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Mycobacterial infection: Immune evasion, host susceptibility and immunological markers of diagnostic importance

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Stockholm 2008

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ISBN 978-91-7155-737-7 pp 1-85

Printed in Sweden by Universitetsservice AB, Stockholm 2008
Distributor: Stockholm University Library

SUMMARY

According to the WHO, the interaction between the twin pandemics of human immunodeficiency virus (HIV) and tuberculosis (TB) could soon become a "threat to global health security," particularly with the emergence of almost untreatable strains of *Mycobacterium tuberculosis*. Understanding the mechanisms involved in the host-pathogen interaction; from persistence, to the immunological processes induced by the pathogen, to susceptibility of the host to infection may help in the rational design of more effective drugs and vaccines, as well as the development of better diagnostic techniques.

Toll-like receptors (TLRs) are key sensors of microbial infections, and an important link between innate cellular responses and the subsequent activation of adaptive immune defenses against the invading pathogen. A role for persistent TLR2 signalling as an immune evasive mechanism by mycobacteria in the host has been reported. In the first study, we investigated the functional implications of prolonged TLR signalling on interferon-gamma (IFN-γ) mediated killing of mycobacteria by murine macrophages *in vitro*. Continuous TLR2, but not TLR4 ligation interfered with IFN-γ mediated killing of mycobacteria in macrophages. In terms of mechanisms, neither tumor necrosis factor (TNF) nor nitric oxide (NO) production was significantly affected, and the refractoriness induced could be reversed with increasing amounts of IFN-γ.

Receptor mediated recognition and phagocytosis of mycobacteria culminates in a cascade of immunological events, resulting in the production of chemokines and pro-inflammatory cytokines by innate cells, and the subsequent generation of mycobacteria-specific T- and B-lymphocytes, capable of producing soluble mediators of adaptive immunity. In the second paper, we aimed to identify immunological markers of diagnostic importance in both the respiratory tract and serum during pulmonary mycobacterial infection in mice. We found that increased levels of immunological markers in the respiratory tract, but not in serum, correlated better with active mycobacterial infection in the lungs, suggesting that the immune response in the respiratory tract is more reflective of the infection status and pathology than the systemic response.

Finally, we investigated the level and nature of immune responses to pulmonary mycobacterial infection in BALB/c and C57BL/6 mice, two mouse strains known to exhibit different susceptibilities to infection with several intracellular pathogens, including mycobacteria. We showed that increased susceptibility of BALB/c mice to early mycobacterial infection was associated with reduced Th1 immune responses, and increased sTNFR secretion in the lung. Moreover, BALB/c mice recruited fewer monocytes/macrophages to the lung, and although IFN-γ stimulation of infected bone marrow derived macrophages (BMM) in both mouse strains resulted in induction of antimycobacterial activity, BALB/c mice had a reduced capacity to kill ingested bacteria.

In conclusion, the work presented in this thesis provide further insight into the immune evasive mechanisms utilized by mycobacteria to persist in the host, and strengthen the notion that in TB, the nature and level of immune responses in the respiratory tract is more reflective of disease activity than systemic responses. Furthermore, it provides some immunological basis underlying the differences in host susceptibility to mycobacterial infections.

"*It is not the strongest of the species that survives, or the most intelligent that survives. It is the one that is the most adaptable to change*"

Charles Darwin

To Matilda, Kimberly, Karen and Kevin

ORIGINAL ARTICLES

This doctoral thesis is based on the following papers, which are referred to by their Roman numerals in the text:

I. **Arko-Mensah J,** Julián E, Singh M, Fernández C. TLR2 but not TLR4 signalling is critically involved in the inhibition of IFN-γ induced killing of mycobacteria by murine macrophages. (*Scand J Immunol* 2007; **65**:148-157).

II. **Arko-Mensah J*,** Rahman J M*, Julián E, Horner G, Singh M, Fernández C. Increased levels of immunological markers in the respiratory tract but not in serum correlate with active pulmonary mycobacterial infection in mice. Accepted.

III. **Arko-Mensah J*,** Rahman J M***,** Fernández C. Early immune responses are responsible for the better control of pulmonary mycobacterial infection in C57BL/6 compared with BALB/c mice. Manuscript.

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ABBREVIATIONS

Ag85	Antigen 85 complex
APC	Antigen-presenting cell
BAL	Broncho-alveolar lavage
BCG	*Mycobacterium bovis* Bacillus Calmette-Guérin
BMM	Bone-marrow macrophages
CFP	Culture filtrate protein
CIITA	Class II transactivator
CR	Complement receptor
CTL	Cytotoxic-T lymphocyte
CW_{BCG}	BCG cell wall
$CW_{M.vaccae}$	*M. vaccae* cell wall
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing nonintegrin
DOTS	Directly observed treatment-short course
ESAT-6	Early secretory antigenic target 6
HIV	Human immunodeficiency virus
hk-BCG	Heat killed BCG
HLA	Human leukocyte antigen
IGRA	Interferon-gamma release assay
i.n.	Intranasal
i.m.	Intramuscular
IRAK	Interleukin-1-receptor associated kinase
i.v.	Intravenous
IFN-γ	Interferon-gamma
IL	Interleukin
LAM	Lipoarabinomannan
LN	Lymph node
LPS	Lipopolysaccharide
MDR	Multidrug-resistant
MHC	Major-histocompatibility complex
MyD88	Myeloid differentiation factor 88
MOTT	Mycobacteria other than tuberculosis
NF-kB	Nuclear factor kappa-B
NK	Natural killer
NK-T	Natural killer-T cells

NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NOS	Nitric oxide synthase
Nramp1	Natural resistance associated macrophage protein 1
PAMP	Pathogen-associated molecular pattern
PPD	Purified protein derivative
PRR	Pattern recognition receptor
RNI	Reactive-nitrogen intermediate
ROI	Reactive-oxygen intermediate
s.c.	Subcutanous
sst1	Super-susceptibility to tuberculosis 1
sTNFR	Soluble tumor necrosis factor receptor
TACE	Tumor necrosis factor converting enzyme
TACO	Tryptophan aspartate rich coat protein
TB	Tuberculosis
TCR	T-cell receptor
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TmTNF	Transmembrane tumor necrosis factor
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
TST	Tuberculin skin test
WHO	World Health Organization
WT	Wild type
XDR	Extensively drug-resistant

INTRODUCTION

TUBERCULOSIS

Tuberculosis (TB), also known as the 'white plague'[1], and human immunodeficiency virus (HIV) are the major infectious killers of adults in the developing world, and about 13 million people are infected with these two pathogens. The global epidemic of TB results in 8-10 million new cases every year [2], with an annual projected increase rate of 3%. It is estimated that between 5 and 10% of immunocompetent individuals are susceptible to TB, of which, 85% develop pulmonary disease [3]. In 1993 and also 2002, the World Health Organization (WHO) declared TB a global public health emergency. The resurgence in the incidence of TB in the last two decades has been attributed to the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* [4, 5], the causative organism of TB, coinfection with HIV [6, 7], as well as immigration of infected persons from TB prevalent to less prevalent areas.

The genus Mycobacterium comprises mostly soil dwelling saprophytes, and only a few members of the genus have evolved to adopt a pathogenic lifestyle, causing diseases of diverse nature and varying severity [8]. Tuberculosis is caused by members of the *M. tuberculosis* complex that consists of *M. tuberculosis, M. bovis, M. africanum, M. canettii* and *M. microti*. The mycobacteria grouped in the complex are characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences [9, 10], but differ widely in terms of their host tropisms, phenotypes, and pathogenicity. Some are exclusively human pathogens (*M. tuberculosis, M. africanum, M. canettii*) or rodent *M. microti* whereas *M. bovis* have a wide host spectrum [8]. All members of the complex are slow-growing, with generation time ranging from 12 to 24 hrs depending on environmental and microbial variables.

MYCOBACTERIAL INFECTIONS

M. tuberculosis is an obligate, aerobic, intracellular pathogen, which has a predilection for the lung tissue rich in oxygen. TB occurs almost exclusively from inhalation of aerosol droplet

containing *M. tuberculosis* expelled by an individual with active pulmonary TB through coughing, spitting, singing and other forced respiratory maneuvers. Usually, repeated exposure to a TB patient is necessary for infection to take place. Inhaled droplets are deposited in the alveolar spaces, where the bacteria are taken up by phagocytic cells, mainly alveolar macrophages [11], an event which induces a rapid inflammatory response and accumulation of cells.

Pathogenesis of TB

Infection with *M. tuberculosis* may lead to different clinical outcomes [12]:
Primary TB: Clinical symptoms develop within the first 1–2 years of infection, and represent the majority of pediatric cases. Alternatively, infection can lead to chronic, slowly progressive TB in which clinical symptoms develop after more than 2 years of infection. Finally, in 90% of cases, the infection remains latent and totally asymptomatic. The latter two groups of infected individuals constitute the reservoir of *M. tuberculosis*.

Secondary TB: Seen mostly in adults as a reactivation of previous infection (latent TB), or reinfection, particularly when ones' health status declines. Typically, the upper lung lobes are most affected, and cavitation can occur.

Dissemination of tuberculosis outside the lungs (extrapulmonary TB) is more common in children and HIV infected individuals [13], leading to the appearance of a number of uncommon findings with characteristic patterns [reviewed in 14]: skeletal TB, involves mainly the thoracic and lumbar vertebrae also known as Pott's disease, genital tract TB involves the fallopian tube, prostate and epididymis. Others are: urinary tract TB, TB of the central nervous system, cardiac TB and scrofula (lymphadenitis TB) [15].

IMMUNE EVASIVE MECHANISMS

M. tuberculosis invades and replicates in macrophages, cells of the host innate defense system designed to eliminate pathogenic microorganisms, through a variety of immune evasion strategies. The use of non-activating complement receptors (CR) to enter into macrophages may be advantageous for the bacterium, since engagement of these receptors does not induce

the release of cytotoxic reactive oxygen intermediates (ROI) [16]. The ability of pathogenic mycobacteria to adapt to the hostile environment of macrophages has been instrumental in its success as a pathogen. Mycobacteria interfere with host trafficking pathways by modulating events in the endosomal/phagosomal maturation pathway to create a protective niche, the phagosome [17]. The mycobacteria containing phagosome, while connected to the endocytic pathway, does not fuse with lysosomes or mature into phagolysosomes [18, 19]. By blocking its delivery to lysosomes, the mycobacterium is able to avoid the acidic proteases of lysosomes; avoid exposure to the bactericidal mechanisms within lysosomes; prevent degradation and hence processing and presentation of mycobacterial antigens to the immune system [20]. Another mechanism by which mycobacteria could interfere with phagolysosomal fusion is by retention of an important host protein termed (tryptophan aspartate containing Coat protein (TACO), also known as coronin 1 on the phagosome [18], thereby behaving as self antigens. TACO represents a component of the phagosome coat, and retention of TACO prevents phagosomes from fusing with lysosomes, thereby contributing to the long-term survival of bacilli within the phagosome.

The recognition of infected macrophages by $CD4^+$ T cells depends on constitutively expressed major histocompatibility complex (MHC) class II on professional antigen-presenting cells (APCs), level of which is upregulated upon activation with IFN-γ. One mechanism by which *M. tuberculosis* avoids elimination by the immune system after infection is through the inhibition of MHC II expression or antigen processing or presentation by macrophages [21-24]. Inhibition of MHC II expression or antigen processing does not require viable bacilli and can be achieved by exposure to bacterial lysate [21, 22, 25]. The *M. tuberculosis* 19-kDa lipoprotein (19-kDa) was identified as the predominant ligand involved in inhibiting MHC II expression and antigen processing in a toll-like receptor (TLR) 2 dependent manner [26]. Subsequently, several studies have shown that 19-kDa inhibits the expression of several interferon gamma (IFN-γ) responsive genes, including MHC class II transactivator (CIITA) and MHC II, as well as class II dependent antigen presentation in a TLR2 dependent manner [27-29]. Moreover, we (paper I), and others [30] have demonstrated that signalling through TLR2 by 19-kDa inhibits IFN-γ -mediated killing of ingested mycobacteria by murine macrophages.

It was recently demonstrated that *M. tuberculosis* uses at least two mechanisms to block responses to IFN-γ; one initiated by lipoproteins acting through TLR2/ MyD88 (myeloid

differentiation factor 8), whereas the other is initiated by mycobacterial peptidoglycan (PGN), acting in a TLR2-, MyD88-independent manner [30]. Other immune evasive mechanisms include the secretion of enzymes such as superoxide dismutase or catalases by *M. tuberculosis*, which are antagonistic to ROI [31], or the inhibition of macrophage apoptosis [32]. Furthermore, macrophages infected with *M. tuberculosis* produce inhibitory cytokines, such as transforming growth factor (TGF)-β and interleukin (IL)-10, which reduce macrophage activation, thereby leading to decreased clearance of bacteria [33, 34].

IMMUNITY TO MYCOBACTERIAL INFECTIONS

Innate immunity

It is believed that the host innate immunity provides the initial resistance to infections with intracellular pathogens, such as mycobacteria, before the adaptive type 1 cell-mediated immunity fully develops. The major cellular components involved in innate immunity include phagocytes; macrophages, neutrophils, dendritic cells (DCs); natural killer (NK) cells; γδ T cells, and soluble mediators released by these cells serve as a linker to cell-mediated immunity. During the initial phase of infection, mycobacteria are ingested by resident alveolar macrophages. However, mycobacteria can also be ingested by alveolar epithelial type II pneumocytes [35], found in greater numbers than macrophages in alveoli.

Overall, phagocytic cells play a key role in restricting the multiplication and dissemination of intracellular pathogens, as well as initiation and direction of the adaptive immune response. In addition, DCs, known to be much better antigen presenters than macrophages [36, 37], play an important role in the early stages of infection through presentation of specific mycobacterial antigens to T cells [38]. A number of receptors are critical for *M. tuberculosis* detection and uptake by phagocytes.

Receptor mediated detection of mycobacteria

Entry of mycobacteria into phagocytic cells can occur through binding to multiple receptors. In human macrophages, the primary receptors for *M. tuberculosis* recognition and uptake are the mannose receptors and complement receptors 3 [39, 40].

Other receptors have been shown to interact with mycobacteria: surfactant protein A and its receptors, scavenger receptor class A, mannose binding lectin, and possibly dectin-1 [reviewed in 41, 42]. The mode of entry into macrophages is considered as predetermining the subsequent intracellular fate of mycobacteria. In contrast to human macrophages, human DCs primarily use DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) for mycobacterial detection and uptake, with no significant role for complement or mannose receptors [43]. However, experiments have shown that blocking individual receptors does not significantly alter *M. tuberculosis* intracellular trafficking [41].

Toll-like receptors

One of the earliest indications that the body has been infected with an invading microbe is the activation of signalling pathways upon recognition of specific components conserved among microorganisms, known as pathogen-associated molecular patterns (PAMPs) by evolutionarily ancient germline-encoded receptors, the pattern recognition receptors (PRRs) [44]. The most studied PRRs, the TLRs constitute a family of transmembrane proteins expressed on many cells, including cells of the innate immune system such as macrophages and DC. Messenger RNA expression for TLRs 1-9 has been shown in human lungs, indicating that it is a major site for TLR activity [45]. This is important since lungs are the primary target for infection by many pathogens including *M. tuberculosis*.

Some of the bacterial molecules that are recognized by TLRs include lipopeptides by TLR2 (as a heterodimer with TLR1 or TLR6), lipopolysaccharide (LPS) by TLR4, flagellin by TLR5, and bacterial CpG DNA by TLR9. TLRs, with the exception of TLR3, require the adaptor molecule MyD88 for signal transduction (Figure 1) [46].

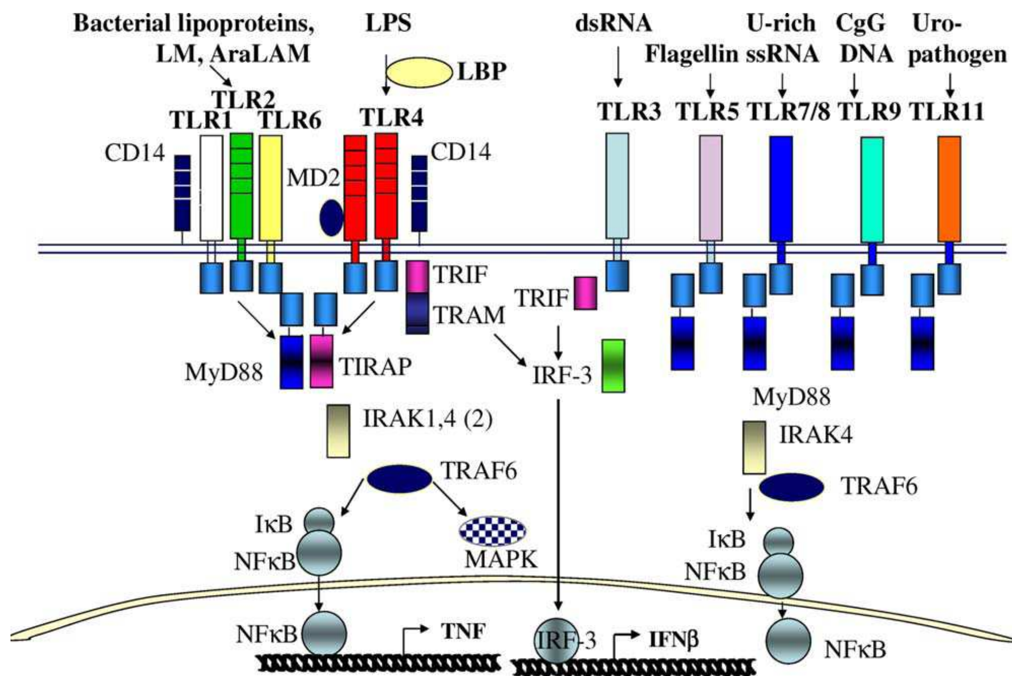

Figure 1. Microbial ligands and association with known TLRs and adaptor molecules. (*Microbes Infect* 2004; **6**:946-959). Reprinted with permission from Elsevier.

TLR signal transduction is mediated by binding of the intracellular adaptor protein MyD88 to the TIR domain of TLRs, followed by the recruitment of IL-1 receptor associated kinases (IRAK), tumor necrosis factor (TNF) receptor associated factor (TRAF), mitogen-activated protein kinase (MAPK) leading to translocation of nuclear factor-κappa B (NF-κB) [46, 47]. Translocation of the cytoplasmic factor NF-κB results in the transcription of several genes, leading to activation of pro-inflammatory and antibacterial effector pathways, which include production of pro-inflammatory cytokines such as TNF and ILs, chemokines, nitric oxide (NO) and defensins [48, 49]. TLR signalling also triggers differentiation of monocytes into macrophages and DCs, thereby generating the cellular populations necessary for a potent innate and adaptive immune response [50]. In human naive B cells, TLRs are expressed at low to undetectable levels, but their expression is rapidly up-regulated upon B-cell receptor

trigerring by microbial PAMPs [51]. In contrast however, memory B cells express several TLRs at consitutively high levels. Although B cell-intrinsic TLR signalling is not required for antibody production, it plays a role in the amplification of the humoral immune response [52]. Therefore, in addition to their role in innate immunity, TLRs are also critically involved in the initiation and enhancement of adaptive immune responses [49].

In experimental TB, TLR2-mediated signalling of APCs by mycobacterial components is the most studied. It was shown that direct activation of TLR2 by the 19-kDa resulted in reduced viability of ingested bacilli in human macrophages in a TNF and NO independent manner [53]. Moreover, TLR2 signalling can also promote apoptosis of *M. tuberculosis* infected macrophages [54]. Other signals also contribute to the pro-inflammatory response; TLR-1/TLR6 and TLR4 have been implicated in responses to *M. tuberculosis* [55]. *In vitro*, mice deficient in TLR2 [56], or the TLR adaptor molecule MyD88 displayed a higher susceptibility to mycobacterial infection [57]. It has also been shown that nucleotide-binding oligomerization domain (NOD) 2 is a nonredundant PRR of mycobacteria, and synergizes with TLRs in the stimulation of cytokine production by phagocytic cells [58, 59]. Furthermore, mannose-capped lipoarabinomannan (LAM), a component of *M. tuberculosis* cell wall, can deliver anti-inflammatory signals through DC-SIGN on DCs, thereby reducing antimycobacterial activity and stimulating the release of IL-10 [43].

Macrophages

A key characteristic of *M. tuberculosis* infection is that this bacterium multiplies intracellularly, primarily in macrophages, evading in this way many host-defense mechanisms [60]. Thus, internalization of the bacterium by alveolar macrophages is a critical step for the establishment of TB infection. In the lung, the bacteria are phagocytosed by alveolar macrophages and induce a localized pro-inflammatory response and rapid production of cytokines such as TNF, IL-1 and IL-6, and chemokines that lead to the recruitment of immune cells to the site of infection [61-63]. After internalization, macrophages process and present antigens on both MHC I and II to T cells, which in turn secrete IFN-γ, required for the induction of antimycobacterial activity.

A major effector mechanism responsible for the antimycobacterial activity of IFN-γ and TNF is the induction of NO and related reactive nitrogen intermediates (RNIs) by macrophages via

the action of inducible form of nitric oxide synthase (NOS) [61, 64]. Whereas the antimycobacterial property of RNI is well documented both *in vitro* and *in vivo* in the murine model [64-66], there has been conflicting data on the role of RNI in human TB. However, recent data support a protective role for these reactive intermediates in human TB as well [67]. Other antimycobacterial mechanisms of macrophages are: phagolysosome fusion, a process which exposes ingested bacteria in the phagosome to lytic enzymes in the lysosome [18, 20]; apoptosis of infected macrophages [54], which removes the niche for growth and therefore restricts multiplication of bacteria. Moreover, recent studies have demonstrated that autophagy; the cellular process by which a cell degrades its own intracellular compartments is a previously unappreciated innate immune defense mechanism [68]. Stimulation of mouse macrophages with IFN-γ induced autophagy which was necessary for antimicrobial activity against *M. tuberculosis*. A separate study demonstrated that lysosomal hydrolyzed ubiquitin peptides have direct antimicrobial activity against *M. tuberculosis*, and are delivered in an autophagy dependent manner to phagosomes harbouring mycobacterium [69].

During *M. tuberculosis* infection, macrophages and their circulating precursors present at or recruited to the site of infection phagocytose bacteria and migrate deeper into lung tissues, thereby playing important roles in immune activation and bacteria dissemination [70]. The zebrafish embryo infection model by *M. marinum* has helped to elucidate in real time the step-by-step processes: from macrophage migration to the site of infection, to phagocytosis of mycobacteria, to migration of infected macrophages to deeper tissues in the lungs, to growth of mycobacteria within individual macrophages, to granuloma formation [70, 71].

Dendritic cells

It is now established that DCs are also involved in an effector role against *M. tuberculosis* infection [72, 73], and are central to the generation of acquired immunity after carriage of antigens to draining lymph nodes (LN), where recognition by T cells can be maximized [37, 38, 74]. To optimally prime pathogen-specific Th1 responses, DCs require stimulation through TLRs [75] by the pathogen as well as host-derived factors such as type I and type II IFNs, cytokines, and chemokines [76]. For example, *M. tuberculosis* dependent TLR2 ligation can promote the maturation of DCs via upregulation of costimulatory molecules and production of IL-12 essential to prime optimal Th1 responses [77]. However, ligation of DC-SIGN with the *M. tuberculosis*-derived LAM could lead to suppression of immune function

through induction of IL-10 secretion [33, 43, 78]. In DCs, TLR9 signalling results in remodeling at the IL12p40 promoter leading to a robust IL-12 release [79].
The immune response limiting and switching off infection during primary TB is presumably initiated when, upon exposure to *M. tuberculosis*, the efficient antigen-capturing immature DCs [80], are transformed into mature T cell stimulating DCs, which migrate with high efficiency into draining LN. In these compartments, the stimulatory capacity of mature DCs ultimately leads to effector T cell differentiation and memory T cell expansion, which in turn, confer protection against *M. tuberculosis* in the lungs [81, 82]. In contrast to macrophages, DCs have poor mechanisms to eliminate internalized mycobacteria [83, 84]. Rather, it has been suggested that DCs offer a niche for long-term survival of intracellular bacteria [83, 84]. Thus, accumulation of DCs at the granuloma site during pulmonary infection with *M. tuberculosis* may provide niches where the bacteria can survive.

NK (T) cells

NK cells are a type of cytotoxic lymphocyte that is a major component of the innate immune system. These cells have been implicated in early immune responses to a variety of intracellular pathogens, including mycobacteria, through their capacity to rapidly produce IFN-γ and other immunoregulatory cytokines [85-87]. In mycobacterial infections, previous studies on the role of NK cells in host resistance have involved the use of antibodies that deplete NK populations. Although mice depleted of NK cells by this procedure were initially reported to be more susceptible to *M. avium* infection [88], this finding could not be reproduced in a later, more comprehensive study [89]. In contrast to these studies involving mice with an intact T cell compartment, severe combined immunodeficiency mice infected with *M. avium* were shown to be capable of forming hepatic granulomas, the response of which was demonstrated to be dependent on both IFN-γ and TNF [90]. More importantly, T cell receptor (TCR) αβ-deficient mice infected with *M. tuberculosis* were shown to survive longer than IFN-γ-deficient mice [91], a finding strongly suggestive of NK cell involvement. NK cells recruited to the lungs during mycobacterial infection are known to expand and become a primary source of IFN-γ [92]. In line with this, a protective role for NK-produced IFN-γ in T-independent host resistance to aerogenic *M. tuberculosis* infection has been demonstrated [93].

Among the cell types that have been postulated to link the two arms of the immune system; the innate and adaptive immune responses, CD1d-restricted NKT cells are compelling candidates, being able to respond rapidly and subsequently to activate other cell types [94]. Because of their apparent self-reactivity and ability to quickly release large amounts of cytokines such as IFN-γ, NKT cells are hypothesized to be important in the initiation and regulation of various immune responses [94]. NKT cells are a subset of T cells that co-express an αβTCR, but also express a variety of molecular markers that are typically associated with NK cells, such as NK1.1. Unlike conventional αβ T cells, their TCRs are far more limited in diversity and recognize lipids and glycolipids presented by CD1d molecules, a member of the CD1 family of antigen presenting molecules, rather than peptide-MHC complexes. In mice, NKT cells are mainly represented by Vα14 NKT cells, while in humans, there is a homologous population of Vα24 NKT cells. A regulatory role for Vα14 NKT cells has been described during the course of mycobacterial infection in mice, through their ability to produce the anti-inflammatory cytokine IL-4, thereby limiting the extent of the inflammatory response [95]. This suggests an important role for this cell subset as a regulator of the balance between protective immune responses and immunopathology. Activated human CD1d-restricted NKT were capable of restricting the growth of *M. tuberculosis* in a granulolysin-dependent manner [96]. Furthermore, it has been shown that NKT cells induce a granulomatous response to a glycolipid fraction of *M. tuberculosis* cell wall [97].

Neutrophils

In infectious inflammation, polymorphonuclear cells, principally neutrophils are the first phagocytes to arrive from circulation and attempt to eliminate invading pathogens via oxygen-dependent and oxygen-independent mechanisms. The former mechanism results from the generation of reactive oxygen species [98], whereas the latter mechanism reflects the capacity of neutrophils to degranulate and release preformed oxidants and proteolytic enzymes from granules [99]. Neutrophils have been implicated in the control of mycobacterial infections [100, 101], but the mechanisms by which they exert direct protective functions are not completely resolved.

Some studies have demonstrated that human neutrophils are able to kill virulent *M. tuberculosis* [102], while others have not [103]. The recruitment of neutrophils to the lung has been described for acute TB in humans [102], and in experimental animals infected with

mycobacteria [104]. A possible indirect role of neutrophils to mycobacteria killing was demonstrated by Tan and colleagues [105]. In this study, mycobacteria-infected macrophages acquired the contents of neutrophil granules and their antimicrobial molecules by the uptake of apoptotic neutrophil debris, which was trafficked to endosomes and co-localized with intracellular bacteria [105].
Both human and animal studies have shown that neutrophils may play an important role in the transition from innate to adaptive immune responses by producing critical cytokines and chemokines [106, 107].

γδ T cells

Human T cells expressing γδTCR represent a unique lymphocyte population with an unusual tissue distribution and antigen recognition pathway. Conditions that lead to responses of γδ T cells are not fully understood, and current concepts of γδ T cells as 'first line of defense', 'regulatory cells', or 'bridge between innate and adaptive responses' [108] only address facets of their complex behavior. The involvement of γδ T cells in the primary immune response to *M. tuberculosis* infection was described as early as 1989 [109]. Upon contact with mycobacteria, Vγ9/Vδ2 T cells have been shown to exhibit cytolytic functions and are hence involved in innate immune effector mechanisms [110, 111]. In these studies, the ability of Vγ9/Vδ2 T cells to kill mycobacteria was dependent on the release of preformed granules, perforin and granulysin [110, 111]. Murine studies have indicated that the induction of γδ T cells in the immune response against TB precedes that of conventional CD4 and CD8 cells, hence plays an important role in modulating the effector response against tuberculosis. For example, intranasal (i.n.) infection of mice with BCG resulted in an early accumulation of γδ T cells in the lungs, and the peak of γδ T cells expansion at 7 days postinfection preceded the 30 day peak of αβ T cells [112], suggesting that γδ T cells in the lungs might help to control mycobacterial infection before the onset of adaptive immunity.

Studies using γδ TCR knockout mice indicate that γδ T cells may be involved in the regulation of granuloma formation, which is critical for the control of mycobacteria [113]. Infection of mice deficient in γδ T cells with high dose *M. tuberculosis* resulted in the formation of pyogenic granulomas, suggesting that a role for these cells is perhaps in cellular traffic during mycobacterial infection [114]. In humans, loss of Vγ9+/Vδ2+ T cells, the major subset of the circulating γδ T cell pool correlated with pulmonary TB [115]. In mice, the

reciprocal stimulation of γδ T cells and DCs was shown to be important for the optimal induction of antimycobacterial CD8 T cell response, indicating that stimulation of γδ T cells and their non-cognate interaction with DCs could be applied as an immune adjuvant strategy to optimize vaccine induced CD8 T cell immunity [116]. Recently, it has been shown that IL-17 production is dominated by γδ T cells rather than CD4 T cells during *M. tuberculosis* infection, thereby implicating them as a main player in the resistance against infection at the early stage [117].

Adaptive immunity

Several studies have shown that protective immunity to TB is dependent on the adaptive Th1 immune responses [61, 81, 118, 119], mediated by macrophages, DCs, T cells and their interactions, and depends on the interplay of cytokines produced by these cells [42, 61]. Clearance of bacteria by macrophages is in part dependent on macrophage activation by the cytokine IFN-γ secreted by $CD4^+$ T cells, $CD8^+$ T cells and NK cells [61, 81, 93, 118-120]. Infected macrophages secrete pro-inflammatory cytokines such as TNF, IL-1 and IL-6, as well as chemokines that leads to the migration of monocyte derived macrophages and DCs to the site of infection [61, 37, 121]. The migration of cells to the site of infection results in the formation of granuloma, which functions to restrict further bacterial dissemination [122, 123]. The adaptive immune response is initiated when mycobacteria infected DCs mature and migrate to local LN, where recognition by T cells takes place [36, 37, 73, 74].

The hallmark of chronic infections such as TB is the significant delay between infection and the induction of the adaptive immune response, which allows early growth of the pathogen and the establishment of persistent infection. Recently, it was demonstrated that activation of *M. tuberculosis*-specific $CD4^+$ T cells is dependent on trafficking of bacteria from the lung to local LN, and that delayed dissemination from the lung to sites of antigen presentation accounts for the lag in the initiation of adaptive immunity [38], (Figure 2) [124].

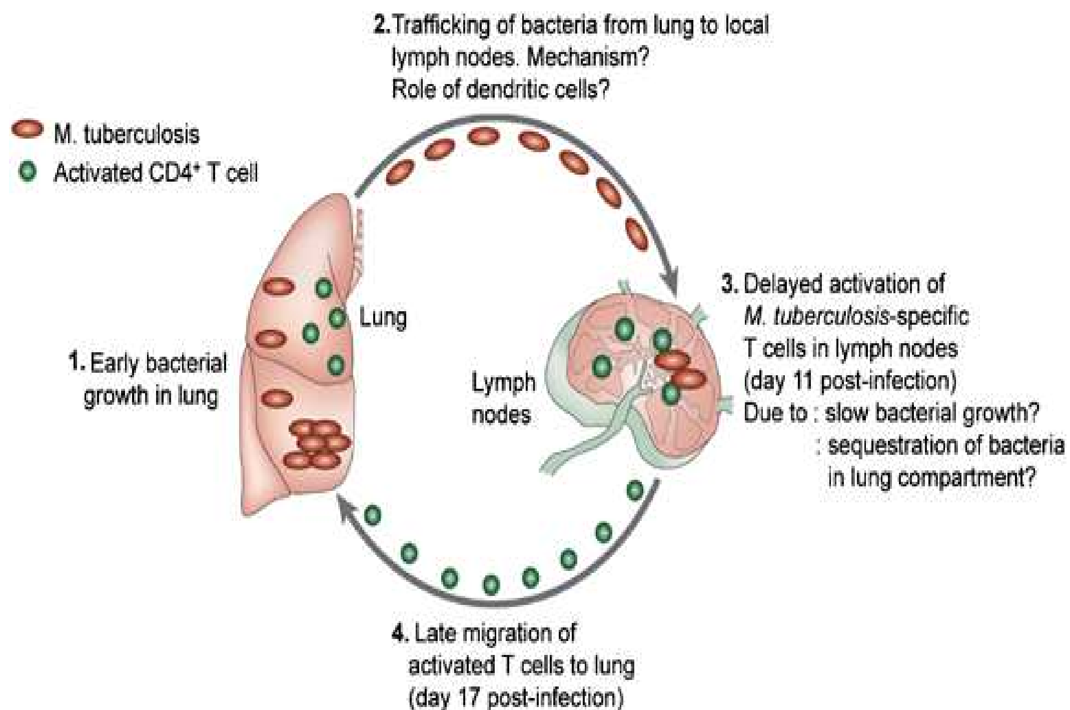

Figure 2. Overview of the events that influence the initiation of the adaptive immune response after infection with *M. tuberculosis* (*Immunol Cell Biology* 2008; **86**:293-294). Reprinted with permission from the Nature Publishing Group.

While the precise mechanisms for this delay are unclear, it has been suggested that low levels of antigen in early infection may help evade immune recognition and that some threshold level of antigen is required to stimulate the T-cell response [38, 125]. On the other hand, late migration of activated T cells to the lung was suggested to contribute to the delay in the onset of adaptive immunity [38].

The granulomatous response

The granulomatous response is a protective immunopathological response of the host following infection with *M. tuberculosis*. Pulmonary granuloma formation is a desperate attempt by the host immune system not only to contain multiplication and further dissemination of bacteria to other organs, but also to localize inflammation and prevent

damage to the lungs. It is postulated that stimulated alveolar macrophages in the airways invade the lung epithelium following internalization of inhaled bacteria [122, 123]. Production of TNF and inflammatory chemokines from infected macrophages drive the recruitment of successive waves of neutrophils, NK cells, $CD4^+$ T and $CD8^+$ T cells, DC and B cells, each of which produces its own complement of cytokines that amplify cellular recruitment and remodeling of the infection site [90, 91, 122, 123, 126].

This inflammatory cascade is regulated and superceded by a specific cellular immune response that is linked to the production of IFN-γ. At this stage, formation of the 'stable' granuloma responsible for immune containment during latent or subclinical infection becomes recognizable and stratification of the structure emerges [127, 128]. The granuloma subsequently develops central areas of necrosis [129] (called caseum, from the word 'cheese' in Latin), resulting in the death of the majority of bacteria and destruction of the surrounding host tissue. The surviving bacilli exist in a latent state and can become reactivated leading to development of active disease. The granuloma serves 3 major purposes; it is a barrier to dissemination of bacteria throughout the lungs and other organs, a local environment in which immune cells can interact to kill bacteria, and a focus of inflammatory cells that prevent inflammation from occurring throughout the lungs [126].

The granuloma maintains a dynamic T cell population reflective of the systemic activated repertoire [123, 130], and are able to accumulate recently activated T cells. Disruption of the granuloma structure or function is therefore detrimental to the control of bacterial replication or immunopathology in the lung. In this regard, the reactivation of latent infection that stems from a failure of tissue granulomas to contain the organism has been reported in experimental models [61].

$CD4^+$ T cells

In the majority of individuals exposed to *M. tuberculosis*, the innate response is not sufficient enough to protect against infection, and the adaptive immune response is necessary to restrict bacterial growth and mediate protection. Although various cells contribute to immunity against *M. tuberculosis*, T cells, notably effector $CD4^+$ T cells play a dominant role [119, 131]. *M. tuberculosis* resides primarily in a vacuole within the macrophage, resulting in MHC II presentation of mycobacterial antigens to $CD4^+$ T cells. Upon activation, $CD4^+$ T cells

secrete IFN-γ and TNF, which in turn induce antimycobacterial mechanisms in macrophages [61, 131, 132].

Studies in mouse models using antibody depletion of $CD4^+$ T cells [133], adoptive transfer [134], or the use of gene-deficient mice [135], have demonstrated that $CD4^+$ T cell subsets are required for the control of the infection. For example, $CD4^+$ T cell-deficient mice infected with *M. tuberculosis* transiently displayed diminished levels of IFN-γ, yet succumbed to the infection [135]. In another study, depletion of $CD4^+$ T cells resulted in reactivation of persistent TB in mice despite continued expression of both IFN-γ and NOS2 [136]. It has been demonstrated that $CD4^+$ T cells are required for the development of a protective granulomatous response to pulmonary TB [137].

The important role of $CD4^+$ T cells in the control of *M. tuberculosis* in humans is illustrated by the strong association of $CD4^+$ T cell impairment and the reactivation of *M. tuberculosis* in patients with HIV infection [138]. Other roles played by $CD4^+$ T cells include induction of apoptosis suggested to be important in controlling *M. tuberculosis* infection [139], conditioning of APCs, help for B cells and $CD8^+$ T cells [140]. $CD4^+$ T cells can also contribute to the control of acute mycobacterial infections through IFN-γ independent mechanisms, which have been demonstrated in experimental models using antibody depletion or mouse strains deficient in either CD4 or MHC class II molecules [136].

$CD8^+$ T cells

Although mycobacteria reside within phagosomes, there is a large body of evidence that $CD8^+$ T cells participate in immunity against *M. tuberculosis* infection [141]. It has been demonstrated that mycobacterial antigens derived from infected cells can be presented by MHC I to $CD8^+$ T cells in both humans and mice, and antigens recognized by these cells have been identified [142]. $CD8^+$ T cells also recognize various antigens from *M. tuberculosis* that are not presented by classical MHC I molecules, but by a closely related group of molecules, the Class Ib molecules. These are non-polymorphic, and include the CD1 molecules [reviewed in 143], as well as H2-M3. CD1 molecules primarily present lipid antigens from *M. tuberculosis* to $CD8^+$ T cells, thereby increasing the possible antigen source greatly. Experimentally, mice deficient in β2-microglobulin, a component of both MHC I and non-classical MHC class Ib molecules were found to be more susceptible to infection with *M.*

tuberculosis than wild type (WT) mice [144]. Moreover, increased susceptibility to mycobacterial infections has been observed in mice deficient in transporters associated with antigen processing, which transport peptides from the cytosol to the endoplasmic reticulum for loading into MHC I molecules [145, 146], indicating a protective role for $CD8^+$ T cells.

In humans, $CD8^+$ T cells can kill intracellular mycobacteria via the release of the antimicrobial peptide granulysin [147]; however, this molecule is not present in the mouse. The fact that no mouse analog of granulysin exists, may in part explain why $CD8^+$ T cells are not as important in the control of infection in mouse models of TB [148]. The cytotoxic potential of $CD8^+$ T cells to kill infected cells (Cytotoxic T cell; CTL activity) has been shown to be dependent on $CD4^+$ T cells in the mouse model, suggesting that the susceptibility of $CD4^+$ T cells knockout mice to *M. tuberculosis* infection might be due in part to impaired CTL activity [140]. $CD8^+$ T cells also produce cytokines (IFN-γ and TNF) during *M. tuberculosis* infection, which probably participate in activation of macrophages [149].

B cells

Presently, there is a growing body of evidence demonstrating that B cells have a greater contribution to TB immunity than previously thought, and play a significant role in optimizing the host response against *M. tuberculosis* infection. For example, the identification of follicle-like B cell dominant structures within TB infected lungs of humans has suggested that B cells may play a previously unappreciated role in local immunity [150, 151]. Moreover, results from previous studies suggest that B cells influence the inflammatory progression in the lungs during *M. tuberculosis* infection [152, 153]. In mice, B cell-deficiency resulted in reduced recruitment of neutrophils, macrophages and $CD8^+$ T cells to the lungs, suggesting a role for B cells in the regulation of chemokines and/or adhesion molecules [152]. A role for B cells in protection against *M. tuberculosis* infection was suggested on grounds of raised bacterial load in the organs of B cell-deficient mice [154]. An additional role for B cells as APCs has also been suggested [154].

Soluble mediators of mycobacterial infections

Innate immune recognition of mycobacteria by phagocytic cells leads to cellular activation and rapid production of pro- and anti-inflammatory cytokines (Figure 3) [42]. These cytokines and chemokines recruit inflammatory cells (T cells, neutrophils and NK cells) to areas of infection, activate transmigrated cells, and coordinate the inflammatory and adaptive immune response to infection. The outcome of mycobacterial infections depends upon cytokine networks established and maintained by innate cells, of which macrophages are of critical importance. In addition to the well-defined cellular immune responses, individuals infected with mycobacteria mount a vigorous humoral immune response. In this thesis, the role of the following cytokines or cytokine receptors and antibodies in immunity to TB have been assessed, and will therefore be discussed; IFN-γ, IL-12, TNF, soluble TNF receptors (sTNFR) and antibodies.

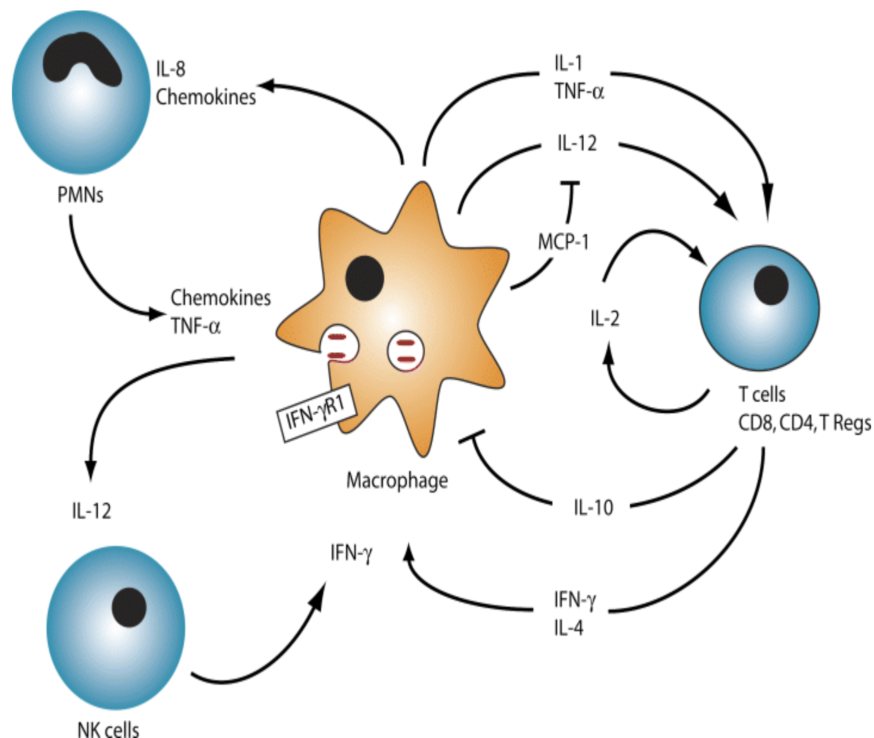

Figure 3: Cellular immune response to *M. tuberculosis* (*Immunological Reviews* 2007; **219**:167-186.). Reprinted with permission from Interscience-Wiley.

IL-12

IL-12 is a product of phagocytic APCs, and acts as a pro-inflammatory cytokine that bridges the innate and adaptive immune responses and skews T cell reactivity toward a Th1 cytokine pattern [155, 156]. The bioactive IL-12p70 is a heterodimeric protein consisting of covalently linked p40 and p35 subunits, both of which are regulated independently [157]. IL-12 was the first cytokine to be described with potent Th1 promoting attributes, followed by IL-23 (shares the p40 component with IL-12), and more recently IL-27. Data from several studies indicate that the three cytokines together orchestrate Th1 responses, with IL-12 being the dominant cytokine that affects both the induction and maintenance of Th1 immunity [158]. Production of IL-12 by *M. tuberculosis*-infected DCs and macrophages is essential for the priming of potent Th1 responses, characterized by IFN-γ production by $CD4^+$ and $CD8^+$ T cells [61, 118, 119, 159, 160]. Humans with mutations in IL-12p40 or the IL-12 receptor genes have reduced capacity for IFN-γ production, and display increased susceptibility to environmental mycobacteria and BCG [161]. Moreover, a role for IL-12 in resistance to *M. tuberculosis* was suggested by the improved clinical outcome observed when the cytokine was combined with drug therapy in a case study [162].

In mice, early administration of IL-12 after *M. tuberculosis* infection resulted in a significantly decreased bacterial burden, and increased mean host survival time [163]. Moreover, neutralization of IL-12 at the initiation of *M. tuberculosis* infection led to increased bacterial loads and reduced granuloma integrity [162]. Furthermore, mice deficient in IL-12p40 were highly susceptible to *M. tuberculosis* infection [164]. It has been shown that the administration of IL-12 could substantially reduce bacterial numbers in mice with a chronic *M. tuberculosis* infection [165], suggesting that the induction of this cytokine is an important factor in the design of TB vaccines.

IFN-γ

IFN-γ produced mainly by $CD4^+$, $CD8^+$ T cells, NK and γδ T cells during *M. tuberculosis* infection [61, 93, 118, 119, 131, 166] is critical for macrophage activation and the subsequent induction of microbicidal mechanisms. Individuals defective in genes for IFN-γ or IFN-γ receptors are susceptible to serious mycobacterial infections, including *M. tuberculosis* [161]. In a large study, it was reported that patients with IFN-γ receptor-deficiency developed

disseminated infection with *M. bovis* BCG or environmental mycobacteria, which in some cases resulted in death of about half of the patients and required continuous antimycobacterial treatment in the survivors [reviewed in 161]. In mice, IFN-γ knockout strains are the most susceptible to virulent *M. tuberculosis* infection [167], with defective macrophage activation and low NOS2 expression [167-169].

M. tuberculosis has developed mechanisms to limit the activation of macrophages by IFN-γ [21-30], suggesting that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine. In this regard, it has been shown that the level of IFN-γ produced by a mouse in response to a candidate vaccine does not always correlate with the effectiveness of the vaccine during *M. tuberculosis* challenge [170]. Similarly, evaluation of the efficacy of human BCG vaccination using several assays demonstrated that mycobacterial growth inhibition did not correlate with IFN-γ response [171]. Thus, although IFN-γ is essential for the development of an immune response that prolongs the life span of an infected animal, it is not sufficient to eliminate an *M. tuberculosis* infection.

TNF and soluble TNF Receptors

TNF is produced primarily by activated monocytes/macrophages in response to pathogens, but can also be expressed by activated T cells, B cells, NK cells, and some tumor cells [172]. TNF is first synthesized as a transmembrane (TmTNF) precursor and cleaved by a membrane-bound metalloprotease disintegrin, TNF converting enzyme (TACE), generating a soluble TNF molecule [173]. Both forms of TNF function physiologically by interacting with one of two receptors; TNFR1 (55 kDa) and TNFR2 (75 kDa) expressed on a diverse range of cell types [172]. Upon stimulation, these receptors could be cleaved from the cell surface, or directly expressed as soluble isoforms lacking the transmembrane domain. TNF mainly binds to TNFR1 while the TmTNF binds to TNFR2 [174, 175].

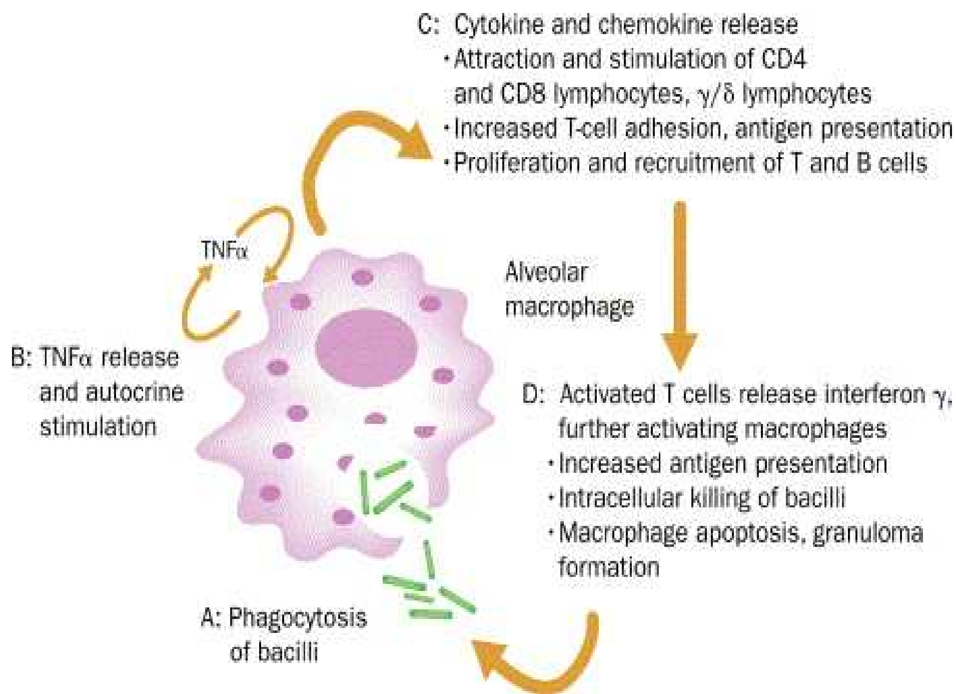

Figure 4: Schematic representation of the central role of TNF in the cellular immune response to *M. tuberculosis* infection (*Lancet Infect Dis* 2003; **3:**148-155). Reprinted with permission from Elsevier.

The importance of TNF in the generation and maintenance of a protective immune response against *M. tuberculosis*, and other bacterial and viral pathogens has been clearly demonstrated [176-178]. Although TNF is not required for the generation of antigen-specific T cell responses, it is essential for controlling the recruitment of inflammatory cells to sites of infection and the development of a protective granulomatous response, resulting in containment of bacillar growth and survival of the infected animals [126, 178-180]. During *M. tuberculosis* infection, TNF is involved in almost every stage of the inflammatory response, from the initial macrophage response, to the attachment, migration, and trafficking of leukocytes through blood vessels, to retention at the site of infection and in immunopathology (Figure 4) [Reviewed in 181]. For example in TNF-knockout mice, the inflammatory response generated following *M. tuberculosis* infection was highly

dysregulated, and T cells recruited to the lungs failed to migrate into central lesions, thereby limiting their contact with infected macrophages [182]. In addition, mice deficient in TNF or sTNFR1 succumbed quickly to *M. tuberculosis* infection, with substantially higher bacterial burden compared to their WT counterparts [176].

TNFR1 signalling is required for the modulation of T-cell responses, because in TNFR1-deficient mice, T-cell dependant granuloma decomposition is observed [180], whereas TNFR2 seems to have a lesser role in granuloma formation and mycobacterial immunity. Although soluble TNF is required for the long term control of *M. tuberculosis* infection, TmTNF was sufficient to control acute, but not chronic infection [183, 184]. However, sTNFR neutralization of TNF is important for homeostasis, since excessive production could lead to exaggerated inflammation resulting in tissue damage.

Antibodies

Historically, the view that protective immunity against TB is mediated exclusively by T cells, involving cytokines, mainly IFN-γ-mediated activation of infected macrophages, rather than antibodies has determined all strategies of TB vaccine research. This view has been sustained by the notion that antibodies cannot reach the bacilli within the phagosomes of infected macrophages [185], and the initial difficulty in demonstrating a consistent protective effect of antibodies in *M. tuberculosis* infection [186]. However, the fact that TB develops despite the presence of abundant T helper immunity [187], coupled with the observation that T-cell targeted vaccination does not always induce optimal protection either in humans or in experimental animals, has made it necessary to investigate alternative immune mechanisms of protection [188].

To this end, the protective role of antibodies in TB has been elucidated recently using modern approaches and tools [reviewed in 189, 190]. IgA is the primary immunoglobulin isotype induced at mucosal sites [191], and is thought to mediate defense functions at these sites [192]. Secretory IgA in mucosal secretions has been shown to prevent the adsorption of pathogens and to neutralize their toxic products at the mucosal epithelium [192]. With regard to TB, previous studies in our group demonstrated that mice deficient in IgA [193], or the polymeric Ig receptor [194], and thereby incapable of actively transporting either IgA or IgM, were more susceptible to mycobacterial infection than their WT counterparts. Furthermore,

the beneficial effect of passively administered IgA or IgA in synergy with IFN-γ on the survival of animals infected with mycobacteria has been demonstrated [185, 188, 195]. In addition, the possible role of antibodies in humans to the natural course of *M. tuberculosis* infection was indicated in clinical studies, which reported higher antibody titres to Ag85 in patients with milder forms of active TB [196].

Mucosal immunity in pulmonary TB

Mucosal immunization has received increasing attention because the respiratory tract is the natural route of *M. tuberculosis* infection, and it is believed that mucosal vaccination provides the best protection from mucosal infectious diseases [197]. Emerging evidence suggests that respiratory mucosal vaccination provides better immune protection against pulmonary TB than parenteral vaccination [198, 199]. For example, respiratory mucosal immunization uniquely elicited higher numbers of antigen-specific $CD4^+$ and $CD8^+$ T cells in the airways capable of IFN-γ production, cytotoxic lysis of target cells, and immune protection against *M. tuberculosis* infection. In comparison, parenteral intramuscular (i.m.) immunization led to activation of T cells, particularly $CD8^+$ T cells, in the peripheral lymphoid organs, but failed to elicit airway luminal T cells or protect the lung from *M. tuberculosis* infection [200]. Furthermore, airway exposure to an otherwise non-immunogenic soluble *M. tuberculosis* antigens resulted in recruitment and retention of antigen-specific T cells in the airway lumen, which were capable of robust protection against pulmonary *M. tuberculosis* challenge [201]. It has been proposed that the failure or success of parenteral immunization hinges critically on T cell geography, whether antigen-specific T cells are within or outside of the mucosal lumen at the right time in order for immune protection to occur [201]. The immunoprotective role of mucosally induced IgA against mycobacterial infection has been demonstrated [193, 202].

The lungs are the site of primary exposure to several pathogenic microorganisms, including mycobacteria, and local immunoregulatory mechanisms are essential to ensure that immune effector mechanisms remain quiescent or are activated as necessary. The major lung accessory cells with immunoregulatory capacity are alveolar macrophages [203] and pulmonary DCs [204]. Alveolar macrophages are professional phagocytes residing within the alveoli and capable of rapidly clearing large numbers of bacteria in the lung when activated [61, 205].

DIAGNOSIS OF TB

The most powerful tool in any TB control program is prompt diagnosis and successful treatment of patients with active contagious disease. In this regard, existing tests for diagnosis of TB vary in sensitivity, specificity, speed and cost.

Sputum smear microscopy

The use of stained-sputum microscopy (Ziehl-Neelsen, Kinyoun, or fluorochrome) for acid-fast bacilli still remains the most available, easy to perform, inexpensive, and rapid diagnostic test for TB [206]. This is especially true for laboratories in developing countries [207], where there are limited resources. The greatest difficulty in diagnosing TB and other mycobacterial infections by sputum microscopy is the test's sometimes lack of sensitivity and specificity [208]. Further, diagnosis of TB by microscopy is difficult especially in children who rarely produce adequate sputum. Currently, the sensitivity of this test has improved considerably with improved techniques and standardization of sputum preparation, and the use of auramine-rhodamine/fluorochrome method instead of the classic Ziehl-Neelsen stain which uses carbol-fuchsin [209]. Identification of smear positive patients is of major importance, because only smear positive pulmonary TB patients are regarded as highly infectious to others [210].

Bacteria cultivation

Mycobacterial culture is the ultimate proof of mycobacterial infection and is often used as a reference method due to its high sensitivity and specificity [211, 212]. However, it takes 4-6 weeks for *M. tuberculosis* to grow on solid culture medium (e.g. agar based Middlebrook 7H10 or 7H11 or the egg-based Lowenstein-Jensson medium), and 3 weeks to grow in liquid 7H9 medium [213]. Notwithstanding the long culture period, it is still a requirement for definitive diagnosis of TB and in drug-susceptibility testing [214].

Biomarkers

A biological marker (biomarker) is defined as a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or

pharmacological responses to a therapeutic intervention [215]. For example with regard to treatment, a good biomarker must measure a factor that is part of the pathological process leading to the clinical endpoint and should be scientifically plausible. TB diagnostic tests that rely on detection of host immunological markers currently in use include the tuberculin skin test (TST) [216, 217] and interferon gamma release assays (IGRAs) [218, 219]. TB infection is controlled by cell-mediated immunity, and the reactivity of sensitized lymphocytes *in vivo* (TST), or IFN-γ release *in vitro* are expected to be strong indicators of exposure, disease progression or treatment.

Tuberculin skin test

The TST or Mantoux test or purified protein derivative (PPD) test has been used for almost a century as the standard test for the diagnosis TB infection and disease [220]. The TST test is based upon the type 4 hypersensitivity reaction, in which a standard dose of 5 Tuberculin units is injected intradermally into the forearm and read 48 to 72 hours later [221]. The TST is based on the principle that T cells of individuals sensitized with mycobacterial antigens produce IFN-γ when they re-encounter these antigens (Figure 5) [218]. The reaction is read by measuring the diameter of induration across the forearm, perpendicular to the long axis in millimeters. No induration is recorded as "0 mm", whereas reactions over 10 mm in size are considered positive in non-immunocompromised persons. The main drawback with the clinical use of the TST is the lack of specificity due to cross-reactivity with proteins present in other mycobacteria, such as BCG or mycobacterium other than tuberculosis (MOTT) [220, 221]. Moreover, several factors may contribute to false-negative results such as age, poor nutrition, acute illness or immunosuppression induced by medication or HIV infection [210].

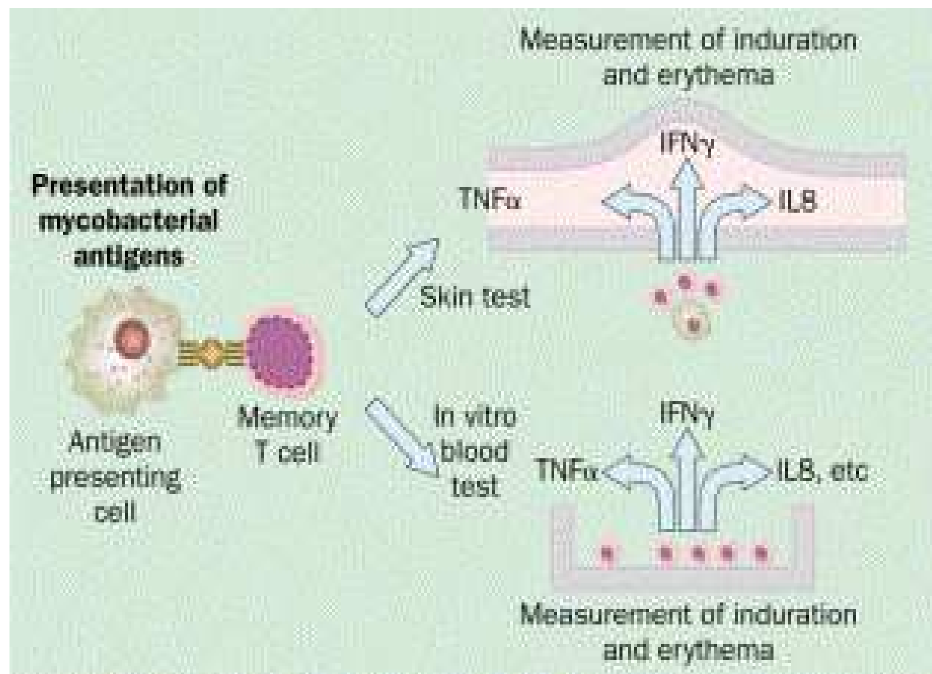

Figure 5. Biological basis of the TST and IFN-γ release assays. (*Lancet* 2000; **356**:1099-1104). Reprinted with permission from Elsevier.

Interferon gamma release assays

As a replacement for the Mantoux test, several other tests are being developed. IGRAs are based on the same principle as the TST, that T cells of individuals sensitized with *M. tuberculosis* produce IFN-γ when they re-encounter mycobacterial antigens (Figure 5). IGRAs quantify the amounts of antigen-specific IFN-γ in blood culture supernatants (QuantiFERON-TB Gold, Cellestis Limited, Carnegie, Victoria, Australia) or determine the frequency of IFN-γ producing blood leukocytes T SPOT-TB assay (Oxford Immunotec, Oxford, UK) in response to specific mycobacterial peptides [222]. These newer assays use antigens specific to *M. tuberculosis*, such as the early secretory antigenic target 6 (ESAT-6) and culture filtrate protein (CFP) 10.

These proteins are encoded by genes located within the region of difference 1 of the *M. tuberculosis* genome, and are not shared with any BCG substrains or most MOTT species, with the exception of *M. kansasii*, *M. marinum, M. flavesence* and *M. szulgai* [218, 223]. The test is used in conjunction with risk assessment, radiography and other medical and diagnostic assays. However, while IGRAs offer comparably high sensitivities and specificities in the diagnosis of TB in immunocompetent patients, there is concern about the sensitivity in immunocompromised patients, especially when using the QFT-TB test [224, 225]. Thus, IFN-γ based assays may give false negative TB diagnosis in endemic areas with high burden of HIV coinfection where reliable diagnostic tools are needed the most.

Serodiagnosis

Mycobacterial infections elicit the production of antibodies to several antigens that may be used as markers of TB infection. Sero-diagnostic tests are usually simple and rapid, and do not involve the use of living cells or direct detection of bacteria in specimens. Different mycobacterial preparations have been evaluated as candidates for the development of serodiagnostic assays, including CFPs [226], purified extracts of glycolipids [227], mycobacterial sonicates [228] and PPD [229]. *In vitro* cultivation of *M. tuberculosis* results in the accumulation of proteins in the extracellular milieu, collectively termed CFPs. By virtue of their immunodominant nature [230], and capacity to activate both humoral and cell-mediated immune responses, these CFPs appear to be the most promising for proteins for use in the diagnosis of TB. Some important CFPs are the 6-kDa antigen or ESAT-6, CFP-10, the 19-kDa, the 30- to 31-kDa Ag85 complex (Ag 85) and the 38-kDa.

A major fraction of the secreted proteins in *M. tuberculosis* culture filtrate is formed by the Ag85, a family of proteins (Ag85A, Ag85B and Ag85C) [231]. The potential use of Ag85 for TB diagnosis has been evaluated by many investigators with varying sensitivities or specificities [232, 233]. The 38-kDa antigen is a surface expressed glycolipoprotein, and is one of the most important antigens used in the development of serodiagnostic tests [234]. In an enzyme-linked immunosorbent assay (ELISA) format, up to 85% of smear-positive cases were detected [235]. However, as a single antigen, the 38-kDa antigen may lack sufficient sensitivity to create an optimal serodiagnostic test, especially for smear-negative individuals, where sensitivity is considerably lower [235]. The 19-kDa has been shown to be recognized

by sera from TB patients [235, 236]. Varying sensitivities have been demonstrated, which corresponded with the disease pathology [236].

The 16-kDa heat shock protein X, is specific to the *M. tuberculosis* complex [237], and essential for mycobacterial persistence within macrophages. It is the dominant protein produced during static growth or under oxygen deprivation [238]. The 16-kDa antigen has been used for the detection of antibody isotypes in the sera of TB patients alone, or in combination with other antigens [239-242]. In addition, both T-cell and B-cell responses to the 16-kDa antigen were found to be associated with latent *M. tuberculosis* infection [243, 244], pointing to the importance of 16-kDa as an antigenic target of immune responses during latent TB infection. This antigen has been incorporated into a commercial kit in combination with the 38-kDa antigen (Pathozyme TB complex, Omega Diagnostics Ltd, Alloa, Scotland).

Molecular methods

Nucleic acid amplification tests, such as polymerase chain reaction have contributed to a more rapid and reliable diagnosis of pulmonary TB. These technologies allow for the amplification of specific target sequences of nucleic acids that can be detected through the use of nucleic acid probes; both RNA and DNA amplification systems are commercially available [245, 246]. Amplification methods for *M. tuberculosis*, however, have low sensitivity, and the absence of specific internal controls for the detection of inhibitors of the reaction means it cannot completely replace the classical diagnostic techniques [246].

THE BCG VACCINE

M. bovis Bacillus Calmette-Guérin (BCG) is the most widely used vaccine in the world, and approximately three billion people have been vaccinated since 1921. Close to 115 million doses are distributed each year [247, 248], providing almost 80% coverage of infants worldwide. Robert Koch (1843-1910) elucidated the etiology of TB, and Calmette (1863-1933), together with Guérin (1872-1961), developed the BCG vaccine in the 20th century, which is still the only vaccine available against TB. The first clinical studies took place from 1921 to 1927 in France and Belgium, and showed that BCG was highly efficient in protecting against TB in children [249]. Unfortunately, despite the early success, the BCG vaccine has

had a limited effect against the TB epidemic in developing countries [250]. Although BCG protects children efficiently against the early manifestations of TB, it hardly offers protection against adult pulmonary TB [251].

The reasons for the varying efficacy of BCG in protection against pulmonary TB are not completely understood. However, potential explanations that have been suggested include: interference with the immune response to BCG caused by previous exposure to environmental mycobacteria [252]; differences among BCG vaccine sub-strains; phenotypic changes in the vaccine during passage from the original cultures and during the manufacturing process. Other factors are; the deletion of protective antigens from BCG; failure of BCG to stimulate adequate, balanced antimycobacterial $CD4^+$ and $CD8^+$ T-cell responses; variability in dose, and route of administration [247]. The current route of vaccination, the intradermal route is thought of as not inducing an optimal immune response. Vaccination via the respiratory tract is believed to be superior to vaccination at other sites in conferring protection against several mucosal infections [253]. In line with this, i.n. vaccination with BCG led to better protection of mice against challenge by *M. tuberculosis* [202] or *M. bovis* [254], which was attributed to an enhanced and more rapid production of IFN-γ by T cells [198]. Also, better protection was observed in the majority of studies using aerosol delivery of BCG [255]. Apart from the immunological advantages offered by i.n. vaccination, there are logistic advantages such as vaccination without needles and syringes, which will make immunization more acceptable, safer and better suited for mass administration.

Development of New TB vaccines

Substantial efforts are currently being put into the development of new TB vaccines to either replace or boost the existing BCG vaccine, albeit the fact that the BCG vaccine provides varying degrees of protection against TB. Among the spectrum of innovative new approaches that have been applied to TB vaccine development during the last decade, some have relied on strengthening the immunogenicity and/or persistence of genetically modified recombinant BCG strains [256], and others on using attenuated mycobacteria such as auxotrophic *M. tuberculosis* strains [257, 258] or less virulent mycobacteria, such as *M. microti*, *M. vaccae*, or *M. smegmatis* that overproduce immunogenic antigens of *M. tuberculosis* [259, 260]. Re-engineering BCG is an interesting approach that relies on the basic premise that the efficacy of BCG could be enhanced through insertion of genes encoding immunodominant antigens or

immunostimulatory cytokines. For example, the reintroduction of the RD1 locus, which encodes the immunodominant and protective ESAT-6 and CFP-10, enhanced the protective efficacy of BCG:RD1-2F9 vaccine compared with BCG [261]. This approach, although increasing the potency of BCG, might also increase its virulence [262] and pose problems for human use.

Subunit vaccines are made up of one or a few mycobacterial antigens, and are generally considered as vaccines to be used on top of BCG as a booster vaccine following a conventional BCG prime vaccination. Through biochemical and proteomic approaches [263], several mycobacterial antigens with good immuno-potentiating properties have been identified. Among these antigens, secreted proteins have received much attention, and today, most subunit vaccine candidates are based on secreted proteins [264, 265]. Vaccination with recombinant fusion proteins such as Ag85B-ESAT-6 (hybrid-1) induced a better protection in mice against *M. tuberculosis* challenge than the individual components [266]. It is conceivable that the two types of vaccines could be combined in the near future: that is, prime with recombinant BCG vaccine and boost with a subunit vaccine. For this to be achievable, it is essential to define *in vivo* correlates of protection [267], which are ill-defined at present. The most advanced TB vaccine candidates were recently reviewed [250, 268, 269].

TREATMENT

The WHO has been tackling the global problem of inadequate TB control for some years and launched a new programme of integrated care in 1994, called directly observed treatment, short course (DOTS) program [270]. A combination of drugs referred to as first line drugs (Isoniazid, rifampicin, pyrazinamide and ethambutol) are used together in initial treatment for 6 months under close supervision. However, the well-designed DOTS program faces higher failure rates, due in part to increasing incidences of multidrug-resistant-TB [271]. Other antibiotics are active against TB, and are used primarily for MDR-TB. The 2 most important classes are the aminoglycosides (streptomycin, kenamycin, amikacin) and fluoroquinolones (levofloxacin, moxifloxacin). The emergence of extensively drug-resistant (XDR) *M. tuberculosis* [272] has presented another setback to the use of chemotherapy. Unfortunately, no new anti-TB agents have been introduced in a long while, and currently, there are no effective vaccines available as yet [273].

ANIMAL MODELS IN TB

The wide spectrum of disease outcomes seen in human TB is difficult to mimic in any single animal model, and therefore experimental infections in different animal models have been used to study different aspects of *M. tuberculosis* pathogenesis [3, 274, 275]. In experimental infections, conditions such as the route of infection, dose, and strain, as well as environmental factors like diet or stress can be tightly controlled.

Undoubtedly, the mouse is the most sophisticated and cost-efficient animal model in biomedical research. The mouse has been used extensively to study the genetics and pathogenesis of susceptibility to TB for a number of reasons. First, mice infected with *M. tuberculosis* develop a fatal pulmonary disease that pathologically resembles that seen in humans. Moreover, the innate and acquired host immune responses to *M. tuberculosis* in mice resemble those seen in humans, including the protective roles played by $CD4^+$ T cells, IFN-γ, TNF and IL-12 [reviewed in 3]. Furthermore, the genetic manipulation of mice is highly advanced. For example, transgene expression, gene knock-out, gene knock-in, both constitutive and conditional, have all become standard technologies and also a large variety of mouse mutants with defined immunodeficiencies are available to researchers studying the role of distinct cells and surface molecules in the *in vivo* setting of TB. In addition, reagents such as monoclonal antibodies against surface markers and cytokines are available. Moreover, the mouse genome has been completely sequenced, making the blueprint for future experiments available [276].

The guinea pig is one of the more extensively studied animal models of TB, because of the significant ways in which it resembles both the normal human physiology and the pathophysiology of pulmonary TB [277]. For example, low-dose aerosol infection of the guinea pig with *M. tuberculosis* produces a well-characterized disease that shares important morphologic and clinical features with human TB [274, 277, 278]. Moreover, granulomatous lesions in guinea pigs are very similar to those seen in human TB patients. In addition, guinea pigs respond quite well to anti-TB antibiotics, and have been successfully used to test the efficacy of new drugs or drug combinations [279].

In recent times, the zebrafish model of mycobacterial infection has been used to study the pathogenesis of *M. marinum*, a close genetic relative of *M tuberculosis* [280]. *M. marinum* is

a natural pathogen of ectotherms, and like *M. tuberculosis*, replicates in host macrophages and produces a chronic granulomatous infection [281]. Moreover, the zebrafish has both innate and adaptive immunity, and similar to mammals, both are involved in protection against TB [282]. Also, important cells of human immunity such as macrophages, granulocytes, T and B lymphocytes have all been identified in zebrafish [282].

SUSCEPTIBILITY TO TB

The natural history of TB shows that most humans are resistant, presumably because of an ability to generate a successful immune response against *M tuberculosis* infection. It has been estimated that only 10% of persons infected with *M. tuberculosis* will ever develop clinical disease [283]. This implies that the great majority of infected persons are capable of preventing infection from becoming established after implantation in the lung. Of the 10% who are susceptible, half of them develop active disease within one year, and the rest do so thereafter owing to reactivation, suggesting that predisposed humans show a spectrum of susceptibilities [3]. In this regard, there is little doubt that host genetic factors explain, at least in part, why some people resist infection more successfully than others. For example, in 1926, an accidental administration of live *M. tuberculosis* (in place of BCG) to babies in Lubeck, Germany left some babies unaffected, whereas others developed severe disease resulting in death [284], thus supporting the notion that majority of the population have effective resistance to TB.

Convincing evidence exists from twin studies that host genetic factors are important in determining who will succumb to *M. tuberculosis* infection and who will not [285, 286]. In these studies, there was a higher concordance for TB among monozygotic twins compared to dizygotic twins. The association of host genetic factors, such as human leukocyte antigens (HLA) and non-HLA genes that are associated with susceptibility or resistance to TB, has been studied using various methods such as case control studies, candidate gene approach, family-based and genome-wide linkage studies [287-290]. One important reason attributed to the existence of racial differences in susceptibility to TB is polymorphisms in genes involved in resisting infectious pathogens [287, 290]. With regard to non-HLA, polymorphisms in genes for the natural resistance-associated macrophage protein 1 (*Nramp1*), now known as the

solute carrier family 11 member 1 gene (*SLC11A1*), vitamin D receptor and mannose-binding protein have all been associated with TB susceptibility [291-295].

The *Nramp1* gene has been shown to be directly involved in determination of the resistance of mice to infections with several unrelated pathogens, including *Leishmania donovani*, *Salmonella typhimurium*, and BCG [296-299]. In the case of infection with BCG, susceptibility to infection was based on permissiveness to microbial replication in the spleen during the early phase of infection after intravenous (i.v.) injection with low dose bacteria [300, 301]. Susceptibility is inherited as a recessive trait that segregates as a single locus (*Bcg*). Experiments *in vitro* demonstrated that *Nramp1* controls the degree of intracellular microbial replication in macrophages [302]. *Nramp1* is expressed in the membrane of lysosomes in macrophages, as well as in tertially granules in neutrophils [303, 304]. However, unlike infection with BCG, the super-susceptibility to tuberculosis 1 (*sst1*) locus controls resistance to *M. tuberculosis* infection. This resistance is attributable to a transcript designated intracellular pathogen resistance 1 on the *sst1* locus [305].

Polymorphisms in cytokine or cytokine receptor genes are known to influence host susceptibility to TB [161, 306]. More generally, there is a correlation between the functional severity of mutations that disrupt IFN-γ-mediated immunity and the extent of susceptibility to mycobacterial infections [307]. The role of IL-12 in mycobacterial infections has been firmly established by the presence of patients with uncommon polymorphisms or mutations that predispose them to severe disseminated mycobacterial infection [161]. Furthermore, mice deficient in IL-12p40 and IL-12p35 show enhanced susceptibility to mycobacterial infection [164, 308].

TLRs are important for host responses to mycobacteria [43, 44], and mice deficient in TLR2 [56] or the adaptor protein, MyD88 have increased susceptibilities to mycobacterial infections [310, 311]. Polymorphisms in human TLR2 have been associated with enhanced susceptibility to leprosy and TB [312-314].

PRESENT STUDY

AIMS

TB has been declared a major public health problem worldwide, due to the emergence of almost untreatable strains of *M. tuberculosis*, and coinfection with HIV. In addition, the only existing vaccine against TB, the BCG vaccine, fails to protect against pulmonary TB, the most important form of disease manifestation. Therefore, the overall aim of this study was to increase our understanding of the host-pathogen interaction, with regard to mycobacteria persistence, host immune response and susceptibility to infection. Dissection of these parameters is a prerequisite not only for accurate diagnosis, but effective treatment and design of better vaccines:

The specific objectives were:

Paper I

To investigate the role of TLR signalling as an evasive mechanism for mycobacterial survival and persistence in the host.

Paper II

To identify immunological markers (biomarkers) of diagnostic importance in active pulmonary TB.

Paper III

To investigate the contribution of host genetic background to susceptibility or resistance to pulmonary mycobacterial infection.

MATERIALS AND METHODS

The materials and methods used in this work are described in the individual papers (I-III).

RESULTS AND DISCUSSION

PAPER I

IFN-γ is critically important for the control of mycobacterial infections, and acts primarily through regulation of gene expression [315]. The functional consequences include up-regulation of MHC II and co-stimulatory molecule expression, and the production of antimicrobial effectors such as oxygen radicals and NO by macrophages [316]. IFN-γ is found at the site of *M. tuberculosis* infection and even within the granuloma [317, 318]. Nonetheless, *M. tuberculosis* persists within macrophages and neither infected humans nor mice are able to completely clear the infection [319]. This suggests that *M. tuberculosis* has evolved mechanisms to avoid elimination by the hosts' immune response [14, 320], potentially contributing to its persistence in the infected host. In this regard, several studies have shown that the 19-kDa lipoprotein of *M. tuberculosis* inhibits IFN-γ responsiveness in macrophages in a TLR2 dependent manner [26-30, 321].

In this paper, we investigated the functional implications of continuous TLR signalling on IFN-γ mediated killing of internalized mycobacteria by murine macrophages. As a first step, we investigated the effect of chronic exposure of the murine macrophage cell line, J774 cells to 19-kDa on IFN-γ mediated killing of phagocytosed mycobacteria. To model the impact of prolonged TLR signalling in chronic infection with *M. tuberculosis*, macrophages were pretreated with the 19-kDa for 24 h prior to IFN-γ stimulation. We found that macrophages previously exposed to 19-kDa before IFN-γ stimulation were less able to kill internalized bacteria, compared to non-treated, but IFN-γ stimulated macrophages. To further investigate

the critical role of TLR2 signalling on inhibition of IFN-γ mediated killing of mycobacteria by macrophages, we pretreated J774 cells as before with 19-kDa, but also with zymosan, which is of yeast origin [322], but signals through TLR2 [323], or LPS, a known TLR4 ligand. We found that prior exposure of J774 cells to zymosan, but not LPS, impaired their ability to kill ingested mycobacteria after IFN-γ stimulation, thus reinforcing the important role of TLR2 signalling in this context. Furthermore, we demonstrated that exposure of macrophages to the cell wall of BCG (CW_{BCG}), known to express the 19-kDa lipoprotein, but not *M. vaccae* cell wall ($CW_{M.\ Vaccae}$), which lacks this antigen, interfered with IFN-γ mediated killing of mycobacteria. Consistent with the observation in J774 cells, both 19-kDa and zymosan failed to interfere with IFN-γ induced killing of mycobacteria in TLR2-deficient, but not TLR4-deficient bone marrow derived macrophages (BMM). In contrast, TLR4 deficient BMM behaved similarly to WT macrophages, indicating a critical role for TLR2, but not TLR4 signalling as an important immune evasive mechanism by mycobacteria.

Similarly to infection with *M. tuberculosis* [21-24, 324], the 19-kDa antigen is known to inhibit multiple IFN-γ dependent responses in macrophages, including induction of CD64, MHC-II, interferon regulatory factor 1, and CIITA [22, 23, 26-29], which regulates MHC II expression [325]. Indeed, it has been shown that the inhibitory effect is independent of live bacteria, as gamma-irradiated *M. tuberculosis* or bacterial components could also inhibit IFN-γ responsiveness similarly to live bacteria [21]. Inhibition of macrophage MHC II expression was suggested to be based on several PAMPs, since such inhibition could be induced by LPS or CpG DNA [326]. However, it has been demonstrated that macrophages from TLR2, but not TLR4-deficient mice are largely resistant to inhibition of MHC II antigen processing following infection with *M. tuberculosis* [26], indicating that *M. tuberculosis*-mediated inhibition of MHC II antigen processing is dependent on TLR2, and does not require TLR4. Thus, it is conceivable that the interaction between TLR4 and LPS does not translate to functional interference with IFN-γ mediated killing of mycobacteria.

The fact that exposure of macrophages to zymosan induced a state of refractoriness to IFN-γ similar to 19-kDa, indicates that continuous signalling through TLR2 is more important than the nature of antigen. Intriguing though, is the fact that zymosan, unlike the 19-kDa is a yeast cell wall particle containing mainly polysaccharides, of which β-glucan and mannan are the major constituents [323]. Although we cannot adduce reasons in support of this observation, there is growing evidence that many mycobacterial lipoproteins are also glycosylated. For

example, the 19-kDa has been shown to contain carbohydrate modifications on several threonine residues close to the N terminus of the protein [327]. It is, however, not known how glycosylation affects interactions between pathogens and TLRs. Whereas many acute bacterial pathogens fail to exploit this inhibitory mechanism, it is conceivable that the chronicity of most fungal infections, like *M. tuberculosis*, make them particularly well adapted to exploit this mechanism *in vivo*. It has been demonstrated that multiple mycobacterial cell wall components, including other lipoproteins, and non-lipoprotein components such as phosphatidylinositol mannan and PGN inhibit macrophage responses to IFN-γ [28, 30, 321]. In the case of PGN, the inhibition was independent of TLR2 [30]. Consistent with this, it was shown that 19-kDa lipoprotein-null BCG was as capable as the 19-kDa lipoprotein-replete strain in inhibiting MHC-II antigen presentation by IFN-γ stimulated macrophages [28]. However, it is of importance to also point out that not all TLR2 ligands of mycobacteria exhibit this inhibitory effect. For example, it was shown that mannosylated LAM, a major component of the *M. tuberculosis* cell wall was not responsible for the inhibition of IFN-signalling by unfractionated cell wall [21].

We next studied the direct effect of TLR2 ligation on bacterial killing, as well as production of effectors of antimycobacterial activity, such as TNF and NO. We did not find any direct relationship between TLR2 signalling and cell proliferation or induction of antimycobacterial activity in macrophages, which is in contrast to the finding by Thoma-Uszynski *et al* [53]. A plausible explanation for the difference in results could be the significantly lower concentration of the 19-kDa antigen used in our study. In terms of mechanisms, neither TNF nor NO production by IFN-γ activated macrophages was significantly affected by exposure to TLR2 ligands. Although NO plays a significant role in the induction of antimycobacterial activity, at least in murine macrophages [61], it has been demonstrated that 19-kDa could inhibit IFN-γ signalling through mechanism(s) other than the production of NO [30]. We finally demonstrated that the refractoriness induced in macrophages after prolonged TLR2 ligation could be reversed with increasing amounts of IFN-γ. Although infections with certain pathogens have been shown to down-regulate IFNR expression at the cell surface, the general consensus is that exposure of macrophages to mycobacteria or to 19-kDa antigen affects neither the expression of IFN-γ receptors, nor the IFN-γ proximal signalling steps [21, 324]. Presently, we cannot explain the mechanism(s) underlying this observation. It is possible that certain IFN-γ responsive genes are upregulated with increasing amounts of IFN-γ, or other PRR are involved. Since PGN, shown to inhibit IFN-γ activation, also signals through

nucleotide-binding oligomerization domain (NOD) proteins [328], it is possible that these NOD receptors are partly involved in this inhibitory mechanism.

At this point, it is important to emphasize the predominant role of TLR2 in immune recognition of *M. tuberculosis*, as well as in the activation of sentinel cells like macrophages and dendritic cells. For example, ligation of TLR2 by the 19-kDa lipoprotein is known to induce TNF, IL-12 and NO production, important mediators of macrophage antimycobacterial activity in both murine and human macrophages [329]. *In vivo*, it has been demonstrated that TLR2-deficient mice are more susceptible to early mycobacterial infection than their WT counterparts [56]. Inhibition of IFN-γ induced responses through prolonged TLR signalling may seem discordant with the roles of TLRs in acute immune activation, but this inhibition may represent a counterregulatory mechanism to promote immune homeostasis. Although PAMP-induced TLR signalling acutely produces pro-inflammatory innate immune responses that contribute to host defense, some aspects of these responses may have to be downregulated after the acute phase to prevent exaggerated inflammation and limit damage to vital tissues or organs. For many acute bacterial infections, the necessary innate immune functions are induced during the acute phase.

Our observation, together with those of others, may help to explain recent findings that BCG vaccination induced T cell responses do not always translate to better protection against TB in both humans [171] and mice [330]. Together, these observations suggest that efforts to develop new vaccines for TB should not solely rely on production of IFN-γ by T cells as the *in vitro* correlate of potential efficacy of the candidate vaccine. Moreover, further understanding of the mechanisms underlying the ability of *M. tuberculosis* to suppress macrophage responsiveness to IFN-γ may help develop means to circumvent these mechanisms, and thus achieve more effective containment or eradication of the bacteria by cellular immune responses.

PAPER II

Accurate and timely diagnosis of *M. tuberculosis* infection is important for the success of any TB control program. The currently existing diagnostic tools vary in sensitivity, specificity, speed and cost. For example, the TST which is the standard tool for detecting latent TB fails to distinguish between immunization with BCG or exposure to MOTT [220, 221]. Thus, the development of new diagnostic tools is imperative. The fact that TB is a disease of the lung means that levels of immunological markers in the respiratory tract are likely to reflect on the infection status or pathology.

In paper II, we aimed to identify immunological markers of diagnostic importance in both the respiratory tract and serum during active mycobacterial infection. First, to model the natural route of infection with *M. tuberculosis*, mice were infected i.n. with BCG, or treated with heat killed BCG (hk-BCG) or BCG lysate (the last two as examples of non-replicating BCG). We demonstrated that active pulmonary infection, but not presence of mycobacterial antigens, induced production of sTNFR, IL-12 or IFN-γ in the lung microenvironment (broncho-alveolar lavage, BAL). Moreover, levels of sTNFR, IL-12, and to a lesser extent IFN-γ in BAL correlated positively with bacterial growth in the lungs, but not in the spleen or liver.

During mycobacterial infection, macrophages and their circulating precursors present at or recruited to the site of infection phagocytose bacteria and migrate deeper into lung tissues, thereby playing important roles in immune activation and bacterial dissemination [70]. In the lung tissues, mycobacteria continue to replicate, at least until the induction of the adaptive immune response. In addition, mycobacteria-infected DCs transport mycobacteria from the lungs to regional LN [37], where the stimulatory capacity of DCs ultimately leads to effector T-cell differentiation and memory T-cell expansion [38]. After initial proliferation in the LN, effector $CD4^+$ T cells traffic rapidly to the lung, and are capable of secreting immune mediators such as IFN-γ and TNF. Indeed, Daugelat and colleagues have demonstrated that immunization of mice with live, but not hk-BCG resulted in induction of a specific set of mycobacterium-reactive T cells capable of recognizing secreted proteins [331]. Also, replicating mycobacteria present both secreted and somatic antigens, and are therefore likely to activate a wide range of immune cells, compared to non-replicating antigens. The reasons adduced may explain the inability of non-replicating BCG to induce detectable levels of IL-12, IFN-γ or sTNFR in the respiratory tract, compared to live bacteria.

In experimental animal TB models, the route of bacterial administration can influence the level or nature of immune responses generated. Therefore, we infected mice via the i.v. route, and assessed the bacterial load in the lung, spleen and LN, as well as sTNFR levels in the respiratory tract and serum. Similarly to the observation with the i.n. route of infection, infected mice were unable to control infection in the lung until week 3 postinfection, after which the bacterial load declined significantly. This is in agreement with the observation that the protective adaptive immune response in mice requires a minimum of two weeks to develop after aerosol mycobacterial infection, thus allowing bacterial growth at the early phase of infection [332]. Consistent with the observation using the i.n. route of infection, sTNFR levels correlated strongly with bacterial load in the lung, but not the spleen or liver, strengthening the notion that irrespective of the route of infection, mycobacterium has a particular tropism for the lung. These findings suggest that the nature or level of immune responses in the respiratory tract is dependent on successful colonization and growth of bacteria in the lungs, but not in other organs like the spleen or liver.

Receptor-mediated recognition and phagocytosis of mycobacteria generates an inflammatory response, characterized by the production of pro-inflammatory cytokines such as IL-12 or TNF that influence the development of the adaptive Th1 immune response, characterized by IFN-γ production by mycobacterium-specific T cells [61]. This makes it logical to associate increased pro-inflammatory cytokine concentrations in the respiratory tract to disease activity in the lung, since cytokines have been ascribed an important role in TB pathogenesis. Consistent with this, broncho-alveolar cells from TB patients expressed higher levels of IL-12 and IFN-γ mRNA [333], or released higher amounts of TNF, IFN-γ and IL-1β [334] than healthy controls. In addition, nucleic acid amplification test accuracy for the diagnosis of TB was far superior when applied to respiratory samples as opposed to other body fluids [335]. Although bacterial growth in the lungs resulted in elevated levels of sTNFR and IL-12 in serum, non-replicating BCG induced secretion of similar levels in serum as live BCG. This clearly demonstrates that in TB, the local immune response in the respiratory tract is more indicative of the infection status, compared to systemic responses, supporting the generally held notion that immune markers in the respiratory tract are more reflective of disease pathology in the lungs.

In addition to cytokines, increased levels of soluble cytokine receptors, such as sTNFR, have been associated with mycobacterial infections. In this study, we found that the highest sTNFR

secretion coincided with the peak bacterial growth in the lungs. This is in good agreement with the fact that TNF production is a requirement for granuloma formation, important for restriction of mycobacteria growth and dissemination [126]. Indeed, reactivation of controlled mycobacterial infection in the lung resulted in an increase in sTNFR levels in the respiratory tract. Consistent with our finding, transgenic mice expressing high serum sTNFR levels exhibited reduced bactericidal activity and succumbed to BCG infection [336]. In a study aimed to identify immune markers that are independently associated with HIV infection or TB *in vivo*, increased sTNFR1 levels were associated with TB rather than HIV infection [337]. In this study, increased levels of sTNFR in both BAL and serum may explain the minimal amounts of TNF detected, as overexpression of these receptors may result in TNF neutralization.

Elevated levels of pro-inflammatory cytokines in general are a general characteristic of the inflammatory response and not specific for mycobacterial infection. We reasoned that detection of mycobacterial-specific antibodies together with sTNFR could be useful in distinguishing active infection from latency. Indeed, active pulmonary infection but not the presence of non-replicating BCG (hk-BCG or BCG lysate) induced the production of BCG-specific IgG or IgA in the respiratory tract. Moreover, IgA was detected in BAL but not serum. Indeed, IgA is the predominant immunoglobulin isotype induced at mucosal sites, where it is believed to mediate defense mechanisms [338]. Although active infection resulted in the production of IgG in serum at similar levels as in BAL, non-replicating BCG also induced detectable levels of IgG. This confirms our earlier observation that the immune response in the respiratory tract is more specific than the systemic. Until recently, the prevailing opinion has been that antibodies have little or no role in protection against TB. However, several studies have provided data on the protective role of antibodies [Reviewed in 188-190]. In this study, our particular interest was to find the relationship between antibody production and infection. In conclusion, a combinatorial detection of mycobacterium-specific antibodies, especially IgA, together with sTNFR in the respiratory tract may indicate active infection, whereas increased antibodies levels alone may indicate chronic or latent infection.

PAPER III

Host genetic background is known to influence resistance or susceptibility to infection with many pathogens. The availability of numerous inbred mouse strains has allowed the establishment of models of resistance or susceptibility to infections with different microbial pathogens. For example, infection with the protozoan parasite *Leishmania major* in BALB/c and C57BL/6 mice led to the *in vivo* demonstration of the Th1/Th2 paradigm, and the relationship between T cell subsets and susceptibility to this pathogen [339]. Resistance in C57BL/6 mice has been attributed to a bias towards Th1 cytokine production, and BALB/c mice to less efficient production of IFN-γ and a strong Th2 response [339]. BALB/c and C57BL/6 mice have genetic differences not only in the H-2 locus and other H-2 associated genes, and have been used to establish resistance or susceptibility to infection by several pathogens, including mycobacteria [339-343]. In the past, studies to dissect genetic differences in inbred mouse strains to BCG used the i.v. route of infection, and susceptibility was defined as permissiveness to bacterial replication in the spleen rather than the lung [300, 301]. It is therefore not surprising that in these studies, mycobacterial replication in the spleen was similar between BALB/c and C57BL/6 mice. Nonetheless, differences in susceptibility to *M. bovis* BCG [342, 343] or *M. avium* [341] have been demonstrated, with BALB/c mice displaying a higher susceptibility to infection than C57BL/6 mice.

In paper III, we investigated the nature of immune responses in the two mouse strains during primary pulmonary mycobacterial infection. As described earlier, we infected mice via the respiratory tract in order to mimic the natural route of infection with mycobacteria, and analyzed the bacterial growth in the lung until week 9 postinfection. We found that BALB/c mice were less able to control bacterial growth during the early phase of infection than C57BL/6 mice, although both mouse strains successfully controlled infection by week 9 postinfection. Since the difference in susceptibility occurred early during infection, (day 3 to week 3), we focused on these early time points, and analyzed the levels and nature of immune responses, especially in the lung during this period. Increased susceptibility in BALB/c mice was associated with a delay in IFN-γ, TNF and to some extent IL-12 production in the lung microenvironment, but high sTNFR levels. This observation was confirmed *ex vivo*, where infected lung cells from BALB/c mice released lower amounts of IFN-γ, TNF and IL-12, but

higher sTNFR levels compared to C57BL/6 mice. Furthermore, BALB/c mice exhibited decreased IFN-γ recall responses to mycobacterial antigens than C57BL/6 mice.

We ascribe the decreased ability of BALB/c, compared to C57BL/6 mice to control early pulmonary mycobacterial infection, partly to a delay in innate responses (week 1-2), known to be critical in shaping the development of Th1 adaptive immunity (week 2 or more) [332, 344]. Our reasoning is based on the fact that during mycobacterial infections, immune stimulated T cells do not accumulate in the lungs until 2-3 weeks postinfection [345]. Moreover, it is believed that the innate immunity provides the initial resistance in the first 1-3 weeks after infection before the adaptive Th1 cell-mediated immunity fully develops [44]. There is compelling evidence that IFN-γ, TNF and IL-12 play a critical role in the development of Th1 immunity against intracellular pathogens. For example, resistance of C57BL/6 mice to *Listeria monocytogenes* infection was associated with early production of IL-12 by DCs, whereas susceptibility of BALB/c mice was linked to delayed production of this cytokine [340]. Furthermore, it is believed that IFN-γ can supersede the attempts of *M. tuberculosis* to foil phagosome maturation and enable the host to control bacterial replication.

TNF is produced mainly by activated monocytes/macrophages in response to pathogens, but also expressed by several other cells including T cells, B cells, NK cells. We believe that neutralization of TNF as a result of increased shedding of sTNFR in the lungs of BALB/c mice may partly explain their inability to control pulmonary mycobacterial infection, compared to C57BL/6 mice. The fact that patients receiving anti-TNF therapy for chronic inflammatory diseases have increased risk of developing TB [346] is compelling evidence that TNF is critical in the control of TB. Indeed, it has been demonstrated that for an optimal IL-12-driven Th1 development, BALB/c, but not C57BL/6 mice require TNF as an additional co-factor [347].

In order to investigate the possible sources of pro-inflammatory cytokines, including IFN-γ during the early phase of infection (week 1-2), especially in C57BL/6 mice, we analyzed the cellular subset distribution in the lung, spleen and LN in the two mouse strains in their naive state, and also after infection. Whereas infection resulted in a global increase in the proportion of all cells analyzed, there was particularly a significant increase in $CD11b^+$ cells (monocytes/macrophages) in the lungs of C57BL/6 as compared to BALB/c mice at day 3

postinfection. We speculate that macrophages could be a possible source of IFN-γ production during the early phase of infection. Indeed, mycobacteria-infected macrophages have been shown to be capable of producing IFN-γ, which further regulated the production of TNF and IL-12 [348]. Although IFN-γ produced by NK cells has been shown to play a protective role during the early phase of mycobacterial infection, in this study, it is not clear whether NK cells contributed to the early IFN-γ production in C57BL/6, as both mouse strains displayed similar percentages in the lungs. Perhaps, independent of the proportions displayed, NK cells in C57BL/6 mice have an increased tendency to produce IFN-γ upon activation with mycobacteria.

Given the ability of IFN-γ to stimulate antimycobacterial activity in murine macrophages, we analyzed the capacity of IFN-γ-activated BMM from the two mouse strains to kill intracellular BCG. Unstimulated macrophages from the two strains displayed similar abilities to phagocytose mycobacteria or produce soluble mediators like TNF, IL-12 or NO. However, even if IFN-γ stimulation of infected BMM in both mouse strains resulted in induction of antimycobacterial activity, BALB/c mice had a somewhat reduced capacity to kill ingested bacteria. With regard to IFN-γ activation, a possible explanation for the increased capacity of C57BL/6 mice to kill bacteria as compared to BALB/c mice could be differences in the level of activation during mycobacterial infection, which could affect their responsiveness to IFN-γ. At this point, it is noteworthy to mention that IFN-γ, although important, is not the sole correlate of protection in TB [171, 330].

BALB/c mice have been described as Th2 biased, and C57BL/6 as Th1 biased, and susceptibility to intracellular pathogens has been ascribed to these Th1/Th2 differences. However, it has been demonstrated that the capacity to produce Th1 cytokines, especially IFN-γ, rather than the presence of IL-4, determines the degree of resistance or susceptibility to intracellular pathogens, such as *L. donovani* [349]. In this study, the susceptibility of BALB/c and C57BL/6 mice to the course of infection correlated with the potency of T cells to produce IFN-γ. C57BL/6 mice, developed a marked IFN-γ response within the first 30 days of infection, and rapidly recovered from the infection after the acute stage. Furthermore, it has been shown in experimental leishmaniasis that development of Th2 responses is dependent on the infection dose; low parasite doses induced a Th2 response in C57BL/6 mice, whereas high doses induced a Th1 response [350].

At this point, it is noteworthy to mention that naive BALB/c and C57BL/6 mice have been shown to differentially express certain TLRs. DCs isolated from the spleen of C57BL/6 mice preferentially expressed TLR9 mRNA, whereas DCs from BALB/c mice strongly expressed TLR2, -4, -5, and -6 mRNA [351]. Consequently, C57BL/6 DCs produced a higher level of IL-12p40 in response to ligands for TLR2 (lipoprotein), and TLR9 (CpG), whereas BALB/c DCs responded to these ligands by producing higher amounts of monocyte chemoattractant protein 1. It is plausible that differences in reactivities of DCs to microbial molecules through TLRs may be associated with susceptibility or resistance to microbial infections in BALB/c and C57BL/6 mice.

The fact that both BALB/c and C57BL/6 mice successfully controlled infection at week 9 is consistent with the notion that protective immunity to mycobacterial infections is mediated by the adaptive Th1 immune responses. In addition to the factors discussed, other factors are known to contribute to antimycobacterial immunity. For example, in humans, vitamin D-mediated induction of antimicrobial peptides appears to be an important player in combating mycobacterial infections [352]. Moreover, autophagy has been demonstrated as an important innate immune defense mechanism, at least in mice [68]. In this regard, lysosomal hydrolyzed ubiquitin peptides displayed a direct antimicrobial activity against mycobacteria after delivery to phagosomes harbouring mycobacteria in an autophagy dependent manner [69]. Further understanding of these defense mechanisms will help the development of new interventional strategies to prevent and treat the disease.

CONCLUDING REMARKS

The BCG vaccine has been in existence for eight decades, and currently, a vast majority of the world's population has been vaccinated with BCG. Despite this, TB remains the second leading cause of death by an infectious disease worldwide, and is also the major complication in HIV infections**.** Whether or not exposure to *M. tuberculosis* infection will result in disease development is dependent on the outcome of the host-pathogen interactions, which generates a complex immune response locally in the lungs as well as the periphery. We have shown that in TB, analysis of multiple immunological markers such IL-12, IFN-γ, sTNFR and antibodies especially IgA in the respiratory tract may be useful in distinguishing active from latent infection or immunization. Furthermore, we have demonstrated that TLR signalling of macrophages by mycobacteria is an important evasive mechanism used for survival in the host. In addition, we have shown that the host genetic background may influence the nature of immune response generated during the early stages of mycobacterial infection and impact on the successful control of infection.

FUTURE PERSPECTIVES

Our study (paper II) showed a strong correlation between sTNFR levels in the lung microenvironment, and bacterial burden in the lungs. Similarly, there was an association between IgA levels in the respiratory tract and infection status. It will be of clinical importance to analyze in more detail these immunological markers in humans with active or latent TB. Assessment of immunological parameters in biological fluids can be accomplished within days of sample collection, and may be particularly useful in settings where the activity of short-term administration of new drugs (early bactericidal activity studies) is being tested. The obtention of BAL through bronchoscopy might be invasive, and therefore difficult to apply serially during treatment of TB. The use of sputum or saliva will be a more practical choice. Indeed, evidence from other pulmonary conditions, such as asthma and chronic obstructive pulmonary disease, indicates that sputum may provide a ready alternative to BAL for serially evaluating factors involved in lung immunity. Identification of biomarkers in saliva that correlate with pathology or treatment will be the ultimate "magic-bullet" in TB diagnosis and therapy.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Immunology, Stockholm University. I would like to express my sincere gratitude to all who have contributed in any and many ways in making this work possible. In particular, I want to acknowledge:

Carmen Fernández, my supervisor, for accepting me as a PhD student in the first instance, for introducing me to the fascinating world of tuberculosis, for all the interesting discussions and the results we achieved together.

Marita Troye-Blomberg, the Head of Department, for your dedication to the training of many a young scientist in immunology, especially, from less privileged countries for which reason this thesis has become a reality.

The “seniors” at the department; **Klavs Berzins**, **Eva Severinson** and **Eva Sverremark-Ekström**. Thank you for sharing your vast knowledge in immunology, and for being pleasant to everybody.

The late **Peter Perlmann,** and **Hedvig Perlmann** for their dedication to research, and immense contributions to the advancement of immunology.

My co-authors **Esther Julian**, **Mahavir Singh** and **Gudrun Horner,** for the excellent discussions.

All colleagues at the department; especially my co-author and “lab-mate” **Muhammad J Rahman**, for being such a nice person to work with, and for all the numerous discussions we had about research and family. **Halima Balogun,** for being the first to show me around Stockholm, I remember the “Bakarby” days. **Shanie Saghafian-Hedengren,** for your positive energy and encouragement when the going got tough. **Nancy Awah,** for all the interesting discussions we had whenever we met on the bus. **Yvonne Sundström,** for being the coolest C-course partner and for the mutual encouragement we gave each other. **Jacqueline Calla,** for being such a pleasant person, for the gift you brought from Bolivia for my kids.

Nora Bachmayer, Olga Flores, Salah Eldin Farouk, Pablo Giusti, Nnaemeka Iriemenam, Lisa Israelsson, Maria Johansson, Valentina Mangano, Amre Nasr, Irene Román Dégano, Ylva Sjögren, Ebba Sohlberg, Luis Fernando Tordoya, Stefania Varani, Bakary Maiga, Judith Anchang and **Charles Arama** for providing the friendship and right atmosphere needed for research.

Former students at the department; **Khosro Masjedi, Manijeh Vafa Homann, Petra Amoudruz, Anna Tjärnlund, Camilla Rydström, Jacob T Minang, Qazi Khaleda Rahman, Ariane Rodríguez, Magdi M. Ali, Nina-Maria Vasconcelos, Anna-Karin Sigfrinius (Larsson) and Ahmed Bolad.**

Margareta Hagstedt, affectionately called **"Maggan"** and **Ann Sjolund,** for being such nice personalities, and for all your help during these years.

Gelana Yadeta, for making the department run smoothly and for all your assistance when it comes to "paperwork". **Anna-Leena Jarva**, for being such a pleasant person, for lending a helping hand in all administrative matters, and for organizing some of the best social events at the department. **Elizabeth Bergner**, for your assistance and professional advice on every issue.

The staff at the animal house, **Eva Nygren** and **Solveig Sundberg**, for your ever readiness to help, and for taking such good care of my mice.

To all the wonderful friends in Sweden, who have been like family to me, especially:

John and **Lydia Owusu-Ansah**, for your friendship and encouragement. My daughters found a sister in your "lil" daughter **Ewura Esi**. Many thanks to **Patience Akwetteh**, affectionately called "Auntie Pat" for playing the role of a big auntie, and for all the delicious food you made for me and my family. **Amilcar Reis** for being such a nice personality, and for all the interesting stuff we talked about. **Ruby, Gertrude, Wendy, Joseph, Mavis, Dominic, Fiifi, Saliu, Ali** and **Rashid** for your friendship and warmth.

William and **Beatrice van der puije** and “lil” **Nathan,** I cherish our friendship that dates back to the “stone age”. Thanks for everything**.** To **Roselyn Mills**, I say many thanks for being such a sweet friend.

My appreciation to **Ben** and **Vida Gyan**, your two boys **Christodea** and **Josiah** for everything you have done for me. To the rest of the people at the department of Immunology, Noguchi in Ghana: **BD Akanmori, D Doodoo, J Tetteh, M Ofori, C Danquah, Gerald** and **Eric** for all your support.

Many thanks to my family; **Rose, Stephen, Emmanuel, Paul, Esther, Samuel, Robert, Agnes** and **Prince** for all your support, goodwill and prayers. I cannot thank you enough. To my late father **John** snr, and my dear mother **Grace**, affectionately called “Auntie” for your love and sacrifice for me, I can never repay you for all the things you have done for me.

Finally, to **Tilly**, our princesses **Kim** and **Karen**, and “lil” master **Kevin**, I say a big thank you for being the best company I could ever ask for. I am grateful for providing a shoulder to lean on during these years, and for believing in me. Without your support and encouragement, life would have been difficult.

These studies were financially supported by **Hjärt-och Lungfonden**, the **Swedish Institute** and the **European Commission program QLK2-CT-2002-2006**.

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