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Host immunity increases *Mycobacterium tuberculosis* reliance on cytochrome bd oxidase [preprint]

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1 Title: Host immunity increases *Mycobacterium tuberculosis* reliance on cytochrome *bd* oxidase

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28 **Abstract:**

29 In order to sustain a persistent infection, *Mycobacterium tuberculosis* (*Mtb*) must adapt to a changing
30 environment that is shaped by the developing immune response. This necessity to adapt is evident in the
31 flexibility of many aspects of *Mtb* metabolism, including a respiratory chain that consists of two distinct terminal
32 cytochrome oxidase complexes. Under the conditions tested thus far, the *bc₁/aa₃* complex appears to play a
33 dominant role, while the alternative *bd* oxidase is largely redundant. However, presence of two terminal
34 oxidases in this obligate pathogen implies that respiratory requirements might change during infection. We
35 report that the cytochrome *bd* oxidase is specifically required for resisting the adaptive immune response.
36 While the *bd* oxidase was dispensable for growth in resting macrophages and the establishment of infection in
37 mice, this complex was necessary for optimal fitness after the initiation of adaptive immunity. This requirement
38 was dependent on lymphocyte-derived interferon gamma (IFN γ), but did not involve nitrogen and oxygen
39 radicals that are known to inhibit respiration in other contexts. Instead, we found that Δ *cydA* mutants were
40 hypersusceptible to the low pH encountered in IFN γ -activated macrophages. Unlike wild type *Mtb*, cytochrome
41 *bd*-deficient bacteria were unable to sustain a maximal oxygen consumption rate (OCR) at low pH, indicating
42 that the remaining cytochrome *bc₁/aa₃* complex is preferentially inhibited under acidic conditions. Consistent
43 with this model, the potency of the cytochrome *bc₁/aa₃* inhibitor, Q203, is dramatically enhanced at low pH.
44 This work identifies a critical interaction between host immunity and pathogen respiration that influences both
45 the progression of the infection and the efficacy of potential new TB drugs.

46
47 **Author Summary:**

48 Tuberculosis, caused by *Mycobacterium tuberculosis* (*Mtb*) is a serious global health problem that is
49 responsible for over one million deaths annually, more than any other single infectious agent. In the host, *Mtb*
50 can adapt to a wide variety of immunological and environmental pressures which is integral to its success as a
51 pathogen. Accordingly, the respiratory capacity of *Mtb* is flexible. The electron transport chain of *Mtb* has two
52 terminal oxidases, the cytochrome *bc₁/aa₃* super complex and cytochrome *bd*, that contribute to the proton
53 motive force and subsequent production of energy in the form of ATP. The *bc₁/aa₃* super complex is required
54 for optimal growth during infection but the role of cytochrome *bd* is unclear. Here we report that the cytochrome

bd oxidase is required for resisting the adaptive immune response, in particular, acidification of the phagosome induced by lymphocyte-derived IFN γ . We found that the cytochrome *bd* oxidase is specifically required under acidic conditions, where the *bc₁/aa₃* complex is preferentially inhibited. Additionally, we show that acidic conditions increased the potency of Q203, a cytochrome *bc₁/aa₃* inhibitor and candidate tuberculosis therapy. This work defines a new link between the host immune response and the respiratory requirements of *Mtb* that affects the potency of a potential new therapeutic.

Introduction

Tuberculosis (TB) is responsible for an estimated 1.4 million deaths annually and remains one of the most deadly infectious diseases (1). The causative agent of TB, *Mycobacterium tuberculosis* (*Mtb*), is an obligate aerobe and relies on oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC) and glycolysis for energy production. The mycobacterial ETC has two terminal oxidases, the cytochrome *bc₁/aa₃* super complex that is related to mitochondrial complex III and IV, and the cytochrome *bd* oxidase which is unique to prokaryotes. These terminal oxidases transfer electrons from the ETC to O₂ and contribute to the proton motive force (PMF) gradient that powers the production of ATP by ATP synthase. In *Mtb*, cytochrome *bc₁/aa₃* is required for optimal growth and persistence in macrophage and infection mouse models using both genetic (2) and chemical inhibition of the complex (3-5).

In the absence of cytochrome *bc₁/aa₃*, electrons are rerouted through the cytochrome *bd* oxidase (6). The latter complex in *Mtb* is encoded in a single operon, *cydABDC*, which produces both the *cydAB* oxidase complex and *cydDC*, a putative ABC-transporter that has not been studied in *Mtb*, but is necessary for assembly of the cytochrome in *Escherichia coli* (7, 8). Genetic deletions in the *cydABDC* operon produce hyper-susceptibility to cytochrome *bc₁/aa₃* inhibitors, demonstrating a partially-redundant role for the terminal oxidases (4, 6, 9). However, the specific role played by the cytochrome *bd* oxidase in *Mtb* remains unclear. In *E.coli*, the cytochrome *bd* oxidase detoxifies peroxide radicals and maintains respiration under hypoxic conditions (10, 11). Similarly, studies in the saprophyte, *Mycobacterium smegmatis*, show that *cyd* mutants are hyper-susceptible to peroxide stress and expression of the *cydAB* operon is induced in hypoxic conditions (12, 13). While it is plausible that these properties contribute to *Mtb* fitness during infection, the role played by the cytochrome *bd* oxidase in the mouse model of TB remains unclear. Some studies report no effect of

82 *cydABDC* mutation, while others describe a fitness defect at the later stages of infection (2, 4, 14). Thus, while
83 it is clear that the cytochrome *bd* oxidase is active in mycobacteria, the non-redundant role of this system
84 during infection is unknown.

85 As an obligate aerobe it is likely that this flexible respiratory chain has evolved to adapt to changing
86 oxygen gradients encountered during infection. During the initial days after infection of the lung, *Mtb* replicates
87 in macrophages, but once these cells are stimulated by T cell-derived cytokines, they restrict *Mtb* growth.
88 Although *Mtb* is exposed to a variety of host pressures during infection, the stressors associated with activation
89 of the macrophage cause a number of specific alterations in the bacterial environment that may alter
90 respiratory requirements. In particular, IFN γ induces antimicrobial responses in the macrophage, including the
91 production of the known respiratory poison, nitric oxide (NO) (15) via nitric oxidase synthase 2 (Nos2).
92 Additionally, inhibition of respiration alters the sensitivity of *Salmonella* to IFN γ -induced superoxide production
93 via the NADPH-dependent phagocyte oxidase (Phox) system (16). Lastly, IFN γ promotes the maturation of the
94 pathogen-containing vacuole, promoting both its acidification and fusion with more degradative compartments.
95 The observation that *cydAB* expression peaks with the onset of the adaptive immune response in the mouse
96 model of infection further suggests an association between T cell cytokines, such as IFN γ , and alterations in
97 the respiratory requirements of *Mtb* (14).

98 In this work, we investigated the interactions between macrophage activation and the mycobacterial
99 respiratory chain. We report that cytochrome *bd* oxidase is specifically required for the bacillus to resist IFN γ -
100 induced macrophage function. In particular, cytochrome *bd* oxidase is necessary in acidic environments similar
101 to those encountered in the phagosome of IFN γ activated macrophages. These compartments can reach pH
102 levels as low as 4.5 (17, 18), which we show preferentially inhibits the function of cytochrome *bc₁/aa₃* complex.
103 The relative acid-resistance of the cytochrome *bd* oxidase explains its specific function in counteracting IFN γ -
104 dependent immunity.

105 **Results**

106 **Δ *cydA* mutant is susceptible to IFN γ -activation of macrophages independent of Nos2 and Phox**

107 To investigate the effects of macrophage activation state on the requirement for the cytochrome *bd*
108 oxidase in *Mtb*, we constructed a Δ *cydA* deletion mutant in H37Rv (19). Consistent with previous studies, there

109 was no difference in growth between H37Rv and $\Delta cydA$ mutants in broth culture (2, 9) (Figure 1A). We
110 compared the fitness of H37Rv and $\Delta cydA$ mutants in bone marrow-derived macrophages (BMDMs) from
111 C57BL/6J (wildtype) mice. Initially, we used flow cytometry and fluorescent live/dead reporter strains of *Mtb* to
112 estimate relative bacterial growth and viability. The *Mtb* strains expressed a constitutively expressed GFP
113 marker and an anhydrotetracycline (ATc)-inducible RFP marker. GFP intensity was used to estimate total
114 infected cell number and ATc-induced RFP intensity served as a surrogate measure of viability and correlates
115 with CFU (20). By these metrics, the growth and viability of H37Rv and the $\Delta cydA$ mutant were not appreciably
116 different in unstimulated BMDMs (Figure 1B-C).

117 In other bacterial systems, the cytochrome *bd* oxidase is important for resistance to NO and oxidative
118 stress, which are major mediators of IFN γ -dependent antimicrobial activity (10, 21-23). To determine if IFN γ or
119 these reactive species alter the requirement for $\Delta cydA$, we stimulated BMDMs with IFN γ , and included cells
120 from *Nos2*^{-/-} and *Cybb*^{-/-} mice which lack functional *Nos2* and *Phox* systems, respectively. In wildtype BMDMs,
121 addition of IFN γ significantly reduced the number of cells harboring live H37Rv and $\Delta cydA$ bacteria (Figure
122 1B). IFN γ treatment had no effect on the viability of H37Rv in *Nos2*^{-/-} BMDMs, indicating that IFN γ -mediated
123 inhibition of H37Rv is primarily dependent on NO, consistent with previous studies (18, 22) (Figure 1B). In
124 contrast, *Nos2* and *Cybb* were not necessary for IFN γ to inhibit $\Delta cydA$ mutants (Figure 1B).

125 These observations were confirmed by CFU enumeration (Figure 1C). The magnitude of IFN γ -
126 dependent inhibition was smaller in the CFU assay than the flow cytometry study, likely reflecting increased
127 sensitivity of the live/dead reporter for bacterial fitness. Regardless, the CFU assay also showed that IFN γ
128 treatment reduced the viability of H37Rv in a *Nos2*-dependent, *Cybb*-independent manner, whereas the
129 suppression of $\Delta cydA$ mutants was independent of both mediators. To verify that these mutant phenotypes
130 were due to a lack of *cydABDC* function, we tested a $\Delta cydD$ mutant and found that it was similarly attenuated
131 in IFN γ -stimulated *Nos2*^{-/-} cells as the $\Delta cydA$ strain (Figure 1D). Both of these mutants could be
132 complemented *in trans* by expressing the *cydABDC* operon. These observations indicated the cytochrome *bd*
133 oxidase was necessary to resist an IFN γ -induced stress that is independent of *Nos2* and *Cybb*.

136 **IFN γ but not iNOS or Phox is necessary for the attenuation of Δ *cydA* in mouse lungs**

137 The interaction between IFN γ and Δ *cydA* was then assessed in the mouse model. To evaluate the
138 relative fitness of the Δ *cydA* mutant we performed competition infections using a mixture of H37Rv:Kan and
139 Δ *cydA*:Hyg. To test the importance of lymphocyte-derived IFN γ , the relative fitness of the Δ *cydA* mutant was
140 assessed in wild type C57BL/6J mice and animals lacking adaptive immunity (Rag^{-/-}), IFN γ receptor (Ifngr^{-/-}),
141 Nos2 (Nos2^{-/-}) and Phox (*Cybb*^{-/-}) (Figure 2A). At each timepoint, lung homogenates were plated on kanamycin
142 and hygromycin and CFU were enumerated to compare the fitness of H37Rv and Δ *cydA* (Figure 2B). On day
143 15 post infection, before the onset of adaptive immunity, there was no significant difference in CFU between
144 H37Rv and Δ *cydA* across all five mouse genotypes (Figure 2B). Once the adaptive response was established
145 after 30 days of infection, we observed a significant decrease in Δ *cydA* lung CFU compared to H37Rv in
146 wildtype, Nos2^{-/-}, and *Cybb*^{-/-} mice. However, there was no difference between Δ *cydA* and H37Rv lung CFU in
147 *Ifgr*^{-/-} and Rag^{-/-} mice demonstrating that the attenuation of the Δ *cydA* strain is dependent on lymphocytes and
148 IFN γ (Figure 2B). Complementation of the Δ *cydA* mutant with *cydABDC* rescued the fitness defect observed in
149 the mutants at the 30-day timepoint in wildtype mice (Figure 2C). These observations were consistent with the
150 *ex vivo* macrophage infections, both indicating that the IFN γ -dependent attenuation of Δ *cydA* is Nos2 and
151 Phox independent.

152 **Δ *cydA* mutants are defective for growth at low pH**

153 Beyond stimulating the production of RNS and ROS in macrophages, IFN γ also promotes the
154 maturation and acidification of the mycobacterial phagosome. To determine if the hyper-susceptibility of
155 Δ *cydA* to IFN γ could be due to this reduction in pH, we investigated the fitness of Δ *cydA* mutants under acidic
156 conditions. H37Rv and Δ *cydA* were grown in modified 7H9 media at pH values that span those encountered in
157 the maturing phagosome (17, 18). The growth rate of Δ *cydA* was significantly reduced at low pH in comparison
158 to H37Rv or the complemented mutant strain (Figure 3A-C). We next sought to determine if the IFN γ -
159 dependent acidification of the phagosome in macrophages could account for the intracellular growth defect of
160 the Δ *cydA* mutant. BMDMs from wildtype, Nos2^{-/-} and *Cybb*^{-/-} mice were infected with either H37Rv or the
161 Δ *cydA* mutant, and we determined if the inhibitory effect of IFN γ was affected with bafilomycin A1, an inhibitor
162 of the vacuolar-type H⁺-ATPase that is responsible for the acidification of the phagosome. Using the live/dead

163 reporter assay, we confirmed that *Nos2* was required for IFN γ to inhibit H37Rv, but not the Δ *cydA* mutant,
164 providing a situation where the NO-independent inhibitory effect of IFN γ on *bd* oxidase-deficient *Mtb* could be
165 assessed. In these *Nos2*^{-/-} macrophages, BAF had no effect on the growth of H37Rv, but completely reversed
166 the inhibitory effect of IFN γ on the Δ *cydA* mutant, restoring fitness to levels equivalent to unstimulated BMDMs
167 (Figure 3D). In all three macrophage genotypes, BAF treatment restored the fitness of the Δ *cydA* mutant to
168 wild type levels in the presence of IFN γ . While BAF treatment had a modest effect on the fitness of wild type
169 *Mtb*, the preferential effect on the Δ *cydA* mutant was consistent with the hypersensitivity of this strain to low
170 pH. Together these observations indicate that the NO-independent inhibitory effect of IFN γ on *bd* oxidase-
171 deficient *Mtb* could primarily be attributed to the lowered pH of the phagosome.

172 **The *bd* oxidase is necessary for optimal respiration under low pH conditions**

173 Next, we hypothesized that the fitness defect of the Δ *cydA* at low pH was due to reduced activity of the
174 remaining cytochrome *bc*₁/*aa*₃ under these conditions. To evaluate OXPHOS in H37Rv and Δ *cydA* at low pH,
175 we used extracellular flux analysis (Agilent Seahorse XFe96) to measure the oxygen consumption rate (OCR).
176 There was no difference in OCR between H37Rv exposed to pH 7.4 and pH 4.5 media (prior to CCCP
177 addition), suggesting that the respiratory chain in wildtype *Mtb* is able to adapt to reduced pH (Figure 4A).
178 Q203 (Telacebec), an inhibitor of the QcrB subunit of the cytochrome *bc*₁/*aa*₃ complex (5), was used to
179 specifically measure the acid sensitivity of cytochrome *bd* oxidase in H37Rv. At pH 7.4, consistent with
180 previous studies, addition of Q203 led to increased OCR through the re-routing of electrons through
181 cytochrome *bd* (6, 9) (Figure 4A). At pH 4.5, Q203-treated cells displayed an even higher OCR than at neutral
182 pH, potentially indicating further inhibition of the cytochrome *bc*₁/*aa*₃ complex and increased reliance on
183 cytochrome *bd* (Figure 4B).

184 The same experiments were performed with the Δ *cydA* mutant to more specifically probe the effects of
185 low pH on cytochrome *bc*₁/*aa*₃ (Figure 4C). First, we verified that Q203 abrogated OCR in the Δ *cydAB* strain,
186 indicating that both terminal oxidases are inhibited under these conditions. More importantly, decreasing the
187 pH from 7.4 to 4.5 reduced OCR in the Δ *cydA* mutant strain (following media addition – green line and bar),
188 where only the *bc*₁/*aa*₃ complex is active (Figure 4C and D). As this decrease in OCR at low pH was seen only
189 in the Δ *cydAB* mutant and not wild type (Figure 4A), these data indicate that cytochrome *bc*₁/*aa*₃ activity is

190 preferentially reduced under these conditions (Figure 4D). Given this pH-dependent decrease in activity, we
191 hypothesized that acid stress would also increase the sensitivity of cytochrome *bc₁/aa₃* to inhibition. Indeed,
192 reducing the pH from 7.4 to 5.5 enhanced the potency of Q203, lowering the MIC₅₀ by almost 20-fold (Figure
193 4E). In sum, we observed that low pH both decreases *bc₁/aa₃*-dependent OCR and increases the potency of a
194 *bc₁/aa₃*-specific inhibitor. These findings indicate that cytochrome *bc₁/aa₃* activity is inhibited at low pH, and
195 that the cytochrome *bd* oxidase is preferentially required to maintain respiration under acidic conditions, such
196 as those found in the phagosome of IFN γ -stimulated macrophages.

197 **Discussion**

198 The flexibility of bacterial respiratory chains facilitates adaptation to changing environments, and in
199 many situations the *bd* oxidase becomes critical under conditions where the *bc₁/aa₃* complex is inhibited. In
200 pathogens such as *E.coli*, *Listeria monocytogenes*, and *Salmonella typhimurium*, the requirement for the
201 cytochrome *bd* oxidase in bacterial virulence has been attributed to its role in resisting hypoxia and nitrosative
202 and oxidative stress (23-26). While previous studies in *M. marinum* and *M. smegmatis* found that the
203 mycobacterial *bd* oxidase can also confer resistance to hypoxia and peroxide, the specific roles played by the
204 cytochrome *bd* and *bc₁/aa₃* oxidases of *Mtb* during infection has been less clear. Our work indicates that the
205 flexibility of the *Mtb* respiratory chain facilitates adaptation to changes in the immune response. As outlined
206 below, our findings specifically suggest that the cytochrome *bd* oxidase provides resistance to IFN γ -mediated
207 immunity by facilitating respiration under the acidic conditions encountered in the phagosomes of IFN γ -
208 stimulated macrophages.

209 Both in *ex vivo* macrophage cultures and intact animals, we found that the *bd* oxidase was required to
210 resist IFN γ -dependent immunity. These data are consistent with those of *Shi et al.*, who showed that *Mtb*
211 cytochrome *bd* oxidase mutants were specifically attenuated in C57BL6 mice only after 50 days of infection.
212 (14). Conversely, other studies have concluded that the cytochrome *bd* oxidase is dispensable for growth in
213 C57BL/6J and BALB/C mice (2, 4). We suspect that these differing conclusions were caused by variations in
214 the infection models. Specifically, our study used a competitive infection model, which ensures that both wild
215 type and mutant bacteria are exposed to identical immune pressures and captures even transient differences

216 in fitness. As a result, we consider competitive studies, such as ours, to be a sensitive approach to detect
217 differences in fitness.

218 While IFN γ stimulation induces large transcriptional changes and antimycobacterial functions in
219 macrophages, we attributed the *bd* oxidase requirement to alterations in phagosomal pH. *Nos2* and *Phox* are
220 strongly induced by IFN γ , and the requirement for the *bd* oxidase in other bacterial pathogens has been
221 attributed to the resulting NO and ROS (21). As a result, it was somewhat surprising that the sensitivity of
222 Δ *cydA* mutant bacteria to IFN γ treatment was independent of these mediators. Instead, multiple lines of
223 evidence indicated that this mutant was sensitive to the low pH encountered in the phagosome of IFN γ -
224 stimulated macrophages. Firstly, we found that that *bd* oxidase-deficient bacteria grew poorly and respired at
225 reduced rates at low pH. These findings that are consistent with previous transposon mutant screening data
226 suggesting that the *cydABDC* operon was required for optimal growth at pH 4.5 (27). While the reduction in
227 OCR that we observed in the Δ *cydA* mutant at low pH was modest, we speculate that even a small deficit in
228 respiratory rate could cause the observed decrease in growth. These *in vitro* growth defects were related to
229 the intracellular growth environment by demonstrating that inhibition of vacuolar acidification with BAF
230 abrogated the relative fitness difference between Δ *cydA* and wild type *Mtb* in IFN γ -stimulated macrophages.
231 While these data are consistent with a primary role for the *bd* oxidase in adaptation to low pH conditions, we
232 note that inhibition of phagosome acidification can have pleiotropic effects on processes such as phagosome-
233 lysosome fusion and autophagy. Thus, while it remains possible that additional stresses play a role, our
234 observations are consistent with a model in which the *bd* oxidase promotes resistance to the adaptive immune
235 response by promoting respiration in the low pH environment of the IFN γ -stimulated macrophage.

236 While the requirement for *bd* oxidase activity at low pH can be attributed to the reduced activity we
237 detected for the *bc₁/aa₃* complex under these conditions, the mechanism by which low pH inhibits the activity of
238 the cytochrome *bc₁/aa₃* is unclear. The cytochrome *bc₁/aa₃* super complex is tightly coupled to the transport of
239 protons (28). For every O₂ molecule reduced by the super complex, 4 protons are pumped into the periplasm
240 and contribute to the proton motive force (PMF) (28). While the cytochrome *bd* oxidase also contributes
241 protons to PMF, it does not have proton pumping capabilities and contributes half of the protons for every
242 molecular oxygen reduced as the super complex (7, 29). It is possible that the tight coupling between proton

243 pumping and electron transfer for the cytochrome *bc₁/aa₃* complex results in its inhibition when extracellular
244 proton concentrations are high. However, acid stress induces a wide variety of transcriptional and physiological
245 responses in *Mtb* and it is also possible that pH has additional indirect effects on the cytochrome *bc₁/aa₃*
246 complex (30-32).

247 The success of bedaquiline, a mycobacterial ATPase inhibitor, has made respiration an attractive target
248 for new therapeutics. Multiple small molecule inhibitor screens have identified drugs that target the QcrB
249 component of the proton-pumping cytochrome *bc₁/aa₃* (3, 5, 33, 34). Most notably is Q203 (Telacebec) which
250 is currently in clinical trials (5). However, the flexibility of the mycobacterial respiratory chain has raised
251 concerns about the potential efficacy of this drug (2, 4, 35). One strategy to enhance the efficacy of respiratory
252 inhibition is to simultaneously target both the *bc₁/aa₃* and *bd* oxidase complexes, which produces a bactericidal
253 effect (4, 6, 9). Our data suggest that immunity is another important factor that determines the relative
254 importance of terminal oxidases and the ultimate efficacy of these agents. The concept is similar to the
255 previously described synergy between IFN γ -induced tryptophan depletion and the efficacy of *Mtb* tryptophan
256 synthesis inhibitors (27). These examples highlight the importance of understanding the interactions between
257 bacterial physiology and immunity for evaluating and optimizing new therapies.

258 **Experimental Methods**

259 ***Bacterial growth and strain generation***

261 *Mycobacterium tuberculosis* strains were cultured at 37°C in complete Middlebrook 7H9 medium containing oleic
262 acid-albumin-dextrose-catalase (OADC, Becton, Dickinson), 0.2% glycerol, and 0.05% Tween 80 or 0.02%
263 Tyloxapol. Hygromycin, kanamycin, and zeocin were added as necessary at 50 ug/mL, 25 ug/mL, and 25 ug/mL,
264 respectively. All *Mtb* mutant strains were derived from the wildtype H37Rv. Δ *cydA*, Δ *cydD* and Δ *cydABDC*
265 operon were deleted by allelic exchange as described previously (19). The gene deletions were confirmed by
266 PCR verification and sequencing of the 5' and 3' recombinant junctions and the absence of an internal fragment
267 within the deleted region. An L5attP-zeoR-cydABDC-operon complementing plasmid was assembled by
268 Gateway reaction (Invitrogen) and transformed into the *hygR cydA* mutant to generate the *cydABDC*-
269 complementing strain. The Live/Dead reporter strains were generated by transforming *Mtb* with the replicating

270 Live/Dead plasmid that contains a constitutively expressed GFP and a tetracycline-inducible TagRFP fluorescent
271 protein.

272 ***Ethics Statement and Experimental Animals***

273 C57BL/6 (stock no. 000664), *Cybb*^{-/-} (B6.129S-Cybb^{tm1Din}/J stock no. 002365), *Nos2*^{-/-} (B6.129P2-Nos2^{tm1Lau}/J,
274 stock no. 002609), *Ifngr*^{-/-} (B6.129S7-Ifngr1^{tm1Agt}/J), and *Rag*^{-/-} (B6.129S7-Rag1^{tm1Mom}/J, stock no. 002216) were
275 purchased from the Jackson Laboratory. Housing and experimentation were in accordance with the guidelines
276 set forth by the Department of Animal Medicine and University of Massachusetts Medical School Institutional
277 Animal Care and Use Committee (IACUC). Animals used for experimentation were between 6 and 8 weeks old.

278 ***Macrophage infection***

279 Bone marrow derived macrophages (BMDMs) were isolated from C57BL/6, *Cybb*^{-/-} or *Nos2*^{-/-} mice by
280 culturing bone marrow cells in DMEM supplemented with 20% conditioned medium from L929, 10% FBS, 2
281 mM L-glutamine and 1 mM sodium pyruvate for 7 days. BMDMs were seeded and left unstimulated or
282 stimulated with IFN- γ (25ng/mL, PeproTech) overnight and then infected with *Mtb* at an MOI of 5. After 4 h
283 incubation, macrophages were washed twice with PBS to remove extracellular bacteria and incubated in fresh
284 complete medium with or without IFN- γ . In some conditions, bafilomycin A (100ng/mL, Sigma) or Q203 (at
285 specified concentrations) was added. Cells were lysed with 1% Saponin/PBS (Sigma) at 120 h after infection
286 and then plated on 7H10-OADC plates in serial dilutions. CFUs were counted after 3 weeks of incubation at
287 37°C.

288 ***Flow Cytometry***

289 For flow cytometry, BMDMs pretreated with or without IFN- γ were infected with Live/Dead reporter *Mtb* strains.
290 At day 3 post-infection, tetracycline (500 ng/ml) was added to medium. Macrophages were harvested after 24
291 hours tetracycline addition and fixed with 4% PFA for 45 minutes, then run on an LSR II flow cytometer.

292 ***Mouse Infection***

293 Prior to infection, *Mtb* strains were resuspended and sonicated in PBS containing 0.05% Tween80. Δ *cydA*
294 mutant fitness *in vivo* was determined by inoculating mice with a ~1:1 mixture of Δ *cydA* (hygromycin resistant)

295 and H37Rv (harboring pJEB402 chromosomally integrated plasmid encoding kanamycin resistance) strains via
296 the respiratory route using an aerosol generation device (Glas-Col). At the indicated time points, mice were
297 sacrificed and CFU numbers in lung homogenate were determined by plating on 7H10-OADC agar containing
298 Kanamycin (25 ug/mL) or Hygromycin (50 ug/mL).

299 ***Acid sensitivity assays.***

300 The early log-phase of *Mtb* strains were wash twice with PBS and inoculated to a starting optical density at 600
301 nm (OD₆₀₀) of 0.01 in 96-well plates with 7H9-Tyloxapol-7.4, 7H9-Ty-6.0, 7H9-Ty-5.5, 7H9-Ty-5.0 and 7H9-Ty-
302 4.5, respectively. The desired media pH was achieved by adding 2.5N HCl and 1N NaOH. In some conditions,
303 Q203 (gifted from Professor Barry Clifton) was added. Growth was monitored by OD₆₀₀ daily. Growth rate was
304 determined by comparing OD600 under different pH in day 5.

305 ***Extracellular Flux Analysis***

306 The OCR of *Mtb* bacilli adhered to the bottom of an XF cell culture microplate (Cell-Tak coated) (Seahorse
307 Biosciences), at 2x10⁶ bacilli per well, were measured using a XF96 Extracellular Flux Analyser (Seahorse
308 Biosciences)(6). All XF assays were carried out in unbuffered 7H9 media (pH 7.4 or pH 4.5 for acidic
309 conditions) without a carbon source. Basal OCR was measured for ~ 25 min before the addition of compounds
310 through the drug ports of the sensor cartridge. After media or Q203 addition (0.9 μM), OCR was measured for
311 ~ 40 min, followed by the addition of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (2
312 μM) and the OCR measured for a further ~20 min. All OCR Figures indicate the approximate point of each
313 addition as dotted lines. OCR data points are representative of the average OCR during 4 min of continuous
314 measurement in the transient microchamber, with the error being calculated from the OCR measurements
315 taken from at least three replicate wells by the Wave Desktop 2.2 software (Seahorse Biosciences). The
316 microchamber is automatically re-equilibrated between measurements through the up and down mixing of the
317 probes in the wells of the XF cell culture microplate.

318 ***MIC assay***

319 Log-phase H37Rv was washed twice with PBS + 0.02% tyloxapol and used to inoculate 10mL cultures of 7H9-
320 Ty-7.0, 7H9-Ty-6.5, and 7H9-Ty-5.5 to an OD₆₀₀ of 0.02. As stated before, media pH was achieved by the
321 addition of 2.5N HCl or 1N NaOH. To determine the MIC of Q203 (HY-101040, MedChemExpress), 3-fold serial

322 dilutions from 24nM to 0.3 nM were performed at pH 7.0, 6.5, and 5.5 with a vehicle (DMSO) control. Cultures
323 were incubated at 37°C with shaking. The Syngery HXT microplate reader was used to measure daily OD₆₀₀ of
324 100uL aliquots in a 96 well plate. The MIC values were calculated on day 6 of growth using nonlinear regression
325 analysis.

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1. Organization WH. Global tuberculosis report 2018. 2018.
2. Beites T, O'Brien K, Tiwari D, Engelhart CA, Walters S, Andrews J, et al. Plasticity of the Mycobacterium tuberculosis respiratory chain and its impact on tuberculosis drug development. *Nat Commun.* 2019;10(1):4970.
3. Foo CS, Lupien A, Kienle M, Vocat A, Benjak A, Sommer R, et al. Arylvinylpiperazine Amides, a New Class of Potent Inhibitors Targeting QcrB of Mycobacterium tuberculosis. *mBio.* 2018;9(5).
4. Kalia NP, Hasenoehrl EJ, Ab Rahman NB, Koh VH, Ang MLT, Sajorda DR, et al. Exploiting the synthetic lethality between terminal respiratory oxidases to kill Mycobacterium tuberculosis and clear host infection. *Proc Natl Acad Sci U S A.* 2017;114(28):7426-31.
5. Pethe K, Bifani P, Jang J, Kang S, Park S, Ahn S, et al. Discovery of Q203, a potent clinical candidate for the treatment of tuberculosis. *Nat Med.* 2013;19(9):1157-60.
6. Lamprecht DA, Finin PM, Rahman MA, Cumming BM, Russell SL, Jonnala SR, et al. Turning the respiratory flexibility of Mycobacterium tuberculosis against itself. *Nature Communications.* 2016;7:12393.
7. Borisov VB, Gennis RB, Hemp J, Verkhovsky MI. The cytochrome bd respiratory oxygen reductases. *Biochim Biophys Acta.* 2011;1807(11):1398-413.
8. Cook GM, Cruz-Ramos H, Moir AJ, Poole RK. A novel haem compound accumulated in Escherichia coli overexpressing the cydDC operon, encoding an ABC-type transporter required for cytochrome assembly. *Arch Microbiol.* 2002;178(5):358-69.
9. Moosa A, Lamprecht DA, Arora K, Barry CE, 3rd, Boshoff HIM, Ioerger TR, et al. Susceptibility of Mycobacterium tuberculosis Cytochrome bd Oxidase Mutants to Compounds Targeting the Terminal Respiratory Oxidase, Cytochrome c. *Antimicrob Agents Chemother.* 2017;61(10).
10. Lindqvist A, Membrillo-Hernandez J, Poole RK, Cook GM. Roles of respiratory oxidases in protecting Escherichia coli K12 from oxidative stress. *Antonie Van Leeuwenhoek.* 2000;78(1):23-31.
11. D'Mello R, Hill S, Poole RK. The cytochrome bd quinol oxidase in Escherichia coli has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity in vivo by oxygen inhibition. *Microbiology.* 1996;142 (Pt 4):755-63.
12. Lu P, Heineke MH, Koul A, Andries K, Cook GM, Lill H, et al. The cytochrome bd-type quinol oxidase is important for survival of Mycobacterium smegmatis under peroxide and antibiotic-induced stress. *Sci Rep.* 2015;5:10333.
13. Aung HL, Berney M, Cook GM. Hypoxia-activated cytochrome bd expression in Mycobacterium smegmatis is cyclic AMP receptor protein dependent. *J Bacteriol.* 2014;196(17):3091-7.
14. Shi L, Sohaskey CD, Kana BD, Dawes S, North RJ, Mizrahi V, et al. Changes in energy metabolism of Mycobacterium tuberculosis in mouse lung and under in vitro conditions affecting aerobic respiration. *Proc Natl Acad Sci U S A.* 2005;102(43):15629-34.
15. Voskuil MI, Schnappinger D, Visconti KC, Harrell MI, Dolganov GM, Sherman DR, et al. Inhibition of respiration by nitric oxide induces a Mycobacterium tuberculosis dormancy program. *J Exp Med.* 2003;198(5):705-13.
16. Husain M, Bourret TJ, McCollister BD, Jones-Carson J, Laughlin J, Vazquez-Torres A. Nitric oxide evokes an adaptive response to oxidative stress by arresting respiration. *J Biol Chem.* 2008;283(12):7682-9.
17. Ohkuma S, Poole B. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc Natl Acad Sci U S A.* 1978;75(7):3327-31.
18. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science.* 2003;302(5645):654-9.
19. Murphy KC, Nelson SJ, Nambi S, Papavinasasundaram K, Baer CE, Sassetti CM. ORBIT: a New Paradigm for Genetic Engineering of Mycobacterial Chromosomes. *mBio.* 2018;9(6).
20. Martin CJ, Booty MG, Rosebrock TR, Nunes-Alves C, Desjardins DM, Keren I, et al. Efferocytosis is an innate antibacterial mechanism. *Cell Host Microbe.* 2012;12(3):289-300.

- 405 21. Ehrt S, Schnappinger D, Bekiranov S, Drenkow J, Shi S, Gingeras TR, et al. Reprogramming of the
406 macrophage transcriptome in response to interferon-gamma and Mycobacterium tuberculosis: signaling roles
407 of nitric oxide synthase-2 and phagocyte oxidase. *J Exp Med*. 2001;194(8):1123-40.
- 408 22. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide
409 synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A*. 1997;94(10):5243-8.
- 410 23. Mason MG, Shepherd M, Nicholls P, Dobbin PS, Dodsworth KS, Poole RK, et al. Cytochrome bd
411 confers nitric oxide resistance to *Escherichia coli*. *Nat Chem Biol*. 2009;5(2):94-6.
- 412 24. Jones-Carson J, Husain M, Liu L, Orlicky DJ, Vazquez-Torres A. Cytochrome bd-Dependent
413 Bioenergetics and Antinitrosative Defenses in *Salmonella* Pathogenesis. *mBio*. 2016;7(6).
- 414 25. Jones SA, Chowdhury FZ, Fabich AJ, Anderson A, Schreiner DM, House AL, et al. Respiration of
415 *Escherichia coli* in the mouse intestine. *Infect Immun*. 2007;75(10):4891-9.
- 416 26. Corbett D, Goldrick M, Fernandes VE, Davidge K, Poole RK, Andrew PW, et al. *Listeria*
417 *monocytogenes* Has Both Cytochrome bd-Type and Cytochrome aa₃-Type Terminal Oxidases, Which Allow
418 Growth at Different Oxygen Levels, and Both Are Important in Infection. *Infect Immun*. 2017;85(11).
- 419 27. Zhang YJ, Reddy MC, Ioerger TR, Rothchild AC, Dartois V, Schuster BM, et al. Tryptophan
420 biosynthesis protects mycobacteria from CD4 T-cell-mediated killing. *Cell*. 2013;155(6):1296-308.
- 421 28. Wiseman B, Nitharwal RG, Fedotovskaya O, Schafer J, Guo H, Kuang Q, et al. Structure of a functional
422 obligate complex III₂IV₂ respiratory supercomplex from *Mycobacterium smegmatis*. *Nat Struct Mol Biol*.
423 2018;25(12):1128-36.
- 424 29. Safarian S, Rajendran C, Muller H, Preu J, Langer JD, Ovchinnikov S, et al. Structure of a bd oxidase
425 indicates similar mechanisms for membrane-integrated oxygen reductases. *Science*. 2016;352(6285):583-6.
- 426 30. Bansal R, Anil Kumar V, Sevalkar RR, Singh PR, Sarkar D. *Mycobacterium tuberculosis* virulence-
427 regulator PhoP interacts with alternative sigma factor SigE during acid-stress response. *Mol Microbiol*.
428 2017;104(3):400-11.
- 429 31. Rohde KH, Abramovitch RB, Russell DG. *Mycobacterium tuberculosis* invasion of macrophages: linking
430 bacterial gene expression to environmental cues. *Cell Host Microbe*. 2007;2(5):352-64.
- 431 32. Tan S, Sukumar N, Abramovitch RB, Parish T, Russell DG. *Mycobacterium tuberculosis* responds to
432 chloride and pH as synergistic cues to the immune status of its host cell. *PLoS Pathog*. 2013;9(4):e1003282.
- 433 33. Lu X, Williams Z, Hards K, Tang J, Cheung CY, Aung HL, et al. Pyrazolo[1,5- a]pyridine Inhibitor of the
434 Respiratory Cytochrome bcc Complex for the Treatment of Drug-Resistant Tuberculosis. *ACS Infect Dis*.
435 2019;5(2):239-49.
- 436 34. van der Westhuyzen R, Winks S, Wilson CR, Boyle GA, Gessner RK, Soares de Melo C, et al.
437 Pyrrolo[3,4-c]pyridine-1,3(2H)-diones: A Novel Antimycobacterial Class Targeting Mycobacterial Respiration. *J*
438 *Med Chem*. 2015;58(23):9371-81.
- 439 35. Berney M, Hartman TE, Jacobs WR, Jr. A *Mycobacterium tuberculosis* cytochrome bd oxidase mutant
440 is hypersensitive to bedaquiline. *mBio*. 2014;5(4):e01275-14.

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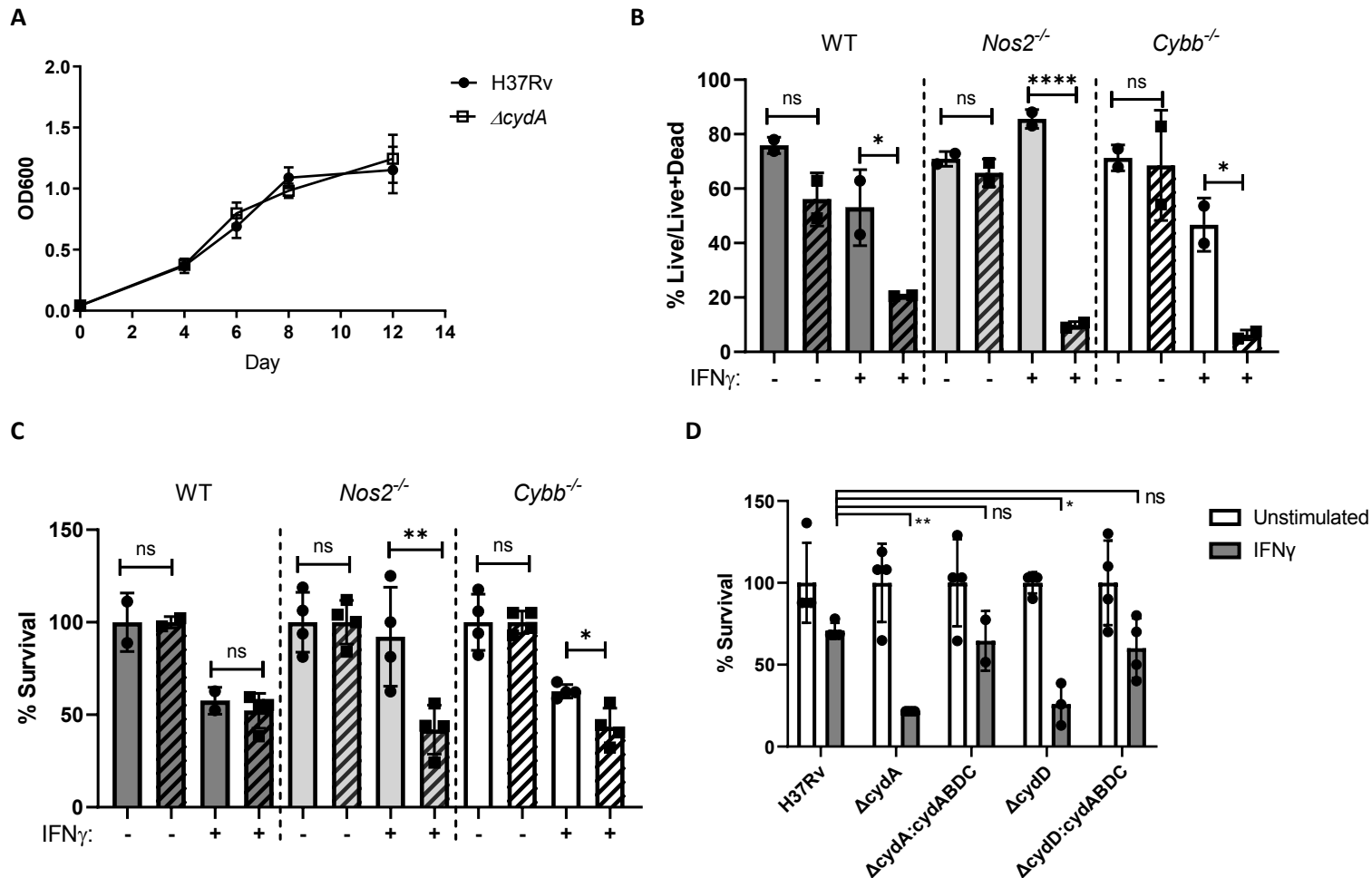
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Figures

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Figure 1: $\Delta cydA$ mutant is susceptible to IFN γ -activation of macrophages independent of iNOS and Phox

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Figure 1: $cydA$ is required to resist IFN γ -mediate immunity independent of $Nos2$ and $Phox$.

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A) Growth curve of H37Rv and $\Delta cydA$ grown in broth for 12 days.

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B) C57BL/6J (WT), $Nos2^{-/-}$ and $Cybb^{-/-}$ BMDMs were

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left untreated or treated with 25ng/mL of IFN γ for 18 h. Macrophages were infected with H37Rv (solid) or

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$\Delta cydA$ (diagonal bars) live-dead reporter strains (MOI=5). Y-axis represents the fraction BMDMs with live

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bacteria (RFP $^{+}$ GFP $^{+}$) over total infected BMDMs (GFP $^{+}$) determined by flow cytometry. C) IFN γ treated or

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untreated BMDMs were infected (MOI=5) with H37Rv (solid) or $\Delta cydA$ (diagonal bars). CFU determined 4 days

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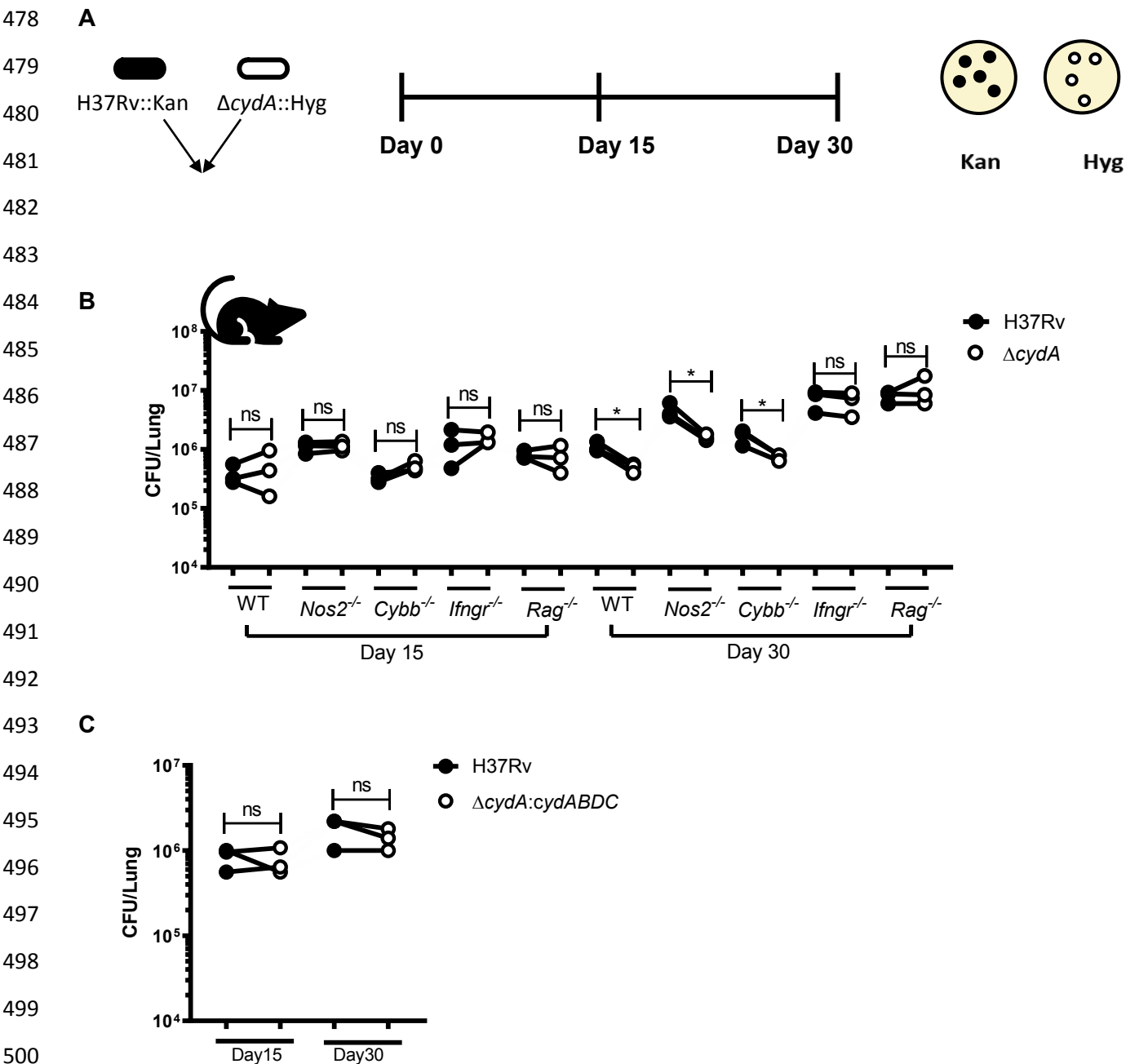
post-infection. Represented as percent survival relative to untreated. D) IFN γ treated or untreated $Nos2^{-/-}$

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BMDMs infected with H37Rv, $\Delta cydA$, $\Delta cydD$, $\Delta cydA:\Delta cydABDC$, or $\Delta cydD:\Delta cydABDC$ (MOI=5). CFU

473 determined 4 days post-infection. Represented as percent survival relative to untreated. Analysis of B and C
 474 was performed using a two-way ANOVA with Sidak post-test. Analysis of D was performed using a Dunnett's
 475 multiple comparisons test (H37Rv stimulated versus other stimulated conditions). * p-value < 0.05, ** p-value <
 476 0.01, **** p-value <0.0001.

477 **Figure 2: IFN γ but not iNOS or Phox is necessary for attenuation of Δ cydA mutants in mouse lungs**

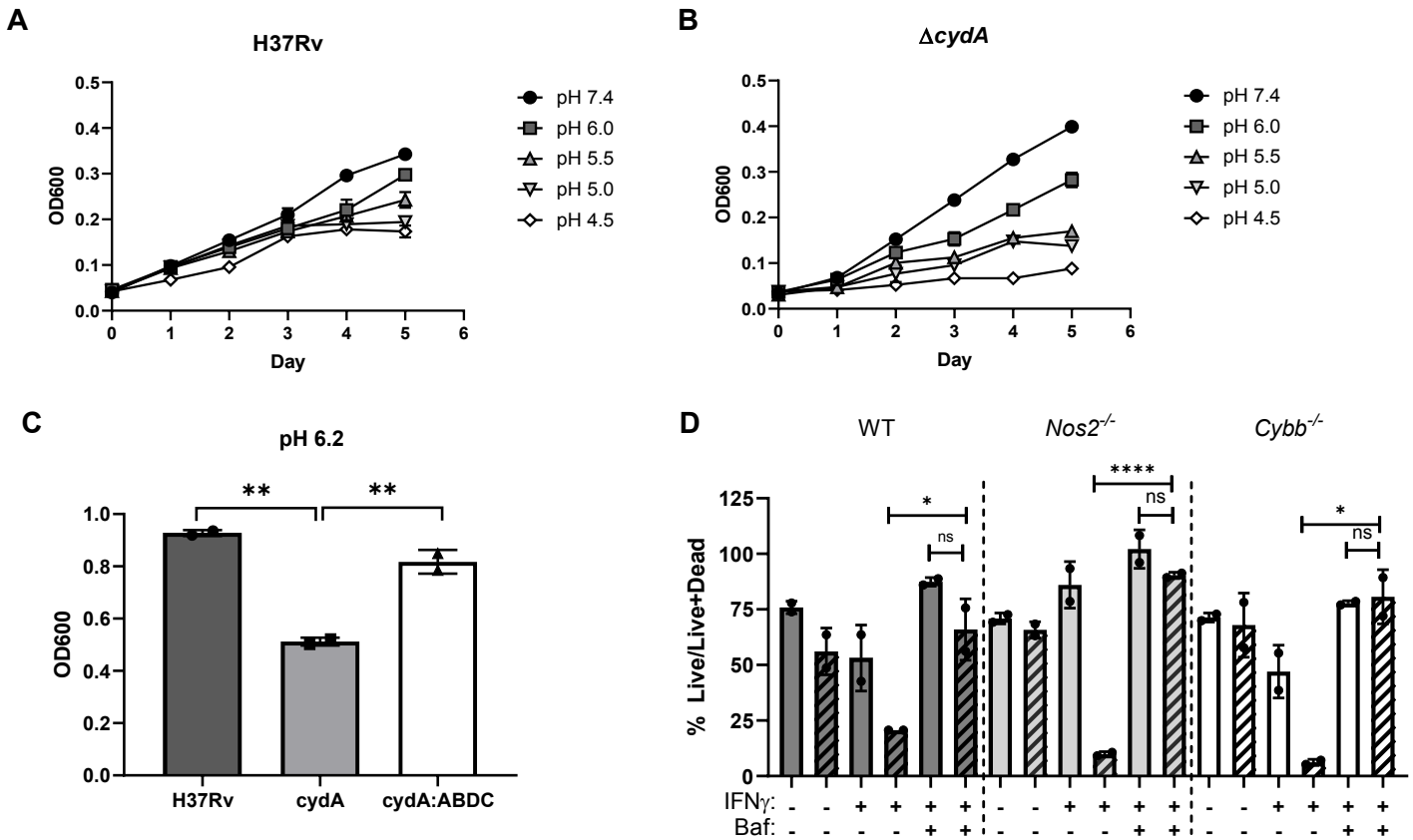


502 **Figure 2: cydA is required for persistence in IFN γ competent mice independent of Nos2 or Phox. A)**

503 Schematic of experimental design for 1:1 co-infection with H37Rv::Kan and Δ cydA::Hyg. Lungs were collected

504 on day 15 and day 30 post infection and dilutions of lung homogenates were plated on both 7H10+kanamycin
 505 and 7H10+hygromycin. B) Colony forming units (CFU) of H37Rv (black) and Δ cydA (open) in the lungs of
 506 C57BL/6J (WT), *Nos2*^{-/-}, *Cybb*^{-/-}, *Ifngr*^{-/-}, and *Rag*^{-/-} mice were enumerated at day 15 and day 30 post-
 507 infection. C) Δ cydA mutant can be complemented *in vivo* in C57BL/6J. Lung CFU of H37Rv and Δ cydA:ABDC
 508 shown at day 15 and day 30 post-infection.

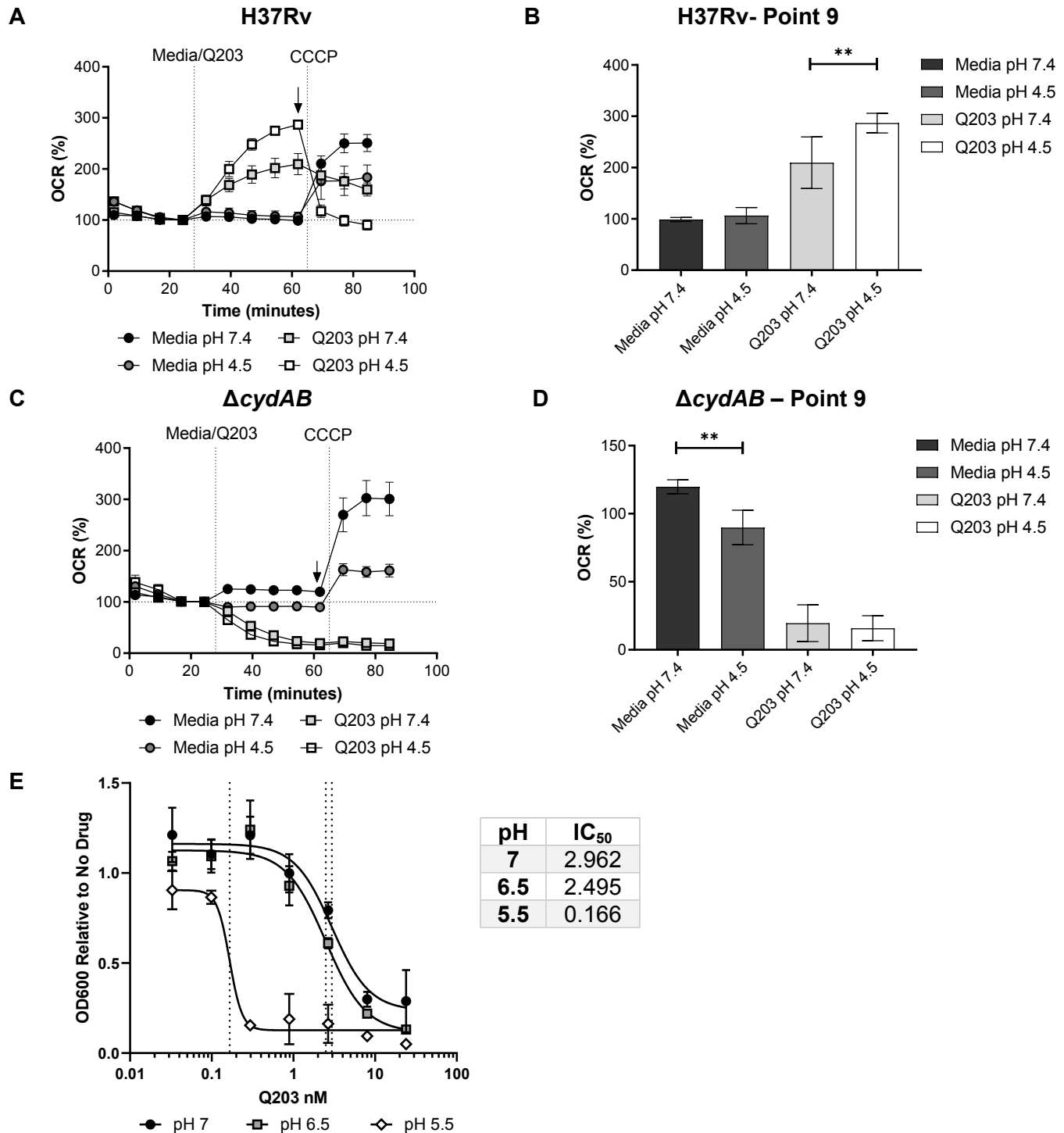
509 **Figure 3: Δ cydA are defective for growth at low pH**



525 **Figure 3: *cydA* is required for growth in acidic conditions.** A, B) Growth of H37Rv (A) and Δ cydA (B) in
 526 7H9-tyloxapol at pH 7.4, pH 6.0, pH 5.5, pH 5.0, and pH 4.5 measured by OD600 for 5 days in a 96 well plate.
 527 C) Δ cydA mutant is complemented (Δ cydA:ABDC) at pH 6.2. Cultures grown for 7 days in inkwells. D) WT,
 528 *Nos2*^{-/-} and *Cybb*^{-/-} BMDMs were left untreated or treated with IFN γ (25ng/mL). Macrophages were infected
 529 with H37Rv (solid) or Δ cydA (diagonal bars) live-dead reporter strains (MOI=5). Post-infection BMDMs were
 530 left untreated, treated with IFN γ , or treated with IFN γ and Bafilomycin A (100ng/mL). The fraction of
 531 macrophages harboring live bacteria (%Live/ Live+Dead) was determined using flow cytometry. Analysis of C

was performed using a one-way ANOVA. Analysis of D was performed using a two-way ANOVA with Sidak post-test. Dunnett's. * p-value < 0.05, ** p-value < 0.01, **** p-value < 0.0001

Figure 4: Low pH alters bacterial oxygen consumption rate in Q203 treated WT and Δ cydAB strains



562 **Figure 4: Low pH reduces cytochrome *bc₁/aa₃* dependent oxygen consumption.** A) H37Rv was exposed
563 to media at pH 7.4 and pH 4.5 for ~30 minutes and then treated with Q203 (10nM), followed by the uncoupler,
564 CCCP. The oxygen consumption rate (OCR) was measured using the extracellular flux analyser B) Bar graphs
565 are plotted from point 9 (indicated by the black arrow in A) before the addition of CCCP. C) $\Delta cydAB$ mutant
566 was exposed to the same conditions described in A. D) Bar graph of point 9 (black arrow in C). E) IC50 curves
567 of H37Rv treated with Q203 at pH 7.0, pH 6.5 and pH 5.5- day 6. IC50 values for Q203 (nM) at each pH show
568 in the table.

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