Innate and adaptive host responses and their genetic control in tuberculosis : studies in Indonesia, a highly TB endemic setting

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STOP TB before it's too late
Free! Treatment in PUSKESMAS
Photo of billboard in front of Dinas Kesehatan Jakarta Timur

1 Introduction

Tuberculosis (TB) is a chronic infectious disease that is caused by a single pathogen *Mycobacterium tuberculosis* (MTB). TB is known as one of the oldest diseases in human history as established by paleomicrobiologists.[1] For example, ancient Egyptian doctors already described a chronic tuberculous node in the neck, and various archaeological art findings and human remains showed spinal TB, known also as Pott's disease.[2] Though many old manuscripts described features of mycobacterial infection in humans, it was not until 1882 that Robert Koch identified its causative bacterial pathogen, MTB.

I. *Mycobacterium tuberculosis* infection and disease: an overview

Based on tuberculin skin test (TST) surveys, MTB is estimated to have infected one-third of the global population, accounting for 8 million new cases and 2 million deaths per year. TB is mostly concentrated in developing countries,[3] and is the second most common cause of death due to an infectious disease after acquired immunodeficiency syndrome (AIDS) by human immunodeficiency virus (HIV). TB is a growing international health concern. In particular, the increasing prevalence of multi-drug resistant (MDR) and even extremely drug resistant MTB strains combined with co-infection with HIV have greatly contributed to the increasing difficulties in the control of TB. TB is not only a problem in developing but also in developed countries, affecting immunocompromised patients (AIDS, transplantation, immunosuppressive therapy such as anti-TNF treatment). Moreover, global migration is raising the likelihood of MTB transmission from endemic to non-endemic areas.[4,5]

The outcome of the battle between MTB and the human host is determined by complex host-, environmental- and pathogen-factors, and clearly is a multi-factorial disease. Much of the considerable variation in outcome to exposure and infection with MTB,[6] and also in TB disease severity can probably be attributed to variations in the interplay between pathogen, host and environment (**Figure 1**). When the interactions shift the balance in favor of MTB, TB disease will develop. On the other hand, when the balance is in favor of the host, MTB can be killed or contained by the host immune system. The coordinated response of the innate and adaptive immune systems is required for an effective host defense.

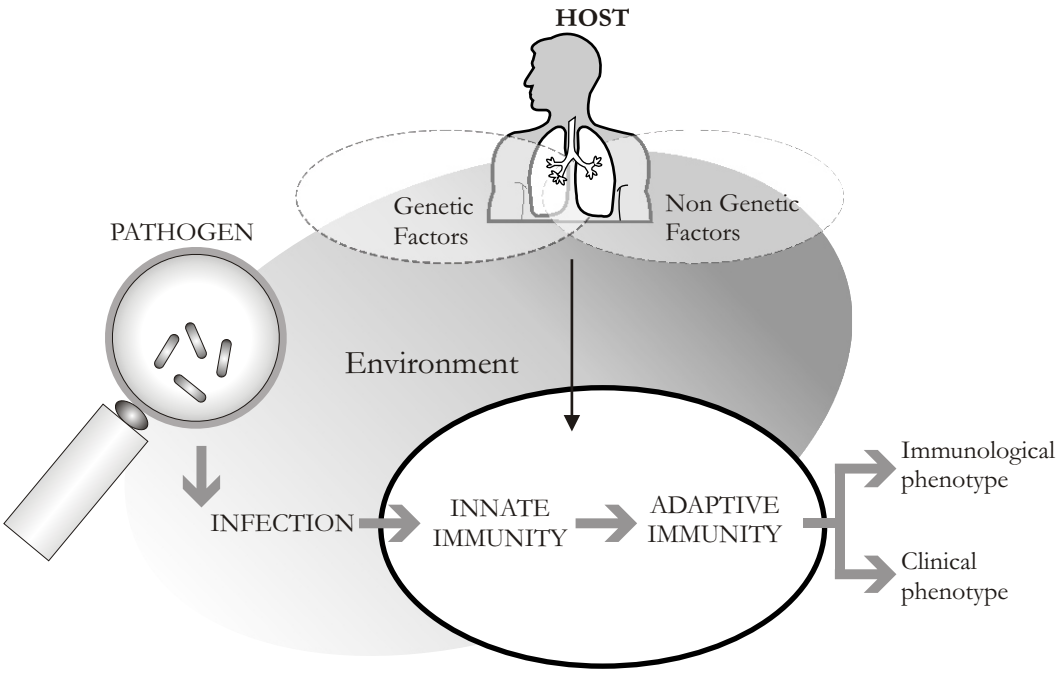


Figure 1. The interaction of host factors, pathogen factors and environmental factors play a key role in outcome of *M. tuberculosis* infection. Whether infection becomes symptomatic depends on innate immunity, with or without involvement of adaptive immunity.

Mycobacterium tuberculosis transmission

MTB is transmitted solely by direct human-to-human spread through the air via inhalation.[7] The bacilli can remain in the air for prolonged periods of time. Dried bacteria may even survive for days to months if protected from sunlight.[8] The bacteria can be inhaled by any bystander individuals (young and old) when active TB patients cough, sneeze or speak, especially in the close or intimate environment of the patients; e.g. family members and close contact at home, work or school (**Figure 2A**). Intensity of exposure (the load/concentration of the mycobacteria inhaled) is related to the infectivity of the case. A study in The Gambia, West Africa showed that TB infection in children was directly related to the intensity of exposure of the child to the individual with infectious TB, mostly in the form of parent-to child or vertical transmission.[9] Infection in infants and young children up to 5 years may thus

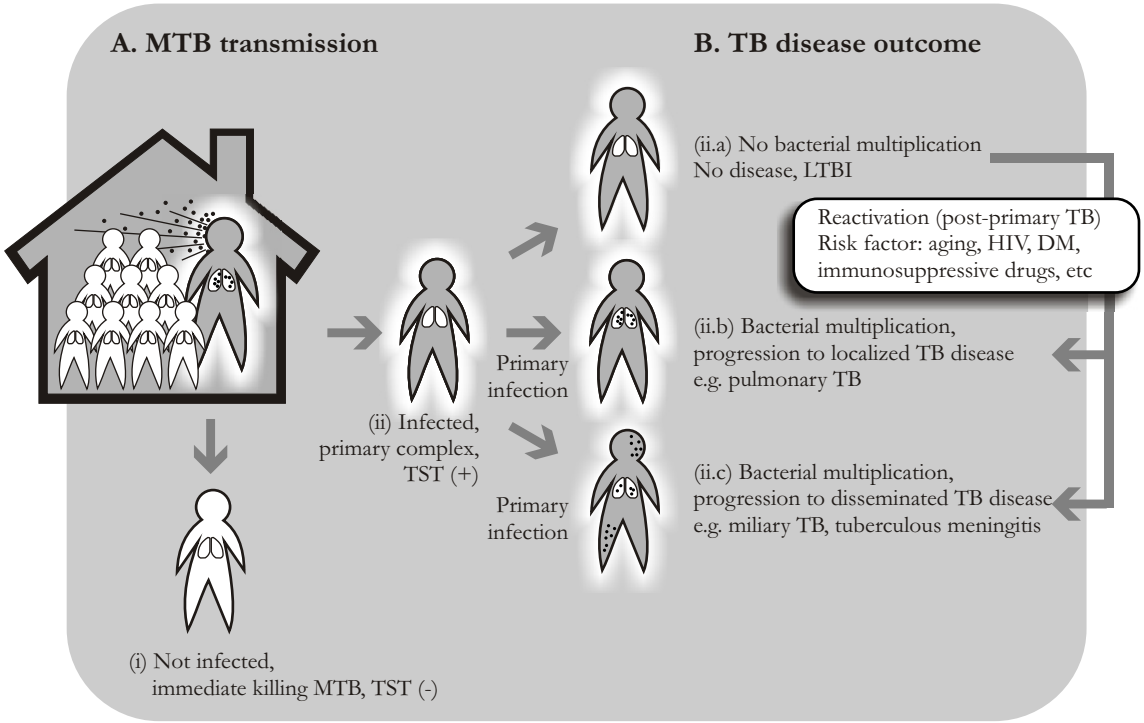


Figure 2. (A) *M. tuberculosis* **transmission.** MTB carried by aerosols from an infected individual is transmitted to bystander individuals resulting in various outcomes. **(B) TB disease outcome. (i)** A subset of exposed individuals does not seem to become infected, or at least does not develop adaptive immunity, as the tuberculin skin test (TST) is negative. This may be due to lack of infection or adequate control of infection by the innate response. **(ii)** In the infected group with TST reactivity **(ii.a)** in the vast majority of individuals, no disease will develop since the immune system contains or controls the bacteria effectively, leading to latent TB infection (LTBI). LTBI can be maintained for a lifetime with no clinical symptoms or alternatively disease can reactivate (post-primary TB infection) which can be triggered by several factors. In a small percentage of the infected people bacterial multiplication will progress to active TB (primary infection) within 1-2 years. The final outcome of clinical TB disease can be either **(ii.b)** localized e.g. pulmonary TB or **(ii.c)** disseminated e.g. miliary TB, meningitis TB, both in the form of severe of less severe TB.

indicate recent transmission. Infected children represent a large proportion of the pool from which TB cases will arise. The World Health Organization (WHO) reported that 75 % of TB cases are adults in their most productive life years.[10] Recent studies in three countries in West Africa showed that the environmental risk factors for TB are associated with single marital status, crowding, and living in a rented house.[11,12] As TB transmission occurs in a closed environment, reduction of crowding and improvements in housing with better ventilation are likely to have an essential impact.[13] Furthermore, virulence of the MTB is also associated with the development of TB. Recent epidemiological data suggest that differences in transmission and virulence among MTB strains are related to the genetic background of the organisms.[14,15] For instance, MTB of the Beijing genotype which is highly prevalent in Asia and the former USSR is considered to be more virulent and elicits a non-protective immune response. In contrast, *M. canettii* is associated with a more favorable course while other genotypes caused intermediate clinical and pathological effects.[16] A better understanding of differences in virulence between MTB genotypes could have implications for TB control once established more precisely.

Transmission of MTB can be limited by proper TB control strategies, including active TB case detection (the so-called contact tracing), supervised treatment of sputum smear-positive cases and *M.bovis* Bacillus Calmette-Guérin (BCG) vaccination for neonates. BCG, discovered in 1921 by Albert Calmette and Camille Guérin, is the only vaccine currently available against TB (*see below*). It is, however, difficult to control TB due to difficulties in TB diagnosis, (extreme) multi-drug resistance, lack of treatment compliance, resource-poverty and HIV co-infection. Knowledge of the factors that influence progression of TB infection to disease will obviously be important to identify susceptible individuals and understand transmission patterns in the community.

TB disease outcome

After being exposed to MTB, there are several ways in which the host can respond to infection (**Figure 2B**). A spectrum of possible clinical manifestations can occur at any stage of life in MTB infected individuals. Of the infected individuals only 5 to 10%, a relatively small subset, may develop active TB within one or two years after infection (primary TB).[17] When active TB develops, disease presentation (disease localization and -severity) can be quite variable. Because the vast majority of infected individuals will not develop disease, the host immune system apparently is able to contain or even

eradicate the pathogen. However, at least a significant proportion continues to harbor MTB and develops latent TB infection (LTBI). Later during their lifetime, these latently infected individuals will have a risk of reactivating their latent infection, resulting in the development of clinical active TB (post-primary TB). The estimated lifetime risk of reactivation for a 25-year old with LTBI has been estimated to be around 7%, at least in certain populations.[18] Several risk factors for TB have been reported, either affecting the risk of infection or the risk of developing disease after infection, including socio-economic factors (e.g. crowding, malnutrition), behavioral factors (e.g. smoking, alcohol abuse), age, gender, and history of contact with infectious TB.[11] Concomitant diseases and infections such as diabetes mellitus (DM), HIV, or rheumatoid arthritis and Crohn's disease patients who are treated with an immunosuppressive drug anti-TNF antibodies, have a significantly increased risk of reactivation of LTBI and developing disseminated TB disease,[19] showing that there obviously is a constant battle between MTB and the latently infected individual's host immune system.[20] Clearly, breakdown of the immune response through various factors can thus result in reactivation and replication of the mycobacteria.[21] Control of risk factors which can trigger reactivation of LTBI may be an important TB control strategy. As there is an enormous reservoir of individuals with LTBI that are at risk for developing active TB, and therefore for potentially infecting other individuals, diagnosis and treatment have been recommended not only for active TB but also for LTBI.[22]

Since the first encounter between MTB and man usually happens within the airways, the disease occurs mainly in the lungs, known as pulmonary TB. The microorganisms can, however, seed any organ via hematogenous spread, resulting in extrapulmonary TB either in a localized form (e.g. lymph nodes in the neck) or disseminated (e.g. miliary TB), both presenting with various severities (mild, moderate or severe forms). The most severe form of extrapulmonary TB is TB meningitis that can lead to death. The whole spectrum of clinical presentation ranging from subclinical to rapidly fatal can occur, and may reflect the balance between the bacilli and host defense mechanisms. According to the WHO definition, TB cases are patients which have been bacteriologically confirmed, or have been diagnosed by a clinician. The definite TB cases are patients with positive culture for MTB. In countries where mycobacterial culture is not routinely available patients with two sputum smears positive are also considered as definite TB cases. In case of pulmonary TB, the WHO has set three major criteria to diagnose active pulmonary TB; e.g. clinical appearance, chest X-ray signs and detection of the pathogen.

Clinical appearance

Individuals with MTB infection are in most cases asymptomatic and noninfectious. In active pulmonary TB, patients may present in the clinic with a wide clinical appearance spectrum. The pathognomic symptoms such as coughing and night sweating are the most common complaints reported by TB patients; coughing with blood (hemopthysis), and shortness of breath (dyspnea) are also reported. Systemic manifestations include wasting or weight loss (phtisis), fever, loss of appetite, and fatigue. Nonspecific hematological changes e.g. anemia, increase in the peripheral blood leukocyte count (leukocytosis), and low albumin are also common.[23] The presence of an inflammatory process can be monitored by the increase of erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) level as these are markers for an acute phase response of inflammation and tissue injury.[24] In lymphocytic pleural effusions, CRP can be used as a tool for diagnosis of TB pleuritis.[25] TB with other concomitant diseases such as HIV-infection, DM or malignancy may present with different clinical presentations. For example, HIV-positive TB patients develop a negative TST and cutaneous anergy indicating a strong association with immune dysfunction.[26] Patients with AIDS and TB are more likely to develop multifocal extrapulmonary disease and atypical chest X-ray radiography (CXR).[27] TB patients with concomitant DM develop more cavitation nodules,[28] and tend to have higher body mass index (BMI) compared with TB patients without concomitant DM. Of clinical importance, the prominent weight loss (wasting) in TB disease can be masked by the fact that concomitant DM leads to higher BMI compared with healthy individuals.[220]

Chest X-ray examination

The airway is the usual port d'entrée of MTB in humans. Lung tissue destruction such as lung infiltrates with or without cavities can be observed by CXR and is indicative of active TB. The severity of pulmonary TB can be classified based on the CXR using the criteria from The National (USA) Tuberculosis and Respiratory Disease Association.[29] Briefly, minimal lung disease or *mild-TB* is defined when CXR shows infiltrates of slight to moderate density, present in a small portion of both lungs with a total volume of infiltrate(s) of one lung, and no cavitations present. *Moderate-TB* is defined as scattered lesions of slight to moderate density present in a total volume of infiltrate(s) of one lung and /or dense, confluent infiltrates present in one third of the volume of one lung, with or without cavities not greater than 4 cm. *Advanced -TB* is defined as lesions more extensive than moderate TB.

Granuloma formation is the pathological hallmark of the host response to MTB, characterized by an accumulation of infected phagocytes surrounded by activated monocytes, macrophages and lymphocytes[30] (*see below*). When the infection is successfully contained, the granuloma shrinks and may eventually calcify. This calcification can be observed in CXR of individuals who did not develop active TB in the past indicating a successful containment of MTB. On the other hand, destroyed macrophages and lymphocytes release large amounts of proteolytic enzymes (liquefaction process) which form a place where MTB can grow extracellularly. Cavitation is a process in which liquefied granulomas breach the mucosal surface by tissue destruction, resulting in MTB spread to other regions of the lung or to the environment by coughing.[31] CXR is, thus, supportive for TB diagnosis, especially in a poor area where microscopy or culture of MTB is lacking.

Detection of MTB

The gold standard for diagnosis of course is the pathogen detection. It is, for diagnosis of TB, essential to obtain two consecutive sputa positive for acid-fast bacilli. The bacilli are easy to detect under the microscope, however, it is difficult to obtain sputum from TB patients. Better patient instruction may help to improve the volume and the quality of sputum samples which may increase the yield of sputum microcopy, and thus improve TB diagnosis.[32] The culture of MTB, which takes 4 to 6 weeks, gives a definite diagnosis. In poor areas where culture of MTB is difficult or not routinely available, a patient with two sputum smears positive for the bacilli is also considered a definite case. There are some other techniques to detect MTB such as polymerase chain reaction (PCR) based assays.[15] This technique cannot, however, give any information on whether or not TB is in an active phase, because DNA from dead mycobacteria is detected as well.

Childhood TB

TB in children poses diagnostic particular challenges because children often present with vague and aspecific signs and symptoms, and clinical specimens are difficult to obtain, as well as the lack of specific radiographic features at the stage of primary infection.[33] TST is widely used in pediatric practices as evidence of recent infection. History of contact with active TB cases is, therefore, worth noting. In TB-endemic countries the TST is, however, not reliable as this may be false positive due to BCG vaccination or infection with environmental mycobacteria. In these countries neonates are BCG-vaccinated in line with WHO recommendations. Co-infection with helminths in childhood may negatively influence outcome of BCG vaccination.[34]

The role of host genetic in TB susceptibility

The outcome of mycobacterial infection is partly influenced by host genetic factors that control activation of innate and adaptive immune responses. There is growing evidence that a large number of host genes are involved in susceptibility to TB and other infectious diseases.[35] This is not surprising, because only a minority of infected individuals is known to develop TB during their lifetime and many TB patients do not have any obvious underlying risk factors. The first evidence for genetic susceptibility in TB comes from twin studies, showing a higher concordance for TB among monozygotic twins compared to dizygotic twins.[36] Furthermore, several studies showed patients who suffered from severe infections due to otherwise poorly pathogenic mycobacteria and salmonellae. Rare immune deficiencies were identified due to abnormalities of genes that encode the type-1 cytokine network: *IL12B* (encoding IL-12p40), *IL12RB1* (encoding IL-12Rβ1), *IFNGR1* and *IFNGR2* (encoding IFN-γR1 and IFN-γR2 chains, respectively) or *STAT1* (encoding the IFN-γ-associated signal-transducer and activator-of-transcription).[37-41] These unfortunate experiments of nature underline the crucial role of the type-1 cytokine network in optimal host defense against mycobacterial pathogens. Subtle variations in genes involved in host responses have been described in several populations with different results. Polymorphisms, defined as genetic variations occurring in more than 1% of the population, are sufficiently common to contribute to the risk of mycobacterial infection at the population level. Some of these polymorphisms are functional, but for many of these no functional or immunologic changes have been demonstrated yet. The particular polymorphisms may be linked to other, as yet unidentified susceptibility loci, and these associations therefore need further confirmation and investigation. To date, many candidate gene polymorphisms have been identified to be associated with susceptibility to TB in various populations (summarized in **Table 1**).

The impact of environmental as well as tuberculous mycobacterial exposure on the population's immunity may differ significantly, resulting in the molecular genetic variants associated with disease susceptibility or resistance among ethnic groups.[42] The varying results in different racial groups may reflect the differential contribution to genetic susceptibility of different genes or alleles in a polygenic model. This finding highlights the importance of recording accurate data on ethnic origin.[43] Several studies in different populations are needed to dissect the possible subtle variations among populations, e.g. in many populations there is an excess of TB cases in males,

Gene polymorphism	Population		
	Susceptibility	Resistance	No association
HLA class II			
HLA-DRB	Asians: South Indians HLA DRB1*1501 [122] South Americans: Mexicans [213]		
HLA-DQB	Asians: Cambodians DQB*0503,[121] South India DQB1*0601[122]		
HLA-DQP		Asians: South India DPB1*04 [122]	
Cell surface receptors			
TLR2			
GT repeat intron II	Asians: Koreans [79]		
VDR			
Taq1	Asians: female South Indians [145]	Africans: Gambians [106]	Asians: London Gujarati,[146] Cambodians [42]
Fok1	Asians: Han Chinese [214]		Asians: London Gujarati,[146] Cambodians [42]
MBL Exon 1 G54D		Africans: South Africans [215]	
Exon 1 G57D	Africans: Gambians [216]		
P2X7			
-762 T>C		Africans: Gambians [148]	
Phagosome			
NRAMP1			
-236 SNP			Africans: Gambians [106] Asia: Cambodians [42]
5'promotor (CA)n	Africans: Gambians,[106] South Africans [108]	Asians: Japanese [109]	Africans: Guineans [107] Europeans: Danes [113]
D543N	Africans: Gambians [106] Asians: Japanese,[109] Han Chinese [214]	Asians: Cambodians, [42] Japanese [110]	Africans: Guineans,[107] Asians: Taiwanese,[114] **Indonesians** (this thesis) Europeans: Danes [113]
Intron 4 (469+14G>C)	Africans: Gambians,[106] Guineans [107]	Asians: Japanese [110]	Asians: Cambodians [42] Europeans: Danes [113]
3'UTR(1729+55del4)	Africans: Gambians,[106] Asians: Koreans,[111,112] Han Chinese [214]	Asians: Cambodians,[42] Japanese [110]	Asians: Taiwanese,[114] **Indonesians** (this thesis) Europeans: Danes [113]

Cytokines and their receptors:			
IFNG			
Intron 1 (+874A>T)	Africans: South Africans [157] Europeans: Spanish [158]		
IL1B			
-511 SNP			Asians: Indians,[130] Cambodians [42] Africans: Gambians [106]
+3953 SNP	Asians: Indians [130]		
IL1RA			
INT2 (VNTR 86 bp)	Asians: Indians [130]	Africans: Gambians [132]	
IL10			
-1082 SNP	Asians: Cambodians [42]		Africans: Gambians [106]
-819, -592 linked SNPs			Africans: Gambians [106] Asians: Cambodia [42]
IL12p40			
INT2 (ATT)8	Asians: Hongkong Chinese [217]		
IL12RB1			
-2 C>T	Africans: Moroccans [218]		
TNF			
-1030 SNP			Asians: Cambodians [42]
-862 SNP			Asians: Cambodians [42]
-856 SNP			Asians: Cambodians [42]
-375 SNP			Asians: Cambodians [42]
-307 SNP	Asians: Indians [219]		Asians: Cambodians [42]
-243 SNP			Asians: Cambodians [42]
-237 SNP	Asians: Indians [219]		Asians: Cambodians [42]

Table 1.
Reported associations (susceptible, resistance/protection, or no association to TB) of gene polymorphisms with TB in various populations.

suggesting a link between regions of the chromosome X and susceptibility to TB, as shown in a genome-wide linkage study.[44] Certain human leukocyte antigen (HLA) variants have been associated with TB (*see section Antigen Presentation*). HLA polymorphisms may explain the vulnerability of certain populations to TB,[45] as also evidenced in leprosy.[46] Therefore, future studies that define haplotype patterns in different populations and evaluate the effect of haplotypes, as in the case of HLA genes, may be more informative than the study of individual genotypes. The determination of ethnic-specific genetic associations with TB susceptibility may impact on the design of improved strategies to enhance host resistance, for example by vaccination and may also help to identify better correlates of protection and disease.

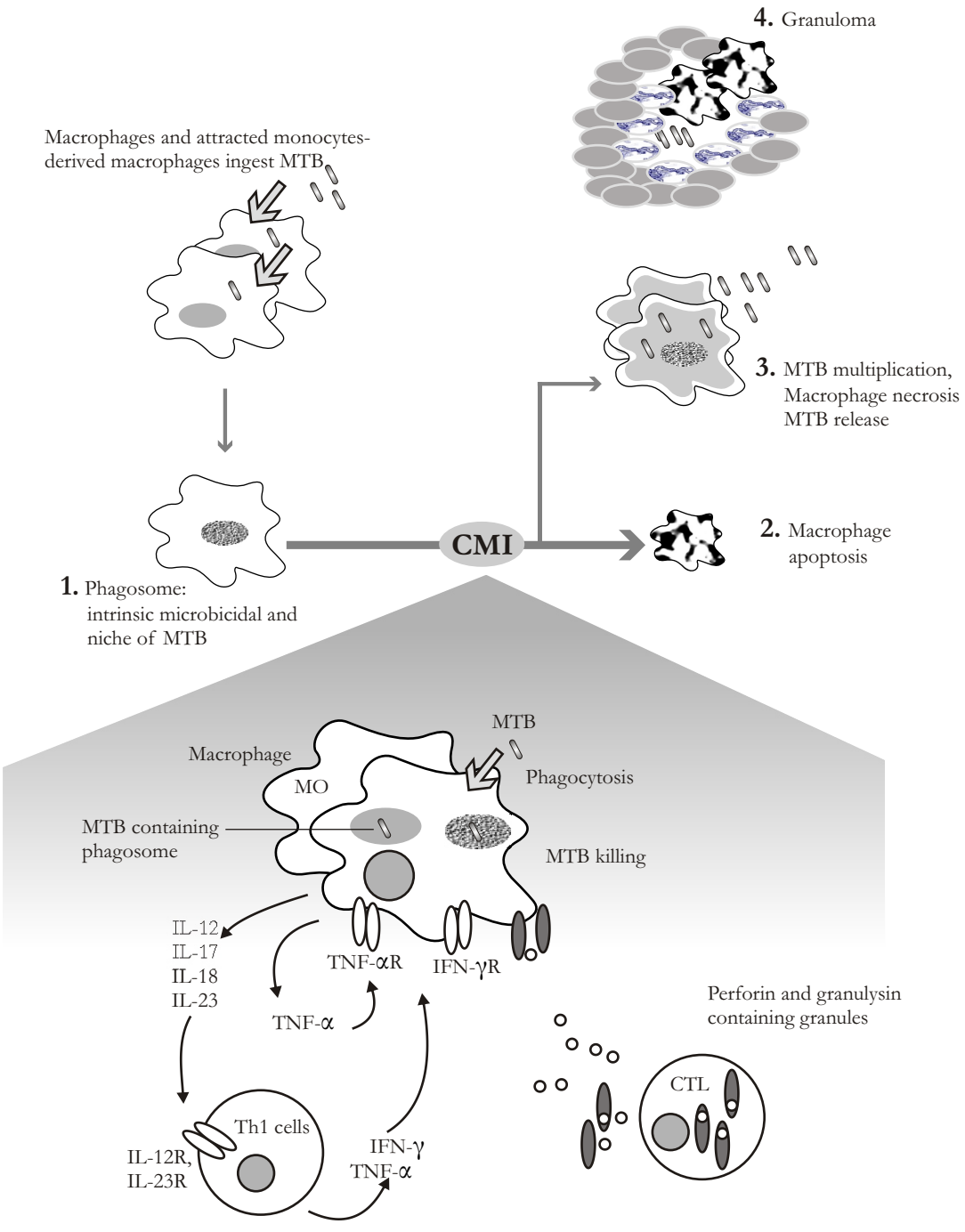
From the pathogen point of view, studies have reported that human pathogens have geographically structured population genetics. Some of the MTB lineages have been linked to ancient human migrations.[47] A recent publication showed a predominance of ancestral MTB genotypes in the Indian subcontinent, which supports the hypothesis that India was one of the ancient endemic foci of TB.[48] It is believed that the exposure in history has shaped resistance to TB in present-day populations.[35] The susceptibility to mycobacterial infection in this model has changed from being the norm for all humans to being confined to certain populations, due to the natural selection of resistance among ancestors who survived the infection during the pre-antibiotic era.[49] Thus, certain populations would have increased susceptibility to such infections while others would be more resistant.[43] Such environmental and natural selective factors may well have resulted in population-specific immunogenetic adaptations to TB[42] as also described in malaria,[50] where ethnic-specific adaptations of common erythrocytic variants and hemoglobinopathies are associated with resistance to plasmodial parasite infections in populations from Africa, Asia, and the Mediterranean.

The identified host genes and mycobacterial genes that are expressed after infection, could subtly influence the interplay between the host immune defenses and the bacterial escape mechanisms.[51] Identifying and characterizing the genetics of both host and pathogen factors involved are a great challenge and will help to understand disease susceptibility and TB resistance.

II. Host immune responses to *Mycobacterium tuberculosis* infection: cross-talk between innate and adaptive immunity

Much of the host defense mechanisms and TB pathogenesis remain unclear. Human host defense mechanisms are considered key elements in the pathogenesis of and protection from mycobacterial infection, including MTB and *M. leprae*, the causative agent of leprosy. Protection is dependent on the cellular immune system (CMI),[52,53] involving activated macrophages, T cells, and type-1 cytokines (**Figure 3**). From the first exposure to MTB in the airways, a series of immune responses are triggered. Briefly, MTB infects and replicates within the phagosome of alveolar resident macrophages, macrophages that differentiate from blood monocytes that are recruited to the site of infection,[54] as well as dendritic cells (DCs).[55] These cells, responsible as the first barrier in the lungs of the host, develop natural defense mechanisms to eliminate the mycobacteria, and, in the case of DC, migrate to draining lymph nodes to prime or boost specific T cells. The intrinsic microbial capacity of phagocytes and the virulence factors of the ingested mycobacteria will determine intracellular mycobacterial survival (**Figure 3.1**). In most cases, mycobacteria can evade intracellular destruction, resulting in mycobacterial multiplication and disruption of the macrophages. Blood monocytes and other inflammatory cells that are attracted to the site of infection, however, could subsequently ingest the extracellular mycobacteria released from the disrupted macrophages.

T cell immunity develops two to three weeks after infection and activates macrophages to eliminate the intracellular mycobacteria.[17] These activated infected-macrophages undergo apoptosis, which may prevent dissemination of infection and reduce viability of intracellular mycobacteria (**Figure 3.2**). Moreover, apoptosis may facilitate DC mediated cross priming of CD8 effector cells. Necrosis of infected cells in contrast, does not and allows MTB release extracellular[56] (**Figure 3.3**). The released bacteria are taken up by activated macrophages within the granuloma (*see below*), and will be contained or destroyed[57] (**Figure 3.4**). The state of the mycobacteria within the granuloma (the so-called tubercle) in latent infection is not known. The latent stage of infection is associated with a few bacteria surviving and perhaps replicating inside the granuloma. This latent stage might also be in a dormant non-replicating state with a low metabolic activity for years and MTB could be contained as long as the individuals remain immunocompetent. The establishment of latent infection coincides with nutrient limitation, low pH, hydrolytic enzymes, reactive nitrogen and oxygen species and reduced oxygen tension.[58]


4. Granuloma
Macrophages and attracted monocytes-derived macrophages ingest MTB
3. MTB multiplication, Macrophage necrosis MTB release
CMI
2. Macrophage apoptosis
1. Phagosome: intrinsic microbicidal and niche of MTB
MTB
Macrophage
MO
Phagocytosis
MTB containing phagosome
MTB killing
IL-12
IL-17
IL-18
IL-23
TNF-αR
IFN-γR
TNF-α
Perforin and granulysin containing granules
CTL
Th1 cells
IL-12R, IL-23R
IFN-γ
TNF-α

Upon triggering of microbial pattern recognition receptors (PRR); e.g. Toll-like receptors (TLR),[52] mannose receptors,[59] C-type lectins like DC-SIGN,[60] and NOD/NACHT receptors,[61] phagocytes including macrophages and dendritic cells[62] are activated to produce an array of cytokines that may act in concert for optimal effector function of macrophages. These cytokines e.g. interleukin (IL)-12, IL-18, and IL-23 are recognized by complementary receptors (IL-12R, IL-18R, and IL-23R respectively) on type-1 helper T (Th1) cells and natural killer (NK) cells. The major pro-inflammatory cytokine, IL-12, links innate and adaptive immunity by driving the development of T cells and NK cells to produce Th1 pro-inflammatory cytokines, including IFN-γ and TNF, and regulates IL-17 production.[53,63,64] IFN-γ is the key activating cytokine and induces, in synergy with TNF, e.g. inducible isoform of nitric oxide synthetase (iNOS) expression in macrophages as the major antimycobacterial mechanism, at least in the mouse model. Thus, the elimination of MTB mainly depends on the success of the interaction between infected macrophages and T cells.

While studies in TB clearly showed that CMI plays an essential role in the control of infection, the humoral immune response in contrast is considered not to be associated with protection to TB. However, Th1 cells induces B cells to release antibodies of the immunoglobulin (Ig) G2 isotype, responsible for phagocyte activation and antibody-dependent cellular cytotoxicity.[65] A strong antibody response is generated in the infected host, and B cells or antibodies may nevertheless assist in the control of infection although clearly playing only an accessory role. The humoral immune response facilitates immunodiagnosis of active TB.[66] Several components of MTB are known targets for B cells, e.g. crude cell sonicates, culture filtrate, purified protein antigens, and cell wall lipids.[67] The lipoprotein 38 kD, the most frequently studied serologically recognized antigen, is a component in different commercial TB serological tests. The practical use of serodiagnosis

Figure 3. MTB killing mechanisms of host macrophages. After being ingested by macrophages (MO) and attracted monocytes-derived macrophages, MTB resides in the preferred niche in phagosome, a hostile environment with intrinsic antimicrobicidal capacity (**1**). Cell-mediated immunity (CMI) will be triggered in attempt to eliminate MTB. The final clinical outcome will be determined by the balance in the host factors which are orchestrated by CMI, consisting of activated macro-phages, T cells and Th1 cytokines. Activated macrophages induce the production of pro- and anti-inflammatory cytokines. T cells migrate to the site of infection and interact with macro-phages, resulting in various effector functions leading to (**2**) apoptosis, a mechanism of MTB killing or (**3**) necrosis with MTB spread. The released MTB will be taken up in granuloma (**4**).

for active TB has, however, not been widely appreciated, partly because the reliability of current tests using protein antigens is not very satisfactory. In a recent report by Azzurri *et al.*, IgG antibody levels in plasma against ESAT-6, LAM and 38kDa Ag were higher in untreated patients than in community controls, representing serological correlates of active disease. These serological markers might be predictive in treatment outcome.[68] However, these levels may vary greatly depending on the stage of disease and depending on the structure of the antigens. Combinations of multiple selected antigens might give a higher sensitivity and specificity for screening strategy.[69]

Mycobacteria induce regulatory cells and target multiple important signaling pathways to promote infection and survival. At many stages in the host response, MTB has developed mechanisms to circumvent or antagonize protective immunity: e.g. its ability to arrest phagolysosome biogenesis, avoiding direct microbicidal mechanisms in macrophages, and blocking efficient antigen processing and presentation. Below, the host innate and adaptive response will be described in more detail together with the possible variations in host genetic factors.

1. Innate immunity

A. MTB recognition and uptake by macrophages

(i) MTB recognition leading to immune activation

The outer surface of MTB contains a number of molecules, termed pathogen-associated molecular pattern molecules (PAMP), that can interact with host cells through ligation to pattern recognition receptors (PRR).[70] The recognition of MTB and/or MTB components by receptors on the surface of macrophages is an essential initial step in phagocytosis and activating the anti-mycobacterial innate immune response PRR include various members of the toll-like receptor (TLR) family,[71,72] cytosolic nucleotide-binding oligomerization domain 2 (NOD2),[61] dendritic cells specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN),[60] and mannose receptors (MR).[59] TLR and NOD2 pathways are nonredundant MTB recognition mechanisms that synergize for the induction of pro-inflammatory cytokines.[61] TLRs are transmembrane proteins with leucine-rich repeats in the extracellular domain, that play important roles in early innate immune

extracellular domain, that play important roles in early innate immune recognition and inflammatory responses.[73] The cytoplasmic portion of TLRs shows high similarity to the IL-1 receptor family, referred as Toll/IL-1 receptor (TIR) domain, and TLR signaling pathways are finely regulated by TIR domain-containing adaptors, such as MyD88, TIRAP/Mal, TRIF and GRAM. Of interest, MyD88 is homologous to the signaling domain of IL-1 receptor and links to IRAK (IL-1R associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines. MyD88 has been reported to be essential for macrophage activation,[74] thus play a role in innate immune signaling.

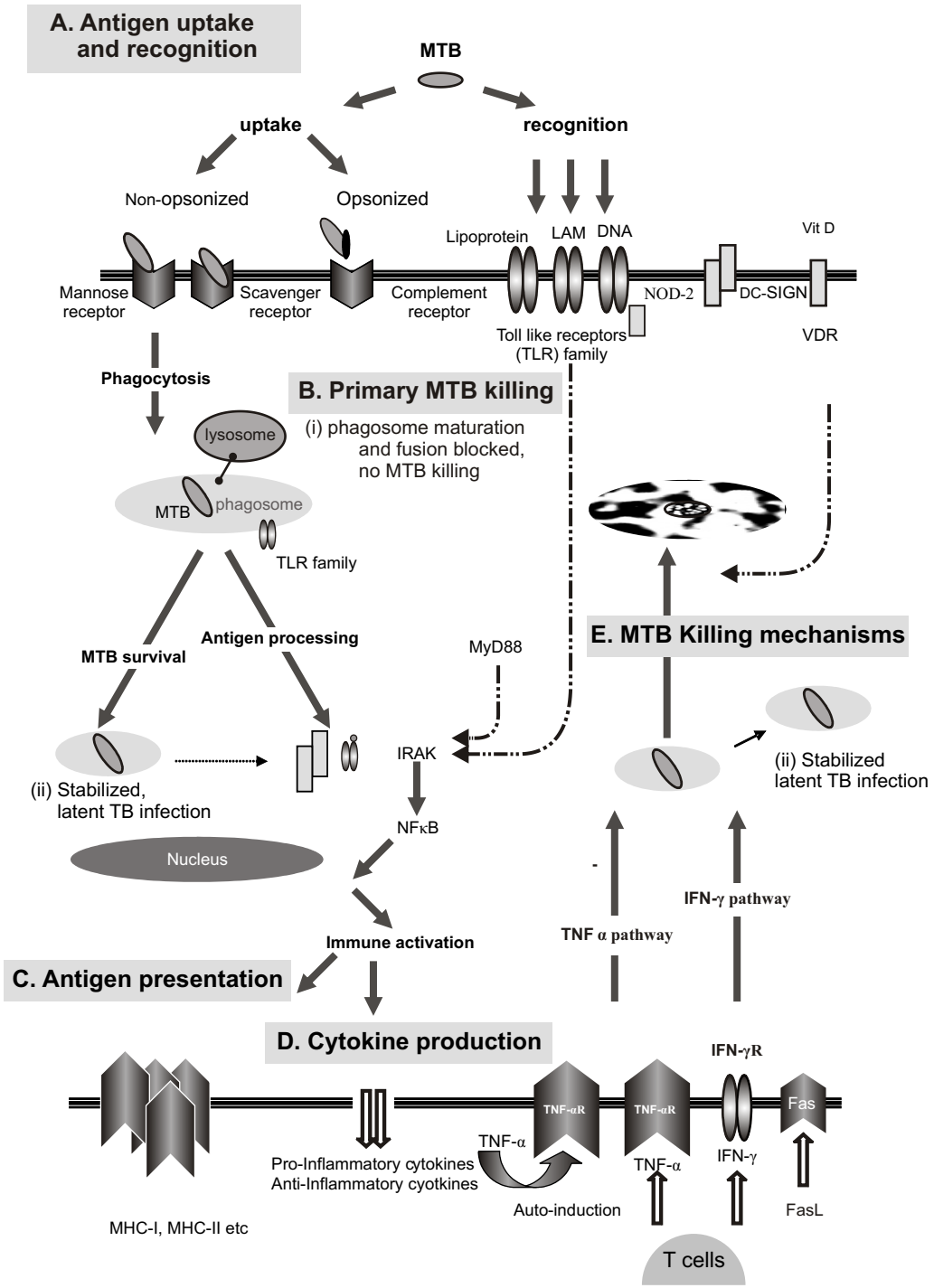
TLR2, in combination with TLR1 or TLR6 as heterodimers, seems to be responsible for TLR2-dependent cellular activation upon recognition of a variety of microbial components, including lipoarabinomannan from mycobacteria.[75] TLR2 ligands induce moderate amounts of TNF and IL-1β and no production of IL-12p40 or IFN-γ inducible protein 10 (IP10),[76] meaning a bias toward Th2 or T regulatory responses. Prolonged exposure to MTB 19-kDa lipoprotein and cell wall peptidoglycan inhibits IFN-γ-induced regulation of genes (e.g., CD64), via both TLR/MyD88-dependent and TLR/MyD88 independent mechanisms.[77] TLR2 deficient mice are highly susceptible to MTB infection, suggesting that mutations affecting TLR2 expression may impair host response to MTB.[74,78] Subtle variations which result in reduction of TLR2 expression make humans more prone to development of TB as reported in several study populations. For instance, genotypes with shorter GT repeat in intron two (INT2) were more common among TB patients in Korea (**Table 1**), and this is associated with weaker promoter activities and lower TLR2 expression on $CD14^{+}$.[79] It may be anticipated that genetic polymorphisms, or perhaps mutations, in the relevant TLR or the downstream signaling proteins will affect the performance of the innate host response to mycobacteria.

TLR4 binds endotoxin of gram-negative bacteria and immune recognition of the major mycobacterial cell wall component, lipoarabinomannan (LAM), and appears to resemble that of gram-negative bacterial lipopolysaccaride (LPS).[80] Stimulation of TLR4 leads to release of large amounts of TNF, IL-1β, IL-12p40 and IP10, thus inducing potent Th1 responses. Some other TLRs are tought to be localized intracellularly, expressed in the endoplasmic reticulum and phagolysosomes, which means that the ligands need to be internalized and transported to the endosome before signaling is initiated,[81] e.g. TLR8 with antiviral activity and single-stranded RNA,[82] and TLR9 that recognizes bacterial and viral DNA sequences that contain unmethylated CpG motifs.[83]

NOD2 recognizes peptidoglycans in the cell wall of MTBs.[61] Similar to TLR, NOD2 has an intracellular domain containing leucin-rich repeats, that activates the NF-κB signaling pathway to induce inflammatory response including production of pro-inflammatory cytokines. Polymorphisms in the NOD2 gene have been reported to be associated with Crohn disease, however, these variants are not associated with susceptibility to pulmonary TB in the Gambians.[84]

DC-SIGN, receptor in the C-type-lectin family encoded by the CD209 gene and expressed by myeloid DCs and macrophages, recognizes a large range of pathogens, including MTB. Increased DC-SIGN expression levels may result in better capture and processing of mycobacterial antigens, leading to a stronger and wider T cell response, or alternatively, to their regulation, since MTB capture through DC-SIGN may promote production of IL-10.[85] The CD209 gene is highly polymorphic. Variation in this gene thus might have a broad range of effects on the pathogenesis of a number of infectious diseases, including TB. A study in a South African colored population showed that the -336A allele is associated with TB protection. This polymorphism has been shown to affect an Sp1-like binding site and to modulate transcriptional activity *in vitro* by increasing levels of DC-SIGN expression. In contrast, however, a study in a Colombian population failed to replicate this association.[86] The cohort in this study was however much smaller than the study in South Africa, which may attribute to the inability to detect an association.

Figure 4. Host responses in macrophages. (A) Antigen uptake and recognition by several pattern recognition receptors. **(B)** Primary killing mechanisms in phagosome. MTB is contained in early endosomal stage, resulting in stabilized latent TB infection. Cell activation by processed MTB/antigen, resulting in **(C)** presentation of antigen at the surface of the cells and **(D)** cytokine production **(E)** MTB killing mechanisms can be directed via TNF pathways (auto-induction and adaptive immunity) or IFN-γ pathways (adaptive immunity). See text for detail on processed macrophages. Figure is adapted from van Crevel *et al.*[17]


A. Antigen uptake and recognition
MTB
uptake
recognition
Non-opsonized
Opsonized
Lipoprotein
LAM
DNA
Vit D
Mannose receptor
Scavenger receptor
Complement receptor
NOD-2
DC-SIGN
Toll like receptors (TLR) family
VDR
Phagocytosis
B. Primary MTB killing
(i) phagosome maturation and fusion blocked, no MTB killing
lysosome
MTB
phagosome
TLR family
MTB survival
Antigen processing
MyD88
E. MTB Killing mechanisms
IRAK
(ii) Stabilized, latent TB infection
(ii) Stabilized latent TB infection
NFκB
Nucleus
IFN-γ pathway
TNF α pathway
Immune activation
C. Antigen presentation
D. Cytokine production
IFN-γR
TNF-αR
TNF-αR
Fas
TNF-α
Pro-Inflammatory cytokines
Anti-Inflammatory cyotkines
TNF-α
IFN-γ
Auto-induction
FasL
MHC-I, MHC-II etc
T cells

(ii) MTB uptake by phagocytosis

Distinct routes of entry of MTB may lead to differences in signal transduction, immune activation and intracellular survival of MTB. There are multiple mechanisms for the uptake of MTB, involving different host cell receptors. Complement receptors (e.g. CR1, CR3, CR4) are responsible for uptake of MTB after opsonisation with C3.[87] Mannose-binding lectin (MBL), a calcium-dependent serum lectin, acts as an opsonin to promote phagocytosis by activating the complement cascade. Single-base substitutions in codon 52, 54 and 57 of the MBL gene have been reported to result in reduced serum MBL concentrations in pulmonary TB.[88]

Mannose receptors (MR) and Scavenger receptors (SR)[89] are receptors for non-opsonin-mediated phagocytosis. MR recognize terminal mannose residues on MTB which leads to MTB uptake.[59] Virulent strains of MTB are phagocytosed through MR, while attenuated strains are not, suggesting that this route of entrance is advantageous to pathogenic mycobacteria.[90] MR is only one of the many members of the large and heterogenous family of C-type lectins, which also include DC-SIGN.

MTB may also bind to human surfactant protein A (Sp-A), Sp-D, and fibronectin, which are produced by pulmonary epithelial cells, where they act as opsonins and regulators of cell receptor activity in enhancing phagocytosis by macrophages.[91] Moreover, soluble circulating factors may also transfer mycobacterial products to macrophages, including their targeting to CD14, which is expressed as a glycosylphosphatidylinositol-linked membrane protein, forming a receptor complex with TLR4. Thus, TLR4 requires CD14 to participate in the process of LPS-induced signaling, including NF-κB activation. CD14 may thus promote cell adherence, binding, and phagocytosis.[92]

B. The mycobacterial phagosome as hostile environment and as an essential niche of MTB

MTB is a slow-growing organism that needs an aerobic environment. Though the bacilli can remain in the air for a long period, MTB can only grow or multiply inside the human phagocytes or on a special synthetic culture medium. In general, mature phagosomes are kill intracellular organisms by various mechanisms which include acidification of phagosomes, phagosome-lysosome fusion and the limitation of intraphagosomal iron.[93] Lysosomes are

highly acidic organelles and contain numerous hydrolytic enzymes. Fusion of the lysosome with a phagosome containing ingested MTB is the primary mechanism by which macrophages kill MTB. MTB is, however, able to evade the antimicrobicidal effect of the lysosome by inhibiting the phagosome-lysosome fusion and prohibiting the phagosome to mature.[94,95] MTB produce sulfatides and ammonia that alkalize the phagosome environment.[96] In murine macrophages, phagosome and lysosome fusion can be blocked by retention of TACO (tryptophan aspartate-containing coat) or human coronin, a 50-kDa phagosome-specific polypeptide.[97]

Iron is essential for both the pathogen and the host as a cofactor for basic metabolic pathways. For the host, iron is involved as an important component of the innate immune response through its role in the generation of toxic oxygen and nitrogen intermediates; and for the mycobacteria to grow and survive.[98] Since iron is not freely available in the host, mycobacteria must actively compete for this metal to establish an infection. Mycobacteria produce iron-like siderophores that appear to be important for growth,[99] and are equipped with iron binding molecules, including mycobactins and exochelins, for iron uptake.[100] MTB also exploit the host cell's iron uptake system e.g. the transferrin-transferrin receptor,[101] and can also access iron directly from cytoplasm.[102] It is clear that the shared requirement of mycobacteria and the host for this important nutrient has shaped the pathogen-host relationship during evolution.[98] There is rising evidence that the natural-resistance-associated macrophage protein 1 (NRAMP1) is involved in susceptibility or resistance to TB.[42, 103] NRAMP 1 is an integral membrane protein expressed exclusively in the lysosomal compartment of monocytes and macrophages.[104] This protein translocates to the membrane of the phagosome following phagocytosis and might function as a metal transporter of especially iron.[105] NRAMP1 may control intracellular microbial replication by actively removing iron or other divalent cations from the phagosomal space.[104] To date at least four variants of *NRAMP1* (INT4, D543N, 5'CA repeat, and 3'UTR) have been reported to be associated with TB disease susceptibility in various populations (**Table 1**), but with discrepant results, e.g. D543N is associated with TB susceptibility in some populations,[106-113] while in other populations it is associated with resistance to TB[42] or no association was found.[114] In TB patients, alveolar macrophages show increased production of NRAMP as well as iNOS, involved in macrophage activation and generation of toxic antimicrobial radicals.

C. Initiation of adaptive immunity

(i) Antigen presentation to T cells

After processing of mycobacterial proteins into smaller fragments called peptides, macrophages as well-known antigen presenting cells (APC) present the peptides bound to specialized antigen presentation molecules, encoded by the human major histocompatibility complex or HLA. These molecules are highly polymorphic, and present bound peptide on the cell surface to T cells, activating the latter to respond (**Figure 4**). Antigens derived from endosomes or phagosomes (e.g. exogenous antigens) are mostly presented by HLA class II and will be recognized by $CD4^+$ T cells. It remains, however, unclear how MTB is able to evade antigen presentation to $CD4^+$ T cells, but macrophages infected with MTB *in vitro* can block induction of a subset of IFN-γ-responsive genes including Fcγ receptor type I and the MHC class II transactivator (CIITA), which controls MHC class II expression.[115,116]

Furthermore, MTB antigens from the phagosome can undergo proteosomal degradation in the cytosol.[117] Although foreign antigens are normally presented by MHC class II, MTB antigens can elicit an MHC class-I-dependent $CD8^+$ T cell response, a process likely involving 'cross presentation', although also the canonical MHC class I presentation may be involved in part of the CD8 response. Macrophages infected with MTB or BCG have been shown to facilitate presentation of ovalbumin through the MHC class I presentation pathway via a TAP-dependent mechanism. TAP (transporter associated with antigen processing) is a molecule that is required for transport of peptide from the cytosol to the endoplasmic reticulum for loading onto MHC class I molecules. Peptides derived from this cytosolic-origin can thus be bound to MHC class I following the classical route, and are recognized by $CD8^+$ T cells.[45,118] Nonpolymorphic MHC class 1 molecules such as type I CD1 (-a, -b, and -c) molecules are able to present mycobacterial lipoproteins to CD1-restricted T cells. Some of the known antigens that are presented by MHC class I to $CD8^+$ T cells are the lipoprotein 19kD and Ag85B by TAP independent mechanisms.[119]

MHC class I and II are essential to present antigens to the adaptive immune system in MTB infection, and polymorphisms of HLA class I or class II may thus contribute to differences in disease susceptibility or outcome.[120,121] Certain allelic HLA variants have been associated with TB, e.g. the HLA class II variant DR2 which is encoded by alleles DRB1*15 and DRB1*16 and is associated with TB in several populations,[121,122] although not in others.[123,124] Lack of recognition and consequently activation of infected macrophages may therefore contribute to the inability of T cells to eliminate intracellular bacteria.

(ii) Cytokine production by macrophages

Recognition and/or uptake of MTB by phagocytic cells leads to their activation and the subsequent production of cytokines. As briefly mentioned above, pro-inflammatory cytokines such as IL-12, IL-18, IL-23, TNF and IL-1β are produced, and particularly in the case of DC-SIGN also anti-inflammatory cytokines including IL-10 and TGF-β.[52] IL-12 is a heterodimer composed of a p40 and a p35 subunit,[125] that binds to a receptor at the surface of Th1 and NK cells. The receptor for IL-12 is also a heterodimer consisting IL12Rβ1/ IL12Rβ2 where IL-12p40 binds to IL12Rβ1 and IL-12p35 to IL12Rβ2.[38] The importance of IL-12/IL-23 in resistance to TB was provided by studies in mice deficient for IL-12p40. These mice were susceptible to infection and had increased bacterial burden due to the substantially reduced IFN-γ production.[126] Similarly, humans with mutations in IL-12p40 or the IL-12Rβ1 genes presented with strongly reduced IFN-γ production from T cells and developed severe BCG-itis, including disseminated BCG-osis, as well as severe disease due to otherwise weakly pathogenic mycobacteria.[53] IFN-γ, a key activating cytokines produced by T cells, is needed to activate macrophages to eliminate MTB (*see below*). IL-12 and IL-18 synergize in the IFN-γ production.

TNF (previously known as TNF-α), secreted by T cells and also by macrophages and DCs, plays multiple roles in both immune and pathologic responses in TB. TNF, in synergy with IFN-γ, induces macrophage activation and the expression of isoforms of nitric oxide synthetase (iNOS) as a major antimycobacterial mechanism through the production of NO. TNF is also required for induction of apoptosis (*see below*), a process of natural cell self-killing, and granuloma formation (*see below*) to control acute infection. Absence of TNF or TNF receptor signaling may result in disorganized granulomas. In terms of pathologic responses, high levels of TNF may lead to destructive inflammation and tissue necrosis. However, since rheumatoid arthritis and Crohn's disease patients with anti-TNF antibody treatment significantly more often develop severe and sometimes fatal disseminated TB, TNF clearly also in humans is involved in protection against mycobacteria.[127] The dual role of TNF in both protection and immunopathology in TB needs further study. Variants in TNF are associated with susceptibility to TB in some populations but not in others (**Table 1**).

Interleukin (IL)-1 consists of IL-1α and IL-1β, and is mainly produced by macrophages, monocytes and dendritic cells.[128] Studies in knock-out mice[129] suggest an important role of IL-1β in models of TB by showing an increased bacterial outgrowth and defective granuloma formation. Variants in *IL1B* have been reported to be associated with TB susceptibility.[130]

Whereas a subset of cytokines is essential for control of the infection, others have been suggested to contribute to the destructive pathology of TB. Anti-inflammatory cytokines antagonize the pro-inflammatory responses induced by MTB infection. Anti-inflammatory cytokines include, amongst others: IL-1 receptor antagonist (IL-1ra), IL-10 and transforming growth factor (TGF)-β. IL-1ra is an important inhibitor of IL-1,[131] and variants in *IL1RA* are associated with resistance to TB.[132] IL-10 is generally considered to be an anti-inflammatory cytokine, and is produced by alternatively activated macrophages,[133] DC subsets, as well as Th2-, Th3- and subsets of T-regulatory cells[134,135] (*see below*). IL-10 antagonizes the pro-inflammatory cytokine response by downregulation of pro-inflammatory cytokine production. For example, IL-10 downregulates IL-12 production and directly inhibits $CD4^+$ T cells, including through downregulation of HLA class II expression, both resulting in a decrease of IFN-γ production.[136,137] Furthermore, IL-10 production counteracts the TNF production. The TNF/IL-10 ratio may control the balance between apoptosis and macrophage survival, and therefore impact on the control of MTB infection.[138] TGF-β as well as IL-10 are produced in excess during TB and seem to synergize in the suppression of IFN-γ.

D. Effector mechanisms of MTB killing by macrophages

(i) Oxidative and non-oxidative antimicrobial mechanisms

Macrophages, when activated, are able to eliminate or at least control mycobacteria. As mentioned above, putative mechanisms involved in killing of MTB by activated macrophages include the production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). The role of RNI in TB remains a matter of debate. *In vitro*, human alveolar macrophages infected with *M. bovis* BCG display increased inducible nitric oxide synthase (iNOS) mRNA, and inhibition of iNOS is followed by increased bacterial outgrowth. In mice, the major antimycobacterial mechanism is the production of NO by nitric oxide synthase (NOS2) as part of the RNI-generating pathway.[139,140] These antimycobacterial effects are induced by immune activation via different pathways. The activity of these antimycobacterial effects is increased when the macrophage is activated by IFN-γ. TNF synergizes with IFN-γ to induce the expression of the RNI-generating pathway. In humans, increased NOS2 expression was reported to be induced by the active form of vitamin D [1,25-dihydroxy vitamin D3;1,25-(OH)2D3], alone or in combination with IFN-γ and TNF.[141] The active form of vitamin D

activates monocytes through binding to the vitamin D receptor (VDR).[142] TLR activation also up-regulate expression of the VDR and the vitamin D-1-hydroxylase genes, leading to induction of the cathelicidin, a peptide that are capable of mediating antimicrobial activity.[143] Mutations in VDR impair the VDR function and this was found to be associated with frequent and severe episodes of infection.[144] Some polymorphisms of the VDR gene are associated with low bone mineral density.[44,145] These epidemiologic evidences support a link between vitamin D deficiency and susceptibility to TB, indicating an effect of active metabolite of vitamin D on mycobacterial growth,[146] and the important role of vitamin D in the TB treatment.[141]

Moreover, human cathelicidin-derived peptide LL37 was found to stimulate IL-1β secretion from monocytes by activation of the $P2X_7$ receptor on the surface of macrophages.[147] Activation of a purigenic $P2X_7$ receptor by extracellular ATP causes an immediate opening of a cation-selective channel, resulting in the influx of Ca^{2+}, and inducing caspase cascade dependent apoptosis, independently of primary effector mechanisms in macrophages such as ROI or RNI. Polymorphisms in $P2X_7$ receptor have been reported to increase the susceptibility to extrapulmonary TB in the Gambia.[148] The link between TLRs and vitamin D-mediated innate immunity is likely complemented by T cell-dependent adaptive immune mechanisms, including macrophage activation by cytokine and granulysin release as described below.

(ii) Apoptosis of macrophages

Apoptosis is a physiological process that is essential in regulation of immune responses.[149] Several *in vitro* studies have shown that alveolar macrophages undergo apoptosis following infection with MTB, which is associated with effective killing of intracellular mycobacteria.[56] During early mycobacterial infection, there is a rapid apoptosis of activated macrophages that may prevent dissemination of infection.[150] Infected macrophages express Fas, a member of the TNF receptor family that interacts with Fas ligand (FasL), which is expressed on the surface of amongst others $CD4^+$ T cells (*see below*). Binding of Fas to FasL results in signal transduction activating a suicide pathway leading to regulated apoptosis, which inhibits the growth of attenuated MTB strains in human macrophages *in vitro*.[151] Only apoptotic but not necrotic cell death reduces bacterial viability inside human macrophages. Moreover, infected macrophage apoptosis can also be mediated by TNF, suggesting that apoptosis-related reduction of MTB in macrophages is not restricted to one particular apoptosis pathway. Reduction of bacterial viability can also occur in a granule-dependent mechanism e.g. granulysin in cytotoxic T cells,[152] while lack of $CD4^+$ restricted cytolytic activity against mycobacterial infected phagocytes has bee associated with higher bacillary loads and disseminated TB.

2. Adaptive immunity

Since MTB primarily resides within phagocytic cells, celular rather than humoral immunity is required to eliminate the bacilli. T cell effector mechanisms are essential to control MTB. T cell responses involve several phenotypic T cell subsets, multiple mechanisms of antigen recognition and distinct effector functions. Phenotypically, T cell subsets are recognized based on the T cell receptor (TCR) they express, e.g. αβ chain or γδ chain. Contributing to protective immunity in TB includes the αβ T cell subsets $CD4^+$, $CD8^+$, and Double Negative (DN, $CD4^-CD8^-$) T cells. The latter express either αβ or γδ TCR.[152] $CD4^+$ T cells are important in early MTB infection, whereas $CD8^+$ T cells may be more important in controlling persistent infection. A major effector function of $CD4^+$ T cells is the production of pro-inflammatory cytokine IFN-γ in response to MTB antigens.[153] Therefore, optimal function of $CD4^+$ T cells and macrophages is critically important for immunity against mycobacteria. Loss of $CD4^+$ T cells, as seen in individuals infected with HIV, leads to an increased susceptibility to both acute and reactivation TB.[154]

There are different ways by which T cells could participate in the control of bacterial infections. The main mechanism of T cells in TB protection is to activate antimicrobial capacities in infected macrophages via the release of cytokines (*see under A*) and the second function of T cells and NK cells is their cytotoxic activity (*see under B*).

A. Cytokine releasing T cells

In response to microbial pathogens, $CD4^+$ T cells differentiate into Th1 or Th2 cells, adapted to the type of infectious agents. The Th1 subset is characterized by IFN-γ production, and induces B cells to release antibodies of the immunoglobulin G2 isotype, responsible for phagocyte activation and antibody-dependent cellular cytotoxicity. The Th-2 subset is characterized by IL-4, IL-5 and IL-10 production, and induces production of immunoglobulin E antibodies, responsible for immunity against parasitic infection. Both subsets develop from naïve T cells; the T cell differentiation is influenced by IL-12, produced by macrophages and DC in case of Th1, and by IL-4 and IL-10 in case of Th2.

The balance between Th1 and Th2 subsets may influence disease outcome. The key activating Th1 cytokine IFN-γ, produced by both $CD4^+$ and $CD8^+$ T cells,[155] as well as by NK cells, binds to the IFN-γ receptor (IFN-γR) present on macrophages and DCs. IFN-γ, in synergy with TNF, induces NOS2

expression in macrophages. IFN-γ gene knockout mice are highly susceptible to MTB,[156] and individuals with a defect in IFN-γ or IFN-γ receptor are prone to mycobacterial infections, including MTB.[37] Subtle variations in IFN-γ and IFN-γR have been reported to be associated with susceptibility to TB in some populations.[157,158] Of note, Th 1 mediated IFN-γ production and IFN-γ signaling are strongly downregulated during active TB and normalize during TB cure, underlining the role of Th1 cells and type 1 cytokine signaling in protective immunity against MTB. In addition, humans with IL-12/IL-12Rß1 gene defects present with strongly reduced IFN-γ production from T cells and often develop severe disseminated to otherwise weakly pathogenic mycobacteria.[53] IFN-γ production is often used in *ex vivo* assays to measure CMI responses by quantifying IFN-γ released by T cells or the frequency of IFN-γ producing T cells in response to stimulation by MTB antigens (*see below*).

It is clear that TB is associated with reduced Th1 type responses. An enhanced Th2 type response with high IL-4, IL-5, IL-10 and TGF-β[159-161] can reduce the Th1 response. Increased IL-4 production in TB patients[162] can suppress IFN-γ production and macrophage activation.[163] In addition, overexpression of IL-4 results in excessive tissue damage in the lung.[164] TGF-β, which is present in granulomatous lesions, is produced by human monocytes after stimulation with MTB. TGF-β has been described to inhibit proliferation and IFN-γ production by T cells, antagonizes antigen presentation and pro-inflammatory cytokine production by macrophages.[165] On the other hand, TLR2 stimulation may result in a Th2-biased response.[166] Th2 responses are also elicited by helminth infections. It is worth noting that in developing countries where both TB and helminth infections are common, helminth infections induce potent Th2 responses and may suppress Th1 responses, suggesting that helminth infections could impact on protective immunity to TB. Helminth infections have been added to the list of risk factors for developing active TB,[167] and may influence the efficacy of vaccines against TB in the tropics.[34] In advanced stages of the infectious process, another subset of T cells; T regulatory cells characterized by $CD4^{+}CD25^{(high)}$, may control excessive inflammation by producing large amounts of the anti-inflammatory cytokines IL-10 and TGF-β that suppress Th1 type responses or cytotoxic T cells and interfere with effector T cell activation.[168]

B. Cytotoxic T cells

CTLs can lyse infected host cells via two major pathways. The first pathway is that CTLs express the death-inducing molecule Fas ligand (FasL) which induces apoptosis of Fas expressing target cells. Also $CD4^{+}$ CTL kill target cells via FasL-induced apopotosis.[169] The second pathway is that CTLs, both

$CD4^+$ [170] and $CD8^+$,[171] release cytoplasmic granules containing various effector molecules with cytolytic activity, e.g. perforin, granzymes, and granulysin.[172] Granulysin, a cytolytic protein localized to granules of human CTLs and NK cells,[173] is released into the intercellular space upon TCR stimulation via a granule exocytosis pathway.[174] It has been demonstrated that granulysin has tumoricidal and antiviral activities and it is also able to inhibit the growth of pathogenic bacteria, fungi and parasites *in vitro*. Furthermore, granulysin can directly affect the mycobacterial membrane integrity and thereby kill MTB, both extra and intracellularly.[172,175] For granulysin to reach and kill intracellular MTB perforin is thought to be required to create a pore in the target cells membrane. In mouse models, $CD8^+$ T cells can contain MTB, and $CD8^+$ T cell deficiency resulted in susceptibility to tuberculosis.[176] $CD8^+$ T cell counts were markedly decreased in active TB and recovered after therapy.[177] CTL may complement the effector functions of IFN-γ secreting effector cells by the specific killing of infected cells. CD8 CTL may also kill HLA class II negative infected target cells, thus providing an unique effector arm distinct from that of the CD4 system.[168]

3. Granuloma formation

In response to MTB infection, inflammatory cells migrate to the lungs to organize into a structure known as granuloma. One of the major roles of the granuloma is to localize and contain not only the bacteria but also the inflammatory response to the bacteria, by walling off the organisms from the rest of the lung as well as providing a local environment for the action of the immune cells. The granuloma is initially composed of $CD4^+$ and $CD8^+$ T cells, but a complex array of T cells including $CD4^+$, $Cd8^+$, γδ T cells and CD1 restricted αβT cells are also involved.[52,178] Recruitment and retention of T cells, macrophages as well as B cells within inflamed tissue depends on adhesion to the extra cellular matrix (ECM). T cells use integrins to adhere to the ECM; and fibronectin is one of its major components.[179] TNF is essential in formation and maintaining the integrity of tuberculous granulomas.[18] Mice deficient in TNF or its receptor develop disorganized granulomas and neutralization of TNF in MTB-infected mice results in progressive disease.[52] It is clear that the Th1 immune response in the granuloma is essential to contain the infection and prevent active disease in these individuals, but it is -at least in many cases- unable to completely eradicate the infection, thus inducing latent infection which makes individuals vulnerable to re-activation of the disease when host immune defenses become compromised. When in the process of establishing

latency, MTB enters a stationary phase; it adapts to its intracellular habitat in granulomas which is characterised by hypoxia, nutrient scarcity and the presence of microbicidal molecules such as reactive oxygen and nitrogen intermediates.[180] MTB cultured *in vitro* under low oxygen condition upregulate the expression of a-crystallin, resulting in a stage of non-replicating persistence (NRP) and to a low-dose NO stimulation.[181] In this Wayne-model, the strategies employed by MTB to permit hypoxic NRP include restriction of biosytnthetic activity to conserve energy, induction of alternative energy pathways, and stabilization of essential cell components to lessen the need for repair or replacement. a-crystallin, a small heat-shock protein (Hsp) termed as HspX, is part of the so-called dormancy (DosR) regulon, consisting of 48 genes,[182] Indeed, DosR regulon was found to encode a number of MTB genes with strong T cell and IFN-γ inducing capacity,[183] and may contribute to natural protection against TB disease. High levels of HspX mRNA were found in lungs of patients with chronic active TB,[184] providing new target antigens for vaccination against reactivation of latent mycobacterial infection (*see below*).

III. TB control strategies: reducing the global burden

Despite the discovery of TB therapy and BCG vaccination, TB has not been eradicated and continues to cause chronic persistent as well as latent infection. TB is the leading cause of death from a single bacterial infectious disease worldwide. Due to its growing incidence TB has become a re-emerging disease, and the WHO has declared TB a global health emergency.[10] TB develops very rapidly and fatally in the context of HIV/AIDS. Ineffective TB control programs are leading to the development of multi-drug resistant bacilli. The goal of TB control strategy is to cure and prevent TB.

TB treatment and multi-drug resistancy

The era of TB chemotherapy begun in the 1950's with the finding of Para-Aminosalicylate (PAS) by Lehmann (1944), followed by other findings including streptomycin by Waksman and Schatsz (1943), and isoniazid by Domagk (1952).[185] Though an efficient TB treatment exists which makes TB a curable disease, TB nevertheless kills 5000 people every day.[186] The main therapeutic strategy, as defined and recommended by the WHO, is to use a combination of three or four different anti-TB drugs: isoniazid, rifampin, pyrazinamide and/or ethambutol.[10] The course of TB treatment is lengthy (4-6 months), because there are several goals that need to be achieved e.g. to

rapidly kill the massive numbers of bacilli, to prevent the emergence of clinically significant strains of drug-resistant mutants, and to effectively sterilize the disease sites.[187] In many parts of the world, access to TB drugs is limited, and compliance with the drug regimen is poor. The complicated regimen, the drugs' side effects, and the lengthy treatment can trigger patients to fail to complete therapy.[188] For the latter reason, a directly observed TB therapy short-course (DOTS) has been widely implemented. However, new drugs are urgently needed. Global surveillance has shown that multi-drug resistance (MDR) is becoming widespread and is a threat to TB control programs in many countries.[189,190] MDR-TB is defined as being resistant to the first-line TB drugs e.g. isoniazid and rifampin. MDR-TB treatment therefore requires the use of second-line drugs which are less effective, more toxic and more expensive than the first-line. In a recent report summarized by the Centers for Disease Control (CDC) and the WHO, a survey of several international TB laboratories during 2000 - 2004 had determined that of 17,690 TB isolates, 20% were MDR and 2% were even extremely or extensively drug resistant (XDR).[191] XDR-TB has been identified in all regions of the world, prevalent mostly in Asia and in Eastern Europe, but so far only or mostly in HIV co-infected individuals. XDR-TB may emerge as a worldwide threat, raising concerns of future untreatable TB epidemics. No new anti-TB drugs have been licensed in the past decades. Ideally, new drugs should have a high activity to reduce the duration of treatment, the ability to kill persistent bacilli that might otherwise reactivate later in life and activity against multi-drug resistant TB strains.[192] Recent findings reported the possibility of breakthroughs in the field of anti-TB drug discovery, thanks to whole genome sequencing and comparative genome analysis.[193] R207910, a diarylquinoline, has been proposed as a novel drug that inhibits both drug-sensitive and drug-resistant MTB *in vitro* by targeting the proton pump of adenosine triphosphate (ATP) synthase.

The search of TB preventive vaccines

Although drug treatment is very effective, a vaccine that would prevent infection and/or disease will be necessary to control or eliminate TB worldwide. To date, more than 3 billion people in the world have been BCG vaccinated, a vaccine against TB that was developed in the early 20th century by Albert Calmette and Camille Guérin. BCG vaccine consist an attenuated strain of *M. bovis*, the etiologic agent of TB in cattle. According to WHO recommendation, infants should be BCG immunized soon after birth as part of routine health-care in all countries with a high risk of TB infection. In some

countries BCG vaccination is given again as a booster during childhood. BCG is believed to reduce the incidence of childhood TB, particularly TB-meningitis,[194-196] but unfortunately does not lead to eradication of MTB. Moreover, BCG seems ineffective to protect adults from TB as has been shown in several human trials in which protection varied from 0% to 80%.[197] The low efficacy of the BCG vaccine may relate to pre-existing immune responses to antigens that are present in both environmental mycobacteria and MTB,[198] or other co-infections e.g. helminth infection.[34] Clearly, a more effective vaccine against adult pulmonary TB is needed and the development of several new TB vaccine candidates is underway.[199]

Effective vaccine development is dependent on a clear understanding of the host immune response to MTB. There is evidence to suggest that a vaccine effective against primary infection may not necessarily stimulate an immune response effective against latent infection.[200] Therefore, the ability of T cells to release IFN-γ has been used as a critical criterion for the identification of candidate antigens. The strategies used for candidate vaccines against TB are the use of subunits, recombinant BCG vaccines, and the use of attenuated gene deletion mutant of viable mycobacteria. Only the first two will be described below. These vaccines can be given as pre-exposure (prophylactic) vaccination to prevent infection or disease, or as post-exposure vaccine, given after infection (in latent phase) to eliminate or contain LTBI and prevent re-activation (**Figure 5**).

The availability of the MTB genomic sequence[201] has facilitated development of new tools to probe for genes involved in pathogenesis. Identification of mycobacterial genes or gene products which can interact with the host's immune system has become an international research priority; e.g. the identification of MTB proteins that are essential for virulence, or antigens that are immunodominant, are the main goal for vaccine research and diagnostics. The subunit vaccines, including antigen-adjuvant formulations, naked DNA vaccine constructs, and recombinant carriers expressing antigen, are based on protein antigens. They can be given alone or in addition to BCG. Alternatively, glycolipid antigens can be also included in the vaccine formulations since these could serve as antigens in the context of CD1 and as adjuvant for TLRs.[199]

Most antigens investigated are early produced secreted antigens such as early secretory antigenic 6 kDa (ESAT-6) and Ag85. These antigens are highly reactive T cell antigens, that are strongly recognized by the majority of individuals with active TB,[202] indicating that these genes are expressed during the early stages of infection. MTB's survival in phagosomes may depend on

specific MTB virulence factors and secretion of ESAT-6 correlates closely with virulence and immunogenicity of MTB.[203] Comparative studies between avirulent *M. bovis* BCG and virulent MTB led to the identification of several genetic regions of difference (RD). The current vaccine BCG lacks approximately 130 genes that are clustered in 16 RDs, of which in part involved in pathogeneicity and persistence. As mentioned earlier, the virulent MTB and related pathogenic mycobacteria require ESAT-6 and culture filtrate protein 10 (CFP-10), as part of the ESAT antigen family. These small proteins, encoded by the RD1 genetic locus, play a role in preventing phagolysosomal fusion, thus potentiating the virulence of pathogenic mycobacteria.[204] Entire RD1 region of MTB had been introduced by Pymm *et al*, comprising at least 11 genes.[205] Clearly, the RD1 region play an important role in virulence as also demonstrated by the attenuation of *M. bovis* or MTB strains which carry knockout mutations in the RD region. In murine models, deletion of the 9.5kb RD1 region from MTB -abrogating the synthesis of ESAT-6 or CFP-10- attenuated the virulence of MTB similar to BCG.[206]

Reintroduction of deleted genes expressing RD1 or Ag85 to BCG, termed as recombinant BCG (r-BCG), may increase immunogeneicity, antigencity or both without reverting it to a pathogen, resulting in the stimulation of a profound immune response. The RD1-restored mycobacterium strain persisted longer in immunocompetent mice,[205] while r-BCG expressing Ag85 induced substantially higher protection than parental BCG against TB in guinea pigs.[207] Antigens that recognized by the immune system during the first stage of infection are probably adequate for pre-exposure vaccination. The r-BCG vaccine candidates with higher immunogenicity may comprise strain that express human cytokine involved in TB protection. On the other hand, understanding the dynamic transition of MTB from active multiplication, dormancy and resuscitation has stimulated attempts to identify components for post-exposure vaccine. This containing dormancy antigens would be targeting the remaining dormant bacteria (latency antigens or resuscitation antigens), and thus might contribute to control an important part of the reservoir of MTB, and to protect against new cases that arise from reactivation of latent infection.[208] As described above, DosR regulon is expressed *in vitro* during hypoxia and low-dose NO stimulation and MTB encounter these conditions during latent infection. Recently, 25 strongly induced DosR encoded proteins have been tested, confirming the expression of DosR regulon antigens by MTB that were recognized by the host immune system during natural infection.[183] The best-known dormancy antigen HspX represents promising candidate antigen for post-exposure vaccine (**Figure 5**).

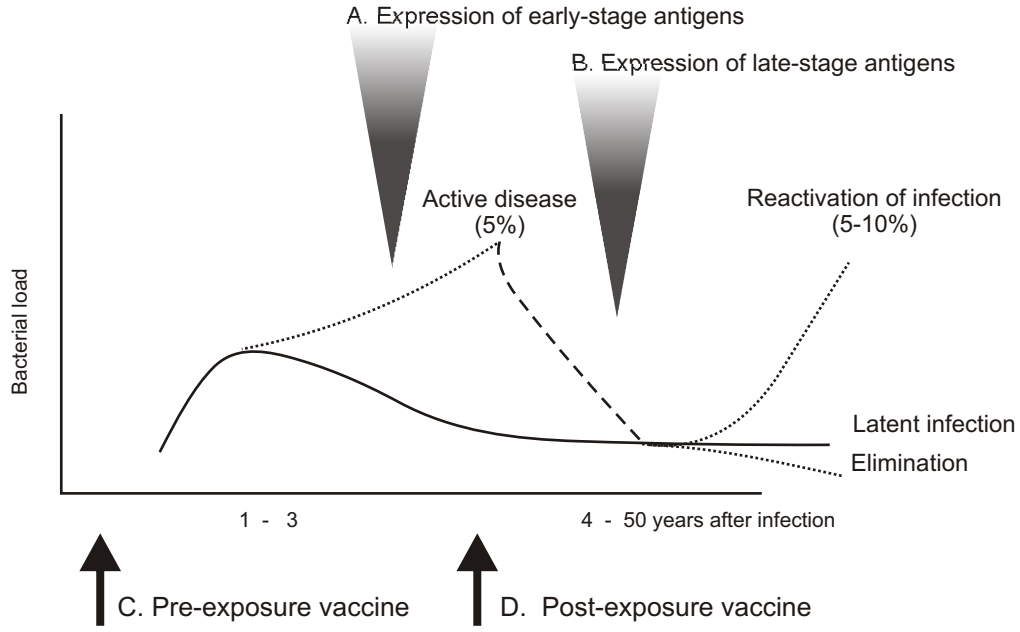


Figure 5. Antigens expressed during the different cycles of MTB infection.
(A) Expression of early antigens secreted by replicating MTB in active TB
(B) Expression of late-stage antigens induced during latent phase (dormancy or resucitation)
(C) Pre-exposure (prophylactic) vaccine to prevent infection or disease
(D) Post-exposure vaccine, given after infection or in latent phase, to eliminate or contain latent TB infection and prevent re-activation

The development of diagnosis of LTBI

Identifying individuals with LTBI faces many challenges. TST or Mantoux test was, until recently, the only tool to assess LTBI.[209] This test is based on a type-IV delayed type hypersensitivity reaction to purified protein derivatives (PPD) that involves recruitment of macrophages and T cells to the skin. PPD is a crude mixture of several antigens taken from dead mycobacteria; *M. bovis* BCG

and several non-tuberculous mycobacteria (NTM). A positive TST result could therefore be due to true infection with MTB, prior BCG vaccination or due to exposure to NTM. The TST reaction is read by measuring induration size of the skin at the site of the intradermally injected PPD after 48-72 h. The TST cut-off point of <5mm is considered negative, ≥5mm of induration is considered positive for those at highest risk, ≥10mm is positive for those at intermediate risk, and ≥15mm is positive for those at low risk.[22] Although a TST+ (or PPD+ for *in vitro* use) is an indicator of CMI responses to MTB, it is not correlated with protection against TB disease. It remains unclear which aspects of the CMI response determine protection against TB disease. In low TB endemic countries, TB control strategies are targeted to LTBI which makes the diagnosis of LTBI of major interest. With the advances in immunology and genomics, a new generation of *in vitro* tests of cellular immunity has been developed in the form of T cell based IFN-γ release assays (IGRA). These *ex vivo* assays measure CMI response by quantifying IFN-γ released by T cells in response to stimulation by MTB antigens. A high level of IFN-γ response is likely indicative of previous sensitization with MTB, but does not necessarily point to active TB. A new development in IGRA has been achieved using the antigens that are highly specific for MTB, including ESAT-6 and CFP-10. These RD1-proteins, are not shared with the BCG strains and most NTM species. Another assay to detect IFN-γ responses is by counting the number of IFN-γ producing T cells that has been developed in the enzyme linked immunospot (ELISPOT). To date, there are two RD1-based assays available commercially; e.g. the QuantiFERON-TB-GOLD® (Cellestis Ltd, Carnegie, Australia) for the first assay mentioned, and T SPOT-TB® (Oxford Immunotec, UK) for the latter. IGRA can not, however, completely replace the currently most common used TST yet, since the performances of both tests have advantages and limitations. Several studies have evaluated both analyzing the agreement and discordance between TST and IGRA,[210,211] however, it is worth noting that neither TST nor IGRA can distinguish between LTBI and active TB disease which has important implications for high endemic countries. The decision to select one over the other will depend on the population, the goal of testing and the resources available,[212] especially the cost and technical considerations may favor the selection of the TST in rural settings.

Concluding Remarks

One third of the global population is infected with MTB. The vast majority who harbor the pathogen will not develop tuberculosis, indicating that MTB lives in a fairly balanced coexistence with the human host in most cases, and has developed the capacity to evade killing mechanisms. The interaction between pathogen, host and environmental factors will determine the disease outcome. Protection from developing TB disease, and the possible elimination of MTB mainly depends on effective cell-mediated immunity which includes activated macrophages, T cells and Th1 cytokines. Macrophages play multiple roles during mycobacterial infection; as principal host cells for intracellular replication of the bacilli, as antigen presenting cells for activation of T-lymphocytes at the site of infection, and as effector cells responsible for the killing of mycobacteria. It is clear that innate immunity plays a crucial role not only in recognizing the pathogen, but also in directing many aspects of the subsequent host response, including the ensuing acquired or adaptive response. Interindividual differences in outcome after MTB infection may, in part, be explained by the efficiency of various innate host defense mechanisms and by subtle variations of genetic polymorphisms, which are associated with increased susceptibility or severity of TB. Despite the availability of TB drugs and vaccination, TB control is still far from reach, because other problems have arisen; e.g. MDR/XDR strains, spread of HIV, and the lack of BCG efficacy. Knowledge of the factors that influence progression of TB infection to TB disease will be of importance to evaluate transmission of infection in the community and to adapt TB control activities.

The scope of the thesis

The thesis consists of two different but related sets of studies. The first part of the thesis comprises immunological and genetic studies that aim at dissecting essential mechanisms of host defense and the role of host genetic variations, particularly in cytokine- and cytokine receptor encoding genes, in relation to susceptibility to and clinical disease manifestation of TB. The second part of the thesis deals with disease presentation in active TB in relation to other concomitant disease, e.g. DM. The clinical outcomes of mycobacterial infection vary greatly, ranging from infection with no clinical symptoms to active TB. Our overall study has therefore used a large case-control cohort design, which allowed us to examine differences between cases (those with active TB infections) and controls (individuals with exposure but no evidence of TB infections). Moreover, part of the patients were included in a longitudinal study, and analyzed in relation to treatment outcome.

Part 1.
Immunological and immunogenetic analysis of host immunity to TB.
MTB has evolved efficient ways to evade host defense by down-regulating various key elements of the cell-mediated immune system. Immunity to mycobacteria is critically dependent on type-1 immunity, involving the IL-12/23/IFN-γ/IFN-γR, NF-κB and TNF-α/TNF-αR-axes. We therefore hypothesized that impairment of these pathways is related to TB disease. Our study design was partly aimed at analyzing the integrity of these pathways in TB patients, both before, during and following completion of microbiological cure through chemotherapy. Its setting in Indonesia, which is a highly TB endemic area, enabled us to analyse these questions in the context of environmental as well as tuberculous mycobacterial exposure, which are considered to impact significantly on the population's immunity.

In **Chapter 2** the dynamic of the immune response as evaluated by innate and adaptive pro- and anti-inflammatory cytokine secretion was studied in TB patients compared with their neighborhood controls. We evaluated the capacity of TB patients' peripheral blood cells before, during and after treatment to produce pro-inflammatory (TNF-α, IL-12/23p40, IL-23 and IFN-γ) and anti-inflammatory (IL-10) cytokines, in response to MTB as well as unrelated stimuli (lipopolysaccharide (LPS), phytohemagglutinin (PHA)). In view of recent evidence that MTB infection inhibits IFN-γR signaling pathways in *in vitro* systems, we also examined the ability of the above cells to respond to IFN-γ. Finally, cytokine responses and profiles were analyzed in relation to clinical severity and to treatment outcome.

Granulysin is a cytolytic protein localized to granules of human cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. It is the major known cytolytic protein responsible for $CD8^+$ CTL mediated antimicrobial activity after granule release upon T cell receptor (TCR) stimulation. To obtain better insight into the role of granulysin in tuberculosis, plasma granulysin levels were measured in **Chapter 3**, and the levels of plasma granulysin were correlated to disease activity and clinical outcome after successful therapy. Plasma granulysin levels were compared with IFN-γ levels, and their cellular source was studied.

Part of the considerable variation in outcome to MTB infection can likely be explained by human genetic variations, but their identities remain largely unknown. In **Chapter 4** the role of genetic polymorphisms and mutations in genes *IL12B*, *IL12RB1*, *IFNG*, and *IFNGR1* which encode type-1 cytokine and type-1 cytokine receptors that are essential in controlling specific immunity to MTB is described. Identification of genetic factors underlying host susceptibility to TB will help to elucidate the relevant molecular and cellular mechanisms that mediate host immune response to this bacterial infection.

One of the key components in determining MTB infection is its recognition by a wide-range of pattern recognition receptor molecules, expressed at the surface of macrophages or dendritic cells. Ligation to these receptors provide a first step in the innate immune response. A comprehensive association study of genes involved in innate immune recognition is described in **Chapter 5**.

Part 2.
TB-disease presentation

A characteristic disease symptom of active TB patients is the development of anemia as a result of chronic infection. The hematological and iron metabolism state were examined during treatment of TB. The effect of polymorphisms of the macrophage phagosome iron transporter (NRAMP1) on susceptibility to TB and their relation to disease severity are described in **Chapter 6.**

Diabetes mellitus (DM) is a well-known risk factor for TB. The association between TB and DM is receiving more attention, due to the globally growing prevalence of type-2 DM in developing countries where TB is endemic. Indonesia has the third highest number of TB cases and the fourth highest number of people with DM worldwide. DM was found as a

concomitant disease in TB and has a strong relation with TB in Indonesia as reported by our group recently. **Chapter 7** describes the disease presentations and clinical severity of TB patients with concomitant DM, compared with TB patients without DM.

Finally, the main findings of the studies are summarized and discussed in chapter Summary and General Discussion.

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