

1 **CD4 T cells and CD8 $\alpha$ + lymphocytes are necessary for intravenous BCG-induced protection**  
2 **against tuberculosis in macaques**

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29

## 30 **Abstract**

31 Tuberculosis (TB) is a major cause of morbidity and mortality worldwide despite widespread  
32 intradermal (ID) BCG vaccination in newborns. We previously demonstrated that changing the  
33 route and dose of BCG vaccination from  $5 \times 10^5$  CFU ID to  $5 \times 10^7$  CFU intravenous (IV) resulted  
34 in prevention of infection and disease in a rigorous, highly susceptible non-human primate model  
35 of TB. Identifying the immune mechanisms of protection for IV BCG will facilitate development  
36 of more effective vaccines against TB. Here, we depleted select lymphocyte subsets in IV BCG  
37 vaccinated macaques prior to Mtb challenge to determine the cell types necessary for that  
38 protection. Depletion of CD4 T cells or all CD8 $\alpha$  expressing lymphocytes (both innate and  
39 adaptive) resulted in loss of protection in most macaques, concomitant with increased bacterial  
40 burdens ( $\sim 4$ - $5 \log_{10}$  thoracic CFU) and dissemination of infection. In contrast, depletion of only  
41 adaptive CD8 $\alpha\beta^+$  T cells did not significantly reduce protection against disease. Our results  
42 demonstrate that CD4 T cells and innate CD8 $\alpha^+$  lymphocytes are critical for IV BCG-induced  
43 protection, supporting investigation of how eliciting these cells and their functions can improve  
44 future TB vaccines.

45

## 46 **One Sentence Summary**

47 Antibody depletion of lymphocytes in rhesus macques demonstrates key roles for CD4 T cells and  
48 innate-like CD8 $\alpha^+$  lymphocytes in conferring sterilizing immunity against tuberculosis following  
49 intravenous BCG vaccination.

50

## 51 **Introduction**

52 *Mycobacterium tuberculosis* (Mtb) caused over 10.5 million new cases of active tuberculosis (TB)  
53 in 2022 and 1.6 million deaths, with most of the global burden of disease seen in low- and middle-  
54 income countries (1). Efforts at reducing TB were limited by disruptions to medical infrastructure  
55 and research interests due to the COVID-19 pandemic (2, 3). To combat the TB epidemic, new  
56 vaccine approaches are critical (4-6). However, despite decades of research, there are still major  
57 gaps in our understanding of the mechanisms of effective control of Mtb infection and which  
58 immune factors should be targeted in a vaccine strategy (7-9), in part due to a lack of a fully  
59 protective vaccine. Currently, the live attenuated Bacille Calmette-Guérin (BCG) vaccine is the  
60 only licensed vaccine in use and is administered intradermally (ID) at birth in much of the world,  
61 including high-burden regions (10). ID BCG confers protection against disseminated disease in  
62 children but has variable efficacy against pulmonary disease and protection generally wanes  
63 through adolescence (11). BCG vaccination in adults has little efficacy (12). Despite limited  
64 durable immunity against TB disease, BCG has been used for over 100 years due to a lack of  
65 success by alternative vaccine candidates and regimens (13, 14).

66  
67 Experimental vaccine strategies that provide exceptional protection in an animal model that  
68 recapitulates all important aspects of human TB are essential to dissecting the immune factors  
69 required for prevention of infection or disease. This knowledge can lead to development of  
70 vaccines suitable for clinical trials. Macaques infected with a low dose of Mtb develop TB with  
71 similar outcomes and pathologies to humans (15, 16). Rhesus macaques (*Macaca mulatta*),  
72 specifically, are extremely susceptible to progressive disease following Mtb infection and thus  
73 they serve as a rigorous model for evaluating vaccine efficacy (17).

74

75 Recent studies in rhesus macaques from our group have demonstrated that the efficacy of BCG  
76 can be greatly enhanced by changing the administration method from the conventional low dose  
77 ID route (18). High dose intravenous (IV) high dose BCG elicited a significantly higher number  
78 of CD4 and CD8 T cells in the airways and lung tissue in macaques compared to the same dose  
79 given by ID or aerosol immunization. Following Mtb challenge, IV BCG vaccination resulted in  
80 90% of animals being protected (<100 total CFU recovered), with 60% developing sterilizing  
81 immunity, and minimal lung inflammation by PET CT.

82  
83 Correlates analyses of macaques immunized with a wide dose range of IV BCG revealed a highly  
84 integrated and coordinated immune response in the airway, some features of which could be  
85 predicted by early (day 2) innate signatures in whole blood (19, 20). Antigen-specific cytokine-  
86 producing CD4 T cells (IL-2, TNF, IFN $\gamma$ , IL-17) and natural killer (NK) cell numbers in the airway  
87 were among features most strongly correlated with protection. A parallel systems serology analysis  
88 of the study showed that humoral signatures, including complement fixing IgM and NK cell  
89 activating antibody, associated with protection (21, 22). Together, these data suggest that IV BCG  
90 elicits a multi-faceted immune response that mediates high-level protection against infection and  
91 disease. Here, to define the potential immune mechanisms of protection, we explored the  
92 contribution of specific lymphocyte subsets to IV BCG-induced immunity using an in vivo  
93 depletion strategy in vaccinated rhesus macaques.

94  
95 In this study, the immune response to IV BCG immunization developed over 5 months before  
96 individual lymphocyte subsets were depleted a month prior to and during the Mtb challenge period.  
97 Vaccinated NHPs were depleted of lymphocyte subsets using anti-CD4, anti-CD8 $\alpha$  or anti-

98 CD8 $\beta$  antibodies(23). CD8 is expressed as a dimer on the surface of a wide variety of immune  
99 cells, across innate and adaptive populations (24). Conventional adaptive CD8 T cells express both  
100 CD8 $\alpha$  and CD8 $\beta$  as a heterodimer, while subsets of innate cells, such as NKs,  $\gamma\delta$  T cells, NKTs  
101 and mucosal-associated invariant T cells (MAITs), often express a CD8 $\alpha\alpha$  homodimer. Thus,  
102 CD8 $\alpha$  depletion depletes all CD8+ populations, while CD8 $\beta$  depletion primarily targets the  
103 adaptive subset. Our results demonstrate that depletion of CD4 T cells or all CD8 $\alpha$ + lymphocytes  
104 substantially diminished IV BCG-induced protection against Mtb. By pairing Mtb outcome data  
105 with immunological assays and bacterial barcoding, we identified spatial immune bottlenecks to  
106 controlling Mtb. Our study not only supports the importance of CD4 T cell responses in vaccine-  
107 induced protection against Mtb, but also raises the intriguing possibility that innate CD8 $\alpha$ +  
108 lymphocytes also play a critical role in the robust protection afforded by IV BCG.

109

## 110 **Study design**

111 Rhesus macaques were vaccinated IV with  $1.7-4.5 \times 10^7$  colony forming units (CFU; target dose:  
112  $5 \times 10^7$ ) of BCG Danish (n = 65), as described previously (18, 19). Five months after vaccination,  
113 the NHPs were allocated to treatment groups receiving biweekly infusions of antibodies targeting  
114 either CD4 (n = 16), CD8 $\alpha$  (n = 17), or CD8 $\beta$  (n = 14) to deplete cells expressing these markers  
115 (**Fig. 1A**)(23). The final group (n = 18) of vaccinated animals received IgG or saline infusions as  
116 a non-depleting control. Cellular composition and function of peripheral blood mononuclear cells  
117 (PBMCs), airway cells from bronchoalveolar lavage (BAL), and peripheral lymph node (LN)  
118 biopsies were assessed at baseline, following vaccination and after depletion. Notably, subsets of  
119 innate (e.g. NK cells,  $\gamma\delta$  T cells, MAITs, NKTs, etc.) and adaptive CD8+ T cells express CD8 $\alpha$ .  
120 Previous studies from our group and others have shown that CD8 $\alpha$  expression on NK cells and  $\gamma\delta$

121 T cells is variable across macaques (23, 25, 26). NK cell phenotype differs by compartment (i.e.  
122 blood vs tissue) but are mostly CD8 $\alpha\alpha^+$ .  $\gamma\delta$  T cells are split between CD8 $\alpha\alpha^+$  and DN, while  
123 MAITs are 50% CD8 $\alpha\beta$ . However, unlike innate CD8 $^+$  lymphocytes which express a CD8 $\alpha\alpha$   
124 homodimer, adaptive CD8 T cells express a CD8 $\alpha\beta$  heterodimer and are therefore selectively  
125 targeted by anti-CD8 $\beta$  antibody (**fig. S1**). A small subset of CD4 T cells can also express CD8 $\alpha$ ;  
126 these CD4+CD8 $\alpha^+$  double positive T cells may represent an activated population, although our  
127 previous studies on granuloma T cells did not show notable differences in function between CD4+  
128 and CD4+CD8 $\alpha^+$  T cells (27). The efficiency of antibody depletion and composition of targeted  
129 cell types has been studied in depth using IV BCG vaccinated, Mtb naïve animals (23).

130  
131 One month after initiating depletion antibody infusions (6 months post-IV BCG), macaques were  
132 challenged intrabronchially with a low dose (5-39 CFU, median: 15) of genetically barcoded Mtb  
133 Erdman, as previously described (15, 28). Antibody infusions were continued biweekly for the  
134 duration of the study. The infection was monitored clinically and by serial positron emission  
135 tomography and computed tomography (PET CT) scans using  $^{18}\text{F}$ -fluorodeoxyglucose (FDG) as  
136 a PET probe for 8 weeks. FDG activity indicates increased cellular metabolism, a sign of localized  
137 inflammation, which we use as a proxy for TB disease (29). At necropsy (8 weeks post-challenge),  
138 the final PET CT scan was used as a map to identify individual granulomas and other pathologic  
139 lesions which, along with lung lobes and LNs, were excised and homogenized into single cell  
140 suspensions for microbiological and immunological analysis.

141  
142 This study was done in two cohorts, each with multiple BCG vaccination and Mtb challenge  
143 groups. Of note, the anti-CD8 $\beta$  antibody was only included in the second cohort. Specific cohort

144 information, including vaccination and infection doses for each animal are reported in **table S1**.  
145 Control groups included IV BCG vaccinated macaques (positive control for protection) treated  
146 with saline or non-specific IgG and unvaccinated macaques (negative control). Our previous IV  
147 BCG protection studies had a challenge phase duration of 12 weeks. However, due to the concern  
148 that T cell depletion could lead to heightened and more rapid disease, we chose a challenge phase  
149 duration of 8 weeks for this study.

150

#### 151 **IV BCG induces robust immune responses**

152 Similar to our findings in prior studies, IV BCG vaccination induced a pronounced increase in  
153 viable cells in the airways, due mostly to an increase in T cells (**Fig. 1B,C**)(18, 19). Early and  
154 transient increases in innate lymphocytes (V $\gamma$ 9+  $\gamma\delta$  T cells, MAIT cells, and NK cells) were  
155 observed in BAL at 2 and 4 weeks post-BCG (**Fig. 1D**). Between 4 and 8 weeks, CD4 and CD8  
156 T cells became the dominant cell populations in the airways, comprising 60-75% of airway  
157 leukocytes. NK cells, B cells, and neutrophils also increased at early timepoints (2-4 weeks) after  
158 IV BCG (**Fig. 1C**). Flow cytometry on BAL and PBMCs showed that antigen specific T cells  
159 making cytokines (IFN $\gamma$ , TNF, IL-2, and/or IL-17) after restimulation peaked in the first month  
160 post-vaccination and plateaued (**Fig. 1E,F; fig. S2A,B**).

161

162 Humoral responses were also induced in BAL following vaccination, with mycobacterial specific  
163 IgG, IgA, and IgM increasing by 4 weeks post immunization (**fig. S2C**). Although titers partially  
164 waned over time, they remained above baseline and the unvaccinated group.

165

#### 166 **Antibody-mediated depletion was profound in blood and tissues**



167 Using flow cytometry, we monitored the extent of antibody-mediated depletion of individual  
168 lymphocyte populations in blood (via PBMCs) throughout the depletion phase of the study, as well  
169 as in the airways (via BAL) and peripheral LNs (via biopsy) after two infusions. Depletion by each  
170 antibody was >90% of the expected populations in PBMCs (**Fig. 2A, fig. S1**). While depletion was  
171 most efficient in the blood, results from the airways (CD4: 89% depletion of CD4 T cells, CD8 $\alpha$ :  
172 99.9% depletion of all CD8 T cells, and CD8 $\beta$ : 97% depletion of CD8 $\alpha\beta$  T cells) and LNs (CD4:  
173 81% depletion, CD8 $\alpha$ : 99% depletion, and CD8 $\beta$ : 99% depletion) showed that these antibodies  
174 were effective even in tissues. The airway, where Mtb first interacts with the host, had a  
175 fundamentally altered profile in each depletion group (**Fig. 2B**). These data align with more  
176 extensive depletion analyses performed in IV BCG vaccinated rhesus macaques that were not  
177 challenged with Mtb (23). As expected, a substantial reduction in CD4<sup>+</sup> T cells was only observed  
178 in each tissue compartment in the anti-CD4 group. The increase in frequency of CD4s seen in both  
179 CD8 depletion groups is a result of the missing subsets from the CD3<sup>+</sup> population and not  
180 indicative of an increase in cell numbers (**Fig. 2A,B, fig. S3**). CD8 $\alpha$ <sup>+</sup> T cells, including both  
181 CD8 $\alpha\alpha$  and CD8 $\alpha\beta$  cells in the CD3<sup>+</sup> $\gamma\delta$ TCR<sup>-</sup> population, were depleted in both anti-CD8 $\alpha$  and  
182 anti-CD8 $\beta$  groups. Distinguishing targeted effects in each of these populations required more  
183 detailed analysis of the CD3<sup>+</sup>CD8 $\alpha$ <sup>+</sup> population (**fig. S4**). Classical adaptive CD8 T cells (i.e.  
184 CD8 $\alpha\beta$  T cells) were depleted in the airway by both anti-CD8 antibodies. Populations that express  
185 the CD8 $\alpha\alpha$  homodimer were only depleted by anti-CD8 $\alpha$  and not anti-CD8 $\beta$  antibody.

186

187 In macaques, it is not feasible to target singular cell types without depletion effects in other  
188 lymphocyte subsets. For example, CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> double positive T cells were reduced in both the  
189 anti-CD4 and anti-CD8 $\alpha$  groups (**figs. S3,S4**). While the exact classification of these cells is

190 debated between a distinct T cell subset and an activated CD4 phenotype, their absence across  
191 both depletion groups is important for contextualizing disease outcomes of the two experimental  
192 groups.

193  
194 There is no clear consensus on an exact definition of NK cells in NHPs, including methods of  
195 distinguishing them from other innate lymphoid cell (ILC) populations. Further complicating  
196 evaluation of these subsets is the fact that NK cells in blood and tissues are phenotypically  
197 heterogeneous (25, 30, 31). A conservative and inclusive definition of NK cells as CD3-CD20-  
198 lymphocytes expressing CD8 $\alpha$ , NKG2A, and/or CD16 expression was used across tissues. This  
199 could include ILCs, but it ensures a complete analysis of a potentially key cell type. Depletion of  
200 NK cells with the anti-CD8 $\alpha$  antibody was not completely effective (**fig. S3**). NK cells were  
201 characterized into different subsets based on expression of CD8 $\alpha$ , NKG2A, or CD16. Pre-  
202 depletion, the NK population in the airway was dominated by CD8 $\alpha$ +NKG2A-CD16- and  
203 CD8 $\alpha$ +NKG2A+CD16- subsets (**Fig. 3A**). Intracellular cytokine staining of BAL from vaccinated  
204 but undepleted animals by flow cytometry showed that CD8 $\alpha$ + subsets produce high levels of IL-  
205 17 and TNF, while NKG2A+ subsets were associated with elevated IL-2 (**Fig. 3B**). Following  
206 CD8 $\alpha$  depletion, CD8 $\alpha$ + NKs were largely replaced by NKG2A (CD159a) single positive cells  
207 (**Fig. 3A**), but the functional profile of NKG2A+ single positive cells did not change to compensate  
208 for lost cytokine production, with the potential exception of more IFN $\gamma$  (**Fig. 3C**). This led to a  
209 significant shift in overall NK functionality, as the number of IL-17 and TNF expressing NK cells  
210 in the airways dropped by roughly 10-fold, a ~90% decrease (**Fig. 3D**).

211

212 **CD4 and CD8 $\alpha$ + lymphocytes are necessary for IV BCG-induced protection**

213 Serial PET CT scanning was performed to monitor Mtb infection trajectory and disease over time  
214 (**fig. S5A**). The positive control, undepleted (IgG/saline group) IV BCG vaccinated animals had  
215 lower total lung FDG activity (inflammation) compared to unvaccinated animals, consistent with  
216 previous studies (18, 19). While the depleted groups showed minimal inflammation at 4 weeks  
217 post-infection, most CD4 and CD8 $\alpha$  depleted macaques had high lung FDG activity by 8 weeks  
218 post-Mtb, with significantly higher total lung FDG activity compared to IgG/saline vaccinated  
219 animals (**Fig. 4A**). In contrast, CD8 $\beta$  depleted vaccinated animals were similar to undepleted  
220 vaccinated animals in total lung FDG activity. The majority of the CD8 $\beta$  depletion group did not  
221 show increased lung FDG activity, with only 2 NHPs having high PET signal in the lungs at 8  
222 weeks post-infection, in contrast to 9 of 15 in the CD8 $\alpha$  depletion group.

223  
224 The number of granulomas identified on PET CT scans 4 weeks post-infection indicated that  
225 vaccinated animals formed relatively few lesions at 4 weeks post-infection, regardless of depletion  
226 (0-14 granulomas in vaccinated animals with or without depletion compared to 6-19 granulomas  
227 in unvaccinated animals) (**fig. S5B**). By 8 weeks post-infection, granulomas increased in all  
228 animals in the unvaccinated group, and in a subset of animals in the CD4 and CD8 $\alpha$  depleted  
229 groups. This was not observed in the IV BCG animals that received IgG/saline or anti-CD8 $\beta$   
230 antibody infusions.

231  
232 Detailed necropsies were performed using the final PET CT scan as a map of disease.  
233 Unexpectedly, the numbers of granulomas grossly identified at necropsy in many of the depleted  
234 vaccinated macaques were lower than unvaccinated animals (median: 25) (**Fig. 4B**). Still, the CD4  
235 depleted group (median: 4.5) and CD8 $\alpha$  depleted group (median: 3) had significantly higher

236 numbers of granulomas than the non-depleted vaccinated controls (median: 0), and a small number  
237 of clearly unprotected animals in each group had extensive disease (e.g. TB pneumonia). CD8 $\beta$ -  
238 depleted animals had very few granulomas at necropsy, similar to undepleted animals (median: 0).  
239 Gross pathology was scored by evaluating the number and size of granulomas and other lung  
240 pathologies, size and granuloma involvement of LNs, and evidence of extrapulmonary (EP)  
241 dissemination (16). As seen previously, IV BCG vaccination reduced the gross pathology score  
242 compared to unvaccinated animals (median: 9 vs 29; **Fig. 4C**) (18). Both anti-CD4 depleted  
243 (median: 16) and anti-CD8 $\alpha$  depleted (median: 18) macaques had significantly higher gross  
244 pathology scores compared to undepleted vaccinated controls. In contrast, CD8 $\beta$ -depleted animals  
245 were not significantly different from IgG/saline treated vaccinated controls.

246  
247 Multiple individual tissue homogenates (all lung granulomas and other pathologies, multiple  
248 random samples of each lung lobe, and all thoracic LNs) were plated and counted to calculate total  
249 thoracic bacterial burden as Mtb colony forming units (CFU). Peripheral LNs, spleen and liver  
250 were also plated. IV BCG vaccination resulted in a ~10,000-fold reduction in bacterial burden  
251 compared to unvaccinated controls (median IV BCG: 166 CFU vs median unvaccinated  $1.08 \times$   
252  $10^6$  CFU) (**Fig. 4D**), consistent with previous studies (18). Despite the small number of lesions  
253 observed by PET CT or recovered during necropsy of depleted NHPs, plating tissues for CFU  
254 revealed significant thoracic bacterial burden in both CD4 depleted (median:  $3.5 \times 10^5$  CFU) and  
255 CD8 $\alpha$  depleted (median:  $2.7 \times 10^5$  CFU) groups, which were significantly higher compared to the  
256 vaccinated controls and similar to unvaccinated animals. CD8 $\beta$ -depleted animals were similar to  
257 undepleted vaccinated controls in bacterial burden with several sterile (no Mtb recovered)  
258 macaques. In striking contrast, no CD4 depleted NHPs (0/16) showed sterilizing immunity,

259 although one animal had only 20 total CFU, so would still be considered to be protected. Two  
260 CD8 $\alpha$  depleted animals (2/17) had no Mtb recovered from any sites. CD4-and CD8 $\alpha$ -depleted  
261 animals were significantly higher than vaccinated controls in both lung CFU and thoracic LN CFU.

262  
263 Previously, our data supported that there is a limit to the bacterial capacity of granulomas before  
264 failure and dissemination occur (32). IV BCG vaccination reduced the number of bacteria  
265 recovered from granulomas (median undepleted IV BCG: 411 CFU vs median unvaccinated: 4.1  
266  $\times 10^4$  CFU), indicating effective early control. A significant increase in CFU per granuloma was  
267 observed in the CD4 depletion group (median:  $5.8 \times 10^4$  CFU) (**Fig. 4E**). Most granulomas in CD4  
268 depleted animals contained higher bacterial burden than those from undepleted animals,  
269 implicating CD4 T cells in limiting bacterial growth within granulomas (**fig. S5C**). There was a  
270 trend toward increased bacterial burden in granulomas from CD8 $\alpha$  depleted animals ( $p = 0.1494$ )  
271 (**Fig. 4E**). The low numbers of granulomas in the CD8 $\beta$  depleted animals precluded this analysis  
272 for that group.

273  
274 Dissemination, or spread of Mtb infection, is a useful indicator of a lack of immune containment.  
275 In CD4 depleted animals, grossly uninvolved lung tissue had 48-fold more CFU than undepleted  
276 vaccinated controls, indicating dissemination through the lung tissue (**Fig. S5D**); CD8 $\alpha$  depleted  
277 animals had 2.25 fold more CFU in uninvolved lung tissue compared to undepleted vaccinated  
278 controls. Thoracic LNs are common destinations for dissemination events, illustrated by  
279 unvaccinated animals having a median of 6 involved (Mtb positive) thoracic LNs (**Fig. 4F**). Since  
280 infection is generally controlled early in the lung following IV BCG precluding dissemination,  
281 there was little spread of Mtb from lung to thoracic LNs in undepleted vaccinated controls. We

282 observed significant increases in the number of CFU+ LNs following both CD4 and CD8 $\alpha$   
283 depletions, implicating these lymphocytes in limiting disease progression. Despite significant  
284 impact on dissemination to LNs, depletion did not appear to affect extrapulmonary dissemination,  
285 or spread to non-thoracic tissues (**Fig. 4G**). However, this could change in the case of infections  
286 lasting longer than the eight week timepoint chosen for this study.

287

### 288 **CD4 and CD8 $\alpha$ depletion leads to increased Mtb dissemination**

289 To gain further insight about Mtb infection establishment and dissemination following depletion  
290 in vaccinated macaques, we analyzed data from the genetically barcoded strain of Mtb Erdman  
291 used for infection in conjunction with serial PET CT scans to quantify how many bacilli initiated  
292 infection and the extent and sites of dissemination (**data file S1**) (26, 28). We previously  
293 demonstrated that each granuloma is seeded by a single bacterium (28, 32). The number of unique  
294 barcodes found in vaccinated animals was reduced compared to unvaccinated animals (**Fig. 5A**).  
295 Paired with minimal granuloma numbers and Mtb CFU in the vaccinated control group, these data  
296 corroborate our previous results that IV BCG has a robust effect on limiting Mtb infection (18,  
297 19). CD4 depletion led to a modest yet significant increase in unique barcodes compared to  
298 undepleted vaccinated macaques, indicating a slight loss of control over establishment. Depletion  
299 of CD8+ cells by either anti-CD8 $\alpha$ - or CD8 $\beta$ - antibodies did not result in increased numbers of  
300 unique barcodes, which aligns with the limited early (4 week) granuloma formation observed by  
301 PET CT (**fig. S5B**).

302

303 To quantify dissemination, we evaluated the percentage of barcodes that were found in multiple  
304 sites (i.e. barcodes shared by granulomas, lung tissue and/or LNs). Specifically, we used a cut-off

305 of 3 or more shared sites to capture widespread dissemination, avoiding one-off dissemination  
306 events from granuloma to a LN that are a general feature of the macaque model. Undepleted  
307 vaccinated macaques had no or minimal dissemination, yet both CD4 and CD8 $\alpha$  depletion resulted  
308 in significantly increased dissemination (**Fig. 5B**). Barcodes shared between a lung or lung  
309 granuloma and a LN increased in CD4 and CD8 $\alpha$  depleted animals, with most or all established  
310 barcodes spreading to LNs (**Fig. 5C**). Unique barcodes in thoracic LNs also increased in CD4 and  
311 CD8 $\alpha$  depleted animals relative to undepleted vaccinated controls (**Fig. 5D**). CD8 $\beta$  depletion  
312 resulted in minimal dissemination, similar to undepleted vaccinated animals.

313  
314 We developed methods to quantify inflammation (via FDG avidity and PET CT scans) in the  
315 thoracic LNs (see Materials and Methods). Our previously published data showed that thoracic  
316 LNs with high FDG avidity were very likely to contain Mtb bacilli (33). Both CD4 and CD8 $\alpha$   
317 depleted macaques had significantly elevated FDG activity in LNs compared to undepleted  
318 vaccinated controls, while the CD8 $\beta$  depleted group was not significantly different than controls  
319 (**Fig. 5E**). The maximum FDG avidity, quantifying the most inflamed LN per animal, significantly  
320 increased following CD4 and CD8 $\alpha$  depletion (**Fig. 5F**). Historically, we have seen that  
321 substantially diseased and necrotic LNs can infiltrate the airway and rupture, rapidly spreading  
322 large numbers of bacilli throughout the lung. Prevention of LN disease progression by vaccines is  
323 important to limit overall disease progression.

324  
325 CD4 and CD8 $\alpha$  depleted animals were less capable of controlling Mtb disseminating to and  
326 replicating in LNs (**Fig. 5G, fig. S5E**). Mtb bacterial burden in involved LNs following either CD4  
327 (median:  $2.7 \times 10^4$  CFU) or CD8 $\alpha$  (median:  $8.7 \times 10^4$  CFU) depletion was significantly higher

328 than undepleted vaccinated controls (median: 75 CFU). However, we saw a 3-fold increase in  
329 median CFU in the CD8 $\alpha$  depleted group compared to the CD4 depleted group, suggesting more  
330 bacterial growth in LNs in the absence of CD8 $\alpha$ + lymphocytes. These data support a role of  
331 vaccine-induced, CD4 T cell and innate CD8 $\alpha$ + lymphocyte mediated control of dissemination to  
332 and bacterial control in thoracic LNs.

333

### 334 **High Mtb burden in depleted lung tissue leads to increased effector molecule production**

335 IFN $\gamma$  release assays, or IGRAs, are a key component in diagnosing Mtb infection. We used IFN $\gamma$   
336 ELISpots to assess Mtb-specific responses in blood following Mtb challenge using ESAT-6 and  
337 CFP10 peptide pools as stimulators. ESAT-6 and CFP10 are not expressed by BCG due to its  
338 attenuation, enabling a differentiation of vaccine- and infection-induced responses. Five of six  
339 unvaccinated macaques showed a positive ELISpot result (>10 spot forming units (SFU)/200,000  
340 PBMC) at necropsy (**fig. S6A**). In the undepleted vaccinated group, 11 of 17 of the animals had  
341 negative Mtb-specific responses, as in our previous study (18). This also held true in the CD8 $\beta$   
342 depletion group, with 12/14 animals returning negative results. These animals had not developed  
343 significant disease and likely controlled infection effectively before an adaptive T cell response  
344 specific to Mtb could be formed. Approximately half (7/13) of CD4 depleted macaques and most  
345 (9/13) CD8 $\alpha$  depleted macaques had positive ESAT6/CFP10 ELISpots at necropsy. While the  
346 proportion of CD4 and CD8 $\alpha$  depleted animals testing positive was similar, the amplitude of  
347 response was lower in the CD4 depleted group (median 10.5 SFU/200,000 cells) than the CD8 $\alpha$   
348 depleted group (median 148.5 SFU/200,000 cells). These assays are largely driven by CD4 T cell  
349 IFN $\gamma$  production, so it is reasonable to expect stunted responses after CD4 depletion despite high  
350 bacterial burden.



351  
352 While IGRAs are a useful clinical tool, granulomas in the lung are the primary battleground  
353 between the immune system and the invading pathogen. To determine how these cell populations  
354 may be influencing infection dynamics, flow cytometry was used to analyze the populations  
355 present within these tissues and their functionality. IV BCG vaccinated, undepleted animals  
356 generally have fewer cells collected from granulomas (**fig. S7A**), likely because they controlled  
357 the infection early and are healing by the time of excision at necropsy. The number of cells in  
358 granulomas did not change following CD4 or CD8 $\beta$  depletion, but significantly increased  
359 following depletion of all CD8 $\alpha$ + lymphocytes. Relative frequencies of lymphocyte subsets in  
360 excised granulomas were consistent between unvaccinated and undepleted vaccinated groups (**fig.**  
361 **S7B**). Populations not targeted by depletion (e.g. CD4 T cells in CD8 $\alpha$  depletion) were found in  
362 higher frequencies within lesions.

363  
364 CD4+CD8 $\alpha$ + and  $\gamma\delta$  T cells are cell types lost following both CD4 and CD8 $\alpha$  depletion that might  
365 be playing an important role in restricting Mtb infection at the granuloma. We hypothesized that  
366 CD4+CD8+ double positive T cells could be playing a role in IV BCG-induced protection since  
367 this population was at least partially depleted in both groups. However, we did not identify a  
368 correlation with the number of cytokine producing double positive T cells granulomas with  
369 thoracic CFU (**fig. S7C**).  $\gamma\delta$  T cells, which are predominantly CD4-CD8 $\alpha$ - or CD8 $\alpha$ + in naïve  
370 animals (34), were not efficiently depleted and were abundant in CD8 $\alpha$  depleted granulomas. The  
371 CD4/CD8 composition of this population revealed an influx in  $\gamma\delta$  T cells that were negative for  
372 CD4 or CD8 $\alpha$  following both CD4 and CD8 $\alpha$  depletions (**fig. S7D**).

373

374 Successful depletion was also observed in grossly uninvolved lung tissue, where we are able to  
375 sample larger amounts of tissue and therefore recover more cells than from individual granulomas  
376 (**Fig. 6A**). Despite depletion of >90% of CD4 T cells in the lung, a number of cytokine positive  
377 CD4 T cells were detected by flow cytometry (**Fig. 6B**). As it has been demonstrated previously  
378 that effector/memory populations are effectively depleted in tissue by antibody infusions, it is  
379 unlikely that this represents a change in functional profile of the cell types, such as CD4 T cells  
380 adopting a cytotoxic phenotype in the absence of CD8 $\alpha$ <sup>+</sup> cells (23, 26). Rather, it is likely the  
381 result of heightened disease burden in depleted animals leading to increased activation of those  
382 remaining CD4 T cells. CD8 $\alpha$  depletion was nearly complete in its elimination of cytokine  
383 producing cells. It appears as though the population not targeted by the depletion antibody (e.g.  
384 CD4 T cells following CD8 $\alpha$  depletion) provides some level of compensatory cytokine  
385 production, again likely due to high bacterial burden rather than a phenotypic change, however our  
386 study was not powered to evaluate such subtle differences. In the CD8 $\beta$  depleted lung tissue, the  
387 remaining functional CD8 $\alpha$ <sup>+</sup> T cells are primarily CD8 $\alpha\alpha$  (**Fig. 6C**). Ex vivo stimulation with  
388 whole cell lysate (WCL) showed vaccine-induced memory responses, especially in CD4 T cells  
389 (**fig. S6B**). Stimulation with a peptide pool of ESAT-6 and CFP10 showed negligible Mtb-specific  
390 responses in either CD4 or CD8 $\alpha$  T cells regardless of depletion, despite the increase in disease  
391 observed in depleted animals. Eight weeks may be too early to see significant tissue resident Mtb  
392 antigen specific populations, which are slow to develop in TB, illustrated by a lack of Mtb-specific  
393 functionality in the unvaccinated group. Further, any responses, whether to WCL or ESAT-6 and  
394 CFP10, may be masked by in vivo stimulation from residual antigen in the lung tissue (i.e. effector  
395 molecule production without ex vivo stimulation).

396

## 397 **Discussion**

398 IV BCG vaccination is highly effective against Mtb infection and disease in non-human primates,  
399 providing a model to assess immune correlates and mechanisms of protection. IV BCG results in  
400 a large influx of antigen-specific T cells to the airway and our correlates analysis found that CD4  
401 T cells producing Th1 or Th17 cytokines and the number of NK cells in the airways were primary  
402 correlates of protection in this model (18, 19). These data corroborate rodent and macaque models  
403 and human observational studies that demonstrate a key role for CD4+ T cells in anti-  
404 mycobacterial protection (7, 35-37). Of note, the expansion of mycobacteria-specific CD8+ T cells  
405 following IV BCG suggested that a multicellular mechanism could be also be necessary for  
406 protection. Our study of the influence of depletion of CD4+, CD8 $\alpha$ + or CD8 $\alpha\beta$ + lymphocytes on  
407 the establishment, progression and dissemination of Mtb following IV BCG vaccination  
408 demonstrates a requirement for CD4 T cells and CD8 $\alpha$ + lymphocytes, but not CD8 $\alpha\beta$  T cells, for  
409 IV BCG-induced protection in macaques.

410  
411 Here, either CD4 or CD8 $\alpha$ , but not CD8 $\beta$ , antibody-mediated depletion led to abrogation of IV  
412 BCG-induced immunity in most animals. This supports non-redundant roles for CD4+ T cells and  
413 CD8 $\alpha$ + lymphocytes in protection mediated by IV BCG by the primary outcome measure of  
414 thoracic Mtb bacterial burden. These results are consistent with other data supporting a key  
415 protective role for T cells against Mtb infection. While CD4 T cells are canonically associated  
416 with both vaccine responses and natural immunity to Mtb infection (7, 35), we recently showed  
417 that CD8+ lymphocytes are also critically important to controlling early Mtb infection (26). We  
418 expected that, given the influx of antigen-specific CD8+ T cells in airways and return of rapidly  
419 expanded  $\gamma\delta$  T cell and MAIT populations to pre-vaccination baselines, we would see similar

420 effects between the two CD8 depletion groups. Anti-CD8 $\alpha$  antibody targets both CD8 $\alpha\alpha$  and  
421 CD8 $\alpha\beta$  expressing cells, including NK cells,  $\gamma\delta$  T cells, MAITs, and CD4+CD8 $\alpha$ + double positive  
422 T cells, in addition to conventional adaptive CD8 $\alpha\beta$  T cells. Anti-CD8 $\beta$  antibody, however,  
423 primarily targets conventional CD8 T cells, which express CD8 $\alpha\beta$  heterodimers. In contrast to  
424 CD8 $\alpha$  depletion, CD8 $\beta$  depletion did not diminish the IV BCG-induced protection. Our results  
425 raise the possibility that innate-like CD8+ lymphocyte subsets, possibly NK/ILCs,  $\gamma\delta$  T cells, or  
426 MAITs, are critical for BCG IV-induced protection. This conclusion is supported by the  
427 demonstration that the primary correlates of protection following IV BCG are the number of CD4  
428 T cells and NK cells in the airways (19, 38). Although antibody responses were elevated in  
429 response to IV BCG, in a separate study we depleted macaques of B cells during the early  
430 vaccination phase which greatly diminished antibody levels for the duration of the study, yet this  
431 had little to no effect on IV BCG-induced protection (39).

432  
433 We have considered potential experimental factors that could confound our ability to see a loss of  
434 protection in CD8 $\beta$  depleted animals. Insufficient depletion is unlikely, according to our own  
435 analysis and a recent study which clearly demonstrated the effectiveness of CD8 $\beta$  depletion in IV  
436 BCG vaccinated but unchallenged macaques (23). Further, CD8 $\beta$  depletion via antibody infusion  
437 is capable of disrupting control of infections in other disease models (40-43). Therefore, our data  
438 support that the mycobacteria-specific airway or lung adaptive CD8 T cells are not required for IV  
439 BCG-induced early protection. It is possible that since MHC Class I primarily presents ESX-1  
440 antigens on Mtb-infected human macrophages in vitro (44), CD8 T cells may need to recognize  
441 infected macrophages presenting ESX-1 antigens to be protective in primates. ESX-1 antigens are  
442 not expressed by BCG due to the RD1 deletion, so CD8 $\alpha\beta$  T cells recruited to the lung following

443 IV BCG vaccination likely recognize mycobacterial antigens expressed by BCG that are presented  
444 less efficiently by Mtb infected macrophages.

445

446 The simplest interpretation of our data is that CD4 T cells and innate-like CD8 $\alpha$ <sup>+</sup> lymphocytes are  
447 both critical for IV BCG-induced protection. However, it could be that removal of a singular cell  
448 type from the system is responsible for the effect seen in both groups. This would most likely  
449 suggest that CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> (double positive) T cells are primarily responsible for protection  
450 following IV BCG. CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells have been described as both an activated CD4 subset and  
451 a distinct population (45, 46). However, our data show no correlation between the level of  
452 depletion of CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells in granulomas and outcome (i.e. bacterial burden) in either  
453 depletion group, suggesting that disease burden was independent of this population. Thus, while  
454 CD4<sup>+</sup>CD8 $\alpha$ <sup>+</sup> T cells may be involved in IV BCG-induced protection, they are not likely to be the  
455 sole source of protection; a key role for these cells cannot be ruled out without targeting them in  
456 isolation. A single source of protection could also be  $\gamma\delta$  T cells, the majority of which are CD8 $\alpha$ <sup>+</sup>  
457 in granulomas. The rest of the  $\gamma\delta$  T cells are primarily CD8 $\alpha$ -CD4<sup>-</sup>, although some CD4<sup>+</sup>  $\gamma\delta$  T  
458 cells were observed.  $\gamma\delta$  T cells previously were shown to have protective potential in  
459 macaques (47, 48). Depletion with anti-CD8 $\alpha$  antibody or anti-CD4 antibody did not change the  
460 total  $\gamma\delta$  T cell population, with CD4<sup>-</sup>CD8 $\alpha$ -  $\gamma\delta$  T cells expanding to fill the gap due to CD8 $\alpha$   
461 depletion. Thus, we conclude that although  $\gamma\delta$  T cells certainly may have a role in IV BCG-induced  
462 protection, the primary role may be during initiation of the immune response, hence the short-lived  
463 expansion following vaccination, rather than during the effector phase of protection.

464

465 Our data support that one or more subsets of CD8 $\alpha$ <sup>+</sup> innate-like lymphocytes, in addition to CD4  
466 T cells, are needed for IV BCG-induced protection. Recent studies have shown that CD8 $\alpha$ <sup>+</sup>  
467 lymphocytes play a key role in control of early Mtb infection in macaques (26, 49). Here, the  
468 question remains which CD8 $\alpha$ <sup>+</sup> lymphocytes are necessary for the protection. Memory-like NK  
469 cells have been shown to exhibit therapeutic potential in vaccine models (50, 51). Relevant to our  
470 study, NK cells are affected by CD8 $\alpha$  depletion, although CD8 $\alpha$ -negative CD16<sup>+</sup> and NKG2A<sup>+</sup>  
471 NKs are still present after CD8 $\alpha$  depletion. Specifically, CD8 $\alpha$ <sup>+</sup>CD16<sup>-</sup>NKG2A<sup>-</sup> were the primary  
472 population affected by depletion. The CD8 $\alpha$ <sup>+</sup> NK subsets are sources of IL-17 and TNF in the  
473 airway after IV BCG vaccination, cytokines that have been implicated in control of Mtb infection  
474 (52, 53). IL-17 produced specifically by innate lymphocytes has been implicated in pro-  
475 inflammatory responses in the lung and mucosal immunity against pathogenic bacteria (54, 55).  
476 CD8 $\alpha$ <sup>-</sup>CD16<sup>-</sup>NKG2A<sup>+</sup> cells, which produce less IL-17 and TNF, filled the void left by the loss  
477 of CD8 $\alpha$ <sup>+</sup> NK cells after depletion. NK cells may also be responsible for priming adaptive T cell  
478 responses during IV BCG vaccination. Another possibility to consider is that unconventional T  
479 cell subsets are playing a role in BCG IV-induced protection, including MAIT (MR-1  
480 restricted)(56), NKT (CD1d restricted)(57), and GEM (CD1b restricted)(58) T cells, all of which  
481 can express CD8 $\alpha$ . It is important to note that there are key differences between species, such as  
482 most MAITs being CD4<sup>-</sup>CD8<sup>-</sup> double negative in mice and CD8 $\alpha$ <sup>+</sup> or CD8 $\alpha$  $\beta$ <sup>+</sup> in humans and  
483 non-human primates. An early expansion of MAITs was observed following IV BCG, suggesting  
484 that their role may be in priming adaptive responses, rather than combating the infection directly.  
485 It should also be noted that we did not have the ability to further characterize CD8 $\alpha$ <sup>+</sup> T cell  
486 populations present in granulomas as MAITs, iNKTs or CD1b-restricted due to low event rates  
487 and therefore cannot determine whether these niche populations could contribute to protective

488 capacity following CD8 $\beta$  depletion. At this time, depletion of specific innate-like or  
489 unconventional T cell subsets in macaques is not feasible due to lack of appropriate reagents.

490

491 Both CD4 and CD8 $\alpha$  groups developed a small number of lesions, while still experiencing high  
492 bacterial burden. Thus, the infection establishment bottleneck in the airways was not greatly  
493 affected by CD4 or CD8 $\alpha$  depletion, although the few Mtb that entered the lung parenchyma were  
494 uncontrolled in the absence of CD4 T cells or CD8 $\alpha$ + lymphocytes and disseminated within the  
495 lung and to the lymph nodes. We posit that five months of IV BCG-induced T cells in airways  
496 interacting with and activating macrophages in the presence of high levels of antigen prior to  
497 depletion is critical in priming a robust first line of defense. Single cell RNAseq analysis and  
498 murine studies support a model of macrophage training that is T cell dependent and contributes to  
499 limiting establishment of infection (59, 60). It is possible that there is redundancy in the activation  
500 of macrophages by these lymphocyte subsets, such that removal of one subset did not affect the  
501 bottleneck for infection.

502

503 Limitations to this study include our inability to determine which types of innate-like CD8+  
504 lymphocytes contribute to IV BCG protection, due to the lack of reagents for in vivo selective  
505 depletion of specific cell types. In addition, the small number of granulomas obtained from the  
506 CD8 $\beta$ -depleted group limited our analysis of these samples. Finally, the level of protection due to  
507 high dose IV BCG in our previous experiments was extremely high, with 90% protection (<100  
508 Mtb CFU/animal)(18, 19). In this study, we used the same strain and cryopreserved culture stock  
509 of BCG (SSI), and although there was still a 10,000 fold reduction in Mtb burden compared to  
510 unvaccinated animals in the undepleted BCG IV group, we did not obtain the same overall level

511 of sterilizing immunity that we saw previously. The reasons for this are unclear, but might be as  
512 simple as statistical variation, as the first study had only 10 NHP per group and rhesus macaques  
513 are an outbred animal model.

514  
515 In conclusion, these results support multiple, non-redundant cell-mediated mechanisms of  
516 protection involved in protection against TB provided by IV BCG. Further work must still be done  
517 to elucidate the critical CD8 $\alpha\alpha$ + subset(s) that are complementary to CD4 T cells in conferring  
518 robust protection by IV BCG vaccination. The findings here may be relevant for defining  
519 successful vaccine response metrics in future candidate screening. Canonical CD4 T cell responses  
520 are important in combating Mtb, as we and others have found, but they are independently incapable  
521 of effective control in the absence of their innate-like CD8+ counterparts, at least with IV BCG.  
522 This model system provides significant insight into key cellular mechanisms that can be used to  
523 evaluate future candidates.

524

## 525 **Materials and Methods**

### 526 Animals and Handling

527 This research involved the use of Indian-origin rhesus macaques (*Macaca mulatta*), between 3 and  
528 7 years of age. Cohort 1 (n = 40) was split evenly between males and females (n = 20 each), while  
529 Cohort 2 (n = 32) was all males due to limited availability. All experimental procedures involving  
530 care of animals complied with ethical regulations at the respective institutions (Animal Care and  
531 Use Committees of the Vaccine Research Center, NIAID, NIH, and of Bioqual, Inc., and of the  
532 University of Pittsburgh School of Medicine Institutional Animal Care and Use Committee).  
533 Macaques were housed and cared for in accordance with local, state, federal, and institute policies



534 in facilities accredited by the American Association for Accreditation of Laboratory Animal Care  
535 (AAALAC), under standards established in the Animal Welfare Act and the Guide for the Care  
536 and Use of Laboratory Animals as mandated by the U.S. Public Health Service Policy. Macaques  
537 were monitored for physical health, food consumption, body weight, temperature, complete blood  
538 counts, and serum chemistries. Vaccinations and pre-challenge blood draws and BALs were  
539 performed at the NIH and Bioqual. Animals were transported to University of Pittsburgh for Mtb  
540 challenge and analyses. All Mtb infections were performed a biosafety level 3 facility. Veterinary  
541 staff regularly monitored clinical signs following challenge, including appetite, behaviour and  
542 activity, weight, erythrocyte sedimentation rate, Mtb growth from gastric aspirate and coughing.  
543 Input from these examinations, along with serial PET-CT imaging, were used to determine whether  
544 a macaque met criteria for the humane end point before the pre-determined study end point of 8  
545 weeks post-infection.

546

#### 547 Sample size and statistics

548 The sample size for this study was determined using bacterial burden (measured as log<sub>10</sub>-  
549 transformed total thoracic CFUs) as the primary outcome variable. Comparisons included in the  
550 analysis were between the undepleted vaccinated controls (IgG/Saline) and each depletion group  
551 (anti-CD4, anti-CD8 $\alpha$ , and anti-CD8 $\beta$ ). A standard deviation of 1.8 was conservatively estimated  
552 from prior studies using BCG vaccination (18, 19). Using this standard deviation, the group sizes  
553 provide power to detect a mean difference of 2.5 log for two-sided t tests between un-depleted  
554 immunized controls and CD4-depleted animals (87.3%), CD8 $\alpha$ -depleted animals (92.9%) and  
555 CD8 $\beta$ -depleted animals (87.3%) with an  $\alpha=0.05/3=0.0167$  to account for 3 pairwise  
556 comparisons. Unvaccinated infection controls were included in each challenge cohort to ensure

557 Mtb infection was achieved, but these animals were not included in statistical analyses. Depletion  
558 groups were compared to vaccinated IgG/Saline controls using the Kruskal-Wallis test, with  
559 Dunn's multiple comparison adjusted p-values shown. All statistical tests were run in GraphPad  
560 Prism for macOS (version 10.1.1). For numbers of granulomas found at necropsy and counted on  
561 PET CT, areas of TB pneumonia and consolidations were considered to be too numerous to count  
562 (TNTC) and numerically represented as 100. For all graphs with zero values that were log<sub>10</sub>-  
563 transformed, the number 1 was added to the entire data set.

564

#### 565 Vaccination

566 Macaques were randomized into vaccinated (n=65) and unvaccinated (n=7) groups based on age,  
567 weight and gender. The macaques were vaccinated under sedation. Cryopreserved aliquots of BCG  
568 Danish Strain 1331 (Statens Serum Institute, Copenhagen, Denmark) were thawed immediately  
569 before vaccination and diluted in cold PBS containing 0.05% tyloxapol (Sigma-Aldrich) to a target  
570 dose of  $5 \times 10^7$  CFU. The solution was delivered intravenously into the saphenous vein in a volume  
571 of 2 mL. Actual BCG doses were quantified by dilution-plating and are reported in **table S1**.

572

#### 573 Lymphocyte depletion

574 Vaccinated macaques were randomly assigned to four depletion groups. IgG (n = 6) and Saline (n  
575 = 12) served as a combined undepleted control group, with anti-CD4 (n = 16), anti-CD8 $\alpha$  (n = 17),  
576 and anti-CD8 $\beta$  (n = 14) as experimental groups. The Anti-CD4 [CD4R1] antibody (NIH  
577 Nonhuman Primate Reagent Resource Cat#PR-0407,RRID:AB\_2716322), Anti-CD8 alpha  
578 [MT807R1] antibody (NIH Nonhuman Primate Reagent Resource Cat#PR-  
579 0817,RRID:AB\_2716320), and Anti-CD8 beta [CD8b255R1] antibody (NIH Nonhuman Primate

580 Reagent Resource Cat# PR-2557, RRID:AB\_2716321) were engineered and produced by the  
581 Nonhuman Primate Reagent Resource. Starting 5 months post vaccination and 4 weeks prior to  
582 Mtb challenge, the macaques were administered depletion antibodies at 50 mg/kg/dose  
583 intravenously every two weeks, continuing through Mtb challenge until necropsy, as previously  
584 described (23, 26, 61). Cohort 1 did not include any macaques assigned to CD8 $\beta$  depletion. Cohort  
585 2 consisted of primarily CD8 $\beta$  depleted animals, with an additional 4 animals in each of the other  
586 groups as internal controls for any cohort effect (**table S1**).

587

### 588 Infection

589 Examination of animals was performed in quarantine upon arrival at the University of Pittsburgh  
590 to assess physical health and confirm no previous *M. tuberculosis* infection was detected via  
591 ELISpot assays.

592 All macaques were challenged via bronchoscopic instillation with 5-39 CFU of barcoded Mtb  
593 (Erdman), as previously described (**table S1**) (15, 16). This range of doses has resulted in  
594 comparable levels of progressive TB in unvaccinated rhesus macaques in this and previous studies  
595 (17, 18). Infection groups included animals from multiple experimental groups to reduce dose bias.

596

### 597 PBMC, bronchoalveolar lavage and lymph node biopsy processing

598 PBMCs were isolated from whole blood draws using Ficoll-Paque PLUS gradient separation, as  
599 previously described (9). Bronchoalveolar lavage (BAL) samples were centrifuged and the  
600 supernatant was collected and frozen. Remaining cells were resuspended in warm warm R10  
601 (RPMI 1640 with 2 mM L-glutamine, 100 U ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin, and 10%  
602 heat-inactivated FBS). Biopsied LNs were mechanically disrupted and filtered through a 70  $\mu$ m

603 cell strainer. Single-cell suspensions were resuspended in warm R10 to be counted and for use in  
604 same-day assays. If necessary, cells were cryopreserved in FBS containing 10% DMSO between  
605  $5 \times 10^6$  and  $2 \times 10^7$  cells mL<sup>-1</sup>.

606

#### 607 Electrochemiluminescent Antibody ELISA

608 IgG, IgM, and IgA were batch-analyzed from cryopreserved 10x concentrated BAL (in PBS) or  
609 plasma. A 384-well MA6000 high binding plate (MSD L21XB) was washed with PBS/Tween 20  
610 (0.05%) and coated with 5-10µg/ml H37Rv Mtb whole cell lysate (WCL) at 4C overnight. All  
611 reagents were brought to room temperature (RT) and plates were washed prior to blocking with  
612 MSD blocking buffer A (5% BSA) for 1h at RT (shaking,900rpm) and then washed. Samples were  
613 diluted (1:20 and then serially 1:5 for a total of 7) in diluent 100 (MSD), added to plates and  
614 incubated with shaking (900rpm) for 2h at RT, then washed. MSD-Sulfo-Tag-Conjugated anti-  
615 hu/NHP secondary antibodies (0.5µg/mL; IgG cat#D20JL-6, IgA cat#D20JJ-6, IgM cat#D20JM-  
616 6) for 1h at RT (900rpm) and washed. Gold Read Buffer A (HB) was added, plates were run on  
617 the MSD Meso Sector S 600MM reader and analyzed using Methodical Mind® software v2.0.5.  
618 AUC (ECL, arbitrary units) of individual binding curves for each sample were calculated in Prism  
619 v10.1.1.

620

#### 621 ELISpot assays

622 IFN $\gamma$  ELISpot assays were performed, as previously described, after vaccination prior to depletion,  
623 following initiation of depletion infusions before Mtb challenge, and at necropsy (18).  
624 Hydrophobic high protein binding membranes 96-well plates (Millipore Sigma) were hydrated  
625 with 40% ethanol, washed with sterile water, and coated with anti-human/monkey IFN $\gamma$  antibody

626 (15  $\mu\text{g ml}^{-1}$ , MT126L, MabTech) overnight at 4°C. Plates were washed with PBS and blocked  
627 with R10 media for 2 hours at 37°C with 5% CO<sub>2</sub>.  $2 \times 10^5$  PBMCs per well were incubated in R10  
628 media only, or containing 1  $\mu\text{g mL}^{-1}$  each of ESAT-6 or CFP-10 peptide pools (BEI Resources)  
629 for 40–48 hours. To develop, plates were washed with PBS and biotinylated anti-human IFN $\gamma$   
630 antibody (2.5  $\mu\text{g mL}^{-1}$ , 7-B6-1, MabTech) was added for 2 hours at 37°C. After washing,  
631 streptavidin-HRP (1:100, MabTech) was added for 45 min at 37°C. Spots were stained using AEC  
632 peroxidase (Vector Laboratories, Inc.) and counted on a plate reader. Assays were performed using  
633 frozen PBMCs thawed 1 day prior to the assay start and rested in R10 media at 37°C overnight.  
634 Assays were validated using PDBU and ionomycin (P+I) as a positive control stimulation  
635 condition, guaranteeing cellular functionality after a freeze/thaw cycle. If no spot formation was  
636 observed with P+I stimulation, the assay was repeated with a different frozen vial. Results at given  
637 timepoints are not reported from animals for which the assay was never successful.

638

### 639 Necropsy procedures and tissue processing

640 Macaques were euthanized by sodium pentobarbital injection and necropsied between 7 and 11  
641 weeks post-challenge, as previously described (18, 28, 32, 62). Gross pathology observed in lungs  
642 (including number and size of granulomas, consolidations, and pneumonias), LNs (including  
643 swelling, lesions, and necrosis) and extrapulmonary compartments (such as peripheral LNs,  
644 spleen, liver and gastrointestinal tract) was quantified using a published scoring system (16, 17).  
645 Individual lesions were indentified by PET-CT mapping before excision. These tissues, along with  
646 regions of uninvolved lung tissue from each of the seven lobes, were excised and homogenized  
647 into single cell suspensions for CFU quantification, barcoding analysis, single cell RNA  
648 sequencing and various immune assays. Homogenization was accomplished by mechanical

649 disruption and passing the suspension through a 70 µm cell strainer. Lung lobes and other tissues  
650 designated for scRNAseq were subjected to enzymatic dissociation (GentleMACS; Miltenyi).  
651 Sections of lung lobes, thoracic LNs and large granulomas (>2 mm in diameter) were also  
652 processed for formalin fixation and paraffin embedding for histological analysis.

653

#### 654 Bacterial burden quantification

655 Colony forming units (CFU) were calculated from plating serial dilutions of homogenized tissues  
656 excised during necropsy on 7H11 agar plates, as previously described. Quantification of bacterial  
657 burden was performed as previously described (16). Plates were incubated for 21 days at 37°C in  
658 5% CO<sub>2</sub> before CFU counting.

659

#### 660 Mtb CFU and barcode determination

661 To track the establishment and dissemination of Mtb, barcoded Mtb Erdman was used for infection  
662 and barcodes were determined after necropsy as previously described (28). A library of digitally  
663 barcoded plasmids containing 7-mer barcodes and adjacent 74-mer library identifier sequences  
664 was stably transformed into the bacterial chromosome of Mtb Erdman. Each library contained  
665 ~16,000 unique barcodes and three independently generated libraries were combined into a master  
666 library to increase barcode diversity, thereby ensuring a <2% chance that a barcode would be  
667 represented twice if 20 bacteria were randomly selected. Mtb colonies on plates from necropsy  
668 tissues were DNA extracted using phenol–chloroform methods. After DNA purification, samples  
669 were subjected to amplicon-based sequencing to identify all the barcode tags present and shared  
670 across tissues. Q-tags and barcodes were identified and quantified as previously described (26).  
671 Distribution of barcodes in CFU+ tissues is shown in **data file S1**.

672

673 Flow cytometry

674 Flow cytometry was performed on BALs prior to Mtb challenge, PBMCs throughout the study,  
675 and a subset of lung lobes, LNs (peripheral and thoracic) and granulomas processed at necropsy.  
676 Additionally, cryopreserved PBMCs were processed as a batch. Immediately after processing, up  
677 to  $5 \times 10^6$  cells from single cell suspensions allocated for intracellular cytokine staining were  
678 stimulated by incubation with R10 media only,  $20 \mu\text{g ml}^{-1}$  H37Rv Mtb whole cell lysate (WCL),  
679 or  $1 \mu\text{g ml}^{-1}$  each of ESAT-6 and CFP-10 peptide pools for 2 hours at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Necropsy  
680 tissue samples were then incubated with  $10 \mu\text{g ml}^{-1}$  BD GolgiPlug (BD Biosciences) overnight for  
681 up to 12 hours. BAL and PBMC samples were incubated with either GolgiPlug or 1:500  
682 eBioscience Protein Transport Inhibitor Cocktail (500X) (Life tech) for 6-12 hours. After  
683 stimulation and blocking, samples were stained with a fixable viability dye, followed by antibody  
684 panels for surface markers and intracellular antigens using standard protocols (18). Ten PBMC  
685 samples spanning all groups, including unvaccinated were not stained with viability dye at the 10  
686 week timepoint. Staining panels are detailed in **table S2**. We confirmed our CD8 $\beta$  flow cytometry  
687 antibody was not being blocked by the depletion antibody, which would skew our ability to detect  
688 and characterize remaining cells (**fig. S8**). Cytometry was performed on a 5 laser Aurora with  
689 SpectroFlo software (16UV-16V-14B-10YG-8R; Cytex) and a LSR Fortessa X-50 Cell Analyzer  
690 with DiVA software. Analysis of flow cytometry data was performed in FlowJo (v10.9.0), with  
691 gating strategy shown in **figs. S9-12**. Unless otherwise noted, T cell subsets are  $\gamma\delta\text{TCR}$ -negative.  
692 NK cells in BAL and necropsy tissues are defined as CD20-CD3- cells that are CD8 $\alpha$ +,  
693 CD16(Fc $\gamma$ RIIIa)+ and/or NKG2A(CD159a)+. Samples with fewer than 100 total lymphocyte  
694 events were excluded from associated frequency analysis to avoid skewing, but still included for

695 cell count. For analysis of specific cell types (e.g. cytokine production by CD4 T cells), samples  
696 with fewer than 50 events in parent cell type gate (e.g. CD4 T cells) were excluded for frequency  
697 calculations but included for cell counts.

698

#### 699 PET-CT scanning

700 We obtained PET-CT images with a Mediso MultiScan LFER 150 integrated preclinical PET CT  
701 (63). Scans were obtained following *Mtb* infection at 4 and 8 weeks. Prior to each scan, animals  
702 were weighed and injected with a roughly mCi/kg dose of PET tracer 2-deoxy-2-(<sup>18</sup>F)Fluoro-D-  
703 glucose (FDG). FDG is a glucose analog that collects non-specifically in metabolically active  
704 tissue, tracking overall inflammation (27, 64). Prior to obtaining PET scans, an uptake period of  
705 50 minutes was observed to allow the FDG to be taken up by active tissue. In the intervening time,  
706 animals were intubated, and anesthetized. CT scans were obtained during the uptake period.  
707 During CT acquisition (about 40 seconds in duration), the animal's breath was held via a  
708 mechanical ventilator. This step ensures a clear CT image of the lungs.

709

710 All imaging was performed according to biosafety and radiation safety requirements within the  
711 Biosafety Level 3 (BSL3) facility at the University of Pittsburgh. Scans were analyzed using  
712 OsiriX DICOM viewer (65) by in-house trained PET-CT analysts (29, 66, 67).

713

#### 714 PET-CT analysis

715 Measurements described below were detailed previously (29). "Total Lung FDG Activity  
716 (SUV<sub>R</sub>)" is calculated as the sum of all PET signal contained in the lungs above a background  
717 threshold of SUV=2.3 and divided by the average PET signal in an area of back muscle directly



718 adjacent to the vertebrae. The division by PET uptake in resting muscle is included to account for  
719 variations in baseline metabolic activity between animals. “Number of Granulomas” gives the total  
720 number of tuberculosis lesions observed in the lungs via CT. For thoracic LNs observed via PET  
721 to have a peak SUV  $\geq 5$ , “Thoracic LN FDG Activity” is the sum of all the peak PET signal values  
722 measured in each thoracic LN. As in the total lung FDG activity, the summed SUV values are  
723 divided by the average PET signal from resting back muscle. This measurement represents the  
724 total metabolic activity of the thoracic LNs for each animal.

725

## 726 **Supplementary Materials**

727 Figs. S1 to S12

728 Tables S1 to S2

729 Data file S1

730

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749 Formal analysis: AWS, JJZ, ANB, MCC, AJM, CLA, HC, HJB, PM

750 Investigation: AWS, JJZ, ANB, SP, FH, MRC, AJV, MSS, CGW, CLA, RK, BK, LEH, JL, CCL,

751 MK, JT, RD, EK, PLL

752 Visualization: AWS, ANB, MCC, CLA, LEH, HJB, PM, PAD

753 Funding acquisition: JLF, RAS, PAD, MR, SF

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757

758 Competing Interests

759 Authors declare that they have no competing interests.

760

761 Competing Interests

762 Data are available in the main text, supplementary materials or through IMPAc-TB's SEEK

763 database housed at MIT.

764

765

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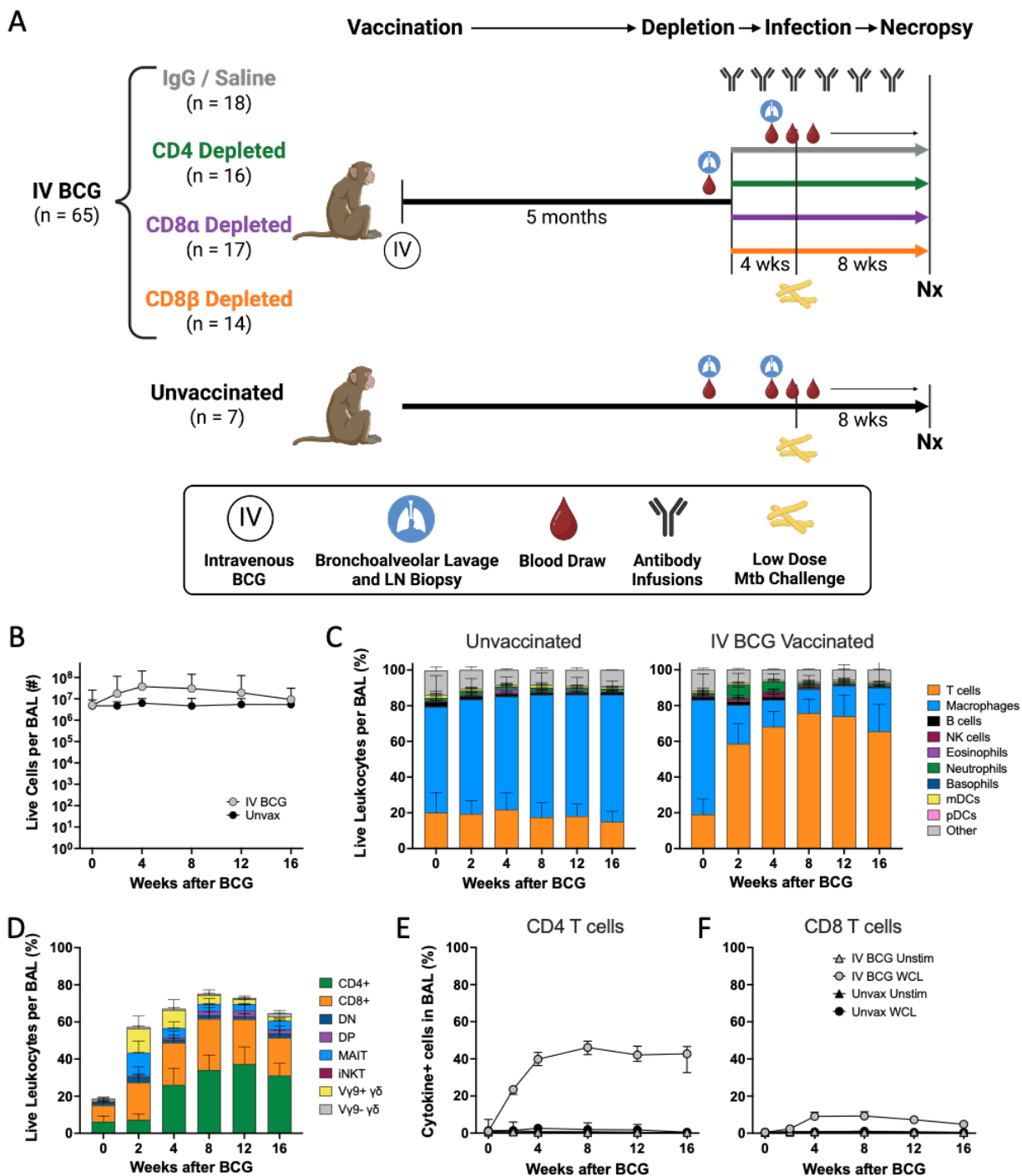
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918  
919



920 Fig. 1



921

922 **Fig. 1. A robust immune response is induced by IV BCG vaccination.** (A) Schematic outlining

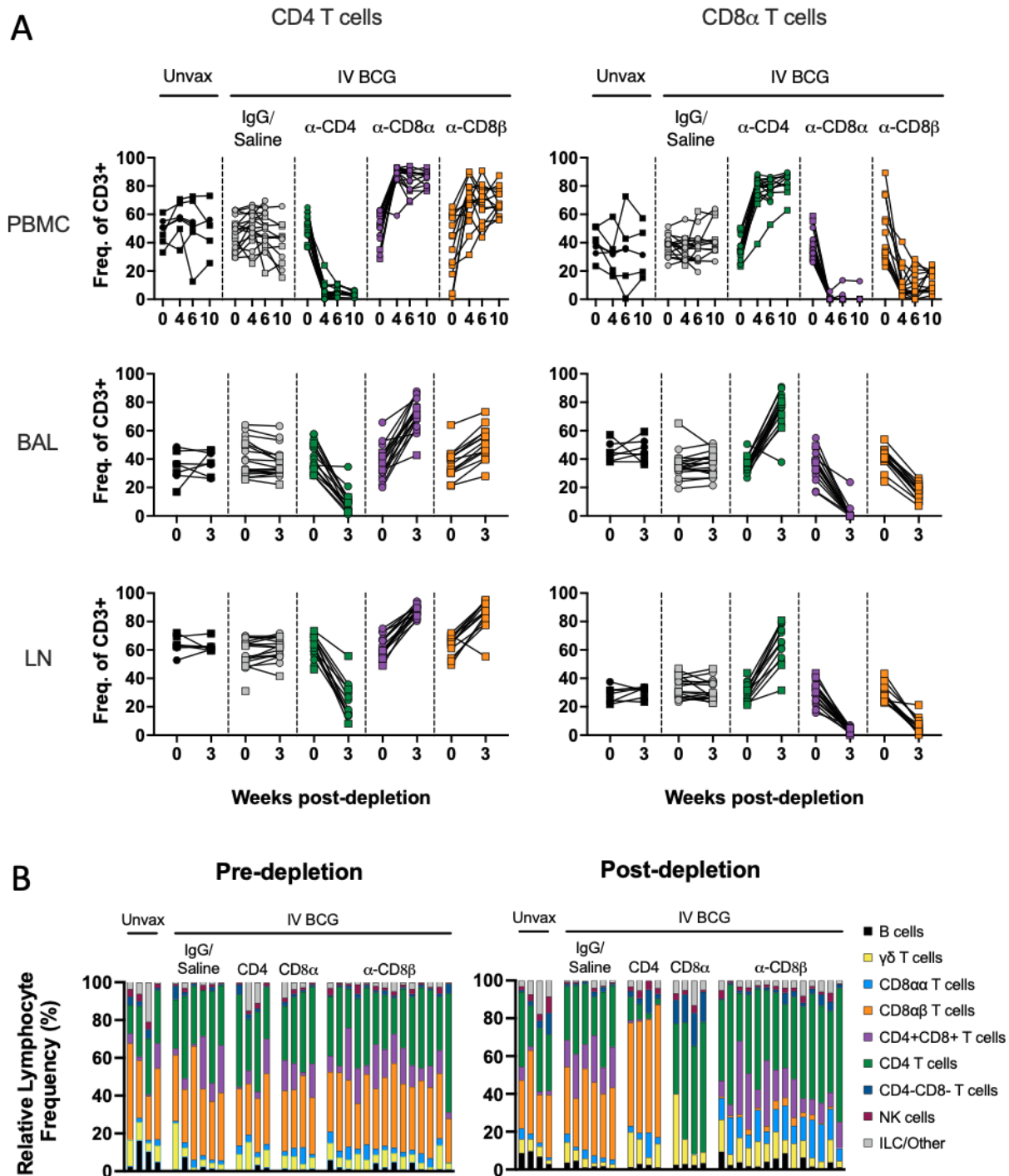
923 general timeline of the study. (B) Number of live cells recovered from BAL over time in IV BCG

924 vaccinated and unvaccinated animals. (C) Relative abundance of cell types in BAL of unvaccinated



925 (*left*) and IV BCG vaccinated (*right*) macaques, reported as frequency of live leukocytes. **(D)**  
926 Relative abundance of T cell subsets in BAL, reported as the frequency of live leukocytes. **(E,F)**  
927 Frequency of antigen specific CD4 (E) and CD8 (F) T cells in BAL producing IFN $\gamma$ , TNF, IL-2,  
928 and/or IL-17 in response to whole cell lysate (WCL) or no stimulation in IV BCG vaccinated or  
929 unvaccinated macaques.  
930

931 **Fig. 2**



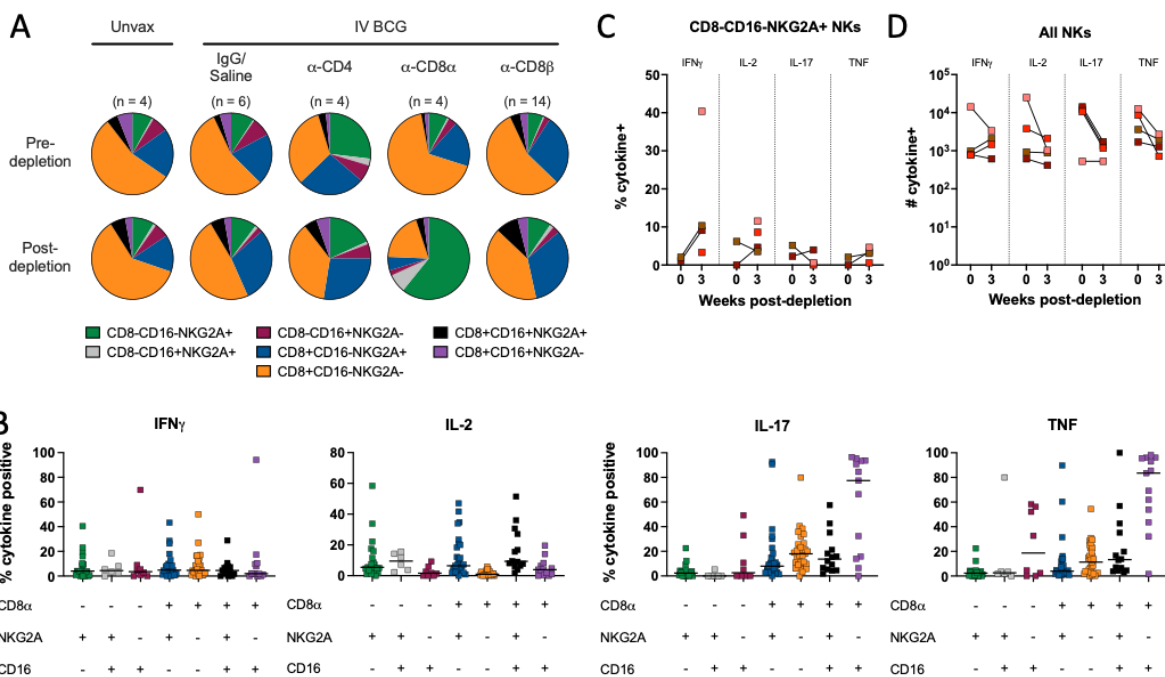
932

933 **Fig. 2. Target cell populations are depleted by antibody infusions in vivo. (A)** Flow cytometry

934 to assess T cell depletion in blood (PBMC, *top*), airway (by BAL, *middle*), and peripheral LNs (by

935 biopsy, *bottom*). For each depletion group, conventional CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD20<sup>-</sup>γδTCR-  
936 CD8α<sup>-</sup>) are shown on the left and CD8α<sup>+</sup> T cells (CD3<sup>+</sup>CD20<sup>-</sup>γδTCR-CD4<sup>-</sup>) are shown on the  
937 right; vaccination status is at the top of the plot and antibody infusion designates depletion group.  
938 Each symbol represents an animal, lines connect each animal across timepoints. **(B)** Population  
939 composition in BAL, shown as a relative frequency of lymphocytes. Pre-depletion is shown of the  
940 left, post-depletion is shown on the right. Each bar represents an animal. Only animals in the  
941 second cohort are included, as anti-CD20 and anti-CD8β antibodies were not included in the flow  
942 cytometry panels for the first cohort.  
943

944 **Fig. 3**



945

946 **Fig. 3. CD8 $\alpha$  depletion shifts phenotypic and functional profile of natural killer cells in the**

947 **airway. (A)** Relative frequency of NK (CD3 negative) subtypes in BAL pre- (*top*) and post-

948 depletion (*bottom*). **(B)** Cytokine (IFN $\gamma$ , IL-2, IL-17, TNF) production by NK subtype using

949 CD8 $\alpha$ , NKG2A and CD16 markers in BAL of animals pre-depletion, represented as the frequency

950 of cytokine positive NK cells by flow cytometry. Each symbol represents an animal, lines represent

951 group median. If subtype was below event count threshold by flow cytometry, that animal will not

952 appear as a symbol for that specific cell type. Subtypes are same colors in panels A and B. **(C)**

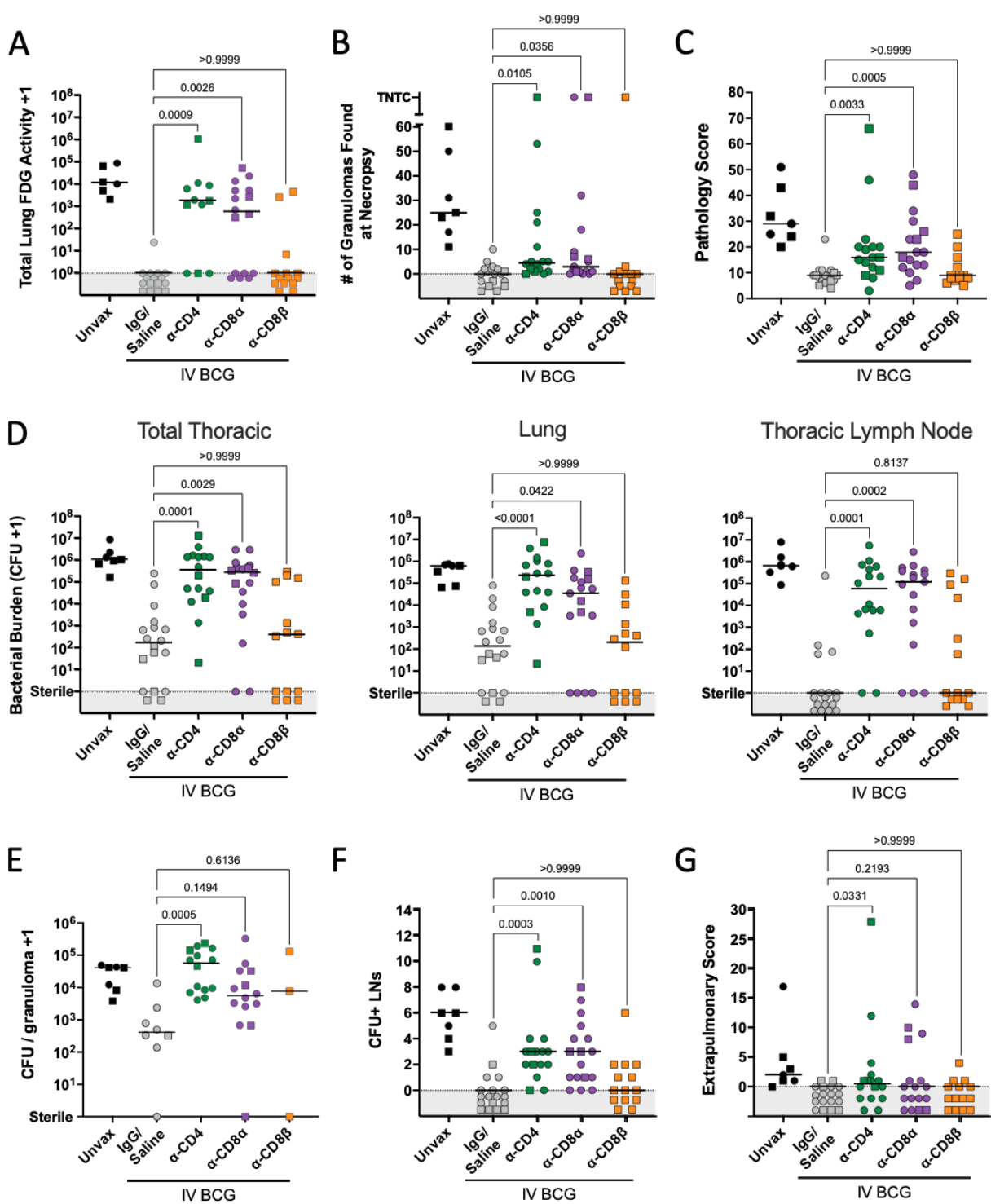
953 Frequency of cytokine-positive NKG2A single positive NK cells (CD8 $\alpha$ -CD16-) before and after

954 CD8 $\alpha$  depletion. **(D)** Number of NK cells expressing IFN $\gamma$ , IL-2, IL-17 or TNF before and after

955 CD8 $\alpha$  depletion. Each symbol in panels C and D represents an animal.

956

957 Fig. 4



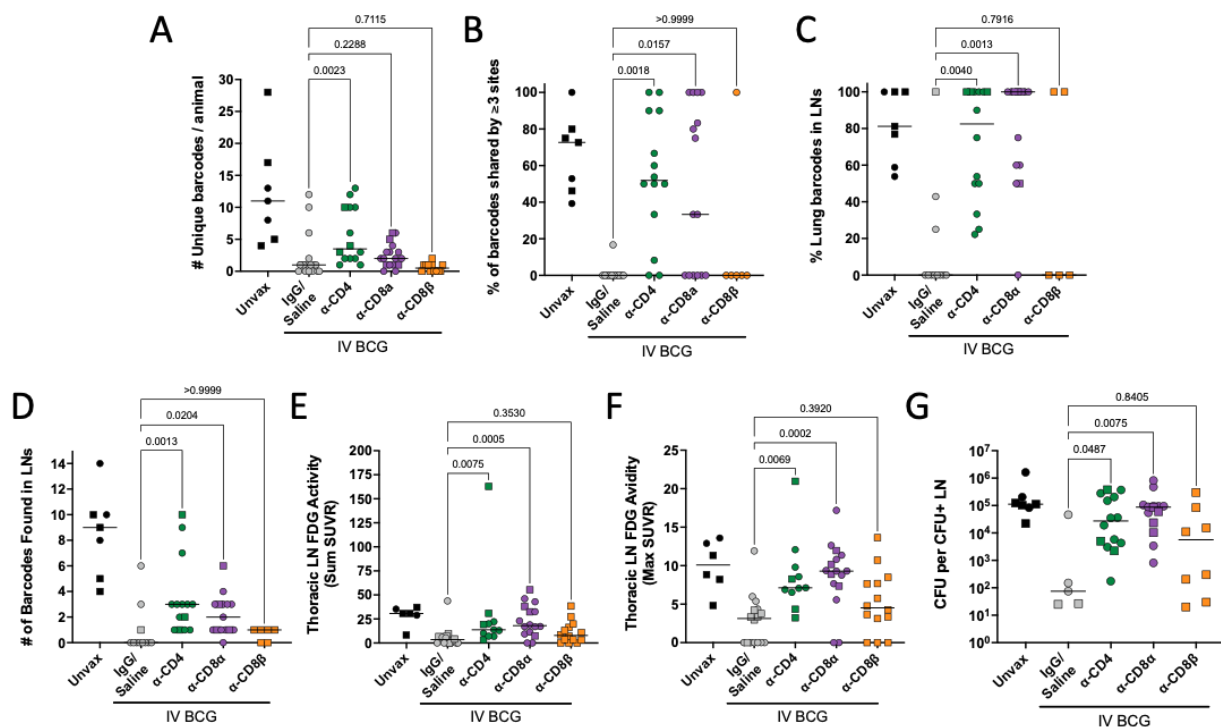
958

959 **Fig. 4. CD4 and CD8 $\alpha$  depletion lead to increased disease and bacterial burden (A) Total lung**

960 **FDG activity at necropsy. Animals with missing PET scans were not included. Unvax: n = 6;**

961 IgG/Saline: n = 16;  $\alpha$ -CD4: n = 11;  $\alpha$ -CD8 $\alpha$ : n = 16;  $\alpha$ -CD8 $\beta$ : n = 14. **(B)** The number of  
962 granulomas found at necropsy. TB pneumonia and consolidations are represented as too numerous  
963 to count (TNTC). **(C)** Gross pathology score, as described in Ref. (17). **(D)** Bacterial burden (CFU)  
964 of all thoracic tissues (*left*), lung (*middle*) and thoracic LNs (*right*). **(E)** The bacterial burden per  
965 granuloma. Symbols represent a mean of all granulomas within an animal, and animals with no  
966 granulomas are not included. **(F)** The number of CFU+ thoracic LNs per animal. **(G)** The  
967 extrapulmonary score, as described in Ref. (17). For all panels, each symbol represents an animal,  
968 line represents group median; circles represent cohort 1, squares represent cohort 2. All symbols  
969 in gray region are of equal value (0 or sterile) and were spread for better visualization. Groups  
970 were compared using the Kruskal-Wallis test, with Dunn's multiple comparison adjusted p-values  
971 shown, comparing IgG/Saline group against each depletion group.  
972

973 **Fig. 5**



974

975 **Fig. 5. CD4 and CD8 $\alpha$  depletion results in increased dissemination within lung and to lymph**

976 **nodes.** (A) The total number of unique barcodes identified in each animal. Sterile animals are

977 shown as a value of 0. (B) The frequency of barcodes found in three or more sites, regardless of

978 tissue. (C) The frequency of barcodes found in lung tissue or granulomas that were also identified

979 in thoracic LNs. (D) The number of barcodes found in any thoracic LN. Sterile animals are not

980 included in panels B-D. (E) The total FDG activity in thoracic LNs as a sum of all measured LNs.

981 (F) The single thoracic lymph node with maximum FDG avidity per animal. In panels E and F, if

982 no LNs were distinguishable on the scan, a 0 is reported; animals missing PET scans were not

983 included. (G) CFU per non-sterile thoracic LN showing the mean of all CFU+ LNs in an animal.

984 All groups (excluding unvaccinated animals) were compared using the Kruskal-Wallis test with

985 Dunn's multiple comparison adjusted p-values reported, comparing between IgG/Saline group and

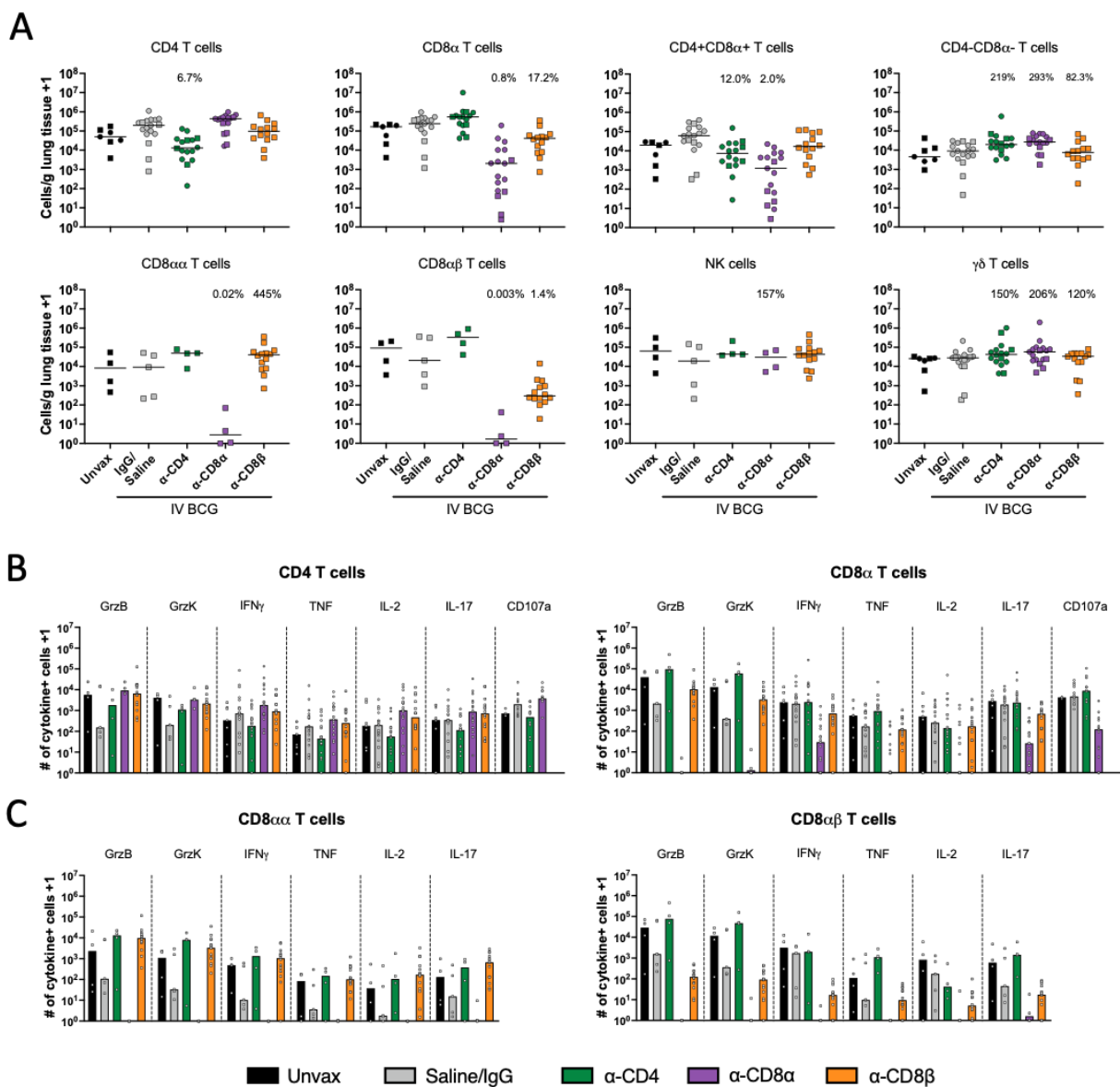
986 each depletion group. Symbols represent an animal, line represents median of group. Circles

987 represent cohort 1, squares represent cohort 2.

988



989 **Fig. 6**



990

991 **Fig. 6. Functional lymphocytes are depleted in lung tissue. (A)** Number of each lymphocyte

992 subset per gram of lung tissue, characterized by flow cytometry. Each symbol represents an animal.

993 Percentages shown in each plot represent population size relative to IgG/Saline group, calculated

994 by group median (line). Only cohort 2 was included for CD8 $\alpha\alpha$ , CD8 $\alpha\beta$  and NK cell

995 quantification, as anti-CD20 and anti-CD8 $\beta$  antibodies were not included in the flow cytometry

996 panels in cohort 1. **(B)** Number of cytokine producing CD4 and CD8 $\alpha$ + T cells in lung tissue. **(C)**

997 CD8 $\alpha$ + T cell responses categorized by CD8 $\alpha\alpha$  and CD8 $\alpha\beta$  T cells as subsets of the CD8 $\alpha$  T  
998 cells in B. Granzymes B and K were only analyzed in cohort 2. CD107a was only analyzed in  
999 cohort 1. Each symbol represents a mean per animal of 2-4 lung lobes sampled, bar represents  
1000 group median. Circles represent cohort 1, squares represent cohort 2.  
1001