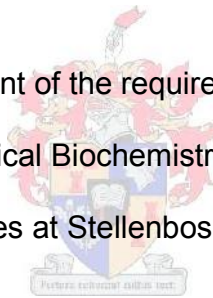


EVALUATION OF ANTI-TUBERCULOSIS RESPONSES IN HUMANS USING DIFFERENT COMPLEMENTARY IMMUNOLOGICAL TECHNIQUES

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Science in Medical Science (Medical Biochemistry) in the Faculty of Medicine and
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DECLARATION

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ABSTRACT

Background

The QuantiFERON In-Tube (QFT IT) assay is an Interferon-gamma release assay (IGRA) which is currently used to detect *Mycobacterium tuberculosis* (*M. tb*) infection. It however cannot differentiate between latent infection and active tuberculosis (TB) disease. In an attempt to improve this tool to accurately diagnose active TB, the release of a variety of markers should be assessed in combination with Interferon gamma (IFN- γ). Luminex analysis was previously done on QFT plasma and promising candidates were identified which could be of great value in treatment response studies. IFN- γ ELISpot, are not only used to detect *M.tb* infection, but is also implicated in vaccine trials to assess immunogenicity. The IFN- γ ELISpot and flow cytometry are the most common assays to assess these phenomena during clinical trials. Our aim therefore was to develop a multi platform immune analysis assay using the QFT IT system.

Study design and method

The first approach of this study was to optimize the QFT IT assay for flow cytometry applications. The following questions formed part of the optimization study: How does the QFT whole blood assay (QFT-WBA) compare to the currently used WBA? Is antigen re-stimulation required after the initial incubation time and for how long should cells be re-stimulated in the presence of Brefeldin A? The second approach was to use the optimized QFT-WBA for community controls (CTRL), household contacts (HHC) and TB cases, which were recruited from the high TB incidence areas Ravensmead, Uitsig and Elsie's River. The infection status of each participant was determined by IFN- γ

ELISA and Luminex analysis was performed to measure a wide range of cytokine expression. In addition immune cell markers like CD14, CD4, CD8, CD19, and T cell receptor gamma delta (TCR $\gamma\delta$) were characterized; polyfunctional characteristics (IFN- γ , Tumor necrosis factor-alpha (TNF- α) and Interleukin-2 (IL-2)) and proliferation (Ki-67+) of T cells determined by flow cytometry.

Results

After stimulating the whole blood of the study participants for 22 hours with the *M. tb* specific antigens, early secreted antigenic target 6 kDa (ESAT-6), culture filtrate protein-10 kDa (CFP-10) and TB7.7 the levels of TNF- α producing CD4 T cells were elevated in TB cases compared to HHCs. After stimulating the whole blood for 6 days TNF- α producing T cells declined in TB cases and HHC showed a higher expression. CD40L+CD4+ ($p=0.0225$) was increased in HHC while IL-9+CD8+ (0.3230) was decreased in HHC compared to TB cases. Other markers such as IL-5_(Ag-NIL), IL-13_(Ag-NIL), FGF basic_{Ag}, GM-CSF_{NIL}, VEGF_{NIL/(Ag-NIL)}, MIP-1 β _{Ag} and MCP-1_{Ag/(Ag-NIL)} showed significant differences between HHC and TB cases.

Conclusions

The responses in the QFT-based assay were generally comparable to the WBA that is routinely used. The differences of TNF- α expression seen in QFT-WBA and QFT-LPA could be explained by the fact that effector T cell responses were measured in the short term assay and the central memory T cell responses in the long term assay. Our study therefore shows that the QFT-based tests can be used to simultaneously assess a wide range of immunological markers and not only IFN- γ expression.

OPSOMMING

Agtergrond

Die QuantiFERON In Tube (QFT IT) toets is 'n Interferon-gamma vrystellingstoets (IGRA) wat huidiglik dien as 'n maatstaf van *Mycobacterium tuberculosis* (*M. tb*) infeksie. Hierdie toets kan egter nie onderskei tussen latente infeksie en aktiewe tuberkulose (TB) nie. 'n Noemenswaardige verbetering in die vermoë van hierdie toets om aktiewe TB te diagnoseer, berus op die studie van 'n verskeidenheid vrygestelde merkers, insluitend Interferon gamma (IFN- γ). In vorige Luminex studies op QFT plasma, is belowende kandidate geïdentifiseer wat van groot waarde kan wees vir studies wat fokus op die reaksie tot behandeling. Die IFN- γ ELISpot dien nie net as 'n maatstaf van *M.tb* infeksie nie, maar word ook in vaksienproewe betrek om die aard van immuniteit te ondersoek. Die IFN- γ ELISpot toets sowel as vloeisitometriese toetse, is van die mees algemene toetse om hierdie verskynsels te meet, tydens kliniese proewe. Die doel van hierdie studie was dus om die QFT IT sisteem te ontwikkel as 'n basis vir 'n multi-platform immunologiese analiseringstoets.

Studie ontwerp en metode

Die inleidende benadering van hierdie studie was die optimisering van die QFT IT toets, vir vloeisitometrie doeleindes. Die volgende vrae het die deel uitgemaak van die optimiseringstudie: Hoe vergelyk die QFT heelbloedtoets (QFT-WBA) met huidige WBAs wat in gebruik is? Word meermalige antigeenstimulasies benodig na die oorspronklike inkubasieperiode en hoe lank moet die tydperk wees vir sellulêre opvolgstimulasie, in die teenwoordigheid van Brefeldin A? As 'n tweede benadering, was om die

geoptimeerde QFT-WBA te gebruik vir gemeenskapskontroles (CTRL), huishoudelike kontakte (HHC) en TB gevalle. Al drie hierdie groepe was opgeneem uit Ravensmead, Uitsig en Elsie's Rivier, areas met betreklik hoë vlakke van TB infeksie. Elke persoon in die studie se vlak van infeksie is vasgestel met behulp van die IFN- γ ELISA en Luminex analiese was uitgevoer, om 'n wye verskeidenheid uitdrukkingsvlakke van sitokiene te meet. Dies meer, was immuunselmerkers soos CD14, CD4, CD8, CD19 en T sel reseptor gamma delta (TCR $\gamma\delta$) gekarakteriseer. Meervuldige funksionele karakteristieke (IFN- γ , Tumor nekrose faktor-alpha (TNF- α) en Interleukin-2 (IL-2)) en vermenigvuldiging van T-selle, was vasgestel deur middel van vloesitometrie.

Resultate

Nadat die heelbloed van studiedeelnemers gestimuleers was met *M. tb* spesifieke antigene, vroeë afscheidings antigeniese teiken 6kDa (ESAT-6), kultuurfiltraatproteïen 10kDa (CFP-10) en TB7.7, vir 22 uur, was gevind dat vlakke van TNF- α produserende CD4 T selle hoër was in TB pasiënte, in vergelyking met HHCs. Nadat die heelbloed vir 6 dae gestimuleer was, het die vlak van TNF- α produserende T-selle afgeneem in TB pasiënte, terwyl dit hoër was in HCC. CD40L+CD4+ ($p=0.0225$) het hoër vlakke bereik in HHC, terwyl IL-9+CD8+ (0.3230) vlakke afgeneem het, in vergelyking met TB pasiënte. Ander merkers soos, onder andere, IL-5_(Ag-NIL), IL-13_(Ag-NIL), FGF basic_{Ag}, GM-CSF_{NIL}, VEGF_{NIL/(Ag-NIL)}, MIP-1 β _{Ag} and MCP-1_{Ag/(Ag-NIL)}, het noemenswaardige verskille geopenbaar tussen HHC en TB pasiënte.

Gevolgtrekking

Die reaksies waargeneem in die geval van die QFT gebaseerde toets, was in die algemeen vergelykbaar met dié van die WBA. Die verskille wat waargeneem was vir die uitdrukkingsvlak van TNF- α in QFT-WBA en QFT-LPA , kan moontlik toegeskryf word aan die feit dat effektor T-sel reaksies in die korttermyn toets gemeet was, terwyl die sentrale geheue T-sel reaksie gemeet was in die langtermyn toets. Hierdie studie het dus gewys dat die QFT gebaseerde toets gebruik kan word vir die gesamentlike ondersoek van 'n wye verskeidenheid immunologiese merkers en nie net vir die uitdrukking van IFN- γ nie

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*Dedicated to
My loving parents*

TABLE OF CONTENTS

DECLARATION	ii
ABSTRACT	iii
OPSOMMING	v
ACKNOWLEDGEMENTS	viii
TABLE OF CONTENTS	x
LIST OF TABLES	xii
LIST OF FIGURES	xiii
ABBREVIATIONS	xv
1 CHAPTER ONE - INTRODUCTION	1
1.1 EPIDEMIOLOGY OF TUBERCULOSIS	2
1.2 PATHOGENESIS OF <i>MYCOBACTERIUM TUBERCULOSIS</i> (<i>M. TB</i>)	3
1.2.1 <i>M. TB</i> : CAUSATIVE AGENT OF TB	3
1.2.2 TUBERCULOSIS INFECTION IN HUMANS.....	4
1.3 DIAGNOSIS OF TB	5
1.4 TREATMENT	8
1.5 HOST IMMUNE RESPONSE	9
1.5.1 INNATE IMMUNITY	10
1.5.2 ADAPTIVE IMMUNITY	11
1.5.3 IMMUNE REGULATION THROUGH CYTOKINES	15
1.6 TB VACCINATION	16
1.7 HYPOTHESIS.....	20
1.8 AIM OF THE STUDY	20
1.9 OBJECTIVE OF THE STUDY	20
2 CHAPTER TWO – METHODS	21
2.1 STUDY PARTICIPANTS	22
2.1.1 CONSENT AND ETHICAL APPROVAL	22
2.1.2 INCLUSION CRITERIA	22
2.1.3 EXCLUSION CRITERIA	22
2.1.4 SELECTION OF PARTICIPANT COHORT	23
2.2 BLOOD COLLECTION AND HARVESTING OF SAMPLES	23
2.2.1 BLOOD COLLECTION.....	24
2.2.2 HARVESTING OF SAMPLES	24
2.3 CYTOKINE EXPRESSION IN SUPERNATANT.....	27
2.3.1 QUANTIFERON®-TB GOLD ELISA.....	27
2.3.2 BIO-PLEX PRO ASSAY (LUMINEX).....	31
2.4 FLOW CYTOMETRY.....	34
2.4.1 PHENOTYPING	35

2.4.2	INTRACELLULAR CYTOKINE STAINING	36
2.5	STATISTICS.....	37
3	CHAPTER THREE - RESULTS.....	38
3.1	INTRODUCTION.....	39
3.2	OPTIMIZATION OF QFT ASSAY FOR USE IN FLOW CYTOMETRY.....	39
3.2.1	REMOVAL OF 300ML OF WHOLE BLOOD FROM THE QFT TUBES DOES NOT AFFECT IFN- γ ELISA RESULTS	40
3.2.2	COMPARISON OF WBA VS. QFT-WBA IN FLOW CYTOMETRY.....	42
3.2.3	COMPARISON OF 4H VS. 6H RE-STIMULATION OF QFT-WBA	45
3.2.4	DIFFERENT CONCENTRATION OF PMA/ IONOMYCIN USED FOR QFT-WBA.....	46
3.2.5	DIFFERENT RE-STIMULATION METHODS OF QFT-WBA.....	48
3.2.6	LYMPHOCYTE PROLIFERATION ASSAY	49
3.2.7	SETTING UP FACSCANTO II FOR FLOW CYTOMETRY.....	50
3.3	STUDY POPULATION	54
3.4	ASSESSING THE IFN- γ SECRETION IN QFT SUPERNATANT.....	55
3.5	CORRELATION BETWEEN QFT AND LUMINEX.....	56
3.6	LUMINEX RESULTS.....	57
3.7	FLOW CYTOMETRY.....	60
3.7.1	PHENOTYPING	60
3.7.2	ASSESSING POLYFUNCTIONAL T CELLS IN QFT WBA.....	63
3.7.3	ASSESSING POLYFUNCTIONAL T CELLS IN QFT LYMPHOCYTE PROLIFERATION ASSAY (QFT LPA)	71
4	CHAPTER FOUR - DISCUSSION	75

LIST OF TABLES

Table 1.1: New Vaccines in pipeline.....	18
Table 2.1 Reagents and Consumables for Blood Collection and harvesting of samples.....	23
Table 2.2 Reagents for QuantiFERON®-TB Gold ELISA.....	27
Table 2.3: Reagents for Bio-Plex Pro Assay (Luminex).....	32
Table 2.4: Reagents and Consumables for Flow Cytometry.....	35
Table 2.5: Antibody master mix for phenotypic analysis.....	36
Table 2.6: Antibody master mix for WBA and LPA.	37
Table 3.1: QuantiFERON results of lab controls.....	41
Table 3.2: Titration of surface antibodies and antibodies against ICS.	52
Table 3.3: Study population.	55
Table 3.4: QuantiFERON results.....	56

LIST OF FIGURES

Figure 1.1: Estimated incident rate of tuberculosis worldwide. (WHO 2012)	3
Figure 1.2: Phagocytosis of <i>Mycobacterium tuberculosis</i>	4
Figure 1.3: The innate and adaptive immune system.....	9
Figure 2.1: Sample Layout for QuantiFERON ELISA using NIL, TB-Antigens and Mitogen.....	28
Figure 2.2: Preparation of Standard dilutions	29
Figure 2.3: Interpretation Flow Diagram	31
Figure 2.4: Plate layout for Bio-Plex Pro Assay.....	33
Figure 3.1: Comparison of QuantiFERON results between normal QFT and modified QFT.	41
Figure 3.2: Gating Strategy used for all optimization steps..	43
Figure 3.3: Comparison of IFN- γ expressing CD4 and CD8 T cells in WBA and QFT-WBA.	44
Figure 3.4: Comparison of IFN- γ expressing CD4 and CD8 T cells in QFT cells stimulated for 4h and 6h.	46
Figure 3.5: Different concentrations of Ionomycin in stimulated whole blood.	47
Figure 3.6: Different re-stimulation methods of QFT-WBA after harvesting of plasma. .	49
Figure 3.7: Antibody titration plots.....	53

Figure 3.8: Correlation between Luminex and QFT results.	57
Figure 3.9: Luminex Results.	59
Figure 3.10: Gating strategy for phenotyping.....	61
Figure 3.11: Phenotyping.....	62
Figure 3.12: Gating strategy for polyfunctional T cells.	64
Figure 3.13: Median Cytokine expression in antigen stimulated CD4 T cells with and without restimulation.	64
Figure 3.14: Polyfunctional CD4 T cells in QFT WBA.	67
Figure 3.15: Polyfunctional CD8 T cells in QFT-WBA.	68
Figure 3.16: Median expression of IL-9 in CD4 and CD8 T cells.....	69
Figure 3.17: Median expression of CD40L in CD4 and CD8 T cells.....	70
Figure 3.18: Gating strategy for proliferating T cells.	72
Figure 3.19: Polyfunctional CD4 T cells in QFT LPA.	73
Figure 3.20: Percentage of proliferating CD4 T cells.	74

ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µL	Microlitre
µm	Micrometer

A

AD	Anno Domini
AFB	acid-fast bacilli
Ag	Antigen
am	Ante Meridiem
APC	antigen presenting cell
APC	Allophycocyanin
ART	antiretroviral therapy

B

BCG	Bacillus Calmette-Guérin
BrefA	Brefeldin A

C

CCR	Chemokine receptor
CD	Cluster of Differentiation
CFP-10	culture filtrate protein-10 kDa
CFSE	Carboxyfluorescein succinimidyl ester
CLR	C-type lectin receptors
CMI	Cell Mediated Immune
CO ₂	Carbon dioxide
CTL	cytotoxic T lymphocytes
CTRL	Community control
CV	coefficient of variation
Cy	cyanine

D

DC	dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTH	delayed type hypersensitivity

E

ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunosorbent spot
EMB	Ethambutol
ESAT-6	early secreted antigenic target 6 kDa
et al	et alia

F

FACS	Fluorescence Activated Cell Sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FGF	fibroblast growth factor
FITC	Fluorescein isothiocyanate
FSC-A	forward scatter-area
FSC-H	forward scatter-height

G

G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good Manufacturing Practice

H

h	Hour
HHC	Household contact
HIV	Human Immunodeficiency Virus
HTF	High-Throughput Fluidics

I

ICS	intracellular staining
IFN- γ	Interferon gamma
IGRA	interferon gamma release assays
IL	Interleukin
INH	isoniazid
IU	International unit

K

kDa	kilodalton
-----	------------

L

LC	Lab control
LTBI	latent <i>M. tb</i> infection

M

<i>M. tb</i>	<i>Mycobacterium tuberculosis</i>
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
min	minutes
MIP	Macrophage inflammatory protein
mL	millilitre
MTBC	<i>M. tb</i> complex
MVA	modified vaccinia Ankara

N

NaHep	Sodium Heparin
neg	Negative
NK cells	natural killer cells
NTM	Non-tuberculosis mycobacteria

O

OD	Optical Density
OG	Oregon Green

P

PAS	para-aminosalicylic acid
PBMC	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
pm	Post Meridiem
PMA	Phorbol myristate acetate
pos	Positive
PPD	purified protein derivative
PRR	pattern-recognition receptors
PZA	Pyrazinamide

Q

QFT	QuantiFERON
QFT IT	QuantiFERON In Tube
QFT-LPA	QuantiFERON lymphocyte proliferation assay
QFT-WBA	QuantiFERON whole blood assay

R

r	correlation coefficient
RANTES	Regulated and normal T cell expressed and secreted
RMP	Rifampicin
RNA	Ribonucleic acid
RNI	reactive nitrogen intermediates
RPMI	Roswell Park Memorial Institute

S

S	Standard
sec	Second
SSC	side scatter

T

TB	tuberculosis
T _{CM}	central memory T cells
TCR	T cell receptor
TCR $\gamma\delta$	T cell receptor gamma delta
T _{EM}	effector memory T cells
T _{EMRA}	terminally differentiated effector memory T cells
TGF- β	transforming growth factor-beta
TH	T helper
TLR	Toll-like receptor
T _{naïve}	naïve T cells
TNF- α	Tumor necrosis factor-alpha
Treg	Regulatory T cell
TST	tuberculin skin test

V

VEGF	Vascular endothelial growth factor
VPM	Vakzine Projekt Management GmbH

W

WBA	Whole blood assay
WHO	World Health Organization

CHAPTER ONE – INTRODUCTION

There are two ways to life:

One is the common, direct, and brave.

The other is bad, leading through death, and that is the genius way.

“Magic Mountain” Thomas Mann

1.1 Epidemiology of Tuberculosis

Tuberculosis (TB) has a long history. The disease was first documented 5,000 years ago in Egyptian mummies, which showed pathological signs of tubercular decay in their spinal column (102). Subsequent discoveries were made in India 3,300 years ago and in China 2,300 years ago. The prevalence of TB escalated long before colonization and slavery, spreading in Rome, in Borneo, before any European contact and even in the American natives before Columbus (58). The outbreak among the American natives was established only around 1880 when they were forced to live in reservations or prisons (63;83). In Europe the epidemic started in 17th century and was present for the next 200 years. Europeans who conquered Africa brought TB into the small villages that had never been exposed before. Similarly, African slaves taken to America got exposed to TB and transmitted the disease into their homes upon returning, which resulted in increasing of TB mortality in the villages (58).

Today one third of the population worldwide is infected with TB. It remains a major global health problem (26;68;113) and is the leading cause of death in low- and middle-income countries (60)(Figure 1.1). In 2011, the WHO reported an estimated 8.7 million incident cases, of which 1.1 million are reportedly co-infected with HIV, and 1.4 million people died of TB disease. Looking at absolute numbers of TB incident cases in 2010, South Africa is ranked in third position with 0.4-0.6 million, following India and China. Africa accounts for the highest number of people co-infected with HIV with 39% of TB cases co-infected with HIV. This accounts for 79% of the global TB-HIV co-infection statistics. About 6% of TB cases involve children (114). In an environment where TB occurs prolifically, infection of children occurs mainly via transmission of *M. tb* by adults

(61). Predictably, children living in low socio economic settings are at even higher risk of infection due to increased transmission in crowded living conditions (25).

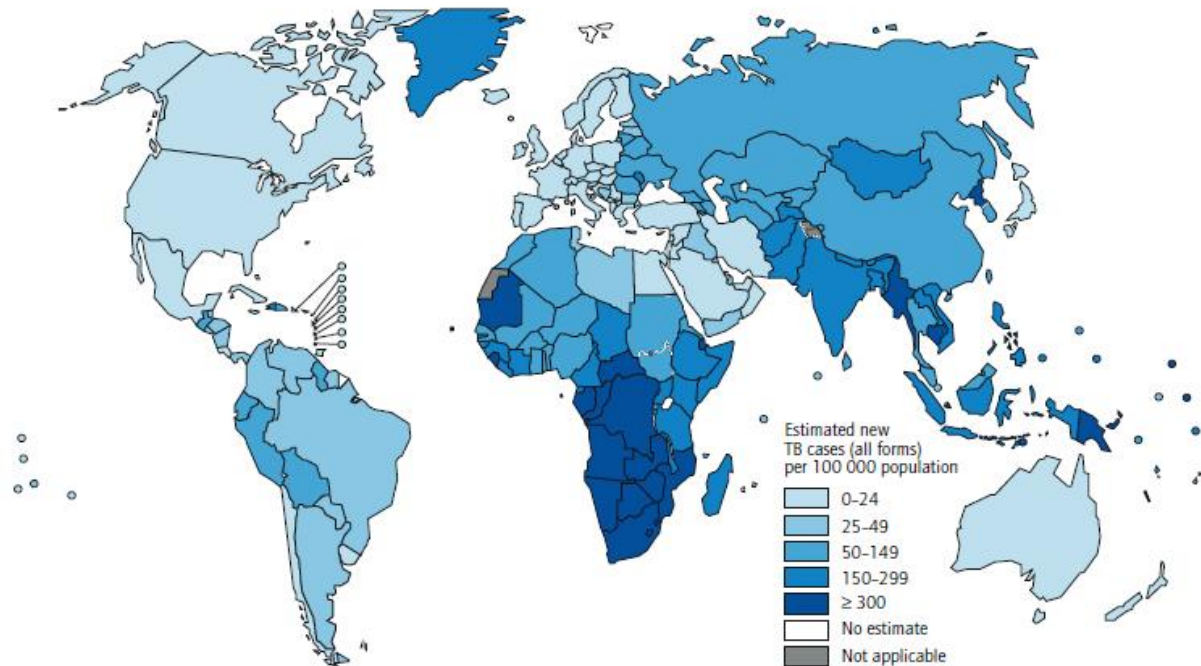


Figure 1.1: Estimated incident rate of tuberculosis worldwide. (WHO 2012)

1.2 Pathogenesis of *Mycobacterium tuberculosis* (*M. tb*)

1.2.1 *M. tb*: Causative agent of TB

M. tb, which causes TB, is a slow growing acid-fast bacterium with about a 12 hour replication time, while most of other bacteria only need 30min (47;102). *M. tb* is a rod-shaped bacterium approximately 0.3-0.6 μ m in width and 1.0 μ m in height (24) (Figure 1.2). The resistant and unique cell wall, composed of the covalently attached glycolipids, arabinogalactans, peptidoglycans and other free lipids and protein molecules, allows the bacterium to survive inside the macrophage, without getting phagocytosed (24).

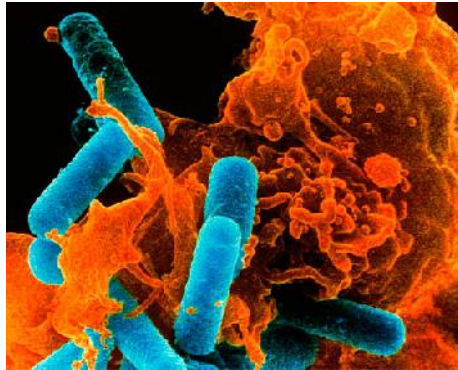


Figure 1.2: Phagocytosis of *Mycobacterium tuberculosis*. Colored scanning electron micrograph of a white blood cell (orange) engulfing bacteria (blue rods). (Credit: Dr Kari Lounatmaa / Science Photo Library. <http://www.nobelprize.org>)

1.2.2 Tuberculosis infection in humans

Pattern of TB

TB infection typically occurs during childhood upon first exposure to tubercle bacilli, hence the description of childhood TB or primary TB. Although *M. tb* infections has been associated with erythema nodosum and fever, the initial exposure to *M. tb* is usually asymptomatic (109). If the infection does not lead to disease, it can enter a latent stage of infection. Post-primary TB refers to reactivation of latent bacilli, while TB disease due to re-infection is described as secondary TB (40). Post primary TB can either affect the lungs (pulmonary TB) or other body parts such as the spine, joints, genitourinary tract, nervous system or abdomen (extra-pulmonary TB) (72).

Progression of TB

TB is a chronic infection with tubercular bacilli, which get transmitted through coughing, sneezing or singing by a patient with active disease. Following inhalation, the bacilli are inoculated into the respiratory bronchioles and alveoli. In immunocompetent hosts, a cell mediated T cell response is induced which leads to activation and

recruitment of macrophages to the site of infection (102). Alveolar macrophages and phagocytes such as dendritic cells (DCs) and neutrophils are the first cells to interact with the bacilli (18). *M. tb* replicate in naïve macrophages and remain confined in the intracellular compartment for extended periods (69). Bacilli can however be released at any time following immune suppression (102). *M. tb* infected macrophages are transported via the lymph and blood vessels to organs such as the spleen, liver and lymph nodes (69) where T cells are primed and clonally expanded (23). Due to the release of chemotactic factors, circulating monocytes will infiltrate the infection site and differentiate into mature macrophages which are able to kill free bacteria (69). Ultimately, progression from infection to disease, from latent TB infection (LTBI) to active TB, is dependent on infection dose, immune status and other factors such as under nutrition and toxins (alcohol, tobacco) (25).

1.3 Diagnosis of TB

In 100 - 200 AD the cause of TB was unknown and the disease was called (amongst other names) phthisis, which means 'wasting away', and consumption. Ulceration of the lung, chest or throat, together with coughs and fever were observed. Since the discovery of X-Rays by Wilhelm Konrad von Roentgen in 1895, the progression of disease and its severity could be well documented in TB patients (58). In 1891 a compound called tuberculin, prepared from liquid culture of tubercle bacilli, was isolated by Robert Koch in order to use it as a therapeutic vaccine against TB. Although tuberculin treatment failed, its use as diagnostic tool for *M. tb* infection was discovered. Today Koch's description of the preparation of tuberculin is used in the production of purified protein derivative (PPD) of tuberculin which is used in the Mantoux test, known as the tuberculin skin test (TST) (58). The TST is an *in vivo* test in which tuberculin is

injected intradermally and, within 48-72 hours, a positive reaction, such as induration and swelling, which is caused by delayed type hypersensitivity (DTH) reaction, is used as measurement of infection. PPD consists of secreted and somatic proteins of *M. tb* specific antigens, which is also found in *M. africanum*, environmental non tuberculosis mycobacteria (NTM) and *M. bovis* Bacillus Calmette-Guérin (BCG) vaccine. Consequently, the TST has a high sensitivity, but poor specificity for infection with *M. tb* (96;105). Furthermore the TST cannot differentiate LTBI from active TB, which is a problem particularly in high incidence areas (82). In developing countries the identification of acid-fast bacilli (AFB) in sputum samples by smear microscopy, which is based on the high lipid content of the cell wall of mycobacteria, is used as a standard test to diagnose TB. This diagnostic method is however problematic in HIV positive TB cases, as they produce smear negative results, and children who fail to produce enough sputum (21).

While smear microscopy needs 10 thousand bacilli per mL of sputum, the GeneXpert only requires 150 bacilli per mL of sputum (57). The GeneXpert is a polymerase chain reaction (PCR) based assay which only detects Deoxyribonucleic acid (DNA) of the *M. tb* complex (MTBC) and not NTMs. The GeneXpert can be used on smear positive and negative samples. While the GeneXpert can only detect resistance to Rifampicin (RMP), other polymerase chain reaction (PCR) tests such as the Line Probe Assay can detect both RMP and isoniazid (INH) resistance. The disadvantage of the Line Probe Assays is that it can only detect *M.tb* on smear and culture positive samples, which makes it necessary to culture the samples first (57;114).

Other assays rely on proliferation of lymphocytes after exposure to *M. tb* antigens. Accurate detection of *M. tb* infection, without detection of NTMs or *M. bovis* BCG, antigens specific for *M. tb* had to be used. The region of difference 1 (RD1) contains antigenic proteins which are restricted to the *M. tb* complex; the 6-kDa early secreted antigen target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) (60;105). These antigens have been used in two commercial interferon gamma release assays (IGRAs), the QuantiFERON-TB GOLD (together with TB7.7) and the T-SPOT.TB test. These tests determine the amount of interferon gamma (IFN- γ) secreted by T cells stimulated with TB specific antigens (74). Only T cells of sensitized individuals who have re-encountered *M. tb* antigens will produce IFN- γ . The T-SPOT.TB test is an *in vitro* test in which mononuclear cells are stimulated with ESAT-6 and CFP-10. Effector cells, T cells which have recently been exposed to the TB antigens, will be able to produce IFN- γ (54), while memory T cells are less likely to produce IFN- γ due to the short incubation time (96).

The QuantiFERON-TB GOLD test is an Enzyme Linked Immunosorbent Assay (ELISA) based test whereby IFN- γ in culture supernatant is measured by ELISA (96).

Even though both these tests show high sensitivity and specificity (105) neither differentiates between active and latent TB and the performance remains poor in children (17) and immunosuppressed individuals (60).

Discordance between TST and IGRA results has also been shown. While the IGRA is a short time assay and only effector T cells are detected, the T cells in the TST test have time to expand into memory T cells (17). Overall it is believed that the IGRA is more specific than the TST (74). The TST leads to more indeterminate results in HIV

positive individuals and in young children (105), which is a problem as those groups are of much higher risk to progress to active disease from LTBI (1). It has been shown that a decrease of CD4 T cells leads to more indeterminate results, which makes it less sensitive in HIV positive individuals (1;78;103).

1.4 Treatment

In the 19th century very little information about TB and its appropriate treatment was available. In 1854 Hermann Brehmer, who suffered from TB, travelled to the Himalayas where he was exposed to the specific climate of the area and got cured. He wrote his medical dissertation "Tuberculosis is a Curable Disease", propagating good nutrition, bed-rest and fresh air as a cure, and subsequently sanatoria were introduced all over Europe (64). After 1919 more drastic methods were applied such as lung volume reduction by artificial pneumothorax and surgery, but those methods became dangerous and controversial. By 1943 the antibiotic streptomycin was found to be effective against TB. Other drugs such as para-aminosalicylic acid (PAS) and INH also have been shown to cure TB, but mycobacteria quickly developed resistance and only a combination of the three drugs proved better than in a single dose (58). Today INH is still employed as a first line drug together with RMP, Pyrazinamide (PZA) and Ethambutol (EMB) in the 2-month intensive phase of treatment, followed by 4 months of INH and RMP. TB drugs must be bactericidal, bacteriostatic, or have the ability to prevent resistance. INH is bactericidal after 24 hours and kills over 90% of the rapid and intermediate growing bacilli in the first 2 days. RMP and PZA are also bactericidal and are used as sterilizing agents, whereas EMB is bacteriostatic and used to minimize the emergence of drug resistance (72).

1.5 Host immune response

The growth of *M. tb* can be controlled in healthy people by the innate and adaptive immunity which results in the secretion of appropriate chemokines and cytokines (Figure 1.3). Due to primary progression or reactivation some people will develop active TB (37). T cells play a vital role in the immune response to *M. tb* as they lead to activation or cytolytic activity of macrophages (46).

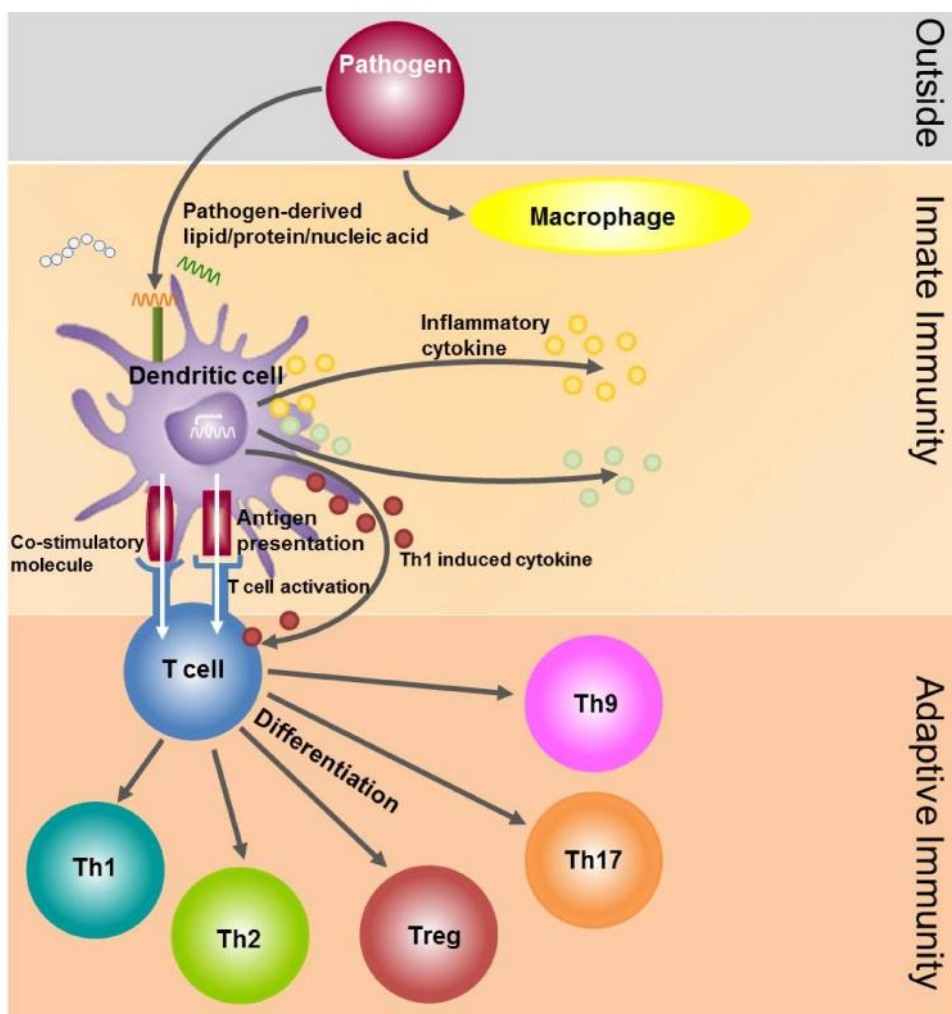


Figure 1.3: The innate and adaptive immune system. (Figure adapted from <http://www.rikenresearch.riken.jp>)

1.5.1 Innate Immunity

Innate phagocytes serve as antigen presenting cells (APCs) and contain several receptors (pattern-recognition receptors; PRRs) able to recognize *M. tb* components. Such receptors include Toll-like receptors (TLRs), C-type lectin receptors (CLRs) and cytosolic pattern recognition receptors, and induce expression of pro-inflammatory cytokines, chemokines and adhesion receptors after stimulation (27). In an attempt to contain the infection, granulomas are formed which consist of different cell populations such as alveolar macrophages, Langhans giant cells, T cells and DCs. In the granuloma, macrophages and Langhans giant cells surround the intracellular mycobacteria and present processed *M. tb* antigens to the T cells via major histocompatibility complex (MHC) class II molecules. T cells are activated upon antigen recognition and secrete cytokines and chemokines which recruit cells from circulating blood, and activate macrophages and APCs to kill the bacteria. *M. tb* activated macrophages release IL-12, leading to the development of T helper 1 (TH1) cells (77) and their release of TH1 cytokines (14;23;30). The formation of the granuloma wall is mediated by Tumor necrosis factor-alpha (TNF- α) and transforming growth factor-beta (TGF- β) secretion by CD4 T cells and macrophages which prevents pathogen dissemination (30;46;104). Together with IFN- γ , TNF- α induce antimycobacterial effects, such as the production of nitric oxide and related reactive nitrogen intermediates (RNI) by macrophages (27).

1.5.2 Adaptive immunity

Innate immune responses to *M. tb* infection establish an environment that allows development of adaptive immunity. The adaptive immune responses consist of cell mediated and humoral immunity.

Cell mediated immunity

During *M. tb* infection, activated antigen-specific CD4 T cells (30) play an important role in the protection against *M. tb* (38;46). Protective cell mediated immunity to *M. tb* is associated with the development of TH1 responses and production of TH1 cytokines such as IFN- γ , IL-2, TNF- α (3). On the other hand, the release of IL-4 leads to development of TH2 responses, such as IL-4, IL-5, IL-13 and IL-10 and support of B cell growth and differentiation (51). TH2 responses can induce suppression of TH1 immunity, thereby impairing control of *M. tb* infection (23). While TH2 cells drive the humoral immunity through up-regulation of antibody production (particularly immunoglobulin G1 and immunoglobulin E) to subsequently fight extracellular organisms, the TH1 cells drive the cellular immunity to overcome viruses and intracellular pathogens, to raise DTH and fight cancer (22;49;116). CD4 T cells can also develop into TH17 cells due to the release of IL-23, IL-6, and IL-21, and produce IL-17 and IL-22, which stimulates defensin production and the recruitment of monocytes to the inflammatory site. *Chen et al.* have shown that patients with tuberculosis show a suppressed TH17 response, which is associated with the clinical outcome of *M. tb* infection (13).

The role of CD8 T cells in TB less compelling, but increasing evidence suggests they promote immunity against *M. tb* by contributing to long-term protection (54). Some

CD8 T cells possess CD4 receptors, and are referred to as precursor T cells, mainly observed in neonates. *M. tb* antigens are presented to CD8 T cells via MHC class I molecules (14;54). Recognition of *M. tb* infected cells by CD8 T cells induce secretion of IFN- γ and lysis of infected cells via exocytosis of granules containing perforin and granzymes (46;98). Lysed cells release mycobacteria in the extracellular environment, facilitating uptake by activated macrophages (14). Due to the high frequency of CD8 T cells in peripheral blood their effector function is observed as early as 12 hours after exposure to antigen, highlighting their effective role as killer cells during *M. tb* infection (30;47).

Following proliferation and differentiation, a subset of effector T cells develop into memory T cells which are able to respond faster after re-exposure to an antigen. The expression of several surface markers distinguishes between naïve and memory T cells (71). Memory T cells can be distinguished from naïve T cells ($T_{\text{naïve}}$) via the memory marker CD45RO. Through the expression of homing receptors such as CD62L or CCR7, memory T cells can be further divided into central memory (T_{CM} , CD62L+) and effector memory T cells (T_{EM} , CD62L-)(79;88). T cells expressing these homing receptors are able to migrate to secondary lymphoid organs (88). T_{CM} and T_{EM} are also distinguished by their expression of the IL-7 receptor alpha (CD127). CD127 is expressed upon exposure of naïve T cells to an antigen, which gets cleared and leads to a re-expression of CD127 on memory cells. After antigenic stimulation T_{CM} , which produce a large amount of IL-2, proliferate to T_{EM} and are able to produce multiple cytokines such as IFN- γ , IL-2, TNF- α , Granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-4. T_{CM} are mainly found as CD4 T cells, while T_{EM} have also been found as CD8 T cells. Due to the expression of CD45RA a fourth subpopulation,

terminally differentiated effector memory T cells (T_{EMRA}), are found in the in CD8 T_{EM} subset. These cells do not express CD62L and down regulate CD45RO (88). Regulatory T cells (Treg) develop from CD4 T cells through the release of IL-2 and high concentrations of TGF- β , which in return produce IL-10 and TGF- β (23). A dominating phenotype is the expression of CD25 and CD25 high on CD4 T cells, which is increased in active TB cases (81;99). The detrimental role of Tregs in TB was shown by various studies such as *Ribeiro et al.* (81), reporting their suppression of IFN- γ production and increase in IL-10 and TGF- β , leading to decreased T cell function.

T cells contain two types of T cell receptors (TCR), composed of α and β chains, or γ and δ chains (42;89). While α and β chain containing CD4 and CD8 T cells make up the majority of T cells in peripheral blood, $\gamma\delta$ T cells represent only 1-5% percent of peripheral blood lymphocytes. $\gamma\delta$ T cells act as non-classical T cells that cross link innate and adaptive immunity and are, along with natural killer (NK) cells, the first cells to express IFN- γ , well before IL-12 secreting APCs initiate the expression of IFN- γ in adaptive T cells (66). It is well established that patients with pulmonary TB express increased frequencies of $\gamma\delta$ T cells in peripheral blood (41). The presence of $\gamma\delta$ T cells in alveolar spaces of pulmonary TB patients, also suggests that alveolar macrophages serve as APCs for $\gamma\delta$ T cells (92).

Since the discovery of the characteristic TH1, TH2, TH17 and Treg polarized T cell subsets (70), other subsets such as TH9 have been described. Initially, IL-9 was thought to be expressed by TH2 cells, but *Veldhoen et al.* (108) showed that IL-9 producing T cells were distinct from TH1, TH2, Treg and TH17 cells. They showed that TGF- β alters TH2 cells, resulting in loss of IL-4 and, IL-5 and IL-13 production and initiation of IL-9

production. It has been shown that peripheral blood mononuclear cells (PBMCs) from TB patients express increased levels of IL-9 when stimulated with ESAT-6 and that the expression of IFN- γ could be decreased by neutralization of IL-9. These findings support the hypothesis that TH1 responses are induced by IL-9 production (115).

The interaction of CD40 and CD40L (CD154) plays a role in cellular and humoral immunity. CD40 is a surface receptor first identified on B lymphocytes, while its ligand CD40L, was recognized on activated CD4 T cells including TH0, TH1 and TH2 cells (106). More recent evidence shows that CD40L is also expressed on cytotoxic T lymphocytes (CTL) (90), memory T cells (86), NK cells (9), macrophages (5), basophils (34), and eosinophils (33), and CD40 on activated T cells (86), macrophages (5) and dendritic cells (76). The activation of naïve T cells requires antigen-specific signaling via TCR as first signaling pathway, co-stimulatory molecules CD80/ CD86 on APCs and the presence of cytokines. The co-stimulatory molecule interacts with CD28 on T cells and forms the second signaling event (91). Only in the presence of a third signal which is provided by essential cytokines such as IL-12, a full activation and differentiation can be achieved (73). CD40L is subsequently expressed on memory cells, which produce TNF- α , IL-2 or IFN- γ (10).

Humoral immunity

While CD4 T cells mainly target intracellular microbes, antibodies can recognise extracellular microbes. Antibodies are important in neutralization and prevention of invasive pathogens, especially at the mucosal surface (56). Since TB is primarily a respiratory mucosal disease, there is renewed interest in the potential protective role of *M. tb*-specific antibodies, against TB (80;110). *Senol et al.* showed that the humoral

antibody response to mycobacterial antigens could be a significant diagnostic indicator of active pulmonary TB. They showed that the serological response to combined 38-kDa and 16-kDa *M. tb* antigens was higher in actively diseased TB patients compared to those previously treated for TB with a negative a sputum smear and culture (95).

1.5.3 Immune regulation through Cytokines

All cytokine responses are interconnected and shape the outcome of host infection as they regulate all cells of the immune system (30). Although much is known on the role on each individual cytokine, insufficient information exists on the combined role of the cytokine repertoire and their linked interactions. TNF- α is an proinflammatory cytokine (44) activating chemokine production which attracts leukocytes to inflamed tissues and normal secondary lymphoid organs (94). TNF- α is produced by macrophages, DCs and T cells and its primary role is to limit replication of *M. tb* (111). For this reason, TNF- α is particularly relevant during the early stages of infection to control acute *M. tb* infection (31). As described earlier, TNF- α is also important in granuloma formation (30) as it forms the granuloma wall together with TGF- β and is responsible for caseous necrosis (31). Together with IFN- γ it plays an important role in defense against intracellular pathogens such as *M. tb* (30). IFN- γ is an important mediator of macrophage activation in *M. tb* (55), is secreted from activated T cells and NK cells and is also responsible for the formation of granulomas and killing of cells (23). IL-2 is a potent T cell growth factor and plays a critical role in clonal expansion of memory T cells (43). A study by *Lalezari et al.* has shown that treating HIV infected participants with low dose IL-2 leads to increase of CD4 count, and expansion of NK cells and naïve T cells (53). GM-CSF stimulates growth of granulocytes, activates macrophage functions (15) and inhibits bacterial growth (101). *M. tb* H37Rv infection

models in mice have shown that treatment with IL-2 and GM-CSF decreases the bacterial load in the lungs and spleen and those mice showed a higher survival rate compared to untreated mice (117).

1.6 TB vaccination

BCG vaccine, an attenuated *M. bovis* strain developed by Albert Calmette and Camille Guérin in 1921 (47;72), is widely used and routinely given to infants at birth in countries with high burden of TB (112). BCG is ineffective in preventing TB in adulthood, but has been shown to prevent TB meningitis and miliary TB in children (72). The effectiveness of BCG depends on the age of the vaccine recipient, virulence of the infecting *M. tb* strain, co-infection with other pathogens such as helminthes or HIV, exposure to environmental mycobacteria and other factors that influence the immune response such as malnutrition (23). The variability and poor efficacy of BCG steered research into the development of improved vaccines against TB. However, opposing views exist in the scientific community on the most suitable replacement or booster vaccine for BCG. Some suggest development of a vaccine which neutralizes the production of an *M. tb* protein essential for virulence (73). Others endorse development of a vaccine containing the attenuated and avirulent strain of *M. tb* to induce an immune response (16). The question remains: if BCG shares approximately 95% homology with *M. tb*, why is it still not effective (30)?

Currently many TB vaccines, based on the various suggestions mentioned above, are in the pipeline (Table 1.1). Most of these can be divided into subunit vaccines, which can further be divided into adjuvant- or viral vector based and whole mycobacterial based vaccines. Subunit vaccines present one or more immunogenic *M. tb* antigen(s).

Examples of such vaccines are Aeras-402 (2) and MVA85A (65) consisting of a modified vaccinia Ankara. Adjuvant based vaccines such as the GlaxoSmithKline M72 and Aeras-404 , rely on adjuvant fusion proteins (85). Modified strains of BCG are also used in whole mycobacterial vaccines. These modified strains have been manipulated to overexpress TB antigens, such as antigen 85B in rBCG30, altering the immune response which leads to overexpression of listeriolysin in VPM1002, or Aeras-422 which overexpresses listeriolysin, antigens 85A, 85B and 10.4 (11).

Status	Vaccine	Vaccine description	Type of vaccine	Target population
Phase III	Mw [M. indicus pranii (MIP)]	Whole cell saprophytic non-TB mycobacterium	Whole cell, Inactivated or Disrupted	
Phase III (completed)	M. vaccae	Inactivated whole cell non-TB mycobacterium; phase III in BCG-primed HIV+ population completed; reformulation pending	Whole cell, Inactivated or Disrupted	BCG-vaccinated HIV+ adults
Phase IIb	MVA85A/Aeras-485	Modified vaccinia Ankara vector expressing Mtb antigen 85A	Viral Vectored	BCG-vaccinated infants and adolescents; HIV infected adults
	Aeras-402/Crucell Ad35	Replication-deficient adenovirus 35 vector expressing Mtb antigens 85A, 85B, TB10.4	Viral Vectored	BCG-vaccinated infants, children and adults
Phase II	M72 + AS01	Recombinant protein composed of a fusion of Mtb antigens Rv1196 and Rv0125 & adjuvant AS01	Recombinant Protein	Adolescents/adults, infants
	Hybrid-I+IC31	Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT-6	Recombinant Protein	Adolescents; adults
	VPM 1002	rBCG Prague strain expressing listeriolysin and carries a urease deletion mutation	Recombinant Live	
	RUT1	Fragmented Mtb cells	Whole cell, Inactivated or Disrupted	HIV+ adults, LTBI diagnosed
Phase I	AdAg85A	Replication-deficient adenovirus 5 vector expressing Mtb antigen 85A	Viral Vectored	Infants; adolescents; HIV+
	Hybrid-I+CAF01	Adjuvanted recombinant protein composed of Mtb antigens 85B and ESAT-6	Recombinant Protein	Adolescents, adults
	Hybrid 56 + IC31	Adjuvanted recombinant protein composed of Mtb antigens 85B, ESAT-6 and Rv2660	Recombinant Protein	Adolescents, adults
	HyVac 4/Aeras-404, + IC31	Adjuvanted recombinant protein composed of a fusion of Mtb antigens 85B and TB10.4	Recombinant Protein	Infants
	ID93/GLA-SE	Subunit fusion protein composed of 4 Mtb antigens	Recombinant Protein	Adolescents, adults
Phase I [completed]	Aeras-422	Recombinant BCG expressing mutated PfoA and overexpressing antigens 85A, 85B, and Rv3407	Recombinant Live	Infants
	rBCG30	rBCG Tice strain expressing 30 kDa Mtb antigen 85B	Recombinant Live	Newborns, adolescents, and adults
	M. smegmatis	Whole cell extract	Whole cell, Inactivated or Disrupted	

Table 1.1: New Vaccines in pipeline. List of tuberculosis vaccine candidates which have been in clinical trials in 2011. (http://www.stoptb.org/wg/new_vaccines/documents.asp)

The vaccines listed above are designed to induce a CD4 or CD8 T cell response which can be assessed using different immunological assays. These assays can measure cell mediated responses or the level of gene expression. The most common

assay to measure immunogenicity in vaccine trials is the IFN- γ ELISpot. With the growing and better understanding of flow cytometry, it has become a widely used assay. Flow cytometry makes it possible to evaluate T cell responses in short term and long term assays by re-stimulating cells with the antigen of interest. Flow cytometry has the advantage over ELISPOT assays in that the researcher can identify the specific cell populations producing the resultant cytokines (e.g. IFN- γ). Activation markers and innate cell markers can be assessed by phenotyping, determining the proportion of T cells expressing one, two, three or more cytokines/chemokines using a short term assay, and the kinetics of immune responses can be assessed using proliferation assays, mostly over 4-6 days (20). During vaccine trials, a blood for safety analysis has to be drawn before blood for immunogenicity. Immunogenicity assays require a large amount of blood, depending on the assays and which source of sample will be used (PBMCs or whole blood) (28;39). Clinical phase I trials will be conducted in adults, where there is enough blood available. Going further along the pipeline (phase II and III trials), the blood volume becomes limited as those trials are done in infants (babies). These trials follow specific guidelines on how much and how often blood can be drawn from infants, which have to be strictly followed (39).

1.7 Hypothesis

Taking all of the mentioned aspects into account I hypothesize that the QuantiFERON in-tube assay system can be used to assess the immunogenicity of vaccines using the one-tube for multiple immunogenicity assays with comparable results to the established assays.

1.8 Aim of the study

The aim of the study is to develop a multi platform immune analysis assay using the QuantiFERON in-tube assay system. Adaptations of the QFT assay to incorporate antigen-specific cellular characterization (by flow cytometry) and soluble host marker production (multiplex cytokine arrays) may have the advantage that multiple complimentary immunological readouts can be obtained from a commercially available, highly standardized *M. tb* antigen stimulated whole blood culture assay.

1.9 Objective of the study

To fulfill the aim of the study the following objectives have to be met:

- i. IFN- γ ELISA must be performed to determine QFT status of community controls, household contacts and TB cases.
- ii. Luminex experiments to assess the expression of different cytokines in QFT plasma from community controls, household contacts and TB cases.
- iii. Phenotyping of QFT cells by flow cytometry.
- iv. Polyfunctional T cells are assessed using QFT blood in short term whole blood assay (QFT-WBA) and long term lymphocyte proliferation assay (QFT-LPA).
- v. Ribonucleic acid (RNA) isolation of QFT cells.

CHAPTER TWO – METHODS

The most exciting phrase to hear in science,

The one that heralds new discoveries,

Is not 'Eureka!' but 'That's funny...'

Isaac Asimov

2.1 Study Participants

2.1.1 Consent and Ethical Approval

Informed written consent was obtained from all participants. All participants were volunteers and could terminate their participation in the study at any time without any negative effect on their treatment.

Community Controls (CTRL), Household contacts (HHC) and TB index cases were recruited at public health care clinics in urban areas in the Western Cape, specifically in Ravensmead, Uitsig and Elsies River.

2.1.2 Inclusion Criteria

Study participants had to be willing to give written informed consent. They had to be available for a TST reading after 48-72 hours after administration of the test and had to be willing to undergo HIV testing. Participants had to be 15 years of age or older.

HHCs had to be in contact with a household member who had been diagnosed with sputum smear positive TB in the past 4 months. The TB cases did not have to be part of the study. A HHC had to have a positive TST.

A TB index case had to be newly diagnosed with active pulmonary TB (positive sputum culture), or retreated for TB.

2.1.3 Exclusion Criteria

Participants who were on TB treatment for more than 7 days, were currently on antiretroviral therapy (ART), participated currently or recently (past 3 months) in drug or vaccine trials or were pregnant, were excluded from this study.

2.1.4 Selection of Participant Cohort

The final participant cohort for the study consisted of three groups between 15 and 59 years of age. The first group included 16 household contacts, second group 19 TB index cases and the third group 10 community controls. For all optimization steps six laboratory controls (LC) were used with a mean age of 35.5 years. Two of the lab controls have had previous TB, which also showed a positive QuantiFERON response.

2.2 Blood Collection and harvesting of samples

Items	Company	Catalog number
QuantiFERON®-TB Gold IT Blood Collection tubes	Cellestis	T0590 0301
15mL tube	LASEC	PGRE188261
50mL tube	LASEC	PGRE227261
2mL Screw cap tubes	LASEC	PSOR12980
2mL Cryo vials	LASEC	PGRE126263
RNA later	Ambion	AM7021
Fetal Bovine Serum (FBS)	Lonza	DE14-80F1
10X Phosphate Buffered Saline (PBS)	Lonza	BE17-517Q
Roswell Park Memorial Institute (RPM 1640)	Sigma	R0883
L-Glutamin	Sigma	G7513
Dimethyl sulfoxide (DMSO)	Sigma	D8418
Brefeldin A	Sigma	B7651
Ionomycin	Sigma	I0634
Phorbol myristate acetate (PMA)	Sigma	P8139
FACS™ Lysing Solution	BD	349202
BD Pharm Lyse™ lysing solution	BD	555899

Table 2.1 Reagents and Consumables for Blood Collection and harvesting of samples

2.2.1 Blood Collection

QuantiFERON® blood collection tubes are pre-coated with Sodium Heparin (NaHep) and the antigens ESAT-6, CFP-10 and TB7.7 (p4) in the 'Antigen' tube. The 'NIL' tube serves as negative control to determine the background or nonspecific binding. The 'Mitogen' tube is coated with Phytohaemagglutinin (PHA), and serves as positive control as an indication for correct blood handling and incubation. About 1mL of blood was drawn directly into each tube. To ensure proper mixing of blood with tube contents, the tubes were shaken up and down 10 times. The QuantiFERON tubes were transported to the laboratory at room temperature within 3 hours. To assure a constant incubation time of 18h at 37°C the tubes were put in the incubator at 2pm and processed at 8am the next morning.

2.2.2 Harvesting of samples

After 18h incubation of the QFT tubes, blood is mixed by inverting the tube.

Harvesting blood for RNA

At first 200µL of blood is removed and transferred into a 2mL tube containing 600µL RNA^{later}® Solution. Samples are then frozen at -80°C.

Harvesting blood for phenotyping

100µL of QuantiFERON blood were transferred into a 15mL tube containing 5mL of 1X BD FACS™ Lysing Solution, incubated for 10 minutes at room temperature and spun down for 10 minutes at 400g. The supernatant was discarded and the pellet resuspended in 0.5mL Roswell Park Memorial Institute (RPMI) media. 0.5mL of 20%

DMSO in FBS was added drop wise to the tube and the content of the tube was transferred into a 2mL cryo vial and frozen away in a Nalgene® Mr. Frosty container at -80°C overnight, and thereafter transferred into liquid nitrogen.

Harvesting of plasma

After removing blood for RNA the tubes were centrifuged at 3000g for 10 minutes. The gel plug in each tube separates the blood from the plasma and plasma can be harvested. For each sample 3X 3 tubes were prepared (three for each stimulus). 80µL of plasma was harvested into the first two tubes. The remaining plasma was harvested into the last tube. All tubes were stored at -80°C until human IFN-γ ELISA and Luminex were performed.

Harvesting of blood cells

In order to harvest the blood cells the QFT tubes were put upside down in a 50mL centrifugation tube. The tubes were spun down for 1min at 400g. The whole blood is now on top of the gel and can be removed carefully. The blood cells were harvested into a 15mL centrifugation tube containing 5mL of 1X PBS. After removing the blood cells the tubes were washed with the 1X PBS to harvest any remaining cells. After spinning down the tubes for 10min at 400g the supernatant was removed carefully. About 500µL of blood remained in the tubes and was split between the QFT-WBA and QFT-LPA.

For the QFT-WBA six 2mL tubes containing 10µg/mL Brefeldin A were prepared and three of these tubes also contained PMA/ Ionomycin. 200µL of blood from each of the NIL, Antigen and Mitogen tube were transferred into one of the tubes containing Brefeldin A only and one of the tubes containing Brefeldin A, PMA and Ionomycin. The

tubes were vortexed and incubated for another 4h at 37°C. After the incubation the blood was transferred into 15mL tube containing 10mL of 1X BD FACS™ Lysing Solution and incubated for 10min at room temperature in the dark following a centrifugation step at 400g for 10min. Supernatant was discarded and cell pellet were resuspended in 0.5mL RPMI. 0.5mL of 20% DMSO in FBS was added drop wise to the tube and the content of the tube was transferred into a 2mL cryo vial and frozen away in a Nalgene® Mr. Frosty container at -80°C overnight, and thereafter transferred into liquid nitrogen.

For the QFT-LPA a 96 well plate was prepared. Six wells were allocated to each patient sample. PMA and Ionomycin were added to half of the wells. Following the same principle as in the WBA, 50µL blood of each of the NIL, Antigen and Mitogen tube was transferred into a well with and without PMA/ Ionomycin. 150µL of RPMI containing 1% L-Glutamine was added to each well. The plate was incubated for 6 days at 37°C. On day 6 Brefeldin A was added into each well and PMA/ Ionomycin into stimulated wells, followed by 4h incubation. After the incubation the blood was transferred into 15mL tube containing 5mL of 1X BD FACS™ Lysing Solution and incubated for 10min at room temperature in the dark following a centrifugation step at 400g for 7min. Supernatant was discarded and cell pellet was resuspended in 1mL BD Pharm Lyse™ lysing solution, filled up to 5mL with the same buffer, incubated for 10min at room temperature and spun down at 400g for 7min. Supernatant was discarded and pellets resuspended with 0.5mL RPMI. 0.5mL of 20% DMSO in FBS was drop wise added to the tube and the content of the tube was transferred into a 2mL cryo vial and frozen away in a

Nalgene® Mr. Frosty container at -80°C overnight, and thereafter transferred into liquid nitrogen.

2.3 Cytokine expression in Supernatant

2.3.1 QuantiFERON®-TB Gold ELISA

The QuantiFERON®-TB GOLD ELISA test is a test for Cell Mediated Immune (CMI) responses against mycobacterial proteins. It is used as an *in vitro* diagnostic test of latent *M. tb* infection by measuring the amount of IFN- γ produced by stimulated cells in whole blood.

Items	Company	Catalog number
QuantiFERON®-TB Gold IT kit	Cellestis	0594-0201

Table 2.2 Reagents for QuantiFERON®-TB Gold ELISA

One hour before starting the ELISA, plasma was thawed and reagents from the QuantiFERON®-TB GOLD ELISA kit, except 100X concentrate was brought to room temperature. Assay strips were labeled to prevent switching of samples. In this study 'NIL', 'TB Antigen' and 'Mitogen' tubes were used. Therefore a Sample Layout (Figure 2.1) for 27 Samples and 1 internal lab control was used, including a four concentration standard series in triplicate. Once the reagents have reached room temperature the standard was made up by reconstitution with distilled water to give a concentration of 8 IU/mL. The vial was mixed gently to minimize frothing and the freeze-dried Kit Standard was dissolved completely. Furthermore a 1:4 dilution series in Green Diluent was produced. Standard 1 contains 4 IU/mL, Standard 2 contains 1 IU/mL, Standard 3 contains 0.25 IU/mL, and Standard 4 contains 0 IU/mL (Green Diluent alone). The

standard dilution series was made up as follows (Figure 2.2): 4 tubes were prepared and labeled with S1, S2, S3 and S4. Green Diluent was added into the tubes. S1 contains 150 μ L while S2-S4 contains 210 μ L. 150 μ L of the reconstituted Kit Standard was added to tube S1 and mixed thoroughly. From S1 70 μ L were transferred to S2 and mixed thoroughly. From S2 70 μ L were transferred into S3 and mixed thoroughly. S4 only contains green Diluent and serves as zero standard.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1N	1A	1M	S1	S1	S1	13N	13A	13M	21N	21A	21M
B	2N	2A	2M	S2	S2	S2	14N	14A	14M	22N	22A	22M
C	3N	3A	3M	S3	S3	S3	15N	15A	15M	23N	23A	23M
D	4N	4A	4M	S4	S4	S4	16N	16A	16M	24N	24A	24M
E	5N	5A	5M	9N	9A	9M	17N	17A	17M	25N	25A	25M
F	6N	6A	6M	10N	10A	10M	18N	18A	18M	26N	26A	26M
G	7N	7A	7M	11N	11A	11M	19N	19A	19M	27N	27A	27M
H	8N	8A	8M	12N	12A	12M	20N	20A	20M	28N	28A	28M

Figure 2.1: Sample Layout for QuantiFERON ELISA using NIL, TB-Antigens and Mitogen. S1-S4 indicates the Standard Series; N indicates NIL control plasma; A indicates TB-Antigen plasma; M indicates Mitogen control plasma, 1-27 indicated the different samples. An internal control was used for Sample 28.

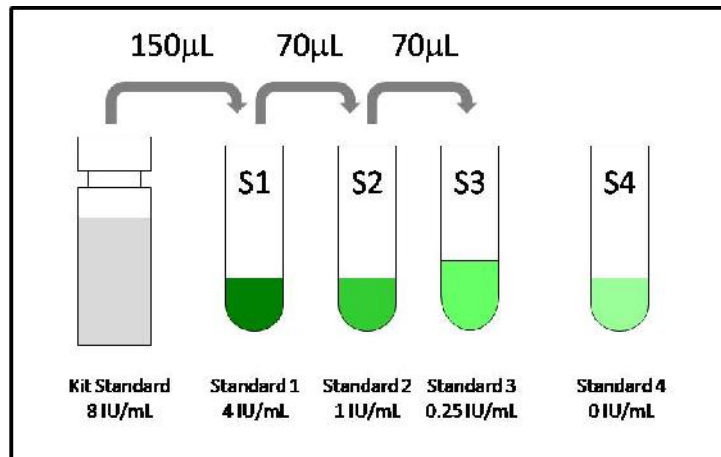


Figure 2.2: Preparation of Standard dilutions (adapted from Cellestis QuantiFERON®-TB Gold handbook)

The freeze-dried Conjugate 100X Concentrate was reconstituted with 0.3mL distilled water and mixed well until dissolved completely. A working solution was prepared by pipetting 60µL of reconstituted 100X Conjugate into 6.0mL of Green Diluent in a 15mL tube.

50µL of working solution was added into each well using a multichannel pipette. Plasma was mixed and 50µL added into the specific wells using a multichannel pipette. The tips were discarded after each pipetting step. Finally 50µL of the standards 1 to 4 were added to each well S1 to S4. The plate was mixed carefully and incubated for 2 hours at room temperature in the dark.

While incubating a 1X washing buffer was made up using 100mL Wash Buffer 20X Concentrate and 1900mL distilled water. The ELISA plate was washed 10 times per hand with 1X wash buffer. For the performance it is really important that the wells get filled with wash buffer. The plate was tapped upside down on an absorbent towel. At this step it can happen that the strips fall off. Therefore it is important that they have been

labeled properly. 100µL of Enzyme Substrate Solution were added to each well and mixed. The plate was incubated for 30min at room temperature while kept in the dark.

While incubating the micro plate the ELISA reader was started up. 50µL of Enzyme Stopping Solution was added to each well and the Optical Density (OD) was measured within 5min.

Raw data were analyzed and calculated using the QuantiFERON®-TB GOLD IT Analysis Software from Cellestis. As a quality control the software calculates and reports the following parameters (from Cellestis hand book; Figure 2.3):

- The mean OD value for Standard 1 must be ≥ 0.600 .
- The % coefficient of variation (CV) for Standard 1 and Standard 2 replicate OD values must be $\leq 15\%$.
- Replicate OD values for Standard 3 and Standard 4 must not vary by more than 0.040 OD units from their mean.
- The correlation coefficient (r) calculated from the mean absorbance values of the standards must be ≥ 0.98 .
- The mean OD value for the Zero Standard should be ≤ 0.150 .

If any of these criteria are not met the run is invalid and has to be repeated.

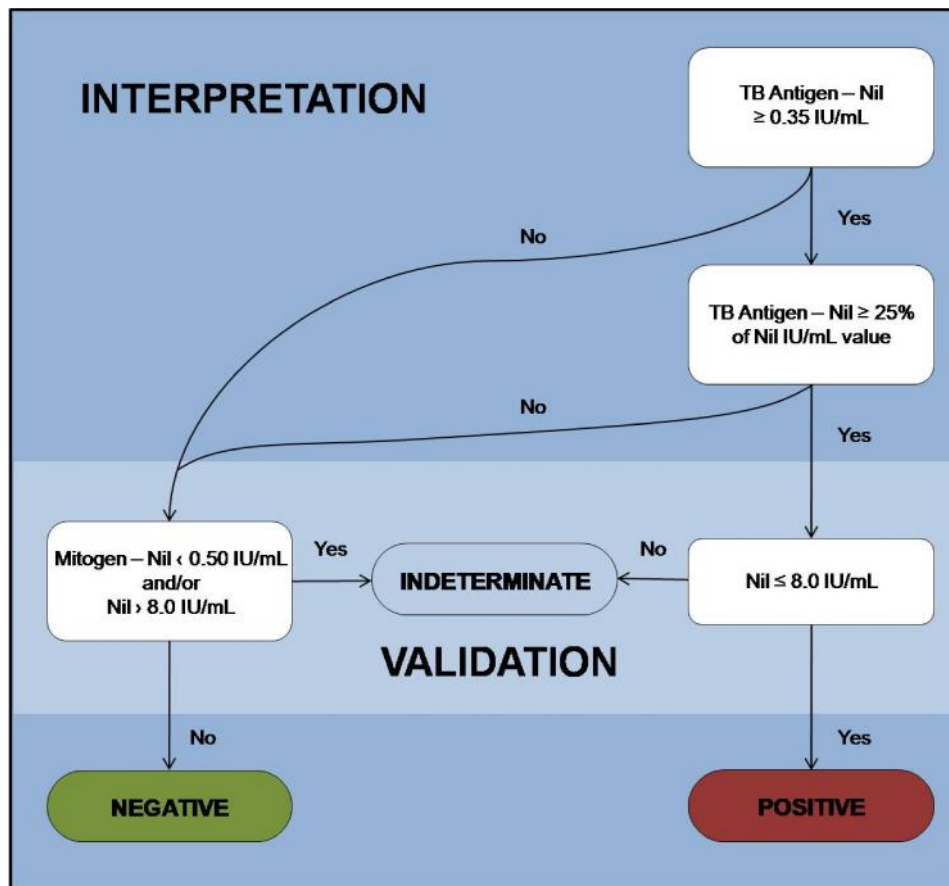


Figure 2.3: Interpretation Flow Diagram (adapted from Cellestis QuantiFERON®-TB IT Gold handbook); when the result is positive *M. tb* infection is likely, when the result is negative *M. tb* infection is NOT likely, when the results indeterminate it is indeterminate for TB-Antigen responsiveness.

2.3.2 Bio-Plex Pro Assay (Luminex)

Bio-Plex Pro Assay is an assay to quantify multiple protein biomarkers, which are secreted by many cell types, in a single well with as little as 12-25 μ L supernatant or plasma. In the present study a 27-Plex assay was used containing the following marker: IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, Basic fibroblast growth factor (FGF), Eotaxin, Granulocyte colony-stimulating factor (G-CSF), Granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , IP-10, Monocyte chemotactic protein (MCP)-1, Macrophage inflammatory protein (MIP)-1 α ,

MIP-1 β , Platelet-derived growth factor (PDGF)-BB, Regulated and normal T cell expressed and secreted (RANTES), TNF- α and Vascular endothelial growth factor (VEGF).

Items	Company	Catalog number
Bio-Plex Pro Human Cytokine 27-Plex Assay	Bio-Rad	M10-0KCAF0Y

Table 2.3: Reagents for Bio-Plex Pro Assay (Luminex)

Preparation of standard and reagents

Before starting the assay the samples and standards were brought to room temperature.

For the reconstitution of the standard it was ensured that the pellet was at the bottom of the vial by tapping the vial gently on the lab bench. 500 μ L of standard diluents was added to the vial, vortexed for 1-3 sec and incubated for 30min on ice. While the standard was incubating, the samples were prepared. For the preparation of the dilution series, nine 1.5mL tubes were labeled S1 to S8 and Blank. 150 μ L of standard diluents were added to tubes S2 to S8 and Blank, while S1 only got 72 μ L. The reconstituted standard was vortexed gently for 1-3 sec and 128 μ L were transferred into tube S1, containing 72 μ L of standard diluents. After each dilution the tube was vortexed for 1-3 sec and a new pipette tip was used to transfer 50 μ L from S1 to S2, S2 to S3 up to S8. The diluted standard was used immediately.

For this assay QuantiFERON plasma was used, which was diluted 1:4 by adding 20 μ L sample to 60 μ L Bio-Plex sample diluent. The samples were kept on ice until usage.

For the preparation of the beads, 5,175 μ L of assay buffer was added to a 15mL tube. The beads were vortexed for 30 sec at medium speed. The cap was opened carefully and remaining liquid in the cap was pipette back into the tube. 575 μ L of 10X beads were transferred into tube containing the assay buffer. The beads had to be protected from light at all time and adjusted to room temperature before use.

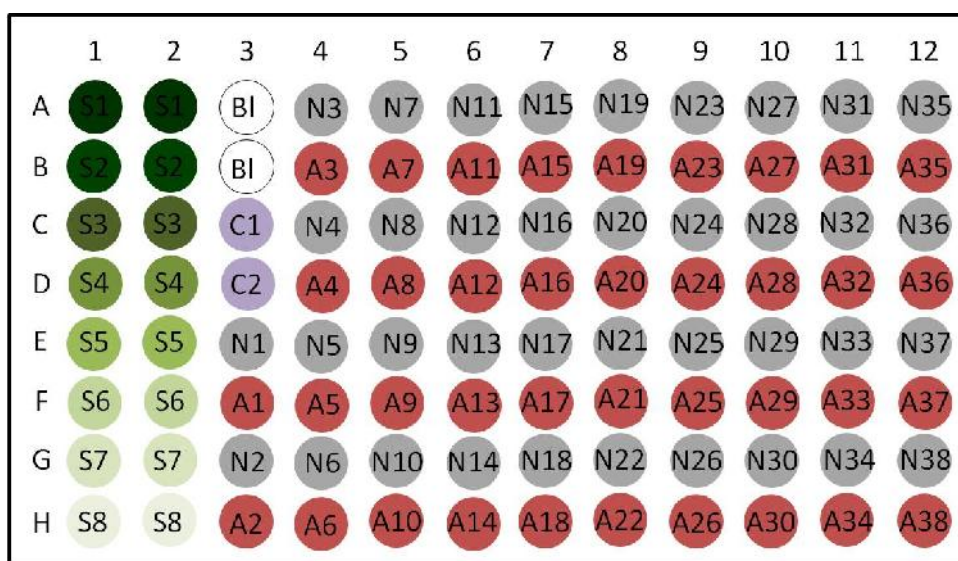


Figure 2.4: Plate layout for Bio-Plex Pro Assay. Standards (S1-S8) and blank (BI) were added in duplicates while control (C1/C2) and samples (1-38, NIL (N) and Antigen (A)) were added as single wells.

Assay Procedure

The diluted beads were vortexed for 30 sec at medium speed, poured into a reagent reservoir and 50 μ L were added into each well using a multichannel pipette. The wells were washed twice using the Bio-Plex Pro™ Wash Station. The standard dilutions, samples and controls were vortexed gently for 1-3 sec and 50 μ L were added to the appropriate wells (Figure 2.4). The plate was sealed with a cover and incubated on a shaker at room temperature for 30min. During the last 10min of the incubation time 1X

detection antibody was prepared by adding 300 μ L of 10X detection antibody (vortexed for 15-20 sec at medium speed) to 2,700 μ L of detection antibody diluent in a 15mL tube. Once the sample incubation was over, the sealing tape was removed and the plate was washed three times. The diluted detection antibody was vortexed for 1-3 sec, poured into a reagent reservoir and 25 μ L were added into each well using a multichannel pipette. The plate was covered with a new sealing tape and incubated on a shaker for 30min at room temperature. During the last 10min of the incubation 1X Streptavidin-Phycoerythrin (PE) was prepared by adding 60 μ L 100X Streptavidin-PE (vortexed for 15-20 sec at medium speed) to 5,940 μ L assay buffer in a 15mL tube. After incubation of the detection antibody, the sealing tape was removed and the plate washed three times. The diluted Streptavidin-PE was vortexed for 3-5 sec, poured into a reagent reservoir and 50 μ L were added into each well using a multichannel pipette. The plate was covered with a new sealing tape and incubated on a shaker for 10min at room temperature. Once the incubation time was over, the sealing tape was removed, plate washed three times and 125 μ L of assay buffer was added to each well. The plate was covered with a new sealing tape, incubated on shaker at room temperature at 1,100rpm for 30 sec. The plate cover was removed and the plate read using Bio-Plex High-Throughput Fluidics (HTF) System.

2.4 Flow Cytometry

Flow Cytometry is a method to characterize single cells using different parameters such as granularity, size, surface and intracellular structure as well as functional characteristics like intracellular staining (ICS) and proliferation.

Items	Company	Catalog number
FBS	Lonza	DE14-80F1
10X PBS	Lonza	BE17-517Q
IC Fixation Buffer	eBioscience	00-8222-49
10X Permeabilization buffer	eBioscience	00-8222-56
BD™ CompBeads Set Anti-Mouse Ig, κ	BD	552843
BD™ CompBeads Set Anti-Rat/Hamster Ig, κ	BD	552845
BD Cytometer Setup and Tracking Beads	BD	641319

Table 2.4: Reagents and Consumables for Flow Cytometry.

For all flow cytometry analysis a 96-well plate was used. 15mL tubes containing 2% FBS in PBS were prepared. The cryo tubes were taken out from liquid Nitrogen and placed in a 37°C water bath to thaw. The thawing process was observed and as soon only a small bit of ice was left, the tubes were taken out. 1mL of 2% FBS/ PBS was taken out of 15mL tube and slowly added to the cryo tube. The content of the cryo tube was transferred into a 15mL tube and spun down for 10min at 400g. The supernatant was discarded, the remaining sample transferred into a well of a 96-well plate and used for phenotyping or ICS, depending on sample.

2.4.1 Phenotyping

The plate containing the samples was spun down for 5min at 400g. The supernatant was discarded and the remaining cells stained with 20µL of the antibody mix (Table 2.5) and incubated for 20min at 4°C. Cells were washed twice with 200µL of 2% FBS/ FCS, resuspended in 200µL and run on a BD FACSCanto II.

Antibody	Fluorochrom	Volume per sample [μL]
CD3	Pacific Blue	0.75
CD4	Horizon V500	1.00
CD8	Phycoerythrin-cyanine (PE-Cy)7	0.25
CD14	PE	0.25
CD19	Allophycocyanin (APC)	0.25
TCR $\gamma\delta$	Fluorescein isothiocyanate (FITC)	0.75
	2% FBS/ PBS	16.75

Table 2.5: Antibody master mix for phenotypic analysis. Antibody mix was prepared during the last washing step and 20 μ L were added into each well using a multi channel pipette.

2.4.2 Intracellular Cytokine Staining

The plate containing the samples was spun down for 5min at 400g. The supernatant was discarded; the remaining cells resuspended in 100 μ L IC Fixation buffer and incubated for 20min at room temperature. The plate was spun down for 5min at 400g and washed twice with 1X Permeabilization buffer. The supernatant was discarded; the remaining cells were stained with 20 μ L of the antibody mix (Table 2.6) and incubated for 20min at room temperature in the dark. Cells were washed twice with 1X Permeabilization buffer, resuspended in 200 μ L and run on a BD FACSCanto II.

Antibody	Fluorochrome	WBA Volume per sample [μL]	LPA Volume per sample [μL]
CD3	Pacific Blue	0.75	0.75
CD4	Horizon V500	1.00	-
CD8	PE-Cy7	0.25	0.25
CCR7	APC	-	0.50
CD45RA	Horizon V500	-	0.75
Ki-67	FITC	-	2.00
CD40L	FITC	0.50	-
TNF- α	PerCP-Cy5.5	1.00	1.00
IFN- γ	APC-Cy7	0.50	0.50
IL-2	PE	0.75	0.75
IL-9	Alexa Fluor 647	0.75	-
1X Permeabilization buffer		14.50	13.50

Table 2.6: **Antibody master mix for WBA and LPA.** Antibody mix was prepared during the last washing step and 20 μ L were added into each well using a multi channel pipette

2.5 Statistics

In all studies data were analyzed using GraphPad Prism 5. Multiple comparison analysis using the Kruskal-Wallis test was done to determine differences between study groups. Datasets which are not independent were analyzed using Wilcoxon matched pairs test, such as data used for optimization, where same lab controls were used for different experiments. Statistical significant differences were recorded as follow:

$p > 0.05$ not significant (ns)

$p \leq 0.05$ *

$p \leq 0.01$ **

$p \leq 0.001$ ***

CHAPTER THREE – RESULTS

Science is wonderfully equipped to answer the question "How?"

But it gets terribly confused when you ask the question "Why?"

Erwin Chargaff

3.1 Introduction

This chapter describes the development of additional applications of the QFT assay to incorporate antigen-specific cellular characterization (by flow cytometry) and soluble host marker production (multiplex cytokine arrays). The advantage of this approach would be that multiple complimentary immunological readouts can be obtained from a commercially available, highly standardized *M. tb* antigen stimulated whole blood culture assay.

3.2 Optimization of QFT assay for use in Flow Cytometry

With one third of the population infected with *M. tb* (114), TB remains a global health problem. Diagnosis of TB is often a problem especially due to the lack of sensitivity of the most widely used test, sputum smear microscopy, or high costs of more sensitive diagnostic tools, like X-rays or GeneXpert, or due to difficulty in obtaining adequate sputum sample especially in children. More cost efficient diagnostic tools are necessary. In 2007, QFT-G was approved by Food and Drug Administration (FDA), which measures IFN- γ responses to *M. tb* specific antigens (ESAT-6 and CFP-10) and is used to detect asymptomatic or LTBI but cannot discriminate between LTBI and active TB. By assessing QFT supernatants by Luminex, *Chegou et al.* (12) have shown that 3-marker combination can differentiate between LTBI and active TB. It has also been shown that cellular immunity plays a role in the control of *M. tb* infection and the analysis of polyfunctional T cells by flow cytometry can help to understand the mechanism (8;30;45). The hypothesis of the present work was that the QFT tubes would be useful to evaluate a range of complementary antigen-specific immunological readouts, including soluble marker expression, whole blood cell characterization by flow cytometry and gene

expression analysis. The advantage of such an approach would be that a highly standardized assay system would be used that requires only 3mL of blood, a major benefit in pediatric participants of clinical trials, where blood volumes that are safely obtainable are very limited.

The optimization for the usage of flow cytometry on cells from QFT tubes will be discussed by comparing whole blood cells obtained from the QFT-WBA directly to cells from a standard whole blood assay, whereby the optimal stimulation times and re-stimulation methods will be addressed.

For all optimizations, whole blood from laboratory controls (LC) was used. In order to compare the different assays, the same participant was used for WBA and QFT-WBA.

3.2.1 Removal of 300 μ L of whole blood from the QFT tubes does not affect IFN- γ ELISA results

When using QFT for RNA isolation and phenotyping, blood was taken off before harvesting the supernatant. It had to be assessed whether the removal of blood would affect the QFT ELISA result.

1mL of blood from six lab controls was drawn into two sets of QFT tubes, each set containing NIL, TB Antigen and Mitogen tubes. The tubes were incubated for exactly 18h at 37⁰C. After incubation the two QFT tube sets were split and one set was labeled 'normal' and the other 'modified'. The normal QFT tubes were processed as per manufactures guidelines, whereas 300 μ L were taken off from the modified QFT tubes before processing. The supernatant was harvested into three tubes each, and frozen at -80⁰C. For the QFT ELISA one NIL, TB Antigen and Mitogen tube from each set was

processed as per manufactures guidelines. The results (Table 3.1) were obtained using the QuantiFERON-TB Gold analysis software. To compare the results directly the values for the normal and modified QFT were analyzed using GraphPad Prism 5 (Figure 3.1).

Lab Control Number	QFT	NIL [IU/mL]	TB Antigen [IU/mL]	TB Ag - NIL [IU/mL]	Result
LC 004	NORMAL	0.18	0.26	0.08	NEGATIVE
	MODIFIED	0.23	0.35	0.12	
LC 005	NORMAL	0.13	0.49	0.36	POSITIVE
	MODIFIED	0.16	0.68	0.52	
LC 006	NORMAL	0.05	8.62	8.57	POSITIVE
	MODIFIED	0.04	9.22	9.18	
LC 007	NORMAL	0.04	0.04	0.00	NEGATIVE
	MODIFIED	0.03	0.03	0.00	
LC008	NORMAL	0.04	0.04	0.00	NEGATIVE
	MODIFIED	0.05	0.04	-0.01	
LC 009	NORMAL	0.11	0.07	-0.04	NEGATIVE
	MODIFIED	0.04	0.04	0.00	

Table 3.1: QuantiFERON results of lab controls. The QuantiFERON-TB Gold analysis software calculates the expression of IFN- γ in plasma by obtaining a standard curve. All the criteria described in the method chapter were met and the test was declared as valid. The concentration of NIL, TB Antigen and TB Antigen minus NIL are shown. Two out of six LCs were tested positive. There is no significant difference between the normal and modified QFT.

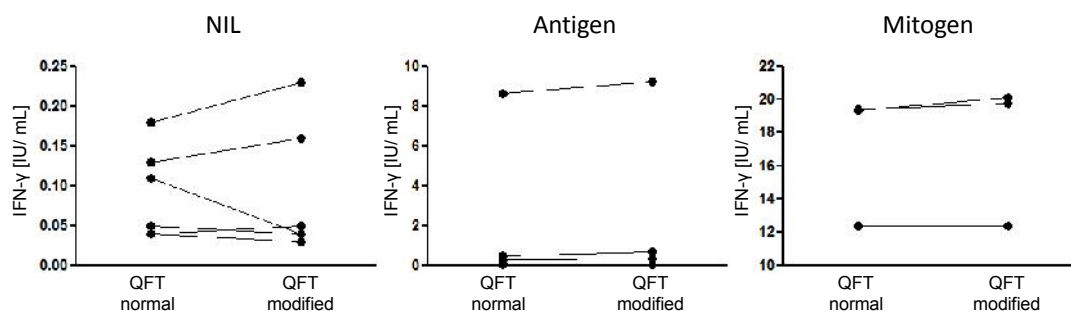


Figure 3.1: Comparison of QuantiFERON results between normal QFT and modified QFT. 2 sets of QFT tubes were filled with 1mL of blood from lab controls. Plasma was harvested as per manufactures guideline (QFT normal) from one half while 300 μ L of blood were taking off from the other half (QFT modified) before processing. ELISA was performed as per manufactures guideline.

The comparison was done on six LCs. Two of the LCs had positive QFT tests with both approaches. The concentration of IFN- γ for each stimulus between the normal and modified assays was compared using the Wilcoxon matched pairs test. P values of 0.92 in NIL, 0.31 in Antigen and 0.50 in Mitogen tubes showed no significant differences and the slight differences found probably reflect assay variability or pipetting errors.

The use of plasma and blood cells from the QFT can therefore be performed without altering the QFT test result.

3.2.2 Comparison of WBA vs. QFT-WBA in flow cytometry

When following the manufacturer's guideline the blood has to pass through a gel layer. The constituents of the gel layer are not shared by the manufacturer and it is unknown whether this layer damages the white blood cells. To assess white cell viability the QFT-WBA had to be compared directly to a WBA. Blood from LCs were drawn into QFT tubes directly and 3mL was drawn into a NaHep tube. The whole blood from the NaHep tube was transferred into a 24- well plate, each well containing 1mL of blood. The blood was treated the same way as the QFTs, by adding 1 drop of ESAT-6 and CFP-10 into the TB Antigen well, and 1 drop of Mitogen into the Mitogen well. The Antigens and Mitogen were obtained from reagents of the QuantiFERON®-TB Gold assay which has been discontinued and replaced by QuantiFERON®-TB Gold IT. The blood was incubated for 18h at 37°C followed by harvesting of supernatant. The blood cells from the QFT tubes were obtained by centrifuging the tubes upside down. The blood cells were transferred into 2mL tubes, 200 μ L each, and incubated together with 10 μ g/mL Brefeldin A for another 4h. After the incubation period the red blood cells were lysed, white blood cells were fixed and permeabilized, followed by staining with

antibodies against human CD3, CD4, CD8 and IFN- γ . The cells were acquired on a FACSCanto II instrument and analyzed with the FlowJo 7.6.5 and the percentage of IFN- γ producing CD4 and CD8 T cells was determined using the gating strategy in Figure 3.2. At first single cells were identified by gating on forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). The lymphocyte population was selected and defined as CD3 T cells, which then was divided into CD4 and CD8 T cells. Further, the expression of IFN- γ was determined by gating on those subpopulations. The data obtained from FlowJo 7.6.5 were analyzed by Excel and GraphPad Prism 5 using the Wilcoxon matched pairs test.

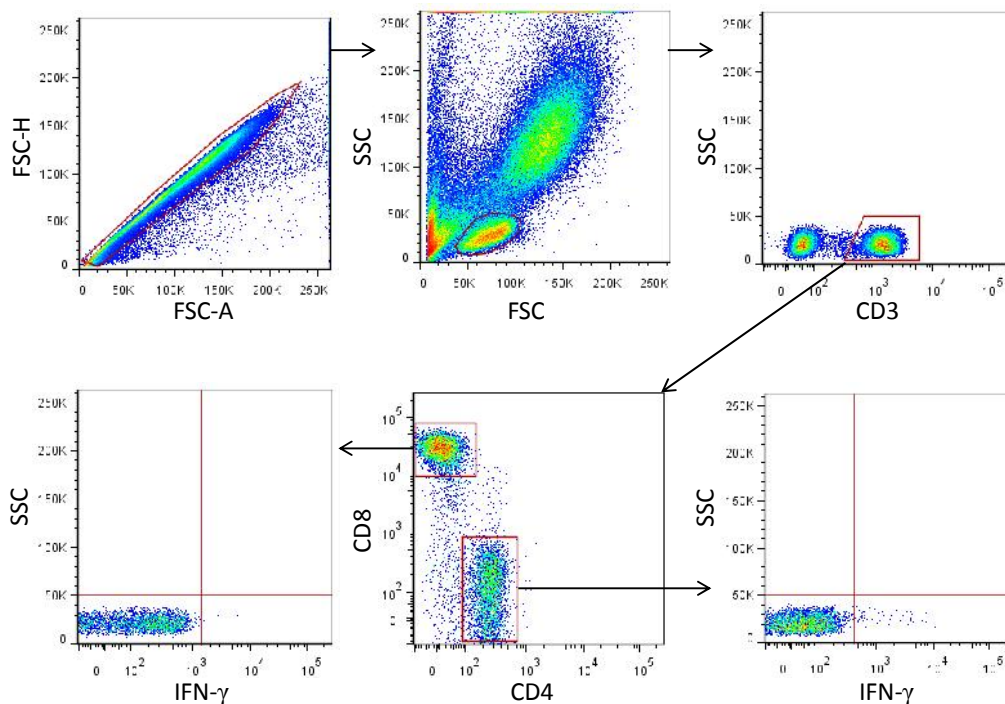


Figure 3.2: Gating Strategy used for all optimization steps. Flow cytometric analysis of IFN- γ expression of T cells in stimulated whole blood cells. Firstly, cell doublets were excluded using forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). Using forward scatter (FSC) versus side scatter (SSC) the lymphocyte population could be determined followed by identification of CD3 T cells, which is further subdivided into CD4 and CD8 T cells. Cell specific expression of IFN- γ was subsequently analyzed.

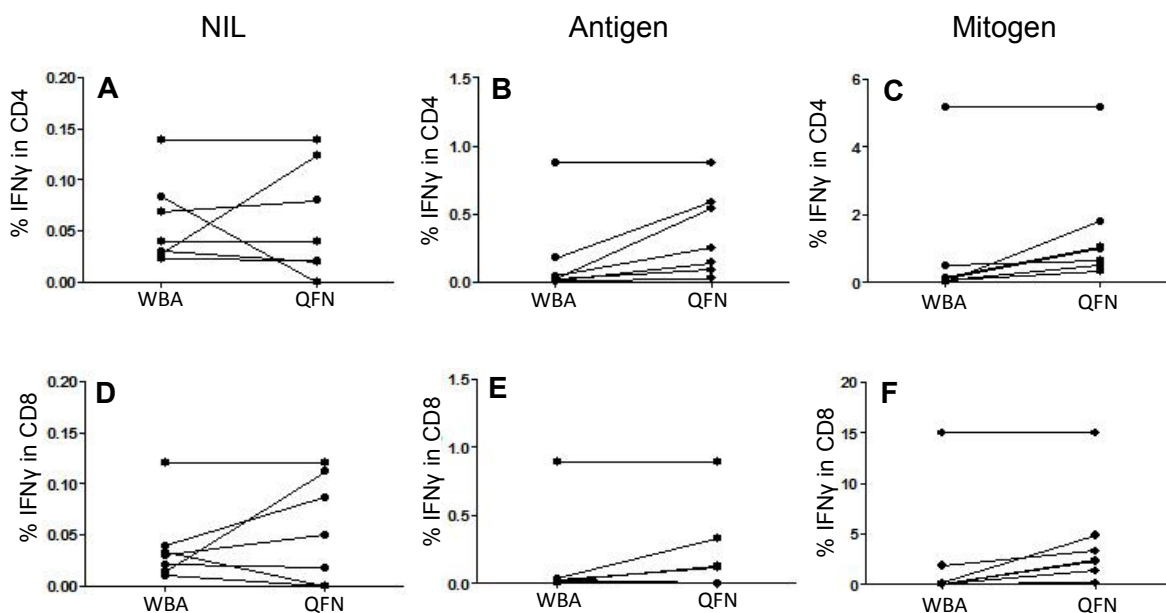


Figure 3.3: Comparison of IFN- γ expressing CD4 and CD8 T cells in WBA and QFT-WBA. 1mL of whole blood from LCs was stimulated with the same culture conditions as used for the QuantIFERON assay. One set of QFT tubes were incubated for 22h. For the last 4h, Brefeldin A [10 μ g/mL] was added to NIL control (A, D), Ag tubes containing ESAT-6/ CFP-10 (B, E) and MIT tubes containing PHA (C, F). Intracellular staining was performed using antibodies against human CD3, CD4, CD8 and IFN- γ . Concentration of IFN- γ expression in CD4 T cells (A, B, C) and CD8 T cells (D, E, F) was determined by flow cytometry.

In the NIL control two of the six LCs show differences in IFN- γ expression, with one sample showing an increase in the QFT-WBA while the other one shows a decrease (Figure 3.3). The differences in the NIL tube are not significant, showing a p value of 1.00/ 0.56 (CD4/ CD8). In the Ag and Mitogen tube the QFT-WBA shows a higher expression of IFN- γ compared the WBA. Significant differences were observed in the IFN- γ expression of antigen stimulated CD4 T cells ($p=0.03$) and Mitogen stimulated CD8 T cells ($p=0.3$). The higher expression of IFN- γ in QFT could be due to the presence of TB7.7 as additional peptide, which may be responsible for the stronger immune response in QFT. Overall the QFT-WBA shows good correlation with the WBA method and can be used for flow cytometry.

3.2.3 Comparison of 4h vs. 6h re-stimulation of QFT-WBA

The QuantiFERON tubes were incubating for 18h before harvesting the plasma. To determine ICS, the transport of proteins to the cell surface has to be blocked by using Brefeldin A. Brefeldin A leads to accumulation of cytokines at the Golgi complex which then can be measured via ICS. To determine the optimal re-stimulation time, the blood was incubated for another 4h and 6h in the presence of Brefeldin A. After the standard 18h incubation for the QFT test following the method described in the methods chapter, the blood was harvested and divided into two tubes. One tube was incubated for 4h in the presence of Brefeldin A; the second tube was incubated for 6h, whereby Brefeldin A was added for the last 4h. After the incubation the red blood cells were lysed and ICS was performed using antibodies against human CD3, CD4, CD8 and IFN- γ . The percentage of IFN- γ expressing CD4 and CD8 T cells was determined and analyzed in GraphPad Prism 5 using the Wilcoxon matched pairs test.

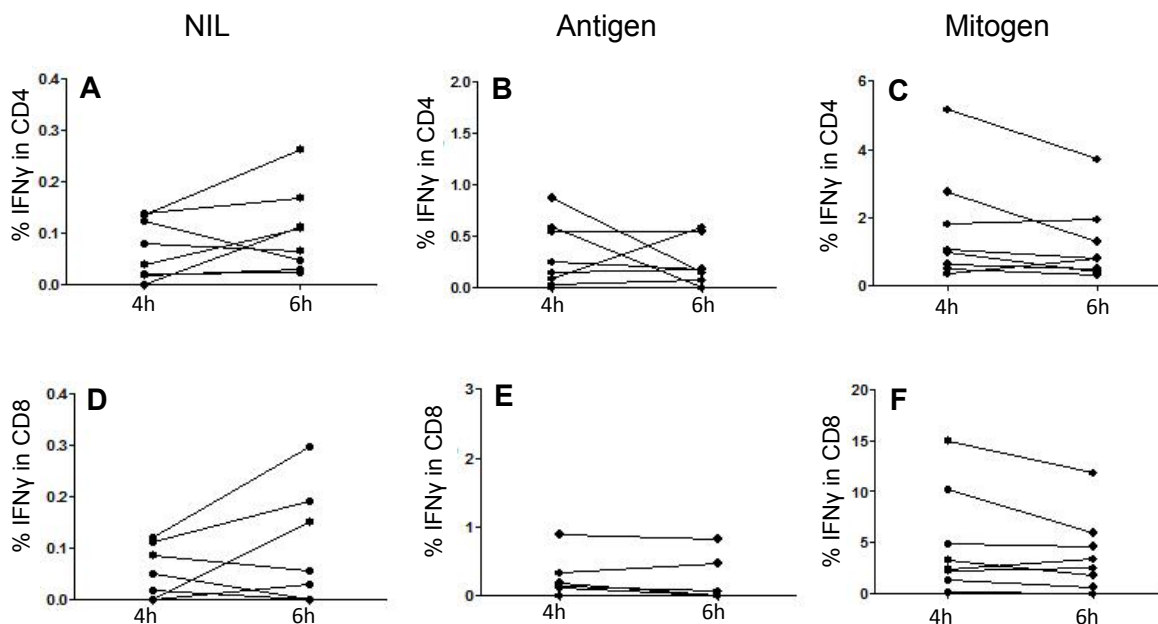


Figure 3.4: Comparison of IFN- γ expressing CD4 and CD8 T cells in QFT cells stimulated for 4h and 6h. After the initial 18h incubation, the QFT tubes were incubated for another 4h or 6h, whereby for the last 4h Brefeldin A [10 μ g/mL] was added to NIL control (A, D), Ag tube containing ESAT-6/ CFP-10 (B, E) and MIT tube containing PHA (C, F). Intracellular staining was performed using antibodies against human CD3, CD4, CD8 and IFN- γ . Concentration of IFN- γ expression in CD4 T cells (A, B, C) and CD8 T cells (D, E, F) was determined by flow cytometry.

The NIL tube showed an increase in most of the LCs in the 6h re-stimulation (Figure 3.4

Figure 3.4), while a few LCs showed a decrease in the Ag and MIT tube. Statistical analysis does not show significant differences between the 4h and 6h assay with P values of 0.25/ 0.38 (CD4/ CD8) in NIL, 0.69/ 0.44 (CD4/ CD8) in Ag and 0.15/ 0.20 (CD4/ CD8) in MIT. It was thus decided to use the 4h re-stimulation assay for all further assays.

3.2.4 Different concentration of PMA/ Ionomycin used for QFT-WBA

Following an 18h incubation significant IFN- γ production and secretion into the supernatant would have taken place. To ensure that meaningful additional production of

this and other cytokines takes place after the addition of Brefeldin A, re-stimulation had to be considered. One possible re-stimulation method would be through PMA and Ionomycin, which stimulates the production of cytokines. To determine the optimal concentration, whole blood was incubated with 2ng to 20ng of PMA and 200ng to 2µg of Ionomycin, by maintaining a PMA/ Ionomycin ratio of 1/100, for a further 4h in the presence of Brefeldin A. The optimization was done for an unrelated vaccine study, performed by myself, where BCG and PHA were used as primary stimulus.

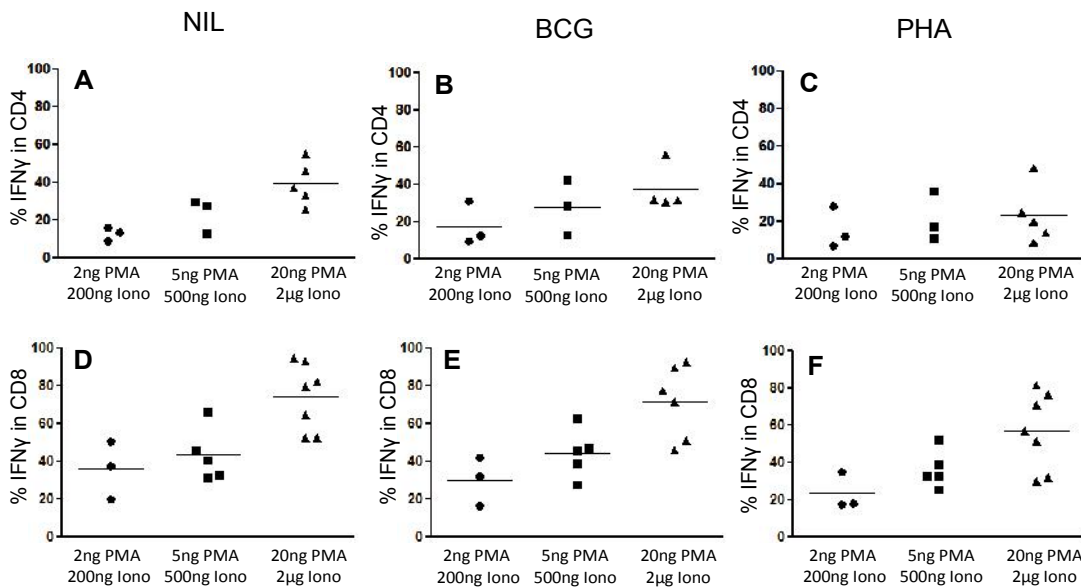


Figure 3.5: Different concentrations of Ionomycin in stimulated whole blood. Whole blood from LCs was stimulated with BCG (B, E) and PHA (C, F) and incubated for a total of 22h, whereby different concentrations of PMA/ Ionomycin and Brefeldin A [10µg/mL] were added for the last 4h. Blood was lysed and intracellular staining was performed using antibodies against human CD3, CD4, CD8 and IFN-γ. Concentration of IFN-γ expression in CD4 T cells (A, B, C) and CD8 T cells (D, E, F) was determined by flow cytometry.

The graph shows that in all samples the expression of IFN-γ increases with increasing PMA/ Ionomycin concentration (Figure 3.5). Because the PMA/ Ionomycin should only serve as a boost the high re-stimulation concentrations may also

overshadow antigen-specific cytokine production, the lowest concentration, 2ng/mL PMA and 200ng/mL Ionomycin, was used for further assays.

3.2.5 Different re-stimulation methods of QFT-WBA

To improve the cytokine expression after 18h culture in QFT tubes different re-stimulation methods using anti CD28/ 49d, PMA/ Ionomycin and Ag or MIT from the QuantiFERON®-TB Gold assay were investigated. Therefore QFT bloods from LCs were incubated for 18h following a 4h re-stimulation in the presence of Brefeldin A plus the different restimulation agents. One sample of each QFT tube was left without restimulation to serve as control. The red blood cells were lysed and ICS was performed using antibodies against human CD3, CD4, CD8 and IFN- γ . The percentage of IFN- γ expressing CD4 and CD8 T cells was determined and analyzed in GraphPad Prism 5. Differences between the restimulated sample and their unstimulated control were calculated using the Mann-Whitney test.

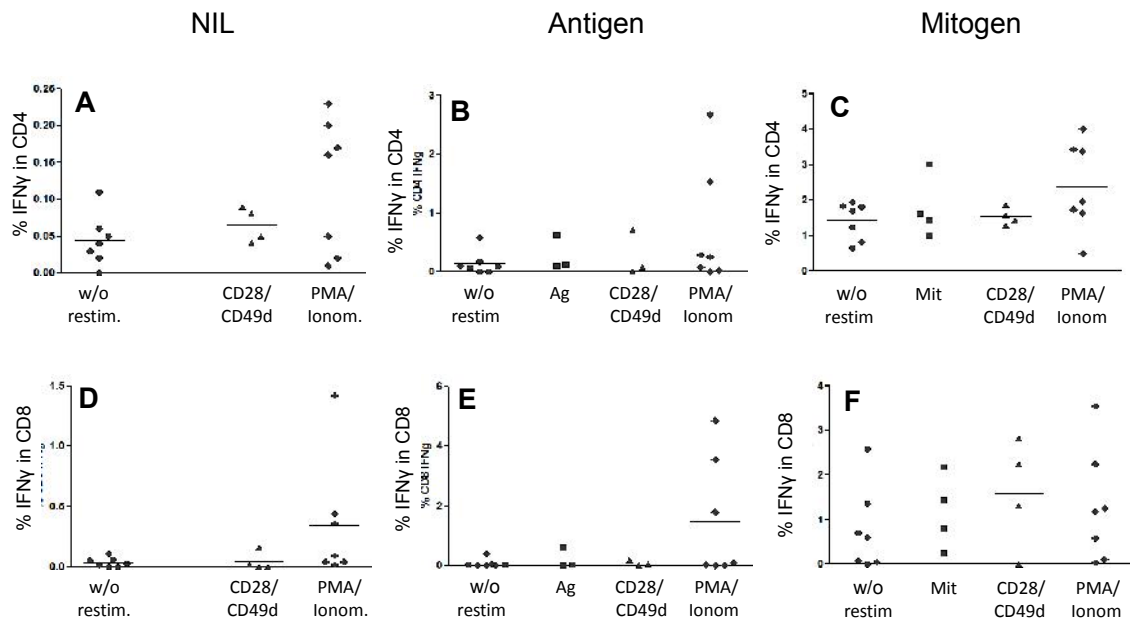


Figure 3.6: Different re-stimulation methods of QFT-WBA after harvesting of plasma. QFT from LCs were incubated for 18h. After harvesting of plasma, blood cells were obtained and stimulated for another 4h with CD28/ CD49d, PMA/ Ionomycin and Antigen or Mitogen (depending on stimulus) in the presence of Brefeldin A. Blood was lysed and intracellular staining was performed using antibodies against human CD3, CD4, CD8 and IFN- γ . Concentration of IFN- γ expression in CD4 T cells (A, B, C) and CD8 T cells (D, E, F) was determined by flow cytometry.

While the CD28/49d re-stimulation does not show differences for samples without restimulation, a statistically not significant increase can be seen after Ag or MIT and PMA/ Ionomycin addition (Figure 3.6). Because the Ag and MIT are not available anymore (QFT Gold test was replaced by the QuantiFERON®-TB Gold IT test), all further assays were done using PMA/ Ionomycin as re-stimulation.

3.2.6 Lymphocyte Proliferation Assay

Proliferation in T cells can be assessed using ^3H -thymidine (a radioactive substance) (12;75), and fluorescent dyes like Carboxyfluorescein succinimidyl ester (CFSE) or Oregon Green (OG). OG is a visible light–excitable intracellular calcium

indicator that is cell-permeate and loads into living cells. With each cell division the relative fluorescent intensity of OG decreases by half. Oregon Green is excited at 488nm and emits light at 518nm, and shares the emission wavelength with FITC. Therefore using flow cytometric analysis the different cell cycles can be assessed. In order to use CFSE or OG, PBMC's have to be isolated, whereas Ki-67 allows the use of WB cells and is not limited to the FITC channel. Ki-67 is a protein which is expressed during cell division and can be measured after stimulation combined with other markers for phenotyping or ICS. Soares *et al.* (97) compared proliferation assays using OG in PBMCs and Ki-67 in WB cells. They showed similar frequencies of proliferating T cells and high reproducibility between the assays. They proposed Ki-67 as marker for antigen-specific T cells proliferation. The advantage of using Ki-67 is the limited blood volume required and that it requires no incubation or washing steps prior to incubation. Using the above assay whole blood cells obtained from QFT tubes were stimulated for 6 days with PMA/ Ionomycin followed by re-stimulation with PMA/ Ionomycin for 4h after addition of Brefeldin A.

3.2.7 Setting up FACSCanto II for Flow Cytometry

Many different reagents and fluorescent antibodies are available which react differently under different conditions and therefore have to be optimized. The optimal usages of marker have to be investigated in order to get the best results. Protocols have to be adapted as the conditions in each laboratory are not the same and temperature and humidity may influence the results. The Fluorescence Activated Cell Sorting (FACS) Canto II is a sensitive machine. Equipped with three lasers it needs special attention as the laser power varies from machine to machine and optimization has to be done

specifically for the use of the machine otherwise it could result in false analysis of the samples.

3.2.7.1 *Antibody titration*

For each experiment the antibodies had to be titrated to ensure optimal signal compared to background noise. Too high concentration may result in non specific binding. Whole blood cells from LCs were stained with different concentrations of surface markers (Table 3.2). To assess the concentrations for ICS whole blood cells were stimulated with PHA for 12h, followed by fixation and permeabilization. The data were analyzed by determining the mean of the positive (signal) and negative population (noise) for each antibody, and by calculating the signal/noise ratio.

After calculating the signal/noise ratio, antibody titration curves were plotted to determine the saturating concentration (Figure 3.7). Most of the antibodies (human CD3, CD4, CD8, CD40L, CD19, TCR $\gamma\delta$, Ki-67, IL-2 and IFN- γ) show a saturation point. At this point the antibody shows the optimal separation between negative and positive population. Antibodies like human CD45RA, Chemokine receptor 7 (CCR7), CD14 and IL-9 show first a decrease in signal/noise ratio, followed by an increase until it reaches saturation and decreases again. The decrease in those antibodies is only visible in volumes lower than 0.5 μ L. Too few cells binding the antibody at the lower limit of detection could affect the signal/noise ratio. Good separation was achieved when reaching saturation. The only antibody which did not show a good signal/noise titration curve was anti human TNF- α . The titration curve showed a continuous increase and did not reach saturation even though the dot plot showed distinctive negative and positive population.

	Antibody	Fluorochrom	I	II	III	IV	V
Surface staining	CD3	Pacific Blue	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	CD4	V500	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	CD8	PE-Cy7	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	CD40L	FITC	0.25 µL	0.50 µL	1.00 µL	2.00 µL	5.00 µL
	CCR7	APC	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	CD45RA	V500	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	Ki-67	FITC	0.25 µL	0.50 µL	1.00 µL	2.00 µL	5.00 µL
	CD14	PE	0.125 µL	0.25 µL	0.50 µL	1.00 µL	2.00 µL
	CD19	APC	0.125 µL	0.25 µL	0.50 µL	1.00 µL	2.00 µL
	TCR γδ	FITC	0.25 µL	0.50 µL	0.75 µL	1.00 µL	
ICS	IFN-γ	PE-Cy7	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	IL-2	PE	0.25 µL	0.50 µL	0.75 µL	1.00 µL	1.25 µL
	IL-9	Alexa Fluor 647	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL
	TNF-α	PerCP Cy5.5	0.25 µL	0.50 µL	0.75 µL	1.00 µL	2.00 µL

Table 3.2: Titration of surface antibodies and antibodies against intracellular cytokines. Unstimulated whole blood was stained with different Titer of surface makers while PHA stimulated whole blood was used for ICS. Bold values indicate optimal performance of the assay.

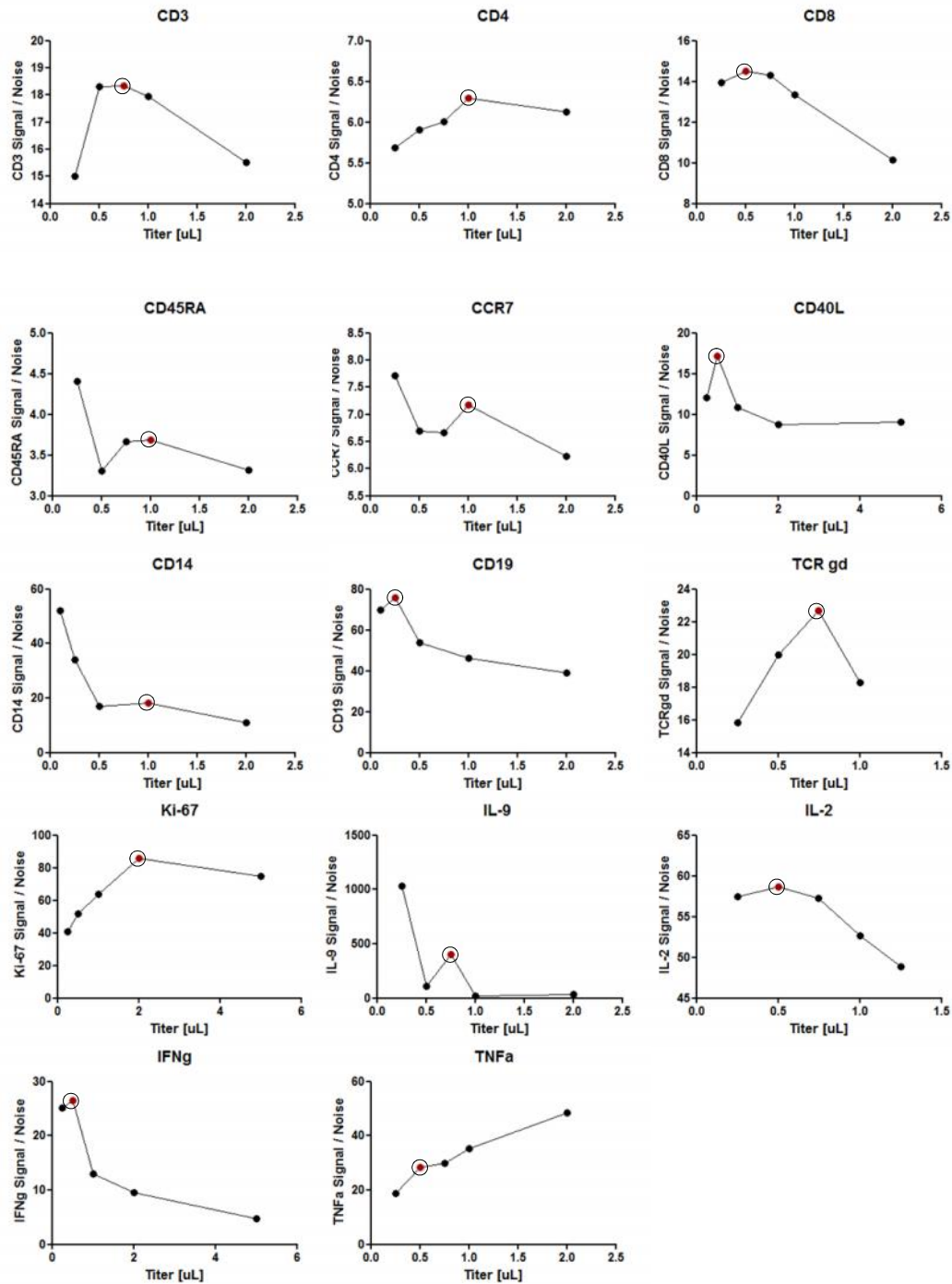


Figure 3.7: Antibody titration plots. Evaluation of different antibody volumes by staining whole blood from LCs with each antibody. The frequencies of positive populations (signal) and negative populations (noise) was assessed by plotting the signal to noise ratio into antibody titration curves. The circled point in each graph shows the optimal titer, which was chosen for further flow cytometry assays.

3.2.7.2 Compensation

Using 8-color flow cytometry requires good compensation as there is spill over between emission spectra of different fluorochromes. To correct for the spillover compensation had to be done, which allowed quantifying each single dye by subtracting the portion of the spillover from one particular parameter into the other. For this study compensation beads were used due to the many advantages; no cells are needed which in some cases might be limited, dimly expressed antigens can still be compensated for, guaranteed negative and positive population.

Compensation was done for all three assays (phenotyping, WBA and LPA). The compensation beads kit contained negative and positive beads. The negative beads were uncoated and had no binding capacity whereas the positive beads were coated with IgG kappa and bind to the immunoglobulin. For an 8-color experiment nine tubes were prepared. One tube served as negative control and contained only negative and positive bead, whereas the specific antibody was added to the other tubes containing the negative and positive beads. The tubes were incubated for 15min, followed by a washing step and compensation was run on FACSCanto II using FACS Diva software. After defining the negative and positive population for each antibody the spectral overlap was calculated. The compensations were used for all further assays.

3.3 Study population

The study groups used for the QFT assay consist of Community Controls (CTRLs), Household Contacts (HHCs) and TB cases (TB) that were recruited from Ravensmead and Uitsig. QFT from 10 CTRLs, 16 HHCs and 19 TBs were used for QFT IFN- γ ELISA, Luminex and flow cytometry for immune cell phenotyping and ICS and QFT from 6

CTRLs, 9 HHC and 11 TBs were used for LPA and RNA analysis. The demographic characterization is shown in Table 3.3.

	Community Controls	Household Contacts	TB cases
Number of participants			
For Luminex, WBA, Phenotyping	10	16	19
For LPA and RNA	6	9	11
Gender			
Female	4	11	5
Male	6	5	14
Age in years			
Mean	31.7	34.4	34.5
Age range	22-49	18-58	16-59
QFT results			
Positive	5	13	16
Negative	5	3	1
not done			2

Table 3.3: Study population. Demographic characterization of study groups used for QFT assay.

3.4 Assessing the IFN- γ secretion in QFT supernatant.

The IFN- γ secretion in QFT supernatants was assessed using the QuantiFERON®-TB GOLD ELISA assay as per manufacturer guidelines. The ELISA showed that the distribution of positive and negative results in the community controls was equal, whereas few participants were negative in the household contact (19%) and TB groups (6%) (Table 3.4). For further analysis only QFT negative CTRLs (n=5), QFT positive HHCs (n=13) and QFT positive TB cases (n=16) were assessed.

COMMUNITY CONTROLS				HOUSEHOLD CONTACTS				TB INDEX CASES			
	RESULT	Ag [IU/mL]	Ag-NIL [IU/mL]		RESULT	Ag [IU/mL]	Ag-NIL [IU/mL]		RESULT	Ag [IU/mL]	Ag-NIL [IU/mL]
001	POS	0.55	0.42	04	POS	0.57	0.35	01	POS	14.68	14.51
002	POS	3.75	3.55	05	POS	19.44	19.22	03	POS	12.53	12.37
003	NEG	0.51	-0.28	09	POS	16.81	16.16	04	POS	12.01	11.80
004	NEG	0.77	0.26	11	POS	0.87	16.81	05	POS	3.07	2.93
005	POS	1.93	1.76	12	POS	10.93	10.79	06	POS	0.75	0.62
007	POS	5.65	5.31	18	POS	7.06	6.87	07	POS	0.63	0.51
008	NEG	0.23	0.11	19	POS	2.72	0.61	08	POS	2.21	1.74
009	POS	17.91	17.77	20	NEG	0.27	0.12	12	POS	1.27	1.14
010	NEG	0.29	0.12	21	POS	11.40	11.26	13	POS	3.50	3.37
012	NEG	1.16	-0.80	23	POS	1.28	1.02	001	POS	13.61	13.41
				26	POS	5.95	5.23	002	POS	4.25	3.79
				29	POS	4.58	4.11	003	POS	2.92	2.59
				30	POS	12.32	11.99	004	POS	6.44	6.11
				31	NEG	0.15	0.01	007	NEG	0.56	0.24
				32	NEG	0.14	0.00	008	POS	5.42	5.23
				34	POS	0.56	0.42	009	POS	18.83	18.57
								010	POS	2.30	9.43

Table 3.4: QuantiFERON results. IFN- γ secretion in QFT supernatant in CTRLs, HHCs and TBs using QuantiFERON®-TB GOLD ELISA.

3.5 Correlation between QFT and Luminex

QFT results are measured in IU/mL. Those values were transformed into pg/mL by multiplying with the factor 40. The correlation of IFN- γ secretion obtained by QFT ELISA and Luminex were assessed (Figure 3.8). No correlation was seen in QFT negative subjects as all levels approached zero. In QFT positive and TB subjects a good correlation between QFT ELISA and Luminex (as part of a 27 plex kit) was observed (r 0.75-0.96; p 0.0020 and p <0.0001).

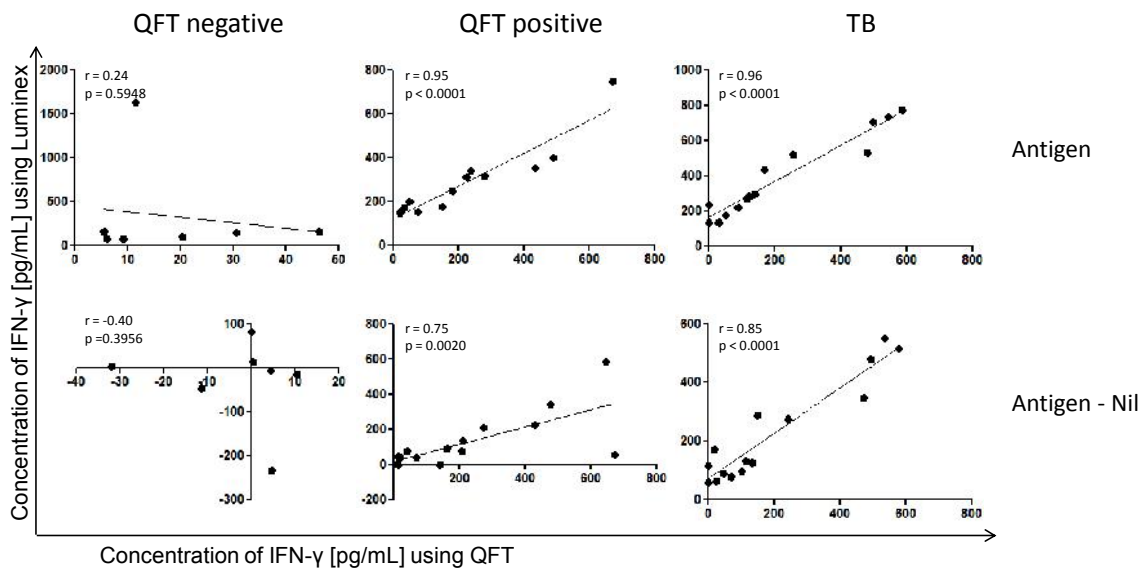


Figure 3.8: Correlation between Luminex and QFT results. Linear regression curve between IFN- γ expression using Luminex vs. QFT ELISA. r shows the Spearman rank correlation coefficient and p the p value. A good correlation was observed in QFT positive and TB subjects.

3.6 Luminex results

Using the Biorad multiplex assay, 27 markers in QFT supernatant could be assessed simultaneously. *Breen et al.* have shown that there is no major difference between extrapolated and not extrapolated datasets (7). Extrapolated data is routinely used in the analysis of Luminex data and reported as valid results by the accompanying analysis software. Values that fell outside the acceptable ranges appeared as OOR or were set to Zero. While most of the markers did not show any differences, five markers (FGF basic, GM-CSF, MCP-1, MIP-1 β and VEGF) showed differences between HHCs and TB cases (Figure 3.9). The unstimulated levels (NIL) of GM-CSF and VEGF were higher in TB compared to HHC and were down regulated after stimulating with ESAT-6 and CFP-10 (Ag). The expression of those two cytokines was lower in TB cases than in HHCs when unstimulated background was subtracted from antigen-stimulated values.

FGF basic and GM-CSF showed an antigen specific increase in TB cases while MCP-1 and MIP-1 β showed decreases in TB cases compared to HHC. When subtracting the unstimulated background from antigen-stimulated values, a decrease was seen in TB compared to HHC for MCP-1, MIP-1 β and VEGF (significant with $p=0.0099$).

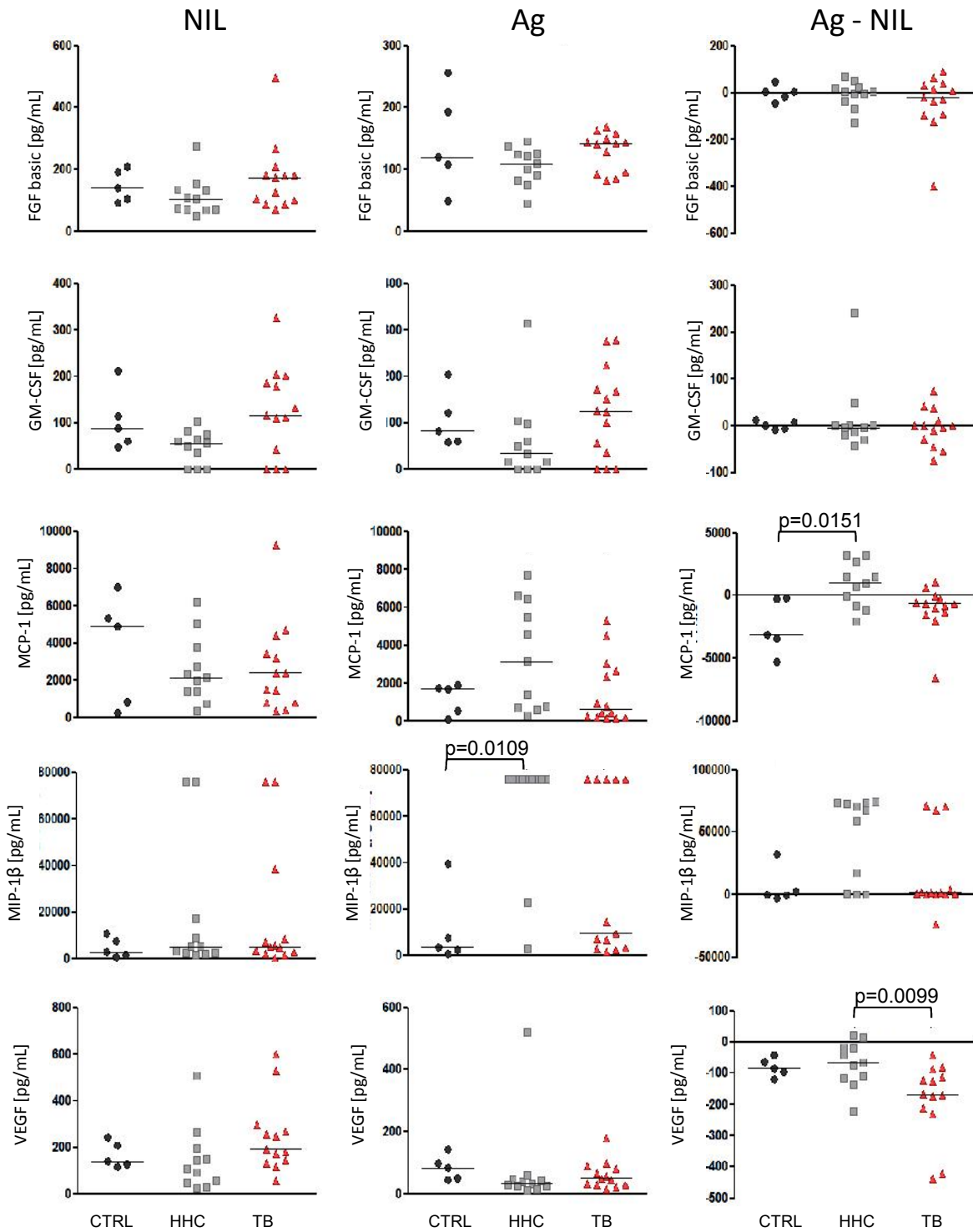


Figure 3.9: Luminex Results. Results of expression of analytes in QFT supernatant after 18h stimulation with ESAT-6 and CFP-10 (Ag). This figure represents median levels and ranges of analytes which show significant differences between HHCs and TB cases. The Kruskal-Wallis test was used to calculate the p value between groups.

3.7 Flow Cytometry

3.7.1 Phenotyping

In this section the pattern of peripheral blood cell populations in CTRLs, HHC and TB cases was assessed. The unstimulated QFT whole blood cells were phenotypically assessed, defining monocytes, B cells, CD4 and CD8 T cells, and TCR $\gamma\delta$ expressing CD4 and CD8 T cells. 100 μ L of QFT blood was incubated with the specific markers for 20min and analyzed on a FACSCanto II. First, single cells were defined using forward scatter-height (FSC-H) versus forward scatter-area (FSC-A) (Figure 3.10). The lymphocyte and monocyte population could be determined according to the size (FSC) and granularity (SSC) of the cells. The lymphocyte population was subdivided into B cells using anti-human CD19, CD4 and CD8 T cells using combination of anti human CD3, CD4 and CD8, and TCR $\gamma\delta$ expressing CD4 and CD8 T cells using combination of anti human CD3, CD4, CD8 and TCR $\gamma\delta$. Monocytes were identified using anti human CD14. The percentage of each population was determined and differences between the groups were assessed using Man Whitney test in GraphPad Prism 5.

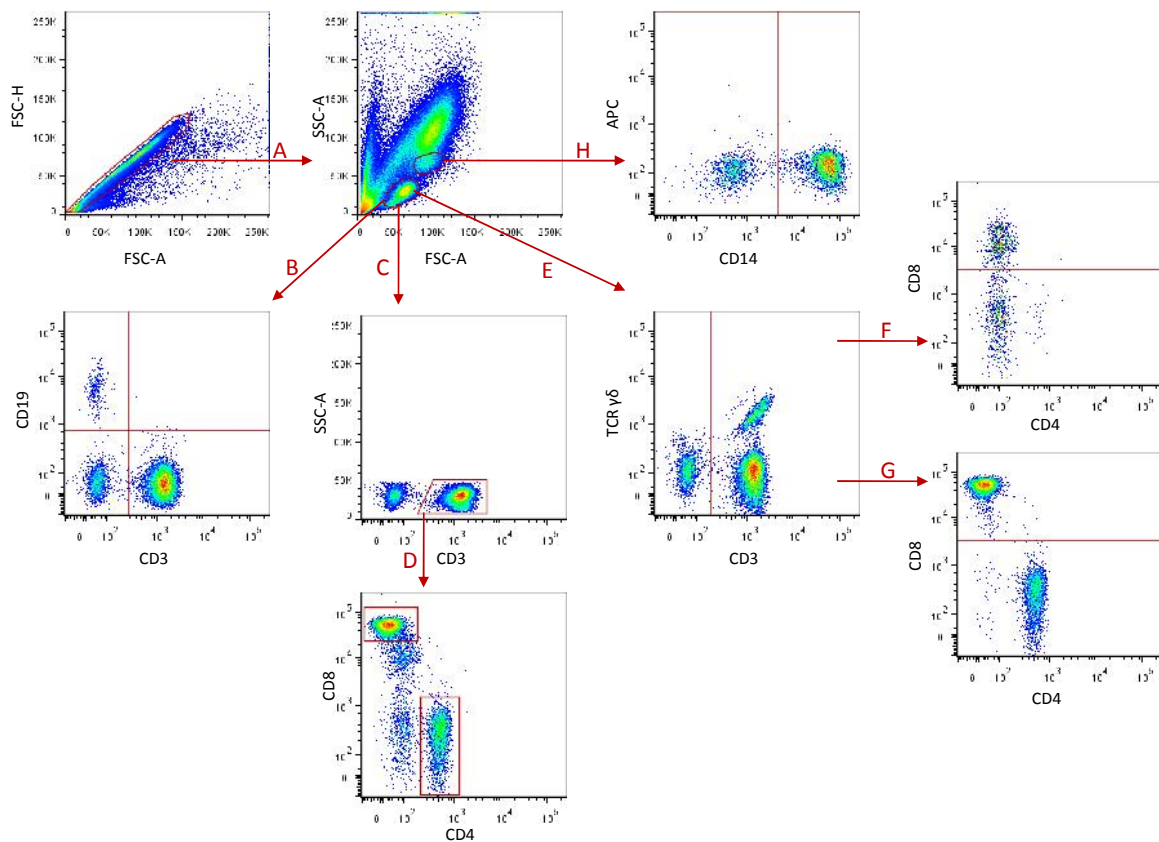


Figure 3.10: Gating strategy for phenotyping. Single cells (A) were included using forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). Using forward scatter (FSC) versus side scatter (SSC) the lymphocyte and monocyte population could be determined followed by identification of B-cells (B); identification of CD3 T cells (C), which is further subdivided into CD4 and CD8 T cells (D); identification of TCR $\gamma\delta$ T cells (E), which is further subdivided into TCR $\gamma\delta$ + expressing CD4 and CD8 (F) and TCR $\gamma\delta$ - expressing CD4 and CD8 (G); identification of monocytes using CD14 (H).

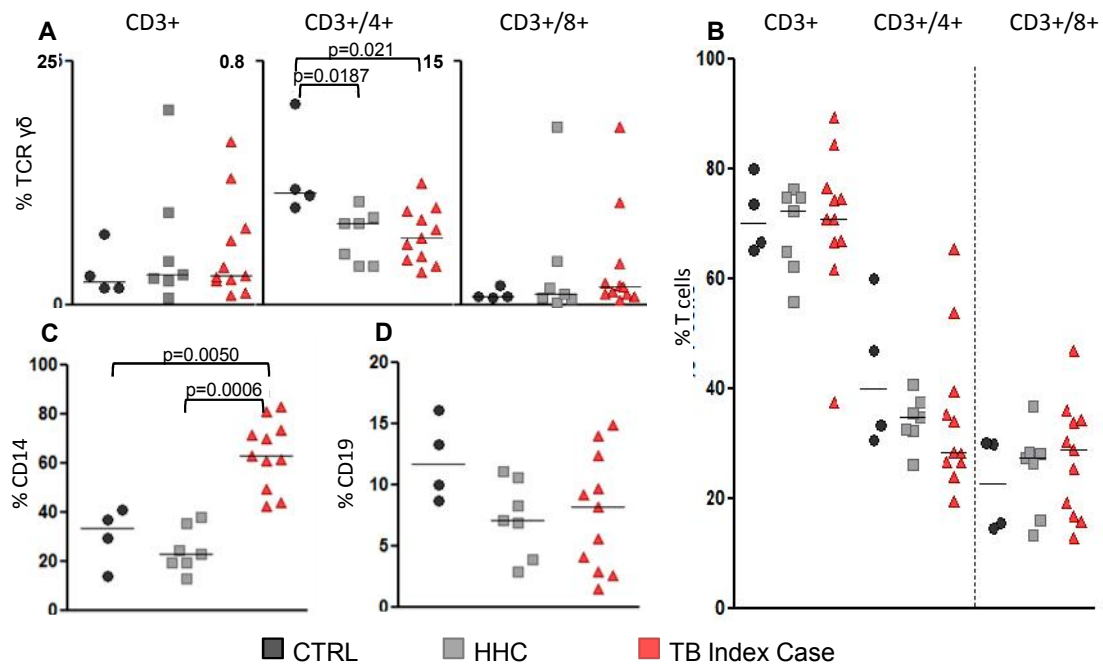


Figure 3.11: Phenotyping. QFT cells after 18h stimulation were phenotypically assessed using the gating strategy above. The Mann Whitney test was used to calculate the p value between any two groups.

The percentage of $\gamma\delta$ T cells was similar in HHCs and TB cases (Figure 3.11-A). CTRLs showed a lower number of $\gamma\delta$ expressing CD3 T cells. When subdividing $\gamma\delta$ expressing CD3 T cells further the CTRLs showed a significant higher number of $\gamma\delta$ expressing CD4 T cells ($p=0.021-0.0187$) and lower number of $\gamma\delta$ CD8 T cells. While the percentages of CD3 T cells were similar between the three groups, differences were seen in CD4 and CD8 T cells (Figure 3.11-B). The CTRL group had higher numbers of CD4 cells than the HHCs and TB cases and no difference was seen between the HHCs and TB cases. The percentage of CD8 T cells was also different among the groups, and lower in CTRLs compared to the other two groups. The percentage of CD8 T cells was slightly higher in TB cases than HHCs, which could be due to the low number of subjects. The percentage of B cells was lower in HHCs and TB cases compared to

CTRLs (Figure 3.11-C). TB cases showed a significantly higher percentage of CD14 compared to CTRLs ($p=0.0050$) and HHCs ($p=0.0006$) (Figure 3.11-D).

3.7.2 Assessing polyfunctional T cells in QFT WBA

Harari et al. (36) have shown that TNF- α expressing CD4T cells discriminate between latent infection and active disease when stimulating with ESAT-6 and CFP-10. Using their strategy it was determined whether the QFT WBA could replicate this result. Therefore the QFT blood was re-stimulated with PMA/ Ionomycin for another 4h in the presence of Brefeldin A. PMA/ Ionomycin were used at low concentration (2ng/ mL and 200ng/ mL respectively) to activate the cells. Samples with and without restimulation were run in parallel. ICS was performed and cells analyzed using a FACSCanto II. Polyfunctional T cells were assessed using the gating strategy below (Figure 3.12). In short, single cells and lymphocytes were identified by forward and side scatter. CD3 T cells were determined and subdivided into CD4 and CD8 T cells. A Boolean gating strategy was used to assess the cytokine pattern in CD4 and CD8 T cells. The expression of IL-9 and CD40L was determined by gating on CD4 and CD8 T cells.

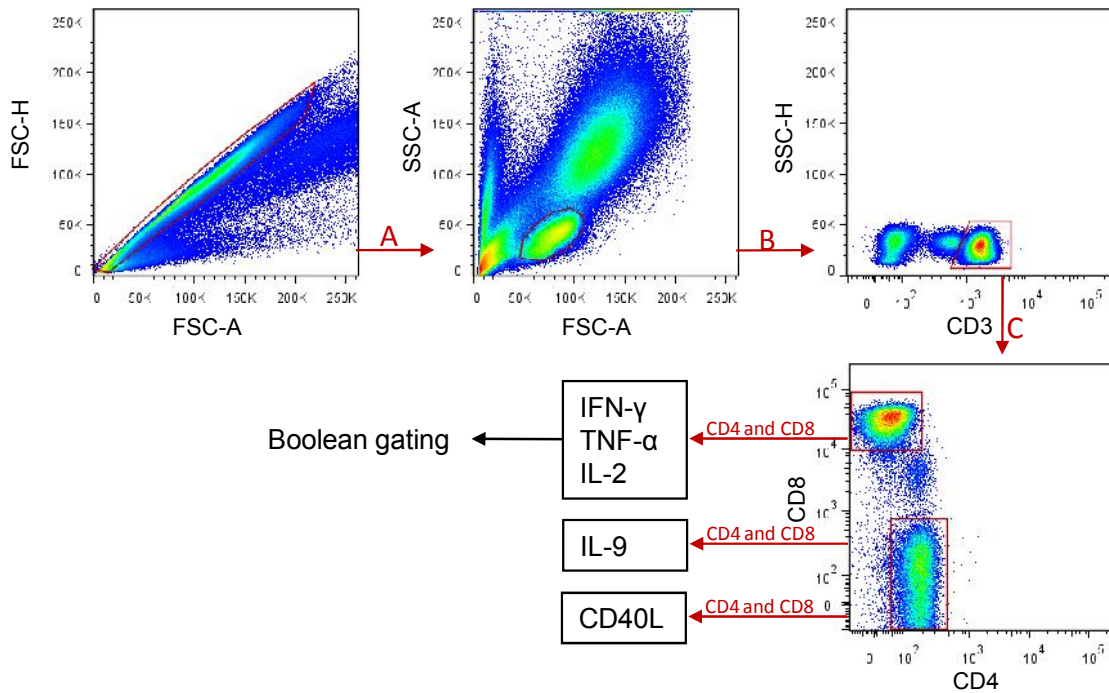


Figure 3.12: Gating strategy for polyfunctional T cells. Single cells (A) were included using forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). Using forward scatter (FSC) versus side scatter (SSC) the lymphocytes could be determined followed by identification of CD3 T cells (B), which is further subdivided into CD4 and CD8 T cells (C). The Boolean gating strategy was used to assess the cytokine patterns in CD4 and CD8 T cells. Further, IL-9 and CD40L expression was assessed in CD4 and CD8 T cells.

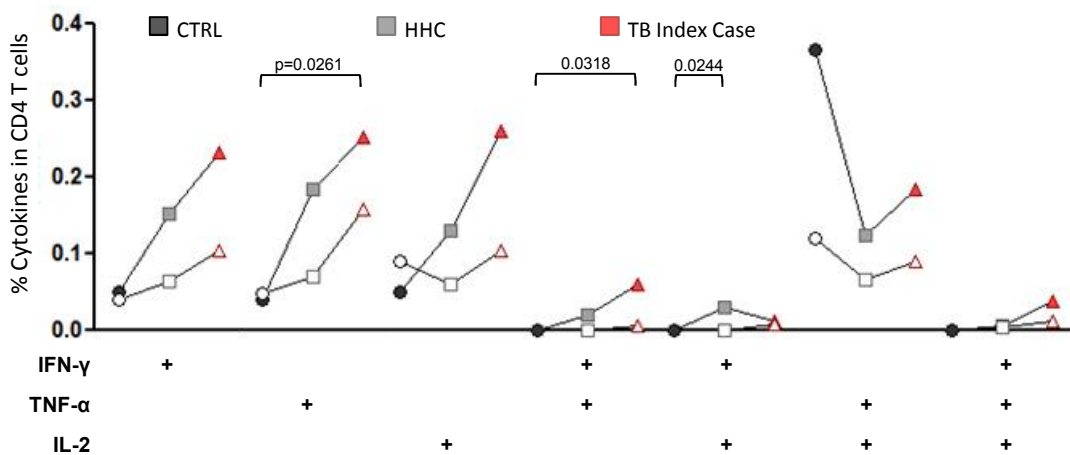


Figure 3.13: Median Cytokine expression in antigen stimulated CD4 T cells with and without restimulation. QFT tubes were left without restimulation (open symbols) or were stimulated with PMA/Ionomycin (solid symbols) for another 4h in the presence of BrefA.

Overall the cytokine profiles were similar in samples with and without PMA/Ionomycin stimulation. For instance, TB cases showed a higher cytokine response than HHC in both restimulated and unstimulated samples. Significant differences were seen between CTRL and TB ($p=0.0261/ 0.0318$) or CTRL and HHC ($p=0.0244$) only after PMA/Ionomycin restimulation (Figure 3.13). The cytokine expression in CTRL, which were all QFT negative, did not increase when restimulated with PMA/ Ionomycin apart from TNF- α / IL-2 double positive cells. It appears that only cells, which are already activated by antigens, respond to PMA/ Ionomycin at this low concentration. PMA/ Ionomycin restimulation therefore increases cytokine production to a level where it becomes more easily measurable without altering differences between groups.

The cytokine profile in CD4 T cells did not show significant differences between HHCs and TB cases (Figure 3.14). Significant differences were only seen between CTRLs and TB cases in the single-cytokine expressing cells, TNF- α ($p=0.0261$), or two-cytokine expressing cells, IFN- γ /TNF- α ($p=0.0318$) after stimulation with ESAT-6/CFP-10. Significant differences disappeared when subtracting the unstimulated background. As observed in the Luminex assay some cytokines show a higher background. In the QFT-WBA, CTRLs showed a higher IL-2 response in NIL than antigen stimulated tubes. The percentage of TNF- α expressing CD4 T cells was increased in TB cases compared to HHCs, although not significantly so, after Ag stimulation, but this difference disappeared after subtraction of background. Figure 3.14 also shows that TB cases and HHCs usually had a higher background cytokine production than CTRLs. TB cases showed a slightly lower number of IFN- γ /TNF- α and IFN- γ /IL-2 expressing CD4 T cells compared to HHCs.

The cytokine profile in CD8 T cells did not show significant differences between HHCs and TB cases (Figure 3.15). Significant differences were only seen between CTRLs and TB cases in expressing the single cytokine TNF- α ($p=0.0129$). As observed in CD4 T cells, those significant differences disappeared when subtracting the unstimulated background. When subtracting the background a statistically not significant increase of CD8 T cells expressing TNF- α or IL-2 is seen in TB cases compared to HHCs.

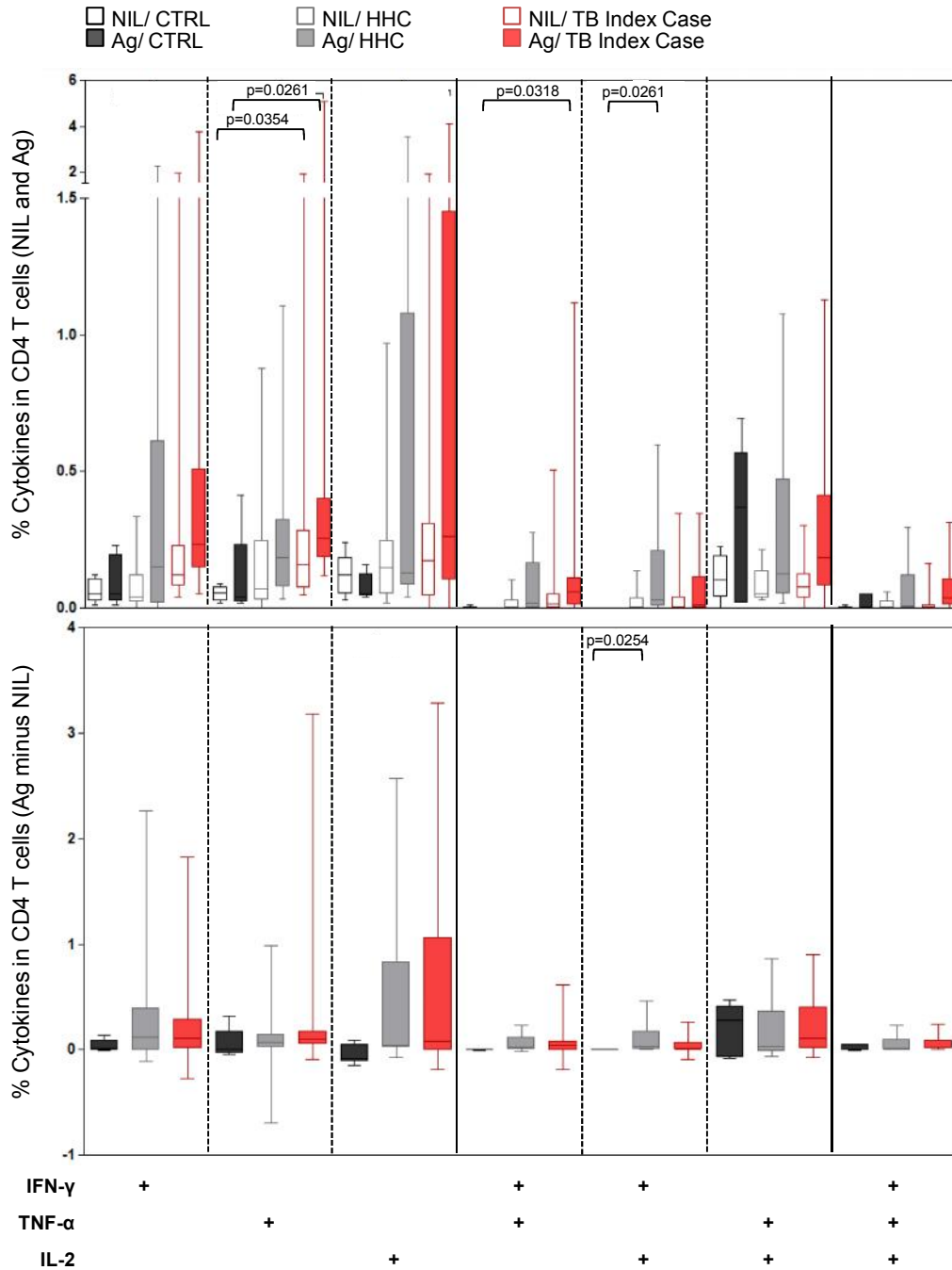


Figure 3.14: Polyfunctional CD4 T cells in QFT WBA. Cytokine profile of polyfunctional CD4 T cells on the basis of IFN- γ , TNF- α and IL-2 production. Responses to M. tb specific Ag (solid box) and NIL (blank box) values from CTRLs (black), HHCs (grey) and TB cases (red) are shown. The upper graph shows cytokine profiles in NIL and Ag stimulated samples and the lower graph shows cytokine profiles in Ag stimulated samples minus background. All possible combinations of cytokines are shown below the graphs.

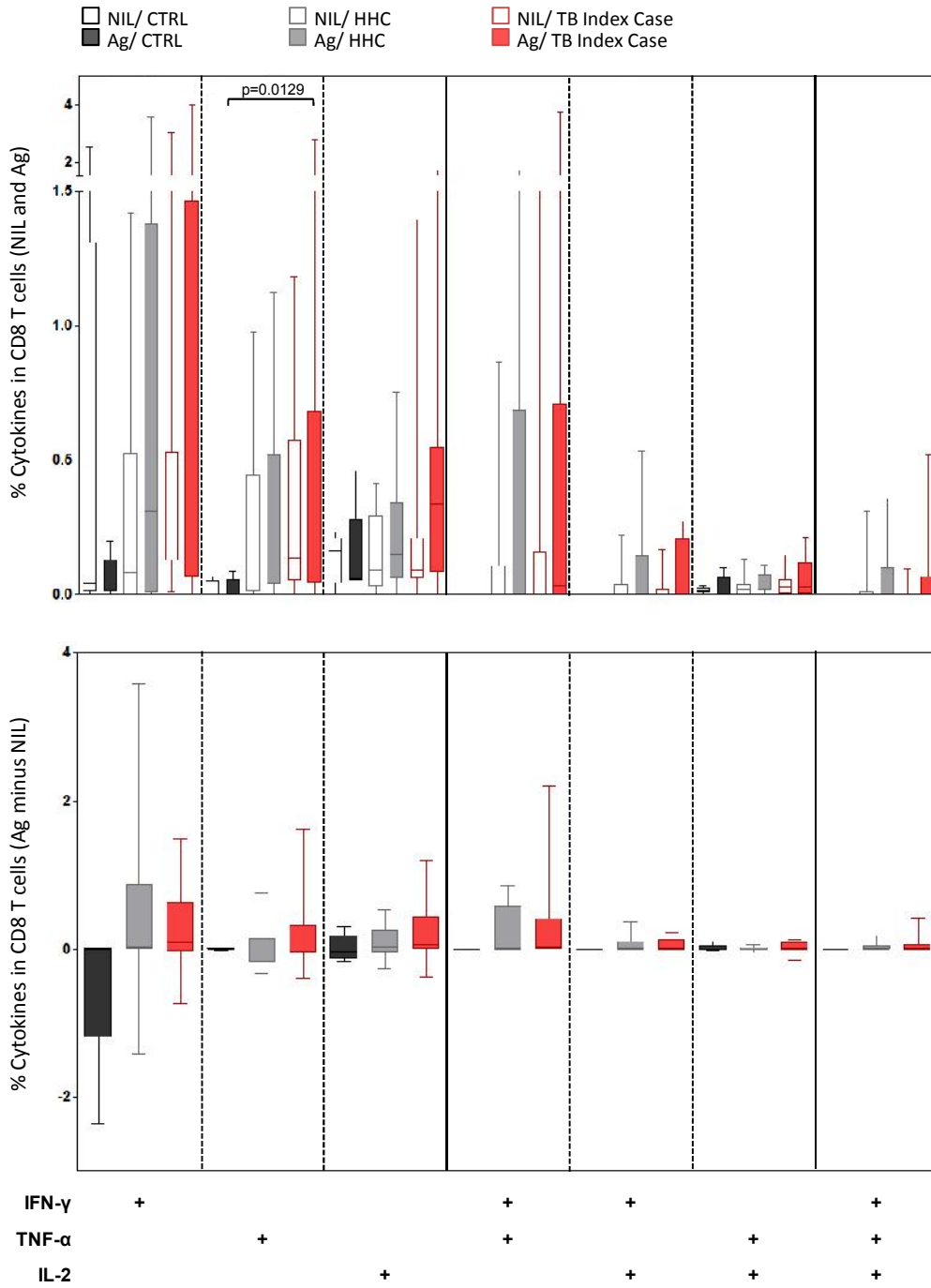


Figure 3.15: Polyfunctional CD8 T cells in QFT-WBA. Cytokine profiles of polyfunctional CD4 T cells on the basis of IFN- γ , TNF- α and IL-2 production. Responses to *M. tb* specific Ag (solid box) and NIL (blank box) values from CTRLs (black), HHCs (grey) and TB cases (red) are shown. The upper graphs shows cytokine profiles in NIL and Ag stimulated samples and lower graphs show cytokine profiles in Ag stimulated samples minus background. All possible combinations of cytokines are shown below the graphs.

IL-9 was thought to be expressed by TH2 cells, but *Veldhoen et al.* (108) showed that IL-9 producing T cells were distinct from TH1, TH2, Treg and TH17 cells. Whereas IL-9 levels were not statistically different in the Luminex experiments, TB cases show a significantly higher background than HHCs in CD4 T cells in the ICS experiments ($p=0.0289$) (Figure 3.16). When subtracting the background from the Ag stimulated tube the expression level of IL-9 expression in CD4 T cells was similar in TB cases and HHCs. The percentage of IL-9 producing CD8 T cells was elevated in TB cases compared to HHCs. Statistically significant differences were only seen between CTRLs and TB cases in the Ag tube ($p=0.0119$) and the Ag minus NIL tube ($p=0.0118$). In general CD8 T cells showed a higher expression of IL-9 than CD4 T cells.

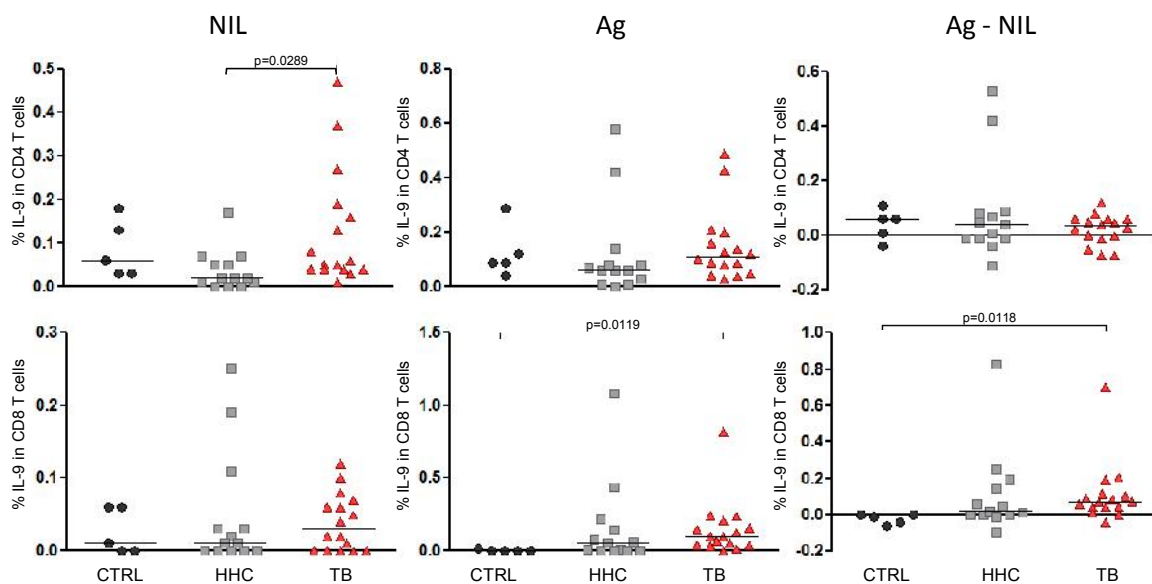


Figure 3.16: Median expression of IL-9 in CD4 and CD8 T cells. QFT blood was incubated for 22h in the presence of Brefeldin A for the last 4h. The expression of IL-9 in CD4 and CD8 T cells was analyzed using FlowJo 7.6.5 and GraphPad Prism 5.

Soon after activation, T cells express CD40L (CD154) and its interaction with CD40 regulates the expression of co-stimulatory factors, which leads to activation of naïve T cells. CD40L is not only restricted to T cells and B cells. It can also be expressed by monocytes, dendritic cells, endothelial cells and other cells types (91). In this study I looked at the expression of CD40L in T cells.

The expression of CD40L in antigen specific CD4 and CD8 T cells respectively was significantly lower in TB cases (0.0020/ 0.0101) compared to HHCs. CTRLs and HHCs show a similar CD40L expression on CD4 T cells, while TB cases and CTRLs showed a similar pattern in CD8 T cells. The expression of CD40L in T cells was higher on CD4 than CD8 T cells.

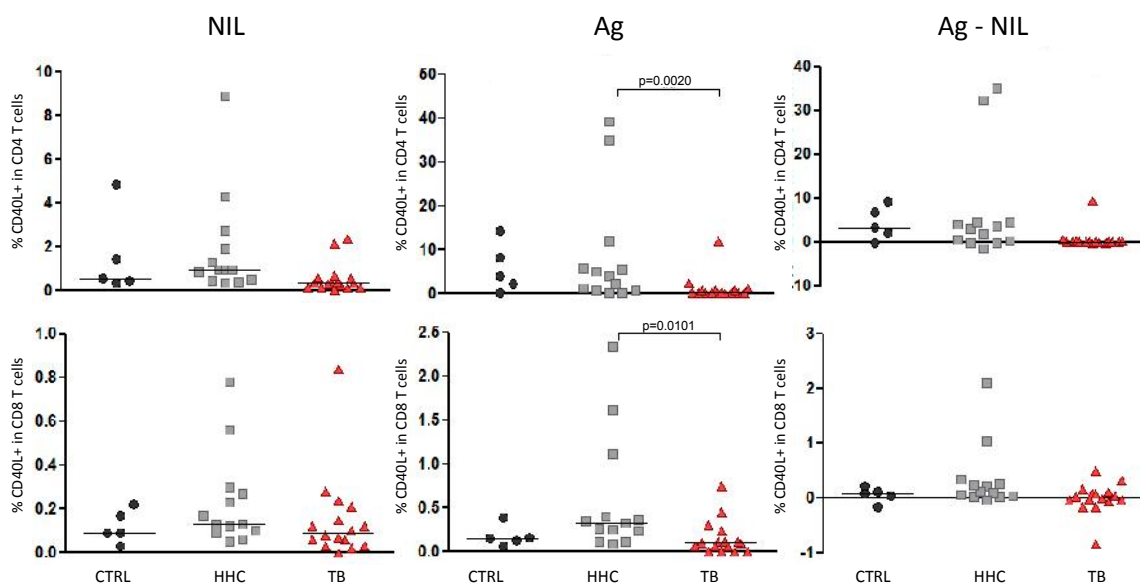


Figure 3.17: Median expression of CD40L in CD4 and CD8 T cells. QFT blood was incubated for 22h in the presence of Brefeldin A for the last 4h. The expression of CD40L in CD4 and CD8 T cells was analyzed using FlowJo 7.6.5 and GraphPad Prism 5.

3.7.3 Assessing polyfunctional T cells in QFT Lymphocyte Proliferation Assay (QFT LPA)

The present study showed that TB cases expressed higher levels of TNF- α than HHC in QFT-WBA. After six days of stimulation, the expression of TNF- α increased and resulted in a higher expression in HHC than in TB cases (Figure 3.19 – A). In a next step the proliferating T cells were assessed using the gating strategy below. In short, single cells and lymphocytes were identified by forward and side scatter. CD3 T cells were determined and subdivided into proliferating CD4 and CD8 T cells, using Ki-67 as proliferation marker. A Boolean gating strategy was used to assess the cytokine pattern in proliferating T cells. Differences between the groups were analyzed using Kruskal-Wallis test.

Looking at proliferating CD4 T cells (Figure 3.19 – B), HHC were still expressing a high level of TNF- α . While there was no difference in the expression level of TNF- α / IL-2 between HHC and TB cases in CD4 T cells, a difference was seen in proliferating CD4 T cells. HHCs showed a higher expression of TNF- α / IL-2 than TB cases, but not significant.

To analyze if the T cells were proliferating the NIL and Ag tube were compared for each group. In each group the percentage of proliferating CD4 T cells increased when stimulated with Ag. Significant differences were seen in HHC ($p=0.0313$) and TB cases ($p=0.0029$).

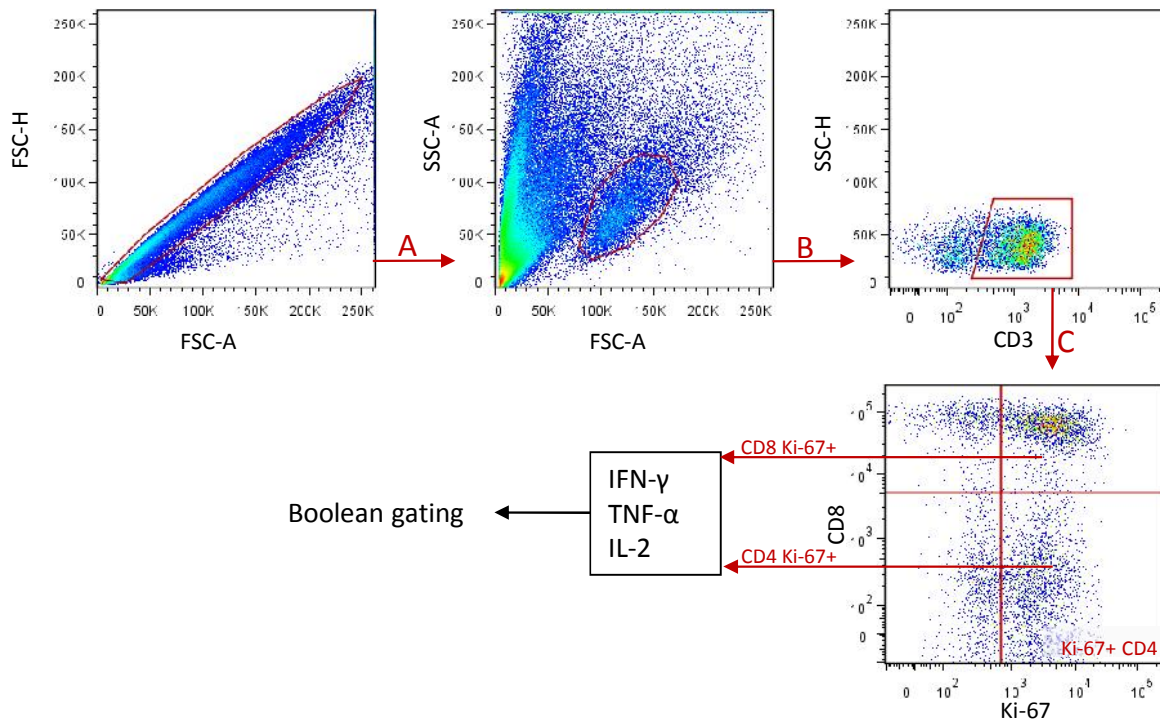


Figure 3.18: Gating strategy for proliferating T cells. Single cells (A) were included using forward scatter-height (FSC-H) versus forward scatter-area (FSC-A). Using forward scatter (FSC) versus side scatter (SSC) the lymphocytes could be determined followed by identification of CD3 T cells (B), which is further subdivided into proliferating CD4 and CD8 T cells (C). The Boolean gating strategy was used to assess the cytokine patterns in proliferating CD4 and CD8 T cells.

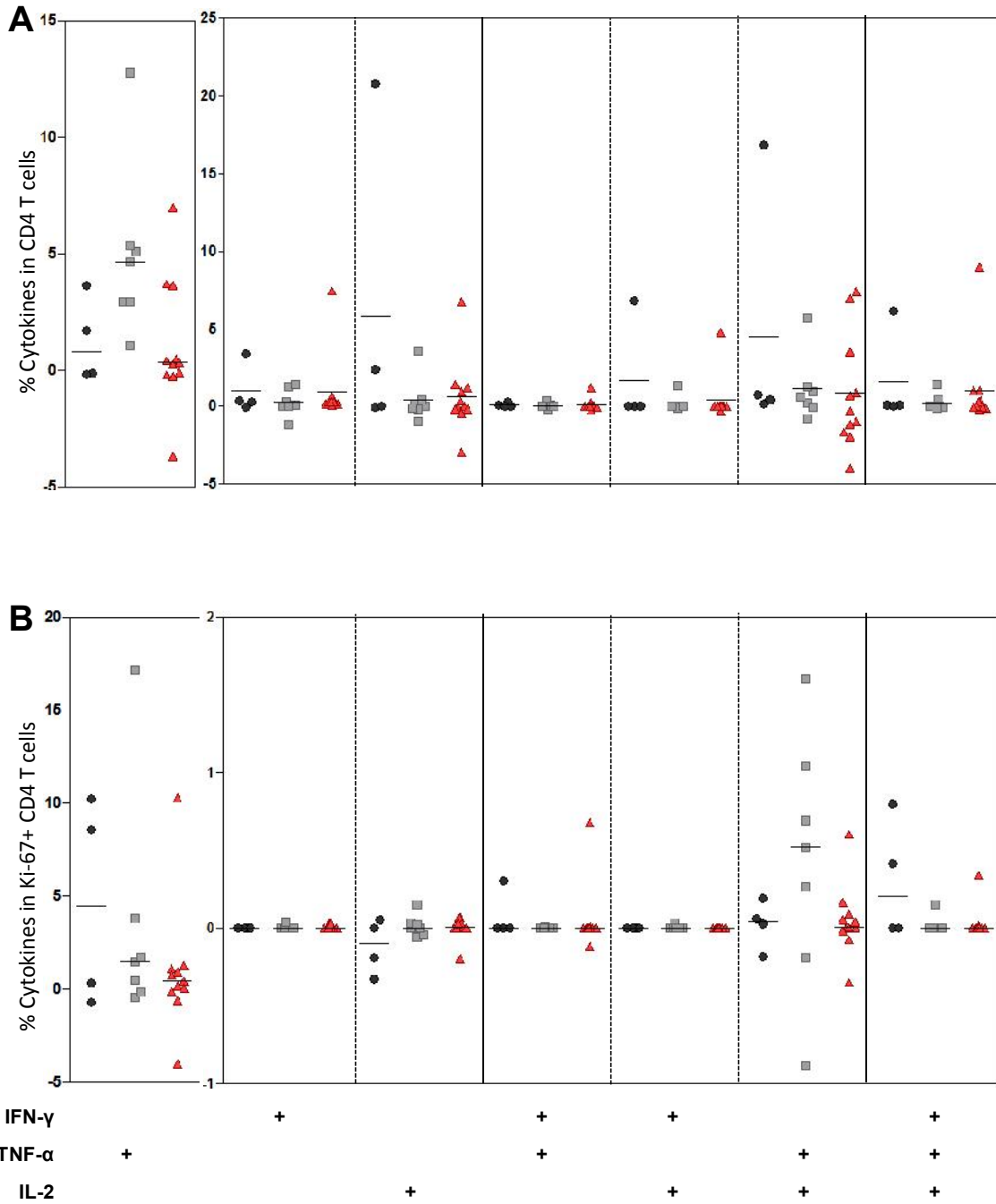


Figure 3.19: Polyfunctional CD4 T cells in QFT LPA. Cytokine profile of polyfunctional CD4 T cells on the basis of IFN- γ , TNF- α and IL-2 expression. Responses to M. tb specific Antigens minus background from CTRLs (black), HHCs (grey) and TB cases (red) are shown. Upper graph shows cytokine profile in CD4 T cells only, while the lower graph shows the cytokine profile in proliferating CD4 T cells. All possible combinations of cytokines are shown below the graph.

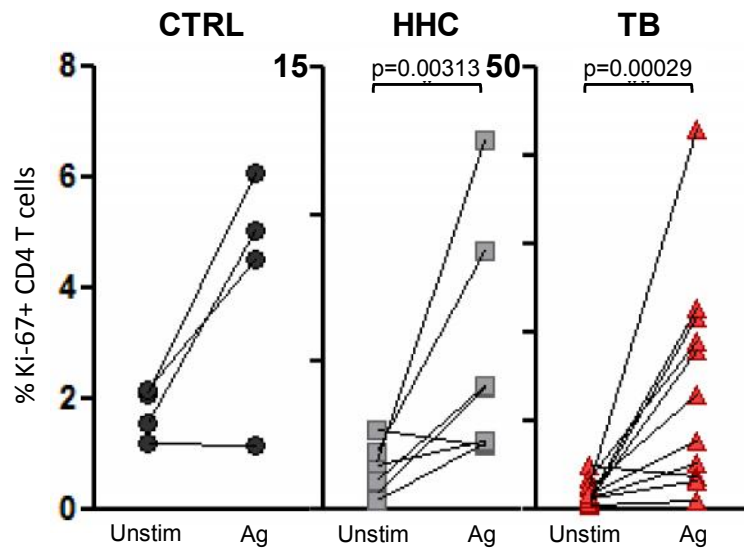


Figure 3.20: Percentage of proliferating CD4 T cells. Percentage of Ki-67 expressing CD4 T cells are shown after 6 day stimulation of NIL and Ag. Differences were calculated using the Wilcoxon matched pairs test.

CHAPTER FOUR – DISCUSSION

*When you take stuff from one writer it's plagiarism;
but when you take it from many writers, it's research.*

Wilson Mizner

In this thesis, the development of a QFT-WBA was shown step by step. The responses in the QFT-based assay were generally comparable to the WBA that is routinely used for vaccine immunogenicity assays. Re-stimulation methods, including duration of stimulation and antigens were optimized. The new QFT-WBA was put in place to answer immunological questions. Flow cytometry was used to look at multifunctional and proliferating T cells and to characterize certain immune cells while multiplex cytokine arrays can be used to investigate secreted host markers.

An estimated one third of the world population is infected with *M. tb* (114), which can lead to active disease, when not controlled by the immune system (29;30). The cellular immunity mediates the protective immune response due to expression of diverse cytokines (50), such as IFN- γ , TNF- α and IL-2 (31;43;55;111), and T cells, which lead to activation or cytolytic activity of macrophages (46).

The aim of this study was to develop a multi-platform immune analysis assay using the QFT IT assay system. In this thesis it was shown that the QFT IT can be used not only for IFN- γ ELISA. *Chegou et al.* (12) have already shown that it is suitable for multiplex cytokine array. This thesis added to this work by using the QFT cell pellet for flow cytometry. One set of QFT tubes will result in both plasma and white blood cells from unstimulated and Ag stimulated samples, using only 1mL blood each. The plasma was used for IFN- γ ELISA and Luminex looking at one or multiple cytokines at a time, while the cells can be used for flow cytometry to characterize specific cell types, assess polyfunctional and proliferating T cells.

The issue of PMA/Ionomycin restimulation after initial antigen stimulation may be contentious as it induces antigen independent stimulation of cytokine. However, in the present study I have shown that a low concentration of the restimulants does not increase cytokine production in participants who are naïve to the MTB antigens but merely amplify existing responses to such a degree that the responses become more easily measurable. Differences between groups were not affected. However, future studies will include larger numbers of participants to thoroughly evaluate if restimulation with receptor independent stimulants introduces artifacts or aid the analysis.

Although it was shown that the QFT assay could be used in flow cytometry, the study has some limitations. Firstly the numbers in the groups were small. There were only 5 participants available for the QFT negative CTRL group, 13 in the QFT positive HHC group and 16 in the TB group.

Another limitation is the kinetics of the cytokine production. One study had shown that in flow cytometry, the expression of IFN- γ and IL-2 reach a peak at 8h, TNF- α already after 2h, and declined thereafter. They also showed that the cytokine level decreases in naïve cells and increases in memory cells (62), suggesting that naïve T cells develop a memory type. Another study showed that the optimum time to detect TNF- α and IFN- γ was after 8h and 16h respectively (4). The optimum incubation times seem to vary between different laboratories. Therefore it is clear that further work is needed to investigate the optimal incubation times for different cytokines as the current work only provides a snap-shot of cell function. The kinetics of cytokine production was also different in supernatants than in single cell measurements. The peak expression of each cytokine was much later than in flow cytometry. Some cytokines such as IFN- γ , IL-

2, TNF- α , IL-1 β , IL-6 and IL-10 showed the highest expression after 48h, while other cytokines (TNF- β and IL-12) showed an increase after that time (52). This may be due to a steady accumulation of cytokines that are secreted into the medium.

All results in this study were obtained by stimulating blood from adults. Taking the QFT-WBA forward, it has to be addressed in infants and children and in HIV infected adults as these are the populations of special interest for vaccine studies and also where the QFT assay has been shown to result in increased indeterminate results (1;84).

Despite these limitations the new developed assay has several advantages. 6-12h WBA assays have been developed which require a stimulation step in the absence of Brefeldin A, followed by addition of this Golgi apparatus inhibitor to trap the markers intracellularly. Depending on the total stimulation time Brefeldin A is usually added after 2-5h (35). Often laboratories have to deal with late sample arrival and delayed processing, which results in late evening work. After the first incubation step, samples often get transferred into a programmable water bath, which switches off after a given time. Using the QFT tubes, samples can be processed in batches and tubes can be left at room temperature until all samples have been collected for the day. Once all samples have been received the tubes can be transferred into an incubator. There is no statistically significant difference in mRNA expression levels between tubes which have been incubated immediately or after a delay of 3h (6). Although the effect of different pre-incubation times on single cell marker expression, including intracellular cytokine production, has not been specifically measured in the present study the published mRNA work suggests that synchronized starting times of the incubation step at 37°C

would not affect the results. This will be confirmed in future studies. In the present study, after the 18h incubation, plasma was frozen, and cells were harvested, re-stimulated for another 4h in the presence of Brefeldin A, red blood cells were lysed, and white blood cells cryopreserved. This workflow, which takes place 18 hours after the start of incubation, can be completed in 6h and represents a user-friendly assay system. *Hanekom et al.* (35) have shown that stimulated WBA cells can be stored in liquid nitrogen for 12 months, with comparable results to baseline assessment. With the new QFT-WBA limited resources are required, as it only needs an incubator without a programmable water bath, further simplifying the assay.

We need to confirm that different pre-incubation times (as mentioned above) and the absence of CO₂ enriched incubation at 37°C, as is generally practiced for the conventional QFT assay, do not affect single cell cytokine production. This would add another big advantage to the new assay as the time window for delivery of samples from remote field research sites to more advanced laboratories capable of processing for ICS would increase dramatically.

Multiplex cytokine arrays on QFT supernatants were conducted in the present study and the results are in concordance with many other studies that employed different functional assays. The Luminex data suggest that unstimulated values (NIL) and not only antigen stimulated values minus the background (Ag-NIL) show differences between groups. It is at present unclear whether the unstimulated QFT values correlate with serum levels and this needs to be assessed in future studies. The expression of VEGF and GM-CSF was higher in unstimulated samples. After stimulation with ESAT-6,

CFP-10 and TB10.4 the production of these markers was downregulated. The same was reported in a study from *Chegou et al.* (12).

FGF basic_{Ag}, GM-CSF_{NIL}, VEGF_{NIL/(Ag-NIL)}, MIP-1 β _{Ag} and MCP-1_{Ag/(Ag-NIL)} showed differences between QFT pos HHC and TB cases. *Chegou et al.* (12) reported statistically significant increases of MIP-1 β and VEGF in HHC compared to TB cases. Overall the present study showed that TB cases express less cytokines/chemokines than HHC. This may be due to immune suppression in active disease or due to compartmentalization of antigen-specific effectors to the site of disease. Several studies have shown that MCP-1 is higher expressed in TB cases than HHC (32;48;87), which is contradictory to the present study, but results compared in higher expression of MCP-1 in TB cases compared to CTRLs.

Similarly, flow cytometric analysis of the new QFT-WBA is in concordance with published studies. Monocytes are important during innate immune response and circulate in the blood (69). As expected the number of monocytes is statistical significant higher in TB cases compared to CTRLs and HHC, which had also been shown by *Veenstra et al.* (107).

The expression of CD40L is associated with activation of T cells. This study showed significantly higher expression of CD40L in HHC than TB which is contrary to some studies which have reported a significantly higher expression of CD40L in pulmonary TB patients (67) and with tuberculosis pleurisy (59). However, the present results are in accordance with a study from *Chegou et al.* (12) in which a higher number of CD40L was found in peripheral blood of HHC compared to TB cases.

While Luminex analysis showed a higher expression of IL-9 in HHC, flow cytometry data showed that IL-9 expressing CD8 T cells were increased in TB cases compared to HHC, suggesting that cells other than T cells also express IL-9 which was measured by Luminex. A study by *Wu et al.* (114) showed that PBMCs of TB cases stimulated with ESAT-6 express higher level of IL-9 mRNA than LTBI and TST-, which compares with the findings in the present study.

In this study differences in the expression of TNF- α between QFT-WBA and QFT LPA (6-day assay) were shown. In the QFT-WBA, TB cases show an increased expression of TNF- α , which correlates with published findings, in which high frequencies of TNF- α were found in TB cases stimulated with ESAT-6 or CFP-10 (36). When stimulating QFT whole blood for six days, the HHC showed high frequencies of TNF- α compared to TB cases. Further investigation showed that proliferating CD4 T cells express higher levels of TNF- α in HHC. A study from *Sutherland et al.* (100) showed increase of TNF- α in TB cases compared to TST negative and TST positive people. The discrepancy in these findings may be explained by the different assays used in these studies as the Sutherland group employed Luminex in plasma. In their study the measured cytokine levels were not only produced by T cells, but also macrophages (111). The same was seen in the expression of IFN- γ /IL-2, where TB cases showed higher expression in the short term assay, and lower expression after six days. A study by *Davids et al.* (19) also showed differences between those two assays, suggesting an *in vitro* expansion of T cells from effector T cells in the short term assay and proliferating central memory T cells in the long term assay.

Future studies and implications

The QFT-WBA could be used in vaccine immunogenicity studies to assess memory T cell induction. Many studies used whole blood stimulated with *M. tb* specific antigens, which are also part of the trial vaccine (2;93). In those studies, approximately 1mL of blood per condition is needed, and at least 3 conditions have to be assessed, (unstimulated, positive control and the antigen of interest). For studies where ESAT-6, CFP-10 or TB7.7 antigens were used in the vaccine, including modified *M. tb*-based vaccines, the QFT assay can be used directly. For other vaccines, the antigen of interest can be added to a separate NIL tube and stimulation can be done as in this study described. When assessing short term and long term assays even more blood is needed. The QFT-WBA would assess immune responses against immunodominant *M. tb* antigens but only require 2.4-3mL of blood to measure a range of immune responses. This blood volume is feasible in studies on neonates, where allowable blood volumes are small (28;39). Processing of the QFT-WBA is simplified, requires less advanced laboratory equipment or procedures and may allow longer time windows for transport of stimulated samples from field sites to immunogenicity laboratories. The first part of the assay, the QFT tubes with optimized antigenic formulations, is produced under Good Manufacturing Practice (GMP) standards and represents a widely available and simple testing procedure. Adaptations as performed in the present study may allow widespread application in larger, including phase III, trials with comparability of results across different studies. In addition, the assay may be useful for other TB-related biomarker investigations, including TB treatment response evaluation and diagnostic studies. Further studies are underway to optimize mRNA extraction for transcriptome analysis in

200 μ L of QFT whole blood in addition to the soluble marker and cell-specific phenotypic and functional assessment.

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