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Phenotypic Characterization of Peripheral B cells in *Mycobacterium tuberculosis* infection and disease in Addis Ababa, Ethiopia

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This is to certify that the thesis prepared by Tigist Girma, entitled: **Phenotypic Characterization of Peripheral B cells in *Mycobacterium tuberculosis* infection and disease in Addis Ababa, Ethiopia** and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

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List of abbreviations

AACAHB	Addis Ababa City Administration Health Bureau
AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immunodeficiency Syndrome
BCG	Bacillus Calmette-Guerin
BCR	B cell Receptor
Bregs	Regulatory B cells
CD	Cluster of differentiation
CFP	Culture filtrate protein
CSR	Class Switched Recombination
CMI	Cell-mediated immunity
CVID	Common variable immunodeficiency
DCs	Dendritic cells
ESAT	Early secretory antigenic target
ELISA	Enzyme-linked Immuno-Sorbent Assay
FACS	Fluorescence Activated Cell Sorting
HCs	Healthy controls
HIV	Human Immunodeficiency Virus
IFN- γ	Interferon-gamma
IGRAs	Interferon-gamma Release assays
IL	Interleukin
LJ	Lowenstein Jensen Media
LTB	Latent tuberculosis infection
CMZ	Circulating Marginal Zone
MDR-TB	Multidrug-resistant tuberculosis
MHC	Major Histocompatability Complex
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NK	Natural Killer

rpoB	Gene encodes the β subunit of RNA polymerase
sIg	Surface immunoglobulin
SHM	Somatic Hypermutation
TGF	Transforming growth factor
TLRs	Toll-like receptors
TST	Tuberculin skin test
PBMC	Peripheral blood mononuclear cell
PTB	Pulmonary tuberculosis
QFN	QuantiFERON
VCT	Voluntary Counseling and testing
WHO	World Health Organization
XDR-TB	Extensively Drug-Resistant Tuberculosis

Abstract

Background: *Mycobacterium tuberculosis* (MTB) remains an unresolved threat resulting in great annual loss of life. The role of B cells during the protective immunity to MTB is still unclear. B cells have been described as effector cells in addition to their role as antibody producing cells during disease.

Objective: to identify and characterize the frequency of peripheral B-cell subpopulations during active and latent tuberculosis.

Methods: A total of 52 participants comprising 16 active untreated pulmonary TB (PTB) cases, 17 Latent Tuberculosis Infection (LTBI), and 19 Healthy Controls (HCs) were enrolled in the study. Peripheral blood samples were collected to do QuantiFERON (QFT) assay to diagnose LTBI and to isolate Peripheral Blood Mononuclear Cells (PBMC). PBMCs were stained with monoclonal antibodies. Expressions Cluster of differentiation (CD) markers was assessed on B cells subsets using multicolor flowcytometry. A difference in the frequency of B cell subsets among the groups was analyzed using the One-way Anova and Kruskal-Wallis tests was used for comparison between two groups. Data is presented as median (Interquartile range) and a P value of less than 0.05% was taken as a statistically significant difference.

Results: Tissue-like memory B cells ($CD19^{+}CD27^{-}CD21^{-}$); p = 0.0024 and class switched memory B cells ($CD19^{+}CD27^{+}CD21^{+}$ IgM^{-} IgD^{-}); p = 0.0065 had significantly higher proportion in active PTB when compared to LTBI and HCs. On the contrary, resting memory B cells ($CD19^{+}CD27^{+}CD21^{+}$); p <.0001, non-class switched memory B cells ($CD19^{+}CD27^{+}CD21^{+}$ IgM^{+} IgD^{+}); p = 0.0011, marginal zone B cells (MZCs) ($CD19^{+}CD27^{+}CD21^{+}IgM^{+}IgD^{+}CD23^{-}$); p = <0.0001 and regulatory B cells (Breg) ($CD19^{+}CD24^{hi}CD38^{hi}CD5^{+)}$; p = 0.0043 had significantly lower proportion in active PTB group as compared to LTBI and HCs. There were no statistically significant difference observed in percentage of total B cells ($CD19^{+}$); p = 02407, Naïve B cells ($CD19^{+}CD27^{-}CD21^{+}$); p = 0.1156 and activated memory B cells ($CD19^{+}CD27^{+}CD21^{-}$); p = 0.1134 among the three study groups.

Conclusions: The findings of this study showed that B cells markers distinguish active untreated TB cases from LTBI, that indicate B cells phenotype is promising for clinical application as potential biomarker for TB disease and LTBI identification. A large scale longitudinal study is required to confirm and translate this finding into clinically applicable tests.

Keywords: B cells, Tuberculosis, Immuno-phenotyping

1. Background

1.1. Introduction

Tuberculosis (TB) is a communicable bacterial disease caused by *Mycobacterium tuberculosis*. The microbe is an obligatory aerobic, intracellular pathogen that has a predilection for the lung tissue rich in oxygen supply. Even though it typically affects the lungs causing pulmonary TB, it can also affect other sites leading to extra pulmonary TB(1, 2). *Mycobacterium tuberculosis* is a member of the *M. tuberculosis* complex (MTBC) which includes *M. africanum, M. canettii,M. bovis, M. microti, M. orygis, M. caprae, M. pinnipedii, M. suricattae* and recently recognized *M. mungi*. The MTBC members are genetically closely related, the genome of *M. tuberculosis* shows <0.05% difference with *M. bovis*, where the latter species primarily infects cattle but can also cause TB in other mammals including humans(2). All MTBC members cause TB but they exhibit distinct phenotypic properties and host range (2-4).

Non-tuberculosis Mycobacteria (NTM) are ubiquitous in the environment with the heaviest concentrations found in soil and water sources. They are associated with biofilm formation which contributes to disinfectant and antibiotic resistance. The hydrophobicity of NTM results in preferential aerosolization from water and many of these organisms are resistant to high temperature and are relatively resistant to low pH (5). NTM infections have also increased in many regions of the world along with MTBC infections (4).

The *Mycobacterium tuberculosis* presentations in the human host can be separated into three main stages: latent infection, reactivation and primary active disease. Each stage represents differences in MTB gene expression (6).

Latent *tuberculosis* infection (LTBI) is defined as being infected with the causative agent but lacking clinical symptoms, and without being exempted from future development into clinical disease. MTB interacts with the host immune system in a very complex fashion. Not all people who are infected progress to active disease; latently infected individuals have a 10% lifetime risk of developing active disease with clinical symptoms (7). The reactivation of a latent infection requires the activation of the quiescent bacilli. Several factors can trigger the development of active disease from reactivation of latent infection, which usually involves the decline of the immune response. HIV infection is the most important risk factor for progression to active disease due to depletion of $CD4^+$ T cells. Older age, malnutrition, and medical conditions that

compromise the immune system are also risk factors for the reactivation(8). Immunosuppressive triggers such as anti-tumour necrosis factor (TNF) therapy for unrelated diseases greatly enhances this risk. The symptoms of active TB disease of the lung are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever and night sweats (9,10)

A greater understanding of the interactions and complementing effects between the different arms of the immune system against TB and the ways by which *M. tuberculosis* manipulates these responses is important for optimal protection against infection and development of disease (11, 12).

B-lymphocytes are characterized by the expression of CD19 surface antigen, which is present early on bone marrow progenitor cells and persists during all stages of B-cell maturation. This cell population does, however, include phenotypically and functionally different subgroups. The largest subgroup is formed of B2 cells, or conventional B cells, which account for about 10% of peripheral blood lymphocytes in human. The remaining B cells are described as B1 cells and can be distinguished by the presence of CD5; which are sub-classified into as B1-a cells (CD5+) and B1-b cells (CD5–). Regarding the functional differences of these B cell populations, B2 lymphocytes are largely mediators of adaptive immunity and are located in the follicular zone. They respond to conventional protein antigens, with the help of T cells, and undergo antibody class switching and affinity maturation. An additional marker on these cells, CD27, indicates the transition from naïve B cells to memory B cells. B1 cells, on the other hand, are considered to be effectors of innate immunity; they respond to T-cell independent antigens, mainly carbohydrates, which are common in various infectious agents. In particular, B1-a cells spontaneously secrete IgM (natural antibodies) while B1-b cells are source of long-lasting antibody production (in T independent manner) (13).

Protection against intracellular pathogens is often generalized as exclusively T cell mediated, with B cells and antibodies playing a limited role than they are perceived to play during extracellular infections (3, 12, 14). This has led to the use of highly T cell-centric strategies for the development of vaccines against intracellular pathogens including *M. tuberculosis.*

The *tuberculosis* granuloma contains both CD4+ and CD8+ T cells that contains the infection within the granuloma and prevent reactivation (3). B cells are also prominent components of TB pulmonary granulomatous inflammation, with large aggregates of these lymphocytes as

prominent histological characteristics of the infection, although there are ongoing debates about their relevance (11, 14). B cells can conceivably shape anti-*tuberculosis* immunity through a variety of means including direct effects of antibody upon the pathogen, cytokine production, as well as influencing the intracellular killing mechanisms of leukocytes (14). B cells are professional antigen-presenting cells with notable impact upon T cell responses by capturing and internalizing antigens *via* surface immunoglobulins. These antigens are then processed and presented on the surface as peptide; MHC class II complexes. Then B lymphocyte activation is initiated. Subsequent progression through cellular interactions with $CD4^+$ helper T cells includes antigen-presentation events which stimulate T cells to produce the cytokines that reciprocally regulate the antibody responses of B cells. B cells down regulate T cell function either directly via IL-10 or TGF- β production or by augmentation of the regulatory T cell pathway. Importantly; the regulatory B cells have been shown to play a role in the control of autoimmunity, inflammation, and cancer (12, 14).

1.2. Statement of the Problem

Recent studies have broadened the views of biomarker research to diagnose tuberculosis on a serological basis by combining different immunoglobulin (Ig) classes against selected MTB targets. This raises the possibility of utilising B cells as biomarkers in tuberculosis disease, not only by measuring their capacity as effectors/regulators in expressing cytokines, but also on a phenotypic level and observing dynamic changes in population groups(10).

The need for tuberculosis biomarkers arises, in part, from the lack of suitable tests to detect *M. tuberculosis* or its products in host samples. Diagnosing TB is not easy especially latent TB. The diagnosis of active TB is critical for controlling the disease. Conventional diagnostic methods of active TB include sputum smear microscopy (in pulmonary TB a low bacillary burden often leads to a missed diagnosis) and *M. tuberculosis* isolation in bacteriological culture (currently the gold standard for definitive diagnosis of pulmonary and extra-pulmonary TB). Although these methods are widely used for diagnosing TB, they lack specificity and sensitivity and microbiological culture takes several weeks to confirm a clinical diagnosis and also require highly skilled personnel and specialized laboratory infrastructure. In addition to the above conventional diagnostic methods there is also advanced molecular tools (Gene X-pert) test that allows automated sample processing and produces results within two hours with excellent sensitivity (15-17) .

Better understanding of protective immunity components and their interactions is necessary to control *Mycobacterium tuberculosis* (MTB) infection in humans which is critical for tuberculosis (TB) therapy and vaccine development strategies (11).

Since *Mycobacterium tuberculosis* is an intracellular pathogen, role of B cell or antibody in protection against TB is underestimated, inferior to cellular immunity; therefore, most studies done on TB focused on T cells. But, emerging studies showed significance of B cells in fighting against *M. tuberculosis* (3, 11, 14), although some studies reported conflicting results on the role of B cells in TB (18-20). B and T cell percentage determinations are not routine tests in diagnostic laboratories. However, considering that the use of flowcytometry is increasing through time, determining B and T cell percentages by FACS analysis is an easy and useful determination that could contribute to a better management of TB patients(18).

There is no previous works that determine phenotypic analysis of B cells among TB patients in Ethiopia. Therefore, this study focuses on identification of B cells subsets during *Mycobacterium tuberculosis* infection and disease. The finding may be used to determine significance of B cells against tubercle bacilli.

1.3. Significance of the study

Studies showed Profile change in B cells subsets during *Mycobacterium tuberculosis* infection and disease indicating role of B cells in TB. But, there is a controversial idea on the contribution of B cells in TB, some studies reported that B cells are unaltered, while others report level of B cells decrease or increase of peripheral blood B cells populations in latent and actively infected patients (18-20). As a result, currently significance of B cells against TB is not clearly known. This study provides information on peripheral B cells and their subpopulation during *Mycobacterium tuberculosis* disease. Therefore, it is useful for greater understanding of B cells role in TB disease which may help in controlling *Mycobacterium tuberculosis* disease.

2. Literature review

Tuberculosis (TB) has existed for millennia and remains a major global health problem. It causes ill-health for approximately 10 million people each year and is one of the top ten causes of death worldwide(1). The effective cell-mediated immune response to MTB infection, involving mainly the $CD4^+$ and $CD8^+$ T cell subsets, plays an essential role in the pathogenesis of TB (21). Recently studies suggest that B cells and humoral immunity can also modulate the immune response to MTB infection (12, 19, 21).

2.1. Epidemiology of tuberculosis

Tuberculosis is the ninth leading cause of death worldwide from a single infectious agent, ranking above HIV/AIDS. In 2016, there were an estimated 1.3 million TB deaths among HIV-negative people (down from 1.7 million in 2000) and an additional 374 000 deaths among HIV-positive people. Most of the estimated number of incident cases in 2016 occurred in the WHO South-East Asia Region (45%), the WHO African Region (25%) and the WHO Western Pacific Region (17%); smaller proportions of cases occurred in the WHO Eastern Mediterranean Region (7%), the WHO European Region (3%) and the WHO Region of the Americas (3%). According to WHO 2017 report, Ethiopia is among the 30 high-burden country lists for TB, TB/HIV and MDR-TB (1).

2.2. Transmission and Pathogenesis of *Mycobacterium tuberculosis*

Tuberculosis is infectious disease and patients with pulmonary TB are the most important source of infection. Infection is initiated by inhalation of droplet nuclei, which are particles of 1–5 μm in diameter containing *M. tuberculosis*, expectorated by patients with active pulmonary TB (open TB), typically when the patient coughs. The droplet nuclei, due to their small size, can remain suspended in the air for several minutes to hours. The risk of infection is dependent on several factors such as the infectiousness of the source case, the closeness of contact to the patient, the bacillary load inhaled, and the immune status of the potential host. The primary route of infection involves the lungs. Inhaled droplet nuclei avoid the defenses of the bronchi due to their small size and penetrate into the terminal alveoli where they are engulfed by phagocytic immune cells (macrophages and dendritic cells). *M. tuberculosis* can also infect non-phagocytic cells in the alveolar space including M cells, alveolar endothelial, and type 1 and type 2 epithelial cells (pneumocytes). In the early phase of infection, *M. tuberculosis*, internalized by phagocytic immune cells, replicates intra-cellularly, and the bacteria laden immune cells may cross the

alveolar barrier to cause systemic dissemination. The intracellular replication and simultaneous dissemination of the pathogen to the pulmonary lymph nodes and to various other extra-pulmonary sites occur prior to the development of the adaptive immune responses. This exemplifies the extraordinary ability of *M. tuberculosis* to establish a protected niche where they can avoid elimination by the immune system and to persist indefinitely (1, 2).

2.3. Diagnosis of tuberculosis

The diagnosis of active Tuberculosis is critical for controlling the disease. Detection of *Mycobacterium tuberculosis* by microbiological methods remains the gold standard for the diagnosis of tuberculosis (TB) disease in humans, also referred to as active TB. Conventional diagnostic methods of active TB include sputum smear microscopy for pulmonary TB and *M. tuberculosis* isolation from bacteriological culture is currently the gold standard for definitive diagnosis of pulmonary and extra-pulmonary TB. Both methods require highly skilled personnel and culture requires a specialized laboratory infrastructure (17, 22).

The Gene X-pert is molecular assays for the detection of *M. tuberculosis*, based on the polymerase chain reaction (PCR) principle. Gene X-pert is a cartridge-based, automatic nucleic acid amplification test which has the advantage of significantly shortening the time needed to confirm suspected TB disease. The assay is based on a qualitative, nested real-time PCR, which allows the detection of *M. tuberculosis* complex in clinical samples, and simultaneously detects mutations in the *rpoB* gene, which are associated with rifampicin resistance. However, its use is restricted to the identification of active pulmonary tuberculosis (15-17, 22).

Tuberculin skin test (TST) could be used for clinical diagnosis of TB together with other clinical findings. However, the tests cross reacts with antigens, which are common in other mycobacteria than *Mycobacterium tuberculosis.* Thus, the test is prone to give false positive results following BCG vaccination and environmental exposition to different mycobacterial strains (2, 15). More sensitive and specific tests such as cell-mediated immunity-based interferon-gamma (IFN-γ) release assays (IGRAs) have also been developed that detect T cell responses after stimulation by two *M. tuberculosis*-specific antigens, early secreted antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10). One major limitation with IGRAs, however, is that it cannot differentiate active tuberculosis from latent tuberculosis infection (LTBI). The IGRAs have excellent specificity as the antigens (ESAT-6 and CFP-10) used in these assays are encoded by genes deleted in the vaccine strain *M. bovis* BCG and majority of environmental mycobacteria of

clinical relevance. Another variation of conventional IGRAs has also been developed by using flow cytometry. Although flow cytometric approach has an advantage over conventional IGRAs as a smaller blood volume (<1ml) is required for testing, the assay has limited utility in much of the developing world due to the requirement of technical expertise and the high cost of flow cytometer (2).

There is no immunodiagnostic test that can accurately diagnose active TB, distinguish LTBI from active TB, or tell apart asymptomatic forms of infection that are associated with a high risk of disease progression (16).

2.4. Immunology of tuberculosis

Alveolar resident macrophages are the primary cell type involved in the initial uptake of *M. tuberculosis.* After this first encounter, DCs and monocyte-derived macrophages also take part in the phagocytic process. Besides phagocytosis, recognition of *M. tuberculosis* or mycobacterial products is a crucial step in an effective host response. Toll-like receptors (TLRs) are phylogenetically conserved mediators of innate immunity which are essential for microbial recognition on macrophages and DCs. Phagocytic cells also play a key role in the initiation and direction of adaptive T-cell immunity by presentation of mycobacterial antigens and expression of co-stimulatory signals and cytokines (23).

The cytokine interferon-gamma (IFN- γ) produced by both $CD4^+CD8^+$ T cells, as well as by natural killer (NK) cells plays a pivotal role in protective immunity against *Mycobacterium tuberculosis* (MTB) infection, IFN- γ is an important mediator of macrophage activation. Humans deficient in either the gene for IFN- γ or the IFN- γ receptor show enhanced susceptibility to mycobacterial infections (3,15,24). Another cytokine is interleukin-12 (IL-12), which stimulates IFN- γ production. A lack of this cytokine increases susceptibility to mycobacterial infections in humans (15).

The tuberculosis granuloma is a site of immunological priming, which occurs at the interface between the macrophage-rich inner layer and the surrounding T-cell-rich outer layer. The granulomas contain both $CD4^+$ and $CD8^+$ T cells that contains the infection within the granuloma and prevent reactivation (3, 16). B cells are also prominent components of TB pulmonary granulomatous inflammation, with large aggregates of these lymphocytes as prominent

histological characteristics of the infection, although there are ongoing debates about this relevance (11, 14).

2.4.1. B cells in *Mycobacterium tuberculosis* infection and disease

T helper cells greatly impact the development and specialization of B cell responses and B cells conversely impact T cell activation by acting as or upon antigen-presenting cells. Within this intricate relationship between both arms of immunity, cellular and humoral responses together determine the outcome of intracellular infection (14).

The functional versatility of B cells is remarkable and lymphocyte subset can shape the development of an immune response through multiple mechanisms. B cells are proficient antigen presenting cells, a rich source of a wide array of cytokines, and the only generator of immunoglobulins. Each of these attributes can impact the differentiation and functions of a wide variety of immune cells such as T cells, neutrophils, macrophages, and dendritic cells. Due to the highly active and dynamic interaction among various cells in the immune network, prediction of the relative contribution of a specific cell type is not currently possible or straightforward (11).

B lymphocytes, along with T cells, constitute the adaptive immune compartment. B cells may also play roles in recruiting cells to the lungs through secretion of chemokines or inducing the activation of T cells. Thus, B cells have a plethora of functions that regulate the course of immune response and inflammation (24). B-cells have a regulatory phenotype as well, where they produce cytokines such as IL10 and TGF-alpha. Several phenotypes of B-cells with regulatory functions have been termed Bregs, which include $CD19^{+}CD5^{+}CD1d^{+}$ B cells, as well as the recently described $CD19^{+}CD24^{hi}CD38^{hi}$ B-cells (26).

Joosten SA *et al* observed that the frequency of $CD19^{+}CD24^{hi}CD38^{hi}$ Bregs is not significantly different between controls and treated and untreated TB cases. However, a study conducted by Zhang M *et al* reported TB patients were found to have significantly higher frequencies of $CD19^{+}CD5^{+}CD1d^{+}$ B cells with stronger suppressive activity than such cells from healthy donors. They also showed the frequency of $CD19^{+}CD5^{+}CD1d^{+}$ B cells in peripheral blood was inversely correlated with that of Th17 cells in TB patients (26, 27).

A study done by Hernandez J *et al* and Joosten SA *et al* indicated significant reduction in the percentage and total number of B cells ($CD19^{+}$) in pulmonary TB patients compared with healthy donors at time of diagnosis (18, 19). Barcelos W *et al* and Zhang M *et al* studies showed

that a significant increase in the marker (CD19) at the end of the TB treatment. It was observed that after the treatment, these cells were significantly increased, probably due to the endogenous booster that caused an increased reaction to *Mycobacterium* soluble antigens released by the action of the chemotherapic agents that stimulate the proliferation of these cells and also Dubaniewicz *et al* found similar data strengthened by the significant increase of IgG class antibody levels after healing (26-28).

Another study conducted in South Africa reported that CMZ B cells ($CD19^{+}$ $IgM^{+}CD23^{-}CD27^{+}$) and memory phenotypes are able to distinguish between TB diagnosis and end of treatment. Frequencies of mature B cells from TB cases are lower than that of other-lung diseases at diagnosis. Subpopulations of activated memory B cells ($CD19^{+}IgM^{+}CD23^{+}CD27^{+}$) are present at the end of TB treatment (29).

So far as to my knowledge, there is no published report on the role of B cells in TB patients in Ethiopia. Thus, in our study we showed peripheral B cells as distinguishing phenotype between active TB, LTBI and HCs.

3. Objectives

3.1. General objective

To determine B cells phenotype in peripheral blood of *Mycobacterium tuberculosis* infected and active TB disease cases as compared to healthy controls from selected health centers in Addis Ababa, Ethiopia**.**

3.2. Specific objectives

1. To determine total peripheral B cells frequencies in active PTB cases prior to initiation of anti-tuberculosis treatment.
2. To identify total peripheral B cells frequencies in LTBI cases.
3. To compare peripheral B cells subsets between TB patients, LTBI and HCs groups.

4. Hypothesis

4.1. Null hypothesis

Peripheral total B cells and their subset frequency is the same for active PTB cases, LTBI and HCs.

5. Materials and Methods

5.1. Study area

The study was conducted from February.2018 to April.2018 in Lomi Meda, Woreda 06 (Hiwot Amba), Arada, Philipos, and Hidase health centers and their respective voluntary counseling and testing (VCT) centers, Addis Ababa, Ethiopia.

Administratively, Addis Ababa is divided into 10 sub-cities, which are in turn divided into 116 Woredas, the smallest government administrative units. Health centers are managed by Addis Ababa City Administration Health Bureau (AACAHB) and mainly focused on primary health care services. The following health centers were randomly selected and their TB patient and VCT flow per day during the study period is described below.

Woreda 06 health center: Found in kirkos sub-city, minimum of 3 suspected TB cases seen per day. 5-6 individuals were coming to this health center for VCT per day.

Arada health center: Found in Arada sub-city, minimum of 4 suspected TB cases seen per day. 8-10 individuals were coming to this health center for VCT per day.

Philipos health center and: Found in Kolfe keraniyo sub-city, minimum of 5 and 4 suspected TB cases seen per day. 4-6 individuals were coming to this health center for VCT per day.

Lomi Meda health center: Found in Kolfe keraniyo sub city, minimum of 4 suspected TB cases seen per day. 3-7 individuals were coming to this health center for VCT per day.

Hidase health center: Found in Gulele sub city, minimum of 4 suspected TB cases seen per day. 5-7 individuals were coming to this health center for VCT per day.

5.2. Study design and period

A cross sectional study was conducted from February.2018 to April.2018 to determine B-cell phenotype in the peripheral blood of adult participants during *Mycobacterium tuberculosis* infection and clinical TB diseases; recruited from selected health centers, Addis Ababa, Ethiopia.

5.3. Populations

5.3.1. Source population

The source populations were all patients who visited Woreda 06, Arada, Philipos, Lomi Meda and Hidase health centers and as controls, apparently healthy participants were recruited from the respective VCT centers in Addis Ababa, Ethiopia.

5.3.2. Study population

All newly diagnosed patients who were smear positive by Ziehl Neelson staining method recruited prior to initiation of anti-TB treatment from TB clinics found in Woreda 06, Arada, Philipos, Lomi Meda and Hidase Health centers. Apparently healthy participants were recruited from the respective VCT centers in the selected health centers.

5.4. Inclusion and Exclusion criteria

5.4.1. Inclusion criteria

- Age 18-65 years old.
- Voluntary individuals who gave consents

For TB cases:

- Patients with clinical symptoms of TB including a chronic cough, weight loss, night sweats.
- Chest radiograph with suggestive signs of TB.
- Positive smear microscopy and sputum culture.

Latent TB:

- Apparently healthy individuals.
- Positive for interferon gamma release assay (IGRA) using QuantiFERON (QFN) blood test.

For Healthy uninfected control

- Apparently healthy individuals.
- Negative for interferon gamma release assay (IGRA) using QuantiFERON (QFN) blood test.

5.4.2. Exclusion criteria

- Previously treated TB cases.

- Individuals undergoing treatment with immunosuppressive drugs.
- HIV Positive individuals
- Mentally ill and unconscious patients.
- Chronic disease

5.5. Study Variables

5.5.1. Dependent variables

- Peripheral B cells Phenotype in active untreated PTB cases.
- Peripheral B cells Phenotype in LTBI and HC group.

5.5.2. Independent variables

- Socio demographic characteristics (including Age, Sex)
- Tb infected groups: clinical and LTBI cases.
- Non Tb infected apparently healthy control.

5.6. Measurement and Data collection

5.6.1. Sample size determination and Sampling method

The sample size was determined by convenient sampling where fifty two study participants were recruited purposefully (PTB, N =16, LTBI, N=17 and healthy control, N=19).

5.6.2. Data collection tools and procedures

All study participants were properly informed about the study and also asked for consent. Then, data was collected from the study participants using an interview with a structured questionnaire. The interview was conducted by principal investigator and trained data collector. Ten ml of blood and 5 ml of sputum from TB patients and 15ml of blood from apparently healthy individuals were collected at the health centers and transported to AHRI for laboratory investigations.

5.6.3. Specimen collection procedure

5.6.3.1. Sputum collection and processing

Two spot sputum specimen of at least 5 ml volume in clean, leak-proof, with screw cap, wide-mouth, disposable containers were collected by the laboratory personnel working at TB laboratory of each health facilities in the study area from patients who were suspected to have PTB prior to initiation of anti-TB treatment. AFB microscopy was done for each sample at the

health facilities. Transportation of samples was carried out by principal investigator. Sputum samples were digested and/or decontaminated by N-acetyl L-cysteine- sodium hydroxide (NALC-NaOH) method (See Annex XI**)**

5.6.3.2. Smear Microscopy

AFB Smears were prepared by taking a small portion of the purulent part (cheesy, necrotic, bloody-tinged) of sputum with an applicator stick, and smeared on a microscope slide and air dried. The standard procedure of Ziehl Neelson method was employed to confirm the presence of Acid Fast Bacilli. Smears were examined using a light/electrical microscope by scanning up to 100 oil immersion fields before reporting a smear as negative. Acid Fast Bacilli (AFB) in specimens appeared as red rod shaped, but the back ground and other cells stained blue.

5.6.3.3. Culture and Identification

Two LJ media tubes with glycerol were prepared for every sputum sample inoculation to maximize mycobacterial isolation. Culture tubes were incubated at 37°C and growth was observed weekly for a maximum of 8 weeks. Mycobacterial growth was confirmed by typical colony morphology and AFB staining. Growth was checked twice a week for the first one week starting on day-3 post inoculation, then once a week. All specimens showing growth on culture were confirmed by smear microscopy with ZN staining and positive by SD BIOLINE MPT64 Rapid test were reported as culture positive for *Mycobacterium tuberculosis* complex. *M. tuberculosis* colonies develop well within 3 to 4 weeks as white creamy appearance on LJ media and results were reported immediately after detection and culture media kept up to 8 weeks if no growth was detected (See Annex XI).

5.6.3.4. SD Bioline TB Ag MPT64

SD BIOLINE TB Ag MPT64 is a rapid immuno-chromatographic identification test for the *M. tuberculosis* complex. It detects MPT 64 antigen in *M. tuberculosis* isolates using mouse monoclonal MPT 64 antibody (See Annex XI).

5.6.3.5. Whole blood collection and PBMC isolation

Ten and 15 ml of whole blood were collected from TB patients and apparently healthy individuals, respectively. PBMC were isolated by Ficoll density centrifugation from heparinized blood samples. Collected peripheral bloods from apparently healthy individuals were also used for QFT assay (See Annex XI).

5.6.3.6. Flowcytometry

Freshly isolated PBMC were stained with surface antibodies (CD19-Percp, CD21- PE-Cy7, CD27-FITC, CD23-Pacific Blue, CD24-Pacific Blue, CD5-PE, sIgM-APC, CD38-APC, sIgD-PE). All antibodies were purchased from (BD Bioscience, San Jose, CA, USA).Analyzed by BD FACS Canto II with Diva software and FlowJo (Version 9.6.2) (See Annex XI).

Table 1: Definitions of B cells subsets

Parameter	Subset
$CD19^{+}$	B cell
$CD19^{+}CD27^{-}CD21^{+}$	Naïve B cell
$CD19^{+}CD27^{-}CD21^{-}$	Tissue-like (Atypical) memory B cell
$CD19^{+}CD27^{+}CD21^{-}$	Activated memory B cell
$CD19^{+}CD27^{+}CD21^{+}$	Resting (Classical)Memory B cells
$CD19^{+}CD27^{+}CD21^{+}sIgM^{+}sIgD^{+}$	Non-class switched memory B cell
$CD19^{+}CD27^{+}CD21^{+}sIgM^{-}sIgD^{-}$	Class switched memory B cell
$CD19^{+}CD27^{+}CD21^{+}sIgM^{+}sIgD^{+}CD23^{-}$	Circulating Marginal Zone
$CD19^{+}CD24^{hi}CD38^{hi}$	Regulatory B cells
$CD19^{+}CD24^{hi}CD38^{hi}CD5+$	Regulatory B cells

5.6.3.6.1. Gating strategy

Flow cytometry data analysis is fundamentally based upon the principle of gating. Gates and regions are placed around populations of cells with common characteristics, usually forward scatter, side scatter and marker expression, to investigate and quantify these populations of interest (30).

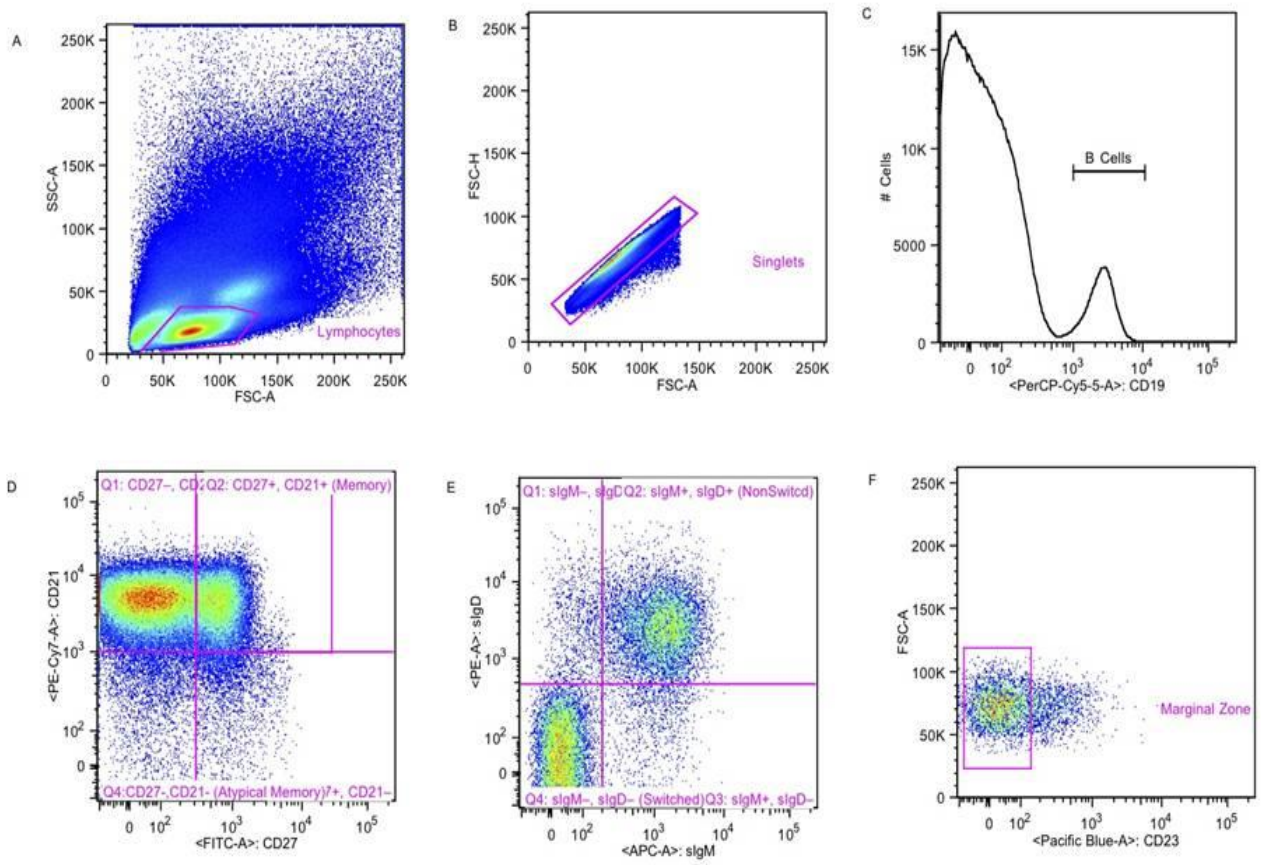

Figure 1: Typical example of gating Strategy memory B cells subsets: (**A**) the first step is lymphocyte gate using forward scatter (FSC) and Side Scatter (SSC,) **(B)** followed by gate on singlets based on FSC-height and area. **(C)**Subsequently, B-cells are identified using CD19. (**D)** Memory B cell subsets are identified using CD27 and CD21 within a total B-cell gate. (**E)** Class switched and non-class switched memory B cell gate on resting memory B-cell subsets. (**F)** Circulating Marginal zone B cells gate on non-class switched memory B cell

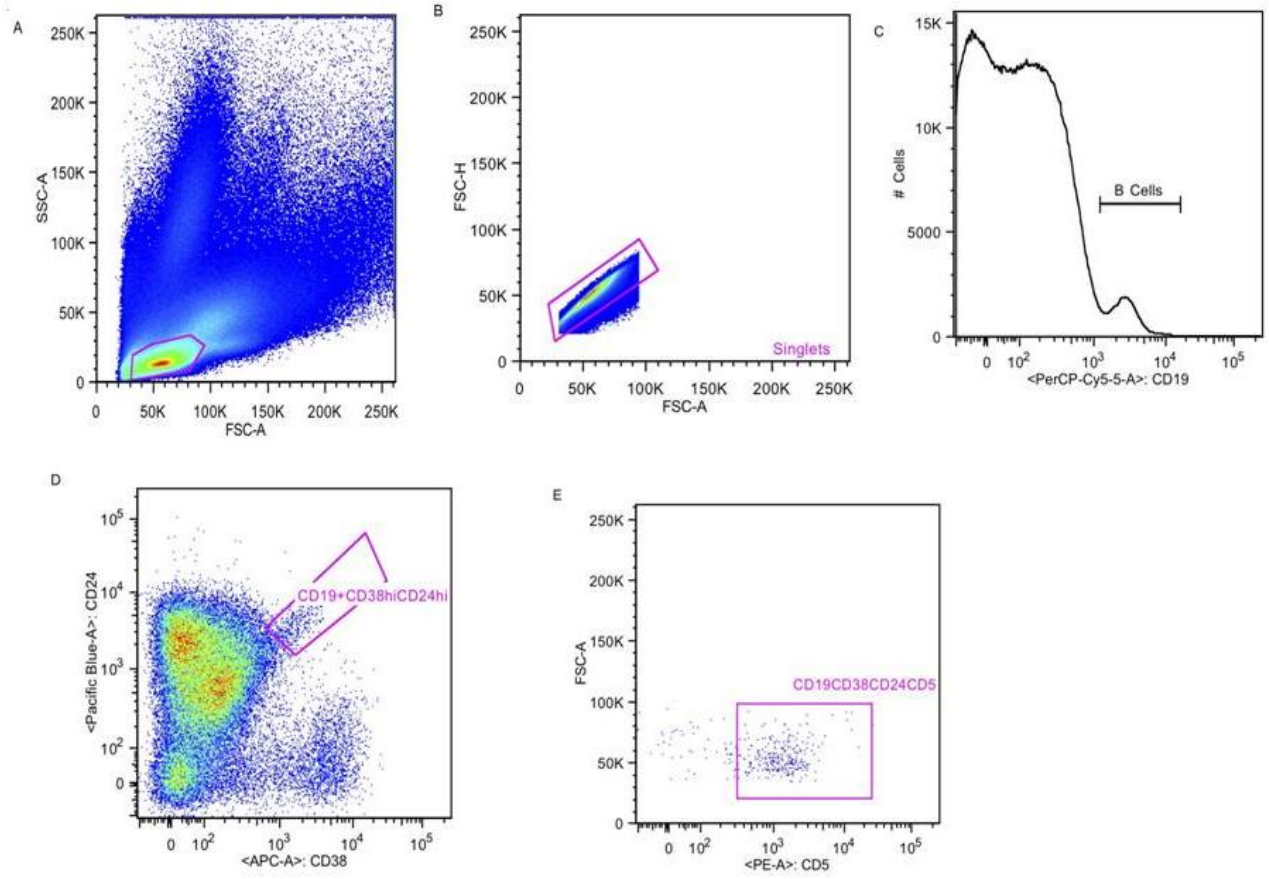

Figure 2: Typical example of gating for Strategy for regulatory B cell: (A) The first step is lymphocyte gate using FSC and SSC, **(B)** followed by gate on singlet based on FSC-height and area. **(C)** Subsequently, B-cells are identified using CD19. **(D)** Regulatory B cell subsets are identified using CD24 and CD38 within a total B-cell gate.**(E)** Alternative subset of regulatory B cells gate on CD24 and CD 38($CD19^{+}CD24^{hi}CD38^{hi}CD5^{+}$).

5.6.3.7. QuantiFERON TB Gold Plus assay

The QuantiFERON-TB Gold Plus (QFT-Plus) assay is an *in vitro* diagnostic test using a peptide cocktail simulating ESAT-6 and CFP-10 proteins to stimulate cells in heparinized whole blood obtained from healthy donors. The assay was performed according to the manufacturer's instructions using the QFT-TB Gold Plus blood collection tubes (622222) composed of Nil, TB 1 antigen, TB 2 antigen and Mitogen tubes and the QFT-TB Gold plus ELISA kit (5501188).

Optical density was read using microplate reader with 450 nm filter and with a 620 nm to 650nm reference filter. The data was analyzed using the QFT-TB Gold plus Analysis Software (See Annex XI).

5.7. Data quality Assurance

Data quality was addressed by following standard operating procedures carefully for all laboratory tests. Every laboratory tests for this study performed by skilled and trained laboratory technologists and the quality of this study was followed by supervisors. Reagents and PBMC were optimized for flowcytometry. Quality of LJ medium was assured by sterility checking and inoculating of known MTB isolate. The original data collected from each of health centers were pooled to AHRI data base and data entry was held by trained professionals. The quality of generated data was assured by double entry. The data is confidential and also maintained at the data base of AHRI.

5.8. Data analysis and interpretation

Data was analyzed by JMP software. A difference in the frequency of B cell subsets among the groups was analyzed using the One-way Anova and Kruskal-Wallis tests was used for comparison between two groups. Data is presented as median (Interquartile range) and a P value of less than 0.05% was taken as a statistically significant difference.

5.9. Ethical considerations

Ethical approval was obtained from AAU department of SMLT, AACAHB and / AHRI/ ALERT Ethics Review Committee. All participants gave informed consent prior to data and specimen collection. For all study participants, ethical issues, privacy, potential risk, safety, confidentiality, beneficence and justice was strictly observed in order to ensure the study is under the legal frame-work of AAU department of MLT, AHRI and AACAHB Ethics Review Board.

5.10. Dissemination of results

The findings of this study will be presented to AAU department of MLT and AHRI. The information will be presented in different national and international scientific seminars and workshops for dissemination and it will also be submitted to journals for possible publication.

5.11. Operational definitions

Latent Tuberculosis infection: LTBI group in this study is defined as apparently health and a positive QFT test result.

Healthy Control: QFT negative and apparently healthy participants.

Active pulmonary TB: Active TB patients in this study were those diagnosed as smear positive by microscopy and culture.

Subsets of B-cells: specific phenotypes of B cells generated through the differentiation of early B-cell precursors into mature B-lymphocytes in the bone marrow (BM) and antigen-triggered maturation of germinal center B-cells into memory B-lymphocytes and plasmablasts in lymphoid tissues.

Biomarkers: broad sub-classification of medical signs (outside observations from the patient that objectively quantifies its medical state) which can be measured in an accurate and reproducible fashion.

TB biomarker: are ideally markers capable of distinguishing between active TB disease and latent tuberculosis infection, returning to basal levels during treatment, reproducibly predicting clinical outcomes (cure, relapse or sterilizing cure of *M. tuberculosis* infection) in diverse population settings and finally, predicting the efficacy of vaccines and serve as end points in clinical trials.

6.0. Results

6.1. Demographic and clinical characteristics of study participants

A total of 16 TB cases, 17 LTBI cases and 19 health controls were involved in this study. There were more males than females in each group with no statistically significance difference among the groups (p = 0.8727). The median age was 26, 29 and 26 years for TB cases, LTBI and HC, respectively. Body Mass Index (BMI) was significantly lower in active TB cases than LTBI and HCs (p = 0.0007).

Table 2: Demographics characterization of study participants, in selected health centers, Addis Ababa, Ethiopia, 2018

Variables	TB (n=16)	LTBI (n=17)	HC (n=19)	P value
Sex				0.8727
Male	10	10	12	
Female	6	7	7	
Age				
Median (IQR)	26(21-35)	29(26-33)	26(22-28)	0.2344
BMI				
Median (IQR)	18.75(17.1-20.5)	21.7(20-25.35)	22(20-24)	0.0007

TB: Tuberculosis; HC: Healthy control; LTBI: Latent tuberculosis infections; IQR: Interquartile Range; BMI: Body mass index

6.2. Analysis of B cells and B cell subset frequencies during Mtb infection and disease

We analyzed B cell frequencies and B cell subset frequencies to evaluate whether peripheral B cell subset compartment alters between the study groups. Total percentages of peripheral B cells between the three groups were not statistically significant. However, decreased frequencies of B-cells in the circulation of TB patients were observed as compared to HCs group (fig. 3 A).

6.2.1. Naïve B cells frequency among the three groups

Frequencies of naïve B-cells did not significantly differ between the evaluated study participants (p = 0.1156). However, the frequency of naïve B-cells was decreased in patients with active TB and trended a decreased frequency in individuals with LTBI compared to controls (Fig. 3B).

6.2.2. Memory B-cell subsets during TB disease and infection

The percentages of tissue-like (atypical) memory B cells were significantly higher in the TB group when compared to the HC group ($p = 0.0036$) and LTBI group ($p = 0.0017$) (Table 3**)**. Resting memory B-cell frequencies were significantly decreased in active TB patients when compared to LTBI and HC; $p<.0001$ (fig.3C) and the relative fraction of B-cells with class switched memory phenotype was increased in active TB as compared to HC ($p = 0.0118$) (fig. 3G).There were no significant difference between active TB patients and LTBI ($p = 0.4937$). In contrast, significantly decreased proportions of non-class switched memory B cells were observed in the TB group as compared to the HC group ($p = 0.0021$) and there was no statistically significant difference between TB and LTBI group ($p = 0.1213$) (fig. 3F). There was no statistically significant difference in percentage of activated memory B cells among the three groups. However, Proportions of activated memory B cells in active TB patients were higher than LTBI ($p = 0. 4280$) and HC ($p =0.0569$) (fig. 3 D).

6.2.3. Circulating Marginal Zone (CMZ) B-cells distinguish active TB from LTBI

The relative fraction of CMZ B cells within non-class switched memory B cells were significantly decreased in the TB group as compared to the HC group ($p<.0009$**)** and LTBI ($p = 0.001$) respectively. There were no statistically significant difference observed between LTBI and HC ($p= 0.7393$) (fig. 3 I).

6.2.4. Lower regulatory B-cell (Breg) subsets in active tuberculosis

The proportions of Breg ($CD19^{+}CD24^{hi}CD38^{hi}$) were similar among the three groups. We also assessed the alternative ($CD19^{+}CD24^{hi}CD38^{hi}CD5^{+}$) and observed significantly lower Bregs among active TB patients when compared to LTBI ($p = 0.0042$) and also between TB and HCs ($p = 0.0044$) and there were no statistically significant difference between HCs and LTBI ($p= 0.5792$)(fig. 3 H).

Table 3: Percentage of B cells subsets [Median (IQR)] in the study participants Addis Ababa, Ethiopia, 2018.

B cells subsets %	HCs	LTBI	TB	P value
Total B cell	3(1.6-5.3)	1.9(1.0-3.3)	1.5(0.8-5.2)	0.2407
Naïve B cell from total B cells	70.7(64-78)	64.7(56.6-71.9)	60.9(45-74.7)	0.1156
TLM from total B cells	11.5 (6.4-16.2)	10.5(8.5-15.3)	20.8(13.6-34.3)	0.0024
AMB from total B cells	5.3 (3.8-7.3)	6.9(4.8-11.3)	9.9(4.1-16.7)	0.1134
RMB from total B cells	10.9 (8.3-14)	15(11.2-19.5)	3.2(2.2-5.3)	<.0001
CSMB from RMB cells	62.7 (54.9-68.7)	71.4(65.6-76.3)	70.4(66.3-84.4)	0.0065
NCSMB from RMB cells	27.6 (21.4-35)	19.5(14.1-23.2)	12.9(6.1-25.6)	0.0011
CMZ from total cells	2.3(1.98-3.7)	2.1(1.49-3.9)	0.3(0.2-0.7)	<.0001
Breg from total B cells	13(7.0-17.8)	7.64(3.6-14.2)	7.47(3.7-10.5)	0.1635
($CD19^{+}$ $CD24^{hi}CD38^{hi}$ $CD5^{+}$) Breg subset from Breg cells	49.3(41.367.6)	47.9(41.3-58.2)	34.5(25-42.5)	0.0043

IQR: Interquartile Range; HC: Healthy Control; LTBI: latent TB infection; TB: tuberculosis; TLM: Tissue like memory B cells; AMB: Activated memory B cells; RMB: Resting memory B cells; CSMB: Class switched memory B cells; NCSMB: Non-class switched memory B cells; CMZ: Circulating marginal zone; Breg: Regulatory B cells

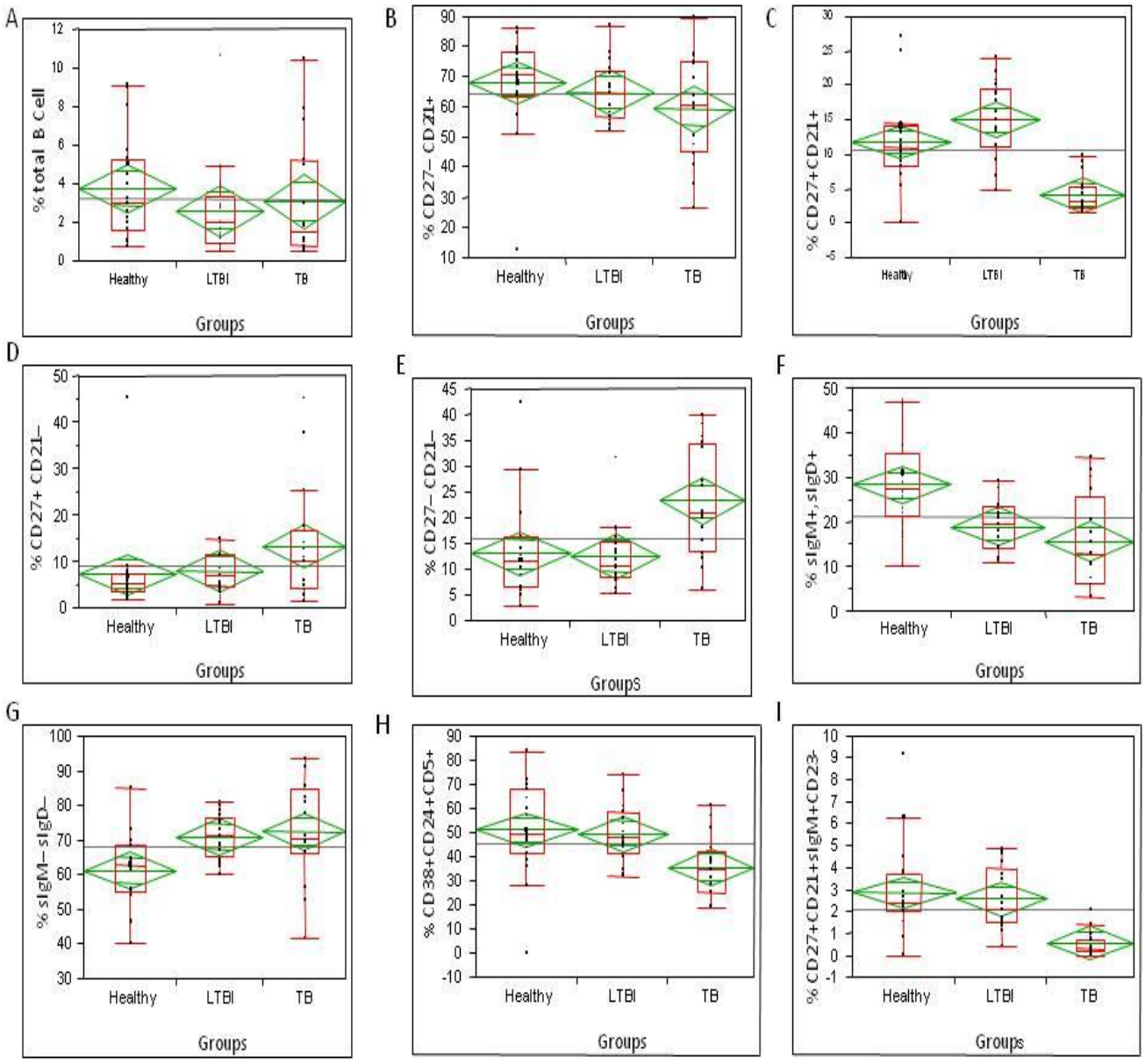

Figure 3: Frequency of B cell subsets by groups. **A:** The proportions of total B cells in healthy controls (HCs), Latent TB infection (LTBI) and Tuberculosis (TB) **B**: The proportions of naïve B cells in HCs, LTBI and TB. **C:** The proportions of resting memory B cells in HCs, LTBI and TB. **D:** The proportions of activated memory B cells in HCs, LTBI and TB. **E:** The proportions of tissue-like memory B cells in HCs, LTBI and TB**. F:** The proportions of non-class switched memory B cells in HC, LTBI and TB. **G:** The proportions of class switched memory B cells in HCs, LTBI and TB. **H:** The proportions of regulatory B cells in HCs, LTBI and TB and **I:** The proportions of circulating marginal zone B cells in HCs, LTBI and TB

7.0. Discussion

The significance of B-cells in the control of MTB infection in humans has been under studied compared to the large number of studies assessing the contribution of T-cells. Literature on B-cells in TB has suggested that these cells, apart from the antibodies they produce, may be involved in disease pathogenesis but data are often contradictory (12, 14, 18-20).We therefore decided to analyze phenotype of B-cells during MTB infection and disease; active pulmonary TB, LTBI and healthy control individuals. In order to investigate the role of B cell and their subsets in TB infection, we characterized frequencies of B cells and B cell subsets defined by ten markers and compared levels between TB patients, latently infected persons, and healthy controls.

Most of the previous studies done on TB patients characterized B cells percentage and total number as ($CD19^{+}$) and majority reported a lower frequency in active untreated TB patients than healthy controls (18, 19) and few showed higher frequency of B cells in PTB patients (20). However, we did not observe any significant difference in the frequency of total B cells among the groups.

Memory B-cells are subtypes of B-cells that are formed within the germinal centers following infection. They proliferate and differentiate into antibody producing plasma cells also called effectors B-cells in response to re-infection (31). Levels of memory B cells were assessed in our study among the evaluated groups. We observed a significant decrease in Resting (Classical) memory B-cell frequencies in active TB patients when compared to LTBI and HCs. In line with a previous study on TB (29) and contrary to a report on common variable immuno-deficiency (CVID) (32); we found increased frequencies of class switched memory B cells in the circulation of active TB patients than in HCs. This could be due to rapid differentiation of memory B cells into plasmablasts that produce class-switched antibodies which are capable of clearing the infection far more quickly than naïve B-cells (31). A significant difference was not observed with regard to the frequency of activated memory B cells among the groups.

Tissues like memory B cells are found in healthy individuals, at low numbers, suggesting in disease settings these cells are an expansion of normally occurring cells. However, tissue like memory B cells do not actively secrete Abs or differentiate into Antibody-secreting cells (ASCs) and also, they exhibit reduced B cell receptor (BCR)-mediated signaling resulting in markedly

impaired BCR-triggered $Ca2^{+}$ mobilization, proliferation and cytokine production (33,34). In line with our report, phenotypic studies revealed that $CD21^{-}CD27^{-}$ tissue like memory B cells, similar in phenotype to $CD11c^{+}$ B cells, are also increased in the peripheral blood of patients with active TB than LTBI and HC, and also with the frequency of this population returning to control levels following treatment (19). Moreover, increased proportion of tissue like memory B cells have been reported in chronic infections such as in hepatitis C virus (35) and malaria infections (36). In contrary to our findings, decreased proportions of TLM B cells have been reported in untreated erythema nodosum leprosum (ENL) (31).

In line with study reported by du Plessis WJ *et al* (29) we found significantly lower fraction CMZ B cells in active untreated TB patients than HCs. This could have been due rapidly differentiation of CMZ B cells into plasmablasts that produce large amounts of IgM after interacting with antigens (MTB) exposed on macrophages, DCs or neutrophils (37).

A distinct set of cell surface markers to specifically identify regulatory B cells (Breg) has not been elucidated. Because numerous cell surface markers have been associated with regulatory B cells (Breg) the use of a combination of markers has been suggested as a better approach to isolating Breg populations. However, IL-10 secretion is crucial for the identification of Breg, and is thought to contribute to their suppressive function (38).Therefore, in our study we assessed $CD19^{+}CD24^{hi}CD38^{hi}$ Breg and alternative $CD19^{+}CD24^{hi}CD38^{hi}CD5^{+}$Breg marker which are main source of IL-10. In consistent with a previous report by Joosten and colleagues (19) we found $CD19^{+}$CD24hiCD38hi Breg were not significantly different among the three study groups. However, we found that $CD19^{+}CD24^{hi}CD38^{hi}CD5^{+}$were significantly decreased in active TB patients compared to LTBI ($p = 0.0102$) and as well as HCs ($p = 0.0039$). In contrary to our finding, Zhang M *et al* reported significantly higher frequencies of $CD19^{+}CD5^{+}CD1d^{+}$ B cells with stronger suppressive activity in TB patients than healthy donors (27). Study conducted in *M. tuberculosis*-infected mice indicated that there was enhanced lung cell production of IL-10(14). Therefore, the decreased frequencies of Breg cells in the circulation of TB patients could have been the result of B-cell sequestration to the site of disease.

8.0. Strength and Limitations of the study

8.1. Strength

Most studies mainly focus on total B cells (CD19+) however, in this study B cells subpopulations were included. As showed in the results section, B cell subsets distinguish active TB from LTBI.

8.2. Limitation

This study is not supported by functional study. However, characterization of B cells and their subset populations in PTB is the first study in Ethiopia.

9. Conclusion and Recommendations

9.1. Conclusion

The study has shown that the percentages of resting memory B cells, non-class switched memory B cells and CMZ B cells and Bregs ($CD19^{+}$ $CD24^{hi}CD38^{hi}$ $CD5^{+}$) were significantly lower in active untreated TB cases than in LTBI and healthy controls (HCs) while class switched memory B cells and atypical memory B cells had higher proportions in active untreated TB cases when compared with LTBI and HCs. The findings of this study showed that B cells markers distinguish active untreated TB cases from LTBI. Profile changes in B cells subsets during *Mycobacterium tuberculosis* infection and disease indicated that the markers are promising for clinical application as potential biomarkers for active TB disease and LTBI identification.

9.2. Recommendations

A large scale longitudinal study is required to confirm and translate this finding into clinically applicable tests.

10. References

1. World Health Organization. Global Tuberculosis Report 2017.
2. Ahmad S. Pathogenesis, immunology, and diagnosis of latent *Mycobacterium tuberculosis* infection. *Clin Dev Immunol*. 2011 Dec;2011:814943.
3. Raja A. Immunology of tuberculosis. *Indian J Med Res*. 2004 Oct 1; 120(4):213-32.
4. Sinha P, Gupta A, Prakash P, Anupurba S, Tripathi R, Srivastava GN. Differentiation of *Mycobacterium tuberculosis* complex from non-tubercular mycobacteria by nested multiplex PCR targeting IS6110, MTP40 and 32kD alpha antigen encoding gene fragments. *BMC Infect Dis.* 2016;16:123.
5. Johnson MM, Odell JA. Non-tuberculous mycobacterial pulmonary infections. *J Thorac Dis.* 2014;6(3):210-20.
6. Sutherland JS, Lalor MK, Black GF, Ambrose LR, Loxton AG, Chegou NN, *et al.* Analysis of host responses to *Mycobacterium tuberculosis* antigens in a multi-site study of subjects with different TB and HIV infection states in sub-Saharan Africa. *PLoS One*. 2013;8(9):e74080.
7. Helbig S, Rekhtman S, Dostie K, Casler A, Schneider T, Hochberg NS *et al* . B cell responses in older adults with latent tuberculosis: Considerations for vaccine development. *Glob Vaccines Immunol*. 2016; 1(2):44-452.
8. Cavalcanti YV, Brelaz MC, Neves JK, Ferraz JC, Pereira VR. Role of TNF-Alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis. *Pulm Med*. 2012;2012: 745483.
9. Campbell IA, Bah-Sow O. Pulmonary tuberculosis: diagnosis and treatment. *BMJ*. 2006;332(7551):1194-7.
10. Plessis WJd, Gerhard Walzl, Loxton AeG. B cells as multi-functional players during Mycobacterium tuberculosis infection and disease. *Tuberculosis.* 2016:118e25.
11. Achkar JM, Chan J, Casadevall A. B cells and Antibodies in the Defense against *Mycobacterium tuberculosis* infection. *Immunol Rev*. 2015 March; 264(1):167–81.
12. Kozakiewicz L, Phuah J, Flynn J, Chan J. The role of B cells and humoral immunity in *Mycobacterium tuberculosis* infection. *Adv Exp Med Biol*. 2013;783:225-50.
13. Veneri D, Ortolani R, Franchini M, Tridente G, Pizzolo G, Vella A. Expression of CD27 and CD23 on peripheral blood B lymphocytes in humans of different ages. *Blood Transfus*. 2009;7(1):29-34.

14. Maglione PJ, Chan J. How B cells shape the immune response against Mycobacterium tuberculosis. *Eur J Immunol*. 2009;39(3):676-86.

15. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. Immunological biomarkers of tuberculosis. *Nat Rev Immunol*. 2011;11(5):343-54.

16. Kunnath-Velayudhan S, Gennaro ML. Immunodiagnosis of tuberculosis: a dynamic view of biomarker discovery. *Clin Microbiol Rev*. 2011;24(4):792-805.

17. Tucci P, Gonzalez-Sapienza G, Marin M. Pathogen-derived biomarkers for active tuberculosis diagnosis. *Front Microbiol*. 2014;5:549.

18. Hernandez J, Velazquez C, Valenzuela O, Robles-Zepeda R, Ruiz-Bustos E, Navarro M, *et al*. Low number of peripheral blood B lymphocytes in patients with pulmonary tuberculosis. *Immunol Invest*. 2010;39(3):197-205.

19. Joosten SA, Meijgaarden KEv, Nonno Fd, Baiocchini A, Petrone L, Vanini V, *et al.* Patients with Tuberculosis Have a Dysfunctional Circulating B-Cell Compartment, Which Normalizes following Successful Treatment. *PLoS Pathog*. 2016;12(6). e1005687.

20. Wu YE, Zhang SW, Peng WG, Li KS, Li K, Jiang JK, *et al*. Changes in lymphocyte subsets in the peripheral blood of patients with active pulmonary tuberculosis. *J Int Med Res.* 2009 Dec; 37(6):1742-9.

21. Li XX, Chen JX, Wang LX, Sun J, Chen SH, Chen JH, *et al.* Profiling B and T cell immune responses to co-infection of *Mycobacterium tuberculosis* and hookworm in humans. *Infect Dis Poverty*. 2015;4:20.

22. Tebruegge M, Ritz N, Koetz K, Noguera-Julian A, Seddon JA, Welch SB, *et al.* Availability and use of molecular microbiological and immunological tests for the diagnosis of tuberculosis in europe. *PLoS One*. 2014;9(6):e99129.

23. van Crevel R, Ottenhoff THM, van der Meer JWM. Innate Immunity to *Mycobacterium tuberculosis*. *Clinical Microbiology Reviews*. 2002;15(2):294-309.

24. Lalvani A, Millington KA. T Cells and Tuberculosis: Beyond Interferon-gamma. *J Infect Dis.* 2008;197(7):941-3.

25. Vani J, Shaila MS, Rao MK, Krishnaswamy UM, Kaveri SV, Bayry J. B lymphocytes from patients with tuberculosis exhibit hampered antigen-specific responses with concomitant overexpression of interleukin-8. *J Infect Dis*. 2009;200(3):481-2.

26. van Rensburg IC, Loxton AG. Killer (FASL regulatory) B cells are present during latent TB and are induced by BCG stimulation in participants with and without latent tuberculosis. *Tuberculosis* (Edinb). 2018;108:114-7.

27. Zhang M, Zheng X, Zhang J, Zhu Y, Zhu X, Liu H, *et al*. CD19+CD1d+CD5+ B cell frequencies are increased in patients with tuberculosis and suppress Th17 responses. *Cellular Immunology*. 2012;274: 89–97.

28. Barcelos W, Martins-Filho OA, Guimaraes TM, Oliveira MH, Spindola-de-Miranda S, Carvalho BN, *et al*. Peripheral blood mononuclear cells Immunophenotyping in pulmonary tuberculosis patients before and after treatment. *Microbiol Immunol.* 2006; 50(8):8.

29.du Plessis WJ, Keyser A, Walzl G, Loxton AG. Phenotypic analysis of peripheral B cell populations during *Mycobacterium tuberculosis* infection and disease. *J Inflamm*. 2016;13:23.

30. BIO-RAD. Flow Cytometry Basics Guide.

31. Negera E, Walker SL, Bekele Y, Dockrell HM, Lockwood DN. Increased activated memory B-cells in the peripheral blood of patients with erythema nodosum leprosum reactions. *PLoS Negl Trop Dis*. 2017; 11(12):e0006121.

32. Vodjgani M, Mirahmadian M, Srrafnejad A, Aghamohammadi A, Parvaneh N, Samadi M, *et al*. Analysis of Class-Switched Memory B Cells in Patients with Common Variable Immunodeficiency and Its Clinical Implications. *J Investig Allergol Clin Immunol*. 2007; Vol. 17 (5):321-8.

33. Karnell JL, Kumar V, Wang J, Wang S, Voynova E, Ettinger R. Role of CD11c (+) T-bet(+) B cells in human health and disease. *Cell Immunol*. 2017; 321 :40-5.

34. Portugal S, Tipton CM, Sohn H, Kone Y, Wang J, Li S, *et al*. Malaria-associated atypical memory B cells exhibit markedly reduced B cell receptor signaling and effector function. *Elife*. 2015 May 8;4: e07218.

35. Charles ED, Brunetti C, Marukian S, Ritola KD, Talal AH, Marks K, *et al*. Clonal B cells in patients with hepatitis C virus-associated mixed cryoglobulinemia contain an expanded anergic CD21low B-cell subset. *Blood*. 2011;117(20):5425-37.

36. Weiss GE, Crompton PD, Li S, Walsh LA, Moir S, Traore B, *et al.* Atypical memory B cells are greatly expanded in individuals living in a malaria-endemic area. *J Immunol*. 2009; 183(3):2176-82.

37. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol*. 2013;13(2):118-32.

38. Jiao Y, Wang X, Zhang T, Sun L, Wang R, Li W, *et al*. Regulatory B cells correlate with HIV disease progression. *Microbiol Immunol*. 2014; 58(8):449-55.

39. Global laboratory initiative. Microbiology laboratory manual .1st ed. 2014.

40. Brown M, Wittwer C. Flowcytometry: principles and clinical applications in hematology. *Clin Chem*. 2000 Aug 1; 46(8):1221-9.

41. QuantiFERON®-TB Gold plus (QFT® ELISA Package Insert. Available from: http://www. QuantiFERON.com

11. Annexes

Annex I: English version of Participants' information sheet for presumptive TB patients

Principal Investigator: Tigist Girma

Name of Organization: AAU, College of Health Sciences, School of Allied Health Sciences, Department of Medical Laboratory Sciences

Name of the sponsors: AHRI and AAU School of Graduate studies.

Title: Phenotypic Characterization of Peripheral B cells in *Mycobacterium tuberculosis* infection and disease in Addis Ababa, Ethiopia.

Aim: B cells are part of protective immune cells against MTB. However, there is a controversial idea on the contribution of B cells in TB. Therefore we are going to assess peripheral B cells phenotype as biomarker for *Mycobacterium tuberculosis* infection which is used to determine significance of B cells.

Study Participants: In this study active TB patients, Latent TB and healthy control age of 18-65 both male and female who fulfill inclusion criteria will participate.

Samples Required: If you agree to participate in the study, you are requested to provide sputum and 10ml of venous blood, before starting the medicine and at end of six month of treatment.

Duration: From Feb. 2018 till stated number of study participants will be recruited.

Procedures to be carried on: For this study to be successful we need your participation. If you are voluntary to participate, you are expected to understand and sign the informed consent. Then, socio demographic and clinical information related TB disease which is important for this study will be taken.

Risk: We are asking you to share with us some personal and confidential information, and you may feel uncomfortable talking about some of the topics. You do not have to answer any question or take part in the interview if you don't wish to do so. We do not expect a definitive risk as a result of sample collection or participating in the study. However, during blood

collection, you may experience some pain and discomfort. Taken blood will be used only for the indicated purpose.

Benefits: In this study, you are not directly benefited however; the result of this study may provide better management of TB in the future.

Compensation: Fifty birr will be given for study participants to compensate transport cost.

Confidentiality: All your personal information collected for the purpose of this study will be kept confidential.

Right: Participation in the study is voluntary, and refusal to participate involves no penalty or loss of benefits to which you are otherwise entitled. The study participants have a right to withhold information, decline to cooperate in the study and refuse provision of specimens.

Approval: This research project will get ethical clearance from Addis Ababa University Department of medical laboratory sciences, AHRI and AACAHB ethics review Board.

Whom to contact: If you have any question or description about this study, you can communicate on the following address:

1. AAU (SMLT) Tel: +251-112-75-51-70.
2. AHRI/ALERT Ethics Review Committee: Tel: +251-113-48-12-89.
3. Principal Investigator: Tigist Girma: Tel: +251-9841048/912371290.

Annex II: Participant information sheet (Amharic version) for presumptive TB patients

ለጥናቱ ተሳታፊ ታካሚዎች የሚሰጥ መረጃ

የተመራማሪዉ ስም፡ ትዕግሰት ግርማ

የተቋሙ ስም፡ አአዩ ፣ የጤና ሳይንስ ኮሌጅ፣የአላይድ ጤና ሳይንስ ት/ት፣ ህክምና ላቦራቶሪ ሳይንስ ክፍል

የጥናቱ በጀት የሚሸፈነው፡ አዲስ አበባ ዩኒቨርስቲ፣አርማውር ሃንሰን የምርምር ተቋም

የጥናቱ አርዕስት፡- በደም ዝውውር ውስጥ የሚገኘውን የቢ ህዋስን ዝርያ ለ ተንቀሳቃሽ (ተላላፊ)የቲቢ በሽታ እና ለ ተዳፈነ ቲቢ እንደ አመልካች እንዲውል፣የቲቢ በሽታ ሁልጊዜ በሚከሰትበት አካባቢ፣አዲስ አበባ፣ ኢትዮጵያ።

የጥናቱ ዋና አላማ፡-ሰዉነታችን የሳንባ ነቀርሳን (ቲቢ፡TB) ከሚከላከልባቸው የደም ህዋሶች አንዱ የሆነውን የቢ ህዋስ ነው።ነገር ግን ቢ ህዋስ አስተዋጽዎ አሻሚ ነው።ስለሆነም የቢ ህዋስን ዝርያ በተለያየ የሳንባ ነቀርሳ ማለትም በ ተንቀሳቃሽ የቲቢ በሽታ እና ለ ተዳፈነ ቲቢ እንደ አመልካች እናያለን።እሱም የቢ ህዋስን ጥቅም ያሳየናል።

የጥናቱ ሂደት፡- ለዚህ ጥናት እዉን መሆን የእርስዎን ተሳትፎ እንፈልጋለን። በዚህ ጥናት ለመሳተፍ ፈቃደኛ ከሆኑ የስምምነት ቅጹን መረዳትና መፈረም ይጠበቅብዎታል።ከዛም ለጥናቱ አስፈላጊ የሆኑ ህብረተሰብ ነክ እና የህክምና መረጃዎችን እንወስዳለን ።

የጥናቱ ተሳታፊዎች፡- ተንቀሳቃሽ (ተላላፊ) የቲቢ በሽታ፤ ለተዳፈነ ቲቢ እና ጤናማ ከሆኑ የህብረተሰብ አባላት እድሜቸዉ 18-65የሆኑ ወንድ እና ሴት የማሳተፊያውን መሰፈርት የሚያማሉ ተሳታፊ ይሆናሉ።

የሚፈለገው ናሙና፡- በዚህ ጥናት ለመሳተፍ ከተስማማው አክታ እንድሰጥ እጠየቃለው ።ከዚያም አክታ ሰጥቼ የቲቢ በሽታ መኖሩ ከተረጋገጠ መድሀኒት ከመጀመረ በፊት እና በድጋሚ በስድስት ወር 10 ሚሊ ሊትር ደም እንደምሰጥ ተነግሮኛል።

የጥናቱ ጊዜ፡-እንደ ኢትዮጵያ አቆጣጠር ከ የካቲት 2010 ጀምሮ የተጠቀሰው የጥናቱ ተሳታፊዎች ቁጥር እስከ ሞላ ድረስ።

ሊከሰቱ ስለሚችሉ ስጋቶችና የምችት መጓደሎች፡- ስለግልህይወትዎ የተወሰነ ቃለ-መጠየቅ እናረግሎታለን፤ምንአልባት የተወሰኑት ጥያቄዎች ምቾት ላይሰጡት ይችላሉ፤ ሁሉንም ጥያቄ መመለስ አይጠበቅቦትም፤አለመመለስ ይችላሉ።ለጥናቱ በሚወሰደዉ ናሙና ምክንያት ከትንሽ ስሜት ዉጪ የተለየ ችግር/ስጋት አይኖረዉ ምክንያቱም የጥናቱ ናሙና አወሳሰድ ከተለመደዉ የአገልግሎት አሰጣጥ የተለየ አይደለም። ምንአልባት ደም በሚወሰድበት ጊዜ ትንሽ ህመም /አለመመቸት ሊያጋጥም ይችላል። የሚወሰደው ደም የሚያገለግለው ለተገለጸለት አላማ ብቻ ነው።

ጥቅሞች፡- በዚህ ጥናት ላይ እርሰዎ ቀጥተኛ ተጠቃሚ አይሆኑም ነገርግን ይህ ጥናት ለወደፊት የተሻለ የቲቢ አያያዝ/ህክምና እንዲኖር ሊያደርግ ይችላል።

ማካካሻ፡ የትራንስፖርት ክፍያን ለማካካስ ሀምሳ ብር ለትናቱ ተሳታፊዎች ይሰጣል ።

ሚስራጥዊነት፡- ለጥናቱ ተብለው የተሰባሰቡ የግልዎ መረጃ ሚስጢራዊነቱ የተጠበቀ ነው።

የተሳታፊዉ መብት፡- ፈቃደኛ ካልሆኑ በዚህ ጥናት ያለመሳተፍ መብትዎ የተጠበቀ ነዉ። በዚህም ምክንያት ከጤና ጣቢያዉ በሚያገኙት አገልግሎት ላይ ምንም አይነት ተጽዕኖ አይደርስብዎትም።መረጃ ለራሳችው መያዝም ሆነ ናሙና አለመስጠትም ትችላላችው።

የጥናቱ ፈቃድ/ህጋዊነት፡- የዚህ ጥናት ፍቃድ በ አዲስ አበባ ዩኒቨርስቲ (ህክምና ላቦራቶሪ ሳይንስ ዲፓርትመንት)፤በአህሪ/አለርት እና በአዲስ አበባ ከተማ አስተዳደር ጤና ቢሮ ስነ ምግባር ቅኝት ኮሚቴ ሂጋዊነቱን ያገኛል።

መረጃ ስለማግኘት፡- ይህን ጥናት አስመልክቶ ምንም አይነት ጥያቄ ወይም ማብራሪያ ቢያስፈልግዎት፤

1. አዲስ አበባ ዩኒቨርሲቲ(ህክምና ላቦራቶሪ ሳይንስ ዲፓርትመንት)፤ስ.ቁ.፤- +251-112-75-51-70

2. አህሪ/አለርት ምርምር ስነ ምግባር ቅኝት ኮሚቴ ጽ/ቤት ፤ስ.ቁ፤ +251-11-3481289/ +251-11-348 3752

3.የጥናቱተመራማሪ፡-ትዕግሰትግርማ፤ስ.ቁ፤0912841048/091237129

Annex III: English version of Participants' information sheet for apparently healthy control

Principal Investigator: Tigist Girma

Name of Organization: AAU, College of Health Sciences, School of Allied Health Sciences, Department of Medical Laboratory Sciences

Name of the sponsor/s: AAU School of Graduate studies and AHRI

Title: Phenotypic Characterization of Peripheral B cells in *Mycobacterium tuberculosis* infection and disease in Addis Ababa, Ethiopia

Aim: B cells are part of protective immune cells against *MTB*. Therefore we are going to assess peripheral B cells phenotype as biomarker for *Mycobacterium tuberculosis* infection which is used to determine significance of B cells.

Study Participants: In this study active TB patients, Latent TB and healthy control age of 18-65 both male and female who fulfill inclusion criteria will participate.

Samples Required: If you agree to participate in the study, you requested to provide 15ml of venous blood only at the time of enrollment.

Duration: From Feb. 2018 till stated number of study participants will be recruited.

Procedures to be carried on: For this study to be successful we need your participation. If you are voluntary to participate, you are expected to understand and sign the informed consent. Then, socio demographic and clinical information related TB infection which is important for this study will be taken.

Risk: We are asking you to share with us some personal and confidential information, and you may feel uncomfortable talking about some of the topics. You do not have to answer any question or take part in the interview if you don't wish to do so. We do not expect a definitive risk as a result of sample collection or participating in the study. However, during blood collection, you may experience some pain and discomfort. Taken blood will be characterized only for the indicated purpose.

Benefits: In this study, you are not directly benefited however; the result of this study may provide better management of TB in the future.

Compensation: Fifty birr will be given for study participants to compensate transport cost.

Confidentiality: All your personal information collected for the purpose of this study will be kept confidential.

Right: Participation in the study is voluntary, and refusal to participate involves no penalty or loss of benefits to which you are otherwise entitled. The study participants have a right to withhold information, decline to cooperate in the study and refuse provision of specimens.

Approval: This research project will get ethical clearance from Addis Ababa University Department of medical laboratory sciences, AHRI and AACAHB Ethics Review Board.

Whom to contact: If you have any question or description about this study, you can communicate on the following address:

1. AAU (SMLT) Tel: +251-112-75-51-70.

2. AHRI/ALERT Ethics Review Committee: Tel: +251-113-48-12-89.

3. Principal Investigator: Tigist Girma: Tel: +251-9841048/912371290.

Annex IV: Amharic version of participant information sheet for apparently healthy control

ለጥናቱ ተሳታፊዎች የሚሰጥ መረጃ

የተመራማሪዉ ስም፡ ትዕግሰት ግርማ

የተቋሙ ስም፡ አአዩ ፣ የጤና ሳይንስ ኮሌጅ፣የአላይድ ጤና ሳይንስ ት/ት፣ ህክምና ላቦራቶሪ ሳይንስ ክፍል

የጥናቱ በጀት የሚሸፈነው፡ አዲስ አበባ ዩኒቨርስቲ፣አርማውር ሃንሰን የምርምር ተቋም

የጥናቱ አርዕስት፡- በደም ዝውውር ውስጥ የሚገኝውን የቢ ህዋስን ዝርያ ለ ተንቀሳቃሽ (ተላላፊ)የቲቢ በሽታ እና ለ ተዳፈነ ቲቢ እንደ አመልካች እንዲውል፣የቲቢ በሽታ ሁልጊዜ በሚከሰትበት አካባቢ፣አዲስ አበባ፣ ኢትዮጵያ።

የጥናቱ ዋና አላማ፡- ሰዉነታችን የሳንባ ነቀርሳን (ቲቢ፡TB) ከሚከላከልባቸው የደም ህዋሶች አንዱ የሆነውን የቢ ህዋስን ዝርያ በተለያየ የሳንባ ነቀርሳ ማለትም በ ተንቀሳቃሽ የቲቢ በሽታ እና ለ ተዳፈነ ቲቢ እንደ አመልካች እናያለን፡፡እሱም የቢ ህዋስን ጥቅም ያሳየናል፡:

የጥናቱ ሂደት፡- ለዚህ ጥናት እዉን መሆን የእርስዎን ተሳትፎ እንፈልጋለን፡፡ በዚህ ጥናት ለመሳተፍ ፈቃደኛ ከሆኑ የስምምነት ቅጹን መረዳትና መፈረም ይጠበቅብዎታል፡፡ከዛም ለጥናቱ አስፈላጊ የሆኑ ህብረተሰብ ነክ እና የህክምና መረጃዎችን እንወስዳለን ፡፡

የጥናቱ ተሳታፊዎች፡-ተንቀሳቃሽ (ተላላፊ) የቲቢ በሽታ፤ ለ ተዳፈነ ቲቢ እና ጤናማ ከሆኑ የህብረተሰብ አባላት እድሜቸው 18 -65 የሆኑ ወንድ እና ሴት የማሳተፊያውን መሰፈርት የሚያማሉ ተሳታፊ ይሆናሉ፡፡

የሚፈለገው ናሙና፡- በዚህ ጥናት ለመሳተፍ ከተስማማው 15 ሚሊ ሊትር ደም አንድ ጊዜ እንድሰጥ እጠየቃለው ፡፡

የጥናቱ ጊዜ፡-እንደ ኢትዮጵያ አቆጣጠር ከ የካቲት 2010 ጀምሮ የተጠቀሰው የጥናቱ ተሳታፊዎች ቁጥር እስከ ሞላ ድረስ ፡፡

ሊከሰቱ ስለሚችሉ ስጋቶችና የምችት መጓደሎች፡- ስለግልህይወትዎ የተወሰነ ቃለ-መጠየቅ እናረግሎታለን፤ምንአልባት የተወሰኑት ጥያቄዎች ምቾት ላይሰጦት ይችላሉ፤ ሁሉንም ጥያቄ መመለስ አይጠበቅቦትም፤አለመመለስ ይችላሉ፡፡ለጥናቱ በሚወሰደዉ ናሙና ምክንያት ከትንሽ ስሜት ዉጪ የተለየ ችግር/ስጋት አይኖረዉ ምክንያቱም የጥናቱ ናሙና አወሳሰድ ከተለመደዉ የአገልግሎት አሰጣጥ የተለየ አይደለም፡፡ ምንአልባት ደም በሚወሰድበት ጊዜ ትንሽ ህመም /አለመመቸት ሊያጋጥም ይችላል፡፡ የሚወሰደው ደም የሚያገለግለው ለተገለጸለት አላማ ብቻ ነው፡፡

ጥቅሞች፡- በዚህ ጥናት ላይ እርሰዎ ቀጥተኛ ተጠቃሚ አይሆኑም ነገርግን ይህ ጥናት ለወደፊት የተሻለ የቲቢ አያያዝ/ህክምና እንዲኖር ሊያደርግ ይችላል፡፡

ማካካሻ፡ የትራንስፖርት ክፍያን ለማካካስ ሀምሳ ብር እንሰጥዎታለን፡፡

ሚስራጥዊነት፡- ለጥናቱ ተብለው የተሰባሰቡ የግልዎ መረጃ ሚስጢርነቱ የተጠበቀ ነው፡፡

የተሳታፊዉ መብት፡- ፈቃደኛ ካልሆኑ በዚህ ጥናት ያለመሳተፍ መብትዎ የተጠበቀ ነዉ፡፡ በዚህም ምክንያት ከጤና ጣቢያዉ በሚያገኙት አገልግሎት ላይ ምንም አይነት ተጽዕኖ አይደርስብዎትም፡፡ መረጃ ለራሳችው መያዝም ሆነ ናሙና አለመስጠትም ትችላላችው፡፡

የጥናቱ ፈቃድ/ህጋዊነት፡- የዚህ ጥናት ፍቃድ በ አዲስ አበባ ዩኒቨርስቲ (ህክምና ላቦራቶሪ ሳይንስ ዲፓርትመንት) በአህሪ/አለርት እና አዲስ አበባ ከተማ አስተዳደር ጤና ቢሮ ስነ ምግባር ቅኝት ኮሚቴ ህጋዊነቱን ያገኛል፡፡

መረጃ ስለማግኘት፡- ይህን ጥናት አስመልክቶ ምንም አይነት ጥያቄ ወይም ማብራሪያ ቢያስፈልግዎት፡

1. አዲስ አበባ ዩኒቨርሲቲ(ህክምና ላቦራቶሪ ሳይንስ ዲፓርትመንት)፤ስ.ቁ.፤- +251-112-75-51-70፡፡

2. አህሪ/አለርት ምርምር ስነ ምግባር ቅኝት ኮሚቴ ጽ/ቤት ፤ስ.ቁ፤ +251-11-3481289/ +251-11-348 3752፡፡

3.የጥናቱተመራማሪ፡-ትዕግሰትግርማ፤ስ.ቁ፤0912841048/0912371290፡፡

Annex V: English version of Study Participant informed Consent Form for presumptive TB patients

Name of the participant: _____Age _____Sex___

Code _____Study site/Health center _____

I confirm that I have been given adequate information about the research project; Phenotypic Characterization of Peripheral B cells in *Mycobacterium tuberculosis* infection and disease in Addis Ababa, Ethiopia. I have been requested to provide sputum and 10ml of venous blood, before starting the medicine and at end of six month. The researchers informed that there is no major risk associated with participating in the study or providing the requested samples. I have also understood that the results of clinical and laboratory diagnosis will be used for research purposes and the information related to myself/my family will be kept strictly confidential. I am well informed that participation in the study is fully voluntary and I can withdraw anytime without giving any reason. I confirm that all the information provided to me is very clear and has been conveyed by the language that I fully understand. Finally, I declare that I have been given enough time to deliberate before I agree to participate in the study, and I signed this informed consent.

Name of participant.....Signature.....Date……

Name of the physician obtaining consent.....signature: …… date

Name of witness ……Signature……Date…….

Annex VI: Amharic version of Study Participant informed Consent Form presumptive TB patients

ለጥናቱ ተሳታፊ ታካሚዎች የሚሰጥ መረጃ

የተሳታፊው ስም _____እድሜ _____ጾታ ____

የተሳታፊ ቁጥር _____ጤና ጣቢያ ስም_____

በደም ዝውውር ውስጥ የሚገኘውን የቢ ህዋስን ዝርያ ለ ተንቀሳቃሽ (ተላላፊ)የቲቢ በሽታ እና ለ ተዳፈነ ቲቢ እንደ አመልካች እንዲውል፣በሚል የተዘጋጀ ጥናት ላይ እድሳተፍ ተጠይቄ ስለ ጥናቱም ለመረዳት በቂ መረጃ ማግኘቴን አረጋግጣለው። አክታ ሰጥቼ የቲቢ በሽታ መኖሩ ከተረጋገጠ መድሀኒት ከመጀመረ በፊት እና በድጋሚ በስድስት ወር 10 ሚሊ ሊትር ደም እና አክታ እንደምሰጥ ተነግሮኛል።የህክምና መረጃዎች እና የላቦራቶሪ ውጤት ለጥናት እንደሚውል እንዲውም ከእኔ እና ከቤተሰቦቼ ጋር የተያያዙ ጥያቄዎች ሚስጢራዊነቱ እንደሚጠበቅ ተገንዝቤአለው።በጥናት ላይ መሳተፍ በፍቃደኝነት ላይ የተመሰረተ ሲሆን በፈለኩ ጊዜ ምክንያቴን ሳላሳውቅ ማቋረጥ እንደምችል ተረድቻለሁ። የተነገሩኝ ሁሉም መረጃዎች ግልጽ ናቸው እንዲውም በሚገባኝ ቋንቋ ተብራርተውልኛል። በመጨረሻም በጥናት ላይ መሳተፍ ከመስማማቴ በፊት እንዳስብበት በቂ ጊዜ የተሰጠኝ ሲሆን የስምምነት ቅጹ ላይ ፍቃደኝነቴን በፊርማዬ አረጋግጫለሁ።

የተሳታፊ ስም_____ ፊርማ _____ቀን_____

የጥናቱ አስከያጅ ስም_____ ፊርማ _____ቀን_____

ምስክር (ማንበብና መፃፍ ለማይችሉ) _____ቀን_____

Annex VII: English version of Study Participant informed Consent Form for apparently healthy control

Name of the participant: _____Age _____Sex___

Code_ _____Study site/Health center _____

I confirm that I have been given adequate information about the research project; Phenotypic Characterization of Peripheral B cells in *Mycobacterium tuberculosis* infection and disease in Addis Ababa, Ethiopia**.** I have been requested to provide 15ml of venous blood. The researchers informed that there is no major risk associated with participating in the study or providing the requested samples. I have also understood that the results of clinical and laboratory diagnosis will be used for research purposes and the information related to myself/my family will be kept strictly confidential. I am well informed that participation in the study is fully voluntary and I can withdraw anytime without giving any reason. I confirm that all the information provided to me is very clear and has been conveyed by the language that I fully understand. Finally, I declare that I have been given enough time to deliberate before I agree to participate in the study, and I signed this informed consent.

Name of participant.....Signature.....Date……

Name of the physician obtaining consent..... signature: …… date

Name of witness …….Signature……Date…….

Annex VIII: Amharic version of Study Participant informed Consent Form for apparently healthy control

ለጥናቱ ተሳታፊዎች የሚሰጥ መረጃ

የተሳታፊው ስም _____እድሜ _____ጾታ ____

የተሳታፊ ቁጥር _____ጤና ጣቢያ ስም_____

በደም ዝውውር ውስጥ የሚገኘውን የቢ ህዋስን ዝርያ ለ ተንቀሳቃሽ (ተላላፊ)የቲቢ በሽታ እና ለ ተዳፈነ ቲቢ እንደ አመልካች እንዲውል በሚል የተዘጋጀ ጥናት ላይ እድሳተፍ ተጠይቄ ስለ ጥናቱም ለመረዳት በቂ መረጃ ማግኘቴን አረጋግጣለው።በዚህ ጥናት ለመሳተፍ ከተስማማው 15 ሚሊ ሊትር ደም አንድ ጊዜ እንድሰምሰጥ ተነግሮኛል ። የህክምና መረጃዎች እና የላቦራቶሪ ውጤት ለጥናት እንደሚውል እንዲውም ከእኔ እና ከቤተሰቦቼ ጋር የተያያዙ ጥያቄዎች ሚስጢራዊነቱ እንደሚጠበቅ ተገንዝቤአለው።በጥናት ላይ መሳተፍ በፍቃደኝነት ላይ የተመሰረተ ሲሆን በፈለኩ ጊዜ ምክንያቴን ሳላሳውቅ ማቋረጥ እንደምችል ተረድቻለሁ። የተነገሩኝ ሁሉም መረጃዎች ግልጽ ናቸው እንዲውም በሚገባኝ ቋንቋ ተብራርተውልኛል። በመጨረሻም በጥናት ላይ መሳተፍ ከመስማማቴ በፊት እንዳስብበት በቂ ጊዜ የተሰጠኝ ሲሆን የስምምነት ቅጹ ላይ ፍቃደኝነቴን በፊርማዬ አረጋግጫለሁ።

የተሳታፊ ስም_____ ፊርማ _____ቀን_____

የጥናቱ አስከያጅ ስም_____ ፊርማ _____ቀን_____

ምስክር (ማንበብና መፃፍ ለማይችሉ) _____ቀን_____

Annex IX: English version of questionnaire for presumptive TB patients

Questioner: Subject identification and Clinical Examination form (TB patients)

STUDY SITE:_____ Date (EC): _____/_____/_____

1. Socio-demographic data

Patient ID ☐☐☐☐☐ Medical Record No ☐☐☐☐☐

Gender: 1. M ☐ 2. F ☐ Age (years): ☐☐

Education: 1. No formal education ☐ 2. Elementary ☐ 3. Secondary ☐ 4. Diploma& above ☐

Occupation: 1. Employed ☐ 2. Self employed ☐ 3. Not employed ☐

BMI: Height (cm) ☐☐☐ Weight (kg) ☐☐

Vital sign: Temp (^{0}C) ☐☐.☐ PR ☐☐ RR ☐☐ BP: SP ☐☐☐ DP ☐☐☐

2. TB status :

BCG scar: 1. Yes ☐ 2. No ☐

Symptoms: 1. Cough ☐ 2. Fever ☐ 3. Loss of weight ☐ 4. Shortness of breath ☐

5. Loss of appetite ☐ 6. Night sweats ☐ 7. Other, _____

AFB smear: 1. P ☐ 2. N ☐ If Pos, Bacillary load: 1+ ☐ 2+ ☐ 3+ ☐ 4+ ☐

Gene X-pert: 1. P ☐ 2. N ☐ If Pos, RIF: S ☐ R ☐

X- ray: 1. Normal ☐ 2. Abnormal ☐

Final diagnosis: 1. TB negative ☐ 2. Pulmonary TB ☐ 3. Extra Pulmonary TB ☐

3. Disseminated TB ☐

4. HIV Status : 1. P ☐ 2.N ☐

Sample collected 1. Blood heparin tube ☐ 2.Sputum ☐

Annex X: English version of questionnaire for Apparently Healthy Controls

Questioner: Subject identification and Clinical Examination form (Healthy Control**)**

STUDY SITE:_____ Date (EC): _____/_____/_____

1. **Socio-demographic data**

Patient ID ☐☐☐☐☐ Medical Record No. ☐☐☐☐☐☐☐

Gender: 1. M ☐ 2. F ☐ Age (years): ☐☐

Education: 1. No formal education ☐ 2. Elementary ☐ 3. Secondary ☐ 4. Diploma& above ☐

Occupation: 1. Employed ☐ 2. Self employed ☐ 3. Not employed ☐

BMI: Height (cm) ☐☐☐ Weight (kg) ☐☐

Vital sign: Temp (^{0}C) ☐☐ . ☐ PR ☐☐ RR ☐☐ BP: SP ☐☐☐ DP ☐☐☐

2. **TB status :**

BCG scar: 1. Yes ☐ 2. No ☐

Symptoms: 1. Cough ☐ 2. Fever ☐ 3. Loss of weight ☐ 4. Shortness of breath ☐

5. Loss of appetite ☐ 6. Night sweats ☐ 7. Other, _____

Close contact with persons known or suspected to have active TB

1 .Yes ☐ 2.No ☐

X- ray: 1. Normal ☐ 2. Abnormal ☐

3. HIV Status: 1. Negative ☐ 2. Positive ☐

4. Other symptoms: 1. Intestinal Parasite: Yes ☐ No ☐

2. Chronic disease: Yes ☐ No ☐

5. On drug: 1. Antibiotics: Yes ☐ No ☐

2. Others ☐

Sample collected 1. Blood heparin tube ☐

Annex XI: Standard operating procedures for laboratory tests

Objective: To describe laboratory tests performed for the present study.

Personnel

Data was collected by principal investigator and health workers. All laboratory tests for this research were performed by skilled and trained researcher.

Sputum specimen

Lowenstein Jensen (LJ) Media

A. Media Preparation (preparation of a total volume of 1600ml)

1. Weigh 37.2g of LJ medium base powder (DIFCO/Fluka).
2. Measure 600 ml of distilled water into a 1000ml beaker and dissolve the Lowenstein-Jensen Medium base in the water.
3. Mix well and bring to the boil into a microwave oven with constant agitation until the reagents are completely dissolved.
4. Using a single sterile pipette (25ml), measure 12ml of glycerol and add it to the mixture.
5. Mix and autoclave the mixture at 121°C for 15 minutes and let cool to room temperature.

Egg Fluid

1. Break 1000ml of eggs into a sterile beaker (2000ml) and add the fluid eggs to the autoclaved mineral solution.

2. Using a Homogeniser, mix until homogenious
3. Stir for at least 10 minutes before dispensing and Filter the mixture through sterile muslin into a sterile round glass flask (2000ml) containing a sterile magnetic bar.
4. Add: 3.0ml of Polymyxin B (100,000 Iu/ml) and Fungizone (5mg/ml) Mix well and slowly on a magnetic mixer for at least one hour under UV light in a biosafety cabinet (without running the cabinet).
5. Decant into two separate sterile Duran glass flasks and close each flask with sterile lids surmounted by a sterile dispenser.

Aliquoting the medium

1. Dispense 8ml into each sterile test tube or 10ml into each universal tube. Avoid air bubbles.
2. Clean the dispenser top with paper tissue impregnate with 70% ethylic alcohol after every set of 10 tubes and transfer the tubes containing the medium into a sterile crate.
3. After dispensing the entire medium, range the tubes on a special rack and lean them to give a slope when placing in an oven.
4. Tighten screw caps, slant them and coagulate by inspissations at 85 ^{0}C for 45min.

B. Sputum Decontamination Procedure

NALC-NAOH METHODS

Reagents

Sodium hydroxide (NaOH) solution, 4% NaOH autoclaved at 121^{o}C for 15 min

Sodium citrate solution (2.9%) autoclaved at 121^{o}C for 15 min

Procedure

1. Mix 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%)
2. Add 0.25gm of NALC in to the 50 ml NAOH-sodium citrate solution and mix it well
3. Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of NaOH-NALC-sodium citrate and tighten the screw-cap and Vortex to digest.
4. Allow to stand for 15 minutes at room temperature.
5. Fill the tube to within 2 cm of the top (e.g. to the 50-ml mark on the tube) with phosphate buffer and Centrifuge at 3000g for 15 minutes.

6. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant.
7. Re-suspend the sediment with pre-measured 2 ml phosphate buffer. Vortex to mix (1-2 seconds)
8. Inoculate deposit on two slopes of egg-based medium labeled with the ID number. Use a pipette to inoculate each slope with 3 - drops (approximately 0.1 - 0.15 ml).
9. Smear one drop on a slide, marked with the ID number, for microscopic examination.

Incubation of cultures

Incubate tubes/vials at 36 ±1 ^{0}C.Tubes should be incubated in a slanted position, with screw-caps loose, for at least 1 week to ensure even distribution and absorption of inoculums. After 1 week of incubation, caps are tightened to minimize evaporation and drying of the media. Tubes may then stand upright to save space in incubators.

Reading and reporting

Check colony formation every week, preferably twice within the first week, to allow rapid detection of contamination and a timely request for another specimen if necessary. Contaminated cultures and rapidly growing mycobacteria (colonies apparent in less than 7 days) are removed. Report results immediately and ask for another specimen. *M. tuberculosis* colonies should be well developed within 3- 4 weeks. Report results immediately after detection and identification**.** Cultures should be kept for up to 8 weeks before being reported as negative (39).

SD MPT64 Rapid test

SD MPT64 Rapid test was performed according to the manufacturer's guidelines. Three colonies of mycobacterial strains grown in Löwenstein-Jensen media were emulsified in 200 µL of extraction buffer. Next, 100 µL were placed in sample wells of the test and the results were visually assessed based on color development after incubating at room temperature for 15 min.

HIV rapid testing

HIV was tested following the national testing algorithm. Pre and post test counseling was performed by health professional. Samples tested positive by WANTAI rapid test was repeated with the second test uni-gold if positive provide post-test counseling and disclosure support and direct client to enroll in HIV care. Sample tested positive by WANTAI was repeated with second test uni-gold if negative repeated by Vikia (tie breaker) then the result of tie breaker taken. HIV tested negative individuals with the screening test were included in this study. HIV testing was performed at health centers by health professional in VCT and TB clinics.

Peripheral blood mononuclear cells Isolation

Peripheral blood mononuclear cells (PBMC) were isolated from blood collected in heparinized tubes by density gradient centrifugation using Ficoll-Paque^{TM}PLUS solution (GE Healthcare). Briefly, whole blood was layered onto Leucosep tubes (BD) containing 15 ml of Ficoll and centrifuged for 20 min at 1700 rpm. The fluid above the separation membrane of the Leucosep tube which contains the PBMC was transferred into a new tube and first washed with phosphate buffered saline (PBS) and then washed with FACS buffer (PBS, Heat-inactivated FBS and Na2 EDTA) for 10 min at 1700 rpm .

Principle of Flowcytometry

Flowcytometry measures optical and fluorescence characteristics of single cells or any other particle, including nuclei, microorganisms, chromosome preparations, and latex beads. Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labeled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation

wavelengths and different emission wavelengths (or “colors”), allows several cell properties to be measured simultaneously (40).

Flowcytometry Procedure

Flowcytometry staining was performed on fresh PBMC. Surface staining done by incubating cells with a cocktail of antibodies containing 5 µl CD19 PerCP (Clone 4G7 , BD, Catalog No. 345778), 3 µl CD21 PE-CY7 (Clone B-ly4, BD, Catalog No. 561374), 20 µl CD27 FITC (Clone Ll28, BD, Catalog No. 340424), 3 µl CD23 BV421 (Clone M-L233, BD, Catalog No. 562707), 10 µl sIgD (Clone IA6-2, BD, Catalog No. 555779) , 10 µl sIgM (Clone G20-127, BD, Catalog No. 551062**)** in panel one and 5 µl CD19 PerCP (Clone 4G7 , BD, Catalog No. 345778), 3 µl CD21 PE-CY7 (Clone B-ly4, BD, Catalog No. 561374), 20 µl CD27 FITC (Clone Ll28, BD, Catalog No. 340424),10 µl CD 5(Clone Ll7F12, BD, Catalog No. 345782) , 3 µl CD38 (Clone HB7, BD, Catalog No. 345807) and 3 µl CD24 BV421 (Clone ML5, BD, Catalog No. 562789) in panel two. All incubations were done for 20 min at room temperature in the dark. Data was acquired on a BD FACS Canto II with Diva software and analyzed using FlowJo (Version 9.6.2).

QuantiFERON blood test

Principle

The QFT-Plus assay uses specialized blood collection tubes, which are used to collect whole blood. Incubation of the blood occurs in the tubes for 16 to 24 hours, after which, plasma is harvested and tested for the presence of IFN-γ produced in response to the peptide antigens.

The QFT-Plus test is performed in two stages. First, whole blood is collected into each of the QFT-Plus Blood Collection Tubes, which include a Nil tube, TB1 tube, TB2 tube, and a Mitogen tube. Alternatively, blood may be collected in a single generic blood collection tube that contains lithium heparin as the anticoagulant, and then transferred to QFT-Plus tubes. The Mitogen tube is used with the QFT-Plus test as a positive control. This may be important where there is doubt as to the individual’s immune status. The Mitogen tube also serves as a control for correct blood handling and incubation. The QFT-Plus tubes should be incubated at 37°C as soon as possible, and within 16 hours of collection. Following a 16 to 24 hour incubation period, the tubes are centrifuged, the plasma is removed and the amount of IFN-γ (IU/ml) is measured by ELISA.

QFT test procedure

Stage 1 — incubation of blood and harvesting of plasma

1. If the blood is not incubated immediately after collection, re-mixing of the tubes by inverting 10 times must be performed immediately prior to incubation.

2. Incubate the tubes UPRIGHT at 37°C ± 1°C for 16 to 24 hours. The incubator does not require CO2 or humidification.

3. After incubation at 37°C, blood collection tubes may be held between 4°C and 27°C for up to 3 days prior to centrifugation.

4. After incubation of the tubes at 37°C, harvesting of plasma is facilitated by centrifuging the tubes for 15 minutes at 2000 to 3000 x *g* RCF (*g*). The gel plug will separate the cells from the plasma. If this does not occur, the tubes should be re-centrifuged.

5. After centrifugation, avoid pipetting up and down or mixing plasma by any means prior to harvesting. At all times, take care not to disturb material on the surface of the gel.

Stage 2 — IFN-γ ELISA

1. All plasma samples and reagents, except for Conjugate 100x Concentrate, must be brought to room temperature (22°C ± 5°C) before use. Allow at least 60 minutes for equilibration.

2. Reconstitute the IFN-γ Standard with the volume of deionized or distilled water indicated on the label of the vial. Mix gently to minimize frothing and ensure complete solubilization. Reconstitution of the standard to the stated volume will produce a solution with a concentration of 8.0 IU/ml. Prepare four standard dilutions

- Use the reconstituted kit standard to produce a 1 in 2 dilution followed by a 1 in 4 dilution series of IFN-γ in Green Diluent . S1 (Standard 1) contains 4.0 IU/ml, S2 (Standard 2) contains 1.0 IU/ml, S3 (Standard 3) contains 0.25 IU/ml, and S4 (Standard 4) contains 0 IU/ml (GD alone). The standards must be assayed at least in duplicate.

3. Reconstitute lyophilized Conjugate 100x Concentrate with 0.3 ml of deionized or distilled water. Mix gently to minimize frothing and ensure complete solubilization of the conjugate.

4. Add 50 µl of freshly prepared working strength conjugate to the required ELISA wells using a multichannel pipet.

5. Add 50 µl of test plasma samples and 50 µl standards to appropriate wells .Mix using shaker.

6. Incubate for 120 ± 5 minutes at room temperature

7. Dilute one part Wash Buffer 20x Concentrate with 19 parts deionized or distilled water and mix thoroughly. Wash wells with 400 µl of working strength wash buffer for at least 6 cycles. An automated plate washer is recommended.

8. Add 100 µl of Enzyme Substrate Solution to each well, cover each plate with a lid and mix thoroughly using a microplate shaker. Incubate for 30 minutes at room temperature.

9. Add 50 µl of Enzyme Stopping Solution to each well and mix**.** Mix using shaker.

10. Measure the Optical Density (OD) of each well within 5 minutes of stopping the reaction using a microplate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filter. OD values are used to calculate results. Analyze data using the Quanti-FERON TB Gold plus Analysis Software.

QFT result interpretation

Either TB Antigen tube that is significantly above ≤8.0 of the Nil IFN-γ IU/ml value and TB1 minus Nil (IU/ml) or TB2 minus Nil (IU/ml) ≥0.35 IU/ml and ≥25% of Nil IU/ml is considered positive for an IFN-γ response. The plasma sample from the Mitogen tube serves as an IFN-γ positive control for each specimen tested. TB1 minus Nil (IU/ml) or TB2 minus Nil (IU/ml) <0.35 OR ≥0.35 and < 25% of Nil and Mitogen minus Nil (IU/ml) ≥0.5 is considered negative for an IFN-γ response. TB1 minus Nil (IU/ml) or TB2 minus Nil (IU/ml) <0.35 OR ≥0.35 and < 25% of Nil and low response to Mitogen (<0.5 IU/ml) indicates an indeterminate result when a blood sample also has a negative response to the TB antigens. This pattern may occur with insufficient lymphocytes, reduced lymphocyte activity due to improper specimen handling, incorrect filling/mixing of the Mitogen tube, or inability of the patient's lymphocytes to generate IFN-γ. The Nil tube adjusts for background (e.g., excessive levels of circulating IFN-γ or presence of heterophile antibodies). The IFN-γ level of the Nil tube is subtracted from the IFN-γ level for the TB Antigen tubes and Mitogen tube (41)**.**

Declaration

I, the undersigned, declare that this M.Sc. research thesis is my original work, has not been presented for a degree in this or any other university and that all sources of materials used for the research proposal have been duly acknowledged.

M.Sc. candidate: **Tigist Girma (B.Sc.)**

Signature: _____

Date of submission: _____

Place Addis Ababa, Ethiopia.

This research thesis has been submitted with our approval as advisors.

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