2003, Mathema et al., 2017). Although *M.tb* is transmitted by air, the abundance of bacilli in sputum has been used as an indicator of infectiousness (Sepkowitz, 1996a). However, although individuals with detectable acid fast bacilli in their sputum are more likely to cause infection (Zelner et al., 2014), sputum smear acid fast bacilli negative individuals have also been shown to contribute to *M.tb* transmission (Behr et al., 1999, Hernandez-Garduno et al., 2004). In addition, not every person with TB can produce sputum and it is an assumption that such individuals are not transmitting. Therefore, other sensitive and inexpensive methods of measuring infectiousness that do not rely on sputum are required. As previously stated, a recent case-contact study reported that the risk of IGRA conversion was higher in individuals who were exposed to TB patients with the *M.tb* Beijing strain compared to other strains (Verrall et al., 2020b), thus, highlighting that infectiousness of the source case could be *M.tb* strain dependent. TB patients who are coinfected with HIV, particularly individuals with low CD4 counts, are considered less infectious than TB patients without HIV infection (Huang et al., 2014). TB-HIV patients generally have a low sputum bacillary load and are often smear negative, have a lower likelihood of cavitation and have a shorter period of infectiousness (Dye and Williams, 2010, Corbett et al., 2004, Alpert et al., 1997). The shorter period of infectiousness is thought to be due to more rapid rates of progression to TB disease and treatment or death than HIV-negative TB patients (Corbett et al., 2004, Ku et al., 2021). The clinical presentation of TB disease in TB-HIV patients is related to their immunological status. It has been shown that CD4 counts positively correlate with cavitation in TB-HIV patients, in addition, HIV-infected TB patients with high CD4 counts have symptoms (and potentially infectiousness) similar to those individuals without HIV infection (Kwan and Ernst, 2011).

Cough is considered to be the primary mechanism whereby infectious aerosols are generated. However, breathing, talking and other activities may also be significant

contributors to transmission (Turner and Bothamley, 2015, Loudon and Roberts, 1968, Loudon and Roberts, 1967, Patterson et al., 2021, Dinkele et al., 2021a). A recent study by Dinkele et al. used a modified version of the respiratory aerosol sampling chamber (RASC), with an improved sampling protocol (discussed in detail below) and were able to compare aerosolisation of *M.tb* between tidal breathing and cough in TB patients. Viable *M.tb* was captured in 66% of tidal breathing samples and 65% of cough samples. The authors concluded that although coughing produced 3 fold more *M.tb* aerosols than tidal breathing, because of the infrequent nature of coughing, tidal breathing is likely a large contributor to *M.tb* aerosol expulsion and transmission (Dinkele et al., 2021a). A 1969 study by Loudon et al. measured cough frequencies in untreated TB patients for an 8-hour period (23:00 - 07:00) and attempted to link cough frequency to infection rates [measured by TST reactivity] in HHCs of TB patients. The investigators observed that cough counts decreased over time in patients on TB treatment. However, there were no significant associations between cough and infection rates in HHCs (Loudon and Spohn, 1969). It is important to note that, in this study, cough counts were strictly measured at night and only TB-HHCs who were 14 years and younger were included in the analysis. A recent study in Peru showed that cough frequencies were highest in TB patients from 13:00 to 14:00. It was also shown that cough frequencies were higher among patients who had a higher sputum bacillary load (measured as time to microbiological culture positivity) (Proano et al., 2017). Another study by the same investigators showed that cough frequencies in TB patients on treatment were higher in those individuals with larger cavities and cavities closer to the airways (Proano et al., 2018). A study in the UK also reported that 24-hour cough frequencies were higher in TB patients in the day than at night. In addition, it was reported that 24-hour cough frequencies were associated with *M.tb* infection in TB-HHCs (Turner et al., 2018). These studies demonstrate the link between TB disease severity, bacillary load and infectiousness.

ii. Proximity and duration of exposure

The dose of *M.tb* aerosols inhaled by an individual is an important driver of *M.tb* transmission. It has been proposed that the quantity and size (aerosols <5 μm are infectious) of *M.tb* aerosols inhaled by humans are major determinants of the likelihood of establishment of infection and subsequent immunopathology following infection

(Fennelly and Jones-López, 2015). Early studies in guinea pigs estimated that one infectious dose equals one droplet nucleus, however, in humans this might differ based on host, bacterial and environmental factors (Nardell, 2016). The proximity and duration of exposure to the source case are considered surrogates for the dose of infectious *M.tb* aerosols inhaled (Fennelly and Jones-López, 2015). In an outbreak in 1966 on a United States (US) Navy ship, individuals were more likely to be infected with *M.tb* and progress to TB disease if they were in close proximity and spent more time with the source case (Houk et al., 1968a, Houk et al., 1968b). In a recent study it was shown that HHCs of TB patients were less likely to convert their IGRA test (from a negative to positive test) if they had a lower measure of exposure to *M.tb* (Verrall et al., 2020a). It has also been reported that individuals sharing a bed with a TB patient have a higher risk of infection than individuals who sleep in the next room (Lienhardt et al., 2003). Studies in the UK and South Africa (SA) among health-care workers (HCW) showed that individuals working in high TB exposure settings had higher rates of *M.tb* infection than those individuals working in low TB exposure settings in hospitals (van Rie et al., 2013, Sepkowitz, 1996b). Also, HCWs working in an overcrowded emergency department in Peru were reported to have IGRA conversion rates of 30% whereas hospital employees such as cleaners and security guards did not convert their IGRA in the 12-month study period (Escombe et al., 2010).

iii. Environmental factors

The environmental factors conducive for TB transmission include enclosed spaces with poor air circulation and lack of exposure to ultraviolet light (Nardell, 1993). Humidity may also affect the effectiveness of air disinfectant measures and thus influence *M.tb* transmission (Riley and Kaufman, 1972, Xu et al., 2005).

iv. Transmission settings

Historically *M.tb* transmission studies focused on households. Interestingly, it has been shown that in high burden settings, less than 20% of source cases transmit to contacts within a household (Verver et al., 2004, Andrews et al., 2014, Middelkoop et al., 2015, Buu et al., 2010). However, most cases of *M.tb* transmission in children younger than 15 years of age are the result of a HHC (Zelner et al., 2014, Middelkoop

et al., 2014). In a high incidence area in Cape Town, SA, investigators performed deoxyribonucleic acid (DNA) fingerprinting on TB patients and determined that the proportion of TB transmission that took place in households was 19% (Verver et al., 2004). Another study in SA where participants were asked to wear carbon dioxide (CO_2) monitoring devices (used to infer the amount of rebreathed air that individuals were exposed to as a proxy for potential *M.tb* exposure) and keep records of their social settings throughout the day, showed that less than 16% of infections occurred in the household (Andrews et al., 2014). In fact, in many high burden settings such as Malawi, the majority of infections occur due to unknown contacts (Glynn et al., 2015, Crampin et al., 2006). A better understanding of the social dynamics and places where transmission takes place in TB endemic settings is required, so that targeted interventions to halt transmission may be applied.

Places which have been identified as high TB transmission settings include hospitals (Gandhi et al., 2013, Ikeda et al., 1995), homeless shelters (Curtis et al., 2000) and prisons (often overcrowded in under-resourced countries) (Telisinghe et al., 2014). Community settings such as public transport (Andrews et al., 2013), schools (Andrews et al., 2014) and bars (Murray et al., 2009) have also been identified. In addition, occupations in settings within healthcare (Escombe et al., 2010), mining (Hanifa et al., 2009) and the transport sector (minibus drivers) (Horna-Campos et al., 2011) have also been identified as high risk.

v. Host susceptibility

HIV infection is the strongest risk factor for TB disease (Kwan and Ernst, 2011, Corbett et al., 2003). Other host risk factors include malnutrition (Cegielski and McMurray, 2004), diabetes mellitus (DM) (Stevenson et al., 2007), drug and alcohol abuse (Lonnroth et al., 2008, Oeltmann et al., 2009) and smoking (Lin et al., 2007). Individuals with the beforementioned risk factors are more likely to progress to TB disease once infected and contribute to the TB burden and the possible spread of *M.tb*.

6.1.3. TB aerobiology studies

Pivotal work by Riley, Wells and colleagues in the 1950s showed that *M.tb* is transmitted through the airborne route (Riley et al., 1995). In their experimental model, guinea pigs were exposed to air exhausted from a TB hospital ward, and monthly TSTs were performed to measure acquisition of *M.tb* infection by the guinea pigs. Their work also highlighted the variability of *M.tb* transmission: approximately 4% of TB patients were responsible for more than 73% of infections in the guinea pigs (Riley et al., 1995). In a follow up study, the investigators showed that 13% of TB patients were responsible for all the infections in guinea pigs (Riley et al., 1962). The guinea pig infection model was replicated for the first time by Escombe et al. in HIV-infected TB patients in Peru (Escombe et al., 2007, Escombe et al., 2008). TB transmission was also shown to be highly variable, with 8.5% of patients accounting for 98% of infections, which was determined by DNA fingerprinting. Interestingly, 90% of infections were caused by inadequately treated multidrug resistant TB patients (Escombe et al., 2008). Other studies since have used the guinea pig infection model to investigate the effects of interventions such as upper room ultraviolet lights, negative air ionisation, wearing of surgical masks and TB treatment on TB transmission (Escombe et al., 2009, Dharmadhikari et al., 2012, Dharmadhikari et al., 2014).

Figure 36. Cough aerosol sampling system (CASS). View inside the sampling system with two Six-stage Viable Anderson Cascade Impactors (VACIs) and a collection plate (left) and the outside view of the CASS set up in the procedure room (right). Adapted from Fennelly et al., 2012.

In recent years, novel experimental systems to better capture *M.tb* bacilli and understand *M.tb* transmission have been developed (Fennelly et al., 2004, Wood et al., 2016, Williams et al., 2014). Fennelly et al. created a cough aerosol sampling system (CASS) **(Figure 36)** and asked individuals with TB to cough for two 5-minute sessions into the sampling chamber. The CASS is a chamber containing two six-stage Viable Anderson Cascade Impactors (VACI)s **(Figure 37)**, which allow the capture of *M.tb*-containing aerosols onto agar plates based on particle size, followed by microbiological culture and quantification of the bacilli (Fennelly et al., 2004, Fennelly et al., 2012)**.** The investigators also showed the variability of TB transmission, reporting that between 25-45% of culture positive TB patients were able to produce culturable *M.tb* aerosols (Fennelly et al., 2004, Fennelly et al., 2012, Jones-Lopez et al., 2013). Despite the small proportion of culturable cough aerosols, TB patients with high *M.tb* aerosol counts were better predictors of new infection to a HHC than smear microscopy and time to culture positivity (Jones-Lopez et al., 2013). The VACI sampling device has been also used to quantify *M.tb* in aerosols from TB patients in other studies (Wood et al., 2016, Patterson et al., 2017, Theron et al., 2020, Shaikh et al., 2019). Wood et al. developed the RASC to quantify particles expelled by TB patients during normal breathing (Wood et al., 2016). This sampling method, described in detail below, was used to generate results reported in this chapter. Another study investigated *M.tb* aerosols expelled during normal breathing by asking TB patients to wear a mask aerosol sampling system (MASS) for an hour out of every 3 hours for 24 hours. The MASS is a filtering facepiece mask modified to contain a gelatine filter (pore size 0.3 μm), which allows the detection of *M.tb* DNA by polymerase chain reaction (PCR) (Williams et al., 2014, Williams et al., 2020). Interestingly, both RASC and MASS systems could detect bacilli by PCR from aerosols expelled by individuals with no recorded cough, indicating that although cough may be the main mechanism whereby *M.tb* is spread, other activities may also contribute to transmission (Patterson et al., 2017, Williams et al., 2020). Although, as discussed above, recent studies indicate that tidal breathing may be the major contributor to TB transmission (Dinkele et al., 2021a)

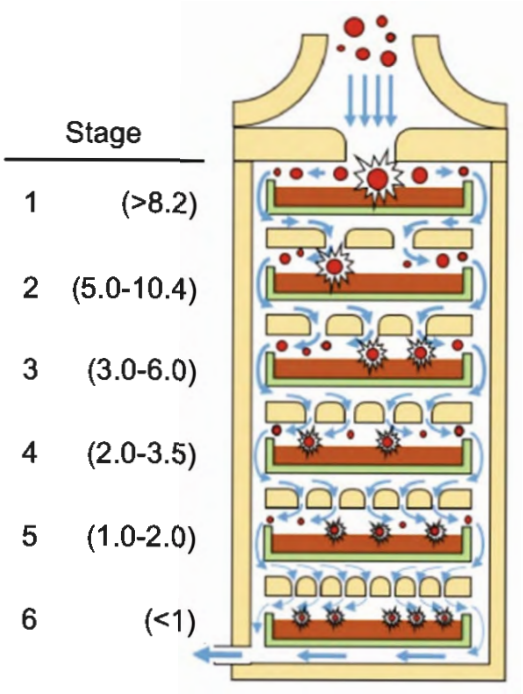


Figure 37. The Six-stage Viable Andersen Cascade Impactor (VACI). A graphical representation of the VACI showing the approximate diameter (μm) of droplets that are allowed to pass through at each stage. Adapted from Theron et al., 2020.

6.1.4. Immunological determinants of TB transmission

The progression to TB disease and the level of infectiousness of the source case are thought to be driven by inflammation and immunopathology in the lung after failed containment of *M.tb* infection by the host immune system (Ehlers and Schaible, 2012). Scriba et al. undertook a longitudinal study in adolescents and performed whole blood transcriptomic, proteomic and cellular analyses in samples from peripheral blood to investigate the biological mechanisms underpinning the progression from *M.tb* infection to TB disease. The investigators reported that type I/II IFN signalling and the complement cascade were increased in those adolescents who progressed to TB disease (progressors) 18 months before diagnosis compared to adolescents who remained healthy (controls). The expression genes in the Zak 16 correlates of risk signature (discussed below) also increased positively, the closer to TB diagnosis. It was also shown that proportions of neutrophils and monocytes were increased while T and B cells were decreased in progressors closer to TB diagnosis (Scriba et al., 2017). Contribution of host immune cells and their responses in peripheral blood in the context of TB disease is discussed in detail below.

The successful control of *M.tb* infection depends on complex interactions between host innate and adaptive immune cells in tissues and the bacilli (BoseDasgupta and Pieters, 2014). It is important that a balance between the induction of inflammation to restrict *M.tb* growth and the prevention of excessive pathological inflammation is achieved. Patients co-infected with TB and DM are more likely to develop cavitary disease, whereas this is less likely in TB-HIV patients (Gil-Santana et al., 2016). DM and HIV acts on the immune system differently, although both lead to immune dysregulation (either due to an excessive inflammatory response or inappropriate immunosuppression) and therefore increase the likelihood of progression to TB disease in *M.tb* infected individuals.

Immune cells and their responses in blood have also been associated with TB outcomes. High levels of neutrophils in peripheral blood during TB disease was associated with immunopathology and poor disease outcomes (Nandi and Behar, 2011, Lowe et al., 2013). It was reported that a neutrophil-driven IFN-stimulated gene expression signature was present in the blood of active TB patients and correlated with extent of lung pathology measured by chest X-ray (discussed below) (Berry et al., 2010). Interestingly a decrease in monocytes, granulocytes and $\gamma\delta$ T cells was observed in HHCs of TB patients who were persistently IGRA negative, which was not the case for IGRA converters of TB-HHCs (Verrall et al., 2020c),

A study in The Gambia reported that the granulocyte to lymphocyte ratio (GLR) was higher in TB patients compared to LTBI-HHCs. Interestingly, high granulocyte and low NKT cell frequencies were able to classify TB patients from HHCs with an 88% accuracy (Sutherland et al., 2009b). Using complete blood count-derived measurements, the neutrophil to lymphocyte ratio (NLR) and the monocyte to lymphocyte ratio (MLR) have been investigated as diagnostic markers for different diseases and are considered useful indicators of inflammation. complete blood counts are easily obtained and routinely performed in healthcare settings. Studies have reported that NLR is elevated in TB patients compared to healthy controls (Iliaz et al., 2014, Jeon et al., 2019). High NLR has also been associated with increased risk of developing TB disease in HIV-infected individuals (Miyahara et al., 2019) and pre-treatment NLR values can predict retreatment in those individuals who have already received anti-TB treatment (Yin et al., 2017). The NLR can also be used to differentiate

TB disease from other diseases such as community acquired pneumonia and sarcoidosis (Yoon et al., 2013, Iliaz et al., 2014, Jeon et al., 2019). Interestingly, in a recent study, the neutrophil to monocyte plus lymphocyte ratio was able to differentiate TB from other infectious lung diseases with greater accuracy than NLR (Jeon et al., 2019). It has also been shown that individuals with active TB have higher monocyte to lymphocyte ratios compared to healthy controls (La Manna et al., 2017, Wang et al., 2015) which return to normal levels after TB treatment (Wang et al., 2015, Wang et al., 2019). It has also been reported that MLR can be used to identify HIV-infected individuals coinfected with *M.tb* who are likely to develop TB upon initiating ART (Naranbhai et al., 2014a). Also, a study reported that high MLR in SA infants at approximately 3 months of age correlated with an increased risk of developing TB in the first 2 years of life (Naranbhai et al., 2014b). In early studies, an increased MLR was linked to TB disease severity in animals (Sabin et al., 1926) and humans (Rogers, 1928). The proportion of monocytes in peripheral blood also increases during TB disease (Sanchez et al., 2006, Rogers, 1928, Scriba et al., 2017). It has been proposed that elevated monocytes and TST values could be used to identify HHC of TB patients who are more likely to develop active TB disease (Rakotosamimanana et al., 2015). $CD16^{+}$ monocytes (intermediate monocytes: $CD14^{++}CD16^{+}$; non-classical monocytes: $CD14^{+}CD16^{++}$) increase substantially during inflammatory conditions and infectious diseases (Wong et al., 2012), including TB (Castano et al., 2011, Balboa et al., 2011). The intermediate monocyte subset, in particular, has been shown to positively associate with disease severity in inflammatory diseases (Wong et al., 2012). A study in young children also reported elevated frequencies of intermediate, non-classical and $CD163^{+}$ monocytes in children with TB disease compared to controls (Cetin et al., 2017). In adults with active TB the expansion of $CD16^{+}$ monocyte numbers in peripheral blood was associated with disease severity and soluble TNF levels (Balboa et al., 2011). Overall, GLR, MLR and/or $CD16^{+}$ monocyte numbers could be an important biomarker of TB patients with severe disease pathology and thus be more likely to transmit *M.tb*.

The role of other immune cells, including donor unrestricted T cells, in TB pathogenesis and infectiousness should be further explored (Ruibal et al., 2021, Rijnink et al., 2021). The abundance of immune subsets in the blood and/ or at sites of infection during TB infection, disease and recovery, could point to the role that these

immune cells play in protection or immunopathogeneses. As stated in chapter 3, higher frequencies of NK cells were observed in healthy *M.tb*-infected individuals compared to uninfected controls (Chowdhury et al., 2018). Interestingly, NK cell frequencies decreased during progression from latent infection to active TB disease and were re-established after successful treatment (Chowdhury et al., 2018). This result suggests that NK cells may play a role in controlling *M.tb* infection. Studies investigating B cell frequencies in TB patients compared to healthy controls, however, have yielded conflicting results (Joosten et al., 2016, Chowdhury et al., 2018, Hernandez et al., 2010, Wu et al., 2009). One of the few studies investigating HLA-E restricted T cells showed higher frequencies in TB individuals with and without HIV compared to healthy controls (Prezzemolo et al., 2018). An increase in $\gamma\delta$ T cells has also been reported (Ito et al., 1992, Balbi et al., 1993) while in other studies a decrease has been observed in peripheral blood from TB patients compared to healthy controls (Gioia et al., 2002, Yan et al., 2013). The decrease in frequencies of $\gamma\delta$ T cells in peripheral blood from TB patients also correlated with increased disease severity as measured by chest X-ray (Yan et al., 2013). As previously stated, MAIT cells are decreased in the periphery of active TB patients compared to healthy controls (Le Bourhis et al., 2010, Gold et al., 2010, Sharma et al., 2015, Jiang et al., 2014, Kwon et al., 2015, Balfour et al., 2021) and are believed to be enriched at the site of infection (Wong et al., 2019). However, a recent study showed no differences between TB patients and controls (Suliman et al., 2020). In the case of CD1-restricted T cells, CD1b-restricted T cells were not different between TB patients and controls (Lopez et al., 2020), while the type I iNKT (CD1d-restricted) cells were shown to be lower in numbers in active TB patients compared to controls in peripheral blood (Snyder-Cappione et al., 2007, Im et al., 2008). To our knowledge, the link between the frequencies of host immune subsets in peripheral blood and the infectiousness of a TB patient has not been investigated before.

In this chapter we sought to determine if immune cell subsets in blood are associated with *M.tb* aerosolisation. A test measuring immune cells in peripheral blood as a biomarker for infectiousness would allow targeted treatment of potential super-spreaders. Peripheral blood is an easily accessible human sample whereas a sputum sample (currently used to diagnose TB and assess treatment success) is not produced by all TB patients and is particularly difficult to obtain in populations such as young

children, HIV-infected individuals and patients with disseminated disease. Also, infection control measures are required to collect sputum samples, which can be difficult to put in place in under-resourced settings.

6.1.5. Host transcriptomics

Host transcriptional profiles measured in peripheral blood have promising diagnostic performance between TB patients and healthy controls and/ or other diseases (Berry et al., 2010, Kaforou et al., 2013, Anderson et al., 2014, Sweeney et al., 2016, Roe et al., 2016, Suliman et al., 2018, Penn-Nicholson et al., 2020). Many transcriptomic signatures have been developed in different cohorts (children and adults), geographical locations and in individuals with differing co-infections, and their performance for the diagnosis and prognosis of TB have been assessed (Turner et al., 2020, Gupta et al., 2020). The aim of this growing body of work is to develop a non-sputum based, concise TB transcriptomic signature that can be used as a point-of-care diagnostic or triage test, which would allow accurate and targeted treatment, and/or transcriptomic signatures with excellent prognostic performance for identifying those at high risk for incident TB to allow biomarker-targeted preventive therapy. Host transcriptomic signatures have also improved our understanding of the immune response and the mechanisms underpinning TB immunopathogenesis. Berry et al. developed a signature comprising 393 transcripts, using whole genome microarray, which was present in individuals with active TB but absent in *M.tb* infected individuals and healthy controls. The 393-transcript signature was enriched in neutrophil-driven IFN transcripts that correlated with disease severity and down-regulated with TB treatment (Berry et al., 2010). Thus, this work pointed towards a role of the IFN response in the pathogenesis and outcome of TB disease. Another study in adolescents from Cape Town, SA reported that a 47 transcript (16 gene) correlate of risk signature, Zak 16, could identify individuals who were at high risk of developing TB disease. Adolescents were monitored for two years and blood draws taken every 6 months for RNA sequencing, which allowed the discovery of transcripts that could differentiate between progressors and non-progressors. The signature was later adapted to a quantitative PCR-based method for easier application in future studies. The Zak16 correlates of risk signature is composed of type I IFN-related genes and

was able to identify individuals at high risk of developing TB up to 18 months prior to TB diagnosis, in addition to being a good diagnostic of prevalent TB (Zak et al., 2016). This signature was later refined to the smaller RISK11 signature, which is composed of 38 transcripts (11 genes), with equivalent performance, (Darboe et al., 2018). Results from application of this RISK11 signature are reported in this chapter. The overarching goal was to determine whether the RISK11 signature could be used as an inflammatory marker, and associated with determinants of TB transmission

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6.2. Aims

We sought to identify host immune cell subsets associated with inflammation, pathology and infectiousness in TB patients. We hypothesised that TB patients with excessive inflammation and cavitation would expel higher levels of *M.tb*-containing aerosols (a surrogate for infectiousness) than TB patients with minimal inflammation and pathology. We also hypothesised that immune subsets would be perturbed by inflammatory processes and that levels of immune cell subsets in the blood could serve as an indicator of infectiousness of TB patients (**Figure 38**).

The following objectives were addressed:

1. Identify host immune cell subsets associated with transcriptional signatures of TB-associated inflammation.
2. Characterise host immune cell subsets associated with radiological evidence of TB lung pathology.
3. Determine host immune cell subsets associated with high or low levels of *M.tb* aerosol expulsion.

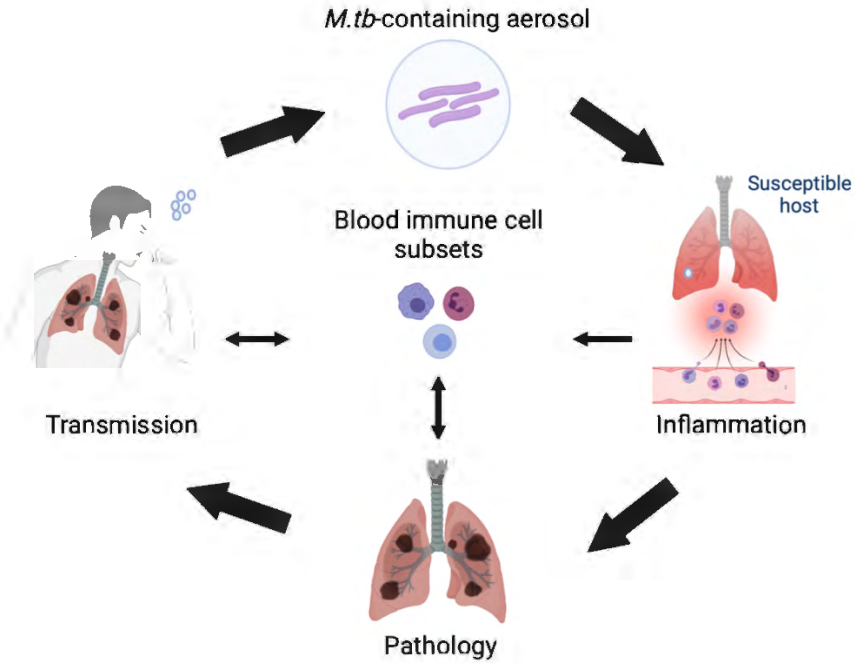

Figure 38. Proposed mechanism of immune determinants of TB transmission. *M.tb*-containing aerosols are inhaled by a susceptible host, leading to *M.tb* recognition and pathogen and host interactions. A lack of immune control leads to host-driven inflammation, the perturbation of immune cell subsets and lung pathology such as cavitation. This creates an environment for *M.tb* growth and transmission to a new host through the expulsion of infectious *M.tb* containing aerosol droplets. Illustration created in BioRender.com.

6.3. Materials and methods

6.3.1. Study participants

Study participants were recruited from Masiphumelele, a peri-urban township in Cape Town, SA by a study team of the Desmond Tutu Health Foundation, as part of a study funded by the Strategic Health Innovation Partnerships of the South African Medical Research Council (Principal Investigator: Robin Wood). Patients who presented for health care at a public clinic and who tested positive for drug-sensitive pulmonary TB by Xpert MTB/RIF were approached and consented prior to start of TB treatment. Initiation of chemotherapy was delayed 1-2 hours in order to complete sampling. Healthy controls were contacted and recruited in the surrounding Masiphumelele community. Blood samples were collected from TB patients from March 2015 until May 2017 and from healthy controls from October 2016 until November 2016. The demographic characteristics of the cohort are summarised in **Table 9**. The ethnicity of study participants was not captured. All participants provided written, informed consent. The UCT Human Research Ethics Committee reviewed and approved the study protocol (REF: 680/2013).

Table 9. Demographic characteristics of the adult cohort.

Variable	TB patients	Controls
	n = 48	n = 52
Female, n (%)	24 (50)	29 (56)
HIV positive, n (%)	25 (52)	21 (40)
Median age, years (range)	33.5 (18-66)	34.5 (20-56)

6.3.2. RASC

The RASC **(Figure 39)** has previously been described (Wood et al., 2016, Patterson et al., 2017). Briefly, the RASC is a custom-built clean room which allows the quantification of aerosolised particles expelled by a study participant during normal breathing. Study participants were asked to wear a full-body DuPont Tyvek suit, to prevent background particle detection, and remain seated in the RASC for 1 hour. Once the RASC was sealed, the air was purged using high efficiency particulate air filters for 10 minutes in order to remove background particles. During sampling a fan maintained air circulation [**Figure 39**, (4)]. In addition, temperature, humidity and CO_2 levels were monitored throughout sampling [**Figure 39**, (5)]. An aerodynamic particle size spectrometer (APS Model 3321, TSI, Shoreview, MN USA) [**Figure 39**, (1)] and a laser scattering particle size spectrometer (Model 3016, Lighthouse Worldwide Solutions, Medford, Oregon, USA) detected aerosol particle concentrations and particle size from the last minute of the air wash throughout the sampling protocol. After the air wash phase, the participant contamination phase commenced in which exhaled air from the participant was allowed to rise to a 10% threshold defined by a CO_2 concentration of 40 000 parts per million. After this, air from the RASC was sampled at various flow rates depending on the capturing device. Particles expelled by participants were captured by the VACI (Model 10830-EPD, Thermo Scientific, USA) [**Figure 39**, (3)] for 10 minutes and analysed by microbiological culture. A 0.4 μm polycarbonate membrane filter (Sterlitech Corporation, WA, USA) [**Figure 39**, (2)] was used for the direct capture of particles for 10 minutes. The polycarbonate filter was cut in two, with one half analysed by microbiological culture and the other half by droplet digital PCR. A gelatine filter (Model 12602-37-ALK, Sartorius, Goettingen, Germany) was placed nearby [**Figure 39**, (2)] and air sampled for 10 minutes to capture particles that were analysed by microbiological culture. In addition, air was also sampled for 10 minutes and particles captured by a Dekati three-stage impactor (PM 10 impactor, Dekati, Kangasala, Finland) [**Figure 39**, (6)] for imaging by scanning electron microscopy. The final 10 minutes were used to capture aerosol particles using a 47 mL sterile felt filter with a 1.0 μm pore size (American Felt and Filter company, New Windsor, New York; Lockheed Martin, Alexandria, VA, USA) to be analysed by droplet digital PCR. In this chapter, we report data from the RASC in *M.tb* CFU detected by microbiological culture of particles expelled by study participants.

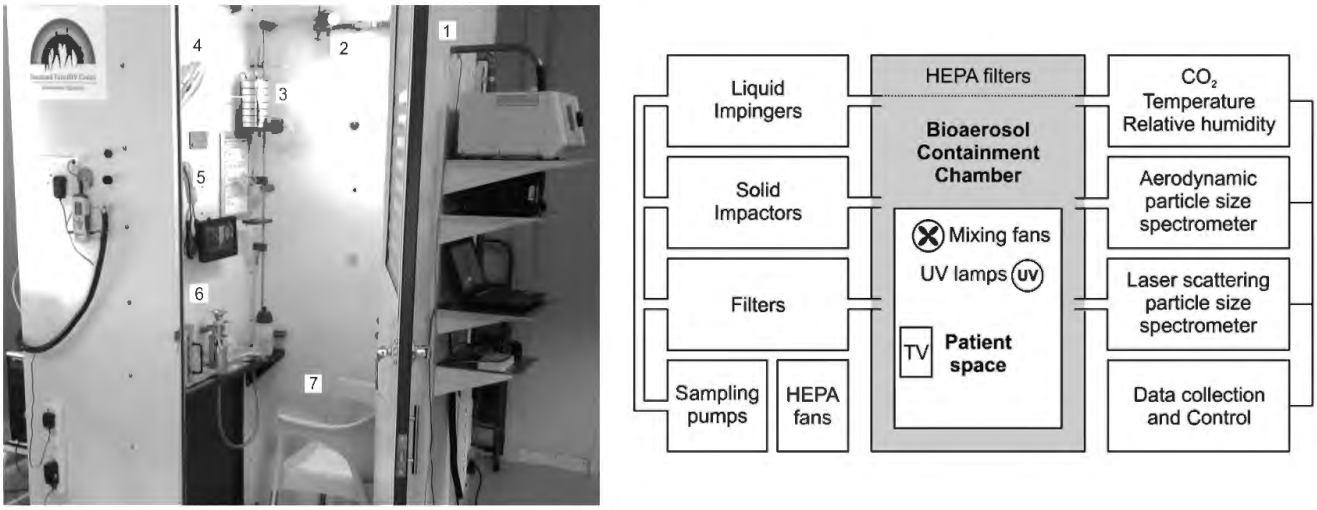

Figure 39. The Respiratory Aerosol Sampling Chamber (RASC). **(A)** View of the RASC (1) aerodynamic particle size spectrometer, (2) filter samplers, (3) six-stage Viable Andersen Impactor, (4) mixing fan (5) carbon dioxide, temperature and relative humidity, (6) Dekati three-stage impactor, (7) chair for study participant. **(B)** A diagram showing the configuration of the RASC. Adapted from Wood et al., 2016.

6.3.3. Six-stage Viable Andersen Cascade Impactor (VACI)

The majority of *M.tb* CFU detected from aerosols expelled by TB patients were from the VACI, at stages 4 and 5, which corresponded to aerosol diameters in the approximate range of 1.0 μm to 3.5 μm (Patterson et al., 2017) (**Figure 37**). An infectious droplet is thought to be in the range of 1 μm to 5 μm, as they are small enough in size to allow their passage through the upper airways and eventually to the alveoli of the lung (Fennelly and Jones-López, 2015). The VACI allows capture of aerosolised particles based on size using six stages of plates with solid Middlebook 7H10 medium. The captured particles were cultured (described below) to determine the number of *M.tb* bacilli expelled by study participants.

6.3.4. Microbiological culture and detection

The quantification of aerosol *M.tb* CFU was previously described (Wood et al., 2016, Patterson et al., 2017). Briefly, plates from the VACI were placed in a biosafety cabinet and filters that captured aerosolised particles for microbiological culture were placed on solid Middlebrook 7H10 agar plates. Agar plates were supplemented with 0.2%

glycerol and oleic acid/albumin/dextrose/catalase complex and 0.05% Tween 80, PANTA (BD) antibiotic mixture and incubated at 37°C for 4-6 weeks. The number of CFU with expected *M.tb* colony morphology and rate of formation *in vitro* was then recorded, followed by genomic DNA extraction for quantitative PCR confirmation with the primer set, RD9F (5'-gtgtaggtcagccccatcc-3') and RD9R (5'-gctaccctcgaccaagtgtt-3') using a protocol previously described (Parsons et al., 2002).

6.3.5. Radiological scoring

Study participants returned to the study site for a chest X-ray approximately seven days after the start of treatment. Chest X-rays were evaluated independently by two pulmonologists, who were blinded to study participant information. The pulmonologists completed a standardised chest radiograph reading and recording system (CRRS) (**Appendix B**). In addition, we created a modified scoring algorithm using the results from the CRRS for each patient (**Appendix C**).

6.3.6. Immunophenotyping on cryopreserved ex-vivo whole blood

500 μL of venous blood was collected in sodium heparin tubes and processed using a protocol previously described (Nemes et al., 2015), with modifications. Briefly, red blood cells were lysed by adding 500 μL of blood to 4.5 mL FACS Lysing solution (BD Biosciences), followed by an incubation period in the dark at room temperature for 15 minutes. Samples were then centrifuged and white blood cells cryopreserved in cryosolution. Cryopreserved cells were then thawed, washed in PBS and stained for 45 minutes at 4°C in PermWash (BD Biosciences) with antibody Panel 4 (**Table 10**). Flow-count Flourosphere beads (Beckman Coulter) were added at 50 μL to each sample. Stained cells were acquired on a LSR II flow cytometer (BD Biosciences). The flow cytometer configuration is described in chapter 2 (**Table 5**).

6.3.7. RNA isolation and RISK11 signature score measurement

Blood was collected in PAXgene® tubes, incubated at room temperature for approximately 3 hours and stored at -20°C. RISK11 signature gene expression was quantified using a protocol as previously described (Zak et al., 2016, Darboe et al., 2018). Briefly, RNA was isolated using the PAXgene™ blood RNA kit (QIAGEN), complimentary DNA was created by reverse transcription and pre-amplified using PCR. Signature transcripts were then quantified by microfluidic qRT-PCR using TaqMan primer-probes on 96.96 Gene Expression Chips (Fluidigm) on a Biomark HD multiplex microfluidic qRT-PCR instrument (Fluidigm). The RISK11 signature scores were calculated as previously described (Zak et al., 2016, Darboe et al., 2018).

Table 10. Markers and fluorochrome combinations for Panel 4. Optimal antibody titers and manufacture details are provided.

Fluorochrome	Antigen	Clone	Manufacturer	Volume (μL/50μL)
APC-H7	CD45	2D1	BD	0.125
FITC	CD66	B1.1/CD66	BD	0.016
Brilliant Violet 650	CD14	M5E2	BioLegend	0.5
Brilliant Violet 785	CD11c	3.9	BioLegend	0.625
Brilliant Violet 510	CD16	3G8	BioLegend	2.5
APC	CD56	HCD56	BioLegend	0.625
AlexaFlour700	CD19	HIB19	BioLegend	0.078
Brilliant Violet 421	CD3	UCHT1	BD	0.16
ECD	CD4	SFCI12T4D11	Beckman Coulter	0.125
Brilliant Violet 605	CD8	RPA-T8	BD	2.5
PeCy7	CD26	BA5b	Biolegend	0.05
PeCy5	CD161	DX12	BD	2.5
PE	HLADR	L243	BioLegend	0.5

6.3.8. Data analysis

As shown in **Figure 40** flow cytometry files were analysed using FlowJo version 9.7-9.96 (Treestar, Ashland, OR). Flow cytometry analyses were undertaken as described in Chapter 2. Absolute cell counts were calculated using the formula: [(cell event count/bead count) x #beads/ test/initial blood volume)] (Nemes et al., 2015). Statistical

analyses were performed and graphs created using GraphPad Prism version 6-8 or R (http://www.r-project.org). The Venn diagram was created using the VennDiagram package (Chen and Boutros, 2011). The DeLong's test was used to compare differences between receiver operating characteristics-area under the curve (ROC-AUC) values using the pROC package (Robin et al., 2011). The Mann-Whitney U test was applied to compare different groups. The Fisher's Exact test was used to compare binary indicators of disease and infectiousness. To measure the degree of association between two variables Spearman rank correlation was applied. The p values were shown unadjusted and bolded values represent those comparisons that are significant after corrections for multiple comparisons using the Bonferroni correction method.

Figure 40. Gating strategy for identification of innate and adaptive immune cell subsets from ex-vivo whole blood.

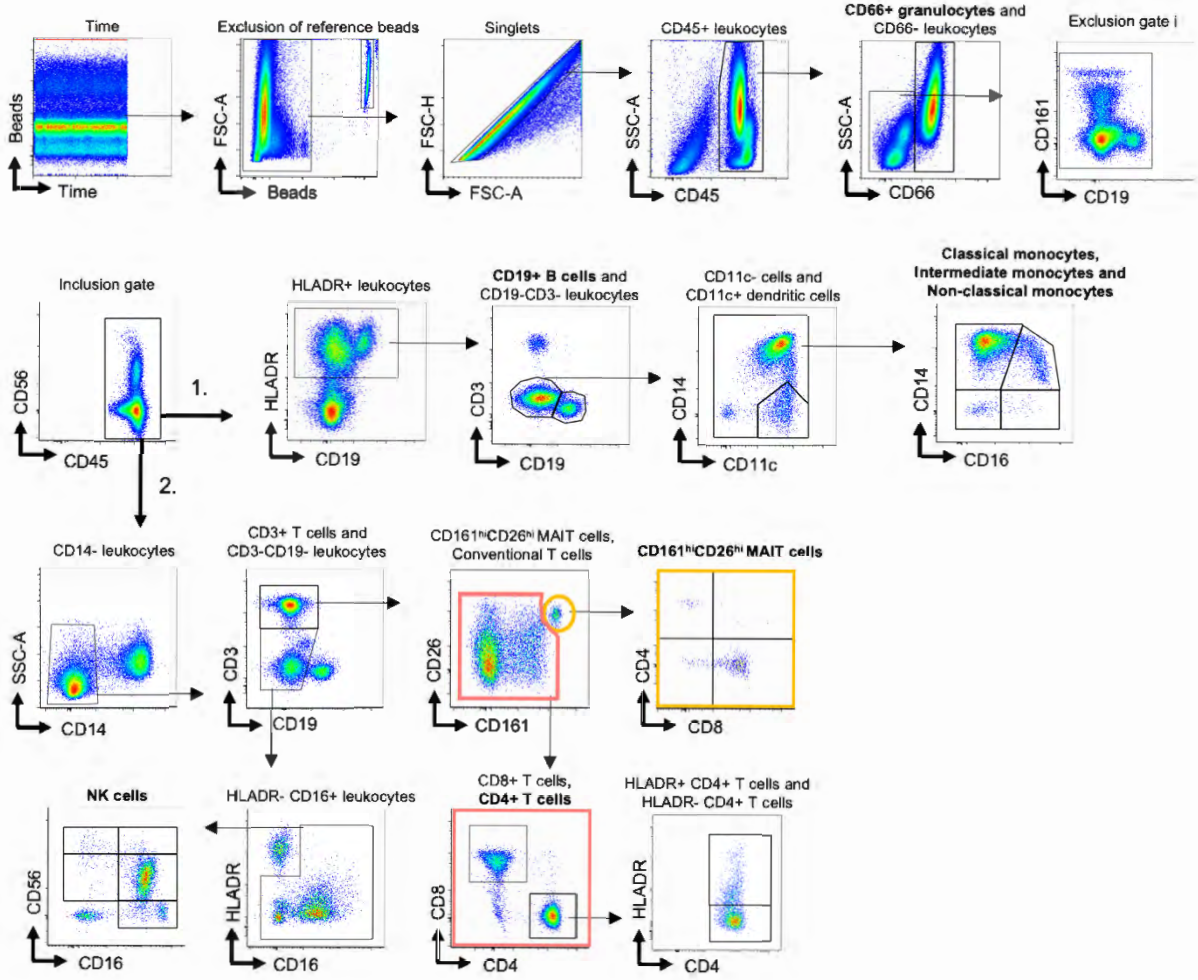

Figure 40. Gating strategy used for Panel 4. The first gate was set on time to ensure that uniform fluorescence signal was obtained during acquisition of the sample. Reference beads were added in order to quantify the number of cells per μL of whole blood (absolute cell counts). The next gate excluded reference beads from cells. Singlets were then selected followed by a gate on all CD45+ leukocytes. Next, granulocytes were gated on using CD66 and the CD66- used for the rest of the gating strategy. An exclusion gate was used on the CD66- cells to exclude antibody aggregates and autofluorescent cells. This was followed by an inclusion gate, on which two different pathways were chosen in order to get the best separation and resolution of the immune subsets of interest. Pathway one: HLADR+ cells were gated on, followed by the selection of CD19+ B cells and CD19-CD3- cells. Monocytes were selected using CD14+/-CD11c- cells from the CD19-CD3- cells and then characterised based on CD14 and CD16 expression (classical monocytes (CD14+CD16-), intermediate monocytes (CD14+CD16+) and non-classical monocytes (CD14-CD16+). Pathway 2: CD14- cells were selected from which CD3+ T cells and CD3-CD19- cells were selected. $CD161^{hi}CD26^{hi}$ phenotypic MAIT cells and conventional CD3+ T cells were gated from the CD3+ T cell gate and the MAIT cells further characterised based on CD4 and CD8 expression. Conventional CD3+ T cells were also further separated based on CD4 and CD8 positivity and the conventional CD4+ T cells further subsetted into HLADR+ and HLADR- CD4+ T cells. In addition, CD16-$HLADR^{hi}$ cells were excluded from CD3-CD19- cells and NK cell subsets selected based on CD56 and CD16 expression.

6.4. Results

6.4.1. Sample availability

A total of 48 TB patients and 52 controls were recruited for the immunological component of the RASC study **(Table 9)**. However, we were unable to collect all respiratory aerosol sampling data for each participant from the different assays, which made analyses challenging. Since this is one of the first studies to measure aerosolised *M.tb* with this technology, the study design was not locked down before recruitment commenced, thus the *M.tb* aerosol collection and detection method using the RASC changed somewhat during the study. Here, we included the first batch of participants recruited in this study for whom the same experimental protocol was followed. For the X-ray data, two participants did not return after seven days for radiological imaging. Lastly, for the gene expression and flow cytometry data, some samples were of poor quality and did not meet our quality thresholds and were excluded from the relevant analysis as indicated below.

M.tb aerosol CFU detected using microbiological culture were available for 35/48 (73%) of the TB patients. Chest X-ray data was available for 46/48 (96%) of the TB patients (**Figure 41)**. Gene expression data was available for 46/48 (96%) of the TB patients **(Figure 41)** and 45/52 (87%) of the controls. Finally, flow cytometry data on immune subset frequencies was available for 46/48 (96%) **(Figure 41)** of the TB patients and 42 of the controls 42/52 (81%) recruited.

6.4.2. RISK11 signature distinguishes TB patients from healthy controls

The RISK11 signature, a blood-based biomarker, has promising diagnostic performance for TB disease (Darboe et al., 2018, Scriba et al., 2021, Mendelsohn et al., 2021, Mulenga et al., 2021). In our cohort, RISK11 scores differentiated between TB patients and controls with a ROC-AUC of 0.952 (95% CI 0.910 to 0.994, $p<0.001$) in all participants, an AUC of 0.984 (95% CI 0.953 to 1.000, $p<0.001$) in the HIV- only group and 0.923 (95% CI 0.840 to 1.000) in the HIV+ only group **(Figure 42A and Figure 42B)**. Although the AUC value was lower in the HIV+ only group compared to

the other group, there was no significant difference in the performance between HIV+ only group and the HIV- only group (p=0.179).

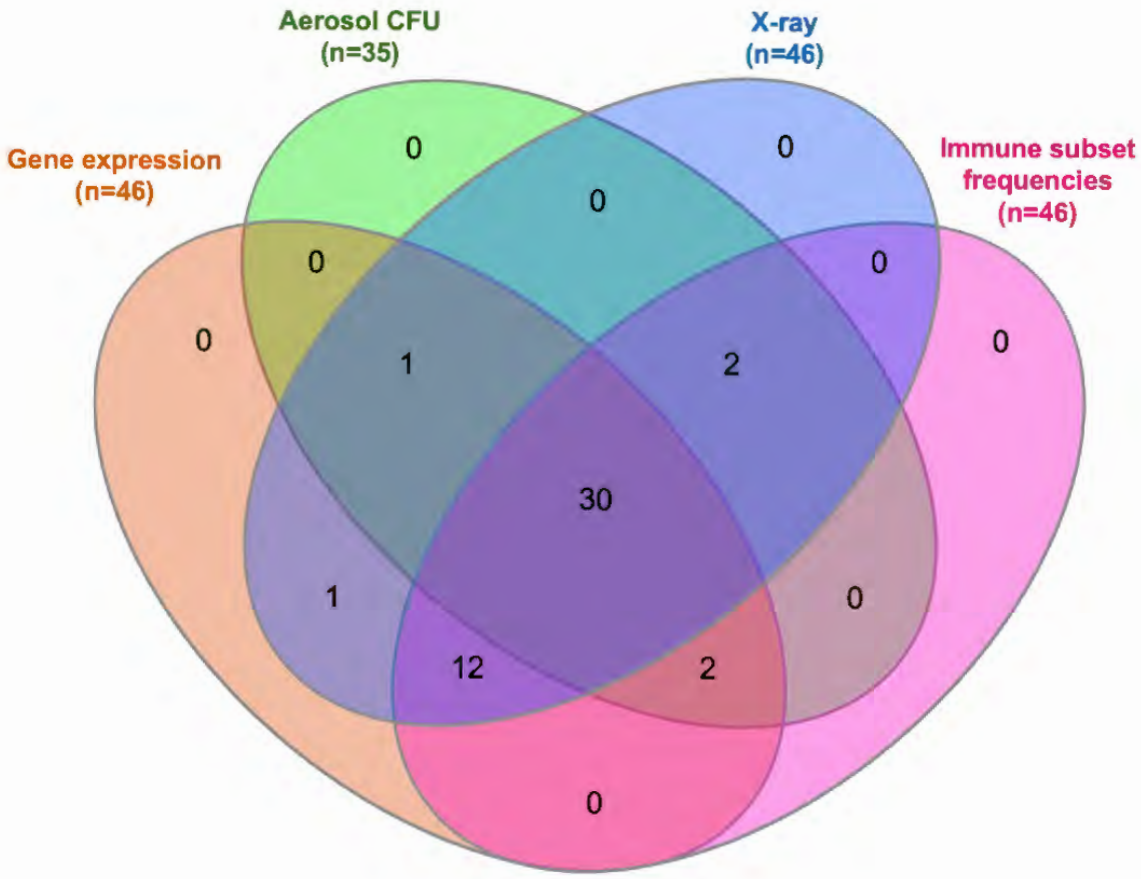

Figure 41. Venn diagram of TB patients sampled. A graphical depiction of the number of TB patients sampled for each experimental assay performed.

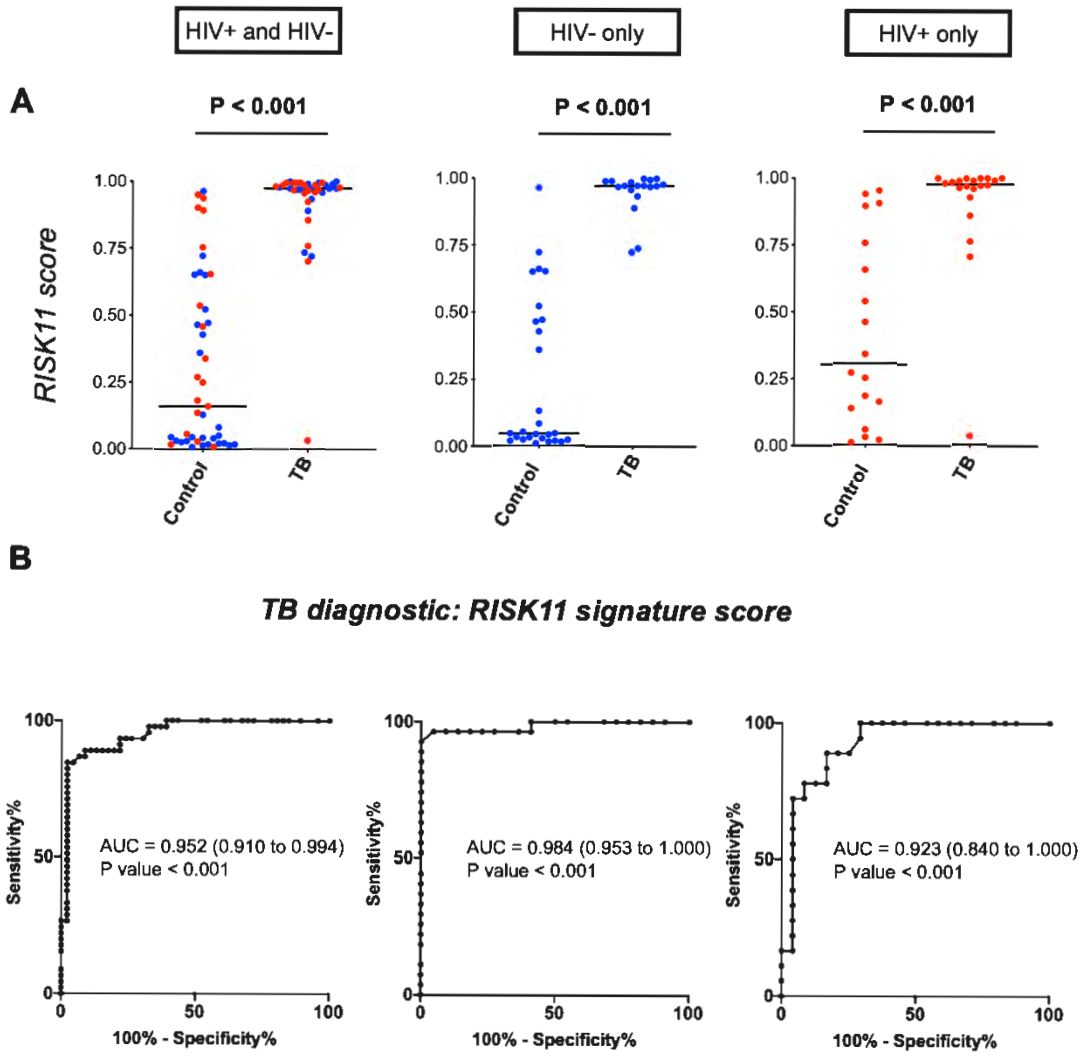

Figure 42. Diagnostic performance of RISK11 signature as a TB classifier. (A) RISK11 signature scores in TB patients and healthy controls; red symbols (HIV+). **(B)** ROC AUCs depicting the discrimination of TB patients and healthy controls using RISK11 signature scores in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals.

6.4.3. M.tb aerosol associations with cavitation status in TB patients

It is thought that TB patients with inflammation and lung pathology are highly infectious (Rodrigo et al., 1997, Kaplan et al., 2003, Mathema et al., 2017). Thus, we investigated whether there were any associations between TB disease severity (cavitation) and infectiousness (aerosol CFU). We correlated radiological scores from the CRRS system (**Appendix B and Appendix C**) with the other outcomes (RISK11 signature and *M.tb* aerosol CFU) but found no associations (not shown). We therefore only report on cavitation as an indicator of radiological disease severity. Aerosol CFU was

higher in patients with cavitation compared to those without when we combined individuals with and without HIV. However, the p-values were above 0.017, which was the threshold after adjusting for multiple testing using the Bonferroni method **(Figure 43A)**. When we compared aerosol positive and aerosol negative individuals based on cavitation status, there was a minor difference in the HIV negative patient group, which also did not meet the p-value threshold after correction for multiple comparisons **(Figure 43B)**.

6.4.4. Detection of acid-fast bacilli and associations with infectiousness and cavitation in TB patients

Individuals with smear microscopy-positive TB are typically more infectious than smear-negative, culture-positive patients (Zelner et al., 2014)**.** We therefore determined whether aerosol CFU and cavitation were associated with smear positivity. There were no differences in aerosol CFU and cavitation between individuals who were smear positive or smear negative **(Figure 44A and Figure 44B)**. We also compared smear grade results with the other outcomes (RISK11 signature, *M.tb* aerosol CFU and cavitation) and observed no differences among individuals with TB (data not shown).

6.4.5. RISK11 score is higher in TB patients with cavitary disease compared to those without cavitation

We next sought to determine whether there were any associations between the RISK11 score (a blood transcriptomic signature of Type I/II IFN-driven inflammation) and other measures of disease severity and infectiousness (cavitation, aerosol CFU, aerosol positivity) **(Figure 45)**. RISK11 scores were higher in TB patients with cavitary disease compared to those without cavitation, a difference that was observed only in the HIV-negative group **(Figure 45A)**. There were no associations between the RISK11 score and *M.tb* aerosol CFU **(Figure 45B and Figure 45C)**. In addition, no differences were detected in the RISK11 signature scores between individuals who were smear positive or smear negative (**Figure 46**).

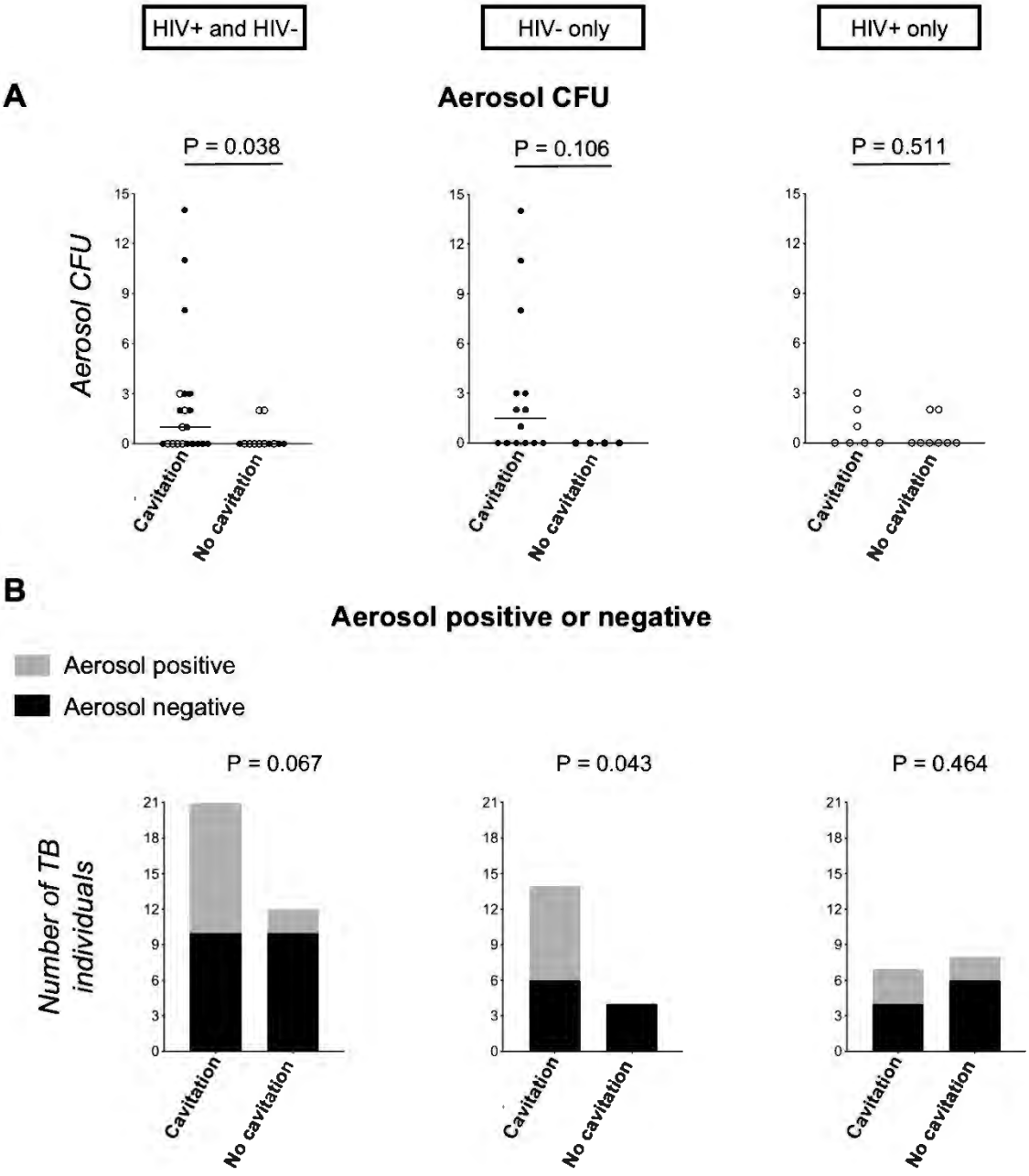

Figure 43. *M.tb* aerosol associated with cavitation status in TB patients. (A) Aerosol CFU counts in individuals with and without cavitation in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. Solid symbols represent HIV- individuals and open symbols represent HIV+ individuals. **(B)** Aerosol positivity (aerosol+ was defined as any detectable *M.tb* while aerosol- was defined as no detectable *M.tb* by microbiological culture from any capturing device from the RASC) in individuals with and without cavitation in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. The p values were calculated with the Mann-Whitney U test. The p values < 0.017 were considered statistically significant (three comparisons).

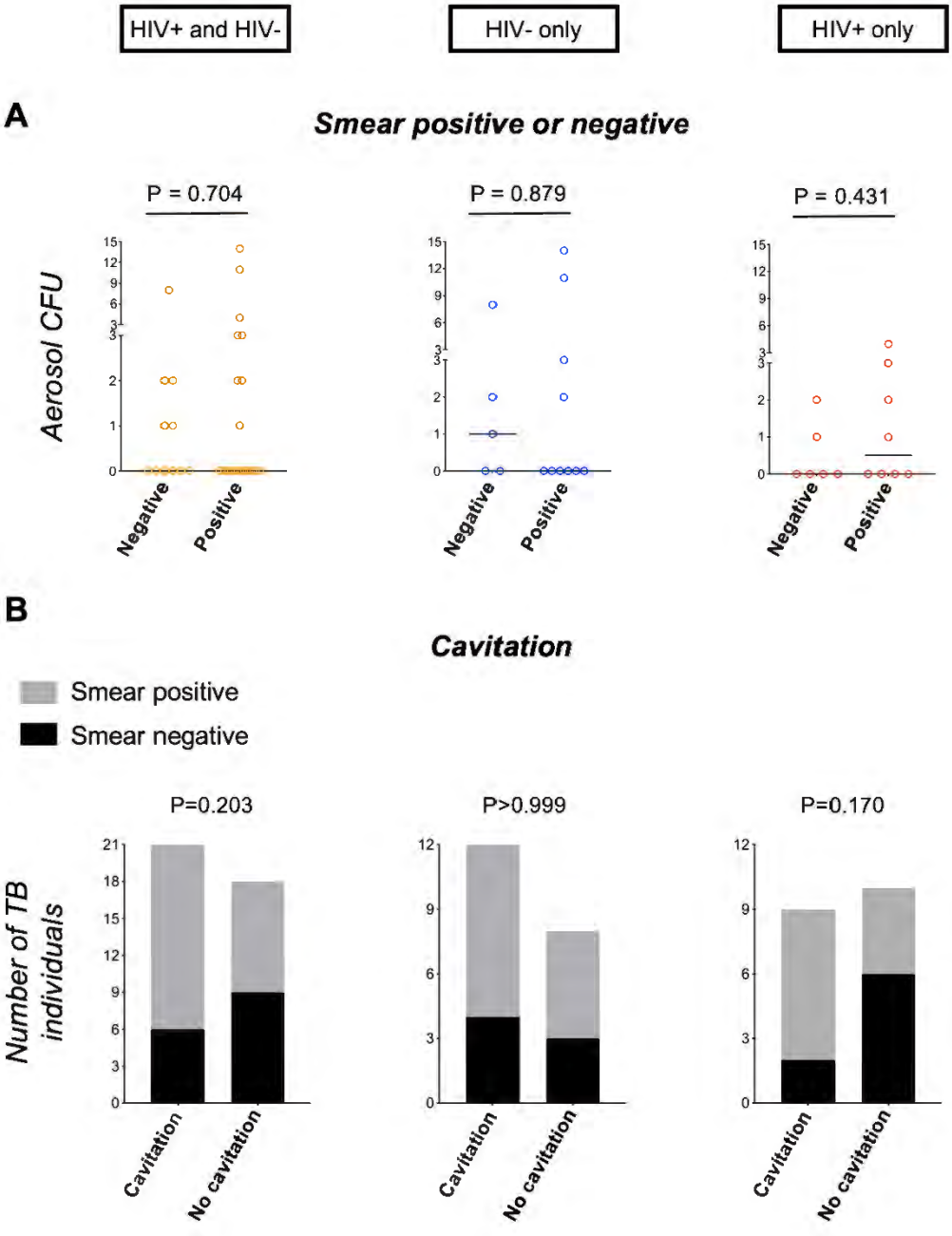

Figure 44. Acid-fast bacilli smear positivity and associations with infectiousness and cavitation in TB patients. (A) Aerosol CFU counts in smear negative and positive individuals in the HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) group. **(B)** Smear positivity in individuals with and without cavitation in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. The Mann-Whitney U test was used to calculate p values in **A** and the Fischer's exact test was used to calculate p values in **B**. The p values < 0.017 were considered statistically significant (three comparisons).

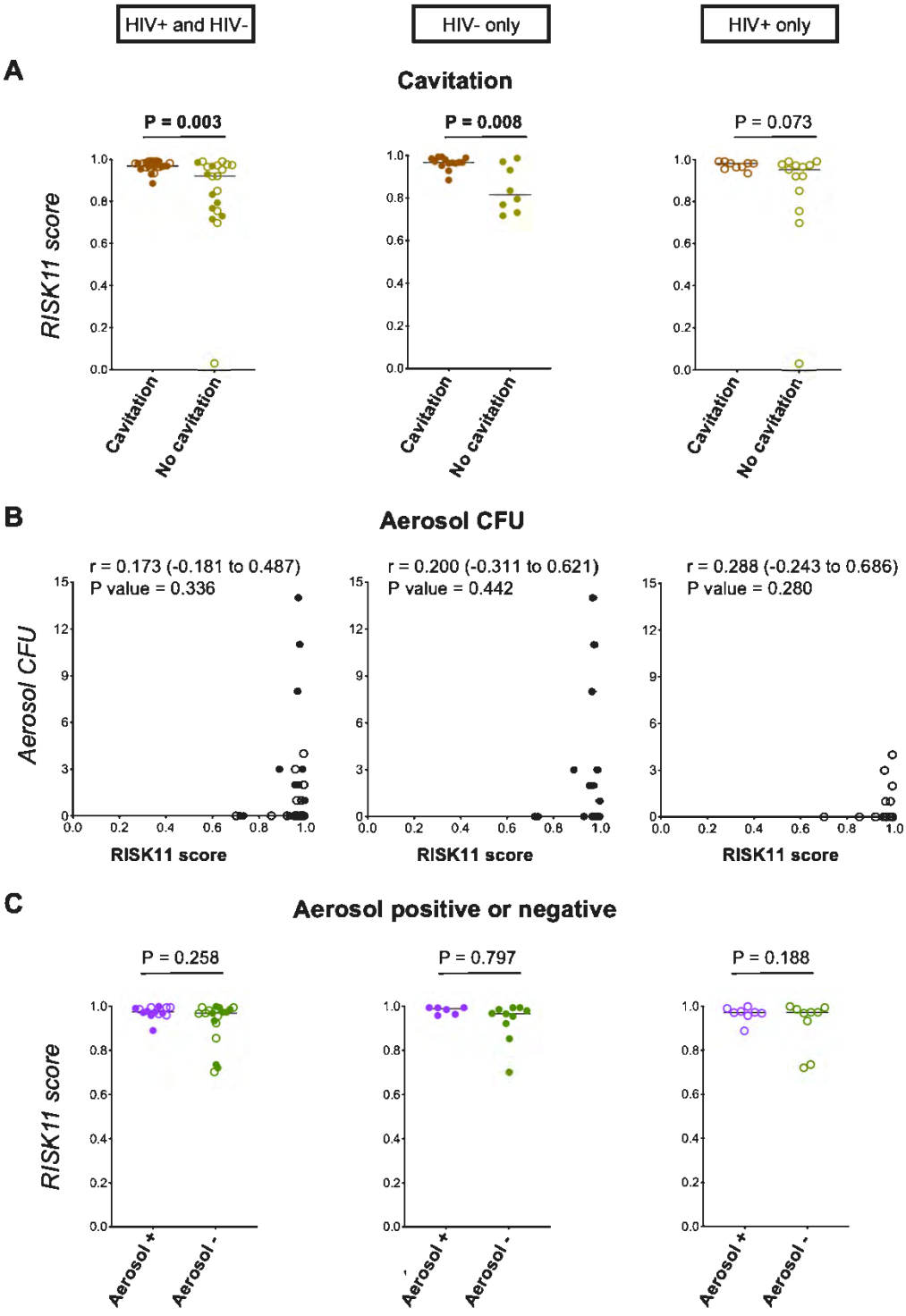

Figure 45. RISK11 signature score associations with cavitation and aerosol CFU. (A) RISK11 scores in TB patients with and without cavitation in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. **(B)** Correlations between aerosol CFU and RISK11 scores in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. **(C)** RISK11 scores in aerosol+ and aerosol- TB patients (aerosol+ was defined as any detectable *M.tb* while aerosol- was defined as no detectable *M.tb* by microbiological culture from any capturing device from the RASC) in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. The p values were calculated with the Mann-Whitney U test. The p values < 0.017 were considered statistically significant (three comparisons). Solid symbols represent HIV- individuals and open symbols represent HIV+ individuals.

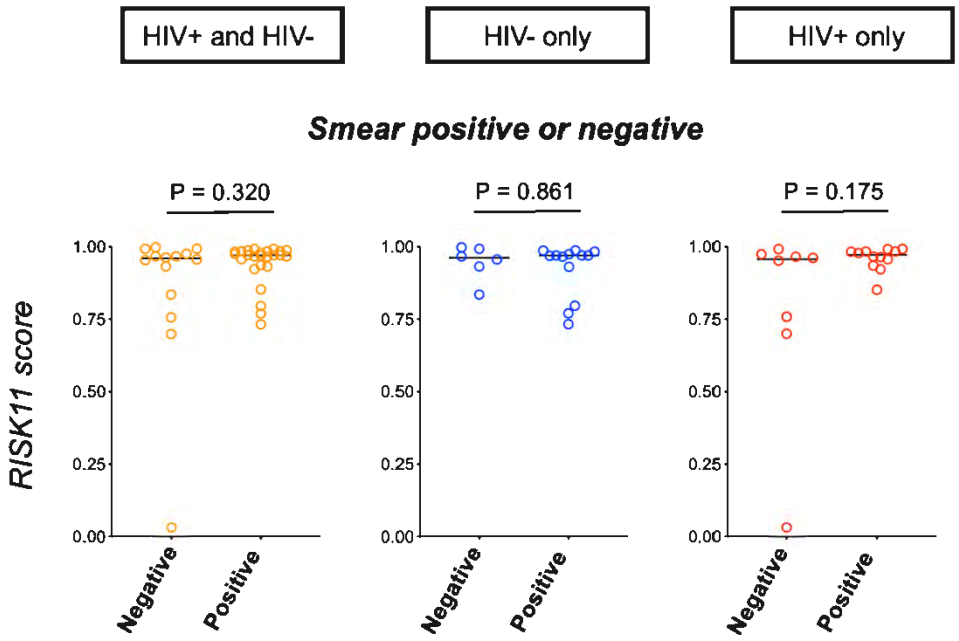

Figure 46. Acid-fast bacilli smear positivity and associations with RISK11 signature score. RISK11 signature scores in smear negative and positive individuals in the HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) group. The p values were calculated with the Mann-Whitney U test and $p < 0.017$ (three comparisons) were considered statistically significant.

6.4.6. Frequencies of immune subsets in TB patients and controls

Next, we measured the abundance of immune subsets in peripheral blood from TB patients and controls. We measured both relative proportions as well as absolute cell counts and observed very similar results, hence we mainly report on the relative proportions (frequencies) of host immune cell subsets in this chapter. Studies have reported higher GLR and MLR in TB patients compared to controls (La Manna et al., 2017, Wang et al., 2015, Sutherland et al., 2009b). In our study GLR and MLR were indeed higher in TB patients compared to healthy controls in all the comparisons (all individuals, HIV- individuals only and HIV+ individuals only) **(Figure 47)**. To unpack this finding, we further measured the relative abundance of neutrophils, monocyte, $CD4^+$ T cells, $CD19^+$ B cells, NK cells and MAIT cells. Granulocytes (**Figure 48A**) and intermediate monocytes **(Figure 48B)** were higher in TB patients compared to controls regardless of HIV status. An increase in frequency of non-classical monocytes was observed in individuals with TB compared to controls (HIV-positive and HIV-negative individuals and HIV-positive only individuals), while no differences were detected in

classical monocytes **(Figure 48B)**. Among lymphocytes, a decrease in frequencies of the $CD56^{dim}CD16^{-}$ and $CD56^{bright}CD16^{-}$ NK cell subsets was observed in TB patients compared to controls regardless of HIV status **(Figure 48C)**. A decrease was also detected for the $CD56^{bright}CD16^{dim}$ NK cell subset in TB patients compared to controls, but only in the analyses which included both HIV-positive and HIV-negative individuals **(Figure 48C)**.

Frequencies of $CD19^{+}$ B cells and $CD4^{+}$ T cells were lower in TB patients compared to healthy controls, regardless of HIV status **(Figure 49A and Figure 49B)**. $CD4^{+}$ T cell absolute counts were also lower in TB patients compared to controls (**Figure 49C**). All MAIT cell subsets were decreased in individuals with TB compared to controls in the analyses that included individuals with and without HIV **(Figure 49D)**. However, total MAIT (regardless of CD4/CD8 expression) and $CD8^{-}CD4^{-}$ MAIT cells were lower in TB patients compared to healthy controls in the HIV negative group while total MAIT, $CD8^{-}CD4^{+}$ MAIT and $CD8^{-}CD4^{-}$ MAIT cell subsets were lower in TB patients compared to controls in the HIV-positive group **(Figure 49D)**. There were no differences detected for the other immune subsets between TB and control individuals.

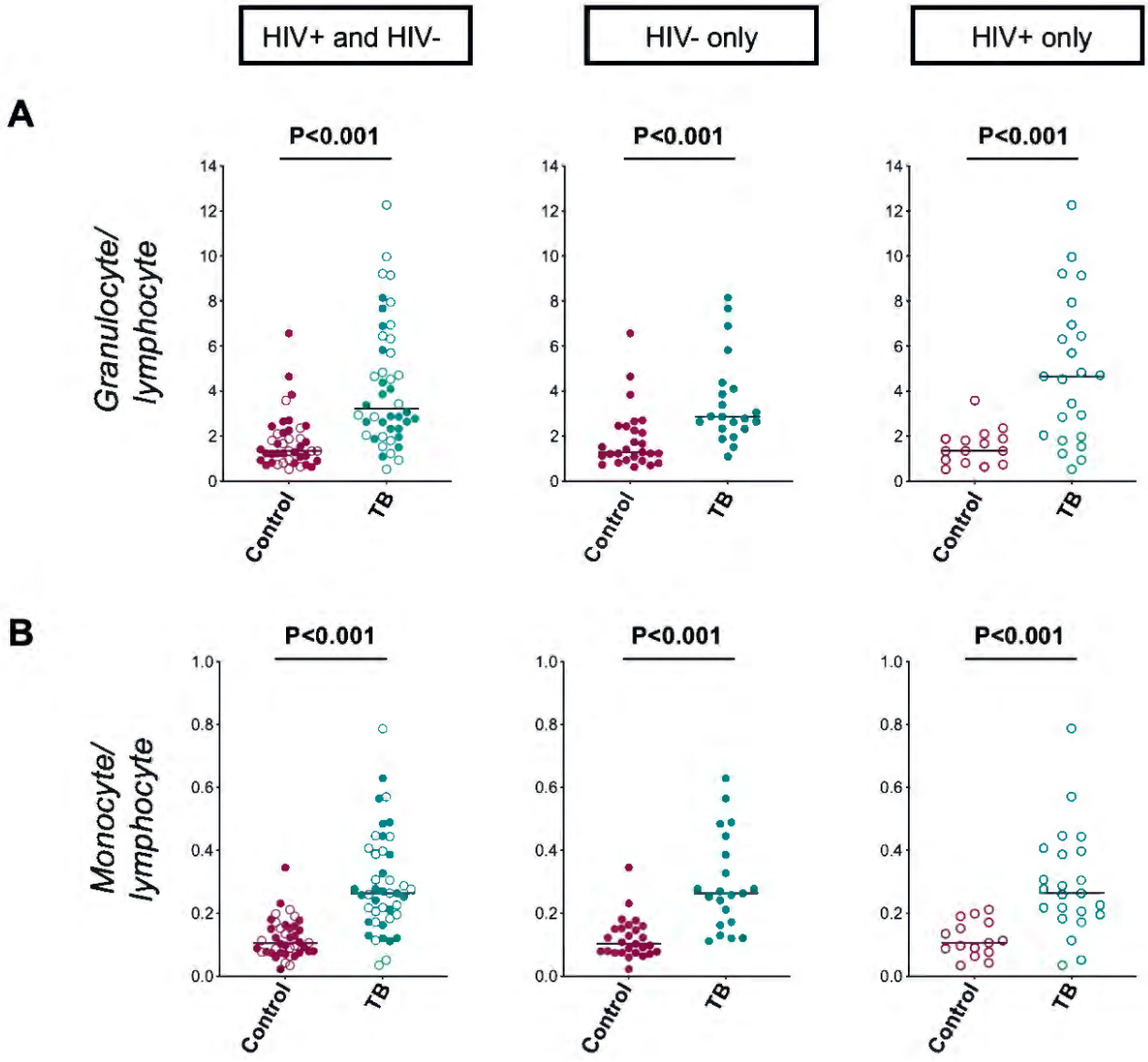

Figure 47. Granulocyte to lymphocyte ratios and monocyte to lymphocyte ratios in TB individuals and healthy controls. (A) Granulocyte to lymphocyte ratios and **(B)** monocyte and lymphocyte ratios in TB individuals in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals**.** The p values were calculated with the Mann-Whitney U test; $p < 0.017$ were considered statistically significant (three comparisons). Solid symbols represent HIV- individuals and open symbols represent HIV+ individuals.

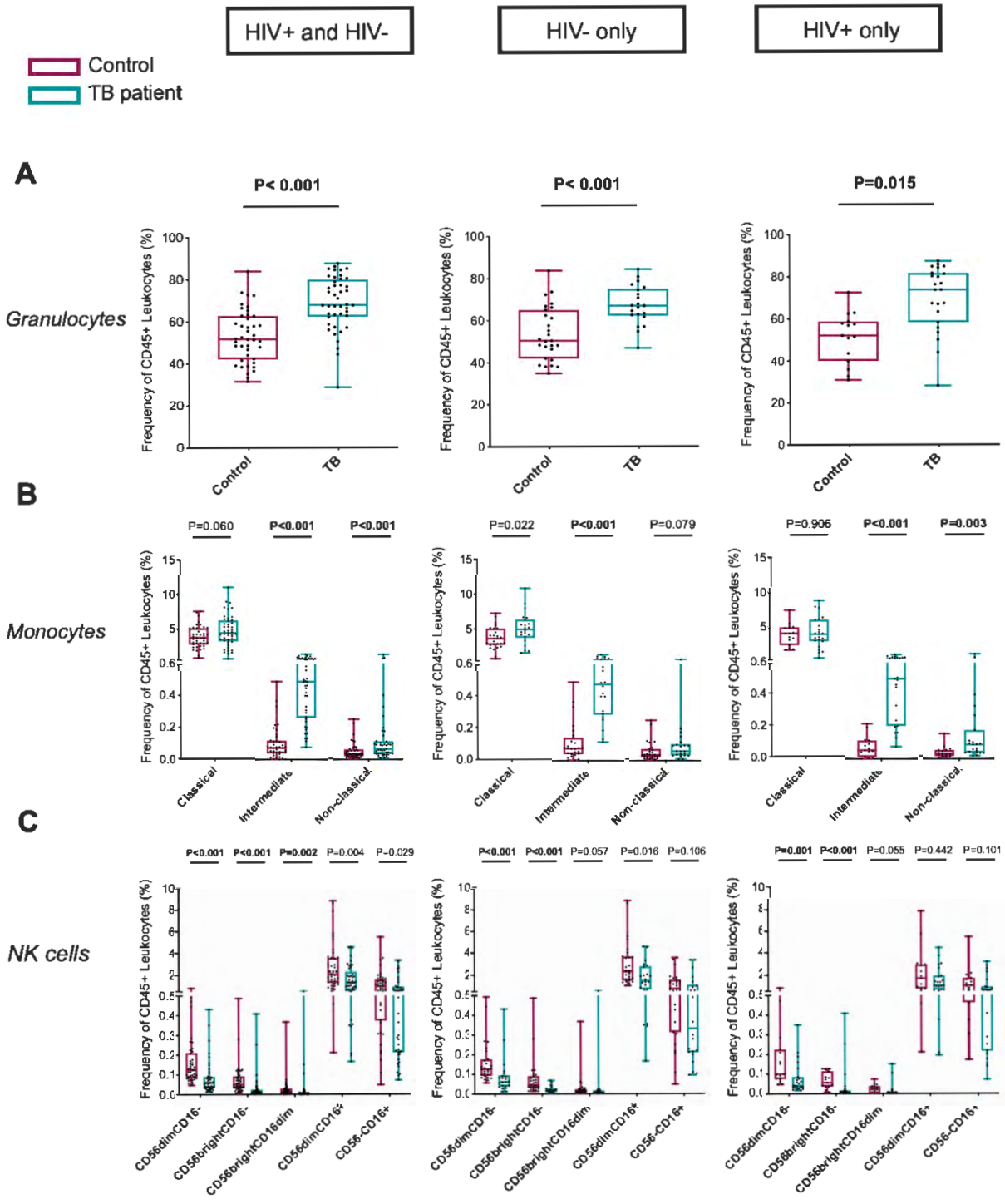

Figure 48. Frequencies of innate immune subsets in TB patients and healthy controls. Frequencies of **(A)** granulocytes **(B)** monocytes and **(C)** NK cells in TB patients and healthy controls in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. In **A** $p < 0.017$ (three comparisons), in **B** $p < 0.0056$ (nine comparisons) and in **C** $p < 0.003$ (fifteen comparisons) were considered statistically significant.

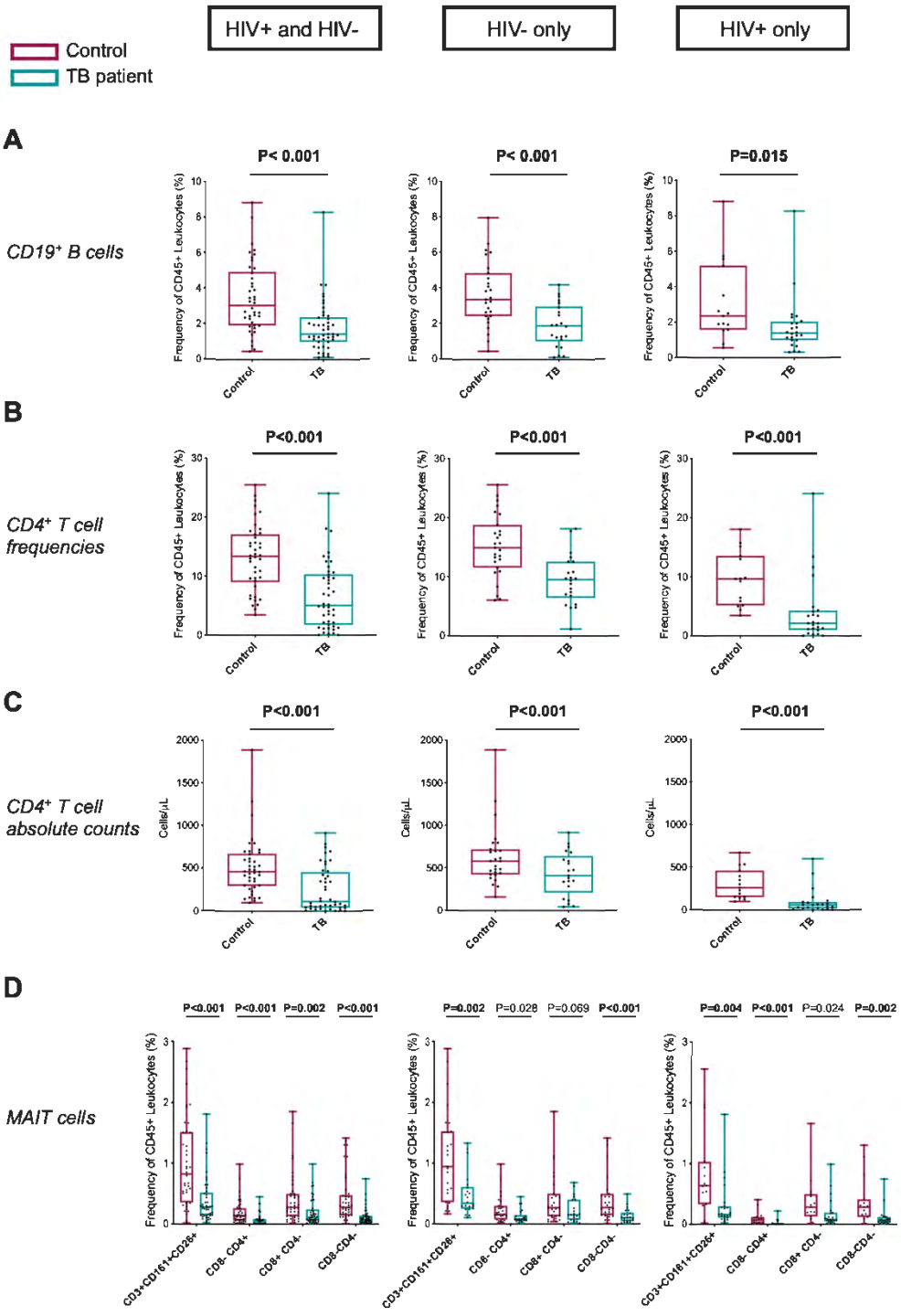

Figure 49. B and T cell immune subsets in TB patients and healthy controls. F **(A)** CD19$^+$ B cell frequencies **(B)** CD4$^+$ T cell frequencies **(C)** CD4$^+$ T cell absolute counts and **(D)** MAIT cell frequencies in TB patients and healthy controls in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals. Horizontal lines represent medians, boxes represent the interquartile range, and the whiskers represent the range. The p values were calculated with the Mann-Whitney U test. In **A** $p < 0.017$ (three comparisons), in **B** $p < 0.0042$ (twelve comparisons) and in **C** $p < 0.017$ (three comparisons) were considered statistically significant.

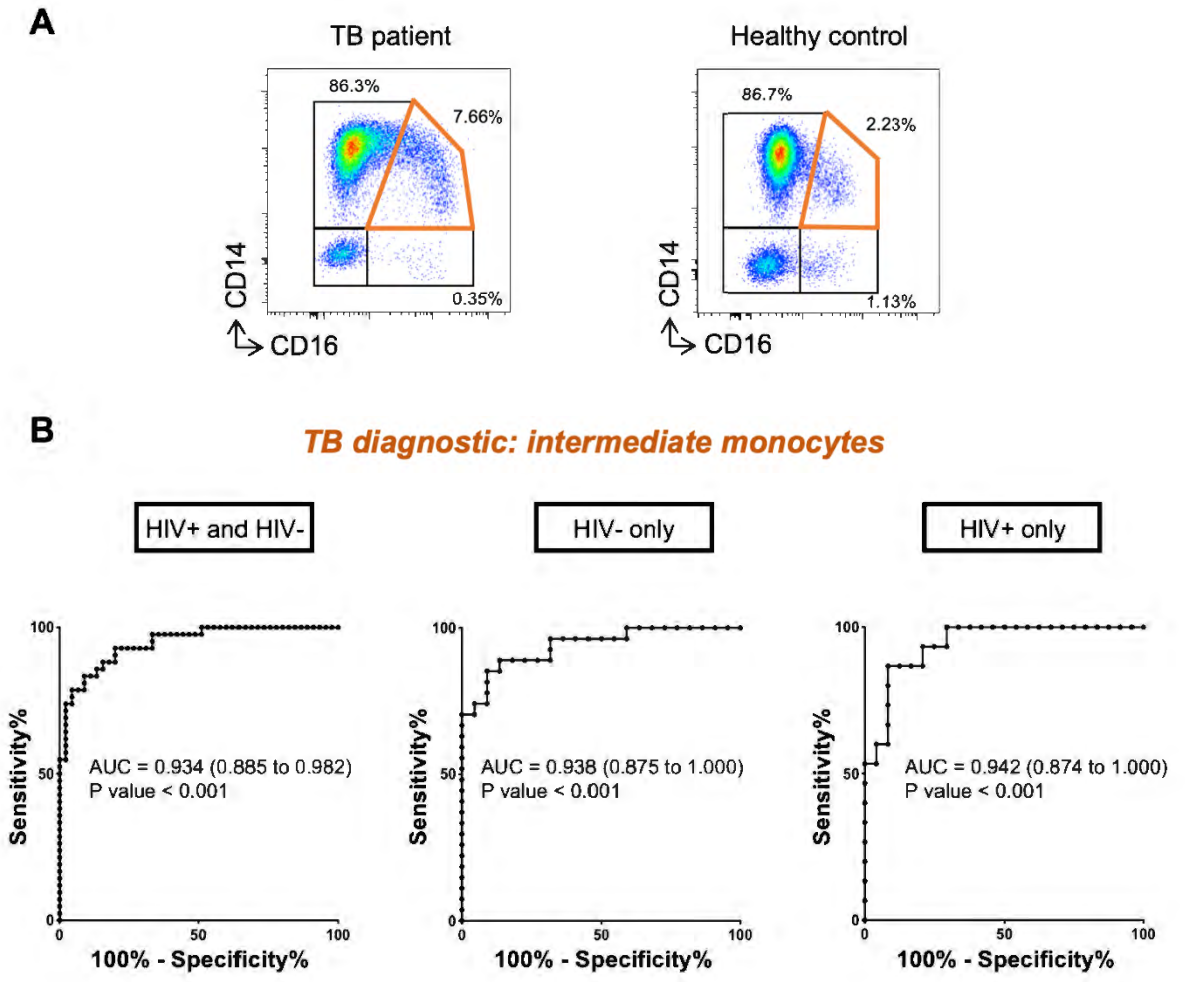

Figure 50. Performance of frequencies of intermediate monocytes as a TB classifier. (A) Representative flow cytometry plots showing monocyte subsets based on CD14 and CD16 expression in a TB patient (left) and a healthy control (right). Gating of the intermediate monocyte subset is shown in orange. **(B)** ROC AUCs depicting the discrimination of TB patients and healthy controls using intermediate monocyte frequencies in HIV+ and HIV- (left), HIV- only (middle) and HIV+ only (right) individuals.

6.4.7. Frequencies of intermediate monocytes distinguish TB patients from healthy controls

Among all the leukocyte subsets measured, frequencies of intermediate monocytes **(Figure 50A)** were able to differentiate between TB patients and controls in all analyses, with an AUC of 0.934 (95% CI 0.885 to 0.982, p<0.001) in all participants, an AUC of 0.938 (95% CI 0.875 to 1.00, p<0.001) in the HIV- only group and an AUC of 0.942 (0.874 to 1.00, p<0.01) in the HIV+ only group (**Figure 50B**). Although the

AUC value was higher in the HIV+ only group compared to the other group, there was no difference in overall performance between the HIV+ only group and the HIV- only group (p=0.933).

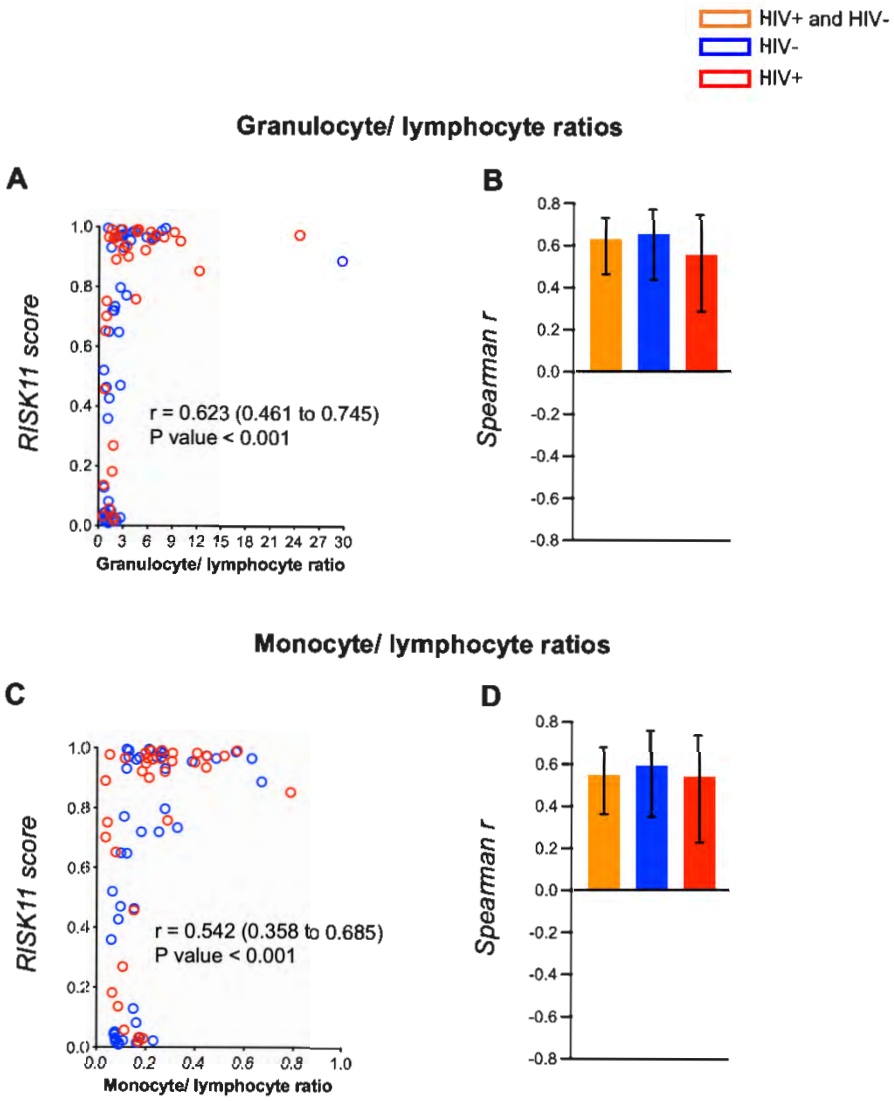

Figure 51. Granulocyte and monocyte to lymphocyte ratios associate with RISK11 signature score in TB individuals and healthy controls. Spearman correlation between RISK11 signature scores and **(A)** granulocyte to lymphocyte ratios and **(C)** monocyte to lymphocyte ratios in HIV+ and HIV- individuals. Spearman correlation values between RISK11 signature scores and **(B)** granulocyte to lymphocyte ratios and **(D)** monocyte to lymphocyte ratios in HIV+ and HIV- (yellow), HIV- (blue) and HIV+ (red) individuals. Bars represent Spearman correlation coefficient values and whiskers represent 95% confidence intervals.

6.4.8. Host immune subset frequencies associate with the RISK11 signature score in TB patients and controls

To ascertain associations between immune subsets and a measure of TB-associated inflammation, we correlated frequencies of immune subsets with the RISK11 signature score. GLR and MLR were positively correlated with the RISK11 signature scores in TB patients and controls regardless of HIV status **(Figure 51A and Figure 51B)**. Overall, frequencies of granulocytes and monocytes positively correlated while lymphocyte immune subsets negatively correlated with RISK11 signature scores **(Figure 52A).** Frequencies of granulocytes were strongly correlated with the RISK11 signature scores regardless of HIV status (**Figure 52B**). After correcting for multiple comparisons, intermediate monocytes was the only monocyte subset that was positively correlated with RISK11 signature scores **(Figure 52C)**. For the NK cell subsets, $CD56^{dim}CD16^{-}$ NK cells (all individuals and HIV- individuals), $CD56^{bright}CD16^{-}$ NK cells (all individuals and HIV- individuals), $CD56^{bright}CD16^{dim}$ (all individuals) and $CD56^{dim}CD16^{+}$ NK cells (all individuals and HIV- individuals) were negatively correlated with RISK11 signature scores **(Figure 52D)**. $CD4^{+}$ T and $CD19^{+}$ B cells were negatively correlated with RISK11 signature scores **(Figure 52E)**. Finally, $CD3^{+}$, $CD4^{+}$ (all individuals and HIV+ individuals) and $CD8^{-}CD4^{-}$ (all individuals and HIV- individuals) phenotypic MAIT cells were also negatively correlated with RISK11 signature scores **(Figure 52F)**.

6.4.9. Immune cell subset frequencies are not different between individuals with cavitary disease and those without cavitation

Next, we compared the frequencies of immune subsets (and absolute CD4 counts, not shown) in TB patients with and without cavitation and observed no differences when all individuals were combined **(Figure 53),** nor when HIV- individuals and HIV+ individuals were analysed separately (data not shown).

6.4.10. MAIT cell frequencies in peripheral blood negatively associate with M.tb aerosol CFU in TB patients

Finally, we performed correlation analyses between frequencies of immune subsets and aerosol CFU, in order to identify immune subsets in peripheral blood that are associated with levels of aerosolised *M.tb* expelled by TB patients **(Figure 54)**. Total phenotypically defined MAIT and $CD8^+$ MAIT cells negatively correlated with *M.tb* aerosol CFU, although the correlation coefficients were weak. Also, p-values did not meet the 0.05 threshold for significance after multiple correction testing **(Figure 54A and Figure 54F)**. However, frequencies of $CD8^-CD4^-$ $CD26^+CD161^+$ MAIT cells (HIV-positive and HIV-negative individuals and HIV-negative individuals only) were negatively correlated with levels of *M.tb* aerosol CFU **(Figure 54A and Figure 54F).** There were no associations between frequencies of the other immune subsets and levels of *M.tb* aerosol CFU **(Figure 54B-54E)**.

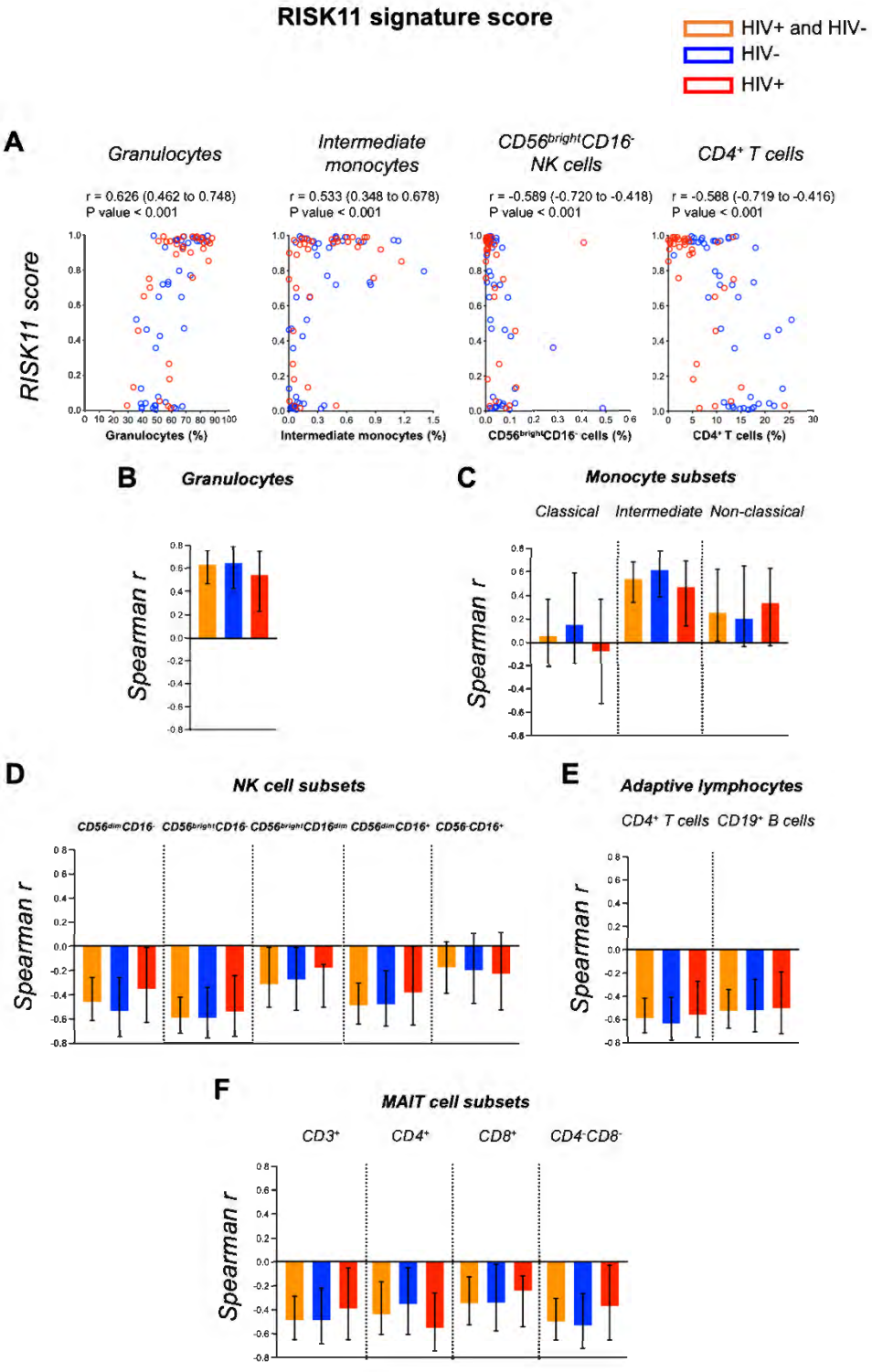

Figure 52. Immune cell subset associations with RISK11 signature scores in TB patients and controls. (A) Spearman correlations between RISK11 signature scores and frequencies of innate and adaptive immune cell subsets. Blue symbols represent HIV- individuals and red symbols represent HIV+ individuals. Spearman correlations of **(B)** granulocytes **(C)** monocyte subsets (classical, intermediate and non-classical) **(D)** NK cell subsets ($CD56^{dim}CD16^{-}$, $CD56^{bright}CD16^{-}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$) **(E)** adaptive lymphocytes ($CD4^{+}$ T cells and $CD19^{+}$ B cells) and **(F)** MAIT cell subsets ($CD3^{+}$, $CD4^{+}$, $CD8^{+}$ and $CD8^{-}CD4^{-}$) with RISK11 signature scores. Bars represent spearman coefficient values and whiskers represent the 95% confidence intervals.

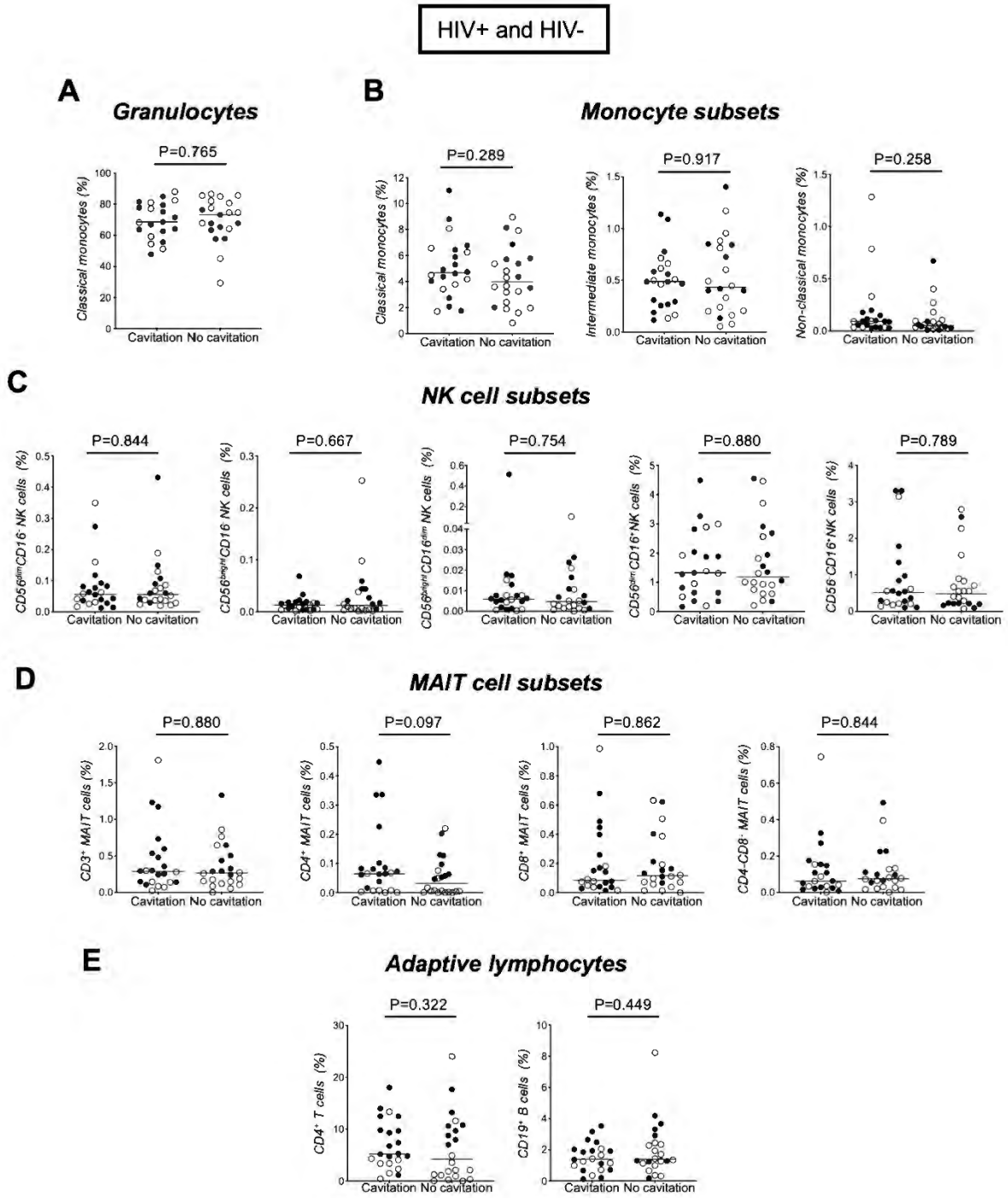

Figure 53. Frequencies immune subsets in individuals with and without cavitation. (A) Granulocytes **(B)** monocyte subsets (classical, intermediate and non-classical) **(C)** NK cell subsets ($CD56^{dim}CD16^{-}$, $CD56^{bright}CD16^{-}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$) **(D)** MAIT cell subsets ($CD3^{+}$, $CD4^{+}$, $CD8^{+}$ and $CD8^{-}CD4^{-}$) and **(E)** adaptive lymphocytes ($CD4^{+}$ T cells and $CD19^{+}$ B cells) with and without cavitation in HIV+ and HIV- TB individuals. Solid symbols represent HIV- individuals and open symbols represent HIV+ individuals. The p values were calculated with the Mann-Whitney U test.

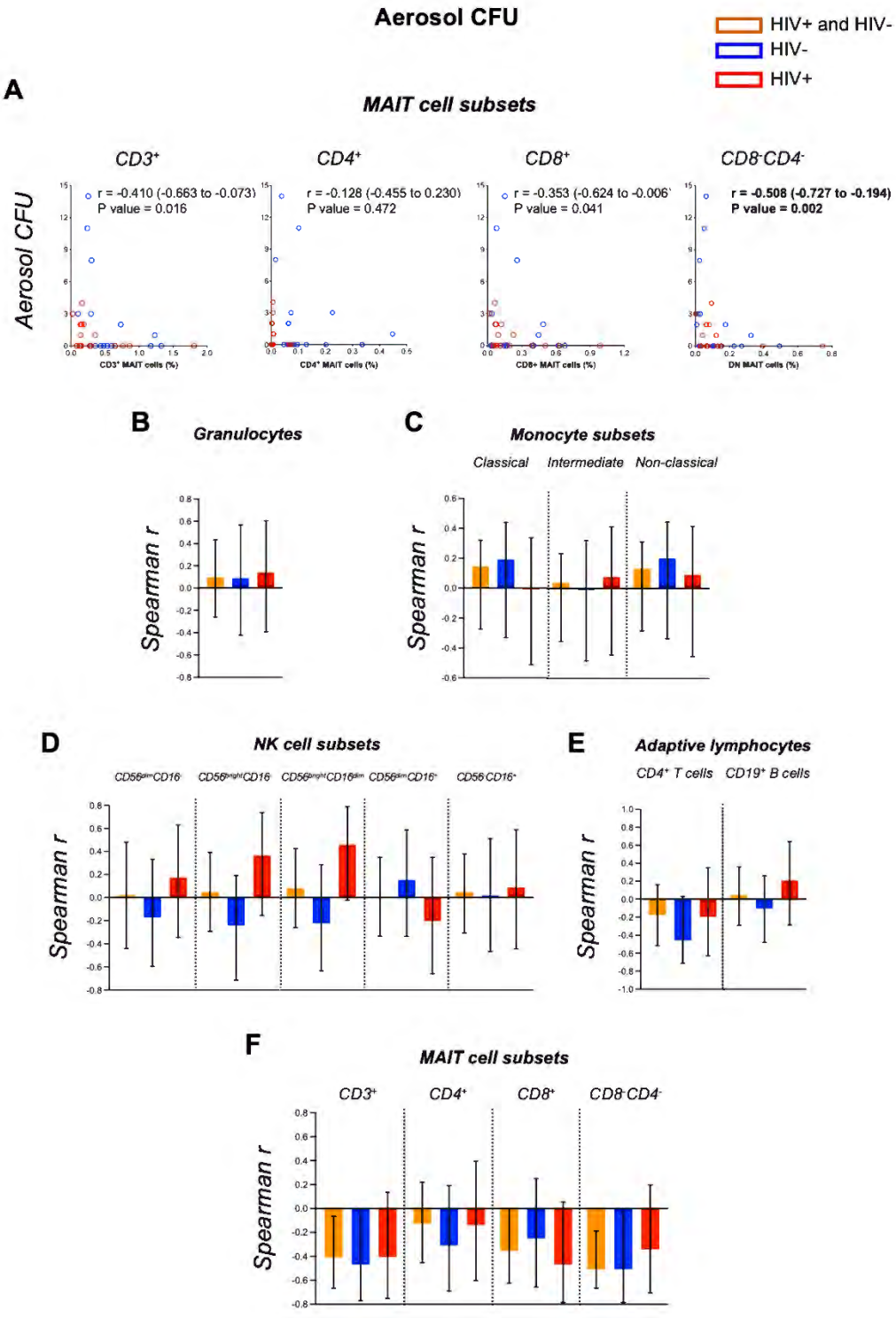

Figure 54. (A) Spearman correlations between aerosol CFU and frequencies of innate and adaptive immune cell subsets. Blue symbols represent HIV- individuals and red symbols represent HIV+ individuals. Spearman correlations of **(B)** granulocytes **(C)** monocyte subsets (classical, intermediate and non-classical) **(D)** NK cell subsets ($CD56^{dim}CD16^{-}$, $CD56^{bright}CD16^{-}$, $CD56^{bright}CD16^{dim}$, $CD56^{dim}CD16^{-}$ and $CD56^{-}CD16^{+}$) **(E)** adaptive lymphocytes ($CD4^{+}$ T cells and $CD19^{+}$ B cells) and **(F)** MAIT cell subsets ($CD3^{+}$, $CD4^{+}$, $CD8^{+}$ and $CD8^{-}CD4^{-}$) with RISK11 signature scores. Bars represent spearman coefficient values and whiskers represent the 95% confidence intervals.

6.5. Discussion

Our goal was to identify host immune cell subsets that associate with inflammation and aerosolised *M.tb*, a measure of infectiousness, in TB patients. A simple, whole blood host biomarker that identifies those TB patients who are infectious would allow studies to enhance our understanding of the biology underlying TB transmission. It would also facilitate development of targeted treatment approaches, potentially disrupting TB transmission. Our main findings include: 1. The RISK11 signature score was higher in HIV-negative TB patients with cavitation compared to those without cavitary disease; 2. RISK11 signature scores positively correlated with the GLR and MLR; 3. MAIT cell frequencies negatively correlated with *M.tb* containing aerosols and; 4. Frequencies of intermediate monocytes differentiated TB patients from controls regardless of HIV status with good diagnostic performance.

The overarching study was designed to understand *M.tb* transmission dynamics and measure aerosol expulsion. The immunology component, presented in this chapter, was an exploratory outcome and thus subject to limitations. As previously stated, we report on the first batch of participants who were recruited into this study before significant changes in the study protocol were implemented. Therefore, the sample size was small and we were underpowered to detect small differences, particularly in the HIV-infected group. Overall, study participants were very unwell, as detected by the high RISK11 signature scores and shown by radiological evidence, therefore most of measured outcomes showed a narrow dynamic range, which has likely hampered the possibility to observe immune features with small effect sizes. We were particularly interested in identifying a robust immune marker that identifies infectious individuals in a population with high HIV prevalence. The HIV-infected group, however, was heterogenous, with some individuals on ART while others were not, likely resulting in a large range of CD4 counts and viral loads. Unfortunately, the CD4 counts and viral loads were not captured for all participants in this group and, therefore, we couldn't account for differences in both CD4 counts and viral loads. However, we were able to measure the absolute cell counts for $CD4^+$ T cells in our cohort. Another limitation is the lack of a validation cohort, to determine if the biomarkers identified here reproducibly associate with the outcomes measured.

We were able to measure *M.tb*-containing aerosols expelled by TB patients during tidal breathing using a novel technology. Sampling methods such as the RASC allows quantification of *M.tb*-containing aerosols over a short time period. The RASC could potentially be used to assess infectiousness of TB patients before and during TB treatment with high accuracy. In addition, the RASC measures *M.tb*-containing aerosols expelled during normal breathing; study participants were not asked specifically to cough or to perform particular vocal tasks, which was the case for other studies (Fennelly et al., 2012, Williams et al., 2014, Shaikh et al., 2019, Theron et al., 2020), thus our results are more representative of real-life transmission dynamics. On the other hand, the majority of *M.tb* captured from aerosols in this study were cultured on agar plates from the six-stage Viable Anderson Cascade Impactors (Patterson et al., 2017). It is possible that the culturing method could enrich for *M.tb* strain subtypes that are not representative of bacilli that survive in the air and cause infection once inhaled by a susceptible host. To address this, Patterson et al. recently improved the RASC by modifying the *M.tb*-aerosol sampling protocol by utilising continuous liquid collection of *M.tb*-aerosols using a high efficiency cyclone collector instead of the VACI as the major capturing device. In addition, a non-culture based *M.tb* detection method was used: bacilli in the liquid were concentrated and detected by fluorescence microscopy utilising DMN-trehalose, a dye that becomes fluorescent when incorporated into the cell wall of metabolically active *M.tb* (Patterson et al., 2020). The detection of viable *M.tb* improved from 43% (range: 1-14 CFU) using the original RASC to 95% (range: 2-155 DMN-trehalose positive bacilli) using the modified RASC (Patterson et al., 2020). The improved protocol of the RASC could, in future, allow the detection and visualisation of *M.tb* collected from aerosols expelled by a range of TB patients, such as sputum smear negative or those with subclinical TB (Dinkele et al., 2021a, Dinkele et al., 2021b).

In our study, the RISK11 signature was able to differentiate between TB patients and controls, with a performance that was not diminished in the HIV-infected group. However, it was previously shown in a different case-control study that the discriminatory power of RISK11 was lowered in HIV-infected individuals (Darboe et al., 2019). The RISK11 signature is composed of type I/II IFN-stimulated genes, which are typically induced by viral infections, such as HIV and may lead to higher RISK11 signature scores and false positive test results (Darboe et al., 2019). In a recent study,

individuals in a community setting were recruited in two prospective studies to determine if the RISK11 signature could identify individuals with undiagnosed TB who ordinarily would not seek healthcare or those at high risk of progression to incident TB. It was shown that the RISK11 signature overall had good diagnostic and prognostic performance (although the discriminatory power of RISK11 was reduced in asymptomatic individuals), with similar performances in individuals with and without HIV (Scriba et al., 2021, Mendelsohn et al., 2021). However, the signature fell short of the World Health Organisation's target product profiles for a TB triage test (WHO, 2014). It is possible that the average viral load of HIV-infected individuals in this and previous studies differed (higher viral loads could lead to higher RISK11 signature scores) to the extent that it may account for the contradictory results on the performance of the RISK11 signature between all individuals.

We measured *M.tb*-containing aerosols expelled by TB patients during normal breathing as a surrogate for potential infectiousness. Previous studies have shown that cough-generated *M.tb* aerosols are a better predictor of infectiousness than sputum bacillary load measured by smear microscopy (Jones-Lopez et al., 2013). Interestingly, *M.tb* aerosol CFU was higher in those individuals with cavitation compared to those without cavitary disease only when the HIV+ and HIV- groups were pooled (all individuals). However, this difference was not significant after corrections for multiple testing. This result supports the thinking that TB patients with severe lung pathology are more likely to expel infectious *M.tb* aerosols into the air (Rodrigo et al., 1997, Kaplan et al., 2003, Mathema et al., 2017). Thus, the likelihood that the result is a false positive is low. In addition, when we analysed cavitation in aerosol positive and negative individuals, there was a small difference in the HIV-negative group only, which was significant based on a p-value threshold of 0.05, but not after adjustment for multiple testing.

Traditionally, sputum smear microscopy has been used as an indicator of infectiousness despite it not being sensitive (Sepkowitz, 1996a). We sought to determine whether our outcomes of inflammation and infectiousness associated with smear microscopy results. No differences were detected for the RISK11 signature score, aerosol CFU or cavitation between sputum positive and negative individuals. We speculate that low sensitivity of sputum does not account for the full range of

infectiousness and therefore no association was observed between sputum smear positivity and the RISK11 signature score, aerosol CFU and cavitation. However, as stated above, our sample size was small, and we were likely underpowered to detect minor differences between sputum positive and sputum negative individuals in this heterogenous cohort. It is also important to note that we use *M.tb* aerosolisation as a proxy for infectiousness, but do not show that individuals in our cohort go on to transmit (showing IGRA conversion in a case contact study). This could account for the discordant results we observed in this study.

Although RISK11 scores were higher in HIV- patients with cavitary disease, we did not observe an association between aerosol CFU and the RISK11 scores in any group. Considering that the TB patients in our cohort overall had very high RISK11 signature scores, it is possible that the dynamic range of RISK11 scores was insufficient to detect weak correlations with infectiousness. Additionally, several TB patients had a high RISK11 signature score and negative aerosol culture results, further reducing the power to detect associations in such a small sample size. Studies have shown that less than 45% percent of TB patients have culturable *M.tb*-containing aerosols using the VACI as a sample capturing method (Patterson et al., 2017, Fennelly et al., 2012, Jones-Lopez et al., 2013). This result could be a product of the relatively poor sensitivity of the sampling apparatus and/ or could be indicative of the variability of transmission. It would be interesting to perform this analysis in individuals with subclinical TB, who most likely would show a greater range in RISK11 scores and may expel *M.tb*-containing aerosols (Scriba et al., 2021, Mendelsohn et al., 2021).

We aimed to identify an immune subset in peripheral blood that could be used as an indicator of TB infectiousness. Although targeted treatment of potential TB super-spreaders is unlikely to be a feasible development, our results pave the way for improved studies of *M.tb* transmission. We were particularly interested in evaluating immune subsets that have been understudied in the context of TB, such as NK cells, B cells and MAIT cells.

Consistent with previous studies, peripheral blood frequencies of lymphocytes were lower while granulocytes and monocytes were higher in TB patients compared to controls (La Manna et al., 2017, Wang et al., 2015, Sutherland et al., 2009b).

Interestingly, among all the immune subsets measured, intermediate monocytes were able to differentiate TB patients from controls, regardless of HIV status. This may prove to be an invaluable TB biomarker, since enumerating intermediate monocytes in blood does not require extensive sample processing and the discriminatory ability did not diminish in HIV-infected individuals. It has been shown that $CD16^{+}$ monocytes increase in numbers in individuals with TB and that this increase is more striking in the intermediate monocyte subset than non-classical monocytes (Castano et al., 2011). Our study builds on this work, with the inclusion of HIV-infected individuals in our cohort, which was not the case in the Castano et al. study. Increased intermediate monocyte numbers positively correlating with disease severity in inflammatory diseases has also been reported (Wong et al., 2012), but this needs to be further studied in the context of TB and trained immunity. It will be important to assess how other inflammatory conditions affect this biomarker. A CD14-based TB biomarker has been proposed, but large prospective studies are required to validate this as a viable option (Sampath et al., 2018).

In previous studies, MLR was positively associated with IFN-related gene transcripts from monocytes, which were enriched in TB disease and other inflammatory conditions (Naranbhai et al., 2015). Our results showing that MLRs were higher in TB patients compared to controls and positively correlated with RISK11 scores, indicating upregulation of IFN-related genes, are therefore consistent with previous reports. Additionally, we observed a strong positive association between GLR and RISK11 signature scores. The high GLR, MLR and the RISK11 signature scores in TB patients compared to controls is consistent with increased inflammatory processes that occur during ongoing disease. In addition, strong associations were observed between frequencies of immune cell subsets and RISK11 signature scores, with granulocyte and monocyte subsets positively associated and lymphocyte subsets negatively associated with RISK11 signature scores, showing that these inflammatory processes are associated with a general lymphopenia which is not restricted to a specific cell subset.

Surprisingly, we found no differences in immune cell subset frequencies between individuals with and without cavitary disease regardless of HIV status. A study in Russia showed that band (immature) neutrophils were positively and strongly

associated with lung destruction (measured by chest X-ray) and bacteria excretion (measured by sputum smear microscopy and PCR) (Panteleev et al., 2017). A possibly important difference is that in the Russian study band neutrophil numbers were enumerated by haematology tests while in our study we measured granulocytes (which mainly comprise of neutrophils) by flow cytometry. It is possible that only certain neutrophil subsets, that we did not measure, are associated with lung pathology. It must also be noted that NHP models suggest that there is extreme heterogeneity in sterilisation of individual granulomas within a host (Lin et al., 2014), and it is likely that the relative abundance of immune cells in the periphery does not account for the heterogeneity of lesions and cavitation at the site of infection.

Frequencies of MAIT cell subsets, in particular $CD8^{-}CD4^{-}$ MAIT cells, were negatively associated with *M.tb* aerosol CFU in HIV- participants. MAIT cell depletion in peripheral blood of TB patients compared to controls has been shown, and a recent study suggests that MAIT cells leave the periphery to migrate to the site of infection (Wong et al., 2019). In this study we expand on these findings by showing that more pronounced depletion of MAIT cells from peripheral blood is observed in presumably more infectious TB patients. These findings suggest that MAIT cells are present during lung inflammation and damage, which results in *M.tb* aerosolisation. Depletion of MAIT cells from the peripheral blood of TB patients could be a candidate biomarker of infectiousness, which warrants further studies. It is important to note, however, that the depletion of MAIT cells in the periphery is not specific to TB disease and occurs during various diseases (Serriari et al., 2014, Wong et al., 2013, van Wilgenburg et al., 2016). In a previous study it was reported that MAIT cell numbers were depleted in peripheral blood of individuals with active TB and non-tuberculosis mycobacterial lung disease. In this same study, MAIT cell numbers in the periphery negatively correlated with bacterial load and extent of disease (Kwon et al., 2015). MAIT cells are known for their ability to respond rapidly once activated and produce proinflammatory cytokines and cytotoxic molecules (Dusseaux et al., 2011, Le Bourhis et al., 2010), which could contribute to promoting excessive inflammation and failed bacterial containment. However, as stated before in chapter 3, MAIT cells have been shown to contribute to protection against mycobacterial infection in animal models (Chua et al., 2012, Sakala et al., 2015). The exact role of MAIT cells, whether protective or not, is still unknown in the context of TB disease.

Our results shed some light on the pathophysiology of infectiousness. Indicators of systemic inflammation (RISK11 signature, GLR, MLR and intermediate monocyte frequencies) were higher in TB patients compared to controls. Also, among HIV-negative TB patients, the RISK11 signature scores were higher in patients with cavitation compared to those without cavitation. Our results support the hypothesis that individuals with systemic inflammation are more likely to transmit *M.tb*.

In conclusion, we show that frequencies of intermediate monocytes can differentiate between TB patients and controls regardless of HIV status and that MAIT cell frequencies negatively associate with *M.tb*-containing aerosols expelled by TB patients prior to start of treatment. Frequencies of certain immune cell subsets in peripheral blood may be useful biomarkers of TB disease and transmission.

6.6. Contributions to this chapter

Aerosol CFU, X-rays and blood was collected by the Desmond Tutu Health Foundation UCT TB aerobiology study team under the supervision of Dr. Carl Morrow and Prof. Robin Wood.

M.tb-containing aerosol CFUs were cultured and enumerated by the Molecular Mycobacteriology Research Unit UCT TB aerobiology study team under the supervision of Prof. Digby Warner.

Gene expression experiments were carried out by the SATVI correlates of risk targeted intervention study (CORTIS) team under the supervision of Dr. Adam Penn-Nicholson and Prof. Thomas J. Scriba.

X-ray data was analysed using the CRRS evaluations by Prof. Rodney Dawson and Prof. Richard van Zyl and the modified scoring algorithm was created by Prof. Thomas J. Scriba and Melissa Murphy with the assistance of Prof. Mark Hatherill and Dr. Simon Mendelsohn.

Flow cytometry experiments were carried out by Melissa Murphy.

All analyses were carried out by Melissa Murphy under the supervision of Dr. Sara Suliman, Assoc. Prof. Elisa Nemes and Prof. Thomas J. Scriba.

Chapter 7: General conclusions

We investigated immune responses other than conventional T cells in the context of BCG vaccination in infants and of TB disease in adults. The following are novel findings in this thesis, that will further our understanding in the research fields of TB whole cell vaccines, TB diagnostics and *M.tb* transmission:

1. BCG vaccination modulates $\gamma\delta$ T, $CD8^{-}CD26^{hi}CD161^{hi}$ T and NK cell functional responses in infants.
2. The induction of BCG-reactive IFNγ-expressing NK cells after BCG vaccination could not be linked to NK cell maturation, differentiation, or cytokine receptor expression changes. However, BCG-reactive NK cell responses correlated with levels of innate and adaptive pro-inflammatory cytokines in BCG vaccinated infants only.
3. Frequencies of $CD16^{+}$ intermediate monocytes differentiated between TB patients and HCs, regardless of HIV status in adults and were thus a promising TB biomarker.
4. MAIT cell frequencies negatively correlated with *M.tb*-containing aerosols in TB patients.

For the work done in infants, we analysed a valuable SA cohort in whom BCG vaccination was either administered at birth, as is routine, or delayed, for the purpose of this study, allowing cross-sectional comparisons between vaccinated and unvaccinated infants from the same community. This is an important component of the study design, since many studies on delayed BCG vaccination are based on opportunistic sampling. Different vaccination practices may apply to different populations based on their epidemiological risk of TB and delaying vaccination for purposes other than research might introduce confounders that could lead to differences in the two groups that are unrelated to BCG vaccination.

We made use of our standardised 12-hour WB-ICS assay to carry out a comprehensive characterisation of the phenotype and function of understudied immune subsets in the context of BCG vaccination in infants and optimised several

flow cytometry panels. We also carried out multiplex immunoassays on stimulated supernatants harvested after 7 hours during the WB-ICS assay protocol. The WB-ICS assay allows assessment of a more biologically relevant sample than purified PBMCs, since it contains all the immune subsets and secreted analytes present in peripheral blood. However, it is a short-term, 12h assay which was originally optimised to detect conventional T cell responses. The fixation step in the WB-ICS protocol does not allow for stimulation of cells after cryopreservation. This limitation makes it impossible to modify protocol conditions (stimulants and duration of stimulation) after collection of blood to optimally investigate immune subsets based on new findings in the literature. Also, we could only access small volumes of blood from our infant cohort. Therefore, we could not include many stimulation conditions and differing incubation time points (prior to fixation and cryopreservation) depending on the immune subset of interest. Despite these limitations, we were able investigate a wide array of cytokines produced by various innate and adaptive immune cell subsets in response to mycobacterial stimulation in infants.

While several studies with a strong design have investigated classical T cell responses to BCG in infants (Burl et al., 2010, Kagina et al., 2010, Blakney et al., 2015, Tchakoute et al., 2015)**,** few studies have explored the contributions of other cell types, but the design either did not include a control group (Zufferey et al., 2013) or may have been affected by selection bias if vaccination was not delayed specifically for study purposes (Smith et al., 2017)**.** We showed in **Chapter 3** that frequencies of CD4 Th1 cells were higher in the BCG compared to the no BCG group. These results agree with prior studies indicating that BCG vaccination elicits a strong Th1 phenotype in infants (Marchant et al., 1999, Vekemans et al., 2001, Soares et al., 2008).

We observed a transient increase in frequencies of BCG-reactive IFNγ-producing $\gamma\delta$ T cells in 5-week-old infants, however this effect waned in 9-week-old infants. Other studies have either only investigated frequencies of $\gamma\delta$ T cells following BCG in infants and adults (Mazzola et al., 2007, Tastan et al., 2005, Hoft et al., 1998) or have investigated intracellular cytokine responses to PMA or specific mycobacterial antigens (Papadopoulou et al., 2020). As discussed in **Chapter 2,** the $\gamma\delta$ B1 antibody clone used in our experiments does not stain all $\gamma\delta$ T cells, however, the alternative

11F2 pan-$\gamma\delta$ clone was affected by fixation. These experiments highlight the limitations of fixation on optimal staining using certain antibody clones in flow cytometry experiments and should be considered during experimental study design. Based on these results and to overcome some of these limitations, we are planning further characterisation of $\gamma\delta$ T cells in PBMCs collected from our infant cohort, to define the transcriptional state and TCR sequences of $\gamma\delta$ T cells in BCG vaccinated and unvaccinated infants.

Phenotypic MAIT cells (defined as $CD8^+TRAV1\text{-}2^+CD26^+CD161^+$ T cells) were not modulated by BCG vaccination in infants, including those defined by MR1-5-OP-RU-tetramer staining (Gela et al., 2022). It was also shown that other DURT cell subsets such as CD1-restricted T cells were not modulated by BCG vaccination in infants (Gela et al., 2022). However, we observed a peculiar subset of BCG-reactive $TRAV1\text{-}2^-CD8^-CD26^{hi}CD161^{hi}$ T cells, which were found at higher frequencies in the BCG group compared to the no BCG group in 5-week and 9-week-old infants. We speculate that these immune cells might represent a BCG-responsive DURT cell type (Fergusson et al., 2014, Godfrey et al., 2019) that should be further investigated in the context of TB vaccination studies in infants. Ongoing collaborative studies where single cell RNA and TCRs are sequenced from sorted MAIT cells should clarify whether these cells are indeed a DURT cell subset and whether BCG vaccination modulates other features of MAIT cells that we did not measure in these initial experiments.

We observed no differences in the cytokine expression and memory phenotype of $CD20^+$ B cells between the two groups of infants. We do not think that these results are conclusive, because we experienced several challenges during our optimisation experiments of B cell markers for Panel 3 (discussed in **Chapter 2**). We discovered that fixation resulted in variable CD19 staining between individuals, thus these experiments also highlight the effect of fixation on optimal antibody staining of certain immune markers. In addition, we also showed that our 12-hour WB-ICS assay interferes with the optimal staining of IgD and CD27 in infants (this was not the case for adults). These factors should be carefully considered during experimental design using similar WB-ICS assays.

In contrast to our expectations based on the increasing literature showing BCG-mediated trained immunity of monocytes, we observed no differences in the frequencies of BCG-reactive $CD33^+$ myeloid cells between infants who were BCG vaccinated and those who were not. We were particularly interested in investigating monocyte responses, but due to the physiological down-regulation of CD14 expression on monocytes stimulated with BCG, we opted to use the CD33 myeloid marker, which seemed less affected by activation during the assay optimisation. Using characteristics of the cells (size and CD16 expression) we excluded granulocytes and are confident that the vast majority of $CD33^+$ cells that we analysed from peripheral blood samples were monocytes, with a very small population of dendritic cells. These are distinct cell subsets, and we cannot exclude the possibility that minor differences in monocyte responses between the two groups of infants could have been masked by the contribution of dendritic cells.

We undertook a comprehensive investigation of NK cell phenotype and function in response to mycobacterial stimulation in BCG-vaccinated and unvaccinated infants. Our results indicate that BCG does not modulate the proportion or cytotoxic potential of the different NK subsets. We did, however, observe higher frequencies of BCG-reactive IFNγ-expressing NK cells in BCG-vaccinated compared to unvaccinated infants. This finding was not associated with changes in NK cell maturation, differentiation or IL-2, IL-18 and IL-12 cytokine receptor expression (which could indicate responsiveness to bystander activation).

We also measured several secreted innate and adaptive cytokines in response to BCG stimulation of whole blood, but observed no differences in the levels of cytokines, except for IFNγ, between the BCG and no BCG group. Interestingly, there were strong positive correlations between BCG-reactive IFNγ-expressing NK cells and key adaptive (IL-2) and innate (IL-6, IL-1β and TNF) secreted cytokines in the BCG-vaccinated infants only. We postulate that, although there were no differences in secreted cytokine levels for the majority of measured analytes, BCG vaccination was able to enhance cross-talk between innate and adaptive immune cells. This finding is corroborated by the positive correlation between BCG-reactive adaptive cells ($CD4^+$ T cells and $CD20^+$ B cells) and BCG-reactive $CD33^+$ myeloid cells. These results

indicate that NK cells may be better suited to contribute to antimycobacterial immunity following BCG vaccination.

Our data does not fully elucidate the underlying mechanism resulting in the higher frequencies of BCG-reactive NK cells in BCG-vaccinated compared to BCG-unvaccinated infants and further studies are warranted in this area of research. The role of trained immunity contributing to the adaptive features of NK cells in this study is not known, but our results strongly support the investigation of NK cells in trained immunity studies. Due to delays caused by the COVID-19 pandemic, we could not complete key experiments that were planned in collaboration with Stanford University, to answer critical questions raised by our results by the time of submission. These experiments are now under-way and include measurement of functional responses to heterologous stimuli and single cell profiling of the epigenetic landscape by mass cytometry (Cheung et al., 2018). These experiments will aim to answer whether NK cell cytokine production is higher in BCG vaccinated compared to unvaccinated infants in response to related and unrelated stimuli. In addition, we will aim to answer whether these functional responses are associated with BCG-induced epigenetic modifications. In light of our work and that of others, we propose that BCG vaccination is able to amplify monocyte and NK cell effector functions in infants through trained immunity, leading to an enhanced immune response to related and unrelated infections (**Figure 55**).

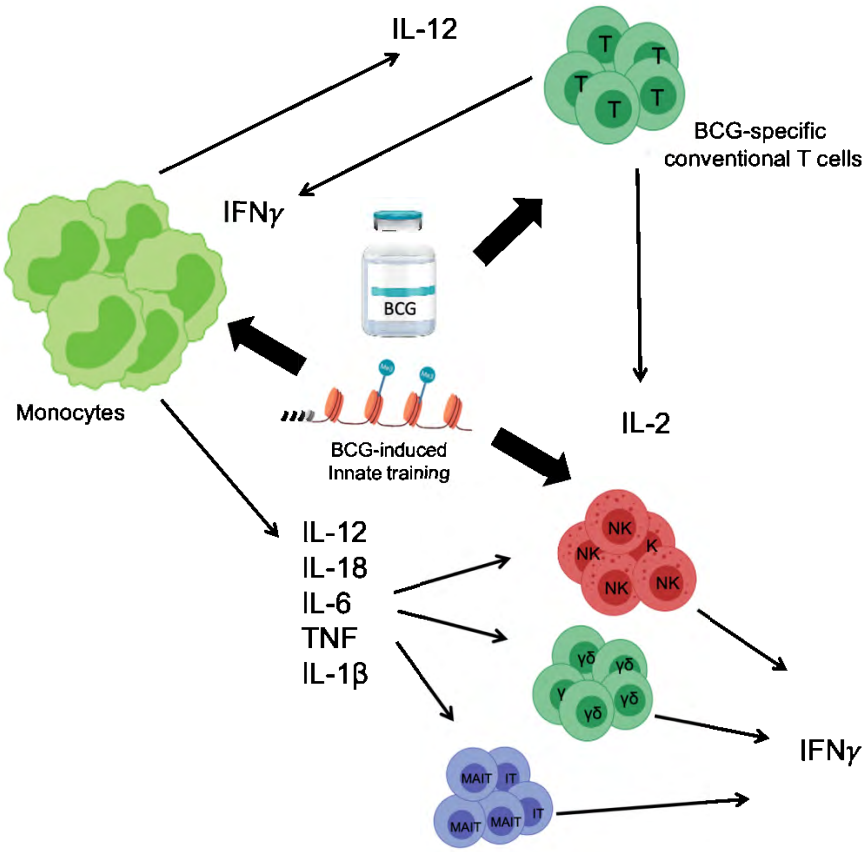

Figure 55. Proposed mechanism for BCG-induced enhanced immune responses in non-conventional T cell subsets in infants. After BCG vaccination, in addition to the initiation of BCG-specific T cell immunity, epigenetic reprogramming of monocytes and NK cells occur (trained immunity). This leads to enhanced cytokine production by monocytes which act on (cytokine-mediated activation) other immune subsets such as NK cells and some DURT cell subsets. NK cells themselves are also in a more permissive state for cytokine mediated activation due to epigenetic reprogramming. Altogether, this leads to an overall enhanced immune response to related and non-related infections. Illustration created in BioRender.com.

The immunological work done in HIV-infected and uninfected adult TB patients and controls was an exploratory component of a larger study investigating *M.tb* transmission dynamics and aerosol expulsion. Using the first iteration of the RASC, we were able to detect aerosolised *M.tb* in 43% of TB patients (Patterson et al., 2017). The sensitivity of the RASC has since increased by improving sampling methods and protocols (Patterson et al., 2020). We hypothesised that TB patients with excessive inflammation (high RISK11 scores) and lung pathology (cavitation) would expel higher levels of *M.tb*-containing aerosols, which we used as a surrogate of infectiousness. We also hypothesised that inflammation would modulate immune subsets in the blood, which could serve as a biomarker of infectiousness in individuals with TB disease (**Chapter 6, Figure 38**). In line with these hypotheses, we observed that *M.tb* aerosol

CFUs were higher in individuals with cavitation compared to those without cavitary disease, but only when the HIV+ and HIV- individuals were pooled. However, this difference was not significant after corrections for multiple testing. In the first round of experiments, using *M.tb* aerosol CFU data from the RASC, we were also able to show that MAIT cell frequencies negatively associated with *M.tb*-containing aerosols and could potentially be used as an indicator of infectiousness. This result also highlights the potential role of MAIT cells in inflammation and potential infectiousness of TB individuals. This is of particular importance, as *M.tb* transmission is a major driver of the TB epidemic in many parts of the world. We primarily used the RISK11 score in this study as an indicator of type I/II IFN-driven inflammation, but we also reported, in line with other studies, that RISK11 was able to differentiate TB patients from controls (Darboe et al., 2019, Scriba et al., 2021, Mendelsohn et al., 2021, Mulenga et al., 2021). RISK11 signature scores were higher in HIV-negative TB patients with cavitation compared to those without cavitation. RISK11 signature scores also positively correlated with the GLR and MLR, which has been previously shown to be higher in TB patients compared to healthy controls (La Manna et al., 2017, Wang et al., 2015, Sutherland et al., 2009b). Interestingly, among all the immune subsets measured, frequencies of CD16 intermediate monocytes were able to differentiate individuals with TB from healthy controls. The diagnostic performance of intermediate monocyte frequencies did not diminish in HIV+ individuals, making it a promising TB biomarker. More studies will be required to determine a TB-specific cut-off for classifying TB patients from healthy controls, also considering that this is a general response to inflammation, which has been reported in other types of inflammatory diseases (Wong et al., 2012). Due to the exploratory nature of this study, there were several limitations, including a small sample size and the fact that CD4 counts and HIV viral load data were not available for all HIV-infected individuals. However, we were still able to gain several useful insights on host immune subsets from peripheral blood that associate with inflammation and aerosolisation of *M.tb.*

In conclusion, our results indicate that innate lymphocytes are modulated by BCG vaccination in infants and therefore represent attractive targets for vaccination and should be further explored in the context of trained immunity. MAIT cell frequencies could potentially be used as an indicator of infectiousness and allow targeted treatment of TB super-spreaders. Lastly, the frequency of intermediate monocytes in

peripheral blood differentiates TB disease and healthy individuals and might be a useful TB biomarker.

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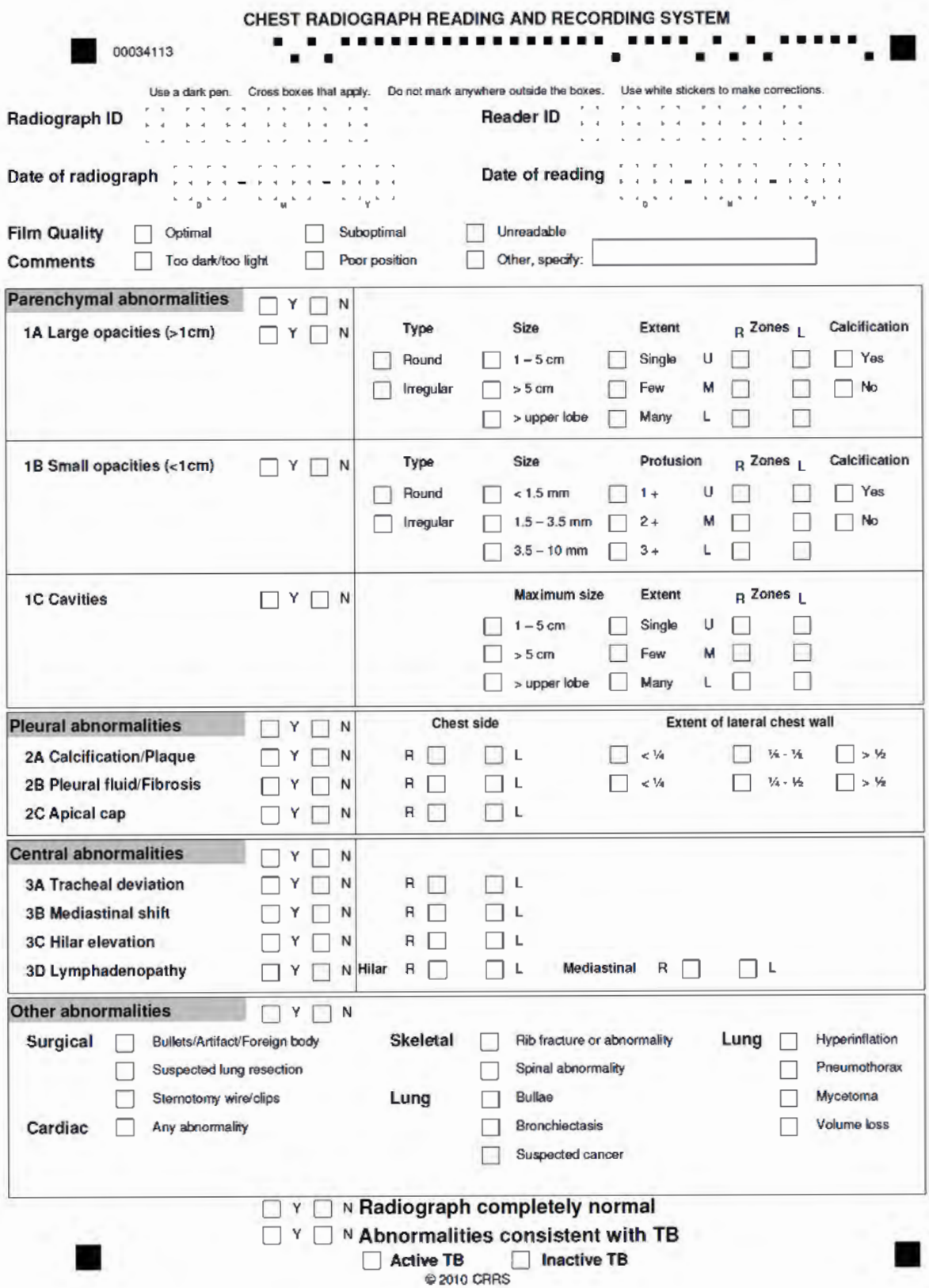
Appendices

Appendix A

Spearman correlation coefficients and corresponding p values shown for host secreted cytokines and BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants (no BCG= 23: n=13 5-week-olds, n= 10 9-week-olds; BCG= 43: n=15 5-week-olds, n=28 9-week-olds). Correlation coefficients in bold indicate corresponding p values that were significant after correcting for multiple comparisons using the FDR method.

Host secreted cytokine	CD56+CD16- NK			
	No BCG		BCG	
	r	FDR p	r	FDR p
IFNγ	0.10	0.76	**0.73**	<0.0001
IL-18	0.42	0.12	**0.49**	0.0039
IL-2	0.0053	0.99	**0.66**	<0.0001
IL-6	0.17	0.59	**0.58**	0.00046
IL-1β	0.29	0.32	**0.63**	0.00011
TNF	0.29	0.33	**0.70**	<0.0001
IL-10	0.10	0.76	**0.37**	0.044
GM-CSF	0.16	0.61	**0.53**	0.0015
IL-5	0.076	0.83	**0.49**	0.0041
Host secreted cytokine	**CD56++ NK**			
	No BCG		BCG	
	r	FDR p	r	FDR p
IFNγ	0.40	0.14	**0.60**	0.00025
IL-18	0.41	0.13	**0.44**	0.010
IL-2	0.18	0.58	**0.62**	0.00012
IL-6	0.21	0.52	**0.51**	0.0030
IL-1β	0.41	0.13	**0.57**	0.00060
TNF	0.48	0.06	**0.62**	0.00012
IL-10	0.37	0.18	0.35	0.059
GM-CSF	0.16	0.61	**0.48**	0.0054
IL-5	0.14	0.66	**0.45**	0.0096
Host secreted cytokine	**CD56+CD16+NK**			
	No BCG		BCG	
	r	FDR p	r	FDR p
IFNγ	0.064	0.84	**0.54**	0.0014
IL-18	0.20	0.54	0.25	0.20
IL-2	0.0023	0.99	**0.38**	0.034
IL-6	-0.11	0.74	0.31	0.11
IL-1β	0.22	0.50	**0.42**	0.018
TNF	0.12	0.70	**0.44**	0.011
IL-10	-0.17	0.60	0.19	0.38
GM-CSF	0.010	0.99	0.30	0.12
IL-5	0.16	0.61	0.27	0.16
Host secreted cytokine	**CD56+CD16- NK**			
	No BCG		BCG	
	r	FDR p	r	FDR p
IFNγ	-0.32	0.27	**0.44**	0.011
IL-18	0.12	0.70	0.20	0.32
IL-2	-0.18	0.57	0.32	0.095
IL-6	0.0057	0.99	0.23	0.27
IL-1β	0.19	0.55	**0.37**	0.040
TNF	0.15	0.62	0.36	0.053
IL-10	-0.25	0.41	0.14	0.54
GM-CSF	-0.049	0.88	0.19	0.37
IL-5	-0.069	0.83	0.15	0.50
Host secreted cytokine	**Rainbow NK**			
	No BCG		BCG	
	r	FDR p	r	FDR p
IFNγ	0.30	0.32	**0.63**	0.00011
IL-18	0.41	0.13	**0.46**	0.0093
IL-2	0.069	0.83	**0.56**	0.00065
IL-6	0.18	0.58	**0.46**	0.0079
IL-1β	0.38	0.17	**0.51**	0.0030
TNF	0.32	0.27	**0.57**	0.00060
IL-10	0.19	0.55	0.26	0.17
GM-CSF	0.046	0.88	**0.45**	0.010
IL-5	0.045	0.88	**0.39**	0.034

Appendix B



CHEST RADIOGRAPH READING AND RECORDING SYSTEM

00034113

Use a dark pen. Cross boxes that apply. Do not mark anywhere outside the boxes. Use white stickers to make corrections.

Radiograph ID

Reader ID

Date of radiograph D M Y

Date of reading D M Y

Film Quality ☐ Optimal ☐ Suboptimal ☐ Unreadable

Comments ☐ Too dark/too light ☐ Poor position ☐ Other, specify:

Parenchymal abnormalities ☐ Y ☐ N

1A Large opacities (>1cm) ☐ Y ☐ N

Type	Size	Extent	R Zones L	Calcification
☐ Round	☐ 1 – 5 cm	☐ Single	U ☐ ☐	☐ Yes
☐ Irregular	☐ > 5 cm	☐ Few	M ☐ ☐	☐ No
	☐ > upper lobe	☐ Many	L ☐ ☐	

1B Small opacities (<1cm) ☐ Y ☐ N

Type	Size	Profusion	R Zones L	Calcification
☐ Round	☐ < 1.5 mm	☐ 1+	U ☐ ☐	☐ Yes
☐ Irregular	☐ 1.5 – 3.5 mm	☐ 2+	M ☐ ☐	☐ No
	☐ 3.5 – 10 mm	☐ 3+	L ☐ ☐	

1C Cavities ☐ Y ☐ N

Maximum size	Extent	R Zones L
☐ 1 – 5 cm	☐ Single	U ☐ ☐
☐ > 5 cm	☐ Few	M ☐ ☐
☐ > upper lobe	☐ Many	L ☐ ☐

Pleural abnormalities ☐ Y ☐ N

		Chest side	Extent of lateral chest wall
2A Calcification/Plaque	☐ Y ☐ N	R ☐ ☐ L	☐ < ¼ ☐ ¼ - ½ ☐ > ½
2B Pleural fluid/Fibrosis	☐ Y ☐ N	R ☐ ☐ L	☐ < ¼ ☐ ¼ - ½ ☐ > ½
2C Apical cap	☐ Y ☐ N	R ☐ ☐ L	

Central abnormalities ☐ Y ☐ N

3A Tracheal deviation	☐ Y ☐ N	R ☐	☐ L			
3B Mediastinal shift	☐ Y ☐ N	R ☐	☐ L			
3C Hilar elevation	☐ Y ☐ N	R ☐	☐ L			
3D Lymphadenopathy	☐ Y ☐ N	Hilar R ☐	☐ L	Mediastinal R ☐	☐ L	

Other abnormalities ☐ Y ☐ N

Surgical	☐ Bullets/Artifact/Foreign body	Skeletal	☐ Rib fracture or abnormality	Lung	☐ Hyperinflation
	☐ Suspected lung resection		☐ Spinal abnormality		☐ Pneumothorax
	☐ Sternotomy wire/clips	Lung	☐ Bullae		☐ Mycetoma
Cardiac	☐ Any abnormality		☐ Bronchiectasis		☐ Volume loss
			☐ Suspected cancer		

☐ Y ☐ N **Radiograph completely normal**

☐ Y ☐ N **Abnormalities consistent with TB**

☐ **Active TB** ☐ **Inactive TB**

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Appendix C

Modified scoring		
1- Large opacity score	**If there's a large opacity don't add small opacities for the same lobe**	
	No	0
	If yes	
	small (1-5cm)	0.5
	>5	1
	multiply by number of lobes	
2- Small opacity score		
	No	0
	If yes	
	<1.5mm	0.1
	1.5-3.5mm	0.2
	3.5-10mm	0.3
	multiply by profusion number	
	multiply by number of lobes	
3- Cavities		
	No	0
	If Yes	
	small (1-5cm)	0.5 per lobe
	>5	1 per lobe
4- Pleural abnormalities		
	No	0
	If Yes	
	Effusion	
	No	0
	If Yes	
	one side	1
	bilteral	2
	Apical cap	
	No	0
	If Yes	
	one side	1
	bilteral	2
TOTAL		
5- Pure extent	Extent calc (%)	
6- Additional parameters (with/without) Extent Pleural effusion Cavities		

Effects of BCG vaccination on donor unrestricted T cells in two prospective cohort studies

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Summary

Background Non-protein antigen classes can be presented to T cells by near-monomorphic antigen-presenting molecules such as CD1, MR1, and butyrophilin 3A1. Such T cells, referred to as donor unrestricted T (DURT) cells, typically express stereotypic T cell receptors. The near-unrestricted nature of DURT cell antigen recognition is of particular interest for vaccine development, and we sought to define the roles of DURT cells, including MR1-restricted MAIT cells, CD1b-restricted glucose monomycolate (GMM)-specific T cells, CD1d-restricted NKT cells, and γδ T cells, in vaccination against *Mycobacterium tuberculosis*.

Methods We compared and characterized DURT cells following primary bacille Calmette-Guerin (BCG) vaccination in a cohort of vaccinated and unvaccinated infants, as well as before and after BCG-revaccination in adults.

Findings BCG (re)vaccination did not modulate peripheral blood frequencies, T cell activation or memory profiles of MAIT cells, CD1b-restricted GMM-specific and germline-encoded mycolyl-reactive (GEM) cells or CD1d-restricted NKT cells. By contrast, primary BCG vaccination was associated with increased frequencies of γδ T cells as well as a novel subset of $CD26^{+}CD161^{+}TRAV1\text{-}2^{-}$ IFN-γ-expressing $CD4^{+}$ T cells in infants.

Interpretation Our findings, that most DURT cell populations were not modulated by BCG, do not preclude a role of BCG in modulating other qualitative aspects of DURT cells. More studies are required to understand the full potential of DURT cells in new TB vaccine strategies.

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Keywords: BCG; Tuberculosis; Vaccination; Unconventional T cells; Memory

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Introduction

Current approaches to develop vaccines against tuberculosis (TB), one of the top 10 causes of death globally, are based primarily on the induction of conventional,

Research in Context

Evidence before this study

Bacillus Calmette-Guerin (BCG), the only licensed tuberculosis vaccine, provides variable protection against tuberculosis, the deadliest disease caused by a single infectious agent. While many efforts have been made to investigate the role of Th1 cytokine-producing, MHC-restricted T cells in protection against TB, other arms of immunity, including donor-unrestricted T (DURT) cells, have not been substantially explored. Evidence from non-human primates and human studies suggest that DURT cells are activated during natural infection with *Mycobacterium tuberculosis*, providing a rationale to investigate if BCG vaccination can modulate these T cell populations in humans.

Added value of this study

We aimed to determine if BCG vaccination modulates peripheral blood frequencies, activation and expression of memory markers of MR1-restricted MAIT cells, CD1b-restricted glucose monomycolate (GMM)-specific T cells, CD1d-restricted NKT cells, and $\gamma\delta$ T cells. We performed flow cytometric analysis in unvaccinated and BCG vaccinated infants and BCG re-vaccinated adults to profile DURT cells. Our findings show that neonatal BCG vaccination was associated with an increase in $\gamma\delta$ T cells as well as a novel subset of BCG-reactive IFN-γ-producing $CD26^{+}CD161^{+}TRAV1{-}2^{-}$ T cells that express CD4. However, BCG (re)vaccination did not modulate the other three DURT cell subsets.

Implications of all the available evidence

Our results lend further support to prior evidence that $\gamma\delta$ T cell responses play a role in immunity induced by whole cell vaccination against TB to BCG, and highlight a new BCG-reactive IFN-γ-producing CD4 T cell subset that deserves investigation.

major-histocompatibility complex (MHC)-restricted, Th1 cytokine-producing T cells to protein antigens. While proteins constitute the majority of known antigens recognized by $\alpha\beta$ T cell receptor (TCR)-bearing cells, other chemical classes including lipids, small-molecule metabolites and specially modified peptides are also antigenic and can be presented to T cells by the non-polymorphic molecules, CD1, MR1, and butyrophilin 3A1, respectively.[1-3] Unlike MHC-restricted T cells, these T cell subsets generally bear T cell receptors that show limited diversity, and are therefore referred to as donor-unrestricted T (DURT) cells.[3] DURT cells are activated through their TCR in a manner that is unrestricted by donor origin: response is mediated by antigen presentation molecules of limited polymorphism, such that DURT responses can be shared among genetically diverse individuals.[1] This nature of antigen recognition by DURT cells is of particular interest for vaccine development, since immunogens could be designed to elicit universal, population-wide T cell responses, irrespective of host MHC-encoded genetic factors.

Studies in mouse and non-human primate models have demonstrated the importance of T cells in conferring protection against challenge with *Mycobacterium tuberculosis* (M.tb).[4-6] However, it remains unclear which and how many mycobacteria-derived antigens should be targeted by protective T cell responses, and which T cell functional, homing and memory attributes are required for protection.[7] Given the complexity of host-pathogen interactions, TB vaccination strategies that exploit immunological diversity and target additional arms of the immune system, including DURT cells, such as the CD1d-restricted NKT cells, CD1b-restricted glucose monomycolate (GMM)-specific T cells and germline-encoded mycolyl-reactive (GEM) cells, $\gamma\delta$ T cells, and MR1-restricted MAIT cells, should be explored.[1]

Current studies provide extensive evidence that DURT subtypes are activated during natural M.tb infection in humans and non-human primates. Analysis of the T cell response during human M.tb infection indicates that infected individuals have increased CD1-restricted T cell responses to lipid antigens compared to M.tb naïve controls.[8-10] A recent study in non-human primates also reported that glucose monomycolate (GMM)-specific CD1c-restricted T cells expanded after intravenous bacille Calmette-Guerin (BCG) administration.[11] Vaccination of guinea pigs with mycobacterial lipids formulated in liposomes was also associated with a reduction in the number of lesions, severity of pathology, and reduction of bacterial load upon challenge with M.tb.[12]

Activation of MR1-restricted MAIT cells, which recognize vitamin B metabolites presented by the monomorphic MHC class 1-related molecule (MR1), has been shown in response to a variety of bacteria including BCG, *Francisella tularensis, Klebsiella pneumonia*, and M.tb.[13] Despite previous evidence that MAIT cells can respond to M.tb infection, there is conflicting data on the importance of MAIT cells in controlling infection. In the murine model, endogenous MAIT cell responses have been implicated in early innate responses to M.tb infection,[14] but more recently additional studies suggest they are less important for adaptive host defenses against M.tb infection.[15-17] Moreover, early MAIT cell responses impeded the priming and induction of conventional peptide-specific CD4 T cell responses.[15]

Butyrophilin 3A1 facilitates recognition of phosphoantigens, small molecules like isopentyl pyrophosphate, by $\gamma\delta$ T cells, which rapidly respond to pathogen infection in mucosal tissues, and are induced by both M.tb infection and BCG vaccination in humans and non-human primates.[18-22] Importantly, induction of $V\gamma2V\delta2$ T cells specific for the phosphoantigen, (E)−4-

hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), in non-human primates was associated with increased Th1-like Vγ2Vδ2 T cells in the airways, facilitated earlier recruitment of conventional Th1 cytokine-expressing CD4 and CD8 T cell to the lungs, and was associated with containment of M.tb after pulmonary challenge.[20]

Another feature of DURT cells that makes them attractive as vaccine targets is their inherent immediate effector function, such as secretion of inflammatory cytokines and cytotoxic molecules upon recognition of microbial antigens.[23] Immediate cytokine secretion allows DURT cells to modulate the antimicrobial function of other cells before recruitment or induction of conventional MHC-restricted T cells has occurred. For example, MAIT cell secretion of CCL4 mediates recruitment of NK cells, monocytes, and other inflammatory cells to infected tissues.[24] Similarly, immediate or early secretion of pro-inflammatory cytokines that can facilitate the development of adaptive immunity has been described for NK cells,[25] γδ T cells,[26] and NKT cells.[27] The relatively high baseline precursor frequency in blood and tissues of DURT cells allows them to recognize antigens and respond in large numbers without requiring extensive clonal expansion, implying that they can act simultaneously with innate cells as sensors of infection or immune dysregulation. Despite these attributes, it remains unknown whether DURT cells possess immunological memory such that they can be selectively expanded by vaccination to provide long-term, antigen-specific protective immunity.

In this study, we sought to determine if a live attenuated vaccine, BCG, modulates frequencies, phenotypes or functions of DURT cells in humans. The key premise of this study is that live BCG contains peptide and non-peptide antigens that can be recognized by DURT cells. We employed a unique study design in which BCG was given at birth, as is routine, or in which BCG was delayed until sampling, to allow comparison of a vaccinated and unvaccinated group of infants. We also evaluated BCG effects on DURT cells in M.tb-infected adults who received investigational BCG revaccination. This allows ascertainment of the modulatory effect of BCG vaccination on DURT cells to inform whether changes are durable such that they constitute immunological memory. Ours is the first study that aimed to systemically profile DURT cells in a case/control design.

Methods

Study participants

All participants were enrolled at the South African Tuberculosis Vaccine Initiative Field site in Worcester near Cape Town, South Africa. We collected blood from two cohorts of participants who were vaccinated with BCG.

The first cohort comprised two groups of healthy, 9-week-old infants. In one group (BCG vaccinated), infants received BCG at birth as is routine according to the World Health Organization Expanded Program on Immunization (EPI) in South Africa. In the second group (BCG naïve), BCG vaccination was delayed until after blood collection at 9 weeks, for whole blood stimulation and PBMC cryopreservation. In the BCG naïve (with delayed BCG vaccination) cohort, mothers and infants were closely monitored by our clinical team to maximize the likelihood of identifying those with TB exposure, in order to provide clinical care. Mothers were also sensitized about TB signs and symptoms and had access to the study team for any medical concern. Infants who were born preterm (<37 weeks of gestation), with a low birth weight (<2.5 kg), had congenital malformations or perinatal complications, were in close contact with someone with TB disease or had suspected TB, received isoniazid preventive therapy or immunosuppressant therapy, or had any acute or chronic disease were excluded.

The second cohort, the TBRU adult BCG revaccination trial,[28, 29] comprised healthy tuberculin skin test (TST)-positive, HIV-negative adults who received at least 6 months of isoniazid preventive therapy before, or 7 months after, BCG revaccination (NCT01119521). Peripheral blood was collected before BCG revaccination and on days 21, 35, and 365 post-vaccination and peripheral blood mononuclear cells (PBMCs) cryopreserved.

Sample size simulations indicate that the study was well powered to detect a standardized effect size of 2 or more (see Data Analysis for details) using a Mann-Whitney test under a variety of distributions with varying degrees of skewness. Power was 67% under the statistical distribution with the least power (gamma distribution) when the family-wise type I error rate was controlled at 0.05 across five tests (Supplementary Figure 1); however, typically the data were more normally distributed. Depending on the distribution and the number of tests across which the type I error rate was controlled, power varied around 50% for a standardized effect size of 1, and was negligible when the standardized effect size was 0.1.

Ethical approvals

The studies were conducted in accordance with Good Clinical Practice (GCP) and guidelines set out by the World Medical Association's Declaration of Helsinki. The studies, cohort protocols and all procedures were reviewed and approved by the Human Research Ethics Committee (HREC) of the University of Cape Town as follows: BCG revaccination trial (Ref. 387/2008), infants vaccinated at birth (Ref. 126/2006) and infants with delayed BCG vaccination (Ref. 177/2011). The BCG revaccination study was also reviewed and approved by the University Hospital Cleveland Medical Center Institutional Review Board. Written informed consent was

obtained from all adult participants and from a parent or legal guardian of infants before enrollment.

Flow cytometry assays

PBMC staining. Cryopreserved PBMCs were thawed in a 37 °C water bath, washed in PBS, and then stained with LIVE/DEAD dye according to the manufacturer's instructions. Thereafter, PBMCs were stained with the fluorochrome-conjugated tetramers (CD1b-GMM,[30] CD1d-PBS57, MR1-5-OP-RU, and their respective controls) for 45–60 min at room temperature, followed by CCR7 staining at 37 °C for 30 min, and thereafter stained for other phenotypic markers (antibody panels described in Supplemental Table 1) at 4 °C for another 30 min.

Whole blood intracellular cytokine staining (WB-ICS) assay and staining. Heparinized blood was processed, within 75 min of blood collection, using a standardized 12 h WB-ICS assay protocol.[31] Briefly, blood was stimulated with BCG Vaccine SSI (Biovac, Cape Town, South Africa) reconstituted with RPMI (final concentration 1.2×10^6 CFU/ml), PHA (Sigma-Aldrich; positive control at 5 μg/ml) or RPMI (negative control). For all stimulation conditions, co-stimulants anti-CD28 and anti-CD49d (BD Biosciences; San Diego, USA) were added at 0.25 μg/ml. Blood was stimulated for 7 h at 37 °C, after which Brefeldin-A (Sigma-Aldrich) was added at a concentration of 10 μg/ml for the remaining 5 h of stimulation. At the end of the stimulation, 2 mM of EDTA (Sigma-Aldrich) was added, red blood cells were lysed using 1:10 FACS Lysing solution (BD Biosciences) and fixed white blood cells were cryopreserved in liquid nitrogen. Cryopreserved fixed white blood cells from infant participants were thawed, washed in PBS, permeabilized in Perm/Wash buffer (BD Biosciences) and stained with antibody panels (Supplemental Table 2, 3) for 30 min at 4 °C.

Flow cytometry. Stained samples were acquired on a BD-LSR-II flow cytometer configured with 4 lasers: Solid state Blue (488 nm; 100 mW; 3 detectors), Solid state Violet (405 nm; 25nW; 8 detectors), HeNe gas Red (635 nm; 70 mW; 3 detectors), and Diode-pumped Coherent Compass (532 nm; 150 mW; 8 detectors). We used mouse κ chain BD CompBeads stained with each individual antibody conjugate (for tetramer reagents, fluorochrome-matched antibody conjugates were used) to compensate all parameters. Samples were acquired with optimal photomultiplier tube voltages calibrated daily using targets for SPHERO Rainbow Fluorescent Particles (Spherotech, Inc.).

Data analysis

Flow cytometric data were analyzed using FlowJo (version 10.5.3). Statistical analyses were performed in R (version 3.6.3). Within a particular age and vaccination status, as well as the effect of BCG revaccination after a certain number of days since revaccination, estimates of the median and 95% confidence interval thereof were performed by quantile regression using the quantreg package.[32, 33] The effect of BCG within infants and the effect of age (adults versus infants) within BCG-vaccinated individuals was calculated using quantile regression and tested against the null hypothesis using a Wald test,[34] controlling for sex and ethnicity. Other measured potential variables were deemed to have little effect on the response based on graphical assessment and prior experience. When comparing a single time point since vaccination to pre-vaccination, the effect of BCG within adults was assessed using the Wilcoxon signed-rank test. The test for an effect of BCG revaccination at any time-point was a likelihood ratio test for linear mixed-effects models,[35] using race and sex as confounders. The type I family-wise error rate was controlled throughout using the Bonferroni procedure, with an adjusted p-value of less than 0.05 considered statistically significant.

The standardized effect size (and associated standardized confidence interval) was estimated by dividing the estimated effect size (and associated confidence interval bounds) by the standard deviation of the raw residuals, after accounting for vaccination status (as well as ethnicity and sex, where appropriate).

Role of funding source

The study was funded by Aeras and the Bill and Melinda Gates Foundation. The delayed BCG infant study was funded by NIH R01 grant AI087915 and the adult BCG revaccination study was funded by NIH grant NO1-AI70022. CD1b tetramers, ligands and their validation were supported by R01 AI049313. Anele Gela was supported by postdoctoral fellowships from the Claude Leon Foundation and the Harry Crossley Foundation. Melissa Murphy was supported by a Masters and Doctoral Innovation Scholarship from the National Research Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Results

Participant enrollment and demographic data

We enrolled two cohorts from communities residing in a TB endemic setting, in South Africa. The infant cohort comprised two groups of healthy 9-week-old infants, born to HIV negative mothers, who either received routine BCG at birth as part of the expanded program on

Variable	Infants		Adults (*N*=25)
	No BCG (*N*=25)	BCG (*N*=50)	
BCG (re)vaccination	No	Yes	Yes
Median age	9 weeks	9 weeks	24 years
Range	(8–11)	(8–11)	(19–39)
Sex, n (%)			
Male	13 (52)	25 (50)	7 (28)
Female	12 (48)	25 (50)	18 (72)
Ethnicity, n (%)			
African	4 (16)	13 (26)	5 (20)
Caucasian	0 (0)	0 (0)	1 (4)
Mixed ancestry	21 (84)	37 (74)	19 (76)

***Table 1*: Demographic characteristics of participants.**

immunization (EPI) by the World Health Organization, or in whom BCG vaccination was delayed until after blood collection at 9 weeks. There were no significant differences in sex or ethnicity distribution between the vaccinated and the delayed BCG arm. The adult cohort included tuberculin skin test (TST)-positive, HIV-negative adults who received BCG revaccination after pretreatment with isoniazid as part of the TBRU adult BCG revaccination trial.[28, 29] Peripheral blood samples were analyzed before revaccination and on days 21, 35, and 365 post-vaccination. The demographics of the study populations are summarized in Table 1.

DURT cell subset characterization by flow cytometry

We quantified CD1b-, CD1d-, MR1-restricted, and $\gamma\delta$ T cells in peripheral blood using a combination of tetramers and monoclonal antibody staining against phenotypic markers. DURT cell subsets were defined by flow cytometry as MR1-5-OP-RU$^+$TRAV1-2$^+$(or CD26$^+$CD161$^+$) MAIT cells, CD1d-PBS57$^+$NKT cells, $\gamma\delta$-T cells, and CD1b-GMM$^+$CD4$^+$TRAV1−2$^+$GEM cells (Figure 1).

Frequencies of γδ T cell, but not other DURT subsets, increase after BCG vaccination in infants

Previous studies showed that neonatal BCG vaccination boosts antigen-specific T cell responses that peak 6–10 weeks of age,[36, 37] while BCG revaccination in adults transiently boosts frequencies of antigen-specific T cells, including $\gamma\delta$ T cells, that peak 3–5 weeks post-vaccination.[38] An unresolved question is whether BCG revaccination induces durable changes in frequencies of DURT cells in peripheral blood. To determine if BCG vaccination alters DURT cell abundance, we measured frequencies of DURT cell subsets in infants and adults at 35 days post revaccination. Frequencies of the different DURT subsets were generally lower in infants compared to adults, but there was high heterogeneity among the adults, especially for frequencies of MAIT and $\gamma\delta$ T cell populations (Figure 2a-e). Frequencies of MAIT cells, CD1d- restricted NKT cells, CD1b-restricted GMM-specific and germline-encoded mycolyl-reactive (GEM) cells were not significantly modulated by primary BCG vaccination, nor by BCG re-vaccination at all time-points (Fig 2a-d, data not shown for day 21 and 365). However, frequencies of $\gamma\delta$ T cells were significantly higher in BCG-vaccinated infants compared to their unvaccinated counterparts (fold change 1.47, 95% CI 1.27–2.24; p=0.012 by Wald test, q=0.083) (Fig 2e). No difference in $\gamma\delta$ T cell frequencies was observed before and after BCG revaccination in adults at any of the post-vaccination time points (Fig 2e, data not shown for day 21 and 365).

Infants also had higher frequencies of bulk CD4 T cells than adults, but these were not modulated by BCG in either age group (Figure 2f). In contrast to these DURT cell subsets, antigen-specific CD4 T cells were significantly modulated by BCG vaccination, as expected for peptide-responsive, MHC-restricted T cells; primary BCG vaccination induced significant increases in BCG-reactive IFN-γ+ CD4 T cells in infants (fold change 5.34, 95% CI 5.01 – 6.05; p < 0.0001 by Wald test, q < 0.0001), while BCG re-vaccination boosted pre-existing BCG-reactive CD4 T cells in adults (fold change 1.43, 95% CI 1.15 – 1.7; p=0.0009 by Wilcoxon signed-rank test, q=0.0064) (Figure 2g). Taken together, these results show that with the exception of a significant increase in total $\gamma\delta$ T cells in BCG-vaccinated infants, DURT cell frequencies in peripheral blood were not modulated by BCG vaccination.

BCG vaccination does not modulate the activation status of DURT cells in peripheral blood

We next sought to determine if BCG vaccination results in activation of DURT subsets in peripheral blood, since evidence of T cell activation may indicate that DURT cells sense and respond to the vaccine, or its

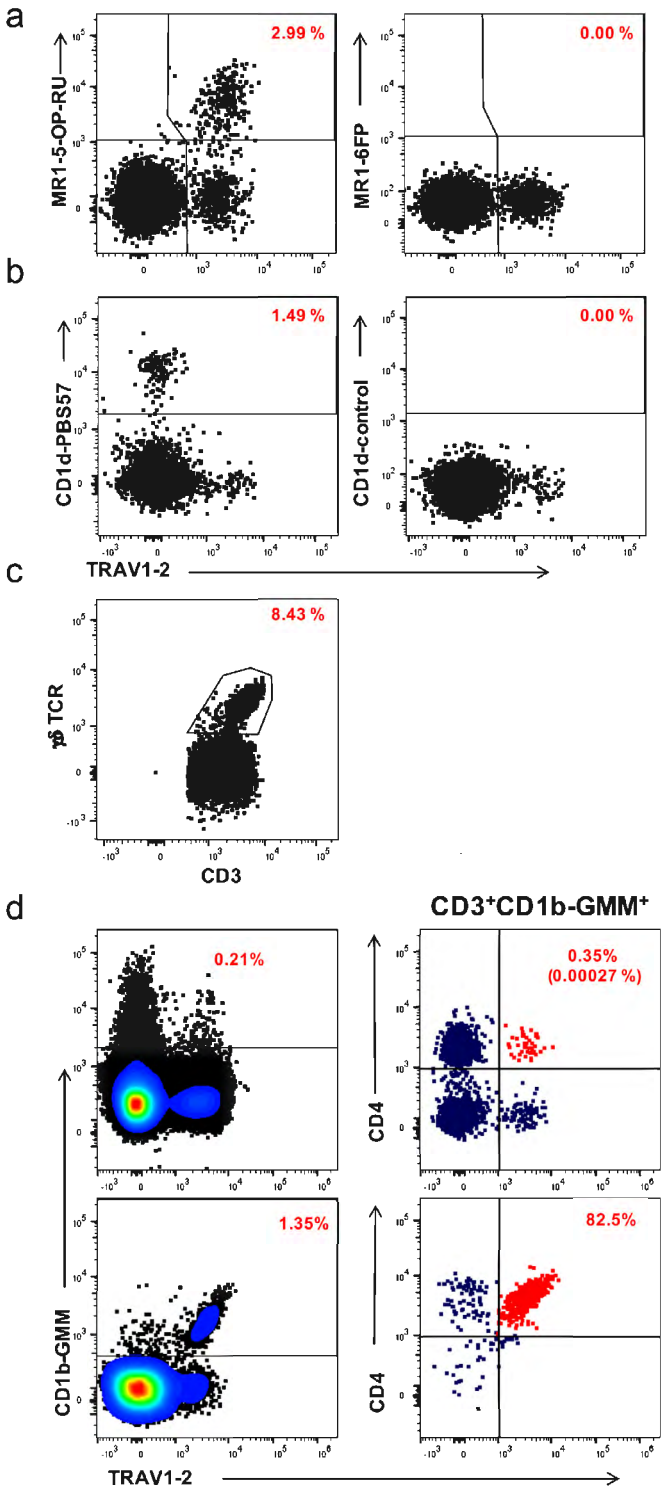

Figure 1. Representative flow cytometry plots demonstrating detection of DURT cell populations in a typical infant. Plots represent live, CD3+ T cells co-stained with the indicated antibody-conjugate on the X-axis and the indicated tetramer reagent or antibody-conjugate on the Y-axis. (a) MR1-restricted MAIT cells (MR1-5-OP-RU tetramer on the left and MR1-6FP tetramer as negative control on the right). (b) CD1d-restricted NKT cells (CD1d-PBS57 tetramer on the left and unloaded [empty] CD1d tetramer as negative control on the right). (c) $\gamma\delta$ T cells, defined as CD3 T cells that stained positive with anti-pan $\gamma\delta$ TCR antibody. (d) Definition of CD1b-restricted glucose monomycolate (GMM)-specific T cells and germline-encoded mycolyl-reactive (GEM) cells in an infant

downstream effects. Our previous study of T cell response kinetics after neonatal BCG vaccination indicated that antigen-specific CD4 T cell activation also peaked around 6 weeks after vaccination.[39] Consistent with this, BCG-specific IFN-γ-expressing CD4 T cells detected at 9 weeks of age in the BCG vaccinated infants expressed significantly higher levels of the *in-vivo* activation marker, HLA-DR, than bulk CD4 T cells (Figure 3a). BCG-specific CD4 T cell responses were not detected in unvaccinated infants, precluding analysis of activation status of these CD4 T cells in this group (Figure 3a). By contrast, at the corresponding post-BCG time point, measured at 9 weeks of age, HLA-DR expression levels on all DURT cell subsets were not different between BCG-vaccinated and unvaccinated infants (Figure 3b-e). Similarly, levels of HLA-DR expression by DURT cell subsets or bulk CD4 T cells were not different between pre- and post- BCG-revaccination time points in BCG-revaccinated adults (Figure 4a-e). Taken together, these data suggest that DURT cells in the peripheral blood were not activated by BCG vaccination at the time points that we assessed.

BCG vaccination modulates a novel CD26$^+$CD161$^+$ CD4 T cell subset in infants

We also sought to evaluate functional characteristics of DURT subsets. In a subgroup of the infant cohort, we quantified IFN-γ-expressing BCG-reactive $\gamma\delta$ and phenotypically defined MAIT cells using a 12-h whole blood intracellular cytokine-staining (WB-ICS) assay. Although a number of BCG-vaccinated infants appeared to have markedly higher frequencies of BCG-reactive IFN-γ-expressing $\gamma\delta$ T cells than the range of IFN-γ-expressing $\gamma\delta$ T cells in unvaccinated infants, no significant difference between the two groups was observed (Figure 5a-b). On the other hand, frequencies of BCG-reactive IFN-γ-expressing CD26$^+$CD161$^+$ CD3$^+$ T cells, which phenotypically resemble the definition for MAIT cells,[40] were significantly elevated in BCG vaccinated compared to BCG naïve infants (fold change 3.46, 95% CI 2.28 – 4.72; p=0.0084 by Wald test, q=0.017) (Figure 5c-d). Further phenotypic characterization revealed that this result was driven by an IFN-γ-expressing, BCG-reactive CD4-positive CD26$^+$CD161$^+$ T cell subset (fold change 9.69, 95% CI 6.19 – 13.4; p < 0.0001 by Wald test, q=0.00017), and not the typical CD8$^+$ subset that characterizes MAIT cells (Figure 5e-f). These BCG-reactive CD4$^+$CD26$^+$CD161$^+$ T cells also did not express TRAV1–2, the canonical TCRα variable gene associated with MAIT cells (fold change 0.001, 95% CI 0.001 - 0.001; p < 0.0001 by Wald test, q < 0.0001) (Figure 5g). Unfortunately, the MR1 tetramer was not included in the flow cytometry panel used to analyze stimulated whole blood, and therefore we could not determine whether these cells were MR1-restricted. In summary, these data indicate that neonatal BCG vaccination modulates a functional subset of CD4$^+$ T cells with a CD26$^+$CD161$^+$TRAV1–2$^-$ phenotype.

T cell memory profiles of DURT subsets differ from conventional CD4$^+$ T cells

DURT cells are known to display immediate effector functions, such as secretion of inflammatory cytokines and cytotoxic molecules.[41] By comparison, conventional MHC-restricted T cells fully develop effector functions only after antigen-induced priming and differentiation into memory and effector cells. To investigate possible effects of BCG on differentiation and memory marker expression, we assessed proportions of DURT cells expressing CCR7 and/or CD45RA.

In both infants and adults, MAIT and NKT cells predominantly expressed a CCR7$^-$CD45RA$^-$ phenotype at all time points, consistent with effector memory T cells (Figure 6a-b). In infants, the proportion of CD45RA$^-$CCR7$^+$ MAIT cells was marginally higher in the BCG vaccinated group (fold change 2.41, 95% CI 1.81 – 3.02; p=0.009 by Wald test, q=0.14) with a concomitant decrease in CD45RA$^-$CCR7$^-$ MAIT cells (fold change 0.95, 95% CI 0.89 – 0.99; p=0.11 by Wald test, q=1). However, the standardized effect size confidence intervals were at most consistent with minor to moderate effect sizes, indicating minor effects that do not change the overall phenotypic profile. Regarding adults, our data did not reveal any BCG-associated effects. The "effector-like" CD45RA$^-$CCR7$^-$ phenotype of these cell subsets was as prominent in infants as in the adult population, suggesting early development of this phenotype of MAIT and NKT cells in infants with little change to its predominance thereafter. Longitudinal analysis of memory profiles of MAIT and NKT cells in the adult cohort before and after BCG-revaccination suggested that BCG does not modulate CCR7 or CD45RA expression up to one year post-vaccination (Suppl. Figure 2).

$\gamma\delta$ T cells, on the other hand, exhibited a more mixed phenotype comprising CCR7$^-$CD45RA$^-$ (consistent with effector memory) and CCR7$^-$CD45RA$^+$ (terminally differentiated effector) T cells (Figure 6c). Contrasting with NKT and MAIT cells, vaccination in infants was associated with a reduction in the proportions of CD45RA$^-$CCR7$^-$ $\gamma\delta$ T cells (fold change

sample (top plots) or a PBMC sample that was spiked with GEM T cell clone cells (bottom plots). GMM-specific T cells (left plots) were defined as CD1b-GMM tetramer$^+$ T cells and GEM cells were defined as CD1b-GMM tetramer$^+$ T cells that co-expressed CD4 and the TCR variable chain TRAV1–2 (red dots in right plots). Red numbers in all plots denote the percentages of T cells in that plot that stain positive for each population-defining marker out of CD3+ T cells. The red numbers in parentheses in the top, right plot in panel d denote the percentage of GEM cells out of all CD3+ T cells.

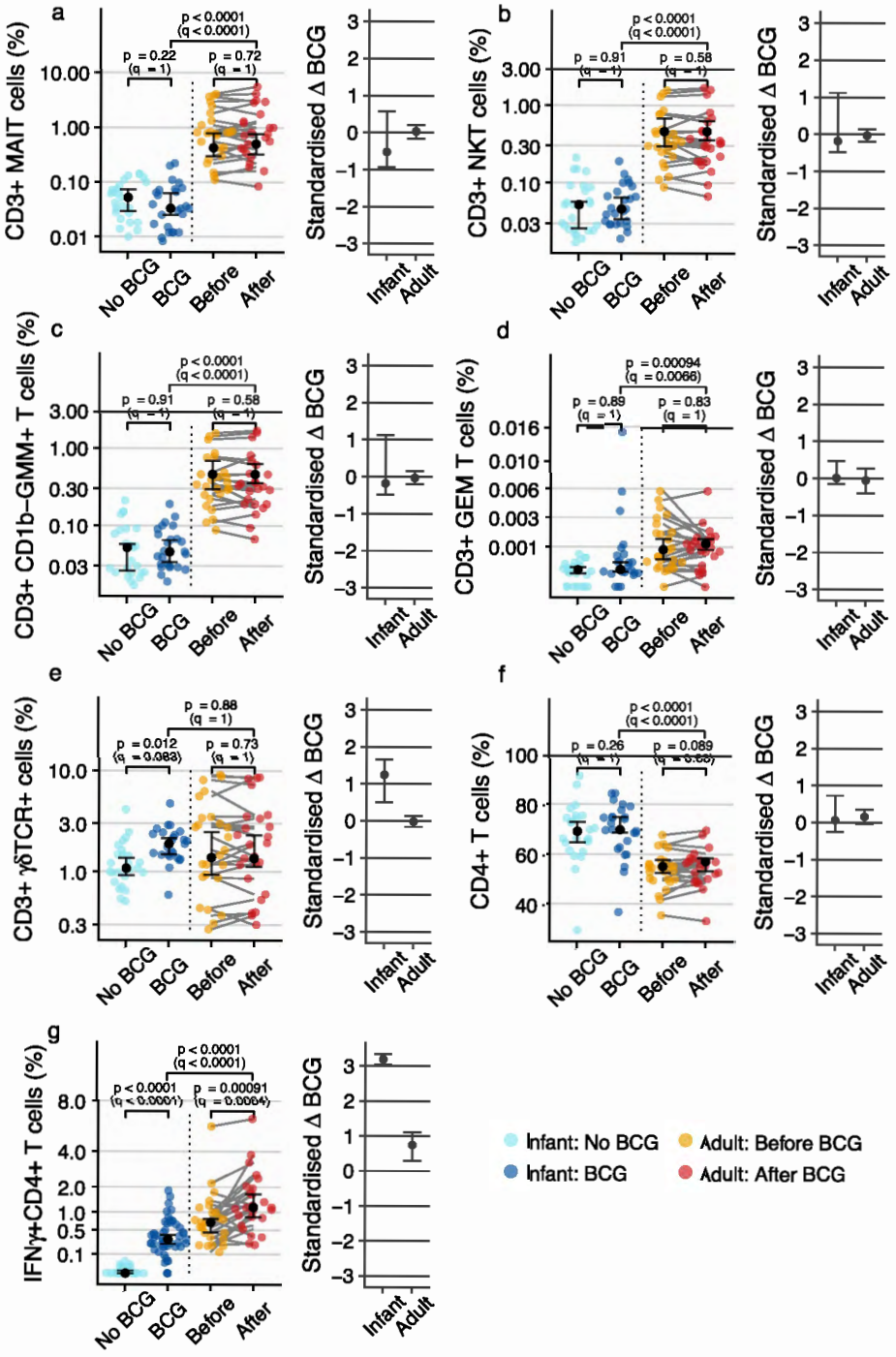

Figure 2. T cell subset frequencies measured by flow cytometry in BCG-vaccinated or unvaccinated infants or before and after BCG revaccination in adults. Peripheral blood frequencies and standardized BCG effect sizes for (a) MR1-5-OP-RU tetramer$^+$ MAIT cells, (b) CD1d-PBS57 tetramer$^+$ NKT cells, (c) CD1b-GMM tetramer$^+$T cells, (d) CD1b-GMM tetramer$^+$ CD4$^+$TRAV1$-$2$^+$ GEM cells, (e) $\gamma\delta$ T cells and (f) "conventional" CD4$^+$ T cells in individual infants (blue colors) or adults before (yellow) or 35 days after (red) BCG re-vaccination. (g) Peripheral blood frequencies of BCG-reactive "conventional" CD4 T cells expressing IFN-γ in individual infants (blue colors) or adults (yellow and red). For frequency plots, the black dots represent the estimated median while error bars represent 95% confidence intervals. For standardized effect size plots, black dots represent the standardized estimated difference in medians between infants who were vaccinated with BCG and those who were not, or in adults between the pre- and post- BCG revaccination time-points. Error bars represent 95% confidence intervals. Q-values are Bonferroni-adjusted *p*-values, with $q < 0.05$ considered statistically significant.

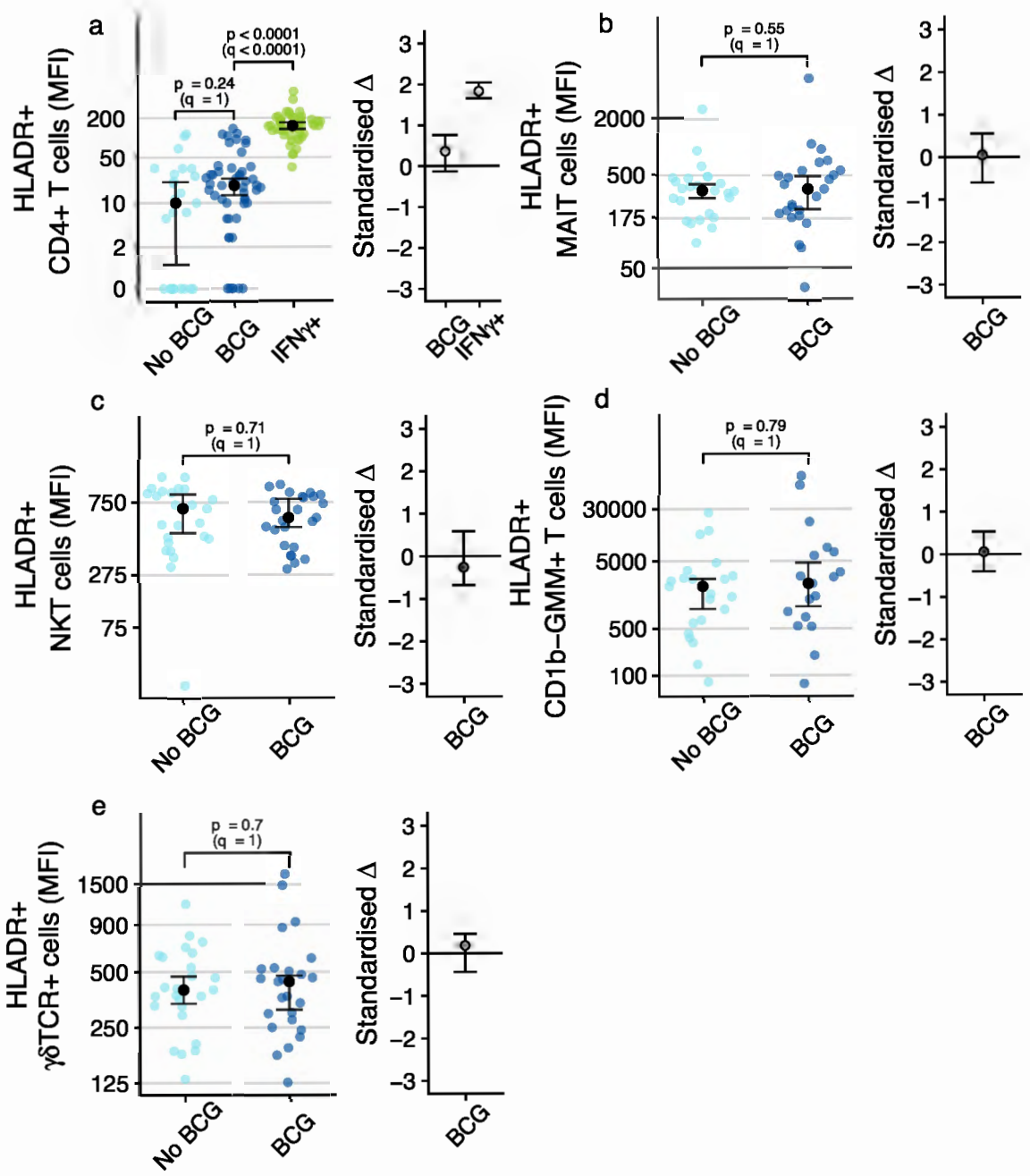

Figure 3. Assessment of BCG-associated T cell activation of the various cell subsets in BCG-vaccinated or unvaccinated infants. (a) T cell activation, measured by expression levels of HLA-DR, by total $CD4^+$ T cells in BCG-vaccinated or unvaccinated infants or by IFN-γ-expressing, BCG-reactive $CD4^+$ T cells in BCG-vaccinated infants (IFN- γ-expressing $CD4^+$ T cells in unvaccinated infants were too infrequent to quantify HLA-DR expression). T cell activation of (b) MR1-5-OP-RU $tetramer^+$ MAIT cells, (c) CD1d-PBS57 $tetramer^+$ NKT cells, (d) CD1b-GMM $tetramer^+$T cells or (e) $\gamma\delta$ T cells. CD1b-GMM $tetramer^+$ $CD4^+TRAV1{-}2^+$ GEM cells were too infrequent to reliably quantify HLA-DR expression. MFI, median fluorescence intensity. For frequency plots, the black dots represent the estimated median; for standardized effect size plots, the black dots represent the standardized estimated difference in medians. Error bars represent 95% confidence intervals. Q-values are Bonferroni-adjusted p-values, with $q < 0.05$ considered statistically significant.

0.79, 95% CI − 0.7 − 0.96; p=0.0029 by Wald test, q=0.047) and increased the proportions of $CD45RA^+CCR7^+$ cells (fold change 1.66, 95% CI 1.3 − 1.76; p = 0.0011 by Wald test, q=0.017) and possibly $CD45RA^+CCR7^-$ cells (fold change 1.25, 95% CI 0.98 − 1.44; p=0.019 by Wald test; q=0.3). Again,

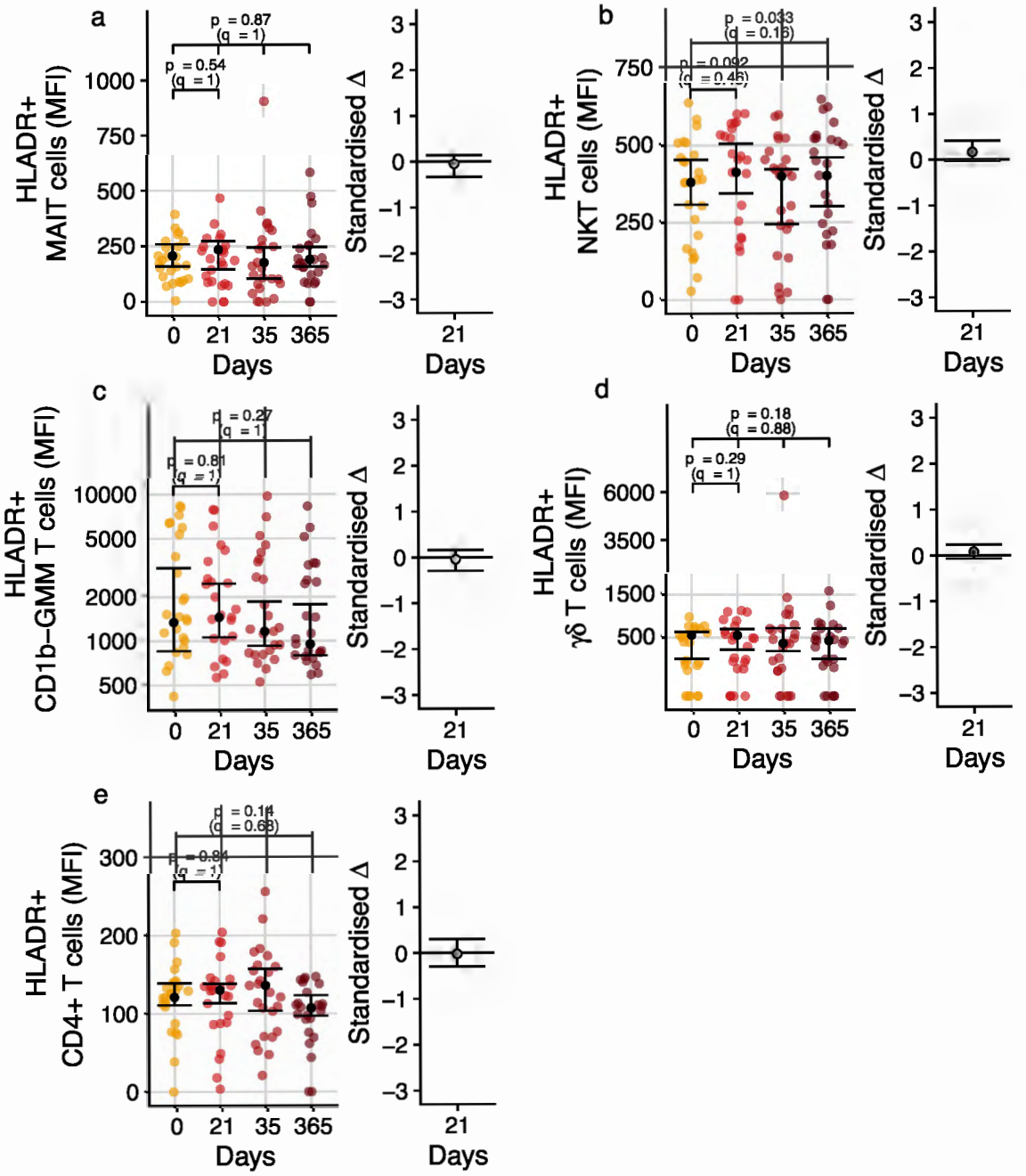

Figure 4. Longitudinal analysis of DURT cell activation profiles in adults before and after BCG revaccination. T cell activation, measured by expression levels of HLA-DR, on MR1-5-OP-RU tetramer$^+$ MAIT cells (a), CD1d-PBS57 tetramer$^+$ NKT cells (b), CD1b-GMM tetramer$^+$T cells (c), $\gamma\delta$ T cells (d) or total CD4$^+$ T cells (e). CD1b-GMM tetramer$^+$ CD4$^+$TRAV1$-$2$^+$ GEM cells were too infrequent to reliably quantify HLA-DR expression. MFI, median fluorescence intensity. For frequency plots, the black dots represent the estimated median; for standardized effect size plots, the black dots represent the standardized estimated difference in medians between the pre- and post- BCG revaccination time-points. Error bars represent 95% confidence intervals. Q-values are Bonferroni-adjusted p-values, with $q < 0.05$ considered statistically significant.

vaccination in adults was associated with little to no effect on the phenotypic profile.

As expected from previous work, bulk CD4$^+$ T cells in infants were comprised predominantly of naïve (CCR7$^+$CD45RA$^+$) and some central memory (CCR7$^+$CD45RA$^-$) T cells, whereas adults had comparatively lower proportions of naïve and higher proportions of central memory and effector memory CD4$^+$ T cells

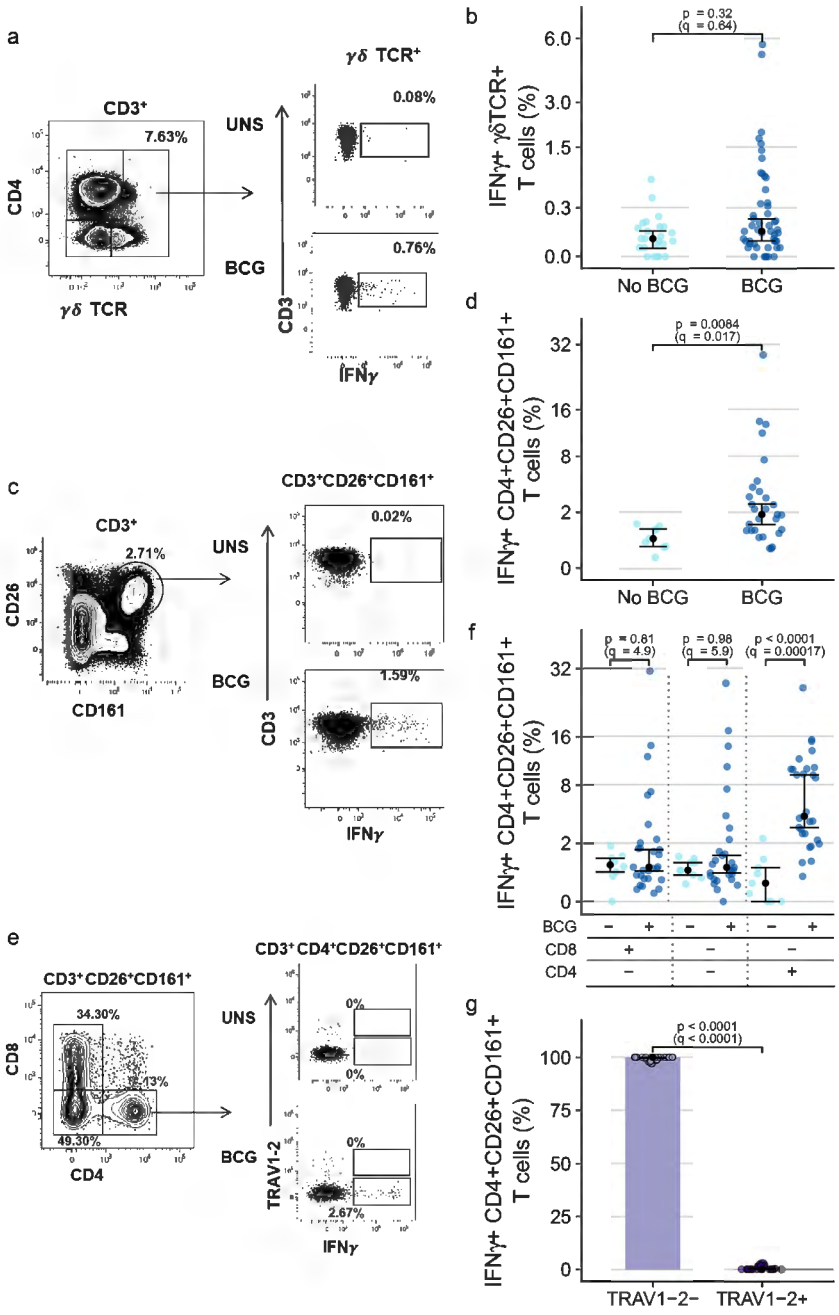

Figure 5. IFN-γ-expressing unconventional T cells elicited by infant BCG vaccination. (a) Representative flow cytometry plots depicting $\gamma\delta$ TCR expressing CD3$^+$lymphocytes (left) and IFN- γ expression in unstimulated and BCG-stimulated $\gamma\delta$ T cells (right). (b) Frequencies of BCG-reactive $\gamma\delta$ T cells expressing IFN-γ in BCG-vaccinated (dark blue) and unvaccinated (light blue) infants. (c) Representative flow cytometry plots of (left) CD26 and CD161 expression by CD3$^+$lymphocytes to identify CD26$^+$CD161$^+$ T cells producing IFN-γ in unstimulated and BCG-stimulated infant blood samples (right). (d) Frequencies of BCG-reactive IFN-γ-expressing CD3$^+$CD26$^+$CD161$^+$ T cells in BCG-vaccinated (dark blue) and unvaccinated (light blue) infants. (e) Representative flow cytometry plot depicting CD8 and CD4 staining and gating in CD3$^+$CD26$^+$CD161$^+$ T cells (left). Plot depicting TRAV1-2 and IFN-γ staining among CD4+CD26+CD161+ T cells (right). (f) Proportions of BCG-reactive IFN-γ^+CD3$^+$CD26$^+$CD161$^+$ T cells that are CD8+, double negative for CD8 and CD4 or that are CD4+ in BCG-vaccinated (dark blue) and unvaccinated (light) infants. (g) Proportions of BCG-reactive IFN-γ^+CD4$^+$CD26$^+$CD161$^+$ T cells that express TRAV1$-$2 in BCG-vaccinated infants. The black dots represent the estimated median; the error bars represent 95% confidence intervals. Q-values are Bonferroni-adjusted p-values, with $q < 0.05$ considered statistically significant.

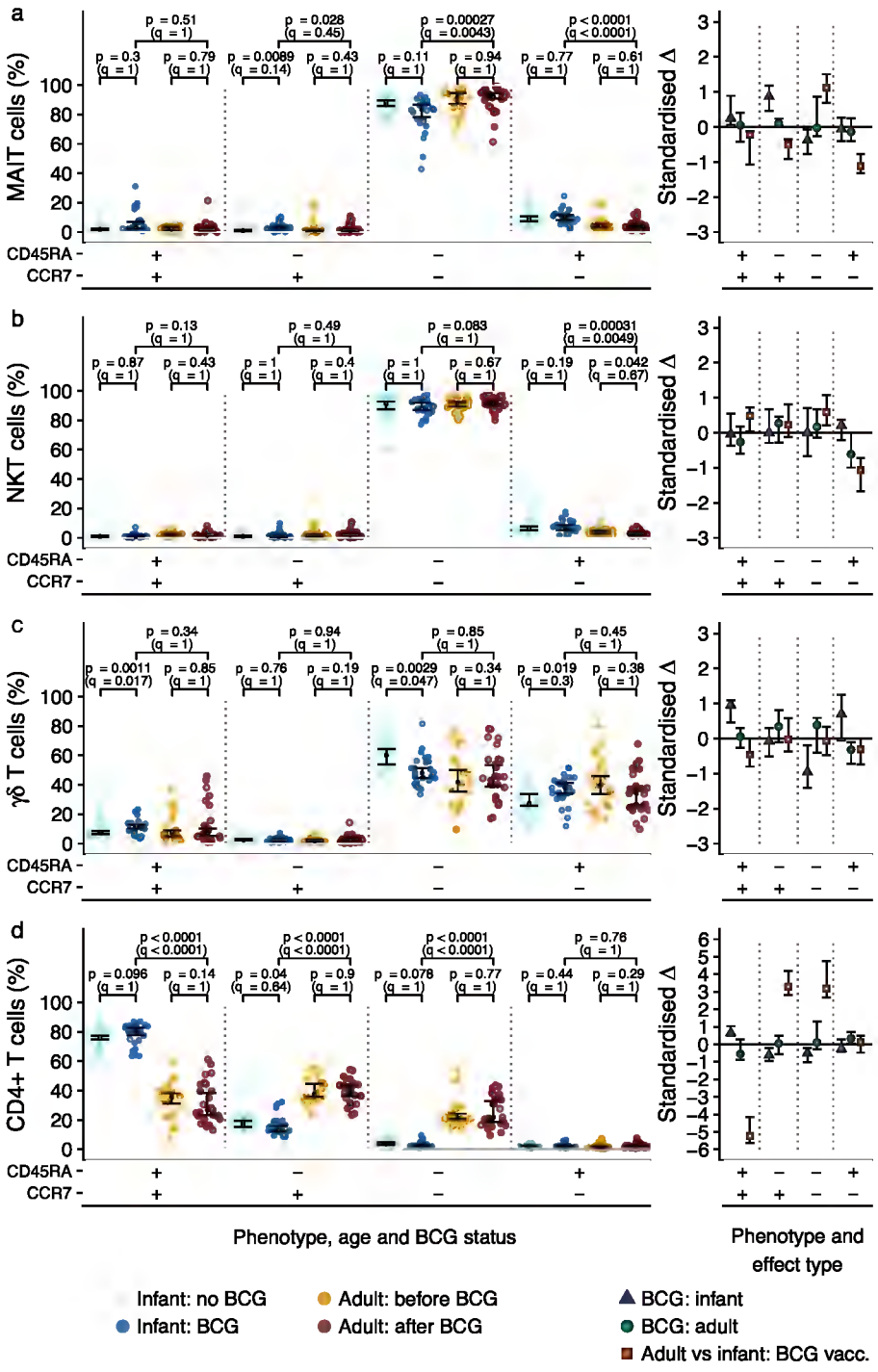

Figure 6. Memory phenotypes of DURT cell subsets or CD4 T cells in infants or adults. T cell memory profiles, measured by CCR7 and CD45RA co-expression patterns, on (a) MR1-5-OP-RU tetramer$^+$ MAIT cells, (a) CD1d-PBS57 tetramer$^+$ NKT cells, (c) $\gamma\delta$ T cells or (d) total CD4 T cells in BCG-vaccinated and unvaccinated infants or in adults before and 365 days after BCG re-vaccination. Relative proportions of cells that fall into each of the possible combinations of CCR7 and CD45RA are represented as percentages. For percentage plots, the black dots represent the estimated median; for standardized effect size plots, the black dots represent the standardized estimated difference in medians. Error bars represent 95% confidence intervals. Q-values are Bonferroni-adjusted p-values, with $q < 0.05$ considered statistically significant.

($q < 0.0001$ for all phenotypes except terminally differentiated) (Figure 6d).

Discussion

DURT cells and their roles in protective and pathogenic immune responses have received much attention in recent years.[42] Because these cell populations are not restricted by donor genotype, are relatively abundant and are intrinsically poised for rapid effector function, there is growing interest in whether DURT cells can be harnessed to improve efficacy of vaccination against organisms that express ligands for DURT TCRs.[1] We characterized frequencies, phenotypic and functional characteristics of DURT cell populations in the peripheral blood following BCG vaccination to explore their potential as possible immunological targets for TB vaccination. Evidence that $\gamma\delta$ T cells, MAIT, NKT and GEM T cells can respond to mycobacteria[15,43-47] provides a strong rationale to investigate if BCG vaccination can modulate these T cell populations.

A key finding was that frequencies of $\gamma\delta$ T cells, and perhaps frequencies of IFN-γ-expressing $\gamma\delta$ T cells detected by intracellular cytokine staining after in vitro BCG stimulation, were increased in BCG-vaccinated compared with unvaccinated infants. We also previously reported that BCG revaccination induced an increase of BCG-reactive IFN-γ-expressing $\gamma\delta$ T cells in adults,[38] however, here we show that $\gamma\delta$ T cell frequencies were not significantly modulated. Our results are also consistent with data from non-human primates, in which a rapid and robust expansion of $\gamma\delta$ T cells has been observed after primary BCG vaccination or M.tb-infection.[18] In cattle, $\gamma\delta$ T cells have been shown to accumulate in the early phases of *M. bovis* infection, but quickly dissipate in peripheral blood upon the arrival of other cells. Moreover, circulating $\gamma\delta$ T cells from these animals produced increased amounts of IFN-γ and CCL2, and expressed high amounts of cytolytic molecules and lysed BCG-infected target cells with greater efficiency compared to cells from uninfected animals.[48] Our findings are thus consistent with the previously reported role of $\gamma\delta$ T cells as early responders to BCG vaccination, and may contribute to or enhance the ensuing immune response by recruiting or activating other key immune and effector response players. However, these results are at odds with findings of another recent study of $\gamma\delta$ T cell development in early life. Frequencies and differentiation profiles of $\gamma\delta$ T cells, assessed in cord blood and at 10 weeks of age, revealed a rapid expansion of Vγ9Vδ2 T cells with cytotoxic phenotypes after birth.[49] Notably, this early and robust change in T cell responses was not attributable to newborn BCG vaccination, since Vγ9Vδ2 T cell responses between BCG-vaccinated and unvaccinated infants were not different. The data suggest a possible role of environmental phosphoantigen exposure in the priming of these cells in early life.[49] The discrepancy between the BCG-attributable effects on $\gamma\delta$ T cells between this study and our present one most likely relate to the different subsets of $\gamma\delta$ T cells detected in each study. Although Vγ9Vδ2 are the main $\gamma\delta$ T cell subset, in this study we measured the total $\gamma\delta$ T cell population, and we cannot exclude that other minor subsets may have contributed to the differences observed here.

We found that infants generally had lower peripheral blood frequencies of MAIT, NKT, GMM-specific and GEM T cells compared to adults, a finding consistent with our previous study.[50] By contrast, frequencies of $\gamma\delta$ T cells were not different between infants and adults. As expected, BCG vaccination markedly induced conventional, IFN-γ-expressing CD4 T cell responses. However, we show that frequencies of MAIT, NKT, GMM-specific and GEM T cell populations were not modulated by intradermal BCG administration in either infants or adults. BCG vaccination also did not affect DURT cell activation and only induced minor alterations in the expression of the T cell memory markers CCR7 and CD45RA on DURT populations in infants. DURT cell activation was assessed by quantifying HLA-DR expression by the different DURT subsets, with the rationale that TCR-mediated recognition of BCG antigens in vivo would lead to increased HLA-DR expression. Despite the use of HLA-DR as a marker to measure vaccine-induced activation in studies of MHC-restricted T cells,[37,51,52] it is not known if this marker is ideal for measuring DURT cell activation. Inclusion of additional activation markers, such as Ki67, would have allowed a more comprehensive ascertainment of the activation status on these cells. The memory phenotypes observed for the DURT subsets is not surprising given that the DURT subsets exhibited very dominant CCR7$^-$ CD45RA$^-$ effector-like phenotypes in infants and adults, consistent with the well-described intrinsic effector function of these innate-like T cell populations.[1]

The finding that BCG vaccination did not modulate DURT subsets can be explained by the well-described intrinsic innate-like and poor proliferative capacity generally attributed to DURT cells, which prescribe that these cells do not adapt to antigenic stimulation in the same manner as conventional, adaptive CD4 and CD8 T cells do. A recent study illustrated this elegantly by employing transcriptomic analyses of conventional T cells, MAIT cells, iNKT cells, $\gamma\delta$ T cells and NK cells to investigate the apparent trade-off between potential for cellular proliferation and rapid effector function.[53] The analyses revealed that these cell subsets can be ranked according to their "innateness" with conventional, adaptive CD4 T cells the least innate and NK cells the most, and that this innateness was characterized by pre-formed mRNA encoding effector molecules, while impaired proliferation was marked by decreased baseline expression of ribosomal genes.

We acknowledge the possibility that the timing of our analyses, performed at 9 weeks after neonatal BCG vaccination and 3, 5 weeks and 1 year after adult BCG revaccination, may have missed transient DURT cell responses to BCG vaccination. In a controlled human Salmonella challenge model, MAIT cells were shown to be activated at the peak of infection (day 10) and this activation state was maintained even after antibiotic treatment.[54] By contrast, there was an early decrease in MAIT frequencies after bacterial challenge (day 8), which recovered after antibiotic treatment (day 28).[54] Greene et al.[55] also reported significant, but transient activation of MAIT cells in peripheral blood of rhesus macaques in response to BCG, which peaked around 3 weeks after vaccination. Notably however, no significant modulation of MAIT cell frequencies in the peripheral blood was observed, highlighting that clonal expansion may be subtle and easy to miss. We also note that the adult BCG revaccinated cohort enrolled individuals with tuberculin skin test (TST) indurations exceeding 15 mm, consistent with prior M.tb infection. These individuals therefore had high levels of baseline anti-mycobacterial immune responses, which may have led to a masking effect such that subtle changes in BCG-induced immune responses were not detected. Future studies should also include TST or IGRA-negative individuals which are difficult to enroll in a high TB incidence setting like South Africa, to allow investigation of the effects of prior sensitization on vaccine-induced DURT responses. Our findings do not preclude that BCG vaccination may shape other qualitative aspects of DURT cells, such as their homing capacity, redistribution of the clonal TCR repertoire and/or proliferative potential.

Neonatal BCG vaccination was associated with a marked increase of an interesting and novel subset of BCG-reactive, IFN-γ-expressing $CD26^+CD161^+$ CD4 T cells. Further phenotypic characterization of this subset revealed that these cells did not express the canonical MAIT TCR alpha variable gene segment, TRAV1–2. MAIT cells have traditionally been phenotypically identified as $TRAV1\text{–}2^+CD161^+$ or $CD26^+CD161^+$ $CD8^+$ cells, and most studies have focused exclusively on adults. This peculiar cell subset was not detectable before and after BCG revaccination in adults. We recently showed that MR1–5-OP-RU tetramer-positive MAIT cells in neonates and infants do also include $CD4^+$ and TRAV1–2-negative T cells.[50] In addition, in a TCR transgenic MAIT cell murine model, $CD4^+$ MAIT cells were associated with pulmonary protective immunity, recruited into the lung in response to M.tb aerosol challenge.[56] However, these BCG-reactive, IFN-γ-expressing $CD26^+CD161^+$ CD4 T cells could also be a subset of conventional, activated CD4 T cells or a novel DURT subset that are induced by BCG vaccination. Interestingly, these cells share characteristics with a recently described $CD4^+CD26^+CD161^+CCR6^+$cell subset that expresses IL-17 and IL-22 and was preferentially enriched in a Peruvian cohort of TB non-progressors.[57] These findings warrant further clinical and mechanistic investigation of these cells in clinical studies and animal models.

In conclusion, our study suggests that intradermal BCG vaccination does not modulate MAIT, NKT, GMM-specific and GEM T cell frequencies. Newborn BCG vaccination was associated with increased frequencies of $\gamma\delta$ T cells; however, there was no significant increase in IFN-γ expressing $\gamma\delta$ T cells in response to BCG. More studies are required to understand the full potential of DURT cells for TB vaccination and whether the modulation of $\gamma\delta$ T cells is durable. Given their immediate effector properties, the monoclonal or oligoclonal use of a TCR, and their role in regulating other key immune cell subsets and functions, future research should also explore their potential adjuvant effects.

Contributors

AG, DFH, WAH, WHB, JLJ, DFH, SAJ, THMO, SS, DBM, DL, MH, CS, EN and TJS designed the experiments or clinical studies. WAH, HB, and MH performed clinical investigations. AG, MM, MR, KH and SS processed samples, performed assays, and/or analyzed data. AG, MM, MR, KH, SS, EN and TJS verified and interpreted the data. AG, MM, MR, CS, EN and TJS wrote the manuscript. All authors reviewed and approved the manuscript.

Declaration of interests

No competing interests.

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Data sharing statement

All datasets and metadata have been deposited in Zivahub (https://figshare.com/s/91b7b121ea2dcf7b5b36), an open access data repository hosted by the University of Cape Town's institutional data repository powered by Figshare for Institutions.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.103839.

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Newborn BCG vaccination induces robust infant IFNγ-expressing NK cell responses to mycobacteria

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¶Equal contribution

Short title: BCG vaccination and NK cells in infants

Abstract

Infants are highly susceptible to disease caused by infection with intracellular pathogens, such as *Mycobacterium tuberculosis*. The Bacille Calmette-Guérin (BCG) vaccine is usually administered at birth to protect against severe forms of tuberculosis in children. BCG also has non-specific protective effects against heterologous infections, thought to be mediated by enhanced innate cell functions. We aimed to determine whether newborn BCG vaccination modulates myeloid and NK cell responses to mycobacteria.

We enrolled 130 South African infants in whom BCG vaccination was either administered at birth or delayed to 6 or 10 weeks of age. CD4 T, myeloid and NK cell responses to whole cell mycobacteria were measured by flow cytometry. Levels of cytokines secreted upon whole blood stimulation with BCG were measured by multiplex bead array.

Newborn BCG vaccination was associated with significantly higher frequencies of BCG-reactive, cytokine-expressing $CD4^+$ T cells and IFNγ-expressing NK cells, compared with unvaccinated infants at 5-weeks and 9-weeks of age. However, cytokine-expressing $CD33^+$ myeloid cells were not different between vaccinated and unvaccinated infants. Induction of BCG-reactive IFNγ-expressing NK cells was not associated with enhanced NK cell maturation, differentiation or cytokine receptor expression. Although levels of secreted cytokines were not different in vaccinated and unvaccinated infants, BCG-reactive NK cell responses correlated directly with levels of IL-2 and IFNγ, and the innate pro-inflammatory cytokines IL-6, IL-1β and TNF in BCG vaccinated infants only.

Our data show that BCG-reactive IFNγ-expressing NK cells are strongly induced by BCG vaccination in infants and are likely amplified through by-stander cytokine-mediated activation. Further studies in infants are needed to improve our understanding of the possible role BCG-induced NK cell responses may play in mediating protection against tuberculosis and other infectious diseases.

Introduction

Newborns and infants have distinct immune responses that markedly differ from those of adults, including lower proinflammatory innate responses, antimicrobial proteins, Th1-inducing cytokines and higher IL-10 production (1-3). The infant immune response is also known to be Th17 and Th2-biased (1, 4, 5). These unique features are thought to be necessary to protect young infants against excessive inflammation when transitioning from the intrauterine to the non-sterile environment after birth. However, it appears that this can also lead to insufficient immunity against pathogenic challenge (6). Thus, infants are highly susceptible to intracellular pathogens, including *Mycobacterium tuberculosis* (*M.tb),* and have a high risk of developing tuberculosis (TB) disease (7). The TB vaccine, Bacille Calmette-Guérin (BCG), is routinely administered at birth to protect infants against severe forms of TB, such as miliary TB and meningitis (8, 9). Previous studies investigating mycobacteria-specific immune responses that may mediate protection elicited by BCG in infants have largely focused on T cells (10-15). This is informed by small animal models, which show that $CD4^+$ and $CD8^+$ T cells are necessary for control of *M.tb* (16-18). The importance of intact $CD4^+$ T cell responses for immunity against mycobacteria in humans is highlighted by the increased risk of TB disease after *M.tb* infection in people living with human immunodeficiency virus (HIV) (19). However, we showed that frequencies and functional attributes of BCG-specific Th1 responses in 10-week-old infants did not differ between infants who progressed or did not progress to active TB disease (13). Also, the MVA85A vaccine, designed to boost the immune response primed by BCG, induced durable Th1 and Th17 responses, but failed to enhance protection against TB disease in BCG-vaccinated infants. These studies are consistent with the view that Th1 responses alone do not seem to be sufficient to control *M.tb* infection, or that such responses may target the wrong antigens (20).

In vitro stimulation of blood leukocytes with BCG can activate immune subsets beyond classical T cells in infants, including NK cells (21). NK cells are a major source of IFNγ in the immune response to mycobacteria in newborns and infants (21-23). A study in the United Kingdom (UK) showed that NK cells from BCG-vaccinated infants displayed enhanced expression of the CD69 activation marker in response to Pam3Cys and *M.tb* lysate compared to unvaccinated infants (24), suggesting that BCG may modulate NK cell activity. NK cells are activated through a complex network of germline-encoded receptors or through bystander activation by cytokines such as IL-2, produced by antigen-specific T cells, and IL-18 and IL-12, produced mainly by phagocytes. This activation leads to rapid proliferation and production of effector molecules (25-28). In addition, following stimulation with haptens, viruses and cytokines, NK cells can exhibit memory-like properties and enhanced recall responses (29-32). The modulatory effects and underlying mechanisms of BCG vaccination on infant NK cells in a TB endemic setting have not been previously investigated.

Experimental evidence suggests that innate cells such as monocytes, macrophages and NK cells, can be "trained" or programmed by initial exposure to certain stimuli to retain the ability to respond with greater (or lesser) magnitude or function to subsequent heterologous exposures (33, 34). For example, initial exposure to certain pathogens or vaccines can lead to metabolic and epigenetic changes in monocytes that result in higher production of pro-inflammatory cytokines, such as IL-1β, TNF and IL-6, in response to subsequent challenge with unrelated organisms (33, 35). BCG is a potent mediator of such monocyte training, which has been extensively studied (35-38). Other whole cell vaccines, such as measles and the molecule β-glucan, can also mediate trained immunity through a IL-1β-dependent mechanism (39, 40). BCG-mediated training of neutrophils and NK cells has also been reported in adult volunteers in non-TB endemic settings (34, 41). These heterologous effects of BCG are clinically significant, as shown by clinical trials in Guinea-Bissau, where BCG administration at birth

was associated with significant reductions in all-cause mortality in low birthweight infants (42-44). BCG-mediated trained immunity has been proposed as the underlying mechanism for these beneficial heterologous effects of vaccination (39). A recent study in Uganda also showed a decrease in incidence of non-TB infectious diseases in BCG vaccinated 6-week-old infants compared to infants of the same age in whom BCG vaccination was delayed. This protective effect had waned at 10 weeks, 4 weeks after the delayed group received BCG (45), and evidence for trained innate immunity was less clear compared to previous studies.

Here, we determined if neonatal BCG vaccination modulates myeloid and NK cell responses to mycobacteria, by comparing vaccinated and unvaccinated infants from a setting endemic for TB. We hypothesized that myeloid cell expression and whole blood secretion of pro-inflammatory molecules would be enhanced after BCG vaccination, compared to unvaccinated infants. We further hypothesized that BCG vaccination would increase frequencies of functional BCG-reactive NK cells and lead to differentiation and enhanced effector functions.

Materials and Methods

Study participants

Study participants were recruited from Worcester in the Western Cape, South Africa. We sought to enrol infants in which BCG vaccination was either administered at birth, as is routine, or delayed to 6 or 10 weeks of age **(Fig. 1)**. For the birth-vaccination cohort, mothers were approached for consent to participate at public and private vaccination clinics. In the delayed BCG-vaccination cohort, pregnant women were contacted and approached for consent to participate in Worcester communities, ante-natal care clinics or at hospitals. In both the birth-vaccination and the delayed BCG-vaccination cohorts, exclusion criteria included: 1. Mothers who had known chronic or acute infections during the last trimester of pregnancy; 2. Mothers who were HIV+ or who refused an HIV test; 3. Infants who were enrolled in another experimental protocol; 4. Infants born through Caesarean section and those with delivery complications; 5. Infants born before 37 weeks of gestation and those with low birth weight (<2500g); 6. Infants who had congenital malformations and those with perinatal complications; 7. Infants who had a history of TB, or close contact with someone with TB disease; 8. Infants who had received isoniazid (INH) therapy; 9. Infants with any chronic disease or any acute disease within 2 weeks of blood collection; 10. Infants who were on chronic or immune-modifying drugs. Infants who had not received BCG within 48 hours of birth, as is routine, were excluded from the birth-vaccination cohort. In the delayed-BCG cohort, infants were excluded if their mothers had a high chance of relocating or had TB disease or contact with someone with TB disease. Infants received intradermal BCG Danish strain 1331 (Statens Serum Institut, SSI) at the standard dose of 1-4 x 10^5 colony forming units. Venous blood was collected from infants in the birth or delayed BCG cohorts at 5 or 9 weeks of age **(Fig. 1)**, after which BCG was administered in those not yet vaccinated. Demographic characteristics of the infant cohorts are summarised in **Supplementary Table 1.**

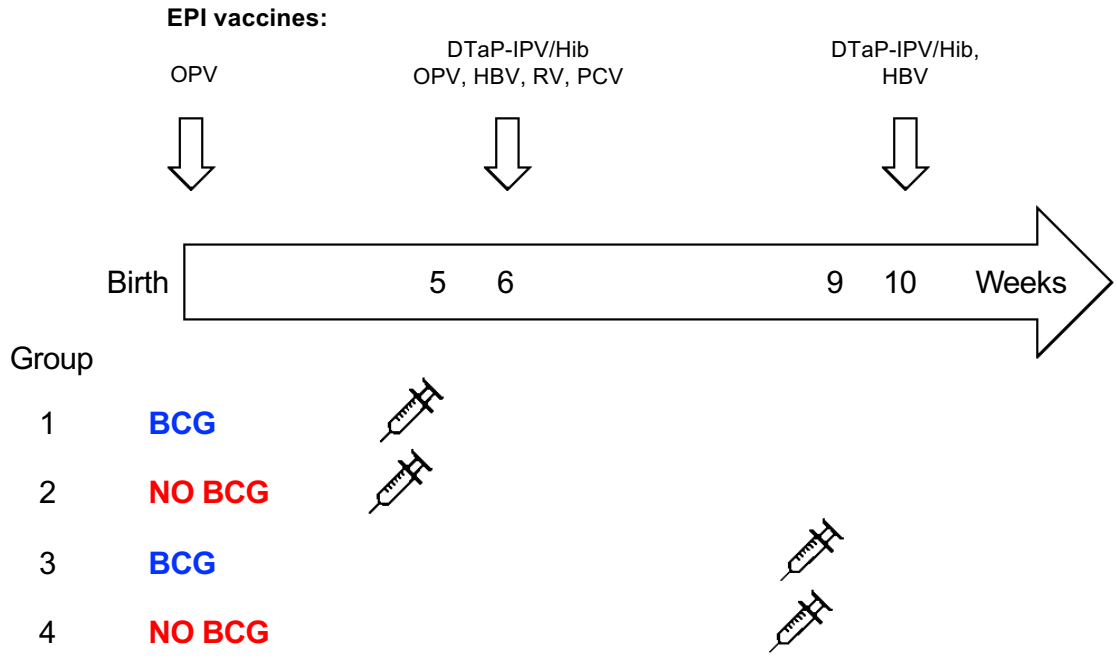


Figure 1. Delayed BCG Infant study design. Schematic representation of administered vaccines and blood draws in four cohorts of infants. Group 1 and 3 received BCG vaccination at birth. Group 2 and 4 received BCG vaccination at 6-weeks and 10 weeks of age respectively. Syringes indicate when blood draws were taken. The arrows indicate when the respective vaccines were administered. BCG, Bacille Calmette-Guérin vaccine; OPV, oral polio vaccine; DTaP-IPV//Hib, diphtheria, tetanus, acellular pertussis, inactivated polio vaccine, haemophilus influenzae type b; PCV, pneumococcal conjugate vaccine; RV, rotavirus vaccine; HBV, hepatitis B virus.

Supplemental table 1. Demographic characteristics of infant cohorts

Variable	5-week-old	5-week-old	9-week-old	9-week-old
BCG vaccination	Yes	No	Yes	No
Participants, n	26	30	50	24
Age, median (week, range)	5 (4-8)	5 (4-5)	9 (8-11)	9 (8-11)
Female, n (%)	10 (38)	15 (50)	25 (50)	11 (46)
Ethnicity, n (%) Black African Mixed ancestry	 4 (15) 22 (85)	 5 (17) 25 (83)	 13 (26) 37 (74)	 4 (17) 20 (83)

Ethics statement

Parents or legal guardians provided written, informed consent before enrolment in the study. The Human Research Ethics Committee at the University of Cape Town approved protocols for infants vaccinated at birth (REF: 126/2006) and infants who had delayed BCG vaccination (REF: 177/2011). Good Clinical Practice procedures and the World Medical Association Declaration of Helsinki guidelines were adhered to.

Whole Blood Intracellular Cytokine Staining (WB-ICS) assay

Whole blood was collected in heparin blood tubes and processed within 75 minutes from phlebotomy, as previously described (71, 72). Briefly, blood was stimulated with either BCG Vaccine SSI (1.2 x 10^6 CFU/ml; Biovac, Cape Town, South Africa), phytohemagglutinin (PHA; 5 μg/ml; Sigma-Aldrich; positive control) or RPMI alone (negative control). Co-stimulants anti-CD28 and anti-CD49d (0.25 μg/ml; BD Biosciences, San Diego, USA) were added to all stimulation conditions. Blood was stimulated for 7 hours at 37℃, after which

100μL of plasma was removed and stored at -80℃ for later quantification of secreted cytokines (described below). Brefeldin-A (10 μg/ml; Sigma-Aldrich) was added for the remaining 5 hours of the stimulation. At the end of the stimulation, adherent cells were detached using EDTA (2mM; Sigma-Aldrich) and red blood cells lysed using 1:10 FACS Lysing solution (BD Biosciences) after which fixed cells were cryopreserved in liquid nitrogen.

Antibodies and flow cytometry

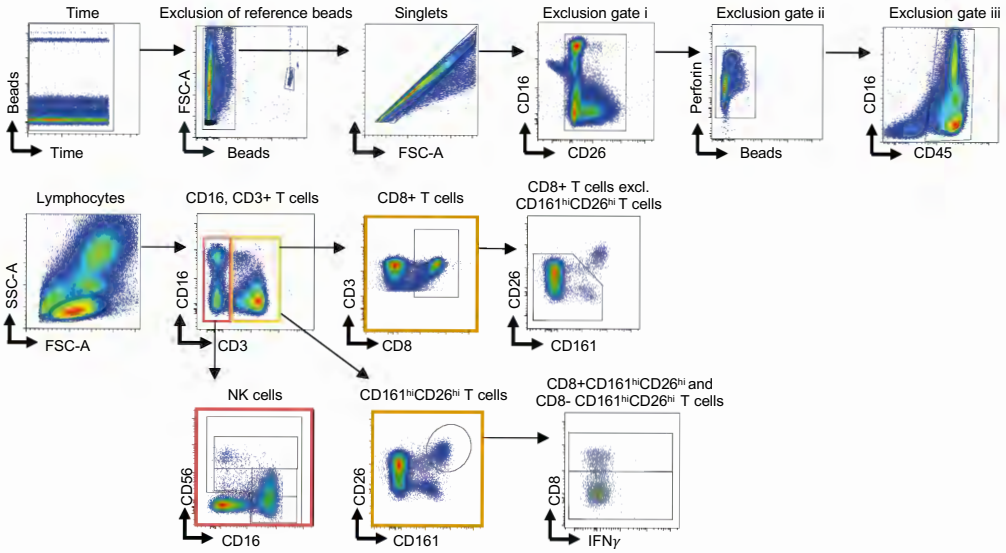
Cryopreserved cells from infant participants were thawed, washed in PBS and stained with antibody panels **(Supplementary Table 2)** in Perm/Wash buffer (BD Biosciences) for 45 minutes at 4℃. Flow-count Flourosphere beads (50μL; Beckman Coulter) were added to each sample in Panel 1 and Panel 2 **(Supplementary Fig. 1)**. Stained cells were acquired on a LSR II flow cytometer (BD Biosciences) equipped with solid state blue (488nm; 100mW; 3 detectors), solid state violet (405nm, 25nW; 8 detectors), HeNe gas red (635nm, 70mW; 3 detectors), and diode-pumped coherent compass green (532nm; 150mW; 8 detectors) lasers. Single-stained rat κ-chain BD CompBeads (BD Biosciences) were used to compensate the IL-6-PE antibody and single-stained mouse κ-chain BD CompBeads (BD Biosciences) were used to compensate all other parameters. Cytometer Setup and Tracking beads were run daily to ensure consistent median fluorescent intensity (MFI) and signal CVs were maintained. Photomultiplier tube voltages were calibrated daily using SPHERO Rainbow Fluorescent Particles (Spherotech; Lake Forest, USA) (73).

Supplemental table 2. Antibody panels used for flow cytometry experiment

Fluorochrome	Antigen	Clone	Manufacturer
Panel 1			
FITC	CD45	2D1	BD
Brilliant Violet 510	CD16	3G8	BioLegend
Brilliant Violet 711	CD56	HCD56	BioLegend
APC-H7	CD3	SK7	BD
Brilliant Violet 650	CD8	RPA-T8	BD
APC	CD26	BA5b	BioLegend
PeCy5	CD161	DX12	BD
Brilliant Violet 605	CD57	NK-1	BioLegend
PE	CD158b	CH-L	BD
AlexaFlour700	IFNγ	B27	BD
PeCy7	TNFα	Mab11	eBioscience
Brilliant Violet 421	Perforin	B-D48	BioLegend
Panel 2			
Brilliant Violet 510	CD16	3G8	BioLegend
Brilliant Violet 711	CD56	HCD56	BioLegend
APC-H7	CD3	SK7	BD
ECD	CD4	SFCI1274011	Beckman Coulter
Brilliant Violet 650	CD8	RPA-T8	BD
APC	CD26	BA5b	BioLegend
PeCy5	CD161	DX12	BD
Brilliant Violet 605	TRAV1-2	3C10	BioLegend
BB515	CD212	2.4E6	BD
PeCy7	CD122	TU27	BioLegend
PE	CD218a	H44	BD
AlexaFlour700	IFNγ	B27	BD
Brilliant Violet 421	Perforin	B-D48	BioLegend
Panel 3			
BV650	CD33	VM53	BD
PeCy5	CD16	3G8	BD
APC-H7	CD3	SK7	BD
Brilliant Violet 786	CD4	SK3	BD
Brilliant Violet 421	TCR-$\gamma\delta$	B1	BioLegend
Brilliant Violet 711	CD20	2H7	BioLegend
BB15	IgD	IA6-2	BD
PE-CF594	CD27	M-T271	BD
Brilliant Violet 605	HLADR	L243	BioLegend
Brilliant Violet 510	Granzyme B	GB11	BD
PE	IL-6	MQ2-13A5	BD
AlexaFlour647	IL-1β	JK1B-1	BioLegend
AlexaFlour700	IFNγ	B27	BD
PeCy7	TNFα	Mab11	eBioscience

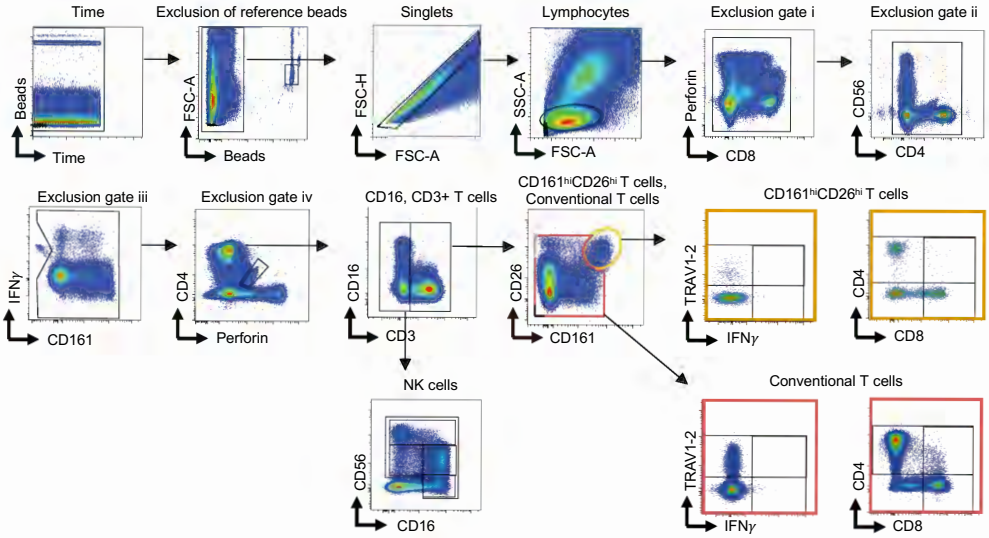
A.

Panel 1



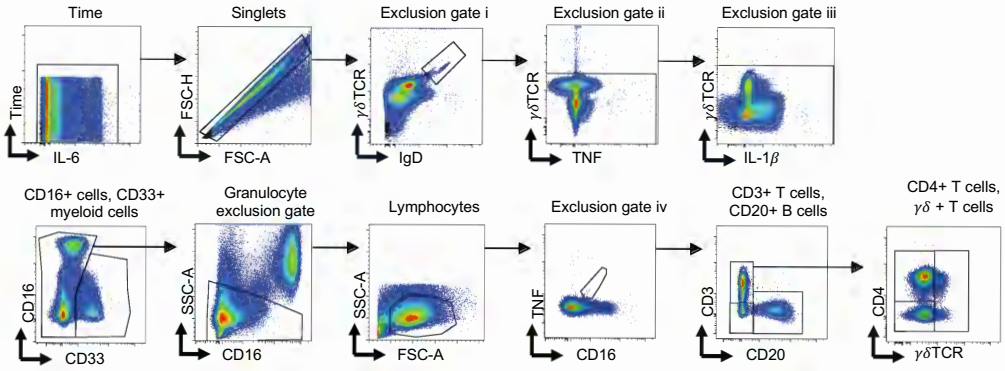
B.

Panel 2



C.

Panel 3



Supplementary figure 1. Gating strategies. Gating strategies for analysis of flow cytometry **(A)** Panel 1 **(B)** Panel 2 and **(C)** Panel 3.

Multiplex bead array assay

Plasma samples collected from stimulated whole blood were thawed and levels of IFNγ, TNF, IL-12p70, IL-18, IL-1β, IL-6, IL-2, GM-CSF, IL-10, IL-13, IL-17A, IL-21 IL-23, IL-27, IL-4, IL-5 and IL-9 quantified using the Th1/ Th2/ Th9/ Th17 Cytokine 18-Plex ProcartaPlex™ Panel (Thermo Fisher Scientific; Waltham, USA) on the Bio-Plex® 200 System (Bio Rad laboratories; Hercules, CA, USA) in accordance with the manufacturer's instructions (Thermo Fisher Scientific; Waltham, USA). Each sample was run in duplicate. Following optimisation experiments, all samples were diluted 1:3 in assay buffer. The standard curve for all analytes ranged from 1.78-60,900 pg/mL. Concentration values within the linear range of quantification are reported for secreted cytokines; fluorescence intensity values were assigned a corresponding concentration value based on the standard curve. Analyte concentrations detected in unstimulated samples were subtracted from those measured after BCG stimulation (BCG-UNS). No values were above the upper limit of detection. Values below the lower limit of detection of each analyte's standard curve and BCG-UNS values that were zero or negative were assigned the value 0.89, derived using the lowest standard value in the kit (1.78) divided by 2.

Data analysis

As shown in **Supplementary Fig. 1**, flow cytometry data were analysed using FlowJo version 9.7- 9.9.6 (Treestar; Ashland, OR). Background subtractions were performed in Pestle version 1.7-2.0 and boolean cytokine combinations were analysed in Spice version 5.3-6.0 (Roederer, Nozzi and Nason, 2011). Absolute cell numbers were calculated using the formula: [(cell event count/bead count) x #beads/test/initial blood volume)] (74). Acquisition and analysis for multiplex bead array experiments were performed using the Bio-Plex Manager Software. Statistical analyses were performed and graphs created using GraphPad Prism version 6-8 or

R (http://www.r-project.org). The heat map was generated using the ComplexHeatmap package (75). The Mann-Whitney U test was applied to compare different vaccination groups. The Wilcoxon signed-rank test was used for paired comparisons in the same vaccination group. To measure the degree of association between two variables Spearman rank correlation was applied. Unadjusted p-values are shown; p-values that were significant after correction for multiple comparisons are shown in bold. The false discovery rate (FDR) or the Bonferroni correction method were used, where appropriate, to correct p-values for multiple comparisons.

Results

Newborn BCG vaccination is associated with higher frequencies of BCG-reactive CD4$^+$ T cells but not CD33$^+$ myeloid cells.

To determine the modulatory effects of BCG vaccination on adaptive and innate cell frequencies and functions, we characterized BCG-reactive CD4$^+$ T and CD33$^+$ myeloid cells in response to BCG stimulation in 5-week-old and 9-week-old infants who did (BCG) or did not (no BCG) receive BCG vaccination at birth. CD4$^+$ T cells expressing IFNγ or TNF were significantly higher in BCG vaccinated compared to the delayed BCG group in both age groups **(Fig. 2 A and B)**.

In the myeloid cell compartment, BCG stimulated samples expressed high levels of IL-6, IL-1β and TNF compared to the unstimulated samples **(Fig. 2 C and D)**. However, there were no differences in frequencies of cytokine-expressing myeloid cells between the study groups **(Fig. 2 E)**. There were also no differences in cytokine MFI on myeloid cells (data not shown). Taken together, our results indicate that BCG vaccination induces IFNγ and TNF producing CD4$^+$ T cells, as expected, but GrnB-producing CD4$^+$ T cells did not change. Similarly, IL-1β, IL-6 or TNF-expressing myeloid cells were not modulated by neonatal BCG vaccination.

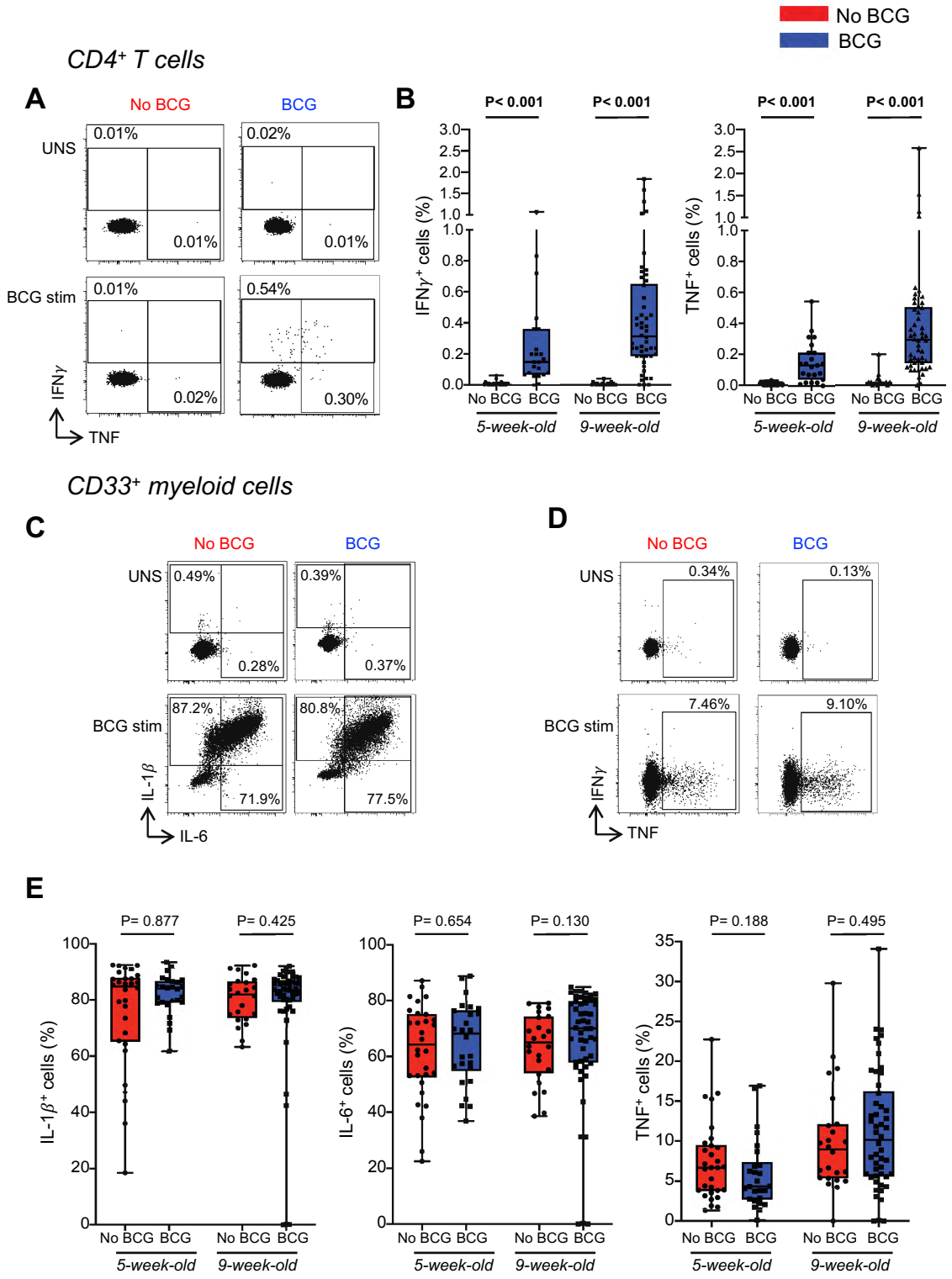


Figure 2. Frequencies of BCG-reactive CD4⁺ T cells and CD33⁺ myeloid cells in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing IFNγ versus TNF expression by CD4⁺ T cells in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(B)** Frequencies of BCG-specific IFNγ+ (left) and TNF+ (right) CD4⁺ T cells in 5-week-old and 9-week-old infants. Representative flow cytometry plots showing IL-6 versus IL-1β **(C)** and IFNγ versus TNF **(D)** expression by CD33⁺ myeloid cells in unstimulated or BCG-stimulated samples from an

unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(E)** Frequencies of BCG-reactive IL-1β^+ (left), IL-6$^+$ (middle) and TNF$^+$ (right) CD33$^+$ myeloid cells in 5-week-old and 9-week-old infants. Horizontal lines represent medians, boxes represent the interquartile range, and whiskers represent the range. P-values were calculated with the Mann-Whitney U test. Bolded p-values are considered significant after correcting for multiple comparison testing using the Bonferroni method. P-values < 0.025 (two comparisons) were considered statistically significant.

BCG-reactive IFNγ-expressing NK cells are induced after BCG vaccination.

Next, we evaluated whether newborn BCG vaccination modulates NK cell responses. We defined four distinct NK subsets: CD56$^+$CD16$^-$, CD56^{++}, CD56$^+$CD16$^+$ and CD56$^-$CD16$^+$ in addition to total NK cells **(Fig. 3A)**. Five NK subsets have previously been defined based on CD56 and CD16 expression (46). We, however, did not separate the CD56^{++} population into two separate subsets (CD56^{bright}CD16$^-$ and CD56^{bright}CD16^{dim}) as previously described, because these subpopulations were variable (**Fig. 3A**). In addition, due to the small number of events, the two populations were pooled together to ensure that there were enough cell numbers to allow for downstream functional and phenotypic analyses. There were no differences in frequencies **(**among CD3- cells, **Supplementary Fig. 2)** or proportions **(**among total NK cells, **Fig. 3B and C)** of NK cell subsets between the BCG vaccinated and delayed BCG groups at 5-weeks. Although the composition of NK cell subsets in 9-week-old infants was different by Permutation Test **(Fig. 3B)**, the proportions of each individual subset was not significantly different between the infant groups **(Fig. 3C)**. Absolute cell counts were also calculated for all immune subsets, but no differences in counts between the BCG and no BCG groups were observed (not shown).

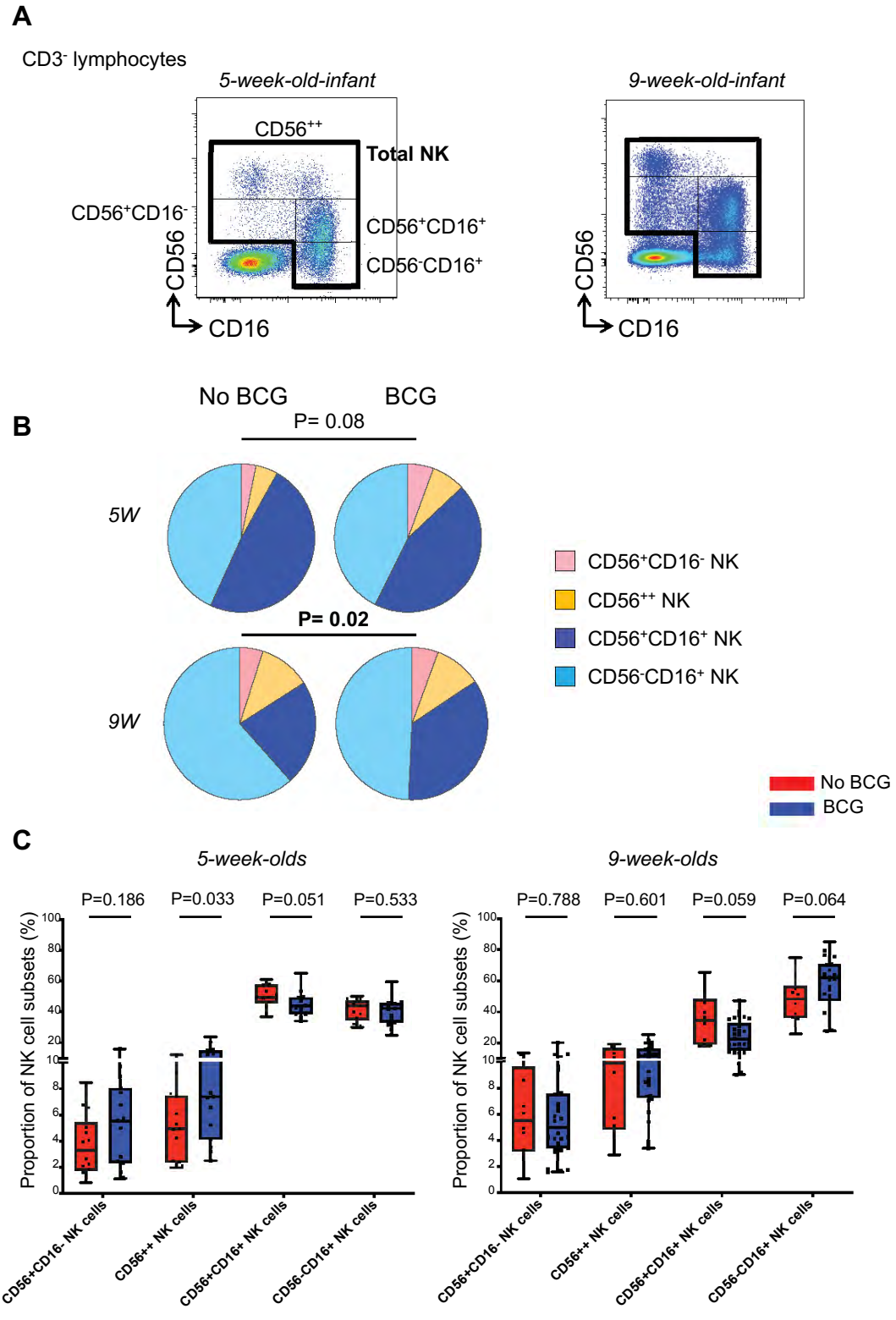
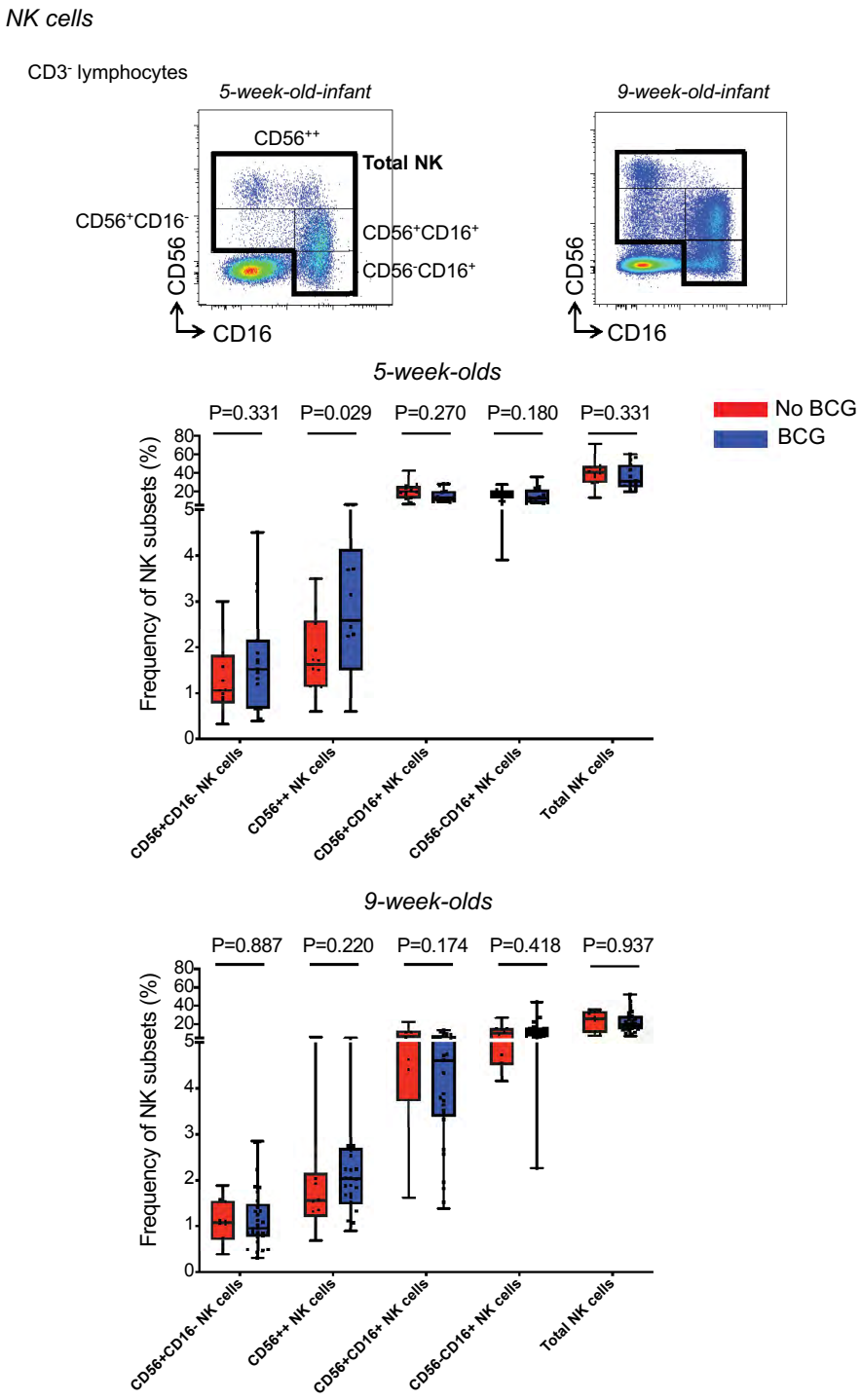


Figure 3. Proportions of NK subsets in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots depicting the gating of NK subsets in 5-week-old (left) and 9-week-old (right) infants based on CD56 and CD16 expression. **(B)** Pie charts showing proportions of NK subsets ($CD56^{+}CD16^{-}$, $CD56^{++}$, $CD56^{+}CD16^{+}$ and $CD56^{-}CD16^{+}$) among total NK cells. P-values were computed with a Permutation Test. **(C)** Box-and-whisker plots of proportions of NK cell subsets in 5-week-old (left) and 9-week-old (right) infants.

Horizontal lines represent medians, boxes represent the interquartile range and whiskers represent the range. P-values were calculated with the Mann-Whitney U test. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.0125, four comparisons).



Supplementary figure 2. Frequencies of NK cell subsets in CD3- lymphocytes in 5-week-old and 9-week-old infants. (Top panel) Representative flow cytometry plots depicting the gating of NK subsets in 5-week-old (left) and 9-week-old (right) infants based on CD56 and CD16 expression. Box-and-whisker plots of frequencies of NK cell subsets in 5-week-old

(middle panel) and 9-week-old (bottom panel) infants. Horizontal lines represent medians, boxes represent the interquartile range and the whiskers represent the range. The p values are calculated with the Mann-Whitney U test. Bolded p values are significant after correcting for multiple comparison testing using the Bonferroni method. The p values < 0.0125 are considered statistically significant (four comparisons).

NK cells are a major source of IFNγ in response to mycobacterial stimulation in infants (21, 22). Therefore, we measured the frequencies of BCG-reactive NK cells expressing IFNγ in the BCG-vaccinated and delayed BCG infant groups **(Fig. 3A and Fig. 4A)**. Among 5-week-old infants, frequencies of $CD56^{++}$ and $CD56^{-}CD16^{+}$ IFNγ-expressing NK cells were significantly higher in BCG vaccinated compared to delayed BCG (unvaccinated) infants **(Fig. 4B)**. Among 9-week-old infants, frequencies of all IFNγ-expressing NK subsets were significantly higher in the BCG-vaccinated compared to the delayed BCG group **(Fig. 4B)**, although frequencies of total IFNγ-expressing NK cells did not reach significance after adjustment for multiple testing.

NK cells also produce other pro-inflammatory cytokines in response to mycobacteria, such as TNF and IL-22 (41, 47, 48). We measured the frequencies of TNF-expressing NK cells in 5-week-old infants. The frequencies were very low overall and there were no differences between the two infant groups (not shown).

Overall, these results indicate that BCG vaccination does not modulate NK cell abundance in peripheral blood but leads to increased frequencies of IFNγ-producing NK cell subsets.

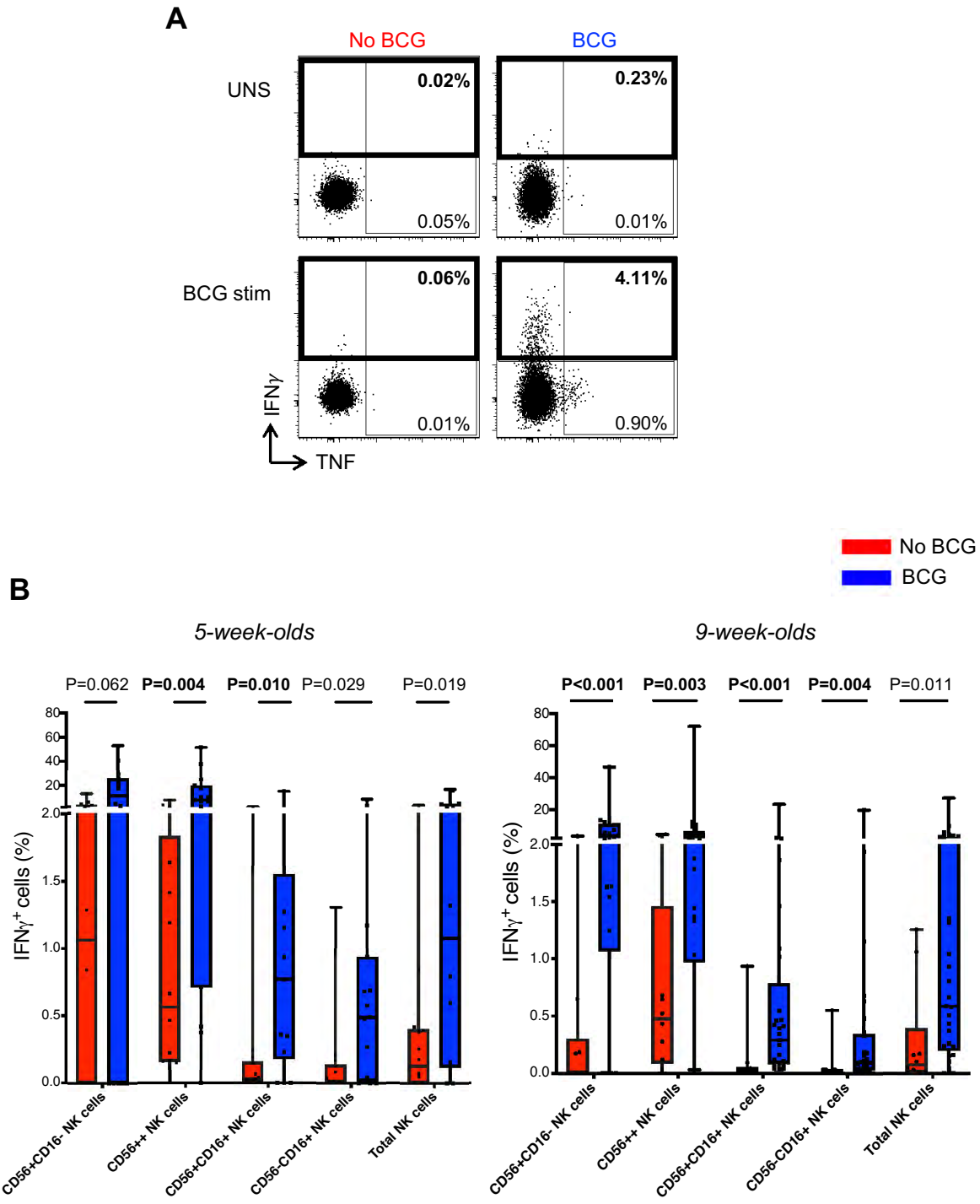


Figure 4. Frequencies of BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing TNF versus IFNγ expression in the total NK subset in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 5-week-old infant. **(B)** Frequencies of IFNγ-expressing NK subsets in 5-week-old (left) and 9-week-old (right) infants. Horizontal lines represent medians, boxes represent the interquartile range and the whiskers represent the range. P-values were calculated with the Mann-Whitney U test. Bolded p-values were significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.010, five comparisons).

Perforin expression by NK subsets is not modulated by BCG vaccination.

Cytotoxic activity is a major NK cell function and the $CD56^{bright}$ subset typically has less cytotoxic activity than the $CD56^{dim}$ $CD16^{+}$ subset, (49, 50). To determine if BCG vaccination influences the cytotoxic potential of NK cells, we measured perforin expression by NK cell subsets at 5 and 9 weeks of age **(Fig. 5)**. The majority of NK cells expressed high levels of perforin (median >80%), which clearly differed to the expected low perforin expression by $CD3^{+}$ T cells **(Fig. 5A)**. Neither proportions of perforin-expressing NK cell subsets (not shown) nor perforin MFI were different between the BCG vaccinated and delayed BCG groups in 5-week-old or 9-week-old infants **(Fig. 5B)**. As expected, the $CD56^{+}CD16^{+}$ and $CD56^{-}$ $CD16^{+}$ NK cell subsets had higher perforin expression than the $CD56^{+}CD16^{-}$ and $CD56^{++}$ NK subsets **(Fig. 5B)**. Interestingly, when we compared perforin expression between the two age groups, irrespective of vaccination status, 9-week-old infants had higher perforin expression than 5-week-old infants in the $CD56^{++}$, $CD56^{+}CD16^{+}$, $CD56^{-}CD16^{+}$ and total NK subsets **(Fig. 5C)**. Also, a sub-analysis in 9-week-olds revealed that expression of perforin was lower in IFNγ-expressing total NK cells, compared to bulk total NK cells in both infant groups **(Fig. 5D)**. In summary, our results show that NK cells in infants express high levels of perforin overall, which is not modulated by BCG vaccination.

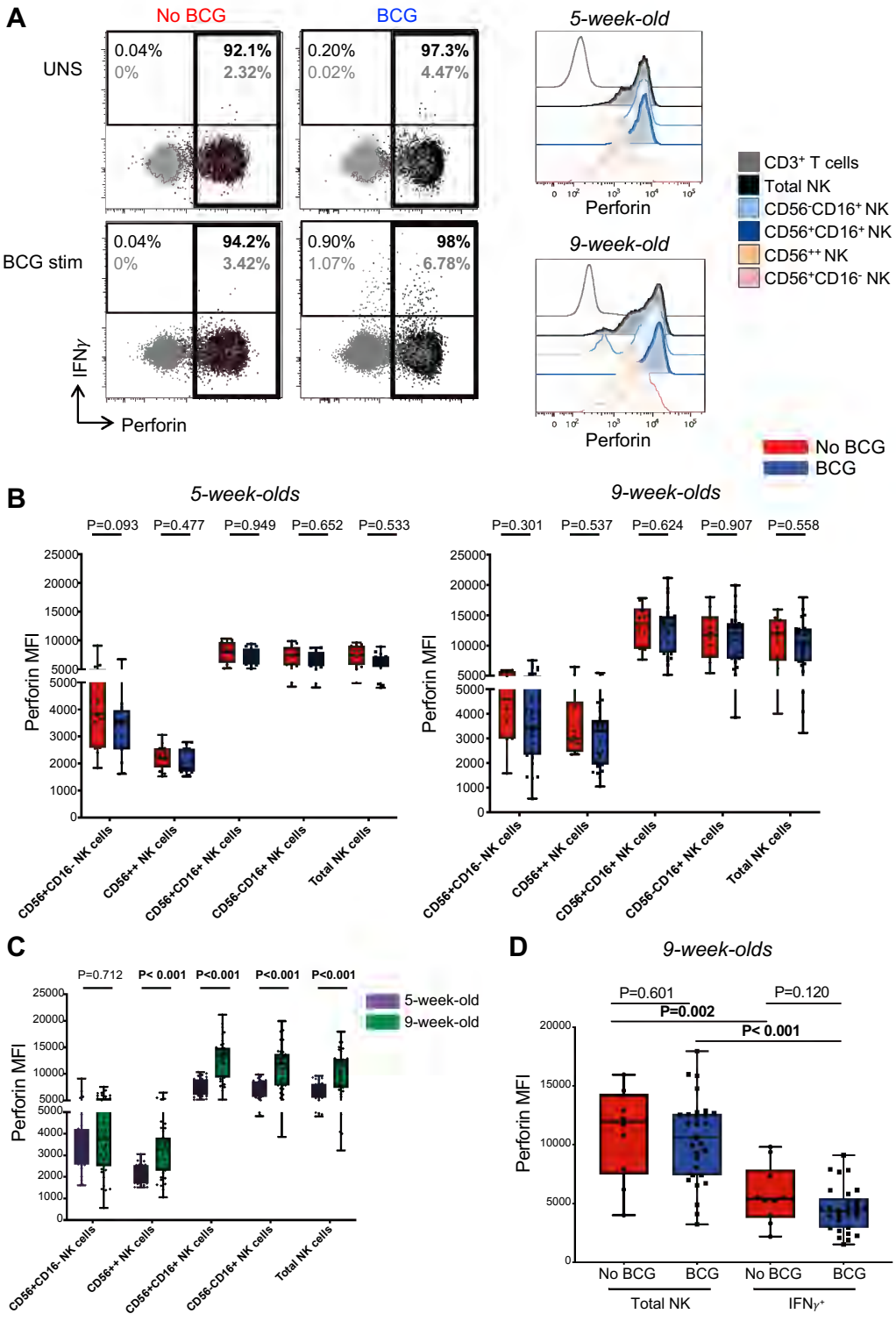


Figure 5. Perforin expression by NK subsets in 5-week-old and 9-week-old infants. (A) Representative flow cytometry plots showing perforin versus IFNγ expression in the total NK subset in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) 9-week-infant (left) and histograms showing relative expression of perforin in NK subsets ($CD56^+CD16^-$, $CD56^{++}$, $CD56^+CD16^+$, $CD56^-CD16^+$ and total NK) and $CD3^+$ T cells in a 5-week-old (top right) and 9-week-old (bottom right) infant. **(B)** Perforin median

fluorescence intensity (MFI) in NK subsets in 5-week-old (left) and 9-week-old (right) infants. **(C)** Perforin MFI in 5-week-old and 9-week-old infants irrespective of BCG vaccination status (BCG and no BCG groups combined). **(D)** Perforin MFI in bulk NK and IFNγ^+ total NK cells in 9-week-old infants. Horizontal lines represent medians, boxes represent the interquartile range and whiskers represent the range. P-values were calculated with the Mann-Whitney U or Wilcoxon signed-rank tests. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.010 in **B** and **C**, five comparisons; p values < 0.0125 in **D**, four comparisons).

Expression of NK cell differentiation markers is not modulated after BCG vaccination.

We next sought to determine if IFNγ responses by NK cells were associated with NK phenotypic and/ or differentiation marker expression. We measured CD57, CD158b, CD161 and CD8a expression on bulk cells and IFNγ^+ CD56^{hi}CD16^{lo} and CD56^{dim}CD16$^+$ NK cells **(Fig. 6)**, the two major NK subsets selected because they were sufficiently frequent to allow reliable combinatorial analysis **(Fig. 6A and B)**. No differences in expression of CD8, CD57, CD158b and CD161 on bulk or IFNγ^+ CD56^{dim}CD16$^+$ and CD56^{hi}CD16^{lo} NK cells between the BCG-vaccinated and delayed BCG groups at 5 weeks of age were observed **(Fig. 6C)**.

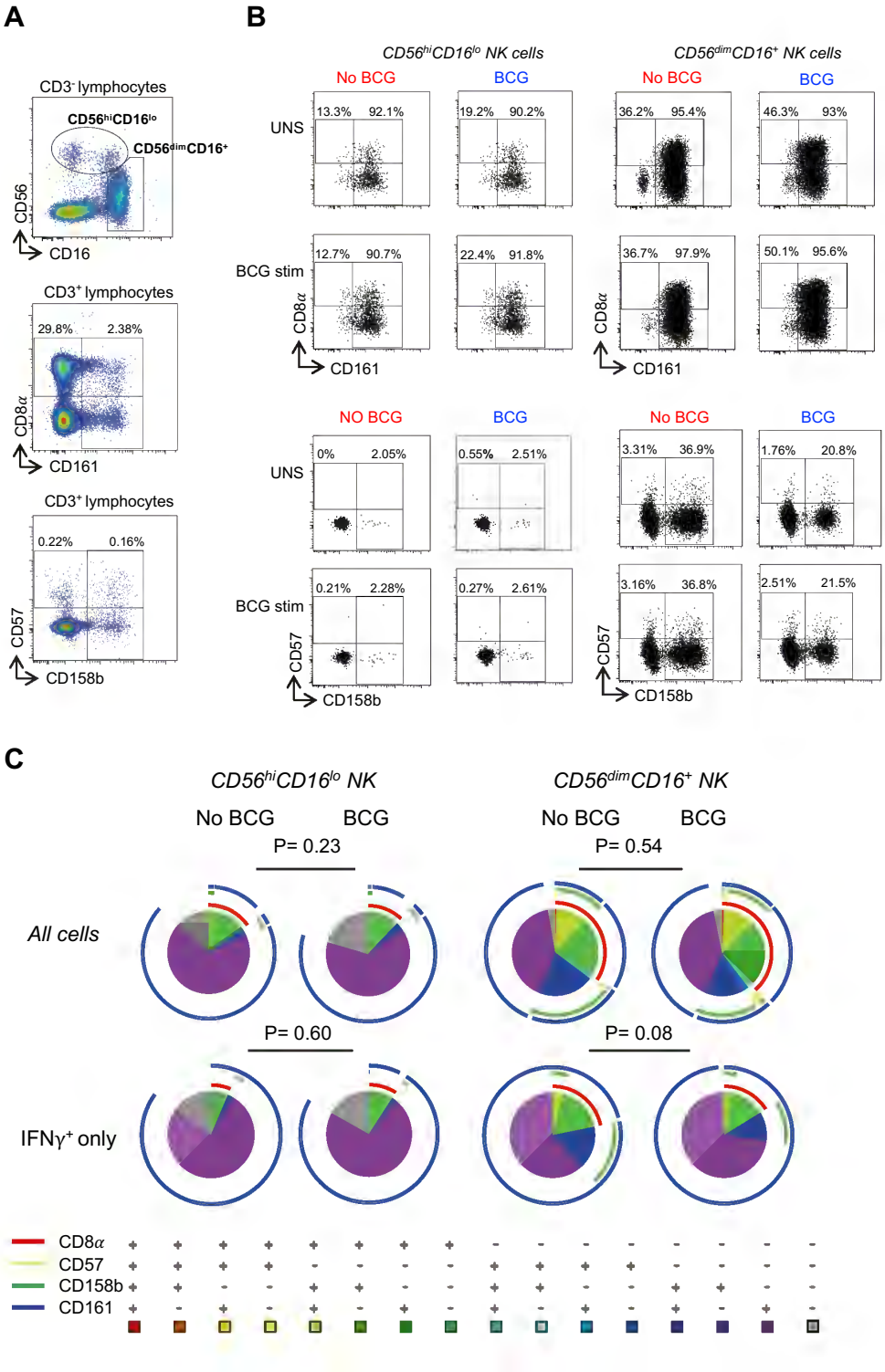


Figure 6. Expression of NK differentiation markers in 5-week-old infants. (A) Representative flow cytometry plots depicting the gating of NK subsets ($CD56^{hi}CD16^{lo}$ and $CD56^{dim}CD16^{+}$) based on CD56 and CD16 expression in a 5-week-old infant (top), CD8 versus CD161 in $CD3^{+}$ lymphocytes (middle) and CD57 versus CD158b in $CD3^{+}$ lymphocytes (bottom). **(B)** Representative flow cytometry plots showing CD8 versus CD161 expression in $CD56^{hi}CD16^{lo}$ NK (top left panel) and $CD56^{dim}CD16^{+}$ NK (top right panel) and CD158b versus CD57 in $CD56^{hi}CD16^{lo}$ NK (bottom left panel) and $CD56^{dim}CD16^{+}$ NK (bottom right panel) in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated

(BCG) infant. **(C)** Pie charts showing combinatorial expression of phenotypic markers (CD8, CD57, CD158b and CD161) as median proportions of $CD56^{hi}CD16^{lo}$ NK and $CD56^{dim}CD16^{+}$ NK cells. P-values < 0.05 were considered statistically significant.

Expression of cytokine receptors on NK cells is not modulated by BCG.

Since NK cell function can be modulated by cytokine stimulation, we also determined whether BCG vaccination leads to changes in cytokine receptor expression on NK cell subsets. Expression of IL-18Rα (CD218a), IL-2Rβ (CD122) and IL-12Rβ1 (CD212) were measured in 9-week-old infants **(Fig. 7A and B)**. As expected, the expression of CD218a was higher in the $CD56^{++}$ subset compared to the other NK subsets **(Fig. 7C)** (51, 52). CD218a, CD122 and CD212 expression were not different between the BCG vaccinated and delayed BCG groups for any of the NK subsets **(Fig. 7D)**. Interestingly, CD218a expression was higher in IFNγ-expressing total NK cells compared to bulk total NK cells in both infant groups **(Fig. 7E)**. CD122 expression was also higher in IFNγ-expressing total NK cells than bulk total NK cells, but only in the BCG-vaccinated group **(Fig. 7E)**. CD212 levels on NK subsets was low overall and indistinguishable between NK subsets **(Fig. 7C and 7D)** but was higher than total $CD3^{+}$ T cells **(Fig. 7C)**, confirming successful CD212 antibody staining. Overall, there were no differences in expression of key cytokine receptors between the two vaccine groups in 9-week-old infants.

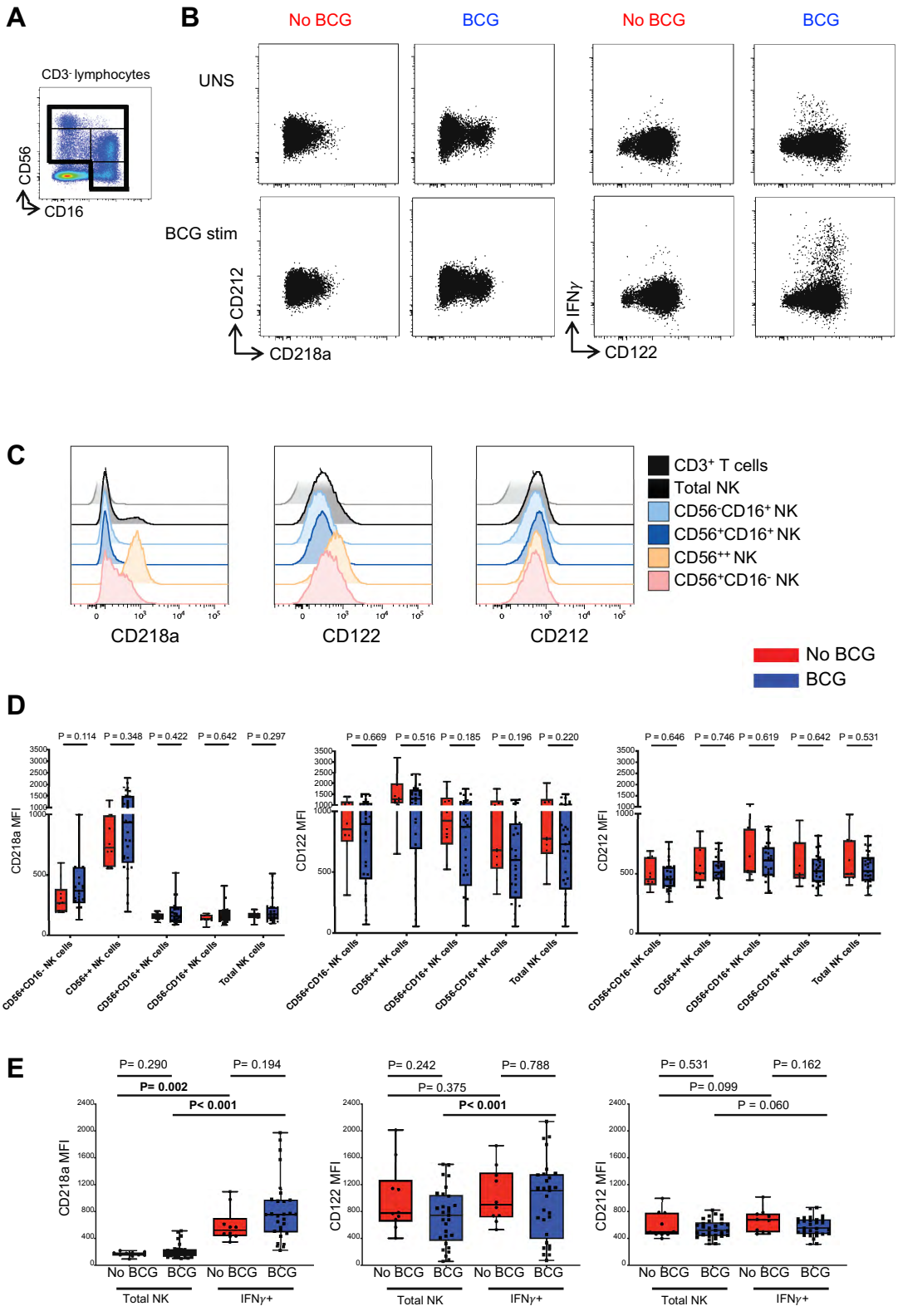


Figure 7. Cytokine receptor expression by NK cells in 9-week-old infants. (A) Representative flow cytometry plot depicting the gating of NK subsets based on CD56 and CD16 expression. **(B)** Representative flow cytometry plots showing CD212 versus CD218 (left panel) and IFNγ versus CD122 in the total NK subset in unstimulated or BCG-stimulated samples from an unvaccinated (no BCG) or vaccinated (BCG) infant. **(C)** Histograms showing relative expression of CD218a (left), CD122 (middle) and CD212 (right) in NK subsets and

$CD3^+$ T cells in a 9-week-old infant. **(D)** CD218 (left) CD122 (middle) and CD212 (right) median fluorescence intensity (MFI) in infant NK subsets. (**E)** CD218a (left) CD122 (middle) and CD212 (right) MFI in bulk NK and IFNγ^+ total NK cells. Horizontal lines represent medians, boxes represent the interquartile range and whiskers represent the range. P-values were calculated with the Mann-Whitney U or Wilcoxon signed-rank tests. Bolded p-values were considered significant after correcting for multiple comparison testing using the Bonferroni method (p values < 0.010 in **D**, five comparisons; p values < 0.0125 in **E**, four comparisons).

Host secreted Th1 cytokines are modulated by BCG vaccination.

We next sought to determine whether BCG vaccination modulates release of innate cytokines that could lead to downstream activation of other immune subsets. We measured levels of 18 secreted cytokines in BCG-stimulated (above levels in unstimulated blood, which were generally below the limit of detection) plasma samples from all study groups. Nine analytes were too low to be detected, including IL-12p70 (not shown). We initially analysed the 5-week-old and 9-week-old infants separately because the two groups not only differ in age but have also received different types and numbers of childhood vaccinations as part of the Extended Programme for Immunization **(Fig. 1)**. Since the same trends were detected for each analyte in the two age groups, and there were no age-related differences, we combined the data for the secreted cytokines from 5- and 9-week-old infants in the no BCG and BCG groups to maximise statistical power. As expected, IFNγ secretion was higher in infants who received BCG vaccination compared to those who did not receive BCG **(Table 1)**. There were no other differences between the BCG vaccinated and delayed BCG infant groups for the other analytes measured **(Table 1)**.

Table 1. Host secreted cytokine concentration in range values in vaccinated (no BCG) and vaccinated (BCG) infants.

Host secreted cytokines (pg/ml)	No BCG	BCG	*p*	Bonferroni *p*
	n= 23	n=43		
Higher in BCG-vaccinated infants Median (LQ-UQ)				
IFNγ	0.89 (0.89-2.95)	26.78 (0.89-89.68)	< 0.001	0.011
No statistically significant difference Median (LQ-UQ)				
IL-18	7.74 (0.89-20.38)	13.39 (0.89-58.10)	0.18	1
IL-2	51.8 (41.75-66.38)	71.86 (47.63-171.05)	0.020	0.36
IL-6	8231.72 (3250.70-10741.68)	9272.25 (3250.70-10741.70)	0.38	1
IL-1β	605.51 (336.64-1961.32)	737.84 (341.23-2347.61)	0.72	1
TNF	1442.90 (607.74-2753.98)	1409.54 (648.66-4322.26)	0.52	1
IL-10	52.58 (14.22-123.15)	40.31 (24.02-102.12)	0.76	1
GM-CSF	63.45 (19.94-151.15)	61.48 (0.89-194.23)	0.86	1
IL-5	28.79 (22.04-80.92)	28.84 (10.58 – 109.15)	0.84	1

Host secreted cytokines were measured on thawed plasma samples from whole blood incubated with BCG or medium for 7 hours (no BCG= 23: n=13 5-week-olds, n= 10 9-week-olds; BCG= 43: n=15 5-week-olds, n=28 9-week-olds) using the Bio Plex™ platform. Concentration in range values were used and values from unstimulated samples were subtracted from those values measured after BCG stimulation (BCG-UNS).
IL-12p70, IL-13, IL-17A, IL-21, IL-23, IL-4, IL-9, IL-22 and IL-27 were too low to be detected.
Cytokine values presented as median with interquartile range (IQR): median (IQR).
The p values are calculated with the Mann-Whitney U test and corrected for multiple comparisons with the Bonferroni method.

Host secreted cytokines positively correlate with BCG-reactive NK cells only in BCG vaccinated infants.

Next, we examined associations between BCG-reactive IFNγ^+ NK cells and levels of secreted cytokines. Interestingly, although there were no differences in secreted cytokine concentrations, except for IFNγ, between the infant groups **(Table 1 and Fig. 8)**, frequencies of BCG-reactive IFNγ^+ NK subsets correlates with secreted cytokines in the BCG vaccinated group, but not in the delayed BCG group **(Table 2 and Supplementary Table 3)**. In the BCG group only, IFNγ^+CD56$^+$CD16$^+$ NK cells positively correlated with all detectable analytes, **(Fig. 8, Table 2 and Supplementary Table 3)**, IFNγ^+CD56^{++} NK cells and IFNγ^+ total NK

cells positively correlated with all detectable analytes except IL-10, while IFNγ^{+}CD56^{+}CD16^{+} NK cells and IFNγ^{+}CD56^{-}CD16^{+} NK cells positively correlated with only some of the cytokines (**Table 2 and Supplementary Table 3**). Defining the four distinct NK subsets based on their expression of CD56 and CD16 showed that CD56 expression by BCG-reactive NK cell subsets was an indicator of their responsiveness (association with) to soluble secreted cytokines in the BCG-vaccinated infant group (**Table 2 and Supplementary Table 3**).

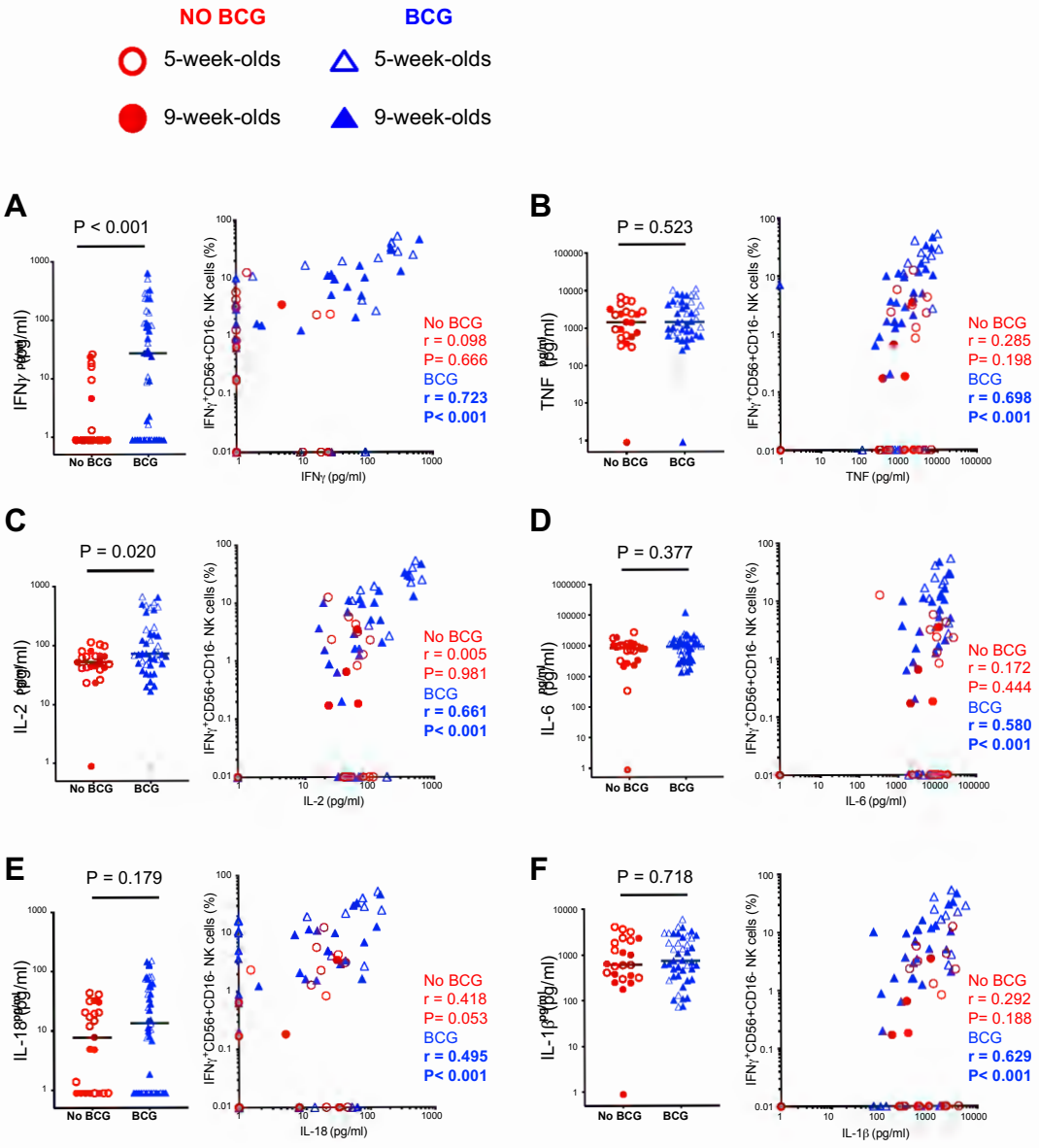
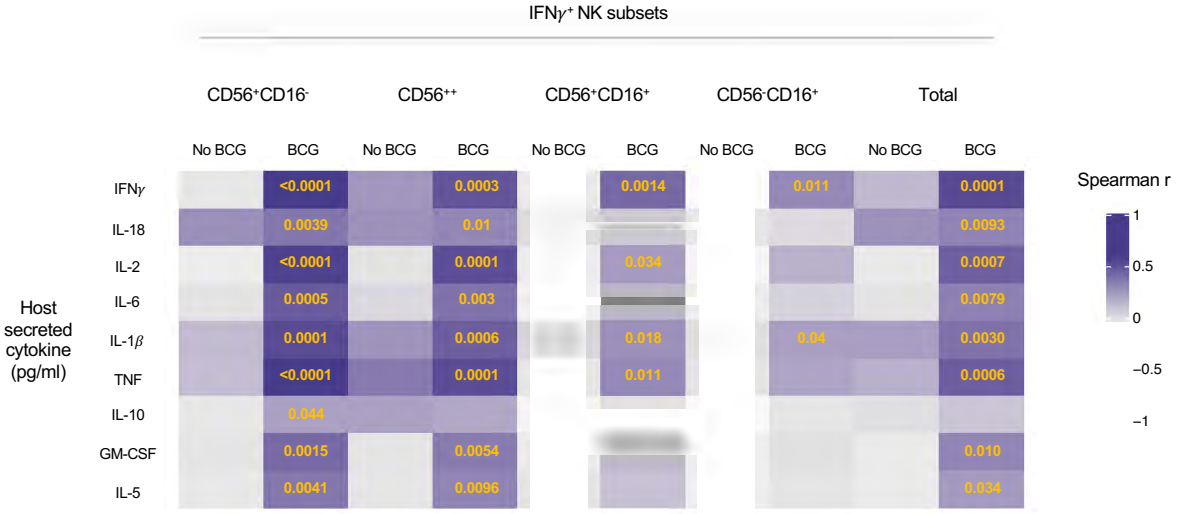


Figure 8. Host secreted cytokines correlate with CD56^{+}CD16^{-} NK cells in 5-week-old and 9-week-old infants. (**A**) IFNγ (**B**) TNF (**C**) IL-2 (**D**) IL-6 (**E**) IL-18, (**F**) IL-1β levels (left)

and correlations with BCG-reactive IFNγ+ CD56^{+}CD16^{-} NK cells (right) in unvaccinated (no BCG, red) and vaccinated (BCG, blue) infants. Comparisons between the delayed (no BCG) and BCG vaccinated groups were done using the Mann-Whitney U test. Correlation coefficients and respective p-values were calculated using the nonparametric Spearman Correlation test. Unadjusted p-values are reported (see Supplementary Table 3 for FDR-adjusted p values).

Table 2. Associations between host secreted cytokines and BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants.



Heat map showing Spearman correlation coefficients for host secreted cytokines and BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants (no BCG= 23: n=13 5-week-olds, n= 10 9-week-olds; BCG= 43: n=15 5-week-olds, n=28 9-week-olds). The coefficients with p values that were significant after correcting for multiple comparisons using the FDR method are shown.

Supplemental table 3. Spearman correlation coefficients and corresponding p values shown for host secreted cytokines and BCG-reactive IFNγ-expressing NK subsets in 5-week-old and 9-week-old infants (no BCG= 23: n=13 5-week-olds, n= 10 9-week-olds; BCG= 43: n=15 5-week-olds, n=28 9-week-olds). Correlation coefficients in bold indicate corresponding p values that were significant after correcting for multiple comparisons using the FDR method**.**

Host secreted cytokine	IFNγ^+CD56$^+$CD16$^-$ NK			
	No BCG		BCG	
	r	FDR p	r	FDR p
IFNγ	0.10	0.76	**0.73**	<0.0001
IL-18	0.42	0.12	**0.49**	0.0039
IL-2	0.0053	0.99	**0.66**	<0.0001
IL-6	0.17	0.59	**0.58**	0.0005
IL-1β	0.29	0.32	**0.63**	0.0001
TNF	0.29	0.33	**0.70**	<0.0001
IL-10	0.10	0.76	**0.37**	0.044
GM-CSF	0.16	0.61	**0.53**	0.0015
IL-5	0.076	0.83	**0.49**	0.0041
Host secreted cytokine	**IFNγ^+CD56^{++} NK**			
	No BCG		**BCG**	
	r	FDR p	r	FDR p
IFNγ	0.40	0.14	**0.60**	0.0003
IL-18	0.41	0.13	**0.44**	0.01
IL-2	0.18	0.58	**0.62**	0.0001
IL-6	0.21	0.52	**0.51**	0.003
IL-1β	0.41	0.13	**0.57**	0.0006
TNF	0.48	0.06	**0.62**	0.0001
IL-10	0.37	0.18	0.35	0.059
GM-CSF	0.16	0.61	**0.48**	0.0054
IL-5	0.14	0.66	**0.45**	0.0096
Host secreted cytokine	**IFNγ^+CD56$^+$CD16$^+$NK**			
	No BCG		**BCG**	
	r	FDR p	r	FDR p
IFNγ	0.064	0.84	**0.54**	0.0014
IL-18	0.20	0.54	0.25	0.20
IL-2	0.0023	0.99	**0.38**	0.034
IL-6	-0.11	0.74	0.31	0.11
IL-1β	0.22	0.50	**0.42**	0.018
TNF	0.12	0.70	**0.44**	0.011
IL-10	-0.17	0.60	0.19	0.38
GM-CSF	0.010	0.99	0.30	0.12
IL-5	0.16	0.61	0.27	0.16
Host secreted cytokine	**IFNγ^+CD56$^-$CD16$^+$ NK**			
	No BCG		**BCG**	
	r	FDR p	r	FDR p
IFNγ	-0.32	0.27	**0.44**	0.011
IL-18	0.12	0.70	0.20	0.32
IL-2	-0.18	0.57	0.32	0.095
IL-6	0.0057	0.99	0.23	0.27
IL-1β	0.19	0.55	**0.37**	0.04
TNF	0.15	0.62	0.36	0.053
IL-10	-0.25	0.41	0.14	0.54
GM-CSF	-0.049	0.88	0.19	0.37
IL-5	-0.069	0.83	0.15	0.50
Host secreted cytokine	**IFNγ^+ Total NK**			
	No BCG		**BCG**	
	r	FDR p	r	FDR p
IFNγ	0.30	0.32	**0.63**	0.0001
IL-18	0.41	0.13	**0.46**	0.0093
IL-2	0.069	0.83	**0.56**	0.0007
IL-6	0.18	0.58	**0.46**	0.0079
IL-1β	0.38	0.17	**0.51**	0.0030
TNF	0.32	0.27	**0.57**	0.0006
IL-10	0.19	0.55	0.26	0.17
GM-CSF	0.046	0.88	**0.45**	0.010
IL-5	0.045	0.88	**0.39**	0.034

Discussion

We investigated myeloid and NK cell immune responses to BCG vaccination in infants and found that, 1. frequencies of BCG-reactive IFNγ-expressing NK cells were higher in BCG-vaccinated compared to unvaccinated 5-week-old and 9-week-old infants; 2. BCG vaccination did not modulate expression of NK cell differentiation markers, perforin or cytokine receptors; 3. levels of secreted pro-inflammatory cytokines were not different between the infant groups but correlated directly with frequencies of BCG-reactive IFNγ-expressing NK cells only in BCG-vaccinated infants and, 4. functional responses of $CD33^{+}$ myeloid cells to in vitro BCG stimulation were not different between vaccinated and unvaccinated infants.

The finding that frequencies of BCG-reactive IFNγ-expressing NK cells were elevated by BCG vaccination in infants is consistent with our previous study of BCG revaccination in healthy, tuberculin skin test–positive South African adults (48). In this study, BCG revaccination boosted frequencies of IFNγ–expressing BCG-reactive $CD56^{++}$ NK cells, which remained elevated above pre-vaccination levels for at least one year. Other studies have reported increased IFNγ expression by NK cells after *in vitro* BCG stimulation (22, 23, 53-55). In addition, NK cells from BCG vaccinated individuals had enhanced function following stimulation with mycobacterial and unrelated stimuli (24, 41). Possible mechanisms underlying BCG-mediated induction of BCG-reactive IFNγ-expressing NK cells remain unknown but may include: 1. vaccination-induced NK cell maturation, augmenting IFNγ expression; 2. BCG-mediated upregulation of cytokine receptors on NK cells; 3. Increased sensitivity by NK cells to bystander activation by BCG-specific IL-2-expressing $CD4^{+}$ T cells and/ or pro-inflammatory cytokines expressed by innate cells, such as IL-12 and IL-18; or 4. direct training of NK cells through epigenetic modifications by BCG.

We measured perforin expression as a proxy for NK cell cytotoxic potential and show that NK cell subsets expressed high levels of perforin, which were not modulated by BCG vaccination. Frequencies of NK cells expressing perforin and granzyme B have previously been shown to be similar in cord and adult blood, indicating that NK cells are inherently highly cytotoxic and do not transition through a maturation or differentiation process during early childhood (56). Consistently, expression of NK cell differentiation markers were not modulated by BCG vaccination in 5-week-old infants, similarly to what we reported in BCG revaccinated adults (48).

Expression of the key cytokine receptors, CD122, CD218a and CD212, which may indicate protentional responsiveness of NK cells to cytokines, was not altered by BCG vaccination in 9-week-old infants. A study in mice infected by aerosol with *M.tb* reported unchanged expression of CD122 on NK cells in the lung (57), indicating that mycobacterial infection may not alter total cytokine receptor expression on NK cells. The observation that CD218a was more highly expressed on IFNγ^+ NK cells compared to bulk NK cells could indicate that cells with higher levels of CD218a were preferentially activated and produced IFNγ (28, 58). Interestingly, higher expression of CD122 on IFNγ^+ NK cells compared to bulk NK cells was observed in the BCG group only, suggesting a vaccine-specific response.

The finding of higher levels of soluble IFNγ in the supernatant of BCG-stimulated whole blood is consistent with the increased T and NK cell responses observed in BCG vaccinated infants. However, the lack of difference in the concentration of secreted IL-6, TNF and IL-1β levels between the BCG vaccinated and unvaccinated groups was unexpected, since other studies have reported enhanced production of these cytokines following BCG vaccination (35, 41). We also hypothesized that concentrations of IL-12 and IL-18, potent activators of NK cells (26-28), would be increased in the BCG group, resulting in stronger by-stander activation of NK

cells in vaccinated infants. This hypothesis is supported by studies showing that BCG vaccination induced trained immunity in monocytes, leading to enhanced functional responses including higher expression of cytokines, upon *in vitro* re-stimulation to mycobacteria and unrelated organisms (33, 35). However, it is important to note that these studies were mostly performed on purified monocytes isolated from adults in settings where TB is not endemic. It is plausible that baseline responses in non-endemic settings are lower, thus the magnitude of change that BCG can induce is higher and more easily detectable**.** In a study in UK infants, levels of secreted IL-6 and TNF after 48 hour stimulation of whole blood with *M.tb* lysate were higher in BCG-vaccinated infants compared to unvaccinated infants, while no differences were found for IL-1β (24). In a recent study conducted in Uganda, where BCG vaccination was associated with lower incidence of heterologous infectious diseases, the authors attempted to link these effects to trained immunity (45). They measured histone modifications at promoters of inflammatory cytokine genes in total PBMCs, as well as levels of secreted cytokines upon a 24-hour stimulation of whole blood with mycobacteria and unrelated organisms. Surprisingly, they did not find any changes in histone modifications at *IL-6* or *IL-1*β promoters and found a decrease in both activating and inhibitory histone modifications at the *TNF* promoter in vaccinated compared to unvaccinated infants, differing from previous results (35-37). In addition, there were no differences in levels of secreted TNF, IL-6, IL-1β, IL-10 or IFNγ in response to unrelated organisms in the BCG compared to the no BCG groups, however, differences were found in response to *M.tb* purified protein derivative (PPD) stimulation.

It is possible that the 7-hour stimulation time point at which supernatants were harvested in our WB-ICS assay protocol was suboptimal to detect differences in secreted cytokine levels. In other studies of delayed BCG vaccination in which cells were stimulated for 7 hours, few analytes, mainly Th1 and Th2 cytokines, were measured, showing that levels of secreted TNF (59) were higher in the BCG-vaccinated infants while IL-10 (60) was higher in BCG-

unvaccinated infants. Despite differences in study design and location, other studies in delayed BCG cohorts measuring heterologous effects in infants (with larger bead array panels and in which the duration of stimulations were 24 hours or longer) showed higher levels of some of the same innate and Th1 cytokines in vaccinated compared to unvaccinated infants after mycobacterial stimulations (24, 45, 61, 62). Although these technical differences may explain the discrepancies between our results and those published by others, we were mostly interested in myeloid-derived cytokine responses, which would peak at an earlier time-point than 24 or 48 hours.

A limitation of our study was that we did not include stimulations with other pathogens, which would allow a broader assessment of innate training. The small volume of blood that can be safely collected from infants for immunological studies did not allow additional time points or stimuli. Despite these limitations, levels of IL-2 secreted upon whole blood stimulation with BCG were strongly correlated with frequencies of BCG-reactive IFNγ-expressing NK cell subsets in the BCG group, but not in unvaccinated infants. This is in line with other studies that have reported an association between IL-2 expressing $CD4^+$ T cells and NK cell functional responses after vaccination (63-66). We also found strong positive correlations in vaccinated infants between BCG-reactive IFNγ-expressing NK cells and IL-6, IL-1β and TNF, hallmark innate cytokines of trained immunity (35). We speculate that, despite observing no differences between the BCG vaccinated and unvaccinated infants for these key cytokines, BCG vaccination does enhance crosstalk between innate and adaptive immune cells, resulting in strong correlations observed between BCG-reactive functional NK cells and the secreted cytokines. Our previous report that BCG revaccination of *M.tb* infected adults was associated with increased frequencies of BCG-reactive IFNγ^+ NK cells up to 1 year post vaccination, and that functional NK responses were dependent on IL-12 and IL-18 (48), supports this

hypothesis. However, in the present infant analyses, correlation between IL-18 and IFNγ-expressing NK cells was weak, and soluble IL-12 levels were not detected with the kit we used. Another limitation is the cross-sectional design of our study, which precluded paired comparisons before and after BCG administration.

Studies investigating innate immune responses in infants have largely focused on stimulation with purified TLR ligands (1, 3, 67-69). To our knowledge, this is the first study to investigate myeloid cell intracellular cytokine expression in response to whole cell mycobacterial stimulation in BCG vaccinated and unvaccinated infants from a TB-endemic setting. We did not observe a difference in the frequencies of cytokine-expressing myeloid cells in response to BCG in vaccinated compared to unvaccinated infants. It is possible that our study sample size was too small and therefore underpowered to detect small differences in cytokine production in the two groups of infants. Also, the high multiplicity of infection (MOI) of BCG used in the assay, which is a potent stimulus, could mask subtle differences that may have been detectable at sub-saturating doses of BCG. On the other hand, the stimulation time of 12 hours was probably too long to detect the peak myeloid response. Indeed, in previous studies conducted in our lab, it was found that 6-hour stimulation was optimal for intracellular detection of cytokine produced by myeloid cells (70).

Our data suggest that BCG vaccination induces an increase in frequencies of BCG-reactive IFNγ-expressing NK cells in infants and that, among the vaccinated infants only, the abundance of these cells correlated with levels of Th1 cytokines and innate pro-inflammatory cytokines. These data support the potential contributing role for BCG-induced NK responses in protective immune responses against mycobacterial and heterologous infections. Further studies are needed to better understand the mechanisms underlying BCG-mediated protection against heterologous infections in newborns, and its non-specific beneficial effects.

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