Rethinking Tuberculosis Host Responses and Treatment Outcome Evaluation

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ISBN: 978-94-6361-115-2
Layout and printed by: Optima Grafische Communicatie (www.ogc.nl)

Printing of this thesis was financially supported by the Netherlands Society of Medical Microbiology (NVMM) and the Royal Netherlands Society for Microbiology (KNVM).

Publication of this thesis was financially supported by KNCV Tuberculosis Foundation.

Rethinking Tuberculosis Host Responses and Treatment Outcome Evaluation

Heroverweging van de gastheer respons
en de evaluatie van behandeluitkomsten in tuberculose

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus
Prof. dr. R.C.M.E Engels
en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op
Vrijdag 7 September om 13.30 uur

door

Bas Christiaan Mourik
geboren te Utrecht

Erasmus University Rotterdam

Erasmus

PROMOTIECOMMISSIE:

Promotor:	Prof. Dr. A. Verbon
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‘Comfort is the enemy of progress’
P.T. Barnum

CONTENTS

1

Introduction and thesis outline

THE HISTORICAL CONTEXT

A long time ago, on the African continent *Homo Erectus* distinguished itself from all other species as it started to use fire in a controlled setting (1). Ironically, this milestone approximately 300 - 400.000 years ago might have been the event that triggered the evolutionary emergence of one of the most lethal infectious diseases known to man (2). Social gathering around fire in combination with smoke-induced airway damage has been hypothesized to provide the ideal environment for the emergence of *Mycobacterium tuberculosis* as a specialized human-specific pathogen causing TB (3).

M. tuberculosis co-evolved with mankind at every step of human evolution. The great migration of *Homo sapiens* out of Africa 100.000 years ago and its subsequent spread across the globe can be reconstructed based on the seven phylogeographical lineages of *M. tuberculosis* (4). Strains from each of these specific lineages continue to show increased transmissibility among their geographically associated human population, indicating optimal host adaptation (5).

During the Neolithic demographic transition around 10.000 years ago, agricultural advances and animal domestication gradually replaced our hunter-gatherer lifestyle, which resulted in massive population expansions. This steered *M. tuberculosis* co-evolution from a slowly progressive disease that benefits from host survival into a 'crowd disease' in which pathogen transmissibility equals evolutionary success and host survival becomes less important (4).

M. tuberculosis virulence increased throughout history. It burdened Egyptians around 5000 years ago (6) and plagued ancient Greeks in the form of 'phthisis' according to Hippocrates' *Of the epidemics* around 2500 year ago. It reached devastating proportions in Europe during the industrial revolution between the 18th and 19th century. Overcrowded cities, poor hygiene and smog exposure created a perfect combination for TB to thrive and no less than one in five human deaths was caused by it (7). Rich and poor alike were slowly dying of 'consumption', a mysterious disease with no cure that killed young people in the prime of their life.

The disease was viewed upon as a romantic disease that inspired artists through '*spes phthisica*', a phenomenon in which the physically wasting body inspired the creative soul and turned prosaic humans into poets (8). A famous example of this was John Keats with poems such as 'Ode to a Nightingale'. The romantic aspects of TB quickly vanished after Robert Koch identified the bacterium *M. tuberculosis* in 1882 as its causative agent.

In a relatively short period of time TB was degraded from a poet's disease to a contagious pathogen that was associated with 'the poor man's sputum'.

Despite the identification of its cause in 1882, an effective cure for TB remained to be found and treatment was limited to a combination of liver cod, sunlight and (perhaps most importantly) isolation from society in sanatoria (9). This changed from the 1950s onwards with the discovery and use of effective antibiotics. Streptomycin and para-aminosalicylic acid (PAS) were the first agents with moderate antimycobacterial efficacy (10). While promising, the first clinical report on these drugs already encountered two important aspects of TB treatment which still apply today: drug resistance and treatment side effects (10). A cure became possible with the discovery of isoniazid as TB drug (11). For over a decade, treatment with oral isoniazid and PAS for 18 to 24 months combined with intramuscular injections of streptomycin during the first 6 months became the standard TB treatment (12). The introduction of agents such as pyrazinamide in 1955, ethambutol in 1961 and rifampicin in 1966 further improved cure rates, while reducing treatment duration (12). Eventually, in 1979, a six months treatment course with the oral antibiotics isoniazid, rifampicin, pyrazinamide and ethambutol became, and still is, the standard of care in TB treatment (9, 13).

Unfortunately, the progression in TB treatment was overshadowed by an infectious disease crisis that started in the 1980's. The Human Immunodeficiency Virus (HIV) manifested itself and caused mortality on an unprecedented scale. Where TB caused approximately 20% mortality among affected individuals in Europe during the industrial revolution, HIV was accountable for over 50% of adult mortality in most African countries during the early 90's (14). HIV-induced immunodeficiency was quickly recognized to act as a TB-catalyzer and vice versa. The coinfection of the two diseases was termed 'the cursed duet' (14). An immunocompetent, latently infected individual has a 5-10% life-time risk of progressing to active TB (15). These chances increase substantially when this same individual is also infected with HIV, which is now the most important predisposing factor for the development of active TB disease (16). The increased progression and transmission of TB amongst HIV-infected individuals caused an increase in TB incidence in sub-Saharan Africa between 1990 and 2005, while stabilization or steady decrease of TB incidences was observed in countries outside Africa during this period (17).

Another impact of HIV on TB is of a more indirect nature. During the last 30 years, enormous global efforts have resulted in rapid development and implementation of anti-retroviral HIV therapy. Unfortunately, these efforts appear to have been at the cost of funds for TB treatment, as TB treatment has remained virtually unchanged compared to pre-HIV times. In 2016, TB claimed more victims than HIV and malaria combined (18).

Nevertheless, the global fund disbursed 40.4% of its funding for HIV, 29.7% for malaria and 22.4% for tuberculosis (19). In absolute terms: in 2016 a worldwide total of 19.1 billion dollar was available for HIV treatment and prevention compared to 6.3 billion for TB (18, 20). A potential explanation for this discrepancy is best embodied by the words of the director of the WHO global TB program, stating that "HIV is a disease that involves one of the most important aspects of life – sex. Tuberculosis involves the sputum of poor people, and the poor are without voice in most societies" (21).

Nowadays, TB treatment, in combination with improved sanitation, housing and nutrition, screening programs and outbreak prevention measures has resulted in a decline of TB incidence in developed countries. Nevertheless, in the developing world TB still has a profound impact and claimed an estimated 1.7 million lives in 2016 (18). This makes TB one of the top 10 causes of death worldwide and places it above road injuries. New threats are present on the horizon in the form of more extensive drug resistance and the emergence of *M. tuberculosis* genotypes with increased virulence. Combined with the current major funding gaps for TB diagnosis, treatment and research, it remains to be seen how long our 1979 drug regimen can contain this evolutionary giant.

From the microbe's point of view: the template for success

M. tuberculosis is a slow-growing, rod-shaped, facultative intracellular bacterium that is primarily spread through aerosols coughed up by infected individuals. Detailed description of the evolutionary success of *M. tuberculosis* can be divided into three different categories: mycobacterial factors, host factors and treatment factors, which will be described in more detail.

Mycobacterial factors in M. tuberculosis' evolutionary success

The unique features of *M. tuberculosis* start with the composition of its cell wall. The thick, lipid-rich combination of mycolic acids, lipomannan arabinogalactan and peptidoglycans prevents regular Gram staining and requires specific stains such as Ziehl-Neelsen (acid-fast) or auramine-rhodamine staining for identification (22). The composition of the mycobacterial wall stimulates rapid contact with innate leukocytes such as macrophages and subsequent phagocytosis (15). The unique inflammation-inducing capacity of the mycobacterial cell wall is best exemplified by complete Freund's adjuvant, a common immunopotentiator used to enhance vaccination efficacy in experimental animals, which primarily consists of inactivated mycobacteria. Upon phagocytosis, *M. tuberculosis* prevents acidification of the phagosomal compartment caused by phagolysosomal fusion (15). Subsequently, pore-forming virulence factors such as early secreted antigenic target 6 kDa (ESAT-6) enable translocation to the cytosol (23). It has been demonstrated that in the intracellular compartment, *M. tuberculosis* can prevent further degradation

and use the macrophage as a shielded niche for survival, replication and persistence (15).

Under influence of environmental stress factors such as antibiotic pressure or adaptive immunity, *M. tuberculosis* can further alter the composition of its cell wall as part of a transition into a slow-growing, non-replicating state in which it is highly resistant to host responses and antibiotics (24, 25). The ability of *M. tuberculosis* to progress to this resilient state is one of the main reasons for the long treatments, as metabolism is low and the thickened cell wall prevents entry of antibiotics into the bacterium (26). In its persistent state, *M. tuberculosis* is also able to effectively circumvent immunity and cause the clinical phenomenon termed 'latent TB' in which mycobacteria are present in the body, but do not cause active disease at that moment (15).

Mycobacterial strain variance is a virulence factor in TB pathogenesis that is gaining interest among TB researchers. Due to its slow-growing character, *M. tuberculosis* was initially viewed upon as a genetically conserved organism for which strain variation played a minor role in disease outcome (27). This assumption could be one of the reasons why the mycobacterial H37Rv strain, isolated from a patient in 1905 remains one of the most commonly used strains in preclinical TB research to date (28). Advances in genotyping technologies and clinical observations over the last two decades have proven this assumption to be false. H37Rv is deemed a laboratory strain as it is no longer isolated from patients, while strains from other genotypes have emerged at an alarming rate (29, 30). The best example of this is the Beijing genotype, identified in 1995 (31). Strains of the Beijing genotype show increased virulence and drug resistance compared to strains from other lineages, as illustrated by exceptionally high rates of drug resistance in Eurasia (32-40). Given the current high TB incidences in East-Asian countries, Beijing genotype strains are the second-most common strains responsible for TB after strains from the East-African Indian (EAI) genotype (29). Thus, an important consequence of strain variance that will also be discussed in this thesis is that the strains that currently cause the major burden of TB in patients are clearly distinct from those most frequently used for screening of novel anti-mycobacterial drugs in preclinical TB models.

Host factors in M. tuberculosis' evolutionary success

Worldwide, a huge human reservoir of latently infected individuals exists of which most will most likely never progress to active TB. Latent TB poses a significant challenge to the global eradication of TB as an estimated 30% of the world population can be classified as having latent TB (15). However, an immune-compromised state significantly increases the risk of TB reactivation (15). In developing countries this is best exemplified by HIV co-infection as discussed above. In the developed world, major risk factors for TB include

type 2 diabetes, alcohol use and smoking (41). Another increasing population of individuals at risk for TB reactivation comprise those receiving deliberate immunosuppression for treatment of auto-immune diseases, malignancies and organ transplants (16). A notorious example is the introduction of anti-TNF-α monoclonal antibody therapies for rheumatoid arthritis, which caused increased rates of TB reactivation in latently infected individuals (42). The association of such specific interventions like anti-TNF-α with TB reactivation does provide insight into their role in TB pathogenesis (43). Another classic example is the discovery of genetic defects as observed in Mendelian Susceptibility to Mycobacterial Disease (MSMD) (44). Patients with MSMD have genetic mutations resulting in defective IL-12 production or IFN-γ responsiveness, which renders them extremely susceptible for mycobacterial disease (44).

Anti-TNF-α treatment and MSMD highlight the importance of an intact IL-12 / T-helper 1 immune response/ IFN-γ in TB. However, this axis alone is not sufficient for an optimal host response. The current vaccine for TB, Bacillus Calmette-Guérin (BCG), induces a strong Th1 response but provides highly variable protection between 0-80% due to unknown causes (41, 45, 46). Further boosting of the Th1-inducing potential of BCG by using a modified Ankara virus did not improve efficacy (47, 48). BCG offers higher protection rates in young children. In adults, however, BCG vaccination not only has a lower efficacy for protection against TB, but might even have been a selective force contributing to the spread of virulent Beijing strains, which circumvent vaccine-mediated immunity more efficiently (41, 49). With increasing incidences of Beijing strain infections, this might even call for more selective vaccination strategies. Also, the basic principle of vaccination is that once the immune system has encountered a pathogen, it will form a more effective and efficient adaptive immune response upon re-infection. In the case of TB, it should be noted that reinfection after successful TB treatment frequently occurs and actually increases the chances of developing active TB instead of offering protective immunity (50, 51). Thus, in contrast to most other infectious diseases, survival after primary infection provides limited protection against future exposure. Combined with the variable efficacy of BCG, this indicates the complexity of TB immunology and the need for better understanding and identification of protective host responses.

Immune-compromised individuals have an increased risk of developing active TB, but the vast majority of TB patients are non-immune compromised adults, capable of inducing robust host responses (18). Thus, a final important host factor to consider is the contribution of our own immune system to disease progression. In other words: To what extent does our own immune system contribute to a detrimental course of TB? Gene expression signatures in TB have greater overlap with auto-immune diseases than with other infectious diseases (52). Also, preclinical studies show that boosting protective

T-cell-mediated IFN-γ production in TB promotes disease progression due to hyper-inflammation (53). So it appears that both immune suppression and stimulation can cause disease progression in TB. Unraveling the exact host factors and immunological mechanisms responsible is crucial for the development of host-directed therapies as possible adjunct to antibiotic treatment.

Treatment factors in M. tuberculosis' evolutionary success

Current strategies for global TB treatment revolve around DOTS, i.e. 'Directly Observed Treatment, Short course'. The success of DOTS depends on five distinct elements: (i) sustained political and financial commitment, (ii) diagnosis by quality-ensured microscopy services, (iii) a secured supply of high quality TB drugs, (iv) standardized recording and of course (v) Directly Observed Treatment (DOT) (18). DOT has been proven to be important to complete the 6-months treatment course successfully. TB treatment eliminates nearly all mycobacteria and most of the clinical symptoms in the first 2 months of treatment. However, longer treatment durations are required to eliminate persistent populations of mycobacteria. In these last 4 months, in which low numbers of persistent mycobacteria are treated, compliance to therapy is essential to prevent the development of drug-resistant TB. Drug resistance currently occurs in 4.1% of all new TB cases and 19% of previously treated cases (18). The impact of drug resistance in TB is substantial: treatment of drug-susceptible TB comprises a 6-months course with daily oral first line TB drugs, has a cure rate of approximately 83% and costs around 1200 dollar (18). In contrast, treatment of multi-drug resistant TB requires at least 18 months of treatment with second-line TB drugs, has a cure rate of approximately 55% and costs almost 10.000 dollar (18, 54, 55).

Probably one of the best ways to increase compliance and prevent drug resistance is to shorten treatment duration, but chemotherapeutic advancements that may shorten TB treatment have been scarce. After 40 years of silence, delamanid and bedaquiline have recently been approved as new agents for TB treatment, but remain reserved for the treatment of drug-resistant forms of TB (56, 57). Fortunately, the need for new TB treatment has been recognized and the current clinical pipeline for new TB drugs looks more promising than ever (41). Meanwhile, reducing duration of TB treatment through repurposing of other chemotherapeutic agents proved difficult. In 2014, a large phase III clinical trial to reduce treatment duration to 4 months through implementation of moxifloxacin in the multidrug regimen essentially failed (58). Although this clinical trial did not achieve treatment reduction of TB, it did provide essential information to rethink current methods and improve future drug development programs. It showed that early surrogates for treatment efficacy assessments as measured in clinical phase IIa/b trials are unreliable predictors for cure in TB (59, 60). More relevant for this thesis, it

also pointed out that current preclinical TB models require further optimization in order to increase their translational value (61).

Current research on new drugs, drug regimens and treatment duration primarily occurs in mouse TB models (62). These are readily available models that allow testing in large groups, but have the drawback that infected mice do not develop necrotizing granulomas. These structures are the hallmark for disease in human TB and are believed to play a central role in mycobacterial persistence (62, 63). For the experiments described in this thesis we use the BALB/c mouse model, because, despite the absence of necrotizing granulomas, the course of infection and treatment in the BALB/c mice resembles the clinical situation remarkably (64). After several months of treatment no mycobacteria can be cultured from the lungs, but a full 6-months course with the current TB drug regime is necessary to eradicate persistent mycobacteria and prevent relapse of disease (64). Eradicating these persistent mycobacteria more efficiently is the key to shortening treatment duration and their proven presence in the BALB/c mouse model indicates its usefulness as preclinical model.

OUTLINE OF THIS THESIS

The aim of this thesis is to increase our understanding of TB pathogenesis and improve its treatment. Therefore, mycobacterial-, host-, and treatment factors are studied.

Mycobacterial factors

To what degree does mycobacterial strain diversity influences treatment outcome and host responses in mouse TB models? The mycobacterial strain H37Rv is still commonly used in preclinical TB research, but it is more than 100 years old and no longer isolated from patients, can we therefore still use it as model organism? In **Chapter 3** we analyze host-responses against H37Rv compared to two recently isolated clinical strains from the Beijing and East-African Indian genotype to evaluate how currently circulating strains evade protective immunity more efficiently. To evaluate the impact of strain diversity on TB treatment, we assess bactericidal drug activity and treatment outcome against recent clinical isolates in **Chapter 5**.

Host factors

Both impaired host responses and boosting immunity can result in disease progression in TB, indicating the duality and importance of our immune system in TB pathogenesis. In the current paradigm, IL-12 stimulates IFN-γ-mediated macrophage activation and mycobacterial killing, which is essential in TB as observed in patients with MSMD. How-

ever, this does not explain the recently identified functional role of antibodies and B-cells in TB (65, 66). Also the emerging s of type 1 interferons and/or Th17 immunity in patients need to be incorporated into our current understanding of TB pathogenesis. Therefore we have performed a review of the current literature on these factors in **Chapter 2**. We also evaluate the feasibility of altering host responses through host-directed therapy adjunct to antibiotic treatment to improve treatment outcome in **Chapter 4.**

Treatment factors

Poor outcomes of recent clinical phase III trials evaluating novel TB treatment regimens have shown that the predictive value of preclinical models needs to be further optimized (58). In **Chapter 5** we validate the efficacy of conventional TB drugs in our own mouse TB model using a mycobacterial Beijing genotype strain and assess the predictive value of early bactericidal activity, i.e. during the first months of treatment, on treatment outcome. Finally, in **Chapter 6** we present a new approach for treatment outcome evaluation by combining observational data with mathematical modeling in order to evaluate the potency of (new) TB drug regimens.

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2

Interactions between type 1 interferons and the Th17 response in tuberculosis: Lessons learned from auto-immune diseases

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Published in Frontiers in Immunology 2017 Apr 5;8:294. doi: 10.3389/fimmu.2017.00294. eCollection 2017

ABSTRACT

The classical paradigm of TB immunity, with a central protective role for Th1 responses and IFN-γ-stimulated cellular responses, has been challenged by unsatisfactory results of vaccine strategies aimed at enhancing Th1 immunity. Moreover, preclinical TB models have shown that increasing IFN-γ responses in the lungs is more damaging to the host than to the pathogen. Type 1 interferon signaling and altered Th17 responses have also been associated with active TB, but their functional roles in TB pathogenesis remain to be established. These two host responses have been studied in more detail in auto-immune diseases and show functional interactions that are of potential interest in TB immunity. In this review we first identify the roles type 1 interferons and Th17 immunity in TB, followed by an overview of interactions between these responses observed in systemic auto-immune diseases. We discuss (i) the effects of GM-CSF-secreting Th17.1 cells and type 1 interferons on $CCR2^{+}$ monocytes; (ii) convergence of IL-17 and type 1 interferon signaling on stimulating B-cell activating factor (BAFF) production and the central role of neutrophils in this process; (iii) synergy between IL-17 and type 1 interferons in the generation and function of tertiary lymphoid structures and the associated follicular helper T-cell responses. Evaluation of these auto-immune-related pathways in TB pathogenesis provides a new perspective on recent developments in TB research.

1. INTRODUCTION

Tuberculosis (TB) has been responsible for an estimated one billion deaths worldwide over the last 200 years (1), which is more than any other infectious disease caused by a single pathogen. Given its global magnitude, it has been hypothesized that TB particularly contributed to the genetic selective pressure that predisposes for development of auto-immune diseases (AID) (2). This is supported by polymorphism studies of the *TNF* gene, which show an opposite association between susceptibility to TB versus susceptibility to several AID (3). Additionally, a gender-dependent predisposition to either TB or AID exists with a male predominance among TB patients (4) opposed to increased AID incidences in women (5). The general concept of an inverse relation between infectious diseases and AID is best described by the hygiene hypothesis, which states that diminished exposure to infectious pathogens during childhood increases the chances of developing auto-immune diseases (AID) and allergies (6) (7). Also, epidemiologically, the decline in burden of infectious diseases over the last century in industrialized countries is accompanied by increasing rates of auto-immune diseases (AID) (8).

Despite support for an inverse relation, similarities between TB and AID have also been identified. TB is even hypothesized to be an infection-induced AID based on the observation that diverse clinical autoimmune phenomena frequently occur in TB patients (9, 10). Furthermore, up to 32% of patients with active TB have elevated autoantibody titers (11, 12). Rational explanations for these findings could be that either TB and AID activate common immunological pathways (10), or protective immunity in TB increases the chance to develop AID (2). In both scenarios, key findings in AID immunology could potentially contribute to our understanding of TB pathogenesis.

The current paradigm of the host response to Mtb infection is summarized in **Fig. 1**. The indispensable role of IL-12/IFN-γ-mediated Th1 immunity against Mtb has long been recognized (13). However, stimulating Th1 immunity in TB can also result in excessive inflammation (see **Box 1**). More recently the contributions of additional immune pathways have been explored, especially the role of T1-IFNs, Th17 immunity (14, 15) and unconventional T cell immunity (16-18). Little is known about the potential interaction between T1-IFNs and Th17 responses in TB, but interesting observations in this regard have been reported for multiple AID (19-21). To determine if these findings are relevant for the understanding of TB pathogenesis, we first review the separate involvements of T1-IFNs and Th17 responses in TB pathogenesis in section 2 and section 3, respectively. Next, their known interactions in AID are discussed in section 4. Lastly, in section 5 the potential relevance of these interacting pathways in TB is assessed and integrated into the current understanding of TB pathogenesis.

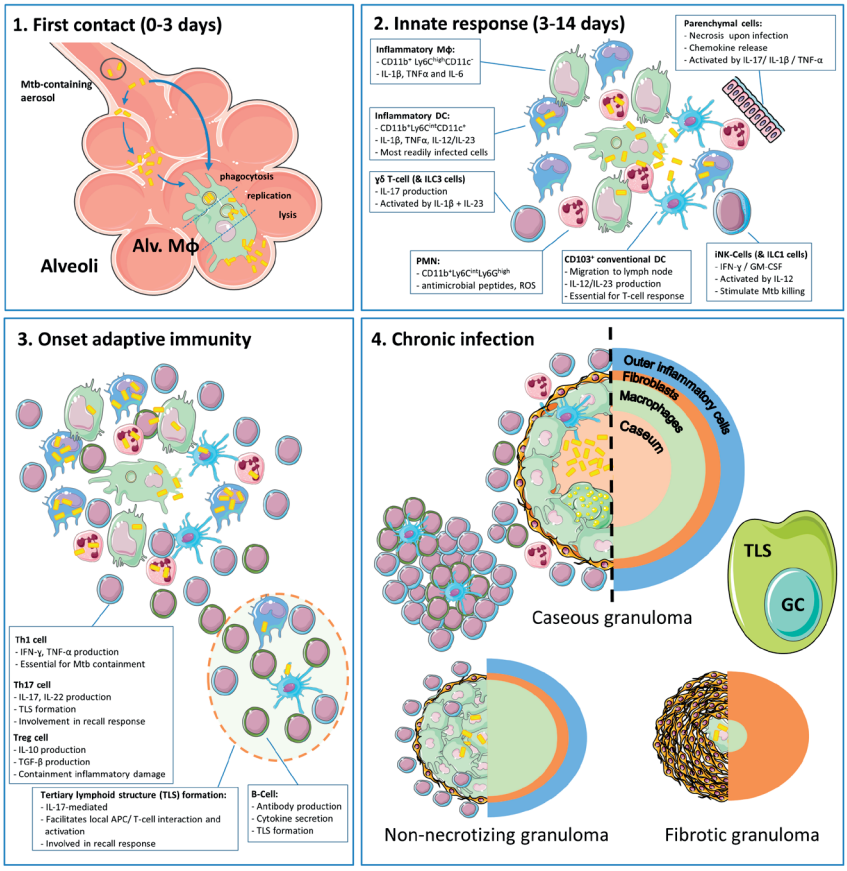


Figure 1. The phases and cell types involved in the immune response to TB in the lungs

1) Inhaled Mtb-containing aerosols are deposited deep into the lung, reaching the alveoli (24). Within the alveoli, Mtb are phagocytosed by alveolar macrophages (Alv. Mϕ) or infect alveolar epithelial cells prior to ending up in alveolar macrophages (25). Within Alv. Mϕ, the bacteria are able to inhibit phagosome-lysosome fusion and replicate until cell lysis ensues, which takes approximately 3-5 days (26). **2)** After the initial contact, Mtb encounters infiltrating myeloid cells of which inflammatory dendritic cells (iDC) and PMN are most readily infected (13, 27). During these early phases, invariate natural killer (iNK)-cells and type 1 innate lymphoid cells (ILC1) produce IFN-γ in response to IL-12 and stimulate myeloid cells to kill phagocytosed Mtb. In addition, γδ T-cells and ILC3 produce IL-17. There is increasing appreciation for the role of tertiary lymphoid structures that arise under influence of IL-17 and facilitate optimal activation of myeloid cells and efficient recall-responses. During this process loosely aggregated 'innate granulomas' are already formed (28). It should be noted that the roles of ILC1s and ILC3s are based on their general function, which has not yet been formally demonstrated in TB (29). **3)** Onset of adaptive immunity in Mtb infection is delayed to circa 14 days in mice and up to 6 weeks in humans (13, 24). At this point distinct T-cell subsets and B-cells migrate to the site of infection and execute their different effector functions. **4)** After onset of adaptive immunity, 90-97% of infected individuals develop sustained infection without clinical symptoms termed 'latent TB infection' (LTBI) (13). LTBI was initially considered a static phase, but it is now known that this stage is hallmarked by the presence of granulomas in various stages (caseous, non-caseous, fibrotic) and an ongoing balance between anti-mycobacterial activity and regulatory mechanisms to minimize immunopathology (13, 30). Cell phenotypes are as present in mouse TB models.

2. TYPE-I INTERFERON IN TB

Type I interferons (T1-IFNs) comprise a family of 13 IFN-α subtypes, IFN-β, IFN-ε, IFN-κ, and IFN-ω, which have the shared ability to bind to the IFN-α/β receptor (IFNAR) (22). Other interferons include the single type II interferon, interferon-γ, and the type III interferon family covering three IFN-δ types. All nucleated cell types are capable of both producing T1-IFNs and responding to them, while type II/III interferons are mostly produced by leukocytes (22). The main function of T1-IFNs is to 'interfere' with intracellular infections. Therefore, T1-IFN expression is primarily induced through cytoplasmic pattern recognition receptors (PRRs) and endosomal Toll-like receptors (TLRs), which activate distinct interferon regulatory factors (IRFs) that act as transcription factors enabling expression of interferon-responsivegenes (23). In contrast, extracellular pathogens trigger surface-bound TLRs that preferentially induce IL-1β and TNF-α through activation of NF-κB.

The role of T1-IFNs in infectious diseases is complex (15, 31-33). T1-IFNs boost the immune system upon pathogen encounter by activating dendritic cells and NK-cells and by stimulating both B-cell responses and $CD4^+$/$CD8^+$ T-cell responses. However, T1-IFNs can also induce anti-inflammatory responses to control immune-mediated tissue damage during chronic infections. These contradictory effects of T1-IFNs in different situations can likely be ascribed to the heterogeneity of the T1-IFNs family, downstream activation of different STAT homo/heterodimers after binding to IFNAR (23, 34) and to differential priming of cells prior to induction of T1-IFN signaling (35).

2.1. T1-IFNs in human TB

When recombinant or purified T1-IFNs became available as therapeutic agents in the 1980s, different applications have been established based on their antiviral, immune-stimulating and -suppressive effects. These include treatment of viral infections (e.g. IFN-α treatment of hepatitis B/C infections), auto-immune diseases (e.g. IFN-β treatment for multiple sclerosis) and various malignancies (44). Based on their well-described immune-stimulating effect, the use of T1-IFNs as adjuvant to antibiotic treatment for patients with active TB has also been explored (see **Table 1**). All studies found a positive influence of adjuvant T1-IFN therapy on clinical outcomes in active TB (45-49). Conversely, IFN-α treatment without concomitant antibiotic treatment, e.g. for hepatitis C, has been described to cause reactivation of latent TB (50-57). While reactivation of latent TB and treatment of active TB are two distinct clinical situations, the latter finding suggests an unfavorable role for T1-IFNs in TB pathogenesis

In 2010, an interferon-inducible transcriptional signature was reported in circulating leukocytes of TB patients, thus linking increased T1-IFN signaling with active disease

Box 1. The dual faces of IFN-γ in TB immunity

In the current paradigm of a successful host response, lung DCs migrate to the draining lymph node after Mtb contact and induce a robust IL-12-mediated Th1 response (13). This results in migration of IFN-γ-producing $CD4^+$ T-cells to the site of infection. Subsequently, activation of macrophages by IFN-γ results in killing of intracellular Mtb, while activated $CD8^+$ T-cells lyse infected host cells. Conversely, unsuccessful clearance of infection is due to poor activation of adaptive immunity. This can result from insufficient antigen presentation (36), or from the action of regulatory factors that interfere with Th1 responses such as IL-10 or PDL1-PD1 interaction (13). Paradoxically, the current vaccine Bacillus Calmette-Guérin (BCG) induces a strong Th1 response, but is only partially effective in protecting against TB (37). Boosting the Th1-inducing potential of BCG by using a modified Ankara virus also has yielded disappointing results (38, 39). Thus, solely stimulating Th1 immunity might not be the solution in TB prevention. This is confirmed in a mouse TB study showing that increasing IFN-γ production by T-cells in the lungs is detrimental to the host due to hyper-inflammation that requires PD-1- mediated suppression to limit pathology (40). In line with this, Mtb-infected mice deficient in PD-1, or mice in which PD-1 is selectively inhibited display excessive inflammation and disease progression (41, 42). Lastly, *ex vivo* studies in human monocyte-derived macrophages show that protective effects of IFN-γ are dependent on multiple factors including time of contact, concentration, and the magnitude of the ensuing microbial challenge (43). Based on these observations it can be concluded that boosting IFN-γ production and Th1 immunity in TB, besides potentially enhancing protection, can also result in unbalanced inflammation in the lungs that is more harmful to the host than to the pathogen. This emphasizes the need for involvement of additional immunological pathways for optimal protection.

(58). This finding has been validated in several independent studies (59-62). A meta-analysis confirmed statistical significance but found a less dominant role for T1-IFN-related genes than expected (63). This is ascribed to the involvement of signaling components downstream of the T1-IFNs receptor in multiple overlapping intracellular pathways. Also, association studies do not necessarily implicate a causally detrimental effect of T1-IFNs in TB pathogenesis. In line with this, T1-IFN responses show potential as biomarkers or diagnostic tool for risk of active disease, but their functional involvement during TB progression in patients is not yet understood (62).

2.2. Preclinical studies in mice support a detrimental role of T1-IFNs during acute TB

A causal relationship between T1-IFN signaling and TB disease severity was first suggested in 2001 when IFN-α levels in the lungs of Mtb-infected mice were shown to be associated with Mtb strain virulence (64). Several approaches have been used to verify this relationship between increased T1-IFN signaling and unfavorable disease outcome. Blocking the T1-IFN signaling pathway through use of IFN-α/β receptor knockout ($IFNAR^{-/-}$) improves survival, but only when applied on the background of mouse strains in which acute TB is lethal, such as the A129 strain (65). In $IFNAR^{-/-}$ mice with a relatively TB-resistant C57BL/6 background, survival rates were similar to wildtype mice, but mycobacterial loads in the lungs were lower(66-69). One study actually observed increased loads in the lungs (70) (**Table 2**).

Table 1. Effect of T1-IFN supplementation in human TB

Study design	Regimen	Outcome	Side effects	Ref
Open parallel, susceptible Mtb strain, HIV(-), N=20 (2x10), 2 mo treated	HRZE vs. HRZE+ IFN-α	-Less fever on d3&4 after start treatment in HRZE+IFNα group -Increases in total lymphocytes and HLA DR1+ cells after 2 months only in HRZE+IFN-α group -Reduction in HRCT-score only in HRZE+IFN-α group -Stronger reduction of pro-inflammatory cytokines in BALF after 2 months treatment in HRZE+IFN-α group	No adverse effects reported	(45)
Patients treated prior for 3-12 years, MDR strain, HIV(+), N=5, 12 wks treated	Anti-TB treatment +IFN-α	-2/5 complete response -1/5 partial response -2/5 no response -Increase of NK (% cytotoxicity) in all patients after 12 weeks	Flu-like symptoms in 4/5 patients, not needing treatment	(46)
Patients treated prior for 6 mo, MDR strain, HIV (-) N=7, 9 wks treated	DOT+ IFN-α	-Significant drop ($p = 0.02$) in Mtb-loads at the end of a 9-weeks IFN-α treatment course -Significant increase ($p = 0.03$) in Mtb-loads after stop of IFN-α treatment -Significant drop in IL-1β, IL-6, TNF-α and IFN-γ proinflammatory cytokines; IL-4 & IL-10 showed inconsistent changes.	No adverse effects reported	(47)
Parallel, patients treated prior for 6 mo with DOT, MDR strain, HIV (-) N=12 (2x6), 8 wks treated	1. DOT 2. DOT + IFN-α	-After 8 weeks, all five subjects of the case group became sputum smear-negative; the control group remained smear-positive ($p = 0.012$) -Evaluation of smear results after 6 months showed two smear-negative subjects in the case group while all controls were smear-positive ($p = 0.132$)	4 subjects mild arthralgia and myalgia, flu-like symptoms in all subjects	(48)
Case report, MDR strain, HIV (-), N=1, 2 mo treated	HRZE+ IFN-α	-Two months after initiation of therapy, sputum smears became negative, the patient's clinical and radiological findings strikingly improved. During 4-year follow-up, all consecutive sputum cultures remained negative.	No adverse effects reported	(49)

BALF= Bronchoalveolar lavage fluid, DOT= Directly Observed Therapy (antibiotic TB treatment), HRCT= high-resolution Computed Tomography, HRZE= Isoniazid, Rifampicin, Pyrazinamid & Ethambutol, MDR= Multi-drug resistant, Mo= months.

In a second approach Mtb-infected mice were supplemented with T1-IFNs after start of infection or treated with the TLR3-ligand poly-ICLC, which stimulates T1-IFN production and signaling (64, 71). Both studies showed increased mortality and higher mycobacterial loads in the supplemented groups, which were not observed when T1-IFNs or poly-ICLC were administered to Mtb-infected $IFNAR^{-/-}$ mice. Lastly, in a third approach, mice were primed with a T1-IFN-inducing influenza virus prior to TB infection, which led to enhanced mycobacterial growth and reduced survival (72).

Table 2. Interference with T1-IFN signaling in preclinical TB studies

Mouse back ground	Intervention	Mtb strain	Survival	Mtb load	Ref
A129	IFNAR$^{-/-}$	HN878, W4, CDC1551, 100-200 CFU, aerosol	Better survival against CDC1551. Trend towards better survival against HN878	No data	(65)
B6D2/ F1	anti-IFN-α/β antibody	HN878, 100-200 CFU, aerosol	Better survival against HN878	No differences up to d.100	(65)
B6/129	IFNAR$^{-/-}$	H37Rv, HN878, CSU 93, CSU 123 50-100 CFU, aerosol	No differences in survival after infection with all strains	Lower Mtb loads in lungs after infection with all strains up to d.150	(66)
B6	IFNAR$^{-/-}$	Erdman, 10^6 CFU, i.v. injection	No data	No differences in lung until d.20 Lower Mtb loads in spleen at d.10 and d.20	(67)
B6	IFNAR$^{-/-}$	H37Rv, 100 CFU, aerosol	No differences up to d.70	Lower Mtb loads in lungs at d.18, no differences at d.25	(68)
129S2	IFNAR$^{-/-}$	H37Rv, 200 CFU, aerosol	Improved survival	Lower Mtb loads at d.21	(69)
B6	IFNAR$^{-/-}$	H37Rv, 500 CFU, aerosol	No differences in survival up to d.90	Lower Mtb loads at d.21	(69)
B6.SJL	IFNAR$^{-/-}$	H37Rv, 100-150 CFU, aerosol	No differences in survival up to d.90	No data	(73)
B6/129	IFNAR$^{-/-}$	Erdman, 100 CFU, aerosol	No data	Higher Mtb loads in lungs on d.10, d.20 and d.40. Equal loads at d.80.	(70)

i.v.: intravenous

Enigmatically, reduced mycobacterial loads in IFNAR$^{-/-}$ mice are primarily observed in the acute phase of infection in which T1-IFNs are considered immune stimulating. No differences in survival or long term control of infection were found in C57BL/6 IFNAR$^{-/-}$ mice compared to wild-type. In support of this notion, T-cell analyses in several of the above-mentioned studies convincingly excluded an effect of increased or decreased T1-IFN signaling on the adaptive immune response (68, 69, 71). Notably, none of these studies addressed the effect of T1-IFNs as adjunct treatment to antibiotics, which was shown to be beneficial in TB patients (**Table 1**).

2.3. Mtb actively induces T1-IFNs

Multiple studies indicate that Mtb employs both active and passive mechanisms to induce T1-IFNs (74-76). The mycobacterial ESAT-6 secretion system (ESX-1) and its 6 kDa early secretory antigenic target (ESAT-6) are essential in this process, as mycobac-

teria lacking ESX-1 fail to induce T1-IFN production (67, 77-81). ESAT-6 can disrupt the phagosomal membrane, which allows translocation of mycobacteria and mycobacterial products from the phagosome into the cytosol (78, 82).

Mycobacteria actively secrete several T1-IFN-inducing compounds, including double-stranded (ds)DNA and the bacterial second messenger cyclic-di-AMP (83). These compounds are recognized by different cytosolic PRRs, including cGAS (81), IFI-204 (78), AIM2 (84) and possibly NOD2 (77), although data on the latter are conflicting (67, 78). Activation of these cytosolic PRRs converges to activate 'STimulator of INterferon Genes' (STING), which subsequently forms a complex with TANK-binding Kinase 1 (TBK-1) (79). This STING-TBK1 complex activates IRF3, leading to IFN-β production in mice (80) as well as human dendritic cells (74). IRF3$^{-/-}$ mice are poor producers of IFN-β and more resistant to Mtb infection, which supports a negative role for T1-IFNs in TB pathogenesis (78).

However, the overall picture is more complex. IRF3$^{-/-}$ mice are more resistant to Mtb infection, but mice deficient in the cytosolic PRR cGAS, upstream of IRF3, show diminished control of chronic Mtb infection (79). This can be traced back to a concomitant reduction in autophagy, which is also dependent on the cGAS-induced activation of the STING-TBK1 axis, but independent of IRF3. In line with this, mice infected with an Mtb-strain that induces higher amounts of cyclic-di-AMP, thus stimulating both IRF3-mediated IFN-β production and STING-TBK1-mediated autophagy, show improved survival compared despite increased IFN-β levels (83). Taken together, this suggests that pro-mycobacterial effects of stimulating the cytosolic PRR/STING/IRF3/IFN-β-axis by mycobacteria might be outweighed by the anti-mycobacterial effects of the PRR/STING/autophagy pathway.

Autocrine or paracrine IFN-β-signaling induces IRF-7 and leads to the production of IFN-α in human dendritic cells (74). In line with this, injection of recombinant IFN-β in mice induces IFN-α production (85). Alternatively, myeloid cells and particularly plasmacytoid dendritic (pDC) cells are capable of directly activating IRF7-mediated IFN-α production after recognition of Mtb, particularly by endosomal TLR9 (86). In TB, this TLR9-IRF7 pathway is studied to lesser extent than the cytosolic PRR-IRF3 axis (87). This is possibly due to the dependence of T1-IFN-mediated pathogenic effects in mice on ESX1, which induces IRF3 rather than IRF7 as explained above (67). However, IRF7 is recognized as commonly induced transcription factor by multiple clinical Mtb strains in alveolar epithelial cells (88). Moreover, TLR9$^{-/-}$ mice succumb earlier to high-dose Mtb infection than wildtype mice, which suggests a role for the TLR9/IRF7/IFN-α-axis in TB as well (89).

2.4. T1-IFNs drive the influx of Mtb-permissive myeloid cells during acute infection

Most studies in mouse TB models found significant functional effects of T1-IFNs specifically on $CD11b^{+}Gr1^{int}$ myeloid cell populations (68, 69, 71). This population comprises monocyte-derived $Ly6C^{high}CD11c^{-}CCR2^{high}$ inflammatory macrophages (iM) and $Ly6C^{int}CD11c^{+}CCR2^{int}$ inflammatory dendritic cells (iDC), but not $CD11b^{+}Gr1^{high}$ PMN (90). This is an important distinction, as T1-IFNs actively inhibit PMN influx, as discussed in more detail below in **paragraph 2.4.3.**

iM and iDC have been identified as major contributors to disease progression in mouse TB models (91-93). Several lines of evidence suggest that T1-IFNs regulate the influx of these cells and play a role in their functional impairment to resist Mtb. This interference with protective immunity is multifaceted and concerns four important interactions, which will be reviewed separately:

1) T1-IFNs mediate the influx of iM and iDC. 2) T1-IFNs inhibit IL-1β responses by these cells, which are essential in the initial host-responses to Mtb. 3) Prolonged IL-1β signaling can also cause excessive inflammation and thus requires regulation during later phases. This can be mediated by T1-IFNs, but also by IFN-γ through functionally different routes. 4) T1-IFNs and IFN-γ show a complex interplay in the activation of iM and iDC.

2.4.1. T1-IFNs mediate the influx of iM and iDC

Mtb-infected mice treated with the T1-IFN-inducing compound poly-ICLC show increased numbers of iM and iDC in the lungs, which are ten times more permissive to Mtb infection than their counterparts in PBS-treated mice (71). Others confirmed that signaling through IFNAR indeed augments the recruitment of Mtb-permissive iM and iDC into the lungs (69). Mechanistically, IFNAR-dependent expression of the chemokine CCL2 mediates the influx of $CCR2^{+}$ monocytes that differentiate into iM and iDC (71). Both myeloid and parenchymal cells can produce CCL2 in response to T1-IFNs, but parenchymal cells appear the main source of this chemokine (94-96). Expression of CCL2 is reduced in the lungs of $IFNAR^{-/-}$ mice and the pathogenic effects of poly-ICLC treatment are absent in Mtb-infected $CCR2^{-/-}$ mice (71). Thus, preclinical TB studies indicate that T1-IFNs stimulate the influx of $CCR2^{+}$ monocytes, but not PMN, to the site of infection in a CCR2-dependent way via the induction of CCL2 in parenchymal cells (74-76).

2.4.2. T1-IFNs inhibit IL-1β responses during acute TB

T1-IFNs not only stimulate the influx of $CCR2^{+}$ monocytes, but also stimulate their differentiation into Mtb-permissive iM and iDC (71, 75, 76). This can be traced back to a crosstalk between T1-IFNs and IL-1β (73, 90). iM and iDC are the major sources of IL-1β

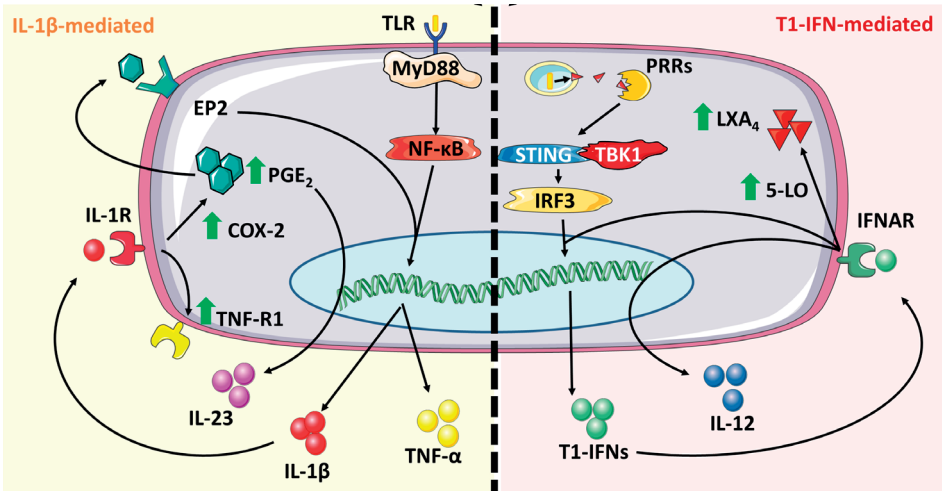
in the lungs Mtb-infected mice and IL-1β plays a crucial role in the acute host response to Mtb-infection (73, 90). IL-1β augments TNF-α-stimulated Mtb-killing and increases prostaglandin E_2 (PGE_2) production by upregulating cyclooxygenase-2 (COX2/PTGS2) (73, 97, 98). PGE_2 is involved in control of intracellular Mtb replication, but also prevents necrotic host cell death (99). In accordance, *Ptgs2*$^{-/-}$ mice, unable to produce PGE_2, are more susceptible to Mtb infection than wild type mice, but to a lesser degree than IL1$^{-/-}$ mice. Further information on PGE_2 in TB is given in **Box 2**.

T1-IFNs inhibit the expression and production of IL-1β and simultaneously increase the expression of 5-lipoxygenase (5-LO), which is a competitive enzyme for COX2 in the arachidonic acid metabolism (73, 90, 114, 115). As a result, IFNAR signaling causes a shift from COX2-mediated PGE_2 production to an increase in the 5-lipoxygenase (5-LO) products such as lipoxin A_4 (LXA_4) and leukotriene B_4 (LTB_4), which render cells more susceptible to necrotic cell death (73, 116). Pharmacological intervention in this process by administrating the 5-LO inhibitor Zileuton to Mtb-infected mice, improved disease outcomes during acute infection to similar extent as observed in IFNAR$^{-/-}$ mice(73). An overview on the balance between IL-1β and T1-IFNs is given in **Figure 2**.

2.4.3. Prolonged IL-1β signaling causes PMN-mediated tissue damage and is regulated by both T1-IFNs and IFN-γ.

The cross-talk between T1-IFNs and IL-1β influences disease outcome in TB (73). However, this does not fully explain the harmful effects of T1-IFNs observed in TB. Most importantly, although IL-1β production is essential for protective immunity in the acute phase of disease in TB, it requires strict regulation as unchecked IL-1β signaling in TB can result in excessive PMN-mediated tissue damage (120, 123). Also, as explained in **Box 2**, IL-1β-mediated PGE_2 production is protective during acute disease, but appears to have a detrimental effect during chronic disease. Lastly, inflammatory mediators associated with continuing infection, e.g. GM-CSF, predispose for IL-1β production over T1-IFNs by iM and iDC (43, 94, 124-126). This reflects an increasing need over time to limit IL-1β-mediated inflammatory responses.

To prevent PMN-mediated inflammation caused by excessive IL-1β signaling, the expression and production of IL-1β is inhibited not only by T1-IFNs, but also by IFN-γ (90, 120). In line with this, both T1-IFNs and IFN-γ can inhibit PMN influx (127-131). T1-IFNs and IFN-γ can both reduce pro-IL-1β gene expression and increase the expression of soluble antagonists for the IL-1 receptor (114, 132).



	IL-1β-mediated inflammation	T1-IFN-mediated inflammation
Effect on cell	IL-1β stimulates TNF-α-mediated Mtb-killing [1] PGE_2 promotes apoptosis over necrosis [2] PGE_2 stimulates IL-23 over IL-12 production [3]	PRR/STING/TBK1 pathway stimulates autophagy [10] LXA_4 promotes necrosis over apoptosis [2] T1-IFNs stimulate IL-12 over IL-23 production [11] T1-IFNs inhibit IL-1β production [12]
Effect on parenchymal cells	IL-1β+TNF-α stimulate production of PMN-attracting chemokines [4]	T1-IFNs induce CCL2 expression, promoting CCR2-dependent monocyte influx [13] Paracrine amplification of T1-IFN responses
Effect on ILC	IL-1β+IL-23 stimulate IL-17 production by γδ T-cells (and ILC3 cells) [5]	T1-IFNs and IL-12 stimulate IFN-γ and GM-CSF production by iNK-cells (and ILC1 cells) [14]
Effect on T-cells	PGE_2 promotes Th17/Th2 over Th1 [6] IL1β+IL-23 stimulate inflammatory Th17.1 cells [7]	T1-IFNs promote Th1 immunity [15]
Effect suppl	Not available	Increased frequency of IL-10^{high} permissive myeloid cells [16]
Effect KO	$IL1R^{-/-}$: Severely compromised immunity to Mtb [8]	$IFNAR^{-/-}$: Improved control of TB through reduced influx of permissive myeloid cells [17]
Prolonged signaling	Inflammatory phenotype [9] PMN-mediated tissue destruction (see also **Fig. 4**)	Regulatory phenotype [18] Inhibition of IL-12 / Th1 response (see also **Fig. 3**)

Figure 2. Inflammatory responses during acute infection in naïve inflammatory macrophages and dendritic cells

Green text indicates a beneficial host effect during Mtb infection, red indicates a detrimental effect. Mtb: Mycobacterium tuberculosis, PRR: Pattern recognition receptor, STING: Stimulator of interferon genes, TBK1: Tank-binding kinase 1, IRF3: interferon regulatory factor 3, 5-LO: 5-lipoxygenase, COX-2: cyclo-oxygenase 2, PGE_2: Prostaglandin E2, EP2: Prostaglandin E2 receptor 2. ILC3: Innate Lymphoid Cells type 3. [1](97), [2](116), [3](107), [4](98), [5](117), [6](106),[7](118) [8](119), [9](120), [10](80), [11](94), [12](73), [13](71), [14](121), [15](122), [16](64), [17](65), [18](90).

Despite the above mentioned functional similarities between T1-IFNs and IFN-γ in IL-1β inhibition, mechanistic differences exist between these IFN types in mediating this effect.

Ex vivo studies in human iM and iDC demonstrate that IFN-β inhibits IL-1β production more potently than IFN-γ (90, 114). One explanation might be that IFN-γ inhibits IL-10, while T1-IFNs induce IL-10, which contributes to the inhibition of IL-1β production (90,

Box 2. The dual faces of PGE_2 in TB immunity

PGE_2 is generally considered a pro-inflammatory mediator and indispensable for the induction of fever, which is a hallmark symptom of active TB (100, 101). The anti-inflammatory effects of prostaglandin synthase (COX-) inhibitors such as NSAIDs underline this notion. However, high levels of PGE_2 can also exert immunosuppressive effects as they stimulate alternative activation of macrophages (102), inhibit bactericidal activity (103) and promote production of IL-10 (104). Moreover, high PGE_2 levels can stimulate the development of myeloid-derived suppressor cells (MDSC) with inhibitory effects on adaptive immune cells (104, 105). Lastly, PGE_2 inhibits IL-12 production by DCs and IFN-γ production by T-cells, thereby promoting Th2/Th17 immunity (106, 107). In the serum and broncho-alveolar lavage fluid of TB patients PGE_2 levels were found to be elevated (73, 108, 109) and polymorphisms in the PGE_2 receptor EP2 are associated with TB-susceptibility (110). Experimentally, one mouse study showed that low PGE_2 levels in the acute phase of infection are essential for iNOS-mediated control of Mtb (111). Also, PGE_2 plays an important role during acute TB since the PGE_2-producing enzyme COX2 competes for arachidonic acid substrate with 5-lipoxygenase, which produces leukotrienes and lipoxins. Hereby, PGE_2 prevents necrotic cell death thus benefiting the host (73). Opposed to the protective role of low PGE_2 levels during acute disease, PGE_2 levels are higher during the chronic phase of TB and these concentrations contribute to disease by suppressing IFN-γ, TNF-α and iNOS (111). Notably, the cellular source of PGE_2 appears to differ between acute and chronic TB. During the acute phase of infection inflammatory myeloid cells are the main source of PGE_2, while foamy macrophages are strong producers of PGE_2 during the chronic phase of disease (112). In line with a detrimental effect of high PGE_2 levels in the chronic phase, foamy macrophages are typically associated with disease progression(113).

114, 115). Additionally, an IL-10-independent inhibition of IL-1β by T1-IFNs was recently identified (129). T1-IFNs induce cholesterol 25-hydroxylase, which potently reduces IL-1β transcription and broadly represses IL-1-activating inflammasomes. In contrast, IFN-γ inhibits Il-1β by increasing intracellular nitric oxide (NO) in an iNOS-dependent way (120)., 2013). This prevents NLRP3 inflammasome activation and cleavage of pro-Il-1β into IL-1β. In contrast to the mechanisms exerted by T1-IFNs, IFN-γ-induced iNOS not only limits IL-1β-mediated inflammation, but also markedly enhances the bactericidal potential of iM (120). Conversely, T1-IFNs suppress iNOS production (90). Based on the stimulation of iNOS by IFN-γ and the inhibition of iNOS by T1-IFNs, it appears that iDC are more sensitive to T1-IFN signaling and iM to IFN-γ when both types of interferon are present. T1-IFN-mediated inhibition of iNOS appears to occur primarily in iDC, since iDC only expressed iNOS in $IFNAR^{-/-}$ mice during viral infection, while iM appear more sensitive to IFN-γ and are the main source of iNOS in wild-type mice (131).

When taken together, these data suggest that IL-1β inhibition by either T1-IFNs or IFN-γ has strong implications on the bactericidal potential of iM and iDC. Furthermore, T1-IFNs interfere with the induction of iNOS by IFN-γ, particularly in iDC. This fits the observation that IFN-γ only inhibits IL-1β production by iM but not iDC in mouse TB models (90). Notably, iDC are the most readily infected cells in the lungs of Mtb-infected mice (27) and are present in larger numbers than iM during Mtb infection (71, 133).

2.4.5. The interplay between T1-IFNs and IFN-γ

During direct contact with Mtb through Toll-like receptors , endogenous T1-IFN signaling through IRF3 promotes IL-12 production by iDC over IL-23 (see also: **Fig. 2** and **Box 3**) (94, 134, 135). This early IL-12 signaling is required to induce IFN-γ production by innate lymphoid cells (ILC) such as NK-cells and possibly ILC1s (136, 137). However, exogenous T1-IFNs or T1-IFN signaling in the absence of TLR stimulation can also inhibit IL-12 production by iDC (115, 138). This inhibition of IL-12 by T1-IFNs occurs particularly through induction of IL-10 (15). T1-IFNs also inhibit the responsiveness of iDC to IFN-γ-mediated activation, which is required for Mtb-killing. This occurs partially by reducing the expression level of IFN-γ-receptor on the cell surface, but primarily through induction of an IL-10^{high} regulatory phenotype in which antimicrobial pathways by IFN-γ are not readily activated, as discussed below (90, 115, 131, 139-141).

Recent findings might explain the mechanism behind this paradox where T1-IFNs initially support IL-12-mediated IFN-γ production by NK-cells, but can also induce an IL-10^{high} phenotype in iDC, which interferes with IL-12 production and prevents IFN-γ-mediated activation. It has been observed in different mouse models, including Mtb-infected mice, that T1-IFNs can only induce an IL-10^{high} regulatory phenotype in monocyte-derived DCs (iregDC) if these cells have been primed previously by IFN-γ (35). IFN-γ-primed DCs that did not receive T1-IFN signaling differentiated into iDC that stimulated robust T-cell responses. This phenomenon of monocyte-priming by IFN-γ has been demonstrated to occur in the bone marrow (142). During gut infections, local production of IL-12 in mucosa-associated lymphoid tissue stimulates bone-marrow resident NK-cells to produce IFN-γ as early as 3 days post infection (142). This results in a uniform presence of an IFN-γ-primed signature of Ly6C^{high} monocytes in the circulation at day 6. Furthermore,

Box 3. IL-12 or IL-23 production by dendritic cells?

IL-12 and IL-23 are heterodimeric cytokines composed of a common p40 subunit, coupled with either a p35 subunit in IL-12 or a p19 subunit in IL-23. Both IL-12 and IL-23 are produced in particular by stimulated dendritic cells and to lesser degree by macrophages. The preferential production of IL-12 or IL-23 by these cells is multifactorial. Increased levels of PGE2 support IL-23 production over IL-12 (106, 107, 143). Activation of TLR-2 and TLR-4 also stimulates IL-23 production over IL-12, especially when NOD2 is simultaneously activated (144, 145). On the other hand, TLR9 and TLR3 agonists preferentially induce IL-12 (134, 146, 147). Downstream of PRRs, activation of IRF 4 and 5 favor induction of IL-23, while IRF 1, 3 and 7 induce IL-12 (134, 148). In line with this, T1-IFN-mediated IRF-3 activation and IFN-γ-mediated IRF-1-activation both favor IL-12 production (94, 134, 149).IL-4 also favors IL-12 production and inhibits IL-23 production, especially in combination with IFN-γ or GM-CSF (150, 151). Lastly, an important pathway that promotes IL-12 over IL-23 is ligation of the co-stimulatory molecule CD40 by CD40L on activated T-cells or by agonist antibodies (152). Taken together, IL-23 is induced in the presence of pathogens and innate signaling in the acute phase of infection, while onset of adaptive immunity with increased levels of IFN-γ and/or IL-4 shifts the balance towards IL-12 (153).

IFN-γ indeed primed these monocytes towards a regulatory phenotype, as they more effectively produced IL-10 in response to bacterial ligands (142). We speculate that a similar mechanism of IFN-γ priming is likely to be involved in pulmonary infections.

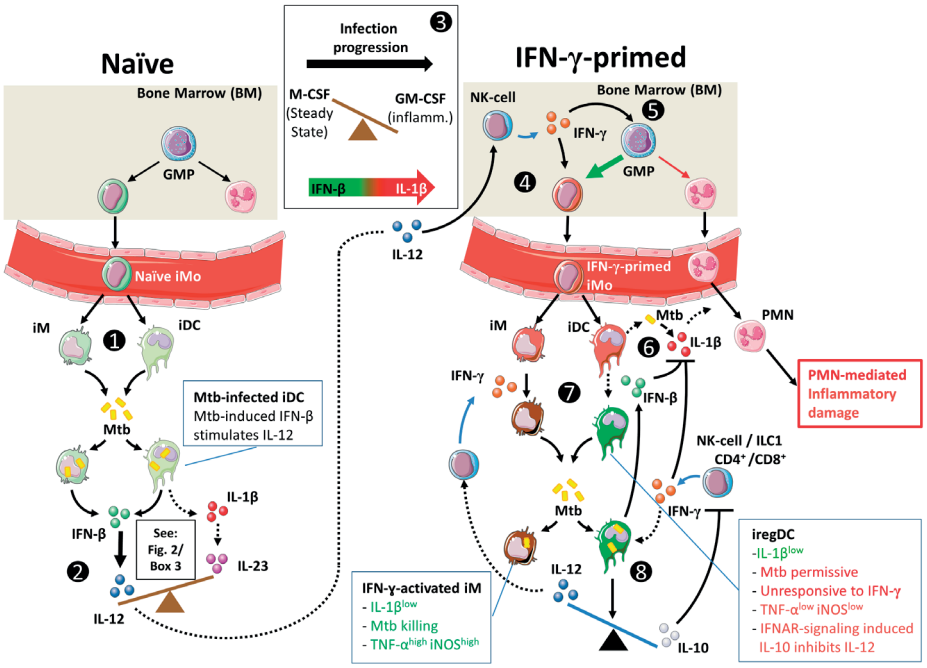


Figure 3. Hypothetical interplay between T1-IFNs and IFN-γ in monocyte priming and shaping of the immune response in Mtb infection

Dotted lines indicate speculations in the context of pulmonary Mtb infection; solid lines indicate shown pathways in human and/or in animal models. **1)** T1-IFN induces migration of $CCR2^+$ monocytes (iMo) from the bone marrow to the lungs of Mtb-infected mice under influence of CCL2 (71). Locally, these cells develop into $CD11b^+Ly6C^{high}$ inflammatory macrophages (iM) and $CD11b^+Ly6C^{int}$ inflammatory dendritic cells (iDC) (35). **2)** As shown in **Fig. 1**, naïve iM and iDC can initiate either IL-1β-mediated inflammation or T1-IFN-mediated inflammation. Mtb actively triggers intracellular PRRs to induce a T1-IFN-mediated response. **3)** Additionally, iM and iDC in the naïve situation have differentiated under influence of M-CSF, which makes them more responsive to T1-IFN signaling (94). During progression of Mtb-infection, GM-CSF levels rise and increase the potential for IL-1β production by iM and iDC (94, 125, 154, 155). **4)** Similar to the situation in gut infection, we propose that in TB IL-12 production in the lungs stimulates IFN-γ production by bone marrow-resident NK-cells, which locally primes monocytes (142). IFN-γ priming of monocyte-derived iDC is necessary for T1-IFNs to induce a regulatory (iregDC) phenotype in iDC in the lungs (35). **5)** Additionally, IFN-γ stimulates monopoiesis over granulopoiesis by granulocyte/macrophage progenitor cells (128). **6)** As Mtb infection progresses and GM-CSF levels increase, iM and iDC readily produce IL-1β (see also 3) (124, 154), which can lead to PMN-mediated inflammatory damage in TB (120). **7)** IL-1β production can be inhibited in response to either IFN-γ or IFN-β through mechanistically distinct pathways that differently affect Mtb-killing **8)** Signaling through IFNAR in IFN-γ-primed iDCs induces IL-10 production (35, 115, 131), inhibits IL-12 production (115) and makes these cells unresponsive to activation by IFN-γ (35, 115, 141), which together interfere with protective immunity during acute Mtb infection.

These data suggest interplay between T1-IFNs and IFN-γ as proposed in **Fig. 3**. T1-IFNs initially induce IFN-γ responses by promoting IL-12 production in naïve cells as shown in **Fig. 2**. These IL-12 responses stimulate IFN-γ production by innate lymphoid cells not only locally, but also systemically, which results in IFN-γ priming of monocytes in the bone marrow. Once IFN-γ production is initiated, T1-IFNs mediate a regulatory function by inducing an IL-10^{high} phenotype in IFN-γ-primed iDC. This prevents further production of IL-12 by these cells, inhibits their activation by IFN-γ and results in an Mtb-permissive phenotype.

2.5 Summary: The role of T1-IFNs in TB

Several modest clinical successes have been shown with IFN-α supplementation adjunct to antibiotic TB treatment (**Table 1**). However, case reports of TB reactivation under IFN-α treatment without concomitant antibiotics have put T1-IFNs in a negative spotlight (50-57). Furthermore, a T1-IFN transcriptional signature in circulating leukocytes is associated with active TB. Nevertheless, the functional role of T1-IFNs in TB patients remains to be determined (62).

Preclinical studies in mice support a detrimental role for T1-IFN in the acute phase of Mtb infection. T1-IFN signaling was associated with increased mortality in Mtb-susceptible mouse strains and higher Mtb loads in the lungs in most studies (**Table 2**). However, it should be noted that most of these preclinical studies do not unequivocally support a harmful effect of T1-IFNs during the chronic phase of disease based on mortality, Mtb-loads or differences in adaptive immunity.

In support of a pathogenic role of T1-IFNs during acute infection, Mycobacteria actively induce T1-IFNs by triggering cytosolic PRRs. This leads to IFN-β production in an IRF3-dependent way. Subsequently, T1-IFNs mediate the CCL2/CCR2-dependent migration of iM and iDC into the lungs (71). In these cells, interference of T1-IFNs with IL-1β and PGE_2 as shown in **Fig. 2** can lead to an altered metabolism of arachidonic acids that leaves cells more vulnerable to necrotic cell death (73). However, sustained IL-1β signaling itself carries the risk of excessive inflammation in TB and not only T1-IFNs, but also IFN-γ inhibits IL-1β to prevent excessive PMN-mediated inflammation (120). T1-IFNs inhibit IL-1β more effectively than IFN-γ, but stimulate an IL-10^{high} Mtb-permissive phenotype (71, 90).

Next to their shared ability to inhibit IL-1β, an interesting interplay between T1-IFNs and IFN-γ exists in TB as summarized in **Fig 3**. Two recent findings that are of particular interest include the observation that T1-IFNs can only induce an IL-10^{high} phenotype in IFN-γ-primed cells (35) and the inductive role of T1-IFNs in early IL-12 signaling, which is

required for IFN-γ priming in the bone marrow (142). Further research into this complex interplay between T1-IFNs and IFN-γ during early host responses in TB would be highly interesting given the T1-IFN-inducing capacities of Mtb and the shaping effect of early T1-IFN or IFN-γ signaling on the ensuing immune response.

3. THE TH17 RESPONSE IN TB

As discussed in the previous paragraph, T1-IFNs induce IL-12 production by iDC, while IL-1β induces IL-23. Other factors also influence production of IL-12 or IL-23 (see: **Box 3).** IL-12 is essential for the induction of IFN-γ responses in TB, but IL-1β is protective during acute TB despite inducing IL-23 over IL-12. Similar to the requirement of IL-12 for Th1 responses, IL-23 is essential for establishing Th17 immunity (156-158). Here we review the effect of IL-23 signaling and the Th17 response in TB.

3.1. introduction to the Th17 response

The Th17 response is distinct from classical cell-mediated Th1 immunity or B-cell-stimulating Th2 responses and is often associated with a potent inflammatory response and tissue damage (158). Th17 cells display a high degree of plasticity and their ability to express signature markers of other T-helper lineages makes it difficult to establish their exact role in disease. Four different subsets of Th17 cells have been described to date with ranging inflammatory potential (159). On one side of the spectrum are highly inflammatory and often pathogenic IFN-γ /GM-CSF-producing Th17.1 cells that result from prolonged IL-1β and IL-23 signaling (Kara et al., 2015). On the other side are IL-10-producing Th17 cells, which can even trans-differentiate into regulatory T-cells and contribute to resolution of inflammation (160).

Despite the plasticity in cytokine production, IL-17 remains the hallmark cytokine of the Th17 response. Next to Th17 cells, innate lymphoid cells such as γδ T-cells and ILC3 cells can also produce IL-17 in response to IL-23 and IL-1β (29, 117, 161). IL-17 exerts its effects primarily on nearby parenchymal cells and to lesser extent on hematopoietic cells, which is distinct from Th1 and Th2 cytokines like IFN-γ and IL-4 (158). In parenchymal cells, IL-17 primarily stimulates the production of the chemokines that attract PMN (162). However, it should be noted that IL-17 alone is a poor inducer of these chemokines and that synergistic activation by inflammatory ligands such as IL-1β, TNF-α or GM-CSF markedly increases the effects of IL-17 (162, 163).

Table 3. IL-17 responses in patients with acute TB (ATB) compared to Latent TB

	Increased in ATB	No difference	Reduced in ATB
% of IL-17$^+$CD4$^+$ T-cells			
Short incubation (0-48h)	(171)*	(172, 173)	(174, 175)
Long incubation (72-144h)	(173, 176)	(172, 177)	(175, 178)
***Ex vivo* IL-17 production**	(176, 179)	(177, 180, 181)	(182-184)

*only in MDR-TB

3.2. The Th17 response in human TB infection

The exact role of the Th17 response in human TB remains a topic of debate (13, 14, 164). Polymorphisms in genes encoding IL-17 are associated with susceptibility to pulmonary TB, which indicates a role for this cytokine in TB (165-168). However, these findings could not be reproduced in different demographic settings (169, 170). Analyses of Th17 responses in peripheral blood mononuclear cells (PBMC) from TB patients do not show uniform results either. Direct *ex vivo* analyses of unstimulated circulating CD4$^+$ T-cells show that active TB (ATB) is associated with reduced frequencies of circulating Th17 cells compared to latent TB infection (LTBI) (177, 185). However, serum IL-17 levels do not differ between ATB and LTBI and IL-17 is undetectable in the broncho-alveolar lavage fluid during both stages of disease (174, 177). Different studies report PBMC stimulation assays with Mtb-specific antigens showing either increased or reduced Th17 responses in ATB compared to LTBI (**Table 3**). These diverse findings are similar to those observed in IFN-γ response assays (IGRA), in which the levels of IFN-γ often also cannot discriminate between ATB and LTBI (186, 187). Interestingly, both Th1 and Th17 cells appear functionally inhibited in ATB patients by a PD-1-mediated immunosuppressive state (188-191). In accord, reductions in PD-1 expression under TB treatment restored both Th1 and Th17 responses (184).

Taken together, systemic Th17 responses in TB patients demonstrate similar variability as observed for IGRA studies. Both are unable to distinguish ATB from LTBI. How these systemic responses relate to local host responses in the lungs has not been characterized in TB patients.

3.3. Preclinical studies in mice support a protective role for IL-23 and IL-17 in TB

Based on mortality and mycobacterial loads, studies in Mtb-infected mice support a protective role for IL-23 and IL-17 in TB, but only during later stages of disease (**Table 4**). Interestingly, these late protective effects result from effects induced during the initial phases infection (143, 192). This is due to the essential roles of IL-23 and IL-17 in the local formation of tertiary lymphoid structures (TLS) (193, 194). These structures are formed during early infection, but can persist for longer periods of time and are associated with

protective immunity in Mtb-infected mice (193, 195) (**Table 4 / Fig. 4**). Furthermore, IL-17 and IL-23 increase the expression of the chemokine CXCL13 (196, 197). This chemokine stimulates the influx of TLS-associated $CXCR5^+$ follicular helper (T_{fh})-cells, which facilitate optimal localization of effector T-cell populations within the lung parenchyma, thereby promoting efficient T-cell-dependent macrophage activation and intracellular Mtb killing (195, 196).

Table 4. Th17-related effects in preclinical TB studies in mice

Mice (age, wks)	Inter-vention	Mtb strain, route	Survival	Mtb load (vs. wildtype mice)	Immunological effect	Ref
B6 (6-12)	IL23 p19 $^{-/-}$	H37Rv (100 CFU), aerosol	No data	No difference in lungs 1 log higher Mtb load in spleen at d.150	No IL-17-producing cells in lungs up to d.150	(157)
B6 (6-12)	IL23 p19 $^{-/-}$	H37Rv (100 CFU), aerosol	No data	d.120 and onwards, 0.5-1 log higher Mtb load in lungs	Reduced no. of B-cell follicles at d.200 (Cxcl13-mediated) Strongly impaired IL-17, IL-22 production in lungs up to d.250	(196)
B6 (6-12)	IL22 $^{-/-}$	H37Rv (100 CFU), aerosol	No data	No effect up to d.200	Suboptimal B-cell follicle development (Cxcl13-mediated)	(196)
B6 (6-9)	IL-17 RA $^{-/-}$	H37Rv (1.10^3 CFU), i.t.	higher mortality (median survival: 18 vs. 35 wk)	1.5 log higher Mtb load at w. 12 and w. 20 in lungs	Impaired cell recruitment (PMN, Lymphocytes, Mo/DC) Increased IL-1β Decreased TNF-α, IL-6 & IL-10	(192)
B6 (6-12)	IL-17 RA $^{-/-}$	H37Rv (100 CFU), aerosol	No data	No effect up to d. 200	Suboptimal B-cell follicle development (Cxcl13-mediated)	(196)
B6 (8-12)	IL-17 $^{-/-}$	H37Rv (1.10^3 CFU), i.t	No data	1.5 log higher Mtb load	Reduced no. of granulomas at d.28	(201)
B6 (6-8)	IL-17 $^{-/-}$	HN878 (100 CFU), aerosol	No data	1 log higher Mtb load in lungs at d.30 0.5 log higher Mtb load in lungs at d.60	Infection with HN878 showed robust production of IL-1β through TLR2, which supported increased IL-17 production compared to H37Rv and CDC1551	(197)
B6 (6-8)	IL-17 $^{-/-}$	H37Rv, CDC1551 (100 CFU), aerosol	No data	No difference at d.30 and d.60		(197)
B6 (8-12)	IL-17$^{-/-}$	H37Rv, (1.10^3 CFU), i.t	Higher mortality	1.5 log higher Mtb loads in lungs at d. 30, 1 log higher Mtb loads at d.60 d.120	Impaired granuloma formation, γδ T-cells primary source of IL-17	(200)

CFU: Colony forming Units, IL-17RA: IL-17 receptor A, i.t: intra-tracheal instillation.

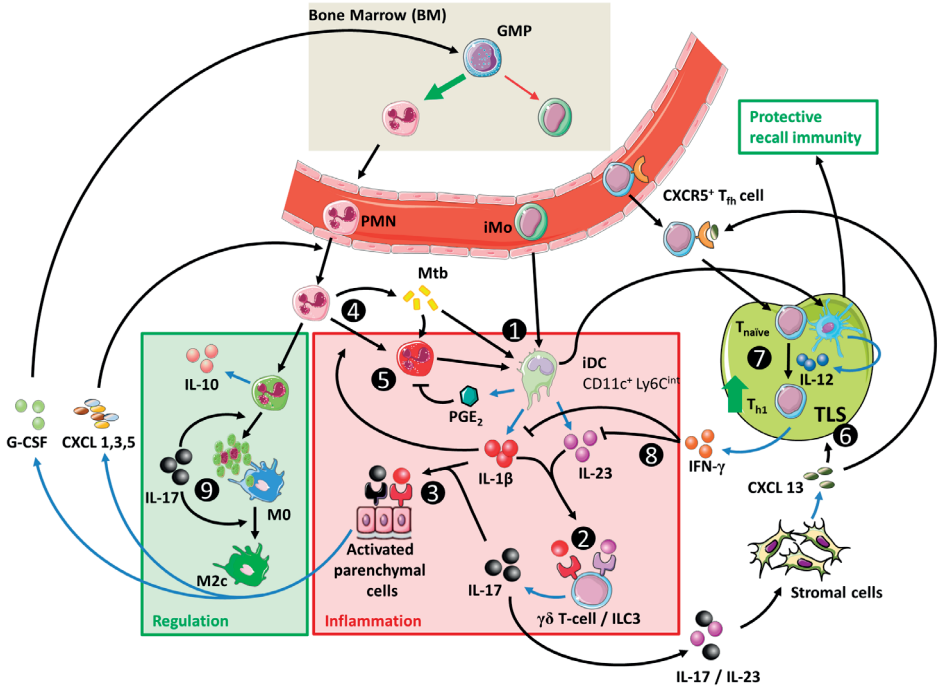


Figure 4. The IL-23/IL-17 axis in acute TB

When iDC recognize Mtb through membrane-bound TLRs, they can secrete IL-1β, IL-23 and PGE_2 (see: **Fig. 1**). This occurs more efficiently if iDC are activated through contact with Mtb-infected PMN, which also stimulates their migratory capacity to TLS and promotes recall immunity (202-206). **2)** The combination of IL-1β and IL-23 induces IL-17 production by γδ T-cells and possibly ILC3 (29, 117). **3)** Activation of parenchymal cells by IL-17 in combination with IL-1β or other inflammatory mediators ultimately results in PMN influx. **4)** PMN contribute to inflammation when stimulated by extracellular Mtb or inflammatory cytokines. **5)** Activated PMN readily cause tissue damage through production of ROS and proteases; this effect is suppressed by activated iDC in a PGE_2-dependent way (112, 207, 208). **6)** IL-23 and IL-17 stimulate the local production of CXCL13 by stromal cells (193, 196). This promotes TLS formation and follicular helper T-cell migration to the site of infection. **7)** CD40 ligation in the interaction between (i)DC and $CD4^+$ T-cells is a strong stimulus for IL-12 production over IL-23 (**Box 3**) and leads to Th1 formation and IFN-γ production. **8)** IFN-γ inhibits IL-1β production and shifts IL-23 production to IL-12, thus inhibiting Il-17 production and reinforcing the Th1 response (209). **9)** In the absence of inflammatory stimuli, PMN can produce IL-10 and undergo apoptosis. Phagocytosis of apoptotic PMN induces an IL-10-producing regulatory M2c phenotype in macrophages and further contributes to resolution of inflammation.

On account of their ability to induce TLS formation, boosting IL-23 and IL-17 production is also an interesting strategy for vaccine-induced protection against TB. In this regard, IL-17 production by Th17 cells during recall responses is indeed dependent on IL-23 and could reduce mycobacterial loads in the lungs of Mtb-infected mice (198). Th17 cells preferentially migrate to the lungs and are better contained in the lungs compared to Th1 cells upon adoptive transfer to naïve mice (198, 199). The developmental flexibility of Th17 cells is illustrated in experiments where Mtb-antigen-primed Th17 cells have

been adoptively transferred to naïve mice (198). Initially, these Th17 cells produce IL-17. However, upon recall immunity against Mtb, they primarily produce IFN-γ, with or without IL-17. Paradoxically, the latter switch results in a less effective reduction in bacterial loads compared to IL-17-producing Th17 cells that are adoptively transferred from IFN-$\gamma^{-/-}$ mice. This tendency of Th17 cells to produce IFN-γ instead of IL-17 during recall responses might explain the observation that IL-17 production during later phases of Mtb infection is dominated by γδ T-cells rather than $CD4^+$ cells (117, 200).

When taken together, initial shaping of the local inflammatory environment by IL-17 and IL-23 during acute infection stimulates local TLS formation. This facilitates the development of more robust Th1 responses by improving contact between antigen-presenting cells and lymphoid cells (**Fig. 4**). Furthermore, Th17 cells confer protective immunity during recall responses by their enhanced capacity to migrate to the lungs and stimulate T_{fh} responses compared to other $CD4^+$ T-helper cell populations.

3.4. The Th17 response, PMN and inflammatory damage

IL-17 stimulates granulopoiesis in the bone marrow and increases PMN influx to the site of infection by inducing G-CSF, CXCL1, CXCL3 and CXCL5 expression by parenchymal cells in mice or G-CSF and IL-8 in humans (158). These effects of IL-17 are markedly enhanced through synergistic activation by inflammatory mediators such as IL-1β, TNF-α or GM-CSF (162, 210, 211). In this regard, IL-17 is not a strong inducer of inflammation by itself, but rather amplifies pre-existing inflammation.

This IL-17-mediated 'inflammatory boost' can positively shape adaptive immunity, but prolonged or repeated antigen exposure can also lead to PMN-mediated pathological inflammation (212). Since IL-17 signaling is inevitably linked to PMN influx, the role of PMN in TB provides an additional perspective on the effects of IL-17 signaling in TB.

Review of available literature on the role of PMN in TB yields a complex picture with seemingly conflicting effects (14, 164, 213). In patients with active TB, PMN are the predominantly infected cells in the airways and provide a permissive site for a burst of active mycobacterial replication prior to transmission (214). On the other hand, PMN from healthy individuals, especially when stimulated with TNF-α, show a strong bactericidal effect (215). In preclinical TB models, highly susceptible mouse strains such as I/St, CBA/J or DBA/2 show an enhanced influx of apoptosis-resistant, highly phagocytic neutrophils that negatively affect survival compared to more TB-resistant C57BL/6 and BALB/c mice (216-218). Moreover, PMN are poor producers of essential cytokines such as IL-1α/β and IL-12p40 in the anti-TB response (90, 219). These effects in preclinical models primarily suggest a negative contribution of PMN to acute disease. However,

increasing evidence suggests a supportive role for PMN in protective immunity. PMN can indirectly augment IL-1β-mediated inflammatory responses in macrophages after contact with Mtb (202, 204, 205). Also, and consistent with Th17 responses, PMN play an essential role in generation of protective recall responses in Mtb-infected mice (192, 206, 220). Early, but not late PMN recruitment is essential for IL-17-mediated long-term control of Mtb infection (192). This can be explained by the finding that DCs that acquire Mtb through uptake of infected PMN are better able to activate T-cells (203, 220). The importance of this mechanism is recently highlighted in Mtb-infected mice, showing that PMN-depletion during vaccination prevented the generation of specific Th1 and Th17 responses (206).

A second emerging protective role of PMN is their contribution to initiating inflammation resolution (221). In mouse TB models, PMN are the main producers of IL-10 in the lungs and can dampen inflammatory damage (222). In this regulatory role, PMN inhibit Th17 responses, but do not interfere with IFN-γ-mediated Th1 immunity due to relative insensitivity of Th1 cells to IL-10 (222, 223). Another regulatory effect of PMN concerns their apoptosis and subsequent phagocytosis by macrophages in the absence of extracellular Mtb. This inhibits IL-23 production by these macrophages and induces a regulatory IL-10^{high} M2c phenotype under influence of IL-17 and IL-10 (see **Fig. 4**) (224, 225). IL-17 can further contribute to this process by attenuating the anti-apoptotic effect of GM-CSF on PMN and by stimulating PMN apoptosis (226, 227).

Taken together, PMN recruitment to the site of infection is largely dependent on IL-17, but only in synergy with innate inflammatory cytokines such as IL-1β. Locally, these recruited PMN contribute to inflammation if pathogens are still present, improve dendritic cell function and contribute to the formation of recall responses, or initiate resolution of inflammation in the absence of inflammatory or microbial stimuli.

3.5. Summary: The role of Th17 immunity in TB

The roles of IL-23 and IL-17 in TB are more subtle than the effects of Th1-related cytokines or T1-IFNs. Patient data are mostly limited to studies in PBMC. These show inconclusive results that are possibly confounded by the dynamics and heterogeneity of the Th17 response, which can range from highly pro-inflammatory IFN-γ/GM-CSF-producing Th17.1 cells to IL-10-producing regulatory Th17 cells.

Preclinical mouse TB models provide evidence for a protective role of the Th17 cytokines IL-23 and IL-17 in TB. These protective effects become apparent in the chronic phase of infection, but result from IL-23/IL-17-mediated effects in the earlier, acute phase of infection. This is associated with early protective effects of IL-1β, which is a strong

inducer of IL-23 and IL-17 (**Fig. 4**). Mechanistically, evidence for the protective effects of IL-17 and IL-23 primarily points towards their role in the development of TLS during the acute phase of disease, which provide protective effects during later stages (193, 228). Additionally, IL-23 and IL-17 induce CXCL13 expression that mediates the influx of TLS-associated T_{fh} cells. TLS and T_{fh} responses facilitate optimal interactions between adaptive and innate immunity, contribute to granuloma formation and improve the quality of T-cell recall responses in TB (195). In TB patients, TLS have also been associated with immune control, but more in-depth research is needed to establish their exact functional role and contribution to protective immunity (195).

Next to TLS formation and function, IL-23 and IL-17 mediate the influx of PMN into the lungs and the contribution of these cells to protective immunity in TB is increasingly recognized (206). Early, but not late PMN recruitment is essential for IL-17-mediated long-term control of Mtb infection (192) and DCs that acquire Mtb through uptake of infected PMN are better able to activate T-cells than DCs that directly interact with Mtb themselves (203, 220). The ability of IL-17 to induce the production of PMN-attracting chemokines in parenchymal cells is markedly improved when IL-17 signals in synergy with inflammatory mediators such as IL-1β, which again indicates synergy between IL-1β and IL-17 responses during acute TB. Prolonged activation of IL-1β and IL-17 responses can lead to massive accumulation of PMN and their local necrotic death can also be damaging to the host. However, in the absence of inflammatory stimuli, PMN are an important source of IL-10 in the lungs and can initiate resolution of inflammation (**Fig. 4**).

4. T1-IFNS, THE TH17 RESPONSE AND THEIR INTERACTIONS IN AUTOIMMUNE DISEASE

AID comprise a wide range of organ-specific and systemic disorders. Most systemic AID are considered classical B cell-mediated diseases, typified by circulating autoreactive antibodies against intracellular self-antigens. The clinical presentation of different AID varies, but evidence from genome-wide association studies points towards common immunogenetic mechanisms, as many systemic AID share disease-associated genes (229). Another trait particularly shared amongst different antibody-driven AID is the expression of a T1-IFN signature in both blood and disease-affected tissue (230-232), the strength of which generally correlates with disease activity and severity (233-236). Vice versa, T1-IFN immunotherapy as treatment for other diseases is known to cause symptoms similar to those observed in AID, such as development of psoriatic lesions in multiple sclerosis (MS) or hepatitis C-infected patients (237, 238).

T-cells also have a major impact on the development and progression of AID and increasing evidence points towards crucial involvement of the Th17 response in the pathogenesis of multiple AID (159, 239) (Martin et al., 2014;Lubberts, 2015). Th17 cells have been shown to be critical in the pathogenesis of MS and rheumatoid arthritis (RA) (19, 159). However, Th17 cells have also been associated with disease severity in autoimmune diseases characterized by a T1-IFN signature, such as systemic lupus erythematosus (SLE), (20, 231, 240, 241). Since T1-IFN signatures and Th17 responses are both associated with disease in AID, the question arises whether these two pathways act in concert to sustain and amplify autoimmune responses, or control each other (20, 21, 242). Therefore, we will discuss below the involvement of the T1-IFN and Th17 responses in AID individually as well as their interaction. We refer readers who are familiar with the contributions of T1-IFN and Th17 in AID to continue at paragraph 4.3 where we discuss the interaction between these pathways.

4.1. The contribution of T1-IFNs to the pathogenesis of AID

Most insight into the role of T1-IFNs in the pathogenesis of AID has been obtained in SLE, which was the first disease in which a T1-IFN transcriptional signature was identified in 2003 (233). Since then it has become clear that 60-80% of adult SLE patients and nearly 100% of pediatric SLE patients express a T1-IFN signature in their blood (243). Several mechanisms through which T1-IFNs contribute to disease in SLE, outlined below, have been elucidated.

4.1.1. Induction of T1-IFNs in AID

Specifically IFN-α appears to play a central role in SLE pathogenesis (243, 244). As mentioned in **paragraph 2.3**, IFN-α is produced in an IRF7-dependent way by pDC and other myeloid cell types. In accordance, pDC have been found to be a major source of T1-IFNs in SLE (245, 246). Immune complexes (IC), consisting of antibodies bound to self-DNA, are a major trigger for IFN-α production by pDC in AID (247) . However, pDC are not activated by self-DNA under steady state conditions, which indicates that additional stimuli are required. One such stimulus is the PMN-derived antimicrobial peptide LL37 (247), which converts inert self-DNA into a potent activator of endosomal TLR9 (248). Another stimulus is the nuclear protein High Mobility Group Box 1 protein (HMGB1), which is secreted by activated myeloid cells and passively released by necrotic, but not apoptotic cells (249). HMGB1 binds DNA and the formed complexes bind with high affinity to RAGE (receptor for advanced glycation end-products), which facilitates internalization into the endosome where TLR-9 can be activated (247). Extracellular HMGB1 also triggers the recruitment of PMN and stimulates their formation of Neutrophil Extracellular Traps (NETs) (250). NETs contain large amounts of nucleic acids and LL37, and are also a major driving factor behind chronic pDC activation and IFN-α production in SLE (251).

It deserves mention that NET-formation is driven by reactive oxygen species (ROS), which in PMN are particularly produced by NADPH-oxidase and subsequently processed by myeloperoxidase (252). Paradoxically, despite the capacity of NETs to induce T1-IFNs and the pathogenic role of T1-IFNs in SLE, NADPH oxidase appears to be protective in SLE (253). Lupus-prone mice deficient in NADPH-oxidase develop more severe SLE (253). Moreover, autoimmunity with T1-IFN signatures can still develop in individuals with chronic granulomatous disease, who lack NADPH-oxidase activity (254). This seeming contradiction has been partially explained by the observation that IgG auto-antibody-mediated NETosis, which is most relevant in SLE, is specifically reliant on mitochondrial ROS, while NETosis induced by e.g. TLR4 signaling is NADPH-dependent (254). In line with this, NETs from SLE patients have been shown to contain mitochondrial DNA (254). Thus the way NETs are induced and the type of DNA that is present on NETs probably also influences their ability to induce T1-IFNs and their role in disease.

Taken together, TLR9-mediated IFN-α production by pDC in response to immune complexes and NETs appears the major driving factor behind T1-IFN production in autoantibody-mediated AID. Additionally, the way NETs are induced and the type of DNA present on NETs can influence disease outcomes.

4.1.2. Disease-promoting effects of T1-IFN in AID

T1-IFNs exert a detrimental effect in AID through different pathways. In monocyte-derived cells T1-IFNs stimulate maturation, increase phagocytic capacities (255) and increase the expression of co-stimulatory molecules (256). Also, T1-IFNs have a direct stimulating effect on T-cells. Together these effects promote the generation of autoreactive T-cells which support autoreactive B-cell responses (255, 257).

At cytokine level, T1-IFNs can induce the production of B-cell Activating Factor (BAFF) by myeloid cells (236, 258, 259). BAFF induction confers a significant proportion of T1-IFN-mediated damage in SLE as supported by the observation that IFN-α administration induces disease in SLE-prone mice, but fails to do so in B-cell-deficient and BAFF-deficient mice on the same background (260). BAFF plays a central role in the development and selection of autoreactive B-cells (258). In line with this, increased BAFF expression correlates with disease severity in SLE (21, 258, 261). BAFF also induces class switch recombination in B-cells, leading to preferential expression of IgG and IgA over IgM, which is important for Fc-receptor-mediated NETosis induction in PMN (262). The clinical relevance of BAFF in SLE pathogenesis is illustrated by the current use of Belimumab, a monoclonal antibody against BAFF, as treatment for SLE (263). Interestingly, targeting BAFF is effective in SLE patients, while B-cell depleting therapies using CD-20-targeting Rituximab show disappointing results in phase III clinical trials (264, 265). This suggests

effector functions of BAFF other than B-cell activation. In this regard, BAFF can act as a co-stimulatory molecule for T-cells and promote Th17 development (266, 267). BAFF can also directly activate plasma cells, which are not depleted by rituximab (268, 269).

4.2. The contribution of Th17 in the pathogenesis of AID

4.2.1. GM-CSF-secreting Th17.1 cells

Pathogenic effects of Th17-mediated immunity in AID have been studied most detailed in MS and RA and their respective mouse models, experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) (159, 240). MS was long believed to be primarily driven by an IL-12/Th1 response, but this concept was challenged by observations in the EAE mouse model for MS showing that the IL-23p19 subunit instead of IL-12p35 (see **Box 3**) caused disease (270). In addition, the classic cytokines of Th1 and Th17 immunity, i.e. IFN-γ and IL-17, respectively, were found dispensable in EAE and instead GM-CSF appeared to be the effector cytokine responsible for IL-23-induced encephalopathy (118). Notably, while most studies agree on a central pathogenic role for GM-CSF in MS, conflicting results are reported regarding its cellular source (19, 271-273). One study shows that GM-CSF expression in MS patients is promoted by the IL-12/T-bet/Th1 axis, instead of IL-23 as observed in mouse EAE (271). Other publications report that B-cells are a major source of GM-CSF and specifically act in concert with Th17 cells (272, 274). In accord with these discrepant results, MS is shown to be a heterogeneous disease that can be driven by either Th1 or Th17 immunity (240), which also has implications for therapy as will be discussed in **paragraph 4.3.1.**

One interesting observation in this regard is the development of 'hybrid' Th17.1 cells that express markers of both Th17 cells and Th1 cells. Naïve $CD4^+$ T-cells in both mice and man do not express the IL-23 receptor and can either differentiate into T-bet^+ Th1 cells under influence of IL-12 or differentiate into $CCR6^+$ Th17 cells under influence of IL-6 and TGF-β (275). These IL-6/TGF-β-differentiated Th17 cells have low inflammatory potential and are prone to adopt an IL-10-producing regulatory phenotype. However, IL-6 also induces STAT3-dependent upregulation of IL-23 receptor (276). Subsequent (re)activation of such IL-6-primed Th17 cells by IL-23 increases Th1-associated T-bet expression and generates inflammatory IFN-γ/GM-CSF-producing Th17.1 cells (275). These cells can also switch their chemokine receptor profile and become $CCR2^+$ instead of $CCR6^+$ (275). Expression of CCR2 by Th17.1 cells can contribute to their inflammatory potential as it can divert their migration to sites without concomitant influx of regulatory T-cells, which depend on CCR6 for their migration (277).

Mechanistically, it was shown in a mouse EAE model that GM-CSF exerted its pathogenic effector function by stimulating IL-1β production by monocyte-derived cells (278). This

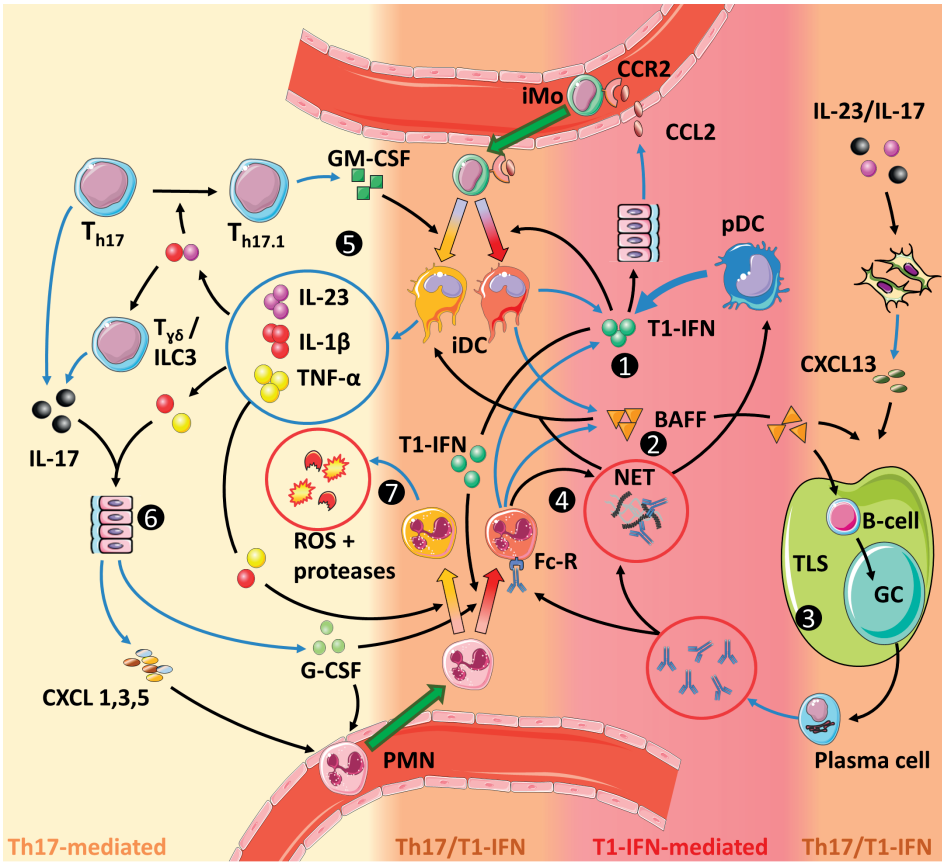


Figure 5. interactions between T1-IFN and Th17 immunity in AID

The color grading in the figure indicates the level of involvement of either Th17 immunity or T1-IFN-associated signaling. **1)** T1-IFNs, primarily produced by pDC, but also by iDC and PMN, prime the latter cells towards a T1-IFN/BAFF-producing phenotype, promote NETosis by PMN and stimulate monocyte migration by inducing CCL2 production. **2)** BAFF activates B-cells, stimulates TLS formation together with CXCL13, directly promotes Th17 differentiation (not shown) and stimulates the release of IL-1β by iDC. **3)** TLS facilitate optimal interaction between activated B-cells and antigen presenting cells (APC), while necrosis, NETs and T1-IFN increase the chance that these APC present self-antigens. Subsequent germinal center (GC) reactions within these TLS result in B-cells differentiating into plasma cells that produce large quantities of auto-antibodies. These auto-antibodies can mediate tissue damage and sustain a self-amplifying loop by inducing NETosis through binding the Fc-receptor on PMN. B-cells can also contribute to Th17 immunity by their ability to secrete IL-6 and GM-CSF (not shown and uncertain if this is BAFF-dependent). **4)** NETs trap antibodies. This facilitates their Fc-receptor-mediated internalization by pDC in which they stimulate T1-IFN production through endosomal TLR9 activation. Circulating NETs also stimulate IL-1β production by iDC and can mediate tissue damage. **5)** In a pro-inflammatory feedback loop, IL-23 stimulates the development of GM-CSF-producing Th17 cells (Th17.1), which in turn, together with BAFF and/or NETs stimulate an inflammatory phenotype in iDC. **6)** IL-1β and IL-23 stimulate IL-17 production by γδ T-cells, while concomitant stimulation with IL-1β and TNF-α is required for IL-17-induced G-CSF and chemokine production in parenchymal cells. **7)** IL-1β and TNF-α activate PMN to release reactive oxygen species (ROS) and proteases that cause tissue damage. Furthermore, GM-CSF increases longevity of PMN (not shown). Lastly, the priming of PMN and monocytes prior to entering the site of disease is important for their eventual effector function. For monocytes this is shown in more detail in **Fig. 3**.

suggests a positive inflammatory feedback loop, since IL-1β in turn promotes IL-23 production and development of Th17.1 cells (118). A similar pathogenic Th17.1 response is observed in RA, which was the first AID in which IL-1β inhibition was approved for clinical use (279). Also, regarding the distinction between Th17 and Th17.1 responses in RA, it should be noted that anti-GM-CSF therapy shows more promise than anti-IL-17 in clinical phase I/II trials (159, 280).

4.2.2. The contribution of IL-17-producing Th17 cells to AID pathogenesis

Next to GM-CSF-secreting Th17.1 cells, regular Il-17-producing Th17 cells also have been identified as pathogenic in other AID. This is best exemplified by the clinical successes of targeting IL-17 in psoriasis (281). Th17-associated pathogenic effects in SLE also appear to be driven by IL-17 rather than GM-CSF (21, 282). This is further supported by the specific contribution of PMN to disease in SLE, which is dependent on IL-17, opposed to GM-CSF which primarily influences the inflammatory potential of monocytes in MS and RA.

4.3. Interactions between T1-IFNs and the Th17 response in AID

SLE and other auto-antibody-mediated AID show a pathogenic role for T1-IFNs, while T-cell-mediated AID, such as MS, are driven primarily by GM-CSF-stimulated IL-1β production. With the functional dichotomy of IL-1β and T1-IFNs in mind, as shown in **Fig. 2**, MS and SLE seem to be opposite ends of the disease spectrum in AID instead of demonstrating interactions between T1-IFNs and the Th17 response. However, the existence of different Th17 subsets might explain this seeming disparity and suggest roles for GM-CSF-producing Th17.1 cells in MS and regular IL-17-producing Th17 cells in SLE. Both Th17 responses interact differently with T1-IFNs as will be discussed here. We identify three relevant interactions: **1)** Th17.1 responses are fueled by T1-IFN-stimulated influx of $CCR2^+$ inflammatory monocytes; **2)** a pathological IL-17/T1-IFN/BAFF axis driven by NET-forming PMN; and **3)** Th17 immunity and T1-IFNs collaborate in the generation and function of TLS. An overview of these pathways is presented in **Fig. 5**.

4.3.1. T1-IFNs can contribute to Th17.1-mediated AID

Among MS patients treated with IFN-β, approximately 30-50% do not respond favorably to treatment (283). It was shown that IFN-β suppresses Th1-mediated inflammation in MS, but is ineffective and may even exacerbate Th17-mediated inflammation (19). This is one of the first studies that report a detrimental interaction between T1-IFNs and Th17 responses. Given the importance of Th17.1 cells in MS, this negative outcome might be explained by the observation that IFN-β therapy in MS increases CCL2 production (284). Expression of this chemokine in the brain recruits inflammatory $CCR2^+$ monocytes as well as Th17.1 cells, which switch their chemokine receptor profile from $CCR6^+$ to $CCR2^+$

upon terminal differentiation (275). Moreover, Th17.1 cells stimulate IL-1β production in $CCR2^+$ monocytes (278, 285). Inflammatory monocytes may differentiate locally into dendritic cells further stimulating Th17 responses (286). Thus, a strongly pro-inflammatory condition is created in Th17.1-mediated MS. Since regulatory T lymphocytes rely on CCR6 rather than CCR2 (278), recruitment of these anti-inflammatory cells does not appear to hold pace with the influx of inflammatory monocytes and Th17.1 cells in MS.

4.3.2. A pathological IL-17/T1-IFNs/BAFF axis in AID

IL-17 induces PMN influx through induction of G-CSF and chemokines (**section 3.4**), which contribute to the production of IFN-α by pDC via the NETosis process (**section 4.1.1**). However, increasing evidence suggests a more prominent contribution of IL-17 and PMN to T1-IFN-mediated disease in SLE. Firstly, besides being major inducers of IFN-α production by pDC upon NETosis, PMN also appear to be a significant source of IFN-α themselves (287, 288). This was related to their sheer numbers, as circulating pDC were 27 times more efficient in secreting IFN-α, but PMN were 100 times more frequent (287). Secondly, both T1-IFNs and IL-17-induced G-CSF prime PMN for NETosis (248, 289). In accord, circulating PMN of SLE-patients are also the main cells expressing the transcriptional T1-IFN signature and release more NETs than PMN from healthy individuals (248, 251, 287, 288, 290, 291). Thirdly, T1-IFNs stimulate BAFF production, which is essential for T1-IFN-mediated pathogenic effects in mouse SLE (259, 260, 292). It is recently shown that IL-17 also induces BAFF production and that IL-17-driven, G-CSF-dependent PMN recruitment drives plasma cell responses during emergency granulopoiesis in a BAFF-dependent way (268).

Also, therapeutically administered G-CSF, which is physiologically induced by IL-17, increases BAFF production by PMN (293). These interactions indicate a prominent role for IL-17-mediated PMN influx in T1-IFN-production and induction AID and synergistic induction of BAFF production by IL-17 and T1-IFNs. In support of this, IL-17 and Th17 cells are associated with disease severity in SLE to similar extent as T1-IFNs (20, 21, 239, 242). In turn, BAFF can promote Th17 responses (266, 267). This further suggests an inflammatory loop with a central role for PMN in which IL-17, T1-IFNs and BAFF continuously increase each other's production and contribute to auto-antibody-mediated responses.

4.3.3. T1-IFNs, Th17 responses and TLS

Lastly, T1-IFNs and Th17 responses converge onto the development and functioning of TLS. In these structures, T_{fh} cells support germinal center reactions in which B-cells differentiate into antibody-producing plasma cells and memory cells (294). As expected from their function, TLS and T_{fh} cells are essential components in the pathogenesis of multiple auto-antibody-mediated AID (295-302). The cytokines IL-17 and IL-22 secreted by ILC3,

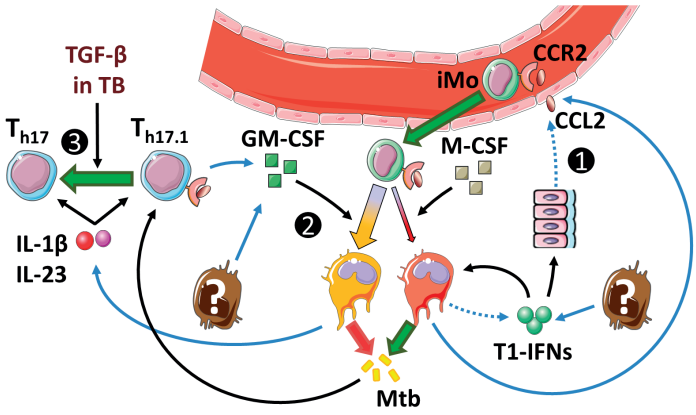


Figure 6. Th17.1 responses in TB

1) T1-IFNs induce CCL2 production in parenchymal cells and MDM, but not GMDM. This induces the influx of CCR2+ monocytes that mediate detrimental effects in TB as Mtb-permissive cells develop upon T1-IFN stimulation. **2)** GM-CSF increases IL-1β production, limits responsiveness to T1-IFNs and increases Mtb-killing potential. However, the exact cellular source of GM-CSF in TB is unknown. **3)** Patients with active TB overexpress TGF-β, which may drive Th17 development over Th17.1 in the presence of IL-1β and IL-23. Dotted lines implicate mechanisms shown in AID that have not been confirmed in TB.

Outstanding questions:

1) What is (are) the cellular source(s) of T1-IFNs in TB?
2) What is the ratio between different Th17 subsets in TB?
3) Do T-cells contribute to GM-CSF production in TB?

γδ T-cells and Th17 cells are required for local TLS-formation (193, 228, 303). T1-IFN and IL-17-induced BAFF promotes the formation and integrity of germinal centers within TLS and stimulates T_{fh} development (304, 305). T1-IFNs directly induce the expression of the T_{fh}-markers CXCR5 and PD-1 on T cells (306, 307). Also, T1-IFNs promote the survival of aberrantly selected B-cells in the GC reactions during SLE directly and indirectly through BAFF-induction as discussed in **4.2.2**. Thus, it appears that by stimulating TLS development, the Th17 response facilitates an environment that promotes selection of autoreactive B-cells under influence of T1-IFNs and BAFF.

Taken together, several lines of evidence exist for interactions between the Th17 response and T1-IFNs in systemic AID. Current data support a scenario in which Th17 immunity fuels T1-IFN-related pathology by mediating PMN influx and driving TLS formation, which facilitates T1-IFN/BAFF-mediated plasma cell responses and auto-antibody production. In turn, T1-IFNs can support pathogenic Th17.1 responses in AID by driving the influx of $CCR2^+$ inflammatory monocytes and potentially $CCR2^+$ Th17.1 cells themselves, which locally drive IL-1β mediated inflammation. An overview of these interacting pathways is shown in **Fig. 5**.

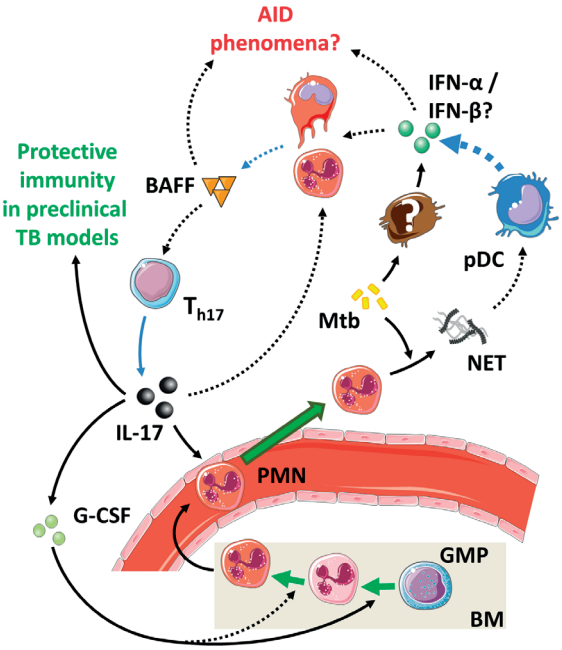


Figure 7. The IL-17/T1-IFNs/BAFF axis in TB

Mtb actively induces NET formation by PMN, but subsequent activation of IFN-α production by pDC appears less relevant in TB than in AID. IL-17 levels from TB patients vary (**Table 3**), but preclinical models support a protective role. Dotted lines implicate mechanisms present in AID that have not been confirmed in TB.

Outstanding questions:

1) What are the specific contributions of IFN-α vs. IFN-β to disease in the different phases of TB?

2) Do BAFF and T1-IFNs promote the observed auto-immune phenomena in TB?

5. INTERACTIONS BETWEEN T1-IFNS AND TH17 IMMUNITY IN TB

In the previous section we have outlined how T1-IFNs and Th17 immunity interact in AID (illustrated in **Fig. 5**). These interactions primarily concern **1)** Th17.1 responses fueled by T1-IFN-stimulated influx of $CCR2^+$ monocytes; **2)** The IL-17/T1-IFNs/BAFF axis driven by NET-forming PMN; and **3)** Synergism between Th17 immunity and T1-IFNs in TLS formation and function. In this section we assess the relevance of these three pathways in TB based on cell types and effector molecules involved. Each subsection contains a part of **Fig. 5**, supplemented with relevant finding and outstanding questions in TB.

5.1. Th17.1 responses in TB

Studies in MS and RA emphasize the difference between GM-CSF/IFN-γ-producing Th17.1 cells and regular IL-17-producing Th17 cells. The former primarily increase the inflammatory potential of monocytes (**Fig. 7**), while the latter are more closely associated with PMN. Data on subtypes of Th17 cells and particularly Th17.1 cells in human TB

are limited. One study shows that circulating GM-CSF$^+$ T-cells are not increased in ATB compared to LTBI, but it is unclear if this concerns Th17.1 cells or Th1 cells (308). Interestingly, GM-CSF production by both granuloma-associated T-cells and circulating CD4$^+$ T-cells in TB patients only occurs after mycobacterial antigen stimulation (308, 309). In mice, adoptively transferred Mtb-primed Th17 cells that produce IL-17 upon transfer, predominantly produce IFN-γ upon subsequent contact with Mtb, which is suggestive of a Th17.1 phenotype (198).

Th17.1 cells in AID result from prolonged innate IL-1β and IL-23 signaling. With regard to the role of IL-1β and IL-23 in human TB, IL-1β is essential for the expansion of both IFN-γ$^-$ IL-17$^+$ Th17 cells and IFN-γ$^+$ IL-17$^+$ Th17 cells (310, 311). IL-23 promotes the development of IFN-γ$^+$ IL-17$^+$ Th17 cells, but promotes IFN-γ$^-$ IL-17$^+$ Th17 cells if TGF-β is concomitantly present (310). Since active TB is associated with elevated TGF-b levels (188, 312, 313), it is possible that Th17.1 cell differentiation does not play a major role, but this remains to be demonstrated.

Th17.1-derived GM-CSF exerts a pathogenic effect in AID by stimulating IL-1β production in CCR2$^+$ monocytes. Although the role of Th17.1 cells in TB is uncertain, other cells such as NK-cells and Th1 cells can also produce GM-CSF in TB and during the course of infection GM-CSF levels progressively increase in the lungs of Mtb-infected mice (125, 314). The functional role of GM-CSF is of interest in TB, because it importantly impacts on CCR2$^+$ monocytes, which play a central role in T1-IFN-mediated pathogenic effects. T1-IFNs stimulate the influx of inflammatory CCR2$^+$ monocytes, but inhibit their IL-1β production and stimulate their differentiation into Mtb-permissive cells (see: **Fig. 3**). In contrast, GM-CSF is protective during acute TB, which is in line with the protective effects of IL-1β in this phase of disease. Mice deficient in GM-CSF succumb rapidly to infection due to their inability to mount Th1 responses (315, 316). Transgenic mice that overexpress GM-CSF in the lungs, but are GM-CSF-deficient in all other organs can develop Th1 responses, but still succumb to infection more rapidly than wild-type mice due to their inability to develop a normal granulomatous response (315, 316). Evidence from *in vitro* studies suggests that GM-CSF exerts its protective effect in TB by countering the effects of T1-IFNs in CCR2$^+$ monocytes (43, 94). Under physiological conditions monocytes differentiate under influence of M-CSF into monocyte-derived macrophages (MDM). These MDM have a CCR2^{low} phenotype, readily produce CCL2 and IL-10 in response to T1-IFNs and have a low Mtb-killing capacity (94, 155, 317, 318). Conversely, monocytes that differentiate under influence of GM-CSF (GMDM) are CCR2^{high}, relatively unresponsive to T1-IFN signaling, produce small amounts of CCL2 and IL-10 and have better Mtb-killing capacities than MDM in response to activation by IFN-γ (43, 126).

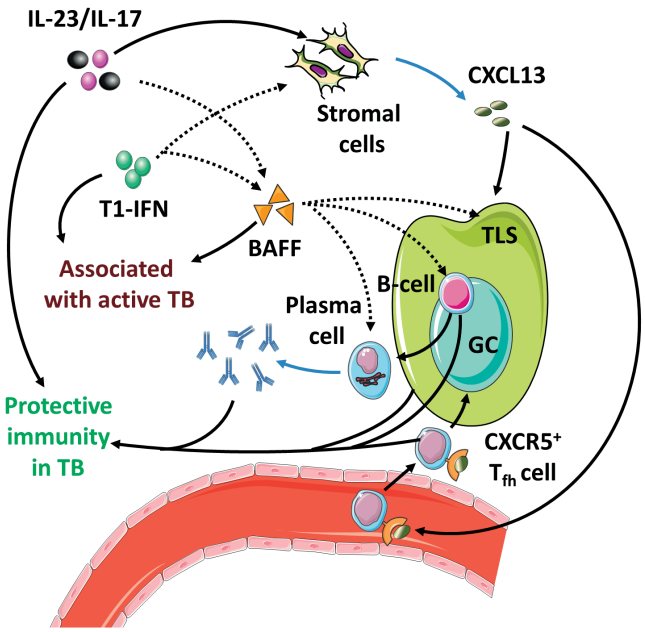


Figure 8. Tertiary lymphoid structures in TB

TLS, Tfh cells, B-cells and antibodies are all associated with protective immunity in TB. Preclinical TB models show that TLS induction and CXCL13 production are driven by IL-17 and IL-23. T1-IFNs and BAFF support TLS function and Tfh responses in AID. In TB, T1-IFNs and BAFF are associated with active disease, but their functional role remains to be identified. Dotted lines implicate mechanisms present in AID that have not been confirmed in TB.

Outstanding question:

1) What are the functional roles of T1-IFNs and BAFF in TLS function and humoral immunity in Mtb infection?

The relative unresponsiveness of GMDM to T1-IFNs might explain why preclinical studies primarily show effects of T1-IFNs during acute TB when the GM-CSF/M-CSF ratio in the lungs is relatively low, but less pronounced effects during later stages when GM-CSF-levels progressively increase (**section 2.4.3 / Fig. 3**) (125). However, similar to IL-1β, prolonged GM-CSF signaling also appears detrimental in TB. In particular, GM-CSF contributes to foamy macrophage development during later stages of infection, which can sustain persistent mycobacteria and contribute to inflammation (125, 319).

In summary, relatively few data are available on Th17.1 cells or T-cell-derived GM-CSF in TB. The requirement for antigen stimulation of T-cells to induce expression of GM-CSF is interesting. However, elevated TGF-β levels in TB patients suggest a limited contribution of Th17.1 cells to disease, as TGF-β favors the development of regular IL-17-producing Th17 cells. Regardless of its cellular source, preclinical TB studies support a protective role for GM-CSF during acute infection. GM-CSF causes monocytes to differentiate into cells with decreased T1-IFN responsiveness and increased Mtb-killing potential compared to

their M-CSF-differentiated counterparts. However, during chronic Mtb infection, high GM-CSF levels appear detrimental as they stimulate foamy macrophage development and inflammation.

5.2. The IL-17/T1-IFNs/BAFF axis in TB

In the previous paragraph it was discussed that regular IL-17-producing T-cells are more likely to play a role in TB than Th17.1 cells. Opposed to Th17.1 cells, regular Th17 cells exert their effect primarily through PMN instead of $CCR2^+$ monocytes in AID. Particularly in SLE, this was shown to be part of a pathogenic axis together with T1-IFNs and BAFF. The roles of T1-IFNs and IL-17 in TB have been discussed already in **section 2 & 3**. In this section we assess the roles of the other components of the IL-17/T1-IFNs/BAFF axis in TB, which include PMN-derived NETs, pDC and BAFF (**Fig. 7**).

5.2.1. PMN, NETs and pDC in TB

PMN isolated from SLE patients are the primary cells that express the transcriptional T1-IFN signature. Furthermore, a specific subclass of PMN, termed low-density granulocytes (LDG) have been identified in SLE that express a pro-inflammatory phenotype, have increased T1-IFN-production and more readily form NETs than PMN from healthy individuals (290, 291). Similar to SLE, the transcriptional T1-IFN signature in TB patients is mostly expressed in PMN (58). Moreover, LDG are also present in TB patients and correlate with disease severity, but it is unclear if these cells also have a similarly increased tendency for NETosis as their SLE counterparts (320). Nevertheless, NETosis does occurs in TB, as Mtb readily induces NETosis itself in PMN in an ESX-1-dependent way and can even stimulate extracellular trap formation in macrophages (205, 321-323).

NETs are strong inducers of IFN-α production in pDC in SLE (254). Conversely, pDC produce only small amounts of IFN-α and appear of minor clinical significance in TB (324). In accord, circulating pDC are elevated in SLE (325), but reduced in TB patients (326).

Final support for a limited role of pDC in TB pathogenesis comes from the observation that pDC produce IFN-α after endosomal TLR-activation, while it is shown that Mtb primarily induces IFN-β through activation of cytoplasmic PRRs (see **section 2.3**) (80, 248). While both IFN-α and IFN-β signal through IFNAR, this diversification in cellular source and type of T1-IFN that is induced can have important consequences for TB pathogenesis (see: **Box 4**).

5.2.2. BAFF in TB

Both T1-IFNs and IL-17 can induce BAFF expression, which contributes to disease in SLE as illustrated by the clinical successes of BAFF-inhibition (259, 268, 327). BAFF increases

B-cell numbers and antibody titers (292, 328) and treatment with anti-BAFF in SLE patients reduces serum IgG levels (327). The role of BAFF in TB has been explored to a much lesser extent, with currently one paper demonstrating BAFF levels to be elevated in patients with active TB without elaborating on its functional contribution to the host-response (329).

Box 4: IFN-α or IFN-β: which is relevant in TB?

IFN-α and IFN-β both exert their effect by binding to IFNAR, but increasing evidence from AID and viral infections suggests divergent effector functions (336, 337). In AID this is illustrated by the pathogenicity of IFN-α in SLE opposed to the therapeutic application of IFN-β as immunosuppressive treatment in MS. Recently, these different immunoregulatory roles of IFN-α and IFN-β in SLE and MS have been confirmed by more detailed analysis of blood transcriptional profiles in patients (338). The molecular explanation for the differential function of IFN-α and IFN-β traces back to subtle differences in receptor binding, signaling cascades and feedback mechanisms initiated, and has been reviewed in detail elsewhere (30, 339).

The specific contributions of IFN-α and IFN-β to the host response in infectious disease have been studied particularly in mice infected with lymphocytic choriomeningitis virus (LCMV). This work supports an immune-stimulating, antiviral role for IFN-α as opposed to an immunosuppressive effect by IFN-β (336, 339, 340). IFN-β specifically inhibits anti-viral T-cell responses and promotes viral persistence (339). In contrast, IFN-α-signaling associates with tissue damage and antiviral activity (339, 340).

In TB, evidence for the involvement of both T1-IFNs is present. Reactivation of TB has been reported specifically after treatment of patients with IFN-α, but not IFN-β(50-57). Also, mice infected with virulent Mtb strains specifically show higher IFN-α levels in the lungs compared to less virulent strains (64, 65). However, IFN-α-producing pDC seem to be of minor significance in TB patients (324, 326) and preclinical studies show that Mtb preferentially induces IFN-β through cytoplasmic PRRs and IRF3 instead of IFN-α through endosomal TLRs and IRF7 (79-81). Mycobacterial persistence in patients with TB is a major clinical problem and in line with the immunosuppressed state in active TB primarily supports a role for IFN-β (188, 341). However, exaggerated innate responses are also observed in TB where IFN-α might be involved. This is supported by recent evidence showing that IRF7 drives excessive innate inflammation during bacterial infections and provides an interesting therapeutic target (342). Taken together, little is known about the separate effects of IFN-α and IFN-β in TB, but clinical and preclinical studies support a role for both in different disease contexts. The diversification of IFN-α and IFN-β responses in transcriptional signatures observed in AID patients and the distinct effects of IFN-α and IFN-β in experimental LMCV infection therefore provide highly interesting perspectives for TB.

The functional role of BAFF in TB might be of particular interest given its stimulation of humoral immunity and the recently demonstrated protective effects of antibody-mediated immunity in TB patients (330, 331). Next to antibody-mediated protection, B-cells also essentially support T-cell responses in TB, but circulating B-cells are dysfunctional and reduced in absolute numbers in patients with active TB (332). The protective effects of Mtb-specific antibodies and B cells in TB suggest that increased BAFF levels may supports host responses by stimulating antibody production and perhaps other B cell functions such as stimulating T cell responses (330). However, high BAFF levels also predispose for the development of autoreactive B-cells in AID (333). Thus, elevated BAFF

levels in TB could relate to the observation that up to 32% of patients with active TB have elevated auto-antibody levels (12). Such correlations between elevated BAFF-levels and autoimmunity have been demonstrated in other chronic infections (334).

Additional support for a supposed protective role in BAFF in TB comes from its interaction with IL-17, which shows protective effects in preclinical early infection phase TB models, as discussed in **section 3**. IL-17 stimulates the migration of PMN to lymphoid structures where they can produce large quantities of BAFF that directly drive plasma cell responses. Also, IL-17-induced G-CSF primes PMN for BAFF-production upon activation (268, 293). Vice versa, elevated BAFF levels have been reported to increase Th17 immunity in AID and infection (266, 267, 335).

Taken together, preliminary pieces of evidence support the presence of interactions between IL-17, T1-IFNs and BAFF in TB, similar to those demonstrated in AID. This primarily includes the presence of NETs and elevated BAFF levels. However, despite the T1-IFN signature observed in TB, NET-induced IFN-α production by pDC appears less relevant in TB than in AID, and the specific contributions of IFN-α and IFN-β are of high interest in TB, but currently largely unknown. Studies in TB patients show protective effects of antibody-mediated immunity, but also elevated titers of autoantibodies. This supports a view in which BAFF is protective in TB, but excessive BAFF levels, driven by either T1-IFNs or IL-17 can also increase the chances of developing auto-immunity in TB patients.

5.3. Tertiary lymphoid structures in TB

As a third place of interaction, IL-17, T1-IFNs and BAFF converge in the local formation and functioning of TLS. In these structures, T_{fh} cells support germinal center reactions in which B-cells differentiate into plasma cells and memory cells (294). As discussed in **section 4.3.4** observations in AID suggest that Th17 responses drive TLS development and facilitate an environment that promotes development of autoreactive B-cells under influence of T1-IFNs and BAFF. Conversely, both TLS and T_{fh} cells are associated with immune control in TB patients and preclinical TB models, which is in line with the protective role of humoral immunity in TB discussed in the previous section (195, 196, 343, 344). Here we discuss how TLS and T_{fh} responses are associated with protective immunity in TB and how interactions between IL-17, T1-IFNs and BAFF may contribute to this immune response.

Migration of $CXCR5^+$ T_{fh}-cells into TLS is largely dependent on CXCL13, which is primarily induced by IL-23 and IL-17 in mouse TB models, but can also be induced by T1-IFNs, as demonstrated in viral infections (196, 345). Mechanistically, $CXCR5^+$ T_{fh}-cells mediate their protective effect in Mtb-infected mice by facilitating optimal localization of

effector T-cell populations within the lung parenchyma, thereby promoting efficient T-cell-dependent macrophage activation and intracellular Mtb killing (195, 196).

Another interesting observation regarding T_{fh} responses concerns the induction of PD-L1 expression on antigen-presenting cells and PD1 on T-cells by T1-IFNs (35, 136, 306). In TB circulating PMN primarily express the T1-IFN signature, but also overexpress PD-L1 (58, 346). The interaction of PD-L1 with PD1 on $CD4^+$ T-cells is a key immunological checkpoint in TB that limits excessive T-helper responses (40, 42). In line with this, PD1-deficient mice are extraordinarily susceptible to TB (41). T_{fh} cells constitutively express $PD1^+$, which distinguishes them from conventional $CD4^+$ T_h cells. Interestingly, while increased PD1/PD-L1 interaction suppresses conventional T-helper responses, the opposite is observed for T_{fh} responses (347). Interaction between $PD1^+$ T_{fh} cells with PD-L1 has a stronger suppressive effect on the regulatory subset of T_{fh} cells than on stimulatory T_{fh} cells and results in a net increase of T_{fh} activity (347).

Taken together, IL-17, T1-IFNs and BAFF act in concert to drive TLS formation and T_{fh} responses. These responses support the development of autoreactive B-cells and the subsequent production of autoantibodies in AID, but confer protective immunity in TB by improving the interaction between adaptive and innate cells and facilitating antibody production, while simultaneously inhibiting excessive inflammation by conventional $CD4^+$ T-cell responses.

CONCLUDING REMARKS

The notion that complex mechanisms beyond Th1 immunity are at play in TB immunity is supported by 1) the unsatisfactory results of vaccine strategies aimed at boosting Th1 immunity in TB patients (38); 2) The inflammatory damage associated with increasing IFN-γ production by T-cells in the lungs of Mtb-infected mice (40); and 3) The host-detrimental effect of targeting the Th1-inhibiting PD1/PD-L1 interaction in mice (41, 42).

Patients with active TB express a T1-IFN transcriptional signature in their circulating leukocytes, but the exact identity and functional role of T1-IFNs in patients remains to be elucidated (62). Others have speculated that deleterious effects of T1-IFN-signaling during bacterial infections are tolerated because of their ability to suppress myeloid cell responses (31). This review highlights two additional aspects of T1-IFNs that are of interest in TB. The first concerns the preconditioning of myeloid cells prior to their contact with T1-IFNs. IFN-γ priming appears essential for the induction of an Mtb-permissive phenotype, and monocytes that differentiate under GM-CSF are less responsive to T1-

IFNs than their M-CSF-differentiated counterparts (**Fig. 3**) (35, 94). The second aspect is the diversification of IFN-α and IFN-β responses on a transcriptional and functional level as explained in **Box 4.** We propose that the inflammatory effects of IRF7-mediated IFN-α might contribute to excessive innate inflammatory responses in TB, while the immunosuppressive effects of IFN-β are more likely to support mycobacterial persistence.

Determination of the role of the Th17 response in TB is impeded by its heterogeneity, reflected in the presence of different of Th17 subsets with ranging inflammatory potentials. Observations in AID emphasize the difference between IFN-γ/GM-CSF-producing Th17.1 cells and regular IL-17-producing Th17 cells. The exact role of T-cell-derived GM-CSF in TB remains to be determined, but preclinical TB studies show a protective role for GM-CSF on monocyte differentiation in the acute phase of TB. In contrast, IL-17 and PMN appear more relevant in chronic control of Mtb-infection and recall immunity.

Immunological similarities between TB and AID may result from commonly activated pathogenic pathways. Alternatively, compensatory mechanisms induced by one disease might predispose for the development of the other. Interactions between IL-17, T1-IFNs and BAFF form a pathological axis in AID that promotes autoantibody-mediated autoimmunity.

The newly appreciated functional roles of antibodies, B-cells and T_{fh} cells in TB provide suggestive evidence that pathogenic mechanisms in AID confer protective immunity to TB. Further insight into these mechanisms as discussed in **Fig 6-8** may generate leads for immune-directed therapies adjunct to current and newly developed antimicrobial treatment protocols.

ACKNOWLEDGEMENTS

We thank Ko Hagoort for his critical reading of the manuscript and Servier Medical Art (http://servier.com/Powerpoint-image-bank) for providing base images for the figures.

EL acknowledges the Dutch Arthritis Association (12-02-409; 13-3-403; 14-02-201; 15-2-206). THMO acknowledges EC FP7 ADITEC (Grant Agreement No. 280873); EC HORIZON2020 TBVAC2020 (Grant Agreement No. 643381); EC FP7 EURIPRED (FP7-INFRA-2012 Grant Agreement No. 312661); The Netherlands Organization for Scientific Research (NWO-TOP Grant Agreement No. 91214038); The Bill & Melinda Gates Foundation Grand Challenges in Global Health (Grant GC6-2013); the National Institute Of Allergy And Infectious Diseases of the National Institutes of Health under Award Number R21AI127133.

The content is solely the responsibility of the authors and does not necessarily represent the official views of any funder. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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3

Modern day Mycobacterium tuberculosis Beijing and East-African Indian strains cause B-cell influx into the lungs compared to an H37Rv-induced T-cell response

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ABSTRACT

To identify how virulence among different *Mycobacterium tuberculosis* lineages can influence host responses, we infected BALB/c mice with Beijing-1585, EAI-1627 or the less virulent laboratory strain H37Rv. Disease progression was monitored up to 28 days post infection. Beijing-1585 and EAI-1672 infection resulted in higher mycobacterial loads at earlier time points. They induced a marked influx of B-cells and elevated IL-4 protein levels in the lungs compared to H37Rv, which induced a T-cell influx with higher γγ and IL-17 levels. During infection with Beijing-1585 and EAI-1627, myeloid cells in the lungs appeared functionally impaired with reduced iNOS and IL-12 expression levels compared to H37Rv infection. In the bone marrow of mice infected with Beijing-1585 and EAI-1627 we found reduced expression of IFN-γ, TNF-α and IFN-β, essential for myeloid cell priming, from the third day post infection onwards. Our findings indicate that the increased virulence of two clinical isolates compared to H37Rv is characterized by a fundamentally different systemic immune response, which already can be detected early during infection.

Submitted for publication

INTRODUCTION

Tuberculosis (TB) is a leading cause of death among infectious diseases worldwide and claimed more victims in 2015 than HIV and malaria combined (1). While global efforts have resulted in a steady decline in TB-related deaths over the years, new threats are present in the form of drug resistance and the emergence of more virulent *Mycobacterium tuberculosis* (Mtb) genotypes (1-3).

Mycobacterial strains belonging to the Beijing genotype particularly have shown an aggressive global spread over the last century and have been associated with higher rates of treatment failure and disease relapse compared to other genotypes (4-10). One major explanation for this clinical impact of the Beijing genotypes seems to be their increased capacity to acquire drug resistance (11). A less well-defined characteristic concerns their hypervirulence (12-14). Clinical studies on immunology and pathogenicity of strains belonging to the Beijing genotype are challenging due to varying outcome parameter definitions (15, 16). Nonetheless, Beijing strains cause higher mycobacterial loads, more lung damage and earlier mortality compared to strains from other lineages in preclinical TB models (13, 17, 18). Mechanistic studies have suggested that Beijing strains have enhanced capacity to inhibit protective immunity in the lungs through induction of higher levels of type-I interferons, leading to lower IL-12 and TNF-α levels and reduced T-cell activation (19, 20). Increased Beijing virulence also has been attributed to bacterial phenolic glycolipid (PGL)-production, which suppresses the production of IL-12, IL-6 and TNF-α by host immune cells (21, 22). Lastly, Beijing strains may induce a stronger regulatory T-cell response compared to other strains, thereby down-regulating protective immunity (17, 23).

One factor hampering Beijing genotype-specific immunological characterization is the genetic diversity among Beijing strains, which can substantially affect virulence (3, 24). In this study we evaluate the host response during acute infection against the well-defined, highly virulent Beijing-1585 strain. This strain has previously demonstrated similar infection and mortality kinetics as other virulent Beijing strains (18, 24). Furthermore, Beijing-1585 was found associated with drug resistance and treatment failure (18, 25). We compare Beijing-1585 with a clinical isolate from the East-African/Indian (EAI) lineage that displays similar virulence as Beijing-1585 in our model (18), and with the less virulent laboratory strain H37Rv (26).

Previous studies in our (BALB/c) mouse TB model showed that mice infected with the laboratory strain H37Rv reach maximal mycobacterial loads and start developing progressive pneumonia 28 days post infection (dpi). Next, they enter a phase of chronic

infection and become moribund between 22 and 38 weeks post infection (26). In contrast, mice infected with Beijing-1585 or EAI-1627 reach peak infection at 14 dpi with histopathological signs of pneumonia comparable to H37Rv at 28 dpi and rapidly become moribund between three to five weeks post infection if left untreated (26, 27). In this study we aim to identify the underlying host responses that might contribute to this marked difference in virulence.

MATERIALS AND METHODS

Mycobacterial strains

We used the *Mycobacterium tuberculosis* H37Rv strain (ATCC 27294) and two strains isolated from patients in Vietnam in 2002, Beijing-1585 and EAI-1627, as representatives for their respective lineage based on genotyping results (27).

Mice and infection

Female specific pathogen-free BALB/c mice aged 10-11 weeks and weighing 22-24 grams (Charles River, Les Oncins, France) were infected by intra-tracheal instillation under general anesthesia as described previously (25). Inoculum sizes were confirmed by plating and were $1.0.10^5$ colony forming units (CFU) for Beijing-1585, $1.3.10^5$ CFU for EAI-1627 and $1.8.10^5$ CFU for H37Rv. Mice infected with Beijing-1585 or EAI-1627 rapidly become moribund between 3-5 weeks (18), therefore mycobacterial loads and other parameters for these two clinical strains were measured up to the peak of infection at 14 dpi, while measurements on H37Rv-infected animals were continued up to peak of infection at 28 dpi. All protocols were approved by the institutional animal ethics committee (DEC number 117-12-13, EMC-number 3005) and adhered to the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 201/63/EU.

Determination of mycobacterial load

Lungs and spleens were removed aseptically and homogenized in 2 mL PBS using the gentleMACS Octo Dissociator (Miltenyi Biotec BV, Leiden, the Netherlands) according to the manufacturer's protocol. From each tissue homogenate 10-fold serial dilutions were made. Next, 200 µL aliquots were plated on 7H10 agar culture plates supplemented with 10% OADC. Plates were incubated for up to 42 days at 37 °C and 5% CO_2 before colonies were counted.

Flow cytometry, Real-time quantitative PCR and Cytokine assessment

The flow cytometry protocol, fluorescent antibody panels, Real-time quantitative PCR and Cytokine assessment were essentially as described previously (29). An additional

fluorescent antibody panel used in this study is described in **supplementary Table 1**. Primer sequences and manufacturers are listed in **supplementary Table 2**.

Data analysis and statistics

Flow cytometry data were analyzed using Flowjo 7.6.5. Analyses were done and graphs were made using PRISM Graphpad 7. All data are expressed as mean ± SEM. Student's t-test, followed by Bonferroni correction for multiple comparisons where applicable, was used to calculate significance, except for **Fig**. **7B**. Here we used two-way, repeated measure ANOVA. P-values less than 0.05 were considered statistically significant.

RESULTS

Beijing-1585 and EAI-1627 lead to higher mycobacterial loads than H37Rv at earlier time points

We found no significant differences in mycobacterial load between Beijing-1585, EAI-1627 and H37Rv at 1 dpi and 3 dpi, indicating that all groups received a similar inoculum of mycobacteria (**Fig. 1A**). At 7 dpi, mice infected with Beijing-1585 had significantly higher mycobacterial loads than mice infected with EAI-1627 or H37Rv and at 14 dpi, Beijing-1585 and EAI-1627 caused almost 2 log higher loads than H37Rv. Mycobacterial loads for H37Rv at peak infection (28 dpi) were still 1 log lower than those observed for Beijing-1585 and EAI-1627 at 14 dpi. These findings are in agreement with previous studies monitoring mycobacterial loads for Beijing strains and H37Rv (13, 28).

To check whether the higher mycobacterial loads in the lungs caused by Beijing-1585 and EAI-1627 were associated with more rapid dissemination to other organs, we determined mycobacterial loads in the spleen (**Fig. 1B**). No significant differences in culture-conversion were found. At 7 dpi the Beijing-1585 group did show culture-positive spleens in all mice with higher loads compared to other groups.

Beijing-1585 and EAI-1627 induce lung influx of B-cells, while H37Rv induces T-cell influx

To explore whether the distinct mycobacterial growth profiles in our model correlated with differences in adaptive immune responses, we evaluated the numbers of B- and T-cells recruited to the lungs by the three different strains. Most notably, Beijing-1585 and EAI-1627 induced a strong influx of B-cells at 14 dpi, which was not observed for H37Rv at either 14 dpi or 28 dpi (**Fig. 2A**). In contrast, H37Rv induced the recruitment of $CD4^+$ and $CD8^+$ T-cells at 28 dpi, which was not observed upon infection with the clinical strains (**Fig. 2B/C**). Despite the marked T-cell increase in the H37Rv group at 28

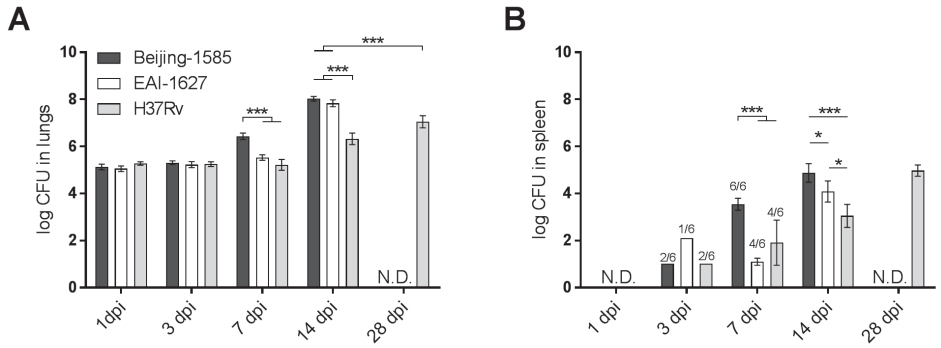

Figure 1. Mycobacterial loads in lungs and spleen

A) Mycobacterial loads in the lungs after intratracheal infection with Beijing-1585 (black bars), EAI-1627 (open bars) or H37Rv (grey bars). At 14 dpi, Beijing-1585 and EAI-1627 cause higher loads than H37Rv at either 14 dpi or 28 dpi (peak infection H37Rv). After 14 days Beijing-1585 and EAI-1627-infected mice rapidly become moribund, therefore no later analyses for these strains are possible. **B)** Mycobacterial loads in the spleen. Dissemination rates from the lungs to the spleens are not significantly different. Beijing-1585-infected mice showed higher loads at 7 dpi and 14 dpi compared to the other strains. Three mice were used for each group at 1 dpi and 6 mice for each group at the remaining time points. Inoculum sizes were $1.0.10^5$ CFU for Beijing, $1.3.10^5$ CFU for EAI and $1.8.10^5$ CFU for H37Rv. * $p < 0.05$ ** $p < 0.01$ *** $p<0.001$ after Bonferroni correction.

dpi, Foxp3$^+$ regulatory T-cells percentages of total lung single cell suspension remained lower compared to Beijing-1585 and EAI-1627 at 14 dpi (**Fig. 2D**). The total lung single cell suspension included parenchymal cells, which function as an internal control as it is reasonable to assume that their numbers remain constant during infection.

Figure 3 shows the associated cytokine protein levels in the lungs for each time point and genotype strain. In accordance with the increase in B-cells, Beijing-1585 and EAI-1627-infected mice showed elevated protein levels of IL-4 at 14 dpi, which were 4-5 fold higher than IL-4 levels observed for H37Rv at 14 and 28 dpi (**Fig. 3A**). Although Beijing-1585 and EAI-1627 also caused elevated protein levels of IFN-γ and IL-17a at 14 dpi, these remained 2-fold and 6-fold lower respectively, compared to the H37Rv group at 28 dpi (**Fig. 3B/C**).

TNF-α protein levels in the lungs closely correlated with strain-dependent differences in mycobacterial loads over time. At 7 dpi, TNF-α levels were significantly induced only in mice infected with Beijing-1585- and EAI-1627, which were almost 2-fold higher at 14 dpi compared to H37Rv at 28 dpi (**Fig. 3D**). This is in line with the role of TNF-α as general inflammation marker. In support of this, the inflammation marker IL-6 showed similar kinetics as TNF-α over time (**Fig S1**). IL-10 and IL-23 levels were also measured in the lung homogenates, but were below the limit of detection of our assay (data not

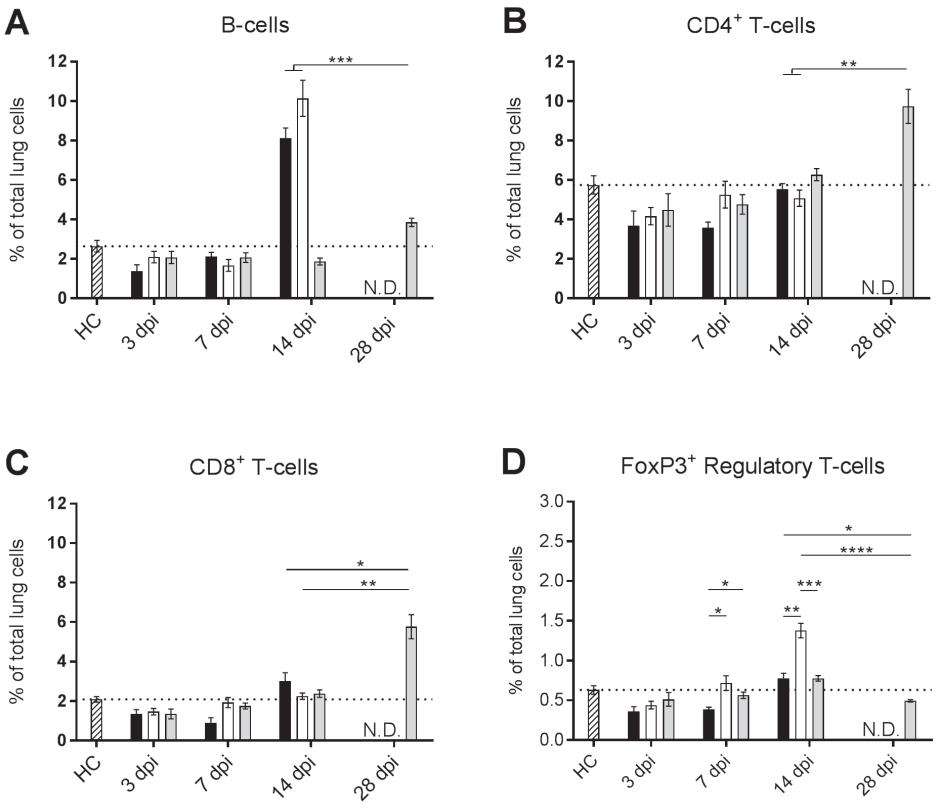

Figure 2. Lymphoid cell populations in the lungs of mice infected with different Mtb strains
Lymphoid cells were determined in the lungs of mice infected with Beijing-1585 (black bars), EAI-1627 (open bars) or H37Rv (grey bars) and compared to healthy control mice (HC, striped bars). **A)** B-cells are significantly higher for Beijing-1585 and EAI-1627 at 14 dpi compared with H37Rv at 14 and 28 dpi. **B/C**) Only H37Rv shows an increase in both $CD4^+$ and $CD8^+$ T-cells at 28 dpi. **D**) Despite the increase in T-cells caused by H37Rv infection at 28 dpi, $Foxp3^+$ regulatory T-cells are significantly lower compared to Beijing-1585- and EAI-1627-infected mice at 14 dpi. Gating strategies were similar as described previously (29). N=6 mice per group per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after Bonferroni correction. Data are shown as % of total lung single cell suspension including parenchymal cells. These cells function as an internal control as it is reasonable to assume that their numbers remain constant during infection.

shown). Quantitative PCR measurements of IFN-γ, IL-17a TNF-α, IL-6 in the lungs were also performed with outcomes comparable to those on protein level as shown in **Figure 3** (**Fig. S2**). IL-10 expression levels were above the lower limit of detection, but did not show strain-specific differences (**Fig. S2**).

Beijing-1585 and EAI-1627 induce a qualitatively impaired myeloid response compared to H37Rv.

Next, we evaluated potential differences in $CD11b^+$ myeloid cells in the lungs that could explain the observed differences in lymphoid cell responses and cytokine levels. Lung polymorphonuclear leukocytes (PMN) percentages were increased in the Beijing-1585 and EAI-1627 group compared to H37Rv at 7 dpi and 14 dpi, which was in line with the elevated mycobacterial loads and inflammation markers TNF-α and IL-6 at these time points (**Fig. 4A**). However, at 28 dpi the PMN frequency in the H37Rv group was comparable with that in the Beijing-1585 group and EAI-1627 group at 14 dpi.

Inflammatory macrophages / dendritic cells (iM/DC) showed a similar trend as PMN (**Fig. 4B**). Monocyte-like cells were present to a lesser extent than PMN and iM/DC, and were only higher in the EAI-1627 group at 14 dpi compared to the H37Rv group at 28 dpi (**Fig. 4C**). Alveolar macrophages (AM) were reduced over time in all groups, associated with inflammatory cell influx, but most prominently at 14 dpi in the Beijing-1585 and EAI-1627 groups compared to the H37Rv group (**Fig. 4D**). We also evaluated lung eosinophils in each group since these cells are known IL-4 producers, but levels of these cells were not elevated in the Beijing-1585 and EAI-1627 groups compared to the H37Rv group at any time point evaluated (**Fig. S3**).

AM and iM/DC are important cellular sources of IL-12 in the lungs (30), which is essential for initiation of T-cell responses (31). To determine potential functional differences between the infiltrating iM/DC and the lung-resident AM in the different groups, we measured the expression of IL-12p35 and IL-12p40. Most notably, Beijing-1585 caused the strongest down-regulation of IL-12p35 in the lungs compared to healthy control mice and did not induce any IL-12p40 expression at all time points evaluated (**Fig 5A and 5B**).

We also measured the expression of inducible nitric oxide synthase (iNOS) in both cell populations at each time point evaluated. iNOS expression is induced by IFN-γ and TNF-α and associates with bactericidal activity (30, 32). The AM from mice infected with Beijing-1585 failed to up-regulate iNOS at any point during the course of infection (**Fig. 5C**). In contrast, iNOS expression was already significantly higher in AM from H37Rv-infected mice at 3 dpi and its expression continued to increase up to 14 dpi. The expression of iNOS could not be determined in AM of H37Rv-infected mice at 28 dpi as the AM-population at this point was too small to be clearly separated from other cell populations (**Fig 4D/ Fig S4A**).

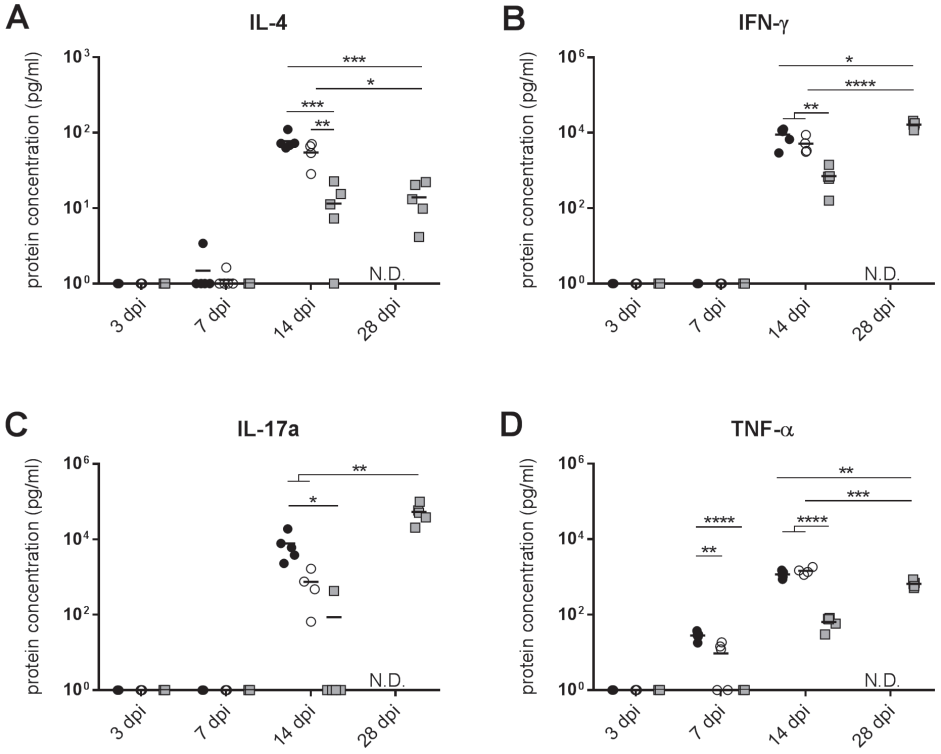

Figure 3. Cytokine protein levels in the lungs of mice infected with different Mtb strains
Protein levels were determined in lung tissue homogenates of mice infected with Beijing-1585 (black dots), EAI-1627 (open circles) or H37Rv (grey squares). **A)** IL-4 levels are 4-5 fold higher for Beijing-1585 and EAI-1627 at 14 dpi compared to H37Rv at 14 dpi or 28 dpi. **B)** IFN-γ levels are elevated for Beijing-1585 and EAI-1627 at 14 dpi, but are 2-fold lower compared to H37Rv at 28 dpi. **C)** Beijing-1585 and EAI-1627 induced circa 7-fold lower levels of IL-17a at 14 dpi compared to H37Rv at 28 dpi. **D)** TNF-α levels are circa 2-fold higher for Beijing-1585 and EAI-1627 at 14 dpi compared to H37Rv at 28 dpi. N=6 mice per group per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ after Bonferroni correction.

iM/DC in lungs of mice infected with Beijing-1585 or EAI-1627 also showed significantly lower iNOS expression compared to H37Rv-infected mice at 3 dpi and 7 dpi (**Fig 5D**). At 14 dpi, when IFN-γ and TNF-α levels were high in the lungs of mice from the Beijing-1585 and EAI-1627 group (**Fig. 3C**), iNOS expression by iM/DC was increased accordingly. Nevertheless, iNOS expression by iM/DC in H37Rv-infected mice was significantly higher at 14 dpi despite lower levels of IFN-γ and TNF-α in the lungs compared to Beijing-1585- and EAI-1627-infected mice and iNOS expression levels increased even further at 28 dpi.

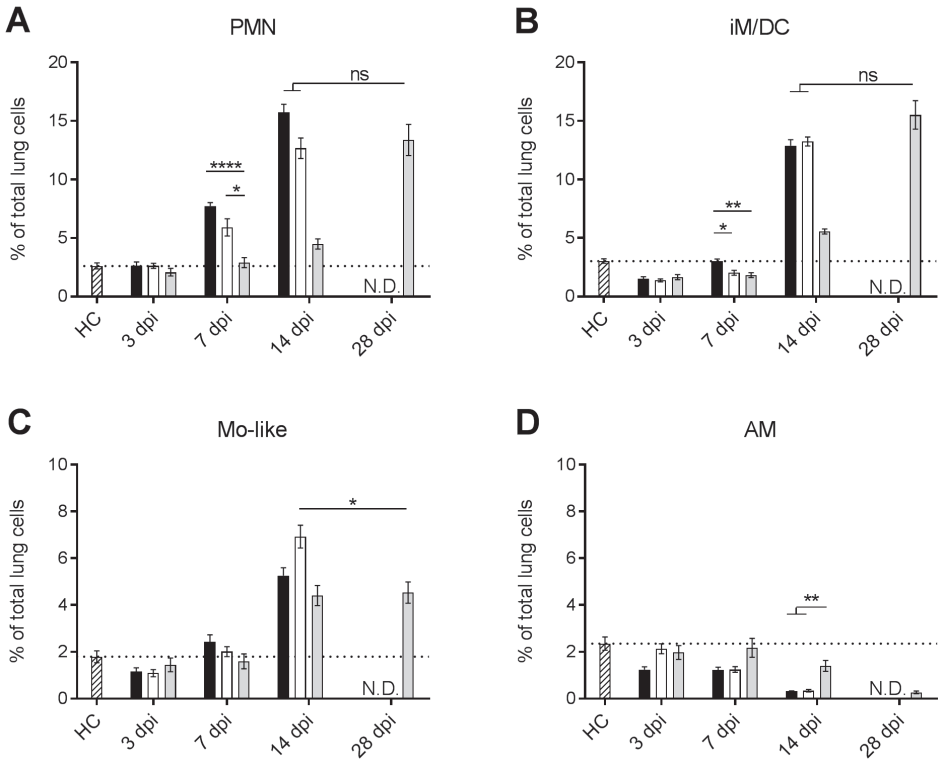

Figure 4. Myeloid cell populations in the lungs of mice infected with different Mtb strains
CD11b^{+} cells were distinguished as PMN, iM/DC, monocyte-like cells (Mo-like) and AM in the lungs of mice infected with Beijing-1585 (black bars), EAI-1627 (open bars) or H37Rv (grey bars) compared to healthy control mice (HC, striped bars). **A)** PMN (CD11b^{+}Ly6G^{high}) cells showed a more rapid increase for Beijng-1585 and EAI-1627 compared to H37Rv. **B)** iM/DC (CD11b^{+}Ly6C^{int}CD11c^{high}) showed kinetics comparable to PMN for the different groups. **C)** Monocyte-like cells (CD11b^{+}Ly6C^{high}CD11c^{low}) are only higher in the EAI-1627 group at 14 dpi compared to the H37Rv group at 28 dpi. **D)** Lung alveolar macrophages (CD11b^{int}CD11c^{high}SiglecF^{+}) are lower in the Beijing-1585 and EAI-1627 group compared to the H37Rv group at 14 dpi. N=6 mice per group per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after Bonferroni correction.

Infection with Beijing-1585 induces less expression of inflammatory cytokines in the bone marrow compared to H37Rv

To assess if iM/DC from the H37Rv-infected group were primed differently at an earlier developmental stage, we determined cytokine mRNA expression in the bone marrow in the course of infection.

Similar to the lungs, IL-12p35 mRNA expression in the bone marrow was down-regulated most effectively by Beijing-1585 infection compared to uninfected mice at all time points evaluated (**Fig**. **6A**). Interestingly, Beijing-1585 infection also showed a lack of induction, or even reduced expression of inflammatory cytokines IFN-γ, IL-17a and

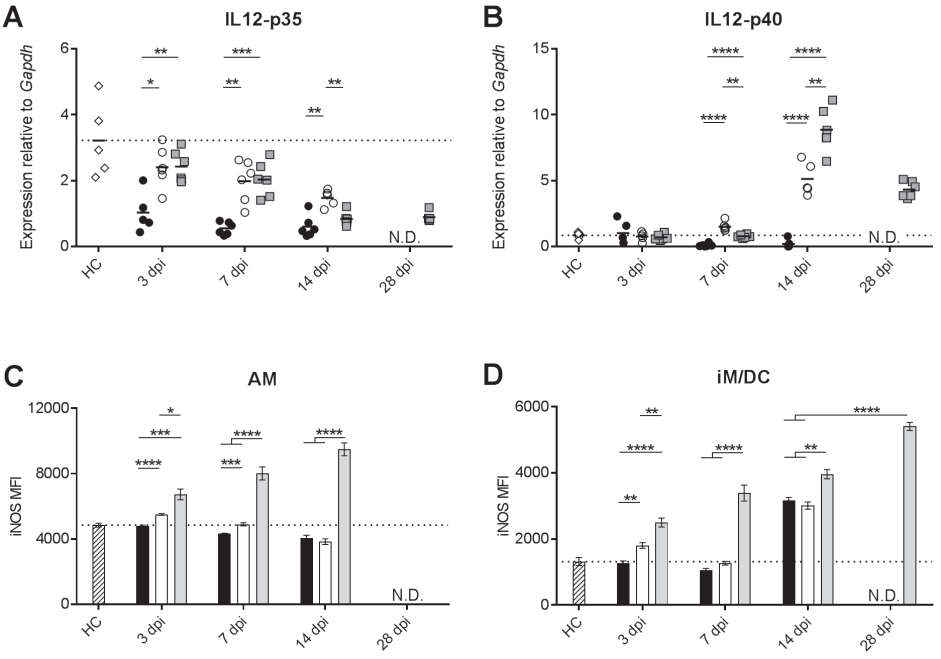

Figure 5. IL-12 production and myeloid cell populations iNOS expression in the lungs of Mtb-infected mice

IL-12 mRNA expression in total lung homogenate (**A,B**) and iNOS expression in alveolar macrophages (AM) and inflammatory macrophages/dendritic cells (iM/DC) in the lungs (**C,D**) of mice infected with Beijing-1585 (black bars/dots), EAI-1627 (open bars/dots) or H37Rv (grey bars/squares) are compared to healthy control mice (HC, striped bars/open diamonds). **A)** IL-12p35 expression levels are lower in the Beijing-1585 group compared to the EAI-1627 and H37Rv group. **B)** IL-12p40 expression is induced in EAI-1627- and H37Rv-infected mice, and reached its peak for both strains at 14 dpi with higher expression in the H37Rv group. Beijing-1585 does not induce any notable expression of either IL-12p35 or IL-12p40 at all time points evaluated. **C**) AM in the lungs of Beijing-1585-infected mice fail to induce iNOS expression beyond levels observed in healthy control mice. **D)** iM/DC in H37Rv-infected mice show higher iNOS expression than iM/DC in the lungs of mice from the Beijing-1585 or EAI-1627 group at all time point evaluated. N=6 mice per group per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after Bonferroni correction. For experiments depicted in this figure, iM/DC are defined as $CD11b^{high}CD11c^{high}MHC\text{-}II^{+}$ cells and AM were defined as $CD11b^{int}CD11c^{+}F4/80^{+}CD200R^{+}$ cells (gating strategies: **Fig. S4A**), based on a distinct panel of antibodies. Population frequencies through this gating were highly comparable to those in **Figure 4** (**Fig. S4B**).

TNF-α compared to H37Rv as early as 3 dpi (**Fig. 6B-D**). Especially for TNF-a, expression levels differed markedly between BM cells from Beijing-1585- and H37Rv-infected mice over time with a decreased expression for Beijing-1585 at 14 dpi compared to 3 dpi, as opposed to a 34-fold increase for H37Rv. Measurement results for the EAI-1627 group consistently were intermediate between those for the Beijing-1585 and H37Rv groups, but tended to be more similar to results from Beijing-infected mice.

Induction of type 1 IFN signature genes in the lungs of infected mice essentially correlates with expression of IFN-β

Lastly, we tested the mRNA expression of IFN-α genes (subtypes 1,2,5,6 and 7) in the lungs with methods described by Manca et al (20). We only found a limited expression of the tested IFN-α genes at 3 dpi for all strains, which decreased upon progressing infection and showed no significant inter-strain differences (**Fig. 7A**).

Since IFN-α and IFN-β share the ability to bind to, and signal via the IFN-α/β receptor, we evaluated IFN-β mRNA expression in the lungs. IFN-β expression in the H37Rv group was significantly higher than that in the EAI-1627 group at 3 dpi, and also higher than that in the Beijing-1585 group, but without statistical significance (**Fig. 7B**).

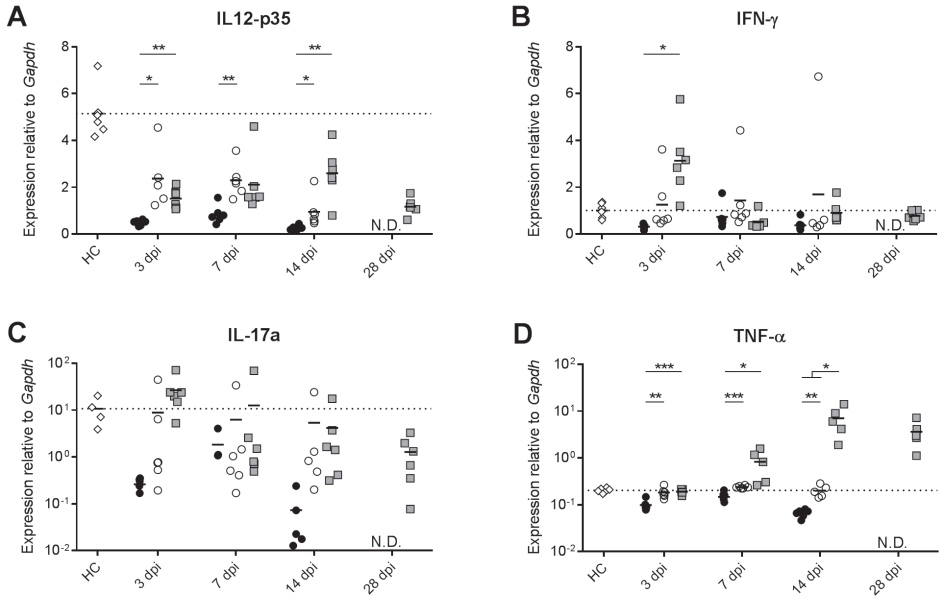

Figure 6. Cytokine mRNA expression levels in the bone marrow of mice infected with different Mtb strains.

Expression levels of target cytokine mRNA are shown relative to *Gapdh* in the bone marrow of mice infected with Beijing-1585 (black dots), EAI-1627 (open circles) or H37Rv (grey squares). **A)** IL-12p35 expression at 3 dpi and 7 dpi was lower in the Beijing-1585 group compared to H37Rv. **B)** IFN-γ expression was lower for Beijing-1585 at 3 dpi compared to H37Rv. **C)** IL-17a levels were markedly lower in the Beijing-1585 group compared to the H37Rv group (mean of 0.26 vs. 26.7), but without statistical significance (p= 0.06 after Bonferroni correction) due to the high spread in the H37Rv group (5.3 – 71.8). **D)** TNF-α levels were higher in the bone marrow of H37Rv-infected mice compared to Beijing-1585-infected mice at all time points evaluated. EAI-1585 consistently showed intermediate results between Beijing-1585 and H37Rv for all cytokines. N=6 mice per group per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ after Bonferroni correction.

Type I interferons comprise several more subtypes than those for which we could test expression by qPCR and their expression is often transient. Therefore we decided to test the type 1 interferon response, represented by expression of *Mx1*, *IFI44* and *CCL2*, which are known type 1 interferon-inducible genes (33-35). Expression levels of such genes can be combined into a type 1 interferon signature, which provides an indication of type 1 interferon responsiveness in a tissue (36). Our type 1 interferon signature showed different kinetics between Beijing-1585 and H37Rv, with EAI-1627 again showing intermediate results (**Figure 7C,** see **Fig S5** for individual graphs). Most notably, the Beijing-1585 group showed the strongest induction of type 1 interferon inducible

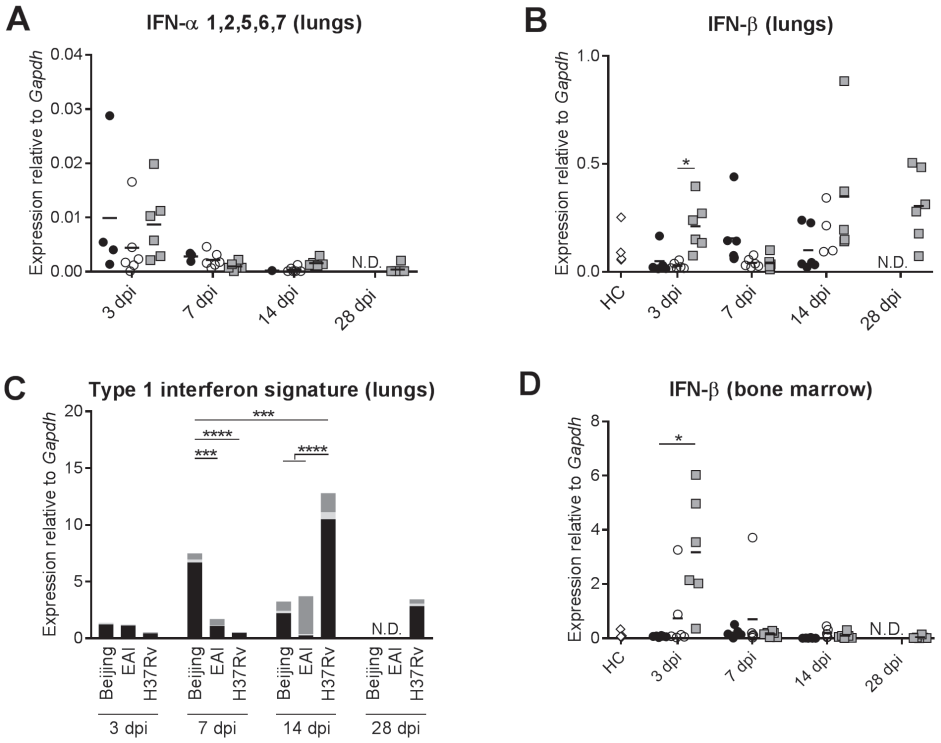

Figure 7. Expression of IFN-α, IFN-β and type 1 interferon-inducible genes in lung and BM during infection with different Mtb strains.

A) Combined expression of IFN-α 1,2,5,6,7 mRNA in lung homogenate did not show significant differences between groups **B)** Expression of IFN-β mRNA in lung homogenate is higher in the lungs of H37Rv-infected mice compared to EAI-1627-infected mice at 3 dpi. Other differences did not reach significance. **C)** Combined expression in the lungs of type 1 interferon-inducible genes *Mx1* (black), *IFI44* (light grey) and *CCL2* (dark grey), represented in a type 1 interferon signature, showed the highest level in the Beijing-1585 group at 7 dpi and in the H37Rv group at 14 dpi. **D)** Expression of IFN-β in the bone marrow is higher in the H37Rv group compared to Beijing-1585 at 3 dpi. N=6 mice per group per time point, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ after Bonferroni correction. Two-way, repeated measure ANOVA followed by Bonferroni correction was used to calculate significance for Figure 7C.

genes in the lungs at 7 dpi, while H37Rv-infected mice showed a higher peak induction at 14 dpi. The observed kinetics for the type 1 interferon-inducible genes at 7 dpi and 14 dpi closely matched the trends observed for IFN-β expression at these time points, and not IFN-α, except for the absence of a type 1 interferon signature in the lungs of mice infected with H37Rv 4 days before. This suggests that in this model IFN-β is more relevant for the induction of type 1 IFN-regulated genes during acute infection than the tested IFN-α subtypes.

To compare the findings on IFN-β in the lungs at 3 dpi with differential systemic effects observed previously in the bone marrow, we measured IFN-β mRNA expression in the bone marrow. This showed a significantly increased expression of IFN-β in the H37Rv group compared to the Beijing-1585 group at 3 dpi (**Fig. 7D**).

DISCUSSION

This study shows that infections with two clinical isolates of *Mycobacterium tuberculosis* belonging to the Beijing and EAI lineages is characterized in the lungs by an influx of B-cells, higher IL-4 protein levels and recruitment of myeloid cells that appear functionally impaired with low IL-12 and iNOS expression levels. Moreover, especially Beijing-1585 infection is associated with a reduced expression of inflammatory cytokines in the bone marrow as early as from 3 dpi onwards, suggesting the hampered priming of developing myeloid cells for subsequent responses.

Our model suggests a pathogenic effect of B-cells during acute infection, thereby contrasting an earlier study that showed that B-cells reduced neutrophilia by limiting IL-17 responses (37). We found lower IL-17 protein levels in the lungs of Beijing-1585- and EAI-1627-infected mice compared to H37Rv and a similar influx of PMN into the lungs at peak infection. These findings were in line with more recent studies demonstrating a protective effect of IL-17 during acute infection, particularly against Beijing strains (38, 39). Another study in non-human primates showed that B-cell depletion resulted in lower levels of inflammation and increased bacterial burdens during acute infection (40). Similarly, we found that the higher percentages of B-cells were associated with higher levels of inflammation, as expressed by inflammation markers TNF-α and IL-6, but also with higher bacterial burdens. Potential interactions between B-cells, IL-17 and inflammation during acute TB are complex and reviewed elsewhere (41). However, based on our data it appears that the combination of a B-cell influx and high IL-17 protein levels in the lungs during acute infection causes excessive inflammatory damage without having

an effective bactericidal effect. Interestingly, during chronic infection a protective role was recently demonstrated for B-cells and antibodies in TB patients (42, 43).

Beijing-1585 and EAI-1627 infection elicited significant IL-4 protein levels in the lungs, thus matching the observed increase in B-cells. The presence of IL-4 was previously shown to exert a pathogenic effect during Mtb infection by diverting the role of TNF-α from myeloid cell activator to tissue damage mediator (44). In line with this, *in vitro* studies showed that Beijing-HN878, another highly virulent Beijing strain, preferentially induced IL-4 expression in human peripheral blood mononuclear cells compared to the CDC1551 strain (45), and that virulent Beijing strains induced higher IL-4 mRNA expression levels in the lungs of mice at 14 dpi compared to non-virulent Beijing strains (24). This adds to the support for a negative role of IL-4 and B-cell responses during acute infection.

The lack of T-cell influx into the lungs observed for Beijing-1585 and EAI-1627 compared to H37Rv can potentially be explained by differences found in IL-12 expression, which is essential for dendritic cell migration from the lungs to the lymph node and the initiation of T-cell responses (31, 46). Our findings that Beijing-1585 induced lower lung IL-12p35 and IL-12p40 mRNA expression levels compared to H37Rv, are in line with previous studies (20, 21, 45, 47). Also the observed lower iNOS-expression levels in AM and iM/DC of mice infected with Beijing-1585 compared to H37Rv were described previously (13, 24, 48). However, these earlier studies found that low iNOS expression was accompanied by low expression levels of iNOS-inducing IFN-γ and TNF-α mRNA (32). In sharp contrast, we observed lower iNOS expression by iM/DC in the lungs at 14 dpi in Beijing-1585- and EAI-1627-infected mice accompanied with significantly higher lung protein levels IFN-γ and TNF-α compared to H37Rv.

Low iNOS expression in AM and iM/DC in the lungs despite high IFN-γ protein levels could be due to inhibition or prevention of iNOS induction at the site of infection. Alternatively, these myeloid cells recruited to the lung might have been primed differently in an earlier stage of development. Gut mucosal immunology studies have shown how local IL-12 production can exert a systemic effect by stimulating bone marrow resident NK-cells to produce IFN-γ early during infection, thereby priming immature myeloid cells to inhibit pathological inflammatory responses in the periphery (49). We have previously reasoned that a similar mechanism might be present in TB (41). In the current study we provide experimental evidence that reduced IL-12 expression in the lungs of Beijing-1585-infected mice is associated in the bone marrow with reduced IL-12 and IFN-γ, as well as reduced IL-17a and TNF-α expression. This occurs already in the early stage of infection before widespread bacterial dissemination. While not conclusive, this

suggests that IL-12-mediated differential bone marrow priming of myeloid cells might be an important factor early during infection with *Mycobacterium tuberculosis*. In this case, lung IL-12 present for 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.

Next to differential expression of IFN-γ in the bone marrow, we also found significant differences in type 1 interferon expression and responses in both bone marrow and lung. Manca *et al*. have associated virulent Beijing-HN878 infection with elevated IFN-α mRNA expression in the lungs at 28 dpi in a low-dose BALB/c infection model (19, 20). We were unable to reproduce this preferential increase in IFN-α mRNA upon infection with the virulent Beijing strain in the lungs, which could be due to using a high-dose infection model and/or measurements at different time points. In our subsequent analysis of IFN-β expression and type 1 interferon-inducible genes we found lower expression levels for Beijing-1585 and EAI-1627 compared to H37Rv, thus refuting the association of type 1 interferon activity and Mtb virulence in our model.

ACKNOWLEDGMENTS

The authors thank S. van den Berg and M. T. ten Kate from the Department of Medical Microbiology and Infectious Diseases, M. A. W. Smits, A. van Oudenaren and L. Hogenkamp from the Department of Immunology for their technical support, and J. Hagoort from the Department of Communication for his critical reading of the manuscript. Research for this manuscript was (in part) performed within the framework of the Erasmus post-graduate school Molecular Medicine.

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4

Immunotherapy added to antibiotic treatment reduces relapse of disease in a mouse model of tuberculosis

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ABSTRACT

Rationale

Immune-modulating drugs that target myeloid-derived suppressor cells or stimulate Natural Killer T-cells have been shown to reduce mycobacterial loads in tuberculosis. We aimed to determine if a combination of these drugs as adjunct immunotherapy to conventional antibiotic treatment could also increase therapeutic efficacy against tuberculosis.

Methods

In our model of pulmonary tuberculosis in mice, we applied treatment with isoniazid, rifampicin and pyrazinamide for 13 weeks alone or combined with immunotherapy consisting of all-trans-retinoic acid, 1,25(OH)2-vitamin D3 and α-galactosylceramide. Outcome parameters were mycobacterial load during treatment (therapeutic activity) and 13 weeks after termination of treatment (therapeutic efficacy). Moreover, cellular changes were analyzed using flow cytometry and cytokine expression was assessed at mRNA and protein level.

Results

Addition of immunotherapy was associated with lower mycobacterial loads after 5 weeks of treatment and significantly reduced relapse of disease after a shortened 13-weeks treatment course compared to antibiotic treatment alone. This was accompanied by reduced accumulation of immature myeloid cells in the lungs at the end of treatment and increased TNF-α protein levels throughout the treatment period.

Conclusion

We demonstrate in a mouse model of pulmonary tuberculosis that immunotherapy consisting of three clinically approved drugs can improve the therapeutic efficacy of standard antibiotic treatment.

Published in the American Journal of Respiratory Cell and Molecular Biology
2017 Feb;56(2):233-241. doi: 10.1165/rcmb.2016-0185OC.

INTRODUCTION

Annually, an estimated 9 million people worldwide develop active tuberculosis (TB) and rates of multidrug-resistant and extensively drug-resistant TB continue to rise (1). In the search for improved and shortened treatment regimens to counter the TB pandemic, immunotherapy as an adjunct to antibiotic treatment is gaining interest (2).

Several strategies in this regard are currently being explored, including the use of immunoglobulins, statins and metformin (3). Another option that may contribute to anti-TB treatment is targeted inhibition of myeloid-derived suppressor cells (MDSC) (4). MDSC are immature myeloid cells that accumulate during pathological inflammatory conditions and have the functional ability to suppress T-cell proliferation and to inhibit IFN-g production (5). In mice, MDSC are broadly defined as $CD11b^+$ $Gr1^+$ cells. However, as they phenotypically resemble their non-suppressive counterparts developing under steady state conditions, MDSC can only be identified unequivocally based on functional testing (5, 6).

The detrimental function of MDSC in TB has been demonstrated in both mouse models (4, 7-9) and in patient populations (10, 11). Moreover, in mouse TB, MDSC that infiltrated the lungs phagocytosed Mycobacterium tuberculosis, thereby creating a potential niche for pathogen survival.

The inhibitory function of MDSC can be targeted with all-trans-retinoic acid (ATRA) (12). This vitamin A-derivative was shown to induce maturation and thereby functional depletion of MDSC (13). In experimental TB in mice and rats, ATRA therapy reduced the number of MDSC (4), lowered mycobacterial loads in the lungs in vivo (4, 14), and stimulated antimicrobial activity against *Mycobacterium tuberculosis* (Mtb) in vitro (15, 16). The maturation effect of ATRA on MDSC could be enhanced by adding 1,25$(OH)_2$-vitamin D3 (DiOH-VD3) (17), which in itself also stimulated antimicrobial activity against Mtb that is mechanistically distinct from ATRA's mode of action (15).

Lastly, ATRA increased the expression of CD1d on antigen presenting cells, which is required for activation of Natural Killer T-cells (NKT cells) (18). CD1d-dependent activation of NKT cells with the CD1d ligand α-GalactosylCeramide (α-GalCer) reduced mycobacterial loads and improved survival in mice with TB (19). Since the combination of ATRA and α-GalCer has been found to convert MDSC into immunogenic antigen-presenting cells (20), we added α-GalCer to our treatment so as to combine differentiation therapy with activation of CD1d-restricted NKT cells.

The contributions ATRA and α-GalCer to therapy in TB are limited to therapeutic activity studies, determining reduction in mycobacterial load (4, 19). However, therapeutic activity was shown to be a poor predictor of therapeutic efficacy in TB, which can be determined by survival or relapse studies (21, 22). Therefore, to test the above-mentioned therapeutics in a setting more similar to clinical conditions, we determined if a combination of ATRA, DiOH-VD3 and α-GalCer could increase the therapeutic efficacy of antibiotic treatment, allowing shortening of TB treatment duration from 26 weeks to 13 weeks. We performed these studies in our model of pulmonary TB in mice, optimized to simulate the full course of TB treatment in humans according to WHO recommendations (23, 24).

MATERIALS AND METHODS

Mice, bacteria and infection

Female specific pathogen-free BALB/c mice aged 10-11 weeks and weighing 22-24 grams (Charles River, Les Oncins, FR) were infected with the H37Rv Mtb strain (ATCC 27294) by intra-tracheal instillation of 1.0-2.9x10^5 mycobacteria under general anesthesia as described previously (25). Inoculum sizes were confirmed by plating. All protocols were approved by the institutional animal ethics committee and adhered to the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 201/63/EU, DEC number 117-12-07, EMC-number 2737.

Treatment regimens

Choice of antibiotic drugs and dosage schedules were in accordance with the WHO guidelines for treatment of TB as described previously (25). In short, treatment started 4 weeks post infection (p.i.) and consisted of isoniazid (H, 25 mg/kg), rifampicin (R, 10 mg/kg) and pyrazinamide (Z, 150 mg/kg) administered subcutaneously in the neck once daily for five days a week (Mo-Fri). The DiOH-VD3 (Sigma Chemical Co, St. Louis, MO, USA) was frozen as 10% stock solution in 100% ethanol and was diluted in sterile 0.9% NaCl prior to administration. DiOH-VD3 was injected intraperitoneally in a volume of 500 µL containing 0.05 µg DiOH-VD3 three days per week (Mo-We-Fri). Dose and route of administration resulted in human pharmacokinetic equivalent dosage (26). α-GalCer (Tebu-bio, Le Perray-en-Yvelines, France) was dissolved as described previously (27) and was injected intraperitoneally at 500 µg/kg in a total volume of 300 µL at day 1 of treatment and subsequently once every 30 days conform to treatment of TB in an earlier study (19). ATRA was administered using slow release drug pellets (4 or 10 mg, 90-days sustained release pellets; Innovative Research of America, Sarasota, FL, USA). Pellets were implanted subcutaneously in the flank under general anesthesia.

Real-time quantitative PCR

RNA from mouse lung homogenate was purified and processed as described previously (28). Sequences for primers and reference numbers for probes (Universal Probe Library; Roche Applied Science) are listed in **Table E1** in the supplementary data. RNA levels were calculated relative to RNA levels of household gene *Gapdh*.

Flow cytometry

The flow cytometry protocol is explained in detail in the online data supplement. To eliminate live mycobacteria prior to flow cytometry analysis, cell suspensions were fixed for 30 min in fix/perm solution (Ebioscience, Vienna, AT). Cells were stained with different mAb mixes as described in **Table E2** in the supplementary data and measured on a FACS Canto II flow cytometer (BD Biosciences, Breda, NL).

Data analysis and statistics

Flow cytometry data were analyzed using Flowjo 7.6.5. Analyses were done and graphs were made using PRISM Graphpad 6. All data are expressed as mean ± SEM. Student's t-test, followed by Bonferroni correction where applicable, was used to calculate significance unless stated otherwise. P-values less than 0.05 were considered statistically significant.

Supplemental data

Tissue handling, mycobacterial load determination, ATRA and Ca^{2+} serum level quantification and lung supernatant cytokine level quantification are described in detail in the online data supplement.

RESULTS

TB leads to increased ATRA serum concentration during treatment

Since ATRA has a narrow therapeutic window (29), we first performed dose finding in uninfected mice with ATRA as single adjunct to treatment with isoniazid, rifampicin and pyrazinamide (HRZ) before starting combination therapy. Based on loss of bodyweight and supported by animal wellbeing scores, ATRA therapy in a dose of 5 mg/kg/day (10 mg pellets) was well tolerated and addition of HRZ did not influence ATRA serum concentrations in uninfected mice (**Fig. 1A**). However, in Mtb-infected mice we found ATRA serum concentrations 2-3-fold higher compared to uninfected ATRA-treated mice, which was associated with excessive weight loss (**Fig. 1B**). A lower ATRA dose of 2 mg/kg/day (4 mg pellets) was well tolerated by Mtb-infected mice and was still associated with a 10-fold increase of physiological ATRA serum concentrations to 11.9 ± 0.2 pmol/

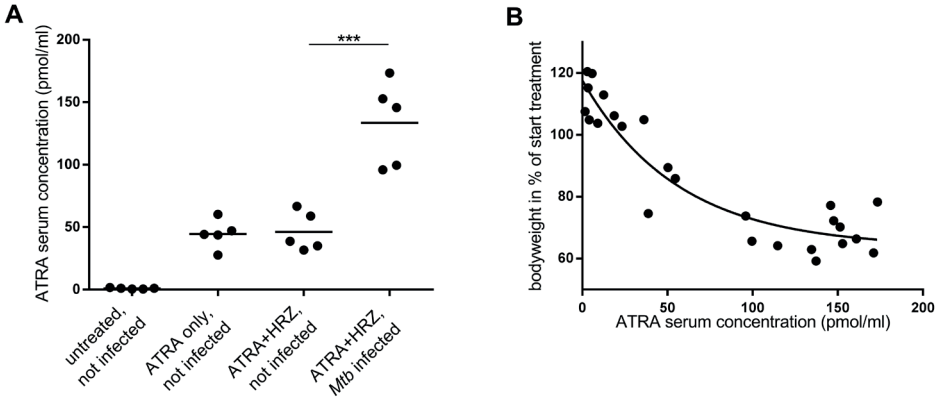

Figure 1. ATRA serum concentrations increase upon ATRA-treatment during Mtb infection and are associated with excessive body weight loss.

A) After 25 days of treatment, ATRA serum concentrations were measured in uninfected and Mtb-infected mice treated with ATRA, eventually supplemented with antibiotics (HRZ). ATRA was applied as 10 mg, 90-days release pellets. ATRA serum concentrations were not affected by addition of HRZ, but were significantly increased when treatment was started 4 weeks after infection with Mtb (***$p < 0.001$). **B)** Pooled data from different experiments for ATRA serum concentrations versus body weight loss in Mtb-infected mice after 21 to 35 days of treatment with ATRA+HRZ using 10 mg 90-days sustained release pellets. ATRA serum concentration was strongly correlated with body weight loss (R^2: 0.88).

ml (**Fig. E1** in the supplementary data). Therefore, ATRA was applied in this lower dose in our experiments.

Adjunct immunotherapy is well tolerated and marginally increases therapeutic activity

Next, we determined the therapeutic activity of immunotherapy consisting of ATRA, DiOH-VD3 and α-GalCer (ADG) adjunct to antibiotic treatment with isoniazid, rifampicin and pyrazinamide (HRZ). To this aim, we used the BALB/c mouse model of Mtb infection. In this model, mice reach the peak of infection at 4 weeks post infection with Mtb loads of 10^6-10^8 in the lungs and of 10^4-10^6 in spleen and liver. Mycobacterial loads subsequently stabilize and untreated mice become moribund between 22 and 38 weeks after infection (25).

At the peak of infection treatment was initiated with HRZ or HRZ+ADG for a period of 5 weeks. After termination of treatment, HRZ+ADG-treated mice had three-fold lower mycobacterial loads in the spleen ($p<0.05$) and showed a trend towards lower mycobacterial loads in the lungs (2.7-fold lower, $p=0.08$) compared to HRZ-treated mice (**Fig. 2A**).

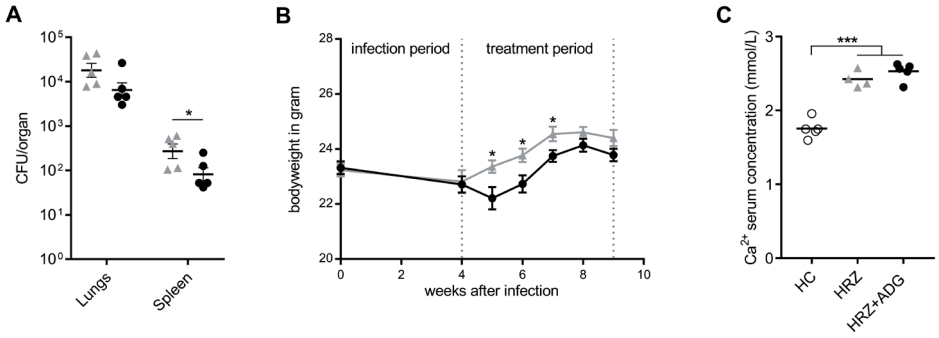

Figure 2. Addition of ADG to HRZ therapy is well tolerated and enhances mycobacterial killing during active disease.

A) Mycobacterial loads in the lungs and spleen after 5 weeks of treatment (9 weeks p.i.) with HRZ (grey triangles) or HRZ+ADG (black dots), n=5 mice per group. **B)** Bodyweight in grams during infection and treatment. **C)** Serum concentrations of Ca^{2+} in Mtb-infected mice treated with HRZ or HRZ+ADG after 5 weeks of treatment (9 weeks p.i.) compared to healthy controls. In untreated mice mycobacterial loads in the lungs and spleen at 9 weeks p.i. are 10^7 and 10^5 respectively (published earlier in (25).Data are derived from a single experiment and shown as mean ± SEM. Mycobacterial loads in antibiotic-treated mice are in agreement with previous historic controls (25). *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$. HC=healthy control, HRZ= isoniazid, rifampicin and pyrazinamide, ADG= ATRA, DiOH-VD3 and α-GalCer.

Regarding tolerability, HRZ+ADG-treated mice weighing >20 grams at start of treatment transiently lost weight compared to HRZ-treated mice (**Fig. 2B**). However, mice weighing ≤20 gram 4 weeks p.i. showed excessive weight loss in the first week of treatment and had to be euthanized as they had reached humane endpoints. Therefore, mice weighing ≤20 gram at start of treatment were excluded in both treatment groups (2 mice from each group).

Treatment with ATRA- or DiOH-VD3, but also TB itself, can all cause hypercalcaemia. Therefore, serum Ca^{2+} was determined at end of treatment. The Ca^{2+} levels in both HRZ or HRZ+ADG treatment groups were significantly higher than in uninfected mice, but did not differ between the two treatment groups (**Fig. 2C**).

Adjunct immunotherapy reduces relapse of disease

In order to determine the effect of ADG adjunct therapy on therapeutic efficacy, our next step was to measure relapse of TB. In previous experiments in our mouse TB model, mycobacterial loads in infected tissues became undetectable after 13 weeks of HRZ treatment (25). However, when treatment was terminated at this point instead of completing the WHO-recommended 26 weeks period, relapse of disease still occurred leading to mycobacterial loads of circa 10^3 CFU in the lungs (25). To determine the effect of ADG therapy against these persistent Mtb populations responsible for relapse of

disease, we treated Mtb-infected mice for 13 weeks with HRZ or HRZ+ADG. Next, at 13 weeks after termination of treatment, i.e. at 30 weeks p.i., we measured mycobacterial loads in lungs and spleen (**Fig. 3A**). We validated our data with regard to infection kinetics by comparing it to earlier findings in this model. In line with previous findings, mice from both groups had low or undetectable mycobacterial loads in the lungs after 13 weeks of treatment (25). At 30 weeks p.i., relapse of disease with mycobacterial loads of 10^3 in the lungs was observed in only 1 out of 8 HRZ+ADG-treated mice versus 6 out of 8 HRZ-treated mice ($p < 0.05$). In addition, one HRZ-treated mouse had Mtb in the spleen ($10^{2.4}$ CFU, data not shown) versus none of the HRZ+ADG-treated mice. In 3 mice from the HRZ+ADG-treated group, minimal mycobacterial loads were still detectable at 30 weeks p.i., but none exceeded those found 17 weeks p.i. (**Fig. 3B**). Interestingly, serum Ca^{2+} levels in the HRZ+ADG-treated group were significantly lower than in HRZ-treated mice at the end of the post-treatment period, but still higher than in healthy control mice (**Fig. 3C**).

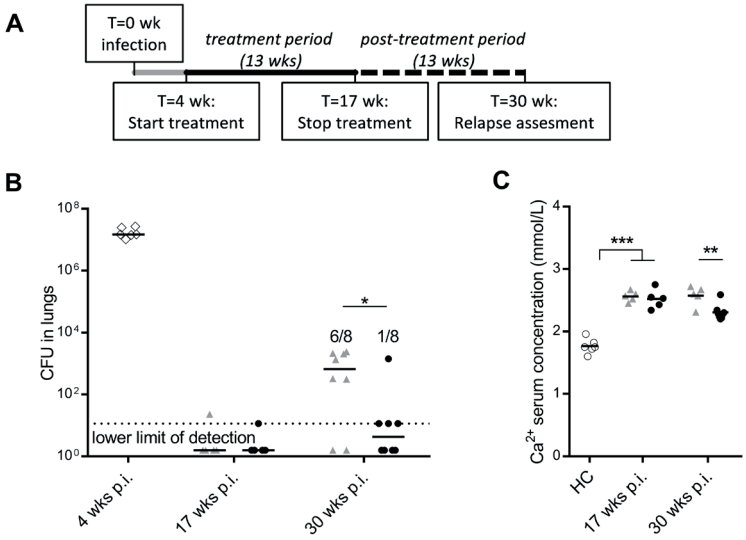

Figure 3. Addition of ADG to HRZ therapy significantly reduces relapse of disease.
A) Outline of the experimental design. **B)** Mice were treated with HRZ (grey triangles) or HRZ+ADG (black dots) starting 4 weeks p.i. (open diamonds). Lung mycobacterial loads were determined by plating. At 17 weeks p.i. both treatment groups still had 1 mouse with a detectable mycobacterial load in the lungs (HRZ only: $10^{1.4}$ HRZ+ADG: $10^{1.1}$). At 30 weeks p.i. 3 mice from the HRZ+ADG treated group had detectable mycobacterial loads (all: $10^{1.1}$). Fisher's exact test was used to calculate significance between relapse vs. no relapse at 30 weeks p.i. Of note: in untreated mice mycobacterial loads in the lungs remain constant between 10^6-10^8 from week 4 up to week 30 (25) **C)** Serum concentrations of Ca^{2+} at each time point as shown in **B** in both treatment groups compared to healthy controls (open circles). Data are derived from a single experiment and shown as mean ± SEM, n=5 mice per group at 4 and 17 wk p.i., n=8 mice per group at 30 wk p.i. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, HRZ= isoniazid, rifampicin and pyrazinamide, ADG= ATRA, DiOH-VD3 and α-GalCer.

To verify adequate release of ATRA throughout the treatment period the remaining amount of ATRA in the pellets of sacrificed mice 17 weeks p.i. was measured. These still contained 100 ± 18 µg of ATRA (2.5% of original content). Serum concentrations of ATRA at 17 weeks p.i. were elevated compared to concentrations earlier found in healthy control mice, but were no longer elevated at 30 weeks p.i. (**Fig. E1** in the supplementary data). The small amounts of ATRA found in the pellets at 17 weeks p.i. as well as serum levels over time suggest an adequate release of the 4 mg ATRA content over the treatment period.

Addition of ADG to HRZ therapy modulates the cellular immune response in TB.

To assess the immune-modulating effects associated with ADG we measured the composition of immune cell populations in the lungs during infection and treatment. Since the components of ADG primarily target myelomonocytic cells, we focused on the myeloid populations in the lungs, identified primarily by high level CD11b expression.

First we identified the different $CD11b^+$ cell populations in the lungs during steady state and infection (**Fig. 4A**). These consisted of alveolar macrophages, inflammatory macrophages/dendritic cells, PMN-like cells, monocyte (Mo)-like cells and eosinophils (see: **Table 1)**. A small $CD11b^+CD68^-Ly6G^-$ population, earlier shown to consist mainly of NK-cells and CD11b+ T-cells, was also identified (30). For the non-myeloid cell populations gating strategies can be found in **Fig. E2** in the supplementary data.

Table 1. Identified cell populations in the lung

Cell type	Identification
CD4+ T-cells	$CD3^+/CD4^+$
T-reg cells	$CD3^+/CD4^+/FoxP3^+/CD25^+$
CD8+ T-cells	$CD3^+/CD8^+$
B-cells	$CD45R^+/MHC\text{-}II^+/Ly6C^-$
Alv. Mϕ	$CD11b^{int}/Siglec\text{-}F^+/CD11c^{high}$
iM/DC	$CD11b^+/MHC\text{-}II^+/CD11c^{int}/Ly6C^{int}$
Mo-like cells	$CD11b^+/MHC\text{-}II^-/CD11c^-/Ly6C^{high}$
PMN-like cells	$CD11b^+/Ly6G^+/Ly6C^{int}$
Eosinophils	$CD11b^+/$ $Siglec\text{-}F^+$

In the acute phase of infection until the start of treatment at week 4 p.i. almost all myeloid and non-myeloid cell populations increased in the lungs of infected mice (**Fig. 4B**). Focusing on immature myelomonocytic cells as potential MDSC we found a strongly diminished expression of Ly6G in our PMN-like population (pop. IV) at 4 weeks p.i. compared to healthy controls (**Fig. 4C**), similar to earlier described MDSC identified

as CD11b^{+} Ly6G^{dim} cells (7, 8). Upon treatment, Ly6G expression on the PMN-like cells increased in both groups, but more rapidly in the HRZ+ADG-treated group than in the group receiving only HRZ.

Upon 5 weeks of treatment (9 weeks p.i.) all myeloid cell populations rapidly decreased to below their steady state, with the exception of PMN-like cells (**Fig. 5A**). The latter returned to steady state level in the HRZ+ADG-treated group, while being markedly reduced in the HRZ-treated group. At the end of treatment (17 weeks p.i.), PMN-like cell

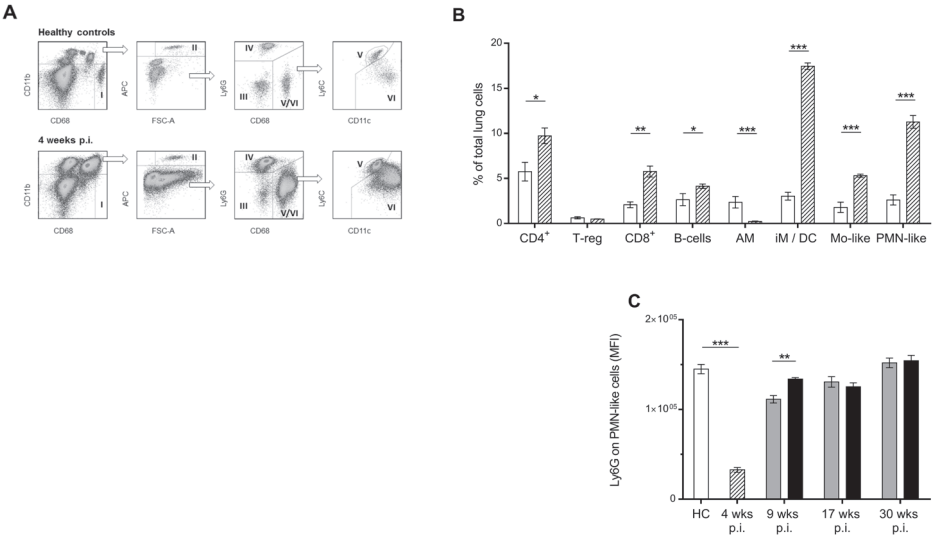

Figure 4. Addition of ADG to HRZ therapy is associated with faster recovery of Ly6G expression on PMN-like cells

A) Separation of the different CD11b+ cell populations in whole lung single cell suspension in steady state and in Mtb-infected mice at 4 weeks p.i. We first separated the AM (I) based on their reduced expression of CD11b and high expression of macrosialin (CD68), followed by eosinophils (II) based on their high auto fluorescence in the APC channel (CD117), but also based on their expression of Siglec-F (see **Fig. E2B** in the supplementary data). Next, based on their lack of expression of both Ly6G and CD68, a population known earlier to exist of T-cells and NK-cells (III) was identified. The PMN-like cells (IV) were separated based on Ly6G expression and lack of CD68 expression. Finally the CD68+ population was divided into Mo-like cells (V) and iM/DC (VI) based on their differential expression of Ly6C and CD11c. **B)** Quantitative comparison of the different cell populations as shown in **Table 1** in whole lung single cell suspension between steady state (HC, open bars) and 4 weeks p.i. (striped bars) shows a sharp increase of all inflammatory myeloid cells, a reduction in AM and an increase of mainly CD8+ cells and B-cells in the lymphoid cell compartment. **C)** Ly6G expression on PMN-like cells during infection and under treatment with HRZ (grey bars) or HRZ+ADG (Black bars). Cell populations are shown as mean ± SEM, ***p < 0.001, **p < 0.01, *p < 0.05, n=5 per group at 9 weeks p.i. and 17 weeks p.i., n=8 per group 30 weeks p.i. Data are from the same mice used for experiments shown in **Fig. 2-3**. HC= healthy controls, AM=alveolar macrophages, Mo= monocytic, PMN= polymorphonuclear, iM/DC= inflammatory macrophage/dendritic cells, HRZ= isoniazid, rifampicin & pyrazinamide, ADG= ATRA, DiOH-D3 and α-GalCer.

numbers were similar in both groups. However, at this point the iM/DC and their precursor population of Mo-like cells were reduced in the HRZ+ADG-treated group (**Fig. 5B**). At the end of the post-treatment and potential relapse period (30 weeks p.i.) there were no differences in myeloid cell populations between the two treatment groups and healthy control mice (**Fig. 5C**).

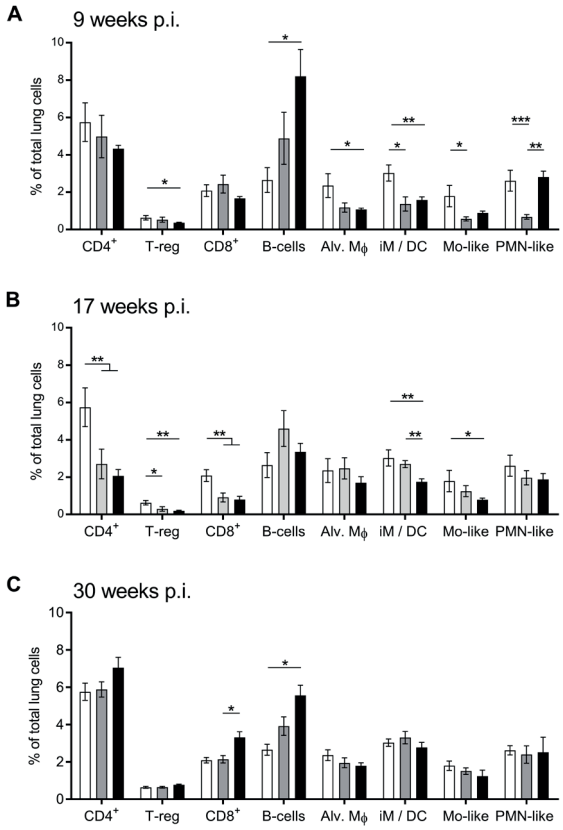

Figure 5. Addition of ADG to HRZ therapy is associated with fewer iM/DC at end of treatment and more CD8$^+$ T-cells at 30 weeks p.i.

A) Quantitative comparison of the different cell populations as shown in **Table 1** in whole lung single cell suspension between the two treatment groups at 9 weeks p.i. The PMN-like cells in the HRZ-treated group (grey bars) are markedly suppressed compared to HRZ+ADG-treated mice (black bars) and uninfected mice (open bars). **B)** At 17 weeks p.i. iM/DC are reduced in HRZ+ADG-treated mice compared to HRZ-treated mice **C)** At 30 weeks p.i. CD8$^+$ cells are increased in the HRZ+ADG-treated mice compared to HRZ-treated mice. Cell populations are shown as mean ± SEM, ***p < 0.001, **p < 0.01, *p < 0.05 after Bonferroni correction, n=5 per group at 9 weeks p.i. and 17 weeks p.i., n=8 per group at 30 weeks p.i. Data are from the same mice used for experiments shown in **Fig. 2-3**. HC= healthy controls, AM=alveolar macrophages, Mo= monocytic, PMN= polymorphonuclear, iM/DC= inflammatory macrophage/dendritic cells, HRZ= isoniazid, rifampicin & pyrazinamide, ADG= ATRA, DiOH-D3 and α-GalCer.

Concerning non-myeloid cells there were no significant differences between the two treatment groups during treatment at 9 weeks p.i. (**Fig. 5A**). At the end of treatment T-cells were significantly suppressed in both groups compared to steady state (**Fig. 5B**). At 30 weeks p.i. the HRZ+ADG-treated group had significantly higher percentages of $CD8^+$ T-cells compared to the HRZ-treated group (**Fig. 5C**).

Addition of ADG to HRZ therapy increases TNF-α protein levels.

To identify the cytokine response associated with ADG adjunct therapy we measured cytokine expression at mRNA and protein level during infection and treatment. At the peak of infection, 4 weeks p.i., mRNA expression of IFN-g, IL-6 and IL-17a was increased, but expression of TNF-α was reduced compared to steady state (**Fig. 6A**). Protein levels

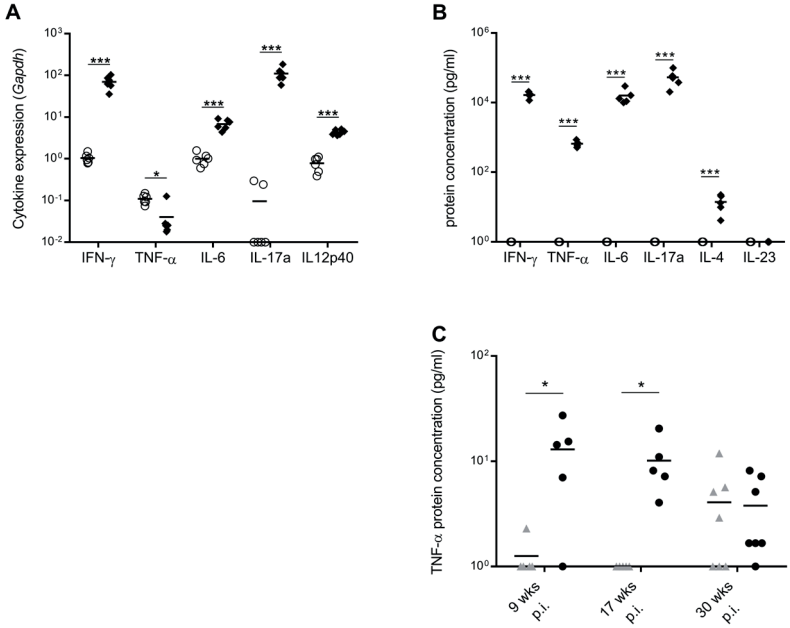

Figure 6. Addition of ADG to HRZ treatment is associated with increased TNF-α protein levels during treatment

A) Gene expression analysis of key cytokines in whole lung homogenate normalized to the expression of *Gapdh*. 4 weeks p.i. (black diamonds) compared to steady state (open circles). **B)** Cytokine protein concentration analysis in whole lung homogenate supernatant 4 weeks p.i., **C)** TNF-α protein concentrations after 5 weeks of treatment (9 wks p.i.), 13 weeks of treatment (17 wks p.i.) and 13 weeks after termination of treatment (30 wks p.i.) with HRZ (grey triangles) or HRZ+ADG (black dots). Significance for TNF-α was corrected for 1 outlier at 9 wks p.i. (with outlier included: p= 0.03, NS after Bonferroni correction, with outlier excluded: p=0.004, * after Bonferroni correction) *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$ after Bonferroni correction, n=5 per group at 9 weeks p.i. and 17 weeks p.i., n=8 per group 30 weeks p.i. Data are from the same mice used for experiments shown in **Fig. 2-3**.

correlated with mRNA expression for IFN-g, IL-6 and IL-17a**,** but TNF-α protein levels were substantially increased (**Fig. 6B**) despite reduced mRNA expression.

During and after treatment, TNF-α mRNA expression did not differ significantly between both groups (**Fig. E3** in the supplementary data). However, protein levels of TNF-α in HRZ+ADG-treated mice were significantly increased compared to HRZ-treated mice at 9 weeks p.i. and 17 weeks p.i. (**Fig. 6C**).

Messenger RNA (mRNA) expression of IFN-g, IL-17a and IL-6 remained increased in both treatment groups at 9, 17 and 30 weeks p.i., but these increases were not observed at protein level and no significant differences were found between the two treatment groups (**Fig. E3-4** in the supplementary data). IL-4 protein levels were increased 4 weeks p.i. (**Fig 6B**) and persisted at this level throughout the examination period (**Fig E4** in the supplementary data). Lastly, IL-10 mRNA expression and IL-10 protein levels were both below our limit of detection (data not shown).

DISCUSSION

We determined if adjunct immunotherapy with drugs known to reduce mycobacterial loads in the lungs could increase long term therapeutic efficacy of the WHO-recommended antibiotic treatment. In our mouse TB model we have found that a combination of ATRA, DiOH-D3 and α-GalCer was tolerated at the given dosage, increased antibiotic-mediated reduction of mycobacterial loads in infected organs after 5 weeks of treatment, reduced the levels of immature myeloid cells in the lungs at the end of treatment, and was associated with 10-fold increased levels of TNF-α protein in the lungs throughout the treatment period. Even more, addition of ADG significantly reduced relapse of disease and was associated with nearly twice the levels of $CD8^+$ T-cells, compared to HRZ alone 13 weeks after termination of treatment.

To our knowledge, this is the first study in which serum concentrations of ATRA were determined when added as a supplement to therapy in TB and we found an important association between fulminant TB and pathologically elevated ATRA serum concentrations. This can probably be ascribed to altered ATRA pharmacokinetics during infection. In our in vivo TB model, Mtb have already spread from the lungs to the liver 4 weeks p.i. (23). Local inflammation and bacterial infection are known to impair CYP450-mediated drug metabolism in the liver, resulting in increased ATRA levels (38). The use of ATRA as a single therapeutic agent for TB has been tested earlier (4, 14). In those two studies ATRA

treatment was started earlier in the course of infection, i.e. before bacterial dissemination to the liver occurred, and ATRA-associated weight loss was not reported.

Other studies have demonstrated that functionally suppressive MDSC accumulate during acute TB in the lungs of mouse strains that are either less or more susceptible than the currently used BALB/c mice (4, 7, 9) and that these MDSC could be targeted successfully with ATRA (4). These findings inspired us to evaluate the therapeutic efficacy of ATRA in TB in combination with two synergistic immune-modulating drugs (17, 20). Both ATRA and α-Galcer as single drug therapy earlier lowered mycobacterial loads in the lungs with a range between log 0.5 and log 2 after different treatment regimens and durations (4, 14, 19). For α-GalCer it has been shown that its effect is CD1d-dependent, but ATRA also has a direct bacteriostatic effect that could contribute to the reduced mycobacterial loads observed in both the reported (4) and the present study. However, this direct bacteriostatic effect was only demonstrated *in vitro* with ATRA concentrations well above serum concentrations found in our study (16). For α-GalCer it has been shown that reductions in CFU persist when isoniazid is concomitantly administered (19). We demonstrate that when tested as adjuvant to the full HRZ regimen, a combination of ATRA, α-GalCer and DiOH-VD3 marginally improves therapeutic activity. We do not consider it likely that this could be of major clinical significance. Elaborating on this, clinical studies indicate that anti-mycobacterial activity, as measured in early bactericidal activity assays (EBA), is a poor predictor of therapeutic efficacy (21, 22).

With regard to therapeutic efficacy, it has been demonstrated previously that α-GalCer prolongs survival of Mtb-infected mice (19). However no data are available in the context of the most clinically relevant scenario, which is concomitant administration adjunct to the current antibiotic anti-TB regimen. Using the BALB/c mouse model as a well-recognized experimental approach for efficacy studies (31, 32), our relapse data demonstrate that adjunct ADG therapy improved the therapeutic efficacy of HRZ treatment, but was not sufficient to eliminate all Mtb during a 13 week treatment course. This is supported by the observation that at 30 weeks p.i., Ca^{2+} levels, which correlate with disease activity in TB (33), were significantly higher in the HRZ-treated group, but also remained elevated in the HRZ+ADG-treated group compared to healthy controls. In addition, both treatment groups showed elevated IFN-g mRNA expression and increased IL-4 and TNF-α protein levels compared to steady state 30 weeks p.i. Lastly, mycobacteria were still detectable in the lungs of some mice from the HRZ+ADG group that did not relapse. Taken together, these observations suggest that HRZ+ADG-treatment was not able to clear infection completely in this TB model but did significantly improve containment of infection compared to treatment with antibiotics only.

Based on published results of single-drug experiments and synergy reported between the individual components (17, 20), we chose ADG to potentiate host immunity, in particular targeting MDSC activity. However, we did not prove the latter to be the main mechanism of action by performing functional MDSC testing. Hence, we only describe correlations between treatment modality and changes in immune parameters. At the peak of infection the $CD11b^{+}Ly6G^{dim}$ PMN-like cells from our model phenotypically resembled the $CD11b^{+}Ly6G^{dim}$ MDSC population found earlier (7). However, during treatment these immature cells with reduced Ly6G expression could no longer be found. Given the sharp reduction of PMN-like cells in the lungs during antibiotic treatment and their rapid change in phenotype towards more mature, conventional PMN, we consider it likely that functional MDSC activity by the PMN-like cell population is concomitantly reduced. In line with this, increased numbers of MDSC have been found in patients after recent TB infection and active disease, but successful anti-TB treatment significantly reduced MDSC numbers and coincided with increased MDSC maturation (10). On the other hand, overall adaptive immune cells are increased at 30 weeks p.i. in mice from the HRZ+ADG-treated group with significantly more $CD8^{+}$ cells in the lungs, but without differences in the myeloid compartment or cytokine profile between the two treatment groups.. This suggests reduced functional MDSC-activity in the HRZ+ADG-treated group, which is supported by studies showing that MDSC inhibit $CD8^{+}$ T-cells primarily in a contact-dependent way and reduce $CD8^{+}$ T-cell infiltration (5, 34). However, in the context of the current study this remains speculative.

Apart from the potential role of MDSC, we observed increased levels of TNF-α protein in the HRZ+ADG-treated group during treatment, which could have contributed to the improved therapeutic efficacy. TNF-α is a critical immune mediator in TB and holds a central protective position in the balance between an adequate anti-TB response, excessive inflammation-induced pathology, and reactivation of latent disease (35).. We observed discrepancies between TNF-α mRNA expression and protein levels that were not found for other cytokines tested. This is likely due to post-transcriptional regulation to which TNF-α is exceptionally prone (36), as is also observed in TB (37). If enhancing TNF-α production is indeed a key factor mediated by ADG adjunct therapy, treatment initiation should be timed carefully, since increased TNF-α levels during the active phase of disease might also explain the initial bodyweight loss in the HRZ+ADG-treated group in the first 3 weeks of treatment. In support of this ambiguous role of TNF-α, a recent in vivo study showed increased therapeutic efficacy of TNF-α inhibition (38), further demonstrating the complexity of the interplay between TB and immune modulation strategies. These data together suggest that it might be more beneficial to treat TB initially with antibiotics only and add ADG later in order to increase sterilizing immune activity.

In conclusion, we have shown that a combination of three clinically approved drugs as an adjunct to current HRZ treatment can modulate the immune response and improve therapeutic efficacy in TB. This adds to the possibility of adjunct immunotherapy as viable treatment modality in TB. In the current study we demonstrate a proof of principle with clinical potential. However, based on the experimental design and data obtained, the therapeutic mechanism of ADG on a cellular and molecular level and the role of MDSC were not identified unequivocally, which should be addressed in forthcoming studies.

ACKNOWLEDGEMENTS

The authors thank S. van den Berg, M.T. ten Kate, M.A.W. Smits, A. van Oudenaren and M. Schreuders-Koedam for their technical support, and J. Hagoort and D. Drevets for their critical reading of the manuscript.

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5

Assessment of bactericidal drug activity and treatment outcome in a mouse tuberculosis model using a clinical Beijing strain

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ABSTRACT

Objectives

Mycobacterium tuberculosis Beijing strains are associated with lower treatment success rates in tuberculosis patients. In contrast, laboratory strains such as H37Rv are often used in preclinical tuberculosis models. Therefore, we explored the impact of using a clinical Beijing strain on treatment outcome in our mouse tuberculosis model. Additionally, the predictive value of bactericidal activity on treatment outcome was assessed.

Methods

BALB/c mice were infected with a Beijing strain and treated with one of ten different combinations of conventional anti-TB drugs. Bactericidal activity was assessed by determining reductions in mycobacterial load after 7, 14 and 28 days and after 2, 3 and 6 months of treatment. Treatment outcome was evaluated after a 6-months treatment-course and was based on lung culture-status 3 months post-treatment.

Results

reatment success rates in Beijing-infected mice were consistently lower than treatment success observed for similar anti-TB drug regimens in multiple previous studies using H37Rv-infected mice. Treatment outcome depended critically on rifampicin. Four non-rifampicin-containing regimens showed 0% treatment success compared to success rates ranging between 80-95% for six rifampicin-containing regimens. Bactericidal activity was only predictive for treatment outcome after 3 months of treatment.

Conclusion

Our data advocate the use of Beijing strains to increase the translational value of mouse TB models evaluating treatment outcome. Additionally, our findings support the notion that bactericidal activity in the first two months of treatment, as measured in clinical phase IIa/b trials, has limited predictive value for tuberculosis treatment outcome, thus emphasizing the need for better parameters to guide future phase-IIII trials.

Published in Antimicrobial Agents and Chemotherapy
2017 Sep 22;61(10). pii: e00696-17. doi: 10.1128/AAC.00696-17. Print 2017 Oct.

INTRODUCTION

With 1.8 million deaths in 2015, tuberculosis (TB) surpassed HIV as leading cause of death amongst infectious diseases (1). One factor contributing to this ongoing burden of TB is the rapid emergence of *Mycobacterium tuberculosis* strains of the Beijing genotype (2, 3). These strains specifically contribute to the spread of drug-resistant TB and are clinically associated with increased rates of treatment failure (3-8).

To overcome this new challenge in TB treatment, novel treatment strategies with increased efficacy are urgently needed. However, clinical trials evaluating TB treatment outcome are expensive, involve large numbers of patients, and may take up to 10 years from drug design to clinical use (9). Moreover, phase IIa and IIb trials, which rely on early bactericidal activity (EBA) and surrogate endpoints such as two month sputum culture status respectively, cannot predict treatment outcome in phase III trials in TB to a satisfying degree (9-11).

Phase III trials can also be guided by preclinical testing of anti-TB drugs, which is often performed in mouse TB models (12-15). However, recent disappointing results of phase III clinical trials on moxifloxacin for anti-TB treatment, which were partly based on promising results from mouse experiments, have also raised skepticism regarding the predictive value of preclinical TB models and emphasize the need for their improvement (9). This has led to the formation of multiple international consortia, such as PreDiCT-TB and CPTR, aimed at improving the translational value of preclinical TB models (12).

Approaches that are currently being evaluated include the development of specific *in vitro* models that allow drug activity assessment against *Mycobacterium tuberculosis* in different metabolic states (14), increased appreciation of the pharmacokinetic aspects of treatment(9) and the use of mouse models that develop cavitating lesions, thus better representing human pathology (12).

Most mouse TB models that evaluate treatment outcomes use *Mycobacterium tuberculosis* laboratory strains such as H37Rv and Erdman, which are originally derived from clinical isolates in 1905 and 1945 respectively, but are no longer found in patients (12, 14).

Given the significant clinical impact of Beijing strain infections on treatment outcome, the use of Beijing strains in preclinical mouse TB models should increase their translational value. Therefore, the primary aim of this study was to assess treatment outcome, as measured in clinical phase III trials, in mice infected with an Beijing genotype strain (16-18).

Additionally, we evaluated the predictive value of bactericidal activity-based parameters, as measured in clinical phase IIa/b trials, on treatment outcome at multiple time points throughout the full 6-months treatment course.

MATERIALS AND METHODS

Bacterial strain

For all experiments, the previously described Beijing VN 2002-1585 (BE-1585) *Mycobacterium tuberculosis* genotype strain (18) was used. This strain was isolated from a patient in Vietnam in 2002 and was verified as a typical Beijing strain based on single nucleotide polymorphism analysis (19). Susceptibility assays performed according to CLSI guidelines (20) showed minimal inhibitory concentrations for rifampicin of 0.25 mg/L, for isoniazid of 0.125 mg/L, for ethambutol of 5 mg/L and for streptomycin of 2 mg/L.

Mice

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France) and acclimatized at least 7 days prior to starting experiments. Mice received food and water ad libitum. At the day of infection, animals were 13-15 weeks old and weighed 20-25 grams. Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act and were in concordance with the EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus MC approved the present protocols (117-12-08 and 117-12-13).

Infection

A suspension of *Mycobacterium tuberculosis* stored at -80°C was thawed at room temperature for 30 min and centrifuged for 10 min at 14.000*xg*. The pellet of mycobacteria was resuspended and diluted in fresh phosphate buffered saline (PBS). Mice were infected under general anesthesia using a mixture of medetomidine (Sedator®, 0.5 mg/kg; Eurovet Animal Health, Bladel, the Netherlands), midazolam (Midazolam, 5 mg/kg; Actavis, Baarn, the Netherlands) and fentanyl (Fentanyl, 0.05 mg/kg; Hameln Pharmaceuticals, Hameln, Germany), by intratracheal installation of a 40µl suspension containing 1.4×10^5 ($0.3 – 2.0 \times 10^5$) CFU of BE-1585, using a repeating dispenser (Hamilton company; Bonaduz, Switzerland), a 1 mL syringe and a 22-Gauge mouse gavage feeding needle (Fine Science Tools; Heidelberg, Germany), followed by proper inhalation. Mice were antagonized using a mixture of atipamezole (Antisedan®, 2.5 mg/kg; Orion Corporation, Espoo, Finland), flumazenil (Flumazenil, 0.5 mg/kg; Pharmachemie, Haarlem, the Netherlands) and naloxon (Naloxon, 1.2 mg/kg; Orpha-Devel Handels und Vertriebs, Purkersdorf,

Germany). Anesthetic and antagonistic agents were administered intraperitoneally, in a total volume of 175 µL and 250 µL, respectively.

Antibiotic treatment

All treatment schedules started 14 days after infection. In the experiments assessing bactericidal activity of single anti-TB drugs, mice received 0.5x, 1x or 2x the human pharmacokinetic equivalent dose (HED) (21) with rifampicin (R) (5, 10 or 20 mg/kg), isoniazid (H) (12.5, 25 or 50 mg/kg), streptomycin (S) (100, 200 or 400 mg/kg), ethambutol (E) (50, 100 or 200 mg/kg) or pyrazinamide (Z) (75, 150 or 300 mg/kg), for 5 days a week, up to 28 days. In the experiments assessing bactericidal activity and treatment outcome of the different anti-TB drug regimens, mice received treatment up to 6 months with different regimens of 1x the HED of each antibiotic 5 days a week. All drugs were administered via oral gavage except streptomycin, which was administered via subcutaneous injections. The different drug regimens are shown in **Table 1.**

Table 1. schematic overview of the experiments

		D0 [a]	D7	D14	D28	M2[b]	M3	M6	M6+3[c]
R		3 [d]	3	3	3				
H		3	3	3	3				
Z		3	3	3	3				
S		3	3	3	3				
E		3	3	3	3				
RE	(6 RE)	3	3	3	3	3	3	3	20
RZ	(2 RZ / 4 R) [e]	3	3	3	3	3	3	3	15
RH	(6 RH)	3	3	3	3	3	3	3	15
RHZ	(2 RHZ / 4 RH)	3	3	3	3	3	3	3	21
RHZE	(2 RHZE / 4 RH)	3	3	3	3	3	3	3	21
RHZS	(2 RHZE / 4 RH)	3	3	3	3	3	3	3	21
HS	(2 HS / 4 H)	3	3	3	3	3	3	3	9
HZ	(2 HZ / 4 H)	3	3	3	3	3	3	3	20
HE	(6 HE)	3	3	3	3	3	3	3	18
ZES	(2 ZES / 4 E)	3	3	3	3	3	3	3	20

[a] D0= day 0 (start of treatment), [b] M2= 2 months after start of treatment, [c] M6+3= 3 months after stop of a 6-months treatment course, [d] number of mice used for determination of mycobacterial loads in the lungs,[e] 2 RZ / 4 R= two months of RZ treatment followed by 4 months of R treatment. Drugs were administered in their human pharmacokinetic equivalent dose, mice were infected at day -14.

Assessment of mycobacterial load in the lungs

In order to assess the mycobacterial load in the lungs, mice were sacrificed by CO_2 exposure. To prevent carry-over of anti-TB drugs on subculture plates, treatment was stopped

72 hours before sacrificing the mice. In addition, activated charcoal (0.4%) was added to the agar to inhibit the antibiotic residue from the tissue samples (22). The lungs were removed aseptically and homogenized according to protocol using the gentleMACS Octo Dissociator (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 mL PBS. From each tissue homogenate 10-fold serial dilutions were made. Next, 200 µL per dilution was cultured on drug-free 7H10 Middlebrook agar and incubated for 28 days at 37^0C with 5% CO_2 followed by colony enumeration. The time points at which mycobacterial loads were evaluated are shown in the schematic overview of the experiments in **Table 1**.

Data analysis and statistics

Analyses were performed and graphs were made using PRISM Graphpad 6 (Graphpad software, La Jolla, CA). All data are expressed as median ± range. Student's t-test was used to calculate significance in figure 1. Two-way ANOVA followed by Bonferroni correction was used to calculate significance in table 3. P-values less than 0.05 were considered statistically significant.

Ethical Approval

Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act and are in concordance with the EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus MC approved the present protocols (117-12-08 and 117-12-13).

RESULTS

Mortality and bactericidal activity after single drug exposure

Mice infected with the Beijing strain were treated with isoniazid, rifampicin, ethambutol, pyrazinamide, or streptomycin in 3 different doses**. Figure 1** shows mortality and bactericidal activity after 7, 14 and 28 days of single drug exposure. Earlier observations in our model have shown that untreated Beijing-infected mice uniformly become moribund after 3-4 weeks of infection(16). Treatment with rifampicin, isoniazid or streptomycin was able to prevent mortality, whereas mice treated with pyrazinamide or ethambutol showed similar mortality as untreated mice.

Rifampicin effectively reduced mycobacterial loads in the lungs and showed a significant dose-dependent bactericidal effect after 28 days (**Fig. 1A**). Isoniazid also showed bactericidal activity, but significant dose-dependent effects were only observed at day 7 (**Fig. 1B**). Streptomycin reduced mycobacterial loads, but did not show dose-dependent effects (**Fig. 1C**). Ethambutol showed bactericidal activity after 7 days that was compa-

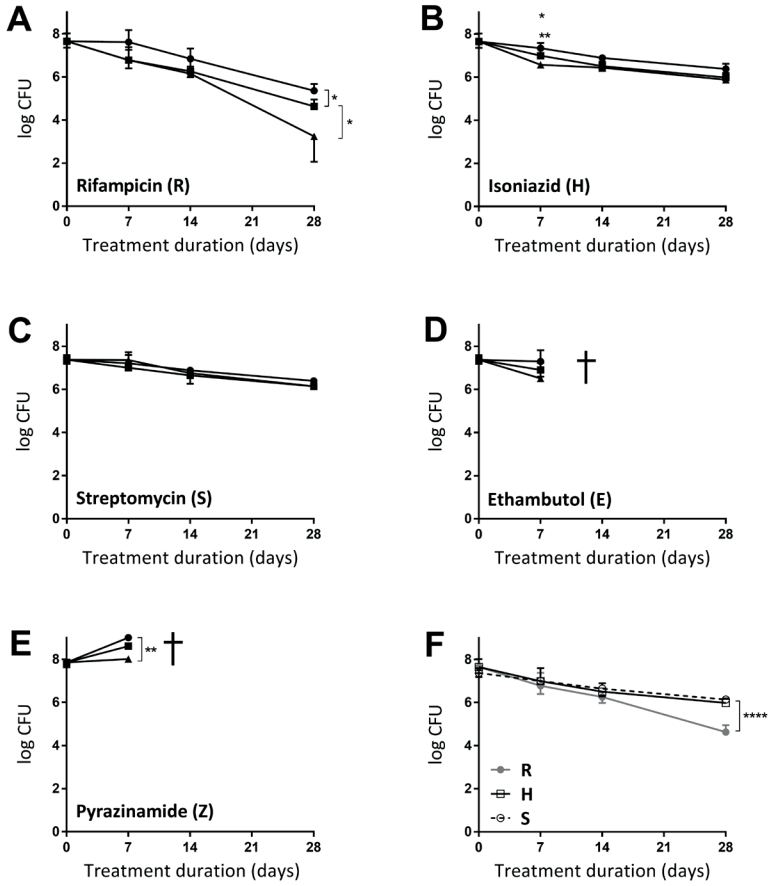

Figure 1. Bactericidal activity after single drug exposure

Mycobacterial loads in the lungs after single drug exposure over a 28-days treatment course, using 0.5x (dots), 1x (squares) and 2x (triangles) the human pharmaco-equivalent dose (HED) of the selected drugs. Data are shown as median with ranges with n=3 mice per time point. **A)** Rifampicin showed significant dose responses after 28 days of treatment between 0.5x, 1x HED and 2x HED (*) **B)** Isoniazid showed significant dose responses after 7 days of treatment between 0.5x and 2x HED (**) and between 1x and 2x HED (*). **C)** Streptomycin showed limited bactericidal activity, but prevented mortality **D)** Ethambutol showed no dose responses and could not prevent mortality. **E)** Pyrazinamide showed a significant dose-response between 0.5x and 2x HED after 7 days (**), but none of the administered dosages could prevent mortality. **F)** Comparison of 1x HED treatment with rifampicin, isoniazid and streptomycin shows significantly stronger bactericidal activity of rifampicin compared to the other two drugs after 28 days (****), * p< 0.05, ** p< 0.01, **** p<0.0001.

rable to rifampicin or isoniazid, but failed to prevent mortality (**Fig. 1D**). Pyrazinamide did not display bactericidal activity in any of the dosages tested and did not prevent mortality (**Fig. 1E**).

Comparison of the bactericidal activity of rifampicin, isoniazid and streptomycin over the 28-days exposure window showed no significant differences between the different drugs after 7 and 14 days of treatment (**Fig. 1F**). However, after 28 days rifampicin showed markedly stronger bactericidal activity than the other two drugs.

Treatment outcome and bactericidal activity after treatment with different anti-TB drug regimens

Treatment outcome after a six-months treatment course for ten different anti-TB drug regimens is shown in **Table 2**. Interestingly, none of the regimens achieved 100% treatment success.

Table 2. Treatment outcome against a Beijing strain

Drug regimen		Treatment success[a]	
RZ	(2 RZ / 4R)[b]	95%	(20/21)[c]
RHZS	(2 RHZS / 4 RH)	95%	(20/21)
RHZE	(2 RHZE / 4 RH)	90%	(19/21)
RH	(6 RH)	87%	(13/15)
RE	(6 RE)	85%	(17/20)
RHZ	(2 RHZ / 4 RH)	80%	(17/21)
HS	(2 HS / 4 H)	0%	(0/9)
HZ	(2 HZ / 4 H)	0%	(0/20)
HE	(6 HE)	0%	(0/18)
ZES	(2 ZES / 4 E)	0%	(0/20)

[a] percentage of mice with culture-negative lungs 3 months after stop of a 6-months treatment course, [b] (2 RZ / 4 R) = 2 months RZ treatment followed by 4 months treatment with R only, [c] (20/21) = 20 mice with culture-negative lungs out of 21 mice assessed. All rifampicin-containing regimens are marked grey. R = rifampicin, H = isoniazid, Z = pyrazinamide, S = streptomycin and E = ethambutol.

Another finding was that treatment success depended critically on rifampicin. The six rifampicin-containing-regimens showed treatment success rates between 80-95%, compared to 0% treatment success of all four non-rifampicin containing regimens (**Table 2**). Among the different rifampicin-containing regimens themselves, no significant differences in treatment success could be detected. This indicates limited contribution of anti-TB drugs other than rifampicin on treatment outcome. Notably, the rifampicin-pyrazinamide regimen even appeared to perform better than the rifampicin-isoniazid-pyrazinamide regimen, which suggests potential antagonism between anti-TB drugs.

Next, we determined whether the degree of bactericidal activity after any given treatment duration could predict the impact of rifampicin on treatment outcome as observed in **Table 2**. To this aim, we ranked the bactericidal activity of the different rifampicin-

containing regimens and non-rifampicin-containing regimens after 7, 14 and 28 days and after 2, 3 and 6 months. The results are shown in **Table 3**.

After 7 and 14 days, no significant differences in mycobacterial load could be found between rifampicin-containing regimens and non-rifampicin-containing regimens. After 28 days, the rifampicin-containing regimens started to show a trend towards stronger bactericidal activity compared to non-rifampicin-containing regimens, which is in line with the single drug exposure kinetics as shown in **Fig 1F**. At this time point, two out of six rifampicin-containing regimens showed significantly lower mycobacterial loads in the lungs compared to all non-rifampicin-containing regimens tested.

After two months of treatment, four out of six rifampicin-containing regimens showed significant lower mycobacterial loads compared to all non-rifampicin-containing regimens. However, a clear distinction in bactericidal activity between all rifampicin-containing regimens compared to all non-rifampicin-regimens could only be made after three months of treatment (**Table 3**).

Table 3. Bacterial loads over a 6-months treatment course

D0		Intensive phase (all drugs administered)								Continues phase (no Z/S/E)[b]			
		D7[a]		D14		D28		M2		M3		M6	
RHZE	7,3	RH	6,7	RHZ	6,1	RHZS	4,3*	RHZS[c]	0,9****	RE	1,4****	RE	0
RZ	7,7	HS	6,8	HS	6,1	RE	4,3*	RHZE	2,3****	RH	1,4****	RH	0
HS	7,7	RHZE	6,9	RHZS	6,1	RZ	4,5	RZ	3,0**	RHZS	1,6****	RHZS	0
RH	7,7	RHZ	6,9	ZES	6,2	RH	4,7	RE	3,1*	RZ	1,8****	RHZ	0
HZ	7,7	RHZS	7,0	RH	6,2	RHZ	4,9	RHZ	3,8	RHZE	2,1****	RHZE	0
HE	7,9	HE	7,2	RHZE	6,3	RHZE	5,3	RH	3,9	RHZ	2,3****	HS[d]	0,4
RHZ	8,0	HZ	7,2	RZ	6,4	ZES	5,3	ZES	4,3	HS	3,9	RZ[d]	1,1
RE	8,0	ZES	7,3	HZ	6,6	HS	5,5	HS	4,3	ZES	4,0	HE	3,0
RHZS	8,0	RE	7,3	HE	6,7	HE	5,8	HZ	4,9	HZ	4,0	HZ	3,4
ZES	8,0	RZ	7,6	RE	6,7	HZ	6,2	HE	5,2	HE	4,9	ZES	4,9

The different anti-TB drug regimens were ranked based on the mean log value of colony forming units (CFU) of mycobacteria in the lungs of n=3 mice per time point. Rifampicin-containing regimens are marked grey. Mice were infected at day -14 and treatment was started at day 0. [a] D7=7 days after start of treatment, M2= 2 months after start of treatment, etc. [b] after 2 months Z, S and E were stopped, with the exception of the ZES, RE and HE regimen. [c] 2/3 mice of the RHZS group were culture negative at M2,[d] 2/3 mice of the HS and RZ group were culture negative at M6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ after Bonferroni correction for multiple comparisons. Significance for rifampicin-containing regimens was calculated against all non-rifampicin-containing regimens at that point. R = rifampicin, H = isoniazid, Z = pyrazinamide, S = streptomycin and E = ethambutol

At the end of the six-months treatment course no mycobacteria could be cultured from the lungs of nearly all mice treated with rifampicin-containing regimens. One exception was the RZ group, in which one out of three mice still had culture-positive lungs. Of the non-rifampicin-containing regimens, all mice treated with isoniazid (H) in the continuous phase (HE, HZ and HS) showed reductions in mycobacterial load, but mycobacteria could still be cultured from the lungs. One exception was the HS group, in which two out of three mice had culture-negative lungs. All mice of the ZES group, which were treated with E in the continuous phase of treatment, showed an increase in mycobacterial loads compared to three months of treatment.

DISCUSSION

Two important findings in this study were that infection with a *Mycobacterium tuberculosis* Beijing genotype strain in mice is associated with lower treatment success rates compared to other strains in literature (13, 17, 23, 24) and that bactericidal activity is an unreliable predictor for treatment outcome in TB when assessed in the first 2 months of treatment.

Infections with Beijing strains are associated with treatment failure in TB patients (3, 5-8). The data obtained in our mouse TB model reflect these clinical findings. None of the regimens tested, including the standard of care regimen 2RZH/4RH, achieved 100% treatment success. In contrast, at least four different studies using *Mycobacterium tuberculosis* H37Rv strains, including one previous study in our own model, showed 100% treatment success of the 2RHZ/4RH regimen in BALB/c mice (13, 17, 23, 24). In TB patients, treatment success rates with 2RHZE/4RH in controlled trial settings are 92% or less (25). This indicates that the repeatedly found 100% treatment success rates in preclinical mouse TB models using H37Rv might overestimate clinical treatment success rates. The 90% treatment success rate for 2RHZE/4RH observed in our model using a Beijing strain might approach clinical observations better.

The difference in treatment outcome between Beijing and H37Rv strains could potentially be explained by the observation that only Beijing strains constitutively express proteins belonging to the DosR dormancy regulon (26, 27). These proteins regulate the mycobacterial metabolic state in response to stressors induced by the host-response. This might result in a more rapid or more profound conversion by Beijing genotype strains to a metabolic state in which the mycobacteria are less susceptible to anti-TB drugs. Other possibilities include the ability of Beijing strains to circumvent and ma-

nipulate host-responses more effectively than H37Rv (28, 29), thus resulting in better localization in (intracellular) niches, shielded from anti-TB drugs (30).

In TB, clinical phase IIa trials were found to be a poor predictor for treatment outcome (9, 31). These studies measure early bactericidal activity (EBA) in patient sputum samples between 2-7 days or between 2-14 days in case of the extended EBA (11). Our mouse TB model clearly supports this clinical finding, as it is impossible to distinguish the rifampicin-containing regimens from the non-rifampicin-containing regimens after 7 or 14 days of treatment, despite their markedly different treatment outcome after 6 months of treatment.

Our single-drug exposure experiments showed that rifampicin only starts to show significantly stronger bactericidal activity compared to other anti-TB drugs after a minimum of 28 days of treatment. Two clinical studies that continued EBA measurements up to 28 days indeed found a markedly stronger association between bactericidal activity and treatment outcome for anti-TB drug regimens containing pyrazinamide and rifampicin (32, 33). These studies indicate that extending EBA to 28 days might be a better predictor for treatment outcome. In our regimen-experiments we found that after 28 days the rifampicin-containing regimens showed a trend towards lower mycobacterial loads in the lungs compared to the non-rifampicin-containing regimens. However, a significant distinction in bactericidal activity between all rifampicin-containing regimens and all non-rifampicin-containing regimens could still not be made. Moreover, after 28 days the standard of care RHZE-regimen showed similar mycobacterial loads in the lungs as the non-rifampicin-containing ZES-regimen, while having markedly different treatment outcomes. Thus, based on our data we conclude that extending EBA for up to 28 days is more informative compared to 7 or 14 days, but remains an unreliable parameter for predicting treatment outcome.

Clinical phase IIb trials measure bactericidal activity over a 2-months period with sputum culture status as surrogate endpoint for treatment outcome (31). These studies were initially thought indicative for phase III trial outcomes in TB (31), but the disappointing results of the recent phase III REMox trials show otherwise (9, 25). Our study shows that after 2 months of treatment, the rifampicin-containing regimens RH and RHZ still do not show significant differences in lung mycobacterial loads compared to the non-rifampicin-containing regimens. The inability at this time point to significantly distinguish between regimens with a markedly different treatment outcome after 6 months supports the limited predictive value of measuring bactericidal activity during longer treatment durations in TB.

Our mouse TB model did show a clear distinction in lung mycobacterial loads between rifampicin-containing regimens and non-rifampicin-containing regimens after 3 months of treatment. However, the value of such a late time point in clinical studies is highly questionable, especially when phase III trials strive to shorten treatment duration to 4 months (25).

In conclusion, multiple approaches are currently evaluated for their potential to further increase the translational value of preclinical TB models. Examples such as implementation of mouse strains that better mimic human disease and integration of advanced biostatistics to generate more informative models are likely to improve future anti-TB drug research. Our data complement these developments by advocating the use of *Mycobacterium tuberculosis* Beijing genotype strains to increase the translational value of preclinical models assessing treatment outcomes. Also, our data in this mouse TB model support the notion that bactericidal activity in the first 2 months of treatment as measured in clinical phase IIa/b trials has limited predictive value for treatment outcome, which emphasizes the need for better biomarker to guide future phase III trials.

ACKNOWLEDGMENTS

Authors thank Carla Roodbol, Marian ten Kate, Aart van der Meijden and Sanne van den Berg for their technical assistance. Research was conducted on behalf of the PreDiCT-TB Consortium (http://predict-tb.eu).

FUNDING

This work was supported by the Innovative Medicines Initiative Joint Undertaking (115337), resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution.

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6

Improving treatment outcome assessment in a mouse tuberculosis model

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ABSTRACT

Preclinical treatment outcome evaluation of tuberculosis (TB) occurs primarily in mice. Current designs compare relapse rates of different regimens at selected time points, but lack information about the correlation between treatment length and treatment outcome, which is required to efficiently estimate a regimens' treatment-shortening potential. Therefore we developed a new approach. BALB/c mice were infected with a *Mycobacterium tuberculosis* Beijing genotype strain and were treated with rifapentine-pyrazinamide-isoniazid-ethambutol (R_pZHE), rifampicin-pyrazinamide-moxifloxacin-ethambutol (RZME) or rifampicin-pyrazinamide-moxifloxacin-isoniazid (RZMH). Treatment outcome was assessed in n=3 mice after 9 different treatment lengths between 2-6 months. Next, we created a mathematical model that best fitted the observational data and used this for inter-regimen comparison. The observed data were best described by a sigmoidal E_{max} model in favor over linear or conventional E_{max} models. Estimating regimen-specific parameters showed significantly higher curative potentials for RZME and R_pZHE compared to RZMH. In conclusion, we provide a new design for treatment outcome evaluation in a mouse TB model, which (i) provides accurate tools for assessment of the relationship between treatment length and predicted cure, (ii) allows for efficient comparison between regimens and (iii) adheres to the reduction and refinement principles of laboratory animal use.

Published in Nature Scientific Reports
2018, 5714. doi:10.1038/s41598-018-24067-x

INTRODUCTION

Tuberculosis (TB) claimed 1.7 million lives in 2016, which is more than any other infectious disease caused by a single pathogen (1). The global treatment success rate for drug-susceptible TB is 83%, which falls short of the $\geq$ 90% target rate set by the WHO (1). Moreover, treatment success rates against multi-drug resistant (MDR; 52%) and extensively drug resistant (XDR; 28%) TB are markedly lower (1). These rates emphasize the need for more effective anti-TB drug regimens that can improve treatment success. In addition, new anti-TB regimens should allow for shortening of the current 6-months treatment length to increase compliance and minimize further drug resistance development.

Recently, a large clinical Phase III trial failed to reduce anti-TB treatment length from six to four months by substituting conventional anti-TB drugs with moxifloxacin (2). This trial was conducted based on promising results from clinical Phase IIa/b trials and preclinical experiments in mouse TB models (3-5). Overall, this has led to the conclusion that early surrogates for treatment efficacy assessments as measured in clinical phase IIa/b trials are unreliable predictors for cure in TB (6, 7). This has further inspired efforts to improve preclinical mouse TB models aimed at evaluating treatment outcomes (8).

Preclinical evaluation of TB treatment outcome occurs primarily in mouse models (9). The conventional design involves a two-step approach. During the first step, early treatment efficacy is measured by determining mycobacterial load reductions in the lungs of small groups of mice (n=3-5) at preset time points until culture conversion is reached (10-15). In the second step, relapse of infection is evaluated for regimens that resulted in culture negativity. This occurs by determining lung culture status three months after treatment has ended in larger groups of mice (n=12-30), after 1-3 selected treatment lengths) (10-15).

This conventional design seems to have several drawbacks. Most importantly, it 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. The conventional design also has limited screening potential for regimens with unknown efficacy, as prior knowledge on when a regimen will reach culture-conversion is required before relapse can be evaluated. Lastly, recent clinical and preclinical observations suggest that early treatment efficacy assessment as measured in step one of the conventional design has limited predictive value for treatment outcome after a full course of anti-TB treatment (6, 7, 16).

In the current study we propose an alternative design for treatment outcome assessment in our mouse TB model. We increase the number of treatment schedules assessing outcome three months after the end of treatment regardless of culture status at the end of treatment, but decrease the number of mice per treatment length (n=3 instead of n=12-30). This way of data collection allows for mathematical modeling of the observational data optimized for establishing a robust and informative link between treatment length and cure.

The mathematical modeling is based on conventional logistic regression, but is designed to be more informative. This approach differs from survival-, or time-to-event analysis, because the bacterial burden is determined after a fixed period of time after stop of treatment. Therefore the time of culture-conversion relative to stop of treatment is unknown.

In silico simulations of the mathematical model can be used to visualize and accurately quantify the association between treatment length and predicted treatment outcome for each regimen. Advantages include the possibility to compare the curative potential of different anti-TB regimens with each other over time instead of at selected time points only and simultaneously assess the treatment-shortening potential of each individual regimen.

MATERIAL AND METHODS

Mice, infection and mycobacterial strain

Specified pathogen-free female BALB/c mice, aged 13-15 weeks, were infected by intratracheal installation of 1.0-1.8 x 10^5 drug-susceptible *Mycobacterium tuberculosis* Beijing VN 2002-1585 (BE-1585) under general anesthesia as described previously (16, 17). The mice were housed and experiments were conducted in the Erasmus MC animal biosafety level III facility.

Ethical approval

All protocols were approved by the Erasmus MC animal ethics committee under DEC number 117-12-13 and EMC number 2887, and were in accordance with the rules laid down in the Dutch Animal Experimentation Act and the EU Animal Directive 201/63/EU.

Treatment

Treatment consisted of either of three regimens: (i) rifapentine, pyrazinamide, isoniazid and ethambutol (R_pZHE), (ii) rifampicin, pyrazinamide, moxifloxacin and ethambutol

(RZME) or (iii) rifampicin, pyrazinamide, moxifloxacin and isoniazid (RZMH). The first two months of each regimen consisted of treatment with all four drugs (intensive phase) followed by four months of treatment with rifapentine and isoniazid for the R_pZHE regimen, rifampicin and moxifloxacin for RZME and rifampicin, moxifloxacin and isoniazid for RZMH. All drugs were administered 5 days a week via oral gavage in their human pharmacokinetic equivalent dose: rifampicin: 10 mg/kg, rifapentine: 10 mg/kg, moxifloxacin: 200 mg/kg, isoniazid: 25 mg/kg, ethambutol: 100 mg/kg, pyrazinamide: 150 mg/kg (18, 19).

Treatment outcome evaluation

Treatment was initiated 2 weeks after infection and was stopped between 2 and 6 months with intervals of 2 weeks (i.e. nine different treatment lengths per drug regimen). The protocol was designed to include three (n=3) mice per treatment length. A sample size of n=3 was found to be sufficient to detect a 50% difference in potency between different treatments and was expected to give reasonably high precision in model parameters, according to a statistical power calculation (described in **supplementary data file S1**).

One 'backup' mouse was added per regimen to reduce the impact of unexpected animal loss. All mice were sacrificed 3 months post-treatment to determine mycobacterial loads in the lungs as described previously (16).

Statistical analysis

The statistical analysis involved the development of a logistic regression model based on the observational data. These data were treated as a binary outcome variable of either cure (defined as a negative solid culture 3 months post-treatment) or failure (defined as a positive solid culture 3 months post-treatment). The independent variable was treatment length. The data were analyzed using the non-linear regression software NONMEM (version 7.3) with simultaneous estimation of all model parameters (20). Only if parameters were estimated close to a parameter boundary (as described below) they were fixed to the value of the respective boundary. NONMEM maximizes the likelihood of a model to fit the observational data. In NONMEM the model fit (defined as the likelihood of the model to describe the observational data) was assessed using the objective function value (OFV), which is equal to -2 times the log value of the likelihood. In order to generate a model that best described (fitted) the data, the OFV between models was compared using the likelihood ratio test (LRT). To this aim, for each model comparison a reduced model and a full model were evaluated where the full model always included more model parameters than the reduced model. The null hypothesis was that the full model did not provide better fit than the reduced model. Testing was performed at the

5% significance level which corresponds to a drop in the OFV of at least 3.84 points with one degree of freedom. Data handling and graphical analysis were conducted in R (version 3.3.0) (21).

The model development was divided into two parts; in the first part, an appropriate relation between probability of cure and treatment length was identified (regardless of drug regimen). In the second part we explored if this relation between probability of cure and treatment length was significantly different between the drug regimens.

The starting point for the first part of model development was a base model which assumed that the probability of cure was identical regardless of treatment lengths according to:

Equation 1: $p_{failure} = 1 - p_{cure} = p_{base}$

In this model, $p_{failure}$ and p_{cure} are the predicted probabilities of failure and cure respectively and p_{base} is the base probability of failure. The p_{base} parameter was constrained to be between 0 and 1. First, this base model was compared to a model assuming linear increase in cure rate with treatment length according to:

Equation 2: $p_{failure} = 1 - p_{cure} = p_{base} \times (1 \times Slope \times T)$

In this model 'Slope' is the linear increase in probability of cure with treatment length (T). The 'Slope' parameter was constrained to be between 0 and 1 divided by the maximum treatment duration of 6 months. Secondly, an E_{max} model was tested according to:

Equation 3: $p_{failure} = 1 - p_{cure} = p_{base} \times (1 - \frac{E_{max} \times T}{T_{50} + T})$

In this model 'E_{max}' is the maximal achievable probability of cure and 'T_{50}' is the treatment length at which half the E_{max} is seen. The E_{max} parameter was constrained to be between 0 and 1. Lastly, a sigmoidal E_{max} model was tested according to:

Equation 4: $p_{failure} = 1 - p_{cure} = p_{base} \times (1 - \frac{E_{max} \times T^{\gamma}}{T_{50} + T^{\gamma}})$

In this model 'γ' is a shape parameter controlling the steepness of the curve produced by the E_{max} equation.

In the second part of model development, we explored if the identified relation between cure and treatment length from the first part of model development was significantly different for the different drug regimens by comparing the model parameters of the different drug regimens (Slope, E_{max}, T_{50} or γ, depending on the model). This was done in a step-wise approach, here exemplified for a sigmoidal E_{max} model, which includes the three model parameters E_{max}, T_{50} and γ. Firstly, one model was fitted to explore if E_{max} for each regimen was significantly different from the other two regimens. This procedure was repeated for the T_{50} and γ parameters, thus resulting in nine different models. Secondly, the models that did not significantly improve the fit (OFV drop of less than 3.84 points) were not evaluated further. Of the remaining models that did result in an OFV drop of at least 3.84 points, the model with the lowest OFV was accepted. Thirdly, the accepted model with the greatest drop in OFV was combined with the remaining models that also improved the fit significantly (i.e. whose OFV drop was lesser than the accepted model but at least 3.84 points). If this combination improved the fit significantly, it was accepted as the new model. This whole three-step procedure was repeated until no significant improvement was seen anymore, which was defined as the final model.

In addition to assessment of OFV, model selection was guided by parameter uncertainty and visual predictive checks (VPC) generated using PsN (http://psn.sourceforge.net/ [cited 19-12-2016]) and Xpose (http://xpose.sourceforge.net/ [cited 19-12-2016]) using 1000 simulated datasets. The VPC is a visual diagnostic which shows how well data simulated from a model agree with the observed data.

Simulations

The observational data using n=3 animals can only theoretically generate cure rates of 0%, 33%, 67% or 100%. Therefore, we used the mathematical model to simulate treatment outcome from 1000 mice per time point to increase the resolution in the predicted cure rates (i.e. to allow cure rate to continuously range between 0-100%). Simulations were performed using Monte Carlo sampling from a random uniform distribution ranging from 1 to 0. This was also used to determine the model-predicted treatment length required for each regimen to achieve 85%, 90% or 95% cure, respectively.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary data files).

RESULTS

Observed treatment outcome

A schematic overview of our method of data collection compared to the conventional design is found in **Figure 1**. The observed proportions of cured animals for the different treatment lengths for the R_pZHE, RZME and RZMH regimens are shown in **Table 1**. R_pZHE started to show cure rates above 0% after 2.5 months of treatment and showed 100% cure after 4 months. RZME displayed similar kinetics and also showed complete cure rates from 4 months of treatment onwards. In contrast, RZMH only started to show cure rates above 0% after 4 months of treatment and did not reach complete cure even after 6 months of treatment.

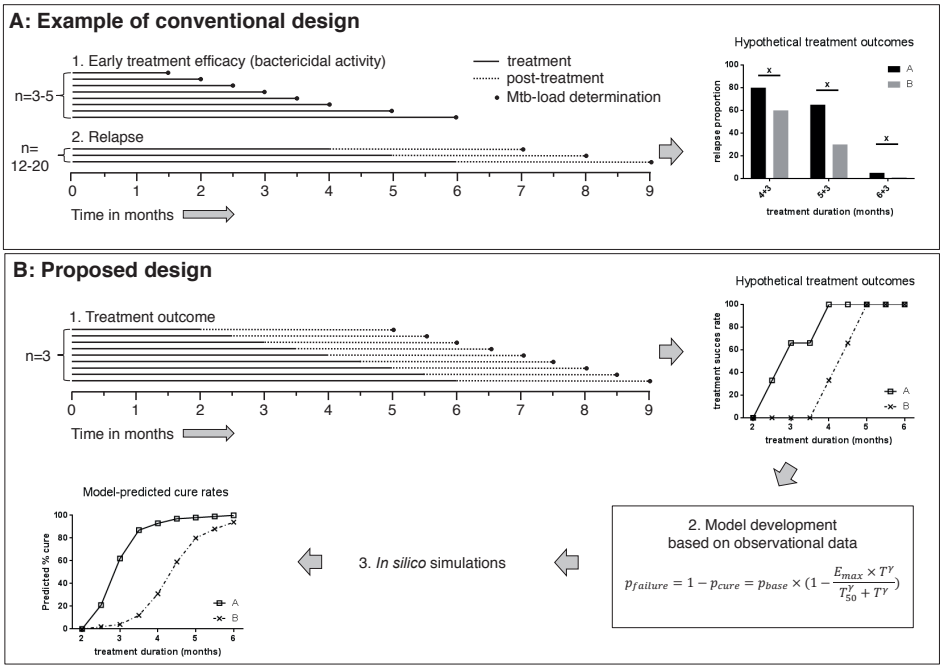

Figure 1. Schematic examples of the conventional design and the proposed design for treatment outcome evaluation in mouse TB models

A) Shows an example of the conventional design in which bactericidal activity is determined by measuring reductions in Mtb-loads in the lungs until culture negativity is reached, followed by cross-sectional evaluation / comparison of relapse rates 3 months post-treatment (x in upper right figure).

B) Shows our proposed design in which treatment outcome is determined regardless of lung culture-status at stop of treatment. This allows for more informative mathematical modeling of the data and subsequent simulation of large numbers of mice to generate a high resolution correlation between treatment duration and treatment success.

Model development

Part I: Relation between treatment length and probability of cure

The observational data from **Table 1** were first converted into a dataset used for modeling (**supplementary data file S2**). Compared to the base model which does not assume any relationship between cure and treatment length, a linear relationship between treatment length and cure gave a significant improvement in model fit compared to the base model ($p<0.001$, OFV drop of 32.2 points). An E_{max} relationship between treatment length and cure did not improve model fit compared to a linear relationship (OFV increased with 15.9 points) and was rejected. However, a sigmoidal E_{max} relationship improved model fit significantly compared to a linear relationship between treatment length and probability of cure ($p=0.001$, OFV drop of 13.3 points). Thus, the sigmoidal E_{max} model was identified as appropriate and was brought forward to the second part of model development.

Notably, the baseline probability (p_{base}) in this sigmoidal E_{max} model was estimated very close to 1, which resulted in an unstable model (not possible to obtain any parameter uncertainty). Fixing p_{base} to 1 could correct for this without affecting the OFV.

Table 1. Observational data on cure

Treatment length	R_pZHE	RZME	RZMH
2 months	0/3	0/3[a]	0/3
2.5 months	1/3	0/3	0/3
3 months	2/3	3/3	0/3
3.5 months	2/3	2/3	0/3
4 months	3/3	2/2[b]	1/3
4.5 months	2/2[c]	3/3	2/3
5 months	3/3	3/3	3/3
5.5 months	3/3	2/2[b]	3/3
6 months	3/3	3/3	3/4[d]

[a] 0/3 = 0 of 3 mice was cured (culture-negative lungs 3 months post-treatment) after indicated treatment duration; [b] Animal died of a non-tuberculosis cause prior to time point; [c] The plates for colony counting were contaminated and no counting could be performed; [d] The backup mouse included for the RZMH regimen was still alive at the 6 month time point. R=rifampicin, R_p= rifapentine, Z= pyrazinamide, M= moxifloxacin, H=isoniazid, E= Ethambutol

Part II: inter-regimen differences

The generated sigmoidal E_{max} relationship in part 1 of model development assumed a similar relationship between treatment length and probability of cure for all three regimens tested. In order to detect if the selected relationship deviated significantly between the different regimens, we determined if implementing drug regimen-specific

model parameters (including γ, E_{max} and T_{50}) improved model fit and could detect significant differences between the different regimens.

Initially, the following models improved the model fit to the observational data significantly: Model 1: separate T_{50} for RZMH ($p<0.001$, OFV drop of 18.6 points), Model 2: separate E_{max} for RZMH ($p=0.00298$, OFV drop of 8.82 points), Model 3: separate γ for RZMH ($p=0.0408$, OFV drop of 4.18 points) and Model 4: separate T_{50} for R_pZHE ($p=0.0377$, OFV drop of 4.32 points). Model 1 (separate T_{50} for the RZMH regimen) had the lowest OFV and was therefore accepted. When combined with model 2-4, no significant improvements were observed. Therefore only model 1, which included simultaneous estimation of separate T_{50} parameters for RZMH only and for RZME and RpZHE, respectively was selected.

Notably, The E_{max} parameter for the sigmoidal E_{max} model with a separate T_{50} for the RZMH regimen was estimated very close to 1 which also resulted in an unstable model. Fixing E_{max} to 1 could correct for this and improved the model fit slightly ($p=0.827$, OFV drop of 0.048 points).

Taken together, the final model included a sigmoidal E_{max} relationship where the probability of cure increased with treatment length. The E_{max} parameter had the value of 1 which implies that all included regimens can achieve 100% cure if the treatment length is sufficiently long. The baseline probability (p_{base}) also had a value of 1 which implies that at very short or no treatment duration at all (i.e. T=0) treatment failure will occur in all mice. Apart from p_{base} and E_{max}, all parameters were simultaneously estimated.

Importantly, our finding that estimating a separate T_{50} for the RZMH regimen significantly improved our model fit indicates that RZMH has reduced curative potential compared to the other regimens. The final model parameters are shown in **Table 2**. The final model code is supplied in **supplementary data file S3**.

Model validation

To verify the model, a visual predictive check (VPC) was performed where the observational data and simulated data (presented as 95% confidence interval based on 1000 simulated datasets) were compared in the same plot (**Figure 2**). As can be seen in the VPC, the observed proportions of cure fell within the 95% confidence of the simulated data. The confidence intervals may appear large at some time points which is due to the relatively low number of animals per time point and thus, given the data, the model can describe the observed data well.

Table 2. Final parameter estimates

Parameter	Description	Parameter estimate	Standard error (%CV)[a]
p_{base}	Baseline probability of no cure	1 FIX	-
E_{max}	Maximum achievable probability of cure	1 FIX	-
$T50_{RpZHE/RZME}$ (months)[b]	The treatment time at which half the E_{max} is reached for R_pZHE and RZME	2.87	5.4
$T50_{RZMH}$ (months)[b]	The treatment time at which half the E_{max} is reached for RZMH	4.35	6.0
γ	Shape factor	9.82	23.0

R_p= rifapentine, Z= pyrazinamide, M= moxifloxacin, H=isoniazid, E= Ethambutol; CV coefficient of variance; [a] The standard errors were calculated using the covariance step in NONMEM; [b] T50 was significantly different between treatment arms (no statistically significant differences were found in other parameters)

Part I: Cure rate predictions based on model simulation

Simulations of high numbers of mice (n=1000 per arm) using the developed model enabled us to provide a high-resolution estimate of the predicted cure rates of each regimen for different treatment lengths as shown in **Figure 3.** For R_pZHE and RZME this estimates that mice must be treated at least 3.5 months to reach 85% cure and 4 months to reach 90% or 95% cure. In contrast, mice must be treated with RZMH for at least 5.5 months to reach 85% or 90% cure and a full 6 months to reach 95% cure.

Part II: Model comparison to conventional relapse assessment other mouse TB models

Next, we aimed to evaluate if the predicted cure rates generated in our model were comparable to observational data obtained from other mouse TB models using pulmonary infection. These data are shown in **Table 3** (5, 12, 13, 22, 23). A direct advantage of our model-based approach is the possibility to compare our predicted cure rates for any treatment length evaluated in other mouse TB models (**Fig. 3** and **Table 3**).

Our model predicted a cure rate of 60% for RZME after three months of treatment. This was lower than the observed cure rates from three other mouse TB models, which were 80-100% (**Table 3**: models 2, 4, 5), but higher than the observed cure rate of 40% in model 6 (12). After four months of treatment no data on RZME was available in other mouse TB models and we could only compare our data to the RZM regimen. A 97% predicted cure rate for RZME in our model showed similar cure rates as observed for RZM in models 1 and 4 and a 13% higher cure rate than RZM in model 3 (**Table 3**). After five months of treatment, results were similar compared to one other mouse TB model, which also showed 100% cure.

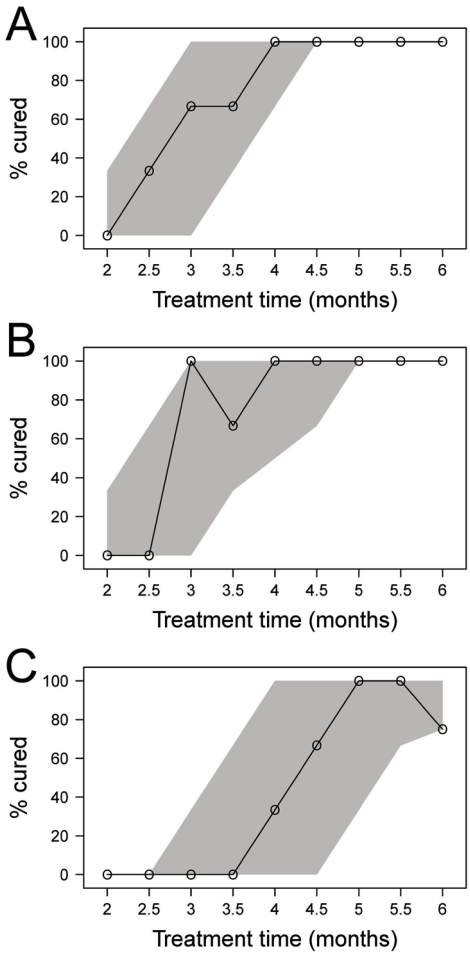

Figure 2. Visual predictive check (VPC) of the final model for each regimen
A) rifapentine, pyrazinamide, isoniazid and ethambutol (R_pZHE), **B)** rifampicin, pyrazinamide, moxifloxacin and ethambutol (RZME) and **C)** rifampicin, pyrazinamide, moxifloxacin and isoniazid (RZMH). The open circles connected by the solid black lines are the observed probabilities of cure following different treatment lengths and the shaded areas are the 95% non-parametric confidence interval of the predicted cure rates following different treatment lengths.

For RZMH, our predicted cure rate of 2% after three months of treatment was lower compared to the observed cure rates of 27%, 93% and 80% in models 2, 4 and 5, respectively, but higher than the 0% cure observed in model 6 (**Table 3**). After four months, RZMH in our model could only be compared to RZM in other mouse TB models. Our predicted cure rate of 29% for RZMH at this point was markedly lower than the cure rates observed for RZM of 100-95%, 84% and 95% in models 1, 3 and 4 respectively. In this regard it is of note to mention that after three months of treatment RZMH also showed inferior results compared to RZM in mouse TB models 1 and 4 and inferior results compared to RZME in mouse TB models 2,4 and 6 (**Table 3**).

Taken together, our finding that RZMH has significantly lower curative potential compared to RZME is reflected in trends observed in other mouse TB models. Moreover,

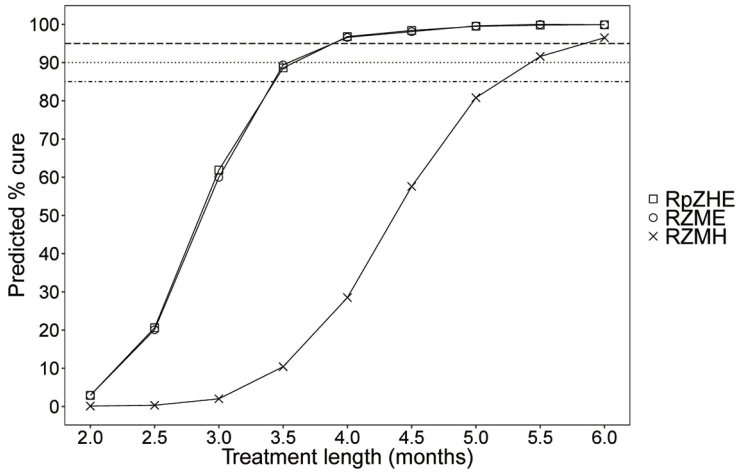

Figure 3. Model-predicted cure at different treatment lengths for each regimen
The black horizontal lines indicate 95% (dashed line), 90% (dotted line) and 85% (dashed-dotted line) cure rates. R=rifampicin, R_p= rifapentine, Z= pyrazinamide, M= moxifloxacin, H=isoniazid, E= Ethambutol

Table 3. Comparison of our model-based predictions of cure rates with observational data

		% cured at:			
	Regimen	**3 months**	**4 months**	**5 months**	**Ref.**
Our model BALB/c, Beijing, HDIT	2 R_pZHE/ 1,2,3 R_pH[a]	62%	97%	100%	
	2 RZME/ 1,2,3 RM[a]	60%	97%	100%	
	2 RZMH/ 1,2,3 RMH[a]	2%	29%	81%	
Model 1 BALB/c, H37Rv, HDA	2 R_pZM/ 1,2 R_pM	100%			[22]
	3,4,5 RZM	75%	100%	100%	[5]
	2 RZM/ 1,2,3 RM	83%	100%	100%	[5]
	2 RZM/ 2,3 RM		95%	100%	[23]
Model 2 BALB/c, H37Rv, LDA	2 RZME/ 1 RM	80%			[12]
	2 RZMH/ 1 RMH	27%			[12]
Model 3 BALB/c, Erdman, HDA	2 RZM/ 2 RM		84%		[13]
Model 4 BALB/c, Erdman, LDA	3 RZME	100%			[12]
	3 RZMH	93%			[12]
	2 RZM/ 1 RM, 2 RM	100%	95%		[13]
Model 5 C3HeB/FeJ[b], H37Rv, LDA	2 RZME/ 1 RM	80%			[12]
	2 RZMH/ 1 RMH	80%			[12]
Model 6 C3HeB/FeJ[b], Erdman, LDA	3 RZME	40%			[12]
	3 RZMH	0%			[12]

[a] Predicted cure for 3,4 and 5 months of treatment is shown as estimated in **Figure 3,** [b] C3HeB/FeJ mice can develop cavitating lesions that more closely resemble human disease, Abbreviations for route of infection: HDIT = high dose intratracheally, HDA = high dose aerosol, LDA = low dose aerosol, R=rifampicin, R_p= rifapentine, Z= pyrazinamide, M= moxifloxacin, H=isoniazid, E= Ethambutol

the discrepancy between our predicted cure rates for RZMH compared to RZM in other mouse TB models, suggests a negative effect of H on the efficacy of RZM in mouse TB models.

DISCUSSION

In this study we demonstrated that a model-based analysis of observational *in vivo* data on TB treatment outcomes can be used to generate a high resolution association between treatment length and probability of cure. The developed model could detect statistically significant differences in the curative potentials of R_pZHE and RZME compared to RZMH, which could not have been identified based on the observational data alone. Validation of our model against other mouse TB models supported a negative effect of isoniazid on the efficacy of RZM in mouse studies.

In our model RZMH showed significantly reduced curative potential compared to RZME. Interestingly, a similar trend was observed in other mouse TB models where RZMH consistently showed a trend towards inferior results compared to RZME and/or RZM (12). One explanation for this phenomenon might be a species-dependent, antagonistic effect of isoniazid on the therapeutic efficacy of rifampicin. Rifampicin is more essential for cure than isoniazid in mice (16). It has been demonstrated that concomitant administration of isoniazid negatively affects the pharmacokinetics of rifampicin by lowering the highest observed plasma concentration (C_{max}) and area under the plasma concentration-time curve (AUC) (24). However, pharmacokinetics is an unlikely cause in our model as isoniazid co-administration previously did not affect rifampicin C_{max} and AUC compared to rifampicin monotherapy (25). Also in patients no clinically significant pharmacokinetic interactions between isoniazid and rifampicin have been reported (26). Nevertheless, addition of isoniazid (H) to the combination of rifampicin and pyrazinamide (RZ) significantly reduced bactericidal activity and cure in other mouse TB models (24, 27, 28). In addition, an earlier study in our mouse TB model showed that RZ-treated mice had higher cure rates than mice treated with RH or RHZ after a six-months treatment course (95% vs. 87% and 80%, respectively) (16). This previous comparison between RZ, RH and RHZ using the conventional design as shown in **Figure 1** did not yield significant differences, but the observed inferiority of RZMH compared to RZME in the current study supports earlier observations of an antagonistic effect of isoniazid on the therapeutic efficacy of rifampicin in mice.

Advantages of the combination of animal research and mathematical modeling are the ability to detect significant differences in the curative potential of different regimens,

and the ability to compare our data with other studies that evaluated treatment outcome after any given treatment length as demonstrated in **Table 3**. In addition, animal research experiments should always strive towards the 3R-principles of replacement, reduction and refinement (29). Our method adheres to the reduction and refinement principles. Firstly, the implementation of mathematical modeling and simulations can be considered a refinement as it enabled us to detect significant differences between regimens and allowed efficient comparison with other mouse TB models, which could not be derived from our observational data alone. Secondly, our approach enables assessment of treatment outcome without requiring early treatment efficacy data. This reduces the total number of mice required (**Fig. 1**).

Early treatment efficacy as measured through bactericidal activity might be of limited predictive value for treatment outcome in TB (16). However, it remains an important screening tool in the setting of early drug discovery. The similar principle of observational data and mathematical modeling can be applied to bactericidal activity experiments as well using 'culture negativity' as outcome parameter in order to improve data interpretation.

One initial concern with the proposed design was that with only n=3 mice per time point, the treatment outcome in a single mouse on a crucial time point might have a disproportional impact, e.g. if in the RZMH group 4/4 mice would be cured after 6 months or if only 2/3 mice would be cured in the RZME group after 6 months (**Table 1**). However, sensitivity analysis of such scenarios did not alter the conclusions based on the model (results not shown). This can be explained by the notion that the fit of a model involves all mice evaluated at all time points and thus reduces the impact of potential outliers at a single time point.

A common method to analyze binary data is standard logistic regression but in this work we applied a new alternative to standard logistic regression. The main advantage with our new method is that it is more widely applicable than standard logistic regression. Observational data may not always behave similar to a logistic curve and in such situation our new method will outperform logistic regression. Additionally our new method can detect differences in the maximum probability of cure which standard logistic regression cannot provide. Furthermore, if different mouse models are compared, the treatment failure rate at no treatment may be different (i.e. different p_{base} between mouse models) which is another example of a scenario that can be handled using our approach but not using conventional logistic regression.

A potential improvement of our model in its current form might be evaluation of the (re) growth curve of *M. tuberculosis* during treatment failure. In the current design, the data were analyzed as a binary outcome because cure or failure was based on the absence or presence of mycobacteria in the lungs at a single time point three months after stop of treatment. If mycobacterial loads were measured at multiple time points after stop of treatment, e.g. after one, two and three months, as opposed to only three months, a time-to-event approach could have been used to analyze the data. A time-to-event analysis is considered more informative than analyzing the data as a binary outcome because it can provide information on the time course of cure or relapsing treatment failure. This could allow for better estimation of treatment success rates, but would also require substantially more mice.

In conclusion, we provide a new design for treatment outcome evaluation in our mouse TB model, which (i) provides accurate tools for assessment of the relationship between treatment length and predicted cure, (ii) allows for efficient comparison between regimens, (iii) can be readily compared to other studies and (iv) adheres to the reduction and refinement principles of laboratory animal use.

ACKNOWLEDGEMENTS

Authors thank Carla Roodbol, Marian ten Kate, Aart van der Meijden and Sanne van den Berg for their technical assistance and Sake de Vlas for his scientific comments and critical reading of the manuscript. Research was conducted on behalf of the PreDiCT-TB Consortium (http://predict-tb.eu).

FUNDING

This work was supported by the Innovative Medicines Initiative Joint Undertaking (115337), resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies' in kind contribution.

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7

Summarizing discussion and future perspectives

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

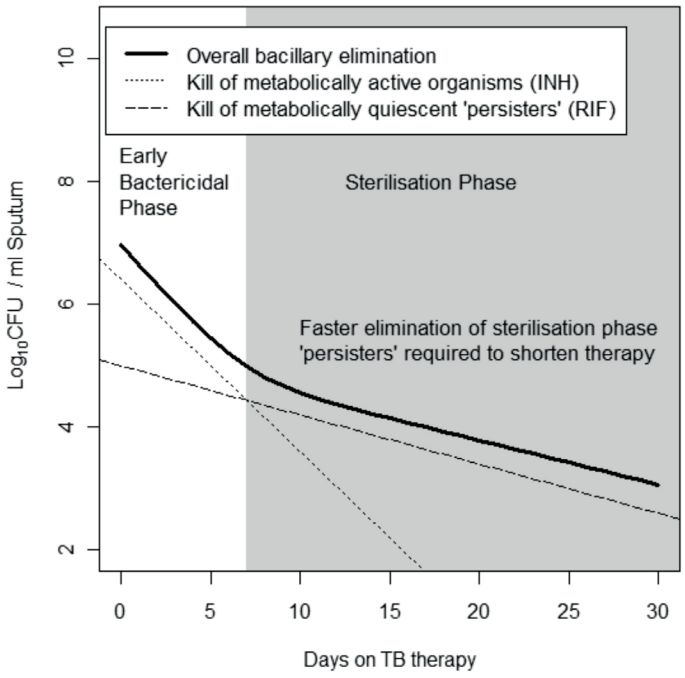

Figure 1. Biphasic kinetics of TB treatment
Adapted from Sloan et al (74), sputum colony counting data from three patient cohorts.

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).

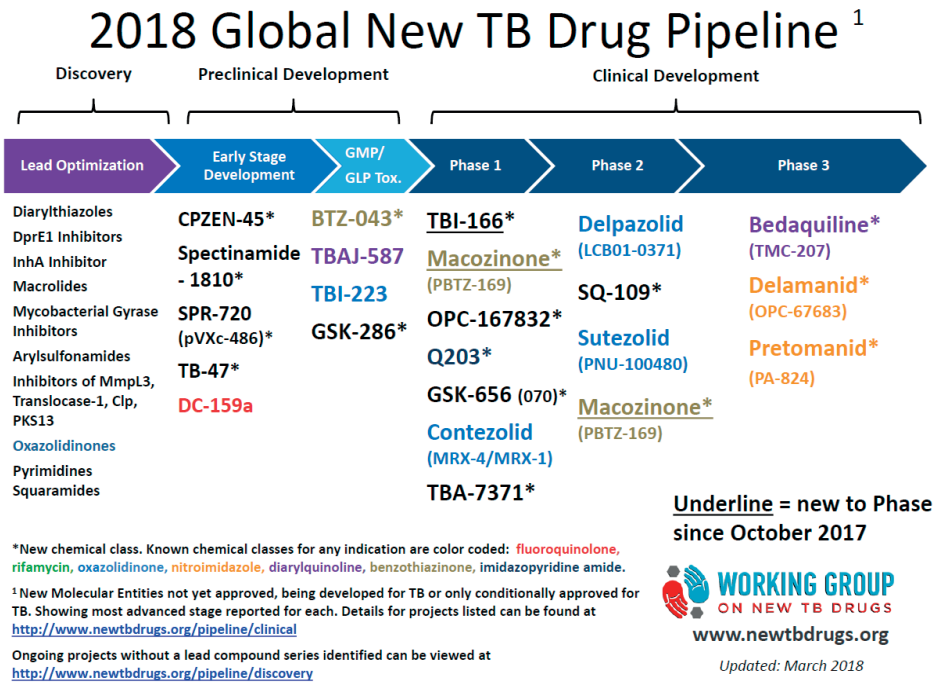

Figure 2. an overview of the TB drug pipeline, available from http://www.newtbdrugs.org/pipeline/clinical

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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8

Nederlandse samenvatting

SAMENVATTING VAN DE BESPREKING

Tuberculose (TBC) brengt al millennia lang een forse ziektelast met zich mee voor de mensheid en blijft dit tot op heden doen, ondanks het feit dat curatieve behandeling inmiddels beschikbaar is. Dit relatieve 'succes' van TBC kan worden verklaard door een combinatie van mycobacteriële-, gastheer- en behandelingsfactoren. Belangrijke mycobacteriële factoren die bijdragen aan deze persisterende ziektelast zijn nieuw opkomende stammen met verhoogde virulentie en de toenemende mate en ernst van antibioticaresistentie onder deze stammen. Een menselijke (gastheer) factor die een belangrijke rol speelt is de grote hoeveelheid individuen die de bacterie *Mycobacterium tuberculosis* bij zich dragen zonder hier ziek van te zijn, maar wel actieve TBC kan ontwikkelen. Een laatste belangrijke factor is de huidige (te) lange behandelduur van TBC van gemiddeld zes maanden, die noodzakelijk is om persisterende bacteriën te elimineren. Belangrijke vragen waarop TBC onderzoek zich momenteel richt, zijn:

- Hoe en waar persisteren de mycobacteriën die de behandeling van TBC zo langdurig maken?
- Hoe draagt ons eigen immuunsysteem bij aan hun voortbestaan en reactivatie?
- Hoe kunnen we persisterende subpopulaties van mycobacteriën meten?
- Hoe ontwikkelen en screenen we nieuwe middelen specifiek gericht op deze persisterende populaties?

Het werk in dit proefschrift heeft als doel om de behandeling van TBC te verbeteren door; (i) meer inzicht te krijgen in de pathofysiologie van TBC om nauwkeuriger te bepalen welke factoren van ons eigen immuunsysteem mogelijk gemoduleerd kunnen worden om behandeling te bespoedigen, en (ii) De translationele waarde van preklinische muismodellen voor TBC als screeningsinstrument voor potentiële nieuwe middelen of combinaties van middelen te optimaliseren. Hieronder volgt een samenvatting van het verrichte werk onderverdeeld in relevatie ten aanzien van mycobacteriële factoren, gastheer factoren en behandelingsfactoren.

MYCOBACTERIËLE FACTOREN

Vorderingen in genotypering in combinatie met klinische observaties over de afgelopen twintig jaar hebben laten zien dat de verschillen tussen *M. tuberculosis* stammen die TBC veroorzaken groter zijn dan aanvankelijk werd gedacht. Deze vormen een belangrijke factor in de pathogenese van TBC. Het beste voorbeeld betreft de stammen van het Beij-

ing genotype. Beijing stammen hebben een prominente rol gespeeld bij de verspreiding van multiresistente TBC in Eurazië en zijn geassocieerd met falen van de behandeling en opvlamming van actieve TBC in patiënten in andere delen van de wereld. Het is niet moeilijk voor te stellen hoe antibioticaresistentie kan bijdragen aan het falen van de behandeling. Beijing stammen beschikken echter ook over een aantal andere unieke pathogene eigenschappen die beter in kaart gebracht moeten worden om hun klinisch succes te begrijpen. Om deze reden is de mycobacteriële factor die nader bestudeerd zal worden in dit proefschrift de variatie tussen *M. tuberculosis* stammen en hun invloed op de behandeling en pathogenese van TBC in muismodellen.

In tegenstelling tot de meeste andere *M. tuberculosis* stammen brengen stammen van het Beijing genotype ook onder normale omstandigheden een groep eiwitten tot expressie die behoren tot het DosR regulon. Dit bestaat uit circa 50 genen die onder controle staan van de DosR transcriptie factor. Dit regulon is een belangrijke virulentiefactor voor *M. tuberculosis*, omdat een essentiële rol speelt in de mycobacteriële overgang naar een persisterende staat onder invloed van hypoxie. Aangezien mycobacteriën in deze persisterende staat beter bestand zijn tegen antibiotica kan de expressie van het DosR regulon onder niet-hypoxische condities een mogelijke verklaring zijn voor het verschil in behandeluitkomst tussen Beijing stammen en andere stammen.

Preklinische muismodellen voor TBC die behandeluitkomst meten gebruiken vaak *M. tuberculosis* stammen als H37Rv en Erdmann. Deze stammen waren klinisch relevant ten tijde van hun isolatie meer dan honderd jaar geleden, maar worden tegenwoordig niet meer geïsoleerd uit patiënten. Om deze reden was ons specifieke doel in **Hoofdstuk 5** om uit te zoeken wat het effect was op de behandeluitkomst van het gebruik van een Beijing stam in ons muismodel om op deze manier de translationele waarde van ons proefdiermodel te verhogen.

Onze evaluatie van behandeluitkomsten in muizen geïnfecteerd met de Beijing-1585 stam liet zien dat geen van de tien geteste combinaties van antibiotica, inclusief de huidige standaardbehandeling van TBC bestaande uit 2RZH/4RH (twee maanden rifampicine-pyrazinamide-isoniazide gevolgd door vier maanden rifampicine-isoniazide), 100% genezing kon bewerkstelligen. Dit is interessant, omdat ten minste vier eerdere vergelijkbare studies waarin de H37Rv stam werd gebruikt 100% genezing liet zien van 2RHZ/2RH in BALB/c muizen, waaronder één studie met H37Rv in ons eigen muismodel voor TBC. Dit geeft aan dat het succespercentage van behandeling in muismodellen waarin H37Rv wordt gebruikt mogelijk overschat wordt. Op basis hiervan lijkt het er dus op dat het gebruik van stammen die recent uit patiënten zijn geïsoleerd, zoals Beij-

ing-1585, in plaats van een laboratoriumstam zoals H37Rv de translationele waarde van preklinisch muismodellen voor TBC kan verbeteren.

Een andere belangrijke eigenschap van Beijing stammen betreft hun virulentie. Infectie met virulente Beijing stammen resulteert in hogere aantallen mycobacteriën, meer schade aan de longen en verhoogde mortaliteit in vergelijking met andere stammen en niet-virulente Beijing stammen. Eerdere studies in ons muismodel voor TBC bevestigden deze virulentie voor Beijing-1585, maar ook voor EAI-1627, een ander klinisch isolaat dat veel voorkomt in Zuidoost-Azië en toebehoort aan het 'East-African/Indian'genotype. Dit eerdere evidente verschil in virulentie tussen recent geïsoleerde klinische stammen in vergelijking met H37Rv inspireerde ons om de immunologische reactie op elk van deze stammen nader te onderzoeken. De resultaten beschreven in **Hoofdstuk 3** laten zien dat de immuunreactie uitgelokt door H37Rv overeen komt met het huidige paradigma van TBC pathogenese, gekarakteriseerd door een IL-12 / T-Helper 1 / Interferon (IFN)-γ reactie in de longen zoals uitgelegd in **Figuur 1** en **Box 1** in **Hoofdstuk 2**. De immuunreactie tegen Beijing-1585 en EAI-1627 bleken echter geassocieerd met een toename van B-cellen in plaats van T-cellen in de longen op het hoogtepunt van infectie. Myeloïde cellen waren in vergelijkbare frequenties aanwezig bij Beijing-1585 en EAI-1627 in vergelijking met H37Rv, maar leken minder functioneel met lage expressie van iNOS en IL-12. Daarnaast vonden we in het beenmerg van muizen geïnfecteerd met Beijing-1585 en EAI-1627 verminderde expressie van IFN-γ, TNF-α en IFN-β, cytokines die essentieel zijn voor de ontwikkeling van myeloïde cellen. Dit was al meetbaar vanaf de derde dag na infectie, voordat andere systemische effecten gedetecteerd konden worden. Deze combinatie van myeloïde cellen in de longen die waarschijnlijk verminderd functioneel zijn, in combinatie met de verminderde expressie in het beenmerg van cytokines essentieel voor de ontwikkeling van cellen met een bactericide fenotype is suggestief voor een eerder niet beschreven belangrijke rol van deze processen in stamgerelateerde virulentie in TBC.

In **Hoofdstuk 2, Figuur 3** wordt een gedetailleerde hypothese beschreven over de invloed van cytokines op de ontwikkeling van myeloïde cellen. Interleukine (IL)-12, wat aanwezig is in de longen tijdens infectie met H37Rv, maar niet bij Beijing-1585 of EAI-1627 infectie, zou systemisch kunnen zorgen voor een IFN-γ-gemedieerde ontwikkeling van myeloïde cellen met een fenotype dat excessieve inflammatoire schade door het aangeboren immuunsysteem in de longen voorkomt, maar het adaptieve immuunsysteem stimuleert. Een andere bevinding beschreven in **Hoofdstuk 3** betreft de rol van type 1 interferon in de pathogenese van TBC tijdens acute infectie. Eerdere studies in een ander muismodel voor TBC speculeerden dat toegenomen type 1 interferon signalering een bijdrage leverde aan de verhoogde virulentie van Beijing stammen in

vergelijking met H37Rv. In die studies werd er gevonden dat de Beijing HN878 stam een hoger IFN-α mRNA expressie-niveau induceerde in de long, wat geassocieerd was met lagere inductie van IL-12 en TNF-α en verminderde T-cel activatie in vergelijking met H37Rv. Wij vonden ook lagere inductie van IL-12 voor Beijing-1585, maar konden dit niet associëren met verhoogde type 1 interferon activiteit. Onze conclusie was gebaseerd op directe meting van IFN-α en IFN-β expressie, vergelijkbaar met de eerdere studie, maar verder versterkt door metingen van drie type 1 interferon-geïnduceerde genen.

Samengevat pleiten onze bevindingen uit **Hoofdstuk 5** over de invloed van *M. tuberculosis* stamverschillen op de uitkomst van behandeling en onze bevindingen uit **Hoofdstuk 3** over de invloed van stamverschillen op de immuunreactie tijdens acute infectie ervoor dat klinische mycobacteriële stammen in preklinische muismodellen gebruikt moeten worden. Dit kan de translationele waarde van deze modellen verbeteren en onze basale kennis over de immuunrespons tegen TBC uitbreiden. Een belangrijk gegeven bij dit laatste punt betreft dat veel van onze fundamentele kennis over de immuunreactie in TBC gebaseerd is op experimentele data van muisstudies die H37Rv gebruikten, terwijl wij laten zien dat de gemeten uitkomsten voor H37Rv niet representatief zijn voor de immuunreactie tegen stammen die op dit moment TBC veroorzaken in patiënten.

GASTHEER FACTOREN

Onze bevindingen zoals beschreven in **Hoofdstuk 3** lieten een verschil zien in de regulatie van IL-12 tussen H37Rv en de klinische stammen. Daarnaast vonden we ook verschillen in B-cellen, IL-4 en de type 1 interferon respons die niet goed passen binnen het huidige IL-12 / T-helper (Th) 1 / IFN-y paradigma, maar wel van belang kunnen zijn in TBC pathogenese. Redenen om te geloven dat immunologische reacties naast Th1 immuniteit van belang zijn komen voort uit (i) onbevredigende resultaten van vaccinatie studies gericht op het stimuleren van Th1-immuniteit, (ii) verhoogde inflammatoire schade geassocieerd met interventies leidend tot toegenomen IFN-y productie door T-cellen in de longen van *M. tuberculosis*-geïnfecteerde muizen en (iii) de toename van ziektelast geassocieerd met het blokkeren van systemen die de effectiviteit van Th-1 immuniteit remmen in muizen. Om deze redenen was het doel in **Hoofdstuk 2** om de inzichten in de rollen van B-cellen, IL-17 en type 1 interferon beter te integreren in ons huidige begrip van TBC pathogenese. De belangrijkste hypothese die we hierbij hebben gevormd is dat de type 1 interferon respons, IL-17 en hun interactie convergeren en leiden tot een stimulerend effect op B-cellen door: (i) de inductie van B-cell activating factor (BAFF), (ii) stimulatie van vorming en functie van tertiaire lymfoïde structuren

(TLS) en (iii) stimulatie van Th17.1 cellen die door hun hoog inflammatoire karakter sterk pathogeen kunnen zijn. (**Hoofdstuk 2,** Fig. 5).

Type 1 interferonen worden over het algemeen beschouwd als negatieve regulators in TBC pathogenese, omdat: (i) ze een regulatoir fenotype induceren in myeloïde cellen, waardoor intracellulaire mycobacteriën in deze cellen eerder persisteren dan geëlimineerd worden, (ii) er beschreven is dat virulente stammen type 1 interferonen sterker induceren en (iii) dat een interferon genexpressie handtekening in RNA geïsoleerd uit perifere bloedcellen correleert met actieve TBC. Gebaseerd op ons literatuurreview zoals beschreven in **Hoofdstuk 2** kan er geconcludeerd worden dat de effecten van type 1 interferon divers zijn en sterk afhankelijk van de ontwikkeling van myeloïde cellen onder invloed van IFN-γ, M-CSF en/of GM-CSF voorafgaand aan het contact met type 1 interferon (**Hoofdstuk 2, Fig. 3**). De belangrijkste overweging is dat ontwikkeling onder invloed van IFN-γ essentieel is voor het induceren van een regulatoir fenotype van myeloïde cellen door type 1 interferon. Dit wordt ondersteund door twee studies waarvan de resultaten laten zien dat type 1 interferonen juist een beschermend effect hebben in de acute fase van *M. tuberculosis* infectie in de afwezigheid van IFN-γ in muismodellen voor TBC. Een tweede overweging is dat type 1 interferonen alleen een regulatoir fenotype kunnen induceren in myeloïde cellen die gedifferentieerd zijn onder invloed van M-CSF, omdat GM-CSF myeloïde cellen minder gevoelig maakt voor type 1 interferon. Dit kan een mogelijke verklaring zijn voor het feit dat de effecten van type 1 interferonen vooral gezien worden tijdens acute infectie, maar afnemen als de infectie doorzet en de systemische hoeveelheid GM-CSF toeneemt. Ten slotte kunnen IFN-α en IFN-β een verschillend effect bewerkstelligen waarbij IFN-α voornamelijk pro-inflammatoir lijkt te werken en IFN-β juist regulatoir (**Hoofdstuk 2, box 4** & **Hoofdstuk 3).**

De rol van B-cellen in TB pathogenese is de laatste decennia naar de achtergrond verdrongen door de aangetoonde centrale rol van T-cellen, maar heeft de afgelopen jaren hernieuwde aandacht ontvangen. In patiënten is aangetoond dat antilichaamgemedieerde immuniteit een actieve rol speelt in TBC en dat patiënten met actieve TBC over lagere aantallen, dysfunctionele B-cellen beschikken. Deze bevindingen suggereren dat B-cellen een beschermend effect hebben tijdens chronische TBC infectie in patiënten. In ons muismodel van TBC in **Hoofdstuk 3** zien we echter dat acute infectie met Beijing-1585 en EAI-1627 geassocieerd is met hogere aantallen B-cellen en IL-4 eiwit levels in de longen in vergelijking met de minder virulente H37Rv. Deze ogenschijnlijke discrepantie tussen humane en muisbevindingen suggereert dat B-cellen beschermend zijn tijdens chronische TBC, maar eventueel bij kunnen dragen aan de ernst van de ziekte tijdens acute infectie.

Het belang van een verbeterd begrip van TBC pathogenese is benadrukt door de centrale rol die ons eigen afweersysteem speelt in de behandeling van TBC. Het is een effectieve en efficiënte eerste barrière, gezien het feit dat 'slechts' 5-15% van alle niet immuun-gecompromitteerde patiënten geïnfecteerd met *M. tuberculose* daadwerkelijk actieve TBC ontwikkelt. Echter, op het moment dat deze barrière geen stand houdt en een actieve TBC zich ontwikkelt zal 50-70% van de patiënten binnen twee jaar overlijden als zij geen behandeling krijgen. Paradoxaal kan dezelfde granulomateuze afweerreactie en de intracellulaire verblijfplaats van mycobacteriën in macrofagen en andere myeloide cellen juist bescherming bieden tegen antibiotica en mycobacteriën ondersteunen hun ontwikkeling tot een persisterend fenotype. Om deze reden kan de ziekte in het geval van TBC niet los gezien worden van de gastheer. Deze essentiële rol van ons eigen afweersysteem in de pathogenese van TB geeft het belang aan van het integreren van gastheerfactoren in de ontwikkeling van nieuwe behandelopties.

In de studie beschreven in **Hoofdstuk 4** onderzoeken we of het moduleren van ons eigen afweersysteem, naast het toedienen van antibiotica, kan leiden tot betere behandeluitkomsten in ons muismodel voor TBC. Immuuntherapie in TBC kan bestaan uit strategieën gericht op het verbeteren van de capaciteit van myeloïde cellen om intracellulaire mycobacteriën te doden. Onze strategie was gebaseerd op de hypothese dat immunotherapie opgebouwd uit all-trans retinoic acid, α-galactosylceramide en 1,25 dihydroxyvitamine D de ontwikkeling van myeloïde cellen stuurt naar een bactericide fenotype in plaats van een regulatoir fenotype. Muizen werden geïnfecteerd met *M. tuberculosis* H37Rv en behandeld met isoniazide, rifampicine en pyrazinamide (RHZ) of een combinatie van RHZ met immunotherapie. De resultaten laten zien dat RHZ in combinatie met immunotherapie leidt tot significant minder opvlamming van ziekte na 12 weken behandeling in vergelijking met alleen RHZ (**Hoofdstuk 4, Fig. 3**). De belangrijkste conclusie die hieruit getrokken kan worden is dat adjuvante immunotherapie gericht op het verhogen van de effectorfunctie van myeloïde cellen kan helpen in de specifieke eliminatie van persisterende mycobacteriën die opvlamming van ziekte op een later moment kunnen veroorzaken.

BEHANDELINGSFACTOREN

Klinische implementatie van nieuwe behandelopties, zoals immunotherapie of nieuwe antibiotica die goede resultaten hebben laten zien in preklinische studies is een langdurig en kostbaar proces. In de context van TBC heeft de negatieve uitkomst van de REMox studie uit 2014 ons een belangrijke les geleerd: vroege surrogaat eindpunten gebaseerd op bactericide activiteit of de aanwezigheid van mycobacteriën in het sputum zoals

gemeten in klinische fase IIa/b studies zijn onbetrouwbare voorspellers voor de uiteindelijke behandeluitkomst. In andere woorden: de capaciteit van (nieuwe) middelen om actief delende mycobacteriën te doden zoals aanwezig in de eerste fase van infectie en behandeling garandeert geen effectiviteit tegen persisterende mycobacteriën zoals aanwezig in de tweede fase van behandeling. Klinische fase III studies zijn duur, afhankelijk van grote groepen patiënten en kunnen wel 10 jaar duren van het ontwerpen van de studie tot het publiceren van de resultaten. Om deze reden moeten deze klinische studies gebaseerd zijn op preklinisch onderzoek met maximale translationele waarde. Voor de REMox studie waren de resultaten van de preklinische studies in muismodellen mogelijk te optimistisch geïnterpreteerd. De noodzaak blijft om deze reden aanwezig om de huidige preklinische modellen verder te optimaliseren om hun voorspellende waarde voor behandeluitkomst in klinische studies te verbeteren.

Met dit specifieke doel voor ogen hebben wij een deel van het onderzoek uit dit proefschrift verricht als onderdeel van het PreDiCT-TB consortium. Dit bestaat uit 19 publieke en private wetenschappelijke partners binnen de Europese Unie. Het doel van PreDiCT-TB is het genereren van een geïntegreerd en gevalideerd preklinisch traject voor nieuwe behandelopties voor TBC. Dit wordt bereikt door huidige en nieuwe antibiotica in meerdere *in vitro* en *in vivo* preklinische modellen te valideren tegen uitkomsten van klinische studies. Onze bijdrage bestond uit de evaluatie van diverse behandelmethoden in het Beijing-1585-geïnfecteerde BALB/c muismodel voor TBC. Een algemeen punt van kritiek op het BALB/c muismodel is het feit dat de granulomen die gevormd worden in de longen geen necrotische kern bevatten. Deze necrotische, of verkazende, kern lijkt in humane TBC een belangrijke rol te spelen, omdat het mycobacteriën kan beschermen tegen antibiotica, omdat het de weefselpenetratie beïnvloedt en kan fluctueren in mate van hypoxie en pH. De klinische relevantie van deze laesies kan het beste worden geïllustreerd door de huidige toename van chirurgische resectie hiervan in patiënten met refractoire ziekte. De afwezigheid van necrotische granulomen is de belangrijkste reden geweest voor het testen van behandeling in cavia's in plaats van muizen en de ontwikkeling van het C3HeB/FeJ muismodel voor TBC, waarin zulke laesies zich wel vormen. Echter laten andere studies zien dat de behandeluitkomst in modellen met of zonder necrotische granulomen identiek was. Ook de Ph-afhankelijke antibiotica pyrazinamide moet vermeld worden dat de mate van weefselpenetratie identiek was voor BALB/c en C3HeB/FeJ muizen. Ten slotte wijzen onder andere onze eigen studies uit dat, ondanks de aanwezige limitaties van het BALB/c muismodel voor TBC, er nog steeds sprake is van persisterende subpopulaties van mycobacteriën en dat de behandeluitkomsten vergelijkbaar zijn met humane studies. Dit blijkt ook uit de resultaten beschreven in **Hoofdstuk 5** waarin de combinatie RZHE een succespercentage had van 90% vergeleken met

de gevonden 92% in de REMox studie. Concluderend kan er dus gesteld worden dat het BALB/c muismodel, ondanks zijn limitaties, een bruikbaar preklinisch TBC model is.

Een andere belangrijke bevinding beschreven in **Hoofdstuk 5** was dat bactericide activiteit tijdens de eerste fase van behandeling niet voorspellend was voor behandeluitkomst, net zoals eerder is besproken voor klinische TBC studies. Daarnaast zijn de huidige methoden die gebruikt worden om verschillende regimes met elkaar te vergelijken gebaseerd op relatief simpele Chi-square testen tussen grote groepen muizen na een vooraf bepaalde behandelduur. Op deze manier kan er gekeken worden naar verschillen in behandeluitkomst na één specifieke behandelduur, maar kan er geen correlatie gemaakt worden tussen behandelduur en behandeluitkomst. Deze correlatie is juist nodig om nauwkeurig in te schatten met welke mate een nieuw getest regime de behandelduur eventueel kan verkorten. Om deze reden was het doel van de studie beschreven in **Hoofdstuk 6** om de huidige methoden van evaluatie van behandeluitkomst te verbeteren. In deze studie hebben we de behandeluitkomst bepaald in n=3 muizen na 9 verschillende behandelduren en combineerden dit met modelgebaseerde analyses om een methode te ontwikkelen waarin de relatie tussen behandelduur en voorspelde kans op genezing accuraat voorspeld kon worden. Implementatie van deze methode stelde ons in staat om aan te tonen dat de behandeling met rifapentine-pyrazinamide-isoniazide-ethambutol (RpZHE) en rifampicine-pyrazinamide-moxifloxacin-ethambutol (RZME) effectiever is in vergelijking met rifampicine-pyrazinamide-moxifloxacine-isoniazide (RZMH). Dit verschil kon niet aangetoond worden op basis van alleen de observationele data. Deze uitkomsten waren vergelijkbaar met trends geobserveerd in andere muismodellen voor TBC en wekken de suggestie dat isoniazide een negatief effect heeft op de effectiviteit van RZM in muismodellen. Helaas bleek de translationele waarde van onze nieuwe methode voor RZME en RZMH beperkt in vergelijking met de humane data na vier maanden behandeling (97% genezing in muizen versus 80% in mensen voor RZME en 29% in muizen versus 85% in mensen voor RZMH). Deze discrepantie tussen data afkomstig van humane studies en muisstudies geeft aan dat de translationele waarde van ons muismodel voor TBC nog verder moet worden verbeterd. Een recente studie laat zien dat correctie voor additionele factoren, waaronder geavanceerde farmacokinetische factoren en farmacodynamische modelering, soort-specifieke eiwitbinding en pathologie de translationele waarde van het BALB/c muismodel voor TBC verder verbetert. Correctie voor deze factoren in combinatie met onze beschreven methodologie en integratie van mycobacteriële stamverschillen in het BALB/c muismodel voor TBC zal de translationele waarde van dit model ongetwijfeld verbeteren zodat er nauwkeuriger en efficiënter gezocht kan worden naar nieuwe regimes die geïncludeerd kunnen worden in toekomstige klinische onderzoeken.

9

Appendices

CURRICULUM VITAE

Bas Mourik was born on February 18th, 1987 in Utrecht. He completed his VWO In 2005 at the Thorbecke Lyceum in Rotterdam where he attended the top sport academy program for basketball. After high school he joined the Royal Netherlands Marine Corps for two years and was stationed at the Royal Naval Academy in Den Helder and the van Ghent Marine Corps training center in Rotterdam. Afterwards he pursued a short career as a bouncer in which he gathered sufficient funds to spend the next seven months crossing the African continent from Cairo to Cape town with a backpack. Upon his return to the Netherlands in 2008 he started his Bachelor in medicine at Erasmus University, for which he graduated *with honours* in 2011. In parallel with his bachelor he enrolled in the Molecular Medicine Postgraduate School research master 'Infection & Immunity'. He set up his own research on immunotherapy in TB under supervision of Pieter Leenen from the Erasmus MC department of immunology. This work later became the first scientific contribution to this thesis. In 2012 he was offered to continue his research at the department of Medical Microbiology and Infectious Diseases under the supervision of Jurriaan de Steenwinkel in close collaboration with Pieter Leenen. Between 2012 and 2016 he combined medical school, internships and PhD research. He graduated *cum laude* for his MSc. Infection & Immunity in 2015 and graduated in March 2016 for his MSc. in Medicine after his final internship at the surgical ward of the Academic Hospital in Paramaribo, Suriname. After completing medical school he worked full time as a PhD student until December 2016. In January 2017 he started his medical career as a surgical resident in the Franciscus Gasthuis hospital in Rotterdam and used the weekends and late evenings to finish his PhD research. After one and half year he succeeded in this. In July 2018 he started his current 5-year residency program at the Medical Microbiology department of Leiden University Medical Center under the supervision of Prof. Dr. A.C.M (Louis) Kroes.

PHD PORTFOLIO

Courses	
17-09-2010	Study design
01-10-2010	Genetics for dummies
15-10-2010	Biomedical research techniques
17-04-2011	SPSS
27-05-2011	4th symposium & Masterclasses on mucosal immunology
13-09-2011	Photoshop and Illustrator
12-10-2011	InDesign
27-10-2011	Writing successful grant proposals
21-11-2011	Course on animal experimentation
26-04-2012	Presentation Skills
28-06-2012	Biomedical English writing
13-06-2013	MolMed Get out of you lab days (13-15 juni)
19-04-2016	Scientific integrity course

Presentations	
27-09-2012	Presentation Unit meeting MMIZ
05-10-2012	Presentation MSc. I&I research period 1
15-10-2012	Presentation Unit meeting Immunology
11-06-2013	Presentation research meeting immunology
15-07-2013	Presentation Unit meeting Immunology
07-11-2013	Presentation Unit meeting MMIZ
14-02-2014	Journal club MMIZ
11-03-2014	Oral presentation MolMed grant application
17-03-2016	Presentation ABSL-III lab information meeting
24-03-2016	Presentation ABSL-III lab information meeting

Symposia	
28-02-2011	Attendance 16th MolMed Day
13-02-2012	Poster presentation 17th MolMed Day
13-02-2013	Poster presentation 18th MolMed day
16-04-2013	Poster presentation NVMM spring meeting 16+17 april
17-06-2013	Poster presentation International PreDiCT Amsterdam Meeting (17+18 juni)
20-02-2015	Oral presentation MDR-TB BOG Surinam
03-03-2016	Poster presentation 20th MolMed Day
09-04-2016	Poster presentation ECCMID 2016 Amsterdam

Teaching	
06-11-2012	Research talk - MSc. Students lab rotation
08-11-2012	Research talk - MSc. Students lab rotation
13-11-2012	Research talk - MSc. Students lab rotation
01-07-2013	Supervision Lab rotations MSc students
30-08-2013	Presentation Summercourse MSc. I&I
24-10-2013	Supervision Lab rotations MSc students
07-11-2013	Supervision Lab rotations MSc students
06-08-2015	Lecture at department of Gynaecology Erasmus MC: basic immunology

Community	
15-01-2013	Introduction day viruskenner, function: coach Kaj Munk college
11-02-2013	Viruskenner introduction+STD talk, basisschool de Triangel
02-04-2013	Judge viruskenner project Kaj Munk+reviewing 6 research reports
09-04-2013	Attendance finals viruskenner project Triangel in NEMO Amsterdam
24-01-2014	Presentation Viruskenner project introduction day
17-05-2015	Presentation Viruskenner project Surinam
17-06-2015	Judge final day Viruskenner project Nederland
18-03-2016	Evaluation student projects Viruskenner Kaj Munk college
08-04-2016	Judge student project Viruskenner Kaj Munk college
26-10-2016	Scientific input exposition antibiotics resistance Natuurhistorisch Museum
26-10-2016	Information video exposition antibiotics resistance Natuurhistorisch Museum

DANKWOORD

De eerste stappen van dit proefschrift heb ik gezet in 2011, als onstuimige, wereldreizende geneeskundestudent die tropenarts wilde worden, maar ook wel wat onderzoek wilde doen. Deze laatste stap van mijn proefschrift in de vorm van het dankwoord schrijf ik als getrouwde man en vader van twee kinderen. In de tussenliggende zeven fantastische jaren zijn er een hoop mensen op mijn pad voorbij gekomen die allen op hun eigen unieke manier een positieve bijdrage hebben geleverd aan de totstandkoming van dit proefschrift. Allen betrokkenen persoonlijk bedanken is lastig, maar graag zou ik de volgende personen hier willen noemen.

Allereerst zou ik het dankwoord graag willen richten aan de twee onmisbare pijlers tijdens mijn onderzoeksperiode: mijn twee co-promotoren **Pieter Leenen** en **Jurriaan de Steenwinkel**. Beste Pieter, ontzettend bedankt voor het geduld en de flexibiliteit waarmee je mij hebt aangestuurd deze jaren. Jouw vertrouwen in mijn ideeën en de hulp in de praktische invulling ervan zijn onmisbaar geweest. Zelfs als ik voor de 5^{e} keer in de week enthousiast met een nieuw plan of paper je kamer binnen kwam stormen zonder afspraak wist je nog tijd te vinden voor een kritische discussie. Beste Jurriaan, zonder jouw voorwerk op het TB lab, de verworven fondsen en kennis over de muis-TBC-modellen (vooral geen muismodel ;) had dit proefschrift nooit kunnen bestaan. Specifiek bedankt voor de vrijheid en het vertrouwen wat je me hebt gegeven in het uitvoeren en inrichten van mijn promotieonderzoek.

Mijn promotor, **Annelies Verbon**, beste Annelies, hartelijk dank voor je begeleiding, de sterke inhoudelijke feedback op de manuscripten en jouw enthousiasme over het onderzoeksproject en de creatieve invulling ervan.

De leden van de kleine commissie, beste **Jon Laman, Marleen Bakker** en **Tom Ottenhoff**. Hartelijk dank voor het feit dat jullie de tijd hebben gevonden in jullie drukke levens om dit proefschrift kritisch door te lezen en te beoordelen. Beste Tom, daarnaast natuurlijk ook ontzettend bedankt voor de samenwerking en het brainstormen tijdens het schrijven van het review, ons magnus opus, en de steeds extreem snelle feedback.

De leden van de grote commissie, hartelijk dank voor de tijd die jullie hebben geïnvesteerd om mijn werk kritisch te beoordelen, te bediscussiëren en op waarde te schatten.

Vanaf hier ga ik chronologisch de verschillende mensen langs met wie ik gewerkt heb. Te beginnen met de persoon die mij heeft laten zien dat de wetenschap een prachtig iets is. **Maarten Frens**, bedankt voor de inspiratie en dat ik onderdeel mocht uitmaken van

de allereerste honours class, al was het maar deels omdat 'als we alleen de allerslimste kandidaten hadden gekozen het maar een saaie groep zou worden'.

De volgende halte was de research master 'Infection & Immunity'. Beste **Jan Nouwen** en **Frank van Vliet**, bedankt voor jullie geweldige opleiding. De researchmaster was voor mij dé manier om met alle onderzoeksafdeling in contact te komen en samen te werken. Bedankt voor de schat aan kennis, jullie steun, enthousiasme en flexibiliteit. Daarnaast moet je het ook hebben van je klasgenoten waarbij ik specifiek wil noemen **Marco Goeijenbier**. Marco, ouwe tijger, wat een heerlijke tijd hebben we gehad, eindeloos trainen tot de meest extreme trainingschema's, mudmasters en alle fantastische dingen eromheen. Om een of andere reden ben ik alleen maar minder door in vorm geraakt en jij steeds meer.. Je arbeidsethos en discipline zijn bewonderenswaardig ik ben blij dat ik je tegen ben gekomen. **Lennert vd Dries**, nog zo'n mooie baas, trouwen en promoveren in één maand, wie verzon het, maar jij deed het gewoon met Tjitske, veel geluk samen.

Tijdens mijn eerste onderzoeksstage onder supervisie van Pieter Leenen bij de immunologie was daar **Adri van Oudenaren,** of zoals iedereen hem kent **A3.** Beste A3, bedankt alle instructies en onmisbare hulp in het lab en het inwerken op de FACS, maar vooral bedankt voor je geduld. Verder zou ik graag van de immunologie alle collega's van de werkgroep willen bedanken. Mijn 'thuisstation' was natuurlijk de microbiologie, vandaar dat ik minder vaak bij jullie in de buurt was, maar bedankt voor de collegialiteit en attentheid. Specifiek nog **Ruth Huizinga**, beste Ruth, ontzettend bedankt voor de leuke samenwerking en de hulp bij alle PCR experimenten. **Marjan Versnel,** de type 1 interferon autoriteit, bedankt voor de fijne gesprekken. Ik ben blij dat je pols zo mooi hersteld is.

Vanuit de immunologie weet ik nog goed dat ik aan kwam kloppen bij de TB werkgroep van Irma en Jurriaan met de vraag of we de TB muizen misschien immunotherapie mochten geven. Beste **Irma Bakker-Woudenberg**, ontzettend bedankt voor je altijd aanwezige interesse en enthousiasme, niet alleen over werk-gerelateerde zaken, maar ook daarbuiten. **Marian ten Kate**, lieve Marian, bedankt voor alle ondersteuning in het lab en het EDC en natuurlijk ook alle gezelligheid eromheen, onmisbaar voor een lab-vreemde geneeskunde student, je bent fantastisch! **Gerjo de Knegt**, voor de eindeloze uurtjes in het TB lab en in de door jou geliefde kantoortuin. Ontzettend bedankt voor alle hulp, co-auteurschap en humor. Veel geluk, zowel met de nieuwe carriere als thuis! **Sanne v/d Berg**, voor jou hetzelfde, bedankt voor alle hulp en gezelligheid en veel plezier met Daag en de kleintjes! **Corné de Vogel**, ouwe warlock, het was gezellig. **Aart v/d Meijden**, voor mij blijf je altijd de persoon op wie ik het charcoal-plate-validation-experiment heb kunnen afschuiven, veel succes in je onderzoekscarriére. **Hannelore**

Bax, bedankt voor het kritisch meedenken en schrijven van de PreDiCT manuscripten en excuses voor de soms wat verhitte discussies hierover. **Elise Pieterman**, de hollow fiber heldin, succes met het afronden van je PhD, gaat helemaal goed komen. **Heleen v/d Spek**, ondanks het beperkte contact weet ik dat je vaak valt met de fiets na het uitgaan. **Maarten Sarink**, eigelijk geen TB werkgroep meer, maar vooruit, opvallend veel aanwezig in de weekenden zo vroeg in het onderzoek, succes met je PhD. Jullie waren een geweldige groep om mee samen te werken!

Vanuit de TB werkgroep grijp ik graag de kans om door te pakken naar de collega's van de Medische Microbiologie met wie ik heb samengewerkt. Collega's van de befaamde kantoortuin Na 9.02. Allen bedankt voor alle humor, steun, collegialiteit en gezelligheid die ik bij jullie heb gevonden. Het was fantastisch samenwerken. Bij deze een tevens een collectief sorry, ik heb in de weekenden ongetwijfeld van jullie allemaal wel een keer iets opgegeten wat jullie vergeten waren op te bergen. Specifiek nog **Astrid Heikema**, heel veel succes met de verdere postdoc carriére, ik ben benieuwd hoe het afloopt met die B-cellen. **Michiel Bexkens**, de man van 18 Februari, succes met de afronding van het onderzoek. **Wendy Kaman**, altijd mooie verhalen, bedankt voor de samenwerking. Hetzelfde geldt natuurlijk voor alle collega's van het lab van de B-toren, jullie ook bedankt voor de gezelligheid tijdens de koffiepauzes en de samenwerking!

Een groot gedeelte van het onderzoek is gedaan in het EDC, graag zou ik ook alle EDC medewerkers willen bedanken voor hun hulp, specifiek **Mathieu Sommers** in de hulp met het rondkrijgen van de autorisatie, zodat ik überhaupt zelf mijn experimenten mocht uitvoeren. Vanaf de Reumatologie ook dank aan **Erik Lubberts** voor de discussies en het gezamelijk werken aan het review.

Ten slotte voor een ieder op werkgebied die ik vergeten ben te noemen, alsnog bedankt!

Naast werkgebied zijn er uiteraard ook nog vrienden en familie die ik specifiek wil bedanken en benoemen. Allereerst **mijn ouders**, lieve papa en mama, volgens jullie was ik al van kinds af aan een wetenschapper en was het doen van promotie onderzoek de meest logische keuze. Bedankt voor jullie onvoorwaardelijke steun. Papa, samen naar Parijs om de FEDEX samples terug te halen was een van de beste verhalen van mijn promotie onderzoek. **Sarah Mourik,** mijn grote zus en gynaecoloog, bedankt voor de gezellige lunches in het EMC, ik ben trots op jou en blij dat je mijn paranimf bent! **Dirk Mourik**, de entrepeneur van de familie, heel veel succes met Decorista, het is fantastisch wat jullie aan het doen zijn. **Daan Mourik**, mijn andere paranimf en de nieuwe contentheld van T-Mobile, heel veel succes met je nieuwe job. **Denise Wijngaarden**, lieve schoonmoeder, bedankt voor je prachtige dochter en je steun en hulp met de kinderen

als we door alle drukte weer eens klem zaten met de planning. **Maarten Kloek**, bedankt voor de afwisselingen en uitdagingen tijdens de soms drukkere perioden en de backpacktrips in de rustigere perioden van het onderzoek. Altijd goed om iemand te hebben die perspectief biedt. **Novaro Mourik**, mijn kleine sprinter, bedankt dat je papa zoveel geluk geeft in zijn leven en met je komst hebt benadrukt wat de belangrijke dingen zijn in het leven. **Ché Mourik**, mijn kleine krijger, bedankt dat je samen met je broer zo lief bent geweest voor mama als papa in het weekend weer eens op het Erasmus MC aan het werken was. Ik ben nu al ongelooflijk trots op jullie allebei. Als allerlaatste, lieve **Genelva Mourik-Lo-Kioeng-Shioe**, zonder jouw onvoorwaardelijke steun, flexibiliteit en kracht de afgelopen jaren had ik mijn promotie onderzoek nooit af kunnen maken. Ontzettend bedankt voor je geduld en je begrip, je bent een fantastische vrouw. Ik bewonder je en ik hou van jou.