# A "suicide" BCG strain provides enhanced immunogenicity and robust protection against *Mycobacterium tuberculosis* in macaques

Authors

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**Abstract** (Limit: 150 words. Current: 150)

Intravenous (IV) BCG delivery provides robust protection against *Mycobacterium tuberculosis* (Mtb) in macaques but poses safety challenges. Here, we constructed two BCG strains (BCG-TetON-DL and BCG-TetOFF-DL) in which tetracyclines regulate two phage lysin operons. Once the lysins are expressed, these strains are cleared in immunocompetent and immunocompromised mice, yet induced similar immune responses and provided similar protection against Mtb challenge as wild type BCG. Lysin induction resulted in release of intracellular BCG antigens and enhanced cytokine production by macrophages. In macaques, cessation of doxycycline administration resulted in rapid elimination of BCG-TetOFF-DL. However, IV BCG-TetOFF-DL induced increased pulmonary CD4 T cell responses compared to WT BCG and provided robust protection against Mtb challenge, with sterilizing immunity in 6 of 8 macaques, compared to 2 of 8 macaques immunized with WT BCG. Thus, a

33 “suicide” BCG strain provides an additional measure of safety when delivered intravenously and robust protection
34 against Mtb infection.

35 **Introduction**

36 *Mycobacterium bovis* BCG, also known as Bacille Calmette-Guérin or BCG, is a live *Mycobacterium bovis* strain
37 that was attenuated by serial passaging in vitro and remains the only tuberculosis (TB) vaccine approved for use
38 in humans. BCG protects children against miliary TB and TB meningitis but is only partially protective against
39 pulmonary TB (Trunz, Fine and Dye, 2006). Typically, BCG is delivered by intradermal injection, but studies in
40 non-human primates revealed that endobronchial instillation and high-dose intravenous administration result in
41 improved protection against *M. tuberculosis* (Mtb) infection (Dijkman *et al.*, 2019; Darrah *et al.*, 2020). Mucosal
42 and intravenous delivery of BCG pose the risk of developing disseminated BCGosis, with potentially fatal
43 outcomes in immunocompromised individuals (Yamazaki-Nakashimada *et al.*, 2020), such as HIV infected
44 children (Hesseling *et al.*, 2006), children with Mendelian susceptibility to mycobacterial disease (MSMD)
45 (Jouanguy *et al.*, 1996) and patients with chronic granulomatous disease (Conti *et al.*, 2016). Furthermore,
46 intravesical BCG therapy is the most successful immunotherapy for bladder cancer but is also associated with
47 severe and possibly fatal complications in approximately 5% - 8% of patients (Alexandroff *et al.*, 1999; Gonzalez
48 *et al.*, 2003; Kawai *et al.*, 2013; Liu *et al.*, 2019).
49
50 We sought to construct a BCG strain that can be killed by addition or removal of a small molecule, such as a
51 tetracycline (Ehrt *et al.*, 2005; Klotzsche, Ehrt and Schnappinger, 2009; Kim *et al.*, 2013) and would be safer
52 than BCG so that it could be used with non-conventional delivery approaches and increased dosage.
53 Mycobacteriophages use lytic enzymes to kill their host cells including holin proteins that permeabilize the
54 cytoplasmic membrane, endolysins that cleave peptidoglycan and lipid hydrolases/esterases that degrade the
55 outer membrane (Catalão and Pimentel, 2018). We took advantage of the lysin operons from the
56 mycobacteriophages D29 and L5 (Ford *et al.*, 1998) to construct regulated kill switches for BCG. We
57 hypothesized that killing by inducing cell lysis may make BCG more immunogenic due to the release of
58 intracellular antigens. In a previous study 5 x $10^7$ CFU of BCG (SSI strain) were delivered intravenously to rhesus
59 macaques which resulted in substantial (~10,000 fold) reduction of live Mtb in the animals by 4 weeks, with 6 of
60 10 macaques showing sterile protection (Darrah *et al.*, 2020). Here, we assessed whether doxycycline-controlled

61 lysins can kill BCG (Pasteur strain) in mice and macaques. We evaluated the level of persistence of BCG kill
62 switch strains and the induced immune responses, and whether these strains administered intravenously could
63 provide protection against Mtb challenge in mice and macaques.

64 **Results**

65 **Construction and in vitro characterization of BCG kill switch strains**

66 We evaluated the lysin operons of the mycobacteriophages L5 (L5L) and D29 (D29L) to construct BCG strains
67 that could be efficiently killed by adding or removing tetracyclines such as anhydrotetracycline (atc) or
68 doxycycline (doxy). We first tested functionality of these lysin operons in BCG using a TetON expression system
69 (Ehrt *et al.*, 2005). Lysin transcription is repressed by a wild-type (WT) tetracycline repressor (TetR) that binds
70 to a WT tetO positioned between the -10 and -35 elements of the lysin promoter. Addition of atc/doxy inactivates
71 TetR and turns on lysin expression (**Fig. S1a**). As expected, atc addition prevented growth of BCG strains that
72 carried TetON-D29L or TetON-L5L integrated into the chromosome (**Fig1a, Fig. S1b**). The optical densities (OD)
73 of BCG cultures carrying both TetON-D29L and TetON-L5L (BCG TetON-DL) rapidly declined when atc was
74 added indicating that death occurred by cell lysis (**Fig. 1b, Fig. S2a**). The impact of atc on OD was similar
75 regardless at what time after inoculation it was added. Cytosolic enolase and proteasome subunit B accumulated
76 in the culture filtrate of BCG TetON-DL following treatment with atc, which demonstrated that atc-induced lysin
77 expression indeed resulted in bacterial lysis (**Fig. S2b**). The combined induction of two lysins reduced the fraction
78 of escape mutants by almost two orders of magnitude compared to those observed with single lysin strains (**Fig.**
79 **S2c**).
80 To test whether L5L/D29L could also be used to generate atc/doxy-addicted BCG, which would require a
81 tetracycline to grow, we cloned the lysin operons into two expression systems that are both silenced by atc/doxy.
82 L5L was cloned into a TetPipOFF system (Boldrin *et al.*, 2010), in which WT TetR represses a second
83 transcriptional repressor, PipR, that in turn represses L5L. Here, atc inactivates TetR, which turns lysin
84 expression off via production of PipR. D29L was cloned into an expression system controlled by a reverse TetR
85 (revTetR), which requires atc/doxy to efficiently bind to its operator DNA ($tetO_{4C5G}$) (Kim *et al.*, 2013).
86 Heterodimerization of TetR and revTetR produced in the same cell was prevented by using single-chain versions
87 of each of these repressors (Krueger *et al.*, 2003). Both BCG-TetOFF lysin strains were killed when atc was

88 removed from the cultures (**Fig. 1c, Fig S2d**). The onset of killing was delayed compared to the TetON strains,
89 which is likely due to the time required to dilute intracellular atc from the bacteria. Death of the BCG-TetOFF
90 strains was accompanied by cell lysis (**Fig. S2b**), and expression of two lysins led to enhanced killing and
91 reduced the fraction of escape mutants (**Fig. S2e**). Analysis of the dual TetOFF lysin strain, BCG-TetOFF-DL,
92 with fluctuation assays detected ~2 x $10^{-9}$ escape mutants per cell division (**Fig. 1d**).

93 **Lysin induction in intracellular BCG promotes proinflammatory cytokine production**

94 We infected bone marrow derived mouse macrophages (BMDM) with BCG and BCG-TetON-DL and treated
95 them with rifampin (RIF) or atc (**Fig. 1e**). RIF killed both intracellular BCG and BCG-TetON-DL effectively. Atc
96 had no effect on BCG but killed BCG-TetON-DL presumably via lysin induction. BCG and BCG-TetON-DL both
97 induced TNF, IL12 p40 and IL-6 production by BMDMs. However, cytokine production was significantly increased
98 when killing was mediated by phage lysin expression (**Fig. 1f-h**). These data suggest that intracellular lysis of
99 BCG-TetON-DL enhanced cytokine production compared to that stimulated by live BCG or by BCG and BCG-
100 TetON-DL that had been killed by RIF.

101 **Lysin induction kills BCG in immune competent and immune deficient mice**

102 In C57BL/6 mice BCG-TetON-DL established infection in lungs and spleens and persisted similar to BCG
103 following intravenous injection (**Fig.2 a,b**). When mice were treated with doxy starting seven days post infection,
104 BCG-TetON-DL was killed in lungs and spleen, while doxy had a modest impact on WT BCG. BCG-TetOFF-DL
105 similarly established infection in C57BL/6 mice that received doxy containing chow (**Fig. 2c,d**); its titers declined
106 slowly in the lungs even when mice received doxy, likely due to doxy levels that were insufficient to maintain
107 persistence in the context of host immunity, but remained stable in spleens. When doxy was eliminated from the
108 mouse chow starting 14 days post infection, BCG-TetOFF-DL lost viability in lungs and spleens and was cleared
109 from the lungs within 6 weeks of doxy withdrawal.
110 To assess safety of BCG-TetOFF-DL, we infected immunocompromised SCID mice (**Fig. 2c,d**). In SCID mice
111 that received doxy, BCG-TetOFF-DL replicated in lungs during the 84 week-long infection. In spleens the strain
112 replicated until it reached a titer of ~$10^6$ and then persisted at a 10-fold higher burden than in C57BL/6 mice. In
113 SCID mice that received doxy only for 14 days, BCG-TetOFF-DL was cleared from the lungs although slower
114 than in immunocompetent C57BL/6 mice (**Fig. 2c**). In spleens, BCG-TetOFF-DL titers declined with kinetics like

115 those observed in C57BL/6 mice (**Fig. 2d**). These data demonstrate that the dual lysin BCG kill switch strains
116 recapitulate vaccination with BCG but can be killed by lysin expression via doxy administration or withdrawal.
117 We did not detect escape mutants in any of the animal experiments and BCG-TetOFF-DL proved to be safe in
118 immunocompromised SCID mice.

119 **BCG kill switch strains provide similar protection as wild type BCG against Mtb infection in mice**

120 We vaccinated C57BL/6 mice by intravenous administration of $1x10^6$ BCG and BCG-TetOFF-DL and measured
121 CFU in lungs and spleens 21, 56 and 84 days post vaccination (**Fig. S3a**). The mice did not receive any doxy,
122 so that lysin expression was induced in BCG-TetOFF-DL soon after infection. Both strains lost viability in lungs,
123 but BCG-TetOFF-DL was eliminated faster than BCG and cleared from the lungs by 56 days post vaccination.
124 In spleens, BCG persisted at approximately $2x10^4$ CFU, while BCG-TetOFF-DL steadily lost viability, with 20
125 CFU remaining on day 84.
126 We examined pulmonary T cell responses in vaccinated mice and in mice injected with PBS (**Fig. 3a-e**). BCG
127 and BCG-TetOFF-DL elicited effector memory CD4 T cells (**Fig. 3a**) and CD8 T cells (**Fig. 3b**) whose frequencies
128 were reduced on days 56 and 84 in BCG-TetOFF-DL vaccinated mice compared to BCG vaccinated mice.
129 CD153 expressed on CD4 T cells has been identified as immune mediator of host protection against Mtb infection
130 (Sallin *et al.*, 2018; Du Bruyn *et al.*, 2021). In mice vaccinated with BCG or BCG-TetOFF-DL, CD153 positive
131 CD4 T cells were similarly enriched in the lungs on day 56 and 84 post vaccination (**Fig. 3c**). Lung resident
132 memory CD4 T cells were also induced by vaccination, although the responses in mice vaccinated with BCG-
133 TetOFF-DL were slightly lower than in mice vaccinated with BCG (**Fig. 3d**). Finally, we measured the frequency
134 of pulmonary cytokine (TNF, IFNγ, IL2, IL17A) expressing CD4 T cells and observed similar responses in BCG
135 and BCG-TetOFF-DL vaccinated mice (**Fig. 3e**). Collectively these data indicate that vaccination with BCG-
136 TetOFF-DL resulted in robust pulmonary T cell responses that were similar to those elicited by BCG vaccination,
137 despite the absence of doxy from the beginning of vaccination and faster clearance of BCG-TetOFF-DL from the
138 lungs (**Fig. S3a**).
139 We challenged unvaccinated and vaccinated mice with Mtb H37Rv via aerosol infection (**Fig. 3f**). Mtb H37Rv
140 carried a hygromycin resistance cassette that allowed us to specifically detect Mtb in BCG and BCG-TetOFF-
141 DL vaccinated mice. On day 28 post aerosol challenge, Mtb H37Rv had replicated to a mean of $4 \times 10^6$ CFU in
142 the lungs and $2 \times 10^5$ CFU in the spleen of unvaccinated mice. In mice vaccinated with BCG or BCG-TetOFF-

143 DL, the Mtb titers were reduced by approximately 10-fold (2.5 x $10^5$) in lungs. In spleens BCG vaccination
144 reduced Mtb burden by more than 100-fold compared to unvaccinated mice, while vaccination with BCG-
145 TetOFF-DL led to a 30-fold reduction. The differences in bacterial burden between BCG and BCG-TetOFF-DL
146 did not reach statistical significance. On day 56 post challenge, neither BCG strain provided significant protection
147 against Mtb in lungs, while levels of protection were largely maintained in the spleens. We repeated the challenge
148 experiment with BCG-TetON-DL with similar outcomes (Fig. S4). Together, these data demonstrate that BCG
149 kill switch strains protect against Mtb infection in mice comparably to BCG, although they are eliminated more
150 rapidly than BCG from lungs and spleens in the mouse model (Fig S3b).
151

152 **BCG persistence study design for NHPs**

153 We used BCG-TetOFF-DL to assess persistence in non-human primates. To determine whether the
154 doxycycline-regulated lysin expression was functional in vivo in macaques and whether BCG-TetOFF-DL
155 persisted or was diminished after removal of doxy treatment, we administered 5x$10^7$ BCG-TetOFF-DL CFU IV
156 to nine Mauritian cynomolgus macaques (MCM)(**Fig. 4a**). Group A was given daily doxy beginning one day prior
157 to BCG inoculation and continued for 2 weeks and was euthanized at 4 weeks post-vaccination. Group B had
158 the same 2-week doxy regimen and was euthanized at 8 weeks. Group C had an 8-week doxy regimen and was
159 euthanized at 8 weeks. Doxy administration was expected to prevent the expression of the two lysin genes,
160 maintaining the ability of BCG-TetOFF-DL to persist and/or grow in the macaques. Withdrawing doxy treatment
161 after two weeks in groups A and B was expected to result in expression of the lysin genes, preventing replication
162 or persistence of BCG-TetOFF-DL. Group C was maintained on doxy for the full 8 weeks of the study as a control
163 group. We performed a bronchoalveolar lavage (BAL) 4 weeks post-vaccination. At necropsy, we plated tissues
164 for BCG-TetOFF-DL on plates containing atc. Single cell suspensions of BAL and tissue samples were assessed
165 via flow cytometry for immune responses induced by BCG.
166

167 **BCG-TetOFF-DL induced robust T cell responses in airways**

168 After IV BCG-TetOFF-DL vaccination, the number of memory (CD45RA+CD28-, CD45RA-CD28+ or CD45RA-
169 CD28-) CD4+ and CD8+ T cells recovered from the airways via BAL increased ~100-fold in all three groups,
170 which is indicative of the generation of an IV BCG-dependent vaccine response, based on our previous studies

171 (Darrah *et al.*, 2020)(**Fig. 4b, Table S1**). This corresponded with an increase in the number of cytokine and
172 cytotoxic molecule producing effector T cells in the BAL (**Fig. 4b**). Cell numbers and effector molecule expression
173 between groups remained consistent, suggesting the duration of the doxy regimen and time of necropsy did not
174 play a significant role in the generation of the immune response. These data suggest that a BCG-dependent,
175 multi-faceted, immune response was generated in all animal groups within 4 weeks post-vaccination. Stimulation
176 with *M. tuberculosis* H37Rv whole-cell lysate did not have a large influence on the number of cytokine producing
177 CD4+/CD8+ T-cells, when compared to unstimulated controls. This is likely due to the systemic spread of BCG-
178 TetOFF-DL when administered via the intravenous route, resulting in common mycobacterial antigens persisting
179 at this early time point post-vaccination.
180
181 **Cessation of doxycycline reduced BCG-TetOFF-DL bacterial burden in macaque tissues**
182 Gross pathology score is a quantitative measure of grossly apparent mycobacterial related lesions at necropsy.
183 It takes into account granuloma numbers and lung lobes involved, lymph node and spleen size and granuloma
184 involvement and any other evidence of infection (Maiello *et al.,* 2018). Gross pathology scores (**Fig. 4c**) were
185 relatively low in all groups, which was expected since the animals were not challenged with Mtb. Two animals in
186 group A, and 1 animal each in groups B and C had a few small granulomas found at necropsy, with 1 granuloma
187 in a group A animal and 1 granuloma in a group B animal positive for BCG-TetOFF-DL CFU. BCG-TetOFF-DL
188 bacterial burden was assessed in multiple tissue samples to assess the efficiency of the doxy dependent self-
189 killing of the BCG-TetOFF-DL strain in all lung lobes, thoracic and peripheral lymph nodes and extrapulmonary
190 organs (spleen and liver) of each animal (**Fig. 4d-g**). BCG-TetOFF-DL CFU was recovered at low levels from
191 the lungs in 2 of 3 animals in group A (doxy stopped at 2 weeks and necropsied 4 weeks post-vaccination), from
192 thoracic lymph nodes of all 3 animals in group A, from peripheral lymph nodes in one animal and spleen from a
193 different animal. For group B animals (necropsied at 8 weeks post BCG-TetOFF-DL which was 6 weeks post-
194 doxy cessation), one was sterile (no CFU recovered), and BCG-TetOFF-DL was recovered from one lung sample
195 in one animal and from the spleen in a different animal, all at low bacterial burdens. For group C animals (treated
196 with doxy throughout the study and necropsied at 8 weeks post-BCG-TetOFF-DL), CFU were recovered from
197 lung lobes in two animals and from a peripheral lymph node in two animals. No culturable bacteria were
198 recovered from the blood at weeks 1 and 2 post vaccination or in the sternum, kidneys or liver of any animals.

199 These data indicate that killing of BCG was accelerated when doxy treatment was stopped at 2 weeks post-
200 vaccination. Group A (doxy treatment for 2 weeks, necropsied at 4 weeks) had a mean total body CFU of 479
201 (range 160-1041); Group B (doxy treatment for 2 weeks, necropsied at 8 weeks) mean total body CFU was 22
202 (range 0-50)(**Fig. 4d**). Group C (doxy for 8 weeks, necropsy at 8 weeks) had a mean total body CFU of 102
203 (range 25-195)(**Fig. 4d**). Thus, there was on average a 4.5-fold reduction in CFU (p=0.2, likely influenced by
204 small sample size) in the animals that were treated with doxy for 2 weeks vs 8 weeks and necropsied at 8 weeks
205 (i.e. comparing groups B and C). However, it is clear that even with continuation of doxcycyline for the 8 weeks
206 of this study (group C), BCG-TetOFF-DL bacterial burden was relatively low. This is consistent with our previous
207 data on wild type SSI BCG (Darrah *et al.*, 2020). Although the animals were vaccinated with >$10^7$ CFU, BCG
208 and BCG-TetOFF-DL appear to be rapidly reduced in macaques (~10,000 fold reduction by 8 weeks), suggesting
209 minimal replication and/or enhanced bacterial killing.
210
211 Non-necrotizing 'microgranulomas' and lymphohistiocytic aggregates were seen throughout the spleen (**Fig.**
212 **S5a**), thoracic lymph nodes (**Fig. S5b**) and livers (**Fig. S5c**) of animals in all groups. BCG-TetOFF-DL CFU was
213 recovered from spleen in only 2/9 animals, liver in 0/9 animals and lymph nodes (peripheral or thoracic) in 6/9
214 animals. Splenomegaly was seen in animals intravenously vaccinated with BCG (Darrah *et al.*, 2020). Comparing
215 spleen size at 4 and 8 weeks post BCG-TetOFF-DL vaccination with an unvaccinated control, the spleen was
216 enlarged in all groups, with the largest spleens being recorded at the 4-week time point (**Fig. S5d**, black
217 symbols). This may suggest a reduction in spleen size over time or with reduced total body bacterial burden.
218
219 **Lymphocyte proportions in lung and lymph nodes were similar from each group post BCG-TetOFF-DL**
220 **vaccination**
221 We compared lymphocyte composition in the lung and thoracic lymph nodes in tissue samples collected at
222 necropsy (**Fig. S5e,f**). Expected levels of animal-to-animal variation was seen in cellular composition, however,
223 populations remained consistent within and across groups. $CD4^-CD8^-$ (double negative) T cells were more
224 prominent in group A lung tissues compared to group B and C, however other populations were similar. Few B
225 cells (CD20+) were found in lung tissue compared to lymph nodes, but a higher proportion of $\gamma\delta$ T-cells and NK

226 cells were present in lung tissue. Cellular populations were similar across all animal groups in thoracic lymph
227 nodes, with CD4+ T-cells being the most prominent cell type.
228
229 **T-cell responses in the lung and lymph nodes at necropsy**
230 Comparing CD4+ and CD8+ T cell numbers in lung tissue at necropsy shows comparable levels of cells in all
231 three groups (**Fig. 4h,i** – No stim). Similar cell numbers from animals on a short doxy regimen and necropsied
232 at 4 weeks (Group A), and from those necropsied at 8 weeks (Group C) on a longer doxy regimen suggest a
233 robust, multi-cellular immune response is generated and resides in the lungs within 4 weeks post vaccination
234 with IV- BCG-TetOFF-DL. Cytokine and cytotoxic molecule producing cells were similar between all 3 groups,
235 further reinforcing the multi-faceted response in lung tissue (**Fig. 4h,i**). A high number of CD4+ cells responded
236 to H37Rv whole cell lysate (WCL) stimulation, resulting in the production of TNF, IFN-γ and IL-2 in the lung
237 tissue. Cytotoxic molecules (granzyme B and K) are preformed molecules, therefore stimulation with H37Rv
238 WCL has minimal effect on the quantities detected compared to unstimulated cells. High levels of these cytotoxic
239 molecules were detected in all groups, in both unstimulated and stimulated samples.
240 In summary, IV BCG-TetOFF-DL vaccination was able to generate a robust, multi-faceted immune response
241 comprising of effector CD4+ and CD8+ T-cells within 4 weeks post vaccination. The 'kill-switch' was successfully
242 induced in a NHP model by removal of doxy, with a reduction in BCG CFU 6 weeks after cessation of doxy
243 administration. Gross pathology scores at necropsy remained low, suggesting minimal negative side-effects of
244 IV BCG-TetOFF-DL vaccination.
245
246 **Protective efficacy study design for NHPs**
247 Following the persistence study, we performed an Mtb challenge study to assess the protective efficacy of BCG-
248 TetOFF-DL and WT BCG Pasteur in NHPs. Protection was assessed by monitoring disease progression over
249 time using PET-CT and quantifying Mtb bacterial burden at necropsy. The immune response generated to each
250 vaccine strain – BCG-TetOFF-DL and WT BCG Pasteur- was characterized over the duration of the study and
251 at necropsy.
252

253 We administered $5x10^7$ BCG-TetOFF-DL or WT BCG Pasteur CFU to 8 MCMs per group, both intravenously
254 (**Fig. 5a**). Two MCMs were unvaccinated as concurrent controls for this study; 8 additional historical
255 unvaccinated MCM controls with the same time point for necropsy were included in this study, resulting in 10
256 unvaccinated control MCMs total. To reduce the possibility of confounding effects, both vaccine groups were
257 given a 2 week doxy regimen post-vaccination which should not affect WT BCG. At 22 weeks post vaccination,
258 we challenged all groups with 9-16 CFU Mtb strain Erdman via intrabronchial instillation (**Fig. 5a**), monitored
259 disease progression over time using PET/CT, and necropsied 12 weeks post-challenge.
260

261 **T-cell responses in airways were similar upon vaccination with BCG-TetOFF-DL or WT BCG Pasteur**

262 We monitored the immune response in the airways for both vaccine strains and unvaccinated control animals
263 using BALs. BALs were performed pre-vaccination and 4, 12 and 20 weeks post IV-vaccination. We assessed
264 the number of memory effector T-cells at each time point (memory CD4+ or memory CD8+ producing either IFN-
265 γ, TNF, IL-17, IL-2, GzmB, GzmK, granulysin or perforin) (**Fig. S6**). Both WT and BCG-TetOFF-DL induced a
266 sustained increase in total T cell numbers in BAL while there was little change in the unvaccinated animals. This
267 corresponded to a sustained increase in T cells producing cytokines and in CD8 T cells producing perforin in
268 vaccinated animals. This confirms and extends our data from the persistence study (**Fig. 4**) that a robust T cell
269 response is generated upon vaccination with both IV BCG-TetOFF-DL and WT BCG Pasteur. At 20 weeks, the
270 final time point before challenge, we did not observe significant differences in effector T-cell numbers in the
271 airway when comparing BCG-TetOFF-DL and WT BCG Pasteur. The similar level of response in both vaccination
272 groups suggests live mycobacteria are only required to be present for a short amount of time for a robust memory
273 response to be generated.
274

275 **BCG-TetOFF-DL and WT BCG had fewer granulomas and less lung inflammation compared to**
276 **unvaccinated macaques**

277 Serial PET-CT allows the assessment of disease trajectory by enumerating numbers of granulomas and lung
278 and lymph node inflammation over time; increasing numbers of granulomas indicates Mtb dissemination which
279 correlates with development of active TB. Thus, greater the granuloma count, the more severe the infection and
280 the less protective the vaccine. NHPs vaccinated with either BCG-TetOFF-DL or WT BCG Pasteur exhibited

281 lower granuloma formation at every time point compared to unvaccinated control animals (**Fig. 5b,c**). One WT
282 BCG Pasteur vaccinated NHP developed a large number of granulomas. Higher levels of the PET probe $^{18}$F
283 fluorodeoxyglucose (FDG) activity in the lung due to increased host cell glucose metabolism indicate an increase
284 in lung inflammation (White *et al.*, 2017). We previously demonstrated that total lung FDG activity is correlated
285 to bacterial burden in Mtb infected macaques (Darrah *et al.*, 2020). Unvaccinated animals had significantly more
286 total lung FDG activity after Mtb challenge compared to vaccinated animals (**Fig. 5d,e**). All animals of the BCG-
287 TetOFF-DL vaccinated group had undetectable levels of inflammation (via lung FDG) just prior to necropsy,
288 whereas 2 of the 8 animals in the WT BCG Pasteur group had elevated levels of total lung FDG activity (**Fig.**
289 **5e**).
290

291 **BCG-TetOFF-DL vaccination leads to enhanced protection against Mtb challenge**

292 At necropsy, gross pathology scores in vaccinated animals were significantly lower than unvaccinated animals
293 (**Fig. 5f**). Our stated primary outcome measure of vaccine efficacy was total thoracic Mtb burden at necropsy.
294 BCG-TetOFF-DL IV vaccinated animals displayed robust protection against Mtb with significantly lower total
295 thoracic CFU compared to unvaccinated animals (p=0.001)(**Fig. 5g**). WT BCG IV vaccinated animals also had
296 lower bacterial burdens compared to unvaccinated macaques (p=.0583)(**Fig. 5g**). Although BCG-TetOFF-DL
297 was not significantly different than WT BCG in terms of total thoracic bacterial burden (p=0.3124), 6 out of the 8
298 animals in the IV-BCG-TetOFF-DL were sterile, defined as 0 Mtb CFU recovered, compared to two of eight for
299 WT BCG (**Fig. 5h**). Thus, there was a trend towards increased sterile protection in the BCG-TetOFF-DL
300 macaques (Fisher's exact test, p = 0.1319). Total thoracic bacterial burden can be separated into lung CFU and
301 thoracic lymph node (LN) CFU. BCG-TetOFF-DL vaccinated animals had significantly reduced lung CFU, with
302 only one of the eight animals with lung CFU, while there was only a trend towards lower lung CFU in the WT
303 BCG animals compared to unvaccinated animals (**Fig. 5i)**. Both BCG-TetOFF-DL and WT BCG IV vaccinated
304 animals had significantly lower thoracic LN CFU compared to unvaccinated animals (**Fig. 5j**), suggesting
305 dissemination from lung to lymph nodes was prevented with both WT and BCG-TetOFF-DL.
306

307 **CD4 T cell responses are enhanced in the lungs of BCG-TetOFF-DL compared to WT BCG vaccinated**
308 **macaques**

309 The bacterial burden data suggest better protection by BCG-TetOFF-DL IV vaccination than by WT BCG using
310 the Pasteur strain (6/8 sterile with BCG-TetOFF-DL vs 2/8 for WT BCG). To investigate the factors that might
311 contribute to this, we analyzed immune cell populations and functions in the tissues of the macaques at necropsy.
312 Multiparametric spectral flow cytometry and Boolean gating revealed a significantly higher frequency of CD4+ T
313 cells in lungs of BCG-TetOFF-DL animals, whereas the CD8+ T cell population was slightly, although not
314 significantly, higher in WT BCG Pasteur vaccinated animals (**Fig. 6a**). There was a significant increase in the
315 frequency of lung memory CD4+ T-cells producing cytokines (IFN-γ, TNF, IL-17 and/or IL-2) in the BCG-TetOFF-
316 DL vaccinated group compared to the WT BCG Pasteur group upon stimulation with mycobacterial WCL (**Fig.**
317 **6b**). There were no statistically significant differences between BCG-TetOFF-DL or WT BCG vaccinated animals
318 in lung T cells producing cytotoxic effector molecules, although slightly higher frequencies were seen in WT BCG
319 vaccinated animals (**Fig. 6b**). In the thoracic lymph nodes, WT BCG vaccinated animals had significantly higher
320 frequencies of CD8+ T cells producing cytotoxic molecules compared to BCG-TetOFF-DL vaccinated animals
321 (**Fig. 6d**). There was an increase in the number of cytokine producing memory CD4+ cells, specifically IFN-γ, IL-
322 2 and TNF, present in the lung tissue of BCG-TetOFF-DL vaccinated macaques compared to those vaccinated
323 with WT BCG (**Fig. S7**), corroborating the significant increase in frequency of CD4 T cells.
324
325 **Spleen pathology**
326 Due to the systemic nature of IV BCG vaccination and the robust immune response induced, splenomegaly was
327 observed in NHPs (Darrah *et al.*, 2020). The duration of the enlargement and the relationship with BCG survival
328 is unknown. Here we show that spleen size appears to correlate with time post-vaccination and BCG CFU (**Fig.**
329 **S5d**). IV-BCG vaccinated and IV-BCG vaccinated/Mtb challenged spleen sizes are larger than the average range
330 of an unvaccinated/unchallenged macaque but are largest at 4 weeks post vaccination. Spleen size reduces
331 over time, with the smallest measurement being recorded 34 weeks post vaccination. Even spleens from
332 unvaccinated but Mtb challenged macaques did not fall within the normal range. We assume that this is due to
333 Mtb infection in these animals, noting that IV BCG vaccinated and Mtb challenged animals had spleen sizes
334 similar to unvaccinated and challenged macaques at this late time point.
335

336 **Discussion**

337 BCG has likely been administered to more humans than any other vaccine designed to prevent infectious
338 diseases and is generally safe (Lange *et al.*, 2022). Though rare (0.1 to 4.3 per one million vaccinated children),
339 complications from, BCG vaccination is one of the most common causes of death in immunocompromised
340 children (Hassanzad *et al.*, 2019). Although BCG is an effective option for treatment of bladder cancer,
341 approximately 8% of patients develop complications which leads to cessation of treatment (Liu *et al.*, 2019). We
342 therefore sought to generate BCG strains whose elimination does not depend on a patient's immune system.
343 Usage of phage lysins to establish conditional kill switches was prioritized over other toxins because we expected
344 these enzymes to not only increase safety but to also increase immunogenicity via the release of cytoplasmic
345 antigens.

346

347 Combining the controlled expression of two phage lysins resulted in BCG strains that either require atc/doxy to
348 grow or are efficiently killed by exposure to atc/doxy and escape from this controlled growth was as low as ~2 x
349 $10^{-9}$ mutants per cell division. Escape was thus not as low as for the Mtb strain described in the accompanying
350 manuscript (Wang et. al.) but likely low enough to allow studies in humans given that BCG is attenuated already.
351 Characterization of the atc/doxy-addicted BCG (BCG-TetOFF-DL) in SCID mice confirmed that induction of lysin
352 expression is sufficient to eliminate the strain and the clearance of the infection no longer depends on an intact
353 immune system. Mice vaccinated with BCG-TetOFF-DL or WT BCG showed similar protection against Mtb
354 challenge, even though the BCG-TetOFF-DL strain was eliminated faster than WT BCG. It seems likely that
355 protection benefited from lysis of BCG-TetOFF-DL as it releases additional antigens. This interpretation is
356 supported by the enhanced stimulation of proinflammatory cytokines we observed in macrophages infected with
357 this strain compared to WT BCG.

358

359 BCG delivered intravenously was shown to provide robust protection against Mtb infection and disease in
360 macaques (Darrah *et al.*, 2020). Here we show, using genetically modified BCG that is killed by cessation of
361 doxy administration in vivo in macaques, that BCG does not need to be alive for more than a few weeks to
362 provide robust protection against Mtb challenge. Our data indicate that this "suicide" BCG strain induces greater
363 CD4 T cell responses in lungs and may provide even more robust protection than WT BCG in macaques. Using

364 a self-killing BCG strain may thus increase the safety of IV BCG vaccination strategies while maintaining
365 remarkable protective efficacy.
366
367 The ability of BCG-TetOFF-DL to generate a robust CD4 response could be vital in its ability to be an efficacious
368 vaccine, as CD4 T cells are known to play an important role in protection against TB (Sakai, Mayer-Barber and
369 Barber, 2014). Our data indicated that BCG-TetOFF-DL induces a stronger CD4 T cell response compared to
370 WT BCG with production of key cytokines including IFN-γ and TNF in lungs. We hypothesize that the lysis of
371 BCG-TetOFF-DL leading to release of mycobacterial proteins and the enhanced cytokine production by
372 macrophages, as shown in vitro, leads to a more robust priming of T cells in vivo, at least in macaques.
373
374 BCG IV was previously noted to result in enlarged spleens in macaques. We considered that reducing the
375 duration of live BCG could mitigate this side effect. In the persistence study, at 4 weeks post-BCG-TetOFF-DL,
376 spleens were quite large, but reduced in size by 8 weeks. Spleen sizes were smaller overall in the BCG-TetOFF-
377 DL vaccinated macaques that received doxy for only 2 weeks compared to those receiving doxy for 8 weeks. In
378 the Mtb challenged animals, both vaccinated and unvaccinated animals had increased spleen sizes compared
379 to “normal” spleens and were slightly smaller than the spleens harvested at 8 weeks post-vaccination, although
380 spleen size can vary with age, sex, and size of macaques. Mtb infection generates immune responses resulting
381 in spleen enlargement, which may suggest the normal spleen size range is not achievable in Mtb infected groups
382 (Chapman, Goyal and Azevedo, 2023). The BCG associated non-necrotizing ‘microgranulomas’ that were
383 present 4- and 8-weeks post-vaccination were not seen in animals necropsied at 34 weeks post-vaccination.
384
385 BCG-TetOFF-DL was generated using a BCG Pasteur background and the WT BCG used as a comparator here
386 was the same Pasteur strain, while our previous IV-BCG studies performed in NHPs used BCG-SSI (Danish) as
387 the vaccine strain (Darrah *et al.*, 2020). Thus, both BCG Pasteur and BCG Danish/SSI can achieve sterilizing
388 levels of protection when delivered IV. This study and one other BCG IV study (Larson *et al.*, 2023) were
389 performed in Mauritian cynomolgus macaques (MCM) while our original study was performed in rhesus
390 macaques. MCM have similar susceptibility to TB disease as rhesus macaques (Maiello *et al.*, 2018; Rodgers *et*
391 *al.*, 2018) and BCG IV provides robust protection in both macaque species (Darrah *et al.*, 2020; Larson *et al.*,

392 2023). In contrast, BCG-TetOFF-DL or WT BCG delivered IV provided only modest protection in mice in the
393 current study, similar to other studies with IV BCG in mice (Pym *et al.*, 2003; Mittrücker *et al.*, 2007). Thus,
394 vaccines that provide robust protection in macaques cannot always be predicted from murine data.
395
396 Limitations to this study are the small samples sizes used in the macaque persistence study which limited
397 statistical analyses. Similarly, larger sample sizes for the macaque protective efficacy study could provide clearer
398 differences between BCG-TetOFF-DL and WT BCG outcomes. Future studies could also assess whether doxy
399 is necessary in vivo, or whether the strain could be eliminated even earlier in the vaccination phase. In mice it
400 remains to be demonstrated whether vaccination efficacy can be improved by prolonging persistence of the BCG
401 kill switch strain by administrating doxy for different periods of time.
402
403 In summary, our data support that a limited exposure to BCG delivered intravenously is effective against Mtb
404 challenge. The use of a strain that lyses itself may provide enhanced protection due to increased immunogenicity
405 in vivo. This “suicide” BCG strain could limit safety concerns that are raised regarding IV administration of a live
406 vaccine as well as provide an option for safer intradermal BCG vaccination of immunocompromised individuals
407 and treatment for bladder cancer.
408

409 **Materials and Methods**

410 **Strains, media and culture conditions.** All *M. bovis* BCG strains are derived from BCG Pasteur TMC 1011
411 obtained from the American Type Culture Collection (ATCC #35734). *M. tuberculosis* H37Rv was used for
412 challenge experiments. Strains were cultured in liquid Middlebrook 7H9 medium supplemented with 0.2%
413 glycerol, 0.05% tween80 and ADN (0.5% bovine serum albumin, 0.2% dextrose, 0.085% NaCl) and on
414 Middlebrook 7H10 agar plates supplemented with 0.5% glycerol and Middlebrook OADC enrichment (Becton
415 Dickinson). Antibiotics were added for selection of genetically modified strains at the following concentrations:
416 hygromycin (50 μg/ml), kanamycin (25 μg/ml), zeocin (25 μg/ml). Anhydrotetracycline was used at 0.5 or 1 μg/ml.

417 **Generation of strains.** To create the TetON single and dual-lysin strains, BCG was transformed with either or
418 both plasmids pGMCK3-TSC10M1-D29L and pGMCgZni-TSC10M1-L5L, integrating the D29-lysin and L5-lysin
419 into BCG L5 and Giles sites, respectively. To create the TetOFF single and dual-lysin strains, BCG was
420 transformed with either or both plasmids pGMCK3-TSC10M-TsynC-pipR-SDn-P1-TsynE-PptR-L5L and
421 pGMCgZni-TSC38S38-TrrnBd2-P749pld-10C32C8C-D29L, integrating the L5-lysin and D29-lysin into BCG L5
422 and Giles sites, respectively. The single lysin strains were cultured in the presence of 0.5 μg/ml atc and the dual
423 lysin strain was cultured in the presence of 1 μg/ml atc.

424 **Analysis of bacterial lysis by immunoblotting.** Culture filtrates were prepared as follows. BCG strains were
425 grown in 7H9 medium with 0.2% glycerol, 0.05% Tween-80, 0.5% BSA, 0.2% dextrose and 0.085% NaCl until
426 the culture reached an OD of 0.6 ~ 0.8. Cultures were then washed three times with PBS to remove BSA and
427 Tween-80. We next suspended the pellet in 7H9 medium supplemented with 0.2% glycerol, 0.2% dextrose and
428 0.085% NaCl. After incubation, culture supernatant was harvested by centrifugation and filtration through 0.22
429 μm filters. Filtrates were concentrated 100-fold by using 3K centrifugal filter units (Millipore) and analyzed by
430 immunoblotting with antisera against Eno and PrcB.

431 **Preparation and infection of murine bone marrow derived macrophages (BMDMs).** Femurs and tibias of
432 female C57BL/6 mice were extracted, and bone marrow cells were aseptically flushed using PBS. Cells were re-
433 suspended in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS)
434 and 20% L929 culture filtrate and incubated for 6 days to allow differentiation into macrophages. Cells were
435 harvested and seeded at 6 x $10^4$ per well in 96-well plates in DMEM supplemented with 10% FBS, glycine and

436 10% L929 cell culture overnight before infection. Mycobacteria were washed in PBS + 0.05% tyloxapol and a
437 single cell suspension was generated by low speed centrifugation to pellet clumped cells. The bacteria were
438 diluted into DMEM, 10% LCM and added to macrophages at MOI of 0.1. After 4 hours, extracellular bacteria
439 were removed by washing the macrophages three times with warm PBS. Infected BMDMs were treated with atc
440 (0.5 μg/ml) or RIF (0.5 μg/ml) starting 16 hrs post infection. Cytokines were quantified using BD OptEIA ELISA
441 kits for mouse TNF, IL-12p40 or IL6 (BD Biosciences). The number of intracellular bacteria was determined by
442 lysing macrophages with 0.01% Triton X-100 and culturing dilutions of macrophage lysates on 7H10 agar plates.

443 **Mouse infections**. Female 8- to 10-week-old C57BL/6 (# 000664, Jackson Laboratory) were vaccinated with ~
444 $10^6$ CFU of the indicated BCG strain by the intravenous route. Mice received doxycycline containing mouse chow
445 (2,000 ppm; Research Diets) for the indicated periods. Mice were infected with Mtb H37Rv using an inhalation
446 exposure system (Glas-Col) with a mid-log phase Mtb culture to deliver approximately 100 bacilli per mouse. To
447 enumerate CFU, organs were homogenized in PBS and cultured on 7H10 agar. Charcoal (0.4 %, w/v) was added
448 to the plates that were used to culture homogenates from doxy treated mice. Agar plates were incubated for 3-
449 4 weeks at 37°C. Mice were housed in a BSL3 vivarium. All mouse experiments were approved by and performed
450 in accordance with requirements of the Weill Cornell Medicine Institutional Animal Care and Use Committee.

451 **Flow cytometry to assess immune responses in mice**. Mouse lungs were isolated and placed in RPMI1640
452 containing Liberase Blendzyme 3 (70 μg/ml; Roche) and DNase I (50 μg/ml; Sigma-Aldrich). Lungs were then
453 cut into small pieces and incubated at 37 C for 1 hour. The cells were filtered using cell strainers, collected by
454 centrifugation, resuspended in ACK hemolysis buffer (ThermoFisher) and incubated for 10 minutes at room
455 temperature. Cells were then washed with PBS and resuspended in splenocyte medium (RPMI-1640,
456 supplemented with 10% FBS, 2 mM GlutaMax 10 mM HEPES, and 50 μM 2-mercaptoethanol). For intracellular
457 staining samples, cells were stimulated with PPD (20 μg/ml) in the presence of anti-CD28 antibody (37.51,
458 BioLegend) for 1.5 hours and 10 μg/ml Brefeldin A (Sigma) and monesin were added and incubated at 37 C for
459 another 3 hrs. Samples were kept on ice in a refrigerator overnight. Cells were stained with Zombie-NIR
460 (BioLegend) to discriminate live and dead cells. Purified anti-CD16/32 antibody (93, BioLegend) was used to
461 block Fc receptor before staining. PerCp-Cy5.5 anti-CD62L (MEL-14, Thermofisher), BV605 anti-CD4 (RM4-5,
462 BioLegend), BV711 anti-CD8 (53-6.7, BioLegend), eFluor450 anti-CD11a (M17/4, Thermofisher), BUV395 anti-
463 CD153 (RM153, BD Biosciences), BV480 anti-CD69 (H1.2F3, BD Biosciences), APCC7 anti-CD44 (IM7,

464 Biolegend) were used to stain cells for 30 minutes at room temperature. The cells were fixed in fixation buffer
465 (BioLegend) for 30 minutes and taken out of BSL3. The cells were incubated for 20 minutes in permeabilization
466 buffer (eBioscience) before intracellular cytokine staining. BV421 anti IL-17A (TC11-18H10.1, Biolegend), BV750
467 anti-IFNγ (XMG1.2, BD Biosciences), PE-C5.5 anti-IL-2 (JES6-5H4, Biolegend), FITC anti-TNF (MP6-XT22,
468 BioLegend), BV785 anti-CD3 (17A2, BioLegend) were used to stain cells for 30 minutes. Cells were washed and
469 resuspend in cell staining buffer (BioLegend). Flow cytometry data were acquired on cytometer (LSR Fortessa
470 TM; BD Biosciences) or cytometer (Cytek Aurora; Cytek Biosciences) and were analyzed with FlowJoTM V10.

471 **Macaques**

472 Mauritian cynomolgus macaques (*Maccaca fasicularis*) used in this study were obtained from BioCulture US (all
473 males, 6-9 years old). All procedures and study design complied with ethical regulations and were approved by
474 the Institutional Animal Care and Use Committee of the University of Pittsburgh. Macaques were housed either
475 singly or in pairs in a BSL2 animal facility and cared for in accordance with local, state, federal, and institute
476 policies in facilities accredited by the American Association for Accreditation of Laboratory Animal Care
477 (AAALAC), under standards established in the Animal Welfare Act and the Guide for the Care and Use of
478 Laboratory Animals. During the Mtb challenge phase, animals were housed in a BSL3 animal facility. Macaques
479 were monitored for physical health, food consumption, body weight, temperature, complete blood counts, and
480 serum chemistries. Full details on macaques in this study are in **Table S2**.

481 **BCG vaccination**

482 To assess persistence of BCG-TetOFF-DL, animals that were intravenously vaccinated were sedated with
483 ketamine (10 mg/kg) or telazol (5- 8 mg/kg) and injected intravenously with 3.74 x $10^7$ CFU BCG-TetOFF-DL
484 vaccine in an injection volume of 1mL. Animals that underwent challenge with *M. tuberculosis* strain Erdman
485 were vaccinated intravenously with 5 x $10^7$ CFU BCG-TetOFF-DL or WT BCG Pasteur.

486 **Macaque Mtb challenge**

487 Macaques were challenged by bronchoscope with 9-16 Mtb strain Erdman 22 weeks post vaccination as
488 previously described (Martin *et al.*, 2017). Control animals (unvaccinated) were challenged at the same time.

489 Historical control unvaccinated and Mtb challenged MCMs (previously published by our group (Larson *et al.*,
490 2023)) were included for statistical analyses and comparison.
491

492 **Blood, BAL and tissue processing**

493 To assess the presence of viable BCG-TetOFF-DL, blood was drawn from each animal 1- and 2-weeks post
494 vaccination. Blood was cultured and the presence of BCG-TetOFF-DL assessed using atc-containing plates.
495 Bronchoalveolar lavages for the persistence study in macaques were performed pre-vaccination, 4 weeks post
496 vaccination (all groups) and 8 weeks post vaccination (for groups B and C). For the immunization and challenge
497 studies in macaques, BAL was performed prior to vaccination and monthly thereafter until the time of challenge.
498 Procedures were performed as previously described (Darrah *et al.*, 2020). PBMCs were isolated from peripheral
499 blood using Ficoll-Paque PLUS gradient separation (GE Healthcare Biosciences) and standard procedures.

500 **PET/CT**

501 PET/CT scans were performed at designated time points throughout the study to assess thoracic cavity
502 inflammation. Animals were sedated with 10 mg/kg ketamine / 0.5 ml atropine before imaging. An intravenous
503 catheter was placed in the saphenous vein and animals were injected with ~ 5 millicurie (mCi) of $^{18}$F-FDG. Once
504 placed on the imaging bed, anesthesia was induced with 2.5 to 3% isoflurane which is reduced to 0.8–1.2% for
505 maintenance. Breathing during imaging was maintained using an Inspiration 7i ventilator (eVent Medical, Lake
506 Forest, CA, USA) with the following settings: PF = 9.0 l/min, respiration rate = 18–22 bpm, tidal volume = 60 ml,
507 O2 = 100, PEEP = 5–8 cm $H_2O$, peak pressure = 15–18 cm $H_2O$, I:E ratio = 1:2.0. A breath hold was conducted
508 during the entirety of the CT acquisition.
509 PET/CT scans were performed on a MultiScan LFER 150 (Mediso Medical Imaging Systems, Budapest,
510 Hungary). CT acquisition was performed using the following parameters: Semi-circular single field-of-view, 360
511 projections, 80 kVp, 670 µA, exposure time 90 ms, binning 1:4, voxel size of final image: 500 x 500 µm. PET
512 acquisition was performed 55 min after intravenous injection of $^{18}$F-FDG with the following parameters: 10 min
513 acquisition, single field-of-view, 1–9 coincidence mode, 5 ns coincidence time window. PET images were
514 reconstructed with the following parameters: Tera-Tomo 3D reconstruction, 400–600 keV energy window, 1–9
515 coincidence mode, median filter on, spike filter on, voxel size 0.7 mm, 8 iterations, 9 subsets, scatter correction

516 on, attenuation correction based on CT material map segmentation. Serial CT or PET/CT images were acquired
517 pre-infection and at 4 and 11 dpi. Animal A2 was CT scanned at 9 dpi instead of 11 dpi, and animal A1 was
518 scanned at 18 dpi in addition to the standard imaging schedule previously described.
519 Images were analyzed using OsiriX MD or 64-bit (v.11, Pixmeo, Geneva, Switzerland). Before analysis, PET
520 images were Gaussian smoothed in OsiriX and smoothing was applied to raw data with a 3 x 3 matrix size and
521 a matrix normalization value of 24. Whole lung FDG uptake was measured by first creating a whole lung region-
522 of-interest (ROI) on the lung in the CT scan by creating a 3D growing region highlighting every voxel in the lungs
523 between -1024 and -500 Hounsfield units. This whole lung ROI was copied and pasted to the PET scan and
524 gaps within the ROI were filled in using a closing ROI brush tool with a structuring element radius of 3. All voxels
525 within the lung ROI with a standard uptake value (SUV) below 1.5 were set to zero and the SUVs of the remaining
526 voxels were summed for a total lung FDG uptake (total inflammation) value. Thoracic lymph nodes were analyzed
527 by measuring the maximum SUV within each lymph node using an oval drawing tool. Both total FDG uptake and
528 lymph node uptake values were normalized to back muscle FDG uptake that was measured by drawing cylinder
529 ROIs on the back muscles adjacent to the spine at the same axial level as the carina (SUVCMR; cylinder-muscle-
530 ratio). PET quantification values were organized in Microsoft Excel and graphed using GraphPad Prism.

531 **Necropsy, pathology scoring, BCG burden and Mtb burden**

532 At necropsy, NHPs were sedated with ketamine and had a maximal blood draw then euthanized by sodium
533 pentobarbital injection, followed by gross examination for pathology. A gross pathology scoring system was
534 employed, assessing lung, lymph node and extrapulmonary compartments (Maiello *et al.*, 2018). Spleen size
535 was also measured. Average spleen size of adult macaques was provided by the Wisconsin Primate Center. A
536 pre-necropsy PET-CT scan was used to map lesions in the thoracic cavity and at necropsy these regions were
537 excised and homogenized to form a single-cell suspension. Uninvolved lung tissue, lymph nodes and spleen
538 were also processed. To recover BCG-TetOFF-DL, the individual cell suspensions were plated on 7H11 agar +
539 atc and incubated at 37°C with 5% $CO_2$ for 3 weeks with atc replenished on the plates after 10 days of incubation.
540 CFU were counted to assess the BCG-TetOFF-DL burden of each animal. To assess Mtb burden post-challenge,
541 single-cell suspensions were plated on 7H11 agar and incubated at 37°C with 5% $CO_2$ for 3 weeks.

542 **Multiparameter flow cytometry**

543 Up to one million viable cells isolated from tissue or BAL were stimulated with 20 µg/ml H37Rv Mtb whole cell
544 lysate (WCL) (BEI Resources), 1 µg/ml each of ESAT-6 and CFP-10 peptide pools (provided by Aeras, Rockville,
545 MD) or R10 media for 2 h before adding 10 µg/ml BD GolgiPlug (BD Biosciences) for a further 12 hours. Cells
546 were surface stained to allow for the assessment of cell composition, followed by intracellular staining (ICS) for
547 the analysis of cytokine and cytotoxic molecule production. Permeabilization for ICS was performed using BD
548 Fixation/Permeabilization Kit. Antibodies used in the study are outlined in Table 1. Surface and ICS antibody
549 cocktails were made in BD Brilliant Stain buffer. Cells were analyzed using a five laser Cytek® Aurora spectral
550 flow cytometer. The flow cytometry results were analyzed using FlowJo™ v10.8 Software (BD Life Sciences).
551 All antibodies used in the flow panels are shown in **Table S1**.

552 **Statistical methods**

553 For mice, generation of graphics and data analyses were performed in Prism version 10.0.2 software
554 (GraphPad). NHP data were tested for normality using the Shapiro-Wilk test. For comparisons between only
555 BCG-TetOFF-DL and WT, Mann-Whitney tests were used. For comparisons including historical unvaccinated
556 macaque controls, Kruskal-Wallis tests were performed and Dunn's p-values (adjusted for multiple comparisons)
557 were reported. For longitudinal data with only 3 animals per group, two-way repeated measure ANOVA was
558 performed (random variable was NHP) with the assumption of sphericity. For BAL longitudinal data, groups
559 were compared at each time point using unpaired t-tests (with Welch correction) and Holm-Šídák adjustment for
560 multiple comparisons. For categorical variables, Fisher's exact test p-value was reported. Statistical tests were
561 not run for any groups with 3 or fewer points. All p-values less than 0.10 are shown.

562

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679 **Figure 1. Induction of phage lysins results in cell lysis with low frequency of escape and promotes**
680 **macrophage proinflammatory cytokine production.**

681 (a) Growth of BCG TetON single and dual lysin kill switch strains with and without atc. Individual CFU data points
682 from two replicate cultures are depicted.

683 (b) Impact of BCG TetON dual lysin kill switch induction at different times of growth.

684 (c) Growth of BCG TetOFF single and dual lysin kill switch strains with and without atc. Data are means ± SD
685 from triplicate cultures. Error bars are frequently too small to be seen.

686 (d) Fraction of resistant mutants per culture and mutation rate per cell division of BCG-TetOFF-DL. The
687 resistance rate in 20 individual cultures was determined in a fluctuation assay. The number of mutations per cell
688 division was calculated using the bz-rates web-tool.

689 (e) CFU quantification of WT BCG and BCG-TetON-DL (plus or minus atc or rifampin) during infection of murine
690 BMDMs. Data are means ± SD from triplicate cultures. The difference in survival of BCG-TetON-DL treated with
691 rif or treated with atc is not statistically significant (ns).

692 (f, g, h) Quantification of TNF (f), IL-12 p40 (g), and IL-6 (h) production by macrophages infected with BCG or
693 BCG-TetON-DL and treated with rifampin or atc for 20 hrs. Data are means ± SD from triplicate cultures.
694 Significance was determined by one-way ANOVA and adjusted for multiple comparisons. ns, not significant; * $P$
695 $< 0.05$; ** $P < 0.01$; ***, $P < 0.005$; ND, not detected.

696 All data are representative of two or three independent experiments.

697

698 **Figure 2. Phage lysin induction kills BCG in immune competent and immune deficient mice.** (a, b) CFU
699 quantification from lungs (a) and spleens (b) of BCG and BCG-TetON-DL infected C57BL/6 mice treated or not
700 with doxycycline (doxy) starting day 7 post infection. Data are means ± SD from 3-5 mice per group and time
701 point BCG CFU +/- doxy and BCG-TetON CFU +/- doxy were analyzed by unpaired t test and p values are
702 indicated. (c, d) CFU quantification from lungs (c) and spleens (d) of BCG-TetOFF-DL infected C57BL/6 and
703 SCID mice treated or not with doxy for the indicated times. Data are means ± SD from 4-5 mice per group and
704 time point.

705

706 **Figure 3. BCG-TetOFF-DL and wt BCG provide similar protection against Mtb infection in mice.** Mice were
707 i.v. vaccinated with BCG or BCG-TetOFF-DL or received PBS.

708 (a-e) Quantification of T cell subsets in mouse lungs from mice vaccinated with BCG or BCG-TetOFF-DL; (a)
709 effector memory CD4 T cells; (b) of effector memory CD8 T cells; (c) CD153 expressing CD4 T cells; (d) lung
710 resident memory CD4 T cells; (e) Cytokine (TNF, IFNγ, IL2, IL17A) expressing lung cells following ex vivo
711 restimulation with PPD prior to intracellular cytokine staining and Boolean OR gating. Symbols are data from
712 individual mice with lines indicating mean ± SD. Statistical significance was assessed by two-way ANOVA with
713 Turkey's multiple comparison test, *$P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

714 (f) Bacterial burden in lungs and spleens of vaccinated and PBS treated mice. Mice were infected with Mtb
715 H37Rv by aerosol 90 days post vaccination. CFU on day one post infection were 90 ± 17. Symbols represent
716 data from individual mice with lines indicating mean ± SEM. Statistical significance was assessed by two-way
717 ANOVA with Turkey's multiple comparison test, *$P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$, ns = not
718 significant.

719

720 **Figure 4 Discontinuation of doxy limits BCG-TetOFF-DL persistence in NHP.** (a) Persistence study design
721 (b) Memory CD4+ pre vaccination, memory effector CD4+ 4 weeks post vaccination, memory CD8+ pre
722 vaccination and memory CD8+ 4 weeks post vaccination (n=3/group) total cell numbers isolated from the BAL,
723 stimulated with H37Rv WCL.Two-way ANOVA was performed. There were no treatment effects, but total cells
724 increased over time in all cell types. Median and range shown. (c) Gross pathology scores at necropsy. (d-g)
725 Bacterial burden (CFU) of BCG-TetOFF-DL CFU recovered from total body of animal (d), thoracic cavity (e),
726 lungs (f) and thoracic lymph nodes (g) (n=3/group, mean and SD shown). Number of effector CD4+ cells (h) and
727 CD8+ cells (i) per gram of lung tissue, stimulated with media or H37Rv WCL (average of 4 lung tissues per
728 animal, n=3 animals/group). Memory is defined as CD45RA+CD28-, CD45RA-CD28+ or CD45RA-CD28-.

729

730 **Figure 5 BCG-TetOFF-DL provides robust protection against Mtb infection in macaques.** (a) Efficacy study
731 design. (b, c) Number of lung granulomas over time (b) and at necropsy (c). (d, e) Total lung FDG activity by

732 PET imaging over time (d) and at necropsy (e). (f) Gross pathology scores at necropsy. (g) Total thoracic
733 bacterial burden at necropsy. (h) Fisher's exact test showing trend of higher levels of sterility in animals
734 vaccinated with BCG-TetOFF-DL versus WT BCG Pasteur; Fisher's exact p-value reported. Total bacterial
735 burden in lungs (i) and thoracic lymph nodes (j). WT BCG (n=8), BCG-TetOFF-DL (n=8) and unvaccinated
736 animals (n=10). Stars represent historical controls. Lines represent the median and range. Statistic for c, e, f, g,
737 i, j Kruskal-Wallis tests were performed and Dunn's p-values (adjusted for multiple comparisons) were reported.
738
739
740 **Figure 6 BCG-TetOFF-DL induces more CD4 T cells producing cytokines in lung tissue compared to WT**
741 **BCG. (a)** Frequency of CD4+ and CD8+ T-cells as a percentage of CD3+ cells isolated from homogenized lung
742 tissue at necropsy (WT BCG , BCG-TetOFF-DL n=8, unvaccinated n = 2). Frequency of effector CD4+ and CD8+
743 T-cells in the lung (b) and thoracic lymph nodes (c) producing cytokines or cytotoxic molecules. Mean with SD
744 shown. Statistics: Mann-Whitney p values reported comparing BCG-TetOFF-DL and WT BCG.
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759 **Figure S1. Single and dual lysin kill switches**. (a) TetON single lysin kill switch schematic. Lysin expression
760 is repressed by tetracycline repressor (TetR) and induced by anhydrotetracycline (atc) or doxycycline (doxy)
761 which bind TetR and prevent it from binding DNA.

762 (b) Impact of atc on growth of BCG-TetON single lysin strains. The paper disc contains 1 μg of atc.

763 (c) TetOFF dual lysin kill switch schematic. Reverse TetR (Rev TetR) binds to DNA in complex with atc or doxy
764 to repress D29L. TetR represses PipR and in the presence of atc/doxy PipR is expressed and represses L5L.

765 (d) Impact of atc on growth of BCG-TetOFF-DL. The paper disc contains 1 μg of atc.

766

767 **Figure S2. In vitro characterization of BCG kill switch strains.**

768 (a) Growth of BCG TetON single and dual lysin kill switch strains with and without atc. $OD_{580}$ data are means
769 from duplicate cultures.

770 (b) Western blot analysis of culture filtrates from BCG-TetON-DL and BCG-TetOFF-DL strains grown in the
771 absence of detergent. A whole cell lysate of WT BCG serves as control. Eno and PrcB were enriched in culture
772 filtrate of BCG-TetON-DL after 6 and 9 days of growth in the presence of atc and in culture filtrate of BCG-
773 TetOFF-DL after 6 and 9 days of growth in the absence of atc indicating cell lysis.

774 (c) Expression of two lysins reduces the fraction of escape mutants compared to expression of single lysins.
775 Cultures were grown in the presence of atc. Symbols represent data from 3-6 individual cultures and means ±
776 SD are depicted. Significance was determined by one-way analysis of variance (ANOVA) and adjusted for
777 multiple comparisons. with Turkey's multiple comparison test; **** $P < 0.0001$.

778 (d) Growth of BCG TetOFF single and dual lysin kill switch strains with and without atc. Data are means ± SD
779 from triplicate cultures. Error bars are too small to be seen.

780 (e) Expression of two lysins reduces the fraction of escape mutants compared to expression of single lysins.
781 Cultures were grown in the absence of atc. Symbols represent data from 6 individual cultures and means ± SD
782 are depicted. Statistical significance was assessed by one-way ANOVA with Turkey's multiple comparison test;
783 *** $P = 0.0008$; **** $P < 0.0001$.

784

785 **Figure S3. Survival of BCG, BCG-TetOFF-DL and BCG-TetON-DL following intravenous vaccination**.

786 (a) CFU from lungs and spleens of mice infected with BCG and BCG-TetOFF-DL not receiving doxy.

787 (b) CFU quantification from lungs and spleens of mice infected with BCG and BCG-TetON-DL treated with doxy.

788 Data are means ± SEM from 4-5 mice per group and time point.

789

790 **Figure S4. BCG-TetON-DL and wt BCG provide similar protection against Mtb infection in mice.** Mice

791 were i.v. vaccinated with BCG or BCG-TetON-DL or received PBS.

792 (a,b) Quantification of effector memory CD4 and CD8 T cells in mouse lungs from mice vaccinated with BCG

793 and BCG-TetON-DL. Symbols are data from individual mice with lines indicating mean ± SD. Statistical

794 significance was assessed by two-way ANOVA with Turkey's multiple comparison test, *$P < 0.05$, ** $P < 0.005$,

795 *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

796 (c) Quantification of cytokine producing antigen specific CD4 T cells from mice vaccinated with BCG and BCG-

797 TetON-DL on day 30 post vaccination. Lung cells were restimulated ex vivo with PPD prior to intracellular

798 cytokine staining.

799 (d) Bacterial burden in lungs and spleens of vaccinated and PBS treated mice. Mice were infected with Mtb

800 H37Rv by aerosol 90 days post vaccination. Symbols represent data from individual mice with lines indicating

801 mean ± SEM. Statistical significance was assessed by two-way ANOVA with Turkey's multiple comparison test,

802 *$P < 0.05$, ** $P < 0.005$, **** $P < 0.0001$, ns = not significant.

803

804 **Figure S5 Histologic analysis indicates microgranulomas in spleen, liver and lymph nodes 8 weeks post-**

805 **BCG-TetOFF-DL.** H&E staining of fixed spleen (a), lymph node (b) and liver (c) tissue. (d) Spleen size at

806 necropsy for macaques in persistence study and macaques vaccinated and challenged with Mtb. Dashed lines

807 represent normal spleen size range of adult male MCMs. (e, f) Lymphocyte composition of lung tissue (e, n=4)

808 and thoracic lymph nodes (f, n=3) from NHP in persistence study at necropsy.

809

810 **Figure S6 T cell responses in airways are similar between BCG-TetOFF-DL and WT BCG after**
811 **vaccination.** Total number of cells, CD4+, CD8+, effector memory CD4+ and CD8+ cells during the vaccination
812 phase with BCG-TetOFF-DL, WT BCG or unvaccinated macaques. BAL samples were obtained pre-vaccination
813 and 4, 12, 20 weeks post vaccination and stimulated with Mtb WCL. Flow cytometry was performed with
814 intracellular staining for effector molecules IFN-γ, TNF, IL-17, IL-2, GzmB, GzmK, granulysin or perforin. Multiple
815 unpaired t-tests were used to compare groups at each time point. Holm-Šídák adjusted p-values are reported.
816 Median and IQR shown.
817
818 **Figure S7 Individual effector molecules produced by T cells in lungs of vaccinated and challenged NHP.**
819 Total number of CD4+, CD8+, effector memory CD4+ and CD8αβ+ cells in the lung at necropsy (n=8). Cells
820 producing either cytokines or cytotoxic molecules (IFN-γ, TNF, IL-17, IL-2, GzmB, GzmK, granulysin or perforin)
821 were analyzed. Mann-Whitney p-values reported. Median shown.
822
823 **Table S1: Flow cytometry panel for NHP samples**
824
825 **Table S2 Full details on macaques used in this study**
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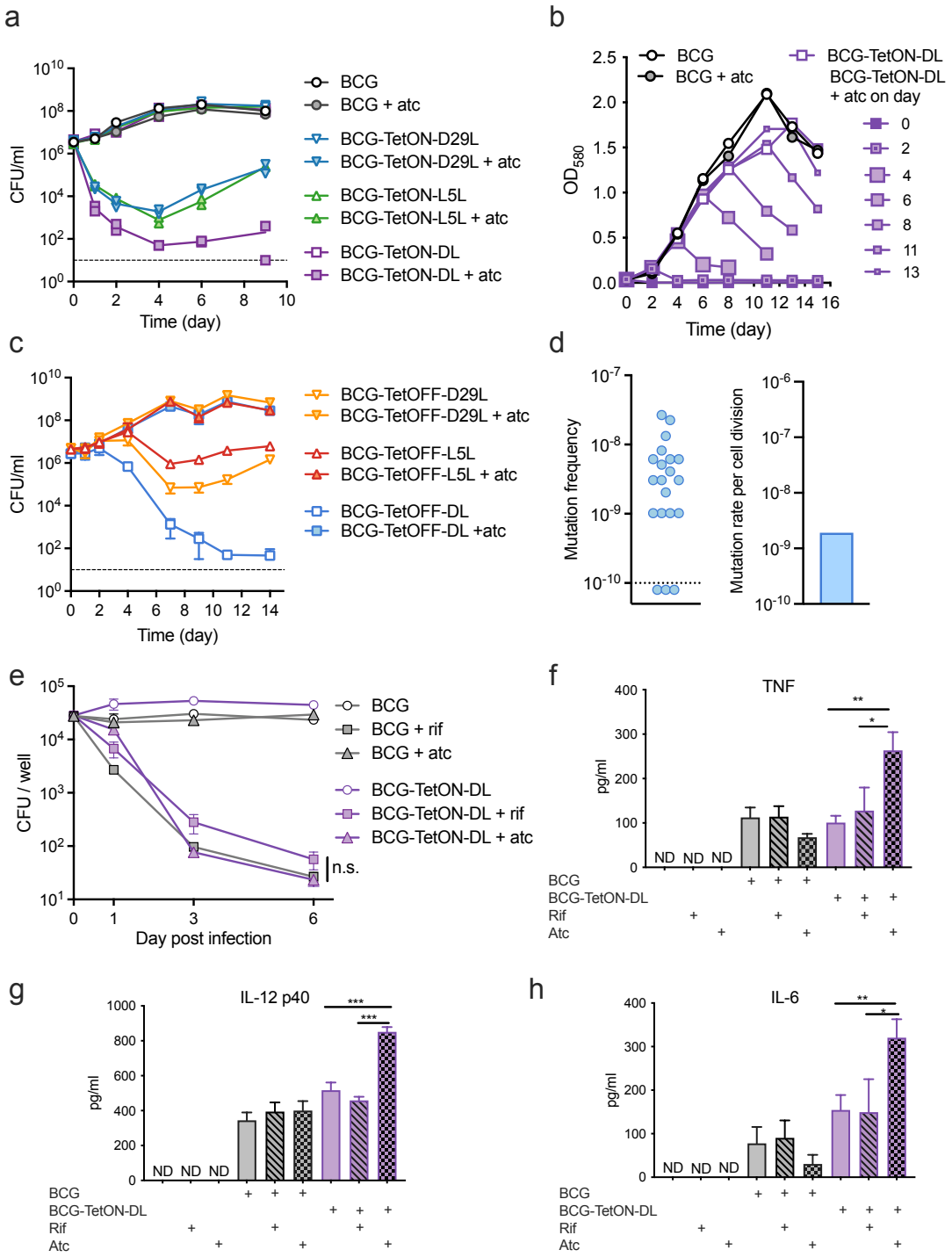
a
CFU/ml
Time (day)
BCG
BCG + atc
BCG-TetON-D29L
BCG-TetON-D29L + atc
BCG-TetON-L5L
BCG-TetON-L5L + atc
BCG-TetON-DL
BCG-TetON-DL + atc
b
OD580
Time (day)
BCG
BCG + atc
BCG-TetON-DL
BCG-TetON-DL + atc on day
0
2
4
6
8
11
13
c
CFU/ml
Time (day)
BCG-TetOFF-D29L
BCG-TetOFF-D29L + atc
BCG-TetOFF-L5L
BCG-TetOFF-L5L + atc
BCG-TetOFF-DL
BCG-TetOFF-DL +atc
d
Mutation frequency
Mutation rate per cell division
e
CFU / well
Day post infection
BCG
BCG + rif
BCG + atc
BCG-TetON-DL
BCG-TetON-DL + rif
BCG-TetON-DL + atc
n.s.
f
TNF
pg/ml
ND ND ND
BCG
BCG-TetON-DL
Rif
Atc
g
IL-12 p40
pg/ml
ND ND ND
BCG
BCG-TetON-DL
Rif
Atc
h
IL-6
pg/ml
ND ND ND
BCG
BCG-TetON-DL
Rif
Atc

**Figure 1**

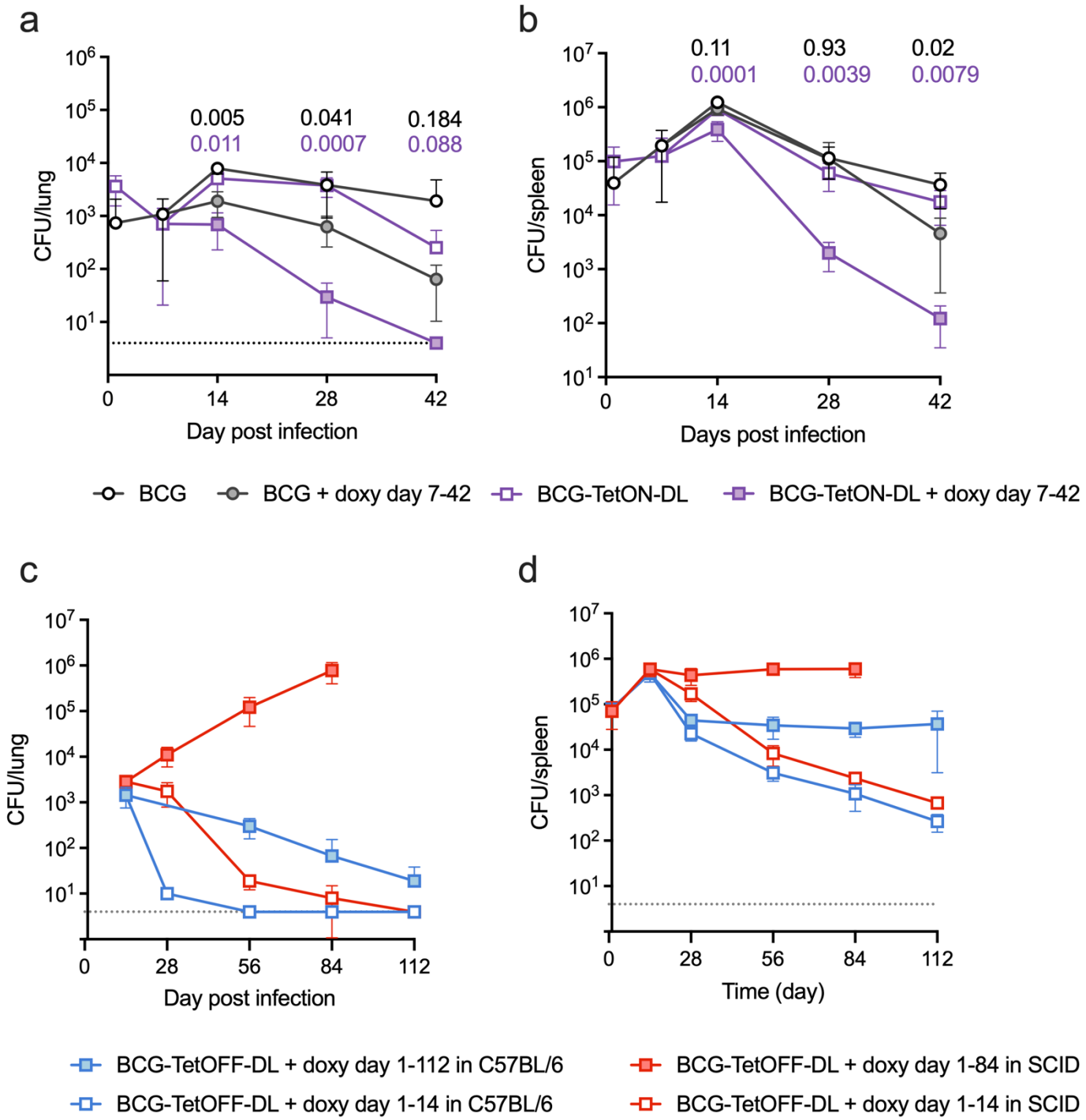
a
CFU/lung
Day post infection
0.005
0.011
0.041
0.0007
0.184
0.088
b
CFU/spleen
Days post infection
0.11
0.0001
0.93
0.0039
0.02
0.0079
BCG
BCG + doxy day 7-42
BCG-TetON-DL
BCG-TetON-DL + doxy day 7-42
c
CFU/lung
Day post infection
d
CFU/spleen
Time (day)
BCG-TetOFF-DL + doxy day 1-112 in C57BL/6
BCG-TetOFF-DL + doxy day 1-14 in C57BL/6
BCG-TetOFF-DL + doxy day 1-84 in SCID
BCG-TetOFF-DL + doxy day 1-14 in SCID

**Figure 2**

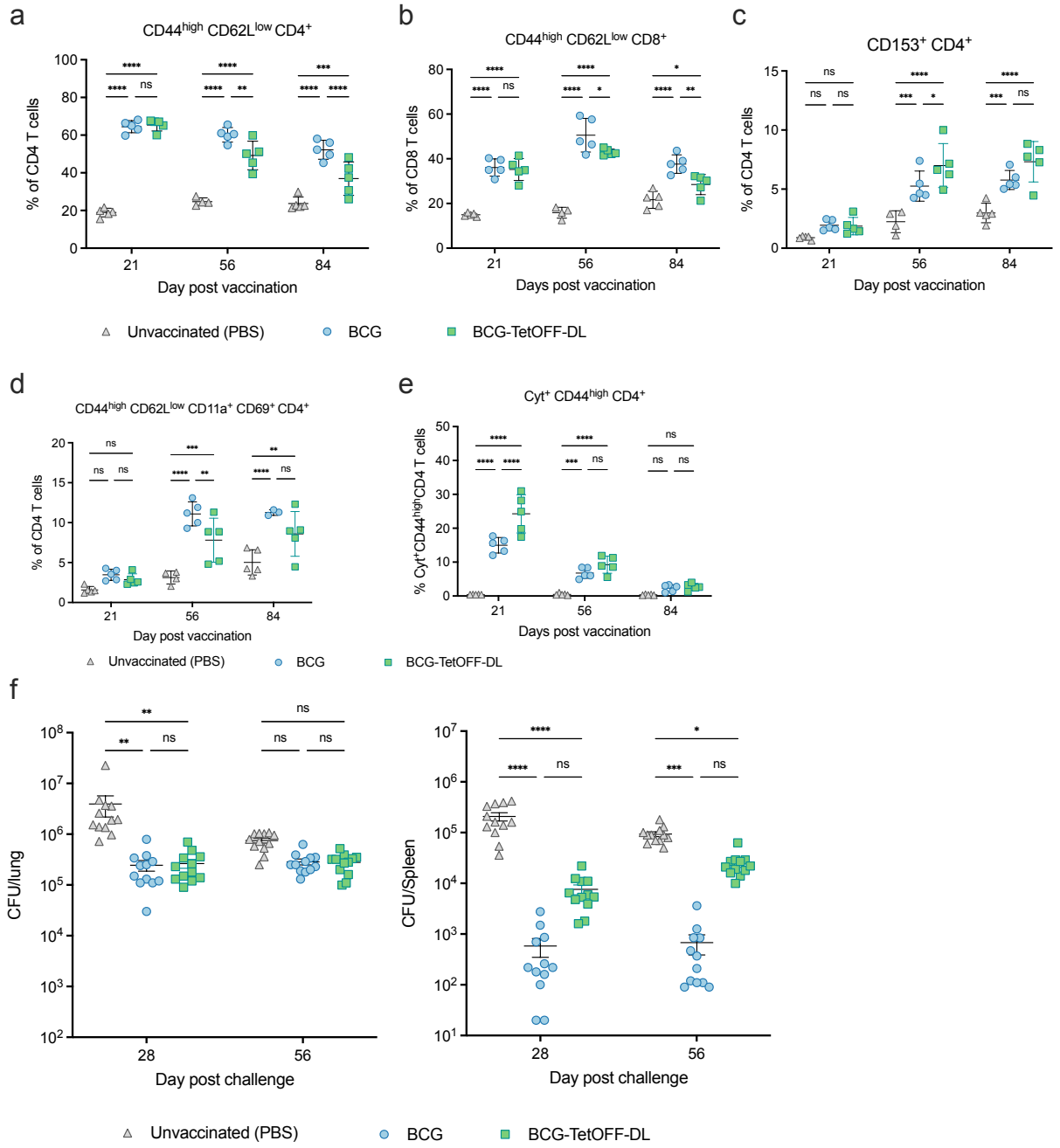
a
CD44high CD62Llow CD4+
% of CD4 T cells
Day post vaccination
b
CD44high CD62Llow CD8+
% of CD8 T cells
Days post vaccination
c
CD153+ CD4+
% of CD4 T cells
Day post vaccination
Unvaccinated (PBS)
BCG
BCG-TetOFF-DL
d
CD44high CD62Llow CD11a+ CD69+ CD4+
% of CD4 T cells
Day post vaccination
e
Cyt+ CD44high CD4+
% Cyt+CD44highCD4 T cells
Days post vaccination
f
CFU/lung
Day post challenge
CFU/Spleen
Day post challenge

**Figure 3**

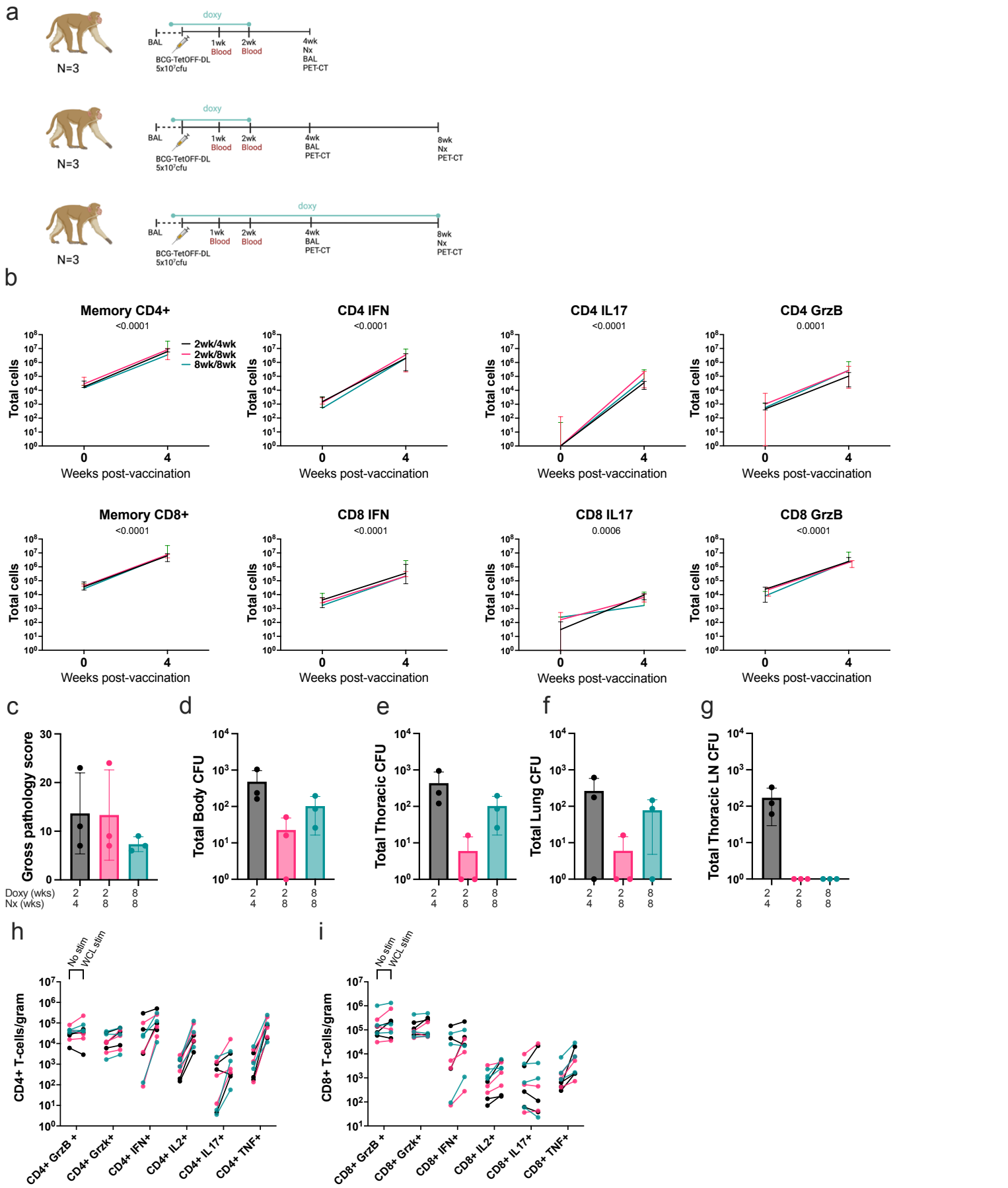
a
N=3
doxy
BAL
1wk Blood
2wk Blood
4wk Nx BAL PET-CT
8wk Nx PET-CT
BCG-TetOFF-DL 5x10⁷cfu
b
Memory CD4+
<0.0001
CD4 IFN
<0.0001
CD4 IL17
<0.0001
CD4 GrzB
0.0001
Memory CD8+
<0.0001
CD8 IFN
<0.0001
CD8 IL17
0.0006
CD8 GrzB
<0.0001
2wk/4wk
2wk/8wk
8wk/8wk
Total cells
Weeks post-vaccination
c
Gross pathology score
d
Total Body CFU
e
Total Thoracic CFU
f
Total Lung CFU
g
Total Thoracic LN CFU
Doxy (wks)
Nx (wks)
h
CD4+ T-cells/gram
No stim
WCL stim
CD4+ GrzB +
CD4+ Grzk+
CD4+ IFN+
CD4+ IL2+
CD4+ IL17+
CD4+ TNF+
i
CD8+ T-cells/gram
CD8+ GrzB +
CD8+ Grzk+
CD8+ IFN+
CD8+ IL2+
CD8+ IL17+
CD8+ TNF+

**Figure 4**

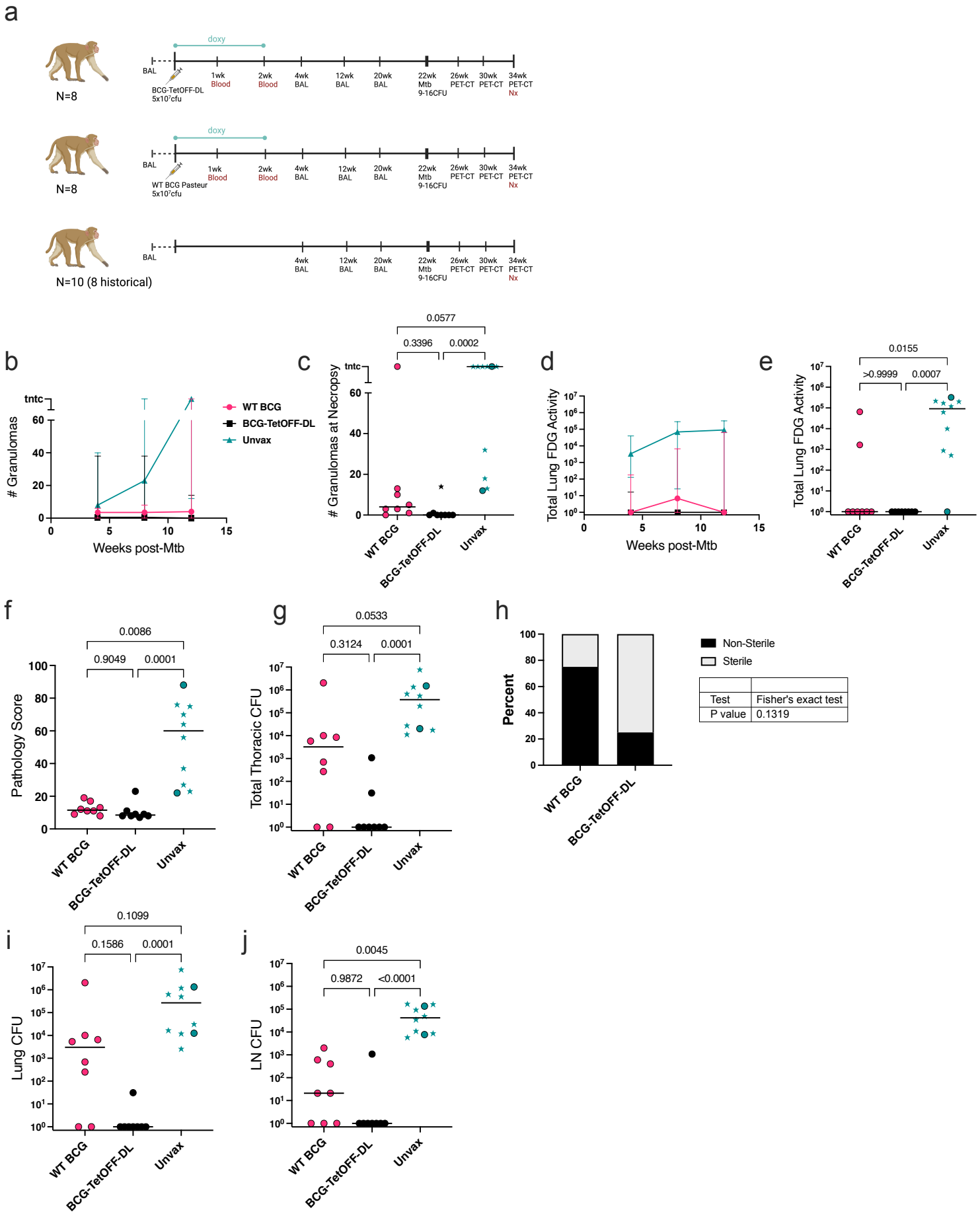
a
N=8
doxy
BAL
BCG-TetOFF-DL
5x10⁷cfu
1wk Blood
2wk Blood
4wk BAL
12wk BAL
20wk BAL
22wk Mtb 9-16CFU
26wk PET-CT
30wk PET-CT
34wk PET-CT Nx
N=8
WT BCG Pasteur
5x10⁷cfu
N=10 (8 historical)
b
tntc
# Granulomas
Weeks post-Mtb
WT BCG
BCG-TetOFF-DL
Unvax
c
0.0577
0.3396
0.0002
# Granulomas at Necropsy
d
Total Lung FDG Activity
Weeks post-Mtb
e
0.0155
>0.9999
0.0007
Total Lung FDG Activity
f
0.0086
0.9049
0.0001
Pathology Score
g
0.0533
0.3124
0.0001
Total Thoracic CFU
h
Percent
Non-Sterile
Sterile
Test
Fisher's exact test
P value
0.1319
i
0.1099
0.1586
0.0001
Lung CFU
j
0.0045
0.9872
<0.0001
LN CFU

**Figure 5**

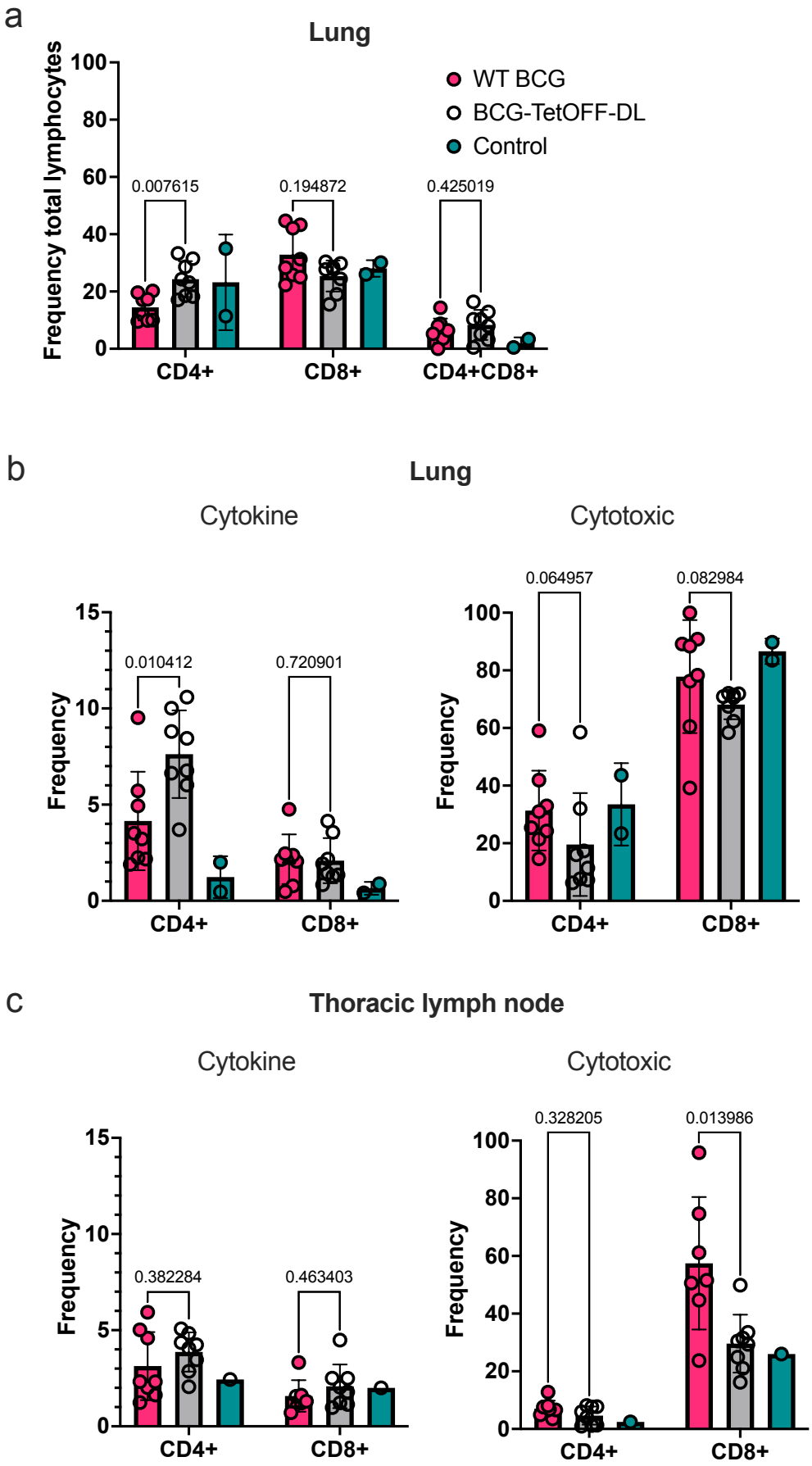
a
Lung
Frequency total lymphocytes
WT BCG
BCG-TetOFF-DL
Control
0.007615
0.194872
0.425019
CD4+
CD8+
CD4+CD8+
b
Lung
Cytokine
Cytotoxic
Frequency
0.010412
0.720901
0.064957
0.082984
CD4+
CD8+
c
Thoracic lymph node
Cytokine
Cytotoxic
Frequency
0.382284
0.463403
0.328205
0.013986
CD4+
CD8+

**Figure 6**