

1 **The Phagocyte Oxidase Controls Tolerance to *Mycobacterium tuberculosis* infection.**

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6 Running Title: Phox protects against TB

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18 **Summary**

19 Protection from infectious disease relies on two distinct mechanisms. “Antimicrobial resistance”
20 directly inhibits pathogen growth, whereas “infection tolerance” controls tissue damage. A single
21 immune-mediator can differentially contribute to these mechanisms in distinct contexts,
22 confounding our understanding of protection to different pathogens. For example, the NADPH-
23 dependent phagocyte oxidase complex (Phox) produces anti-microbial superoxides and
24 protects from tuberculosis in humans. However, Phox-deficient mice do not display the
25 expected defect in resistance to *M. tuberculosis* leaving the role of this complex unclear. We re-
26 examined the mechanisms by which Phox contributes to protection from TB and found that mice
27 lacking the Cybb subunit of Phox suffered from a specific defect in tolerance, which was due to
28 unregulated Caspase1 activation, IL-1 β production, and neutrophil influx into the lung. These
29 studies demonstrate that Phox-derived superoxide protect against TB by promoting tolerance to
30 persistent infection, and highlight a central role for Caspase1 in regulating TB disease
31 progression.

32

33 **Introduction**

34 Protective immunity to infectious disease involves functionally overlapping responses
35 that can be divided into two fundamentally different categories (Medzhitov et al., 2012;
36 Schneider and Ayres, 2008). Infection “resistance” refers to functions that directly target the
37 infecting pathogen to prevent its growth and dissemination. Resistance pathways act by a
38 variety mechanisms including disrupting the bacterial niche, serving as metabolic poisons, and
39 sequestering critical nutrients (Hood and Skaar, 2012; Olive and Sasse, 2016; Pilla-Moffett et
40 al., 2016). In addition, the extent of disease is also influenced by “tolerance” mechanisms that
41 enhance host survival but do not directly impact pathogen growth (Ayres and Schneider, 2008;
42 Jamieson et al., 2013; Weis et al., 2017). Tolerance pathways control a broad range of functions
43 that protect the infected tissues from both the direct cytotoxic properties of the pathogen and
44 inflammation-mediated immunopathology. While it is well appreciated that both resistance and
45 tolerance mechanisms are required to limit disease, the relative importance of these pathways
46 vary for different infections (Medzhitov et al., 2012). Furthermore, since individual immune
47 effectors can promote both tolerance and resistance, the specific role for each mediator can
48 change in different contexts (Jeney et al., 2014; Medzhitov et al., 2012; Meunier et al., 2017;
49 Mishra et al., 2017). In the context of chronic infections, where resistance mechanisms are
50 insufficient and the pathogen persists in the tissue, tolerance is likely to play a particularly
51 important role (Meunier et al., 2017).

52 Like many other chronic infections, the outcome of an encounter with *Mycobacterium*
53 *tuberculosis* (*Mtb*) varies dramatically between individuals (Cadena et al., 2017). Only 5-10% of
54 those that are infected with this pathogen progress to active tuberculosis (TB), and disease
55 progression is influenced by a wide-variety of genetic and environmental factors that could
56 modulate either tolerance or resistance (Chen et al., 2014; Lopez et al., 2003; Tobin et al.,
57 2012). For example, observations from humans and mice indicate that several specific changes
58 in T cell function may contribute to a failure of resistance and disease progression due to loss of

59 antimicrobial resistance (Jayaraman et al., 2016; Larson et al., 2013; Redford et al., 2011). In
60 addition, studies in animal models indicate that a failure of host tolerance, which is necessary to
61 preserve lung function or granuloma structure, influences the extent of disease (Desvignes et
62 al., 2015; Pasipanodya et al., 2010). While these studies suggest that variation in overall
63 tolerance may be an important determinant TB risk, the specific tolerance mechanisms that
64 influence disease progression in natural populations remain ill defined.

65 During many bacterial infections the production of reactive oxygen species (ROS) by the
66 NADPH phagocyte oxidase (Phox) is essential to protect the host from disease (Segal, 2005).
67 Phox is a multi-protein complex, including the subunits Cybb (gp91) and Ncf1 (p47) that
68 assemble in activated immune cells to produce superoxide radicals by transferring electrons
69 from NADPH to molecular oxygen (Panday et al., 2015). Humans with deleterious mutations in
70 the Phox complex develop a clinical syndrome known as chronic granulomatous disease
71 (CGD). Leukocytes from patients with CGD are unable to kill a number of bacterial pathogens,
72 such as *Staphylococcus aureus* and *Serratia marcescens*, and this defect is associated with the
73 susceptibility to infection with these organisms (Johnston and Baehner, 1970). Because ROS
74 contributes to the microbicidal activity of phagocytes, previous studies in *Mtb*-infected mice
75 focused on the role of Phox in antimicrobial resistance. However, mice deficient in Phox are
76 able to restrict *Mtb* growth to levels comparable to wild type animals during the initial stages of
77 infection (Deffert et al., 2014a; Jung et al., 2002); (Cooper et al., 2000; Jung et al., 2002). The
78 lack of an obvious antimicrobial role for Phox has been attributed to the expression of
79 mycobacterial defenses, such as the catalase/peroxidase, KatG, which detoxifies ROS directly,
80 or CpsA which prevents Phox localization to the *Mtb* containing vacuole (Colangeli et al., 2009;
81 Koster et al., 2017; Nambi et al., 2015; Ng et al., 2004). These findings have led to the
82 conclusion that ROS produced by Phox are not required for protection to *Mtb* (Nathan and
83 Shiloh, 2000). In contrast, mutations in the Cybb gene are strongly associated with susceptibility
84 to mycobacterial disease, including tuberculosis (Bustamante et al., 2011; Deffert et al., 2014a;

85 Khan et al., 2016; Lee et al., 2008). Mutations that specifically reduce Cybb activity in
86 macrophages produce a similar clinical presentation, highlighting the importance of the
87 macrophage-derived ROS in protection from pathogenic mycobacteria (Bustamante et al.,
88 2011). The apparently conflicting data from mice and humans regarding the importance of Phox
89 during *Mtb* infection suggest two possibilities. Either Phox is differentially required to protect
90 against *Mtb* in mice and humans or the ROS produced by Phox is necessary to control immune
91 mechanisms that do not directly modulate bacterial replication.

92 Here we re-examined the role of Phox in the context of *Mtb* infection. Consistent with
93 previous reports we found that loss of the Phox subunit Cybb does not alter the growth or
94 survival of *Mtb* during infection. Instead, *Cybb*^{-/-} animals suffered from a hyper-inflammatory
95 disease caused by increased activation of the NLRP3-dependent Caspase-1 inflammasome
96 and IL-1-dependent neutrophil accumulation in the lung. Thus, the protective effect of Phox can
97 be attributed to increased tolerance to *Mtb* infection instead of a direct antimicrobial effect.
98 These studies provide a mechanism to explain the association between Phox expression and
99 TB disease in natural populations, and implicate Caspase-1 as an important regulator of
100 infection tolerance.

101

102 **Results**

103 ***Cybb*^{-/-} mice are susceptible to TB disease, but maintain control of bacterial replication.**

104 In order to re-examine the role of Phox in mediating protection against *Mtb*, we compared
105 disease progression and the immune responses in wild type and *Cybb*^{-/-} C57BL/6 mice after
106 infection via aerosol with 50-100 bacteria. We found no significant difference in the survival or
107 bacterial levels in the lung between groups of mice up to 3 months following infection,
108 confirming that Cybb is not required for surviving the early stages of *Mtb* infection (Figure 1A).
109 However, after 100 days of infection, *Cybb*^{-/-} infected mice lost an average of 10% of their body
110 weight while wild type animals gained weight (Figure 1B). Histopathological inspection of the

111 lungs also indicated a difference in disease between these groups, with *Cybb*^{-/-} lungs containing
112 larger and less organized lesions than wild type (Figure 1C). These data suggested that wild
113 type and *Cybb*^{-/-} animals might tolerate *Mtb* infection differently even while harboring identical
114 levels of bacteria.

115 In order to dissect the mechanisms controlling tolerance to *Mtb* disease in *Cybb*^{-/-} mice,
116 we profiled the infected lungs of animals during infection by flow cytometry. We found no
117 significant differences in the numbers of dendritic cells, macrophages, B cells, as well as total T
118 cells between wild type and *Cybb*^{-/-} mice (Figure S1). In contrast, we observed an early and
119 sustained increase of Ly6G⁺ CD11b⁺ neutrophils in the infected lungs of *Cybb*^{-/-} mice (Figure 1D
120 and 1E). A 3-5-fold increase in the total number neutrophils was observed as early as 4 weeks
121 following infection and was maintained throughout the 12-week study.

122 The cytokine IL-1 β promotes neutrophil-mediated disease during *Mtb* infection of other
123 susceptible mouse strains (Mishra et al., 2017; Mishra et al., 2013). Similarly, when we assayed
124 cytokine levels in lung homogenates, we noted a dramatic and specific increase in IL-1 β
125 concentration in *Cybb*^{-/-} animals compared to wild type at all time points. In contrast, no
126 significant differences were noted for IFN γ or TNF α at any time point between groups. Thus,
127 while the adaptive immune response to *Mtb* appeared to be intact, *Cybb*^{-/-} animals produced
128 excess IL-1 β and the concentration of this cytokine correlated with neutrophil infiltration into the
129 lung.

130 Previous studies have shown that following low dose aerosol infection mice deficient in
131 Phox (both *Cybb*^{-/-} or p47^{-/-}) survive for at least 60 days (Cooper et al., 2000; Jung et al., 2002).
132 However, longer infection is likely necessary to determine whether the enhanced disease we
133 noted in *Cybb*^{-/-} mice would result in a survival defect. These long-term survival experiments
134 following low dose aerosol proved difficult, since uninfected *Cybb*^{-/-} mice develop arthritis as
135 they age (Lee et al., 2011). To avoid this confounder, we quantified the survival of mice in a

136 shorter-term study using a high dose aerosol infection. When mice were infected with ~5000
137 CFU per animal, *Cybb*^{-/-} mice succumbed to disease significantly more rapidly than wild type
138 animals. *Cybb*^{-/-} mice had a median survival time of 88 days while only two out of fifteen wild
139 type mice succumbed during the 120 day study (Figure 1K). In order to distinguish survival
140 effects not related to *Mtb* infection, a cohort of uninfected age-matched *Cybb*^{-/-} mice were
141 maintained for the duration of the experiment. None of these animals required euthanasia over
142 the 120 days and no animals included in this experiment developed arthritis.

143 During this high-dose study, we also examined a cohort of mice 50 days following
144 infection and found identical levels of bacteria in the lungs and spleen between wild type and
145 *Cybb*^{-/-} groups (Figure 1G). Consistent with our earlier findings, *Cybb*^{-/-} mice showed a significant
146 increase in neutrophils and IL-1 β in the lung (Figure 1H-J). We also found minimal levels of IL-
147 1 β and neutrophils in uninfected *Cybb*^{-/-} lungs indicating that these phenotypes are dependent
148 on *Mtb* infection. Therefore, the loss of *Cybb* leads to more severe *Mtb* disease that is
149 associated with increased IL-1 β levels and neutrophil recruitment, even though the number of
150 viable *Mtb* in the lung did not appear to be affected.

151

152 ***Cybb* controls tolerance to *Mtb* infection.**

153 Our initial results suggested that *Cybb* protects mice by promoting tolerance to *Mtb* infection
154 rather than directly controlling bacterial replication. However, while viable bacterial numbers
155 were similar in wild type and *Cybb*^{-/-} mice, we could not rule out that the course of disease was
156 altered by subtle changes in the dynamics of bacterial growth and death. To more rigorously
157 address this question, we employed two additional animal models that allowed us to
158 differentiate tolerance and direct antimicrobial resistance *in vivo*.

159 To more formally exclude the possibility that *Cybb* alters the intracellular growth of *Mtb*
160 during infection, we used a previously optimized mixed bone marrow chimera approach (Mishra

161 et al., 2017). These experiments normalize potential inflammatory differences between wild type
162 and *Cybb*^{-/-} mice allowing us to specifically quantify differences in bacterial control (Figure 2A).
163 Irradiated wild type mice were reconstituted with a 1:1 mixture of CD45.1⁺ wild type and
164 CD45.2⁺ *Cybb*^{-/-} or wild type cells. Five weeks following infection, both CD45.1⁺ and CD45.2⁺
165 cells were sorted from the lungs and the levels of *Mtb* in each genotype was determined by
166 plating and the purity of populations was determined by flow cytometry (Figure 2B and 2C and
167 Figure S2). We found that the relative abundance of wild type and *Cybb*^{-/-} cells was similarly
168 maintained throughout infection in both the myeloid and lymphoid compartments, indicating that
169 *Cybb* does not alter cellular recruitment or survival in a cell-autonomous manner. When *Mtb*
170 was enumerated in sorted cells, we found identical levels of H37Rv in wild type CD45.1⁺ and
171 *Cybb*^{-/-} CD45.2⁺ populations from the same mouse, similar to the results from mice where both
172 populations were reconstituted with congenically mismatched wild type cells. In contrast, when
173 chimeric mice were infected with a ROS-sensitive Δ *katG* mutant of *Mtb*, we found higher levels
174 of bacteria in *Cybb*^{-/-} cells compared to wild type cells from the same mouse. These data show
175 that the assay is able to detect the cell-autonomous antimicrobial activity of ROS against a
176 KatG-deficient *Mtb* strain, but *Cybb*-dependent ROS did not restrict the intracellular replication
177 of wild type *Mtb*.

178 To specifically determine if the loss of *Cybb* decreased tolerance to a given burden of
179 bacteria, wild type and *Cybb*^{-/-} mice were infected with a streptomycin auxotrophic strain of *Mtb*
180 that allows exogenous control of bacterial replication during infection. Streptomycin is provided
181 for the first two weeks of infection, allowing the pathogen to reach the burden observed in a wild
182 type *Mtb* infection. Upon streptomycin withdrawal, the pathogen is unable to replicate but
183 remains viable and able to drive inflammatory responses (Figure 2D) (Honore et al., 1995;
184 Mishra et al., 2017; Mishra et al., 2013). Five weeks after infection, *Cybb*^{-/-} mice lost more
185 weight than wild type animals while harboring identical levels of non-replicating bacteria (Figure
186 2E and 2F). Lungs from *Cybb*^{-/-} mice contained significantly more neutrophils and higher levels

187 of IL-1 β compared to wild type animals (Figure 2G-2I). Thus, even when the need for
188 antimicrobial resistance is obviated by artificially inhibiting bacterial replication, *Cybb* $^{-/-}$ animals
189 continued to exhibit a hyper-inflammatory disease.

190 The granulocytic inflammation observed in *Cybb* $^{-/-}$ mice was reminiscent of several other
191 susceptible mouse strains. However, the neutrophil recruitment in other models is generally
192 associated with a concomitant increase in bacterial growth (Kimmey et al., 2015; Kramnik et al.,
193 2000; Nandi and Behar, 2011) and a transition of the intracellular *Mtb* burden from
194 macrophages to granulocytes (Mishra et al., 2017). We hypothesized that *Cybb* $^{-/-}$ mice may be
195 able to retain control of *Mtb* replication because the bacteria remain in macrophages. To test
196 this hypothesis, we used a YFP-expressing *Mtb* strain to compare the distribution of cells
197 harboring bacteria in wild type and *Cybb* $^{-/-}$ mice with *Nos2* $^{-/-}$ animals in which *Mtb* replicates to
198 high numbers in association with infiltrating granulocytes (Mishra et al., 2017). Four weeks
199 following infection we found that lungs from both *Cybb* $^{-/-}$ and *Nos2* $^{-/-}$ mice contain higher levels
200 of IL-1 β and neutrophils than wild type animals, although the loss of *Nos2* $^{-/-}$ produced a much
201 more severe phenotype than *Cybb* $^{-/-}$. (Figure 2J-2L and Figure S2). However, the cells
202 harboring *Mtb* in these two susceptible mouse strains differed. In wild type and *Cybb* $^{-/-}$ mice,
203 YFP-*Mtb* was evenly distributed between CD11b+/Ly6G+ neutrophils and the CD11b+/Ly6G-
204 population that consists of macrophages and dendritic cells (Wolf et al., 2007). This proportion
205 was dramatically altered in *Nos2* $^{-/-}$ mice, where close to 90% of bacteria were found in the
206 neutrophil compartment. (Figure 2M and 2N). Thus, unlike other susceptible mouse models, the
207 loss of *Cybb* does not alter bacterial replication or the distribution of *Mtb* in different myeloid
208 subsets. Instead, this gene plays a specific role in controlling IL1 β production, neutrophil
209 recruitment to the infected lung, and disease progression. As a result, we conclude that *Cybb*
210 specifically promotes tolerance to *Mtb* infection.

211

212 **Enhanced IL-1 β production by *Cybb*^{-/-} macrophages and dendritic cells is due to**
213 **deregulated Caspase-1 inflammasome activation**

214 To investigate the mechanism underlying increased IL-1 β production in *Cybb*^{-/-} mice, we
215 quantified the release of mature cytokine from bone-marrow derived macrophages (BMDMs)
216 and bone-marrow derived dendritic cells (BMDCs). Compared to wild type, we found that *Cybb*^{-/-}
217 BMDMs and BMDCs produced 4-5 fold more IL-1 β after 24 hours of *Mtb* infection (Figure 3A
218 and 3D). Under these conditions, wild type and *Cybb*^{-/-} cells remained equally viable and
219 produced equivalent amounts of TNF (Figure 3B-F), suggesting that the effect of Cybb on IL-1 β
220 production was specific to this cytokine.

221 The production of mature IL-1 β requires two distinct signals (von Moltke et al., 2013).
222 The first signal induces the expression of *Il1b* mRNA and subsequent production of pro-IL-1 β ,
223 and a second signal activates Caspase1, which is necessary for the processing and secretion of
224 mature IL-1 β . To understand what step of IL1 β production was altered in *Cybb*^{-/-} cells, we
225 quantified these two signals. The expression of *Il1b* mRNA in uninfected and infected BMDMs
226 was unchanged between wild type and *Cybb*^{-/-} BMDMs (Figure 3G). In contrast, under the same
227 conditions, the processing of Caspase1 to its active form was increased in infected *Cybb*^{-/-}
228 BMDMs compared to wild type cells (Figure 3H).

229 These observations suggested that caspase-1 activity is increased in *Cybb*^{-/-} cells, which
230 could allow mature IL-1 β secretion in the absence of an inflammasome activator. To test this
231 hypothesis, we stimulated cells with the TLR2 agonist, PAM3CSK4, to induce pro- IL-1 β
232 production. PAM3CSK4 stimulation induced *Il1b* mRNA to similar levels between wild type and
233 *Cybb*^{-/-} BMDMs, albeit over 100 times higher than infection with *Mtb* (Figure 3I). In wild type
234 cells, this induction of *Il1b* expression produced little mature IL-1 β secretion, consistent with the
235 need for subsequent inflammasome activation. In contrast, induction of *Il1b* expression led to
236 robust secretion of mature IL-1 β from *Cybb*^{-/-} BMDM, consistent with unregulated inflammasome

237 activity in these cells (Figure 3J). Together these data show that loss of Cybb leads to hyper-
238 activation of Caspase1 and increased release of IL-1 β during *Mtb* infection of both BMDMs and
239 BMDCs.

240

241 **Loss of tolerance is reversed in *Cybb*^{-/-} macrophages and mice by blocking the
242 production or activity of IL-1 β**

243 The NLRP3 inflammasome consists of NLRP3, ASC, and Caspase 1. While this
244 complex is generally responsible for IL-1 β processing in *Mtb* infected macrophages (Coll et al.,
245 2015; Dorhoi et al., 2012), it remained unclear whether the enhanced IL-1 β secretion from *Cybb*^{-/-}
246 cells also relied on these components. To identify the responsible complex, we blocked the
247 activation of the NLRP3 inflammasome in several distinct ways. The NLRP3 inflammasome is
248 specifically inhibited by IFN γ stimulation, via the nitric oxide-dependent nitrosylation of the
249 NLRP3 protein (Mishra et al., 2013). Pretreatment of wild type and *Cybb*^{-/-} BMDMs with varying
250 concentrations of IFN γ , inhibited the secretion of IL-1 β from both wild type and *Cybb*^{-/-} BMDMs
251 compared to untreated cells. While this result indicated an important role for NLRP3, IFN γ
252 pretreatment did not completely suppress IL-1 β secretion and there remained significant
253 differences in the IL-1 β release between *Cybb*^{-/-} and wild type cells at all concentrations of the
254 cytokine.

255 To more directly assess the role of NLRP3 and Caspase-1 in IL-1 β production by *Cybb*^{-/-}
256 cells, we employed specific small molecule inhibitors. Treatment of *Mtb*-infected BMDMs with
257 either the NLRP3 inhibitor, MCC950 (Coll et al., 2015), or the Caspase-1 inhibitor, VX-765
258 (Stack et al., 2005) caused a dramatic reduction in IL-1 β in both wild type and *Cybb*^{-/-} BMDMs
259 compared to untreated cells (Figure 4B and 4C). This ten-fold decrease in IL-1 β secretion could
260 not be attributed to inhibition of pro-IL-1 β levels, as none of these inhibitors affected *Il1b* mRNA
261 by more than two-fold. Similarly, the spontaneous IL-1 β secretion observed upon PAM3CSK4

262 stimulation was also inhibited by MCC950 and IFN γ , (Figure 4E). In each case inflammasome
263 inhibition reduced IL-1 β secretion to the same level in both wild type and *Cybb* $^{-/-}$ cells, indicating
264 that the NLRP3 inflammasome was responsible for the enhanced production of this cytokine in
265 *Cybb* $^{-/-}$ BMDM.

266 Based on these studies, we hypothesized that the tolerance defect observed in the intact
267 mouse was due to inflammasome-dependent IL-1 signaling. IL-1 β serves a complex role during
268 infection (Mayer-Barber et al., 2010; Nunes-Alves et al., 2014). Some production of this cytokine
269 is important for the antimicrobial immunity, but persistent production can lead to pathology. In
270 order to focus on the role of over-production of IL-1 β on tolerance, we inhibited IL-1 signaling in
271 mice infected with non-replicating auxotrophic *Mtb* to normalize the bacterial burden. Two
272 weeks after infection, wild type and *Cybb* $^{-/-}$ mice were treated with either an isotype control
273 antibody or an anti-IL1R antibody to block the effect of increased IL-1 β production. As expected,
274 *Mtb* levels were similar in all mice, but more neutrophils accumulated in the lungs *Cybb* $^{-/-}$
275 animals (Figure 4F-H). While anti-IL-1R treatment had no effect in wild type animals, inhibition
276 of IL-1 signaling reduced neutrophil infiltration in *Cybb* $^{-/-}$ mice to the level observed in wild type
277 animals. Taken together, our data show that *Cybb* $^{-/-}$ contributes to protective immunity to *Mtb*
278 not by controlling bacterial replication, but instead by preventing an IL1 dependent inflammatory
279 response that increases neutrophil recruitment to the lung and exacerbates disease
280 progression.

281

282 **Discussion**

283 The role of the Phox complex in protection from TB has presented a paradox (Deffert et al.,
284 2014a). Based on the well-described antimicrobial properties of Phox-derived ROS, previous
285 studies have focused on examining the function of Phox components in controlling *Mtb*
286 replication in mice (Cooper et al., 2000; Deffert et al., 2014b; Jung et al., 2002). The minimal

287 effects observed in these assays suggested that Phox may not play a protective role in TB. This
288 conclusion contrasts with several studies indicating that human CGD patients show increased
289 susceptibility to TB disease (Bustamante et al., 2011; Deffert et al., 2014a; Khan et al., 2016).
290 Our dissection of disease progression in Cybb-deficient mice harmonizes these conflicting
291 observations. While we verify that Phox plays no discernable role in antimicrobial resistance to
292 *Mtb*, we uncovered a previously unknown role for this complex in promoting tolerance to *Mtb*
293 infection and inhibiting TB disease progression.

294 While we were able to clearly delineate the role of Phox during *Mtb* infection, the role(s)
295 played by this complex in any given infection is likely to vary. Phox-deficient mice are unable to
296 control the growth of several bacterial pathogens that are known to cause serious infections in
297 CGD patients, including non-tuberculous mycobacteria (Deffert et al., 2014b; Dinauer et al.,
298 1997; Fujita et al., 2010; Jackson et al., 1995). In the context of these infections, the
299 antimicrobial functions of Phox may predominate. In contrast, for pathogens such as *Mtb* that
300 are resistant to ROS-mediated toxicity and persist in the tissue to promote continual
301 inflammatory damage, the tolerance-promoting activity of Phox appears to play a dominant role.

302 During *Mtb* infection, we found that the ROS produced by Phox are critical to control the
303 activation of the NLRP3 inflammasome. In contrast, mitochondrial ROS are well known to
304 activate inflammatory cascades (Weinberg et al., 2015), suggesting that the context by which
305 ROS are produced influences the inflammatory outcome of activated cells. Despite this
306 complexity, Phox-deficient mice and CGD patients suffer from hyper-inflammatory diseases
307 including arthritis, colitis, and prolonged inflammatory reactions to microbial products, indicating
308 that the dominant immunoregulatory role for Phox-derived ROS is anti-inflammatory
309 (Morgenstern et al., 1997; Schappi et al., 2008; Segal et al., 2010). Several non-mutually
310 exclusive mechanisms could explain this anti-inflammatory effect. For example, the loss of Phox
311 derived ROS has been proposed to promote the production of inflammatory mediators by
312 inhibiting autophagy (de Luca et al., 2014). Another mechanism was described in superoxide

313 dismutase1 (*Sod1*) deficient cells, where the accumulation of ROS inhibits Caspase1 activation
314 through glutathionation of reactive cysteines (Meissner et al., 2008). This latter mechanism is
315 reminiscent of the process by which nitric oxide (NO), inhibits inflammasome activation via S-
316 nitrosylation of NLRP3 (Mishra et al., 2013). The intersection of these two important anti-
317 inflammatory pathways at the NLRP3 inflammasome indicates that this complex may be a
318 critical point of integration where inflammatory cascades are controlled during chronic infections.

319 Our findings are consistent with a growing body of literature suggesting that
320 inflammasome-derived IL-1 promotes TB disease progression. For example, genetic
321 polymorphisms that increase the expression of IL1 β or the production of IL-1 dependent pro-
322 inflammatory lipid mediators are associated with TB disease progression (Mishra et al., 2017;
323 Zhang et al., 2014). Similarly, transcriptional signatures of inflammasome activation have been
324 observed in severe forms of TB disease, such as meningitis (Marais et al., 2017) and TB-
325 associated immune reconstitution syndrome (Tan et al., 2016). Together with our work, these
326 findings imply an important role for infection tolerance in protection from TB disease, and
327 implicate Caspase-1 as a critical point at which tolerance is regulated.

328

329 **Materials and Methods**

330 **Mice**

331 C57BL/6J (Stock # 000664), *Cybb*^{-/-} (B6.129S-Cybb^{tm1Din}/J stock # 002365), *Nos2*^{-/-} (B6.129P2-
332 *Nos2*^{tm1Lau}/j, stock # 002609), B6.SJL-*Ptprc*^a *Pepc*^b carrying the pan leukocyte marker CD45.1
333 (stock # 002014) were purchased from the Jackson Laboratory. Mice were housed under
334 specific pathogen-free conditions and in accordance with the University of Massachusetts
335 Medical School, IACUC guidelines. All animals used for experiments were 6-12 weeks except
336 mixed chimeras that were infected at 16 weeks following 8 weeks of reconstitution.

337

338 **Mouse Infection**

339 Wild type *M. tuberculosis* strain H37Rv was used for all studies unless indicated. This strain was
340 confirmed to be PDIM-positive. Prior to infection bacteria were cultured in 7H9 medium
341 containing 10% oleic albumin dextrose catalase growth supplement (OADC) enrichment
342 (Becton Dickinson) and 0.05% Tween 80. H37Rv expressing msfYFP has been previously
343 described and the episomal plasmid was maintained with selection in Hygromycin B (50ug/ml)
344 added to the media (Mishra et al., 2017). For low and high dose aerosol infections, bacteria
345 were resuspended in phosphate-buffered saline containing tween 80 (PBS-T). Prior to infection
346 bacteria were sonicated then delivered via the respiratory route using an aerosol generation
347 device (Glas-Col). Infections of mice with the streptomycin auxotrophic strain of *Mtb* (18b) have
348 been previously described. In short mice were infected via intra-tracheal infection and treated
349 daily with 2mg of streptomycin daily for two weeks. For anti-IL1R treatment mice were injected
350 with 200ug of anti-IL1R antibody or Isotype control (Bio-xcell) every other day starting at day 14.
351 Both male and female mice were used throughout the study and no significant differences in
352 phenotypes were observed between sexes.

353

354 **Immunohistochemistry**

355 Lungs from indicated mice were inflated with 10% buffered formalin and fixed for at least 24
356 hours then embedded in paraffin. Five-Micrometer—thick sections were stained with
357 haematoxylin and eosin (H&E). All staining was done by the Diabetes and Endocrinology
358 Research Center Morphology Core at the University of Massachusetts Medical School.

359

360 **Flow Cytometry**

361 Lung tissue was harvested in DMEM containing FBS and placed in C-tubes (Miltenyi).
362 Collagenase type IV/DNaseI was added and tissues were dissociated for 10 seconds on a
363 GentleMACS system (Miltenyi). Tissues were incubated for 30 minutes at 37C with oscillations
364 and then dissociated for an additional 30 seconds on a GentleMACS. Lung homogenates were

365 passed through a 70-micron filter or saved for subsequent analysis. Cell suspensions were
366 washed in DMEM, passed through a 40-micron filter and aliquoted into 96 well plates for flow
367 cytometry staining. Non-specific antibody binding was first blocked using Fc-Block. Cells were
368 then stained with anti-Ly-6G Pacific Blue, anti-CD4 Pacific Blue, anti-CD11b PE, anti-CD11c
369 APC, anti-Ly-6C APC-Cy7, anti-CD45.2 PercP Cy5.5, anti-CD3 FITC, anti-CD8 APC-Cy7, anti-
370 B220 PE-Cy7 (Biolegend). Live cells were identified using fixable live dead aqua (Life
371 Technologies). For infections with fluorescent H37Rv, lung tissue was prepared as above but no
372 antibodies were used in the FITC channel. All of these experiments contained a non-fluorescent
373 H37Rv infection control to identify infected cells. Cells were stained for 30 minutes at room
374 temperature and fixed in 1% Paraformaldehyde for 60 minutes. All flow cytometry was run on a
375 MACSQuant Analyzer 10 (Miltenyi) and was analyzed using FlowJo_V9 (Tree Star).

376

377 **Macrophage and Dendritic Cell Generation**

378 To generate bone marrow derived macrophages (BMDMs), marrow was isolated from femurs
379 and tibia of age and sex matched mice as previously described. Cells were then incubated in
380 DMEM (Sigma) containing 10% fetal bovine serum (FBS) and 20% L929 supernatant. Three
381 days later media was exchanged with fresh media and seven days post-isolation cells were
382 lifted with PBS-EDTA and seeded in DMEM containing 10% FBS for experiments.

383 To generate bone marrow derived dendritic cells (BMDCs) Marrow was isolated from
384 femurs and tibia of age and sex matched mice. Cell were then incubated in iMDM media
385 (GIBCO) containing 10% FBS, L-Glutamine, 2 μ M 2-mercaptoethanol and 10% B16-GM-CSF
386 supernatant (Zanoni et al., 2016). BMDCs were then purified on day six using Miltenyi LS
387 columns first using negative selection for F480 followed by CD11c positive selection. Cells were
388 then plated and infected the following day.

389

390 **Macrophage and Dendritic Cell Infection**

391 *Mtb* or *Mycobacterium bovis*-BCG were cultured in 7H9 medium containing 10% oleic albumin
392 dextrose catalase growth supplement (OADC) enrichment (Becton Dickinson) and 0.05%
393 Tween 80. Before infection cultures were washed in PBS-T, resuspended in DMEM containing
394 10%FBS and passed through a 5-micron filter to ensure single cells. Multiplicity of infection
395 (MOI) was determined by optical density (OD) with an OD of 1 being equivalent to 3×10^8
396 bacteria per milliliter. Bacteria were added to macrophages for 4 hours then cells were washed
397 with PBS and fresh media was added. At the indicated time points supernatants were harvested
398 for cytokine analysis and the cells were processed for further analysis. Cell death was assessed
399 using Cell-Titer-Glo luminescent cell viability assay (Promega) following manufacturer's
400 instructions. For inhibitor treatments cells were treated with the indicated concentrations of IFN γ
401 (Peprotech), MCC950 (Adipogen) or VX-765 (Invivogen) or vehicle control overnight prior to
402 infection and maintained in the media throughout the experiment.

403

404 **Mixed Bone Marrow Chimera generation and cell sorting**

405 Mixed bone marrow chimera experiments were done essentially as previously described. Wild-
406 type CD45.1 $^+$ mice were lethally irradiated with two doses of 600 rads. The following day, bone
407 marrow from CD45.1 $^+$ wild-type mice and CD45.2 $^+$ knockout mice (wild-type or Cybb $^{-/-}$) was
408 isolated, red blood cells were lysed using Tris-buffered ammonium chloride (ACT), and the
409 remaining cells were quantified using a haemocytometer. CD45.1 $^+$ and CD45.2 $^+$ cells were then
410 mixed equally at a 1:1 ratio and 10^7 cells from this mixture were injected intravenously into
411 lethally irradiated hosts that were placed on sulfatrim for three weeks. 8 weeks later mice were
412 then infected by low-dose aerosol with *M. tuberculosis* H37Rv. Four weeks following infection,
413 the lungs of chimera mice were processed for flow cytometry. An aliquot of this suspension was
414 saved for flow cytometry analysis of the lung population and overall bacterial levels. The
415 remaining cells were split equally and stained with either anti-CD45.1 APC or anti-CD45.2 PE.

416 Stained populations were then incubated with either anti-APC or anti-PE magnetic beads
417 (Miltenyi) following the manufacturer's instructions and sorted using LS-columns (Miltenyi).
418 Purified cells were divided equally and then plated for *M. tuberculosis* on 7H10 agar or counted
419 and stained for analysis of cellular purity. Cells from the input homogenate, flow through and the
420 positive sort fractions were stained with for purity. Samples with >90% were used for
421 subsequent analysis. At 21 days after plating, colonies were enumerated and the *Mtb* levels per
422 sorted cells were determined.

423

424 **qRT-PCR**

425 Cells were lysed in Trizol-LS (Thermofisher), RNA was purified using Direct-zol RNA isolation
426 kits (Zymogen) and quantified on nanodrop. RNA was diluted to 5ng/µl and 25 ng total RNA was
427 used for each reaction. Ct values for each sample were determined in technical duplicates for β -
428 Actin and IL-1 β using one-step RT-PCR Kit (Qiagen) on a ViiA7 Real-time PCR system (Life
429 Technologies). $\Delta\Delta\text{ct}$ values were then determined for each sample.

430

431 **Immunoblotting and Cytokine quantification**

432 Murine cytokine concentrations in culture supernatants and cell-free lung homogenates were
433 quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D). All
434 samples were normalized for total protein content. Caspase1 activation in macrophage lysates
435 was determined by western blotting with Caspase1 antibody purchased from Adipogen.

436

437 **Author Contributions**

438 AO and CMSassetti conceived of and designed the experiments. AO, CMSmith, and MK

439 performed the experiments and analyzed the data. AO and CMSassetti wrote the initial
440 manuscript. All authors edited the manuscript.

441

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449

450 **Figure Legends**

451

452 **Figure 1. Anti-inflammatory activity of Cybb protects mice from TB disease.**

453 **A.** Following low dose aerosol infection (Day 0 of ~50-100 colony forming units, cfu) total
454 bacterial burden (expressed in cfu, mean \pm s.d.) was determined in the lungs of wild type or
455 *Cybb*^{-/-} mice at the indicated time points.

456 **B.** Percentage weight loss (mean change \pm s.d) from Day 0 to Day 100 was determined for wild
457 type and *Cybb*^{-/-} mice.

458 **C.** Immunohistochemical staining for Haematoxylin and Eosin is shown for representative lung
459 sections from wild type and *Cybb*^{-/-} mice at 8 weeks post-infection at 20x magnification.

460 **D.** Representative flow cytometry plot showing increased Ly6G⁺ CD11b⁺ neutrophil recruitment
461 to the lungs of *Cybb*^{-/-} mice 4 weeks following infection (gated on live/singlets/CD45⁺).

462 **E.** Quantification of neutrophil recruitment to the lungs at the indicated time points following
463 infection for wild type and *Cybb*^{-/-} mice is shown as absolute number of Ly6G⁺ CD11b⁺ cells per
464 lung (mean \pm s.d). * p-value <.05 by unpaired two-tailed t-test.

465 **F.** Lung homogenates from wild type or *Cybb*^{-/-} mice infected for the indicated time were probed
466 for the cytokines IL-1 β , IFN γ , and TNF α by ELISA (mean \pm s.d). Results shown in A-D are
467 representative of 3 independent experiments with 3-5 mice per group. ** p-value <.01 by
468 unpaired two-tailed t-test.

469 **G.** Fifty days following high dose aerosol infection (Day 0 of 5000-7500 CFU) total bacterial
470 burden (expressed in cfu, mean \pm s.d.) was determined in the lungs and spleen of wild type or
471 *Cybb*^{-/-} mice.

472 **H.** Representative flow cytometry plot showing increased Ly6G⁺ CD11b⁺ neutrophil recruitment
473 to the lungs of *Cybb*^{-/-} mice 50 days following infection (gated on live/singlets/CD45⁺).

474 **I.** Quantification of the absolute number of neutrophils recruited to the lungs 50 days following
475 high dose infection for wild type and *Cybb*^{-/-} mice is shown (mean ⁺⁻ s.d). ** p-value <.01 by one-
476 way ANOVA with tukey correction.

477 **J.** Lung homogenates from wild type or *Cybb*^{-/-} mice infected for 50 days following high dose
478 aerosol were probed for IL-1 β by ELISA (mean ⁺⁻ s.d). ** p-value <.01 by unpaired two-tailed t-
479 test. Results shown in G-J are representative of 2 independent experiments with 5 mice per
480 group. ** p-value <.01 by unpaired two-tailed t-test.

481 **K.** Survival of infected wild type and *Cybb*^{-/-} and uninfected *Cybb*^{-/-} mice was determined
482 following high dose infection. Data are representative of two independent experiments with 14-
483 15 mice per group. *** p-value <.001 Mantel-Cox text.

484

485 **Figure 2. The primary protective role of Cybb is anti-inflammatory.**

486 **A.** Schematic for the generation of mixed bone marrow chimeras. Mixed bone marrow chimeras
487 were infected by low dose aerosol with either H37Rv or Δ KatG mutant. Five weeks following
488 infection CFU levels were determined in purified haematopoietic cells of indicated genotypes.

489 **B.** Shown are the normalized CFU per sorted cells in each population from each mouse. * p<.05
490 by unpaired two-tailed t-test.

491 **C.** The fold-increase of bacterial levels in CD45.2 $^+$ cells (experimental) compared to CD45.1 $^+$
492 cells (wild type control) (mean ⁺⁻ s.d.). The results in B and C are representative of three
493 independent experiments with 3-4 mice per group.

494 **D.** Schematic for streptomycin-dependent auxotroph infection. Wild type and *Cybb*^{-/-} mice were
495 infected intratracheally with *Mtb* strain 18b and treated for two weeks daily with streptomycin.
496 Mice were then removed from streptomycin for three weeks halting active growth of the
497 bacteria.

498 **E.** Five weeks after infection the total levels of viable *Mtb* in the lungs was determined by CFU
499 plating on streptomycin (mean \pm s.d.).

500 **F.** Percentage weight loss (mean change \pm s.d) from Day 0 to Day 35 was determined for wild
501 type and *Cybb*^{-/-} mice.

502 **G.** Representative flow cytometry plot showing increased Ly6G⁺ CD11b⁺ neutrophil recruitment
503 to the lungs of *Cybb*^{-/-} mice 5 weeks following infection (gated on live/singlets/CD45⁺).

504 **H.** Quantification of neutrophil recruitment to the lungs at the indicated time points following
505 infection for wild type and *Cybb*^{-/-} mice is shown as absolute number of Ly6G⁺ CD11b⁺ cells per
506 lung (mean \pm s.d). * p-value <.05 by unpaired two-tailed t-test. Data in D-G are representative
507 of 4 independent experiments with 4-5 mice per group.

508 **I.** Lung homogenates from wild type or *Cybb*^{-/-} mice infected with 18b were probed for IL-1 β by
509 ELISA (mean \pm s.d). ** p-value <.01 by unpaired two-tailed t-test.

510 **J.** Following low dose aerosol infection with sfYFP H37Rv (Day 0 of ~50-100 colony forming
511 units, cfu) total bacterial burden (expressed in cfu, mean \pm s.d.) was determined in the lungs of
512 wild type, *Cybb*^{-/-} or *Nos2*^{-/-} mice 4 weeks post-infection. ** p-value <.01 by one-way ANOVA
513 with tukey correction.

514 **K.** Shown are representative flow cytometry plots from each genotype of total Ly6G⁺ CD11b⁺
515 cells in the infected lungs.

516 **L.** Quantification of total neutrophil recruitment to the lungs of the indicated genotypes four
517 weeks following infection (mean \pm s.d.). ** p-value <.01 * p-value <.05 by one-way ANOVA with
518 tukey correction.

519 **M.** Shown are representative flow cytometry plots from each genotype of infected (YFP⁺ Ly6G⁺
520 CD11b⁺) cells in the lung.

521 **N.** Enumeration of infected (YFP⁺) neutrophils (Ly6G⁺ CD11b⁺) or monocytes/macrophages
522 (Ly6G- CD11b⁺) in the indicated genotypes. * p-value <.05 by unpaired two-tailed t-test.

523

524 **Figure 3. Cybb controls Caspase1 activation in macrophages and dendritic cells during**
525 ***Mtb* infection.** Bone marrow-derived macrophages (BMDMs) or Bone marrow-derived dendritic
526 cells (BMDCs) from wild type or *Cybb*^{-/-} mice were left untreated or infected with *Mtb* for 4 hours
527 then washed with fresh media. 18 hours later supernatants were harvested and the levels of **A.**
528 and **B.** IL-1 β and **C.** and **D.** TNF α were quantified in the supernatants by ELISA. Shown is the
529 mean of 4 biological replicates normalized to a standard curve \pm s.d. *** p<.001 by unpaired
530 two-tailed t-test.
531 **E.** and **F.** Viability of remaining cells was determined by quantifying ATP via luminescence and
532 compared to cells at 4 hours post-infection (mean % viability \pm s.d.). Data in A,C and E are
533 representative of five independent experiments with at least 3 biological replicates per
534 experiment. Data in B, D and F are representative of three independent experiments with 4
535 biological replicates per experiment.
536 **G.** Relative RNA expression of *Il1b* (compared to β -Actin) was determined from wild type and
537 *Cybb*^{-/-} BMDMs left untreated or infected for 24 hours with *Mtb* (mean \pm s.d.) by qRT-PCR. Data
538 are representative of two independent experiments with 3-4 biological replicates per group.
539 **H.** Immunoblot analysis was used to assess the activation of Caspase1 from Wild type and
540 *Cybb*^{-/-} BMDMs infected for 24 hours with *Mtb*. Total Caspase1 was used as a loading control.
541 Data are representative of 3 independent experiments with at least 3 biological replicates
542 analyzed per experiment.
543 **I.** Relative RNA expression of *Il1b* (compared to *Actb*) was determined from wild type and *Cybb*⁻
544 [/] BMDMs left untreated or treated with Pam3CSK4 for 24 hours (mean \pm s.d.) by qRT-PCR.
545 Data are representative of two independent experiments with 3-4 biological replicates per group.
546 **J.** Wild type and *Cybb*^{-/-} BMDMs were left untreated or treated with PAM3CSK4 for 12 hours,
547 supernatants were harvested and the levels of IL-1 β were quantified by ELISA (mean \pm s.d.). **

548 p<.01 by unpaired two-tailed t-test. Data are representative of three independent experiments
549 with 4 biological replicates per experiment.

550

551 **Figure 4. Hyper-inflammation in *Cybb*^{-/-} is reversed by inflammasome and IL1 inhibition.**

552 **A.** Wild type (Black Bars) and *Cybb*^{-/-} (Grey Bars) BMDMs were left untreated or treated with
553 with the indicated concentrations of IFN γ for 12 hours. Cells were then infected with *Mtb* for 4
554 hours then washed with fresh media. 18 hours later supernatants were harvested and levels of
555 IL-1 β from each condition were quantified by ELISA (mean \pm s.d.). ** p-value <.01 * p-value
556 <.05 by one-way ANOVA with tukey correction. Data are representative of three independent
557 experiments with at least 3 biological replicates per experiment.

558 **B.** Wild type and *Cybb*^{-/-} BMDMs were left untreated or treated with the indicated concentrations
559 of MCC950 for 2 hours. Cells were then infected with *Mtb* for 4 hours then washed with fresh
560 media with inhibitor. 18 hours later supernatants were harvested and levels of IL-1 β from each
561 condition were quantified by ELISA (mean \pm s.d.). ** p-value <.01 by one-way ANOVA with
562 tukey correction. Data are representative of three independent experiments with at least 3
563 biological replicates per experiment.

564 **C.** Wild type and *Cybb*^{-/-} BMDMs were left untreated or treated with the indicated concentrations
565 of VX-765 for 2 hours. Cells were then infected with *Mtb* for 4 hours then washed with fresh
566 media with inhibitor. 18 hours later supernatants were harvested and levels of IL-1 β from each
567 condition were quantified by ELISA (mean \pm s.d.). ** p-value <.01 by one-way ANOVA with
568 tukey correction. Data are representative of three independent experiments with at least 3
569 biological replicates per experiment.

570 **D.** Relative RNA expression of IL-1 β (compared to b-Actin) was determined from wild type and
571 *Cybb*^{-/-} BMDMs left infected for 24 hours with *Mtb* in the presence or absence of the indicated
572 inhibitors (mean \pm s.d.) by qRT-PCR.

573 **E.** Wild type and *Cybb*^{-/-} BMDMs were left untreated or treated 25ng/ml IFN γ or 1 μ M MCC950
574 overnight. The following day cells were treated with PAM3CSK4 for 12 hours, supernatants
575 were harvested and the levels of IL-1 β were quantified by ELISA (mean \pm s.d.). ** p<.01 by
576 unpaired two-tailed t-test. Data are representative of two independent experiments with 4
577 biological replicates per experiment.

578 **F.** Wild type and *Cybb*^{-/-} mice were infected intratracheally with *Mtb* strain 18b and treated for
579 two weeks daily with streptomycin then were injected every other day for two weeks with 200ug
580 of either isotype control antibody or anti-IL1R antibody. The total levels of viable *Mtb* in the
581 lungs was determined by CFU plating on streptomycin (mean \pm s.d.).

582 **G.** Representative flow cytometry plot showing Ly6G $^+$ CD11b $^+$ neutrophil recruitment to the
583 lungs of *Cybb*^{-/-} mice during control and IL1R blockade conditions (gated on
584 live/singlets/CD45 $^+$).

585 **H.** Quantification of neutrophil recruitment to the lungs at the indicated time points following
586 infection for wild type and *Cybb*^{-/-} mice during control and IL1R blockade conditions is shown as
587 an absolute number of Ly6G $^+$ CD11b $^+$ cells per lung (mean \pm s.d.). *** p-value <.001 ** p-value
588 <.01 by one-way ANOVA with tukey correction. Data in F-G are representative of two
589 independent experiments with 4-7 mice per group.

590

591 **Figure S1.**

592 **A.** Gating strategy used for all flow cytometry analysis. Shown is a representative gating
593 approach for the analysis used throughout the paper. Cells were first gated on live/dead
594 negative cells then forward by side scatter then singlets. We then gated on all CD45 $^+$ cells in
595 and analyzed the subsequent populations using the indicated fluorescent markers.

596 **B.** Quantification of macrophages, B cells, Dendritic cells and T cells recruitment to the lungs at
597 the 28 days following infection for wild type and *Cybb*^{-/-} mice is shown as absolute number of
598 cells per lung (mean ⁺⁻ s.d).

599

600 **Figure S2.**

601 **A.** A representative sample of the purity of the cell populations depicted in “Figure 2b and c”
602 were determined in the input and sorted fraction of MACS purification of the lung leukocytes
603 from mixed-bone marrow chimeric mice.

604 **B.** Lung homogenates from wild type, *Cybb*^{-/-} or *Nos2*^{-/-} mice infected with *Mtb* for 28 days were
605 probed for the cytokines IL-1 β by ELISA (mean ⁺⁻ s.d). Results are representative of 3
606 independent experiments with 3-5 mice per group. ** p-value <.01 by unpaired two-tailed t-test.

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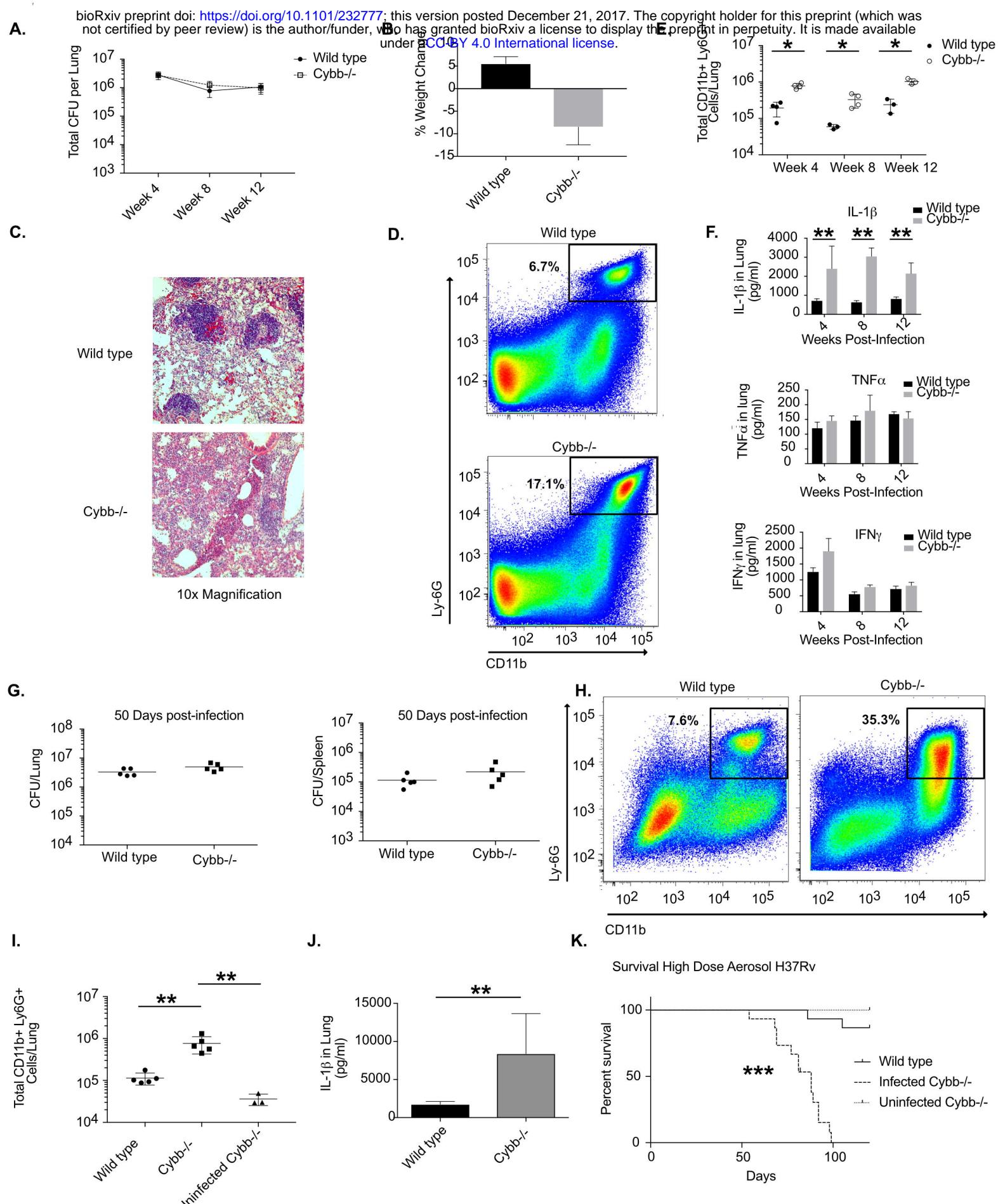


Figure 1

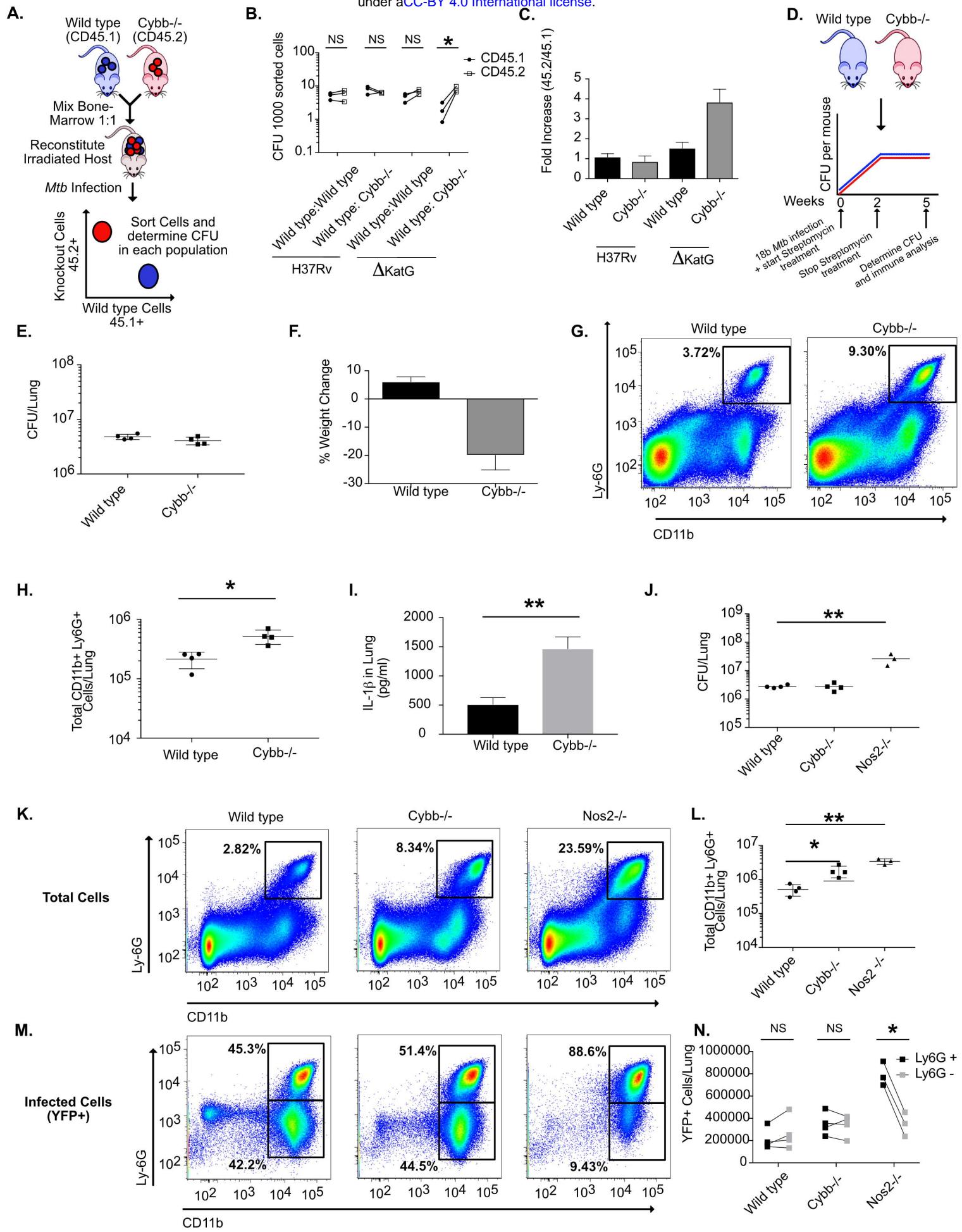


Figure 2

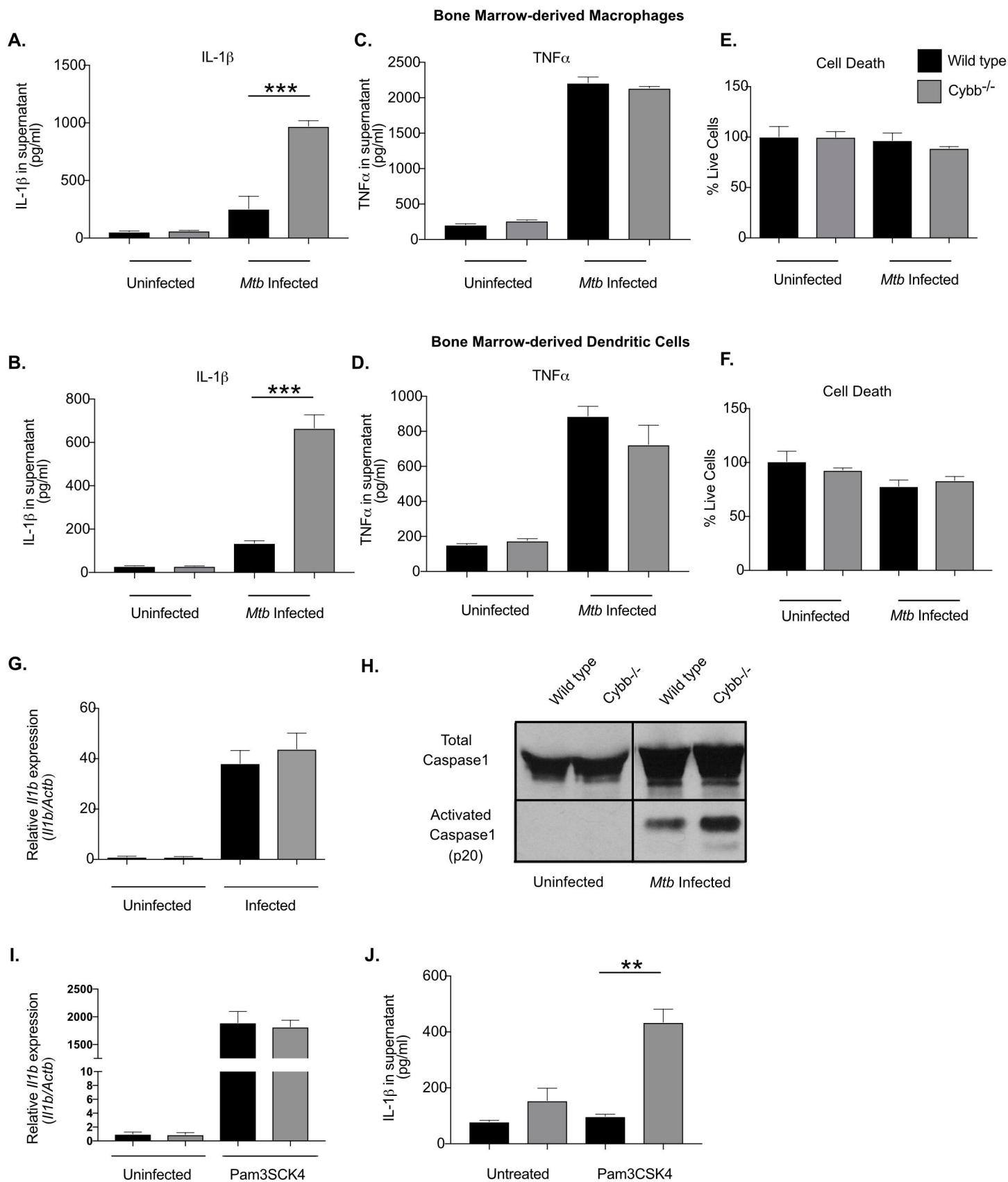


Figure 3

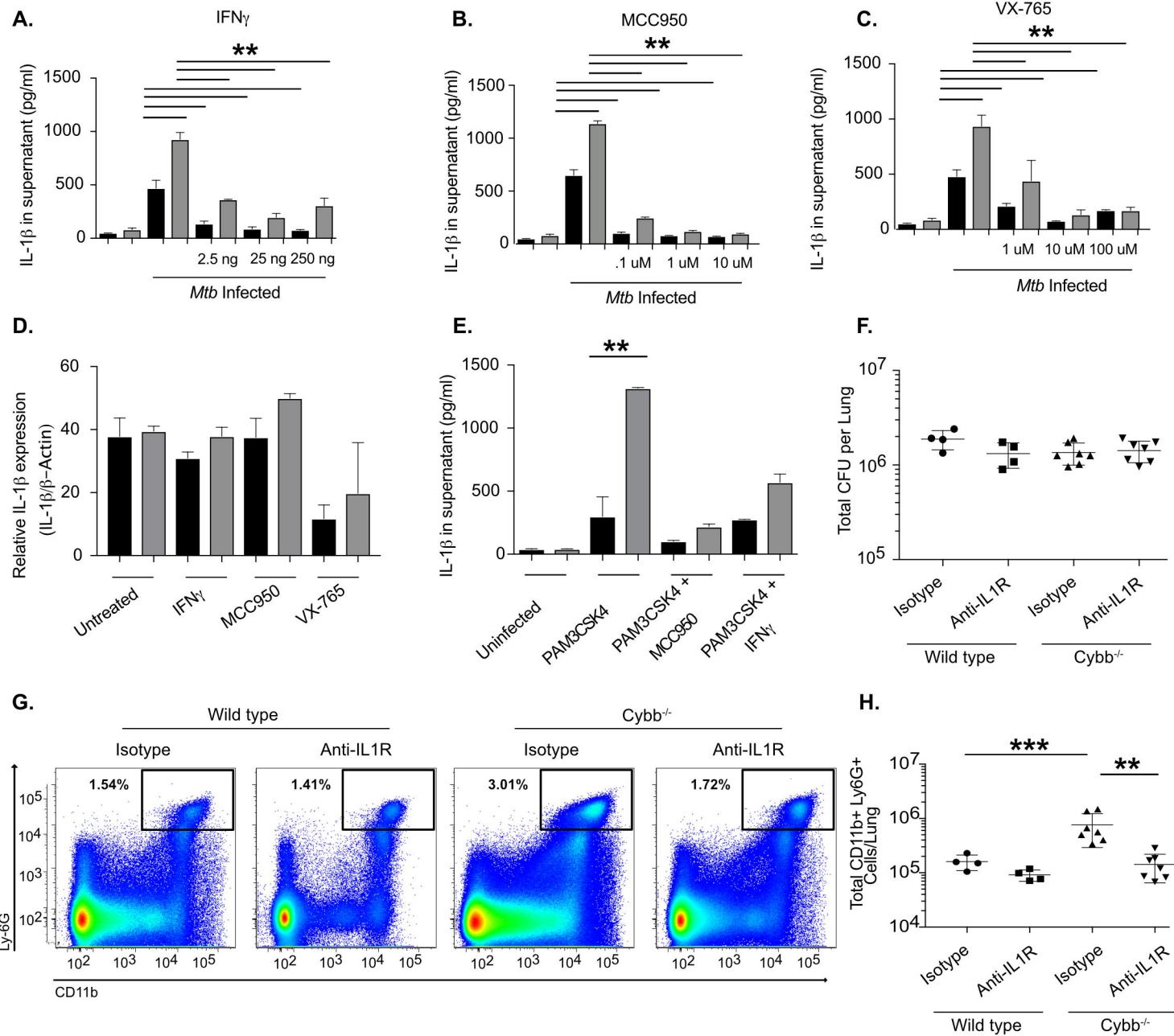


Figure 4