

1 ***Mycobacterium tuberculosis* evasion of Guanylate Binding Protein-mediated host defense in mice**  
2 **requires the ESX1 secretion system.**

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23 **Abstract**

24 Cell-intrinsic immune mechanisms control intracellular pathogens that infect eukaryotes. The  
25 intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*) evolved to withstand cell-autonomous  
26 immunity to cause persistent infections and disease. A potent inducer of cell-autonomous immunity is  
27 the lymphocyte-derived cytokine IFN $\gamma$ . While the production of IFN $\gamma$  by T cells is essential to protect  
28 against *Mtb*, it is not capable of fully eradicating *Mtb* infection. This suggests that *Mtb* evades a subset  
29 of IFN $\gamma$ -mediated antimicrobial responses, yet what mechanisms *Mtb* resists remains unclear. The IFN $\gamma$ -  
30 inducible Guanylate binding proteins (GBPs) are key host defense proteins able to control infections  
31 with intracellular pathogens. GBPs were previously shown to directly restrict *Mycobacterium bovis* BCG  
32 yet their role during *Mtb* infection has remained unknown. Here, we examine the importance of a  
33 cluster of five GBPs on mouse chromosome 3 in controlling Mycobacterial infection. While *M. bovis* BCG  
34 is directly restricted by GBPs, we find that the GBPs on chromosome 3 do not contribute to the control  
35 of *Mtb* replication or the associated host response to infection. The differential effects of GBPs during  
36 *Mtb* versus *M. bovis* BCG infection is at least partially explained by the absence of the ESX1 secretion  
37 system from *M. bovis* BCG, since *Mtb* mutants lacking the ESX1 secretion system become similarly  
38 susceptible to GBP-mediated immune defense. Therefore, this specific genetic interaction between the  
39 murine host and *Mycobacteria* reveals a novel function for the ESX1 virulence system in the evasion of  
40 GBP-mediated immunity.

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## 45 **Introduction**

46  
47 Eukaryotic cells control intracellular pathogens using a variety of cell intrinsic immune pathways (1).  
48 These innate mechanisms allow the cell to rapidly detect, target and destroy invading pathogens,  
49 preventing the spread of an infection. The immune pathways controlling innate immunity arose early in  
50 the evolution of the eukaryota, providing ample time for the selection of pathogens that express  
51 mechanisms to bypass cell-autonomous immunity (2). This long-term arms race has produced a myriad  
52 of interactions between immune effectors and pathogen countermeasures that determine the outcome  
53 of an infection.

54 *Mycobacterium tuberculosis* (*Mtb*) has become highly adapted to its human host, as it spread  
55 globally along with human migrations over tens of thousands of years (3, 4). As much as one-third of the  
56 human population has been exposed to *Mtb*, which causes a persistent infection than can last for years,  
57 or even decades, despite a robust immune response that eradicates less pathogenic mycobacteria (5).  
58 While immunity controls *Mtb* growth and prevents disease in most individuals, a subset will develop  
59 active tuberculosis (TB), a disease that kills an estimated 1.8 million each year (6). Disease progression is  
60 influenced by a variety of genetic and environment factors, but ultimately is determined by the interplay  
61 between host immunity and bacterial virulence systems(5, 7).

62 In the host, the development of a robust T cell response and the production of the cytokine IFN $\gamma$   
63 are important for the control TB infection and disease (5, 8). Humans with inherited mutations affecting  
64 either the development or expression of this response are highly susceptible to mycobacterial infections  
65 including TB (9). This susceptibility is faithfully modeled in mice, where the loss of IFN $\gamma$  signaling  
66 promotes disease by at least two distinct mechanisms (10, 11). IFN $\gamma$  is an important immunomodulatory  
67 cytokine, and its loss results in uncontrolled IL1 production and neutrophil recruitment, driving both  
68 bacterial replication and tissue damage (10, 12). Perhaps more importantly, IFN $\gamma$  stimulates cell-intrinsic

69 immune pathways in phagocytes, which is critical for the control of intracellular bacterial growth (13,  
70 14). Thus, IFN $\gamma$  is a pleotropic cytokine that controls direct antimicrobial resistance and disease  
71 tolerance, both of which are essential to survive *Mtb* infection.

72 While many of the immunomodulatory effects of IFN $\gamma$  have been elucidated, the IFN $\gamma$ -induced  
73 factors that control *Mtb* replication remain comparatively obscure. IFN $\gamma$ -induced oxygen and nitrogen  
74 radical generation limits *Mtb* replication in macrophages *ex vivo*, but appear to serve a limited  
75 antimicrobial role in the intact animal (14-16). Instead, a subset of IFN $\gamma$ -inducible cell-intrinsic immune  
76 mechanisms, known as Guanylate binding proteins (GBPs), target and disrupt the intracellular niche  
77 required for a number of pathogens to grow (2, 17-21). Macrophages lacking GBPs 1, 6,7 or 10 fail to  
78 control the growth of *M. bovis* BCG, an attenuated vaccine strain that is closely related to *Mtb* (22).  
79 Similarly, mice lacking GBP1 or the cluster of 5 GBP proteins on chromosome 3 are more susceptible to  
80 intravenous BCG challenge (22, 23). While the mechanism of BCG control remains unclear, GBPs are  
81 known to bind to pathogen containing vacuoles in other infections and recruit additional effector  
82 molecules (17, 24). The outcome of this recognition can be the direct restriction of pathogen growth  
83 and alterations in cytokine production. While these observations suggest that GBPs may be an  
84 important mediator of IFN $\gamma$  -mediated control of pathogenic mycobacteria, their role in during *Mtb*  
85 infection has remained untested.

86 BCG was attenuated for use as a vaccine via long term serial passage, and as a result, it interacts  
87 with the macrophage quite differently from *Mtb* (25). Most notably, the primary genetic lesion  
88 responsible for the attenuation of BCG is a deletion that disrupts the ESX1 type VII secretion system (26).  
89 ESX1 is specialized protein secretion complex that contributes to pathogen replication by remodeling its  
90 intracellular environment (27). ESX1 is responsible for the disruption of the phagosomal membrane, the  
91 activation of multiple cytosolic immune sensing pathways, and stimulation of both cytokine secretion

92 and autophagy (28). How ESX1 alters other aspects of cell-intrinsic bacterial control remains to be  
93 determined.

94           To understand the mechanisms of IFN $\gamma$ -mediated protection we investigated the role of GBPs  
95 during both BCG and *Mtb* infection. Specifically, we examined the function of a cluster of 5 GBP  
96 proteins that are encoded in a single locus on mouse chromosome 3 (29). While these GBPs restricted  
97 *M. bovis* BCG replication, we found no contribution of these proteins in the control of virulent *Mtb*  
98 infection. The discrepant effects of GBPs during BCG and *Mtb* infection could be attributed to  
99 differential ESX1 function in these two pathogens. ESX1-deficient strains of *Mtb* were better controlled  
100 by GBP-mediated immunity, while GBP deficiency had no effect on either the growth of ESX1 expressing  
101 *Mtb* or the immune response to this virulent strain. Together, these observations indicate that the ESX1  
102 system plays an essential role in the evasion of GBP-mediated cell intrinsic immunity.

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## 105 **Materials and Methods**

106

### 107 **Mice**

108 C57BL/6J (Stock # 000664) and IFN $\gamma$ R<sup>-/-</sup> (Stock # 003288) mice were purchased from the Jackson  
109 Laboratory. The Gbp<sup>chr3-/-</sup> mice were previously described (29). All knockout mice were housed and bred  
110 under specific pathogen-free conditions and in accordance with the University of Massachusetts Medical  
111 School IACUC guidelines. All animals used for experiments were 6-12 weeks.

112

### 113 **Bacterial Strains**

114 Wild type *M. tuberculosis* strain H37Rv was used for all studies unless indicated. This strain was  
115 confirmed to be PDIM-positive. The espA (Rv3616c) deletion strain ( $\Delta$ espA) was a gift from Dr. Sarah  
116 Fortune (30). The eccb1 (Rv3869) deletion strain ( $\Delta$ eccb1) was constructed in the H37Rv parental  
117 background using the ORBIT method as described previously (31). The live/dead strain was built by  
118 transforming the live/dead vector (pmV261 hsp60::mEmerald tetOtetR::TagRFP) into H37Rv and  
119 selected with hygromycin. Protein expression was confirmed via fluorescence microscopy and flow  
120 cytometry. H37Rv expressing msfYFP has been previously described and the episomal plasmid was  
121 maintained with selection in Hygromycin B (50ug/ml) added to the media (32). *Mycobacterium bovis*  
122 BCG Danish Strain 1331 (Statens Serum Institute, Copenhagen, Denmark) was used for all BCG infection  
123 studies. Prior to infection bacteria were cultured in 7H9 medium containing 10% oleic albumin dextrose  
124 catalase growth supplement (OADC) enrichment (Becton Dickinson) and 0.05% Tween 80.

125

### 126 **Mouse Infection**

127 For low dose aerosol infections (50-150 CFU), bacteria were resuspended in phosphate-buffered saline  
128 containing tween 80 (PBS-T). Prior to infection bacteria were sonicated then delivered via the

129 respiratory route using an aerosol generation device (Glas-Col). For mixed infections, bacteria were  
130 prepared then mixed 1:1 before aerosol infection. To determine CFU, mice were anesthetized via  
131 inhalation with isoflurane (Piramal) and euthanized via cervical dislocation, the organs aseptically  
132 removed and individually homogenized, and viable bacteria enumerated by plating 10-fold serial  
133 dilutions of organ homogenates onto 7H10 agar plates. Plates were incubated at 37C, and bacterial  
134 colonies counted after 21 days. Both male and female mice were used throughout the study and no  
135 significant differences in phenotypes were observed between sexes.

136

### 137 **Flow Cytometry**

138 Lung tissue was harvested in DMEM containing FBS and placed in C-tubes (Miltenyi). Collagenase type  
139 IV/DNaseI was added and tissues were dissociated for 10 seconds on a GentleMACS system (Miltenyi).  
140 Tissues were incubated for 30 minutes at 37C with oscillations and then dissociated for an additional 30  
141 seconds on a GentleMACS. Lung homogenates were passaged through a 70-micron filter or saved for  
142 subsequent analysis. Cell suspensions were washed in DMEM, passed through a 40-micron filter and  
143 aliquoted into 96 well plates for flow cytometry staining. Non-specific antibody binding was first blocked  
144 using Fc-Block. Cells were then stained with anti-Ly-6G Pacific Blue, anti-CD11b PE, anti-CD11c APC, anti-  
145 Ly-6C APC-Cy7, anti-CD45.2 PercP Cy5.5, anti-CD4 FITC, anti-CD8 APC-Cy7, anti-B220 PE-Cy7 (Biolegend).  
146 Live cells were identified using fixable live dead aqua (Life Technologies). For infections with fluorescent  
147 H37Rv, lung tissue was prepared as above but no antibodies were used in the FITC channel. All of these  
148 experiments contained a non-fluorescent H37Rv infection control to identify infected cells. Cells were  
149 stained for 30 minutes at room temperature and fixed in 1% Paraformaldehyde for 60 minutes. All flow  
150 cytometry was run on a MACSQuant Analyzer 10 (Miltenyi) and was analyzed using FlowJo\_V9 (Tree  
151 Star).

152

153 **Bone marrow-derived macrophage generation**

154 To generate bone marrow derived macrophages (BMDMs), marrow was isolated from femurs and tibia  
155 of age and sex matched mice as previously described (33). Cells were then incubated in DMEM (Sigma)  
156 containing 10% fetal bovine serum (FBS) and 20% L929 supernatant. Three days later media was  
157 exchanged with fresh media and seven days post-isolation cells were lifted with PBS-EDTA and seeded in  
158 DMEM containing 10% FBS for experiments.

159

160 **Macrophage Infection**

161 *Mtb* or *Mycobacterium bovis*-BCG were cultured in 7H9 medium containing 10% oleic albumin dextrose  
162 catalase growth supplement (OADC) enrichment (Becton Dickinson) and 0.05% Tween 80. Before  
163 infection cultures were washed in PBS-T, resuspended in DMEM containing 10%FBS and centrifuged at  
164 low speeds to pellet clumps. The supernatant was transferred to a new tube to ensure single cells.  
165 Multiplicity of infection (MOI) was determined by optical density (OD) with an OD of 1 being equivalent  
166 to  $3 \times 10^8$  bacteria per milliliter. Bacteria were added to macrophages for 4 hours then cells were washed  
167 with PBS and fresh media was added. At the indicated time points supernatants were harvested for  
168 cytokine analysis and the cells were processed for further analysis. For cytokine treatments cells were  
169 treated with the indicated concentrations of IFN $\gamma$  (Peprotech) or vehicle control four hours following  
170 infection and maintained in the media throughout the experiment. For CFU experiments at the indicated  
171 timepoints 1% saponin was added to each well and lysates were serially diluted in PBS .05% Tween and  
172 plated on 7H10 agar and colonies were counted 21-28 days later. For the Live/Dead reporter  
173 experiments, Anhydrotetracycline (aTc) (Cayman Chemical) was added to a final concentration of  
174 500ug/ml 24 hours before cells were lifted, fixed in 1% Paraformaldehyde and analyzed on a MacsQuant  
175 VYB analyzer.

176



177 **Cytokine quantification by ELISA**

178 Murine cytokine concentrations in culture supernatants and cell-free lung homogenates were quantified  
179 using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D). All samples were normalized  
180 for total protein content.

181 **Statistics.** GraphPad Prism version 7 was used for all statistical analysis. Unless otherwise indicated one-  
182 way ANOVA with a tukey correction was used to compare each condition to each genotype.

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187 **Results**

188

189 ***Mycobacterium bovis* BCG growth is restricted by the Chromosome 3 Guanylate binding protein**  
190 **cluster.**

191 The ten murine GBP genes are encoded in two clusters on chromosomes 3 and 5 (1). To begin to  
192 address the potential redundancy between these genes, we used mice lacking the entire chromosome 3  
193 cluster that contains the Gbp1 and 7 genes that were previously implicated in BCG control, along with  
194 GBPs 2, 3, and 5 (29). To determine if these GBPs contribute to control of mycobacterial infection, bone  
195 marrow-derived macrophages (BMDMs) from wild type and Gbp<sup>chr3-/-</sup> mice were infected with  
196 *Mycobacterium bovis* BCG (Figure 1A). IFN $\gamma$ -treatment, which induces GBP expression, reduced the  
197 intracellular bacterial burden in wild type BMDMs. In contrast, the Gbp<sup>chr3-/-</sup> deletion significantly  
198 reduced the ability of macrophages to control BCG upon IFN $\gamma$  activation. Thus, GBPs on chromosome 3  
199 contribute to IFN $\gamma$ -mediated restriction of BCG in macrophages.

200 We next examined if GBPs controlled BCG infection in the context of an intact immune  
201 response. Wild type, IFN $\gamma$ R<sup>-/-</sup> and Gbp<sup>chr3-/-</sup> mice were infected intravenously with BCG. When CFU were  
202 quantified in the spleen 50 days later we observed twenty-fold more BCG in IFN $\gamma$  mice and five-fold  
203 more BCG in Gbp<sup>chr3-/-</sup> mice compared to wild type controls (Figure 1B). The increased susceptibility of  
204 IFN $\gamma$ R<sup>-/-</sup> compared to Gbp<sup>chr3-/-</sup> mice indicated that the function of these GBPs accounted for some but  
205 not all of the protective effect of IFN $\gamma$ . Together these data show that the GBPs on chromosome 3 are  
206 able to restrict the growth of BCG in IFN $\gamma$  -stimulated macrophages and in the intact animal, which is  
207 consistent with the previously described roles of GBPs in immunity to BCG (22, 23).

208

209 **Chromosome 3 GBP cluster does not control *Mycobacterium tuberculosis* growth in macrophages.**

210 To determine if the chromosome 3 GBPs restricted the intracellular growth of virulent *Mtb*, in addition  
211 to BCG, we quantified the growth of *Mtb* strain H37Rv in BMDMs from wild type,  $\text{IFN}\gamma\text{R}^{-/-}$  and  $\text{Gbp}^{\text{chr}3-/-}$   
212 animals in the presence and absence of  $\text{IFN}\gamma$  (Figure 2A). We observed no differences in the uptake  
213 between genotypes four hours following infection.  $\text{IFN}\gamma$  priming reduced the intracellular *Mtb* growth by  
214 3-4 fold in wild type macrophages, but not  $\text{IFN}\gamma\text{R}^{-/-}$  BMDMs at 5 days post infection (dpi). In contrast to  
215 BCG, we observed no difference in *Mtb* growth in  $\text{Gbp}^{\text{chr}3-/-}$  BMDMs compared to wild type  
216 macrophages in the presence or absence of  $\text{IFN}\gamma$ .

217 To ensure that the relatively insensitive CFU-based intracellular growth assay did not mask  
218 subtle effects of GBP expression on bacterial fitness, we used a fluorescent live/dead reporter as an  
219 orthologous method to measure *Mtb* intracellular viability by flow cytometry. This reporter expresses a  
220 constitutive mEmerald and an anhydrotetracycline (aTc)-inducible tagRFP. BMDMs from wild type,  
221  $\text{IFN}\gamma\text{R}^{-/-}$  and  $\text{Gbp}^{\text{chr}3-/-}$  mice were infected with the Live/Dead *Mtb* reporter and left untreated or were  
222 stimulated with  $\text{IFN}\gamma$ . Four days later aTc was added to induce tagRFP expression in viable intracellular  
223 bacteria. The following day, cells were analyzed by flow cytometry and the mean fluorescence intensity  
224 (MFI) of tagRFP in infected mEmerald+ cells was quantified (Figure 2B and 2C). Similar to the CFU  
225 analysis,  $\text{IFN}\gamma$  activation reduced the intensity of tagRFP to a similar extent in both wild type and  $\text{Gbp}^{\text{chr}3-/-}$   
226  $^{-/-}$  BMDMs, and this reduction depended on the  $\text{IFN}\gamma$  receptor. Thus, we were not able to detect a role  
227 for the chromosome 3 GBP cluster in the  $\text{IFN}\gamma$  mediated restriction of virulent *Mtb* in macrophages.

228

### 229 **Chromosome 3 GBPs have no effect on *Mtb* infection in intact mice**

230 To assess the role of the chromosome 3 GBPs in the more complex setting of the intact animal, we  
231 infected wild type,  $\text{IFN}\gamma\text{R}^{-/-}$  and  $\text{Gbp}^{\text{chr}3-/-}$  mice with *Mtb* by low dose aerosol and quantified bacteria in  
232 the lungs at four and five weeks following infection (Figure 3A and 3B).  $\text{IFN}\gamma\text{R}^{-/-}$  mice harbored more *Mtb*  
233 than wild type controls at both time points in the lung and the spleen. In contrast, the *Mtb* burdens in

234  $Gbp^{chr3-/-}$  mice were indistinguishable from wild type animals at all timepoints. At 110 days after  
235 infection (Figure 3A and 3B), we continued to find no difference in CFU in the lungs between wild type  
236 and  $GBP^{chr3-/-}$  mice.  $IFN\gamma R^{-/-}$  mice required euthanasia within six weeks of infection and were not  
237 included in this late time point.

238 To examine more subtle differences in the extent of infection, wild type,  $IFN\gamma R^{-/-}$  and  $Gbp^{chr3-/-}$   
239 mice were infected with a well-characterized fluorescent H37Rv strain by low-dose aerosol (32). Twenty-  
240 eight days later the number of YFP+ cells in the lung environment were determined by flow cytometry  
241 (Figure 3C). Similar to our CFU results, we found  $IFN\gamma R^{-/-}$  mice contained over 10 times more infected cells  
242 than wild type mice while  $Gbp^{chr3-/-}$  mice showed no significant difference in the number of infected  
243 cells per lung (Figure 3D).

244 We next examined if there were differences in  $Gbp^{chr3-/-}$  mice infected intravenously since this  
245 route matched the BCG infections where the role of GBPs was evident (Figure 1B and (22)). Following  
246 intravenous infection with  $10^6$  *Mtb*, bacterial levels in the spleen were quantified 10 and 28 days later.  
247 Similar to the aerosol infection results, we saw no difference between  $Gbp^{chr3-/-}$  and wild type animals,  
248 while  $IFN\gamma R^{-/-}$  animals had 10 times more *Mtb* growth in the spleen (Figure 3E). Thus, unlike the loss of  
249  $IFN\gamma R$ , the loss of chromosome 3 GBPs does not affect *Mtb* growth in the lungs or spleen, regardless of  
250 infection route, suggesting no major function of these five GBPs in controlling antimicrobial resistance to  
251 *Mtb* in mice.

252

### 253 **$Gbp^{chr3-/-}$ mice continue to regulate inflammatory responses to *Mtb* infection**

254  $IFN\gamma$  protects against disease both by restricting bacterial growth and by inhibiting tissue-damaging  
255 inflammation (11). To determine if the chromosome 3 GBPs contribute to the latter immunoregulatory  
256 function of  $IFN\gamma$ , we profiled the immune responses of infected macrophages and mice. Wild type and  
257  $GBP^{chr3-/-}$  BMDMs were treated with  $IFN\gamma$  then infected with *Mtb* and the following day supernatants

258 were harvested. IFN $\gamma$  signaling showed the expected inhibitory effect on IL-1 $\beta$  secretion, but the  
259 chromosome3 Gbp deletion had no effect on either cytokine (12). We next profiled the host response  
260 in the lungs of mice to determine if chromosome 3 GBPs modulated the recruitment of immune cells or  
261 the production of cytokines during *Mtb* infection. Wild type, IFN $\gamma$ R $^{-/-}$  and Gbp<sup>chr3 $^{-/-}$</sup>  mice were infected  
262 with *Mtb* and four weeks later we quantified the immune populations in the lungs by flow cytometry  
263 (Figure 4B). While we observed the previously described increase in neutrophil recruitment in IFN $\gamma$ R $^{-/-}$   
264 mice, we observed no differences in any myeloid or lymphoid derived cells that were examined between  
265 Gbp<sup>chr3 $^{-/-}$</sup>  and wild type mice (10). We also quantified IL1 $\beta$  and TNF $\alpha$  in the lungs from these infected  
266 animals (Figure 4C). The production of these cytokines in wild type and GBP<sup>chr3 $^{-/-}$</sup>  mice were  
267 indistinguishable while IFN $\gamma$ R $^{-/-}$  mice showed an increase in IL1 $\beta$ . Thus, we were unable to detect a role  
268 for GBPs on chromosome 3 in controlling the host response during virulent *Mtb* infection.

269

### 270 **ESX1 is required for *Mtb* to evade GBPs of chromosome 3.**

271 The reduced virulence of BCG compared to *Mtb* has been largely attributed to the loss of ESX1 function  
272 in BCG. As a result, we hypothesized ESX1 function in virulent *Mtb* provides resistance to GBP-mediated  
273 immunity, and its loss renders BCG susceptible to this mechanism. To test this hypothesis, we  
274 determined whether abrogation of ESX1 function in *Mtb* would result in a strain that was susceptible to  
275 GBP-mediated control. Wild type and Gbp<sup>chr3 $^{-/-}$</sup>  macrophages were infected with H37Rv or two isogenic  
276 mutants each lacking a gene that is necessary for ESX1 function,  $\Delta$ *espA* or  $\Delta$ *eccb1*. We observed that  
277 ESX1 mutants displayed reduced intracellular growth compared to H37Rv in both IFN $\gamma$  stimulated and  
278 unstimulated wild type macrophages, confirming the attenuation of these mutants (Figure 5A and 5B).  
279 When we compared wild type and Gbp<sup>chr3 $^{-/-}$</sup>  BMDMs, we found that the loss of GBP function had no  
280 effect on H37Rv, while it significantly reduced the ability of IFN $\gamma$  to control the growth of both ESX1  
281 mutants.

282           To confirm the specific effect of chromosome 3 GBPs on ESX1 mutants in the setting of intact  
283 immunity, we conducted a competitive infection of wild type and  $Gbp^{chr3-/-}$  mice with an equivalent  
284 number of H37Rv and  $\Delta eccb1$  bacteria. Four weeks later we quantified the competitive index of each  
285 bacterial strain in the lungs (Figure 5C). As anticipated, the ratio of two differentially marked H37Rv  
286 strains stayed constant throughout the infection. While selection in wild type mice resulted in almost  
287 100-fold underrepresentation of the  $\Delta eccb1$  mutant compared H37Rv, the fitness defect of the  $\Delta eccB1$   
288 mutant was significantly reduced in the  $GBP^{chr3-/-}$  mice (Figure 5C). These findings demonstrate that  
289 ESX1 deficiency is sufficient to render *Mtb* susceptible to GBP-mediated immunity.

290

291

## 292 Discussion

293 Understanding the immune mechanisms that restrict the intracellular growth of *Mtb* is essential for the  
294 rational design of interventions. Initial observations demonstrating a role for GBPs in the control of *BCG*  
295 growth suggested that this pathway might represent an important component of IFN $\gamma$ -mediated  
296 immunity to *Mtb* (22, 23). However, while we were able to detect the previously-described role for the  
297 chromosome 3 GBPs in immunity to *BCG*, we found that these proteins have no effect during *Mtb*  
298 infection. By attributing this difference to the ESX1 locus that is present in *Mtb* but not *BCG*, we  
299 discovered a specific role for ESX1 in overcoming GBP-mediated defenses.

300  
301 Our findings question the importance of GBPs in the control of ESX1-expressing *Mtb*. While our results  
302 are strictly in the mouse model, evidence in humans also suggests GBPs may not effectively control TB  
303 progression. For example, the high expression of a subset of GBPs is predictive of patients that are more  
304 likely to progress to active disease (34). However, it is important to note that it remains possible that  
305 the chromosome 5 GBPs, or human-specific GBP functions, can overcome ESX1-mediated bacterial  
306 defenses. In addition, our findings do not rule out an important role for GBPs in resistance to non-  
307 tuberculous mycobacteria (NTM). While all mycobacteria express ESX paralogs, many pathogenic NTM  
308 do not possess a clear ortholog of ESX1, suggesting that they may remain susceptible to GBP immunity  
309 (35).

310  
311 How the ESX1 type VII secretion system allows *Mtb* to evade restriction by GBPs remains to be  
312 investigated. To date, the type III secretion system effector protein IpaH9.8 from *Shigella flexneri* is the  
313 only other described GBP antagonist (36-39). In the cytosol of mammalian cells, where *S. flexneri*  
314 replicates, IpaH9.8 targets GBP1 for degradation thereby interfering with direct GBP binding to the  
315 *Shigella* outer membrane, a process by which GBP1 disrupts the function of a membrane-bound *Shigella*

316 virulence factors required for actin-based motility and bacterial dissemination (36-38, 40). Similar to  
317 IpaH9.8, ESX1 and its substrates may act as direct GBP antagonist to prevent binding to *Mycobacteria*-  
318 containing vacuoles. Alternatively, ESX1 may control the evasion of antimicrobial mechanisms that occur  
319 subsequent to GBP translocation to *Mycobacteria*-containing vacuoles. Future work will need to dissect  
320 these possible mechanisms for ESX1-mediated antagonism of GBP function.

321

322 While IFN $\gamma$  is unquestionably important to survive *Mtb* infection, the IFN $\gamma$ -mediated pathways that  
323 directly control the intracellular replication of *Mtb* remain surprisingly unclear. Our results add to a  
324 growing list of direct antimicrobial pathways that are ineffective during *Mtb* infection. The IFN $\gamma$ -  
325 mediated production of nitric oxide, reactive oxygen species and itaconate kills many pathogens;  
326 however, these mechanisms appear to play a small role in directly controlling *Mtb* growth *in vivo* (32,  
327 33, 41). Instead, these mediators are required to inhibit persistent inflammation and to prevent disease  
328 progression. In addition, the IFN $\gamma$ -regulated immunity related GTPases (IRG) family protein, Irgm1, was  
329 originally described to target the *Mtb* containing vacuole to control pathogen growth, but recent  
330 evidence has questioned whether Irgm1 targets *Mtb* phagosomes (42, 43). Instead, the lymphopenia  
331 observed in Irgm1-deficient mice may be predominantly responsible for their susceptibility to *Mtb*  
332 similar to other pathogens like *Chlamydia trachomatis* (44, 45). While other pathways have been  
333 suggested to play a role in IFN $\gamma$ -mediated control, including the production of Cathepsins, the role of  
334 these mediators in protection *in vivo* remains unclear (46). Overall, our findings add GBP-mediated  
335 immunity to the list of IFN $\gamma$  dependent host defense programs to which *Mtb* has evolved specific  
336 counter immune mechanisms blunting the effectiveness of these antimicrobial effectors and thus  
337 driving pathogen persistence and disease.

338



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341 by grants from the NIH to CMS (AI132130) and the Arnold and Mabel Beckman Foundation to AJO.  
342  
343

344 **Figure Legends.**

345

346 **Figure 1. Guanylate binding proteins contribute to control of *Mycobacterium Bovis* BCG infection. (A)**

347 BMDMs from wild type or Gbp<sup>chr3-/-</sup> mice were infected with *M. Bovis* BCG for 4 hours then washed with  
348 fresh media in the presence or absence of IFN $\gamma$ . Five days later the macrophages were lysed and serial  
349 dilutions were plated to quantify colony forming units (CFU) of viable BCG. Shown is the mean CFU from  
350 four biological replicates +/- SD \*p<.05 \*\*p<.01. Data are representative of four independent  
351 experiments. **(B)** Following IV infection with BCG (1x10<sup>6</sup> bacteria) the total bacterial burden (expressed  
352 in CFU, mean +/- SD) was determined in the spleen of wild type or Gbp<sup>chr3-/-</sup> mice 50 days following  
353 infection. Representative of two independent experiments with 4-5 mice per group, \*p<.05 \*\*p<.01.

354

355 **Figure 2. *Mycobacterium tuberculosis* is resistant to chromosome 3 GBP-mediated control in**

356 **macrophages. (A)** BMDMs from wild type, IFN $\gamma$ R<sup>-/-</sup> or Gbp<sup>chr3-/-</sup> mice were infected with *M. tuberculosis*  
357 for 4 hours then washed with fresh media in the presence or absence of IFN $\gamma$ . Five days later the  
358 macrophages were lysed, serial dilutions were plated to quantify colony forming units (CFU) of viable *M.*  
359 *tuberculosis*. Shown is the mean CFU from four biological replicates +/- SD \*p<.05 \*\*p<.01. Data are  
360 representative of five independent experiments. **(B)** and **(C)** BMDMs from wild type, IFN $\gamma$ R<sup>-/-</sup> or Gbp<sup>chr3-/-</sup>  
361 mice were infected with the live/dead reporter *M. tuberculosis* for 4 hours then washed with fresh  
362 media in the presence or absence of IFN $\gamma$ . Four days later aTc was added to each well at a final  
363 concentration of 500ng/ml. The following day infected cells were lifted and the fluorescence intensity of  
364 the inducible Tet-on TagRFP was determined by flow cytometry. Cells were gated on live and infected  
365 (mEmerald+) cells. **(B)** A representative histogram of tagRFP fluorescence is shown. **(C)** Shown is the  
366 mean fluorescence intensity of tagRFP for three biological replicates. These data are representative of  
367 three independent experiments. \*\*\*p<.001.

368  
369 **Figure 3. Mice lacking chromosome 3 GBPs control *M. tuberculosis* infection.** Following low-dose  
370 aerosol infection with H37Rv (day 0 of ~50-150 CFU), total bacteria (expressed in CFU, mean +/- SD) was  
371 determined in the lungs **(A)** or the spleen **(B)** of wild type, IFN $\gamma$ <sup>-/-</sup> or Gbp<sup>chr3-/-</sup> mice at the indicated time  
372 points with four mice per group. 28 days following low-dose aerosol infection with sfYFP H37Rv, infected  
373 cells in the lungs of wild type, IFN $\gamma$ <sup>-/-</sup> or Gbp<sup>chr3-/-</sup> mice were quantified by flow cytometry. **(C)** Shown is  
374 a representative flow cytometry plot of the infected cells (YFP positive) that are gated on live, single cells  
375 and **(D)** the mean percent of YFP positive cells in the lungs of infected animals. **(E)** Following intravenous  
376 infection with H37Rv (1x10<sup>6</sup> per mouse), total bacteria were determined in the spleen of wild type,  
377 IFN $\gamma$ <sup>-/-</sup> or Gbp<sup>chr3-/-</sup> mice at the indicated time points with 3-5 mice per group. All data are  
378 representative of 3 independent experiments.

379  
380 **Figure 4. Chromosome 3 GBPs do not control the host response to *M. tuberculosis* infection. (A)**  
381 BMDMs from wild type, IFN $\gamma$ <sup>-/-</sup> or Gbp<sup>chr3-/-</sup> mice were stimulated with IFN $\gamma$  overnight then infected with  
382 *Mtb* for 4 hours then washed with fresh media. 18 hours later, supernatants were harvested and the  
383 levels of IL1 $\beta$  and TNF $\alpha$  were quantified in the supernatants by ELISA. Shown is the mean of four  
384 biological replicates normalized to a standard curve +/- SD \*p<.05. **(B)** and **(C)** 28 days following low-  
385 dose aerosol infection with H37Rv (day 0 of ~50-100 CFU), the lungs of wild type, IFN $\gamma$ <sup>-/-</sup>, and Gbp<sup>chr3-/-</sup>  
386 mice were harvested and the immune cell populations were quantified by flow cytometry and cytokines  
387 were quantified by ELISA. For **(B)**, we determined the % of live cells for Granulocytes (CD45<sup>+</sup> CD11b<sup>+</sup>  
388 Ly6G<sup>+</sup>), Macrophages (CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>-</sup>), Dendritic Cells (CD45<sup>+</sup> CD11b<sup>-</sup> Ly6G<sup>-</sup> CD11c<sup>+</sup>), CD4<sup>+</sup> T cells  
389 (CD45<sup>+</sup> CD4<sup>+</sup>), CD8<sup>+</sup> T cells (CD45<sup>+</sup> CD8<sup>+</sup>) and B cells (CD45<sup>+</sup> B220<sup>+</sup>). For **(C)** Cytokines quantified from lung  
390 homogenates. Shown is the mean of three mice per group +/- SD \*p<.05. These data are representative  
391 of 3-4 independent experiments with similar results.

392

393 **Figure 5. *M. tuberculosis* ESX1 mutants are susceptible to GBP-mediated control. (A)** BMDMs from wild

394 type or *Gbp*<sup>chr3-/-</sup> mice were infected with *M. tuberculosis* H37Rv,  $\Delta$ espA (Left) or  $\Delta$ eccb1 (Right) for 4

395 hours then washed with fresh media in the presence or absence of IFN $\gamma$ . Five days later the

396 macrophages were lysed and serial dilutions were plated to quantify colony forming units (CFU) of viable

397 *M. tuberculosis*. Shown is the mean CFU from four biological replicates +/- SD \*p<.05 \*\*p<.01 by two-

398 tailed t-test. Data are representative of two independent experiments with similar results. (B) Following

399 low-dose aerosol infection with a 1:1 mixed infection of either H37Rv (Hyg):H37Rv (Kan) or  $\Delta$ eccb1

400 (Hyg): H37Rv (Kan) (day 0 of ~100-200 CFU), total bacteria in the lungs that were either Kan or Hyg

401 resistant were quantified. The competitive index was calculated (Hyg CFU/Kan CFU) and is shown as the

402 mean for 3 independent mice for each genotype combination. The data are representative of two

403 independent experiments with similar results.

404

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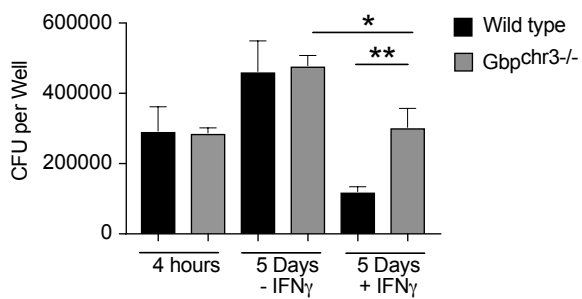
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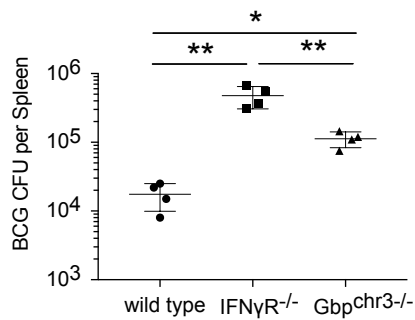
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**Figure 1**

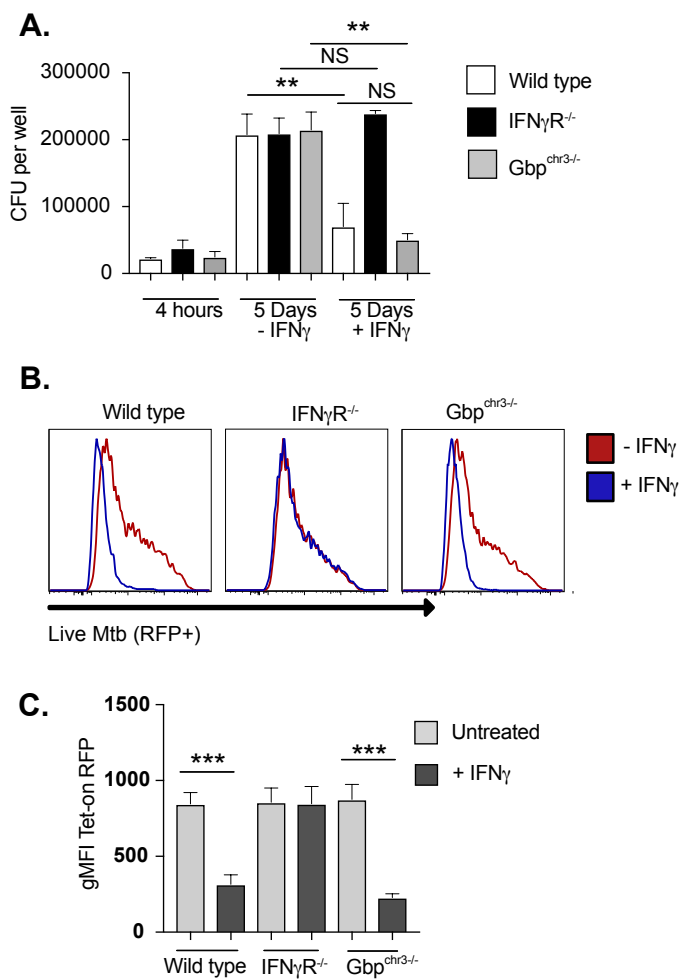
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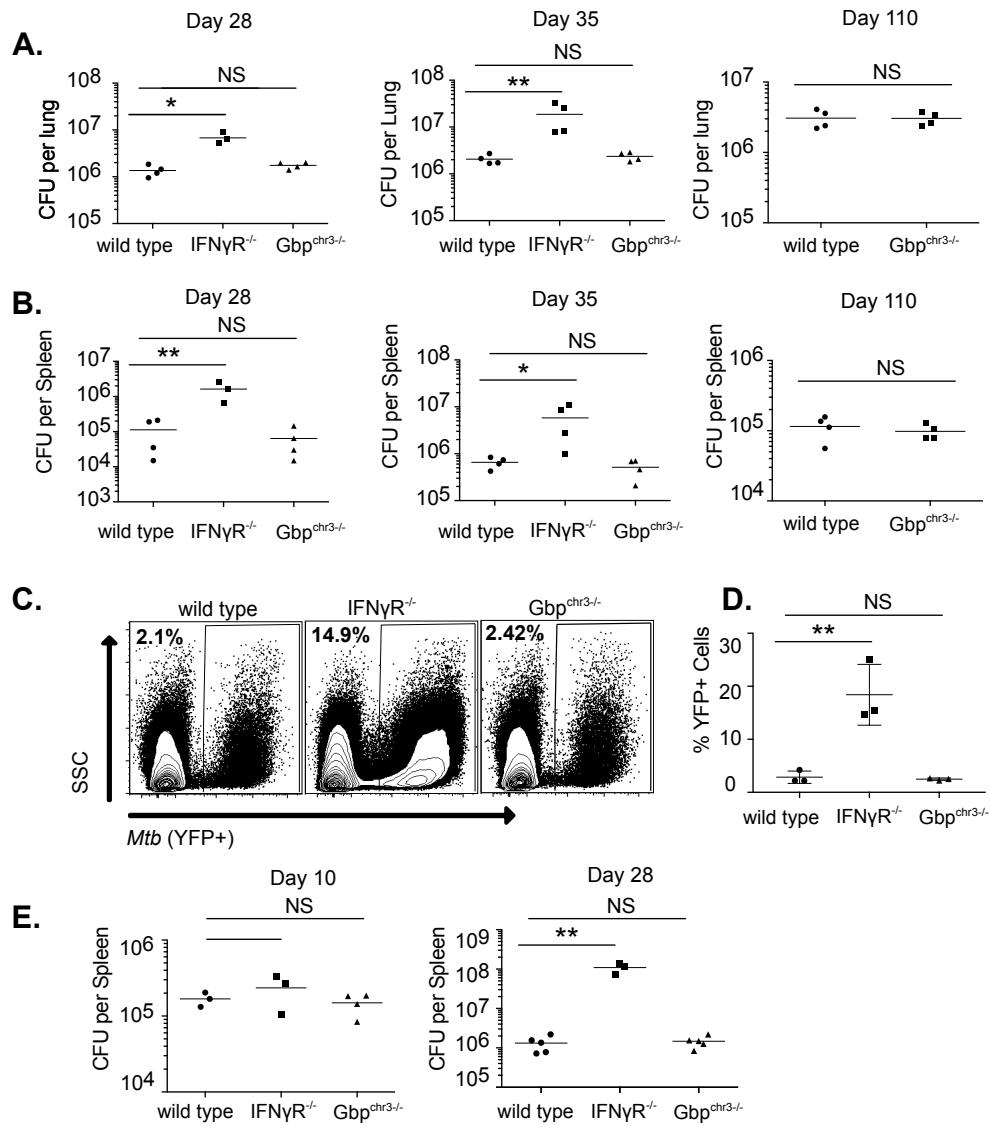
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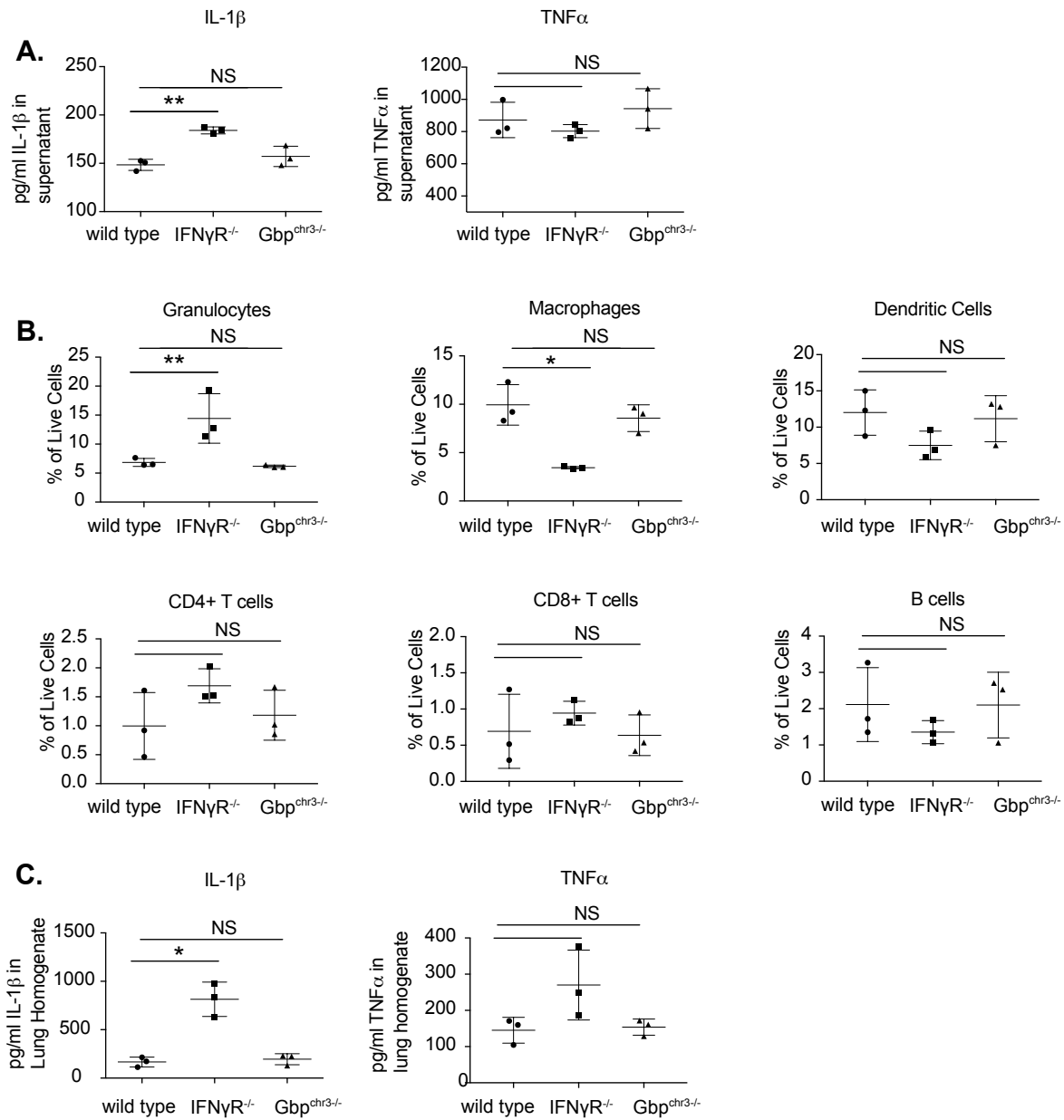
**Figure 2**



**Figure 3**

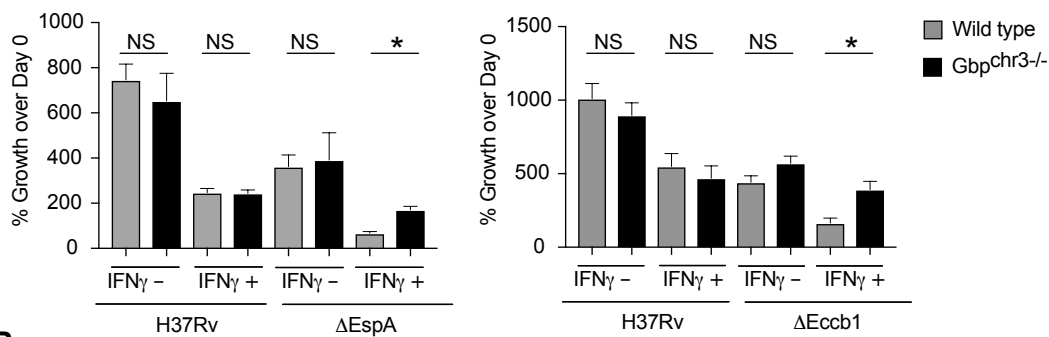


**Figure 4**



## Figure 5

**A.**



**B.**

