Title:

Diverse clinical isolates of *Mycobacterium tuberculosis* develop macrophage-induced rifampin tolerance

Running Title:

Macrophage-induced drug tolerance in MTB

Brief Summary:

Mycobacterium tuberculosis develops tolerance to multiple antibiotics when residing in host macrophages. We demonstrate that macrophage-induced tolerance to rifampin is common across major lineages of *M. tuberculosis* except for Beijing family lineage 2 strains.

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ABSTRACT

The *Mycobacterium tuberculosis* (Mtb) Lineage 4 strains CDC1551 and H37Rv develop tolerance to multiple antibiotics upon macrophage residence. To determine if macrophage-induced tolerance is a general feature of clinical Mtb isolates, we assessed macrophage-induced drug tolerance in strains from lineages 1-3, representing the other predominant Mtb strains responsible for tuberculosis globally. All three lineages developed isoniazid tolerance. While lineage 1, 3 and 4 strains developed rifampin tolerance, lineage 2 Beijing strains did not. Their failure to develop tolerance may be explained by their harboring a loss-of-function mutation in the Rv1258c efflux pump that is linked to macrophage-induced rifampicin tolerance.

KEY WORDS: tuberculosis, drug efflux, Beijing lineage, *Rv1258c*, antibiotic tolerance

BACKGROUND

Mycobacterium tuberculosis (Mtb) enters host macrophages shortly after infection and resides within granulomas, organized macrophage aggregates, for much of its life cycle [1]. Previously we and others showed that Mtb develops tolerance to multiple first and second line anti-tubercular drugs soon after infecting macrophages [2-4]. Moreover, we found that macrophage-induced tolerance to rifampin is mediated via *Tap* (*Rv1258c*), a major facilitator superfamily (MFS) efflux pump [2]. *Rv1258c* expression is induced when bacteria reside within cultured human macrophages [5] as well as in bacteria in sputum of TB patients undergoing treatment with a rifampin-containing regimen [6]. These observations suggest that macrophage induced tolerance to rifampin mediated by *Rv1258c* may contribute to drug tolerance observed in patients.

Based on genomic differences, Mtb is broadly categorized into multiple lineages associated with distinct phenotypes with regard to mutability, drug sensitivity, immunogenicity and virulence [7]. The vast majority of TB worldwide (over 90%) is caused by Mtb lineages 1 (Indo-oceanic), 2 (East Asian), 3 (East African-Indian), and 4 (Euro-American)[7](Supplementary Figure 1). Our prior observations of macrophage induced tolerance were made in H37Rv and CDC1551, both of which represent lineage 4 strains. While lineage 4 is perhaps the most widely distributed geographically, it accounts for only approximately 11% of the global TB burden [7] (Supplementary Figure 1B). Therefore, we sought to determine whether macrophage-induced tolerance to isoniazid and rifampin is a shared feature across the other three lineages that are predominant in high TB-burden areas [7]. In addition, because the Beijing subgroup of lineage 2 strains harbor an inactivating frameshift mutation in *Rv1258c* [8], we were interested to see if

they develop rifampin tolerance. Furthermore, Rv1258c also facilitates bacterial growth within macrophages[2,9], so we assessed both Beijing and non-Beijing strains for growth within macrophages.

METHODS:

Bacterial strains

The sources and antibiotic susceptibilities of the strains used are detailed in Supplementary Table 1. Bacteria were grown to mid log-phase in Middlebrook 7H9 medium (Becton Dickinson) with 0.05% Tween-80 and albumin, dextrose, catalase (Middlebrook ADC Enrichment, Becton Dickson) prior to infection.

Macrophage Growth and Infection

THP-1 cells (ATCC) were grown in RPMI 1640, supplemented with 10% FBS and 2mM L-glutamine (Sigma) in 37^{o}C incubator with 5% CO2. 5x10^{5} THP-1 cells were differentiated into wells of 24-well plates with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 hours, then media was replaced with fresh media without PMA for 24 hours prior to infection. The differentiated cells were infected at a multiplicity of infection (MOI) of 1 for 2 hours. Cells were washed with media and 6 μg/ml streptomycin (Sigma) was added to the media for the remainder of the intracellular growth to eliminate extracellular bacteria; this was defined as the start of infection. Media were changed every 48 hours. For intracellular growth inhibition assays, verapamil HCl (40 μg/ml) (Sigma) was added to the media 48 hours post-infection and streptomycin was omitted.

Macrophage-induced tolerance assay

The work flow for this assay is depicted in Supplementary Figure 2. Briefly, THP1 cells were infected as above and were lysed 2 or 96 hours post-infection to release the bacteria as follows: Cells were washed briefly once with PBS and then with water. Cells were then incubated with 100 µl of water per well at 37°C for 15 minutes. Then 900 µl of 7H9 medium (supplemented with Middlebrook ADC and 0.05% Tween-80) was added and the well bottoms scraped with a pipette tip to ensure complete macrophage lysis, which was microscopically confirmed. Serial dilutions of 150 µL of cell lysates were made in PBS and plated on 7H10 agar (Becton Dickson) to obtain the initial colony forming units (CFU). To measure antibiotic killing, 500 µL of cell lysate was treated with the indicated antibiotic (rifampin 1 µg/ml or isoniazid 0.6 µg/mL, Sigma) for 48 hours at 37C° before making serial dilutions and plating on 7H10 agar. Percent survival was determined by dividing the post-antibiotic treatment CFU by the pre-treatment CFU.

Intracellular growth assay

Infected cells were washed twice with PBS and incubated with 100 µl 0.1% Triton X-100 for 10 minutes. Then 900 µl of PBS was added and the wells scraped with a pipette tip. Dilutions of cell lysates were plated on 7H10 agar as above.

Statistical analyses

GraphPad Prism 6.0 was used for statistical analyses. Means were compared via statistical tests indicated in the figure legends. P values are abbreviated in figures as follows: *, $P<0.05$; **,

P<0.01; *** *P*<0.001, **** *P*<0.0001. Results from one representative experiment is shown in each figure. The number of independent experiments is indicated in the figure legends, with independent experiments defined as experiments setup on different days with different cultures of bacteria or cells.

RESULTS

Macrophage-induced antibiotic tolerance occurs across predominant *Mycobacterium tuberculosis* lineages

Working at two sites, Seattle Children's Research Institute (Seattle, USA) and the National Institute for Research in Tuberculosis (Chennai, India), we used a panel of Mtb strains representing lineages 1-4 assembled from previously published strains (Seattle site) and from recent clinical isolates (Chennai site) (Supplementary Table 1). All strains were confirmed to be susceptible to both isoniazid and rifampin, except strain NIRT203 which was resistant to isoniazid (Supplementary Table 1). The lineage 2 Beijing strains were confirmed to harbor the previously described frameshift mutation in *Rv1258c*, and this mutation was absent in all other strains, including the lineage 2 non-Beijing isolate M4100A (Supplementary Table 1).

To assess development of macrophage-induced antibiotic tolerance, THP-1 macrophages were infected with the clinical MTB strains and then lysed at 2 and 96 hours post-infection. Macrophage lysates were treated with antibiotics for 48 hours and tolerance to antibiotics was assessed by comparing CFU at lysis to the number surviving CFU after antibiotic treatment (Supplemental Figure 2A). Macrophage-induced tolerance was defined as a significant ($p \leq 0.05$) increase in the fraction of bacteria surviving antibiotic treatment between

the 2 hour and 96 hour time points. All of the isoniazid susceptible strains developed macrophage-induced tolerance to isoniazid (Figure 1A and 1B), while NIRT203 was resistant to killing by isoniazid as expected. Strains from lineages 1, 3 and 4 developed tolerance to rifampin (Figure 1C). The capacity of lineage 2 Mtb strains to develop tolerance to rifampin was variable. Both lineage 2 Beijing strains failed to develop rifampin tolerance (Figure 1D). In contrast, M4100A, a non-Beijing lineage 2 strain, developed macrophage-induced rifampin tolerance (Figure 1D).

Lineage 2 Beijing strains grow normally in macrophages

In the CDC1551 strain, Rv1258c mutants not only fail to develop macrophage-induced rifampin tolerance, but also are defective for early growth in macrophages [2,9]**.** However, Beijing strains do not exhibit a macrophage growth defect; indeed, many of them grow more rapidly in macrophages than non-Beijing isolates [10]. This may be one of the reasons that some Beijing strains have been found to be hypervirulent in animal infection models and are spreading globally [7,11].

When we tested the two Beijing strains in our panel for their ability to grow in macrophages, we found that neither manifested an intramacrophage growth defect, confirming prior findings (Figure 2A). Thus, these Beijing strains have evolved compensatory mechanisms that allow them to grow in macrophages. Additional mechanisms that include host immune dysregulation have been invoked to further render them hypervirulent [7]. Indeed, when we assessed the SA161 Beijing strain in a mouse aerosol infection model, we found it to be hypervirulent. Transient early increased bacterial burdens compared to H37Rv were associated

with early lethality (Supplementary Figure 3). Together these findings confirmed that SA161 has not only compensated for any macrophage growth defect due to the loss of Rv1258c but has further evolved additional mechanisms that renders it hypervirulent. Because multiple efflux pumps are reported to be upregulated in Beijing strains [12], we considered the possibility that the mechanisms that compensated for its early growth in macrophages might include the induction of other efflux pumps. Consistent with this possibility, we found that the bacterial efflux pump inhibitor verapamil inhibited SA161's intramacrophage growth similarly to strains from other lineages [13](Figure 2B).

DISCUSSION

Our earlier studies showed that macrophage-induced drug tolerance is a potential contributor to the slow response of Mtb to antimicrobial treatment [2,3]. However, the findings were limited to laboratory strains belonging to a single Mtb lineage. This work shows that macrophage-induced drug tolerance is a feature of the other three predominant Mtb lineages as well. Moreover, the finding that the Beijing strains lack rifampin tolerance while retaining isoniazid tolerance corroborates our previous findings linking the efflux pump to the development of macrophage-induced rifampin tolerance [2].

Our finding that the Beijing strains fail to develop macrophage-induced rifampin tolerance might be seen as presenting a potential quandary given that Beijing lineage TB is more likely to relapse after standard rifampin-containing regimens [14]. However, this increased propensity to relapse may simply be due to the compensated growth in macrophages and hypervirulence traits of the Beijing lineage, as we have shown here for SA161. Furthermore,

194 we demonstrate that treatment with the efflux pump inhibitor verapamil may inhibit
195 intracellular growth of Beijing lineage strains even if they do not appear to develop
196 macrophage-induced tolerance to rifampin.

197 Our finding that the majority of Mtb lineages responsible for disease worldwide exhibit
198 macrophage-induced tolerance to rifampin suggests that strategies to inhibit efflux mediated
199 tolerance may be effective in shortening treatment regimens for the majority of patients.
200 Although, Beijing strains do not demonstrate macrophage-induced tolerance to rifampin, efflux
201 inhibition may still offer benefit for patients infected with these strains because verapamil and
202 possibly other efflux pump inhibitors reduce the Beijing strains intramacrophage survival.

FIGURE LEGENDS:

Figure 1: Macrophage induced tolerance to rifampin is common across clinical lineages of *M. tuberculosis.* A-D, THP-1 macrophages were infected with H37Rv (reference strain) or clinical strains as indicated and lysed at 2 hours (black bars) or 96 hours (white bars) post-infection. The released bacteria were treated for an additional 48 hours with 0.6 μg/ml isoniazid (A and B) or 1 μg /ml rifampicin (C and D) prior to enumeration of colony-forming units (CFU). Results (A, C and D) are representative of three independent experiments and (B) is representative of two experiments. Error bars represent standard deviation. Significance testing was performed using T-test.

Figure 2: Beijing lineage strains of *M. tuberculosis* are not compromised for early macrophage growth and are susceptible to intracellular verapamil treatment. A. THP-1 macrophages were infected with H37Rv or clinical strains of *M. tuberculosis* (MTB) as indicated and lysed at 2 hours (black bars) or 96 hours (white bars) and CFU enumerated at each time-point. B. THP-1 macrophages were infected with MTB strains H37Rv, SA161, M4100A and SG1 for 48 hours and subsequently left untreated or treated for an additional 48 hours with 40 μg/ml verapamil (VER) prior to lysis and enumeration of CFU. Results (A) are representative of at least three independent experiments and (B) is representative of at least two experiments. Error bars represent standard deviation. Significance testing performed using T-test.

FOOTNOTES

Author Contributions:

K.N.A., A.K.V., K.U.D., L.R. and R.E.H. designed experiments. K.N.A., A.K.V., R.G., H.A., D.K.S. and R.E.H. performed experiments. K.N.A., A.K.V., D.R.S., K.B.U., U.D.R., L.R. and R.E.H. analyzed and interpreted data. U.D.R., D.R.S. and S.T. provided project administration and supervision. K.N.A., L.R. and R.E.H. prepared figures and wrote the manuscript. All authors reviewed the manuscript. L.R. conceived the project.

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Potential conflicts of interest

All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

Prior presentation

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