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Studies of immune responses in human tuberculosis

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This thesis is dedicated to all
patients who participated
in the study.

“In the fields of observation chance favors
only the prepared mind’’ Louis Pasteur

ABSTRACT

TB (tuberculosis), caused by *Mycobacterium tuberculosis* (Mtb), continues to be a world-leading killer and a serious global health problem primarily affecting poor people in many developing countries. The difficult situation with TB/HIV co-infection is also a major key challenge to public health. Despite recent advances in TB research, the host- and pathogen-specific factors that lead to protective immunity, particularly in humans, remain unclear. While it is well-established that cell-mediated immunity is required to control TB infection, the role of humoral immunity including Th2 immune responses is debated. This thesis aimed to explore immune responses in human TB and the immunopathogenic mechanisms involved in the progression of clinical TB in both HIV-negative and HIV-positive individuals.

To address the aims of this thesis work, well-defined study cohorts of active TB patients were obtained in close collaboration with the Black Lion University Hospital in Addis Ababa, Ethiopia. We used a novel immunodiagnostic test, Antibodies in Lymphocyte Supernatants (ALS), to demonstrate that IgG-secreting plasmablasts were significantly higher in the peripheral circulation of patients with active TB compared to latent TB cases and non-TB controls. Interestingly, BCG-specific IgG titers were particularly high in blood samples from TB/HIV co-infected patients with CD4 T cell counts <200 cells/ml who produced low levels of Mtb-specific IFN-γ in vitro. A technological platform including quantitative assessments of mRNA and protein expression in tissue (lymph nodes), fluids (bronchoalveolar lavage (BAL) and pleura fluid) and peripheral blood, was also used to investigate antimicrobial effector pathways and adverse immune responses in unique clinical samples obtained from the local site of Mtb infection. The results from these studies revealed that TB disease was associated with extensive tissue remodelling including an altered cellular composition, collagen deposition and granuloma formation. Here, the degree of necrotic granuloma formation was particularly prominent in TB/HIV-co-infected patients. Despite granuloma enrichment of activated $CD68^+$ macrophages containing Mtb-antigens, mRNA levels of IFN-γ, TNF-α and IL-17 remained low in Mtb-infected lymph nodes. Accordingly, $CD8^+$ T cells expressing cytolytic and antimicrobial effector molecules perforin and granulysin were low inside the TB lesions, while $CD4^+FoxP3^+$ regulatory T cells (Treg) and Th2/Treg cell cytokines IL-13 and TGF-β were up-regulated in the Mtb-infected tissues. The observed shift of the immune response from a Th1/Th17 towards an immunoregulatory phenotype was supported by our finding of a Th2 polarized response in the lung of patients with pulmonary TB. Here, multiplex protein analysis of BAL and plasma samples demonstrated low levels of Th1/Th17 cytokines and the T cell-chemoattractant CCL5, but significantly up-regulated levels of pro-inflammatory cytokines and the Th2 cytokine IL-4. The enhanced Th2 response was associated with increased levels of CCL4, suppressors of cytokine signaling-3 (SOCS3) and mycobacteria-specific IgG in BAL fluid from patients with active pulmonary TB. Contrary, IL-4, CCL4, SOCS3 and IgG-responses remained low in patients with less severe extrapulmonary pleural TB disease, who demonstrated up-regulated levels of both IFN-γ and CCL5.

Taken together, our results provide evidence that human TB is associated with impaired Th1 immunity but elevated Th2/immunoregulatory responses and induction of antibody-mediated immunity. Importantly, enhanced Th2 and/or plasmablast responses may be used as relevant biomarkers or immune response signatures of active progressive TB disease that could be explored as potential targets for clinical TB management in the future.

LIST OF PUBLICATIONS

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Progression of clinical tuberculosis is associated with a Th2 immune response signature in combination with elevated levels of SOCS3

Invited revision to Clinical Immunology.

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CONTENTS

1 Introduction to the thesis ... 1
2 Background ... 2
2.1 TB infection: A long journey ... 2
2.1.1 History of TB ... 2
2.1.2 Epidemiology of TB ... 2
2.1.3 Transmission of TB in humans ... 3
2.2 Mtb: A complex bacteria ... 4
2.2.1 The Genus Mycobacterium ... 4
2.2.2 Virulence of Mtb ... 5
2.3 TB immunity: Induction of cellular immune responses ... 6
2.3.1 The innate immune response in TB ... 6
2.3.2 The adaptive immune response in TB ... 7
2.4 TB granuloma: The hallmark of TB disease ... 9
2.4.1 Cell types in the TB granuloma ... 10
2.4.2 Immune mediators in TB ... 16
2.5 TB Disease: Clinical features, diagnosis and treatment ... 22
2.5.1 Common aspects of active TB disease ... 22
2.5.2 Clinical forms of TB ... 22
2.5.3 TB diagnosis ... 26
2.5.4 Treatment of TB ... 29
2.5.5 TB vaccine ... 30
2.6 The future: New drugs and biomarkes for TB ... 31
3 Aims, research design and methods ... 33
3.1 Aims ... 33
3.2 Patients and clinical samples ... 33
3.2.1 Study site ... 33
3.2.2 Study cohorts ... 34
3.2.3 Clinical samples ... 35
3.3 Experimental methods ... 35
3.3.1 Immunohistochemistry (IHC) ... 36
3.3.2 Multiplex luminex assay ... 37
3.3.3 Enzyme-linked immunosorbent assay (ELISA) ... 37
3.3.4 Flow cytometry (FACS) ... 37
3.3.5 Quantitative real-time PCR (qPCR) ... 38
3.3.6 Immunodiagnosis of TB ... 38
3.4 Statistical analyses ... 39
3.5 Ethical considerations ... 40
4 Results and Discussion ... 41
4.1 Clinical and demographic data of the study cohort ... 41
4.1.1 Primarily young individuals are infected with active TB ... 41
4.1.2 TB/HIV co-infection is common in the adult study cohort 41
4.1.3 Low level of BCG vaccination among adult TB patients ... 42
4.2 Novel immunodiagnostic assays of active TB ... 42
4.2.1 Challenges of conventional methods for TB diagnosis ... 42
4.2.2 B cell-based immunodiagnosis ... 43

4.2.3 T cell-based immunodiagnosis 45
4.3 Host immune responses in Mtb infection 47
4.3.1 Altered tissue architecture and cellular composition in active TB 47
4.3.2 Compartmentalization of immune responses in active TB . 50
4.3.3 Polarization of immune responses in active TB 53
5 Concluding remarks 58
6 Acknowledgements 59
7 References 61

LIST OF ABBREVIATIONS

TB	tuberculosis
Mtb	*Mycobacterium tuberculosis*
HIV	Human immunodeficiency virus
BCG	Bacillus Calmette Guerin
AFB	acid fast bacilli
PTB	pulmonary TB
EPTB	extrapulmonary TB
LNTB	lymph node TB
LTBI	latent TB infection
NAATs	nucleic acid amplification tests
ALS	Antibodies in lymphocyte secretion
TST	Tuberculin skin test
IGRA	IFN-gamma release assay
QFTG	Quantiferon-TB Gold In-Tube
WHO	World health organization
PBMC	peripheral blood mononuclear cells
TU	tuberculin unit
mRNA	messenger RNA
qPCR	quantitative real-time PCR
SOCS	suppressors of cytokine signaling
IgG	immunoglobulin G
IFNγ	interferon gamma
PUR	paradoxical upgrading reactions
BAL	bronchoalveolar lavage
LAM	lipoarabinomannan
PRRs	pattern recognition receptors
PAMPs	pathogen-associated molecular patterns
MOI	multiplicity of infection
ADE	antibody-dependent enhancement
iNOS	inducible nitric oxide synthase
NO	nitric oxide
TGF-β	transforming growth factor- β
IL	interleukin
TNF	tumor necrotic factor
FoxP3	fork head box P3
CTLA4	cytotoxic T lymphocyte antigen 4
GITR	glucocorticoid-induced TNFR family related gene
TLR	toll like receptors
MDR	multidrug resistant
XDR	extensively drug resistant
RD1	region of difference 1
Tregs	T regulatory cells
MGC	multinucleated giant cell
APC	antigen presenting cells

Th1	T helper 1
Th2	T helper 2
γ/δ T cells	gamma delta T cells
MAIT cells	mucosa-associated invariant T cells
NK cells	natural killer cells
DC	dendritic cell
ESAT-6	early secreted antigen target 6
MMP-1	matrix metalloproteinase-1
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
Arg-1	Arginase type 1
ART	anti-retroviral therapy
MOTT	mycobacteria other than tuberculosis
CTLs	cytolytic T lymphocytes
MHC	major histocompatibility complex
IRIS	immune reconstitution inflammatory syndrome
HAART	Highly active anti-retroviral therapy

1 INTRODUCTION TO THE THESIS

Tuberculosis (TB) is a serious global health problem and after HIV, TB is ranked as the second leading cause of death from infectious disease worldwide. The global burden of TB is enormous with about 9 million new cases in 2011, accounting for 1.4 million deaths of which 430000 were HIV-associated TB deaths [1]. In parallel with the problems related to TB/HIV co-infection, the emergence of multidrug resistant (MDR) and extensively drug resistant (XDR) TB has increased the need to address the clinical problems with TB more effectively. About 80% of all TB cases are found in developing or low-income countries primarily in Africa, Asia and Eastern-Europe, where the resources to successfully diagnose and treat TB is limited. Importantly, diagnosis of active TB is complicated, particularly among immunosuppressed TB/HIV co-infected patients. A delay in TB diagnosis and proper treatment will lead to a continuous spread of TB.

Mycobacterium tuberculosis (Mtb) was discovered by Robert Koch in 1882 as the bacteria that causes TB. A heat-killed extract generated from the tubercle bacilli was later developed as the first diagnostic method, the Tuberculin Skin Test (TST), by Charles Mantoux in 1907 [2]. Even though the specificity of the TST to detect active TB disease is low, this method is still extensively used in clinical routine practice due to the lack of better diagnostic tests. The only existing TB vaccine is the Bacillus Calmette Guerin (BCG), which was introduced in 1921. Unfortunately, the protective efficacy of BCG is highly variable (0-80%) [3] and thus new and better vaccine regimens are required. Despite a lot of recent advances in TB immunology research, the host response required for protective in human TB has not been clearly defined. Therefore the search for specific biomarkers or immune signatures of active TB will continue in order to develop better diagnostic tests as well as novel concepts for vaccines and drugs that could prevent transmission of TB.

In the Background section of this thesis, I will present the Mtb bacteria, the host immune response in TB, and the clinical characteristics of TB as well as available diagnostic methods to detect active TB disease. Thereafter, I will present the specific aims, study design and methods used in this thesis work, followed by a presentation and discussion of the results of the thesis.

2 BACKGROUND

2.1 TB INFECTION: A LONG JOURNEY

2.1.1 History of TB

TB has existed together with humans for a long time. Skeletons carrying lesions apparently caused by spinal TB have been identified from Stone Age skeletons and Egyptian mummies that are around 5,000 years old [4]. Archaeological evidence of TB scars on the lungs of a mummified body comes from early Han dynasty (206BC-7AD). These diseases were given different names and were thought of as separate diseases. The term pulmonary consumption was used to label pulmonary TB, scrofula described TB of the lymph nodes and Lupus vulgaris was the term used to describe TB of the skin [5]. The idea of a single cause for the different forms of TB was advanced by Laennec at the start of the 19th century. Although it was not until the experimental work performed by Villermin in 1865 and by Koch in 1882 that the present concept of TB as a single disease caused by the tubercle bacillus emerged.

2.1.2 Epidemiology of TB

TB results in an estimated 1.7 million deaths each year and the worldwide number of new cases is all-time high [6]. The World Health Organization (WHO) reported 8.8 million new TB cases in 2010, with about 25% occurring among people living with HIV [7]. In addition, 1/3 of the world population is estimated to be carriers of latent TB infection [8]. Although the reliability of epidemiological assessments has progressively improved, only around 30% of the people suffering from TB receive a confirmed diagnosis of active TB [1].

TB is a disease of poverty that thrives where social and economic determinants of ill health prevail and it affects mostly young adults in their most productive years [1]. Thus, more than 80% of all TB cases in the world are found in 22 low- and middle-income countries; thirteen in Africa and nine in Asia. Due to the devastating effect of HIV on the susceptibility to TB, sub-Saharan Africa has been disproportionately affected and accounts for around 80% of all TB/HIV co-infected cases [6]. In endemic areas, an estimated 15-20% of TB cases are seen in children [7]. According to the WHO, rates of MDR-TB are the highest in Eastern Europe, where around 20 % of all new TB cases are MDR-TB. In some parts of the former Soviet Union, up to 25-30% of new TB cases are MDR-TB.

Ethiopia is ranked as number 7 out of the 22 high-burden countries in the world, with a TB prevalence of 237 per 100,000 populations. In 2011, only 47% of all new pulmonary TB (PTB) cases were sputum-smear positive for Mtb, while most extrapulmonary (EPTB) forms of TB were diagnosed using invasive techniques or clinical diagnosis only, which stress the need for new diagnostic methods. The HIV prevalence in incident TB cases is 17%, while 1,6% of all new TB cases have MDR-TB [1]. Thus, in Ethiopia, TB/HIV co-infection is a larger public health problem than MDR-TB.

2.1.3 Transmission of TB in humans

TB is an airborne disease [9] and Mtb is transmitted through aerosol droplets that remain suspended in the air for prolonged periods of time. Thus, Mtb usually enters the human host via the airways, which will result in one of the following outcomes (Figure 1): a) the host immune response clears the infection, but this is probably very rare, b) the host immune response controls the infection in a latent state, but is not sufficient to eradicate TB infection, c) the host immune response cannot control bacterial replication, which results in progression of active TB disease [10, 11].

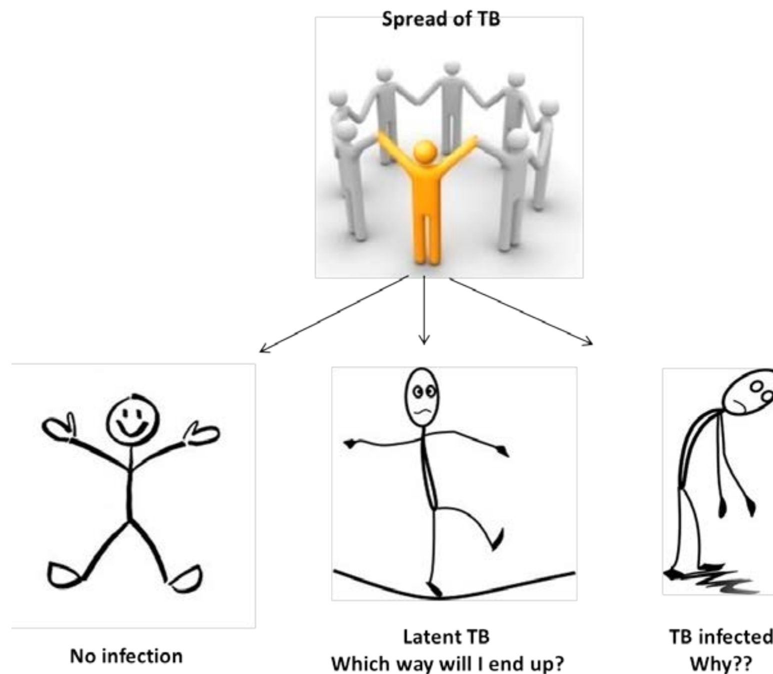

Figure 1. No infection, latent TB infection or active TB disease. Which factors will decide?

The most common form of active disease is PTB, although the EPTB forms can be established in any organ in the body (Figure 2). The infectiousness of a person with progressive TB disease is a multifactorial event and depends on the clinical form of TB (only an open PTB is contagious), the bacterial load in the lungs, the area in which exposure occurs, the time of exposure and the susceptibility of the host [12]. A person with latent TB does not have any clinical symptoms and is not contagious, even though the bacteria persist in a dormant state in the infected organ [12]. Individuals with latent TB are estimated to have a lifetime risk of 5-10% to ever develop active TB disease. Clinical signs and symptoms of active TB will generally develop within 2 years after initial Mtb exposure [13]. Mtb can reside in human tissues for decades without active replication, but the bacteria maintain the ability to resume growth that could break latency and cause active TB. Thus, Mtb can exist in equilibrium with the host immune response for time periods that outcompete almost any other infectious disease [14]. Latent TB represents a significant obstacle to the control and eradication of TB because the non-replicating bacteria may be in a state of “drug indifference”, meaning the bugs cannot be effectively killed by anti-TB drugs [15].

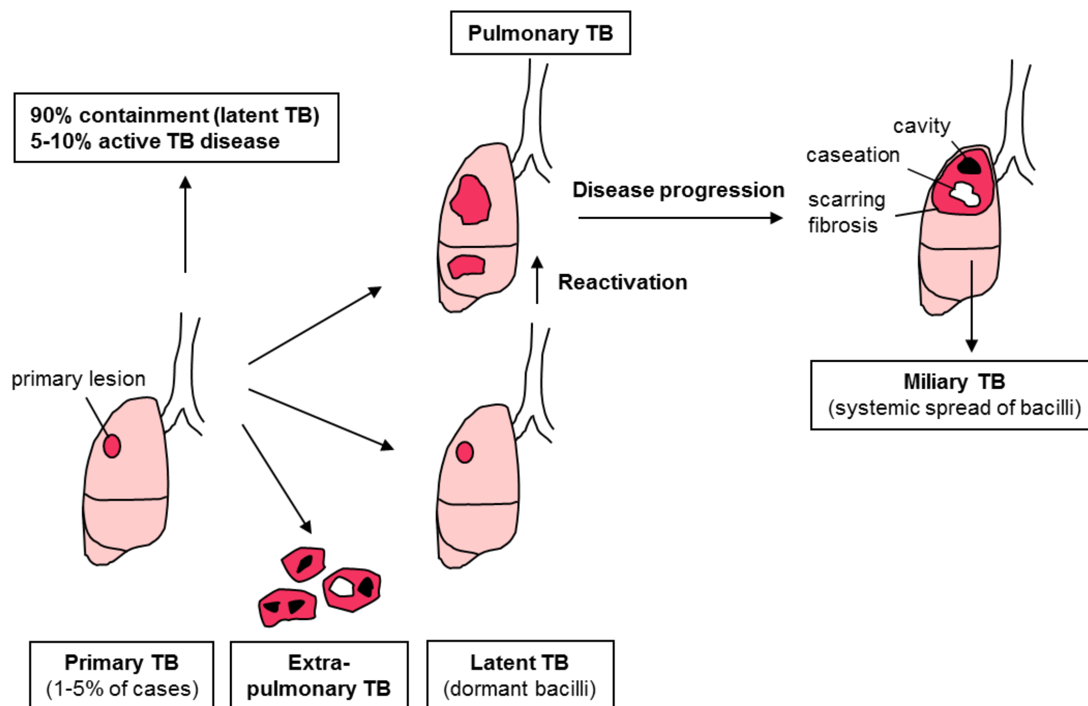

Figure 2. Clinical outcome of TB infection and disease. Mtb is an airborne pathogen that transmits via contagious aerosols. The primary site of infection is the lung, where a primary lesion is formed upon initial Mtb infection. 5-10% of Mtb infected individuals will develop active disease (pulmonary or extrapulmonary TB disease) while the remaining individuals will contain TB infection in a latent state. Reactivation of latent TB occurs if the immune system fails to control bacterial growth. If left untreated, primary TB will progress to more severe form of disease including systemic or miliary TB disease, which involves severe inflammation and tissue destruction in the lung that could also cause cavity formation.

2.2 MTB: A COMPLEX BACTERIA

2.2.1 The Genus Mycobacterium

Mycobacteria are rod-shaped, facultative intracellular bacteria with a size of 2-4µm. There are over 100 mycobacterial species that are wide-spread in nature and the majority of these are non-pathogenic. The *Mycobacterium tuberculosis* complex consists of closely related species that cause disease in both humans and animals. The mycobacteria grouped in this complex differ widely in terms of their host tropisms and pathogenicity. Some are human pathogens (Mtb, *M. africanum*, *M. canettii*), whereas others such as *M. bovis*, have a wide spectrum of hosts [16]. While Mtb is the most pathogenic species in humans, the only existing vaccine against TB, the BCG vaccine, was generated from a live attenuated strain of *M. bovis, M. bovis* BCG [17]. Mtb is also able to infect animals that have contact with humans, although experimental animals such as mice are not natural hosts of Mtb. Members of the genus Mycobacterium have a very complex cell wall envelope, which is responsible for the low permeability that is used as the characteristic differential feature in the staining procedure known as Ziehl-Neelsen acid-fast stain [17]. Microscopic analysis of acid-fast stained clinical specimen, primarily sputum-smear samples, is the most common laboratory test used for TB diagnosis. Mtb is a slow-growing bacteria that replicates with a doubling time of 12 to 24 hours [18], resulting in lengthy cultures of clinical specimens (4-8 weeks) that often cause delays in diagnosis.

2.2.2 Virulence of Mtb

Once Mtb enters the respiratory tract, the bacteria are engulfed by alveolar macrophages [19]. Mtb has evolved strategies to survive inside macrophages even in the presence of inflammation. This involves a number of different virulence factors that have developed in the bacteria in response to the host immune response [17], and by which the bacteria can inhibit immune cell functions [20]. Inhibition of phagolysosomal fusion in Mtb-infected macrophages is a key mechanism used by Mtb to avoid intracellular destruction via the action of lysosomal hydrolases and an acidic environment [19]. It has also been shown that the amount of bacteria plays a role in the ability of the macrophage to control Mtb infection. Human macrophages were able to control infection with Mtb at a low multiplicity of infection (MOI) of 1, but not at a high MOI of 10 [21]. Accordingly, the acidification of mycobacterial phagosomes was also more efficient at MOI 1 compared to 10, which highlights the role of phagosomal acidification in the restriction of Mtb growth [21]. If the pressure from the immune system is too strong, Mtb bacilli stop their active replication and enter a dormant phase. Then the bacilli reside in the protective milieu of the phagosome in a state of non-replicating persistence [22]. Dormant bacilli maintain their ability to resume growth when conditions become favorable.

The thick cell wall of Mtb contains mycolic acids that provide a special lipid barrier responsible for many of the physiological and disease-inducing aspects of Mtb [23]. Common anti-TB drugs such as isoniazid and ethionamide work by inhibiting synthesis of mycolic acid [24]. Another well-characterized virulence factor of Mtb is lipoarabinomannan (LAM), which is an abundant cell wall glycolipid [25]. Mtb has mannose-capped LAM (ManLAM), whereas LAM of less pathogenic mycobacteria lacks the Man-cap [26]. Thus, the type of LAM-capping is crucial for virulence [27]. There are multiple effects of Man-LAM on the host cell, but the interference of Man-LAM with phagosomal maturation in macrophages is the best characterized [28].

Comparisons between *M. bovis* BCG and virulent Mtb have resulted in the identification of several genetic regions of difference [29-31]. Only one of these, region of difference 1 (RD1), is absent from every strain of BCG but present in every virulent Mtb strain tested [29]. Early secreted antigen target 6 (ESAT-6) is a protein encoded by RD1 and is a key component of the type VII secretion system, Esx-1, that has been shown to be a major virulence factor of Mtb [32-35]. Interestingly, virulent Mtb expressing Esx-1 can translocate from the phagosome and replicate inside the macrophage cytoplasm, causing significant cell death within a week [36]. As virulent Mtb reaches a threshold number of bacilli inside the human macrophage, ESAT-6-dependent necrosis occurs [37]. Esx-1 may also be involved in the impairment of autophagy, a physiological process known to inhibit intracellular survival and spread of mycobacteria [38]. Thus, the proteins expressed by ESX-1, are responsible for the phagolysosomal escape seen in macrophages infected with virulent Mtb strains [36]. Furthermore, peptides derived from RD1-encoded proteins including ESAT-6, CFP-10 and TB10.4, have been explored in TB diagnosis [39, 40] as BCG vaccinated individuals or patients with mycobacterial infections other than TB do not respond to these Mtb-specific peptides [41].

Despite the discovery of a number of mechanisms used by Mtb to survive within the host to avoid immune defense mechanisms, the complex nature of TB infection and the

interaction of Mtb with the host, still remains poorly understood [42]. Due to the emergence of Mtb strains which are resistant to almost all available drugs, there is an urgent need to understand the molecular basis of this pathogen's success in causing disease in humans.

2.3 TB IMMUNITY: INDUCTION OF CELLULAR IMMUNE RESPONSES

Mtb is a persistent pathogen that is not easily cleared by the immune system and therefore a chronic disease develops. Control of TB infection depends on the development of efficient innate and adaptive immune responses at the site of infection. However, bacterial control requires an immunological balance and too little as well as too much inflammation can be harmful to the human host [43]. Many of the immune system´s cells and molecules that act during Mtb infection have multiple and redundant roles in a complex network of immune reactions. In active TB, Mtb is able to manipulate certain arms of the immune response to provide a suitable environment for growth while promoting the immune-mediated damage required for transmission of disease [44].

2.3.1 The innate immune response in TB

The ability of the innate immune system to rapidly recognize and respond to invading microbes is essential for controlling infections. This is accomplished by the expression of pattern recognition receptors (PRRs) on macrophages and other leukocytes, which recognize and respond to microbial components [45, 46]. The PRRs recognize a diverse set of molecules generally classified as pathogen-associated molecular patterns (PAMPs) [47]. A particular set of PRRs, the mammalian Toll-like receptors (TLR), play an essential role in innate immunity by recognition of conserved PAMPs that initiate the activation of NF-κB and other intracellular signaling pathways through the adapter protein, MyD88 [45]. This allows the TLRs to detect the presence of infection and to induce activation of inflammatory and antimicrobial innate immune responses.

Mtb primarily infects macrophages and modulate the cell´s bactericidal mechanisms to create a suitable niche for bacterial survival and effective replication [48]. Innate resistance to Mtb is a key factor in disease outcome as only a small percentage of individuals exposed to this pathogen become actively infected [49]. The innate immune system responds to Mtb infection by producing pro-inflammatory cytokines leading to intercellular communication and cell recruitment to the site of infection. Mtb conserved PAMPs trigger the activation of diverse PRRs and innate mediators involved in the phagocytosis of mycobacteria and induction of signaling pathways that trigger production of inflammatory cytokines such as interleukin-12 (IL-12), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) [50, 51]. Mtb enter host macrophages via binding to multiple PRR receptors types that could be used simultaneously [52]. These receptors belong to four main classes: opsonizing receptors (e.g. FcγR and complement receptors), scavenger receptors (e.g. CD36 and MARCO), C-type lectin receptors (e.g. mannose receptor, dectin-1 and 2, and DC-SIGN) and innate immune sensors (e.g. TLRs and NODs) [27, 53, 54].

TLRs are essential for recognition of a broad repertoire of microbes by antigen-presenting cells (APCs) such as macrophages and dendritic cells (DCs). Here, TLR2, TLR4 and TLR9 play an important role in the innate responses against Mtb [55-57].

Accordingly, polymorphisms in TLR2 and TLR9 have been associated with increased susceptibility to TB in humans, confirming the importance of TLRs in the human host defense against mycobacteria [58]. The mycobacterial cell wall consists of a complex array of proteins and lipids, many of which have been shown to have immune modulatory activity [26]. These cell wall components have been shown to be released from infected macrophages as exosomes [59, 60] that stimulate a pro-inflammatory response in a TLR dependent manner [60]. Importantly, Mtb has also developed strategies to interfere with TLR activation and the induction of inflammation.

Upon TB infection in the lung, initial activation of cells of the innate immune defense involves classically phagocytic cells such as resident alveolar macrophages, pulmonary DCs, monocytes and neutrophils [61]. Moreover, Mtb can also bind and interact with non-specialized phagocytic cells such as alveolar epithelial cells [62]. Inhaled mycobacteria are engulfed by alveolar macrophages that will become activated at the site of infection in the lung. Activated macrophages will produce reactive nitrogen intermediates (RNI) [63] as well as antimicrobial peptides that will comprise the first line of defense to limit intracellular bacterial replication [64]. Neutrophils are also acknowledged to confer protection by phagocytosis and killing of Mtb bacilli [65].

Death of the host macrophage is necessary for the bacilli to escape and infect new cells. It has been found that different modes of host cell death play different roles during microbial infections in terms of host defense and microbal survival [66, 67]. Apoptosis is a programmed cell death that is dependent on the induction of caspases, while necrosis is a passive form of cell death that is induced by different types of stress, inflammation or microbial infection [66, 68]. Here, Mtb has developed mechanisms to limit macrophage apoptosis [69, 70] and instead promote host cell necrosis, which can prevent cross-presentation of Mtb-antigens by DCs that could impede and delay T cell priming [71]. Accordingly, infection of macrophages with virulent Mtb above a certain MOI quickly reduces viability of the host cells, in a manner similar to necrosis rather than apoptosis [72, 73].

2.3.2 The adaptive immune response in TB

Successful control of Mtb infection is dependent on the induction of cell-mediated immunity, which involves close interactions between innate and adaptive immune responses (Figure 3). Recognition of Mtb products by TLRs and other PRRs expressed on DCs triggers functional maturation of DCs and leads to initiation of antigen-specific adaptive immune responses [46]. Here, DCs that take up Mtb-infected cells or bacterial products in the lung are capable of migrating to the draining lymph nodes to initiate cross-presentation and activation of specific T cell responses [74]. After arrival of Mtb bacilli in the lung, it takes 7-9 days for the bacteria to be delivered to the draining lymph nodes and an additional 6-10 days for antigen-specific T cells to become activated and migrate to the primary site of infection [75]. A slow induction of the adaptive immune response allows Mtb infection to become well-established before effective bacterial elimination can occur [76]. Ineffective initiation of T cell responses may also be a product of the slow growth of the Mtb bacteria and of the relatively immune-privileged nature of the alveolar tissue [76]. Moreover, early induction of regulatory T cells (Tregs) with suppressive functions may be capable of delaying the priming of effector $CD4^+$ and $CD8^+$ T cells in the draining lymph nodes [77].

It has been shown that the development of effector T cell responses requires the dissemination of live bacteria or bacterial antigens presented by DCs [74, 78]. The function of $CD4^+$ and $CD8^+$ T cells in TB is to activate phagocyte killing of bacteria or to induce direct killing of Mtb-infected cells via expression of cytolytic effector molecules, but also to regulate the inflammatory environment to limit tissue damage [79, 80]. The major effector mechanism of cell-mediated immunity is the activation of infected macrophages by interferon-γ (IFN-γ) produced by $CD4^+$ T cells [81]. The production of IFN-γ is regulated by IL-12, which is released by activated DCs as well as macrophages. IFN-γ cooperates with TNF-α to activate microbicidal mechanisms in macrophages that are responsible for the control and elimination of intracellular Mtb [82]. Moreover, cellular immunity involves killing of Mtb-infected macrophages by $CD8^+$ cytolytic T cells (CTLs) expressing granule-associated effector molecules [82]. Induction of an effective adaptive immune response typically leads to Mtb containment inside highly organized granulomatous lesions.

The importance of $CD4^+$ T cells as the primary mediators of anti-TB immunity in humans was demonstrated by the observation that virus-mediated loss of $CD4^+$ T cells in HIV-infected patients increased the susceptibility to TB [83]. Moreover, inherited deficiencies in the IFN-γ-receptor have been identified in humans that result in lack of IFN-γ signaling, which leads to a severely enhanced susceptibility to infection with mycobacteria of low-grade virulence [82].

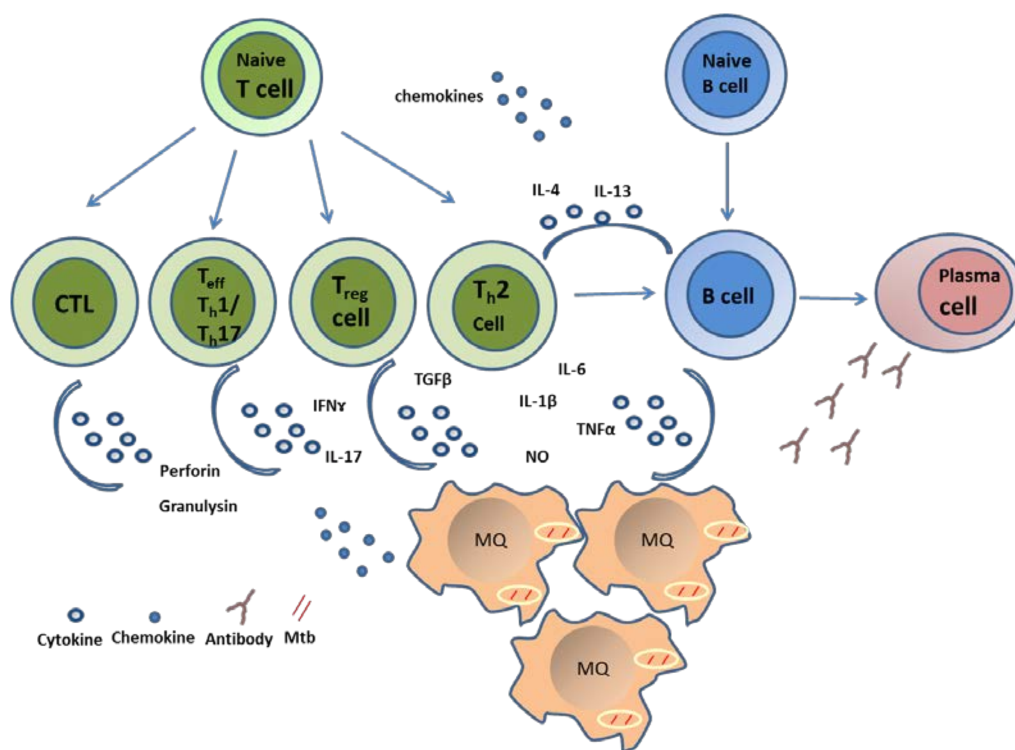

Figure 3. Cellular immune responses in human TB. Mtb bacilli (red rods) primarily persist in the phagosomal system of infected macrophages. Activated macrophages try to combat the infection through the production of antimicrobial compounds but also through activation of Mtb-specific T cells. APCs such as macrophages and DCs take up the bacteria and present peptide and/or lipid antigens to $CD8^+$ and $CD4^+$ T cells as well as non-conventional T cells. While $CD8^+$ T cells differentiate into CTLs, $CD4^+$ T cells differentiate into cytokine-producing Th1, Th17 (T effectors), Th2 cells or immunosuppressive Treg cells. Fine-tuned interactions between pro- and anti-inflammatory responses determine the outcome of TB infection.

2.4 TB GRANULOMA: THE HALLMARK OF TB DISEASE

A consequence of chronic inflammation and a typical hallmark of TB disease is formation of granulomas at the site of Mtb infection (Figure 4). Early granuloma formation is dictated by Mtb-infected macrophages that cluster together to form the core of the granuloma. Continuous activation of macrophages induces the cells to adhere closely together, assuming an epithelioid shape and sometimes fusing to form multinucleated giant cells (MGC) that are also called Langhans giant cells [84]. Apart from the macrophage populations, the granuloma mostly contains other immune cells such as different T cell subsets, B cells, neutrophils and DCs surrounded by an outer sheet of fibroblasts [84, 85]. In human TB, the immune reactivity present in the infected tissue will ultimately result in extensive cell death, detected as characteristic caseous necrosis in the center of the granuloma. Caseous granulomas are typical for human TB, but also other types of granulomas can be seen including non-necrotic granulomas, necrotic granulomas with neutrophils as well as fibrotic granulomas [86, 87]. Typically, Mtb bacilli are released from dying cells into the caseous necrotic material of the granuloma where the bacteria can thrive and persist in an extracellular phase [87]. When liquefied granulomas burst into the bronchial system, extracellular Mtb bacilli can spread via aerosols expelled from the respiratory tract.

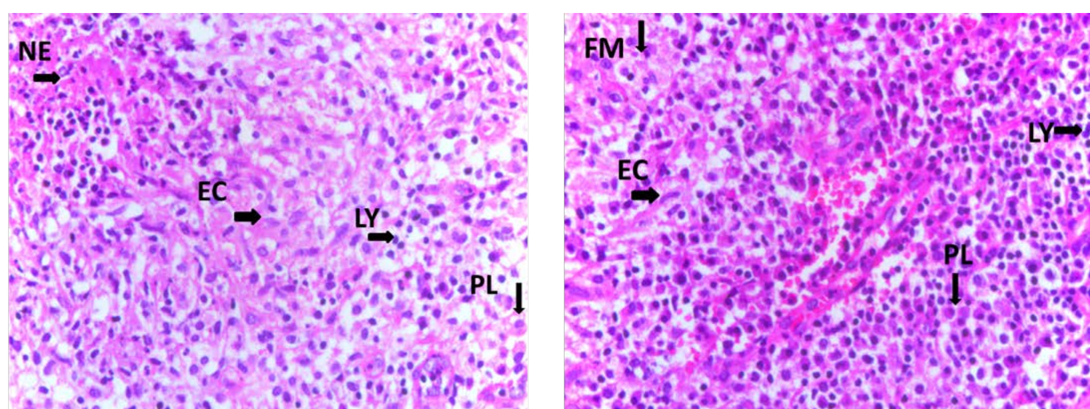

Figure 4. Epithelioid TB granuloma in a human lymph node. EC=epithelioid cells; LY=lymphocytes; PL=plasma cell; FM=foamy macrophages; NE=necrosis.

One of the most important factors required for control of TB infection is a balance between the pro-inflammatory and anti-inflammatory cytokines that are produced to reduce bacterial proliferation but also to reduce tissue damage. Here, TNF-α and IFN-γ have been shown to be particularly important to promote the formation and function of the granuloma [88]. While IFN-γ has an essential role in activation of macrophages and T cells, TNF-α has a major role in the recruitment of different immune cell subsets to the granuloma [89]. The critical role of TNF-α in granuloma formation is demonstrated by the findings that the expression of chemokines is deregulated in TNF-α-deficient mice, which result in impaired recruitment of macrophages and T cells into the granuloma [89]. Although the granuloma provides an immunological microenvironment in which the infection can be controlled, some reports suggest that early granuloma formation may also function as a protective shelter for bacterial replication that can support bacterial dissemination [90, 91].

2.4.1 Cell types in the TB granuloma

Among the numerous cell types and inflammatory mediators involved in TB, the macrophage is the key host cell involved in granuloma formation and control of intracellular Mtb replication [86]. Mtb-infected macrophages recruit uninfected macrophages and other immune cells of both myeloid and lymphoid origin to ultimately form a productive granuloma [92-94].

2.4.1.1 Cells of myeloid origin in TB

Macrophages: After initial infection of alveolar **macrophages** in the lung, **monocytes** migrate to the site of Mtb infection from the blood. Monocytes can differentiate into tissue macrophages that participate in the immune response against Mtb. Most of the macrophages involved in granuloma formation are the **epithelioid cells**, which are activated macrophages with abundant cytoplasm [88]. Recently, M1 and M2 polarization of macrophages have been used to describe the two major and opposing activities of macrophages [95]. The M1 subset is classically activated macrophages with powerful bactericidal properties that are induced by Th1 cytokines (i.e. IFN-γ). M1 express inducible nitric oxide synthase (iNOS), which is an enzyme that catalyzes the synthesis of the potent antimicrobial compound nitric oxide (NO). Thus, the production of iNOS is strongly induced by IFN-γ. Conversely, the M2 subset is alternatively activated macrophages with anti-inflammatory properties that are induced by Th2 cytokines (i.e. IL-4, IL-13). M2 express Arginase type 1 (Arg-1), which is an enzyme that competes with iNOS for the use of arginine as a substrate [96]. Importantly, while iNOS use arginine as a substrate to generate toxic NO, Arg-1 use arginine as a substrate to generate ornithine. Ornithine promotes collagen deposition and contributes to the formation of epithelioid granulomas and tissue fibrosis. Thus, M2 activation allows the bacteria to survive in infected macrophages partly by preventing NO synthesis. M2 also produce anti-inflammatory cytokines including IL-10 and transforming growth factor-β (TGF-β) that could further suppress a bactericidal state of the macrophage. TLR signaling triggered by mycobacteria could induce the production of Arg-1 via IL-10, IL-6 and granulocyte colony stimulating factor (G-CSF) and inhibition of Arg-1 expression has been shown to be beneficial for host survival [97, 98]. The ratio between M1 and M2 macrophages in the granuloma are probably an important determinant for the outcome of TB infection, although the presence of both subsets may be required to maintain a balance between pro- and anti-inflammatory responses in the local environment.

Multinucleated giant cells (MGCs): Chronically activated macrophages may also fuse to generate MGCs, which are characteristic of human TB (Figure 5). The cell-cell fusion process could be triggered by mycobacterial lipomannan in a TLR2-dependent manner [99] or by IL-4 in a STAT6-dependent manner [100]. MGCs may displace the normal tissue and release high concentrations of lytic enzymes, which destroy the surrounding tissue. The functional significance of MGCs during granulomatous inflammation remains to be determined. MGCs may lose their ability to mediate bacterial uptake due to decreased expression of phagocytic receptors such as the mannose receptor and CD11b [101] . However, MGCs seem to have retained the ability to present antigens and may therefore have a function in T cell priming [101].

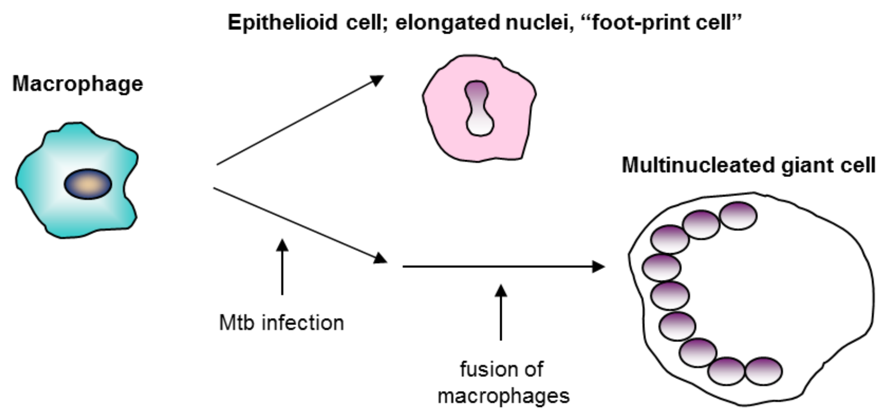

Figure 5. Modified macrophages in TB. Continuous activation of macrophages induces the cells to adhere closely together, assuming an epithelioid shape and sometimes fusing to form multinucleated giant cells (MGC) that are called Langhans giant cells.

Dendritic cells (DCs): DCs do not become productively infected with Mtb, but keep the bacteria in a non-replicating state [102]. Moreover, DCs are not very effective at killing Mtb [103] but these cells are more important in presentation of Mtb-antigens and triggering of specific T cell responses. Thus, DCs containing live mycobacteria could stimulate T cells but may also be used by pathogens as a vehicle for more efficient spreading. DCs present peptide and lipid antigens to T cells in the context of MHCII and CD1molecules, respectively. However, Mtb-induced DC maturation may lead to an uncoordinated presentation of primarily peptide antigens that may affect subsequent T cell activation [104]. There is also evidence that Mtb could inhibit MHCII antigen presentation by DCs without reducing surface expression of MHCII [105]. It is well-known that DCs have an important function in cross-presentation of Mtb-antigens as DCs can engulf apoptotic vesicles from Mtb-infected cells to prime T cells [106]. Importantly, IL-12 production by DCs is pivotal in the induction of IFN-γ and a Th1-mediated immune response that is required for immune protection in TB [107]. Mtb infection may fail to induce effective production of IL-12 by DCs, which could further subvert the induction of a proper Th1 response [103]. Even if DCs are present in chronic granulomatous lesions in the lungs, these cells have down-regulated MHCII and co-stimulatory molecules and consequently DCs isolated from the granulomas fail to induce proper activation of Mtb-specific $CD4^+$ T cells [108, 109].

Neutrophils: Neutrophils have been described as the first line of defense against Mtb. Initial interactions between Mtb and potently bactericidal neutrophils results in rapid apoptosis of the neutrophils that could prevent proper elimination of mycobacteria [110]. These cells can become infected and are directly activated by Mtb products, such as LAM [111]. Neutrophils assist in killing of bacteria, primarily via production of reactive oxygen species, although virulent Mtb has evolved mechanisms to escape oxidative killing [112]. Neutrophils also initiate the inflammatory process through the secretion of chemokines, such as CXCL8 in order to recruit leukocytes and also organize early granuloma formation through the secretion of CXCR3-signaling chemokines [111, 113]. Recently, it has also been found that neutrophils exert early protection in the TB granuloma by oxidative killing of mycobacteria phagocytosed from dying Mtb-infected macrophages [114]. Neutrophils can take up mycobacteria and cluster with human DCs to promote DC activation with less IL-10 and better presentation compared to mycobacteria and DCs alone [115].

2.4.1.2 Cells of lymphoid origin in TB

$CD4^+$ T helper 1 (Th1) cells: The essential role of $CD4^+$ T cells in the control of mycobacterial infections has been highlighted in many studies [81, 116, 117]. Particularly, Th1 cells have a key function in protection against intracellular pathogens, including mycobacteria [117]. Th1 cells typically produce IFN-γ and TNF-α which synergize to activate microbicidal effector mechanisms in human macrophages [118]. In addition, IL-2 is produced that supports the proliferation and clonal expansion of antigen-specific T cells. A Th1 cytokine profile is required for proper activation and differentiation of $CD8^+$ CTLs that participate in killing of Mtb-infected cells [80]. Deficiencies in the Th1 pathway, that occur either as a consequence of HIV infection, cancer, immunosuppressive drugs or genetic defects, greatly increase the susceptibility to mycobacterial diseases [119]. APCs stimulate $CD4^+$ T cells via T cell receptor engagement and co-stimulatory molecules including CD40-CD40L and CD80/CD86-CD28 [120, 121]. Properly activated APCs produce IL-12, which is a crucial cytokine that promotes Th1 polarization including elevated production of IFN-γ. The early $CD4^+$ T cell-dependent burst of IFN-γ production seems to be critical for the effective control of TB infection [81]. In addition, polyfunctional Mtb-specific $CD4^+$ T cells secreting combinations of IFN-γ/TNF-α or IFN-γ/IL-2 have been detected in patients with TB/HIV co-infection [122]. Here, effective anti-TB treatment leads to a shift from IFN-γ single positive $CD4^+$ T cells to predominantly $IFN\text{-}\gamma^+/IL\text{-}2^+$ double-positive T cells [122], which suggests that polyfunctional $CD4^+$ T cells may be involved in immune protection in TB.

$CD4^+$ T helper 2 (Th2) cells: While a Th1 response is required for immune control in TB, active TB disease has been associated with increased activation of Th2 cells in vivo [123]. Th2 cells typically produce IL-4, IL-5 and IL-13, which contribute to initiation of a humoral immune response. However, since Mtb is mostly an intracellular bacterium, antibody-mediated immunity may not provide protective immunity in TB [124] and a pathological role of a Th2 type response involving IL-4 has previously been described [125]. Thus, increasing evidence supports the notion that Th2 polarization could affect disease susceptibility in TB [125-127]. A 100-fold increase of IL-13 and IL-4 has been reported in patients with active TB compared to age- and gender-matched controls [128]. It has also been demonstrated that progressive TB disease might be due to preexisting Th2-like activity that could induce toxicity of TNF-α and/or impair bactericidal functions [129]. Importantly, progressive TB disease may not be solely associated to the absence of a Th1 response, but may rather be caused by co-existing Th1/Th2 or excessive Th2 immunity [123].

$CD4^+$T helper17 (Th17) cells: Th17 cells are pro-inflammatory cells that mediate antimicrobial immunity against bacteria and fungi [130], particularly at mucosal surfaces. Studies have shown that Th17 cells emerge in the early phase of adaptive immunity and contribute to host defense against intracellular pathogens, including Mtb [131]. Th17 cells produce IL-17, IL-21, IL-22 and IL-23 and in addition to the classical Th1 response, a Th17 response is considered to be protective in mycobacterial infection. Th17 cells can trigger the production of chemokines (CXCL9, CXCL10 and CXCL11) that recruit IFN-γ producing CD4+ T cells to the

site of Mtb infection in the lung, which result in restriction of bacterial growth [131]. Th17 cells can also regulate the production of antimicrobial peptides [132] and has been shown to be essential in the formation of mature TB granulomas in the lung [133]. BCG vaccination induce both Th1 and Th17 cells [134] while Th17 cells are reduced in the peripheral circulation of active TB patients [135]. Whether these cells are ultimately necessary for protection or contribute to disease pathology is not clear.

Regulatory T cells (Tregs): Natural Tregs constitute about 5% of peripheral T cells and constitutively express CD25, the transcription factor fork head box P3 (FoxP3) and the T cell inhibitory receptors, Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and the Glucocorticoid-Induced TNFR family related gene (GITR) [136]. Inducible Tregs can also develop from conventional $CD4^+$ T cells that are exposed to immuno-regulatory cytokines or other deactivating signals [137]. The $CD4^+CD25^+FoxP3^+$ Treg compartment has been shown to be expanded in patients with active TB [138] and Tregs also expand in the lung during experimental Mtb infection, which interfere with bacterial control [139]. $CD4^+Foxp3^+$ Tregs are present in the granuloma of humans, non-human primates and mice [139-141]. The immunosuppressive functions of natural and induced Tregs are known to be dependent on negative signals induced by CTLA-4, GITR or Programmed cell Death-1 Ligand (PDL1), but also on soluble factors such as IL-10 and TGF-β [142]. Expansion and accumulation of Tregs has been shown to decrease Th1 cell responses in patients with TB [143]. It has also been shown that the immunosuppressive cytokines that impair immunity to Mtb can be produced by both Tregs and anti-inflammatory M2 macrophages [144, 145].

Excess numbers of Mtb-specific Tregs may restrict and delay T cell effector responses that is detected in TB [146]. This includes delays in priming, expansion and mobilization of both $CD4^+$ and $CD8^+$ effector T cells [77], which allows enhanced Mtb growth. Tregs also counteract Th17 cells, thus inhibiting the early recruitment of IFN-γ-producing $CD4^+$ T cells to the site of infection [131]. Immature DCs, tolerizing DCs and anti-inflammatory macrophages that are targeted by Mtb have all been shown to be capable of inducing Tregs [147, 148]. Although enhanced Treg responses are generally considered detrimental in chronic infections including TB, these cells may also have a function to prevent pathology in persistent infections [86].

$CD8^+$ T cells: Mtb-specific $CD8^+$ T cells have been described in human TB [149] as a subset of T cells that are required to obtain maximum control in TB infection [150]. $CD8^+$ CTLs are specialized to kill Mtb-infected target cells by the release of granules containing cytolytic and bactericidal effector molecules, but can also be activated to produce Th1 cytokines that contribute to immune protection [146]. Thus, there is evidence that $CD8^+$ T cells participate in the control of Mtb infection by lysis of Mtb-infected macrophages combined with killing of intracellular bacteria [151] and also by the expression of effector cytokines [152, 153]. While $CD8^+$ CTLs possess potent anti-mycobacterial activity via granule-mediated cytotoxicity [154], death receptor-ligand induced apoptosis may be less efficient in killing intracellular Mtb [155]. Mtb-specific CTLs have been found in humans [151] and shown to kill Mtb-infected cells using the pore-forming protein perforin in cooperation with the antimicrobial peptide granulysin [156]. Important evidence has been provided that Mtb-reactive $CD8^+$ effector memory T cells expressing high levels of perforin and granulysin, can mediate

antimicrobial activity in human TB [157]. Reduced numbers of these effector T cells were detected in patients with rheumatoid arthritis after treatment with anti-TNF-α inhibitors, which resulted in a significantly enhanced susceptibility of the patients to develop active TB [157].

Polyfunctional Mtb-specific $CD8^+$ T cells have also been described. The peripheral frequency of IFN-γ^+/IL-2^+ double-positive $CD8^+$ T cells has been shown to correlate with host protection and a curative response to anti-TB treatment [149]. In contrast, a regulatory function of $CD8^+$ T cells have been found in the TB granuloma where activated $CD8^+$ T cells produced the chemokine XCL1 (lymphotactin), which negatively regulates IFN-γ production by $CD4^+$ T cells [158]. In addition, analyses of $CD8^+$ T cell repertoires in children with TB have identified clonal expansion of terminally differentiated $CD8^+$ effector T cells in severe forms of disease [159]. Hence, $CD8^+$ T cells may also serve as targets of immune evasion by Mtb.

B cells: Humoral immune responses are vital for protective immunity to many pathogens and thus antibody production by **B cells** is one of the most important effector functions of these cells. However, B cells also carry out a variety of other effector and regulatory functions during the course of an immune response [160]. B cells mediate protection from pathogens via antigen presentation, co-stimulation and antibody secretion, but also by producing cytokines that regulate the quality and magnitude of humoral as well as T cell responses [161]. It is becoming clear that the different effector functions of B cells are tightly controlled and that there are functionally and phenotypically distinct effector and regulatory B cell subsets that can be sub-divided also based on their cytokine profile [160]. The functional relevance of the different cytokine profiles is illustrated by the ability of distinct effector B cell subsets to polarize the development of T cells along the Th1 or Th2 lineage [162, 163], via production of IFN-γ and IL-4 [162]. Lately, it has also become evident that **regulatory B cells** producing IL-10 can maintain $FoxP3^+$ Tregs and limit Th1 and Th17 responses in healthy individuals, but not in patients with an autoimmune rheumatoid arthritis [164]. Contrary, patients with active TB were found to have significantly higher frequencies of regulatory B cells with stronger suppressive activity compared to healthy controls [165]. Interestingly, the frequency of regulatory B cells in peripheral blood was inversely correlated with that of Th17 cells in these TB patients [165].

While a protective immune response in TB infection is mostly based on cellular immunity, the role of B cell mediated immunity in TB remains controversial [166, 167]. B cells have been shown to have a significant impact on the inflammatory response and disease outcome upon aerosol challenge with Mtb in experimental animal models [168]. B cells have also been shown to be present in granulomatous TB lesions in the lung [85, 169, 170], which may indicate that B cells are involved in the organization and development of the TB granuloma [169]. Thus, B cells may potentially use their diverse functions to regulate other immune cells present in the granulomas including macrophages and T cells [86].

Since Mtb is an intracellular bacterium, the role of antibodies in TB remains debatable. However, extracellular Mtb bacilli and bacterial products are released during progressive TB disease, which will result in significant amounts of bacterial

antigens in the local tissue environment that will leak out into the peripheral circulation. This will promote activation and differentiation of **plasma B cells** and some studies have shown a protective function of antibodies in Mtb infection [171]. Several mechanisms have been proposed for this protection including antibody-mediated inhibition of Mtb attachment to human macrophages [172], enhancement of phagolysosomal fusion inside Mtb-infected cells, neutralization of bacterial products and cellular- or complement-mediated bacterial lysis [173]. By virtue of their ability to produce antibodies, B cells are also essential to form immune complexes with potent immunoregulatory functions [174]. Here, ligation and activation of Fcγ receptors (FcγRs) on macrophages by immune complexes can have prominent immunological effects including induction of phagocytosis [175]. On the contrary, the ligation of monocyte or macrophage Fcγ receptors by IgG immune complexes lead to an increased production of IL-10 [175] and promote a shift of Th1 to Th2 responses, which result in an increased infectious output in parasite infected cells [176]. This intrinsic antibody-dependent enhancement (ADE) facilitates microbial entry into host cells that could modulate the severity of infectious diseases [176]. Accordingly, the ligation of FcγRs by antibody-coated Leishmania resulted in increased IL-10 and decreased IL-12 production by macrophages, thus enhancing leishmanial growth in macrophages [176]. Immune complexes are known to exist in Mtb infection [177, 178], but whether ADE is applicable in human TB remains to be determined.

Other T cells subset: Apart from the interaction with conventional lymphocytes of the adaptive immune system, mycobacteria can interact with human innate lymphoid cells and modulate their function. **Gamma-delta T cells (γδ T cells)** are non-conventional T cells that can perform a vast array of immune effector functions and appear to play important roles in antimicrobial immunity as well as in chronic inflammatory reactions [179]. The early source of IL-17 is mostly from γδ T cells, which bridge innate and adaptive immunity and play an essential role in early host defense in TB [180]. The γδ T cell compartment has been shown to expand after mycobacterial infection in humans [181]. This compartment also expands rapidly to recall responses, to Mtb antigens and live infection, in a similar manner to memory cells [181, 182]. Human DCs infected with *M. bovis* BCG activate γδ T cells to kill mycobacteria-infected primary macrophages but not infected DCs by a perforin-mediated mechanism [183].

Other studies have identified non-classical Mtb-reactive T cell subsets that are enriched in the lung mucosa. These T cell subsets recognize antigen presented by non-classical MHC molecules [146]. The best known are **CD1 restricted T cells** that recognize mycobacterial lipids [184], although their role in protective immunity in TB remains incompletely defined. Another recently discovered T cell subset is **mucosa-associated invariant T (MAIT) cells**, which also recognize antigen in the context of the MHC class-Ib molecule MR1 (MHC class I related) expressed on phagocytes and epithelial cells [185]. MAIT cells are enriched in the human lung and recognize epithelial cells infected by a broad spectrum of bacterial species including Mtb [186]. MAIT cells respond rapidly, are cytolytic, secrete multiple cytokines (IFN-γ and IL-4) and appear to be important effector cells in mucosal immunity. [146]. The precise ligands presented by MR1 remain unidentified, but may involve vitamin B metabolites that are produced by bacteria [187].

Natural killer (NK) cells: It is well-known that NK cells are important in the innate defense against pathogens [188]. Previous studies have demonstrated that human NK cells can recognize and lyse Mtb-infected macrophages using the NKp46 receptor [189]. In addition to the role of activated NK cells in granule-mediated killing of infected cells, these cells also produce significant amounts of cytokines. In the presence of IL-15 and IL-23, $CD3^{-}CD56^{+}$ NK cells produced vast amounts of IL-22 when exposed to autologous monocytes and gamma-irradiated Mtb. Addition of recombinant IL-22 to Mtb-infected macrophages enhanced phagolysosomal fusion and reduced growth of Mtb, suggesting that NK cells can contribute to immune defenses against Mtb through production of IL-22 [190].

Natural killer T (NKT) cells: NKT cells are known to play a protective role in the immune responses against a variety of infectious pathogens [191]. The proportion of NKT cell and their proliferative responses were significantly lower in the peripheral blood of PTB and EPTB patients compared to latent TB subjects and healthy controls [191]. A poor NKT cell response in Mtb-infected patients was found to be due to increased NKT cell apoptosis, reduced CD1d expression and an elevated expression of the inhibitory receptor PD-1 on NKT cells [191].

2.4.2 Immune mediators in TB

A network of different immune mediators that are produced by the different cells involved in TB will ultimately determine the outcome of Mtb infection. Both poor and exaggerated inflammatory responses will lead to disease. This fine balance requires bacterial orchestration of both innate and adaptive immunity [87]. The cytokine balance during mycobacterial infection is the key to control bacterial growth and to maintain tissue function [192]. In addition to the role of cytokines in the formation and stability of the granuloma, the presence of chemokines is crucial for the recruitment of inflammatory cells to form granulomas [193].

2.4.2.1 Pro-inflammatory and effector cytokines in TB

TNF-α: TNF-α is produced by a variety of cells including macrophages, lymphocytes, neutrophils, mast cells and endothelial cells. Several important functions have been attributed to TNF-α [194, 195], including control of bacterial replication in the host by activation of antimicrobial effector functions in macrophages and T cells and also by organization of granuloma formation [196, 197]. There is experimental data indicating that TNF-α promotes autophagy but also phagolysosomal maturation in an IFN-γ-dependent manner [198-200]. In addition, TNF-α induces early production of chemokines by macrophages, which initiate recruitment of immune cells to the site of Mtb infection. Accordingly, TNF-α-deficient mice produce reduced levels of chemokines that result in defective granuloma formation [89]. Likewise, blockade of TNF-α during the chronic phase of experimental Mtb infection leads to breakdown of the granuloma and an enhanced pro-inflammatory response, which suggest that TNF-α may also exert an anti-inflammatory effect on certain inflammatory mediators [201].

Intricate regulation of the timing, site and extent of TNF-α production is required in response to mycobacterial infection, as this fine-tuning will have a significant influence on the net effect of bacterial control [87]. Too little TNF-α will fail to induce proper TB immunity and distort granuloma formation while too much TNF-α will exacerbate

tissue damage and compromise lung function. The importance of TNF-α in human TB was confirmed by the finding that TNF-antagonists increased the risk of active TB 4-10-fold among patients with rheumatoid arthritis [202-204]. The TB disease pattern in these patients is characterized by a high rate of extrapulmonary (56-62%) and disseminated TB disease (24-28%) [205]. Previous reports demonstrate that anti-TNF treatment resulted in a decreased frequency of a subpopulation of memory $CD4^+$ T cells [206] and also of $CD8^+$ effector memory T cells [157], which confirm that this treatment has severe effects also on Mtb-specific T cell responses.

IL-1β: IL-1β is produced by monocytes, macrophages and DCs [207]. Expression of this highly pro-inflammatory cytokine is tightly controlled through the combination of two distinct triggers: Signal 1 typically involves activation via a TLR ligand while signal 2 involves the assembly of a protein complex known as the inflammasome that activates caspase-1, which cleaves the proform of IL-1β into the mature form [208, 209]. Infection with both Mtb and *M. bovis* BCG induces IL-1β mRNA in human macrophages but only virulent Mtb can activate type I IFN signaling and selectively limit the production of IL-1β [210]. In addition, decreased caspase-1-dependent IL-1β activation was seen in the BCG-infected macrophages [210]. Mtb can also limit IL-1β expression by inducing Zn^{2+} metalloproteases, which inhibits the inflammasome [211].

Whereas IL-1β is an important mediator of the inflammatory response in TB, high levels of IL-1β may also be exploited by Mtb to cause severe inflammation and tissue destruction. Here, both IL-1β and TNF-α can induce the expression of the protease, matrix metalloproteinase (MMP)-1, as shown in lung fibroblasts in vitro as well as in human TB granulomas [212]. MMP-1 transgenic mice develop more severe alveolar destruction and collagen breakdown during Mtb infection, which suggest that MMP-1 promotes tissue destruction in TB [213]. MMP-1 function can be regulated by specific inhibitors that are down-regulated by Mtb [212, 214]. Accordingly, high levels of MMP-1 and low levels of inhibitors have been found in the bronchoalveolar lavage (BAL) of TB patients [213].

IL-6: IL-6 is secreted by different cell types including activated T cells, macrophages, fibroblasts and endothelial cells [215]. This is a cytokine with a wide variety of functions that can exert pro- or anti- inflammatory effects, but mostly IL-6 is critical for the development of an early inflammatory response [194]. IL-6 is also involved in hematopoiesis [216]. In addition, IL-6 and IL-12 are critical in the development of efficient anti-mycobacterial responses by T cells, as both IL-6 gene-disrupted mice and depletion of IL-6 and IL-12 with neutralizing antibody showed reduced antigen specific proliferation and IFN-γ secretion after vaccination with Mtb culture filtrate [217]. Although IL-6 may support IFN-γ production in the early phase of TB infection, IL-6 does not seem to be essential for the development of protective immunity in TB [218].

IL-12: The production of IL-12 by DCs and macrophages is essential for the development of host Th1 immunity and resistance against intracellular pathogens [219]. This is primarily accomplished by IL-12-mediated induction of IFN-γ production in NK, NKT and T cells. Thus, IL-12 promotes the differentiation of naive $CD4^+$ T cells into Th1 cells that produce IFN-γ and aid cell-mediated immunity [220]. IL-12-deficiency strongly increases the susceptibility to mycobacterial disease in humans,

which underlines the importance of IL-12 to trigger Th1 immunity [221]. IL-12 is rapidly produced by mature DCs after interaction of Mtb with TLRs on the DC. Studies have suggested that induction of IL-12 production is dependent on TLR9 on DCs and TLR2 on macrophages [221]. It has also been found that both human and murine macrophages release significantly lower amounts of IL-12 than DCs in response to Mtb infection, showing that DCs are better IL-12-producers compared to macrophages [222, 223]. Previously, it was shown that the Mtb cell wall component ManLAM were able to inhibit TLR-induced IL-12 production by human DCs [224], which may prevent proper DC maturation and T cell activation.

IFN-γ: IFN-γ, produced by activated T cells, NK and NKT cells, is a key effector cytokine that promotes the activation of phagocytes, cellular proliferation, cell adhesion and apoptosis [225]. The function of IFN-γ in response to pathogens has been extensively studied and it has been shown to be critical in the regulation of cellular immune responses in mycobacterial disease [226]. IFN-γ activates macrophages, and enables them to overcome Mtb-induced arrest of phagosomal maturation and enhance MHCII-restricted antigen presentation [227]. In addition, IFN-γ induces autophagy in infected macrophages enabling them to inhibit intracellular survival of Mtb [228]. In macrophages, IFN-γ also induces the respiratory burst pathway that contributes to the production of RNIs and oxygen intermediates (ROIs) [226]. The production of large amounts of ROIs and NO by innate immune cells is considered to be one of the most important effects of IFN-γ in the early immune defense against TB. $CD4^+$ T cells play a central role in containment of Mtb infection by secreting IFN-γ that will activate macrophages as well as Mtb-specific CTLs [229]. Accordingly, there is an enhanced susceptibility to mycobacterial infection in IFN-γ-knockout mice [230]. IFN-γ-deficient mice also fail to develop granulomas following aerosol infection with Mtb and show significant impairment of macrophage activation [231]. The importance of IFN-γ in human TB has been discovered in patients with severe mycobacterial infections, who had a mutation in the gene for IFN-γ receptor 1 that resulted in reduced macrophage activation [232]. Together, these studies confirm an important role of IFN-γ in response to intracellular pathogens such as mycobacteria.

IL-17: IL-17 is primarily produced by γδ T cells and CD4+ T cells and has a function as a pro-inflammatory cytokine that supports the recruitment of effector cells such as neutrophils and activated T cells to disease sites [233]. Thus, the role of IL-17 in the development of antimicrobial responses, chemokine production and recruitment of inflammatory cells has been described in several studies [133, 234-236]. Particularly the IL-23/IL-17 pathway has been demonstrated to be involved in the development of protective and regulatory immune responses in mice and humans infected with Mtb [131, 237]. Here, IL-23 may not be a determining factor for the early control of Mtb infection but is required for the development and maintenance of Th17 responses [236]. It has been found that IL-17 participates in the activation of macrophages and IL-17-producing $CD4^+$ T cells are present in mycobacterial granulomas [238].

IL-4: The Th2 cytokines, IL-4 and IL-13, are produced by Th2 cells and basophils [239, 240]. Both IL-4 and IL-13 are typically involved in antibody-mediated humoral immunity with limited protective effects in intracellular Mtb infection [125, 241]. IL-4 is a Th2 signature cytokine and is implicated in the activation of anti-inflammatory type

of macrophages (M2) through IL-4R signaling [242]. Both IL-4 and IL-13 have been shown to be detrimental in the control of intracellular Mtb infection, as these cytokines suppress IFN-γ production and IFN-γ-mediated effects [243]. Furthermore, IL-4 can inhibit autophagy, which is known as a physiological mechanism to enhance intracellular degradation of dysfunctional cell components including intracellular microbes as Mtb [126]. IL-4 and IL-13 also induce in vitro expansion of antigen-specific $FoxP3^+$ Treg cells from both human and mouse cells [244]. Moreover, IL-4 has been shown to antagonize host defenses and lead to tissue necrosis in patients with TB [245]. Increased Th2 responses in the lung augment immunopathology by induction of pulmonary fibrosis and cavitation, which compromise lung function in TB patients [125, 245].

2.4.2.2 Chemokines in TB

CC and CXC chemokines: Chemokines produced during mycobacterial infections augment the host's innate resistance while shaping the ultimate adaptive immune response [246]. Chemokines are small (around 8-14 kDa) cytokines that are grouped as CC and CXC chemokines, depending on the amino acid present between the first two out of four cysteine residues [247]. Similar to cytokines, these small signaling molecules play a central role in the orchestration of tissue homeostasis and inflammation and deregulated chemokine production have been implicated in several human infections [248, 249]. Chemokines play an important role in leukocyte recruitment to the site of inflammation [250, 251] and also regulate cell activation, migration and survival [252-254]. The contribution of chemokines in the control of Mtb infection has been described by several in vitro and in vivo studies [255-257]. Chemokines promote migration of monocytes, DCs, activated macrophages, neutrophils and T lymphocytes to bronchoalveolar spaces during PTB [258] and play a significant role in the recruitment of effector T cells to the site of Mtb infection [259].

CC chemokines are a sub-group of chemokines that include 27 members. In addition to their well-characterized roles in the regulation of inflammation and immune homeostasis, some members of this chemokine family such as **CCL2** have been associated with increased susceptibility to PTB [260]. High concentrations of CCL2 have been shown to inhibit production of IL-12 in cultures of Mtb-stimulated monocytes, which may increase the likelihood of the infection to progress to active PTB [261]. **CCL5** is a chemokine produced by a variety of cells including macrophages, fibroblasts, eosinophils, endothelial cells and platelets. CCL5 exerts chemotactic activity on NK cells, T cells, DCs and macrophages to inflamed or infected tissues [262]. The expression and functional activities of CCL5 have been studied in experimental models of type 1 and type 2 granulomas, elicited by *M. bovis* and schistosoma infection, respectively. These results demonstrated higher levels of CCL5 in type 1 lesions [263]. Consequently, the recruitment of APCs and T cells and also the formation of granulomas induced by Mtb, have been shown to be reduced in CCL5 knockout mice [264]. CCL5 is especially important in the early responses to Mtb due to its role in recruitment of IFN-γ-producing T cells to form lymphocyte enriched granulomas at the site of infection [264].

CXC chemokines are a sub-group of chemokines that includes 17 members. *M. bovis* BCG-infected human epithelial cells can play an active role in local inflammatory immune responses via the secretion of CXC chemokines [265]. It has also been shown that neutralization of TNF-α in Mtb-infected macrophages resulted in a reduction of many inflammatory chemokines such as CCL5, **CXCL9** and **CXCL10**, both in vitro and in vivo [266]. **CXCL8** is a proinflammatory chemokine with the ability to recruit neutrophils and T cells in response to a variety of stimuli [267]. It has been shown that IL-4 can selectively reduce the levels of CXCL8, while it did not have a significant effect on CXCL10 levels. This down-regulation of CXCL8 secretion may be important to prevent pathological inflammation in human TB, but may simultaneously reduce imperative cell-mediated immune responses, since CXCL8 is required to induce neutrophil and T cell migration [265]. CXCL9 and CXCL10 are induced by IFN-γ during infection, injury or inflammation, to participate in leucocyte trafficking of mainly activated $CD4^+$ Th1 cells, $CD8^+$ T cells and NK cells [268]. CXCL10 secretion is induced in Mtb-stimulated macrophages [269] and attracts T cells but not neutrophils [270].

2.4.2.3 Cytolytic and antimicrobial effector molecules in TB

Reactive nitrogen species: ROS and RNI are produced by macrophages and neutrophils and effectively kill various bacteria [271]. Of greater importance in TB is RNI and particularly **NO**, which is produced upon activation of iNOS using L-arginine as a substrate [272]. In the mouse model of Mtb infection, it has been confirmed that NO is essential in TB control by killing of Mtb in mononuclear phagocytes [63]. In iNOS-deficient mice, TB infection has a rapid progression and results in a higher rate of mortality compared to wild-type mice [273, 274]. However, it has been much more difficult to confirm a role of NO in humans, partly because human macrophages cannot produce NO upon stimulation or Mtb-infection in vitro [275]. However, both iNOS and the NO-metabolite nitrotyrosine, have been detected in situ in tissue from human TB granulomas [141], which may suggest that macrophages are activated to produce NO in vivo at the site of Mtb-infection.

Cytolytic effector molecules: As described above, the granule-exocytosis pathway of cytolytic lymphocytes is crucial for immune surveillance and homeostasis in TB as well as other infectious and non-infectious diseases. Cytolytic granules typically contain cytolytic and antimicrobial proteins that execute the function of the CTL. Here, the membrolytic pore-forming protein **perforin** was originally purified from cytolytic granules and identified as a key effector molecule for T cell and NK cell mediated cytolysis [276]. Perforin is released, via the granule-exocytosis pathway, into the immunological synapse of the CTL and the target cell where it forms pores in the target cell membrane to trigger cell lysis [277-279]. In this process, other effector molecules including **granzymes** and **granulysin** may also be released from the lytic granules to enter specific target cells. Granzymes are serine proteases that cleave specific substrates in the cytosol to promote rapid and efficient apoptosis by various pathways [280, 281] Elevated levels of circulating extracellular granzymes A and B have been associated with various inflammatory diseases [282-284].

Perforin plays a major role in CTL function and the regulation of intracellular bacterial infections like TB [285, 286]. Consequently, patients with a deficiency in perforin

show impaired lymphocyte cytotoxicity [287]. Furthermore, perforin-deficient mice also show reduced killing of Mtb-infected target cells, which correlates with diminished protection [288]. Perforin-deficient mice also show defective granzyme-mediated cytotoxicity in both CTLs and NK cells [289, 290].

The antimicrobial peptide granulysin is a human polypeptide that is constitutively expressed in NK cells and induced in CTLs upon activation [291] with activity against a broad range of microbes [292]. The lytic effect of granulysin towards mycobacteria was initially discovered to decrease the viability of intracellular Mtb in cooperation with perforin [156]. Granulysin is a small cationic molecule with the ability to interact with the negatively charged surface of the mycobacteria through ionic strength [156]. The bacterial membranes are disrupted upon leakage of fluids that will end in osmotic lysis of bacterial cells [293]. Granulysin can also lyse human cells through the mitochondrial pathway of apoptosis [294]. Reduced levels of granulysin have been shown to be associated with progression of clinical disease caused by both *M. leprae* [295] and Mtb [296]. The mycobactericidal activity of granulysin constitutes an attractive pathway of protective immunity in humans, which could be targeted by vaccination [297].

2.4.2.4 Negative regulators in TB

Anti-inflammatory cytokines: IL-10 and **TGF-β** are immunoregulatory cytokines produced by macrophages and certain T cell subsets such as Tregs [146]. These cytokines are typically involved in the down-regulation of Th1 responses and IL-10 is a well-known antagonist of IFN-γ [298]. Similar to Th2 cytokines, IL-10 and TGF-β possess antagonistic effects on cellular immunity by inhibition of T cell proliferation, reduced production of IFN-γ and pro-inflammatory cytokines, reduced antigen presentation and reduced activation of bactericidal macrophages [299]. However, IL-10 possesses a stimulatory capacity on Th2 cells and also promotes B cell maturation and antibody production [300]. Likewise, TGF-β is believed to be important for the differentiation of FoxP3+ Treg cells and Th17 cells [301]. Both IL-10 and TGF-b are known to be involved in the pathogenesis of active TB and transient overexpression has previously been observed in TB patients [302]. Together, TGF-β and IL-10 potentiate the down-modulatory effects on Mtb-induced T cell production of IFN-γ, and TGFβ enhances IL-10 production [303]. Mycobacterial components have also been shown to induce TGF-β production in peripheral blood monocytes from TB patients [304]. In addition, TGF-β supports the production and deposition of macrophage collagenases [299] and collagen matrix [305] that may alter tissue morphology and promote tissue fibrosis in Mtb-infected organs.

Suppressors of cytokine signaling (SOCS): SOCS molecules act as feedback inhibitors induced by different stimuli including cytokines and TLR ligands [306]. The SOCS family comprises eight members and at least four of these, namely CISH, SOCS1, SOCS2, and SOCS3, are expressed in T cells [307]. The mycobacterial cell wall possesses a variety of lipopeptides and liposaccharides that act as TLR ligands. TLR engagement by these ligands during bacterial phagocytosis can lead to inhibition of cytokine synthesis [308] and decreased cytokine responsiveness through induction of SOCS [309]. Common cytokine receptors use the Janus kinase (JAK) and signal

transducers and activators of transcription (STAT) signaling pathway [310] that are regulated by several molecules including members of the SOCS family [307].

Some of the SOCS molecules have been found to be raised in patients with active TB, which may indicate an important role of SOCS in the regulation of TB-specific immunity [311]. SOCS-mediated regulation of cytokine production may ultimately affect immune polarization and immune cell function. Mtb expresses different antigens with the ability to inhibit IL-12 secretion and induction of oxidative burst by inducing increased expression of **SOCS1** [312]. SOCS1 could also regulate IFN-γ expression and function [313]. **SOCS3** overexpression in mice has been shown to promote the generation of Th2 cells [314]. It has also been shown that SOCS3 seems to be preferentially expressed in Th2 cells [315] while SOCS3 overexpression inhibits differentiation of Th17 cells [316].

2.5 TB DISEASE: CLINICAL FEATURES, DIAGNOSIS AND TREATMENT

2.5.1 Common aspects of active TB disease

Active TB is the common name for a broad spectrum of different forms of clinical TB disease described below. Even though lung or PTB is the most common form of disease, TB may affect any organ in the body and cause EPTB. Mtb bacilli may also escape from infected foci in the lung and enter the blood stream and cause miliary TB, which may develop into a fatal condition. The greatest risk for development of active disease is between 1-5 years after primary exposure to Mtb. The risk decreases for every year and on average the risk is 10% in a life-time for HIV-negative individuals. The risk of active disease is greatest in infants that are younger than 4 years and slowly declines from age 5-10 years. During adolescence, age 15-19 years, the risk rapidly increases with a second peak between the ages of 20-30 years [317]. Age-related differences in disease risk are accompanied by differences in the response to TB infection and clinical features of disease. In early childhood, disseminated forms of disease such as miliary TB and TB meningitis are common. With increasing age, these features become less common with a sudden shift in disease pathology observed during adolescence. At this stage, the features of TB disease resemble the hallmark of adult disease including severe inflammation, tissue destruction and lung cavitation [317-319]. Both ageing and HIV infection result in markedly reduced function of the immune system as does other types of immunosuppressive conditions or treatments such as poor nutrition, drug abuse, anti-TNF-α inhibitors, cancer, diabetes and smoking [320]. Similar to elderly people, HIV-infected patients tend to develop a more disseminated disease with less lung cavitation. The clinical symptoms of TB can be many but primarily include cough, chest pain, fever, weakness, chills, night sweats and weight loss. The infectiousness of a person with TB is a multifactorial event depending on the clinical form of TB, the bacterial load in the lungs, the environment in which the exposure occurs, duration of exposure, virulence of the Mtb strain and also the susceptibility of the person to whom the infection is spread to.

2.5.2 Clinical forms of TB

2.5.2.1 Pulmonary TB (PTB)

PTB is the primary site of TB disease and represents the majority of all active TB cases reported annually. Cough is the most common clinical symptom of PTB and thus long-

term cough over 3-4 week is strongly indicative of TB, especially in high-endemic countries. Early in the course of illness there may not be production of sputum, but after initiation of inflammation and tissue damage, sputum and sometimes bloody sputum is produced by the TB patient. Children do not produce sputum as they cannot cough with a force similar to adults, and therefore PTB in children is rarely infectious. Clinical symptoms of PTB are generally not specific enough to confirm an active TB diagnosis; while PTB nearly always causes abnormalities in the lung that could be detected with chest X-ray. These abnormalities could be visible as inflammatory infiltrates or small nodular lesions, but large cavities may also be detected in patients with an untreated progressive PTB. Lung cavitation is typically considered an open form of TB disease, which results in efficient transmission of Mtb to other people [321]. Cavitation is probably the effect of a robust immune response that lead to severe tissue destruction in the lung [321]. Thus, PTB can also be divided into sub-groups including non-cavitary and cavitary forms of PTB. PTB mostly occurs as a local infection where the disease is isolated to the lung, but severe forms of PTB may also spread into the systemic circulation and cause miliary TB that involves the lung as well as other organs.

2.5.2.2 Extrapulmonary TB (EPTB)

EPTB can occur as a local disease primarily in the lymph nodes or pleura, but EPTB can also be presented together with PTB, either in a localized or in a disseminated form. EPTB is usually considered a more difficult diagnostic problem than PTB because the disease involves relatively inaccessible sites where few bacilli can cause great damage. The paucibacillary nature of EPTB in combination with inaccessible disease sites, causes bacteriological confirmation of TB to be more difficult and invasive procedures are often required to confirm a TB diagnosis [322].

2.5.2.3 Lymph node TB

It has previously been shown that local TB lymphadenitis accounts for about 10% of all TB cases. Although lymph node TB has been considered as a disease of children, the peak age for lymph node TB in recent years has been 30-40 years [323]. In addition, lymph node TB is more common among women and patients of Asian descent [323]. Studies from non-endemic countries have revealed that immigrants from South-east Asia, India and Africa appear to have a special predilection to develop TB lymphadenitis [324-329]. The basis for the enhanced risk among women, Asians and Africans is not known. Possibly, determining host factors involve occupation or cultural practices favoring oro-pharyngeal exposures to strains of the Mtb complex (e.g. exposure to *M. bovis* or Mtb from milking cows), genetically determined organ tropism, hormonal influences, effects related to BCG immunization and differences in health-seeking behavior [323]. In addition, genetic differences in the virulence of different strains of Mtb may play a role [330, 331].

TB lymphadenitis usually presents as a slowly progressive, painless swelling of a single group of lymph nodes, usually in the cervical region [332, 333]. The duration of symptoms at the time of presentation is typically 1-2 months and chest X-ray findings may be positive in 10%–40% of the patients [325, 332, 333]. A definitive diagnosis of TB lymphadenitis can be made by Mtb culture, polymerase chain reaction (PCR) or histopathological examination of fine-needle aspirations to demonstrate the presence of

Mtb or Mtb-infected cells in the affected lymph node. Excisional biopsy is the most invasive approach in diagnosis; however, it has the highest sensitivity and may produce a more rapid and favorable symptomatic response [332].

Mtb is the most common cause of TB lymphadenitis [334]. Accordingly, PCR-based studies have shown that Mtb is the main etiological agent of lymph node TB in Ethiopia [335, 336]. In some patients with TB lymphadenitis, the response to therapy may be slow or paradoxical. Such paradoxical upgrading reaction (PUR) has been reported in around 20% of HIV-negative TB lymphadenitis patients [325, 337, 338]. One definition of PUR is the development of enlarged nodes, new nodes or a new draining sinus in patients who have received at least 10 days of anti-TB treatment [338]. These features are consistent with the initiation of a robust immune response to Mtb upon administration of antibiotic therapy, which leads to release of mycobacterial antigens [323]. Steroids have been considered as a means to reduce the robust immune response in PUR, but their use is controversial; even though some reports describe a beneficial effect of steroid treatment [339, 340]. Instead, surgical excision combined with standard antibiotic therapy has been shown to produce favorable outcomes [341].

2.5.2.4 Pleural TB

The proportion of TB patients with pleural effusions has varied markedly from county to country ranging from 20-25% [342, 343]. The pleural effusions secondary to TB pleurisy are usually unilateral and can be of any size. Approximately 20% of TB patients with pleural effusions have co-existing parenchymal disease detected on chest X-rays [344]. The pathogenesis of pleural TB and the formation of pleural effusion is thought to be related to the initiation of a delayed hypersensitivity reaction (DTH) and the subsequent rupture of a sub-pleural caseous focus in the lung into the pleural space [345]. Evidence for the role of hypersensitivity was shown in studies where Mtb protein was injected into the pleural spaces of guinea pigs sensitized to purified protein derivative (PPD), whereupon an exudative pleural effusion rapidly develops [346]. Most patients with TB pleurisy have more than 50% small lymphocytes in their pleural fluid and many have more than 90% [344, 345]. Accordingly, in vivo administration of an anti-lymphocyte serum to the sensitized guinea pigs prevented the development of the pleural effusion, which suggests that lymphocytes are important mediators of this inflammatory reaction when Mtb components gains access to the pleural space [347]. Mycobacterial cultures of pleural fluid samples obtained from pleural TB patients are usually negative [348, 349], which further support the notion that Mtb components, rather than viable bacteria, promote the development of the pleural effusion.

The chain of immunological events leading to accumulation of fluid in the pleural cavity is assisted by an increased permeability of the pleural capillaries to proteins, which results in a much higher rate of pleural fluid formation. In addition, the lymphocytic pleuritis obstructs the lymphatic drainage in the parietal pleura, which prevents proper clearance of the pleural fluid. Thus, the accumulation of pleural effusion is a result of an increased formation of pleural fluid and the decreased removal of this fluid [350]. The most common diagnostic method of TB pleurisy is the demonstration of TB granulomas in the parietal pleura in tissue biopsies from the pleura [344]. TB diagnosis can also be based on elevated levels of adenosine deaminase (ADA) or IFN-γ in the pleural fluid [351-354]. Without treatment, TB pleurisy usually

resolves spontaneously, but the patient frequently develops active TB at a later time-point [355]. Similar to PUR in TB lymphadenitis patients, paradoxical worsening of the pleural effusion occurs in few patients after the initiation of anti-TB therapy [356].

2.5.2.5 TB/HIV co-infection

TB/HIV co-infection is one of the main factors that fuel the TB epidemic worldwide [357]. The prevalence of HIV infection in patients with TB is highest in the African region, which accounts for almost 80% of all TB/HIV co-infected cases worldwide. Among all TB patients with an HIV test result, 44% were tested positive in 2011, ranging from 8% in Ethiopia to 77% in Swaziland [358]. According to WHO, TB remains the leading cause of death among HIV-infected patients and those with latent Mtb infection are at high risk of progression to active TB in the absence of preventive therapy. A study done on South African gold miners, from 1990 to 2002, showed TB as the cause of death in almost 50% of HIV-seropositive patients [359]. Furthermore, evidence of TB was found in almost 50% of post-mortem autopsies performed on HIV-infected individuals in Kenya including disseminated TB in 80% of the cases [360]. Within areas of high TB incidence, the risk of developing active TB is significantly increased, even during the first year after HIV transmission, when total CD4 T cell counts are still quite high [361, 362]. Thus, the dramatic increase in active TB associated with HIV infection suggests that Mtb-specific immunity might be particularly vulnerable to HIV-associated immune defects [363]. Mtb-specific $CD4^+$ T cells are depleted in peripheral blood early after HIV infection, whereas these do not change significantly over a 6-12 month time in HIV-negative subjects with latent TB infection [364]. Remaining CD4+ T cells may be functionally inactivated or develop immunosuppressive functions. A consequence of the HIV-induced negative effects on T cell as well as macrophage responses includes defective granuloma formation (Figure 6). Destruction of the TB granuloma ultimately results in extensive dissemination of Mtb in TB/HIV co-infected patients.

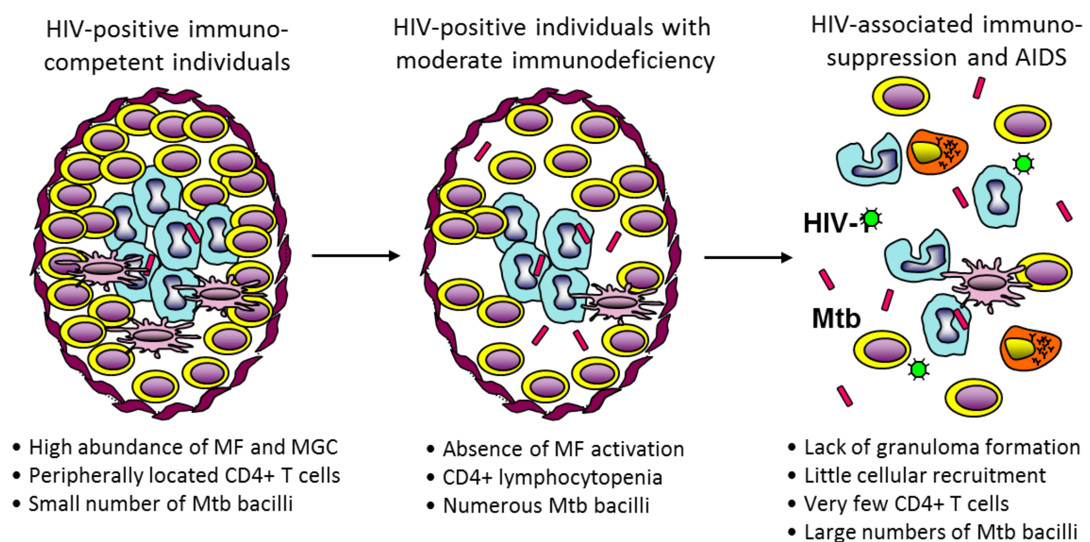

Figure 6. Defective granuloma formation in TB/HIV co-infected patients. As illustrated, deficient TB granuloma formation is dependent on the degree of immunosuppression in HIV-infected individuals.

HIV infection will affect both the immune response as well as the clinical presentation of active TB among patients with different forms of disease. In TB/HIV co-infected patients with PTB, atypical chest X-ray findings are more common compared to HIV-negative patients with PTB [322]. Here, the nature of the X-ray findings often depends on the degree of HIV-associated immunosuppression. TB that develops relatively early in the course of HIV infection tends to present with more typical radiographic findings [365, 366], but with more advanced HIV disease, the radiological findings become more atypical and cavitation is uncommon [322].

Before the beginning of the HIV epidemic, approximately 85% of reported TB cases were limited to the lungs and the remaining 15% involved extrapulmonary or both pulmonary and extrapulmonary sites [367]. This proportional distribution is substantially different among persons with HIV infection. One large retrospective study of TB in patients with advanced HIV infection reported that only 38% had PTB while 30% had a local EPTB and 32% presented both pulmonary and extrapulmonary involvement [368]. Thus, EPTB is more common among HIV-infected patients [369, 370]. The pathogenesis of PUR in HIV-positive TB lymphadenitis patients is complex, because HIV patients who started antiretroviral therapy (ART) sometimes develop immune reconstitution inflammatory syndrome (IRIS) that also contributes to an exaggerated inflammatory response [371]. It has also been shown that the proportion of thoracic TB patients who developed pleural effusions was higher among HIV-positive patients compared to immunocompetent TB patients [343, 372, 373]. The clinical manifestations of TB pleurisy also tend to be different in HIV-infected patients with a longer duration of illness [374] and thus systemic signs and symptoms are more common [375]. Pleural fluid obtained from TB/HIV co-infected patients, is more likely to be smear- and culture-positive for mycobacteria [374, 376] and if the peripheral CD4 T cell count is less than 100 cells/mm^3, approximately 50% of patients will show detectable AFB in their pleural fluid [374]. Moreover, HIV viral load is usually higher in the pleural fluid compared to the peripheral serum, which may indicate active HIV replication at the site of TB infection [377].

2.5.3 TB diagnosis

2.5.3.1 Commercial methods for TB diagnosis

TB is unique among the major infectious diseases because of the lack of an accurate rapid point-of-care diagnostic test. Failure to control the spread of TB is largely due to our inability to detect and treat all infectious cases of PTB, allowing continued transmission of Mtb in the society. Early case detection is dependent on test accuracy, accessibility, cost and complexity [42]. A combination of epidemiological, clinical, radiological, microbiological and histopathological features is used to verify a diagnosis of active TB (Figure 7) [378]. This battery of methods contributes to diagnostic delays with serious consequences for public health efforts to control TB disease [379]. In Africa only about 60% of the estimated number of TB cases are detected and thus close to half of active TB cases remain undetected and continue to transmit contagious TB disease [7]. Unrecorded numbers of active TB cases are particularly high among TB/HIV co-infected patients. The clinical examination is considered to be the first step in TB diagnosis [380]. Imaging techniques like chest X-ray are used to detect signs consistent with immune cell infiltrates, dense TB lesions and cavitation.

Histopathology can be useful to detect epithelioid granulomas and caseous necrosis that are typical findings in tissue or cell samples from patients with EPTB. In addition, microbiological techniques including AFB microscopy of sputum samples, Mtb-culture of sputum or tissue/body fluid specimens (golden standard) and molecular tests such as Mtb-specific PCR are used to detect the presence of Mtb. Mtb-specific Nucleic Acid Amplification tests (NAATs) can be a valid surrogate for direct isolation of Mtb bacilli [6]. One of the weak points of the microbiological techniques is that diagnostic performance relies on the presence of Mtb bacilli in the analyzed specimen. A test capable of diagnosing TB independently of the presence of live Mtb or mycobacterial components in the analyzed specimen would significantly improve the diagnosis of EPTB and smear-negative PTB cases [381].

In contrast to direct identification of Mtb using bacteriological methods, indirect methods that measure the immune response to Mtb can be helpful. Immunological diagnostic tests such as the Tuberculin Skin Test (TST) and the whole blood based Interferon-Gamma Release Assay (IGRA) including T-SPOT TB or QuantiFERON, are currently used in health care to assess the immune response against Mtb [382]. However, neither TST nor IGRA can separate active TB from latent TB or an old TB infection and thus these tests are less useful in high-endemic countries where latent TB is common. Furthermore, serological tests showed unsatisfying diagnostic performance (low specificity and sensitivity) to detect active TB in randomized controlled trials and therefore the clinical use of serology has been questioned by the WHO [383].

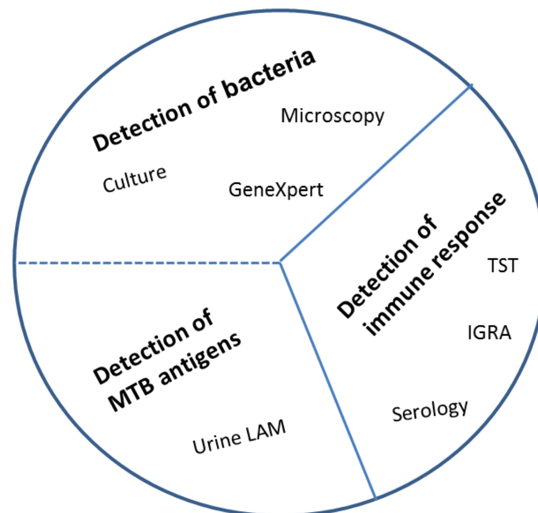

Figure 7. Pie chart illustrating the principal methods used in TB diagnosis.

2.5.3.2 *Poor performance of TB diagnostic methods in certain patient groups*

The only group of TB patients where a definite diagnosis can be both rapid and cheap is patients with sputum-smear positive PTB. But sputum-microscopy is used mainly for suspected PTB cases and performs poorly in young children [319] and in immunosuppressed individuals [379]. Accordingly, the sensitivity of sputum-microcopy is low, 60% at best and only 30-40% in HIV-infected patients with PTB [384]. TB diagnosis also becomes challenging among patients with **smear-negative PTB** or **EPTB**, due to presentation of diffuse clinical manifestations, low bacterial

loads or it is difficult to obtain clinical specimen from the site of Mtb infection, which complicates TB diagnosis using commercially available tests. Therefore, diagnosis of smear-negative PTB and EPTB is often delayed and sometimes based on the clinical response to empiric anti-TB treatment without microbiological confirmation [385, 386]. Similarly, **childhood TB** is associated with more problematic diagnosis because of atypical clinical manifestations [380, 387], low mycobacterial loads and difficulties to obtain sputum samples that are suitable for microbiological analysis [380, 388]. As a result, a golden standard for TB diagnosis of childhood TB is still missing [389]. Furthermore, upon development of **TB in HIV-infected or AIDS patients**, TB disease is not only more severe at the clinical level, but it also becomes more difficult to diagnose TB because of the higher proportion of atypical cases. Because TB/HIV co-infected patients often fail to form productive granulomas [390], bacteria less frequently enter the airway system and thus the proportion of sputum-negative TB cases paradoxically increases.

2.5.3.3 Challenges in TB diagnosis

The diagnostic challenges described above are most problematic in TB endemic countries. It is therefore important that a diagnostic technique intended for TB control at a global level should be suitable for use in a setting where it is most needed, namely in resource-poor high-burden countries in Africa and Asia [42]. In industrialized countries, radiology advanced imaging techniques, rapid and automated systems for mycobacterial growth (ie. BACTEC Mycobacterial Detection System), and NAATs are used to supplement light and light-emitting diode (LED) microscopy for the diagnosis of active TB disease [6]. Unfortunately, many of these technologies are too advanced and too expensive to be used in routine diagnosis in the developing world. In resource-limited settings, microscopic examination of Ziehl-Neelsen stained sputum specimens is often the only TB test available. The ideal TB test would provide an on-the-spot accurate diagnosis of : 1) active TB in both HIV-negative and HIV-positive TB patients, 2) active TB in both adults and children, 3) both PTB and EPTB, 4) both sputum-positive and sputum-negative patients [391]. The ability of an efficient diagnostic test to distinguish between active TB disease and latent TB infection is also critical [42].

2.5.3.4 Recent advances in TB diagnosis

IGRA: UK National Institute for Health and Clinical Excellence guidelines recommends the use of the IGRA tests combined with traditional TST and other routine TB diagnostic tests in the screening of patients with suspected TB. IGRAs that are based on the detection of Mtb-specific T cells, are an improvement over the TST since these assays produce less false positives caused by mycobacteria other than tuberculosis (MOTT) or BCG vaccination, have an internal control and do not require a follow-up visit to assess the reaction.

GeneXpert: The Xpert MTB/RIF assay is a major advance among a new generation of automated easy-to-use nucleic acid amplification tests. This PCR-based assay is a very sensitive diagnostic test for detection of Mtb bacilli in sputum and other clinical samples and is also useful to detect rifampicin resistance in high-risk populations as well as low-risk populations [392-394]. Despite the high sensitivity and specificity of

the GeneXpert among sputum-positive patients with PTB, the performance of this test in sputum-negative high risk groups is still sub-optimal. Therefore GeneXpert cannot be universally applied as a definitive test for point-of-care.

Urine-based diagnostic tests: Commercial assays are available that can detect LAM in the urine of patients with active TB, ie. the Determine TB-LAM is a commercially available, lateral-flow urine 'strip test' assay [395]. Although the sensitivity of this test has been disappointing in HIV-negative TB patients, moderate sensitivity and high specificity has been observed in HIV-infected TB patients with advanced immunodeficiency [396].

Serological tests: Commercial serological tests for detection of active TB have been a disappointment so far, showing overall low quality including inconsistent and imprecise estimates of sensitivity and specificity. Furthermore, there is no evidence that existing serological assays improve patient-important outcomes. Studies have found considerable variation in Mtb-specific antibody responses detected in TB patients, suggesting that multiple targets will be needed if antibody-based tests are to achieve the required sensitivity [397, 398].

Flow cytometric assays: In a study performed on Mtb-specific T cell responses using flow cytometry, the proportion of TNF-α single-positive Mtb-specific $CD4^+$ T cells were found to be the strongest predictor of active TB disease. The sensitivity of the $CD4^+$ T cell signature was 100% and the specificity was 96% for these data that were generated from a European cohort [399].

2.5.4 Treatment of TB

2.5.4.1 TB chemotherapy

Drug treatment is the corner stone of TB control and a clinical cure if followed properly. A review of the pre-chemotherapy era showed that HIV-negative patients with an untreated smear-positive TB has a 10-year case fatality of 53-86% (weighted mean of 70%) [400]. The duration of TB from onset of disease to cure or death is approximately 3 years and appears to be similar for smear-positive and smear-negative cases [400]. Before effective antibacterial treatment was developed, the historical medical therapy for TB was based on isolation of the TB patient in a sanatorium where the patients could rest in fresh cold air [401]. Some of the TB patients were indeed cured at the sanatorium. It has been speculated that their clinical recovery was enhanced after exposure to sun light, which is known to promote the production of vitamin D in the skin that can enhance anti-mycobacterial effector functions in human macrophages [402]. The first TB patient was treated with streptomycin in 1944 and she was cured from her life-threatening disease! Further trials confirmed that streptomycin was indeed effective in the treatment of TB. Selman Waksman was awarded the Nobel Prize in Physiology or Medicine in 1952 for his discovery of streptomycin (Nobel prize.org, tuberculosis). Nowadays, standard drug regimen for effective treatment of chronic TB involves a combination therapy with first- and/or second-line drugs for 6-8 months. Recommended first-line anti-TB drugs includes rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months, followed by rifampicin and isoniazid for 4 months [403].

2.5.4.2 Drug resistant TB

The extended multidrug therapy that is typically used to treat patients with active TB prevents the emergence of drug resistance, but also contributes to a low compliance among the TB patients. Importantly, many patients feel better already a couple of weeks after initiation of anti-TB chemotherapy and therefore stop taking their drugs, which also has many unwanted side-effects. This opens up an opportunity for residual bacteria remaining in the infected organ to start to replicate and grow out a drug-resistant population of Mtb that will be much more difficult to treat. Recently, increasing numbers of MDR-TB, and also extensively drug-resistant (XDR) TB and totally drug-resistant cases threaten TB control all over the world [404, 405].

2.5.5 TB vaccine

2.5.5.1 BCG vaccine

BCG is the first TB vaccine and is still being used as the only TB vaccine in routine clinical practice. In 1908, Albert Calmette and Camille Guerin at the Institute of Pasteur, made the attenuated form of *M. bovis* from a virulent *M. bovis* strain which was then used for the development of the first TB vaccine [406, 407]. BCG is the most extensively used vaccine with more than 4 billion doses administered worldwide [408]. BCG is a most debated vaccine as it provides varying protection against TB which varies extensively comparing different populations and geographic locations in the world [409, 410]. Although BCG confers protection against severe forms of TB in children such as meningitis and disseminated TB, the estimated protection ranges from 0-80% in adult PTB [3]. Several factors are likely to influence the inconsistency in BCG-induced protection. Some of these are batch differences in the many strains of BCG in use, which differ in immunogenicity [411]. Moreover, BCG differs from Mtb in that it lacks the RD1 region that encodes for many of the specific virulence proteins expressed by Mtb. The absence of RD1 in BCG will probably result in a failure of BCG to induce Mtb-specific T cell responses [412, 413].

2.5.5.2 Improvement of the BCG vaccine

The development of efficient memory $CD4^+$ and $CD8^+$ T cell responses is the main requirement of a novel vaccine against Mtb [194]. In an attempt to improve BCG vaccination, it could be possible to boost the initial BCG response by delivery of the RD1-specific antigen(s) (i.e. ESAT-6 or CFP10) together with an adjuvant. Boosting BCG-primed mice with a recombinant adenovirus expressing Mtb antigen (Ag)85A by the intra-nasal route was associated with the induction of immune protection that correlates with the presence of polyfunctional T cells expressing high levels of Th1 cytokines (IFN-γ, IL-2 and TNF-α) [414]. Boosting of BCG-induced responses in humans also results in expansion of long-lived polyfunctional T cells expressing IFNγ, IL-2 and TNF-α [415]. Current knowledge of the immune response to Mtb has not allowed for a dramatic improvement in the design of vaccines, even though many new vaccine constructs are currently being tested in clinical trials. Once TB disease has developed, a delicate balance between protective and pathological immune responses is the key to survival. Thus, T cell functional data has to be integrated with an analysis of the consequences of TB infection itself to improve rational vaccine design [192].

2.6 THE FUTURE: NEW DRUGS AND BIOMARKES FOR TB

It is generally accepted that reasonable control of TB on a global scale depends on new interventions including a better vaccine that protects adults against PTB, better drugs that act on dormant Mtb and shorten treatment of active TB as well as better diagnostic tests with the ability to rapidly detect active TB. Due to the extended duration of anti-TB drug treatment that spans over months, the benefits of potential resolution mediators, that could lower antibiotic requirements, merit attention [43]. Here, a study also showed a potential role of adjunctive treatment with vitamin D to standard treatment of PTB that could accelerate resolution of inflammatory responses associated with increased risk of mortality [416]. After many years, a new anti-TB drug has been approved in the US for restricted use and this drug is in the late phases of clinical development and is being tested in patients with MDR-TB. This is bedaquiline, a diarylchinolone, which blocks the ATP synthase in the bacteria and probably also acts on dormant Mtb [417, 418]. Currently, a dozen preventive TB vaccine candidates and a few therapeutic ones are undergoing clinical trial assessment [418].

Moreover, studies of novel biomarkers, comparing gene expression profiles in patients with active TB with latently infected healthy individuals, have provided proof of principle for the discovery of bio-signatures that reliably discriminate active from latent TB [43]. Besides the primary goal of identifying new diagnostic tools, biomarkers also provide deep insights into biological mechanisms operative in TB disease and latent TB infection [43]. A whole-blood microarray gene expression analyses performed in TB patients, showed that FcγR was one of the most discriminative markers giving a high degree of accuracy in discriminating TB patients and latently infected donors [419]. Fc receptors bind to antibodies and stimulate phagocytic or cytolytic cells to destroy microbes, by phagocytosis or cytotoxicity. On the contrary some viruses use Fc receptors to infect cells by ADE [420]. FcγR are the most important Fc receptors for inducing phagocytosis of opsonized microbes, on the other hand they can also be released in cell supernatant and circulate in biological fluid and bind to immunoglobulin’s, interfering with their function [421]. A study performed in a cohort from Gambia, showed that the gene expression of FcγR1 was one of the most differentially expressed genes in active TB compared to latent TB [422]. Similar results were provided in cohorts from both Europe and South Africa [419, 423]. Elevated levels of immune complexes accompanied by defective immune complex solubilization have also been documented in serum of TB patients as potential attractive biomarkers of active TB [424].

In the context of intracellular microbes such as Mtb and also HIV, for which effective vaccines are lacking, an increased understanding of how B cells regulate the immune response to pathogens and how antibody-dependent immunity interacts with the cellular arm of the host response to mediate protective effects will likely aid in the development of strategies to enhance antimicrobial immunity and vaccine efficacy [425]. The large plasticity of the human immune system including an integrative understanding of how immune cells cooperate to control infection is essential to improve vaccine design and immune response monitoring, because no single cell or effector function can be solely related to immune protection in TB [146]. The search for specific signature molecules of active TB disease continues to be essential.

3 AIMS, RESEARCH DESIGN AND METHODS

In this chapter, an overview of the aims, experimental design and methods used in this thesis work will be explained. Details regarding individual experiments can be found in the Materials and Methods sections in each of the papers included in this thesis.

3.1 AIMS

This thesis focused to explore immune responses in human TB in order to increase the understanding of the immunopathogenic mechanisms associated with progression of active TB disease in HIV-negative and HIV-positive individuals. The aim was to use this new knowledge to identify relevant biomarkers or immune response signatures specific for clinical TB.

- **Study I:** To dissect the antimicrobial effector pathways and immunoregulatory mechanisms at the site of infection in children with a local lymph node TB.
- **Study II:** To evaluate a new diagnostic plasma B cell test for detection of active TB in adult patients with different clinical forms of TB.
- **Study III:** To explore the expression and regulation of cytokine/chemokine profile in adult patients with pulmonary and pleural TB.
- **Study IV (ongoing):** To study the effects of HIV infection on tissue inflammation and remodeling in adult patients with a local lymph node TB.

3.2 PATIENTS AND CLINICAL SAMPLES

3.2.1 Study site

All study subjects were recruited at the Black Lion University Hospital located in Addis Ababa, Ethiopia (Figure 8). The children were recruited from the Department of Pediatrics (**Study I**), while adult patients were recruited from the Department of Internal Medicine (Chest unit) at the hospital (**Study II-IV**). The Black Lion Hospital is the largest, general specialized and referral teaching hospital in Ethiopia that cares for many TB-infected and TB/HIV co-infected patients every year. A control group of children with tonsil hyperplasia (**Study I**) as well as healthy blood donors (**Study III**) were also recruited at the Karolinska University Hospital in Huddinge, Sweden. The laboratory analyses were performed at the Armauer Hansen Research Institute (AHRI) in Addis Ababa, Ethiopia (Figure 8) and also at the Center for Infectious Medicine (CIM), Karolinska Institutet, Sweden.

Figure 8. The Black Lion University Hospital (left) and the Armauer Hansen Research Institute (right) in Addis Ababa, Ethiopia.

3.2.2 Study cohorts

This thesis study was designed to explore immune responses in patients with a sputum-negative active TB as compared to appropriate control groups:

- **Study I:** Ethiopian children with a local persistent TB lymphadenitis, age 3-10 years (n=11).
  Controls: Age-matched children:
  - Ethiopian children with a non-specific reactive lymphadenitis (n=10).
  - Swedish children with a non-infectious tonsil hyperplasia (n=10).

  HIV status: All study subjects were HIV-negative.
- **Study II:** Ethiopian adult patients >18 years with different clinical forms of TB:
  - Pulmonary TB (n=35)
  - Pleural TB (n=23)
  - Lymph node TB (n=26)

  Controls: Ethiopian adult patients >18 years:
  - Asymptomatic individuals with latent TB (n=45)
  - Asymptomatic individuals with neither latent or active TB (n=40)
  - Symptomatic patients with other diseases than TB (n=12)

  HIV status: The study subjects included both HIV-negative and HIV-positive individuals.
- **Study III:** Ethiopian adult patients >18 years with different clinical forms of TB:
  - Pulmonary TB (n=35)
  - Pleural TB (n=23)

  Controls: Adult patients >18 years:
  - Individuals with a bronchial wall irritation (noninflammatory) (n=10)
  - Patients with pleural diseases other than TB (n=5)

  HIV status: The study subjects included both HIV-negative and HIV-positive individuals.
- **Study IV (ongoing):** Ethiopian adult patients >18 years with a local persistent TB lymphadenitis (n=18).
  Controls: Ethiopian adult patients >18 years with a non-specific reactive lymphadenitis (n=10).
  HIV status: The study subjects included both HIV-negative and HIV-positive individuals.

These are well-defined study cohorts where all subjects had a clinical (typical clinical symptoms including persistent cough, fever, sweating and weight loss), radiological (chest X-ray abnormality consistent with TB) and laboratory diagnosis (culture of bronchoalveolar lavage (BAL), histopathological examination of cells or tissue biopsies and/or Mtb-specific PCR) of active TB. At the time of inclusion, all patients were treatment naïve for both TB (standard anti-TB chemotherapy) and HIV drugs (antiretroviral therapy). Patients with a diagnosis of TB were treated with anti-TB drugs and followed for 8 months. In cases of clinical TB (patients without bacteriological confirmation of Mtb), the response to anti-TB treatment (radiological resolution of pulmonary or pleural lesions and clinical improvement) was used as a retrospective diagnostic criteria for TB.

3.2.3 Clinical samples

At the time of TB diagnosis, clinical samples were obtained both from the site of infection (pleura or lymph node tissue, BAL or pleural fluid) and from the peripheral circulation (blood) (Figure 9). All tissue biopsies were cryopreserved in liquid nitrogen and stored in -85°C until laboratory analysis. Cells isolated from BAL and pleura fluid and peripheral blood mononuclear cells (PBMCs) isolated from blood samples were stored at -150°C. BAL and pleura fluid supernatant and plasma obtained from blood were stored at -85°C.

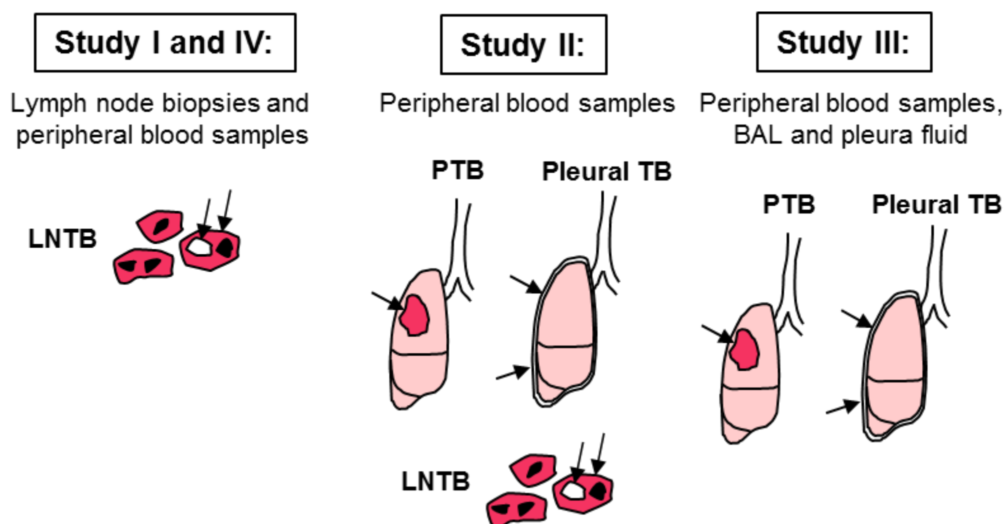

Figure 9. Schematic illustration of the clinical samples collected from the study subjects. LNTB=lymph node TB and PTB=pulmonary TB.

3.3 EXPERIMENTAL METHODS

This thesis is based on comprehensive analysis of clinical samples obtained from TB patients and controls. In summary the following techniques were used to study immune responses in human TB:

- Immunohistochemistry and in situ computerized image analysis: Study I and IV
- Multiplex Luminex Assay: Study III
- Enzyme Linked Immunosorbent Assay (ELISA): Study II and III
- Flow cytometry: Study II
- Quantitative real-time PCR (qPCR): Study I, III, IV
- Immunodiagnosis of TB (ALS, TST and QFTG): Study II

All work with Mtb-infected cell and tissue samples have been performed at biosafety level 3 (BSL-3) laboratories at AHRI in Ethiopia and at the Swedish Institute for Communicable Diseases Control (SMI) in Sweden. After chemical inactivation of Mtb or filtration of body fluids, the samples could be transferred to BSL-2 laboratory for continued analysis. A theoretical description of the key methodologies is provided below.

3.3.1 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a method used to detect protein expression and distribution in tissue using phenotypic and functional markers. IHC and in situ computerized image analysis is a well-established technology in our laboratory and has been used to study immune responses in TB, HIV and Streptococcus infection [426-429]. In **Study I** and ongoing **Study IV**, IHC provided us with a unique advantage to study the spatio-temporal organization of cells and effector molecules in the intact tissue. Thus, tissue inflammation and architecture including granuloma formation could be studied in a physiologically relevant milieu at the site of Mtb infection.

3.3.1.1 Cryosectioning of Mtb-infected tissue samples

Lymph node biopsies from TB patients were cryopreserved in order to prevent destruction and loss of sensitive epitopes. Since Mtb is resistant to cold, freezing and desiccation [430], work with frozen biopsies from Mtb-infected patients is not without risk. To be able to perform this work, our group has established a facility suitable for cryosectioning of Mtb-infected tissues in the BSL-3 laboratory at SMI. In this facility, a cryostat is covered by a custom-made ventilation hood that prevents potential spread of infectious aerosols. This special construction allows safe and efficient processing of Mtb-infected frozen tissue samples. Ultrathin sections of 8 microns each are cut from the tissue biopsies and mounted on microscope slides before fixation and inactivation in 4% formaldehyde.

3.3.1.2 In situ computerized image analysis

Antimicrobial and immunoregulatory pathways in TB were investigated using IHC and in situ computerized image analysis, which provides an excellent technological platform to explore local immune responses in clinical tissue samples. The Avidin-Biotin Complex (ABC) method was used for IHC detection. IHC is a semi-quantitative method to study protein expression in cryopreserved tissue. Immunostainings enable visualization of tissue morphology, expression and distribution of cells and effector molecules, specific cell-cell interactions as well as the anatomical location of mycobacteria or mycobacterial antigens. Thus, IHC enables functional assessment of various proteins at the single-cell level, including cell surface, cytoplasmic and nuclear as well as granule-associated and secreted proteins in the complex environment of real tissue. High-resolution images can be taken at different magnifications using a digital camera that is connected to the microscope (Leica Microsystems). Furthermore, protein expression can be quantified at the single-cell level using a highly sensitive computerized image analysis program (Leica Qwin). The Leica Qwin software can differentiate an extensive range of colors (up to 16.7 million) and thus support detailed assessment of different proteins in the tissue. It is also possible to choose different applications of the software, such as tissue-excluder or tissue-includer analysis. These functions can be used to exclusively analyze certain areas of the tissue, such as a TB granuloma. Co-expression of surface-expressed and/or intracellular proteins can also be detected using multicolor labeling (immunofluorescence) and confocal microscopy that can be manually quantified. In summary, this method can be widely used to study the expression of many different proteins in human tissues.

3.3.2 Multiplex luminex assay

The multiplex luminex is a microplate bead-based protein detection immunoassay that was used to examine the cytokine and chemokine profiles in active TB patients and controls (**Study III**). The aim of this analysis was also to compare the cytokine/chemokine profile at the site of Mtb infection (BAL or pleural fluid) to peripheral blood (plasma). The advantage with the multiplex luminex assay is that it detects several cytokines and chemokines in the same clinical samples, which provides the opportunity to map the relative expression levels of many proteins simultaneously.

3.3.2.1 Protein assessment using multiplex luminex

The multiplex luminex system enables to detect up to 100 different analytes in a small test sample, allowing sensitive quantification of many different cytokines and chemokines in the same sample. Microscopic beads with different color codes (spectral addresses) permit discrimination of individual assays. In addition, the beads are internally dyed with different ratios of fluorophores and are thus classified into unique bead regions. Beads with different spectral addresses are selected and coupled to antibodies against a different target. These antibody-coupled beads are added to a 96-well plate before addition of the test sample. The targets, cytokines and chemokines, in the sample are detected by biotin-labeled detection antibodies specific for secondary epitopes on each target. A fluorescent-labeled streptavidin reporter is used to bind to the biotin-labeled antibodies, where after the reaction is read on a Bio-plex reader. Dyed beads are identified by their bead region and the level of target is indicated by intensity of the reporter signal. All multiplex data are reported simultaneously and analyzed by the Bio-plex manager 4.0 curve fitting software using the five parametric algorithms.

3.3.3 Enzyme-linked immunosorbent assay (ELISA)

A standard ELISA was performed to assess BCG-specific IgG antibodies produced in lymphocyte supernatants (**Study II**) and also in plasma, BAL and pleural fluid (**Study III**) from TB patients and controls. A Japan BCG vaccine was used as coating antigen and after incubation with the clinical samples, the ELISA was developed using a rabbit anti-human IgG horseradish peroxidase (HRP) conjugate and an O-phenylenediamine (OPD) substrate.

3.3.4 Flow cytometry (FACS)

In **Study II**, multicolor flow cytometry (FACS) was applied to detect and quantify the presence of IgG-expressing plasmablast in the peripheral circulation of TB patients and controls. FACS is a laser-based technique used for cell counting and sorting, which allows simultaneous detection and analysis of multiple markers expressed on the same cell and can be used to detect rare subsets of cells. Here, FACS analysis of PBMC samples included a panel of plasmablast markers (CD3, CD20, CD27, CD38, CD19 and IgG) that were evaluated using a Gallios flow cytometer and the Kaluza software. Plasmablasts were identified as $CD3^{-}CD19^{+}CD20^{-}CD27^{high}CD38^{high}$ cells that expressed membrane-bound IgG.

3.3.5 Quantitative real-time PCR (qPCR)

A convenient method has been developed in our research group to quantify mRNA expression of different host molecules in cells and tissues using quantitative real-time PCR (qPCR). qPCR analysis is a sensitive method that provides the possibility to study the relative expression of transcripts in rare clinical samples. In **Study I, III and IV**, qPCR was used to measure different effector and inhibitory molecules at the mRNA level in human TB. mRNA was extracted from human cells (PBMCs, BAL and pleural cells preserved in RNAlater) using a phenol-based compound (TRI Reagent), which also inactivates any mycobacteria present in the sample. A very small amount of clinical material is required to perform the qPCR and once the mRNA has been converted to cDNA, the sample could be stored at -20°C for long periods of time.

3.3.6 Immunodiagnosis of TB

3.3.6.1 Antibodies in Lymphocyte Supernatant (ALS)

The Antibodies in Lymphocyte Supernatants (ALS) assay is an ELISA-based method that detects BCG-specific IgG antibodies secreted by PBMCs cultured in vitro (Figure 10). In **Study II**, we used the ALS assay as novel diagnostic method to improve detection of active TB among TB patients and controls. The ALS is a novel test that has previously been developed by our collaborator Dr. Rubhana Raqib at the International Centre for Diarrhoeal Disease Research (icddr,b), Bangladesh [431, 432]. The theoretical basis for this assay is that in a steady-state, migratory plasmablasts among PBMCs are very low, but significantly elevated upon continuous antigen exposure ie. during active TB infection [433, 434]. In the ALS assay (48-well format), PBMCs are isolated from peripheral blood and cultured (2x2,5 million cells) for 72 h in the absence of exogenous stimulation. The spontaneous release of antibodies from in vivo activated plasmablasts results in an accumulation of Mtb-specific IgG in culture supernatants that are measured using ELISA and the BCG vaccine as antigen. Thus, the ALS assay is an in vitro assay to detect in vivo activated IgG-secreting plasmablasts. Pooled sera from sputum-smear positive TB patients are used as positive control while cell culture medium is used as negative control. The potential use of the ALS assay as a diagnostic test was evaluated in comparison to clinical diagnosis as well as immunodiagnosis using the commercially available TST and QFTG test.

3.3.6.2 QuantiFERON (QFTG)

The QuantiFERON or Quantiferon-TB Gold In-Tube (QFTG) is an ELISA-based test to assess Mtb-specific IFN-γ production in whole blood samples from TB patients and controls (**Study II**) (Figure 10). In the QFTG assay, whole blood aliquots are collected in tubes pre-coated with a cocktail of three Mtb-antigens, ESAT-6, CFP-10 and TB7.7. Importantly, these antigens are absent from BCG and MOTT. IFN-γ production in the Mtb-stimulated blood sample is compared to a positive (mitogen) as well as a negative control (nil) after incubation for 16-24 hours at 37°C. Individuals that have been exposed to Mtb will have memory T cells in their circulation that will respond to the Mtb-antigen cocktail by producing IFN-γ. An individual is considered TB-positive if the QFTG result is above the test cut-off (>0.35 IU/ml), after subtracting background production of IFN-γ in the negative control. Even though the QFTG test does not

distinguish active TB from latent TB, the sensitivity of this test is high. In addition, since this is a standardized commercial assay, the reader bias is very small, which makes QFTG a superior method as compared to TST.

3.3.6.3 Tuberculin Skin Test (TST)

The Tuberculin Skin Test (TST) is a diagnostic assay for TB that has been routinely used for the last century. The TST is based on the presence of a delayed-type hypersensitivity reaction in the skin after intradermal injection of 0.1ml (5TU) of Tuberculin Purified Protein Derivative (PPD) (Figure 10). The induration of the skin reaction (swelling caused by local inflammation) is read after 48-72 hours. The TST is considered positive when the transverse diameter of the reaction is ≥10mm in HIV-negative and ≥5mm in HIV-positive individuals. This test has the capacity to reveal exposure to mycobacteria, but it cannot differentiate Mtb infection from MOTT or previous BCG vaccination [435]. Despite this obvious drawback, the TST is still one of the most common diagnostic tests used in routine clinical practice to detect active TB.

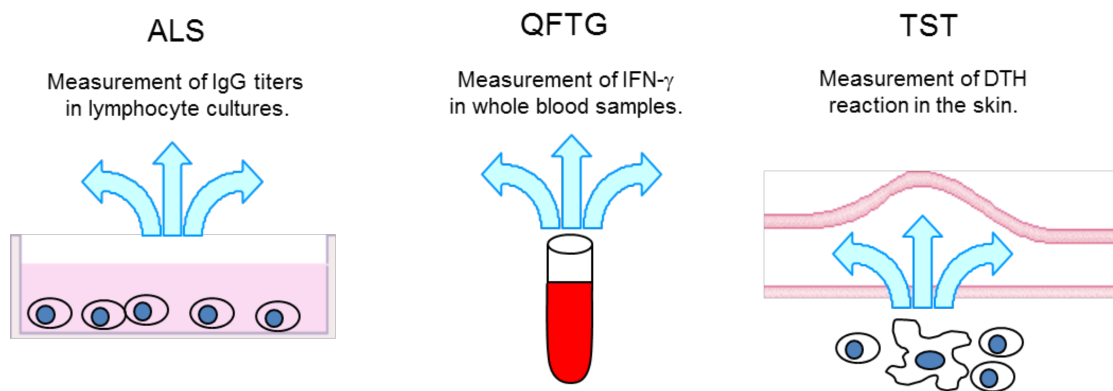

Figure 10. Schematic illustration of the immunodiagnostic assays used in this thesis work.

3.4 STATISTICAL ANALYSES

Non-parametric analyses were used to calculate the p-values for groups of a small sample size (n<10) or for non-normally distributed data (D'Agostino and Pearson omnibus normality test) and included the Kruskal-Wallis test and Dunn's post-test (comparing more than two unmatched groups), the Mann-Whitney U test (comparing two unmatched groups) or the Wilcoxon signed-rank test (comparing two matched groups). A p-value <0.05 was considered significant (*), a p-value <0.01 was considered very significant (**), a p-value <0.001 was considered extremely significant (***), whereas a p-value >0.05 was considered not significant (ns). Spearman's correlation test was used for the correlation analysis. A value of r=1, indicates a perfect positive correlation whereas r=-1 indicates a perfect negative correlation. Receiver operating characteristics curves (ROC) were used to determine the relation between sensitivity and specificity at various cut-off levels of BCG-specific IgG titers and to determine the cut-off point based on the level of maximum accuracy. Statistical analyses were performed in Graph Pad Prism (**Study I, II, III and IV**) and SPSS V.12 (**Study II**).

3.5 ETHICAL CONSIDERATIONS

Lymph nodes from Ethiopian patients and tonsils from Swedish uninfected controls were obtained after ethical approval from both Sweden (EPN dnr: 365/00 and 2007/141-32) and Ethiopia (**Study I**). Patients were recruited into the study after parent's or guardian's approval and signed informed consent. Clinical samples from Ethiopian adult patients and controls were obtained after ethical approval from Sweden (EPN dnr: 2007/675-31/4, 2011/1014-31/1 and 2010/603-31/4) and Ethiopia (**Study II-IV**). Patients and controls were recruited into the study after obtaining signed informed consent.

4 RESULTS AND DISCUSSION

In this chapter, the clinical and demographic characteristics of the study population will be presented, the experimental results of the thesis will be analyzed and their implications discussed.

4.1 CLINICAL AND DEMOGRAPHIC DATA OF THE STUDY COHORT

4.1.1 Primarily young individuals are infected with active TB

The demography of the study subjects included in this thesis work is outline in Table 1. The mean age of the adult study cohort was 31 years for all forms of clinical TB disease (**Study II-IV**). This is similar compared to a study in South Africa where the highest TB notification rates were found in the age group of 30-49 years, which typically indicates a shift in the disease burden to individuals in the economically productive age groups [436]. Another study on lymph node TB in USA also detected a peak in TB disease in the age group of 30-40 years [323]. Interestingly, we observed a higher mean age of TB patients who were HIV-positive (36 years) compared to HIV-negative (29 years) TB patients. This age difference was also observed in HIV-positive (33 years) compared to HIV-negative (28 years) control subjects, which indicates that this deviation may be related to HIV rather than TB infection. This finding is in agreement with a study in Burundi where the mean age of HIV-positive patients with intra-thoracic TB (35,8 years) was higher compared to HIV-negative TB subjects (29,4 years) [342]. Another study also showed that HIV-positive individuals with (33,4 years) or without active TB (34,1 years) were generally older than HIV-negative controls (25,7 years) [437].

Similar numbers of males and female were included in the adult study cohort (**Study II-IV**), while there were more females included in the children cohort (**Study I**) (Table 1). Our finding in children is consistent with reports showing that TB lymphadenitis is seen more frequently in females compared to males in both children and adults [323, 438].

Table 1. Demography of the study subjects

	Groups:	Numbers (%)	Mean age (years)	Females (%)	HIV (%)
Children	Lymph node TB	11	5.7 (3-9)	78	0
	Controls	10	6.1 (3-10)	50	0
Adults	Pulmonary TB	35 (42)	31 (>18)	41	34
	Pleural TB	23 (27)	32 (>18)	52	30
	Lymph node TB	26 (31)	29 (>18)	46	31
	HIV-neg TB	57	29	26	68
	HIV-pos TB	27	36	13	32
	Controls	85	32	46	39

4.1.2 TB/HIV co-infection is common in the adult study cohort

HIV-infected individuals and other patient groups with impaired cell-mediated immunity are much more likely to develop active TB disease, both from a new infection and from reactivation of latent TB. In this study cohort (**Study II-IV**), TB/HIV co-infection was observed in 32% of the adult TB patients (Table 1), which is in agreement with a global TB/HIV co-infection prevalence of 39% [1]. Likewise, an

Ethiopian study previously showed that 30% of urban patients with TB were HIV-positive [439]. In contrast, TB/HIV co-infection was very low in our Ethiopian child cohort (Table 1) (**Study I**). Out of a total of 49 children with a suspected TB lymphadenitis, only two HIV-positive children with a local TB lymphadenitis were found (unpublished observation). Thus, this finding may be explained by the fact that we selected children with a local lymph node TB without pulmonary involvement or signs of systemic disease. Children are known to be particularly vulnerable to severe disease following TB infection, partly because the immune system is not fully mature in children [440]. Therefore, TB/HIV co-infection may be more frequently associated with systemic disease in children compared to adults [322].

The clinical data on the adult study cohort also showed that parasite infections were rare (11%) and mostly associated to TB/HIV co-infection (**Study II**), which is consistent with the notion that immunosuppressed individuals are more susceptible to opportunistic intestinal infections [441]. This finding is also in agreement with a previous study from Ethiopia showing that parasitic infections are more common in HIV patients [442].

4.1.3 Low level of BCG vaccination among adult TB patients

Even though BCG vaccination is known to induce a very variable protection against TB in different populations and in different age groups, BCG efficiently protects children from severe childhood TB. Since the children were recruited from a pediatric unit where children are routinely BCG vaccinated, all children with lymph node TB were BCG vaccinated (**Study I**). Instead, BCG vaccination (detected by a BCG scar) was only observed in 20% of our adult TB cohort (**Study II-IV**). A lower level of BCG vaccination among adult TB patients compared to the children cohort could be explained by a very low BCG immunization coverage in Ethiopia before 1980, which has increased to 72% after 2006 (BCG World Atlas).

4.2 NOVEL IMMUNODIAGNOSTIC ASSAYS OF ACTIVE TB

4.2.1 Challenges of conventional methods for TB diagnosis

As described in the Background section of this thesis, diagnosis of TB is difficult and rapid bacteriological confirmation of Mtb is only possible in 30-60% of all TB patients. High-risk groups that are particularly difficult to diagnose include sputum smear-negative PTB, TB/HIV co-infection, extrapulmonary forms of TB as well as TB in small children. In this thesis work, we decided to include adult sputum-smear negative TB cases including both PTB and EPTB cases with and without a concomitant HIV infection. Consequently, all of the adult TB patients included in this thesis work were sputum-smear negative and comprised 42% PTB cases and 58% EPTB cases (**Study II-IV**). Bacteriological confirmation using BAL samples collected with bronchoscopy have been shown to improve the diagnosis of patients with a sputum-negative TB [443]. In this study cohort, 69% of the BAL samples obtained from PTB patients were culture positive for Mtb (**Study II-III**), which is consistent with a study observing 75% of BAL-culture confirmed TB cases among sputum-negative TB patients [444]. Accordingly, the proportion of BAL-culture negative PTB patients in this study was 31% (**Study II-III**). These patients were diagnosed based on clinical symptoms, abnormal chest X-ray findings and clinical improvement and X-ray resolution of lesion

after treatment with anti-TB drugs (**Study II-III)** [445]. Importantly, bronchoscopy is an invasive procedure and not suitable for routine diagnosis in resource-poor settings. Instead, better diagnostic assays are urgently required to improve TB diagnosis among patients with sputum- and/or BAL-negative TB as well as clinical TB.

4.2.2 B cell-based immunodiagnosis

4.2.2.1 The ALS assay can improve TB diagnosis in selected high-risk groups

In **Study II**, we aimed to investigate if Mtb-specific plasmablast responses, assessed with the novel ALS assay, could be used to detect active TB among sputum smear negative TB cases and differentiate different forms of active TB (PTB and EPTB) from latent TB and healthy controls. The aim was also to study the diagnostic performance of the ALS assay among TB/HIV co-infected patients. For the first time, we were able to demonstrate that the ALS assay can be used to improve diagnosis of active TB in these high-risk groups (**Study II**). The majority of patients with active TB had a positive result with the ALS assay (91%), but also with the TST (89%) and QFTG (79%) tests (**Study II**). The ALS assay has previously been shown to discriminate active TB from TB-exposed contacts and also subclinical TB disease [431, 432, 446]. Here, we compared the ALS response of active TB patients to a well-defined cohort of latent TB controls as well as a control group without either latent or active TB. Importantly, the ALS was the only assay that could differentiate active TB from latent TB, while neither TST nor QFTG could discriminate active from latent TB (**Study II**). This result suggests that the ALS may be useful to diagnose active TB disease, especially in TB endemic areas where latent TB is very common. Some latent TB cases (16%) presented a positive ALS response that could be indicative of subclinical TB [432], which is associated with an increased risk of developing active TB infection. Previous studies of the ALS assay performed by our colleagues in Dhaka, Bangladesh, have demonstrated efficient diagnostic capacity of ALS in patients with sputum-positive TB [431] and also in children [447]. Interestingly, Mtb-specific plasmablast responses decrease after 2-6 months of successful standard chemotherapy [432], while patients with incurable MDR-TB maintain high IgG titers [448]. Thus, the ALS assay may also be used to monitor the response to anti-TB treatment in order to predict the development of MDR-TB early on.

It has been shown that BCG is the superior antigen to detect Mtb-specific plasmablasts responses compared to PPD, LAM or selected RD1-encoded antigens [449]. However, work is in progress to try and improve the format of the ALS assay including selection of a new antigen that could further enhance the specificity of the ALS assay.

4.2.2.2 The ALS assay is particularly useful to detect TB in HIV-infected cases

It is difficult to diagnose active TB in TB/HIV co-infected patients, especially in patients with CD4 T cell counts less than 200 cells/mm^3. In **Study II**, we demonstrated that BCG-specific IgG titers were significantly higher in TB/HIV co-infected patients compared to latent TB (Figure 11) and non-TB controls, but also higher compared to HIV-negative patients with active TB (**Study II**).

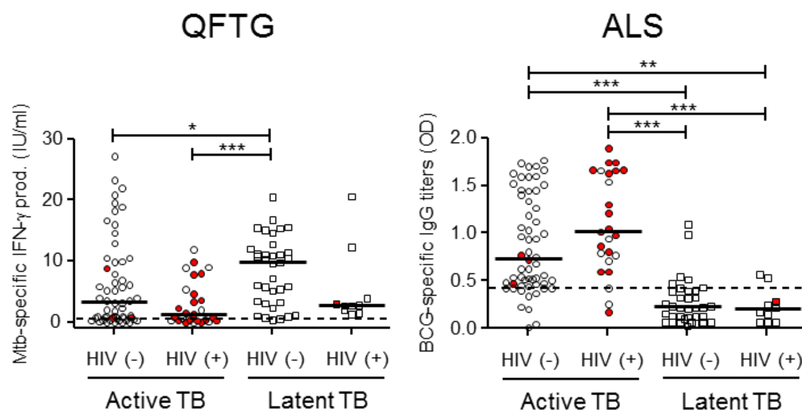

Figure 11. Mtb-specific IFN-γ production (QFTG) and BCG-specific plasmablasts (ALS) in HIV-negative and HIV-positive individuals with active TB (circles) or latent TB (squares). Red symbols represent patients with a CD4 T cell count < 200 cells/ml. The solid bars indicate the median values for each group while the dashed line indicates the cutoff.

Accordingly, the sensitivity of the ALS assay was slightly higher in TB/HIV co-infected (91%) compared to HIV-negative patients with active TB (86%) (Table 2). These results are very promising and suggest that the ALS could be used for effective diagnosis of active TB also among HIV-infected patients with low CD4 T cells counts.

Table 2. Diagnostic performance of the ALS assay

Compared with healthy controls	All TB	Pulmonary TB	Pleural TB	Lymph node TB
Sensitivity HIV-neg (%)	86	80	87	84
Sensitivity HIV-pos (%)	91	90	80	100
Specificity HIV-neg (%)	94	94	94	94
Specificity HIV-pos (%)	94	94	91	91

HIV infection could give rise to different types of B cell abnormalities; including selective loss of antigen-specific memory B cells [450] but also hypergammaglobulinemia and the production of polyclonal antibodies by activated naive B cells [451, 452]. Hypergammaglobulinemia has also been observed in TB and leprosy infections both in the presence and absence of HIV infection [453]. Hypergammaglobulinemia in HIV has been suggested to involve T follicular helper cells producing the B cell stimulatory cytokine IL-21 as well as IL-10 that may promote the differentiation of IgG-secreting plasma cells [454]. Interestingly, we have found up-regulated mRNA levels of total IgG as well as IL-21, particularly in PBMC samples obtained from the TB/HIV co-infected patients included in **Study II** (unpublished observations). Moreover, significantly higher serum levels of total IgG have been found in patients with chronic HIV-1 infection compared to healthy controls and raised IgG levels were also found in HAART-naïve compared to HAART-treated HIV patients [455]. This increase in serum IgG correlated with high serum levels of the immune activation marker, soluble CD27, which could promote IgG-secretion by recently antigen-experienced B cells in vitro [455]. Thus, the continued exposure to microbial antigens during chronic infection with TB and/or HIV may enhance B cell activation and antibody-mediated immunity, especially in patients with impaired T cell responses. It will be important to continue and study the mechanisms behind the increased level of BCG-specific plasamblast responses in TB-infected as well as TB/HIV co-infected patients. Here, it would also be interesting to study the presence of different immunoglobulin isotypes (IgE, IgG1-4, IgM, IgA), the specificity of these antibodies and the cytokine profile in the ALS supernatants.

4.2.2.3 The basis of the ALS assay is different compared to conventional serology

The theoretical basis for the ALS assay is that in a steady-state, migratory and IgG-secreting plasmablasts among PBMCs are very low, but significantly elevated upon continuous antigen exposure ie. during active TB infection [433, 434]. HIV infection is also associated with an increased frequency of antibody-secreting short-lived plasmablasts [454]. Antibody-secreting cells leave the B cell follicles of the secondary lymphoid tissues as plasmablasts [433] and are later found in the bone marrow with the phenotype of mature plasma cells [456, 457]. Accordingly, antibody-secreting cells are temporarily present in the peripheral circulation of patients with inflammatory diseases [458]. Consistent with the findings of increased BCG-specific IgG titers detected in the ALS supernatants, we found significantly elevated levels of circulating $CD3^{-}CD19^{+}CD20^{-}CD27^{high}CD38^{high}$ plasmablasts expressing membrane-bound IgG in PBMC samples from patients with active TB disease (**Study II**). It has been shown that plasmablasts migrate toward CXCL9, CXCL12 [433], CXCL10, and CXCL11 into the bone marrow and/or inflamed tissues [459], which is interesting as we found significantly elevated levels of CXCL9 and CXCL10 both in the lung and in the peripheral blood of patients with PTB (**Study III**).

There are considerable differences between the ALS assay and conventional serology (Table 3). In contrast to the ALS assay, stable antibodies in serum are continuously present in the circulation because these are primarily produced by non-migratory plasma cells residing in bone marrow [460]. High levels of serum antibodies were previously shown in TB patients with severe disease [453, 461]. However, serology-based studies generally show highly variable results including suboptimal sensitivity and specificity to detect active TB [462-465].

Table 3. Comparison of the ALS assay and conventional serology

	ALS assay	Serology
Assay methodology	In vitro	In vivo
Clinical specimen	Circulating plasmablasts in PBMC sample	Circulating antibodies in serum sample
Positive response in latent TB infection	No	Yes
Positive response in BCG-vaccination	No	Inconsistent
Positive response in sputum-negative TB patients, EPTB,TB/HIV co-infection, pediatric TB	Yes	Inconsistent
Monitor antimicrobial treatment efficacy	Yes	Inconsistent

In **Study III**, we used conventional serology to study the induction of mycobacteria-specific IgG responses in patients with PTB and pleural TB. BCG-specific IgG titers in plasma as well as BAL fluid was significantly higher in active TB patients, particularly in TB/HIV co-infected patients, compared to healthy controls (**Study III**). Enhanced IgG titers were associated to increase IL-4 levels in HIV-negative patients, but not in HIV-positive patients, which may suggest that humoral immunity are differentially regulated in TB-infected and TB/HIV co-infected patients.

4.2.3 T cell-based immunodiagnosis

4.2.3.1 TST and QFTG are dependent on cellular immunity

As described above, mycobacteria-specific plasmablasts should only be present in the circulation during active TB infection and not during latent TB infection or in healthy

individuals. In contrast, peripheral Mtb-specific effector memory T cells will persist [466] in patients with active TB and also in individuals with latent TB and therefore neither QFTG nor TST can discriminate active TB from latent TB [12]. Thus, the relevance of these tests become small in high-endemic countries, whereas the TST and QFTG assays can be successfully used to screen TB-exposed contacts in low-endemic countries where latent TB is rare [435]. It has also been shown that a combined use of TST and QFTG may increase the sensitivity of these assays to detect active TB [467]. Compared to TST, QFTG has increased specificity to detect TB in BCG-vaccinated individuals and in patients with exposure to or infection with non-tuberculous mycobacteria [468, 469]. However, the diagnostic performance of both TST and QFTG is dependent on the number of peripheral CD4 T cells [437]. We observed significantly reduced levels of $CD3^+$ and $CD4^+$ T cell numbers in blood from active TB patients compared to both latent TB cases and non-TB controls (**Study II**). Accordingly, IFN-γ production was significantly higher in HIV-negative individuals with latent TB and we found a positive correlation between IFN-γ and CD4 T cell counts in latent TB cases and also in patients with a local lymph node TB (**Study II**). In contrast to QFTG, active TB patients with low CD4 T cell counts had higher ALS responses and demonstrated an inverse correlation between BCG-specific IgG titers and blood CD4 T cell counts (**Study II**). A schematic outline of the differences between the ALS, QFTG and TST assays are shown in Table 4.

Table 4. B cell- vs T cell-based immunodiagnostic assay

ALS	QFTG	TST
In vitro assay	In vitro assay	In vivo assay
PBMC samples	Whole blood	Skin test
Spontaneous release of IgG	Mtb-stimulated IFN-γ	PPD-stimulated DTH
BCG-ELISA	IFN-γ-ELISA	Measure skin induration
Incubation time 24-72hours	Incubation time 16-24hours	48-72 hours
Positive in active and subclinical TB, TB/HIV co-infection, pediatric TB.	Positive in active and latent TB. Inconsistent in TB/HIV and pediatric TB.	Positive in active and latent TB, MOTT, BCG vaccination, old TB infection. Inconsistent in TB/HIV and pediatric TB.
Negative in latent TB, MOTT and BCG vaccination	Negative in MOTT and BCG vaccination	Negative in healthy individuals

In our Ethiopian study cohort, 21% QFTG-negative (15% false negative and 6% indeterminate) results were found among patients with active TB (**Study II**), which is in agreement with a meta-analysis performed in 2011 [470, 471]. As expected TB/HIV co-infected patients have a lower sensitivity of the QFTG [472] and consequently all our indeterminant QFTG-results were from TB/HIV co-infected patients. Interestingly, the performance of the QFTG as well as the T-SPOT.TB assays could be improved by using cells from the site of Mtb infection including BAL [473] and pleural [474] fluid samples instead of whole blood, since effector T cells are accumulated at the disease sites [473] .

4.2.3.2 IP-10 is a promising biomarker that could improve diagnosis of TB

Another immunodiagnostic biomarker of TB that has been suggested to be superior of IFN-γ, is detection of IFN-γ inducible protein10, IP-10 or CXCL10, which has been shown to be particularly more robust to detect active or latent TB in adults [475], young children [476] and in HIV-infected individuals with low CD4 cell counts [477-480]. Here, IP-10 may be a better biomarker of active TB disease compared to IFN-γ, since IP-10 is mostly produced by monocytes and macrophages rather than

CD4+ T cells. Importantly, even if IP-10 is an amplified signal of IFN-γ [481], a small number of IFN-γ-secreting cells is enough to induce large quantities of IP-10 secretion. In line with this notion, we found significantly elevated levels of IP-10 in both BAL and plasma samples from patients with active PTB, even though the levels of IFN-γ were low in these patients (**Study III**). Here, we also found a strong negative correlation ($r=-0.75$; $p<0.0001$) between IP-10 and peripheral CD4 T cell counts and particularly high IP-10 levels in TB/HIV co-infected patients (**Study III**). Consistent with our results, IP-10 was shown to generate indeterminate results only with CD4 T cell counts < 50 cells/ml, whereas QFTG generated indeterminate results with CD4 T cell counts < 200 cells/ml [479]. Accordingly, the IP-10-based assay produced around 50% less indeterminate results when compared with QFTG [479]. IP-10 can be assessed in the same Mtb-stimulated and unstimulated plasma samples obtained using the QFTG kit, which provide the possibility to directly compare the diagnostic performance of IP-10 and IFN-γ in the patient cohorts [482]. Recently, it was reported that in vivo levels of IP-10 can also be measured in dried plasma spots placed on filter paper with a diagnostic accuracy comparable to the QFTG [483]. Such a simplified method is preferable to use in field studies and also provide the possibility to send samples via conventional mail over long distances for analysis without affecting the results.

4.3 HOST IMMUNE RESPONSES IN MTB INFECTION

Deregulated inflammation represents a central pathogenic feature of numerous microbial infections including TB [484]. To explore these immunopathogenic events in human TB, immune responses were studied at the local site of infection in Ethiopian children with lymph node TB (**Study I**) and also in adult Ethiopian patients with lymph node TB (**ongoing Study IV**) (Table 5), PTB or pleural TB (**Study III**).

Table 5. Demography of adult patients with lymph node TB (ongoing Study IV)

	Active TB (HIV-neg)	TB/HIV co-infection	Control (HIV-neg)	Control (HIV-pos)
Number	10	8	5	5
Median age in years (range)	24 (18-46)	35 (26-49)	22 (18-30)	40 (25-40)
Male:Female	8:2	5:3	3:2	2:3
TB lymphadenitis (histopathology)	10 (100%)	8 (100%)	0 (0%)	0 (0%)
Reactive hyperplasia (histopathology)	0 (0%)	0 (0%)	5 (100%)	5 (100%)
Mean CD4 (range)	669 (355-1233)	203 (52-488)	609 (336-1051)	320 (230-459)
Mean CD8 (range	572 (315-1928)	818 (368-1528)	1608 (389-1746)	1146 (639-1961)

4.3.1 Altered tissue architecture and cellular composition in active TB

In chronic infections such as TB, it is of significant relevance to study host-pathogen interactions in the infected tissue because effector T cells are recruited to and accumulate at the local site of bacterial replication [474, 485, 486]. Thus, our research group has developed a tissue-based technological platform to study expression and distribution of immune cells and effector molecules in Mtb-infected tissue biopsies (**Study I and IV**).

4.3.1.1 Granuloma formation and tissue remodeling is enhanced in lymph node TB

Mtb induces formation of granulomas, collections of inflammatory myeloid cells and effector lymphocytes at the site of infection, which are important for the control of bacterial replication [86]. Therefore, it is important to study granulomatous inflammation in the physiological context of the Mtb-infected tissue. In **Study I and IV**, we had the opportunity to dissect local immune responses in Mtb-infected lymph nodes using microscopy and computerized image analysis. As expected, the lymphoid structure was replaced by confluent clusters of $CD68^{+}$ macrophages surrounded mostly by $CD3^{+}$ and $CD4^{+}$ T cells forming granulomas of different sizes and shapes (**Study I and IV**). Accordingly, the cellularity of the Mtb-infected tissues was significantly lower compared to the reactive lymphadenitis controls (**Study I and IV**). Granuloma formation was typically accompanied with different types of tissue remodeling features such as necrosis, fibrosis and collagen deposition, ranging from solid to necrotic granulomas [43] (**Study I and IV**). In adult Mtb-infected lymph nodes, hematoxylin and eosin stained tissue sections were used to classify the TB granulomas into four stages (Table 6) depending on the morphological features of the granuloma (**Study IV**).

Table 6. Histopathological staging of granulomas in active TB

	Appearance:	Necrosis:
Stage 1	Irregular clusters of epithelioid cells interspersed with lymphocytes.	No
Stage 2	Epitheloid cell aggregates with sharp border.	Minimal, if present.
Stage 3	Clusters of epithelioid cells with sharp borders.	Yes
Stage 4	Large, irregular, multi-centric epithelioid cell clusters with prominent necrosis.	Necrosis is occupying greater part of the TB lesion.

Most of the granulomas in HIV-negative TB patients were non-necrotic (stage 1 and 2), whereas 66% of the granulomas in TB/HIV co-infected lymph nodes had extensive necrosis (stage 3 and 4) (**Study IV**) (Figure 12). This finding is in agreement with an IHC-based study performed with human lung tissue, which revealed extensive necrosis in TB/HIV co-infected patients [487]. Now, we plan to continue to dissect the local inflammatory responses in the tissue to determine which host factors could contribute to the fibrotic and necrotic process.

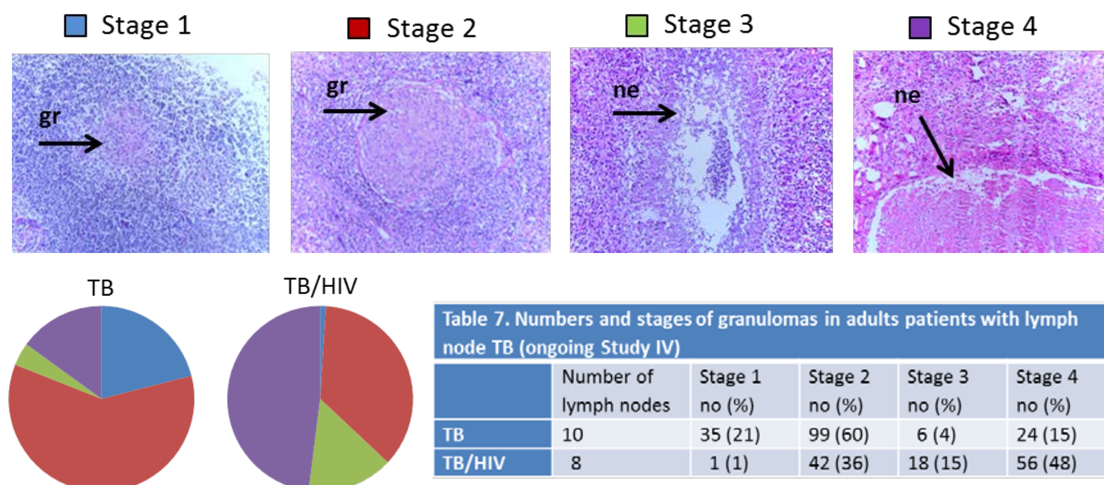

Table 7. Numbers and stages of granulomas in adults patients with lymph node TB (ongoing Study IV)

	Number of lymph nodes	Stage 1 no (%)	Stage 2 no (%)	Stage 3 no (%)	Stage 4 no (%)
TB	10	35 (21)	99 (60)	6 (4)	24 (15)
TB/HIV	8	1 (1)	42 (36)	18 (15)	56 (48)

Figure 12. Hematoxylin/eosin stain of paraffin-embedded lymph nodes showing the stages of granuloma formation in TB. The pie charts illustrate the relative granuloma staging in HIV-negative TB patients compared to TB/HIV co-infected patients. gr=granuloma; ne=necrosis.

In contrast to lymph node TB in children, $CD3^+$ T cells including $CD4^+$ and $CD8^+$ T cells were significantly reduced in lymph node tissue from both TB single-infected as well as TB/HIV co-infected patients (**Study IV**) (Figure 13). This may be because of more severe necrosis and fibrosis observed in adult compared to children´s lymph nodes (**Study I and IV**). $CD68^+$ macrophage-rich granulomas contained an accumulation of Mtb-complex specific antigens including MPT64 (**Study I**) and *M. bovis* BCG (**Study IV**), which has previously been shown to be present in granulomas of both human lung [170] and lymph nodes [488]. Quantitative image analysis demonstrated significantly elevated levels of $CD68^+$ macrophages and memory $CD45RO^+$ T cells but decreased levels of $CD20^+$ B cells in Mtb-infected lymph nodes from children (**Study I**). Similar to children, adult lymph nodes had reduced levels of $CD20^+$ B cells but increased levels of $CD68^+$ macrophages (**Study IV**) (Figure 12). Thus, B cell follicles were distorted in the Mtb-infected tissue and the normal lymphoid tissue was displaced by the granulomatous inflammation. Contrary, development of B cell containing secondary lymphoid structures have been described in close proximity to TB granulomas in the lung that may play a role in the control of local host-pathogen interactions [170].

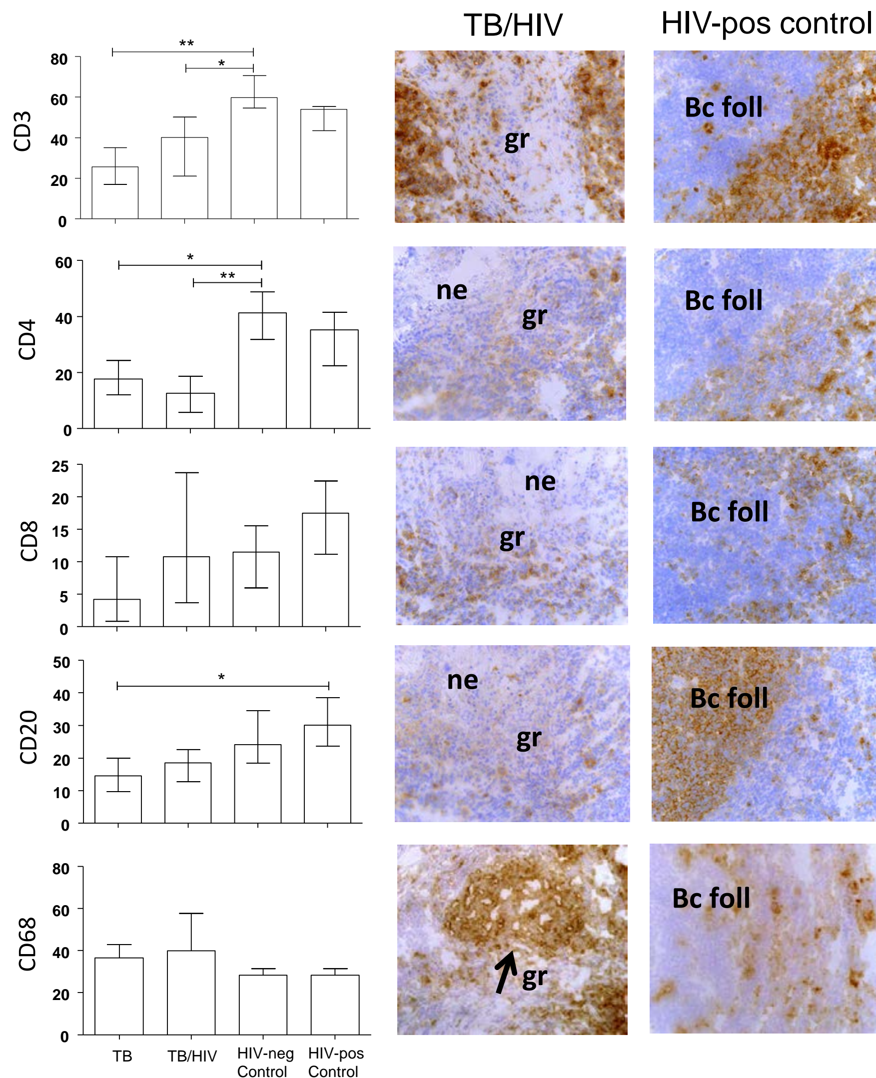

Figure 13. In situ image analysis of CD3, CD4, CD8, CD20 and CD68 protein expression (% positive area of total cell area) in lymph node tissue from adult TB patients. Representative IHC images are shown to the right. gr=granuloma; ne=necrosis; Bc foll=B cell follicle.

4.3.1.2 A shift towards a Th2 and/or Treg response is evident in lymph node TB

Protective immunity in TB has been shown to be dependent on Th1 $CD4^+$ T cells producing IFN-γ and TNF-α that support the differentiation of CTLs producing granule-associated cytolytic effector molecules [489]. mRNA analysis of cytokine profile, demonstrated a weak induction of Th1 cytokines in the Mtb-infected tissue including IFN-γ, TNF-α and IL-17 (**Study I**). Consequently, there was no up-regulation of cytolytic and antimicrobial effector molecules, perforin and granulysin, in TB lymph nodes compared the control group, either at the mRNA or protein level (**Study I**). These results were consistent with our previous findings showing an impaired expression of perforin and granulysin in $CD8^+$ T cells present in TB lung lesions of patients with chronic TB [490]. Instead, we found significantly elevated levels of IL-13 and TGF-β in TB compared to the controls (**Study I and IV**), which are known to support a shift towards a Th2 or Treg immune response. An anti-inflammatory cytokine milieu may promote alternative macrophage activation and support the function of Treg cells [86]. Whereas IFN-γ promotes classical M1 macrophage activation and a hostile milieu in the Mtb-infected tissue, Th2 cytokines such as IL-4 and IL-13 could promote activation of TGF-β-producing M2 macrophages that contribute to collagen deposition in the inflamed tissue [96], which are typical traits of advanced TB disease. Here, TGF-β secretion by macrophages has been shown to be primarily associated with increased collagen synthesis by fibroblasts in chronic lung inflammation [491]. It has also been described that initial induction of iNOS expression in alveolar macrophages in the lung was followed by arginase expression, which would support a switch in macrophage polarization upon progression of TB disease [492].

mRNA of Mtb-infected lymph node tissue also demonstrated a significant increase in FoxP3 (**Study I and IV**) and IL-10 (**Study IV**) compared to the control groups. $CD4^+CD25^+FoxP3^+$ Treg cells suppress Th1 cell responses in patients with TB [143], partly via the secretion of IL-10 and TGF-β [142]. Thus, the frequency of Treg cells in blood has been found to be higher in active TB patients compared to healthy controls and Tregs were also higher in sputum-positive compared to sputum-negative TB patients [493]. Successful anti-TB treatment reduced the peripheral Treg cell frequency to control levels in active TB patients [493].

4.3.2 Compartmentalization of immune responses in active TB

Most studies on human TB involve cells from the peripheral blood, which may not provide a representative image of the specific immune responses present at the site of the infected organ or in the microenvironment of the granulomatous lesions [494]. Here, we were able to perform exclusive analysis of immune responses in lymph node granulomas (**Study I and IV**) and also in BAL and pleura from patients with active TB (**Study III**).

4.3.2.1 Impaired $CD8^+$ CTL responses are associated with elevated Treg cells in the TB granuloma

The TB granuloma is a dynamic structure and the balance of local immune responses in the granuloma will dictate bacterial control and pathology. In **Study I**, we used our software programme (Leica Qwin) to specifically assess protein expression in the TB

granulomas compared to the total lymph node tissue obtained from children with a TB lymphadenitis (Figure 14). Microscopy and in situ image analysis demonstrated that $CD8^+$ T cells expressing perforin and granulysin were scarce inside the granulomas, while granzyme A levels were expressed at similar levels inside and outside the granulomas (**Study I**). The importance of perforin and granulysin in killing intracellular Mtb has been previous been established [156]. Impaired CTL responses in the granulomas were associated to an increased proportion of both $FoxP3^+$ Treg cells and TGF-β producing cells inside the granulomas (**Study I**). Here, TGF-β may be produced by Treg cells as well as Mtb-infected macrophages inside the granuloma. A higher abundance of activated $CD8^+$ T cells around the granuloma may indicate defective trafficking of effector T cells into the lesions. Mtb-infected macrophages and $CD4^+$ T cells may fail to provide proper chemokine responses to attract functional CTLs to the site of Mtb infection [495]. Interestingly, high local concentrations of iNOS were found inside the TB granuloma (**Study I**), which may contribute to bacterial control [496]. However, high levels of NO may also reduce IL-1-mediated inflammation indicating that NO has a dual role including antimicrobial activity and immunoregulatory functions in TB infection [497]. The relevance of NO in human TB remains to be fully elucidated.

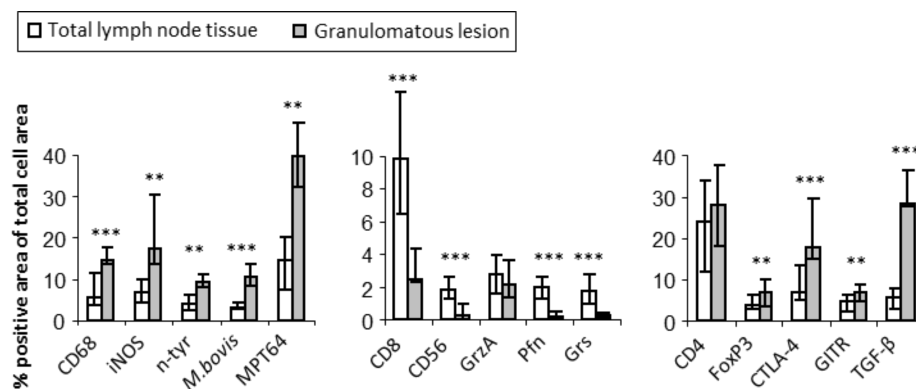

Figure 14. In situ image analysis of protein expression inside lymph node granulomas compared to total lymph node tissue. Expression of CD68, iNOS, nitrotyrosine, *M. bovis* BCG as well as the Mtb-specific antigen MPT64 was significantly increased in the granuloma, which suggests that Mtb-infected macrophages expressing NO are accumulated inside the granuloma. Low expression of $CD8^+$ CTLs and $CD56^+$ NK cells as well as low perforin and granulysin inside the granuloma correlated with an enhanced expression of the Treg cell markers FoxP3, CTLA-4, GITR and TGF-β, suggesting active immunosuppression at the site of Mtb infection.

While previous studies have shown that the granuloma is dominated by macrophages and $CD4^+$ memory T cells, lower numbers of $CD8^+$ T cells are mostly found in the peripheral rim surrounding the TB granuloma [85]. The functional importance of this lymphocyte arrangement is currently unclear. Elevated levels of $FoxP3^+$ Treg cells have previously been observed inside granulomas in both murine and primate TB [139, 140]. Hence, a substantial proportion of the $CD4^+$ T cells may be Treg cells and not IFN-γ-producing T effector cells. Migration of Treg cells but also Th2 cells to sites of bacterial replication could be important to control local inflammation, but may simultaneously inhibit essential Th1 responses. Such compartmentalization of local immune responses may result in impaired antimicrobial effector T cell responses and thus reduce contact-dependent killing of Mtb-infected cells inside the granuloma [498].

4.3.2.2 Pro-inflammatory and Th2 responses are higher at the site of infection in the Mtb-infected lung compared to peripheral blood

Previous studies on TB patients have demonstrated that similar T cell subsets are present in lung tissue and BAL fluid [499]. Multiplex protein analysis of BAL fluid samples (Figure 15) revealed that the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α were significantly elevated in BAL fluid, while only IL-6 was up-regulated in blood from active PTB patients compared to uninfected controls (**Study III**). On the contrary, there was no induction of Th17 or Th1 effector cytokines, IFN-γ, IL-12 and IL-2, in the lung or peripheral circulation, while the Th2 cytokine IL-4 was significantly elevated at both these sites (**Study III**). Even though IFN-γ was only up-regulated in plasma from TB/HIV co-infected patients, we could detect an elevated level of the IFNγ-inducible inflammatory chemokines, CXCL9 and CXCL10, both in lung and blood samples from patients with active TB (**Study III**). This may suggest the presence of an earlier peak of IFN-γ that had already declined, before proper immune control could be established. Interestingly, regulatory DCs selectively recruit Th1 cells via CXCL10 to inhibit Th1 proliferation [500]. Consistent with low levels of IFN-γ, we could not detect an increase in the T cell-chemoattractant CCL5 (**Study III**). IFN-γ may contribute to increased mRNA stability and expression of CCL5 [501, 502]. Instead, the only CC chemokine that was significantly up-regulated in BAL fluid from PTB patients was CCL4 (**Study III**). There is evidence that vaccination induces IL-17-producing $CD4^+$ T cells that populate the lung and, after Mtb challenge, trigger the production of chemokines that recruit IFN-γ-producing $CD4^+$ T cells that contribute to control of bacterial growth [131]. IFN-γ promotes the development of a Th1 cell response [503] and synergizes with TNF-α to activate iNOS-production in macrophages. Moreover, IFN-γ-producing $CD4^+$ T cells are required to induce robust $CD8^+$ T cell responses [79]. Thus, a lack of IFN-γ may lead to a deficiency in both frequency and function of $CD8^+$ CTL in TB. CCL5 is a key chemokine in the recruitment of $CD4^+$ T cells and especially $CD8^+$ T cells [504, 505] to the lung upon microbial infection and has been implicated in classical IFN-γ dominated Th1 responses. IFN-γ cooperates with CCL5 to induce proliferation and activation of certain cytolytic lymphocyte subsets [506].

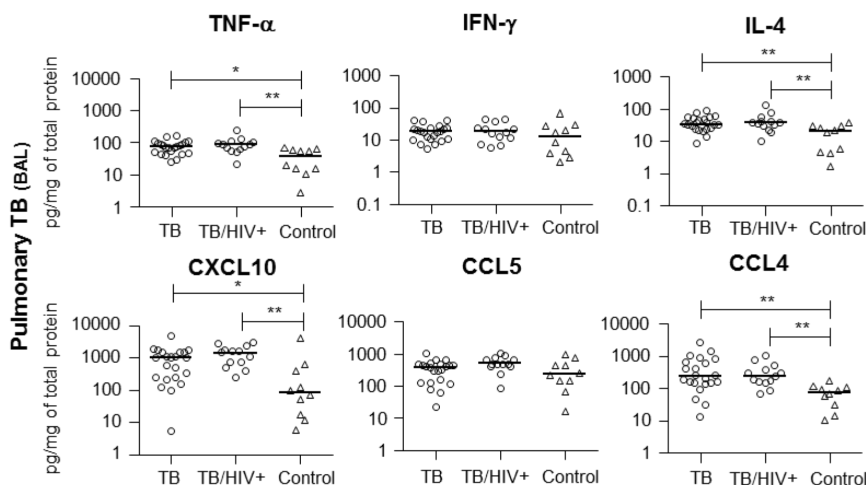

Figure 15. Elevated levels of TNF-α and IL-4 together with CXCL10 and CCL4, while IFN-γ and CCL5 remain low in BAL fluid from patients with pulmonary TB. The solid bar indicate the median for each group.

The above findings suggest an association between low IFN-γ at the site of infection (**Study I and III**) and impaired CD8 T^+ cell responses (**Study I**). Instead, a strong up-regulation of pro-inflammatory cytokines and chemokines in TB disease may promote severe inflammation in the lung (**Study III**). Up-regulation of TNF-α together with IL-4 may lead to tissue destruction rather than immune control, since IL-4 has been reported to be involved in TNF-α-mediated toxicity and tissue fibrosis in patients with PTB [125]. In line with our results, TNF-α levels higher than 29 pg/ml and IL-4 levels higher than 13 pg/ml were considered to signal a high risk for TNF-α induced cytotoxicity and excess inflammation [507]. Interestingly, IL-1β has been shown to directly augment TNF-α signaling in macrophages through the up-regulation of TNF secretion and TNFR1 cell surface expression [508]. IL-1β and TNF-α can also induce the expression of MMP-1 in lung fibroblasts [212], which is the principal mediator of tissue destruction in the lung required to promote dissemination of disease [44]. Furthermore, it has been shown that the protective function of TNF-α can be inhibited by soluble TNFR that has been shown to be elevated in the lungs of BCG-infected mice [509].

4.3.3 Polarization of immune responses in active TB

Although a wide range of cytokines may contribute to the immunological balance in TB infection, a Th1 response dominated by TNF-α and IFN-γ are known as the principal mediators of protective immunity in TB [510], while IL-4 has the opposite effect. It has been shown that IL-4-producing T cells were increased in PBMCs of patients with untreated TB, which declined after two weeks of effective anti-TB treatment [511]. Thus, a delicate balance of Th1 and Th2 responses is of crucial importance to prevent the progression of TB disease [510] .

4.3.3.1 Polarization of a Th2 cytokine response at the site of Mtb infection

As described above, our findings demonstrated that neither Th1 (IFN-γ) or Th17 (IL-17) cytokines were up-regulated in Mtb infected lymph nodes (**Study I**) nor in BAL fluid samples from patients with PTB (**Study III**). Moreover, Mtb-specific IFN-γ production was significantly decreased in blood from active TB patients as compared to individuals with latent TB (**Study II**). Instead, there was a significant up-regulation of IL-13 and TGF-β in tissue from lymph node TB (**Study I and IV**) while IL-4 was significantly up-regulated in BAL and blood from patients with lung TB (**Study III**). Elevated plasma levels of IL-4 and also IL-10 have been shown to coincide with increased levels of both TNF-α and IFN-γ in HIV-negative TB patients and also in TB/HIV co-infected patients as compared to healthy individuals [507]. This implies coexistence of elevated Th1 and Th2 responses in patients with TB. Decreased IFN-γ in active TB patients has also been associated to enhanced mRNA levels of FoxP3, TGF-β and IL-4 in blood cells [512]. We found a modest up-regulation of IFN-γ levels in blood of TB/HIV co-infected patients compared to the uninfected controls (**Study III**). TB/HIV patients may be able to maintain levels of IFN-γ, irrespective of their CD4 T cell counts or viral loads [507].

Although a pathological role of a Th2 response dominated by IL-4 has been described in human TB [125], the properties of such a response has not been fully characterized. Apparently, it is difficult to distinguish IL-4 from IL-4delta2, a splice variant and

inhibitor of IL-4 [513]. mRNA analysis of blood and BAL cells obtained from TB patients showed that radiological findings correlated with a high IL-4/IFN-γ ratio [513]. After chemotherapy, IL-4 mRNA levels remained unchanged, whereas IL-4delta2 increased in parallel with IFN-γ [513]. While HIV/TB co-infected patients showed increased levels of IL-4 and IFN-γ in both blood and BAL, an increase in IL-4delta2 was only detected in BAL [514]. Moreover, healthy individuals with latent TB exhibited a selective increase of IL-4delta2, compared with both TB patients and non-infected individuals [515]. Collectively, these data suggest that an increase in Th1 responses has to be accompanied with an inhibition of Th2 responses in order to acquire long-term control of Mtb infection.

It has been suggested that infection with helminth parasites could promote polarization of Th2 and Treg responses that could suppress imperative Th1 and Th17 responses upon concomitant infection with Mtb [516]. Parasitic gut infections are frequent in most regions where Mtb is endemic and a previous study from Ethiopia have shown that the TST result is affected by parasite infection [517]. However, deworming could improve the responsiveness to PPD [518]. In Africa, active TB has been shown to be associated with a low Th1 and a high Th2 response, whereas healthy exposed individuals have a high Th1/Th2 ratio [123]. In addition, TB patients with a favorable treatment outcome exhibit a higher Th1/Th2 ratio compared to patients with a poor clinical outcome [123]. The exact contribution of parasite-mediated Th2 polarization in human TB needs to further investigated.

4.3.3.2 Th2 polarization may support the induction of CCL4 in active TB

While we could not detect an up-regulation of CCL5 or the monocyte-chemoattractants CCL2 and CCL3, there was a significant up-regulation of CCL4 in BAL fluid from patients with active PTB (**Study III**). It is tempting to speculate that the lack of CCL5 up-regulation (**Study III**) can partly explain low numbers of $CD8^+$ T cells expressing perforin and granulysin in lymph node TB granulomas (**Study I**). It has been described that human T cells co-expressing CCL5 together with perforin and granulysin provide important protection in TB as these cells can kill Mtb-infected cells and also attract immune cells to the site of infection [519]. Instead, the CCL4 gene demonstrates significant association with increased susceptibility to TB [520] and accumulating data provide evidence of an immunoregulatory role of CCL4. Here, it was recently shown that BCG-activated $CD8^+CD39^+$ Treg cells expressed CCL4 and suppressed the proliferative response of antigen-specific $CD4^+$ Th1 cells [521]. Such CCL4-producing $CD8^+$ Tregs were found to be enriched in human lymph node granulomas [522]. Interestingly, IL-4 specifically elevates CCL4 expression and support polarization of regulatory Th2 cells in vivo that suppress Th1-mediated pathogenesis by decreasing the recruitment of activated $CD8^+$ T cells into the pancreatic lesions of diabetic mice [523]. Our findings of elevated levels of CCL4 in the presence of a Th2 shift at the site of Mtb infection may suggest a synergistic interaction between CCL4 and IL-4 also in TB.

4.3.3.3 Th2 polarization is associated to induction of humoral immunity in active TB

Our findings suggest that loss of Th1-mediated cellular immune control is associated with enhanced activation of humoral immune responses in human TB. The shift towards a Th2 cytokine profile in patients with active TB, were found to correlate with

elevated antibody responses determined using the ALS assay (**Study II**) or conventional serology (**Study III and IV**) (Figure 16). These results are supported by previous studies showing that some pathogens elicit a mixture of protective, non-protective and disease-enhancing antibodies [524]. Several studies suggest that B cells and antibodies are required to control mycobacterial infections [166, 525-528], while other studies fail to show any protective effects of antibodies [167]. Possibly, B cell responses may play a role in early protection and the induction of adaptive immunity in TB [529]; however, in the chronic phase of TB infection, enhanced antibody-responses may instead be a consequence of exacerbated TB disease. Humoral immunity is driven by soluble Mtb-antigens that are released and spread from destructive lesions present at the site of Mtb infection, especially in patients with extensive pulmonary TB including cavitary TB [530]. Accordingly, high levels of total and Mtb-specific serum antibodies have previously been shown in patients with advanced TB disease [453, 461].This is in agreement with the results from **Study II**, demonstrating that elevated levels of BCG-specific IgG-secreting cells in the peripheral circulation of patients with active TB, were associated to reduced Mtb-specific IFN-γ production and more severe forms of TB disease. Similar to TB, *Mycobacterium leprae*-specific serum IgG1 antibodies in patients with leprosy show a direct correlation with bacterial load [531] Antibody responses and antibody-secreting CD138$^+$ plasma B cells were also elevated at the site of *M. leprae* infection in skin lesions from patients with advanced disseminated forms of disease [532] which suggest that humoral immunity is not protective but rather associated to progression of mycobacterial disease.

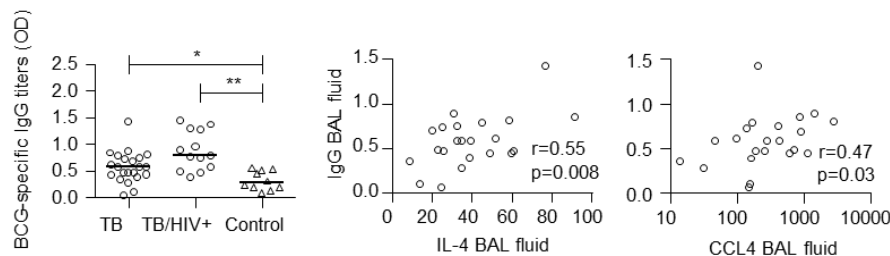

Figure 16. Mycobacteria-specific IgG titers were increased in the BAL fluid of patients with pulmonary TB. There was a positive correlation between IgG titers and protein levels of both IL-4 and CCL4 in the BAL fluid from HIV-negative PTB patients. The solid bars indicate the median for each group.

4.3.3.4 SOCS3 may contribute to Th2 polarization in progressive TB disease

To explore the mechanisms of cytokine polarization in human TB, we assessed mRNA expression of SOCS1 and SOCS3 proteins in BAL cells and PBMCs from patients with active TB (**Study III**). Similarly to IL-4 and CCL4, we found a local up-regulation of both SOCS 1 and SOCS 3 in in the lung of patients with PTB (**Study III**). Accordingly, in vitro Mtb infection of human primary macrophages induced a rapid and strong mRNA expression of both SOCS molecules (**Study III**). Interestingly, we found a positive correlation between both IL-4 and CCL4 with SOCS3 (**Study III**), but not with SOCS1, which may support a functional link between up-regulation of SOCS3 and Th2 cytokine polarization in patients with PTB. It has previously been determined that SOCS3 expression is significantly higher in Th2 than in Th1 cells [533]. Higher expression of SOCS3 have been detected in

whole blood from TB patients and patients with recurrent TB as compared to latent TB and this elevated SOCS3 expression was found to decrease simultaneously as IFN-γ increased after successful chemotherapy [534]. Moreover, SOCS3 overexpression in mice promotes immune polarization of Th2 cells [314] and suppression of Th17 responses [316]. Likewise, T cells from patients with Th2-mediated allergy have a high SOCS3 expression that correlates with disease pathology including the induction of antibody-mediated immunity [535]. Additional longitudinal studies may determine the usefulness of SOCS3 as a prognostic biomarker for the risk of TB in latent TB cases [536].

4.3.3.5 Th2 polarization is enhanced in patients with severe forms of TB disease

For patients most severely affected by TB, morbidity and mortality of the infection is partly the result of a pathological host immune response, which leads to tissue damage and loss of lung function [124, 537]. To investigate the role of a Th2 polarized immune response in severe forms of TB disease (**Study III**), an experienced radiologist graded disease severity in patients with PTB using chest X-ray findings into mild and moderate-to-severe TB disease according to the criteria described in Table 8. Thus, chest X-ray was used to grade disease severity in PTB but also to identify accumulation of fluid in the pleural space (Figure 17), which is consistent with extrapulmonary pleural TB. In the absence of pulmonary infiltrates or lesions, pleural TB is considered a milder form of TB disease [538].

Table 8. Chest X-ray grading to determine severity of TB disease in the lung of PTB patients

Mild	Non-confluent unilateral or bilateral infiltrates without noticeable cavitations. The extent do not exceed the volume of the lung on one side above the 2nd costochondral junction.
Moderate-to-severe	Disseminated lesions of slight to moderate density that may extend throughout the total volume of one lung or equivalent volume in both lungs. Alt. dense confluent lesion(s) that is limited to 1/3 of the volume of one lung lobe. Diameter of any cavitation must be <4cm.

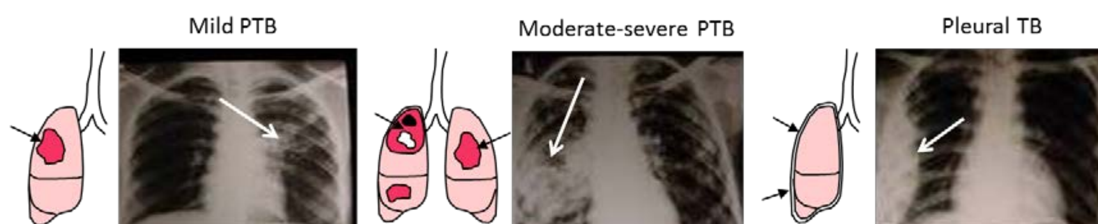

Figure 17. Representative chest X-ray images from PTB patients showing mild and moderate-to-severe TB disease. To the right, also chest X-ray image from a patient with pleural TB showing a collection of pleural fluid in the pleural cavity. The arrows indicate the lesion/infiltrate or pleural effusion.

We found significantly elevated levels of IL-4, CCL4 and SOCS3 in HIV-negative patients with severe-to-moderate compared to mild TB disease (**Study III**) (Figure 18). This difference was not evident in TB/HIV co-infected patients. Atypical chest X-ray findings are common in TB/HIV co-infected patients and therefore radiological grading may become less powerful in this group of patients.

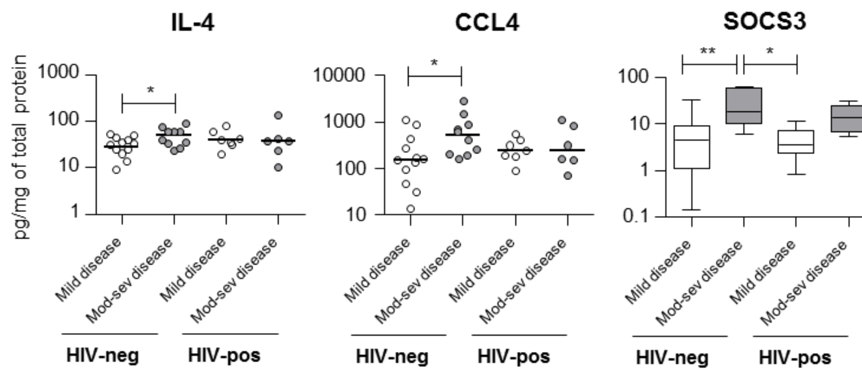

Figure 18. Increased expression of IL-4, CCL4 and SOCS3 in the BAL fluid of HIV-negative patients with moderate-to-severe PTB compared to patients with mild TB disease. The solid bars indicate the median for each group.

Previously, IL-4 levels were shown to be relatively higher in patients with cavitary TB (moderate-to-advanced disease) [539]. In addition, patients with miliary TB (extensive TB disease), had significantly lower levels of IFN-γ but higher IL-4 levels in BAL fluid cells compared to peripheral blood [540]. These results suggest that there may be a skewing of the local cytokine profile toward a Th2 response, especially in patients with severe forms of TB disease [540]. This notion is supported by analysis of pleural fluid from patients with pleural TB, showing a significant up-regulation of both IFN-γ and CCL5 in the absence of Th2 skewing including low levels of IL-4, CCL4 and SOCS3 (**Study III**). Accordingly, significantly higher IFN-γ levels have been shown in pleural fluid as compared to peripheral blood from patients with pleural TB, which confirm the presence of a predominant Th1 response in patients with a localized, less severe form of TB [540].

A role of humoral immunity in severe forms of TB disease, is also supported by our findings of elevated BCG-specific plasmablasts responses (**Study II**) as well as higher plasma IgG titers (**Study III**) in TB/HIV co-infected patients with low CD4 T cell counts (< 200 cells/mm^3). In addition, BCG-specific IgG titers in plasma were significantly higher in PTB patients (particularly in TB/HIV co-infected patients) compared to patients with extrapulmonary pleural TB (**Study III**) or lymph node TB (**Study IV**) (Figure 19). Collectively, these results suggest that we could use different forms of clinical TB as important tools or model systems to study immune polarization in human TB.

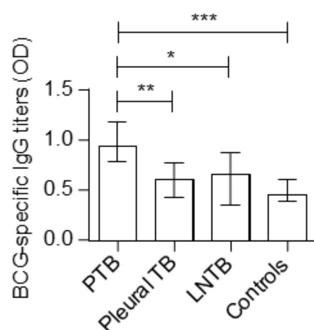

Figure 19. Mycobacteria-specific IgG titers were increased in plasma from patients with PTB (n=35) compared to patients with either pleural TB (n=23) or lymph node TB (n=18) as well as uninfected controls (n=10). Results are presented as median +/- interquartile range.

5 CONCLUDING REMARKS

This thesis provides novel insight into the immunopathogenic events that occur at the site of infection and in the peripheral circulation of patients with active TB. Based on studies of two clinical cohorts from Ethiopia, we were able to demonstrate that:

1.) the novel ALS test can be used to detect active TB in both HIV-negative and HIV-positive sputum-negative patients with PTB as well as EPTB.

- Elevated BCG-specific IgG titers as well as IgG-expressing plasmablasts in samples from active TB patients compared to latent TB and non-TB controls.
- Particularly high BCG-specific IgG titers in TB/HIV co-infected patients with low CD4 T cell counts and low Mtb-specific IFN-γ production.
- Inverse correlation between BCG-specific IgG titers and peripheral T cell counts in patients with active TB.

2.) progression of active TB is associated with polarization of immune responses from a Th1 towards a Th2 and/or immunoregulatory profile at the site of Mtb infection.

- Low Th1/Th17 (IFN-γ, IL-17) responses and impaired perforin and granulysin expression in CD8+ CTLs.
- Elevated Th2 (IL-4 or IL-13) responses as well as elevated IgG antibody responses and enhanced expression of CCL4 and SOCS-3.
- Enhanced Treg cell (FoxP3, CTLA-4, GITR) responses and elevated levels of TGF-β and/or IL-10.

The shift from a Th1 towards a Th2 cytokine/chemokine profile may explain the impaired CTL responses but enhanced antibody responses observed in patients with progression of clinical TB. Similarly, enhanced plasmablast responses detected with the ALS assay, were associated with lower IFN-γ responses and progression of TB, which suggest that the ALS assay represents an adverse immune response resulting from impaired cellular immunity in chronic TB. To improve clinical management of TB, we need new specific biomarkers ie. Mtb-specific plasmablasts, to facilitate an early and rapid diagnosis of active TB. Methods to follow disease prognosis in patients with active TB may also involve a combination of markers ie. IL-4/CCL4/SOCS3 used as novel immune response signatures to predict disease progression and outcome.

In June 2009, our **Study I** was selected for press release by the American Journal of Pathology: "*TB: hiding in plain sight*", that stress the importance to perform studies at the site of Mtb infection as we found a compartmentalization of immune responses in the granulomas. Likewise, it is an advantage to compare findings from peripheral blood to clinical samples obtained from the disease sites i.e. lung (BAL) or pleura. We aim to continue to explore the Th1/Th2 balance in the clinical material obtained from this thesis and to combine this work with functional experiments performed using in vitro model systems and virulent Mtb strains. In addition, the results from **Study II** were highlighted in the editorial of Thorax, 68(3):204-6, 2013: "*TB immunodiagnosis: delving below the surface*". Now, we continue the important work with the ALS assay and currently test the diagnostic performance of the ALS among TB suspects recruited in a low-endemic setting in Sweden. In addition, the ALS assay is currently used to monitor treatment efficacy in a clinical trial in Ethiopia, where we test the antimicrobial effects of adjunctive immunotherapy with vitamin D and phenylbutyrate in HIV-negative patients with PTB (ClinicalTrials.gov Identifier: NCT01698476).

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