Chapter 2
Immunology
Immunology in Tuberculosis: Challenges in Monitoring of Disease Activity and Identifying Correlates of Protection

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ABSTRACT: Humans have always lived with tubercle bacilli. Host susceptibility – both inherited and acquired – determines whether an individual infected with Mycobacterium tuberculosis will eventually fall ill and develop tuberculosis (TB). After infection with M. tuberculosis, a latent TB infection may ensue reflected by immune recognition of specific antigenic epitopes expressed by M. tuberculosis – the Region of Difference 1 proteins ESAT-6 and CFP-10 leading to interferon gamma release in vitro. Multi-Drug-Resistant TB has emerged as an enormous infectious threat in certain regions in the world, and the Acquired immunodeficiency by co-infection with HIV has accelerated the TB epidemic even further. A paradoxical response – or Immune Response Inflammatory Syndrome in the context of treatment of HIV co-infection - is an increased inflammatory reaction during effective reduction in the bacterial load. This should be differentiated from treatment failure. A huge challenge is to develop novel markers that can differentiate paradoxical responses from treatment failure.

We discuss the role of protection of vaccines – especially BCG, iron metabolism and the role of vitamin D.

KEYWORDS: Mycobacterium tuberculosis, immune response, interferon gamma, BCG, vitamin D, iron, immune response inflammatory syndrome.

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INTRODUCTION
Humans have struggled with tuberculosis (TB) for thousands of years. *Mycobacterium tuberculosis*–specific gene insertions (including insertion segment 6110) have been retrieved from bone lesions in mummies in Egypt dating back as far as 2000 BC [1]. Several studies around the world support the concept that lineages of *M. tuberculosis* closest to its ancestral origin are *M. africanum* (I, II) hail from the African continent [2]. Within the *M. tuberculosis* complex, several other pathogens may affect humans. *M. bovis* should be considered a zoonosis. This micro-organism shares a common ancestor with *M. tuberculosis* and is an important pathogen in several different other mammals than man [3].

In this paper we briefly review host-pathogen interactions, give a summary of the current concepts of local and systemic immune response to *M. tuberculosis*, and focus on potentially modifiable factors (i.e., the micro-nutrients iron, and vitamin D); we discuss BCG and newer TB vaccines currently in development; and provide an update on monitoring of disease activity. Clinicians are increasingly challenged by patients who do not respond favourably to antituberculosis-treatment. Failure to respond to treatment may be clinically indistinguishable from a paradoxical response. We define the term ‘paradoxical response’ here as clinical worsening with increasing inflammation, but with decreasing mycobacterial load suggesting a beneficial response at the bacteriological level but paradoxically, a clinical deterioration. In the context of immune recovery as a result of treatment for HIV co-infection as well as TB treatment, the pro-inflammatory response is referred to as Immune Response Inflammatory Syndrome or IRIS. We discuss current assays and tools to differentiate paradoxical response from treatment failure, and discuss inherited as well as environmental and nutritional factors that confound this response.

TUBERCULOSIS: HOST-PATHOGEN INTERACTIONS
The concept that in the *M. tuberculosis* complex, *M. bovis* should be considered a subset most closely related to the ancestor of modern *M. tuberculosis* strains has been abandoned [4, 3]. Although *M. bovis* infection is indeed a zoonosis, TB is not. Humans are indeed the almost exclusive reservoir of *M. tuberculosis*. The interplay between the pathogen and its usual host has provided genetic selection pressure that has both impacted on *M. tuberculosis* and on its human host [5]. Selection pressure by the human host appeared to reduce the variability of genes of *M. tuberculosis* that are essential for survival and duplication but also the epitopic repertoire of antigens that are recognized by human T-cells [6]. An important feature of *M. tuberculosis* is its capacity - under environmental pressure such as low oxygen pressure and immune surveillance by the human host – to go into a dormant phase by expression of special genes [7, 8]. This unique feature allows *M. tuberculosis* to latently infect one third of the world’s human population. Only in the last 60 years, the battle with *M. tuberculosis* could change in favor of the human host, with the
introduction of effective antimycobacterial treatment [9]. Not immunity alone, but highly effective drugs could now fight TB [10]. This was of course a historical revolution, and when Selman Waksman was honored with the Nobel Prize in Physiology and Medicine in 1952 for his discovery of streptomycin, the first drug shown to be effective against TB, he believed that TB control could be expected. In his Banquet Speech he said: ‘The Great White Plague, which only 10 years ago was thought to be immune to drug therapy, is gradually being eliminated. Even persons afflicted with those forms of tuberculosis, such as meningitis and miliary, which were nearly always fatal, now have a better than even chance of recovery. Streptomycin pointed a way. Later supplemented with PAS and more recently with isoniazid, it has brought the control of this disease within sight’. With the introduction of rifampicin and pyrazinamide, two decades later, short course therapy had become available [11] and the world’s attention for TB waned. These successes were however followed by the emergence of HIV/AIDS that weakened the immune defense of individuals latently infected with M. tuberculosis (LTBI) [12, 13, 14, 15, 16]; and the development of multi-drug resistant TB (MDR-TB) and extensively-drug resistant TB (XDR-TB) [17]. TB has remained and even re-emerged as a major deadly disease with an evolving burden of drug resistance. In 2008 alone, 9.3 million new cases world-wide, and 1.3 million people killed 2 and among these, 440,000 individuals with MDR-TB [18]. The concept that drug resistant strains would represent strains that are less transmissible or less ‘fit’ or virulent has largely been abandoned [19]. Indeed, MDR-TB and XDR-TB (Table 1) appear to be both transmissible and virulent [14]. Although previous TB treatment has been identified as an independent risk factor for MDR-TB, most patients presenting with MDR-TB have no treatment history and appear newly infected [20]. One of the clade 1 strains, the Beijing strain [21], has been shown to be highly transmissible, replacing other lineages and strains in certain parts of the world. The Beijing strain has also appeared more virulent than comparator strains, and it is associated with MDR-TB [22]. One important aspect is that in areas in the world where the epidemics of TB and HIV/AIDS overlap, such as in certain townships in the Cape Town area in South Africa, TB has emerged unprecedentedly [23]. For instance, prevalence data reported from the Kayelisha Township, an area with a high HIV/AIDS burden, were as high as 1,000 bacteriologically confirmed TB cases per 100,000 population [24] which is currently around the highest prevalence figures reported around the world.
**TABLE 1. Antituberculosis drugs - definitions of MDR- and XDR-TB**

<table>
<thead>
<tr>
<th>First-line oral agents</th>
<th>Injectable</th>
<th>Fluoroquinolones</th>
<th>Other second line agents</th>
<th>Agents with unclear efficacy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (H)</td>
<td>Kanamycin (Km)</td>
<td>Moxifloxacin (Mfx)</td>
<td>Etionamide (Eto)</td>
<td>Clofazimin (Cfz)</td>
</tr>
<tr>
<td>Rifampicin (R)</td>
<td>Amikacin (Am)</td>
<td>Levofloxacin (Lfx)</td>
<td>Protonamide (Pto)</td>
<td>Linezolid (Lzd)</td>
</tr>
<tr>
<td>Pyrazinamide (Z)</td>
<td>Capreomycin (Cm)</td>
<td>Ofloxacin (Ofx)</td>
<td>Cycloserin (Cs)</td>
<td>Amoxiclav, Imipenem</td>
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<tr>
<td>Ethambutol (E)</td>
<td>PAS</td>
<td></td>
<td>Clarithromycin (Clr)</td>
<td></td>
</tr>
</tbody>
</table>

NB: The use of the fluoroquinolone ciprofloxacin is no longer encouraged

Multi-Drug Resistant TB (MDR-TB) denotes resistance to the two most powerful TB drugs currently in use: isoniazid (H) and rifampicin (R). Extensively Drug Resistant TB (XDR-TB) denotes MDR-TB plus resistance to one group 2, and one group 3-drug. With the advent of MDR-TB and XDR-TB, and especially in HIV co-infected patients, clinicians need novel tools to discriminate increase in mycobacterial load with failing treatment from ongoing or increasing inflammation despite effective bacterial killing, a condition referred to as paradoxical response [25, 26, 27, 28, 29]. Interestingly, certain anti-tuberculosis drugs notably PAS have an immuno-modulatory effect, apart from their anti-mycobacterial action [30].

As explained earlier, the context of immune reconstitution in patients with HIV co-infection, treated with combined Anti-Retroviral Therapy (cART), the phenomenon of paradoxical increase in inflammation is referred to as IRIS [31, 32]. Here we will refer to IRIS also in case of ‘unmasking’ TB co-infection in HIV patients started on cART [33, 34]. TB accounts for 20% of the reported IRIS cases and is common in resource limited settings [35].

**LOCAL AND SYSTEMIC IMMUNE RESPONSES TO M. TUBERCULOSIS**

Most *M. tuberculosis* infections do not result in clinical TB. *M. tuberculosis* bacilli persist in mononuclear phagocytes and Dendritic Cells (DCs) where they may survive [7]. The macrophage is the primary host cell for mycobacterial infection and is also the source for the cytokines. These cytokines play an important role in recruiting other immune cells for the formation of the granuloma. The *M. tuberculosis* bacilli, during their residence in the phagosome secrete proteins, which after degradation are presented by MHC class II molecules to CD4+ T cells. The MHC class I molecules present to the CD8+ T cells. CD4+ T cells are polarized into Th1 and Th17 cells, which perform effector functions. CD8+ T cells contribute to protection by cytolytic mechanisms and IFN-γ production. Upon activation,
macrophages also express the cytokines IL-10 and IL-12. These molecules counteract each other as IL-12 helps in the expression of IFN-γ from lymphocytes and IL-10 down regulates macrophage activation. The MHC class I-restricted CD8+ T cells also contribute to protective immunity against TB. These CD8+ T cells secrete perforin and granulysin, which lyse host cells and attack *M. tuberculosis* directly. T regulatory cells (Treg) are CD4+ T cells involved in regulation of self-tolerance, autoimmunity and suppression of immune responses during infections [36]. These Treg cells secrete regulatory cytokines, notably interleukin-10 (IL-10) and Transforming Growth Factor-β (TGF- β), which suppress Th1 immune responses [37] (see Fig. 1).

**FIG. (1).** Cross-talk between cytokines.

Post infection of tuberculosis in the lung and the macrophages and DC’s stimulate specific CD4+ and CD8+ T cells. The initial interaction between the TB bacilli and macrophage results in the expression of cytokines the IL-12 and TNF- α which act upon the other cells of the immune system such as the NK cells and DC cells. Infected macrophages respond in an autocrine manner to the cytokines derived from the antigen specific CD4 cells. CD4+ cells are differentiated into Th1 and Th17 cells to perform effector functions. Memory T cells develop, with co-expression of multiple cytokines, notably IL-2. TNF, and IFN- γ and co-expression of cytolytic molecules (38). Treg cells produce transforming growth factor-beta (TGF- β) and IL-10, which tries to suppress the function of Th1 cells (36, 37).

Abbreviations: NK - natural killer cells. Teff - effector T cells; Th- T helper cell; Mφ - macrophage; CTL - cytolytic T lymphocyte; TNF - Tumor necrosis factor; IFN- γ - interferon-gamma; TGF-β - transforming growth factor - beta; IL - interleukin; MHC - major histocompatibility complex; PNG - polymorphonuclear granulocyte.
When *M. tuberculosis* bacilli are engulfed by macrophages, intra-cellular killing may result if the vacuole fuses with the lysosome to allow acidification to occur [38]. Although certain mutants of *M. tuberculosis* are unable to block fusion due to altered expression in acyltrehalose glycolipids [39], most wild-type *M. tuberculosis* are able to arrest this process resulting in persistence of *M. tuberculosis* in macrophages and latent infection. Protective cellular immune responses are elicited by antigen processing to Thelper 1 cells (Th1) orchestrating the immune response by inflammatory mediators, cytokines and chemokines. Interferon-gamma (IFN-γ) is the dominant cytokine reflecting Th1 protective immune response. Generally, Th1-type pro-inflammatory mediators are in turn set off by counteracting regulatory T-cells producing IL-10 and TGF-β [37]. A localized infectious focus may result that is contained in a granulomatous inflammatory response. A granuloma is a complex host response pattern with macrophages providing a secured niche with a necrotic centre in which live but dormant *M. tuberculosis* may persist. Tumor Necrosis Factor-alpha (TNF-α) is an essential cytokine for granuloma formation and maintenance, and interference with TNF-α (fusion, receptor blockade) jeopardizes the host granulomatous immune response. Interleukin 12 (IL-12) is an important cytokine in the Th1 response too. The granulomas reflecting contained TB infection are known as the Ghon focus, usually situated in the lung [31, 40]. Recently a CD4 cell associated, non IFN-γ driven pathway has been shown to be important too [41].

Latent Tuberculosis Infection (LTBI) reflects the situation in which the infection is contained and controlled without elimination of live *M. tuberculosis*. LTBI may either persist indefinitely [42], or change to complete eradication of live *M. tuberculosis*; or fail, with progression to overt TB. Clinical data suggest that during LTBI hardly any replication seems to occur, as evidenced by the fact that strains within one cluster originally isolated and subsequently emerging after LTBI appear genetically identical [43]. Recent evidence from whole *M. tuberculosis* genome sequence comparisons in a macaque model however has emerged that in fact replication continues during latency at a rate at least as fast as during fast replication [44]. The authors have explained this by assuming DNA change as a result of oxidative stress and they produce some evidence for this hypothesis in their paper. Most of the knowledge on the local immune response with granuloma formation has been derived from murine animal models [45]. Few studies have used non-human primate animal models in exploring the complex process of granuloma formation in TB [46]. The concept that granuloma formation merely provides protection has been challenged [47, 48].

Although most of the evidence was derived from a zebrafish model of *M. marinum* infection, the authors found that the presence of the critical virulence region ESX1 - that contains the Region of Difference-1 (RD-1) genes, are essential to induce host macrophage cell death by the apoptotic pathway. The tuberculous granulomas have long been
considered host-protective structures formed to contain infection. However, work in zebrafish infected with *M. marinum* suggests that granulomas contribute to early bacterial growth [47, 48]. Early Secretory Antigenic Target – 6 (ESAT-6) appears to induce matrix metalloproteinase-9 (MMP-9) in epithelial cells neighboring infected macrophages. MMP-9 enhanced recruitment of macrophages, which contributed to nascent granuloma maturation and bacterial growth. Disruption of MMP-9 function attenuated granuloma formation and bacterial growth [49]. IFN-γ activated macrophages are able to kill *M. tuberculosis* by inducing NO-dependent apoptosis [50]. Apoptosis allows the host to fight *M. tuberculosis* effectively by containing the micro-organism [51]. On the other hand, apoptotic host cells contain viable *M. tuberculosis* bacilli, that are able to escape host IFN-γ immune defense by reduced expression of Ag85B [52]. When these micro-organisms in turn are engulfed by newly attracted host macrophages, this process might allow *M. tuberculosis* to replicate and survive in the host. Although this may occur in human disease, and may explain some of the epidemiology and the intricate host-pathogen interaction, failed granuloma formation as is seen in immuno-compromised hosts is a risk factor for multiplication of the organisms and the subsequent spread of disease as well. Apart from apoptosis, *M. tuberculosis* also significantly induces host cell death by necrosis, by causing plasma membrane micro-disruptions. Resealing of these lesions, a process crucial for preventing necrosis and promoting apoptosis, requires translocation of vesicles derived from the lysosomal and Golgi apparatus to the plasma membrane. Plasma membrane repair depends on prostaglandin E2, which regulates synaptotagmin 7, the calcium sensor involved in the lysosome-mediated repair mechanism. By inducing production of lipoxin A4, which blocks prostaglandin E2 biosynthesis, virulent *M. tuberculosis* prevents membrane repair and induces necrosis [53, 54]. Apart from MMP-9, MMP-1 is also an important inflammatory mediator that is upregulated during *M. tuberculosis* infection. As mentioned earlier, certain drugs – PAS being the most clear example – may have immune-modulatory effects. PAS being chemically closely related to acetosal, appears to modulate the immune response in *M. tuberculosis* – infected isolated human macrophages. MMP-1 (and not MMP-7) appears to be stimulated through the p38 mitogen activated protein kinase (MAPK) signal transduction pathway, and the signaling by p38 MAPK is inhibited by PAS [30]. Necrosis resulting in the release of many live *M. tuberculosis* bacilli escaping the host immune surveillance, with unlimited spread and multiplication presents the greater risk for the host. In summary, a granuloma in the context of LTBI is no longer considered a passive quiescent lesion with low cell turnover and low metabolic activity, but rather a dynamic, immunologically active battlefield of immune cells and replicating bacilli. An ever expanding array of newly detected pro-inflammatory cytokines and chemokines appears involved in this struggle between the host and the pathogen, in a delicate balance with apoptotic cell signalling and necrosis.
IMPORTANT OF RD-1 GENES

Over the last decade, the RD-1 genes have been shown to be highly specific for *M. tuberculosis* [55, 56]. The RD-1 genes coding for TB-specific proteins - ESAT-6 and Culture Filtrate Protein-10 (CFP-10) are not present in *M. bovis* BCG, nor in most of the environmental mycobacteria. The only non-tuberculosis mycobacteria currently known to contain RD-1 genes are *M. szulgai*, *M. marinum* and *M. kansasii*, making tests based on detection of RD-1 proteins potentially useful in clinical practice. Three commercial tests have been developed for use in detection of LTBI – with one also marketed for active TB. The tests are known as IFN-γ Release Assays (IGRA) and either measure the number of IFN-γ positive cells (TSPOT-TB; Oxford Immunotec, UK), or the concentration of IFN-γ after stimulation with Mtb-specific antigens (Quantiferon TB Gold; and Quantiferon TB Gold-in-Tube; both manufactured by Cellestis, Carnegie, Australia). IGRA provide a sensitivity approaching 90% for TB diagnosis using culture of *M. tuberculosis* as the reference test [31, 57, 58, 59, 60]. The problem with IGRA is that these tests do not differentiate between TB and LTBI; and their use as a marker of success or failure in TB has yielded controversial and generally disappointing results (reviewed in [57]); [61, 62]. Genetic expression patterns derived from whole genome analysis studies in two different cohorts of TB patients appear to reflect a highly specific pattern for active TB, differentiating it from LTBI, and healed TB, after completion of treatment [63]. The sophistication of this study is high, and although the study provides much detailed information, a general pattern that emerges from this study is that it largely confirms the importance of upregulation of many genes in the IFN-γ post-signal transduction pathways (JAK-2, STAT-1).

Besides, also IFN-γ/δ pathways appear upregulated, including their post-signalling downstream pathways (STAT-2). This powerful approach might lead to detecting novel useful markers for disease activity and treatment response in the future.

ACQUIRED IMMUNOLOGICAL SUSCEPTIBILITY – ROLE OF HIV

The interplay of *M. tuberculosis* and HIV has been reviewed in detail [16, 15, 9, 14, 31]. Here, we summarize the most important research findings. HIV replication is enhanced at sites with active TB. Reactivation of TB in granulomas where viable dormant *M. tuberculosis* bacilli persist, can at least partly be explained by failure of CD4 cells to secrete IFN-γ and TNFβ. TNFα is essential for formation and maintenance of granuloma formation, and lack of TNFα results in decay and resolution of granulomas. Weakened macrophage killing of intracellular *M. tuberculosis* bacilli and impaired specific anti- *M. tuberculosis* CD4 cell function reflected by impaired secretion of IFN-γ, TNFα and IL2 to *M. tuberculosis* specific stimuli all contribute to the devastating effects of HIV coinfection in TB patients. During TB treatment, paradoxically increased inflammatory responses may mimic failure to respond to treatment and this paradoxical reaction is even worse following cART in which
case the immune phenomenon is referred to as IRIS. Until recently the treatment of HIV infection was postponed at least until the initial intensive phase of TB treatment had been completed, this policy has recently been challenged. In a retrospective analysis, patients in whom cART was postponed until at least two months after start of TB treatment had a significantly decreased chance of survival compared to individuals who were started on cART earlier on [64]. Recently a randomized controlled trial was reported that confirmed that early start of cART in HIV co-infected TB patients is beneficial [65], and also further evidence has emerged in favour of starting cART early on during treatment of TB in HIV-coinfected TB patients [66].

**IMMUNE GENETIC SUSCEPTIBILITY**

Several genetic studies in different founder populations have shown that immunological defense against development of TB is genetically driven [67, 68]. Many different genetic polymorphisms are associated with development of disease or LTBI, but different genes emerged from different genetic founder populations [69, 70, 71, 72, 73, 74]. Genetic polymorphisms in the SLC11A gene that is believed to regulate macrophage bactericidal function by metal (iron) transport have been shown to be associated with susceptibility to TB [75]. Not all genetic polymorphisms that have been shown to be associated with TB have a clear biological explanation yet. In a recent genome-wide association analysis from Ghana and The Gambia, 11,425 individuals were studied. In this combined study, rs4331426, located in a gene-poor region on chromosome 18q11.2, was associated with disease (combined \( P = 6.8 \times 10^{-9} \), odds ratio =1.19, 95% CI = 1.13–1.27) [76].

**VACCINATION; THE ROLE OF BCG**

BCG has not been shown to provide protection against the development of pulmonary TB [77]. In the 1970s, the Indian government ordered a trial to identify the possible protective role of BCG. This largest study ever was conducted in Chingleput, India, with nearly 270,000 participants receiving either a locally manufactured (‘Madras strain’) BCG, a French product of BCG, or placebo vaccination, with a follow-up of 15 years. After 7.5 years of follow-up, the actively vaccinated groups had similar rates of pulmonary TB proven by sputum smear microscopy and culture [78, 79]. International WHO teams confirmed that the study was meticulously conducted. At 15 years follow-up, BCG still failed to show any protection against bacillary-proven TB [80].

A large BCG study conducted later in Malawi likewise failed to show any protection against TB [81]. A post-hoc analysis of the Chingleput trial revealed a low level of overall protection against TB meningitis and other lethal forms of TB in infants and young children (27%, 95% CI - 8 to 50%). In a more recent meta-analysis the protective effect of BCG for infants and young children to develop severe forms of TB is estimated to be at around
50% [82]. In the analysis of an outbreak of TB among infants and children in a nursery in London resulting from a nursery teacher who had had symptomatic pulmonary TB for several months, BCG-vaccinated children appeared protected against LTBI by 66% using IGRA [83] but in this small-sized study the confidence intervals overlaps the much smaller effects reported in earlier studies [84].

The WHO recommends BCG vaccination in the newborn [85] but in newborns and infants with HIV co-infection BCG should not be given [86] as there is considerable risk for severe and potentially lethal disseminated BCG infection [87]. In a recent study from Tanzania, an international research team found evidence that TB patients with previous BCG vaccination as evidenced by a scar had better sputum conversion rates at two months after start of treatment than those without previous BCG vaccination [88]. One problem in the interpretation of this finding is that scar formation following BCG intra-dermal vaccination is highly variable, and does not necessarily correlate with immune protection against TB [84].

BCG vaccination in infants results in complex patterns of cytokine responses, with clearly IFN-γ being the dominant response [89]. Although BCG induces a large array of cellular immune responses in humans and experimental animal models, good correlates of protection have not been identified [84, 90, 91].

Obviously there is a dire need for new, better vaccines, and some 12 novel components are now considered for clinical trials. Most of these moieties aim at boosting a primary vaccination with BCG [91, 92, 93]. An important challenge is to develop correlates of protection so that the response to novel vaccines can effectively be monitored. Potential candidates to be explored as possible surrogate markers for beneficial response should best be identified during follow-up of individuals enrolled in prophylactic vaccine trials, but surrogate parameters might also be identified in patient cohorts during treatment.

THE ROLE OF IRON

Iron is known to play an important role in host-pathogen interactions. The withholding of intracellular iron has been a host defense strategy against intracellular pathogens such as mycobacteria [94]. Iron is essential as a cofactor of enzymes involved in vital cellular functions ranging from respiration to DNA replication. It is not freely available to the intracellular organisms for in the presence of oxygen and at physiological pH, it exists mainly in insoluble ferric complexes. In higher organisms iron is available in bound forms such as transferrin, lactoferrin and ferritin, thereby limiting the levels of free iron required for bacterial survival [95, 96]. The concentration and the availability of iron are important in determining the outcome of the infection with pathogenic mycobacteria [97, 98]. Supplementing iron to patients suffering from various infections may enhance infections by viruses, other bacteria and various parasitic organisms [99]. Excess iron
(Fe), from dietary iron, causes individuals to be more susceptible to TB. Iron overload of both macrophages and hepatocytes is common in sub-Saharan African populations. A plausible reason is drinking of a traditional beverage of high iron content that is brewed in non-galvanized steel containers. Increased iron supply will result in *M. tuberculosis* growth, and iron overload is a known risk factor for infections, as this may worsen the course of disease [100]. Mild iron deficiency causes a significant impairment in the immune status and reduces the capacity to control infections [101]. More severe iron deficiency due to chronic peptic ulceration and blood loss may on the other hand protect against TB. The high prevalence of Helicobacter pylori in populations where TB and other lethal infections remain endemic suggests the host-pathogen interplay of these infections has coevolved. *H. pylori* infection appears to be associated with protection against TB [102].

Mycobacteria acquire iron by secreting high-affinity iron chelators called siderophores that sequester ferric iron. The ferric–siderophore complexes are transported into the bacteria and iron is released in the cytoplasm, probably by reduction. Mycobacteria produce two classes of siderophores, the intra-cellular mycobactins and the extra-cellular exochelins or the carboxymycobactins (also called exomycobactins). Mycobactin is the major intracellular hydrophobic siderophore of most mycobacteria. By virtue of its hydrophobic nature mycobactin acts as a repository for holding iron within the cell envelope before its release into and through the cytoplasmic membrane [103]. A ten-gene cluster designated mbt A-J, contains the core components necessary for mycobactin biogenesis. Mutant *M. tuberculosis* lacking a gene from mycobactin biosynthesis has decreased ability to grow in human macrophages thereby establishing that iron acquisition from the host iron sources is an essential pre-requisite for mycobacteria to be pathogenic [104]. Enzymes involved in mycobactin biosynthesis are important targets for the design of specific inhibitors for TB treatment [105]. One of the oldest anti-TB drugs is p-aminosalicylic acid (PAS) that is believed to exert its effect by the inhibition of mycobactin biosynthesis [94, 105]. *M. tuberculosis* is physically deprived of iron when it is within macrophages [106]. Unless the bacterium is able to acquire iron by synthesizing mycobactin and carboxymycobactin, its virulence is greatly diminished. Ferric-siderophore complexes are recognized by specific surface receptors and translocated through the plasma membrane by ABC-type transporters that are energy dependent [107]. Inactivation of *M. tuberculosis* irtA (Rv1348) or irtB (Rv1349) genes, which encode membrane proteins of the ABC transporter family, results in decreased ability of *M. tuberculosis* to replicate in low-iron medium and to utilize ferric-exomycobactin as the sole iron source [108]. Probably, IrtA-NTD functions as a flavin/ferric reductase that reduces iron in the imported Fe3+-exomycobactin complex for its assimilation [109]. Iron regulates not only the iron acquisition machinery but also the expression of virulence factors / toxins in several other bacterial systems [95, 110].

*M. tuberculosis* scavenges iron from the host-cell through the transferrin - iron
acquisition pathway. IFN-γ, important for the protection against TB, also influences cellular iron status. IFN-γ activation of human monocytes down-regulates transferrin receptor numbers on the cell surface and the rate of macrophage iron acquisition from holotransferrin thereby decreasing iron availability to intracellular microorganisms that utilize transferrin iron [98].

The siderophore-dependent iron acquisition pathways in *M. tuberculosis* were well established as discussed above. Recent studies [111, 112] demonstrate a newly characterized pathway, whereby *M. tuberculosis* can use free heme and heme from hemoglobin as an iron source. The genomic region, Rv0202c-Rv0207c was identified to be responsible for the passage of heme iron across the mycobacterial membrane. The discovery of a unique mycobacterial heme acquisition pathway could open new avenues for drug targets.

In summary, the role of iron in TB is complex, with nutritional factors as well as genetic polymorphisms in iron-handling transport systems (e.g. SLC11A1, formerly called: NRAMP-1) playing a complex role [100, 97].

**THE ROLE OF VITAMIN D**

Vitamin D (vit D) is known to play an important role in host immune defense against *M. tuberculosis*, and vit D deficiency is closely associated with active TB [113]. 1-α 25(OH)2 vit D, the active form, targets various immune cells modulating both innate and adaptive immune responses. The enzyme 25-OH- vit D 1-α-hydroxylase converts the 25-OH- vit D to the active 1-α 25(OH)2vit D. IFN-γ potentiates this effect by up-regulating 1α – hydroxylase [114, 115]. Several mechanisms of anti-mycobacterial activity for 1-α 25 (OH)2 vit D have been described. Exogenous 1-α 25 (OH)2 vit D induces a superoxide burst and enhances phagolysosome fusion in *M. tuberculosis* -infected macrophages [116]. TLR activation of human macrophages, by mycobacterial antigens, results in the up-regulation of expression of vit D receptor (VDR) and the vit D -1 α -hydroxylase genes. This leads to induction of the antimicrobial peptide cathelicidin and killing of *M. tuberculosis* [117]. The cathelicidin antimicrobial peptide is cleaved to LL-37, which restricts the growth of *M. tuberculosis*. Interestingly, several genes classically regarded as being essential for protection against TB are actually down-regulated by 1-α 25 (OH)2 vit D, including IL-12, tumor necrosis factor, and IFN-γ [118]. This is indicative that vit D therapy could both switch on beneficial microbicidal mechanisms, and at the same time decrease the expression of inflammatory mediators that may be contributing to pathology [119, 120].

Serum concentrations of vit D in TB patients are generally but not invariably lower than in healthy controls. In a genetically homogeneous immigrant population of Gujarati Asians in the London area with high rates of TB there was an association between TB and vit D deficiency [121]. In a double-blinded randomized controlled trial, a single dose of 100,000 U (2.5 mg) of vit D3 enhanced anti-mycobacterial immunity in healthy tuberculin
skin test-positive donors [122]. VDR polymorphisms determine the activity of the receptor and might represent potential markers of host susceptibility to TB. The association between vit D physiology and infectious disease is also supported by genetic studies implicating polymorphisms in the gene encoding the VDR in disease susceptibility [123]. There are numerous VDR polymorphisms, including a common Fok1 restriction fragment length polymorphism (RFLP) that shifts translational initiation to an ATG three codons downstream. The Taq1 and Bsm1 RFLPs are present in the 3’ untranslated region. Genetic studies have linked VDR polymorphisms with susceptibility to *M. tuberculosis* infection and treatment outcome. In the past few years, more studies addressing the impact of VDR polymorphisms on TB susceptibility were conducted in different populations. In a case control study of 2,015 African subjects, homozygotes for Taq1 polymorphism (genotype tt) were significantly underrepresented in TB patients [124]. In the study on Gujarati Asians [121], the ff genotype of the Fok1 RFLP was associated with the extent of pulmonary TB in vit D deficient patients. The same study suggested a synergistic association between the T allele and vit D deficiency. The results show that 23% of healthy contacts were of the genotype non-ff and vit D deficient as compared with 46% of TB patients and increasing to 52% of individuals with severe disease. Sunlight exposure modulates skin production of vit D, and studies with vit D supplementation conducted in Africa may therefore not necessarily apply to pigmented individuals with TB in moderate climate zones with less sunlight exposure [125, 126]. In moderate climates, 25-OH-D levels among TB patients are typically low [127] and supplementation of vit D in TB patients may be advantageous; in a small subset of subjects with tt Taq1 VDR genotype it was associated with enhanced sputum culture conversion rates [128]. Interestingly, vit D may be enhanced by BCG vaccination as has been suggested in a study in infants with and without previous BCG vaccination [129].

In summary, elucidation of the mechanisms by which antimicrobial peptides restrict the growth of *M. tuberculosis* could lead to novel pharmacologic approaches. The interaction between vit D, VDR, and effector molecules such as IFN-γ is complex and further studies are warranted to understand metabolic, nutritional and immunological factors in the pathogenesis of TB.

**MONITORING DURING TB TREATMENT**

Diagnostic and disease monitoring assays including the detection of drug-resistant strains of *M. tuberculosis* have been reviewed recently [57, 130]. The adherence to treatment is supported by directly observed therapy (DOT) with witnessed drug ingestion [131]. The response to treatment is monitored clinically and by cultures, side effects are mainly monitored by liver tests. Liver test abnormalities although relatively uncommon are the most frequent reason for treatment interruption [132, 133, 134, 135]. In the WHO guidelines
[131], the suggestion is made to monitor sputum microscopy after the intensive phase – and with smear-positive sputum, a repeat microscopy after three months should be made, with culture and drug sensitivity testing if sputum smear microscopy is still positive after three months. Drug intolerance and liver toxicity are important but relatively infrequent events during follow-up [31]. MDR-TB is a rapidly emerging epidemic. In 2008, 440,000 new cases were detected with an alarming death toll of 150,000 [18, 9], and treatment outcome and treatment duration in XDR-TB are increasingly challenging the health care system [136, 137, 17]. At the level of individual health care, clinicians are faced with only few tools to monitor treatment.

In pulmonary TB (PTB), serial sputum culture may conceivably help to detect treatment failure. Simply testing for sputum microscopy and even culture at any one time point after start of treatment [138] was neither sensitive nor specific enough to guide therapy, but it also failed as a continuous monitoring tool [139] and the technique is not helpful in extra-pulmonary TB (EPTB) [57]. Sputum microscopy is insensitive to differentiate live bacilli from dead bacilli, and PCR-based techniques are similarly insensitive. Real-time quantitative PCR has the potential to measure the presence of \textit{M. tuberculosis} DNA semi-quantitatively by the number of cycle times required for a positive signal. Electronic nose technology has not yet been developed to a level that makes it a promising tool in the near future [140, 141]. Lipo-arabinomannan (LAM) is a cell wall constituent of mycobacteria. Although not entirely specific for \textit{M. tuberculosis}, serial urinary secretion of LAM might provide a tool for monitoring especially when the antigen load is high, such as in EPTB in HIV-co-infected individuals with low <200 cells/μL) [142]. IGRA has been proposed as a monitoring tool but it has largely failed to live up to expectations in this respect as the test remained positive in individuals responding to therapy [57, 61]. Neopterin is a metabolic product of guanosine-triphosphate and is released by macrophages following stimulation by IFN-γ. It has been considered a marker of cellular immune system activation.

Neopterin has been suggested as a marker for diagnosis of TB [143, 144] either alone or in a panel of markers [145, 144].

\textbf{FUTURE DIRECTIONS}

The number of individuals infected with MDR-TB and XDR-TB is increasing. In the absence of novel powerful drugs [146, 9, 147], immune enhancing therapies like therapeutic vaccines, and modalities of treatment interfering with the iron and vitamin D should be addressed and controlled for in future studies. Nutritional intake as well as ultraviolet-induced skin production of vit D are important to consider. Immune-based monitoring will need to be further developed before it can be introduced in clinical practice [63]. Rapid detection of MDR-TB with molecular tools has been possible since almost two decades [148] but an elegant, rapid, robust, and easy-to-use point-of-care system has
only recently been reported [149, 150]. Differentiating failing treatment caused by MDR-TB from paradoxical responses, notably, IRIS in HIV-co-infected patients is critically important [32, 151] especially as evidence is now emerging that corticosteroids may be beneficial in IRIS [152]. New insights into the pathogenesis of IRIS help identify biomarkers that could be useful in predicting or diagnosing IRIS. Studies on immunopathogenesis of IRIS have shown a significant activation of both innate and adaptive immune responses with elevation of plasma or serum chemokines and cytokines. Markers of inflammation such as C-reactive protein, interferon-inducible protein 10 or IFN-γ may be helpful as predictors of IRIS events. In addition, TB-associated IRIS is associated with a prominent Th1 response that can be heightened even prior to ART initiation in cases of unmasking TB, and may assist in early diagnosis. Future studies are needed to elucidate the diagnostic value of IRIS biomarkers [153, 154].
# ABBREVIATIONS

| **M. tuberculosis** = Mycobacterium tuberculosis |
| TB = Tuberculosis |
| PTB = Pulmonary TB |
| EPTB = Extra-pulmonary TB |
| LTBI = Latent Tuberculosis Infection (immune recognition of - and assumed infection with *M. tuberculosis* without clinical disease) |
| MDR-TB = Multi-drug resistant TB (i.e., drug resistant to at least isoniazid and rifampicin) |
| XDR-TB = MDR-TB, with additional resistance to: one of the injectable agents amikacin, kanamycin, or capreomycin - and to one of the fluorquinolones |
| IGRA = Interferon gamma release assay - reflecting immune recognition of region of difference-1 coded proteins of *M. tuberculosis*: ESAT-6 and CFP-10 |
| TNF-α = Tumor necrosis factor alpha |
| TGF-β = Transforming Growth-Factor β |
| IFN-γ = Interferon-gamma |
| IL-10, IL-12 = Interleukin 10, and 12 |
| HIV = Human immunodeficiency virus |
| IRIS = Immune response inflammatory syndrome (denotes paradoxical response following cART in HIV-co-infected TB patients) |
| cART = Combined anti-retroviral therapy (HIV-treatment) |
| Vit D = Vitamin D |
| VDR = Vitamin D receptor |
| BCG = Live-attenuated *M. bovis* Bacille Calmette Guerin. |
| ESAT-6 = Early Secretory Antigenic Target-6 |
| CFP-10 = Culture Filtrate protein-10 |
| RD-1 = Region of Difference-1 (region in the ESX-1 gene coding for ESAT-6 and CFP-10) |
| ESX-1 = Genetic region in *M. tuberculosis* genome interrupting fusion of phagolysosome |
| MMP-1, -7, 9 = Matrix metalloproteases 1,7 and 9 |
| P38 MAPK = P38 mitogen activated protein kinase (several classes have been described) |
| LAM = Lipo-arabinomannan |
| TGF-β = Transforming growth factor - β |
| DOT = Directly Observed Therapy |
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