



## Human tuberculosis I. epidemiology, diagnosis and pathogenetic mechanisms

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# Human Tuberculosis I. Epidemiology, diagnosis and pathogenetic mechanisms

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**Abstract:** *Mycobacterium tuberculosis* (*M. tuberculosis*), a genetically monomorphic pathogen with substantial genomic diversity, is an obligate human parasite, transmitted only by humans and causes tuberculosis (TB), disease firmly associated to poverty, which is responsible for nearly two million deaths annually. Some steps of the progress of our knowledge of *M. tuberculosis* physiology and of its interactions with humans, are reviewed here. This progress has provided fertile ground for improving diagnosis and cure of TB infection. For TB diagnostics laboratories in high-burden countries, primary isolation is the first step before performing drug susceptibility testing (DST) of *M. tuberculosis*. IGRA (interferon- $\gamma$  release assay)-based tests for diagnosis of active TB are sufficiently fast, specific and sensitive to allow to contain infection and distinguish among latent TB infection and BCG vaccination individuals from those who have clinically resolved *M. tuberculosis* infection after anti-TB treatment.

**Keywords:** *Mycobacterium tuberculosis*, tuberculosis, tuberculosis diagnosis, IGRA (interferon- $\gamma$ -release assay), drug susceptibility testing (DST), multi-drug resistant (MDR) tuberculosis, vitamin D, nitric oxide (NO).

## 1. EPIDEMIOLOGY AND DIAGNOSIS OF TB: THE MULTIDRUG RESISTANT *M. TUBERCULOSIS*

The Gram-resistant, acid fast *M. tuberculosis*, a genetically monomorphic pathogen with substantial genomic diversity, is an obligate aerobe, human parasite and can infect many species, but it is transmitted only by humans and has no environment reservoir. It causes tuberculosis (TB), which is responsible for nearly two million deaths annually. This disease is firmly associated to poverty [1-6] and under-developed healthcare infrastructure [7] In eastern countries of the United States of America a highly transitive social networks established by crack users may contribute to TB transmission [8].

### 1.1. Epidemiology

An estimated 1.32 million people who were not infected with the human immunodeficiency virus (HIV) died of TB in 2007 as did an estimated 456,000 people who were HIV-positive; 1.8 million deaths were reported in 2008 and in 2009 they amounted to 1.7 million. Without effective treatment, tuberculosis is associated with substantial morbidity and mortality.

Among sputum-smear-positive cases of pulmonary tuberculosis in HIV-negative patients, the estimated 10-year case fatality rate is 70% [9]. The global burden of TB remains enormous. In 2011 8.7 million incident cases of TB (13% co-infected with HIV) occurred and 8.6 million in 2012, along with 1.4 million deaths in 2011 (990 000 deaths among HIV-negative individuals and 430 000 among people who were HIV-positive) and 1.3 million in 2012 (940 000 deaths among people who were HIV-negative and 320 000 among people who were HIV-positive). These deaths included 0.5 million among women, making TB one of the top killers of women worldwide. About one million cases involve children, with 130,000 deaths per year in the pediatric population, making TB among the top ten causes of death in childhood [10]. Developing countries are at the hotspots where 94% of TB cases and 98% of deaths take place. Migration from countries with a high burden of infection has significantly affected TB epidemiology in western, low-incidence countries that receive substantial numbers of migrants [11]. The impact of immigration on TB in a low-incidence area of Italy, i.e. Tuscany (6-10 bacteriologically confirmed TB cases x 10<sup>5</sup> persons in the last 25 years) has been analyzed [12]. With use of a molecular epidemiological approach about 13 % of the Italian TB cases (614 italian-born out of a total of 1080 TB patients hospitalized in Tuscany during a four year period) was attributed to transmission from immigrants to the local population.

Estimates say one third of the world population – about two billion people – are currently infected with TB without

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manifesting clinical signs of the disease, a condition known as “latent infection” that has allowed *M. tuberculosis* to maintain its reservoir [13]. Among these, only 10% suffer from active TB. The disease often remains latent in the lungs of unknowing victims for years, usually manifesting if and when the victim’s immune system is stressed (which is one reason TB is a leading cause of death among HIV/AIDS sufferers). Because *M. tuberculosis* readily fly through the air, as when an afflicted person coughs, it’s estimated that each victim will infect 10 to 20 more other people – in whom the disease will likely remain latent - before finally getting successfully treated.

## 1.2. Diagnosis

The gold standard to diagnose active pulmonary TB infection remains clinical examination, combined with microscopic analysis of sputum to visualize *M. tuberculosis*, and culture of bacteria. A positive microscopic diagnosis is achieved after spreading a sputum sample on a microscope slide, fixing, applying a stain, decolorizing with acid, counterstaining, and visualizing the acid-fast tubercle bacilli. However, this method is not very sensitive and detects 50 - 60% of culture-positive patients with active pulmonary TB [14, 15]. Moreover, culture of *M. tuberculosis* can take up to 8 weeks (see below), and in 10-20 % of cases the bacillus is not successfully cultured [16]. Diagnosis in these patients depends on clinical findings and radiographic examination. This delays early diagnosis and can result in substantial lung damage and transmission of the disease-causing pathogen before therapy starts.

Tuberculin skin testing *in vivo* (TST or Mantoux skin test, see below) with purified protein derivatives of *M. tuberculosis* has been the major means of diagnosing TB infection for almost the last half century. The principle upon which this test is based, is the assessment whether patients’ memory T cells have been exposed to, and sensitized by, antigens specific to *M. tuberculosis*. When mycobacterial antigens (tuberculin Purified Protein Derivatives, PPD) are injected below the epidermal layer antigen-specific lymphocytes infiltrate the tissue and elaborate inflammatory cytokines, thus promoting induration and erythema of the skin (delayed type hypersensitivity reaction, see below). This assay, however, suffers from two drawbacks represented by the fact that bacillus Calmette-Guerin (BCG, *M. bovis*) vaccination and exposure to non-tuberculosis mycobacteria also elicit a positive response and that it requires two visits for each subject (first for PPD intradermal injection and second to have three days later a skilled reader measure and record the result). Since 10-25 % of TB patients do not react to PPD, test specificity decreases to less than 50% [17]. Moreover, TST does not distinguish between latent TB infection and active TB disease. *M. tuberculosis* genomics has identified regions that are not present in BCG and non-tuberculous mycobacteria (for a review, [18]). This has brought to the discovery that two secretion protein products ESAT-6 and CFP-10 are typically present in all strains of virulent *M. tuberculosis* and

immunogenic in humans (see below). This has led to set up two new *in vitro* diagnostic assays requiring fresh blood samples that are termed interferon- $\gamma$  (IFN- $\gamma$ ) release assays (IGRAs). QuantiFERON-TB Gold (Cellestis) uses enzyme-linked immunosorbent assays (ELISA) to measure differences in the concentration of IFN- $\gamma$ , while T-SPOT.TB (Oxford Immunotec) uses enzyme-linked immunospot assay (ELISpot) to measure differences in the number of cells that produce IFN- $\gamma$  after incubation of whole blood or peripheral blood mononuclear cells (PBMCs) with *M. tuberculosis* antigens. Initially, IGRAs assessed response to PPD or culture filtrate of *M. tuberculosis* [19, 20]. Thereafter, proteins that are present in *M. tuberculosis* but absent from BCG vaccine strains and most nontuberculous mycobacteria were found to improve IGRA specificity [18, 21-24]. ESAT-6, CFP-10 and TB7.7 (also called Rv2654) proved to be able to induce IFN- $\gamma$  release, and manufactured peptides representing these proteins are used in various combinations as antigenic stimuli in commercially available IGRAs designed to detect TB infection [25-27](for a review, [15]). The development of these methods for the *in vitro* immunological diagnosis of TB infection (latent or active) has made possible the rapid laboratory diagnosis of patients with suspected TB and to distinguish responses due to the infection with *M. tuberculosis* from responses due to vaccination with BCG or infections by non-tuberculosis mycobacteria (high estimates of IGRA specificity for TB infection) [28-33]. Estimates of IGRA sensitivity vary widely and range from 70 to 90% based on pooled data from published reviews involving adults with culture-confirmed TB (CCTB) [25, 34]. However, since the sensitivity of IGRAs in culture-positive TB may be as low as 60% - especially in high TB-endemic countries [34], negative results cannot be used to exclude a diagnosis of TB [35, 36]. IGRAs have been approved for the detection of latent TB infection owing to their greater sensitivity and specificity than the traditional TST [37-39] and their higher reliability for the exclusion of *M. tuberculosis* infection [40]. On a sample of CCTB adults (n=21) in comparison with a sample of controls (n=24), measurement of multiple pro-inflammatory and anti-inflammatory cytokines, chemokines and growth factors released by whole blood cells challenged with a combination of antigens (ESAT-6, CFP-10 and TB7.7) was performed (multiplexed assay) [41]. Compared with measurement of IFN- $\gamma$  alone, this assay can improve diagnostic sensitivity for TB infection by assessing simultaneously multiple cytokine responses, using smaller sample volumes and reducing the cost of testing compared to ELISA and other methods. Moreover, the use of combinations of specific *M. tuberculosis* antigens may increase the diagnostic sensitivity by increasing the magnitude of cytokine responses as compared to individual antigens.

Multidrug-resistant (MDR) TB patients harbor strains of *M. tuberculosis* resistant to at least rifampicin (RIF) and isoniazid (INH), two essential drugs in the treatment of TB and constitute a serious problem for the efficient control of the disease (**Figure 1**). Preventing initial infection with

MDR strains of *M. tuberculosis* and managing the treatment of existing cases appropriately are the keys to containing the spread of this disease. Worldwide in 2011, there were an estimated 630 000 cases of MDR-TB among the world's 12 million prevalent cases of TB. Globally, 3.7% of new cases and 20% of previously treated cases may have MDR-TB. Levels of MDR-TB remain worryingly high in some parts of the world, notably countries in Eastern Europe and central Asia. In several of these countries, 9–32% of new cases have MDR-TB and more than 50% of previously treated cases have MDR-TB. Almost 60,000 cases of MDR-TB were notified to WHO in 2011 –against the 30,000 cases of MDR-TB reported in 2008–, mostly by European countries and South Africa. The number of cases reported by 27 high MDR-TB burden countries almost doubled between 2009 and 2011.

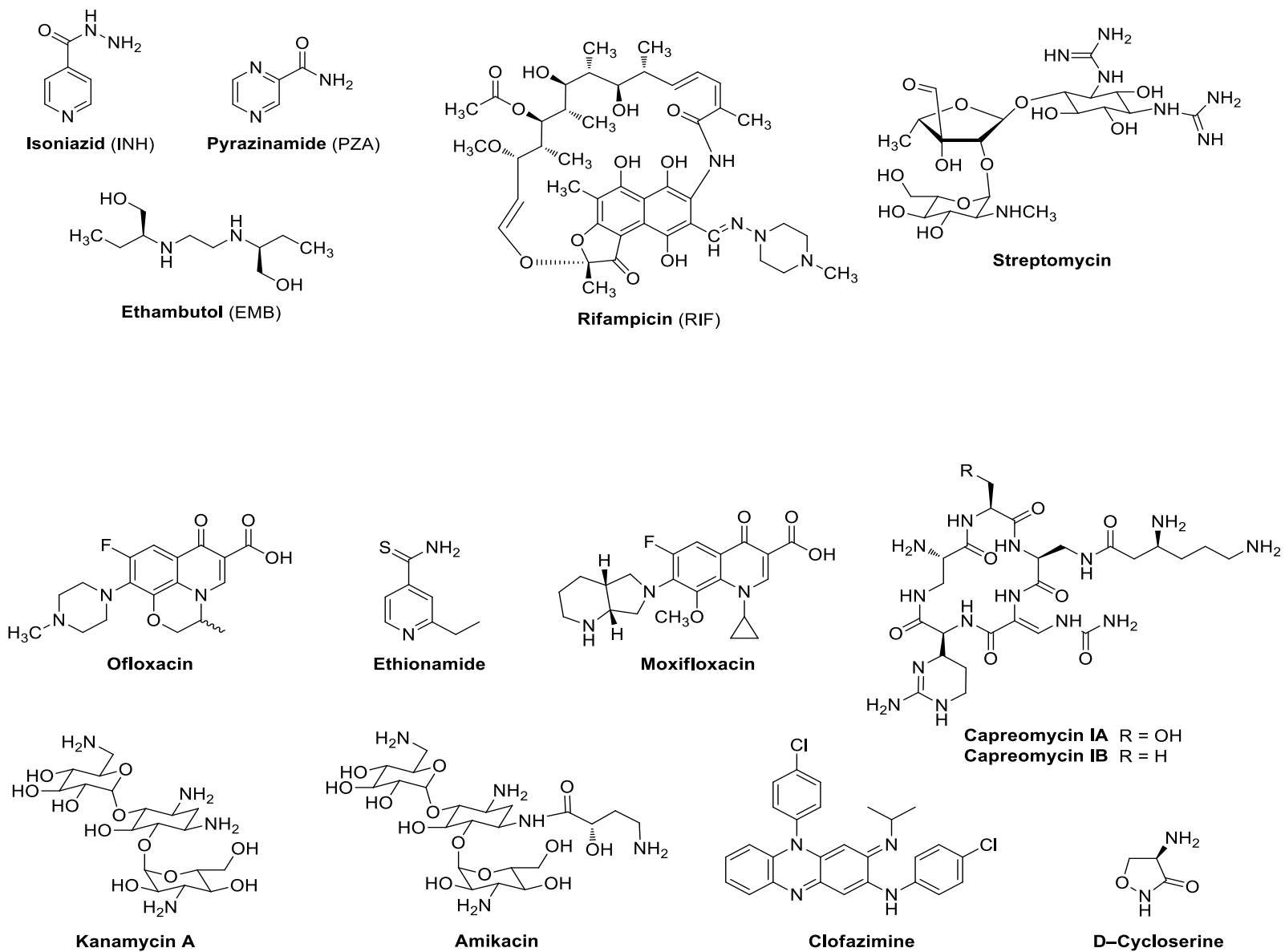
Despite progress, the number of MDR-TB cases notified in 2011 represented only 19% of the estimated 310,000 cases of MDR-TB among reported TB patients with pulmonary TB, and less than 10% in the two countries with the largest number of cases, China and India. Extensively drug-resistant TB (XDR-TB) is defined as TB resistance to at least INH and RIF, in addition to resistance to any fluoroquinolone and to at least one of the three injectable drugs used in the treatment of TB capreomycin, kanamycin and amikacin [42]. The occurrence of XDR-TB involves 84 countries globally. A total of 65 countries and 3 territories reported representative data from continuous surveillance or special surveys on the proportion of XDR-TB among MDR-TB cases. Combining their data, the proportion of MDR-TB cases with XDR-TB was 9.0%. Since 2007, only 13 out of 68 (19.1%) countries and territories have reported more than 10 XDR-TB cases in a single year. Among them, the proportion of MDR-TB cases with XDR-TB was highest in Azerbaijan (Baku city, 12.7%), Belarus (11.9%), Estonia (18.7%), Latvia (12.6%), Lithuania (16.5%) and Tajikistan (Dushanbe city and Rudaki district, 21.0%). Recently, clinical strains of *M. tuberculosis* that are totally drug resistant (TDR-TB) have been isolated in India [43]. Less than 4% of new, bacteriologically-positive cases, and 6% of previously treated cases were tested for MDR-TB in 2011, with particularly low levels of testing in the African and South-East Asia regions. In the European Region, 56% of new cases and 27% of previously treated cases were tested for MDR-TB. China has a serious epidemic of drug-resistant TB, with the highest annual number of cases of MDR TB in the world, a quarter of the cases worldwide. MDR-TB in China appears linked to inadequate treatment in both the public health system and the hospital system, especially TB hospitals; however, primary transmission accounts for most cases [44].

In 2001 an estimated 35% of people did not take their medications correctly, whether for TB or any ailment. The patient who is being treated with several antibiotics and decides to only take one at a time to cut down on side effects is providing the bacillus with ideal conditions for becoming progressively resistant to a series of medications. Anti-TB drugs are widely available over the counter in retail

pharmacies in many countries. This encourages self-treatment and the purchase of inadequate quantities and combination of medicines [45]. In the context of TB, directly observed therapy involves a trained observer watching patients take their TB medications in order to improve adherence and achieve a cure. Directly observed therapeutic short-course (DOTS) programs, which underpin the WHO-recommended Stop TB [46] were introduced in 1992 and show heartening improvements in detection and cure rates, but are not likely to have an impact on existing MDR cases. Use of combination therapy (two or three antibiotics in one capsule) in Europe turned out to be an effective way to circumvent this behavior. It is mandatory that prescription and dispensing of medicines in general, of antibiotics in particular, are well monitored and regulated in the interested countries. Since many of the TB infections in high incidence countries were transmitted recently, the failure to contain MDR- and XDR-TB also reflects the inability to diagnose the problem quickly enough to prevent transmission while continuing to prescribe an ineffective standardized regimen. Individualized therapy could optimize multidrug treatment and limit the further acquisition of resistance. However, in the face of limited resources for the necessary testing and decision making, it has been necessary to adopt a standardized approach, which contributes to further treatment failure in MDR-TB [45]. Optimizing disease management and care of TB drug-resistance needs early detection of drug-resistance to first- and second-line drugs in *M. tuberculosis*, which represents a major priority for TB control programs.

### **1.3 Methods for detecting drug-resistance in *M. tuberculosis***

Conventional methods for detecting drug-resistance in *M. tuberculosis* are slow and cumbersome. Moreover, laboratory diagnosis is complicated by the tedious growth of the tubercle bacillus; the doubling time of *M. tuberculosis* in rich broth media or during logarithmic growth *in vivo* is approximately 24 hours. For TB diagnostics laboratories in high-burden countries, primary isolation is the first step before performing drug susceptibility testing (DST) of *M. tuberculosis*. For primary isolation, the most commonly used proportion method on Löwenstein-Jensen medium or Middlebrook agar requires a minimum of 3-4 weeks whilst the automated mycobacterial culture needs 13 days to produce results. In Peru a microscopic-observation drug susceptibility (MODS) assay was developed which combines broth culture in 24-well tissue culture plates with early growth detection by microscopic examination of *M. tuberculosis* forming the characteristic cords [47]. MODS was proven to provide positive results in a median of 7 days with a sensitivity exceeding that of automated mycobacterial culture or culture on Löwenstein-Jensen medium; moreover, with MODS the time to obtain drug susceptibility results was shorter as compared to the other methods mentioned



**Fig. (1).** First- (upper panel), and second- and third-line (lower panel) anti-tuberculosis drugs.

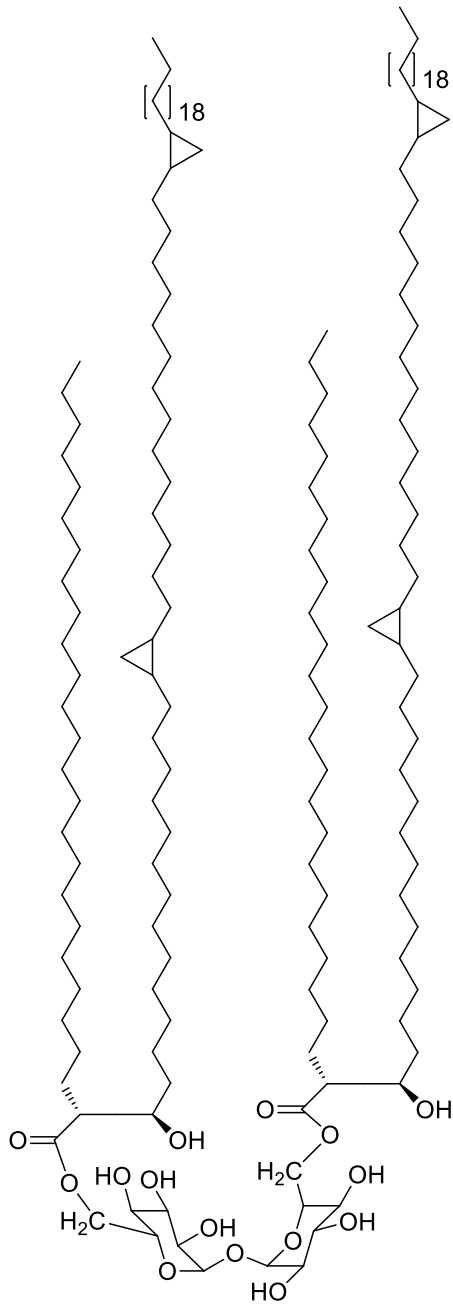
above [48]. In recent years several new approaches have been proposed for the rapid and early detection of drug resistant *M. tuberculosis* which include both phenotypic and genotypic methods [49]. The former relate to detection of growth in the absence or the presence of antibiotics in liquid or solid culture (for a review, [50]). Some of them have reduced the turnaround time to 2-3 days. Generally, they are simple to perform, allow several drugs being tested simultaneously, and should be applicable to all drugs. The latter relate to genetic determinants, involving nucleic acid amplification, to detect gene mutations known to be associated with drug resistance. The costs involved in setting up these methods and their requirement for skilled personnel have precluded their wider implementation in clinical microbiology laboratories, especially in low-resources countries where the TB situation and the problem of drug-resistance are of major concern. Today, however, automated, molecular tests for MDR-TB are available which provide detection of TB and rifampicin (RIF) resistance directly from untreated sputum in less than 2 hours [51]. These tests (e.g., “Xpert”, Cepheid, Inc., Sunnyvale, California, USA, endorsed by WHO to improve the sensitivity of TB diagnosis) use heminested real-time polymerase-chain-reaction assay to amplify an *M. tuberculosis*-specific sequence of *rpoB* gene, that is probed with molecular beacons for mutations within the RIF-resistance determining region [52, 53]. Moreover, they have minimal human resources requirements, and have the ability to detect 70% of TB that is negative by sputum smear. In a compartmental model of a generalizable African TB epidemic using ordinary differential equations, combining the two strategies (i.e., same day microscopy plus Xpert MTB/RIF) proved to have the potential to avert substantial morbidity and mortality in communities within the WHO African Region over a ten-year period [54]. The line probe assays (LPA) such as the INNO-LiPARif.TB test (INNOGENETICS, Belgium) [55] and the GenoType MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) [56] probe assays contain probes specific for *M. tuberculosis* complex, as well as probes for common RIF resistance-conferring mutations, and a subset of the mutations conferring resistance to INH. These assays have been validated in several countries in Africa [57-59] and abroad [60-63]. It is widely recognized that community-based is preferable to clinic-based care. Transmission of MDR *M. tuberculosis* occurs in the community, as indicated by the high frequencies of MDR-TB among previously untreated patients in some countries. In most countries with limited resources, patients with MDR- or XDR-TB must complete two unsuccessful courses of treatment with first-line anti-TB drugs before being eligible for treatment with second-line drugs. Moreover, in many countries treatment of MDR-TB starts only after confirmation of diagnosis, a process that takes months when conventional methods are used. Outbreaks of MDR-TB have occurred in hospitals, and patients with TB have a higher risk of acquiring MDR-TB

(re-infection) when hospitalized than do outpatients. Treating MDR-TB in a hospital is more expensive than doing so on an ambulatory basis. Hospital treatment is also more socially and economically disruptive for most patients. Community-based care has proved to be feasible and effective in several countries. However, the effectiveness of outpatients care depends on the availability of primary care facilities, qualified health care workers, and social support networks to promote adherence to treatment [44].

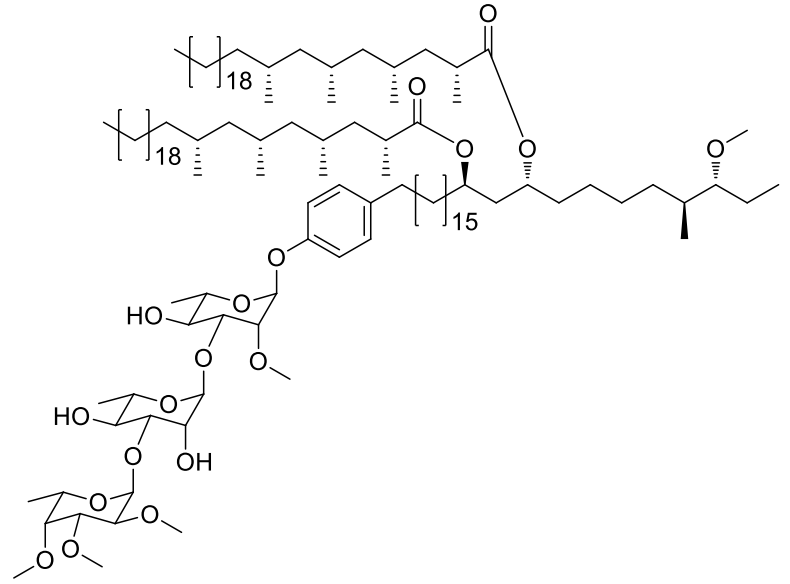
The success or failure of antimicrobial therapy in bacterial infections can be predicted by drug susceptibility testing (DST) performed with radiometric or fluorimetric assay systems; afterwards, microorganisms are divided into treatable and non-treatable categories based on the minimal inhibitory concentration (MIC) breakpoints. MIC breakpoints generally divide bacteria into three categories of susceptibility: susceptible (where the antimicrobial activity is associated with a likelihood of therapeutic success), intermediate (where the antimicrobial activity is associated with an uncertain therapeutic effect) and resistant (where the antimicrobial activity is associated with a higher than expected likelihood of therapeutic failure). Low-level resistance starts at the upper limit of the epidemiological cut-off values [64, 65]. Under-dosed antibiotics by causing low concentrations in tissues of active principles, far below the MIC values, may facilitate the formation of bacterial strains of *Staphylococcus aureus* (*S. aureus*) or *Escherichia coli* (*E. coli*) with altered antibiotic susceptibility or tolerance, which reflect on small MIC changes. Sub-lethal antibiotic treatment of *E. coli*, in fact, has been shown to lead to accumulation of gene mutations at a large target size that cause increase in either susceptibility or resistance to the antibiotics of the same class or of other classes and this may have important clinical implications [66]. The mechanism by which sub-lethal antibiotic treatment leads to multidrug resistance has been investigated in *E. coli* and shown to proceed *via* radical-induced mutagenesis through the formation of ROS. Noteworthy, these effects can lead to mutant strains that are sensitive to the applied antibiotic but are resistant to other antibiotics [67].

#### 1.4 *M. tuberculosis* cell wall structure and its intrinsic resistance to antimicrobial agents

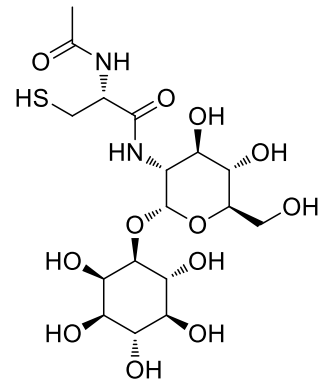
*M. tuberculosis* displays marked intrinsic resistance to a variety of antimicrobial agents, and this property is caused mostly by their unique cell wall structure, which is rich in lipids which in mycobacteria comprise approx. 60% of the dry weight, much higher than Gram-negative (3%) and Gram-positive (0.5%) bacteria [68]. The mycobacterial cell wall contains unique long chain (C<sub>70</sub> to C<sub>90</sub>),  $\alpha$ -alkyl,  $\beta$ -hydroxy fatty acids called mycolic acids, often in the form of trehalose-6,6'-dimycolate (TDM) (**Figure 2**). The synthesis of these fatty acids requires the coenzyme A



**Trehalose-6,6'-di- $\alpha$ -mycolate (TDM)**



**Phenolic glycolipid-1 (PGL-1)**



**Mycothiol (MSH)**

**Fig.(2).** Structure of some cell wall components of *M. tuberculosis*

(CoA)-dependent fatty acid synthase type I (FASI) and the enoyl-acyl carrier protein (ACP)-dependent multi-enzyme fatty acid synthase type II (FASII) system. FASI produces fatty acids of up to C<sub>16</sub> and C<sub>26</sub> chain lengths, and FASII elongates these fatty acids to meromycolates. Acyl chains bound to the ACP which is part of the multienzyme FASII complex, are elongated by repetitive cycles of condensation, keto-reduction, dehydration and enoyl-reduction catalyzed by a synthase, a dehydrase and two reductases of the FASII complex [69-74]. Condensation of meromycolates with the end product of FASI generates mycolic acids [74-76]. Structures of these lipids undergo changes during early pro-inflammatory phases *via cis*-cyclopropanation of mycolic acids on TDM, specifically mediated by proximal cyclopropane synthase activity on alpha mycolates. While this *cis*-cyclopropanation process of mycolic acids appears to be crucial in chronic *M. tuberculosis* persistence and inflammation-associated immune pathology [77], *trans*-cyclopropanation of mycolic acids suppresses *M. tuberculosis*-induced inflammation and virulence [78]. Mycolic acids bind covalently to the peptidoglycan-associated polysaccharide arabinogalactan (**Figure 3**). The FASII enzymes are viable targets for drug development, since eukariotic cells use only a FASI-type enzyme to synthesize fatty acids [79]. The genes encoding FASII enzymes are located in two loci [70, 80-81]. One locus is an operon which includes genes encoding two discrete  $\beta$ -ketoacyl-ACP synthases (*kasA* and *kasB*) and the acyl carrier protein (*acpM*). The ketoreductase (*mabA*) and the enoyl reductase (*inhA*) genes are present in another operon [70, 82]. Depletion of KasA in *M. smegmatis* leads to mycobacterial cell lysis [83]. The natural product thiolactomycin is effective in mouse infection models and inhibits KasA and KasB, with KasA being the most sensitive [72, 84]. Binding of thiolactomycin (**Figure 4**) to crystal structures of *M. tuberculosis* KasA has been described [85]. INH blocks the biosynthesis of mycolic acids, resulting in cell death by lysis [71, 82], by inhibiting InhA [71, 82, 86]. Lipids of *M. tuberculosis* contain also various non-covalently attached lipids and glycolipids. Two structurally related families of non-covalently attached cell wall and capsular lipids, phthiocerol and phenol-phthiocerol esters, have retained special attention. These complex lipids are composed of a mixture of long-chain  $\beta$ -diols, esterified with multi-methyl-branched fatty acids (C<sub>16</sub>-C<sub>22</sub>). Depending on the stereochemistry of chiral centers bearing the methyl branches, the resulting fatty acids are called mycocerosic or phthioceranic acids. Phthiocerol dimycocerosates (DIM) and diphthiocerانات are normal constituents of *M. tuberculosis* and other mycobacterial species [87].

The main structural element of *M. tuberculosis* cell wall above the plasma membrane consists of a cross-linked network of peptidoglycan (PG) in which some of the muramic acid residues are esterified with a complex polysaccharide, arabinogalactan (AG). The AG is bound to

PG through a unique diglycosylphosphoryl bridge, and in turn is acylated at its distal end to PG with mycolic acids (**Figure 3**). The entire complex, abbreviated as the mAGPG (mycolyl-arabinogalactan-peptidoglycan) is essential for viability in *M. tuberculosis* and other mycobacteria [88]. Moreover, mycobacterial porins, the water-filled channel proteins which form the hydrophilic diffusion pathways, are sparse and much less numerous than those of Gram-negative bacteria. Thus, the mycobacterial cell wall functions as an even more efficient protective barrier than the outer membrane of Gram-negative bacteria and limits the access of drug molecules to their cellular targets [89].

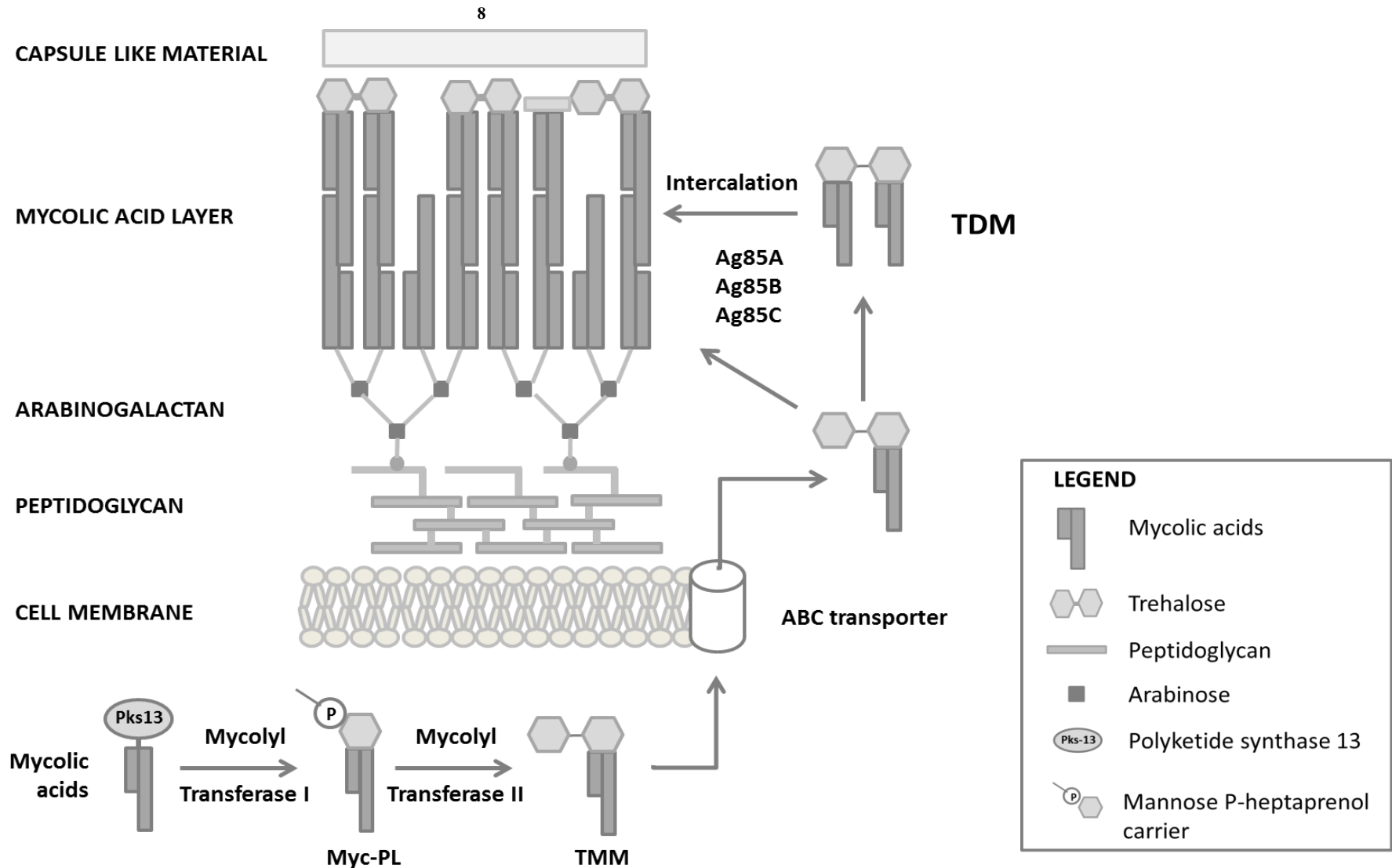
## 2. MYCOBACTERIUM TUBERCULOSIS-HOST INTERACTION: OUTLINES

In 1934, W.F. Wells [90] postulated and demonstrated that “respiratory droplets generated by human coughs and sneeze would desiccate before impacting on surfaces, becoming particles so small they remain airborne as ‘droplet nuclei’, carrying infectious human pathogens from person to person”. The diameter of an infectious ‘droplet nucleus’ is approximately 1 to 3  $\mu$ m, and its content is 1 to 3 bacilli [91]. It is unknown whether a single inhaled ‘droplet nucleus’ is sufficient to cause infection in humans [92]. Inhalation of less than 50 bacilli is sufficient to cause lung infection in guinea pig (see below). Cynomolgus macaques are infected with a low dose (about 25 colony forming units) of *M. tuberculosis* [93] *via* bronchoscope. Although majority of TB is pulmonary, *M. tuberculosis* can disseminate to and infect most organs and tissues causing a variety of TB diseases.

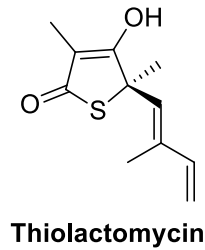
### 2.1 How *M. tuberculosis* enters the lung and gives rise to granuloma formation: Mycobacterium secretion systems and secreted proteins

*M. tuberculosis* enter lungs of healthy individuals by inhalation, where they are phagocytosed by alveolar macrophages and dendritic cells that eliminate most of invading mycobacteria generating potent cell-mediated immunity (CMI) and granuloma formation in lung tissue (see below). Among the many lipids present in the wall of *M. tuberculosis*, chord factor (the factor considered responsible for the growth of mycobacteria in culture forming chains or chords), structurally solved as TDM [94] is very abundant, the most toxic and most potent activator of the innate immune system in mammals [95]. TDM, a highly water-insoluble, wax-like substance, is a potent immune-adjuvant for enhancing CMI through the recognition by the macrophage inducible C-lectin (Mincle) receptor and *in vivo* Mincle-deficient mice are incapable of forming granulomas after TDM injection [96]. At variance with this finding, however, these mutant mice are capable of mounting an





**Fig.(3).** Diagrammatic representation of the mycobacterial cell wall and proposed pathway involved in mycolic acid processing and export. The cartoon depicts some components of the cell wall while primarily focusing on trehalose monomycolate (TMM) and trehalose dimycolate (TDM). Arrows indicate hypothetical or proposed steps. The inset legend represents the shapes used to depict the components shown. Note that the diagram is not to scale and does not include detailed structures or all components of the mycobacterial cell wall. Antigen 85 proteins (Ag85A, -B, and -C), mediate mycolate transfer from one TMM molecule to another to give TDM, and, also, from TMM to the cell wall arabinogalactan. Adapted with permission from [286] (permission to be requested).



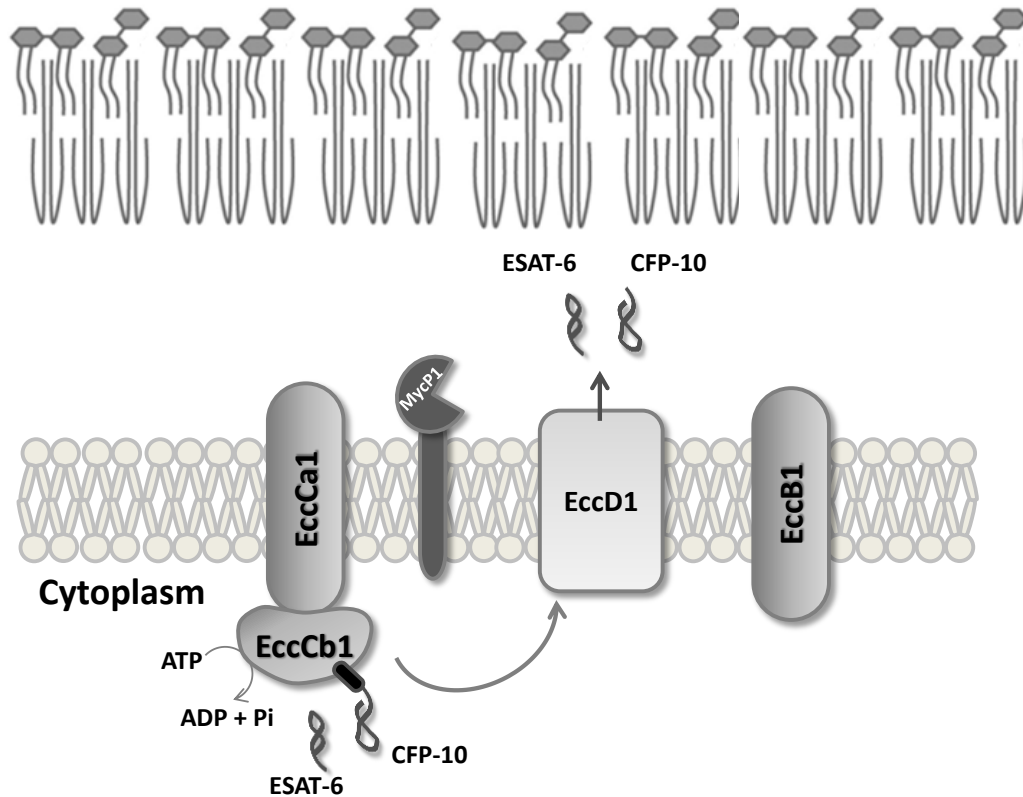
**Fig. (4).** Thiolactomycin structure

efficient granulomatous and protective immune response after low and high dose infections with *M. tuberculosis*, thus indicating the dispensability of Mincle and the possible involvement of other receptors [97]. Other mechanisms by which *M. tuberculosis* may invade cells other than phagocytes (e.g. epithelial cells) include a surface exposed adhesin termed HBHA (heparin-binding hemagglutinin); *M. tuberculosis* strains lacking HBHA were found to be defective in colonizing spleen and liver, indicating that HBHA is critical for dissemination from the lung to other organs [98].

Tuberculous granuloma formation is promoted by a mycobacterium virulence determinant, elaborated by a secretion system encoded by RD (region of difference)1 locus [99], – the loss of which is the main molecular mechanism of attenuation of BCG [100, 101]-, identified as the bacterial secreted, already mentioned, protein ESAT-6 (early secreted antigenic target, 6 kDa protein). This interacts with host epithelium where it induces matrix metalloproteinase-9 (MMP-9), thus contributing to nascent granuloma maturation and bacterial growth by enhancement of macrophage recruitment [102]. The Mincle pathway is also involved in neutrophil-promoted, early phase of TDM-induced lung inflammation by enhancing neutrophil integrin expression, cytoskeleton remodeling and cell adhesion. Furthermore, neutrophils enhance TDM-induced lung inflammation by producing pro-inflammatory cytokines/chemokines [103]. Other mycobacterial secreted proteins which ethero-dimerize with ESAT-6 are those related to CFP-10 (culture filtrate protein, 10 kDa) family [104] (for a review, [15]). CFP-10 and ESAT-6 are potent T-cell antigens, recognized by TB patient sera [28]. ESAT-6, CFP-10 and their complex modulate the macrophage signalling pathway, and in particular the ERK 1/2 MAP kinase pathway [105] through a strong inhibition of the phosphorylation and subsequent activation of extracellular signal-regulated kinases 1/2 (ERK1/2) in the nucleus. This inhibition is mediated by an increase in phosphatase activity in the nucleus, which in turn causes de-phosphorylation of pERK1/2 coming from the cytoplasm. The limitation of ERK1/2 activation affects the expression of c-Myc, a key factor in macrophage activation, and thus down-regulates the expression of LPS-inducible *c-myc* gene. CMI

orchestrated by T-cell-derived lymphokines and carried out by the effector cells, TDM-activated macrophages [106], is so potent that about 90% of the immunocompetent humans infected with *M. tuberculosis* are able to contain the infection (latent TB) and avoid progression to clinical disease during their lifetime [107].

*M. tuberculosis* has several systems to export proteins. In addition to the well-known, highly conserved Sec and Tat, mycobacteria possess specialized SecA2 and ESX protein export systems (reviewed in [108]). ESX or type VII protein secretion systems are encoded by (identified so far) five genetic loci, named ESX-1 to ESX-5. The best studied system, ESX-1, encoded by *esx-1* genomic locus located in RD-1, is responsible for the secretion of 10 substrates, including ESAT-6 and CFP-10 (**Figure 5**), and is required for full virulence of *M. tuberculosis*. ESAT-6 and CFP-10 are exported together as a 1:1 complex which is formed in the cytoplasm and each protein depends on the other for its export [109, 110]. CFP-10 has a targeting element at its C-terminus that directs it to interact with cytosolic AAA ATPase EccCb1, which delivers CFP-10 to an ESX-1 export channel [111]. ESX-5, the next best studied ESX system after ESX-1, exports PE/PPE proteins that contain N-terminal Pro-Glu or Pro-Pro-Glu repeats, after which they are named [112], and are implicated in virulence and/or elicitation of host immune responses (reviewed by [113]). The triacylglycerol lipase LipY in slow growing mycobacteria has a PE or PPE domain. *M. tuberculosis* PE-LipY and *M. marinum* PPE-LipY are both secreted to the bacterial surface in an ESX-5-dependent fashion and, after transport the PE/PPE domains are removed by proteolytic cleavage [114]. This suggests that the PE or the PPE domain of the LipY homologues functions as a secretion signal that is recognized by the ESX-5 secretion system. After secretion, processed LipY is loosely associated with the bacterial surface where it can hydrolyze triacylglycerols (**Figure 6**). Both the innate and adaptive immune systems, alveolar macrophages, neutrophils, T cells, and numerous cytokines and chemokines are important regulators of the immune response to *M. tuberculosis* and granuloma formation in both the human and the guinea pig model of primary TB ( for reviews [14, 115, 116]). The cascade of immunological events shows first the engulfment of *M. tuberculosis* by alveolar macrophages. The bacilli, in turn, may inhibit phago-lysosomal fusion within macrophages (see below). Macrophages produce cytokines (IFN- $\gamma$ , TNF $\alpha$ , IL1 [CXCL8], IL10, IL12, TGF $\beta$ , MCP1 [CCL2], GM-CSF, and RANTES [CCL5]). By means of the major histocompatibility complex (MHC) *M. tuberculosis* antigens are presented to CD4+, CD8+ T helper cells, as well as to CD1 and  $\gamma\delta$ T cells. After presentation also to dendritic cells, antigens are transferred from lungs to draining lymph nodes. CD4+ and, over time, CD8+ T helper cells become activated in lymph node tissues. CD4+ cells produce IL2 to increase



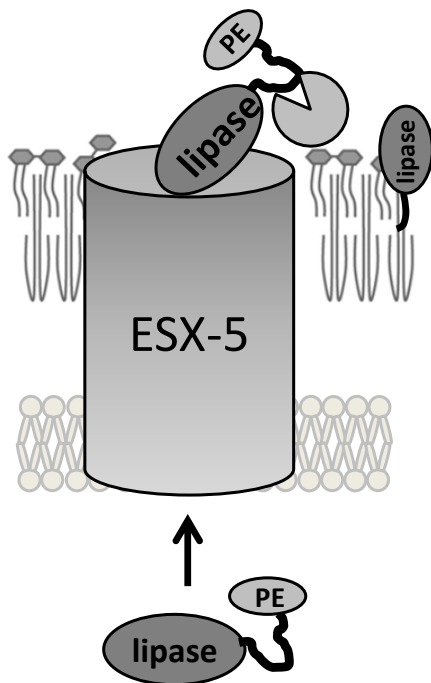
**Fig. (5).** A model of the core components of the ESX-1 export system. ESX-1 exported proteins CFP-10 and EspC have a targeting element at their C-terminus (black ovals) that directs them to interact with the cytosolic AAA ATPases EccCb1 and EccA1, respectively. Through interactions with cytoplasmic membrane proteins, these AAA ATPases seem able to deliver proteins to an ESX-1 export channel. EccD1 is a leading candidate for being a component of this channel. ESAT-6 lacks a C-terminal targeting element and must form a complex with CFP-10 in order to be exported. The functions of EccCa1 and EccB1 are unknown, but all are predicted cytoplasmic membrane proteins and core components of the ESX-1 pathway. MycP1 is a membrane protein with protease activity with a serine protease domain located on the cell wall side of the membrane. Adapted with permission from [108] and [114] (permissions to be requested).

the pool of lymphocytes specific for antigen. The primed T cells migrate back to the site of infection within the lungs and cause granuloma formation.

Within eight weeks after human infection and coincident with the development of delayed-type hypersensitivity (DTH, tuberculin conversion, i.e. TST positive), these granulomas undergo caseous (caseum= cheese) necrosis, resulting in the destruction of surrounding host tissue and bacillary death. However, a small proportion of bacilli mainly extracellular and particularly in the caseous pulmonary lesions live in an environment characterized by anoxic conditions [117] nutrient limitation and acidic pH [118] and the presence of numerous enzymes released from the dead cells. They can persist for many years in the host by entering a dormant, hypo-metabolic state of growth arrest and antibiotic indifference.

## 2.2 Evolution of TB: the caseous necrosis

In untreated patients developing of post-primary TB begins as a lipid pneumonia accompanied by bronchial obstruction in which infection is restricted to foamy macrophages; cavities result from a combination of caseation of tuberculous pneumonia and microvascular occlusion characteristic of DTH [119]. Pulmonary caseous necrotic material in humans is composed primarily of host lipid [102, 121]. Mice treated with high doses of *M. tuberculosis* develop a chronic infection that, like most human TB, is restricted to the lung where large amounts of lipid (especially cholesterol) appear to be concentrated in alveoli, residing in foamy alveolar macrophages [122, 123]. Noticeably, in both humans and mice caseous necrosis is associated with localization of *M. tuberculosis* within lipid droplets [123]. Mycobacteria induce the transformation of *in*



**Fig. (6).** LipY secretion and processing. The model shows how PE or PPE domain of the LipY homologues functions as a secretion signal detected by the ESX-5 secretion system. After recognition, LipY is transported across the mycobacterial cell envelope by the ESX-5 system, and during or after translocation the PE or PPE domain is proteolytically removed, possibly by the mycosin associated with the ESX-5 system. Maturation only occurs in strains with a functional ESX-5 system, and no further processing takes place during extended incubation of lysed bacteria. After secretion, processed LipY is loosely associated with the bacterial surface, where it can hydrolyze TAGs. Adapted with permission from [108] and [114] (permissions to be requested).

*in vitro* grown human macrophages into foamy macrophages within 6 days, and even more rapidly (3-4 hours) in cultured macrophages [124]. Developing caseation is anatomically associated with ingestion of fat by macrophages that die to form caseation. The onset of caseous necrosis of granulomas coincides with CMI and DTH. CMI is the capacity of activated macrophages to kill *M. tuberculosis* and CMI and DTH are associated to tissue damage (the “Koch phenomenon”) [125]. That these elements are the opposite sides of the same coin becomes evident when reduction of both components in HIV-positive patients with TB coincides with CD4 counts decline, and when the signs and symptoms of TB in some HIV-positive patients receiving effective antiretroviral therapy paradoxically worsen [126, 127]. Caseation is the “solid” necrosis of the exudative initial alveolar lesion and of the lung tissue surrounding the lesion. Whilst the bacillary content of the lesion in which the caseation process is beginning is high, the number of viable bacilli in old caseous *foci* is limited or null. Caseous necrosis correlates to DTH. All factors that increase DTH, for example the mixture of bacilli and oil or TDM in an oil-in-water emulsion [95], enhance caseation; moreover, animal species that have high DTH responses develop strong caseation and *vice versa* (see below). Activated

cytolytic T lymphocytes kill *M. tuberculosis*-infected macrophages, leading to destruction of surrounding tissue. During the process, the majority of tubercle bacilli die, while some survive extracellularly in the caseous material but are unable to multiply (see below). The necrosis can become more or less organized or it can soften. In up to 90% of the infected individuals, highly activated macrophages surround the caseous center. The bacillary antigens released by the dead bacilli expand T-cell populations. These T cells release interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-6 and other lymphokines that activate local macrophages. Such macrophages ingest and destroy the bacilli that escape from the edge of the caseum.

In humans with untreated pulmonary TB, caseous granulomas in the lungs contain lipid-loaded foamy macrophages harboring acid-fast bacilli [119]. Such lipid-loaded macrophages inside the hypoxic environment of the tuberculous granuloma contain abundant stores of triacylglycerol (TAG) and provide a lipid-rich microenvironment for *M. tuberculosis* [124]. Human macrophages cultured under hypoxia (1% O<sub>2</sub>) accumulate TAG in lipid droplets [128]. Genome-wide microarray analysis was exploited to assess whether genes for lipid sequestration and metabolism were highly expressed in caseous human TB granulomas [129]. Immune-histological analysis of these granulomas confirmed the disproportionate abundance of the proteins involved in lipid metabolism in cells surrounding the caseum; namely, adipophilin, acyl-CoA synthetase, long-chain family member 1 and saposin C. Mass-spectral analysis of the lipid material within the caseum of granuloma identified cholesterol, cholesteryl esters, triacylglycerols and lactosylceramide, which implicated low-density lipoprotein-derived lipids as the most likely source. The abundance of lipid and cholesterol within the granuloma finds a reflection in the accumulation of foamy macrophages. These cells result from excess lipid uptake. This originates from an imbalance of the export of low-density lipoprotein (LDL) through macrophage-associated efflux pumps like ABCA-1, and the excess uptake of LDL through scavenger receptor A (SRA) and CD36, in addition to other mechanisms like pinocytosis [130]. These findings provide evidence that development to caseation of human TB granuloma correlates with pathogen-mediated dysregulation of host lipid metabolism and excess intake of endogenous lipids by macrophages. The progression of TB to active disease and transmission involves the development of a caseous granuloma that hollows and releases infectious *M. tuberculosis* bacilli.

The relationship between macrophages and *M. tuberculosis* as to their lipid metabolism and the development in *M. tuberculosis* of dormancy/drug tolerance are matter of the section “Mechanisms of mycobacterial phenotypic resistance to anti-TB drugs” (Sgaragli & Frosini. Human tuberculosis II, this issue).

### 2.3 Caseous lesions in resistant hosts and the “latency” phenomenon

In a resistant host, the caseous lesion is capsulated. At the early phase of TB studies, such lesions seemed to be devoid of *M. tuberculosis* [131, 132]. Along with lesions that comprise healing scars and progressing ones, some caseous lesions can persist for long periods of time without a defined capsule or softening of the caseous center. Viable extracellular bacilli persist in these caseous lesions in a state of “latency”. In up to 10% of the infected individuals [133], the hard caseum softens. Because of softening infection with *M. tuberculosis* progresses into TB, the disease [131]. In some cases, especially when the softened caseum lesion is not open to the bronchi, the softening of the caseum is not associated with an increase in number of the bacilli. In most cases, however, the softening of the caseum is associated with emptying of the softened material through a communication with the bronchial tree (the same happens in the rabbit), the formation of a lung cavity and explosive growth of tubercle bacilli in the newly oxygen-enriched environment [131, 134]. For the first time during the course of active TB, the bacilli are free to multiply extracellularly. With a cough, the softened caseous material with bacilli flows out into the bronchi and to other parts of the lung and to the outside environment. Usually, the tuberculous cavity does not heal spontaneously. The outcomes depend on the competency of the patient’s immune system. The bacilli, discharged from the cavity, are ingested by non-activated macrophages in which they temporarily grow until tissue-damaging hypersensitivity process kills the bacilli-containing macrophages and destroys nearby tissues. A new caseous lesion is then created and if the caseation process is renovated a large part of the lung is destroyed and the patient eventually dies; 50% of patients with cavitary lung TB died before the advent of anti-TB drugs. However, in a host with good CMI, immunocompetent T cells activate macrophages that become able to kill the intracellular bacilli without tissue damage. In such TB patients (25%, before the antibiotic era) continuous destruction of host tissue is not necessary to contain the growth of bacilli and the lesions become more or less stable. Intermediate between these two extreme events, another 25% of untreated patients experienced a chronic waxing-and-waning course of their cavitary TB [92, 133].

#### 2.4 Role of macrophages in TB

The macrophage is the most important player in TB where it has two mutually contradictory roles. On one hand, activated macrophages are capable of killing or at least controlling the growth of *M. tuberculosis*. Traditionally, the organized aggregates of immune cells, granulomas, restrict mycobacterial growth [135]. On the other hand, macrophages provide the prime growth niche for this intracellular organism. In a study performed on zebrafish embryos infected with *M. marinum*, however, it is proposed that granulomas may help to promote infection, rather than simply contain it [102, 136]. By means of quantitative intravital microscopy to reveal distinct steps of granuloma

formation and assess their consequence for infection, intracellular mycobacteria were shown to use the *ESX-1/RDI* virulence locus to induce recruitment of new macrophages to, and their rapid movement within, nascent granulomas. This motility enables multiple arriving macrophages to efficiently find and phagocytose infected macrophages undergoing apoptosis, leading to rapid, iterative expansion of infected macrophages and thereby bacterial numbers. The primary granuloma then seeds secondary granulomas via egress of macrophages infected by mycobacteria that turned out to be genetically drug-tolerant. In the same experimental model also INH treatment facilitates growth of tolerant bacteria inside macrophages, that can give rise to granulomas by recruiting new macrophages and/or migrate to disseminate infection. This observation provides insight into how these pathogenic, tolerant mycobacteria exploit the granuloma during the innate immune phase for local expansion and systemic dissemination [137].

The delivery of *M. tuberculosis*, and of particles in general, to the phagosome and the initiation of bactericidal responses requires an encounter with macrophages and ingestion through a receptor-mediated and actin-dependent process called phagocytosis. At the interface between mycobacteria and macrophages, cell wall probably plays a role in facilitating host cell entry. Some envelop molecules have been shown to interact with macrophage phagocytic receptors. Mycobacterial lipoarabinomannan [138] and phosphatidylinositol mannosides, as well, [139] bind to the mannose receptor, one of the major receptors for mycobacterial entry via the glycosylated part of the molecule [140, 141]. Another important phagocytic receptor of macrophages, the complement receptor 3 [142], recognizes outer mycobacterial capsular polysaccharides through its lectin site [143] and phosphatidylinositol mannosides, as well [144]. In a study with use of *M. tuberculosis* mutants deleted for genes involved in dimycolate (DIM) biosynthesis, it was shown that DIM participates both in the receptor-dependent phagocytosis of *M. tuberculosis* and the prevention of phagosome acidification [145].

#### 2.5 Phagosomes and the role of NO

Throughout infection, mycobacteria are either intracellularly residing largely within phagosomes - but also in autophagous vacuoles [146] where they may be exposed to peptides derived by endo-proteolysis of ubiquitin that can be mycobactericidal *in vitro* [147] - of the infected cells or very few into the cytosol and extracellularly. The controversy of the intracellular localization of *M. tuberculosis*, i.e. inside or outside the phagosome, has been intense in recent times. Once bacteria are ingested by resident human alveolar macrophages, the phagosome undergoes a process of maturation (endocytic pathway) thereby acquiring antimicrobial properties. These include acidification (about pH 4.5 in mice), production of antimicrobial peptides, activation of NADPH oxidase (NOX) and inducible nitric

oxide synthase (iNOS) leading to generation of reactive oxygen species (ROS) and nitrogen intermediates (RNI), and finally degradative enzymes such as cathepsins and other lysosomal hydrolases. That iNOS has a role in controlling murine TB is evidenced by the fact that the primary product NO either from activated murine macrophages or exogenous sources exhibits anti-mycobacterial properties and can irreversibly damage bacteria [148]. NO, in fact, acts as a potent oxidant and inhibitor of cellular respiration [149-152]. Other lines of evidence for the role of iNOS in controlling TB [153] here are summarized: Immunologically activated, iNOS expressing mouse macrophages can kill *M. tuberculosis in vitro*, but not if macrophages are treated with iNOS inhibitors [154], nor if they bear disrupted NOS2 alleles [155]. iNOS is expressed in infected mouse tissues in which the growth of *M. tuberculosis* is restrained, whereas iNOS is scant or absent when immunosuppressive drugs or genetic interventions impair host resistance (reviewed in [156]). Healthy mice infected with *M. tuberculosis* succumb abruptly to TB after treatment with specific iNOS inhibitors [156, 157]. Similarly, mice with disrupted NOS2 alleles die with fulminant TB in a few weeks whereas wild type mice survive the infection for about 9 months [156, 158].

Once the macrophage becomes capable of killing bacilli, however, *M. tuberculosis* for its survival and growth in the macrophage seems capable of developing a strategy which consists in both escape from the phagosome and inhibition of phagosome maturation (for a review, [159]). The pH of phagosomes in naïve macrophages ranges from 6.3 to 6.5 [160], whereas the pH of activated macrophages ranges from 4.5 to 4.8 [161]. To survive inside phagosomes and lyso-phagosomes, mycobacteria inhibit the acidification of phagosomes by producing and secreting ammonia [162] or by inhibiting the vacuolar proton pumping ATPase through secretory factors [163]. *M. tuberculosis* urease may contribute to this strategy by producing ammonia, known to inhibit phagosome-lysosome fusion in macrophages [164]. The alkalinizing effect of urease activity within the mycobacterium-containing vacuole in resting macrophages has been confirmed, but not in the more acidic phago-lysosomal compartment of activated macrophages [165]. Gaseous ammonia generated also by the catabolism of L-aspartate in *E.coli* may influence multidrug resistance between physically distant colonies of Gram-positive and Gram-negative bacteria, where it increases the intracellular content of polyamines that leads to alterations in membrane permeability to different antibiotics and enhances protection against oxidative stress [166]. Whether this holds true also with *M. tuberculosis* needs assessment.

Studies have shown that immune-activation of murine bone marrow derived macrophages with IFN- $\gamma$  and/or LPS releases the arrest of phagosome maturation by mycobacteria [167, 168]. TDM coated beads are capable of slowing down phagosome maturation, thus suggesting that TDM is contributing to mycobacterial survival in bone marrow-derived macrophages; TDM effect is abolished in immune-activated (by IFN- $\gamma$  and/or LPS) wild-type, but not

in activated nitric oxide synthase deficient macrophages. Confirmation that NO counteracts TDM's virulence function was revealed by experiments showing that specific nitric oxide synthase-2 inhibition by N(G)-nitro-L-arginine methyl ester prevented inactivation of TDM in activated macrophages. Noteworthy, when TDM coated beads are treated with NO donors, e.g. sodium nitro-prusside and 3-morpholininosydnonimine, phagosomes quickly traffick to phago-lysosomes indicating that TDM is inactivated. NMR analysis reveals alteration of TDM's hydroxyl groups by nitric oxide, thus indicating that intact hydroxyl groups are essential moieties of TDM in inhibition of phagosome maturation [169].

In cryo-immunogold TEM (transmission electron microscopy) images, increasing numbers of *M. tuberculosis* are into the cytoplasm of *in vitro* infected human dendritic cells and macrophages when cell infection proceeds beyond two days. This indicates that bacterial replication is enhanced once *M. tuberculosis* have escaped from the lysosome-associated protein 1- (LAMP1-) and cathepsin-rich phagosome [170]. A mycobacterial, virulence gene cluster extending RD1, is required for macrophage cytolysis, bacterial spreading and ESAT-6 secretion [171]. The best studied function of ESAT-6 is its role in plasma membrane lysis, which seems to be essential for the spread of *M. tuberculosis* from one macrophage to another [171, 173]. Moreover, the ESAT-6/CFP-10 complex can inhibit the production of ROS, thus interfering with LPS-induced ROS production. As a consequence, the down-regulation of LPS-induced nuclear factor-kB (NF-kB), DNA binding activity [174], causes a reduced expression of several pro-immune mediators (TNF- $\alpha$ , IL-2 and IFN- $\gamma$ ) and nitric oxide synthase [175, 176].

One explanation for the presence of cytosolic *M. tuberculosis* is that the phagosomal membrane is degenerated when bacilli lyse the cell and spread to adjacent cells [177]. The possible function and significance of *M. tuberculosis* escape from the phagosome and the context in which the bacterium escapes remain unresolved.

## 2.6 Role of vitamin D

Macrophage anti-mycobacterial responses involve vitamin D, as already mentioned. Cholecalciferol (vitamin D3) and its metabolite 1,25-dihydroxy vitamin D have immunomodulatory activity *in vitro* in a human macrophage-like cell line with induction of nitric oxide synthase and suppression of growth of *M. tuberculosis* [178]. Vitamin D3 has been shown to be involved in the killing of *M. tuberculosis* inside human macrophages through the induction there of production of cathelicidins, multifunctional antimicrobial peptides, that are key components of innate host defences against mycobacteria [179-181]. This is achieved upon Toll-Like Receptors (TLRs) stimulation to upregulate in human macrophages the vitamin D receptor and the vitamin D-1-hydroxylase gene [183, 183] or through simultaneous stimulation with pro-inflammatory cytokines [14, 184] as discussed above. T

cells by the release of IFN- $\gamma$  or CD40L induce autophagy, phagosome maturation, the production of antimicrobial peptides such as cathelicidins and antimicrobial activity against *M. tuberculosis* in human macrophages via a vitamin D-dependent pathway [185, 186].

The guinea pig model of TB has been used to evaluate the effects of malnutrition on TB [187], and the ensuing vitamin D deficiency that has been considered since long time a risk factor for TB among human populations [188]. African-Americans have significantly decreased amounts of 25-hydroxyvitamin D because their skin melanin content diminishes ultraviolet-dependent cutaneous vitamin D3 synthesis [189]. In addition, African-Americans have increased susceptibility to TB [190]. The importance of nutritional (i.e. vitamin D intake) as well as environmental factors (i.e. exposure to sun light) in human TB is outlined by a systematic review and meta-analysis of seven studies. They show that low serum vitamin D levels are associated with higher risk of active TB [191]; moreover, low serum vitamin D level is a good predictor of prolonged clinical course in patients with active pulmonary TB [192]. Supplementation with vitamin D3 to patients treated for TB in Guinea Bissau showed no clinical effect [193]. In a similar study in London there was a significant effect on time to sputum conversion, but only in a subgroup of TB patients with a specific vitamin D receptor (VDR) polymorphism [194]. Although malnutrition may have great impact on the TB epidemic, it has been difficult to show convincingly a beneficial effect of vitamin D supplementation in clinical studies, as described in a Cochrane review [195]. A clinical investigation was performed recently on ninety-five patients receiving antimicrobial therapy for pulmonary TB. They were randomized to receive adjunctive high-dose vitamin D or placebo. This study allowed to appreciate a role for vitamin D supplementation in accelerating resolution of inflammatory responses during TB treatment by enhancing treatment-induced resolution of lymphopenia, monocytosis, hyper-cytokinaemia and hyper-chemokinaemia, and accelerated sputum smear conversion [196].

## 2.7 Antimicrobial responses to TB: role of TNF- $\alpha$ and NO

The maturation of phagosomes containing mycobacteria and the control of infection (*M. tuberculosis* dormancy or growth suppression) are driven by IFN- $\gamma$ , secreted by Th1 and NK cells, which stimulates in the macrophage the arginine-derived NO pathway. Th1 and NK cells, as well, undergo stimulation by *M. tuberculosis*-infected, antigen presenting cells. Interaction of *M. tuberculosis* with TLRs in monocytes and macrophages leads to a cascade of anti-mycobacterial responses which include TNF- $\alpha$  production and upregulation of the vitamin D3-cathelicidin-LL37 pathway (for a review, [197]). Mice deficient in TNF- $\alpha$  production are highly susceptible to primary infection with *M. tuberculosis* [198], and depletion of TNF- $\alpha$  by treatment

with anti-TNF- $\alpha$  antibodies – but not with the TNF receptor fusion molecule etanercept, as shown in the murine model of TB [199] - results in reactivation of latent disease [200-203]. The treatment of patients affected by chronic inflammatory diseases with monoclonal anti-TNF- $\alpha$  antibodies (infliximab) has led to a 4-fold increased incidence of TB [204], thus representing a major complication of anti-TNF therapy. Since latent TB is reactivated in anti-TNF- $\alpha$ -treated individuals [205, 206], its detection before and during treatment with anti-TNF- $\alpha$  antibodies is mandatory. However, since this treatment is known or suspected to decrease responsiveness to the TST, and it also might decrease production of IFN- $\gamma$  in the IGRAs, as with a negative TST result, negative IGRAs results alone might not be sufficient to exclude *M. tuberculosis* infection in these persons [36]. Since IL-6 is not as essential as TNF- $\alpha$ , therapeutic targeting of IL-6 – and especially, of IL-6-trans-signaling – has been proposed as a promising approach for the therapy of inflammatory diseases without compromising cell-mediated immunity in TB [207]. Anti-TNF- $\alpha$  treatment impairs T-cell directed antimicrobial activity against *M. tuberculosis* and is associated with reduced numbers of CD8<sup>+</sup> T<sub>EMRA</sub> (granulysin-rich CD8<sup>+</sup>CD45RA<sup>+</sup>CCR7 effector memory T) cells. These contribute to the killing of intracellular or extracellular *M. tuberculosis* by releasing inside the macrophage their granules containing perforin and granulysin [208, 209]. The depletion of T<sub>EMRA</sub> cells results in suboptimal control of mycobacterial growth, leading to the potential spread of *M. tuberculosis* infection. Although the effect of adding cytokines to *M. tuberculosis*-containing human macrophages is not fully established, some studies show that IFN- $\gamma$  and TNF- $\alpha$  stimulation lead to more effective phagosomal maturation [210]. Additional TNF inhibition with etanercept, however, enhances bacterial clearance in a murine model of necrotic tuberculous granulomas [211]. Mouse macrophages can be stimulated with IFN- $\gamma$  to inhibit the growth of mycobacteria. In the mouse, the control of mycobacterial growth is dependent on the production of NO [184, 212-214]. This puts iNOS under consideration as a protective locus against TB [156]. NO and NRI are endogenous products of the mammalian cells metabolism that can kill tubercle bacilli *in vitro* with a potency comparable to that of some anti-TB agents [148]. The role of NO in human TB is unsettled owing to the lack of iNOS in macrophages that differentiate *in vitro* from normal donors' monocytes. However, the lines of evidence that NO is an important component in human TB control follow. Granulomas from surgically resected lungs of some (eight) patients with TB contain higher amounts of iNOS, endothelial-NOS, but not neuronal NOS, and nitro-tyrosine –a marker of NO expression-, as compared to the other lung tissues not affected by TB [153, 215]. iNOS is expressed in pulmonary alveolar macrophages from patients with TB [216]. Moreover, the ability of human alveolar macrophages to kill *M. tuberculosis* being dependent on the activity of iNOS and the human macrophages taken from healthy subjects latently infected with *M. tuberculosis* producing

NO, thus controlling the growth of the bacteria [217], further support this hypothesis. NO, in fact, is mycobactericidal when produced at relatively high concentrations and bacteriostatic, promoting the non-replicative, persistent state of *M. tuberculosis* by induction of the Dos regulon, when produced at low concentrations. The presence of NO within human granulomas could contribute to host resistance since *in vitro* experiments demonstrate direct RNI-mediated bacteriostatic activity [151, 218, 219].

### 3. MYCOBACTERIAL BIOCHEMICAL SYSTEMS TOWARDS THE HOST-PROMOTED OXIDATIVE AND NITROSATIVE STRESS

#### 3.1 NADPH oxidase

In addition to NO and related RNI the macrophage can eliminate *M. tuberculosis* by employing a battery of enzymes producing ROS which includes NOX (NADPH oxidase). NOX, in particular NOX2/gp91<sup>phox</sup>, extracts electrons from NADPH and transfers them to oxygen to generate superoxide radical. This leads through the Fenton reaction to the production of hydroxyl radicals (OH<sup>•</sup>) and other toxic ROS such as H<sub>2</sub>O<sub>2</sub> and hypochlorite.

*M. tuberculosis* is extraordinarily sensitive *in vitro* to killing by a vitamin-C induced Fenton reaction [220]. On the other hand, superoxide and NO form the highly toxic peroxynitrite [221]. The amount of peroxynitrite (formed by the reaction between equimolar amounts of NO and superoxide within the phagosome) produced per hour per macrophage is approximately 6 fmol [222]. *M. tuberculosis* is susceptible to the cidal and growth inhibitory activity of ROS owing to their potential to damage cell lipids, proteins and DNA. DNA toxicity is further aggravated in *M. tuberculosis* by the absence of important DNA repair pathways such as mismatch repair [223] although a direct DNA protection is provided by the multifunctional histone-like protein Lsr2 [224]. Alveolar macrophages and blood monocytes obtained from active lung TB patients produce significantly reduced levels of ROS compared with cells from healthy individuals [225, 226], the reduced ROS formation being related to the decreased activities of NOX and the enzymes of the hexose monophosphate shunt [225]. Further evidence for a role of NOX in TB pathogenesis comes from the observation that NOX2 is essential for TLR2-dependent inflammatory response and 1,25-dihydroxyvitamin D<sub>3</sub>-mediated antimicrobial activity against *M. tuberculosis* via cathelicidin expression [227]. *In vitro* experiments, however, demonstrate no effect on *M. tuberculosis* intracellular growth in Phox-deficient macrophages, suggesting wild-type *M. tuberculosis* is resistant to the inhibitory effects of the macrophage oxidative burst [154]. At variance, mice deficient in Phox are partially defective in controlling *M. tuberculosis* growth, in an aerosolized infection model before the onset of specific immunity,

suggesting a role for ROS in the control of *M. tuberculosis* early in the infectious process [228].

#### 3.2 Superoxide dismutases

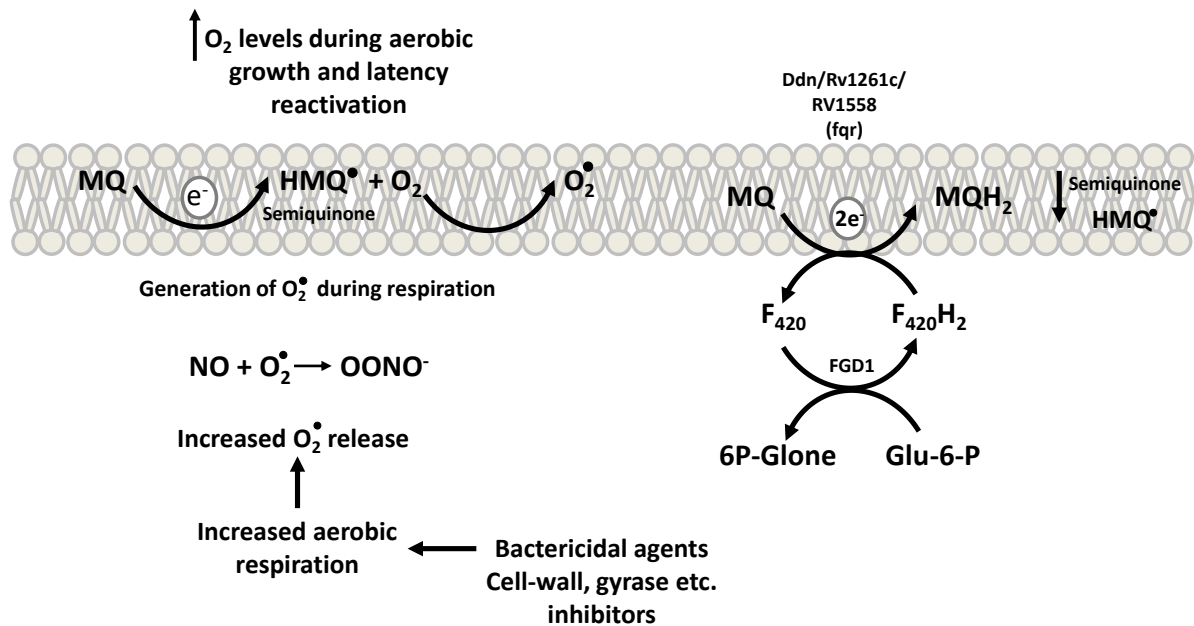
*M. tuberculosis*, however, is provided with protective mechanisms and enzymes –superoxide dismutases (SodA and C, [229-231]) catalase (KatG, [232, 233]), alkyl hydroperoxidase and peroxynitrite reductase complex of AhpC, AhpD, SucB (DlaT) and Lpd [234] and peroxythioredoxins [235] - conferring the ability to neutralize the redox stress generated by the macrophage. The resistance of *M. tuberculosis* to ROS is partly due to its thick cell wall containing lipoarabinomannan (LAM) and cyclopropanated mycolic acids, as well as phenolic glycolipid I (PGL-1) (**Figure 2**), which act as potent scavengers of oxygen radicals [236]. The low-molecular weight mycothiol (**Figure 2**), a substitute for glutathione, found in many bacteria, is important in maintaining a reducing environment and resistance to oxidative stress [237].

#### 3.3 The F<sub>420</sub>-dependent anti-oxidant mechanism

Among the diverse mechanisms evolved by *M. tuberculosis* to combat and survive oxidative and nitrosative stress, a novel F<sub>420</sub>-dependent anti-oxidant mechanism has been described which protects *M. tuberculosis* against these stresses and the bactericidal agents such as INH, moxifloxacin and clofazimine (**Figure 1**) that act by enhancing oxidative stress. F<sub>420</sub> is a redox active enzyme cofactor and *M. tuberculosis* F<sub>420</sub>-dependent enzymes are a glucose 6-phosphate dehydrogenase (FGD1) and a deazaflavin-dependent nitroreductase (Ddn). Rv1261c and Rv1558 encode for an F<sub>420</sub>H<sub>2</sub>-dependent quinone reductase function (Fqr) leading to dihydroquinones possibly from endogenous quinones. Thus, this F<sub>420</sub>-dependent antioxidant pathway sustained by Fqr protein family brings to an F<sub>420</sub>H<sub>2</sub>-specific obligate two-electron reduction of quinones, thereby competing with the harmful one-electron reduction pathway (**Figure 7**); thus, the formation of cytotoxic semiquinones is avoided [238]. Deletion of genes that encode F<sub>420</sub> biosynthesis renders *M. tuberculosis* hyper-susceptible to RNI (*fbtC*, [239]). The importance of F<sub>420</sub> system in the generation of proton motive force in hypoxic non-replicating mycobacteria is clearly established.

The mycobacterial sulphur metabolism plays a role in oxidative and nitrosative stress as a *cysH* mutant deficient in cysteine and methionine synthesis is more sensitive to these stresses [240]. Moreover, deletion of the gene encoding methionine sulfoxide reductase renders mycobacteria more susceptible to killing by hypochlorite and nitrite [221, 241]. Additional RNI resistance of *M. tuberculosis* derives from the catalytic detoxification of NO operated by the truncated hemoglobin (trHbN) in an oxygen-dependent reaction [219, 242].





**Fig. (7)** . Proposed model for an  $F_{420}$ -dependent anti-oxidant pathway by Fqr protein family in *M. tuberculosis*. Fqr proteins catalyse  $F_{420}H_2$ -specific obligate two-electron reduction of quinones, thereby competing with the harmful one-electron reduction pathway, and avoiding the formation of cytotoxic semiquinones. Thus,  $F_{420}^-$  as well as FGD $^-$  strain is hypersensitive to oxidative stress; hypersensitivity of  $F_{420}^-$  strains to nitrosative stress is probably due to peroxynitrite formation. Ddn: deazaflavin-dependent nitroreductase. Rv1261c and Rv1558: Ddn homologues which encode for  $F_{420}H_2$ -dependent quinone reductase (Fqr) function. FGD1:  $F_{420}$ -dependent glucose-6-phosphate dehydrogenase. Adapted with permission from [238] (permission to be requested).

### 3.4 *M. tuberculosis* transcriptional responses to the oxidative stress

One would expect that under oxidative or nitrosative stress *M. tuberculosis* responds transcriptionally through the regulators of the genes encoding these mechanisms by upregulating them, as it happens in enteric bacteria. In *E. coli* the defence against oxidative stress is largely controlled by OxyR and SoxR regulators [243], the catalase gene *katG* for detoxification of  $H_2O_2$ , and the alkyl hydroperoxide reductase gene, *ahpCF*, for detoxification of alkyl hydroperoxides being regulated by the gene repressor factor OxyR. The genes of *katG*, *sodA*, *ahpCDE*, thioredoxin and thioredoxin reductase are present in *M. tuberculosis* and are considered important in resistance to oxidative and nitrosative stress [244]. However, their regulation differs markedly from that in enteric bacteria, being many of these genes not induced *in vitro* by  $H_2O_2$  or NO and not upregulated early in a mouse model of TB when the oxidative burst is occurring [245]. This is the consequence of the absence of a functional OxyR in *M. tuberculosis*, as it is inactive owing to multiple mutations [24]; on the contrary, a functional copy of *oxyR* is present in *M. leprae*, *M. bovis* and *M. marinum* [247]. SoxR regulates expression of *sodA* and other ROS responsive genes in *E. coli*, but no apparent homologue of *soxR* exists in the *M. tuberculosis* genome [80]. FurA [248-250], IdeR [251, 252], CarD [253],

SigmaH [254, 255] and the genes involved in iron acquisition [256] are alternative transcriptional *M. tuberculosis* regulators involved in ROS defence.

The bacteriostatic and bactericidal effects and the transcriptional response of *M. tuberculosis* to ROS and RNI were investigated in growth and survival conditions, and by whole genome expression analysis of bacilli exposed to a range of concentrations of  $H_2O_2$  and diethylenetriamine-NO adduct, respectively [257]. Low-level and intermediate exposure to  $H_2O_2$  resulted in the selective induction of genes involved in DNA repair and recombination, along with that of several genes of the PE and PPE family whose functions remain largely unknown; in contrast, high exposure resulted in a muted transcriptional response and eventual death. Low concentrations of NO increased the expression of the majority of genes in the dormancy regulon, which seems to mediate the physiological transition and adaptation of mycobacteria to dormancy, and caused dose-related bacteriostatic activity without killing. At higher NO concentrations several genes with functions related to oxidative stress were also induced concurrently with dormancy regulon. Stress regulons controlled by Ide R, Sigma H, Sigma E comprised a large portion of the response to both stresses, along with the induction of gene encoding iron-sulphur cluster repair functions including iron acquisition. Noticeably, the expression of several oxidative stress defence genes was constitutive or increased

moderately from an already elevated constitutive level, suggesting that bacilli are continually primed for oxidative stress defence.

NO and H<sub>2</sub>S, however, products of both Gram-positive and Gram-negative bacteria, act synergistically as diffusible, protective agents by elevating the antioxidant capacity of bacteria through the suppression of the Fenton reaction, which brings to the production of toxic ROS, and stimulating SOD and catalase production [258, 259]. Furthermore, in mutant bacteria deficient in the starvation-signaling stringent response, active starvation response induces tolerance to several bactericidal antibiotics, acting through a common oxidative damage mechanism based on the production of ROS, by increasing antioxidant enzyme production and blocking the production of pro-oxidant molecules, thus reducing toxic OH [260]. These represent two convergent strategies used by bacteria to combat ROS produced through the antibiotic effect [261]. Whether this holds true also for *M. tuberculosis* is still matter of investigation.

#### **4. RECENT ADVANCES ON INTERFERON- $\gamma$ RELEASE ASSAYS (IGRAS)**

A major challenge in TB research is development of new immunological tests that can help distinguish, among subjects responsive to IGRA, those who are able to control *M. tuberculosis* replication from those who cannot. Patients of the former group are subjects with “remote latent TB infection”, “recent TB infection”, and “past TB infection”, evaluated from 6 months up to 2 years after treatment and scored positive to QFT-IT at the time of observation. Patients of the latter one are subjects with “active TB disease”, i.e. patients diagnosed either by a positive culture for *M. tuberculosis* from sputa or with a positive *M. tuberculosis*-specific RNA amplification test from biopsy specimens and/or biological fluids, who started a specific treatment within a month and scored positive to QFT-IT [262]. This discrimination would be very useful because it would allow for targeting patients needing a rapid full-course therapy, as it is the case of health care workers [263], and thus avoiding dissemination of the infection in the community. In the last few years, several components of *M. tuberculosis* cell wall have been challenged as modulators of the host immune response, worth to sustain an IGRA system (for a review, [264]). Some of these components are already in clinical use as immune reagents for IGRAs, such as the already mentioned ESAT-6, TB 7.7 and CFP-10; however, neither the actual IGRAs nor TST can separate acute TB from latent TB infection and there is concern that responses in IGRAs may decline with time after infection. The novel antigen heparin binding hemagglutinin (HBHA) purified from *M. tuberculosis* was previously shown to stimulate high levels of IFN- $\gamma$  secretion by peripheral blood mononuclear cells (PBMC) [265, 266]. It has been evaluated for *in vitro* detection of latent TB infection in a sample formed by 82 subjects with suspected latent TB infection, 102 patients with suspected active TB and 51

negative controls living in Belgium, a country with low TB prevalence [267]. Based on well-standardized TST reactions, 89 patients turned out to have active TB and 65 latent TB infection. When compared to PPD and ESAT-6 in IGRAs on PBMC purified from fresh blood samples, HBHA was significantly more sensitive than ESAT-6 or QFT-IT and more specific than PPD for detection of latent TB infection. The HBHA IGRA was unaffected by prior BCG vaccination, and, in contrast to the QFT-IT test, could detect remote infections as well as recent infections. In a more recent study [262], when IFN- $\gamma$  release was stimulated from whole blood by methylated HBHA produced by *M. smegmatis*, it was possible to discriminate between active TB and latent TB infection among individuals at different stages of TB who scored positive to QFT-IT. It is now clear that T cell-based diagnostics (QFT-IT and T-SPOT.TB), although capable of identifying latent TB infection in BCG-vaccinated populations thus eliminating issues of BCG cross-reactivity [25, 268], do not distinguish latent TB infection individuals from healthy individuals who have been previously treated for TB. That latent TB infection and BCG vaccination shape in a different way long-lived memory CD4<sup>+</sup> T cells is an hypothesis assessed recently. The study was performed on eighty healthy asymptomatic individuals with BCG vaccination histories, twelve individuals previously treated for pulmonary, active TB and five non-BCG-vaccinated healthy individuals with no history of exposure to *M. tuberculosis*, as controls [269]. It was shown that CD27 (member of tumour-necrosis-factor-receptor superfamily) and PD-1 expression on antigen-specific memory CD4<sup>+</sup> T cells not only could predict latent TB infection versus BCG status in healthy individuals but, remarkably, could also distinguish latent TB infection individuals from those who have clinically resolved *M. tuberculosis* infection after anti-TB treatment. Thus, CD27 and PD-1 have the potential to improve the evaluation of *M. tuberculosis* infection status in healthy individuals.

#### **5 *M.TUBERCULOSIS*: A GENETICALLY MONOMORPHIC PATHOGEN WITH SUBSTANTIAL GENOMIC DIVERSITY**

From the first half of the 20<sup>th</sup> century studies on *M. tuberculosis* guinea pig virulence – using either pathogen injections or, more recently, aerosol infections – showed that many strains isolated from TB patients in India were less virulent than strains from the UK (for a review, [270]). The theory that genetic variability in *M. tuberculosis* translates into important phenotypic differences found later further support from two observations. The first relates to the strain CDC1551 that caused an outbreak in a rural community near the Kentucky-Tennessee border of the USA, characterized by a high skin test conversion rate among patient contacts and a higher virulence towards mice compared with the Erdman laboratory strain [271]. The second to the strain HN878 which caused outbreaks of TB in Los Angeles and Houston in the USA [272, 273] and shown to exhibit a hyper-virulent phenotype in various

experimental models (for a review, [270]). HN878 belongs to a strain lineage associated to East Asia, which includes the “Beijing family of strains, and its hyper-virulence and inhibition of the innate immune response depend on the production and secretion of a specific phenolic glycolipid [274].

Initial comparative sequencing of *M. tuberculosis* has revealed very low sequence diversity compared with other bacteria [272, 275]. Genetic studies showed that this organism exhibits such a highly clonal population structure [276-278] to be considered a genetically monomorphic pathogen [279]. Recent advances in mycobacterial genomics, however, have shown that substantial genetic variation exists, which makes possible to obtain a phylogenetically robust strain classification and make possible to differentiate between disease relapse and exogenous reinfection. The discovery of unique and irreversible mutations identified in *M. tuberculosis*, mostly in the form of large sequence polymorphisms (LSPs) and single nucleotide polymorphisms (SNPs), by various molecular genotyping methods developed to differentiate, on a global scale, specific strains, strain families and evolutionary lineages of the *M. tuberculosis* complex (for a review, [280]), has been fundamental. Massively paralleled whole-genome sequencing was applied to two isolates (K-1 and K-2) from a high-incidence region in Uzbekistan that were part of a large cluster of closely related organisms, the Beijing K-family, representing the dominant, expanding *M. tuberculosis* variant in that region [281]. These isolates were virtually identical with respect to all standard genotyping markers, including IS6110 RFLP (Restriction Fragment Length Polymorphism) fingerprinting and SPOLIGOTYPING (Spacer Oligotyping). K-1 and K-2 differed in only one of 24 VNTR-MIRU (Variable Number of Tandem Repeats-Mycobacterial Interspersed Repetitive Units). However, whilst K-1 was fully drug-susceptible, K-2 was resistant to all first-line anti-TB drugs. As to genomic diversity, 130 SNPs and 1 deletion were identified which differentiate K-1 and K-2, whilst 1,209 SNPs were present in both isolates. Compared to K37Rv, K-1 encoded 55 specific SNPs and one specific deletion, while the MDR variant K-2 had 75 specific SNPs, including the five resistance-conferring mutations in the *katG*, *rpoB*, *embB*, *pncA*, and *rpsL* genes that are known to be altered in resistance to INH, RIF, EMB, PZA and SM, respectively [279].

Some typing methods used by molecular epidemiologists provide markers sufficiently strain-specific. These make possible to identify individual *M. tuberculosis* strains, track epidemics, detect new outbreaks, and determine the rate and dynamics of TB transmission in population studies. Other typing methods targeting more conserved and less discriminatory polymorphisms (for example, LSPs) allow to track the phylo-geographical diversification of the *M. tuberculosis* complex and to describe TB diffusion across different geographic areas and human populations [282]. The global population structure of *M. tuberculosis* now consists in six phylo-geographical lineages, each associated

with specific, sympatric human populations [283] and characterized by different degrees of virulence and immunogenicity.

## 6. CONCLUSIONS

The great progress of our knowledge of both *M. tuberculosis* genetic and physiology and how human host and bacilli interact has provided fertile ground for improving diagnosis and, consequently, cure of TB infection.

Tests so far developed for diagnosis of active TB proved to be sufficiently fast, specific and sensitive to allow avoiding the spread of infection and distinguishing among latent TB infection and BCG vaccination individuals from those who have clinically resolved *M. tuberculosis* infection after anti-TB treatment. To combat poor diagnosis of TB, which is a key contributor to the burden of morbidity and mortality of this disease, the WHO has endorsed the “same day microscopy” protocol. This recommends that systems be developed to collect sputum, perform microscopy, report results, and initiate treatment on the day of initial presentation [284]. To improve the sensitivity of diagnosis, the WHO has endorsed an automated molecular assay with minimal human-resource requirements and the ability to detect 70% of TB that is negative by sputum smear [51]. Scaling up novel diagnostic tests for TB and optimizing existing ones are complementary strategies that, when combined, may have substantial impact on TB epidemics in developing countries. WHO estimates that there are 3 million men, women and children globally with active TB who are currently being missed by health services and remain undiagnosed and untreated. Most of these three million people live in the world’s poorest countries, and include the most vulnerable members of the community such as displaced populations and homeless people, individuals with immune-suppression, pregnant women, migrants, prisoners, drug users and sex workers [287].

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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