

Targeting multidrug-resistant tuberculosis (MDR-TB) by therapeutic vaccines

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Abstract Tuberculosis (TB) has scourged humankind for millennia, and latent infection affects nearly one-third of today's world population. The emergence of multidrug-resistant (MDR)-TB is a major global threat and reflects treatment failure of drug-sensitive disease. MDR-TB management is a burden for patients and society; success rates are unacceptably low with prolonged treatment duration. *Mycobacterium tuberculosis* (*Mtb*) possesses the ability to transform into a dormant state in which it can

persist in the face of antimicrobial treatment and host defense. This sub-population of persisters is largely responsible for lengthy and difficult treatment. Targeting persistent bacilli could eventually improve the treatment success rate (currently 50–65 %) and shorten duration of treatment. A subset of therapies in the pipeline, termed therapeutic vaccines, use the host immune response to attack *Mtb*. The historical occurrence of an exacerbated host response has resulted in a negative perception of therapeutic vaccines. Thus, a renewed concept of immunotherapy is needed. We review current perspectives of immunotherapy in MDR-TB based on the knowledge of

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TB immunology and briefly discuss the profiles of several therapeutic vaccine products.

Keywords Tuberculosis · Immunology · Drug-resistant · Persistent bacteria · Therapeutic vaccine · Immunotherapy · Review

Tuberculosis (TB) has been a scourge to humankind for thousands of years. With the advent of effective chemotherapy and declining incidence rates in affluent societies, interest in the disease waned [1]. In 1994, however, drug-resistant TB emerged [2, 3], and the World Health Organization (WHO) declared TB a Global Emergency [4]. Reports from New York show that drug-resistant TB was readily transmitted in the early 1990s among hospitalized AIDS patients. In 2010 alone, nearly 9.2 million new cases of TB occurred and an estimated 1.4 million deaths were caused by it [5]. The spread of HIV/AIDS accelerated the TB epidemic in large parts of the world, as the risk of developing the disease is markedly increased in HIV-infected persons [6].

MDR-TB and XDR-TB

When susceptibility to the two most powerful anti-tuberculosis drugs, isoniazid (H) and rifampicin (R), is lost, short-course treatment is no longer an option. HR resistance is referred to as multidrug-resistant (MDR)-TB. When resistance develops to not only HR, but also to two major second-line TB drug classes, the condition is referred to as extensively drug-resistant (XDR)-TB. By 2010, the WHO estimated the global prevalence of MDR-TB to be around 650,000 among the world's 12.0 million cases of TB and 58 countries around the world have reported at least one case of XDR-TB [7, 8]. Countries of the Former Soviet Union have been among the most severely affected by the MDR-TB epidemic. A representative survey conducted in 2010 in Minsk, Belarus, showed alarming levels of drug resistance with nearly one out of two tuberculous patients being affected by MDR-TB [9]. Surveillance data from South Africa indicate a high prevalence of drug-resistant TB in the region [10], with a hall-mark paper reporting an alarmingly high mortality rate in XDR-TB patients co-infected with HIV [11]. Recent data from China have raised even greater concern. In a nation-wide survey on drug-resistant TB, 5.7 % of culture-positive pulmonary treatment-naïve cases appeared to have MDR-TB [12]. An independent survey in China revealed that 12 % of TB cases in one particular province were MDR-TB [13]. China has the second highest TB incidence in the world with India having the highest [7]. The case of China underlines

the fact that MDR-TB has become an important global health threat. Increasing mobility and cross-border travel demands radical changes in the approach to combat TB [14, 15]. Currently available treatment for MDR- and XDR-TB requires administration of longer treatment with less effective, more costly, and more toxic drugs in comparison with the standard 6-month short-course chemotherapy for drug-susceptible TB: Indeed, the current treatment for MDR-TB is a burden for patients as well as for society [16, 17]. Nevertheless, treatment success with MDR-TB is low compared with that of drug-sensitive TB [18–20]. The emergence of a type of TB resistant to all currently available first- and second-line drugs—totally drug-resistant (TDR)-TB or very extensively drug-resistant (XXDR)-TB—has been reported in the last few years [21–23]. In summary, the emergence of ever-increasing resistance represents a major challenge for TB management in the future.

Risk factors for MDR-TB

Inadvertent monotherapy in multi-bacillary TB has been considered the driving mechanism in the bacillary repopulation of lesions by drug-resistant mutants, leading to monodrug resistance. When this process is repeated with another drug, MDR-TB results [24]. Previously defaulted or retreated cases, prolonged treatment time (>180 days), delayed initiation of chemotherapy, and misconducted treatment have all been associated with the development of MDR-TB [13, 25]. All of these factors have been alluded to as mistakes made by patients, doctors, and pharmacists. An alternative explanation was proposed by the group of Tawanda Gumbo, suggesting the role of interindividual pharmacokinetic (PK) variability of a single drug in the regimen—particularly H or R—as a major contributing factor in the development of MDR-TB rather than inappropriate treatment [26]. This would imply that drug resistance cannot be prevented even by well-designed standardized directly observed treatment, and that in one way or another, PK variability should be taken into account as an essential component in treatment and control programs.

Pathogenesis and immunology of TB

Several reviews on pathogenesis and immunology of TB have recently been published [27–31]. For the purpose of this review, we have summarized the main findings. Following aerogenic transmission from an individual with active disease, infectious droplets of *Mycobacterium tuberculosis* (*Mtb*)—the bacilli causing TB—reach the

alveolar spaces. During its residency in lung parenchyma, *Mtb* can be phagocytosed either by alveolar macrophages (AM), dendritic cells (DC), epithelial cells, or neutrophils. These phagocytes are able to recognize the molecular components of *Mtb*—referred to as pathogen-associated molecular patterns—through pattern recognition receptors (PRR) on their cell surfaces. Much work has been done to reveal the PRR involved in the recognition process, which includes several receptor families such as toll-like receptors, nucleotide oligomerization domain-like receptors, C-type lectins, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (also known as CD209 or DC-SIGN), and dectin-1. Among the phagocytes expressing PRR, AM play a major role. AM possess the capability to produce essential cytokines and also act as a reservoir for *Mtb* bacilli. Once phagocytosed by these AM, *Mtb* may resist bactericidal actions by inhibiting phagolysosome function. Typically, macrophages acidify their phagosomes to pH 5.2 and kill the bacteria by the production of antimicrobial peptides (i.e., cathelicidin), activating a vitamin-D-dependent pathway [32]. The release of nitric oxide (NO) also constitutes an important defense mechanism in mouse models, but evidence in human studies has been conflicting [33, 34]. Several different mechanisms have been hypothesized and identified in halting phagosome maturation. Reduced recruitment of vacuolar H-ATPases hampers the acidification process and thus enables *Mtb* to persist inside the phagosomal vacuole [35, 36]. While lysosome fusion is altered, the vacuoles still fuse with other early endosomal vesicles while acquiring some lysosomal molecules by the conventional synthetic pathway [37]. GTPases of the Rab protein family are involved in lysosome fusion. Normally, Rab 5 is replaced by Rab 7, but Rab 5 is retained in phagosomes that contain *Mtb* [37]. Lipid bodies (LB)—dynamic and functionally active lipid-rich organelles—are the sites for these GTPases, and they play a role in transporting Rab into and from the phagosome in order to procure phagosome maturation [38]. LB also supply neutral lipids to the phagosome which can be utilized as an energy source by *Mtb* during its residency inside AM [39, 40]. Calcium-binding calmodulin is involved in early endosome fusion and calmodulin inhibitors inhibit fusion [41]. Suppression of the actin-binding coronin I (TACO in humans), which associates with early phagosomes, results in defective phagosome fusion [42]. In summary, *Mtb* inhibits phagosome maturation by several different mechanisms, and the pathogen persists intracellularly while avoiding macrophage apoptosis. During its residency in macrophages, *Mtb* can either multiply and eventually kill the host cell or alternatively once the infected macrophage is activated, *Mtb* can be destroyed. A third possibility, a unique feature of *Mtb* and leprosy bacilli, is that they fall into a stage of

dormancy with low to absent metabolic and replicative activity.

If macrophages become necrotic, the bacilli enter the extracellular space where they are phagocytosed by other macrophages. This cycle ends only after the cell-mediated immune response is elicited with interferon-gamma (IFN- γ) released by CD4⁺ Th1 lymphocytes [43]. Upon recognition of major histocompatibility (MHC) class II molecules expressed by the infected macrophages, CD4⁺ T lymphocytes are able to polarize into distinct types of effector cells [44]. Th1 and Th17 T-lymphocytes play an important role in immunity to *Mtb*. Th1 lymphocytes produce several cytokines, including IFN- γ which is strongly associated with protective immunity. IFN- γ promotes macrophage activation by reviving phagosomal maturation, inducing NO-dependent apoptosis, and modulating autophagy thus enhancing *Mtb* clearance [45, 46]. IFN- γ as an immunogenic marker has been recommended by WHO as an assessment tool for new TB vaccine trials although it does not suffice as a marker of protection [47]. Th17 lymphocytes are involved in the early phase of host defense as they produce cytokines essential for neutrophil and monocyte recruitment to the site of infection. Th2 lymphocytes produce interleukin (IL)-4 that downregulates the Th1 response, thereby contributing to the development of progressive disease [48]. Treg lymphocytes produce transforming growth factor-beta (TGF- β) and IL-10, which inhibit all other CD4⁺ T cells. Apart from these CD4⁺ T lymphocytes, CD8⁺ T lymphocytes, which are MHC I-restricted, also contribute to protective immunity by directly lysing *Mtb* and producing Th1 type cytokines. Several innate immune cells, such as natural killer (NK) cells and gamma-delta ($\gamma\delta$) T cells, also play a role in the immune response against *Mtb*. As disease progresses, surface density of MHC class II molecules is decreased [49]. Costimulatory molecules are downregulated reducing cellular immune responses even further. *Mtb* also alters the cytokine profile around the macrophage. IL-6 attracts B-lymphocytes but reduces T-lymphocyte proliferation [49]. Hence, *Mtb* not only reduces the initiation of an effective host immune response, it also minimizes its effects.

The dormancy and persister stage of *Mtb*

Mtb and possibly leprosy bacilli have the unique capability to turn off their cellular metabolism, halt replication, and transform into a dormant stage under stress conditions (see Fig. 1) [50, 51]. While dormancy is typically induced by stress imposed by the host on *Mtb*, persistence refers to the survival of *Mtb* under harsh conditions, be they caused by drug treatment or host immunity. This makes it difficult for antituberculosis drugs to eliminate these organisms, which are therefore called “phenotypic persisters” or “phenotypic

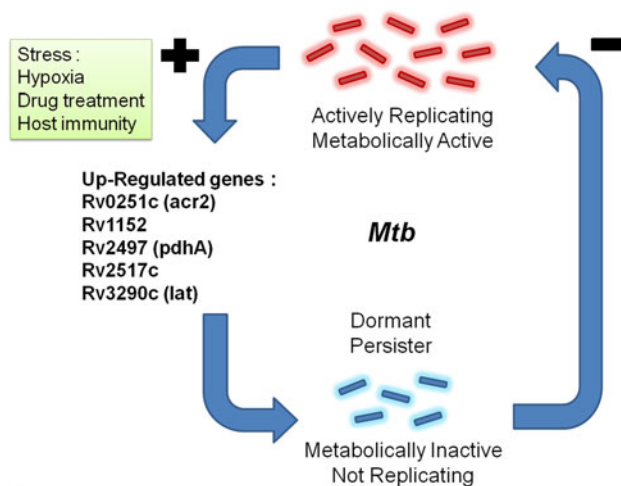


Fig. 1 *Mtb* changes phenotype by changing the genetic program during stress

resistance” [52]. The sub-population of persistent *Mtb* organisms—though genetically identical to susceptible, fast-replicating, metabolically active bacteria—resist antimicrobial treatment and host immunity. At the same time, these dormant *Mtb* may not cause major harm to the host. This is the very reason why treatment is so lengthy and difficult [53]. *Mtb* enter the persister state expressing a range of different genes challenged by cellular immunity [54]. Through dormancy, the bacillus escapes the host immune response by many mechanisms [29, 53]. Dormant *Mtb* can persist in healthy individuals in a stage termed latent TB infection (LTBI).

Granulomas act as a suitable microenvironment where persisters of *Mtb* reside and persist [55], although earlier reports failed to show the presence of TB bacilli in lung granulomatous lesions [56]. Several other possible locations of these persistent *Mtb*, including adipose tissue, normal lung parenchyma, and several other organs, have been tentatively identified [57–59]. Tumor necrosis factor- α (TNF- α) plays a major role in granuloma formation and preservation [29], possibly with the involvement of other factors [60–62]. Granulomatous inflammation presents a complex cellular interplay to protect the host against invasion of organisms by walling *Mtb* off and attracting specific lymphocytes at the site of infection [63, 64]. An alternative hypothesis toward this assumption has however been proposed [65]. The *Mtb* region of difference-1 (RD-1) encoded ESX-1 secretion system enhances host expression of matrix metalloproteinase 9 (MMP-9), which is essential in initiating macrophage recruitment to the granuloma [66]. An increase in RD-1 expression by *Mtb* has been associated with an increased bacterial load [60]. If this is true, granuloma formation in its early stage—prior to

T cells’ arrival—should be considered a pathogen-initiated response favoring the bacilli rather than a host defense mechanism.

The granuloma environment imposes several stress factors—such as low pH, NO, hypoxia, and limited nutrients—driving *Mtb* into dormancy. During this *Mtb* persister stage, stress-related genes are upregulated, while several central metabolism genes are downregulated [52]. The dormancy survival regulator (DosR)—transcription factors of *Mtb* responsible for increased expression of dormancy genes—upregulates the expression of several proteins crucial for *Mtb* survival under stress conditions. Some of these DosR regulon-encoded *Mtb* proteins evoke immunogenic responses when administered in a latently TB-infected individual [67]. Subsequent to phagocytosis, *Mtb* expresses DosR-encoded genes in the phagosome [68]. Transcriptome analysis from human smear-positive sputum samples revealed that *Mtb* persisters express signals of the DosR regulon [69]. IFN- γ production upon stimulation with some of these DosR regulon-encoded antigens may differentiate active and latent TB [70, 71]. Of the different stress factors, hypoxia is probably more critical than low pH, or increased NO and carbon monoxide production, in inducing activation of the DosR regulon [72, 73]. Disruption of DosR genes only slightly impairs the survival of *Mtb* in several animal models [52], suggesting that it might not be the only factor involved in the switch into dormancy. DosR is important for the initial hypoxic adaptation but not for the survival of *Mtb* during chemotherapy [74]. Genes encoded by sigma E transcription regulator are also involved in the stress response, and *Mtb* persistence inside macrophages [75].

Across a range of different stressor models studied, a set of five genes appear consistently upregulated. These genes are Rv0251c (*acr2*), encoding an α -crystallin heat shock protein (Hsp); Rv1152, a transcriptional regulator of the *gntR* family; Rv2497c (*pdhA*), encoding a possible pyruvate dehydrogenase component; Rv2517c, encoding a hypothetical protein; and Rv3290c (*lat*), encoding an L-lysine-epsilon-aminotransferase [74]. In a mouse model of *Mtb* infection, six other genes (*fadE5*, *sigE*, Rv2030c, Rv2660c, *sigB*, and *ppsD*) were consistently expressed—both during early and late stages of infection. One of these genes, Rv2660c—the most upregulated gene in an in vitro starvation model—encodes a hypothetical protein essential for adaptation to lack of nutrition and hypoxia [75]. A new multistage vaccine based on the Rv2660c antigen combined with two other early secreted antigens (Ag85B and early secreted antigenic target-6, or ESAT-6) appeared to prevent TB disease in a post-exposure TB infection model [76]. To what extent these persister genes are important in humans remains to be further elucidated, but their potential

role in the switch of *Mtb* into dormancy may be important as a novel target for therapy.

Targeting persisters by drugs: old and new

Targeting the persister stage of *Mtb* may eventually lead to shorter drug regimens resulting in enhanced treatment success. Several old and new TB drugs are currently being tested for their activity against persistent bacteria, with the hope of shortening current regimens [53, 77]. Only a few new powerful drugs are in the pipeline [78], such as delamanid [79], bedaquiline [80], and the linezolid derivative sutezolid [81].

Animal models have been developed to assess elimination of persisters by using sterilization as an endpoint [82] but pharmacokinetics/pharmacodynamics (PK/PD) as well as the immune response in these animals is quite different from that of humans. Apart from novel therapeutic agents and novel therapeutic schedules, an important goal is to improve and optimize available second-line drugs by therapeutic drug monitoring and modeling [83–87]. Interindividual differences in PK/PD may be a more important driving force in the development of drug resistance than mistakes in medications [26, 88]. The strategy to optimize the use of current and novel drug therapy may however ultimately fail.

TB vaccines

Bacille Calmette–Guérin (BCG)—the only currently available TB vaccine—is a live attenuated strain of *Mycobacterium bovis*. It has been an important part of the Expanded Program on Immunization since the 1970s and has since been given more than 4 billion times. Its safety record is astonishing as few serious adverse events have been reported [89]. Yet, this 90-year-old vaccine does not protect against pulmonary TB in adults, its efficacy is minimal (0–80 %) in adults, and it provides only limited protection in children [90]. BCG remains controversial, and different policies and practices can be seen across nations [91]. Moreover, the current HIV epidemic places immune-compromised patients, who are being BCG vaccinated, at risk of developing BCG disease [92]. Promising progress can be seen in new TB vaccine development as many candidates have entered first clinical safety and immunogenicity evaluation. Eleven candidates have passed phase I clinical trials [93, 94]. Even more products are in the pipeline of preclinical development. Although these candidate vaccines represent potential improvements compared with BCG [95–97], they have not been shown to provide sterilizing immunity in a mouse model [98], and

their potential value as therapeutic vaccines has not been assessed [99].

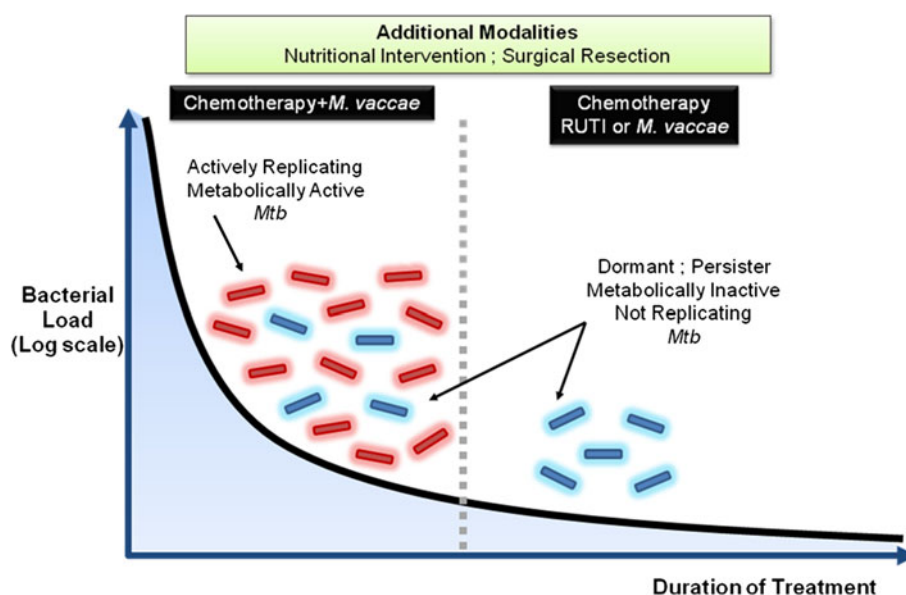
Therapeutic TB vaccine

Early initiation of anti-retroviral therapy provides benefit in preventing TB in HIV-infected individuals, highlighting the importance of enhanced cellular immunity to control LTBI [100]. Few vaccines use the host immune response to target active TB; these are termed therapeutic vaccines, though this is a misnomer. Two inactivated or fragmented mycobacterial formulations are considered for TB immunotherapy. Heat-killed *Mycobacterium vaccae* is an inactivated environmental mycobacterium. In phase III clinical trials, it was shown to be safe in HIV patients who had previously received BCG [92, 101]. *M. vaccae* immunotherapy enhances host defense against *Mtb* by promoting Th1 and suppressing Th2 response. When added to chemotherapy, *M. vaccae* improves sputum conversion and chest radiographic resolution [102, 103]. RUTI is composed of detoxified fragments representing a whole range of inactivated latency-associated antigens. In phase I clinical trials, its safety and immunogenicity has been demonstrated [104]. A phase II study for RUTI was recently completed, and a phase III trial is envisaged. Both are by far the most advanced therapeutic vaccines today. *M. smegmatis* and *M. indicus pranii* (MIP) are other saprophytic non-TB mycobacteria (NTM) that also share antigens with *Mtb*, similar with *M. vaccae* [105]. V-5 immunitor (V5)—an oral therapeutic vaccine initially developed for management of chronic hepatitis—has been shown in several studies to be beneficial when administered to TB patients [106, 107]. The authors suggest it could contain latency-associated *Mtb* antigen, resembling RUTI.

Current concept of immunotherapy

The use of therapeutic vaccines in TB was first suggested by Robert Koch in 1890. He believed that the use of repeated injections of supernatants of *Mtb* cultures—known as old tuberculin—could act as a potential remedy for TB [108]. In the absence of available treatment for TB, his ideas attracted many physicians around the world, and soon the old tuberculin was widely used. Tuberculin immunotherapy failed—in some instances it probably killed patients as a result of an exacerbated immune response [109]. With today's knowledge, it is likely that administration of these antigens to already infected individuals resulted in a cytokine storm, a tissue-damaging, harmful response involving exacerbated release of TNF- α , as well

Fig. 2 The current concept of immunotherapy in MDR-TB management



as more down-stream pro-inflammatory cytokines [110]. Yet, no well conducted study has addressed the question whether, and under which circumstances, the old Tuberculin lacked safety.

The introduction of chemotherapy proved highly successful, especially after combination therapy was introduced [111]. With the emergence of drug-resistant TB, effectiveness of chemotherapy was challenged, and immunotherapy has been revisited [112]. To overcome the argument presented by this exacerbated immune response against the use of therapeutic vaccines, a modified concept is needed before they can be reconsidered.

There are two areas in which immunotherapy need to be applied. One, exemplified by *M. vaccae*, is that of immunomodulation, in which the Th2 milieu induced and maintained by active tuberculous infection is corrected toward Th1, increasing the efficacy of chemotherapy. The other is elimination of persisters after a course of chemotherapy or in cases of latent tuberculosis that have never had clinical disease, for which the RUTI vaccine may be most effective.

Immune modulation needs to be applied as soon as diagnosis is confirmed, and chemotherapy started to correct the immune response away from that responsible for the pathology. The mechanism for this does not require antigens specific to *Mtb*, but reversion to Th1 restores cellular immune responses to antigens common to all mycobacteria, some of which are shared with human stress proteins expressed in tissues involved in chronic inflammation [113]. It is through this correction that immunotherapy with *M. vaccae* and some organisms related to it, results in its applicability to a range of chronic inflammatory diseases, many of them not mycobacterial in their etiology [114–121].

Eradication of persistent *Mtb*, which is virtually resistant to chemotherapy (see Fig. 2), is a modern concept. By reducing bacterial load, the cytokine storm which causes the Th2-related exacerbated immune response can be prevented [122]; this is essential for therapeutic vaccination. Indeed for this purpose, the focus should be on therapeutic vaccines that express latency-associated antigens as found in dormant *Mtb*. Such vaccination should assist the host immune system in boosting the immune response directed at these latency antigens, eventually resulting in complete eradication of persisting *Mtb*. They could be used to enhance the immune response during the continuation phase of TB treatment, in which the remaining bacteria are poorly sensitive, if not refractory, to antimycobacterial agents, and potentiate chemotherapy.

The greatest benefits for both types of immunotherapy would be for MDR-TB or XDR-TB as it would potentially improve the relatively low treatment success rate. Therapeutic vaccines do not interfere directly with the causative organism, and hence, they are not involved in the development of drug resistance [123]. Therapeutic vaccination would also be beneficial for drug-sensitive TB as it could potentially shorten the current 6-month standard therapy and help diminish the development of drug resistance. Finally, success in MDR-TB treatment could pave the way for a latency-targeted (post-exposure) vaccine. Indeed, reducing the huge reservoir of *Mtb*—drug-susceptible or not—by vaccination strategies could ultimately accelerate elimination of the disease.

Chemotherapy combined with immunotherapy should be the frontline approach in the management of MDR-TB. Early bactericidal activity of currently available second-line regimens is essential for the elimination of most

replicating metabolically active *Mtb* bacilli in the early phase of treatment.

After significant reduction of the initial bacterial load with chemotherapy and *M. vaccae*, therapeutic vaccine(s) such as RUTI should be introduced in order to overcome the remaining persistent *Mtb* with slow replication rate and reduced drug sensitivity. The timing of immunotherapy initiation with RUTI is critical to prevent the emergence of an exacerbated immune response; chemotherapy, with or without *M. vaccae*, must have significantly reduced the bacterial load in advance.

Clinical, radiological, and bacteriological outcome, for example, using the vital stain fluorescent diacetate [124, 125], must be assessed in order to monitor true reduction in bacterial burden. In vitro susceptibility, fast molecular assays, and PK, using limited sampling of dried blood spots, should be incorporated to enhance the chance of drug treatment success. Eventually, additional modalities, such as nutritional intervention and surgical resection, should also be considered during the course of treatment.

Outlook

The classical approach using combined drug regimens remains the key modality in MDR-TB management. Additional support, such as nutritional intervention (by micronutrients and macronutrients) [126–128], and in selected cases, surgical resection [129, 130] are also important for beneficial outcome. However, considering the current low rate of success as well as the long duration of therapy in MDR-TB treatment, immunotherapy should now be explored in a formal randomized fashion. Current understanding of immune responses against *Mtb* should lead the way to further developing the concept of immunotherapy. The immunogenicity and safety of two therapeutic TB vaccines, *M. vaccae* and RUTI, have been demonstrated in preclinical work and in human trials. *M. vaccae* evokes optimal therapeutic results when given in multiple doses, while RUTI should probably best be given after significant reduction of bacterial load. For the RUTI product, further safety studies are necessary to reduce the risk of a pathologic immune response. *M. vaccae* restores Th1/Th2 balance, while RUTI exposes the host with specific sets of inactivated latency or dormancy antigens. Hence, administration of both vaccines in combination might be worth pursuing. Indeed, immunotherapy is now ready for prime time.

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