

1 **Title:**

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

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5 **Running Title:**

6 Macrophage-induced drug tolerance in MTB

7

8 **Brief Summary:**

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

12

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51 **ABSTRACT**

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

60

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

62 BACKGROUND

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

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

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

87

88 **METHODS:**

89 **Bacterial strains**

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

94

95 **Macrophage Growth and Infection**

96 THP-1 cells (ATCC) were grown in RPMI 1640, supplemented with 10% FBS and 2mM L-
97 glutamine (Sigma) in 37°C incubator with 5% CO₂. 5x10⁵ THP-1 cells were differentiated into
98 wells of 24-well plates with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 48 hours,
99 then media was replaced with fresh media without PMA for 24 hours prior to infection. The
100 differentiated cells were infected at a multiplicity of infection (MOI) of 1 for 2 hours. Cells were
101 washed with media and 6 µg/ml streptomycin (Sigma) was added to the media for the
102 remainder of the intracellular growth to eliminate extracellular bacteria; this was defined as the
103 start of infection. Media were changed every 48 hours. For intracellular growth inhibition
104 assays, verapamil HCl (40 µg/ml) (Sigma) was added to the media 48 hours post-infection and
105 streptomycin was omitted.

106

107 **Macrophage-induced tolerance assay**

108 The work flow for this assay is depicted in Supplementary Figure 2. Briefly, THP1 cells were
109 infected as above and were lysed 2 or 96 hours post-infection to release the bacteria as follows:
110 Cells were washed briefly once with PBS and then with water. Cells were then incubated with
111 100 µl of water per well at 37°C for 15 minutes. Then 900 µl of 7H9 medium (supplemented
112 with Middlebrook ADC and 0.05% Tween-80) was added and the well bottoms scraped with a
113 pipette tip to ensure complete macrophage lysis, which was microscopically confirmed. Serial
114 dilutions of 150 µL of cell lysates were made in PBS and plated on 7H10 agar (Becton Dickson)
115 to obtain the initial colony forming units (CFU). To measure antibiotic killing, 500 µL of cell
116 lysate was treated with the indicated antibiotic (rifampin 1 µg/ml or isoniazid 0.6 µg/mL, Sigma)
117 for 48 hours at 37C° before making serial dilutions and plating on 7H10 agar. Percent survival
118 was determined by dividing the post-antibiotic treatment CFU by the pre-treatment CFU.

119

120 **Intracellular growth assay**

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

124

125 **Statistical analyses**

126 GraphPad Prism 6.0 was used for statistical analyses. Means were compared via statistical tests
127 indicated in the figure legends. P values are abbreviated in figures as follows: *, $P < 0.05$; **, $P < 0.01$.

128 $P < 0.01$; *** $P < 0.001$, **** $P < 0.0001$. Results from one representative experiment is shown in
129 each figure. The number of independent experiments is indicated in the figure legends, with
130 independent experiments defined as experiments setup on different days with different
131 cultures of bacteria or cells.

132

133 **RESULTS**

134 **Macrophage-induced antibiotic tolerance occurs across predominant *Mycobacterium*** 135 ***tuberculosis* lineages**

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

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

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

157

158 **Lineage 2 Beijing strains grow normally in macrophages**

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

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

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

180

181 **DISCUSSION**

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

189 Our finding that the Beijing strains fail to develop macrophage-induced rifampin
190 tolerance might be seen as presenting a potential quandary given that Beijing lineage TB is
191 more likely to relapse after standard rifampin-containing regimens [14]. However, this
192 increased propensity to relapse may simply be due to the compensated growth in macrophages
193 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.

203 **FIGURE LEGENDS:**

204 **Figure 1: Macrophage induced tolerance to rifampin is common across clinical lineages of *M.***

205 ***tuberculosis*.** A-D, THP-1 macrophages were infected with H37Rv (reference strain) or clinical

206 strains as indicated and lysed at 2 hours (black bars) or 96 hours (white bars) post-infection. The

207 released bacteria were treated for an additional 48 hours with 0.6 µg/ml isoniazid (A and B) or

208 1 µg /ml rifampicin (C and D) prior to enumeration of colony-forming units (CFU). Results (A, C

209 and D) are representative of three independent experiments and (B) is representative of two

210 experiments. Error bars represent standard deviation. Significance testing was performed using

211 T-test.

212

213 **Figure 2: Beijing lineage strains of *M. tuberculosis* are not compromised for early macrophage**

214 **growth and are susceptible to intracellular verapamil treatment.** A. THP-1 macrophages were

215 infected with H37Rv or clinical strains of *M. tuberculosis* (MTB) as indicated and lysed at 2 hours

216 (black bars) or 96 hours (white bars) and CFU enumerated at each time-point. B. THP-1

217 macrophages were infected with MTB strains H37Rv, SA161, M4100A and SG1 for 48 hours and

218 subsequently left untreated or treated for an additional 48 hours with 40 µg/ml verapamil (VER)

219 prior to lysis and enumeration of CFU. Results (A) are representative of at least three

220 independent experiments and (B) is representative of at least two experiments. Error bars

221 represent standard deviation. Significance testing performed using T-test.

222

223 **FOOTNOTES**

224 **Author Contributions:**

225 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
226 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
227 interpreted data. U.D.R., D.R.S. and S.T. provided project administration and supervision.
228 K.N.A., L.R. and R.E.H. prepared figures and wrote the manuscript. All authors reviewed the
229 manuscript. L.R. conceived the project.

230

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241

242 **Potential conflicts of interest**

243 All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for
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245 **Prior presentation**

246 Portions of this work were previously presented at the Tuberculosis Drug Discovery and
247 Development Gordon Research Conference at Lucca, Italy (June 2017) and at the PacTB
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262 **REFERENCES**

- 263 1. Cambier CJ, Falkow S, Ramakrishnan L. Host evasion and exploitation schemes of
264 *Mycobacterium tuberculosis*. *Cell* **2014**; 159:1497–1509.
- 265 2. Adams KN, Takaki K, Connolly LE, et al. Drug tolerance in replicating mycobacteria
266 mediated by a macrophage-induced efflux mechanism. *Cell* **2011**; 145:39–53.
- 267 3. Adams KN, Szumowski JD, Ramakrishnan L. Verapamil, and its metabolite norverapamil,
268 inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-
269 tubercular drugs. *J Infect Dis* **2014**; 210:456–466.
- 270 4. Liu Y, Tan S, Huang L, et al. Immune activation of the host cell induces drug tolerance in
271 *Mycobacterium tuberculosis* both in vitro and in vivo. *J Exp Med* **2016**; 213:809–825.
- 272 5. Schnappinger D, Ehrt S, Voskuil MI, et al. Transcriptional Adaptation of *Mycobacterium*
273 *tuberculosis* within Macrophages: Insights into the Phagosomal Environment. *J Exp Med*
274 **2003**; 198:693–704.
- 275 6. Walter ND, Dolganov GM, Garcia BJ, et al. Transcriptional Adaptation of Drug-tolerant
276 *Mycobacterium tuberculosis* During Treatment of Human Tuberculosis. *J Infect Dis* **2015**;
277 212:990–998.
- 278 7. Gagneux S, Small PM. Global phylogeography of *Mycobacterium tuberculosis* and
279 implications for tuberculosis product development. *Lancet Infect Dis* **2007**; 7:328–337.

- 280 8. Villellas C, Aristimuno L, Vitoria MA, et al. Analysis of Mutations in Streptomycin-
281 Resistant Strains Reveals a Simple and Reliable Genetic Marker for Identification of the
282 Mycobacterium tuberculosis Beijing Genotype. J Clin Microbiol **2013**; 51:2124–2130.
- 283 9. Lin W, de Sessions PF, Teoh GHK, et al. Transcriptional Profiling of Mycobacterium
284 tuberculosis Exposed to In Vitro Lysosomal Stress. Infect Immun **2016**; 84:2505–2523.
- 285 10. Theus S, Eisenach K, Fomukong N, Silver RF, Cave MD. Beijing family Mycobacterium
286 tuberculosis strains differ in their intracellular growth in THP-1 macrophages. Int J Tuberc
287 Lung Dis **2007**; 11:1087–1093.
- 288 11. Merker M, Blin C, Mona S, et al. Evolutionary history and global spread of the
289 Mycobacterium tuberculosis Beijing lineage. Nat Genet **2015**; 47:242–249.
- 290 12. Li G, Zhang J, Guo Q, et al. Efflux pump gene expression in multidrug-resistant
291 Mycobacterium tuberculosis clinical isolates. PLoS One **2015**; 10:e0119013.
- 292 13. Li XZ, Nikaido H. Efflux-mediated drug resistance in bacteria: an update. Drugs **2009**;
293 69:1555–623.
- 294 14. Huyen MNT, Buu TN, Tiemersma E, et al. Tuberculosis relapse in Vietnam is significantly
295 associated with Mycobacterium tuberculosis Beijing genotype infections. J Infect Dis
296 **2013**; 207:1516–1524.