

1 **BCG overexpressing an endogenous STING agonist provides**
2 **enhanced protection against pulmonary tuberculosis**

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23 **ABSTRACT**

24
25 Stimulator of interferon genes (STING) has emerged as a key signaling receptor that
26 induces proinflammatory cytokines, and small molecule STING agonists are being
27 developed as anticancer and antiviral agents. Here we report a strategy of delivering a
28 STING agonist from within live BCG. We generated a recombinant BCG (BCG-*disA*-OE)
29 that overexpresses the endogenous mycobacterial diadenylate cyclase gene and
30 releases high levels of the STING agonist c-di-AMP. In macrophages BCG-*disA*-OE
31 elicited statistically significantly stronger TNF- α , IL-6, IL-1 β , IRF3, and IFN- β levels than
32 BCG-WT. In a 24-week guinea pig vaccination-*Mtb* challenge model, BCG-*disA*-OE
33 reduced lung weights, pathology scores, and *Mtb* CFU counts in lungs by 28% ($p < 0.05$),
34 34%, and 2.0 log₁₀ CFU units ($p < 0.5$) compared with BCG-WT, respectively.
35 Overproduction of the STING agonist c-di-AMP significantly enhanced the protective
36 efficacy of BCG against pulmonary and extrapulmonary tuberculosis. Our findings
37 support the development of BCG-vectored STING agonists as a TB vaccine strategy.

38 INTRODUCTION

39 Recent studies have identified a key role for the stimulator of interferon genes
40 (STING) intracellular sensor in mediating innate immune responses cellular stress or
41 pathogen infection [1,2]. STING is a cytosolic receptor for both pathogen-associated
42 molecular pattern (PAMP) molecules such as cyclic dinucleotides c-di-AMP or c-di-GMP
43 produced by bacteria, and for mammalian endogenous danger signaling DAMP
44 molecules such as 2',3' cyclic GMP-AMP (cGAMP) which is synthesized by cGAS (cyclic
45 GMP-AMP synthase) in response to microbial or self-derived cytosolic double-stranded
46 DNA [1-4]. Activation of STING induces numerous interferon-stimulated genes including
47 type I interferons (IFN α/β) and is associated with co-activation of NF- κ B and STAT6
48 transcription factors that follows or parallels the induction of interferon regulatory factors
49 (IRF) transcription factors. Thus endogenous and exogenous cyclic dinucleotides (CDNs)
50 are strong TLR-independent mediators of innate host defenses [5,6]. Based on the key
51 role of STING signaling, pharmacological stimulation of the pathway using small molecule
52 STING agonists is currently being tested for enhancement of antitumor immunity and as
53 potential anti-viral therapy [5,7].

54 As they are capable of inducing potent cytokine and cellular immune responses
55 against pathogens, CDN STING agonists also exhibit attractive vaccine adjuvant
56 properties. They increase expression of MHC class II, co-stimulatory molecules
57 (CD80/CD86) as well as activation (CD40) and adhesion (CD45) markers; in addition
58 STING agonists have been shown to enhance antigen processing and presentation to T
59 cells [8-10]. Among microbial-derived CDNs, c-di-AMP has emerged as an efficient
60 activator of macrophages and leads to robust Th1, Th17 and CD8 T cell responses [11].

61 Efforts to harness small molecule CDNs themselves as vaccine adjuvants to
62 enhance systemic immunity, however, may be limited by rapid systemic clearance and
63 by the fact that as negatively charged molecules, CDNs do not effectively cross cell
64 membranes of macrophages and professional antigen presenting cells [6]. Hence there
65 is a need to develop a cost-effective formulations of CDNs and related STING agonists
66 that allow for sustained release to the cytosolic compartment of antigen-presenting cells.

67 Our previous findings showed that *Mycobacterium tuberculosis* (*Mtb*), an
68 intracellular pathogen, possesses di-adenylate cyclase enzyme, *disA* (MT3692), that
69 synthesizes and secretes c-di-AMP into the host cell cytosol. We showed that an *Mtb*
70 strain engineered to overexpress c-di-AMP (*Mtb-disA-OE*) was significantly attenuated in
71 lethality, ability to proliferate, and cause disease pathology in a mouse model [12]. In a
72 separate study, we showed that mutation of the *cdnP* gene encoding a cyclic dinucleotide
73 phosphodiesterase (CdnP) that hydrolyzes c-di-AMP lead to a mutant *Mtb* strain that
74 accumulates c-di-AMP and is similarly attenuated for virulence [13].

75 Based on these results we constructed a c-di-AMP–overexpressing bacillus
76 Calmette–Guérin (BCG), an attenuated strain of *Mycobacterium bovis* widely used as a
77 TB vaccine globally, and found that it induced a significantly higher IRF and IFN- β
78 response than BCG itself, indicating that bacterial-derived c-di-AMP gains access to the
79 host cell cytosol despite the fact that BCG lacks the ESX-1 protein secretion system found
80 in *Mtb*. These findings strongly encouraged us to evaluate whether BCG-induced immune
81 responses against TB are boosted by overexpression of the STING agonist c-di-AMP.

82 A number of candidate vaccines for TB are currently being evaluated in clinical
83 trials, prime boost strategies that include BCG have failed to show improved protective

84 efficacy in humans over BCG alone [14]. In the paper we report the development of a
85 recombinant BCG which is boosted by endogenous overexpression of a STING agonist.
86 This c-di-AMP–overexpressing recombinant BCG strain (BCG-*disA*-OE) exhibited
87 increased IRF induction, IFN- β synthesis, and release of the pro-inflammatory cytokines
88 IL-6, TNF α and IL1 β in vitro than were observed with BCG-WT. Importantly, guinea pigs
89 vaccinated with BCG-*disA*-OE were significantly better protected against aerosol
90 challenge with virulent *Mtb* than with BCG-WT suggesting improved protective efficacy
91 over the existing BCG strain.

92

93 **Results**

94 **Induction of type I IFN responses by BCG-*disA*-OE**

95 We constructed a recombinant BCG strain (BCG-*disA*-OE) overexpressing the
96 endogenous diadenylate cyclase gene *disA* (also known as *dacA*) encoded by MT3692
97 in the CDC1551 genome or Rv3568 in the H37Rv genome. The *disA* genes of *Mtb* and
98 BCG are 100% identical at the nucleotide level. DisA catalyzes the conversion of 2 ATP
99 molecules to c-di-AMP (**Supplementary Figure 1a, 1b and 1c**). The overexpression
100 construct was generated by fusing the *disA* gene to the strong mycobacterial promoter
101 *hsp60* within the episomal mycobacterial overexpression vector pSD5-*hsp60*. Gene
102 expression profiling by real time PCR showed ~50-fold upregulation of *disA* expression
103 in BCG-*disA*-OE as compared to the parental strain (**Supplementary Figure 1d**).

104 Next we evaluated whether strong *disA* expression would result in c-di-AMP-
105 mediated IRF3 activation and consequent elevations in IFN- β levels in mouse
106 macrophages. Raw Blue™ reporter macrophage cells when infected with BCG-*disA*-OE

107 strains showed a significant 2-fold induction of IRF3 as compared to that observed with
108 the wild-type parental strain BCG-Pasteur-WT (**Supplementary Figure 2a**). These
109 preliminary results suggested that heightened levels of c-di-AMP release from
110 BCG-*disA*-OE produced significant activation of the STING/IRF3 axis, an observation
111 similar to our earlier findings with *Mtb-disA*-OE [12].

112 Next we infected primary murine BMDMs with BCG-*disA*-OE and BCG-WT and
113 quantified *Ifnb* gene expression using qPCR. BCG-*disA*-OE-infected macrophages
114 showed a 2-fold induction of *Ifnb* ($p < 0.005$, **Supplementary Fig 2b**) during an early
115 temporal window. The notion that CDNs such as c-di-AMP can induce STING-dependent,
116 but c-GAS independent induction of type I IFN responses (even in absence of
117 extracellular DNA) was validated earlier, and these results were in accordance with our
118 previous findings with recombinant *M.tb* overexpressing *disA* [12]. Previous studies
119 suggest that viral infection of non-phagocytic cells lead to cytosolic penetration by leaking
120 CDNs thus resulting into IFN β production [18]. Since BCG lacks a functional Esx-1
121 secretion system required to release bacterial DNA, these results further reinforce the
122 idea that phagosomes harboring mycobacteria are rather dynamic leaky structures or
123 really do not require Esx-1 for membrane disruption [19]. Our data indicate that bacterial-
124 derived c-di-AMP is detected in the macrophage cytosol and leads to STING-dependent
125 IFN- β synthesis. Increased levels of type I IFNs in macrophages in response to increased
126 c-di-AMP production in genetically modified BCG was the first step towards generation of
127 a vaccine strain with an increased antigenic repertoire and ability to stimulate STING.

128

129

130 **Macrophage activation by c-di-AMP overexpressing BCG (BCG-*disA*-OE)**

131 Although, the binding affinity of c-di-AMP for STING is weaker than that for
132 cGAMP, ligation of c-di-AMP with STING appears sufficient to induce co-activation of
133 transcription factors other than IRF-3 and, hence induction of pro-inflammatory cytokines
134 [20,21]. Identification of another bona-fide physiological sensor for c-di-AMP, an
135 endoplasmic adaptor, ERAdP, that binds to c-di-AMP with higher affinity, suggests
136 ERAdP-dependent initiation of activation of NF- κ B signaling in innate immune cells during
137 bacterial infection [20]. We previously showed that *disA*-OE strains of *M.tb* induce a
138 strong pro-inflammatory cytokines, such as TNF- α , IL-6 and IL-1 β , suggesting
139 macrophage activation and a complex link between c-di-AMP-based STING activation
140 and induction of pro-inflammatory cytokines and other interferon stimulated genes (ISGs)
141 [12]. Bone marrow-derived primary murine macrophages infected with BCG-*disA*-OE
142 showed significant increased levels of TNF- α , IL-6 and IL-1 β in culture supernatants as
143 compared to uninfected or BCG-WT-infected controls (**Figure 1**). These results reveal a
144 robust macrophage activation phenotype in response to c-di-AMP overproducing BCG
145 with increased levels of M1 or Th1 cytokines (TNF- α , IL-1 β and IL-6) that is more
146 pronounced than that seen with BCG-WT.

147

148 **CDN-adjuvanted recombinant BCG offers better protection against virulent *M.tb*** 149 **challenge in guinea pigs**

150 Our *in vitro* studies accessing macrophage response due to increased levels of
151 c-di-AMP encouraged us to test the vaccine potential of BCG-*disA*-OE in the guinea pig
152 model of tuberculosis infection (**Supplementary Figure 3**). Groups of twelve guinea

153 pigs were vaccinated intradermally with 0.1 ml of PBS (sham vaccination), 10^5 CFU of
154 BCG-*disA*-OE or BCG-WT and held for six weeks before challenge with aerosol
155 challenge with 10^2 CFU of *M. tuberculosis* H37Rv. As described in the Methods, lungs
156 from one set of infected animals were obtained on day 1 after challenge to confirmed
157 this implantation dose of *M. tuberculosis* (**Supplementary Figure 4**). Separate groups
158 of infected animals were euthanized 14 and 18 weeks post-challenge to determine the
159 protective efficacies of BCG-*disA*-OE and BCG-WT by organ weight, gross pathology,
160 and bacterial loads in lungs and spleen.

161 At 14-week post-challenge, both BCG-WT and BCG-*disA*-OE vaccinated animals
162 showed significantly lower lung weight, gross pathology scores, and the bacillary loads
163 in the lungs, relative to saline-treated controls (**Figure 2A** and **2B**). Vaccination with
164 BCG-*disA*-OE resulted in the highest reduction in lung gross pathology score, which
165 was even more pronounced at 18-week post-challenge (**Figure 3A** and **3B**). While the
166 impact of BCG-*disA*-OE vaccination on lung CFU counts was modest at the 14 week
167 time point, by the 18 week time point lung CFU counts in the BCG-*disA*-OE vaccinated
168 guinea pigs were 2.0 \log_{10} units lower than in animals vaccinated with BCG-WT. In
169 fact, two out of six guinea pigs in BCG-*disA*-OE group had lung CFU counts below the
170 limit of detection which is ~3-5 bacilli.

171 Additionally, vaccination with BCG-*disA*-OE effectively controlled the
172 hematogenous spread of *M.tb* to the spleen as evident from significant reduction in
173 spleen weights, spleen pathology scores, and spleen bacterial burdens when compared
174 with the sham-immunized animals at both 14 and 18 week post-infection
175 (**Supplementary Figure 5** and **Figure 4**, respectively). While spleen pathology scores

176 were comparable between animals vaccinated with wild-type and *disA* overexpressing
177 BCG at 14-weeks post-challenge (**Supplementary Fig 5**), by 18 weeks after challenge
178 significantly spleen lower pathology scores was observed in the latter group (**Figure 4**).
179 In addition, there was a trend towards lower spleen CFU in animals vaccinated with
180 *disA* overexpressing strain compared to BCG, especially when examined at 18-weeks
181 post-challenge (**Supplementary Figure 4**). Indeed, at 18 weeks post-challenge, three
182 out of six guinea pigs in BCG-*disA*-OE group had spleen CFU below the limits of
183 detection. These findings clearly indicate that administration of BCG-*disA*-OE effectively
184 controlled *M.tb* replication in the lungs and its dissemination to the spleen.

185

186 **DISCUSSION**

187 The cytosolic danger sensor STING has emerged as a central Toll-like receptor-
188 independent mediator of host innate immune responses. STING is activated by binding
189 CDNs either secreted by bacteria (such as c-di-AMP or c-di-GMP) or distinct host CDNs
190 (such as cGAMP) generated by host cell receptor following recognition of cytosolic
191 double-stranded DNA [22-24]. As a relatively new class of immunomodulatory molecules,
192 CDNs exhibit strong potential to promote protective immunity and increase vaccine
193 potency through STING-dependent signaling that involve transcription factors IRF-3, IRF-
194 7 and NF- κ B [6,10,11]. STING agonists are therefore regarded as promising immune
195 adjuvants for promoting immune responses against tumors and infections. However, the
196 efficacy of small molecule STING agonists as vaccine adjuvants may limited since they
197 are rapidly cleared and may not gain long-lived access to the cytosol for STING activation.

198 Here we tested the hypothesis that BCG strains overexpressing the STING agonist
199 c-di-AMP might offer sustained intracellular delivery of c-di-AMP and thereby improve the
200 vaccine potential of BCG against TB. We constructed a c-di-AMP-producing
201 recombinant BCG strain by over-expressing the *disA* (MT3692) gene which is identical at
202 the nucleotide level in both *M.tb* and BCG. Since BCG survives and replicates
203 intracellularly for several weeks post-vaccination, over-expression of *disA* under the
204 influence of strong constitutive mycobacterial *hsp60* promoter allows sustained
205 intracellular exposure of the STING agonist in the cytosol of phagocytic cells. By utilizing
206 BCG as the vector for STING agonist delivery, BCG-*disA*-OE offers enhanced innate
207 immune activation via the STING pathways in addition to the full antigenic repertoire of
208 BCG.

209 Complex genomic rearrangements in BCG strains are one of the major
210 contributors of immunological and phenotypic differences that in turn contribute to the
211 variability in the degree of protection offered by BCG [16,25]. A global resurgence of
212 MDR-TB, HIV-TB co-infection has heightened the need for an improved TB vaccine that
213 provides better protection than that of BCG. Additionally, new vaccines must be safe
214 enough to be used in immunocompromised HIV-TB co-infected individuals. Rational
215 modification of live BCG to increase its antigenic repertoire, and with a prior knowledge
216 of attenuation factors and immunity is critically needed [14,17]. While our study did not
217 directly assess the virulence of BCG-*disA*-OE to that of BCG-WT, based on the finding
218 that *Mtb-disA*-OE showed a median time to death in BALB-c mice of 321.5 days compared
219 to 150.5 days for *Mtb*-WT following an aerosol infection of 3.5 log₁₀ CFU [12], we
220 anticipate that BCG-*disA*-OE is a weaker pathogen than BCG-WT.

221 Inside the host, both CD4+ and CD8+ T cells are essential for protective immunity
222 against TB. Dendritic cells (DCs) migrating from the alveoli to the draining lymph nodes
223 are crucial for activation of *M.tb* antigen-specific CD4+ and CD8+ T cells and contribute
224 to resistance to *M.tb* [26]. The requirement for a Th1-like T cells response for host
225 immunity against *M.tb* is clear, and recent vaccine development has sought to stimulate
226 both CD4 and CD8 T-cell responses to produce Th1 cytokines [15]. Hence, elicitation of
227 enduring Th1 responses is a desirable feature of candidate TB vaccines. Not only are
228 STING-activating adjuvants known to elicit antigen-specific Th1 responses, but they also
229 elicit Th17 responses and have been shown to confer improved protection against *M.tb*
230 [10,27]. A recent report published while this manuscript was in preparation suggested that
231 the protection efficacy of protein subunit vaccine adjuvanted with small molecule CDNs
232 was durable for up to 12 weeks after *M.tb* challenge in mice, suggesting that a CDN-
233 adjuvanted vaccine can reduce TB progression in mice through T cell-dependent
234 mechanisms [27].

235 Guinea pigs are highly susceptible to *M.tb* infection, and the model provides an
236 important pre-clinical evaluation of potential vaccine candidates [28]. Immunization with
237 a single dose of BCG-*disA*-OE resulted in a marked reduction in the gross pathology and
238 bacterial loads in both lungs and spleens of *M.tb* challenged animals when compared to
239 sham treatment or vaccination with BCG alone. Thus our studies highlight the improved
240 potential of BCG-*disA*-OE over BCG to impart protection against *M.tb* infection and
241 disease dissemination.

242

243 This study is an important step forward towards implementing CDN-based STING
244 agonists as novel vaccine adjuvants into vaccine strategies for TB. Our results provide
245 proof-of-concept data for utilizing BCG as the vector for producing STING agonist(s)
246 naturally from within the intracellular compartment in a sustained fashion. Our results
247 suggest that STING agonist overexpressing BCG may offer greater efficacy as TB
248 vaccine than BCG alone and that this approach may have utility as an immunotherapeutic
249 tool against other diseases including cancer.

250

251 **MATERIALS AND METHODS**

252 **Animals:** All procedures involving live animals were performed in agreement with the
253 protocols approved by the Institutional Animal Care and Use Committee at the Johns
254 Hopkins University School of Medicine. Pathogen-free female outbred guinea pigs (300
255 g) and C57BL/6 mice were purchased from Charles River Laboratories (North
256 Wilmington, Mass.). Uninfected guinea pigs were housed under pathogen-free conditions
257 at BSL3 animal facility without cross-ventilation. C57BL/6J mice were housed in BSL2
258 animal facility at the School of Medicine, Johns Hopkins University. Animals were given
259 free access to water and standard mouse or guinea pig chow, respectively. The general
260 behavior and appearance were monitored by veterinary specialists.

261 **Bacterial strains and cell culture:** Details of all bacterial strains are provided in
262 **Supplementary Table 1**. BCG Pasteur was the kind gift of Frank Collins from the FDA,
263 and *M. tuberculosis* H37Rv and CDC1551 were obtained from ATCC. Frozen vials of
264 bacterial strains were revived and subsequently sub-cultured in 7H9 Middlebrook liquid
265 medium (B271310, Fisher Scientific) supplemented with oleic acid-albumin-dextrose-

266 catalase (OADC) (B11886, Fisher Scientific), 0.5% glycerol (G5516, Sigma) and 0.05%
267 Tween-80 (BP338, Fisher Scientific) in BSL3 facility. Murine bone marrow was isolated
268 from 4-6 weeks old female C57BL/6J mice. Approximately 10^8 cells were stored in
269 cryopreservation media made of 10% DMSO (D2650, Sigma) in heat inactivated FBS
270 (10082-147, Fisher Scientific) overnight at -80°C followed by transfer to deep
271 cryopreservation in liquid nitrogen. For differentiation of bone marrow cells into primary
272 macrophages, bone marrow cells were differentiated for 7 days in presence of RPMI-
273 Glutamax (61870-036, Fisher Scientific) supplemented with 10% heat inactivated fetal
274 bovine serum and antibiotics (Penicillin-Streptomycin solution) (15140-122, Fisher
275 Scientific) and 30% (vol/vol) L929 conditioned media. Mouse fibroblast L929 cells
276 (ATCC® CCL-1™) were maintained in RPMI-1640 medium supplemented with 10% FBS
277 and antibiotics.

278 **Overexpression of MT3692 in BCG Pasteur:** High molecular weight genomic DNA was
279 isolated using CTAB method from log phase cultures of *M.tb*-CDC 1551. Using gene-
280 specific primers (**Supplementary Table 2**), pSD5hsp60.MT3692 (F) and
281 pSD5hsp60.MT3692 (R), the *disA* (MT3692) gene of *M.tb*, was PCR amplified from *M.tb*-
282 derived genomic DNA. Gene amplicons were cloned into mycobacterial shuttle vector
283 pSD5-hsp60 (**Supplementary Table 1**) at the NdeI and MluI restriction sites. The clone
284 (pSD5-hsp60-MT3692) was confirmed by insert release and sequence analyses. The
285 construct pSD5-hsp60-MT3692 was subsequently used to transform BCG.
286 Recombinants clones (BCG-*disA*-OE) selected against kanamycin (25 $\mu\text{g}/\text{mL}$) and further
287 conformed using colony PCR using kanamycin-specific primers (**Supplementary Table**

288 **2).** MT3692 overexpression phenotype of BCG-*disA*-OE strain was further confirmed by
289 mRNA expression using quantitative real time PCR (qPCR).

290 **Quantitative real-time PCR (qPCR):** Late log phase BCG culture pellets were bead
291 beaten using Zirconium beads (KT03961-1-102.BK, Berlin Technologies) before
292 performing total RNA isolation. Trizol reagent (15596026, Fisher Scientific) was used for
293 RNA isolation from both bacterial and mammalian cells. Quantification of mRNA,
294 amplification, and quantification of cDNA was carried out using SYBR Fast green double
295 stranded DNA binding dye (4085612, Applied Biosystems, USA) and ABI StepOnePlus
296 Real Time PCR System (Applied Biosystems, USA). Amplification of *sigH* and mouse
297 beta actin was used as internal controls for BCG and mouse BMDMs respectively. Melt
298 curve analyses confirmed formation of desired and specific PCR product. Experiments
299 were performed in triplicate using three independent biological samples and results were
300 analyzed and presented using $2^{-\Delta\Delta CT}$ method. Details of NCBI gene identifiers and primer
301 sequences are mentioned (**Supplementary Table 2**).

302 **Macrophage infection, IRF3 activation assay and cytokine ELISAs:** Infection assays
303 were performed in resting mouse BMDMs in 24 well plates in triplicates. Briefly, early log-
304 phase cultures of BCG strains were washed, diluted appropriately to pre-defined
305 concentrations using macrophage infection media (DMEM with 10% FBS) and deposited
306 on the monolayer of cells at a precalibrated MOI (1:20). Infection was allowed continue
307 for 5 h, following which uninfected extracellular bacilli were removed by repeated washing
308 using Dulbecco's PBS (DPBS). This time point was considered 0 h and the cells were
309 incubated for desired number of hours till the end points were met. To access accurate
310 bacterial counts of infection and internalized bacterial numbers, serial dilutions of the

311 bacterial suspension and 0.025% SDS lysed macrophage were plated on 7H9 plates.
312 RAW-Blue ISG (InvivoGen) reporter cells, derived from the murine RAW 264.7
313 macrophage cell line by stably integrating an interferon regulatory factor (IRF)-inducible
314 secreted embryonic alkaline phosphatase (SEAP) reporter construct were used for IRF
315 activation assay. The cells were infected with wild-type and BCG-*disA*-OE strains. Cells
316 were incubated for 18 h in fresh macrophage infection media (DMEM with 10% FBS), and
317 culture supernatants were harvested for determination of IRF activation by a SEAP
318 colorimetric assay using QUANTI-Blue reagent (InvivoGen). Following macrophage
319 infection, culture supernatants were isolated, filtered and immediately frozen at -80 for
320 cytokine quantification. Mouse DuoSet ELISA kits for TNF (DY410), IL-6 (DY206-05) and
321 IL1 β (DY201-05) were used for cytokine quantification. The absolute concentrations were
322 determined by referring to a standard curve and expressed as pg/mL. For quantification
323 experiments were performed in triplicates.

324 **Guinea pig immunization and determination of protective efficacy:** To test the
325 prophylactic potential of BCG-*disA*-OE as a vaccine candidate, guinea pigs (n=12 per
326 group) were immunized intradermally using 10⁵ cfu/100 μ l of wild-type parental BCG
327 Pasteur or BCG-*disA*-OE strains. Guinea pigs were sham immunized with saline. Animals
328 were challenged with ~100 cfu of *M.tb* H37Rv strain by the aerosol route 6 weeks
329 **(Supplementary Figure 1)** after primary immunization. Lungs from one set of infected
330 animals were harvested, and homogenates were plated on day 1 after to check for
331 established implantation. Infected animals from each group were euthanized 14 and 18
332 weeks later to determine the protective efficacy of the BCG-*disA*-OE. Gross-pathological
333 features and bacillary burden in lungs and spleen of sham and BCG-immunized guinea

334 pigs after *M.tb* challenge were measured as described previously [29] . **Supplementary**
335 **Figure 3** shows the details of experimental plan and different animal groups used in the
336 study and **Supplementary Table 1** depicts the bacterial strains and plasmid used in the
337 study.

338 **Statistical analyses:** Fold-expression (qRT-PCR and ELISA) were represented as mean
339 value \pm standard error mean (SEM). Differences between individual test groups were
340 analyzed using by applying unpaired Student's t-test. All statistics was performed using
341 GraphPad Prism Version 5.01. P values < 0.05 were considered statistically significant.

342

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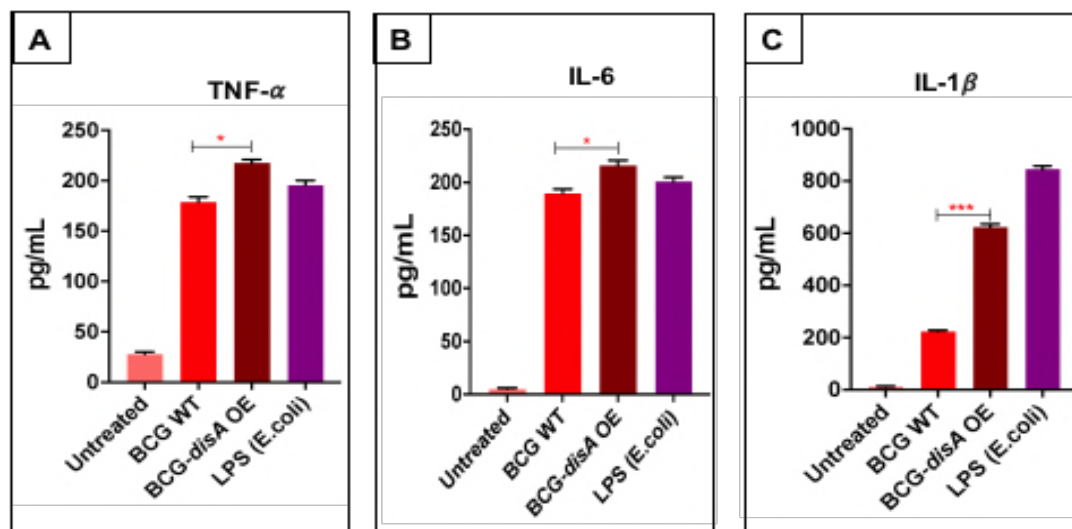
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Figure 1

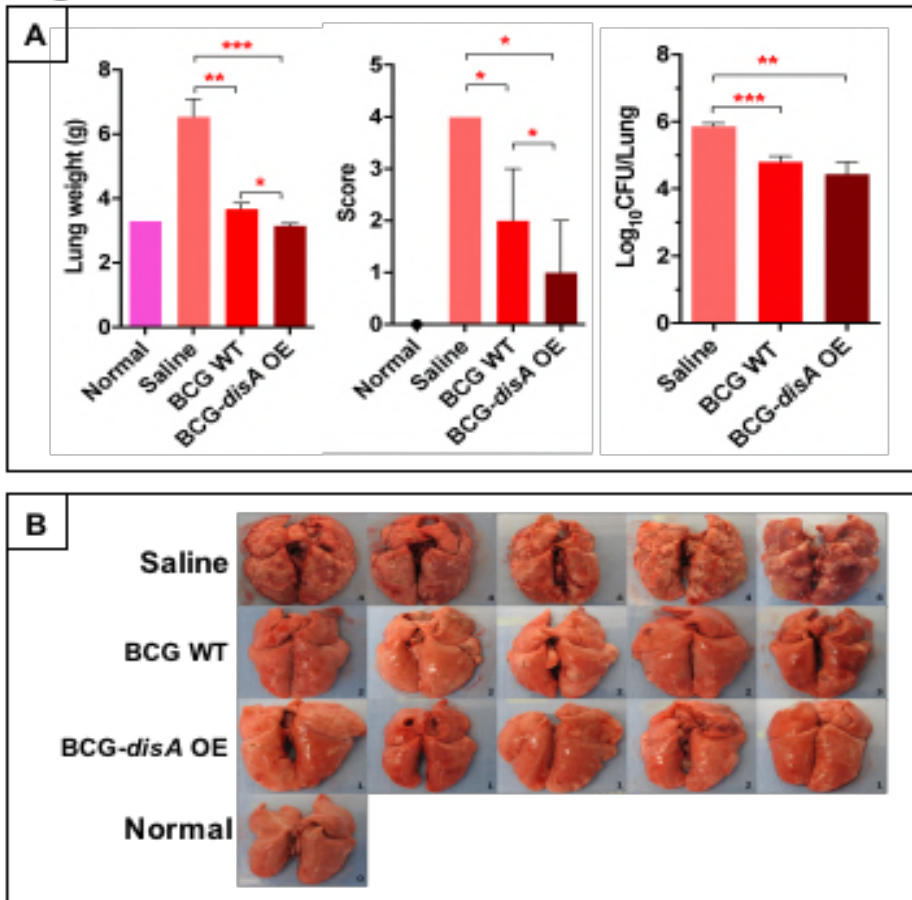


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418 **Figure 1. Modulation of pro-inflammatory cytokines in response to *disA***
419 **overexpression.** Differential induction of (a) TNF- α , (b) IL-6 and (c) IL1 β in mouse
420 BMDMs challenged with wild-type and *disA* overexpression strains of *Mycobacterium*
421 *bovis* BCG-Pasteur. BMDMs were challenged with wild-type and *disA* OE strains at an
422 MOI of 1:20 for 5 h to establish the infection. Uninfected bacteria were washed using ice-
423 cold DPBS and cells were subsequently incubated for another 24 h. Culture supernatants
424 were assayed by ELISA for different cytokines. The graphical points represent mean of 3
425 independent experiments \pm standard error mean (SEM). Student's t test (*P < 0.05 **P <
426 0.01, ***P < 0.001). MOI (multiplicity of infection).

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Figure 2



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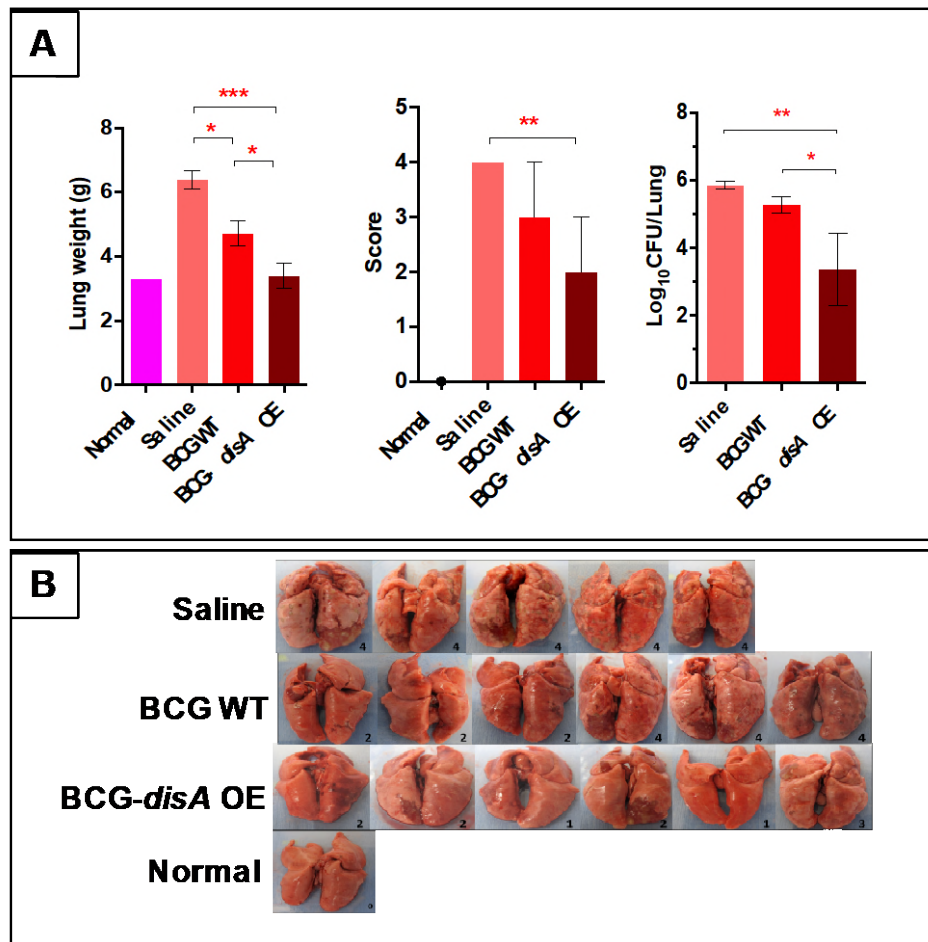
433 **Figure 2. Effect on lung weight, gross lung pathology scores and lung gross-**
434 **morphological features in guinea pigs 14 weeks post-challenge with *M.***
435 ***tuberculosis* H37Rv following vaccination with BCG-WT or BCG-disA-OE. A) Lung**
436 **weights and gross pathology scores at 14 weeks post-*M. tuberculosis* challenge. B)**
437 **Images of lungs at necropsy. ***P < 0.001, **, P< 0.01; *, P<0.05; Non-parametric Man**
438 **Whitney Test.**

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Figure 3

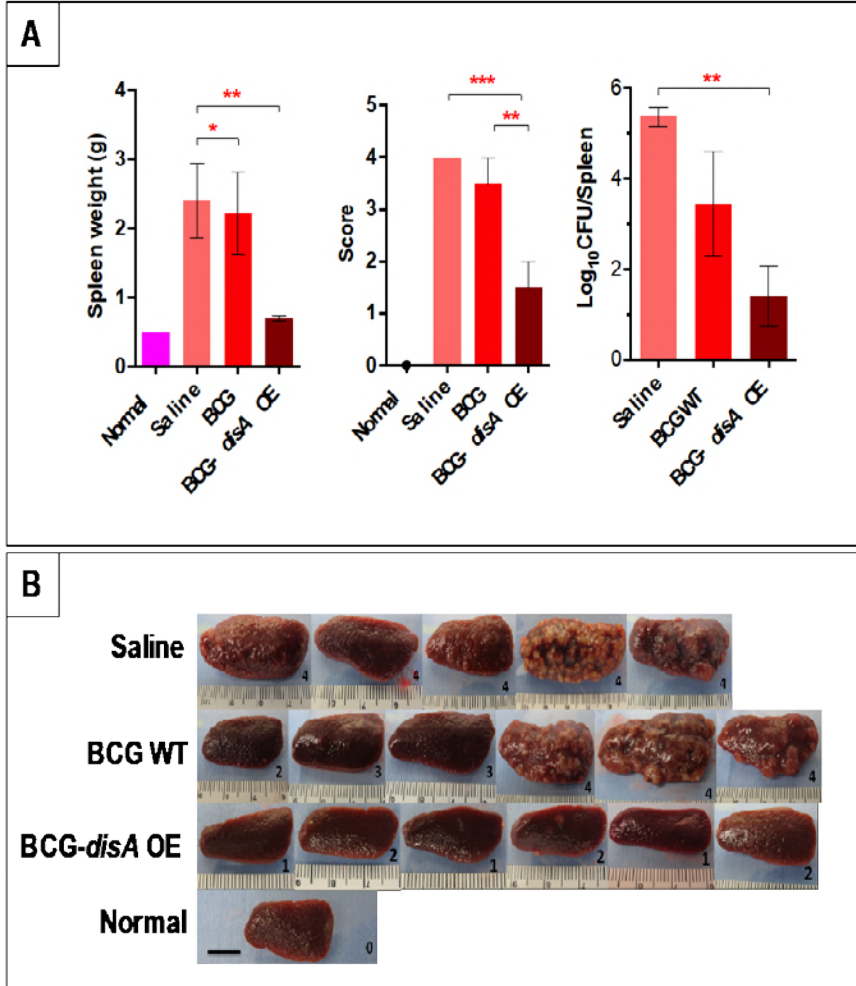


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Figure 3. Effect on lung weight, gross lung pathology scores, bacterial burden, and lung gross-morphological features in guinea pigs 18 weeks post-challenge with *M. tuberculosis* H37Rv following vaccination with BCG-WT or BCG-*disA*-OE. A) Lung weights, gross pathology scores, and *M. tuberculosis* bacterial burden at 18 weeks post-*M. tuberculosis* challenge. Two guinea pigs in BCG-*disA*-OE group had lung CFU counts below the limit of our detection. B) Images of lungs at necropsy. ***P < 0.001, **, P < 0.01; *, P < 0.05; Non-parametric Man Whitney Test.

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Figure 4

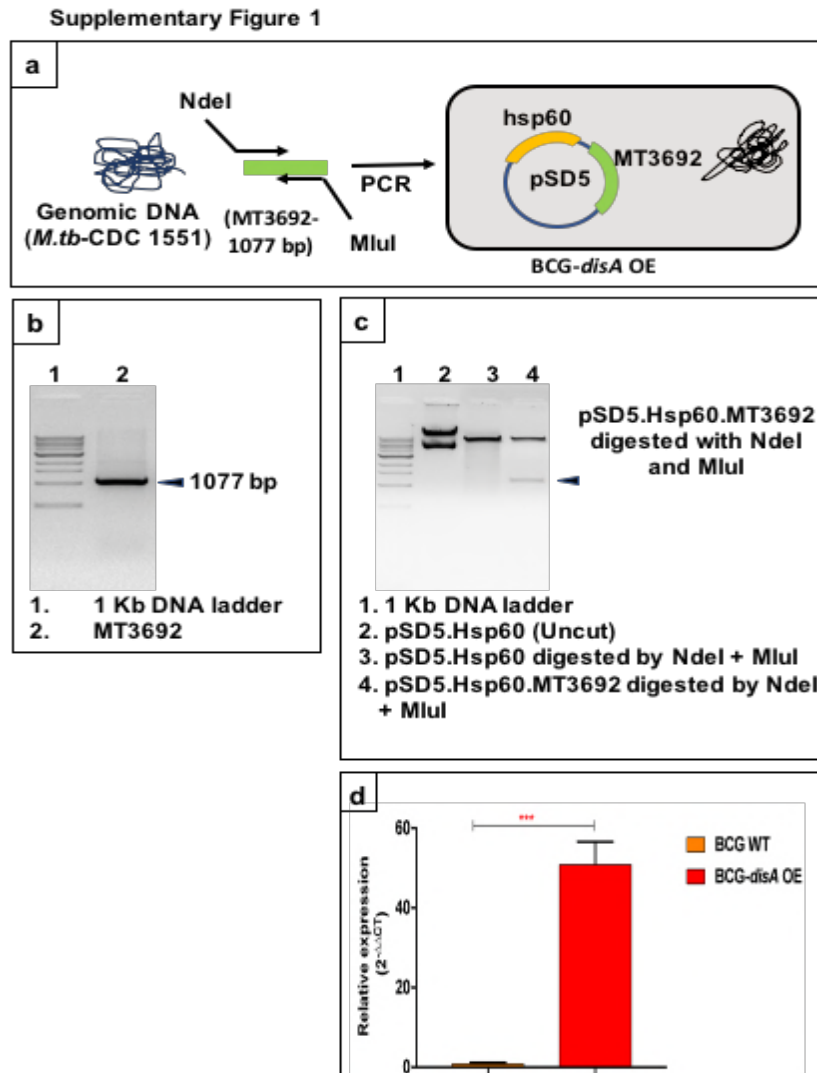


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Effect on spleen weight, gross spleen pathology scores, bacterial burden, and spleen gross-morphological features in guinea pigs 18 weeks post-challenge with *M. tuberculosis* H37Rv following vaccination with BCG-WT or BCG-*disA*-OE. A) Spleen weights, gross pathology scores, and *M. tuberculosis* CFU counts at 18 weeks post-*M. tuberculosis* challenge. Three guinea pigs in BCG-*disA*-OE group had spleen CFU counts below the limit of our detection. B) Images of spleens at necropsy. Scale bars indicate 1 cm. The number in the box is gross pathological score. *** $P < 0.001$, **, $P < 0.01$; *, $P < 0.05$; Non-parametric Man Whitney Test.

466 **SUPPLEMENTARY FIGURES AND TABLES**

467



468

469 **Supplementary Figure 1. Generation of the BCG-*disA*-OE strain and confirmation**

470 **of c-di-AMP overexpression. (a)** The MT3692 (*disA*) gene of *M. tuberculosis* was PCR-

471 amplified from *M. tuberculosis* CDC 1551 genomic DNA using gene-specific cloning

472 primers. **(b)** The amplicons were cloned into the mycobacterial shuttle expression vector

473 pSD5-hsp60 at the NdeI and MluI restriction sites. The construct (pSD5-*hsp60*-MT3692)

474 generation was confirmed using restriction analyses and sequencing. Constructs were

475 used to transform wild-type BCG Pasteur strain and recombinant clones were selected
476 against Kanamycin (25 µg/mL). **(c)** Differential expression of *disA* in wild-type and
477 BCG-*disA*-OE strains. Gene expression was measured in total RNA isolated from the late
478 log phase cultures using SYBR based quantitative real-time PCR. The graphical data
479 points represent the mean of 3 independent experiments ± standard error mean (SEM).
480 *M. tuberculosis sigA* (Rv2703) was used an internal control. Data analysis was performed
481 using $2^{-\Delta\Delta CT}$ method. Student's t test (**P < 0.0001).

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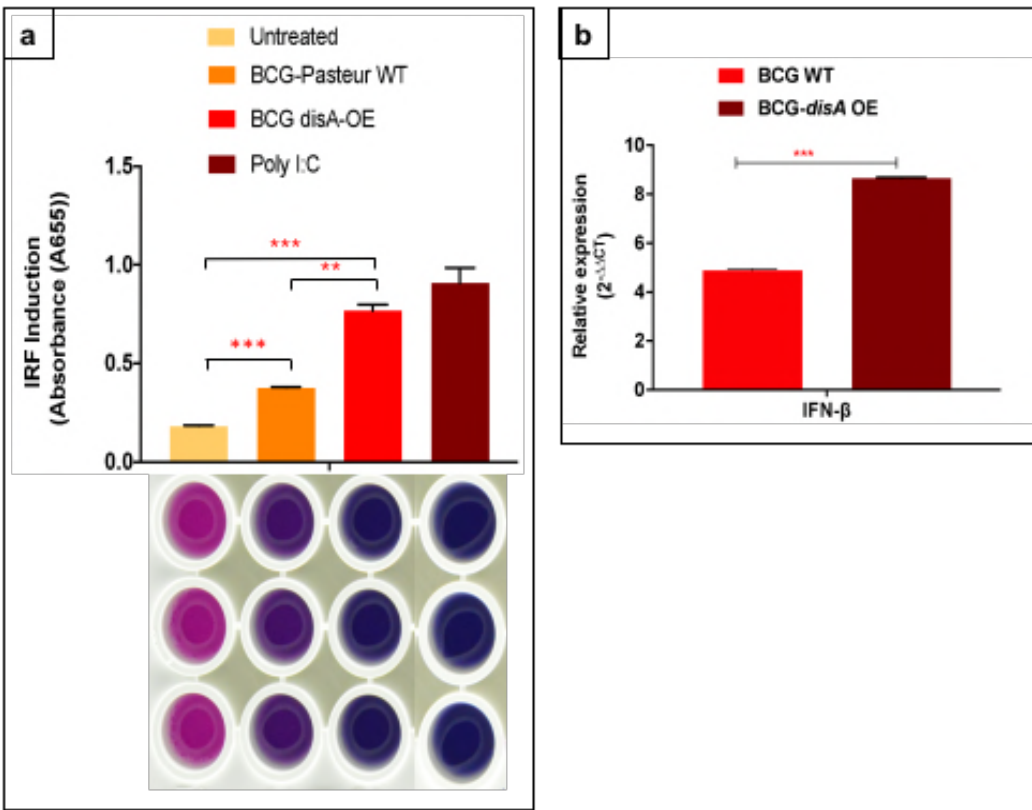
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Supplementary Figure 2



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499 **Supplementary Figure 2. BCG-*disA*-OE overexpressing c-di-AMP gives more**

500 **potent IRF3 and Type I IFN stimulation that BCG-WT: (a) Effect of *disA* overexpression**

501 on activation of IRF pathway measured by IRF-SEAP QUANTI Blue reporter assay.

502 RAW-Blue ISG cells were challenged with wild-type and BCG-*disA*-OE strains at an MOI

503 of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed out using

504 ice-cold DPBS and subsequently incubated for another 18-24 hours. The culture

505 supernatants of infected RAW-Blue ISG cells were assayed for IRF activation. The image

506 below the IRF-activation graph represents QUANTI Blue assay plate and sample wells;

507 treatment parameters for column of wells correspond to those defined for the bars above

508 aligned with the wells. The graphical points represent mean of three independent

509 experiments \pm standard error mean (SEM). Student's t test (**P < 0.0005, **P < 0.001).

510 **(b)** Differential expression of IFN β : Mouse BMDMs were challenged with wild-type and
511 BCG-*disA*-OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected
512 bacteria were washed using ice-cold DPBS and cells were subsequently incubated for
513 another 6 hours. Expression levels of mRNA was measured using a SYBR green-based
514 quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages
515 was used for data normalization and hence to access relative expression. β -actin was
516 used as an internal control. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The
517 graphical points represent mean of 3 independent experiments \pm standard error mean
518 (SEM). Student's t test (**P < 0.0005, **P < 0.001). MOI (multiplicity of infection).

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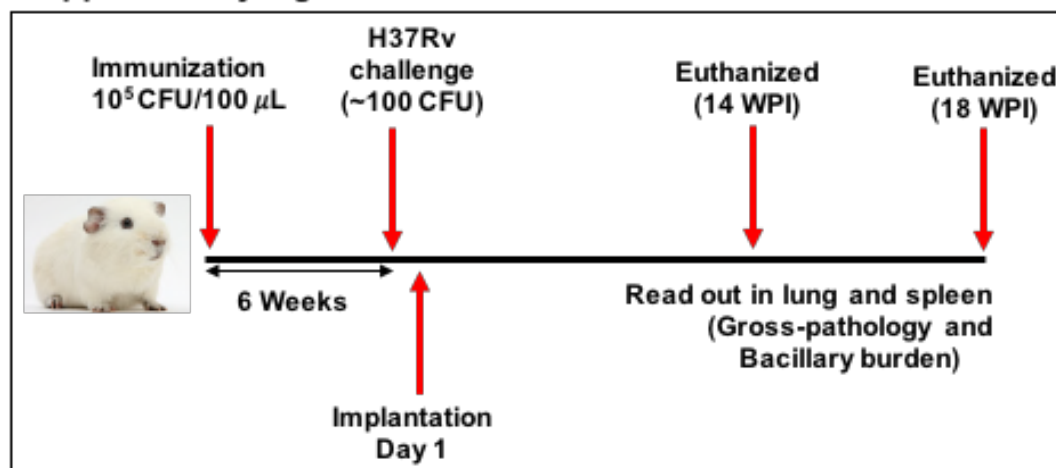
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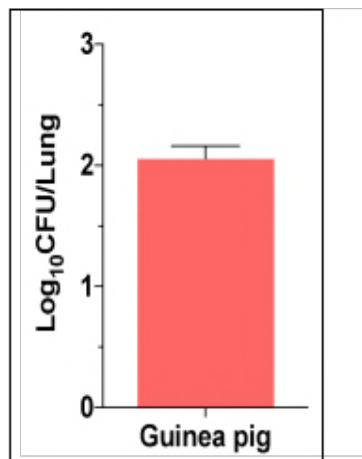
Supplementary Figure 3



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532 **Supplementary Figure 3.** Time line showing the experimental strategy of BCG
533 immunization and challenge in the guinea pig model of vaccination followed by *M.*
534 *tuberculosis* aerosol infection challenge.

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Supplementary Figure 4



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549 **Supplementary Figure 4 Bacillary load in the guinea pig lung following *M.***

550 ***tuberculosis* challenge.** The bacillary load in the lung of guinea pigs (n=3) were

551 determined at day 1 post aerosol challenge. Briefly, animals were euthanized, and lungs

552 were aseptically removed and homogenized in saline. The homogenates were serially

553 diluted and plated in duplicates on 7H11 medium supplemented with appropriate

554 antibiotics. To determine colony forming unit (CFU), Log₁₀ CFU were graphically

555 represented as dot plot, wherein median values ± standard error mean (SEM) are

556 denoted by horizontal line.

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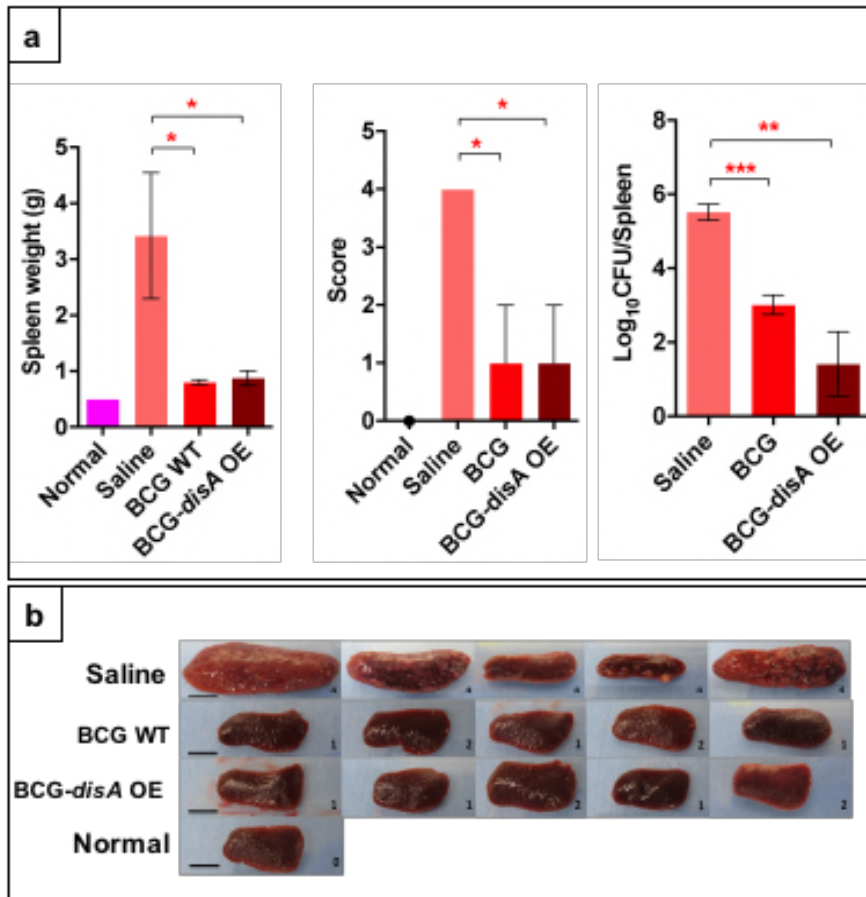
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Supplementary Figure 5



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566 **Supplementary Figure 5.**

567 **Effect on spleen weight, gross spleen pathology scores, bacterial burden, and**
568 **spleen gross-morphological features in guinea pigs 14 weeks post-challenge with**

569 ***M. tuberculosis* H37Rv following vaccination with BCG-WT or BCG-*disA*-OE. A)**

570 **Spleen weights, gross pathology scores, and *M. tuberculosis* CFU counts at 14 weeks**

571 **post-*M. tuberculosis* challenge. B) Images of spleens at necropsy. Scale bars indicate 1**

572 **cm. The number in the box is gross pathological score. **, P< 0.01; *, P<0.05; Non-**

573 **parametric Man Whitney Test.**

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576 **Supplementary Table 1: Bacterial strains and plasmids used in this study**

Name	Description
<i>M. tuberculosis</i> strains	
Mtb-CDC1551	Wild-type <i>M. tuberculosis</i>
<i>Mtb</i> -H37Rv	Wild-type <i>M. tuberculosis</i>
<i>M. bovis</i> BCG strains	
BCG	<i>M. bovis</i> BCG Pasteur
BCG- <i>disA</i> -OE	BCG Pasteur strain overexpressing <i>disA</i> (MT3692) of <i>M.tb</i>
Plasmids	
pSD5.hsp60	Mycobacterial expression plasmid with hsp60 promoter
pSD5hsp60.MT3692	<i>disA</i> over-expression plasmid

577
578 **Supplementary Table 2: Cloning and PCR primers used in the study**

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List of primer sequences used for gene expression analysis		
Accession Number	Gene	Primer Sequence 5'-3'
	pSD5hsp60.MT3692 (F)	GGGCATCATATGCACGCTGTGACTCGTC
	pSD5hsp60.MT3692 (R)	GGGACGCGTTATTGATCGCTGATGGTCG ATT
	Kanamycin cassette (F)	GAGAAACTCACCGAGGCAG
	Kanamycin cassette (R)	GTATTTTCGTCTCGCTCAGGC
32287254	<i>M.tb</i> sigH (F)	GCGATGGTGGCTTCTCCCTCG
	<i>M.tb</i> sigH (R)	CCATCTTGACACAGCTCGCGTAG
11461	Mouse.β actin (F)	TAAGGCCAACCGTGAAAAGATG
	Mouse.β actin (R)	CTGGATGGCTACGTACATGGCT
15977	Mouse. IFNβ (F)	CCACAGCCCTCTCCATCAAC
	Mouse. IFNβ (R)	CTCCGTCATCTCCATAGGGA
922803	<i>M.tb-disA</i> (F)	GCGATGGTGGCTTCTCCCTCG
	<i>M.tb-disA</i> (R)	CCATCTTGACACAGCTCGCGTAG

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