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# Summarizing discussion and future perspectives

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## SUMMARIZING DISCUSSION

Tuberculosis (TB) has been around for millennia and remains a serious health threat to date, despite the availability of curative treatment. Reasons for this persistent burden of disease include (i) mycobacterial factors such as newly emerging strains with increased virulence and the increasing rates and degrees of drug resistance; (ii) host factors such as large reservoir of latently infected individuals with the potential to progress to active disease and, lastly, (iii) treatment factors, including long treatment durations necessary to eradicate persistent populations of mycobacteria. Major questions that are currently in focus in TB research are:

- How and where do populations of mycobacteria persist that make anti-TB treatment so lengthy?
- How can we measure these persisting (perhaps dormant) subpopulation(s) of mycobacteria?
- How do host responses contribute to their persistence or reactivation?
- How do we develop and screen new anti-TB drugs to target these subpopulations?

The aim of this thesis was to improve TB treatment by (i) gaining insight into TB pathophysiology in search for factors that can be modulated to our advantage during treatment, and (ii) optimizing and increasing the translational value of the preclinical mouse TB model as screening tool for new TB drugs and regimens.

### Mycobacterial factors

Advances in genotyping technologies and clinical observations over the last two decades have shown mycobacterial strain variance to be an important factor in TB pathogenesis. The most illustrative example of this concerns the emergence of Beijing genotype strains, which have been a driving force behind the spread of multidrug-resistant TB in Eurasia and have been associated with elevated treatment failure rates and disease relapse compared to other genotypes in other parts of the world (1-8). It is not difficult to imagine how drug resistance contributes to treatment failure, but other unique pathogenic mechanisms specific to Beijing strains such as their differential gene expression and immune modulating capacities are also thought to contribute to their clinical success. Therefore, strain diversity and its influence on treatment and pathogenesis is the mycobacterial factor that is addressed most prominently in this thesis.

In contrast to most other *Mycobacterium tuberculosis* lineages, Beijing strains constitutively express genes belonging to the DosR dormancy regulon (9-11), which comprises approximately 50 genes and is controlled by the DosR transcription factor (12). This regulon is an important virulence factor for *M. tuberculosis* as it is believed to play a pivotal role in mycobacterial progression to a persistent state under influence of (hypoxic) stress (13). Since mycobacteria in this persistent state are less affected by TB drugs, differential expression of DosR regulon genes by Beijing strains could explain differences in treatment outcome (14, 15).

Preclinical mouse TB models evaluating treatment outcome often use mycobacterial strains such as H37Rv or Erdmann, which are no longer found in patients (16-20). Our specific aim in **Chapter 5** was to evaluate the impact of using a Beijing genotype strain on treatment outcome and increase translational value of our mouse TB model. Our characterization of the treatment response in Beijing-1585-infected mice showed that none of the ten regimens tested, including the standard of care regimen 2RZH/4RH (two months of rifampicin-pyrazinamide-isoniazid followed by 4 months of rifampicin-isoniazid), achieved 100% treatment success. In contrast, at least four different studies using *M. tuberculosis* H37Rv strains, including one previous study in our own model, showed 100% treatment success of the 2RHZ/4RH regimen in BALB/c mice (16, 19, 21, 22). This indicates that treatment success rates in mouse TB models using H37Rv probably overestimate clinical efficacy, as Beijing strains show increased survival within the host. Thus, the use of recent clinical isolates like Beijing-1585 instead of the laboratory H37Rv strain has the potential to increase the translational value of mouse TB models.

Another characteristic of the Beijing strain concerns the increased virulence, even called hyper-virulence. Virulent Beijing strains cause higher mycobacterial loads, more lung damage and earlier mortality in preclinical models compared to strains from other lineages and non-virulent Beijing strains (14, 23, 24). Previous studies in our mouse TB model confirmed these virulence factors for Beijing-1585, but also for EAI-1627, another clinical isolate prevalent in Southeast Asia and belonging to the East-African/Indian lineage (14, 25). This marked difference in virulence in our mouse TB model between currently circulating clinical strains compared to H37Rv inspired us to evaluate host responses for each strain more thoroughly. In the study described in **Chapter 3** we found that host responses against H37Rv were in line with the current basic paradigm of TB immunity characterized by an IL-12/Th1/IFN-γ response in the lungs as explained in **Figure 1** and **Box 1** in **Chapter 2**. In marked contrast, host responses against Beijing-1585 and EAI-1627 were associated with an influx of B-cells rather than T-cells into the lungs at the peak of infection. Myeloid cell populations were present in the lungs at similar proportions upon Beijing-1585-and EAI-1627- compared to H37Rv infection, but appeared

functionally impaired in infection with the Beijing- and EAI- strains with low iNOS and IL-12 expression. In addition, in the bone marrow of Beijing-1585- and EAI-1627-infected mice reduced expression was found of IFN-γ, TNF-α and IFN-β, cytokines essential for myeloid cell priming. This effect at distant site already became apparent on the third day post infection, before other systemic effects were detected. This combination of impaired myeloid cells in the lungs of mice infected with Beijing-1585 and EAI-1627 and reduced expression of essential priming cytokines in their bone marrow suggests a previously unrecognized role for myeloid cell priming in the bone marrow with regard to strain-related virulence.

In **Chapter 2, figure 3** a detailed hypothesis is shown for such a mechanism. IL-12 expression in the lungs, which is present during infection with H37Rv, but absent for the clinical strains, could cause IFN-γ-mediated myeloid cell development in the bone marrow towards a regulatory phenotype that prevents excessive innate inflammatory damage upon their migration to the lungs, while concomitantly stimulating protective adaptive responses. Another finding in **Chapter 3** concerned the role of type 1 interferons in acute TB. Previous studies speculated on a detrimental role for type 1 interferons in the pathogenesis of Beijing strains compared to H37Rv (26, 27). They found that the Beijing HN878 strain induced higher IFN-α mRNA expression levels in the lungs, which was associated with lower induction of IL-12 and TNF-α levels and reduced T-cell activation compared to H37Rv (27, 28). We also found lower induction of IL-12 by Beijing-1585, but refuted an association with elevated type 1 interferon levels. This was based on direct measurement of IFN-α/β mRNA expression similar to those previous studies, but was further substantiated by measurement of type 1 interferon-inducible genes.

Taken together, our combined findings from **Chapter 5** on the influence of strain variance on treatment outcome and **Chapter 3** on the influence of strain variance on host responses during acute infection suggest that integration of clinical mycobacterial strains into preclinical mouse TB models should be pursued as it will increase their translational value and expand our basic knowledge on TB immunity. It is especially important to realize that much of our fundamental knowledge on TB immunity during acute infection is based on experimental data from mouse studies involving H37Rv, which we show not to be the best representative for host responses against modern day strains. This indicates that our basic paradigm of TB pathogenesis might be outdated.

**Host factors**

Our findings in **Chapter 3** on mycobacterial strain variance indicate differential regulation of IL-12, which is part of the classical IL-12/T-helper 1/IFN-γ immune response in TB. However, we also found differences in B-cells, IL-4 and type 1 interferon responses

upon infection with different strains that do not fit the conventional paradigm so easily, but might be of influence in TB pathogenesis. Reasons to believe that such additional mechanisms beyond Th1 immunity are at play in TB immunity include unsatisfactory results of vaccine strategies aimed at boosting Th1 immunity (29), the inflammatory damage associated with increasing IFN-γ production by T-cells in the lungs of *M. tuberculosis*-infected mice (30) and the host-detrimental effect of blocking Th1-inhibiting pathways in mice (31, 32). Therefore, the aim in **Chapter 2** was to review and integrate the role of increasingly recognized immunological elements in TB pathogenesis such as B-cells, IL-17 and type 1 interferons into our current understanding of TB immunity. The major hypothesis that we formed is that type 1 interferon responses, the IL-17 pathway and their interaction converge onto a stimulatory effect on B-cells through the induction of B-cell activating factor (BAFF), stimulation and functioning of tertiary lymphoid structures (TLS) and stimulation of the Th17.1 response (**Chapter 2,** Fig. 5). How this affects disease progression is multifactorial and dependent on the phase of disease as will be explained below.

Type 1 interferons are generally regarded as negative regulators of TB immunity because: (i) they induce a regulatory phenotype in myeloid cells, which favors mycobacterial persistence over eradication (33), (ii) they have been described to be upregulated by virulent strains (28, 34) and (iii) an interferon signature in blood cell RNA corresponds with active disease (35, 36). Based on our literature review, we conclude that the effects of type 1 interferon are diverse and depend on prior priming of myeloid cells by either IFN-γ and M-CSF or GM-CSF (**Chapter 2**, Fig. 3).The most important consideration is that IFN-γ priming at precursor stage appears to lead to the induction by type 1 interferons of a regulatory phenotype in myeloid cells (37). This regulatory myeloid phenotype might favor mycobacterial persistence (33), but could also ameliorate destructive inflammation as the results described in **Chapter 3** suggest. A second consideration is that type 1 interferons can only induce a regulatory phenotype in myeloid cells differentiated under M-CSF, as GM-CSF renders myeloid cells less responsive to type 1 interferon signaling (38). This might explain why effects of type 1 interferons are observed during acute infection and wane while infection progresses and GM-CSF levels rise. Lastly, the effects of type 1 interferon might differ between IFN-α and IFN-β and potentially serves as a mechanism to prevent excessive immune-mediated tissue damage (**Chapter 2**, box 4 and **Chapter 3**).

The role of B-cells in TB immunity has been neglected over the past decades due to the established central role of protective T-cell mediated responses, but regained interest in the past years (39). Most notably, a functional role for antibody-mediated immunity in TB was demonstrated and circulating B-cells were shown to be dysfunctional and

reduced in absolute numbers in patients with active TB (39-41). Despite the potentially beneficial effects of B-cells and antibody-mediated immunity in chronic TB, we show in **Chapter 3** that acute infection with virulent Beijing-1585 and EAI-1627 in our mouse TB model is associated with increased B-cell influx and higher IL-4 protein levels in the lungs compared to infection with the less virulent H37Rv as outlined above. This suggests that, while protective during chronic infection, B-cells might also contribute to disease severity during acute infection, again emphasizing the complexity of TB immunity.

The importance of elucidating TB pathophysiology is emphasized by the central role that our own immune system plays in TB treatment. It is an effective and efficient first-line barrier against TB as only 5-15% of all individuals with intact immunity infected with *M. tuberculosis* will progress to active disease (42). However, once that barrier fails and infection progresses to active disease, without treatment 50-70% of TB patients will die within two years (43). Paradoxically, upon disease progression, the same immunological barriers that can prevent active disease may hinder successful treatment. The granulomatous immune responses and intracellular residence of mycobacteria in macrophages and other myeloid cells shield mycobacteria from TB drugs and favor their persistence (44). Thus, the disease cannot be viewed separate from the host. This pivotal role of our own immune system in TB pathogenesis indicates the importance of integrating host factors into the exploration of new treatment modalities.

In **Chapter 4** we investigated if modulation of host responses adjunct to antibiotic treatment could lead to improved treatment outcome in our mouse TB model. Host-directed therapies for TB include (i) strategies aimed at increasing macrophage effector function, such as metformin or high-dose immunoglobulins and (ii) strategies aimed at reducing inflammatory damage such as NSAIDs and statins (45). We followed the first strategy based on the hypothesis that host-directed therapy consisting of all-trans retinoic acid, α-galactosylceramide and 1,25 dihydroxyvitamin D could skew myeloid cell development away from a *M. tuberculosis*-permissive and immune suppressive myeloid-derived suppressor cell (MDSC) phenotype towards a bactericidal phenotype. Mice were infected with *M. tuberculosis* H37Rv and were treated with isoniazid, rifampicin and pyrazinamide (HRZ), or HRZ in combination with host-directed therapy. We showed that HRZ in combination with host-directed therapy resulted in a significantly lower frequency of disease relapse after a shortened 12-weeks treatment course compared to HRZ alone (**Chapter 4,** Fig. 3). The most important conclusion that can be drawn from this study is a proof of principle that adjuvant immunotherapy aimed at increasing macrophage effector function can aid in the specific elimination of persistent mycobacteria that are responsible for relapse of disease.

**Treatment factors**

Clinical implementation of novel treatment modalities identified in preclinical research, such as host-directed therapy or new drugs with anti-TB potential is a lengthy and expensive process. In the context of TB, the 2014 REMox trial taught an important lesson: early surrogate endpoints for treatment efficacy based on early bactericidal activity or sputum culture conversion as measured in clinical phase IIa/b trials are unreliable predictors for cure in TB (46, 47). In other words: the capacity of (new) anti-TB drugs to eliminate actively replicating mycobacteria during the initial phase of treatment does not guarantee efficacy against persistent mycobacteria in the second phase of treatment. Clinical phase III trials are costly, require large numbers of patients and may take up to 10 years from study design to publication (48). Therefore, such trials should be based on preclinical evidence with maximum translational value. For the REMox trial the interpretation of preclinical data from mouse TB models might have been too optimistic (48). Therefore, current preclinical models require further optimization to improve their predictive value for treatment outcome in human trials.

To address this problem, a part of the work in this thesis was conducted on behalf of the PreDiCT-TB consortium, which consists of 19 public and private scientific partners in the European Union. The aim of PreDiCT-TB is to generate an integrated and validated preclinical pathway for new treatment options. This is achieved by validating multiple *in vitro* and *in vivo* preclinical TB models by testing currently used and new TB drugs and drug regimens and subsequent comparison of the results with clinical trial data. Our specific contribution was the Beijing-1585-infected BALB/c mouse TB model. A general criticism on the BALB/c mouse TB model is that the granulomas that are formed do not have a caseous center, which is thought to play a central role in human disease (49). The caseous center harbors mycobacteria, influences drug penetration and also results in different degrees of hypoxia and acidity (50-52). The clinical relevance of these lesions is best illustrated by the current resurgence of surgical resection in the context of drug resistance or poor treatment response (53, 54). The presence of caseous granulomas has been the major reason for preclinical testing in guinea pigs instead of mice and the development of the C3HeB/FeJ mouse model, in which such lesions do develop (55). This model is currently validated within the framework of 'Critical Paths to TB Drug Regimens (CPTR)' the American counterpart of the European PreDiCT consortium. Interestingly, by comparing BALB/c and C3HeB/FeJ mice, it was show that sterilizing activity was shown not to be affected by the presence or absence of caseous granulomas (56). Also, for drug penetrance of pyrazinamide it should be mentioned that, despite the formation of different types of granulomas, this was similar for BALB/c and C3HeB/FeJ mice (57). Within the same study it was also found that the pH within C3HeB/FeJ mouse granulomas was outside the range in which efficacy was observed *in vitro*, indicating that acidification

within the granuloma does not alter drug efficacy . Lastly, persistent populations of mycobacteria are present in BALB/c mice despite the abcence of caseous granulomas and treatment failure rates approximate human clinical trial data. This is shown in **Chapter 5**, where HRZE in our model had a treatment success rate of 90% compared to 92% as found in the REMox trial (58). Taken together, this indicates that the BALB/c model is still a valuable preclinical TB model.

Another important finding in **Chapter 5** was that bactericidal activity of the antibiotic regimen early during treatment did not predict treatment outcome to a satisfying degree, similar to clinical phase IIa/b trials (46, 47). Also, the current methods applied for treatment outcome evaluation itself in TB are of a relatively basic nature and involve simple Chi-square testing between large groups of mice after predetermined treatment lengths (59-63). This allows for relapse rate comparison between regimens at selected time points, but does not provide an individual regimen's correlation between treatment length and treatment outcome. This correlation is required to efficiently estimate a regimen's treatment-shortening potential. Therefore, our next aim in **Chapter 6** was to improve the current methods of data collection for treatment outcome experiments. In this study we evaluated treatment outcome in n=3 mice after 9 different treatment durations and combined this with model-based analyses to create an accurate tool for assessment of the relationship between treatment length and predicted cure. Implementation of this model-based approach allowed us demonstrate that treatment with rifapentine-pyrazinamide-isoniazid-ethambutol (RpZHE) and rifampicin-pyrazinamide-moxifloxacin-ethambutol (RZME) resulted in significantly better treatment outcome compared to rifampicin-pyrazinamide-moxifloxacin-isoniazid (RZMH). This could not have been identified based on the observational data alone. These data are in line with other mouse TB studies (**Chapter 6,** table 3) and suggest a negative effect of isoniazid on the efficacy of RZM in mouse studies. Unfortunately, on a translational level our generated data for RZME and RZMH were not comparable to the human clinical trial data after 4 months (97% cure in mice versus 80% in humans for RZME and 29% in mice versus 85% in humans for RZMH) (58). This discrepancy between human and mouse data indicates that the translational value of mouse TB models should be further improved. Indeed, a recent study showed that correcting for additional factors including advanced pharmacodynamics and pharmacokinetic modeling, species-specific protein binding and species-specific pathology further improved the translational value of the BALB/c mouse model (64). Correction for these factors in combination with our integration of mycobacterial strain variance is likely to increase the translational value of mouse TB models further in order to guide the selection of new regimens eligible for future clinical phase III trial testing.

## FUTURE DIRECTIONS

The summarizing discussion emphasizes the complexity of the triangle between mycobacteria, host and treatment in TB, which only appears to increase as our understanding of each factor advances. The work described in this thesis is only a small contribution to the ongoing TB research efforts which result in approximately one hundred new TB-related articles on a weekly basis. In this section I would like to present an integrated view on elements I believe to be essential for advancing our understanding of TB pathophysiology and improving TB treatment.

### 1. Shaping the host response

#### 1.1. Prevention

The best initial step in prevention is maximizing the efficacy of host responses in uninfected individuals. Th1-stimulating BCG vaccination offers variable rates of protection in adults. Based on our analysis of the role of Th17 immunity in TB, an interesting alternative would be to develop a TB vaccine that stimulates Th17 immunity instead. A recent study showed suppressed Th17 responses in young adults progressing to active disease compared to latently infected controls (65). This result was confirmed in an independent cohort that received BCG revaccination, which was also associated with suppressed Th17 responses. There are (at least) three additional reasons why an IL-17-skewed initial host response might be beneficial in TB. The first reason is a specific protective effect of IL-17 against infection with a Beijing genotype strain during acute infection in a mouse TB model (66) (see **Chapter 2, Table 4**). Increased efficacy against currently circulating strains would pose a major benefit, especially since current BCG vaccination appears to act as a selective force contributing to the spread of virulent Beijing strains (15). The second reason is that IL-17 inhibits the development of hypoxic necrotic granulomas and reduces disease severity, which has been demonstrated recently in a mouse TB model (67). A third reason why targeting IL-17 might be a promising approach concerns stimulation of the IL-23/IL-17/CXCL13/Tertiary Lymphoid Structure (TLS)-axis as shown in **Chapter 2, Fig. 4**. Studies in mouse TB models indicate that IL-17 responses might not be essential during acute infection, but that the mentioned axis shapes an efficient micro-environment during acute infection that confers more efficient long-term protection. This is due to efficient recall immunity through the locally formed TLS (68). While speculative, this might also to some degree regulate the increasingly recognized role of B-cell responses in TB. Thus, with regard to preventive treatment, further development of Th17-inducing vaccines is of interest. Important considerations in developing such a vaccine based on our findings in **Chapter 2** section 3 are mucosal delivery, as this favors tissue-resident Th17 responses over systemic Th1 responses and TLS formation

(69, 70), specific attention to the plasticity of Th17 cells, which can alter their cytokine production upon recall immunity (70) and the role of neutrophils, which have shown to be essential in the induction of vaccine-elicited T-helper responses (71).

### 1.2. Treatment

Immunotherapy has the potential to improve treatment outcomes (**Chapter 4**). Effective adjunct therapy, however, requires detailed knowledge of TB pathogenesis and specifically of the failing parts of immunity in case of active disease. Clinical studies continue to show involvement of type 1 interferon-related pathways in blood RNA signatures used for monitoring disease stage or progression in TB (35, 36). Since immunotherapy is primarily targeted at myeloid effector cells such as macrophages, a logical first step for future directions would be to establish the functional consequences of type 1 interferon exposure on myeloid cells more clearly. In **Chapter 2** the currently available literature on this topic has been reviewed. It would be interesting to test the hypothesis that IFN-γ priming of myeloid cells in the bone marrow indeed is required for type 1 interferons to induce a regulatory phenotype in the lungs in the context of *M. tuberculosis,* as has been demonstrated during viral infection and depicted in **Chapter 2**, Fig 3 (37). This would indicate that a systemic effect lies at the base of the locally impaired immune response in the lungs observed in TB (72). In further support of the potential relevance of this systemic pathway, we found in **Chapter 3** that Beijing-1585 and EAI-1627 markedly affect cytokine expression in the bone marrow, indicating that this pathway might be an essential factor in strain-dependent virulence.

Mycobacterial strain variance in general is still a relatively undervalued factor that can be used to improve our understanding of TB pathogenesis. Comparative experimental studies such as **Chapter 3** with a specific focus on host responses are still scarce and need to be verified and complemented with additional analyses such as gene signature studies and characterization/functional testing of the identified cell populations in the lungs. A specific research question here is why B-cells and IL-4 appear to play a detrimental role in our mouse TB model of acute infection, while beneficial effects of antibodies and B-cells have been described in patients. A second interesting development is a recently described sequential association between type 1 interferon and the Th17 response. It was demonstrated that systemic interferon responses preceded and occurred concomitantly with Th17 inhibition which was observed prior to the development of active TB in young adults (65). I believe that particularly the sequential association is relevant. In the context of TB drug evaluation, we find in **Chapter 5** that bactericidal activity during acute disease is not representative for treatment outcomes. Similar principles should be implemented for immunological studies, which should not only focus on characterization and modulation of host-pathogen interactions during

acute infection, but also during the chronic phase of infection. This could also be done in mouse TB models through similar methods as used in **Chapter 3**, albeit with lower infection load of *M. tuberculosis* or less virulent strains, since mice infected with Beijing-1585 and EAI-1627 become moribund after three weeks in our high-dose inoculum model.

A final point of interaction between host and pathogen relevant to our understanding of TB pathogenesis would a mycobacterial subpopulation study. We still do not know precisely where specific populations of mycobacteria in their respective metabolic states reside in different stages of infection. This knowledge would already be of high value in the BALB/c mouse model, where mycobacteria can persist for long durations of time without the presence of necrotizing granulomas. A specific alternative niche worthwhile exploring in this regard is the bone marrow (73). A pilot study within the framework of this thesis was performed to pursue this research question with fluorescent dyes to stain the mycobacterial wall and metabolic activity followed by flow cytometric analysis. Further optimization of this technique will likely provide answers to this fundamental question. Alternatively, culturing isolated samples in the presence of resuscitation-promoting factor proteins might increase the sensitivity of our assays and reveal the populations of mycobacteria that are currently not cultured, but do cause relapse of infection.

### 2. Creating the perfect TB treatment

How would the perfect TB drug regimen look like? TB treatment exists of a short phase in which rapidly dividing mycobacteria are targeted, followed by a longer phase aimed at the low number of persistent mycobacteria that have adopted a metabolic state that is more resistant to killing as shown in **Figure 1.**

In the study described in **Chapter 5** we show that most current TB drugs possess bactericidal activity, but that only rifampicin-containing regimens target persistent subpopulations efficiently enough to achieve cure within 6 months. This persistent state is a mycobacterial stress response caused by antibiotic pressure on the one hand and immunological pressure (e.g. granuloma-associated hypoxia) on the other. Each of these stress-related factors can be manipulated and future TB treatment should make use of all these factors instead of antibiotic pressure alone. Starting with host factors, we show in **Chapter 3** that increasing immunological pressure has the potential to improve treatment outcome. This is based on the hypothesis that latent mycobacteria reside within permissive myeloid cells and that boosting these cells increases their bacterial killing capacities. Alternatively, reducing immunological pressure on mycobacteria through immune suppression, e.g. through adjunct therapy with TNF-α blockers has also shown to improve treatment outcomes (45). This is based on the hypothesis that host responses

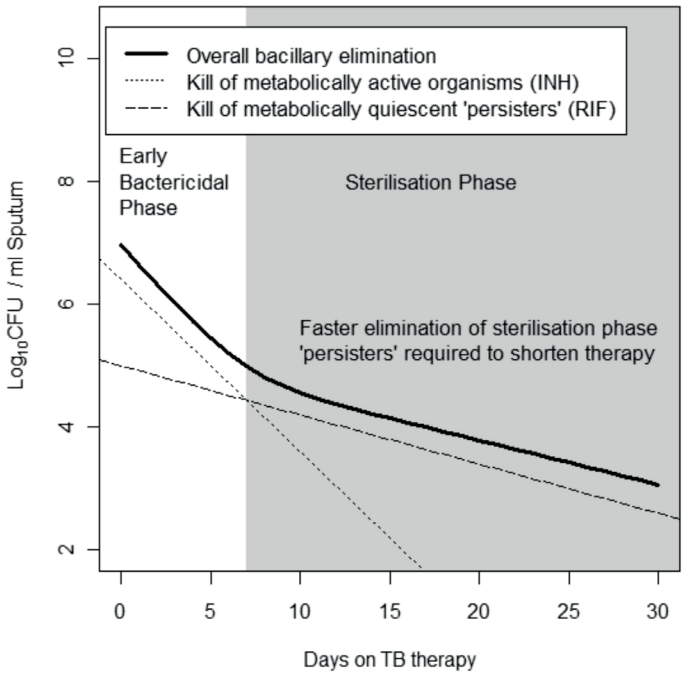


**Figure 1. Biphasic kinetics of TB treatment**
Adapted from Sloan et al (74), sputum colony counting data from three patient cohorts.

such as granuloma formation interfere with optimal drug efficacy. To determine which of these principles is more effective as adjunct to antibiotic treatment, comparative studies in mouse TB models would be a valuable first step. Also, specific attention should be given to the phase in which adjunct treatment should be initiated in the treatment process. When striving towards patient-specific treatment regimes, other factors, such as the degree mycobacterial resistance to antibiotic treatment and the immunological status of the individual patient might also influence the decision whether to increase or reduce host-originated stress.

With regard to the mycobacterial stress response, it might be worthwhile to explore in TB the 'Shock and Kill' principle applied in HIV research (75-77). This would mean treatment with latency-disrupting compound in order to force mycobacteria from latency into a metabolically active state. This might increase susceptibility to currently used anti-TB drugs. Such a strategy has become hypothetically possible with the discovery of *M. tuberculosis* Resuscitation Promoting Factors (RPFs) (78), which have been shown to be major virulence factors (79). Current research on RPFs for *M. tuberculosis* primarily shows practical potential in culturing dormant / persistent mycobacterial populations

that do not propagate in conventional cultures (80). Therapeutic use of RPFs adjunct to antibiotic treatment has been hypothesized to be effective in TB, as its mechanism of action differs from antibiotic treatment and host-directed therapy (81). However, this remains to be tested *in vivo*.

The last, and clinically most relevant factor is antibiotic treatment, which can be optimized through (1) the introduction of new drugs, (2) altering the dose of currently used drugs and (3) changing the treatment schedule.

The TB drug pipeline in particular shows exciting new developments. Bedaquiline is the most clinically advanced example of a drug that appears to have increased efficacy against persistent mycobacteria, but also other new compounds including pretomanid, teixobactin and CPZEN-45, a capreomycin derivate have shown potential specifically against non-actively replicating mycobacteria (82). An overview of the current global TB drug pipeline is shown in **Fig. 2**. Especially for the TB drugs pretomanid and bedaquiline multiple phase III clinical trials are currently ongoing to study their most optimal use in a TB drug regimens (74).

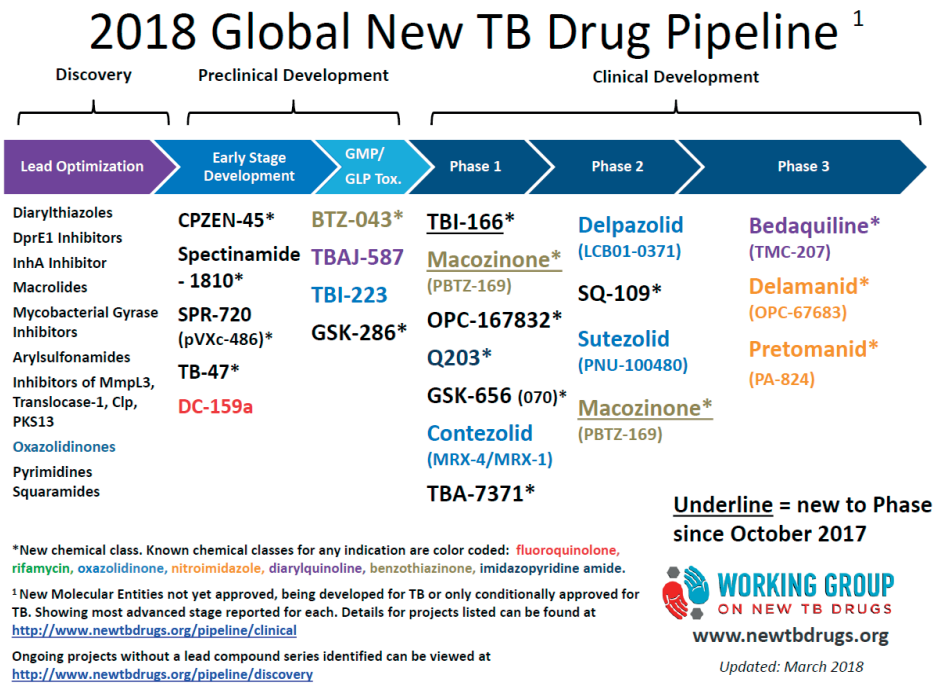


**Figure 2. an overview of the TB drug pipeline, available from http://www.newtbdrugs.org/pipeline/clinical**

Improving the dosing of current TB drugs has received attention in the last years, with a specific focus on the ryfamycins (83, 84). As shown in **Chapter 5**, this is an essential component of the current TB drug regimen to achieve cure in our mouse TB model. Recently, a large clinical trial indeed showed that increasing the dose of rifampicin from 10 mg/kg to 35 mg/kg was safe and reduced the time to culture conversion (85). A final interesting finding in both **Chapter 5** and **Chapter 6** is the role of isoniazid. This is one of the most effective drugs against actively replicating mycobacteria in humans during the first days of treatment. However, we found in **chapter 5** that its efficacy against persisting mycobacteria is limited in mice. Also in **Chapter 6** we found that the isoniazid-containing regimen was less effective in achieving cure compared to the other regimens in mice. A potential antagonistic effect of isoniazid on the efficacy of other drugs has also been described in other mouse TB models (64). As mentioned in **Chapter 6**, this might be a species-dependent flaw for mice that requires correction on a translational level. However, it could also be that the high efficacy of isoniazid against actively replicating bacteria causes a relative increase of mycobacteria progressing to a metabolically less active state, which reduces the efficacy of other drugs. If this hypothesis is true, it might be more effective to use isoniazid only during the first weeks of treatment and replace it later during the course with drugs that are more effective against persistent mycobacteria. This is a hypothesis worthwhile exploring for which the newly designed treatment outcome evaluation as presented in **Chapter 6** is particularly useful. If this outcome then persists in mouse TB models it could be implemented in clinical studies that test new TB drugs as mentioned above.

Taken together, the perfect TB drug regimen in theory would consist of an initial antibacterial multidrug treatment to kill all actively replicating mycobacteria. This should then be followed by a TB drug regimen with specific efficacy against metabolically less active mycobacteria combined with immunotherapy to alter host-induced stress on the mycobacteria. This combination could hypothetically be improved further by adding compounds that prevent mycobacterial transition to a persistent state. Such drugs remain to be developed and tested *in vivo,* preferably in preclinical TB models with high translational value.

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