IMPROVING TREATMENT OF MYCOBACTERIAL INFECTIONS

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Improving Treatment of Mycobacterial Infections

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Mycobacteriële Infecties

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CHAPTER 1

General introduction

The family of mycobacteriaceae comprises a group of small, rod-shaped, acid fast bacilli and can be classified into 3 main groups: nontuberculous mycobacteria (NTM), *Mycobacterium tuberculosis* complex (including *Mycobacterium tuberculosis*) and *Mycobacterium leprae.* As one of the neglected tropical diseases, leprosy caused by *M. leprae* is a very distinct disease entity and is not addressed in this thesis. Although infections with NTM and *M. tuberculosis* differ with respect to epidemiology, transmission, clinical picture, diagnosis and treatment, they have overlapping features. As to treatment, both infections require long-term treatment with multiple antibiotics which is associated with drug toxicities, drug-drug interactions, patient incompliance and the emergence of drug resistance. There is a strong need to improve treatment efficacy of both infections which could include similar strategies.

NONTUBERCULOUS MYCOBACTERIA (NTM)

NTM are ubiquitous in the environment, mainly in water and soil. So far, over 140 different NTM species have been identified with large differences in pathogenicity (1). Although there is considerable geographic diversity in distribution of NTM species, *Mycobacterium avium* is the most frequently isolated species worldwide (2). The global incidence of NTM infections is rising and is even exceeding the prevalence of tuberculosis in developed countries including the Netherlands (3). NTM can cause severe disseminated infections in immunocompromised patients and the widespread use of immunosuppressive agents is a major contributor to the increasing NTM incidence (4). Another important disease manifestation is pulmonary infection in patients with underlying structural lung diseases such as chronic obstructive pulmonary disease and cystic fibrosis (5). Other disease manifestations include lymphadenitis (mainly cervical adenitis in children) and skin, soft tissue and bone infections. In addition, it is important to consider NTM in the context of health care related infections, especially postoperatively. As such, NTM infections should be part of the differential diagnosis in patients with persistent, refractory postoperative infections, particularly when related to cosmetic surgery. Early diagnosis is crucial to prevent long-term use of inappropriate antibiotics as well as too many unnecessary radiological and surgical procedures (6). Several immune defects **predispose** to NTM infections, such as advanced infection with the human immunodeficiency virus (HIV) (7), the use of immunosuppressive agents (8) as well as Mendelian susceptibility to mycobacterial disease (MSMD) (3). MSMD comprises a rare group of primary immunodeficiencies with a defective interleukin 12 - interferon gamma (IFN-γ) signaling pathway, which is crucial for appropriate mycobacterial defense. Most of these defects are associated with some degree of impairment in IFN-γ production or responsiveness. So far, mutations in 9 different genes have been identified including Signal Transducer

and Activator of Transcription 1 (*STAT1*) 1 (3). Besides increased susceptibility to NTM infections, these patients are also at risk for infections with certain other pathogens including *Salmonella* spp*, Histoplasma* spp*, Coccidioides* spp and some viruses (3). Additionally, mutations in *GATA2* as well as the acquisition of auto-antibodies against IFN-γ are associated with increased susceptibility to NTM infections (3). Recognition and identification of underlying defects in IFN-γ signaling is crucial as long-term, usually life-long treatment with anti-NTM agents is required and a subset of these patients benefit from additional immunotherapy. In general, treatment of NTM infections is a major clinical challenge involving a complex interplay between host and pathogen factors. In patients with immune disorders, NTM infections usually persist as the immune system is unable to assist in the clearance of infection (3). In patients with structural lung diseases, normal lung architecture is disrupted providing an optimal micro-environment for infection and persistence of NTM. Good penetration of anti-NTM agents into this compartment is difficult. With respect to the nontuberculous mycobacterial properties, the complex lipid architecture of the mycobacteria forms an effective permeability barrier limiting anti-NTM drug efficacy, in this way contributing to the intrinsic resistance of NTM to many antibiotics (9). Additional factors complicating effective treatment design are the lack of correlation between *in vitro* antibiotic susceptibility of NTM and clinical response (10) as well as the lack of scientific evidence supporting the preferred use of one anti-NTM drug regimen over another (8). In addition, proper species identification is crucial as treatment options differ considerably between NTM species. Current anti-NTM treatment is long-lasting containing multiple antibiotics, which is associated with drug-drug interactions, drug toxicities, the emergence of drug resistance and therapy failure. Although the introduction of macrolides as cornerstone agents in the treatment of the majority of NTM infections led to improved outcome, overall prognosis is still poor (5). This is especially the case for infections with *Mycobacterium abscessus,* which is one of the most difficult to treat species due to its multi-drug resistant character (2, 11). Particularly complicated is the treatment of *M. abscessus* subsp. *abscessus*, which is characterized by the presence of a functional erythromycin ribosomal methylase gene, *erm* (41), conferring inducible resistance to macrolides including clarithromycin, which negatively impacts treatment outcome (12)**.** Therefore, **novel treatment strategies** for NTM are needed aiming for the enhancement of drug activity as well as the prevention of emerging drug resistance.

MYCOBACTERIUM TUBERCULOSIS

Tuberculosis (TB) caused by *M. tuberculosis* (Mtb) is still a major global health problem with 9.6 million cases of active disease worldwide in 2014. Even nowadays TB is one of

the most fatal infectious diseases killing 1.5 million people each year (13). In contrast to NTM infections, TB spreads from person-to-person usually through inhalation of Mtb-infected aerosols and is therefore contagious. TB can occur both in immunocompetent and immunocompromised patients, this latter category being at increased risk (14). Globally, 12% of TB patients are co-infected with HIV underlining the importance of HIV testing upon TB diagnosis (13). While pulmonary infection is the main disease manifestation of TB, infection can occur in all organs, especially in immunocompromised patients (15, 16). Current anti-TB treatment is complex requiring at least 6 months of duration with multiple anti-TB agents. The treatment duration of multi-drug resistant TB (MDR-TB), comprising 3.3% of the new and 20% of the relapse cases, is even longer (around 20 months) and less effective with only 50% of patients treated successfully (13). MDR-TB is defined as resistance to isoniazid and rifampicin, the most powerful first-line anti-TB drugs. Approximately 10% of patients with MDR-TB have extensively drug-resistant TB (XDR-TB), defined as MDR-TB plus resistance to at least one of the fluoroquinolones and at least one of the second-line injectables. The treatment outcome of XDR-TB is even worse with reported success rates of a measly 25% (13). To end the global TB epidemic, a shorter treatment duration resulting in higher patient compliance is of utmost importance. Therefore, **novel potent treatment strategies** with increased sterilizing capacity are needed for improving cure rates and reducing the selection of anti-TB drug resistance and relapse of disease. Appropriate (preclinical) assessment of the activity of novel drugs and drug combinations is complex. Complicating factors are the alleged heterogeneity of the Mtb population at the infected sites representing Mtb with high and low metabolic activity as well as mycobacteria being present extracellularly and intracellularly inside macrophages (17). Anti-TB drugs differ with respect to their preferred activity against highly- or low metabolically-active Mtb as well as the difference in their capacity to penetrate infected target cells. The optimal anti-TB drug regimen contains a variety of different anti-TB agents with combined activity against the heterogeneous Mtb population. Many different preclinical models exist to assess the *in vitro* activity and therapeutic efficacy of anti-TB drugs, but it is yet unclear which (combination of) models are most predictive of clinical response during a full treatment course. This was recently illustrated by studies on the activity of moxifloxacin as anti-TB drug. The promising results from *in vitro* studies and animal models could not be translated into daily clinical practice as the expected shortening of anti-TB treatment duration in clinical trials was not found (18, 19). These observations make clear that **optimizing preclinical modeling** is crucial to achieve rapid and reliable identification of potential novel anti-TB drug regimens which can be translated into clinical practice. An important preclinical *in vitro* model that can be used to study the capacity of (novel) antimycobacterial drugs is the time-kill kinetics (TKK) assay (20). This assay provides unique information as to the concentration- and time-dependent mycobacterial killing capacity of antimycobacterial

agents alone and in combination as well as to the selection of drug resistance. As such, the TKK assay has been shown to detect differences in anti-TB drug activities that would not have been detected with the use of classical susceptibility assays, such as the Minimal Inhibitory Concentration (MIC) determining only mycobacterial growth inhibition (21). Therefore, the *in vitro* TKK assay is used in three studies of this thesis to investigate the antimycobacterial drug activity of different antibiotics alone and in combination.

THESIS OUTLINE

The studies described in this thesis are focused on three different topics, all related to improving antimycobacterial treatment success.

The first topic involves the **genetic predisposition to NTM infections (chapter 2)**. A novel autosomal dominant mutation in the SH2 domain of *STAT1* is described in a patient with disseminated *M. avium* infection. Additionally, the functional consequences of this novel mutation in terms of IFN-γ and interferon alpha (IFN-α)-related immune response are shown **(2.1)**.

The second topic involves different strategies to **increase antimycobacterial drug activity in NTM infections and TB (chapter 3).** The first study investigates the role of IFN-α treatment in patients with disseminated mycobacterial infections and describes the functional overlap between the different interferon pathways **(3.1).** The second study highlights a possible role of tigecycline as a novel agent in the treatment of *M. avium* infections **(3.2).** The third study concerns the role of colistin as membrane destabilizer, in this way increasing the mycobacterial cell wall permeability and the activity of currently used anti-TB drugs. In this study, highly- and low metabolically-active Mtb populations are involved **(3.3)**.

The third topic of this thesis concerns the importance of **optimization of preclinical anti-TB drug modeling (chapter 4).** Central theme is to underline the relevance of an *in vitro* assay investigating the concentration- and time-dependent killing activity of anti-TB agents over time instead of determination of mycobacterial growth inhibition as an endpoint (MIC). The role of the TKK assay as a component of a preclinical modeling framework predicting clinical anti-TB drug efficacy is discussed **(4.1)**.

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CHAPTER 2

Genetic predisposition to mycobacterial infections

CHAPTER 2.1

A novel STAT1 mutation associated with disseminated mycobacterial disease

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ABSTRACT

STAT1 is a key component of interferon (IFN)-γ and IFN-α signaling and mediates protection against mycobacterial, fungal and viral infections and against cancer. Dominant negative inhibitory as well as gain of function heterozygous STAT1 mutations demonstrate that IFN-γ driven cellular responses need to be tightly regulated to control infections. We describe an autosomal dominant mutation in the SH2 domain of STAT1 that disrupts protein phosphorylation, c.1961 T>A (M654K). The mutant allele does not permit STAT1 phosphorylation, and impairs STAT1 phosphorylation of the wild type allele. Protein dimerization is preserved but DNA binding activity, IFN-γ driven GAS-luciferase activity, and expression of IFN-γ target genes are reduced. IFN-α driven ISRE response, but not IFN-α driven GAS response, are preserved when cells are co-transfected with wild type and the mutant STAT1 constructs. M654K exerts a dominant negative effect on IFN-γ related immunity and is recessive for IFN-α induced immune function.

INTRODUCTION

Signal transducer and activator of transcription (STAT) family members are latent transcription factors sharing a similar structure, containing N-terminal, central DNA binding, carboxy-terminal SH2-containing, and transactivation domains (1). STAT1 is critical to both interferon (IFN)-γ and IFN-α signaling. Following IFN-γ binding to its receptor, STAT1 is phosphorylated resulting in homodimerization and nuclear translocation. Binding to specific DNA sequences known as gamma-activating sequences (GAS) in the promoters of IFN stimulated genes induces transcription (2). Binding of IFN-α to its receptor results in heterodimerization of phosphorylated STAT1 and STAT2 molecules followed by association with another signaling molecule, Interferon Regulatory Family-9 (IRF9), or p48. This heterotrimer then translocates to the nucleus, and binds to specific DNA sequences, the interferon stimulating response element (ISRE), and induces gene transcription (2). Although IFN-γ and IFN-α signaling pathways seem quite distinct, there is considerable overlap (3). Dominant negative mutations in *STAT1* cause increased susceptibility to weakly virulent intracellular pathogens, such as Bacillus Calmette-Guérin (BCG) and nontuberculous mycobacteria (NTM) due to impaired IFN-γ activity (4); patients with heterozygous mutations that are dominant negative for GAS activation and recessive for ISRE activation, mostly present with only mycobacterial disease and the clinical course of their infections is usually milder (5, 6). On the other hand, autosomal recessive *STAT1* mutations typically cause more profound defects in STAT1 and are therefore associated with impairment of both IFN-γ and IFN-α related immunity. The clinical picture of patients with recessive mutations is typically more severe and characterized by both viral and mycobacterial infections (7, 8). We report a novel autosomal dominant negative mutation in the SH2 domain of STAT1 in a patient who presented with disseminated mycobacterial infection.

METHODS

Blood cell isolation and mutational analysis

All blood samples were collected under NIAID IRB-approved protocol. The parents of the patient provided written informed consent for study participation. Blood of healthy volunteers was obtained through the NIH Blood Bank (Dept. of Transfusion Medicine, National Institutes of Health, Bethesda, MD) in accordance with an NIAID IRB-approved protocol of the National Institutes of Health. For sequencing, genomic DNA and total RNA were extracted from EBV-transformed B cell lines or polymorphonuclear leucocytes. Primers spanning exons and flanking splice sites of human *STAT1* and full-length cDNA were designed using Primer Select (DNAstar Lasergene). Genomic amplification was performed with Platinum PCR Supermix High Fidelity (Invitrogen). Sequencing

was performed with Big Dye Terminators v3.1 (Applied Biosystems, Foster City, CA), run on an Applied Biosystems 3730XL sequencer and aligned to the consensus sequence NM_007315.3 using Sequencer software (Gene Codes). The mutation in the STAT1 coding sequence was created using a STAT1 expression vector (OriGene Technologies, Rockville, MD) as template (BioInnovatise Inc., Rockville, MD). STAT1-Myc tag or GFP-tagged constructs were created from the untagged STAT1 expression vector (BioInnovatise). STAT1-FLAG tag (Addgene plasmid 8691) was purchased from Addgene, Cambridge, MA (deposited by Dr. Jim Darnell) (9). Plasmids encoding wild type (WT) STAT1 and the mutant constructs were isolated using the QIAprep maxiprep kit (QIAGEN) according to the manufacturer's recommendations; all mutations were verified by sequencing.

Cell lines

EBV-transformed B cell lines derived from patients and normal donors were maintained in RPMI 1640 with 20% fetal calf serum (FCS; Gibco BRL, Carlsbad, CA), 2mM L-glutamine, penicillin 100U/ml, 100µg/ml streptomycin (Gibco), at 37°C in a humidified 5% CO2 incubator. STAT1 deficient U3A cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FCS, 2mM L-glutamine and antibiotics. Transient transfection of U3A cells was done using the Amaxa nucleofector (Lonza, Walkersville, MD). Culture media were replaced 24 h post-transfection and cells were either left untreated or stimulated with IFNs as indicated.

Flow cytometry

To assay STAT1 activation, EBV transformed B cells or transfected U3A cells (Amaxa nucleofector; Lonza, Walkersville, MD) were stimulated with IFN-γ (R&D System, Minneapolis, MN) 400 IU/ml or IFN-α (IFN-α2b, PBL Biomedical Laboratories, Piscataway, NJ) 1000 IU/ml for 15 min, when cells were recovered, fixed and permeabilized in methanol. Cells were stained for total (Alexa647 conjugated anti-STAT1) and phosphorylated tyrosine Y701 STAT1 (Alexa488 conjugated anti-pSTAT1; BD Biosciences). For U3A cells, the levels of phosphorylation were assessed in the cells gated for the expression of total STAT1. Data were collected using FACS Caliber (BD Biosciences) and analyzed using FlowJo (Treestar).

Immunoprecipitation and immunoblotting

For Western blot analysis (WB), EBV-B or transfected U3A cells, stimulated as described above, were lysed in buffer containing protease and phosphatase inhibitors (Calbiochem, Gibbstown, NJ). Samples were sonicated, equal amounts of proteins were run on a 10% SDS polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Invitrogen). After blocking, the membranes were incubated with the primary antibody, anti-pSTAT1 Tyr701 (Cell Signaling Technology, Danvers, MA) or anti-pSTAT2 Tyr690 (Abcam, Cambridge, MA), as indicated. Membranes were washed, incu-

bated in the horseradish peroxidase-conjugated secondary antibody and signal detected using an enhanced chemiluminescence system (ECL; Amersham Biosciences, Piscataway, NJ). Blots were stripped and re-probed with anti-total STAT1 (Cell Signaling) or STAT2 (Millipore, Billerica, MA) antibodies, respectively, to assess protein loading. For detection of dimerization, STAT1 co-immunoprecipitation (IP) was evaluated using U3A cells co-transfected with FLAG- or myc-tagged WT or mutant STAT1 constructs and stimulated or not with IFN-γ (400 IU/ml, 30 min). Cell lysates were then precipitated with anti-myc or anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO) followed by protein G-Sepharose binding and immunoblotting. Blots were probed with anti-FLAG, anti-Myc or anti-STAT1 antibodies. To evaluate STAT1/STAT2 association, transfected U3A cells (WT and M654K STAT1) were stimulated with IFN-α (1000 IU/ml, 30 min) and IP:WB STAT1:STAT2.

Confocal microscopy

U3A cells were seeded onto coverslips in the 12-well plates (Costar), followed by transfection of plasmids encoding WT STAT1 and/or its mutant M654K with lipofectamine (Invitrogen). The following day, culture media was replaced and cells were either untreated or treated with IFN-γ (400 IU/ml, 15 min). Cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% (w/v) Triton X-100 in PBS. Coverslips were incubated with the mouse anti-human STAT1 (BD biosciences), followed by a secondary staining with goat anti-mouse IgG conjugated to Alexa Flour-568. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Co-localization studies were done in a Leica SP5 confocal microscope (Leica Microsystems, Exton, PA) using a 63×-oil immersion objective NA 1.4. The images were collected sequentially and the data were analyzed using Leica software. For data presentation, the images were assembled in Adobe Photoshop CS3.

Nuclear extracts and nuclear complex binding

Nuclear extracts from transfected U3A cells stimulated or not with IFN-γ (400 IU/ml) or IFN-α (1000 IU/ml), were prepared using the Panomics kit (Panomics, Fremont, CA). For determination of DNA binding activity, an ELISA-like colorimetric assay (TRANSAM, Active Motif, Carlsbad, CA) using a plate coated with a STAT1 binding oligonucleotide derived from the GAS sequence, was used according to the manufacturer's protocol. Absorbance was measured on a spectrophotometer at 450nm.

Reporter gene assay

U3A cells were transiently transfected with WT and/or mutant STAT1 expression constructs and a plasmid containing tandem IFN-response elements driving a luciferase reporter gene (1μg; HSV-thymidine kinase promoter; Panomics, Fremont, CA). A Renilla expression vector (0.03 μg/ml) was co-transfected to serve as an internal control for transfection efficacy. Following overnight incubation, media were replaced and cells

were stimulated or not with IFN-γ or IFN-α (1000 IU/ml) for 6 h. Cells were resuspended in lysis buffer and luciferase activity was evaluated using a dual luciferase assay (Promega, Madison, WI). Relative luciferase units were normalized to Renilla activity. Data are expressed as fold increase in response to IFN over the non-stimulated samples.

Real time PCR

Total RNA was extracted from cultured cells with the RNeasy mini kit (QIAGEN). For RT-PCR, 1 µg of total RNA was reverse transcribed and the resulting cDNA amplified by PCR using the ABI 7500 Sequencer using Taqman expression assays (Applied Biosystems). GAPDH was used as normalization control. The data were analyzed using the 2-ΔΔCT method.

Statistical analysis

Results are reported as mean ± standard deviation (SD) unless otherwise stated. Differences between groups were assessed by the unpaired two-tailed Student's *t*-test using GraphPad Prism Software (San Diego, CA). The statistical significance level adopted was $p < 0.05$.

RESULTS

Case description and mutation

The patient was a 5-year-old boy, born to unrelated parents, who first presented at age 1 week with recurrent pneumonia. At 1.5 years, he presented with cervical lymphadenitis, fever and respiratory complaints. Computed tomography (CT) of the chest indicated massive mediastinal lymphadenopathy. *Mycobacterium avium* complex (MAC) was isolated from the cultures of lymph nodes, lung and bone marrow. Disseminated MAC infection was treated with ethambutol, azithromycin, rifampin, ciprofloxacin and amikacin. Amikacin was subsequently discontinued due to ototoxicity. IFN-γ was added by subcutaneous injection three times weekly. At 2.5 years he was referred to the National Institutes of Health (NIH) for further evaluation. His CT scan showed right bronchial mucus plugging with atelectasis that improved on subsequent imaging; there was no significant lymphadenopathy. Repeated mycobacterial blood cultures were negative. In addition to IFN-γ injections, azithromycin, rifampin, and ciprofloxacin were continued. Ethambutol was discontinued due to concern for optic neuritis. He has remained well. He has been hospitalized once for an asthma exacerbation and received additional antibiotics for bacterial pharyngitis and otitis media. He has had typical childhood viral infections without complications. He had normal lymphocyte numbers and quantitative immunoglobulins.

Full length sequencing of *STAT1* genomic and cDNA identified a heterozygous mutation c.1961 T>A in the SH2 domain, resulting in methionine to lysine at position 654 (M654K) (Figure 1). His parents were mutation free.

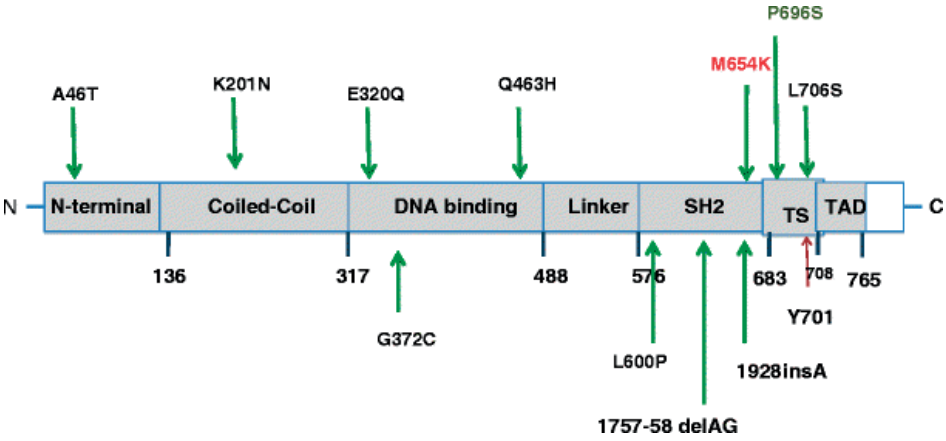
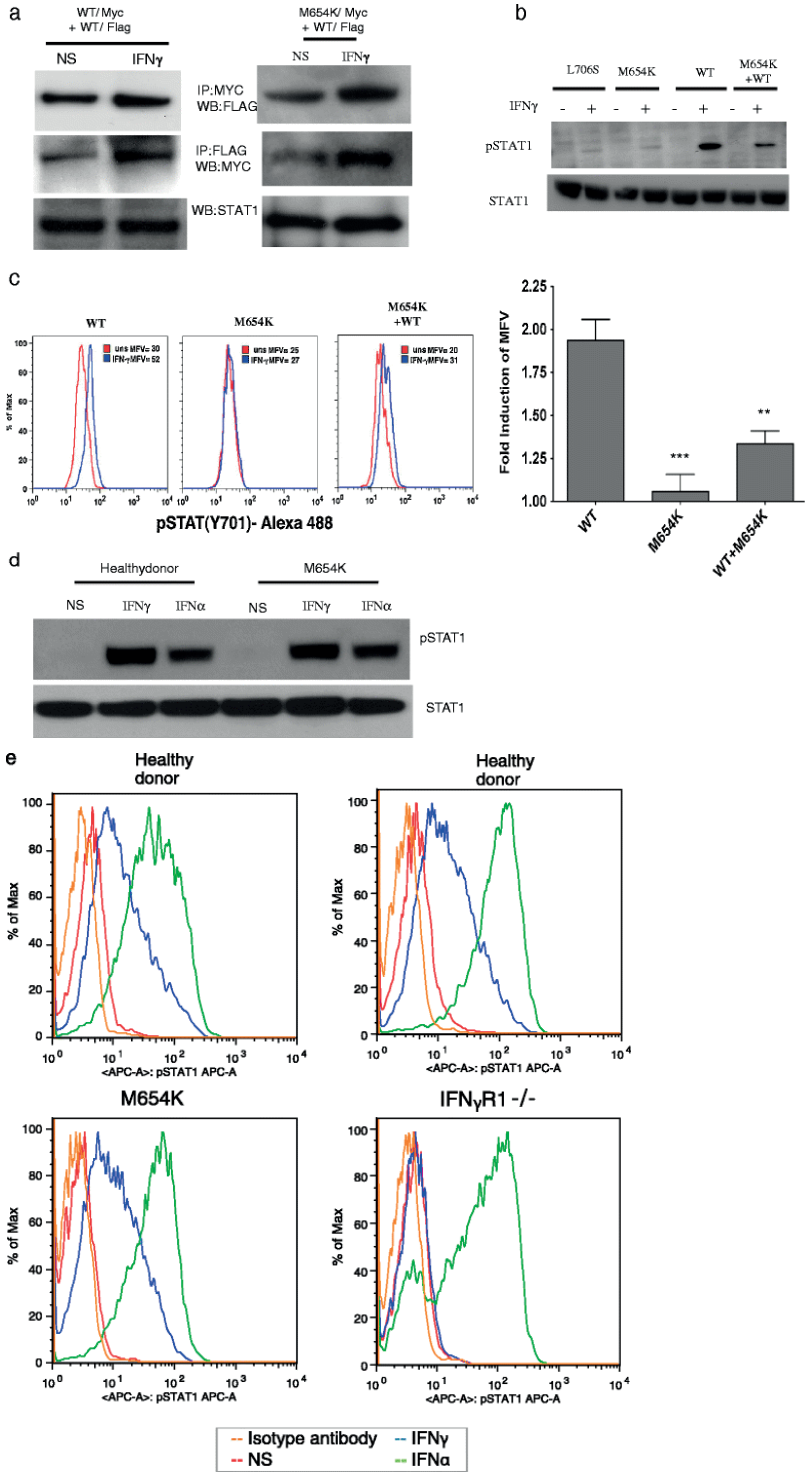


Figure 1. *STAT1* dominant negative and loss of function mutations. The N-terminal domain, coiled-coil domain, DNA binding domain, linker domain, SH2 domain, tail segment domain (TS), and transactivation domain (TAD) are represented; Y701, site of tyrosine phosphorylation. The dominant negative mutants are indicated above the protein and all recessive or loss of function mutations are indicated below the protein. M654K is show in red.

STAT1 phosphorylation and DNA binding

To investigate the ability of the M654K mutant to dimerize with WT STAT1 protein, U3A cells were co-transfected with Myc- and FLAG-tagged constructs followed by co-immunoprecipitation and immunoblotting. Cells stimulated or not with IFN-γ showed normal dimerization of the mutant protein to WT STAT1 (Figure 2a).

Activation of STAT1 was assayed in STAT1 deficient U3A cells. When transfected alone into the cells, M654K STAT1 was not phosphorylated (Tyr701) following stimulation with IFN-γ whereas WT STAT1 was (Figure 2b). The previously described STAT1 mutation L706S STAT1, which disrupts tyrosine phosphorylation and predisposes to mycobacterial disease [6], also inhibits STAT1 phosphorylation. Co-transfection of M654K and WT STAT1 constructs partially restored IFN-γ responsiveness, consistent with this dominant mutant's inhibition of WT STAT1 activity. These results were confirmed by flow cytometry in the transfected cells (Figure 2c). Experiments using EBV-B cells, immunoblotting (Figure 2d) and flow cytometry (Figure 2e), showed STAT1 phosphorylation (Y701) after stimulation with IFN-γ in the heterozygous patient cells (M654K; 27.2% positive cells) vs. healthy control cells (34.6%) as opposed to one patient with homozygous complete IFNGR1 deficiency (1.06%) (Figure 2e), who had disseminated mycobacterial disease (10). Activation of STAT1 in response to IFN-α occurred to similar extents in each group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IFN-γMFVs 52
uns MFVs 25
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% of Max
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M654K
WT+M654K
d
Healthydonor
M654K
NS
IFNγ
IFNα
NS
IFNγ
IFNα
pSTAT1
STAT1
e
Healthy
donor
Healthy
donor
% of Max
<APC-A>: pSTAT1 APC-A
M654K
IFNγR1 -/-
Isotype antibody
NS
IFNγ
IFNα

← **Figure 2.** Dimerization and activation of STAT1 protein. **a** For evaluation of STAT1 dimerization, U3A cells were transfected with WT and patient M654K STAT1 constructs tagged with either Myc or Flag. Proteins obtained from non-stimulated (NS) or IFN-γ stimulated (400 IU/ml) cells were co-precipitated with antibodies against Myc or Flag and blotted for the detection of Myc or Flag. Blots were stripped and reprobed with anti-total STAT1 antibody; **b** For Western blot analysis, whole cell lysates obtained from transfected U3A cells stimulated or not (NS) with IFN-γ were blotted with anti-phosphorylated STAT1 (pSTAT1). Blots were stripped and reprobed with anti-STAT1 antibody; **c** Evaluation of U3A cells by flow cytometry confirmed the absent phosphorylation in the M654K transfected cells in response to IFN-γ. Lower phosphorylation is sustained in the co-transfected cells (M654K+WT). Histograms from one representative experiment is presented (right panel). The bars represent mean fold change (±SEM) in mean fluorescence value (MFV) observed in the stimulated cells relative to the unstimulated controls (uns) (n=3). **p<0.01; ***p<0.001 when compared to WT; **d** EBV transformed B cells obtained from healthy controls and from the STAT1 deficient patient (M654K) were stimulated with IFN-γ or IFN-α. Lysates were blotted with anti-phospho STAT1. Blots were treated as described above and are representative of three individual experiments. NS=non stimulated; **e** Flow cytometry assayed in EBV-B cells from patients (M654K STAT1 deficient and one patient with complete IFNGR1 deficiency) and two healthy donors. Histograms are included for each experimental condition (non-stimulated, NS; IFN-γ 400 IU/ml; IFN-α 1000 IU/ml). One representative experiment out of three is presented.

(82.9%, 81.2% and 70.2%, respectively). STAT2 tyrosine (Tyr690) phosphorylation was preserved in patient cells (not shown). Interestingly, IFN-γ induced nuclear translocation in the transfected cells was reduced when using the M654K construct alone, and it was detectable in co-transfection with WT STAT1 (Figure 3).

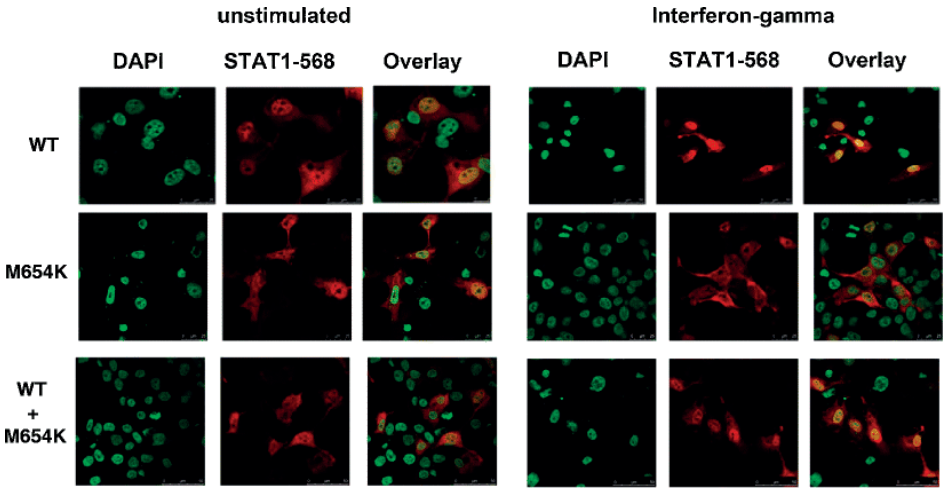

Figure 3. Evaluation of nuclear translocation. Nuclear translocation of the mutant STAT1 proteins was assessed in U3A cells transfected with WT STAT1 or M654K mutant, stimulated or not with IFN-γ (400 IU/ml, 15 min). The distribution of STAT1 in the nucleus was identified by primary staining against total STAT1 followed by a secondary staining with Alexa Fluor 568, together with the nuclear DAPI. Following stimulation, WT and WT+M654K STAT1 but not the M654K alone were detected at the nucleus as shown in the overlay image.

DNA binding activity of M654K STAT1 to the GAS oligonucleotide was determined in lysates of U3A cells transfected with the M654K construct and stimulated with IFN-γ or IFN-α. M654K showed markedly reduced GAS binding compared to WT (Figure 4a). The dominant negative effect of M654K was confirmed following co-transfection with WT STAT1, which sustained the reduced DNA binding in response to IFN-γ and IFN-α stimulation.

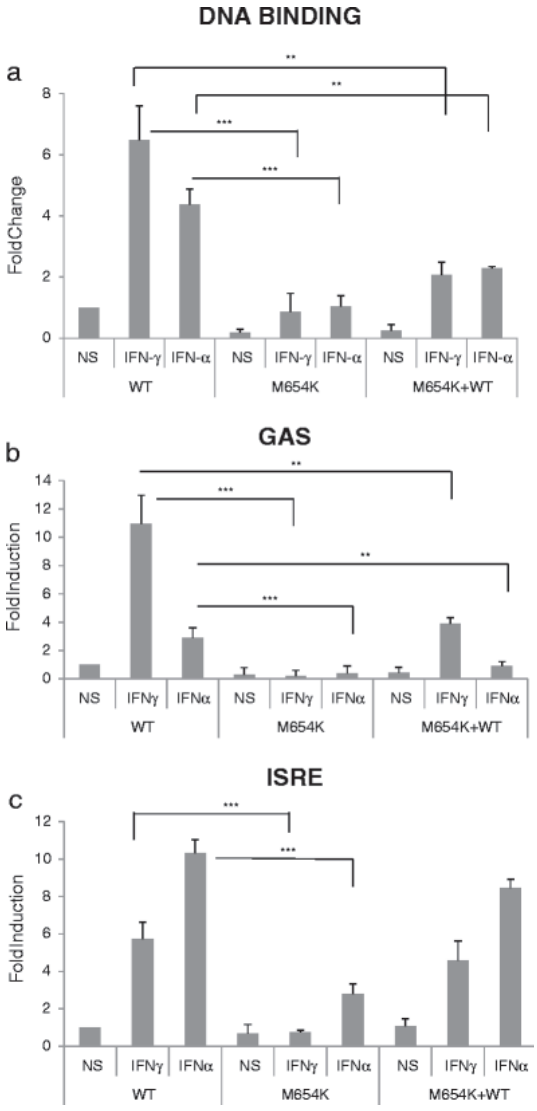

Figure 4. Effect of STAT1 mutation on DNA binding and transcriptional activity. **a** STAT1 DNA-binding activity was assayed in nuclear extracts isolated from U3A cells transfected with STAT1 WT, STAT1 mutant (M654K) or STAT1 mutant co-transfected with WT (M654K+STAT1) and stimulated or not with IFN-γ or IFN-α 1000U/ml (30 min). Data are mean fold increase (± SD) detected in the stimulated cultures relative to the WT non-stimulated (NS) from a total of five experiments; **b** STAT1 expression vectors (STAT1 WT, mutant M654K, M654K+WT) were transiently co-infected with luciferase GAS reporter plasmids or **c** with luciferase ISRE reporter plasmids into U3A cells. Following stimulation with IFN-γ or IFN-α, cells were harvested and assayed using the dual luciferase reporter assay. Experiments were done in triplicate and results are mean fold increase (± SD) in the stimulated cells relative to the WT non-stimulated (NS) (n=4). $^{**}p<0.01$; $^{***}p<0.001$ when compared to WT.

Luciferase activity and gene induction

The transactivation activity of M654K STAT1 was evaluated in U3A cells co-transfected with WT and mutant constructs using GAS and ISRE response elements. In agreement with its diminished ability to bind DNA, the M654K construct alone showed no transactivation activity on GAS reporter constructs after stimulation with IFN-γ or IFN-α (Figure 4b). The ISRE response was induced about 3-fold, indicating more preserved IFN-α than IFN-γ responsiveness (Figure 4c). When WT and M654K constructs were co-transfected, normal GAS-driven luciferase activity was diminished, whereas ISRE driven luciferase activity to IFN-α and IFN-γ was essentially normal. Accordingly, experiments exploring STAT1/STAT2 association performed in transfected U3A cells showed preserved association when comparing WT and M654K STAT1 constructs (Figure 5).

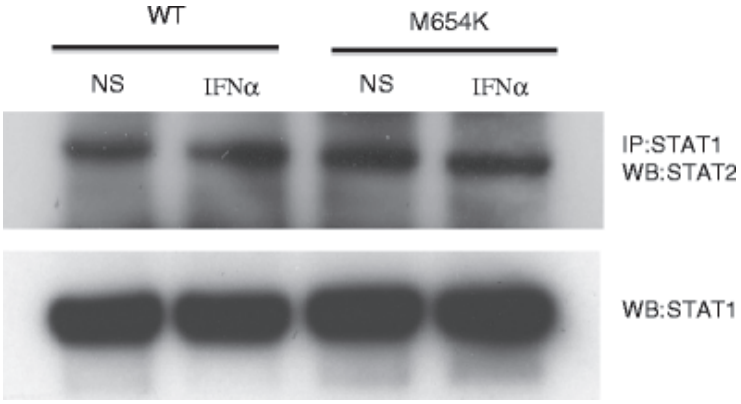

Figure 5. For evaluation of STAT1/STAT2 association, U3A cells were transfected with WT STAT1 or M654K mutant constructs, left non-stimulated (NS) or stimulated with IFN-α (1000 IU/ml) for 30 min when cell lysates were prepared and co-precipitated with anti-STAT1 antibody and blotted for STAT2. Blots were stripped and reprobed with anti-total STAT1. One representative experiment out of two is presented.

In agreement with these data, mRNA expression of typical IFN-γ target genes (*CXCL-9 and CXCL-10*), evaluated by qRT-PCR, was reduced in U3A cells transfected with the mutant construct alone or when co-transfected with the WT STAT1 and stimulated with IFN-γ (400 IU/ml) or IFN-α (1000 IU/ml) for 3 h (Figure 6a). On the other hand, the expression of the typical IFN-α induced genes (*MX-1* and *OAS-2*) was sustained in the heterozygous cells (Figure 6b).

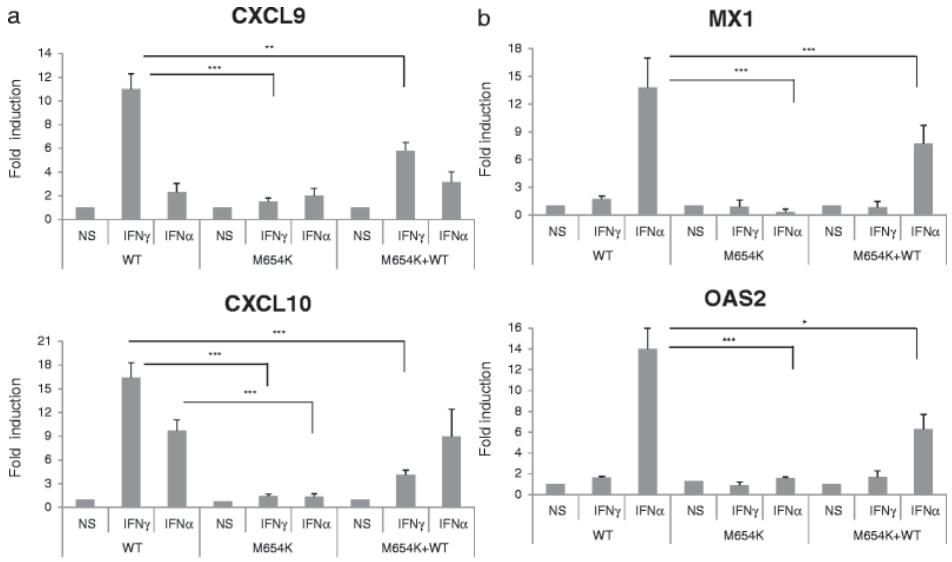

Figure 6. Analysis of gene expression. U3A cells co-transfected with WT and/or patient construct (M654K) were stimulated or not with IFN-γ or IFN-α for 3 h. Expression of IFN target genes, **a** *CXCL9, CXCL10*, **b** *MX1, OAS2* was evaluated in the transfected cells by real time PCR. Results are expressed as mean fold induction (± SD) of four individual experiments. **p* <0.05; ***p* < 0.01; ****p* < 0.001 when compared to WT.

DISCUSSION

We report a patient with a novel heterozygous mutation in the SH2 domain (M654K) of STAT1 associated with disseminated MAC infection. Transfection of U3A cells with the mutant construct alone showed no STAT1 phosphorylation in response to IFN-γ but preserved response to IFN-α and preserved STAT1:STAT2 association. In co-transfected U3A cells (WT + mutant construct M654K), IFN-γ induced STAT1 phosphorylation and activation was partially affected, despite sustained capacity for protein dimerization and normal response to IFN-α. This phenotype is easily explained since STAT1 homodimers are formed in response to both IFN-γ and IFN-α (2). Around 25% of STAT1 dimers formed in response to IFN-γ stimulation are dimers between WT molecules. Therefore it is not surprising that some nuclear translocation and also other functional effects can be observed following stimulation. In addition, 50% of the dimers formed between STAT1 and STAT2 in response to IFN-α are dimers containing WT-STAT1. These data reinforce the dominant negative effect of the mutation on IFN-γ / GAS related immunity. Similarly, the ISRE driven luciferase activity following IFN-α was compromised in U3A cells transfected with the mutant construct alone. Co-transfection of mutant and WT constructs resulted in recovery of the IFN-α response.
The SH2 domain of STAT1 is required for the recruitment of latent STAT1 molecules to the activated interferon receptors (11). In addition, the domain is thought to be crucial

for dimerization of activated STAT1 molecules via reciprocal interactions between SH2 domains and phosphorylated Tyr701 residues (1). Following cell activation and nuclear translocation, STAT1 dimers trigger high affinity DNA binding and gene transcription (1). Accordingly, mutations in the SH2 domain of STAT1 have been associated with compromised STAT dimerization and nuclear translocation *in vitro* (11). Moreover, mutations affecting the SH2 domain of STAT3 have been reported to diminish tyrosine phosphorylation (12), confirming that this domain plays a crucial role in the activation of STATs.
STAT1 mutations can lead to a wide range of clinical manifestations (4, 13). Recently, dominant gain of function mutations in the coiled coil domain of *STAT1* have been described as causing susceptibility to chronic mucocutaneous candidiasis (14, 15). On the other hand, the previously identified dominant negative or loss of function mutations comprise 10 mutations in *STAT1* reported in 13 patients, involving the tail segment (5), DNA binding domain (6), the coiled coil domain (16), and the N-terminal region (17). Another mutation is located between the SH2 domain and tail segment (18). The three previously reported recessive mutations in the SH2 domain, 1758_1759delAG, L600P, 1928insA (7, 8), and a recently described *STAT1* splicing mutation (19) were all associated with severe impairment of GAS and ISRE responses. In line with these observations, the clinical picture of those patients was characterized by mycobacterial as well as viral infections. Interestingly, one of the patients was able to clear the attenuated polio type II virus vaccine that was given at 2 months of age as well as a parainfluenza II and rhinovirus infections (8).
We describe here the first autosomal dominant mutation in the SH2 domain of STAT1 associated with a dominant negative effect on IFN-γ related immunity but clinically adequate IFN-α induced immune function. The M654K mutation led to relatively mild mycobacterial infection responsive to antimycobacterial therapy with IFN-γ supplementation. The patient cleared his usual childhood viral infections, including influenza A, which all ran benign clinical courses.

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CHAPTER 3

Increasing antimycobacterial drug activity

CHAPTER 3.1

Interferon alpha treatment of patients with impaired interferon gamma signaling

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ABSTRACT

Patients with deficiency in the interferon gamma receptor (IFN-γR) are unable to respond properly to IFN-γ and develop severe infections with nontuberculous mycobacteria (NTM). IFN-γ and IFN-α are known to signal through STAT1 and activate many downstream effector genes in common. Therefore, we added IFN-α for treatment of patients with disseminated mycobacterial disease in an effort to complement their IFN-γ signaling defect. We treated four patients with IFN-γR deficiency with adjunctive IFN-α therapy in addition to best available antimicrobial therapy, with or without IFN-γ, depending on the defect. During IFN-α treatment, *ex vivo* induction of IFN target genes was detected. In addition, IFN-α driven gene expression in patients' cells and mycobacteria induced cytokine response were observed *in vitro*. Clinical responses varied in these patients. IFN-α therapy was associated with either improvement or stabilization of disease. In no case was disease exacerbated. In patients with profoundly impaired IFN-γ signaling who have refractory infections, IFN-α may have adjunctive antimycobacterial effects.

INTRODUCTION

Type I (IFN-α/β) and type II interferons (IFN-γ) are important immunomodulatory cytokines classically associated with protection against viruses and intracellular pathogens, respectively. In patients with defects in IFN-γ signaling, *M. tuberculosis* (Mtb), environmental nontuberculous mycobacteria (NTM), Bacillus Calmette-Guérin (BCG), dimorphic yeasts and *Salmonella* spp can cause infections which are typically extensive and can be fatal (1, 2).

Although the signaling pathways and the immunological functions of type I and type II interferons are thought to be somewhat distinct, they overlap through the common use of Janus kinase (JAK) 2 and Signal Transducer and Activator of Transcription (STAT) 1. Binding of IFN-γ to its specific receptors (IFN-γR1 and IFNγR2) activates JAK1 and JAK2, leading to the phosphorylation of STAT1, the formation of active STAT1 homodimers, and the induction of IFN-γ target genes. Similarly, IFN-α/β bind to their shared receptors (IFNAR1 and IFNAR2) leading to activation of JAK1 and tyrosine kinase (Tyk) 2, the phosphorylation of STAT1 and STAT2, and the formation of STAT1 homodimers and STAT1 / STAT2 heterodimers which activate both common IFN-γ and IFN-α target genes, respectively (3). Interferon regulatory factor (IRF) 1 is important in regulating immune responses and is commonly induced by type I and II interferons. The chemokines CXCL9 (monokine induced by interferon-gamma or MIG), CXCL10 (interferon-inducible protein-10, IP-10) and CXCL11 (interferon-inducible T cell alpha-chemoattractant, I-TAC) are structurally and functionally related molecules. CXCL10 and CXCL11 are induced by IFN-α/β as well as IFN-γ, whereas CXCL9 induction is mostly restricted to IFN-γ (4). These chemokines are best known for their roles in leucocyte trafficking, mainly on activated CD4+ Th1 cells, CD8+ T cells and NK cells. The enzyme indoleamine 2,3-dioxygenase (IDO) catabolizes the essential amino acid tryptophan and is induced by interferons. It plays a role in inhibiting replication of pathogens and also has immunoregulatory functions (5, 6).

IFN-γ is approved for prophylaxis in chronic granulomatous disease (CGD), osteopetrosis (7, 8), and has been used in patients with refractory mycobacterial diseases (1). IFN-α is approved for viral infections such as hepatitis (9), cystic hygroma (10) and chronic myelogenous leukemia (11). IFNs modulate the production of inflammatory cytokines, such as TNF-α, which has antimicrobial properties. The importance of this pathway is evidenced by anti-TNF therapies, which increase susceptibility to mycobacterial infections, such as Mtb, *M. abscessus, M. avium*, *M. leprae* and intracellular fungi (12-16). The IFN pathway also impacts on the IL-1 response (17, 18), and mice deficient in IL-1 succumb to Mtb infection (19, 20).

We describe four patients with mutations in the IFN-γ receptor whose disseminated mycobacterial infections were refractory to best available therapy. Adjunctive treat-

ment with IFN-α was associated with variable clinical responses, some of which were extremely beneficial. Moreover, none had major toxicities or increased mycobacterial burdens while on IFN-α. Gene expression *in vitro* and *ex vivo* showed activation of both typical IFN-α and IFN-γ inducible genes in response to IFN-α, as well as the sustained production of mycobacterium-induced TNF-α and IL-1β *in vitro*. IFN-α may be able to overcome some aspects of impaired IFN-γ signaling and to confer clinical benefits to a selected group of patients.

MATERIALS AND METHODS

Subjects

All patients (Table 1) were followed and treated at the National Institutes of Health, NIH. Patients or their guardians provided informed consent on approved protocols of the National Institutes of Health. Whole blood was obtained from patients before and after IFN-α administration. Blood from healthy volunteers and elutriated monocytes were obtained under appropriate protocols through the Department of Transfusion Medicine, NIH. Alveolar macrophages (AM) were isolated from bronchoalveolar lavage fluid obtained from normal donors on NIAID IRB approved protocols.

Cell culture and stimulation

Peripheral blood mononuclear cells (PBMCs) obtained from whole blood by gradient density centrifugation (BioWhittaker, Walkersville, MD) and elutriated monocytes were plated in ($3x10^6$ cells/well) in RPMI 1640 with 5% human AB serum, (Gibco BRL), 2mM L-glutamine, penicillin 100U/ml, 100µg/ml streptomycin, at 37°C. For monocyte differentiation, cells were kept in culture for 6-7 days, and allowed to differentiate into monocyte-derived macrophages (MDM). For AM, bronchoalveolar lavage fluid was immediately cooled (4°C) and filtered through a cell strainer to remove particulate debris before centrifugation. Cells were counted and plated for stimulation as above. PBMCs were either left unstimulated or stimulated with human IFN- 400 IU/ml (R&D System, Minneapolis, MN) or IFN-α2b 1000 IU/ml (PBL Biomedical Laboratories, Piscataway, NJ). MDM and AM cultured in supplemented media without antibiotics were stimulated with live mycobacteria (*M. avium*, ATCC 35717) at a multiplicity of infection (MOI) of 5, in the presence or absence of IFN-γ or IFN-α for 3 h (for evaluation of gene expression) or 20 h (for detection of cytokine release). Supernatants were recovered and frozen at -20°C until use. *Ex vivo* evaluation of gene expression was assayed in cells (PBMCs) obtained from patients before and after (14-20 h) IFN-α injection and left unstimulated.

Cytokine determination

Culture supernatants were further analyzed for cytokine levels using a custom bead based cytokine assay for IL-1β, TNF-α and of the chemokine CXCL10/IP-10 (Bio-Plex assay, BioRad, Hercules, CA), processed according to the manufacturer's specifications.

Real time PCR

Total RNA was extracted from isolated cells with the RNeasy mini kit (QIAGEN). For RT-PCR, 1 μg of total RNA was reverse transcribed (Invitrogen) and the resulting cDNA amplified by PCR using the ABI 7500 Sequencer and Taqman expression assays (Applied Biosystems). GAPDH was used as a control for normalization.
Data were analyzed using the $2^{-\Delta\Delta CT}$ method and results expressed as mean fold induction.

Statistical analysis

Results are presented as mean ± standard deviation (SD). Statistical comparisons were made using Student's *t*-test (GraphPad Prism Software, San Diego, CA). The statistical significance level adopted was $p < 0.05$.

RESULTS

Patient 1 is a 22-year-old Caucasian woman born in the US to unrelated parents. Flow cytometry identified absent expression of IFN-γR1 on monocytes and lack of STAT1 phosphorylation in response to IFN-γ *in vitro* (not shown). She is compound heterozygous for IFNGR1 mutations: paternal allele, 373(+1)g→t in intron 3 resulting in complete deletion of exon 3; maternal allele, 201(-1)g→c in intron 2 generating a cryptic splice site in the middle of exon 3 causing an in frame deletion of 34 aa. She presented at 1 year with disseminated *Mycobacterium avium* complex (MAC) involving bone, bone marrow, lung, lymph nodes and liver. While on antimycobacterial therapy, she had recurrent lymphadenopathy and osteomyelitis growing MAC, *M. kansasii* and *M. fortuitum*. At age 17, persistent pulmonary and mediastinal MAC (Figure 1a) led to referral to the NIH and IFN-α2b, 3 million units three times weekly subcutaneously, was added. After three months on IFN-α, chest CT showed marked reduction of pulmonary disease, she reported increased energy, her temperature normalized, and after one year her lesions had resolved completely (Figure 1b). Two years later she presented with abdominal pain and increased retroperitoneal lymphadenopathy due to *M. abscessus*. IFN-α was stopped and antimycobacterial regimen included carbapenems and tigecycline resulting in clinical improvement.

Table 1. Patient characteristics

Patient	Age	Sex	Immunodeficiency	Mutation	Country of origin	Age at disease onset	Isolated mycobacterial strains	Disease localization	Interferon treatment
1	22	Female	Complete IFNγR1 deficiency	Compound heterozygous 373(+1)g →t 202(-1)g →c	US	1 yr	MAC *M. fortuitum* *M. kansasii* *M. abscessus*	Bone Bone marrow Liver Lung Lymph nodes	IFNα2b
2	19	Male	Complete IFNγR1 deficiency	Homozygous 22delC	Pakistan	2 yr	MAC *M. abscessus*	Blood Liver Lung Lymph nodes Skin	IFNα2b PEG IFNα
3	5	Male	Partial IFNγR1 deficiency	Homozygous I87T	Chile	2 mo	*M. bovis* BCG	Bone Brain Lung Lymph nodes Pleura Skin	IFNα2b & IFNγ
4	52	Female	Partial IFNγR1 deficiency	Heterozygous 818del4	US	20 yr	MAC *M. fortuitum* *M. avium* X-cluster	Maxillary Sinus Skin Palate Lymph nodes	IFNα2b & IFNγ

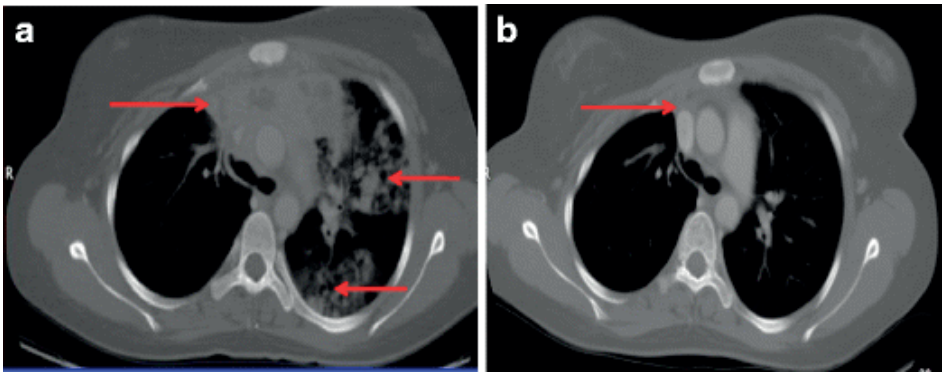

Figure 1. Patient 1. **a** CT chest showing necrotizing mediastinal lymphadenitis and extensive parenchymal disease due to refractory MAC infection; **b** After 1 year of treatment with IFN-α, cervical, mediastinal and parenchymal disease had resolved completely.

Patient 2 was a 19-year-old Pakistani male living in Norway whose cells had absent IFN-γR1 surface expression and lack of responsiveness to IFN-γ *in vitro*. He had homozygous IFNGR1 22delC leading to a frameshift and premature stop codon. He first presented to NIH at 4 years with a history of disseminated MAC (21). At 13 years new submandibular and axillary lymphadenopathy grew *M. abscessus,* which was persistent and disseminated despite antimycobacterial therapy. After two years, with failure to thrive, weight loss and lymphadenopathy, linezolid was changed to tigecycline and IFN-α2b (3 million units three times weekly subcutaneously) was added. At age 18 he was re-admitted with increasing hepatomegaly and deteriorating liver function tests. Meropenem was introduced and short acting changed to pegylated IFN-α. Blood cultures on pegylated IFN were reduced to <1 colony/ml *M. abscessus*. Three months later, new liver and lung lesions proved to be B-cell lymphoma; biopsies showed no mycobacteria. He died due to disseminated B-cell lymphoma at 20 years. No autopsy was performed.

Patient 3 is a 7-year-old Chilean male who received BCG immunization at birth. At age two months he presented with hydrocephalus and cerebral lesions that grew *M. bovis* BCG with involvement of bone, lung and lymph nodes. At 2 years while on antimycobacterial therapy, partial IFN-γR1 deficiency due to homozygous I87T (22) was diagnosed. Two months of IFN-γ 150μg/m^2 subcutaneously daily led to worsening hydrocephalus and fever. He was referred to the NIH at 3 years with severe failure to thrive, persistent hydrocephalus, intra-cerebral lesions (Figure 2a.1), a draining head wound at the site of brain biopsy (Figure 2a.2) and a large right pleural mass (Figure 2b). The pleural mass biopsy grew multidrug resistant *M. bovis*. IFN-γ was re-started (150μg/m^2 subcutaneously) three times weekly. His antimycobacterial regimen was extended to levofloxacin, linezolid, clofazimine, azithromycin and capreomycin. After three months, he remained ill and IFN-α2b, 0.1 million units subcutaneously three times weekly, was alternated with IFN-γ. On this

regimen, he started gaining weight, his head wound healed (Figure 2a.3, after 8 months on IFN-α therapy; Figure 2a.4, after 21 months), his pleural mass decreased (Figure 2b,2c) and laboratory parameters slowly improved. He is now well without serious hospitalizations for over 3 years. His therapy has been reduced to azithromycin and IFN-γ alone.

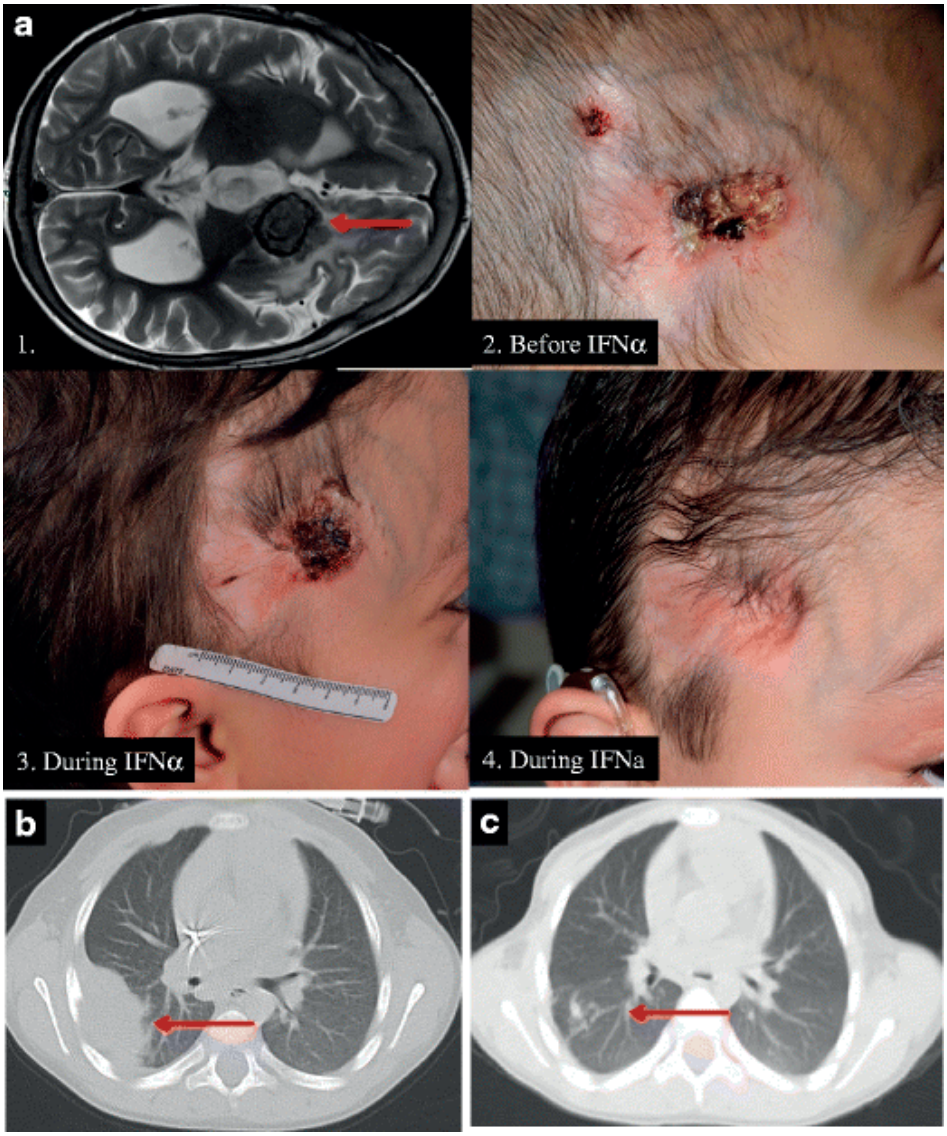

Figure 2. Patient 3. **a1**. Multidrug resistant disseminated *M. bovis* BCG infection with intracerebral lesions; **a2**. A draining head wound; **a3** and **a4**. The lesion progressively healed while on combined IFN-α and IFN-γ therapy; **b** A large right pleural mass before IFN-α which (**c**) completely resolved on combined IFN-α and IFN-γ treatment.

Patient 4 is a 52-year-old Caucasian female with recurrent lymphadenopathy, rashes and hospitalizations since childhood. She has been treated with prolonged antibiotics and intermittent corticosteroids. In her 20s *M. fortuitum* was isolated from a lymph

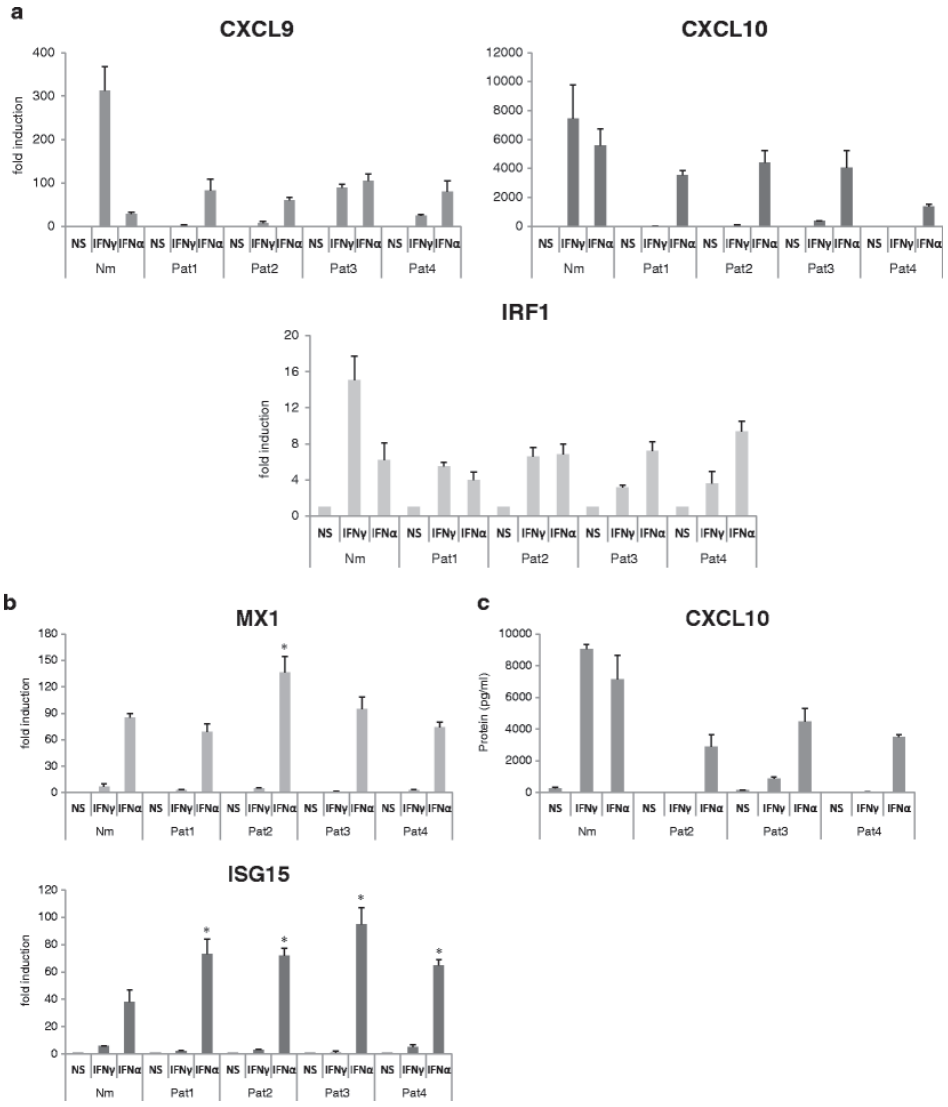

Figure 3. Gene expression *in vitro*. PBMCs from normal donors (Nm) and IFN-γR deficient patients were stimulated with IFN-γ (400 IU/ml) or IFN-α (1000 IU/ml) for 3 h and assayed for the **e**xpression of target genes **a** *CXCL9, CXCL10, IRF-1* and **b** *MX1, ISG15*. **p*<0.05 vs. the response observed in healthy controls; **c** Levels of CXCL10 protein were assayed in the 20 h culture supernatants. Results are mean ± SD of five independent experiments.

node. She was diagnosed with partially responsive IFNGR1 818del4 (23) after her son had osteomyelitis secondary to MAC and was found to have IFNGR1 818del4. Another son had died of disseminated MAC infection. At 50 years she presented with cervical and supraclavicular lymphadenopathy and her maxillary sinuses growing *M. avium*-X cluster. Therapy included doxycycline, clarithromycin, trimethoprim / sulfamethoxazole, rifampin and IFN-γ 100μg/m^2 subcutaneously three times weekly. At 52 years new skin lesions and a hard palate fistula arose. In view of persistent and progressive disease, IFN-α2b 3 million units subcutaneously was added three times weekly alternated with IFN-γ. Her lymphadenopathy and skin infiltration improved and her fistula closed. Since that time she has had no new infections. She continues on both interferons and oral antibiotics.

Analysis of gene expression in primary human cells in vitro

Cells from patients with partial or complete IFN-γR deficiency showed reduced or absent responses to stimulation with IFN-γ. Expression of *CXCL9* and *CXCL10* was strikingly diminished in patients 1, 2, 3, and 4 compared to controls. Induction of *IRF1* in response to IFN-γ was about half normal (Figure 3a). On the other hand, stimulation of the same cells with IFN-α raised the expression of *CXCL10* (except patient 4), *CXCL9* and *IRF1* to levels close to normal (Figure 3a). Induction of the more IFN-α specific target genes, *MX1* and *ISG15*, was normal to high in patients' cells (Figure 3b). CXCL10 protein levels in stimulated culture supernatants correlated with message (Figure 3c).

Ex vivo analysis of gene expression in patients' cells following IFN-α treatment

Ex vivo mRNA expression was assayed in PBMCs from patients 1, 2 and 4 during treatment with IFN-α, looking at expression of *ISG15*, *CXCL9*, *CXCL10*, *CXCL11* and *IDO* before and one day after injection. As expected, transcription of the typical IFN-α regulated gene *ISG15* was enhanced after IFN-α injection (Figure 4). Induction of *CXCL9* and *CXCL10*, which are typically impaired in response to IFN-γ in patients with defective IFN-γ signaling, and of *CXCL11* and *IDO*, genes modulated by both IFN-γ and IFN-α, were robustly up-regulated in patients' PBMCs 18-20 h following IFN-α injection (Figure 4).

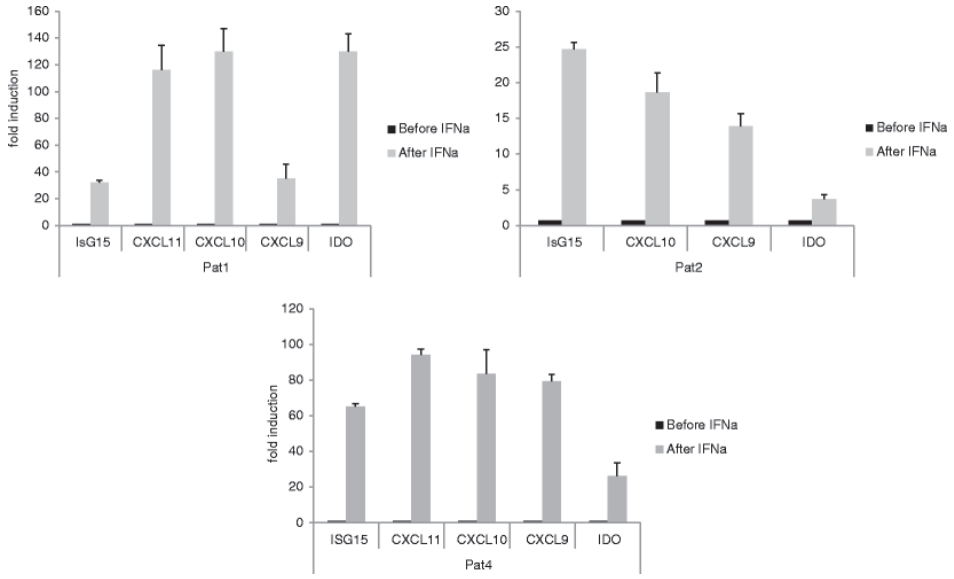

Figure 4. Gene expression *ex vivo*. Gene expression in freshly isolated unstimulated PBMCs obtained from IFN-γR deficient patients was assayed before and 20 h after IFN-α injection. Results for each patient represent mean fold induction ± SD of triplicate wells for each condition compared to samples obtained before IFN-α injection.

Mycobacteria induced cytokine response in vitro is sustained by IFN-α

Evaluation of the effect of IFN-α on *in vitro* cytokine production was assayed in MDM obtained from healthy donors and patients following incubation with mycobacteria. *M. avium*-induced TNF-α and IL-1β secretion was modestly to markedly enhanced following stimulation with IFN-α (Figure 5a). Similar responses were observed when cells were stimulated with *M. abscessus* (not shown). In comparison to normals and to the findings on protein expression, patient 1 cells showed robust induction of IL-1β message in response to mycobacteria, which was not much altered by IFN-α (Figure 5b). A similar effect of IFN-α on *M. avium* induced responses was also detected in alveolar macrophages (Figure 5c), suggesting that the modulatory action of IFN treatment may be active in the lung tissue environment.

Macrophage differentiation is known to be modulated by many factors, such as GM-CSF/CSF2, and M-CSF/CSF1, which lead to specific response profiles, cell morphologies and phenotypes (M1 and M2 macrophages, respectively) (24, 25). The induction of CSF2 in MDM cultures (but not CSF1, not shown) triggered by the mycobacteria (*M. avium*, 342 ± 98.1 fold induction) was remarkable and sustained by IFN-α (*M. avium* + IFN-α, 756 ± 208).

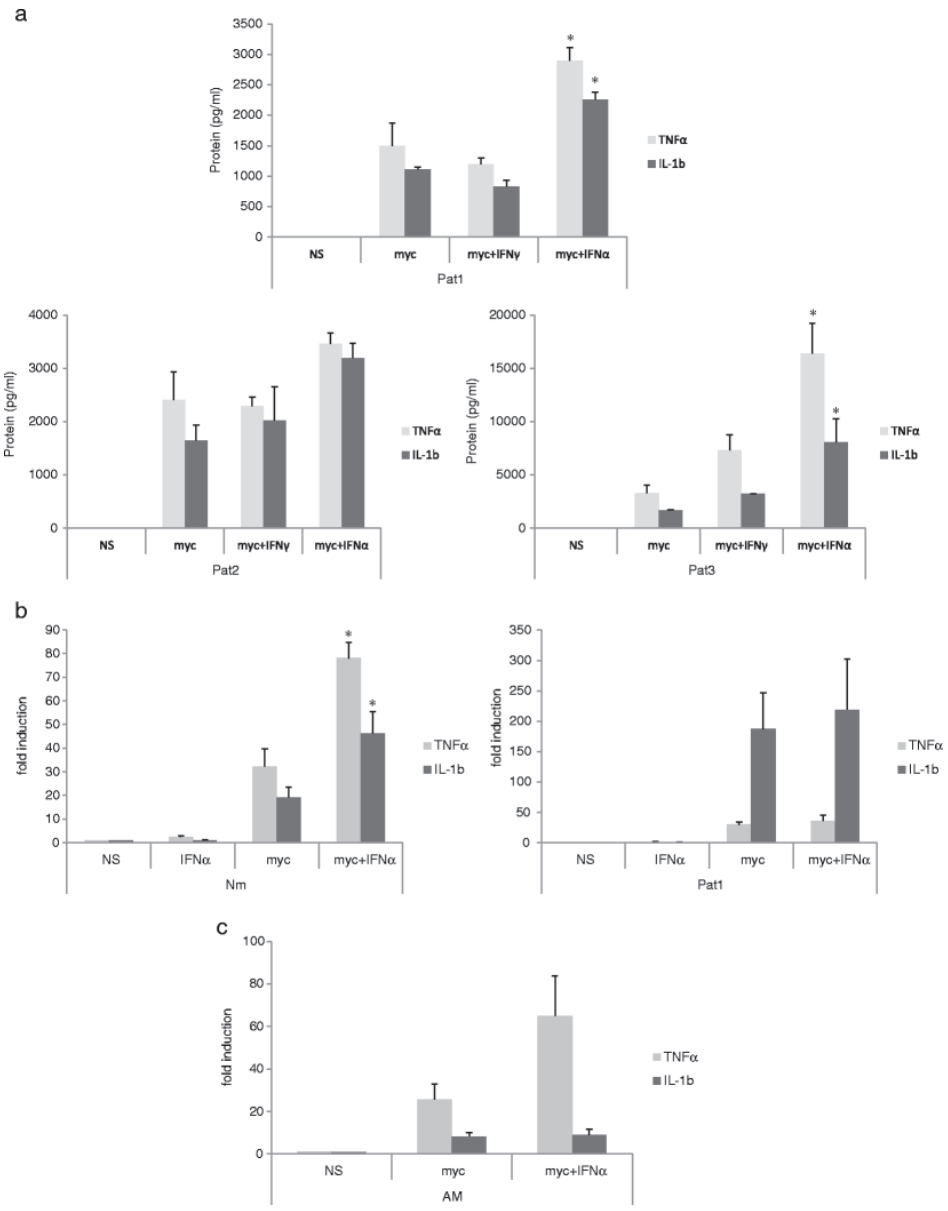

Figure 5. Gene expression following mycobacterial stimulation. **a** MDM obtained from patients were stimulated with mycobacteria (myc = *M. avium*, MOI 5) and IFN-α (1000 IU/ml) added simultaneously to the wells. Supernatant levels (pg/ml) of TNF-α and IL-1β were assayed 20 h after culture stimulation; **b** Gene expression was evaluated in MDM 3 h after stimulation; **c** Alveolar macrophages (AM) obtained from normal donors were plated, stimulated and assayed as above. Results are mean ± SD of three independent experiments. **p*<0.05 when compared to cells stimulated with mycobacteria alone.

DISCUSSION

We hypothesized that IFN-α could bypass defective IFN-γ signaling, since both use STAT1 and induce phospho-STAT1 homodimers. Further, low dose IFN-γ followed by IFN-α has been demonstrated to lead to STAT1 dependent up-regulation of typical IFN-γ inducible genes, shifting the IFN-α response to a more pro-inflammatory phenotype (26, 27).

IFN-α therapy seemed to be useful in our patients with IFN-γ signaling defects who had resistant intracellular infections. These four IFN-γR deficient patients suffered from extensive refractory disseminated mycobacterial infections due to compromised IFN-γ response. Although the extent of the clinical effect of IFN-α varied between patients, all showed some degree of clinical response with either improvement or stabilization. Importantly, in no case did IFN-α therapy exacerbate disease.

Signaling through both the IFN-γ and the IFN-α receptors is both specific and promiscuous. In addition to the canonical STATs (STAT1, STAT2), others are activated following the same signals (STAT3, STAT4) (1, 28). The activation of STAT1 dependent genes is reinforced here since *ex vivo* mRNA expression after IFN-α injection in patients 1, 2, and 4 clearly showed the induction of both common IFN genes and more classical IFN-γ specific genes, supporting the hypothesis that IFN-α activation, at least at pharmacologic doses and administrations, overlaps molecularly to some extent with IFN-γ activation. *In vitro* expression of IFN-γ and IFN-α induced genes observed in these patients following IFN-α stimulation were similar to or higher than those levels induced in normals. It remains unclear to what extent IFN-α can replace IFN-γ in the setting of IFN-γR deficiency, but it clearly fails to do so spontaneously.

Patient 1 showed marked clinical and radiological response to IFN-α within two months of treatment, followed by further improvement over the next year. It is unlikely that this effect was caused by the change of azithromycin for clarithromycin in her antimycobacterial regimen. Interestingly, after two years on IFN-α therapy she developed disseminated *M. abscessus* infection, suggesting that conventional dose IFN-α was able to control MAC but insufficient to prevent *M. abscessus* infection. Patient 2 had persistent *M. abscessus* infection in the lung, blood and liver despite conventional IFN-α therapy. The use of pegylated IFN-α with its different pharmacokinetic and pharmacodynamic properties may have been effective in this condition, as it is in hepatitis B and C (29, 30). Following introduction of pegylated IFN-α, the patient's liver biopsy and culture showed no mycobacteria and his last set of blood cultures were negative for MAC. Patient 3 had impaired IFN-γ signaling, but had enough residual function that high dose daily IFN-γ led to fever, inflammation and hydrocephalus. We reduced his IFN-γ to every other day, which he tolerated better and then added IFN-α because his extensive multidrug resistant BCG infection persisted despite aggressive antimycobacterial treatment with end organ toxicity. The combination of IFN-α and IFN-γ treatment led to rapid and

sustained clinical improvement. Despite persistent infection over many years, patient 4 had significant clinical improvement only following the combination of IFN-α and IFN-γ therapy. Two other patients treated with IFN-α in the setting of IFN-γR deficiency have been previously reported (31-33). These patients showed mild transient clinical improvement even though IFN-α therapy was introduced late in the course of severe disseminated disease.

Type I interferons are constitutively produced in a broad range of tissues and are critical mediators of the innate and adaptive immune responses and modulate macrophage function (18, 34). IL-1 has important proinflammatory effects and contributes to host defense. Moreover, macrophage differentiation and release of cytokines, such as TNF-α and GM-CSF, are critical for control and resolution of infection and are induced by mycobacteria. We found that MDM from normals and patients co-stimulated with *M. avium* and IFN-α (or IFN-γ) showed no downregulation of TNF-α or IL-1 responses.

The clinical impact of IFN-α therapy on these patients and the *in vitro* data are in sharp contrast to reports of enhanced Mtb growth in mice associated with induction of type I interferons (19, 20, 35). The use of the potent stimulator of IFN-α production, Poly-IC, led to suppression of IL-1 production in Mtb infected mice, arguing against a beneficial effect of IFN-α in mycobacterial control (19, 20). However, the differences between these studies and our observations include host and pathogen species, as well as the potentially complex nature of the Poly-IC itself. Other studies described enhanced mycobacterial growth with IFN-α administration (36) or enhanced IFN gene signature in patients with active tuberculosis (37, 38). Some studies have shown increased mycobacterial burden in IFN-α deficient mice (39), decreased mycobacterial burden in mice treated with IFN-β (40), and increased chemotaxis of T cells activated by IFN-α and mycobacterial infection (41). One recent publication showed that IFN-α was unable to up-regulate LPS-induced TNF-α secretion in human MDM and was not associated with *in vitro* killing of *M. smegmatis* (42). The effects of IFNs on macrophage responses will likely be dependent on cell differentiation and activation state. It is feasible to postulate that patients 1 and 2 reported here had some baseline levels of IFN-γ response *in vivo*, which allowed some effective clinical responses to IFN-α. The beneficial effects of

IFN-α treatment may rely on the priming effects that IFN-γ and IFN-α exert on each other in mutual responses (27). Recently, Bogunovic et al. (43) described patients with inherited ISG15 deficiency who had associated mycobacterial disease. ISG15 is induced by IFN-α and activates T and NK cells to more effective production of IFN-γ, a mechanism that could also enhance control of mycobacterial disease in our setting. The investigation of mechanisms by which IFN-α helps these patients to control infection need to be extended to investigate the role of other cytokines and microbicidal assays.

Since mycobacterial species can behave differently in their induction of inflammatory responses as well as their responses to treatment, adjunctive IFN-α therapy may drive

different effects depending on the type of mycobacteria and on the patient's clinical condition. Despite the variability of response, IFN-α induces typical IFN-γ responsive genes *in vivo* and may boost therapeutic antimycobacterial effects in patients with NTM infections caused by IFN-γ signaling disorders. The mechanisms, optimal dosing regimen and optimum time to initiate this therapy remain to be determined.

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CHAPTER 3.2

Tigecycline potentiates clarithromycin activity against *Mycobacterium avium in vitro*

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ABSTRACT

The *in vitro* activities of clarithromycin and tigecycline alone and in combination against *Mycobacterium avium* were assessed. The activity of clarithromycin was time-dependent, highly variable and often resulted in clarithromycin resistance. Tigecycline showed concentration-dependent activity and mycobacterial killing could only be achieved at high concentrations. Tigecycline enhanced clarithromycin activity against *M. avium* and prevented clarithromycin resistance. Whether there is clinical usefulness of tigecycline in the treatment of *M. avium* infections needs further study.

Although the introduction of macrolides improved the success of treatment of *Mycobacterium avium* infections, the overall prognosis is still poor (1). Therefore, new powerful treatment strategies are needed. Tigecycline has been shown to have good *in vitro* activity against rapidly growing nontuberculous mycobacteria (NTM) (2-4) and success in clinical use has been reported when the drug is combined with macrolides (5, 6). In contrast, little is known about tigecycline activity against slowly growing NTM including *M. avium* and *in vitro* activity has not been demonstrated so far (7). In the present study, the bactericidal activities of clarithromycin and tigecycline alone and in combination against *M. avium* were assessed.

Suspensions of *M. avium* complex (MAC) 101 strain (kindly provided by L.S. Young, Kuzell Institute for Arthritis and Infectious Diseases, San Francisco, CA) were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI), supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Baltimore Biological Laboratories, Baltimore, MD), 0.5% glycerol (Scharlau Chemie SA, Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co., St. Louis, MO) under shaking conditions at 96 rpm at 37ºC. Cultures on solid medium were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol, for 14 days at 37ºC with 5% CO_2. The susceptibility of the *M. avium* strain in terms of MICs determined according to the guidelines of the Clinical and Laboratory Standards Institutes (CLSI) (8) were 2 mg/L for clarithromycin and 16 mg/L for tigecycline. The concentration- and time-dependent killing capacities of clarithromycin and tigecycline was determined as previously described (9, 10). Briefly, *M. avium* cultures were exposed to antimicrobial drugs at 4-fold increasing concentrations for 21 days at 37°C under shaking conditions. At days 1, 3, 7, 10 and 21 during exposure, samples were collected, centrifuged at 14000*xg* and subcultured onto antibiotic-free solid medium. Subculture plates were incubated for 14 days at 37°C with 5% CO_2 to determine the number of cfu. The lower limit of quantification was 5 cfu/mL (log 0.7). To assess selection of clarithromycin-resistant *M. avium*, subcultures were also performed on solid medium containing 32 mg/L of clarithromycin. The stabilities of clarithromycin and tigecycline in mycobacterium-free Middlebrook 7H9 broth at 37°C were determined using two test concentrations of clarithromycin and tigecycline (8 and 32 mg/L). Antimicrobial activity over time was assessed using the standard large-plate agar diffusion assay as previously described (11). In contrast to clarithromycin (showing no decline during 21 days of exposure), tigecycline concentrations fell to 20% of the original concentrations within the first 24 hours, and thus 80% of the original tigecycline concentrations was added daily. The two endpoints of this study were drug synergy and the prevention of the emergence of clarithromycin-resistance. Synergistic activity was defined as a ≥100-fold ($2\log_{10}$) increase in mycobacterial killing with the combination compared to the most active single drug or when the drug combination achieved elimination of *M. avium* after 21 days of drug exposure, which was not achieved during single drug exposure (12, 13).

Clarithromycin showed time-dependent bactericidal activity toward *M. avium* (Figure 1a). At the intermediate concentrations (2 and 8 mg/L) high inter-experimental variability was observed showing mycobacterial killing in 2 out of 4 experiments and mycobacterial regrowth in the other 2 experiments, which was associated with the selection of clarithromycin resistance. Only at the highest concentration tested (32 mg/L) was ≥99% killing consistently observed. Tigecycline showed concentration-dependent bactericidal activity toward *M. avium* (Figure 1b). At tigecycline concentrations of ≥8 mg/L, ≥99% killing was observed, and mycobacterial elimination was achieved only at the highest concentration tested (32 mg/L).

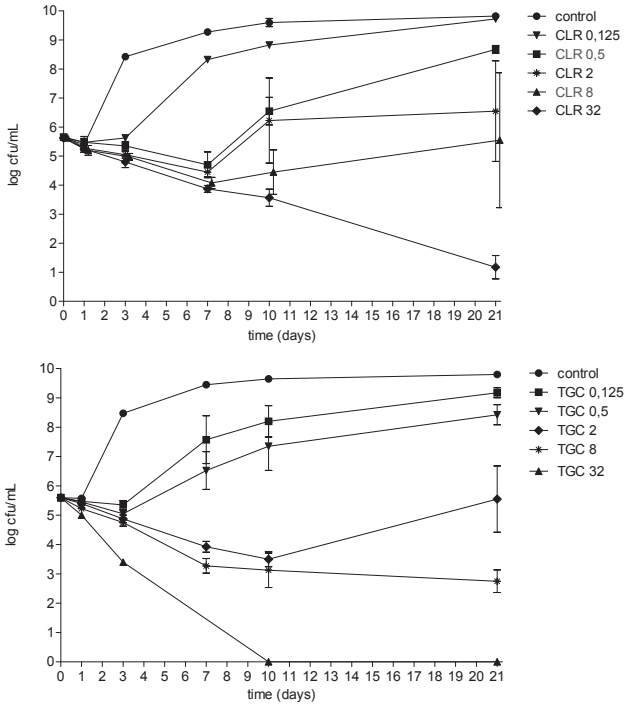

Figure 1. a Concentration- and time-dependent bactericidal activity of clarithromycin (CLR) toward *M. avium.* **b** Concentration- and time-dependent bactericidal activity of tigecycline (TGC) toward *M. avium.* The drug concentrations are milligrams per liter.

A concentration-dependent enhancement of clarithromycin activity by tigecycline was observed (Figure 2 and Table 1). Whereas a low concentration of tigecycline (0.125 mg/L) effected synergy in combination with clarithromycin at ≥2 mg/L, tigecycline at 0.5 mg/L effected synergy with clarithromycin at ≥0.5 mg/L and tigecycline at 2 mg/L effected synergy with clarithromycin at ≥0.125 mg/L, except with clarithromycin 2 mg/L. All

tested tigecycline concentrations prevented the emergence of clarithromycin resistance when combined with clarithromycin at 0.125 to 8 mg/L.

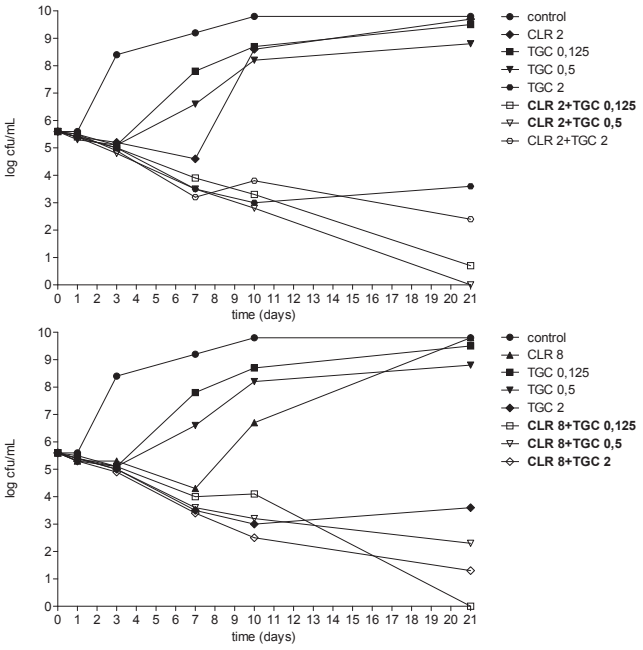

Figure 2. a Concentration- and time-dependent bactericidal activity of clarithromycin (CLR) at 2 mg/L combined with tigecycline (TGC) at various concentrations (milligrams per liter) toward *M. avium*. **b** Concentration- and time-dependent bactericidal activity of clarithromycin at 8 mg/L combined with tigecycline at various concentrations (milligrams per liter) toward *M. avium.*

	no TGC	TGC 0.125		TGC 0.5		TGC 2	
	Res	Syn	Res	Syn	Res	Syn	Res
CLR 0	1:4.5E7*						
CLR 0.125	0.31%	-	1:1.2E7*	-	1:1.5E7*	+	0%
CLR 0.5	0.06%	-	1:1.0E7*	+	0%	+	0%
CLR 2	80%	+	nd	+(E)	0%	-	0%
CLR 8	54%	+(E)	0%	+	0%	+	0%

Table 1. Synergistic activity (Syn) and prevention of selection of clarithromycin resistance (Res) in *M. avium* after 21 days of drug exposure. CLR, clarithromycin; TGC, tigecycline; *, spontaneous mutation frequency; +, synergy; -, no synergy; E, elimination (< 5 cfu/mL = limit of quantification); nd not determined.

To our knowledge, this is the first study showing that the addition of tigecycline to clarithromycin increased bactericidal activity against *M. avium* and prevented the selection of clarithromycin resistance. Recently, Huang *et al.* showed that tigecycline could potentiate clarithromycin activity against rapidly growing mycobacteria (RGM) *in vitro* (2) supporting the use of this combination in the treatment of infections caused by RGM. In humans, the steady-state maximum serum concentration at clinical dosages of tigecycline is around 0.6 mg/L (14). Although this concentration is far below

the tigecycline concentrations needed to achieve mycobacterial killing in our *in vitro* study, it approximates the concentrations effecting synergy in combination with clarithromycin in our study. Moreover, tigecycline has been shown to accumulate in tissues and human macrophages (14, 15). Further studies including macrophage-infection and pharmacodynamic/pharmacokinetic models, and *in vivo* models are needed to establish to what extent tigecycline can contribute to killing and elimination of *M. avium* and whether tigecycline can indeed effect synergy in combination with clarithromycin as we have shown in the present study. Although clarithromycin is considered the cornerstone agent in the treatment of *M. avium* infections, consistent mycobacterial killing was only seen at the highest concentration tested (32 mg/L). Importantly, at clinically relevant concentrations clarithromycin activity against *M. avium* was highly variable between experiments, alternating mycobacterial killing and mycobacterial regrowth. These results might explain the fact that despite macrolide-containing regimens, overall treatment success of *M. avium* infections is still unpredictable and disappointing (1). The results of our study also illustrate that the dynamic time-kill kinetics assay can detect important differences in antibacterial drug behaviour that cannot be detected when static susceptibility assays such as MICs are used. This is in line with our previous studies (10, 16) as well as with another recent study assessing the activities of several antibacterial drugs against *Mycobacterium abscessus* (17). The results of our *in vitro* study underline the need for potentiation of clarithromycin activity against *M. avium*. Whether tigecycline might be clinically useful when added to clarithromycin based regimens needs further study.

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CHAPTER 3.3

Colistin as a potentiator of anti-TB drug activity against *Mycobacterium tuberculosis*

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ABSTRACT

Objectives: The mycobacterial cell wall is an effective permeability barrier that limits intracellular concentrations of anti-TB drugs and hampers the success of treatment. We hypothesized that colistin might enhance the efficacy of anti-TB drugs by increasing mycobacterial cell wall permeability. In this study, we investigated the additional effect of colistin on the activity of anti-TB drugs against *Mycobacterium tuberculosis in vitro*.
Methods: The concentration- and time-dependent killing activity of isoniazid, rifampicin or amikacin alone or in combination with colistin against *M. tuberculosis* H37Rv was determined. Mycobacterial populations with both high and low metabolic activity were studied, and these were characterized by increasing or steady levels of ATP, respectively.
Results: With exposure to a single drug, striking differences in anti-TB drug activity were observed when the two mycobacterial populations were compared. The addition of colistin to isoniazid and amikacin resulted in sterilization of the mycobacterial load, but only in the *M. tuberculosis* population with high metabolic activity. The emergence of isoniazid and amikacin resistance was completely prevented by the addition of colistin.
Conclusions: The results of this study emphasize the importance of investigating mycobacterial populations with both high and low metabolic activity when evaluating the efficacy of anti-TB drugs *in vitro*. This is the first study showing that colistin potentiates the activity of isoniazid and amikacin against *M. tuberculosis* and prevents the emergence resistance to anti-TB drugs. These results form the basis for further studies on the applicability of colistin as a potentiator of anti-TB drugs.

INTRODUCTION

TB is still a major global health problem, with 9.6 million people affected worldwide. It is the second most common cause of mortality related to infectious diseases, being responsible for 1.5 million deaths each year (1). Long-term treatment with multiple anti-TB agents is necessary and is often accompanied by drug toxicities, drug-drug interactions, a lack of patient compliance and the emergence of drug resistance. Powerful new treatment strategies are needed that aim to improve the potency of antimycobacterial drugs and prevent the emergence of drug resistance while minimizing drug toxicities.

The mycobacterial cell wall is known for its complex lipid architecture, forming an effective permeability barrier that contributes to the intrinsic resistance to multiple antimicrobial agents and limited efficacy of the currently used anti-TB drugs. In general, high concentrations of anti-TB drugs are needed to prevent the selection of drug-resistant mutants. In clinical practice, high dosing of anti-TB drugs is usually limited by toxic side effects. Increasing the permeability of the lipid bilayer in the mycobacterial cell wall could be a way to increase intrabacterial anti-TB drug concentrations. Different studies have shown that changes in the mycolate composition of the mycobacterial cell wall result in increased antimycobacterial drug susceptibility in both *Mycobacterium tuberculosis* (Mtb) and *Mycobacterium avium* (2, 3).

Colistin, a member of the polymyxin group of drugs, is currently used in the treatment of drug-resistant Gram-negative bacterial infections. The proposed mechanism of action of colistin is through interaction with the Gram-negative bacterial membrane (4). This includes electrostatic interactions between positively charged groups on the polymyxins and negatively charged components of LPS, as well as interactions between the polymyxin fatty acid tail and the lipids of the bacterial membrane (4). Destabilization of the cytoplasmic bacterial membrane occurs, resulting in leakage of intracellular contents and apoptosis (4). Although the composition of the mycobacterial cell wall is different from that of Gram-negative bacteria, an interaction with colistin and colistin-like particles has been shown, resulting in increased permeability (5, 6). We therefore hypothesized that colistin might enhance anti-TB drug efficacy by increasing mycobacterial cell wall permeability, allowing increased intracellular concentrations of anti-TB drugs.

In the present study we investigated the activity of various combinations of anti-TB drugs and colistin against extracellular Mtb *in vitro*. We compared the activity against Mtb populations with high and low metabolic activity, as we previously showed that the activity of anti-TB drugs depends on the metabolic activity of the mycobacteria (7). In most *in vitro* studies, anti-TB drug activity is determined using the MIC assay, providing endpoint data and determining only the inhibition of mycobacterial growth. In the

present study, we used the time-kill kinetics assay, providing unique information on the concentration-dependent and time-dependent killing capacity of anti-TB drugs.

MATERIALS AND METHODS

Bacterial strain and culture

The Mtb strain used was Mtb H37Rv (ATCC 27294), a clinical isolate and reference strain commonly used in TB studies. Mtb suspensions were cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 10% OADC (Baltimore Biological Laboratories, Baltimore, MD, USA), 0.5% glycerol (Scharlau Chemie SA, Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co., St Louis, MO, USA), under shaking conditions at 96 rpm at 37°C. Vials with Mtb suspensions were stored at -80°C. Cultures on solid medium were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 21 days at 37°C with 5% CO_2. The susceptibility of the Mtb strain in terms of MICs, determined at the Dutch National Reference Laboratory according to the CLSI guidelines (8), was 0.125 mg/L for isoniazid, 0.125 mg/L for rifampicin, 2 mg/L for amikacin and >1024 mg/L for colistin.

Anti-TB drugs and colistin

Isoniazid was purchased from Sigma Chemical Co., rifampicin was purchased from Aventis Pharma BV (Hoevelaken, the Netherlands), amikacin was purchased from Hospira Benelux BVBA (Brussels, Belgium) and colistin sulphate was purchased from Spruyt Hillen (IJsselstein, the Netherlands, 20400 IU/mg).

Time-kill kinetics assay

The concentration-dependent and time-dependent killing capacity of the different anti-TB drugs alone or in combination with colistin was determined as previously described (7). Mtb populations with high and low metabolic activity were prepared for testing the activity of anti-TB drugs. The metabolic activity of the Mtb cultures was assessed in a previous study by measuring the ATP level using the firefly luciferase bioluminescence assay (7). The Mtb population with high metabolic activity, starting at a density of $7.2x10^5$ cfu/mL (range 5.2-10x10^5), showed a 6-fold increase in ATP concentration per viable Mtb after 3 days of incubation. Mtb cultures with low metabolic activity obtained after 4 days of incubation started at a density of $1.8x10^7$cfu/mL (range 0.8-3.4x10^7) and showed steady ATP levels. Mtb cultures with high and low metabolic activity were exposed to anti-TB drugs and/or colistin for 6 days at 37°C under shaking conditions at 96 rpm at 2-fold increasing concentrations, ranging from 0.031 to 1024 mg/L for isoniazid, rifampicin and amikacin, and from 1 to 1024 mg/L for colistin. On days 1, 2, 3 and 6

during exposure, samples were collected. These were centrifuged at 14000*xg* to avoid drug carry-over and subcultured onto solid media. The plates were incubated for 21 days at 37°C with 5% CO_2 to determine the number of cfu representing viable bacteria. The lower limit of detection was 5 cfu/mL (log 0.7).

Selection of drug-resistant Mtb

In order to assess the selection of drug resistance after 6 days of drug exposure, subcultures were also performed on solid media containing anti-TB drugs (7). The drug concentrations in the subculture plates were 4-fold the critical concentrations, i.e. 0.8 mg/L isoniazid, 4 mg/L rifampicin and 20 mg/L amikacin (8).

Endpoints

The two endpoints of this study were (i) synergy between the anti-TB drug and colistin; and (ii) the prevention of the emergence of anti-TB drug resistance. Synergistic activity of a drug combination was defined as ≥99% killing compared with the killing obtained with the most active single drug or when the drug combination achieved sterilization of Mtb after 6 days of drug exposure that was not achieved during single-drug exposure (9).

RESULTS

Concentration- and time-dependent bactericidal activity of anti-TB drugs and colistin at single-drug exposure in relation to the metabolic activity of the mycobacteria

As shown in Figures 1-4 (all tested concentrations are depicted in Figures S1-S4, available as Supplementary data at *JAC* online), the anti-TB drugs and colistin differed with respect to their concentration-dependent and time-dependent killing capacity. In the absence of anti-TB drugs or colistin, the Mtb population with high metabolic activity showed an average increase from $7.7x10^5$ cfu/mL to $6.1x10^7$ cfu/mL within 6 days of incubation. The Mtb population with low metabolic activity showed only a modest increase (on average from $2.1x10^7$ to $7.2x10^7$ cfu/mL) within 6 days.

Isoniazid showed a concentration-dependent effect on the Mtb populations with high and low metabolic activity (Figure 1 and Figure S1), and ≥99% killing was rapidly achieved at low concentrations in the Mtb population with high metabolic activity. Sterilization was observed only at extremely high concentrations (≥128 and ≥256 mg/L, respectively). Both Mtb populations became 100% isoniazid resistant at concentrations ranging from 0.031 to 64 mg/L and 0.125 to 128 mg/L, respectively.

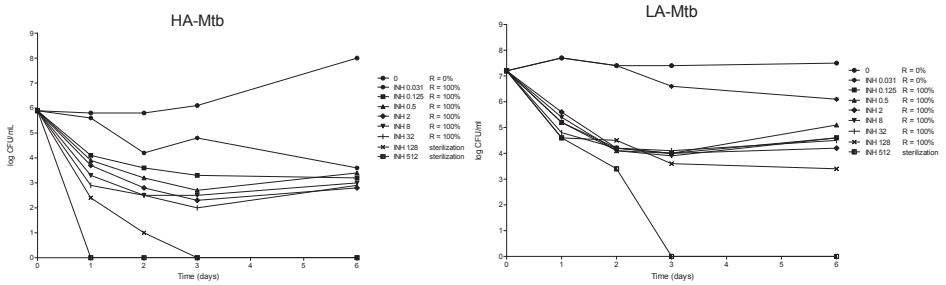

Figure 1. Concentration-dependent and time-dependent bactericidal activity of isoniazid against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb). Mtb cultures were exposed to 2-fold increasing isoniazid concentrations for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free and isoniazid-containing solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. INH, isoniazid; R, isoniazid-resistant mutants.

Rifampicin showed a concentration-dependent effect, particularly in the Mtb population with high metabolic activity (Figure 2 and Figure S2). In both Mtb populations, mycobacterial killing was less rapid than with isoniazid. Sterilization was achieved at ≥8 mg/L in the Mtb population with high metabolic activity and at 1024 mg/L in the Mtb population with low metabolic activity. Rifampicin-resistant mutants were selected only in the Mtb population with low metabolic activity, and resistance increased slowly over concentrations ranging from 0.25 to 512 mg/L.

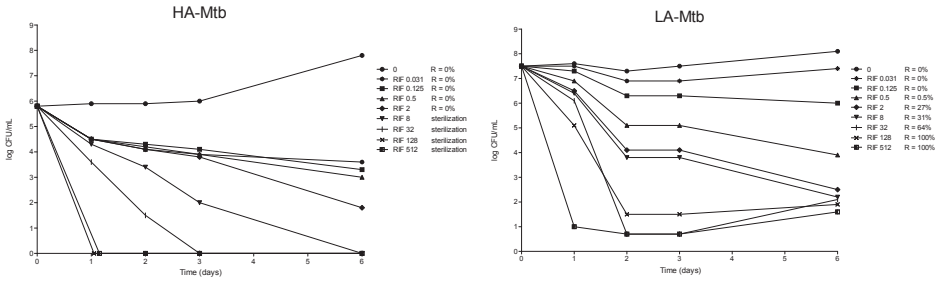

Figure 2. Concentration-dependent and time-dependent bactericidal activity of rifampicin against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb). Mtb cultures were exposed to 2-fold increasing rifampicin concentrations for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free and rifampicin-containing solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. RIF, rifampicin; R, rifampicin-resistant mutants.

Amikacin showed a strong concentration-dependent effect on the Mtb population with high metabolic activity (Figure 3 and Figure S3). In both Mtb populations, ≥99% killing was rapidly achieved. Sterilization was observed at ≥1 mg/L in the Mtb population with

high metabolic activity and at 1024 mg/L in the Mtb population with low metabolic activity. Amikacin-resistant mutants were selected only in the Mtb population with low metabolic activity, and resistance increased slowly over concentrations ranging from 1 to 512 mg/L.

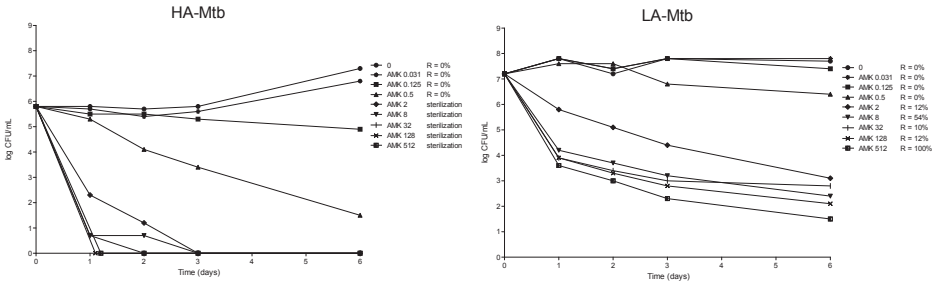

Figure 3. Concentration-dependent and time-dependent bactericidal activity of amikacin against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb). Mtb cultures were exposed to 2-fold increasing amikacin concentrations for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free and amikacin-containing solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. AMK, amikacin; R, amikacin-resistant mutants.

The effect of colistin on Mtb was modest in both Mtb populations (Figure 4 and Figure S4). In the two populations, ≥99% killing was achieved only at high concentrations and sterilization never occurred.

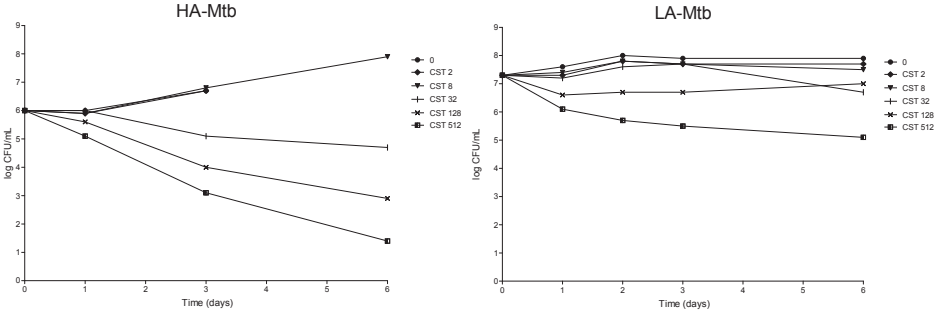

Figure 4. Concentration-dependent and time-dependent bactericidal activity of colistin against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb). Mtb cultures were exposed to 2-fold increasing colistin concentrations for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. CST, colistin sulphate.

Tables 1 and 2 summarize the comparative bactericidal activity of the anti-TB drugs and colistin at single-drug exposure.

	Lowest concentration (mg/L) resulting in ≥ 99% killing of Mtb					
	day 1		day 3		day 6	
	HA-Mtb	LA-Mtb	HA-Mtb	LA-Mtb	HA-Mtb	LA-Mtb
Isoniazid	1	32	0.125	0.063	0.031	0.063
Rifampicin	32	128	2	0.5	0.031	0.25
Amikacin	2	4	0.5	2	0.25	1
Colistin	>1024	>1024	256	1024	64	512

Table 1. Concentration-dependent bactericidal activity (≥ 99% killing) against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb) during 6 days of exposure to anti-TB drugs or colistin.

Lowest concentration (mg/L) resulting in sterilization (100% killing) after 6 days of drug exposure		
	HA-Mtb	LA-Mtb
Isoniazid	128	256
Rifampicin	8	1024
Amikacin	1	1024
Colistin	>1024	>1024

Table 2. Concentration-dependent sterilizing activity against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb) after 6 days of exposure to anti-TB drugs or colistin. Sterilization was defined as < 5 cfu/mL as this was the lower limit of detection of our time-kill kinetics assay.

Effect of colistin on the concentration- and time-dependent bactericidal activity of anti-TB drugs in relation to the metabolic activity of the mycobacteria

The colistin concentrations used in the combination experiments were 8 and 32 mg/L, based on the activity against Mtb at single-drug exposure. Colistin at 8 mg/L showed no activity against Mtb and colistin at 32 mg/L showed only a modest, inhibitory effect on mycobacterial growth (Figure 4). The anti-TB drug concentrations in the combination experiments studying the Mtb population with high metabolic activity were based on the MIC values of the anti-TB drugs. Because sterilization was achieved at the MIC of amikacin, a lower amikacin concentration was used (1/8x MIC). In the combination experiments studying the Mtb population with low metabolic activity, we also used higher concentra-

tions of anti-TB drugs, which were associated with the selection of drug resistance after single-drug exposure (isoniazid and amikacin, 4x MIC; rifampicin, 8x MIC).

The concentration- and time-dependent killing capacities of isonazid, rifampicin, and amikacin combined with colistin are depicted in Figures 5-7.

In the Mtb population with high metabolic activity, a synergistic effect was observed when high colistin concentrations (32 mg/L) were combined with low concentrations of isoniazid (0.125 mg/L) or amikacin (0.25 mg/L), resulting in sterilization (Figures 5 and 7). In the presence of colistin, isoniazid and amikacin achieved sterilization at lower concentrations (0.125 and 0.25 mg/L, respectively) compared to isoniazid and amikacin at single-drug exposure (128 and 1 mg/L, respectively). Synergy was not observed when colistin was combined with rifampicin (Figure 6). In the Mtb population with low metabolic activity, there was no synergistic effect of colistin and anti-TB drugs (Figures 5-7).

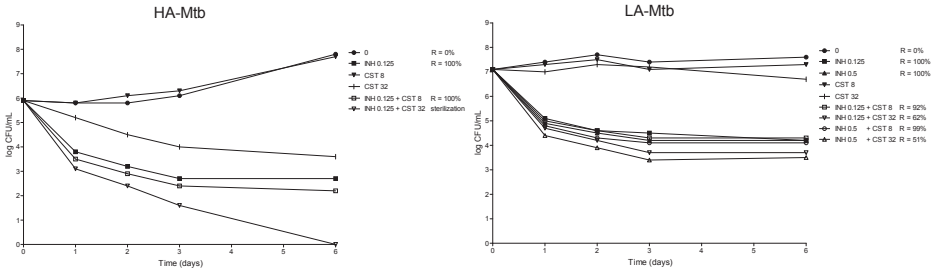

Figure 5. Concentration-dependent and time-dependent bactericidal activity of isoniazid combined with colistin against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb). Mtb cultures were exposed to isoniazid or colistin alone or in combination for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free and isoniazid-containing solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. INH, isoniazid; CST colistin sulphate; R, isoniazid-resistant mutants.

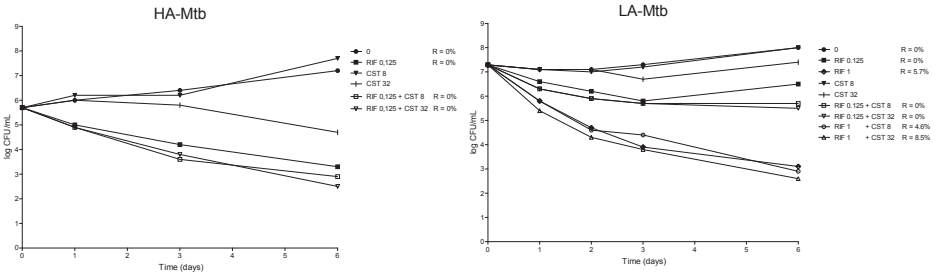

Figure 6. Concentration-dependent and time-dependent bactericidal activity of rifampicin combined with colistin against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity (LA-Mtb). Mtb cultures were exposed to rifampicin or colistin alone or in combination for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free and rifampicin-containing solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. RIF, rifampicin; CST colistin sulphate; R, rifampicin-resistant mutants.

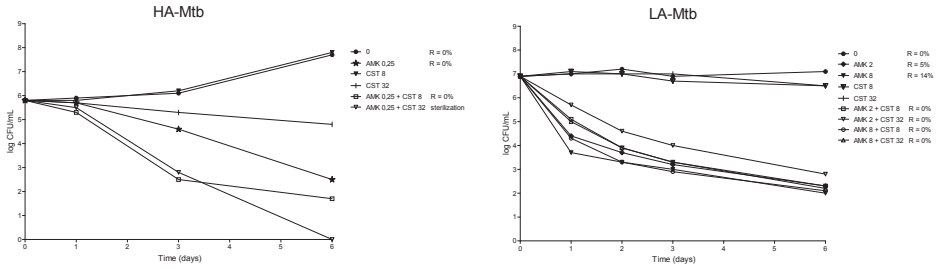

Figure 7. Concentration-dependent and time-dependent bactericidal activity of amikacin combined with colistin against the Mtb population with high metabolic activity (HA-Mtb) and the Mtb population with low metabolic activity Mtb (LA-Mtb). Mtb cultures were exposed to amikacin or colistin alone or in combination for 6 days at 37 °C under shaking conditions. On day 1, 2, 3 and 6, samples were collected, centrifuged and subcultured onto antibiotic-free and amikacin-containing solid media and incubated for 21 days at 37 °C with 5% CO_2 to determine the cfu count. AMK, amikacin; CST colistin sulphate; R, AMK-resistant mutants.

Tables 3 and 4 summarize the effect of colistin on the bactericidal activity of the anti-TB drugs.

	HA-Mtb										
	no CST			CST 8			CST 32				
	day 6	day 1	day 2	day 3	day 6		day 1	day 2	day 3	day 6	
	Res(%)	Syn	Syn	Syn	Syn	Res(%)	Syn	Syn	Syn	Syn	Res(%)
INH 0.125	100	-	-	-	-	100	-	-	-	+ (S)	0
RIF 0.125	0	-	-	-	-	0	-	-	-	-	0
AMK 0.25	0	-	-	+	-	0	-	-	-	+ (S)	0

Table 3. Synergistic activity (Syn) and prevention of selection of drug resistance (Res) in the Mtb population with high metabolic activity (HA-Mtb). CST, colistin sulphate; INH, isoniazid; RIF, rifampicin; AMK, amikacin; +, synergy; -, no synergy; S, sterilization.

Selection of drug resistance after exposure to anti-TB drugs combined with colistin

The selection of isoniazid resistance observed after single-drug exposure was completely prevented by the addition of high-dose colistin in the Mtb population with high metabolic activity and was reduced in the Mtb population with low metabolic activity (Tables 3-4). There was no amikacin resistance after single-drug exposure in the Mtb population with high metabolic activity. In the Mtb population with low metabolic activity, the addition of colistin to amikacin completely prevented the selection of amikacin resistance (Table 4). Rifampicin resistance did not occur after single-drug exposure in the Mtb population with high metabolic activity. In Mtb population with low metabolic activity, colisitin was not able to prevent the selection of rifampicin resistance (Table 4).

	LA-Mtb										
	no CST			CST 8			CST 32				
	day 6	day 1	day 2	day 3	day 6		day 1	day 2	day 3	day 6	
	Res(%)	Syn	Syn	Syn	Syn	Res (%)	Syn	Syn	Syn	Syn	Res (%)
INH 0.125	100	-	-	-	-	92	-	-	-	-	62
INH 0.5	100	-	-	-	-	99	-	-	-	-	51
RIF 0.125	0	-	-	-	-	0	-	-	-	-	0
RIF 1	5.7	-	-	-	-	4.6	-	-	-	-	8.5
AMK 2	5	-	-	-	-	0	-	-	-	-	0
AMK 8	14	-	-	-	-	0	-	-	-	-	0

Table 4. Synergistic activity (Syn) and prevention of selection of drug resistance (Res) in the Mtb population with low metabolic activity (LA-Mtb). CST, colistin sulphate; INH, isoniazid; RIF, rifampicin; AMK, amikacin; -, no synergy.

DISCUSSION

In this study, we assessed the concentration- dependent and time-dependent activity of anti-TB drugs in combination with colistin against Mtb. We identified two new potent anti-TB drug combinations that induced strong and rapid killing of Mtb and prevented the emergence of drug resistance. To our knowledge, this is the first study to provide evidence that colistin can potentiate the activity of isoniazid or amikacin against Mtb *in vitro*. The addition of colistin to low concentrations of isoniazid and amikacin resulted in sterilization of the mycobacterial load, which is one of the main goals in TB treatment. Interestingly, synergy was only achieved in Mtb population with high metabolic activity. In addition, in the presence of colistin, the emergence of isoniazid resistance was completely prevented in the Mtb population with high metabolic activity, but not in the Mtb population with low metabolic activity. Colistin combined with amikacin completely prevented the emergence of amikacin resistance in the Mtb population with low metabolic activity. The results of our study emphasize the importance of investigating Mtb populations with both high and low metabolic activity when evaluating anti-TB drug activity *in vitro* as these drugs can behave differently depending on the metabolic activity of the mycobacteria. This is supported by striking differences in the anti-TB drug activities of isoniazid, rifampicin and amikacin at single-drug exposure as well as differences in the emergence of anti-TB drug resistance between the two Mtb populations. The observation that the potentiating effect of colistin on isoniazid and

amikacin activity was only observed in the Mtb population with high metabolic activity might be related to the fact that higher drug concentrations were needed to achieve sterilization of the Mtb population with low metabolic activity. It can be speculated that these concentrations cannot be achieved even with the addition of colistin, explaining the lack of synergy when studying the Mtb population with low metabolic activity. Distinguishing between the two different Mtb populations might be clinically relevant as the Mtb population with high metabolic activity is supposed to be responsible for spreading of TB, whereas the Mtb population with low metabolic activity better represents Mtb that is present in deep-seated tissue infection, which is probably responsible for TB relapse. Our study also showed pronounced differences in strength and rate of mycobacterial killing when comparing the different anti-TB drugs, which is in line with our previous results (7).

It is surprising that, in the present study, the addition of colistin did not result in enhanced efficacy of rifampicin; neither did it prevent the emergence of rifampicin resistance. An explanation might be that the influence of colisitin on anti-TB drug activity depends on the lipophilicity of the anti-TB drug. It has been suggested that the lipophilicity of antimycobacterial agents is correlated with their ability to invade the mycobacterial cell wall and therefore with antimycobacterial drug activity (10). Rifampicin is known for its lipophilic properties, whereas isoniazid and amikacin are hydrophilic agents (11, 12). It has been shown that the activity of isoniazid against *M. avium* can be improved by using a more lipophilic isoniazid derivate (12), strengthening the hypothesis that a lipophilic nature is associated with increased efficacy. The pronounced ability of rifampicin, compared with isoniazid and amikacin, to cross the mycobacterial lipid bilayer might explain the fact that the increasing mycobacterial cell wall permeability brought about by colistin was not beneficial when it was combined with rifampicin. In addition, it has been proposed that cationic peptides, such as colistin, form micelle-like aggregates spanning the cytoplasmic membrane and providing water channels for the movement of ions and hydrophilic molecules across the bacterial membrane (4). This mechanism might also contribute to the synergistic effect observed with colistin and hydrophilic compounds such as isoniazid and amikacin as opposed to the lipophilic rifampicin. However, our results are in contrast to the findings of Korycka-Machala *et al.,* which showed increased susceptibility to rifampicin in *Mycobacterium vaccae* treated with polymyxin B nonapeptide (PMBN), which was linked to changes in the lipid composition of the mycobacterial cell wall (5). Since the cell wall of *M. vaccae* is known to be different from that of most other mycobacterial species, it is unclear whether similar results would have been obtained with Mtb or whether structural differences between PMBN and colistin account for these differences. The study by Yuan *et al.* also showed that changes in cell wall lipid composition of a mutant Mtb strain were associated with increased rifampicin susceptibility, whereas susceptibility to isoniazid remained unchanged (3). Although

colistin has been shown to increase the permeability of the mycobacterial cell wall (6), a role for other mechanisms in colistin's potentiation of anti-TB drug activity cannot be excluded. For instance, colistin has recently been shown to induce hydroxyl radical production (13). The hypothesis that colistin causes increased intramycobacterial drug concentrations needs to be confirmed by measuring intramycobacterial anti-TB drug concentrations.

In the present study, the concentration of colistin that was needed to potentiate anti-TB drugs was relatively high, at 32 mg/L. When used intravenously, colistin is administered as the prodrug colistin methane sulfonate (CMS), which is rapidly converted to its active component *in vivo*. It has been reported in two studies in critically ill patients that intravenous clinical dosages of CMS resulted in suboptimal maximum plasma concentrations of active colistin of 2.21 $\pm$ 1.08 and 2.93 $\pm$ 1.24 mg/L (14, 15). Colistin concentrations were undetectable in the bronchoalveolar lavage fluid (14). These concentrations in serum and bronchoalveolar lavage fluid are far below the colistin concentration that effected synergy *in vitro* in the present study. In relation to this, administration of colistin via inhalation should achieve attention. Colistin administered by inhalation is already successfully used in current clinical practice for the treatment of pneumonia caused by resistant Gram-negative bacteria in patients with cystic fibrosis and in patients with (ventilator-associated) pneumonia (16). The potential advantage of inhalation therapy is that higher local concentrations can be achieved while minimizing systemic drug concentrations and associated drug toxicities. It has recently been shown that colistin sulphate nebulization in rats resulted in concentrations in the pulmonary epithelial lining fluid that were around 1800 times higher than those achieved by intravenous colistin administration (17). Another recent study confirmed these findings in patients with cystic fibrosis, showing that dry powder nebulization of CMS resulted in colistin sputum concentrations of ≥128 mg/L in 38% of patients. Importantly, minimal systemic exposure was observed with inhalation therapy (18). However, whether the pulmonary colistin concentrations that can be achieved via inhalation are high enough to achieve synergy in combination with anti-TB drugs remains to be determined, especially since pulmonary surfactant has been shown to impair *in vitro* colistin activity (19).

Based on the results obtained in the present study we suggest that inhalational colistin when combined with anti-TB drugs could be a potential new strategy for the treatment of TB. In general, the concept of pulmonary drug delivery of anti-TB drugs is very attractive and has recently been attracting interest. In addition to achieving enhanced anti-TB drug efficacy against susceptible Mtb while minimizing systemic drug toxicity, the increase in local drug concentrations at the primary infected site could overcome drug resistance in (extensively) drug-resistant TB (20) and perhaps decrease the contagiousness of the disease (21). Several anti-TB drugs have already been studied in animal models for pulmonary delivery, including the first line agents isoniazid, rifampicin and

pyrazinamide; the results have been promising (22-25). However, the translation of these experimental animal data to clinical practice remains to be determined as data on clinical efficacy and safety in humans are scarce (26). Further studies are needed to assess the clinical applicability of anti-TB drug inhalation, and the additional value of colistin.

In conclusion, this is the first study to show that colistin potentiates the activity of isoniazid and amikacin against Mtb *in vitro* and prevents resistance to anti-TB drugs. These results form the basis for further studies on the applicability of colistin as a potentiator of anti-TB drug activity against intracellular Mtb hiding inside macrophages.

ACKNOWLEGEMENTS

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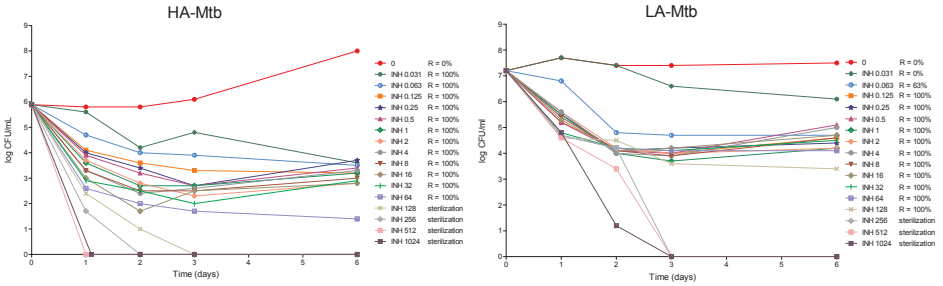
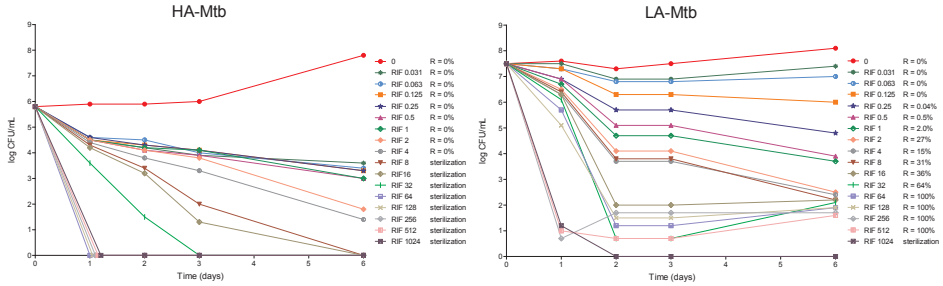
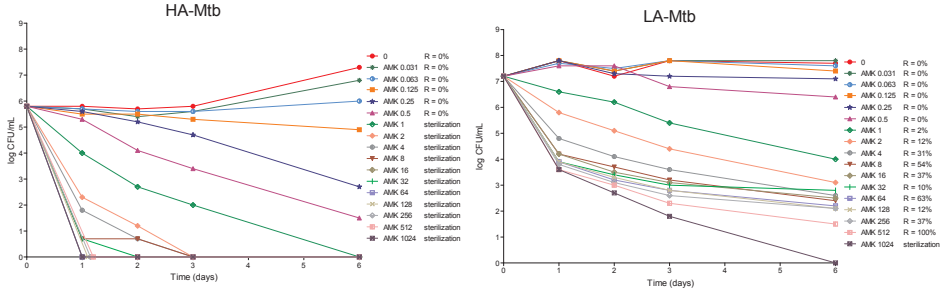
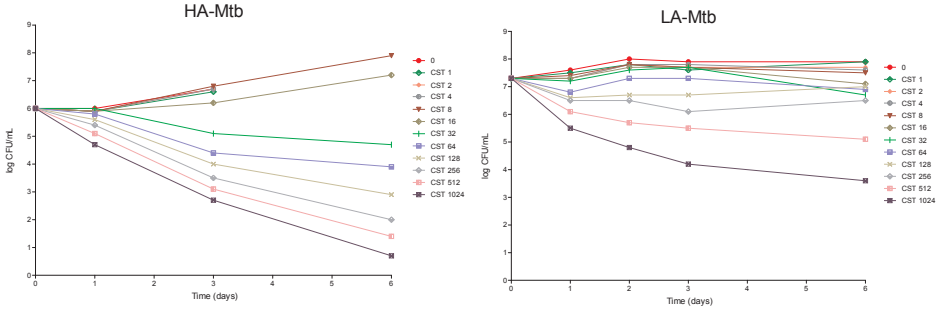
SUPPLEMENTARY FIGURES









CHAPTER 4

Optimizing preclinical antimycobacterial drug development

CHAPTER 4.1

The role of the time-kill kinetics assay as part of a preclinical modeling framework for assessing the activity of anti-tuberculosis drugs

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submitted

ABSTRACT

Novel treatment strategies for tuberculosis are urgently needed. Many different preclinical models assessing anti-tuberculosis drug activity are available, but it is yet unclear which combination of models is most predictive of clinical treatment efficacy. The aim of this study was to determine the role of our *in vitro* time kill-kinetics assay as an asset to a predictive preclinical modeling framework assessing anti-tuberculosis drug activity. The concentration- and time-dependent mycobacterial killing capacities of six anti-tuberculosis drugs were determined during exposure as single drugs or in dual, triple and quadruple combinations towards a *Mycobacterium tuberculosis* Beijing genotype strain and drug resistance was assessed.

Streptomycin, rifampicin and isoniazid were most active against highly metabolically active *Mycobacterium tuberculosis*. Isoniazid with rifampicin or high dose ethambutol were the only synergistic drug combinations. The addition of rifampicin or streptomycin to isoniazid prevented isoniazid resistance. *In vitro* ranking showed agreement with early bactericidal activity in tuberculosis patients for some but not all anti-tuberculosis drugs. The time-kill kinetics assay provides important information on the mycobacterial killing dynamics of anti-tuberculosis drugs during the early phase of drug exposure. As such, this assay is a valuable component of the preclinical modeling framework.

INTRODUCTION

Although the incidence and mortality of tuberculosis (TB) are declining worldwide, the actual numbers are still impressive with over 9 million new cases and 1.5 million deaths in 2014 (1). To end the global TB epidemic, a shorter treatment duration is required (1). Therefore, novel treatment strategies with increased sterilizing capacity are needed to improve cure rates and to reduce the emergence of anti-TB drug resistance and disease relapse. The correct (preclinical) assessment of the activity of novel drugs and drug combinations is complex. Many different preclinical models to determine the activity and therapeutic efficacy of anti-TB drugs are available, but it is yet unclear which combination of models are most predictive of clinical treatment response with an acceptable cost-effectiveness. Optimizing preclinical modeling is important, enabling rapid and reliable identification of novel potentially powerful anti-TB regimens and their translation into clinical practice.

PreDiCT-TB is a European multidisciplinary consortium focusing on anti-TB drug development (www.predict-tb.eu). The main goal of PreDiCT-TB is to find the combination of preclinical models that is most representative of response in TB patients. As a first step, PreDiCT-TB focuses on studying the most important anti-TB drug combinations in a number of preclinical models, the results of which are compared to historical clinical efficacy data. In this way, an integrated modeling framework will be constructed, serving as a reliable method to study novel anti-TB drug regimens and forming the basis for clinical trial design. In the context of the PreDiCT-TB consortium the present study determined the concentration- and time-dependent killing activity of six anti-TB drugs alone and in combination *in vitro*. The aim of this study was to establish the role of our *in vitro* time kill kinetics assay as an asset to a predictive preclinical modeling framework assessing anti-TB drug activity and therapeutic efficacy.

METHODS

Bacterial strain and culture

The *Mycobacterium tuberculosis* (Mtb) genotype strain Beijing VN 2002-1585 (BE-1585) was cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% oleic acid-albumin-dextrose-catalase enrichment (OADC, Becton, Dickinson and Company (BD), Sparks, MD, USA), 0.5% glycerol (Scharlau Chemie SA, Sentmenat, Spain) and 0.02% Tween 20 (Sigma Chemical Co., St Louis, MO, USA), under shaking conditions at 96 rpm at 37ºC. Vials with Mtb suspensions were stored at -80°C. Cultures on solid medium were grown on Middlebrook 7H10 agar (Difco), supplemented with 10% OADC and 0.5% glycerol for 28 days at 37ºC with 5% CO_2. Antibiotic suscepti-

bility in terms of Minimal Inhibitory Concentration (MIC) was determined according to the guidelines of the Clinical and Laboratory Standards Institutes (CLSI) (2). The BE-1585 strain was found to be susceptible to isoniazid (MIC 0.125 mg/L), rifampicin (MIC 0.25 mg/L), streptomycin (MIC 2 mg/L), ethambutol (MIC 5 mg/L), and para-amino salicylic acid (MIC 0.125 mg/L). The BE-1585 strain was also found to be susceptible to pyrazinamide, which was tested by the radiometric method (MIC <100 mg/L).

Anti-TB drugs

All anti-TB drugs were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Time-kill kinetics assay

The concentration- and time- dependent killing capacities of isoniazid, rifampicin, streptomycin, ethambutol, para-amino salicylic acid and pyrazinamide were determined as previously described (3). In brief, highly metabolically active (exponential phase) Mtb cultures were exposed to anti-TB drugs at 4-fold increasing concentrations for 6 days at 37°C under shaking conditions at 96 rpm. The metabolic activity of the mycobacteria was assessed in a previous study by determination of the adenosine-5'-triphosphate (ATP) level using the firefly luciferase bioluminescence assay (3). The anti-TB drug concentrations ranged from 0.01 to 40 mg/L for isoniazid, from 0.002 to 32 mg/L for rifampicin, from 0.02 to 96 mg/L for streptomycin, from 0.008 to 32 mg/L for ethambutol and para-amino salicylic acid and from 0.02 to 80 mg/L for pyrazinamide. The tested concentrations were based on the maximum free drug concentrations (*f*Cmax) of the individual anti-TB drugs ranging from 1/1024x *f*Cmax to 4x *f*Cmax comprising a representative range of clinically achievable drug concentrations for studying *in vitro* drug activity. As for rifampicin, a concentration of 16x *f*Cmax was added considering the high protein binding of this agent. When pyrazinamide was included, the medium pH was reduced from 6.6 to 5.6, which is considered to be essential for detection and evaluation of pyrazinamide activity (4, 5). At various time intervals during antibiotic exposure, samples were collected, centrifuged at 14000*xg* to avoid drug carry-over and subcultured onto solid medium. Plates were incubated for 28 days at 37°C with 5% CO_2 to determine colony forming units (cfu) counts. The lower limit of detection was 5 cfu/mL. All experiments were performed in duplicate. The bactericidal activity of the anti-TB drugs is expressed as the lowest concentration resulting in ≥ 99% killing of Mtb and as the mean daily fall in log 10 cfu/mL during the first two days and during six days of drug exposure, which is an approach similar to calculation of the early bactericidal activity (EBA) in TB patients (6). The *in vitro* sterilizing activity of the anti-TB drugs is expressed as the lowest concentration resulting in 100% killing of Mtb.

In the combination experiments, the anti-TB drugs were tested using combinations of isoniazid 0.01, 0.63 and 40 mg/L, rifampicin 0.002, 0.125 and 8 mg/L, streptomycin 0.02,

1.5 and 96 mg/L, ethambutol and para-amino salicylic acid 0.008, 0.5 and 32 mg/L and pyrazinamide 0.02, 1.25 and 80 mg/L. These concentrations were based on 1/1024x, 1/16x and 4x *f*Cmax, which was considered an appropriate range of concentrations for synergy testing. In the dual combinations, isoniazid was combined with rifampicin, streptomycin, ethambutol or pyrazinamide. Rifampicin was also combined with ethambutol or pyrazinamide. In the triple combinations, isoniazid and rifampicin were combined with streptomycin, ethambutol or pyrazinamide. The other triple combinations analysed were isoniazid - streptomycin - para-amino salicylic acid and streptomycin - ethambutol - pyrazinamide. In the quadruple combinations, isoniazid, rifampicin and pyrazinamide were combined with streptomycin or ethambutol.

Selection of drug-resistant Mtb

In order to assess selection of drug resistant mutants after 6 days of drug exposure, subcultures were also performed on solid media containing anti-TB drugs (3). The drug concentrations in the subculture plates were a 4-fold of the critical concentrations, i.e 0.8 mg/L for isoniazid, 4 mg/L for rifampicin, 40 mg/L for streptomycin, 20 mg/L for ethambutol, and 8 mg/L for para-amino salicylic acid (2). Pyrazinamide resistance was not determined due to technical issues related to the reduced medium pH required for pyrazinamide activity.

Endpoints for assessment of the activity of anti-TB drug combinations

The two endpoints were 1) synergy and 2) prevention of the emergence of drug resistance. Synergistic activity was defined as a ≥100-fold ($2\log_{10}$) increase in mycobacterial killing with the 2-drug combination compared to the most active single drug (or with 3- and 4-drug combinations compared to 2-drug and 3-drug combinations, respectively). The definition of synergy was also met when a 2-drug combination achieved elimination of Mtb after 6 days of drug exposure which was not achieved during single drug exposure (or 3-drug and 4-drug combinations compared to 2-drug and 3-drug combinations, respectively) (7, 8).

RESULTS

Concentration- and time-dependent bactericidal activity of anti-TB drugs at single drug exposure

Tables 1-3 summarize the comparative bactericidal and *in vitro* sterilizing activity of the anti-TB drugs at single drug exposure. Tables 1 and 2 illustrate that although isoniazid showed the highest mycobacterial killing rate, this agent failed to achieve elimination of Mtb at a concentration of 1x *f*Cmax (10 mg/L). The killing rate of rifampicin was less

rapid, but elimination of Mtb at day 6 was achieved at a concentration of 1x *f*Cmax (2 mg/L). Streptomycin was the only agent showing both rapid killing and elimination of Mtb at concentrations far below 1x *f*Cmax (< 24 mg/L). The bactericidal activity of ethambutol was moderate and no elimination was achieved. Both para-amino salicylic acid and pyrazinamide showed little activity. Lowering the pH of the medium from 6.6 to 5.6 as required for pyrazinamide activity resulted in inhibition of mycobacterial growth of the unexposed control sample (from $7.9x10^5$ to $2.6x10^5$cfu/mL in 6 days) compared to the growth of the control sample at a normal pH (from $6.2x10^5$ to $3.2x10^7$cfu/mL in 6 days). Ranking based on mean daily fall in cfu count at 1x *f*Cmax (Table 3) confirmed that streptomycin, rifampicin and isoniazid had the most prominent anti-TB drug activity at the end of drug exposure (day 6) with particularly rapid bactericidal activity (day 2) observed for streptomycin and isoniazid.

Lowest concentration (mg/L) resulting in ≥ 99% killing of Mtb during drug exposure			
	day 1	day 3	day 6
Isoniazid	0.63	0.16	0.63
Rifampicin	32*	0.5	0.008
Streptomycin	1.5	0.38	0.38
Ethambutol	>32*	8	2
PAS	>32*	>32*	>32*
Pyrazinamide	>80*	>80*	>80*

Table 1. Concentration- and time-dependent bactericidal activity (≥ 99% killing) of anti-TB drugs against *Mycobacterium tuberculosis* BE-1585 (Mtb) at 4-fold increasing concentrations during 6 days of drug exposure. Results shown are from experiments in duplicate.
*maximum concentration tested; PAS para-amino salicylic acid.

Lowest concentration (mg/L) resulting in elimination after 6 days of drug exposure	
Isoniazid	>40*
Rifampicin	2
Streptomycin	0.38
Ethambutol	>32*
PAS	>32*
Pyrazinamide	>80*

Table 2. Concentration-dependent sterilizing activity (100% killing) of anti-TB drugs against *Mycobacterium tuberculosis* BE-1585 (Mtb) at 4-fold increasing concentrations after 6 days of drug exposure. Elimination was defined as < 5 cfu/mL as this was the lower limit of detection of our time-kill kinetics assay. Results shown are from experiments in duplicate.
*maximum concentration tested; PAS para-amino salicylic acid.

	BA 0-2	BA 0-6
Isoniazid 10 mg/L	1.79	0.70
Rifampicin 2 mg/L	0.75	0.95
Streptomycin 24 mg/L	2.87	0.96
Ethambutol 8 mg/L	1.04	0.61
PAS 8 mg/L	0.07	0.17
Pyrazinamide 20 mg/L	0.05	0.15

Table 3. Bactericidal activity of different anti-TB drugs against *Mycobacterium tuberculosis* BE-1585 based on the mean daily fall in log10 cfu/mL at 1x *f*Cmax (*in vitro* bactericidal activity, BA) during the first 2 days (BA 0-2) and during 6 days (BA 0-6) of drug exposure. Results shown are from experiments in duplicate. PAS para-amino salicylic acid.

Selection of drug resistance only occurred upon single drug exposure to isoniazid at concentrations ranging from 0.04-40 mg/L. Regarding the other anti-TB drugs, single drug exposure did not result in selection of drug-resistant mutants.

Concentration- and time-dependent bactericidal activity of anti-TB drugs in dual combinations

The value of the addition of anti-TB drugs to isoniazid is shown in Table 4a,4b and Figure 1. Rifampicin was the only agent achieving synergy when added to isoniazid at low concentrations. Additionally, both rifampicin and streptomycin prevented the selection of isoniazid resistance. Ethambutol achieved synergy when combined with isoniazid and prevented the emergence of isoniazid resistance, but only at a high ethambutol concentration of 4x *f*Cmax (32 mg/L) (data not shown). Regarding other dual combinations of anti-TB drugs, rifampicin combined with ethambutol or pyrazinamide showed no synergistic activity at the required experimental conditions.

	none	Rifampicin 0.002		Rifampicin 0.125		Rifampicin 8	
	Res	Syn	Res	Syn	Res	Syn	Res
Isoniazid 0.01	*	-	*	-	*	-	*
Isoniazid 0.63	31%	-	0%	+(e)	0%	-	0%
Isoniazid 40	80%	-	0%	-	0%	-	0%

Table 4a. Synergistic activity (Syn) and prevention of selection of isoniazid resistance (Res) after 6 days of drug exposure in *Mycobacterium tuberculosis* BE-1585. Results shown are from experiments in duplicate. Isoniazid and rifampicin concentrations in mg/L.

* spontaneous mutation frequency of isoniazid ($1.6x10^5$); +, synergy; -, no synergy; e, elimination.

	none	Streptomycin 0.02		Streptomycin 1.5		Streptomycin 96	
	Res	Syn	Res	Syn	Res	Syn	Res
Isoniazid 0.01	*	-	*	-	*	-	*
Isoniazid 0.63	3%	-	0%	-	0%	-	0%
Isoniazid 40	100%	-	25%	-	0%	-	0%

Table 4b. Synergistic activity (Syn) and prevention of selection of isoniazid resistance (Res) after 6 days of drug exposure in *Mycobacterium tuberculosis* BE-1585. Results shown are from experiments in duplicate. Isoniazid and streptomycin concentrations in mg/L.

* spontaneous mutation frequency of isoniazid ($0.98x10^5$); -, no synergy.

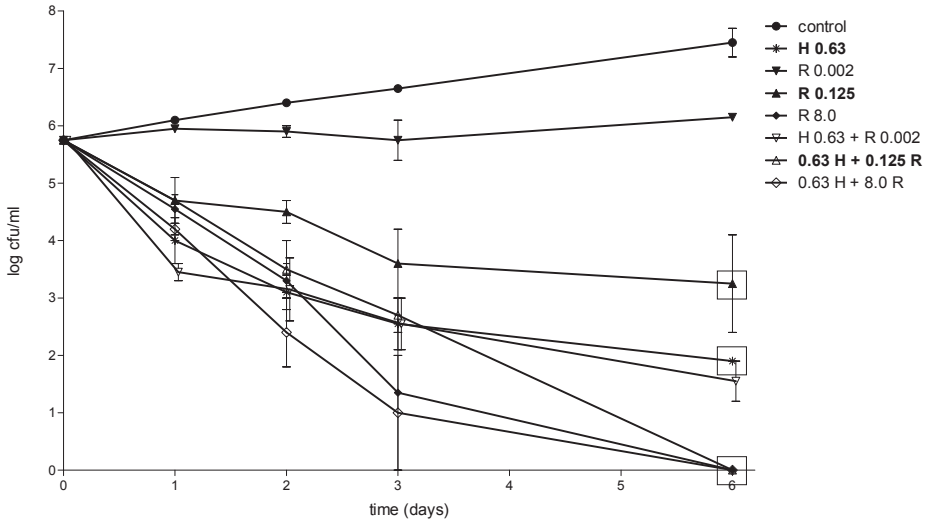

Figure 1. Concentration- and time-dependent bactericidal activity of isoniazid (H) combined with rifampicin (R) towards highly metabolically active Mtb. Mtb cultures were exposed to H or R alone or in combination for 6 days at 37°C under shaking conditions. On day 1, 2, 3 and 6 samples were collected, centrifuged and subcultured onto antibiotic-free and H- and R-containing solid media and incubated for 28 days at 37 °C with 5% CO_2 to determine colony forming units (cfu). Results shown are from experiments in duplicate.

Concentration- and time-dependent bactericidal activity of anti-TB drugs in triple and quadruple combinations

In none of the tested triple or quadruple combinations, the addition of a third or fourth agent resulted in synergy (data not shown).

DISCUSSION

In the present *in vitro* study, the concentration- and time-dependent bactericidal activities of six anti-TB drugs against the Mtb genotype strain Beijing 1585 were investigated at single drug exposure or in 2-, 3- or 4-drug combinations. At single drug exposure, we observed important differences in strength and rate of anti-TB drug activity between the different drugs. This is in line with previous *in vitro* studies and is confirming the distinctive added value of using the time-kill kinetics assay measuring bactericidal activity throughout the duration of drug exposure over classical *in vitro* susceptibility tests, such as the determination of the MIC assay measuring the bacteriostatic activity at the end of drug exposure (3, 9). Ranking based on strength and rate of mycobacterial killing *in vitro* showed that streptomycin, rifampicin and isoniazid were the most powerful anti-TB drugs. Streptomycin and isoniazid showed the most rapid bactericidal activity, while streptomycin and rifampicin showed sterilizing capacity against highly metabolically active, extracellular Mtb. Isoniazid plus rifampicin was the only combination showing synergistic activity at clinically relevant concentrations, whereas in none of the other dual, triple or quadruple drug combinations synergy was achieved in this model. Both rifampicin and streptomycin were able to prevent the selection of isoniazid-resistant mutants at clinically achievable concentrations.

Most of the previous studies assessing the *in vitro* activity of anti-TB drugs used the Mtb H37Rv/a genotype strain (3, 9-12), whereas the present study was conducted with the globally emerging Beijing genotype strain. The genetically distinct Beijing family of strains compared to other Mtb strains differ with respect to antibiotic susceptibility and the selection of anti-TB drug resistant mutants (13, 14). In this context, Beijing genotype TB infections have been associated with large outbreaks, increased virulence and a less favourable treatment outcome related to (multi)drug resistance. Our study is unique in testing the activities of six different anti-TB drugs against the Mtb Beijing genotype strain not only at single drug exposure, but also in multiple dual, triple and quadruple drug combinations. This is important for proper evaluation of the value of the time-kill kinetics assay as an asset to a preclinical modeling framework assessing the potency of anti-TB drugs. Our data obtained with single drug exposure in the Beijing genotype Mtb strain are in agreement with our previous studies using the H37Rv Mtb strain indicating that the activity of isoniazid, rifampicin and ethambutol behave similarly in both strains

under the conditions of high metabolic activity (3, 9). However, the existence of any Mtb strain-related differences regarding anti-TB drug activity of 2-, 3- and 4-drug combinations using the present or other preclinical models remains to be determined (13).

To evaluate the predictive value of our *in vitro* drug activity data for drug efficacy during treatment in TB patients, we consider early bactericidal activity (EBA) the most appropriate parameter for comparison. The EBA is calculated as the mean daily fall in Mtb cfu count during the first two days of therapy (EBA 0-2) (6, 15). The EBA 0-2 is thought to represent early bactericidal activity against rapidly growing, highly metabolically active, predominantly extracellular Mtb in the first phase of infection, which is also the Mtb population studied in the present *in vitro* assay.

Comparing the data at single drug exposure, the most striking similarity is the rapid bactericidal activity of isoniazid observed both *in vitro* and in EBA 0-2 (6, 15). In addition, isoniazid could not achieve elimination of Mtb *in vitro,* in contrast to both rifampicin and streptomycin. These *in vitro* observations seem in line with the observations in EBA 2-14, which is believed to investigate the next phase of infection requiring the activity of anti-TB drugs against Mtb with lower metabolic activity, which possibly represents sterilizing capacity in the clinical setting (15). It cannot be excluded that low active Mtb subpopulations are also present in our *in vitro* assay. As to rifampicin, higher bactericidal activity compared to isoniazid was observed *in vitro*, but its activity was less rapid which is in agreement with its EBA 0-2 (6, 15). The most striking discrepancy between our *in vitro* activity data and EBA 0-2 data is the activity of streptomycin. Although streptomycin was the only anti-TB drug with (extremely) rapid bactericidal activity *in vitro*, including elimination of Mtb*,* the EBA 0-2 of streptomycin was low (6, 15). In general, the poor intracellular penetration capacity of aminoglycosides is well known (16), however cannot explain the discrepancy between our *in vitro* data and the EBA 0-2 data as in both studies predominantly extracellular Mtb is targeted. Possibly the poor penetration of aminoglycosides in the infected lung tissue after intravenous administration contributes to the discrepancy in streptomycin activity *in vitro* compared to clinical EBA 0-2 (17).

Regarding pyrazinamide, it can be concluded that the present assay is unsuitable for studying pyrazinamide activity against the highly active Mtb subpopulation as lowering the pH required for pyrazinamide activity compromised mycobacterial growth. It could be argued that studying pyrazinamide activity against this particular Mtb subpopulation is not desirable as pyrazinamide is believed to lack activity against highly active Mtb, which is in line with its EBA 0-2 (5, 6). However, a recent study using continuous cultures in chemostats showed similar pyrazinamide activity in both highly and low active Mtb subpopulations at a constant pH of 6.3 (18), suggesting that studying both Mtb subpopulations might provide useful information. As to the limited activity of pyrazinamide observed in the present study, this is in line with previous *in vitro* studies showing that pyrazinamide activity was highest in 3-months old static cultures underlining the

importance of selecting the proper Mtb subpopulation when studying different anti-TB drugs (4, 19). Finally, ethambutol performed better in our *in vitro* assay compared to its EBA 0-2 (6, 15). Interestingly, increasing the ethambutol dose of 15 mg/kg to 25 mg/kg resulted in a substantial increase in EBA 0-2. Further exploration of clinical dosing is required to establish whether the currently recommended dose of ethambutol requires adjustment. The observations described above emphasize that detailed knowledge on drug characteristics and mode of action as well as on mycobacterial growth rate is crucial for optimal design of *in vitro* studies to evaluate the activity of novel anti-TB drugs. This means that the decision to discard the potential of novel anti-TB drugs should not be based solely on disappointing results of *in vitro* assays.

Comparing the data obtained with anti-TB drug combinations, there was no correlation between the results of our *in vitro* study and the results on EBA 0-2. Probably the strong EBA 0-2 of isoniazid does not leave room for improvement with other anti-TB drugs. Observations regarding prevention of the emergence of anti-TB drug resistance could not be compared as in EBA studies, no drug resistance was observed at single drug exposure, in contrast to the selection of isoniazid resistance *in vitro*.

When evaluating the predictive value of preclinical models assessing different combination anti-TB drug regimens, the outcome of clinical trials are ultimately the most reliable comparators. In clinical trials, combination drug regimens are investigated during the different phases of a full treatment course requiring multiple anti-TB drugs with distinct properties and activities against Mtb subpopulations with both high and low metabolic activity. The main outcome parameters in these clinical studies are the prevention of the emergence of drug resistance, the percentage of culture negative sputum samples after two months of therapy and the prevention of disease relapse (cure), the latter two parameters reflecting the sterilizing capacity of anti-TB drug regimens (20). In the present *in vitro* assay, bactericidal activity against highly metabolically active Mtb was determined, reflecting drug activity during the first days of anti-TB treatment in the clinical setting while not measuring drug activity against low metabolically active Mtb more likely associated with sterilization. In that respect, *in vitro* activity against Mtb with low metabolic activity and non-replicating Mtb might provide important additional information, which has already been shown in previous studies (3, 9, 11, 12).

In summary, our *in vitro* assay provides valuable information as to the bactericidal activity of anti-TB drugs during the first (early) phase of therapy. The results emphasize the relevance of investigating anti-TB drug activity at single drug exposure or in dual combinations only. By generating data on anti-TB drug activity, suitable for incorporation into a predictive modeling framework, the time-kill kinetics assay may serve as the basis for preclinical anti-TB drug development.

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CHAPTER 5

Summarizing discussion and future perspectives

SUMMARIZING DISCUSSION

Infections with nontuberculous mycobacteria (NTM) and tuberculosis (TB) are distinct mycobacterial diseases with clear differences in epidemiology, transmission, clinical picture, diagnosis and therapy. Nevertheless, they share several characteristics including the complexity of treatment. Improving treatment success of mycobacterial infections requires a multifactorial approach, including recognition of underlying genetic or acquired host predispositions (**chapter 2**) as well as different treatment strategies to increase antimycobacterial drug efficacy (**chapter 3**). In this context, it is crucial to compose an optimal predictive preclinical modeling framework, allowing rapid and reliable identification of the potency of novel antimycobacterial agents and their translation into clinical practice. Such a modeling framework is currently under construction for anti-TB drugs (**chapter 4**).

In **chapter 2 (2.1)** a novel genetic defect in Signal Transducer and Activator of Transcription 1 (*STAT1*) is described in a patient suffering from disseminated *Mycobacterium avium* infection. STAT1 is a key component of interferon gamma (IFN-γ) and interferon alpha (IFN-α) signaling and mediates protection against intracellular pathogens including mycobacteria and NTM in particular (1). A novel, autosomal dominant mutation in the SH2 domain of *STAT1* is described with a dominant negative effect on STAT1 phosphorylation and DNA binding activity reducing IFN-γ driven gene expression, while preserving IFN-α related immunity. The importance of unravelling the underlying genetic defect in this patient is illustrated by the fact that with adequate and ongoing antibiotic therapy as well as with IFN-γ supplementation, no recurrence of NTM disease occurred.

The SH2 domain of STAT1 is important for the recruitment of STAT1 molecules to the activated IFN-γ receptors and for dimerization of activated STAT1 molecules and subsequent binding to particular DNA sequences resulting in gene transcription (2). Although homozygous mutations in the SH2 have been described compromising both IFN-γ and IFN-α responses (3), in **2.1** we described the first heterozygous mutation in the SH2 domain compromising IFN-γ responses, but preserving responses to IFN-α. After this publication, two other heterozygous, dominant negative mutations in the SH2 domain were identified affecting STAT1 phosphorylation and DNA binding activity and thereby reducing IFN-γ induced responses while preserving responses to IFN-α (4). Recently, the first heterozygous gain-of-function mutation in the SH2 domain of *STAT1* was described enhancing STAT1 phosphorylation, nuclear translocation and STAT1-driven gene expression and protein production (5). This hyper-responsiveness to IFNs inhibits differentiation of T helper 17 cells explaining the association with chronic mucocutaneous candidiasis, which is the main disease manifestation (6). In addition, *STAT1* gain-of-function mutations have been associated with viral and disseminated fungal infections (7) as well as with clinical pictures resembling IPEX (immune dysregulation, polyendocri-

nopathy, enteropathy, X-linked) (8). In patients with disseminated fungal infections and *STAT1* gain-of-function mutations, other potential explanatory mechanisms have been described, including lower STAT1 methylation, enhanced STAT1/PIAS1 (protein inhibitor of activated STAT1) association and an impaired response to IFN-γ re-stimulation (7).

In **chapter 3,** we focused on several strategies for improving the success of antimycobacterial treatment.

In **3.1** we described four patients suffering from refractory disseminated mycobacterial infections due to mutations in the IFN-γ receptor, who were treated with IFN-α. Although the extent of the clinical response varied between patients, IFN-α treatment was associated with either disease improvement or disease stabilization. Importantly, in no case did IFN-α exacerbate the infection. We showed *in vitro* and *ex vivo,* that IFN-α stimulated both classically IFN-α and more classically IFN-γ regulated genes. In addition, sustained mycobacteria-induced cytokine production was observed *in vitro*. Although the signaling pathways and immunological functions of IFN-γ and IFN-α are thought to be distinct, they overlap through the common use of signal transducers Janus kinase (JAK) 2 and STAT1, both forming STAT1 homodimers and activating some of the same genes (1). Interestingly, it has been shown that low dose IFN-γ followed by IFN-α stimulation leads to upregulation of typical IFN-γ inducible genes, shifting the IFN-α response to a more pro-inflammatory phenotype (9). Furthermore, ISG15 deficiency in patients has been associated with mycobacterial infections (10). ISG15 is induced by IFN-α and activates T- and NK cells to produce IFN-γ, supporting valuable crosstalk between both IFNs with regards to mycobacterial defense. We concluded that IFN-α may overcome some aspects of impairment in IFN-γ signaling and may confer clinical benefits in a subset of patients with refractory disseminated mycobacterial infections related to impaired IFN-γ signaling. Our results are in agreement with several other studies supporting a beneficial role of IFN-α or interferon beta (IFN-β) (both comprising the group of type 1 IFNs) in antimycobacterial defense. A few small studies in TB patients showed clinical resolution when conventional anti-TB drug regimens were accompanied by IFN-α supplementation (11, 12). *In vitro,* IFN-β has been shown to promote dendritic cell maturation and IL-12p70 production in response to infection with *Mycobacterium bovis* bacillus Calmette Guérin (BCG) (13). In *Mycobacterium tuberculosis* (Mtb) infected mice, a protective role of type 1 IFNs against TB was shown by limiting the number of recruited Mtb infected macrophages (14). Similarly, other studies in mice confirmed the importance of type 1 IFNs for the protection against infections with *M. avium* and *M. bovis* BCG (15, 16). In contrast to these observations, it should be recognized that there are multiple studies arguing against a beneficial role of IFN-α in mycobacterial defense. From a clinical perspective, several reports were published on the association of active TB and IFN-α treatment in the context of hepatitis C (17). However, the lack of a control group of hepatitis C patients not on treatment impedes drawing definite conclusions regarding a causal relationship. In fact, hepatitis C and active TB

share several risk factors such as homelessness, alcoholism, incarceration and infection with the human immunodeficiency virus (HIV). In addition, hepatitis C as well as advanced liver disease have been identified as independent risk factors for active TB (18, 19). In line with these observations, a recent cohort study comparing hepatitis C patients with and without IFN-α treatment did not demonstrate a significant association between IFN-α treatment and active TB (20). An insignificantly higher TB incidence rate in patients on IFN-α was reported, but confidence intervals were wide. Interestingly, a type 1 IFN transcriptional signature in patients with active TB has been described, suggesting a role for these cytokines in TB control (21, 22). It should be noted that in one of these studies IFN-γ related genes were also upregulated. In connection with this it could be argued that gene upregulation during active infection does not necessarily prove causality, but could also reflect induction of responsive defense mechanisms (21). Besides clinical reports, several preclinical studies reported on type 1 IFNs inhibiting IFN-γ induced signaling. As such, *in vitro* studies have shown that type 1 IFNs significantly reduced IFN-γ induced expression of CD64 and CD54 as well as production of the cytokines IL-1b, TNF-α and IL12p40, which are believed crucial for mycobacterial defense (23, 24). Similarly, type 1 IFNs were shown to inhibit the ability of human macrophages to control the growth of *M. bovis* BCG and *M. avium* (25, 26). Studies in mice treated with type 1 IFN (or polyinosinic-polycytidylic acid, polyIC, a potent type I IFN inducer) showed aggravation of TB and improved control in IFN-α receptor knock out mice (27, 28). Possible mechanisms underlying this antagonism postulated are IFN-γ downregulation, prevention of STAT1 homodimer formation and induction of the STAT1 inhibitor PIAS1 (23). The question arises why the data on the role of IFN-α in mycobacterial defense are contradictory. Some of the discrepancies in preclinical studies could be explained by differences in experimental design, such as the type and activation state of cells and the mycobacterial species investigated, since these factors have been shown to influence immune responses (29, 30). In addition, both pro- and anti-inflammatory properties of type I IFNs during TB infection have been demonstrated (14). The transition from pro- to inflammatory effects has been shown to be time-dependent and influenced by the balance between IFN-α and IFN-γ production. As such, the effect of IFN-α on mycobacterial defense has been shown to be different in early versus late infection (16, 31). In this respect, it has been shown that mice lacking both the IFN-γ and the IFN-α receptors died significantly earlier from TB compared to mice lacking the IFN-γ receptor alone (14). It could be speculated that the effect of IFN-α supplementation depends on the specific patient population (with or without proper IFN-γ signaling) as well as on the particular mycobacterial species involved. Considering the conflicting data it should be concluded that IFN-α treatment seems not advisable in the vast majority of patients with mycobacterial infections.

In **3.2** we investigated the *in vitro* potentiation of the activity of the macrolide clarithromycin against *M. avium*. Clarithromycin is the cornerstone drug in the treatment of many

NTM infections, including infections with *M. avium* (32). Although the introduction of macrolides including clarithromycin improved the success of treatment of *M. avium* infections, the overall prognosis is still poor (33). We investigated whether the addition of tigecycline could enhance the activity of clarithromycin. Tigecycline is a broad-spectrum glycylcycline antimicrobial agent already used in clinical practice for other indications (34). Remarkably, our study showed that at clinically relevant concentrations, clarithromycin activity against *M. avium* was highly variable, was unable to achieve mycobacterial elimination and often resulted in clarithromycin resistance. These observations might explain the currently unpredictable and disappointing efficacy of macrolide treatment of *M. avium* infections (33). The results also make quite clear that our dynamic *in vitro* time-kill kinetics assay, determining the drug concentration-dependent mycobacterial killing over time, can detected important differences in antibacterial dug behavior that cannot be detected when using static susceptibility assays such as determination of the Minimal Inhibitory Concentration (MIC). This is in line with our previous observations (35-37). Interestingly, we showed that tigecycline enhanced clarithromycin activity against *M. avium* and prevented clarithromycin resistance. To assess the value of the potentiating effect of tigecycline in the treatment of *M. avium* infections, further studies including pharmacokinetic/pharmacodynamic models, intracellular infection models as well as *in vivo* mouse models are needed.

In **3.3** another strategy for potentiating antimycobacterial drug activity was investigated *in vitro* in Mtb. In this study we focused on increasing intracellular concentrations of anti-TB drugs by using colistin, which is known for its destabilizing effect on the bacterial membrane. The complex lipid architecture of the mycobacterial cell wall forms an effective permeability barrier limiting the efficacy of current anti-TB drugs. Colistin, a member of the polymyxin group of drugs is already used in clinical practice for the treatment of drug-resistant Gram-negative bacterial infections. The proposed mechanism of action of colistin is through interaction with the Gram-negative bacterial membrane resulting in destabilization and leakage of intracellular contents and apoptosis (38). Although the mycobacterial cell wall composition is different from Gram-negative bacteria, its interaction with colistin and colistin-like particles has been shown to result in increased permeability (39, 40). Therefore we hypothesized that colistin might enhance anti-TB drug activity by increasing the mycobacterial cell wall permeability allowing increased intramycobacterial concentrations of anti-TB drugs. In order to enhance the clinical relevance of our *in vitro* studies, drug activity against Mtb with high as well as low metabolic activity was investigated. Several important observations came from this study. First, we identified two new potent anti-TB drug combinations inducing rapid and strong killing of Mtb. It is the first study providing evidence that colistin can indeed potentiate the activity of isoniazid as well as amikacin *in vitro*. We also showed that colistin could prevent the emergence of isoniazid and amikacin resistance. The

results of our study have recently been confirmed by another study showing that the prodrug colistin methanesulfonate could enhance isoniazid activity against Mtb *in vitro* (41). Ultrastructure analyses showed disruption of the outer polysaccharide layer of Mtb supporting the hypothesis that the effect of colistin could be mediated by increasing intramycobacterial anti-TB drug concentrations (41). This hypothesis needs to be confirmed by measuring intramycobacterial drug concentrations. A second important observation from this study is that the degree of drug activity in terms of mycobacterial killing and prevention of the emergence of drug resistance depended on the metabolic state of Mtb, which is in line with previous studies (36, 42, 43). Interestingly, the potentiating effect of colistin was only observed in the Mtb population with high metabolic activity. These observations emphasize the importance of investigating mycobacterial subpopulations with different metabolic activities when assessing the activity of anti-TB drugs *in vitro*. Distinguishing between the different Mtb populations might be clinically relevant as Mtb with high metabolic activity is supposed to be responsible for spreading of TB, whereas Mtb with low metabolic activity better represents Mtb that is present in deep-seated tissue infection and is probably responsible for relapse of TB. In view of our observation that relatively high colistin concentrations were needed to potentiate anti-TB drugs, and data from literature that colistin concentrations in broncheoalveloar lavage fluid are rather low after intravenous administration (44), we discuss the potential advantage of the administration of colistin via inhalation. Colistin inhalation is already successfully used in clinical practice for the treatment of pneumonia by resistant Gram-negative bacteria in patients with cystic fibrosis and (ventilator associated) pneumonia (45). Through inhalation, higher local drug concentrations at the primary infected site can be achieved while minimizing systemic drug toxicity. This could not only lead to enhanced activity against drug-susceptible Mtb, but the increased local drug concentrations might also exhibit activity against less susceptible Mtb strains (46).

In **chapter 4,** the need of developing novel treatment strategies against mycobacterial infections, and the challenge to establish which preclinical models are most predictive of clinical efficacy are discussed for anti-TB drugs. Although many different preclinical models determining the activity and therapeutic efficacy of anti-TB drugs are available, it is yet unclear which models provide the highest translational value. PreDiCT-TB is a European multidisciplinary consortium focusing on anti-TB drug development (www.predict-tb.eu). The main goal of PreDiCT-TB is to find the combination of preclinical models that is most representative of response in TB patients. As such, the results of the PreDiCT studies are back-validated using historical clinical data on the most important drug combinations in TB patients. In this way, an integrated modeling framework will be constructed, serving as a reliable method to study novel anti-TB drug regimens and forming the basis for the clinical trial design.

In **4.1** we studied the role of the *in vitro* time-kill kinetics assay as an asset to a predictive preclinical modeling framework assessing anti-TB drug activity. The concentration- and time-dependent killing capacities of six anti-TB drugs used in clinical practice alone or in combination against a Beijing genotype Mtb strain were determined *in vitro*. Ranking based on rate and strength of mycobacterial killing showed that streptomycin, rifampicin and isoniazid were most active against Mtb with high metabolic activity. Isoniazid in combination with rifampicin or high dose ethambutol were the only synergistic drug combinations. The addition of a third or fourth anti-TB drug did not result in further synergy. Both rifampicin and streptomycin were able to prevent the selection of isoniazid resistance. The challenging question is how to relate these data on *in vitro* anti-TB drug activity to historical clinical data on therapeutic drug efficacy. In this respect, the results of clinical trials are ultimately the most reliable comparators when evaluating the predictive value of preclinical models assessing different combination anti-TB drug regimens. It is well known that during TB treatment, different phases can be distinguished (47). The first days of therapy are characterized by a rapid reduction of rapidly growing mycobacteria. The next phase is characterized by a much slower decrease in mycobacterial load in which the persisting mycobacteria with a lower replication rate, including those mycobacteria that are non-replicating (dormant), are killed, which process is referred to as sterilization (47). In anticipation of this fact, anti-TB treatment requires a combination of drugs showing activities against Mtb with both high and low metabolic activity as well as non-replicating Mtb. Clinical trials investigate combination anti-TB drug regimens during different phases of a full treatment course. The main outcome parameters are the prevention of the emergence of drug resistance, the percentage of negative sputum samples after two months of therapy and disease relapse, the latter two parameters reflecting the sterilizing capacity of anti-TB drug regimens. The present *in vitro* assay evaluates drug activity during the first few days of TB treatment in the clinical setting by assessing the bactericidal activity against highly metabolically active (rapidly growing) Mtb, while not measuring drug activity against Mtb with low metabolic activity, related to sterilization. In that respect, determination of *in vitro* activity against Mtb with low metabolic activity and non-replicating Mtb may provide important additional information, which has already been shown in previous studies (36, 42, 43). Moreover, during a full course of anti-TB drug therapy, other factors not included in our *in vitro* assay play a role in clinical success, such as drug penetration into macrophages harbouring intracellular Mtb and into TB lesions (48) as well as drug tissue distribution, pharmacokinetic/pharmacodynamic properties and host-pathogen interactions. We concluded that the time-kill kinetics assay provides important information on the mycobacterial killing dynamics of anti-tuberculosis drugs during the early phase of drug exposure. As such, this assay is a valuable component of the preclinical modeling framework

FUTURE PERSPECTIVES

I. Infections with nontuberculous mycobacteria (NTM)

One of the most important findings of our *in vitro* study on drug activity against *Mycobacterium avium* **(3.2)** is that the activity of the current cornerstone drug clarithromycin is unreliable, underlining the importance of investigating novel strategies to improve anti-NTM drug activity against *M. avium,* especially since this is the leading cause of NTM disease worldwide. Besides *M. avium,* there is a strong need to identify novel treatment strategies for *Mycobacterium abscessus* as this is one of the most difficult to treat NTM species due to its multi-drug resistant character.

One of the major challenges in current anti-NTM treatment is to achieve appropriate drug levels at the site of infection. Improving drug efficacy should include strategies to increase drug concentrations within mycobacteria, in infected target cells (macrophages) as well as in the lung compartment. These strategies could be used to improve the activity of the current cornerstone drugs as well as the activity of novel anti-NTM drugs. Several strategies are worthwhile exploring based on studies performed by our research group as well as by others.

A. Increasing mycobacterial cell wall permeability

Studies with different mycobacterial species including *M. avium* have shown that changing or destabilizing the mycobacterial cell wall resulted in increased mycobacterial drug activity (49, 50). Based on our previous results in **3.3**, showing that colistin could potentiate the activity of two anti-tuberculosis (anti-TB) drugs, it is interesting to investigate the potential of colistin to increase the activity of anti-NTM drugs towards *M.avium* and *M. abscessus in vitro.* Next, the most promising combinations should be further studied in our intracellular infection model as well as in our mouse model of disseminated NTM infection (51). When successful, we might consider the application of colistin for pulmonary NTM infections which step would be relatively uncomplicated as inhalational colistin is already used in current clinical practice (45).

B. Inhibition of mycobacterial efflux pumps

Another potential strategy increasing the potency of antimycobacterial drugs and decreasing the emergence of drug resistance is the use of efflux pump inhibitors (EPIs). Accumulating evidence suggests an important role for mycobacterial efflux pumps in the extrusion of antimycobacterial drugs before reaching their target (52). In *Mycobacterium tuberculosis* (Mtb) overexpression of mycobacterial efflux pumps is considered a major factor leading to drug resistance (53, 54). It has been shown that EPIs inhibit the expression of genes encoding efflux pumps in Mtb, and thereby also reduce the activity of existing efflux pump proteins (53, 55). As such, EPIs have been shown to reduce

anti-TB drug resistance (56-60) and to increase the activity of current as well as novel anti-TB drugs both *in vitro* and in *in vivo* mouse models (56, 60-62). Only a few studies investigated efflux pumps and EPIs in the context of NTM. Knocking out the efflux pump gene *lfrA* resulted in accumulation of ethidium bromide and increased susceptibility and decreased resistance to ethambutol and ciprofloxacin in *Mycobacterium smegmatis* (63, 64). Similarly, EPIs have been shown to increase ethidium bromide accumulation in *M. avium* which was associated with reduced macrolide resistance (65). Between the different EPIs investigated, verapamil is of particular interest. Verapamil has been shown to be a potent mycobacterial EPI increasing the activity of multiple anti-TB drugs *in vitro* and in intracellular infection models (56, 66) and was also used in *M. avium* studies (65). Verapamil is already approved by the Food and Drug Administration (FDA) for the treatment of certain cardiovascular diseases facilitating clinical application in mycobacterial infections. Moreover, verapamil has been shown to accumulate in lung tissue in experimental animals (67), which is an attractive property for the intended use in pulmonary mycobacterial infections. Recently, an inhalable dry power consisting of verapamil combined with the anti-TB drug rifapentin was shown to increase mycobacterial killing capacity against Mtb inside macrophages (68). The data obtained further promote verapamil as a candidate in the examination of the role of EPIs in increasing anti-NTM drug activity. However, we should keep in mind that the calcium channel blocking property of verapamil making it a useful drug in the treatment of cardiac arrhythmia's, is undesirable in terms of safety and tolerability when used in patients with mycobacterial infections. Importantly, the efflux pump blocking effects of verapamil have been suggested to be unrelated to its calcium blocking activity (66, 69) stimulating further investigation of the applicability of verapamil derivates with decreased calcium blocking activity, such as R-verapamil or norverapamil (66).

Next to verapamil, other EPIs should be explored as well such as thioridazine, which has been extensively studied in the context of Mtb (53). Although thioridazine toxic side effects are a major drawback, current studies are exploring strategies to reduce toxicity in this way reinstating thioridazine as a candidate EPI (70, 71). In the presence and absence of EPIs, the activity of different anti-NTM drugs against *M. avium* and *M. abscessus* should be assessed. Next, the most promising drug combinations should be selected and further investigated in our intracellular infection model and in our mouse NTM infection model.

C. Increasing drug concentrations in infected tissues and infected macrophages

Besides increasing antimycobacterial drug concentrations of anti-NTM drugs, future attempts should focus on increasing drug concentrations in the infected lung compartment (through inhalation) as well as inside infected macrophages. As to enhancing intra-macrophage drug concentrations, the use of nanocarriers encapsulating antimy-

cobacterial drugs is of potential interest, as nanoparticles are effectively phagocytized by macrophages (72). In addition, following encapsulation, the drugs will be protected from degradation and the release (slow versus fast) can be influenced depending on the type of nanoparticles, which factors are all contributing to increased efficacy (73, 74). Although most published studies using nanocarriers were performed with Mtb, also for *M. avium* the beneficial effect of nanocarrier formulations regarding anti-NTM drug activity has been demonstrated in intracellular infection models and in *in vivo* animal models (51, 75-77). Nanocarriers can also be used to exploit the synergistic interaction of a specific drug combination by co-encapsulation of the drugs in the same nanocarrier (78). In this way, parallel distribution in tissues or cells of both drugs may be ensured and the synergistic activity at the infectious focus guaranteed. In this respect, co-encapsulation of antimycobacterial drugs in nanocarriers may open new perspectives in the treatment mycobacterial infections.

To support the efforts of increasing intramycobacterial concentrations of anti-NTM drugs, tools to measure those concentrations are needed. In this context, the use of confocal fluorescence microscopy as an innovative tool for measuring intramycobacterial drug concentrations should receive attention. An important subject of research is to investigate whether increased anti-NTM drug activity as observed in extracellular and intracellular mycobacteria can be related to increased drug concentrations in mycobacteria and in infected macrophages, respectively. In addition, fluorescence recovery after photobleaching (FRAP) might be used to determine efflux rates of anti-NTM drugs when investigating the effect of different EPIs. Super resolution microscopy might provide information on the specific localization and accumulation of different anti-NTM drugs within infected macrophages in our intracellular infection model. Preliminary results generated in a collaborative project with the Erasmus MC Optical Imaging Centre showed that different anti-NTM drugs could be visualized in labeled extracellular *M. avium*. Intramycobacterial antibiotic concentrations can now be quantified in the presence and absence of different drug potentiators, such as EPIs and perhaps in a later stage cell wall destabilizers, such as colistin. Recently obtained data showed that although increased anti-NTM drug activity in the presence of EPIs was observed *in vitro*, this could not be linked to increased intramycobacterial drug concentrations by fluorescence microscopy. This might be due to the fact that the antibiotic concentrations needed to achieve a substantial fluorescent signal were much higher than the concentrations used in our *in vitro* assay. In future studies we should proceed with fluorescent-labeling of antibiotics, enabling the investigation of much lower (clinically relevant) anti-NTM drug concentrations with and without different potentiating agents.

In summary, future NTM research should focus on improving anti-NTM drug efficacy by increasing exposure of mycobacteria to anti-mycobacterial drugs at three different levels: 1) the infected tissue, 2) the extracellular mycobacteria and 3) the intracellular mycobacteria inside macrophages. These strategies could be used to increase the activity and therapeutic efficacy of the current cornerstone anti-NTM drugs as well as potential novel anti-NTM compounds.

II. Tuberculosis (TB)

The main reason for the current lengthy TB treatment duration is the difficulty to eradicate the non-replicating (or dormant) Mtb subpopulation, which is responsible for disease persistence and relapse. In order to shorten treatment duration, anti-TB drug development should focus on improving drug activity against the Mtb subpopulation in the low metabolic state. For this purpose, identifying the dormant Mtb subpopulation in different preclinical models is important, allowing appropriate investigation of the required properties of novel anti-TB drugs, which is sterilizing activity against this particular Mtb subpopulation. This could significantly add to the predictive value of preclinical models as illustrated by our main finding in **4.1** demonstrating that the predictability of our current *in vitro* time-kill kinetics assay assessing drug killing activity against actively multiplying mycobacteria is limited to the early phase of TB treatment in the clinical setting. However, identification of dormant Mtb in preclinical models is difficult as so far, this subpopulation could not be cultured *in vitro*. In this context, investigating the potential of resuscitating promoting factors (RPFs) to help revival of the non-replicating Mtb subpopulation should receive attention. RPFs are a family of proteins produced by Mtb acting on the mycobacterial cell wall and stimulating growth of dormant, otherwise non-culturable Mtb in *in vitro* and in mouse models of TB as well as in sputum samples of TB patients (79, 80). However, the methods used in these studies are complex, time-consuming using one read-out only based on estimates of mycobacterial load. Therefore, different techniques for investigating the potential of RPF to revive the non-replicating Mtb subpopulations should be used and compared including mycobacterial staining, determination of the time to positivity (TTP), molecular detection as well as traditional mycobacterial cultures. If successful, the combination of assays might be used to compare current and novel anti-TB drugs for their activity against the dormant Mtb subpopulation. In addition, further insight could be gained by assessing the sterilizing activity of anti-TB drugs in the RPF knockout Mtb strain (81). Proper identification of the dormant Mtb subpopulation *in vitro* allows further research in our intracellular infection model and in our mouse TB model (82, 83).

Other strategies focusing on the dormant Mtb subpopulation include the use of a streptomycin-dependent Mtb strain or the use of potassium depletion. The streptomycin-dependent Mtb strain 18b typically requires streptomycin for its growth (84). Im-

portantly, the strain maintains viability when kept in streptomycin-free culture medium and growth is recovered when streptomycin is added, which approach has been shown to mimic dormancy in animal TB models (84). Potassium depletion has recently been shown to trigger Mtb dormancy *in vitro,* which could be reverted by potassium repletion and as such could be an elegant way to study dormancy (85).
Besides focusing on the dormant Mtb population, it is also informative to establish the activity of anti-TB drugs in the culturable Mtb population with high versus low metabolic activity. In addition, most helpful in improving predictability of preclinical models is to include the hollow fiber system model of TB as a novel tool, approved both by the FDA and the European Medicines Agency (EMA). This pharmacokinetic/pharmacodynamic model has been shown particularly suitable for determining optimal drug exposure in terms of dosage and time in anticipation of clinical use in TB patients (86).
Regarding novel treatment strategies, as discussed in the NTM section, efflux pumps are interesting targets to increase intramycobacterial drug concentrations, also in the context of Mtb. As such, efflux pump inhibition has already been investigated by our research group and should be further explored (60, 87). The concept of the use of colistin as a potentiator of anti-TB drug activity should be further substantiated in our models of intracellular infection, hollow fiber system and mouse TB infection. Other strategies worthwhile exploring are optimization of drug dosing schedules (82, 88) as well as mode of administration, including antibiotic inhalation and nanocarrier medicated drug targeting. Next to a better use of existing anti-TB drugs, investigating the potential of novel compounds is crucial with particular interest in targeting the dormant Mtb subpopulation.

In summary, future TB research should focus on improving the sterilizing capacity of anti-TB drug regimens in order to shorten treatment duration. To that aim, identification and targeting the dormant Mtb subpopulation in different preclinical models is crucial, thereby improving their predictive value and forming a reliable basis for future clinical trial design.

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CHAPTER 6

Nederlandse samenvatting

Infecties met non-tuberculeuze mycobacteriën (NTM) en tuberculose (TB) zijn twee verschillende ziektebeelden, maar worden beiden gekenmerkt door een complexe behandeling. Dit is het resultaat van een nauwgezet samenspel tussen de gastheer (de patiënt) en de ziekteverwekker (mycobacteriën). Het verbeteren van de behandeling van mycobacteriële infecties vereist een multifactoriële aanpak waarvan verschillende facetten in dit proefschrift worden belicht. Enerzijds is het van belang om patiënt gerelateerde factoren te identificeren, die van invloed kunnen zijn op het succes van de behandeling. Zo is het herkennen en aantonen van verschillende predisponerende factoren van belang waarvan er een in **hoofdstuk 2** wordt besproken**.** Anderzijds is het van belang om strategieën te ontwikkelen die leiden tot verbetering van de effectiviteit van de huidige antibiotica tegen mycobacteriën. In **hoofdstuk 3** worden drie verschillende strategieën besproken. Dat verbetering van therapie noodzakelijk is, wordt algemeen onderkend. Het is op dit moment echter nog onduidelijk welke combinatie van preklinische modellen om antibiotica activiteit tegen mycobacteriën te onderzoeken, het beste voorspelt hoe effectief deze middelen daadwerkelijk zijn in patiënten met mycobacteriële infecties. Dit komt in **hoofdstuk 4** aan bod. De bevindingen van de verschillende studies worden hier kort uiteengezet.

In **hoofdstuk 2 (2.1)** wordt een nieuwe mutatie in het Signal Transducer and Activator of Transcription 1 (*STAT1*) beschreven in een patiënt met een gedissemineerde NTM infectie. Het *STAT1* gen codeert voor een eiwit, dat essentieel is voor de werking van interferon gamma (IFN-γ) en van belang is voor de afweer tegen verscheidene bacteriën, waaronder mycobacteriën, virussen en schimmels/gisten. Een nieuwe, autosomaal dominante mutatie in het SH2 bindend domein van *STAT1* wordt geïdentificeerd, die geassocieerd is met gestoorde STAT1 fosforylering en binding van STAT1 aan het DNA in de celkern van geïnfecteerde cellen resulterend in verminderde transcriptie van IFN-γ gereguleerde genen. Het belang van identificatie van deze onderliggende immuundeficiëntie wordt onderstreept door het feit dat met langdurig antibiotica gebruik als ook IFN-γ suppletie, de infecties van deze patiënt over de jaren goed onder controle zijn gebleven.

In **hoofdstuk 3** worden verschillende strategieën besproken om de effectiviteit van antimycobacteriële therapie te verbeteren.

In **3.1** beschrijven we vier patiënten met gedissemineerde mycobacteriële infecties door mutaties in de IFN-γ receptor, die met interferon alfa (IFN-α) behandeld werden. De klinische respons op IFN-α behandeling varieerde, maar resulteerde bij alle patiënten in verbetering danwel stabilisatie van het ziektebeeld. Hoewel de signaal transductie routes en immunologische functies van IFN-γ en IFN-α verschillend zijn, is er overlap door de gemeenschappelijke activatie van de signaal transductie moleculen Janus kinase (JAK) 2 en STAT1. We tonen zowel *in vitro* als *ex vivo* aan dat IFN-α zowel genen stimuleert die met name door IFN-α aangezet worden, als ook genen, die voornamelijk

door IFN-γ aangezet worden. We concluderen dat IFN-α mogelijk gedeeltelijk kan compenseren voor het tekort aan IFN-γ. Bovendien stellen we dat IFN-α gunstig zou kunnen zijn voor een geselecteerde groep patiënten met gedissemineerde, therapie resistente NTM infecties met een onderliggend defect in de functie van interferon gamma.

In **3.2** onderzoeken we de potentiëring van de activiteit van claritromycine tegen *Mycobacterium avium in vitro.* Claritromycine is een macrolide antibioticum en de hoeksteen van de behandeling van vele NTM infecties, waaronder infecties met *M. avium*. We onderzoeken de rol van het toevoegen van tigecycline, een breed spectrum antibioticum behorend tot de groep van glycylcyclines, dat in de klinische praktijk reeds gebruikt wordt voor andere indicaties. Opvallend genoeg blijkt de *in vitro* activiteit van claritromycine zeer variabel bij klinisch relevante concentraties. Dit speelt wellicht een rol in het feit dat het succes van de behandeling van *M.avium* infecties nog steeds marginaal en onvoorspelbaar is. Onze resultaten laten zien dat onze dynamische *in vitro* time-kill kinetics assay waardevolle informatie oplevert over de concentratie- afhankelijke activiteit van antimycobacteriële middelen in verloop van de tijd. Deze informatie wordt niet verkregen wanneer klassieke gevoeligheidsbepalingen worden ingezet, zoals de minimaal inhiberende concentratie (MIC). Er wordt bovendien aangetoond dat de toevoeging van tigecycline leidt tot toegenomen claritromycine activiteit tegen *M. avium.* Ook wordt door deze toevoeging het selecteren van claritromycine resistentie voorkomen. Of claritromycine daadwerkelijk klinisch toepasbaar is in de behandeling van *M.avium* infecties zal verder onderzocht moeten worden in verschillende preklinische modellen.

In **3.3** wordt een andere strategie onderzocht om de *in vitro* activiteit van antimycobacteriële middelen te verbeteren tegen *Mycobacterium tuberculosis* (Mtb). Er wordt gekeken naar het effect van het verhogen van intramycobacteriële concentraties door colistine. De ingewikkelde samenstelling van de mycobacteriële celwand beperkt de doorlaatbaarheid voor antibiotica aanzienlijk. Hierdoor kunnen antimycobacteriële middelen niet goed doordringen in mycobacteriën zoals Mtb waardoor hun werkzaamheid vermindert. Colistine behoort tot de polymyxine antibiotica en wordt klinisch reeds toegepast in de behandeling van infecties met resistente Gram-negatieve bacteriën. Colistine dankt de werking aan de interactie met de bacteriële membraan waar het zorgt voor membraan destabilisatie, lekkage van de intracellulaire inhoud en celdood. Hoewel de samenstelling van de mycobacteriële celwand anders is dan die van Gram-negatieve bacteriën, is interactie met colistine en op colistine gelijkende middelen beschreven leidend tot een toegenomen doorlaatbaarheid. Hierop is onze hypothese gebaseerd dat colistine de activiteit van antimycobacteriële middelen kan verbeteren door verhoging van de intramycobacteriële concentraties. Dit onderzoeken we in een Mtb populatie met hoge en lage metabole activiteit. Deze studie heeft een aantal belangrijke bevindingen. Ten eerste ontdekken we twee nieuwe potente combinaties van middelen met een snelle en sterke bactericide werking tegen Mtb. Voor het eerst

laten we zien dat colistine de *in vitro* activiteit van twee antimycobacteriële middelen, isoniazide en amikacine, tegen Mtb kan versterken en resistentie voorkomt. Of verhoging van de intramycobacteriele antibiotica concentraties hieraan ten grondslag ligt zal onderzocht moeten worden door het meten van intramycobacteriële concentraties. Ten tweede laten we aanzienlijke verschillen zien in *in vitro* activiteit van de verschillende antimycobacteriële middelen in de Mtb populatie met hoge versus lage metabole activiteit. Bovendien treedt de potentiëring door colistine alleen op in de Mtb populatie met hoge metabole activiteit. Deze resultaten illustreren het belang van het bestuderen van Mtb populaties met verschillende metabole activiteit wanneer de activiteit van (nieuwe) antimycobacteriële middelen onderzocht wordt. We bespreken de toediening van colistine per inhalatie. Dit wordt reeds succesvol toegepast in de klinische praktijk bij patiënten met een pneumonie veroorzaakt door resistente Gram-negatieve bacteriën bij patiënten aan de beademing of met cystic fibrosis. Het voordeel van inhalatie is dat lokaal hogere concentraties bereikt kunnen worden van de antimicrobiële middelen terwijl de systemische bijwerkingen beperkt blijven.

In **hoofdstuk 4** wordt het belang van preklinisch modelleren besproken**.**

In **4.1** onderzoeken we de *in vitro* activiteit van zes antimycobacteriële middelen tegen Mtb met hoge metabole activiteit. Deze studie is uitgevoerd in het kader van het PreDiCT-TB consortium. Dit is een Europees consortium, dat als doel heeft om te bepalen welke (combinatie van) preklinische modellen de beste voorspellende waarde heeft voor hoe effectief antimycobacteriële middelen zijn in TB patiënten. Binnen PreDiCT-TB worden verschillende preklinische modellen bestudeerd waarvan de resultaten vergeleken worden met historische data die beschikbaar zijn over de effectiviteit van deze middelen in TB patiënten. Op deze manier wordt een geïntegreerd model gecreëerd waarmee op een betrouwbare manier de activiteit van potentiële nieuwe middelen en combinaties van middelen preklinisch onderzocht kunnen worden. We hebben de *in vitro* activiteit van de verschillende middelen gerangschikt waarbij streptomycine, rifampicine en isoniazide het meest actief zijn. Isoniazide in combinatie met rifampicine of hoge dosis ethambutol zijn de enige synergistische combinaties. Rifampicine en streptomycine kunnen isoniazide resistentie voorkomen. De vraag is hoe we deze resultaten kunnen relateren aan historische gegevens over de effectiviteit van deze middelen in TB patiënten. We concluderen dat ons *in vitro* model belangrijke informatie levert over de activiteit van antimycobacteriële middelen in de eerste (vroege) fase van de behandeling als ook over voorkomen van resistentie. Hiermee is onze *in vitro* assay een belangrijk onderdeel van een geïntegreerd preklinische model voor het bestuderen van antimycobacteriële activiteit en effectiviteit.

CHAPTER 7

Appendices

Dankwoord

Curriculum vitae

List of publications

Portfolio

DANKWOORD

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CURRICULUM VITAE

Hannelore Iris Bax werd geboren op 16 april 1977 te Rotterdam. Ze behaalde in 1995 haar Gymnasium diploma aan het Stedelijk Gymnasium te Utrecht. In datzelfde jaar begon ze met haar studie Geneeskunde aan de Rijksuniversiteit Groningen. Haar afstudeeronderzoek met als onderwerp ANCA-geassocieerde vasculitis verrichtte ze op de afdeling Nefrologie van het Massachusetts General Hospital te Boston, MA, USA onder leiding van dr. J.L. Niles. In 2002 behaalde ze cum laude het artsexamen. In 2003 startte zij met de opleiding tot internist (opleiders prof. dr. H.A.P. Pols en prof. dr. J.L.C.M. van Saase) en in 2007 met de subspecialisatie infectieziekten (opleider dr. J.L. Nouwen). Beide opleidingen rondde zij in 2009 af waarna ze een jaar onderzoek deed op the Laboratory of Clinical Infectious Diseases van het National Institute of Allergy and Infectious Diseases als onderdeel van de National Institutes of Health te Bethesda, VS. Onder leiding van dr. S.M. Holland deed zij onderzoek naar non-tuberculeuze mycobacteriële infecties bij patiënten met stoornissen in de interferon gamma signalering waarvan dit proefschrift een aantal publicaties bevat. In 2010 keerde ze terug naar Nederland en sindsdien is ze werkzaam als internist-infectioloog binnen de Sectie Infectieziekten van het Erasmus Medisch Centrum. Vanaf 2011 combineert ze het klinische werk met preklinisch onderzoek binnen de mycobacteriële onderzoeksgroep van de afdeling Medische Microbiologie en Infectieziekten met prof. dr. A. Verbon als promotor en dr. J.E.M. de Steenwinkel en dr. I.A.J.M. Bakker-Woudenberg als co-promotoren en waarvan de resultaten in dit proefschrift beschreven worden.

LIST OF PUBLICATIONS

Publications related to this thesis:

Bax HI, Bakker-Woudenberg IAJM, de Vogel CP, van der Meijden A, Verbon A, de Steenwinkel JEM. The role of the time-kill kinetics assay as part of a preclinical modeling framework for assessing the activity of anti-tuberculosis drugs. *submitted*

Bax HI, Bakker-Woudenberg IAJM, ten Kate MT, Verbon A, de Steenwinkel JEM. 2016. Tigecycline potentiates clarithromycin activity against *Mycobacterium avium in vitro.* Antimicrob Agents Chemother **60**:2577-2579.

Bax HI, de Steenwinkel JEM, van der Meijden A, Verbon A, Bakker-Woudeberg IAJM. 2015. Colistin as a potentiator against anti-TB drug activity against *Mycobacterium tuberculosis.* J Antimicrob Chemother **70**:2828-2837.

Bax HI, Freeman AF, Ding L, Hsu AP, Marciano B, Kristosturyan E, Jancel T, Spalding C, Pechacek J, Olivier KN, Barnhart LA, Boris L, Frein C, Claypool RJ, Anderson V, Zerbe CS, Holland SM, Sampaio EP. 2013. Interferon alpha treatment of patients with impaired interferon gamma signaling. J Clin Immunol **33**:991-1001.

Sampaio EP*, Bax HI*, Hsu AP, Kristosturyan E, Pechacek J, Chandrasekaran P, Paulson ML, Dias DL, Spalding C, Uzel G, Ding L, McFarland E, Holland SM. 2012. A novel STAT 1 mutation associated with disseminated mycobacterial disease. J Clin Immunol **32**:681-689. ** joint first authorship*

Other publications:

Bax HI, van Ingen J, Dwarkasing RS, Verbon A. 2014. Lipotourism, not without risks: a complication of cosmetic surgery abroad. Ned Tijschr Geneeskd **158**: A7926.

Sampaio EP, Hsu AP, Pechacek J, Bax HI, Dias DL, Paulson ML, Chandrasekaran P, Rosen LB, Carvalho DS, Ding L, Vinh DC, Browne SK, Datta S, Milner JD, Kuhns DB, Long Priel DA, Sadat MA, Shiloh M, De Marco B, Alvares M, Gillman JW, Ramarathnam V, de la Morena M, Bezrodnik L, Moreira I, Uzel G, Johnson D, Spalding C, Zerbe CS, Wiley H, Greenberg DE, Hoover SE, Rosenzweig SD, Galgiani JN, Holland SM. 2013. Signal transducer and activator of transcription 1 (STAT1) gain-of-function mutations and disseminated coccidioidomycosis and histoplasmosis. J Allergy Clin Immunol **131**:1624-1634.

Bax HI, Freeman AF, Anderson VL, Vesterhus P, Laerum D, Pittaluga S, Wilson WH, Holland SM. 2013. B-cell lymphoma in a patient with complete interferon gamma receptor 1 deficiency. J Clin Immunol **33**:1062-1066.

de Jongste AH, Tilanus AM, Bax H, Willems MH, van der Feltz M, van Hellemond JJ. 2010. New insights in diagnosing Schistosoma myelopathy. J Infect **60**:244-247.

Bax HI, van Veelen ML, Gyssens IC, Rietveld AP. 2007. Brucellosis, an uncommon and frequently delayed diagnosis. Neth J Med **65**:352-355.

Bax HI, Kuipers EJ, Van Saase JL. 2004. Discussion on the rates of dyspepsia one year after Helicobacter pylori screening and eradication in a danish population. Gastroenterology **126**:1223-1224.

Bax HI*, Vriesendorp TM*, Kallenberg CG, Kalk WW. 2002. Fatigue and immune activity in Sjögren's syndrome. Ann Rheum Dis **61**:284. ** joint first authorship*

PORTFOLIO

Presentations

2004	The role of somatostatin and cortistatin in human T-cell development. International Congress of Endocrinology, Lisbon, poster
2009	The role of combination therapy in the treatment of severe pseudomonas infections, National Conference of the Dutch Working Party on Antibiotic Policy (SWAB), Utrecht, invited lecture
2010	STAT1:STAT3 cross-regulation in patients with defective interferon gamma signaling. Federation of Clinical Immunology Societies (FOCIS), Boston, poster
2011	Interferon alpha treatment in patients with defective interferon gamma signaling. Internal Medicine Science Days (Erasmus University Medical Center), Antwerp, oral
2013	Novel treatment strategies for immunocompromised patients infected with nontuberculous mycobacteria. Internal Medicine Science Days (Erasmus University Medical Center), Antwerp, oral
2015	Colistin potentiates antimicrobial activity against *Mycobacterium tuberculosis*. Internal Medicine Science Days (Erasmus University Medical Center), Antwerp, poster
2015	Tigecycline potentiates clarithromycin activity against *Mycobacterium avium*. European Symposium on Non-Tuberculous Mycobacteria, Borstel, poster
2015	Emerging Antibacterial Resistance: consequences for empirical sepsis therapy. 2nd Netherlands International Sepsis Symposium, Amsterdam, invited lecture

(Inter)national conferences and courses

2010	Federation of Clinical Immunology Societies (FOCIS), Boston
2011	Boerhaave Nascholingscursus Infectieziekten, Noordwijkerhout
2011	Klinische Avond Interne Geneeskunde, Regio Rotterdam, Rotterdam
2011	European Congress on Clinical Microbiology and Infectious Diseases (ECCMID), Milan
2011	6th International AIDS Society (IAS) conference on HIV pathogenesis, treatment and prevention, Rome
2012	Implementatietraining, Rotterdam
2012	HIV Management - the New York Course, New York
2012	Hepatitis Management - State of the Art, New York
2012	Teach-the-teacher III, Rotterdam

2012	2nd Workshop of the European Network of Young HIV Experts (ENYHE), Paris
2013	Conference on Retroviruses and Opportunistic Infections (CROI), Atlanta
2013	14th European AIDS Conference (EACS), Brussels
2014	Nascholingscursus Mycobacteriën, een familie van witte en zwarte schapen, Groesbeek
2014	Infectious Diseases (ID) Week, Philadelphia
2014	10th International Workshop on HIV & Hepatitis Co-infection, Paris
2014	26ste Internistendagen, Maastricht
2014	Nationaal Congres Antibiotic Stewardship, Lunteren
2015	Boerhaave Nascholingscursus Infectieziekten, Noordwijkerhout
2015	European Congress on Clinical Microbiology and Infectious Diseases (ECCMID), Copenhagen
2016	12th International Workshop on HIV & Hepatitis Co-infection, Berlin

Teaching activities

2011	Webcast presentation in Dutch refresher course on empirical therapy of adult patients with sepsis (Dutch Working Party on Antibiotic Policy,SWAB)
2012	Supervision of research internship of medical student Project: risk factors and outcome of disseminated *M. avium* infections in HIV-positive patients
2013-2014	Supervision of master student (Research Master Infection and Immunity) Project: activity of moxifloxacin with and without the efflux pump inhibitor verapamil against *Mycobacterium avium* and measurement of intramycobacterial drug concentrations
2015-2016	Co-supervision of master student (Research Master Infection and Immunity) Project: identification of the non-replicating (dormant) subpopulation of *Mycobacterium tuberculosis*
2015-present	Co-supervision of PhD-student Project: studying *Mycobacterium tuberculosis* subpopulations
Ongoing	Education of residents Internal Medicine and Medical Microbiology and Infectious Diseases Main topic: nontuberculous mycobacteria

Ongoing	Yearly elective course 2nd year medical students: Infecties in de grote stad Topics: HIV, HPV and cervical carcinoma; HIV and tuberculosis

Other activities

2010	Coordinator of the revised Dutch guidelines on antibacterial therapy in adult patients with sepsis (Dutch Working Party on Antibiotic Policy, SWAB)

Grants

2009	Research Fellowship National Institutes of Health (NIH), Bethesda, USA Project: interferon gamma and alpha cross talk
2011	European Society for Immunodeficiencies (ESID) long-term fellowship Project: novel treatment strategies for patients infected with non-tuberculous mycobacteria
2012	Erasmus MC Grants, pilot project Project: novel treatment strategies for patients infected with non-tuberculous mycobacteria