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LOCAL IMMUNE RESPONSES IN TUBERCULOSIS: CYTOLYTIC EFFECTOR FUNCTIONS AT THE SITE OF *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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MY FATHER
MY LIFETIME HERO

"But I have promises to keep
And miles to go before I sleep……."
-Robert Lee Frost

ABSTRACT

Despite recent advances in tuberculosis (TB) research, shortage of knowledge still exists that limits the understanding of host-pathogen interactions in human TB. Cell-mediated immunity has been shown to confer protection in TB, although the relative importance of cytolytic T cells (CTLs) expressing granule-associated effector molecules perforin and granulysin is debated. A typical hallmark of TB is granuloma formation, which includes organized collections of immune cells that form around *Mycobacterium tuberculosis* (Mtb)-infected macrophages to contain Mtb infection in the tissue. This thesis aimed to increase insights to the immunopathogenesis involved in the progression of clinical TB, with an emphasis to explore antimicrobial effector cell responses at the local site of Mtb infection.

A technological platform including quantitative PCR and *in situ* computerized image analysis was established to enable assessment of local immune responses in tissues collected from lung or lymph nodes of patients with active pulmonary TB or extrapulmonary TB. The results from this thesis revealed enhanced inflammation and granuloma formation in Mtb-infected organs from patients with active TB disease. CD68+ macrophages expressing the Mtb-specific antigen MPT64 were abundantly present inside the granulomas, which suggest that the granuloma is the main site of bacterial persistence. Macrophages expressed nitric oxide, while the antimicrobial peptide LL-37 was very low in TB lung lesions compared to distal lung parenchyma. Mtb-infected tissues and particularly the granulomas were enriched with CD3+ T cells, CD4+ T cells and FoxP3+ regulatory T cells (Treg), while the numbers of CD8+ CTLs expressing perforin and granulysin were very low inside the granulomatous lesions. We further observed that mRNA expression of important Th1/Th17 cytokines were not up-regulated in the Mtb-infected tissues. Instead, IL-13 and TGF-β were elevated in lymph node TB, which may suggest a shift of the cytokine response towards a Th2 or immunoregulatory profile. We also detected elevated levels of the B cell stimulatory cytokine IL-21, but also IL-10 in TB lesions from patients with pulmonary TB. Accordingly, chronic TB was associated with an increased expression of CD20+ B cells and IgG-secreting cells as well as FoxP3+ Treg cells in the TB lung lesions. This may suggest that adverse immune responses in progressive TB disease involve enhanced activities of plasma B cells and Treg cells. Next, our findings of impaired CTL responses in human TB were applied to evaluate a novel TB vaccine candidate in a non-human primate model of TB. Our *in situ* technology was used to show that CD8+ T cells as well as perforin, granulysin and the survival cytokine IL-7, were induced locally in the lungs but also spleens of animals that were primed with the novel TB vaccine before Mtb challenge. Thus, immune correlates of protection discovered in human TB could be used as potential biomarkers to evaluate the immunogenicity of novel TB vaccine candidates.

Taken together, our results provide evidence of an impaired CD8+ CTL response at the site of Mtb infection that involves deficient expression of perforin and granulysin. Instead, chronic TB is associated with enhanced levels of antibody-producing B cells with little documented protection in TB. We propose that the induction of Th2 or immunoregulatory cytokines and FoxP3+ Treg cells represents potential immunopathogenic processes that may contribute to impaired cytolytic and antimicrobial effector cell responses in human TB.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their roman numerals.

I. Jan Andersson, Arina Samarina, Joshua Fink, **Sayma Rahman** and Susanna Grundstrom Brighenti.

Impaired expression of perforin and granulysin in CD8+ T cells at the site of infection in human chronic pulmonary tuberculosis

Infection and Immunity, Nov;75(11):5210-22, 2007.

II. **Sayma Rahman**, Berhanu Gudetta, Joshua Fink, Anna Granath, Senait Ashenafi, Abraham Aseffa, Milliard Derbew, Mattias Svensson, Jan Andersson and Susanna Brighenti.

Compartmentalization of immune responses in human tuberculosis: few CD8+ effector T cells but elevated levels of FoxP3+ regulatory T cells in the granulomatous lesions

American Journal of Pathology, Jun;174(6):2211-24, 2009.

III. **Sayma Rahman**, Isabelle Magalhaes, Jubayer Rahman, Raija K. Ahmed, Donata R. Sizemore, Charles A. Scanga, Frank Weichold, Frank Verreck, Ivanela Kondova, Jerry Sadoff, Rigmor Thorstensson, Mats Spångberg, Mattias Svensson, Jan Andersson, Markus Maeurer and Susanna Brighenti.

Prime-boost vaccination with rBCG/rAd35 enhances CD8+ cytolytic T cell responses in lesions from *Mycobacterium tuberculosis*-infected primates

Molecular Medicine, Feb; 18: 647-658, 2012.

IV. **Sayma Rahman**, Anders Rehn, Jubayer Rahman, Jan Andersson, Mattias Svensson and Susanna Brighenti.

Enrichment of FoxP3+ regulatory T cells and IgG-secreting B cells at the local site of infection in human pulmonary tuberculosis

Manuscript

ADDITIONAL PUBLICATIONS

I. Alexander Y. Persson, Robert Blomgran-Julinder, **Sayma Rahman**, Limin Zheng, and Olle Stendahl.

Mycobacterium tuberculosis-induced apoptotic neutrophils trigger a pro-inflammatory response in macrophages through release of heat shock protein 72, acting in synergy with the bacteria

Microbes and Infection, 10(3): p. 233-40, 2008.

II. Lalit Rane, **Sayma Rahman**, Isabelle Magalhaes, Raija Ahmed, Mats Spångberg, Ivanela Kondova, Frank Verreck, Jan Andersson, Susanna Brighenti and Markus Maeurer.

Increased (6 exon) interleukin-7 production after *Mycobacterium tuberculosis* infection and soluble interleukin-7 receptor expression in lung tissue

Genes and Immunity, 12(7): p. 513-22, 2011.

CONTENTS

LIST OF ABBREVIATIONS

TB	Tuberculosis
Mtb	*Mycobacterium tuberculosis*
ManLAM	Mannose-capped Lipoarabinomannan
MDR	Multidrug Resistant
XDR	Extensively Drug Resistant
DOTS	Directly Observed Treatment Short-Course
TST	Tuberculin Skin Test
PPD	Purified Protein Derivative
PCR	Polymerase Chain Reaction
qPCR	Quantitative PCR
BCG	Bacillus Calmette Guerin
rBCG	Recombinant BCG
RD1	Region of Difference 1
AIDS	Acquired Immunodeficiency Syndrome
HIV	Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
MQ	Macrophage
DC	Dendritic cell
APC	Antigen Presenting Cell
NK	Natural Killer Cell
Th	T helper cell
CTL	Cytolytic T lymphocyte
Treg	Regulatory T cell
Breg	Regulatory B cell
MR	Mannose Receptor
CR	Complement Receptor
TLR	Toll like receptor
FasL	Fas Ligand
MMP	Matrix Metalloproteinase
AMP	Antimicrobial Peptide
ROI	Reactive Oxygen Intermediates
RNI	Reactive Nitrogen Intermediates
NO	Nitric Oxide
iNOS	Inducible Nitric oxide Synthase
MIP-1α/β	Macrophage Inflammatory Protein-1 alpha/beta
RANTES	Regulated on Activation Normal T cell Expressed and Secreted
MCP-1	Monocyte Chemotactic Protein-1
CTLA-4	Cytotoxic T lymphocyte Associated Molecule-1
GITR	Glucocorticoid Induced Tumor Necrosis Factor Receptor
FoxP3	Forkhead Box P3
HSP	Heat Shock Protein
EPI	Expanded Programme on Immunization
MGIT	Mycobacterial Growth Indicator Tubes
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme-linked Immunosorbent Spot

1 INTRODUCTION

TUBERCULOSIS: AN ENDURING DANGER TO HUMAN RACE

Tuberculosis (TB) is primarily a chronic lung infection that is one of the most potent and wide-spread human infections today, and a major cause of death from bacterial pathogens [1]. Historically, TB disease has killed more human beings than any infectious disease. In 2012, WHO estimated 1.4 million deaths from TB and 8.7 million new TB cases, which mostly (80%) affect vulnerable populations in 20 high-burden countries [2]. Although TB is a serious global health problem, several medical advances have been made in the past 150 years to facilitate prevention and control of TB. The discovery of ***Mycobacterium tuberculosis* (Mtb)** as the etiological agent of TB was done by Robert Koch in 1882 and enabled the development of the diagnostic Tuberculin Skin Test (TST), which is extensively used in clinical practice. The Bacillus Calmette Guerin (BCG) vaccine was introduced in 1921 and has been administrated in over 4 billion doses world-wide. In addition, the first anti-TB drugs were introduced to the market in 1944, when streptomycin was successfully used to treat TB disease [3]. In general, TB mortality started to decrease in most industrialized countries during the 20th century, probably due to a better socioeconomic status including improved nutrition and living conditions [4]. TB re-emerged during the 1990s both in developing and several industrialized countries partly due to the HIV/AIDS pandemic and also because of an increased emergence of drug resistant Mtb strains [5-7].

Despite several medical advances, we need to increase and improve research on human TB in order to discover new diagnostic methods, more efficient vaccines and novel therapeutic interventions including better drugs. Pharmacological management of TB is extremely resource intensive, especially in developing countries and treatment of multidrug-resistant TB (MDR-TB) increases the costs several fold compared to drug-susceptible TB [8]. Therefore, additional resources are needed to achieve higher treatment completion rates by more intensive follow-up programs like DOTS (Directly Observed Treatment Short-Course) etc. [9], but also by continued investments in research. So far, the most vital questions in understanding disease progression in human TB remain unanswered and we need to learn more about the protective host responses that are accountable for control of Mtb infection in order to develop effective therapy against TB.

2 BACKGROUND

2.1 THE GENUS *MYCOBACTERIUM*

TB infection and disease are most commonly caused by Mtb which is pathogenic in humans and belongs to the genus *Mycobacterium* of the *Mycobacteriaceae* family. Over 100 mycobacterial species have been identified, but the majority of these species are non-pathogenic. The disease-causing myobacteria in mammals with close genetic similarity are categorized in the Mtb-complex, which comprises seven mycobacterial species: *M. tuberculosis*, *M. bovis*, *M. africanum, M. canettii, M. caprae, M. microti and M. pinnipedii* [10]. Mycobacteria are aerobic, non-motile, hydrophobic, rod-shaped, facultative intracellular bacteria with a size of 2-4 µm. The pathogenic species typically replicate slowly with a doubling time of 12 to 24 hours [11], resulting in lengthy cultures of clinical specimens (4-8 weeks) that often cause delays in TB diagnosis.

The lipid-rich cell wall of Mtb is complex and consists of peptidoglycans, unique mycolic acids, arabinogalactan and lipoarabinomannan (LAM) as well as free lipids, and scattered proteins. Interestingly, the mycobacterial cell wall is about twice as thick compared to gram-positive and gram-negative bacteria. The very unique properties of the thick mycobacterial cell wall make it impermeable to many toxic compounds and also deliberate acid-fastness, which can be used for detection of mycobacteria in clinical specimen such as sputum, cell- or tissue samples [1] [Figure 1].

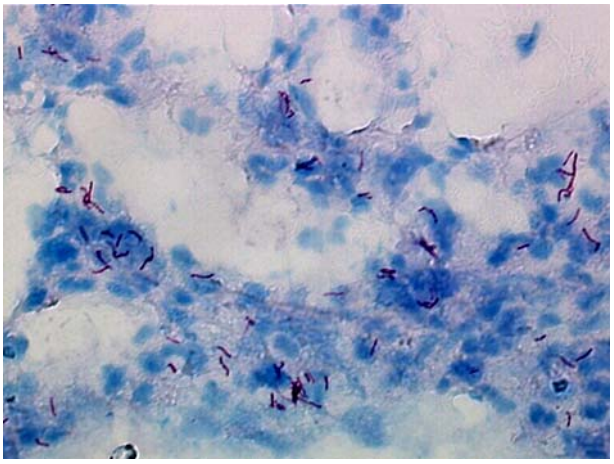

Figure 1. Microscopic image (x125) of acid-fast stained bacilli (red rods) in a tissue section from a TB lung lesion obtained from a patient with chronic pulmonary TB (provided by Susanna Brighenti, Karolinska Institutet).

2.2 INTRACELLULAR UPTAKE AND SURVIVAL OF MTB

Mtb is an intracellular bacterium that primarily infects and reproduces in host **macrophages (MQs)**. Mtb bacilli can bind to different cell surface receptors to ensure intracellular access through phagocytosis. These receptors comprise Toll-like receptors (TLR), complement receptors (CR), mannose receptors (MR), scavenger receptors and DC-SIGN. TLRs play a vital role in the induction of innate immune responses against Mtb and primarily involve Mtb recognition by TLR2, TLR4, TLR9 and also TLR1 or TLR6 that form a heterodimer with TLR2 [12]. Accordingly, TLR engagement has been shown to trigger intracellular killing of Mtb in human MQs [13]. Experimental data also suggest that several receptors may be simultaneously involved during phagocytosis of Mtb [14]. Here, it has been postulated that Mtb uptake through distinct receptors directs the intracellular fate of the bacilli [15]. For example, engagement of mycobacterial mannose-capped LAM (ManLAM) with MR results in restricted phagolysosomal fusion, while TLR2 engagement by Mtb components leads to vitamin D dependent production of antimicrobial peptides that may facilitate phagosomal maturation [16, 17].

Phagocytosis of Mtb initiates innate inflammatory responses that can either result in pathogen clearance or progress to promote the induction of adaptive immune responses including a typical granulomatous type of inflammation associated with chronic infections such as TB [18]. It is well-known that Mtb can survive in infected MQs for extended periods of time, even in the presence of inflammation. Mtb have developed mechanisms to prevent the fusion between phagosomes and lysosomes and thus the bacilli can persist in the endosomal system of the MQ [Figure 2], secure from the toxic contents of the lysosomes.

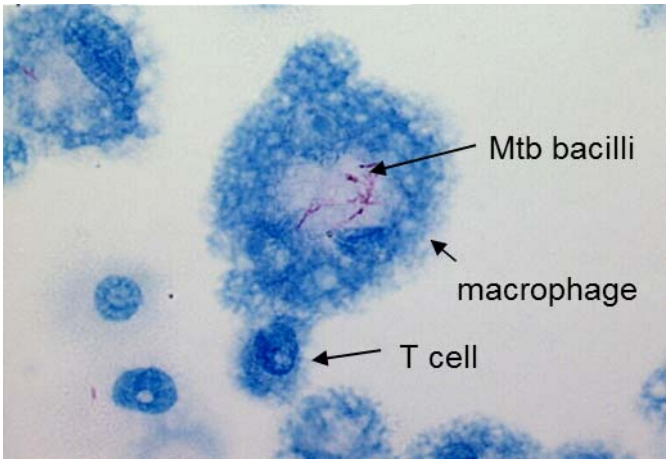

Figure 2. Microscopic image (x600) of acid-fast stained bacilli (red rods) in a culture of human primary blood-derived macrophages and T cells. Note the phagosomal location of intracellular bacilli and the smaller T cells interacting with the large macrophage (provided by Susanna Brighenti, Karolinska Institutet).

2.3 TB INFECTION IN HUMANS

TB is an airborne infection and therefore the lung is the primary site of infection, although infection and disease can be established in any organ in the body [1]. Mycobacteria usually enter the host through inhalation of tiny aerosol droplets expelled from patients with open or active pulmonary TB [19, 20]. Once the bacteria are inside the alveolar space, a cascade of host immune responses starts the battle against the infection including the induction of a granulomatous inflammatory reaction. Approximately 10% of infected individuals will ever develop active disease, while the majority of infected cases contain the mycobacteria in a latent or sub-clinical state [21-23]. Thus, Mtb infection is rarely completely eradicated from the host, but rather persists in a latent state. The outcome of infection is strictly dependent on the balance between the pathogen and the host immune system. Immunocompromised individuals such as HIV infected patients, have a significantly increased risk to develop active TB compared to immunocompetent individuals. Other risk factors for development of active TB disease are poverty and overcrowding living conditions, immunosuppressive treatments including TNF-α inhibitors, diabetes, cancer, malnutrition, age (elderly and children are the most vulnerable groups), alcohol abuse and smoking [24-27]. The probability to develop active TB is highest during the first 1-5 years after the initial exposure. But there are also examples where latent infection has persisted for decades before active TB disease finally progresses.

Human TB is a complex disease with various clinical features. As described above, Mtb primarily infects the lung and cause **pulmonary TB**, while infection of other organs such as lymph nodes or pleura is called **extrapulmonary TB**. Mtb infection can also disseminate throughout the body causing **systemic or miliary TB**, but this is not as common as a localized infection in the lung or other organs. Disease manifestations differ considerably among various age groups. Seemingly, young children do not show typical clinical symptoms of TB, which complicates diagnosis and treatment. Children are more susceptible to develop active TB disease and thus disseminated forms of TB are also more common in children than adults [28]. In contrast, clinical symptoms of active pulmonary TB are more typical in adult patients, including lengthy cough, weight loss, fever, malaise and night sweats.

2.4 THE HUMAN IMMUNE RESPONSE IN TB

2.4.1 Cross-talk between innate and adaptive immune responses

2.4.1.1 Phagocytes

Reciprocal interaction between innate and adaptive immune responses is vital to achieve protective immunity against most pathogens. **Cell-mediated immunity** involving the activation of phagocytes, antigen-specific T cells and the release of specific cytokines is crucial in host defense against Mtb infection [Figure 3]. Upon TB infection in the lung, initial activation of cells of the innate immune defense involves classically phagocytic cells such as resident alveolar MQs, pulmonary

dendritic cells (DCs), monocytes and neutrophils [29]. Mtb can also bind and interact with non-specialized phagocytic cells such as alveolar epithelial cells [30]. Inhaled mycobacteria are engulfed by alveolar MQs that will become activated at the site of infection in the lung. Activated MQs will produce reactive oxygen and nitrogen intermediates (ROI/RNI) as well as antimicrobial peptides (AMPs), which will comprise a first line of defense to limit intracellular bacterial replication and to execute the clearance of bacilli [31-34]. Neutrophils are also acknowledged to confer protection by phagocytosis and killing of Mtb bacilli [35]. Since neutrophils are relatively short-lived, apoptotic neutrophils containing mycobacterial material can be engulfed by and activate MQs and DCs [35, 36]. Importantly, it has been shown that human neutrophil-derived peptides contribute to growth arrest as well as killing of mycobacteria [37]. MQs can also attain the neutrophil-derived antimicrobial peptide lactoferrin and execute bacterial killing [38].

2.4.1.2 Antigen presenting cells (APCs)

MQs and DCs are professional antigen-presenting cells (APCs) that constitute a bridge between innate and adaptive immunity. Activated MQs can present antigens directly to T cells, while DCs may have a more important function in cross-presentation of Mtb antigens [39]. Here, DCs capture Mtb antigens and cell debris from apoptotic Mtb-infected MQs in the local environment and present these antigens to T cells via MHC-I and CD1b molecules [40]. DCs will migrate to the draining lymph nodes to cross-prime naïve T cells [41-43], that will become activated effector T (Teffector) cells. Protein antigens will be presented through MHC-I and MHC-II pathways to activate αβ T cells including Mtb-specific CD4+ and CD8+ T cells that are essential for protective immunity in TB [44]. Because of the lipid-rich nature of the mycobacterial cell wall, lipids and glycolipids will be presented through CD1 molecules to active non-classical T cell subsets such as γδ T cells [45] and CD1 restricted T cells [46-48] that are also known to confer protection against Mtb infection.

2.4.1.3 T cells

Primed T cells egress from the lymph nodes and trace mycobacterial foci in the lung in response to pro-inflammatory cytokines and chemokines produced by Mtb-infected MQs. Subsequent production of a protective cytokine response primarily includes IFN-γ and TNF-α that will be instrumental in the organization of a granulomatous immune response with the aim to prevent continued bacterial growth at the site of infection [49]. In this process, proper activation of microbicidal MQs as well as IFN-γ producing T cells and cytolytic T cells producing cytolytic and antimicrobial effector molecules such as perforin, granulysin and granzymes, are essential to mediate immune protection in TB [50, 51]. Importantly, initiation of the adaptive immune response is delayed in human TB infection and thus the mycobacteria are allowed to increase significantly in numbers already at the early stages of infection [52]. Studies have suggested that priming of Mtb-specific effector T cells may be delayed because of reduced trafficking of DCs carrying Mtb antigens from the lung to the lymph nodes or

because of reduced cross-presentation of Mtb antigens to DCs as a consequence of inhibited apoptosis of infected MQ [53].

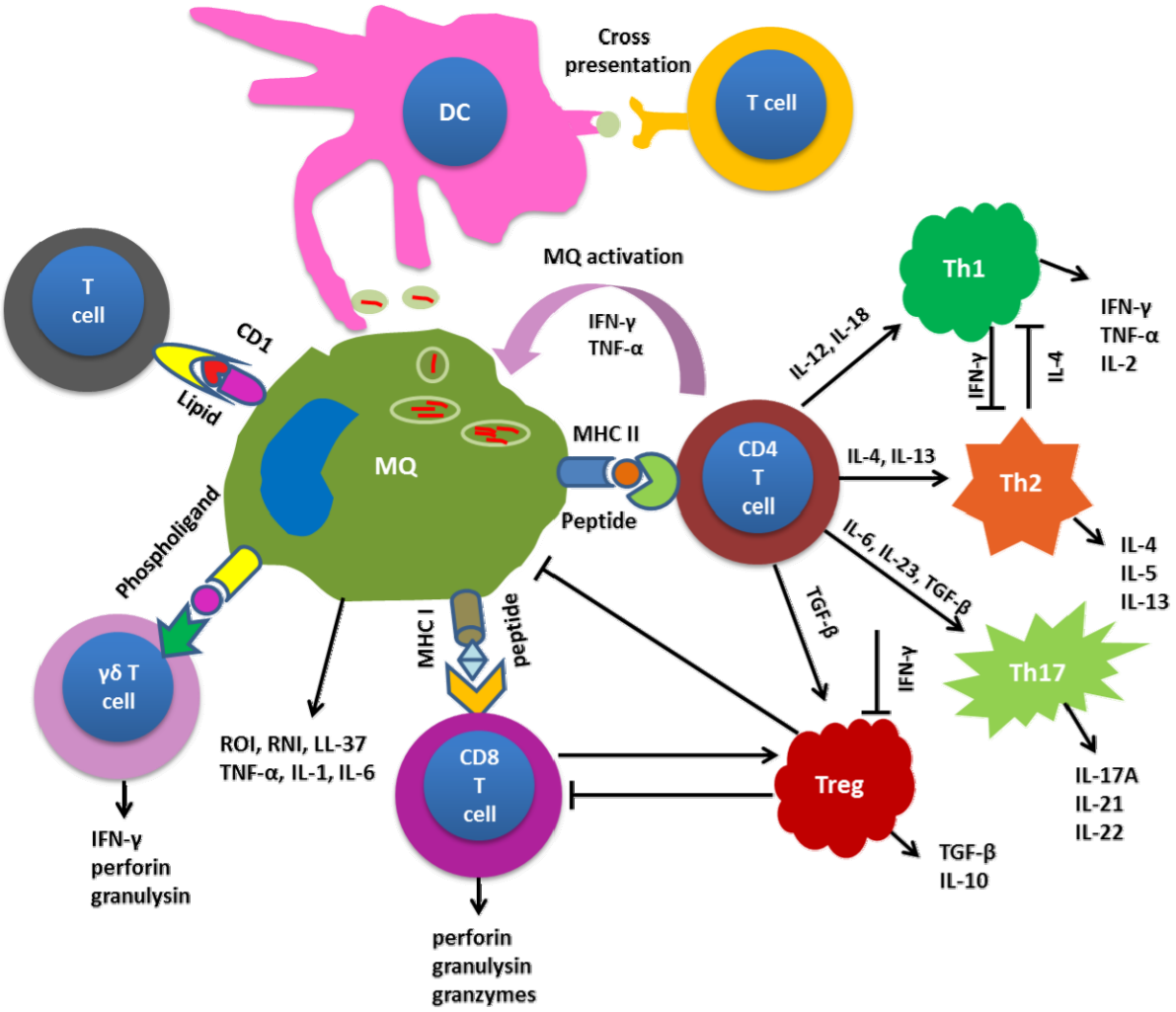

Figure 3. Schematic illustration of the host immune response in human TB. Mtb bacilli primarily persist in the phagosomal system of infected MQ. Activated MQ try to combat the infection through the production of antimicrobial compounds but also through activation of Mtb-specific T cells. APCs such as MQs and DCs take up the bacteria and Mtb-infected cells and present peptide antigens through MHC-I and MHC-II to CD8+ and CD4+ T cells, respectively. While CD8+ T cells differentiate into CTLs, CD4+ T cells differentiate into Th1, Th2 or Th17 cells. Lipid antigens are presented to CD1 restricted T cells and non-peptide phosphate moieties to γδ T cells. A network of protective cytokines and cytolytic effector molecules are produced upon antigen-specific stimulation of the different T cell subsets. However, anti-inflammatory mediators (Th2/Treg cytokines) are also produced to counteract the inflammatory immune response. Fine-tuned interactions between pro- and anti-inflammatory responses determine the outcome of TB infection.

2.4.2 T cell subsets in TB

2.4.2.1 CD4+ T cells

Different subsets of T cells including CD4+, CD8+, γδ and CD1 restricted T cells, are required to control TB infection and prevent clinical progression of TB disease. **$CD4^+$ T cells or T helper (Th) cells** primarily produce cytokines that support proper activation and differentiation of various immune cells including CD8+ cytolytic T cells, B cells, phagocytes and APCs [54-56]. Based on their specific cytokine production, CD4+ Th cells differentiate into one of the major subsets known as **Th1, Th2 or Th17** [57]. Th cells can also become specialized to produce anti-inflammatory or immunoregulatory cytokines [57]. CD4+ Th1 cells mainly producing IFN-γ and TNF-α have been shown to contribute significantly to protective immune responses against Mtb both in humans and rodents [58-60]. Therefore, CD4+ T cell mediated control of TB infection is mainly dependent on CD4+ Th1 and also Th17 cells but not Th2 cells [61]. Importantly, reduction of peripheral CD4+ T cell numbers in HIV infected patients, results in progression of primary TB infection, reactivation of latent TB infection and may also complicate clinical manifestations in TB/HIV co-infected patients [62]. Impairment of CD4+ T cell function including reduced expression of MHC-II molecules increases the susceptibility to TB, which support a pivotal role of CD4+ T cells in TB infection [63]. Thus, Mtb may avoid elimination by limiting the activation of CD4+ effector T cells at the site of Mtb infection in the lungs [64]. Apart from their major function as cytokine producers, CD4+ T cells with cytolytic activities have also been reported in mycobacterial infections [46]. CD4+ cytolytic T cells mediate target cell lysis through the Fas-FasL death-receptor pathway as well as the granule exocytosis pathway [65]. Importantly, a cross-talk between distinct effector T cell subsets in the Mtb-infected lungs has been shown to be crucial to maintain control of TB infection [66]. Thus, a deficiency in CD4+ T cells may impair CD8+ T cell function and increase susceptibility to TB infection [66].

2.4.2.2 CD8+ T cells

CD8+ T cells or cytolytic T cells (CTLs) are the major effector T cell subset that execute killing of Mtb-infected target cells and participate in the memory response to Mtb [67, 68]. Importantly, activated CD8+ CTLs mainly use granule-dependent mechanisms and not the Fas-FasL pathway to induce target cell lysis and intracellular killing of Mtb [69-71]. CTLs are armed with granule-associated cytolytic and antimicrobial effector proteins, perforin and granulysin, which cooperate to eliminate Mtb-infected MQ and bacteria [70, 72]. Although CD8+ T cells are generally considered to be less important than CD4+ T cells to induce protective immunity in TB, failure to induce functional MHC-I restricted T cells in mice with a disruption in the β2 microglobulin gene, provide evidence that CD8+ T cells are important in TB control [73]. It has also been shown that Mtb infection trigger expansion and recruitment of Mtb-specific CD8+ T cells with cytolytic functions to the site of infection in the lungs [74]. In human TB, screening of MHC-I peptides from Mtb proteins resulted in recognition by specific CD8+ CTLs with potent antimicrobial activities [75]. Likewise, reduced numbers of perforin- and

granulysin-expressing effector memory CD8+ T cells in humans significantly enhanced the susceptibility to develop active TB, which also support an essential role of CD8+ T cells in the host defense against Mtb [76].

2.4.2.3 Multifunctional T cells

Antigen-specific polyfunctional or **multifunctional T cells** have been suggested to have a superior functional capacity to provide immune protection in intracellular infections such as Mtb and HIV [77, 78]. Multifunctional T cells may be essential in generating proper cellular immunity and are characterized by the coordinated expression of multiple effector functions, including Th1 and Th17 cytokines, the chemokine MIP-1β and markers for degranulation [77, 78]. Similarly, a coordinated T cell expression of perforin, granulysin and the chemokine CCL5 has been suggested to promote host immunity in human TB [79].

2.4.2.4 Regulatory T cells

Naturally occurring **regulatory T cells (Treg)** regulate peripheral tolerance, control autoimmune diseases and restrict chronic inflammation in order to prevent immunopathology and subsequent tissue damage. Thus, Treg cells may exert both beneficial and detrimental effects [80]. Inducible Treg cells can also develop from conventional CD4+ T cells that are exposed to immunoregulatory cytokines or other deactivating signals [81]. Natural Treg cells constitutively express CD25, the unique transcription factor Foxp3 as well as the T cell inhibitory receptors CTLA-4 and GITR [80]. Pathogen-specific Treg cells suppress Th1 immunity and may be expanded and overexpressed at the site of infection during chronic infections [80, 82]. Here, growing evidence from both humans [83-85] and mice [86] suggests that Mtb can induce Treg cells with immunosuppressive functions that interfere with protective responses in TB. Interestingly, mycobacterial ManLAM can expand human Treg cells *in vitro* [87, 88], which suggest that mycobacteria can use these cells to evade cellular immunity.

2.4.3 Specific cytokine responses in TB

2.4.3.1 Cytokines in general

Cytokines are small, soluble immune mediators or signaling molecules that the immune system uses for intercellular communication. Cytokines with a chemotactic function are called chemokines. Cytokines are produced and secreted by distinct cells in the body in response to activating stimulus and exert their immunomodulatory functions by binding to definite receptors [89]. Cytokine responses are defined as pro- or anti-inflammatory based on the nature of the stimulus. Based on their cytokine production, effector T cells differentiate into one of the subsets known as Th1, Th2, Th17 or Treg. Here, Th1 cells are involved in cellular immunity, Th2 cells induce humoral immune responses, Th17 cells are involved in mucosal immunity and autoimmune inflammation and Treg cells participate in the regulation of inflammatory immune responses.

2.4.3.2 IFN-γ

Protective immunity in TB is characterized by a Th1-mediated immune response that is necessary for the induction of cellular immunity. Various pro-inflammatory and Th1 cytokines are produced and released upon Mtb infection including the classical Th1 cytokines IFN-γ and TNF-α but also IL-1α/β, IL-6, IL-12 and IL-2 [90-95]. **IFN-γ** is a key cytokine that contributes to a protective immune response in TB [96-98]. Importantly, patients with a genetic defect in the IFN-γ receptor have a significantly increased susceptibility to develop active TB disease, which support a protective role of IFN-γ in humans [99]. Activated T cells and NK cells produce IFN-γ, which is critical for the activation of MQs, enhanced antigen presentation as well as expansion Mtb-specific T cells [12]. IFN-γ promotes classical MQ activation (M1) and enhances bactericidal activity of Mtb-infected MQs by the induction of respiratory burst including the production of RNI [100, 101]. Importantly, IFN-γ promotes autophagy [102], which is a physiological process that counteracts the phagosomal maturation block and thus inhibits intracellular growth of mycobacteria [103]. Besides, IFN-γ promotes activation of specific effector functions in both CD4+ and CD8+ T cells. It has also been shown that IFN-γ can induce regulatory effects of non-hematopoietic cells to reduce pathological inflammation and mediate protective responses in TB [104]. Because of its well-known protective effects, IFN-γ has been widely studied as a potential biomarker or correlate of immune protection in TB. However, the number of IFN-γ secreting T cells does not always correlate with enhanced immune control [105, 106], which may suggest that more complex immune signatures are required to define protective immunity in human TB.

2.4.3.3 TNF-α

Another key cytokine known to confer protective immunity in TB is **TNF-α***,* a crucial pro-inflammatory mediator produced by different cells such as mononuclear phagocytes, lymphocytes, neutrophils, mast cells and endothelial cells [12, 107]. TNF-α primarily facilitates the recruitment of immune cells to the site of Mtb infection and is thus central in the organization of a granulomatous response to limit and contain Mtb infection [108]. TNF-α controls cellular recruitment by altering expression of adhesion molecules, chemokines and chemokine receptors [109]. Besides, TNF-α acts in synergy with IFN-γ and promotes activation of Mtb-infected MQ [49]. Absence of TNF-α results in defective granuloma formation and enhanced bacterial growth, that is a consequence of defective cellular recruitment and immune cell activation at the site of infection [108, 110-112]. Reactivation of latent TB is typically observed in rheumatoid arthritis patients receiving anti-TNF therapy, which also underline the importance of TNF-α in TB control [26, 76]. However, the expression of TNF has to be tightly balanced as excess production of this cytokine can cause severe immunopathology and increased morbidity [113].

2.4.3.4 IL-17

A novel T cell subset is Th17 cells that secrete cytokines such as IL-17A, IL-17F, IL-21 and IL-22 [114]. Evidently, **IL-17** (IL-17A) plays an important role in the regulation of chronic inflammatory diseases and autoimmune disorders [115,

116]. Th17 cells have also been described to be critical for host defenses against pathogens primarily at mucosal surfaces [57, 114] and enhance expression of antimicrobial peptides [117]. A key role of IL-17 in intracellular Mtb infection is to promote recruitment and accumulation of IFN-γ producing CD4+ T cells in the Mtb-infected lung [118, 119]. IL-17 has also been shown to be particularly important to induce chemokine production and recruitment of neutrophils that take part in initial granuloma formation upon mycobacterial infection [120].

2.4.3.5 IL-7

IL-7 is a polyfunctional cytokine that can be produced by many cells including MQs, follicular DCs, B cells, fibroblastic reticular cells, epithelial cells, keratinocytes, endothelial cells and smooth muscle cells [121]. Production of IL-7 is induced by IL-1, IFN-γ, and TNF-α and is essential for T cell survival and homeostasis [122, 123]. In chronic infections such as TB, persistent antigen exposure will promote the generation of memory T cells and the maintenance of these cells will be greatly influenced by both IL-7 and IL-15 [124, 125]. Addition of IL-7 and IL-15 as adjuvants in novel vaccination regimens have been shown to broaden the immune responses to less dominant antigens and improve the survival of antigen-specific CD8+ memory T cells [126].

2.4.3.6 Th2 cytokines

Th2 cytokines are typically involved in antibody-mediated humoral immunity with limited protective effects in intracellular Mtb infection [127, 128]. *In vitro* studies with live Mtb strains and their lipid components have been shown to enhance production of Th2 cytokines including IL-4, IL-5, IL-10 and IL-13, which may suggest that virulent mycobacteria promote the differentiation of Th2 cells [129, 130]. Here, **IL-4** and **IL-13** have been shown to be detrimental in the control of intracellular Mtb infection, as these Th2 cytokines suppresses IFN-γ production and IFN-γ mediated effects including MQs activation [29, 131]. IL-4 impairs antimicrobial activities by reducing TNF-α mediated apoptosis of infected cells, decreasing RNI expression and increasing iron availability to support the growth of intracellular Mtb [128]. Furthermore, Th2 cytokines can inhibit autophagy, which is known to enhance intracellular degradation of Mtb bacilli [132]. Instead, IL-4 and IL-13 may induce expansion of antigen-specific FoxP3+ Treg cells [133]. Th2 cytokines also induce alternative MQ activation (M2) that involves a less bactericidal state of the MQ [134]. Ultimately, increased Th2 responses in the lung augment immunopathology by induction of pulmonary fibrosis and cavitation, which compromise lung function in TB patients [128, 135].

2.4.3.7 Anti-inflammatory cytokines

The immunoregulatory cytokines **IL-10** and **TGF-β** are produced by anti-inflammatory MQs and Treg cells [57] and inhibit potent Th1 responses. Both of these cytokines are known to be involved in the pathogenesis of active TB and transient overexpression has been observed in TB patients [136]. Upon Mtb infection, TGF-β selectively induces IL-10 and these cytokines act in a synergistic manner to suppress IFN-γ production [137]. Mycobacterial components have also

been shown to induce TGF-β production in peripheral blood monocytes from TB patients [138]. Similar to Th2 cytokines, IL-10 and TGF-β possess antagonistic effects on cellular immunity by inhibition of T cell proliferation, IFN-γ and pro-inflammatory cytokine production, reduced antigen presentation and reduced activation of bactericidal MQs [139]. In addition, TGF-β supports the production and deposition of MQ collagenases [139] and collagen matrix [140] that may alter tissue morphology and promote tissue fibrosis in Mtb-infected organs.

2.4.4 Innate and adaptive effector molecules in TB

2.4.4.1 ROI and RNI

Reactive oxygen and nitrogen species including both ROI and RNI are produced by MQs and neutrophils and effectively kill various bacteria [141]. Oxidative stress generated by Mtb-infected activated MQs produces a substantial amount of toxic oxygen and nitrogen radicals with the ability to kill the bacillus. H_2O_2 and $O_2^{\cdot -}$ are two common forms of ROI that Mtb encounters inside phagocytes. However, several mycobacterial products including LAM may be able to scavenge ROIs; thereby making Mtb somewhat resistant to killing by ROIs [29]. Of greater importance in TB is RNI and particularly **nitric oxide (NO)**, which is produced upon activation of **inducible nitric oxide synthase (iNOS)** using L-arginine as a substrate [142] [Figure 4]. Data from murine TB provide evidence that iNOS/NO represents an important innate effector molecule that can provide immune protection in TB [31]. However, the protective role of NO in human TB remains controversial [143], even though iNOS has been described to be expressed at the local site of Mtb infection in patients with active TB [144, 145].

2.4.4.2 Cathelicidin, LL-37

Antimicrobial peptides are commonly found in many living organisms including bacteria, fungi, plants, invertebrates and vertebrates, as frontline effector molecules of the innate immune defense [146]. This includes a range of human peptides with broad antimicrobial activity such as defensins, histatin and **cathelicidin** [147]. Cationic human antimicrobial peptides are acknowledged as important players in the barrier function of mucosal and epithelial surfaces and display a wide range of activities against bacteria, fungi, parasites, and viruses [148]. Apart from their antimicrobial function, these molecules may also possess immunostimulatory functions. The expression of antimicrobial peptides can be both constitutive and regulated. Human cathelicidin, also named **LL-37** or hCAP-18, is pre-formed as a 18 kDa protein, produced by neutrophils, mast cells, eosinophils, MQs, DCs, keratinocytes and epithelial cells [149-152]. Cathelicidin peptides are retained inside granules as inactive forms, which are processed into active peptides after induction by various stimuli. LL-37 efficiently perturb membrane integrity of bacterial membranes and thus exhibit potent activity against microbes such as Mtb [153]. LL-37 has also been described as a chemotactic factor for different immune cells [148]. Interestingly, vitamin D-mediated induction of autophagy in monocytes/MQs has been shown to be dependent on LL-37 [154]. Importantly, LL-37 has the ability to directly kill and restrict the growth of intracellular mycobacteria [37, 155].

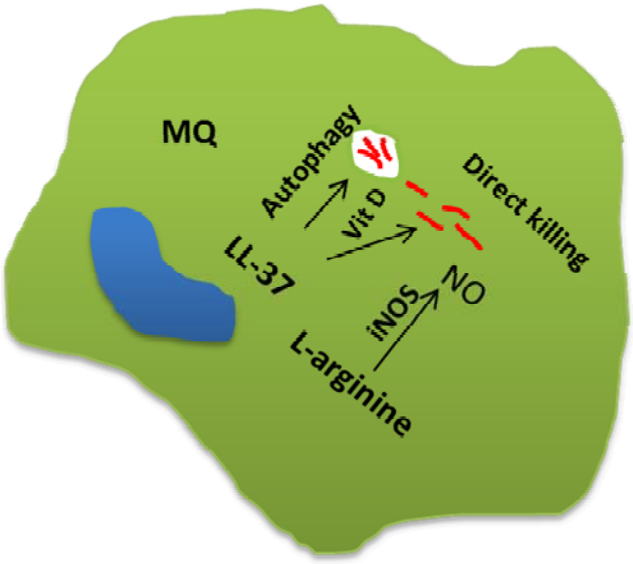

Figure 4. Schematic illustration of important anti-TB innate effector molecules expressed by activated MQs. LL-37 promotes autophagy and direct killing of Mtb bacilli. iNOS catalyzes the enzymatic conversion of the amino acid L-arginine into toxic NO.

2.4.4.3 Granzymes

Granule-mediated exocytosis are the main pathway involved in killing of Mtb-infected target cells [Figure 5]. Primarily CD8+ CTLs and NK cells are armed with cytolytic granules effective in killing of pathogen-infected cells as well as tumor cells [156, 157]. CTLs express granule-associated serine proteases named **granzymes** [158, 159] and so far, five different granzymes (A, B, H, K and M) have been described in humans [160]. Primarily granzyme A and granzyme B are abundantly expressed in activated CTLs to execute target cell death [161]. Here, granzyme B induces apoptosis by cleavage of caspases, while granzyme A induces caspase-independent nuclear damage by generation of single-stranded DNA nicks, which facilitates apoptosis [162, 163].

2.4.4.4 Perforin

In 1985, the membranolytic 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 [164]. Perforin is released via the granule-exocytosis pathway into the immunological synapse of the CTL and the target cell and generates pores in the target cell membrane in order to induce cell lysis but also to facilitate entry of other effector molecules including granzymes and granulysin [159, 162, 165]. Here, perforin has been shown to deliver granzymes to the target cell using two possible mechanisms: either perforin forms pores in the cell membrane through which granzymes are delivered, or perforin forms pores in endosomal membranes and delivers granzymes to the cytosol [156, 166]. Both CTL and NK cells from perforin-deficient mice are defective in granzyme-mediated cytotoxicity, which support the conclusion that perforin is required for granzyme

trafficking [167, 168]. Consequently, lack of perforin increases susceptibility to malignancies and various infections [169, 170]. Similarly, perforin plays a central role in CTL function and the regulation of intracellular bacterial infections like TB [171, 172]. Here, Mtb-infected perforin-deficient mice demonstrated reduced target cell killing and TB protection *in vivo* [173].

2.4.4.5 Granulysin

Another important component of cytolytic granules is the antimicrobial peptide **granulysin**, which is constitutively expressed in NK cells and induced in CTLs upon activation [174]. More recently, granulysin has been given significant scientific attention, as it exhibits cytolytic activity on a variety of pathogens including extracellular and intracellular bacteria, fungi and parasites, as well as on tumor cells [72, 175]. Granulysin is expressed in two forms: a 15 kDa precursor protein and a 9 kDa active cytolytic protein [176]. Similar to human cathelicidin, granulysin is a small cationic molecule that can interact with the negatively charged mycobacterial surface through ionic strength [72]. Granulysin disrupts bacterial membranes and mediates osmotic lysis of bacterial cells [177, 178]. Granulysin can also inhibit viral replication and trigger apoptosis of infected cells [179]. Here, it has been shown that granulysin can lyse human cells via the mitochondria pathway of apoptosis [180]. Interestingly, evidence suggests that elevated levels of granulysin were associated to an improved clinical prognosis of both *M. leprae* and Mtb disease [181, 182]. Recently, granulysin has also been identified as the first lymphocyte-derived protein acting as an alarmin, able to promote APCs recruitment and an antigen-specific immune response [183].

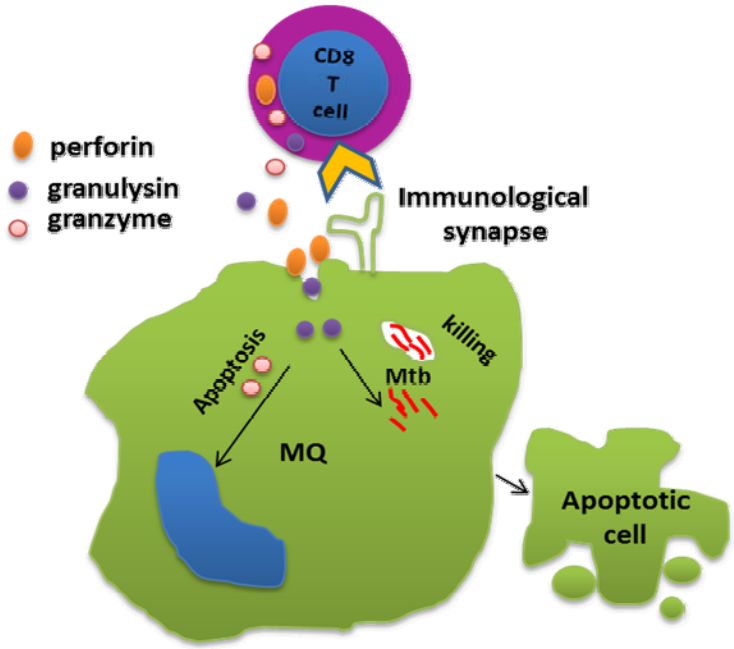

Figure 5. Schematic illustration of important anti-TB effector molecules expressed by activated T cells. The membranolytic protein perforin facilitates target cell access of both granzymes and granulysin, which result in target cell apoptosis and/or microbicidal killing.

2.4.4.6 Antibodies

Antibodies or immunoglobulins (Ig) are Y-shaped proteins that are produced and secreted by plasma B cells with the main function to eliminate extracellular microorganisms. Upon stimulation with specific antigen, B cells become activated and some differentiate into antibody-secreting plasma cells. Plasma cell development is promoted by CD4+ Th2 cells secreting cytokines such as IL-4 and IL-6 [184], but also IL-21 can stimulate antibody secretion by plasma B cells [185]. In mammals, there are five different isoforms including IgA (involved in mucosal protection), IgD (membrane-bound antigen receptor on B cells), IgM (involved in the early stages of humoral immunity), IgG (IgG1-4, protect against most invading pathogens) and IgE (involved in allergy and parasite infections). IgG antibodies provide the majority of protection upon infection and is the most abundant antibody (75% of serum Ig) distributed in blood and in tissue fluids. Antibody-mediated humoral immunity is usually considered as non-protective in TB, as intracellular Mtb bacilli mostly remain inaccessible to soluble antibodies [127]. In TB, glycolipid and polysaccharide antigens are released from dead mycobacteria that are broken down at the site of infection as a consequence of vigorous inflammation. These antigens are responsible for elevated humoral responses (IgM, IgG, IgA) that usually peak after the T cell-mediated immune responses have declined. In addition to Mtb infection, BCG vaccination also induces antibody responses that seem to be inefficient to limit intracellular mycobacterial replication. Although humoral immune responses probably have little clinical relevance to eradicate intracellular Mtb, some animal studies suggest that antibodies have a protective role in TB [186-189].

2.4.5 The TB granuloma: A host shield or a bug shelter?

2.4.5.1 Host shield

The specific immune cell subsets and effector molecules involved in human TB are unable to successfully clear the infection, but instead contribute to containment of the bacteria by the formation of a microenvironment called a **granuloma** [190]. The granuloma is a spherical structure that is a very distinct histopathological hallmark of human TB [191, 192]. It is defined as an organized collection of immune cells which form when the immune system attempts to wall off substances that it perceives as foreign but is unable to eliminate. Initial granuloma formation in TB is characterized by a collection of tightly clustered MQs. Continuous activation of MQs induces the cells to adhere closely together, assuming an epithelioid shape and sometimes fusing to form multinucleated giant cells (MGC) [15]. The function of MGCs in TB remains to be fully elucidated. The cellular core of infected MQs and MCGs is typically surrounded by T and B cells, neutrophils, eosinophils and fibroblasts [192-194] [Figure 6]. The structure and function of the granuloma are regulated by the complex interplay between an array of different cytokines (e.g. IL-12, TNF-α, IFN-γ, IL-8, IL-1, and IL-17) and chemokines (e.g. RANTES, MIP-1α/β, MCP-1, CXCL8-11) [191]. Instead, immunoregulatory cytokines such as IL-10 and TGF-β undermine granuloma maintenance [112, 191].

The granuloma is a dynamic structure with a variety of appearances such as solid, necrotic and caseous that can be found in active as well as latent TB [195, 196]. Small cellular aggregates will progress and mature into productive granulomas as TB disease develops [193] [Figure 6]. High immunoreactivity may lead to caseous necrosis in the center of the granuloma, which is a typical trait of TB granulomas in humans. Upon progression of TB disease, non-necrotic granulomas will advance to form large necrotic granulomas where extracellular bacteria persist in the caseous necrotic fluid [193, 197]. Rupture of necrotic granulomas will result in spread of mycobacteria to the airways, which are expelled from pulmonary TB patients as contagious aerosols.

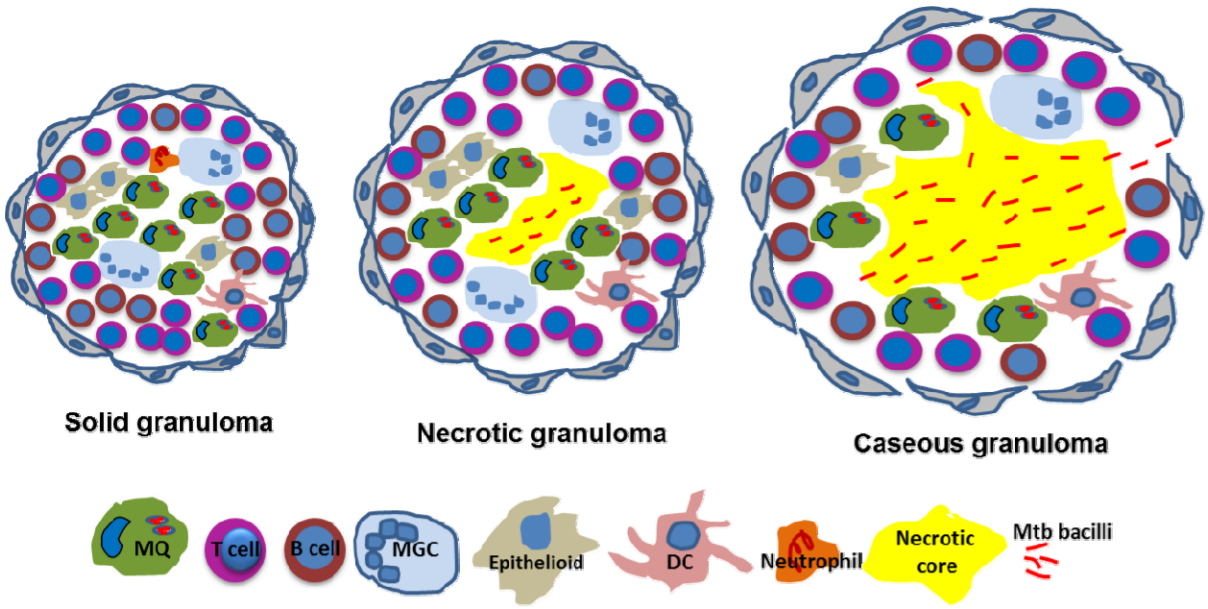

Figure 6. Schematic illustration of granuloma development in TB: i) A compact solid granuloma with infected MQs, epithelioid MQs, MGC, T and B cells enclosed by a coat of fibroblasts, ii) a necrotic granuloma contains a central core of necrosis with few extracellular bacilli, iii) a caseous granuloma comprises an extensive caseous necrotic core with plenty of extracellular bacteria and reduced numbers of immune cells.

A balance of pro- and anti-inflammatory mediators is required for a productive granuloma to restrict bacterial growth and to simultaneously limit immunopathology which is unfavorable to the host [17, 198]. This balance probably occurs in latent infection, where infection rarely progress into active TB disease in immunocompetent individuals. The immune system forces the mycobacteria to alter their metabolic activity and to reduce their replication rate, which only will allow a few dormant mycobacteria to remain within the hostile granuloma [199]. Once the immunological balance fails as a result of impaired immune responses; enhanced tissue damage, necrosis and bacterial dissemination will ensue [200, 201]. Ultimately, the failure of granuloma containment results in reactivation or progression of TB disease [202]. This is clearly evident in HIV-

infected individuals who have a significantly increased risk to develop active TB or to reactive latent TB infection. Granuloma formation is seemingly intact in TB/HIV co-infected individuals with low-moderate levels of immunodeficiency, while granuloma formation is nearly absent in TB/HIV co-infected patients with HIV-associated immunosuppression and AIDS [203].

2.4.5.2 Bug shelter

Mtb virulence factors that constitute the so called region of difference, RD1, have been described to exhibit an essential function in granuloma development [204]. One of these mycobacterial virulence proteins is ESAT-6, which initiates granuloma formation by inducing matrix metalloproteinase-9 (MMP-9) in epithelial cells adjacent to Mtb-infected MQs [205]. MMP-9 enhances the recruitment of new MQ to the site of infection that promotes granuloma maturation as well as bacterial growth [204, 205]. In this way, virulent mycobacteria can induce an RD1-dependent aggregation of macrophages into granulomas that is tightly linked to intercellular bacterial dissemination and increased bacterial numbers [206]. Therefore, it has recently been suggested that virulent mycobacteria that express RD1, may exploit early granuloma formation and promote spreading of bacteria to uninfected MQs that are recruited to the early granuloma [207]. Egress of these Mtb-infected MQs from the primary granuloma may seed secondary granulomas in the infected host and thus propagate the infection [207]. Thus, it is debated whether the granuloma may actually provide a nursery for the mycobacteria in the early stages of infection while the host protective function of the granuloma become more evident at later stages of the infection, after induction of adaptive immunity. Since most of these studies have been performed in the zebra fish model of TB that lack an adaptive immune response, the clinical relevance of these findings needs to be confirmed in more complex models of TB.

2.5 IMMUNE EVASION MECHANISMS IN TB

2.5.1 The mycobacterial cell wall as a protective shield

Efficient clearance of Mtb mostly depends on the well-tuned interplay between infected MQs and other APCs and Mtb-specific T cells. But like other intracellular pathogens, Mtb has developed resourceful survival strategies to evade host immune attacks and to establish a productive infection. First of all, the unique characteristics of the mycobacterial cell wall inherently favor the pathogen to escape host killing mechanisms. The thick lipid-rich cell wall of mycobacteria provides protection against effective antimicrobial responses including osmotic lysis via complement deposition, lethal oxidations and also resistance to many antibiotics and killing by acidic and alkaline compounds. Thus, the Mtb cell wall will enhance bacterial survival inside the hostile MQ environment and promote long-term persistence. It has been suggested that mycobacterial cell wall integrity is crucial for the survival inside the host, as depletion of cell wall components diminishes bacterial virulence [208]. Thus, the cell wall components, especially ManLAM, contribute to Mtb virulence and have been shown to interfere with

phagosomal maturation and the induction of protective cytokine responses [16, 209-211]. Interestingly, dormant Mtb bacilli can alter bacterial metabolism but also cell wall composition as evidenced in latent TB infection, which enable long-term survival within the host [212].

2.5.2 Block of phagosomal maturation

Another well-known evasion strategy used by pathogenic mycobacteria is to block phagosomal maturation in Mtb-infected MQs. Mycobacteria manipulate phagolysosome biogenesis by blocking accumulation of phosphatidylinositol 3-phosphate on the phagosomal membrane [213], and also prevent lysosomal acidification [48, 214]. This will provide the opportunity for mycobacteria to survive and grow inside the phagosomes at a fairly high pH (5.5). Furthermore, it was recently demonstrated that virulent Mtb expressing the type VII secretion system, Esx-1, can translocate from the phagosome and replicate inside the MQ cytoplasm, causing significant cell death within a week [215]. Interestingly, Esx-1 may also be involved in the impairment of autophagy, which promotes intracellular survival and spread of mycobacteria.

2.5.3 Manipulation of immune cells

Upon TB infection, classically activated M1 MQs become highly bactericidal and produce antimicrobial effector molecules such as NO and LL-37. However, mycobacterial virulence factors may interfere with M1 polarization and instead promote polarization of alternatively activated M2 MQs that produce anti-inflammatory cytokines which are immunomodulatory and maintain a poorly microbicidal state of the MQ [216]. Mtb can also avoid or reduce immune recognition by effector T cells through inhibition of the antigen presenting molecules MHC-II [217] or CD1 [48] expressed on the surface of APCs. Interestingly, Mtb express antigens that can induce inflammatory as well as anti-inflammatory responses [218]. Excess production of anti-inflammatory mediators early in the infection may promote mycobacterial growth and survival.

2.6 BIOMARKERS IN HUMAN TB

2.6.1 Biomarkers to monitor human diseases

A biomarker is defined as a biological marker that is an indicator of a biological state, such as a pathogenic process or correlate of protection in a particular disease. It is attractive to discover biomarkers that could be used in the diagnosis of malignancies, chronic inflammatory or infectious diseases. At best, such biomarkers could also be used to predict disease outcome. Our understanding of what constitutes protective immunity against TB remains incomplete. We know that most TB infected individuals contain the mycobacteria without development of active disease, but we do not know what specific factors are responsible and essential for this containment. Thus, many research groups have an interest to identify specific host factors or correlates of immune protection that are involved in the control of TB infection and that could be used as biomarkers to diagnose,

predict disease outcome, and monitor vaccine-induced immune responses or the efficacy of anti-TB treatment. Thus, appropriate biomarkers in TB could potentially be used in a number of areas including both basic and applied activities.

2.6.2 Potential biomarkers of immune protection in TB

Evidence from both experimental and clinical studies confirms a critical role of both CD4+ T cells and IFN-γ production in the immune defense against TB [58, 219]. Thus, assessment of IFN-γ production by CD4+ T cells has been extensively used in TB diagnosis and also to evaluate vaccine-induced immune responses. However, it is evident that IFN-γ production does not always correlate to protective immunity [220]. Here, increased IFN-γ but decreased granulysin levels have been observed in plasma samples from newly diagnosed and relapsed TB patients [221], which suggest that assessment of IFN-γ only may not provide an accurate reflection on the clinical progression of TB disease. Other Th1 cytokines including TNF-α and IL-2 [110, 222], and also CD8+ T cells [73, 76, 223], that are involved in TB protection can also be measured following transient stimulation of whole blood or peripheral blood lymphocytes using Mtb antigens such as Ag85B and TB10.4 [224-227]. Evaluation of novel TB vaccines in experimental animal studies have shown that mycobacteria-specific multifunctional T cells co-expressing IFN-γ, TNF-α, and IL-2 at the site of infection were associated to immune protection in TB [228, 229]. Similarly, CD8+ T cells expressing perforin and granulysin have been shown to correlate with immune protection following BCG vaccination of cattle [230]. In addition, plasma granulysin levels were demonstrated to correlate with clinical recovery in patients with active pulmonary TB [231]. These findings have resulted in an increased interest to evaluate effector responses by different T cell subsets in clinical trials. However, other studies have failed to show that T cell frequencies and cytokine expression correlate with protective immune responses in BCG vaccinated new born [232]. Conclusively, the battle to find novel and specific biomarkers in human TB continues. Possibly, a combination of multiple markers would enhance the probability to establish relevant biomarkers of immune protection in TB.

2.6.3 Potential biomarkers of active TB disease

During the end of the last century, scientists started to reconsider the beneficial role of serum therapy to ameliorate TB disease [233, 234]. Several studies suggest that antibodies have a protective role in TB, while other studies report that antibodies fail to improve control of TB disease [235-237]. It has been demonstrated that production of Mtb-specific IgG was significantly elevated in serum from patients with active TB disease [238-240]. Additionally, we recently described that circulating IgG-secreting plasmablasts were significantly higher in patients with active TB compared with latent TB cases and non-TB controls [241], which suggest that Mtb-specific peripheral plasmablasts could be successfully used as a host-specific biomarker to improve diagnosis of active TB [241, 242]. Interestingly, IgG-secreting plasmablasts were particularly high among TB/HIV co-infected patients and correlated to progression of clinical TB disease [241]. These

findings implicate that antibodies or antibody-secreting cells could be useful biomarkers for active TB and/or as biomarkers of disease progression [241].

2.7 DIAGNOSIS OF TB

2.7.1 Conventional diagnosis

Diagnosis of TB is complex and usually based on several methods including medical history of the patient, clinical examination (cough, fever, weight loss etc.), chest X-ray findings, sputum-smear microscopy, culture of clinical specimen (golden standard), histopathological examination of biopsies or cell samples, Mtb-specific PCR, tuberculin skin test (TST) and IFN-γ release assays (Quantiferon or T-SPOT.TB) [1]. Most of these methods have important limitations and are often slow, expensive and require advanced equipment or invasive procedures. In addition, none of the methods can clearly separate active TB disease from latent TB infection.

2.7.2 Bacteriological diagnosis

Sputum-smear microscopy (detection of acid-fast stained bacilli in sputum samples) is the most widely used and cost-effective diagnostic method. However, about 50% of culture-confirmed pulmonary TB patients are sputum smear-negative and thus microscopy is insufficient to provide an accurate diagnosis [243], even less in areas with a high HIV incidence. Moreover, the sputum test cannot distinguish Mtb from other non-tuberculous mycobacteria. Culture of Mtb from clinical specimen is considered as the golden standard to confirm a TB diagnosis, but it is time-consuming as it takes 4-8 weeks to receive the results. The automatable mycobacterial growth indicator tubes (MGIT) are presently the preferred culture system in high-throughput settings as it shortens the culture time with around 10% increased sensitivity compared to the conventional solid and agar based culture methods [1]. Furthermore, genotype based (PCR) methods are novel advancements for rapid and more specific results in TB diagnosis [244]. However, detection of Mtb using culture methods or the genotype-based assays provides high specificity but variable sensitivity. These methods are also reliant on high bacterial loads in clinical samples, which complicate the diagnosis of sputum-negative patients or patients who cannot provide sputum samples including children.

2.7.3 Immunodiagnosis

So far, immunodiagnosis is considered a promising alternative or complement to the bacteriological methods described above. The immunological tests detect mediators released by specific host immune cells. The TST is the oldest immunodiagnostic test based on measurement of the delayed type hypersensitivity (DTH) reaction (induration) in the forearm after intradermal injection of the heat-killed mycobacterial extract, purified protein derivative (PPD). Usually a strong (>10 mm) skin reaction is indicative of active TB, however, the TST cannot separate an ongoing active infection from latent TB infection or

previous BCG vaccination. Neither can it discriminate Mtb from other environmental mycobacteria. More recently, whole blood tests that are based on rapid detection of Mtb-specific IFN-γ producing memory T cells are commercially available [245, 246]. These tests, the ELISA based Quantiferon TB gold and ELISPOT based T-SPOT.TB, have an advantage in that the assay kits contain a cocktail of Mtb-specific antigens that increases the sensitivity of these assays significantly [247]. However, since these tests cannot discriminate between active and latent TB, their use in routine clinical practice in high-endemic countries is difficult.

2.7.4 Mtb antigens in TB diagnosis

Specific Mtb-antigens are of great interest in TB research since these proteins can be considered as potential diagnostic biomarkers, vaccine candidates and/or targets for drug development. In addition to cell wall components of Mtb such as LAM or PIM, secreted antigens contribute to pathogenicity [248]. Notably, the virulence proteins of the type VII secretion system, ESAT-6 and CFP-10, are strongly immunogenic and induce T cell-mediated IFN-γ production upon recognition [248-250]. Therefore, these antigens are suitable targets to be evaluated in TB diagnosis and as vaccine candidates [251]. In addition, three more immunogenic secretory antigens 38 (Ag85A), 30 (Ag85B) and cytosolic α-crystallin (16 kDa) are currently being assessed for use in TB diagnosis [239, 252]. The mycobacterial cytosolic antigen, 65 kDa heat shock protein (HSP65), has been recognized as a major antigen of Mtb with clinical relevance and is considered to be applicable for use in a novel subunit vaccine against mycobacteria [253]. Furthermore, MPT64 (earlier MPB64) is a 26 kDa Mtb complex-specific antigen secreted by actively replicating bacteria and encoded in the RD2 genomic region [254, 255]. It was first detected in *M. bovis* and Mtb culture filtrates, but not in attenuated BCG [256]. MPT64 contributes to Mtb virulence by inhibition of apoptosis of infected cells [257] and induces potent immunogenic responses [258, 259]. Several studies have reported that MPT64 has a major diagnostic potential both in human and bovine TB [260-262].

2.8 PROPHYLACTIC THERAPY

2.8.1 The BCG vaccine

The only existing vaccine against TB, Bacillus Calmette-Guerin (BCG), is made from a live attenuated *M. bovis* strain. BCG is the most extensively used vaccine with more than 4 billion doses administered worldwide [263, 264]. In 1908, Albert Calmette and Camille Guerin at the Institute of Pasteur, initiated the challenge to attain the attenuated strain from a virulent *M. bovis* strain to be used for the development of the first TB vaccine [265, 266]. They cultured the mycobacteria in ox-bile containing media with glycerol supplement and continued sub-culturing for 230 passages [265]. An attenuated bacillus incapable to form advanced TB in several animal models was established, and the first successful human BCG

vaccination took place 13 years later [267]. The vaccine was first administered orally to an infant of a mother who died from pulmonary TB [3]. In the care of his grandmother who also had pulmonary TB, the child stayed alive and never developed TB [3]. Since then, BCG has been part of the expanded program on immunization (EPI) for at least 40 years and is considered to be a relatively safe vaccine. However, recently it has become apparent that individuals with genetic defects or HIV-infected children are extremely vulnerable to the development of overwhelming BCG disease [268, 269]. This finding poses a high risk of BCG vaccination in HIV-burdened populations in TB endemic countries.

2.8.2 Reasons behind BCG failure

BCG is a most debated vaccine as it provides varying protection against TB (average 35-65%), which also varies extensively comparing different populations and geographic locations in the world [270-272]. 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 pulmonary TB including time-dependent waning of vaccine efficacy [273, 274]. Generally, BCG-induced immunity decreases after about 10 years and a second boost with the vaccine has no effect on the protective efficacy [1]. Several factors are likely to influence the inconsistency in BCG-induced protection, for instance strain disparity among different BCG preparations, genetic and nutritional disparities in populations, environmental effects like sunlight contact, temperature variations upon preservation as well as immunological cross-reactivity between BCG and environmental mycobacterial strains [267, 275, 276]. Interestingly, BCG is a potent inducer of CD4+ T cell responses but fails to generate strong MHC-I restricted CD8+ T cell responses. This is probably because BCG is unable to translocate from the phagosome into the cytosol of the MQ to enhance antigen processing via the MHC-I pathway [215, 277]. In addition, BCG immunity is short-lived. Other reasons may involve helminth infections that could contribute to a decline in BCG efficacy and/or increase the probability of TB among young adults [278]. Parasitic infections shift the immune response towards a Th2 or anti-inflammatory response, which may impede Th1-dependent immune protection [279, 280]. In this regard, attempts have been made to treat helminth infections in TB patients to prevent inappropriate Th2 responses that may reduce the cure rates of their TB disease [281].

2.8.3 New TB vaccine candidates

Almost a century after the discovery of the BCG vaccine, no new TB vaccine has been successfully developed. There is an obvious challenge to develop vaccines against intracellular pathogens like Mtb and HIV, since these infections depend on cellular immunity [282]. Most successful vaccines available today are based on the induction of a powerful antibody response, while T cell-based vaccines are much more difficult to develop. Even so, the complicated mission to create a novel TB vaccine with the ability to induce potent T cell responses is currently ongoing [283-287]. Most of the novel concepts are prophylactic vaccines, however, therapeutic vaccines are also developed as adjunct therapy to Mtb-infected

individuals [284]. Two basic approaches have been exploited in order to generate new TB vaccines candidates [283, 288]. The first set of TB vaccine candidates is intended to replace BCG either by enhancement of BCG using a recombinant BCG (rBCG) or by a genetically attenuated Mtb. Different rBCG variants can be generated by improvement of BCG involving 1.) insertion of a pore forming toxin such as listeriolysin, to enable phagosomal escape, 2.) introduction of potent immunogenic Mtb-specific antigens that are normally missing in BCG, such as ESAT6 and CFP10 (RD1 encoded antigens), 3.) over-expression of existing BCG antigens such as the Ag85 complex [283]. The second set of TB vaccine candidates involves subunit booster vaccines that are able to potentiate the immune response obtained by a prior BCG vaccination. Subunit vaccines have been tested either as a single antigen (e.g. HBHA) or a combination of antigens (e.g. Ag85B-TB10.4) [228, 289].

2.8.4 Novel vaccination strategies

An improved vaccine should be superior in respect of safety, immunogenicity, maintenance of long-term memory and protection against highly virulent clinical stains as well as drug resistant strains. Vaccine-induced immune responses are commonly assessed in animal models like mice, rabbits and non-human primates, but several vaccine candidates are also being studied in clinical trials [220, 225, 290]. The preferred model vaccines should trigger potent immunogenic responses after one vaccination but in practice this aim is unrealistic, particularly with vaccines constructed to induce protective T cell responses. Important steps in the process to discover a potent TB vaccine are to find appropriate Mtb-specific antigens as vaccine targets, a powerful adjuvant configuration, the optimal route of administration, and also the number and timing of vaccinations to be given [291]. Among the categories of potential TB vaccine strategies, prime-boost vaccination is considered the best option as observed in experimental animal models [292-295]. Moreover, considering the vast number of BCG doses that have been administered worldwide, it would be an advantage if the BCG response could be augmented. Out of a combination of multiple prime-boost vaccinations, administration of rBCG and boosting with protein antigens is well appreciated, as this heterologous combination induces expansion of Mtb-specific memory T cells with reactivity against some common epitopes shared by the prime and booster antigens [296]. Current TB vaccine candidates are mostly designed to express early expressed Mtb-antigens to protect against infection. However, such vaccines cannot protect against persistent latent TB infection or reactivation of latent TB, which is a crucial requisite to reduce further spread of TB disease. To overcome these problems, multistage TB vaccines that express both early and late Mtb-antigens have been constructed that evidently confer protection against latency and reactivation in animal models of TB [297, 298].

2.9 ANTI-TB THERAPY

2.9.1 Chemotherapy

Drug treatment is vital for TB control and clinical cure. Without effective drug treatment, WHO has estimated the mortality among TB patients to be around 66%. Anti-TB drugs rapidly reduce bacterial loads in the lung and thus break the chain of transmission when the drug regimen is followed properly. The global situation of TB changed drastically after the introduction of the first anti-mycobacterial agents. In 1944, the first TB patient was successfully treated with streptomycin and in the coming decade, three drugs, streptomycin, para-aminosalicylic acid and isoniazid, became available on the market. This was a significant breakthrough, since these drugs substantially improved the prognosis for patients with active TB disease, especially when taken in combination for a sufficient length of time. Today, the cornerstone for treatment of chronic TB disease is multidrug therapy for at least 6 months. Recommended first-line anti-TB drugs includes rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months, followed by rifampicin and isoniazid for 4 months [299]. This extended multidrug therapy prevents the emergence of drug resistance, but simultaneously creates problems with compliance among the TB patients. Once the patients begin to improve within a few weeks of drug treatment, medication is often interrupted, which increases the risk to develop drug resistant TB. Recently, increasing numbers of MDR-TB, but also extensively drug-resistant (XDR) and totally drug-resistant cases threaten TB control worldwide [300, 301]. To defeat the problem with antibiotic resistance, WHO has installed a program, called DOTS (Directly Observed Treatment Short-Course). DOTS enable the patients to receive their TB drugs daily, under supervision at a hospital or local health care center.

2.9.2 Immunotherapy

Before effective chemotherapy was available, TB patients were isolated in sanatoria and were often placed out on the porch in the fresh air. Good food, rest and sunlight were the only ways to clinical recovery among these patients. Probably, the sunlight promoted conversion of vitamin D in the skin of the TB patients, which has recently been shown to enhance autophagy [154] and the expression of human cathelicidin in Mtb-infected MQs [13]. Consistent with this assumption, vitamin D deficiency have been shown to increase the susceptibility to develop active TB [13, 34, 302]. Clinical trials to further test the beneficial effects of vitamin D, either as adjunctive immunotherapy to patients with active TB [303, 304] or as prophylactic treatment to risk populations with latent TB are required. Similarly, studies including nutritional supplementation with arginine-rich peanuts to TB patients have been performed to explore the possibility that arginine would promote the conversion of NO in Mtb-infected MQs [305]. Hence, several attempts are being made to treat TB by enhancement of important cytolytic and antimicrobial effector molecules that would assist intracellular killing of Mtb bacilli.

2.10 EXPERIMENTAL ANIMAL MODELS OF TB

2.10.1 Pros and cons

Although there are several established experimental animal models to study TB disease, no animal model can entirely imitate human disease [306]. This is particularly true for TB, which involves several complex diagnoses such as latent infection and active disease; active pulmonary TB, extrapulmonary TB, miliary TB and also TB/HIV co-infection. These different conditions are very hard to reproduce and study in experimental models. Human TB disease is also influenced by Mtb strain virulence, host genetics and host immunity, as well as challenging environmental factors. Since the period of Robert Koch, TB research has been conducted in animal models including primarily mice, rabbits, guinea pigs, zebra fish and non-human primates. Although mice are not a natural host for Mtb, this is the most extensively used experimental animal model for TB, probably because it has several technical advantages. A genetically homogenous population can be studied, antibodies and other specific research reagents are commercially available and it is possible to study the development of TB infection in many animals during a defined period of time. Mostly, standard laboratory strains are used for animal infections such as H37Rv or Mtb Erdman, or avirulent strains such as the BCG vaccine strain or H37Ra. However, high numbers of bacteria are usually required to establish active TB infection and these are sometimes injected intravenously, instead of the natural aerosol route. Importantly, the TB disease that develops in mice are very different compared to human TB as mice do not typically develop chronic TB and a DTH reaction [306]. Neither do mice form organized granulomas upon Mtb infection, but only cell aggregates without the presence of epithelioid cells or MGC are formed. Mice also differ in antimicrobial host defenses. It has been shown that iNOS/NO is crucial for TB control in mice, although the relevance of NO to control human TB is debated. In addition, mice lack a homologue of the antimicrobial effector molecule granulysin, which has been shown to be central for immune protection in human TB. Even though it is sometimes necessary to perform well controlled experiments to study TB in mice, these studies will have to be compared and properly evaluated to research on TB infection and disease performed in humans. Other animal models may develop a TB disease with closer resemblance to human TB, but experiments are often more difficult to conduct due to higher costs and lack of commercial reagents.

2.10.2 Non-human primate model of TB

The non-human primate (NHP) model are considered the most appropriate animal alternative to study human TB [307]. NHPs are genetically comparable to humans, since they share analogous physiological and immunological systems with humans [308]. Both Cynomolgus and Rhesus macaques can develop primary TB and latent TB upon mycobacterial infection [308]. Furthermore, the complete spectrum of granuloma types can be detected and studied in Mtb-infected NHPs [309, 310]. The NHP model can also be used to study co-infection with Mtb and simian immunodeficiency virus (SIV) [311]. Since most human reagents are cross-

reactive with NHPs, these can mostly be used to investigate TB in NHPs. However, the overall costs and difficulties of handling, limit the use of the NHP model and therefore NHPs are mostly used in applied research to test novel vaccines or drug candidates. In addition, concerns from animal rights activists, and the general perception that monkeys were intricately vulnerable to TB have limited the use of this model. Spread of TB disease can become problematic in primate colonies, and containing the outbreaks of natural infection within a population can also be expensive.

3 AIMS OF THE THESIS

This thesis aimed to explore the immunopathogenic mechanisms involved in the development of clinical TB disease with a focus to investigate the cytolytic and antimicrobial effector pathways at the local site of infection in human TB.

The specific aims of the studies were:

- ✓ **Study I:** To investigate the expression of cytolytic and antimicrobial effector molecules, granzyme A, perforin and granulysin, at the site of infection in human pulmonary TB.

- ✓ **Study II:** To study potential alterations in the expression of cytokines and antimicrobial effector molecules in human lymph node TB and to determine whether Treg cells could play a role in disease progression.

- ✓ **Study III:** To evaluate the immunogenicity of a new TB vaccine candidate in the lung and secondary lymphoid organs obtained from a non-human primate (NHP) model of TB.

- ✓ **Study IV:** To explore adverse immune responses including the functional expression of T and B cell subsets and antimicrobial effector molecules at the site of infection in human pulmonary TB.

4 EXPERIMENTAL SET-UP

4.1 STUDIES OF MTB AT THE LOCAL SITE OF INFECTION

Despite decades of research on TB, studies of host-pathogen interactions in humans have lagged behind. Local TB pathogenesis in humans remains elusive in many aspects including the expression and distribution of different immune cells and inflammatory mediators. Studies on human TB are almost exclusively performed using cells from the peripheral blood from TB infected individuals, since it is the most easily accessible clinical sample. However, TB infection is mainly an organ-specific disease, which means that the Mtb-specific immune responses are focused at the local site of infection such as the lung or lymph nodes [53]. Thus, *in vitro* analysis of cells obtained from the peripheral circulation may not necessarily provide a proper reflection of the immune responses occurring in the Mtb infected tissue [312-315]. Accordingly, several studies collectively provide evidence of significant differences in T cell responses at the site of Mtb infection as compared to peripheral blood, which stress the need to study TB pathogenesis at the local site of infection [91, 316-318].

Immune cells continuously pass through blood and rapidly migrate to the local site of infection [319]. Under normal conditions, only 2% of all lymphocytes are present in the circulation, while the remainder reside in lymphoid organs as well as in non-lymphoid tissue such as the lung and bone marrow [320]. Importantly, the Mtb-specific host immune response is strongly characterized by granuloma formation involving various immune cell subsets that cannot be imaged using cells from peripheral blood samples. In this respect, appropriate animal models of TB such as the NHP model, may provide an advantage since it is possible to study immune responses at the local site of Mtb infection.

As listed in **Table 1**, there are several important advantages to study local immune responses and host-pathogen interactions locally, in the Mtb infected tissue [53]. Obviously, both practical and technical factors limit the possibilities to study human TB at the site of infection. Collection of human tissues or biopsy materials from infected organs such as lung, lymph nodes and pleura, certainly requires invasive procedures including surgery and biopsy sampling. It may be difficult to obtain and analyze data from small quantities of tissue and thus fine-needle aspirations or small punch biopsies may not provide enough material to perform more extensive immunological or molecular analyses. The only option is to obtain larger biopsy materials as part of the routine practice such as surgery of Mtb infected patients. Another problem follows, as it is crucial to obtain similar clinical materials from uninfected individuals or from patients with other diseases than TB. This is necessary for the overall analysis and the interpretation of the scientific data, but nevertheless, it is very difficult to receive control samples from appropriate study cohorts.

Table 1. Differences in local and systemic immune responses

Local site of infection:	Systemic circulation:
✓ Cells kept in a physiological milieu in the presence of stromal cells and soluble factors	✓ Lack of three-dimensional tissue-organ structure
✓ Close cellular interactions, paracrine signalling, granuloma formation, necrosis and caseation in the tissue	✓ No granuloma formation or other organized cellular interactions
✓ Presence of Mtb bacilli and infected cells	✓ Lack of Mtb bacilli and infected cells (except for miliary TB)
✓ Compartmentalization of different immune cell subsets	✓ Migration of naive immune cells to local sites
✓ Morphological modifications of immune cells including epithelioid and giant cells	✓ No epithelioid or giant cell formation
✓ Tissue macrophages with high bactericidal activity or regulatory function	✓ Undifferentiated monocytes with low bactericidal activity
✓ High frequencies of *in vivo* activated Mtb-specific T cells that express different effector functions	✓ Low frequencies of Mtb-specific T cells that require *in vitro* restimulation with antigen to become activated
✓ Snap shot of a specific temporal window of TB disease	✓ Easily accessible clinical samples, possible to perform longitudinal analysis

Adapted from S. Brighenti, Journal of Infectious Diseases, May 15, 2012

4.2 METHODOLOGY

4.2.1 Project outline

This thesis is based on advanced analysis of tissues obtained from patients or experimental animals infected with Mtb. For this purpose, tissue biopsies have been collected from the lung or lymph nodes from patients with active pulmonary or extrapulmonary TB. In addition, lung, spleen and lymph nodes have been collected from Mtb-infected NHPs. All biopsies have been cryopreserved and stored at -85°C in order to prevent destruction and loss of sensitive epitopes, which is common upon conventional formalin fixation used for histopathological analyses. A key technology has involved assessment of protein expression and distribution in Mtb infected tissue biopsies using microscopy and *in situ* computerized image analysis. Another key method has involved quantification of mRNA expression in the tissues.

In summary, the following techniques have been used to study specific immune responses at the local site of Mtb infection:

- ✓ Immunohistochemistry and *in situ* computerized image analysis: Study I-IV
- ✓ Immunofluorescence and confocal microscopy: Study I-III
- ✓ Quantitative real-time PCR (qPCR): Study I, II and IV
- ✓ Enzyme Linked Immunosorbent Assay (ELISA): Study IV
- ✓ Flow cytometry: Study III

All work with Mtb-infected cryopreserved tissue samples have been performed at a biosafety level 3 (BSL-3) laboratory at the Swedish Institute for Communicable Disease Control; Smittskyddsinstitutet (SMI). After chemical inactivation of Mtb, the samples could be transferred to a BSL-2 laboratory for continued analysis.

A brief methodological description of the patients and key technologies is provided below. A more detailed description of the 'Materials and methods' can be found in each of the papers included in this thesis.

4.2.2 Patients and tissue samples

Tissue biopsies used in this thesis were obtained from the following study subjects:

- ✓ Lung biopsies from TB lesions obtained from Russian adult patients (n=19) with chronic incurable pulmonary TB.
  Controls: Lung biopsies from distal parenchyma obtained as part of the resected Mtb-infected lung (internal control, n=19) as well as lung biopsies obtained from uninfected patients with pulmonary malignancies (n=10).

- ✓ Lymph node biopsies obtained from Ethiopian children (n=10) with a local persistent lymph node TB (lymphadenitis).
  Controls: Lymph node biopsies obtained from age-matched children with a reactive non-specific lymphadenitis (n=10) as well as tonsil biopsies from age-matched children with a non-infectious tonsil hyperplasia (n=10).

- ✓ Lung, spleen and lymph node biopsies obtained from NHPs (n=6) that were given a prime-boost vaccination with a novel rBCG/rAd35 vaccine before challenge with virulent Mtb.
  Controls: Lung, spleen and lymph node biopsies obtained from BCG vaccinated and Mtb challenged NHPs (n=6) as well as corresponding tissue biopsies from unvaccinated and Mtb challenged NHPs (n=6).

A schematic illustration of the biopsies obtained is shown in Figure 7. All tissue biopsies were immediately frozen in liquid nitrogen after excision and preserved in OCT-compound at -85°C for future experiments.

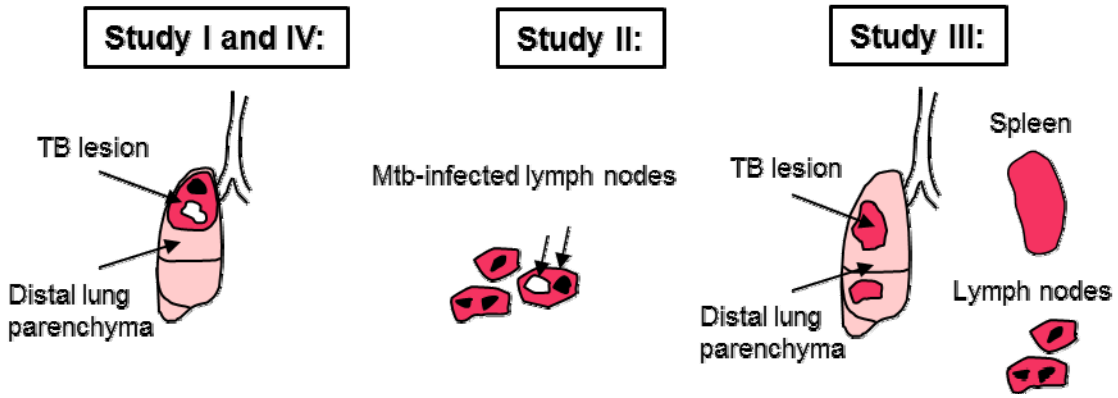

Figure 7. Biopsies were taken from lung and lymphoid tissue of Mtb-infected humans and NHPs. *Study I and IV:* Lung biopsies from TB lesion and unaffected distal lung parenchyma (internal control) from patients with cavitary and non-cavitary forms of TB. *Study II:* Lymph nodes from children with a local TB lymphadenitis. *Study III:* Lung biopsies from TB lesion and unaffected distal lung parenchyma, spleen and lymph nodes from rhesus macaques infected with Mtb by the aerosol route.

4.2.3 Cryosectioning of Mtb-infected tissue samples

Since Mtb is resistant to cold, freezing and desiccation [321], work with frozen biopsies from Mtb-infected patients or NHPs, is not without risk. To be able to perform this work, our group has established a facility suitable for cryosectioning of Mtb-infected materials in the BSL-3 laboratory at SMI. In this facility, the cryostat is covered by a custom-made ventilation hood [Figure 8]. 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.

Figure 8. Cryostat covered with a custom-made air filter (HEPA) in the BSL-3 laboratory at SMI to allow safe sectioning of Mtb-infected tissue samples.

4.2.4 *In situ* computerized image analysis

Immune responses including cytolytic and antimicrobial effector pathways in TB were investigated using immunohistochemistry and *in situ* computerized image analysis. This technology provides an excellent platform to explore local immune responses in clinical tissue samples. This is a semi-quantitative method to study protein expression in cryopreserved tissue. In addition, immunostainings enable visualization of tissue morphology, distribution of cells and effector molecules, and specific cell-cell interactions as well as the anatomical location of mycobacteria. Functional expression and distribution of various proteins including cell surface, cytoplasmic and nuclear as well as granule-associated and secreted proteins can be assessed 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 by quantified at the single cell level using a highly sensitive computerized image

analysis programme (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. It is also possible to choose different macros or 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. The image analysis procedure is illustrated in Figure 9. Co-expression of surface-expressed and/or intracellular proteins can also be detected using multicolor labeling and confocal microscopy. Quantification of immunofluorescent stainings is usually manual. In summary, this methodolgy can be widely used to study the expression of many different proteins in human as well as in animal tissues. *In situ* image analysis is a well-established technology in our laboratory and has been used to study immune responses in TB, HIV and Streptococcus infection [322-325].

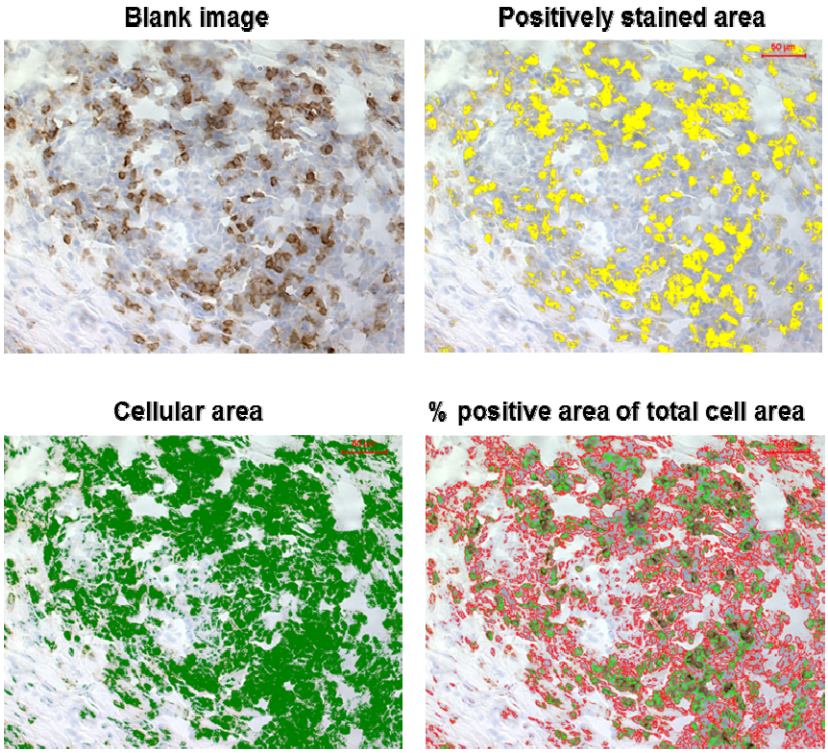

Figure 9. *In situ* computerized image analysis is used to quantify protein expression in tissue. i) A representative blank image of immunostaining of $CD3^+$ T cells in a lymph node granuloma. Positive staining is detected as brown color (diaminobenzidine) while negative staining is detected as blue color (hematoxylin). ii) The blank image is used to set the threshold for the intensity of the positive staining (yellow label). iii) Next, the blank image is used to set the threshold for the intensity of the total cellular area (green label), which is the positive (brown) + negative (blue) staining in the tissue. iv) After the thresholds are set, the digital software is able to determine the percent positive area of the total cell area as well as the total mean intensity of the staining.

4.2.5 Quantitative mRNA analysis

A convenient method has also been developed to quantify mRNA expression in frozen tissue biopsies using qPCR. mRNA analysis has been used as a complementary method to protein analysis and/or as a sensitive method to measure the relative change of markers that are expressed at low levels in the tissue. mRNA could also be used as a rapid and efficient method to screen the expression of multiple markers of interest, before protein analysis of selected markers. Typically, two thin sections of 50 microns each are cut in the cryostat and immediately transferred into an eppendorf tube. mRNA is extracted from the frozen tissue sections using a phenol-based compound (TRI Reagent), which also inactivates the mycobacteria in the sample. qPCR analysis provides the possibility to study the relative expression of many different markers in rare clinical samples. A very small amount of clinical material is required and once the mRNA has been converted to cDNA, the sample could be stored at -20°C for long periods of time.

4.3 STATISTICS

If the sample size >10 and the data passed a normality test, a parametric analysis was used to determine the statistical significance. Data were presented as mean ± standard error (SE) or median and range. If the sample size was ≤10, a non-parametric analysis was used to determine the statistical significance. Data were presented as median ± interquartile range (IQR).

When two groups were compared, one of the following tests was used:

- ✓ unpaired or paired t-test (parametric analyses)
- ✓ Mann-Whitney test or Wilcoxon Rank-Sum test (non-parametric analyses)

When more than two groups were compared, one of the following tests was used:

- ✓ ANOVA (parametric analysis)
- ✓ Kruskal-Wallis test and Dunn´s post-test or Friedman test (non-parametric analyses)

Spearman´s correlation test was used for the correlation analyses. The statistical analyses were performed in GraphPad Prism.

4.4 ETHICAL CONSIDERATIONS

Study I and IV: Lung biopsies from Russian TB patients and Swedish control malignancy patients were obtained after ethical approval from both Sweden (EPN dnr: 238/02) and Russia. Patients were recruited into the study after informed consent. **Study II:** 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. Patients were recruited into the study after parent's or guardian's approval and informed written consent. **Study III:** Lung, spleen and lymph nodes from Mtb-infected rhesus macaques were obtained after ethical approval from both Sweden (EPN dnr: N141/06) and the Netherlands. Housing and care for the animals were according to the general guidelines of the Swedish Animal Welfare Agency.

5 RESULTS AND DISCUSSION

5.1 TISSUE INFLAMMATION AND GRANULOMA FORMATION AT THE LOCAL SITE OF MTB INFECTION

5.1.1 Cellular dynamics

Mtb infection initiates a granulomatous inflammation, which is a critical component of the host immune defense when Mtb-infected MQs fail to eradicate intracellular bacilli [49]. As expected, Mtb-infected tissues from lung, lymph nodes and spleen were characterized by enhanced local inflammation including granuloma formation to wall off and contain the infection (**Paper I-IV**). Microscopic analysis of Mtb-infected lung tissues revealed that the gross TB lesions contained heterogeneous inflammation ranging from large necrotic granulomas to cellular infiltrates with high cellularity (**Paper I, III and IV**) [Figure 10]. Accordingly, mean cellularity of the Mtb-infected tissue including the presence of lymphoid aggregates (LA) was increased in the TB lesions as compared to distal lung parenchyma and uninfected control lung (**Paper I**). Quantitative image analysis was used to determine that the pulmonary infiltrates consisted of CD3+ T cells (both CD4+ and CD8+ T cells) and also CD20+ B cells (**Paper I, III and IV**). In contrast, cells of myeloid origin including monocytes/MQs and neutrophils were reduced in the TB lung lesions from patients with chronic pulmonary TB (**Paper I and IV**). Importantly, granulomatous inflammation was absent in tissue samples from the distal lung parenchyma of the Mtb-infected lung, which instead showed a similar spacious non-inflammatory morphology compared to uninfected control lung (**Paper I and IV**).

In Mtb-infected lymph nodes, we found confluent granulomas that contained CD68+ MQs expressing the Mtb-antigen MPT64, which suggested that the granuloma is the main site of bacterial persistence (**Paper II**) [Figure 10]. In contrast to TB lung lesions, the cellularity in the Mtb-infected lymph nodes was reduced and particularly involved lower levels of CD8+ T cells and CD20+ B cells compared to non-specific bacterial lymphadenitis (**Paper II**). Thus, granulomatous inflammation may involve enhanced cellular infiltration in the lung but disrupt normal lymphoid structure and result in reduced cell numbers in lymph node TB (**Papers I and II**). Both Mtb-infected lungs and lymph nodes had an increased deposition of collagen type I (**Paper II and III**), which suggests that fibrosis and scar tissue formation is enhanced in TB lesions with an active granulomatous inflammation. Consequently, even though both MQs and T cells are important to induce inflammation and immune protection in TB, uncontrolled activation of these cells results in necrosis and loss of normal tissue architecture which also can lead to cavity formation in pulmonary TB.

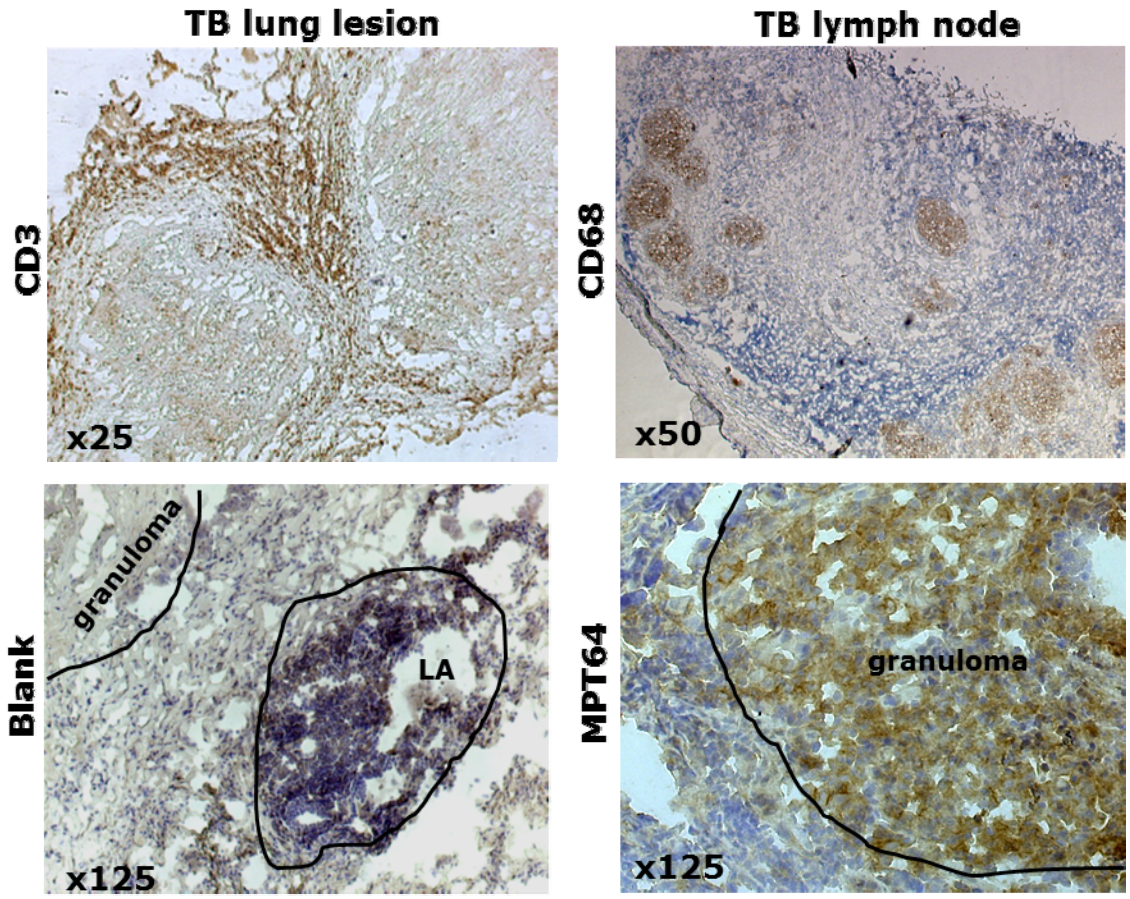

Figure 10. Tissue morphology and granuloma formation in TB lung lesions and lymph node TB. The left images show granulomatous inflammation in a TB lung lesion; note the peripheral expression of CD3 around the necrotic granulomas. Lymphoid aggregates (LA) are located in close proximity to granulomatous lesions. The right images show confluent granulomas in a TB lymph node that are abundant in CD68 staining as well as positive staining for the Mtb-specific antigen MPT64. Brown staining indicates positive cells (DAB); blue staining indicates negative cells (hematoxylin).

5.1.2 Detection of Mtb-specific antigens

Since it is difficult to detect acid-fast bacilli in clinical specimen, Mtb-complex specific antigens including *M.bovis* BCG as well as the Mtb-specific MPT64 protein were used to locate the bacilli in Mtb-infected tissues (**Paper II, III and IV**). It has previously been determined that cross-reactive *M. bovis* BCG as well as MPT64 antibodies can be successfully used to detect Mtb-specific proteins in infected lung and lymph nodes [197, 326]. Here, we were also able to quantify the expression of mycobacterial antigens in the tissue to study the abundance as well as the distribution of the bacteria at the local site of infection. In pulmonary TB, we identified a significantly increased expression of MPT64 (**Paper III**) as well as the *M. bovis* BCG antigen (**Paper IV**) in the TB lung lesions compared to the control groups. Similarly, we found that CD68+ MQs expressing *M. bovis* BCG or MPT64 accumulated in the granulomas of Mtb-infected lymph nodes (**Paper II**), while no expression of Mtb antigens was observed in the control groups. Two-color immunofluorescence labeling further confirmed co-expression of the MPT64

antigen in CD68+ MQs (**Paper II**). Accordingly, expression of MPT64 has been shown to be confined to MGC and modified MQs within the granulomatous lesions of Mtb-infected human lymph nodes [255]. It has also been proposed that immunocytochemical detection of MPT64 could be used to improve diagnosis of primarily extrapulmonary TB [326, 327].

5.2 IMPAIRED CYTOLYTIC AND ANTIMICROBIAL EFFECTOR CELL RESPONSES AT THE LOCAL SITE OF MTB INFECTION

5.2.1 Innate effector molecules: NO and LL-37

The role of NO in controlling mouse TB is well established [31, 101] whereas its role in human TB remains unclear [32, 328]. We were able to detect *in situ* expression of iNOS in both lung and lymph nodes from patients with active TB (**Papers I, II and IV**). Importantly, NO expression in both TB lung lesions and the distal sites were substantially higher compared to the uninfected control lung (**Paper I**). In addition, we detected particularly high expression of both iNOS and the NO-metabolite nitrotyrosine inside lymph node granulomas (**Paper II**), which may suggest that MQs are activated to produce NO *in vivo* at the site of Mtb-infection. Expression of catalytically active iNOS has previously been demonstrated in human alveolar MQs from pulmonary TB patients [329]. In addition, MQs and MGC expressing iNOS as well as nitrotyrosine were detected in TB granulomas from human lung [330], lymph node and pleura biopsies [144]. In contrast, it has been found difficult to induce NO production in human MQs *in vitro*. This may be a result from lack of essential co-factors in *in vitro* cultures [331], or perhaps human blood-derived MQs only produce very low levels of NO upon inflammatory stimuli *in vitro* [332]. However, killing of mycobacteria in human alveolar MQs was shown to be markedly reduced in the presence of a potent NO-inhibitor, which would support a major role for NO in control of intracellular mycobacterial growth [333]. Interestingly, clinical evidence proposed that levels of exhaled NO are lower in pulmonary TB patients compared to exposed household TB contacts [334]. Another study also showed that NO levels were lower in peripheral blood monocytes from patients with chronic MDR-TB compared to patients with a newly diagnosed pulmonary TB [335]. Interestingly, the expression of iNOS was similar in TB lung lesions and distal lung parenchyma, despite a significant reduction of CD68+ MQs in the lesions (**Paper I and IV**). The explanation for this may be that there is a relatively higher expression of iNOS per cell in the remaining CD68+ MQs in the TB lesions compared to distal sites. Alternatively, iNOS could be expressed by other cells that MQs, including DCs [336] or pulmonary epithelial cells [337] that have been shown to be able to express NO.

It is well-established that the antimicrobial peptide LL-37 is important in killing of intracellular Mtb in human MQs [13, 34, 338], both by direct bacterial killing and via the induction of autophagy [154]. Thus, we studied functional expression of LL-37 in human Mtb-infected lung (**Paper IV**). While there was no change in the mRNA levels of LL-37 in the TB lesions compared to distal lung parenchyma, *in*

situ image analysis demonstrated significantly reduced expression of LL-37 secreting cells in the TB lesion site (**Paper IV**). Reduced numbers of CD68+ and MAC387+ cells in the TB lesions compared to the distal sites could explain lower levels of LL-37. Low LL-37 expression could also be the result from posttranscriptional modifications that fail to up-regulate LL-37 peptides in Mtb-infected MQs or other cells such as monocytes, neutrophils or epithelial cells. Hence, despite the presence of iNOS in activated macrophages in the Mtb-infected lung tissue, these cells may not be able to induce proper expression of antimicrobial functions including LL-37. Interestingly, LL-37 protein levels were lower in TB lesions from patients with sputum-positive TB compared to patients with sputum-negative TB, which may suggest that reduced levels of LL-37 are associated to progressive TB disease (**Paper IV**). It has previously been reported that LL-37 expression is absent from pulmonary TB granulomas as well as lung tissue from uninfected individuals, while LL-37 is strongly elevated in inflammatory infiltrates from patients with acute pneumonia [339]. LL-37 is an early effector molecule and may not be expressed at high levels in more advanced stages of TB disease. However, LL-37 is evidently expressed by inflammatory cells in chronic TB lesions, although the expression is significantly higher in the distal sites of the Mtb-infected lung (**Paper IV**). Probably, both NO and LL-37 are most important early upon establishment of Mtb infection, while TB disease will progress if these innate effector responses fail to eradicate or limit intracellular growth of Mtb.

5.2.2 CTL effector molecules: perforin, granulysin and granzyme A

Both CD4+ Th1 cells [60] and CD8+ CTLs [79, 340] play a vital role in the regulation of host-Mtb interactions. In 1998, Steffen Stenger and co-workers were able to demonstrate that the CTL-expressed antimicrobial peptide granulysin, could kill Mtb bacilli by osmotic lysis [72]. Stenger also showed that granulysin was dependent on the pore-forming protein perforin in order to enter Mtb-infected cells and kill intracellular Mtb [72]. This work defined a novel mechanism by which a coordinated expression of perforin and granulysin in the granules of CTLs could contribute to immune protection against intracellular Mtb infection in humans. Thus, the aim with Paper I and II of this thesis was to explore if the progression of active TB in Mtb-infected patients was associated to a reduced antimicrobial activity of CTLs at the site of infection. First, we were able to show that despite an increased inflammation and infiltration of both CD4+ and CD8+ T cells in the TB lesions from patients with chronic pulmonary TB, mRNA as well as protein levels of perforin and granulysin remained low in the TB lesions (**Paper I**) [Figure 11]. Thus, despite an increased infiltration of CD3+ T cells in the TB lesion site, the relative expression of granule-associated effector molecules in CD3+ T cells was lower in the TB lesions compared to the unaffected lung parenchyma and control lung (**Paper I**).

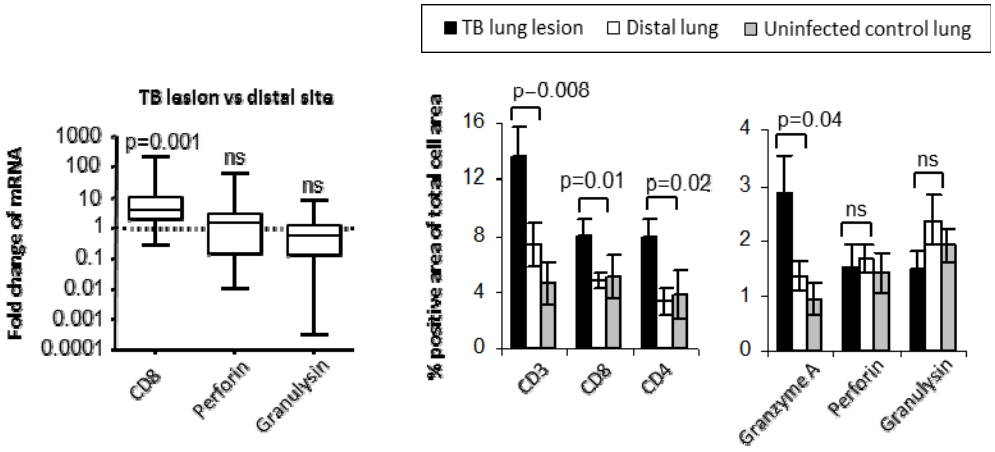

Figure 11. Decreased mRNA (left graph) and protein (right graph) expression of perforin and granulysin in TB lung lesions compared to distal lung parenchyma and control lung despite elevated levels of CD3+, CD4+ and CD8+ T cells in the TB lesion site.

Secondly, in patients with lymph node TB, the levels of CD8+ T cells and perforin were up-regulated compared to the uninfected control, while the expression of granulysin remained low (**Paper II**). Interestingly, CD8+ T cells as well as perforin and granulysin expression were particularly low inside TB granulomas in both lung and lymph nodes (**Papers I and II**). Instead, we found elevated levels of granzyme A in the TB lung lesions and TB lymph nodes compared to the control groups (**Paper I and II**), which may suggest a selective down-regulation of perforin and granulysin in Mtb-infected granulomatous tissues. Accordingly, co-expression of granzyme A in CD8+ T cells was relatively high in the TB lesions while the proportion of CD8+ T cells expressing perforin and granulysin was substantially lower (**Paper I and II**) [Figure 12]. Co-expression of granule-associated effector molecules was mainly confined to the CD8+ T cell subset, although 15-30% of granzyme A-expressing cells were CD8-negative (**Paper I and II**). This is in line with the notion that CD8+ T cells have a primary function in CTL-mediated killing while CD4+ T cells may be more involved in cytokine production. Increased levels of granzyme A but low perforin in granulomatous TB lesions may prevent access of granzyme A into the target cell and instead contribute to extracellular tissue destruction [341]. A similar impairment of perforin in the presence of enhanced granzyme A levels at the site of infection has also been observed in the progression of HIV infection [342, 343].

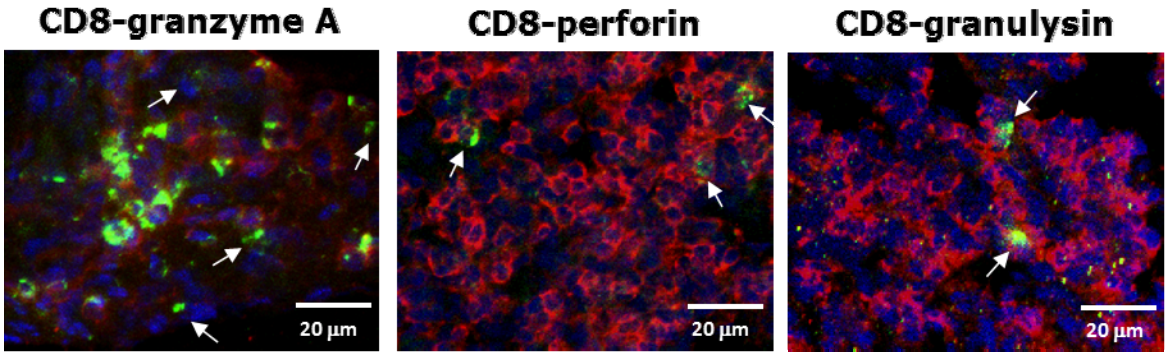

Figure 12. Co-expression of granzyme A, perforin and granulysin in CD8+ T cells present in a TB lung lesion. Single-positive cells are red (CD8) or green (granular markers), whereas double-positive cells are yellow (arrows).

The importance of perforin in CTL-mediated killing of mycobacteria has been illustrated both in mice [71, 167, 173, 344] and humans [345-347]. In contrast, there is no murine homologue to granulysin and thus evidence to support a role for granulysin in CTL-mediated killing of Mtb mainly involves *in vitro* studies of human cells [177, 348, 349]. Interestingly, it has been found that a poor lifestyle significantly decreases the numbers of perforin, granulysin, and granzymes A/B-expressing cells in blood lymphocytes [350], which could contribute to an imbalanced immune response in TB. Clinical studies have previously suggested that CTLs using perforin- and granulysin-mediated killing of intracellular Mtb could contribute to a protective host response in TB patients [351, 352]. Recently, important clinical evidence was also provided that perforin- and granulysin-expressing CD8+CD45RA+CCR7– effector memory T cells (TEMRA), were selectively depleted from rheumatoid arthritis patients treated with anti-TNF therapy, which caused reactivation of latent TB and progression of active TB disease [76]. Moreover, it has been shown that multifunctional CD8+ T cells expressing perforin and granulysin as well as CCL5, may be important to attract Mtb-infected MQs and kill intracellular Mtb bacilli [79]. Expression of CCL5 is also important for optimal T cell priming and control of Mtb infection [353]. Importantly, no other studies apart from the papers included in this thesis work, have been performed to explore the relevance of CD8+ T cells expressing perforin and granulysin at the local site of Mtb-infection. However, it has been discovered that T cell release of perforin and granulysin in skin lesions from patients infected with the Mtb-relative, *M. leprae*, contributes to host protection in humans [181].

5.2.3 B cell effector molecules: antibodies

Cell-mediated mechanisms involving innate immunity and T cells are of crucial importance for the control of intracellular Mtb infection. By contrast, the role of B cells during intracellular bacterial infection is controversial. We were interested to understand if impaired antimicrobial effector functions could be associated to an enhanced humoral immune response in patients with active TB. In pulmonary TB, we could detect clearly enhanced levels of CD20 and total IgG at both the mRNA and protein levels in the TB lesions compared to the distal lung parenchyma

(**Paper IV**) [Figure 13]. CD20+ B cells were typically organized in lymphoid aggregates that were scattered in the granulomatous tissue but not in the distal sites (**Paper IV**). In addition, IgG-secreting cells were also spotted in the lymphoid aggregates in the TB lesions. The presence of lymphoid aggregates in close proximity to TB granulomas have been described both in mice [354, 355] and human [197, 356] pulmonary tissue, which may suggests that such secondary lymphoid structures play a role in the control of local host-pathogen interactions. Both CD20+ B cells and IgG-secreting cells were higher in TB lesions from patients with sputum-positive TB compared to patients with sputum-negative TB (**Paper IV**), which may suggest that enhanced antibody-responses may be a consequence of exacerbated TB disease, especially in sputum-positive patients with extensive TB disease including cavitary TB [357]. Mycobacteria-specific IgG titers were also elevated in serum samples from pulmonary TB patients compared to uninfected healthy controls (**Paper IV**) [Figure 13]. Likewise, high levels of total and Mtb-specific serum antibodies have previously been shown in patients with advanced TB disease [358, 359] and several studies propose that Mtb-specific antibody responses may be used as potential diagnostic biomarkers of active TB disease [241, 360-362]. These findings indicate that humoral immunity may be the consequence of impaired cellular immunity at the local site of infection that contributes to an adverse immune response in chronic TB infection.

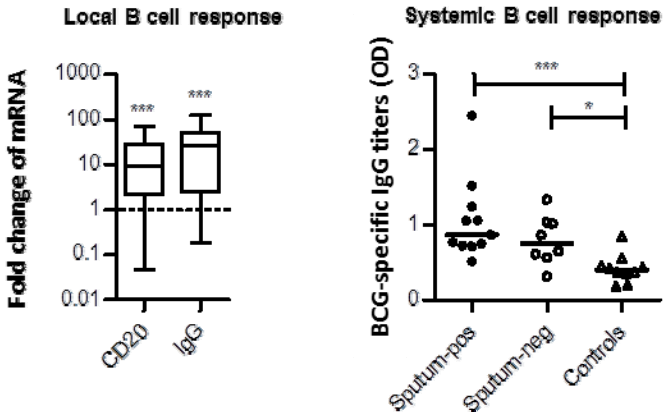

Figure 13. mRNA expression of both CD20 and total IgG was significantly up-regulated in TB lung lesions compared to distal lung parenchyma. BCG-specific IgG titers were also elevated in serum samples from both sputum-positive and sputum-negative TB patients compared to uninfected controls.

B cells are also professional antigen-presenting cells that may support the activation of local T cell responses in Mtb-infected tissues [189]. Hence, B cell responses may play a role in early protection and the induction of adaptive immunity in TB [237, 363], while enhanced antibody-responses in the chronic phase of TB infection may instead be associated to disease progression. Interestingly, it has recently become evident that regulatory B cells (Breg) from peripheral blood of healthy individuals can suppress Th1-mediated immune responses and also promote the expansion of functionally suppressive FoxP3+ Treg cells in an IL-10 dependent manner [364, 365]. One study also reports that elevated levels of a functionally suppressive B cell subset have been found in peripheral blood of TB patients [366]. It could be very interesting to explore the

potential link between certain subsets of Breg cells and FoxP3+ Treg cells at the site of infection in human TB.

5.3 IDENTIFICTAION OF IMMUNOPATHOGENIC PROCESSES AT THE LOCAL SITE OF MTB INFECTION

5.3.1 Altered cytokine responses

Next, we explored the potential immunopathogenic processes that may be involved in the impairment of antimicrobial effector cell responses. Since cytokines are key mediators that initiate and regulate immune responses during TB [49, 367], we assessed the expression of both inflammatory and anti-inflammatory cytokines in the Mtb-infected tissues to investigate if an altered cytokine profile could explain low levels of perforin and granulysin in CD8+ CTLs. Quantitative mRNA analysis revealed that the central Th1 cytokines IFN-γ and TNF-α as well as the Th17 cytokine IL-17, were not up-regulated in either TB lung lesions or Mtb-infected lymph nodes from patients with active TB (**Paper II and IV**). Instead, mRNA expression of the Th2 cytokine IL-13 and also the immunoregulatory cytokine TGF-β were significantly elevated in Mtb-infected lymph nodes compared to the controls (**Paper II**). *In situ* protein analysis confirmed that TGF-β was elevated in the Mtb-infected lymph nodes and involved a particularly high TGF-β expression inside the TB granulomas (**Paper II**). In patients with active pulmonary TB, we found an up-regulation of primarily the B cell stimulatory cytokine IL-21 but also IL-10 in the TB lesions compared to distal lung parenchyma (**Paper IV**). Interestingly, increased IL-21 mRNA levels correlated with mRNA expression of both CD20 and total IgG in the TB lung lesions (**Paper IV**). Accordingly, it has recently been demonstrated that IL-21 is mandatory for B cell activation, plasma cell differentiation and Ig-production [368]. In addition, *in vivo* development of functional IL-10-producing Breg cells have been shown to be dependent on the production of IL-21 [369].

Our results suggest that important Th1 and Th17 cytokines are not properly induced at the site of infection in patients with active TB, while there may be a shift towards a Th2 or immunoregulatory cytokine profile in Mtb-infected tissues [Figure 14]. An unbalanced cytokine response may contribute to an uncoordinated effector T cell response that reduces granule-mediated killing of Mtb-infected cells.

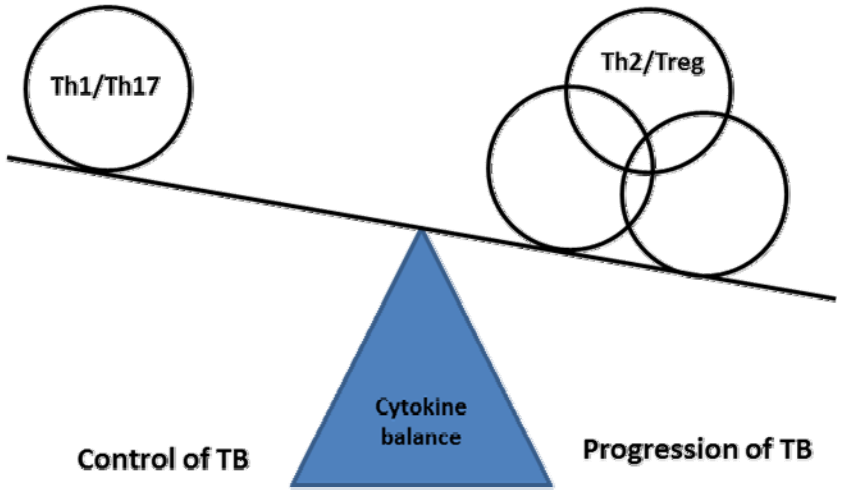

Figure 14. The cytokine balance may be skewed towards a Th2/Treg response in active TB disease.

Growing evidence suggests that a Th1/Th2 balance is crucial to control progression of active TB disease [91, 318, 370, 371]. Mtb-specific Th1 (IL-12, IFN-γ, TNF-α) and Th17 (IL-17) cells activate antimicrobial effector functions in infected MQs as well as in Mtb-specific CTLs to promote bacterial killing and containment of TB infection through granuloma formation [49, 372]. Instead, development of a Th2 (IL-4, IL-13) or anti-inflammatory (IL-10, TGF-β) cytokine response efficiently antagonizes protective cytokine responses, which could result in loss of TB control [128, 373]. Th2 cytokines have been shown to induce alternative MQ activation that involves a less bactericidal state of the MQ [134]. Th2 cytokines can also inhibit Th1-induced autophagy and thus reduce the intracellular degradation of Mtb bacteria [132]. Contrary, Th2 responses promote antibody-mediated immunity that fails to confer resistance in intracellular Mtb infection [371, 374-376]. Likewise, the T cell derived cytokine IL-21 is an important promoter of humoral immunity including the differentiation of human B cells into antibody-secreting cells [377]. In fact, IL-21 has been shown to be considerably more effective to stimulate plasma B cell development compared to both IL-4 and IL-10 [378]. The role of IL-21 in human TB is currently unknown but may involve enhanced plasma B cell responses in TB patients with progressive disease. Although IL-21 modulates a broad range of both myeloid and lymphoid immune cells, B cells are known to express the highest levels of the IL-21 receptor thus making B cells highly responsive to IL-21 [379].

Evidence have been provided that excess local production of IL-10 and TGF-β that occurs already at an early stage of Mtb infection may also suppress Th1 and CTL-mediated immunity and hamper control of TB infection [139, 380-385]. Thus, an early and enhanced Th1 response in Mtb-infected IL-10 deficient mice is associated to enhanced protection [386]. Interestingly, mycobacterial products including LAM, have been shown to induce TGF-β expression in human monocytes, which may promote bacterial survival [387]. Thus, inhibition of TGF-β in murine

TB was shown to increase the expression antimicrobial NO which effectively enhanced bacterial killing of Mtb in the lungs [388]. Although most evidence supports a role of IL-10 and TGF-β in TB disease progression, these cytokines also have a function to dampen chronic inflammation and detrimental immunopathology that may develop upon active TB infection [315, 389].

5.3.2 Induction of regulatory T cell responses

Recent studies provide evidence that a subset of Treg expressing the transcription factor Foxp3, plays a critical role to control the balance of the immune response to Mtb [390]. Thus, we studied the presence of FoxP3+ Treg cells in Mtb-infected tissues and if these cells were associated to suppression of antimicrobial effector T cell responses at the local site of infection. Our observations from lymph node TB revealed significantly elevated mRNA and protein levels of FoxP3 in Mtb-infected tissue compared to both non-specific TB lymphadenitis and the uninfected control (**Paper II**). Corresponding analysis of Mtb-infected tissue from patients with pulmonary TB showed that Foxp3+ Treg cells were also up-regulated in the TB lesions as compared to distal lung parenchyma (**Paper IV**). Importantly, our *in situ* findings suggest that lymph node granulomas contained increased numbers of CD4+FoxP3+ Treg cells compared to areas outside the granulomas (**Paper II**). Confocal microscopy revealed that co-expression of the Treg cell markers, CTLA-4 and GITR, were also particularly high inside the granulomas (**Paper II**), which support the conclusion that functional Treg cells are accumulated at the site of bacterial persistence.

Early induction of Treg cells delays local effector T cell responses in the lung, either by inhibition of DC function or by direct suppression of T cell effector functions [86, 391]. Similarly, premature induction of FoxP3+ Treg cells as well as TGF-β and IL-10 producing cells in SIV infection have been shown limit important SIV-specific CTL responses [392]. Importantly, Mtb may induce the expansion of Treg cells and use Treg cell-mediated immunosuppression to sustain bacterial replication at the site of infection [390]. Previously, increased levels of CD4+CD25+FoxP3+ Treg cells have been shown to suppress Th1 mediated immunity in patients with active TB [84, 85, 393-395]. Highly virulent Mtb Beijing strains also suppress Th1 cytokines responses that are replaced by FoxP3, IL-10 and TGF-β expression in Mtb-infected lungs [396]. Recently, it has been demonstrated that Mtb can promote the generation of Treg cells through the PD1-PDL1 pathway [397, 398]. Treg cells could mediate suppressive functions either by direct cell-cell contact or by the production of immunoregulatory cytokines such as IL-10 and TGF-β [399]. A better understanding of the mechanisms by which Treg cells counteract protective immune responses in TB could support the design of new therapeutic approaches aimed at inducing highly efficient T cell responses in the absence of simultaneous generation of Treg cells [400, 401].

5.4 INSIDE AND OUTSIDE THE TB GRANULOMA

While granulomas are formed by the cells of the immune system to aid Mtb containment, the specific host and bacterial factors that regulate granuloma development and function are still poorly defined. Our initial findings suggested that granulomas in gross TB lung lesions were abundant in both CD3+ and CD4+ T cells, while only few $CD8^+$ T cells were dispersed in the periphery of the granuloma, despite an overall increased number of CD8+ T cells in the TB lesions (**Paper I**). In addition, both perforin and granulysin were scarce inside the granulomas, while granzyme A levels were expressed at similar levels inside and outside the granulomas (**Paper I**). In lymph node TB, we were able to use image exclusion analysis to study specific expression of different proteins in the granulomas compared to the total Mtb-infected tissue (**Paper II**) [Figure 15]. These results suggest that CD68+ MQs expressing NO and Mtb-antigens were collected inside the granuloma at the site of bacterial persistence. Interestingly, high local concentrations of NO inside the TB granuloma may have negative effects on T cell responses [402]. Impaired CTL responses including low numbers of CD8+ T cells and CD56+ NK cells as well as low levels of perforin and granulysin in granulomas were inversely correlated with an increased proportion of both FoxP3+ Treg cells and TGF-β producing cells inside the granulomas. Here, TGF-β may be produced by Treg cells as well as Mtb-infected MQs 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. Perhaps a deficient chemokine response prevents the recruitment of CD8+ T cells into the granulomas. Here, Mtb-infected MQs and CD4+ T cells may fail to provide proper chemokine responses to attract functional CTLs to the site of Mtb infection [403, 404].

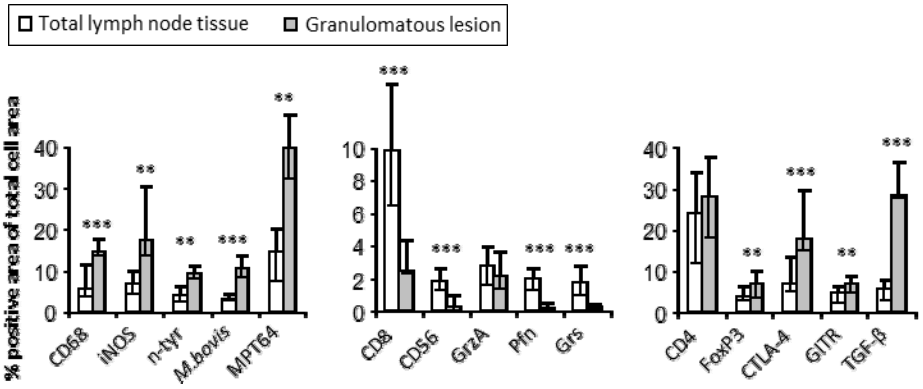

Figure 15. *In situ* image analysis of protein expression inside lymph node granulomas compared to total lymph node tissue. Expression of CD68+, iNOS, nitrotyrosine, *M. bovis* BCG as well as the Mtb-specific antigen MPT64 was significantly increased in the granuloma, which suggests that Mtb-infected MQ 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 enhanced expression of FoxP3, CTLA-4, GITR and TGF-β, suggesting active immunosuppression at the site of Mtb infection.

Whereas Mtb-infected MQs cluster together to form the core of the TB granuloma, a number of studies have shown that the predominating lymphocyte subset in the center of the granuloma is memory CD4+ T cells. Lower numbers of CD8+ T cells are mostly found in the peripheral rim surrounding the TB granuloma [197, 405, 406], while elevated levels of FoxP3+ Treg cells have previously been observed inside granulomas in both murine and NHP TB [407, 408]. The functional importance of this lymphocyte arrangement is currently unclear. It is possible that IFN-γ producing CD4+ T cells promote MQ activation and microbicidal effector functions inside the granuloma [90, 409]. However, as a substantial proportion of the CD4+ T cells may be Treg cells and not T effector cells, a shift in the Teffector/Treg ratio may promote active immunosuppression at the local site of infection. Accordingly, both Mtb-specific IFN-γ producing T cells as well as FoxP3+ Treg cells were previously found to be accumulated at the disease sites [84, 85]. 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 also result in impaired antimicrobial effector T cell responses and thus reduce contact-dependent killing of Mtb-infected cells inside the granuloma [410] [Figure 16].

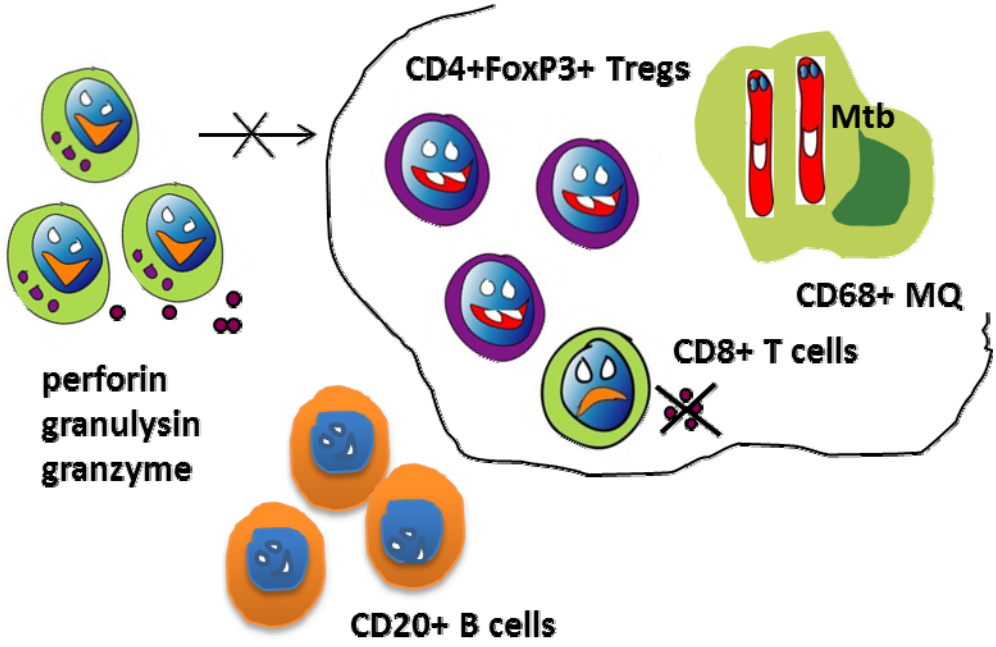

Figure 16. Compartmentalization of immune responses in human TB. Mtb-infected CD68+ MQs accumulate in the granulomas together with CD4+ T cells and FoxP3+ Treg cells. CD8+ CTLs expressing perforin and granulysin are very low inside the granulomas but mostly confined to areas outside the granulomas. CD20+ B cells are organized in lymphoid aggregates in close proximity to the granulomatous lesions.

The TB granuloma is a dynamic structure and thus the function of the granuloma may vary depending on the cellular composition in the granulomatous microenvironment [53, 194]. The balance of local immune responses in the granuloma will finally dictate bacterial control and pathology. Accordingly, classically activated MQs (M1) produce NO and control Mtb replication, while alternatively activated MQs (M2) produce IL-10 and TGF-β and are poorly microbicidal [411]. A predominance of M2 MQs in the granulomas may promote local expansion of Treg cells and impair the host ability to control Mtb infection. Thus, a shift in M1/M2, Th1/Th2 or Teffector/Treg cell ratio could promote immunosuppressive pathways and bacterial persistence in the TB lesions.

5.5 EVALUATION OF CTL INDUCTION IN RESPONSE TO A NOVEL TB VACCINE

Based on the knowledge and expertise we had generated through our studies on CTL responses in human TB, we had the opportunity to test if CD8+ CTLs and the granule-associated effector molecules perforin and granulysin, could be used as potential immune correlates to evaluate the protective efficacy of a novel TB vaccine, rBCG/rAd35 (**Paper III**). The rBCG/rAd35 vaccine, provided by the Aeras Global TB Vaccine Foundation, was designed to specifically enhance MHC-I-restricted CD8+ CTL responses. The novel vaccine was evaluated in a NHP model of TB and involved heterologous prime/boost immunization with the rBCG/rAd35 vaccine compared with parent BCG and an unvaccinated control group. The immunogenicity of the rBCG/rAd35 was evaluated in tissue biopsies from lung, spleen and lymph nodes after challenge with virulent Mtb. *In situ* image analysis revealed that Mtb-antigen load (MPT64) and the level of fibrosis was significantly reduced in the TB lung lesion and spleen tissue from rBCG/rAd35 primed animals compared to BCG/rAd35 and the unvaccinated control group. Importantly, increased numbers of CD3+ and CD8α/β+ T cells as well as increased expression of perforin, granulysin and the protective cytokine IL-7 was observed in the TB lung lesions and spleen tissues from the rBCG/rAd35 group. Consistent with our findings, it was previously shown that Mtb-peptide stimulation of peripheral blood from rBCG/rAd35 vaccinated NHPs increased CD8α/α+ T cell proliferation and IFN-γ production [412]. T cell homeostatic cytokines including IL-7 and IL-15 can enhance survival of Mtb infected mice [413], possibly by enhancing CD8+ CTL activity including up-regulation of serine esterases and perforin [414]. We also found that reduced Mtb-antigen levels was inversely correlated with enhanced CD3+ T cell numbers while perforin and granulysin expression correlated with the expression of CD8+ T cells [Figure 17]. Moreover, survival of Mtb-infected animals was associated with reduced MPT64 antigen expression and elevated CD8+ CTL responses in TB lung lesions. Together these data suggest that a TB vaccine construct that promotes the generation of functionally active CTLs, could reduce bacterial antigen load and destructive inflammation and instead enhance the survival of vaccinated and Mtb-infected animals.

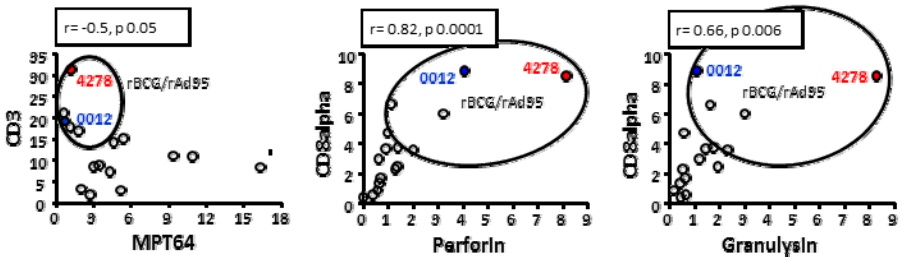

Figure 17. Inverse correlation between Mtb-specific antigen MPT64 and CD3+ T cells but positive correlation between CD8+ T cells and perforin and granulysin expression in the TB lung lesions from Mtb-infected animals. Data from animals in the rBCG/rAd35-vaccinated group are encircled in the graphs. In addition, data from two animals in the rBCG/rAd35-vaccinated group that also presented multifunctional T cell responses in peripheral blood [412] are given in red (ID 4278) and blue (ID0012) symbols.

Vaccine development requires reliable correlates of immune protection to evaluate vaccine-induced immunogenicity in humans and experimental animals. Currently, there are no sufficiently validated immune correlates of protection in human TB, although experimental animal models of TB suggest that IFN-γ secreting CD4+ and CD8+ T cells as well as multifunctional T cells may be used as immune correlates [73, 98, 229, 415]. However, the presence of CD4+ T cells and IFN-γ does not always correlate with immune protection and enhanced vaccine-induced immunity [220, 416]. Among the TB vaccine candidates that are currently being evaluated, most studies focus on the induction of cytokine responses to elicit protective immunity [298, 417-419]. However, it has also been suggested that granule-associated effector molecules including perforin and granulysin could be used as biomarkers of protective immunity following *M. bovis* vaccination of cattle [230]. Perhaps more complex combinations of immune mediators are required to better predict vaccine-induced protective responses in TB.

6 CONCLUDING REMARKS

This thesis provides novel insights into the cytolytic and antimicrobial effector functions and the immunopathogenic events that occur at the site of infection in human TB. We generated a technological platform to study local immune responses in tissue samples obtained from patients with active TB using qPCR and *in situ* computerized image analysis. Our main findings showed that:

- ✓ Inflammation in the Mtb-infected lung involved infiltration of CD3+, CD4+ and CD8+ T cells while tissue remodeling including granuloma formation and reduced cellularity was evident in lymph node TB.
- ✓ TB granulomas were enriched with CD68+ MQ expressing the Mtb-specific antigen MPT64 and also iNOS/NO, while the antimicrobial peptide LL-37 was expressed at low levels in the TB lung lesions.
- ✓ Impaired expression of the cytolytic and antimicrobial effector molecules perforin and granulysin, but not granzyme A, was evident in the Mtb-infected tissues and particularly inside the granulomas.
- ✓ Induction of FoxP3+ Treg cells and a shift in the cytokine balance from a Th1/Th17 towards a Th2/Treg immunoregulatory profile was found in the Mtb-infected tissue.
- ✓ Adverse immune responses in chronic TB were found to be associated with enhanced levels of CD20+ B cells and antibody-secreting cells in lymphoid aggregates detected in the TB lung lesions.
- ✓ Immune correlates of protection discovered in active human TB disease could be used as potential biomarkers to evaluate the immunogenicity of a novel TB vaccine candidate in a NHP model of TB.

In summary, key players of innate and adaptive immune responses in human TB involves antimicrobial effector pathways that we define as: **(1.)** activation of MQs and control of Mtb growth, mainly through the production of NO and the antimicrobial peptide LL-37, **(2.)** activation of CD8+ CTLs that trigger target cell and bacterial killing by the coordinated secretion of cytolytic and antimicrobial effector molecules, perforin and granulysin. These effector functions are regulated by a complex network of cells and immune mediators including Treg cells and immunosuppressive cytokines. Induction of suppressive immunoregulatory pathways could disturb the balance of protective host immunity and result in progression of TB disease and also fuel adverse immune responses including enhanced humoral immunity.

It will be helpful to continue to explore our clinical findings with *in vitro* experiments to investigate the functional link between impaired antimicrobial effector cell responses and immunopathogenic processes including Treg cells or improper cytokine profiles. The findings from this thesis may have clinical implications for novel diagnosis, as biomarkers of vaccine-induced immune responses or to monitor treatment efficacy.

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8 REFERENCES

1. Lawn, S.D. and A.I. Zumla, *Tuberculosis.* Lancet, 2011. **378**(9785): p. 57-72.
2. WHO, *Global tuberculosis report*, 2012, World Health organization.
3. Daniel, T.M., *The history of tuberculosis.* Respir Med, 2006. **100**(11): p. 1862-70.
4. Lienhardt, C., et al., *Global tuberculosis control: lessons learnt and future prospects.* Nature reviews. Microbiology, 2012. **10**(6): p. 407-16.
5. Raviglione, M.C., D.E. Snider, Jr., and A. Kochi, *Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic.* JAMA : the journal of the American Medical Association, 1995. **273**(3): p. 220-6.
6. Dheda, K., et al., *The immunology of tuberculosis: from bench to bedside.* Respirology, 2010. **15**(3): p. 433-50.
7. Bloom, B.R. and C.J. Murray, *Tuberculosis: commentary on a reemergent killer.* Science, 1992. **257**(5073): p. 1055-64.
8. Schnippel, K., et al., *Costs of inpatient treatment for multi-drug-resistant tuberculosis in South Africa.* Tropical medicine & international health : TM & IH, 2013. **18**(1): p. 109-16.
9. Steffen, R., et al., *Patients' costs and cost-effectiveness of tuberculosis treatment in DOTS and non-DOTS facilities in Rio de Janeiro, Brazil.* PloS one, 2010. **5**(11): p. e14014.
10. Forrellad, M.A., et al., *Virulence factors of the Mycobacterium tuberculosis complex.* Virulence, 2012. **4**(1).
11. Sakamoto, K., *The pathology of Mycobacterium tuberculosis infection.* Veterinary pathology, 2012. **49**(3): p. 423-39.
12. Zuniga, J., et al., *Cellular and humoral mechanisms involved in the control of tuberculosis.* Clinical & developmental immunology, 2012. **2012**: p. 193923.
13. Liu, P.T., et al., *Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response.* Science, 2006. **311**(5768): p. 1770-3.
14. Ernst, J.D., *Macrophage receptors for Mycobacterium tuberculosis.* Infection and immunity, 1998. **66**(4): p. 1277-81.
15. Philips, J.A. and J.D. Ernst, *Tuberculosis pathogenesis and immunity.* Annual review of pathology, 2012. **7**: p. 353-84.
16. Kang, P.B., et al., *The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis.* The Journal of experimental medicine, 2005. **202**(7): p. 987-99.
17. Korbel, D.S., B.E. Schneider, and U.E. Schaible, *Innate immunity in tuberculosis: myths and truth.* Microbes and infection / Institut Pasteur, 2008. **10**(9): p. 995-1004.
18. Ottenhoff, T.H., *The knowns and unknowns of the immunopathogenesis of tuberculosis.* The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 2012. **16**(11): p. 1424-32.
19. Kaufmann, S.H., *Is the development of a new tuberculosis vaccine possible?* Nature medicine, 2000. **6**(9): p. 955-60.

20. Gordon, S.V., et al., *Pathogenicity in the tubercle bacillus: molecular and evolutionary determinants.* BioEssays : news and reviews in molecular, cellular and developmental biology, 2009. **31**(4): p. 378-88.
21. Pieters, J., *Mycobacterium tuberculosis and the macrophage: maintaining a balance.* Cell host & microbe, 2008. **3**(6): p. 399-407.
22. Young, D. and C. Dye, *The development and impact of tuberculosis vaccines.* Cell, 2006. **124**(4): p. 683-7.
23. Barry, C.E., 3rd, et al., *The spectrum of latent tuberculosis: rethinking the biology and intervention strategies.* Nature reviews. Microbiology, 2009. **7**(12): p. 845-55.
24. Pawlowski, A., et al., *Tuberculosis and HIV co-infection.* PLoS pathogens, 2012. **8**(2): p. e1002464.
25. Faurholt-Jepsen, D., et al., *The role of diabetes on the clinical manifestations of pulmonary tuberculosis.* Tropical medicine & international health : TM & IH, 2012. **17**(7): p. 877-83.
26. Keane, J., et al., *Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent.* The New England journal of medicine, 2001. **345**(15): p. 1098-104.
27. Brassard, P., et al., *Inhaled corticosteroids and risk of tuberculosis in patients with respiratory diseases.* American journal of respiratory and critical care medicine, 2011. **183**(5): p. 675-8.
28. Alcais, A., et al., *Tuberculosis in children and adults: two distinct genetic diseases.* The Journal of experimental medicine, 2005. **202**(12): p. 1617-21.
29. van Crevel, R., T.H. Ottenhoff, and J.W. van der Meer, *Innate immunity to Mycobacterium tuberculosis.* Clin Microbiol Rev, 2002. **15**(2): p. 294-309.
30. Bermudez, L.E. and J. Goodman, *Mycobacterium tuberculosis invades and replicates within type II alveolar cells.* Infection and immunity, 1996. **64**(4): p. 1400-6.
31. Scanga, C.A., et al., *The inducible nitric oxide synthase locus confers protection against aerogenic challenge of both clinical and laboratory strains of Mycobacterium tuberculosis in mice.* Infection and immunity, 2001. **69**(12): p. 7711-7.
32. Chan, E.D., J. Chan, and N.W. Schluger, *What is the role of nitric oxide in murine and human host defense against tuberculosis?Current knowledge.* American journal of respiratory cell and molecular biology, 2001. **25**(5): p. 606-12.
33. Liu, P.T. and R.L. Modlin, *Human macrophage host defense against Mycobacterium tuberculosis.* Current opinion in immunology, 2008. **20**(4): p. 371-6.
34. Liu, P.T., et al., *Cutting edge: vitamin D-mediated human antimicrobial activity against Mycobacterium tuberculosis is dependent on the induction of cathelicidin.* Journal of immunology, 2007. **179**(4): p. 2060-3.
35. Persson, Y.A., et al., *Mycobacterium tuberculosis-induced apoptotic neutrophils trigger a pro-inflammatory response in macrophages through release of heat shock protein 72, acting in synergy with the bacteria.* Microbes and infection / Institut Pasteur, 2008. **10**(3): p. 233-40.
36. Hedlund, S., et al., *Dendritic cell activation by sensing Mycobacterium tuberculosis-induced apoptotic neutrophils via DC-SIGN.* Human immunology, 2010. **71**(6): p. 535-40.
37. Martineau, A.R., et al., *Neutrophil-mediated innate immune resistance to mycobacteria.* The Journal of clinical investigation, 2007. **117**(7): p. 1988-94.

38. Silva, M.T., M.N. Silva, and R. Appelberg, *Neutrophil-macrophage cooperation in the host defence against mycobacterial infections.* Microbial pathogenesis, 1989. **6**(5): p. 369-80.
39. Winau, F., S.H. Kaufmann, and U.E. Schaible, *Apoptosis paves the detour path for CD8 T cell activation against intracellular bacteria.* Cellular microbiology, 2004. **6**(7): p. 599-607.
40. Schaible, U.E., et al., *Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis.* Nature medicine, 2003. **9**(8): p. 1039-46.
41. Wolf, A.J., et al., *Mycobacterium tuberculosis infects dendritic cells with high frequency and impairs their function in vivo.* Journal of immunology, 2007. **179**(4): p. 2509-19.
42. Cooper, A.M., *Cell-mediated immune responses in tuberculosis.* Annual review of immunology, 2009. **27**: p. 393-422.
43. Khader, S.A., et al., *Interleukin 12p40 is required for dendritic cell migration and T cell priming after Mycobacterium tuberculosis infection.* The Journal of experimental medicine, 2006. **203**(7): p. 1805-15.
44. Mogues, T., et al., *The relative importance of T cell subsets in immunity and immunopathology of airborne Mycobacterium tuberculosis infection in mice.* The Journal of experimental medicine, 2001. **193**(3): p. 271-80.
45. Dieli, F., et al., *Vgamma9/Vdelta2 T lymphocytes reduce the viability of intracellular Mycobacterium tuberculosis.* European journal of immunology, 2000. **30**(5): p. 1512-9.
46. Bastian, M., et al., *Mycobacterial lipopeptides elicit CD4+ CTLs in Mycobacterium tuberculosis-infected humans.* Journal of immunology, 2008. **180**(5): p. 3436-46.
47. Ulrichs, T., et al., *T-cell responses to CD1-presented lipid antigens in humans with Mycobacterium tuberculosis infection.* Infection and immunity, 2003. **71**(6): p. 3076-87.
48. Stenger, S., K.R. Niazi, and R.L. Modlin, *Down-regulation of CD1 on antigen-presenting cells by infection with Mycobacterium tuberculosis.* Journal of immunology, 1998. **161**(7): p. 3582-8.
49. Kaufmann, S.H., *Protection against tuberculosis: cytokines, T cells, and macrophages.* Ann Rheum Dis, 2002. **61 Suppl 2**: p. ii54-8.
50. Brighenti, S. and J. Andersson, *Induction and regulation of CD8+ cytolytic T cells in human tuberculosis and HIV infection.* Biochemical and biophysical research communications, 2010. **396**(1): p. 50-7.
51. Kaufmann, S.H., *How can immunology contribute to the control of tuberculosis?* Nature reviews. Immunology, 2001. **1**(1): p. 20-30.
52. Urdahl, K.B., S. Shafiani, and J.D. Ernst, *Initiation and regulation of T-cell responses in tuberculosis.* Mucosal immunology, 2011. **4**(3): p. 288-93.
53. Brighenti, S. and J. Andersson, *Local immune responses in human tuberculosis: learning from the site of infection.* The Journal of infectious diseases, 2012. **205 Suppl 2**: p. S316-24.
54. Green, A.M., R. Difazio, and J.L. Flynn, *IFN-gamma from CD4 T cells is essential for host survival and enhances CD8 T cell function during Mycobacterium tuberculosis infection.* Journal of immunology, 2013. **190**(1): p. 270-7.
55. Serbina, N.V., V. Lazarevic, and J.L. Flynn, *CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection.* Journal of immunology, 2001. **167**(12): p. 6991-7000.

56. Femke Broere, S.G.A., Michail V. Sitkovsky and Wilem van Eden, *T cell subsets and T cell-mediated immunity,* in *Principles of Immunopharmacology,* F.P.a.P. Nijkamp, M.J., Editor 2011. p. 15-27.
57. Ottenhoff, T.H., *New pathways of protective and pathological host defense to mycobacteria.* Trends in microbiology, 2012. **20**(9): p. 419-28.
58. Scanga, C.A., et al., *Depletion of CD4(+) T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon gamma and nitric oxide synthase 2.* The Journal of experimental medicine, 2000. **192**(3): p. 347-58.
59. Orme, I.M. and F.M. Collins, *Protection against Mycobacterium tuberculosis infection by adoptive immunotherapy. Requirement for T cell-deficient recipients.* The Journal of experimental medicine, 1983. **158**(1): p. 74-83.
60. Boom, W.H., et al., *Human immunity to M. tuberculosis: T cell subsets and antigen processing.* Tuberculosis, 2003. **83**(1-3): p. 98-106.
61. Gallegos, A.M., et al., *A gamma interferon independent mechanism of CD4 T cell mediated control of M. tuberculosis infection in vivo.* PLoS pathogens, 2011. **7**(5): p. e1002052.
62. Lawn, S.D., R. Wood, and R.J. Wilkinson, *Changing concepts of "latent tuberculosis infection" in patients living with HIV infection.* Clinical & developmental immunology, 2011. **2011**.
63. Caruso, A.M., et al., *Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis.* Journal of immunology, 1999. **162**(9): p. 5407-16.
64. Bold, T.D., et al., *Suboptimal activation of antigen-specific CD4+ effector cells enables persistence of M. tuberculosis in vivo.* PLoS pathogens, 2011. **7**(5): p. e1002063.
65. Lewinsohn, D.M., et al., *Human purified protein derivative-specific CD4+ T cells use both CD95-dependent and CD95-independent cytolytic mechanisms.* Journal of immunology, 1998. **160**(5): p. 2374-9.
66. Bold, T.D. and J.D. Ernst, *CD4+ T cell-dependent IFN-gamma production by CD8+ effector T cells in Mycobacterium tuberculosis infection.* Journal of immunology. **189**(5): p. 2530-6.
67. Woodworth, J.S. and S.M. Behar, *Mycobacterium tuberculosis-specific CD8+ T cells and their role in immunity.* Critical reviews in immunology, 2006. **26**(4): p. 317-52.
68. Serbina, N.V. and J.L. Flynn, *CD8(+) T cells participate in the memory immune response to Mycobacterium tuberculosis.* Infection and immunity, 2001. **69**(7): p. 4320-8.
69. Yasukawa, M., et al., *Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicity mediated by alloantigen-specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans.* Blood, 2000. **95**(7): p. 2352-5.
70. Stenger, S., et al., *Differential effects of cytolytic T cell subsets on intracellular infection.* Science, 1997. **276**(5319): p. 1684-7.
71. Canaday, D.H., et al., *CD4(+) and CD8(+) T cells kill intracellular Mycobacterium tuberculosis by a perforin and Fas/Fas ligand-independent mechanism.* Journal of immunology, 2001. **167**(5): p. 2734-42.
72. Stenger, S., et al., *An antimicrobial activity of cytolytic T cells mediated by granulysin.* Science, 1998. **282**(5386): p. 121-5.

73. Flynn, J.L., et al., *Major histocompatibility complex class I-restricted T cells are required for resistance to Mycobacterium tuberculosis infection.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(24): p. 12013-7.
74. Kamath, A.B., et al., *Cytolytic CD8+ T cells recognizing CFP10 are recruited to the lung after Mycobacterium tuberculosis infection.* The Journal of experimental medicine, 2004. **200**(11): p. 1479-89.
75. Cho, S., et al., *Antimicrobial activity of MHC class I-restricted CD8+ T cells in human tuberculosis.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(22): p. 12210-5.
76. Bruns, H., et al., *Anti-TNF immunotherapy reduces CD8+ T cell-mediated antimicrobial activity against Mycobacterium tuberculosis in humans.* The Journal of clinical investigation, 2009. **119**(5): p. 1167-77.
77. Betts, M.R., et al., *HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells.* Blood, 2006. **107**(12): p. 4781-9.
78. Loxton, A.G., et al., *Heparin-binding hemagglutinin induces IFN-gamma(+) IL-2(+) IL-17(+) multifunctional CD4(+) T cells during latent but not active tuberculosis disease.* Clinical and vaccine immunology : CVI, 2012. **19**(5): p. 746-51.
79. Stegelmann, F., et al., *Coordinate expression of CC chemokine ligand 5, granulysin, and perforin in CD8+ T cells provides a host defense mechanism against Mycobacterium tuberculosis.* Journal of immunology, 2005. **175**(11): p. 7474-83.
80. Belkaid, Y. and B.T. Rouse, *Natural regulatory T cells in infectious disease.* Nature immunology, 2005. **6**(4): p. 353-60.
81. Bluestone, J.A. and A.K. Abbas, *Natural versus adaptive regulatory T cells.* Nature reviews. Immunology, 2003. **3**(3): p. 253-7.
82. Suffia, I.J., et al., *Infected site-restricted Foxp3+ natural regulatory T cells are specific for microbial antigens.* The Journal of experimental medicine, 2006. **203**(3): p. 777-88.
83. Ribeiro-Rodrigues, R., et al., *A role for CD4+CD25+ T cells in regulation of the immune response during human tuberculosis.* Clinical and experimental immunology, 2006. **144**(1): p. 25-34.
84. Guyot-Revol, V., et al., *Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis.* American journal of respiratory and critical care medicine, 2006. **173**(7): p. 803-10.
85. Chen, X., et al., *CD4(+)CD25(+)FoxP3(+) regulatory T cells suppress Mycobacterium tuberculosis immunity in patients with active disease.* Clinical immunology, 2007. **123**(1): p. 50-9.
86. Shafiani, S., et al., *Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis.* The Journal of experimental medicine, 2010. **207**(7): p. 1409-20.
87. Garg, A., et al., *Mannose-capped lipoarabinomannan- and prostaglandin E2-dependent expansion of regulatory T cells in human Mycobacterium tuberculosis infection.* European journal of immunology, 2008. **38**(2): p. 459-69.
88. Periasamy, S., et al., *Programmed death 1 and cytokine inducible SH2-containing protein dependent expansion of regulatory T cells upon stimulation With Mycobacterium tuberculosis.* The Journal of infectious diseases, 2011. **203**(9): p. 1256-63.

89. Jafari, C., et al., *Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot.* Eur Respir J, 2008. **31**(2): p. 261-5.
90. Fenhalls, G., et al., *Distribution of IFN-gamma, IL-4 and TNF-alpha protein and CD8 T cells producing IL-12p40 mRNA in human lung tuberculous granulomas.* Immunology, 2002. **105**(3): p. 325-35.
91. Herrera, M.T., et al., *Compartmentalized bronchoalveolar IFN-gamma and IL-12 response in human pulmonary tuberculosis.* Tuberculosis, 2009. **89**(1): p. 38-47.
92. Kellar, K.L., et al., *Multiple cytokines are released when blood from patients with tuberculosis is stimulated with Mycobacterium tuberculosis antigens.* PloS one, 2011. **6**(11): p. e26545.
93. Guler, R., et al., *Blocking IL-1alpha but not IL-1beta increases susceptibility to chronic Mycobacterium tuberculosis infection in mice.* Vaccine, 2011. **29**(6): p. 1339-46.
94. Unsal, E., et al., *Potential role of interleukin 6 in reactive thrombocytosis and acute phase response in pulmonary tuberculosis.* Postgraduate medical journal, 2005. **81**(959): p. 604-7.
95. Zhang, Y. and W.N. Rom, *Regulation of the interleukin-1 beta (IL-1 beta) gene by mycobacterial components and lipopolysaccharide is mediated by two nuclear factor-IL6 motifs.* Molecular and cellular biology, 1993. **13**(6): p. 3831-7.
96. Cooper, A.M., et al., *IFN-gamma and NO in mycobacterial disease: new jobs for old hands.* Trends in microbiology, 2002. **10**(5): p. 221-6.
97. Vogt, G. and C. Nathan, *In vitro differentiation of human macrophages with enhanced antimycobacterial activity.* The Journal of clinical investigation, 2011. **121**(10): p. 3889-901.
98. Flynn, J.L., et al., *An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection.* The Journal of experimental medicine, 1993. **178**(6): p. 2249-54.
99. Newport, M.J., et al., *A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection.* The New England journal of medicine, 1996. **335**(26): p. 1941-9.
100. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nature reviews. Immunology, 2008. **8**(12): p. 958-69.
101. Herbst, S., U.E. Schaible, and B.E. Schneider, *Interferon gamma activated macrophages kill mycobacteria by nitric oxide induced apoptosis.* PloS one, 2011. **6**(5): p. e19105.
102. Fabri, M., et al., *Vitamin D is required for IFN-gamma-mediated antimicrobial activity of human macrophages.* Science translational medicine, 2011. **3**(104): p. 104ra102.
103. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages.* Cell, 2004. **119**(6): p. 753-66.
104. Desvignes, L. and J.D. Ernst, *Interferon-gamma-responsive nonhematopoietic cells regulate the immune response to Mycobacterium tuberculosis.* Immunity, 2009. **31**(6): p. 974-85.
105. Leal, I.S., et al., *Failure to induce enhanced protection against tuberculosis by increasing T-cell-dependent interferon-gamma generation.* Immunology, 2001. **104**(2): p. 157-61.

106. Majlessi, L., et al., *An increase in antimycobacterial Th1-cell responses by prime-boost protocols of immunization does not enhance protection against tuberculosis.* Infection and immunity, 2006. **74**(4): p. 2128-37.
107. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Tumor necrosis factor signaling.* Cell death and differentiation, 2003. **10**(1): p. 45-65.
108. Bean, A.G., et al., *Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin.* Journal of immunology, 1999. **162**(6): p. 3504-11.
109. Raja, A., *Immunology of tuberculosis.* The Indian journal of medical research, 2004. **120**(4): p. 213-32.
110. Flynn, J.L., et al., *Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice.* Immunity, 1995. **2**(6): p. 561-72.
111. Roach, D.R., et al., *TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection.* Journal of immunology, 2002. **168**(9): p. 4620-7.
112. Cooper, A.M., K.D. Mayer-Barber, and A. Sher, *Role of innate cytokines in mycobacterial infection.* Mucosal immunology, 2011. **4**(3): p. 252-60.
113. Jacobs, M., et al., *Tumor necrosis factor is critical to control tuberculosis infection.* Microbes and infection / Institut Pasteur, 2007. **9**(5): p. 623-8.
114. Khader, S.A., S.L. Gaffen, and J.K. Kolls, *Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa.* Mucosal immunology, 2009. **2**(5): p. 403-11.
115. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages.* Nature immunology, 2005. **6**(11): p. 1123-32.
116. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17.* Nature immunology, 2005. **6**(11): p. 1133-41.
117. Liang, S.C., et al., *Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides.* The Journal of experimental medicine, 2006. **203**(10): p. 2271-9.
118. Khader, S.A. and R. Gopal, *IL-17 in protective immunity to intracellular pathogens.* Virulence, 2010. **1**(5): p. 423-7.
119. Khader, S.A., et al., *IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge.* Nature immunology, 2007. **8**(4): p. 369-77.
120. Okamoto Yoshida, Y., et al., *Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung.* Journal of immunology, 2010. **184**(8): p. 4414-22.
121. Maeurer, M.J. and M.T. Lotze, *Interleukin-7 (IL-7) knockout mice. Implications for lymphopoiesis and organ-specific immunity.* International reviews of immunology, 1998. **16**(3-4): p. 309-22.
122. Morrissey, P.J., et al., *Recombinant interleukin 7, pre-B cell growth factor, has costimulatory activity on purified mature T cells.* The Journal of experimental medicine, 1989. **169**(3): p. 707-16.

123. Sprent, J. and C.D. Surh, *Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells.* Nature immunology, 2011. **12**(6): p. 478-84.
124. Baumann, S., A. Nasser Eddine, and S.H. Kaufmann, *Progress in tuberculosis vaccine development.* Current opinion in immunology, 2006. **18**(4): p. 438-48.
125. Cui, W. and S.M. Kaech, *Generation of effector CD8+ T cells and their conversion to memory T cells.* Immunological reviews, 2010. **236**: p. 151-66.
126. Melchionda, F., et al., *Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool.* The Journal of clinical investigation, 2005. **115**(5): p. 1177-87.
127. Dunlap, N.E. and D.E. Briles, *Immunology of tuberculosis.* The Medical clinics of North America, 1993. **77**(6): p. 1235-51.
128. Rook, G.A., *Th2 cytokines in susceptibility to tuberculosis.* Current molecular medicine, 2007. **7**(3): p. 327-37.
129. Freeman, S., et al., *Mycobacterium tuberculosis H37Ra and H37Rv differential growth and cytokine/chemokine induction in murine macrophages in vitro.* Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research, 2006. **26**(1): p. 27-33.
130. Manca, C., et al., *Differential monocyte activation underlies strain-specific Mycobacterium tuberculosis pathogenesis.* Infection and immunity, 2004. **72**(9): p. 5511-4.
131. Ghadimi, D., et al., *Lactic acid bacteria enhance autophagic ability of mononuclear phagocytes by increasing Th1 autophagy-promoting cytokine (IFN-gamma) and nitric oxide (NO) levels and reducing Th2 autophagy-restraining cytokines (IL-4 and IL-13) in response to Mycobacterium tuberculosis antigen.* International immunopharmacology. **10**(6): p. 694-706.
132. Harris, J., et al., *T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis.* Immunity, 2007. **27**(3): p. 505-17.
133. Skapenko, A., et al., *The IL-4 receptor alpha-chain-binding cytokines, IL-4 and IL-13, induce forkhead box P3-expressing CD25+CD4+ regulatory T cells from CD25-CD4+ precursors.* Journal of immunology, 2005. **175**(9): p. 6107-16.
134. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions.* Immunity, 2010. **32**(5): p. 593-604.
135. van Crevel, R., et al., *Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities.* The Journal of infectious diseases, 2000. **181**(3): p. 1194-7.
136. Hirsch, C.S., et al., *Cross-modulation by transforming growth factor beta in human tuberculosis: suppression of antigen-driven blastogenesis and interferon gamma production.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(8): p. 3193-8.
137. Othieno, C., et al., *Interaction of Mycobacterium tuberculosis-induced transforming growth factor beta1 and interleukin-10.* Infection and immunity, 1999. **67**(11): p. 5730-5.
138. Toossi, Z., et al., *Enhanced production of TGF-beta by blood monocytes from patients with active tuberculosis and presence of TGF-beta in tuberculous granulomatous lung lesions.* Journal of immunology, 1995. **154**(1): p. 465-73.

139. Toossi, Z. and J.J. Ellner, *The role of TGF beta in the pathogenesis of human tuberculosis.* Clinical immunology and immunopathology, 1998. **87**(2): p. 107-14.
140. Sporn, M.B., et al., *Transforming growth factor-beta: biological function and chemical structure.* Science, 1986. **233**(4763): p. 532-4.
141. Bogdan, C., M. Rollinghoff, and A. Diefenbach, *Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity.* Current opinion in immunology, 2000. **12**(1): p. 64-76.
142. Schon, T., et al., *Arginine as an adjuvant to chemotherapy improves clinical outcome in active tuberculosis.* Eur Respir J, 2003. **21**(3): p. 483-8.
143. Yang, C.S., J.M. Yuk, and E.K. Jo, *The role of nitric oxide in mycobacterial infections.* Immune Netw, 2009. **9**(2): p. 46-52.
144. Schon, T., et al., *Local production of nitric oxide in patients with tuberculosis.* The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 2004. **8**(9): p. 1134-7.
145. Rahman, S., et al., *Compartmentalization of immune responses in human tuberculosis: few CD8+ effector T cells but elevated levels of FoxP3+ regulatory t cells in the granulomatous lesions.* Am J Pathol, 2009. **174**(6): p. 2211-24.
146. Zasloff, M., *Antimicrobial peptides of multicellular organisms.* Nature, 2002. **415**(6870): p. 389-95.
147. De Smet, K. and R. Contreras, *Human antimicrobial peptides: defensins, cathelicidins and histatins.* Biotechnol Lett, 2005. **27**(18): p. 1337-47.
148. Scott, M.G., et al., *The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses.* Journal of immunology, 2002. **169**(7): p. 3883-91.
149. Agerberth, B., et al., *The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations.* Blood, 2000. **96**(9): p. 3086-93.
150. Bals, R., et al., *The peptide antibiotic LL-37/hCAP-18 is expressed in epithelia of the human lung where it has broad antimicrobial activity at the airway surface.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(16): p. 9541-6.
151. Di Nardo, A., A. Vitiello, and R.L. Gallo, *Cutting edge: mast cell antimicrobial activity is mediated by expression of cathelicidin antimicrobial peptide.* Journal of immunology, 2003. **170**(5): p. 2274-8.
152. Dorschner, R.A., et al., *Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus.* The Journal of investigative dermatology, 2001. **117**(1): p. 91-7.
153. Thennarasu, S., et al., *Antimicrobial and membrane disrupting activities of a peptide derived from the human cathelicidin antimicrobial peptide LL37.* Biophys J. **98**(2): p. 248-57.
154. Yuk, J.M., et al., *Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin.* Cell host & microbe, 2009. **6**(3): p. 231-43.
155. Mendez-Samperio, P., *The human cathelicidin hCAP18/LL-37: a multifunctional peptide involved in mycobacterial infections.* Peptides. **31**(9): p. 1791-8.
156. Voskoboinik, I., M.J. Smyth, and J.A. Trapani, *Perforin-mediated target-cell death and immune homeostasis.* Nature reviews. Immunology, 2006. **6**(12): p. 940-52.

157. Cullen, S.P., M. Brunet, and S.J. Martin, *Granzymes in cancer and immunity.* Cell death and differentiation. **17**(4): p. 616-23.
158. Bots, M. and J.P. Medema, *Granzymes at a glance.* Journal of cell science, 2006. **119**(Pt 24): p. 5011-4.
159. Brahmi, Z., et al., *Synergistic inhibition of human cell-mediated cytotoxicity by complement component antisera indicates that target cell lysis may result from an enzymatic cascade involving granzymes and perforin.* Nat Immun, 1995. **14**(5-6): p. 271-85.
160. Grossman, W.J., et al., *The orphan granzymes of humans and mice.* Current opinion in immunology, 2003. **15**(5): p. 544-52.
161. Zhu, P., et al., *Granzyme A, which causes single-stranded DNA damage, targets the double-strand break repair protein Ku70.* EMBO reports, 2006. **7**(4): p. 431-7.
162. Smyth, M.J., S.E. Street, and J.A. Trapani, *Cutting edge: granzymes A and B are not essential for perforin-mediated tumor rejection.* Journal of immunology, 2003. **171**(2): p. 515-8.
163. Lieberman, J. and Z. Fan, *Nuclear war: the granzyme A-bomb.* Current opinion in immunology, 2003. **15**(5): p. 553-9.
164. Podack, E.R., J.D. Young, and Z.A. Cohn, *Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules.* Proceedings of the National Academy of Sciences of the United States of America, 1985. **82**(24): p. 8629-33.
165. Bovenschen, N. and J.A. Kummer, *Orphan granzymes find a home.* Immunological reviews. **235**(1): p. 117-27.
166. Praper, T., et al., *Human perforin employs different avenues to damage membranes.* The Journal of biological chemistry, 2011. **286**(4): p. 2946-55.
167. Kagi, D., et al., *Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice.* Nature, 1994. **369**(6475): p. 31-7.
168. Lowin, B., et al., *A null mutation in the perforin gene impairs cytolytic T lymphocyte- and natural killer cell-mediated cytotoxicity.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(24): p. 11571-5.
169. van Dommelen, S.L., et al., *Perforin and granzymes have distinct roles in defensive immunity and immunopathology.* Immunity, 2006. **25**(5): p. 835-48.
170. Cullen, S.P. and S.J. Martin, *Mechanisms of granule-dependent killing.* Cell death and differentiation, 2008. **15**(2): p. 251-62.
171. Samten, B., et al., *CD40 ligand trimer enhances the response of CD8+ T cells to Mycobacterium tuberculosis.* Journal of immunology, 2003. **170**(6): p. 3180-6.
172. De La Barrera, S.S., et al., *Specific lytic activity against mycobacterial antigens is inversely correlated with the severity of tuberculosis.* Clinical and experimental immunology, 2003. **132**(3): p. 450-61.
173. Woodworth, J.S., Y. Wu, and S.M. Behar, *Mycobacterium tuberculosis-specific CD8+ T cells require perforin to kill target cells and provide protection in vivo.* Journal of immunology, 2008. **181**(12): p. 8595-603.
174. Jongstra, J., et al., *The isolation and sequence of a novel gene from a human functional T cell line.* The Journal of experimental medicine, 1987. **165**(3): p. 601-14.
175. Siano, A., et al., *Bactericidal and hemolytic activities of synthetic peptides derived from granulysin.* Protein and peptide letters, 2010. **17**(4): p. 517-21.

176. Hanson, D.A., et al., *Biosynthesis of granulysin, a novel cytolytic molecule.* Molecular immunology, 1999. **36**(7): p. 413-22.
177. Ernst, W.A., et al., *Granulysin, a T cell product, kills bacteria by altering membrane permeability.* Journal of immunology, 2000. **165**(12): p. 7102-8.
178. Wang, Z., et al., *Bactericidal and tumoricidal activities of synthetic peptides derived from granulysin.* Journal of immunology, 2000. **165**(3): p. 1486-90.
179. Hata, A., et al., *Granulysin blocks replication of varicella-zoster virus and triggers apoptosis of infected cells.* Viral immunology, 2001. **14**(2): p. 125-33.
180. Pardo, J., et al., *A role of the mitochondrial apoptosis-inducing factor in granulysin-induced apoptosis.* Journal of immunology, 2001. **167**(3): p. 1222-9.
181. Ochoa, M.T., et al., *T-cell release of granulysin contributes to host defense in leprosy.* Nature medicine, 2001. **7**(2): p. 174-9.
182. Di Liberto, D., et al., *Decreased serum granulysin levels in childhood tuberculosis which reverse after therapy.* Tuberculosis, 2007. **87**(4): p. 322-8.
183. Tewary, P., et al., *Granulysin activates antigen-presenting cells through TLR4 and acts as an immune alarmin.* Blood. **116**(18): p. 3465-74.
184. Potian, J.A., et al., *Preexisting helminth infection induces inhibition of innate pulmonary anti-tuberculosis defense by engaging the IL-4 receptor pathway.* The Journal of experimental medicine, 2011. **208**(9): p. 1863-74.
185. Ettinger, R., et al., *IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells.* Journal of immunology, 2005. **175**(12): p. 7867-79.
186. Williams, A., et al., *Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs.* Immunology, 2004. **111**(3): p. 328-33.
187. Reljic, R., et al., *Intranasal IFNgamma extends passive IgA antibody protection of mice against Mycobacterium tuberculosis lung infection.* Clinical and experimental immunology, 2006. **143**(3): p. 467-73.
188. Hamasur, B., et al., *A mycobacterial lipoarabinomannan specific monoclonal antibody and its F(ab') fragment prolong survival of mice infected with Mycobacterium tuberculosis.* Clinical and experimental immunology, 2004. **138**(1): p. 30-8.
189. Maglione, P.J. and J. Chan, *How B cells shape the immune response against Mycobacterium tuberculosis.* European journal of immunology, 2009. **39**(3): p. 676-86.
190. Russell, D.G., *Who puts the tubercle in tuberculosis?* Nature reviews. Microbiology, 2007. **5**(1): p. 39-47.
191. Silva Miranda, M., et al., *The tuberculous granuloma: an unsuccessful host defence mechanism providing a safety shelter for the bacteria?* Clinical & developmental immunology, 2012. **2012**: p. 139127.
192. Ulrichs, T. and S.H. Kaufmann, *New insights into the function of granulomas in human tuberculosis.* The Journal of pathology, 2006. **208**(2): p. 261-9.
193. Fenhalls, G., et al., *In situ detection of Mycobacterium tuberculosis transcripts in human lung granulomas reveals differential gene expression in necrotic lesions.* Infection and immunity, 2002. **70**(11): p. 6330-8.
194. Flynn, J.L., J. Chan, and P.L. Lin, *Macrophages and control of granulomatous inflammation in tuberculosis.* Mucosal immunology, 2011. **4**(3): p. 271-8.
195. Gengenbacher, M. and S.H. Kaufmann, *Mycobacterium tuberculosis: success through dormancy.* FEMS microbiology reviews, 2012. **36**(3): p. 514-32.

196. Gideon, H.P. and J.L. Flynn, *Latent tuberculosis: what the host "sees"?* Immunologic research, 2011. **50**(2-3): p. 202-12.
197. Ulrichs, T., et al., *Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung.* Journal of Pathology, 2004. **204**(2): p. 217-228.
198. Ulrichs, T., et al., *Differential organization of the local immune response in patients with active cavitary tuberculosis or with nonprogressive tuberculoma.* The Journal of infectious diseases, 2005. **192**(1): p. 89-97.
199. Ducati, R.G., et al., *The resumption of consumption -- a review on tuberculosis.* Memorias do Instituto Oswaldo Cruz, 2006. **101**(7): p. 697-714.
200. Ehlers, S., *Immunity to tuberculosis: a delicate balance between protection and pathology.* FEMS immunology and medical microbiology, 1999. **23**(2): p. 149-58.
201. Russell, D.G., *Mycobacterium tuberculosis and the intimate discourse of a chronic infection.* Immunological reviews, 2011. **240**(1): p. 252-68.
202. Co, D.O., et al., *Mycobacterial granulomas: keys to a long-lasting host-pathogen relationship.* Clinical immunology, 2004. **113**(2): p. 130-6.
203. Lawn, S.D., S.T. Butera, and T.M. Shinnick, *Tuberculosis unleashed: the impact of human immunodeficiency virus infection on the host granulomatous response to Mycobacterium tuberculosis.* Microbes and infection / Institut Pasteur, 2002. **4**(6): p. 635-46.
204. Ramakrishnan, L., *Revisiting the role of the granuloma in tuberculosis.* Nature reviews. Immunology, 2012. **12**(5): p. 352-66.
205. Volkman, H.E., et al., *Tuberculous granuloma induction via interaction of a bacterial secreted protein with host epithelium.* Science, 2010. **327**(5964): p. 466-9.
206. Volkman, H.E., et al., *Tuberculous granuloma formation is enhanced by a mycobacterium virulence determinant.* PLoS biology, 2004. **2**(11): p. e367.
207. Davis, J.M. and L. Ramakrishnan, *The role of the granuloma in expansion and dissemination of early tuberculous infection.* Cell, 2009. **136**(1): p. 37-49.
208. Makinoshima, H. and M.S. Glickman, *Regulation of Mycobacterium tuberculosis cell envelope composition and virulence by intramembrane proteolysis.* Nature, 2005. **436**(7049): p. 406-9.
209. Briken, V., et al., *Mycobacterial lipoarabinomannan and related lipoglycans: from biogenesis to modulation of the immune response.* Molecular microbiology, 2004. **53**(2): p. 391-403.
210. Pathak, S.K., et al., *Mycobacterium tuberculosis lipoarabinomannan-mediated IRAK-M induction negatively regulates Toll-like receptor-dependent interleukin-12 p40 production in macrophages.* The Journal of biological chemistry, 2005. **280**(52): p. 42794-800.
211. Fratti, R.A., et al., *Mycobacterium tuberculosis glycosylated phosphatidylinositol causes phagosome maturation arrest.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(9): p. 5437-42.
212. Seiler, P., et al., *Cell-wall alterations as an attribute of Mycobacterium tuberculosis in latent infection.* The Journal of infectious diseases, 2003. **188**(9): p. 1326-31.
213. Chua, J., et al., *A tale of two lipids: Mycobacterium tuberculosis phagosome maturation arrest.* Current opinion in microbiology, 2004. **7**(1): p. 71-7.

214. Bruns, H., et al., *Abelson Tyrosine Kinase Controls Phagosomal Acidification Required for Killing of Mycobacterium tuberculosis in Human Macrophages.* Journal of immunology, 2012. **189**(8): p. 4069-78.
215. van der Wel, N., et al., *M. tuberculosis and M. leprae translocate from the phagolysosome to the cytosol in myeloid cells.* Cell, 2007. **129**(7): p. 1287-98.
216. Redente, E.F., et al., *Differential polarization of alveolar macrophages and bone marrow-derived monocytes following chemically and pathogen-induced chronic lung inflammation.* Journal of leukocyte biology, 2010. **88**(1): p. 159-68.
217. Hestvik, A.L., Z. Hmama, and Y. Av-Gay, *Mycobacterial manipulation of the host cell.* FEMS microbiology reviews, 2005. **29**(5): p. 1041-50.
218. Abebe, M., et al., *Modulation of cell death by M. tuberculosis as a strategy for pathogen survival.* Clinical & developmental immunology. **2011**: p. 678570.
219. Ottenhoff, T.H., et al., *Control of human host immunity to mycobacteria.* Tuberculosis, 2005. **85**(1-2): p. 53-64.
220. Aagaard, C., et al., *TB vaccines: current status and future perspectives.* Immunology and cell biology, 2009. **87**(4): p. 279-86.
221. Pitabut, N., et al., *Decreased plasma granulysin and increased interferon-gamma concentrations in patients with newly diagnosed and relapsed tuberculosis.* Microbiology and immunology. **55**(8): p. 565-73.
222. Ottenhoff, T.H., D. Kumararatne, and J.L. Casanova, *Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria.* Immunology today, 1998. **19**(11): p. 491-4.
223. Chen, C.Y., et al., *A critical role for CD8 T cells in a nonhuman primate model of tuberculosis.* PLoS pathogens, 2009. **5**(4): p. e1000392.
224. Tena-Coki, N.G., et al., *CD4 and CD8 T-cell responses to mycobacterial antigens in African children.* American journal of respiratory and critical care medicine, 2010. **182**(1): p. 120-9.
225. Hoft, D.F., et al., *A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers.* The Journal of infectious diseases, 2008. **198**(10): p. 1491-501.
226. Sander, C.R., et al., *Safety and immunogenicity of a new tuberculosis vaccine, MVA85A, in Mycobacterium tuberculosis-infected individuals.* American journal of respiratory and critical care medicine, 2009. **179**(8): p. 724-33.
227. Scriba, T.J., et al., *Modified vaccinia Ankara-expressing Ag85A, a novel tuberculosis vaccine, is safe in adolescents and children, and induces polyfunctional CD4+ T cells.* European journal of immunology, 2010. **40**(1): p. 279-90.
228. Aagaard, C., et al., *Protection and polyfunctional T cells induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis is highly dependent on the antigen dose.* PloS one, 2009. **4**(6): p. e5930.
229. Forbes, E.K., et al., *Multifunctional, high-level cytokine-producing Th1 cells in the lung, but not spleen, correlate with protection against Mycobacterium tuberculosis aerosol challenge in mice.* Journal of immunology, 2008. **181**(7): p. 4955-64.
230. Capinos Scherer, C.F., et al., *Evaluation of granulysin and perforin as candidate biomarkers for protection following vaccination with Mycobacterium bovis BCG or M. bovisDeltaRD1.* Transbound Emerg Dis, 2009. **56**(6-7): p. 228-39.

231. Sahiratmadja, E., et al., *Plasma granulysin levels and cellular interferon-gamma production correlate with curative host responses in tuberculosis, while plasma interferon-gamma levels correlate with tuberculosis disease activity in adults.* Tuberculosis, 2007. **87**(4): p. 312-21.
232. Kagina, B.M., et al., *Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guerin vaccination of newborns.* American journal of respiratory and critical care medicine, 2010. **182**(8): p. 1073-9.
233. Casadevall, A. and M.D. Scharff, *Return to the past: the case for antibody-based therapies in infectious diseases.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 1995. **21**(1): p. 150-61.
234. Glatman-Freedman, A. and A. Casadevall, *Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis.* Clinical microbiology reviews, 1998. **11**(3): p. 514-32.
235. Abebe, F. and G. Bjune, *The protective role of antibody responses during Mycobacterium tuberculosis infection.* Clinical and experimental immunology, 2009. **157**(2): p. 235-43.
236. Teitelbaum, R., et al., *A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival.* Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(26): p. 15688-93.
237. Turner, J., et al., *The progression of chronic tuberculosis in the mouse does not require the participation of B lymphocytes or interleukin-4.* Experimental gerontology, 2001. **36**(3): p. 537-45.
238. Macedo, G.C., et al., *Human T cell and antibody-mediated responses to the Mycobacterium tuberculosis recombinant 85A, 85B, and ESAT-6 antigens.* Clinical & developmental immunology. **2011**: p. 351573.
239. Mattos, A.M., et al., *Increased IgG1, IFN-gamma, TNF-alpha and IL-6 responses to Mycobacterium tuberculosis antigens in patients with tuberculosis are lower after chemotherapy.* International immunology, 2010. **22**(9): p. 775-82.
240. Gupta, S., et al., *IgG subclass antibody response to mycobacterial serine protease at different stages of pulmonary tuberculosis.* Medical science monitor : international medical journal of experimental and clinical research, 2005. **11**(12): p. CR585-8.
241. Ashenafi, S., et al., *BCG-specific IgG-secreting peripheral plasmablasts as a potential biomarker of active tuberculosis in HIV negative and HIV positive patients.* Thorax, 2012.
242. Thomas, T., et al., *A new potential biomarker for childhood tuberculosis.* Thorax, 2011. **66**(8): p. 727-9.
243. Siddiqi, K., M.L. Lambert, and J. Walley, *Clinical diagnosis of smear-negative pulmonary tuberculosis in low-income countries: the current evidence.* The Lancet infectious diseases, 2003. **3**(5): p. 288-96.
244. Pai, M., et al., *Novel and improved technologies for tuberculosis diagnosis: progress and challenges.* Clinics in chest medicine, 2009. **30**(4): p. 701-16, viii.
245. Lalvani, A., et al., *Rapid detection of Mycobacterium tuberculosis infection by enumeration of antigen-specific T cells.* American journal of respiratory and critical care medicine, 2001. **163**(4): p. 824-8.

246. Pai, M., L.W. Riley, and J.M. Colford, Jr., *Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review.* The Lancet infectious diseases, 2004. **4**(12): p. 761-76.
247. Chegou, N.N., et al., *Potential of novel Mycobacterium tuberculosis infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting.* BMC infectious diseases, 2012. **12**: p. 10.
248. Samten, B., X. Wang, and P.F. Barnes, *Immune regulatory activities of early secreted antigenic target of 6-kD protein of Mycobacterium tuberculosis and implications for tuberculosis vaccine design.* Tuberculosis, 2011. **91 Suppl 1**: p. S114-8.
249. Shams, H., et al., *Characterization of a Mycobacterium tuberculosis peptide that is recognized by human CD4(+) and CD8(+) T cells in the context of multiple HLA alleles.* Journal of immunology, 2004. **173**(3): p. 1966-1977.
250. Simeone, R., D. Bottai, and R. Brosch, *ESX/type VII secretion systems and their role in host-pathogen interaction.* Current opinion in microbiology, 2009. **12**(1): p. 4-10.
251. Ganguly, N., I. Siddiqui, and P. Sharma, *Role of M. tuberculosis RD-1 region encoded secretory proteins in protective response and virulence.* Tuberculosis, 2008. **88**(6): p. 510-7.
252. Kumar, G., et al., *Serodiagnostic efficacy of Mycobacterium tuberculosis 30/32-kDa mycolyl transferase complex, ESAT-6, and CFP-10 in patients with active tuberculosis.* Archivum immunologiae et therapiae experimentalis, 2010. **58**(1): p. 57-65.
253. Shiryaev, S.A., et al., *Matrix metalloproteinase proteolysis of the mycobacterial HSP65 protein as a potential source of immunogenic peptides in human tuberculosis.* The FEBS journal, 2011. **278**(18): p. 3277-86.
254. Chen, J., et al., *Novel recombinant RD2- and RD11-encoded Mycobacterium tuberculosis antigens are potential candidates for diagnosis of tuberculosis infections in BCG-vaccinated individuals.* Microbes and infection / Institut Pasteur, 2009. **11**(10-11): p. 876-85.
255. Mustafa, T., et al., *Differential expression of mycobacterial antigen MPT64, apoptosis and inflammatory markers in multinucleated giant cells and epithelioid cells in granulomas caused by Mycobacterium tuberculosis.* Virchows Archiv : an international journal of pathology, 2008. **452**(4): p. 449-56.
256. Andersen, P., et al., *Specific immune-based diagnosis of tuberculosis.* Lancet, 2000. **356**(9235): p. 1099-104.
257. Mustafa, T., et al., *Reduced apoptosis and increased inflammatory cytokines in granulomas caused by tuberculous compared to non-tuberculous mycobacteria: role of MPT64 antigen in apoptosis and immune response.* Clinical and experimental immunology, 2007. **150**(1): p. 105-13.
258. Kozak, R.A., et al., *Region of difference 2 contributes to virulence of Mycobacterium tuberculosis.* Infection and immunity, 2011. **79**(1): p. 59-66.
259. Andersen, P., *Host responses and antigens involved in protective immunity to Mycobacterium tuberculosis.* Scandinavian journal of immunology, 1997. **45**(2): p. 115-31.
260. Al-Attiyah, R., et al., *Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of Mycobacterium tuberculosis.* FEMS immunology and medical microbiology, 2006. **47**(2): p. 254-61.

261. Roche, P.W., C.G. Feng, and W.J. Britton, *Human T-cell epitopes on the Mycobacterium tuberculosis secreted protein MPT64.* Scandinavian journal of immunology, 1996. **43**(6): p. 662-70.
262. Tavares, R.C., et al., *Interferon gamma response to combinations 38 kDa/CFP-10, 38 kDa/MPT-64, ESAT-6/MPT-64 and ESAT-6/CFP-10, each related to a single recombinant protein of Mycobacterium tuberculosis in individuals from tuberculosis endemic areas.* Microbiology and immunology, 2007. **51**(3): p. 289-96.
263. Dietrich, G., J.F. Viret, and J. Hess, *Mycobacterium bovis BCG-based vaccines against tuberculosis: novel developments.* Vaccine, 2003. **21**(7-8): p. 667-70.
264. Kaufmann, S.H., G. Hussey, and P.H. Lambert, *New vaccines for tuberculosis.* Lancet, 2010. **375**(9731): p. 2110-9.
265. Sakula, A., *BCG: who were Calmette and Guerin?* Thorax, 1983. **38**(11): p. 806-12.
266. Daniel, T.M., *Leon Charles Albert Calmette and BCG vaccine.* The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 2005. **9**(9): p. 944-5.
267. Agger, E.M. and P. Andersen, *A novel TB vaccine; towards a strategy based on our understanding of BCG failure.* Vaccine, 2002. **21**(1-2): p. 7-14.
268. Hesseling, A.C., et al., *The risk of disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children.* Vaccine, 2007. **25**(1): p. 14-8.
269. Mak, T.K., et al., *Making BCG vaccination programmes safer in the HIV era.* Lancet, 2008. **372**(9641): p. 786-7.
270. Young, D.B., et al., *Confronting the scientific obstacles to global control of tuberculosis.* The Journal of clinical investigation, 2008. **118**(4): p. 1255-65.
271. Fine, P.E., *Variation in protection by BCG: implications of and for heterologous immunity.* Lancet, 1995. **346**(8986): p. 1339-45.
272. Kaufmann, S.H., *Future vaccination strategies against tuberculosis: thinking outside the box.* Immunity, 2010. **33**(4): p. 567-77.
273. Andersen, P. and T.M. Doherty, *The success and failure of BCG - implications for a novel tuberculosis vaccine.* Nature reviews. Microbiology, 2005. **3**(8): p. 656-62.
274. Sterne, J.A., L.C. Rodrigues, and I.N. Guedes, *Does the efficacy of BCG decline with time since vaccination?* The international journal of tuberculosis and lung disease : the official journal of the International Union against Tuberculosis and Lung Disease, 1998. **2**(3): p. 200-7.
275. Behr, M.A., *BCG--different strains, different vaccines?* The Lancet infectious diseases, 2002. **2**(2): p. 86-92.
276. Brandt, L., et al., *Failure of the Mycobacterium bovis BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis.* Infection and immunity, 2002. **70**(2): p. 672-8.
277. Hess, J. and S.H. Kaufmann, *Live antigen carriers as tools for improved anti-tuberculosis vaccines.* FEMS immunology and medical microbiology, 1999. **23**(2): p. 165-73.
278. Hatherill, M., et al., *The potential impact of helminth infection on trials of novel tuberculosis vaccines.* Vaccine, 2009. **27**(35): p. 4743-4.
279. Doherty, T.M. and G. Rook, *Progress and hindrances in tuberculosis vaccine development.* Lancet, 2006. **367**(9514): p. 947-9.

280. Elias, D., et al., *Schistosoma mansoni infection reduces the protective efficacy of BCG vaccination against virulent Mycobacterium tuberculosis.* Vaccine, 2005. **23**(11): p. 1326-34.
281. Rafi, W., et al., *'Coinfection-helminthes and tuberculosis'.* Current opinion in HIV and AIDS, 2012. **7**(3): p. 239-44.
282. Reece, S.T. and S.H. Kaufmann, *Rational design of vaccines against tuberculosis directed by basic immunology.* Int J Med Microbiol, 2008. **298**(1-2): p. 143-50.
283. Ottenhoff, T.H. and S.H. Kaufmann, *Vaccines against tuberculosis: where are we and where do we need to go?* PLoS pathogens, 2012. **8**(5): p. e1002607.
284. Cayabyab, M.J., L. Macovei, and A. Campos-Neto, *Current and novel approaches to vaccine development against tuberculosis.* Frontiers in cellular and infection microbiology, 2012. **2**: p. 154.
285. Kaufmann, S.H., *Tuberculosis vaccine development: strength lies in tenacity.* Trends in immunology, 2012. **33**(7): p. 373-9.
286. Pitt, J.M., et al., *Blockade of IL-10 Signaling during Bacillus Calmette-Guerin Vaccination Enhances and Sustains Th1, Th17, and Innate Lymphoid IFN-gamma and IL-17 Responses and Increases Protection to Mycobacterium tuberculosis Infection.* Journal of immunology. **189**(8): p. 4079-87.
287. Raviglione, M., et al., *Scaling up interventions to achieve global tuberculosis control: progress and new developments.* Lancet, 2012. **379**(9829): p. 1902-13.
288. Ottenhoff, T.H., *Overcoming the global crisis: "yes, we can", but also for TB ... ?* European journal of immunology, 2009. **39**(8): p. 2014-20.
289. Temmerman, S., et al., *Methylation-dependent T cell immunity to Mycobacterium tuberculosis heparin-binding hemagglutinin.* Nature medicine, 2004. **10**(9): p. 935-41.
290. Whelan, K.T., et al., *Safety and immunogenicity of boosting BCG vaccinated subjects with BCG: comparison with boosting with a new TB vaccine, MVA85A.* PloS one, 2009. **4**(6): p. e5934.
291. Rahman, M.J. and C. Fernandez, *Neonatal vaccination with Mycobacterium bovis BCG: potential effects as a priming agent shown in a heterologous prime-boost immunization protocol.* Vaccine, 2009. **27**(30): p. 4038-46.
292. Ruberti, M., et al., *Prime-boost vaccination based on DNA and protein-loaded microspheres for tuberculosis prevention.* Journal of drug targeting, 2004. **12**(4): p. 195-203.
293. Cervantes-Villagrana, A.R., et al., *Prime-boost BCG vaccination with DNA vaccines based in beta-defensin-2 and mycobacterial antigens ESAT6 or Ag85B improve protection in a tuberculosis experimental model.* Vaccine, 2012.
294. Bivas-Benita, M., et al., *Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice.* Vaccine, 2009. **27**(30): p. 4010-7.
295. Cayabyab, M.J., S.S. Kashino, and A. Campos-Neto, *Robust immune response elicited by a novel and unique Mycobacterium tuberculosis protein using an optimized DNA/protein heterologous prime/boost protocol.* Immunology, 2012. **135**(3): p. 216-25.
296. Skeiky, Y.A. and J.C. Sadoff, *Advances in tuberculosis vaccine strategies.* Nature reviews. Microbiology, 2006. **4**(6): p. 469-76.

297. Aagaard, C., et al., *A multistage tuberculosis vaccine that confers efficient protection before and after exposure.* Nature medicine. **17**(2): p. 189-94.
298. Lin, P.L., et al., *The multistage vaccine H56 boosts the effects of BCG to protect cynomolgus macaques against active tuberculosis and reactivation of latent Mycobacterium tuberculosis infection.* The Journal of clinical investigation, 2012. **122**(1): p. 303-14.
299. Ramachandran, G. and S. Swaminathan, *Role of pharmacogenomics in the treatment of tuberculosis: a review.* Pharmacogenomics and personalized medicine, 2012. **5**: p. 89-98.
300. Gandhi, N.R., et al., *Multidrug-resistant and extensively drug-resistant tuberculosis: a threat to global control of tuberculosis.* Lancet, 2010. **375**(9728): p. 1830-43.
301. Zumla, A., et al., *Drug-resistant tuberculosis--current dilemmas, unanswered questions, challenges, and priority needs.* The Journal of infectious diseases, 2012. **205 Suppl 2**: p. S228-40.
302. Martineau, A.R., et al., *Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(47): p. 19013-7.
303. Martineau, A.R., et al., *High-dose vitamin D(3) during intensive-phase antimicrobial treatment of pulmonary tuberculosis: a double-blind randomised controlled trial.* Lancet, 2011. **377**(9761): p. 242-50.
304. Wejse, C., et al., *Vitamin D as supplementary treatment for tuberculosis: a double-blind, randomized, placebo-controlled trial.* American journal of respiratory and critical care medicine, 2009. **179**(9): p. 843-50.
305. Schon, T., et al., *Effects of a food supplement rich in arginine in patients with smear positive pulmonary tuberculosis--a randomised trial.* Tuberculosis, 2011. **91**(5): p. 370-7.
306. Flynn, J.L., *Lessons from experimental Mycobacterium tuberculosis infections.* Microbes and infection / Institut Pasteur, 2006. **8**(4): p. 1179-88.
307. Kirschner, D.E., D. Young, and J.L. Flynn, *Tuberculosis: global approaches to a global disease.* Current opinion in biotechnology, 2010. **21**(4): p. 524-31.
308. Kaushal, D., et al., *The non-human primate model of tuberculosis.* Journal of medical primatology, 2012. **41**(3): p. 191-201.
309. Capuano, S.V., 3rd, et al., *Experimental Mycobacterium tuberculosis infection of cynomolgus macaques closely resembles the various manifestations of human M. tuberculosis infection.* Infection and immunity, 2003. **71**(10): p. 5831-44.
310. Lin, P.L., et al., *Quantitative comparison of active and latent tuberculosis in the cynomolgus macaque model.* Infection and immunity, 2009. **77**(10): p. 4631-42.
311. Anandaiah, A., et al., *Novel developments in the epidemic of human immunodeficiency virus and tuberculosis coinfection.* American journal of respiratory and critical care medicine, 2011. **183**(8): p. 987-97.
312. Wilkinson, K.A., et al., *Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2005. **40**(1): p. 184-7.

313. Nemeth, J., et al., *Recruitment of Mycobacterium tuberculosis specific CD4+ T cells to the site of infection for diagnosis of active tuberculosis.* Journal of internal medicine, 2009. **265**(1): p. 163-8.
314. Mazzarella, G., et al., *T lymphocyte phenotypic profile in lung segments affected by cavitary and non-cavitary tuberculosis.* Clinical and experimental immunology, 2003. **132**(2): p. 283-8.
315. Flynn, J.L., *Immunology of tuberculosis and implications in vaccine development.* Tuberculosis, 2004. **84**(1-2): p. 93-101.
316. Hirsch, C.S., et al., *Augmentation of apoptosis and interferon-gamma production at sites of active Mycobacterium tuberculosis infection in human tuberculosis.* The Journal of infectious diseases, 2001. **183**(5): p. 779-88.
317. Barnes, P.F., et al., *Cytokine production at the site of disease in human tuberculosis.* Infection and immunity, 1993. **61**(8): p. 3482-9.
318. Gerosa, F., et al., *CD4(+) T cell clones producing both interferon-gamma and interleukin-10 predominate in bronchoalveolar lavages of active pulmonary tuberculosis patients.* Clinical immunology, 1999. **92**(3): p. 224-34.
319. Salmi, M. and S. Jalkanen, *Cell-surface enzymes in control of leukocyte trafficking.* Nature reviews. Immunology, 2005. **5**(10): p. 760-71.
320. Blum, K.S. and R. Pabst, *Lymphocyte numbers and subsets in the human blood. Do they mirror the situation in all organs?* Immunology letters, 2007. **108**(1): p. 45-51.
321. Barrera, L., *The basics of clinical bacteriology*, in *Tuberculosis 2007 From basic science to patient care*, J.C. Palomino, Leao,S.C., and Ritacco, V., Editor 2007.
322. Andersson, J., et al., *Perforin is not co-expressed with granzyme A within cytotoxic granules in CD8 T lymphocytes present in lymphoid tissue during chronic HIV infection.* AIDS, 1999. **13**(11): p. 1295-303.
323. Zuber, B., et al., *Detection of macaque perforin expression and release by flow cytometry, immunohistochemistry, ELISA, and ELISpot.* Journal of immunological methods, 2006. **312**(1-2): p. 45-53.
324. Lore, K., et al., *Accumulation of DC-SIGN+CD40+ dendritic cells with reduced CD80 and CD86 expression in lymphoid tissue during acute HIV-1 infection.* AIDS, 2002. **16**(5): p. 683-92.
325. Johansson, L., et al., *Cathelicidin LL-37 in severe Streptococcus pyogenes soft tissue infections in humans.* Infection and immunity, 2008. **76**(8): p. 3399-404.
326. Purohit, M.R., et al., *Immunohistochemical diagnosis of abdominal and lymph node tuberculosis by detecting Mycobacterium tuberculosis complex specific antigen MPT64.* Diagn Pathol, 2007. **2**: p. 36.
327. Purohit, M.R., et al., *Rapid diagnosis of tuberculosis in aspirate, effusions, and cerebrospinal fluid by immunocytochemical detection of Mycobacterium tuberculosis complex specific antigen MPT64.* Diagn Cytopathol. **40**(9): p. 782-91.
328. Thoma-Uszynski, S., et al., *Induction of direct antimicrobial activity through mammalian toll-like receptors.* Science, 2001. **291**(5508): p. 1544-7.
329. Nicholson, S., et al., *Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis.* The Journal of experimental medicine, 1996. **183**(5): p. 2293-302.

330. Choi, H.S., et al., *Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis.* American journal of respiratory and critical care medicine, 2002. **166**(2): p. 178-86.
331. Bertholet, S., et al., *Expression of the inducible NO synthase in human monocytic U937 cells allows high output nitric oxide production.* Journal of leukocyte biology, 1999. **65**(1): p. 50-8.
332. Jagannath, C., J.K. Actor, and R.L. Hunter, Jr., *Induction of nitric oxide in human monocytes and monocyte cell lines by Mycobacterium tuberculosis.* Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society, 1998. **2**(3): p. 174-86.
333. Nozaki, Y., et al., *Mechanism of nitric oxide-dependent killing of Mycobacterium bovis BCG in human alveolar macrophages.* Infection and immunity, 1997. **65**(9): p. 3644-7.
334. Idh, J., et al., *Nitric oxide production in the exhaled air of patients with pulmonary tuberculosis in relation to HIV co-infection.* BMC infectious diseases, 2008. **8**: p. 146.
335. Sharma, S., et al., *Mycobacterium tuberculosis induces high production of nitric oxide in coordination with production of tumour necrosis factor-alpha in patients with fresh active tuberculosis but not in MDR tuberculosis.* Immunology and cell biology, 2004. **82**(4): p. 377-82.
336. Stenger, S., et al., *Reactivation of latent leishmaniasis by inhibition of inducible nitric oxide synthase.* The Journal of experimental medicine, 1996. **183**(4): p. 1501-14.
337. Robbins, R.A., et al., *Expression of inducible nitric oxide in human lung epithelial cells.* Biochemical and biophysical research communications, 1994. **203**(1): p. 209-18.
338. Sonawane, A., et al., *Cathelicidin is involved in the intracellular killing of mycobacteria in macrophages.* Cellular microbiology. **13**(10): p. 1601-17.
339. Rivas-Santiago, B., et al., *Expression of cathelicidin LL-37 during Mycobacterium tuberculosis infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells.* Infection and immunity, 2008. **76**(3): p. 935-41.
340. Stenger, S. and R.L. Modlin, *T cell mediated immunity to Mycobacterium tuberculosis.* Current opinion in microbiology, 1999. **2**(1): p. 89-93.
341. Buzza, M.S. and P.I. Bird, *Extracellular granzymes: current perspectives.* Biol Chem, 2006. **387**(7): p. 827-37.
342. Shacklett, B.L., et al., *Abundant expression of granzyme A, but not perforin, in granules of CD8+ T cells in GALT: implications for immune control of HIV-1 infection.* Journal of immunology, 2004. **173**(1): p. 641-8.
343. Andersson, J., et al., *Low levels of perforin expression in CD8+ T lymphocyte granules in lymphoid tissue during acute human immunodeficiency virus type 1 infection.* The Journal of infectious diseases, 2002. **185**(9): p. 1355-8.
344. Kagi, D., et al., *CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity.* European journal of immunology, 1994. **24**(12): p. 3068-72.
345. Klucar, P., et al., *Characterization of effector functions of human peptide-specific CD4+ T-cell clones for an intracellular pathogen.* Human immunology, 2008. **69**(8): p. 475-83.
346. Caccamo, N., et al., *Phenotypical and functional analysis of memory and effector human CD8 T cells specific for mycobacterial antigens.* Journal of immunology, 2006. **177**(3): p. 1780-5.

347. Temmerman, S.T., et al., *Effector functions of heparin-binding hemagglutinin-specific CD8+ T lymphocytes in latent human tuberculosis.* The Journal of infectious diseases, 2005. **192**(2): p. 226-32.
348. Gansert, J.L., et al., *Human NKT cells express granulysin and exhibit antimycobacterial activity.* Journal of immunology, 2003. **170**(6): p. 3154-61.
349. Toro, J.C., et al., *Enhanced susceptibility of multidrug resistant strains of Mycobacterium tuberculosis to granulysin peptides correlates with a reduced fitness phenotype.* Microbes and infection / Institut Pasteur, 2006. **8**(8): p. 1985-93.
350. Li, Q., et al., *Healthy lifestyles are associated with higher levels of perforin, granulysin and granzymes A/B-expressing cells in peripheral blood lymphocytes.* Prev Med, 2007. **44**(2): p. 117-23.
351. Dieli, F., et al., *Granulysin-dependent killing of intracellular and extracellular Mycobacterium tuberculosis by Vgamma9/Vdelta2 T lymphocytes.* The Journal of infectious diseases, 2001. **184**(8): p. 1082-5.
352. Semple, P.L., et al., *Induction of granulysin and perforin cytolytic mediator expression in 10-week-old infants vaccinated with BCG at birth.* Clinical & developmental immunology. **2011**: p. 438463.
353. Slight, S.R. and S.A. Khader, *Chemokines shape the immune responses to tuberculosis.* Cytokine & growth factor reviews, 2012.
354. Maglione, P.J., J. Xu, and J. Chan, *B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with Mycobacterium tuberculosis.* Journal of immunology, 2007. **178**(11): p. 7222-34.
355. Kahnert, A., et al., *Mycobacterium tuberculosis triggers formation of lymphoid structure in murine lungs.* The Journal of infectious diseases, 2007. **195**(1): p. 46-54.
356. Tsai, M.C., et al., *Characterization of the tuberculous granuloma in murine and human lungs: cellular composition and relative tissue oxygen tension.* Cellular microbiology, 2006. **8**(2): p. 218-232.
357. Demkow, U., et al., *Heterogeneity of antibody response to myobacterial antigens in different clinical manifestations of pulmonary tuberculosis.* Journal of physiology and pharmacology : an official journal of the Polish Physiological Society, 2007. **58 Suppl 5**(Pt 1): p. 117-27.
358. Sousa, A.O., et al., *IgG subclass distribution of antibody responses to protein and polysaccharide mycobacterial antigens in leprosy and tuberculosis patients.* Clinical and experimental immunology, 1998. **111**(1): p. 48-55.
359. Da Costa, C.T., et al., *Immunoglobulin G subclass responses to mycobacterial lipoarabinomannan in HIV-infected and non-infected patients with tuberculosis.* Clinical and experimental immunology, 1993. **91**(1): p. 25-9.
360. Davidow, A., et al., *Antibody profiles characteristic of Mycobacterium tuberculosis infection state.* Infection and immunity, 2005. **73**(10): p. 6846-51.
361. Raja, A., U.D. Ranganathan, and R. Bethunaickan, *Improved diagnosis of pulmonary tuberculosis by detection of antibodies against multiple Mycobacterium tuberculosis antigens.* Diagnostic microbiology and infectious disease, 2008. **60**(4): p. 361-8.
362. Raqib, R., et al., *Detection of antibodies secreted from circulating Mycobacterium tuberculosis-specific plasma cells in the diagnosis of pediatric tuberculosis.* Clinical and vaccine immunology : CVI, 2009. **16**(4): p. 521-7.

363. Bosio, C.M., D. Gardner, and K.L. Elkins, *Infection of B cell-deficient mice with CDC 1551, a clinical isolate of Mycobacterium tuberculosis: delay in dissemination and development of lung pathology.* Journal of immunology, 2000. **164**(12): p. 6417-25.
364. Blair, P.A., et al., *CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients.* Immunity, 2010. **32**(1): p. 129-40.
365. Flores-Borja, F., et al., *CD19+CD24hiCD38hi B Cells Maintain Regulatory T Cells While Limiting TH1 and TH17 Differentiation.* Science translational medicine, 2013. **5**(173): p. 173ra23.
366. Zhang, M., et al., *CD19(+)CD1d(+)CD5(+) B cell frequencies are increased in patients with tuberculosis and suppress Th17 responses.* Cellular immunology, 2012. **274**(1-2): p. 89-97.
367. Cooper, A.M. and S.A. Khader, *The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis.* Immunological reviews, 2008. **226**: p. 191-204.
368. Kuchen, S., et al., *Essential role of IL-21 in B cell activation, expansion, and plasma cell generation during CD4+ T cell-B cell collaboration.* Journal of immunology, 2007. **179**(9): p. 5886-96.
369. Yoshizaki, A., et al., *Regulatory B cells control T-cell autoimmunity through IL-21-dependent cognate interactions.* Nature, 2012. **491**(7423): p. 264-8.
370. Sharma, S.K., et al., *Cytokine polarization in miliary and pleural tuberculosis.* Journal of clinical immunology, 2002. **22**(6): p. 345-52.
371. Lienhardt, C., et al., *Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity in vivo.* European journal of immunology, 2002. **32**(6): p. 1605-13.
372. Munk, M.E. and M. Emoto, *Functions of T-cell subsets and cytokines in mycobacterial infections.* The European respiratory journal. Supplement, 1995. **20**: p. 668s-675s.
373. Newcomb, D.C., et al., *A functional IL-13 receptor is expressed on polarized murine CD4+ Th17 cells and IL-13 signaling attenuates Th17 cytokine production.* Journal of immunology, 2009. **182**(9): p. 5317-21.
374. Sanchez, F.O., et al., *Immune responsiveness and lymphokine production in patients with tuberculosis and healthy controls.* Infection and immunity, 1994. **62**(12): p. 5673-8.
375. Seah, G.T. and G.A. Rook, *High levels of mRNA encoding IL-4 in unstimulated peripheral blood mononuclear cells from tuberculosis patients revealed by quantitative nested reverse transcriptase-polymerase chain reaction; correlations with serum IgE levels.* Scandinavian journal of infectious diseases, 2001. **33**(2): p. 106-9.
376. Seah, G.T., G.M. Scott, and G.A. Rook, *Type 2 cytokine gene activation and its relationship to extent of disease in patients with tuberculosis.* The Journal of infectious diseases, 2000. **181**(1): p. 385-9.
377. Konforte, D., N. Simard, and C.J. Paige, *IL-21: an executor of B cell fate.* Journal of immunology, 2009. **182**(4): p. 1781-7.
378. Borte, S., et al., *Interleukin-21 restores immunoglobulin production ex vivo in patients with common variable immunodeficiency and selective IgA deficiency.* Blood, 2009. **114**(19): p. 4089-98.

379. Sondergaard, H. and K. Skak, *IL-21: roles in immunopathology and cancer therapy.* Tissue antigens, 2009. **74**(6): p. 467-79.
380. Rojas, R.E., et al., *Regulation of human CD4(+) alphabeta T-cell-receptor-positive (TCR(+)) and gammadelta TCR(+) T-cell responses to Mycobacterium tuberculosis by interleukin-10 and transforming growth factor beta.* Infection and immunity, 1999. **67**(12): p. 6461-72.
381. Cyktor, J.C., et al., *IL-10 Inhibits Mature Fibrotic Granuloma Formation during Mycobacterium tuberculosis Infection.* Journal of immunology, 2013. **190**(6): p. 2778-90.
382. de la Barrera, S., et al., *IL-10 down-regulates costimulatory molecules on Mycobacterium tuberculosis-pulsed macrophages and impairs the lytic activity of CD4 and CD8 CTL in tuberculosis patients.* Clinical and experimental immunology, 2004. **138**(1): p. 128-38.
383. Redford, P.S., P.J. Murray, and A. O'Garra, *The role of IL-10 in immune regulation during M. tuberculosis infection.* Mucosal immunology, 2011. **4**(3): p. 261-70.
384. Bonecini-Almeida, M.G., et al., *Down-modulation of lung immune responses by interleukin-10 and transforming growth factor beta (TGF-beta) and analysis of TGF-beta receptors I and II in active tuberculosis.* Infection and immunity, 2004. **72**(5): p. 2628-34.
385. Hirsch, C.S., et al., *Enhancement of intracellular growth of Mycobacterium tuberculosis in human monocytes by transforming growth factor-beta 1.* The Journal of infectious diseases, 1994. **170**(5): p. 1229-37.
386. Redford, P.S., et al., *Enhanced protection to Mycobacterium tuberculosis infection in IL-10-deficient mice is accompanied by early and enhanced Th1 responses in the lung.* European journal of immunology, 2010. **40**(8): p. 2200-10.
387. Dahl, K.E., et al., *Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of Mycobacterium tuberculosis.* Infection and immunity, 1996. **64**(2): p. 399-405.
388. Rosas-Taraco, A.G., et al., *Local pulmonary immunotherapy with siRNA targeting TGFbeta1 enhances antimicrobial capacity in Mycobacterium tuberculosis infected mice.* Tuberculosis, 2011. **91**(1): p. 98-106.
389. Higgins, D.M., et al., *Lack of IL-10 alters inflammatory and immune responses during pulmonary Mycobacterium tuberculosis infection.* Tuberculosis, 2009. **89**(2): p. 149-57.
390. Larson, R.P., S. Shafiani, and K.B. Urdahl, *Foxp3(+) regulatory T cells in tuberculosis.* Advances in experimental medicine and biology, 2013. **783**: p. 165-80.
391. Kursar, M., et al., *Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis.* Journal of immunology, 2007. **178**(5): p. 2661-5.
392. Estes, J.D., et al., *Premature induction of an immunosuppressive regulatory T cell response during acute simian immunodeficiency virus infection.* The Journal of infectious diseases, 2006. **193**(5): p. 703-12.
393. Joosten, S.A. and T.H. Ottenhoff, *Human CD4 and CD8 regulatory T cells in infectious diseases and vaccination.* Human immunology, 2008. **69**(11): p. 760-70.
394. Hougardy, J.M., et al., *Regulatory T cells depress immune responses to protective antigens in active tuberculosis.* American journal of respiratory and critical care medicine, 2007. **176**(4): p. 409-16.

395. Roberts, T., et al., *Immunosuppression during active tuberculosis is characterized by decreased interferon- gamma production and CD25 expression with elevated forkhead box P3, transforming growth factor- beta , and interleukin-4 mRNA levels.* The Journal of infectious diseases, 2007. **195**(6): p. 870-8.
396. Shang, S., et al., *Increased Foxp3 expression in guinea pigs infected with W-Beijing strains of M. tuberculosis.* Tuberculosis, 2011. **91**(5): p. 378-85.
397. Trinath, J., et al., *Mycobacterium tuberculosis promotes regulatory T-cell expansion via induction of programmed death-1 ligand 1 (PD-L1, CD274) on dendritic cells.* The Journal of infectious diseases, 2012. **205**(4): p. 694-6.
398. Wang, L., et al., *Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(27): p. 9331-6.
399. Taams, L.S., et al., *The generation and antigen-specificity of CD4+CD25+ regulatory T cells.* Inflammation & allergy drug targets, 2006. **5**(3): p. 149-56.
400. Singh, A., et al., *Foxp3+ regulatory T cells among tuberculosis patients: impact on prognosis and restoration of antigen specific IFN-gamma producing T cells.* PloS one, 2012. **7**(9): p. e44728.
401. Chen, C.Y., et al., *IL-2 simultaneously expands Foxp3+ T regulatory and T effector cells and confers resistance to severe tuberculosis (TB): implicative Treg-T effector cooperation in immunity to TB.* Journal of immunology, 2012. **188**(9): p. 4278-88.
402. Nabeshima, S., et al., *T-Cell hyporesponsiveness induced by activated macrophages through nitric oxide production in mice infected with Mycobacterium tuberculosis.* Infection and immunity, 1999. **67**(7): p. 3221-6.
403. Chensue, S.W., et al., *Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production.* Journal of immunology, 1996. **157**(10): p. 4602-8.
404. Ferrero, E., et al., *Macrophages exposed to Mycobacterium tuberculosis release chemokines able to recruit selected leucocyte subpopulations: focus on gammadelta cells.* Immunology, 2003. **108**(3): p. 365-74.
405. Barnes, P.F., et al., *Compartmentalization of a CD4+ T lymphocyte subpopulation in tuberculous pleuritis.* Journal of immunology, 1989. **142**(4): p. 1114-9.
406. Gonzalez-Juarrero, M., et al., *Temporal and spatial arrangement of lymphocytes within lung granulomas induced by aerosol infection with Mycobacterium tuberculosis.* Infection and immunity, 2001. **69**(3): p. 1722-8.
407. Green, A.M., et al., *CD4(+) regulatory T cells in a cynomolgus macaque model of Mycobacterium tuberculosis infection.* The Journal of infectious diseases. **202**(4): p. 533-41.
408. Scott-Browne, J.P., et al., *Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis.* The Journal of experimental medicine, 2007. **204**(9): p. 2159-69.
409. Fuller, C.L., J.L. Flynn, and T.A. Reinhart, *In situ study of abundant expression of proinflammatory chemokines and cytokines in pulmonary granulomas that develop in cynomolgus macaques experimentally infected with Mycobacterium tuberculosis.* Infection and immunity, 2003. **71**(12): p. 7023-34.

410. Kaplan, G., et al., *Mycobacterium tuberculosis growth at the cavity surface: a microenvironment with failed immunity.* Infection and immunity, 2003. **71**(12): p. 7099-108.
411. Lugo-Villarino, G., et al., *Emerging trends in the formation and function of tuberculosis granulomas.* Frontiers in immunology, 2012. **3**: p. 405.
412. Magalhaes, I., et al., *rBCG induces strong antigen-specific T cell responses in rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector.* PloS one, 2008. **3**(11): p. e3790.
413. Maeurer, M.J., et al., *Interleukin-7 or interleukin-15 enhances survival of Mycobacterium tuberculosis-infected mice.* Infection and immunity, 2000. **68**(5): p. 2962-70.
414. Smyth, M.J., et al., *IL-7 regulation of cytotoxic lymphocytes: pore-forming protein gene expression, interferon-gamma production, and cytotoxicity of human peripheral blood lymphocytes subsets.* Cellular immunology, 1991. **138**(2): p. 390-403.
415. Cooper, A.M., et al., *Disseminated tuberculosis in interferon gamma gene-disrupted mice.* The Journal of experimental medicine, 1993. **178**(6): p. 2243-7.
416. Derrick, S.C., et al., *Immunization with a DNA vaccine cocktail protects mice lacking CD4 cells against an aerogenic infection with Mycobacterium tuberculosis.* Infection and immunity, 2004. **72**(3): p. 1685-92.
417. Wang, Q., et al., *Improved cellular immune response elicited by a ubiquitin-fused DNA vaccine against Mycobacterium tuberculosis.* DNA and cell biology, 2012. **31**(4): p. 489-95.
418. Nicol, M.P. and L.A. Grobler, *MVA-85A, a novel candidate booster vaccine for the prevention of tuberculosis in children and adults.* Current opinion in molecular therapeutics, 2010. **12**(1): p. 124-34.
419. Kolibab, K., et al., *Highly persistent and effective prime/boost regimens against tuberculosis that use a multivalent modified vaccine virus Ankara-based tuberculosis vaccine with interleukin-15 as a molecular adjuvant.* Clinical and vaccine immunology : CVI, 2010. **17**(5): p. 793-801.