IMPACT OF HELMINTH INFECTION ON ANTIMYCOBACTERIAL IMMUNE RESPONSES IN UK MIGRANTS

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Declaration

'I, Shaheda Anwar, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.'

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Abstract

Tuberculosis and helminth infections are co endemic in many parts of the world. This geographical overlap has led to the hypothesis that helminth infections could exacerbate the effects of *Mycobacterium tuberculosis* (*Mtb*) infection. Anthelmintic treatment has been observed to be associated with improved mycobacterial cellular responses and decreases in the frequency of Treg cells. The consequence of this immunomodulation may affect the ability of the host to restrict the growth of mycobacteria or mycobacterial killing. This study aims at investigating the modulations of the immune response profile of latent tuberculosis (LTBI) and helminth co-infected patients and whether these modulations are associated with a decrease in mycobacterial growth inhibition using a mycobacterial growth inhibition assay (MGIA).UK migrants attending University College Hospital London, UK with eosinophilia or suspected/diagnosed helminth infection (Strongyloides spp and Schistosoma spp) and/or LTBI were bled at recruitment (before anthelmintic treatment) and 4 months after completing anthelmintic treatment. Helminth infected patients displayed poor growth inhibition on MGIA which was improved after anthelmintic treatment which indicated this immunomodulation might be helminth mediated. The percentage of CD4+ T cells expressing IFNγ, TNFα and IL-2 were quantified by flow cytometry in PPD and ESAT-6/CFP-10 stimulated PBMC and anthelmintic treatment was observed to increase the frequency of CD4+IFNγ response. LTBI-helminth coinfection was associated with significantly elevated levels of pro-inflammatory and lower levels of anti-inflammatory cytokines after they were treated. IP-10 was significantly upregulated and MCP-1 was significantly downregulated in LTBI-helminth coinfected patients after anthelmintic treatment. The effect of IL-10 and TGFβ on MGIA were observed and suggested an immunoregulatory role in helminth infected patients. Gene expression analysis by qRT-PCR showed varied responses and showed significant fold changes of CXCL-10, arginase 1 and CD163 after the treatment. MGIA and multiple immune parameters have shown that helminth infection can modulate a variety of *Mtb* specific immune responses.

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List of Abbreviations

AAM	Alternatively activated macrophage
ADCC	Antibody dependent cellular cytotoxicity
AFB	Acid Fast Bacilli
ANOVA	Analysis of variance
APC	Antigen presenting cell
Arg 1	Arginase 1
ARI	Annual risk of infection
APTB	Acute pulmonary tuberculosis
ATP	Adenosine triphosphate
AWA	Adult worm antigen
BCG	Bacille Calmette-Guérin
BMDM	Bone marrow-derived macrophage
BMM	Bone marrow macrophage
BSL3	Biosafety level 3
CAM	Classically activated macrophage
CAT	Category
CCL	Chemokine legand
CCR	CC Chemokine Receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFA	Complete Freunds Adjuvant
CFP10	Culture filtrate protein 10kDa
CFU	Colony forming unit
CLR	C-type lectin receptor
CO	Carbon monoxide
COPD	Chronic obstructive pulmonary disease
CoA	Coenzyme A
CR	Complement receptor
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte-associated molecule
DAMP	Danger-associated molecular patterns
DC	Dendritic cell
DCregs	Regulatory dendritic cells
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DosR	Dormancy survival regulator
DTH	Delayed-type hypersensitivity
DTP	Differential time to positivity
ECP	Eosinophil cationic protein
EDN	Eosinophil derived neurotoxin

EDTA	Ethylenediaminetetraacetic acid
EHR	Enduring hypoxia response
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-linked ImmunoSpot
EPO	Eosinophil peroxidase
ESAT-6	Early Secreted Antigenic Target 6kDa
FACS	Fluorescence assisted cell sorter
FBC	Full blood count
FBS	Foetal bovine serum
FDG	Flurodeoxyglucose
FMO	Fluorescence minus one
FSC	Forward scatter
FoxP3	Forkhead box P3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-monocyte colony stimulating factor
HBHA	Heparin binding haemagglutinin adhesion
HCW	Health care worker
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HI	Heat inactivated
HIV	Human Immunodeficiency Virus
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
HTD	Hospital for Tropical Diseases
HTLV-1	Human T cell lymphocytic virus 1
HuPO	Human acidic ribosomal protein
ICS	Intracellular cytokine staining
IDA	Indoleamine 2,3-dioxygenase
IFN	Interferon
IgA	Immunoglobulin A
IgG4	Immunoglobulin G4
IgE	Immunoglobulin E
IGRA	Interferon gamma release assay
IL	Interleukin
ILC	Innate lymphoid cells
iNOS	Inducible nitric oxide synthase
INH	Isoniazid
kDa	Kilodalton
KO	Knock-out
LAM	Lipoarabinomannan
LF	Lymphatic filariasis
LM	Lipomannan
LSHTM	London School of Hygiene and Tropical Medicine
LTBI	Latent TB infection

MA	Mycolic acid
MAN-LAM	Mannose-capped lipoarabinomannan
MBL	Mannose binding lectin
MBP	Major basic protein
MCP-1	Monocyte chemotactic protein-1
MGIA	Mycobacterial growth inhibition assay
MGIT	Mycobacterial growth indicator tube
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mRNA	Messenger Ribonucleic Acid
ML	Monocyte to lymphocyte
MR	Mannose receptor
Mtb	*Mycobacterium tuberculosis*
NADH	Nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NICE	National Institute for Health and Clinical Excellence
NK	Natural killer
NLR	Neutrophil/lymphocyte ratio
NO	Nitric Oxide
NOD	Nucleotide oligomerization domain
NOS	Nitric oxide synthase
NRAMP	Natural resistance associated protein
NRP	Non-replicating state of persistence
NTD	Neglected Tropical Diseases
OADC	Oleic acid, albumin, dextrose and catalase
OD	Optical density
PAMPS	Pathogen-Associated molecular pattern
PANTA	Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDL	Programmed death ligand
PET-CT	Positron emission tomography and computed tomography
PIM	Phosphatidylinositol mannoside
PPD	Purified protein derivative
PRR	Pattern recognition receptor
PZA	Pyrazinamide
QFT-GT	Quantiferon TB Gold-In Tube
qRT-PCR	Quantitative real time polymerase chain reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RCT	Randomized controlled trial
RD-1	Region of difference-1

RELM	Resistin like molecule
RIF	Rifampicin
RNA	Ribonucleic acid
RNIs	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
rIL1-10	Recombinant IL-10
rTGFβ	Recombinant transforming growth factor β
SAFE	Surgery, antibiotic, facial cleanliness, environmental improvements
SR	Scavenger receptor
SDH	Succinate dehydrogenase
SEA	Soluble egg antigen
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SOCS-1	Signal transducer of cytokine signalling 1
SSC	Side Scatter
SSI	Statens Serum Institute
STAT6	Signal transducer and activator of transcription 6
STH	Soil transmitted helminth
TAG	Triglyceride
TB	Tuberculosis
TBP	TATA box binding protein
TCM	T central memory cell
TEM	T effector memory cell
TCR	T cell receptor
TDM	Trehalosedimycolate
TGF	Transforming growth factor
Th	Helper T cell
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
Tregs	Regulatory T cells
TSLP	Thymic stromal lymphoprotein
TST	Tuberculin skin testing
TTP	Time to positivity
UCL	University College London
UIN	Unique identification number
UNICEF	United Nations International Children's Emergency Fund
WASH	Water, sanitation and hygiene
WBA	Whole blood bactericidal activity
WHO	World Health Organization
YLD	Years lived with disability

Chapter - 1   Introduction

1.1   Tuberculosis

Tuberculosis (TB) is an airborne infectious disease caused by organisms of the *Mycobacterium tuberculosis* complex. TB is a major public health care problem and remains a major cause of morbidity and mortality worldwide (1). In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children. Six countries accounted for 60% of the new cases: India, Indonesia, China, Nigeria, Pakistan and South Africa (2).

Only 5-10% of these infected groups develop active disease during their lifetimes. The remaining 90-95% remain latently infected without developing active disease or becoming infectious. The biological risk factors that increase their susceptibility to TB include malnourishment, poor housing, HIV infection, diabetes, prison, tobacco smoking, etc. Host genetic factors, geographic and ethnic clustering of tuberculosis, strain variability and genetic diversity of *Mycobacterium tuberculosis* (*Mtb)* contribute to the risk of developing tuberculosis (3).

It was estimated that in 2014, the global burden of LTBI was 23.0% (95% uncertainty interval: 20.4%– 26.4%), amounting to approximately 1.7 billion people. WHO South-East Asia, Western Pacific, and Africa regions had the highest prevalence and accounted for around 80% of those with LTBI (4). Among this large reservoir of asymptomatic LTBI patients, 8-10 million active TB cases emerge each year resulting in the deaths of nearly 1.7 million people (5).

1.1.1 **Tuberculosis in the United Kingdom**

In 2015, a total of 5,758 TB cases were notified in England, a rate of 10.5 cases/100,000 population. The number of cases and rate of TB in the non-UK born population in England have declined year-on-year since 2011, with a decrease of more than 10% each year since 2012.The majority (60%) of non-UK born cases occurred among those who have lived in the UK more than six years, whereas, the rate of TB in the UK born population declined in 2015, with a decrease of 23% since the peak in 2012. Of the UK

born TB cases notified in 2015 where the ethnic group was known, the majority (60.0%, 928/1,547) were from the White ethnic group, 21.5% (332/1,547) from South Asian ethnic groups (Indian, Pakistani and Bangladeshi) and 13.3% (206/1,547) from Black ethnic groups (Black-Caribbean, Black-African and Black-Other).
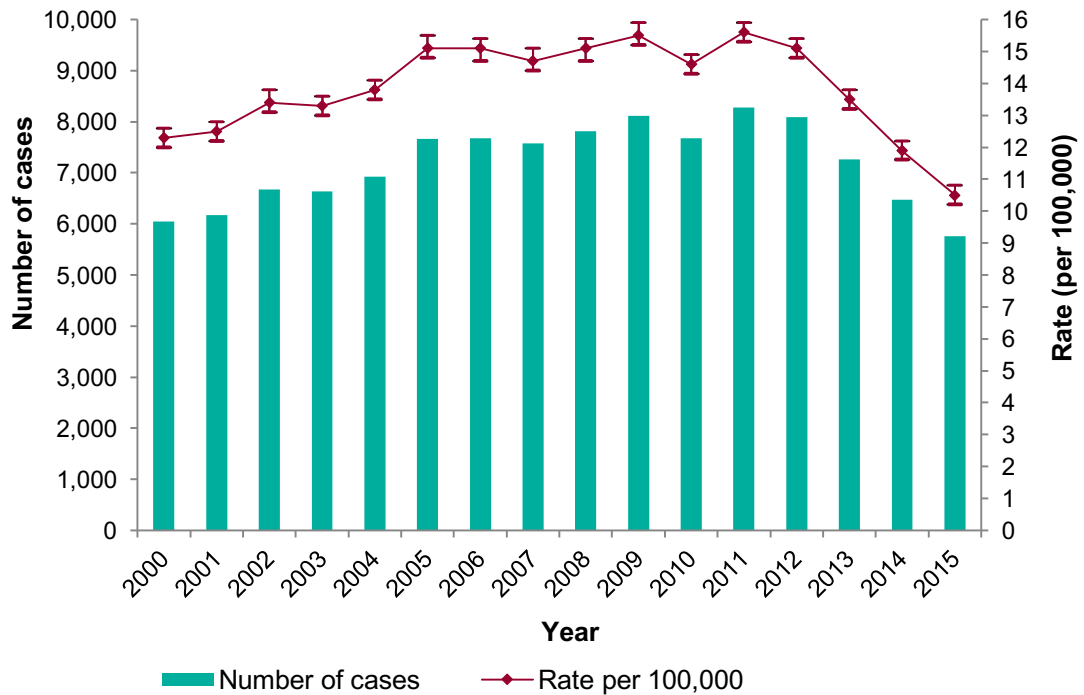
Figure 1.1:   Tuberculosis case reports, rates and annual percentage change, UK, 2000-2015
            Source: Enhanced Tuberculosis Surveillance system (ETS), Office for National Statistics (ONS). Data extracted: April 2016. Prepared by: TB Section, National Infection Service, Public Health England (6).

In 2015, as in previous years, the most frequent countries of birth for non-UK born cases were India, Pakistan, Bangladesh and Somalia (26.3%, 15.9%, 5.2% and 4.4%, respectively).
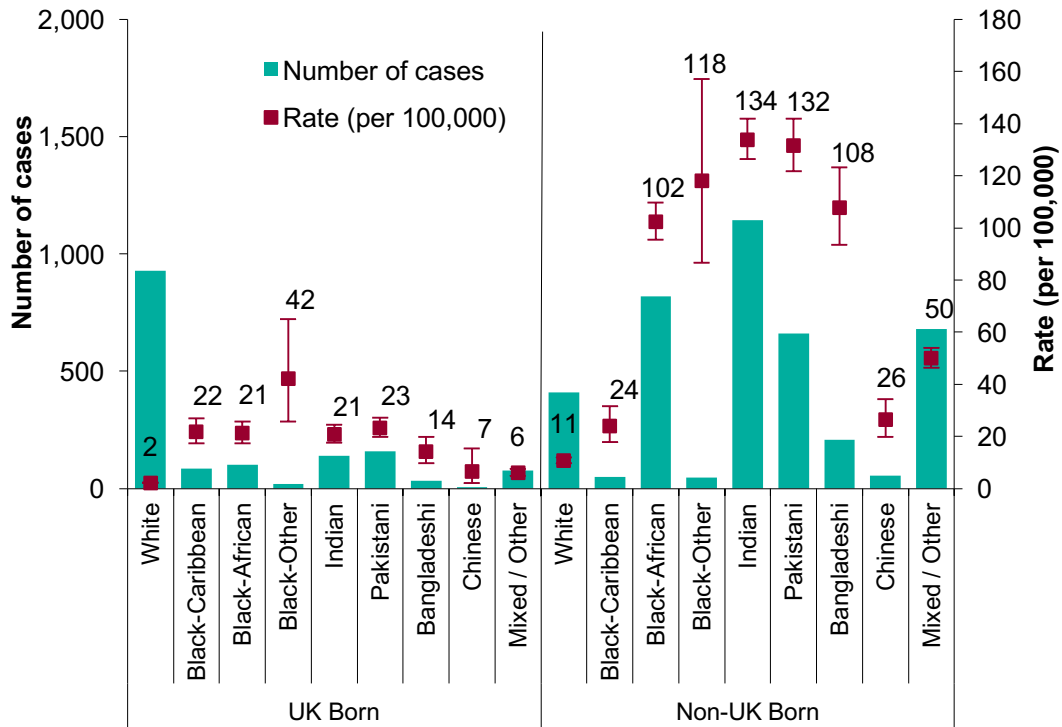
Figure 1.2: Tuberculosis case notifications and rates by ethnic groups, UK. (2016)
Source: Enhanced Tuberculosis Surveillance system (ETS), Office for National
Statistics (ONS) Data extracted: April 2016. Prepared by: TB Section, National
Infection Service, Public Health England (6).

1.1.2 Pathogenesis of tuberculosis

Mtb is transmitted by the aerosol route from person to person. When inhaled, *Mtb* are
deposited into the lower respiratory tract and reach alveoli where they initially infect the
alveolar macrophages. Although macrophages phagocytose and destroy the bacilli, still
Mtb has adapted to the intracellular environment which renders them able to survive and
replicate within these phagocytic cells (7).

Recognition of mycobacterial components by innate immune cells through different
pattern recognition receptor (PRR),TLRs,Nucleotide Oligomerization Domain (NOD)
like receptors, and C type lectin have all been implicated in recognition of
mycobacteria and in the initiation of cytokine responses (8). The most important *Mtb* cell
surface ligands that interact with TLRs and other receptors include the 19, 27 kDa
lipoproteins, 38 kDa glycolipoprotein, glycolipids like phosphatidylinositol mannoside
(PIM), lipoarabinomannan (LAM) and mannose capped lipoarabinomannan (Man-

LAM), trehalose dimycolate (TDM) and lipomannan (LM)(9). Signals generated through TLR promote proinflammatory immune responses including activation of nuclear transcription factor (NF)-κB and production of cytokines and chemokines and recruitment of DC-SIGN that induces suppression of immune responses.TLR activation leads to direct killing of bacilli by nitric oxide (NO) dependent and NO independent pathways (8)(10)(11)(12). The macrophage mannose receptor can facilitate phagocytosis of *Mtb*. The alveolar macrophage can also induce cytokine production–including Interleukins (IL)12, IL23, IL27, IL35, interferon gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) and can also aid in the production of reactive metabolites such as NO that have antimycobacterial properties (13).

Mtb reside in the phagosome and priming of the CD4+T cell response requires the presentation of *Mtb* antigens through the MHC class II pathway. Class I pathway presentation of *Mtb* antigen may be a result of the exocytosis of phagosomes, uptake of apoptotic blebs or recycling of MHC molecules with peptide replacement. NO and RNI generated by iNOS in the macrophages exert its toxic effects on the bacilli. iNOS recruitment into the phagosome is inhibited by *Mtb*. Hypoxia and respiration inhibition by NO induce a dormant state in *Mtb* leading to their persistence (14).

Antimycobacterial mechanisms of the macrophage include lysosomal killing of *Mtb* mediated by ubiquitin derived peptides that destroy *Mtb* by autophagy, impairing the membrane integrity and allowing NO to kill *Mtb* effectively. During this time bacilli resist the bactericidal mechanisms of macrophages by preventing phagosome-lysosome fusion, by escape from phagosomes/ phagolysosomes and by causing macrophage necrosis (13). The escape from the phagosome is aided by Early Secreted Antigenic Target 6kDa (ESAT-6) protein and culture filtrate protein 10 (CFP-10) encoded by region of difference (RD) 1, that is present in all virulent *Mtb* and absent from all BCG strains. ESAT-6 also helps in apoptosis of macrophages, cytolysis of alveolar epithelial cells and in dissemination of TB (15). The released bacilli multiply extracellularly and are phagocytosed by another macrophage that fails to control their growth and is then destroyed.

In the majority of cases, the necrotized lesions heal with scarring and calcification. The pathogen can remain silent inside the body without causing any symptoms and less than 10% of the latent TB cases become reactivated infections. If the patient fails to contain

the initial infection, or if it is reactivated in a latently infected individual, the patient develops active TB.

Possible outcomes of TB infection are illustrated in Fig. 1.3.
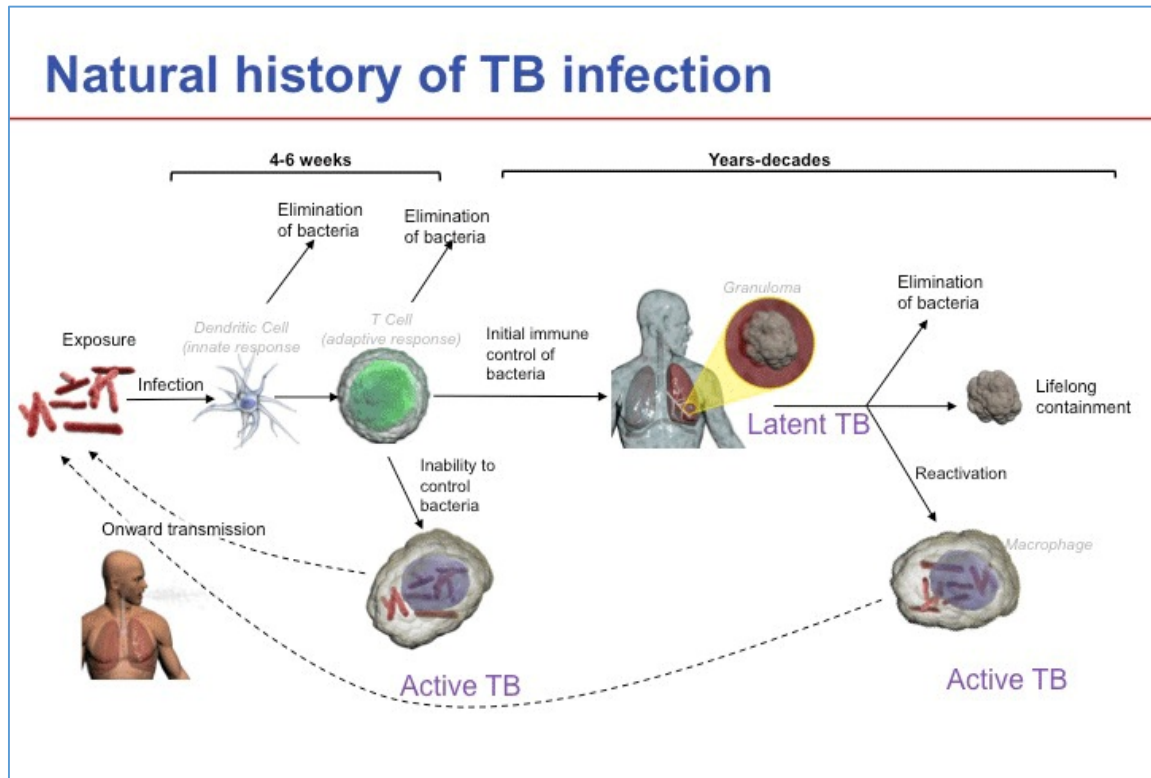
Figure 1.3: Outcome of *Mtb* infection. Picture taken from http://www.oxfordimmunotec.com/international/products-services/healthcare-professionals/tuberculosis/ (16).

1.1.3 The innate immune response to *Mtb*

The major innate cell types involved in dealing with *Mtb* infection are macrophages, neutrophils, dendritic cells and natural killer cells.

1.1.3.1 Macrophages and dendritic cells

Alveolar macrophages and dendritic cells are the first line defence against inhaled particles and microorganisms that deposit in the lung. Alveolar macrophages of susceptible individuals undergo alternative activation following infection with *Mtb*. This phenomenon is associated with reduced oxidative radical generation, altered cytokine responses and reduced microbial action (17). Macrophages express a variety of pathogen

recognition receptors, such as the mannose receptor (MR), dectin receptor (beta-glucan receptor), scavenger receptors (SRs), complement receptors (CRs), mannose binding lectins (MBL), DC-SIGN, CD14 and TLRs (18)(19)(20). Apart from recognizing their ligands and mediating phagocytosis, macrophage receptors are involved in activating several downstream pathways and influencing the ensuing inflammatory responses (21). Subsequently, the pathogenic bacteria engage with vesicular trafficking machinery inducing inhibition of phagosomal maturation. Inhibiting phagosome-lysosome fusion and acidification of the phagosome degrade and can avoid killing and degradation (22). The natural resistance associated membrane protein (NRAMP) is found on the surfaces of the lysosomes of macrophages and migrates to the phagosome compartment after phagocytosis, where NRAMP plays an important role in controlling infection by removing iron and other divalent cations from the phagosomal environment (23). Activated macrophages produce cytokines and according to the pattern of cytokine secretion they are classified as M1 and M2 phenotypes. The innate response against *Mtb* begins with the recognition of the bacterial components leading to IFNγ, reactive oxygen species (ROS), and reactive nitrogen intermediates (RNI). However, in susceptible individuals, the infected macrophages display an alternative activation of macrophages which is characterized by diminished RNI and ROS production and increased secretion of Th2 cytokines like IL-4, IL-5, IL-10, and IL13. This cytokine profile may inhibit Th1 responses by antagonizing IFNγ and decreasing IL-1, IL-6 and TNFα(24). Unlike macrophages, dendritic cells express CRs, MRs, and DC-SIGN that mediate uptake of *Mtb* in the alveoli of the lungs. DCs act as antigen presenting cells in *Mtb* infection, which helps in bridging between innate and adaptive immunity. Once infected, the dendritic cells then migrate to the regional lymph nodes, where they prime naïve T cells leading to T cell expansion and secretion of cytokines (25)

1.1.3.2 Neutrophils

Neutrophils are less well studied than other immune compartments of the host response to *Mtb* infection. Neutrophils are the most commonly infected phagocyte in the lungs in human TB (26). The interaction of neutrophils and macrophages and the downstream effects of T cell activity, could result in a range of outcomes from early clearance of infection to dissemination of viable bacteria and also an attenuated immune response. It is controversial as to whether human neutrophils can kill *Mtb*. Both killing and the failure of killing are possible outcomes *in vivo* depending on the host and the mycobacteria.

Efficient killing could allow the neutrophil to halt the infection at an early stage while the absence of killing would allow the disease to progress locally and also to traffic the organism to distant sites including the systemic circulation (27). Neutrophils influence the development of acquired immunity through the production of IL-12, MCP and other cytokines, which can attract T cells and help their maturation (28). The role of neutrophils is still complex and under investigation by the researchers.

1.1.3.3 Natural killer cells

Natural killer (NK) cells and macrophages are major components of the body's innate immune system, contributing significantly to the body's ability to synergistically inhibit the growth of *Mtb* in immune compromised individuals lacking a sufficient T cell response. Direct mechanisms of control by NK cells are largely through the secretory products perforin, granulysin, and granzymes, as well as multiple membrane-bound death receptors that facilitate target directed lysis. NK cells also have a role in indirectly stimulating an immune response through IFNγ mediated activation of macrophages and monocytes with multiple signalling pathways, including both reactive oxygen species and reactive nitrogen species (29).

1.1.4 Roles of immune cells and cytokines in *Mtb* infection

Activation of CD4+T cells leads to secretion of cytokines such as IFNγ and TNFα that induce macrophage activation. It causes apoptosis of infected macrophages through Fas/Fas ligand interactions, and induction of other immune cells to produce immunoregulatory cytokines such as IL-10, IL-12, and IL-15. It is critical for CD4+T cell function and can also control the intracellular growth of *Mtb* by a NO dependent mechanism(30).

CD8+T cells may be cytotoxic for *Mtb* infected macrophages. They can directly kill *Mtb* via granulysin, perforin, granzyme, hydrolases and extracellular ATP and facilitate the control of acute and chronic infection. The cytotoxicity can lead to intracellular killing of *Mtb* and also releases the viable intracellular mycobacteria, which may lead to their uptake by effective microbicidal macrophages (31). Unconventional T cells such CD1 restricted T cells, and γδ T cells also confer protection.

B cells may play a role in priming the immune response. B-cell deficient mice have been shown to be more susceptible to primary TB infection and had increased bacterial loads (32).

IFNγ is the key component of protective immunity against TB. IFNγ activates macrophages to kill intracellular bacilli. It augments antigen presentation leading to recruitment of CD4+ and CD8+T cells and participates in mycobacterial killing. TNFα provides a major protective role in TB by its involvement in both immunity and immunomodulation. It also initiates cell migration and formation of granulomas. TNFα induces the expression of chemokines such as IL-8, MCP-1, RANTES and sends signals to immune cells for recruitment (33).

Additional cytokines with important roles include IL-12 and IL-17, produced by Th-17 cells. In a human study it was shown that 1L-17 producing cells also made IFNγ and had the phenotype of Th1 central memory or effector memory T cells (33). IL-4 production is induced during clinical TB and indicative of active disease. Immunoregulatory cytokines such as IL-10 and TGFβ exert inhibitory responses against TB.

Granulomas are formed as consequences of the interaction between pulmonary inflammation and host immune responses. Granulomas are initiated by resident macrophages that phagocytose the bacilli and release proinflammatory cytokines-helping in recruitment of immune cells. These are surrounded by collars of lymphocytes including CD4+T cells which may enhance the anti-mycobacterial responses by releasing IFNγ. The centre of this type of granuloma is caseous, a necrotic state that probably consists of dead macrophages and other cells. This area is hypoxic. Mycobacteria in this granuloma can be found in macrophages (either in contact with T cells or not) in the hypoxic centre or possibly even in the fibrotic rim; this provides the mycobacteria with different microenvironments. The other type of granuloma is the non-necrotizing granuloma which is usually seen in active disease and consists primarily of macrophages and some lymphocytes. *Mtb* bacilli are found within macrophages in these lesions. Fibrotic lesions are seen mostly in latent tuberculosis but also in active disease and are composed almost completely of fibroblasts, with a minimal number of macrophages. Although it is possible to culture bacilli from some fibrotic lesions, it is not clear where the bacilli reside (possibly in macrophages or in the fibrotic area) or what the microenvironment is like.

Well-organized granulomas are entirely dependent on an effector Th1 response and can restrict *Mtb* growth. Non-necrotic and fibrous granulomas are observed in LTBI with fewer inflammatory cells. Proper functioning of granulomas can determine the outcome of infection.

a Caseous granuloma
b Non-necrotizing granuloma
c Fibrotic granuloma

B cell
CD8⁺ T cell
CD4⁺ T cell
Neutrophil
Caseum
Fibroblast
M. tuberculosis
Macrophage

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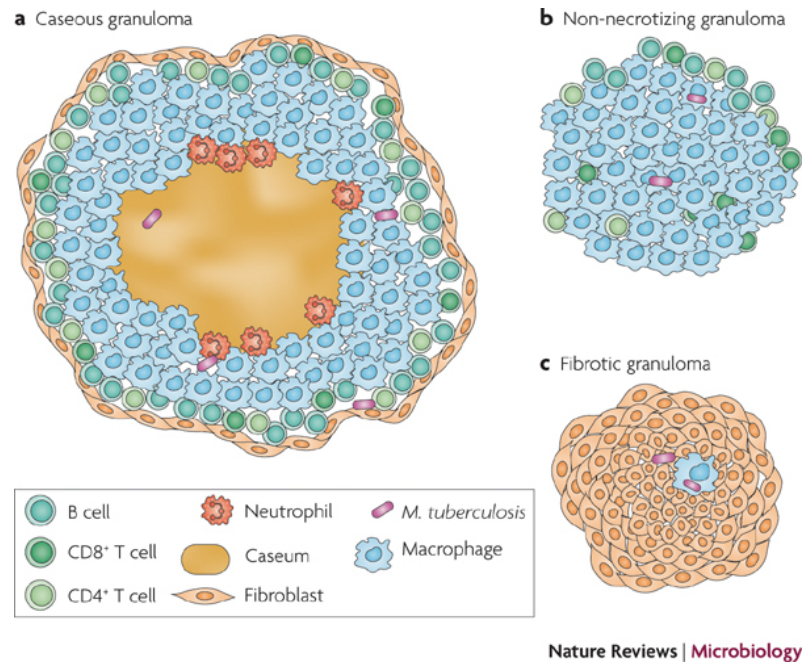
Figure 1.4: The granuloma. The figure shows a) the classic tuberculous granuloma, b) non-necrotizing granuloma c) fibrotic granuloma taken from (34)

1.2 Latent TB

Latent tuberculosis infection (LTBI) is classically defined as measurable immune sensitization to *Mtb* in the absence of active disease manifestations, such as fever, chills, night sweats, weight loss, cough, haemoptysis, or a new opacity on chest radiograph. Immune reactivity to *Mtb* is assessed by either tuberculin skin testing (TST) or interferon-gamma release assay (IGRA), with a positive result by either method indicating LTBI. LTBI can be viewed as an equilibrium between host and bacillus. In most cases, the host response is sufficient to combat active disease for life time, however, occasionally the immune response fails to protect and the infection reactivates to cause active disease (35). Latent TB is generally equated with bacterial containment in some inactive form and includes a diverse range of individuals from those who have completely cleared the infection with substantial memory T cell responses to TB antigens to individuals who are incubating actively replicating bacteria in the absence of clinical symptoms (36). The

basic mechanisms involved in maintaining a latent *Mtb* infection or the causes of reactivation are still difficult to explain, due to the difficulty in developing and manipulating animal models of latent tuberculosis. The design of an adequate animal model of latent *Mtb* infection is hampered by the lack of knowledge about the biological characteristics of both the tubercle bacilli and host immunity during human latent tuberculosis (37).

1.2.1 The global burden of LTBI

The population carrying a LTBI is commonly quoted as "one third" as one third of the global population, a reservoir of approximately 2.3 billion individuals (38). Houben and Dodd constructed trends in annual risk of infection (ARI) for countries between 1934 and 2014 using a combination of direct estimates of ARI from LTBI surveys and indirect transmission of ARI calculated from World Health Organisation (WHO) estimates of smear-positive TB prevalence. In 2014, the global burden of LTBI was estimated to be 23% amounting to approximately 1.7 billion people. WHO South East Asia, Western Pacific and Africa regions were all estimated to have LTBI prevalence in the general population of above 20%, whereas the WHO Eastern-Mediterranean, Europe and Americas regions all had LTBI prevalence of below 17%. Around 80% of the world's latently infected TB patients are in the WHO South-East Asia, Western Pacific and Africa regions (39).
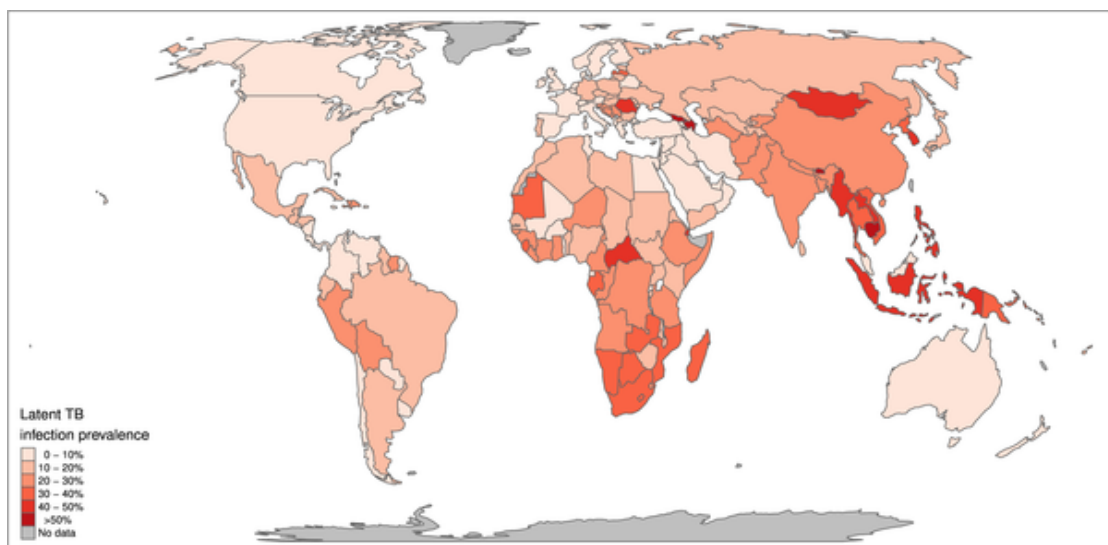
Figure 1.5: Global map of prevalence of LTBI(39)

China and India had the highest LTBI burden, having approximately 350 million infections, followed by Indonesia at about 120 million infections. The substantial increases in TB burden in Africa and South-East Asia are rapidly increasing in the younger age groups compared to other ages. It is estimated that 56 million people worldwide are at high risk of developing TB disease because of reinfection. Assuming the current LTBI activation rate of 0.15% per year, LTBI incidence will be 16.5/100000 per year in 2035, which is above the 10/100000 per year target in the End TB strategy (39).

Health care workers (HCWs) are at increased risk of acquiring LTBI in comparison to the rest of the healthy population. The risk of TB in HCWs is higher in the low and middle income countries that account for 80% of global TB cases. The estimated annual incidence of LTBI is 3.8% to 8.4% among HCWs with varying TB incidence. In country-wise subgroup analysis, the pooled prevalence of LTBI was lowest in Brazil (37%), followed by India (43%), China (54%), and South Africa (64%) among countries with higher TB incidence(40).

Stratified pooled estimates for the LTBI rate for countries with low (<50 cases/100,000 population), intermediate (50–100/100,000population), and high (>100/100,000 population) TB incidence were 3.8% (95% confidence interval [CI] 3.0%–4.6%), 6.9% (95% CI 3.4%–10.3%), and 8.4% (95% CI 2.7%–14.0%), respectively. Increased exposure and suboptimal infection control measures in high burden countries and also cough etiquette and respiratory hygiene can predispose to transmission of TB infection at the physician-patient interface especially in high burden countries. HCWs with active TB are also a potential source of infection transmission to other HCWs (40).

Studies of immigrants moving from high to low TB burden countries can also be another potential risk group for LTBI. Risk of TB is especially high in the first few years of migration, although they remain at higher risk of infection for decades after entry to low income countries. It is difficult to establish whether this infection is acquired as a delayed reactivation or by reinfection following recent transmission from visiting the endemic country or from the local community. McCarthy *et al.,* has demonstrated that TB diagnosis is rare after 15 years of immigration if individuals havea low risk of reinfection. About 230 migrants from high burden countries of South-East Asia who migrated to UK were studied. About 10.4% had arrived in UK 11-15 years previously and 5.6% more than 15 years previously (41).

1.2.2 Models of LTBI

The actual "state" of TB bacilli during LTBI continues to be a question for research. Autopsy studies in the early 20th century suggested that viable bacteria could be isolated from tissue samples in patients who died of TB in endemic countries (42). In LTBI, the bacillus stops growing and enters a state of stationary phase and eventually becomes non-replicating bacilli that retains the ability to resume growth within the granuloma (43). These bacilli are often referred to as dormant, persistent and non-replicating bacilli. It is assumed that the dormant and nonreplicating bacilli are contained within the granuloma but autopsy also suggested that viable bacteria may be present in apparently normal lung tissue in patient with tuberculous infection but no clinical disease (44). The physical location of the bacilli and the metabolic status of the bacilli in LTBI remains poorly understood.

The first and most well-characterized murine model of LTBI was the Cornell model and it was first described in the 1950s (45). In this model, mice were inoculated intravenously with $1x10^6$ to $3x10^6$ viable bacilli of *Mtb* H37Rv, followed by treatment with pyrazinamide (PZA) and isoniazid (INH) anti-mycobacterial chemotherapy for 12 weeks beginning within 20 minutes after infection to reduce the bacterial load to very low or undetectable bacilli levels. In this drug-induced model of LTBI, very low or no bacilli were detected for several months after this treatment, analogous to LTBI in humans. But in many mice, the latent infection spontaneously reactivated and caused active disease. The administration of cortisone reduced the time for spontaneous reactivation for 50% of the mice from 7 months to 2.5 months (46). This model was adapted to use an aerosol route of delivery of *Mtb* in order to mimic the natural route of infection (47). This model focused more on the ability of mycobacteria to persist or replicate following antibiotic regimens than on the host immunity involved in maintaining a latent infection. The modified Cornell model showed that reactivation occurs if the production of reactive nitrogen intermediates (RNI) is blocked by a nitric oxide synthase inhibitor-aminoguanidine, which has relative specificity for NOS2 (48). There is no standard protocol for establishing latency with the Cornell model. Scanga *et al.,* examined the Cornell model variants for their applicability to immunologic studies of LTBI. They evaluated the rate of spontaneous reactivation following the antibiotic regimen or reactivation with immunosuppressive regimens that included NOS2 inhibition, in vivo

neutralization of IFNγ, in vivo neutralization of TNFα and pharmacologic pan-immunosuppression using glucocorticoids. They showed that the outcome of the Cornell model was highly dependent on the parameters used to establish the latency and that each variant of the model has certain limitations (37).

Before the Cornell model, there were two other mouse models of LTBI. In the first model, mice were aerogenically infected with a low dose of *Mtb* (5 to 10 CFU), and within 3 months the pulmonary bacillary burden stabilized at 3 to 4 log10 (47). This clinically quiescent phase of the infection was maintained for 15 to 18 months but after that the infection began to reactivate and the mice succumbed to tuberculosis. This low-dose model has the important advantage of mimicking natural latency in that it relies solely on the host immune response for control of the infection, but it has the disadvantage of a high bacillary burden that is unlikely to be found in human LTBI. Using a modified low-dose model of murine latent tuberculosis, it was previously demonstrated that RNI play an important role in preventing reactivation (49).

A non-human primate model of latency is currently being developed, which would potentially be very relevant to the human clinical condition (50). Cynomolgus macaques infected with a low dose of *Mtb* are the only animal model in which both active disease and latent infection have been described and characterized (51). Gross signs of disease are observed as early as 4 weeks post infection (52). By the sixth week post infection, greater production of mycobacteria-specific IFNγ in PBMC was observed among monkeys who developed active disease later than in the monkeys with latent infection (51). About 40% of cynomolgus macaques infected with such low dose aerosol challenge did not go on to get disease and remained latently infected (53).

A dynamic model of latent infection has been proposed in which endogenous reactivation as well as damaging response occurs constantly in immunocompetent individuals. The model suggests that during initial stages of infection, *Mtb* grows well inside the phagosome and then escapes from the phagolysosome and released to the extracellular milieu due to macrophage necrosis (43)(54)(55)(56). Some of these extracellular bacteria stop replicating due to the hypoxic environment, nutrient deprivation and the presence of bactericidal enzymes released from immune cells. The metabolically inactive, non-replicating /dormant bacilli resist killing and may survive in the face of a strong immune response (43). The model also suggested a role for foamy macrophages that emerge

during the chronic inflammatory process due to phagocytosis of cellular debris rich in fatty acids and cholesterol in the dissemination of infection. The model suggested that the foamy macrophages phagocytose metabolically inactive, dormant extracellular bacilli. At the same time, tubercle bacilli do not grow in the intracellular environment, as the macrophages are activated (57)(58)(59)(60). The foamy macrophages containing phagocytosed bacilli then drain from the lung granuloma towards the bronchial tree and lodge the bacilli into different regions of the lung parenchyma due to aerosols generated by inspired air and in this process bacilli may also be lodged in the upper lobe of lungs, where they are exposed to higher oxygen pressure. This oxygen pressure in the upper lobes also supports rapid extracellular bacillary growth. The subsequent inflammatory response leads to tissue destruction, liquefaction and extracellular bacillary growth (60).

All these models are potentially very useful in modelling human LTBI to evaluate the immunological factors important for controlling such infection and also to evaluate potential therapeutic options. But these models are very expensive, complex, variable and time consuming (61). The outcome of these models depends on certain variables like antibiotic regimen, length of the treatment period, dosage of mycobacterial challenge used, the length of antibiotic-free rest period and strains of mice used. Botha and Ryffel described the limitations of these models which includes spontaneous reactivation, difficulties in inducing reactivation and generating phenotypically altered mycobacteria (62). Also these models have high inter-group variability and depend on group size, cost and availability of a BSL3 containment facility (61).

1.2.3 Pathogenesis of LTBI

In the majority of TB cases, *Mtb* infection induces the development of specific acquired cell mediated immunity inhibiting the growth of mycobacteria without their eradication (63). In such individuals, tuberculosis bacilli live in a dormant state. Well organised granulomas are entirely dependent on an effector Th1 response and mediate restriction of mycobacterial growth either within IFNγ activated macrophages or within the adverse conditions of the necrotic caseum (64). Granulomas are dynamic lesions with cells dying, debris being removed and new cells entering. It is assumed that, within granuloma, there is an equilibrium of actively dividing *Mtb* and *Mtb* adapted to the stress generated within activated or foamy macrophages that do not kill *Mtb* but are capable of inhibiting their

growth. Thus the bacilli are thought to enter a state of non-replicating persistence within the necrotic part of the lesion (65).

The persistence of TB bacilli in granuloma is accompanied by the changes in bacterial metabolism and in host metabolism that are driven by *Mtb* effector proteins and glycolipids. In the granuloma the bacilli are subjected to various stress conditions like nutrition deficiency, acidic pH and inhibition of respiration by nitric oxide. The transcription factor Rv3133c, named dormancy survival regulator (DosR) directly coordinates the expression of approximately 50 genes and DosR induces the metabolic changes that allow the mycobacteria to enter dormancy. All these factors lead to the transformation of *Mtb* into a dormant state (42). The dormant bacilli are able to minimize their metabolic and replicative activity and also inhibit their growth and development. They become resistant to immune attack and can avoid elimination by the effector immune cells (66).

The "Wayne" model of hypoxia induced dormancy, where bacteria were grown in liquid medium in sealed tubes with limited head space gradually reducing oxygen supply, showed a non-replicating state of persistence (NRP) which was characterized by reduced metabolism and increased drug tolerance. In this state, bacterial viability remained unchanged for weeks to months with synchronized replication resuming following reactivation (67). The resultant similarities between bacteria grown in vitro under hypoxic conditions and clinical cases of LTBI have made the model an important tool for investigating the molecular basis of mycobacterial dormancy (68).

Activation of DosR, is mediated through two classic transmembrane sensor histidine kinases, DosS and DosT. DosS senses cellular redox status and DosT senses dissolved oxygen concentration (69). Despite its dominance of gene expression under hypoxia, varying responses, such as genetic inactivation of DosR resulted in a mild loss of viability in vitro(70)(71)(72). Upregulation of the DosR regulon is not specific to hypoxic changes and it can also be activated by CO and NO.

Studies have shown that, while the DosR regulon is strongly induced at the initiation of anaerobiasis, this level is not maintained for a long time. With sustained periods of hypoxia another set of genes, called the enduring hypoxia response (EHR) are

upregulated. The relevance of the EHR overall bacterial adaptation to the hypoxic condition is yet not understood (73)(74).

A reduction in net carbon flux is a hallmark of hypoxia-induced dormancy in *Mtb*, whereas a source of carbon and energy are required for prolonged survival and later resumption of growth (68).

Under hypoxia and stress, *Mtb* experiences significant decreases in ATP and increases in its NADH/NAD+ ratio with a blocked electron transport system, whereas ATP synthesis via the electron transport system is an important requirement for bacterial survival under these conditions. This suggests that despite cessation of replication, *Mtb* maintains both an energized membrane and constitutive ATP production, even in the absence of molecular oxygen (75).

Macrophages undergo substantial phenotypic changes when exposed to reduced oxygen tension and it has been suggested that hypoxia modulates the central effector functions of macrophages with secretion of significantly enhanced amounts of the major proinflammatory cytokines IL-1β and TNFα, chemokines, proangiogenic factors and eicosanoids (76)(77). HIF1α plays a key role in macrophage adaptation to low oxygen tension and helps in the production of key immune effector molecules like granule proteases, antibacterial peptides, TNFα and NO. Mi *et al.,* showed that pattern recognition receptor-dependent stimulation of macrophages under hypoxia led to increased expression of NOS2 which is very important for antibacterial immunity (78).

It has been concluded that macrophages cultured at low oxygen tension are less supportive of mycobacterial growth. A low PO_2 closer to that found in tissue, did not affect the free living bacteria but significantly reduced the growth of intracellular mycobacteria. This growth inhibitory effect was said not be due to differential response to IFNγ and TNFα at low oxygen condition but rather it might be due to a shift from oxidative towards glycolytic metabolism. This was supported by other studies (79)(80).

Macrophages in granulomas are both antimycobacterial effectors but also the host cell for *Mtb*. Immunohistochemical analysis of granulomatous lesions from the *Mtb* infected NHP model suggested that macrophages with anti-inflammatory phenotypes (CD163+iNOS+ Arg1 high macrophages) localized to the outer regions of granulomas, whereas the inner

regions were more likely to contain macrophages with proinflammatory and bactericidal phenotypes (CD11c+ CD68+ CD163 ^{dim}, iNOs+ eNOS+ Arg1 ^{low} macrophages) (68). The highly hypoxic granuloma centre is the focus of antimicrobial activity and is surrounded by an area of reduced proinflammatory activity and gradually increasing oxygen tension. The foamy macrophages are located mainly in the interface region surrounding the central necrosis and this location may reflect the perfect niche and prime location for *Mtb* to initiate a new infection. Histologically, a latent lesion shows thick fibrosis, mineralization and/or central caseation as shown in chronically infected cynomolgus macaques (81)(82).

An insufficient upregulation of adhesion molecules on circulating lymphocytes may hinder the localization of antigen-specific lymphocytes within the lungs and this might affect the formation of granulomas (83). Decreased outer membrane permeability also protects *Mtb* from killing by ubiquitin-derived peptides. Thus some non-replicating bacilli may escape elimination by the immune system and so may persist (84).

Several *Mtb* components such as 19-kDa lipoprotein, Man-LAM, cord factor etc can modulate antigen processing and presentation by mycobacterial protein and glycolipid antigens by MHC class I, MHC class II and CD1 molecules. *Mtb* bacilli may suppress the presentation of antigens to T lymphocytes and macrophages (58). An insufficient activation of effector CD4+, CD8+ and γ/δ T cells, CD1 restricted and cytotoxic T cells, will result in defective microbicidal functions of macrophages and other immune cells leading to tissue damage and dissemination (85)(86)(87).

A particular subset of regulatory T cells, named CD4+CD25+FoxP3+D4GDI+T cells, contribute to the containment of *Mtb* in both human and murine models. These cells secrete Rho GDP dissociation inhibitor (D4GD1), instead of immunoregulatory cytokines, such as TGFβ or IL-10. This factor acts on macrophages, enhancing the production of IL-1β, TNFα and reactive oxygen species and indirectly contributes to DosR regulon activation , and the arrest of mycobacterial growth (88).

1.2.4 Risk of infection progressing to disease

Based on epidemiological studies, known risk factors for reactivation of LTBI include: HIV, malnutrition, tobacco smoke, renal failure, alcoholism, indoor air pollution,

alcoholism, silicosis, diabetes, malignancy and immunosuppressive treatment such as with glucocorticoids (38)(89)(90).
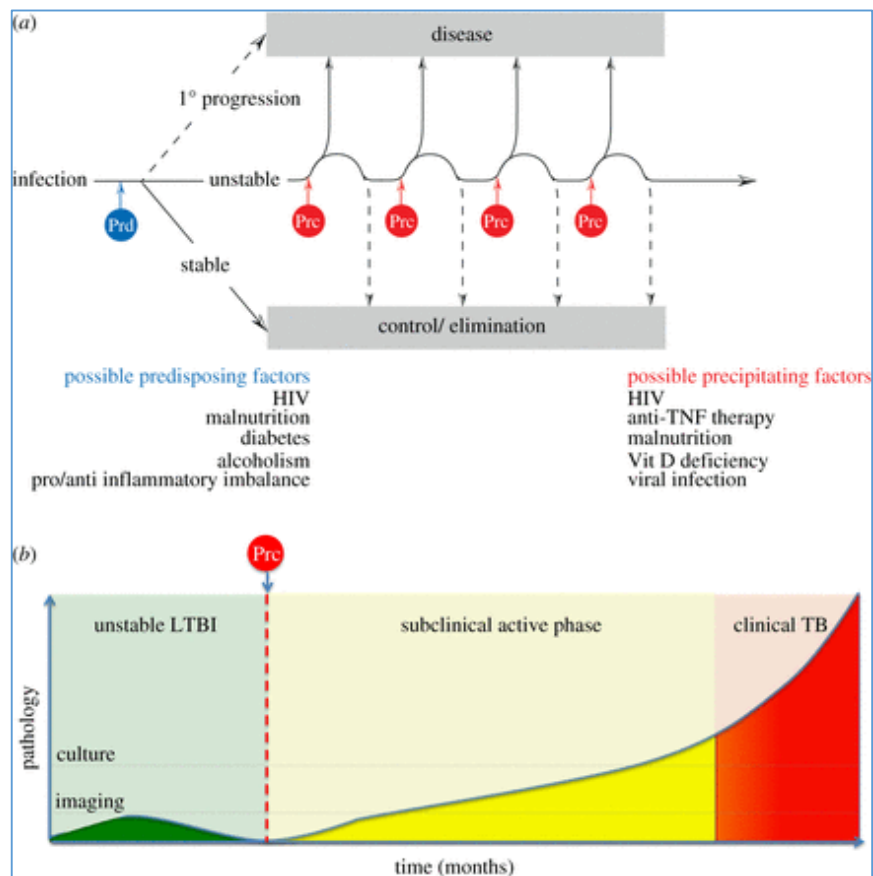
Figure 1.6: Progression of LTBI to active disease. The figure shows-a) The fate of TB infection is determined by predisposing factors influencing the outcome. b) Precipitating factors that may lead to progression of latent TB infection to active TB (91).

The effect of anti-TNFα therapy is prominent in the macaque model of LTBI, where anti-TNFα resulting in almost complete reactivation in animals with no previous signs or symptoms of active disease for at least six months from the time of infection (62). In patients treated with Infliximab, the risk of TB is increased initially up to 20 fold with 43% of TB cases presenting within 90 days of administration of anti-TNFα therapy (92).

HIV remains the most common risk factor for reactivation of TB. The risk of reactivation among HIV patients is almost 10 fold higher than for non HIV patients (93). A higher rate of disseminated, extra pulmonary disease was also observed in HIV-infected patients with TB. A CD4+T cell count of less than 200 cells/ml regardless of antiretroviral therapy was associated with risk of TB progression. In HIV and TB co-infection, there is HIV induced loss of mycobacterial specific CD4+T cells, *Mtb* induced increases in HIV load in serum

and macrophages, a shift from a Th1 to a Th2 response via alteration in IL-10, Tregs, IL-12, IL-4 and TNFα, loss of granuloma integrity and alterations in apoptotic mechanisms (94).

1.2.5 Preventive therapy for LTBI

Drugs that contribute most to treatment for latent TB (Rifampicin, pyrazinamide) have the greatest sterilizing ability, target persisting organisms and provoke resuscitation of non-replicating bacilli and couple this with rapidly bactericidal therapies such as with isoniazid (95). Monotherapy is used in treating latent tuberculosis as the bacillary load is so low that the chance of developing resistant mutants is very unlikely. INH inhibits synthesis of mycolic acids, the major constituents of the cell wall, and displays a biphasic killing with rapid and early bactericidal activity against actively replicating bacilli but it has less efficacy in killing bacilli with low metabolic activity (96). However, this therapy does not predict sterilizing activity (efficacy of preventing relapse in humans and in animal models). Rifampicin and pyrazinamide have more potent sterilizing activity and newer anti TB drugs such as rifapentine, TMC-207 and moxifloxacin also have also potent sterilizing activity (97). Martinson *et al.,* demonstrated that 12 doses of rifapentine and INH given weekly for 3 months is an effective preventive therapy for LTBI (98). The challenge is to evaluate novel LTBI regimens. The only endpoint for clinical trials is the absence of disease and to demonstrate clinical efficacy needs large sample sizes and prolonged follow up. A surrogate marker may allow rapid evaluation of the therapy. An alternative approach is PET/CT imaging to evaluate treatment response. 18F-Fluorodeoxyglucose (FDG), a marker for metabolic activityis taken up by activated neutrophils and macrophages. Sites of TB even in the absence of clinical sign symptoms accumulate FDG avidly and this is reduced following anti-TB therapy (99)(100)(101)(102). Whereas, Stephanus *et al.,* showed that even after receiving full TB treatment, some APTB patients in South Africa and South Korea were not able to eradicate TB completely and showed marked heterogeneity in treatment responses. They have showed that the PET/CT scan of these patients at the end of treatment exhibited lesions with an ongoing inflammatory response and some patients showed intensified or new TB lesions (103). Similar results were observed in a study conducted by Stetzmueller *et al.,* (104). Further research is required to unravel the interaction between the local host response and TB, disease progression and anti-TB treatment responses (105).

1.2.6 Current drug regimens for LTBI

Drugs	Dose(mg)	Duration	Frequency	Comment
INH	300	6–9 months	Daily	Standard 1st line in HIV−/HIV+
INH	300	36 months	Daily	Recent evidence for greater efficacy in HIV+
INH	900	6–9 months	Twice weekly	Alternative regimen- allows directly observed therapy
RIF	600	4 months	Daily	Alternative regimen
RIF+INH	600+300	3 months	Daily	Alternative regimen
RPE+INH	900+900	3 months	Once weekly	Recently reported as effective in HIV+ and HIV−
RIF+PZA	600+2000	2 months	Daily	Hepatotoxicity issues in HIV- no longer routinely recommended

Table 1.1: Drug regimen for LTBI (106).INH = Isoniazid, RIF = Rifampicin, PZA = Pyrazinamide, RPE = Rifapentine

The NICE guidelines contain detailed recommendations for persons with LTBI in the UK. All HIV-negative persons aged 16 to 65 years of age, migrants, aged 16-65 years, who have entered the UK from a high incidence country (≥150 per 100,000 or Sub-Saharan Africa, within the last 5 years and had been previously living there for ≥6 months are eligible for LTBI testing and those who are found to have LTBI are offered chemoprophylaxis- either a combination of Rifampicin and INH for three months or INH on its own for six months. HIV-positive persons of all age groups with LTBI are offered six months of INH and children (positive TST or IGRA and who have not been BCG vaccinated) are also offered chemotherapy if they are aged 1 to 15 years of age if they have been contacts of patients with smear-positive pulmonary TB. All individuals who are eligible for chemotherapy but decline should be given information and advised about

symptoms of active TB. NICE previously did not recommend chemoprophylaxis for any individuals above the age of 35 years old, unless they were HCWs or had an underlying illness with a high risk of reactivation (eg HIV). One reason for this age cut off is that the risk of hepatoxicity increases with age (107).

Until now, TB control has focused on detection and management of active disease, but to reach the WHO End TB strategy to reduce the TB death rate by 95% and TB incidence rate by 90% by the year 2035, it is also imperative to understand and manage the important reservoir of LTBI patients. Safer and more effective treatment of latent TB and effective pre or post-exposure vaccines would be the best ways to reduce the incidence of TB and to move forward towards the 2035 End TB target.

1.3 Biomarkers for LTBI identification

Biomarkers are measurable characteristics that can form the basis of surrogate end points. Biomarkers can be indicators of disease or indicative of host immunity. In some cases, these biomarkers can be indicative of the protective efficacy of a vaccine as correlates of protection. Biomarkers of infection or of risk of progression to disease could also contribute to both the development of diagnostic assays for TB and help to identify those at greater risk of progression (108).

Several efforts have been undertaken to distinguish between active and latent TB. LTBI is characterized by a high heterogeneity of lesions; some of the latently infected TB patients exhibit only the remnants of a waning infection, while some of them present with a slowly progressing form of the disease or may progress to a chronic non-progressing infection (109). What actually constitutes a protective immune response in this chronic infection still remains unclear. There is a need to develop new diagnostic tools and systemic screening based on newer biomarkers and/or newer antigens which are associated with latent TB, but not with active TB which may direct more efficient treatment to the infected individuals. Tools are also needed to screen the treatment response to LTBI, or to a post-exposure vaccine, or a screening test that detects individuals with LTBI, who are likely to progress in the next 5–10 years, all of which are part of Pillar 3 of the WHO End TB strategy (110).

We need to identify biomarkers for people who are at risk of progressing towards active TB disease, in a subclinical phase with minimally active pathology or immunopathology. These

predictive biomarkers could be mycobacterial products or markers of host response within blood, urine or through skin testing. Careful follow-up of the LTBI population at risk over time might allow the identification of some useful biomarkers and correlates of risk.

There is no gold standard test for the diagnosis of LTBI. The diagnosis of LTBI is based on reactive tuberculin skin testing (TST) and /or positive interferon gamma release assay (IGRA). IGRA (either QuantiFERON TB Gold In Tubes (Qiagen,the Netherlands; QFT-GIT) or the newer QFT-GIT Plus or T-SPOT.TB (Oxford Immunotec, Marlborough, MA, USA)) measure *in vitro* IFNγ production by whole blood ELISA or an Enzyme-linked ImmunoSpot (ELISpot) assay on peripheral blood mononuclear cells (PBMC) respectively (111). Blood is stimulated with peptides from *Mtb* specific antigens (ESAT-6, CFP10 and TB 7.7 for QFT-GT). The antigens used in this assay are encoded by genes that are deleted in the vaccine strain of BCG and most environmental mycobacteria of clinical relevance. IGRAs cannot distinguish between LTBI and active TB in immunocompetent individuals, in high risk individuals with immunosuppressive conditions or in children but are not affected by prior BCG vaccination and have no cross reactivity with most environmental mycobacteria. Screening for LTBI in low TB incidence countries is aimed towards high risk populations -like recently infected individuals, close contacts of active TB cases, recent immigrants from TB endemic areas or immunocompromised populations (112).

TST triggers a PPD-driven T-cell mediated delayed type hypersensitivity reaction (DTH). Two IU of the PPD preparation is used and skin induration ≥6 mm to ≥10mm (≥10mm in BCG vaccinated, ≥6 mm in HIV infected) is regarded as a positive TST.This response involves monocytes and previously primed T-cells, which are attracted to the skin where they proliferate and secrete cytokines. TST does not differentiate between infection with virulent *Mtb*, exposure to environmental mycobacteria or prior immunization with BCG. Its sensitivity is low in HIV infected or other immunocompromised individuals (113).The TST is also influenced by inter and intrareader variability and the immunosuppression status of the host (114).

The TST and IGRA rely on DTH or T cell immune response following reexposure to *Mtb* specific antigens (115). There is good correlation between TST and IGRA in low TB endemic settings, but high levels of discordance have been observed with individuals having TST negative but IGRA positive results with some reverse reports as well. The discordance

is assumed to be due to BCG vaccination which has effect on TST but not on IGRA.

Neither diagnostic test reflects the activity of any infectious focus or the risk of progression to active disease. Both the tests show evidence of spontaneous reversion over time that might provide a negative result for those patients who are previously exposed and immune sensitized by *Mtb*. A two-step testing strategy may identify these patients, where an initially negative TST boosts the immune response to subsequent TST positive (116).To measure alternative cytokines or to detect central memory populations using flow cytometric assays could provide a better marker of exposure and history of immune sensitization, as the IGRA after its short incubation (16 to 24 hours) identifies primarily ESAT-6/CFP10-specific effector cells (117). Host biomarkers may prove important in stratifying the risk of development of TB (118).

Different immunoassays have been proposed to aid LTBI identification- for example, IFNγ responses in untreated LTBI cases and unexposed subjects, where unexposed individuals displayed lower IFNγ levels by QFT-GT (119). Evaluation of serum pro-inflammatory cytokines like IL-6, MCP-1, plasma concentrations of markers involved in the TLR 4, fractalkine, IL-4 and monocyte chemoattractant protein (MCP) have been described (120)(121).

One potential candidate to help with diagnosis is the interferon inducible protein (IP-10), production of which in response to *Mtb* antigens could be used as a marker of infection. To be used for the diagnosis of LTBI, IP-10 still depends on stimulation with *Mtb* antigens. IP-10 has also been assessed as a potential biomarker to monitor treatment response to TB medication. Biraro*et al.,* measured the IP-10 concentrations in QFN supernatants from latently infected individuals compared to uninfected contacts and active TB. IP-10 was able to differentiate between the uninfected and latently infected contacts with sensitivity of 87.1% and specificity of 90.9%(122). However, IP-10 was not able to differentiate latent infection from active disease except when *Mtb* specific to mitogen specific ratios were used in HIV negative adults. The study showed good agreement between IP-10 and QFN, moderate agreement between IP-10 and TST and poor agreement between QFN and TST. Wergeland *et al.,* also demonstrated how IP-10 differentiated between active TB disease and LTBI or QFN negative controls in a high TB endemic setting. IP-10 therefore has a good potential to diagnose latent TB (123).

DosR regulon encoded antigens such as Rv2626c, Rv2624c and Rv2628 were also validated in different studies as they were recognized by household contacts or TST positive individuals and also could induce the secretion of significant amounts of IFNγ in comparison to non-infected individuals (124). Other latency antigens such as Rv1733c, Rv2029c and HBHA are also promising tools for identifying LTBI individuals and also to distinguish recent LTBI from remote LTBI (125)(126).

Blood transcriptional profiling has the potential to be used as a diagnostic test and has improved our understanding of disease pathogenesis. A study by Berry *et al.,* performing whole blood gene expression profiling reported an 86-transcript type 1 IFN inducible signature that was specific for active TB and the signature was overexpressed in neutrophils. The signature was associated with the radiographic extent of pulmonary TB. Transcriptional profiles of 10-25% of latent TB patients clustered with active TB patients. This proportion was equal to the predicted frequency of patients progressing toward active TB (109). Whole blood transcriptomic analysis has the potential to provide biomarkers for those with LTBI at highest risk of TB and may act as a diagnostic marker for LTBI with sub-clinical active infection or a higher burden latent infection, although longitudinal studies are required to validate this. Sweeney *et al.,* compared gene expression in patients with LTBI and other diseases versus ATB patients using a validated multicohort analysis framework (127). Three public TB gene expression datasets GSE19491, GSE373250 and GSE42834 composed of 1023 whole blood patient samples were chosen to be used if they were statistically differentially expressed in active TB compared with LTBI and other diseases, the diagnostic capability was not affected by HIV status and BCG vaccination, and if there was significant correlation with severity of active disease. The three gene set could improve clinical diagnosis and treatment response monitoring, although this needs to be confirmed by prospective validation with a targeted assay. The three-gene set should ease translation to clinical practice and might prove cost effective in the resource-poor environments in which tuberculosis is prevalent. The correlation of the tuberculosis score with disease severity also suggests that it might be possible to use the test for a predictive enrichment strategy for new drug trials. Zak *et al.,* identified a 16 gene signature of risk in LTBI and household contacts for South African and Gambian cohorts. The signature predicted tuberculosis progression with a sensitivity of 66·1% (95% CI 63·2-68·9) and a specificity of 80·6% (79·2-82·0) in the 12 months preceding tuberculosis diagnosis (128). The risk signature was excellent for differentiating tuberculosis disease from latent

infection and from other disease states. The newly described signature also holds potential for highly targeted preventive therapy.

Multiparameter flow cytometry can be used to evaluate the proportion of T central (TCM) and T effector memory (TEM) subsets and multifunctional T cells- where the ratio of TCM and TEM can serve as a biomarker of the presence of infection as suggested in different studies (129). Studies have analysed the presence of both TCM and TEM in PBMCs of individuals with LTBI, where a response to RD1 stimulation in LTBI was characterized by TCM phenotype. Adekambi *et al.,* have described a distinct effector memory CD4+T cell signature in latent *Mtb* infection, which was differentiated from that in clinically cured TB patients (130). Caccamo *et al.,* suggested that a multifunctional T cell profile is associated with the presence of live replicating TB rather than operating as a marker of protection (129). Multi-parametric flow analysis of T cells thus has the potential to distinguish those with replicating bacteria from those who have naturally cured their infection. This will provide insights to target preventive therapy for LTBI.

Sandgren *et al.,* systemically reviewed data on initiation and completion rates for LTBI treatment and observed that the rates were frequently suboptimal and varied greatly within and across populations (131). Populations with higher risk of TB infection like HCWs, in mates and the homeless were at higher risk of low initiation and completion rates. Improvement in treatment adherence and completion at risk groups might improve the effectiveness of latent TB treatment and stop progression towards active TB disease. An alternative to shortening to treatment can be to target the persisting organisms and their resuscitation. Bactericidal drugs like INH can strengthen this process. This approach will require more refined understanding of the mechanism behind the resuscitation and the complex metabolic states of this organism.

To find a test that can best evaluate novel LTBI regimens is another challenge. Peripheral biomarkers indicative of treatment response of LTBI might prove an important tool. A surrogate marker of clinical response, early (2 months) culture conversion in active TB, 14-day bactericidal activity, etc. may demonstrate the clinical efficacy and selection of appropriate therapy to prevent reactivation. Recently PET/CT imaging has been used to evaluate the response to therapy. This is a very sensitive imaging modality that can detect the early pathological changes in sputum negative asymptomatic individuals and also pathology consistent with subclinical pulmonary TB. Esmail *et al.,* 2016, revealed that

LTBI patients with fibrotic scarring may undergo some repeated episodes of subclinical reactivation, which makes them more susceptible to develop active disease and this outcome can be assessed through PET/CT scan imaging (105). Malherbe and colleagues observed the PET-CT imaging response patterns in HIV negative patients with PTB after 6 months of standard treatment and post treatment follow-up until 1 year. The presence of *Mtb* mRNA in sputum and BAL samples in these patients and intense lesions on PET-CT indicated that even after a full course of treatment was completed, all the bacteria were not eradicated. They suggested that for developing improved and shortened PTB-treatment strategies, it is necessary to identify a better treatment response marker (132).

It is critical to develop a better understanding of the immunological correlates of protection for LTBI in order to design an effective TB vaccine. The vaccine could be administered pre-infection to prevent the infection from occurring or designed to be used post- exposure to prevent reactivation. H56 is a new multistage vaccine comprising the Ag85B, ESAT-6 and Rv2660 antigens which has been designed to be used post-infection in latently infected and uninfected adults. This vaccine is now in phase IIa clinical studies in South Africa (133) .

Antituberculous treatment and immunomodulation might be another alternative approach to shorten the existing therapy. The RUTI vaccine is comprised of heat inactivated, liposome fragments of *Mtb* that are grown in stress and hypoxia conditions. The vaccine is now in clinical phase 2 trials in patients with LTBI in South Africa. This vaccine is designed to be administered after one month of chemotherapy of LTBI with INH to facilitate the immune clearance of the persisting bacilli. Each subject (HIV+/HIV-) will receive two administrations, 28 days apart and will be monitored until one month after the second inoculation with RUTI (134).

Improved understanding of the natural infection, protective immunity and immunopathology and the mechanism responsible for the reactivation in LTBI, might prove effective ways to accelerate the development a vaccine that could prevent reactivation or that was able to eliminate LTBI successfully. Also we need to get effective and representative results from the existing animal models that will represent better the natural history of LTBI in humans.

1.4 Helminths

Helminths are complex eukaryotic organisms, characterized by their ability to maintain long standing chronic infections in humans (135). Approximately two billion people are

infected with intestinal helminth parasites, rendering them the most prevalent infectious agent in the world today and they are responsible for many chronic, debilitating and sometimes disfiguring diseases (136). Helminths are multicellular worms of three taxonomic groups: the nematodes (also known as roundworms) include the major intestinal worms (also known as soil-transmitted helminths) and the filarial worms that cause lymphatic filariasis (LF) and onchocerciasis, whereas trematodes include the flukes,such as the schistosomes, and cestodes include the tapeworms (137).

Helminth parasites have characteristic complex life cycles with different stages of development from direct faecal-oral transmission (in roundworm infection) to development through free living stages (like hookworm larvae in the environment) or dependence on invertebrate vectors (for example snails in schistosome infection) (138). The responses may be compartmentalized depending on the anatomic location of the parasite, for example in the intestinal mucosa and draining lymph nodes or be systemic (135). Helminths may also have different invasion routes including skin (Schistosoma etc.), or by mosquito bite although the most common route is the gastrointestinal tract. The migration patterns of the parasite may elicit cutaneous, pulmonary or intestinal pathology, for example in Strongyloides infection during their lung migratory phase.

Another hallmark of helminth infection is their chronicity–as some parasites live in the host for years. *S.stercoralis* can cause auto-infection and remain in their host to maintain their life cycle for an indefinite period of time (135). Helminth infection can establish long term chronic infections associated with significant down-regulation of the host immune response. The extraordinary prevalence of helminth infections reflects their ability to modulate the immune response in the host (138).

Helminths compete with the host for nutrition, cause mechanical damage during their migration through tissues and in many cases may also drive immune-mediated pathology. Certain species of helminths are responsible for significant disability, morbidity and a reduction in life expectancy (139).There is variation in helminth infections within endemic regions- some people being susceptible to infection whereas others appear to be immune to infection with chronic exposure (140).

The eradication of helminth infection remains a difficult goal due to a lack of effective vaccines, limited pharmacological efficacy, emerging drug resistance, and rapid reinfection in places where transmission cannot be prevented.

1.4.1 Global prevalence of helminth infection

According to WHO, 24% of the world's population are infected with soil-transmitted helminth infections (STHs) world-wide. Infections are widely distributed in tropical and subtropical areas especially where adequate water and sanitation are lacking. The greatest numbers of STH infections occur in sub-Saharan Africa, the Americas, China and East Asia, and South America (141). The prevalence of helminth infection has declined in some part of the world due to improvements in living conditions and expansion of major deworming efforts. However still about one billion people globally are infected with helminths (142).
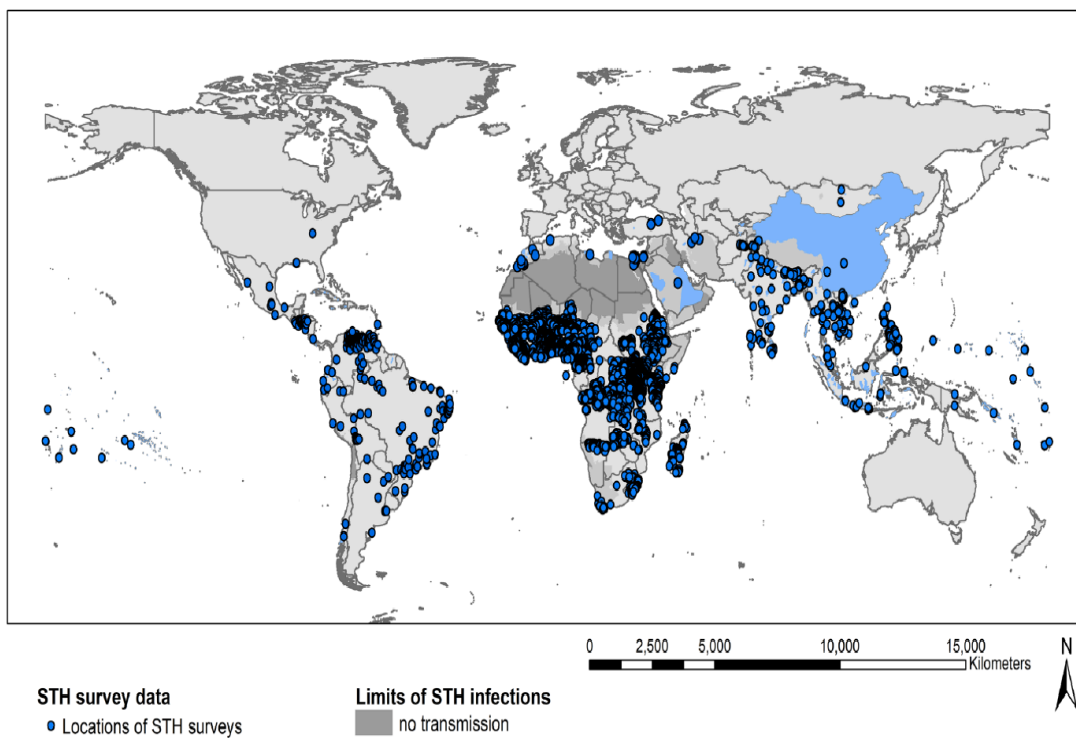
Figure 1.7: Global prevalence of soil transmitted helminth infection (2010) (143).

Helminth	Disease	Global prevalence (in millions)	Regions of prevalence
Ascaris lumbricoides	Ascariasis	807	Developing regions of Asia, Africa, and Latin America
Trichuris trichiura	Trichuriasis	604	
Necator americanus, Ancylostoma duodenale	Hook worm infection	576	
Strongyloides stercoralis	Strongyloidiasis	30-100	
Wuchereria bancrofti	Lymphatic filariasis	120	Developing regions of India, Southeast Asia, and sub-Saharan Africa
S.haematobium, S.mansoni, S.japonicum	Schistosomiasis	207	Sub-Saharan Africa, Eastern Brazil, China, South East Asia
Taenia solium	Cysticercosis	0.4	Developing regions of Asia, Latin America and Sub- Saharan Africa

Table 1.2: The major helminth infections and their distribution and global prevalence (2010) (140).

Pullan *et al.,* built a modelling framework to provide an update on the global situation in 2010, and estimated that 1.45 billion people were infected with at least one species of intestinal nematodes, resulting in 4.98 million years lived with disability (YLDs). The vast majority of infections and burden occurred in Asia, where at least one quarter of the population hosts at least one soil transmitted helminth species (142). There is a website, named "Thiswormyworld" developed by Simon Brooker. The aim of the website is to provide reliable, up-to-date maps on the geographical distributions of NTDs (www.thiswormyworld.org) (144).

1.4.2 Helminth infection in UK migrants

The global burden of helminth infection is greatest among populations who are heavily exposed to helminths specially in low income countries in the tropics (145). However, some chronic helminth infections may persist for years after migration from endemic countries and occasionally may cause severe disease in patients receiving immunosuppressive agents (146). Helminth infection may affect up to 20% of migrants from endemic countries at the time of their arrival in the UK (147), including Schistosomiasis, *S.stercoralis* and other soil transmitted helminth infections.

Strongyloides is common in South East Asia and the Caribbean, whereas Schistosoma infection is acquired by exposure to snail contaminated fresh water in many tropical countries particularly Sub-Saharan Africa where it can affect about 20% of travellers and residents(147).Travelers and expatriates contract schistosomiasis during their leisure activities like water sports, rafting, swimming or showering(148). Helminth infection is the commonest identifiable cause of eosinophilia in the returning traveller or migrant (14% to 64%) (149). Schulte *et al.,* showed that >50% migrants from endemic countries who present with blood eosinophilia harboured helminth infection (150). Smith *et al.,* observed that eosinophilia was associated with African and Asian ethnicity in an inner city gastroenterology service in the UK that serves a migrant population (145). About 40% of the patients in the clinic were born in an endemic country and residing in the UK (a non–endemic country) or had recently travelled to endemic countries and were being investigated for peripheral eosinophilia. The majority of the screened migrants had helminth infection which was detected by the evidence based screening protocol for investigating eosinophilia. They presented with symptoms suggestive of Strongyloidiasis and Schistosomiasis and after anthelmintic treatment the symptoms improved in some of these patients. Eosinophilia on two or more occasion was diagnosed in 6.3% of travellers. The median eosinophil count was higher in those migrants who were parasitology serology and microscopy positive. The most prevalent helminths were *S.stercoralis* and Schistosoma spp. in their study. Another study at the Hospital of Tropical Diseases (HTD), London showed that immigrants have a lower chance of being symptomatic, including in microscopically proven infection although eosinophilia was present in 76% of migrants (151).

The majority of immigrants with Schistosoma infection are also asymptomatic and the worm burden remains low. There are significant differences in immune responses in the immigrants depending on length of exposure to the helminth, IgE level and eosinophil count (12,16).

1.4.3 Immune responses in helminth infection

A complete understanding of immune response to helminths is complicated as these helminths have various lifecycles, tissue tropism and evasion mechanisms. These helminths consequently may activate immunity differently and induce different effector mechanisms. The immunological hallmark of helminth infections is their ability to induce

T helper type 2 (Th2) associated immune responses characterized by the production and marked elevation of cytokines like interleukins IL-4, IL-5, IL-9, IL-10 and IL-13, localized or generalized eosinophilia, production of IgE and IgG4 in humans and mucosal and goblet mast cell hyperplasia (1,18,19). In helminth infection, the initiation, progression and culmination of the type 2 response requires interaction with different cell types, including epithelial cells, innate lymphoid cells, antigen presenting cells-DCs, macrophages, T cells, B cells, eosinophils, mast cells and basophils (153). Naïve CD4 T cells differentiate into Th2 cells in the presence of cytokines such as IL-4, which further strengthens the type 2 response by positive feedback. Activation of cells of the innate and adaptive immune systems together can mediate a potent host protective immune response to helminth infection.

The protective immune response includes mechanisms that mediate helminth and host resistance, resulted in accelerated worm expulsion and also mechanisms for enhancing the tolerance to invasive helminthiasis that reduces the damage to the host without affecting resistance to the pathogen. In the host response to intestinal helminths, tolerance includes direct effects of promoting wound healing and also inhibitory effects on harmful inflammation-which is mediated by the Type 1 immune response with elevation of both Th1 and Th17 cytokines (139).

In helminth infection, the immune regulatory cytokines, namely IL-10 and TGFβ, can be regulated independently of the type 2 response. In a previous study, neutralization of IL-10 and TGFβ resulted in increased production of both IL-4 and IFNγ, suggesting the parasite may induce these anti-inflammatory cytokines to evade the immune response and to prevent host immunopathology(154). In gastrointestinal nematode infection, IL-10 and TGFβ are significantly linked with hyporesponsiveness, susceptibility and patients show a higher frequency of circulating CD4+CTLA-4+Tcells (155)(156).This immunoregulatory state is a common outcome in diverse range of helminth infections (138).

Macrophages may also mediate immune regulatory effects through expression of inhibitory enzymes such as arginase 1, co-inhibitory molecules such as programmed cell death ligand 1 (PDL1), PDL2, IL-10 and suppressor of cytokine signalling 1(SOCS1) (157). Helminths also target these co-inhibitory molecules expressed on T cells and antigen presenting cells (APC) to maintain hyporesponsiveness.

Helminth infection can evade the host immune responses through the action of Tregs. The induction of Treg cells is required for the establishment of chronic infection and depletion of Tregs leads to induction of protective immune response to helminths and worm expulsion (158). Maizels *et al.,* in their study showed that neutralization of Treg derived IL-10 and TGFβ in vivo restored the anti-parasite response, suggesting the immunosuppressive roles of those cytokines which are mediators of Treg-induced immunoregulation in helminth infection (159).

During this acute stage of infection, macrophages have an alternatively activated phenotype (M2 type) which is more consistent with wound healing, but also inducing the differentiation of Tregs. Helminth infection induces alternatively activated macrophages with nitric oxide (NO) synthase (NOS2) suppression, downregulates the expression of Toll- like receptors on APCs and T cells, induces apoptosis of DCs, T cells and NK cells and mediate anergy of cognate T cells (160). The Type 2 response is very important in enabling host tolerance of the extensive tissue damage caused by the helminths during their life cycle and also in their migratory phase to different primary organs. However the prolonged type 2 immune response in chronic infection may also lead to immune pathology and fibrosis (161).

1.4.3.1 Responses to tissue injury

Helminths cause significant tissue damage during feeding and migration in the intestine, which can trigger the release of danger-associated molecular patterns (DAMPs) and cytokine alarmins (IL-25, thymic stromal lymphopoietin (TSLP) and IL-33) (162). Cytokine alarmins are rapidly released by endothelial and epithelial cells as well as necrotic cells following tissue damage and mast cells in response to helminth infection(163). Intestinal epithelial cells form the initial barrier between the lumen and intestinal tissue and may also be a source of cytokine alarmin production(164). These alarmins then activate innate lymphoid cells and other cell types like eosinophils and basophils to secret IL-4, IL-5 and IL-13 resulting in activation and differentiation of Th2 cells and also further amplification of type 2 adaptive immune responses. Activated DCs and other innate immune cells migrate from the lamina propria and Peyers Patches to regional lymph nodes through lymphatic vessel (165).

It is apparent that humans have a robust cytokine alarm system to tissue injury that will activate a type 2 immune response in the absence of a more dominant type 1 trigger (166). Patel *et al.,* in their study linked the cellular damage caused by helminths to the induction of protective immunity in nematode infection and demonstrated that release of extracellular adenosine was associated with induction of a Th2 response (167). Helminth products can induce Th2 responses in the absence of injury (168)(169). The potentially damaging large multicellular organisms may induce a response that resembles a reaction to injury. The three major alarmins are IL-33, IL-25 and TSLP.

1.4.3.2 Interleukin 33 (IL-33)

IL-33 is a member of the IL-1 family and its receptor is ST2, which is expressed on mast cells, Th2 cells and ILCs and can be highly upregulated on macrophages by Th2 cytokines (162)(170)(171). Mice lacking ST2 fail to develop primary Th2 responses or form Th2 dependent lung granulomas in response to intravenous administration of Schistosoma eggs (172). Yin *et al.,* demonstrated that the responses elicited by IL-33 also impact on the repair process and this was evidenced by accelerated wound repair following administration of IL-33 (173). There is also emerging evidence that IL-33 is also playing an important role in epithelial restoration and mucosal healing in the gut. In *Trichuris muris* infection, IL-33 mRNA was observed to be elevated during early infection and administration of rIL-33 resulted in accelerated clearance of the parasites (174). Worm expulsion was mediated by IL-13 production by both ILCs and CD4+T cells which in turn increases production of the anti-worm effector molecule Resistin-like molecule β (RELMβ) by intestinal epithelial cells. RELMβ exerts its anti-helminth effect by interfering with parasite feeding on host tissues or it may block pore-like structures with chemosensory function and chemotaxis. IL-33 is critical in worm expulsion and also acts to minimize host damage early in infection (175).

1.4.3.3 Interleukin 25 (IL- 25)

IL-25 is a member of the IL-17 cytokine family and produced by epithelial cells. Unlike IL-33, it also induces the production of Th2 cytokines by ILCs and it acts as a sensor of epithelial disruption (176). Owyang *et al.,* in their study showed that when mice susceptible to Trichuris spp. were treated with IL-25, they could effectively expel the parasite, while IL-25 deficiency prevented worm expulsion. They also demonstrated that

IL-25 can limit the intestinal inflammation and tissue damage in the colon (177). IL-25 also promotes allergic responses that can lead to tissue damage and remodelling, fibrosis and wound repair (178).

TSLP is a member of the IL-2 cytokine family expressed predominantly by epithelial cells in the lung, gut, liver, central nervous system, etc. where it suppresses inflammatory type 1 responses and promotes Th2 responses in response to tissue injury and is also associated with protection of mucosal surfaces (179). TSLP amplifies type 2 effector responses by enhancing the polarizing effects of IL-13 on macrophages. Mice infected with *T.muris* showed more susceptibility to infection when TSLP was blocked and TSLP receptor-deficient mice have enhanced worm burdens (180). In TSLP receptor deficient mice, liver fibrosis induced by deposition of Schistosoma eggs and reduced IL-13 production was observed (181). Neutralization of TSLP resulted in a significant reduction in the number of fibrocytes and in skin fibrosis and this tissue remodelling function was mostly due to TSLP receptor expression on fibrocytes (182).

1.4.3.4 Innate lymphoid cells (ILCs)

ILCs are a distinct group of innate cells that display transcriptional and functional attributes directly related to the adaptive T helper effector cells, Th1, Th2 and Th17. ILC2s can produce IL-5, IL-9 and IL-13 like Th2 cells and require GATA-3 for lineage commitment. They also play an important role in induction of adaptive Th2 cells after lung allergen exposure and also promote worm expulsion from the gastrointestinal tract (183). IL-13 producing ILC2s promote goblet cell mucus secretion and smooth muscle contraction and help in worm expulsion. ILC2s are the predominant IL-9 producing cells in the lung. Mice lacking the IL-9 receptor failed to repair the lung damage after larval migration, with delayed worm expulsion and more pathological inflammation in the lungs (184).There were also significantly reduced numbers of eosinophils and IL-4Rα activated macrophages as measured by RELMα expression, as a result of reduction of ILC leading to insufficient IL-5 and IL-13 production and failure to induce a sufficient adaptive immune response (51) .

1.4.4 Antibody responses in helminth infection

IgG1 (in mouse), IgG4 (in human) and IgE antibody responses are a feature of helminth infection. Helminth-associated Th2 cytokine responses with IL-4 activation of transcription factor STAT6 , are classically associated with class switching in B cells to produce IgG and IgE (186). IgE is a primary mediator that binds FcRεI on mast cells and basophils.   Antigen crosslinking of FcRεI-bound IgE triggers mast cell degranulation and the release of soluble mediators. In the presence of Th2 responses, there is enhanced IgE production resulting in vascular permeability, smooth muscle contractility and also the recruitment of Th2 type effector cells including eosinophils and Th2 cells (187). There is variable protection conferred by IgE in different studies. Anthony *et al.,* demonstrated that IgE against helminth infection is not essential for protection against helminth infection in mice (188). On the contrary, there are studies such as where following *H.polygyrus* infection in mice, IgE and IgG1 were observed to promote expansion of basophils and provide protective immunity against the helminths (189).

IgM is the primary antibody type that recognizes larval parasites and might be produced in a T-cell independent manner. IgM might be important for macrophage recognition of filarial parasites and macrophages express Fc receptors for IgM. Lack of secretory IgM results in significant impairment of *Brugiya pahangi* larval expulsion and is finding was supported by in vitro data, where IgM was the only antibody isotype able to bind to the parasite's larvae (190). Polyclonal IgG1 and IgE have been shown to be protective during *H.polygyrus* infection; these antibodies reduce parasite reproduction by targeting egg antigens and also halt the development of adult worms (191)(192).

Whether or not antibodies are protective during helminth infection remains controversial, as their function is not as well understood as the prominent Th2 response. In *S.mansoni* infection, administration of IgG2a in mice provides some protection accompanied by decreasing granuloma fibrosis (193). Ligas *et al.,* in their study isolated IgM and IgG from mice vaccinated with *S. stercoralis* larvae and this was administered to naïve mice; they observed a high level of protection with larval killing via ADCC and it was evident that granulocytes and complement were less important (194).

The profile of antibody isotypes is also an indicator of the regulated status of the host. In helminth infection, levels of IgG4 and IgE are important indicator of susceptibility to and

protection from infection. In helminth infection, IgE production by B cells is promoted by IL-4 and IL-13, but in the presence of regulatory cytokines IL-10 and TGFβ, a switch to IgG4 is favoured. IgG4 cannot crosslink receptors on basophils, mast cells and eosinophils, does not activate complement or act as an opsonin. IgG4 antibodies are important markers of the Th2 state, potentially blocking IgE and preventing potentially damaging inflammation (195). In one study it has been shown that IgG4 titres fall rapidly following curative anthelmintic drug treatment. It was suggested that high levels of IgG4 in helminth infected patients reflect a dominant regulatory environment (196).

Although a humoral antibody immune response is typically associated with Th2 type responses during the infection, antibodies do not appear to have an essential role in helminth protective responses. This response varies with the specific parasitic helminth, developmental stages in the host or intensity of infection (197).

1.4.5 Deficient Acquired Immunity

In helminth infection, the immune system is unable to clear chronic infections and immune memory fails to protect against reinfection even after drug mediated clearance. However, effective immunity significantly reduces the parasite burden as is demonstrated by irradiated larval or cercarial vaccines in animal models of schistosomiasis. Deficient immunity reflects inadequate Th2 responses. The resistance of Schistosoma infected patients to reinfection following drug cure correlates strongly with the production of IL-4 and IL-5. During chronic helminth infection, the Th2 response is downregulated and protective immunity is compromised (66)(199)(200). A study on Schistosomiasis showed that protection from infection was associated with both Th1 (IFNγ) and Th2 (IL-4 and IL-13) gene polymorphisms which indicates the possibility of Th1 derived components contributing to immunity to Schistosomes (201). Resistant individuals mount strong IFNγ responses to parasite antigens, indicating that both Th1 and Th2 arms may be modulated in susceptible individuals (202). A link between T cell anergy and the immunoregulatory environment in helminth infections is suggested by the strong correlation between IgG4 levels and unresponsiveness. This hyporesponsiveness can be reversed by the chemotherapeutic removal of the parasite burden, which argues that it reflects a direct effect of live parasites rather than an inherent incapacity of the host to respond to infection (203).

In human Schistosoma infection, CD4+CD25^{high} cell frequencies were significantly reduced after drug cure of *S.mansoni* infection, while in children, CD4+ CD25 ^{high} CD127$^-$Foxp3+ proportions were positively correlated with the *S.haematobium* parasite burden (204). In Schistosoma infection, in adults who were resistant to reinfection to parasite, Treg proportions were negatively correlated with infection intensities. Treg proportions are also reduced upon infection due to the expansion of the T effector population (204).

1.4.6 Dampening bystander responses in humans

Helminth infection has immunoregulatory effects on bystander responses in humans including those of both immunopathogenic and protective natures. Coinfection with helminths suppresses antibacterial, antiviral and antiprotozoal and antitumor immunity leading to increased susceptibility and attenuated immunopathology or if there is high infection burdens there may be exacerbated pathology.

There is a growing concern in developing countries that helminth infection may interfere with childhood vaccination. It has been observed that in Schistosoma infection, there was a weaker IFNγ response to tetanus immunization (205). A similar effect was evident in other helminth infections like onchocerciasis, lymphatic filariasis etc., where higher level of IL-10 was produced. In mouse models, *S.mansoni* infection interferes with the protective effect of BCG vaccination and there were depressed antimycobacterial T cell responses with concomitant increases in TGFβ levels (206).

1.4.7 Cellular basis of immunomodulation

1.4.7.1 Dendritic cells (DCs)

The immunomodulation and stimulation of the host immune system by helminths is predominantly determined by the DC population. The depletion of DCs inhibits the Th2 responses to infection. In Schistosoma infection, lysophosphatidylserine of the parasite acts on human DCs to promote IL-10 producing Tr1 cells and the egg molecule ω-1, drives murine DCs to induce FoxP3 expression in T cells in vitro (207). DCs are also influenced by host "alarmin" such as TSLP. Helminth-specific pattern recognition receptors include TLR, C-type lectin receptors (CLRs) and the class A scavenger receptor

(208).Certain helminth products also interfere with DC function by blocking antigen processing or degrading mRNAs within the host (209).

Helminths can also secrete a variety of excretory/secretory (ES) products that can directly stimulate DCs and macrophages and which can also activate naïve CD4+T cells to differentiate into Th2 cells in the presence of IL-4 and / IL-13 (210). These ES products can also inhibit DC production of IL-12, costimulatory molecules and factors required for Th1 cell differentiation. Helminth derived molecules have been shown to promote tolerogenic DCs that promote T cell anergy and the induction of Tregs cells(139). In a mouse model, Schistosoma infected TLR-2 deficient mice were less capable of inducing Treg cells and exhibited more pronounced immunopathology than the infected wild type mice (211).

1.4.7.2 Alternatively activated macrophages (AAMs)

AAMs are distinct from classical macrophages in expressing higher level of arginase 1, IL-4Rα, the mannose receptor (CD206), the chitinase like molecule Chi3L3 (Ym1) and the resistin like molecule RELM-α (212). IL-4, IL-13, IL-10 and IL-21 trigger AAMs. AAMs regulate the immune response, wound healing and resistance to parasite invasion. In RELM-α deficient mice, there is an exaggerated Th2 response to Schistosome infection (213). AAMs can contribute to wound healing by clearing matrix components, cell debris and by releasing cytokines, growth factors and angiogenic factors that promote fibroplasia and angiogenesis. AAMs may also mediate more direct effects on tissue dwelling nematodes by targeting the glycan chitin. AAMs secrete chitinase and chitinase like secreted proteins like fizz family member proteins (ChaFFS)–all these mediate resistances to helminth infection and promote the Th2 type inflammatory response. When arginase function was inhibited it was observed that protection against the tissue dwelling nematode, *H.polygyrus* was also blocked. This study also demonstrated that Th2 cell-derived IL-4 drives AAMs, which then in turn contributes to parasite clearance through an arginase-1-dependent pathway (214).

1.4.7.3 Regulatory T cells

Nematode infections can induce and expand naturally occurring regulatory Treg cells in humans suggesting a role for these regulatory T cells in helminth-induced modulation of

inflammatory diseases. In mice infected with Schistosoma, FoxP$_3$+Tregs are recruited to the draining lymph nodes and the periphery of egg induced granuloma in the liver and these Tregs can also control granulomatous pathology at the intestinal stage and reduce the size of the granuloma which is correlated with increased CD4+CD25+CD103+ FoxP3+cells (215). In the mouse model, the Th2 responses in later stages of chronic Schistosoma infection are progressively downregulated and granulomas reduce in size and this downregulation is mediated by both regulatory cells and cytokines like IL-10. Treg depletion with anti CD25 antibody results in decreased egg production and increased granulomatous pathology. Th1 and Th17 dominated inflammatory responses can also be fatal in Schistosoma infection.

An expansion of CD4+ FoxP3+Tregs is also seen in gastrointestinal infection with *S.ratti* in the intestine of mice (216). Depletion of Tregs in mice immediately after infection with *S.ratti* resulted in expression of protective immunity. The blockage of both IL-10 and TGFβ increases the resistance to the parasite. In *T.muris* infection, Tregs accumulate in the lamina propria. Depletion of Tregs does not enhance worm expulsion but provokes increased gut pathology, indicating that conventional Tregs in this infection control pathological but not effective anti-parasite immune responses (217).

1.4.7.4 Regulatory B cells

B cells also produce inflammatory and immunoregulatory cytokines, for example as IL-10 producing B cells. In murine *S.mansoni* infection, transfer of *S.mansoni* induced Bregs induced the recruitments of FoxP3+Tregs to the inflammatory airways in an IL-10 dependent manner. This IL-10 in Schistosoma infection, was further shown to be important for expansion of IgG1 producing plasma cells in the liver and also for the suppression of granulomatous responses during chronic infection (218).Thus both immunoregulatory cytokine production and antibody production by B cells are important for immunomodulation in chronic Schistosoma infection.

1.4.7.5 CD4+ T helper effector cells

Effector Th2 cells induced by helminths are characterized by the production of IL-4, IL-5, IL-9, IL-13 and IL-21 and the absence of IFNγ and IL-17 production. Autocrine IL-4 produced by antigen-specific CD4+T cell supports Th2 cell differentiation and expansion

during *N.brasiliensis* infection (188). It has been shown that the expulsion of gastrointestinal nematodes is dependent on a T cell response. During helminth infection, Th2 cells help the activation and expansion of leukocytes primarily through the production of cytokines which is an essential function that serves to amplify and sustain the Th2 type response. Th2 cell-derived IL-4 and IL-13 contribute in a STAT6-dependent manner, which is characteristic of helminth infection, in which increased luminal fluids and muscle contractility, make the intestinal lumen an inhospitable environment for the helminth parasite. This response decreases worm viability and increases worm expulsion, rather than killing the parasite. In Schistosoma infection, the CD4+T cell response stimulates parasite development and results in an inflammatory environment that is required for translocation of helminth eggs from the intravascular compartment into the intestinal lumen (219). In the Th2 polarized environment, the granulomatous inflammation around eggs helps the parasite to complete its life cycle and the same time protects the host from severe, lethal disease. Babu *et al.,*suggested that Th2 cells primarily downregulate harmful Th1 and Th17 type inflammatory response either directly or through innate immune cell populations (220).

1.4.8 Innate effector cells in helminth infections

1.4.8.1 Eosinophils

The innate immune cells that are typically associated with Th2 type responses, eosinophils, basophils and mast cells, have an important role in anti-helminth responses. Eosinophils are equipped with receptors for cytokines, chemokines, immunoglobulin, complement and serine proteases, that enables them to be recruited into affected tissue sites and release granular contents mostly cytotoxic cationic proteins like major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN). In helminth infection, ligation of parasite specific immunoglobulins to Fc receptors is also important for antibody dependent cellular cytotoxicity (ADCC), which results in activation of eosinophils. Following helminth infection, eosinophil numbers increase in the blood and they rapidly migrate to the site of infection, where they degranulate and release secondary granule proteins (ESGPs)(221)(222)(223). In Strongyloides infection, depletion of eosinophils with antibodies specific for CC-chemokine receptor 3 (CCR3) resulted in increased susceptibility to infection (224). Reiman *et al.,* described the regulatory role of eosinophil

by production of cytokines including IL-4 and IL-13 and also to present antigen to *S.stercoralis* (225). Eosinophils also play important roles in tissue remodelling, debris clearance and mediate the wound healing responses following helminth tissue invasion.

1.4.8.2 Mast cells and Basophils

Basophils share many features with mast cells including FcRα1 expression, the TLRs-TLR2 and TLR4 and the capacity to secrete reactive oxygen and nitrogen species, Th2 cytokines and histamine (226). During helminth infection, increased numbers of mucosal mast cells are observed which is dependent on Th2 type cytokines, primarily derived from CD4+ T cells. In *H.polygyrus* infection in mice, an increased number of mast cells were observed in or near granulomas in which larvae develop (227). Karasuyama *et al.,* in their study indicated the important role of basophil in the Th2 polarization of the immune response mainly by secreting IL-4 (228). Basophils contribute to the most of the symptoms of allergic inflammation through the release of leukotrienes and histamines and play an important role in inducing class switching to IgE in B cells.

1.4.8.3 Neutrophils

Neutrophils are also activated and are recruited to sites of infection during tissue invasion by helminths (229). Galioto *et al.,* demonstrated that neutrophils play an important role in the killing of the larval stages of *S.stercoralis* (224), whereas Herbert *et al.,* in their study showed that in infection with *S.mansoni*, neutrophils had little or no effect in their larvae killing (230). Neutrophils are recognized as important cells attracted to sites of helminth invasion.Along with other cell population including eosinophils and macrophages, neutrophils can potentially directly damage tissue dwelling helminths (188). Because of their predominant phagocytic role in terms of microbial pathogens, neutrophils are often overlooked in helminth infections, although this is now being addressed in animal studies.

1.5 Schistosomiasis

Schistosomiasis or bilharzia, is a disease caused by trematodes of the genus Schistosoma. Human Schistosomiasis is caused by five species of Schistosoma: the intestinal species: *S.mansoni, S.japonicum, S.mekongi* and *S.intercalatum* and the urinary species *S.haematobium*.

Human infection is initiated by penetration of intact skin with infective cercariae. These organisms that are released from infected snails in fresh water possess anterior and vertical suckers that attach to the skin and facilitate penetration. Once in the skin, cercariae transform into Schistosomula with morphologic, membrane and immunologic changes. Schistosomula begin their migration within 2-4 days via venous and lymphatic vessels, reaching the lung alveoli and finally the liver parenchyma. Sexually mature worms then descend into the venous system at specific anatomic locations: intestinal veins in *S. mansoni, S.japonicum, S.mekongi* and *S.intercalatum* infection and in the vesical veins for *S.haematobium*. After mating, adult females travel against venous blood flow to the venous wall, where they deposit their ova intravascularly. These ova then move through the venous wall, traversing host tissues to reach the lumen of the intestinal or urinary tract and are voided with stools and urine. The ova that are retained in the tissues, are carried by the venous blood flow to the liver or other organs. Schistosoma ova that reach fresh water hatch, releasing free living miracidia that search for the snail intermediate host and undergo cycles of asexual multiplication. Finally, the infective cercariae are shed from the snails.

The global distribution of Schistosoma infection in human is mostly dependent on parasite and host factors. They are estimated to infect 200-300 million individuals in South America, the Caribbean, Africa, Middle East and Southeast Asia. Intensity of infection follows the increase in prevalence up to the age of 15-20 years and then declines markedly in older age groups. Most infected individuals harbour low worm burdens and only a small portion suffer from high intensity infection depending on the worm infectivity or to a genetic susceptibility in human populations.
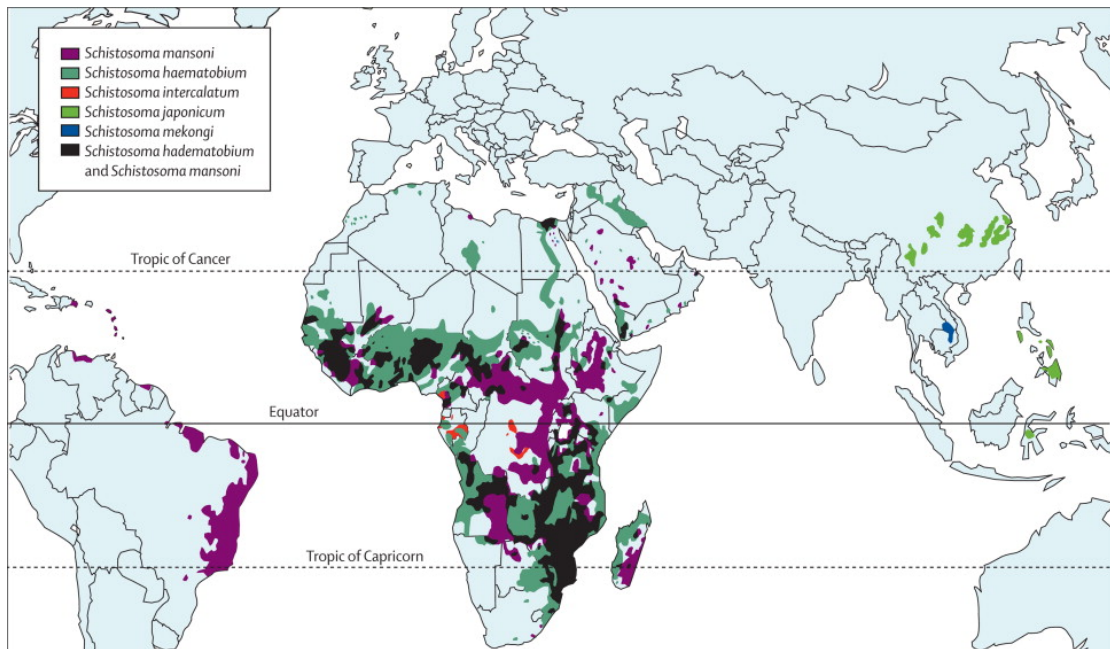
Figure 1.8: Global distribution of Schistosoma infection (2011) (231).

Adult worms residing in the venous environment are protected from immune attack. Multiple factors are important in long-term survival of the worm in the hostile host environment such as their ability to continuously regenerate their outer tegument through unique somatic stem cells (232), molecular mimicry (225), by acquiring host antigen (234)(235), isotypic shifts in antibody specificities and manipulating host immune response immunoregulation (236)(237).

The associated antigen excess results in formation of soluble immune complexes, which may be deposited in different tissues. In chronic infection, most disease manifestations are due to egg retention in host tissues. The granulomatous response around the ova is cell mediated and is regulated by a cascade of cytokine, cellular and humoral responses. Granuloma formation starts with recruitment of inflammatory cells, phagocytes, antigen-specific T cells and eosinophils followed by fibroblasts, giant cells and B cells in response to helminth antigens secreted within by the ova (238).The eggs contain a variety of proteases and toxic moieties, which when they are lodged into the tissues may lead to necrosis. The host defence comes in the form of granuloma formation, to wall off and contain the egg and the proteolytic enzymes they carry (239). The symptology of Schistosomiasis is attributed to the egg induced granulomatous inflammatory response and associated fibrosis and although these granulomas are detrimental to the infected host they also serve an important host protective-function during Schistosoma infection (240).

In areas, endemic for schistosomiasis, in the absence of intervention, this is primarily a chronic disease that can persist even for decades.This chronicity largely depends on the repeated exposure to the cercariae and the longevity of adult worms (241).

1.5.1 Immunological aspects of Schistosomiasis

Animal studies have demonstrated a moderate Th1 response to parasite antigens but there is also a robust Th2 response to egg-derived antigens that dominates and induces fibrogenesis in the liver (242)(243). In immunocompetent wild type (Wt) mice, a shift from a moderate Th1 to a robust Th2-dominant response to Schistosome antigens was observed at the onset of egg laying around 5-6weeks after infection (244). Th2 cytokines, like IL-4, IL-5 and IL-13 have long been related to fibrosis and to much of the pathology (245). In mouse study, vaccination with parasite eggs and IL-12 inhibited the Th1 to Th2 shift and resulted in hepatosplenic pathology following infection. It was also demonstrated in the study that the IL-4/IL-1 mediated STAT6 pathway plays a critical role in the development of a granulomatous response and the resulting fibrosis observed in Schistosoma infection (246). IFNγ may also contribute to granuloma formation, as it was shown that IFNγ deficient mice had reduced granuloma sizes (247).

In IL-10/IL-4 double knockout mice, with polarized Th1 responses, immunization with soluble egg antigen (SEA) in complete Freunds Adjuvant (CFA) also skews the T-cell response, resulting in increased inflammation, larger granuloma, extensive pathology and severe morbidity (248).

Deposition of fibrotic material in the extracellular matrix results from the interaction of T lymphocytes with fibroblasts and elevated levels of cytokines like IL-2, IL-4, IL-1 and TGFβ, IL-5, IL-10 and IL-13, whereas higher IFNγ levels or IL-12 may play a role in modulating the fibrogenesis (249)(250)(251)(252). Maintaining a balanced and controlled Th1 and Th2 response is critical for protective granuloma formation without excessive pathology (244).

Egg deposition in the liver also increases the number of AAMs (253). Mice that were deficient in macrophage specific IL-4 signalling or in IL-10/IL-4 double KO mice, failed to induce arginase expression, suggesting the requirement for Th2 cytokines in arginase production following Schistosoma infection (230)(254). Th1 skewed mice displayed

enhanced iNOS responses that were associated with smaller granuloma sizes and accelerated mortality (254). Lie *et al.,* have demonstrated that Fizz1 expression may be important in fibrogenesis, fibroblast differentiation, increased expression of α-smooth muscle actin and type 1 collagen, as well as also having some anti-inflammatory properties suppressing antigen-specific T cell proliferation in Schistosoma infection *in vitro* (255). Macrophages in Schistosoma infection, play a dual role as an anti-inflammatory cell as well as by sequestering egg products and helping development of granuloma followed by adopting an anti-inflammatory role during chronic infection and by indirectly suppressing the function of other cells like T cells and B cells and decreasing the granuloma size (244).

Newly formed liver granulomas surrounding entrapped eggs decrease in size as the infection progresses into the chronic stage. This 'endogenous desensitization' is the hallmark of granulomatous infection and is critical for host survival in persistent diseases. IL-13Rα plays an important role in granuloma downregulation. *S.mansoni* infected mice that are IL-13Rα2$^{-/-}$ fail to undergo this granuloma downregulation. Their granuloma size continues to increase as the infection becomes chronic. This defect in granuloma modulation suggests that control of IL-13 is required for survival during chronic infection with *S.mansoni* (256)(257).

Regulatory T cell (Tregs) involvement in chronic Schistosoma infection, is related to dampening antihelminth effector responses, allowing chronic infections to develop (258). In Schistosoma infection both inducible and natural Tregs play an important role in suppression of DC cell activation, the organizing and regulation of Th2 effector responses, granuloma development and fibrosis.CD4+CD25+Foxp3+Tcells show prominent expansion especially in liver and spleen at 4 weeks post infection with *S.mansoni* (259). The highly immunogenic SEA may be the main potent inducer of both effector and Tregs cell during infection (260).

IL-10 plays an immunoregulatory role during *S.mansoni* infection and critically regulates the liver pathology. The majority of the IL-10 producing T cells appear to be FoxP3+ and constitute inducible Treg cells and/or Th2 cells (154). Hesse *et al.,* demonstrate that CD4+ cells produce a significant proportion of the IL-10 in *S.mansoni* infection(261). However, non CD4+ derived IL-10 is also an important source of IL-10. IL-10 is very important for the control of DC derived IL-12, generation of Th1 responses during

infection, and also to skew the T cell response towards a Th2 type (262). IL-10 secreting CD4+CD25+T cells were also isolated from the granuloma of chronically infected mice, that could suppress the proliferation of naïve CD4+T cells.The accumulation and suppressive properties of these cells isolated from the liver of infected mice suggested that the roles of Treg cells actively migrate to the inflammatory site to regulate the development of liver granuloma in Schistosoma infection (259)(262).

However, apart from Treg–mediated suppression of T cell function and proliferation in Schistosome infection, Tregs cells may also influence the function of macrophages, CD8+ cells, B cells and eosinophil recruitment. The precise mechanism of regulatory T cells mediated suppression during Schistosoma infection is still unclear (244).

A number of studies have suggested that worm death occurring either naturally or upon treatment, leads to the release of immunogens that stimulate protective responses depending on the exposure histories, different source of transmission, etc. (236)(263)(264)(265). Resistance to reinfection with *S.mansoni* is mostly Th2-associated. An association between eosinophils and parasite-specific IgE was observed in studies of Schistosoma reinfection. Both high and low affinity IgE receptors on eosinophils and B cells were associated with protection against reinfection. Susceptibility to reinfection was also associated with IgG4, which serves as a blocking antibody and inhibits the action of IgE.

1.5.2 Clinical features

During the phase of cercarial invasion, a form of dermatitis called Swimmers' itch is observed in infection with *S.mansoni* and *S.japonicum*. This symptom develops usually in the first 2 or 3 days after invasion as an itchy maculopapular rash on the skin of the affected areas. Cercarial dermatitis is self-limiting. During worm maturation and deposition, (4-8 weeks after skin invasion), acute schistosomiasis or Katayama fever-with serum sickness like symptoms develops. This also presents with fever, generalized lymphadenopathy and hepatosplenomegaly. Individuals with acute Schistosomiasis show elevated levels of eosinophils and parasite specific antibodies may be detected at this stage.

The clinical manifestations of chronic Schistosomiasis are species dependent and may begin after few months of infection, lasting for years. Intestinal species cause intestinal

and hepatosplenic disease. During the intestinal phases, symptomatic patients have colicky abdominal pain, bloody diarrhoea and anaemia. The severity of infection depends mostly on the intensity of the helminth burden.

The clinical manifestation of *S.haematobium* appears early and involves a high percentage of infected individuals. The symptoms manifests as terminal haematuria and dysuria. The manifestation correlates with intensity of infection and the presence of urinary granulomas. In many endemic countries, an association between squamous cell carcinoma and *S.haematobium* has been observed.

1.5.3 Diagnosis

Individuals with Schistosoma infection are diagnosed by endemic country travelling history, characteristic clinical presentation, and presence of Schistosoma ova in urine or faeces, detecting eggs in rectal biopsy or bladder mucosal biopsy. Filtration techniques like Kato-Katz thick smear, Nucleopore filtration etc are used for detecting and quantifying Schistosoma eggs. These methods may provide quantitative data on the intensity of infection, which is important in monitoring tissue damage and effect of chemotherapy. Serological tests using Schistosoma antigens like SEA, AWA antigens are also used for laboratory diagnosis.

1.5.4 Treatment

Treatment of Schistosomiasis depends on the stage of infection and the clinical presentation. The drug of choice is praziquantel that is administered per orally as a total of 40 to 60mg/kg in two or three doses over a single day. Praziquantel treatment results in parasitological cure in 85% of cases and reduces egg counts by >90%. The effect of antischistosomal treatment on disease manifestations varies by stages of the disease. A follow up visit with a health care worker is advised, if there is a history of exposure. Several control measures have been proved to be useful such as application of molluscicides, provision of clean water, sewage disposal, chemotherapy and health education.

1.6 Strongyloidiasis

Strongyloides spp is a nematode that is unique among other helminths in being able to replicate in the human host. This unique capacity permits ongoing cycles of autoinfection as infective larvae are produced internally. Strongyloides spp thus can persist for decades in human hosts without further exposure of the host to endogenous infective larvae(266). The estimated prevalence of Strongyloidiasis has been estimated to be around 370 million infections worldwide (267).

1.6.1 Epidemiology

S.stercoralis is distributed in tropical areas and hot, humid regions of sub-Saharan Africa, Brazil, The Caribbean, Latin America and Southeast Asia. The parasite is found in USA mostly in immigrants, refugees, travellers and military personnel who have lived in endemic areas. A study conducted in the USA, observed that about 46% of 462 Sudanese and 23% of 100 Somali Bantu refugees were infected with *S. stercoralis*(268). This highlights the potential hazards of the transportation of this parasite across borders in mass population movement (269). *S.fuelleborni* can cause human infection and is mainly found in Africa and Papua New Guinea (270). Humans normally acquire Strongyloidiasis when filariform larvae in faecally contaminated soil penetrate the skin or mucus membranes.

Predisposing factors for Strongyloidiasis are living in an endemic region, chronic malnutrition,malignancies, organ transplantation, diabetes mellitus, chronic obstructive pulmonary disease (COPD), alcoholism, chronic renal failure and breast milk from an infected mother (266)(271).

1.6.2 Life cycle

Strongyloides spp have a parasitic cycle of development and can also undergo a free living cycle of development in the soil. Rhabditiform larvae passed in the faeces can transform into infective filariform larvae either directly or after a free living phase of development. The larvae after entering through skin and mucus membranes, travel through the bloodstream to the lungs and alveolar spaces, then they ascend the bronchial tree, are swallowed and then travel to the small intestine. There the larvae mature into adult worms that penetrate the mucosa of the proximal small bowel. The

adult worms reproduce by parthenogenesis. Egg hatch in the intestinal mucosa and release rhabditiform larvae that migrate to the lumen and pass out through the faeces. Alternatively, rhabditiform larvae in the bowel can develop directly into filariform larvae that penetrate the colonic wall or perianal skin and enter the systemic circulation to establish the internal reinfection. This autoinfection cycle allows the nematode to persist for decades (270)(271)(272).

1.6.3 Clinical manifestations

The spectrum of Strongyloidiasis includes acute infection with Loeffler's syndrome, chronic intestinal manifestations, symptomatic autoinfection and hyperinfection with dissemination (273)(274) .

In uncomplicated Strongyloidiasis, many patients are asymptomatic or have mild cutaneous and/or abdominal symptoms. Recurrent urticaria is the most common cutaneous manifestation. Migrating larva can elicit a pathognomonic serpiginous eruption called "larva currens". Adult parasites burrow into the duodenojejunal mucosa and can cause abdominal pain (which resembles peptic ulcer pain), discomfort, bloating, nausea, vomiting, diarrhoea, anorexia etc. Pulmonary symptoms are rare in uncomplicated infection, although intermittent eosinophilia is very common (272).

Hyperinfection syndrome is usually observed in patients who are on corticosteroid therapy or co-infected with Human T Cell Lymphocytic virus 1 (HTLV-1). This invasive form is characterized by a spectrum of gastrointestinal complaints including abdominal pain, dyspepsia, diarrhoea, constipation, intestinal obstruction, enteritis and/or gastrointestinal bleeding. Many patients have worsened pulmonary symptoms like haemorrhagic pneumonitis, respiratory failure, etc.(275). Invading larvae can be transported to the blood and reach the peritoneum, liver, kidney and central nervous system, causing meningitis. In immunosuppression and in HTLV-1 co-infection induces strong Th1 responses and weakens Th2 responses, which leads to increased secretion of type 1 cytokines and decreases in type 2 cytokines. Decreases in IL-5, IgE, diminished mast cell function and reduced eosinophil recruitment all together cause inadequate host responses, which may predispose the co-infected patients to greater parasite burdens, organ damage, bacteraemia and death (274).

1.6.4 Immunopathogenesis

Animal studies have suggested a role for innate and acquired/adaptive immune mechanisms in mediating resistance to Strongyloidiasis. The innate response is primarily mediated by eosinophils and IL-5, with neutrophils and macrophages playing supplementary roles. Messias *et al.,* have shown that in Strongyloides infection human peripheral blood mononuclear cells and neutrophils adhere to *S.stercoralis* larvae following complement activation, but although this could result in decreased motility, it was not sufficient for larval killing (276). However, in *vitro* studies have shown that, when human PBMC or macrophages are complemented with neutrophils, they can kill the larvae, both in humans and mice. Parasite killing was also observed in mice, when BMDM were cultured with PBMC and neutrophils; complement component C3b was observed to be required for larval killing during the primary and secondary immune responses (277).

Complement dependent adherence of macrophages and neutrophils to the larval surface may stimulate the release of cytokines or soluble factors. Neutrophil derived myeloperoxidase and eosinophil derived major basic cationic protein have been shown to be required for cells to kill the parasite (278).

It is hypothesised that the parasite can trigger a potent immune response in the gut that stimulates activated GI-associated DCs to migrate through lymphatic ducts to stimulate Th2 cells and regulatory T and B cell responses in the draining lymph nodes (279).

The adaptive immune response to Strongyloidiasis specifically involves type 2 responses, with Th2 cells secreting Il-4, IL-5 and IL-13, B cells producing IgG and IgE and innate lymphoid cells secreting IL-5 and IL-13 (280). Eosinophils may serve as an antigen presenting cell and are required for an optimal antibody response in Strongyloidiasis. Strongyloides infection can produce a high level of eosinophilia, mucosal mastocytosis and control of Th1 mediated excessive inflammatory reactions. Strongyloides infection can also stimulate Tregs, cells that reduce injurious host inflammatory and immune responses through cell to cell contact, inhibitory cytokines and/or cytokine deprivation. This prevents the immunes responses from causing bystander tissue damage during the host response. Tregs may also blunt Th2 responses such as the IL-5 dependent eosinophil

activation required to kill the parasite. Interplay between Th1, Th2 and regulatory T cell responses appears to be crucial in the immune defence against Strongyloidiasis (281).

Studies have shown that Th2 responses play an important role in the hyperinfection syndrome and individuals with Strongyloidiasis may also develop specific antibodies of IgG, IgA and IgM and IgE isotypes (281)(282). Brigandi *et al.,* showed that IgM and not IgG is protective at 1 week after booster immunization, whereas parasite-specific IgM and IgG are both protective 3 to 5 weeks after booster immunization (283)(284). Neutrophils have been shown to collaborate with IgG and complement in killing larvae. Yung *et al.,* demonstrated that IgM mediated killing of parasites requires the interaction between the IgM on the surface of the parasite and its receptor on the macrophages, activating the cells to release toxic molecules to kill. Also the capacity of IgM to activate the complement system by cleaving C3 may also play an important role in parasite killing (285).

AAMs are associated with Strongyloides infections, these function in modulating the Th2 responses, reduce pathology by controlling fibrosis and also play an important role in wound healing following parasite mediated tissue destruction (286). It was reported in earlier studies that AAM had no role in killing helminths although they have shown to take part in worm expulsion in other nematodes (287). Sandra *et al.,* demonstrated that AAM, but not CAM generated *in vitro* were capable of killing *S.stercoralis* larvae in vivo during primary and secondary immune responses. They have also shown that macrophage and neutrophil function in killing is restricted to BMDM and AAM and that CAM inhibit parasite killing by neutrophils (288). The highly motile larvae of *S.stercoralis* induced a systemic immune response following a subcutaneous injection in presence of AAM in the peritoneal cavity, indicating that AAM are found and maintained in the peritoneal cavity. Polarization of AAM in the peritoneal cavity was also dependent on IL-4/IL-13 signalling like in other helminth infections (289)(290). The signature markers of AAM-Relm-α, Arg-1 and Ym-1 were also studied for their role in Strongyloidiasis. The expression of Arg-1 and Relm-α was not required for parasite killing, but Relm-β was observed to significantly inhibit the chemotaxis of *S. stercoralis* larvae *in vitro* (291).

Rajamanickam *et al.,* examined the systemic cytokine levels of type 1, type 17 and pro-inflammatory cytokines in Strongyloides infection and observed that *S.stercoralis*

infection was associated with diminished proinflammatory cytokine responses and that helminth treatment showed an impact as the majority of these proinflammatory cytokines exhibited enhanced concentrations following treatment. Strongyloidoses was associated with profound alterations of systemic cytokine responses- the finding of which may relate with the auto-infective cycle seen in the nematode (292). ES products of *S.stercoralis* may also help to disseminate the parasites and contribute to the systemic cytokine responses (293). As the larvae migrate from lungs to intestine and propagate their life cycle in the intestine, a local immune response at these various sites may also contribute to the altered systemic responses in Strongyloides infection (292). Toulza *et al.,* investigated the potential impact of helminth infection on immune response to *Mtb* in UK migrants who were LTBI patients with or without helminth infection.Patients with helminth infection showed a significant increase in CD4+FoxP3+T cells (Tregs) compared to those without helminth infection and there was a decrease in the frequency of Treg cells, and an associated increase in CD4+IFNγ+T cells after the anthelmintic treatment showing the potential role of Tregs in the immunopathogenesis in these patients (294).

1.6.5 Diagnosis

In uncomplicated Strongyloidiasis, detection of rhabditiform larvae in faeces is diagnostic- either by direct smear or formal-ether concentration technique. Rhabditiform larvae are 250μm long with a short buccal cavity that distinguishes them from larvae of hookworm microscopically. Serial stool examination increases the sensitivity of the detection. Charcoal agar media is used to detect Strongyloides larva. Strongyloides larvae can also be detected by sampling of the duodenojejunal contents by aspiration or biopsy. An enzyme linked immunosorbent assay (ELISA) for serum antibodies to antigens of *S.stercoralis* is a sensitive method of diagnosing uncomplicated infection (272). Sultana *et al.,*examined Strongyloidiasis in Bangladesh, using serological and faecal examination and observed that in stool specimens, larvae was detected in 23.1% patients by Harada-Mori culture, 10.2% by agar plate culture and 0.7% by microscopy. They also detected Strongyloides specific IgG, IgG1 and IgG4 in 61.2%, 31.3% and 36.1% respectively of patientsby the ELISA method (295). Molecular methods like polymerase chain reaction (PCR) is now also being used for diagnosing Strongyloides infection. In disseminated Strongyloidiasis, filariform larva can be detected in stool and also from the sites of

potential larval migration, including sputum, broncho-alveolar lavage fluid or surgical drainage fluid (272).

1.6.6 Treatment

Even in asymptomatic Strongyloidiasis, it is recommended to treat patients to prevent the subsequent fatal hyperinfection syndrome. Ivermectin (200µg/kg daily for 2 days) is more effective than albendazole (400 mg daily for 3 days). For disseminated cases, the dose for ivermectin is prolonged to 5-7 days or until the parasite is completely eradicated from the body (272).

1.6.7 Prevention

Strongyloidiasis is an emerging global infection. This infection remains an important helminth disease due to increases in travel, migration to endemic and non-endemic countries, lack of adequate sewage disposal system especially in endemic countries and the risk of autoinfection which can lead to persistent disease for years. Patients with travel history to endemic countries, persistent eosinophilia, or signs of pulmonary and gastrointestinal symptoms in immunosuppressed patients should be followed up and early diagnosis is very important to manage this neglected helminth disease. Appropriate practical preventive measures such as health education campaigns on the disease, proper sanitation, regular de-worming, behavioural change through proper disposal of faecal waste and the use of protective foot-wear approaches may reduce the prevalence of Strongyloidiasis.

1.7   Control strategies for soil transmitted helminths (STHs)

The multicomponent integrated control strategy that lead to reductions in STHs includes integrated chemotherapy with anthelmintic drugs, improvement in water, sanitation, and hygiene (WASH), vaccines and vector control strategies.

Chemotherapy is the principal way to achieve rapid, substantial reductions in STH prevalence and intensity through rapid parasite clearance from the human host (296)(297). WASH is an potential means of breaking the STH transmission cycle, although achieving adequate and sustained WASH is a complex and expensive long term objective in the endemic and poorer countries (298).

WHO's strategy for control of STHs is to prevent and control morbidity through periodic treatment of people at risk, such as pre-school aged children, school aged children and women of child bearing age (including pregnant women in the second and third trimesters and breast feeding women) (299). The control strategies include:

- periodic drug treatment (deworming) to all the children living in the endemic countries, once a year in the countries where prevalence of STH infection is over 20% and twice a year when the prevalence of infection intensity in the community is over 50% to reduce the worm burden.
- Health and hygiene education to reduce transmission and reinfection by encouraging healthy behaviour.
- Provision of adequate sanitation.

Four drugs- albendazole, mebendazole, pyrantel, ivermectin are on the WHO model list of essential drugs for STH control (300). The bezimidazoles (albendazole and mebendazole) have been administered to millions of people and usually as a single dose in school children in national helminth control programmes (301). The efficacy however, varies by drug, dose, helminth species, age and intensity of infection. Vercruysse *et al.,* reported the cure rate of albendazole for *A.lumbricoides*, hookworm and *T.trichiura* are 98%, 47% and 8% respectively (296). There are varied reports of their effect on improvements in haemoglobin, weight, height, appetite, cognitive ability, physical fitness and activity levels following chemotherapy in pre-school and school-aged children (302)(303)(304)(305)(306). Improvement in maternal haemoglobin levels, infant birth weight and reduction in infant mortality at 6 months of age was observed when pregnant women were treated for STH infection (307)(308). However, the new Cochrane review concluded that there is substantial evidence that mass treatment of all children in endemic areas does not improve average nutritional status, haemoglobin, cognition. school performance or survival (309).

For schistosomiasis, the WHO recommends treating school-aged children and other at-risk populations with praziquantel, with frequency of administration varying with prevalence of infection.There are two Cochrane reviews of randomized control trials (RCT) of mass drug treatments for schistosomiasis (primarily using praziquantel, though occasionally metrifonate or oxamniquine). They both conclude that praziquantel is effective in treating schistosomiasis (310).

Chemotherapy has disadvantages as well. As treatment cannot prevent reinfection. WHO advocates repeated rounds of chemotherapy, even in areas where chemotherapy programs have ceased infection and/or intensity of infection. Drug resistance emergence can be another outcome after the mass chemotherapy of parasites (311). Reduced efficacy of some of the anthelmintic drugs such as albendazole, mebendazole and pyrantel has been reported. Reduced efficacy might result in from poor drug quality, reduced absorption, parasitological examination performed too early after treatment or poor strain susceptibility (312). Recent mathematical and statistical modelling studies indicate that chemotherapy of school children may break STH transmission cycles in specific epidemiological settings, specially in low transmission settings with strong health systems and drug delivery mechanisms, whereas in highly endemic countries, high coverage, high frequency and broader community based treatment with additional WASH efforts may be needed (313).

WASH is the provision of access to a safe water supply, appropriately conducted sanitation, infrastructure ensuring safe disposal of human excreta, health education and promotion of hygiene.In a RCT, that investigated a school-based integrated WASH program plus albendazole, a significant reduction in *A.lumbricoides* reinfection was observed among school children who received the intervention compared with albendazole alone (314). Improving WASH is enormously challenging as financial, logistic difficulties may impair the process and this may also be subject to culture and environmental influences.

Multicomponent integrated control may augment drug-based integrated chemotherapy. The Chinese approach with chemotherapy, WASH, agricultural mechanization and fencing of water buffaloes has been reported to reduce Schistosoma infection, *S.japonicum*-infected snails and STHs (315). The SAFE (surgery, antibiotics, facial cleanliness, environmental improvements) strategy of the Trachoma elimination programmes and treating animals to control transmission of *Trypanosoma rhodesiense* are also examples of multicomponent integrated NTD control (316).

In large parts of Africa, most children are affected with more than one species of STH. These frequently overlap geographically and impose a great burden on poorer communities.The four major NTDs targeted by WHO through preventive chemotherapy are for Schistosomiasis, STH, onchocersiasis and lymphatic filariasis. In Cameroon,

considering the high prevalence and spread of these diseases and their geographical overlap, an efficient and cost effective integrated control of NTD is being practised. Ivermectin and albendazoleare co-administered during the campaign implemented once per year allowing an integrated treatment of onchocerciasis, LF and STH. Secondly they co-administer mebendazole and praziquantel in school-based deworming campaigns and implement the program annually. In school children aged 1-5 years during child health week campaigns, albendazole and mebendazole are administered and the campaign is implemented twice a year at 6 monthly intervals and targets children up to 5 years (317). In Bangladesh, such interventions are recommended by WHO and UNICEF. Deworming tablets (albendazole) are given every six months to children aged 24 to 59 months (318).

There are still gaps in evidence and challenges in establishing sustainable benefits in health outcomes from STH chemotherapy programs or WASH and other integrated approaches for STH control, as there is a lack of outcome data for STH chemotherapy and WASH. For chemotherapy, research into developing new and effective drugs against STH control is still a priority area. Integrated chemotherapy and control for STH infection requires considerable national and international scale-up funding, drug donations, human and other resource deployment, enhancing diagnostic capabilities and surveillance systems to contribute data, in-country technical expertise and political and programmatic will.

1.8 Helminth –*Mycobacterium tuberculosis* Co-Infection

Tuberculosis and helminth infections are co endemic in many parts of the world, and this geographical overlap has led the hypothesis that helminth infections could also exacerbate the effects of *Mtb* infection. It has been suggested that, by creating an anti-inflammatory environment,helminth co-infection might dampen both protective and immunopathological responses to *Mtb*, and thus alter the TB epidemic and disease severity in endemic countries. Helminth infection induced Th2 and Treg responses may contribute to depressed *Mtb* specific T cell responses, although the precise mechanism is still unknown.

Helminth and *Mtb* co-infection is an issue of particular significance in resource poor environments where co-infection with these pathogens frequently occurs. In such communities, both helminth and TB infection have a tendency to cluster within certain

households or families. Helminths are reported as the most common parasites found in TB patients and TB is also most prevalent in communities where people are helminth infected (319).
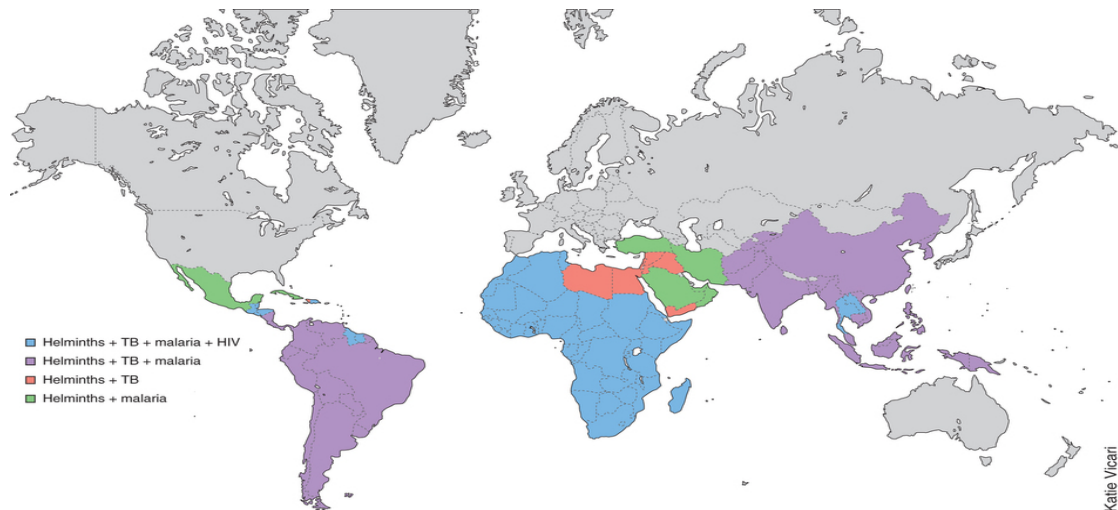
Figure1.9:   World map showing the geographical distribution of co-infection with helminths and tuberculosis (2013)(320).

In this map, it is evident that in Sub-Saharan Africa, there is an extensive overlap of the presence of helminths and TB. In the co-infected patients, there may be conflicting effector cell responses for their control and regulation. It is plausible that, host immune responses to TB are modulated by co-infection with helminths and that helminth infection may potentially impair vaccine efficacy (205)(321).

The larvae of STHs migrate through the lungs in their life-cycle. The localized helminth induced pulmonary response may therefore directly influence the outcome of a concurrent infection with *Mtb* in addition to the systemic effects.

1.8.1 Potential mechanisms of helminth-induced modulation

The immune response induced by most helminth infections is characterized by a type 2 innate and Th2 skewed immune profile, chronic immune activation and regulatory T cell activity. This may modulate the T cell response to effect influence on *Mtb* infection and disease through a variety of mechanisms.

The Th2 immune response in helminth infection may modulate immunity to TB as the Th2 cytokine milieu in the lung facilitates the development of AAMs. CAMs and AAMs induce reciprocal inhibition of iNOS and arginase through competition for the common

substrate L-arginine. M2 macrophages secrete the anti-inflammatory cytokines IL-10 and TGFβ and also have higher arginase 1 levels which promotes wound healing and mediates resistance to helminths (322). These macrophages may impair the immune responses to TB and bacterial control through an IL-4R dependent process. Potian *et al.,* reported that *Nippostrongylus brasilensis* induced Th2 response can enhance intracellular persistence of *Mtb* by mediating the induction of M2 macrophages via the IL-4Rα signalling pathway. Co-infected mice lacking IL-4Rα exhibited an improved ability to control *Mtb* infection and accompanied by a significant reduction in M2 macrophage accumulation. However, the Th2 response in the co-infected animals did not impair the onset and development of the protective *Mtb* specific Th1 cellular immune response (323).

Another mechanism for the downregulation of Th1 responses and cytotoxic T cell responses to *Mtb* is the ability of helminth infection to target the classical activation of DCs. Exposure of DCs to helminth ES products fails to upregulate the surface expression of costimulatory molecules such as CD40, CD80 and CD86 and does not result in the synthesis of proinflammatory cytokines and chemokines such as IL-12, CCL2 (MCP-1) and TNFα (324)(325). After helminth exposure, DCs promote the differentiation of Tregs from naive T cell precursors or the further expansion of a pre-existing Treg subpopulation (326)(327). DC production of TGFβ and /or IL-10 in response to helminth E/S products may be one of the potential mechanisms leading to expansion of Tregs and subsequent down-regulation of Th1 immunity during TB. Talaat *et al.,* reported that exposure to *B.malayi* changes the surface expression of adhesion and costimulatory molecules on macrophages and DCs alter their production of cytokines and chemokines making them less capable of inducing Th1 immune responses (328). Following exposure to microfilaria, there may be down-regulation of the DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) lectin receptor, the receptor which is used by *Mtb* to enter DCs. Concurrent helminth infection may possibly reduce susceptibility of these DCs for infection by *Mtb*. Helminths are also potent inducers of regulatory DCs (DCregs) which are characterized by the expression of IL-10, TGFβ, indoleamine 2,3-dioxygenase (IDO) and cyclooxygenase 2. Possibly these DCregs down-regulate protective Th1 responses to *Mtb*, but this requires further investigation (329).

A number of studies have shown that in mouse models of infection with filarial parasites, there was a persistent type 2 immune response. Sacco *et al.,* showed that a dominant Type 2 response to *Mtb* antigens followed *Mtb* infection (330). Mice infected with *N. brasiliensis* or *S.venezuelensis* also exhibited impaired bacterial growth control and higher bacterial loads in the lungs (323)(331).

1.8.1.1 Th1/Th2 shift

Mutual cross-regulation between the Th1 and Th2 arms of the immune system and the balance between Th1 and Th2 cytokines determines whether the immune response will result in successful elimination or control of an invading pathogen. Early progression of latently *Mtb* infected household contacts of TB diseases to active TB disease was associated with increased Th2 and Treg cytokines such as IL-4 and IL-10 (332). It was also observed that progression towards active TB was associated with reduced Th1 and increased Th2 responses in vivo. TB patients presented with a markedly increased Th1/Th2 (IL-4/IFNγ) mRNA ratio compared to uninfected controls and the presence of Th2 cytokines IL-4 and IL-10 correlated with progression to active TB (333). A study conducted in Ethiopia demonstrated that PBMCs from *S.mansoni* infected individuals displayed poor responsiveness to mycobacterial antigens, characterized by impaired *Mtb* specific IFNγ production and reduced T cell proliferation compared to dewormed controls (334).

Most helminths release large amounts of antigenic material, leading to chronic immune activation, but as the burden or time after infection increases, the worms seem to modify and down-regulate these responses to survive. This chronic immune activation could potentially exhaust the local immune potential of the host, leading to the hyporesponsiveness and anergy that accompany helminth infections. These changes have been reported to revert to normal levels following clearance of the helminth infection (335)(336). Helminth-induced immune hyporesponsiveness may also be explained by IL-10 and TGFβ cytokine production during chronic helminth infection, which mediates antigen-specific hyporesponsiveness and also by impaired signal transduction.

Th17 are another important effector lineage that plays a vital role in protective immunity to mycobacterial infections. IL-17 and IL-23 have been observed to be essential for an accelerated response to limit early bacterial growth and to establish Th17 in the lung

during TB infection. Metenou and colleagues reported that co-infection with filarial worms robustly inhibited mycobacterial-specific IL-17 and IL-23 cytokine responses in *Mtb* infected individuals (337).This downmodulation was partly mediated through the increased expression of the negative costimulatory molecules-cytotoxic T lymphocyte antigen (CTLA)-4 and programmed death (PD)-1 expression. It has also been speculated that increased evidence of TB following helminth infection may be partly due to inhibition of IL-17 production by helminth induced CD4+IL-4+memory T cells (338)(339)(340), which presumably downregulates the expansion of Th1 cells, via a Th1-Th2 crosstalk. Filarial infections also have the additional effect of down modulating the expression and function of TLRs, especially TLR-2 and TLR-9.
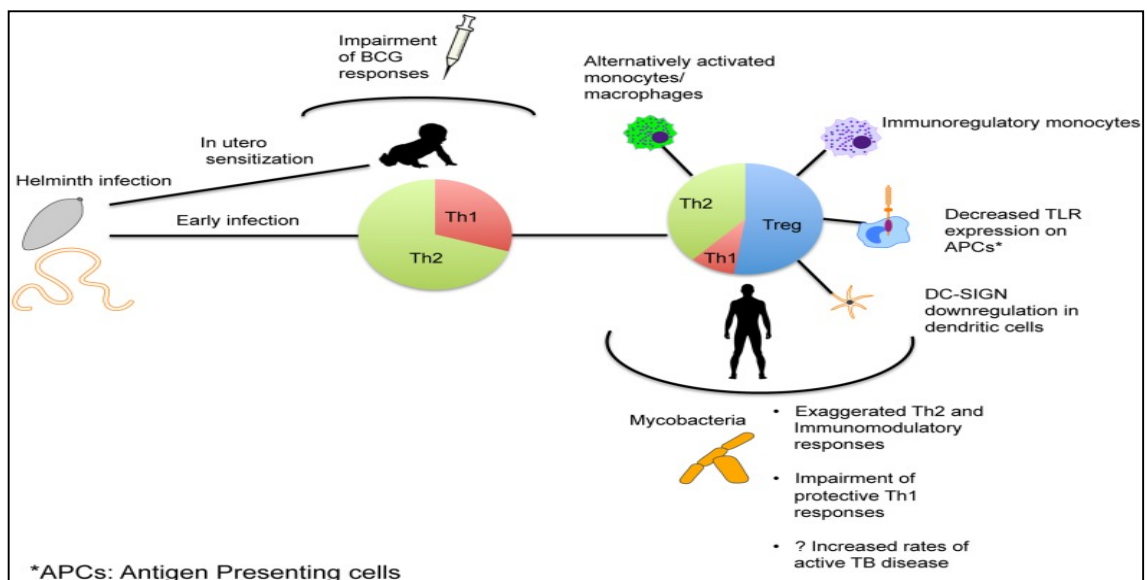
Figure 1.10: Proposed mechanism of immune modulation caused by helminth infections affecting immune responses and susceptibility to TB (341).

1.8.1.2 Regulatory T cells (Tregs)

Helminth driven Treg responses may have an impact on the host's ability to cope with a concurrent *Mtb* infection, benefiting pathogen survival by down-regulating protective Th1 immunity. Increased Treg frequencies and levels of IL-10 and TGFβ mRNA are present in TB patients and both these cytokines are potent inhibitors of *Mtb* specific Th1 responses (326).

1.8.2 Effects of helminth infection on diagnostic tests for LTBI

Helminth infection plays a role in modulating immune response to *Mtb* PPD and other *Mtb* antigens. This immunomodulation may indirectly affect the diagnostic tests used for LTBI. Stewart *et al.,* examined the immune responses of children exposed both to mycobacterial infection and *Onchocerca volvulus* (with microfiladermia) in Cameroon and found that the proliferation of PBMC in response to stimulation with adult female parasite antigen (OvAg) and PPD was down-regulated with increasing intensity of skin MF (342). A similar finding was observed with PPD immunisation of *Brugia malayi* infected mice that exhibited skewed PPD-specific responses towards type 2, but with no change in IFNγ production (343). Onchocerciasis has also been shown to modulate delayed hypersensitivity to tuberculin skin testing in adults in Mali and Chad (344)(345). Thomas *et al.,* conducted a community-based study in Bangladesh, which compared TSTs with QFT-ITs for detection of LTBI among 302 children and revealed a high proportion of indeterminate QFT-GIT results. Risk factors associated with an indeterminate QFT-IT result in this cohort included malnourishment and the presence of infection with *A.lumbricoides* and/or *T.trichiura* (346). Perry *et al.,* screened adult US refugees from TB endemic countries to evaluate cytokine responses in LTBI withconcurrent *Helicobacter pylori* and helminth infection (347). It was observed that, Helicobacter infection was associated with enhanced Th1 type responses to *Mtb* antigens even in the presence of concurrent helminth infection. A cross sectional study in South India was conducted, where individuals aged from 6-65 years were screened for intestinal helminth infection, circulating filarial antigenemia, tuberculin reactivity, active TB and BCG vaccination status. Intestinal infection was not associated with diminished frequencies of PPD positivity, suggesting in this setting that pre-existing intestinal helminth infection or filarial infection does not influence the delayed type hypersensitivity to tuberculin significantly (348).

Helminth infections thus appear to influence the reactivity to *Mtb* antigens, that forms the basis of diagnosis of LTBI in at least some settings. The discrepancy in the results of TST or IGRA may also vary according to the helminth species involved, the methods used for diagnosing the helminth infection or cut-off used for skin test reactivity and due to differences between geographic areas (135).

1.8.3 Effect on progression towards active TB

Helminth infections are known to modulate adaptive immune responses to TB antigens in both latent and active TB and to affect the clinical progression of the disease (349). George *et al.,* in their study described a profound modulatory function of *S.stercoralis* infection on the systemic pathological responses that characterize disease activity and severity of pulmonary TB, an indication that helminth co-infection might dampen the severity of TB disease (350). Co-incident *S.stercoralis* infection in acute pulmonary TB patients was associated with significantly decreased levels of acute phase protein, matrix metalloproteinase, tissue inhibitors of matrix metalloproteinase, the systemic immune activation marker sCD14 and sCD163, all changes specific to ATB. However, animal models of mycobacterial challenge using different helminth species have not provided consistent results. Hübner *et al.,* carried out a study in white cotton rats with or without filarial nematode that were challenged with *Mtb* and after 2 months, the immunological responses, histology and mycobacterial cultures were assessed (351). It was observed that *Mtb* loads and occurrence and size of the granulomas were not increased in co-infected animals suggesting that chronic filarial infections do not exacerbate *Mtb* infection in the cotton rat model (351). Studies in mice showed that the presence of the intestinal helminth *N. brasiliensis* had no effect on the *M.bovis* bacillary load at either 4 or 12 weeks post infection compared with uninfected controls (352).

The different studies that have examined the interaction between helminth infection and TB have been largely cross-sectional and have shown conflicting results, which might be due to ethnic or geographical differences in helminth species or to the limitations of observational cross-sectional study design. Hospital-based studies, conducted in Brazil and Ethiopia, showed a higher prevalence of active TB disease in helminth infected individuals. There was one study in Uganda, that has prospectively examined rates of development of active TB in an HIV-helminth infected cohort which demonstrated an association between having *S.mansoni* infection and developing active TB, though no such association was observed between the presence of intestinal helminth infection and TB incidence rate (353). Chatterjee *et al.,* followed a cohort in South India prospectively over an 8 year period to assess the role of coincident helminth infection on the incidence and severity of adult pulmonary TB (APT) and assessed the smear positivity, bacterial load, PPD responses; they demonstrated that despite the immunomodulatory effects of helminth infection, baseline comorbid infection with these parasites had little effect on

clinical progression from latent TB to active TB (341). In contrast, Elias *et al.,* demonstrated that intestinal helminth infection may be one of the risk factors for the development of APT. They determined the prevalence of helminth infections in active TB patients and their household contacts in a study conducted in North West Ethiopia. They evaluated the association between the number of helminth species a person carried and active TB and showed that the odds of being an active TB patient increased progressively with the number of species of helminth the person carried.In this study a significant association with active TB was found for *A. lumbricoides*, hookworm and *S. stercoralis* (354). Resende *et al.,* showed similar results (355). In their study, the co-infected patients showed depressed anti-*Mtb* immunity and also showed more severe radiological pulmonary disease, indicating that concomitant intestinal helminth infection in patients with newly diagnosed TB skews their cytokine profile toward a Type 2 response, which could potentiate persistent *Mtb* infection and more severe disease progression (355). In contrast, Abate *et al.,* indicated that in patients with asymptomatic helminth infection, active TB was associated with lower sputum positivity, leading to the conclusion that there was a beneficial effect of helminth infection on bacterial burden (356). The influence of helminth infection on the development of active TB or outcome following antiTB treatment is not therefore completely understood. The conflicting results in different studies makes it very difficult to address the influence of helminth infection on progression from latent TB to active TB.

1.8.4 Effect of helminth infection on BCG vaccination

Cellular immune responses to *Mtb* antigens are decreased in individuals with concurrent helminth infection and this coincides the reduced efficiency of BCG in helminth-endemic regions of the world (357). Chatterjee *et al.,*in their study demonstrated that there was no significant difference in BCG vaccination status and TST responses in helminth co-infected patients and patients without helminths (341). A study done in Ethiopia showed that helminth infection influences the outcome of BCG vaccination by improving PPD specific cellular responses in helminth treated individuals compared to untreated controls. A randomized control trial in Ethiopia shown that deworming improved the efficacy of BCG vaccination (358). Elias *et al.,* showed poor immunogenicity of BCG vaccination of PPD negative individuals in a helminth infected population in Ethiopia and concluded that this impairment was associated with strong Th2 bias of the immune responses by chronic helminth infection (358). In a study in Sweden, *S.mansoni* infection, was shown

to reduce the protective efficacy of BCG vaccination against *Mtb* possibly by attenuation of protective immune responses to mycobacterial antigens and /or by polarizing the general immune responses to the Th2 profile (359). However, the effectiveness of BCG vaccination was unaffected by a primary malaria co-infection in a mouse model of *Mtb* infection (360). Th2 like IL-10 responses induced by intestinal helminth infection may also be interfere with BCG induced Th1 like IFNγ responses and may significantly alter the protective immune response to BCG vaccination (361). The impact of helminth infection on BCG vaccination is mostly due to modulation of cell mediated immunity and antigen specific and this low vaccine efficiency may be due to the impairment of immune responses to recall antigens (362).Later it was demonstrated that helminth- induced immune sensitization during gestation persists into childhood and skews subsequent childhood immune responses away from protective Th1 immune responses (363). In another study, BCG vaccination induced T cell responses to *Mtb* were significantly downregulated both before and after BCG vaccination. This impact appears to be reversible since it could be overcome through elimination of intestinal worms during BCG vaccination using broad spectrum anti-helminth treatment (357). Chronic helminth infected individuals have an increased susceptibility to infections normally cleared by Th1 dependent immunity as well as altered immune responses to BCG vaccination.

BCG vaccination provides the least protection in developing countries like such as Malawi and India, where helminths are endemic, in contrast to the UK, where BCG showed 80% protection against TB in the MRC trials. The reasons for this was speculated to be environmental mycobacteria exposure, high prevalence of endemic and chronic infectious diseases including helminth infections (364)(365)(366)(367).

The area of greatest impact of the modulation of the immune responses by helminth infections likely involves attenuation of vaccine-induced immune responses, so it is imperative that the influence of concurrent helminth infection is considered in the vaccine studies in helminth-endemic areas of the world (349).

1.8.5 Deworming and TB-helminth coinfection

The impact of helminth infections can also be assessed by studying the effects of deworming. A reduction or elimination of intestinal helminths using broad spectrum anthelmintic treatment resulted in enhancement of T cell proliferation and IFNγ production

by PBMC stimulated with PPD. T cell responses to PPD were found to be improved in filarial infected subjects after treatment with diethylcarbamazine (323)(343)(368). It was also observed in this study that prior BCG vaccination had no significant effect either on the *in vivo* or the *in vitro* responses to PPD (369). Treatment with an anthelmintic, in this case, albendazole during BCG vaccination was associated with significantly increased proliferative and IFNγ responses to PPD implying that exposure to chronic helminth infection during BCG vaccination may contribute to a decreased T cell response to mycobacterial antigens. It was observed that only albendazole had no direct effect on the cellular immune responses. It was suggested that the removal of intestinal helminths by deworming would remove the inhibitory effects of Th2 cells and cytokines on Th1 responses (355).

Toulza *et al.,* investigated how anthelmintic treatment modulates antimycobacterial immune responses in UK migrants. Patients with helminth infection showed a significant increase in CD4+FoxP3+T cells (Tregs) and a lower frequency of CD4$^+$IFNγ$^+$T cells and these effects were reversed after anthelmintic treatment (370). This was supported by other studies where anthelmintic treatment against Schistosoma infection in Gabon resulted in a marked reduction in CD4+FoxP3+T cells post treatment (371).

Given that the immune changes accompanying helminth infections are profound and universal, and that they revert to normal after eradication of the helminth infection, their role in the interaction between the host and the other infections may be very important (372).

Differential effects were observed in studies on helminth and TB co-infection. These varied responses may be attributed to different species of helminth, their location in the body, different life cycles, different E/S products, or *Mtb* infection. Different strains of Mycobacteria and their route of administration may also contribute. In vitro studies have demonstrated the impact of helminth infection on *Mtb* infection- both on the immune response and the severity of disease, although the clinical outcome is not clearly understood, which may be due to underpowered studies, type or intensity of infecting helminths and the different methodologies used for the detection of helminth infection (135).

1.9 Mycobacterial growth inhibition assay

A recent development in TB biomarker discovery has been the establishment of automated techniques that allow the measurement of mycobacterial growth inhibition *in vitro* (373). Functional assays such as mycobacterial growth inhibition assays (MGIAs) that measure the summative effect of a range of cellular mechanisms might prove to be better surrogates of protection than measuring T cell responses or cytokines as biomarkers (374). MGIA directly measures the ability of heterogeneous populations of human lymphocytes and other mononuclear cells to limit the intracellular growth of mycobacteria. The immune mediated inhibition of mycobacterial growth correlates more directly with protective TB immunity than other immunological responses (375). This assay represents an unbiased surrogate marker of protection against TB and allows for the investigation into the potential roles of immune mechanisms in mediating this protective effect (371). Assays of immune inhibition of mycobacterial growth may provide a more direct measure of *in vivo* resistance against *Mtb*.

The MGIAs which are being evaluated for use in assessing vaccine induced protective immune responses in clinical trials of TB vaccines have varying designs and endpoints (376)(375)(377)(378)(379). BCG and *Mtb* based assays have been used to evaluate vaccine induced immunity in humans.

Youmans *et al.,* demonstrated that lymphokine secreted from splenocytes of immune mice when added to mouse peritoneal macrophages, enhanced inhibition of *Mtb* growth *in vitro* (380).This method was adapted later for use with PBMC isolated from healthy volunteers, both immune (TST positive or BCG vaccinated) and nonimmune. Lymphocytes were cultured for 72 hours with trypsin extracted soluble antigen/tuberculoprotein to induce lymphokine production. Macrophages were then infected with attenuated or virulent mycobacteria (*M. bovis* BCG and *Mtb* Erdman) and cultured for 7 days with or without the addition of immune lymphokine obtained from the lymphocyte stimulation assay. Enhanced inhibition of intracellular bacillary replication was observed in cultures containing lymphokine produced only by lymphocytes of immune subjects, of whom there were three kinds: tuberculin positive naturally immunized, *M. bovis* BCG immunized, and trypsin-extracted bacillary antigen immunized. The human immunity to mycobacteria was measured by inhibition of [3H]thymidine incorporation, counts of AFB, and morphological comparisons of culture

(381). Crowle and May also showed that the active vitamin D3 metabolite 1,25-(OH)$_2$ cholecalciferol had the ability to inhibit mycobacterial growth in macrophages(382). The same group assessed the antimycobacterial effects of lymphokine and recombinant IFNγ using murine peritoneal macrophages and human alveolar lavage cells. This study showed that addition of recombinant IFNγ or crude lymphokine reproducibly activated strong anti-mycobacterial mechanisms in the murine cells, however, these stimuli had weak and variable effects on human alveolar cells. These studies indicated a number of potential reasons for the difficulty in demonstrating inhibition of intracellular growth of *Mtb*; for example host susceptibility, use of high bacterial to cell ratios, higher concentrations of cytokines or synergistic effects of multiple cytokines, and necessity of cell to cell interaction in the containment of intracellular bacteria etc. However, vitamin D and A have some ability to activate *Mtb* infected human mononuclear cells and TNFα may contribute to the in vitro containment of avirulent *Mtb*. But none of the mediators limits the growth of the organism within human mononuclear cells with an efficacy compared to that of the murine cells (383).

Chen *et al.,* studied the mononuclear cells from healthy British school children (aged 13-15 years) who were BCG vaccinated before and 8 weeks post vaccination. The monocytes were then infected with *M.microti* in microtiter trays at a low bacterium/monocyte ratio for 24h to allow phagocytosis and monolayer development. The monolayers then received pulsed exposures to autologous lymphocytes for 2h every day for 3 days. Monocyte monolayers were then infected with *M.microti* for 24h and the bacillary changes in monolayers and supernatants over 4 days were quantified by colony counts. *M.microti* growth was significantly inhibited at days 3 and 4 of the culture using cells from pre and post vaccinated children. Growth rates in lymphocyte-pulsed monolayers were significantly lower after vaccination than before. It was proposed that this difference reflected the protective effect of vaccination (384).

1.9.1 PBMC based mycobacterial growth inhibition assays

1.9.1.1 Primary lymphocyte inhibition assay

This assay measured the ability of freshly harvested blood lymphocytes to inhibit the intracellular growth of virulent *Mtb*. In 1998, Silver *et al.,* developed this assay using low level infection of isolated monocytes with *Mtb* H37Rv for 1 hour followed by a 7 day

culture either alone or with unstimulated autologous lymphocytes (377). At days 0 and 7, co-cultures were lysed with SDS and viable bacteria counted by CFU. Unstimulated lymphocytes induced significant inhibition of mycobacterial growth after 4 and 7 days of culture for both PPD negative and positive subjects and this is indicative that the assay is measuring early innate immune responses. This study also indicated that non adherent cells (NAC) from both PPD-positive and PPD-negative subjects were more effective at limiting the intracellular growth of H37Rv than either cytokines or transferred cytokines. Depletion of CD4+T cells eliminated the growth inhibition capacity of non-adherent PBMC from PPD-positive individuals, but did not reduce the inhibitory effects of NAC from PPD-negative subjects. Depletion of CD8+T cells did not affect the ability of NAC from either group of subjects to contain *Mtb*. Antigen-specific immunologic memory in PPD reactive individuals could not be measured in this study. Addition of rIFNγ and rTNFα to infected monocytes did not show any significant effect on mycobacterial growth inhibition and even additions of blocking antibodies to these cytokines were not able to alter the growth inhibitory capacity.

1.9.1.2 Secondary lymphocyte inhibition assay

Worku and Hoft described this assay(385).They investigated the ability of T cells expanded with mycobacterial antigens from healthy PPD reactive donors prior to co-culture with infected monocytes to inhibit the intracellular growth of BCG (378). Adherent monocytes were cultured for 6 days followed by overnight infection with BCG. PBMC were cultured for 7 days in media alone or with the addition of antigen like mycobacterial whole cell lysate, live BCG or tetanus toxoid (TT) as control. Expanded effector T cells were added to autologous monocyte targets for 72h. After 72 hours, supernatants were aspirated and cells lysed with 0.2% saponin. Viable bacteria were determined by radiolabelling. Lysates were diluted in media and pulsed with [5,6-^{3}H] uridine and incubated at 37ºC for 72 hours, after which BCG bacteria that were incorporating the label were placed on glass-fibre filter mats. The amount of titrated uridine incorporation in the BCG bacteria determined by liquid scintillation counting. The percentage of inhibition of BCG growth was determined by comparing the amount of reactivity and CFU estimations in antigen-expanded effector cell co-cultures with that in medium-rested T cell co-cultures. In this assay, BCG growth was not inhibited by TT expanded lymphocytes which indicated that the effects were mediated by expansion of antigen specific T cells. Interestingly, in this assay γδT cells were the predominant T cell

subset expanded in response to mycobacterial antigens and that were efficient in inhibiting intracellular growth of BCG, suggesting a memory like phenotype but there was no statistically significant mycobacterial growth inhibition in presence of enriched CD4+ and CD8+T cells. Spencer *et al.,* showed that this γδT cell population produce soluble granzyme A in a TNFα dependent manner, which might correlate with their ability to improved mycobacterial growth inhibition (386).

Hoft *et al.,* compared different methods of MGIA (375). In a primary lymphocyte assay, unexpanded lymphocytes were added and that provides an unbiased sampling of peripheral blood responses. The lack of in vitro stimulation, may thus overemphasize the effects of nonspecific defences. In contrast, the secondary lymphocyte inhibition assay, may have higher sensitivity for the detection of memory immunity. Vaccine-induced enhancement of secondary lymphocyte inhibition was statistically more robust in this comparative study . Interestingly, Worku and Hoft investigated the effects of peripheral blood mononuclear cells (PBMC) expanded with irrelevant control and mycobacterial antigens on the intracellular growth of BCG in human macrophages. When T cells were stimulated with purified mycobacterial antigens, levels of resulting growth inhibition varied depending on the antigen used. BCG vaccination was shown to significantly enhance antigen-specific T-cell inhibition and purified mycobacterial antigens were shown to stimulate inhibitory T cells.The results were characteristic of antigen-specific, memory-immune T-cell responses (385).
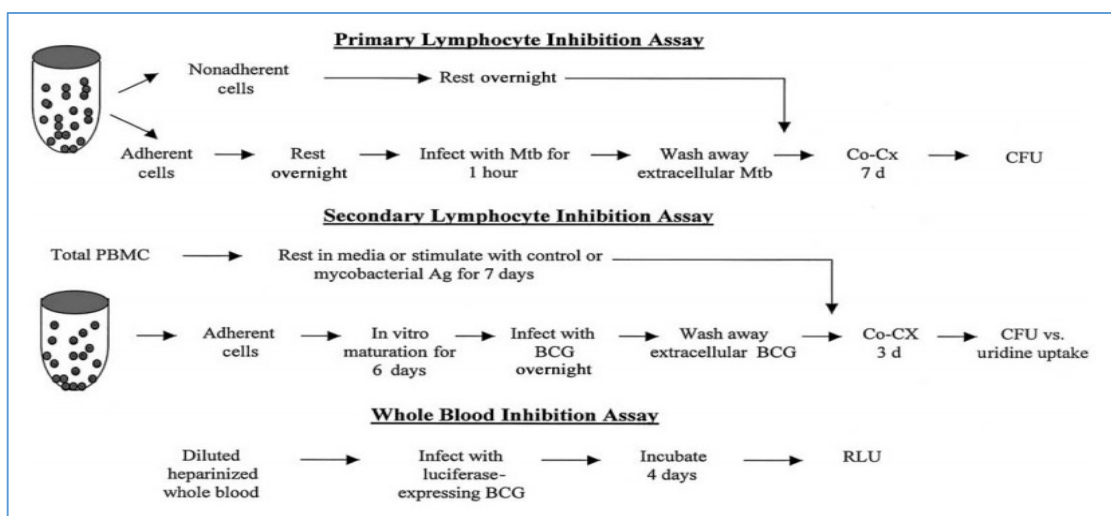
Figure 1.11:Mycobacterial growth inhibition assays (375).

1.9.2 Whole blood mycobacterial growth inhibition assays

Whole blood culture has been widely used to evaluate pathogen-induced immune functions and immune mediated killing of intracellular *M.bovis* BCG. Walis *et al.,* described an *ex vivo* model for measuring the whole blood bactericidal activity (WBA) of drugs-isoniazid, rifampicin, ethambutol, levofloxacin by whole blood culture (387). This WBA is an adaptation of Schlichter and MacLeans's concept of the serum bactericidal assay to monitor therapy for infective endocarditis(388). Blood was collected before, and at intervals after drug administration. *Mtb* was mixed with blood and incubated at 37ºC for 72 hours with constant mixing. The cells were then disrupted by hypotonic lysis and bacilli were sedimented and resuspended in BACTEC medium before adding to MGIT tubes. Mycobacterial survival was determined by comparing the days-to–positivity (DTP) of the control and experimental cultures. In this WBA, growth indices of actively growing BACTEC cultures increase exponentially in proportion to CFUs. BACTEC growth indices were used as a surrogate for CFU to measure intracellular survival of *Mtb* in other studies (389). In this study, DTP was observed to improve accuracy and dynamic range. The inhibition was shown to correlate with sterilizing activity observed *in vivo* during therapy.

Another WBA was developed using BCG transfected with luciferase (BCG-lux) as a reporter (379). Expression of recombinant luciferase enzymes has been widely used as a reporter, to monitor changes in cell viability in bacterial and eukaryotic systems. In mycobacteria, luciferase reporters have also been used mainly to monitor the effect of drugs on viability in earlier studies (389), but they have also found to detect immune-mediated killing in animal models (390)(391). Diluted whole blood was diluted with BCG lux for 96 hour followed by isotonic lysis to obtain a final concentration of RLU/ml. Mycobacterial growth was expressed as a ratio of growth in the sample relative to the control. Silver *et al.,* later optimised the assay for smaller blood volumes to improve suitability for paediatric studies (377). Kampmann *et al.,* suggested that luciferase promoters that are differentially regulated during the course of the infection represent a possible approach to further enhancement of the discriminatory ability of the WBA (379). Tena *et al.,* showed that this WBA demonstrated reduced ability of whole blood from HIV-infected infants to inhibit mycobacterial growth in comparison to healthy control infants (392). In a secondary lymphocyte inhibition assay, it was observed that adding rIFNγ or rTNFα or blocking antibodies against these cytokines did not alter the

mycobacterial growth inhibition (393). But in this BCG Lux assay, mycobacterial growth was partially enhanced, when blocking antibodies were used against these cytokines.

WBA require small volume of blood, which is an advantage for some studies for example, paediatric studies. WBAs cannot distinguish between intracellular and extracellular mycobacterial growth, whereas a cellular assay has the advantage of removing nonphagocytosed bacteria by washing. When attempting to assess a vaccine response, neutrophils in whole blood may be less relevant or a strong effect could mask the functionality of the long term specific responses.

Fletcher *et al.,* adapted the Wallis whole blood MGIA into a PBMC assay (394). Following BCG vaccination, PBMC of British adults exhibited significantly enhanced growth inhibition at both 4 and 8 weeks after vaccination but the growth inhibition had returned to baseline levels by 6 months post vaccination (394). Harris *et al.,* adapted this WBA of MGIA in a BCG challenge study comparing antimycobacterial immunity induced by BCG and MVA85A vaccination. The assay could not detect any significant difference between groups (395).

1.9.3 The BACTEC MGIT assay

The BACTEC MGIT 960 is a fully automated system that contains a modified Middlebrook 7H9 broth with a fluorescence quenching based oxygen sensor that detects the amount of oxygen consumption by growing microorganisms. PBMC were added into 2 ml screw cap micro tubes with a predetermined, optimal quantity of mycobacteria. Tubes were incubated at 37°C with 360º rotation for 96 hours. Following incubations, cells and remaining BCG were pelleted and lysed and are then transferred into corresponding MGIT tubes. Time to positivity (TTP) is determined by using BACTEC MGIT 960. A standard curve enables conversion of TTP into initial mycobacterial volume which is then converted into CFU(394). Smith *et al.,* measured a population of PPD specific, CD4+T cells that demonstrates polyfunctional (IFNγ+TNFα+IL2+) capabilities in infants of UK who have received BCG vaccination at 6 weeks of age. The study demonstrated that PPD specific polyfunctional CD4+T cells secreting IFNγ, TNFα and IL-2 together with the induction of antigen-specific Th-17 cells showed a significant correlation with growth inhibition(396). Fletcher *et al.,* recently conducted the PBMC MGIT assay as a part of a case control analysis of immune correlates of risk of TB

disease in BCG vaccinated infants from South Africa. In the study, reduced risk of TB disease was associated with BCG specific IFNγ ELISpot responses and Ag85A antibodies but was not associated with MGIA. This lack of correlation might be attributed to the low frequency of BCG antigen specific T cells detected in this study.
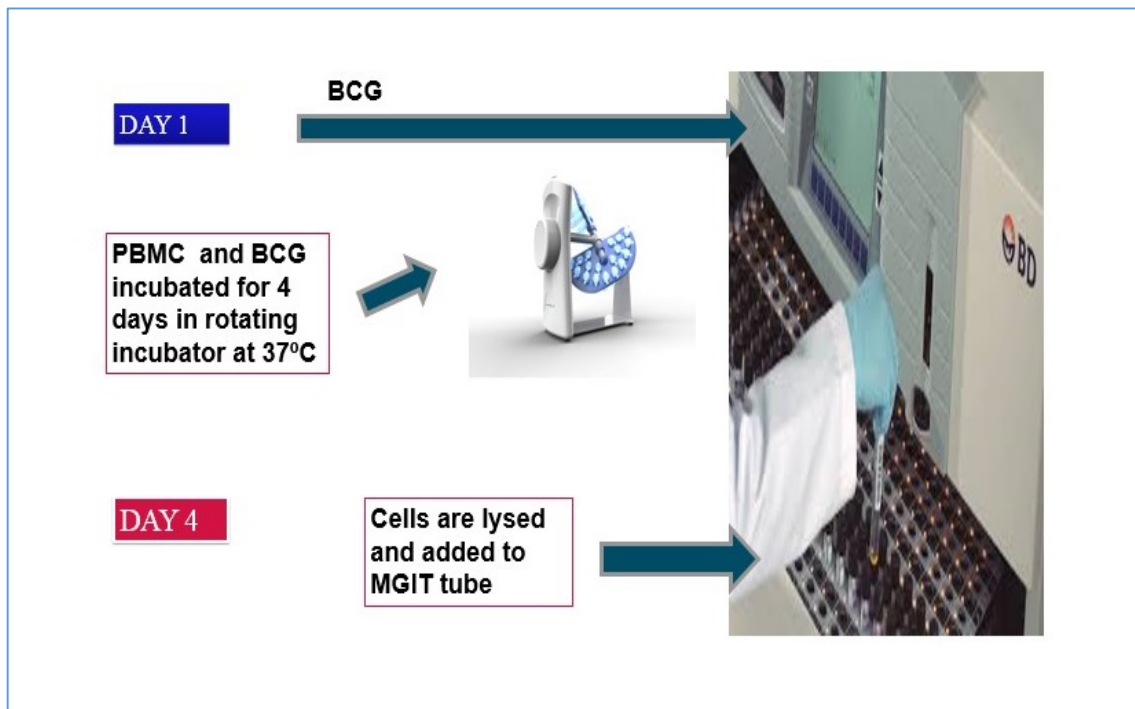
Figure 1.12:MGIA using BACTEC MGIT 960.

1.9.4 MGIAs in preclinical animal models

MGIAs have also used in animal models- especially in cattle, mouse and non-human primate (NHP) models. Developing MGIAs in animal models can provide the ability to validate a vaccine candidate and its correlates of protection.

Reference	Method overview
Cowly and Atkin (397)	Bone marrow macrophages cultured for 7 days and then infected with *Mtb* Erdman for 2 hours. Splenocytes were primed by in vitro *Mtb* infection followed by chemotherapy to clear the infection. Addition of harvested primed splenocytes to the macrophage cultures resulted in significant growth inhibition.
Parra *et al.,*(398)	Adapted the Cowly and Atkin assay to evaluate the vaccine efficacy of novel vaccine. Enhanced growth inhibition of *Mtb* was observed whenco-cultured with bone marrow macrophages from BCG vaccinated mice compared with naïve controls. There was a significant correlation between in vitro growth inhibition and in vivo protection of 5 novel vaccines.
Marsay *et al.,*(374)	Splenocytes were infected with BCG for 96 hours with 360° rotation. Cells were lysed and TTP was calculated by BACTEC MGIT. Inhibition of growth was significantly enhanced in splenocytes from BCG vaccinated mice compared with control mice.
Carpenter *et al.,*(399)	PBMC from immunized cattle expanded by PPD stimulation and added to BCG-infected autologous macrophages. BCG growth was significantly reduced in cultures containing autologous PBMC from both immunized and control cattle.
Yang *et al.,*(400)	Splenocytes were directly infected with *Mtb* from vaccinated mice and quantified mycobacteria using MGIT. Vaccine-mediated immunogenicity detected by this assay correlated with protection.
Zelmer *et al.,*(401)	Optimized *ex vivo* mycobacterial growth inhibition assay using a murine *Mtb* infection model. Assessed the combined ability of host immune cells to inhibit mycobacterial growth in response to vaccination. C57BL/6 mice immunized with BCG and growth inhibition of mycobacteria by splenocytes was assessed.
McShane *et al.,*(394)	Human whole blood, PBMC and mouse splenocytes infected with BCG for 96 hours with 360° rotation. Cells were lysed and mycobacteria quantified by BACTEC MGIT TTP. Significant inhibition of growth was observed at 8 weeks post-BCG in whole blood, 4 and 8 weeks post-BCG in PBMC. There were significant differences in growth inhibition between naïve and BCG vaccinated mice.

Table 1.3: Overview of MGIAs in animal models.

In animal models, mycobacterial growth inhibition can be observed in vitro following BCG immunization in animals and this can be correlated with protection from *Mtb* challenge, although in small animal models the blood volume may impede the use of WBA MGIAs. In different studies it has been concluded that splenocytes are most realistic cells to use, although BCG has a propensity to persist in the spleen, which makes it difficult to study live vaccines and responses in the spleen may not be representative of immunopathogenesis in the periphery or the lung.

1.10 Rationale of the study

The study investigated the effect of helminth coinfection on the ability of patients with LTBI infection to inhibit mycobacterial growth and also observed the impact of anthelminthic treatment on modulation of antimycobacterial immune responses in these coinfected patients. To our knowledge, no other studies have investigated the immune mediated control of the growth of *Mtb* in LTBI and helminth coinfected patients. The results of this study may provide an insight on immune correlates of protection against latent TB in these patients.

1.11 Hypothesis

Anthelminthic treatment modulates the ability of the LTBI and helminth coinfected patients to inhibit the growth of *Mtb*.

1.12 Study Aims

The aims of the study are:
1. To measure the magnitude of IFNγ T cell responses to *Mtb* antigens in these patient groups and controls before and after anthelminthic treatment by ex-vivo ELISpot assay and intracellular cytokine responses of PBMC using flow cytometry.
2. To determine the effect of helminth infections on mycobacterial growth inhibition and to observe whether anthelminthic treatment affects the ability of the cells to control mycobacterial growth.
3. To explore the association of antibodies and macrophage subsets with mycobacterial growth inhibition in the MGIT assay to gain a better understanding of the immune mechanisms underlying LTBI-helminth coinfection.
4. To analyse differential gene expression in helminth and LTBI-helminth coinfected patients after anthelmintic treatment.

Chapter - 2   Materials and Methods

2.1   Study design

To identify and characterize the effects of helminth infection and of anthelmintic treatment on the immune response to TB, UK migrants attending the Hospital of Tropical Diseases (HTD) and TB clinic of University College London (UCL), with eosinophilia or with suspected / diagnosed helminth infection and or latent TB between January 2014 and September 2016 were recruited. All participants were enrolled in this study during their first visit (V1). Blood was drawn to detect helminth infection and to detect LTBI. These patients were given anthelmintic treatment according to their parasitology serological results. All the recruited patients were requested to come back at least 4 months later for a follow-up visit (V2).

2.1.1 Inclusion criteria

Patients who fulfilled the following criteria were eligible to participate in the study and considered as the sample population:

- Migrants in UK or history of living in tropical countries
- UK migrants presenting with eosinophilia
- Male and female aged over 18 years of age
- Suspected or diagnosed helminth infection (no evidence of previous treatment)
- Suspected or diagnosed cases of latent TB (IGRA positive)

2.1.2 Exclusion criteria

Any individual characterized by at least one of the following criteria was not eligible to participate in the study:

- Male and female migrants below the age 18
- Diagnosed cases of HIV or any immunosuppressive condition
- Patients with previous anthelmintic treatment
- Patients with LTBI treatment (ongoing or previous)

2.1.3 Ethical considerations

Research clinical approvals for this study were obtained from the Health Research Authority, National Research Ethics Service Committee (reference 11/H0713/12), London and from the Ethics Committee of London School of Hygiene & Tropical Medicine (LSHTM) (reference number: 7758) (Appendix I).

2.1.4 Study subject's recruitment

At the HTD and the TB clinic at UCLH, patient information leaflets were distributed in the waiting area. A research nurse from the clinical care team identified all patients who met the inclusion/exclusion criteria and approached them to discuss the study. Those patients who had agreed to participate in the study signed an informed consent. Each patient was allocated a unique identification number (UIN) by the research nurse to maintain the anonymity of the samples. Research samples comprised approximately 3 ml blood for Quantiferon Gold in-tube assay to diagnose TB, 6 ml of blood to test for antigens indicative of helminth infection, 3 ml blood for full blood count, 20 ml blood for immunology and 3 ml of blood for RNA preparation for gene expression in a Tempus^{TM} blood RNA tube (Applied Biosystems, USA). All the clinical samples were labelled according to NHS standard operating procedures. Stool and urine samples were collected for microscopy and processed through the routine diagnostic laboratory as per guidelines. All other samples were sent to LSHTM.

2.1.5 Study groups

There are four study groups- controls, helminth infected, LTBI and LTBI and helminth co-infected groups. Blood was also collected from healthy donors from LSHTM to perform different immunological methods and to be used as healthy controls.

Helminth infected patients

Patients with a history of living in endemic countries attending HTD outpatient clinics or inpatients at UCLH with suspected or proven helminth infection belong to this group. The suspected helminth infected persons are those who have symptoms suggestive of helminth infection or with parasitological investigations to diagnose helminthiasis. Some

patients were referred from another clinic as diagnosed helminth infected patients. Those patients were subjected to further investigation and treatment.

Control group

Patient who had no eosinophilia on their full blood count (FBC) and no evidence of helminth infection or no proven TB infection but had a history of living in tropical countries were recruited as controls. They had attended the clinic with gastrointestinal and respiratory symptoms.

LTBI patients

This group comprised of patients who had a history of living in tropical countries who attended the UCLH TB clinic for suspected or proven TB infection. All LTBI patients were IGRA test positive. All the patients were tested for *Mtb* infection, using the QFT-GIT assay. Patients with clinical symptoms of TB were screened for TB disease and excluded from this study if active TB disease was diagnosed.

LTBI- helminth co-infected patients

This group comprised of patients who were IGRA positive or had previously diagnosed LTBI and had evidence of helminth infection based on symptoms or serological examination. Participants to be recruited were grouped according to the result of immunological procedures performed on samples obtained at visit 1.

Group 1: Helminth negative, latent TB negative
Group 2: Helminth negative, latent TB positive
Group 3: Helminth infected, latent TB negative
Group 4: Helminth infected, latent TB positive

2.1.6 Blood collection

Visit 1

Following enrolment, about 25 ml venous blood is collected from each participant into commercial heparinized vacutainer® tubes (BD, Franklin, USA) at the clinic by the phlebotomist. The phlebotomist in charge labelled and documented the date, UIN and

visit number. During recruitment or Visit 1, 3 ml of blood sample for QFT-GIT assay was collected from subjects recruited to diagnose LTB infection and 6 ml blood was used for diagnostic parasitology. Also, stool and urine samples were used for diagnosing helminth infection. Around 20 ml blood in heparinized vacutainer tubes and 3 ml whole blood in a Tempus tube ®were sent to the LSHTM laboratory for immunological analysis and for the gene expression study. Three ml of whole blood in Tempus tubes were kept at room temperature for 2 hours and then stored at -80°C for further gene expression analysis. Four weeks after visit 1 participantwere asked to attend the clinic again. According to the serology results and microscopic helminth diagnostic tests, anthelmintic treatment was chosen, prescribed and supervised by the attending physician. Some patients had another blood sample collection for immunological analysis.

Visit 2

About 16-24 weeks after anthelmintic treatment, participants re-attended for a further blood examination for immunological assays, which also included blood for Quantiferon-Gold In–Tube assay, helminth serology and FBC. About 20 ml of venous blood in the heparinized tube and 3 ml blood in a Tempus tube was sent to LSHTM laboratory for immunological analysis of the post-treatment samples. Four ml of venous blood was collected for a full blood count and sent to the Pathology laboratory.

Blood in heparinized tubes was brought to the laboratory at LSHTM. PBMC isolation was carried out inside a sterile safety cabinet and cells stored in cryovials in -80°C for 24 hours and then the cryovials were transferred to liquid nitrogen for longer storage. Plasma was collected from the blood and stored at -80°C and kept for further immunological analysis.

2.1.7 Clinical data collection

A structured questionnaire was used for clinical and demographic data for each study participant. The data collection sheet included the demographic, clinical and laboratory data information following the standard guidelines. Baseline data included hospital ID, study UIN, age, sex, contact details, referral route, doctor/nurse name, country of origin, and years of stay in UK and history of travelling to tropical/ endemic countries. Clinical data included helminth symptoms, previous TB history, BCG vaccination history and HIV status. Diagnostic data included previous eosinophil count, lymphocyte count,

serology and microscopy results for helminth, IGRA results etc. For all the patients, basic laboratory tests like FBC and haemoglobin concentration were obtained at a baseline visit and at follow-up and recorded in a predesigned data sheet in Microsoft Excel file. All the clinical, socio-demographic, laboratory data, sample collection date and time were collected and stored in a password protected Microsoft Excel file.

2.1.8 Tests for TB latency and helminth infection

Latent TB detection

All the recruited participants were tested for TB. To detect latent TB infection, aQuantiFERON®-TB Gold (QFT®) ELISA test was performed. QFT is a test for cell-mediated immune (CMI) responses to peptide antigens from mycobacterial proteins. These proteins, ESAT-6, CFP10, and TB7.7, are absent from all BCG strains and from most nontuberculous mycobacteria with the exception of *M.kansasii*, *M.szulgai* and *M. marinum.* Individuals infected with *Mtb* complex organisms recognize these mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFNγ. The detection and subsequent quantification of IFNγ form the basis of this test. The test was performed according to the manufacturer's instructions, using equal volumes of plasma (50μl) and conjugate (50μl) in the first step of the assay. OD values were measured using an ELISA reader. The QFT Analysis Software was used to analyze raw data and to calculate results. The software makes all the calculations, generates a standard curve and provides a test result for each subject. For the QuantiFERON-TB Gold test to be valid, the nil value must be less than or equal to 8.0 IU/mL, and the mitogen value (a positive control for IFNγ production) must be at least 0.5 IU/mL higher than the nil value. The QuantiFERON-TB Gold test is considered positive if the IFNγ response if the TB antigen minus nil value is at least 0.35 IU/mL. The tests were performed by the routine diagnostic laboratory at UCH who reported results as QFT positive and negative; this result was used to classify the subjects as LTBI positive or negative.

Tests for helminth infection

Most of the patients in this study were infected with Schistosoma spp and/or Strongyloides spp with a few tapeworms. For Strongyloides spp, serum samples were analysed by a commercial ELISA for anti-Strongyloides antibodies according to the

manufacturer's protocol that uses a variable cut-off based on a low positive serum. This is a solid phase in vitro quantitative assay for the indirect detection of IgG to *S.ratti* somatic larval antigen (Bordier, France). According to the Bordier affinity *S.ratti* kit the test sensitivity is 88% and specificity 100%. Faeces samples were also cultured to detect Strongyloides or hookworm using the charcoal culture method. Afterwards, visual interpretation was also performed microscopically to distinguish whether the larvae were of Strongyloides or hookworm.

For anti-schistosome antibodies, an in-house ELISA assay was performed, using the standard clinical diagnostic tests currently in use in the National Parasite ReferenceLaboratory of the UK, based at the HTD. This in vitro quantitative assay detects IgG antibodies to Schistosoma egg antigens. Stool analysis was performed following formolether/ethyl acetate faecal concentration using a Midi Parasep®fecal concentrator to increase the possibility of detecting ova or cysts that may not be detectable by direct microscopy. Terminal urine microscopy was also performed to see the characteristic eggs of Schistosoma spp. Filarial serology was performed if the patient was from Africa or had clinical evidence of filarial infection. Tapeworms were diagnosed by a routine stool microscopy test. A full blood count was performed to detect eosinophilia.

2.1.9 Immunological methods

PBMC isolation, cryopreservation and thawing

PBMCs were isolated from heparinized whole blood obtained from healthy donors at LSHTM/ patients from HTD or UCL, using density centrifugation on Histopaque (Sigma, UK). Ficoll Histopaque was brought to room temperature. Fifteen ml of whole blood was taken into a 50ml Falcon tube and diluted with PBS to make it up to 50 ml. Fifteen ml of Ficoll Histopaque was added to a 50ml Falcon tube. Twenty-five ml of diluted blood was layered on the top of the Ficoll layer. Then the tube was centrifuged at 673g for 25 minutes without a brake. Ficoll separates blood into layers of plasma, PBMCs, granulocytes and red blood cells. The PBMC layer was collected from the interface between the plasma and Ficoll layers using a Pasteur pipette (Fisher Scientific, UK) and taken into a new Falcon tube and topped up to 45 ml with PBS and centrifuged at 673g for 10 minutes with brake. The supernatants were removed and pellets and resuspended in

45 ml of phosphate buffer saline (PBS-0.01M: NaCl 0.138M, KCL 0.0027M, pH 7.4).The tube was centrifuged at 149g for 15 minutes at 4°C for a further wash.

For cryopreservation, cells were resuspended in cryopreservation medium. The freezing media was prepared with pre-cooled 90% foetal bovine serum, FBS (heat inactivated), (GIBCO®Life technologies, UK) and 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich®, UK) and mixed by inversion. Cells were immediately aliquoted into 2 ml cryovials (Sigma Aldrich, UK) at 1 ml per vial. Each cryovials was labelled with donor ID, date and visit number. The cryovials were transferred to a pre-cooled Mr Frosty freezing container containing isopropanol, which was stored at -80ºC. After 1 to 2 days, vials were transferred to liquid nitrogen for long term storage.

To thaw PBMC, the cryotubes removed from liquid nitrogen were placed on dry ice. About 11 ml of pre warmed RPMI was placed in a 15 ml Falcon tube for each vial of PBMCs. Cells were thawed in a water bath at 37ºC until 70% had melted. The cryotubes were opened under the hood. Around 0.5ml of Roswell Park Memorial Institute-1640 (RPMI) (Sigma Aldrich, UK) HEPES supplemented with 10% L-glutamine and sodium pyruvate (Gibco, UK) was added into the vials. Using a Pasteur pipette, the solution was gently pipetted up and down until the icy portion was completely thawed. Cells from the vial were transferred into the Falcon tube containing RPMI media and centrifuged at 1400 rpm for 7 minutes. The supernatant was discarded and cells were resuspended in RPMI media at approximately $1x10^6$ PBMC per ml (10 ml per vial thawed). About 2µl of Benzonase nuclease (Sigma Aldrich, UK) per ml RPMI was added and incubated for 2 hours at 37°C in an incubator with 5% CO_2 (with the lid of the Falcon tube slightly loosened) before counting the cells.

Cell counting

Twenty µl of cell sample was taken to a well of round well microtiter plate, (Sigma Aldrich, UK) and mixed with 10µl of Trypan blue solution at 0.4% (Sigma Aldrich, UK) to count the live cells. Ten µl of the cell mix was mounted on a Neubauer chamber. Cells in the 25 inner squares of the Neubauer chamber were counted and the total number of cells/ml was obtained by multiplying the counted cell number by the dilution factor (2) and 10^4.

2.2 Mycobacterial Growth Inhibition Assay (MGIA)

BCG Aeras Pasteur was used as the immune target for the Mycobacterial Growth Inhibition Assay (MGIA) in this study. The stock BCG was aliquoted in vials in 0.5ml volumes at a concentration of $1x\ 10^8$/ml. The BCG was grown in shaken flasks in 7H9 medium OADC (Oleic acid, bovine albumin fraction, dextrose and catalase) enrichment and with addition of 0.2% glucose and 0.05% Tyloxapol to an OD of 4. Then the tube was spun down and resuspended in half the volume of formulation buffer (20% glycerol, 0.85% NaCl and 0.05% Tyloxapol).

Mycobacteria stock titration and standard curve for MGIT

Materials required:

- BCG Pasteur Aeras stock (0.5ml)
- Sterile Eppendorf tubes -2 ml (Sigma Aldrich, UK)
- BACTEC MGIT (mycobacterial growth indicator tube) tubes and PANTA enrichment (BecktonDickinson, BD)
- Sterile filter tips (Starlab, UK)
- Sterile PBS-Tween 80 (0.05%) (BD)
- 7H11 agar plates with 10% OADC (BD) and 0.5% glycerol (Sigma Aldrich, UK)
- Cling film (Sphere, UK)

MGIT tube preparation

One bottle of lyophilized MGIT PANTA was reconstituted with 15 ml MGIT growth supplement. It was mixed by turning it over until it was completely dissolved. About 800μl of MGIT PANTA enrichment was added to each BACTEC MGIT tube. For each dilution two tubes were prepared.

Stock titration

BCG Aeras Pasteur was used as the immune target for the MGIAs in this assay. One vial of BCG stock was thawed and brought to room temperature. To make the stock titration for the standard curve, 7 serial 10-fold dilutions of the stock BCG were prepared by adding 1.08ml PBS–Tween to each of the seven Eppendorf dilution tubes, labelled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}. One hundred and twenty µl undiluted stock was added to the first tube and mixed thoroughly by pipetting up and down. From the first tube (1 in 10 dilution), $120\mu l$ was taken out and transferred to the next tube. Seven 10 fold serial dilutions of the stock were prepared. Duplicate MGIT tubes for each dilution were inoculated by adding 500µl of each dilution to the respective MGIT tube. The MGIT tubes were then placed into the BACTEC MGIT instrument.

Agar plates were prepared and used to detect and count colony forming units (CFUs). The 7H11 agar plate was divided into 4 quarters and labelled with different dilutions. Twenty µl of each dilution was inoculated into labelled dilution sectors. The plates were covered with cling film and placed in the incubator at 37ºC with 5% CO_2. Colonies were counted as soon as they were visible, approximately 10-12 days. The number of colonies counted for the 1×10^8 BCG dilution was 100 CFUs. This value was then extrapolated to give the CFUs for each concentration.

Standard Curve

A standard curve was calculated by plotting Time to Positivity (TTP) against the CFU count determined by plating of the equivalent volume of the stock BCG and regression analysis was used to obtain the equation that can then be used to convert any TTP to volume or CFU. The regression analysis provided a R^2 value and the equation describing the line. The equation of the standard curve was x=(y-c)/m, where y=TTP, m=slope, c=y intercept. By inserting the TTP, the number of CFU or $\log_{10}$ CFU initially added to the tube was calculated. The $\log_{10}$ CFU data was plotted in a graph. The number of log10 CFU were divided by the number of log10 CFU in the direct-to-MGIT control to obtain the read-out of relative growth of bacteria.

MGIT standard curve

$R^2 = 0.9953$

$\log(X) = \dfrac{Y - 71.59}{-37.45}$

TTP [hrs] vs Inocula volume [mcl]

MGIT standard curve

$R^2 = 0.9953$

$\log(X) = \dfrac{Y - 277.6}{-37.45}$

TTP [hrs] vs CFU

MGIT standard curve

$R^2 = 0.9953$

$X = \dfrac{Y - 277.5}{-37.44}$

TTP [hrs] vs log10 CFU
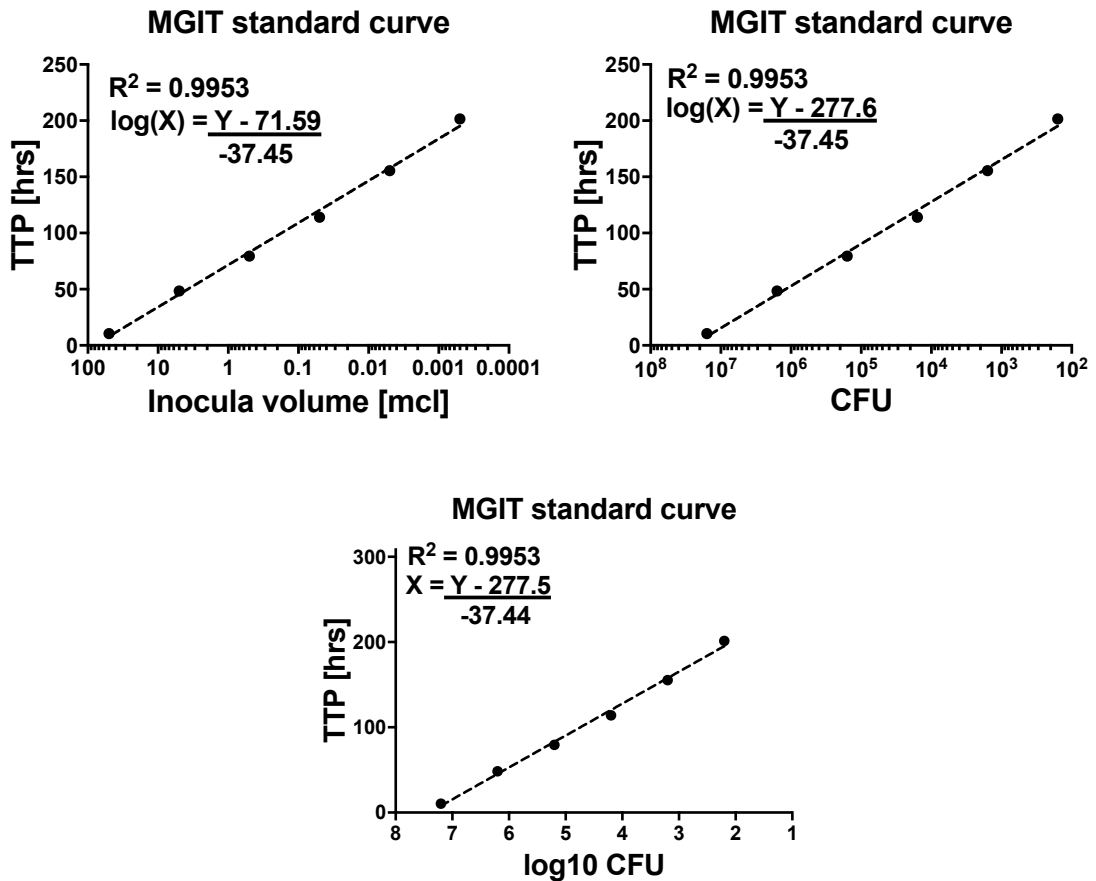
Figure 2.1: MGIT standard curve.

PBMC preparation for the MGIA

Cryopreserved PBMCs were thawed in a water bath at 37°C, washed and resuspended in RPMI-MGIT media (RPMI-1640 medium with 25mM HEPES (Sigma), 2mM L-glutamine, 10% filtered, heat inactivated, pooled Human AB Serum) at a concentration of 1×10^6 cells/ml and incubated for 2 hours with benzonase nuclease (10 units/ml of cell)

(Novagen) in a 37°C incubator with 5% CO_2. Cells were counted, washed and resuspended in RPMI-MGIT media at a concentration of 1×10^6 cells/ml per 300μl medium and added to a 2 ml screw-cap micro tubes. Three hundred μl cell suspension was added to 2 ml screw cap tubes (Sarstedt, Germany) in duplicate.

Preparation of BCG master mix

The total number of tubes to be inoculated was calculated. The total volume of bacterial suspension required from the stock BCG concentration (1×10^8 /ml) for all the samples were also calculated. For each sample, one 2ml screw cap tube is used for the control

(only cells and media) and one for cells with BCG. First 300µl of the RPMI-MGIT media was added in each tube. An aliquot of BCG Pasteur was thawed and the total volume of BCG needed was calculated. The neat stock at 1×10^8/ml was diluted 1 in 10 with RPMI-MGIT media (900µl RPMI+ 100µl of BCG stock) to give a concentration of 1×10^7/ml. Aliquots of 10µl from this 1/10 dilution were taken out and added to 990µl of RPMI-MGIT to make a further 1/100 dilution to give a final 1/1000 dilution of the stock BCG at 1×10^5/ml and then 5µl of the 1 in 1000 dilution was added to 5000µl of RPMI (a further 1/1000 dilution) to prepare the working solution at 1×10^2/ml or 100 CFU/ml. A volume of 300µl of the working stock solution contained 3×10^1/ml (30 CFU).

Preparation of Direct-to-MGIT BCG viability control tubes

Two mycobacterial growth indicator tubes (MGIT) were supplemented with 800µl PANTA enrichment. First, 300µl of BCG master mix was added to each of the two control tubes. Another 200µl of PANTA/OADC enrichment (Becton Dickinson, UK) supplemented Middlebrook 7H9 from a spare tube was added to each tube to make the total volume added to the tube 500µl as per the manufacturer's recommendation. The control tubes were then placed in the BACTEC MGIT 960 instrument. These duplicate MGIT tubes were inoculated on day 0, with the same volume of BCG (300µl) as added to the sample cultures. These tubes act as control tubes, as sample net growth values are normalized to the growth of their corresponding control. This also allows variability between different assay runs using the same BCG stock to be estimated.

BCG and cells co-culture

Three hundred µl of BCG master mix was added in labelled 2ml screw capped tubes and equal volumes of cell suspension containing 1×10^6/ml cells were added and mixed. The tubes were placed on a 360°C tube rotator inside a 37°C incubator for 96 hours. In duplicate tubes, only cells and media were incubated, which acted as the negative control.

Preparing the samples for Mycobacterial Growth Inhibition

After 96-hours incubation, cultures were centrifuged in a bench top microcentrifuge at 12000 rpm for 10 minutes. About 500µl of supernatant were removedmaking sure that the pellet remained intact and the supernatant stored at -80ºC. The PBMCs were then lysed with 100µl tissue culture grade sterile water (Sigma Aldrich, UK). The tubes were

vortexed for 30 seconds and incubated at room temperature for 5 minutes and the process was repeated twice. Four hundred μl of PANTA/OADC enrichment broth were added to the lysate. Tubes were vortexed again for 30 seconds and then the cell suspension was then transferred to a BACTEC MGIT tube supplemented with PANTA antibiotics and OADC enrichment broth. The tube was then placed in a BACTEC MGIT 960 machine and incubated until growth/ time to positivity was detected.

Interpretation and analysis of the assay

To calculate the net growth, the mean TTP for matched duplicates of the samples and controls was converted to an initial mycobacterial inoculum volume. For that a standard curve was prepared based on the BCG stock titration. The equation of standard curve was x= (y-c)/m, where y= TTP, m= slope, c= y intercept. The volume is then converted to CFU by multiplying it by the CFU/μl value of the stock and the result is then log transformed.
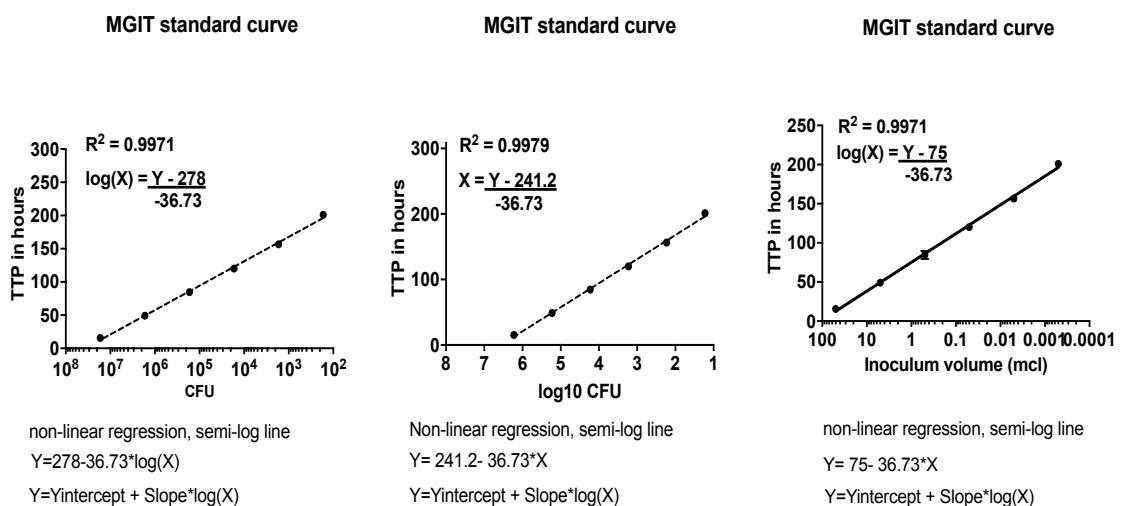
Figure 2.2 : MGIT standard curves for *Mtb*
> A standard curve was created using inoculum volume, CFU and log10 CFU against TTP values. The calculated equation was y=m(lnx)+c, where y= TTP, m=slope and c= y intercept (non-linear regression, semi log line).

	Equation	Slope	Intersect
Curve equation non-linear (volume)	y=-36.73log(x)+ 75	-36.73	75
Curve equation non-linear (CFU)	y=-36.73log(x)+ 278	-36.73	278
Curve equation non-linear (log 10 CFU)	y=-36.73log(x)+ 241.2	-36.73	241.2

Table 2.1: MGIT standard curve equations.

To determine inoculum volume, the following formula is used in a Excel file. $10^{(TTP-y\ intercept)}$/ slope. For example, if TTP is 204 hours, then the inoculum volume will be $10^{(204-75)}$/-36.73. CFU and Log_{10} CFU can also be calculated using the formula generated from the curve. Relative growth is calculated by subtracting the Log_{10} CFU of sample from Log_{10} CFU of the control.

2.3 Flow Cytometry

Intracellular cytokine staining (ICS) of stimulated PBMC followed by flow cytometric analysis was performed to detect the T cell immune responses and cytokine responses. ICS enabled the simultaneous detection of CD4 and CD8 positive T cells and secretion of cytokines such as IFNγ, TNFα, IL-2 in PPD and ESAT-6/CFP10 stimulated PBMC from uninfected individuals, helminth-infected patients, LTBI individuals and patients coinfected with LTBI and helminths.

The aims were

- To measure CD4+IFNγ+T cells in PPD and ESAT-6/CFP10 stimulated PBMCs from uninfected individuals, helminth-infected patients, LTBI individuals and patients coinfected with LTBI and helminths.
- To measure the production of IFNγ, TNFα, IL-2 by CD4+ and CD8+ T cells after stimulation with PPD, ESAT-6/CFP10
- To detect the frequency of CD4+IFNγ+T cells in stimulated PBMCs after anthelmintic treatment in helminth infected and LTBI-helminth infected patients.

Marker	Fluorophore	Clone	Company	Catalogue no
Surface antibody				
CD14	PE	61D3	Ebiosciences	12-0149-42
CD19	Eflour450	HIB19	Ebiosciences	48-0199
CD4	APC-H7	RPA-T4	BDBiosciences	560158
Intracellular antibody				
CD3	Horizon BV510	UCHT1	BD Biosciences	563109
CD8	APC	SK1	BD Biosciences	344722
IFN- γ	Per-CP-Cy5.5	45.B3	Biolegend	502526
TNFα	PE-Cy7	Mab11	BD Biosciences	557647
IL-2	FITC	5344.111	BD Biosciences	340448

Table 2.2: Panel of antibodies used.

FMO controls

Fluorescence minus one (FMO) controls contain all the fluorescent antibodies except the one of interest.

Antibody titrations

To determine the optimum antibody titres required to stain the PBMC samples, all the antibodies were titrated in 5 dilutions with the manufacturer's recommendation as the top dilution. Cells from healthy donors at LSHTM were used for titrating antibodies. Unstained cells were used as negative controls. The median fluorescence intensity (MFI) of positive and negative populations was then calculated. Then the staining index, named as a signal to noise ratio was calculated for each antibody and a scatter plot was obtained for the log antibody concentration versus the staining index.
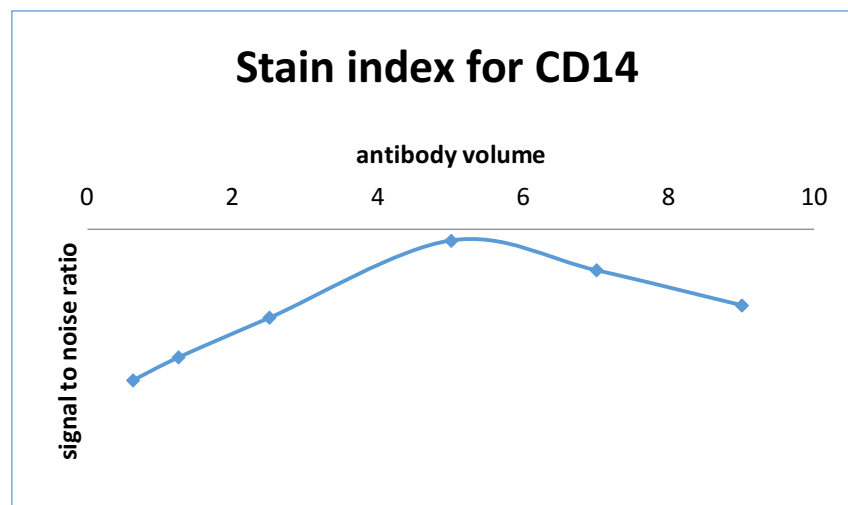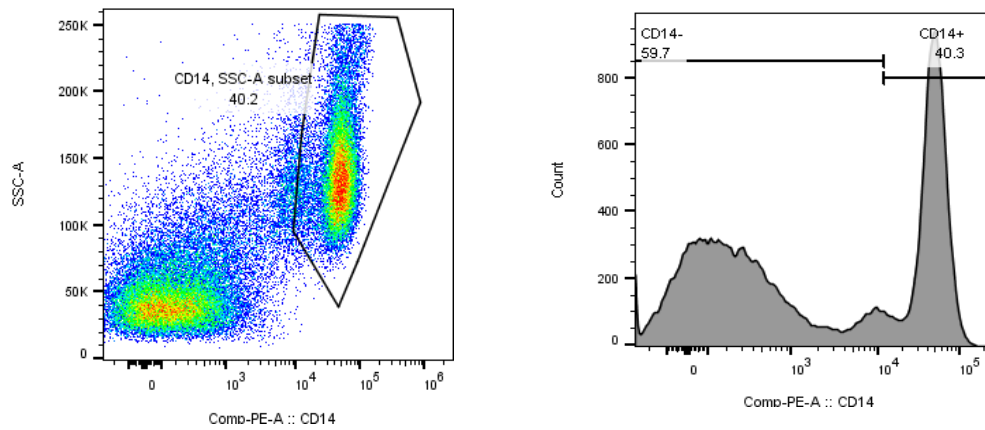
Figure 2.3: Antibody titration to determine the optimum amount of antibody required for single stain. In the example, 5 µl of CD14 antibody is the optimal volume to be used in the 250µl of final volume of staining.

PBMC stimulation assay

Frozen PBMC were thawed and used in this experiment, that were previously cryopreserved at -80ºC in freezing medium (RPMI 1640 (Invitrogen) +20% foetal calf serum (FCS) (Sigma) +10% dimethylsulphoxide (DMSO) (Sigma). PBMCs were resuspended to around 2×10^6 PBMC/ml R10 (RPMI, 10% FCS, Penicillin/streptomycin, L-glutamine and sodium pyruvate) media in 15 ml Falcon tubes. Ten µl Benzonase nuclease (Invitrogen) (2µl/ml) was added to each tube and the cells rested for 2 hours in a 37ºC incubator with 5% CO_2. PBMC were then washed in pre-warmed R10 media and resuspended to 1×10^6 PBMC/ml R10. One ml of PBMC (1×10^6 PBMC/ml) was aliquoted into labelled 5 ml FACS tubes and was stimulated with media alone, purified protein

derivative (PPD; Statens Serum Institute, Copenhagen, Denmark; 20µg/ml concentration from a stock of 1mg/ml), ESAT-6/CFP10 fusion protein (Lionex, Braunschweig; 10µg/ml concentration from a stock of 1mg/ml) and *Staphylococcus* enterotoxin B (SEB; Sigma-Aldrich, St. Louis, MO; 5µg/ml concentration from a stock of 1mg/ml) and kept in the 37ºC incubator with 5% CO_2 for 2 hours. After 2 hours, Brefeldin A (3µg/ml) and monensin (1/2000 final dilution, 2µmol) were added to block the cytokine secretion. The tubes were then incubated for 18 hours in a 37ºC incubator in 5% CO_2.

Cell surface and intracellular staining

Following overnight incubation, the cells were washed with FACS buffer (0.1% bovine serum albumin (BSA) (Sigma), 0.01% sodium azide in PBS (Sigma) and stained with live dead stain (1:40 dilution with dH_2O) (Invitrogen) and incubated at 4ºC in the dark for 10 minutes. Cells were then surface stained with anti CD4-APC-H7, anti CD19-efluor450 and anti CD14-PE for 30 minutes at 4ºC.

Compensation tubes were also prepared with compensation beads (CompBead, BD Biosciences) individually stained for each fluorophore except for CD19 and CD3 (compensated with cells) during surface antibody incubation. After vortexing the beads, 2 drops of positive and negative beads (each) were added with 2µl of antibody suspended in 100µl of FACS buffer. For cells, in 100µl of FACS buffer, 20µl PBMCs from patients were added in tubes. Compensation tubes were incubated in the dark at 4ºC for 20 minutes.

The cells and compensation controls were fixed and permeabilized with a commercial kit (BD Cytofix/Cytoperm™ Fixation/Permeabilization kit, BD Biosciences, UK) following the manufacturer's protocol. After 20 minutes incubation, the compensation control beads were washed and resuspended in FACS buffer. The cells were finally stained intracellularly with anti-CD3-BV510, anti-CD8-APC, anti IFNγ PerCP-Cy5.5, anti TNFα-PE-Cy7, anti-IL-2-FITC for 30 minutes in the dark at room temperature. Cells were washed with PERM wash buffer (Sigma), resuspended in FACS buffer and filtered prior to acquisition on an LSRII flow cytometer (BD Biosciences).

Data acquisition

After staining, cells were analysed on an LSRII flow cytometer. After PMT voltage setup, compensation matrices were calculated using BD FACS DIVA Software V8.0.1 (BD Biosciences, UK). One million events were routinely collected. Doublets were excluded from the analysis by the use of a gate defined by the FSC-A and FSC-H parameters that include only single cells. Viable CD3+ T lymphocytes were gated and from this gate CD4 and CD8 populations were separated and finally a cytokine gate added to derive the frequency of cytokine-positive events. All the data were analysed using FlowJo software version 9 (Treestar, Ashland, OR).

The complete gating strategy is shown below.
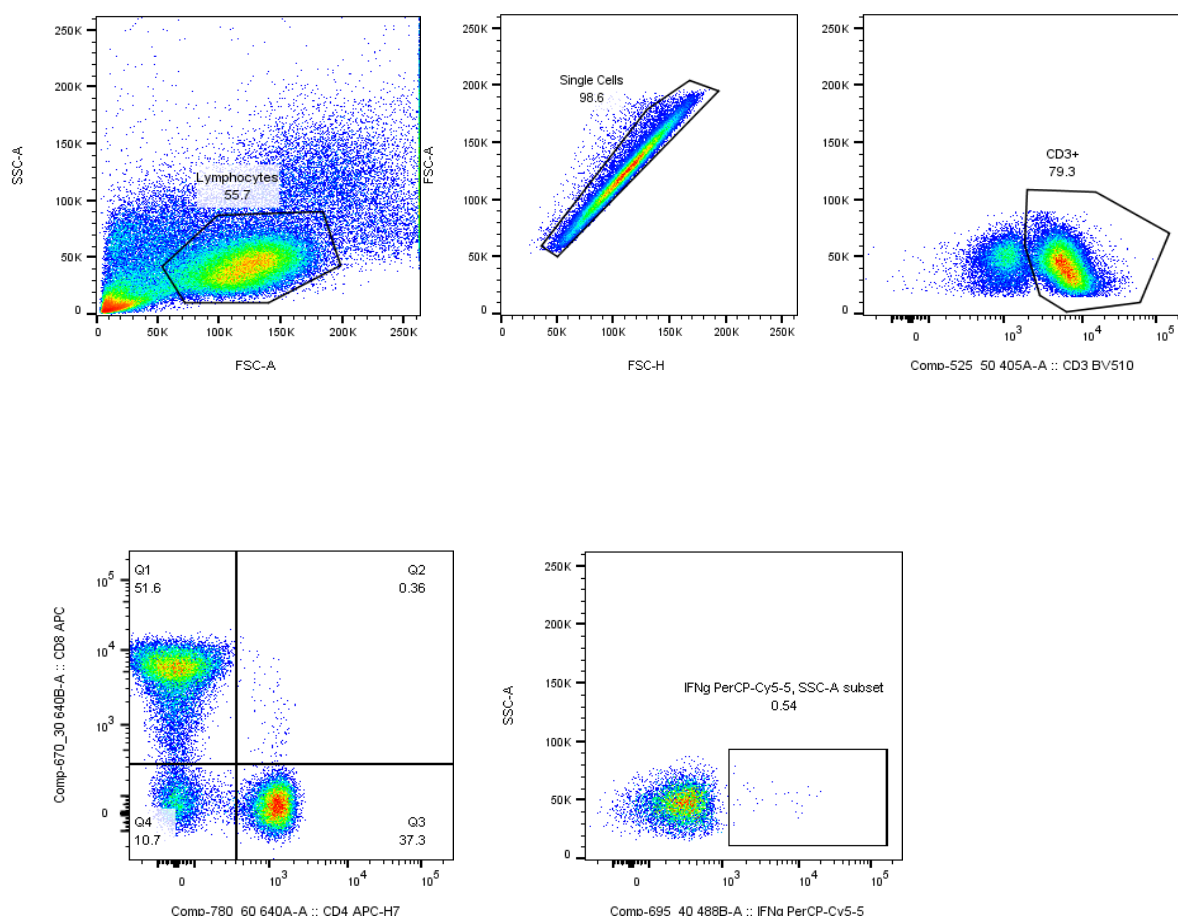
Figure 2.4: The flow cytometry gating strategy. Successive gates were applied to identify lymphocytes, singlet cells, CD3+ T cells and subsequently CD4+ and CD8+ T cells. Evaluation of cytokine production, (IFN γ, TNF α and IL-2) in unstimulated cells and following PPD stimulation was then performed. Figures refer to frequency of cells in the corresponding gate or quadrant.

2.4 *Ex vivo* IFN-γ ELIspot

The ELISpot assay is frequently used for the detection of cytokine production by antigen specific T cells. The ELISpot assay is an effective tool to count the number of cells producing IFN-γ in response to a whole series of antigens, including peptides, peptide pools, proteins and bacterial extracts. Fletcher *et al.,* compared immunological responses in BCG vaccinated individuals with mycobacterial growth inhibition, where IFN-γ responses were measured by ELISpot(394). They showed a significant increase in the IFN- γ ELISpot responses to PPD following primary vaccination and revaccination with BCG.

The ELISpot assay was performed in this study to observe the effect of anthelmintic treatment on the frequency of IFN-γ responses to PPD in LTBI, helminth, LTBI-helminth co-infected and control patients.

The appropriate number of vials were retrieved from liquid nitrogen and put onto ice. To thaw, vials were defrosted quickly in a 37°C water bath and the contents transferred to a 15 ml centrifuge tube containing 12 ml of R10 media. R10 media was prepared using RPMI 1640 (Sigma Aldrich, UK) with 10% L-glutamine, 10% FCS (Biosera, cat S1810), penicillin/streptomycin at 100µg/mL concentration (Sigma Aldrich, UK) and sodium pyruvate (1ml/100ml RPMI) (Gibco, UK). Cells were centrifuged at 439g for 7 minutes and the pellet resuspended in the medium at approximately $1x10^6$ PBMC per ml. Benzonase nuclease (10 units/ml) was added and incubated at 37°C in an incubator with 5% CO_2 for 4 hours.

Ninety-six well polyvinylidene fluoride ELISpot plates (Mabtech, cat MAIPS4510, Millipore, USA) were pre-coated with 15µg/mL of monoclonal antibody 1-D1K against IFNγ (Mabtech, Sweden). Fifty microliters of this solution was added to each well of an ELISpot plate which was tapped until the base of each well was covered. The plate was incubated overnight at 4°C. The following day, the ELISpot plate was washed 5 times with sterile PBS with flicking and blotting between each wash. The plate was blocked by adding 100µl of R10 media at 37°C for 2-5 hours. After 5 hours of blocking, the blocking solution was flicked out and 100µl of PBMC were added at $3x10^6$/well in the plate.

Mtb PPD antigen (Staten Serum Institute) was used at a final concentration of 10µg/ml and SEB (Staphylococcal enterotoxin B) as a positive control at a concentration of

40µg/ml. The final volume was 100µl/well. Two negative control wells contained cells in R10 alone with no antigen.

Next day the media was flicked off and washed 5 times with PBS containing 0.05% Tween 20. Fifty µl of biotinylated anti-IFNγ secondary antibody (Mabtech, Human ELISpot Kit, cat 3420-2A) (diluted 1/1000 in PBS) was added and incubated for 2 hours at room temperature. The plate was then washed 5 times with PBS containing 0.05% Tween 20 (200µl/well). Fifty µl/well of streptavidin (diluted 1/1000 in PBS) was added to the plate, followed by aone hourincubation at room temperature. The plate was washed with wash buffer after one hour and 50µl/well developing buffer was added and incubated for approximately 10 minutes at room temperature until distinct spots developed. The substrate reaction was stopped by rinsing out the plate in tap water 3-4 times and leaving it to dry overnight. Spots were counted on an automated ELISpot reader (AID Germany, software version 5.0.

Results were reported as spot forming cells (SFU) per 1×10^6 PBMC. In the negative control or unstimulated cell wells PBMC must have less than 20 spots/well. For the positive control, ≥ 200 spots was considered as positive. The result was calculated by subtracting the mean number of spots of the unstimulated PBMC from the mean count of duplicate antigen wells. This number is then divided by 0.3 to give a result in SFC/million PBMC. Responses were considered positive if the mean count is at least twice and at least 5 spots more than that of the unstimulated or negative control wells. Data were analysed using Microsoft EXCEL and Graphpad Prism (Version 7.0).

2.5 ENZYME-LINKED IMMUNOSORBENT ASSAY

A sandwich ELISA was used to measure the quantity of total IgE, IgG4, IgA and IFNα. In this technique, the capture antibody is immobilised on a microtiter well. A plasma sample containing unknown amounts of antigen is allowed to react with the immobilized antibody. After the well is washed, enzyme-conjugated secondary antibody is added which is specific for that antibody class and allowed to react with the bound antigen followed by addition of substrate. The coloured reaction product is then quantified using an ELISA reader.

About 20 ml whole blood was collected from each patient during their enrolment visit and during visit 2. From that 1 ml of whole blood was placed in a 2 ml Eppendorf tube and

centrifuged in a micro-centrifuge machine at 5000 rpm for 5 minutes. About 500µl of plasma was collected into a 2 ml tube and stored at -80°C for further analysis. Prior to the ELISA assay, the plasma sample was aliquoted in a micro well plate in volumes of 50µland stored at -80°C to prevent repeated freeze thawing.

A Ready-Set-Go Sandwich ELISA was used for detection and quantification of the antibodies mentioned above in the plasma obtained from the whole blood collected from the clinical samples. A 96 well flat bottom Nunc MaxiSorp® ELISA plate (Affimetrix, eBioscience, UK) was coated with 100µl/well of capture antibody (pre-titrated, purified anti-human IgE/IgG4/IgA/IFNα) in 1:10 dilution of coating buffer (PBS in deionized water) for each antibody. Plates were sealed and incubated overnight at 4°C.

The plates were washed twice with 400µl/well wash buffer with a one-minute interval during each wash step. The plates were blotted on absorbent paper to remove any residual buffer.The plates were blocked with 250µl/well of blocking buffer (Assay buffer concentrate, provided in the kit) for two hours at room temperature. The blocking solution was flicked off and blotted.

Meanwhile, two-fold serial dilutions of a standard were prepared for each antibody and 100µl/well of the serially diluted standard was pipetted into the first two columns of the plates in duplicate. Samples were prepared by diluting test plasma 1:4 and 1:10 in assay buffer. One hundred µl of sample was added per well and 100µl of assay buffer added to the blank wells and the plates sealed and incubated for 2 hours at room temperature on a micro plate shaker at 400 rpm.

The contents were then discarded and the plates were washed 4 times with wash buffer with a one-minute interval between each wash. After blotting, about 100µl/well of diluted detection antibody (pre-titrated, horseradish peroxidase (HRP) conjugated anti-human IgG4/IgE/IgA/IFNα monoclonal antibody) was added, plates were sealed and incubated for 1 hour at room temperature on a micro plate shaker at 400 rpm.

Plates were flicked and washed 4 times as described above followed by adding 100µl/well substrate solution Tetramethylbenzidine (TMB) substrate solution and incubated at room temperature for approximately 15 minutes. About 100µl/well of Stop solution (2N H_2SO_4) was added and optical density (OD) at 450nm was measured using an ELISA plate reader (Microplate Reader, Biorad). Results are shown for samples dilited

1 in 10. As the samples were diluted 1:10, the concentration from the standard curve was multiplied by dilution factor (x10).

Antibody	Sensitivity	Standard curve range	Sample dilution	ELISA Kit and catalog number
IgE	7.8 ng/ml	1000-7.8ng/ml	1:4 and 1:10	eBioscience; 88-50610
IgG4	31.3 ng/ml	2000-31.3ng/ml	1:4 and 1:10	eBioscience; 88-50590-22
IgA	1.6ng/ml	100-1.6ng/ml	1:4 and 1:10	eBioscience; 88-50600-22
IFNα	3.2pg/ml	7.8-500pg/ml	1:4 and 1:10	eBioscience; BMS216

Table 2.3: Antibodies used for ELISA.

2.6 Luminex

The Multiplex MAP Human Cytokine/Chemokine Panel-Premixed 41 Plex-Immunology Multiplex assay was used to analyse plasma /culture supernatants for multiple cytokine and chemokine biomarkers in a Bead-based Multiplex Assayusing Luminex technology. The concentration of 41 analytes in the plasma and culture supernatants included cytokines, chemokine and growth factors: sCD40L, EGF, Eotaxin/CCL11, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFNα2, IFNγ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGFα, TNFα, TNFβ, VEGF. Analyte concentrations were calculated following the manufacturer's instructions (Cat no HCYTMAG-60K-PX41, Merck Millipore, UK).

Plasma and 96 hour MGIT culture supernatants were thawed and aliquoted at 100μl/well in a 96 well micro titre plate and in triplicate. The plate was stored at -80°C. Before use the frozen samples were thawed completely and mixed well by vortexing and centrifuged prior to use in the assay. Neat plasma and culture supernatant was used.

To prepare Antibody-immobilized Beads, the premixed beads in the bottle were sonicated for 30 seconds and then vortexed for 1 minute before use. Sixty μl of the antibody beads was added to the mixing bottle and the volume was made up to 3mL with bead diluents. The beads were then vortexed well.

Before use, Quality Control 1 and Quality Control 2 prepared in 250µl deionized water were prepared. The vials were inverted several times to mix and vortexed. The vial was allowed to sit for 5-10 minutes and then transferred to an appropriately labelled polypropylene microfuge tube.The 10X wash buffer was brought to room temperature and mixed well to bring all salts into solution. Then 60 ml of 10X wash buffer was diluted with 540mL deionized water.

To prepare the serum matrix, 1 ml of deionized water was added to the bottle containing lyophilized serum Matrix, mixed well and left for 10 minutes for complete reconstitution.

Standards were prepared according to the manufacturer's manual by serial dilution. Prior to use, the Human cytokine standard was reconstituted with 250µl deionized water to give a 10,000pg/ml concentration of standard for all the analytes. The vial was inverted several times to mix properly and then vortexed for 10 seconds. The vial was allowed to sit for 5-10 minutes and then transferred to an appropriately labelled polypropylene microfuge tube. Five polypropylene microfuge tubes were labelled as 2000, 400, 80, 16 and 3.2 pg/ml. About 200µl of assay buffer was added to each tube. Serial dilutions were prepared by adding 50µl of the 10,000 pg/ml reconstituted standard to the 2000 pg/ml tube and mixed well. Different standard concentrations were prepared by serial dilution.

Before use in the assay, all the reagents were warmed to room temperature (20-25ºC). A worksheet was prepared to design the sample and standard placement. All the samples were run in duplicate. First, 200µl per well of wash buffer was added to the Luminex plate. The plate was then sealed and mixed on a plate shaker for 10 minutes at room temperature. Wash buffer was decanted by inverting and blotting, and 25µl of each standard was added in the appropriate wells. Two wells of assay buffer were used to determine the background. Twenty five µl of assay buffer was added to all the sample wells and 25µl of serum matrix was then added to the background, standards and control wells and then 25µl of plasma/culture supernatant was added into the appropriate wells. The mixing bottle containing beads was vortexed well and 25µl of the premixed beads were added to each well. The plate was sealed, wrapped in foil and incubated on a plate shaker for 2 hours at room temperature at 400 rpm.

The well contents were then aspirated and the plate rested on a magnet for complete settling of the magnetic beads for 60 seconds before gently decanting and blotting. The

plate was removed from the magnet and washed with 200μl of wash buffer. The wash procedure was repeated twice. Then 25μl of the detection antibody was added to each wells. The plate was sealed, wrapped in foil and incubated on a plate shaker for 1 hour at room temperature at 400 rpm before 25μl of Streptavidin. Phycoerythrin was added to each wells. The plate was sealed, wrapped in foil and incubated on a plate shaker for 30 minutes at room temperature at 400 rpm. The well contents were then carefully aspirated and the plate was washed twice using the magnet as described above. About 150μl of Sheath Fluid was added to each well and the beads were resuspended on the plate shaker for 5 minutes.

The plate was then run on the Luminex^{200} machine and analysed using xPOTENT software. The machine was set to analyse in each well a minimum of 50 analyte-specific beads per region for fluorescence. The MFI was analysed using a 5 parameter logistic (5-PL) algorithm. After the samples were run, a curve fit was applied to each standard curve by adjusting the ratio of the calculated value to the expected value of each standard between 80 and 120% following the manufacturer's instruction. The concentrations of cytokines were then determined by interpolation from the standard curves, those that were outside the standard curve but within the calculable limits was estimated by extrapolation and marked with an asterisk by the software. Concentrations of cytokines that were above or below the available detection limits were marked as Out of Range (OOR) OOR>orOOR< respectively. When the curve fitting was completed, the results were exported into an Excel spreadsheet labelled with the plate number and date of run.

2.7   Real time PCR assay

Using real time quantitative reverse transcriptase (qRT-PCR) assay, the mRNA expression levels for genes- Indoleamine 2,3-dioxygenase 1 (IDO-1), CD14, Arginase 1, Hypoxia inducible factor (HIF) HIF1α, HIF2α, IL-10, CXCL10, CD163 and CD86 were determined in blood samples obtained from controls, helminth infected, LTBI and LTBI-helminth co-infected patients. In qRT-PCR, RNA was first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA. The cDNA was then used as the template for the qRT-PCR reaction. The qRT-PCR assay included total RNA extraction, cDNA synthesis and qRT-PCR assay.

Collection and storage of blood in RNA stabilisation tubes

Following enrolment and at visit 2, 3ml blood were drawn into Tempus™ blood RNA tubes (Applied Biosystems, USA) from patients of all four groups in the clinic. These tubes contain RNA Stabilizing Reagent and RNA expression is, therefore, stable in the collection tube for up to five days at room temperature (18-25°C). Immediately after the Tempus tube was filled, blood was stabilised by shaking the tube vigorously for 10 seconds to ensure that the stabilising agent inside the tube made uniform contact with the blood sample. After collection, these tubes were sent to the LSHTM laboratory (within 4-6 hours of collection) The tubes were then frozen at -80°C for prolonged storage.

Extraction of *ex vivo* RNA

Tempus tube blood RNA extraction was performed using the Tempus Spin RNA Isolation kit (Applied Biosystems, USA). Tempus tubes were thawed for 30 minutes in ice and then brought to normal temperature (18-22ºC). After thawing, the tubes were inverted 5-10 times. The caps from the tubes were removed and the blood was poured into clean 50mL Falcon tubes. Blood samples were washed with PBS and vortexed vigorously for 30 seconds to ensure proper mixing of contents. Then the tubes were centrifuged for 30 minutes at 4ºC at 3000 x g. The supernatant was discarded and tubes were left inverted on absorbent paper for 1-2 minutes. Then 400µl of RNA Purification Resuspension Solution was added to the RNA pellet and vortexed briefly. The resuspended samples were kept on ice until the next steps of purification. A RNA purification filter was inserted into a collection tube. The filter was pre-wetted with RNA purification wash solution. Cell lysate was pipetted directly into the filter and centrifuged at 16,000 x g for 30 seconds and the wash step and centrifugation were repeated twice. DNA digestion was performed as part of the extraction method using RNase-Free DNase Set (Qiagen, Germany). This treatment is required for complete removal of genomic DNA. This set contains RDD buffer and RNase free DNase and contains 1500 Kunitz units of lyophilized enzymes. Eighty ul of the solution (DNase in DNase buffer, 1 in 6) was added to the samples before incubation at room temperature for 15 minutes. DNase was washed away with further washes prior to the elution of RNA. The Nucleic Acid Purification Elution solution was added in tubes which were incubated for 2 minutes at 70°C in Eppendorf Thermomixer Compact (Eppendorf,

USA) without shaking and then centrifuged. Ninety µl of eluted RNA was collected in newly labelled collection tubes. The total RNA extracted by this method was stored at -80°C for further cDNA synthesis and qRT-PCR.

RNA quantification

Extracted mRNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK). This spectrophotometer can measure 1µl RNA elute with high accuracy and reproducibility. The manufacturer's protocol was followed and after quantification sample concentrations are given in ng/µl based on absorbance at 260 and 280 nm. The 260/280 ratio is used to assess the purity of extracted RNA and a ratio of ~2.0 is generally accepted as "pure" for RNA. Samples were diluted to 200ng total RNA in 11µl water for the downstream assay.

First-Strand cDNA synthesis

Complementary DNA (cDNA) was synthesized from the extracted RNA using Superscript III (AB Applied Biosystems, UK). Extracted RNA in tubes were thawed on normal ice. Calculated volumes of water and RNA were added to PCR tubes. Meanwhile, the PCR machine (C1000 thermal cycler, BIO-RAD) was turned on to heat the lid up to 105° C and a program created on the machine. In a nuclease-free microcentrifuge tube (Ambion, USA) Master Mix 1 was prepared using 1µl of 200ng/µl of Oligo(dT) and 1µl of 10mM dNTP each per tube and vortexed briefly. Two µl of Master Mix 1 was added to each PCR tube, making the total reaction volume of 13µl. The PCR tubes were heated for 65° C for 5 minutes and incubated on ice for 1 minute. During that 5 minutes interval Master Mix 2 was prepared using 4µl of 5X First-Stranded Buffer,1µl of 0.1M DTT, 1µl of RNaseOUT (40 units/µl) (Recombinant RNase inhibitor, Invitrogen, USA) and 1µl of SuperScript^{TM} III RT (200 units/µl) (Invitrogen, USA) each per sample. Seven µl of the Master Mix 2 was added to each tube and mixed by brief vortexing. The samples were incubated at 50° C for 1 hour and inactivated at 70° C for 15 minutes and chilled at 4° C. The tubes were then transferred for freezing at -80° C.

Choice of control gene

To perform suitable reliable gene expression quantification analysis, reference genes need to be validated. Reference (or normalizer) genes are defined as those with a stable expression under previously defined conditions, thus appropriate to quantify gene expression levels of specific targets. Previously three reference genes TATA box binding protein (TBP), Human acidic Ribosomal Protein (HuPO) and Hypoxanthine phosphoribosyltransferase (HPRT) were chosen as housekeeping genes. These reference genes have been described in whole blood and PBMC samples to have minimum variability and stable expression (402). In this experiment, HPRT was stably expressed in all the clinical samples and was expressed more than TBP and HuPO. So HPRT was chosen as the housekeeping gene that was assigned as the endogenous control in the experiments. In this experiment, another gene was used as normalizer- CD14. CD14 is stably expressed in monocytes and macrophages and monocytes play an important role in the growth, survival and replication of the mycobacteria. CD14 was used in this experiment to normalize mRNA for monocyte frequency. The variation in the expression of these two genes may reflect that they may be not be constantly expressed or there might be differences in expression stability.
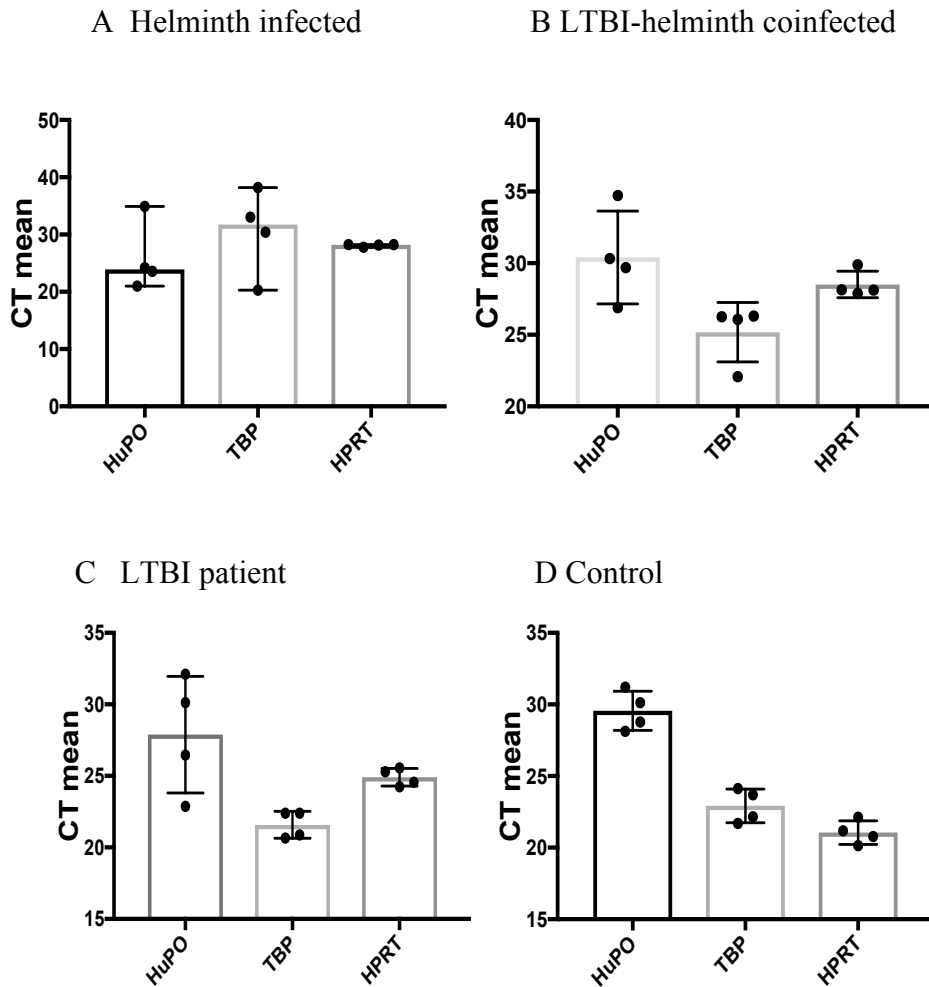
Figure 2.5: Variability in expression of three reference genes (HuPO, TBP and HPRT). Gene expression was assessed in helminth (A), LTBI-helminth coinfected patients (B), LTBI (C) and controls (D). HPRT showed less variability in the pattern and level of expression.

Primers used

All the primers were designed using Primer3 software (403). The primers for CD163 and CD86 were designed by Dr. Jacqueline M Cliff and Dr JiSook Lee and for HPRT was designed by Dr.Martin Holland. The mRNA sequences were obtained from the National Centre for the Biotechnology Information (NCBI) data bank (https://www.ncbi.nlm.nih.gov).Primers between 18-20-24 nucleotides in length were designed across intron/exon boundaries on the mRNA sequence. The optimal design parameter were included as-the Primer Tm 62-63.5-65° C, Primer GC% 30-50-70, maximum self-complementarity 5.00 and maximum 3'self-complementarity 3.00 were

also considered when designing the primers. All primer sequences were blasted on the NCBI data bank to confirm their specificity. A 100μM primer stock solution was prepared according to the Manufacturer's recommendation, by dilution in water the working stock of 10.0μM prepared and kept at -20ºC until used. All the primer pairs were purchased from Sigma-Aldrich.

Primer	Sequence	Length	Tm (ºC)	% GC ratio	Amplicon size (bp)	Accession number
CD163 F	ACTGCAAGAACTGGCAATGG	20	65.2	50	136	NM_004244.5
CD163R	CCATGCTTCACTTCAACACG	20	64.4	50		
CD 86F	CAGGGAGGGGTTTTGGTGAT	20	67.1	55	130	NM_001206924.1
CD86 R	CAGCCAGGAGTGGAAAGTCC	20	66.1	60		
HPRT F	CCTGGCGTCGTGATTAGTGA	20	66.8	55	146	NM_000194
HPRT R	CCCTTTCCAAATCCTCAGCA	24	65.7	41.6		
IDO-1 F	AGGCAACCCCCAGCTATCAG	20	67.4	60	150	NM_002164
IDO-1R	GCATGTCCTCCACCAGCAGT	20	67.8	60		
CD14-F	GGAAGAGAGGTGGGGAGGTG	20	67.2	65	87	NM_001174104
CD14-R	TCGGCTGCCTCTGACAGTTT	20	67.3	65		
Arginase 1 F	ATTCCCGATGTGCCAGGATT	20	67.6	50	89	NM_001244438
Arginase 1R	CCAGGGTCCACGTCTCTCAA	20	67.7	60		
HIF 1α F	GCCGCTGGAGACACAATCAT	20	67.5	55	112	NM_001243084
HIF 1α R	TGGGTGAGGGGAGCATTACA	20	67.7	55		
HIF 2α F	GTCGTCAGCCCACAAGGTGT	20	67.6	60	109	XM_011532698
HIF 2α R	GGGCACGTTCACCTCACAGT	20	67.6	60		
IL-10F	TCCATTCCAAGCCTGACCAC	20	67.4	55	97	NM_000572
IL-10R	GCAGTTAGGAAGCCCCAAGC	20	67.0	60		
CXCL10 F	ACGCTGTACCTGCATCAGCA	20	67.0	55	93	NM_001565
CXCL10R	CGTGGACAAAATTGGCTTGC	20	67.5	50		

Table 2.4: Primer sequences and properties of primers used in the qRT-PCR experiments.

qRT-PCR/ Real-time quantitative PCR

PCR was performed using SYBR-GREEN (Applied Biosystems) on an ABI Prism 7500 Fast machine, followed by melt curve analysis. qRT-PCR was run using the target genes CD163, CD86, IDO-1, CD14, Arginase 1, HIF1α. HIF2α, CXCL10, IL-10 and IL-4 and reference gene HPRT to ensure better quality RNA. During the initial experiments, the primers for IL-4 did not work well for the clinical samples- as they did not amplify as

expected or sometimes the expression was too heterogeneous in individual samples. So IL4 was not included in the experiments afterwards.

For the PCR reaction, a Master Mix was prepared in a 1.5μl Eppendorf tube. The mixture comprised of RNase free water (Ambion, USA),10μl Fast SYBR Green Master Mix (Applied Biosystems, USA) and forward and reverse primers (1μl of 10μM primer concentration) to make a reaction mix volume of up to 15μl. cDNA was diluted to 1 in 10 and 5μl of the diluted cDNA added to the wells of a 96 well PCR plate (FrameStar Fast Plate, 4titude, UK). An Eppendorf Combi-tip Repeating Pipettor was used to dispense 15μl of the reaction mix to each well. Negative controls were included without reverse transcriptase, to exclude genomic DNA contamination. The plate was sealed with adhesive sealing sheets and then spun in the centrifuge very briefly to mix the contents of the wells properly and to remove air bubbles. The plates were first loaded into the plate holder in the instrument. A plate lay out was designed in the software and target genes and endogenous control and reference sample was assigned. The run method in the machine was set. The thermal profile consisted of a holding stage, cycling stage and melting curve stage. The conditions set were 95 for 15 seconds, followed by 40 cycles of 95 C for 15 seconds and 60C for 60 seconds. The primer-dimer formation was checked by melt curve analysis.Relative gene expression quantification was achieved using a relative Ct method and analysed using Fast 7500, version 2.3 (Applied Biosystems 7500 Fast Real Time PCR System).

Calculating the relative gene expression

The relative gene expression was analysed by using the $2^{\Delta\Delta CT}$ method. The C_T value is the threshold number for the amplification of the target gene. The C_T values were obtained for the target gene, and control gene for each patient at two different time points- pre and post treatment. An average of all the Ct values of controls were calculated. The difference in C_T value was obtained by subtracting the C_T of the target gene from the C_T of the control gene and designated as ΔC_T. To compare the relative target gene expression in LTBI, helminth and LTBI-helminth coinfected patients, $\Delta\Delta C_T$ value was calculated by subtracting ΔC_T of the controls from the ΔC_T of LTBI, helminth and LTBI-helminth coinfected groups. The fold change was obtained by using the formula $2^{-\Delta\Delta CT}$.

To compare the modulation in gene expression in helminth and LTBI-helminth coinfected patients after completing the anthelmintic treatment, $\Delta\Delta C_T$ value of pre-treatment samples (V1) were subtracted from the post treatment samples. The fold change for target gene expression in patients from the pre-treatment level was calculated using the formula $2^{-\Delta\Delta CT}$.

Statistical analysis

Differential gene expression in different groups (ΔC_T) were compared using two-way ANOVA. ANOVA was followed by a Bonferroni post–test to compare the mean of all groups with one another. To compare the fold change of each of the target gene in LTBI, helminth and co-infected groups compared to control samples two-way ANOVA was used followed by Dunnett's post-test. The gene expression modulation after anthelmintic treatment in helminth and LTBI-helminth co-infected group were observed using a paired t test- Wilcoxon matched-pairs signed rank test. Heat maps were created using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

Chapter - 3   Characterization of the migrant cohort

3.1   Characterization of the migrant cohort

A total of 155 patients were enrolled in this study since 2013. Of these, 32 were latently infected TB patients (LTBI), 36 helminth infected patients, 16 LTBI- helminth coinfected patients and 32 controls. The patients in this cohort were recruited on their first visit to the clinic when they received anthelmintic treatment if required, and were requested to come back at least 4 months later. Those not treated for helminths were also asked to come back 4 months later. The rate of follow-up at visit 2 was 43.7% for LTBI, 38.8% for helminth infected, 37.5% for controls and 50% for coinfected patients. The time intervals between V1 and V2 were 6 months (4-9 months) for controls, 6 months (3-11 months) for LTBI patients, 4.8 months (4-8 months) for helminth infected patients and 5.7 months (4-8 months) for LTBI-helminth coinfected patients.

This recruitment was a continuation of a project funded by the EU Consortium IDEA that ran from 2011-2015. In that period, 108 helminth infected patients, 48 LTBI-helminth coinfected patients, 58 LTBI patients and 29 controls were recruited till December 2014. The rate of follow-up for this recruitment at visit 2 was 77.5% for LTBI, 73.1% for helminth infected, 58.6% for controls and 85.4% for coinfected patients. Dr. Frederic Toulza was working on the IDEA project and finished the immunological tests for IDEA on those samples in 2014. He investigated the anthelmintic treatment modulation of antimycobacterial immunity in this cohort. He observed the frequency of CD4+IFNγ+T cells measured following stimulation with PPD and ESAT-6/CFP-10 antigen and measured the concentration of IFNγ in culture supernatants using ELISA and multiplex bead array. He also observed the changes in CD4+FoxP3+T cells (Treg) after anthelmintic treatment. The patient recruitment was extended afterwards for this study to use some more immunological methods and to look at the host cell ability to control the growth of mycobacteria in these groups of patients along with other parameters like cytokines, antibodies, etc. Since the patient data for the whole combined cohort was available, the epidemiological data and the haematological parameters were analysed for all these recruited. In this chapter all demographic data and haematological analysis will be discussed for all the patients recruited since 2011 and immunological analyses will be described in the consecutive chapters for the patients recruited since January 2014.

A total of 217 males and 137 females were recruited overall and the male to female ratio was 2:1 (Table 1). The median age for males was 41 years (21-84 years) and for females36 years (20-79 years). The mean ages of the coinfected males and females were higher than the controls and slightly higher in LTBI males, helminth infected male, and females than the controls.

In this cohort, in total 284 patients were BCG vaccinated. Sixteen patients were not vaccinated and 54 could not confirm their BCG vaccination status. Among all the LTBI-helminth coinfected patients,12 could not confirm their BCG vaccination status and the rest were BCG vaccinated. Among the controls, 2 were not vaccinated. In LTBI patients, 5 patients were non vaccinated and the vaccination status was not known for 12 patients. Most of the helminth infected patients were BCG vaccinated but vaccination status was not known for 28 patients (Table 3.1).

Two hundred ninety-eight patients were known to be HIV negative. Sixty-two patients were not sure about their HIV status or had never been tested for HIV. Two male patient with helminth infection and one coinfected patient could not confirm their HIV status, also 2 controls (one male and 2 females) had never been tested for HIV. It is not considered appropriate to test for HIV in this setting unless this is required clinically.

3.1.1 Migrant status

The largest group of patients recruited in this study were from Bangladesh followed by Nigeria, India, Philippines, Kenya, Ghana, Zimbabwe, Somalia, Eretria, Ethiopia, Pakistan, South Africa and Sierra Leone (Figure 3.1). The study population was very heterogeneous ranging from few years' spent in the UK to migrants that had been living in UK for more than 20 years. The mean length of stay in UK for these migrants was 17.3 years for controls and the stay was similar for LTBI and helminth infected patients. However, for LTBI-helminth coinfected patients the mean length of stay in UK was somehow longer than the other groups (23 years).
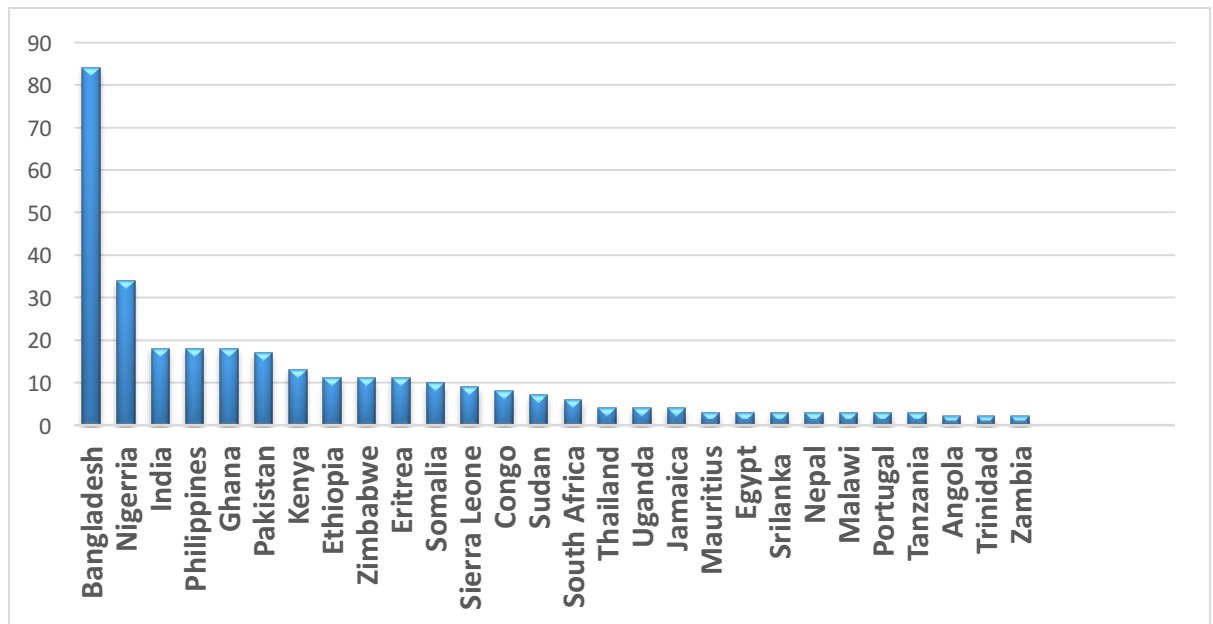
Figure 3.1: The country of origin of migrants recruited in the study (in the combined cohort).

In the control groups, the majority of the patients were from Bangladesh (32%) followed by Nigeria (16%), Ghana (10%) India (8%), Kenya (7%), Pakistan (7%). In the LTBI group of patients, the majority of the patients were from Bangladesh (23%) followed by Nigeria (17%) Philippines (12%), India (6%), Ethiopia (6%).In the helminth infected patients, the majority of the migrants were from Bangladesh (45%), followed by Nigeria (7%), Ghana (6%), Zimbabwe (5%), (Figure 3). In the LTBI-helminth coinfected patients, the migrants were predominantly from Bangladesh (31%), Nigeria (11%) and Ghana (8%).

3.1.2 Clinical data and history of treatment

In this cohort, 14 patients had a previous history of TB. Four patients could not remember their previous TB history and BCG vaccination status. All the other patients were born in endemic countries like Bangladesh, Ethiopia, Somalia, Philippines, and Jamaica, with the majority from Bangladesh. These patients had been living in the UK for more than 10 years. Four of them were coinfected with LTBI and helminth infection and presented with helminth infection in the clinic and were infected with multiple helminths. Two were not BCG vaccinated; they had a previous episode of TB more than 10 years earlier and had treatment.

Of the 90 LTBI patients in this cohort, 33 of them received treatment for LTBI after visit 1. Of the LTBI patients, 25 were HCWs. Twenty-three HCWs received treatment for

LTBI and two were offered treatment but they declined treatment. None of the LTBI patients included in this study was on treatment for their LTBI at visit 1. The average age of LTBI patients, who received treatment were 33.72 years of age and of the HCWs were of 34.7 years. Previous UK policy was to only offer treatment for LTBI to those aged less than 35 years, although this was recently changed to 65 years.

In our cohort, 40.67% were helminth infected and 18.07% were LTBI-helminth coinfected patients. Of the helminth infected group, 66.6% of the patients presented with Strongyloidiasis, 15.27 % with Schistosomiasis, 4.16% with tapeworm infection, 4.16% with both infections and 3% with Strongyloidiasis, Schistosoma infection and filariasis. Of the 64 LTBI-helminth coinfected patients, 78.1% patients presented with Strongyloidiasis, 14.1% with Schistosoma infection and 3.47% with tapeworm infection.

Most of the helminth infected patients presented with symptoms of helminth infection for example fever, abdominal pain, nausea, diarrhoea, bloating, vomiting, respiratory symptoms like cough, wheeze and cutaneous symptoms like itchy urticarial or maculopapular rash. Any history of urinary symptoms like haematuria or dysuria suggestive of *S.haematobium* infection etc. was also investigated. None of these patients were previously treated for helminth infection. Ten patients with latent TB infection, also presented with symptoms consistent with helminth infection, but microscopically and serologically they were not positive for helminths. Twelve of the control patients also exhibited helminth infection symptoms, but were serologically negative. None of these controls received treatment for helminth infection, except for 3 who were empirically treated with Tinidazole as they had eosinophilia and symptoms of helminth infection but were serologically negative for any helminths.

3.1.3 Laboratory diagnosis of helminth infection

Strongyloides infections were diagnosed by faecal microscopy and stool culture for the demonstration of larvae. For Strongyloides spp, serum samples were analysed by a commercial ELISA for anti-Strongyloides antibodies according to the manufacturer's protocol that uses a variable cut-off based on a low positive serum. This is a solid phase in vitro quantitative assay for the indirect detection of IgG to *S.ratti* somatic larval antigen (Bordier, France). According to the Bordier affinity *S.ratti* kit the test sensitivity is 88% and specificity 100%. There is known to be cross reaction between lymphatic

filariasis, onchocerciasis, loiasis and hookworm in ELISA tests. Strongyloides serology showed 73% sensitivity in travellers and 98% in migrants and the specificity was shown to be 94% in non-endemic area and 77% in person with other parasitosis(404). Faeces samples were also cultured to detect Strongyloides or hookworm using the charcoal culture method.

For anti-schistosome antibodies, an in-house ELISA assay was performed, using the standard clinical diagnostic tests currently in use in the National Parasite Reference Laboratory of the United Kingdom, based at the HTD. A minimum of 0.5ml of serum is required.The ELISA is reported to detect about 96% of *S.mansoni* and 92% of *S.haematobium* infections. The test does not distinguish active from treated infections. The actual time taken to become seronegative post- treatment varies, but in some patients the test may remain positive for over two years after treatment. Positive results are reported at Levels 1 to 6. Levels 1 and 2 are regarded as weak positives; levels 5 and over are strong positives. It is known that patients may become seropositive through contact with cercaria from animal species of schistosomes and probably when harbouring unisexual infection with human species. The schistosomal egg antigen used in the ELISA may give a false positive, cross reactive response in trichinosis cases or with those with hepatitis cases in some instances.

For Schistosomiasis definitive diagnosis is by demonstration of the characteristic ova in clinical material. For *S.haematobium*, a terminal urine sample (the last 10 to 20ml of urine passed) is required. Faecal samples are the best specimens for the detection of *S.mansoni* (and *S.japonicum*) but as *S.mansoni* and *S.haematobium* overlap in geographical distribution and can affect both genitourinary and alimentary systems a terminal urine sample and a minimum of three faecal samples should be sent from all patients being investigated for schistosomiasis when serology is positive.

PCR for Strongyloides spp were performed for selected patients using primer for Strongyloides spp (n=67). Forty patients were seropositive, of these one was positive on microscopy and an additional 5 positive on culture. Of these 6 parasite-positive cases, 4 were PCR positive. An additional 11 were negative on microscopy and culture but PCR positive. PCR had the sensitivity of 66% against culture as gold standard and 38% against serology as a gold standard. Of the 27 who were seronegative, none were positive on microscopy or culture, four were positive on PCR. Of the 4 PCR positive patients who

were seronegative, were all presented with asymptomatic eosinophilia. One of them had symptoms of helminth infection. Three of them were treated empirically with anthelmintic and one of them refused treatment as no infection was detected at that stage.

Strongyloidiasis causes mostly asymptomatic infection and diagnosis of latent infections is difficult due to limitations of current parasitological and serological methods. In uncomplicated cases of Strongyloidiasis, the intestinal worm load is very low and the output of larvae is minimal and irregular, hence the sensitivity of direct observation of larvae decreases considerably (405).The detection rate of conventional methods is low and repeated examination of stool over a number of consecutive days is essential for the diagnosis. Serodiagnostic tests ELISA have exhibited variable sensitivity and specificity in studies for diagnosis of Strongyloides infection. Serology may overestimate the burden of disease, because serology remains positive after resolution of the infection and the presence of the cross reactivity. In different studies, agar plate culture of stool samples has reported to be a sensitive method to detect Strongyloides infection. However, this method requires multiple fresh stool samples and an experienced microscopist. Brown *et al.,* conducted a study of interaction of helminth infestation and HIV in a cohort of 412 HIV-infected people in Uganda, where analysis of a single stool sample underestimated the helminth prevalence, especially in low intensity infections. Thirty-nine percent of patients were diagnosed to have *S.mansoni* infection using formol-ether concentration, Kato-Katz and charcoal culture, but the detection was 49% when all three tests were done along with ELISA for *S.mansoni* (406).

In recent years, some PCR based techniques have been developed and used for detection of different intestinal parasites in faecal samples and in some studies, PCR has been stated as a highly sensitive method for detection of helminth infections. Standardization of these techniques is necessary to overcome the limitations of the current diagnostic methods. The difficulty encountered in this method involves the extraction of DNA from larvae of Strongyloides, due to the presence of a resistant cuticle which is difficult to disrupt mechanically.There may be irregular release of larvae in the faeces and presence of inhibitor of DNA in the environment, in addition, small sample size may lead to false negative results, laborious standardization and expensive equipment is required. The detection rate in the current study was very low, which might be due to low concentrations of parasite DNA in presence of PCR inhibitors.

3.1.4 Eosinophilia in helminth infected patients

Helminth infection is one of the commonest cause of eosinophilia in the returning traveller or migrants. In the UK, about 852 asymptomatic returning traveller had eosinophilia (8%) and in Canada, among 1605 individuals returning from the tropics, 10% had eosinophilia (407). Sometimes migrants are infected with multiple species of helminths with more pronounced eosinophilia. There may be some non-infectious causes of eosinophilia in these groups of patients for example, drugs like beta lactam antibiotics, atopy (asthma, eczema, hay fever) and allergy. There are some malignant causes as well but in these cohorts, malignancy or any immune deficiency was excluded. Most travellers who become infected with Schistosoma spp or Strongyloides spp have a very low worm burden and remain asymptomatic-which makes the routine investigation of exposed travellers of limited benefit. Worms can persist long after migration from endemic countries. Although in these patients in non-endemic settings heavy infection is rarely observed early recognition and treatment of these patients may avoid any consequences. A study conducted in the East London Gastrointestinal Clinic on migrants found that Schistosoma and Strongyloides were the most prevalent helminth species in the patients referred to the HTD with eosinophilia and that 56% of Schistosoma infected patients were asymptomatic. Fifty-six percent of Schistosoma infected patients had an absolutely normal eosinophil count (145).

In this cohort 95 patients, out of 353 patients, had eosinophilia (>0.45 x10^9/ml); the rest of all the patients had a normal eosinophil count. Within the 95 patients, 21 were controls, 13 LTBI patients, 52 helminth infected patients and 12 LTBI-helminth co-infected patients. Fourteen helminth infected patients, 4 LTBI, 12 controls and 6 coinfected patients with eosinophilia, had no symptoms of helminth infection. Some of them had presented with respiratory or allergic symptomsin the clinic. Of the patients with eosinophilia, the majority of the patients were from Bangladesh (25), Nigeria (12), Pakistan (10), Ethiopia (7), India (6) and the rest of the patients were from the African and Asian regions. In this cohort, 62% of the migrants had normal eosinophil counts. In returning travellers, this might be due to the "window period" between infection and egg production, so post-travel screening of travellers, especially stool and urine microscopy and serological tests should be delayed for atleast three months after exposure.

Patients who had eosinophilia and confirmed helminth infections were provided with appropriate anthelmintic treatment. Post-treatment eosinophil counts were obtained from the computerized patient records. After assessment of the clinical symptoms in the clinic, patients were ranked as "improvement", "non-improvement" and "some improvement". Information on the helminth infection symptoms, diagnosis, clinical symptoms, eosinophilia, ethnicity were all explored for evidence of improvement after anthelmintic treatment.

Of the 46 helminth infected patients who exhibited eosinophilia (>0.45 x 10^9/ml) and eosinophilia reduced significantly after the treatment (p<0.0001).The median eosinophil count was higher in those with helminth infection than in the controls from the clinic. The median eosinophil count in the helminth infected group was 0.53 x10^9/ml and the median eosinophil count post treatment was 0.2 x 10^9/ml. There were no significant differences in eosinophil count in controls and helminth infection, as most of the controls recruited in this cohort are either allergic or have some respiratory symptoms and moreover most of them had eosinophilia. Similar observations were also obtained in LTBI-helminth co-infected patients. There were no significant changes in eosinophil count in LTBI infected patients at the two points of recruitment. Interestingly, there was a significant change in eosinophil count between controls and LTBI infected patients (0.5 x10^9/ml Vs 0.3x10^9/ml). Breen *et al.,* demonstrated that, many patients with TB presented no abnormalities in their blood inflammation markers- as in the very initial stages of *Mtb* infection, the blood profile may not reflect the characteristic changes during the progression from infection (408). However, these controls are not absolutely healthy controls, they mostly presented with high eosinophil counts and had some allergic or respiratory symptoms.

(A)
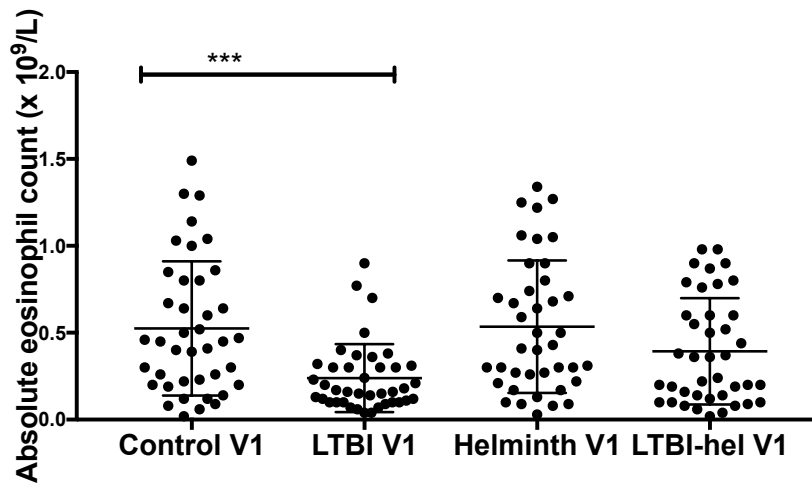
(B)
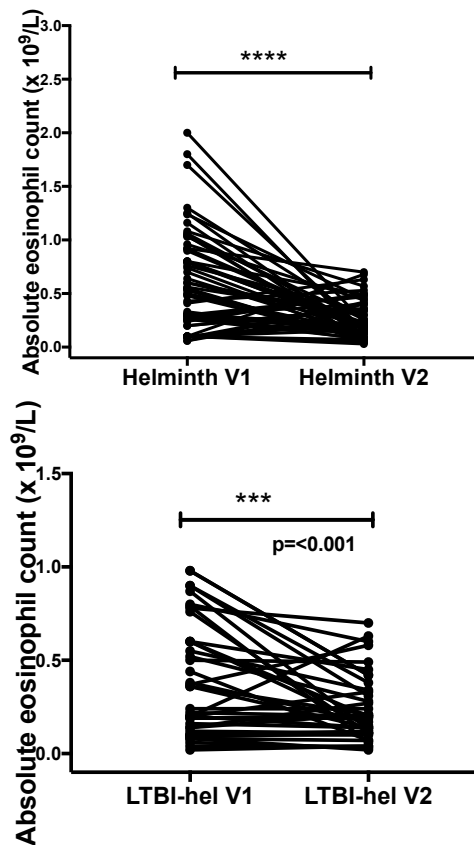
Figure 3.2: Absolute eosinophil count (x 10^9/ml) in controls, LTBI, helminth and LTBI-helminth coinfected patients (n=40). Eosinophil count in controls and LTBI infected patients (n=45) (A), A one-way ANOVA was performed, where *** represents a p value of <0.0007. Bars represent the mean with SD values. (B) Absolute eosinophil count in helminth infections both before and after treatment (n=61) and LTBI-helminth coinfected patients before and after treatment (n=40) (C). A Wilcoxon matched-pairs signed rank test was performed, where *** represents a p value of <0.001 and **** represents a p value of <0.0001.

The patients were tested for IFNγ production to *Mtb* peptides in the QUANTIFERON IGRA test. To determine the correlation between IFNγ production and cell counts, each cell counts are compared with IFNγ production levels (IGRA test). There was no significant correlation between absolute eosinophil count and IGRA values in helminth infected patients (pre-treatment and post treatment) or LTBI infected patients.The absolute eosinophil count was significantly related to the LTBI- helminth co-infected patient's pre-treatment IGRA value (P <0.01). Post treatment IGRA values were not available.
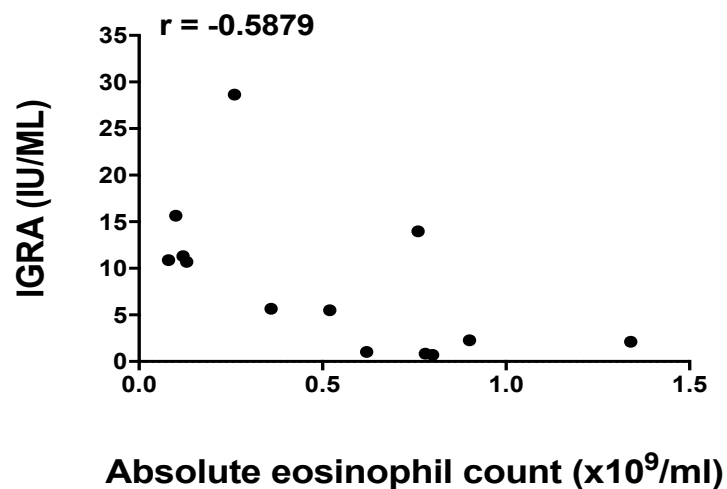
Figure 3.3: Correlation between absolute eosinophil count and IGRA values of LTBI-helminth coinfected patients. Pearson r correlation between absolute eosinophil count and IGRA values of LTBI-helminth coinfected patients. (IGRA test is considered positive if the interferon-gamma response in the TB antigen minus nil value is at least 0.35 IU/mL)

3.1.5 Other blood cell counts in these patients

Polymorphonuclear cells (PMNs) have been implicated in the control of mycobacterial infections, but it is not known whether these cells have direct protective functions. Human and animal studies indicate that neutrophils may play an important role in the transition from innate to adaptive immune responses by producing critical cytokines and chemokines (409)(410) ; although these roles of neutrophils in TB resistance and pathogenesis is still unclear. Monocytes were previously shown to be major effector cells in protecting the host against *Mtb* infection (411).

The absolute count of monocytes was significantly increased in LTBI-helminth co-infected patients (p<0.01). There were no significant changes in the monocyte count in pre and post treatment helminth infected patients, control vs LTBI, helminth and coinfected patients.
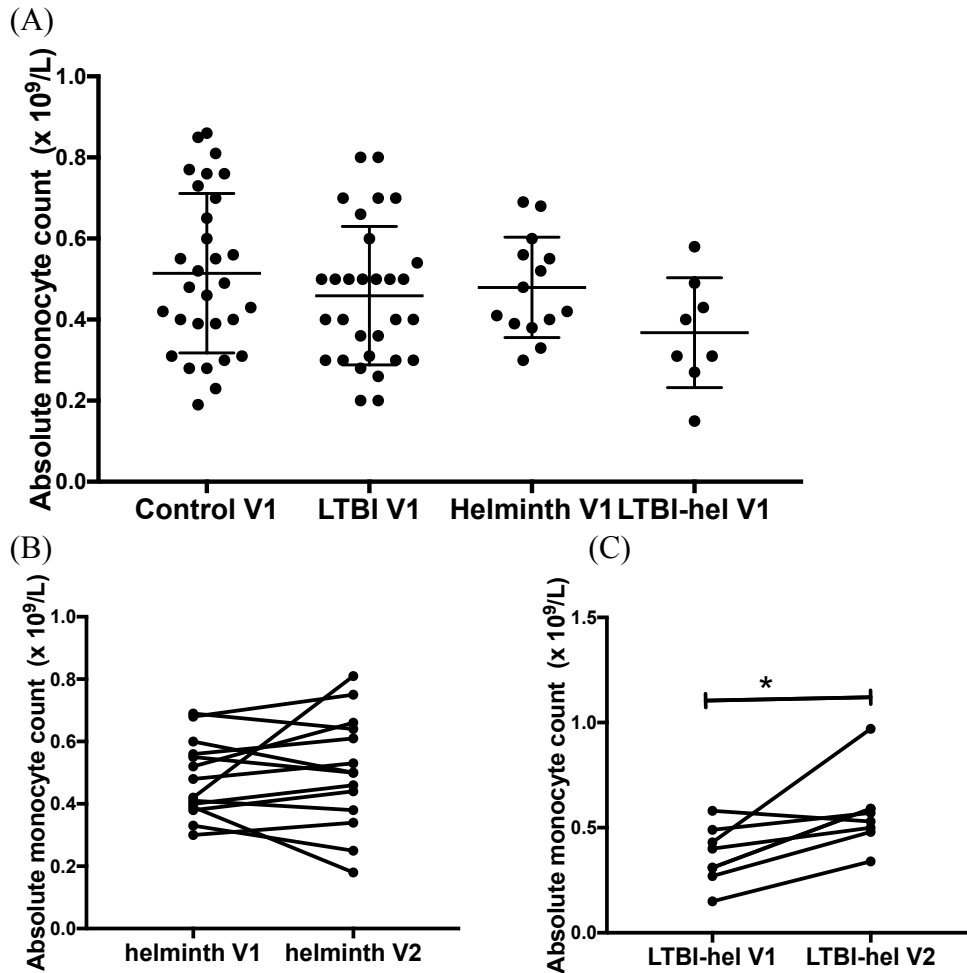
Figure 3.4: Absolute monocyte counts in controls (n=30), LTBI (n=30), helminth infected (n=14) and LTBI-helminth coinfected (n=8) in their visit. A one-way ANOVA was performed. Bars represent the mean with SD values. Absolute monocyte count in patients with helminth infections both before and after treatment (n=14) (B) and in LTBI-helminth coinfected patients before and after treatment (n=8) (C). A Wilcoxon matched-pairs signed rank test was performed, where *represents a p value of 0.01.

The absolute monocyte count was not significantly related to IGRA values in LTBI, coinfected patients (both pre and post treatment, although the number of IGRA results for post treatment co-infected patients was too low to be significant.

Neutrophils are less well studied than other compartment of the host response to *Mtb* infection, probably because of inherent difficulties in working with these cells. The

interaction of neutrophils with macrophages and the downstream effects of T cell activity, could determine the clinical and immunological outcome of infection. This might range from an early innate response to uncontrolled replication, excessive inflammation and primary progressive disease (412).

The absolute neutrophil count was significantly increased following anthelmintic treatment (p<0.01) in LTBI-helminth coinfected patients, although there were no significant changes in the absolute neutrophil count in controls, helminth infected or LTBI patients. The absolute count of neutrophils was not significantly related with any of the clinical groups.

| (A) | (B) | (C) |
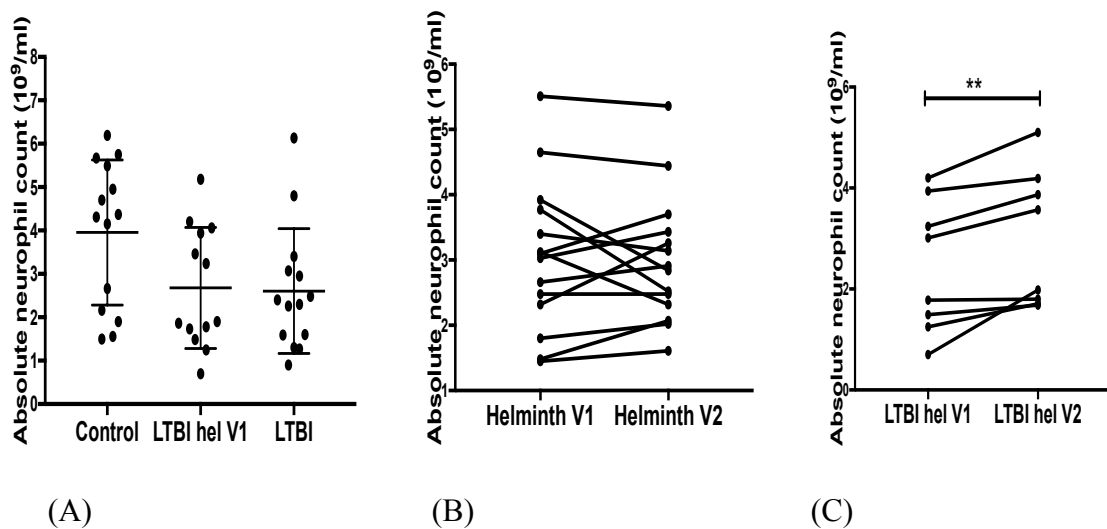
Figure 3.5: Absolute neutrophil counts in the controls, LTBI and LTBI-helminth infected patients before treatment (n=14) (A) in helminth infected patients, both pre and post treatment (n=14) (B), LTBI-helminth coinfected patients before and after treatment (n=8) (C).

There was no significant change in the absolute lymphocyte counts in any of four clinical groups and they were not related to IGRA values in these patients.

3.1.6 Monocyte/lymphocyte (ML) ratio and Neutrophil/lymphocyte (NLR) ratio in the migrants

There are reports that the ratio of monocytes to lymphocytes in peripheral blood correlated with extent of TB disease in both humans and rabbits (413). Sabin and colleagues, between 1921 and 1931 found that the number of monocytes and lymphocytes may be related to TB susceptibilityand showed that the number of monocytes were

increased following experimental infection of rabbits with *M.bovis*(414). We looked at the ML ratio in our cohort. The ML ratio was significantly altered in LTBI-helminth coinfected patients after treatment. There were no significant alterations in any other groups.
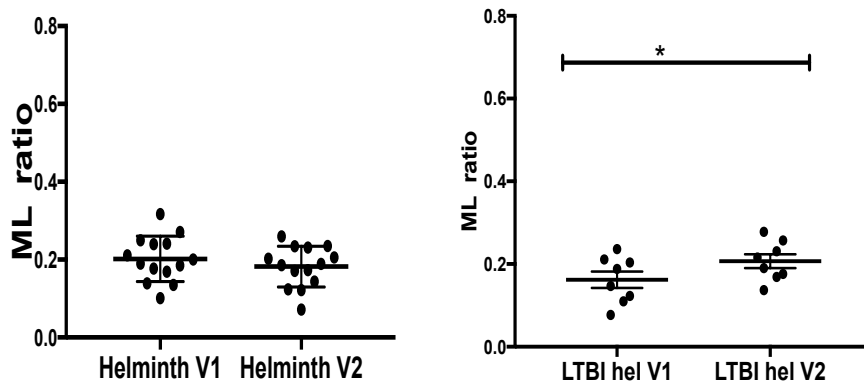
Figure 3.6: ML ratio in controls and helminth infected patients (n=31) (A) in helminth infected patients both before and after treatment (n=14)and in LTBI-helminth coinfected patients before and after treatment (D). Bars represent the mean with SD values. A Wilcoxon matched-pairs signed rank test was performed, where * represents a p value of <0.001.

NLR is determined by dividing the absolute count of neutrophils by the number of lymphocytes in the complete blood count. NLR has emerged as a new marker of inflammation in TB, sarcoidosis etc (415).The NLR in these clinical groups, was not significantly altered in any of four groups and the ratios were not related to IGRA values.

IGRA values were not available for all the post treatment helminth and LTBI-helminth infected patients and so it was difficult to observe a change in the IGRA values in those patients.
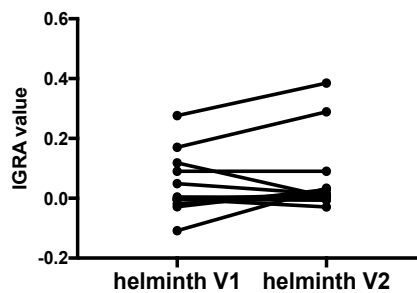
Figure 3.7: IGRA values in IU/ml in helminth infected pre and post treatment patients (n=12). A Wilcoxon matched-pairs signed rank test was performed.

Altered host iron status has been previously identified as a risk factor for progression to TB among HIV-infected individuals, and a number of studies have indicated that *Mtb* iron acquisition plays an important role in TB pathogenesis (416).We looked at the haemoglobin concentrations in these groups of patients. There were no significant changes in the haemoglobin concentration in any of the four of the groups.
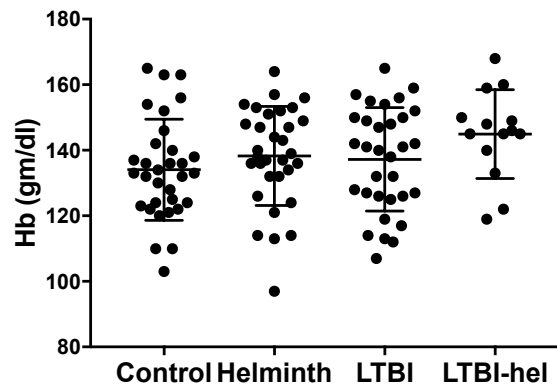
Figure 3.8:   Haemoglobin concentration. Haemoglobin concentrations (gm/dl) are shown in control (n=32), helminth (n=32), LTBI (n=32) and LTBI-helminth coinfected patients (n=14). Bars represent the mean values with SD; a Kruskal-Wallis test was performed where there were no statistically significant differences.

Interestingly, haemoglobin concentration was significantly related to LTBI patients IGRA values (p<0.01) but was not significantly related to any other clinical groups.
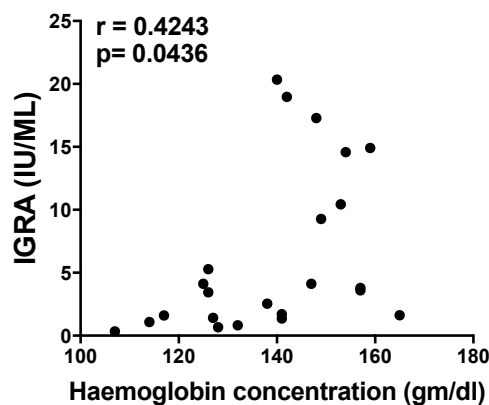
Figure 3.9: Correlation between haemoglobin concentration (gm/dl) and IGRA values of LTBI patients. Pearson r correlation between haemoglobin concentration and IGRA values of LTBI patients.

We also tested if age or sex is related significantly with the IGRA values in these patients. Sex was not significantly related to IGRA values, although age was significantly associated with IGRA results in controls, but not in the other groups.
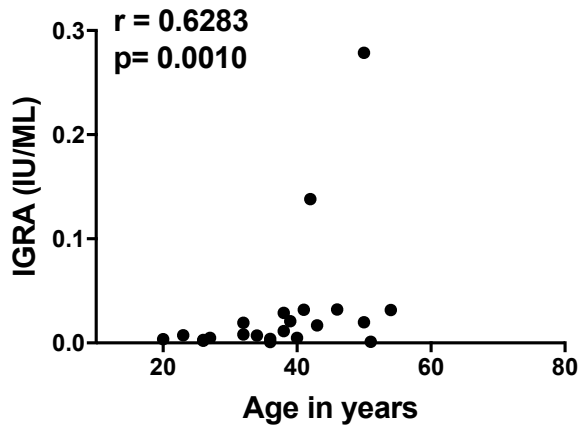
Figure 3.10 : Correlation between age (in years) and IGRA values of controls (n=20). Pearson r correlation between age in years and IGRA values of controls (after outlier correction by ROUT method).

Most of the migrants in this cohort were from Bangladesh followed by Nigeria, India, Philipines, and Ghana. Mean length of the stay in UK was 17-23 years. Twenty-seven percent of the migrants had eosinophila that reduced significantly after anthelmintic treatment. The eosinophil count was correlated significantly with IGRA values in LTBI-helminth coinfected patients. LTBI-helminth infected patients showed significant changes in neutrophil and monocyte count, ML ratio after anthelmintic treatment. Haemoglobin concentration was significantly related to IGRA values of LTBI patients.

Chapter - 4

4.1 T cell responses to *Mtb* in clinical samples

As discussed in the previous chapter, the recruitment in this project was a continuation of the IDEA project funded by the EU Consortium, that ran from 2011 to 2015. In that period Dr. Frederic Toulza was working on the IDEA project and carried out the immunological tests. One hundred eight helminth infected patients, 48 LTBI-helminth coinfected patients, 58 LTBI patients and 29 controls were recruited. He measured the impact of anthelmintic treatment on the immune response to *Mtb* in LTBI in this migrant cohort in London, measuring the frequency of CD4+IFNγ producing T cells, and CD4+FoxP3+Treg cells to assess the potential impact of these cells in helminth infected patients. It was observed that, helminth-infected groups had lower CD4+T cell responses to PPD and ESAT-6/CFP10 than those without diagnosed helminth infection. The memory immune response to TB before anthelmintic treatment and after treatment was also observed and it was shown that anthelmintic treatment increased the memory immune response to *Mtb*. The LTBI-helminth infected group showed a significant increase in the frequency of CD4+ IFNγ+ T cells after anthelmintic treatment when the PBMC from these patients were stimulated with *Mtb* specific PPD and the fusion protein ESAT-6/CFP10. Patients with helminth infection, showed a significant increase in CD4+FoxP3+Treg cells compared to those without helminth infection. There was a significant decrease in the frequency of Treg cells and an associated increase in CD4+IFNγ+T cells and these effects were reversed after anthelmintic treatment (370). With the continuation of the recruitment, it was planned to do more immunological work and to look at how the cells controlled the growth of mycobacteria.

Aims

- To measure the frequency of CD4+IFNγ+T cells in helminth infected patients, and LTBI infected patients.
- To measure the secretion of IFNγ, TNFα and IL-2 by intracellular cytokine staining after stimulating the PBMC overnight with PPD, ESAT-6/CFP10, SEA and SEB.
- To detect the frequency of CD4+IFNγ+T cells in stimulated PBMCs after anthelmintic treatment in helminth infected and LTBI-helminth infected patients.

- To measure the IFNγ responses to PPD, ESAT-6/CFP10 using ELISpot in controls, LTBI, helminth and LTBI-helminth coinfected patients on their pre-treatment visit and second visit.

4.1.1 Results

Intracellular cytokine staining (ICS) of stimulated PBMC followed by flow cytometric analysis was performed to detect the T cell and cytokine immune responses. ICS enabled the simultaneous detection of CD4 and CD8 positive T cells and secretion of cytokines such as IFNγ, TNFα, and IL-2 in PPD and ESAT-6/CFP10 stimulated PBMC from uninfected individuals, helminth-infected patients, LTBI individuals and patients coinfected with LTBI and helminths. PBMC were stimulated with PPD, ESAT-6/CFP or SEB overnight and then stained with a panel of antibodies recognising lymphocyte markers and the cytokines IFNγ, TNFα, and IL-2. Both the pre and post treatment samples were tested at the same time using the same protocol. Helminth infected and LTBI-helminth coinfected patients were requested to come back 4 months after their first recruitment and after they have finished the treatment (V2). The LTBI and control groups were also analysed at least 4 months after the initial visit (V1), although they were not given any anthelmintic treatment.

Following gating on singlet and live cells and the exclusion of CD14 or CD19-expressing cells, single cytokine expression was examined in CD4+T cells. IFNγ expression was detected in all four clinical groups, the median IFNγ expressing CD4 T-cells proportion of subset being 0.31% in controls, 0.41% in helminth infected and 0.19% in LTBI-helminth coinfected patients, which after treatment, was 0.29% in controls, 0.44% in LTBI, and 0.39% in LTBI-helminth coinfected patients in response to PPD (Fig. 4.1).The median IFNγ expressing CD4+T cells in response to ESAT-6/CFP10 in LTBI patients pre and post treatment were 0.23% vs 0.29% and in LTBI-helminth coinfected patients it was 0.18% vs 0.33%.

The frequency of CD4+IFNγ+T cells in LTBI-helminth coinfected patients was lower than that of LTBI infected patients, although the difference was not significantly different. The frequency of CD4+IFNγ+T cells were significantly higher in LTBI patients than in the controls (p<0.005). LTBI-helminth coinfected patients had lower percentages of CD4+IFNγ+T cells before treatment. The result showed that anthelmintic treatment

induces a significant increase in the frequency of CD4+IFNγ+T cells after stimulation with PPD, ESAT-6/CFP10, Schistosoma egg antigen (SEA) or SEB as positive stimulants in LTBI-helminth coinfected patients (p<0.0001). No significant changes were observed in the frequency of CD4+IFNγ+T cells after stimulation by PPD and ESAT-6/CFP10 in LTBI and controls compared to their initial visit. Helminth infected patients had lower proportions of CD4+IFNγ+T cells than the controls in response to PPD before treatment. After anthelmintic treatment the % CD4+IFNγ+T cells increased but this was not significant.
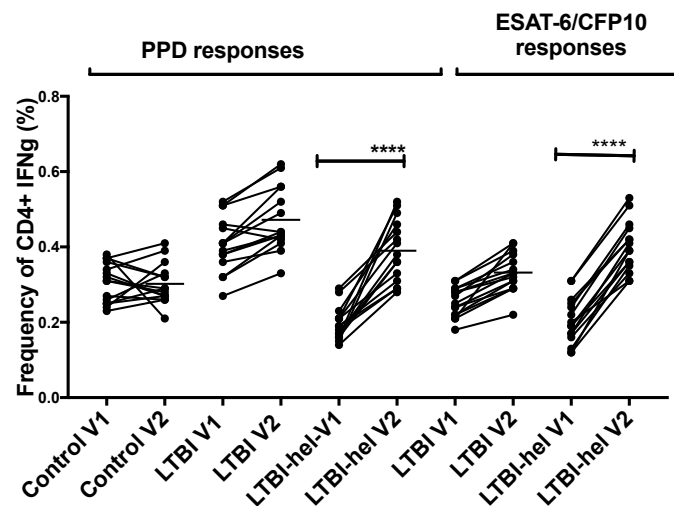
Figure 4.1: Impact of anthelmintic treatment on CD4+IFNγ+T cells in LTBI-helminth coinfected patients. The frequency of CD4+IFNγ+T cells were also measured in LTBI and controls. Antigen stimulated PBMC from fifteen patients from each group were used in this experiment. A non-parametric paired Wilcoxon test was performed, where **** represent a p value of <0.0001.

In helminth infected patients and LTBI-helminth coinfected patients, the PBMC were stimulated with PPD, ESAT-6/CFP10, or SEA antigen overnight and frequency of expression of IFNγ, TNFα and IL-2 was measured (Fig 4.2). In helminth infected patients, IFNγ responses to PPD, ESAT-6/CFP10 and SEA were significantly greater than in the unstimulated cells. TNFα expression was greater with PPD and SEA stimulation than with ESAT-6/CFP10 stimulation. IL-2 expression was greater in response to ESAT-6/CFP10 than PPD or SEA antigen.

(A) helminth infected
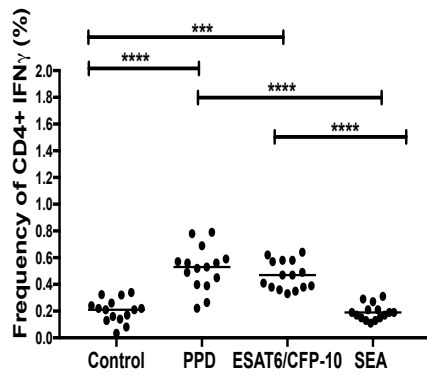
(D) LTBI-helminth coinfected
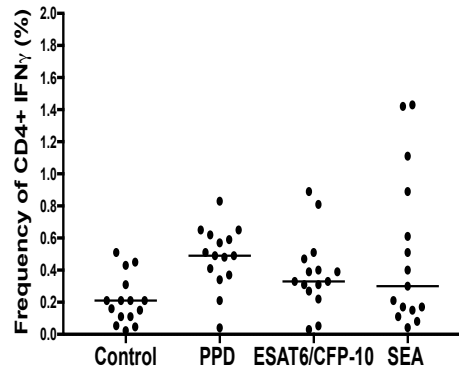
(B) helminth infected
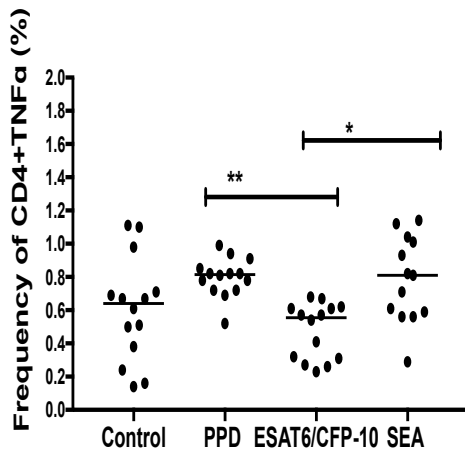
(E) LTBI-helminth coinfected
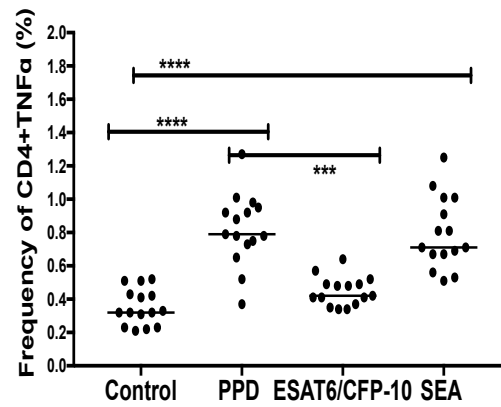
(C) helminth infected
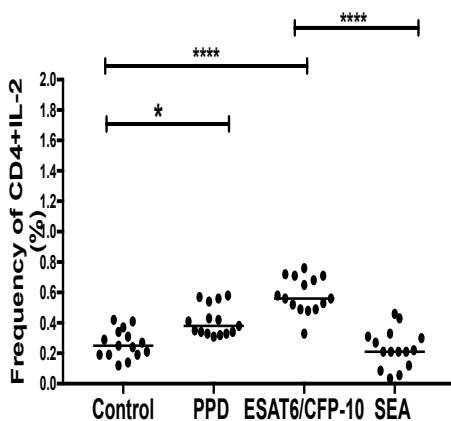
(F) LTBI-helminth coinfected
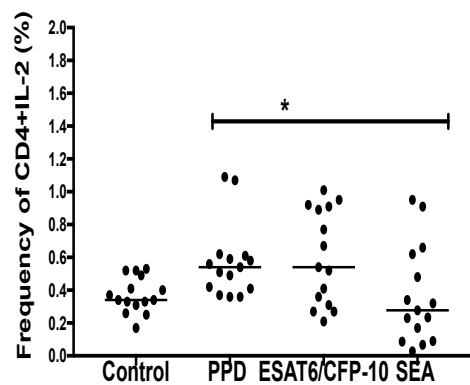
Figure 4.2: PPD, ESAT-6/CFP10 and SEA antigen specific cytokine expression in CD4+ T cells. Frequency of CD4+IFNγ, TNFα and IL-2 measured by intracellular cytokine staining in helminth infected patients (A, B, C) and LTBI-helminth coinfected patients (D, E, F). Line represents the median value. One-way ANOVA was performed, where **** represents a p value of <0.0001, ***represents a p value of <0.0005 and **represents a p value of <0.005.

In LTBI infected patients, TNFα and IL-2 was also measured in PBMC stimulated with PPD, ESAT-6/CFP10, SEA antigen and in unstimulated cells (Fig. 4.3). TNFα expression was detected in LTBI patients, the median TNFα expressing CD4+T cells proportion being 0.38% in unstimulated cells, 0.69% in response to PPD, 0.81% in response to ESAT-6/CFP10 and 0.49% in response to SEA antigen. The median IL-2 expressing CD4+T cells were 0.14% in unstimulated cells, 0.38% in response to PPD, 0.37% in response to ESAT-6/CFP10 and 0.31% in response to SEA antigen. Stimulation of cells with the positive control SEB produced detectable expression of IFNγ, TNFα, and IL-2 (data not shown).
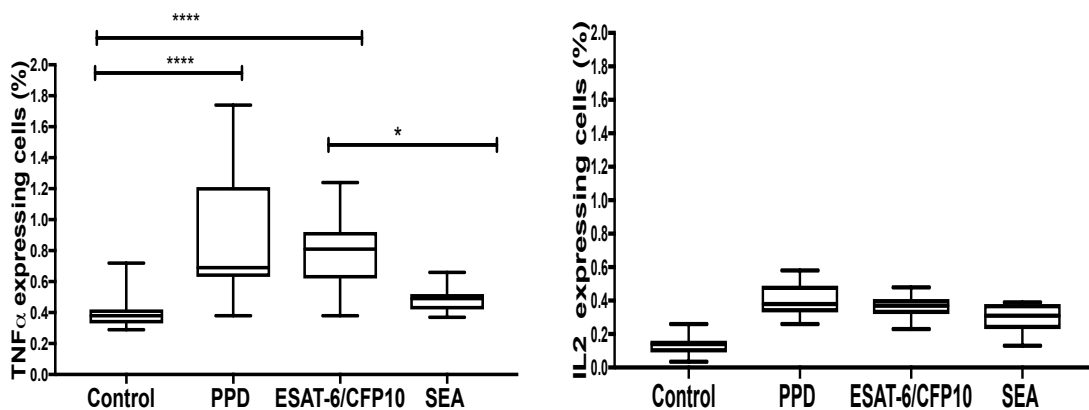
Figure 4.3:   The frequency of CD4+TNFα+T cells and CD4+IL-2+T cells in LTBI patients. Antigen stimulated PBMC from fifteen patients of LTBI was used in this experiment. A non-parametric paired Wilcoxon test was performed. The horizontal line represents the median response, the box represents the interquartile range and the whiskers represent the overall range.

The frequency of CD8+IFNγ+T cells was also measured (Fig. 4.4). There were no significant changes in the IFNγ positive CD8 T cells with the treatment. There were some changes in LTBI-helminth coinfected patients, although the change was not statistically significant (p=<0.99). However, the median proportion of IFNγ positive cells in the CD8 T cells was less than <0.07% in LTBI. <0.05% in helminth infected patients, 0.215% in LTBI-helminth coinfected patients and 0.023% in controls.
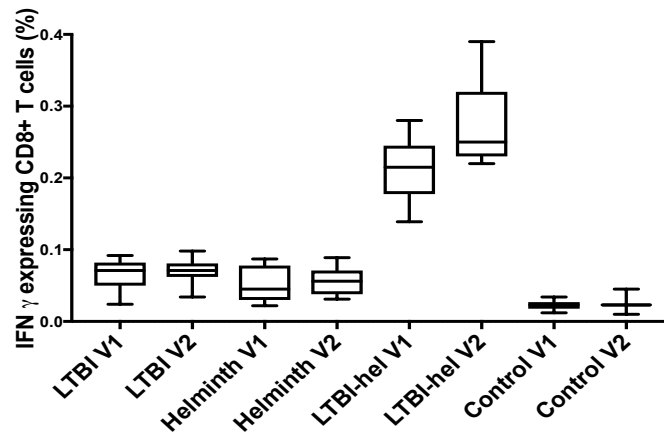
Figure 4.4: The frequency of CD8+IFNγ+T cells was measured in controls, LTBI patients, helminth infected patients and LTBI-helminth coinfected patients. PBMC from fifteen patients per group were stimulated with PPD antigen. A non-parametric paired Wilcoxon test was performed. The horizontal line represents the median response, the box represents the interquartile range and the whiskers represent the overall range.

4.1.2 IFN γ ELISpot response to PPD and ESAT-6/CFP10

PBMC from all the patients were stimulated overnight with PPD and ESAT-6/CFP10 and the IFNγ responses were measured (Fig.4.5). There was a statistically significant increase in the IFNγ responses in LTBI-helminth coinfected and helminth infected patients in comparison to controls both before and after anthelmintic treatment ($p < 0.0001$). The PPD stimulated IFNγ responses were increased after the treatment in LTBI-helminth infected patients ($p = 0.0010$). Interestingly, the response did not change significantly after the anthelmintic treatment in helminth infected patients.
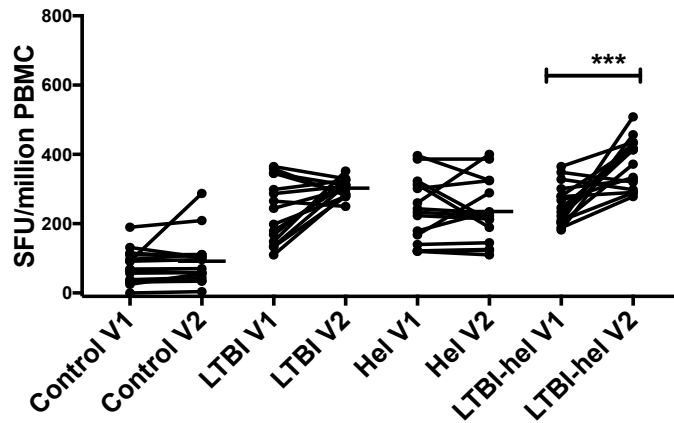
Figure 4.5: IFNγ ELISpot responses to PPD stimulation in controls, LTBI, helminth infected and LTBI-helminth infected patients. An IFNγ ELISpot was performed against PPD using cryopreserved PBMCs from 15 control, LTBI infected, helminth infected and LTBI-helminth infected patients before and after anthelmintic treatment. Bars represent the median values; a Kruskal-Wallis with a Dunn's post-test was performed where **** represents a p value of <0.0001, *** represents a p value of <0.005.

The ESAT-6/CFP-10 stimulated IFNγ responses were significantly greater in LTBI and LTBI-helminth coinfected patients than in controls and helminth infected patients (Fig.4.6). The response was greater in coinfected patients than in the helminth infected patients (p<0.0001). There was no significant difference in IFNγ response between control and helminth infected patients. The *Mtb* specific IFNγ responses were significantly greater in LTBI infected patients in comparison to controls and helminth infected patients (p<0.0001). There was a significant increase in IFNγ responses in LTBI-helminth coinfected patients after anthelmintic treatment, although the treatment did not alter the IFNγ response in helminth infected patients after treatment.
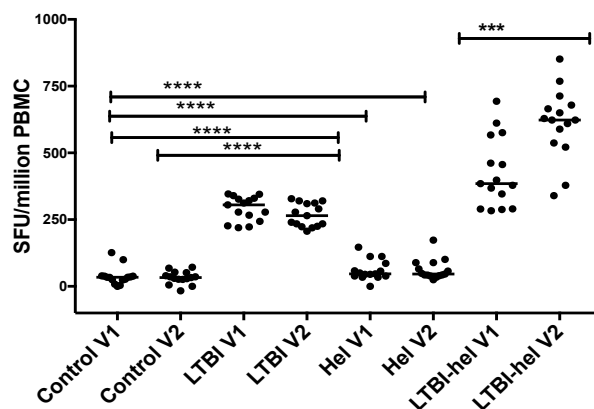
Figure 4.6: IFNγ ELISpot responses to ESAT-6/CFP-10 stimulation in controls, LTBI, helminth infected and LTBI-helminth infected patients. An IFNγ ELISpot was performed against ESAT-6/CFP10 using cryopreserved PBMCs from 15 control, LTBI infected, helminth infected and LTBI-helminth infected patients before and after anthelmintic treatment. Bars represent the median values; a Kruskal-Wallis with a Dunn's post-test was performed where **** represents a p value of <0.0001 and *** represents a p value of <0.005.

4.2 **Discussion**

LTBI is considered a broad spectrum of infection states that differ by the degree of the pathogen replication, host resistance and characteristics of immune activation and inflammation (34)(110). As the bacilli cannot be directly detected *in vivo* during latent asymptomatic *Mtb* infection, a test that could quantify the number of bacteria or the levels of replication might help to define the stages of infection and better understanding of the pathogenesis and immunity during this asymptomatic infection (417). The control of *Mtb* infection requires a Th1 response (IL-2, IFNγ, TNFα and to some extent Th17 response) for the induction and protective immune responses in the control of latent infection (418)(419). Reactivation of latent infection may result from factors inducing suppression of these *Mtb* specific immune response. The severity of TB, where helminth infections are prevalent and the geographical coincidence of these two pathogens has led to the hypothesis that helminth infection could also exacerbate the effects of *Mtb* infection (186). The strong mucosal Th2 immunity and T regulatory cell responses induced by helminths could down modulate the protective immune response against *Mtb* infection (370).

In this study, a London cohort of immigrants who had presented with helminth infection with or without helminth infection were recruited to observe the impact of anthelmintic treatment on the antimycobacterial immune responses. These migrants had negligible chance of risk of exposure and reinfection, as these helminth infections are not prevalent in the UK. This provided an advantage to study the impact of anthelmintic treatment on anti mycobacterial immune responses in helminth infected patients. A previous study of our group in this cohort, looked at the frequency of CD4+IFNγ+T cells following stimulation with PPD or ESAT-6/CFP10 and the concentration of IFNγ in culture supernatants was measured by Luminex and multiplex bead array. It was observed that helminth infected patients had lower responses to PPD and ESAT-6/CFP10 in comparison to other groups and that anthelmintic treatment reversed the CD4+IFNγ+T cell responses and also modulated the frequency of CD4+FoxP3+T cells (370). Following these observations more samples were used to look at the Th1 cytokines like IFNγ, TNFα and IL-2 using intracellular cytokine staining and to observe the effect of anthelmintic treatment on modulation of the protective immune responses against *Mtb* infection.

The frequency of CD4+IFNγ+T cells were 0.31% in controls, 0.41% in LTBI, 0.19% LTBI-helminth coinfected patients and 0.14% in helminth infected patients before they received treatment for helminth infection. At their second visit, the frequency of CD4+IFNγ+T cells increased in helminth and LTBI-helminth coinfected patients (0.26% and 0.39% respectively). The frequency was significantly reduced in helminth infected patients and the coinfected group of patients. This might be due to down modulation of immune responses by concomitant or coexisting helminth infection and TB and is characterized by decreased T cell proliferation and decreased production of IFNγ and IL-2. This was observed in other parasitic diseases like filariasis and hook worm infections(420)(421). In a pulmonary mouse model of *Mtb* infection, *S.mansoni* coinfection reversibly impaired *Mtb* specific T cell responses. These responses were mediated by macrophages expressing arginase-1 and were reversed with anthelmintic treatment(422). There was no significant variation in the frequency of CD4+IFNγ+T cells in controls and LTBI infected patients between their first and second visit.

In the study, The PPD specific response was higher in LTBI than controls. PBMC from all the patients were also stimulated with ESAT-6/CFP10. The response to ESAT-6/CFP 10 was slightly higher than in the controls. These observations were in keeping with the study conducted in Brazilian TB patients, where the levels of IFNγ in response to ESAT-6 in Brazilian controls were much lower than those in TB patients (423). Accordingly, the rate of cells positive to PPD detected by flow cytometry was higher than that of cells positive for ESAT-6 in that study. The majority of TB patients (35 to 92%) can recognize ESAT-6 , as estimated by the production of IFNγ, whereas, healthy unrelated controls from regions of low endemicity cannot(424)(414). Interestingly, some of the BCG vaccinated controls in this study also responded to ESAT-6/CFP10, presumably as they are latently infected accompanied by T cell responses to *Mtb* specific antigens. They had presented in the clinic with eosinophilia and helminth symptoms. However, they were all tested for LTBI and were negative in the IGRA test used.

There was a decrease in CD4+IFNγ+T cells in helminth infected patients and the coinfected groups when stimulated with PPD and ESAT-6/CFP10 both at the baseline in comparison to controls and LTBI infected patients suggesting the complex host pathogen interaction in coinfected patients. Helminth infections appear to influence the reactivity to TB antigens (135). Similarly, in an ex vivo study, Th1 responses to PPD and ESAT-6/CFP10 were significantly lowered in LTBI and filariasis coinfection compared to those

without filarial infection.The potential factors for such down modulation were highlighted as increased expression of the negative co-stimulatory molecule, CTLA-4, PD-1 and also expression of TLRs , specially TLR2 and TLR9 (220)(426).

After clearance of helminths, the PPD response to CD4+IFNγ+T cells increased in helminth infected patients and in the coinfected group. This suggests the impact of anthelmintic treatment on CD4+IFNγ+T cells in helminth infected patients. When the PBMC from these patients were stimulated with ESAT-6/CFP-10, increases in the frequency of CD4+IFNγ+T cells were observed in coinfected patients but no change was observed in controls, confirming the specificity of this antigen for *Mtb*.

The frequency of CD4+T cells and CD8+T cells producing IFNγ, TNFα and IL-2 were measured in helminth infected and LTBI-helminth coinfected patients following stimulation with PPD, ESAT-6/CFP10 and SEA antigen of *S.mansoni* during their first visit. The IFNγ response was more pronounced in both the groups than that to ESAT-6/CFP10. Before treatment, both groups showed lower proportions of IL-2 and higher proportions of TNFα producing cells. A similar pattern was also observed in LTBI infected patients. IFNγ and TNFα are both are well known to play a vital role in the control of *Mtb* infection and mutation of IFNγ or TNFα genes in humans results in increased susceptibility to TB (427)(428). TNFα participates by regulating and maintaining the structural integrity of granuloma and preventing reactivation (429), although TNFα also has a negative impact by promoting growth of *Mtb* in human monocytes and is also known to contribute many of the immunopathological features of TB(427)(431). Studies have shown that CD4+T cells secreting TNFα are more predominant in TB patients than healthy controls or household contacts (432)(433). Although the exact reason and pathology behind the clonal expansion and differentiation of T cells in TB and healthy contacts are still unclear, yet quantification of these cytokines secreted by the T cells can be a promising approach for diagnosis and monitoring of TB(434). Interestingly, the expression of this cytokine was substantially increased in the proportion of TNFα positive cells in the subjects with active disease and was been considered a predictor of diagnosis of active vs latent infection (432). Further prospective works on this signature are required to conclude the roles of these cytokines in helminth and coinfected patients.

Simultaneous measurement of IFNγ and IL-2 at the single cell level identifies T cell cytokine profiles which reflect their memory phenotype and defines three main functional T cell subsets: effector cells that mainly secrete IFNγ only, effector-memory cells primarily secreting both IFNγ and IL-2, and central memory cells secreting only IL-2 (435). Latently infected patients display an immunological signature consistent with persistent low antigen load and long-term immune control, dominated by dual IFNγ/IL-2 secreting effector-memory T cells accompanied by IL-2-secreting central memory T cells. The frequency of LTBI patients in this study finding is consistent with a role in long-term immune control.

In this experiment, TNFα and IFNγ production in response to SEA antigen was higher than that of IL-2. The response was higher in coinfected patients in comparison to helminth infected patients without LTBI. An immunocompetent host mounts immunological response to parasite eggs with the development of a collagen rich granulomatous response around the eggs (244). In immunocompetent wild-type (Wt) mice, immune responses to schistosome antigens causes a shift from a moderate Th1 to a robust Th2 response; which is responsible for most of the immunology (436). Mice deficient in IFNγ signalling have a reduction in granuloma size and this causes transition into the chronic phase of the response suggesting that IFNγ contributes to granuloma formation (437). Patients presenting with severe fibrosis have been associated with production of TNFα, IL-5, IL-10, IL-13, whereas patients with low fibrosis patients had higher IFNγ levels (438).

Although CD4+T cells play a central role in immunity to *Mtb* infection, CD8+T cells are also important in TB immunity. *Mtb* specific CD8+T cell can secrete cytokines such as IFNγ, TNFα, similar to that of CD4+ cells. These cells can also act as cytotoxic effector cells, releasing the cytotoxic granules into the contact zone between CD8+T cells and macrophages (439). The CD8+T cells were also explored in this study to observe the cytokine profile in all the four groups. The median proportion of IFNγ positive cells in the CD8 T cells was less than <0.07% in LTBI. <0.05% in helminth infected patients, 0.215% in LTBI-helminth coinfected patients and 0.023% in controls. TNFα and IL-2 production was very low by CD8+ T cells in all these groups. IFNγ+CD8+T cells significantly increased post-treatment in LTBI-helminth coinfected patients. Although stimulation with PPD showed no difference in cytokine producing CD8+T cells between LTBI, controls and helminth infected patients. Pinxteen *et al.,* in their studies showed that

during the acute phase of the infection, treatment with anti-CD4 exacerbated the pulmonary disease, whereas, the reverse was found during the latent phase of the infection, where treatment with antiCD8 mAb led to a greater increase in bacterial numbers in the lung than did administration of anti-CD4 mAb. This suggests that during the acute phase of disease, CD4+T cells may be more important in controlling bacterial replication. In contrast, CD8+ T cells may have a greater role in maintaining control of the infection during latency, possibly via immunosurveillance of heavily infected cells that have lost the capacity to inhibit bacterial replication (440).

To take the experiments one stage further, individual T cells secreting IFNγ were then measured in all the clinical samples by ELISpot assay. PBMC from all the patients were stimulated overnight with PPD and ESAT-6/CFP10 and the IFNγ responses were measured. There was a statistically significant increase in the IFNγ responses in LTBI-helminth coinfected and helminth infected patients in comparison to controls both before and after anthelmintic treatment (p<0.0001). After anthelmintic treatment the IFNγ responses increased significantly, however, there was no significant changes in helminth infected patients after they received their treatment. Interestingly, PPD responses and EAST-6/CFP10 responses were different in helminth infected patients. IFNγ ELISpot responses to ESAT-6/CFP 10 were lower in helminth infected patients than that of controls.

Although IFNγ is an important component of protective immunity, there is increasing evidence that it does not provide a read out of protective immunity (394). IFNγ that is produced do not parallel control of bacterial growth and also in some animal model studies, greater IFNγ production is associated with more pathology. Polyfunctional T cells (T cells capable of making IFNγ, TNFα and IL-2) are a better indicator of protective immunity than the measurement of IFNγ alone. CD8+ T cells and the cytotoxicity of infected macrophages can also lead to killing of intracellular bacteria and a reduction in CFU (441). Helminth specific antigen stimulation also showed changes in the cytokine profile especially in helminth and LTBI-helminth coinfected patients. Helminth infections appear to play an important role in modulating the immune response to *Mtb* specific PPD and other *Mtb* antigens through a complex immune mechanism, downregulating the expansion of Th1 cells (442). To measure the changes in CFU following the incubation of infected monocytes/macrophages with T cells may therefore provide a highly relevant read-out of protective immunity in this cohort. To explore this, the next step was taken to

perform mycobacterial growth inhibition assays using a commercial BACTEC system. The MGIA provides a measure of a range of different immune mechanisms and their interactions. To assess how the cells in these group of patients control the growth of Mycobacteria and also the impact of anthelmintic treatment on antimycobacterial immune responses- MGIA was performed on all the samples, which will be discussed in the following chapter.

Chapter - 5 Optimization of the MGIT Assay

5.1   Optimization of the MGIT Assay

5.1.1 Introduction

A number of groups have recently investigated the potential use of mycobacterial growth inhibition assays (MGIA) using both whole blood and cryopreserved PBMCs in both *Mtb* exposed and BCG vaccinated cohorts (387)(394)(443). This assay can directly measure the ability of a heterogeneous cell population to limit the intracellular growth of mycobacteria that could correlate directly with protective TB immunity.This is a relatively simple assay in comparison to traditional CFU counting.To quantify mycobacteria in this method is potentially more accurate as it can avoid the pre stimulation steps of cell culture and also avoid the laboursome steps of traditional cultures including serial dilution, clumping, pipetting errors and colony counting. The MGIT system utilizes oxygen depletion as its method to assess the number of the viable bacteria. This fluorescence based technology can utilize the bacterial usage of oxygen as a requirement of growth and metabolism and the computer based system provides a read out as TTP.However, this assay needs to be validated for accuracy, precision, reproducibility,specificity and linearity. Some optimization of the assay has been done, for example- timing of processing the sample, BCG inoculation time, use of serum, antibiotics, BCG inoculum volume, BCG declumping methods etc. However, despite the numerous optimization experiments that have been performed-there is still a large concern about the reproducibility of this assay (Tanner R, Development of Mycobacterial Growth Inhibition Assay for the early evaluation and gating of novel TB vaccine candidates. PhD [Thesis]. Oxford :University of Oxford; 2015).

In this study, MGIA was performed in a LTBI and helminth coinfected cohort for the first time to observe how these subjects can control the growth of mycobacteria and whether the anthelmintic treatment has any impact on the ability of the cells to control mycobacterial growth effectively. In this study PBMC were used to perform the MGIA assay. In this study PBMC were preserved to perform the MGIA and other immunological assays; as it was not possible to perform the MGIT immediately and cells needed to be preserved for testing the paired samples pre and post treatment simultaneously. Although whole blood could have been useful in this study to observe

the role of neutrophils, to make the assay logistically simple, PBMC were used rather than whole blood. Using an optimized assay protocol, different aspects of assay variability at different stages of the assay were tested, for example, pre culture conditions- cell clumping, cell rest, antibiotic use in the medium, serum in the medium, and also individual variation in the MGIA was observed.

Aims

The aims of the experiments detailed in this chapter were

- to compare the variability of the assay using different assay conditions for example-BCG clumping methods, effects of anticoagulants, impact of antibiotics, choice of serum, heat inactivation of serum etc.
- to reduce the variability of the assay and optimize the assay protocol.

5.1.2 BCG declumping to reduce variability

Three different methods of declumping BCG were compared using cultures of cells from ten healthy donors at LSHTM. The methods were- vortexing, vortexing with glass beads, sonicating and the aim was to use a suitable method to reduce the variability of the test. Ten vials of BCG stock were thawed and declumped by these three methods. Vortexing was done on the highest speed for 5 minutes. BCG stock was also taken into a 2mm tube containing sterile glass beads and vortexed on full speed for 3 minutes. The BCG stocks were sonicated for 1 min and placed in an ice box for one minute and the steps were repeated twice more. The BCG were then transferred into BACTEC MGIT tubes and the tubes were placed in a MGIT 960 machine to detect the TTP. Variability was measured as standard deviation. Mycobacterial recovery, as measured by TTP was shortest with vortexing with glass beads (median 243 hours). Other methods resulted in some loss of mycobacteria (the medians for vortexing was 256.5 hours and for sonication was 250 hours). These changes were not statistically significant. Variability between these three methods, was lowest for sonication (CV=9.63%) and was highest for vortexing (CV=12.71%) (Fig 5.1).
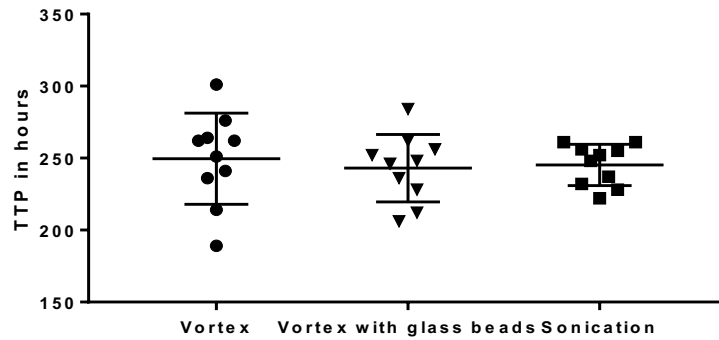
Figure 5.1: Methods of declumping BCG. PBMC MGIT data was obtained using three different methods of declumping BCG Pasteur prior to inoculation into the MGIT tubes for the culture. PBMC were collected from 10 healthy donors at LSHTM. The bars represent the median value. A Kruskal-Wallis non parametric test was performed. There were no significant changes among these three methods of declumping.

Initially during the optimization steps, sonication was used as a method of declumping; but afterwards we started to use BCG Aeras stocks with a concentration 1.1×10^8/ml. The BCG was grown in shaking flasks in 7H9 medium without OADC but with the addition of 0.2% glucose and 0.05% Tyloxapol to an OD of about 4. The BCG was then spun down and resuspended in half the volume of formulation buffer (20% glycerol, 0.85% NaCl and 0.05% Tyloxapol) and frozen in the formulation buffer. Addition of Tyloxapol made it easy to avoid the declumping methods. Afterwards, the BCG stock was thawed and diluted to be used in the MGIT assay- no declumping methods like vortex, sonication etc. were used.

5.1.3 Effect of anticoagulant on MGIA

EDTA and sodium heparin were compared as anticoagulants used for collecting the blood samples. PBMC separated from EDTA anticoagulated blood showed shorter TTP. However statistically there was no difference in these two anticoagulants in terms of cell recovery or viability (Fig. 5.2).
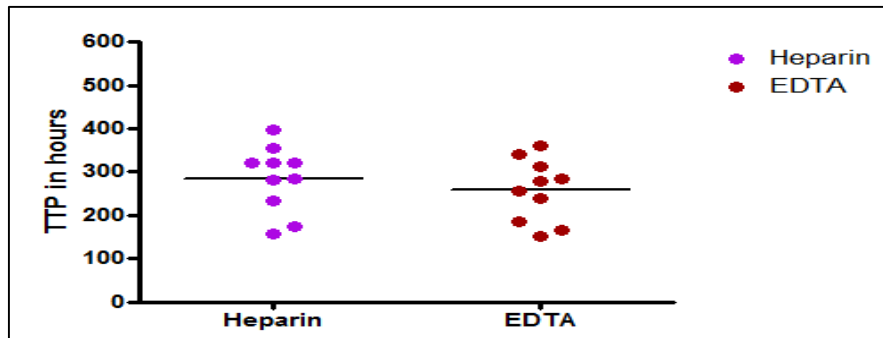
Figure 5.2: Effect of heparin and EDTA on MGIA. PBMC from 10 healthy donors were collected. PBMCs were collected in heparin sprayed and EDTA sprayed tubes. There were no significant differences observed between the groups (p=0.235). The bars represent median values.

5.1.4 Impact of antibiotics on MGIA

In this experiment, cells were rested in standard MGIT media (RPMI containing 10% FBS, L-glutamine and sodium glutamine and HEPES buffer) in post-freezing and pre-thawing steps, before washing and re suspending in culture medium. To see the effect of antibiotics- standard tissue culture concentrations of penicillin-streptomycin (pen-strep) in the culture medium, pen-strep and penicillin alone were used in the post-freezing and pre thawing cells steps. Cryopreserved PBMC from 3 LSHTM donors were tested for this experiment. The pen-strep combination had a pronounced inhibitory effect when the cell rest medium and post-thawing medium contained these antibiotics- even though the cells were washed afterwards (Fig.5.3). The inhibitory effect was not that noticeable when the cell medium only had penicillin in it. To remove this inhibitory effect of antibiotic at the phases of this experiment- no antibiotic was added in the pre culture state.
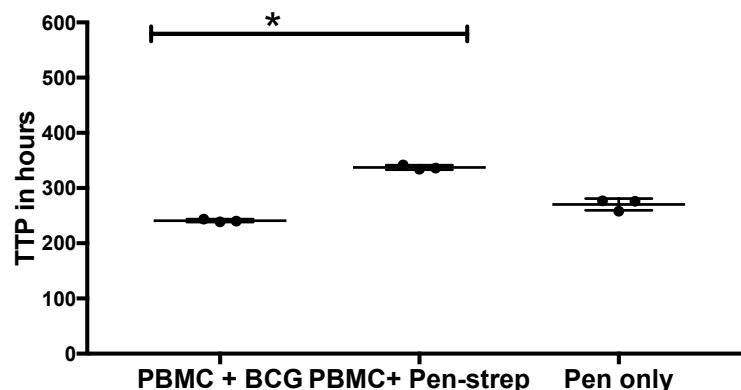
Figure 5.3: Effect of penicillin-streptomycin antibiotics and penicillin alone in the cell rest medium. PBMC from 3 healthy donors were collected. Pen strep antibiotic mix, penicillin alone and no antibiotics were compared in the cell rest medium in post-freezing and pre-thawing steps. The bars represent mean values with S. A one way ANOVA were performed, where * represents a p value of <0.05.

5.1.5 The effect of serum in the cell culture medium

Most protocols with PBMCs use serum to provide optimal conditions for cell viability, reactivity and expansion to provide a more favourable environment for the cells. For this experiment, foetal bovine serum (FBS) and human AB serum (PHS) were used. To determine whether the addition of FBS or PHS effect on mycobacterial growth and the variability, the MGIT assay was performed in 10 healthy donors with either FCS or PHS in the medium. Mycobacterial growth was observed to be significantly higher using human AB serum compared with FBS (Figure -5.4). For the experiments onward, human AB serum was used in the culture medium.
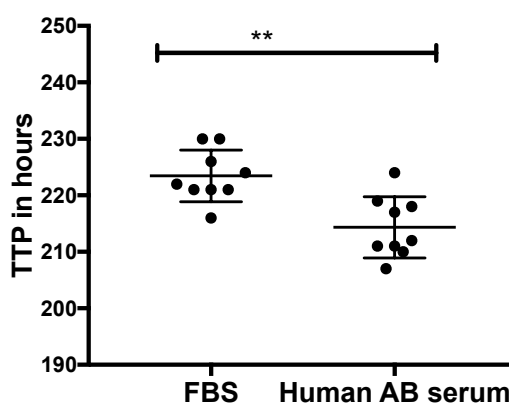
Figure 5.4 : Comparison of human and bovine serum in the culture medium. PBMC were cultured with FBS and human AB serum in the culture medium before adding to MGIT tubes. Bar represents the mean values with SD. A Wilcoxon matched-pairs signed rank was performed where ** represents a p-value of 0.0039.

5.1.6 Impact of heat inactivation of serum

To observe the effect of heat inactivation of human AB serum, PBMC were used from 10 healthy donors at LSHTM and MGIT was performed. The mycobacterial growth was significantly lower when heat-inactivated serum (HI) was used in comparison to normal serum (p<0.001) (Fig.5.5).
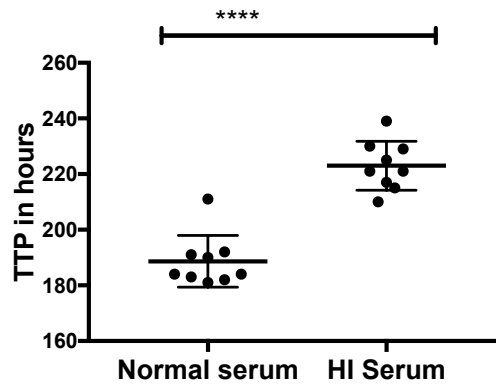
Figure 5.5: Effect of heat inactivation of serum on mycobacterial growth. PBMC from 10 donors were cultured with normal unheated human serum or HI human AB serum in the culture medium. Bars represent the mean values with S. A paired test was performed where **** represents a p-value of <0.0001.

5.1.7 Culture mixing and rotation

The original MGIA protocol included 360º rotation of the cell cultures. The effect of standing and 360º rotation of the cultures were observed. PBMC from 10 healthy donors were used for MGIT experiments to observe the effect. There were significant changes in the mycobacterial growth inhibition when rotating the culture at 360º was compared to standing stationary (p=<0.0001). Mycobacterial growth inhibition was significantly higher when cultures were rotating at 360º compared to stationary cultures (Fig 5.6). The cultures were rotated at 360º rotation for 96 hours in the further experiments.
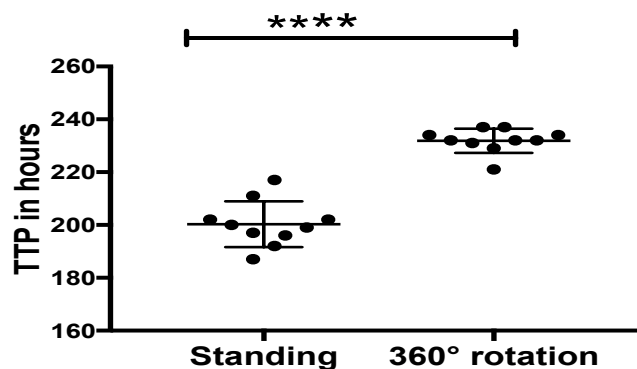
Figure 5.6: Effect of culture mixing and rotation on growth inhibition. MGIT was performed using PBMC from 10 healthy donors, where cultures were kept standing for 96 hours and with 360º rotation. The barsrepresents mean values with SD. An unpaired t-test was performed, where ****represents a p-value of <0.0001.

5.1.8 Cell lysis with sterile water

In this protocol cells are lysed after 96-hour culture to release the intracellular bacteria. The effect of this cell lysis step on the mycobacterial growth inhibition was analysed. For this experiment, PBMC from 8 healthy donors were used and the cells were lysed with water or kept without lysis. After removing the 96-hour culture supernatants, about 100µl of sterile water added to the residual and kept for 5 minutes, and vortexed at the highest speed for 1 min and then kept at room temperature for 5 minutes. The lysis step with sterile water did not have any significant effect on mycobacterial growth when compared to the no lysis cultures (Fig.5.7).
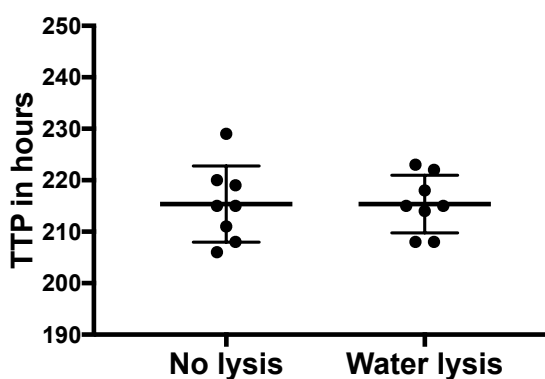
Figure 5.7: Effect of lysing with sterile water on mycobacterial growth inhibition. MGIT was performed using PBMC from 8 healthy donors, where cells were lysed with water at the end of 96-hour culture and compared with no lysis of cells. Bar represents mean value with SD. A Wilcoxon matched-pairs sign rank test was performed. There was no significant difference between the groups.

5.1.9 Individual variation in MGIA

To observe whether the patterns of individual variation were stable over the period of a year, MGIA was performed with PBMC from 7 healthy donors at LSHTM and repeated after one year under the same conditions. There was no significant change in the pattern of mycobacterial growth inhibition after one year. The pattern was similar both in BCG vaccinated and non BCG vaccinated healthy donors (Fig. 5.8).
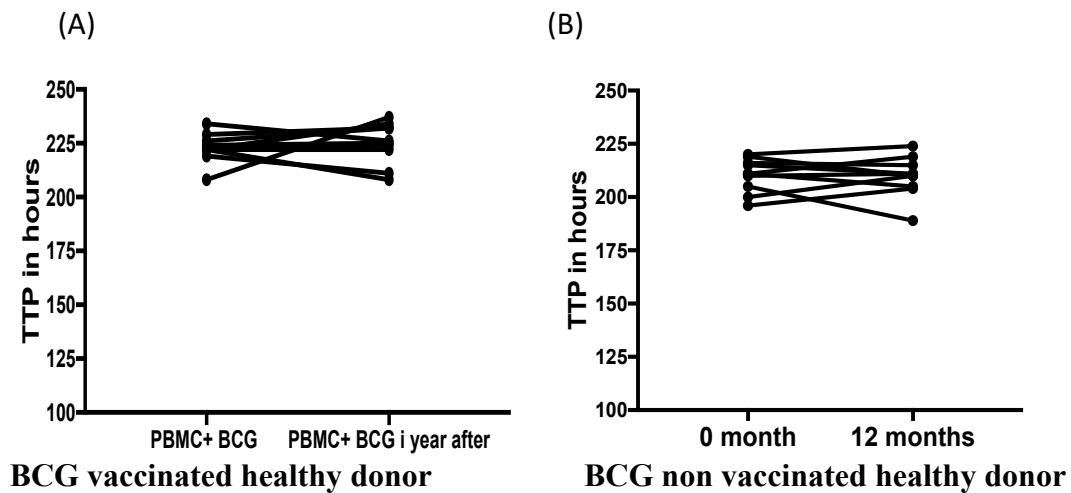
Figure 5.8: Individual variation in MGIA. MGIT was performed using PBMC from 7 healthy donors at two different time points, 1 year apart (0 month and 12 months). There were no significant changes in mycobacterial growth inhibition patterns after 1 year in BCG vaccinated (A) donors or in BCG non-vaccinated (B) donors. A Wilcoxon matched-pairs sign rank test was performed.

5.1.10 BCG vaccination and MGIA

To observe if there are any significant change in the mycobacterial growth inhibition in BCG vaccinated and non-vaccinated individuals, PBMC were collected from 10 BCG vaccinated and 10 BCG non-vaccinated healthy donors at LSHTM. Mycobacterial growth was significantly reduced in the BCG vaccinated healthy donors in comparison to the non-vaccinated healthy donors (p<0.001) (Fig. 5.9).
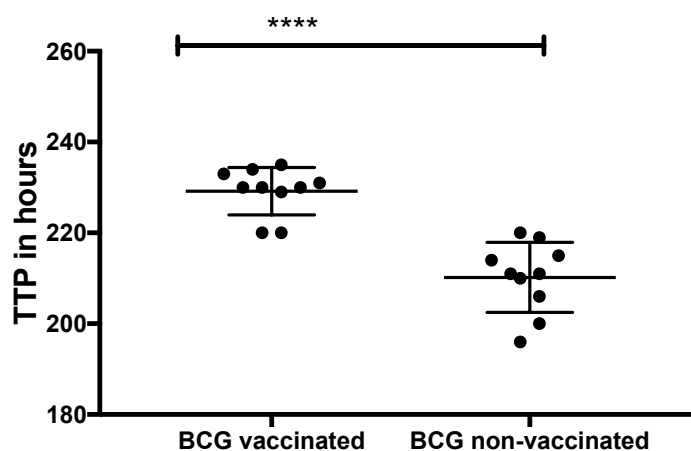
Figure 5.9: MGIA variation in BCG vaccinated and BCG non-vaccinated healthy donors. MGIT was performed using PBMC from 10 BCG vaccinated and 10 BCG non vaccinated healthy donors at LSHTM. A Mann Whitney test was performed, where **** represents the p value of <0.0001.

5.1.11 Effect of PPD stimulation of cells in MGIA

To see the effect of PPD stimulation on MGIA, PBMC from six healthy donors at LSHTM were collected, and PBMC were stimulated with PPD overnight before MGIA. The aim was to see if there was any fold change in growth inhibition if the cells were pre-stimulated with PPD before the culture with BCG. There were no significant changes in the mycobacterial growth inhibition when the cells were stimulated with PPD overnight. This experiment was performed in clinical samples afterwards, which is described in Chapter 7.
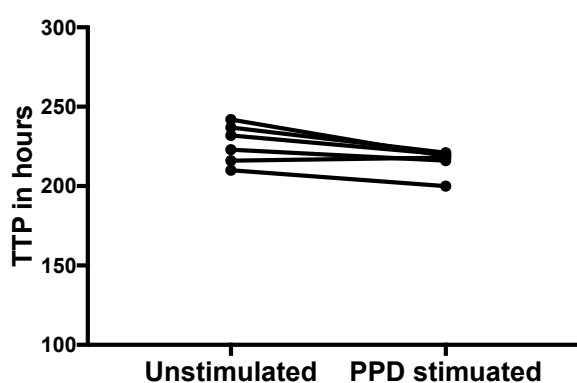
Figure 5.10: Effect of PPD stimulation on MGIA. MGIT was performed using PBMC from 6 healthy donors, where cells were stimulated with PPD overnight. After normalization a Wilcoxon matched-pairs sign rank test was performed. There were no significant changes between the groups (p=0.3215).

5.2 Discussion

Ex vivo MGIA directly assesses the summative ability of the host immune system to inhibit the growth of Mycobacteria. This assay could help to determine the underlying immune mechanism of protection by investigating different common factors in samples with efficient growth inhibition (401). The assays of inhibition of mycobacterial growth previously studied were developed for other research applications and for their ability to detect vaccine-induced immune enhancement. The lymphocyte based growth inhibition assays were developed to study the capacities and mechanisms of various lymphocyte populations to enhance killing of intracellular bacteria and whole blood based assays were developed primarily designed for studies of TB immunity in large populations.This chapter describes several steps to optimize an existing mycobacterial growth inhibition

assay to develop an optimized protocol that could be used for this study in LTBI and helminth coinfected patients, to measure the ability to control the host cell to control the growth of mycobacteria.

There are two main protocols for MGIA-whole blood and PBMC based. The MGIAs encountered significant difficulties with both inter and intra- assay variability, which is still considered an issue with this assay. The PBMC based MGIT assay has also the same variability issue, although the assay is relatively simpler, does not involve the complex pre culture preparation and stimulation of the cells. The BACTEC MGIT assay has some advantages over the conventional CFU counting method, for example avoiding serial dilution, clumping, pipetting error, contamination and subjectivity of the conventional methods (444). The BACTEC MGIT assay is more rapid than colony counting, dependent on the initial bacterial number but is also influenced by the replication characteristics of the particular bacteria and the metabolic rate.

Although the whole blood inhibition assay is simpler to perform, it lacks any cell separation and may reflect contributions by neutrophils, antibodies and red blood cells to mycobacterial immunity. A small volume of blood is required for the assay. However, the whole blood assay cannot distinguish between intracellular and extracellular inhibition of bacterial growth, and it seems less useful for dissecting the mechanisms underlying mycobacterial inhibition than lymphocyte-based assays (375).

For this study the PBMC assay were selected. It was selected as this could gave the strongest experimental design and was logistically simple. The samples were collected at two points and it was planned to test the pre and post treatment samples simultaneously to observe the treatment effect and the other immunological parameters. Running all the samples in a single batch would reduce the variability. Using cryopreserved PBMC helped to evaluate the processing and analysis at a later date.

To optimize the steps of the assay, some of the variables were investigated both pre-culture, and during the culture steps. The individual responses and variation of MGIA was also observed. Cells were stimulated with mycobacteria specific antigen to observe the changes in the pattern of growth inhibition of mycobacteria.

First, heparin and EDTA were compared as anticoagulants and their effect on growth inhibition assay was observed. With EDTA, TTP was shorter than with heparin, but the

difference was not statistically significant. Segura *et al.,* investigated the effect of EDTA in substrate adherence capacity of rat inflammatory macrophages and whether the macrophage function was altered. They concluded that EDTA decreased substrate adherence capacity of macrophages significantly and may inhibit the macrophage function (445). Mouse macrophages possess heparin receptors on the cell surface and heparin has been reported to activate monocyte/macrophages, including the release of IFNγ and promotion of lysosomal enzyme secretion (446). Duvigneau*et al.,* (2007) investigated heparin and EDTA as anticoagulant and their effect on cytokine mRNA level of cultured porcine blood cells. They showed that PBMC isolated from EDTA anticoagulated blood showed a higher cytokine expression capacity (IL-1 IL-2, IL-4, IL-6, IL-10, IFNγ) than PBMC from heparinized blood, though EDTA as anticoagulant is not recommended for the cytokine detection method, as this requires maximal influx of calcium ions for intracellular protein accumulation (447). A different finding was observed in the study by Patil *et al.,* (2013), where they assessed the influence of heparin and EDTA as anticoagulants on various cytokine levels in plasma and concluded that TNFα, IFNγ, IL-4,IL-5 and G-CSF levels were significantly higher in plasma with EDTA, whereas the level of IL-6, IL-8,IL-10, IL-17, MIP-1β, GM-CSF and MCP-1 were found to be higher in plasma with heparin (448). Since there was no statistically significant difference in the anticoagulant used in this study, afterwards heparin containing vacutainer tubes were used to collect blood.

One of the potential causes of variability in these assays can be clumping of BCG. Different methods of resuspension were compared to improve the reproducibility of the test. Although there were no significant differences between the methods, the variability was lowest for sonication other than vortexing in comparison to glass beads with vortexing alone. TTP was shorter with vortexing and the other methods showed some loss of BCG in MGIA protocol. Initially sonication had been used as a method of declumping. When AERAS prepared a large batch of BCG, they added a surface active agent called Tyloxapol. Tyloxapol is a detergent which is commonly used in mycobacterial research to prevent clumping. Tyloxapol is a non-ionic liquid polymer of the alkyl polyether alcohol type and is used as a surfactant to aid liquefaction. Tyloxapol has been used as a more stable and detergent agent in mycobacteria culture than Tween80, which is unstable and more likely to break down to oleic acid. This oleic acid can be used as a carbon source for the growth of mycobacteria (449). Although sonication was the method of choice, the use

of AERAS BCG containing Tyloxapol removed the need for a vortexing or sonication step in further analysis. To avoid pipetting error, BCG was always pipetted into small volumes and then a large dilution prepared to use in the experiment.

Initially when the sample recruitment started in 2011 in the IDEA study, PBMCs were cryopreserved in media which contained pen-strep in the media and also complete medium was used for cell separation and in pre-thawing, pre-freezing media when PBMCs were used for intracellular cytokine assays. When these cryopreserved cells were used for MGIT assays, the growth of mycobacteria was very low or absent. Log CFU values generated in the PBMC based assay were predominantly negative, which indicates more growth inhibition than was expected. To see if there was any inhibitory effect of that pen-strep in the growth inhibition of mycobacteria, an experiment was performed where cells were rested in pen-strep, with only penicillin and without any antibiotic in the washing, pre-thawing and pre-freezing steps of culture.   There was a pronounced inhibitory effect when pen-strep was present in the cell rest medium in the post-thawing stage. There was not such an inhibitory effect if the antibiotic was present in the pre-freezing medium alone. The inhibitory effect was not that pronounced when penicillin alone was used. Penicillin has no established role in antimycobacterial treatment; although amoxicillin-clavulanic acid and meropenem have recently been showed to have some activity against mycobacteria and WHO currently lists amoxicillin-clavulanic acid as a third line (and last line) agent with unclear efficacy for treatment of drug resistant mycobacteria (450).   Streptomycin, which is an effective anti-TB drug, is a protein synthesis inhibitor. It binds to the small 16S rRNA of the 30S subunit of the bacterial ribosome, interfering with the binding of formyl-methionyl-tRNA to the 30S subunit. Drug entry into the bacteria causes the active export of cations out of the cytosol and it hyperpolarizes bacteria inducing an electrophoretic driving force that promotes the entry of aminoglycosidesso they can be sequestered in the lysosomes (451). In bacteria, streptomycin induces mistranslation and inhibition of protein synthesis, resulting in bacterial death (452). It is possible that streptomycin was taken up by the cells and entered their cytosol during the rest period, and was retained there even after the wash step. This might explain the inhibitory effect of the pen-strep during post-freezing and pre-thawing in the growth inhibition assay. Removal of antibiotics from the cell rest medium, reversed the growth inhibition. Although there were not significant changes due to the presence of antibiotics in pre-freezing stage it might be helpful to prevent the

bacterial contamination. Since there was some inhibitory effect still observed and to avoid any individual variation in how the cells take up or retain the antibiotic it is preferable not to use any antibiotic in the cell rest media for MGIT. For the remainder of the samples, no antibiotics were used in the tissue culture medium. Initially in this project the PBMCs from the patients were stored for long term in freezing media containing pen-strep and FBS. Some of the stored samples were tested for MGIT and most of them did not show any growth of mycobacteria. The antibiotic may have some inhibitory effect and also FBS might have some cross reacting antibodies from bovine serum. Other groups in Oxford University performed optimization experiments to reduce the reproducibility issues. They also observed that the pen-strep used in cell rest medium had a pronounced inhibitory effect on mycobacterial growth in the assay (personal communication).

For any effective cell based culture, it is important to choose the most appropriate reagents to promote cellular activity. FBS has been widely used for this purpose. Interestingly, in MGIT it was observed that using FBS rather than pooled human AB serum, resulted in decreased mycobacterial growth. Although viability was not affected by using any of these sera in the cell culture medium, FBS might give some background of non-specific cell stimulation.The thesis of Rachel Tanner, 2015 assessed the cell culture conditions and found that using pooled human AB serum rather than FBS in the culture medium resulted in increased mycobacterial growth suggesting that the FBS may mediate a direct inhibitory effect on mycobacteria. For further MGIT assays, human AB serum was used (R.Tanner PhD Thesis, unpublished).

Heat inactivation of human AB serum resulted in reduced mycobacterial growth in comparison to normal serum. Heat inactivation of serum results in destruction of growth factors, vitamins, amino acid and other protein components like complement. In mycobacterial cultures, heat inactivation of serum markedly reduces the adherence of *Mtb* and also reduces uptake of mycobacteria into the monocytes due to destruction of complement (453). Monocytes are considered to be important for mycobacterial growth and replication. Reduced monocyte invasion might lead to reduced net mycobacterial growth. For further experiments, heat inactivated serum was used.

The final yields of tubercle bacilli in submerged cultures is limited by the rate of diffusion of oxygen through the liquid medium. Increased oxygenation can be obtained by forced aeration or by mechanical agitation of the culture. It is assumed that gentle agitation or

aeration is favourable to the growth of the organisms in liquid media since it helps in breaking up clumps of cells. It has been shown that aeration by continuous rotation used in cultures are preferred as it increases availability of oxygen supply that aids the multiplication of the bacilli (454). In the previous cell based MGIT assay, cultures were done in stationary plates (375)(398). In this study, the cultures were rotated for 96 hours in an incubator with 5% CO_2 Rotating cultures gave significantly greater inhibition of mycobacterial growth. This was greater with 360º rotation in comparison to stationary cultures. In standing cultures cells would have less chance to get mixed with the mycobacteria. In rotating cultures, the mycobacteria would get in contact with more of the monocytes and effector immune cells like T cells would have greater contact with the infected cells and that might explain the increased growth inhibition rate. Jackson *et al.,* showed in their study that agitation enhances the growth of the mycobacteria as it may improve the distribution of the nutrients, and oxygen content in the medium (455). For the assays, cultures continued to be rotated at 360º. There were no significant differences in mycobacterial growth between the lysis steps using sterile water and no lysis state.

To observe if there is any individual variation in MGIT assay responses in healthy donors, 10 donors were bled twice at one year intervals to see the growth inhibitory capacity. All these individuals are healthy, UK born and BCG vaccinated. There was no significant difference over the year in their pattern of growth inhibition. When the growth inhibition pattern was observed among the BCG vaccinated and non-vaccinated healthy donors at LSHTM, there was significantly greater growth inhibition in BCG vaccinated individuals than the non-vaccinated donors. BCG vaccination has variable efficacy though the efficacy is higher in non-endemic countries like UK (456).Using a mycobacterial inhibition assay, Silver showed an association between in vitro lymphocyte responses and inhibition of intracellular *Mtb* growth (377), thus this assay reflects the contribution of mycobacteria-specific T cells to protection. Hoft *et al.,* found that inhibition of intracellular *Mtb* replication significantly increased after BCG vaccination of initially PPD-nonreactive persons (375), thus reflecting BCG-induced protective immunity.We also looked at the effect of PPD pre-stimulation on the MGIT assay. The cells were stimulated with PPD overnight and then used in the cells in MGIT to see if there was any fold change in growth inhibition pattern in PPD stimulated cells compared to non-stimulated cells co-cultured with BCG. PPD responses in individuals may be due to latent TB, prior BCG vaccinations, exposure to environmental mycobacterium-it

indicates some immunity from prior exposure to mycobacterium. Kang *et al.,* in their studies have shown that strongly PPD-reactive TB contacts without ESAT-6/CFP10 responsiveness showed significantly better mycobacterium inhibition activity than ESAT-6/CFP10-responsive TB contacts with the same PPD reactivity. In TB contacts, stronger PPD reactivity was associated with improved mycobacterial killing, whereas ESAT-6/CFP10 responders showed the opposite result (457).

The aim of these optimization steps were to reduce the variability of the test and also to have an optimized protocol to be used for the clinical samples. This optimised protocol (Appendix 4) was taken forward to do the further experiments and analysis.

Chapter - 6 Cellular components in MGIA

6.1 Cellular components in MGIA

6.1.1 Introduction

An understanding of the immune mechanisms underlying mycobacterial growth inhibition in vitro, to identify the mycobacteria specific cytokine responses, how these responses affect the capacity of the cells to control the growth of mycobacteria, and also the contribution of different immune cells in the immunopathogenesis of LTBI-helminth co-infected patients might provide important information on the major immunological effects of helminth coinfection on immune responses to TB. Among the various regulatory mechanisms, IL-10 and TGFβ appear to play the most profound role in influencing the T cell responses to mycobacterial antigens in the context of helminth-TB co-infection (458). Helminth infection is also associated with a decreased frequency of CD4+ IFNγ secreting T cells, as previously shown in a similar group of patients (370). The roles of these cytokines were explored, to observe how they can affect the growth inhibition of mycobacteria.

Aims

The aims of the experiments discussed in this chapter were to:

- investigate the immune mechanisms underlying mycobacterial growth inhibition as measured in the BACTEC MGIT assay.
- investigate the role of monocytes in the control of growth inhibition
- explore the roles of cytokines like IFNγ, IL-10 and TGFβ in growth inhibition in the helminth infected patients and to investigate whether blocking these cytokines with antibodies can reverse the growth inhibition.

6.1.2 Monocyte depletion

Methods

CD14 Micro beads (Miltenyi, Cat no 130-050-201) were used for the depletion of human monocytes from PBMC. PBMC were collected from ten BCG vaccinated and ten BCG

non-vaccinated healthy donors at LSHTM. Afterwards PBMC from 5 more non BCG vaccinated donor PBMC were added to the study. PBMC were counted and placed in Eppendorf tubes at a concentration of 10^7 cells/ml. The cell suspensions were centrifuged at 300g for 10 minutes and the supernatant was aspirated completely. The cell pellet was resuspended in 80μl MACS buffer per 10^7 total cells and 20 μl CD14 micro beads per 10^7 total cells were added. The cell suspension was mixed well and incubated for 15 minutes in the refrigerator (4°C). Cells were washed with MACS buffer and 1 ml of buffer per 10^7 cells was added and centrifuged at 300g for 10 minutes. The supernatant was then aspirated completely. The cell pellet was resuspended in 500 μl of MACS buffer and placed on ice. A MS magnetic column was placed in the magnetic field of the MACS separator. The column was rinsed with 500 μl buffer. The cell suspension was applied onto the column and the effluent collected. The column was washed three times with MACS wash buffer. The column was removed from the separator and was placed on a collection tube. About 500 μl of MACS buffer was pipetted onto the column. Magnetically labelled cells were flushed out by firmly pushing the plunger into the column. Cell solutions were centrifuged at 1800rpm for 5 minutes, supernatant removed and resuspended in 10 ml of RPMI-MGIT for counting. Monocyte-depleted cells were stored in freezing medium and kept at -80°C overnight. The depletions were confirmed by flow cytometry. The monocyte depletion ranged from 76 to 90%. The undepleted and the depleted samples were then thawed and added to MGT tubes to determine the mycobacterial growth.
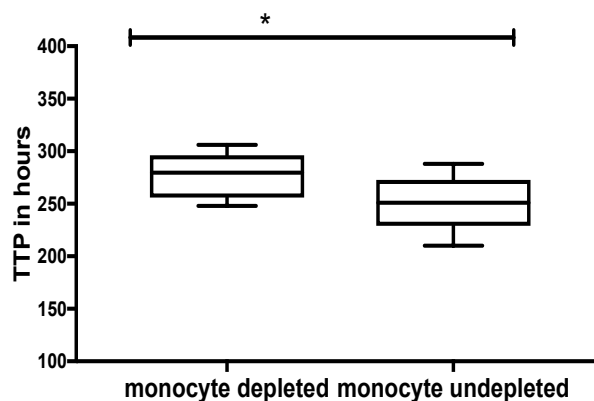
Figure 6.1: Human monocyte depletion. The MGIT assay was performed using depleted and undepleted PBMC (n=10). PBMC were depleted of monocytes using magnetic beads. There were significant differences in growth inhibition between monocyte depleted and undepleted PBMC. A non-parametric paired Wilcoxon test was performed, where * represent a p value of <0.05. The horizontal line represents the median response, the box represents the interquartile range and the whiskers represent the overall range.

The growth inhibition pattern in monocyte-depleted and undepleted PBMC in BCG vaccinated and BCG non-vaccinated healthy donor at LSHTM were observed. There were no significant differences in growth inhibition between monocyte depleted and undepleted PBMC among BCG vaccinated and non-vaccinated healthy donors, although the non-vaccinated group gave varied results (Fig. 6.2).
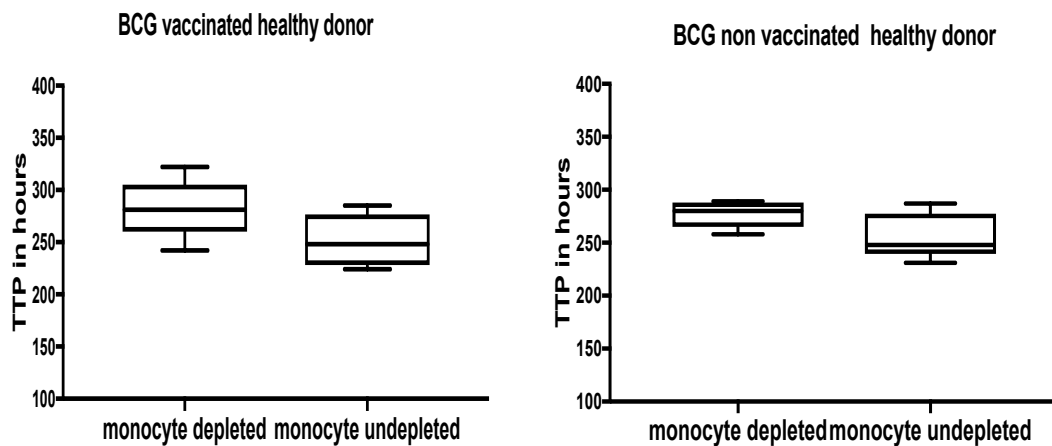
Figure 6.2: Human monocyte depletion in BCG vaccinated and non vaccinated healthy donors at LSHTM. The MGIT assay was performed using monocyte depleted and non depleted PBMC. A non-parametric paired Wilcoxon test was performed. The horizontal line represents the median response, the box represents the interquartile range and the whiskers represent the overall range.

The absolute monocyte counts in these patients was analysed to see if there is any correlation between monocyte count and the IGRA values, which were discussed in previous chapter 4. There were no significant correlations between monocyte count and the mycobacterial growth in LTBI, helminth, LTBI-helminth coinfected patients and the controls. The absolute monocyte counts in these patients at their visit 2 was also available. The result was similar as at visit 1, there was no significant correlation between helminth infected and LTBI-helminth coinfected patients.

6.1.3 ML ratio and MGIA

To further explore the effect of ML ratio, ML ratios calculated from the haematological data of these patients were correlated with the mycobacterial growth. There was a weak trend to positive association between ML ratio and mycobacterial growth in LTBI, LTBI-helminth coinfected patients and helminth-infected patients.

(A) LTBI patients   (B) Helminth infected   (C) LTBI-helminth coinfected
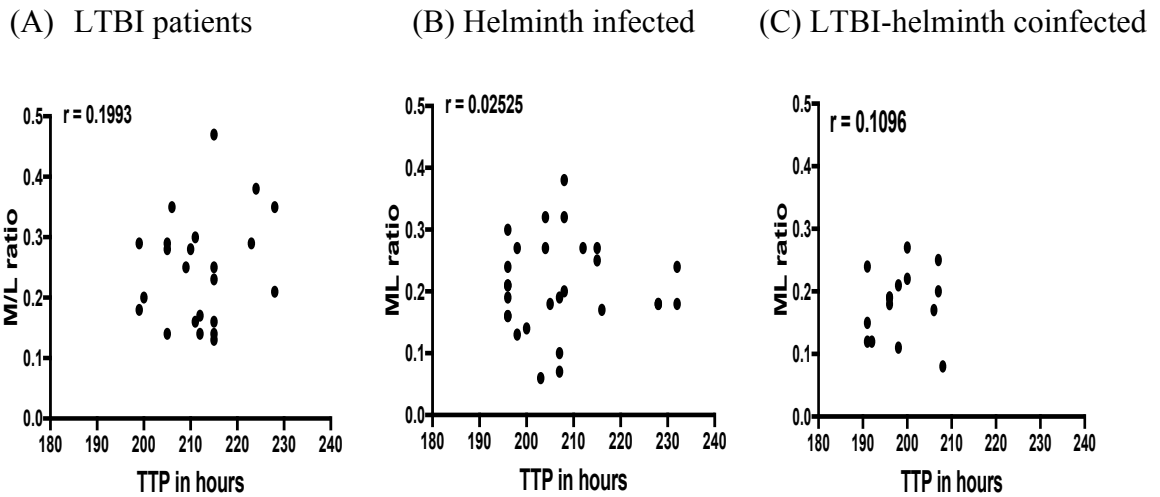
Figure 6.3: Association of ML ratio and mycobacterial growth (visit 1). Mycobacterial growth inhibition was assessed by MGIA using frozen PBMC and ML ratio was calculated from lymphocyte and monocyte count obtained from cell counts performed at UCH. Spearman's correlation between ML ratio and mycobacterial growth measured in the PBMC MGIT assay in (A) LTBI patients (n=23) (p= 0.4386) (B) helminth infected patients (n=28) (p= 0.3245) and (C) LTBI-helminth coinfected patients (n=15) (p=0.273) on their 1st visit (pre-treatment).

After anthelmintic treatment, there was a stronger trend of positive association for the ML ratio and mycobacterial growth in helminth infected and LTBI-helminth co-infected patients during their visit 2, but again the correlation was not statistically significant (Fig. 6.4).

(A)   (B)
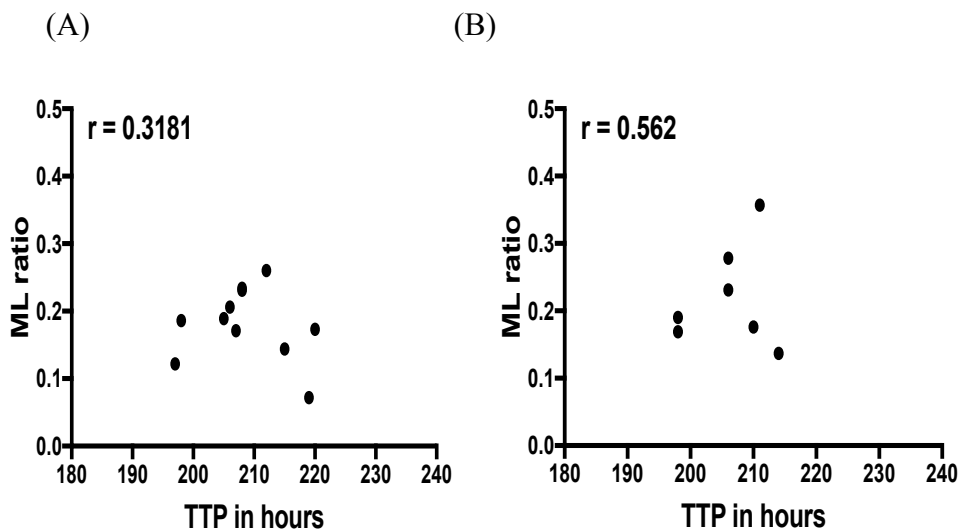
Figure 6.4: Association of ML ratio and mycobacterial growth (visit 2). Mycobacterial growth inhibition was assessed by MGIA using frozen PBMC and ML ratio was calculated from lymphocyte and monocyte count obtained from cell counts performed at UCH. Spearman's correlation between ML ratio and mycobacterial growth measured in the PBMC MGIT assay in (A) helminth infected patients (n=13) (p=0.2966) and (B)

LTBI-helminth coinfected patients (n=8) (p= 0.2316) on their 2nd visit (post-treatment).

6.1.4 The neutrophil:lymphocyte ratio (NLR) and MGIA

The NLR was also explored to see if there is any correlation with the mycobacterial growth in all the four groups. There NL ratio was not significantly associated with mycobacterial growth in LTBI infected patients and helminth infected patients before and after their treatment.

6.1.5 Frequency of eosinophil count and MGIA

The absolute eosinophil count was also explored in all four clinical groups to observe if there was any correlation with the mycobacterial growth inhibition. There was a weak trend of positive association between eosinophilia and mycobacterial growth in LTBI infected patients, helminth infected patients, controls and LTBI-helminth infected patients in their first and second visit but there was a weak negative trend for eosinophilia and mycobacterial growth in LTBI-helminth coinfected patients after their treatment (data not shown).

6.2 The role of cytokines in the mycobacterial growth inhibition assay

6.2.1 The role of IFNγ in MGIA

When associations were explored between IFNγ ELISpot response and mycobacterial growth in the PBMC MGIT, there were no significant associations in all the clinical groups. There was a weaker trend of negative association for IFNγ ELISpot response to PPD and mycobacterial growth in controls, LTBI patients, and coinfected patients in their first and second visit (Fig. 6.5).
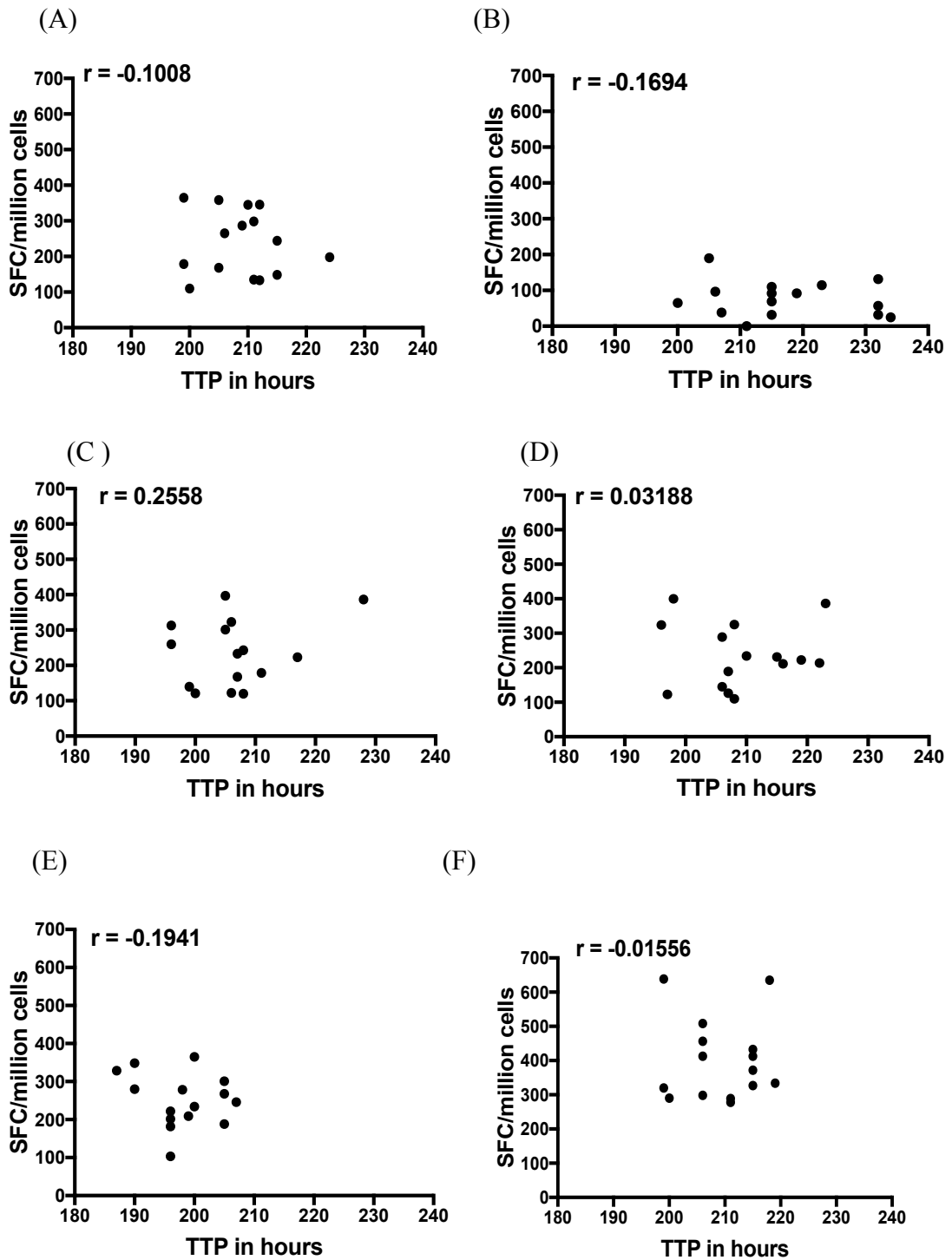
Figure 6.5: Association between IFNγ ELISpot responses to PPD and mycobacterial growth. An IFNγ ELISpot was performed against PPD using cryopreserved PBMCs from control, LTBI infected, helminth infected and LTBI-helminth infected patients before and after anthelmintic treatment (n=15). Spearman's correlation between PPD IFNγELISpot and mycobacterial growth in the PBMC MGIT assay in LTBI (p=0.5335) (A), controls (p= 0.4507) (B), helminth infected on their first visit (p=-0.2081) (C), helminth infected ontheir post-treatment visit (p=0.02736) (D), LTBI-helminth coinfected ontheir first visit (p=0.8065) (E) and on second visit (0.8545) (F).

The association of IFNγ ELISpot responses to ESAT-6/CFP10 and mycobacterial growth was explored in all four clinical groups (Fig. 6.6). The response was very low in controls and helminth infected patients. There was a weak trend of positive association in ESAT-6/CFP10 responses and mycobacterial growth in control and helminth infected patients. In coinfected patients, the ESAT-6/CFP10 response was showed weaker trend of positive association in IFNγ ELISpot responses but after treatment the association was negative, as was also seen in LTBI infected patients.

(A)   (B)

(C)   (D)
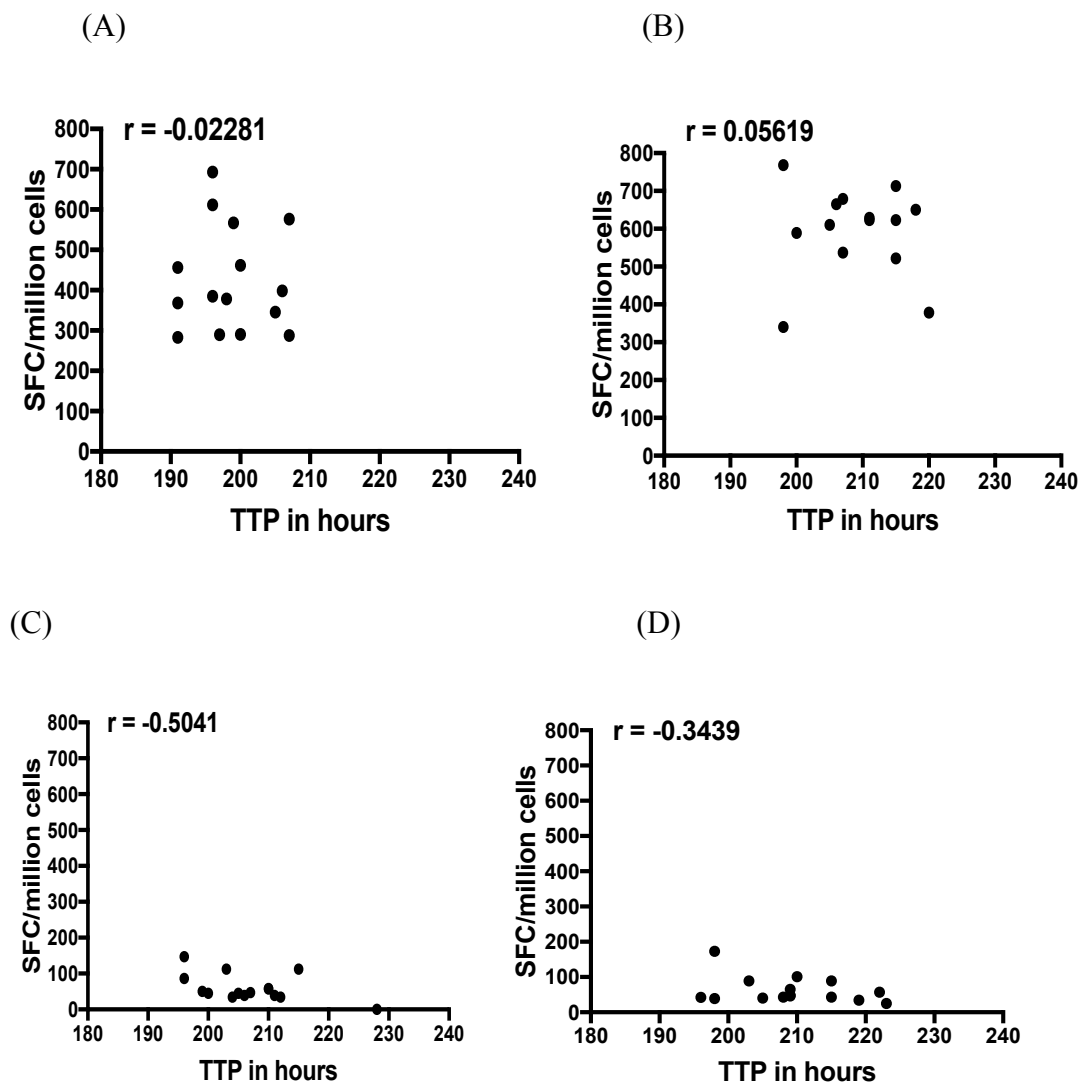
Figure 6.6: Association of IFNγ ELISpot responses to ESAT-6/CFP-10 and mycobacterial growth. An IFNγ ELISpot was performed against ESAT-6/CFP10 using cryopreserved PBMCs from control, LTBI infected, helminth infected and LTBI-helminth infected patients before and after anthelmintic treatment (n=15). Spearman's correlation between ESAT-6/CFP10 IFNγ ELISpot and mycobacterial growth in the PBMC MGIT assay in LTBI-helminth coinfected in V1 (A) and in V2 (B), helminth infected in V1 (C) and in V2 (D).

6.2.2 The role of IL-10 in MGIA

IL-10 inhibits the protective immune response to pathogens by blocking the production of proinflammatory cytokines, such as TNFα and the Th-1-polarizing cytokine IL-12, by directlyacting on antigen-presenting cells such as macrophages andDCs. IL-10 can also inhibit phagocytosis and microbial killing through limiting the production of reactive oxygen and nitrogen intermediates in response to IFNγ all of which are pivotal for mediating immunity to *Mtb*. To further explore the effect of IL-10 on mycobacterial growth, initially recombinant IL-10 (rIL-10) was added to three healthy donors PBMC.

There was significantly increased mycobacterial growth in cultures, when assays were supplemented with rIL-10 in comparison to controls. It was observed that an increased concentration of rIL-10 was associated with increased mycobacterial growth.

(A)
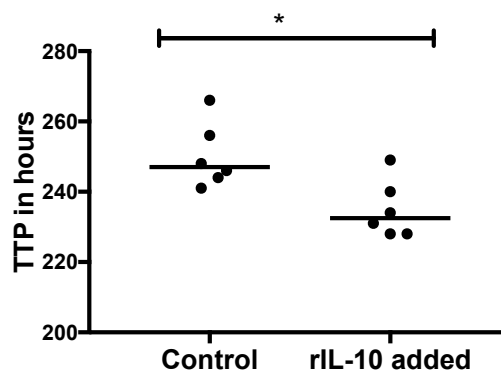
(B)
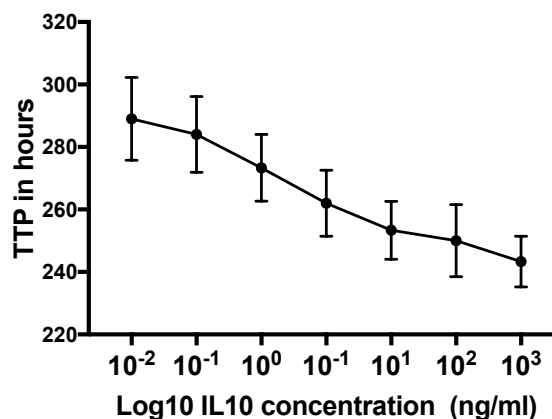
Figure 6.7: Effect of IL-10 on mycobacterial growth. The MGIT assay was performed in PBMC collected from healthy donors at LSHTM (n=6 in figure A and 3 in figure B). The effect was observed in PBMC added with rIL-10 and without rIL-10 (A) (bars represent the median value, a paired t-test was performed, where * represents a p value of <0.005). Increasing concentration of rIL-10 was added to the PBMC of these donors (B), where point represent the mean of duplicate cultures.

6.2.3 The role of TGFβ in MGIA

TGFβ is another cytokine that plays an immunoregulatory role in TB. This cytokine also plays an important regulatory role in helminth infection. Various models of parasite infection have implicated a different number of cytokines and cell types, including IL-10 and TGFβ and FoxP3+Tregs in controlling the host immune response and the resulting immunopathology. The effect of TGFβ on mycobacterial growth was assessed and as for IL-10, three healthy donors PBMC were used for the experiment. Recombinant TGFβ (rTGFβ) was added to the PBMC and then with increasing concentrations, to assess if there is any effect on mycobacterial growth.

There was also a significant increase in mycobacterial growth in cultures supplemented with rTGFβ and like IL-10, with increased concentrations of TGFβ, there was increased mycobacterial growth (Fig. 6.9).
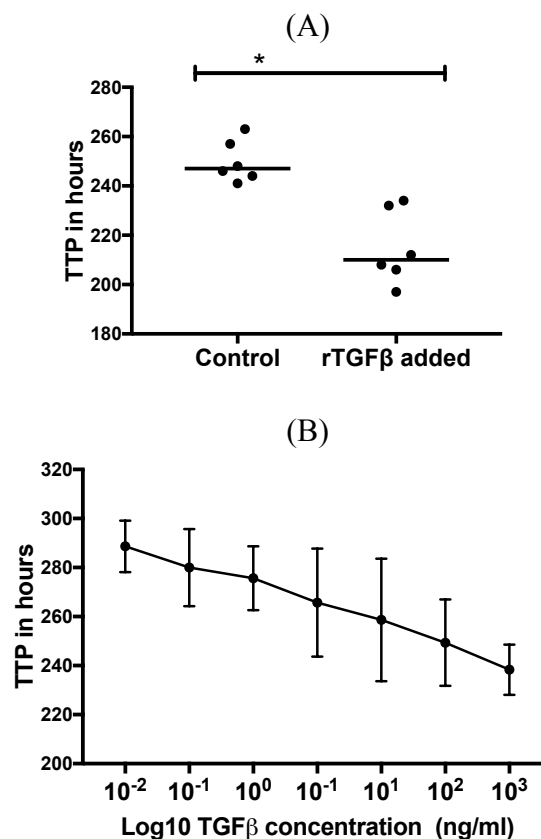
Figure 6.8: Effect of TGFβ on mycobacterial growth. The MGIT assay was performed in PBMC collected from healthy donors at LSHTM (6 in figure A and 3 in figure B). The effect was observed in PBMC with or without rTGFβ. (bars represent the median value, a paired t-test was performed, where * represents a p value of <0.005). Increasing concentration of rTGF-β was added to the PBMC of these donors (B), where points represent the mean of duplicate cultures.

The cytokines IL-10 and TGFβ play immunoregulatory roles in TB and also helminth infections. The experiment also showed that there was significantly increased mycobacterial growth when supplemented with rIL-10 and rTGFβ, suggesting their immunoregulatory role. To explore further the effect of these cytokines on mycobacterial growth, anti-cytokine antibodies were used to block the cytokines. PBMC from 15 helminth infected patients were used for this experiment and anti IL-10, anti TGFβ and anti IFNγ was added to the PBMC. Reduced mycobacterial growth inhibition was observed when IFNγ was blocked using anti-IFNγ antibody indicating IFNγ is needed for better growth control. When IL-10 and TGFβ were blocked there was significantly more growth inhibition suggesting their immunoregulatory role (Fig. 6.10).
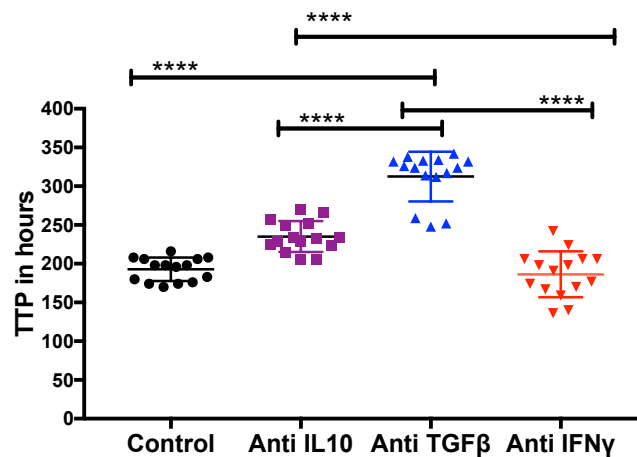
Figure 6.9 :Effect of blocking IL-10, TGFβ, and IFNγ using antibodies on mycobacterial growth. MGIT assay was performed using frozen PBMC in 15 helminth infected patients. Anti-IL-10, anti TGFβ and anti IFNγ were added in the culture supernatant. A two-way ANOVA were performed followed by Bonferroni multiple test,where **** represents a p value of <0.0001.

To further identify the other important cytokines in these group of patients and if there is any correlation with mycobacterial growth, cytokines were measured in supernatants taken at 96 hours. The cytokines were measured using a Luminex assay- which will be discussed in next chapter.

6.3 Discussion

In the previous chapters, the optimization steps of this assay were described to adapt a PBMC based MGIT assayto measure the mycobacterial growth inhibition *in vitro*. This assessment of the capacity of the PBMC compartment to inhibit the growth of

mycobacteria may allow the investigations into the potential roles of immune mechanisms of interest in mediating this protective effect (375)(385)(396). To expand this more, the immune mechanisms underlying growth inhibition including the role of monocytes, eosinophils, ML ratio, NLR, cytokines, antibodies were investigated in controls, LTBI infected patients, helminth infected and LTBI-helminth coinfected UK migrants.

Mtb can establish lifelong, chronic infection in the face of seemingly appropriate immune responses. This extraordinary persistence may be explained by the fact that *Mtb* resides predominantly inside cells of the immune system itself. Monocyte represent the main host cell for mycobacterial survival, growth, replication. Macrophages are the first cells to encounter *Mtb* (459). The signalling program initiated by macrophage receptors, including TLRs,CD14 co-receptor,C-type lectin receptors etc. results in the activation of transcription factors leading to expression of inflammatory mediators. The signals induced by CD14 and TLRs activate feedback inhibitory mechanisms and this transition from a strong inflammatory signalling into a weak activated state may be important in the immune control of *Mtb* infection (460).The MGIT assay was performed using depleted and undepleted PBMC. PBMC were depleted of monocytes using magnetic beads. There were significant differences in growth inhibition between monocyte depleted and undepleted PBMC. In the depleted monocyte state, there was less monocytes present, and lack of target cells for mycobacterial infection might have resulted in an overall reduction in the number of bacteria. Monocyte depletion had similar effects on mycobacterial growth inhibition in BCG and non-BCG vaccinated healthy donors. The non-BCG vaccinated donors exhibited varied results regarding mycobacterial growth. Kleinnijenhus *et al.,* have shown that BCG vaccination in healthy volunteers led to NOD2-dependent epigenetic reprogramming of monocytes, which resulted in an increased expression of cellular receptors on the monocytes, accompanied by higher cytokine production in response to non-related pathogens (461).

The absolute monocyte count obtained from the clinical data from HTD were then explored to see if they have any association with MGIA. There were no significant association between the number of absolute monocyte count in controls and helminth infected patients. A weak trend of positive association was observed for LTBI patients for eosinophil count and mycobacterial growth. The frequency of absolute monocyte count was increased after treatment in LTBI-helminth coinfected patents, but when we explored

the association with MGIA, there was a weak trend of negative association between them. It is unclear to what extent the association in LTBI-helminth coinfected patients may be usefully interpreted, given the small numbers of samples tested (both pre and post treatment).

CD4+T cells and monocytes/macrophages were previously shown to be major effector cells in protecting the host against *Mtb* infection. It was explored whether there were any differences in the monocyte compartment in LTBI and helminth infected patients, measuring their absolute numbers and the effect of ML ratio on their ability to control the growth inhibition of *Mtb*. There was a significant change in ML ratio in coinfected patients but no significant changes from controls, LTBI, or helminth infected patients during their first visit. The ML ratio was not associated with mycobacterial growth in any of the four groups. There was a strong trend for LTBI-helminth coinfected and helminth infected patients for ML ratio and mycobacterial growth to be associated during their second visit. There was a reduction in mycobacterial growth with reduced ML ratio. However, the ML ratio predicted the risk of active TB development in HIV infected patients co-infected with *Mtb* (462). Recent data also have highlighted that an increase of the ML ratio is associated with changes of gene transcription in monocytes that may influence their functional antimycobacterial profiles (463).In an animal study, ML ratio has been associated with inhibition of mycobacterial growth (464). It has been described that human CD14+ monocyte are composed of two subsets based on CD16 expression and that relative percentages of CD16+ monocytes increase along with TB disease severity (463). It would have been interesting to study the functional profile of monocytes and their effect on mycobacterial growth inhibition on these coinfected patients. Turner *et al.,* showed that in *S.mansoni, S.haematobium* or coinfected patients, monocyte recognition of E/S products released by skin invasive cercariae, or eggs, of *S.mansoni* assessed by flow cytometry showed that intermediate CD14brightCD16+monocytes had a significantly enhanced ability to bind to cercarial and egg E/S. This is potentially of significance in the development of schistosome-specific protective immunity or immunopathology (465). Studying the phenotype of monocytes might provide important information regarding the subtypes of monocyte related to immunopathology in helminth infection. The lymphocyte count had no change between visits in all the groups. Manna *et al.,* in their studies have shown that patients with active TB had a very high ML ratio, as compared to LTBI and healthy contacts, as well as cured TB patients. In active TB

patients, ML ratio was significantly correlated with increased monocyte counts and lower lymphocyte count and can be used as an indicator of active TB (466). ML ratio and its association with IFNγ was also explored in the current study, ML ratio changes in blood are likely a marker of changes in the frequency of lineage based haematopoietic stem cells (HSC) and mycobacterial infection has been shown to alter haematopoiesis through IFNγ levels (467). But there was no significant association in LTBI, helminth or LTBI-helminth coinfected patients.

Absolute neutrophil count was observed in the patients and also the treatment effect on neutrophil count was explored. Comparing the values in groups, there were no significant changes in neutrophil count in the clinical samples after anthelminthic treatment. However, in LTBI-helminth coinfected patients, there was a significant reduction in neutrophil count (p<0.05). With neutrophil depletion using specific monoclonal antibody NIMP-R14 during chronic *Mtb* infection, there was a decrease in CFU rather than an increase (468). In humans, the risk of LTBI in contacts of patients with active disease is inversely proportional to the peripheral blood neutrophil count, consistent with the concept that higher neutrophil counts are protective against early infection (469). Absolute neutrophil count was not associated with MGIA in this study. Interestingly, free human neutrophil peptide (HNP) can be taken up by the macrophages and this increases their ability to kill and impair macromolecular biosynthesis of *Mtb* suggesting their potential antimycobacterial role (470). It has been reported that granules or entire apoptotic neutrophils may be phagocytosed by mononuclear cells with trafficking of their contents to endosomes enhance restriction of mycobacterial growth (471). Because of neutrophils predominant role in phagocytosis of microbial pathogens, neutrophils have often been overlooked in helminth infection, although it would be interesting to address the role of neutrophils in the sustained inflammatory milieu in helminth infection (472).

The NLR, another measure of the myeloid: lymphoid cell proportion, has been reported to be associated with cancer and some cardiovascular disease outcome. The NLR of pleural fluid has been evaluated as an adjunctive toll in patients with TB pleurisy and also used as a marker of inflammation and for discriminating patients with pulmonary TB and bacterial pneumonia (473). In this study there was no significant change in neutrophil and lymphocyte counts in the patients during their 2 visits, only a slight reduction in LTBI-helminth coinfected patients after they received treatment.

In the previous chapter, the absolute eosinophil count was explored in all the clinical samples. The count was significantly reduced in LTBI compared to the controls. After treatment, in helminth infected and LTBI-helminth coinfected patients, the eosinophil count was significantly reduced (p<0.0001 and p<0.005). The association of absolute eosinophil count and mycobacterial growth was explored. There was a weak positive trend in all the samples, however, LTBI-helminth coinfected patients showed a reverse association after they were treated with anthelmintic treatment. Eosinophil infiltration of the lungs and peripheral eosinophilia have been associated with tuberculosis disease in *Mtb*-infected patients (474). It was hypothesised that eosinophil influx may exaggerate the disease severity because eosinophils, which have been shown to phagocytose mycobacteria, may provide an intracellular habitat in which BCG could proliferate in an unrestricted manner. BCG-infected eosinophils might also have contributed to haematogenous dissemination of BCG, leading to an increased bacterial load in other tissues (475).

In order to compare immunological responses in the LTBI, helminth and LTBI-helminth coinfected patients with growth inhibition, IFNγ responses were first measured by ELISpot. There was a significant increase in the magnitude of IFNγ ELISpot responses to PPD following LTBI-helminth coinfected patients after they received anthelmintic treatment during their second visit. When the groups were compared, it was found that the IFNγ ELISpot responses were significantly higher in LTBI infected patients in comparison to the controls, helminth and coinfected patients (p<0.0001). When the IFNγ ELISpot responses were observed in response to ESAT-6/CFP10, there was a similar pattern of expression. Interestingly, there was no difference in controls and helminth infected patients, as that of PPD responses. However, in LTBI-helminth coinfected patients, the IFNγ response to ESAT-6/CFP10 was significantly increased after anthelmintic treatment. The relationship between the induction of antigen specific T cell responses in LTBI, helminth and LTBI-helminth coinfected patients and their ability to control mycobacterial growth *in vitro* was explored. There was no significant correlation between mycobacterial growth inhibition and IFNγ ELISpot responses in LTBI, and controls. There was a weak negative trend for helminth and LTBI-helminth coinfected patients for mycobacterial growth inhibition and IFNγ ELISpot responses before they receive anthelmintic treatment and a weak inversion after they received the treatment. This indicates that a T cell response may contribute to the control of mycobacterial

growth after the anthelmintic treatment. After treatment, after removing helminths from the patients, there is induction of a greater number of PPD antigen-specific T cells, although this increased number of T cells does not result in an improved capacity to control mycobacterial growth *in vitro*. IFNγ secreting T cells alone did not reflect the overall capacityof cells to control mycobacterial growth. This was consistent with other studies where increased IFNγ responses were not correlated with MGIA (394)(476)(477).In helminth infected patients the IFNγ response were significantly increased after treatment. A hallmark of helminthic infections, both in experimental models and human infection is the generation of profound Th2 and T regulatory cell responses that have the potential to impede Th1 cell development. Children with onchocerciasis exhibit lowered cellular responses to PPD antigens (478). Coincident helminth infection is also associated with reduced Th1 responses in active and latent TB (355)(426). Studies regarding interaction of helminths with mycobacteria, but none of these studies fully explored the mechanistic basis for how helminth-modulated immune response has an impact on host control of *Mtb* infection. The lack of association between the IFNγ ELISpot responses and MGIT assay in these coinfected patients might necessitate to address the more complex host-pathogen interaction and to look at the other immune parameter for better understanding of the underlying immunopathogenesis.

Infection of various cell types with *Mtb* has been shown to induce IFNα (479). It has been shown that a hyper virulent strain of *Mtb* induces higher levels of IFNα than normal strains and kills infected mice at a faster rate- which is partially caused by the failure to induce a proper Th1 response and partly production of type 1 IFNs and dampening of the immune response by strong IL-10 induction (480). Antonelli *et al.,* shown that type 1 IFN receptor deficient mice chronically infected with *Mtb* strains showed reduced bacterial loads (481). The level of IFNα in all the patients in this cohort was measured. In LTBI and helminth infected patients IFNα concentrations were reduced compared to the controls. There was a significant increase in IFNα concentrations in LTBI-helminth coinfected patients in comparison to LTBI patients. It would be interesting to explore the levels of IFNα after the anthelmintic treatment to see if the treatment leads to the disappearance of this signature and also to measure IL-10, as it has been shown that type 1 IFNs induce the immunosuppressive cytokine IL-10,which might contribute to loss of *Mtb* infection control (482).

IL-10 and TGFβ have diverse roles during mycobacterial infection ranging from immune

evasion to immune protective.In helminth infection, the immune response is specially related to IL-4, IL-10 and TGFβ production, preferentially generated by Th2 or Treg cells (483). The effects of the regulatory cytokines, IL-10 and TGFβ on mycobacterial growth was evaluated in this study. To explore the immunoregulatory effects of IL-10 and TGFβ, the MGIT assay was performed using human PBMC cultured with or without addition of rIL-10 and rTGFβ in helminth infected patients. When cultured with rIL-10 and rTGFβ, there was significantly greater growth of mycobacterium in comparison to culture without rIL-10 and rTGFβ. In animal models, chronic infection with *Fasciola hepatica* enhances IL-10 and TGFβ by PBMCs in response to parasite antigen (484). IL-10 can enhance the intracellular survival of bacilli via inhibiting phagosomal maturation, reducing nitric oxide production and blocking IFNγ signalling in macrophage and can antagonize the development of Th1 responses through suppressing antigen presentation and IL-12 production (485). However, the combination of TGFβ and certain other cytokines, may induce T cells to differentiate into non-regulatory phenotypes such as Th17 effectors in the presence of IL-6. The action of TGFβ on T cells is antagonized by IFNγ and IL-4 (486). In vitro studies have shown that SEA induces the expression of FoxP3 in naïve T cells via TGFβ, which confers Tregs cell-expanding or tolerogenic properties on DC (487). The findings in this experiment, are consistent with the evidence in the literature that IL-10 and TGFβ has immunosuppressive activity and may contribute to mycobacterial disease (488)(489). To further explore the immunoregulatory effects of IL-10 and TGFβ, the cytokines were blocked with anti-IL-10 and anti-TGFβ and also anti IFNγ was also used. Blockage of IL-10 and TGFβ (culture with out cytokines) caused significant reduction in mycobacterial growth in comparison to culture with these cytokines, whereas, blocking IFNγ increased the mycobacterial growth-suggesting the role of IFNγ in controlling mycobacterial growth. Neutralization of IL-10 and TGFβ resulted in increased production of both IL-4 and IFNγ, suggesting the parasite may induce these anti-inflammatory cytokines to evade the immune response also suppression of IFNγ production by parasites may mediate parasite survival the disease. However, the cellular source of these cytokines are needed to be explored (490).

Chapter - 7   MGIA in clinical samples

7.1   MGIA in clinical samples

Helminth infection modulates the immune response by skewing the Th1/Th2 balance and thereby attenuating the effects of the BCG vaccine (356). However, clinical studies investigating the impact of deworming during active TB in humans remain very few in number and the responses obtained were varied (296)(306)(297). A randomized double-blind clinical trial examining the effect of anthelmintic treatment on clinical improvement of TB after 2 months showed no significant effect of anthelmintic (albendazole) treatment over placebo (491). In previous study by Toulza *et al.*, the impact of deworming on the in-vitro T cell memory responses to *Mtb* was observed in the IDEA London cohort of immigrants. The study showed a potential role for Treg cells is downregulating CD4+ IFNγ T cells and Treg numbers decreased and that of CD4+IFNγ+T cells increased after anthelmintic treatment (370). To further explore the effect of anthelmintic treatment on mycobacterial growth inhibition, the MGIT assay was performed in the clinical samples. The aim was to observe the effect of anthelmintic treatment on mycobacterial growth inhibition in helminth-infected and LTBI-helminth coinfected patients before and after anthelmintic treatment. The MGIA was also performed in patients with LTBI and controls at V1 and V2. For most of the experiments described in this project, BCG was used as a surrogate for *Mtb*. To explore how helminth infection affects the growth inhibition of virulent *Mtb*, *Mtb* Erdmann and *Mtb* H37Rv were also tested.

Aims

The aims of the experiments detailed in this chapter were to

- investigate the potential effect of anthelmintic treatment on mycobacterial growth inhibition in helminth and LTBI-helminth coinfected patients
- observe the effect of antigen stimulation (PPD, ESAT-6/CFP-10 and helminth antigen- SEA antigen of *S.mansoni* and *S.haematobium*, AWA antigen of *S.mansoni* and *S.haematobium*) on the pattern of mycobacterial growth control.
- test how helminth infection affects the growth inhibition pattern of *Mtb* Erdman and *Mtb* H37Rv and whether anthelmintic treatment affects the growth inhibition.
- explore if BCG provides an estimation of growth inhibition of *Mtb* by observing if there is any correlation between growth inhibition of BCG and *Mtb* Erdman.

7.1.1 Mycobacterial growth inhibition in controls, LTBI, helminth and LTBI-helminth coinfected patients

The MGIT assay was performed in helminth-infected patients and LTBI–helminth coinfected patients before and after anthelmintic treatment (V1 and V2). The growth inhibition was also observed in controls and LTBI at V1 and V2. In these experiments, the potential effect of anthelmintic treatment on mycobacterial growth inhibition in helminth infected patients was also observed. Cryopreserved PBMC were used for these experiments.

At recruitment, there was significant reduction in mycobacterial growth inhibition in LTBI (p=0.02), in helminth infected patients (p=0.0009) and in LTBI-helminth coinfected patients in comparison to controls (Fig. 7.1). Growth inhibition in coinfected patients, was reduced in comparison to LTBI (p=0.0017) and to helminth infected patients (p=0.04). There were no significant differences in TTP between helminth infected and LTBI.
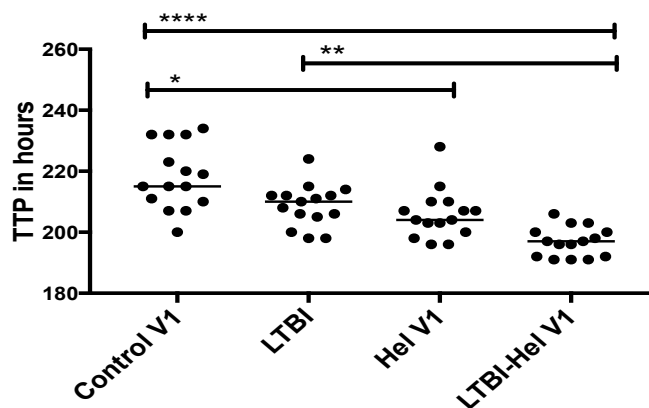
Figure 7.1: Mycobacterial growth inhibition in controls, LTBI, helminth-infected and LTBI-helminth infected patients (visit 1). The MGIT assay was performed pre-treatment (V1) in helminth and LTBI-helminth coinfected patients, LTBI and controls (15 patients per group). Fifteen donors were tested per group. Bar represents median values. A one-way ANOVA was performed, where * represents p value of 0.02, ** represents a p value of <0.005 and **** represents a p value of <0.0001.

A similar experiment was performed in the four clinical groups to see the effect of anthelmintic treatment in helminth infected people. Cryopreserved PBMC were used for the experiment both pre-treatment and post treatment, with the paired samples for each donor. Mycobacterial growth inhibition was significantly reduced in helminth infected and LTBI-helminth coinfected patients, which reversed after anthelmintic treatment

(p<0.001). There was no significant change in MGIA in controls and LTBI patients at two time points (Fig. 7.2). There was significant improvement in mycobacterial growth inhibition in LTBI-helminth coinfected patients (p<0.001). In LTBI-helminth coinfected patients, the lowest growth inhibition was shown pre-treatment, and they had the greatest change post-treatment.
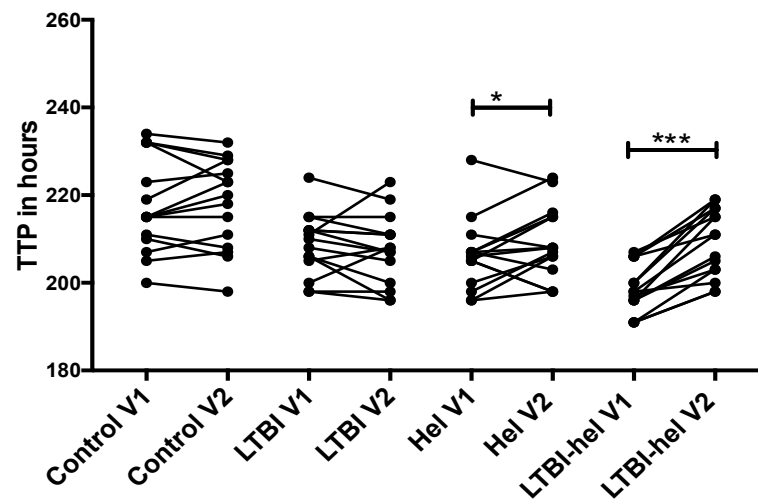
Figure 7.2: Mycobacterial growth inhibition in controls, LTBI, helminth infected and LTBI-helminth infected patients (visit 1 and 2). The MGIT assay was performed in pre and post anthelmintic treatment (V1 and V2) in helminth and LTBI-helminth coinfected patients. PBMC from LTBI and controls were collected at V1 and V2 (without any treatment) and the MGIT assay performed. Fifteen donors were tested per group. A one-way ANOVA was performed, where, * represents a p-value of <0.01 and *** represents a p-value of <0.001.

In this study, 11 patients (4 controls, 2 LTBI, 3 helminth-infected and 2 LTBI-helminth coinfected patients) were bled at 3 points- the first one during their recruitment, the second after one month of recruitment and the third after they finished their anthelmintic treatment- 4 months after visit 1 (recruitment). The log CFU change was observed in all 4 groups at these 3 time points to see the baseline level of variation in immune responses (Fig. 7.3). No significant change was seen between growth inhibition at the first and extra second visit.

(A) Controls   (B) LTBI patients

(C) Helminth infected   (D) LTBI-helminth coinfected
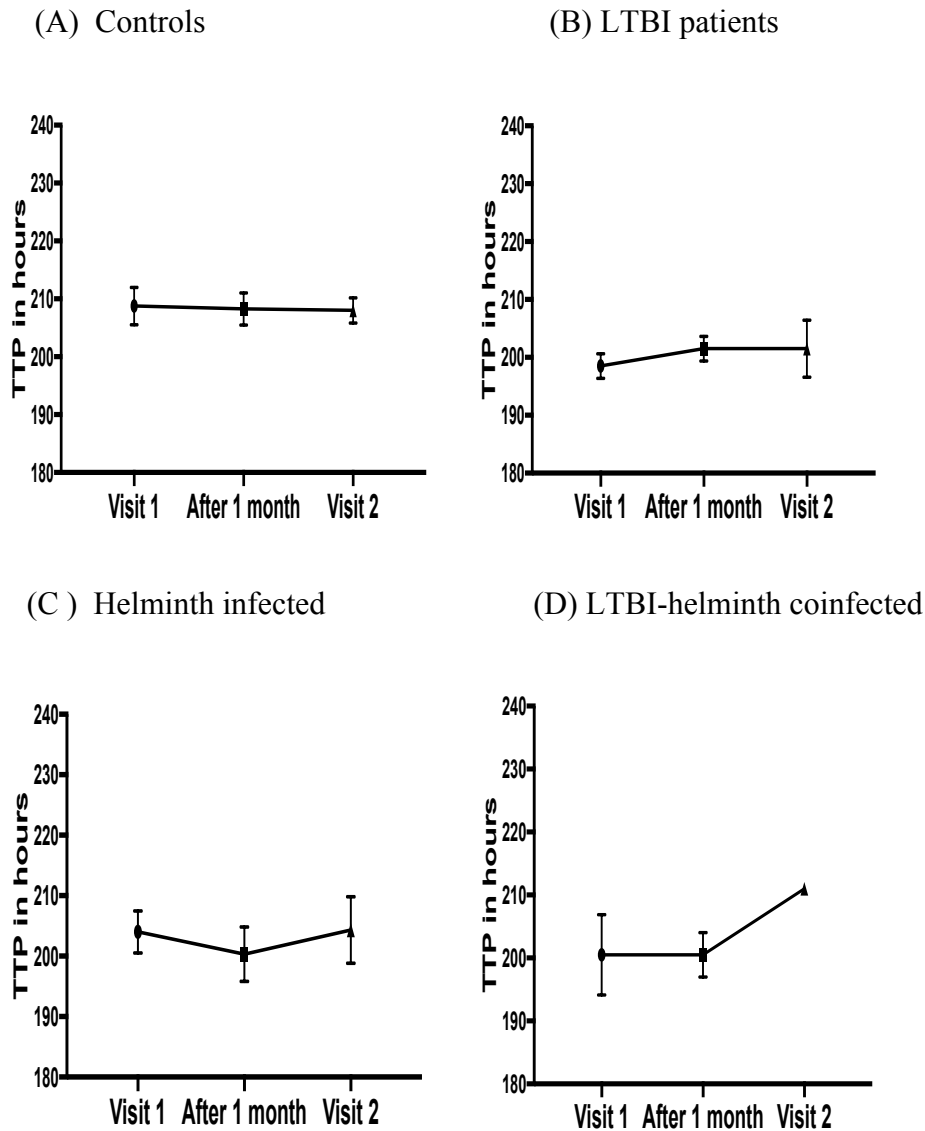
Figure 7.3: Mycobacterial growth inhibition in controls (n=4)(A), LTBI (n=2)(B), helminth infected (n=3)(C) and LTBI-helminth infected patients (n=2)(D). The MGIT assay was performed pre-treatment (visit 1), one month after recruitment and post anthelmintic treatment (Visit 2) in helminth and LTBI-helminth coinfected patients. PBMC from LTBI and controls were also collected at these three points and the MGIT assay performed. A one-way ANOVA was performed.

7.1.2 Effect of antigen stimulation on mycobacterial growth inhibition

The effect of mycobacterial and helminth antigen stimulation was explored in controls, helminth, LTBI, LTBI-helminth coinfected patients using cryopreserved PBMC. Cells were stimulated with PPD, ESAT-6/CFP-10, SEA of *S.mansoni* and AWA antigen of *S. mansoni* added at the same time as the BCG in the culture for 96 hours and the MGIT was performed (Fig 7.4).

In controls there were significant changes in mycobacterial growth inhibition in response to stimulation with SEA and AWA antigen when compared to PPD (p=0.03) and ESAT-6/CFP10 antigen stimulation (p=0.001). When PBMCs were stimulated with SEA and AWA antigen, there was a trend towards significantly increased mycobacterial growth in the LTBI, helminth and LTBI-helminth coinfected patients compared to the control cultures without stimulation and this was statistically significant.
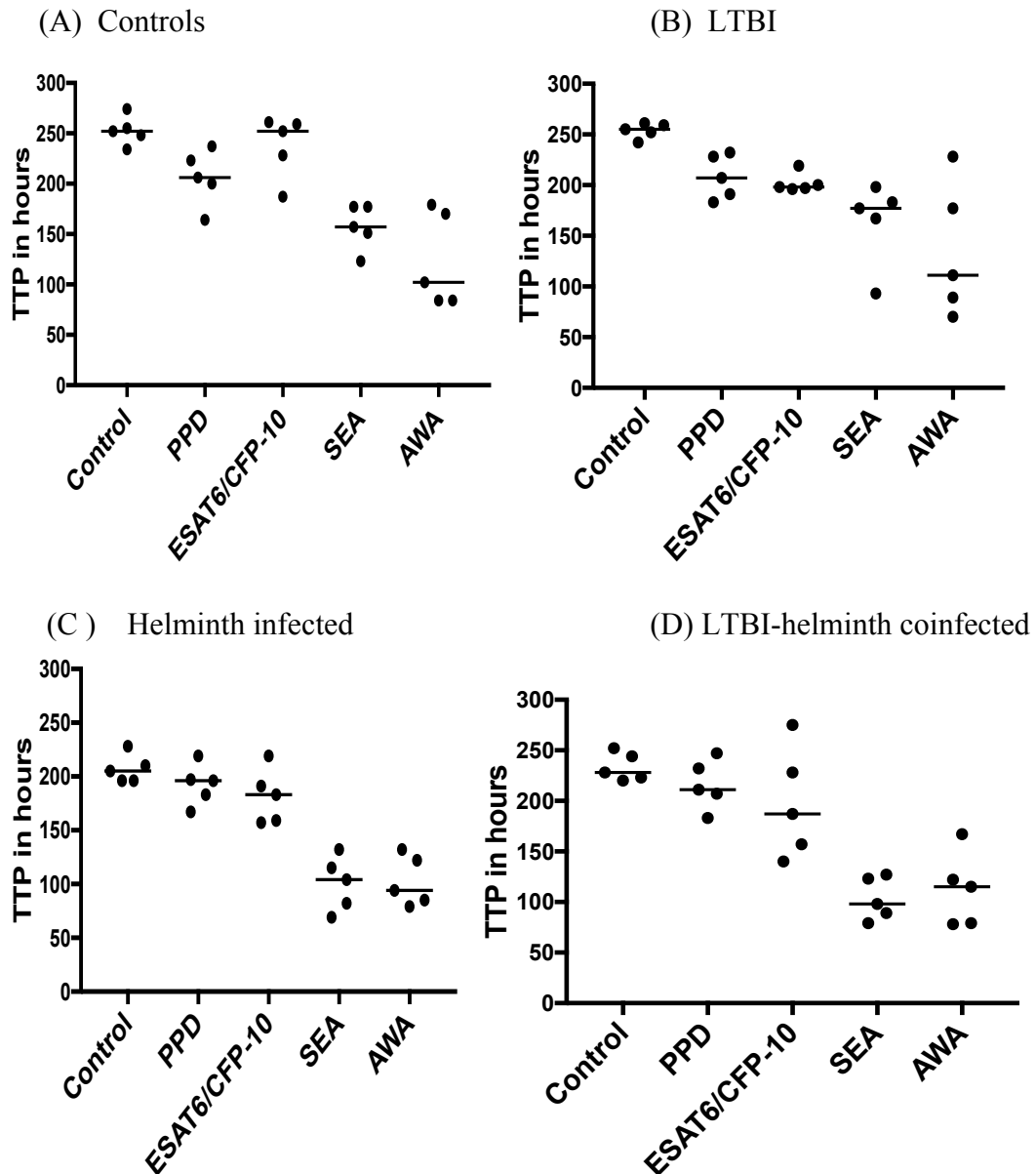
Figure 7.4: Mycobacterial growth inhibition responses with stimulation using PPD, ESAT-6/CFP-10, SEA and AWA antigen. MGIT was performed in PBMC stimulated with PPD, ESAT-6/CFP-10, Schistosoma antigen- SEA and AWA antigen in controls (A), LTBI (B), helminth infected (C) and LTBI-helminth coinfected patients (D). Five donors were tested per group. Bar represents the median values. A Kruskal-Wallis with a Dunn's post-test was performed.

The experiment was repeated. For the MGIA, helminth antigens were dilulted with endotoxin-free PBS and the antigens were used from the stock solution. The helminth antigens were used at 10μg/ml final concentration. Mycobacterial growth in response to addition of SEA and AWA antigen were significantly reduced compared to that of first expeirment. The antigens had been aliquoted in PBS which was not endotoxin free– possibly after using the LPS free PBS, the cytotoxicity was removed.

7.1.3 MGIT using *Mtb* Erdman and *Mtb* H37Rv

For all the optimization steps of the MGIT assay and for most of the tests using the clinical samples, BCG was used. *Mtb* Erdman and *Mtb* H37Rv was used in this experiment, to explore how the growth of virulent *Mtb* is modulated in helminth coinfection and the effect of anthelmintic treatment on their growth. We also wanted to explore whether there was an association between growth inhibition of BCG and of *Mtb* and whether BCG can be used to evaluate growth inhibition of virulent *Mtb*. This work was done in the Category 3 Laboratory with an optimized protocol.

The MGIT assay with *Mtb* was performed using PBMC from healthy controls at LSHTM and from LTBI infected patients. Alhtough the PBMC MGIT assay detected a difference in mycobacterial growth inhibition between LTBI infected patients and healhty controls at LSHTM, the difference was not statistically significant. There was little difference in growth inhibition of *Mtb* H37Rv and *Mtb* Erdman strains between the healthy controls and LTBI infected patients (Fig. 7.5). The reduction in mycobacterial growth observed using *Mtb* H37Rv in LTBI patients was significantly different than that seen in controls (p=0.018). LTBI infected patients exhbited significant differences in growth inhbition pattern when MGIT was performed with H37Rv and *Mtb* Erdman strain (p=0.05). The *Mtb* Erdman strain was selected for the next experiment to see the effect of anthelmintic treatment and to explore the pattern of growth inhibition of BCG and *Mtb*.
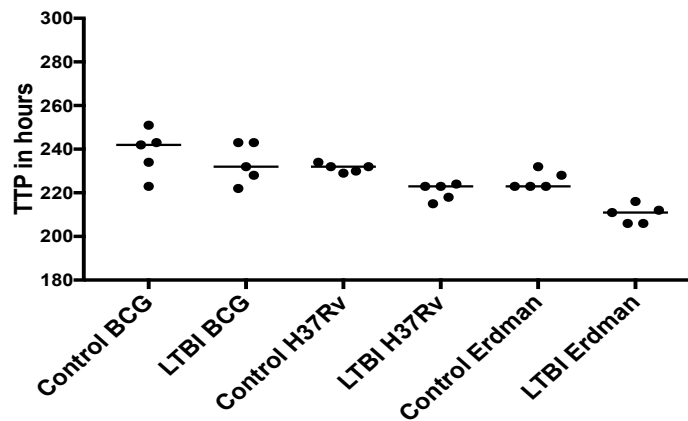
Figure 7.5: MGIT assay in healthy controls and LTBI infected patients using BCG, *Mtb* Erdman and *Mtb* H37Rv. The PBMC MGIT assay was performed in healthy controls and LTBI patients using BCG, *Mt b*Erdman, and *Mtb* H37Rv. Five donors were tested per group. Bars represent the median values. Having passed a normality test, a repeated measures ANOVA was performed followed by a Dunn's post-test.

The MGIT was then performed with BCG and *Mtb* Erdman strain to see whether there was any variation in mycobacterial growth inhibition in helminth and LTBI-helminth co-infected patients. The experiment involved 10 patients per group- and used both pre and post treatment samples. A significant reduction in mycobacterial growth was observed following treatment in helminth infected patients (p=0.0070) and LTBI-helminth coinfected patients (p= 0.0190) when either *Mtb* Erdman strain or BCG were used (Fig. 7.6). Unlike the previous experiment, where we looked at the impact of anthelmintic treatment on mycobacterial growth in all four clinical groups (in 15 patients each group) using BCG, there were significant changes in helminth and LTBI-helminth coinfected patients that reversed after treatment. Similarly, when we explored the *Mtb* Erdman strain, helminth infected and LTBI-helminth coinfected patients showed the similar pattern, but the change was not statistically significant maybe because of the smaller sample size.
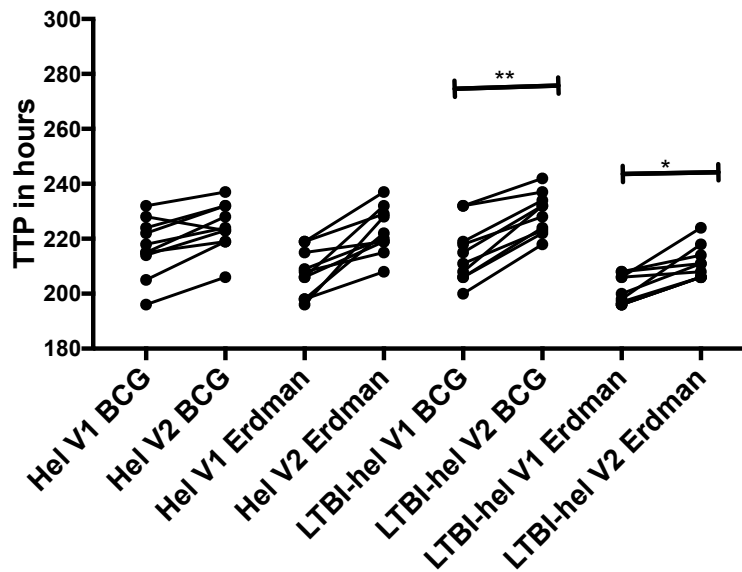
Figure 7.6: MGIT assay in helminth infected and LTBI-helminth co-infected patients using BCG and*Mtb* Erdman. The PBMC MGIT assay was performed in helminth-infected and LTBI-helminth co-infected patients using BCG and *Mtb* Erdman. Ten donors were tested per group. Bars represent the median values. A repeated measures ANOVA was performed followed by a Dunn's post-test, where ** represents a p value of <0.05 and * represents a p value of 0.023.

Next it was explored if the inhibition of BCG provides a satisfactory estimate of *Mtb* growth inhibition and can correlate with the clinical outcome, so that BCG could be used instead of *Mtb*. The experiment was performed with each of the species (BCG and *Mtb*Erdman) to see if there is any correlation between growth inhibition of the two strains in controls, LTBI, helminth-infected and LTBI-helminth coinfected patients both pre-treatment and post-tretament. There was no significant corrrelation between the growth of BCG and Erdman in controls and LTBI infected patients, although there was a weak trend towards a positive association of growth of BCG and *Mtb* Erdman in LTBI-helminth coinfected patients at V1. Interestingly, there were significant positive associations in helminth infected patients between growth inhibition of the two species both pre and post treatment (p= 0.0060 and p=0.0034 respectively) (Fig. 7.7).

(A)   (B)

(C)   (D)
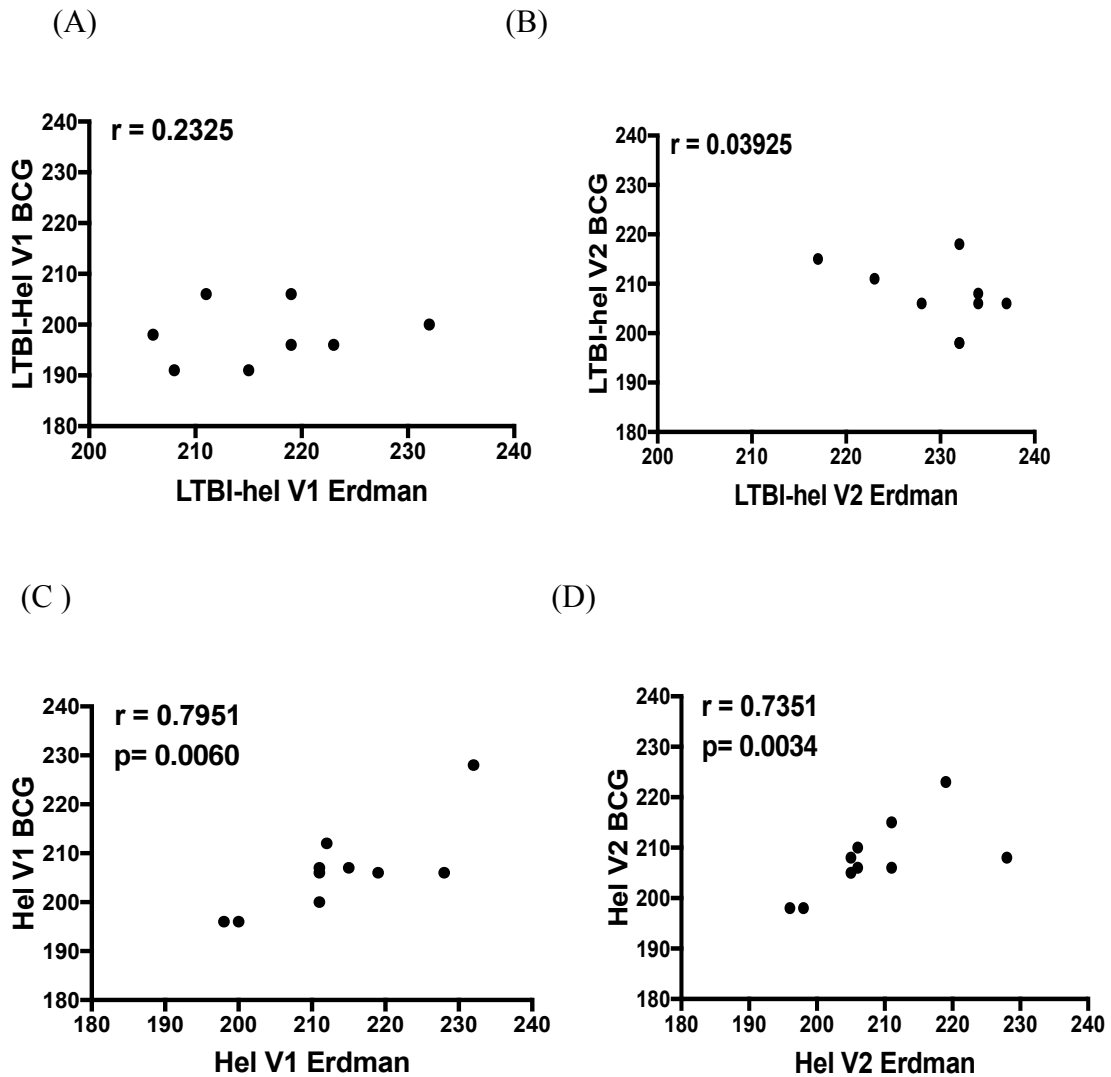
Figure 7.7: Correlation between BCG Aeras and *Mtb* Erdman growth. Spearman's correlation between TTP of BCG Aeras and *Mtb* Erdman in MGIT culture of PBMC from LTBI-helminth coinfected patients both at V1 and V2 (A and B) and helminth infected patients at V1 (C) and V2 (D). Ten donors were tested per group.

The MGIA pattern in patients infected with - Schistosoma spp and Strongyloides spp- were compared in these helminth infected and LTBI-helminth coinfected patients, to see if there is any significance difference in the ability of these helminth species to affect antimycobacterial immune responses. MGIA was performed in 15 Schistosomiasis infected and 15 Strongyloidiasis infected patients, 8 Schistosomiasis and LTBI coinfected patients and 7 Strongyloidiasis and LTBI coinfected patients. There was no significance difference in the mycobacterial growth inhibition in patients infected with Schistosoma or Strongyloides spp (Fig. 7.8).
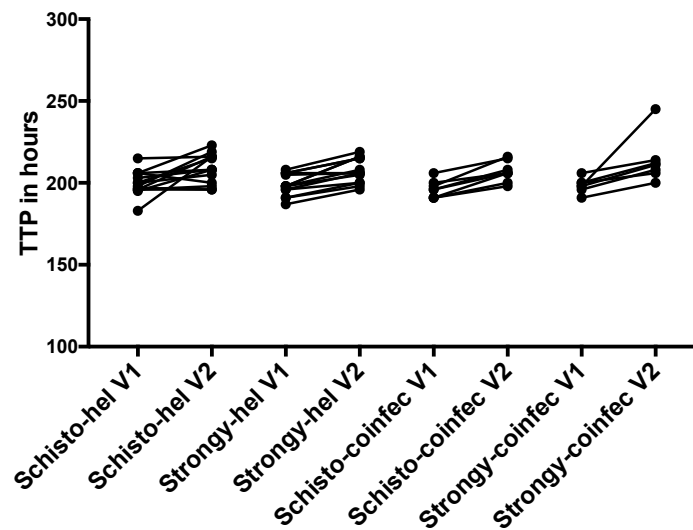
Figure 7.8: MGIT assay in helminth infected and LTBI-helminth co-infected patients infected with Schistosoma and Strongyloides spp. The numbers tested were 15 Schistosomiasis infected and 15 Strongyloidiasis infected patients, 8 Schistosomiasis and LTBI coinfected patients and 7 Strongyloidiasis and LTBI coinfected patients. Bars represent the median values. A repeated measures ANOVA was performed followed by a Dunn's post-test.

7.2 Discussion

Helminth infections modulate the immune system toward a Th2 profile with regulatory responses rather than Th1 responses. Cytokines that mediate humoral immunity can inhibit Th1 immunity, leading to immune polarization and this impacts on the Th1-based immunity required for control of *Mtb* infection (488)

(493). Chronic helminth infestations are also associated with anergic immunosuppressive conditions such as HIV, diabetes mellitus and malnutrition that may be associated with false-negative test results for *Mtb* infection (494)(495)(496). It was hypothesized that anthelmintic treatment in LTBI-helminth coinfected UK migrants would lead to reconstitution of the Th1 response and improve the capacity of the cells to control the growth of mycobacteria. To explore the effect of anthelmintic treatment on mycobacterial growth inhibition, the MGIT assay was performed in the clinical samples. The aim was to observe the effect of anthelmintic treatment on mycobacterial growth inhibition in helminth-infected and LTBI-helminth coinfected patients before and after anthelmintic treatment.

In this experiment, a London cohort of immigrants who had presented with or without LTBI were recruited to evaluate the impact of deworming on the *in vitro* T-cell responses and the ability of the cells to control the growth of mycobacteria. This cohort provided the opportunity to study these responses in a setting where the risk of reexposure and reinfection was negligible and was an ideal situation in which to study the impact of anthelmintic treatment on the antimycobacterial immune responses.

The MGIT assay was performed using PBMC from controls, LTBI, helminth infected and LTBI-helminth co-infected patients at baseline (recruitment). There was significant reduction in mycobacterial growth inhibition in LTBI (p=0.02), in helminth infected patients (p=0.0009) and in LTBI-helminth coinfected patients (p<0.001) in comparison to controls. Helminth infections, in addition to Th2 responses, can induce the suppressive T cell population, Tregs which produce inhibitory cytokines such as IL-10 and TGFβ that suppress Th1 type responses and interfere with effector T cell activation (497). Studies have shown that mice chronically infected with *M.avium* and subsequently infected with *S.mansoni* infection developed granuloma-derived lymphocytes producing PPD-specific IL-4, not IFNγ in coinfected mice, in contrast to the Th1 cytokine pattern by lymphocytes of *M.avium*–only infected mice with impaired resistance against subsequent infection by *Mtb* BCG (498). A similar finding was observed in the mouse model study conducted by Monin *et al.,* where coinfection with *S.mansoni* or immunization with SEA antigens could reversibly impair *Mtb* specific Th1 cell responses but without severely affecting the ability of the macrophages to limit *Mtb* control (422). *S.mansoni* infection resulted in accumulation of arginase-1 expressing macrophages in the lung, forming type 2 granulomas and resulting in increased lung fibrosis and mucus plug formation, exacerbating disease severity and inflammation in *Mtb* coinfected mice. Helminth related alterations of T cell response could also be explained by comparing the immune activation status and cellular response to PPD in chronically helminth infected patients, where PBMC from these people were found to have a greater expression of CTLA-4, a negative regulator of T cell responses that leads to downregulation of Th1 type cytokines (354).

A similar experiment was performed here in the four clinical groups to see the effect of anthelmintic treatment in helminth infected people. Cryopreserved PBMC were used for the experiment both pre-treatment and post treatment, with the paired samples for each donor tested in the same experiment. In helminth coinfected and LTBI-helminth coinfected patients, there was an increase in mycobacterial growth at baseline compared

to that in controls, which reversed after anthelmintic treatment. The anti-schistosome immune response after praziquantel therapy has been characterized in animal models and humans. It has been shown that, damage to tegument exposes antigens that induce strong type 2 immune responses, characterized by increased production of schistosome-specific IL-4, IL-5, IgG and total IgE concentrations, peripheral eosinophilia and histamine concentrations. Increases in such Th2 responses might be expected to affect the immune response, with suppressed Th1 cytokine response and increased Th2 cytokine responses (499). A study conducted in Ethiopia demonstrated that PBMCs from *S.mansoni* infected individuals display poor responsiveness to mycobacterial antigens, characterised by impaired *Mtb* specific IFNγ production and reduced T cell proliferation compared to dewormed controls (358). Helminth induced immune hyporesponsiveness by IL-10 and TGFβ blocks the proliferative responses to recall responses to mycobacterial antigen (500). When occupying the host, most helminths release large amounts of antigenic material, leading to chronic immune activation, but with the increasing burden worms seem to modify and down-regulate these responses to adapt and survive, potential exhausting the local immune potential of the host, leading to hyporesponsiveness. This might be another reason for such responses (501). A reduction or elimination of intestinal helminths using broad spectrum anthelmintic treatment resulted in enhancement of T cell proliferation and IFNγ production by PBMC stimulated with PPD. T cell responses to PPD were found to be improved in filarial infected subjects after treatment with diethylcarbamazine (320)(502). Some studies have shown that there is no significant effect of anthelmintic treatment on clinical outcome of TB (356)(491). These varied responses may be attributed to different species of helminth, their location in the body, different life cycles, different secretory and excretory products, or *Mtb* infection. Different strains of mycobacteria and their route of administration may also contribute. In vitro studies have demonstrated that the impact of helminth infection on *Mtb* infection-andon the clinical outcome is not clearly understood, which may be due to underpowered studies, type or intensity of infecting helminths and the different methodologies used for the detection of helminth infection (135). In this cohort, 7 patients were infected with multiple helminth species, for example- schistosoma spp, Strongyloides, filaria and tapeworm. The CD4+ IFNγ response was lower in those patients and also mycobacterial growth was significantly higher at baseline by MGIA. A case control study from Ethiopia showed an association between helminth and TB coinfection and the association was stronger in patients that were infected with multiple helminth species(503).

MGIA was performed in 11 patients of this cohort, who were bled at three time points-pre-treatment, one month after recruitment (but still pre-treatment) and post-treatment (4 months after anthelmintic treatment). All these patients attended the clinic with diagnosed eosinophilia and or helminth infection and or LTBI. All of them had some symptoms related to helminth infection. The eosinophilia may also be secondary to a number of non-tropical diagnosis like polyarteritis, inflammatory bowel disease, Chrug-Strauss disease, etc, although studies have demonstrated a helminthic aetiology for eosinophilia in 50% patients with travel history (404)(504). The eosinophil count normalised from the previously sustained eosinophilia ($<0.4x$ 10^9/ml) after treatment with anthelmintic drugs. All the helminths were diagnosed with microscopy and serology. Serology positive patients had no other symptoms of filarial or any other helminth infections. The Log CFU in the MGIA was also observed at these three time points. There was no difference in MGIA response between visit 1 and the extra visit one month after visit 1. The CD4+IFNγ response at their pre-treatment visit and one month after did not show any change either. In helminth infected and LTBI-helminth coinfected patients, the CD4+IFN-γ response increased after helminth treatment and also the mycobacterial growth was reduced- suggesting the beneficial impact of anthelmintic treatment.

The effect of mycobacterial and helminth antigen stimulation was explored in controls, helminth, LTBI, and LTBI-helminth coinfected patients using cryopreserved PBMC. When the cells were stimulated with PPD, ESAT-6/CFP-10, SEA of *S. mansoni* and AWA antigen of *S.mansoni*, there was a significant increase in mycobacterial growth in response to SEA and AWA antigens in comparison to PPD, or ESAT-6/CFP10. In response to PPD and ESAT-6/CFP10 the growth control was less in LTBI and LTBI-helminth coinfected patients. The ESAT-6/CFP10 response was observed to be higher in LTBI and LTBI-helminth coinfected patients. Helminth antigens were initially aliquoted in PBS that was not endotoxin free. LPS can stimulate the release of IL-8, CXCL8, CXC ligand and other inflammatory cytokines in various cell types, leading to an acute inflammatory response towards pathogens. These responses are initiated by the activation of the TLR signalling through adaptor proteins, and include induction of gene expression via the activation of the NF-κB and AP-1 signal transduction pathways (505).

Robinson *et al.,* have shown that helminth derived molecule of *F.hepatica* binds directly to LPS preventing its interaction with the TLR4/CD14 complex on the macrophage

surface and prevents the release of inflammatory mediator TNFα and IL-1ß from macrophages (506). The experiment was repeated and cell viability was observed every day while the cells and antigens were in the rotator for 96 hours. There were no changes in viability after 96 hours. The MGIA assay was repeated using the antigens prepared from the stock and diluted in endotoxin free PBS. PPD responses were higher in controls and helminth infected patients in comparison to LTBI infected patients where ESAT-6/CFP10 responses were higher. Strong PPD responders without evidence of LTBI might have distinctly different mycobacterial inhibition activity from LTBI subjects (ESAT-6/CFP-10 responders), with similar PPD reactivity. This is because PPD reactivity may reflect protective immunity in the former group but not the latter. Strong PPD reactivity may paradoxically be associated with poor mycobactericidal activity (457).

For all the optimization steps of MGIT assay and for most of the tests using the clinical samples, BCG was used. *Mtb* Erdman and *Mtb* H37Rv were then compared, to explore how the growth of virulent *Mtb* is modulated in helminth coinfection and the effect of anthelmintic treatment on their growth. The association of growth inhibition of BCG and *Mtb* was also explored. Experiments were run using PBMC from LTBI infected patients and healthy controls from LSHTM , comparing the assay read-out for BCG Aeras, *Mtb* H37Rv and *Mtb* Erdman. Other studies have used BCG as a surrogate marker for *Mtb* in the MGIT assay (387)(375). In LTBI infected patients *Mtb* Erdman showed increased mycobacterial growth compared to that in healthy controls, as well as of BCG and *Mtb* H37Rv. The Erdman strain was selected for the next experiment to see the effect of anthelmintic treatment and to compare the pattern of growth inhibition of the BCG and *Mtb*.The impact of anthelmintic treatment was also observed in helminth infected patients and LTBI-helminth coinfected patients with BCG and *Mtb* Erdman. There was a significant increase in mycobacterial growth with *Mtb* Erdman compared to BCG, which reversed after anthelmintic treatment. *M bovisBCG* and *Mtb* share the same structure and metabolism, and these genomes have >99.5% identity at the nucleotide level (507). Manabe *et al.,* showed in a series of infections in rabbits with three different strains of *Mtb* Erdman, *Mtb* H37Rv and CDC1551, that the *Mtb* Erdman strain appears to be the most virulent for rabbits, requiring the lowest number of inhaled bacilli to make one tubercle. This strain also has the greatest spectrum of disease at 16 to 22 weeks after initial aerosol infection (508). There was a weak trend of positive association in growth inhibition for BCG and *Mtb* Erdman in LTBI, helminth and LTBI-helminth coinfected

patients. A significant correlation was observed between the murine BMMø assay read out using BCG and *Mtb*(509). BCG can therefore be used as potential surrogate for *Mtb* in the MGIA–limiting the use of more virulent strains and Category 3 facilities.

The treatment impact on helminth infected and LTBI-helminth coinfected patients was explored. There was significant reductions in mycobacterial growth after treatment, Evidence suggested that a skewed Th2 response caused due to *S.mansoni* infection protection conferred by *M.bovis* BCG vaccinations in humans and in mice infected with *Mtb* (498). Interestingly, this coinfection increases the mycobacterial burden in the lungs and this increase in burden is often transient and moderate suggesting that the effect of helminth coinfections on *Mtb* control is not significant enough to fully explain the increased severity of TB in helminth coinfected individuals and that *Mtb* control mechanisms are not completely suppressed in coinfected hosts (323). Other immune parameters like systemic cytokine levels, antibodies or the extent of gene expression may help dissecting the immune responses in these coinfected patients. MGIA along with other immune parameters might provide an immune signature for these patients.

The two helminth species –Strongyloides and Schistosoma spp, were compared to see how they might impact the mycobacterial growth inhibition. MGIA was performed to observe if the two helminth- Strongyloides and Schistosoma spp, show any difference in MGIA growth inhibition pattern- but they did not show any significant difference in the pattern of MGIA. The discrepency in the results in terms of reactivity to TB antigens, may be due to type of helminth parasites studied, the methods used for diagnosing helminth infection or the cutoff used for skin reactivity and the differences in geographical regions (135). These two helminth species have different routes and modes of infection, disease pathogenesis and severity, and treatment options. They share some common immune features like skewing of Th2 immune responses, AAMS, similar cytokine profile,etc. In this experiment, PBMC from patients infected with Stongyloides spp showed slightly reduced mycobacterial growth in comparison to those infected with Schistosoma, although the difference was not significant. More exploratory research can be useful regarding these two species,and how they affect antimycobacterial responses.

Chapter - 8

8.1   Antibody and cytokine responses in LTBI, helminth infected and LTBI-helminth infected patients

Cell mediated immunity has been traditionally considered the sole immune mechanism against TB. There are several studies withevidence of multiple attempts to demonstrate the protective role of antibodies (510)(511). Increasing evidence suggests that antibodies could contribute to the defence against *M.tb* (32)(512). However, their role is insufficiently studied because of the general perception that *M.tb*, a largely intracellular pathogen, is outside the reach of extracellularly located Abs (513). Antibody responses to capsular AM(Arabinomannan) and the related cell wall glycolipid LAM (lipoarabinomannan) correlate strongly and significantly in patients with tuberculosis and helminth infections (514). However, a feature of the immune response to parasitic helminth infections, is the production of large amounts of specific and nonspecific IgE. The slow build-up of IgE to high levels and the early production of IgG4 antibodies, which may block IgE pathways were shown to be responsible for delaying the development of protective immunity to *S.haematobium* (515). To explore the quantity of total and parasite specific antibodies–IgE, IgG4 and IgA, plasma samples were used for ELISA. This chapter also describes analysis of the effect of anthelmintic treatment on these antibodies in helminth-infected and LTBI-helminth coinfected patients.

Cytokines and chemokines are also important regulatory factors for protective immunity against tuberculosis. To explore the cytokine production in these patients, 96 hours culture supernatants of the MGIAwas used. The concentrations of the cytokines and the correlation with mycobacterial growth was explored.

Aims:

- To measure the quantity of total and parasite specific IgE, IgG4, ESAT-6/CFP-10 specific IgA in plasma of controls, helminth infected, LTBI-helminth co-infected patients and LTBI patients.
- To explore the association of antibodies with mycobacterial growth inhibition in the MGIT assay
- To measure the cytokines in 96-hour culture supernatants from MGIA cultures

- To explore if there is any correlation between mycobacterial growth inhibition and cytokine concentrations in LTBI, helminth infected and LTBI-helminth coinfected patients.

8.1.1 Methods

At visit 1 (before treatment) and visit 2 (after anthelmintic treatment) 20 ml blood was collected from each patient and from that 1 ml of whole blood was placed in a 2 ml Eppendorf tube and 500µl of plasma collected after centrifuging the whole blood. These plasma samples were used to measure the quantity of total IgE, IgG and IgA.

8.1.1.1 Measuring IgE concentrations in plasma from controls, LTBI, helminth-infected and LTBI-helminth infected patients

A sandwich ELISA was used for detection and quantification of the antibodies in the plasma obtained from the whole blood collected from the clinical samples. To measure the total and antigen specific IgE antibody, plasma from 11 patients from each group, both before and after treatment was used to perform the ELISA. *S.mansoni* SEA antigen and AWA antigen were used as the helminth antigens. There were differences in optical density (OD) between pre and post treatment IgE in helminth infected and LTBI-helminth coinfected patients (Fig. 8.1). Total IgE concentrations were not significantly changed in LTBI infected patients or in controls. But the total IgE was significantly different in helminth infected and LTBI-helminth infected patients both before and after treatment (p= 0.015 and p=0.005 respectively).
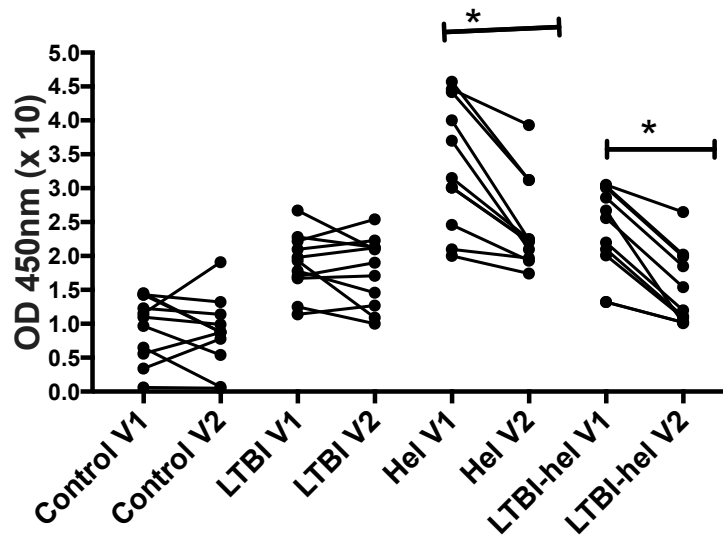
Figure 8.1: Total IgE detected by ELISA in plasma from controls, LTBI, helminth infected patients and LTBI-helminth coinfected patients. Plasma diluted 1 in 10 from eleven patients of each group were used in the ELISA.The OD value at 450nm was measured using an ELISA plate reader. OD values have had the background OD values subtracted. A repeated measures ANOVA was performed followed by a Bonferroni's multiple comparison test, where * represents a p value of <0.05.

To determine whether concentrations of parasite antigen specific IgE antibodies change with anthelmintic treatment, plasma samples were used for the ELISA. *S.mansoni* SEA and AWA antigens were used as antigen (at 10μg/ml) Antigen specific IgE responses were similar like those of total IgE in helminth and LTBI-helminth coinfected patients, although there was an increase in the IgE concentrations in the helminth infected and coinfected patients (Fig. 8.2). There were no significant differences in IgE antibodies to the two antigens in IgE responses.
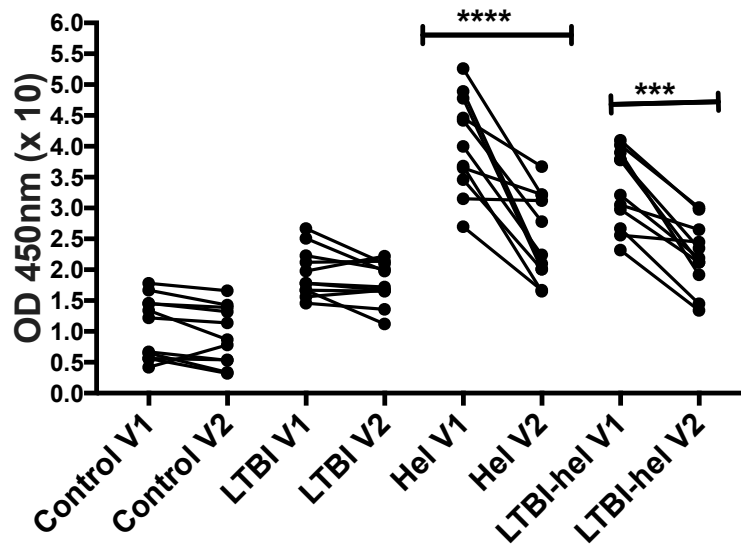
Figure 8.2: Antigen specific IgE responses by ELISA in controls, LTBI, helminth infected patients and LTBI-helminth coinfected patients. Plasma diluted 1 in 10 from eleven patients of each group were used in the ELISA. *S.mansoni* SEA antigen was used as antigen. OD values at 450nm were measured using an ELISA plate reader. A repeated measures ANOVA was performed, where *** represents a p value of <0.005 and **** represents a p value of <0.0001.

To determine whether levels of total and parasite antigen specific IgE antibodies have a significant effect on mycobacterial growth, the relationship between log CFU and ELISA OD were investigated. No significant correlation was observed in assays using PBMC from LTBI and controls. There was a weak trend observed in helminth infected patients in their V1 (before treatment) and similar trend was observed in LTBI-helminth coinfected patients both before and after treatment (Fig. 8.3).

(A) Helminth infected (V1)   (B) Helminth infected (V2)

(C) LTBI-helminth coinfected (V1)   (D) LTBI-helminth coinfected (V2)
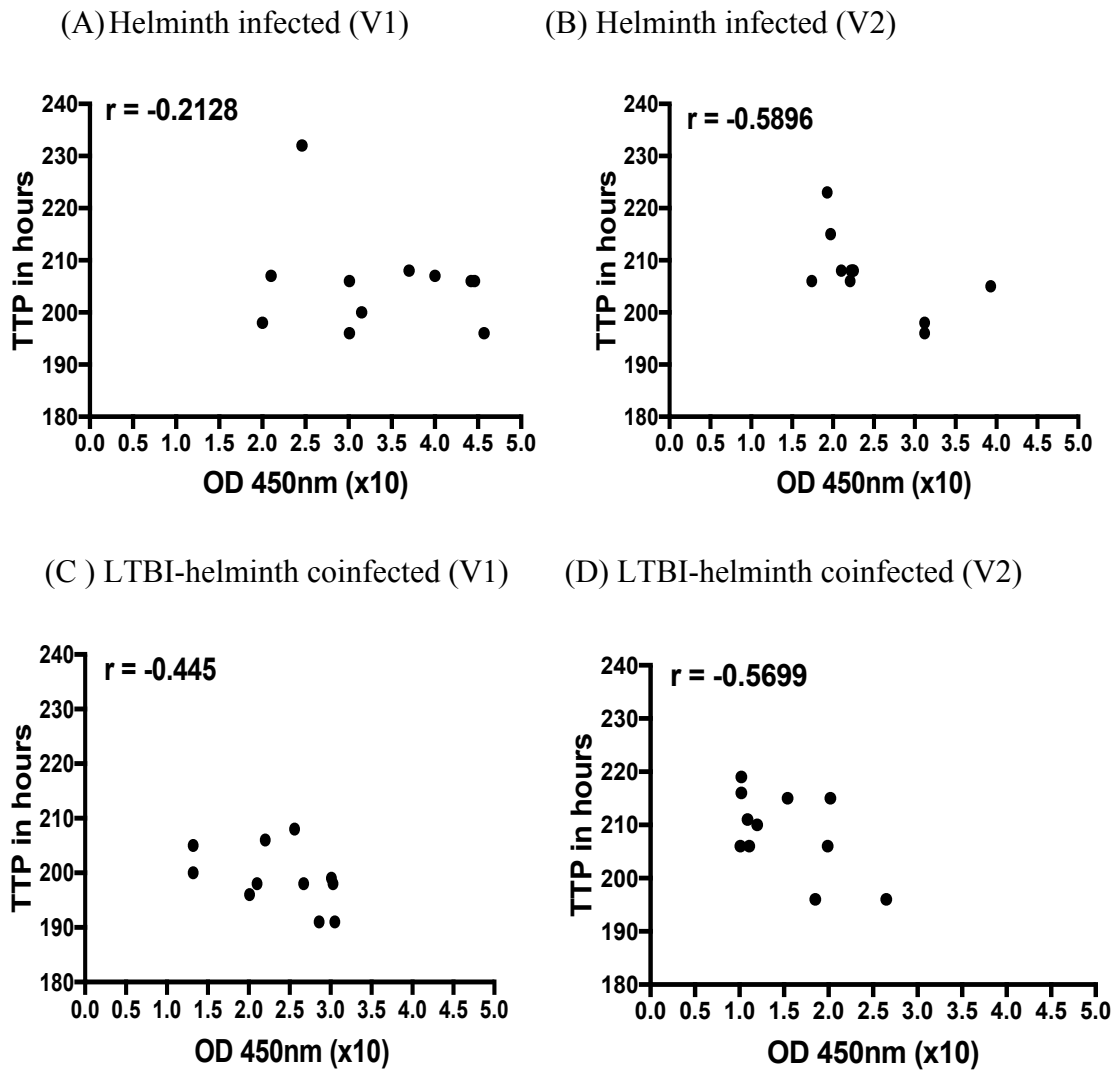
Figure 8.3: Total IgE antibody concentrations and correlation with mycobacterial growth inhibition. PBMC from 11 helminth infected patients both pre and post treatment (A,B), and LTBI-helminth coinfected patients pre and post-treatment (C,D), were used for MGIT. Spearman's correlation between total IgE antibody OD by ELISA and log CFU in the PBMC MGIT assay was performed in the groups. OD value at 450nm was measured using an ELISA plate reader.

Next, whether parasite antigen-specific IgE antibodies have a significant effect on mycobacterial growth was explored, in terms of the relationship between log CFU and ELISA OD. No significant correlation was observed in LTBI and controls, in helminth infected patientsor in LTBI-helminth coinfected patients at their first or second visits (V1 and V2, before and after treatment).

8.1.1.2 IgG4 concentrations in controls, LTBI, helminth infected and LTBI-helminth infected patients

To measure the total and antigen specific IgG4 antibody, plasma from 11 donors from each group, both before and after treatment if appropriate was used to perform the ELISA. *S.mansoni* SEA antigen was used as the helminth antigen.The total IgG4 plasma concentration was not significantly different in LTBI infected patients or in controls (Fig. 8.4). However, the total IgG4 concentrations were significantly higher in helminth infected and LTBI-helminth infected patients both before treatment and the changes reversed after treatment (p= 0.015 and p=0.005 respectively).
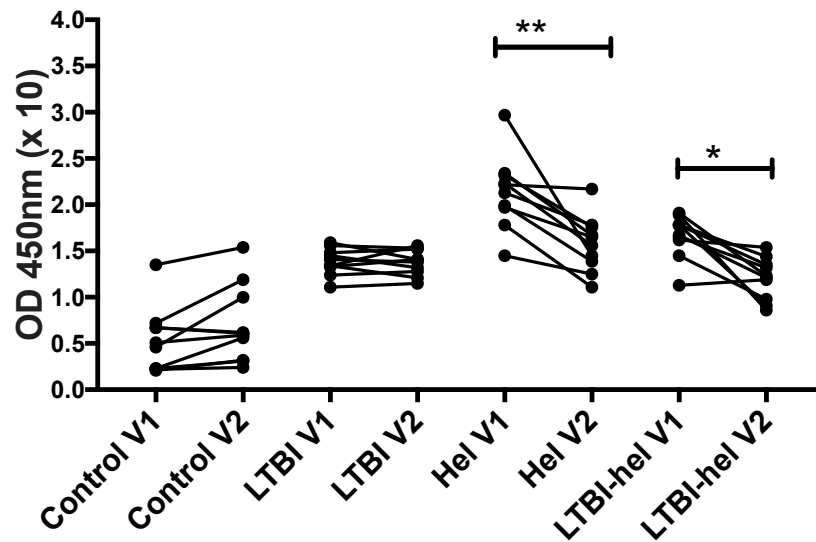
Figure 8.4: Total IgG4 concentrations measured by ELISA in controls, LTBI, helminth infected patients and LTBI-helminth coinfected patients. Plasma diluted 1 in 10 from ten patients of each group were used in the ELISA. OD value at 450nm was measured using an ELISA plate reader. A repeated measures ANOVA was performed, where ** represents a p value of <0.005 and * represents a p value of <0.05.

Next the parasite antigen specific IgG4 responses were measured in all four clinical groups. The antigen specific IgG4 response was mostly similar to that of total IgG4. The antibody response was significantly reduced after anthelmintic treatment in helminth infected and LTBI-helminth infected patients (Fig. 8.5). The level of IgG4 was significantly lower in controls than the helminth infected patients and LTBI-helminth coinfected patients (p<0.0001).
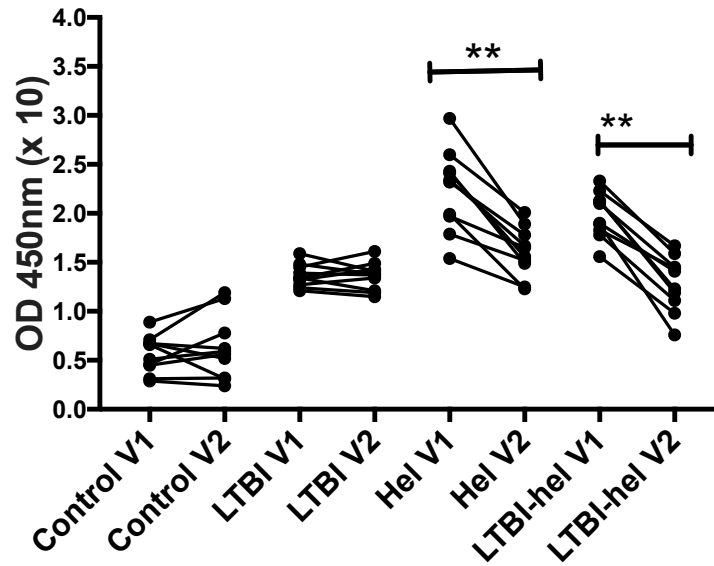
Figure 8.5: Antigen specific IgG4 concentrations measured by ELISA in controls, LTBI, helminth infected patients and LTBI-helminth coinfected patients. Plasma diluted 1 in 10 from eleven patients of each group were used in the ELISA. *S.mansoni* SEA antigen was used as antigen. OD values at 450nm were measured using an ELISA plate reader A repeated measures ANOVA was performed, where ** represents a p value of <0.005.

We explored whether there was any correlation between total IgG4 and antigen specific IgG4 level measured by ELISA and log CFU detected by MGIT assay. There was no significant correlation in any of the groups with either total IgG4 and parasite antigen specific IgG4 and mycobacterial growth.

8.1.1.3 Measuring total IgA and antigen specific IgA in LTBI infected patients

To measure the total and antigen specific IgA antibody, plasma from 10 LTBI infected patients were used to perform the ELISA. ESAT-6/CFP10 fusion protein was used as antigen. Plasma samples from 10 LTBI samples, 10 healthy controls (HC) at LSHTM and 10 controls from the HTD clinic were used in this experiment. There were no significant changes in total IgA between the three groups (Fig. 8.6). The level of antigen specific IgA in controls from the clinic was significantly different from that in healthy controls (HC) at LSHTM.
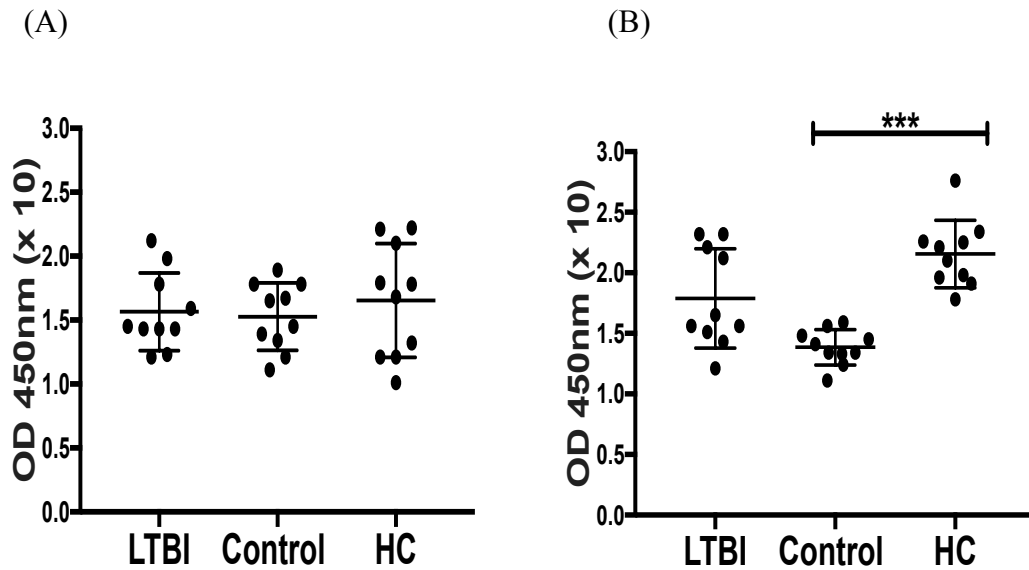
Figure 8.6: Total IgA and ESAT-6/CFP-10 specific IgA responses. Total IgA (A) and ESAT-6/CFP-10 specific IgA responses (B) were measured by ELISA in controls from the clinic, LTBI patients and healthy controls (HC) at LSHTM. Plasma diluted 1 in 10 from 10 donors in each group were used in the ELISA. OD value at 450nm was measured using an ELISA plate reader. Bars represents means with SD. A repeated measures ANOVA was performed followed by a Bonferroni's multiple comparison test, where *** represents a p value of <0.005.

The correlation between LTBI infected patient's IgA level by ELISA and log CFU by MGIT assay was explored and there was no significant correlation between them for both total IgA and antigen specific IgA.

8.2 Cytokine responses

To explore if there was any association between cytokine concentrations and mycobacterial growth and if there is a change in the expression of these cytokines after anthelmintic treatment, cytokines were measured in 96-hour culture supernatants from MGIT cultures. Culture supernatants from eight LTBI-helminth co-infected patients both pre and post treatment were used for this experiment. The concentration of 41 analytes in the culture supernatants included cytokines, chemokines and growth factors measured using the Multiplex MAP human cytokine/chemokine Panel-Premixed 41 Plex-Immunology Multiplex assay. Anthelmintic treatment in LTBI-helminth coinfected patients was associated with increased levels of pro-inflammatory cytokines and decreased levels of anti-inflammatory cytokines (Fig. 8.7). Some cytokines like IL-4, IL-8, IL-5, IL-6, IL-9, chemokines like PDGF-AA, PDGF-BB, RANTES, VEGF etc. were

not detected in these samples. However, when the association between cytokine concentration and mycobacterial growth was assessed, there were no significant associations.

(A)

(B)
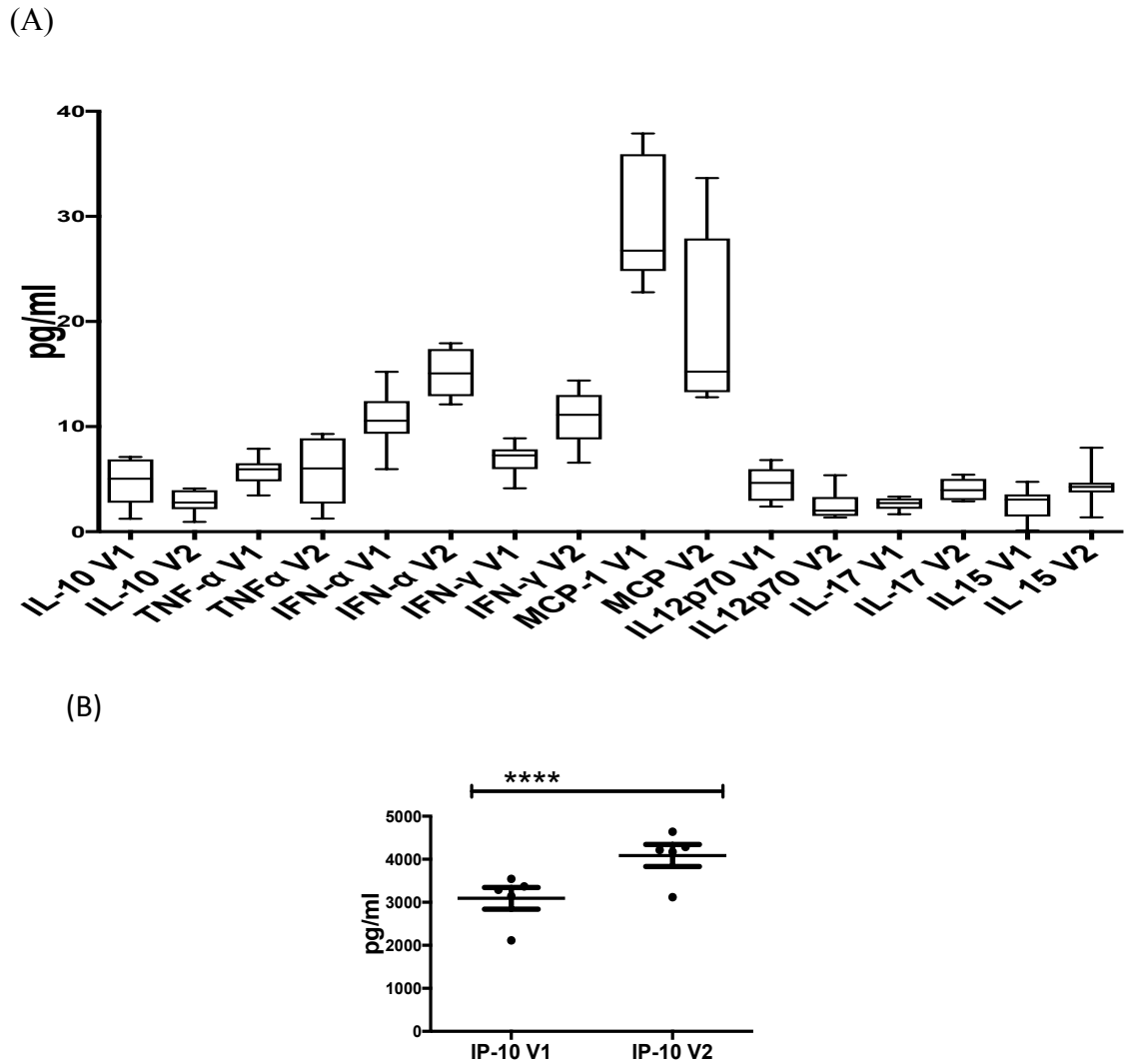
Figure 8.7: Cytokine concentrations in LTBI-helminth coinfected patients (n=8) detected by multiplex assay (A). IP-10 concentration in LTBI-helminth coinfected patients (B) Cytokine concentrations were measured in the supernatants taken from MGIT cultures after 96 hours of culture. A repeated measures ANOVA was performed followed by a Bonferroni's multiple comparison test.

Cytokine production was observed in 8 LTBI-helminth coinfected patients. MGIT culture supernatant from 96 hours was used for the experiment. There were some cytokines and chemokines that showed decreased concentrations after anthelmintic treatment for example, TGFα, IL-10, MCP-3, IL-12p70, PDGF-AA, G-CSF, Flt 3L, Fractalkine, FGF-2, IL-13, IL-1Ra. In contrast concentrations of IFNγ, IFNα, and FGF-2

were increased after treatment. IL-1b, IL-2, IL-33, IL-6, eotaxin, and sCD40L was not detected in the culture supernatants of helminth infected patients. None of the cytokines showed any significant association with mycobacterial growth. IP10 concentration was significantly raised after anthelmintic treatment ($p<0.0001$).

8.3 Discussion

In man, IgE is the least abundant immunoglobulin isotype and provides protection against helminths but also triggers harmful allergic reactions to allergens. IgE recognizes exogenous antigen and triggers an immunological response that is associated with degranulation of mast cells and subsequent release of biogenic amines, lipid mediators and the production of Th2 cytokines, such as IL-4, IL-5 and IL-13 and associated eosinophilia. Chronic immune activation associated with intestinal helminth infection results in an increased and predominantly IgE response. In TB patients specific IgE antibodies are IL-4 dependent. In this study, total and parasite antigen (SEA) specific IgE concentrations were significantly higher in helminth and LTBI-helminth coinfected patients and dropped after anthelmintic treatment ($p<0.05$). When 500 TB patient's sera were tested for IgE antibodies in response to PPD and H37Rv antigens by ELISA, these antigens were shown to be recognized by IgE antibodies. This PPD and H37Rv specific IgE is produced in Warao individuals by chronic exposure to TB, whereas high levels of total IgE may be the result of co-infection with helminth parasites (516). Chronic exposure to TB and coinfection with helminths may contribute to the poor outcome of infection by skewing the immune response to Th2 response and IgE responses, which is IL-4 dependent (517). In a South African community where TB and helminth infections were common, the adolescents exhibited high serum total IgE concentrations. Co-infection with helminths in TB stimulates the production of anti-mycobacterial or anti parasite IgE antibodies but can also non-specifically induce polyclonal IgE which could result in highly elevated levels of total IgE. Th1 cells when stimulated with intracellular bacteria like *Mtb*, secrete IFNγ which can inhibit IgE production by B cells (518). Adams *et al.,* in their studies showed that serum total IgE concentrations decreased after successful treatment for TB in adolescent patients in South Africa. Serum IgE level can also be influenced by parasite burden in the patient (516). In the patients studied here, there was a trend for higher total IgE concentrations in the plasma associated with higher with CFU in helminth infected patients and in LTBI helminth infected patients there was

a weak trend for a positive association between total IgE and mycobacterial growth. Depletion of the Th2 cytokine IL-4 in mice by gene knockout or by neutralizing antibody was shown to reduce the degree of *Mtb* infection as manifested by organ CFU reduction (519).

Total and antigen specific IgG4 was also higher in helminth and LTBI-helminth coinfected patients in this study, although there was no significant correlation with mycobacterial growth. Adjobimey *et al.,* reviewed induction of IgG4 in humans and immunomodulation of the human responses to filarial parasites, and showed that high plasma concentrations of IgG4 in these patients were associated with hyporesponsiveness, high parasite load and high plasma levels of IL-10 and TGFβ (520). Microfilaria induce IL-10 and TGFβ, producing and CD4+CD25+FOXP3+regulatory T cells that might well be responsible for IgG4 production promoting parasite persistence (521). Schistosoma infected Egyptian patients, had elevated levels of IgG4 directed mainly against SEA antigen, suggesting that the IgG4 antibodies interfere with the effector activities of IgE anti-schistosome antibodies and thus inhibit complement activation and mast cell triggering (522)(523).

Total plasma IgA concentrations in healthy controls of LSHTM controls, controls from the clinic and LTBI infected patients did not differ. However, ESAT-6/CFP10 specific IgA was observed to be significantly higher in healthy LSHTM controls than the controls in the clinic. The antigen specific IgA was higher in LTBI patients than the clinic controls. During latent infection, a small number of tubercle bacilli presumably persist inside granulomas, where bacillary multiplication is restricted by the host immune response although some bacterial proteins may induce measurable levels of antibody including to secreted virulence factors such as ESAT-6/CFP10 or components of the bacterial stress response. A higher proportion of individuals with anti-mycobacterial antibodies has been seen in healthy, latently infected patients from TB endemic countries as compared to those with LTBI who live in non-endemic countries (524)(525). Antibodies to ESAT-6/CFP10 have also been found in individuals at risk of reactivation disease due to past TB, history of close contact with infectious cases and migration from TB endemic countries(526). A recent study examined the role of secretory IgA in host defence against mycobacterial infection, in the mouse model. The antibody response induced after intra nasal administration to *Mtb* antigen demonstrated loss of antigen specific IgA responses after immunization with mycobacterial antigen PstS-1 and made

the mice more susceptible to BCG and *Mtb* infection than immunized control wild-type mice, manifested by higher lung CFUs, and reduced production of proinflammatory cytokine responses like IFNγ and TNFα in the lungs (527). However, experiments suggested that IgA could provide a protective role in *Mtb* diseases particularly in the early stages of infection (528). Most of the LTBI patients in this experiment were health care workers (HCWs) who had higher levels of IgA in response to ESAT-6/CFP10 suggesting elevated IgA might have some protective role in IFN-γ positive immunocompetent LTBI individuals. Frequent exposure to tubercle bacilli can possibly stimulate the mucosal immune system. It is also known that commensal bacteria on the mucosal surface induce IgA in an NO dependent manner(529), although it is still unknown if *Mtb* in TB and LTBI has a similar effect in lung mucosa. IgA antibody and IFNγ induce TNFα and NO production inhibit *Mtb* growth in mouse models (530). The association between anti IgA and IFNγ may indicate protective, mucosal immune activities in LTBI in HCWs (531).

There was a lack of significant correlations between the concentrations of antibodies measured and mycobacterial growth.The role of antibodies in TB are still debated. Although there were changes in the antibody concentrations after treatment, this might be due to systemic effects or to other components of the immune system in the population studied or todifferences in host response. Although there is some evidence for a role of antibodies in TB, probably the role of T cells and cellular immunity are more important for host resistance (532).

To explore if the production of cytokines was associated with mycobacterial growth and if there is a change in cytokine secretion after anthelmintic treatment, cytokines were measured in 96-hour culture supernatants from MGIT cultures in LTBI-helminth coinfected patients (both pre and post treatment). Helminth infections have been shown here to modulate the production of some cytokines like IFNγ ,TNFα, IFNα, MCP 1, IL12p40, IL-17, IL-15 etc. and some of them were altered following anthelmintic treatment. Concurrent filarial infection has shown to inhibit the production of potentially protective Th1 immune responses in LTBI-helminth coinfected patients (212). Significant reductions of proinflammatory cytokines were also observed in previous studies in LTBI-helminth coinfected patients(426).Helminth infections establish persistent infection, by mechanisms that dampen the immune responses by Tregs and CD4+T cells expressing

IL-10.In this study, helminth coinfection was associated with a significantly enhanced frequency of IL-10 secretion that reduced after treatment suggesting the immunoregulatory role of IL-10.The concentrations of Th1 (IFNγ,TNFα) and Th17 cytokines were downregulated in coinfected patients before treatment. TNFα and IL-17 have been reported to exhibit antimycobacterial responses either in primary or memory responses to infection. In patients with TB disease, peripheral blood frequencies of these cells are reduced, which may be due to suppression of IFNγ (533). TNFα plays a central part in the host response against tuberculosis, including granuloma formation and containment of disease. Antibodies against TNFα cause a reactivation of tuberculosis in a mouse model of latent infection (534).Both cytokines are important in the induction and maintenance of protective immune responses in TB infection. Downregulation of these cytokines may suggest the modulation of antimycobacterial immune responses in the presence of helminth infection. There was also a change in IP-10 after anthelmintic treatment. IFNγ-inducible protein 10 (IP-10/CXCL10) is a chemokine involved in delayed-type hypersensitivity and attraction of monocytes and activated T lymphocytes at inflammatory foci (535). MCP-1α was found to be significantly reduced after the treatment. This is an important cytokine that can stimulate Th2 polarisation and increased expression of this cytokine is associated with susceptibility to *Mtb* infection inman. Monocytes/macrophages are the major source of CCL2, which regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells (536). There is an alternative activation of macrophages and Th2 polarization in helminth infection, so it would be interesting to see if there is a skewing of immune responses in helminth coinfected patients and whether this alters after anthelmintic treatment. This was investigated in the next chapter.

Chapter - 9 qRT-PCR

9.1 qRT-PCR

As blood samples from the donors studied here had been banked for later RNA extraction, this provided an opportunity to assess the expression of genes associated with classically activated or alternatively activated macrophages and whether there is a potential skewing from M2 to M1 macrophages. Eight genes were selected for analysis using a qRT-PCR assay, that are expressed by M1 (IDO-1, CXCL10, CD86) and M2 macrophages (Arginase 1, CD163) and some other genes identified to be of interest, for example-HIF1α, HIF2α, IL-10 with two reference genes (CD14 and HPRT).This experiment was designed to see the differential expression of these genes in LTBI, helminth and LTBI-helminth coinfected patients, and also to observe if the expression of these genes changes after anthelmintic treatment which might indicate a skewing of immune responses from helminth induced Th2 response to Th1 type and better immune responses for *Mtb*.

9.1.1 Results

In the real time qRT-PCR assay, mRNA expression of the genes- IDO-1, Arginase 1, HIF1α, HIF2α, IL-10, CXCL10, CD163, CD86 was determined in blood samples from controls, helminth infected, LTBI and LTBI-helminth coinfected patients. Fifteen patients from each group were tested. Both the pre and post treatment samples were tested. The expression of these genes were normalized to the CD14 and HPRT genes.

IDO-1 was significantly upregulated in helminth and LTBI–helminth co-infected patients (p=<0.0001), when the genes were normalized to CD14 (Fig. 9.1). This increase of gene expression was observed both before and after anthelmintic treatment. IDO-1 was also upregulated in LTBI patients when compared to controls. The expression pattern was more variable, when the genes were normalized to HPRT. IDO-1 was upregulated in LTBI-helminth coinfected patients and also in helminth infected patients after treatment but not in the pre-treatment phase or in LTBI, relative to HPRT.

(A)
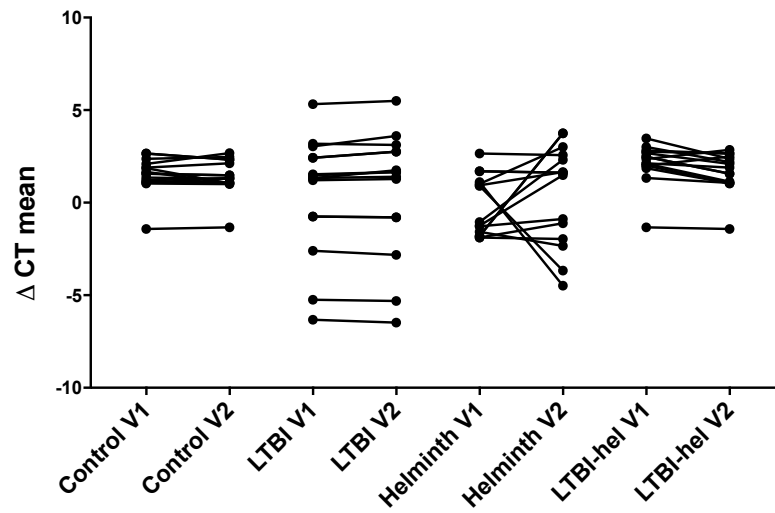
(B)
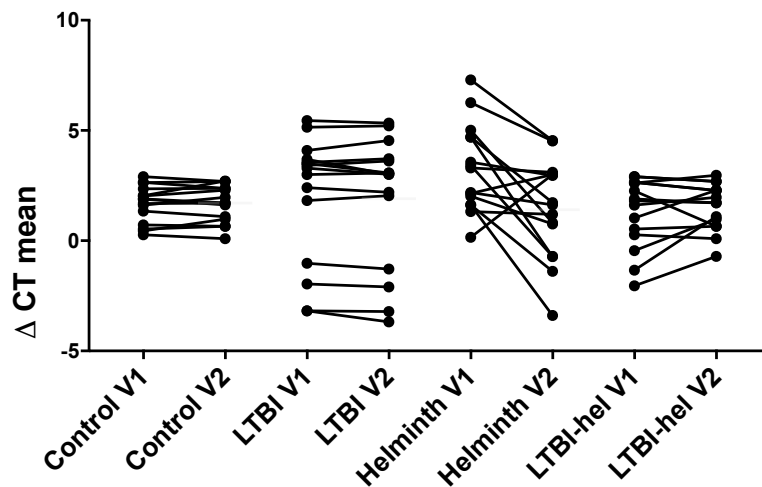
Figure 9.1: Relative IDO-1 expression in controls, helminth, LTBI and LTBI-helminth coinfected patients when normalized to CD14 (A) and HPRT (B). Fifteen patients were tested per group. One way ANOVA test with a Tukey's post-test was performed where * represents a p value of <0.05.

Arginase 1 was observed to be highly significantly upregulated in helminth infected (before treatment p=<0.0001, after treatment p=0.0008) and LTBI-helminth coinfected patients (before treatment p=, <0.0001, after treatment p=0.0068), when the gene expression was normalized against HPRT (Fig. 9.2). A similar finding was also observed when gene expression was normalized to CD14, though the differences between the

groups were not statistically significant. Arginase 1 was downregulated in LTBI patients (p=0.0045) in comparison to controls.

(A)
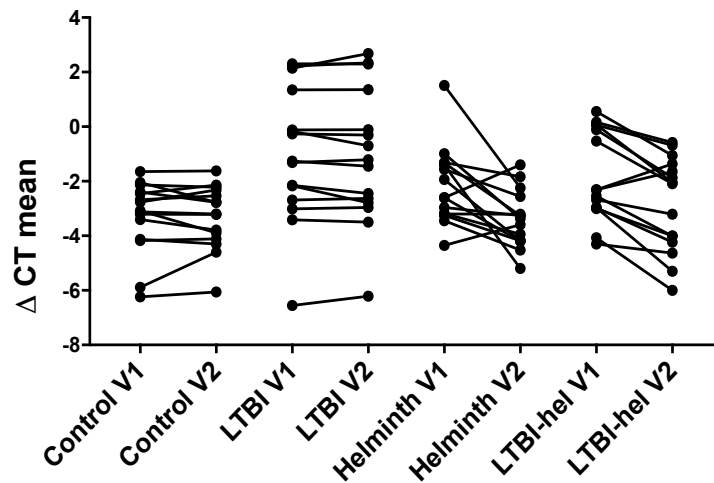
(B)
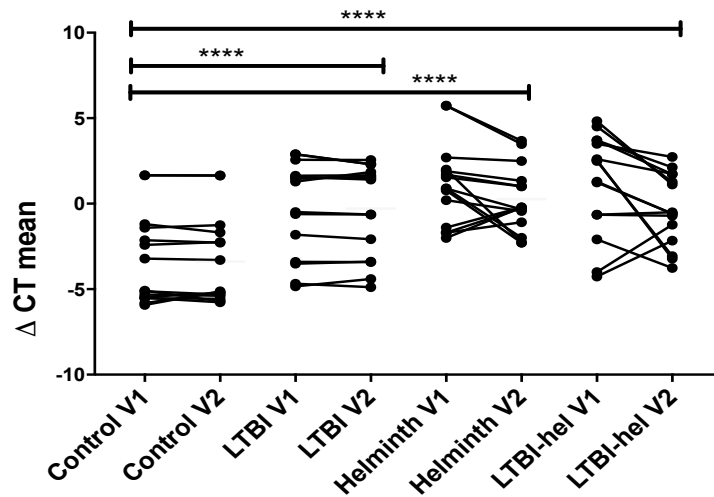
Figure 9.2: Relative arginase-1 expression in controls, helminth, LTBI and LTBI-helminth coinfected patients when normalized to CD14 (A) and HPRT (B). Fifteen donors were tested per group. One-way ANOVA test with a Tukey's post-test was performed where **** represents a p value of <0.0001.

Gene expression in relative to HPRT

Gene expression in relative to CD14
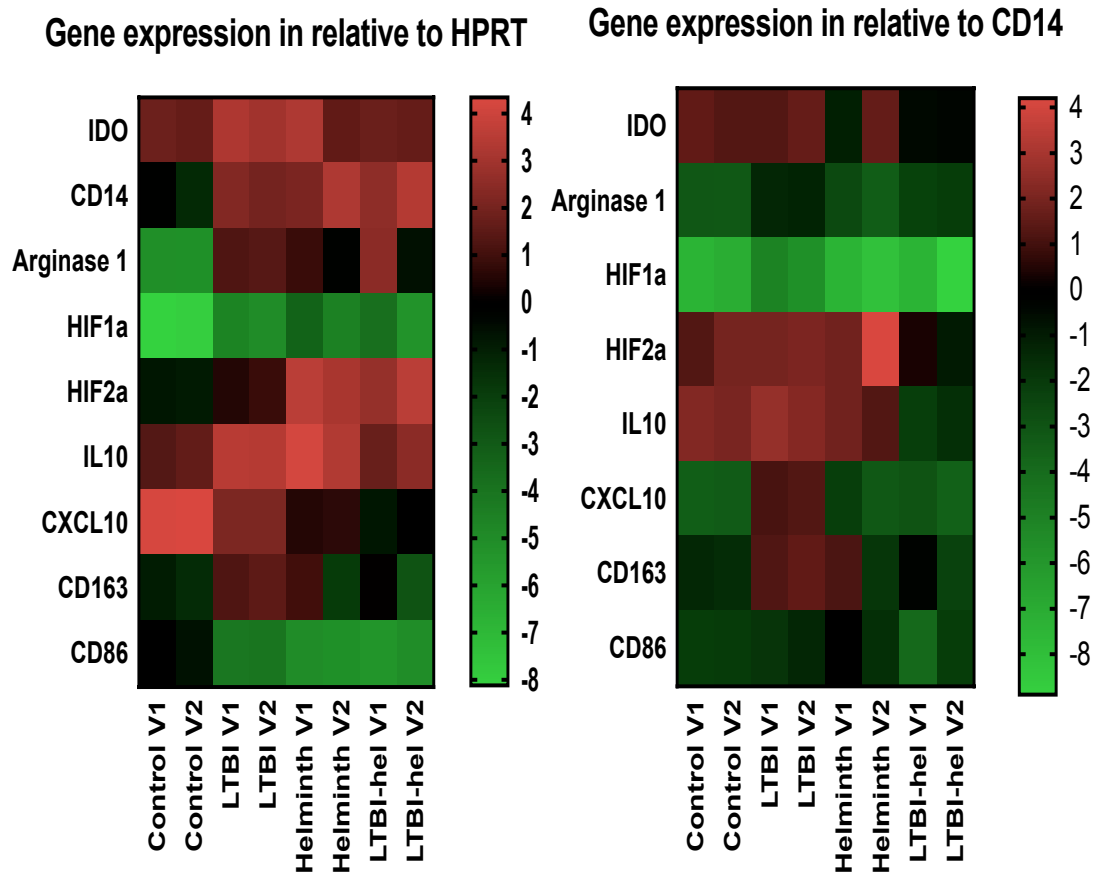
Figure 9.3: Heat map of average gene expression in the control, helminth, LTBI and LTBI-helminth coinfected groups relative to the reference gene HPRT and CD14 at visit 1 and visit 2.

HIF1α gene expression was highly diverse in the different clinical groups and also showed disparity when normalized to CD14 and HPRT (Figure-9.3). Relative to HPRT, HIF1α was significantly downregulated in LTBI-helminth coinfected patients both before and after treatment, in helminth patients before treatment but not after treatment and also in LTBI patients. In contrast, when the genes were normalized to CD14, all the groups showed increased gene expression, except for the LTBI-helminth coinfected pre-treatment group, though these increases in expression were not statistically significant (Table 1).

HIF2α was significantly upregulated in the LTBI-helminth coinfected group, when the genes were normalized against CD14 (before treatment p=<0.01, after treatment p=0.0028). This upregulation was also evident relative to HPRT, though this was not statistically significant. HIF2α was significantly upregulated in helminth infected patients

both before and after treatment relative to HPRT (before treatment p=<0.0068, after treatment p=0.0218).

IL-10 was highly significantly upregulated in LTBI-helminth coinfected patients (before treatment p= <0.0001, after treatment p=0.0001) compared to the controls relative to CD14. Expression of IL-10 gene was also upregulated in helminth infected patients and LTBI patients compared to the controls but this was not statistically significant.

Interestingly, CXCL10 was highly upregulated in LTBI patients when normalized to HPRT but showed a contrasting result relative to CD14 and both the expressions were statistically significant.
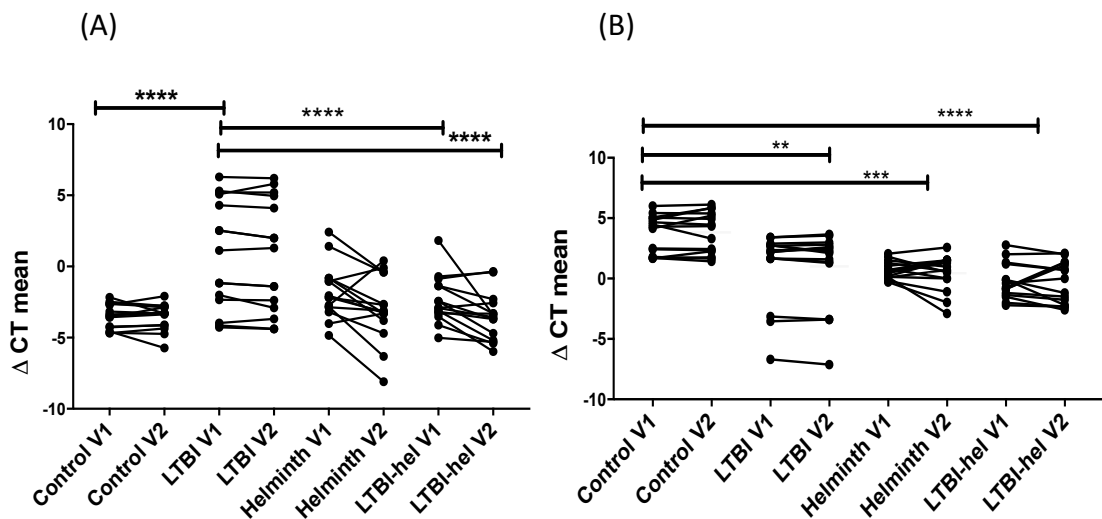
Figure 9.4: Relative expression of CXCL10 in control, LTBI, helminth infected and LTBI-helminth coinfected patients when normalized to CD14 (A) and HPRT (B) (15 patients from each group). One way ANOVA test with a Tukey's post-test was performed where * represents a p value of <0.05.

CD163 did not show any substantial changes in any of the clinical groups in relation to either CD14 or HPRT (Table 1,2). CD86 showed significant upregulation in expression in LTBI-helminth coinfected patients and also in helminth infected patients after their treatment. Unlike CD163, it also did not show any marked changes in LTBI patients in relation to CD14 and HPRT (Table 2, 3).
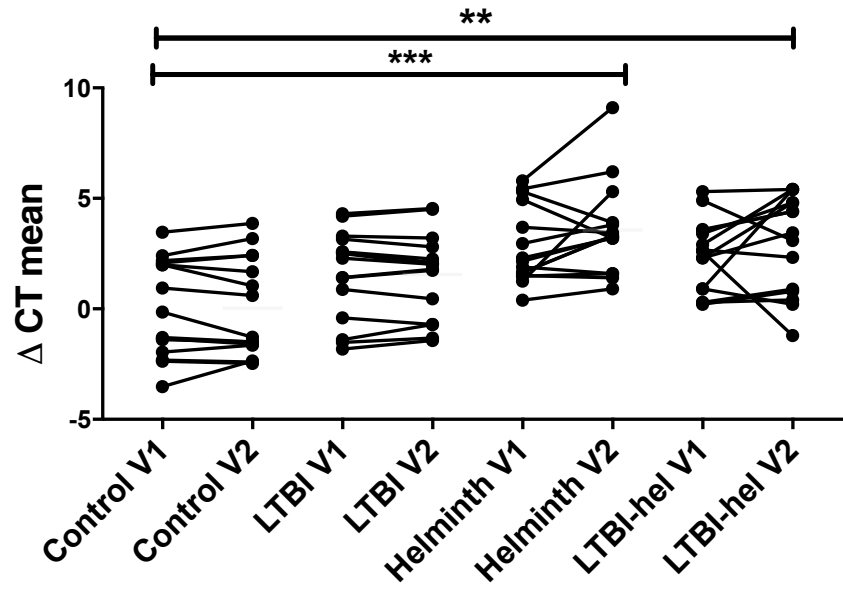
Figure 9.5: Relative CD14 gene expression in controls. LTBI, helminth and LTBI-helminth coinfected patients when normalized to HPRT (Fifteen patients from each group). One way ANOVA test with a Tukey's post-test was performed where * represents a p value of <0.05.

Gene of interest	LTBI			Helminth Visit 1			Helminth Visit 2			LTBI-helminth Visit 1			LTBI-helminth Visit 2		
	ΔΔCT	FC	P value	ΔΔCT	FC	P value	ΔΔCT	FC	P value	ΔΔCT	FC	P value	ΔΔCT	FC	P value
IDO-1	-1.0	2.0	0.75	-1.6	3.0	0.0001****	-0.9	1.9	0.0001****	-1.7	3.2	0.0001****	-1.1	2.1	0.0001****
Arginase 1	2.3	0.2	0.0045**	-1.1	2.1	0.3522	-0.09	1.1	0.9998	-1.5	2.8	0.1158	-0.45	1.3	0.9650
HIF1α	-1.6	3.0	0.3926	-0.3	1.3	0.9999	-1.0	2.0	0.9579	0.6	0.7	0.8734	-2.3	5.2	0.1740
HIF2α	-0.9	1.9	0.7194	-0.5	1.4	0.9943	2.2	0.2	0.1464	-3.2	9.1	0.0135*	-3.7	13.2	0.0028**
IL-10	-0.3	1.2	0.9974	-0.5	1.4	0.9835	-1.0	2.0	0.6684	-3.7	11.3	0.0001***	-3.5	12.0	0.0002***
CXCL10	4.4	0.1	0.0001****	1.7	0.3	0.2505	0.5	0.7	0.9827	1.2	0.4	0.5418	-0.01	1.0	0.9999
CD163	0.3	0.8	0.9957	-2.7	0.2	0.0915	-0.7	1.7	0.9936	-0.5	1.4	0.9935	1.6	0.3	0.5222
CD86	0.4	0.7	0.9796	1.5	0.4	0.4802	1.1	0.5	0.7701	-1.4	2.7	0.4915	-1.01	2.0	0.7966

Table 9.1: Gene expression in LTBI, helminth and LTBI- helminth coinfected patients compared to controls, using CD14 as the endogenous control. The ΔΔCT value was determined from the subtracting the ΔCT value of control samples from ΔCT values of LTBI/ helminth/ LTBI-helminth samples. To compare the fold change of each of the target gene in LTBI, helminth and co-infected group compared to control samples a two-way ANOVA was used followed by Dunnett's post-test.

Gene of interest	LTBI			Helminth V1			Helminth V2			LTBI-hel V1			LTBI-hel V2		
	ΔΔCT	FC	P value	ΔΔCT	FC	P value	ΔΔCT	FC	P value	ΔΔCT	FC	P value	ΔΔCT	FC	P value
IDO-1	0.3	0.8	0.9952	1.6	0.3	0.1557	-0.3	1.2	0.9881	-0.42	1.3	0.9921	-0.10	1.1	0.9998
Arginase 1	3.1	0.1	0.0046	-4.53	0.04	0.0001**	-3.64	0.1	0.0008***	-4.63	0.04	0.0001****	-3.03	0.1	0.0068**
CD14	1.5	0.4	0.1680	2.7	0.1	0.0013**	3.5	0.1	0.0001****	2.4	0.2	0.0086**	2.9	0.1	0.0007***
HIF1α	3.69	0.1	0.0415*	3.87	0.1	0.0262*	2.3	0.2	0.3411	4.63	0.04	0.0049**	4.10	0.05	0.0161*
HIF2α	1.5	0.4	0.9389	-4.68	0.03	0.0068**	-4.08	0.06	0.0218*	-1.41	0.37	0.9857	-1.08	0.47	0.9996
IL-10	0.9	0.5	0.8301	2.64	0.2	0.0410*	3.0	0.12	0.0146*	-0.71	1.64	0.8972	0.45	0.72	0.9977
CXCL10	-2.7	6.2	0.0001****	-3.02	8.1	0.2505	-3.26	9.62	0.9827	-3.98	11.0	0.6418	-3.02	8.1	0.9999
CD163	0.56	0.7	0.9710	1.02	0.5	0.6349	4.26	0.05	0.6465	-0.38	1.3	0.9996	5.19	-1.9	0.1965
CD86	-2.5	5.8	0.1234	-2.4	5.6	0.1654	-3.6	9.64	0.0072**	-3.26	9.6	0.0009***	-2.9	7.5	0.0031**

Table 9.2: Gene expression in LTBI, helminth and LTBI- helminth coinfected patients compared to controls when normalized to HPRT. The ΔΔCT value was determined from subtracting the ΔCT value of control samples from ΔCT values of LTBI/ helminth/ LTBI-helminth samples. To compare the fold change of each of the target gene in LTBI, helminth and co-infected group compared to control samples a two-way ANOVA was used followed by Dunnett's post-test.

9.1.2 Modulation of gene expression after anthelmintic treatment

The fold changes after anthelmintic treatment were also calculated in this experiment. ΔCT value was obtained from subtracting the ΔCT before treatment from the $\Delta\Delta CT$ after treatment. The fold changes were determined using the formula $2^{-\Delta\Delta CT}$. Fifteen helminth and LTBI-helminth coinfected patients were tested to see if there was any modulation of the gene expression after the anthelmintic treatment.

mRNA expression of arginase 1, HIF2α and CD163 was significantly decreased after treatment in helminth infected patients when the genes were normalized to CD14. In LTBI- helminth coinfected patients, expression of arginase 1 and CD163 were also significantly deceased after treatment. There were significant increases in the gene expression of HIF1α and CXCL10 in these group of patient's post-treatment. CXCL10 was also increased in helminth infected patients after they completed their treatment. There was no substantial change in gene expression of CD86, IDO-1 or IL-10 after treatment (Table 9.3).

Gene of interest	Helminth infected				LTBI-helminth coinfected			
	$\Delta\Delta CT$	FC	P value	Gene expression after treatment	$\Delta\Delta CT$	FC	P value	Gene expression after treatment
IDO-1	0.7	0.6	0.3815	Decreased	0.601	0.7	0.2078	Decreased
Arginase 1	1.01	0.5	0.0057**	Decreased	1.05	0.5	0.0020**	Decreased
HIF1α	-0.65	1.6	0.0916	Increased	-2.97	7.5	0.0015**	Increased
HIF 2α	2.69	0.2	<0.0001***	Decreased	-0.53	1.4	0.3805	Increased
IL-10	-0.54	1.4	0.1688	Increased	0.2	0.8	0.2524	Decreased
CXCL10	-1.20	2.3	0.0209*	Increased	-1.21	2.3	0.0043**	Increased
CD163	2	0.3	0.0002***	Decreased	2.1	0.2	<0.0001***	Decreased
CD86	-0.39	1.3	0.3303	Increased	0.42	0.7	0.3813	Decreased

Table 9.3: Fold changes in gene expression following anthelmintic treatment. The genes were normalized to CD14.

When normalized against HPRT, helminth infected patients showed significant changes in gene expression of CD163 (p=<0.0005). Arginase 1, CD14, HIF2α, IL-10, CXCL10 also showed a similar pattern although they were not statistically significant. HIF 1α was significantly increased following helminth treatment in this group. In the LTBI-helminth coinfected group, IDO-1 was significantly increased after treatment (p=0.0067). Expression of arginase 1, IL-10 and CD163 was significantly reduced after treatment in this group. Expression of CD14, HIF2α, CXCL10, CD86 also decreased after treatment but did not show any statistical significance (Table -9.4).

Gene of interest	Helminth infected				LTBI-helminth Coinfected			
	ΔΔCT	FC	P value	Gene expression after treatment	ΔΔCT	FC	P value	Gene expression after treatment
IDO-1	-1.9	3.7	0.5154	Increased	-0.12	1.1	0.0067**	Increased
Arginase 1	0.89	0.5	0.0946	Decreased	1.59	0.3	0.0353*	Decreased
CD14	0.8	0.6	0.1044	Decreased	0.56	0.7	0.1473	Decreased
HIF1α	-1.57	2.9	0.0103*	Increased	-0.53	1.4	0.3088	Increased
HIF 2α	0.63	0.6	0.1179	Decreased	0.33	0.8	0.7028	Decreased
IL-10	0.35	0.7	0.3094	Decreased	1.17	0.4	<0.0001****	Decreased
CXCL10	-0.24	1.2	0.5708	Increased	0.96	0.5	0.4791	Decreased
CD163	3.2	0.1	0.0004***	Decreased	5.6	0.02	0.0054**	Decreased
CD86	-1.21	2.3	0.0833	Decreased	0.4	0.8	0.6387	Decreased

Table 9.4: Fold changes in gene expression following anthelmintic treatment. The genes were normalized to HPRT.

9.2 Discussion

In this experiment, the ΔCT method was employed to compare the relative gene expression of genes expressed in whole blood of controls, LTBI, helminth and LTBI-helminth coinfected patients. The aim was to identify the differential gene expression in these clinical samples at different time points. This method was also used to see the fold changes of gene expression in helminth and LTBI-helminth coinfected patients after they were being treated for their helminth infection.

In qRT-PCR, when comparing gene expression in different samples it is critical to consider the experimental variations such as amount of starting material, RNA extraction method, reverse transcription efficiencies, etc. To account for these, accuracy of qRT-PCR relies on the normalization to an internal control. The housekeeping gene is expected to be adequately expressed in the cells of interest and with minimal variability between samples and under the experimental conditions used. Various studies have shown that the candidate housekeeping genes can vary in their expression or some cases, RNA transcription levels may be too low to quantify accurately or reproducibly. Initially three housekeeping genes- HuPO, TBP and HPRT were studied to identify a housekeeping gene with minimal variability in all the patient groups. These three genes were selected from the previous publications (537)(538) as they had been examined in whole blood and PBMC samples and were stable in expression. Among these three genes examined, HPRT was the most stable housekeeping gene with an average fold change of <2 and maximal variability of approximately 5 fold each. TBP and HuPO showed unexpectedly high variability among the samples, although they had been selected as suitable gene for other studies. But the genes may be variable in this particular experimental model, due to the validation process for each gene or on individual basis. HPRT was therefore selected as the reference gene to be used further in the experiments.

In these experiments the genes were also normalized to CD14. CD14 is primarily expressed on monocytes/macrophages but also by polymorphonuclear leucocytes as well as nonmyeloid cells such as B cells, in intestinal epithelial cells, etc. In humans, the two main subsets of monocytes are defined by their expression of CD14, a co-receptor for bacterial lipopolysaccharide, or CD16, the FcγRIII receptor for the Fc domain of IgG (539). These subpopulations are also characterized by the differential expression of chemokine receptors, especially CCR2 and CX3CR1. The more abundant subset in

humans, termed 'classic monocytes' are CD14+ and express high levels of CCR2 and low levels of CX3CR1.

CD14 is a phosphatidylinositol glycan-linked membrane protein, is the high affinity receptor for lipopolysaccharide of Gram negative bacteria, however CD14 can also bind LAM of *Mtb*. Binding of Mycobacteria to macrophages occurs in cholesterol rich domains of the host cell membrane and involves β_2 integrins, the mannose receptor, CR1 and CD14 (540). In monocytes CD14 has been shown to be a potential gate for entry of mycobacteria. The dominant signalling pathway for LAM involves the membrane receptors CD14 and TLR2. Whether CD14, TLR-2, CR3 cooperate to facilitate the uptake of mycobacteria are still unanswered question. Khalid *et al.,* demonstrated that binding of *M. bovis* BCG to CD14 induces an inside-out signalling pathway which involves TLR-2, PI3K leading to enhanced CR-3 dependent bacterial internalization(541). They have also shown that using transfected THP-1 (a monocyte cell line) and CHO (Chinese hamster ovary cells), CD14 plays a major role in phagocytosis of *M. bovis* BCG. CD14 and other receptors can also sense endogenous signals resulting from tissue damage and necrosis. The signalling pathway induced by CD14 and TLRs activates feedback inhibitory mechanisms that confine the magnitude of inflammatory signalling(542). The dynamics of macrophage transition from an intracellular pathogen driven strong inflammatory signalling into a weaker activated state may be important in the immune control of *Mtb* infection. Druszcynska *et al.,* have demonstrated that active TB patients had more CD14 expression on monocytes than the healthy controls and LTBI patients which might have resulted from the enhanced inflammatory response to mycobacterial components and activity of virulent *Mtb* contributing to TB pathogenesis (460). The interaction of mycobacterial constituents and CD14 (either membrane bound or soluble) can lead to the activation of transcription factors, production of pro-inflammatory cytokines/chemokines and upregulation of cell adhesion molecules that contribute to the development of the granuloma and adaptive immunity (543).

Monocytes represent the main host cell for mycobacterial growth, survival and replication. A monocyte marker was used in this assay to see the level of expression of these target genes. The expression of hypoxia inducible genes, immunoregulatory genes and also macrophage polarization genes in LTBI patients might provide a better understanding of the immune mechanism underlying TB latency and also the treatment modulation of these genes might suggest a potential impact of helminth induced immunomodulation.

Monocytes also play a very important role in immunopathogenesis of helminth infection. Fernandes *et al.,* exhibited that CD14++CD16- or classical monocytes participated in the immunopathogenesis of periportal fibrosis, a consequence of Schistosoma infection that was associated with expression of high levels of proinflammatory and profibrotic cytokines and low levels of regulatory markers (544). CD14 can influence the host immune responses and alternative activation of macrophages during Schistosoma infection (545). Gene expression changes after anthelmintic treatment in this experiment might provide an insight that there might be some skewing of immune responses from M2 dominant genes towards M1 phenotype suggesting the immunoregulatory role of helminth that could modulate the *Mtb* specific responses.

In this experiment, IDO-1 was upregulated in LTBI patients in relation to CD14. Blumenthal *et al.,* demonstrated that IDO-1 is one of the most highly induced gene in human macrophages infected with *Mtb*. In mice IDO activity also increases following *Mtb* infection in an IFN-γ dependent manner (537). Suzuki *et al.,* showed that PTB patients had significantly Increased IDO activity in comparison to controls. In their study 22.4% of the patients died and those who died had significantly higher IDO activity. Li *et al.,* demonstrated that an inhibitor of IDO restored decreased T cell derived cytokine production in the pleural fluid of TB pleurisy patients (546). IDO is also important in protection of the granuloma from T cell attack (547). All these data suggest a role for IDO -1 activity in inhibiting *Mtb* specific adaptive immune responses and that it may potentially aid the persistence of pathogen in the infected host. IDO-1 was also significantly upregulated in helminth (3 fold changes in V1 and 1.9 fold changes in V2) and LTBI-helminth co-infected patients (fold changes 3.2 Vs 2.1 in V1 and V2) in comparison to controls. The immunological hallmark of helminth infection is the ability to induce Th2 immune responses and also Tregs. Helminths are potent inducer of dendritic cell regulatory cells (DCRegs) which are characterized by the expression of IL-10, TGFβ. IDO and cyclooxygenase-2. Such responses could possibly downregulate the protective Th1 to *Mtb* infection (186). IDO- 1 potentially inhibits T cell functions and generates Tregs, leading to immunosuppression or tolerance. IL-4 and IL-13 have been shown to control IDO-1 expression by antagonizing the effects of IFNγ in different cell types (548). IDO-1 was upregulated in LTBI-helminth coinfected patients but not in LTBI and helminth infected patients when the mRNA expression was normalized to HPRT. The result was different when normalized to CD14. Since the monocyte/macrophage is critical

for the bacillary growth and containment, this gene expression may be affected by monocyte frequency in these clinical patients. The fold changes in gene expression in IDO-1 in helminth infected patients in relative to CD14 showed there was downregulation of expression in cured patients and that the IDO-1 was upregulated when normalized against HPRT though this was not statistically significant. These changes can be due to immunomodulation due to helminth infection and after removal of helminth, the level of expression was decreased 0.6 fold. However, the immunomodulation underpinning the changes in gene expression remains to be elucidated and needs to be investigated with larger sample sizes per group.

The next gene of interest investigated was arginase 1. There was a significantly significant upregulation of the arginase 1 genes in helminth infected and coinfected patients compared to controls. Arginase 1 metabolizes the amino acid L-arginine to generate urea and ornithine and the resultant metabolites proline and polyamines drive collagen synthesis and cell proliferation(549). Arginine is a crucial amino acid that modulates immune responses to *Mtb*, as it is a common substrate for both iNOS and arginase 1. Loss of arginase in macrophages is associated with enhanced protection against *Mtb*, probably due to availability of arginine for iNOS activity (550). Monin *et al.,* suggested that a prominent effect of helminth infection during TB is the accumulation of high arginase 1 expressing macrophages within granulomas in the lung. Arginase 1 possesses anti-inflammatory effects on Th1 responses and can also mediate exacerbated inflammation and lung pathology during co-infection. Arginase 1 induction due to helminth co-infections, helminth products and host genetics might mediate the formation of more inflammatory TB granulomas (551). However, in coinfected STAT6-deficient mice, in which no AAMs are recruited to the gut, bacterial infection was not exacerbated. In terms of helminth infection, in mice with a macrophage-specific arginase deficiency (LysM^{cre} Arg-1$^{-/flox}$), schistosoma infection results in the formation of larger granulomas than those in control mice, and these granulomas do not shrink at chronic stages of infection, resulting in death due to increased fibrosis and Th2 cell-mediated inflammation (552). It is evident from the results obtained here, that arginase expression was higher in post treatment patients than the control. Probably these cells can still undergo alternative activation, highlighting the role of arginase-1 in depleting arginine in the local milieu, directly suppressing T cell proliferation (549). After helminth treatment, the level of gene expression was

significantly decreased in helminth infected patients. Helminth removal may modulate the arginase expression, as there might be skewing of immune responses towards Th1.

CD163 did not show any significant difference in the gene expression level in LTBI, helminth and co-infected patients when they were compared to controls. It was downregulated in LTBI patients and relatively upregulated in the helminth infected group. CD163 expression is also suppressed by proinflammatory mediators like LPS, IFNγ, and TNFα, whereas IL-6 and the anti-inflammatory cytokine IL-10 strongly up-regulate CD163 mRNA in monocytes and macrophages- which implies a functional role of CD163 in the anti-inflammatory response of monocytes.The haemoglobin scavenger receptor CD163 is expressed exclusively by monocytes and macrophages and preferentially by macrophages of the M2 phenotype (553)(554). Its expression is amplified by IL-4, M-CSF, IL-6, IL-10, and glucocorticoids, while TNFα, TGFβ, IFNγ, and LPS reduce its expression (555)(556)(554)(557). Although all CD14 monocytes express CD163, the CD14+CD16+ population has the highest expression (558).

In this experiment CD163 showed a highly significant fold change after anthelmintic treatment and the gene expression was decreased after treatment. With helminth infection there is skewing of immune responses towards Th2 with decreased Th1 responses. sCD163 has been shown to directly inhibit T lymphocyte activation and proliferation (559). CD163 may therefore be a potential marker to monitor immunomodulation after the helminth infections has been treated.

Many studies support a dominant role for CD86 in primary T-cell responses (560)(561). CD86 is constitutively expressed on resting human peripheral blood monocytes and is upregulated following stimulation (562). CD86 expression is rapidly upregulated during B-cell and macrophage activation (563)(564). In LTBI, the gene was upregulated in relative to HPRT but downregulated in relative to CD14. CD86 was shown to be significantly upregulated in coinfected patients and its expression decreased with treatment, though it did not show any significant difference in helminth patients before treatment. In murine TB, studies have correlated lower levels of CD86 expression with persistence of *Mtb* or disease progression (562) and within the tubercle granuloma. Flores-Batista and colleagues indicated that HLA-DR and CD86 which are key elements for antigen-specific T cell immune responses against *Mtb*, are diminished on the lung cells of pulmonary TB patients. Low expression of antigen-presenting and costimulatory

molecules by lung cells from tuberculosis (35). Decreased expression of HLA-DR and CD86 might result from direct cell infection by *Mtb* or from a response to antigens released by *Mtb*, or may be indirectly mediated by immunosuppressive cytokines, such as IL-10 and/or TGFβ induced by *Mtb*. The diminished levels of HLA-DR and CD86 molecules may limit T cell immune responses that, in turn, favour *Mtb* persistence and progression to active disease. Deficient expression of CD86 or adhesion molecules could impair antigen presentation by infected cells (565). Bonato *et al.,* showed that *Mtb* H37Rv down-modulated CD86 expression on peritoneal macrophages but did not affect CD80 expression on these cells. In their model, the down-modulation of the CD86 expression on macrophages, impaired the initial steps of T-lymphocyte activation owing to a deficient contact between T cells and APC (566). In contrast, Subauste *et al.,* reported an upregulation of CD80 and CD86 after in vitro infection of human monocytes with *Toxoplasma gondi* (567).

Another two genes that were selected for this study were hypoxia-inducible factor HIF-HIF1α and HIF2α. HIFs are the major regulator of the cellular response to hypoxia and are responsible for optimal functional, metabolic and vascular adaptation to O_2 shortage. HIF1 is expressed in virtually all innate and adaptive immune cell populations including macrophages, neutrophils, dendritic cells and lymphocytes (568). HIF2α is expressed in endothelial cells and certain immune cells like tumor-associated macrophages and CD8+T cells in response to hypoxia and this expression is influenced by cytokine exposure (569). When normalized against CD14, HIF1α and HIF2α both were upregulated in LTBI patients in comparison to controls. Similar expression levels were also observed in helminth and coinfected patients in our cohort. HIF2α showed significant downregulation in helminth infected patients after treatment. HIF1α was significantly upregulated after treatment in helminth and coinfected patients. HIF1α deficient macrophages have an impaired metabolic adaptation to hypoxia, which results in reduced motility and migration and also in a decreased capacity to kill bacteria (570). Peyssonnaux *et al.,* showed that HIF1α was correlated with iNOS expression driving NO and TNFα production and that HIF activity is also important in phagocytic uptake of bacteria by macrophages under hypoxic conditions (571). HIF markedly increases the release of proinflammatory cytokines such as TNFα, IFNγ, IL-1β and expression of co-stimulatory molecules by murine dendritic cells (DCs), enhancing their ability to induce lymphocyte proliferation (572). HIF1α drives the production of chemokines and chemokine receptors that could

influence myeloid recruitment- for example CCL5 and C-X-C motif cytokine 12 (CXCL12)(573). Upon activation, HIF1α is strongly induced in conditions that favour differentiation of CD4+ Th17 cells. HIF1α can increase the expression of RORyt, which in turn promotes IL-17 production and Th17 cell development. HIF1α can bind the FOXP3 protein and impact its function and degradation and promotes FoxP3 mRNA, induction and supports Treg cell function. A HIF1α driven glycolytic shift supports Th17 cell differentiation. HIF1α can also drive metabolic adaptations in CD8+ T cells and control expression of molecules associated with effector function (570). HIF deficient T cells show diminished expression of effector molecules such as perforin and granzymes and altered molecule expression involved in T cell migratory capacity. HIF1α stabilization by activated CD8+T cells causes increased expression of costimulatory/inhibitory molecules (CTLA-4, GITR, etc.), altered migration and chemokine receptor expression.

HIF2α was significantly reduced in helminth infected patients after treatment. HIF2α is also known to have a role in the glycolytic adaptation to hypoxia in macrophages (569). Arginase has an important role in the HIF2α contribution to IL-4 induced M2 polarization. Increased expression of HIF2α outcompetes HIF1, limiting the glycolytic shift associated to M1 polarization (574). To observe the expression of these hypoxia induced gene in helminth and latent TB infected patients would provide important information about the differential expression of these two genes and also if there is any alteration after anthelmintic treatment.

CXCL10 was downregulated in LTBI patients in relative to CD14 but was significantly upregulated when normalized to HPRT in comparison to controls This gene was downregulated in helminth and coinfected patients which was modulated after treatment.CXCL10 or IP-10 is a chemokine expressed in both lymphocytes and monocytes after paracrine stimulation of T-cells by IFNs and other proinflammatory cytokines mainly IFNγ and TNFα, as well as IFNα/β, IL-2, IL-17, IL-27 and IL-1β (124). IP-10 is also induced through cell-surface receptor interaction with T-cell (575). IP-10 is involved in the trafficking of monocytes and activated Th1 cells to inflamed foci through interaction with the CXC chemokine receptor (CXCR) 3(576)(577). IP-10 has been identified as an indicator for both *Mtb* infection and response to treatment, but not activity or severity of pulmonary TB in previous studies (578). IP-10 promotes Th1 immune responses by up regulating the expression of IFNγ and is involved in DTH immune responses (579)(580).

It has been suggested that production of this chemokine in response to *Mtb* antigens could be used as a marker of infection (535)(581). Biaro *et al.,* have demonstrated that IP-10 can perform well in differentiating contacts with either latent or active TB from those who were uninfected and also that IP-10 had the potential to diagnose 'recent TB infection' in persons classified as having LTBI using the TST (122).

IL-10 can be made by Th2 cells, Th1 and Th17 cells, B cells, neutrophils, macrophages, and some DC subsets (582). IL-10 was upregulated in LTBI, helminth and coinfected patients in relative to CD14 and but downregulated expression in relative to HPRT. IL-10 inhibits the protective immune response to pathogens by blocking the production of proinflammatory cytokines, such as TNFα and the Th1 polarizing cytokine IL-12, by directly acting on antigen-presenting cells such as macrophages and DCs(583). One mechanism of *Mtb* killing is mediated by IFNγ with activation of macrophages leading to enhanced production of reactive oxygen and nitrogen intermediates,(584) which can be inhibited by IL-10 (585). IL-10 produced during phagocytosis may also function to block antigen presentation via downregulation of major histocompatibility complex molecules. Inhibitory effects of IL-10 on the production of IP-10 during *Mtb* infection suggest that IL-10 may limit recruitment of Th1 cells to the lungs of infected mice (63). In helminth infection and in LTBI-helminth coinfection IL-10 was upregulated when expressed in monocytes. In helminth infected patients it did not change with treatment but in coinfected patients it was decreased in fold change. In *S.mansoni* infection, Th2 cytokine expression becomes dominant shortly after egg laying begins, with IL-4, IL-5, IL-10, and IL-13 being the principal cytokines secreted by lymphoid cells after stimulation with SEA. The secretion of Th1 cytokines, IFNγ and IL-2, is concurrently down-regulated at the time when Th2 responses are reaching their peak, and it has been postulated that IL-10 production is critical for polarizing the egg-induced Th2 response (587).

Changes in gene expression after helminth treatment was observed for some genes tested, for example, arginase1, HIF1α, HIF2α, CXCL10, CD163 in relation to CD14 and some genes were constitutively expressed in relation to HPRT. When normalized to CD14, IDO-1, HIF 1α, HIF 2α, IL-10 was significantly upregulated in LTBI, helminth infected and LTBI-helminth coinfected patients in comparison to the controls. Arginase 1 and CXCL10 was significantly downregulated in LTBI in comparison to controls. Whereas, when normalized to HPRT, arginase 1 was significantly upregulated in helminth and LTBI-

helminth coinfected patients but not in terms of IDO-1, which was downregulated in LTBI. CXCL10 was significantly upregulated in LTBI patients and CD86 in helminth and LTBI-helminth coinfected patients. CD14 was significantly downregulated in helminth and LTBI-helminth co-infected patients when normalized against HPRT. When the gene expression level after treatment was normalized to CD14 and HPRT, Arginase 1, HIF 1, CD163 was significantly highly expressed and IDO-1 downregulated in helminth and LTBI-helminth coinfected patients. Although the expression level was variable post treatment when normalized to CD14 and HPRT. The level of gene expression and its modulation was different using these two normalizers as HPRT is expressed in all the cells, whereas CD14 is used to normalize for monocyte frequency. CD14 might be more appropriate for observing the gene expression which is constant for a given cell type and to remove non-specific variation of different cell types.

When the gene expression was analysed in helminth-infected patients it was observed that, IDO-1 was significantly expressed in them in relation to CD14. The gene expression pattern was similar to that of LTBI-helminth coinfected patients. However, in coinfected patients HIF2α and IL-10 were also significantly expressed. All these genes are expressed preferentially by M2 macrophages. Such responses could possibly be downregulating the protective Th1 response to *Mtb*. Interestingly with anthelmintic treatment, Arginase 1, HIF2α and CD163 expression was decreased and CXCL10 expression was increased significantly in helminth-infected patients (120). Reduction in the expression of these genes might be associated with enhanced protection against *Mtb* these possesses anti-inflammatory effects on Th1 responses (573) (574). Expression of these genes are amplified by some regulatory cytokines like IL-10, and TGFβ. In MGIA, the immunoregulatory role of IL-10 and TGFβ has been explored in this study in helminth-infected patients. This might explain the immunoregulatory role of these cytokines in mycobacterial growth in coinfected patients before anthelmintic treatment. HIF1α has been linked to the release of proinflammatory cytokines and its expression has been observed to be increased after treatment (564). IP10 expression was significantly upregulated after treatment in helminth and coinfected patients in relation to CD14. IP10 expression is stimulated by IFNs secreted from T cells and other proinflammatory cytokines and is known to have a role in monocyte and Th1 trafficking (573). Increased expression of this gene may thus be associated with increased Th1 cell activation and reduction in mycobacterial growth as observed after treatment. In the Luminex study, it

was also observed that IP10 was increased after treatment, indicating the induction of protective immune activity against TB. Most of these genes are important in immunity against *Mtb*. IDO-1 can potentially inhibit T cell functions, leading to tolerance or immunosuppression and antagonizing the effects of IFNγ (179) and alterations of the expression of this gene after treatment might suggest the skewing of immune responses towards CAM and protective immunity against *Mtb*.

The expression of these genes can be used as a potential bio signature to observe the helminth treatment effect on the helminth and coinfected patients. It would be interesting to explore the gene expression and their modulation in larger sample groups and to explore whether the expression of these correlate with MGIT mycobacterial growth.

Chapter 10

10.1 Summary

Tuberculosis and helminth infection are co-endemic in many parts of the world. The responses induced by the extracellular helminths and those induced by intracellular *Mtb* are often mutually antagonistic and helminths appear to influence the immune response to TB by modulating the immune responses to *Mtb* (135). T cells are the predominant cell types studied in the context of LTBI-helminth coinfection and helminth infection has been shown to reduce the effector T cell responses and increase regulatory T cell responses. Studies have shown varied responses of TB-helminth co-infection in different settings and assays- including effect on acquisition and progression to active disease, effects on the efficacy of BCG vaccination or on the diagnostics of LTBI and even no response depending on the type of helminth species involved, their migratory life cycle phase or chronicity of infection, intensity of helminth infection, patient factors, endemicity, strain of *Mtb* involved, etc (361). Functional assays such as the MGIAs directly measure the ability of heterogeneous populations of human lymphocytes and other cells to limit the intracellular growth of mycobacteria. The immune mediated inhibition of mycobacterial growth correlates more directly with protective TB immunity than other immunological responses. MGIA and other cellular parameters like cytokine profiles, antibodies, and gene expression might provide new information on important aspects of the underlying immunopathogenesis in LTBI-helminth coinfection and how it modulates antimycobacterial immune responses.

In this cohort, the predominant helminth infections were Strongyloidiasis and Schistosomiasis: 26.9% of the patients had eosinophilia with the rest of the patients having normal eosinophil counts. The eosinophilia reduced significantly with treatment and the eosinophil count was significantly correlated with IGRA values in post treatment coinfected patients. Other cellular parameters - monocyte count, ML ratio, and neutrophil count showed significant changes in LTBI-helminth coinfected patients with anthelmintic treatment. The UK migrants in this cohort, had shown a reduced CD4+IFNγ responses, and secretion of IFNγ, TNFα and IL-12, which reversed after anthelmintic treatment. CD4+IFNγ responses to PPD, ESAT-6/CFP10 measured using ELISpot in the helminth infected and coinfected patients improved after anthelmintic treatment. It was therefore of

interest to see how helminth co-infection would affect mycobacterial growth inhibition, and how anthelminthic treatment might affect this.

Before performing the MGIA assay on clinical samples, a protocol was standardized for the reproducibility of the test-BCG declumping methods, impact of antibiotics, heat inactivation serum and various other steps in culture conditions were optimized. Using the optimised protocol, helminth infected and LTBI-helminth coinfected patients exhibited poor mycobacterial growth inhibition before anthelmintic treatment, that reversed significantly with the anthelmintic treatment. *Mtb* Erdman and *Mtb* H37Rv was also used to perform MGIA to see if BCG can provide an estimation of growth inhibition of the more virulent *Mtb*, although the smaller sample size made it difficult to find a significant correlation. Total and antigen specific IgE and IgG4 concentrations were reduced significantly with anthelmintic treatment, although not correlated with MGIA. The complex mixture of Th1, Th17 cytokines and immunosuppressive cytokine like IL-10 and TGF-ß appeared to play important role in the coinfection. The expression of many genes like IDO-1, arginase 1, HIF1α, HIF2α, IL-10, CD163 were significantly changed with anthelmintic treatment indicating an alteration of antimycobacterial immune response in coinfected patients. Mycobacterial growth inhibition did not correlate with CD4+IFNγ+ response, but the MGIA assay along with cytokines, antibodies and gene expression provided more information about the complex host-pathogen interactions involved in the LTBI-helminth coinfected patients, that might indicate that the helminth infection modulating antimycobacterial immune responses.

10.2 Strengths and weaknesses of the study design

The potential impact of helminth infection on immune responses to *Mtb* in patients with LTBI with or without helminth infection (Strongyloides or Schistosoma) and whether anthelmintic treatment modulates the immune responses were explored in this study. The study was performed in migrants in the UK, where reexposure and reinfection following anthelmintic treatment would not occur, as the UK is not a helminth endemic country. This provided a unique opportunity to study the effect of anthelmintic treatment in helminth infected patients. Also the issue of potential drug resistance to anthelmintic treatment as a result of selection through exposure of the worm population would not be an issue in the UK setting. Some previous studies have shown no effect of anthelmintic treatment or clinical improvement of TB and also the effect on the rate of progression from latent to

active TB. All the studies were done in endemic countries, where there was the chance of reexposure and reinfection (354). This study could directly address the influence of anthelmintic treatment on antimycobacterial immune responses.

In various studies, the antimycobacterial effects of ivermectin have been reported, although the MICs of ivermectin were several magnitudes higher than peak plasma concentrations, limiting the use of ivermectin as antimycobacterial agent (588). In the current study 21 Strongyloidiasis patients received ivermectin treatment. However, it would be interesting to see the bactericidal activity of this drug to kill or inhibit the growth of *Mtb*. To avoid any direct effects of the anthelminthic drugs on immune function, or on the BCG, the post-treatment time point was 4 months after the first visit, at which time such effects would not be present.

One issue for such studies is the selection of appropriate controls. Most of the controls in this study that were recruited from the clinic, attended the clinic with eosinophilia and some symptoms of allergic, respiratory and GIT symptoms. In addition, some of the LTBI patients in this cohort, were health care workers, rather than migrants. Of the 90 LTBI patient in this cohort, 33 of them received treatment for LTBI after visit 1. Of the LTBI patients, 25 were healthcare workers. Twenty-two healthcare workers received treatment for LTBI and two were offered treatment but they declined treatment. None of the LTBI patients included in this study were on treatment for their LTBI at visit 1. The average age of LTBI patients, who received treatment was 33.72 years of age and of the healthcare workers was 34.7 years. Previous UK policy was to only offer treatment for LTBI to those aged less than 35 years, although this was recently changed to 65 years.

Helminth infection in this cohort were mostly diagnosed by serological tests, culture and microscopy. The ELISA test detected about 96% of *S.mansoni*and 92%*S.haematobium* infections, although this test does not distinguish between active and treated infections. In some patients the test may remain positive even two years after treatment and there is a chance of a false positive result from cross reacting responses with other helminths like tapeworm, trichinella, etc. In chronic infection, the intestinal worm load is very low and the output of larvae is minimal and irregular, hence the sensitivity of direct observation of larvae decreases. Multiple stool samples would have been helpful in detection of the larvae, which was not possible for this study(404). Also lower larvae yields made it difficult to detect the helminth load and intensity of infection, although with patients in

non-endemic settings heavy infection is rarely found. PCR was performed in Strongyloidiasis patients and it showed a sensitivity of 66% against culture as gold standard and 38% against serology as a gold standard. The detection rate in the present study may be low due to the presence of the resistant cuticle which is difficult to disrupt mechanically, irregular release of larvae and low concentrations of parasite DNA in the presence of PCR inhibitors.

10.3 Features of the MGIA assay

The haematological data collected from the clinic, provided important information on cell counts, IGRA results and their changes after patients had received the treatment. In this cohort, 27% had eosinophilia and the rest had normal eosinophil counts. The eosinophilia in helminth infected and coinfected patients reduced significantly after anthelmintic treatment. Most of the controls also had eosinophilia and many had allergic or respiratory symptoms. The absolute eosinophil count was significantly higher in the LTBI-helminth coinfected patients before they received treatment. IFNγ modifies the effector functions of eosinophils-which had been showed in studies when eosinophils were stimulated with GM-CSF and IL-5(589). Mycobacterial growth inhibition was not related to eosinophilia, neither with other cell counts like neutrophils, lymphocyte count, NLR.

Monocytes are critical for determining the outcome of the MGIA assay. There was reduced mycobacterial growth when monocytes were depleted from the culture. Monocyte frequencies were significantly reduced in LTBI and helminth coinfected patients, although monocyte count failed to show any correlation with mycobacterial growth. A complex association of ML ratio with growth inhibition was observed in coinfected patients. Alteration of the ML ratio was associated with poor growth inhibition, although the correlation was not significant. This finding was consistent with publications on the ML ratio in TB (411). ML ratio changes after anthelmintic treatment might provide an important indicator for progression of LTBI towards active disease.

There was a decrease in CD4+IFNγ+T cells in helminth infected patients and the coinfected groups when stimulated with PPD and ESAT-6/CFP10 both at the baseline in comparison to controls and LTBI infected patients suggesting a complex host pathogen interaction in coinfected patients, similarly with CD8+T cells with lesser magnitude. Potential factors such as negative costimulatory responses and expression of TLRs like

TLR2,9 etc. could be analysed and might be helpful to understand the underlying immunopathogenesis. Mycobacterial growth inhibition did not correlate with IFNγ-ELISpot responses suggesting IFNγ is not a direct correlate of protection but rather MGIA may provide more information about the functional response and also the role of blood components and cytokines may provide more information about the important determinants of mycobacterial growth inhibition.

Mycobacterial growth inhibition was significantly reduced in helminth and LTBI-helminth coinfected patients and this reversed after anthelmintic treatment. Helminth infections, in addition to Th2 responses, can induce inhibitory cytokines such as IL-10 and TGFβ that supress Th1 type responses and interfere with effector T cell activation. Helminths induce strong type 2 immune responses, characterized by increased production of antigen-specific IL-5, IgG4 and total IgE concentration, and peripheral eosinophilia. Increases in such Th2 responses might be expected to affect the immune response, with suppressed Th1 cytokine responses. IL-10 and TGFβ were found to play an important immunoregulatory role in growth inhibition. There was a reduction of Th1 cytokines and the gene expression study showed expression of functionally relevant and polarization associated genes like arginase 1 indicating development of alternatively activated macrophages that may mediate impaired Th1 responses to *Mtb* antigens.

LTBI-helminth coinfection was associated with significantly elevated levels of pro-inflammatory and lower levels of anti-inflammatory cytokines after anthelminthic treatment. Thus potentially modulating the cytokine environment in which TB is controlled in LTBI.

It was hypothesized that chronic helminth infections might worsen the course of TB. This study suggests that helminths not only alter the Th1 and Th2 immune balance but may also adversely affect the ability to control mycobacterial growth. The ability to control the growth of the mycobacteria increases when the helminth infection is treated and patientsare not reexposed. Pre and post treatment IGRA values of helminth infected and LTBI-helminth coinfected patients showed improvement in IGRA values after the treatment, although the change was not significant, perhaps as the sample size was smaller. Helminth infection were associated with inconclusive results in the IGRAs in pregnant mothersin Ethiopia and children in Bangladesh (491). However, a more recent study in

South Africa similarly revealed no significant effect of anthelmintic treatment on tuberculin skin test or IGRAs in children (590).

The influence of helminth infection on the LTBI progression to active TB is not very clearly understood. Among previous studies, some showed no effect of helminths on mycobacterial infection and three observed worsened control. In Uganda, there was a relationship between having Schistosomiasis and progression from LTBI to active TB (336). The observed differences might be due to the helminth species studied, their location in different tissue spaces, different life cycle and the release of different secretory and excretory antigens, intensity of infection and endemicity. However, with longitudinal follow up, these patients who did not receive prophylactic treatment for LTBI could have provided information regarding the clinical outcome.

10.3 Future directions

Various cellular components and their role in MGIA have been observed in this study, but further work is required to investigate the other important cellular compartments, especially neutrophils could be investigated in whole blood assays to see if they also contribute to MGIA variance. NK cells would have been present in the PMBC cultures used in the present study, but were not directly investigated. The MGIA can be performed using whole blood, but this does not allow pre- and post-treatment responses to be directly compared in the same assay. The frequency and cellular subtypes such as neutrophils and their cytokine expression profile can be observed. IL-10 has been proved to be very important immunoregulatory cytokine in MGIA, and the cellular source of IL-10 could be detected by flow cytometry. Various components of complement can also be tested for their role, as when the role of heat inactivation of serum was observed in the MGIA, it was suggested that complement did have a role in mediating the responses. More studies on innate cells including neutrophils, innate lymphoid cells, dendritic cells, B cells and cytokine expression can be conducted.

Antibody concentrations did not correlate with MGIA in this study, although *Mtb* specific antigens like HBHA, Ag85A and other latency antigen could have been used to test the antibody responses, that might provide further important information to show if antibodies are playing any protective role.

Anthelmintic effects on MGIA could be compared with UK migrants and patients from endemic countries to observe the variation in endemic setting. The helminth load and number of helminth species involved can also be useful to evaluate whether individuals with high worm burden have an increased risk of developing TB. Malnutrition is another confounding factor. Data regarding nutritional assessment can also be assessed before and after treatment as this might affect the LTBI diagnostics, although malnutrition was not a factor in the UK cohort.

One question that arose during the study was whether the depressed Th1 responses observed in helminth infected patients might affect the IGRA test used to identify LTBI. IGRA values in these patients were documented in LTBI-helminth coinfected patients only for the pre-treatment visit. There were a few paired samples where both pre and post anthelmintic treatment IGRA values were available; this showed that there was improvement in T cell responses with treatement, although due to the small sample size it was difficult to draw a conclusion. It would be interesting to see if the improvement in T cell responses and IFNγ responses affected the QFT-GIT reading, to see whether the helminth infection is modulating the IFNγ responses and might impact the LTBI diagnosis.

At the start of this work, there was concern that the MGIA assay might not be sufficiently reproducible or robust to provide useful results. Following the optimisation performed here, the MGIA assay was found to be reproducible in the groups who were not treated, and to show reproducible changes in patients whose helminth infections were treated. Further optimization of the MGIA can be assessed like use of PANTA in 96-hour culture, BCG culture in plates and avoiding the use of rotating cultures etc. These changes would reduce the costs of the experiments and make the assay more logistically simple to use in endemic setting.

It has been demonstrated earlier that helminth infection can significantly impair the efficiency of BCG in helminth endemic regions. Observing this effect in non-endemic setting also can be of great importance in the area of greatest impact of the modulation of the immune responses by helminth infection likely involved attenuation of vaccine-induced immune responses.

The influence of helminth infection on the development of active TB is still unanswered. It has been shown previously that patients with active TB were likely to have intestinal

helminth infection. In the present study, helminth infection was shown to modulate LTBI immune responses. Comparing the study of acute and latent TB and helminth coinfection might provide an important information about the immune signature in coinfected patients.

Helminth infected patients in this study displayed poor growth inhibition in MGIA which was improved after anthelmintic treatment, indicating this immunomodulation was helminth mediated.It will be fascinating to detect the effects on progression or pathology in coinfected patients and during their subsequent longitudinal follow-ups as helminths might influence the outcome.

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Appendices

Appendix 1

London School of Hygiene & Tropical Medicine
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United Kingdom
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www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Dr. Shaheda Anwar
LSHTM

16 December 2014

Dear Shaheda,

Study Title: "Investigating the effect of de-worming on the antimicrobacterial immune responses in co infected migrants"

LSHTM Ethics Ref: 7758

Thank you for your letter responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion
Approval is dependent on local ethical approval having been received, where relevant.

Approved documents
The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Investigator CV	cv 2014.docx	28/08/2014	1
Information Sheet	IDEA_consent_V5.docx	28/08/2014	1
Advertisements	IDEA_patientinformationleafletV5 (2).docx	28/08/2014	1
Local Approval	11 H0713 12 Tsang (Brown)Favourable Opinion Susbstantial Amendment Revised 13 1 2014.pdf	28/08/2014	1
Protocol / Proposal	Protocol.docx	29/08/2014	1
Covering Letter	1.docx	26/11/2014	2
Covering Letter	cover letter.docx	05/12/2014	3

After ethical review
Any subsequent changes to the application must be submitted to the Committee via an Amendment form on the ethics online applications website. The Principal Investigator is reminded that all studies are also required to notify the ethics committee of any serious adverse events which occur during the project via an Adverse Event form on the ethics online applications website. At the end of the study, please notify the committee via an End of Study form on the ethics online applications website. Ethics online applications website link: http://leo.lshtm.ac.uk

Yours sincerely,

Professor John DH Porter
Chair
ethics@lshtm.ac.uk
http://www.lshtm.ac.uk/ethics/

National Research Ethics Service

NRES Committee London - Bloomsbury

HRA NRES Centre Manchester
Barlow House 3rd Floor
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09 January 2014 Revised 14 January 2014

Ms Lillian Tsang
Research Nurse
University College London
Department of Infection & Immunity
Room 1.3.1
Cruciform Building
Gower Street
London
WC1E 6BT

Dear Ms Tsang

Study title:	**Investigating the effect of de-worming on anti-mycobacterial immune responses in co-infected migrants.**
REC reference:	**11/H0713/12**
Amendment number:	**Amendment 5**
Amendment date:	**09 December 2013**
IRAS project ID:	**66427**

- The amendment proposes to increase the number of participants to four hundred and twenty.

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date	
Protocol	7	20 December 2013	

Notice of Substantial Amendment (non-CTIMPs)	Amendment 5	09 December 2013

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

11/H0713/12:	**Please quote this number on all correspondence**

Yours sincerely

Signed on behalf of:
Dr Joe Brierley
Chair

E-mail: nrescommittee.london-bloomsbury@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: Ms Anna Jones - Royal Free London NHS Foundation Trust

             Dr Nandi Simpson – UCLH NHS Foundation Trust

             Dr Michael Brown - University College London NHS Trust

NRES Committee London - Bloomsbury

Attendance at Sub-Committee of the REC meeting on 10 January 2014

Name	Profession	Capacity
Dr Joe Brierley (Chair) – Chaired the meeting	Consultant Intensivist	Expert
Michelle McPhail	Lecturer in Management Studies	Lay Plus

Also in attendance:

Name	Position (or reason for attending)
Dr Ashley Totenhofer	REC Manager

Appendix 2

University College London Hospitals **NHS**

NHS Foundation Trust

INFECTIOUS DISEASES IN EUROPE AND AFRICA - IDEA STUDY- INFORMATION FOR PATIENTS

INTRODUCTION

At this hospital we are studying how worm infections influence other infections eg: TB. Worm infections are common in people who have lived in the tropics. We are asking people who come to HTD clinics to be tested for worm infections, and people coming to the UCLH TB clinic to take part in this study. In this leaflet we provide information about the study so that you can decide if you would like to take part.

INVITATION TO PARTICIPATE

In these clinics, the medical staff routinely test people for TB and for worm infections. A research nurse will explain the study to you and answer any questions that you may have. If you agree to be included in the study you will be asked to sign a consent form.

DO I HAVE TO TAKE PART?

No. You do not have to take part. If you do agree to be included you are still free to withdraw at any time without giving a reason. Your decision will not affect your treatment in any way.

WHAT WILL HAPPEN TO ME IF I AGREE TO BE INCLUDED IN THIS STUDY?

Taking part in this study will not affect your treatment in any way. A copy of the signed consent form and this information sheet will be given to you to keep.

If you agree to take part, this will be your first visit (visit one) as a participant, and you will be asked to attend two further appointments. We may send a letter to your GP letting them know that you are taking part.

Visit one: At the same time as taking blood samples that are part of the routine assessment of patients in this clinic, the research nurse will take extra blood up to 33 ml (which is about 3 tablespoons), for the study.

Visit two: Four weeks after your first visit, you will be invited back to the clinic. If tests showed that you had a worm infection, you will be treated for this and a repeat blood sample of 23 ml will be collected. If you did not have a worm infection, a repeat blood sample of 23 ml will be taken.

Visit three: Sixteen to twenty four weeks after your second visit, you will be asked to return to the clinic so that the nurse can collect a final **26 ml** of blood from you. This will be the end of your participation in the study.

ARE THERE ADVANTAGES OR RISKS TO TAKING PART?

No. Being included in this study will not affect your care in any way.

WILL MY TAKING PART IN THE STUDY BE KEPT CONFIDENTIAL?

Yes. All the information about your participation in this study will be kept confidential.

WHO IS RESPONSIBLE FOR THIS STUDY

Dr Mike Brown
The Hospital for Tropical Diseases,
Mortimer Market, Capper Street, Tottenham Court Road,
London, WC1E 6JB
Email: Mike.Brown@uclh.nhs.uk, Tel: 0845 155 5000 ex 5972

WHAT IS THE PURPOSE OF THE STUDY?

Scientific researchers at the London School of Hygiene & Tropical Medicine, and specialists in infectious diseases at this hospital are looking at the way people's bodies respond to infections. They will be measuring the how peoples' bodies respond to TB when they have worm infections, and how this changes when the worm infections are treated. These responses can be seen by using blood cells to measure immune reactions. This research is being done to improve our understanding about the way infections cause disease. The aim is to find new ways to prevent infectious diseases in the future.

WILL ANY INFORMATION BECOME AVAILABLE THAT WILL AFFECT MY CARE?

If results show that you have a worm infection or are infected with TB, you will be treated for these infections. The treatment you will get will be the same as if the infection had been discovered as part of routine care. We will let you know the results of these tests, and we will also tell your GP and the doctors here at the hospital.

WHAT WILL HAPPEN IF I DON'T WANT TO CARRY ON WITH THE STUDY?

If you decide you don't want to continue being part of the study, you can stop at any time. If you decide to withdraw from the study, we will keep any data or samples that have already been collected unless you ask us to destroy these. Your routine health care will not be affected in any way by your decision.

WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?

All information which is collected about you for this study will be kept strictly confidential, this is local NHS policy. Any information about you that is held outside of normal NHS facilities will have your name and address removed so that you cannot be recognised from it. None of your blood samples or data that go to the researchers at London School of Hygiene & Tropical Medicine will have your name on them. Only the clinic staff who have been looking after you will know your identity. All clinic staff and researchers will have a "duty of confidentiality" to you as a research participant, this means that they will not reveal your identity.

DATA ANONYMISATION

At the end of this study, material from the samples you provided will be stored in case further analysis of them is needed for this study. If the samples are used in other approved studies, they will be labelled so that researchers cannot see that they come from you. All research samples and information about you will be securely stored under the custody of Dr Mike Brown All data will be confidential.

EXPENSES AND PAYMENTS

Travel expenses on public transport will be reimbursed.

WHAT WILL HAPPEN TO THE SAMPLES I GIVE?

The blood sample collected for this research study will be given a code number; part of the sample will be analysed at UCLH, the rest will be analysed at the London School of Hygiene & Tropical Medicine. Only research staff working on this project will have access to these specimens. All research samples and information about you will be securely stored under the custody of Dr M. Brown.

WHAT HAPPENS IF A PROBLEM ARISES AS A RESULT OF TAKING PART IN THIS STUDY?

If you have a concern about any aspect of this study, you should ask to speak with Dr M. Brown who will answer your questions (Contact details given in part 1). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital Patient Advice and Liaison Service. In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against University College London Hospitals NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate).

WILL ANYONE OUTSIDE OF THE RESEARCH TEAM BE INVOLVED IN THIS STUDY

A copy of your consent form will be placed in your medical records. No one outside of the named research staff will be involved in this study. This study is part of a European consortium research project, therefore anonymised results from this study will be shared with other consortium members.

WHAT WILL HAPPEN TO THE RESULTS OF THIS STUDY?

The aim is to publish the results of this study in the scientific literature and present them at scientific meetings. You will not be identifiable by any such publication or presentation.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

This research is organised by Dr M. Brown and collaborators within University College London Hospitals NHS Foundation Trust and the London School of Hygiene & Tropical Medicine. This study is funded by an EU FP7 Consortium grant. No doctor or researcher will receive any payment for including you in this study.

WHO HAS REVIEWED THIS STUDY?

This study has been reviewed and approved by the Central London Research Ethics Committee 2 and was given a favourable ethical opinion for conduct by the LSHTM Research Ethics Committee.

Appendix 3

UCLH Project ID number:

INFECTIOUS DISEASES IN EUROPE AND AFRICA
IDEA STUDY -CONSENT FORM

Project title:	Investigating the effect of de-worming on anti-mycobacterial immune responses in co-infected migrants.

Please <u>initial each box</u> to indicate agreement	

1.	I confirm that I have read and understand the information sheet dated **01/10/2013 (version 5)** for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that sections of any of my medical notes and data collected during the study, may be looked at by <u>authorised</u>individuals University College London Hospital or from regulatory authorities where it is relevant to my taking part in this research and that all data will be confidential. I give permission for these individuals to have access to my records.	
4.	I agree to take part in this study.	
5.	I agree to donate blood samples in this study as a gift.	
6.	I consent to the storage and use of material from this study for use in future research studies, subject to approval by Research Ethics Committees.	
7.	I understand that my GP may be informed that I am taking part in this study.	

Name of patient	
Signature		Date	

Name of investigator taking consent	
Signature		Date	

1 Copy to patient		1 Copy to research file		1 Copy to medical notes	

Project title:	Investigating the effect of de-worming on anti-mycobacterial immune responses in co-infected migrants.

Please <u>initial each box</u> to indicate agreement

1.	I confirm that I have read and understand the information sheet dated **01/10/2013 (version 5)** for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3.	I understand that sections of any of my medical notes and data collected during the study, may be looked at by <u>authorised</u>individuals University College London Hospital or from regulatory authorities where it is relevant to my taking part in this research and that all data will be confidential. I give permission for these individuals to have access to my records.	
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6.	I consent to the storage and use of material from this study for use in future research studies, subject to approval by Research Ethics Committees.	
7.	I understand that my GP may be informed that I am taking part in this study.	

Name of patient	
Signature		**Date**	

Name of investigator taking consent	
Signature		**Date**	

1 Copy to patient		**1 Copy to research file**		**1 Copy to medical notes**	

COMMENTS OR CONCERNS DURING THE STUDY
If you have any comments or concerns you may discuss these with the investigator.   If you wish to go further and complain about any aspect of the way you have been approached or treated during the course of the study, you should write or get in touch with the Patients Advice and Liaison Service (PALS). Please quote the UCLH project number at the top this consent form.

Appendix 4

Infectious Diseases in Europe and Africa - IDEA study case record form VISIT 1				
Hospital number			Study UIN	
Gender		DOB		IDEA study group	
Contact details				
Referral route				
Doctor/Nurse			Contact	

Country of origin: _____ In UK since: _____

Inclusion   Exclusion

Age >18   ☐   Age <18   ☐

Lived in Tropics   ☐   Known HIV infection ☐

Eosinophilia   ☐   Previous treatment for helminths ☐ (uninfected)

Helminth infection   ☐

NB: Not all inclusion criteria are necessary for participation, but any of the exclusion criteria disqualifies participation

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Samples collected:

Research samples   ☐

Diagnostic samples for parasitology ☐

Samples for Quantiferon-Gold   ☐

Helminth infection

Symptoms Y ☐ N ☐ ? ☐

Countries of potential exposure: _____

(with date last visited)   _____

Eosinophilia

Historical   date:_____   eosinophils: ☐☐.☐ x 10^9/L

                           lymphocytes: ☐☐.☐ x 10^9/L

Current   date:_____   eosinophils: ☐☐.☐ x 10^9/L

                           lymphocytes: ☐☐.☐ x 10^9/L

Parasitology

Parasitology *(If available)*

Strongyloides

Serology: _____Date: _____

Stool microscopy: _____Date: _____

      charcoal: _____ Date: _____

Schistosoma

Serology: _____Date: _____

Urine: _____Date: _____

Additional helminth serology filaria Y☐N☐

Notes:

TB

Previous history of TB   Y☐N☐?☐

BCG vaccination   Y☐N☐?☐

IGRA
Date tested: _____
Result: _____

Quantiferon-Gold
Date tested: _____
Result: _____
OR:
Samples sent date: _____

HIV Status: Negative/ Unknown*(please delete as appropriate)*

Notes:

Mycobacterial Growth Inhibition Assay

Reagents:

- RPMI-MGIT = Sigma RPMI-1640 Medium HEPES modification with 25mM HEPES (Cat. R5886). Add 5ml of L-glutamine and 50ml of filtered Pooled Human AB Serum (PHS).
- BCG/ Mycobacteria stock.
- BACTEC MGIT tubes containing 7ml media
- PANTA/OADC enrichment broth for MGIT tubes

Equipment:

- BACTEC MGIT 320/960 machine
- 37°C water bath
- Centrifuge and microcentrifuge
- 37°C incubator
- Cell counter/microscope
- 2ml screw-cap tubes
- 360°C tube rotator
- Vortex

Method:

Day 1:

1. Cryopreserved PBMC thawed in a water bath at 37°C, washed twice in RPMI media

2. Count the cells, washed and resuspended in MGIT medium at a concentration of 1 $X10^6$ per 300µl medium

3. Add 300 µl of cell suspension to each of 2 duplicate 2 ml micro tubes per sample

4. Prepare the BCG master mix: Calculate the total number of tubes being inoculated. This will be (number of samples x 2) + 2 control tubes. To make a master mix we would dilute the neat stock 1/10 in RPMI (900ul RPMI and 100ul of BCG stock). take 10ul of your 1/10 dilution above and add it to 990ul of RPMI to make a 1/1000 dilution. We would then add 5ul of the 1/10 dilution to 5000ul of RPMI to make a working stock. 300ul of the working stock contains the equivalent of 0.05ul of the undiluted stock and will give you a TTP of 12 days.

5. Do the same for the TB master mix.

6. Supplement 4 MGIT tubes with 800ul PANTA/enrichment and 200ul of PANTA/Enrichment supplemented Middlebrook 7H9 from a spare tube.

7. Add 300ul of the BCG master mix to each of the 2 positive control tubes, and 300ul of the TB master mix to the other 2. Invert to mix and place on the MGIT machine.

8. Add 300ul of the mycobacteria master mix to each sample 2ml tube.

9. Place tubes on a 360°C tube rotator in a 37°C incubator for 96 hours.

10. Set duplicate direct-to-MGIT viability control tubes. Add 800 µl PANTA/OADC enrichment broth and 300 µl of BCG/*M.tuberculosis* and put into the machine.

Day 2:

1. After 96 hours, remove sample tubes and centrifuge at 12,000rpm for 10 minutes.

2. Remove 500ul of supernatant ensuring the pellet remains intact, and store in a 2ml screw-cap tube. (Some supernatant can remain in the tube).

3. Freeze supernatant at -80 degrees.

4. Add 100µl sterile water to each tube and vortex for 30 seconds

5. Leave at room temperature for 5 minutes, vortex again. And repeat the step twice more.

6. Add 400ul of PANTA and OADC enrichment broth and vortex for 30 seconds.

7. Add 800ul PANTA/enrichment to the same number of MGIT tubes as you have 2ml sample tubes and label accordingly

8. Add the 500ul sample to its corresponding MGIT tube, invert to mix and place on BACTEC MGIT machine and incubate at 37°C until the detection of positivity.