# Deep immunophenotyping of peripheral blood cells in individuals exposed to *Mycobacterium tuberculosis*

**Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester**

**By**

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## Abstract

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is a worldwide public health issue. To control TB, we need prognostic tests to discriminate subjects infected but unlikely to succumb to disease (LTBI) from subjects developing disease (subclinical TB). To address this, we proposed that immunophenotyping could group LTBI from subclinical TB. We reasoned that in a high incidence, low exposure setting such as Leicester we would have a population of people wherein the immune response reflected the state of infection rather than the state of recent exposure to Mtb antigens. We therefore locally recruited 46 subjects designated as healthy unexposed (HC), active TB patients (ATB) or people with evidence of exposure but no clinical symptoms (latent tuberculosis infection, LTBI). We hypothesized that we could separate HC from ATB and that LTBI would be variable, reflecting the broad nature of the LTBI grouping. We compared the frequencies of specific cell types in peripheral blood mononuclear cells both *ex vivo* and post antigen-specific stimulation between these groups. We also compared the expression of inflammatory cytokines in plasma from the subjects. We developed an ELISA to determine the level of a novel biomarker of active TB, ΔTM-IL-12Rβ1. We found some individual characteristics were different for the ATB group compared to the other groups with more regulatory and antigen-specific T cells and fewer naïve CD4 T and NK cells. Principal component analysis showed that HC and ATB subjects could be grouped, while LTBI subjects were spread over the HC and ATB grouping. We have established a set of markers that can be used to group HC and ATB, we can now propose a longer follow up study to see whether any combination of these markers is an indicator of progression from latent to active TB.

**Acknowledgments**

I would like first to thank my supervisors, Professor Andrea Cooper and Professor Manish Pareek for helping me throughout the course of my PhD. I would like to express my appreciation to Andrea who generously provided knowledge, expertise and guidance from the beginning of my research on 2019. Andrea provided her support from experimental design until my final analysis. Additionally, special thanks for Dr John Pearl who provides his expertise and opinions especially in statistics and flow cytometry maintenance during my work in the laboratory. I will never forget the support they provided and I will definitely stay in touch and happy to work with them in the future.

I would also thank Professor Manish Pareek and Dr Christopher Martin. Manish was the chief investigator in my clinical research and he contributed the protocol in ethics approval for my study. Special thanks to Chris who as co-investigator in conjunction with me in this research. Chris and I spent many happy days in the migrant clinic at Leicester royal infirmary to consent and recruit samples from for the study.

I would thank Dr Mrinal Das who as the laboratory postdoc. Mrinal designed ELISA model in conjunction with me, and together we measured the △TM-IL-12Rβ1 in plasma. Special thanks to Benjamin and Ganisha who are PhD candidates when I started my research for the training sessions, they provided to me at the beginning.

I would thank my family for the support they gave me for more than 6 years since I started my studies. Special thanks for my wife who shared journey since we get married on 2022. Special thanks to Husain, my best friend who encouraged me to complete my studies from the beginning. Finally, I would thank Kuwait government, civil service commission and ministry of health in Kuwait which sponsored my study.

**Covid statement**

My experimental programme was impacted by the covid pandemic which limited our cohort to three groups instead of the proposed 6. Because of the pandemic we couldn't gain access to the hospital and recruit more active TB patients, which resulted in the small size in this group. The limited access also meant that the recruiting process was limited to only one research site and there was a significant increase in patients attending their appointments remotely by phone.

**Declaration**

I declare that the work in this thesis titled "immunophenotyping of peripheral blood cells in individuals exposed to *Mycobacterium tuberculosis"* has been carried out by me in the department of Respiratory Science (Previously Infection, Immunity and Inflammation) at the University of Leicester. The information derived from the literature and journal articles has been acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree at this or any other institution.

**Contents**

# Chapter 1: Introduction

## 1.1 Overview of *Mycobacterium tuberculosis*

Tuberculosis (TB) is an airborne infectious disease caused by organisms of the *Mycobacterium tuberculosis* (Mtb) complex. TB remains a major worldwide concern, which results in life threating outcomes to human beings. According to the WHO, 10.6 million people developed active TB disease and 1.3 million died in 2022 (WHO, 2023). Despite Mtb being a pulmonary pathogen, it can cause disease in almost any part of the body from asymptomatic infection to life threating disease (Oliveira de Souza et al, 2019). Indeed, from a clinical and public health perspective, TB can present as a dynamic spectrum from latent through subclinical to active disease (Kumar et al., 2019; O'Shea et al., 2018). TB patients are currently classified as having either latent tuberculosis infection (LTBI) or active TB. LTBI is currently diagnosed by immunological signs of exposure to Mtb without clinical symptoms (Barry et al., 2009; Esmail et al., 2014). There are two tests for the identification of LTBI, the tuberculin skin test (TST) and the interferon gamma release assays (IGRA) (Barry et al., 2009; Esmail et al., 2014). The major issue with these tests is that they do not identify which individuals with LTBI are at increased risk for the development of active TB. Importantly, identification of those individuals most at risk would allow much more focused and effective treatment for LTBI worldwide.

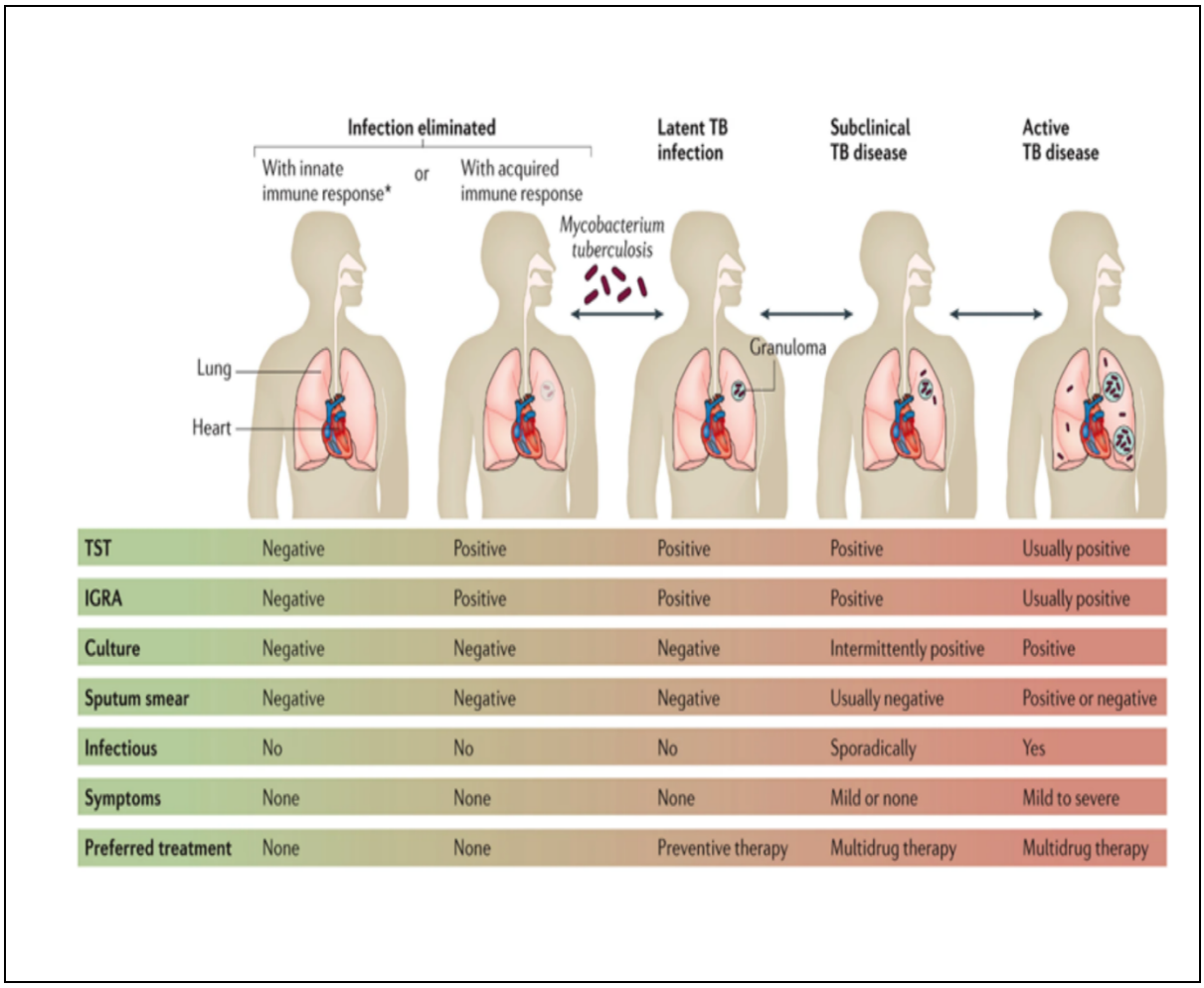
The TSTs and IGRAs are dependent upon an adaptive immune response to Mtb antigens and are thought to be mediated by memory T cells (Pai et al., 2016). Heavily exposed individuals who fail to generate a positive TST or IGRA have been hypothesized to have a strong innate response, which clears Mtb infection without the aid of the adaptive immune response (Pai et al., 2016). Even when an adaptive immune response has been induced, the Mtb is most likely to be in a persistent and non-growing state which means the infection is latent (LTBI) and individuals do not show major symptoms, but have a positive TST or IGRA (Pai et al., 2016). Active TB (ATB), in contrast, is associated with clinical symptoms such as fever, fatigue, cough with blood, weakness or weight loss (Pai et al., 2016). Active TB can be diagnosed by a combination of IGRA test, symptoms, CT scan, X ray, culture based and molecular tests (Pai et al., 2016). There is a growing appreciation for the presence of subclinical patients that are positive for some signs of disease and may be transmitting infection but who remain asymptomatic (Pai et al., 2016).

## 1.2 Host pathogen interaction

### 1.2.1 The spectrum of TB and why we need to understand it

Patients with TB classically have been categorised in two forms, latent tuberculosis infection (LTBI) and active TB disease. O'Garra et al (2013) and Pai et al (2016) have suggested and described a spectrum of disease. Individuals who progress rapidly from early infection with Mtb to active disease are referred to as "primary or primary-progressive TB" whereas those individuals who contain the initial infection are referred to as latently infected; these individuals have "5-10% lifetime risk of developing active TB" (O'Garra et al., 2013). Barry et al (2009) defined LTBI by reactivity to mycobacterial proteins delivered through the tuberculin skin test (TST) or as purified protein derivative (PPD) in the absence of clinical signs and symptoms of active TB disease. Pai and colleagues (Pai et al., 2016) proposed that based on the efficacy of host immunity, either innate responses or acquired T cells responses, patients can progress or reverse within the spectrum of TB (figure 1.1). Indeed, if the pathogen has been eliminated either by innate or adaptive immune responses, the TST and IGRA test could be negative as the memory T cell response may have been lost. Subjects who have been exposed but do not have a sustained TST or IGRA response may then not receive LTBI treatment (figure 1.1). On the other hand, latent TB infected patients who do not report clinical symptoms but for whom both TST and IGRA results are positive, will receive a recommended preventive therapy regiment of rifampicin and isoniazid for 3 months or, if there are other concerns, isoniazid alone for 6 months in many countries (figure 1.1). Subclinical TB disease patients who have reported mild or no clinical symptoms but have confirmed TST, IGRA and culture will receive treatment for active TB disease worldwide (figure 1.1). Active TB disease patients who report clinical symptoms and have confirmed culture/smear results will receive treatment for active TB disease (figure 1.1). Altogether, full understanding of the spectrum of TB is essential to 1) improve diagnosis, 2) to decide which individuals will benefit from treatment and who will not and 3) to provide accurate and sensitive diagnostic and prognostic tests. The complexity of the immune response to Mtb and the inability of this complexity to be represented in a skin test or cytokine release assay, limits the capacity of the current diagnostics to provide insight into the spectrum of TB disease. To

overcome these limitations, we proposed that a detailed analysis of the immune response to Mtb linked to clinical outcome can help in refining diagnostic tools.




| | | | | | |
|---|---|---|---|---|---|
| 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 1.1. The spectrum of TB.** Dynamic spectrum from Mtb infection to active TB disease (Pai et al., 2016. Used with permission, License number 5587700162336)

### 1.2.2 The route of the infection and immune response

Mtb infection is characterized by the formation of granulomas, usually in the lungs and lymph nodes. When Mtb enters the lung via inhalation, the infection begins. Following inhalation, Mtb is translocated to the lower respiratory tract (Pai et al, 2016). During the infection, Mtb encounters the alveolar macrophages, which are the dominant cells that the pathogen infects (Torrelles and Schlesinger, 2017). These cells contribute the internalization of the pathogen by receptor mediated phagocytosis (Pai et al., 2016). After internalization, the fusion of phagosome with the lysosome is blocked by the bacteria for its survival (Conrad et al., 2017).

The importance of this blocking is highlighted by the fact that Mtb activates the ESX-1 secretion system which disrupts the phagosomal membrane and mediates host cell lysis leading to the release of the bacterial products into the cytoplasm of the macrophage (Conrad et al., 2017). Next, Mtb invades lung tissues, either directly by infecting alveolar epithelium, or by the migration of infected alveolar macrophages to the lung parenchyma (Russell, 2011). The infected dendritic cells or inflammatory monocytes migrate to draining lymph nodes. MHC class II is expressed on the surface of antigen presenting cell with the antigenic epitope within the peptide cleft, the TCR of the T cell then recognises the epitope in the context of the MHC and the T cell becomes primed (Orme et al., 2015). The initiation of inflammation locally at the site of infection followed by the activation of T cells in the lymph node and their recruitment back to the site of infection leads to the formation of a granuloma in the lung parenchyma (figure 1.2).

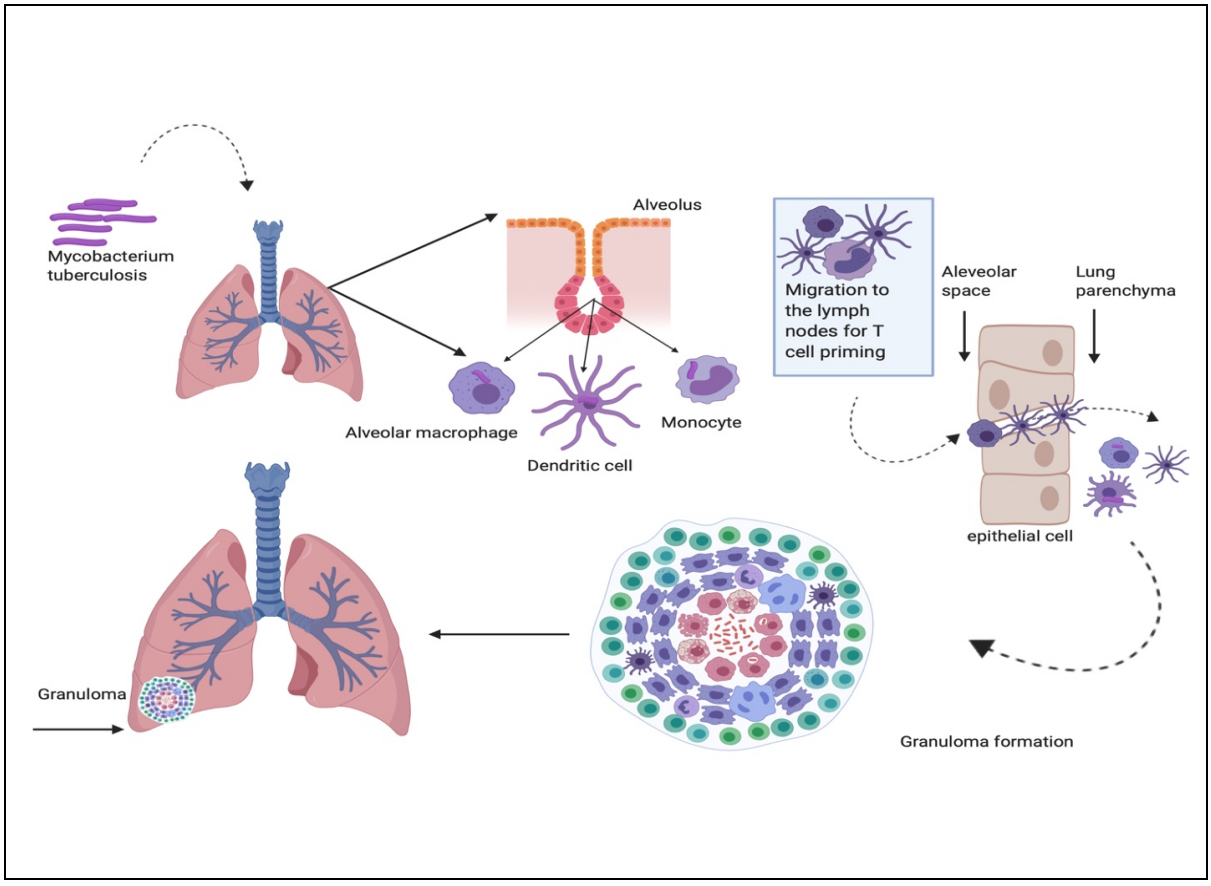



**Figure 1.2. the route of *Mycobacterium tuberculosis* infection.** Mtb enters the lung via inhalation and reach the alveolar space to encounter the macrophages. Then, the infected macrophages and dendritic cells migrate to draining lymph node. T cells become primed and MHC class II expressed on dendritic cells. All immune cells including dendritic, monocyte, neutrophils, multinucleated giant cells, natural killer, T and B cells form the Granuloma. When the granuloma fails to contain the infection, the bacteria enter the bloodstream and disseminate. (Created on Biorender, 2019)

The granuloma is defined as an inflammatory mononuclear cell infiltrate that the bacterial replicate within. The tuberculosis granuloma is an “organized structure of immune cells” that forms in response to Mtb infection, and consists of macrophages, dendritic cells, epithelioid macrophages, multinucleated giant cells, neutrophils, and lymphocytes (Pagan and Ramakrishnan, 2018). If the granuloma fails to contain the infection, the dissemination of bacteria will occur to other organs. If the granuloma grows in size and fails to contain the infection then primary TB and dissemination from the primary site of infection occur (Torrelles and Schlesinger, 2017). Another outcome that contributes to bacterial dissemination is the migration of infected cells into other organs specifically this was shown when a granuloma from an infected mouse was transplanted to a new host, this granuloma released Mtb infected cells expressing CD11c into the host (Schreiber et al., 2011). Davis and Ramakrishnan (2009) also showed that in *Mycobacterium marinum* infected zebrafish embryos Mtb infected macrophages allowed expansion of bacterial growth and expansion into multiple macrophages suggesting that the uninfected macrophages were recruited and were then susceptible to infection.

### 1.2.3 Mtb secretory proteins and how the system works?

#### 1.2.3.a Why is the antigen-specific immune response complex?

According to Cecilia Lindestam Arlehamn et al (2016), human pathogen-specific immune responses are complex, and classified into three factors. The first factor is due to CD4 T cells differentiation into different Th subsets based on the expression and secretion of chemokines receptors and effector cytokines. The second factor is the level of TCR-antigen-MHC restriction. The third factor is the level of antigen and epitope specificity. Lindestam Arlehamn et al (2016) provided deep characterisation of Mtb antigen epitopes recognised within the response-restricting HLA class II molecules in a cohort of Mtb infected population in South Africa. They proposed an alternative approach to characterise global Mtb-specific T cell responses. They reported that pooling of many peptides into megapools may be a more practical approach for response characterisation. They have generated peptide pools of 125 epitopes, 66 epitopes and 300 epitopes of Mtb-derived cell epitopes that have the potential

to stimulate Mtb-specific CD4+ and CD8+ T cells. They then stimulated PBMCs with each of these three pools as well as heat killed Mtb lysate. They found that the frequencies of CD4 T cells producing cytokines in any of three pools were equal to the frequency responding to Mtb lysate in 34 selected donors. Then they measured the Mtb specific CD4+ T cell responses in PBMCs in IGRA positive cohort and a TST+ cohort and found that the response to the 66, 125 and 300 pools supported the ability of peptide pools to detect Mtb specific T cell responses by intracellular staining (ICS). The peptides pools did not stimulate responses in IGRA or TST negative control groups. They expected that these megapools would have "wide applicability irrespective of ethnicity and geographical location".

**1.2.3.b What is MTB300?**

MTB300 is a peptide pool of 300 Mtb epitopes representing most of the HLA restriction worldwide. Mtb peptides captured a large fraction of the Mtb-specific T cells and can be used to characterize this response. The pool includes the known antigens of Mtb. Peptides in MTB300 megapools contain antigens such as culture filtrate protein 10 (CFP10, Rv3874, cell wall and cell process), secretory antigenic target protein 6 (ESAT-6, Rv3875, cell wall and cell process) and the proline-proline-glutamate (PPE protein, Rv3873), TB10.4 (Rv0288 cell wall and cell process), Ag85B protein (Rv1886c, lipid metabolism), Ag85A protein (Rv3084c, lipid metabolism), the Mtb membrane-associated heat-shock protein alpha crystallin (ACR/Rv2031c/HspX, virulence, detoxification and adaptation) and more (Lindestam Arlehamm et al; 2016). MTB300 is 300 epitopes of Mtb-derived cell epitopes that have the potential to stimulate Mtb-specific CD4+ and CD8+ T cells. It has been described in Lindestam Arlehamm et al (2016) that megapools of peptides for antigen stimulation assays might be a "more practical approach for response characterization". They reported that these peptide pools included MTB300 "can be used to detect MTB specific T cell responses by both ELISpot and ICS assays".

### 1.2.3.c Mtb antigens/peptides and what they do

Mtb is a facultative intracellular pathogen and survives within the host macrophages with the alveolar macrophage providing a critical niche for the bacteria (Guo et al., 2012). In general, macrophages provide host defence to most bacterial infection through exerting anti-bacterial activity. On the other hand, some intracellular bacteria can survive within macrophages. Sly et al. (2003) state that Mtb survives and replicate inside host macrophages and does this by modulating the host immune response. This balance of the interaction between the bacteria and the macrophages is a key element determining pathogenesis and virulence of the Mtb. Mtb resists phagosome delivery, disrupts macrophages by cell lysis and survive intracellularly (Tan et al., 2006).

Mtb secreted antigens have an essential role in mediating the interaction between the host and the bacteria. Genomic analysis has revealed that all virulent members of *Mycobacterium tuberculosis* complex including Mtb and *Mycobacterium bovis* have the region of difference RD1 (Guinn et al., 2004). However, all strains of Bacillus Calmette-Guerin lack RD1. According to Stanley et al. (2003), two secretory proteins encoded by RD1 genes are culture filtrate protein 10 (CFP10) and early secretory antigenic target protein 6 (ESAT-6) encoded by the RD1 genes Rv3874 and Rv3875 respectively. Both CFP10 and ESAT6 are essential in Mtb virulence. It has been shown that the expression of these two secretory antigens increases the virulence and immunogenicity of Mtb (Wards et al., 2000). On the contrary, the deletion of the RD1 genes results in the reduction of virulence (Pathak et al., 2007). Furthermore, it has been shown that the ESAT-6/CFP10 complex inhibits the production of cytokines IL-12 and tumor necrosis factor alpha (TNF-$\alpha$) from macrophages, which compromises the innate immune response (Wards et al., 2000). Also, several studies in mouse models of infection show that Mtb lacking RD1 have impaired growth of Mtb in macrophages (Pathak et al., 2007). These studies demonstrate that the secretion of ESAT-6 and CFP-10 is required for RD1-mediated virulence supporting the role of these secretary protein in bacterial intracellular survival within the host.

TB10.4 is a Mtb antigen encoded by Rv0288 gene and located on the esx cluster 3 that is essential for the virulence of Mtb (Hervas-Stubbs et al., 2006). It has been shown that the

expression of Rv0288 is down regulated in avirulent H37Ra strain compared to the expression of this gene in the virulent H37Rv strain (Rindi et al., 1999; Hervas-Stubbs et al., 2006).

In addition to previous, all pathogenic mycobacterial members express Ag85B protein (Wahid et al., 2019). This antigen induces the proliferation of Th cells and the production of Th1 cytokines (Tsujimura et al., 2014). Also, the production of Th17 cytokines IL-17 and IL-22 are induced by Ag85B in mouse model during lung inflammation (Aghababa et al., 2011). Another study found that this antigen stimulates the production of interferon gamma IFN-$\gamma$ and activates lymphocyte proliferation (Wahid et al., 2019). Furthermore, Ag85B secreted protein has major role in cell wall synthesis in combination with Ag85A and Ag85C (Wahid et al., 2019; Tsujimura et al., 2014).

Multiple factors evolved in Mtb to adapt environmental and physiological stress within the host including acidic pH, reactive oxygen, and hypoxia. Therefore, Sun et al. (2017) stated that the hypoxic response protein 1 HRP1 is another protein, which is encoded by Rv2626c and play a role to maintain the survival of mycobacteria induced by hypoxia or NO stress. Also, The Mtb membrane-associated heat-shock protein alpha crystallin (ACR/Rv2031c/HspX) is believed to sustain the bacilli during latent or dormant phase of infection (Demissie et al., 2006). ACR is a potential biomarker for LTBI. Indeed, it is a strong inducer of both T and B cell responses among most immunogenic proteins of Mtb (Geluk et al., 2007; Demissie et al., 2006; Kumar et al., 2020). Demissie et al (2006) investigated the immune response to EAST-6 and RV2031c (Acr/HspX) in clinical cohorts in Africa. They assessed the IFN$\gamma$ responses by ELISA and ELISpot. In an Ethiopian cohort they found that all the three groups (healthy, LTBI and ATB) contained a wide range of ESAT-6-responsive individuals. However, the median responses to RV2031c were significantly higher in LTBI compared to the other groups. Moreover, they reported that in a Gambian cohort the ESAT-6 responses were again not significantly different, but the response to RV2031c was significantly higher in the LTBI group. Geluk et al analyzed the RV2031c response by IFN$\gamma$ ELISpot assays on PBMCs from TB patients, TST positive individuals, BCG-vaccinated individuals, non-BCG vaccinated and healthy control. BCG is the bacille Calmette-Guerin vaccine, which is originally developed from *Mycobacterium bovis* by Calmette and Guerin at the beginning of the last century. It was attenuated by repeated *in vitro* culture and is one of the most widely used vaccine worldwide. They

compared the number of IFNγ producing T cells responding to RV2031c to those responding to secreted Ag85B and found that there were more RV2031c specific IFNγ producing T cells in TB, TST positive and vaccinated BCG individuals. As for the study by Demissie on ESAT-6, this group found that the response to Ag85B was not significantly different between the groups.

To sum up, virulent mycobacterial species secrete these proteins that modulate the behaviour of the macrophage and allow the bacteria to live within it. Table 1.1 shows the critical roles of some major secretory protein in the physiology and pathogenesis of Mtb during the infection**.**

**Table 1.1. a summary description for some Mtb secretory proteins showing the genes encoding the protein and their function**

| Mtb secretory antigen | Description | Application for investigation |
|---|---|---|
| ACR<br>(Rv2031c) | -Shifting of the bacilli from growth to a non-replicating persistent state.<br>-Heat shock protein | 1-Antigen specific T cell stimulation<br>2- T cells assay<br>3- Immune monitoring |
| Ag85B | -Th cells proliferation<br>- Induce cytokines production<br>- Cell wall synthesis | |
| CFP-10<br>(Rv3874) | -Enhance cell surface binding<br>-Modulate host cell<br>-Intracellular survival<br>-Pathogenicity of Mtb | |
| ESAT-6<br>(Rv3875) | -Enhance cell surface binding<br>-Modulate host cell<br>-Intracellular survival<br>-pathogenicity of Mtb | |
| HRP1<br>(Rv2626c) | -adaptation to environmental and physiological stress<br>Mycobacterial survival | |
| Rv1733c | -Membrane protein | |
| TB10.4<br>(Rv0288) | - *Mycobacterium tuberculosis* survival and plays a vital role in mycobacterial pathogenesis<br>- Triggers immune response | |

### 1.3 Role of T cells, monocytes and Dendritic cells

T cells are a type of white blood cell that expresses a unique receptor able to recognise diverse antigens from pathogens, or cancer cells, for clearance. T lymphocytes originate from hematopoietic stem cells in the bone marrow and migrate to the thymus for maturation (Golubovskaya and Wu, 2016). T and B lymphocytes arise from common precursor that becomes committed to the lymphocyte's lineage. The common lymphoid progenitor cell arises from stem cells in the bone marrow and travels through the blood to the thymus for T cells maturation. Cell surface molecules for identifying thymocyte subpopulations are CD4, CD8 and the type of T cell receptor complex (TCR). The earliest cell population in the thymus does not express CD4 or CD8 and is called double negative thymocyte. These cells give rise to two T cell lineages, one is $\gamma\delta$ T cells and one is $\alpha\beta$ T cells. In ontogeny, the production of $\gamma\delta$ T cells predominates over $\alpha\beta$ T cells, but later the majority (more than 90%) of thymocytes express $\alpha\beta$ T cells. In the thymic cortex, the progenitors of αβ T cells begin to express TCRs and CD4 and CD8 coreceptors (double positive thymocyte). These cells interact with self MHC molecules and express either CD4 or CD8 and increase the expression of TCR called single positive thymocyte. After maturation they leave thymus as single positive CD4 or CD8 (Janeway et al., 2001).

In fetal life, precursors of the common myeloid cells arise in the yolk sac, while in adults the precursors arise in the bone marrow given a population of resident and circulating monocytes (Abbas et al., 2015). Based on the stages of macrophages differentiation, human monocytes can be subdivided into three subsets (Passlick et al., 1989). The first subset is the classical monocyte that expresses CD14+ CD16- and represents most of human monocytes that differentiate into M1 (classical activation) macrophages producing pro inflammatory cytokines (Passlick et al., 1989; Wong et al., 2012; Ziegler-Heitbrock et al., 2010). The second subset is non-classical monocytes that express CD14- CD16+ and differentiate into M2 (alternative activation) macrophages (Passlick et al., 1989; Wong et al., 2012; Ziegler-Heitbrock et al., 2010). The third subset is intermediate monocytes that express CD14+ CD16+ and differentiate into M2 macrophages (Passlick et al., 1989; Wong et al., 2012; Ziegler-Heitbrock et al., 2010).

Dendritic cells arise from common precursor cells of the myeloid lineage in the bone marrow and further differentiate into distinct subsets (Abbas et al., 2015). Human DCs contains two functionally separate subpopulations: myeloid (mDCs) and plasmacytoid (pDCs) (O'Doherty et al., 1994; MacDonald et al., 2002). Human pDCs express the cell surface marker CD123 and are characterized by low production of human MHC class I and class II molecule, and high surface expression of Toll-like (TLR) receptors and Type 1 interferons (IFNs) (Chistiakov et al., 2015). Human pDCs have antibacterial and antiviral capacity. Indeed, upon TLR-7 and TLR-9 stimulation results in the production of IFNs which promote wide range of effects such as triggering the natural killer cells (NKs) to produce IFN$\gamma$ (Mckenna et al., 2005). On the other hand, Human mDCs express cell surface markers CD1c and CD141 and they produce a variety of TLR receptors and cytokines (CD141 secretes IL-12 and Type 1 IFNs whereas CD1c secretes TNF-$\alpha$, IL-6, IL-8 and IL-12) (Chistiakov et al., 2015). Figures 1.3 and 1.4 show the key cells of the immune system and the molecules expressed on their surface (which we have used in the subsequent chapters).

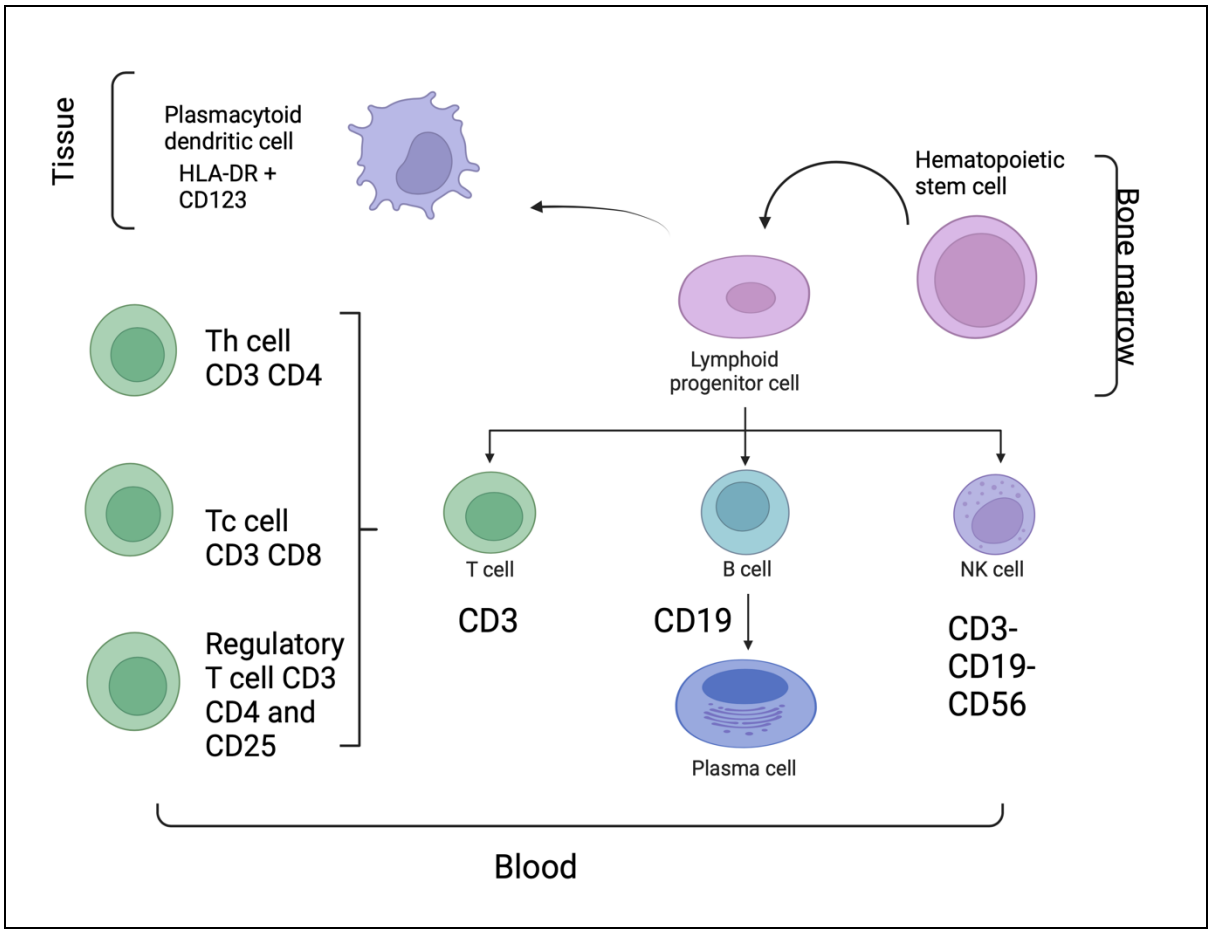



**Figure 1.3. Maturation of lymphocytes.** Precursors of lymphoid progenitor cells migrate from the bone marrow stem cells into the thymus for T cells maturation and into bone marrow for B cells maturation, and then circulate to secondary lymphoid organs. The T cells express either CD4+ T helper lymphocytes or CD8+ cytotoxic T cells and CD4+ regulatory T cells on their surface. Natural killer cells develop and mature in bone marrow and secondary lymphoid tissues (Created on Biorender, 2022).

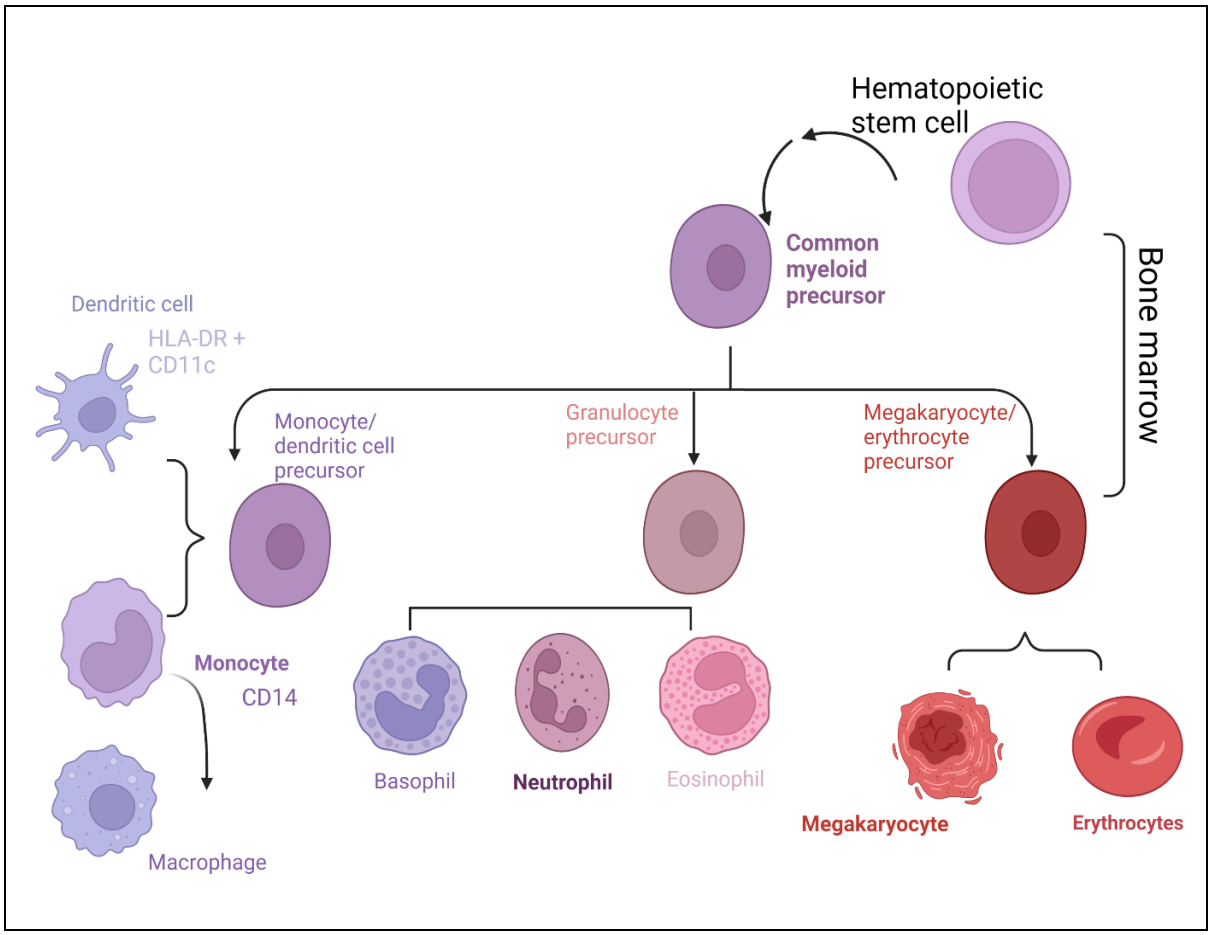



**Figure 1.4. Myeloid linage cells.** In bone marrow, a common precursor cell of the myeloid lineage of hematopoietic cells that can differentiate into monocytes/dendritic cells or Granulocyte precursor that differentiated into neutrophil, basophil or eosinophil cells, or megakaryocyte/erythrocyte precursor differentiated into platelets or erythrocytes respectively.

### 1.3.1 Immunophenotyping

Immunophenotyping is the process of analysing a heterogeneous population of cells for immune function. The role of this analysis is to identify the presence of populations of interest. Early in the 1980s, immunophenotyping of lymphocytes became a diagnostic tool of immunologic disorders such as auto immune disease (Comans-Bitter et al., 1996). To achieve a full characterisation from the peripheral blood, flow cytometry is a critical tool to identify immune cells based on their phenotype. This technology is widely used to characterise different cells type based on the expression of cell surface markers and intracellular molecules. Figure 1.5 represents an example of flow cytometry showing a graph explaining

how to classify the CD3+ population into T helper cells and T cytotoxic cells. We used this tool to determine the frequency and the phenotype of lymphocyte, monocyte and dendritic cells subset for individuals exposed to pathogens, and to measure the antigen induced CD4+ cytokine release and expression of activation induced markers.

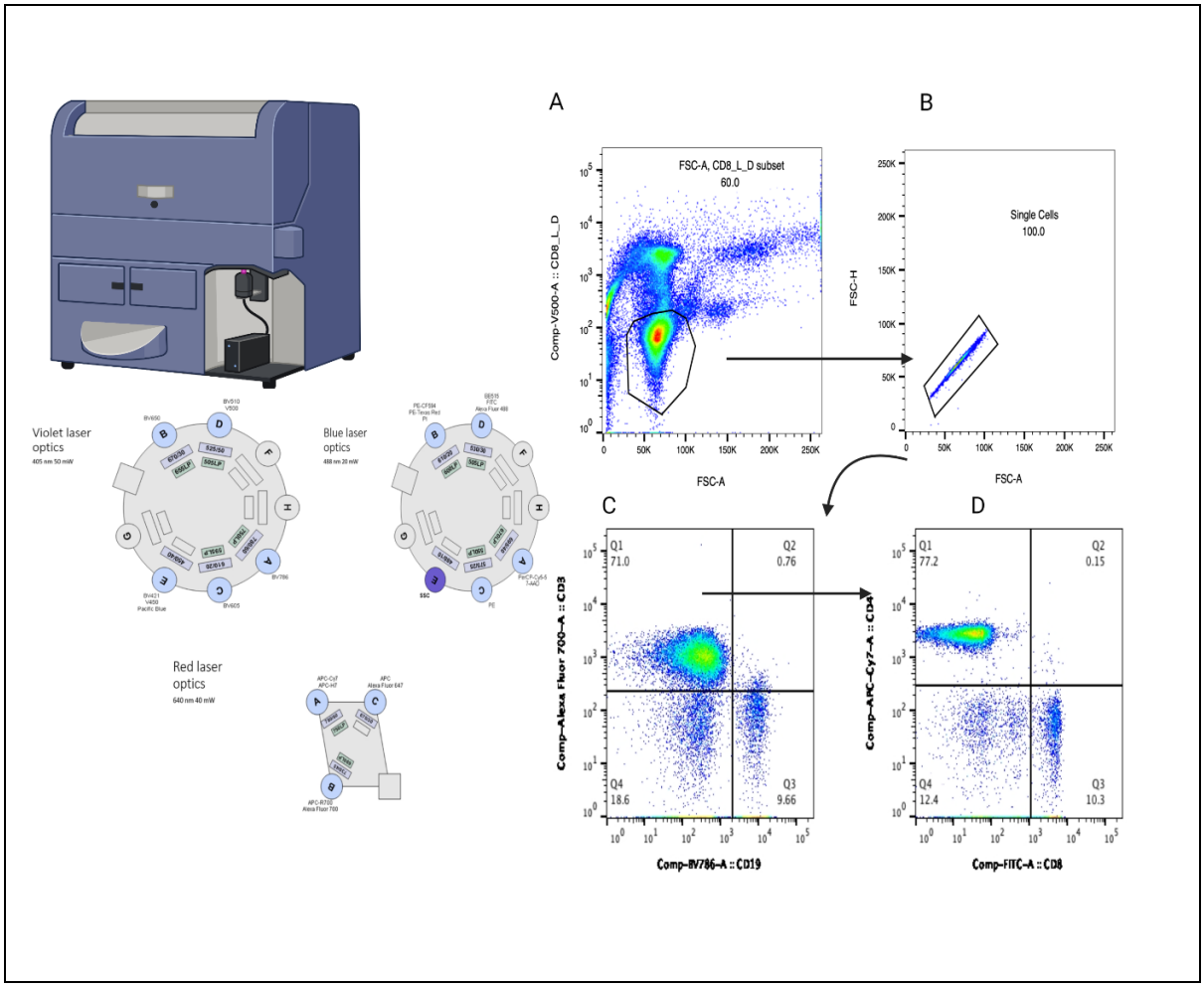



**Figure 1.5. Flow cytometry analysis.** We used the FACSCelesta to analyse cells – the optical layout is shown above. We gated on live single cells and then subdivided populations based on lineage markers. A quadrant plot developed to divide live (A), single (B) cells into CD3+ and CD19+ (C), CD3+ cells were then subdivided into CD4+ T cells and CD8+ T cells (D) (Created on Biorender, 2022).

### 1.3.2 Antigen presenting cell

#### 1.3.2.a Antigen presentation to T lymphocytes

A key role of T lymphocytes is to eliminate infections caused by intracellular microbes inside cells, and to activate other cells such as macrophages and B cells. Cells that capture antigens and present them to T lymphocytes are called antigen-presenting cells (APCs). In this process, antigens are captured from their site of entry and taken to lymphoid organs through which naïve T cells circulate (Abbas et al., 2015). APCs have a key functional role to activate naïve T cells or previously differentiated effector T cells. Different cell types such as dendritic cells, macrophages and B lymphocytes can function as APCs (Haan et al., 2014). Antigens that are recognized by T cells are different from those recognized by B cells. Indeed, T lymphocytes respond to cell associated antigens (Janeway et al., 2001). T cells receptors recognise antigens derived from inside the cell and displayed by cell surface MHC molecule (Janeway et al., 2001). Most T lymphocyte evolved to see and capture only short peptides (Abbas et al., 2015). On the other hand, B lymphocytes, that secrete antibodies, respond to antigens on microbial and host cell surfaces, and soluble cell-free antigens. In contrast to T lymphocytes, B cells can recognize peptides, folded protein, nucleic acid, carbohydrates, lipids, and small chemicals (Abbas et al., 2015). Major histocompatibility complex molecules (MHC) function to display host-cell associated antigens allowing for MHC restricted recognition by $CD4^+$ and $CD8^+$ T cells. MHC recognition is required to ensure efficient maturation of T cell, and to ensure that mature cells are restricted to recognition only MHC molecules with bound antigens (Haan et al., 2016). Peptides displayed by class II MHC are recognised by T helper CD4+; while $CD8^+$ T cells recognise peptides displayed by class I MHC (Geginat et al.,2003; Cole et al.,2012). Indeed, when naïve CD4+ T cells activated following the interaction with TCR-antigen-MHC class II complex they differentiate into effector subtypes that mediate immune response through secretion of specific cytokines (Sallusto et al.,2004; Pepper and Jenkins, 2011). CD8+ cells respond to the signals from the interaction between TCR-p-MHC class I and this interaction triggers T cells activation and induce target cell killing through secretion of cytotoxic granules (Geginat et al.,2003; Cole et al.,2012).

### 1.3.2.b Antigen capture and the functions of Antigen presenting cells

Dendritic cells (DCs) have an essential role in capturing antigen and then migrating and presenting antigens to T lymphocyte cells (Song et al., 2018). Microbial antigens commonly enter through the skin, gastrointestinal and respiratory tract, where they are captured by dendritic cells and transported to regional lymph nodes (Teijeira et al., 2014). Lymph nodes collect antigen from epithelium and connective tissue; however, antigen presenting cells also (APCs) capture antigen that enters the bloodstream in the spleen (Teijeira et al., 2014). Pathogen associated molecules can be recognized by innate pattern recognition receptors and these drive the polarized activation of DCs (Heath and Carbone, 2009). Immature DCs are activated by these signals to mature into potent APCs that transport captured antigen to draining lymph nodes (Bousso, 2008). Figure 1.6 shows the role of dendritic cells in antigen capture and presentation. Activation converts the DCs from primary tissue resting DCs into activated DCs (Abbas et al., 2015). Activated DCs involved in the expression of molecules have a role in T cell activation like cytokines, co-stimulators, and high level of MHC molecules, which all are needed to activate naïve T lymphocytes (Abbas et al., 2015). DCs are the most effective APCs for activating naïve T cells and therefore initiating the T cell responses (Bousso, 2008). Macrophages and B lymphocytes function as APCs for previously activated $CD4^+$ helper T cells (Heath and Carbone, 2009). DCs, macrophages and B lymphocytes express class II MHC molecules and other molecules involved in stimulating T cells and are therefore capable of activating $CD4^+$ lymphocytes (Song et al., 2018).

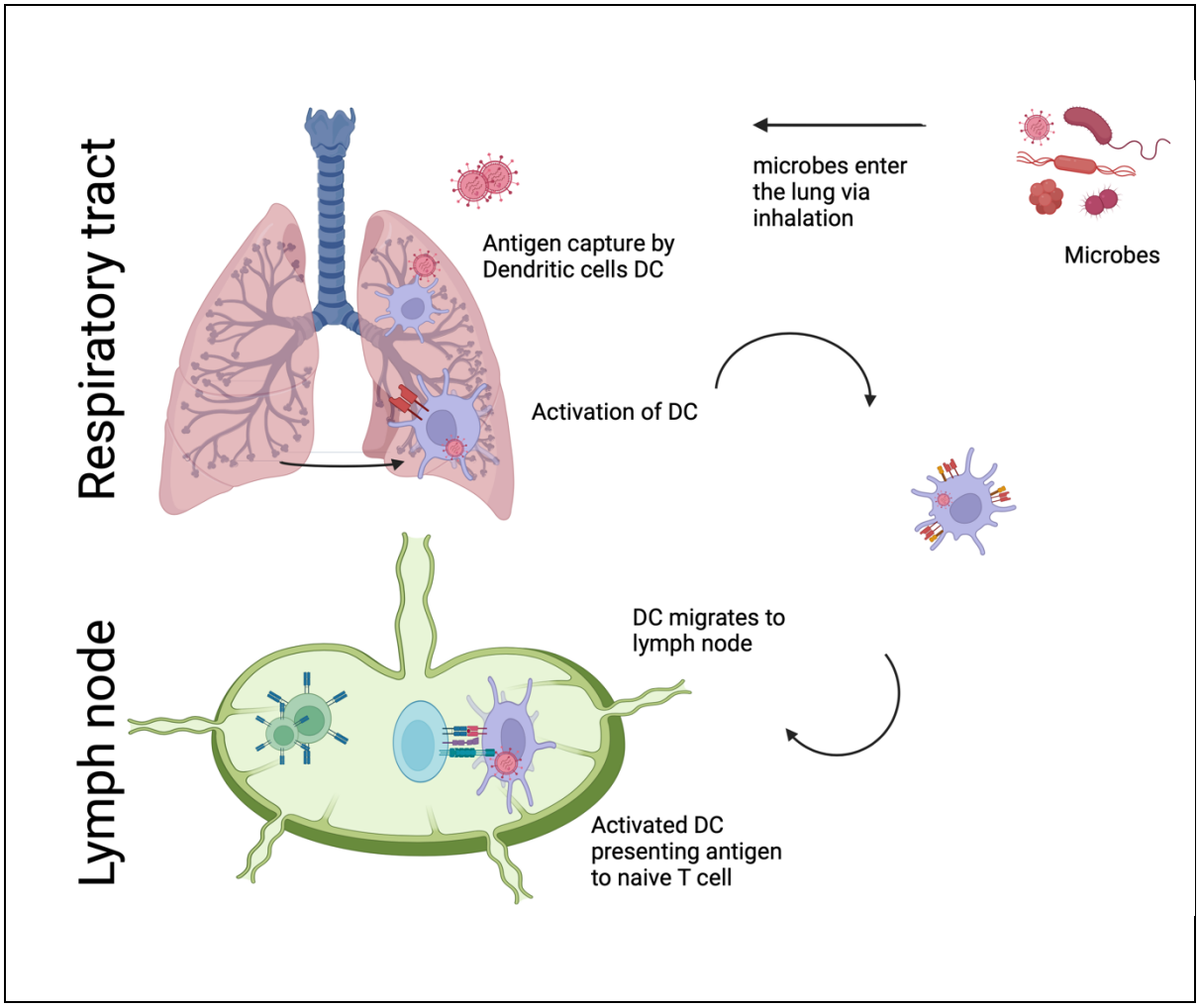



**Figure 1.6. Antigen capture and presentation by dendritic cells.** Microbes and their antigens enter through respiratory tract via inhalation where immature dendritic cells capture them. Dendritic cells activated and mature to become antigen presenting cells APS then transport them to the lymph nodes (Created on Biorender, 2022).

### 1.3.3 Activation of T lymphocytes

There are three signals required for the activation of naïve T lymphocytes into effector and memory cells: (1) antigen recognition, (2) costimulation, (3) and cytokines. The recognition of captured antigens in MHC by T lymphocyte receptors triggers the initial activation of naïve T lymphocytes and cell-mediated adaptive immune responses. The initial activation of naïve T lymphocytes occurs when antigens, which are captured by dendritic cells, encounter naïve T lymphocytes mainly in the peripheral lymphoid organs (Tai et al., 2018). As the antigen recognition occurs, several biological signals in T cells such as cytokine secretion and costimulation pathways are initiated (Borowski et al., 2007), resulting in differentiation of naïve cells into effector and memory cells is induced. Effector T cell are short lived, recognise

antigens and are activated resulting in the elimination of microbes (Wan and Flavell., 2009). On the other hand, memory T cells, which are also generated by T cell activation, exhibit long term survival and have an enhanced ability to react against antigen when it reappears. These memory cells respond rapidly to subsequent encounters with antigen and generate new effectors (Ratajczack et al., 2018). Memory cells are also characterised by increased activity following the re-exposure to same antigen (Chmielewski et al., 2011). According to Sallusto et al (1999) and Sallusto et al (2004) central memory T cells and effector memory T cells were defined based on the presence or absence of immediate effector function and the expression of receptors that migrate to secondary lymphoid organs. Memory T cells can be central memory cells, which have CD45RO and CCR7 on their surface (Sallusto et al., 1999; Roberts et al., 2005: Ratajczack et al., 2018). Another form is effector memory when cell surface expresses CD45RO but lacks CCR7 (Sallusto et al., 1999; Roberts et al., 2005: Ratajczack et al., 2018). Effector memory T cells express rapid effector function and both CD4 and CD8 positive effector memory T cells produce cytokines within hours following antigenic stimulation. On the other hand, central memory T cells produce IL-2, but require sometime following restimulation to express specific cytokines. Effector T cell responses decline after the antigen is eliminated but memory T cell responses are maintained (Abbas et al., 2015). Figure 1.7 shows the summary of the sequence of events in T cells responses from antigen recognition to the proliferation of naïve T lymphocyte into effector and memory cells.

In humans, memory T lymphocytes are heterogenous. Indeed, effector memory $T_{EM}$ cells which express receptors that promote migration to nonlymphoid site of inflammation and secrete cytokines including IFN$\gamma$, IL-5 and IL-4 (Sallusto et al., 1999). Another class of memory cells is central memory $T_{CM}$ cells which produce receptors CCR7 that is involved the migration through lymph nodes (Sallusto et al.,1999). The process of producing CD4+ memory T cells starts when TCR receptors on naïve cells bind to antigen-MHC class II on APCs and differentiate into specific subtypes, while the process of producing CD8+ memory T cells starts when TCR receptors on naïve cells bind to antigen-MHC class I on APCs (Sallusto et al.,2004; Pepper and Jenkins, 2011). CD4+ memory T cells function in protective responsive by making early effector cytokines, enhance other immune cell responses such as B cells and eliminate

infected cells (MacLeod et al., 2009). CD8+ memory cells play role in host defence in antigen recognition and infected cells lysis (Willinger et al.,2005).

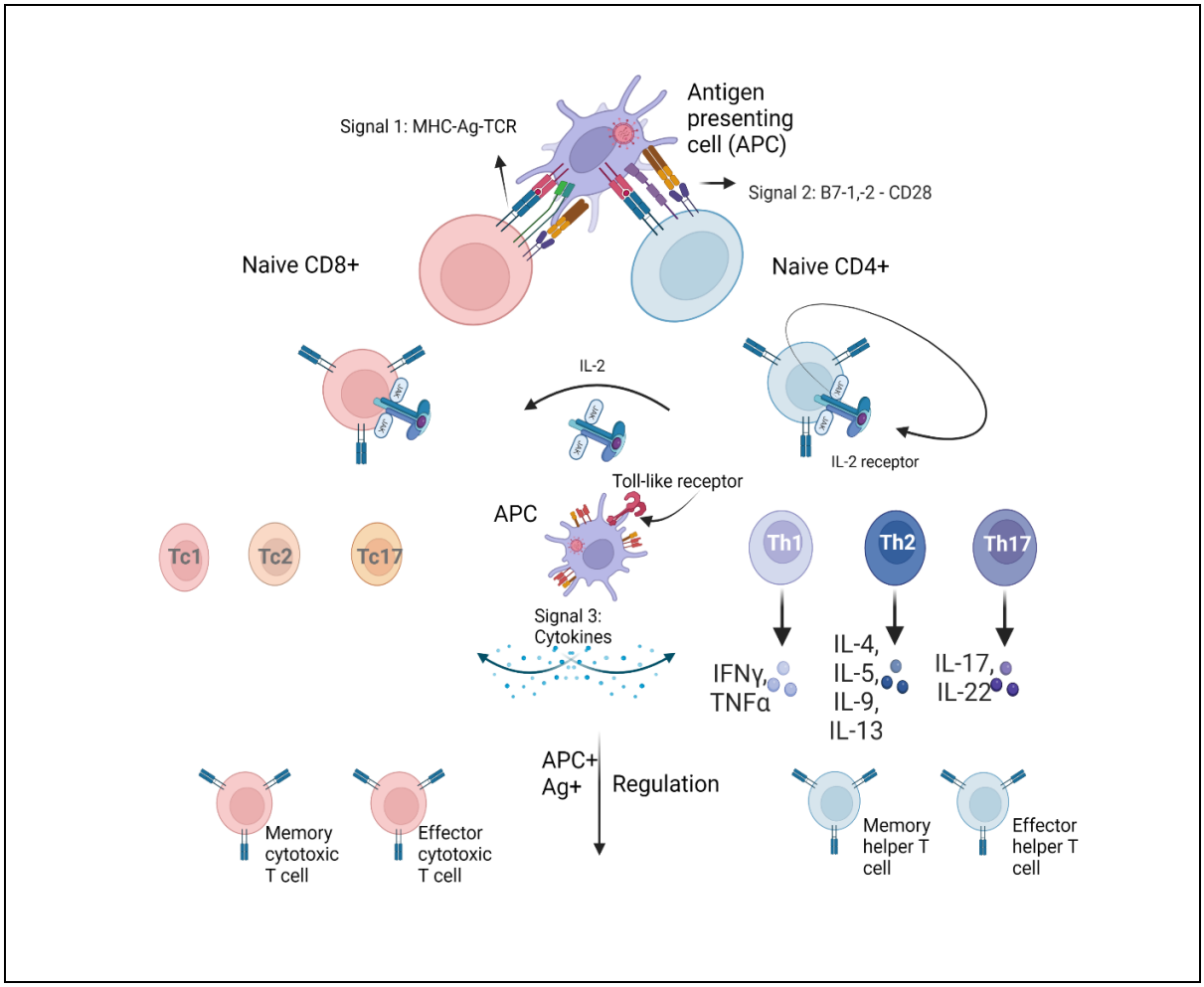



**Figure 1.7. T cell activation.** T cells are generated in and leave the thymus. They circulate through the lymph nodes and can recognise their antigen on the surface of activated APCs. T cell receptors (TCR) on CD4+ T cells bind to the MHC molecule II and TCR receptors on CD8+ T cells bind to the MHC molecule I on the surface of APCs triggering the initial activation of the T cells. Following antigen recognition, both costimulation and cytokines are required for the proliferation and differentiation of T lymphocytes into various types of effector cells. As T cells traffic away from the lymph node and through the tissues they can maintain effector function or can become memory T cells (created on Biorender., 2022).

### 1.3.4 Signals for T lymphocyte activation

#### 1.3.4.a Signal 1

T lymphocytes require specific signals for their proliferation and differentiation into effector and memory cells. These three signals include antigen recognition, costimulation and environmental cytokines (Borowski et al., 2007). The first signal for the activation of lymphocytes is antigen in the context of MHC. Peptide-MHC complexes are recognised by the TCR and the CD4 or CD8 molecule refine the MHC binding thereby initiating a signalling cascade within the cell in response to the antigen (Haan et al., 2016). T cell recognition of antigen is MHC-restricted as binding specificity of an individual T cell receptor is not only to the antigenic epitope but also for the MHC and the complex of peptide-MHC molecule. MHC polymorphism also restricts the nature of the peptides binding the MHC contributing to MHC restriction, defining the specificity of T cell receptor by the peptide it recognises and MHC molecule bound to it (Murphy and Weaver, Janeway's Immunology., 2017). In addition to recognition of antigen presented by dendritic cells, T cells must also receive survival signals from the co-stimulatory molecules that promote expression of IL-2 and IL-2 receptor (Tai et al., 2018).

#### 1.3.4.b Signal 2

Costimulatory signals are required for the T cells proliferation and differentiation. They are provided by molecules on APCs (Borowski et al., 2007). As the first signal for T cells activation is the antigen/MHC recognition, costimulation is the second signal. However, T cells that encounter antigen but fail to receive a costimulatory signal fail to respond (Abbas et al., 2015).

##### 1.3.4.b.1 B7:CD28 Costimulatory pathway

Resting DCs express few or no costimulatory molecules and fail to activate naïve T cells despite presence of antigen/MHC complexes, whereas activated DCs (activated APCs) express the costimulatory molecules on their surface and can therefore support continued survival

and activation (Sanchez-Lockhart et al., 2014). The costimulatory pathway in T cell surface receptor CD28, which binds the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on the surface of APCs. The main B7 molecules are expressed on APCs including DCs, B lymphocytes and macrophages; however, they are expressed and low levels on resting APC (Sanchez-Lockhart et al., 2014). During innate immune responses, stimuli such as microbial products activate the expression of B7 Costimulators by APCs. As a result, APCs become capable of activating naïve T cells into effector cells and memory cells (Sanchez-Lockhart et al., 2014). In the laboratory, Gauduin et al. (2006) reported the use of costimulatory anti CD28/CD49d antibodies to compensate for the lack of APCs and obtained an enhanced detection of antigen specific cytokine production from human peripheral blood mononuclear cells (PBMCs). The function of costimulators in T cell activation was shown in figure 1.8.

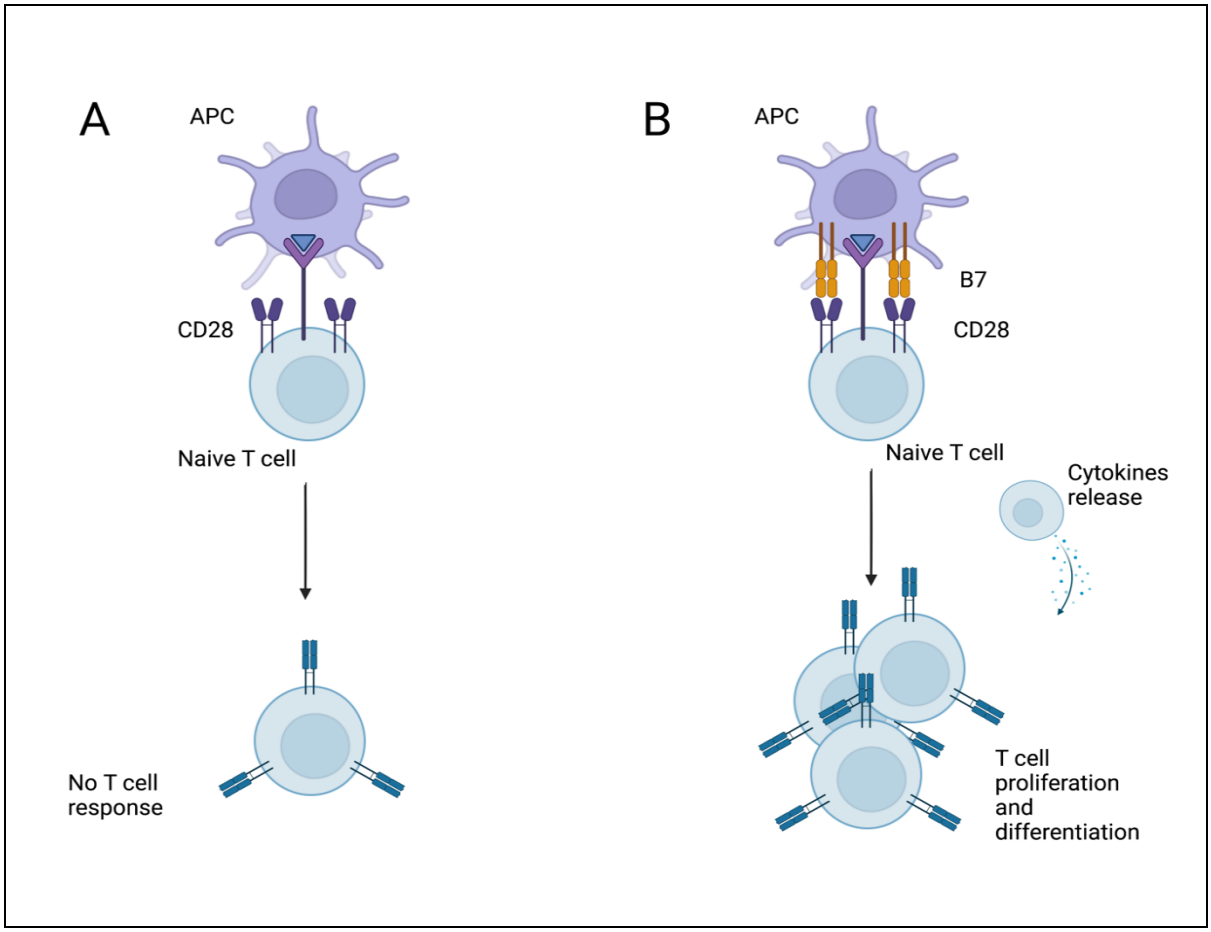



**Figure 1.8. B7:CD28 pathway.** (A) resting APC failed to activate naïve T cell because of the absent of B7 molecule. (B) activated APC expresses B7 molecule that binds to CD28 results in T cells activation and differentiation (created on Biorender, 2022).

#### 1.3.4.b.2 Inhibitory receptors

Immune checkpoints are inhibitory regulators that are essential to regulate the activation of T cells to prevent autoimmunity. Following T cell activation, two inhibitory receptors of the CD28 family, cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD-1) receptors, are expressed to limit the immune response. CTLA-4 and PD-1 immune checkpoint are negative regulators of T cell immune function. CTLA-4:B7 interactions lead to the inhibition of the initial activation of T lymphocytes in secondary lymphoid organs. Indeed, CTLA-4 binds to the B7 molecule providing negative costimulatory signals that lead to blocking of positive B7 molecule signals resulting in prevention of the interaction between CD28 and B7 which thereby inhibits T cell proliferation, IL-2 production and cell cycle progression. PD-1 regulate T cell activation through binding to its B7 family ligand PD-L1 (programmed death ligand-1, B7-H1). PD-1:PD-L1 binding inhibits TCR mediating signalling, leading to reduced proliferation. This interaction inhibits T lymphocyte proliferation and effectors function and without this regulation patients may die from the activation (Buchbinder and Desai, 2016; Murphy and Weaver, Janeway's Immunology., 2017).

#### 1.3.4.c Signal 3

The third signal required for optimal T cell activation is cytokines that promote T cell differentiation into subsets of effector T cells. A naïve T cell receives the required two signals (antigen/MHC recognition on the surface of APC and costimulation through CD28) and becomes activated leading to the secretion of IL-2. Activated T cells induce the secretion and expression of both moderate affinity (IL-2 receptor β and IL-2 receptor $\gamma$) and high affinity (IL-2 receptor α, known as CD25) for IL-2 binding to enhance T cell growth and differentiation. Unlike naïve T cells, which produce IL-2 when activated, effector T cells can produce a variety of cytokines depending on the effector phenotype. APCs including dendritic cells and other innate immune cells provide cytokines that induce the differentiation of naïve CD4 T cells into subsets. IL-12 and IFN$\gamma$ are produced to induce the differentiation of Th1. Innate immune cells such as basophil, eosinophil, mast cells and type 2 innate lymphoid cells ILC2 produce IL-4 to induce the differentiation of Th2 (Keegan et al., 2021). The dendritic cells also produce

TGF-β, IL-23 and IL-6 to induce the differentiation of Th17. These CD4 T cells subsets can regulate each other. For example, Th1 produce IFN$\gamma$ and Th2 produce IL-4 that inhibit the development of Th17. Also, when Th2 produce IL-4 that inhibits Th1 development. Similarly, Th1 produce IFN$\gamma$ that inhibits Th2 development (Murphy and Weaver, Janeway's Immunology., 2017).

### 1.3.5 Cytokines and their role in mediating immunity

Cytokines are group of chemicals released following activation of immune cells and which are involved in both the innate and adaptive immune response. Interleukins are group of cytokines produced in lymphocytes as well as monocytes, macrophages, and granulocytes. They major function is in the development and differentiation of T and B lymphocytes (Wesa and Galy, 2002). Naïve CD4 T cells may differentiate into effector cells in response to antigen (Pastore et al., 2019), thereafter different specific cytokines induce the differentiation of CD4+ subsets and each subsets produce different types of interleukins. Naïve CD4 T cells differentiate into three major subsets called Th1, Th2 and Th17 (Ekkens et al., 2007: Purvis et al., 2010). These function in host defense against both intracellular and extracellular pathogens. CD4+ Th1 is differentiated from naïve CD4 by IL-12 and IFN$\gamma$, CD4+ Th2 is differentiated from naïve CD4 by IL-4 and Th17 is differentiated by IL-1, IL-6 and IL-23. Natural T regulatory cells (nTregs) develop in the thymus and are CD4+ cells, while induced regulatory cells (iTregs) develop in the periphery from naïve CD4 T cells that recognise antigen in the presence of TGF-$\beta$ but no other cytokines. Each set of CD4+ T cells secrete specific cytokines have either pro or anti-inflammatory function. The signature cytokines secreted by CD4+ T cell for Th1 is interferon gamma IFN$\gamma$ and TNF-$\alpha$; Th2 produce IL-4, IL-5 and IL-13; Th17 produce IL-17 and IL-22; and T regulatory produce IL-10 and TGF-β (Ekkens et al., 2007: Purvis et al., 2010: Raphael et al., 2015). Table 1.2 shows the defining the important cytokines explaining the properties of the major role for each one.

**Table 1.2. Signature cytokines secreted by CD4 lymphocytes and their roles**

| cytokine | Cell source | Target | Role in disease |
|---|---|---|---|
| Proinflammatory cytokines | | | |
| IL-1 | Dendritic cells<br>Macrophages | Lymphocyte | - Enhance responses |
| IL-6 | | B cells | - Proliferation |
| TNF-α | | Endothelial cells<br>Neutrophils | - Signaling protein in innate and adaptive immune system |
| Anti-inflammatory cytokines | | | |
| IL-10 | Macrophages | Macrophages<br>Dendritic cells | - Inhibit IL-2 production<br>- Inhibit proinflammatory cytokines synthesis |
| IL-12 | Macrophages<br>Dendritic cells | CD4 T helper<br>NK cells | - Th1 differentiation<br>- IFN$\gamma$ synthesis |
| Cytokines involved in acquired immune responses | | | |
| IL-2 | T cells | T cells<br>B cells<br>NK cells | - Proliferation and activation |
| IL-4<br>IL-5<br>IL-13 | Th2 cells<br>Mast cells | T cells<br>B cells<br>Macrophages | - Th2 cells development and proliferation<br>- Isotype switch to IgE<br>- Control the production, activation and localization of eosinophil<br>- Inhibit IFN$\gamma$ production |
| IFN$\gamma$ | Th1 cells<br>Cytotoxic T cells<br>NK cells | T cells<br>B cells<br>Macrophages | - Th1 cells development<br>- Isotype switch to IgG<br>- Activation |
| IL-23<br>IL-17<br>IL-22 | Th 17 cells | Neutrophils | - Autoimmunity |

## 1.4 Immune response to Mtb infection

### 1.4.1 Innate immune responses

Protective immunity against *Mycobacterium tuberculosis* (Mtb) depends on innate and adaptive immune mechanisms. The activation of innate cells such as dendritic, macrophages, neutrophils and natural killer cells at the infection site is essential to provide protection against TB. Dendritic cells, which are the central for Mtb antigen presentation, mediate the connection between innate and adaptive immunity (Liu et al., 2017). Immature DCs are highly present in the lung mucosa and are attuned for antigen uptake, maturation and then migration to lymphoid organs where they prime T cells (Demangel and Britton., 2000). Mtb-infected DCs release IL-12 and IL-23 and induce both IFN$\gamma$ and IL-17 in CD4 T cells (Khader et al., 2005).

Mtb enters the lung via inhalation and reach the alveolar space to encounter the macrophages, the infected macrophages migrate to the lung parenchyma. All immune cells including DCs, monocytes, neutrophils, T cells and B cells form the granuloma. When the granuloma fails to contain the infection, the bacteria enter the bloodstream and disseminate (Fenton and Vermeulen, 1996; Gonzalez-Juarrero et al., 2003). Monocytes/macrophages mediate host immunity against Mtb infection as a first line of defence (Lugo-Villarino et al., 2011; Flynn et al., 2011). M1 (classical activated) macrophages are characterized by high IFN$\gamma$ production that resist intracellular pathogen growth, while M2 macrophages production of TLRs and immunosuppressants including IL-10 and TGF-β (Lugo-Villarino et al., 2011; Flynn et al., 2011).

Neutrophils are phagocytes that play roles in many infections including Mtb. It is known also that neutrophils are essential for granuloma formation. Eum et al (2010) found that neutrophils are the most abundant cells observed in the sputum and bronchoalveolar lavage (BAL) fluid in 15 patients that received a diagnosis of active TB. Martineau et al (2007) found peripheral blood neutrophil counts in human pulmonary TB are higher than unexposed control at baseline and 6 weeks follow-up. Neutrophils are also involved in tissue damage in TB (Kroon et al., 2018). Dallenga et al (2017) found that Mtb infection induces necrosis of

infected neutrophils in an ESX-1-dependent manner. Also, neutrophils produce reactive oxygen species (ROS) that also promote this necrosis. This necrosis was required for bacterial growth when engulfed by macrophages. The inhibition of ROS and necrosis restored the control of Mtb by macrophage.

Other innate cells such as natural killer (NK) cells also participate in the response to Mtb infection. NK cells are important mediators of cellular cytotoxicity and cytokine signalling in response to antigens. In TB, NK cells participate in granuloma formation and can help control the infection either by cytotoxic activity or signalling to the adaptive system (Garand et al., 2018). Indeed, NK cells have direct mechanisms of cytotoxicity by releasing cytoplasmic granules including perforin, granulysin and granzymes (Allen et al., 2015; Garand et al., 2018). Granzyme B is an enzyme that initiates apoptosis of the target cells and mediates cell destruction in the presence of perforin. Granulysin is a protein produced by activated human NK cells. It is cytolytic against microbial and tumor targets (Allen et al., 2015). Perforin disrupts lipid metabolism, oxidative phosphorylation and facilitates the transportation of granulysin and granzyme into the target cells (Allen et al., 2015). NK cells also are potent producers of IFN$\gamma$ and provide signals to infected macrophages and dendritic cells to assist with mycobacteria elimination (Allen et al., 2015; Garand et al., 2018).

T cells can be activated by non-MHC antigen presenting systems including non-polymorphic MHC related protein1 (MR1), CD1 and butyrophilin 3A1. MR1 presents microbial metabolites such as vitamin B to T cells. CD1 presents microbial and mammalian lipids to T cells. butyrophilin 3A1 present phosphoantigen molecules to $\gamma\delta$ T cells. Donor-unrestricted T cells (DURTs) are specific T cells that recognise antigen presented by these presentation pathways. Studies provided evidence that DURTs subtypes activated during Mtb infection in human and non-human primates. Layton et al (2021) reported that glucose monomycolate-specific CD1c-restricted T cells expanded after BCG administration. Chua et al (2012) reported that activation of MR1-restricted MAIT cells has been shown in response to BCG, and other bacteria. They purified MAIT cells and cultured it with *Mycobacterium bovis* BCG-infected macrophages and compared the intracellular growth to $MR1^{-/-}$ mice. They found that MAIT cells inhibited the bacterial growth by 40% to 80%. Gela et al (2022) characterised DURTs following BCG vaccination in two cohorts of infants (vaccinated/unvaccinated) and adults

(before/after BCG revaccination). They found that there was significant increase in the frequency of γδ T cells in BCG-vaccinated infants compared to the unvaccinated. However, the reported that BCG vaccination did not modulate T cell activation of DURTs subsets.

### 1.4.2 Adaptive immune responses

Human T lymphocytes play an important role in host defense against TB. Antigen recognition and T cell activation occurs when the T cell receptor (TCR) interacts with peptides (or antigens) and the major histocompatibility complex (MHC) molecules (Sallusto et al.,2004; Pepper and Jenkins, 2011). Antigen presenting cells (APC) are involved in the initial T cell response to Mtb antigen. APCs have a functional role to activate naïve T cells or previously differentiated effector T cells. Dendritic cells help to recognise antigen by T cell in the draining lymph nodes, and MHC complexes are functioned to display associated antigens for recognition by $CD4^+$ and $CD8^+$ cells (Banchereau and Steinman, 1998). One cytokine that is released following the activation of the immune system is Tumor necrosis factor alpha (TNF-α). Immune cells such as phagocytes in the lung interact with Mtb upon the initiation of the infection. Consequently, this interaction results in the production of proinflammatory cytokines including TNF-α (Wajant et al., 2003; Domingo-Gonzalez et al., 2016). TNF-α is primarily produced by macrophages, lymphocytes, and endothelial cells. The importance of TNF-α in Mtb infection models was showed in studies; Domingo-Gonzalez et al (2016). Indeed, they stated that mice with TNF-α deficiency results in increased susceptibility to infection within 2 to 3 weeks. They also stated that neutralization of TNF-α leads to loss of granulomas in mycobacterial disease, and decrease the expression of key chemokines that are expressed in innate and adaptive immune cells which results in a loss of communication between immune cells.

Activated CD4+ T and CD8+ T cells secrete cytokines such as interferon-$\gamma$ (IFN$\gamma$), which is the major feature of the adaptive immune response to TB and activate macrophages to destroy bacteria (Garand et al., 2018; Lindestam Arlehamm et al., 2014). Both CD4+ T cells and CD8+ T cells produce IFN$\gamma$ and increase with infected lung however absence of these cells resulted in susceptibility to Mtb infection (Caruso et al., 1999; Domingo-Gonzalez et al., 2017). IFN$\gamma$ provides protective immunity against intracellular pathogens as it mediates macrophage activation and activates phagocytes that contain the pathogen (Kaufmann, 2002). In addition,

CD4+ T cells produce IFN$\gamma$ and TNF-$\alpha$ which is important for forming the granuloma that contain collection of innate and adaptive cells produced to contain the pathogen (Kaufmann, 2002; Rueda et al., 2010; Walzl et al., 2011). IL-2 is a cytokine that provide clonal expansion and differentiation of specific CD4+ and CD8+ T cells (Rueda et al., 2010). The contribution of specific CD4+ providing protective immunity is important. Indeed, humans and mice lack the gene for IFN$\gamma$ are susceptible mycobacterial infection (Cooper et al, 1993). Humans with compromised immune system such as those with HIV+ have deficient in CD4+ are highly susceptible to Mtb infection or re-infection (Rueda et al., 2010). Many studies showed that the production of Mtb specific CD4+ T cells IFN$\gamma$+ is very useful tool which improves the diagnosis of TB. They assessed the ability of these cells to distinguish between those who are infected from healthy individuals. Indeed, it has been reported that the frequencies of IFN$\gamma$ or IL-2 or TNF-$\alpha$ in patient with active TB and LTBI was significantly higher than these frequency in healthy controls, but on the other hand the frequency of these was not significant between LTBI and active TB patients (Lichtner et al., 2014: Adekambi et al., 2015: Esteves et al., 2020). Indeed, T helper 1 cells (Th1) is a subset of CD4+ which secret IFN$\gamma$ and TNF-$\alpha$ that involved in the elimination of intracellular pathogens. IFN$\gamma$. Moreover, during infection with Mtb, antigens from the bacterium are presented to the T cells and this results in the activation of antigen specific T cells. These T cells then proliferate, differentiate and can become memory and regulatory T cells and T regulatory cells (Pathakumari et al., 2017).

Other studies have used flow cytometric approaches to evaluate cell surface markers on Mtb specific CD4+ T cells to differentiate TB stages. The expression of several surface molecules on antigen specific CD4+ T cells such as CD27, CCR7 and CD45RA have been shown to be different proportion among TB patients or LTBI or Healthy control. CD27 is a member of TNF-receptor superfamily that expressed by naïve CD4+ T cells and memory cells (Nikitina et al., 2012). During antigen stimuli, high CD27- effector T cells differentiate from CD27+ (Petruccioli et al., 2015). Indeed, in human studies it has been reported that LTBI patients have a higher proportion of CD45RA- CD27+ than active TB patients in whole blood and PBMCs (Petruccioli et al., 2015; Latorre et al., 2019). They also saw a higher proportion of CD45RA- CD27- in active TB patients compared to these cells in LTBI and a higher CD27 MFI ratio in active TB compared to LTBI.

The programmed cell death-1/ programmed cell death ligand 1 (PD-1/PD-L1) mechanism is involved in negative regulation in TB. The PD-1/PD-L1 interaction is critical to survive in TB. This interaction inhibits T lymphocyte proliferation and effecters function and without this regulation patients may die from the activation. This costimulatory pathway represents negative mediating T cell function (Yang et al.,2022). In human tuberculosis, PD-1/PD-L1 pathway play roles in immune activation and checkpoint inhibitors against the disease (Shen et al., 2016). PD-1 is an inhibitory cell surface receptor that interacts with PD-L1 resulted in inhibition the T cells effector (Shen et al., 2016). Both PD-1 and PD-L1 expressed on activated T, B and myeloid cells (Ishida et al., 1992). Regulatory T cells also involved in the negative regulating in TB (Abbas et al.,2013; in Shen et al., 2016). The association between PD-1, PD-L1 and regulatory T cells was studied among active TB disease and healthy control. Shen et al (2016) reported that the level of PD-1 and PD-L1 on CD4 cells increased significantly in active TB. They reported that the level of these markers on CD4+ including the regulatory T cells are highly produced in active TB patients. Together by showing the information of PD-1 and PD-L1 expression we characterised the PD-L1 CD25 frequency on Mtb antigen specific CD4+ T cells subsets including $T_{CM}$ and $T_{EM}$ among healthy control, LTBI and active TB patients.

Another costimulatory immune checkpoint molecule is the tumor necrosis factor receptor superfamily-4 known as OX40 (CD134). Activated T cells, and NK cells express OX40 after 24 hours during activation (Croft, 2010). The co expression of OX40+ CD25+ is critical for T cell survival during antigen specific activation (Escalante et al.,2015). Zaunder et al (2009) reported that the co expression of OX40 CD25 represents sensitive assay that significantly higher antigen specific T cells including Mtb. Esclante et al (2015) showed that the co expression of these molecule in Tb antigen specific CD4+ was significant between untreated LTBI, treated LTBI and healthy control.

Although adaptive immunity requires T lymphocytes activity for effective containment of Mtb, B cells and their antibodies play a role in host defense the infection. Phuah et al (2012) characterised the B-cell and plasma-cell population in infected animals. They performed flow cytometry on single cells suspension of lung and lymph node tissues of cynomolgus macaques (*Macaca fascicularis*) to assess B cells numbers within infected tissues. They found that CD20+ cells were higher within Mtb-infected lungs compared to healthy lungs. They also found that

plasma cells produced mycobacteria-specific antibodies. Maglione et al (2007) found that the bacterial burden in the lungs of B cell deficient mice was increased suggested that B cell $^{-/-}$ mice have enhanced susceptibility to the infection. Bitencourt et al (2022) investigated the impact of BCG vaccination on purified protein derivative-specific (PPD) antibodies in human and macaques. They found an increase in PPD-specific IgG at 28 and 84 days and IgA at 28 days post BCG vaccination in human. However, they observed an increase in PPD-specific IgG at 56 days post BCG vaccination in macaques. They then investigated the association between level of serum antibodies specific to different mycobacterial fractions. They found that there was association in BCG-induced fold change in IgG between all mycobacterial fractions. They then investigated the impact of BCG vaccination on BCG-specific IgG secreting memory B cells. They found that there was significant increase in memory B cell response at 7 and 70 days post BCG vaccination compared to baseline. These confirmed the role of B cells in the host response against Mtb.

### 1.5 IL-12 receptor structure, function and Isoform 2 (*il12rb1Δtm) in Tb*

IL12RB1 is essential for human resistance to multiple intracellular pathogens, including *Mycobacterium tuberculosis* (Cooper et al., 1995; Cooper et al, 1997). In its absence, the proinflammatory effects of the extracellular cytokines IL-12 and IL-23 fail to occur, and intracellular bacterial growth goes unchecked (Cooper et al., 1995; Cooper et al, 1997). Cytokines IL-12 and IL-23 influence numerous aspects of adaptive and innate human immunity, including the differentiation of helper T and NK cell subsets, enhancing cytolytic T cell responses, promoting B cell production of select Ig isotypes, and regulating dendritic cell activity (Cooper and Khader., 2008; Cooper; 2009). The biological activities of IL-12 and IL-23 are dependent on IL-12Rb1, a type I integral membrane protein that serves as a low-affinity receptor for the p40 subunit of both cytokines (Cooper and Khader., 2008; Cooper; 2009). To relay the immunological effects of IL-12 or IL-23, IL-12Rb1 must both physically associate with each cytokine and signal in complex with either IL-12Rb2 or IL-23R, respectively (Cooper and Khader., 2008; Cooper; 2009).

IL-12Rβ 1 is a receptor chain that combines with IL-12Rβ 2 to form the IL-12 receptor (figure. 1.9) Signalling of IL-12 through the IL-12 receptor is involved the cytoplasmic proteins TYK2, which is directly interact with IL-12Rβ 1. Also, JAK2, which interacts with IL-12Rβ 2. They both are tyrosine phosphorylated (Van de Vosse et al., 2013). Subsequently, the phosphorylated TYK2 and JAK2 are essential for tyrosine phosphorylation and activation of STAT4 that is bound to IL-12Rβ2. STAT4, is a transcription factor, which is translocated to the nucleus, and binds to its target DNA to activate transcription of IFN$\gamma$ and other target genes (Ford et al., 2012; Van de Vosse et al., 201).

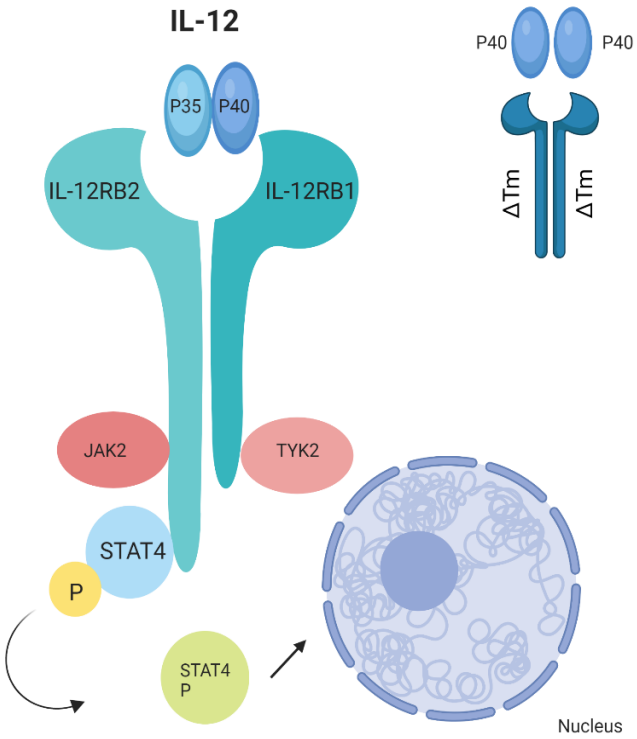



**Figure 1.9. Schematic representation for IL-12.** Binding of cytoplasmic protein JAK2 and TYK2 to IL-12RB2 and IL-12RB1 respectively. Once the JAK2 AND TYK2 is phosphorylated, this result in the phosphorylation, activation and translocation of STAT4 to the nucleus (created on Biorender software, 2019).

According to Robinson et al. (2010), IL-12 contributes TH1 responses to Mtb and other stimuli. Indeed, Cooper et al (1995) demonstrated that IL-12 increased the production of IFN$\gamma$ by splenocytes derived from infected animal or/and antigen specific CD4 T cells from untreated animal. Also, Cooper et al. (1997) published that IL-12 $p40^{-/-}$ mice infected with *Mycobacterium tuberculosis* had reduced IFN$\gamma$ secretion, which is a signature cytokine and play role in Th1 responses. Moreover, Heufler et al. (1996) stated that dendritic cells DCs play a potent role in priming T cells against pathogen stimuli and induce cell mediated immunity. As DCs process antigen then migrate from peripheral tissues to lymph nodes, they can be a critical source of the production of IL-12. IL-12 composed of covalently linked p35 and p40

chains. Also, the importance of the subunit IL-12p40 and the role of IL-12Rβ1 in DCs function during Mtb inflammation have been demonstrated in mouse model studies (Khader et al., 2006). It has been shown in these studies that the subunit IL12p40 is required for the migration of DCs and the initiation of T cells activation (Khader et al., 2006; Robinson et al., 2008). Cooper et al (1997) infected IL-12p40$^{-/-}$ mice with Mtb resulted in the inability of the mice to control the bacterial growth in the spleen, liver and lung. They also reported that the IFN$\gamma$ was reduced in infected mice in the absence of IL-12p40$^{-/-}$. Thus shows the importance role of IL-12p40 in TB disease.

The IL12Rβ1 is a common receptor chain of IL-12. An isoform that called isoform 2 is "transcribed from exon 1 to 9 plus a cryptic exon in intron 9" according to Van de Vosse et al. (2013). Isoform 2 lacks the transmembrane domain that is required for anchoring on the cell surface, as well as the intracellular domain required for signaling (Van de Vosse et al., 2013). Also, in mice, an isoform is known that lacks the transmembrane domain is secreted in response to Mtb exposure (Robinson., 2010). This isoform (△TM-IL-12Rβ1) however is a splice variant of IL-12Rβ 1, which is similar the human produced isoform 2 (DAS et al., 2018; Ray et al., 2015). Furthermore, previous work, which published for DAS et al. (2018), demonstrates how the expression of the splice variant △TM-IL-12Rβ1 limits of the dissemination of Mtb from the lung in a mouse. The aim of the current research is to establish an association between the expression of △TM-IL-12Rβ1 and the clinical parameters of subjects.

### 1.6 Research Rationale and Hypothesis

Regionally, TB remains a significant public health issue and remains a cause for concern in the UK. Most cases of TB in the UK occur amongst non-UK born individuals likely undergoing reactivation of remotely acquired latent tuberculosis infection (LTBI). Migrants arriving from high TB burden settings undergo routine screening for LTBI in primary care with an interferon gamma release assay (IGRA). A positive IGRA results in referral to secondary care for further management. Indeed, The IGRA does not differentiate individuals with active from LTBI, nor does it indicate response to treatment and fails to detect subclinical active TB (radiological evidence of TB in the absence of signs and symptoms). From a clinical perspective, the diagnosis of TB (for both latent and active) can be detected by IGRA test, symptoms, CT scan, X ray, culture based and molecular diagnosis. Data from most migrants who had a positive

IGRA test, shows that half of the active TB cases are asymptomatic (based on unpublished data Prof. Pareek / Dr. Martin, 2019).

In this study we hypothesize that the adaptive immune response *to Mycobacterium tuberculosis* antigens will allow us to differentiate between individuals who are latently infected with Mtb and those who have subclinical or active disease. To test this hypothesis, we undertook deep immunophenotyping of a clinically characterized migrant cohort in Leicester, which is unique in having a high frequency of disease but a low exposure rate. We also hypothesized that the ΔTM-IL-12Rβ1 could augment any diagnostic potential of other immune signals as it is known to be produced in response to live Mtb. To test this hypothesis, we proposed analysis of plasma samples from each subject for both the protein ΔTM-IL-12Rβ1 and other proteins associated with inflammation and TB.

**Aims**

The aim of my work is to test the hypothesis that the adaptive immune response to Mycobacterium tuberculosis antigens will allow us to differentiate between individuals who are latently infected with Mtb and those who have subclinical or active disease.

**Objectives**

1- To generate and use an ethical protocol to develop a local cohort of individuals who are latently infected with Mtb and those who have subclinical or active disease.
2- To develop and use cell-based assays that would allow us to examine the antigen specific cellular response in our local cohort of individuals who are latently infected with Mtb and those who have subclinical or active disease.
3- To determine the frequency and phenotype of both innate and antigen-specific peripheral blood cells in our local cohort individuals who are latently infected with Mtb and those who have subclinical or active disease.
4- To use the developed assays to monitor changes in immune cell populations during anti-TB treatment.

5- To use the plasma from our local cohort of individuals to investigate the association between ΔTM-IL-12Rβ1, a plasma protein with potential to be a biomarker for disease, and other immunological and clinical parameters being measured.

6- To determine whether there is any correlation between the measured immunological variables and any biologic or biomarker signature using multivariate analysis.

# Chapter 2: Optimisation of T cell immunophenotyping and induction and measurement of antigen-specific T cell responses

## 2.1 Introduction

Within the Cellular Immunology laboratory of Prof Cooper, a basic characterization protocol and flow cytometry panel for the analysis of human PBMCs had been established. To test our hypotheses, we needed to develop an in-depth characterization protocol as well as protocols for detection of antigen-specific cellular responses had to be developed. In this chapter I want to outline the processes by which I developed a full immunophenotyping profile for human PBMCs including the phenotype of lymphocytes, monocytes, and dendritic cells panels using healthy control blood from volunteers. By perfecting these panels, I was developing the tools to deeply immunophenotype the subjects recruited for the ISATS study described in Chapter 3. I also had to develop for the first time in the lab the protocols and panels that would allow me to dissect the antigen-specific response. To do this we again used healthy controls and a broad non-specific stimulus of T cell activation to ensure strong measurable responses. We approached the CD4 T cell antigen-specific response both in terms of *ex vivo* expression of cell markers of activation (CD45RO/RA and CCR7, CD25, CD127), and in response to T cell activation the intracellular expression of cytokines (IFN$\gamma$, TNF, IL-2) and expression of activation induced markers (OX-40, CD25). We developed the most appropriate cell preparation protocols, the gating strategies for all the panels and identified the best positive and negative control conditions. We determined that while fresh whole blood could be stimulated, we found that this could not work alongside the longitudinal and sporadic nature of the ISATS trial. We also determined that the impact of isolating PBMC and the impact of freezing were not sufficient to impose the need for immediate analysis of whole blood – this improved feasibility of the study. An important element of the antigen-stimulation studies was optimization of the response so that it would be detectable. To achieve this goal, we included used the published technique of adding costimulatory antibodies and determined that they enhanced the positive signal without increasing the background. Finally, we ensured that the selection of the final gating was based on a positive and specific signal using the fluorescence minus one protocol wherein only the antibody being used to detect the measured parameter is left out of the staining panel. The purpose of this development study was to identify to protocol parameters that would allow reproducible and comparisons between the immune response of healthy and LTBI PBMC in the ISATS study.

**Objectives**

1- To design and develop a cell-based analysis protocol that will allow us to test our hypotheses.
2- To identify panels of antibodies that will allow us to compare the frequency and phenotype of lymphocytes, monocytes and dendritic cells between cohorts.
3- To optimize an intracellular cytokine staining (ICS) assay and an activation induced markers assay in human PBMCs to allow us to identify T cells responding to a signal through the TCR.

## 2.2 Materials and Methods

### 2.2.1 Ethical approval

The use of human blood samples in experiments described in this chapter was approved by the University ethics sub-committee for Medicine and Biological Sciences at University of Leicester (ethic reference 9664-infectionimmunityinflamm). Informed consent for the study titled "Analysis of T cell phenotype and function" was obtained from all participants.

### 2.2.2 Media

**Complete Dulbecco's Modified Eagle Media (DMEM, Sigma-D5671)**

High glucose media was supplemented with 5% fetal bovine serum (FBS, Sigma F4135), 100 u/ml Penicillin-Streptomycin (10000 U/ 10 mg/ml, Sigma P0781), 2mM of L-Glutamine (by adding 5ml of 200mM, Sigma G7513 into 500 ml DMEM), 0.01M Hepes (by adding 5ml of 1M, Sigma H0887 into 500ml DMEM), 1mM of Sodium Pyruvate (by adding 5ml of 100mM, Sigma S8636 into 500ml DMEM) and 1X of MEM amino acids (by adding 10ml of 50x, Sigma M5550 into 500ml DMEM). All the supplements were added at room temperature, the complete media was then sterile filtered and stored at $4^0$ C.

**Freezing media**

Dimethyl sulfoxide (DMSO, SigmaD8418) was diluted in fetal bovine serum (FBS) by adding 10 ml of DMSO to 40 ml of FBS then filtered for sterilization through syringe filter, Filtropur S,

PES, pore size: 0.2 µm (08.1826.001) using IV syringes, lure lock (IVL10). The media was aliquoted in sterile Corning 50 ml polypropylene centrifuge tubes and stored at $4^0$ C.

**Flow cytometry media FSM**

1- **Hank's Balanced Salt Solution**, Sigma H6648 supplemented with 5% FBS, 1X MEM amino acids, 0.01M Hepes, 1mM sodium pyruvate, 2mM L-glutamine, and 1% Penicillin-Streptomycin. All the supplements were added at room temperature, sterile filtered and stored at $4^0$ C.

2- **1% Bovine serum albumin BSA, PB9700** was prepared by dissolving 5 g of BSA in 500ml of 1X Phosphate buffered saline PBS (pH 7.2, Gibco, 20012-019), this media was stored at $4^0$ C.

### 2.2.3 Antigens

**Staphylococcal enterotoxin B SEB, Sigma Aldrich, S4881-1MG** was dissolved in PBS at a final concentration of 0.5 mg/ml, aliquoted and stored at $4^0$ C.

**Dynabeads human T-activator CD3/CD28, Gibco 11131D for activation of human T cells**, were resuspended in 1ml PBS, mixed for 5 seconds and kept on a roller for 5 minutes. Then the tube was placed on a magnet for 1 minute. The supernatant was discarded then the tube was washed with 1 ml of PBS. 2 µl from the pre-washed and resuspended Dynabeads was added to each 96-well cell well containing PBMCs for stimulation.

**Tetanus toxoid (TT, NIBSC, 02/232)** was resuspended in PBS at final concentration 1mg/ml.

**MTB300 (Mtb-300)** is a pool of Mtb peptides synthesized and provided by Dr Cecilia Lindestam Arlehamm, Center for Infectious Disease, La Jolla Institute for Immunology, La Jolla, USA. The mixed peptide pools were re-lyophilized, reconstituted into DMSO, and aliquoted at 0.7mg/ml in tubes and delivered to the Cooper Lab. The tubes were centrifuged to collect everything at the bottom and stored at $-80^0$ C. Each tube has 50 µl and while freeze-thaw cycles have not been shown to reduce activity we did not exceed 20 freeze thaw cycles.

**Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, P1585)** is delivered in a physical state as a soluble clear film on the walls of the vial. PMA has been used in stimulation of IL-2 in

Jurkat cells, T cells stimulation, protein kinase C activator and other molecular biology studies. PMA was resuspended in DMSO at 10 mg/ml and stored in -80$^0$ C.

**Concanavalin A from *Canavalia ensiformis* (Jack bean), Sigma Aldrich, C5275-5MG** is a lyophilized powder suitable for *in vitro* cell culture and lymphocyte proliferation. Concanavalin A stock was diluted in distilled water at 5 mg/ml, aliquoted and stored in -80$^0$ C.

**Ionomycin calcium salt (Sigma Aldrich, I3909)** is an ionophore produced by the bacterium *Streptomyces conglobatus.* It has been used in conjunction with PMA to stimulate the intracellular production of cytokines. 1 mM solution in DMSO was stored in -80$^0$ C.

### 2.2.4 PBMC isolation and storage

#### 2.2.4.a Ficoll approach for human peripheral blood mononuclear cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from blood collected from healthy volunteers at the University of Leicester. Informed written consent and participant information sheet were obtained from all participants. Blood (7.5 ml) was collected into heparin tubes (Sarstedt, 01.1613.100) and then diluted (1:1) in phosphate buffered saline 1X (PBS) in a 50 ml conical tube. 5 ml of density gradient media (Ficoll Paque Plus Sigma alderich, GE healthcare-17144002) was drawn using sterile syringe and needle. The diluted blood was laid down onto Ficoll slowly. The tubes were centrifuged at 350x g for 30 minutes at room temperature with the brake off. The PBMC layer was aspirated onto new 50 ml conical tube using a 3 ml Pasture pipette. 50 ml PBS was then added, and the tubes were centrifuged for 10 minutes at room temperature with the brake on. The supernatant was decanted, and the pellet was flicked, resuspended in half volume of original sample in complete Dulbecco's Modified Eagle Media, supplemented as described above. The cells were counted using a hemocytometer, centrifuged once more and resuspended at a volume to deliver a final concentration of 1 x 10$^7$ cells/ml. An equivalent volume of freezing media (as above) was added and the cells were aliquoted into labeled cryovials, placed in a CoolCell (Biocision, 4929015) and stored at -80$^0$ C overnight, whereupon they were then transferred to -150$^0$ C.

### 2.2.4.b PBMCs isolation using SepMate 50

Whole blood was collected into heparin tubes and then diluted in PBS (1:1). 15 ml of Ficoll Paque Plus (as above) was added to the central hole of the insert within the SepMate 50 tube (SepMate-50, Catalog number 85450). Keeping the tube vertical, the diluted blood was pipetted into the tube and the tube centrifuged at 1200 g for 10 minutes with the brake on. The top layer, which is enriched with plasma and PBMCs was poured into 50 ml falcon tubes. The cells were washed with PBS twice at 300 g for 8 minutes. The pellets were resuspended with complete Dulbecco's Modified Eagle Media (DMEM). The cells were counted using a hemacytometer and resuspended at appropriate volume for approximately 1 x $10^7$ cells/ml and then diluted 1:1 with freezing media and frozen and stored as above. Figure 2.1 illustrates the procedure of isolation of human PBMC from whole blood using Sepmate-50 tubes.

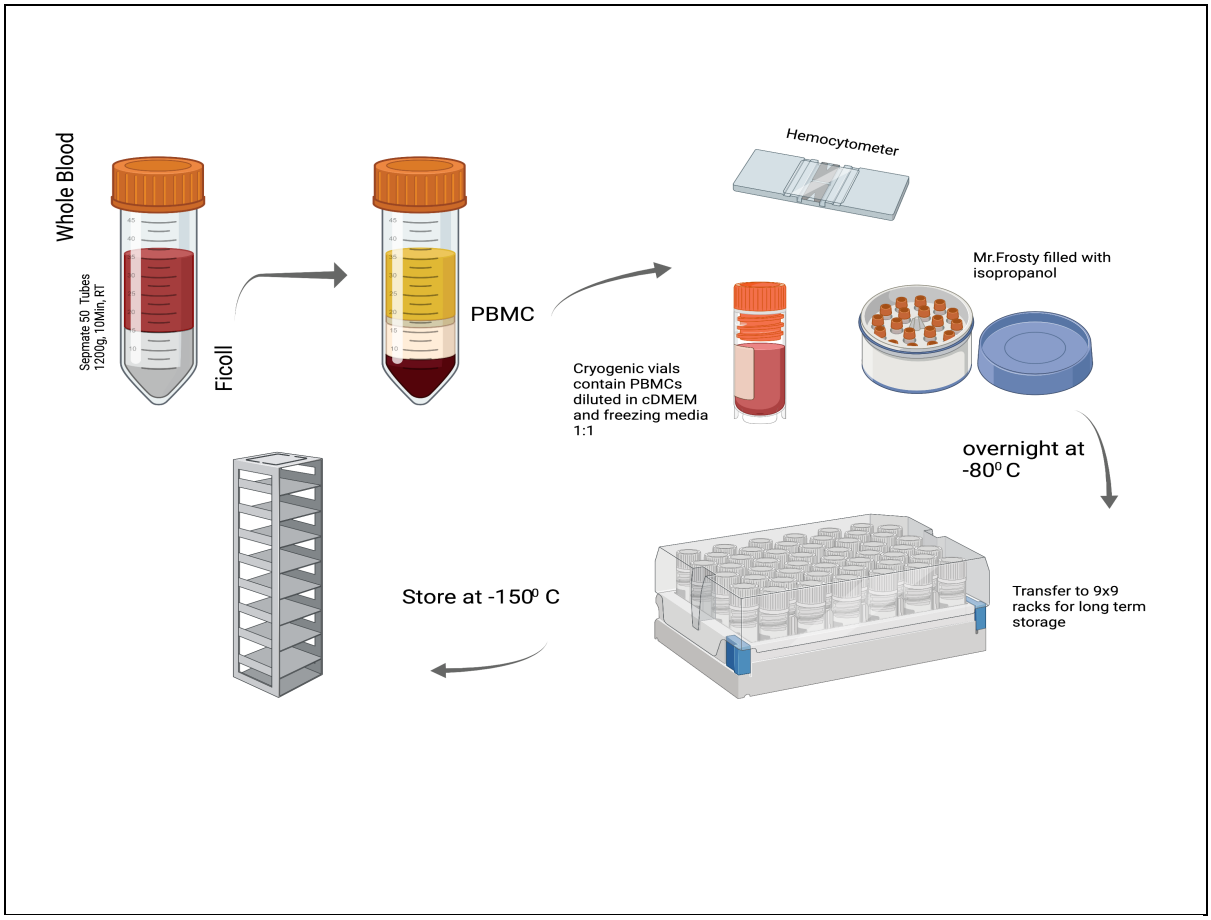



**Figure 2.1 human PBMCs isolation using Sepmate-50 tubes.** Diluted blood was added on Sepmate-50 tube preloaded with Ficoll Paque and centrifuged at 1200g for 10 minutes at room temperature. PBMCs was transferred into new sterile tube, washed twice, and resuspended with freezing media for storage (Created on Biorender.com, 2022).

### 2.2.5 PBMCs thawing process:

Complete DMEM was warmed in the water bath at $37^0$ C, and the cryovials tubes were cleaned with 70% ethanol. Samples were thawed at $37^0$ C no more 2 minutes. The cryovial tubes were cleaned again with ethanol and placed in the class II biosafety cabinet. 1 ml of complete DMEM was added to the cryovials slowly and the diluted and warmed cells transferred into a 15 ml polypropylene centrifuge tube. The tubes were centrifuged at 400 g for 10 minutes at room temperature, the supernatant was decanted, the tubes were flicked to break the pellets up and 10 ml of complete DMEM were added slowly. The tubes were centrifuged as above and the cells were resuspended with 1 ml of media and counted using hemacytometer. Cell suspension (200µl with approximately 800,000 to 1,000,000 cells per well) was added into individual wells of a 96 well U bottom cell culture plate (Greiner, 650180).

### 2.2.6 Analysis of T cells phenotype, monocytes and dendritic cells from human PBMC cells

### 2.2.6.a PBMC cell surface staining assay

After PBMC thawing, the cells were washed once in 200 µl PBS and stained with Aqua live/dead viability fixable dye (Life Technologies, L34957) in PBS (1:1000) for 30 minutes at $4^0$ C. Plates containing cells were then spun at 250 g for 5 minutes and then washed with 200 µl PBS. Cells were spun once more and then resuspended in 50 µl of flow cytometry medium containing 1 µg of Fc block (BD, Biosciences, 10 µg/ml; anti CD16/CD32) for 15 minutes, at room temperature. Two plates were made per sample the first plate was stained with anti-CD3 Alexa Fluor 700 (eBioscience; UCHT1, 56003841), anti-CD4 APC-CY7 (Biolegend; RPA-T4, 300518), anti-CD8a FITC (BD; RPA-T8, 561947), anti-CD19 Brilliant Violet (BV) BV786 (BD,HIB19, 740968), anti-CD56 PE (eBioscience; CMSSB, 12056742), anti-CD45 RA APC (eBioscience; HI100, 17045842), anti-CD45RO BV605 (BD; UCHL1, 562791) anti-CD25 BV 650 (Biolegend; BC96, 302633), anti-CD127 PerCP-Cy 5.5 (BD; HIL-7R-M21, 560551) and anti-CD197 (CCR7) BV421 (Biolegend; G043H7, 353208) diluted in FSM (1:100) and incubated for

30 minutes at $4^0$ C. The second plate was for monocyte/dendritic identification and the cells not required for analysis (i.e. dump cells) were stained with anti-CD3 BV510 (BD; UCHT1, 563109), anti-CD19 BV510 (Biolegend; HIB19, 302242), anti-CD56 BV510 (BD; B159, 740171), and the myeloid cells were identified with anti-CD14 PerCP-Cy 5.5 (BD; M5E2, 561116), anti-CD16 BV650 (BD; 3G8, 563692), anti-HLA-DR APC-H7 (BD; G46-6, 561358), anti-CD123 PE-CF549 (BD; 7G3, 562391), anti-CD1c BV605 (Biolegend; L161, 331538), anti-CD141 APC (BD; 1A4, 564123), anti-CD11c BB515 (BD; B-ly6, 564491). 200 µl FSM was added and then the cells were spun and washed twice with 200 µl FSM at 250 g. Cells were resuspended with FSM:10% formalin (1:1), transferred to flow cytometry tubes and data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

**2.2.6.b Single colour compensation control**

UltraComp eBeads (Invitrogen 01222242) were mixed by using pulse vortixing. Microfuge tubes were labelled and one drop from the beads was added into each tube. Next, 2 µl of each antibody was added and mixed with the beads. The tubes were incubated at $4^0$ C for 15 minutes in the dark. Unstained cells for live/dead discrimination were prepared by mixing unstained cells with pre-heated (i.e., killed) cells ($90^0$ C) and adding 1 µl of aqua viability fixable dye. After the incubation, 1 ml of FSM media was added, and the tubes were centrifuged at 400 g for 5 minutes. Then, the pellets were resuspended within 200 µl of FSM media and transferred to flow cytometry tubes for analysis. Another set of compensation controls was developed using PBMC cells from the same volunteer. A single colour staining for each antibody was used following the same staining protocol described previously.

**2.2.6.c Data acquisition, compensation matrix and sample analysis**

The BD FACSCelesta was set up according to the manufacturers recommendations, and instrument calibration was checked using calibration beads (BD, 655051). Unstained PBMC cells, single-colour stained cells and single stained compensation beads were used to set the PMT voltages and compensation. To achieve this, unstained cells were run to determine the appropriate forward scatter FSC and side scatter SSC setting and PMT voltages for the cells.

Then, each single stained beads were run to assure the positive peak and the same was undertaken with the PBMC cells. For each run, 5000 events were recorded and the compensation was calculated using FACSDiva compensation matrix.

For analysis of the full staining panel, a minimum of $1x10^5$ events were acquired for each sample within lymphocyte gate for lymphoid panel and monocyte gate for monocyte/dendritic cells panel based on FSC and SSC parameters. Flow cytometry standard FSC files were stored at the Cooper Lab R drive at University of Leicester and analysed using FlowJo software. The total percentage of PBMC lymphocyte, monocyte and dendritic cells population was determined by flow cytometry for the healthy volunteers.

### 2.2.7 Jurkat cells stimulated with concanavalin A and PMA-ionomycin

Jurkat cells ($1x10^6$/well) were plated in 24-well culture plate in RPMI in a final volume of 2 ml. Jurkat cells were then either left unstimulated, stimulated with concanavalin A (5 µg/ml) or stimulated with Phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) and ionomycin (500 ng/ml) for 24 hours at $37^0$ C and 5% $CO_2$. After the incubation, the cells were collected into microfuge tubes for flow cytometric processing. Cells were spun at 125 g for 6 minutes. The pellets were flicked and resuspended with 1000 µl PBS containing aqua viability fixable dye. Then, cells were stained firstly with fc block anti-CD16/CD32 for 10 minutes then stained with an antibody cocktail of anti CD69 PE (Biolegend, FN50, 310906), anti-CD25 BV 650 (Biolegend; BC96, 302633), anti-CD127 PerCP-Cy 5.5 (BD; HIL-7R-M21, 560551) and anti CD44 APC/Cyanine 7 (BD, IM7, 103028) diluted in FSM for 30 minutes at $4^0$ C after which the cells were washed twice with FSM media, fixed with 10% formalin, and transferred to flow cytometry tubes. Finally, data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

### 2.2.8 Developing a protocol to stimulate human PBMCs with non-specific or specific T cells antigens for surface staining and intracellular staining for Cytokine's responses:

Using a basic protocol derived from Musvosvi et al, 2018 and Nemes et al, 2018, frozen PBMCs were thawed, spun, and resuspend in complete DMEM media containing 5% fetal bovine serum (FBS). The cells were added into 96 wells U bottom cell culture plate. The plate was labelled as below:

1- **Well 1**: A non-stimulated PBMCs were loaded as negative control.
2- **Well 2**: The cells in were stimulated with Staphylococcus endotoxin B (SEB) 5 µg/ml as positive control.
3- **Well 3**: PBMCs stimulated with Mtb-300 peptides 2 µg/ml.
4- **Well 4**: PBMCs stimulated with Tetanus toxoid 1:100.
5- **Well 5:** PBMCs stimulated with Anti CD3/CD28 to target CD4 T cells.

Cells were incubated at 37°C in 5% $CO_2$ for. After 2 hours, both Golgi stop (BD, CAT. 554724) and Golgi plug (BD, CAT. 555022) were added and cells left to incubate for a total of 6 hours. After the stimulation, prior to staining, the cells were washed once in 200 µl 1x PBS at 250 g for 5 minutes and then stained with Aqua live/dead viability fixable dye (Life Technologies) in PBS (1:1000) for 30 minutes at $4^0$ C and then washed. Cells were then resuspended in 50 µl of flow cytometry medium (PBS supplemented with BSA, 1%) containing 1 µg of Fc block (BD, Biosciences, 10 µg/ml; anti CD16/CD32) for 15 minutes, at room temperature. Cells were then stained with anti-CD3 PE-CF594 (BD; UCHT1, 562310), anti-CD4 APC-eFluor780 (eBioscience; RPA-T4, 47-0049-41), anti-CD8a V500 (BD; RPA-T8, 560775), anti-CD27 APC (BD; M-R271, 561786) and anti-HLA-DR FITC (Biolegend; L243, 307603) antibodies diluted in FSM (1:100) and incubated for 30 minutes at $4^0$ C. Cells were washed twice with FSM at 250 g for 5 minutes. Cells were fixed and permeabilized using BD fixation/permeabilization solution (BD Cytofix/Cytoperm kit, BD 554714) for 20 mins at $4^0$ C. After that, cells were washed in BD Perm/Wash buffer (BD Cytofix/Cytoperm kit, BD 554714, 1:10 diluted in sterile water), then stained with anti-IFN$\gamma$ V450 (BD; B27, 560372), anti-IL-2 PE (BD; MQ1-17H12, 560709) and TNF-$\alpha$ BV650 (BD, MAB11, 563418) antibodies diluted in BD Perm/Wash buffer for 30 mins at $4^0$ C. Cells were washed twice with BD Perm/Wash buffer at 250 g for 5 minutes, resuspended with FSM:10% formalin (1:1), transferred to flow cytometry tubes and data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

### 2.2.9 Fresh vs frozen PBMC

To determine the difference between stimulation of fresh cells from those which are frozen, PBMCs were isolated as described above. Fresh cells were added to 96 well U bottom cell culture plate. The remaining cells were resuspended in freezing media and stored at -150$^0$ C to be used later as frozen PBMCs. The cells were thawed and stimulated as described above. Live/dead viability, cell surface and intracellular staining was performed as described above. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

### 2.2.10 Whole blood vs PBMC

Whole blood assay was adapted from the protocol reported in Hanekom et al, 2004, 0.5 ml of heparinzed whole blood were added to 15 ml polypropylene tubes. 5 µl of CD28/CD49d monoclonal antibody (BD, CAT. 347690) was added into each tube. The tubes were labelled as below:

1- **Well 1**: A non-stimulated PBMCs were loaded as negative control.
2- **Well 2**: The cells in were stimulated with Staphylococcus endotoxin B (SEB) 5 µg/ml as positive control.
3- **Well 3**: PBMCs stimulated with Mtb-300 peptides 2 µg/ml.
4- **Well 4**: PBMCs stimulated with Tetanus toxoid 1:100.
5- **Well 5:** PBMCs stimulated with Anti CD3/CD28 to target CD4 T cells.

The tubes were incubated for 2 hours at 37$^0$ C. 10 µl of diluted stock Brefeldin A 1:10 (BD, CAT. 347688) was added to each tube, vortexed gently and incubated for additional 4 hrs. Then, 50 µl of EDTA solution (BD, CAT. 347689) in PBS were added to each tube. The tubes were vortexed vigorously and incubated for 15 minutes at room temperatures, then vortexed for 10 seconds at high setting. To lyse the cells, 100 µl from each activated tube were aliquoted into new 15 ml tubes. 1 ml of 1X BD FACS lysing solution (10X Diluted it DI water before use at final concentration 1:10, BD, CAT. 349202) were added to each tube, mixed well, and incubated for 10 minutes at room temperature. 2 ml of washing buffer were added to each tube and the cells were centrifuged at 500 g for 5 minutes at room temperature twice. The pellets were stained with live/dead stain and fc block as described above. Cell surface staining were performed for each tube then washed twice as described above. The cells were

fixed and permeabilized for intracellular staining as above. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

To compare the fresh whole blood cells to frozen cells, PBMCs were thawed, spun, and resuspend in complete DMEM media containing 5% FBS. The cells were added in 96 wells U plate. The cells were stimulated as described above. Live/dead viability, cell surface and intracellular staining was performed as described above. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

### 2.2.11 The effect of anti-costimulatory antibodies to induce immune response

To compare the effect of using co-stimulatory antibody to induce immune response in effector cells when antigen presentation cells (APC) are used in culture, PBMCs were thawed and stimulated with antigens as described above and 2 µl per well co-stimulatory antibodies (CD28 and CD49d, BD, 347690, 100 µg/ml) for an initial two hours period. Brefeldin A (BFA) was then added to inhibit the secretion of newly synthesized cytokines and cells were incubated an additional four hours. In the second assay, PBMCs were thawed and stimulated with antigens as described above for initial two hours. Brefeldin A (BFA) was then added, and cells were incubated an additional four hours. In the third assay, PBMCs were thawed and stimulated with antigens as described above for initial two hours. Golgi plug and Golgi plus was then added, and cells were incubated an additional four hours. Cells then stained with Aqua live/dead viability fixable dye, washed then stained with cell surface antibodies as described above. Cells were then washed and permeabilized using BD Cytofix/Cytoperm then stained with antibodies target cytokines IL-2, TNF-$\alpha$ and IFN$\gamma$. Finally, the cells are washed and fixed for analysis on a flow cytometer. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

### 2.2.12 Fluorescence minus one control

To build multicolour flow cytometry panels, the fluorescence minus one (FMO) control is important as it determines where the gates should be set. This is particularly important

when identifying a positive from a negative population and when the expression levels are low. When acquiring data, there is fluorescence spread, especially with brighter fluorophores, which is particularly noticeable after compensation and cross-laser excitation. Careful experimental design and avoiding channels which have a large amount of spreading will help reduce this influence, but FMO controls are still important. FMO controls are the experimental cells stained with all the fluorophores minus one fluorophore.

To test FMO controls on gating cytokines, PBMCs were thawed and stimulated with antigen as described previously. Following the stimulation, the cells were followed the protocol procedures (section 2.2.8) for intracellular staining for cytokines detection. The well number 4, which represent the FMO control and stimulated with SEB, remain unstained with anti IL-2, anti TNF$\alpha$ and anti IFN$\gamma$ antibodies. In addition, the FMO control for cells stimulated with Mtb-300 were obtained to test gating for antigen specific T cell responses. The final FMO control was prepared for CD25 in lymphocyte panel. Similarly, the FMO controls for cells stimulated with Mtb-300 were tested. Cells were thawed and cell surface stained as described in (section 2.2.6.a). The FMO control tube remain unstained with anti CD25 antibody.

### 2.2.13 Developing a final protocol to stimulate human PBMCs with Mtb secretory antigens for intracellular staining for cytokines detection using Co-Stimuli for ISATS study participants:

Frozen peripheral blood mononuclear cells (PBMC) were thawed, spun, and resuspend in complete DMEM media containing 5% FBS. The cells were added in 96 wells U bottom cell culture plate. The plate was labelled as below:

1- **Well 1**: A non-stimulated PBMCs were loaded as negative control.
2- **Well 2**: The cells in were stimulated with Staphylococcus endotoxin B (SEB) 5 µg/ml as positive control.
3- **Well 3**: PBMCs stimulated with Mtb-300 peptides 2 µg/ml.

4- **Well 4**: PBMCs stimulated SEB as fluorescent minus one FMO control.

2 µl of anti-Costimulatory CD28/CD49d antibodies were added to each well then, the cells were incubated at 37°C in 5% $CO_2$ for 6 hours. Brefeldin A was added 2hrs after the stimulation began and remained to complete 6 hours. After the stimulation, prior to staining, the cells were washed once in 200 µl 1x PBS at 250 g for 5 minutes and then stained with Aqua live/dead viability fixable dye (Life Technologies) in PBS (1:1000) for 30 minutes at $4^0$ C and then washed. Cells were then resuspended in 50 µl of flow cytometry medium PBS supplemented with BSA, 1%) containing 1 µg of Fc block (BD, Biosciences, 10 µg/ml; anti CD16/CD32) for 15 minutes, at room temperature. Cells were then stained with an antibody cocktail CD3, CD4, CD8, HLA-DR and CD27 diluted in FSM (1:100) and incubated for 30 minutes at $4^0$ C. Cells were washed twice with FSM at 250 g for 5 minutes. Cells were fixed and permeabilized (cytofix/cytoperm) for 20 mins at $4^0$ C, cells were then washed in BD Perm/Wash buffer) and stained with anti IFN-$\gamma$, IL-2, and TNF-$\alpha$ antibodies diluted in BD Perm/Wash buffer for 30 mins at $4^0$ C. Finally, the cells will be washed twice with BD Perm/Wash buffer, resuspended with FSM:10% formalin (1:1), transferred to flow cytometry tubes and data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

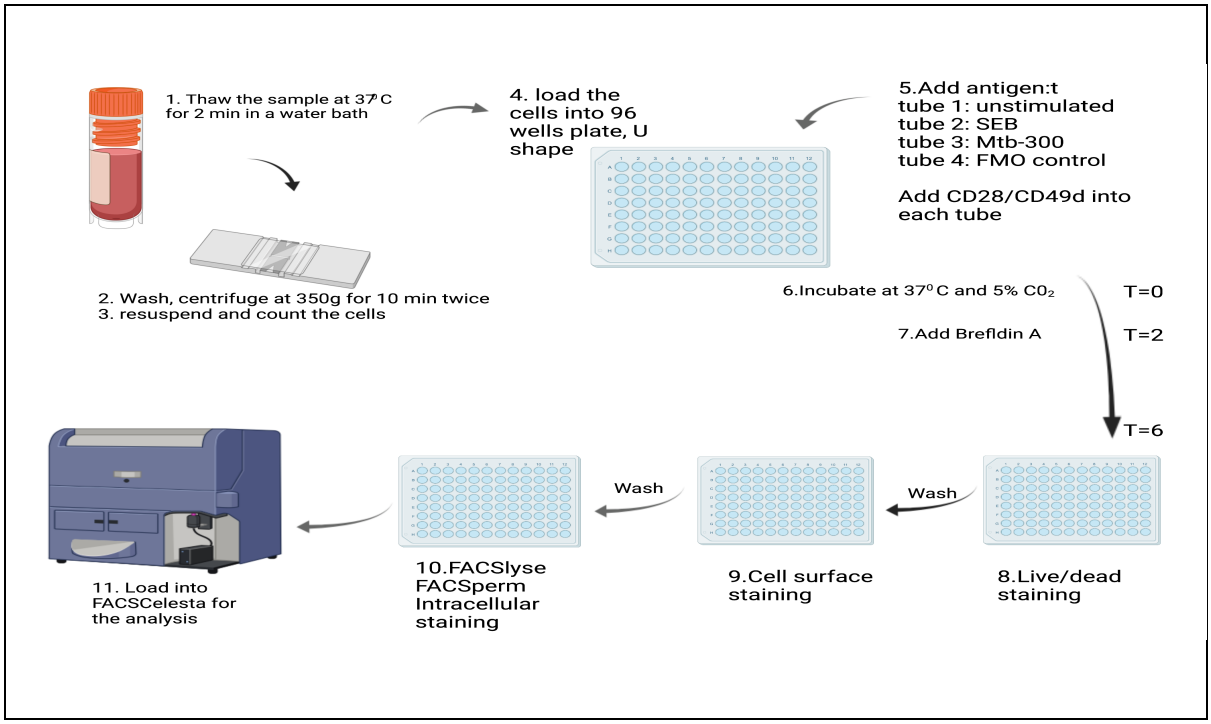



**Figure 2.2. Intracellular cytokines detection following antigen stimulation.** PBMCs were stimulated with SEB 5µg/ml as positive control, second well with Mtb-300 2µg/ml, third well remain unstimulated as negative control and the fourth well with SEB as FMO control for 6 hours at 37$^0$C. Co-stimuli CD28/CD49d were added to each well. Brefeldin A was added two hrs. after the stimulation. Cells were washed then stained with live/dead stain for 30 minutes followed by cell surface staining for 30 minutes. Then, cells were washed and permeabilized for 20 minutes followed by intracellular staining for 30 minutes. Cells were fixed by using 10% formalin and analysed using flow cytometry. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

### 2.2.14 Developing a protocol to stimulate human PBMCs with Mtb secretory antigens to induce activation induced markers (AIM):

Frozen peripheral blood mononuclear cells (PBMC) were thawed, spun, and resuspend in complete DMEM media containing 5% FBS. The cells were added in 96 wells U plate and non-stimulated PBMCs were loaded as negative control, the second well were stimulated with Staphylococcus endotoxin B (SEB) 5 µg/ml as positive control, and third well stimulated with Mtb-300 peptides 2 µg/ml and the fourth well stimulated SEB as fluorescent minus one FMO control. Cells were incubated overnight (around 18-20 hours) at 37°C in 5% $CO_2$ to induce activation markers. After the incubation, live/dead stain was added and incubated for 30 min at $4^0$ C. Cells were washed and stained with Fc block CD16/CD32 for 15 min at room temperature. Then, cells were stained with anti-CD3 Alexa Fluor 700 (Bioscience; UCHT-1, 56-0038-42), anti-CD4 APC-eFluor780 (eBioscience; RPA-T4, 47-0049-41), anti-CD8 V500 (BD, RPA-8, 560775), anti-CD19 V500 (BD; H1B19, 561125), anti-CD14 V500 (BD, M5E2, 561392), anti CD25 FITC (BD; M-A251, 560990), anti-CD45 RA ef450 (Bioscience; HI100, 48-0458-41), ani CCR7 PerCPCY5.5 (Biolegend; 6043H7, 353241), anti OX40 APC (Biolegend; BER-ACT35, 350007) and PD-L1 PE (Biolegend; 29E.2A3, 329705) and incubated for 30 min at $4^0$ C. Cells were washed twice with FSM, fixed by using 10% formalin and run at flow cytometry. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

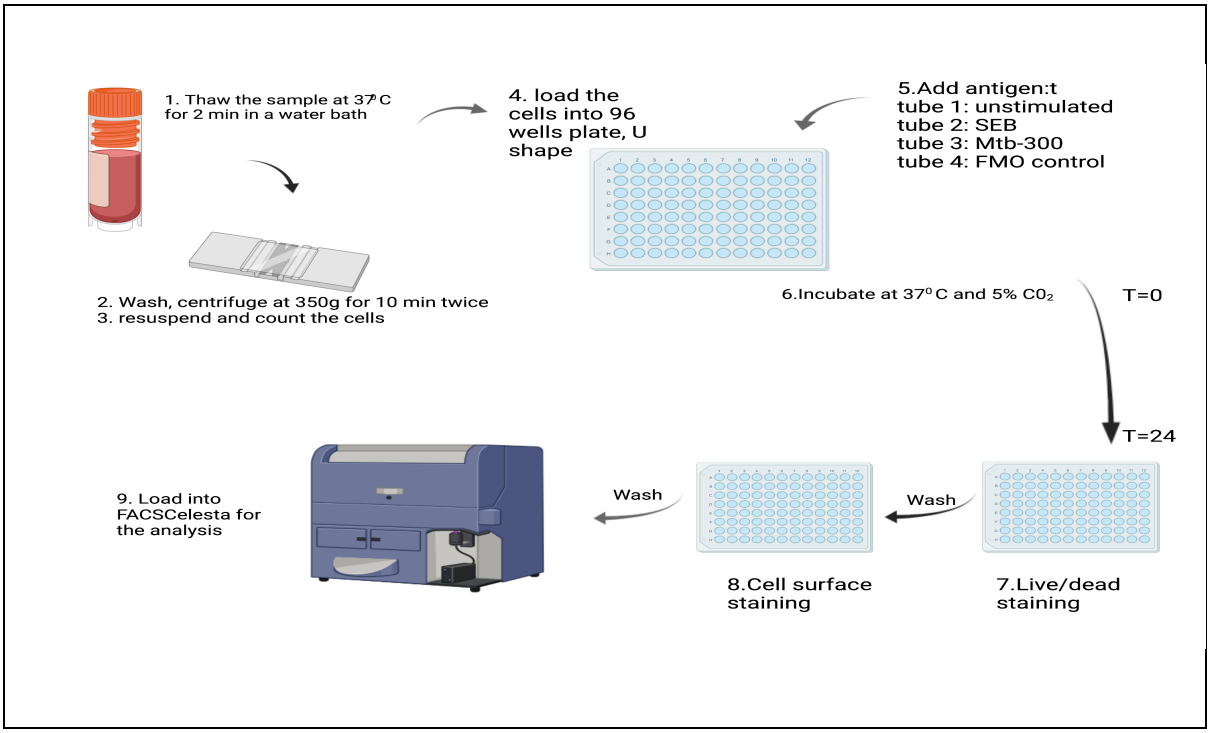



**Figure 2.3. Activation induced marker detection following antigen stimulation.** PBMCs were stimulated with SEB 5µg/ml as positive control, second well with Mtb-300 2µg/ml, third well remain unstimulated as negative control and the fourth well with SEB as FMO control for 24 hours at 37⁰C. Cells were washed then stained with life/dead stain for 30 minutes followed by cell surface staining for 30 minutes. Cells were fixed by using 10% formalin and run at flow cytometry. Data was acquired using a FACSCelesta (BD) and analysed with FlowJo software.

## 2.3 Results

### 2.3.1 Analysis of T cell phenotype and function from human PBMC cells

#### 2.3.1.a Phenotypic identification of lymphocyte

To identify individual cell populations, cells were gated based on FSC and SSC and subsequent gating. Firstly, dead cells were excluded, and single cells were selected (Figure 2.4.A and 2.4.B). Then CD3+ CD19- representing T lymphocyte and CD3- CD19+ representing B lymphocyte were identified by using quadrant gate showing CD19 vs CD3 (figure 2.4.C). Then, CD3 population was selected and from this gate both CD4+ T helper cells and CD8+ T cytotoxic cells were identified. For CD3+ CD4+ cells, a quadrant gate was used to define CD45RO and CD45RA cells (Figure 2.4.F). CD45RA represents naïve CD4+ cells, while CD45RO represent memory CD4+ cells. The same gating was used on CD3+ CD8+ cells (figure 2.4.I). For both CD4 and CD8 the expression of CD45RA (naïve or memory) and CD197 (CCR7, ability to enter lymph node i.e., centrally circulating) was determined (figures 2.4.E, 2.4.G and 2.4.J). The expression of CCR7 (CD197) and CD45RA gated on CD4+ and CD8+ was examined to represent T helper naïve (CD4+ CDRA45+ CD197+), T helper effector (CD4+ CD45RA+CD197-), T cytotoxic naïve (CD8+ CDRA45+ CD197+), T cytotoxic effector (CD8+ CD45RA+ CDAA97-). Also, at the same gates both effector memory (EM) and central memory (CM) can be detectable by T helper EM (CD4+ CDRA45-CD197-), T helper CM (CD4+ CD45RA-CD197+), T cytotoxic EM (CD8+ CDRA45-CD197-), T cytotoxic CM (CD8+ CD45RA- CD197+). Then, regulatory T cells were identified by CD4+ CD25+ CD127- (figure 2.4.H). Finally, natural killer NK cells were identified by the expression of the NK marker (CD56) in the absence of CD3 CD19 (figure 2.4.D).

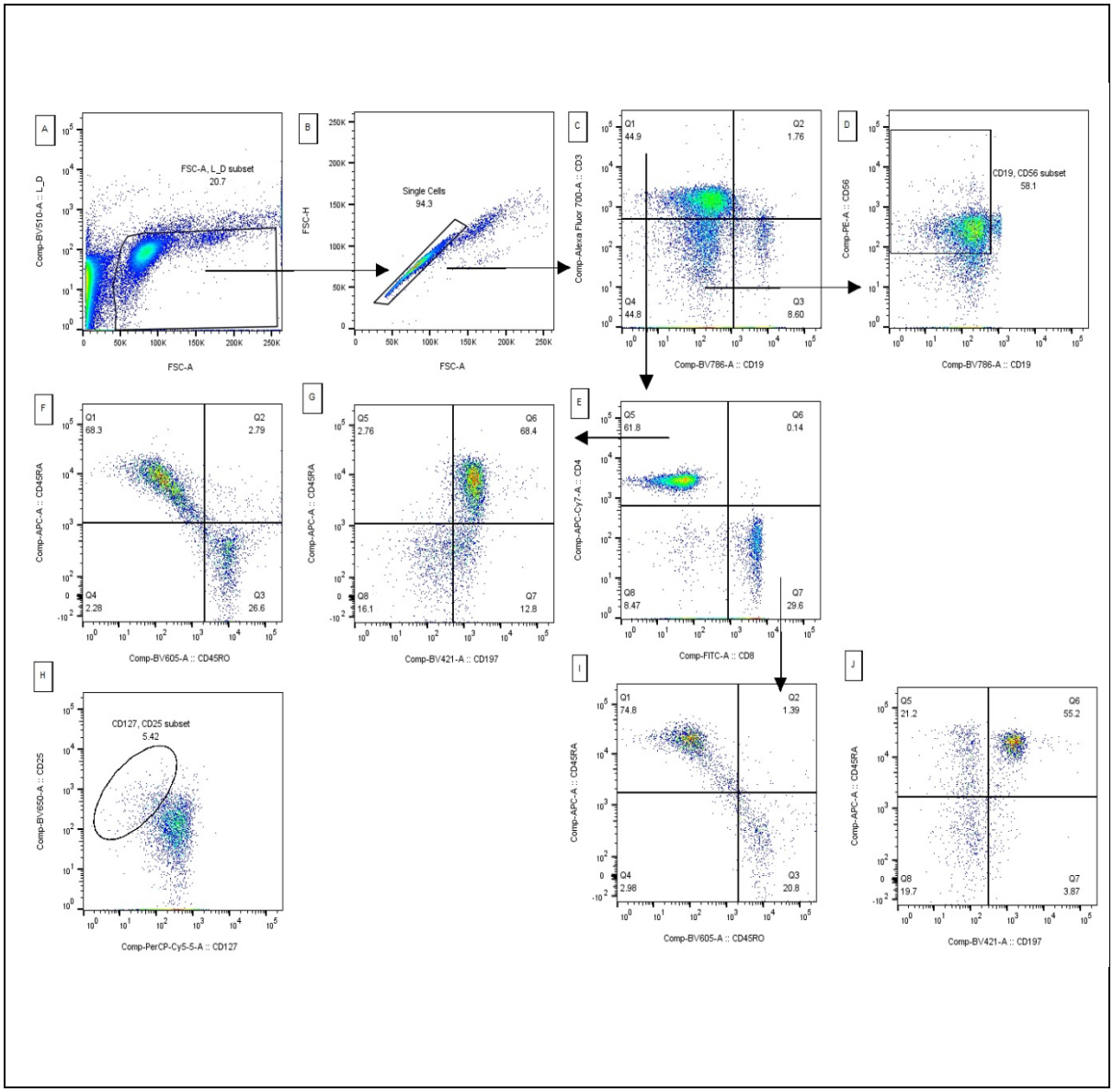



**Figure 2.4. Analysis of lymphocyte population from PBMC using flow cytometry.** First, cells were gated based on FSC and SSC, dead cells were excluded, and single cells selected (A and B). quadrant gate was developed to define CD3+ and CD19+ to define T lymphocyte and B lymphocyte respectively (C). Natural killer NK cells gated as CD3-CD19-CD56+ (D) Gated on CD3+CD19-, a quadrant gate was developed to define CD4+ and CD8+ (E). Gated on CD4+, a quadrant gate was developed to define CD45RA (naïve CD4+ cells) and CD45RO (memory CD4+ cells (F). gated on CD4+ a quadrant gate was developed to define CD45RA- CD197+ (T central memory) and CD45RA- CD197- (effector memory) (G). CD4+ CD25+ CD127- was selected to define CD4+ Treg on CD4+ cells (H). Gated on CD8+, a quadrant gate was developed to define CD45RA (naïve CD8+ cells) and CD45RO (memory CD8+ cells (I). gated on CD8+ a quadrant gate was developed to define CD45RA- CD197+ (T central memory) and CD45RA- CD197- (effector memory) (J).

### 2.3.1.b Phenotypic identifying for monocyte cell surface markers

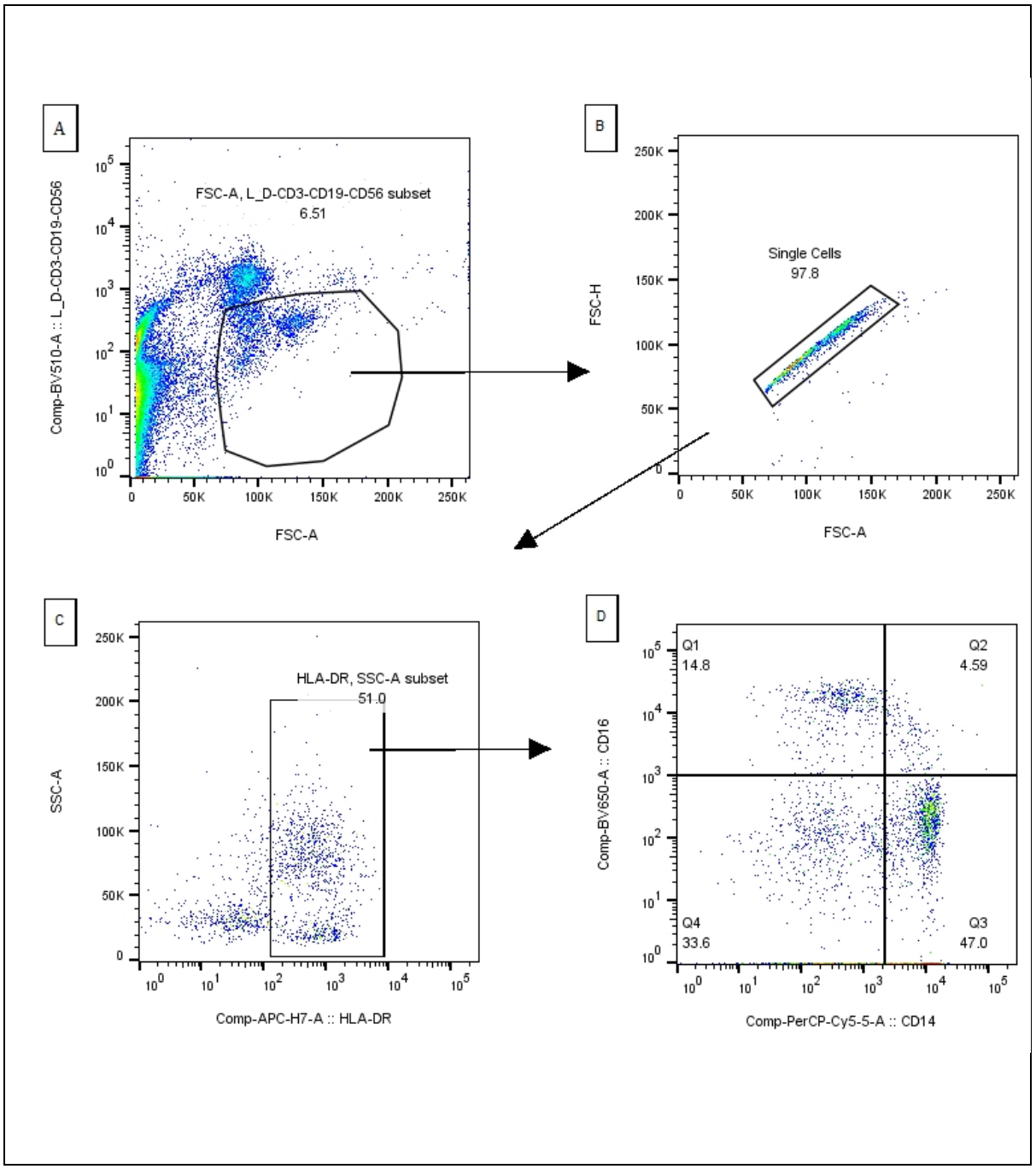



**Figure 2.5. Analysis of monocyte population from PBMC using flow cytometry.** First, cells were gated based on FSC and SSC. Dead cells, CD3+, CD19+ AND CD56+ were excluded (A). Single cells were selected (B). Then, HLA-DR + were selected (C). Three monocyte subsets were gated based on the expression of CD14 and CD16 (D).

To identify monocyte populations, cells were gated based on FSC and SSC with large and granular cells being selected. Figure 2.5 represents the gating strategy used to identify monocyte subsets from PBMC cells. First of all, dead cells, CD3+, CD56+ and CD19+ were

excluded. After that, single cells were selected. Human leucocyte antigen DR (HLA-DR) was selected gated on SSC-A and the expression of surface marker HLA-DR+. Then, a quadrant gate was developed to define classical monocyte subsets (CD14+CD16-), intermediate monocyte subsets (CD14+CD16+), and non-classical monocyte subsets (CD14-CD16+) gated on HLA-DR+.

### 2.3.1.c Gating strategy to identify dendritic cell surface markers

To identify dendritic cells populations, cells were gated based on FSC and SSC, dead cells, CD3+, CD56+ and CD19+ were excluded and single cells were selected (Figure 2.6). Dendritic cells were gated as CD14- HLA-DR+ to avoid monocyte subsets. Cells which are CD14- HLA-DR+, were then characterized as CD11c+ CD123- myeloid dendritic cells (mDC) and CD11C-CD123+ as plasmocytic dendritic cells (pDC) (Autissier et al; 2010). Then mDC were further distinguished by their expression of either CD11c+ CD141+ or CD1c+. Figure 6 represents the gating strategy used to identify dendritic cells subsets from PBMC cells.

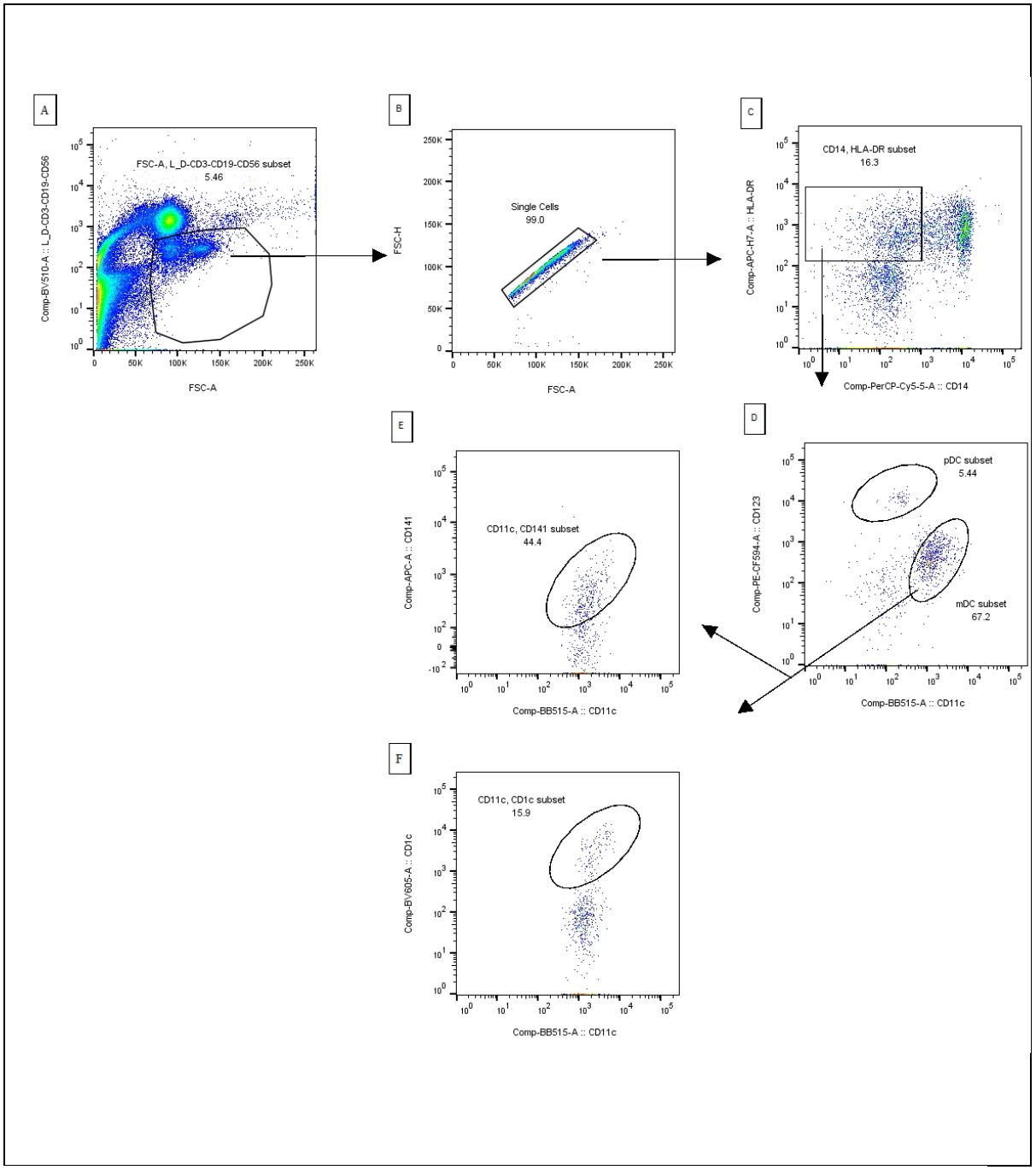



**FIGURE 2.6. Determination of myeloid populations in PBMC population from PBMC using flow cytometry.** First, cells were gated based on FSC and SSC. Dead cells, CD3+, CD19+ AND CD56+ were excluded (A). Single cells were selected (B). HLA-DR+CD14- was selected (C). plasmocytic dendritic cells pDC and myeloid dendritic cells mDC gated based on the expression of CD123 and CD11c respectively (D). mDC subsets were distinguished based on CD141 and CD1c (E) and (F).

### 2.3.2. Human PBMC characterization

Using the above panels, a number of healthy volunteer samples were analyzed and the reproducibility of the compensation settings and FMOs was assessed. Table 2.1 shows the phenotypes of the T cells, B cells, monocytes, NK cells and dendritic cells, in which we are interested to determine in our well characterized cohorts of healthy, latent and active TB subjects in chapter 3.

**Table 2.1. Human PBMC cells characterization**

| | |
|---|---|
| **Total Lymphocyte** | |
| **T cells** | **T cytotoxic Effector memory** |
| Live single CD3+CD19- | Live single CD3+ CD8+ CDRA45- CD197- |
| **T helper Cells** | **T cytotoxic Central memory** |
| Live single CD3+CD4+CD8- | Live single CD3+ CD8+ CD45RA- CD197+ |
| **T cytotoxic cells** | |
| Live single CD3+CD4-CD8+ | |
| **B cells** | |
| Live single CD3- CD19+ | **Monocyte** |
| **Natural killer** | Live single HLA- DR+ CD14+CD16- |
| Live single CD3- CD19- CD56+ | Live single HLA- DR+ CD14+CD16+ |
| **Naïve T cells** | Live single HLA- DR+ CD14-CD16+ |
| Live single CD3+ CD4+ CD45RA+ | |
| Live single CD3+ CD8+ CD45RA+ | |
| **Memory T cells** | **Dendritic cells** |
| Live single CD3+ CD4+ CD45RO+ | Live single HLA-DR+ CD14- |
| Live single CD3+ CD8+ CD45RO+ | Live single HLA-DR+ CD14- CD123+CD11C- |
| **T regulatory cells** | **mDC CD123-CD11C+** |
| Live single CD3+ CD4+ CD25+ CD127- | Live single HLA-DR+ CD14- CD11c+CD141+ |
| **T helper Effector Memory** | Live single HLA-DR+ CD14- CD11c+CD1c+ |
| Live single CD3+ CD4+ CDRA45-CD197- | |
| **T helper Central Memory** | |
| Live single CD3+ CD4+ CD45RA-CD197+ | |

### 2.3.3 Jurkat cells stimulated with concanavalin A and PMA-ionomycin

**Table 2.2. comparison the frequency of cells with high expression of surface marker between unstimulated and stimulated Jurkat cells**

| | Marker | | | |
|---|---|---|---|---|
| **Stimuli** | **Live single CD44+** | **Live single CD25+** | **Live single CD69+** | **Live single CD127+** |
| Unstimulated | 0.81% | 0.27% | 62.30% | 0.56% |
| concanavalin A | 2.25% | 10.40% | 96.30% | 7.77% |
| PMA - ionomycin | 7.64% | 1.70% | 95.60% | 12.10% |

We wanted to identify potential positive controls. To do this we used a cell line to begin the process. We chose stimuli based on the literature and we aimed to assess the changed expression of specific surface markers on Jurkat cells, which are an immortal human T cell line. We found that two known T cell activators had different effects on these cells (Table 2.2). The graphs in figures 2.7 and 2.8 show the variable effects of these stimuli on stimulated Jurkat cells. For example, while PMA/ionomycin which activated protein kinase C directly and bypasses the T cell receptors drives upregulation of CD44. In contrast concanavalin A which irreversibly binds glycoproteins to drive T cell activation does not. In contrast, concanavalin A drives upregulation of CD25 whereas PMA/ionomycin does not. The upregulation of CD69 and CD127 appear similar between the two stimuli. From these variable and artificial data, we decided to pursue a more natural approach using healthy control PBMCs and a more T cell receptor specific stimulus.

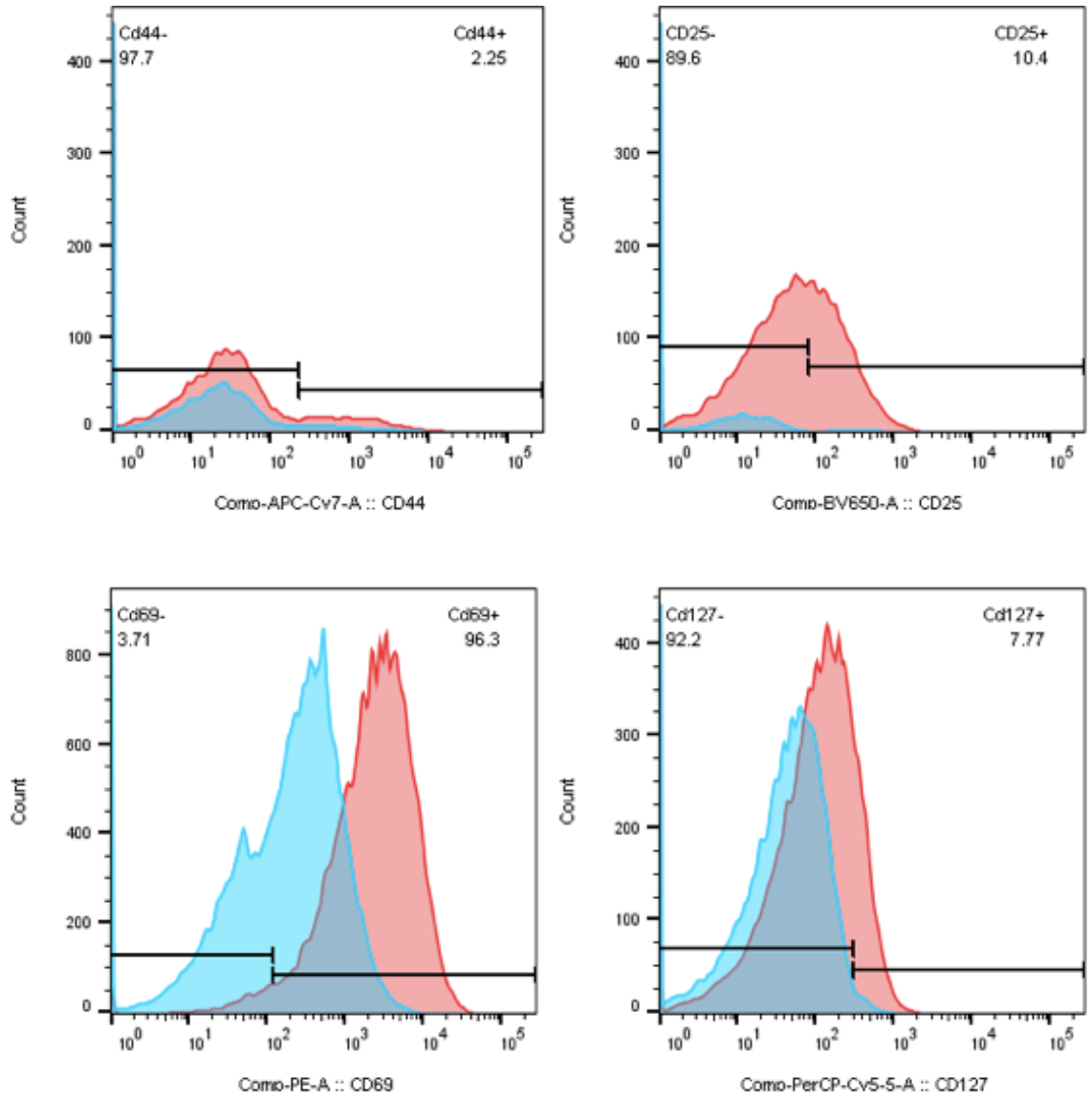



**Figure 2.7. Expression of surface markers by Jurkat cells stimulated with concanavalin A**. The frequency of cells expressing a specific level of surface marker CD44 (top left), CD25 (top right), CD69 (bottom left) and CD127 (bottom right) is represented by the bar and number in panels. The impact of the stimulation can be seen by comparing the blue (unstimulated) curve with the stimulated curve (red).

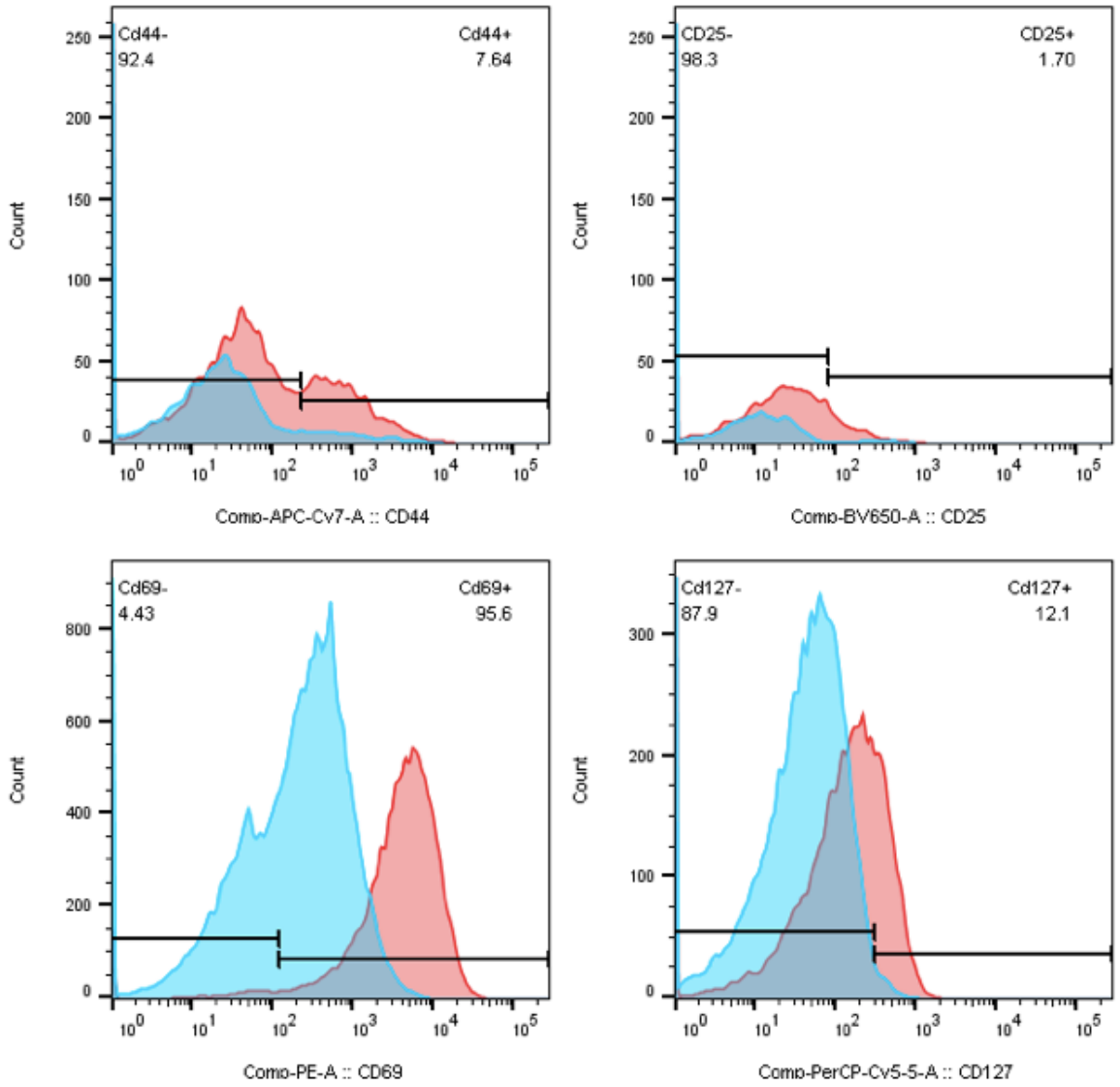



**Figure 2.8. expression of surface markers by Jurkat cells stimulated with PMA/inomycin**. The frequency of cells expressing a specific level of surface marker CD44 (top left), CD25 (top right), CD69 (bottom left) and CD127 (bottom right) is represented by the bar and number in panels. The impact of the stimulation can be seen by comparing the blue (unstimulated) curve with the stimulated curve (red).

### 2.3.4. Gating strategy to detect cytokines released from PBMCs stimulated with SEB

In order to focus on stimulation related to MHC and TCR interactions (rather than nonspecific stimuli) we chose to use SEB, which provides signal 1 and signal 2 to the T cells in a mixed cell culture to induce T cell activation. To do this PBMCs were stimulated with SEB (5µg/ml) for 6 hours at $37^0$C. Golgi plug, and Golgi stop were added two hours after the incubation to ensure sufficient accumulation of protein within the cell. At the end of 6 hours, the cells were stained and analysed using flow cytometry (see panels A-C figure 2.9). CD4+ T lymphocytes were identified using the quadrant gate showing CD3+ CD4+ (figure 2.9.D) and their expression of IL-2, IFN$\gamma$ and TNF-α determined (see panels E-G figure 2.9). The expression of HLA-DR on CD4+ IFN$\gamma$ + was also determined (Figure 2.9.H).

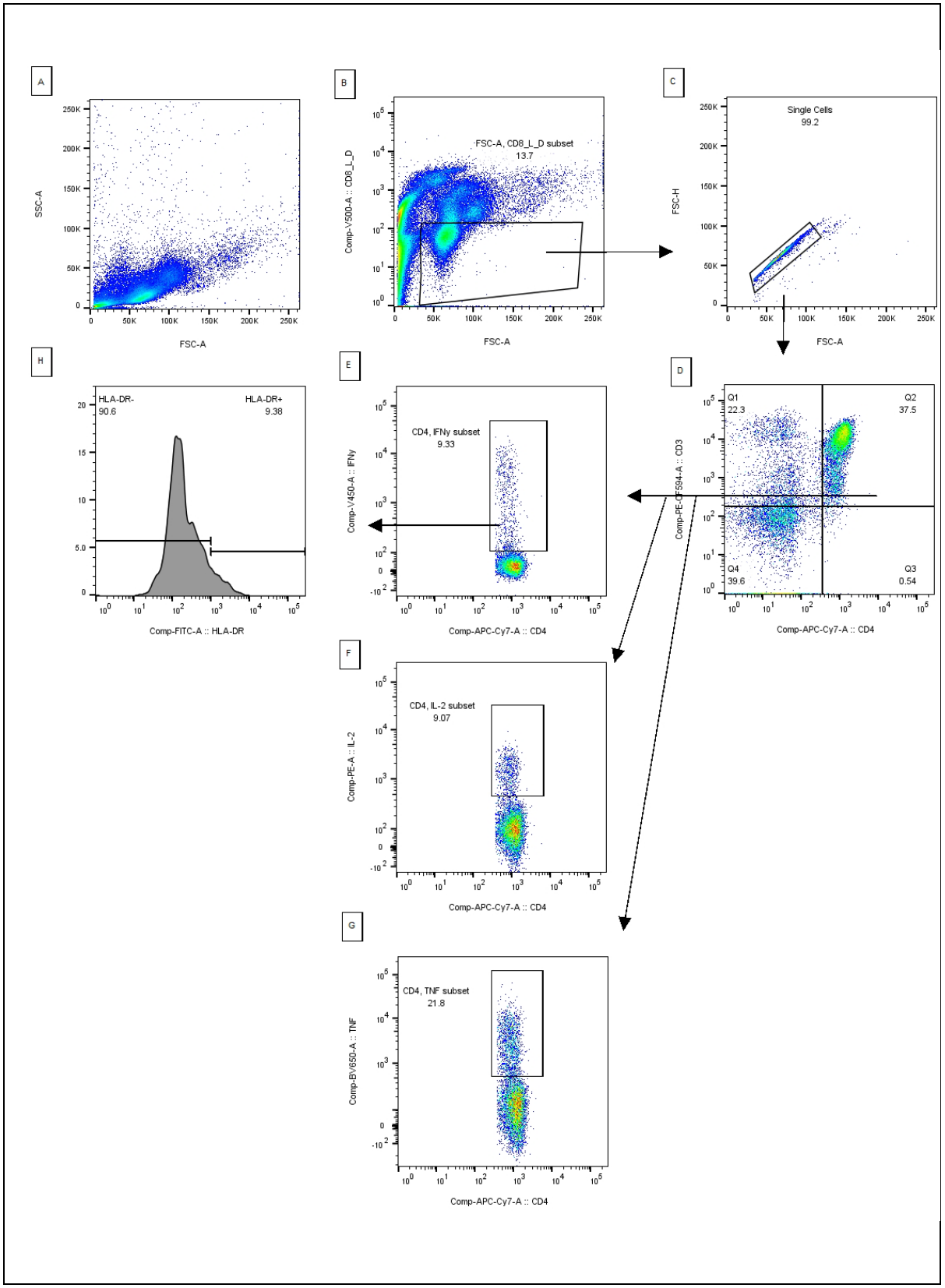



**Figure 2.9. Gating strategy to detect cytokines release.** PBMCs was stimulated with SEB 5µg/ml for 6 hours at 37$^0$C. Golgi plug, and Golgi stop were added two hrs. after the stimulation. Cell's acquisition done on BD FACSCelesta and data analysis on FlowJo software. After dead cells discrimination and single cell selection (A, B and C), CD3+ and CD4+ were gated in (D). live single CD3+ CD4+ is IFN$\gamma$ (E). live single CD3+ CD4+ is IL-2 (F). live single CD3+ CD4+ is TNF-α (G) live single CD3+ CD4+ is IFN$\gamma$+ expressing HLA-DR (H).

### 2.3.5 Gating strategy to characterize activation markers from PBMCs stimulated with SEB

In addition to the cytokine expression in cells we wanted to look at surface expression of activation markers. Firstly, dead cells, cytotoxic T cells, B cells and monocytes were excluded using a dump channel for dead, CD8, CD19 and CD14 expressing cells (figure 2.10.B). Single cells were selected (figure 10.C) and gated for CD3+ CD4+ (figure 2.10.D). The expression of CCR7 (CD197) and CD45RA on the CD4+ CD3+ was determined and represents naive T helper (CD4+ CDRA45+ CD197+), effector T helper (CD4+ CD45RA+CD197-), as well as effector memory (EM) T cells (CD4+ CDRA45-CD197-), and central memory (CM) T cells (CD4+ CD45RA-CD197+) as shown in figure 2.10.E. The expression of the activation induced markers, OX40+CD25+ and PD-L1+ CD25+ were also detected on central and effector memory T cells (see panels F-I figure 2.10.).

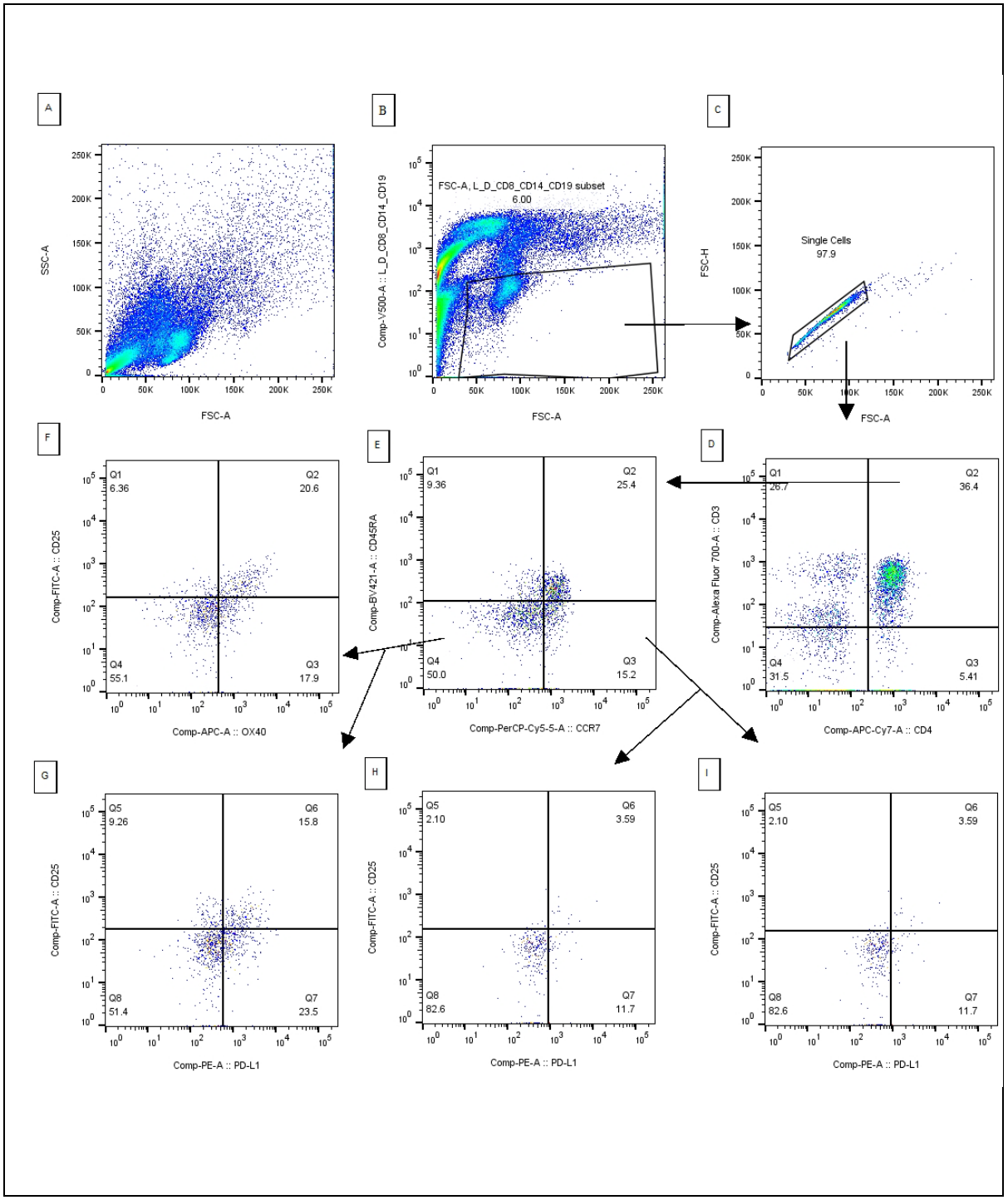



**Figure 2.10. gating strategy to evaluate the expression of activation markers during the stimulation.** PBMCs were stimulated with SEB 5µg/ml for 24hrs at 37° C. a cell surface staining was prepared to detect the activation markers CD25, PD-L1 and OX40. Cell's acquisition done on BD FACSCelesta and data analysis on FlowJo software. after dead cells, B cells and monocytes discrimination, the single cells were selected and CD3+ CD4+ population were gated (A, B, C and D). A quadrant gate was developed to gate both live single lymphocytes are CD4+ CCR7+ CD45RA- and live single lymphocytes are CD4+ CCR7- CD45RA- (E). CD25+, OX40+ and PD-L1+ population gated on CCR7- CD45RA – (F and G) and CCR7+ CD45- (H and I). The figure was created on biorender.com.

### 2.3.6 Developing a protocol to stimulate human PBMCs with antigens for surface staining and intracellular staining for Cytokine's detection:

Human PBMCs were isolated from healthy volunteers at University of Leicester. The cells were stimulated with the TCR T cell stimulus SEB or anti CD3/CD28 and specific T cell stimuli Mtb-300 or Tetanus toxoid. The aim of this is to investigate how best to stimulate, stain and gate markers of T cell activation and cytokines production in human PBMC. Figure 2.11 represents CD4+ T cells IFN$\gamma$ production when PBMCs are stimulated with antigens. Our data shows that both SEB and anti CD3/CD28 derives strong IFN$\gamma$ production compared to Mtb-300 and Tetanus toxoid. Similarly, IL-2 and TNF-$\alpha$ were highly produced when cells stimulated with non-specific stimuli compared to those cells stimulated with specific stimuli figures 2.12 and 2.13 respectively.

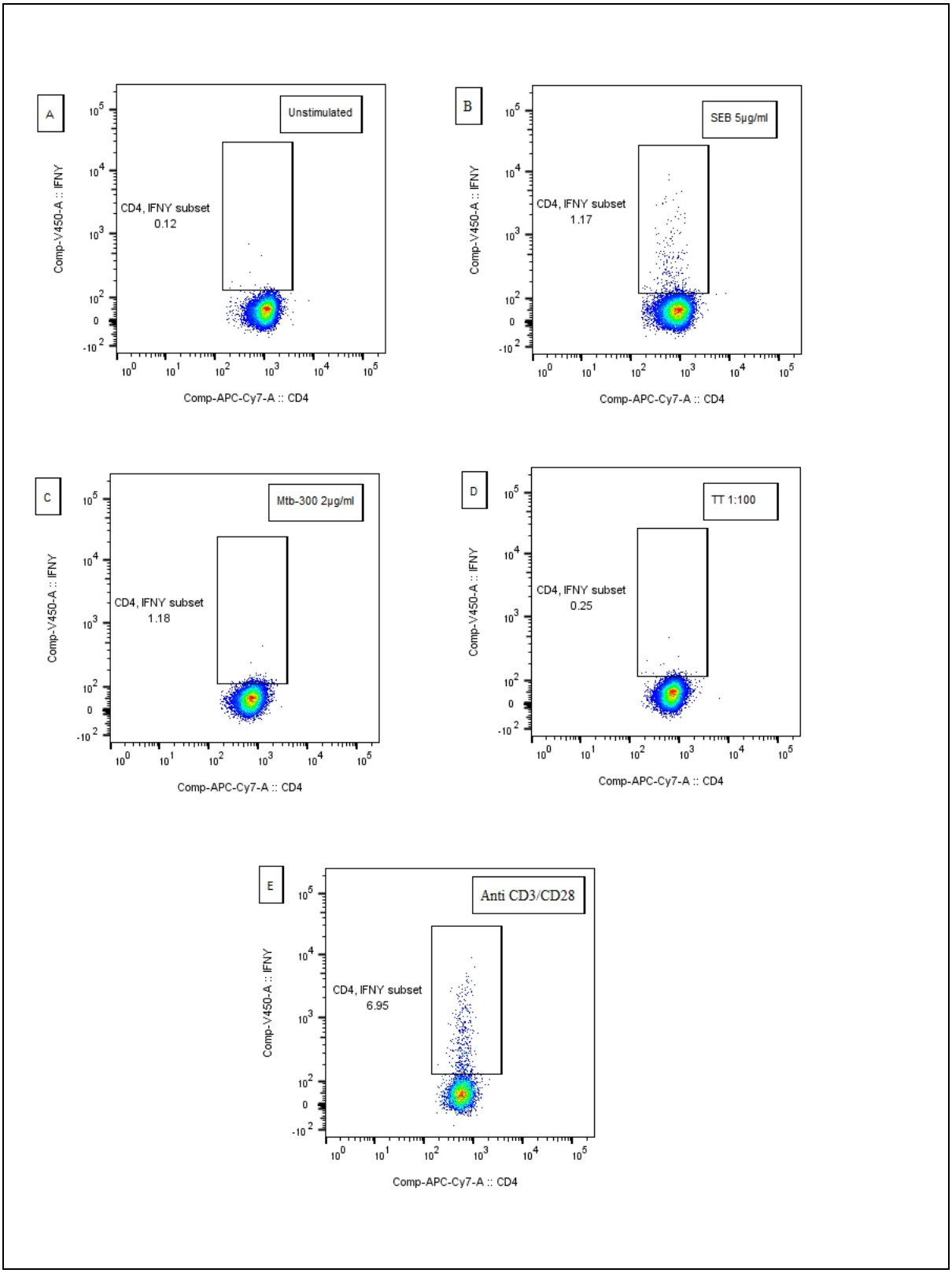



**Figure 2.11. human PBMCs from healthy volunteers stimulated with specific or non-specific T cell antigens to detect the cytokines responses.** (A) The percentage of IFN$\gamma$ gated on CD3+CD4+ from unstimulated cells. B and E showed the percentage of IFN$\gamma$ from cells stimulated with non-specific T lymphocytes antigens with SEB or anti CD3/CD28. C and D represented percentage of IFN$\gamma$ from cells stimulated with specific T lymphocytes antigens Mtb-300 or Tetanus toxoid.

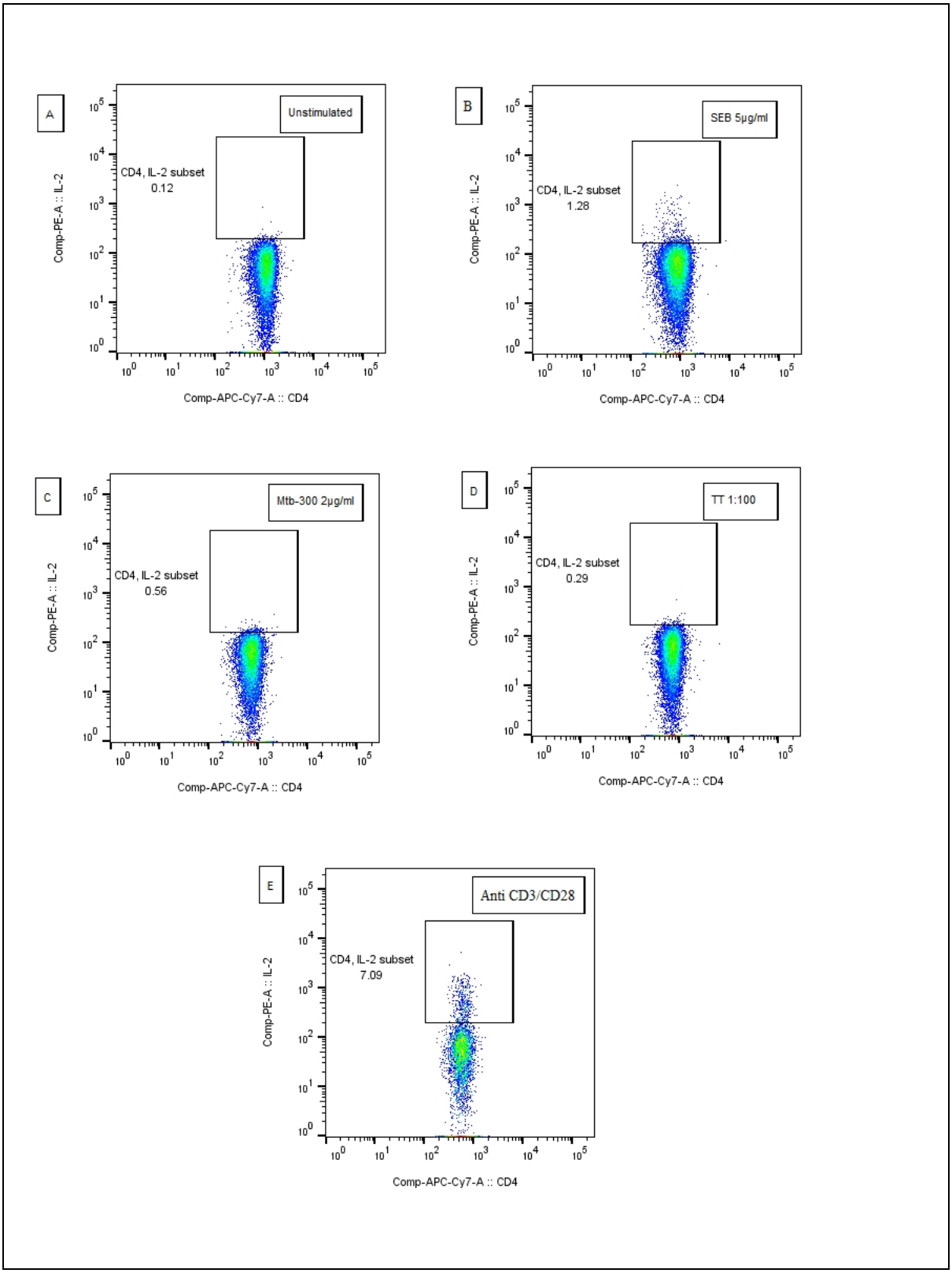



**Figure 2.12. human PBMCs from healthy volunteers stimulated with specific or non-specific T cell antigens to detect the cytokines responses.** (A) The percentage of IL-2 gated on CD3+CD4+ from unstimulated cells. B and E showed the percentage of IL-2 from cells stimulated with non-specific T lymphocytes antigens with SEB or anti CD3/CD28. C and D represented percentage of IL-2 from cells stimulated with specific T lymphocytes antigens Mtb-300 or Tetanus toxoid.

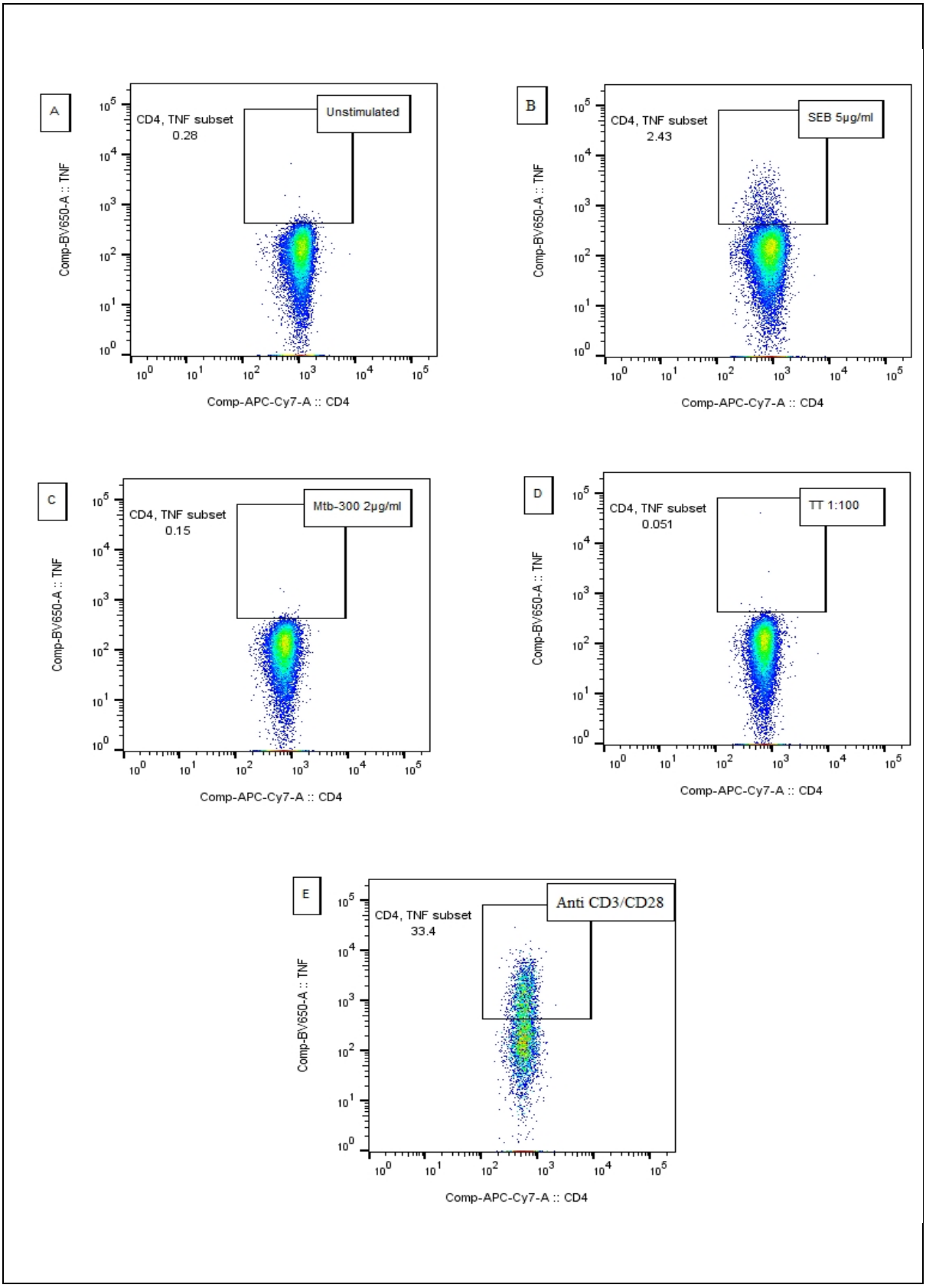



**Figure 2.13. human PBMCs from healthy volunteers stimulated with specific or non-specific T cell antigens to detect the cytokines responses.** (A) The percentage of TNF-α gated on CD3+CD4+ from unstimulated cells. B and E showed the percentage of TNF-α in cells stimulated with non-specific T lymphocytes antigens with SEB or anti CD3/CD28. C and D represented percentage of TNF-α in cells stimulated with specific T lymphocytes antigens Mtb-300 or Tetanus toxoid.

### 2.3.7 Fresh vs frozen PBMCs evaluation

**Table 2.3 Comparison of relative frequency of cytokine production by CD4+ T cells following stimulation with antigens**

| **Cell type** | Live single CD3+ CD4+ TNF-α+ | | Live single CD3+ CD4+ IL-2+ | | Live single CD3+ CD4+ IFNγ+ | |
|---|---|---|---|---|---|---|
| **Stimuli** | Fresh PBMC | Frozen PBMC | Fresh PBMC | Frozen PBMC | Fresh PBMC | Frozen PBMC |
| Unstimulated | 0.14% | 0.33% | 0.08% | 0.12% | 0.14% | 0.12% |
| SEB | 7.95% | 2.49% | 2.14% | 1.28% | 1.72% | 1.17% |
| Mtb-300 | 0.33% | 0.41% | 0.31% | 0.56% | 0.41% | 1.18% |
| Tetanus Toxoid | 0.57% | 0.19% | 0.19% | 0.29% | 0.34% | 0.25% |
| Anti CD3/CD28 | 4.6% | 33.8% | 1.10% | 7.09% | 0.69% | 6.95% |

Because we wanted to be able to freeze samples to compare them longitudinally and against each other, we assessed the effect of freezing on the response of T cells to several stimuli. We found that while freezing could reduce the cytokine response it did not remove it. We also found a much stronger response to anti-CD3/CD28 signalling. Table 2.3 illustrates the frequency of cells capable of producing cytokine in response to each stimulus. The table shows that the response of fresh and frozen PBMCs are different. For example, the frequency of CD4+ T cells expressing TNF-α, IL-2 and IFNγ in fresh PBMCs stimulated with SEB was higher than in similarly stimulated frozen cells. In contrast, anti CD3/CD28 drives strong expression of these cytokines in frozen PBMCs. The response to tetanus was low in both preparations. There was a minor response to Mtb300 antigens for IFN which came up in the frozen sample, which might reflect mycobacterial exposure in the subject or background responsiveness to the peptide pool.

### 2.3.8 Whole blood vs PBMCs stimulated with antigens for cytokines detection:

We aimed in this experiment to determining the impact of cell isolation from whole blood on the expression of cytokines TNF-α, IL-2 and IFNγ following stimulation with SEB, Mtb-300, Tetanus toxoid and anti CD3/CD28. Unstimulated PBMCs were used as negative control. Table 2.4 represents a comparison between whole blood and frozen PBMCs in the expression of these cytokines following stimulation for 6 hours at $37^0$ C. The table shows that the response

of whole blood and frozen PBMCs is different. For example, anti CD3/CD28 drives the production of TNF-α, IL-2 and IFN$\gamma$ in frozen PBMCs to an extent greater than in whole blood, similar to the difference between fresh and frozen PBMC. In contrast, SEB drives the expression of these cytokines in whole blood, and less in PBMCs. Mtb-300 drives a higher frequency of IL-2 production in PBMCs; however, this response is low. Neither Mtb-300 nor Tetanus toxoid stimulated a strong response.

**Table 2.4 comparison of the cytokines production following the stimulation with antigens**

| **Cell type** | Live single CD3+ CD4+ TNF-α+ | | Live single CD3+ CD4+ IL-2+ | | Live single CD3+ CD4+ IFN$\gamma$+ | |
|---|---|---|---|---|---|---|
| **Stimuli** | Whole blood | PBMC | Whole blood | PBMC | Whole blood | PBMC |
| Unstimulated | 0.37% | 0.12% | 0.05% | 0.23% | 0.18% | 0.21% |
| SEB | 7.65% | 3.87% | 4.25% | 1.02% | 5.27% | 1.33% |
| Mtb-300 | 0.57% | 0.92% | 0.16% | 1.14% | 1.11% | 1.24% |
| Tetanus Toxoid | 0.71% | 0.15% | 0.15% | 0.11% | 0.22% | 0.18% |
| Anti CD3/CD28 | 2.70% | 29.5% | 0.08% | 7.94% | 0.29% | 5.61% |

### 2.3.9 Increasing the frequency of PBMCs producing cytokines using co-stimulatory antibodies:

Because we knew we would be looking for very low frequency events, we wanted to augment any signal that might be induced and so determined the effect of addition of co-stimulatory antibodies on the response to specific stimuli. To do this we used the co-stimulatory antibodies anti CD28 and anti CD49d to augment the stimuli specific cytokine response. 2 μl of CD28/CD49d antibody (BD, 347690) was added into each well, then the cells were incubated at $37^0$ C in 5% $CO_2$ for 6 hours (see 2.2.11). Our data shows that SEB resulted in a higher frequency of cytokine producing cells if the costimulatory antibodies were present. We also found that Mtb-300 specific CD4 T cells expressing cytokines were augmented when anti costimulatory antibody was used. Again, this was a healthy control but we do not know the BCG or Mtb exposure history of this healthy control so this could be specific or cross-reactivity being detected. We also wanted to address the role of specific protein secretion inhibitors in driving a detectable signal. We did not see a strong difference between Brefeldin A and Golgi Plug/Golgi Stop and so we continued using Brefeldin A as the chosen reagent (figures 2.14,2.15 and 2.16).

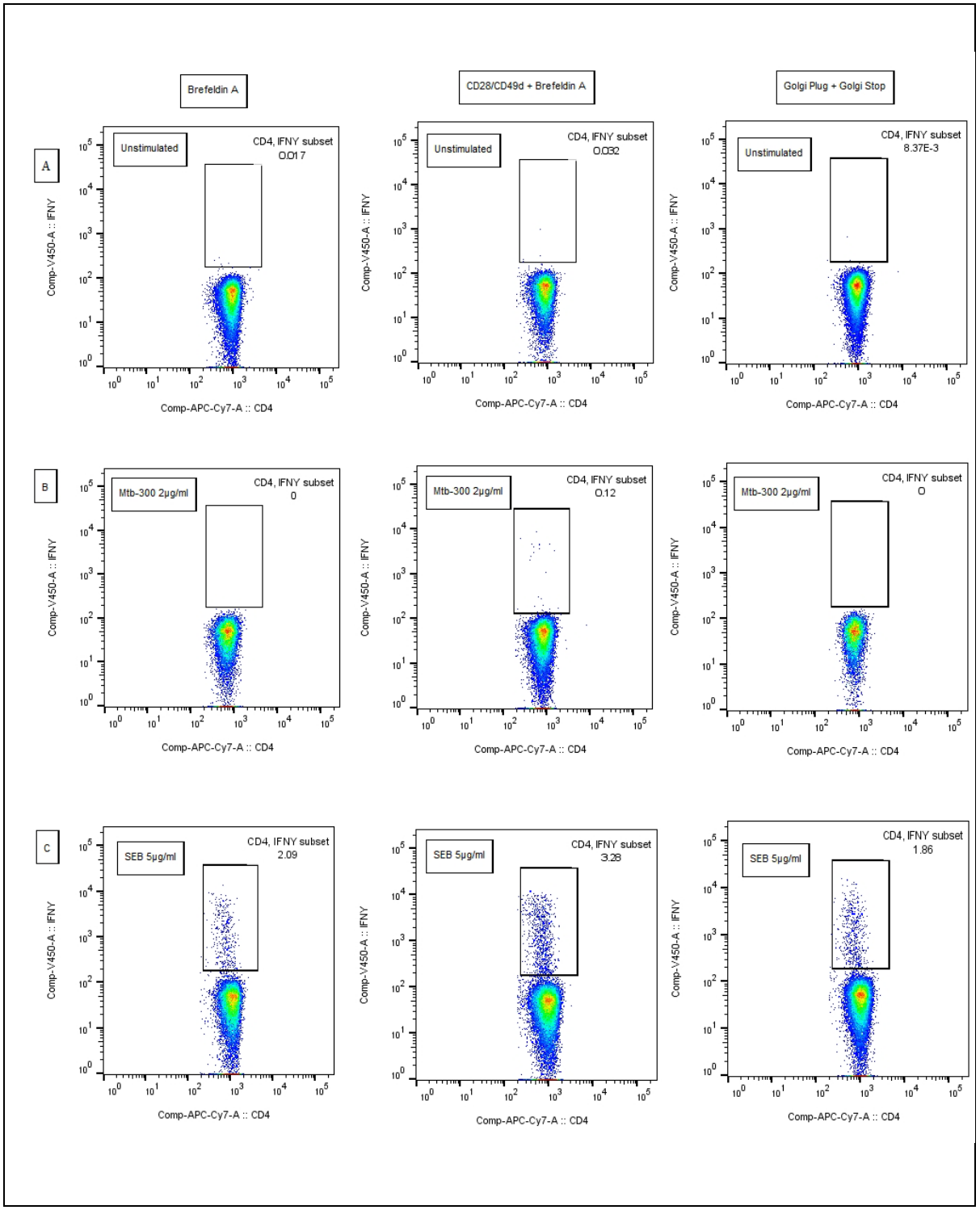



**Figure 2.14. The effect of costimulatory antibodies on antigen specific cytokines detection.** PBMCs were stimulated with SEB or Mtb-300 for 6 hours. Brefeldin A was added after two hours in assay 1. Anti CD28/CD49d was added in assay 2 followed by addition of Brefeldin A after 2 hours. Golgi plug and Golgi stop were added to assays 3 after two hours. A showed the percentage of IFN$\gamma$ gated on CD4+ in unstimulated cells for the three assays. B and C showed the percentage of IFN$\gamma$ from PBMCs stimulated with Mtb-300 or SEB respectively.

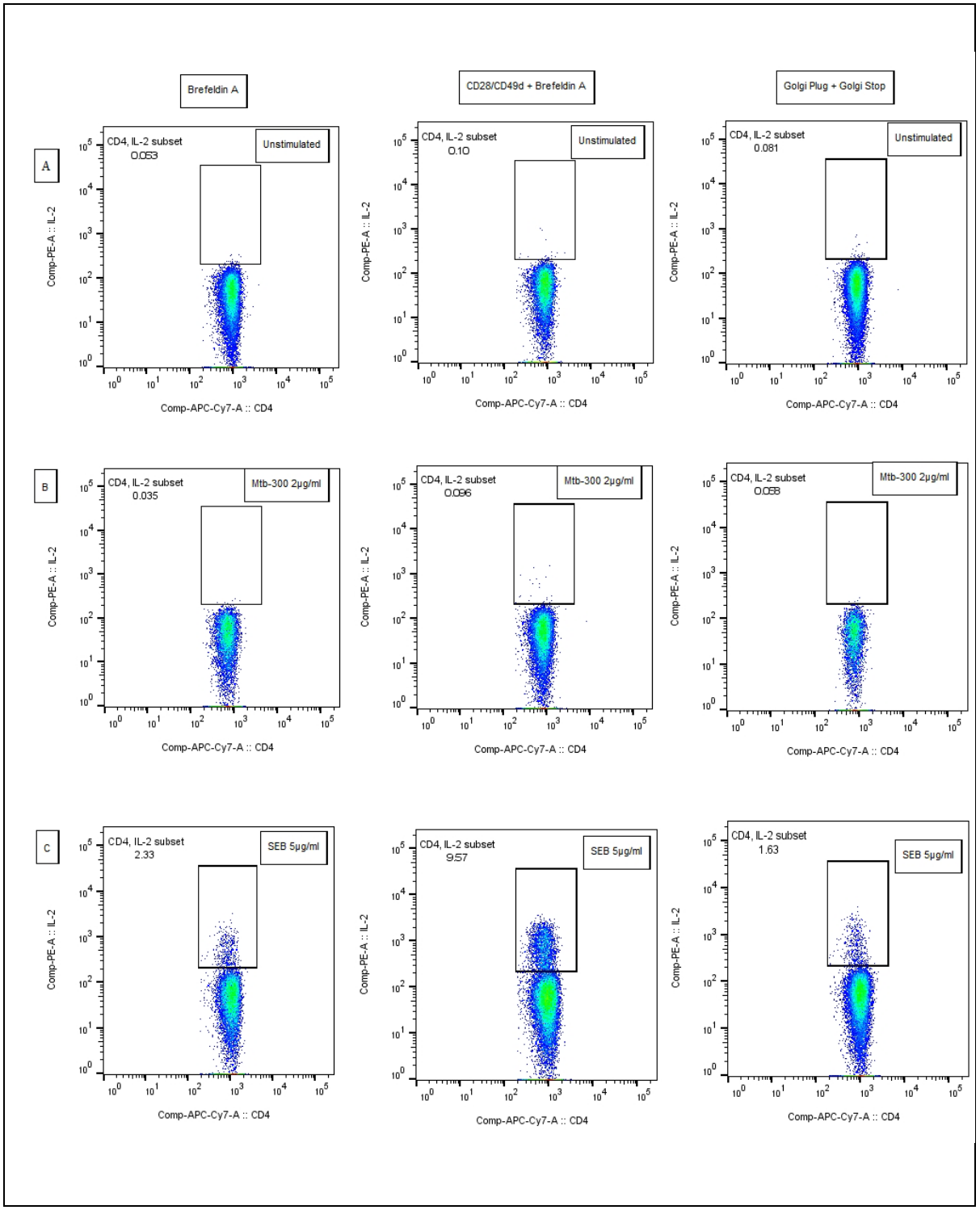



**Figure 2.15. The effect of costimulatory antibodies on antigen specific cytokines detection.** PBMCs were stimulated with SEB or Mtb-300 for 6 hours. Brefeldin A was added after two hours in assay 1. Anti CD28/CD49d was added in assay 2 followed by addition of Brefeldin A after 2 hours. Golgi plug and Golgi stop were added to assays 3 after two hours. A showed the percentage of IL-2 gated on CD4+ in unstimulated cells for the three assays. B and C showed the percentage of IL-2 from PBMCs stimulated with Mtb-300 or SEB respectively.

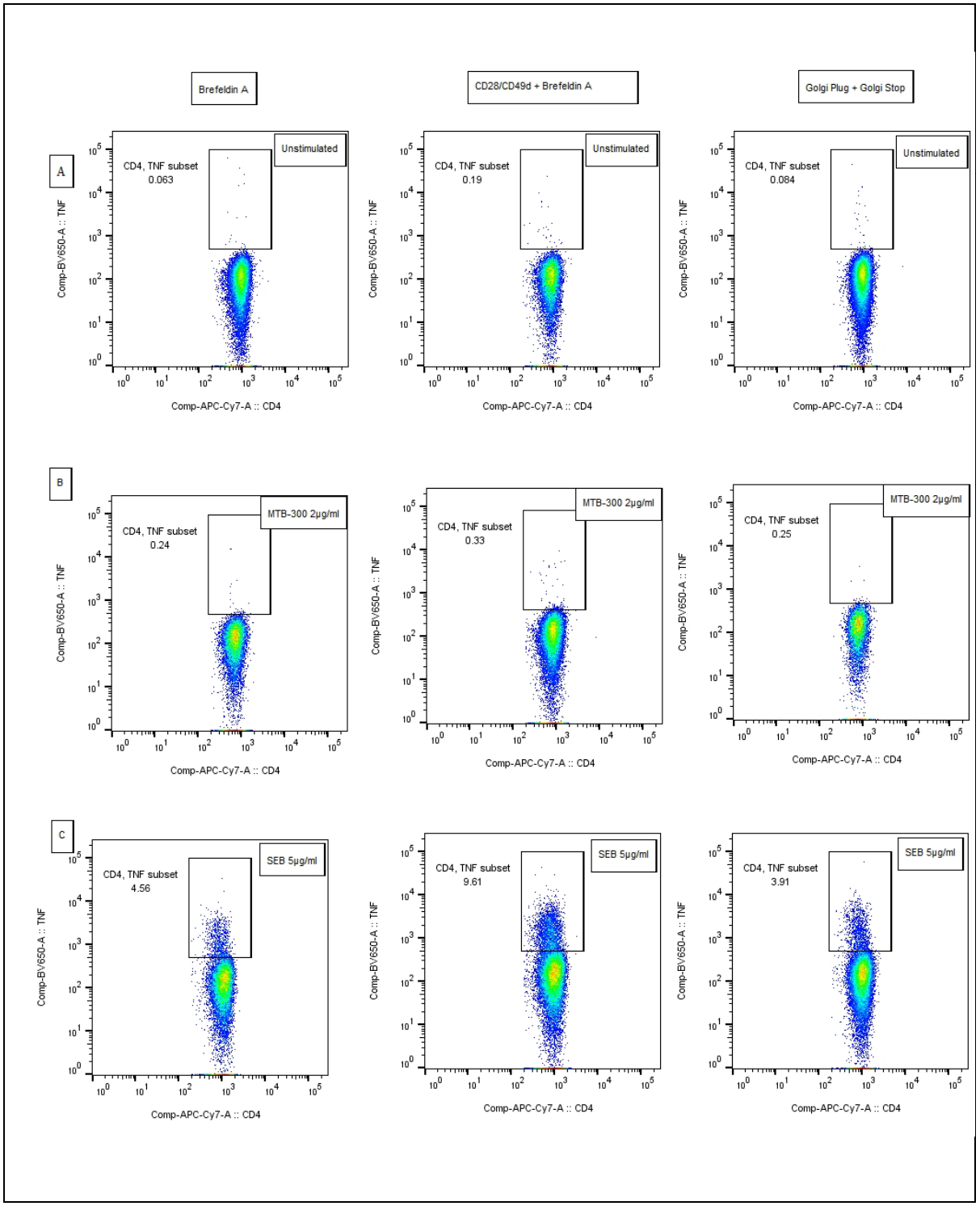



**Figure 2.16. The effect of costimulatory antibodies on antigen specific cytokines detection.** PBMCs were stimulated with SEB or Mtb-300 for 6 hours. Brefeldin A was added after two hours in assay 1. Anti CD28/CD49d was added in assay 2 followed by addition of Brefeldin A after 2 hours. Golgi plug and Golgi stop were added to assays 3 after two hours. A showed the percentage of TNF-α gated on CD4+ in unstimulated cells for the three assays. B and C showed the percentage of TNF-α from PBMCs stimulated with Mtb-300 or SEB respectively.

### 2.3.10 FMOs Control

A Fluorescence Minus One (FMO) control is a tube of cells stained with all fluorochromes used in the experiment except one. During the development of multicolour flow cytometry panels, they are essential to help with where the gates should be set. FMOs are important to identify positive population when the expression level is low or weak.

To test this, FMO control tubes were prepared to examine the positive population of cytokines from PBMCs stimulated with Mtb-300 2 μg/ml compared to unstimulated cells tube and PBMCs stimulated with SEB 5 μg/ml tube. It shows clearly how to discriminate the negative population during the gating on the expression of TNF-α, IL-2 and IFN$\gamma$. Figures 2.17, 2.18 and 2.19 showed the expression of positive cytokines gated on CD3+ CD4+ population from human PBMCs stimulated with Mtb-300 or SEB or unstimulated in the FMO control tubes compared to those cells in the presence of anti TNF-α, IL-2 and IFN$\gamma$ antibodies. In addition to this, Another FMO control experiment was prepared to evaluate the positive expression of CD25, OX40 and PD-L1 to assess the activation induced marker in response to antigens compared to unstimulated sample. Figures 2.20 and 2.21 represented the FMO control for the activation markers for the cells stimulated with antigens compared to the cells stained with anti CD25, OX40 and PD-L1 antibodies. Moreover, FMO control tube for CD25+ marker was examined. Figure 2.22 showed the FMO control for CD25+ marker, which determine the T cell gated on CD4+ CD127- CD25 + following cell surface staining for lymphocyte panel as described previously.

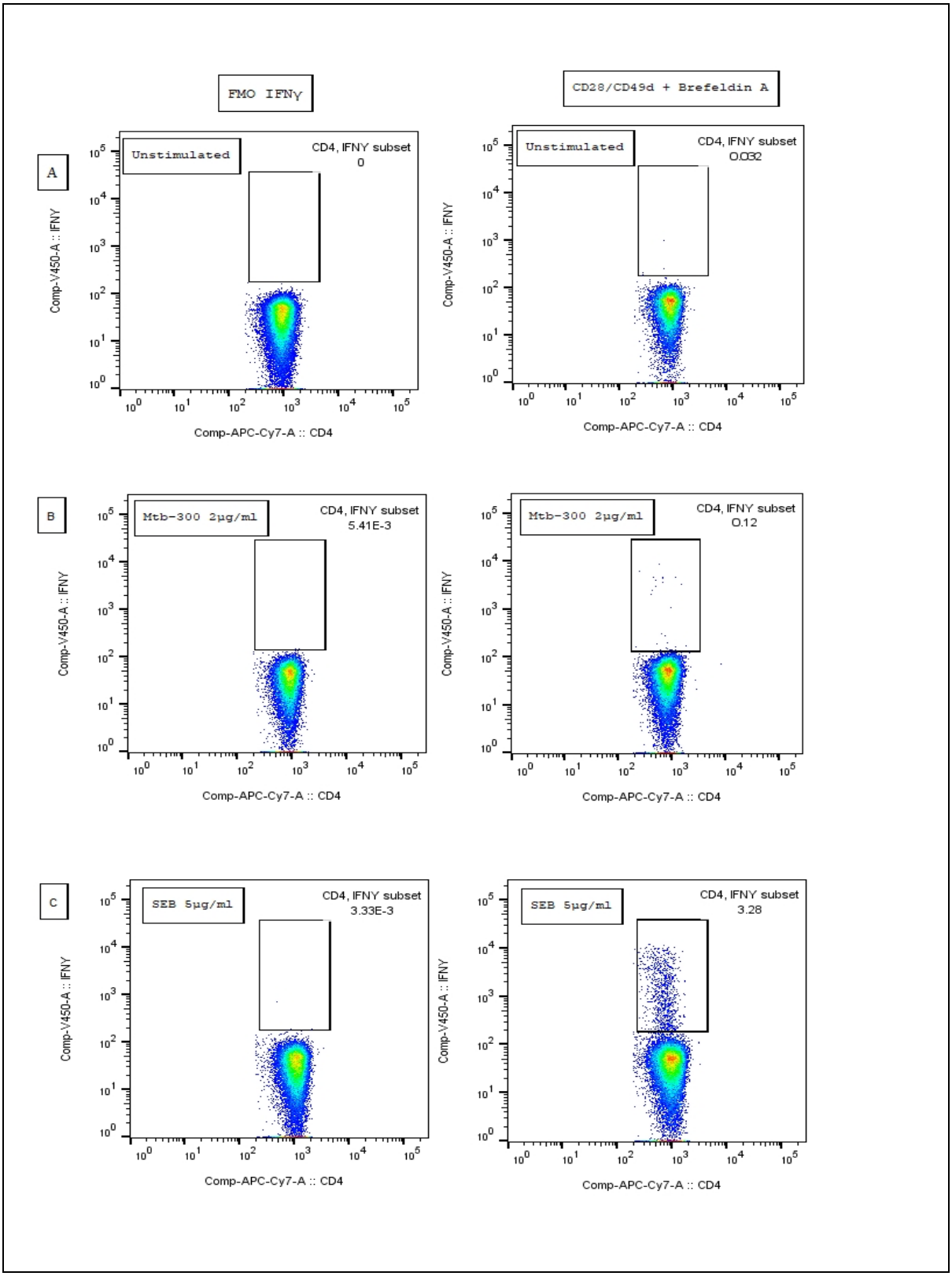



**Figure 2.17. Evaluation of FMO control tubes.** PBMCs stimulated with Mtb-300 or SEB and third tube remained unstimulated with the addition of anti-costimulatory antibodies for 6 hours. During the incubation, Brefeldin A was Added at two hours from stimulation started. The cells stained with anti IFN$\gamma$, IL-2 and TNF$\alpha$ and the FMO tubes remain unstained. A showed the percentage of IFN$\gamma$ gated on CD4+ in unstimulated cells. B and C showed the percentage of IFN$\gamma$ from PBMCs stimulated with Mtb-300 or SEB respectively.

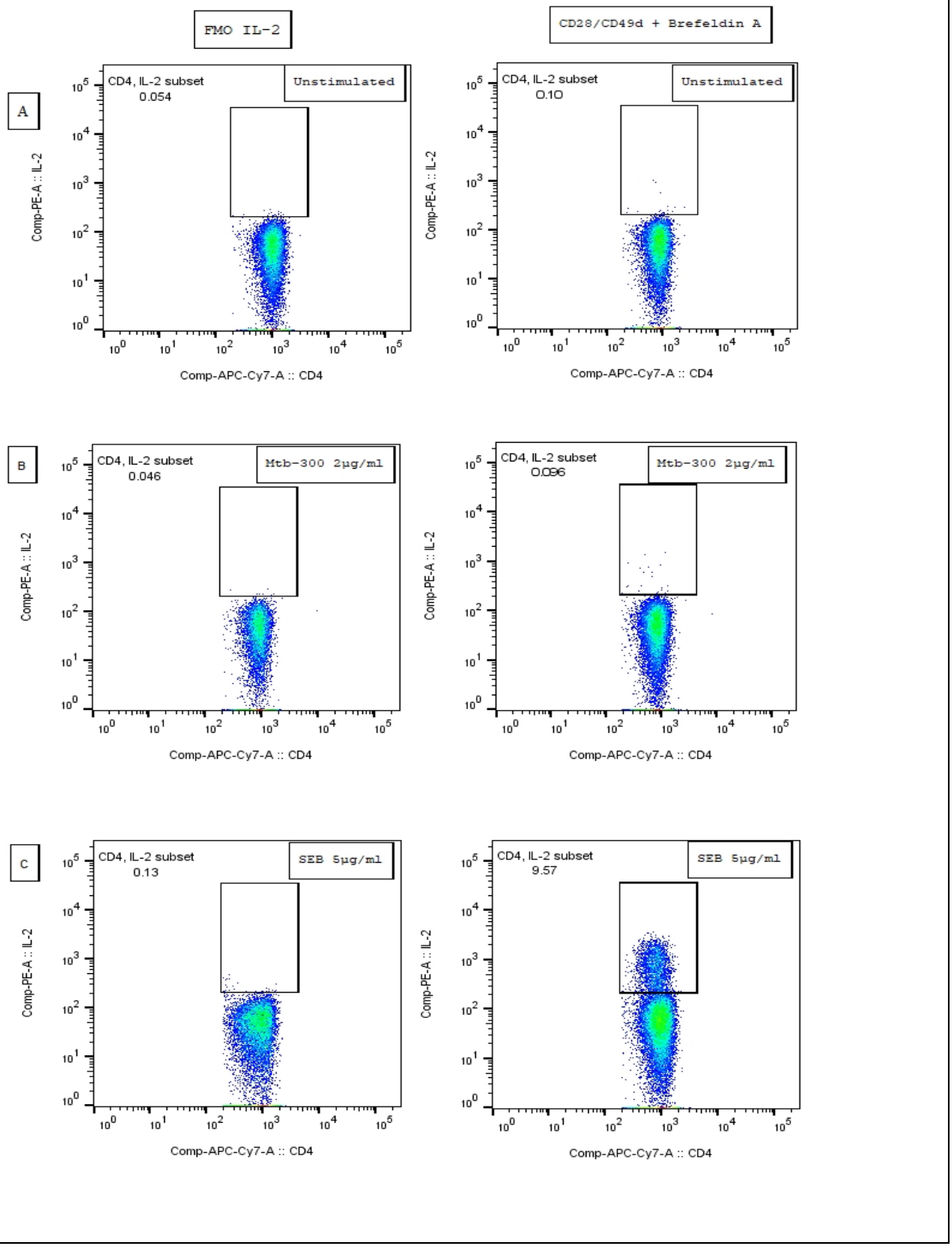



**Figure 2.18. Evaluation of FMO control tubes.** PBMCs stimulated with Mtb-300 or SEB and third tube remained unstimulated with the addition of anti-costimulatory antibodies for 6 hours. During the incubation, Brefeldin A was Added at two hours from stimulation started. The cells stained with anti IFN$\gamma$, IL-2 and TNF-$\alpha$ and the FMO tubes remain unstained. A showed the percentage of IL-2 gated on CD4+ in unstimulated cells. B and C showed the percentage of IL-2 from PBMCs stimulated with Mtb-300 or SEB respectively.

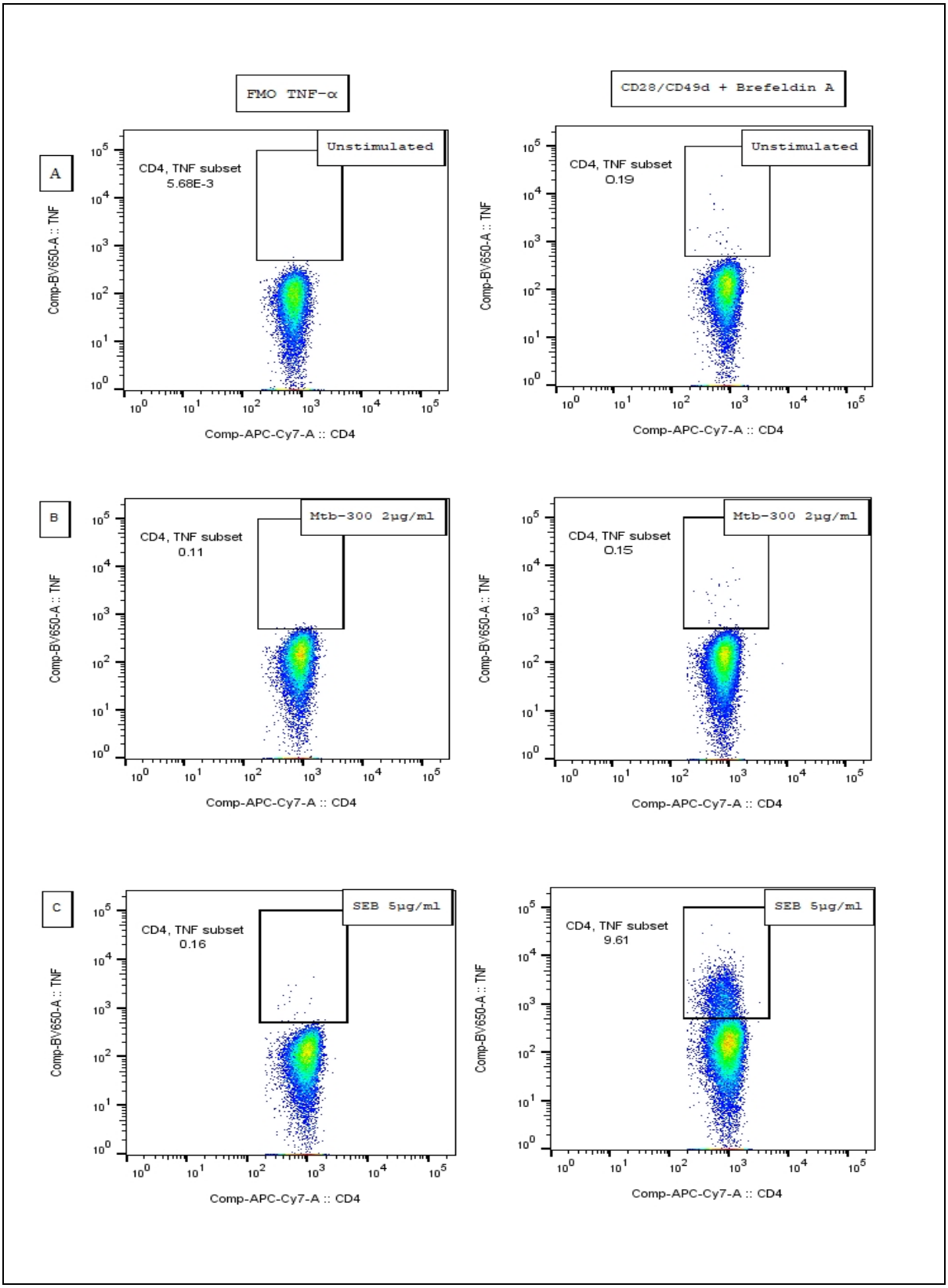



**Figure 2.19. Evaluation of FMO control tubes.** PBMCs stimulated with Mtb-300 or SEB and third tube remained unstimulated with the addition of anti-costimulatory antibodies for 6 hours. During the incubation, Brefeldin A was Added at two hours from stimulation started. The cells stained with anti IFN$\gamma$, IL-2 and TNF$\alpha$ and the FMO tubes remain unstained. A showed the percentage of TNF-$\alpha$ gated on CD4+ in unstimulated cells. B and C showed the percentage of TNF-$\alpha$ from PBMCs stimulated with Mtb-300 or SEB respectively.

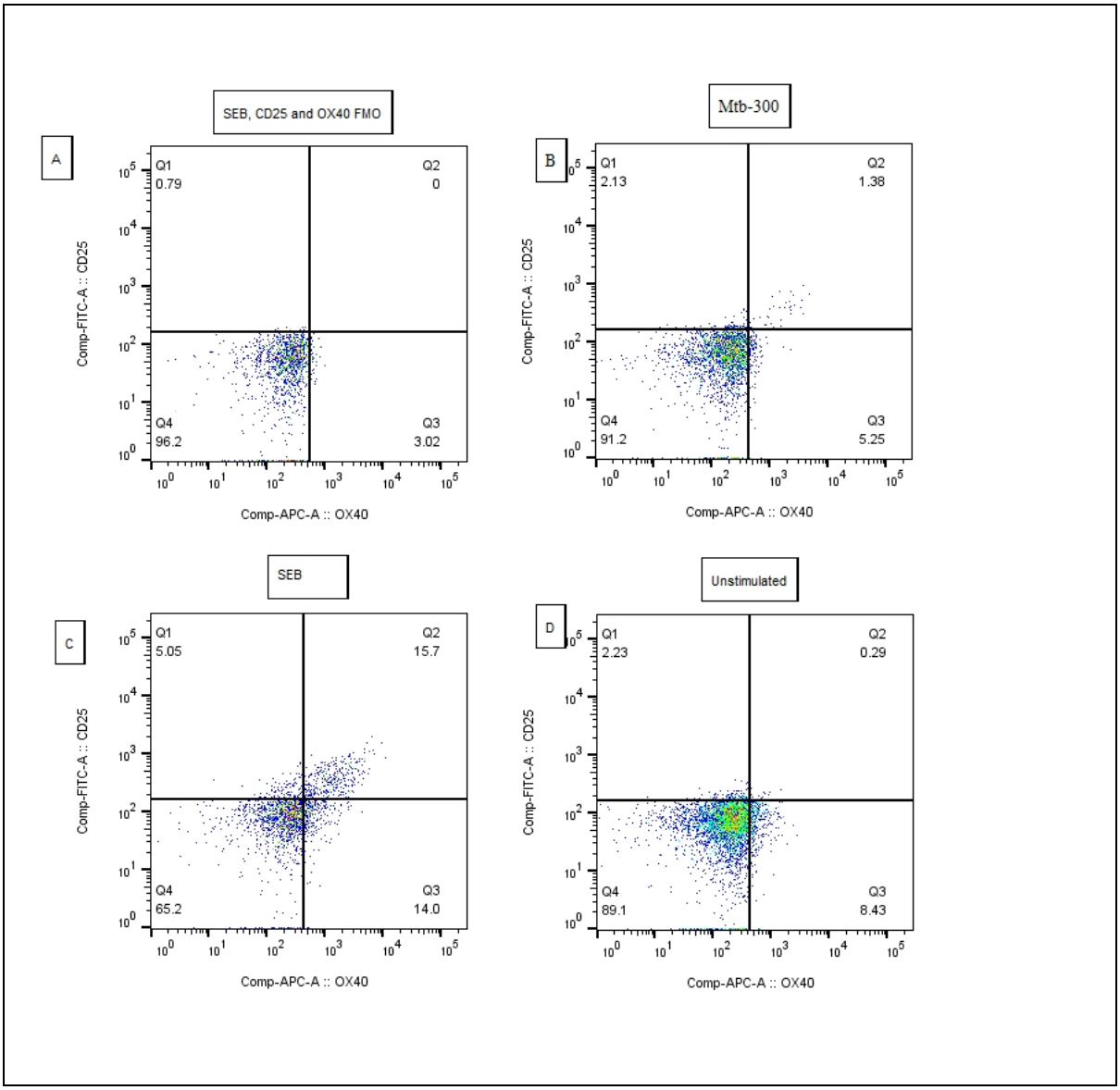



**Figure 2.20. Evaluation of FMO control tubes for the activation markers in effector memory CD45 RA- CCR7-.** PBMCs stimulated with Mtb-300 or SEB and third tube remained unstimulated. The cells were incubated overnight followed by cell surface stating. The FMO tubes were stained in the absence of anti OX40 and CD25 antibodies. A showed the FMO tube. B showed the expression of CD25+ OX40+ from PBMCs stimulated with Mtb-300. C showed the expression of CD25+ OX40+ from PBMCs stimulated with SEB. D showed the expression of CD25+ OX40+ from unstimulated cells.

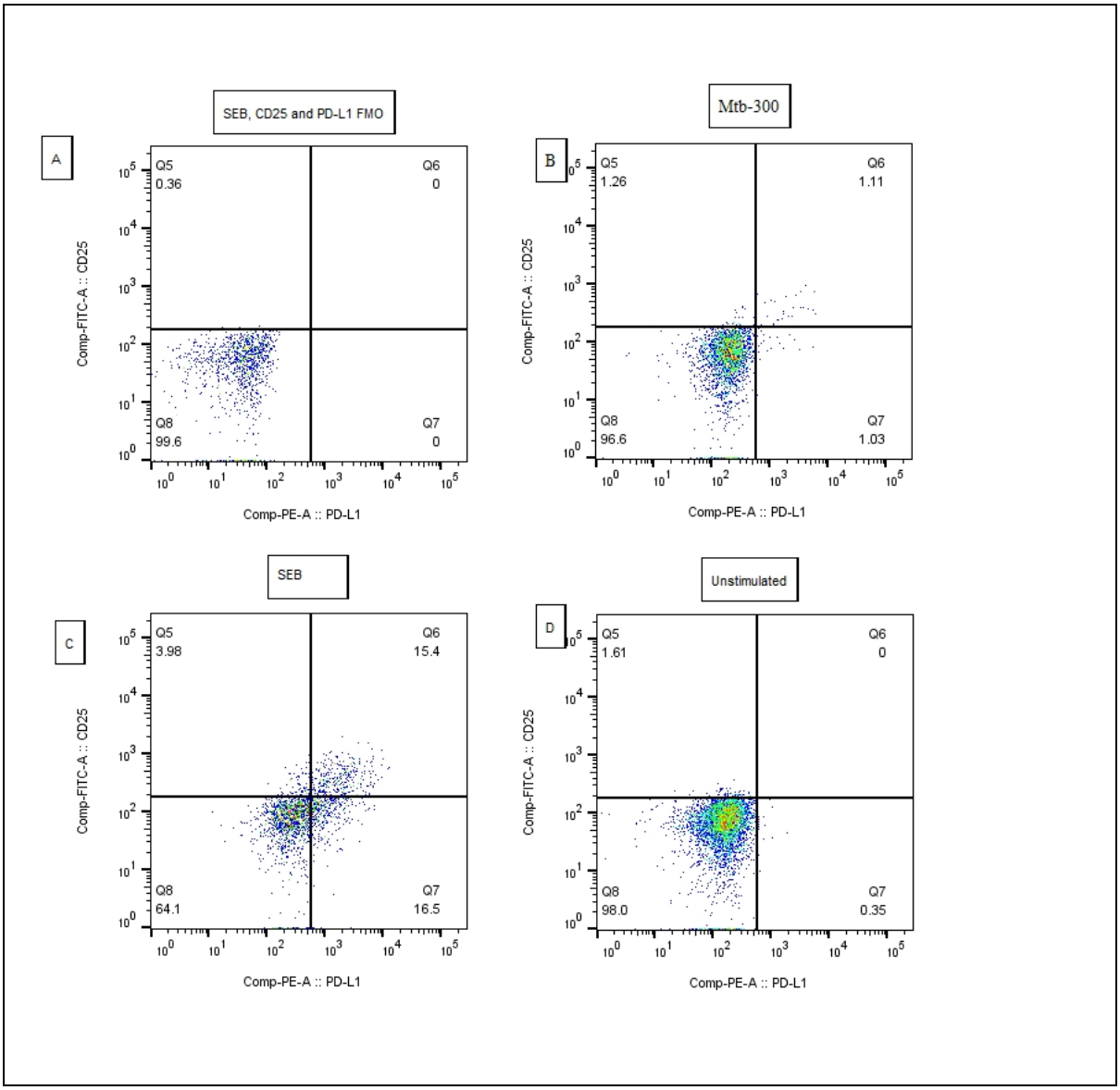



**Figure 2.21. Evaluation of FMO control tubes for the activation markers in effector memory CD45 RA- CCR7-.** PBMCs stimulated with Mtb-300 or SEB and third tube remained unstimulated. The cells were incubated overnight followed by cell surface stating. The FMO tubes were stained in the absence of anti PD-L1 and CD25 antibodies. A showed the FMO tube. B showed the expression of CD25+ PD-L1+ from PBMCs stimulated with Mtb-300. C showed the expression of CD25+ PD-L1+ from PBMCs stimulated with SEB. D showed the expression of CD25+ PD-L1+ from unstimulated cells.

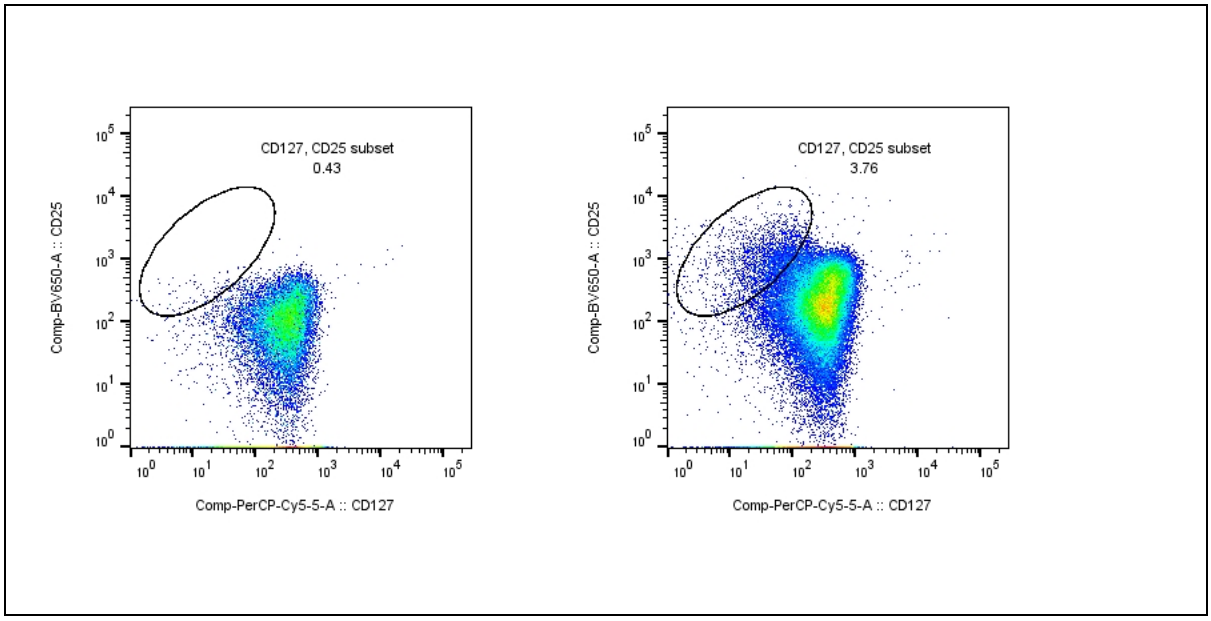



**Figure 2.22. FMO control tube for CD25+.** Human PBMCs were stained following cell surface lymphocyte. The FMO tube was staining in the absence of anti CD25 antibody. CD25+ CD127- gated on CD4+ showed the positive CD25 gating (right). FMO tube showing weak CD25+ CD127- gated on CD4+ (Left).

## 2.4 Discussion

The objective of this chapter was to design and develop a protocol to test our hypotheses. We identified panels of antibodies that allowed us to determine the frequency and phenotype of lymphocytes, monocytes and dendritic cells. The choice of antibodies was based on the existing information available for the local population (Hutchinson (2019) and based on appropriate cells for TB related research. Moreover, our gating strategy was based on papers describing the myeloid and dendritic cells subset (Autissier et al, 2010 and Lee et al, 2017). The data provided in Table 2.1 demonstrate that our panels identified expected frequencies of each population (Autissier et al, 2010). We have developed the protocols to the extent that we can develop a full profile for each participant and to understand the nature of the circulating cell populations during Mtb infection and to compare these profiles between each group. By deeply immunophenotyping the circulating blood cell populations we hope to obtain a correlation between clinical and immunological parameters with experimental measures of these markers. In our protocols and panels, a combination of antibodies tagged with different fluorochromes were used to determine the frequencies and to differentiate the cell types including, lymphocyte CD3+, B cells CD19+ and natural killer cells NK56+. Both T

helper CD4 T cells and T cytotoxic CD8 T cell were defined gated on CD3+ population. Both naïve CD45RA + and memory CD45RO+ were defined gated on CD4+ or CD8+. Another contribution was the activation status of both T helper cells and cytotoxic cell by measuring the expression of CCR7 (CD197) and CD45 RA gated on CD4 and CD8 by using the lymphoid panel. Indeed, by using this gate, the panel can establish both memory effector (CCR7- CD45 RA-) and central memory (CCR7+ CD45 RA -) populations. Also, our myeloid panel demonstrated that there are three major population that can be identified by gating on CD14 vs CD16 to show the classical ($CD14^{+}CD16^{-}$), non-classical ($CD14^{-}$ $CD16^{+}$), and intermediate ($CD14^{+}$ $CD16^{+}$) monocytes (Lee et al 2017). Furthermore, the dendritic cell panel allows us to measure both myeloid (m) DC (CD11c +) and plasmacytoid (p)DC (CD123 +) markers (Autissier et al, 2010). To sum up, the panels allow us to describe and compare the nature of T cells, monocytes and dendritic cells as a function of infection and disease status. This allows us to consider questions about which cells are induced during infection, what are the markers expressed, how different these markers are between the clinical parameters, and what can be detected integrated analysis of the data. We aimed to create a full immune cell profile to test the hypothesis that differences in cell frequencies and phenotype will allow us to find differences between the subjects.

To investigate activation induced markers (both cytokine and surface) we need to identify a positive control protocol. In the first instance we used Jurkat T cell line stimulated with concanavalin A and PMA. While this model allowed us to understand cell responses and characterise the expression of some activation markers and develop some staining protocols, it was not close to the natural situation. To progress our work, we chose to stop using the cell line and to just work with healthy PBMC and also to focus on the use of a positive stimulus that acted through the T cell receptor, thereby mimicking more natural stimulation conditions. We chose to use SEB, which is a superantigen secreted from *Staphylococcus aureus* that induces T cell proliferation via the TCR and represents a biological stimulus (Janik and Lee, 2016). Using this as a positive control we were able to choose a concentration and time of incubation and to then compare the impact of fresh versus frozen and whole blood versus PBMC.

We have optimized an intracellular cytokine staining (ICS) assay in human PBMCs which allows us to identify T cells responding to a signal through the TCR. We considered a variety of experimental protocols to build a workable protocol for our research plan. We compared the response of T cells between fresh and cryopreserved cells as well as the response in whole blood to isolated PBMC. We also determined impact of the addition of brefeldin A or Golgi plug/stop in increasing the signal allowing identification of low frequency responding cells. Finally, we addressed the use of specific panels, gating strategies and the role of fluorescence minus one staining to ensure robust identification of changes in expression of our target markers. As a result, we have developed the parameters and laboratory protocols allowing us to obtain an improved protocol that we can use in our examination for the ISATS study.

Because we realised that our ISATS study would require us to use frozen cells it was necessary to show the difference between using fresh PBMCs and see how they differ from the frozen cells (table 2.3). In addition, we wanted to see if we were changing the response by extracting PBMCs from the whole blood (table 2.4). The analysis shows how the nature of storage could impact on the antigen specific $CD4^+$ response following the stimulation. Although we had repeated the ICS and cell surface staining using frozen PBMCs to optimise our protocol, we only had a few attempts using fresh PBMCs (3 attempts) or whole blood (2 attempts) to do our comparison. Because of the small size of donors, we couldn't summarize our data statistically (mean, median and standard deviation) to draw a statistically analysable conclusion. Also, we found that while freezing could reduce the cytokine response to SEB signalling, it did not remove it. Similarly, there is a trend for greater frequency of cytokines in whole blood compared to the frozen PBMCs in response to SEB. Again, to decide between fresh PBMCs, frozen PBMCs or whole blood we must consider time, type of stimulant and sample handling. However, a stronger response to anti-CD3/CD28 signalling was observed in frozen PBMCs compared to the fresh PBMCs or whole blood. The reason may be that Dynabeads human T-activator CD3/CD28 are intended for *ex vivo* activation and expansion of human T cells, which require human PBMCs or purified T cells isolated from human whole blood for the most optimal activation (the recommended cell type such as PBMCs or isolated T cells available at product manual). Also, we need to consider that the whole blood would result in a variable environment for antibody staining and that may result in variable responses between samples.

As we described in the introduction, there are two signals involved in T cell activation. The binding of TCR to the peptide-MHC complex induces the first signal and the binding of CD28 receptor to B7-1 or B.72 induces the second signal. Naïve T cells therefore requires costimulation for the activation, cytokines production, and provide protection (Xia et al, 2018). Costimulatory antibodies have been described as a key modification for enhancing the detection of antigen specific CD4+ T cell cytokines production (Gauduin et al., 2004). In this study, it was reported that a higher frequency of cytokine producing CD4+ T cells were seen in macaques in response to viral antigen as a result using of costimulatory antibodies. Gauduin (2006) also reported that the frequency of cytokines production CD4+ cells enhanced up to 3-fold by the addition of anti CD28 and anti CD49d following the stimulation with superantigen or specific antigens in macaque. For the human peripheral blood, Waldrop et al, 1998 demonstrated that CMV specific CD4+ T cells showed differences in their costimulatory requirement for inducing cytokines secretion. They reported that the production of IL-2 and IFN$\gamma$ increases in the presence of anti CD28, CD49d or CD5 responding to CMV antigen with "maximum frequencies observed with dual-pathway (e.g., CD28 plus CD49d) costimulation". Indeed, they analysed the costimulatory requirements of antigen specific population and suggested a "spectrum of activation thresholds" divided into cells triggered with no costimulation (or low costimulatory), cells require single or dual pathway costimulation (moderate requirement) and cells requiring optimal dual pathway (high requirement). Other studies of the activation requirement for human CD4+ memory T cells revealed that there is a differential dose response and costimulatory characteristics for cytokine responses (Bitmansour et al., 2002). Indeed, they have reported that IFN$\gamma$ producing cells that also produce IL2 were doubled from when antigen dose was increased in the absence of anti CD28/CD49d; however, the cytokine secretion increased up to 4- to 5- fold in the presence of the costimulatory antibodies in the cells stimulated from low antigen dose to higher one. Our observation on the effect of costimulatory anti CD28 and anti CD49d antibodies on healthy cells stimulated with antigens showed that these molecules enhance the detection of stimuli specific T cells cytokines responses in human cells compared to those cells in the absence of costimulators. Our data revealed that the frequencies of cytokines secreted in Mtb300 specific CD4+ T cells or non-specific SEB (positive control and the negative (unstimulated) tube were almost doubled when we added anti CD28/CD49d in our assay. To

sum up, the evidence from published papers and our data supports the use of costimulation in increasing detection of antigen specific T cell cytokine secretion in intracellular cytokine staining assays using flow cytometry analysis.

Because some of the cell events are at very low frequency, we need to be confident in those positive cells that we see. This means that establishing robust gating is critical to ensuring only true positives are reported. One way to achieve this is by 'fluorescence minus one' (FMO) studies wherein the antibody detecting the key parameter is left out of the staining panel. Some cellular biomarkers are weak in the expression; therefore, FMO control tubes will be essential to identify the positive population of these marker. For example, FMO control for intracellular cytokines discriminates between cells which are moving into the positive gate due to cross over fluorescence rather than actual binding of the key antibody thereby compromising the detection of true production of TNF-$\alpha$, IL-2 and IFN$\gamma$ following stimulation compared to those missing one as shown in figures 2.17, 2.18 and 2.19. In addition to this, the FMO controls have been tested to target the positive population of activation markers OX40, CD25 and PD-L1 (figures 2.20 and 2.21). Obviously, these controls indicate how to develop the quadrant gate correctly showing the positive population.

Although we performed experiments to design a protocol for the study, these optimisation activities have some limitations and technical issues. We have optimized an intracellular staining assay and cell surface staining in human PBMCs from healthy volunteers; however, we didn't know the BCG or Mtb exposure history of each participant in these experiments. The reason of this is the lack of case report form (CRF) for the participants. To address this, we should provide a CRF to include all the required information such as age, ethnicity, BCG history for each participant. We need to update our ethical approval to provide for this in the future.

Another issue we would like to address is the FMO controls. We did FMO controls to accurately discriminate positive signals from negative signals; however, some signals of CD4+ TNF-$\alpha$ and CD4+ IL-2 were detected in FMO controls. This could be due to some technical issues during the staining. Another technical issue is using multiple FMOs at the same time in cell surface staining in activation induced markers assay (figures 2.20 and

2.21). FMO is fluorescence minus one control, and we should not use multiple antibodies in our optimising for activation induced markers assay. We would recommend repeating the FMO control for this assay using single antibody in each well to optimise the FMO control in our AIM assay.

Another technical issue is the separation of positive signals in CCR7 staining (figure 2.4 and 2.10). The reason of this is the impact of temperature on staining intensity and separation of positive population of chemokines receptors CCR7. Wolfswinkel et al (2023) reported that chemokine receptors included CCR7 staining at $37^0$ C increased the sensitivity and separation of positive signal.

Lastly, because of we considered a variety of protocols and gating strategies of Mtb-specific CD4 T cells to build a reproducible protocol for our study, we excluded CD8 T cells in our investigation. However, we should consider also including antigen-specific CD8 T cells in our investigation, which may provide important results.

To sum up, these activities were designed to build a protocol for the subsequent research study, to test the main hypothesis of the thesis. We succeeded in creating a panel allowing us to characterize the whole blood directly *ex vivo* as well as the conditions allowing us to identify stimulus specific responses of T cells, in terms of both cytokine release and cell surface marker expression. This chapter provides the basis for the successful activity described in the following chapters by providing a robust and meaningful laboratory protocol allowing deep immunophenotyping of the subjects and aligning this with their clinical parameters.

## Chapter 3: Immunophenotyping of circulating PBMCs in TB disease

### 3.1 Introduction

Tuberculosis (TB) infection is associated with a “dynamic” and “continuous spectrum” (Pai et al., 2016). The diagnosis of TB is complex. Indeed, in present day the diagnosis of tuberculosis (TB) relies on clinical symptoms, radiological assessments, and detection of mycobacterium tuberculosis in patients. Sputum smear, molecular test such as nucleic acid amplification can confirm the TB diagnosis for patients who experience symptoms like fever and cough. However, 20%-66% of TB cases are smear negative and molecular based test cannot discriminate live from dead Mtb (Davies et al., 2008; in Adekambi et al., 2015). Screening for latent TB (LTBI) involved in tuberculin skin test (TST) and interferon-gamma release assays (IGRAs), which measure interferon gamma (IFN$\gamma$+) in response to Mtb-specific antigens (Adekambi et al, 2015). However, these tests such as T SPOT-TB and QuantiFERON can’t discriminate between LTBI and active TB (Meire et al.,2005; Janssens et al., 2007; Adekambi et al., 2015). Consequently, we need to decide who is at risk to develop active disease or for monitoring treatment response. To address these challenges, studies provided alternative methods to investigate the T cell subsets that involved in Mtb infections. The phenotype of T cells helps us to determine either if the antigen is there then maybe T cells are effectors, or if there is no antigen, then they will be memory and slower to respond. Therefore, we determined phenotypic analysis of lymphocytes in cohorts of healthy, latent and active TB subjects. We performed cell surface staining to determine the frequency of lymphocytes, monocytes and dendritic cells by flow cytometry to determine whether the frequencies of these markers are different in LTBI compared to those who are healthy or active TB to provide better understanding of circulating of PBMCs in TB disease.

It is known that protective immunity against *Mycobacterium tuberculosis* depends on the effective interaction of the innate and adaptive immune systems. Because we need to expand our understanding the relationship between T cells and innate response, we need to examine how myeloid cell are altered in infected individuals. Cell types including dendritic cells, macrophages and natural killer cells involved in the innate responses to Mtb infection within the lung. Each of these types associated with function providing innate immunity against Mtb. Both dendritic cells and macrophages represent the first line defence against the infection. Dendritic cells (DCs) roles are known as superior antigen presenting cells that

capture antigens and migrate to the draining lymph node and expressing of MHC class I and MHC class II which involved in antigen presentation to T cells. Few studies have been involved in the changes of circulating of DCs subsets in peripheral blood in TB patients. It has been shown that total DCs associated with untreated active TB patients but this difference was not significant. Also, it has been shown that the ratio between plasmacytoid DCs which express HLA-DR+ CD123+ and myeloid DCs which express HLA-DR+ CD11C+ is significantly lower in untreated active TB patients compared to healthy control (Gupta et al., 2010). Therefore, we developed a panel of DCs subsets to determine the changes in the frequencies of these markers in our subjects.

Monocyte/macrophages play primary innate responses to Mtb infection. Mtb enters the lung via inhalation and reach the alveolar space to encounter and infect and persist inside macrophages. This allowed to the bacteria adapt to survive and replicate within the macrophages. The changes in circulating of monocytes subsets in peripheral blood in TB patients have been shown. The circulating of CD14+ was increased in patient with active TB; whereas, the expression of HLA-DR+ CD14+ was significantly lower in active TB patients compared to the control group (Sanchez et al., 2006). Active TB patients associate with significantly higher percentage of CD14- CD16+ and CD14+ CD16+ than those in healthy control group (Liu et al., 2019). Therefore, we developed a panel to determine the frequency and phenotype of monocyte subsets to determine the changes in the frequencies of these markers in our subjects.

Adaptive immune responses against Mtb involved T lymphocytes. Naïve CD4 T cells activated following the interaction with antigen-MHC class II complex-TCR and they differentiate into effector subtypes that mediate immune response through secretion of specific cytokines. Naïve CD8 T cells activated following interaction with antigen-MHC class I complex-TCR and this interaction triggers T cells activation and induce target cell killing through secretion of cytotoxic granules. The changes of T lymphocytes circulating in peripheral blood in TB patients have been shown. The frequencies of CD3+ CD4+ was significantly lower in active TB patient; however, the frequency of CD3+ CD8+ was significantly higher in active TB patients (Beck et al., 1985; Wu et al.,2009; EL Kholy et al., 2018 & Venturini et al.,2019). The CD4/CD8 ratio was significantly lower in active TB patients compared to healthy control group. Changes in the

frequencies of CD4+ memory T cells or CD8+ memory T cells in active TB disease has been shown. CD8+ effector T cells is higher in LTBI patients than those active TB patients and CD8+ memory T cells is higher in LTBI than those in healthy control (Tonaco et al.,2017). Moreover, the frequency of CD4+ central memory T cells and CD8+ central memory T cells are higher in LTBI, and the frequency of these population in healthy control is higher than active TB patients (Pathakumari et al., 2017; Esteves et al., 2020). Therefore, we developed a panel to determine the frequency and phenotype profile of CD4+ T cells and CD8+ T cells of memory and effector in subjects.

**Hypothesis:**

We hypothesized that the phenotype and/or relative ratios of PBMCs between samples from defined cohorts of healthy, latent and active TB subjects would allow discrimination between healthy and latent and active TB groups.

**Objectives:**

1- To determine the frequency and phenotype of lymphocytes in well characterized cohorts of healthy, latent and active TB subjects
2- To determine the frequency and phenotype of Monocytes in well characterized cohorts of healthy, latent and active TB subjects
3- To determine the frequency and phenotype of DCs in well characterized cohorts of healthy, latent and active TB subjects
4- To determine the impacts of TB treatment on the circulating PBMCs in well characterized cohorts of healthy, latent and active TB subjects

## 3.2 Materials and methods

### 3.2.1 Ethic statement

The participants in this study were recruited in accordance with ethics approvals for the protocol titled "Determining the immunophenotype of subjects across the spectrum of tuberculosis infection to allow for development of novel biomarkers of active infection (ISATS)". All the documents including the protocol written by the doctoral student Amar Jarkhi and an academic clinical fellow in infection disease Christopher Martin. The documents reviewed under the supervision of the academic supervisor Professor Andrea Cooper and the Chief investigator in this study Dr Manish Pareek. Research conducted for ISATS (protocol number 0770 and IRAS project ID:274087) study was performed in accordance with favourable opinion from London-Central Research Ethic Committee (REC reference 20/LO/0514). Approvals were obtained from Health Research Authority (HRA) and Health and Care Research Wales (HCRW). Sponsor green light was issued to start this study from Research Governance and Integrity in University of Leicester. All individuals aged ≥16 attending recruitment sites will be eligible to participate in the study. Healthy adults from the University population will recruited by poster advertisement. All participants supplied with patient information sheet before recruiting. All participants provided written informed consent. The following procedures was adapted from ISATS protocol $V_4$.

### 3.2.2 Trial design

The treatment of the included participants will be as per routine care and there is no intervention as part of this study. Blood samples will be collected at time 0. For those who are undergoing treatment for TB a further two blood samples (2x7.5 ml at each visit) will be collected at times compatible with routine clinical care. It is anticipated that this will be around 4 – 8 weeks and 12 – 16 weeks into each treatment course. The medical records of each participant (other than those in the healthy control group) will be accessed to collate

routine pathological, histological, microbiological (including whole genome sequencing for Mtb) and radiological outcomes as well as the final clinical diagnosis.

It is anticipated that after clinical assessment, patients will receive one of the following diagnoses which will be used as a categorical variable in data analysis:

1. No infection with Mtb (negative control group): Asymptomatic healthy subjects that are UK born with no history of exposure to TB

2. Low risk of Mtb infection (matched negative controls for migrant LTBI): Migrant subjects lacking TB symptomatology with a negative IGRA and normal CXR

3. LTBI: Migrant subject with positive IGRA, no history of recent TB contact and normal CXR and possibly CT scan.

4. Subclinical active TB: Asymptomatic subject with positive IGRA, no history of recent TB contact and either abnormal CXR or CT scan that leads to either microbiological confirmation of disease or a clinical decision to treat for active TB.

5. Active TB: Symptomatic, abnormal radiology, microbiologically confirmed or clinical decision to treat for active TB.

6. Active community-acquired pneumonia (CAP): Symptomatic, abnormal radiology, decision to treat with conventional antibiotics (clinical and radiological resolution of disease), no microbiological evidence of TB (negative cultures and GX when sputum provided).

### 3.2.3 Trial setting

Subjects will be recruited at two specialist TB clinics operating within the University Hospitals of Leicester NHS Trust - the 'migrant clinic' and the rapid access TB clinic. TB patients may also be recruited from the infectious diseases' unit at UHL. Asymptomatic migrant subjects from non-TB clinics i.e., hepatitis clinic will act as matched TB-negative controls. Recruitment of patients with community-acquired pneumonia (CAP) will take place at the infectious diseases'

unit, at the rapid access TB clinic or an appropriate inpatient setting within UHL. Healthy controls will be recruited from the adult University population.

Those study subjects who receive latent or active TB treatment will also be seen in the clinic as part of routine care for assessment of symptoms and side effects of medications. In addition to blood tests routinely taken as part of these consultations, an additional 2 x 7.5ml tubes of heparinized blood will be taken. Samples will be transported by the research team members to the research site and processed at the Department of Respiratory Sciences, University of Leicester within 24h of collection.

#### 3.2.3.i Participant eligibility criteria

All individuals aged ≥16 attending recruitment sites will be eligible to participate in the study. Healthy adults from the University population will recruited by poster advertisement.

#### 3.2.3.ii Inclusion criteria

1. Willing and able to provide informed, written, consent
2. Age ≥16 years
3. Suspected or confirmed to be in one of the groups explained previously in 3.2.2

#### 3.2.3.iii Exclusion criteria

1. Proven immunosuppression, including infection with human immunodeficiency virus (HIV) or on immunosuppressive therapies, including oral corticosteroids at any dose.
2. Age <16 years
3. Unwilling or unable to provide informed, written, consent.
4. Previous treatment for active or latent tuberculosis.

### 3.2.4 Storage and analysis of clinical samples

Blood samples were collected in a heparin tube (2 x 7.5ml). Once collected, both tubes will be transported to the research site (Prof Cooper's Lab, University of Leicester) in a sealed container, within 24 hours of collection. Transportation will be carried out by a member of the research team.

Blood Samples for all participants were transported securely in a sealed container. Then, the samples were separated, some of the cells were analysed and some were stored at -150$^0$ C in Professor Andrea M Cooper's lab, Department of Respiratory Sciences which is Human and Tissue Act (HTA) compliant and has permission to store human samples at the end of study. Blood samples from healthy volunteers were separated and then stored and reported on the University's HTA licence. A copy of the consent form will be available for HTA compliance for the storage.

### 3.2.5 Study subjects

A total of 48 HIV negative aged ≥16 participants were enrolled into the ISATS screening cohort. 2 of the participants withdrew from the study. For the T cell responses and ΔTM-IL-12Rβ1 measurement, 26 latent Tuberculosis infection LTBI and 4 active TB patients were recruited from TB clinics operating within the University Hospitals of Leicester NHS Trust - the 'migrant clinic' and the rapid access TB clinic. Patients also were recruited from the infectious disease's unit at UHL. Samples from 9 of LTBI patients, who received TB treatment, have been collected around 6 weeks from the time of treatment started. This group was classified as "LTBI following up 1". A further blood samples were collected for 10 patients, who are undergoing for TB treatment around 12 weeks from the time of treatment started. This group was classified as "LTBI following up 2". For the healthy control cohort, 16 healthy adults with no history of exposure to TB were recruited from university population.

### 3.2.6 PBMCs isolation

Human peripheral blood mononuclear cells (PBMCs) for the patients recruited for the ISATS study were isolated according to the instructions described previously in chapter 2. PBMCs from 14 subjects were isolated by standard method for isolating mononuclear cells using Ficoll-Paque media (see chapter 2, 2.2.4.a). PBMCs from 32 subjects were isolated by using Sepmate-50 tube (chapter 2, 2.2.4.b). Serum samples were collected and stored at -80$^0$ C. PBMCs from each patient were counted, cryopreserved, and stored using FBS containing 20% (vol/vol) DMSO at -150$^0$ C as shown previously in chapter 2. Cell culture media and freezing media used in this chapter were prepared as explained in chapter 2.

### 3.2.7 Cell Surface staining and gating strategy

We aim to identify human blood lymphocytes, T cell phenotype, monocytes and dendritic cells subsets using cell surface markers and flow cytometry analysis. Frozen PBMCs for the patients from the ISATS study were thawed as described in chapter 2 (see section 2.2.5). Approximately between 5x10$^5$ to 1x10$^6$ PBMCs per well were loaded into 96 well U bottom cell culture plate. Live/dead viability staining, and cell surface staining was performed following the staining procedure protocols described in method section at chapter 2 (see section 2.2.6.a). Cells in first well were washed and stained with anti CD3, CD4, CD8, CD19, CD56, CD45RA, CD45RO, CD25, CD127 and CD197 antibodies for 30 minutes in the dark at 4$^0$ C. Cells in second well were washed and stained with anti CD3, CD19, CD56, CD16, CD141, CD11c, HLA-Dr, CD1c, CD14 and CD123 antibodies for 30 minutes in the dark at 4$^0$ C. Fluorescent compensation was performed using healthy unstained controls for live/dead viability staining and UltraComp eBeads staining with each fluorochrome separately as described in chapter 2. Compensation values were set to eliminate spectral overlap. Flow cytometry cleaning before and after every experiment was performed using 3ml FACS clean for 5 minutes and followed by 3 ml of FACS rinse for 5 minutes and long clean for 15 minutes was performed at least once in a month.

### 3.2.8 Gating strategy

Flow cytometry tubes and data were acquired using a FACSCelesta (BD, Bioscience) and analyzed with FlowJo software (BD, V10). To determine the frequency and phenotype of lymphocytes we followed the gating strategy described in chapter 2 section 2.3.1.a. To determine the frequency and phenotype of monocytes and dendritic cells followed the gating strategy described in chapter 2 section 2.3.1.b and 2.3.1.c respectively.

### 3.2.9 Statistical analysis

Data were analysed in IBM SPSS Statistics version 28 and GraphPad Prism 9. A nonparametric Kruskal-Wallis test was used to analyse group differences between healthy, LTBI and active TB donors. If we found there was a statistically significant difference between the medians of the independent groups, then Dunn's multiple comparison tests were used to compare the mean rank of LTBI and active TB subjects with mean rank of control subjects (HC). Also, multiple comparisons to compare the mean rank of the LTBI group with mean rank of F1 (4-6 weeks during they start treatment) or F2 (10-12- weeks during they started TB treatment) were tested using a nonparametric Friedman (ANOVA) test and Dunn's multiple comparison test for pairing data. Significance was set as p value < 0.05. data and figures were created using GraphPad Prism 9. Scatter plots were drawn to the median of the distribution.

## 3.3 Results

### 3.3.1 Baseline characteristics for the participants:

Patients enrolled in this study were recruited following REC reference 20/LO/0514, which was approved on 29 April 2020. Subjects were recruited at two specialist TB clinics operating within the University Hospitals of Leicester NHS Trust - the 'migrant clinic' and the 'rapid access' TB clinic. Patients with active TB were recruited from the infectious disease unit at UHL. Healthy controls were recruited from the adult University population. Table 3.1 shows baseline

characteristics for the participants recruited in the study showing their gender, age group, ethnicity and study group based on clinical assessment.

**Table 3.1 Baseline characteristics for the participants of ISATS study**

| Cohort | Healthy control | LTBI | Active TB |
|---|---|---|---|
| Total participant enrolled | 16 | 26 | 4 |
| Male, n (%)<br>Female, n (%) | 10 (21.7%)<br>6 (13%) | 17 (37%)<br>9 (19.6%) | 2 (4.3%)<br>2 (4.3%) |
| Ethnicity, n | | | |
| White | 11 | 3 | 0 |
| Black | 3 | 10 | 1 |
| South Asian | 1 | 10 | 3 |
| Other | 1 | 3 | 0 |
| Median age, years (range) | 28 (21-62) | 32 (17-76) | 43 (25-61) |

### 3.3.2 Analysis of lymphocyte subsets

We performed cell surface staining followed by flow cytometric analysis for the human PBMCs in ISATS participant to create a full profile. Lymphocytes represents 18%-42% of circulating WBC. The main subsets found within the lymphocyte population are B and T cells and natural killer cells (NK). To determine whether latent tuberculosis infection impacts relative levels of specific cellular subsets in the subjects and compared relative cellular levels between healthy, LTBI and active TB subjects. We also followed the LTBI subjects through treatment to assess the impact of treatment on frequencies of specific cell types. In our first analysis we compared the frequencies of CD4 and CD8 T cells within the CD3 positive T cell population. We found that there was no difference between the frequencies of CD4 and CD8 CD3 positive T cells between healthy control (HC) and LTBI subjects or between HC and active TB patients (Figure 3.1.A). The ratio for HC was around 4 with a wide range between 2-10, however the ratio for active TB trended lower around 3 but was not significant difference. The ratio for the LTBI trended lower in the median compared to HC but was not statistically significant.

Our next analysis involved further characterising the CD4 T cells for their ability to bind IL-7 (CD127) or IL-2 (CD25). We found no significant difference between the frequency of CD4+ T cells expressing CD25 but no CD127 between HC and the LTBI group, however we found that the frequency of these cells was significantly lower in HC compared to those with active TB patients (Figure 3.1.B). We found that the distribution of the LTBI subjects was greater than for the HC and that the active TB subjects were less distributed than the LTBI supporting a range of activity for the LTBI covering both the HC and active TB data.

To determine whether non-T lymphocytes were impacted B cells and NK cells were defined by gating on CD3-CD19+ and CD3-CD19-CD56+ respectively. We found no significant differences between HC subjects and either LTBI or active TB subjects in the frequency of B cells (figure 3.1.C). On the other hand, the frequency of NK cells in healthy group was significantly higher compared to the active TB group, but not significantly different to LTBI subjects (figure 3.1.D).

### 3.3.3 Analysis of CD4 and CD8 subpopulation

To determine whether the frequency of memory T cells differed between cohorts of healthy, LTBI and active TB disease, we performed cell surface staining to analyse the live single CD4+ T cells and CD8+ T cells. We used surface markers (CD45 RA and CD45 RO) for phenotypic characterisation of CD4+ naïve cells (CD4+ CD45 RA+), CD4+ memory cells (CD4+ CD45 RO+), CD8+ naïve cells (CD8+ CD45 RA+) and CD4+ memory cells (CD8+ CD45 RO+). We used surface markers (CCR7) for phenotypic characterisation of CD4+ central memory cells (CD4+ CCR7+ CD45 RA-), CD4+ effector memory cells (CD4+ CCR7- CD45 RA-), CD8+ central memory cells (CD8+ CCR7+ CD45 RA-), CD8+ effector memory cells (CD4+ CCR7- CD45 RA-), We found that the frequency of CD4 T cells expressing CD45 RA+ was significantly higher in HC compared to those with active TB, in contrast we found that the difference in frequency of these cells was not significant between HC and LTBI (figure 3.2.A). Also, we found that the frequency of CD4 T cells expressing CD45 RO+ in HC was significantly lower than those with active TB, however we found no significant difference in the frequency of these cells between HC and LTBI (figure 3.2.B). Our next analysis involved to characterise CD4+ expressing memory cells into central memory ($T_{CM}$) and effector memory ($T_{EM}$) to. We compared the frequency of central memory

T cells (cells that express CD45 RO and CCR7) and effector memory T (cells that express CD45 RO with no expression of CCR7) with CD4 positive T cells population. We found that there was no significant difference in the frequency of $T_{CM}$ either between HC and LTBI nor HC and active TB patients (figure 3.2.C). We found that the frequency of $T_{EM}$ was significantly higher in LTBI compared to those in HC, however no significant difference between the frequency of $T_{EM}$ between HC and active TB patients (figure 3.2.D).

Then in our analysis, we determined whether CD8+ was impacted. Naïve and memory cells were defined gating on CD8+ CD45 RA+ and CD8 CD5 RO+ respectively. We compared the frequency of these cells within HC, LTBI and active TB. We found no significant difference between the frequency of CD8+ expressing CD45 RA+ between HC and either LTBI or active patients (figure 3.3.A). Similarly, no significant difference between the frequency of CD8+ expressing CD45 RO+ between HC and either LTBI or active TB patients (figure 3.3.B). Our next analysis involved to characterise the CD8+ expressing $T_{CM}$ and $T_{EM}$. We found that there was no significant difference between the frequency of $T_{CM}$ between HC and either LTBI or active TB patients (figure 3.3.C). Similarly, no significant difference between the frequency of CD8+ expressing $T_{EM}$ between HC and either LTBI nor active TB patients (figure 3.3.D).

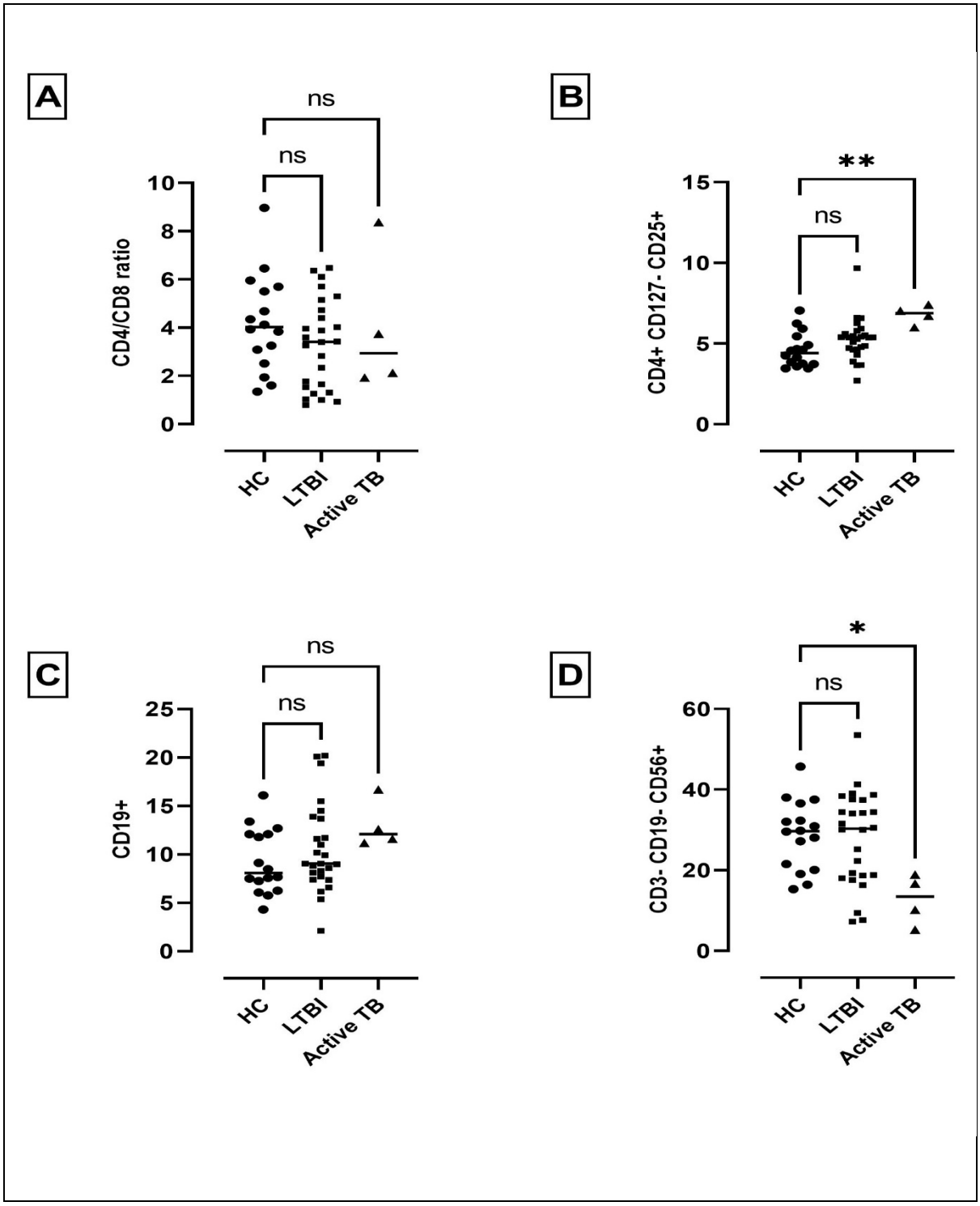



**Figure 3.1. PBMCs T and B cell surface marker expression in LTBI compared to healthy control and active TB patients.** CD4/CD8 ratio within the CD3+ population (A), the frequency (%) of CD3+CD4+ T cells which are CD127- CD25+ (B), frequency (%) of live single lymphocytes that are CD19+ (C) and the frequency (%) of live single lymphocytes that are CD3- CD19- CD56+ (D) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

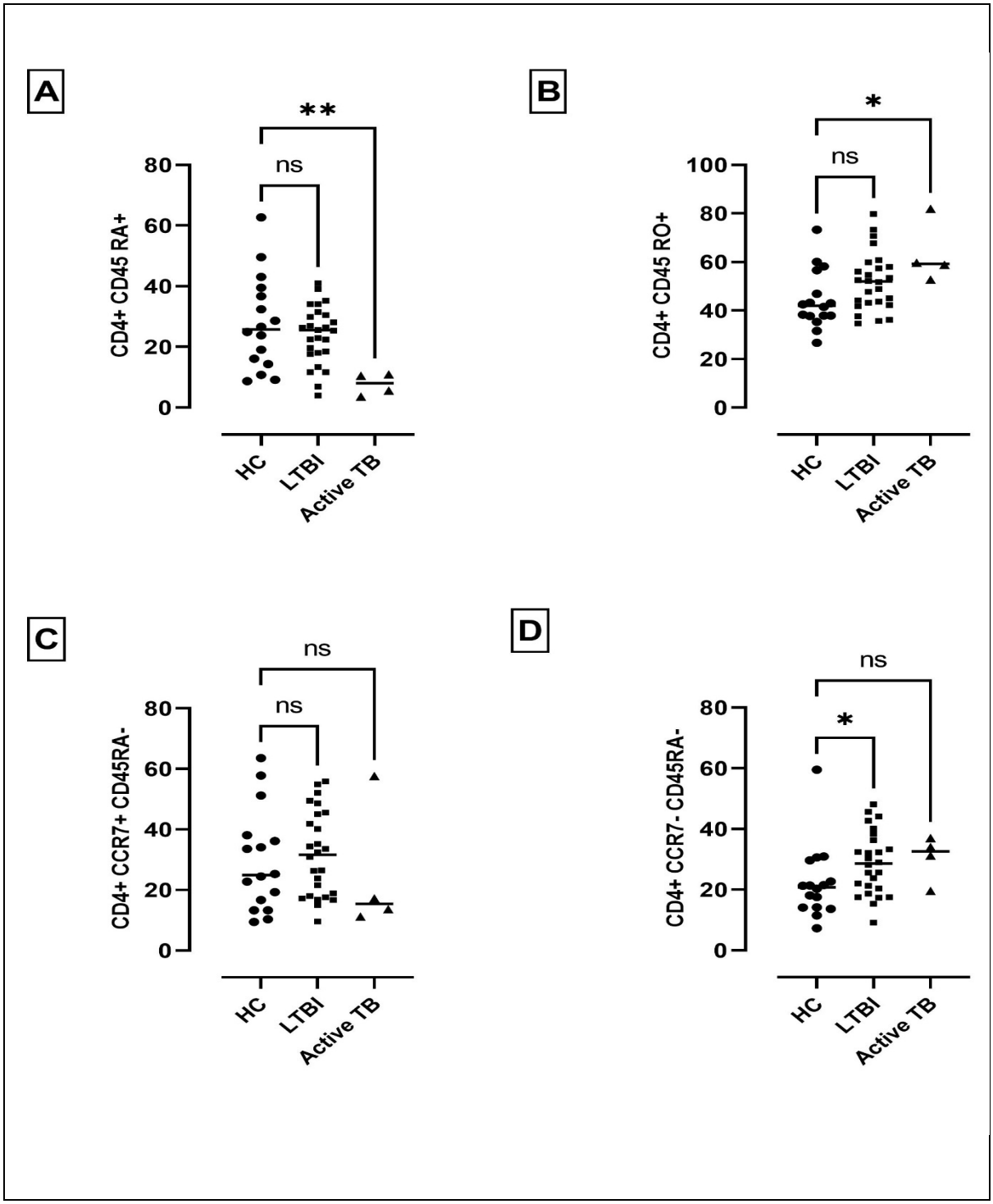



**Figure 3.2. PBMCs CD4+ subsets surface markers expression in LTBI compared to healthy control and active TB patients.** The frequency (%) of live single lymphocyte are CD4+ CD45 RA+ (A), the frequency (%) of live single lymphocyte are CD4+ CD45 RO+ (B), the frequency (%) of live single lymphocyte are CD4+ CCR7+ CD45 RA- (C) and the frequency (%) of live single lymphocyte are CD4+ CCR7- CD45 RA- (D) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

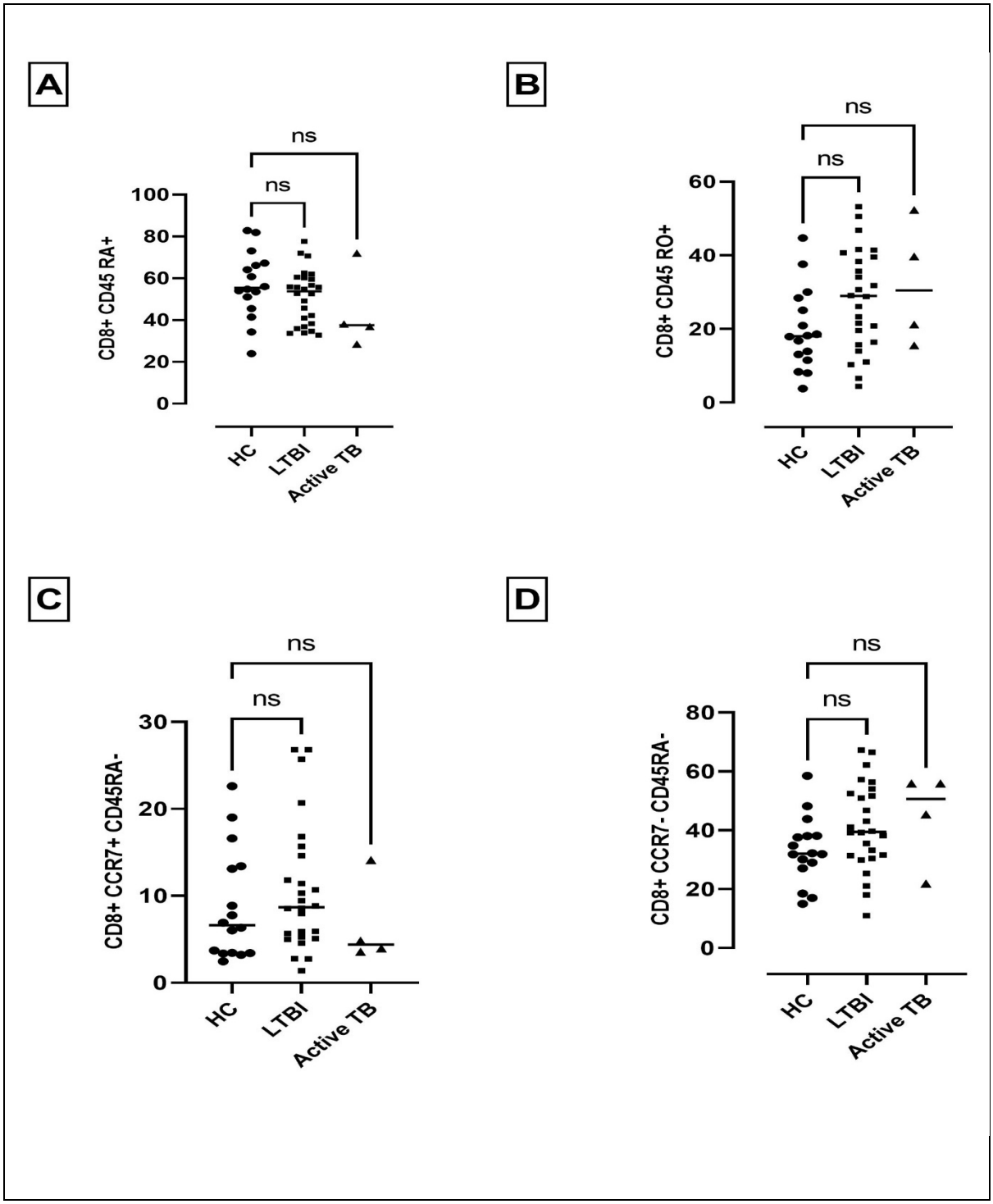



**Figure 3.3 PBMCs CD8+ subsets surface markers expression in LTBI compared to healthy control and active TB patients.** The frequency (%) of live single lymphocyte are CD8+ CD45 RA+ (A), the frequency (%) of live single lymphocyte are CD8+ CD45 RO+ (B), the frequency (%) of live single lymphocyte are CD8+ CCR7+ CD45 RA- (C) and the frequency (%) of live single lymphocyte are CD8+ CCR7- CD45 RA- (D) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

### 3.3.4 Analysis of monocyte subsets

In our next analysis we determined how the level of monocytes subsets impacted in LTBI subjects and compared to the level of monocytes subset in healthy control and active TB patients. We performed cell surface staining for PBMCs to determine the frequency of human blood monocytes subsets in the patients enrolled in the ISATS study. We found that the frequency range of CD14+ CD16- of LTBI was higher than HC or active TB, but not significant. Also, we found that there was no significant difference in these cells between HC and active TB patient (figure 3.4.A). Then we compared the frequency of CD14- CD16+ and we found that there was no significant difference between HC and either LTBI or active TB. Moreover, the frequency range for the HC group was higher than both LTBI and active TB but not significant (figure 3.4.B). These data suggested that although there is an effect on the level of Monocyte subsets however these differences were not significant (figure 3.4.C).

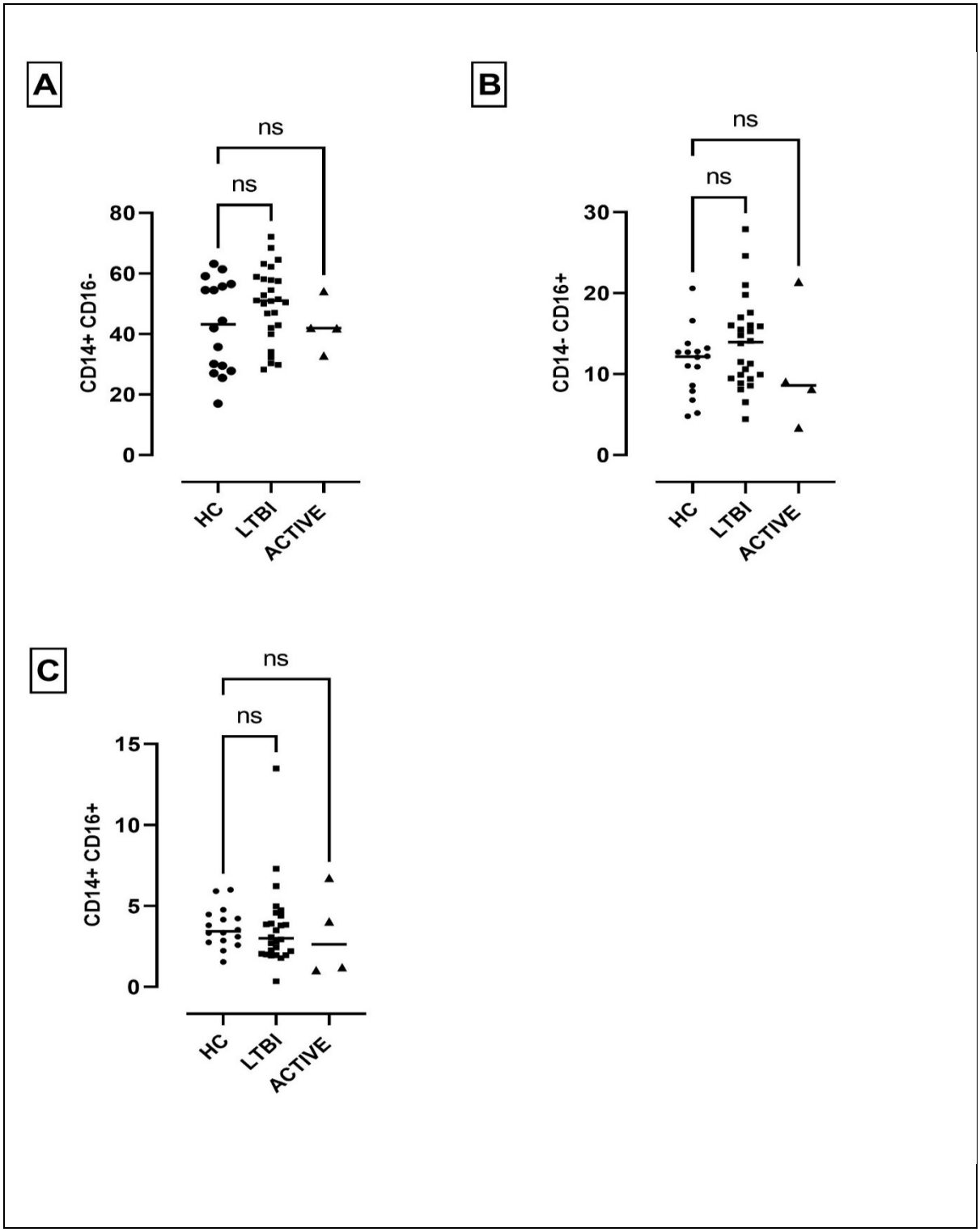



**Figure 3.4. PBMCs monocytes subsets surface markers expression in LTBI compared to healthy control and active TB patients.** The frequency (%) of live single HLA-DR+ are CD14+ CD16- (A), the frequency (%) of live single HLA-DR+ are CD14- CD16+ (B), The frequency (%) of live single HLA-DR+ are CD14+ CD16+ (C) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

### 3.3.5 Analysis of dendritic cells subsets

In our next analysis we performed cell surface staining to determine the frequency of dendritic cells (DCs) subsets. To determine whether the LTBI infection impact the level of specific DCs subsets compared to the relative cellular level between healthy control and active TB, we compared the frequency of DCs in PBMCs. We excluded CD3, CD19 and CD56 then we devolved a gate on HLA-DR+ CD14-. Then, we determine the positive DCs linage markers plasmacytoid DC (pDCs) express CD123+ and myeloid DCs (mDCs) expressing CD11c+. We calculated the mDCs/pDCs ratio and then determine the frequency of mDCs expressing CD1c+ and CD141+ respectively for the HC, LTBI and active TB patients. We found that the ratio range for the three groups was around 10 with no significant difference (1 active patient has mDCs/pDCs ratio > 80) (figure 3.5.A). We also found that the frequency of mDCs expressing CD1c+ in HC was higher compared to those in LTBI or active TB but not significant (figure 3.5.B). In our analysis also we compared the frequency of mDCs expressing CD141+ and we found that no significant difference in the frequency of these subsets between the groups (Figure 3.5.C).

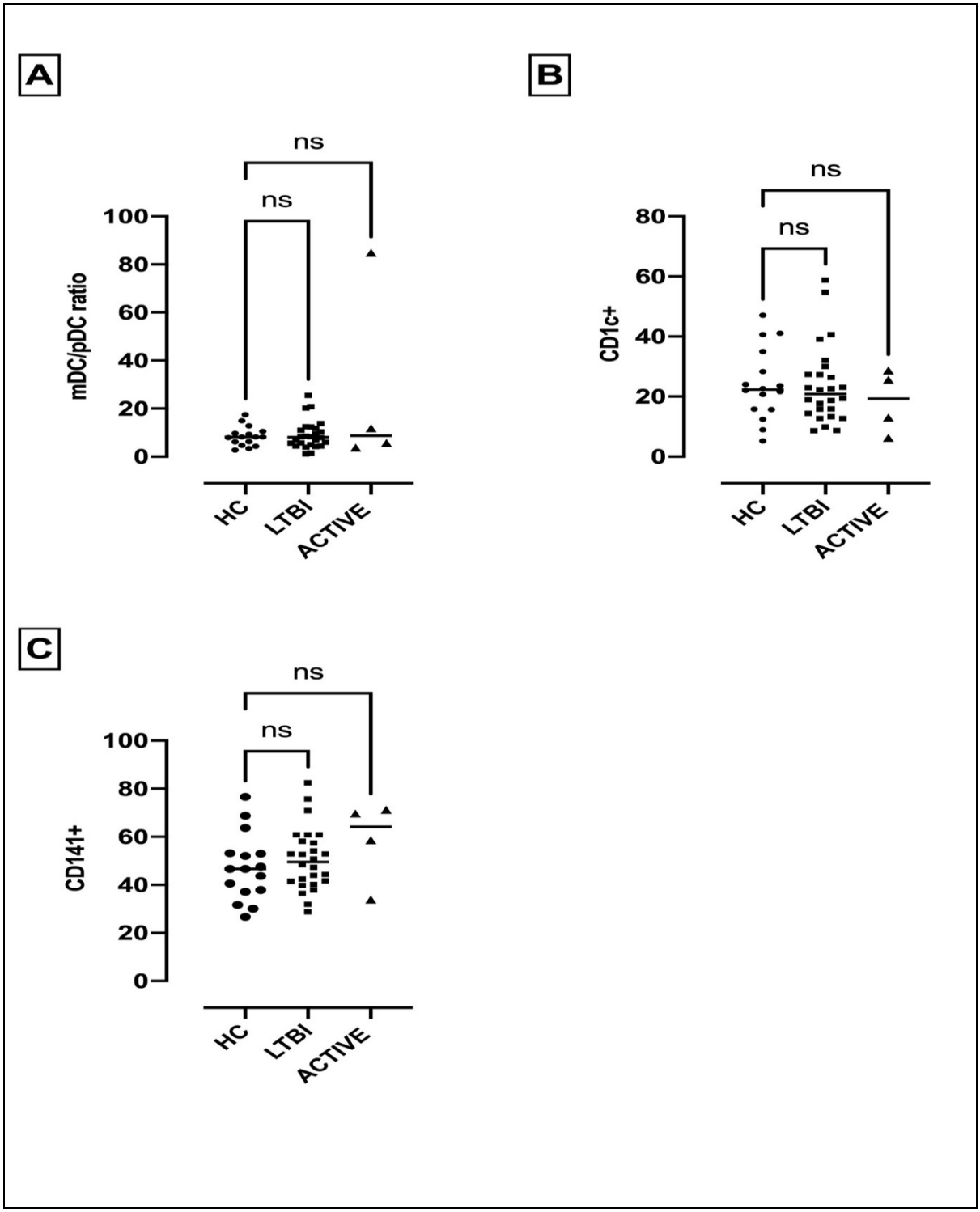



**Figure 3.5. PBMCs dendritic cells subsets surface markers expression in LTBI compared to healthy control and active TB patients.** CD11c+/CD123+ ratio of HLA-DR+ CD14- population (A), the frequency (%) of live single HLA-DR+ CD14- are CD11c+ CD1C (B), the frequency (%) of live single HLA-DR+ CD14- are CD11c+ CD1C (C) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

### 3.3.6 Analysis of lymphocytes subsets for subjects following Mtb treatment

We determine the frequency and phenotype of lymphocytes for the patients who received latent or active TB treatment at the recruiting day and compare the T cells frequency to those who followed the treatment during 4-8 weeks (F1 group) and then during 10-12 weeks (F2 group). In our first analysis we compared the CD4+/CD8+ ration within CD3+ population in the PBMC between LTBI baseline, LTBI F1 and LTBI F2 patients. We found that the ratio ranges for the groups were almost same, however the median of ratio for F2 was lower than F1 or LTBI but not statistically significant figure (3.6.A). Then we compared the frequency of CD4+ expressing CD25+ but not CD127 between the groups. We found that the frequencies of CD4+ CD127- CD25+ for the LTBI F2 group was significantly higher than the LTBI group (figure 3.6.B). In contrast, the frequency of the same cells for the F2 was higher than F1 but not significant (figure 3.6.B). Then, the impact of TB treatment for the LTBI patient for the non-T lymphocytes was determined. We compared the frequency of CD3- CD19- CD56+ and CD3- and CD19+ between the group (figure 3.6.C). We found no significant difference between the frequency of CD19+ between LTBI and either F1 or F2 group. We also found that the frequency of CD3- CD19- CD56+ for F2 group was significantly higher than F1, but not significant compared to LTBI group (figure 3.6.D).

Our next analysis involved further characterisation to determine CD4+ and CD8 expressing naïve or memory T cells to determine whether the frequency of CD4+ or CD8+ expressing CD45 RA+ OR CD45 RO+ was impacted by TB treatment. Then we determine the frequency of CCR7+ CD45 RA- and CCR7- CD45 RA- expressed on CD4+ and CD8+. We compared the frequency of these cells between the LTBI baseline, F1 and F2. We found the frequency of CD4+ CD45RA+ for the F1 group was higher compared to those in LTBI and F2 group, but not significant (figure 3.7.A). We also found the frequencies of CD4+ CD45 RO+ in the F1 group were significantly lower than those in LTBI baseline group (figure 3.7.B). Moreover, the frequency of these subsets in the F1 group was lower than those in the F2 group, but not significant (figure 7.B). Then in our analysis, we found that the frequency of CD4+ expressing CCR7+ but not CD45 RA+ in the F1 group was significantly lower compared to the LTBI baseline and that F1 was lower than the F2 group but not significant (figure 3.7.C). We also found no

significant difference between the frequency of CD4+ CCR7- CD45 RA- between LTBI and either F1 nor F2 (figure 3.7.D).

Similarly, we determined the frequency CD8+ expressing CD45 RA+ or CD45 RO+ and compared these between the groups. We found that the frequency of CD8+ expressing CD45 RA+ in the F1 group was higher than those with the F2 group or LTBI but not significant (figure 3.8.A). We also found the frequency of CD8+ expressing CD45 RO+ in the F1 group was lower than those with the F2 group or LTBI bit not significant. In next our analysis to assess the impact of TB treatment on CD8 expressing central memory or effector memory, we compared the frequency of CD8+ CCR7+ CD45 RA- and CD8+ CCR7- CD45 RA- between the group. We found no significant difference between the frequency of CD8+ CCR7+ CD45 RA- between the groups (figure 3.8.C). We also found no significant difference between the frequency of CD8+ CCR7- CD45 RA- between the group (figure 3.8.D).

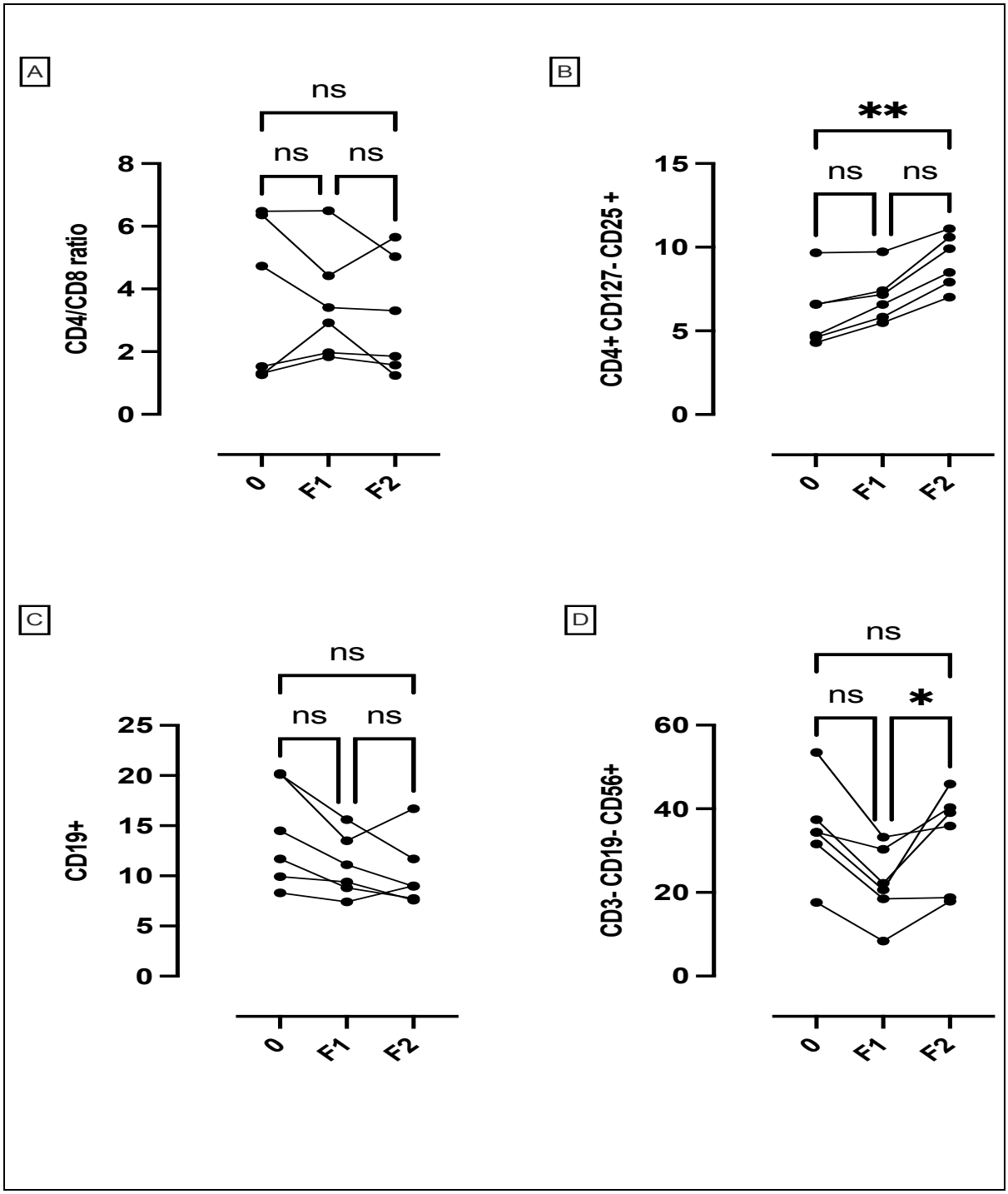



**Figure 3.6 PBMCs T and B cell surface markers expression in LTBI patients compared to the frequencies of the same patients during TB treatment.** CD4/CD8 ratio within the CD3+ population (A), the frequency (%) of CD3+CD4+ T cells which are CD127- CD25+ (B), frequency (%) of live single lymphocytes that are CD19+ (C) and the frequency (%) of live single lymphocytes that are CD3- CD19- CD56+ (D) from untreated LTBI patients (0), 6-8 weeks during the treatment (F1) and at end of treatment (F2). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

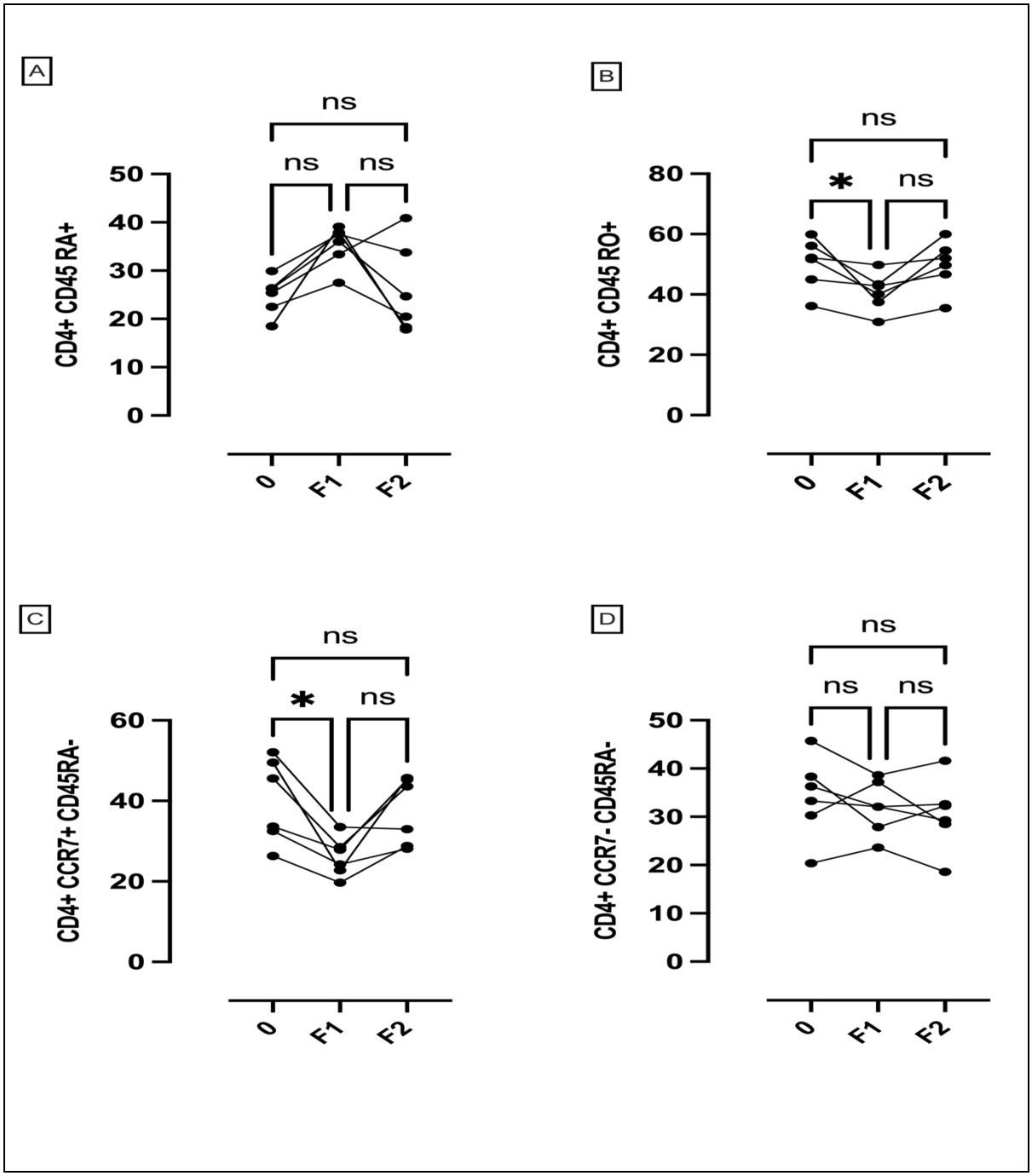



**Figure 3.7 PBMCs CD4+ subsets cell surface markers expression in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) of live single lymphocyte are CD4+ CD45 RA+ (A), the frequency (%) of live single lymphocyte are CD4+ CD45 RO+ (B), the frequency (%) of live single lymphocyte are CD4+ CCR7+ CD45 RA- (C) and the frequency (%) of live single lymphocyte are CD4+ CCR7- CD45 RA- (D) from untreated LTBI patients (0), 6-8 weeks during the treatment (F1) and at end of treatment (F2). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

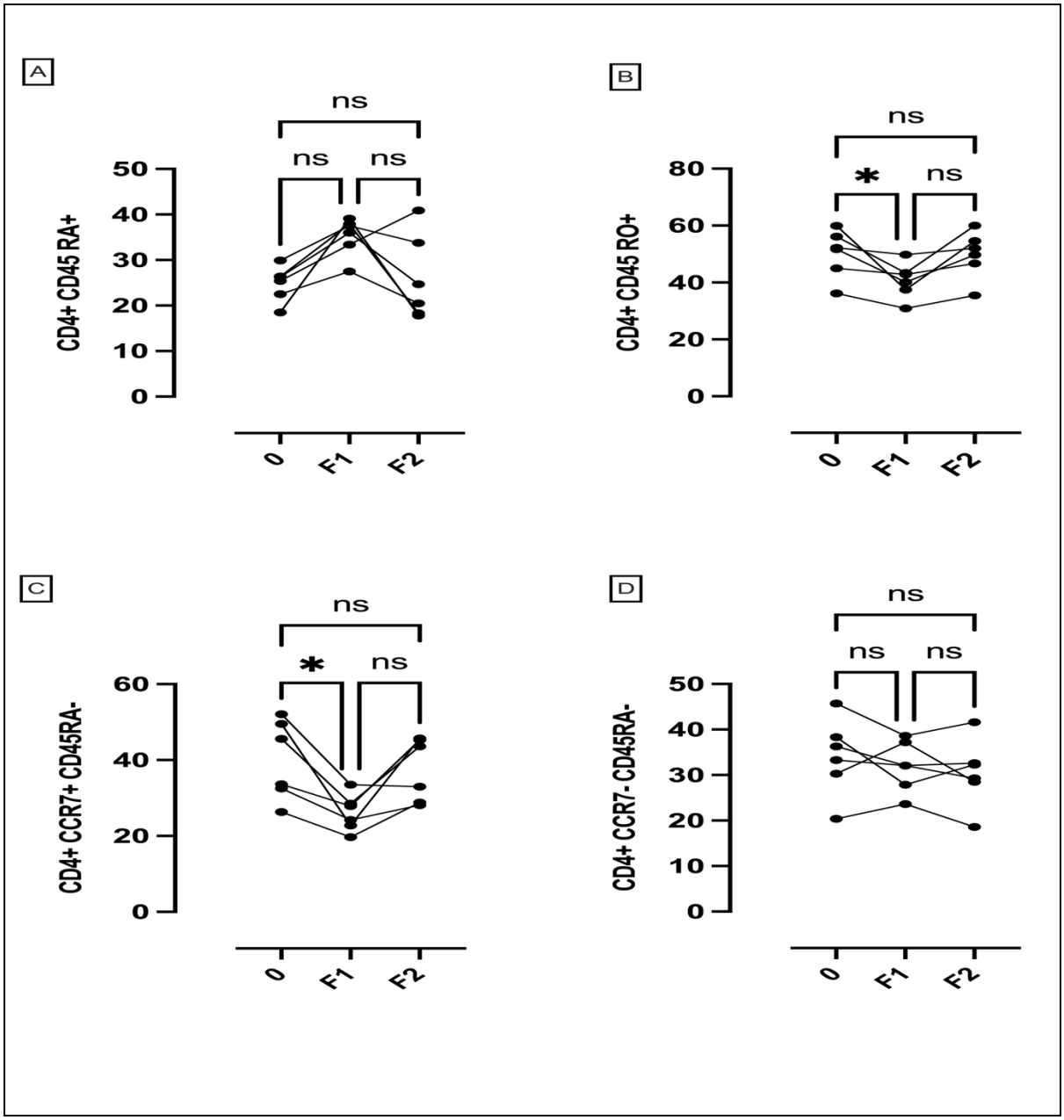



**Figure 3.8. PBMCs CD8+ subsets cell surface markers expression in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) of live single lymphocyte are CD8+ CD45 RA+ (A), the frequency (%) of live single lymphocyte are CD8+ CD45 RO+ (B), the frequency (%) of live single lymphocyte are CD8+ CCR7+ CD45 RA- (C) and the frequency (%) of live single lymphocyte are CD8+ CCR7- CD45 RA- (D) from untreated LTBI patients (0), 6-8 weeks during the treatment (F1) and at end of treatment (F2). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

### 3.3.7 Analysis of monocytes subsets for subjects following Mtb treatment

As well as to the lymphocyte subsets we assessed the effect of TB treatment for the LTBI patients on monocytes subsets. We did cell surface staining in PBMCs for the LTBI F1 and LTBI F2 participants following up their treatment. We determined the frequency of monocytes subsets and compared them between the groups. Our data revealed that there were no significant differences between the frequency of CD14+ CD16- between LTBI and either F1 or F2. The range of frequency of these cells was in F2 group was higher than those in F1, and higher compared to those with LTBI baseline but not significant (figure 3.9.A). In the next analysis, we found that the range of frequency of CD14- CD16+ in LTBI primary PBMCs was higher compared to those with group F1 or group F2 but not significant (figure 3.9.B). We also found that there was no significant difference between the frequency of CD14+ CD16+ between LTBI group and either F1 or F2 (figure 3.9.C).

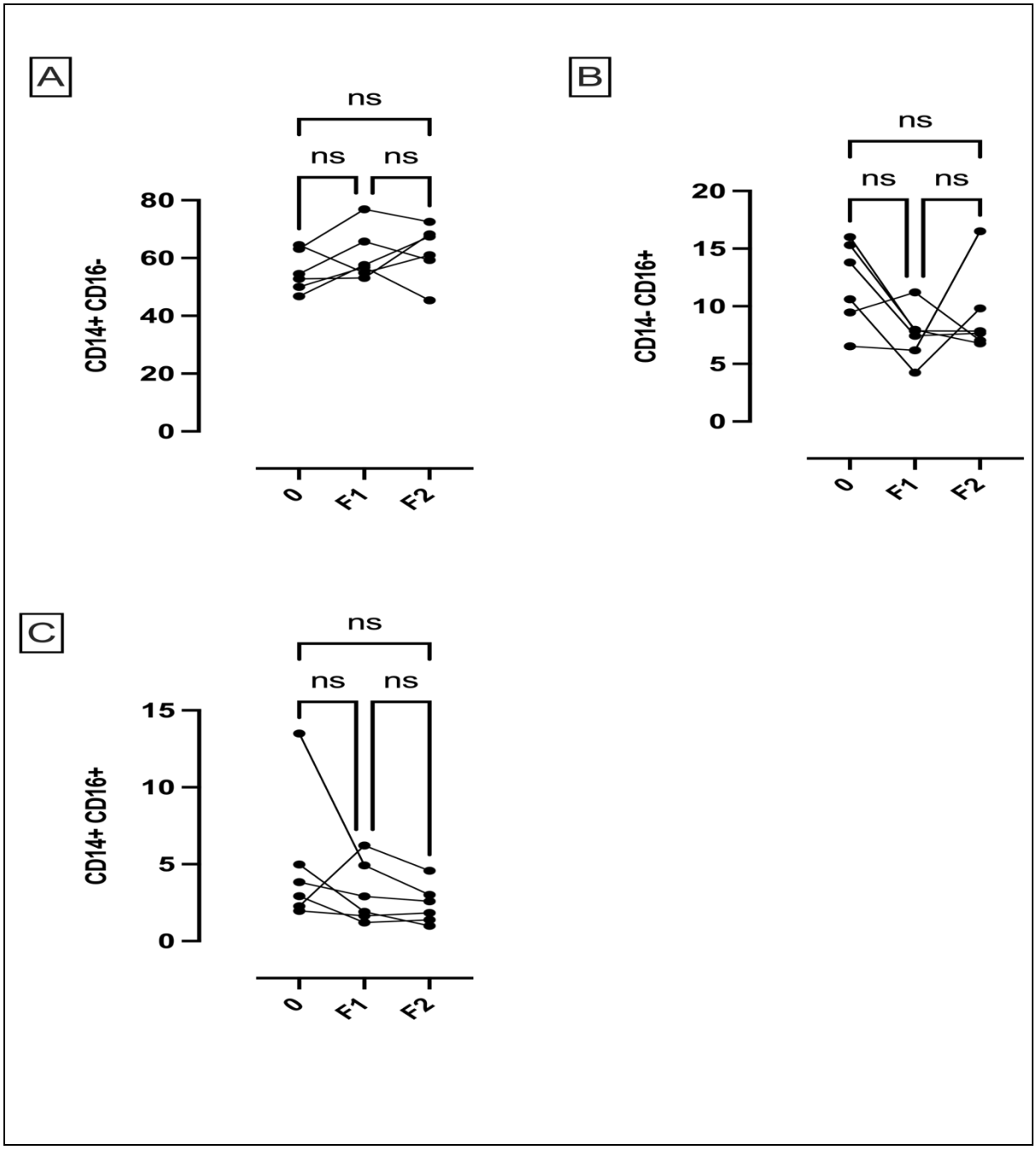



**Figure 3.9. PBMCs monocytes subsets cell surface markers expression in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) of live single HLA-DR+ are CD14+ CD16- (A), the frequency (%) of live single HLA-DR+ are CD14- CD16+ (B), The frequency (%) of live single HLA-DR+ are CD14+ CD16+ (C) from untreated LTBI patients (0), 6-8 weeks during the treatment (F1) and at end of treatment (F2). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

### 3.3.8 Analysis of dendritic cells (DC) subsets for subjects following Mtb treatment

We assessed the effect of TB treatment for LTBI patients on dendritic cells subsets. We did cell surface staining in PBMCs for the LTBI F1 and LTBI F2 participants following up their treatment. We determined the frequency of DCs and compared them between the groups. We found that the mDC/pDC ratio for the LTBI baseline was around 7 and the range was between 4-12 which was higher than the ratio for the F1 group which was around 4 and the range between 3-7 and higher than F2 group which was 6 and range between 4-10 but not significant (figure 3.10.A). The frequency of mDC expressing CD1c+ was in the F1 group significantly higher compared to those with LTBI baseline. However, the frequency of these cells in the F1 group was higher than those in the F2 group but not significant (figure 3.10.B). In our assessment for the impact of TB treatment we compared the frequency of mDCs expressing CD141+ within LTBI baseline, F1 and F2 groups. We found that the frequency of these cells was higher in the F1 group compared to LTBI or F2 group, but not significant (figure 3.10.C).

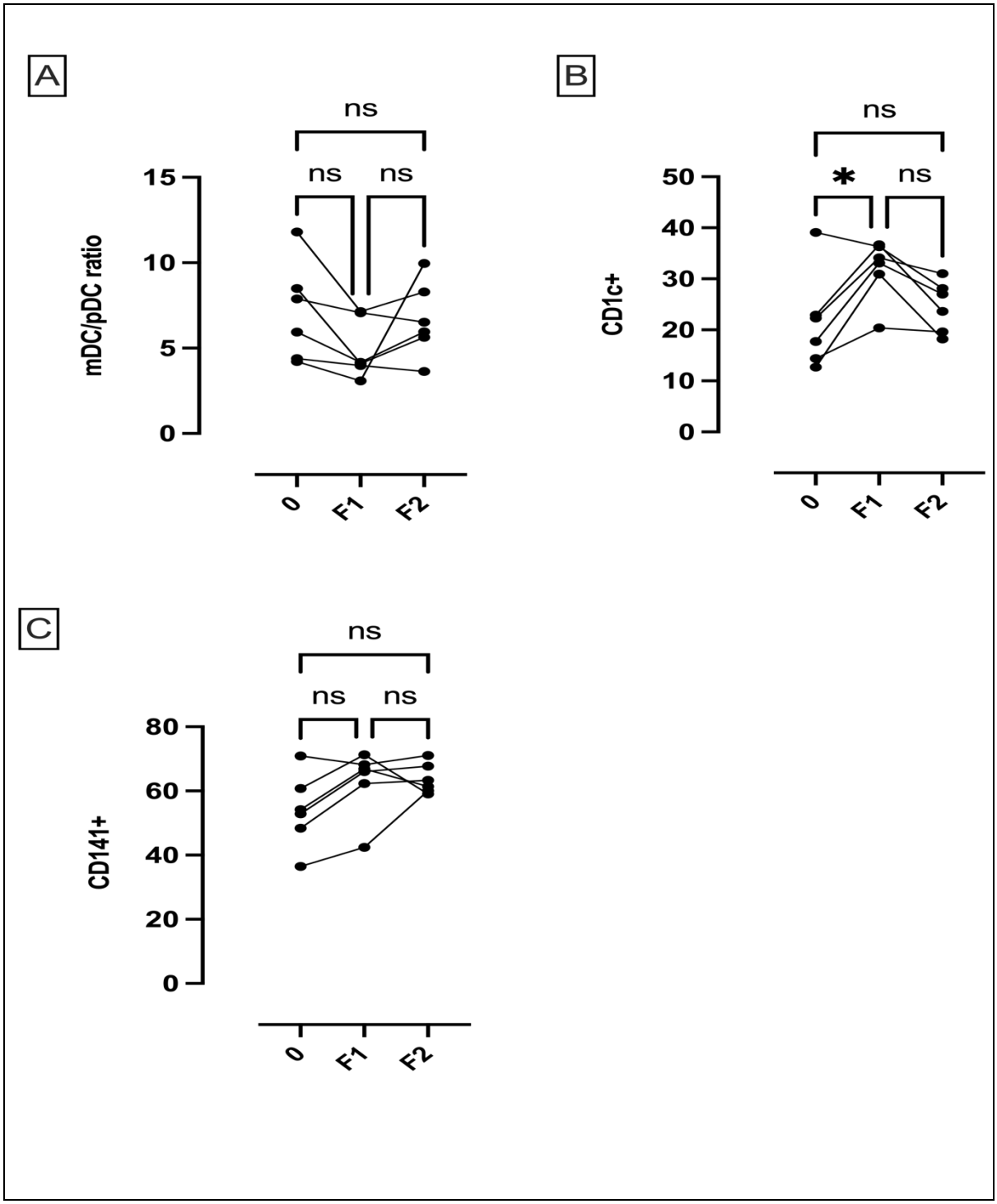



**Figure 3.10. PBMCs dendritic cells subsets cell surface markers expression in LTBI patients compared to the frequencies of the same patients during TB treatment.** CD11c+/CD123+ ratio of HLA-DR+ CD14- population (A), the frequency (%) of live single HLA-DR+ CD14- are CD11c+ CD1C (B), the frequency (%) of live single HLA-DR+ CD14- are CD11c+ CD1C (C) from untreated LTBI patients (0), 6-8 weeks during the treatment (F1) and at end of treatment (F2). The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

### 3.4 Discussion

We determined whether the frequency of T lymphocyte and non-T lymphocytes in the human PBMCs are different between healthy control, LTBI and active TB patients, then we assessed the impact of TB treatment on LTBI subjects following the treatment 4-6 weeks and 10-12 weeks. We found a significant difference between the frequency of CD4+ expressing CD25+ CD127- between HC and active TB. There was a significant difference between the frequency of CD3- CD19- CD56+ between HC and active TB patient. Also, there was a significant difference between the frequency of CD4 + expressing CD45 RA+ and between CD45 RO+ between HC and active TB. However, no significant differences were observed between the frequency of monocytes or dendritic cells subsets between HC and either LTBI or active TB. Overall, our data showed that the study of lymphocyte populations can be used as potential biomarkers to discriminate between different stages. It is not major classifier, but phenotypical profile of CD4+ memory or naïve T cells, Tregs and NK cells are amongst the most promising variables to discriminate active TB from healthy control. On the other hand, the phenotype of NK cells and CD4+ naïve T cells discriminate among the three groups. The scatter plots on the figures for the median distribution of the frequency/ ratio for the groups revealed that some subjects in LTBI groups expressed cells within the frequency range of active TB patients. This suggests that those subjects may be at risk of developing symptomatic active TB in the future. This suggests further investigation including the nature of disease based on the medical records and blood test data to find whether they share common parameters.

We also assessed the impact of TB treatment on the frequency of cells type. We determined the difference between the frequency of cells of LTBI patients who received the treatment at recruiting day compared to their first clinic visit (group F1/4-6 weeks) and second visit (group F2/ 10-12) weeks. We found that there is a significant difference between the frequency of CD4+ expressing CD25+ but not CD127 between the F2 group and LTBI baseline. Also, we found that there is a significant difference between the frequency of CD3- CD19- CD56+ between F2 and F1. Our data showed that there is significant difference between the frequency of CD4+ CD45 RO+ between F1 and LTBI baseline. Similarly, there was a significant difference between the frequency of CD4+ CCR7+ CD45 RA- between F1 and LTBI baseline.

On the other hand, no significant difference was found between either the frequency of monocytes subsets or DCs between the groups, but there was significant difference between the frequency of CD1c+ between F1 and LTBI baseline.

Our data showed the CD4+/CD8+ ratio was lower in active TB patients compared to this ration either in HC or LTBI. The reduction of the CD4+/CD8 ratio was due to the decrease of the CD4+ T cell population rather than the increase in the CD8 T cell population in active TB patients. Changes in lymphocytes subsets in peripheral blood of TB patients had been described previously. For example, The CD4+/CD8+ ratio was reported lower and statistically significant than the healthy control (EL Kholy et al., 2018). However, no significant difference was observed in our study. Moreover, our analysis shows that patients with TB had decreased the frequency of CD3+ CD4+ cells compared with healthy control but this was not significant; also, the frequency of CD3+ CD8+ and B cells were higher in active TB (but not significant). In contrast, Wu et al (2009) noted similar differences, but with statistical difference suggesting we might be able to see differences if we increased the number of subjects.

We investigated functional CD4 and CD8 characterisation and determine whether the disease stage (HC/LTBI/ATB/F1/F2) may impact these parameters. We found higher frequency of CD45RA-CD4+ T cells that express CCR7 (central memory) in both healthy control and LTBI than active TB; however non statistically significant difference was observed. In contrast, CD4+ T cells that express an effector memory phenotype (CD45 RA- CCR7-) was significantly higher in LTBI than healthy control, but not with active TB. Also, we detect that the difference is significant in the expression of CD4+ naïve (or CD4 + memory T cells) between healthy control and active TB patient. Moreover, we found that only naïve T cells were significantly different between LTBI and active Tb. Following TB treatment, the CD4+ T cells that express central memory markers were higher and significantly different in LTBI than F1 group, but not F2. The difference was not significant for CD8+ naïve or memory T cells between the groups. Pathakumari et al, 2017 reported an increased expression of central memory CD4+T cells in LTBI. They performed antigen specific memory markers to differentiate LTBI and active TB (we performed only cell surface staining without stimulation in this chapter, however we will assess the antigen specific activation marker including CD4+ memory T cells later in chapter 4). They also observed higher central memory in healthy control than active TB as we saw in

our data, but this was a significant difference in contrast to our data. Other evidence on human PBMCs suggests that healthy controls have more CD4+ central memory than LTBI (and not significant like our analysis) (Esteves et al., 2020). Furthermore, there was an agreement in that there is difference in CD4+ effector memory between healthy control and active TB but not significant (Esteves et al., 2020; Pathakumari et al., 2017).

In this study, we characterised the Tregs by identifying the extracellular markers used in combination with live singlet CD3, CD4, CD25 and CD127. CD25 is the $\alpha$ chain of IL-2 receptor and upregulated in activated T cells, while CD127 is the $\alpha$ chain of IL-7 and downregulated in activated T cells. To study the association between Tregs and the study groups, the level of $CD4^{+}$ $CD25^{+}$ $CD127^{-}$ was evaluated. We found that the percentage of Tregs was significantly lower in the healthy control group compared to active TB. On the other hand, they are lower but not significant compared to LTBI. Also, the CD4+ CD25+ CD127- percentage was significantly higher in active TB than those in LTBI patients. Moreover, our assessment for the Tregs following Mtb treatment revealed that the level of CD4+ CD25+ CD127- increased with TB treatment (10-12 weeks). The phenotypic characterisation of Tregs in human TB has been discussed and reported. Indeed, Zewdie et al, 2017 reported that CD4+ CD25+ and CD4+ $CD25^{high}$ were found to be higher in TB patients when compared to LTBI. On the other hand, they revealed that there is no difference in the frequencies of other phenotypes such as CD4+ CD25+ CD127- among their study groups. Like our data, Wergeland et al, published in Paula Cardona and Pere-Joan Cardona (2019) noted that there was a lower level of CD4+ CD25+ CD127- in healthy control compared to other groups, but not between LTBI and active TB (in contrast to our data which showed there is significant difference). Guyot-Revol et al, 2006 reported the association between TB patients before the treatment and Tregs, which was noted as the first assessment of Tregs to human TB. They reported that healthy control had lower frequencies of Treg in PBMCs compared to TB patients. Pathakumari et al, 2017 reported an increase in the percentage of antigen specific CD4+ CD25+ in active TB (PBMCs were stimulated with antigen such as ESAT-6 and CFP-10 (Pathakumari et al., 2017). As well as our data to assess the Tregs with TB treatment for 4-6 weeks and 10-12 weeks, Xu et al, 2016 and Whittaker et al, 2017 found that the frequencies of Tregs increased in the PBMCs of subjects who received TB treatment. Alternatively, we should consider the gene expression of the Forkhead box P3 (FoxP3). FoxP3 is a transcription factor used as a marker for Tregs. It

has been reported that TB patients had higher expression of FoxP3 in PBMCs compared to healthy controls (Guyot-Revol et al., 2006). This shows the importance of Tregs markers in human TB that may improve the clinical diagnosis in the future.

Cell types including monocytes/macrophages, DCs and NK cells provide innate immunity in Mtb infection (Liu et al., 2017). In this study we performed cell surface staining to compare the three major monocytes subsets between LTBI patients, active TB patients and healthy control population. We found an increased frequency of the three subsets in healthy control than those active TB patients, but this did not reach significance. Also, we found that the frequency of classical and non-classical monocytes is higher in LTBI than healthy control, but still not significant. Although the frequency of monocytes is higher in LTBI than active TB, no significant difference was observed. Furthermore, there are no significant difference between those LTBI patients who followed TB treatment. In agreement with our data, EL Kholy et al., 2018 reported that no significant differences were observed in the expression of monocytes. Interestingly, studies suggested that there is an association between monocytes subsets and TB disease (Lui et al., 2019). They reported that active TB patients produce higher intermediate monocytes and non-classical monocytes than healthy control and significant. They also reported that the frequency of classical monocytes was significantly higher in healthy control.

Moreover, significant difference was found between healthy control and active TB, and between LTBI and active TB in the CD3- CD19 CD56+ (NK cells). In agreement with previous study, Esteves et al., 2018 reported that the frequency of NK-bright in LTBI is higher than active TB and significant. Also, they noted that the frequency of NK cells is higher in healthy control than active TB, but not significant (in contrast to our analysis which are higher and significant).

Human PBMCs were gated for DC subsets based on HLA DR+ CD14- population. DC is subdivided into myeloid DCs based on the expression of CD11c+ (mDC), and plasmacytoid based on the expression of CD123+ (pDCs). No significant difference was reported between TB patient and healthy control in the total DCs or pDC and mDC (Gupta et al, 2010). However, a significant difference was noted in the mDCs/pDCs ratio, which is higher in healthy control

than TB patients. Similarly, our data showed no significant differences in both pDC and mDC between the groups. Indeed, no significant difference in the expression of myeloid DC subsets including CD1c and CD141 between the groups.

This study has some limitations that impact the interpretation of the research results. We proposed to determine the frequency and phenotype of PBMCs in well characterised cohorts of 6 groups, however we managed to identify and compare the results between only 3 groups. The reason for this was the difficulty of recruiting the community-acquired pneumonia (CAP) patients and those who are negative IGRA test subjects during the COVID-19 pandemic. Another limitation is lack of subclinical active TB group. The reason of that this group is missing because we didn't find patients that matched these criteria during the recruiting for this study (presence of radiographic or microbiologic test results consistent with TB without clinical symptoms). Another limitation is the difference in the ethnicity groups between the latent/active and healthy groups and the insufficient sample size of active TB group. First, although our subjects were recruited at two specialist TB clinics operating within the University of Leicester NHS Trust, some patients were allocated to other studies with more active research nurse commitment. This didn't allow to recruit them to ISATS study. Moreover, due to COVID-19 pandemic, some patients attended their assessment remotely. Also due to the pandemic, the recruiting of healthy volunteer subjects in this study were limited to University of Leicester and NHS Trust staff members. To overcome this limitation, we would suggest extending the ethics approval to recruit more patients and to have more structured collection of healthy controls to more closely match the infected and diseased subjects. These changes would provide a sufficient sample size to draw conclusions on the role of ethnicity and other aspects on the outcomes in the future.

### 3.5 Conclusion

We hypothesized that the phenotype and relative ratios of PBMCs differ could differentiate the complexity of outcomes following Mtb infection. We found that frequency (%) of live, single CD4+ CD25+ but not CD127 is associated with active TB disease. We found that the frequency (%) of live single CD3- CD19- CD56+ is associated with active TB disease. Also, we

found that the frequency (%) of live, single lymphocytes that are CD4+ CD45 RA+ is associated with LTBI patients. We found that the frequency (%) of live, single lymphocytes that are CD4+ CD45 RA+ is associated with active TB but cannot discriminate those from LTBI patients. Finally, the expression of CD56+, CD4+ memory T cells, CD8 + memory T cells, non-classical monocytes markers and intermediate monocytes markers decreased after 6-8 from LTBI treatment, but then the expression of these markers increased at the end of treatment.

**Chapter 4: Mtb specific CD4 T cells responses**

## 4.1 Introduction

As we found in chapter 3 looking at total cells did not provide any major classifier therefore, we wanted to see whether antigen-driven responses differ between the cohorts. It has previously been shown that different Mtb specific T cells express activation markers and/or cytokines and that these can be used to identify peripheral blood host biomarkers for TB diagnosis. This is largely seen as an increase in specific T cells biomarkers in TB patients compared to control. A number of cellular biomarkers have been determined from whole blood to peripheral blood mononuclear cells (PBMCs) to investigate the ability of specific T cells marker to distinguish active TB patient from LTBI. Several protocols and gating strategies of Mtb-specific T cells phenotypes, cytokine production and activation markers have been reported (Graves et al., 2014; Dan et al.,2-16; Musvosvi et al., 2018& Nemes et al., 2018). Nemes et al and Musvosvi work is in South Africa where there is a high pressure of reinfection; however, how do these markers work in Leicester where there is a high incidence but low risk of exposure. We adapted these published protocols and developed cells surface and intracellular staining panels not just to determine the differences, but we want to identify whether there are cellular markers in LTBI patients expressed within the same range of these markers in active TB and then correlate our outcomes to the plasma levels of inflammatory cytokines and our novel biomarker IL-12RB1 ΔTM (later in chapter 5). We also want to be able to correlate the QuantiFERON-plus response with any antigen-specific T cell responses in the blood that may help to identify those subclinical patients or at risk to progress to active TB.

It has been shown that both cytokine production and change in cell surface markers by Mtb-specific CD4+ T cells in response to antigen correlates with TB status. Cell surface expression of CD27 or HLA-DR on Mtb-specific CD4+ T cells has been studied in depth to see whether the detection of these markers was a tool to distinguish between LTBI and active disease. It has been shown that there is higher frequency of Mtb-specific CD4+ T cells expression of both IFN$\gamma$+ TNF-$\alpha$+ in active TB patients relative to healthy controls, but did this signal does not resolve upon cure (Petruccioli et al., 2013). It is also shown that the frequency of single positive TNF-$\alpha$ of Mtb-specific CD4+ T cells was highly associated with active TB patients (Harari et al., 2011). Also, it is shown that Mtb-specific CD4+ T cells expression both IFN$\gamma$+ IL-2+ or single positive IFN$\gamma$+ was associated with LTBI patients relative to active patients

(Caccamo et al, 2010). Moreover, it has been shown that the frequency of Mtb-specific IFN$\gamma$+ producing CD4+ T cells expressing HLA-DR+ is higher in those with active TB relative to LTBI and that the ratio between CD27 expression on IFN positive and negative T cells is greater in active than in latent patients (Musvosvi et al., 2018). Dan et al (2016) have developed activation induced markers (AIM) assay and found that the co-expression of OX40+ CD25+ on Mtb-specific memory CD4+ T cells was increased in LTBI compared to healthy control. Esclante et al (2015) reported that the frequency of TB antigen specific CD3+ CD4+ that express OX40+ CD25+ was significantly higher in untreated LTBI compared to these cells in unexposed healthy control or treated LTBI subject. Moreover, Esclante et al (2020) reported a significant difference in CD25+OX40+ and PD-L1+ CD25+ co-expression between LTBI subjects and unexposed subjects.

By showing the roles of specific T cells secreting cytokines and cellular biomarkers to the immune responses against Mtb, supports the aim of determining the frequencies of Mtb specific T cells among the groups in ISATS study. We therefore determined if there were any measurable differences in the frequencies of antigen-specific T cells between LTBI patients compared to active TB or healthy controls. We chose to address this using a panel of intracellular and cell surface markers which are known to define antigen specific CD4+ T cells responses by flow cytometry. We tested the PBMC's from 26 LTBI patients, 4 active TB and 16 healthy volunteers. Also, to link any observed differences to live Mtb infection, we performed flow cytometric analysis on PBMC from LTBI participants following 4-6 weeks and 10-12 weeks treatment.

**Hypothesis**

We hypothesized that the frequency and phenotype of antigen-specific T cells would allow discrimination of disease phenotype in defined cohorts of healthy, latent and active TB subjects.

**Objectives:**

1- To determine the frequency and phenotype of Mtb300 specific cytokine producing CD4+ T cells in well characterized cohorts of healthy, latent and active TB subjects
2- To determine the frequency and phenotype of Mtb300 specific memory CD4+ T cells responses in well characterized cohorts of healthy, latent and active TB subjects

3- To determine the impact of TB treatment on the T cells responses in LTBI group

4- To correlate the QuantiFERON-plus value with the cellular responses in LTBI

## 4.2 Methods and materials:

### 4.2.1 Study subjects

A total of 48 HIV negative aged ≥16 participants were enrolled into the ISATS screening cohort. 2 of the participants withdrew from the study. Full description of the participants and how we grouped them, and ethics statement for ISATS described in chapter 3.

### 4.2.3 Media

Flow cytometry media (FSM), Dulbecco's Modified Eagle Media (DMEM), and Freezing media were prepared as described in chapter 2. BD fixation/permeabilization solution and BD perm/wash buffer were prepared following the instructions in the BD cytofix/cytoperm kit as described in chapter 2.

### 4.2.3 PBMCs isolation

Human peripheral blood mononuclear cells (PBMCs) for the patients recruited for the ISATS study were isolated according to the instructions described in chapter 2 (see 2.2.4). PBMCs from each patient was counted, cryopreserved, and stored using FBS containing 20% (vol/vol) DMSO at $-150^0$ C as shown in chapter 2. All the preparation of cell culture media including freezing media which used in this chapter was explained in chapter 2.

### 4.2.4 Antigen

**Staphylococcus enterotoxin b (SEB)** see chapter 2 section 2.2.3

**MTB300** see chapter 2 section 2.2.3

**4.2.5.A PBMCs stimulation for intracellular staining to determine the frequency of antigen specific CD4+ T cells cytokines release:**

PBMCs were thawed, spun, and resuspend in complete DMEM media containing 5% FBS. The cells were added in 96 wells U bottom cell culture plate (approximately between $5x10^5$ to $1x10^6$ per well). The plate was as below:

1- **Well 1**: A non-stimulated PBMCs were loaded as negative control.
2- **Well 2**: The cells in were stimulated with Staphylococcus endotoxin B (SEB) 5 µg/ml as positive control.
3- **Well 3**: PBMCs stimulated with Mtb-300 peptides 2 µg/ml.
4- **Well 4**: PBMCs stimulated SEB as fluorescent minus one FMO control.

2 µl of anti-Costimulatory CD28/CD49d antibodies were added to each well then, the cells were incubated at 37°C in 5% $CO_2$ for 6 hours. Brefeldin A was added 2hrs after the stimulation and incubate to complete 6 hours.

**4.2.5.B Intracellular staining protocol**

We determined antigen specific CD4+ T cells cytokines responses in human PBMCs stimulated with Mtb300 using intracellular staining followed by flow cytometric analysis. After the stimulation prior to intracellular staining first we performed Live/dead viability staining, and cell surface staining was performed following the staining procedure protocols described in method section at chapter 2 (see section 2.2.13). Cells were stained with Live/dead viability for 30 minutes in the dark at $4^0$ C. Then, cells were washed and stained for 30 minutes in the dark at $4^0$ C with anti CD3, CD4, CD8a, CD27, and HLA-DR antibodies. Then, cells were washed and fixed (permeabilized) for 20 minutes at $4^0$ C. Then, cells were washed and stained for 30 minutes in the dark at $4^0$ C with anti IFN-$\gamma$, IL-2, and TNF-$\alpha$ antibodies. Then cells were washed twice with BD wash/perm buffer, resuspended with FMS:10% formalin, transferred into flow cytometry tubes and data acquired using FACSCelesta (BD) and analysed using FlowJo software. Fluorescent compensation was performed using healthy unstained control for live/dead viability staining and UltraComp eBeads staining with each fluorochrome separately as described in chapter 2. Compensation values were set to eliminate spectral overlap. Flow

cytometry cleaning before and after every experiment was performed using 3ml FACS clean followed by 3ml FACS rinse for 5 minutes, and long clean for 15 minutes was performed at least once in a month.

#### 4.2.6.A PBMCs stimulation for cell surface staining to determine the induced activation markers:

PBMCs were thawed, spun, and resuspend in complete DMEM media containing 5% FBS. The cells were added in 96 wells U bottom cell culture plate (approximately between $5x10^5$ to $1x10^6$ per well). The plate was labelled as below:

1- **Well 1**: A non-stimulated PBMCs were loaded as negative control.
2- **Well 2**: The cells in were stimulated with Staphylococcus endotoxin B (SEB) 5 µg/ml as positive control.
3- **Well 3**: PBMCs stimulated with Mtb-300 peptides 2 µg/ml.
4- **Well 4**: PBMCs stimulated SEB as fluorescent minus one FMO control.

The cells were incubated at 37°C in 5% $CO_2$ for 18-24 hours.

#### 4.2.6.B Cell surface staining protocol

We aim to determine antigen specific CD4+ T cells cytokines responses in human PBMCs stimulated with Mtb300 using intracellular staining followed by flow cytometric analysis. After the stimulation prior to cell surface staining first we performed Live/dead viability staining, and cell surface staining was performed following the staining procedure protocols described in method section at chapter 2 (see section 2.2.14). Cells were stained with Live/dead viability for 30 minutes in the dark at $4^0$ C. Then, cells were washed and stained for 30 minutes in the dark at $4^0$ C with anti CD3, CD4, CD8, CD19, CD14, CD25, CD45 RA, CCR7, OX40, and PD-L1 antibodies. Then cells were washed twice with FSM media, resuspended with FMS:10% formalin, transferred into flow cytometry tubes and data acquired using FACSCelesta (BD) and analysed using FlowJo software. Fluorescent compensation and flow cytometry cleaning was performed as described previously.

### 4.2.7 Gating strategy

Flow cytometry tubes and data were acquired using a FACSCelesta (BD, Bioscience) and analyzed with FlowJo software (BD, V10). The frequency of antigen specific CD4+ T cells cytokines response was determined following the gating strategy described in chapter 2, section 2.3.4. the frequency of antigen specific CD4+ memory T cells was determined following the gating strategy described in chapter 2, section 2.3.5.

### 4.2.8 Statistical analysis

Data were analysed in GraphPad Prism 9. A nonparametric Kruskal-Wallis test was used to analyse group differences between healthy, LTBI and active TB donors. If we found there is a statistically significant difference between the medians of the independent groups, then Dunn's test will obtained to determine which groups are different. Also, multiple comparisons to compare the mean rank of the LTBI group with the mean rank of F1 (4-6 weeks during the started treatment) or F2 (10-12 weeks during the started treatment) were tested using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Significant was set as p value $< 0.05$. data and figures visualised using GraphPad Prism 9. Scatter plots were drawn to the median of distribution, and column graph bar were drawn for median.

## 4.3 Results

### 4.3.1 Mtb300 specific CD4+ expressing cytokines.

In our analysis, we determined the impact of Mtb300 peptides on the production of IFN$\gamma$, IL-2 and TNF$\alpha$ between the groups. We stimulated the PBMCs with Mtb300 for 6 hours (with addition of anti CD28/CD49d antibodies at time 0 and the addition of Brefeldin A 2 hours from incubation at $37^0$ C). furthermore, we stimulated cells with SEB to provide positive control, and we incubated unstimulated cells as negative control. We performed intracellular staining for the PBMCs to determine the frequency of Mtb300 specific T cells cytokines released in the individuals recruited in this study. Then we compared between the frequency of these cytokines in LTBI patients to those in healthy control (HC) and active TB patients. The results presented in figure 4.1 showed that antigen specific CD4+ T cells produce cytokines, but their frequency is different depending upon the antigen used. Indeed, we found that at 6 hours post stimulation the frequency of CD4+ expressing IFN$\gamma$ were higher in cells stimulated with SEB (positive control, nonspecific T cell superantigen) compared to these frequencies in cells stimulated with Mtb300 (specific T cells antigen) or those unstimulated cells (figure 4.1.A). In our analysis of the responses to Mtb300 specific CD4+ T cells, we found that there was a significant difference between the frequency of CD4+ expressing IFN$\gamma$ in LTBI compared to those in HC (figure 4.1.A). Also, there was a significant difference between the frequency of CD4+ IFN$\gamma$+ between active TB patients and the HC group (figure 4.1.A). The range of the frequency of CD4+ IFN$\gamma$ in the active TB group was significantly higher than the range of these frequency in both HC and LTBI groups and there were 6 subjects in LTBI groups express CD4+ IFN$\gamma$+ within same range of the frequency of these in active TB patients. these 6 subjects were selected based on the expression of CD4+ IFN$\gamma$+ to investigate how they behaved for the Mtb300 specific CD4+ expressing IL-2 and TNF-$\alpha$. Also, we found that at 6 hours post stimulation the frequency of CD4+ expressing IL-2 were higher in cells stimulated with SEB compared to these frequencies in cells stimulated with Mtb300 or those unstimulated cells (figure 4.1.B). In our analysis of the responses to Mtb300 specific CD4+ T cells, we found that there was a significant difference between the frequency of CD4+ expressing IL-2 in LTBI compared to those in HC (figure 4.1.B). Also, there was a significant difference between the frequency of CD4+ IL-2+ between active TB patients and HC group (figure 4.1.B). The range of

the frequency of CD4+ IL-2 in the active TB group was significantly higher than the range of these frequency in both HC and LTBI groups and there were 4 patients in LTBI groups (from the subjects whom express high IFN$\gamma$) express CD4+ IL-2+ higher than range of the frequency of these in active TB patients (figure 4.1.B). The results presented in figure 4.2 showed that antigen specific CD4+ T cells produce TNF-$\alpha$, but their frequency is different depending upon the antigen used. Also, we found that at 6 hours post stimulation the frequency of CD4+ expressing TNF-$\alpha$ were higher in cells stimulated with SEB compared to these frequencies in cells stimulated with Mtb300 or those unstimulated cells (figure 4.2). In our analysis of the responses to Mtb300 specific CD4+ T cells, we found that there was a significant difference between the frequency of CD4+ expressing TNF-$\alpha$ in LTBI compared to those in HC (figure 4.2). Also, there was a significant difference between the frequency of CD4+ TNF-$\alpha$ + between active TB patients and HC group (figure 4.2). The range of the frequency of CD4+ TNF-$\alpha$ in the active TB group was significantly higher than the range of these frequency in both HC and LTBI groups and there were 6 patients in LTBI groups express CD4+ TNF-$\alpha$ within the median of the frequency of these in active TB patients and 1 patient in LTBI was expressed CD4+ TNF-$\alpha$ higher than the range of the frequency of these cells in active TB patients' group (figure 4.2). These data show that there were 6 patients that always expressed cytokines within the same range (or higher for CD4+ IL-2+) of the expression of these in active TB suggesting that they may be subclinical perhaps. Although some other subjects overlap with active TB group, we picked only these who expressed the three cytokines and highlighted them for further investigation (other LTBI subject expressed only either 1 or 2 cytokines within the range of active TB group). We investigated their clinical data and case report forms to see whether if they share any similarities. We found that the only variable they shared is the size of the QuantiFERON-Plus blood test.

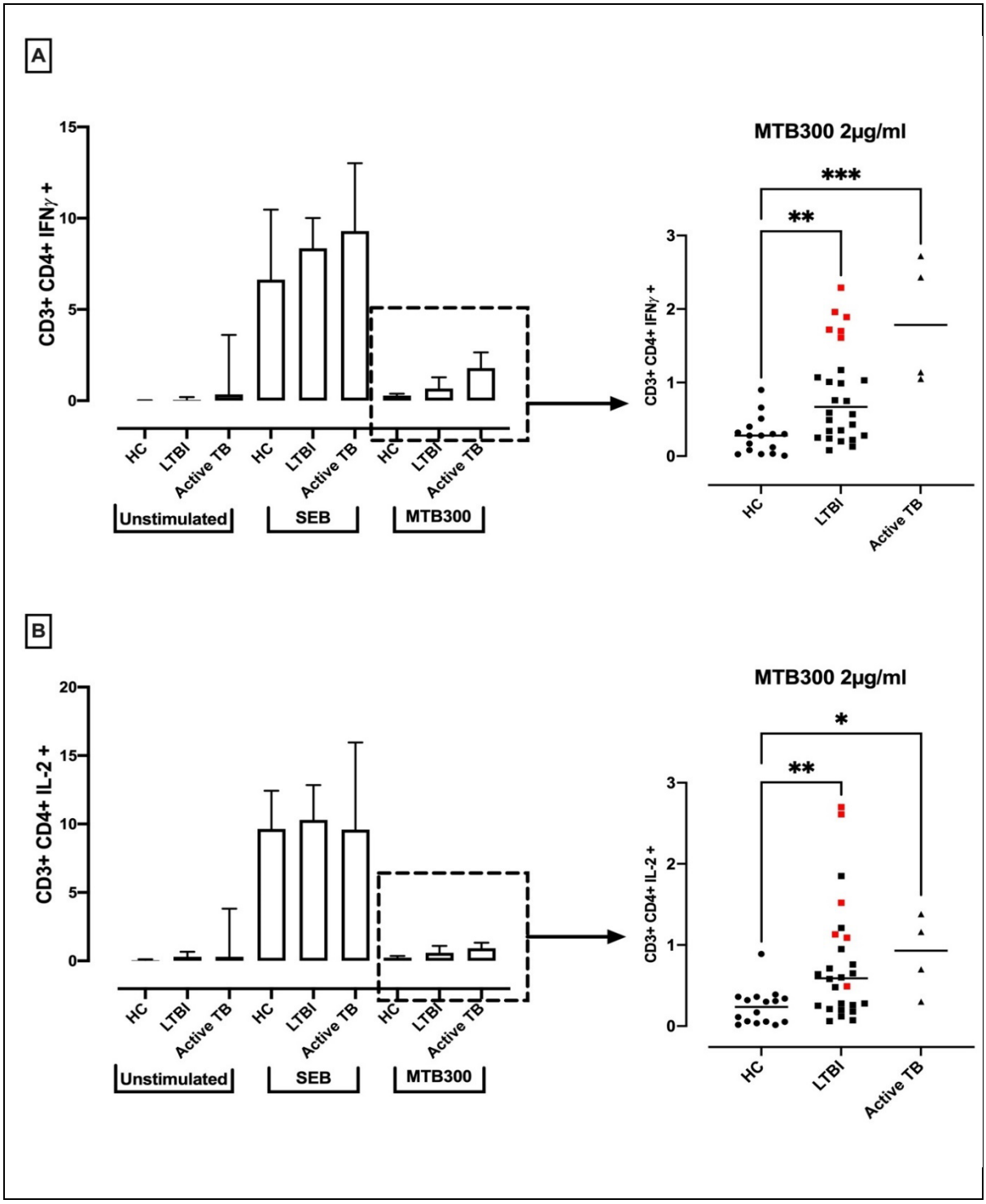



**Figure 4.1. Mtb300 specific CD4+ expressing IFN-$\gamma$ or IL-2 in healthy control, LTBI and active TB subjects.** The frequency (%) of live single lymphocytes are CD4+ IFN-$\gamma$ (A), the frequency (%) of live single lymphocyte are CD4+ IL-2+ (B) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4) stimulated with SEB or Mtb300 or unstimulated negative control **(Left)**. The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test for subjects stimulated with Mtb-300 **(right)**. 6 patients in LTBI group were selected as high live single lymphocytes are CD4+ IFN-$\gamma$ (red colour). Data were analysed in GraphPad Prism 9.

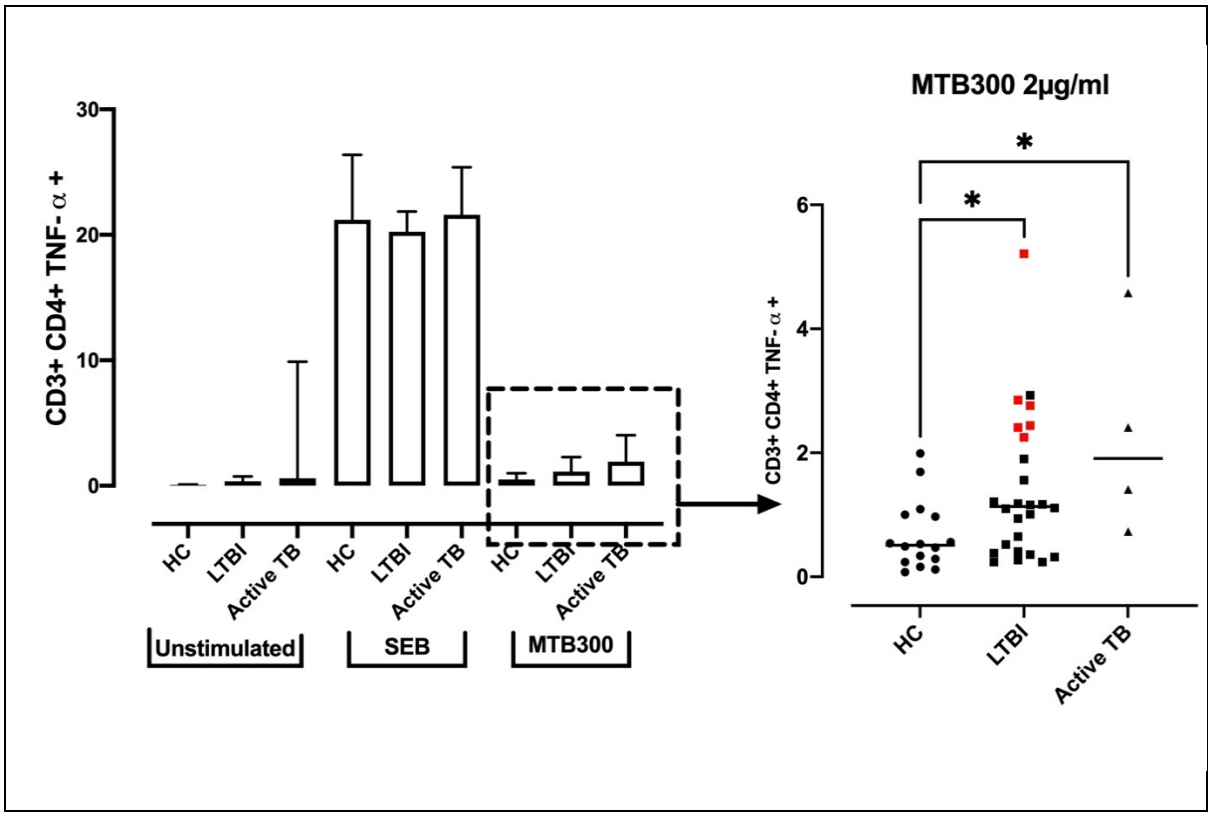



**Figure 4.2. Mtb300 specific CD4+ expressing TNF-α in healthy control, LTBI and active TB subjects.** The frequency (%) of lymphocytes are CD4+ TNF-α+ from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4) stimulated with SEB or Mtb300 or unstimulated negative control **(Left)**. The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test for subjects stimulated with Mtb-300 **(right)**. 6 patients in LTBI group were selected as high live single lymphocytes are CD4+ IFN-$\gamma$ (red colour). Data were analysed in GraphPad Prism 9.

### 4.3.2 Mtb300 specific CD4+ IFN$\gamma$+ expressing HLA-DR and CD27.

In the next analysis, we determined the impact of Mtb300 specific T cells on CD4+ expressing IFN$\gamma$+ CD27+ by comparing the CD27 mean fluorescence index (MFI) ratio between the groups. The CD27 MFI ratio was calculated by dividing the geometric mean of CD27 of total CD4+ T cells by the geometric mean of CD27 on Mtb300 specific CD4 T cells defined by IFN$\gamma$+ expression. We found that the CD27 MFI ratio in the HC groups was significantly lower than the CD27 MFI ratio in the active TB patients. However, the CD27 MFI ratio in the LTBI group

trended lower than those in the HC group but was not significant (figure 4.3.A). We also found that the CD27 MFI ratio in active TB patients was significantly higher to those in LTBI group (p value was calculated using Mann-Whitney for nonparametric test and not showing in figure 4.3.A). Next, we determined the frequency of Mtb300 specific CD4+ T cells defined by IFN$\gamma$ expressing HLA-DR and compare the difference between the frequency of this expression in LTBI patient and these in HC and active TB group. We found that there was no significant difference between the frequency of CD4+ IFN$\gamma$+ expressing HLA-DR between LTBI and HC (figure 4.3.B). Also, there was no significant difference between the frequency of these cells between active TB and HC (figure 4.3.B). The frequency of CD4+ IFN$\gamma$ expressing HLA-DR in active TB was significantly higher than those in LTBI (p value was calculated using Mann-Whitney for nonparametric test and not showing in figure 4.3.B).

### 4.3.3. The association between Mtb300 specific CD4+ expressing cytokines and QuantiFERON-Plus blood test.

In next analysis we assessed the linear relationship between the frequency of mtb300 specific CD4+ expressing cytokines in LTBI subjects and their laboratory QuantiFERON-Plus in blood results QuantiFERON-Plus (QFT-Plus) and QuantiFERON mitogen. QFT-Plus has two distinct TB antigen tubes: TB1 tube (QFN1) and TB2 tube (QFN2). Both tubes contain peptides from Mtb-complex-associated antigen, ESAT-6 and CFP.10. QFN1 tube contains peptides designed to induce CD4+ T lymphocytes; however, QFN2 tube contains peptides designed to induce both CD4+ and CD8+ T lymphocytes. We used GraphPad Prism 9 software to compute Spearman correlation coefficient to assess the linear relationship between these variables. In our analysis we found that the Spearman's r coefficient between mitogen level and MTB300 specific CD4+ T cells expressing IFN$\gamma$+ was r= - 0.07, but not significant (figure 4.4). We found that the Spearman's r coefficient between mitogen and Mtb300 specific CD4+ T cells expressing IL-2 + was r= 0.18, but not significant (figure 4.4). Also, we found that Spearman's r coefficient between mitogen level and Mtb300 specific CD4+ T cells expressing TNF-$\alpha$ was r= 0.13, but not significant (figure 4.4). Furthermore, we found that there was positive correlation between the level of QFN1 and the frequency of CD4+ expressing IL-2+ and the

frequency of CD4+ expressing TNF-α r=0.37 and r= 0.34 respectively. However, this relationship was not statistically significant. On the other hand, there was positive correlation between the level of QFN2 and the frequency of CD4+ expressing IL-2+ and the frequency of CD4+ expressing TNF-α r=0.38 and r= 0.38 respectively. The relationship between these variables (QFN2 and IL-2+) and (QFN2 and TNF-α) are statistically significant. Moreover, there was weak positive relationship between the level of QFN1 and the frequencies of Mtb300 specific CD4+ expressing IFN$\gamma$, and between QFN2 and the frequencies of Mtb300 specific CD4+ expressing IFN$\gamma$. However, this relationship was not significant.

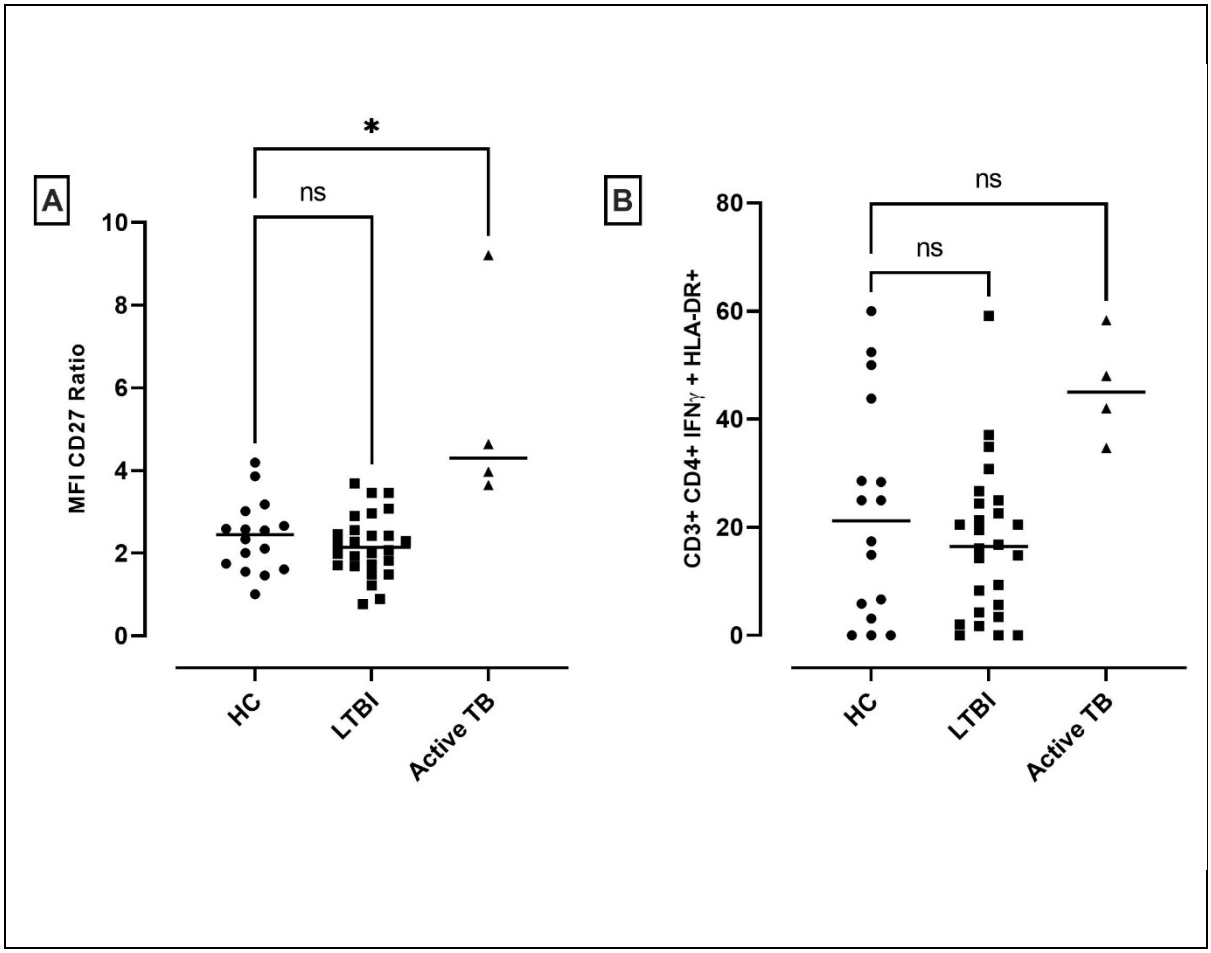



**Figure 4.3. Mtb300 specific CD4+ expressing IFN$\gamma$+ HLA-DR + in healthy control, LTBI and active TB subjects.** CD27 MFI ratio (A) and the frequency (%) of single cells lymphocytes are CD4+ IFN$\gamma$+ HLA-DR + (B) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4) stimulated with Mtb300. The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

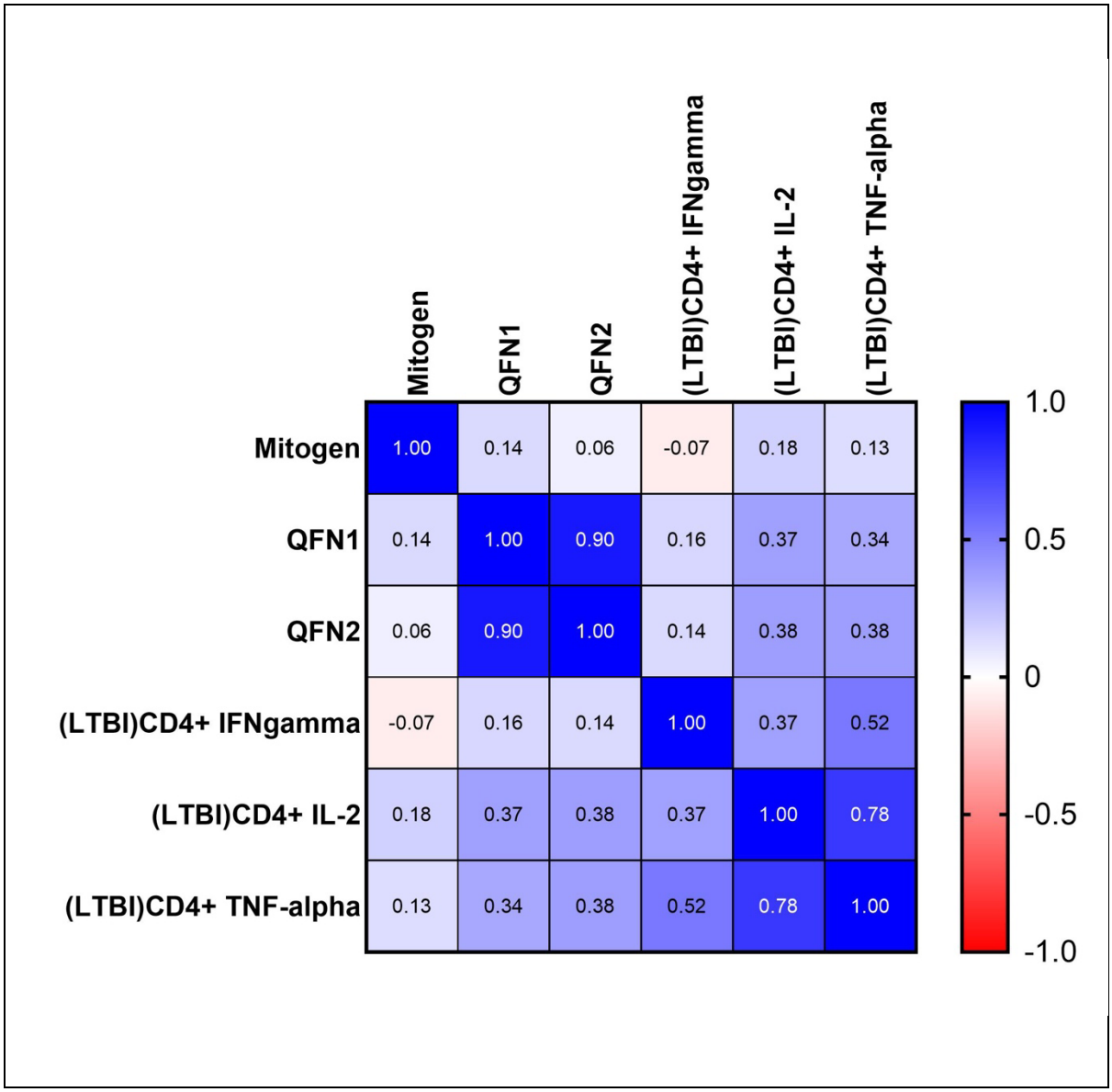



**Figure 4.4. Correlation matrix between blood test and the frequencies of Mtb300 specific CD4+ cytokines expression in LTBI subjects**. Correlation matrix represents nonparametric Spearman correlation r of the level of mitogen, QFN1 and QFN2 vs Mtb300 specific CD4+ T cells expressing IFN$\gamma$, IL-2 and TNF-$\alpha$ in LTBI subjects. Values represented nonparametric Spearman correlation calculated in GraphPad Prism 9.

### 4.3.4 Mtb300 specific CD4+ expressing activation induced markers (AIM)

In next analysis we determined the impact of Mtb300 specific CD4+ memory T cells expressing activation markers CD25+ OX40+ PD-L1 for the latent tuberculosis infection (LTBI) patients and compared them to Healthy control (HC) and active TB patients. To determine whether LTBI infection impacts relative levels of specific cellular subsets in the subject compared relative cellular levels between HC and active TB patients. To do this we stimulated the PBMCs with Mtb300 for 24 hours then we performed cell surface staining using anti CD4, CD45 RA and CCR7 antibodies to define CD45 RA- CCR7+ and CD45 RA- CCR7- gating on CD4 +. We determined the frequency of Mtb300 CD4+ $T_{CM}$ (CD3+ CD4+ CD45 RA- CCR7+) and the frequency of Mtb300 CD4+ $T_{EM}$ (CD3+ CD4+ CD45 RA- CCR7-) and then we compared between the frequency of these cells between LTBI, HC and active TB groups. We found that there was a higher frequency of CD4+ that express CCR7+ alone ($T_{CM}$) in LTBI subjects in response to Mtb300 compared to HC and active TB group but not significant (figure 4.5.A). Then we analyse whether Mtb300 specific effector memory ($T_{EM}$) can discriminate LTBI from HC or active TB. We found that the frequency of these cells was significantly lower in HC compared to these cells in LTBI subject (figure 4.5.B).

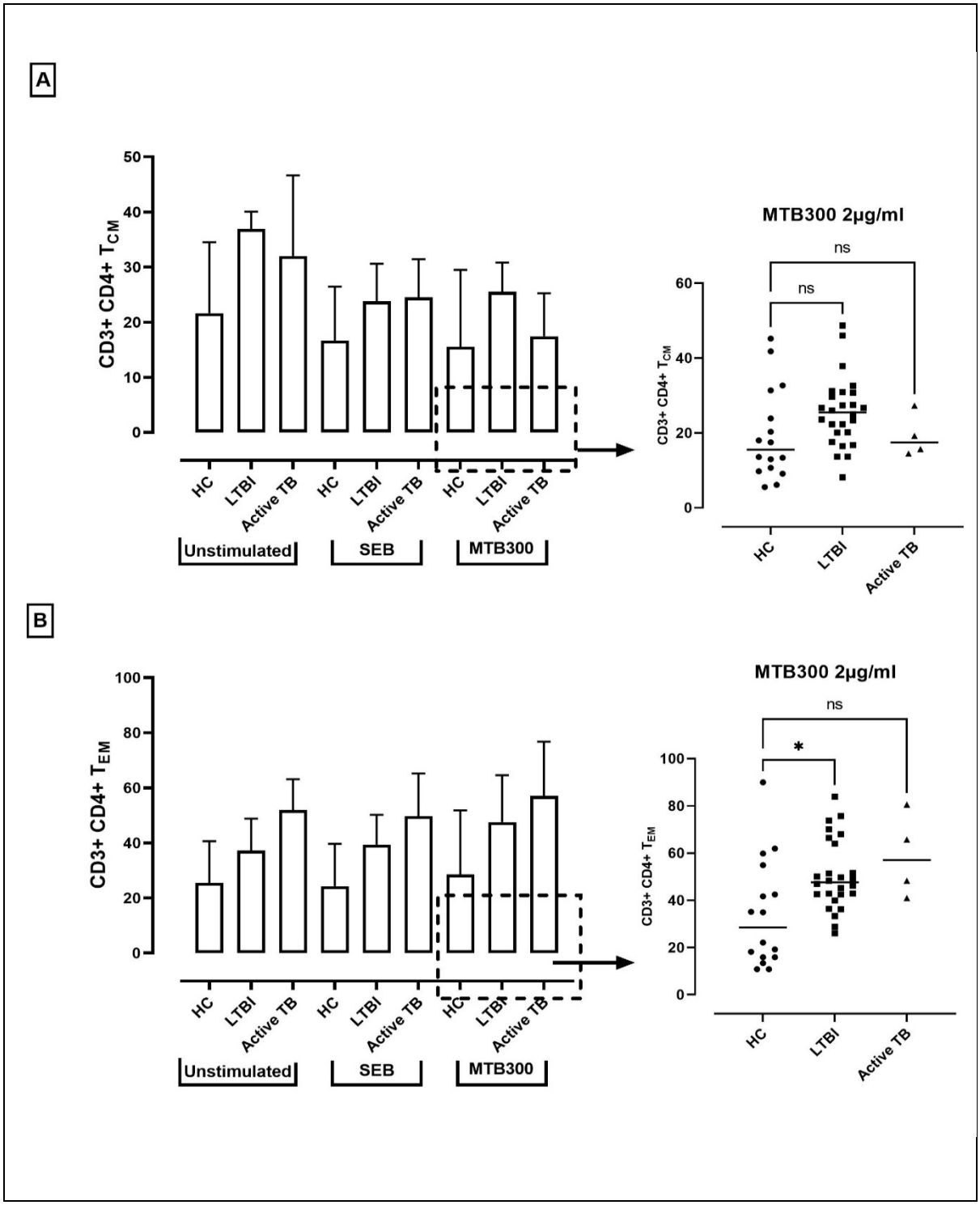



**Figure 4.5. Mtb300 specific CD3+ CD4+ expressing central memory $T_{CM}$ or effector memory $T_{EM}$ in healthy control, LTBI and active TB subjects.** The frequency (%) of live single lymphocytes are CD4+ CCR7+ CD45 RA- (A), The frequency (%) of live single lymphocytes are CD4+ CCR7- CD45 RA- (B) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4) stimulated with SEB or Mtb300 or unstimulated negative control **(Left)**. The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test for subjects stimulated with Mtb-300 **(right)**. Data were analysed in GraphPad Prism 9.

In next analysis we characterised CD4+ memory T cells and their ability to express the activation markers CD25+ OX40+ and PD-L1+. We used anti CD25, OX40 and PD-L1 antibodies to define the co-expression of OX40+ CD25+ and PD-L1+ CD25+ gated on CD4+ CD45 RA- CCR7+ or CD4+ CD45 RA- CCR7- in response to Mtb300. We determine these frequencies and then we compared the frequency of these cells between LTBI, HC and active TB groups. We found that at 18-24 hours post stimulation the frequency of CD4+ $T_{CM}$ cells expressing OX40+ CD25+ were higher in cells stimulated with SEB (positive control, nonspecific T cell superantigen) compared to these frequencies in cells stimulated with Mtb300 (specific T cells antigen) or those unstimulated cells (figure 4.6.A). In our analysis of the responses to Mtb300 specific CD4+ $T_{CM}$ cells, we found that the frequency range of CD4+ central memory T cells expressing OX40+ CD25+ in active TB patients was higher than these cells in HC group or LTBI groups (figure 4.6.A). there was a significant difference between these population between active TB and HC. However, there was no significant difference between these cells between LTBI and HC group (figure 4.6.A). In next our analysis we found that at 18-24 hours post stimulation the frequency of CD4+ $T_{CM}$ cells expressing PD-L1+ CD25+ were higher in cells stimulated with SEB compared to these frequencies in cells stimulated with Mtb300 or those unstimulated cells (figure 4.6.B). In our analysis of the responses to Mtb300 specific CD4+ $T_{CM}$ cells, we found there was no significant difference between the frequency of CD4+ central memory expressing PD-L1+ CD25+ between LTBI and HC (figure 4.6.B). Similarly, there was no significant difference between the frequency of these cells between active TB patients and HC. The frequency PD-L1+ CD25+ in active TB subjects was higher than LTBI or HC but not significant (figure 4.6.B).

In next analysis we determined Mtb300 specific CD4 effector memory T cells expressing activation markers and then we compared between the frequency of these cells between LTBI, HC and active TB groups. We found that at 18-24 hours post stimulation the frequency of CD4+ $T_{EM}$ cells expressing OX40+ CD25+ were higher in cells stimulated with SEB compared to these frequencies in cells stimulated with Mtb300 or those unstimulated cells (figure 4.7.A). In our analysis of the responses to Mtb300 specific CD4+ $T_{EM}$ cells, we found that the frequency of Mtb300 specific CD4+ effector memory expressing OX40+ CD25+ in active TB patients was significantly higher compared to those cells in HC group. However, the frequency

of these cells in LTBI subjects was higher than these cells in HC, but not significant (figure 4.7.A). We also found at 18-24 hours post stimulation the frequency of CD4+ $T_{EM}$ cells expressing PD-L1+ CD25+ were higher in cells stimulated with SEB compared to these frequencies in cells stimulated with Mtb300 or those unstimulated cells (figure 4.7.B). In our analysis of the responses to Mtb300 specific CD4+ $T_{EM}$ cells, we found that the frequency of Mtb300 specific CD4+ effector memory expressing PD-L1+ CD25+ in active TB patients was significantly higher compared to those cells in HC group. However, the frequency of these cells in LTBI subjects was higher than these cells in HC, but not significant (figure 4.7.B). based on our observation, we determined that the frequency of OX40+ CD25+ that express on CD4+ $T_{CM}$ or $T_{EM}$ in response to Mtb300 allows the discrimination of active TB patient from HC and LTBI. On the other hand, we determined that the frequency PD-L1+ CD25+ that express on $T_{EM}$ in response to Mtb300 allow the discrimination of active TB patient from HC and LTBI.

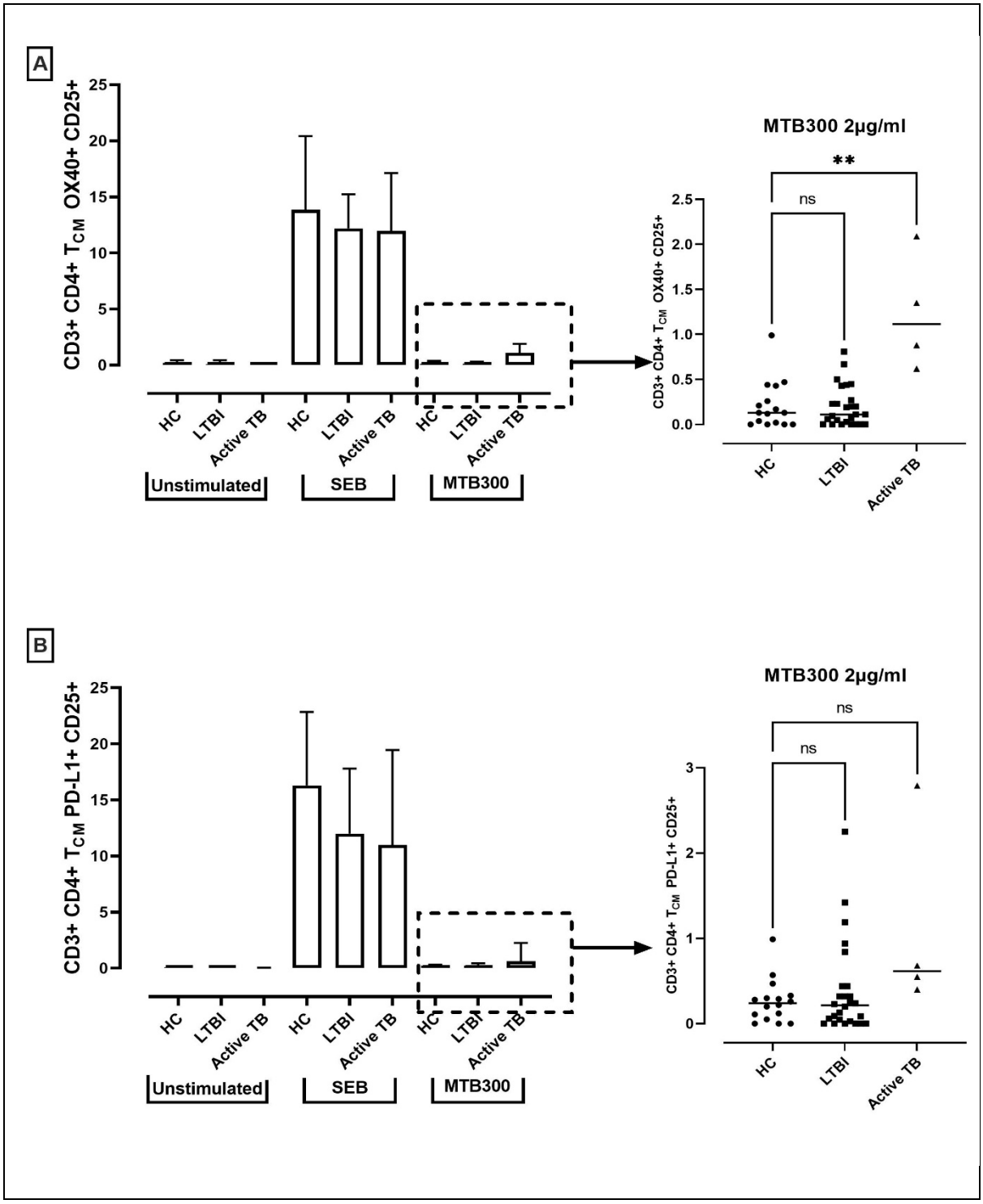



**Figure 4.6. Mtb300 specific CD3+ CD4+ expressing central memory $T_{CM}$ OX40+ CD25+ or PD-L1+ CD25+ in healthy control, LTBI and active TB subjects.** The frequency (%) of live single lymphocytes are CD4+ CCR7+ CD45 RA- OX40+ CD25+(A) and the frequency (%) of live single lymphocytes are CD4+ CCR7+ CD45 RA- PD-L1+ CD25+(B) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4) stimulated with SEB or Mtb300 or unstimulated negative control **(Left)**. The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test for subjects stimulated with Mtb-300 **(right)**. Data were analysed in GraphPad Prism 9.

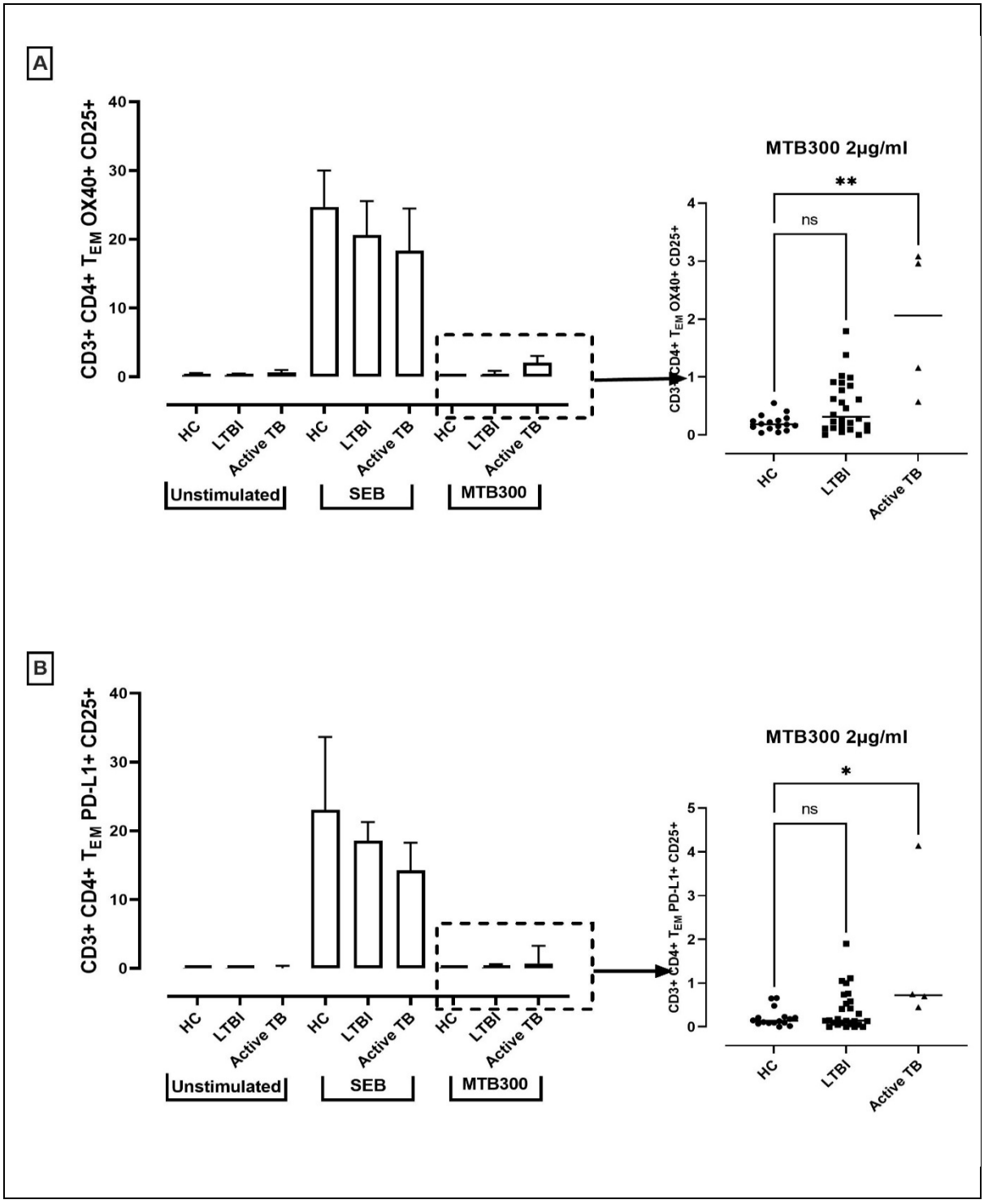



**Figure 4.7. Mtb300 specific CD3+ CD4+ expressing central memory $T_{EM}$ OX40+ CD25+ or PD-L1+ CD25+ in healthy control, LTBI and active TB subjects.** The frequency (%) of live single lymphocytes are CD4+ CCR7- CD45 RA- OX40+ CD25+ (A), the frequency (%) of live single lymphocytes are CD4+ CCR7- CD45 RA- PD-L1+ CD25+ (B) from healthy control (n=16), LTBI patients (n=26) and active TB patients (n=4) stimulated with SEB or Mtb300 or unstimulated negative control **(Left)**. The horizontal bars represent median values for each population and the significance of any difference between each population is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a non-parametric Kruskal-Wallis test for subjects stimulated with Mtb-300 **(right)**. Data were analysed in GraphPad Prism 9.

### 4.3.5 The association between Mtb300 specific CD4 memory T cells and QuantiFERON-Plus blood test

In next analysis we assessed the linear relationship between the frequency of mtb300 specific CD4+ expressing cytokines in LTBI subjects and their laboratory QuantiFERON-Plus in blood results QuantiFERON-Plus (QFT-Plus) and QuantiFERON mitogen. We found that there was negative correlation between the level of mitogen in LTBI subjects and Mtb300 specific CD4+ $T_{CM}$ cells expressing OX40+ CD25+ or PD-L1+ CD25+. Similarly, we found that there was negative correlation between the level of mitogen in LTBI subjects and Mtb300 specific CD4+ $T_{EM}$ cells expressing OX40+ CD25+ or PD-L1+ CD25+. We found that there was negative correlation between the level of QFN1 and the frequencies of Mtb300 specific CD4+ $T_{CM}$ expressing OX40+ CD25+ or CD4 $T_{EM}$ expressing OX40+ CD25+; however, this relationship was not statistically significant. We found that there was weak positive correlation between the level of QFN1 and the frequencies of Mtb300 specific CD4+ $T_{CM}$ expressing PD-L1+ CD25+; however, this relationship was not statistically significant. We found that there was positive correlation between the level of QFN2 and the frequencies of Mtb300 specific CD4 $T_{EM}$ expressing PD-L1+ CD25+; however, this relationship was not statistically significant. We found that there was negative correlation between the level of QFN2 and the frequencies of Mtb300 specific CD4+ $T_{CM}$ expressing OX40+ CD25+ or CD4 $T_{EM}$ expressing OX40+ CD25+; however, this relationship was not statistically significant. We found that there was weak positive correlation between the level of QFN2 and the frequencies of Mtb300 specific CD4+ $T_{CM}$ expressing PD-L1+ CD25+; however, this relationship was not statistically significant. We found that there was positive correlation between the level of QFN2 and the frequencies of Mtb300 specific CD4 $T_{EM}$ expressing PD-L1+ CD25+; however, this relationship was not statistically significant (figure 4.8).

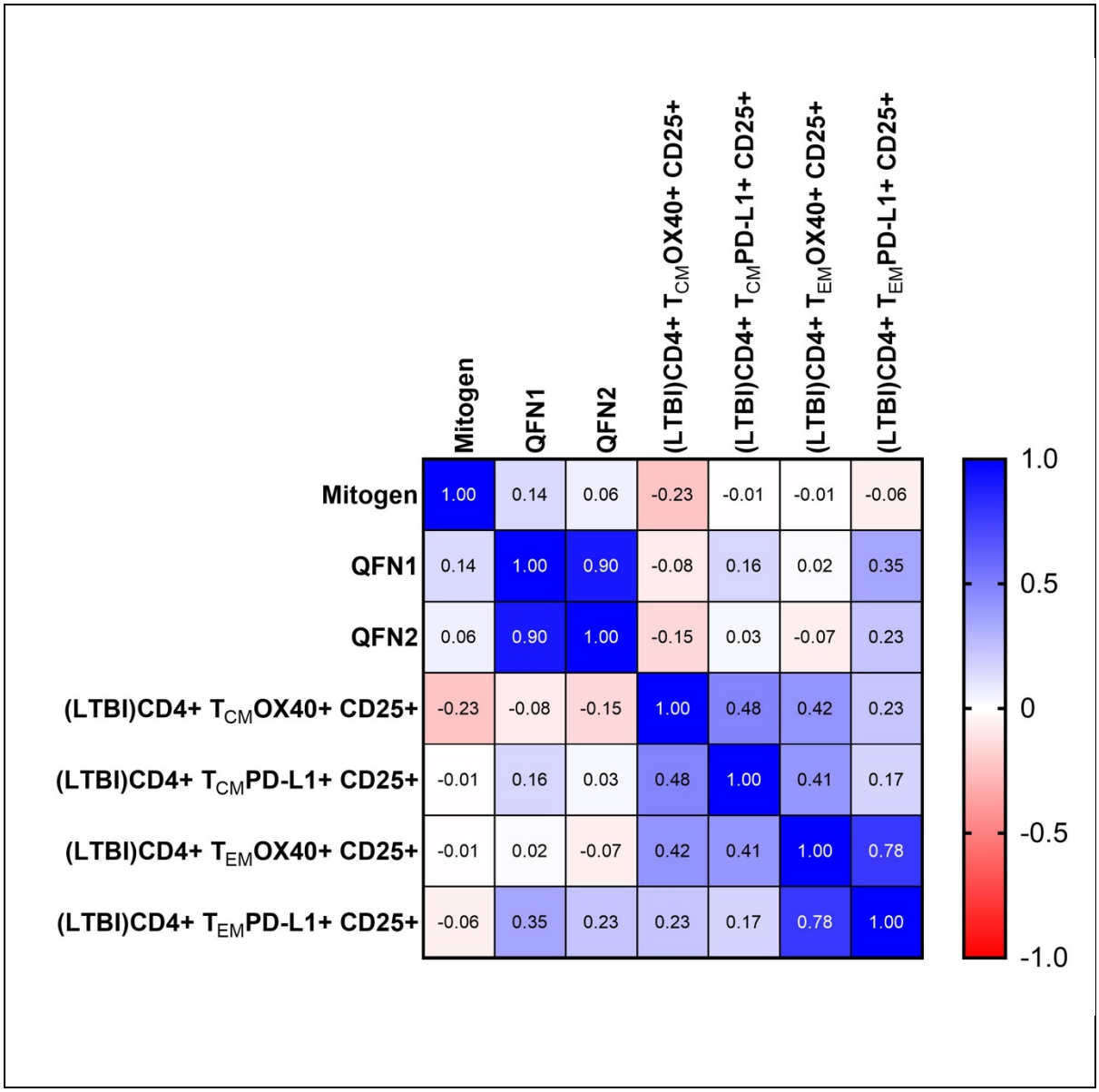



**Figure 4.8. correlation matrix between IGRA test and the frequency of Mtb300 specific CD4+ activation induced marker expression in LTBI subjects**. Correlation matrix represents nonparametric Spearman correlation r of the level of mitogen, QFN1 and QFN2 vs Mtb300 specific CD4+ $T_{CM}$ or $T_{EM}$ cells expressing OX40+ CD25+ or PD-L1+ CD25+ in LTBI subjects. Values represented nonparametric Spearman correlation calculated in GraphPad Prism 9.

### 4.3.6 The effects of TB treatment on Mtb300 specific CD4+ expressing cytokines in LTBI.

In next analysis we followed the LTBI subjects to determine whether the TB treatment impacts on the antigen specific CD4+ T cells expressing cytokines. We determined the ability of CD4+ to express IFN$\gamma$, TNF-$\alpha$ and IL-2 following the 4-6 weeks treatment (F1 group) and 10-12 weeks treatment (F2 group). We stimulated the PBMCs as described previously followed by intracellular staining and we performed flow cytometric analysis to determine the frequency of Mtb300 specific CD4+ T cells expressing IFN$\gamma$+, TNF-$\alpha$+ and IL-2+. Then we compared between the frequency of each cytokine between LTBI subjects before treatment and F1 group and F2 group. The frequency of CD4+ IFN$\gamma$+ in subjects who received treatment for 4-6 weeks trended lower than LTBI subjects before treatment (figure 4.9.A). However, the frequency of these cytokines in subjects who received treatment for 10-12 weeks trended higher than both LTBI and F1 groups (figure 4.9.A). We found that the median of CD4+ IFN$\gamma$+ frequency was higher in F2 group compared to LTBI or F1 group, but the range of frequency of these cytokines remained same (figure 4.9.A). There was no significant difference between the frequency of Mtb300 specific CD4+ IFN$\gamma$+ between LTBI subject and either F1 or F2 groups (figure 4.9.A). The frequency of CD4+ IL-2+ in subjects who received treatment for 4-6 weeks trended lower than LTBI subjects before treatment (figure 4.9.B). However, the frequency of these cytokines in subjects who received treatment for 10-12 weeks trended higher than both LTBI and F1 groups (figure 4.9.B). We found that the median of CD4+ IL-2+ frequency was higher in F2 group compared to LTBI or F1 group, but the range of frequency of these cytokines remained same (figure 4.9.B). There was no significant difference between the frequency of Mtb300 specific CD4+ IL-2+ between LTBI subject and either F1 or F2 groups (figure 4.9.B). The frequency of CD4+ TNF-$\alpha$+ in subjects who received treatment for 4-6 weeks trended higher than LTBI subjects before treatment (figure 4.9.C). However, the frequency of these cytokines in subjects who received treatment for 10-12 weeks trended higher than both LTBI and F1 groups (figure 4.9.C). We found that the median of CD4+ TNF-$\alpha$+ frequency was higher in F2 group compared to LTBI or F1 group, but the range of frequency of these cytokines remained same (figure 4.9.C). There was no significant difference between the frequency of Mtb300 specific CD4+ TNF-$\alpha$+ between LTBI subject and either F1 or F2 groups (figure 4.9.C).

In next our analysis to determine the impact of TB treatment on Mtb300 specific T cells in LTBI subjects, we linked the frequencies for everyone. We found in subject 1 (patient ●) the frequencies of all cytokines were reduced during first visit (4-6 weeks during the treatment), but they were increased following at visit 2 (10-12 weeks). Subject 2 (patient ○) and subject 4 (patient □) had similar responses. They both slightly reduced the expression of CD4+ IFN$\gamma$+ in F1, but massively increased the expression of these in F2. However, the frequency of CD4+ expressing IL-2 and frequency CD4+ expressing TNF-$\alpha$ were slightly increased in F1 and then the frequencies increased massively in F2. In subject 3 (patient■) the frequency of CD CD4+ IFN$\gamma$+ increased in F1, but the frequencies of CD4+ IL-2+ AND CD4+ TNF-$\alpha$ reduced in F1. However, all the frequencies of these cytokines reduced in F2. Subject 5 (patient ▲) and subject 6 (patient △) increased the frequency of CD4+ TNF-$\alpha$ and CD4+ IL-2 in F1 but then there was a reduction in F2. However, the frequency of CD4+ IFN$\gamma$+ lowered in F1 and then reduced further for subject 5 in F2, but remained the same for subject 6.

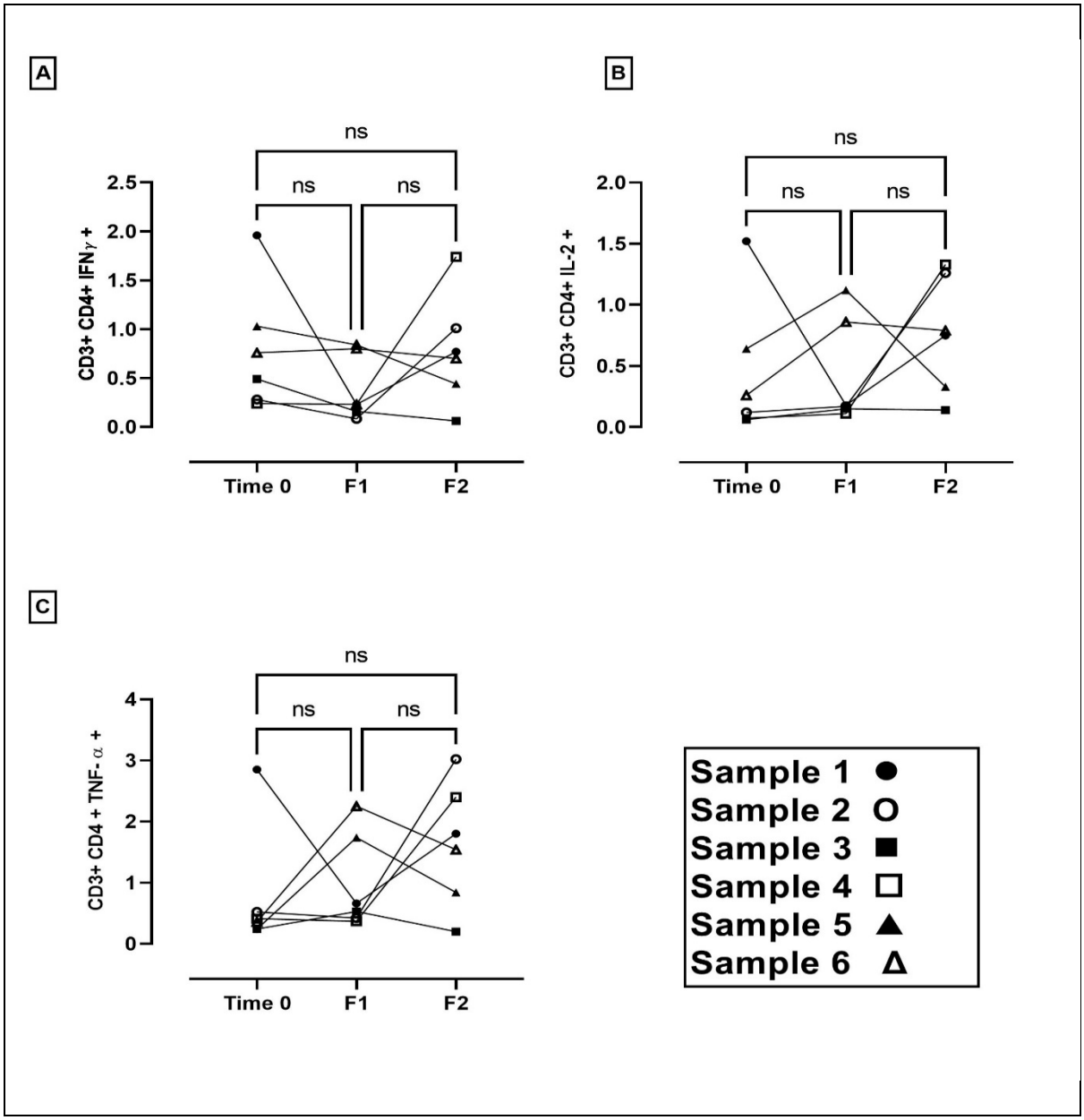



**Figure 4.9. Mtb300 specific CD4+ T cells expressing cytokines in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) live single lymphocytes are CD4+ IFN$\gamma$+ (A), the frequency (%) live single lymphocytes are CD4+ IL-2+ (B) and the frequency (%) live single lymphocytes are CD4+ TNF-$\alpha$+ (C) from LTBI patients before treatment (0), LTBI 6-8 week during the treatment (F1) and LTBI 6-8 week during the treatment (F2). PBMCs were stimulated with Mtb300. The horizontal bars represent median values and significant difference is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

In next analysis we followed the LTBI subjects to determine whether the TB treatment impacts on the antigen specific CD4+ T cells. We determined the impact of Mtb300 specific T cells on the CD27 mean fluorescence index (MFI) in the groups. The CD27 MFI was calculated as described previously for the F1 and F2 groups and then we compared the ratio between LTBI, F1 and F2. We found that the CD27 MFI ratio in F2 group trended higher than F1 and the ratio of LTBI groups was trended higher than F1 group (figure 4.10.A). There was no significant difference between the CD27 MFI ratio between LTBI subject and wither F1 group or F2 group (figure 4.10.A). Then, we determined the impacts of the TB treatment on the frequency of Mtb300 specific CD4+ IFN$\gamma$ expressing HLA-DR+ and we compared the frequency of these cells in LTBI subjects to those in F1 and F2 groups. We found that the frequency of these cells trended higher in F1 group compared to LTBI or F2 group (figure 4.10.B). We also found that there was no significant difference between the frequency of Mtb300 specific CD4+ IFN$\gamma$ expressing HLA-DR+ between LTBI subject and wither F1 group or F2 group (figure 8.B).

In next our analysis to determine the impact of TB treatment on Mtb300 specific CD4+ T cells in expressing IFN$\gamma$ in LTBI subject, we linked the frequencies for everyone. We found that CD27 MFI ratio in subject 1, 2, 3 and 4 was reduced during 4-6 weeks (F1) from they start their treatment. The ratio was increased during 10-12 weeks (F2) from they start their treatment. However, in subject 5 and subject 6 the ratio was decreased in F1, and then ratio was decreased further in F2. In our analysis to characterise Mtb300 specific CD4+ T cells, we found the frequency of CD4+ IFN$\gamma$+ HLA-DR+ was increased in subjects from 1 to subject 4 in during 4-6 weeks. However, the frequency of these cells was reduced (but not in subject 3) during 10-12 weeks. On the other hand, the frequency of CD4+ IFN$\gamma$+ HLA-DR+ was reduced during 4-6 weeks and then reduced further during 10-12 weeks in subject 6 (subject 5 remained the same in F2).

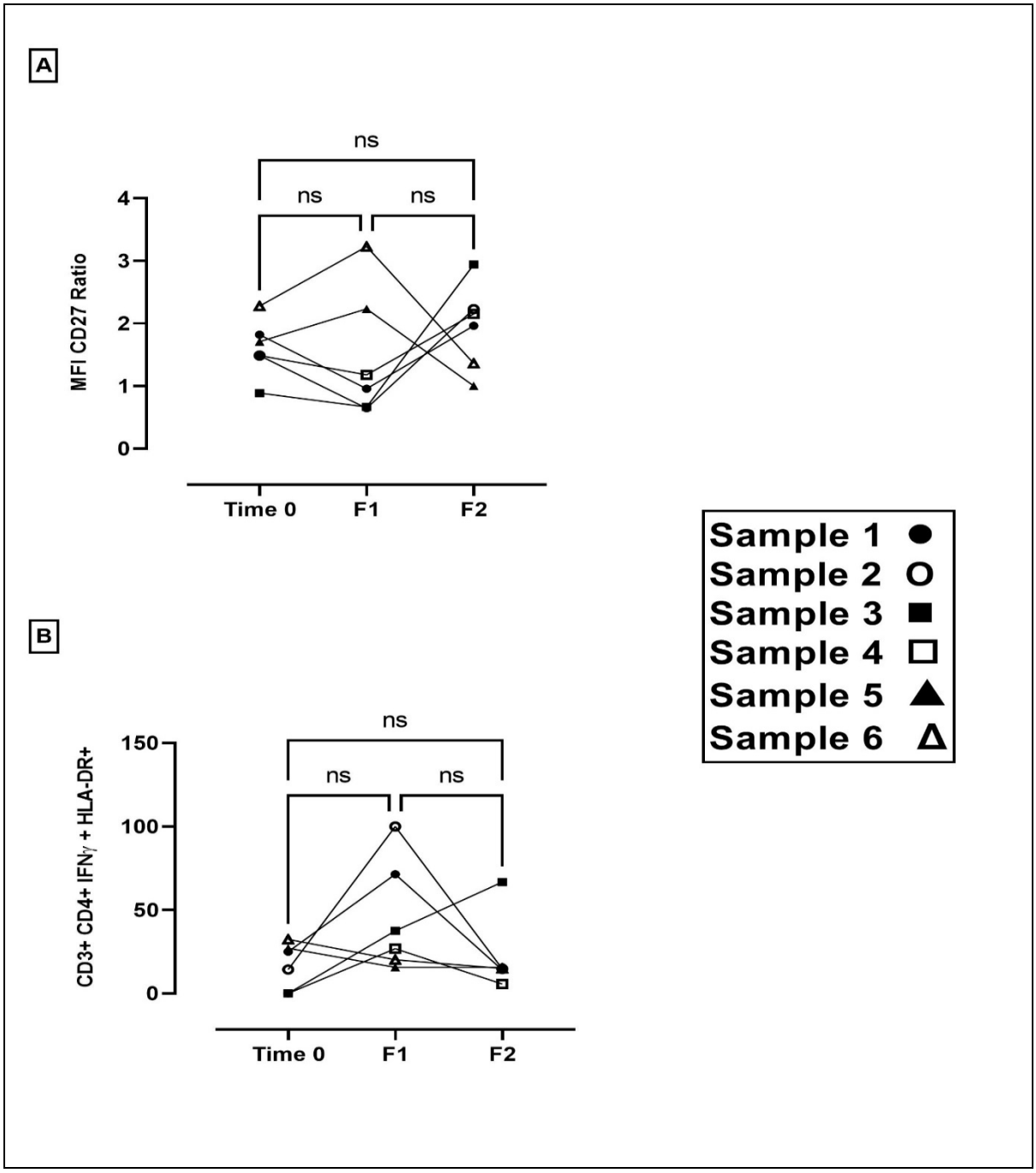



**Figure 4.10. Mtb300 specific CD4+ T cells expressing cytokines in LTBI patients compared to the frequencies of the same patients during TB treatment.** CD27 MFI ratio (A) and the frequency (%) of single cells lymphocytes are CD4+ IFN$\gamma$+ HLA-DR + (B) from LTBI before treatment (n=6), LTBI 6-8 week during the treatment (F1) and LTBI 6-8 week during the treatment (F2). PBMCs were stimulated with Mtb300. The horizontal bars represent median values and significant difference is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in IBM SPSS Statistics version 28 and GraphPad Prism 9.

### 4.3.7 The effects of TB treatment on Mtb300 specific CD4+ expressing AIM in LTBI.

In next analysis we determined the frequencies of Mtb300 specific CD4+ $T_{CM}$ and CD4+ $T_{EM}$ in LTBI subjects who received TB treatment. We found that there was no significant difference between the frequencies of Mtb300 specific CD4+ $T_{CM}$ between untreated LTBI subject and either subjects who received treatment during 4-6 weeks or subjects who received treatment during 10-12 weeks. Similarly, we found that there was no significant difference between the frequencies of Mtb300 specific CD4+ $T_{EM}$ between untreated LTBI subject and either subjects who received treatment during 4-6 weeks or subjects who received treatment during 10-12 weeks. Individually, we found that in subjects from 2 to 6 the frequencies of Mtb300 specific CD4+ $T_{CM}$ was reduced in F1 subjects; moreover, the frequency of these cells was further reduced when they completed their treatment (F2). However, the frequency of Mtb300 specific CD4+ $T_{CM}$ in subject 1 was increased in F1 and then increased further in F2. On the other hand, we found that in subjects 2 to subject 5 the frequencies of Mtb300 specific CD4+ $T_{EM}$ was increased in F1, but then they reduced in F2 except subject 2. However, the frequency of these cells was reduced in subject 1 and 6 in F1, but increased later in subject 6 at the end of treatment.

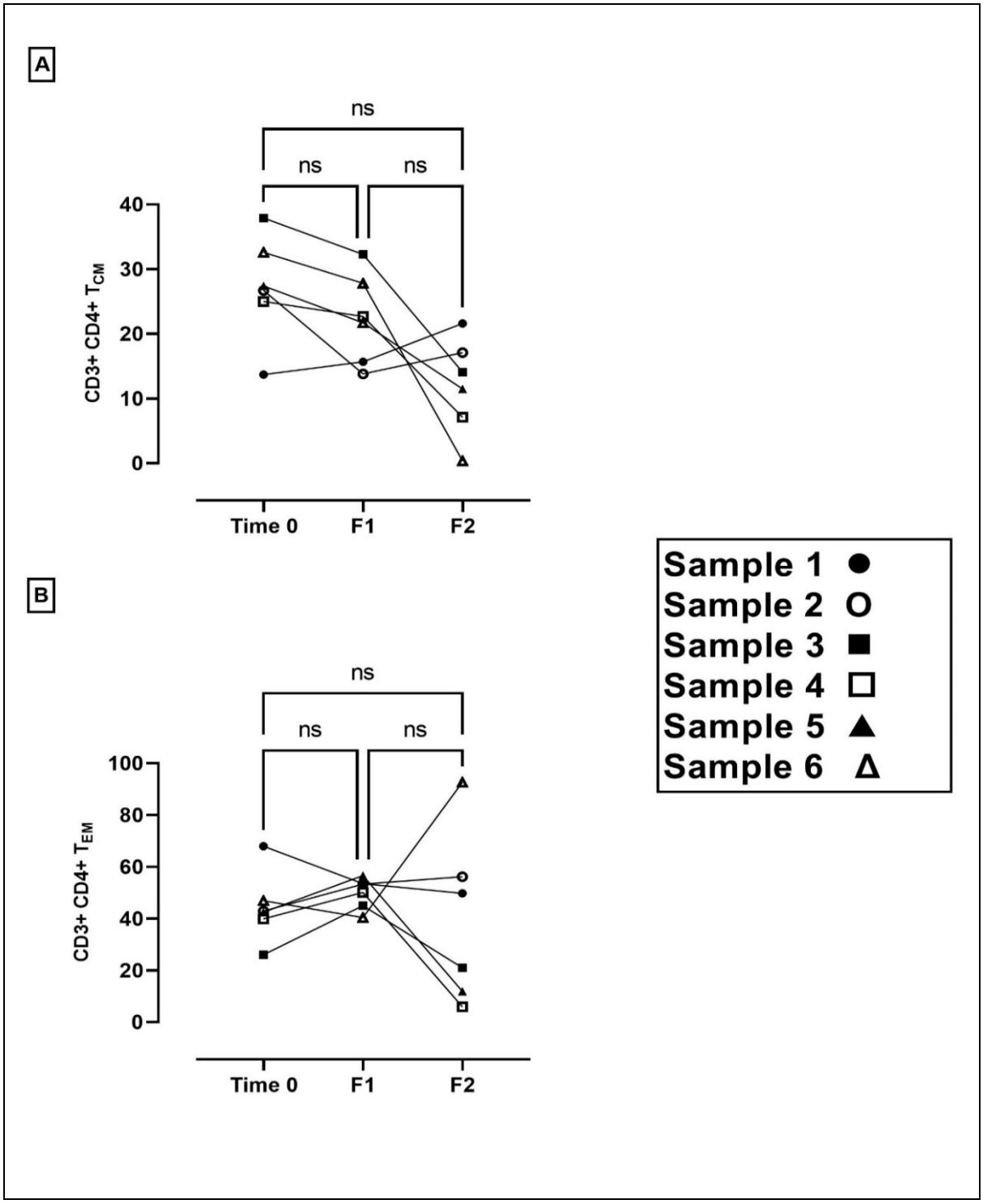



**Figure 4.11. Mtb300 specific CD4+ T cells expressing central memory or central memory in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) of live single lymphocytes are CD4+ CCR7+ CD45 RA- (A), The frequency (%) of live single lymphocytes are CD4+ CCR7- CD45 RA- (B) from LTBI before treatment (n=6), LTBI 6-8 week during the treatment (F1) and LTBI 6-8 week during the treatment (F2). PBMCs were stimulated with Mtb300. The horizontal bars represent median values and significant difference is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

In next analysis we determined the impact of Mtb300 specific CD4+ memory T cells expressing activation markers CD25+ OX40+ PD-L1 LTBI patients who received TB treatment and compared them to 4-6 weeks and 10-12 weeks following their treatment. We characterised CD4+ memory T cells and their ability to produce activation markers CD25+ OX40 and PD-L1+. PBMCs cells was stimulated as described previously followed by cells surface staining and flow cytometric analysis. We found that Mtb300 specific CD4+ T central memory expressing OX40+ CD25+ in F2 group trended higher than those cells in subject before the treatment (LTBI) or F1 group (figure 4.12.A). There was no significant difference between the frequency of CD4+ $T_{CM}$ OX40+ CD25+ between LTBI and either F1 or F2 groups (figure 4.12.A). We found that Mtb300 specific CD4+ T central memory expressing PD-L1+ CD25+ in F2 group trended higher than those cells in subject before the treatment (LTBI) or F1 group (figure 4.12.B). There was no significant difference between the frequency of CD4+ $T_{CM}$ OX40+ CD25+ between LTBI and either F1 or F2 groups (figure 4.12.B). Also, we found that Mtb300 specific CD4+ T effector memory expressing OX40+ CD25+ in in subjects before treatment (LTBI) group trended higher than those cells in F1 or F2 group (figure 4.13.A). There was no significant difference between the frequency of CD4+ $T_{EM}$ OX40+ CD25+ between LTBI and either F1 or F2 groups (figure 4.13.A). We found that Mtb300 specific CD4+ T effector memory expressing PD-L1+ CD25+ in F2 group trended higher than those cells in subject before the treatment (LTBI) or F1 group (figure 4.13.B). There was no significant difference between the frequency of CD4+ $T_{CM}$ OX40+ CD25+ between LTBI and either F1 or F2 groups (figure 4.13.B).

In next our analysis to determine the impact of TB treatment on Mtb300 specific T memory cells in LTBI subject, we linked the frequencies for everyone. We found that in subject 2, 3, 4 and 5 the frequencies of Mtb300 specific CD4+ $T_{CM}$ OX4+ CD25+ reduced after 4-6 weeks during their treatment (F1), and the frequencies increased after 10-12 weeks (F2) during their treatment. The frequency of these cells in subject 6 reduced to 0 in F1 and F2; however, the frequency of these cells increased in subject 1 in F2. We found that in subject 1, 2, 3, 4 and 5 the frequencies of Mtb300 specific CD4+ $T_{CM}$ PD-L1+ CD25+ remained 0 in F1, but increased in F2. However, the frequency of these cells in subject 6 increased in F1, then reduced to 0 in F2. Also, we found that in subjects 1 to 5 the frequencies of Mtb300 specific CD4+ $T_{EM}$ OX4+ CD25+ were reduced in F1 and then increased (except subject 3) in F2. On the other hand, in subject 6 the frequency of these cells increased in F1, and increased in F2. We found that in

subjects 1 to3 the frequencies of Mtb300 specific CD4+ $T_{EM}$ PD-L1+ CD25+ were reduced in F1, but the frequencies of these cells were increased in F2. In subject 4 and subject 6 the frequencies of Mtb300 specific CD4+ $T_{EM}$ PD-L1+ CD25+ were increased in F1, but the frequencies of these cells were reduced in F2. Finally in subject 5, we found that the frequency of these cells increased in F1, and it increased further in F2.

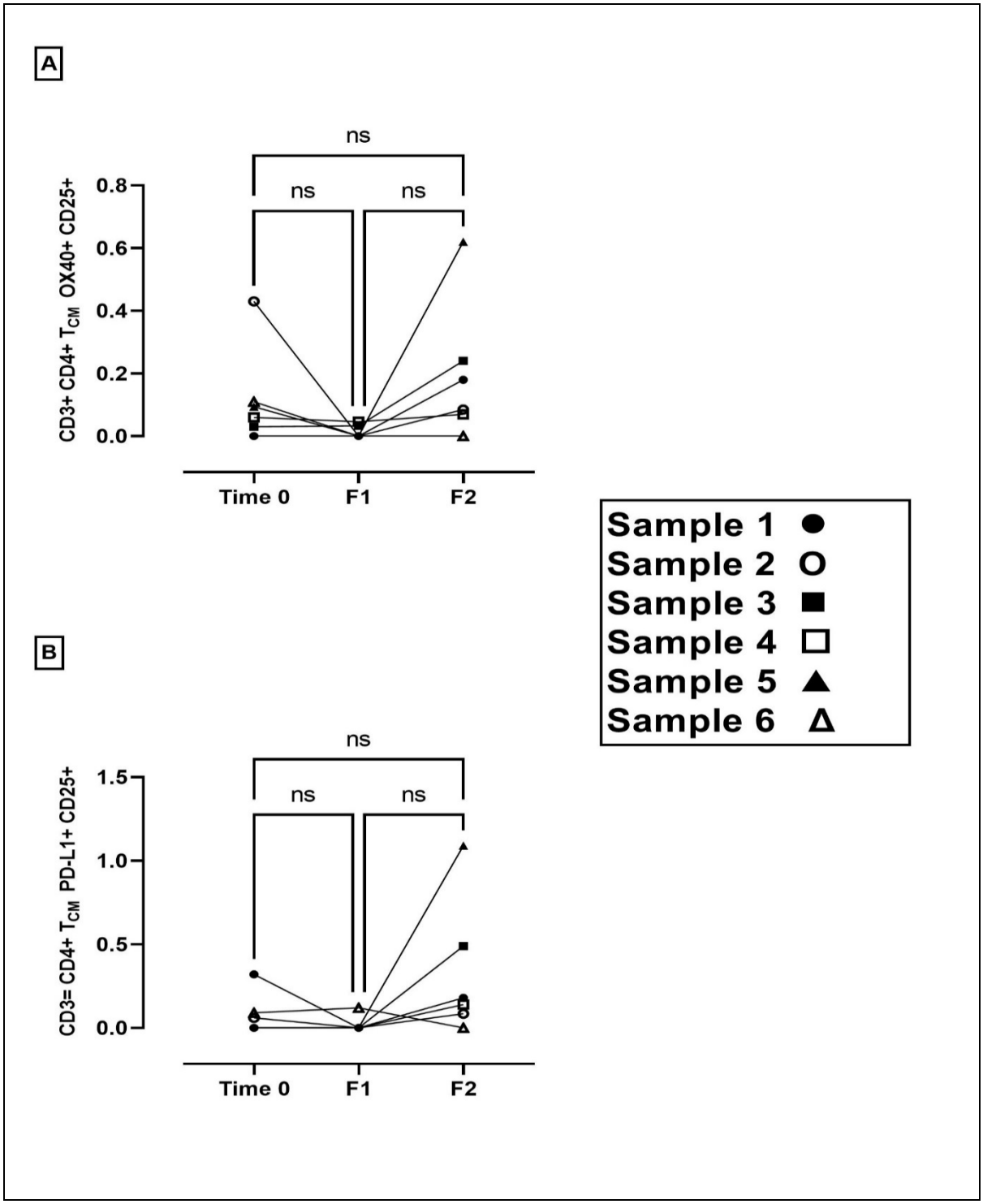



**Figure 4.12. Mtb300 specific CD4+ T cells expressing central memory in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) of live single lymphocytes are CD4+ CCR7+ CD45 RA- OX40+ CD25+(A) and the frequency (%) of live single lymphocytes are CD4+ CCR7+ CD45 RA- PD-L1+ CD25+(B) from LTBI before treatment (n=6), LTBI 6-8 week during the treatment (F1) and LTBI 6-8 week during the treatment (F2). PBMCs were stimulated with Mtb300. The horizontal bars represent median values and significant difference is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

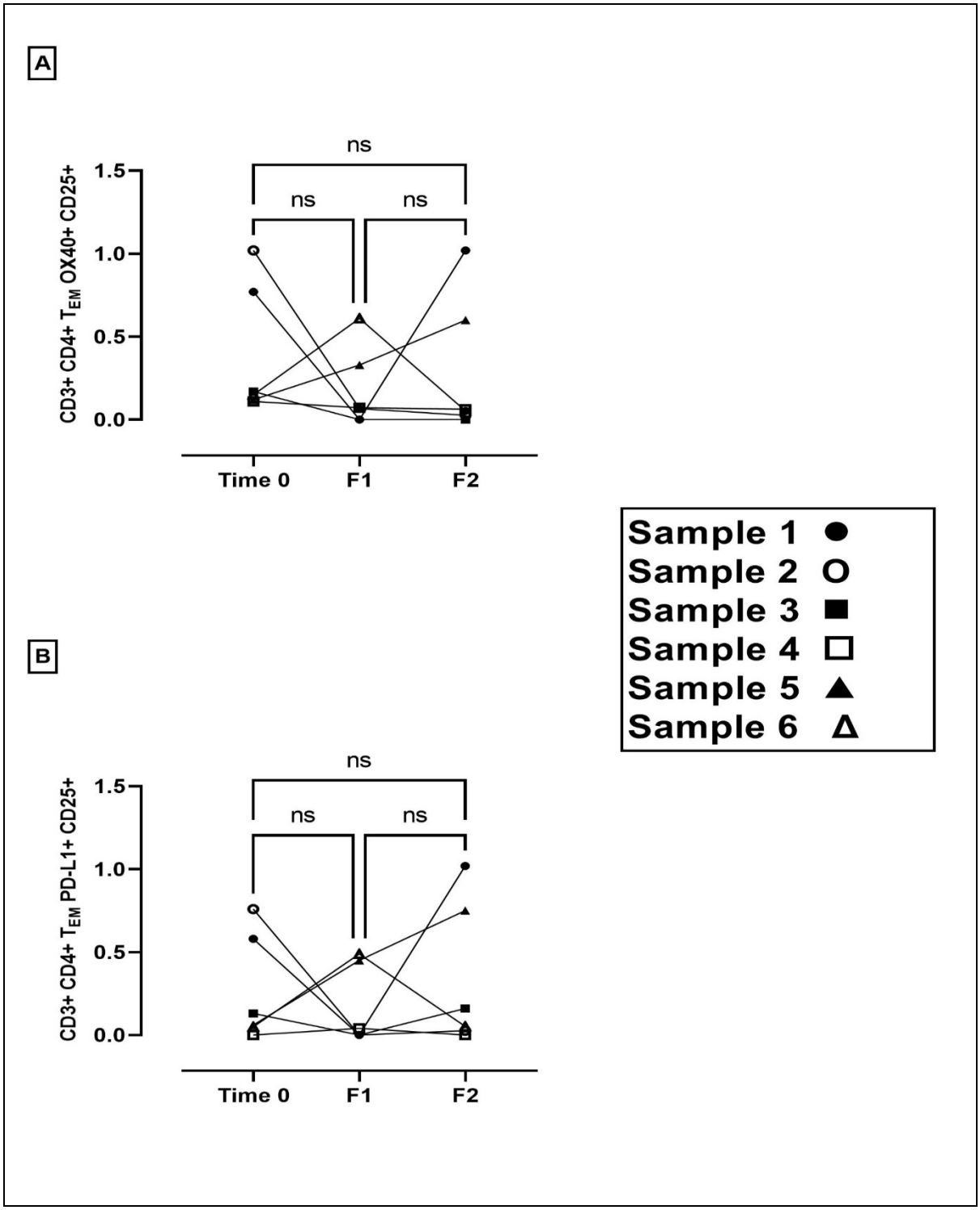



**Figure 4.13. Mtb300 specific CD4+ T cells expressing central memory in LTBI patients compared to the frequencies of the same patients during TB treatment.** The frequency (%) of live single lymphocytes are CD4+ CCR7- CD45 RA- OX40+ CD25+(A) and the frequency (%) of live single lymphocytes are CD4+ CCR7- CD45 RA- PD-L1+ CD25+(B) from LTBI before treatment (n=6), LTBI 6-8 week during the treatment (F1) and LTBI 6-8 week during the treatment (F2). PBMCs were stimulated with Mtb300. The horizontal bars represent median values and significant difference is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Fridman (ANOVA) test and Dunn's multiple comparison test for pairing data. Data were analysed in GraphPad Prism 9.

## 4.4 Discussion

The working model is that the T cell response circulating in subjects reflects the T cells exposure to antigen – in active TB there will be live bacteria and presumably lots of antigen, whereas in latent TB there will be variable antigen and limited live bacteria, hopefully there will be no antigen or live bacteria in the healthy controls. The markers we have used reflect the ability of T cells to respond rapidly *ex vivo* to antigen and would therefore be more likely to indicate circulating T cells that have recently seen antigen or which are ready to respond quickly. Our restimulation models do not allow for expansion of T cells only expression of the cytokines and markers reflecting their current state. In this way we think that the frequency and phenotype of antigen-specific T cells would allow discrimination of disease phenotype in our defined cohorts of healthy, latent and active TB subjects. We did find differences in the cellular responses to Mtb antigens and we did determine the frequencies of Mtb specific CD4+ T cells. Whether we will be able to use these as a prognostic or diagnostic marker has not yet been shown. In order to determine whether the signature derived from our studies is predictive of TB development, we would need a large study allowing us to follow latently infected people through to disease development. Progression is a rare event and needs the subjects to not be treated for latent TB. Some subjects do refuse treatment, but the size of population locally is not large. We would need to perform the progression study in a location where it is not standard of care to deliver treatment to those who have positive IGRA but no symptoms.

We tested our hypothesis using the Mtb300 antigen pool described in the introduction. This antigen pool works to identify T cell responses in many populations worldwide and we expect that it should be useful in the Leicester population – which is representative of broad ethnic diversity. As expected, we found that unstimulated cells showed little change in cytokines or activation induced markers but that the delivery of the broad TCR stimulator SEB resulted in the highest levels of cytokine and activation induced marker changes. The Mtb300 signal was much lower than the SEB signal but substantially higher for some of the LTBI and all of the active subjects. We found differences in antigen-induced cytokine release and activation induced markers in the CD4 T cells between healthy control, LTBI and active TB patients. While

we did not find reproducible differences in changes during treatment for LTBI, we did see that the markers changed similarly within the assays for each patient.

The Mtb300 specific CD4 T cell responses revealed that there were significant differences between the frequency of CD4+ T cells single expressing IFN$\gamma$+, IL-2+ and TNF-$\alpha$+ in HC, LTBI or active TB patients. Furthermore, in our analysis on Mtb300 specific CD4+ IFN$\gamma$+, we observed no significant difference between the frequency of CD4+ IFN$\gamma$ HLA-DR+ in LTBI subjects and between these frequencies either in HC or active TB patients. However, in our analysis of CD27 expression on IFN$\gamma$+ and IFN$\gamma$- cells CD4+ T cells wherein we take the geometric mean of CD27 expression on IFN$\gamma$- CD4 T cells and divide it by the same measure on IFN$\gamma$ + CD4 T cells, we found that CD27 ratio was higher in the active TB patients that the healthy controls. Moreover, in our analysis of the responses to Mtb300 specific CD4+ $T_{EM}$ and CD4 + $T_{EM}$ cells we found that there was significant difference between the frequencies of CD4+ $T_{CM}$ OX40+ CD25, CD4+ $T_{EM}$ OX40+ CD25+ and CD4+ $T_{EM}$ PD-L1+ CD25+ between HC subjects and active TB patients, but not between HC and LTBI patients. However, our data revealed that the frequency of OX40+ CD25+ expressed on Mtb300 specific CD4+ T cells was significantly higher in active TB compared to HC. Finally, in our analysis on the impacts of TB treatment in LTBI subjects we found that the expression of cytokines had the same pattern in each individual. We found that no significant impacts of TB treatment on LTBI subject who follow their treatment.

We evaluated Mtb300 specific CD4+ T cells responses using panel included Th1 cytokines (IFN$\gamma$ and TNF-$\alpha$) and T cells proliferation cytokine (IL-2). Then we further characterized CD4+ T cells by determining the frequencies of Mtb300 specific CD4+ IFN$\gamma$ cells that expressed immune markers HLA-DR and CD27. Studies have identified biomarkers on host Mtb-specific CD4+ T cells that discriminate between active TB from LTBI and healthy control (HC). For example, frequencies of activated CD4+ T cells were higher in subjects with LTBI for IFN$\gamma$+ and TNF-$\alpha$+ but not IL-2+ (Govender et al., 2010). However, our data showed that these cytokines were higher in patients with active TB (TNF-$\alpha$+ and IL-2+ were not significant, p value was calculated using Mann-Whitney for nonparametric test and not shown). Furthermore, Sergentini et al (2009) reported that the distribution of the frequencies of IFN$\gamma$+ T cells upon PBMCs stimulation allowed the discrimination of persons with LTBI or persons with active TB

from healthy control, which agreed with our data. They also reported that in their studies of antigen-specific responses in the diagnosis of TB the frequency of total cytokines IFN$\gamma$ and IL-2 were significant higher in persons with LTBI or persons with active TB compared to healthy control which also agree with our analysis. Caccamo et al (2010) studied the frequencies of Mtb antigen specific CD4+ T cells secreting cytokines. They found that there was no significant difference between the frequencies of Mtb specific CD4+ T cells secreting IL-2 and those secreting TNF-$\alpha$ between LTBI subjects and active TB subjects which agree with our data. In agreement with our data, Adekambi et al (2015) showed that frequencies of Mtb-specific CD4+ IFN$\gamma$+ expressing HLA-DR in human PBMCs were significantly higher in active TB than LTBI. In contrast to our data, they identified that Mtb-specific CD4+ IFN$\gamma$+ was not significant between active TB and LTBI. Estevez et al (2020) reported that the frequencies of Mtb specific CD4+ expressing IFN$\gamma$+ were significantly higher in LTBI or active TB compared to those cells in HC which agree with our data. Latorre et al (2019) reported in their analysis on CD27 MFI on CD4+ T cells that active TB subjects significantly associated with high ratio compared to LTBI subjects in agreement with our analysis and our data also revealed that the ratio was significantly lower in healthy control compared to active TB, but not to LTBI.

In our next analysis we assessed the relationship between the frequency of Mtb300 specific CD4+ expressing cytokines in the LTBI subjects with their QuantiFERON-Plus blood test. We found that while QFN2 or QFN1 are correlated with Mtb300 specific CD4+ expressing either IL-2 or TNF-$\alpha$, the correlation between the QFN2 or QFN1 and Mtb300 specific CD4 expressing IFN$\gamma$ is weak. This was unexpected as both assays measure antigen-specific IFN$\gamma$. The QFN blood test involves whole blood exposure for 16 hours and measures IFN$\gamma$ concentration while our assay measures the frequency of antigen-specific intracellular IFN$\gamma$ production in PBMCs after 6 hours exposure to antigen. The discrepancy may be result of the time of culture or the nature of the measurement, this is an interesting dichotomy which will be interesting to follow up. Interestingly, our data showed that the few LTBI subject who had a high QFN value also expressed higher Mtb300 specific CD4+ IFN$\gamma$+ and this group included those 6 patients we selected them and highlighted them for further investigation.

In next analysis we determined the frequencies of antigen specific CD4+ cytokines secreting in relation to the response to TB treatment for the LTBI patients. The frequencies of Mtb300 specific CD4+ T cells expressing IFN$\gamma$, expressing IL-2, and expressing TNF-$\alpha$ were higher after treatment (10-12 weeks, F2 group) compared with pre-treatment subjects but not this did not reach significance. The frequencies of Mtb300 specific CD4+ secreting these cytokines (IL-2 and IFN$\gamma$) were decreased after 4-6 weeks during the treatment, but the frequencies then increased after 10-12 weeks during the treatment. However, the frequencies of Mtb300 specific CD4+ secreting TNF-$\alpha$ was increased after 4-6 weeks during the treatment, but the frequencies then increased further after 10-12 weeks during the treatment. Our observation that the frequency of cytokine producing CD4+ T cells increased at the end of the treatment is in agreement with Caccamo et al (2010) who reported that functional CD4+ is associated with the changes of bacterial load induced by the therapy. These data may indicate a repressive environment for T cell accumulation or an accumulation of T cells at the active site of disease which are then released back into circulation when bacterial burden is reduced by drug treatment.

We determined the frequencies of Mtb300 specific memory CD4 T cells expressed AIM markers. We determined the frequencies of Mtb300 specific CD4+ $T_{CM}$ and CD4+ $T_{EM}$ between LTBI, active TB and HC. Then, we assessed the co-expression of these biomarkers on $T_{CM}$ and $T_{EM}$ CD4+ T cells to Mtb300 in human peripheral blood. Although the frequency of CD4+ $T_{CM}$ was reduced in PBMCs stimulated with SEB or Mtb300 compared to unstimulated cells, the frequency of CD4+ $T_{EM}$ was slightly increased in induced PBMCs compared to unstimulated cells. The reason of this is that cells may progressively gain effector function with further differentiation because of the stimulation. Esteves et al (2020) reported that CD4+ $T_{EM}$ did was lower in uninfected control subjects compared to LTBI and active TB patients but not significant. However, we observed in our data that the frequencies of these cells were significantly lower in HC compared to LTBI subjects. On the other hand, Esteves et al (2020) reported that CD4+ $T_{CM}$ in LTBI was significantly higher than active TB patients but lower than uninfected control subject. Similarly, our data showed that the frequencies of these cells in LTBI was higher than active TB but not significant. Dan et al (2016) assessed AIM markers to detect Mtb-specific proteins responses and they found that the frequencies of CD25+ OX40+

memory CD4+ T cells in PBMCs in human with LTBI was higher than these cells in cells from healthy control.

In this study we determined the frequencies of antigen specific CD4+ $T_{CM}$ and $T_{EM}$ in relation to the response to TB treatment in LTBI subjects. The frequencies of Mtb300 specific CD4+ $T_{CM}$ was decreased after 4-6 weeks during the treatment, and the frequency reduced further at the end of their treatment. On the other, hand the frequency of Mtb300 specific CD4 $T_{EM}$ was increased after 4-6 weeks of treatment but the frequency decreased dramatically at the end of the treatment, except for 1 subject who remained high. This suggests that patients have a reduction in the frequency of antigen-specific memory T cell by the end of treatment. Furthermore, the frequencies of Mtb300 specific CD4+ expressing OX40+ CD25+ and PD-L1+ CD25+ was decreased after 4-6 weeks during their treatment, but the frequency increased at the end of the treatment.

**4.5 Conclusion**

We hypothesized that the frequency and phenotype of antigen-specific T cells would allow discrimination of disease phenotype in defined cohorts of healthy, latent and active TB subjects. We found that the expression of Mtb300 specific CD4+ expressing IFN$\gamma$, or TNF-$\alpha$, or IL-2 were associated with active TB disease. The expression of Mtb300 specific CD4+ expressing IFN$\gamma$ can discriminate LTBI from active TB patients. The expression of Mtb300 specific CD4 expressing IFN$\gamma$ + HLA-DR+ and the MFI CD27 ratio were associated with active TB patients. The expression of Mtb300 specific CD4 memory T cells expressing OX40+ CD25+ or PD-L1+ CD25+ were associated with active TB patients and can discriminate those population from LTBI patients. Most of the markers were reduced after 6 weeks of LTBI treatment, but the expression of these markers increased by the end of treatment. Finally, there is strong positive correlation between the QFN2 and Mtb300 specific CD4 T cells expressing IL-2+ or TNF-$\alpha$.

# Chapter 5: The relationship between inflammatory markers in plasma from well characterized cohort of healthy, latent and active TB patients with the novel biomarker ΔTM-IL-12Rβ1

## 5.1 Introduction

Cytokines also play protective roles, and the failure to produce specific cytokines fails to control the disease. Interleukin 12 (IL-12) is a key proinflammatory cytokine that promotes innate and adaptive immune responses. A variety of cells including monocytes, neutrophils and B cells produce IL-12, whereas IL-12 is primarily produced by macrophages and dendritic cells (Watford et al., 2004). Functionally, IL-12 promotes CD4+ T cells differentiation, which results in interferon gamma (IFN$\gamma$) production. IL-12 is composed of two subunits (1) IL-12p40 subunit that binds to IL-12Rβ1 receptor (2) IL-12p35 subunit that binds to IL-12Rβ2 receptor (Watford et al., 2004). IL-12 plays a role in controlling infection with Mtb. Indeed, this cytokine is majorly produced by macrophages and dendritic cells and expressed within the lung at the site cells. In mice, those who are genetically lacking IL-12p40 subunit are susceptible to Mtb infection and the absence of these subunits results the loss of antigen specific IFN$\gamma$ production (Cooper et al., 1995; Cooper et al., 1997; Domingo-Gonzalez et al., 2016). Also, IL-12p40 is required in dendritic cells migration from the lung to draining lymph nodes. Khader et al (2006) investigated the role of IL-12p40 and reported the reduction of migration of dendritic cells and reduction of activation of naïve T cells in IL-12p40 deficiency. Moreover, the IL-12p40 subunit along with a p19 subunit result in a cytokine, IL-23. IL-23 is a cytokine that is produced by activated monocytes and activated antigen presenting cells and induces IFN$\gamma$ and IL-17 production (Watford et al., 2004). IL-23 binds a receptor composed of IL-12Rβ that interacts with IL-12p40 subunit, and IL-23R that interacts with p19 subunit (Watford et al., 2004).

IL12Rβ1 is a component receptor chain of the IL-12 receptor. IL-12Rb1 modulates Th1 responses in Mtb and IL-12Rb1 subunit is required for dendritic cells migration in response to bacterial stimuli including Mtb (Khader et al., 2006; Robinson et al., 2008, Robinson et al., 2010). Alternative RNA splicing of IL-12Rβ1 has been demonstrated to produce isoforms with specific function. Mouse leukocytes express two mRNA isoforms from IL-12Rβ1 whereas human leukocytes express 13 mRNA isoforms from IL-12Rβ1 (Robinson et al., 2010; Ford et al., 2012). Robinson et al (2015) reported that an alternative RNA splicing of IL-12Rβ1 isoform called “clone 3” was express with IL-12Rβ1 prior to dendritic cells exposure to *M. tuberculosis.* they stated that “clone 3” isoform which renamed ∆TM-IL-12Rβ1 enhance IL-12p40-

dependent dendritic cells migration to the draining lymph nodes (Robinson et al.,2010; Robinson et al., 2015). Also, in mice, an isoform is known that lacks the transmembrane domain and is secreted in response to Mtb exposure (Robinson., 2010). This isoform (△TM-IL-12Rβ1) however is a splice variant of IL-12Rβ 1, which is similar the human produced isoform 2 (DAS et al., 2018; Ray et al., 2015). Similar to mouse △TM-IL-12Rβ1*,* in human alternative RNA splicing of IL-12Rβ1 express an isoform that called isoform 2 is "transcribed from exon 1 to 9 plus a cryptic exon in intron 9" according to Van de Vosse et al. (2013); Ray et al (2015) and Ford et al (2012); Robinson (2015). Isoform 2 lacks the transmembrane domain that is required for anchoring on the cell surface, as well as the intracellular domain required for signaling (Van de Vosse et al., 2013; Ray et al., 2015). Furthermore, previous work, which published for DAS et al. (2018), demonstrates that the expression of the splice variant △TM-IL-12Rβ1 limits of the dissemination of Mtb from the lung in a mouse.

We aimed to test the ability to diagnose active TB disease from latent infection by combining different biomarkers to distinguish between the two groups. We have addressed the cellular response in chapter 4 and wanted to determine the relationship between the cellular response the circulating soluble factors and the clinical parameters. In this chapter we investigate the correlation between the plasma level of △TM-IL-12Rβ1 and other cytokines in the plasma. AS a result of the importance role of IL-12 including the IL-12/IL23p40 subunit and the role of IL-12Rβ1 receptor and observation of the association between the alternative RNA splicing of this receptor and disease state, we have developed an ELISA assay to detect △TM-IL-12Rβ1 in human plasma samples. We then used this ELISA on plasma samples from the ISATS study and compared expression of these among healthy control, LTBI and active TB. We analyzed the data to try to identify biomarkers capable of discriminating between the healthy control, LTBI and active TB subjects in the ISATS study.

**Hypothesis:**

We hypothesized that ∆TM-IL-12Rβ1 and other inflammatory markers could differentiate between well characterized cohort of healthy, latent and active TB disease subjects.

Objective:

**1-** To measure the plasma level of ∆TM-IL-12Rβ1 in subjects

**2-** To measure the plasma level of cytokines in subjects

3- To determine any correlation among immunological variables that reflect biology or biomarker identification using multivariate analysis

## 5.2 Method and materials

### 5.2.1.A Study subject

A total of 48 HIV negative aged ≥16 participants were enrolled on the ISATS screening cohort. 2 of the participants withdrew from the study. Full description of the participants and how we grouped them, and ethics statement for ISATS described in chapter 3.

### 5.2.1.B Specimen collection and storage

Human plasma samples for the patient who was recruited in the ISATS study were collected from the supernatant during the PBMC isolation (density gradient centrifugation and SepMate 50 tube explained in chapter 2). Samples was stored at $-80^0$ C.

### 5.2.2.A Dot blot method to assess the reactivity of full length ∆TM protein (381 aa) with primary antibody raised in rabbit against ∆TM-IL-12Rβ1 peptide (21 aa)

Transfer PVDF membrane was pre-wetted for 15 seconds in 100% methanol to allow membrane activation and then soaked in distilled water for 2 minutes, then 5 minutes with

TBST solution (20 mM Tris, 150 mM NaCl and 0.05% Tween 20) for equilibration. Then, a white filter paper was soaked in TBST and placed on dry filter paper on top of the towel. The full length recombinant human IL-12 Rβ1 protein (381 aa, Abcam; cat ab152476) sample was diluted 10-fold in a serial dilution from 4ug/µl to 0.4ng/µl and 5µl of each dilution was spotted on the PVDF membrane and left for absorption of the loaded protein. Once the proteins are absorbed, it was soaked in block solution (3% bovine plasma albumin BSA, BP9700-100 Fraction V in TBST) for 1 hour on a rotator at toom temperature. The membrane was washed twice in TBST. Then the membrane had been soaked in 1:500 (in blocking buffer) rabbit polyclonal antibody against ΔTM-IL-12Rβ1 peptide (21 aa, cat SP141_SF) and incubated overnight on ice. Next, the membrane was washed 5 times with TBST for 3 minutes for each wash. Then the membrane had been treated with the secondary antibody (in 1:10000 dilutions in blocking buffer, anti-rabbit IgG alkaline phosphate; Sigmaaldrich, A9919-0.25ML as secondary antibody) for 1 hour and 30 minutes on the rotator at room temperature. Next, the membrane was washed 3 times with TBST for 3 minutes for each wash. The membrane was soaked in substrate solution (NBT/BCIP tablet, 11697471001 Roche, dissolved in 10ml distilled water) and kept in the dark on the rotator at room temperature for 15 minutes. O.1M NaOH was added to stop the reaction.

**5.2.2.B Enzyme-linked immunosorbent assay ELISA to measure the plasma level of ΔTM-IL-12Rβ1 in human participants for ISATS study.**

1- **Coating solution.** Full length IL-12Rβ1 (AF839-SP) antibody at 20 µg/ml diluted in phosphate buffer PBS (1X, pH 7.2). IL-12Rβ1 antibody detects human IL-12Rβ1 in ELISA.
2- **Blocking Buffer.** Blocking buffer was prepared by adding 1.5 g of bovine plasma albumin (BSA) in 50 ml of 1X Tris-buffered saline (pH 7.5), Tween 20 (TBST).
3- **Primary antibody.** Primary detection antibody was prepared in blocking buffer at concentration 1:1000 (rabbit polyclonal antibody against ΔTM-IL-12Rβ1 peptide; 21 aa, cat SP141_SF) in blocking buffer. It was custom generated by Cooper laboratory in collaboration with Generon Ltd.

4- **Secondary antibody.** Secondary detection antibody goat anti-rabbit IgG secondary HRP (Cell signalling technology; 7074S) was prepared in blocking buffer at concentration 1:20000.

5- **TMB substrate solution.** Biolegend cat number 421501

A 96 wells plates with flat bottom was coated with 200 µl per well of coating solution. The plates were covered and incubated overnight at $4^0$ C. The plates were blocked by adding 100 µl of blocking buffer per each well and incubated for 1 hour at room temperature. A standard ΔTM protein dilution was prepared from $10^{-1}$ to $10^{-6}$, and plasma samples were thawed in ice box. 100 µl of each standard diluents and plasma samples were pipetted into designated wells in both plates. The plates were incubated for overnight at $4^0$ C. The plates were washed three times with 100 µl of 1X TSBS. The primary detection antibody 1:1000 rabbit polyclonal IgG diluted in blocking buffer was added into each well (100 µl/well) and incubated for 4 hours at room temperature with gentle continual shaking. The plates were washed three times with 100 µl of 1X TSBS. The secondary detection antibody; goat anti-rabbit IgG secondary HRP, was added into each well (100 µl/well) at 1:20000 in blocking buffer and incubated for 90 minutes at room temperature with gentle continual shaking. The plates were washed three times with 100 µl of 1X TBST. TMB substrate solution (1X, Invitrogen, 00-4201-56) was added to each well (100 µl/well) and the plates were incubated at room temperature for 30 minutes. Stop solution was added to each well (100 µl/well). Absorbance at 450 nm was measured within 30 minutes.

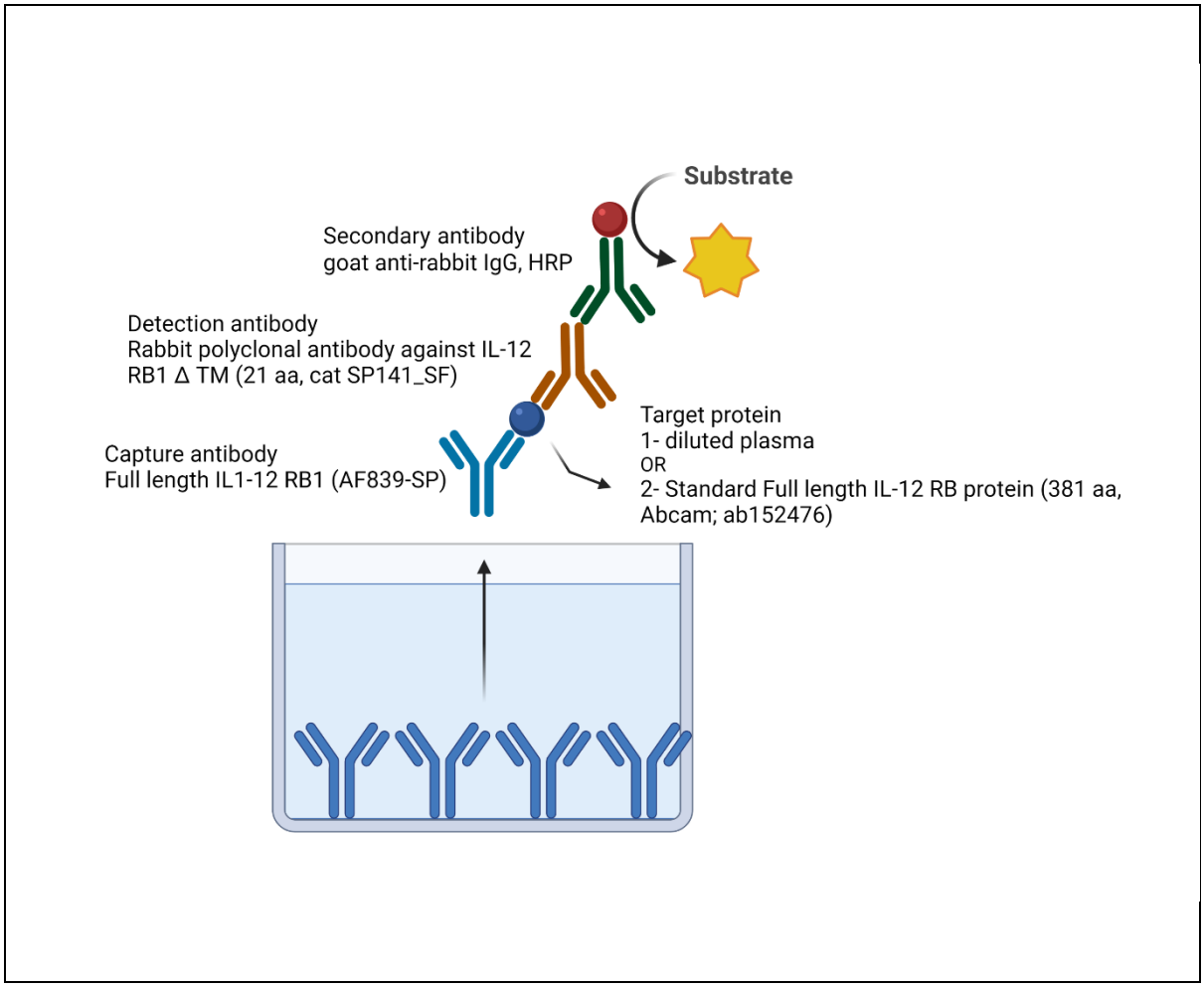



**Figure 5.1. ELISA model.** ELISA model to measure the plasma level of ΔTM-IL-12Rβ1 peptide showing capture antibody, target protein, primary antibody, secondary antibody and substrate.

### 5.2.2 ELISA MAX™ standard set for human cytokines

#### 5.2.3.A Kits and material

1- **Microwell plates** Biolegend cat number 423501

2- **Wash buffer**. Phosphate buffer saline PBS with 0.05% Tween-20

3- **Stop solution**. 2N $H_2SO_4$

4- **Assay diluent.** 5X concentrated phosphate buffered saline solution (PBS recipe: 8.0 g NaCL, 1.16 g $Na_2HPO_4$, 0.2g $KH_2PO_4$ and 0.2 g KCL added to deionized water 1.0 L and filtered through 0.2 µm filter, pH adjusted to 7.4) containing bovine plasma, Biolegend cat number 421203.

5- **Coating buffer A.** 5X concentrated carbonate buffer, pH 9.5, Biolegend cat number 421701

6- **TMB substrate solution.** Biolegend cat number 421501

7- **Deionized water.**

8- **ELISA MAX$^{TM}$ standard set:**

**Table 5.1. Human ELISA MAX$^{TM}$ standard sets**

| Set kit name | Provider (Cat number) | Certificate of analysis lot number |
|---|---|---|
| Human IFN-$\gamma$ | Biolegend (430101) | B345822 |
| Human TNF-$\alpha$ | Biolegend (430201) | B325769 |
| Human IL-2 | Biolegend (431804) | B283656 |
| Human IL-12/IL-23 (P40) | Biolegend (430701) | B337707 |
| Human GM-CSF | Biolegend (432001) | B335244 |
| Human IL-6 | Biolegend (430501) | B338616 |

**5.2.3.B Preparation for reagents**

**ELISA coating buffer 5X.** ELISA coating buffer was diluted to 1X working solution with deionized water.

**ELISA assay diluent 5X.** ELISA assay diluent was diluted to 1X with PBS.

**Human ELISA MAX$^{TM}$ capture antibody (200x).** 60 µl of capture antibody (200x) was diluted with 12 ml of 1X coating buffer A.

**Human ELISA MAX$^{TM}$ detection antibody (200x).** 60 µl of detection antibody (200x) was diluted with 12 ml of 1X assay diluent buffer A.

Avidin-HRP (1000X). 12 µl of Avidin-HRP (1000X) was diluted with 12 ml 1X of assay diluent A.

Human ELISA MAX$^{TM}$ standard kit components were stored between $2^0$ C and $8^0$ C. The lyophilized standard was diluted with assay diluent and aliquoted into polypropylene vials and stored at $-70^0$ C. prior to use, all the components was sat at room temperature.

### 5.2.3.C Standard reconstitution and preparation

**Human IFN$\gamma$ standard.** The lyophilized human IFN$\gamma$ standard was reconstituted by adding 0.2 ml of assays diluent to make 102.5 ng/ml standard stock solution. The reconstituted standard was sat at room temperature for 10-15 minutes and then vortexed. Then 1000 µl of the standard was prepared at 500 pg/ml by adding 4.9 µl of the reconstituted standard to 995.1 µl of assay diluent. Six-fold serial dilution of 500 pg/ml standard was performed in separate tubes. After the diluting the human IFN$\gamma$ standard concentrations were 500 pg/ml, 250 pg/ml, 125 pg/ml 62.5 pg/ml, 31.1 pg/ml, 15.6 pg/ml, and 7.8 pg/ml. Assay diluent was served as zero standard 0 pg/ml. The human standard for TNF-$\alpha$, IL-2, IL-12/IL23 (p40), GM-CSF and IL-6 six-fold serial dilution was prepared followed table 5.2.

**Table 5.2. ELISA MAX$^{TM}$ standard**

| Human standard | Stock solution concentration | Final solution concentration | Six-fold serial dilution |
|---|---|---|---|
| TNF-$\alpha$ | 40 ng/ml | 500 pg/ml | 250 pg/ml, 125 pg/ml 62.5 pg/ml, 31.1 pg/ml, 15.6 pg/ml, 7.8 pg/ml. |
| IL-2 | 150 ng/ml | 500 pg/ml | 250 pg/ml, 125 pg/ml 62.5 pg/ml, 31.1 pg/ml, 15.6 pg/ml, 7.8 pg/ml. |
| IL-12/IL-23 (p40) | 85 ng/ml | 4000 pg/ml | 2000 pg/ml, 1000 pg/ml 500 pg/ml, 250 pg/ml, 125 pg/ml, 62.5 pg/ml. |
| GM-CSF | 65 ng/ml | 500 pg/ml | 250 pg/ml, 125 pg/ml 62.5 pg/ml, 31.1 pg/ml, 15.6 pg/ml, 7.8 pg/ml. |
| IL-6 | 153 ng/ml | 500 pg/ml | 250 pg/ml, 125 pg/ml 62.5 pg/ml, 31.1 pg/ml, 15.6 pg/ml, 7.8 pg/ml. |

### 5.2.3.D ELISA MAX$^{TM}$ protocol

One day prior to running the ELISA, capture antibody was diluted in 1X coating buffer A. 100 µl of this capture antibody solution was added to all wells of a 96-well plate provided in the

kit. The plate was sealed and incubated overnight at 4°C. The plate was washed with 300 µl wash buffer 4 times and blotted upside down on absorbent paper. The wells of the plate were blocked with 200 µl of assay diluent A per will for 1 hour in room temperature on plate shaker. The plate was washed 4 times with washing buffer. Standards and plasma samples were added to the appropriate wells (100 µl / well) and then the plate was sealed and incubated for 2 hours at room temperature. the plate was washed 4 times, and 100 µl of diluted detection antibody was added to each well and incubated for 1 hour at room temperature with shaking. The plate was washed 4 times with washing buffer. 100 µl of diluted Avidin-HRP was added to each well and then incubated for 30 minutes at room temperature with shaking. 100 µl of TMB substrate solution was added into each well and the plate was incubated in the dark for 20 minutes. Stop solution was added to each well. The plate was read within 15 minutes at 450 nm.

### 5.2.4 Statistical analysis

Data were analysed in IBM SPSS Statistics version 28 and GraphPad Prism 9. A nonparametric Kruskal-Wallis test was used to analyse group differences between healthy, LTBI and active TB donors. If we found there was a statistically significant difference between the medians of the independent groups, then Dunn's multiple comparison test was used to compare the mean rank of LTBI and active TB subjects with mean rank of control subjects (HC). Nonparametric Spearman correlation was computed to test the association between the plasma level of ΔTM-IL-12Rβ1 and plasma level of cytokines. Principal component analysis (PCA) was computed for multiple variable analysis. Prior to PCA analysis data were missing value imputed using half of the minimum value of each variable, followed by data transformation into $\log_{10}$ and normalised to achieve a Gaussian distribution. Significance was set as p value < 0.05. data and figures were created using GraphPad Prism 9. Scatter plots were drawn to the median of distribution. We used GraphPad Prism 9 software to compute Spearman rank correlation coefficient to assess the correlation relationship between these variables.

## 5.3 Results

### 5.3.1 The reactivity of full length ΔTM protein (381 aa) with primary antibody raised in rabbit against ΔTM-IL-12Rβ1 peptide (21 aa)

To assess the reactivity of full-length IL-12Rβ1 protein (381 aa) with primary antibody raised in rabbit against ΔTM-IL-12Rβ1 peptide (21 aa) a dot plot assay was developed by Dr Mrinal Das in conjunction with me. We found purple dots that show the full-length peptide does react with the primary antibody (figure 5.2)

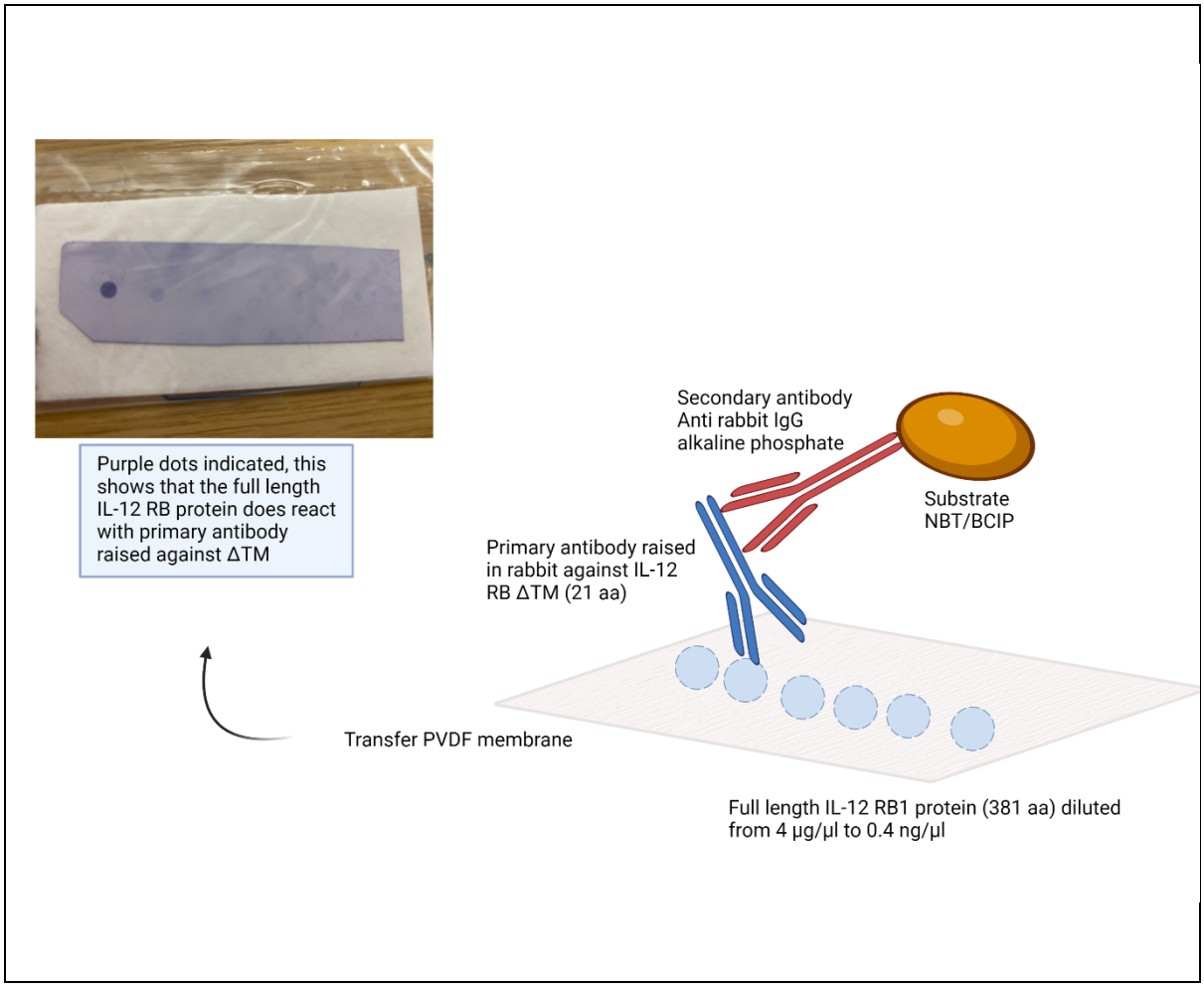



**Figure 5.2. Dot plot assay.** Purple dots indicating the reactivity between full length IL-12RB1 protein at different concentration with the primary antibody raised in rabbit against ΔTM-IL-12Rβ1 peptide.

### 5.3.2 Plasma level of IL-12Rβ delta TM for the individuals

It is known that ∆TM-IL-12Rβ1 enhances IL-12p40-dependent dendritic cell migration to the draining lymph nodes (Robinson et al.,2010; Robinson et al., 2015) and that pulmonary TB is associated with the expression of ∆TM-IL-12Rβ1 (DAS et al., 2018). We therefore measured the plasma level of ∆TM-IL-12Rβ1 for the individuals who participated in ISATS study. We determined the plasma level of ∆TM-IL-12Rβ1 in healthy control (HC), LTBI and active TB patients, then we compared the level of ∆TM-IL-12Rβ1 between the groups. We found that the plasma level of ∆TM-IL-12Rβ1 in individuals was variable (figure 5.3). There was no significant difference between the level of IL-12Rβ delta TM in HC individuals and those level in LTBI or active TB patients.

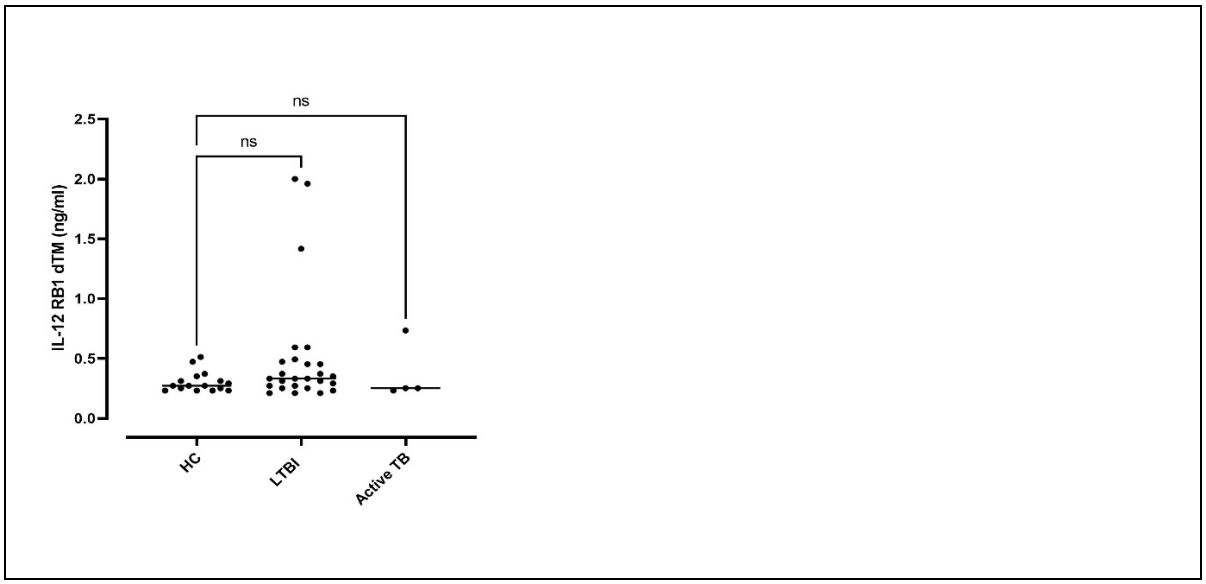



**Figure 5.3. plasma level of ∆TM-IL-12Rβ1**. Representative of the plasma level of ∆TM-IL-12Rβ1 in healthy control, LTBI and active TB patients. The horizontal bars represent median values and significant difference is represented by * *P* value < 0.05, ** *P* value < 0.001, *** *P* value < 0.0001 and ns (not significant) as determined using a nonparametric Kruskal-Wallis test. Data were analysed in GraphPad Prism 9.

### 5.3.3 Plasma level of cytokines

To place the expression of the ΔTM-IL-12Rβ1 in context in the plasma, we determined the level of other inflammatory cytokines in the plasma and looked to see whether there were any associations. We determined the correlation relationship between the plasma levels of ΔTM-IL-12Rβ1 and plasma levels of cytokine production in LTBI patients. To do this, we performed sandwich ELISA assay using BioLegend's ELISA MAX$^{TM}$ standard set. We found that there was no significant difference between plasma level of any cytokines between HC and LTBI patients or HC and active TB.

We found that there was a positive correlation between the plasma level of ΔTM-IL-12Rβ1 and the plasma level of TNF-α with a *p* value of 0.062 and so not quite reaching significance. We found that there was statistically significant positive correlation relationship between the plasma level of ΔTM-IL-12Rβ1 and the plasma level of both IL-6 and IFN$\gamma$. These data suggest that ΔTM-IL-12Rβ1 level in LTBI patients appears to be associated with the level of IL-6 or IFN$\gamma$. Moreover, there was no correlation relationship between ΔTM-IL-12Rβ1 and the plasma level of IL-12/IL-23 (P40) or GM-CSF or IL-2 in LTBI group.

Then we determined the plasma level of cytokines in active TB patient, and we assessed the correlation relationship between IL-12Rβ ΔTM. We found that although there was positive correlation between the plasma level of the following cytokines TNF-α, IL-2, IL-12/IL23p40, IL-6 and GM-CSF) and the plasma level of IL-12Rβ ΔTM. Due to the low number of samples these relationships did not reach significance. The association between plasma IFN$\gamma$ and ΔTM-IL-12Rβ1 was determined in active TB patients. We found that there was no correlation relationship between these cytokines in active TB group. We can conclude that the plasma level of ΔTM-IL-12Rβ1 did not appear to be associated with plasma level of cytokines in active TB patients.

Similarly, we determined the plasma level of cytokines in healthy control participants, and we investigate the correlation relationship between ΔTM-IL-12Rβ1 and these cytokines. We found that there was negative correlation between the plasma level of IFN$\gamma$, IL-2, IL-6 and

GM-CSF and the plasma level of IL-12Rβ delta TM in healthy control individuals, but this was not statically significant. There was positive correlation between the plasma level of TNF-α and IL-12/IL-23p40 and the plasma level of IL-12Rβ delta TM; however, this relationship was not statistically significant. We can conclude that the plasma level of ΔTM-IL-12Rβ1 did not appear to be associated with plasma level of cytokines in healthy subjects.

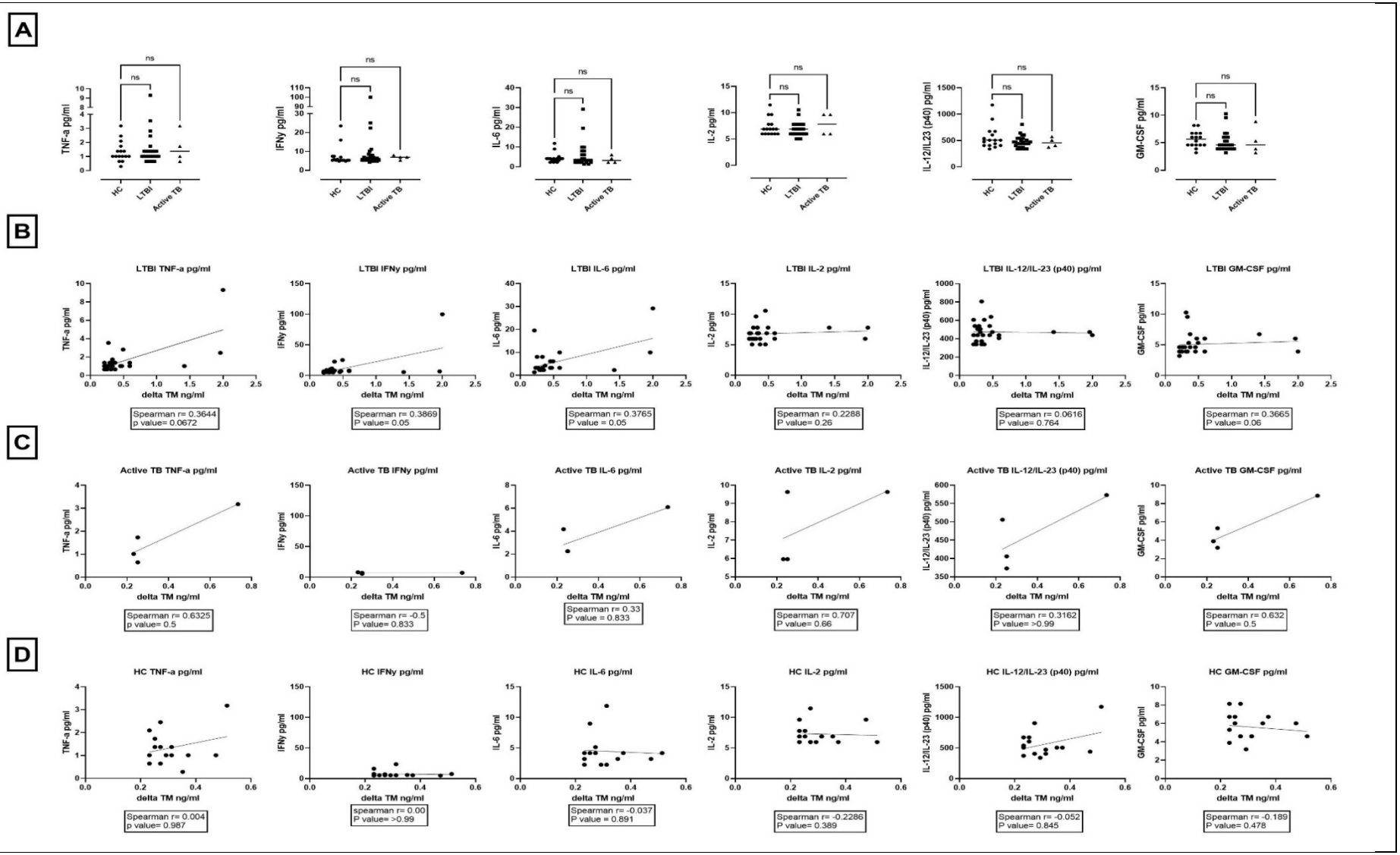

A
TNF-a pg/ml
IFNy pg/ml
IL-6 pg/ml
IL-2 pg/ml
IL-12/IL23 (p40) pg/ml
GM-CSF pg/ml
ns
HC
LTBI
Active TB
B
LTBI TNF-a pg/ml
LTBI IFNy pg/ml
LTBI IL-6 pg/ml
LTBI IL-2 pg/ml
LTBI IL-12/IL-23 (p40) pg/ml
LTBI GM-CSF pg/ml
delta TM ng/ml
Spearman r= 0.3644
p value= 0.0672
Spearman r= 0.3869
P value= 0.05
Spearman r= 0.3765
P value = 0.05
Spearman r= 0.2288
P value= 0.26
Spearman r= 0.0616
P value= 0.764
Spearman r= 0.3665
P value= 0.06
C
Active TB TNF-a pg/ml
Active TB IFNy pg/ml
Active TB IL-6 pg/ml
Active TB IL-2 pg/ml
Active TB IL-12/IL-23 (p40) pg/ml
Active TB GM-CSF pg/ml
Spearman r= 0.6325
p value= 0.5
Spearman r= -0.5
P value= 0.833
Spearman r= 0.33
P value = 0.833
Spearman r= 0.707
P value= 0.66
Spearman r= 0.3162
P value= >0.99
Spearman r= 0.632
P value= 0.5
D
HC TNF-a pg/ml
HC IFNy pg/ml
HC IL-6 pg/ml
HC IL-2 pg/ml
HC IL-12/IL-23 (p40) pg/ml
HC GM-CSF pg/ml
Spearman r= 0.004
p value= 0.987
spearman r= 0.00
P value= >0.99
Spearman r= -0.037
P value = 0.891
Spearman r= -0.2286
P value= 0.389
Spearman r= -0.052
P value= 0.845
Spearman r= -0.189
P value= 0.478

**Figure 5.4. plasma level of cytokines association with plasma level of ΔTM-IL-12Rβ1**. Representative of the plasma level of cytokines in healthy control, LTBI and active TB patients (A). The horizontal bars represent median values and significant difference (p value < 0.05) calculated by a non-parametric Kruskal-Wallis test. (B) representative of plasma level of cytokines association with plasma level of ΔTM-IL-12Rβ1 in LTBI patients. (C) Representative of plasma level of cytokines association with plasma level of ΔTM-IL-12Rβ1 in active TB patients. (D) representative of plasma level of cytokines association with plasma level of ΔTM-IL-12Rβ1 in healthy control. Spearman rank correlation coefficient was computed the correlation relationship between these variables. Data were analysed in GraphPad Prism 9.

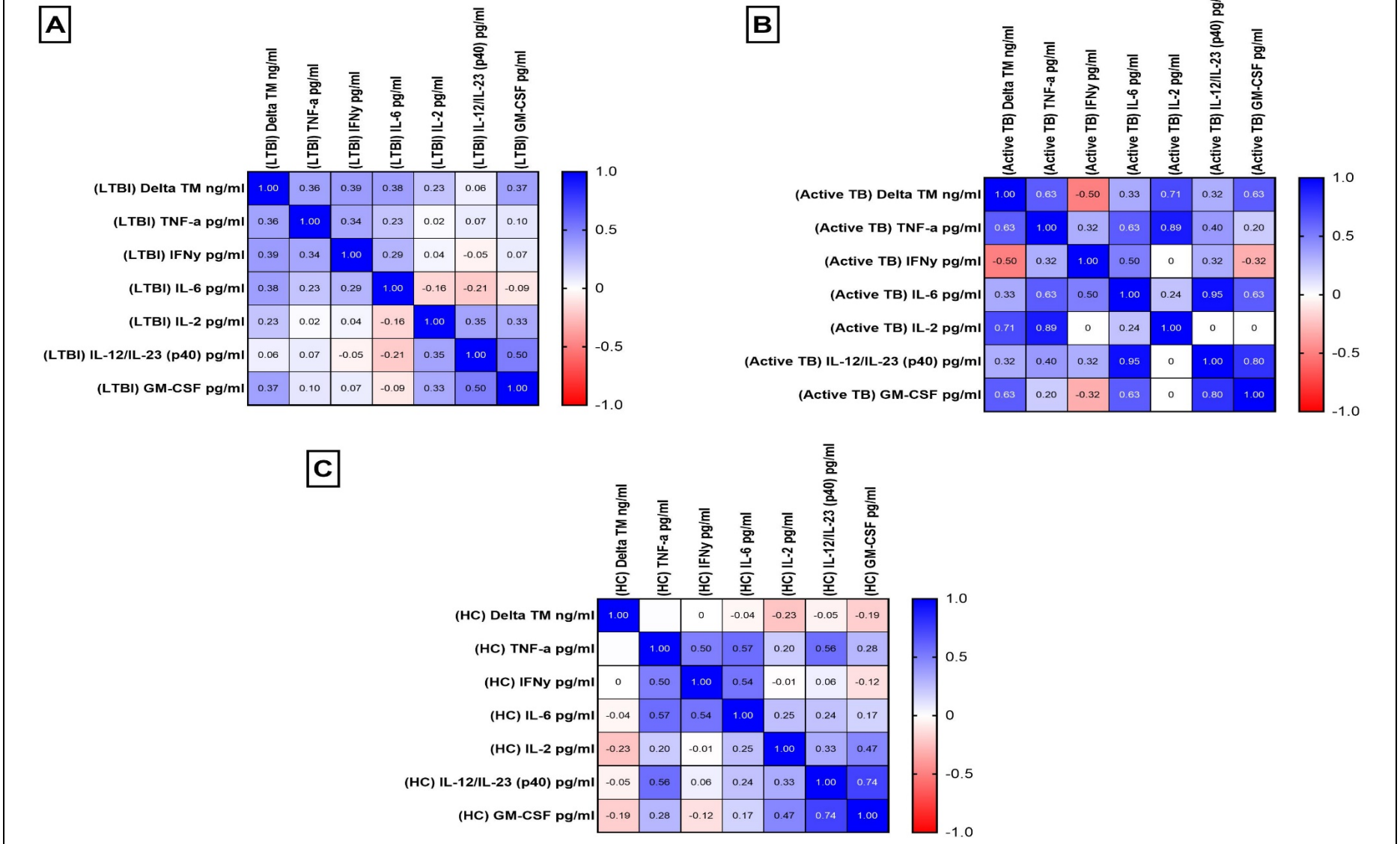

A
(LTBI) Delta TM ng/ml
(LTBI) TNF-a pg/ml
(LTBI) IFNy pg/ml
(LTBI) IL-6 pg/ml
(LTBI) IL-2 pg/ml
(LTBI) IL-12/IL-23 (p40) pg/ml
(LTBI) GM-CSF pg/ml
1.00 0.36 0.39 0.38 0.23 0.06 0.37
0.36 1.00 0.34 0.23 0.02 0.07 0.10
0.39 0.34 1.00 0.29 0.04 -0.05 0.07
0.38 0.23 0.29 1.00 -0.16 -0.21 -0.09
0.23 0.02 0.04 -0.16 1.00 0.35 0.33
0.06 0.07 -0.05 -0.21 0.35 1.00 0.50
0.37 0.10 0.07 -0.09 0.33 0.50 1.00
1.0
0.5
0
-0.5
-1.0
B
(Active TB) Delta TM ng/ml
(Active TB) TNF-a pg/ml
(Active TB) IFNy pg/ml
(Active TB) IL-6 pg/ml
(Active TB) IL-2 pg/ml
(Active TB) IL-12/IL-23 (p40) pg/ml
(Active TB) GM-CSF pg/ml
1.00 0.63 -0.50 0.33 0.71 0.32 0.63
0.63 1.00 0.32 0.63 0.89 0.40 0.20
-0.50 0.32 1.00 0.50 0 0.32 -0.32
0.33 0.63 0.50 1.00 0.24 0.95 0.63
0.71 0.89 0 0.24 1.00 0 0
0.32 0.40 0.32 0.95 0 1.00 0.80
0.63 0.20 -0.32 0.63 0 0.80 1.00
1.0
0.5
0
-0.5
-1.0
C
(HC) Delta TM ng/ml
(HC) TNF-a pg/ml
(HC) IFNy pg/ml
(HC) IL-6 pg/ml
(HC) IL-2 pg/ml
(HC) IL-12/IL-23 (p40) pg/ml
(HC) GM-CSF pg/ml
1.00 0 -0.04 -0.23 -0.05 -0.19
1.00 0.50 0.57 0.20 0.56 0.28
0 0.50 1.00 0.54 -0.01 0.06 -0.12
-0.04 0.57 0.54 1.00 0.25 0.24 0.17
-0.23 0.20 -0.01 0.25 1.00 0.33 0.47
-0.05 0.56 0.06 0.24 0.33 1.00 0.74
-0.19 0.28 -0.12 0.17 0.47 0.74 1.00
1.0
0.5
0
-0.5
-1.0

**Figure 5.5. Correlation matrix between plasma level of ΔTM-IL-12Rβ1 and plasma level of cytokines.** Correlation matrix represents association between plasma level of ΔTM-IL-12Rβ1 and plasma level of IFN$\gamma$, TNF-$\alpha$, IL-6, IL-2, IL12/IL-23 p40 and GM-CSF in (A) LTBI (B) active TB and (C) healthy control. Values represented nonparametric Spearman correlation calculated in GraphPad Prism 9.

### 5.3.4 Principal component analysis (PCA)

Because we determined the frequency and phenotype on the circulating PBMC and we determine the association between ∆TM-IL-12Rβ1 and cytokines in the plasma in characterised cohorts of healthy, LTBI and active TB disease patients, we did PCA analysis to represent a multivariate data table as a small set of variables to observe a trend that may present the relationship among the subjects. To test this, we did log transformation, normalised them on GraphPad Prism software and then we computed the PCA on R software (PCA also computed on Prism software for the PC score and loading score, but not shown). The score plot in figure 5.6 explains the first two principal component and represents a map of 46 participants. The first component is selected as the one that explain the greatest amount of variance in the variables. PC1 explain 18.1% of the variance found in the 42 variables. The second component (PC2) explains an additional 10.9% leading to a combined total of 29% explained with the first two components. The size of the ellipses is in normal probability. The score plot in figure 5.6 showed that a group of subjects trended on the x axis, therefore this may suggest that they share similar immunophenotyping. We found that there were 10 patients sat within same position with those patients with active TB disease. There were 5 patients, which identified with high cytokine expression in chapter 4, sat within the group suggesting that they may be subclinical perhaps.

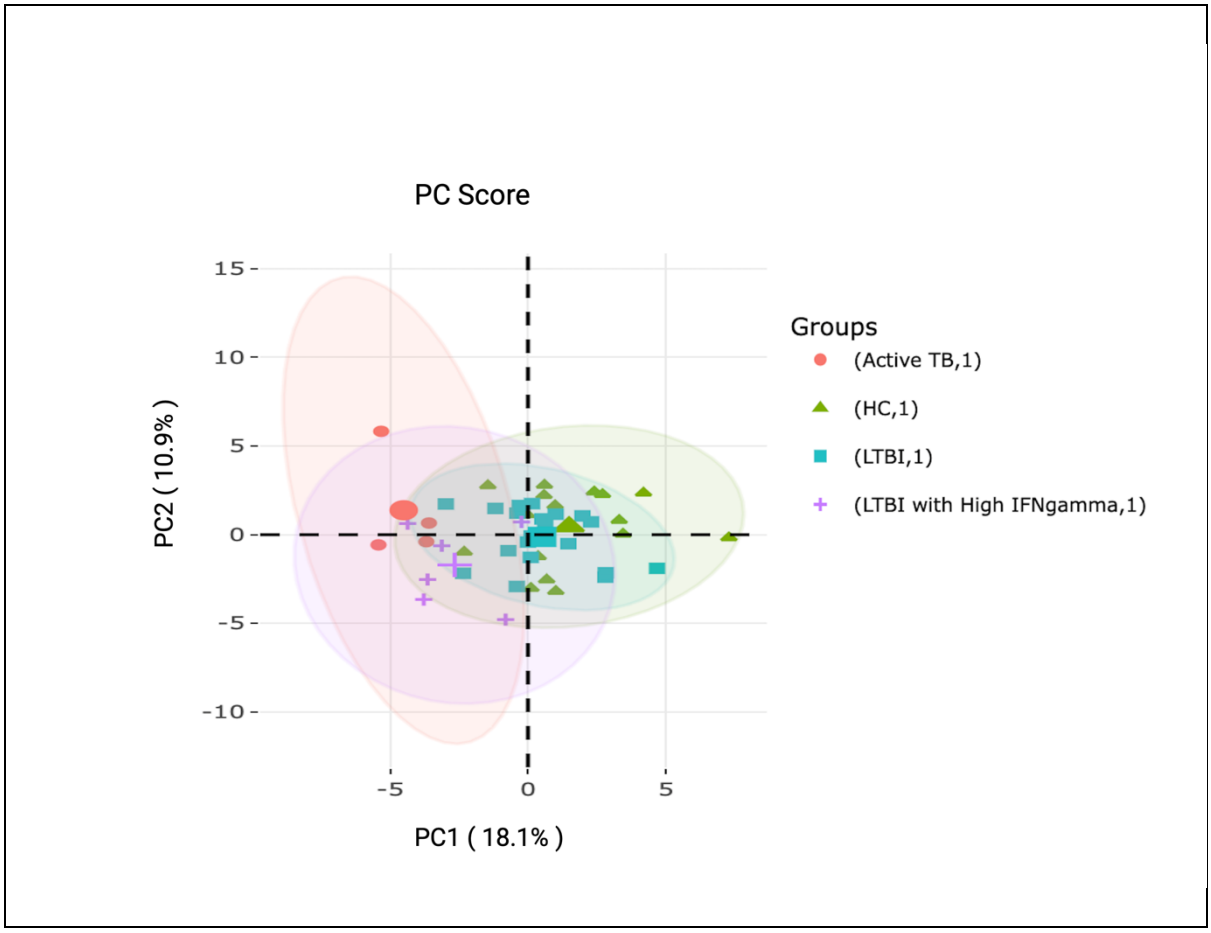



**Figure 5.6. Principal component score plot.** Score plot represents the principal component 1 (PC1) and principal component 2 (PC2). The plot represents a map of 46 participants in the study. Green Triangles represent healthy control, blue squares represent LTBI, violet plus signs represent LTBI patients who expressed high Mtb300 specific CD4+ IFN$\gamma$+ and red dots represent active TB patients. The size of the ellipses is in normal probability.

Next, we have selected 14 variables based on the loading score from the first PCA (loading score computed on prism and not shown). These variables are Live single CD3+ CD4+, CD3+ CD4+ CD127- CD25+, CD3+ CD4 CD45 RA+, CD3+ CD4+ CD45 RO+, CD3+ CD4+ CD45 RA- CCR7- , CD19+, Mtb300-specific CD3+ CD4+ IFN$\gamma$+, CD4+ IFN$\gamma$- CD27 geometric mean, CD4+ IFN$\gamma$+ CD27 geometric mean, CD27 ratio, Mtb300-specific CD3+ CD4+ IFN$\gamma$+ HLA-DR+, Mtb300-specific CD3+ CD4+ IL-2+, Mtb300-specific CD3+ CD4+ TNF-$\alpha$ and plasma level of $\Delta$TM-IL-12R$\beta$1. Each variable is strongly correlated either with PCA 1 or PCA2. Variables which clustered closely are positively correlated. We ran PCA analysis including these selected variables and found that PC1 explain 40% of the variance found in the 14 variables. The second component (PC2) explains an additional 16.1% leading to a combined total of 56.1% explained

with the first two components. The size of the ellipses is in normal probability. The score plot in figure 5.7 showed that a group of subjects trended on the x axis, therefore this may suggest that they share similar immunophenotyping. We found that there were 11 patients sat within same position with those patients with active TB disease. There were 5 patients, which identified with high cytokine expression in chapter 4, sat within the group suggesting that they may be subclinical perhaps.

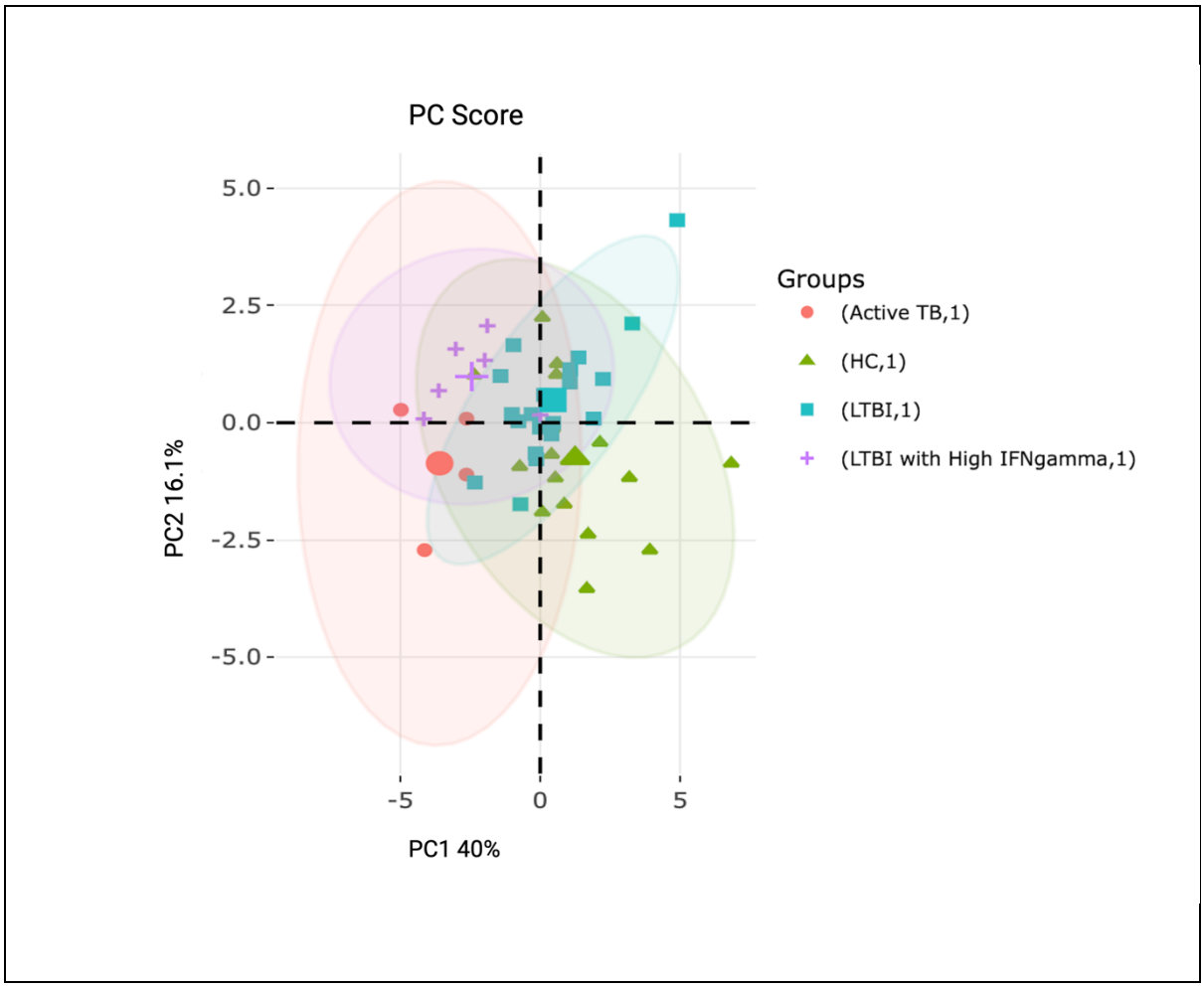



**Figure 5.7. Principal component score plot.** Score plot represents the principal component 1 (PC1) and principal component 2 (PC2). The plot represents a map of 46 participants in the study. Green triangles represent healthy control, blue squares represent LTBI, violet plus signs represent LTBI patients who expressed high Mtb300 specific CD4+ IFN$\gamma$+ and red dots represent active TB patients. The size of the ellipses is in normal probability.

### 5.3.5 Plasma level of ∆TM-IL-12Rβ1 association with Mtb300 specific CD4+ T cells responses in TB patients.

Furthermore, we correlate between the plasma level of ∆TM-IL-12Rβ1 and the frequencies of Mtb300 CD4+ T cells responses in LTBI, active TB and HC individuals. To do this, a Spearman rank correlation coefficient was computed, and correlation matrix was created to assess the relationship between these variables. In LTBI individuals, there was negative correlation relationship between the plasma level of ∆TM-IL-12Rβ1 and the frequencies of Mtb300 specific CD4+ T cells expressing IFN$\gamma$+. We found that the Spearman's r coefficient between the plasma level of ∆TM-IL-12Rβ1 and Mtb300 specific CD4+ T cells responses including CD4+ TNF-$\alpha$, CD4+ IL-2, was r= 0.05, r=0.22 respectively. However, this relationship was not statistically significant. We found that the Spearman's r coefficient between the plasma level of ∆TM-IL-12Rβ1 and Mtb300 specific CD4+ T cells responses including CD4+ $T_{CM}$ OX40+ CD25+, CD4+ $T_{CM}$ PD-L1+ CD25+, CD4+ $T_{EM}$ OX40+ CD25+ and CD4+ $T_{EM}$ PD-L1+ CD25+ was r= 0.02, r=0.11, r=0.14 and r=0.18 respectively. However, the relationship was not statistically significant. Also, the Spearman's r coefficient between the plasma level of ∆TM-IL-12Rβ1 and the blood test QuantiFERON 1, QuantiFERON 2 and mitogen was r=0.37, r=0.34 and r= 0.1 respectively. However, the relationship was not statistically significant.

Furthermore, the correlation matrix in active TB patients showed that there was strong correlation between the plasma level of ∆TM-IL-12Rβ1 and the level of blood test QuantiFERON 1, QuantiFERON 2, mitogen and the frequencies of Mtb300 specific CD4+ T cells expressing IFN$\gamma$+, IL-2. However, the relationship between these were not statistically significant. moreover, we found that there was positive correlation between the plasma level of ∆TM-IL-12Rβ1 and the frequency of Mtb300 specific CD4+ responses including CD4+ TNF-$\alpha$, CD4+ $T_{CM}$ OX40+ CD25+, $T_{CM}$ PD-L1+ CD25+, and $T_{EM}$ PD-L1+ CD25+. However, the relationship between these were not statistically significant. Furthermore, we found negative correlation between the plasma level of ∆TM-IL-12Rβ1 and the frequency of Mtb300 specific CD4 $T_{EM}$ expressing OX40+ CD25+.

Furthermore, the correlation matrix in HC individuals showed that there was no correlation between the plasma level of ∆TM-IL-12Rβ1 and the frequencies of Mtb300 specific CD4+ T

cells expressing IFN$\gamma$+ or TNF-$\alpha$. Similarly, we found that there was no correlation between the plasma level of $\Delta$TM-IL-12R$\beta$1 and the frequency of Mtb300 specific CD4+ $T_{CM}$ OX40+ CD25+, $T_{CM}$ PD-L1+ CD25+, and $T_{EM}$ PD-L1+ CD25+. On the other hand, we found that there was negative correlation between the plasma level of $\Delta$TM-IL-12R$\beta$1 and the frequency of Mtb300 specific CD4+ $T_{EM}$ OX40+ CD25+ or and the frequencies of Mtb300 specific CD4+ T cells expressing IL-2.

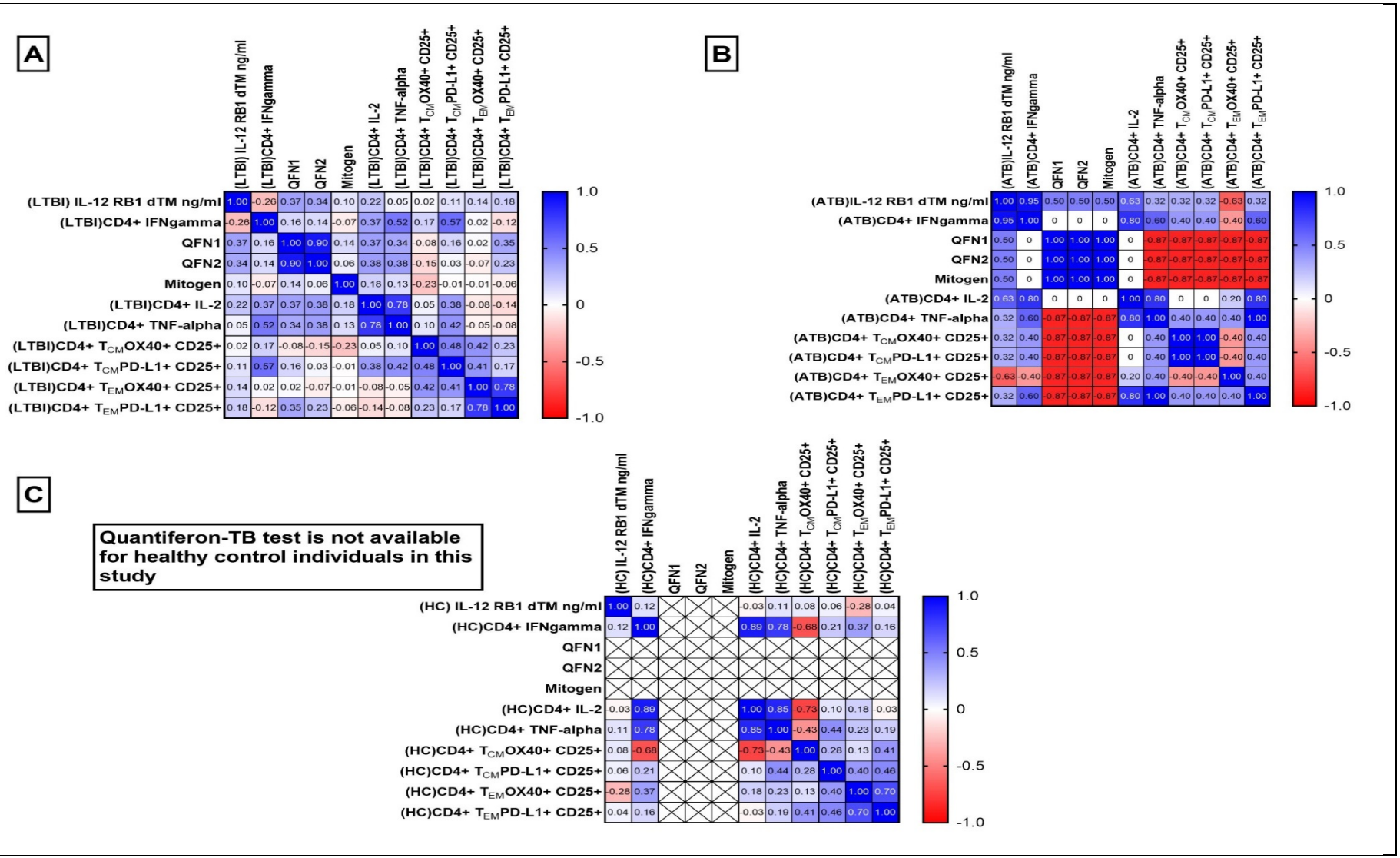

A
(LTBI) IL-12 RB1 dTM ng/ml
(LTBI)CD4+ IFNgamma
QFN1
QFN2
Mitogen
(LTBI)CD4+ IL-2
(LTBI)CD4+ TNF-alpha
(LTBI)CD4+ TCMOX40+ CD25+
(LTBI)CD4+ TCMPD-L1+ CD25+
(LTBI)CD4+ TEMOX40+ CD25+
(LTBI)CD4+ TEMPD-L1+ CD25+
1.00 -0.26 0.37 0.34 0.10 0.22 0.05 0.02 0.11 0.14 0.18
-0.26 1.00 0.16 0.14 -0.07 0.37 0.52 0.17 0.57 0.02 -0.12
0.37 0.16 1.00 0.90 0.14 0.37 0.34 -0.08 0.16 0.02 0.35
0.34 0.14 0.90 1.00 0.06 0.38 0.38 -0.15 0.03 -0.07 0.23
0.10 -0.07 0.14 0.06 1.00 0.18 0.13 -0.23 -0.01 -0.01 -0.06
0.22 0.37 0.37 0.38 0.18 1.00 0.78 0.05 0.38 -0.08 -0.14
0.05 0.52 0.34 0.38 0.13 0.78 1.00 0.10 0.42 -0.05 -0.08
0.02 0.17 -0.08 -0.15 -0.23 0.05 0.10 1.00 0.48 0.42 0.23
0.11 0.57 0.16 0.03 -0.01 0.38 0.42 0.48 1.00 0.41 0.17
0.14 0.02 0.02 -0.07 -0.01 -0.08 -0.05 0.42 0.41 1.00 0.78
0.18 -0.12 0.35 0.23 -0.06 -0.14 -0.08 0.23 0.17 0.78 1.00
1.0
0.5
0
-0.5
-1.0
B
(ATB)IL-12 RB1 dTM ng/ml
(ATB)CD4+ IFNgamma
QFN1
QFN2
Mitogen
(ATB)CD4+ IL-2
(ATB)CD4+ TNF-alpha
(ATB)CD4+ TCMOX40+ CD25+
(ATB)CD4+ TCMPD-L1+ CD25+
(ATB)CD4+ TEMOX40+ CD25+
(ATB)CD4+ TEMPD-L1+ CD25+
1.00 0.95 0.50 0.50 0.50 0.63 0.32 0.32 0.32 -0.63 0.32
0.95 1.00 0 0 0 0.80 0.60 0.40 0.40 -0.40 0.60
0.50 0 1.00 1.00 1.00 0 -0.87 -0.87 -0.87 -0.87 -0.87
0.50 0 1.00 1.00 1.00 0 -0.87 -0.87 -0.87 -0.87 -0.87
0.50 0 1.00 1.00 1.00 0 -0.87 -0.87 -0.87 -0.87 -0.87
0.63 0.80 0 0 0 1.00 0.80 0 0 0.20 0.80
0.32 0.60 -0.87 -0.87 -0.87 0.80 1.00 0.40 0.40 0.40 1.00
0.32 0.40 -0.87 -0.87 -0.87 0 0.40 1.00 1.00 -0.40 0.40
0.32 0.40 -0.87 -0.87 -0.87 0 0.40 1.00 1.00 -0.40 0.40
-0.63 -0.40 -0.87 -0.87 -0.87 0.20 0.40 -0.40 -0.40 1.00 0.40
0.32 0.60 -0.87 -0.87 -0.87 0.80 1.00 0.40 0.40 0.40 1.00
1.0
0.5
0
-0.5
-1.0
C
Quantiferon-TB test is not available for healthy control individuals in this study
(HC) IL-12 RB1 dTM ng/ml
(HC)CD4+ IFNgamma
QFN1
QFN2
Mitogen
(HC)CD4+ IL-2
(HC)CD4+ TNF-alpha
(HC)CD4+ TCMOX40+ CD25+
(HC)CD4+ TCMPD-L1+ CD25+
(HC)CD4+ TEMOX40+ CD25+
(HC)CD4+ TEMPD-L1+ CD25+
1.00 0.12 -0.03 0.11 0.08 0.06 -0.28 0.04
0.12 1.00 0.89 0.78 -0.68 0.21 0.37 0.16
-0.03 0.89 1.00 0.85 -0.73 0.10 0.18 -0.03
0.11 0.78 0.85 1.00 -0.43 0.44 0.23 0.19
0.08 -0.68 -0.73 -0.43 1.00 0.28 0.13 0.41
0.06 0.21 0.10 0.44 0.28 1.00 0.40 0.46
-0.28 0.37 0.18 0.23 0.13 0.40 1.00 0.70
0.04 0.16 -0.03 0.19 0.41 0.46 0.70 1.00
1.0
0.5
0
-0.5
-1.0

**Figure 5.8. Correlation matrix between plasma level of ΔTM-IL-12Rβ1 and Mtb300 specific CD4+ T cells responses**. Correlation matrix represents association between plasma level of ΔTM-IL-12Rβ1 and Mtb300 specific CD4+ T cells expressing IFN$\gamma$, TNF-$\alpha$ and IL-2 or memory CD4+ T cells expressing OX40 CD25/PD-L1 CD25+ or blood test (Mitogen, QFN-1 and QFN-2) in (A) LTBI (B) active TB and (C) healthy control. Values represented nonparametric Spearman correlation calculated in GraphPad Prism 9.

## 5.4 Discussion

We performed ELISA to measure the plasma level of ΔTM-IL-12Rβ1 in LTBI, active TB and healthy control individuals and we compared the these between the groups. Then, we measured the plasma level of cytokines among these groups and correlate between the plasma level of ΔTM-IL-12Rβ1 and the plasma level of cytokines. Then, we correlate between the plasma level of ΔTM-IL-12Rβ1 and Mtb300 specific CD4 T cells responses. Our data showed that plasma level of ΔTM-IL-12Rβ1 was higher in LTBI individuals than those in HC or active TB, but not statistically significant. Our data revealed that in LTBI patients the plasma level of ΔTM-IL-12Rβ1 is associated with the plasma level of IFN$\gamma$ and IL-6. However, there was no association between the plasma level of ΔTM-IL-12Rβ1 and TNF-α, IL-2, GM CSF, and IL-12/23 p40. Although there was positive correlation between cytokines and the plasma level of ΔTM-IL-12Rβ1 in active TB patients, this association was not significant.

Proinflammatory cytokines are signalling molecules that are important in host immunity to tuberculosis, and they are produced by T cells and other immune cells. In this study we measured 6 cytokines in plasma from LTBI, active TB and HC individuals. Quantification the plasma level of cytokines showed that individuals with active TB revealed higher plasma level of IFN$\gamma$, TNF-α and IL-2 compared to with LTBI or HC individuals; however not statistically significant. Many studies examined the association of cytokines with active TB or LTBI individuals. For instance, patients with pulmonary TB (PTB) were associated with significantly higher of most cytokines compared to those with LTBI or HC individuals (Kumar et al., 2019). They stated that these cytokines “could clearly distinguish” PTB form LTBI or HC. Similarity, cytokines including IFN$\gamma$, and IL-6 were significantly higher in tuberculosis patients (Vivekanandan et al., 2022). Zambuzi et al (2016) found that the plasma level of IL-6, TNF-α were elevated in active tuberculosis patient.

Because we determined antigen specific CD4 T cells responses, we correlated our finding in chapter 4 to the plasma level of ΔTM-IL-12Rβ1 to find the association between these markers. our data showed that in LTBI patients, there was positive correlation between the plasma level of ΔTM-IL-12Rβ1 and blood test or Mtb300 specific CD4 T cells responses, whereas this

was not statistically significant. However, there was negative correlation between Mtb300 specific CD4 expressing IFN$\gamma$ and the plasma level of ΔTM-IL-12Rβ1. furthermore, although there was strong positive correlation between the plasma level of ΔTM-IL-12Rβ1 and Mtb300 specific CD4 T cells expressing IFN$\gamma$ and IL-2 in active TB subjects r= 0.95 and r=0.63 respectively, this was not significant. Also, there was correlation between Mtb300 specific CD4 $T_{CM}$ expressing OX40+ CD25+ or CD4 $T_{CM}$ expressing PD-L1+ CD25+ or CD4 $T_{EM}$ expressing PD-L1+ CD25+ and the plasma level of ΔTM-IL-12Rβ1, but not statistically significant. Finally, in healthy control there was no association between the plasma level of ΔTM-IL-12Rβ1 and Mtb300 specific CD4+ T cells responses.

We ran principal component analysis (PCA) for all variables we determined in chapters 3, 4 and 5. The loading score from the PCA showed the variables which strongly correlated with PCA1 or PCA2. We have selected those variables and computed another PCA based on the previous loading score. We found that 11 LTBI subjects and 2 healthy controls sat within same range in a cluster to those who are active TB suggesting that the share similarities. There are 5 patients, which identified with high cytokine expression in chapter 4, sat within the group. Our data in chapter 3 and 4 showed that these subjects shared similarities with active TB patients in expressing some intracellular markers such as Mtb300 specific CD4+ T cells IFN$\gamma$+ or IL-2+ and cell surface markers such as lymphocyte that are CD4+ express CD25+ and Mtb300 specific memory CD4+ T cells OX40+ CD25+. The variables we determined previously and the PCA score plot suggest that those subjects who sat in cluster within active TB may be subclinical.

**5.5 Conclusion**

We hypothesized that ΔTM-IL-12Rβ1 and other inflammatory markers could differentiate between well characterized cohort of healthy, latent, and active TB disease subjects. We found that the level of ΔTM-IL-12Rβ1 in the plasma was more variable and contained some high values in LTBI patients but could not use this single measure to discriminate those populations from active TB disease. We also found there was strong association between the level of ΔTM-IL-12Rβ1 and the level of IL-6 and IFN$\gamma$ in the plasma of LTBI subjects, which was not seen in the HC data. We ran PCA analysis and included all the values in chapters 3, 4, and

5 and then selected 14 variables based on loading score followed by another PCA the PCA score plot for the selected variables showed that there are 7 LTBI patients share similarities with 3 active TB patients, this grouping allows us to hypothesize that those LTBI grouping with the active cases may reflect a subset of LTBI who are subclinical – this would need long term follow up.

# Chapter 6: General discussion and conclusion

## 6.1 TB complexity, understanding the spectrum of TB and developing an accurate and sensitive diagnosis?

Tuberculosis (TB), which is caused by infection with *Mycobacterium tuberculosis* via the lung, remains a global threat to human health. O'Garra et al (2013) mentioned that there are factors that affect control of TB including the lack of effective vaccine, the emergence of drug resistance forms of Mtb and the lack of sensitive and rapid diagnosis. Focusing on the diagnosis factor, we aimed first to understand the spectrum of TB from the infection to active TB disease described in O'Garra et al (2013) and Pai et al (2016) to use laboratory test and experiments that may improve the diagnosis in the future. These activities will help first to describe and discriminate the latent TB infected patient (LTBI) from those active TB patients, thereby leading to a sensitive test may give accurate prognosis. Then, this may impact on the decision to know which asymptomatic individual may progress to active disease and which individual will not. As a results, this will answer the question which patients will benefits from TB treatment? We sought to determine how the immune response to Mtb is variable among the study groups and that may discriminate between them. Consequently, we aimed to determine if there is a biological marker that is associated with one group or not. We attempted to consider the immune responses to Mtb and assessed the biological markers including intracellular cytokines and cellular markers within three study groups. To do this, many studies showed the fundamental role of CD4+ T cells and cytokines such as IFN$\gamma$, TNF-$\alpha$ and IL-12 the control of Mtb infection and we showed how these markers behaved by determining the full profile showing T lymphocyte population and sub-population, monocytes, and dendritic cells sub-population. We attempted to show how Mtb300 specific T cells responses reflected by expression of cytokines and other cellular markers including CD25, OX40 and PD-L1 could represent the antigen experience of these cells within the subjects. We used flow cytometry to study specific cells population and sub-population including cells surface and intracellular protein that provide functional immune characteristics, biological effects related to and clinical diagnosis. Using this method, we compared the frequencies of LTBI patients with active TB or HC volunteers and followed the LTBI individuals through their treatment to assess the impact of treatment on the frequencies of specific cell types.

## 6.2 Determination of CD4 T cells responses, monocytes, and dendritic cells sub-population in human PBMCs

The events of cellular immune response to Mtb were described in chapter 1 showing the early events including the role of dendritic cells (DC). We discussed the fact that CD4+ T cells are critical for control of Mtb in that they activate macrophages to kill the bacteria and they also regulate the overall immune responses to Mtb. This balance between activation and regulation is key to the chronic nature of TB and reflects the body's willingness to accommodate Mtb infection in a latent form to avoid over stimulation of the immune response and tissue damage. This balance also leads to a range of T cell phenotypes that are Mtb antigen-specific, but which have differing cell surface and cytokine producing phenotypes. In our screening and characterizing of the CD4 T cell population and subpopulations, we found that the frequencies of CD4 T cells and T cells sub-population vary considerably from individual to individual and this range was large. Significant differences could be observed between patients with active TB and HC individuals and specifically, the frequencies of CD4 T cells CD45 RA+, CD4+RO+ CD4+, CD127- CD25+ and CD3- CD19- CD56+ allowed the discrimination between ATB and LTBI. The frequencies of CD4 T cells expressing CCR7- CD45 RA- was higher in LTBI individuals compared to HC and statistically significant. On the other hand, frequencies of CD19+, CD4 T cells expressing CCR7+ CD45 RA-, CD8+ CD45 RA+, CD8 CD45 RO+, CD8+ CCR7- CD45 RA-, CD8+ CCR7+ CD45 RA- and CD4/CD8 ratio were not statistically significant between the groups. Furthermore, no significant differences were observed in our analysis for the dendritic cells and monocytes sub-population between the groups.

In our analysis for the T cells populations in the LTBI individual who received TB treatment the frequencies of CD4+ CD127- CD25+ was significantly higher after 10-12 weeks from the treatment compared to the baseline. In addition, the frequencies of CD3- CD19- CD56+ was significantly higher after 10-12 weeks from the treatment compared to the similar individual after 6-8 weeks from the treatment. The frequencies of CD19+ was decreases following the treatment; however, this was not statically significant. The frequencies of CD4+ RO+ and frequencies of CD4+ CCR7+ CD45 RA- was significantly lower after 6-8 weeks from the treatment compared to untreated similar patients, but these frequencies increased later after 10-12 weeks from the treatment. No statistically significant was observed in the frequencies of CD8+ sub-populations and frequencies of monocytes sub-populations in LTBI patients during the treatment. Finally, there was significant difference between

the frequency of CD1c+ between the LTBI individuals who followed the treatment for 6-8 weeks and similar individual before the treatment.

### 6.3 Mtb300 specific CD4+ T cells responses

In our analysis for Mtb300 specific CD4+ T cells responses we found that the frequencies of CD4+ T cells expressing cytokines (including CD4+ IFN$\gamma$, CD4+ TNF-a and IL-2) were significantly higher in individuals with LTBI compared to those in HC group. Moreover, the frequencies of these cytokines were significantly higher in active TB compared to those in HC group. However, there was significant difference between the frequencies of Mtb300 specific CD4+ T cells IFN$\gamma$ between active TB and LTBI individuals; whereas the frequencies of CD4+ TNF-a and CD4 IL-2 was higher in active TB compared to LTBI but not statistically significant. In our analysis to assess the association between Mtb300 specific CD4 T cells expressing activation markers and the spectrum of TB disease we found that the frequencies of these markers were associated with active TB patients. Indeed, the frequencies of Mtb300 specific CD4+ $T_{CM}$ OX40+ CD25+, CD4+ $T_{CM}$ PD-L1 + CD25+ and $T_{EM}$ OX40+ CD25+ were significantly higher in active TB compared to those HC or individuals with LTBI. On the other hand, no significant difference was observed between the frequencies of these markers between LTBI and HC.

Furthermore, the frequencies of Mtb300 specific CD4+ T cells expressing cytokines in the LTBI patient who received TB treatment vary from individual to individual. No significant difference was observed between the those who screened following their treatment after 4-6 week or after 10-12 weeks compared to the same baseline patients. However, we noticed that the T cells responses to Mtb300 behaved. For example, when the frequency of CD4+ IFN$\gamma$ decreased for 4-6 weeks and then increased at the end of treatment, the frequency of CD4+ TNF and CD4 IL-2 responded the same for the same individual.

We can conclude that Mtb300 specific CD4+ T cells responses impacted on the frequencies of LTBI patients. This response was associated with active TB patients and these markers can be discriminate them from LTBI or HC volunteers.

### 6.4 The association between the Plasma level of IL-12Rβ1 ΔTM in human and plasma level of cytokines

IL-12 modulates TH1 responses in Mtb and is required for DCs migration in response to Mtb (Khader et al., 2006). ΔTM-IL-12Rβ1 is an alternative RNA splicing of IL-12Rβ1 has been demonstrated with specific function including the enhancement of IL-12p40-dependent DCs migration to the draining lymph node (Robinson et al., 2010; Robinson et al., 2015). We developed an ELISA assay to measure the plasma level of ΔTM-IL-12Rβ1 among the study group and then correlate these readings with the plasma level of cytokines for everyone for the LTBI, active TB and HC. Although the plasma level was higher in LTBI individuals compared to HC or active TB patient, this was not statistically significant however, the plasma level of ΔTM-IL-12Rβ1 in LTBI was significantly associated with plasma level of IFN$\gamma$ and plasma level of IL-6. On the other hand, there was strong positive correlation between the plasma level of ΔTM-IL-12Rβ1 and most of plasma level of cytokines in active TB patient, but this relationship was not statistically significant due to the small sample size. The correlation matrix between the plasma level of ΔTM-IL-12Rβ1 and Mtb300 specific CD4 T cells responses showed that in LTBI individuals there was positive correlation between these variables while there was strong positive correlation between these variables. However, this relationship was not statistically significant. To conclude, the plasma level of ΔTM-IL-12Rβ1 appeared to be associated with the plasma level of IFN$\gamma$ and the plasma level of IL-6.

Because we found in chapter 3 looking at total cells and chapter 4 looking at antigen-driven cells provided multivariate data we investigated whether any correlation occurred among the immunological variables from chapters 3, 4 and 5 that could reflect biology or biomarker identification using multivariate analysis. Because we found the frequency of MTB300 specific CD4 T cells was high in 6 patients in LTBI group, we used principal component analysis (PCA) score plot to examine the relationship between the immune markers we measured and the disease state. HC and ATB subjects could be identified as separate groups however, the LTBI subjects were spread, and there were 7 LTBI subjects, included 5 subjects with higher live single lymphocytes CD4+ IFN$\gamma$, that shared similarities with the ATB subjects suggesting that those subjects may be subclinical. In order to test the hypothesis that these markers are

prognostic for progression to TB in LTBI individuals we are in a position to propose a longer term study following a larger number of LTBI individuals to determine whether our set of parameters allow for secure diagnosis of progression to TB.

**Future work**

To improve our proposal, we must address the limitations we discussed previously in chapters 2 to 5. First, we need to extend our ethics approval and recruit more participants to increase the small size of active TB group. Then, because of the lack of subclinical group, migrants with negative IGRA and the community acquired pneumonia (CAP) in our cohort, we have to include these groups in a future study to test our proposal and define if there is any cellular markers that may differ in association with the clinical group we investigated in this study. Next limitation of this study is the small number of patients who received treatment. We managed to recruit only 6 LTBI patients who completed their treatment, and it would be important to expand this and recruit ATB patients during their therapy.

Technically, because the separation of positive population was not very clear in CCR7/CD45 RA as expected, we can improve this staining method following the instructions and existing published work as we showed in chapter 2. Also, because we had determined the frequency of cytokines producing CD4+ T cells induced by Mtb300, it is worth investigating these in CD8+ T cells. We would suggest investigating the cytokines producing CD4+ memory T cells and cytokine producing CD8+ memory T cells in our cohort. As our data showed Tregs are a promising variable that differentiate between the groups in this study, we would suggest a further characterization of Tregs by determining CD4+ CD25+ CD127- FoxP3+ induced by Mtb300. This deep characterization will expand our understanding of Mtb-specific responses. Finally, we managed to measure the plasma level of the novel biomarker ΔTM-IL-12Rβ1 in human. Although there were no differences between the groups, both plasma level of IL-6 and IFNγ correlated with ΔTM-IL-12Rβ1 in LTBI subjects. Because of these findings we would increase the sample size and apply this measurement on those who complete their treatment.

To sum up the future of this study we must 1) increase the sample size, 2) resolve some technical issues, 3) expand our investigation and determine a combination of the

phenotypical distribution profile of CD4+ T cells and CD8 T cells that could be used discriminate the active group from LTBI or healthy subjects and 4) measure and compare the plasma level of ΔTM-IL-12Rβ1 in subjects who complete their treatment.

## Appendices

### 1. Antibodies used in lymphoid panel for human PBMC

| Cellular marker | Fluorochrome | Clone | Cat number |
|---|---|---|---|
| CD127 | PerCP 5.5 | HIL-7R-M21 | 560551<br>BD |
| CD56 | PE | CMSSB | 12056742<br>eBioscience |
| CD8a | FITC | RPA-T8 | 561947<br>BD |
| CD4 | APC-CY7 | RPA-T4 | 300518<br>Biolegend |
| CD3 | AF700 | UCHT1 | 56003842<br>Thermo |
| CD45RA | APC | HI100 | 17045842<br>Thermo |
| CD19 | BV786 | HIB19 | 740968<br>BD |
| CD25 | BV650 | BC96 | 302633<br>BD |
| CD45RO | BV605 | UCHL1 | 562791<br>BD |
| CD197 | BV421 | G043H7 | 353208<br>Biolegend |

## 2. Antibodies used in monocytes/dendritic cells panel for human PBMC

| Cellular marker | Fluorochrome | Clone | Cat number |
|---|---|---|---|
| CD14 | PerCP 5.5 | M5E2 | 561116<br>BD |
| CD123 | PE-CF594 | 7G3 | 562391<br>BD |
| CD11c | bb515 | B-ly6 | 564491<br>BD |
| HLA-DR | APC-H7 | G46-6 | 561358<br>BD |
| CD141 | APC | 1A4 | 564123 |
| CD16 | BV650 | 3G8 | 563692<br>BD |
| CD1c | BV605 | L161 | 331538<br>Biolegend |
| CD3<br>CD19<br>CD56 | BV510 | UCHT1<br>HIB19<br>B159 | 563109 BD<br>302242 Biolegend<br>740171 BD |

### 3. Antibodies used in Mtb300 specific CD4+ T cells cytokines

| Cellular markers | Fluorochrome | Clone | Cat number |
|---|---|---|---|
| CD3 | PE-CF594 | UCHIT1 | 562310<br>BD |
| CD4 | APC-eFluor780 | RPA-T4 | 47-0049-41<br>eBioscience |
| CD8 | V500 | RPA-T8 | 560775<br>BD |
| IFN$\gamma$ | V450 | B27 | 560372<br>BD |
| TNF | BV650 | MAB11 | 563418<br>BD |
| IL-2 | PE | MQ1-17H12 | 560709<br>BD |
| CD27 | APC | M-R271 | 561786<br>BD |
| HLA-DR | FITC | L243 | 307603<br>Biolegend |

## 4. Antibodies used in MTB300 specific CD4+ memory T cells expressing AIM

| Cellular markers | Fluorochrome | Clone | Cat number |
|---|---|---|---|
| CD4 | APCef780 | RPA-T4 | 47-0049-41<br>eBioscience |
| CD3 | Alexa Fluor 700 | UCHT-1 | 56-0038-42<br>eBioscience |
| CD8 | V500 | RPA-8 | 560775<br>BD |
| CD19 | V500 | H1B19 | 561125<br>BD |
| CD14 | V500 | M5E2 | 561392<br>BD |
| CD25 | FITC | M-A251 | 560990<br>BD |
| CD45RA | ef450 | HI100 | 48-0458-41<br>eBioscience |
| CCR7 | PerCPCy5.5 | 6043H7 | 353241<br>Biolegend |
| OX40 | APC | BER-ACT35 | 350007<br>Biolegend |
| PD-L1 | PE | 29E.2A3 | 329705<br>Biolegend |

**Publication**

# Ethnic differences in cellular and humoral immune responses to SARS-CoV-2 vaccination in UK healthcare workers: a cross-sectional analysis

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## Summary

**Background** Few studies have compared SARS-CoV-2 vaccine immunogenicity by ethnic group. We sought to establish whether cellular and humoral immune responses to SARS-CoV-2 vaccination differ according to ethnicity in UK Healthcare workers (HCWs).

**Methods** In this cross-sectional analysis, we used baseline data from two immunological cohort studies conducted in HCWs in Leicester, UK. Blood samples were collected between March 3, and September 16, 2021. We excluded HCW who had not received two doses of SARS-CoV-2 vaccine at the time of sampling and those who had serological evidence of previous SARS-CoV-2 infection. Outcome measures were SARS-CoV-2 spike-specific total antibody titre, neutralising antibody titre and ELISpot count. We compared our outcome measures by ethnic group using univariable (*t* tests and rank-sum tests depending on distribution) and multivariable (linear regression for antibody titres and negative binomial regression for ELISpot counts) tests. Multivariable analyses were adjusted for age, sex, vaccine type, length of interval between vaccine doses and time between vaccine administration and sample collection and expressed as adjusted geometric mean ratios (aGMRs) or adjusted incidence rate ratios (aIRRs). To assess differences in the early immune response to vaccination we also conducted analyses in a subcohort who provided samples between 14 and 50 days after their second dose of vaccine.

**Findings** The total number of HCWs in each analysis were 401 for anti-spike antibody titres, 345 for neutralising antibody titres and 191 for ELISpot. Overall, 25.4% (19.7% South Asian and 5.7% Black/Mixed/Other) were from ethnic minority groups. In analyses including the whole cohort, neutralising antibody titres were higher in South Asian HCWs than White HCWs (aGMR 1.47, 95% CI [1.06–2.06], P = 0.02) as were T cell responses to SARS-CoV-2 S1 peptides (aIRR 1.75, 95% CI [1.05–2.89], P = 0.03). In a subcohort sampled between 14 and 50 days after second vaccine dose, SARS-CoV-2 spike-specific antibody and neutralising antibody geometric mean

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titre (GMT) was higher in South Asian HCWs compared to White HCWs (9616 binding antibody units (BAU)/ml, 95% CI [7178–12,852] vs 5888 BAU/ml [5023–6902], P = 0.008 and 2851 95% CI [1811–4487] vs 1199 [984–1462], P < 0.001 respectively), increments which persisted after adjustment (aGMR 1.26, 95% CI [1.01–1.58], P = 0.04 and aGMR 2.01, 95% CI [1.34–3.01], P = 0.001). SARS-CoV-2 ELISpot responses to S1 and whole spike peptides (S1 + S2 response) were higher in HCWs from South Asian ethnic groups than those from White groups (S1: aIRR 2.33, 95% CI [1.09–4.94], P = 0.03; spike: aIRR, 2.04, 95% CI [1.02–4.08]).

**Interpretation** This study provides evidence that, in an infection naïve cohort, humoral and cellular immune responses to SARS-CoV-2 vaccination are stronger in South Asian HCWs than White HCWs. These differences are most clearly seen in the early period following vaccination. Further research is required to understand the underlying mechanisms, whether differences persist with further exposure to vaccine or virus, and the potential impact on vaccine effectiveness.

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**Keywords:** SARS-CoV-2; COVID-19; Vaccine; Ethnicity; Heathcare worker

### Research in context

#### Evidence before this study

We searched PubMed on 4th January 2023 using the following search terms ((((ethnicity) OR (race)) AND ((immune response) OR (antibody))) AND (Vaccine)) AND ((COVID-19) OR (SARS-CoV-2)). The search returned 87 articles. 76 were excluded after abstract screening either because they did not use quantitative immune responses as the outcome, did not assess immune responses to vaccination or they did not stratify results by ethnicity. Of the remaining 11 articles, 6 studies were conducted in populations defined by a particular comorbidity or treatment. 4 of these 6 studies found higher anti-spike antibody titres after vaccination in ethnic minority participants compared to White with 2 studies finding no differences by ethnicity. 4 of the 5 remaining studies were conducted in healthcare worker (HCW) population: 2 Israeli studies demonstrated no difference in antibody titres after vaccination by ethnicity; a small US study found higher antibody responses in 'non-Caucasian' than Caucasian HCW; a UK study found that Black HCWs had lower antibody responses to vaccination than White and found no difference between White and Asian groups. Finally, an observational study in the UK general population found that post-vaccination antibody titres were 16.2% higher in South Asian than White groups. No studies presented data on antibody titres, serum neutralising activity and cellular immune responses.

#### Added value of this study

The evidence base on SARS-CoV-2 vaccine responses by ethnicity is both limited and conflicting. Our study adds considerably to the literature by providing evidence that humoral and cellular immune responses to SARS-CoV-2 vaccine are higher in HCWs from South Asian groups, as compared to those from White groups, particularly in the early phase after vaccine administration. This is the first study to provide data on anti-SARS-CoV-2 antibodies, SARS-CoV-2 neutralising antibodies and cellular responses to SARS-CoV-2 peptides stratified by ethnic group.

#### Implications of all the available evidence

There appear to be differences in the immune response to SARS-CoV-2 vaccination according to ethnicity. The majority of studies that compare Asian or South Asian groups to White have found that vaccine responses are higher in the Asian group. The current work demonstrates that these differences extend to neutralising antibody and T cell responses and that such differences are more apparent in the early stages after vaccination.

## Introduction

The COVID-19 pandemic has led to significant morbidity and mortality globally.[1] Several demographic and occupational risk factors for infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) have been identified as the pandemic has progressed.[2–4]

Studies from the US and the UK have shown that those from minoritised ethnic groups are at higher risk of infection than those of White ethnicity.[5,6] However,

the underlying reasons for this increased risk of infection have not been clearly delineated. Given that ethnicity itself is a complex construct that relates to many facets of one's life (including "language, diet, religion, ancestry and physical features traditionally associated with race"[7]), mechanisms underlying this association are likely to be multi-faceted.[8] Sociodemographic drivers of this increased infection risk have been hypothesised to include: a greater likelihood of working in 'frontline' or 'key-worker' positions that increase exposure to SARS-CoV-2 (particularly whilst 'lockdown' measures were being employed early in the pandemic),[9,10] increased likelihood of living in deprived areas with higher population density making social distancing more difficult; living in smaller more poorly ventilated houses than those living in more affluent areas,[11,12] and a greater propensity for multi-generational living and therefore a greater number of household contacts.[6,11] As well as increased infection risk, there is some evidence to suggest that those from ethnic minority groups face a higher risk of severe COVID-19 (including hospitalisation, intensive care unit admission and death) than White groups.[6,13]

The development of safe and effective vaccines against SARS-CoV-2 has been a significant milestone in the response to the COVID-19 pandemic. Mass vaccination programmes provided a mechanism to reduce the risk of infection and severe disease on a population level.[14,15]

However, despite the observed differences in infection risk and COVID-19 outcome between ethnic groups, evidence concerning differences to vaccine immunogenicity by ethnic group is limited and conflicting. Studies conducted in populations defined by particular diseases or treatment (such as autoimmune conditions treated with immunosuppressive agents, myeloma, lung cancer and dialysis) have provided mixed results relating to the impact of ethnicity on immunogenicity of SARS-CoV-2 vaccine.[16–21] A recent study reporting findings from a large cohort of UK healthcare workers (HCWs) found higher SARS-CoV-2 anti-spike titres in ethnic minority vaccinees than in their White counterparts,[22] which contrasts with another UK HCW study which found lower peak anti-spike antibody titres in Black HCWs compared to White HCWs and did not find differences between the White and Asian groups.[23] A large UK observational study in the general population found South Asians vaccinees to have higher combined IgG, A and M titres against SARS-CoV-2 than those from White groups.[24] Crucially, there are no studies that present data relating to both cellular immune responses and SARS-CoV-2 neutralising activity after vaccination stratified by ethnicity.

Ethnicity has been shown to be a determinant of the immunogenicity of other vaccines, with previous studies showing higher titres of antibody against pertussis toxin in Black children than White children after vaccination,[25] higher post-vaccine measles antibody levels in Innu and Inuit children compared to White children,[26] and higher titres of rubella-specific neutralising antibodies in vaccinees from African ethnic groups compared to those from European ethnic groups.[27]

The aim of this study was to determine whether the humoral and cellular immune profiles of UK HCWs vaccinated against SARS-CoV-2 differ according to ethnicity using baseline data from two cohort studies.

## Methods

### Overview

This cross-sectional study utilises data and samples collected as part of two HCW cohort studies conducted in Leicester, UK. These are:

1) DIRECT (Determining the Immune Response in Ethnic minority healthcare workers to COVID-19 infecTion), which was established with the overarching aim of determining if immune responses to COVID-19 infection and vaccination differ according to ethnicity
2) BELIEVE (Broadening our understanding of Early versus Late InfluEnza Vaccine Effectiveness), which aims to understand whether there is significant waning of influenza vaccination effectiveness during an influenza season (banked serum samples from this study were sent for SARS-CoV-2 serology and neutralising assays. See below).

### Study population and recruitment

Both studies recruited HCWs (including ancillary workers) aged 16 or over who were employed either by University Hospitals of Leicester NHS Trust (UHL), one of the largest acute hospital trusts in the UK, or by Leicestershire partnership NHS Trust (LPT). HCWs could participate regardless of previous SARS-CoV-2 infection or vaccination status.

The studies were advertised in hospital-wide email communications and on the staff intranet. This was supplemented by direct recruitment from clinical and non-clinical areas of the hospital. Sample size calculations were not performed for this exploratory study.

### Study visits

After providing written, informed consent, participants provided information on occupational and demographic characteristics.

DIRECT participants provided blood samples (for SARS-CoV-2 serology, neutralisation activity, and enzyme-linked immunosorbent spot [ELISpot] assays) at a time of their convenience, the only restriction being that the blood sample should not be collected within two weeks of receipt of a SARS-CoV-2 vaccine. Baseline blood samples were collected between 3rd March and 16th September 2021.

BELIEVE participants provided blood samples (for SARS-CoV-2 serology and neutralisation activity) on four occasions which related to timing of influenza vaccination and the peak and end of the influenza season. The samples analysed for this study come from the fourth study visit, between 4th May and 1st June 2021 (this visit was selected in order to align with the period of sample collection for DIRECT).

### Demographic and clinical data

We collected information on self-reported ethnicity (participants could select an ethnic group corresponding to the 18 Office for National Statistics [ONS] ethnic groups[28]), age, sex, type of SARS-CoV-2 vaccine received (BNT162b2 [Pfizer-BioNTech] or ChAdOx1-S [Oxford-AstraZeneca], hereafter referred to as BNT162b2 and ChAdOx1 respectively), number of SARS-CoV-2 vaccine doses received and the dates of receipt. We also collected data on the presence or absence of long-term conditions or medications associated with immunosuppression and body mass index (BMI) for use in sensitivity analyses (see below and Supplementary text 1 for details).

### Laboratory methods

#### *SARS-CoV-2 serology assays*

Anti-spike and anti-nucleocapsid SARS-CoV-2 serology were performed at UKHSA Porton Down on serum samples using the Roche Elecsys anti-SARS-CoV-2 S (Product code: 09203079190) and Roche Elecsys anti-SARS-CoV-2 (Product code: 09289275190) assays, respectively. Samples were considered positive for anti-spike antibodies if $\geq$ 0.8 BAU/ml, and positive for anti-nucleocapsid antibodies if $\geq$ 1 COI.

#### *SARS-CoV-2 neutralising antibody assay*

Plasmid constructs and 293-ACE2 cells were as described previously.[29,30] Sera were screened for neutralising activity against HIV(SARS-CoV-2) pseudotypes bearing the spike glycoprotein of Wuhan D614G.[29,30] Neutralising activity in each sample was measured by a serial dilution approach. Each sample was serially diluted in triplicate from 1:50 to 1:36,450 in complete Dulbecco's Modified Eagle Medium (DMEM) prior to incubation with approximately $1 \times 10^6$ CPS per well of HIV (SARS-CoV-2) pseudotypes, incubated for 1 h, and plated onto 239-ACE2 target cells. After 48–72 h, luciferase activity was quantified by the addition of Steadylite Plus chemiluminescence substrate and analysis on a PerkinElmer EnSight multimode plate reader (PerkinElmer, Beaconsfield, UK). Antibody titre was then estimated by interpolating the point at which infectivity had been reduced to 50% of the value for the no serum control samples.

#### *SARS-CoV-2 ELISpot assay*

To quantify T cell responses, we used T-SPOT® Discovery SARS-CoV-2 platform (Oxford Immunotec), which use ELISpot technology to detect IFN-γ release from immune cells after exposure to SARS-CoV-2 peptides. This test is similar in methodology to the T-SPOT®.TB test which identifies patients infected with *M. tuberculosis*, and has been widely used clinically.

A peripheral venous blood sample of 6 mL was collected from participants and placed in a test tube of heparin anticoagulant. Peripheral blood mononuclear cells were isolated within 32 h of test performance. The T-SPOT *Discovery* SARS-CoV-2 test was performed according to the instructions of the kit. In brief, the peripheral blood mononuclear cells were counted, normalised and 250,000 PBMCs were plated into each well of a T-SPOT® Discovery SARS-CoV-2 plate. Four different but overlapping peptides pools to cover protein sequences of SARS-CoV-2—Spike 1 (S1), Spike 2 (S2), Nucleocapsid and membrane plus negative and positive controls were used (for further details see Supplementary Table S1). Cells were incubated overnight (16–20 h) at 37 °C with 5% $CO^2$, washed with phosphate-buffered saline, and developed using an anti-IFN-γ antibody conjugate and substrate to detect the presence of secreted IFN-γ. Spot-forming cells (SFCs) were counted with an automated spot reader (Cellular Technology Ltd). As our analysis focussed on immune responses to vaccination, we present responses to the spike peptides S1, S2 and spike (S1 + S2).

### Statistical analysis

This analysis focuses on the immune response to vaccination. In order to ensure homogeneity with regard to previous exposure to SARS-CoV-2 we excluded those with a history of SARS-CoV-2 infection (determined by a positive SARS-CoV-2 anti-nucleocapsid antibody assay) and those who had not received the first two doses of SARS-CoV-2 vaccine at the point of sampling. To ensure that sufficient time had elapsed for an immune response to vaccination to develop and that we were not examining data from samples collected during the induction phase of the antibody response, we also excluded those whose blood samples were collected within 14 days of the second dose of vaccine.

For each immune parameter measured, we examined a subcohort who were sampled within 50 days of second vaccine dose. This time period was chosen to include only those close to their peak SARS-CoV-2 anti-spike antibody titre.[31]

We summarised categorical variables as frequency and percentage and non-normally distributed continuous variables and median and interquartile range (IQR). Continuous variables were assessed for normality of distribution by visual inspection.

Comparisons of immune responses between White and South Asian groups were possible as a large proportion of the UHL workforce are from South Asian ethnic groups. In order to maintain statistical power to detect differences by ethnicity whilst preventing

exclusion of particular individuals of minority ethnicity from the analysis, we created a three-level variable collapsing three of the five broad ONS ethnic groups into one (Black/Mixed/Other). Note that this group also includes the low number of those from Chinese ethnic groups.

Antibody titres and neutralising titres were $\log_{10}$ transformed prior to analysis. Raw ELISpot counts were transformed first by subtracting the count from an unstimulated control sample and then multiplied by four to give a value in spot forming units (SFUs) per million peripheral blood mononuclear cells (PBMCs).

For unadjusted comparisons of immune parameters between ethnic groups, we used *t* tests to compare $\log_{10}$ antibody levels and SARS-CoV-2 neutralising titres and Wilcoxon rank-sum test to compare ELISpot results using the White group as the reference. We also presented unadjusted analysis with the cohort stratified by the vaccine they had received (BNT162b2 or ChAdOx1). Geometric mean titres (GMTs) for total anti-spike antibodies and 50% neutralisation are also presented.

We used linear regression to determine the effects of ethnicity on SARS-CoV-2 antibody levels after adjustment for age, sex, vaccine type, time between receipt of second vaccine and collection of sample and time between the first and second doses of vaccine. Regression coefficients were exponentiated for expression as adjusted geometric mean ratios (aGMRs).

After examination of the mean and variance of the ELISpot results, we used negative binomial regression to investigate the impact of ethnicity on an outcome of ELISpot count after adjustment for the same variables used in the linear models. Results were expressed as adjusted incidence rate ratios (aIRRs).

Only those who had serum SARS-CoV-2 neutralising activity greater or equal to 90% at 1:50 dilution underwent further assays to determine the 50% neutralisation titre. Those who did not meet this threshold were excluded from the main analysis of serum neutralising activity. To investigate the impact this had on results we conducted two sensitivity analyses: 1) a comparison of demographic and vaccine related parameters in those excluded and included; 2) an analysis including those not meeting the threshold recoded as a titre of 50.

We conducted further sensitivity analyses to investigate the effect of the 50 day sampling threshold on results. We changed the threshold by −10 and +10 days and repeated the adjusted analyses.

To determine if differences in health factors known to affect vaccine response (long-term conditions and body mass index [BMI]) by ethnicity might have influenced our results, we repeated our multivariable analyses after adjustment for BMI and after exclusion of a small group of those with long-term conditions associated with immunosuppression or those taking immunosuppressive medication. As BMI was not collected in the BELIEVE study we used multiple imputation by chained equations to impute missing BMI data (for further details see Supplementary text 1).

Finally, we repeated our multivariable analyses of SARS-CoV-2 serology and neutralising activity after adjustment for a binary variable indicating which study (DIRECT or BELIEVE) a participant was enrolled in (for those that were enrolled in both studies we used the data collected as part of DIRECT and thus these participants were coded as such in this analysis).

All analyses were conducted using Stata 17 (StataCorp. 2021. Stata Statistical Software: Release 17. College Station, TX: StataCorp LLC.). Figures were created in GraphPad Prism version 9.4.1 for macOS (GraphPad Software, San Diego, California USA, www.graphpad.com). We considered P values < 0.05 to be statistically significant.

### Ethical approval

DIRECT was approved by the Health Research Authority (Brighton and Sussex Research Ethics Committee; ethics reference: 20/HRA/4718). BELIEVE was approved by the Wales National Research Ethics Service, UK (REC number 20/WA/0247). All participants gave informed consent.

### Role of the funding source

The funders had no role in study design, data collection, data analysis, interpretation, or writing of the report. All authors have had the opportunity to access the underlying data used in this study. All authors reviewed the manuscript and approved the final version prior to submission.

## Results

### Formation of the analysed sample

Fig. 1 shows the formation of the analysed cohort and the numbers of individuals included in each analysis.

### Description of the cohort

Table 1 summarises the demographic and vaccine related information gathered for the uninfected and double vaccinated participants included in the analyses. Overall, 401 participants were included in the serology analyses with 102 (25.4%) being from ethnic minority groups (19.7% South Asian and 5.7% Black/Mixed/Other). Median (IQR) age was 45 (33–54) and 78.8% were female. The majority (n = 314, 78.8%) received BNT162b2 vaccine.

In comparison, a greater proportion of the 191 participants included in the ELISpot analyses (n = 73, 38.2%) were from ethnic minority groups (29.3% South Asian and 8.9% Black/Mixed/Other). A similar proportion received BNT162b2 (n = 152, 79.6%).

Supplementary Table S2 contains a detailed cohort description of demographic, health and vaccine related parameters by ethnicity. There was a higher proportion of males in the South Asian (34.2%) and Black/Mixed/

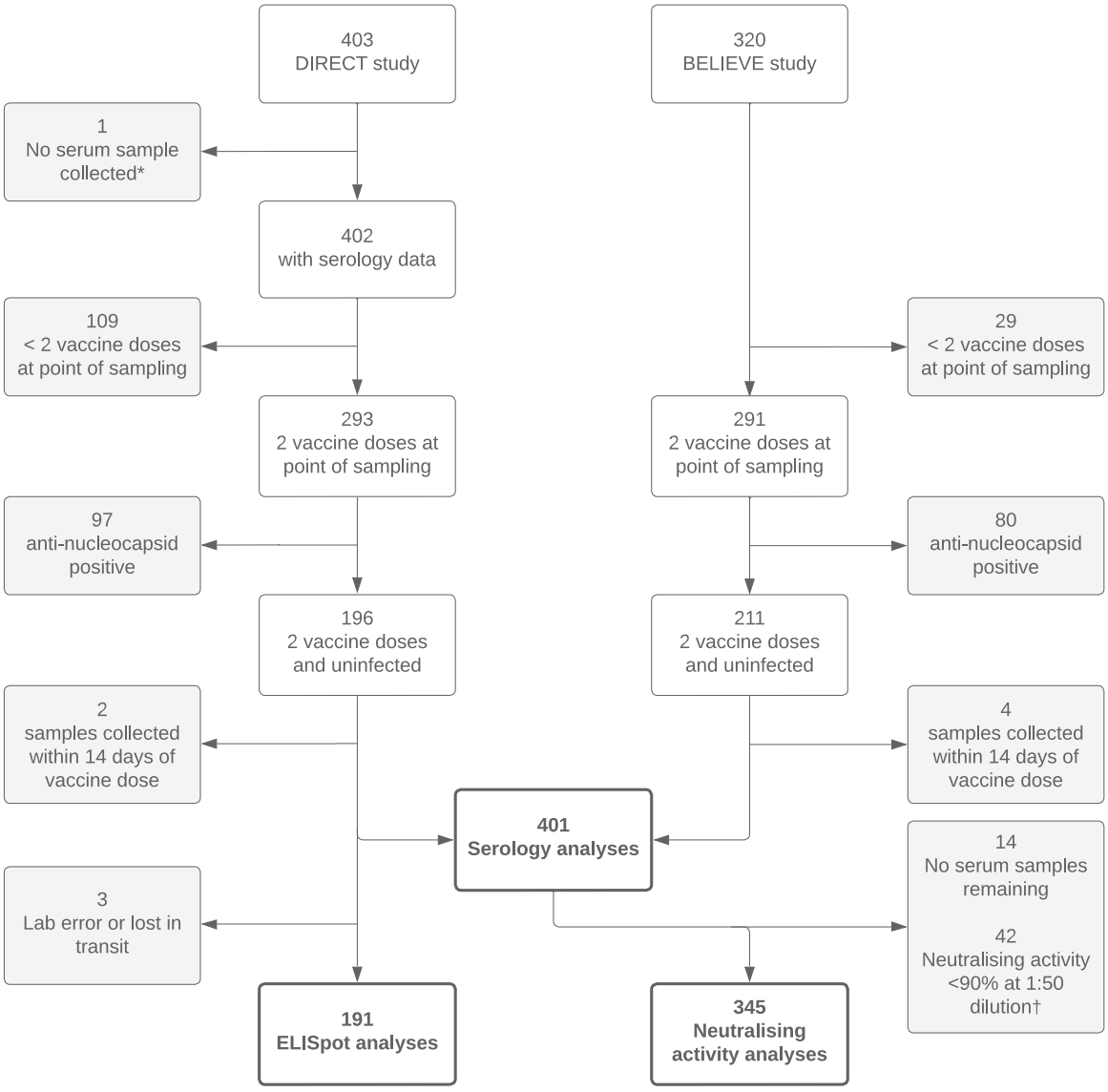



***Fig. 1:* Formation of the analysed cohort**. Fig. 1 shows how the final number of observations in each analysis were derived. The number of observations included in the analyses conducted in the subcohort whose sample was collected within 50 days of a second vaccine dose are detailed in the relevant figures/tables. There are 40 participants who were enrolled into both studies. For clarity, these participants are included in the figures for DIRECT. *Excluded from all analyses as no data on anti-nucleocapsid antibody status. † included in sensitivity analysis of neutralising activity data.

Other cohort (30.4%) compared to the White cohort (17.1%). A greater proportion of those in the South Asian and Black/Mixed/Other cohorts had an interval of <6 weeks between vaccine doses (84.8% and 78.3% vs 94.3% respectively). Prevalence of long term conditions that might impact upon vaccine immunogenicity and distribution across BMI categories did not differ by ethnicity.

### SARS-CoV-2 serology

Scatter plots showing anti-spike antibody titres over time between second vaccine dose and sample collection stratified by ethnic group are shown in Supplementary Figure S1 and dot plots showing anti-spike antibody titres by ethnic group and by vaccine type in the whole cohort and in those sampled within 50 days of second vaccine dose are shown in Fig. 2.

Anti-spike titres decreased with increasing time since second vaccine in all ethnic groups. There were no significant differences in anti-spike titre by ethnicity when considering the whole cohort. However, in the subcohort sampled between 14 and 50 days of second vaccine administration, GMT was higher in South Asian HCWs

| | Serology analyses (n = 401) | ELISpot analyses (n = 191) |
|---|---|---|
| **Ethnicity** | | |
| White | 299 (74.6) | 118 (61.8) |
| South Asian | 79 (19.7) | 56 (29.3) |
| Black/Mixed/Other | 23 (5.7) | 17 (8.9) |
| **Age** | | |
| (years), med (IQR) | 45 (33–54) | 46 (33–55) |
| **Sex** | | |
| Male | 85 (21.2) | 52 (27.2) |
| Female | 316 (78.8) | 139 (72.8) |
| **Healthcare role** | | |
| Non-patient facing | 92 (22.9) | 50 (26.2) |
| Patient facing | 297 (74.1) | 130 (68.1) |
| Missing | 12 (3) | 11 (5.8) |
| **Vaccine** | | |
| BNT162b2 | 314 (78.3) | 152 (79.6) |
| ChAdOx1 | 87 (21.7) | 39 (20.4) |
| **Time between first and second vaccine doses** | | |
| ≤6 weeks | 34 (8.5) | 27 (14.1) |
| >6 weeks | 364 (91.5) | 164 (85.9) |
| Median (IQR) | 77 (70–77) | 74 (68–77) |
| **Time between second vaccine dose and sampling** | | |
| ≥14 days and ≤50 days | 248 (61.9) | 81 (42.4) |
| >50 days | 153 (38.2) | 110 (57.6) |
| Median (IQR) | 43 (31–76) | 65 (31–118) |

Table 1 shows the participants included in the serology (from the DIRECT and BELIEVE studies) and ELISpot (from the DIRECT study only) analyses. Analyses of neutralising activity contain those in the serology cohort less 14 (excluded as there was no serum remaining after the serology assay and a further 42 who were excluded on the basis of neutralising activity <90% at 1:50 dilution (see Fig. 1 and Supplementary Table S3 for details)). All data are n (%) unless otherwise stated. Percentages are computed column-wise. Med–median; IQR–interquartile range. For a detailed description of the cohort by ethnicity, see Supplementary Table S2.

***Table 1:*** **Description of the cohort.**

compared to White HCWs (9616 BAU/ml, 95% CI [7178–12,852] vs 5888 BAU/ml [5023–6902], P = 0.008) and when analysis was further restricted to those receiving BNT162b2 vaccine, GMT was higher in South Asian and Black/Mixed/Other groups compared to White (South Asian: 12,134BAU/ml [9397–15,631], Black/Mixed/Other: 15,524 BAU/ml [9333–25,942] vs White: 9484 BAU/ml [8590–10,471], P = 0.038 and P = 0.027 respectively) (Fig. 2).

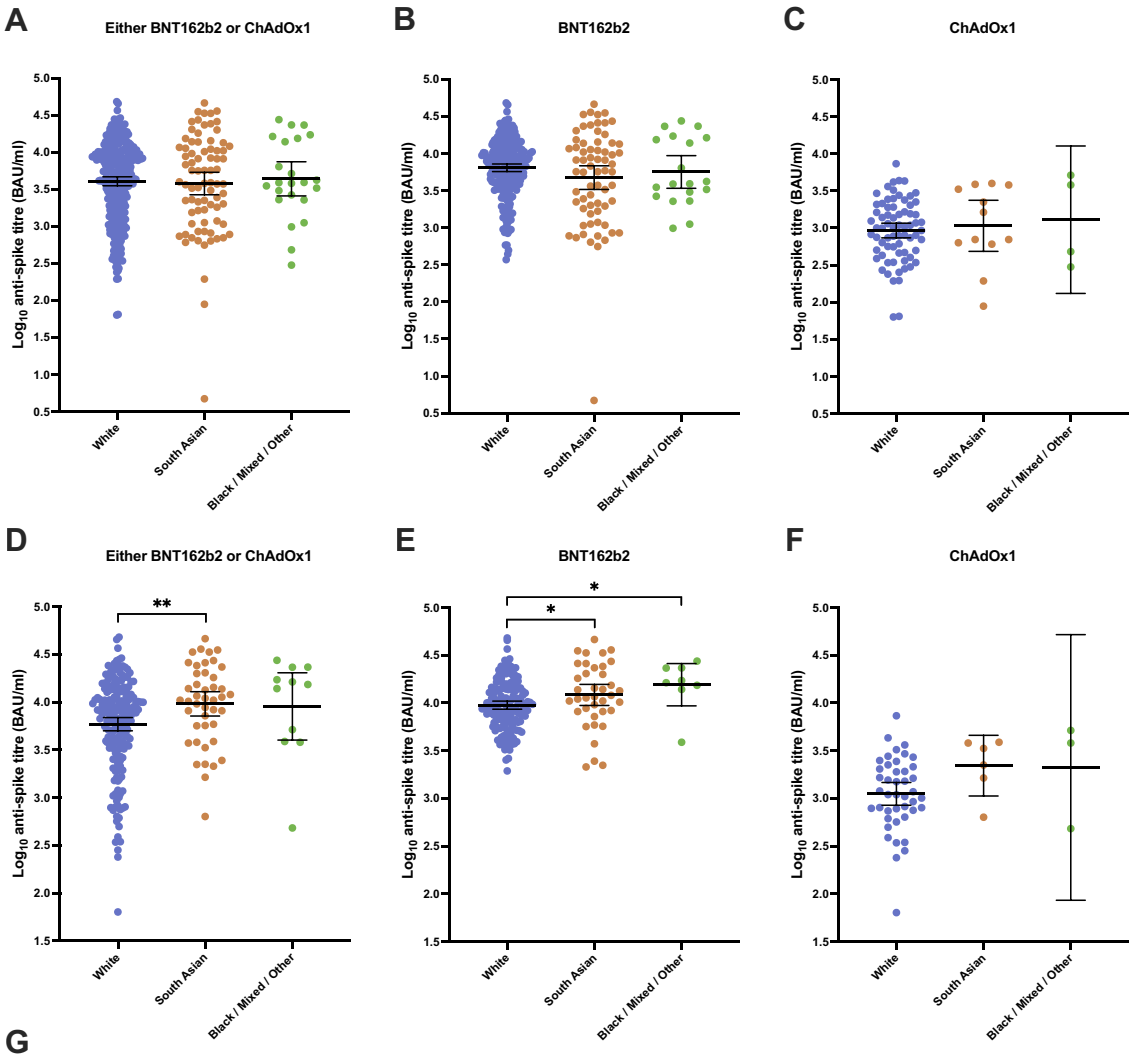
Table 2 shows results from the multivariable linear regression analysis showing the association between ethnicity and anti-spike titre both in the whole cohort and in those sampled within 50 days of second vaccine dose. When the whole cohort are included, there were no significant differences by ethnic group. However, in the subcohort sampled within 50 days of second vaccine administration, anti-spike titres in the South Asian cohort were higher than in the White cohort (aGMR 1.26, 95% CI [1.01–1.58], P = 0.04).

Age was found to be negatively associated with anti-spike titre (aGMR 0.86, 95% CI [0.81–0.93], P < 0.001, per decade increase) as was increasing time between second vaccination and sample collection (aGMR 0.91, 95% CI [0.90–0.92], P < 0.001, per week increase). Anti-spike titre was far lower in those receiving ChAdOx1 compared to those receiving BNT162b2 (aGMR 0.15, 95% CI [0.12–0.18], P < 0.001).

### SARS-CoV-2 neutralising activity

As with the serology assays, neutralising activity decreased with time between second vaccine administration and sample collection in each ethnic group (Supplementary Figure S2). In the unadjusted analysis, no significant differences were seen between mean neutralising titres by ethnicity when the whole cohort were included. In the subcohort sampled between 14 and 50 days of vaccine administration, GMT (for 50% neutralisation) was higher in the South Asian group compared to the White group (2851 [1811–4487] vs 1199 [984–1462], P < 0.001). These differences persisted when the analysis was further restricted to those who had received the BNT162b2 vaccine (South Asian: 3515 [2269–5458] vs White: 1674 [1396–2013], P < 0.001) (Fig. 3).

On multivariable linear regression (Table 2), those from South Asian ethnic groups had higher serum SARS-CoV-2 neutralising activity than those from White ethnic groups (aGMR 1.47, 95% CI [1.06–2.06], P = 0.02). This association was more marked when the

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| Panel | White GMT (95% CI) | South Asian GMT (95% CI) | Black / Mixed / Other GMT (95% CI) |
|---|---|---|---|
| A | 4,055 (3,524 – 4,667), n=299 | 3,784 (2,666 – 5,357), n=79 | 4,365 (2,570 – 7,430), n=23 |
| B | 6,427 (5,701 – 7,228), n=228 | 4,742 (3,281 – 6,839), n=67 | 5,649 (3,396 – 9,375), n=19 |
| C | 924 (737 – 1,161), n=71 | 1,074 (486 – 2,366), n=12 | 1,297 (132 – 12,589), n=4 |
| D | 5,888 (5,023 – 6,902), n=193 | 9,616 (7,178 – 12,852), n=44 | 9,016 (3,999 – 20,323), n=11 |
| E | 9,484 (8,590 – 10,471), n=150 | 12,134 (9,397 – 15,631), n=38 | 15,524 (9,333 – 25,942), n=8 |
| F | 1,114 (847 – 1,465), n=43 | 2,203 (1,059 – 4,581), n=6 | 2113 (85.7 – 52,119), n=3 |

***Fig. 2:* Comparison of total SARS-CoV-2 spike-specific antibody titres by ethnic group and vaccine type**. Fig. 2 shows a comparison of log10 total SARS-CoV-2 anti-spike antibody titres (BAU/ml), stratified by by ethnic group and vaccine type. Panels A, B and C include all participants. Panels D, E and F include only those sampled within 50 days of second vaccine dose. Panel G shows geometric mean titres (GMTs) and their 95% confidence intervals with the number of participants in each ethnic group for panels A–F. Groups were compared (with the White group as reference) using unpaired t tests. *P < 0.05, **P < 0.01.

analysis was restricted to those sampled within 50 days of second vaccine dose (aGMR 2.01, 95% CI [1.34–3.01], P = 0.001).

As with the serology analysis, those who received ChAdOx1 had lower serum SARS-CoV-2 neutralising activity than those who received BNT162b2 (aGMR 0.18 95% CI [0.13–0.25], P < 0.001). Increasing time between second vaccination and sample collection was associated with lower neutralising activity (aGMR 0.92, 95% CI [0.90–0.94], P < 0.001, per week increase) and increasing

| | Outcome—$Log_{10}$ SARS-CoV-2 anti-spike antibody titre (BAU/ml) | | | |
|---|---|---|---|---|
| | In whole cohort (n = 401) | | In those sampled within 50 days of second vaccine dose (n = 248) | |
| | aGMR (95% CI) | P value | aGMR (95% CI) | P value |
| **Ethnicity** | | | | |
| White | Ref | – | Ref | – |
| South Asian | 0.94 (0.77–1.15) | 0.55 | 1.26 (1.01–1.58) | 0.04 |
| Black/Mixed/Other | 1.21 (0.86–1.69) | 0.27 | 1.51 (1.00–2.29) | 0.05 |
| **Age** | | | | |
| per decade increase | 0.86 (0.81–0.93) | <0.001 | 0.88 (0.82–0.95) | 0.001 |
| **Sex** | | | | |
| Male | Ref | – | Ref | – |
| Female | 1.01 (0.83–1.24) | 0.88 | 0.99 (0.78–1.25) | 0.92 |
| **Vaccine** | | | | |
| BNT162b2 | Ref | – | Ref | – |
| ChAdOx1 | 0.15 (0.12–0.18) | <0.001 | 0.13 (0.10–0.16) | <0.001 |
| **Time between second vaccine dose and sampling** | | | | |
| per week increase | 0.91 (0.90–0.92) | <0.001 | 0.89 (0.83–0.95) | 0.001 |
| **Time between first and second vaccine doses** | | | | |
| ≤6 weeks | Ref | – | – | – |
| >6 weeks | 2.16 (1.60–2.94) | <0.001 | – | – |
| | **Outcome—$Log_{10}$ mean titre for 50% neutralisation** | | | |
| | **In whole cohort (n = 345)** | | **In those sampled within 50 days of second vaccine dose (n = 221)** | |
| | aGMR (95% CI) | P value | aGMR (95% CI) | P value |
| **Ethnicity** | | | | |
| White | Ref | – | Ref | – |
| South Asian | 1.47 (1.06–2.06) | 0.02 | 2.01 (1.34–3.01) | 0.001 |
| Black/Mixed/Other | 1.25 (0.71–2.21) | 0.44 | 1.66 (0.76–3.62) | 0.20 |
| **Age** | | | | |
| per decade increase | 0.92 (0.82–1.02) | 0.11 | 0.85 (0.75–0.97) | 0.02 |
| **Sex** | | | | |
| Male | Ref | – | Ref | – |
| Female | 1.25 (0.89–1.75) | 0.20 | 1.19 (0.78–1.81) | 0.42 |
| **Vaccine** | | | | |
| BNT162b2 | Ref | – | Ref | – |
| ChAdOx1 | 0.18 (0.13–0.25) | <0.001 | 0.18 (0.12–0.27) | <0.001 |
| **Time between second vaccine dose and sampling** | | | | |
| per week increase | 0.92 (0.90–0.94) | <0.001 | 0.92 (0.81–1.04) | 0.24 |
| **Time between first and second vaccine doses** | | | | |
| ≤6 weeks | Ref | – | – | – |
| >6 weeks | 2.41 (1.41–4.13) | 0.001 | – | – |

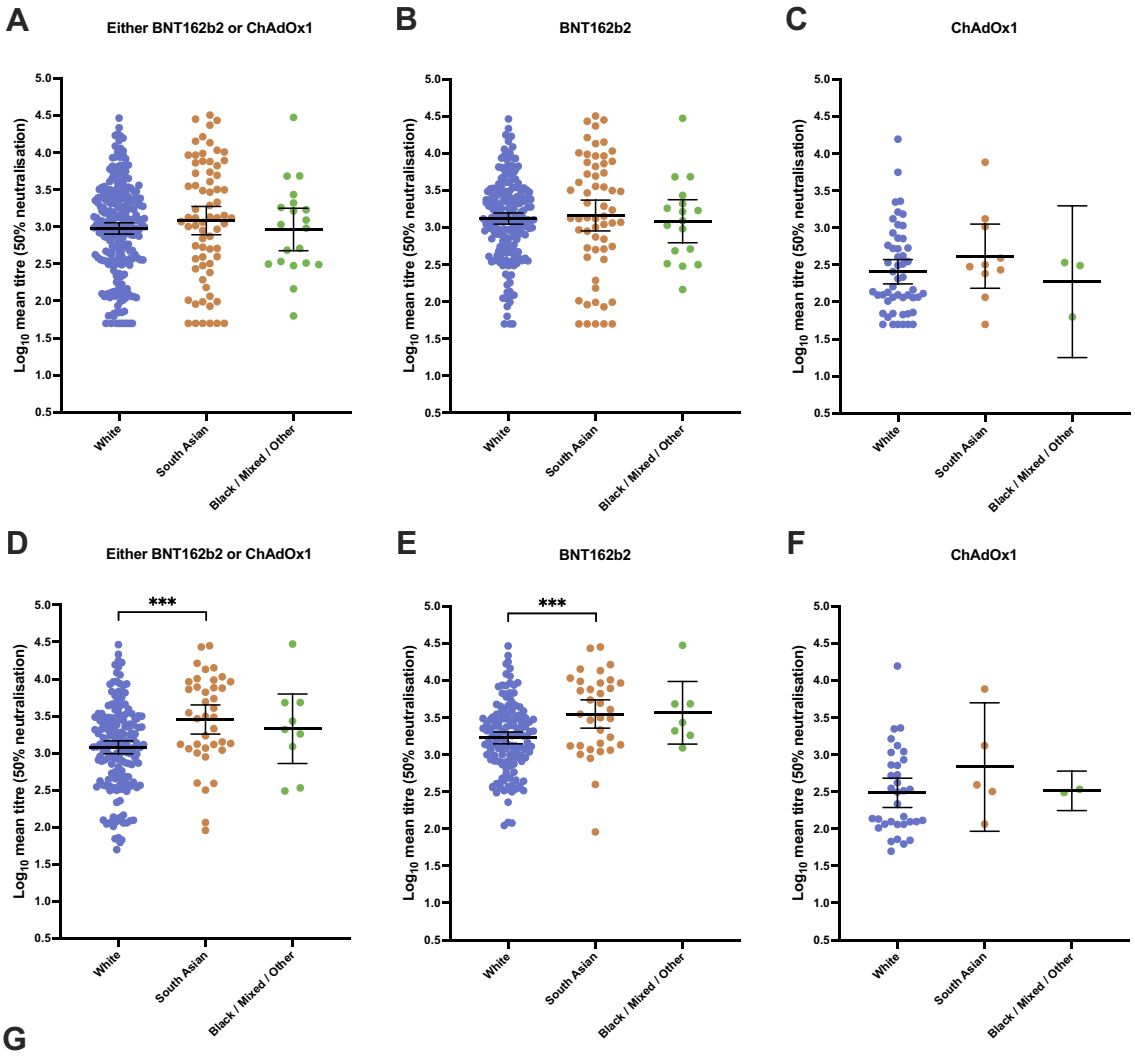
Table 2 shows multivariable linear regression models for the following outcomes: 1. $log_{10}$ total SARS-CoV-2 anti-spike antibody titre (BAU/ml) both in the whole serology cohort and in those sampled between 14 and 50 days of their second vaccine dose (top panel). 2. $log_{10}$ mean titre for 50% neutralisation in a pseudotype-based neutralisation assay against SARS-CoV-2 (Wuhan-Hu-1) both in all those who had samples sent for neutralisation assays and in a subcohort sampled between 14 and 50 days of their second vaccine dose (bottom panel). Coefficients were exponentiated to give adjusted Geometric Mean Ratios (aGMRs). Coefficients were adjusted for all variables in the table. Only 1 participant who was sampled within 50 days of their second dose of vaccine had their initial vaccine doses ≤6 weeks apart, therefore this variable was omitted from the relevant model. Ref–reference group for categorical variable; 95%CI–95% confidence interval.

***Table 2:*** **Linear regression models showing the association between ethnicity and other sociodemographic and vaccine related parameters with $log_{10}$ SARS-CoV-2 total anti-spike titre (top) and $log_{10}$ mean titre for 50% neutralisation (bottom).**

time between vaccine doses with higher neutralising activity (>6 weeks between vaccines: aGMR 2.41, 95% CI [1.41–4.13], P = 0.001 [compared to ≤6 weeks between vaccines]).

## SARS-CoV-2 ELISpot

Fig. 4 shows T cell responses to peptides from SARS-CoV-2 S1 domain, S2 domain and total spike (S1 + S2) by ELISpot. Spot count after stimulation with S1

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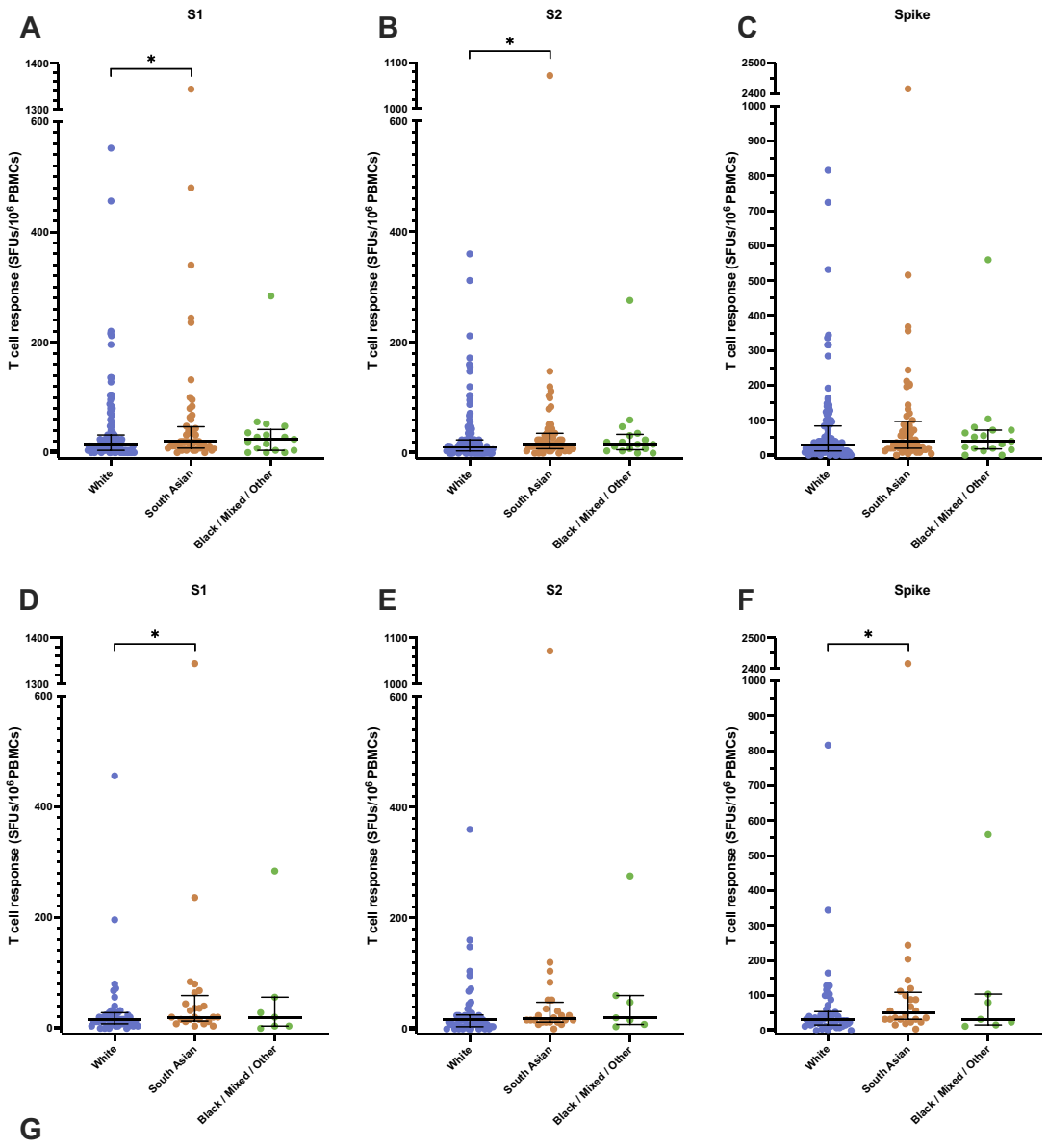
| Panel | White GMT (95% CI) | South Asian GMT (95% CI) | Black / Mixed / Other GMT (95% CI) |
|---|---|---|---|
| A | 948 (796 – 1,122), n=255 | 1,270 (782 – 1,875), n=70 | 558 (111 – 2,818), n=20 |
| B | 1,318 (1,109 – 1,563), n=204 | 1,448 (897 – 2,333), n=60 | 1,213 (622 – 2,371), n=17 |
| C | 256 (175 – 372), n=51 | 413 (153 – 1,119), n=10 | 188 (18 – 1,982), n=3 |
| D | 1,199 (984 – 1,462), n=173 | 2,851 (1,811 – 4,487), n=39 | 2,133 (726 – 6,280), n=9 |
| E | 1,674 (1,396 – 2,013), n=139 | 3,515 (2,269 – 5,458), n=34 | 3,656 (1,384 – 9,661), n=7 |
| F | 305 (194 – 482), n=34 | 680 (93 – 4,989), n=5 | 324 (175 – 598), n=2 |

***Fig. 3:* Comparison of serum SARS-CoV-2 neutralising antibody titre by ethnic group and vaccine type**. Fig. 3 shows a comparison of log10 mean titre for 50% neutralisation from the pseudotype-based neutralisation assay stratified by ethnic group and vaccine type. Panels A, B and C include all participants. Panels D, E and F include only those sampled within 50 days of second vaccine dose. Panel G shows geometric mean titres (GMTs) and their 95% confidence intervals with the number of participants in each ethnic group for panels A–F. Groups were compared (with the White group as reference) with unpaired t tests. ***P < 0.001.

and S2 peptides was higher in the South Asian group than the White group (med 20, IQR [8–46] vs 16 [4–32], P = 0.0498 and 16 [8–36] vs 12 [4–24], P = 0.0413 respectively). When the analysis was restricted to those sampled within 50 days of second vaccine dose, responses to S1 peptides were higher in the South Asian group compared to the White group (20 [14–54] vs 16 [8–28], P = 0.029) as were the responses to spike (32 [16–104] vs 30 [16–52], P = 0.03).

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| Panel | White<br>Med (IQR)<br>A – C, n=118<br>D – F, n=50 | South Asian<br>Med (IQR)<br>A – C, n=56<br>D – F, n=24 | Black / Mixed / Other<br>Med (IQR)<br>A – C, n= 17<br>D – F, n=7 |
|---|---|---|---|
| A | 16 (4 – 32) | 20 (8 – 46) | 24 (4 – 36) |
| B | 12 (4 – 24) | 16 (8 – 36) | 16 (8 – 32) |
| C | 28 (12 – 84) | 40 (20 – 94) | 40 (20 – 72) |
| D | 16 (8 – 28) | 20 (14 – 54) | 20 (4 – 56) |
| E | 16 (4 – 24) | 18 (12 – 44) | 20 (8 – 60) |
| F | 30 (16 – 52) | 50 (32 – 106) | 32 (16 – 104) |

***Fig. 4:* Comparison of T cell responses to SARS-CoV-2 S1, S2 and spike (S1 + S2) epitopes by ethnic group**. Fig. 4 shows results from the ELISpot assay. Results are expressed as spot forming units (SFU/$10^6$ PBMCs) in response to peptides derived from SARS-CoV-2 spike protein (regions S1, S2 and spike [S1 + S2]) stratified by ethnic group. Panels A, B and C include all participants. Panels D, E and F include only those sampled within 50 days of second vaccine dose. Panel G shows median (IQR) SFU/$10^6$ PBMCs for each ethnic group in panels A–F. Groups were compared (with the White group as reference) with Wilcoxon rank sum tests. *P < 0.05.

| | In whole cohort (n = 191) | | | | | |
|---|---|---|---|---|---|---|
| | S1 | | S2 | | Spike | |
| | aIRR (95% CI) | P value | aIRR (95% CI) | P value | aIRR (95% CI) | P value |
| **Ethnicity** | | | | | | |
| White | Ref | – | Ref | – | Ref | – |
| South Asian | 1.75 (1.05–2.89) | 0.03 | 1.23 (0.77–1.96) | 0.38 | 1.50 (0.96–2.35) | 0.08 |
| Black/Mixed/Other | 0.84 (0.38–1.86) | 0.66 | 0.81 (0.39–1.67) | 0.56 | 0.82 (0.40–1.65) | 0.57 |
| **Age** | | | | | | |
| per decade increase | 0.97 (0.82–1.17) | 0.66 | 0.93 (0.79–1.11) | 0.44 | 0.95 (0.81–1.12) | 0.56 |
| **Sex** | | | | | | |
| Male | Ref | – | Ref | – | Ref | – |
| Female | 1.10 (0.66–1.84) | 0.71 | 1.19 (0.75–1.88) | 0.45 | 1.07 (0.68–1.67) | 0.78 |
| **Vaccine** | | | | | | |
| BNT162b2 | Ref | – | Ref | – | Ref | – |
| ChAdOx1 | 0.63 (0.35–1.12) | 0.12 | 0.29 (0.18–0.48) | <0.001 | 0.48 (0.29–0.79) | 0.004 |
| **Time between second vaccine dose and sampling** | | | | | | |
| per week increase | 0.99 (0.96–1.02) | 0.50 | 0.94 (0.92–0.97) | <0.001 | 0.97 (0.95–1.00) | 0.048 |
| **Time between first and second vaccine doses** | | | | | | |
| ≤6 weeks | Ref | – | Ref | – | Ref | – |
| >6 weeks | 0.59 (0.32–1.08) | 0.09 | 0.43 (0.24–0.77) | 0.005 | 0.56 (0.32–0.96) | 0.03 |
| | **In those sampled within 50 days of second vaccine dose (n = 81)** | | | | | |
| | S1 | | S2 | | Spike | |
| | aIRR (95% CI) | P value | aIRR (95% CI) | P value | aIRR (95% CI) | P value |
| **Ethnicity** | | | | | | |
| White | Ref | – | Ref | – | Ref | – |
| South Asian | 2.33 (1.09–4.94) | 0.03 | 1.74 (0.78–3.91) | 0.18 | 2.04 (1.02–4.08) | 0.04 |
| Black/Mixed/Other | 1.21 (0.37–3.98) | 0.76 | 1.47 (0.45–4.75) | 0.52 | 1.34 (0.47–3.81) | 0.58 |
| **Age** | | | | | | |
| per decade increase | 0.93 (0.70–1.26) | 0.66 | 0.90 (0.67–1.21) | 0.48 | 0.93 (0.72–1.20) | 0.58 |
| **Sex** | | | | | | |
| Male | Ref | – | Ref | – | Ref | – |
| Female | 2.75 (1.29–5.85) | 0.009 | 1.79 (0.79–4.06) | 0.16 | 2.21 (1.10–4.42) | 0.03 |
| **Vaccine** | | | | | | |
| BNT162b2 | Ref | – | Ref | – | Ref | – |
| ChAdOx1 | 0.32 (0.13–0.76) | 0.01 | 0.25 (0.10–0.64) | 0.004 | 0.28 (0.13–0.63) | 0.002 |
| **Time between second vaccine dose and sampling** | | | | | | |
| per week increase | 0.98 (0.74–1.31) | 0.92 | 0.97 (0.71–1.32) | 0.85 | 0.98 (0.76–1.28) | 0.91 |

Table 3 shows the results of negative binomial regression analyses for an outcome of ELISpot count in response to peptides derived from SARS-CoV-2 S1, S2 and spike (S1 + S2) for all DIRECT participants meeting inclusion criteria and in a subcohort sampled within 50 days of second vaccine dose. Coefficients were exponentiated to give adjusted incidence rate ratios (aIRRs). Coefficients were adjusted for all variables in the table. There were no participants in the subcohort sampled within 50 days of their second dose of vaccine who had their initial vaccine doses ≤6 weeks apart, therefore this variable was omitted from the relevant model. Ref–reference group for categorical variable; 95%CI–95% confidence interval.

***Table 3*: Negative binomial regression model showing the association between ethnicity and S1, S2 and Spike specific T cell responses after adjustment for demographic and vaccine related factors.**

On multivariable negative binomial regression analyses (Table 3), those from South Asian ethnic groups had higher numbers of circulating T cells responsive to SARS-CoV-2 S1 peptides than those from White groups (aIRR 1.75, 95% CI 1.05–2.89, P = 0.03). In the subcohort sampled within 50 days of second vaccine dose, the number of circulating T cells responsive to both S1 (aIRR 2.33, 95% CI [1.09–4.94], P = 0.03) and spike (aIRR 2.04, 95% CI [1.02–4.08], P = 0.04) peptides were higher amongst South Asian HCWs than White HCWs.

Vaccination with ChAdOx1 was associated with lower S2 and spike T cell responses compared to BNT162b2 (aIRR 0.29, 95% CI [0.18–0.48], P = 0.004 and aIRR 0.48, 95% CI [0.29–0.79], P = 0.002 respectively). An increased time between vaccine doses was associated with reduced T cell responses to S2 and spike peptides (aIRR 0.43, 95% CI [0.24–0.77], P = 0.005 and

aIRR 0.56, 95% CI [0.32–0.96], P = 0.03 respectively). Female HCWs had higher S1 and spike T cell responses than males in the period 14–50 days after second vaccine dose (aIRR 2.75, 95% CI [1.29–5.85] and aIRR 2.21, 95% CI [1.10–4.42] respectively).

### Sensitivity analyses

A comparison of those who were excluded from the neutralising activity analysis due to having a percentage neutralisation <90% at 1:50 dilution by ethnicity is shown in Supplementary Table S3. Exclusion was associated with receiving ChAdOx1 and having samples collected more than 50 days after second vaccine dose. There were no differences in exclusion by ethnicity. Significant findings were unchanged when those with neutralising activity <90% at a dilution of 1:50 were coded as a titre of 50 (Supplementary Table S4).

Changing the upper boundary of the time window for inclusion in the analyses of early immune responses by –10 and +10 days did not have a significant impact on results (see Supplementary Tables S5 and S6).

Findings relating to ethnicity did not change after exclusion of those with immunosuppressive conditions or taking immunosuppressive medications and after adjustment for BMI (Supplementary Tables S7 and S8).

Adjustment for study (DIRECT vs BELIEVE) did not materially change the findings from the analyses of serology or neutralising activity (Supplementary Table S9).

## Discussion

In this cross-sectional analysis of a large multi-ethnic cohort of HCWs who had received two doses of vaccine against SARS-CoV-2 but who had no serological evidence of previous infection, we found that immune responses to SARS-CoV-2 vaccination differed according to ethnicity. In the early period of 14–50 days after vaccination, SARS-CoV-2 spike-specific antibody titre, neutralising antibody titre, and T cell responses to SARS-CoV-2 S1 and spike peptides were all higher in South Asian HCWs compared to White HCWs, associations that persisted after adjustment for other demographic and vaccine related factors. Serum SARS-CoV-2 neutralising activity and T cell responses to S1 peptides were higher throughout the study period in those from South Asian ethnic groups compared to White ethnic groups.

There are few studies that have explored SARS-CoV-2 vaccine induced immune responses by ethnicity. Our serology results are concordant with those from a large cohort study of UK HCWs which found that adjusted anti-spike GMT was higher in infection naïve ethnic minority HCWs than White HCWs after two SARS-CoV-2 vaccine doses and with a large observational study of adults in the UK, which found that combined IgG/IgA/IgM responses to the SARS-CoV-2 spike protein were 16.2% higher in South Asian vaccinees when compared to those from White ethnic groups.[22,32] However, neither study presented data on neutralising activity or cellular responses. In contrast, another UK HCW study found Black HCW to have lower serologic responses to vaccination than their White counterparts and found no differences between the White and Asian groups.[23]

The explanation for the differences in immunogenicity of SARS-CoV-2 vaccination by ethnicity are likely to be complex and multifaceted. Cultural differences between ethnic groups may have an impact on this association, for example diet, which varies by ethnic group,[33] has previously been shown to affect immune response to infection and vaccination.[34] Close contact with COVID-19 has been shown to affect T cell responses even in the absence of seropositivity for anti-nucleocapsid antibody[35] and risk of such contact would be expected to increase with household occupancy, a factor known to differ by ethnicity.[3] Biological/genetic differences between ethnic groups may also play a role.[36] For example, immunoglobulin germline gene polymorphisms have previously been demonstrated to affect neutralising activity of serum after influenza H5N1 vaccination and to differ according to ethnicity[37] and increased transcription of B cell-specific genes compatible with higher antibody responses to influenza vaccine has been shown in younger African Americans compared to their White counterparts.[38]

It is unclear whether the differences in immune response to SARS-CoV-2 vaccination by ethnicity shown in our study and elsewhere translate to differences in the effectiveness of vaccines for reducing the risk of SARS-CoV-2 infection and severe COVID-19. The limited information in the literature may relate to underrepresentation of ethnic minority groups in vaccine trials.[39,40] In a study evaluating efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine, vaccine efficacy at preventing COVID-19 was reported to be 97.5% (87.1–96.4%) in 'communities of colour' (all non-White ethnic groups were combined for statistical power) compared to 93.2% (87.1–96.4%) in the White group.[41] In a study reporting results of the phase 3 trial of the BNT162b2 vaccine, efficacy for preventing COVID-19 was reported as 95.2% (89.8–98.1%) for the White group which was similar to the Hispanic or Latinx (94.4% [82.7–98.9%]) and Non-Hispanic, non-Latinx (95.4% [88.9%–98.5%]) groups. Estimates of efficacy were higher for the Black or African American group (100.0% [31.2%–100.0%]) and lower for the 'All others' group (89.3% [22.6–99.8%]) but confidence intervals were wide due to low numbers of participants in these groups.[42]

Outside of the findings relating to ethnicity our study adds further weight to the accumulating evidence that immunogenicity of SARS-CoV-2 vaccine may be increased by increasing the interval between the first two doses[43,44] and that vaccination with ChAdOx1

compared to BNT162b2 elicits lower antibody and cellular immune responses to SARS-CoV-2 spike protein.[45,46]

This study has several strengths. Our cohort is large, ethnically diverse and has been extensively phenotyped. Therefore, we are able to add significantly to the limited information in the literature concerning ethnic differences in immunogenicity of SARS-CoV-2 vaccines by presenting the first data on differences in serum SARS-CoV-2 neutralising activity and T cell responses by ethnicity. Our study design also allows us to postulate that ethnic differences in immunogenicity of SARS-CoV-2 vaccines may be more marked in the early phase after vaccination. A significant proportion of our cohort are from South Asian ethnic groups which allows for meaningful comparisons between the White and South Asian cohort, rather than restricting analyses to 'White vs non-White'. Our sample is broadly similar to the NHS workforce in terms of age and sex distribution but has a higher proportion of HCWs from minority ethnic groups[47]

Our study also has limitations. The low numbers of participants from ethnic groups other than White and South Asian necessitated collapsing other ethnic groups into a single third group. Whilst this was done to avoid exclusion of participants and to maintain as much granularity as possible, we accept that there is considerable heterogeneity in the Black/Mixed/Other group and suggest that further research is needed to determine whether there are differences in immunogenicity of SARS-CoV-2 vaccines in these ethnic groups. The majority of participants received the BNT162b vaccine and therefore our conclusions are mainly drawn based on responses to this vaccine. Data are from a single centre; however, our results align with the few available studies examining SARS-CoV-2 vaccine immunogenicity by ethnicity suggesting that they are representative. There were differences between ethnic groups in terms of vaccine schedule and sex distribution but these were adjusted for in the multivariable models and thus are unlikely to have affected our conclusions. As with any observational study, we cannot be sure that reported associations are not the result of residual confounding. We used anti-nucleocapsid antibody status to determine which participants had previously been infected with SARS-CoV-2 and as these would be expected to wane with time and we cannot rule out the possibility that, for a participant infected early in the pandemic, anti-nucleocapsid titres have waned to a point below the limit of detection. However the anti-nucleocapsid assay used in this work has been shown to have a sensitivity of 92% 18 months after SARS-CoV-2 infection,[48] a time period that would span from March 2020 (when cases of COVID-19 began to significantly rise in the UK) through to September 2021 when the study closed for recruitment. We also cannot rule out the possibility that a participant was sampled in an early phase of infection prior to seroconversion. However, in a population of HCW who had ready access to SARS-CoV-2 PCR testing and were encouraged to undergo testing as soon as symptoms consistent with COVID-19 developed, we think it unlikely that this had any meaningful impact on our results. Crucially, there is no reason to suspect that either of these antibody related effects would impact differently according to the ethnicity of the participant and introduce bias. We made many comparisons over the course of this analysis, as this is exploratory work we felt it would be unnecessarily restrictive to adjust the alpha level to account for this and we accept that this will increase the risk of type 1 error. We therefore strongly recommend that future studies explore the associations reported here. Follow up work from the current study will also seek to confirm these association in analyses using data from further sampling events.

This cross sectional analysis of an extensively immunophenotyped population provides evidence that serologic response to SARS-CoV-2 vaccination in the early phase after vaccination may be higher in South Asian ethnic groups than White ethnic groups. Our study is the first to demonstrate higher T cell responses to SARS-CoV-2 spike protein epitopes and serum SARS-CoV-2 neutralising activity after vaccination in South Asian ethnic groups compared to White groups. Further research is required to establish: the mechanisms underlying these differences; whether these differences persist over time and with repeated exposure to vaccine; whether these differences impact upon vaccine effectiveness in different ethnic groups; and how natural infection with SARS-CoV-2 might impact upon these differences.

#### Contributors

MP conceived the ideas for DIRECT and BELIEVE and led the applications for funding with input from CAM, JN, PH and AC. Online consent and questionnaire tools were designed and implemented by CAM and LB. Recruitment for the studies was done by CAM, JN, DP, A Ahmed, SB, JM, MM, AM and MP. Sample and additional data collection was done by CAM, JN, AJ, MD, NA, TA, A Asif, NG, MG, RK, MM, VR and DV. SARS-CoV-2 anti-spike antibody assays were performed by BH, ADO and CR. Pseudotype-based neutralisation assays were performed by NL, SS and BJW. ELISpot assays were performed by AT. Data were linked and cleaned by CAM with input from JN, AJ, DP, VR, DV, and MP. Data analysis was by CAM with input from JN, DP, MD, LJG, LT, PM, PH, AC and MP. CAM wrote the first draft of the manuscript with input from JN, DP, KK, AT, BH, ADO, CR, BJW, PH, AC and MP.

All authors have had the opportunity to access the underlying data used in this study. The analysis has been verified by CAM and MP. All authors reviewed the manuscript and approved the final version prior to submission.

#### Data sharing statement

To access data or samples produced by the DIRECT or BELIEVE studies, the working group representative must first submit a request to the Core Management Group by contacting the UK-REACH Project Manager in the first instance. For ancillary studies outside of the core deliverables, the Steering Committee will make final decisions once they have been approved by the Core Management Group. Decisions on granting the access to data/materials will be made within eight weeks. Third party requests from outside the Project will require explicit approval of the Steering Committee once approved by the Core Management Group.

**Declaration of interests**

KK is Chair of the Ethnicity Subgroup of the UK Government Scientific Advisory Group for Emergencies (SAGE) and a member of SAGE. PM has received honoraria from Moderna, AstraZeneca and GSK, support for attending meetings from AstraZeneca and has participated on an advisory board for AstraZeneca. PH received an honorarium for hosting a COVID-19 webinar, on behalf of Oxford Immunotec who are manufacturers of the ELISpot technology used in the manuscript. MP reports grants from UKRI-MRC for the current work and UKRI-MRC, NIHR, Sanofi and Gilead outside the current work and has received consulting fees from QIAGEN.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2023.101926.

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