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

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ABSTRACT

Objectives

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

Methods

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

Results

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

Conclusion

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

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INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Bacterial strain

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

Mice

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

Infection

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

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

Antibiotic treatment

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

Table 1. schematic overview of the experiments

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

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

Assessment of mycobacterial load in the lungs

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

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

Data analysis and statistics

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

Ethical Approval

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

RESULTS

Mortality and bactericidal activity after single drug exposure

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

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

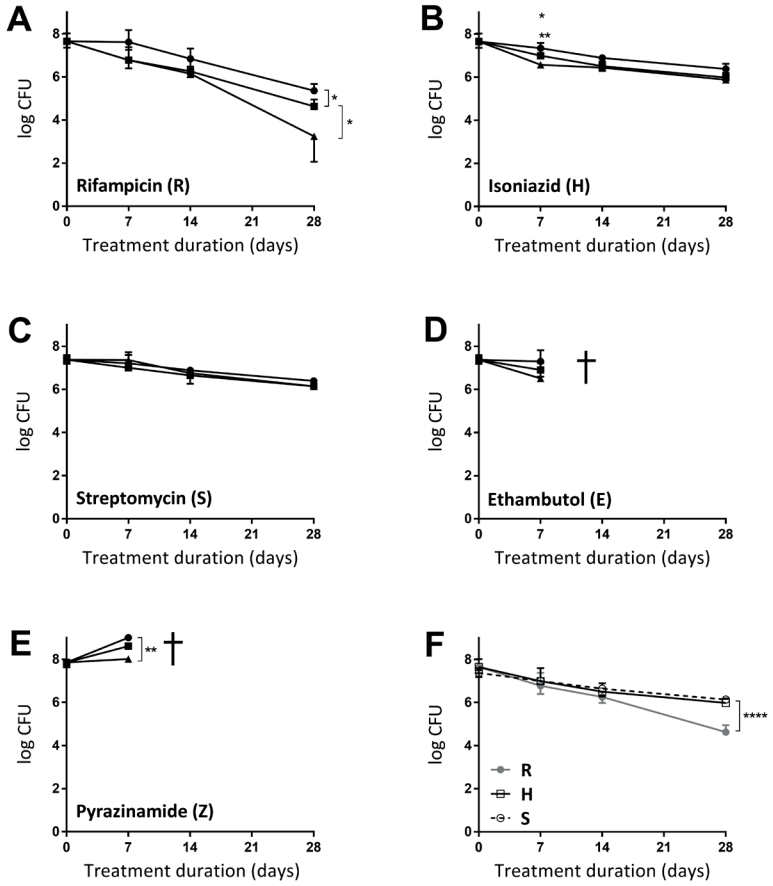

Figure 1. Bactericidal activity after single drug exposure

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

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

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

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

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

Table 2. Treatment outcome against a Beijing strain

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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