

# 1 Efficacy and immunogenicity of different BCG doses in BALB/c and 2 CB6F1 mice when challenged with H37Rv or Beijing HN878

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## 10 Abstract

11  
12  
13 In this study, 2 strains of mice (BALB/c and CB6F1) were vaccinated with a range of Bacille Cal-  
14 mette-Guérin (BCG) Danish doses from  $3 \times 10^5$  to 30 CFU/mouse, followed by either immunogenicity  
15 evaluation or aerosol infection with *Mycobacterium tuberculosis* (a laboratory strain H37Rv or West-  
16 Beijing HN878 strain). The results indicated that both strains of mice when infected with HN878 ex-  
17 hibited significant protection in their lungs with BCG doses at  $3 \times 10^5$  – 3000 CFU (BALB/c) and  
18  $3 \times 10^5$ – 300 CFU (CB6F1). Whereas, both strains of mice when infected with H37Rv, significant pro-  
19 tection was seen in BCG doses at  $3 \times 10^5$  – 300 CFU. Immunological evaluation revealed interesting  
20 results; i) both strains of mice demonstrated a significant increase in the frequencies of BCG-specific  
21  $IFN\gamma^+$   $IL2^+$   $TNF\alpha^+$  CD4 T cells in the BCG doses at  $3 \times 10^5$  – 3000 CFU (BALB/c) and  $3 \times 10^5$ – 300  
22 CFU (CB6F1); ii) secretion of IL2 and  $IFN\gamma$  were correlated with the bacterial burden in the lungs of  
23 HN878 infected CB6F1 mice. The study demonstrated a BCG dose at 3000 CFU (an equivalent single  
24 human dose in the mice by body weight index) is protective in both strains of mice and the use of a  
25 virulent clinical isolate in testing new tuberculosis vaccine/advancing research is recommended.  
26

## 27 Introduction

28 Bacille Calmette-Guérin (BCG) is one of the most widely used injectable vaccines, administered pre-  
29 dominantly in neonates, but confers incomplete and variable protection against pulmonary tuberculo-  
30 sis (TB) in humans <sup>1, 2, 3</sup>. Despite its widespread use, an estimated 1.5 million people in 2018 died due  
31 to the TB disease <sup>4</sup>. Studies in the field of infectious diseases, especially for intracellular pathogens,  
32 acknowledges the complex interplay of the magnitude, speed and qualitative nature of protective im-  
33 mune responses by chronic infections (such as TB, leprosy, leishmaniasis) which is not as straightfor-  
34 ward as an effective antibody response against extracellular pathogens <sup>5</sup>.

35 Although there are many animal models which are used in conducting TB research and testing vac-  
36 cine candidates or drugs against TB, the mouse model is the most preferred choice mainly due to the  
37 small animal size, vast array of commercially available reagents and low cost of running an experi-  
38 ment. Having recognised the limitations of animal models in terms of predicting outcomes in humans,  
39 moving forward, it is vital to improve animal models wherever possible <sup>6, 7</sup>. From the decades of pub-  
40 lished animal TB challenge studies <sup>7, 8, 9, 10</sup>, it is evident to state that protective responses induced by  
41 BCG vaccine against TB in mice varies when compounded by the choice of mouse strain <sup>11</sup>, strains of  
42 *Mycobacteria tuberculosis* (*Mtb*) for infection, route of immunisation and the interval between BCG  
43 vaccination and *Mtb* challenge. Therefore, our aim was to optimise some of these parameters by ana-  
44 lysing the efficacy of different doses of BCG against two strains of *Mtb* namely, laboratory TB strain  
45 H37Rv and a virulent clinical strain, W-Beijing HN878 (HN878) and using each strain of *Mtb* in the  
46 head-to-head comparison using two types of mouse strains, BALB/c and CB6F1 (F1).

47 The study was divided into two parts; 1. H37Rv or HN878 challenge - different doses of BCG were  
48 evaluated by measuring *Mtb* burden in the lungs and spleen of mice infected with either *Mtb* strain  
49 and; 2. Immunogenicity evaluation - cellular immune responses were analysed using flow cytometry,  
50  $IFN-\gamma$  ELISPOT and multiplex cytokine/chemokine assays in equivalent BCG vaccinated and control  
51 mice.

52 The overall results demonstrated differences in the protection and cellular immune responses in both  
53 strains of mice. Our findings will benefit refinement of the TB challenge mouse model for the pre-  
54 clinical testing and selection of new TB vaccines.

55

## 56 **Results**

57 In the initial experiment where BALB/c and F1 mice were vaccinated with a full range of BCG doses  
58 ( $3 \times 10^5$ ,  $3 \times 10^4$ , 3000, 300 or 30 CFU/mouse) followed by H37Rv challenge, a comparable protection  
59 was seen in the lungs of mice vaccinated with BCG dose at  $3 \times 10^4$  and 3000 CFU (Fig. 1A).  
60 Considering the principle of replacement, reduction and refinement (3Rs) in experimental design  
61 using animals, BCG dose at  $3 \times 10^4$  CFU was omitted from HN878 challenge experiments and the  
62 immunogenicity evaluation study. Concentrations of all serially diluted BCG doses (CFU/ml) were  
63 plated on to 7H11 agar plates and confirmed to be within the accepted range (Supplementary Table 1).  
64 An infection inoculum for H37Rv or HN878 were also confirmed using 7H11 agar plates and shown  
65 to be within the expected range of  $3.6\text{-}5 \times 10^6$  CFU/ml or  $4\text{-}7 \times 10^6$  CFU/ml, respectively to achieve  
66 low dose aerosol infection of approximately 100 CFU per mouse. The result for low dose infection in  
67 the lungs of BALB/c mice were, H37Rv - average  $71 \pm 63$  (Std. Dev) CFU and HN878 – average  $144$   
68  $\pm 64$  (Std. Dev) CFU per mouse (Supplementary Table 2).

### 69 ***BCG protection in BALB/c and F1 mice varies when challenged with H37Rv or HN878***

70 Four weeks after BCG vaccination, BALB/c and F1 mice were infected with either H37Rv or HN878.  
71 Animals were sacrificed at 4 weeks post infection and the lungs and spleen were harvested to  
72 determine *Mtb* burden. Fig. 1A shows the graphical representation of the protection data expressed as  
73  $\text{Log}_{10}$  CFU in the lungs and spleen of BALB/c and F1 mice infected with either H37Rv or HN878.  
74 Tables 1A and 1B represent the differences in the mean of  $\text{Log}_{10}$  CFU/organ between all the BCG  
75 vaccinated and the control groups, including within BCG vaccinated groups in the lungs and spleen of  
76 both strains of mice when infected with H37Rv or HN878. For the lowest BCG dose at 30 CFU, *Mtb*  
77 burden ( $\text{Log}_{10}$  CFU/organ) in the lungs of both mouse strains infected with either H37Rv or HN878  
78 were equivalent to the control group (Fig. 1A; Table 1A,1B).

79 H37Rv infected BALB/c and F1 mice vaccinated with BCG at  $3 \times 10^5$  - 300 CFU, displayed significant  
80 protection (Fig. 1A and Table 1) in the lungs when compared to the control group. The protection  
81 within the BCG groups for BALB/c and F1 mice differed. BALB/c mice infected with H37Rv showed  
82 significantly more protection in the lungs of the highest BCG dose  $3 \times 10^5$  CFU (Table 1A) when  
83 compared to all the lower doses of BCG:  $3 \times 10^4$  CFU ( $-0.7 \text{ Log}_{10}$  CFU/lungs), 3000 CFU ( $-0.71 \text{ Log}_{10}$   
84 CFU/lungs), 300 CFU ( $-0.81 \text{ Log}_{10}$  CFU/lungs) and 30 CFU ( $-1.32 \text{ Log}_{10}$  CFU/lungs). In comparison  
85 to BALB/c, H37Rv infected F1 mice displayed comparable protection in the lungs with the highest  
86 dose of BCG  $3 \times 10^5$  CFU when compared to all the lower doses of BCG  $3 \times 10^4$  CFU ( $0.24 \text{ Log}_{10}$   
87 CFU/lungs), 3000 CFU ( $0.16 \text{ Log}_{10}$  CFU/lungs), 300 CFU ( $-0.24 \text{ Log}_{10}$  CFU/lungs), except 30 CFU ( $-$   
88  $1.16 \text{ Log}_{10}$  CFU/lungs) (Table 1A). In the spleen (Fig. 1B), both strains of mice showed significantly  
89 more protection in the groups vaccinated with BCG at  $3 \times 10^5$  - 300 CFU when compared to the control  
90 group. In addition, spleen of F1, but not BALB/c mice, BCG dose at 30 CFU showed significant  
91 protection when compared to the control group, Fig. 1B and Table 1A ( $1.23 \text{ Log}_{10}$  CFU/spleen).

92 When infected with HN878, BALB/c mice only demonstrated significant protection in the lungs with  
93 BCG doses at  $3 \times 10^5$  and 3000 CFU while, F1 mice showed significant protection in the lungs with a  
94 BCG dose as low as 300 CFU when compared to the control group (Fig. 1A and Table 1B). In  
95 addition, F1 mice displayed a dose response pattern in the protection (1.87, 1.42 and 0.78  $\text{Log}_{10}$   
96 CFU/lungs, respectively) when compared to the control group (Fig. 1A, Table 1B). In the spleen (Fig.  
97 1B and Table 1B), F1 mice showed significant protection with BCG at  $3 \times 10^5$  - 300 CFU whereas,  
98 BALB/c mice failed to exhibit protection for all the BCG vaccinated groups when compared to the  
99 control group.

### 100 ***IFN $\gamma$ secreting cells by ELISPOT assay***

101 Given the established critical role of IFN $\gamma$  in controlling TB infection<sup>12, 13</sup>, the frequencies of antigen-  
102 specific IFN $\gamma$  secreting cells in ex vivo isolated cells from the spleen and the lungs of all mice were  
103 evaluated by ELISPOT. All splenocytes and lung cells were stimulated with a defined M7 protein  
104 cocktail. In the spleen and the lungs of BALB/c mice (Fig. 2A), a significantly high frequency of  
105 BCG-specific IFN $\gamma$  secreting cells was evident in the mice vaccinated with BCG at  $3 \times 10^5$  and 3000  
106 CFU whereas, they were equivalent to the control group in the BCG at 300 and 30 CFU groups. In the  
107 spleen of F1 mice, albeit the frequencies of IFN $\gamma$  secreting cells in all the BCG vaccinated groups  
108 were not significantly different, BCG at  $3 \times 10^5$  – 30 CFU displayed higher frequencies of these cells  
109 when compared to the control group. However, in the lungs of F1 mice, only BCG at  $3 \times 10^5$  CFU  
110 found to have significantly higher frequency of IFN $\gamma$  secreting cells whereas, BCG at 3000 and 300  
111 CFU displayed higher responses compared to the control group.

112 Together, these results highlight that in the spleen and the lungs of BALB/c and F1 mice, significant  
113 frequencies of BCG-specific IFN $\gamma$  secreting cells were only detected in the groups that received either  
114 BCG at  $3 \times 10^5$  or 3000 (BALB/c mice only) and not the lower doses.

### 115 ***Multifunctional CD4 T cells producing IFN $\gamma$ , IL2 and TNF $\alpha$***

116 In order to better resolve the functionality of the responding T cells induced by BCG vaccination, M7  
117 stimulated splenocytes from all groups of BALB/c and F1 mice were subsequently interrogated by  
118 intracellular staining (ICS) using 11 colour flow cytometric analysis. The frequency of CD4 T cells  
119 producing any combination of IFN $\gamma$ , IL2, TNF $\alpha$  and IL17a production were assessed. The production  
120 of IL17a was not observed hence, omitted from the results for clarity. As shown in Fig. 3A, BALB/c  
121 and F1 mice induced significantly more multifunctional IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  CD4 T cells with BCG at  
122  $3 \times 10^5$ -3000 CFU and  $3 \times 10^5$ -300 CFU, respectively when compared to the control group. Within the  
123 BCG vaccinated groups,  $3 \times 10^5$  CFU induced a significantly higher frequency of multifunctional  
124 IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  CD4 T cells in BALB/c when compared to 3000 CFU and in F1 mice when com-  
125 pared to 3000 and 300 CFU.

126  
127 For both strains of mice, cytokine producing splenocytes IFN $\gamma^+$ TNF $\alpha^+$  CD4 T cells were significantly  
128 induced in BCG at  $3 \times 10^5$ -3000 CFU when compared to the control group (Fig. 3A). In addition, F1  
129 mice induced IFN $\gamma^+$ TNF $\alpha^+$  CD4 T cells in 300 CFU compared to the control group (Fig. 3A). None  
130 of the mouse strain exhibited multifunctional IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  or IFN $\gamma^+$ TNF $\alpha^+$  CD4 T cells in the  
131 BCG at 30 CFU group. The cytokine producing CD4 T cells detected here, displayed a  
132 CD44<sup>hi</sup>CD62L<sup>lo</sup> phenotype, indicative of CD4 T Effector Memory (TEM), data not shown.

133

134 To understand the significance of correlation between the means of BCG-specific multifunctional  
135 CD4 TEM cells in the spleen and *Mtb* burden in the lungs of H37Rv or HN878 infected BALB/c and  
136 F1 mice in the equivalent vaccine groups, we performed correlation coefficient analysis. As shown in  
137 Fig. 3B and 3C, the correlations were analysed between the mean of IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  or IFN $\gamma^+$   
138 TNF $\alpha^+$  CD4 T cells and the mean of *Mtb* burden as expressed in Log<sub>10</sub> CFU/lungs. Multifunctional  
139 IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  CD4 T cells were significantly inversely correlated with HN878 infected BALB/c ( $r$   
140 = -0.918,  $p$  = 0.0278) and F1 mice ( $r$  = -0.958,  $p$  = 0.0103). However, correlation was not seen with  
141 H37Rv infected BALB/c ( $r$  = -0.8325,  $p$  = 0.0802) and F1 mice ( $r$  = -0.7844,  $p$  = 0.1162), data not  
142 shown. For IFN $\gamma^+$ TNF $\alpha^+$  CD4 T cells, a significantly inverse correlation was exhibited in BALB/c  
143 mice infected with either H37Rv ( $r$  = -0.9038,  $p$  = 0.0353) or HN878 ( $r$  = -0.9043,  $p$  = 0.035). Again,  
144 for IFN $\gamma^+$ TNF $\alpha^+$  CD4 T cells, correlation was not seen in F1 mice infected with H37Rv ( $r$  = -0.7844,  
145  $p$  = 0.1162) or HN878 ( $r$  = -0.8641,  $p$  = 0.0589), data not shown.

146 These results highlight the induction of multifunctional IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  or IFN $\gamma^+$ TNF $\alpha^+$  CD4 T cells  
147 in both strains of mice exhibiting dose response to the vaccine. The highest BCG dose group,  $3 \times 10^5$   
148 CFU induced significantly higher frequencies of these CD4 TEM cells when compared to the BCG at  
149 3000 – 30 CFU groups. In addition, the IFN $\gamma^+$ IL2 $^+$ TNF $\alpha^+$  CD4 T cells inversely correlated with the  
150 HN878 infected BALB/c and F1 mice which demonstrates, the higher the proportion of these cells,  
151 the lower is *Mtb* burden in the lungs of subsequently infected mice. Interestingly, IFN $\gamma^+$ TNF $\alpha^+$  CD4

152 T cells inversely correlated with the *Mtb* burden in the BALB/c mice with both *Mtb* challenge strains,  
153 but not with F1 mice.

#### 154 ***Differential expression of cytokines/chemokines in BALB/c and F1 mice***

155 Splenocytes and lung cells isolated from the BCG vaccinated and control groups were stimulated with  
156 M7 protein cocktail for 3 days and the supernatants were collected. Analyses of cytokines and  
157 chemokines, as listed in the Table 2, were performed in the supernatants of cultured cells (stimulated  
158 and unstimulated) using Bio-Plex Pro Mouse Chemokine Panel. For the data analysis, an estimated  
159 concentration (pg/ml) of cytokine/chemokine were interpolated from the linear part of the 5-parameter  
160 standard curve. Furthermore, the interpolated concentrations from the supernatants of stimulated cells  
161 were subtracted with those of unstimulated cells supernatant and these concentrations were used for  
162 the data analysis. Fig. 4A are the representative graphs of cytokines/chemokines obtained from the  
163 spleen or the lungs of BALB/c or F1 mice and, the concentrations for all cytokines/chemokines from  
164 different samples are presented as the radar plots with the numerical data tabulated in the supplement-  
165 ary Table 3. Groups vaccinated with BCG at  $3 \times 10^5$  CFU exhibited greater variability in the lungs of  
166 BALB/c mice compared to the other BCG vaccinated groups. For the ease of result presentation, sig-  
167 nificance in all the BCG vaccinated groups have been compared with the control group, unless de-  
168 picted otherwise.

169 As expected, secreted IFN $\gamma$  concentration in the splenocytes of BALB/c mice (Fig. 4A) were detect-  
170 able at significantly higher concentration in the BCG at  $3 \times 10^5$  and 3000 CFU groups. However, al-  
171 though IFN $\gamma$  responses were not significantly different, the splenocytes of F1 mice displayed an ele-  
172 vated response in the BCG at  $3 \times 10^5$  – 300 CFU groups (Fig. 4A). Interestingly, a significantly higher  
173 IL2 concentration (Fig. 4A&B) in the BCG at  $3 \times 10^5$  CFU group for the F1 (spleen and lungs) and  
174 BALB/c (lungs) mice were observed. The elevated responses of IL2 were also detected in the spleno-  
175 cytes and the lung cells of F1 (BCG at 3000 – 300 CFU groups) and BALB/c (BCG at 3000 CFU  
176 group) mice.

177 Surprisingly, the concentrations of secreted chemokines, CCL2, CXCL1 and CXCL2 were signifi-  
178 cantly lower from the splenocytes of all the BCG vaccinated compared to the control group for  
179 BALB/c, but not F1 mice. For F1 mice, secreted IL6, TNF $\alpha$ , CCL4 and CCL22 concentrations from  
180 the lung cells were lower in all the BCG vaccinated groups and displaying significant differences in  
181 the BCG at 3000 – 30 CFU groups when compared to the control group. In contrast to the F1 mice,  
182 the lung cells of BALB/c mice secreted significantly higher concentrations of IL6 and CCL4 for  
183  $3 \times 10^5$  CFU BCG group when compared to the control group. The rest of the cytokines/chemokines  
184 shown in Fig. 4A secreted elevated concentrations in the  $3 \times 10^5$  CFU BCG group when compared to  
185 the control and 3000 – 30 CFU BCG groups.

186 An overview of the graphical visualisation, all concentrations of cytokines/chemokines in the lungs  
187 and spleen of BALB/c and F1 mice have been presented in the radar plots (Fig. 4B) except, for  
188 CXCL2 from the supernatants of the lung cells of BALB/c mice which were substantially higher in  
189 concentration. Therefore, for the visual representation in the radar plot for BALB/c (lungs), CXCL2  
190 concentration have been removed.

#### 191 ***Correlation of cytokine/chemokine with TB burden in the lungs of BALB/c and F1 mice***

192 IFN $\gamma$  is the only soluble immunological marker currently used to identify TB infection in the clinical  
193 settings by using an IFN $\gamma$  release assay. Therefore, we sought to investigate the correlation of vaccine  
194 induced IFN $\gamma$  and other cytokines/chemokines with the *Mtb* burden in the lungs of BALB/c and F1  
195 mice subsequently infected with either H37Rv or HN878.

196 Firstly, correlation between the IFN $\gamma$  concentration and other cytokine/chemokine concentrations  
197 from the stimulated cell supernatants were investigated (Supplementary Table 4). For the correlation  
198 analysis, the mean of IFN $\gamma$  concentration was correlated with the mean concentration of other cyto-  
199 kines/chemokines from the supernatants of splenocytes/lung cells of BALB/c and F1 mice. For exam-  
200 ple, the mean of IFN $\gamma$  concentration from BALB/c splenocytes was correlated with the mean concen-  
201  
202  
203  
204



205 trations of other cytokines/chemokines within the same supernatant sample. As shown in the Supple-  
206 mentary Table 4, IFN $\gamma$  responses in the splenocytes of BALB/c mice significantly correlated with IL6  
207 ( $p = 0.0406$ ) and CCL5 ( $p = 0.0229$ ). In the splenocytes of F1 mice, IL2 ( $p = 0.037$ ), CCL1 ( $p =$   
208  $0.0062$ ) and CCL5 ( $p = 0.0441$ ) were significantly correlated with the IFN $\gamma$  responses. In addition,  
209 IL2 responses were strongly correlated with the IFN $\gamma$  responses in the lung cells of BALB/c ( $p =$   
210  $0.0002$ ) and F1 ( $p = 0.0088$ ) mice.

211  
212 To gain further insight for whether the cytokines/chemokines which correlated with the IFN $\gamma$  re-  
213 sponses in the BCG vaccinated groups (Supplementary Table 4) also correlated with the *Mtb* burden  
214 in the lungs of BALB/c or F1 mice, therefore, we investigated using H37Rv or HN878 infected  
215 BALB/c and F1 mice (Fig. 5 and Table 3). The analysis produced an intriguing observation for IL2  
216 correlation. Spleen IL2 (Fig. 5B, Table 3) responses were significantly inversely correlated with the  
217 lungs Log<sub>10</sub> CFU of F1 mice infected with both strains of *Mtb* - H37Rv ( $p = 0.0310$ ) or HN878 ( $p =$   
218  $0.0032$ ) whereas, BALB/c mice did not show any statistically significant correlation with IL2 re-  
219 sponses. As expected, IFN $\gamma$  responses in the lungs (Fig. 5A, Table 3), but not in the spleen (Fig. 5A)  
220 of F1 mice significantly correlated with the *Mtb* burden in the lungs of HN878 infected F1 mice. In  
221 contrast, only HN878 infected BALB/c mice showed significant correlation of *Mtb* burden in the  
222 lungs with the spleen IFN $\gamma$  (Table 3).

223

## 224 Discussion

225 NIBSC have been supporting the international efforts to develop new TB vaccines using a specialised  
226 murine aerosol TB challenge model for testing new vaccine candidates. As a recognised WHO  
227 reference laboratory, NIBSC established the WHO reference reagents for BCG vaccine of various  
228 substrains<sup>14, 15</sup>. Since then, our laboratory has been involved in investigating the mechanism of  
229 protective immune responses elicited by the BCG and a continuous effort in refining TB mouse  
230 models as the first line of screening tool for the selection of new TB vaccine candidates.

231 This study was designed to accommodate and address suppositions or observations as 1. To evaluate  
232 protection of various doses of BCG in BALB/c and F1 mice when infected with H37Rv or HN878  
233 and to understand the protective cellular immunity induced by various BCG doses; 2. For the  
234 refinement of TB mouse model, we were interested to evaluate protective responses of BCG dose at  
235 3000 CFU. BCG dosage at  $1-4 \times 10^5$  CFU is given to infants, preferably at the time of birth. The  
236 commonly used BCG dose in mice is also at  $1-4 \times 10^5$  CFU/mouse. If the body weight of an infant  
237 and an adult mouse is considered, the BCG dose at  $3 \times 10^5$  CFU given to a mouse would be around  
238 150 times in excess compared to the BCG dose in an infant. Therefore, most of the research  
239 conducted in mice using BCG dose at  $1-4 \times 10^5$  CFU would be around 2 Log higher compared to  
240 human and the immune parameters measured would fall under the high dose BCG responses.  
241 According to the study by Bretscher<sup>16, 17</sup> high dose of BCG would tend to skew the immune response  
242 more towards the mixture of Th1 and Th2. Therefore, it was pertinent to evaluate the protection and  
243 immune responses of 3000 CFU in mice, an equivalent dose of BCG  $3 \times 10^5$  CFU in infants; 3. For  
244 the BCG dose sparing in preclinical studies, concerns were raised in 2016<sup>18</sup> for the shortage of global  
245 BCG vaccine and the published study in 2019<sup>19</sup> highlighted the effect of shortage with the rise of TB  
246 meningitis in children. The shortage in global BCG supply has also led to a rethinking of the  
247 established treatment guidelines in rationing of the administration of BCG for bladder cancer<sup>20</sup>.

248 Results from the Log<sub>10</sub> CFU/lungs data demonstrates that the lowest dose at 30 CFU failed to protect  
249 both strains of adult mice when infected via aerosol route with either H37Rv or HN878. However, in  
250 the study conducted by Kiros *et al.*, 2010<sup>17</sup>, BALB/c mice vaccinated with BCG Danish at 30 CFU  
251 protected mice from H37Rv infection via intravenous route of challenge and the study used infant  
252 mice. For BCG doses at  $3 \times 10^5$  to 300 CFU in both strains of mice, protection against TB was  
253 influenced by the type of infecting *Mtb* strain. H37Rv infected BALB/c and F1 mice displayed  
254 significant protection in the lungs when vaccinated with BCG doses at  $3 \times 10^5$  to 300 CFU. However,  
255 for HN878 infected mice, the lowest protective BCG dose for BALB/c mice was BCG at 3000 CFU  
256 and for F1 mice it was 300 CFU. Furthermore, as per the increasing doses of BCG, a decremental

257 trend in the TB burden ( $\text{Log}_{10}$  CFU/lungs) was observed in the lungs of BALB/c and F1 mice when  
258 infected with HN878 whereas, this trend was not seen for H37Rv infected BALB/c and F1 mice.

259 CD4 T cells play a central role for protective immunity against TB, as described by several human  
260 studies and animal models of TB<sup>21,22,23</sup>. The important findings from Sallusto and her colleagues<sup>24,</sup>  
261<sup>25,26</sup> indicated that there were two important subsets of memory T cells - TEM and central memory T  
262 cells (TCM). Differentiation of these memory T cells are based on the clear phenotypic characteristics  
263 - TEM are CD44<sup>hi</sup> CD62L<sup>lo</sup> CCR7<sup>lo</sup> while, TCM are CD44<sup>hi</sup> CD62L<sup>hi</sup> CCR7<sup>hi</sup>. However, recent  
264 evidence depicts a complex interplay and diverse functionality under the broad subsets of memory T  
265 cells<sup>27</sup>. Kaveh *et al.*, 2011<sup>28</sup> reported TEM cells (CD4<sup>+</sup> CD44<sup>hi</sup> CD62L<sup>lo</sup> CD27<sup>-</sup> expressing  
266 IFN $\gamma$ /IL2/TNF $\alpha$ ) persisted for 18 months following BCG vaccination in BALB/c mice which were  
267 strongly associated with the protection against *M. bovis*<sup>29</sup>. When we examined TEM  
268 CD4<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> expressing IFN $\gamma$ /IL2/TNF $\alpha$  in the spleens of BALB/c and F1 mice, the TEM  
269 cells were absent in the BCG 300 and 30 CFU groups for BALB/c mice while, in F1 mice, TEM cells  
270 were absent for BCG 30 CFU group only. The correlation of TEM cells expressing IFN $\gamma$ /IL2/TNF $\alpha$   
271 for BCG dose groups and the correspondent protection data ( $\text{Log}_{10}$  CFU/lungs) in the lungs of  
272 BALB/c and F1 mice, showed significant correlation for both strains of mice when infected with  
273 HN878 but, no correlation when infected with H37Rv. Interestingly, the protection data ( $\text{Log}_{10}$   
274 CFU/lungs) in the lungs of BALB/c mice for BCG vaccinated groups significantly correlated with  
275 TEM cells expressing IFN $\gamma$ /TNF $\alpha$ . Although, for the BCG 300 CFU group in BALB/c mice, TEM  
276 cells were not detected and yet, when infected with H37Rv, the protection was equivalent to BCG 3  
277  $\times 10^4$  - 3000 CFU groups whereas, BCG at 300 CFU group could not protect against HN878 infection.  
278 This is an interesting observation, if the protection in H37Rv infected BALB/c mice was not due to  
279 double or triple cytokine expressing CD4 TEM, this may suggest that either the protection was from  
280 the TEM independent mechanism or due to strain heterogeneity of *Mtb* or the presence of virtually  
281 undetected TEM. BCG at 300 CFU group in BALB/c mice is the lowest protective dose which is  
282 influenced by the type of *Mtb* infecting strain and would be an interesting dose of choice for research  
283 to understand BCG efficacy against different *Mtb* strains.

284 Examination of the cytokine responses in the M7 stimulated cells isolated from the lung and spleen  
285 revealed a distinct pattern of cytokine/chemokine between BALB/c and F1 mice. IFN $\gamma$  is critical for  
286 the TB protection and BCG is the only vaccine whose protective immunity is believed to be depended  
287 upon the induction of CD4<sup>+</sup> T cells that produce IFN $\gamma$ , which in turn activates macrophages to  
288 kill *Mtb*<sup>30,31</sup>. As we expected, IFN $\gamma$  responses in the supernatants of M7 stimulated splenocytes and  
289 lung cells of BCG groups showed correlation with the *ex-vivo* IFN $\gamma$  responses for both strains of mice  
290 (Supplementary Fig. 1). In addition, IL2 responses albeit, low in concentration compared to the IFN $\gamma$ ,  
291 were detected in BCG 3  $\times 10^5$  -300 CFU groups. The IL2 responses from the supernatants of  
292 stimulated splenocytes were significantly inversely correlated with the *Mtb* burden in the lungs of F1  
293 mice infected with either H37Rv or HN878. This suggests that the low concentration of detected IL2  
294 in the spleen would be an indication for the exacerbation of *Mtb* in the lungs of F1 infected mice and  
295 can be used as an immunological marker for F1 mice in preclinical TB vaccine testing. However, in  
296 BALB/c mice, IL2 responses either from the supernatants of stimulated splenocytes or lung cells were  
297 not correlated with the *Mtb* burden in the lungs of infected mice. Interestingly, there were unexpected  
298 and differential responses of the cytokine/chemokine concentrations in the lungs of F1 mice and  
299 spleen of BALB/c mice. In the lungs of F1 and the spleen of BALB/c mice, the concentrations of IL6,  
300 TNF $\alpha$ , CCL4, CCL22, CCL5, CCL17 and CCL2, CXCL1, CXCL2, respectively, were higher in the  
301 control group compared to the BCG vaccinated groups. Surprisingly, the concentrations of  
302 cytokine/chemokine in the lowest dose group, 30 CFU which failed to protect *Mtb* infected BALB/c  
303 and F1 mice followed the cytokine/chemokine responses almost like BCG 300 CFU and not the  
304 control group. High dose of BCG has been implicated to skew immune responses in the mixture of  
305 Th1/Th2, but we could not detect any responses for IL4 in BALB/c or F1 mice.

306 This study has provided some interesting suppositions and questions: 1. Though there were no  
307 protection in the lungs of BALB/c or F1 mice after BCG vaccination with 30 CFU, we were surprised  
308 to find that the cytokine/chemokine responses were not like the control group. In fact, they were more

309 like those in the BCG 300 CFU group, which indeed protected F1 and BALB/c mice infected with  
310 H37Rv. 2. Many animal studies commonly use BCG dose at  $3 \times 10^5$  CFU and have shown to persist  
311 in mice for 18 months<sup>28</sup>, the protection against *Mtb* in these mice is suggested to be from constant  
312 priming of TEM by the presence of live BCG<sup>29</sup>. Does this depicts that the loss of protection in the  
313 lowest dose at 30 CFU is due to the waning of BCG which subsequently ceases constant priming of  
314 TEM cells or is it possible that BCG dose at 30 CFU is so low that the frequency of TEM cells failed  
315 to establish at an optimum level to fight against *Mtb* infection? C. Depending on the research  
316 question, 300 CFU would be an interesting BCG dose to study in BALB/c and F1 mice, for example,  
317 BCG dose at  $3 \times 10^5$  CFU persists in organs for many months in mice and use of BCG dose at 300  
318 CFU may shorten the experimental time and effectively provide a realistic time scale to speed up a  
319 waning experiment.

320 Finally, we conclude that BCG dose at 3000 CFU per mouse, a human equivalent dose, protects  
321 BALB/c and F1 mice when infected with H37Rv or HN878 which also is a realistic window to test  
322 differences in the protection of the new TB vaccine against the benchmark BCG vaccine. The  
323 combination of *Mtb* and mouse strain is an important parameter for evaluating new TB vaccine  
324 against different clinical isolates and must be considered. The spleen IL2 alongside lungs IFN $\gamma$  from  
325 the supernatants of stimulated splenocytes or lung cells may be used as an important immunological  
326 marker for a preclinical TB vaccine testing in F1 mice.

## 327 **Methods**

### 328 *Ethics and animals*

329 All animal procedures were performed in accordance with the UK Home Office (Scientific  
330 Procedures) Act 1986; under appropriate licences, the study protocol was approved by the NIBSC  
331 Animal Welfare and Ethics Review Body.

332 BALB/c and F1 mice, female, 8 weeks old and with approximately 15 -20gm body weight were  
333 obtained from the SPF facilities at the Charles River UK Ltd. All animals were housed in an  
334 appropriate BSL2 (BCG vaccinated) and BSL3 (TB infected) containment facilities at NIBSC. Mice  
335 were checked daily throughout the experiment by trained animal technicians and this frequency could  
336 increase if any adverse reactions were observed. All mice were weighed before TB infection and  
337 subsequently weighed weekly post infection until scheduled experiment termination. No adverse  
338 effect or severe weight loss (more than 20% body weight) were observed in any mice for the entire  
339 duration of the study.

### 340 *Study groups*

341 The study was divided into two parts: 1, H37Rv or HN878 challenge; BALB/c and F1 mice, 5 mice  
342 per group were vaccinated via intradermal route with various doses of BCG Danish 1331 and sterile  
343 saline was administered to the control group. Four weeks post vaccination, all groups (5 mice/group)  
344 were infected with *Mtb* H37Rv or HN878 via aerosol route. BALB/c and F1 mice infected with  
345 H37Rv had BCG at  $3 \times 10^5 - 30$  CFU whereas, in HN878 infected BALB/c and F1 mice, all BCG  
346 vaccinated groups except  $3 \times 10^4$  CFU were selected. 2, Immunogenicity evaluation; BALB/c and F1  
347 mice were receiving BCG vaccination via the intradermal route with various doses except  $3 \times 10^4$  CFU.  
348 At 6 weeks post vaccination, all mice were terminated, the spleen and lungs were harvested for  
349 preparation of single cell suspension for subsequent immunological analyses.

### 350 *BCG vaccination and Mtb Challenge*

351 BCG vaccination for BALB/c and F1 mice for H37Rv challenge experiment was performed using an  
352 expired commercial preparation of Staten Serum Institute BCG vaccine stored at  $-20^\circ\text{C}$ . However, for  
353 HN878 challenge experiment and immunogenicity evaluation, the WHO Reference Reagent for BCG  
354 vaccine of Danish 1331 substrain preparation (NIBSC code 07/270<sup>14</sup>;  
355 [https://www.nibsc.org/products/brm\\_product\\_catalogue/detail\\_page.aspx?catid=07/270](https://www.nibsc.org/products/brm_product_catalogue/detail_page.aspx?catid=07/270)) was used.  
356 Lyophilised BCG preparations were reconstituted and diluted in sterile saline to obtain approximately  
357  $6 \times 10^6$  CFU/ml. Subsequent dilutions (Supplementary Table 1) were prepared in sterile saline to

358 obtain various doses of  $3 \times 10^5$  to 30 CFU/mouse, administered in  $2 \times 25 \mu\text{L}$  injections by bilateral  
359 intradermal route whereas, sterile saline was administered in the control group. All BCG doses ( $3 \times$   
360  $10^5$  to 30 CFU) were checked by plating in duplicate onto 7H11 agar plates (DIFCO) supplemented  
361 with Oleic Acid Dextrose Catalase (OADC, BD) and CFU/ml were estimated at 4 weeks post  
362 incubation at  $37^\circ\text{C}$ .

363 For low-dose aerosol infections, H37RV or HN878 frozen stocks (in 7H9 broth containing 0.05%  
364 Tween 80, OADC and 1% glycerol) were diluted in sterile distilled water to approximately  $5-7 \times$   
365  $10^6$  CFU/ml and placed in a glass nebulizer attached to a Nebuliser system (Walkers, UK) as described  
366 previously<sup>32,33</sup>. The mice were exposed to an aerosol infection in which live bacilli were deposited in  
367 the lungs of each mouse (expected  $\sim 100$  CFU/ lungs). Confirmation of low dose infection for H37Rv  
368 and HN878 challenge experiment was performed by necropsied 4 or 5 BALB/c mice (Supplementary  
369 Table 2) on the same day of challenge. Lung lobes were removed, homogenised and serially diluted  
370 samples were plated onto 7H11 agar (DIFCO) supplemented with OADC (BD). Bacterial colonies  
371 were enumerated 3-4 weeks post incubation at  $37^\circ\text{C}$ . Upon termination of the experiment at 4 weeks  
372 post challenge, mice were necropsied, the spleen and lungs were removed, homogenized and serially  
373 diluted samples were plated in duplicate onto OADC-supplemented 7H11 agar plates. The plates were  
374 incubated at  $37^\circ\text{C}$  and bacterial colonies were enumerated after 3-4 weeks.

### 375 *Splenocytes and lung cells preparations*

376 For the immunogenicity evaluation part; the spleen and lungs were harvested for isolating single cell  
377 suspension. For splenocytes isolation, the spleen was mashed through a  $40 \mu\text{m}$  cell strainer, washed  
378 at 300 g for 5 min and resuspended at  $1 \times 10^7$  cells/ml in the supplemented Dulbecco's Modified  
379 Eagle's Medium (DMEM) (Sigma) containing 10% heat inactivated foetal calf serum (Biosera, UK)  
380 and 2% penicillin/streptomycin (Gibco) for use in subsequent assays.

381 Harvested lung lobes were minced and agitated for 1 hr at  $37^\circ\text{C}$  in supplemented DMEM medium  
382 containing 50 U/ml collagenase I (Gibco) and 10 U/ml DNase II (Sigma), then followed by passing  
383 through a  $40 \mu\text{m}$  cell strainer. Isolated lung cells were washed and resuspended in supplemented  
384 DMEM at  $1 \times 10^7$  cells/ml for use in subsequent assays.

### 385 *Mycobacterial antigens (M7 protein cocktail)*

386 The M7 protein cocktail, which is made of a pool of 7 immunogenic recombinant mycobacterial  
387 proteins - Rv1886c (Ag85B), Rv0251c (hsp20), Rv0287 (TB9.8), Rv0288 (TB10.4), Rv3019c  
388 (TB10.3), Rv3763, Rv3804c (Ag85A) (Lionex, GmbH, Germany) and stored at  $-80^\circ\text{C}$ , was used in  
389 assays where cells required *in vitro* stimulation. Each protein concentration in the cocktail is 50  $\mu\text{g}/$   
390 ml and for stimulating cells, M7 cocktail was diluted to achieve a final concentration of 2  $\mu\text{g}/$  ml for  
391 each protein. M7 protein cocktail can be purchased from the NIBSC website.

### 392 *ELISPOT*

393 Approximately  $2 \times 10^5$  splenocytes or lung cells were incubated in duplicate in 96-well filter plates  
394 (MSIPS4510 Millipore, Ireland) with or without M7 protein cocktail (final conc. 2  $\mu\text{g}/$  ml), or  
395 concanavalin A (10  $\mu\text{g}/$ ml, Sigma) as a positive control for approximately 16 h at  $37^\circ\text{C}$ . The  
396 frequency of IFN $\gamma$  secretors was detected by the AID ISPO (ELR08IFL) reader (AID Autoimmun  
397 Diagnostika, Germany), as per manufacturer's instructions.

### 398 *Flow Cytometry for surface markers and intracellular cytokines*

399 Splenocytes were cultured with 2  $\mu\text{g}/$ ml of M7 protein cocktail, 1  $\mu\text{g}/$ ml of anti-CD28 (BD  
400 Biosciences) for 2 hrs at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ , after which 10  $\mu\text{g}/$ ml Brefeldin A (Sigma) was  
401 added for a further incubation of 14 hrs. Subsequently, cells were washed at 300 g for 5 min and  
402 surface stained with a combination of pre-titrated monoclonal antibody conjugates: CD90.2 - eFluor  
403 450; CD27 - PerCP-eFluor710 (both Life Technologies, UK); CD62L - FITC; CD8 - Alexa Fluor  
404 700; CD44 - Brilliant Violet 785; Zombie Aqua™ Fixable Viability Dye (all BioLegend) and CD4 -  
405 APC-H7 (BD Biosciences). Cells were then washed, fixed, permeabilised and stained intracellularly



406 using BD Cytofix and BD Cytoperm (BD Bioscience), as per manufacturer's instructions, with a  
407 combination of: IL17a – PE; IFN $\gamma$  - PE-Cy7; IL2 - APC; and TNF $\alpha$ -BV605 (all BioLegend). Cells  
408 were analysed immediately after final staining. Data were acquired using a SORP LSR Fortessa (BD  
409 Bioscience), utilizing a 532 nm laser for PE and PE-Cy7, and analysed on Flowjo v.10.1 software  
410 (BD Biosciences). All analyses were gated on a minimum of 100,000 live lymphocytes.  
411 Compensation was performed using UltraComp eBeads (Life Technologies) according to the  
412 manufacturer's instructions. Fluorescence minus one control were used to set gates for cytokine  
413 analyses.

#### 414 ***Multiplex cytokine/chemokine bead assays***

415 Splenocytes and lung cells at  $\sim 2 \times 10^6$  cells/ ml were stimulated with or without M7 cocktail (final  
416 conc. 2  $\mu$ g/ ml) and incubated at 37°C under 5% CO $_2$  for 3 days. Supernatants were harvested and  
417 cytokine/ chemokine beads assays were performed using BioRad's Bio-Plex pro mouse cytokine 34 or  
418 31 - plex assay. BioRad's 34-plex was used for experiments using BALB/c mice and, due to the  
419 availability of the reagent kit, 31-Plex was used for experiments using F1 mice (Table 2). All  
420 multiplex magnetic bead assays were performed according to the manufacturer's protocol.  
421 Concentrations of cytokine/chemokine were extrapolated using manufacturer's provided standard  
422 cocktail in the kits. Reported concentrations of cytokine/chemokine (Supplementary Table 3 and Fig.  
423 4A&B) were obtained from the M7 stimulated cells subtracted by the unstimulated cells.

#### 424 ***Statistical analyses***

425 All data were analysed with the GraphPad Prism 8 software (Graph Pad, USA) using one- or two-way  
426 ANOVA with Dunnett's or Tukey's post-test (3-treatment groups), respectively. Mycobacterial CFUs  
427 were Log $_{10}$  transferred before comparison. Correlation coefficients were assessed using Pearson's  
428 two-tailed correlation test with 95% confidence interval. Differences with a  $p$  value <0.05 were  
429 considered and denoted with, \* $p$ <0.05, \*\* $p$ <0.005, \*\*\* $p$ <0.0005, \*\*\*\* $p$ <0.0001.

430

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543

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545

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### 551 **Author Contributions**

552 B.K. contributed to the experiments, study design, interpretation, analyses and manuscript prepara-  
553 tion. J.K. and B.D. contributed to the experiments and study design. D.A.K contributed in providing  
554 M7 protein cocktail, performing flow cytometry analyses and interpretation. P.J.H contributed to the  
555 study design. M.M.H contributed to the study design and extensive manuscript revision. All authors  
556 approved the final version of the manuscript and have agreed to be personally accountable for their  
557 respective contributions and ensure that questions related to the accuracy or integrity of any part of  
558 the work, even those in which the author was not personally involved, are appropriately investigated,  
559 resolved, and the resolution documented in the literature.

560

### 561 **Competing interests**

562

563 The authors declare no competing interests.

564

### 565 **Additional information**

566 Appended supplementary information as a separate word document - supplementary information.docx

567

### 568 **Figure legends**

569 **Figure 1:** The CFU data for BALB/c and F1 mice vaccinated with various BCG doses and challenged with  
570 HN878 and H37Rv. Four weeks post infection, lungs and spleen were harvested, and bacterial loads were  
571 determined. A, Lungs CFU data as  $\text{Log}_{10}$  protection for the vaccinated BALB/c and F1 mice. B, Spleen CFU  
572 data as  $\text{Log}_{10}$  protection for the vaccinated BALB/c and F1 mice. One-way ANOVA, Tukey's multiple  
573 comparison test was performed for BALB/c and F1 mice infected with H37Rv and HN878 strains. \* $p < 0.05$ ,  
574 \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$ .

575 **Figure 2: IFN- $\gamma$  secreting cells in spleen and lungs of BALB/c and F1 mice.** Splenocytes and lung cells  
576 isolated from six weeks post BCG immunized BALB/c and F1 mice and the frequency of IFN- $\gamma$  secreting cells  
577 evaluated by *ex-vivo* ELISPOT. Approximately  $2 \times 10^5$  cells were cultured with M7 protein cocktail and  
578 developed by ELISPOT assay, bars representing mean ( $\pm$ S.E.) Spot Forming Cells (SFC) per million. Statistical  
579 analysis: One-way ANOVA, Dunnett's test was performed. \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ .

580 **Figure 3: Vaccination induced resident multifunctional CD4<sup>+</sup> cells in the spleen of BALB/c and F1 mice.**  
581 The frequency of multifunctional CD4<sup>+</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> cells at six weeks following BCG immunization. A,  
582 splenocytes from BALB/c and F1 mice for control, BCG groups -  $3 \times 10^5$ , 3000, 300 and 30 CFU were isolated,  
583 stimulated with M7 proteins cocktail and stained by ICS. Bars represent mean ( $\pm$  SE) % frequency of cells of  
584 indicated T cell phenotype as a % of total CD4<sup>+</sup> cells. Two-way ANOVA, Tukey's multiple comparison was  
585 performed, \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$ , \*\*\*\* $p < 0.0001$ . B and C, correlation between the means of  
586 percentages of CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>IL2<sup>+</sup>TNF $\alpha$ <sup>+</sup> (B) and CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>TNF $\alpha$ <sup>+</sup> (C) for BCG groups ( $3 \times 10^5$  – 30 CFU  
587 annotated onto the graphs) and control vs TB burden in the lungs of BALB/c and F1 mice expressed as  $\text{Log}_{10}$   
588 CFU/lungs (as shown in Fig. 1A). Correlation coefficient was assessed using Pearson's two-tailed correlation  
589 test with 95% confidence interval shown as dotted lines on B and C graphs

590 **Figure 4:** Cytokines/ chemokines expression in the M7 stimulated splenocytes and lung cells from BALB/c and  
591 F1 mice vaccinated with various BCG doses. A; representative graphs of cytokines/ chemokines expressed in  
592 pg/ml after 3 days stimulated splenocytes (top graphs) and lung cells (bottom graphs) of BALB/c and F1 mice  
593 for all groups. B; Radar or spider plot with cytokine/chemokine at the corners of the plot. The results of the  
594 mean concentration (n=5) for all cytokines/chemokines measured in the stimulated splenocytes and lung cells of  
595 BALB/c (top and bottom left graphs) and F1 (Top and bottom right graphs). One-way ANOVA, Dunnett's  
596 multiple comparison test, \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.0005$  was performed for A and B.



597 **Figure 5: Correlation of cytokines from supernatants of M7 stimulated splenocytes and lung cells with TB**  
 598 **burden in lungs of H37Rv or HN878 infected F1 mice.**

599 Representative graphs for correlation analysis between the means of cytokines from the supernatants of  
 600 stimulated splenocytes/lung cells and TB burden ( $\text{Log}_{10}$  CFU/ml) in the lungs of H37Rv or HN878 infected F1  
 601 mice. Various doses of BCG vaccines have been annotated onto the graphs. A; The left and right graphs  
 602 represent correlation between TB burden in the lungs of infected F1 mice (HN878 or H37Rv respectively) and  
 603  $\text{IFN}\gamma$  response in the supernatants of stimulated lung cells. B; The left and right graphs represent the correlation  
 604 between the TB burden in the lungs of infected F1 mice (HN878 or H37Rv respectively) and IL2 responses in  
 605 the supernatants of stimulated splenocytes. Correlation coefficient was assessed using Pearson's two-tailed  
 606 correlation test with 95% confidence interval shown as dotted lines in A and B

607 **Tables**

608

**Table 1A: Difference between the mean of CFU/organ ( $\text{Log}_{10}$ ) against all groups for BALB/c and F1 mice when infected with H37Rv.**

Groups	BALB/c – Lungs ( $\text{Log}_{10}$ )					F1 – Lungs ( $\text{Log}_{10}$ )				
	$3 \times 10^5$	$3 \times 10^4$	3000	300	30	$3 \times 10^5$	$3 \times 10^4$	3000	300	30
Control	1.51****	0.81****	0.80****	0.70****	0.20	1.26****	1.50****	1.42****	1.01***	0.10
$3 \times 10^5$	N/A	-0.70****	-0.71****	-0.81****	-1.32****	N/A	0.24	0.16	-0.24	-1.16****
$3 \times 10^4$	N/A	N/A	-0.01	-0.12	-0.62****	N/A	N/A	-0.07	-0.48	-1.36****
3000	N/A	N/A	N/A	-0.11	-0.61***	N/A	N/A	N/A	0.41	-1.32****
300	N/A	N/A	N/A	N/A	-0.50**	N/A	N/A	N/A	N/A	-0.91***

609

Groups	BALB/c – Spleen ( $\text{Log}_{10}$ )					F1 – Spleen ( $\text{Log}_{10}$ )				
	$3 \times 10^5$	$3 \times 10^4$	3000	300	30	$3 \times 10^5$	$3 \times 10^4$	3000	300	30
Control	2.36****	1.66****	1.44****	1.51****	0.54	2.71****	2.66****	2.65****	2.33****	1.23****
$3 \times 10^5$	N/A	-0.69	-0.92**	-0.85*	-1.81****	N/A	-0.05	-0.06	-0.38	-1.48****
$3 \times 10^4$	N/A	N/A	-0.23	-0.15	-1.12***	N/A	N/A	-0.01	-0.33	-1.43****
3000	N/A	N/A	N/A	0.08	-0.89**	N/A	N/A	N/A	0.31	-1.41****
300	N/A	N/A	N/A	N/A	-0.97**	N/A	N/A	N/A	N/A	-1.10***

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**Table 1B: Difference between the mean of CFU/organ ( $\text{Log}_{10}$ ) against all groups for BALB/c and F1 mice when infected with HN878.**

Groups	BALB/c – Lungs ( $\text{Log}_{10}$ )					F1 – Lungs ( $\text{Log}_{10}$ )				
	$3 \times 10^5$		3000	300	30	$3 \times 10^5$		3000	300	30
Control	1.37**		1.06*	0.62	0.30	1.87****		1.42****	0.78**	-0.81
$3 \times 10^5$	N/A		-0.31	-0.75	-1.07	N/A		-0.44	-1.086***	-2.05****
3000	N/A		N/A	-0.44	-0.76	N/A		N/A	-0.65*	-1.61****
300	N/A		N/A	N/A	-0.32	N/A		N/A	N/A	-0.96**

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Groups	BALB/c – Spleen ( $\text{Log}_{10}$ )					F1 – Spleen ( $\text{Log}_{10}$ )				
	$3 \times 10^5$		3000	300	30	$3 \times 10^5$		3000	300	30
Control	0.46		0.74	-0.01	-0.38	1.5***		1.57***	0.96*	0.44
$3 \times 10^5$	N/A		0.29	-0.47	-0.84	N/A		0.07	-0.54	-1.06*
3000	N/A		N/A	-0.76	-1.12	N/A		N/A	-0.61	-1.13**

300	N/A		N/A	N/A	-0.37	N/A		N/A	N/A	-0.52
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**Table 1: Lung and spleen protection data for BALB/c and F1 mice when infected with H37Rv or HN878 strains.** The values in the Log<sub>10</sub> protection data is the difference in the means of control group when compared to the BCG dose groups and the difference within BCG groups. Data is the representation of two independent experiments for H37Rv and one experiment for HN878. Values representing difference for the mean Log<sub>10</sub> of the BCG and control groups (left column) – mean Log<sub>10</sub> of the BCG groups (Top Row). A, Difference between the means of the lung and spleen of Log<sub>10</sub> values for BALB/c and F1 mice infected with H37Rv. B, Difference between the means of the lung and spleen of Log<sub>10</sub> values for BALB/c and F1 mice infected with HN878. One-way ANOVA, Tukey's multiple comparison test was performed for BALB/c and F1 mice infected with H37Rv or HN878. \*p<0.05, \*\*p<0.005, \*\*\*p<0.0005, \*\*\*\*p<0.0001.

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**Table 2: Classification of examined cytokines and chemokines in the M7 stimulated supernatants of splenocytes and lung cells from BCG vaccinated BALB/c and F1 mice.**

<b>Chemokine</b>	CCL	CTACK/CCL27, Eotaxin/CCL11, Eotaxin-2/CCL24, I-309/CCL1, MCP-1/CCL2, MCP-2/CCL8*, MCP-3/CCL7, MCP-5/CCL12, MDC/CCL22, MIP-1α/CCL3, MIP-1β/CCL4, MIP-3α/CCL20, MIP-3β/CCL19, RANTES/CCL5, MCP-3/CCL7 and TECK/CCL25*
	CXCL	BCA-1/CXCL13, Fractalkine/CX3CL1, ENA-78/CXCL5, I-TAC/CXCL11, KC/CXCL1, MIP-2/CXCL2*, SCYB16/CXCL16, SDF-1α/CXCL12, IP10/CXCL10
<b>TNF</b>		TNFα
<b>Interleukins Type I</b>	Th1 cytokines growth factors and Interferons	IFNγ, IL2, IL16, GM-CSF, IL1β
<b>Interleukins Type II</b>	Interleukins	IL4, IL10, IL6

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\*Not analysed for F1 mice (Splenocytes and lung cells) as absent in the Bio-Plex Pro Mouse Chemokine Panel.

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**Table 3: Correlation between the cytokines from supernatants of M7 stimulated splenocytes or lung cells and *Mtb* burden in the lungs (Log<sub>10</sub> CFU/lung) of H37Rv or HN878 infected BALB/c and F1 mice.**

	BALB/c H37Rv Lungs (Log <sub>10</sub> CFU/lungs)	BALB/c HN878 Lungs (Log <sub>10</sub> CFU/lungs)	F1 H37Rv Lungs (Log <sub>10</sub> CFU/lungs)	F1 HN878 Lungs (Log <sub>10</sub> CFU/lungs)	
	-	Spleen IFNγ	Spleen IL2	Lung IFNγ	Spleen IL2
<b>Pearson r</b>	-	-0.9033	-0.9109	-0.8957	-0.9807
<b>P value (2-tailed)</b>	-	0.0356	0.0310	0.0398	0.0032
<b>Sig</b>	-	*	*	*	**
<b>95% confidence intervals (Slope)</b>	-	-1176 to -80.07	-17.46 to -1.595	-11.32 to -0.5208	-10.51 to -4.876

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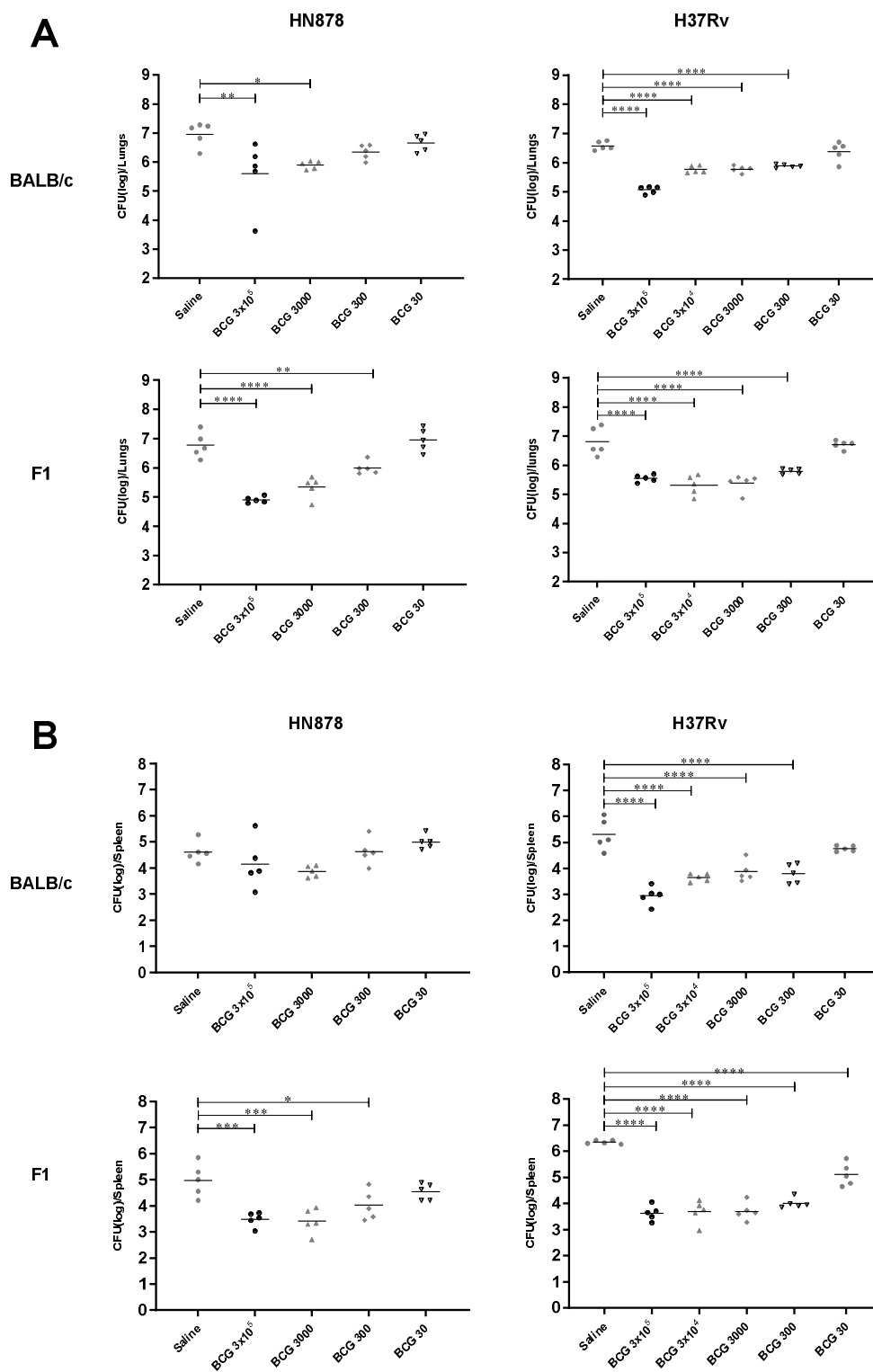


Figure 1

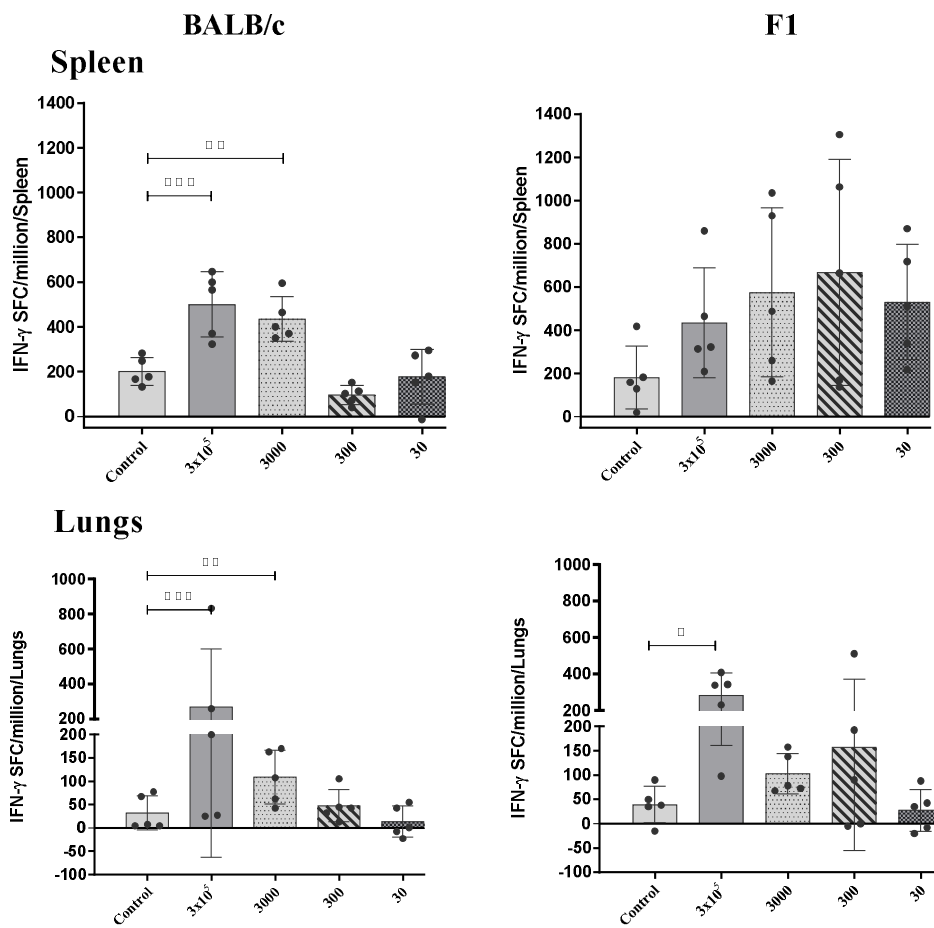
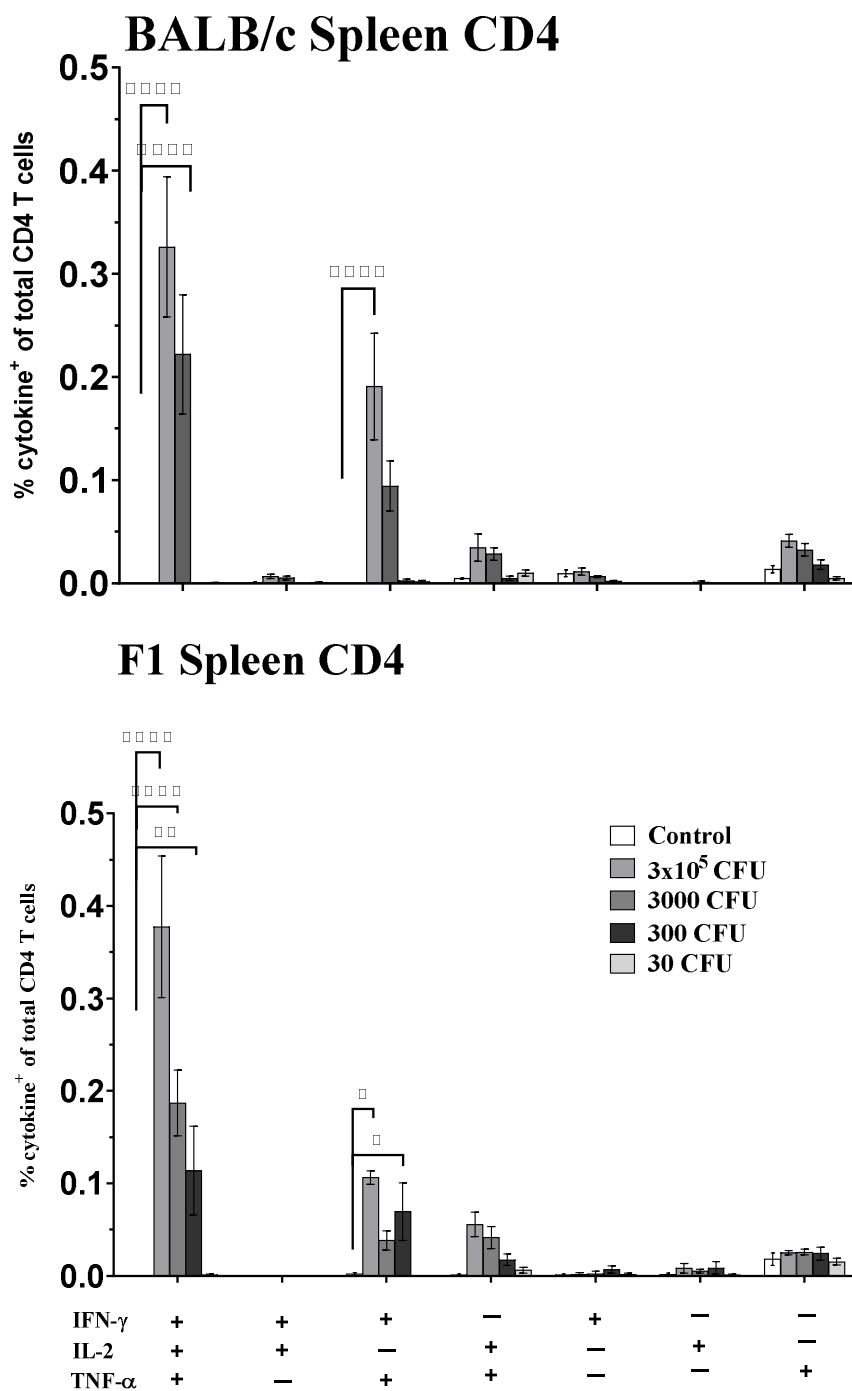


Figure 2



**A**



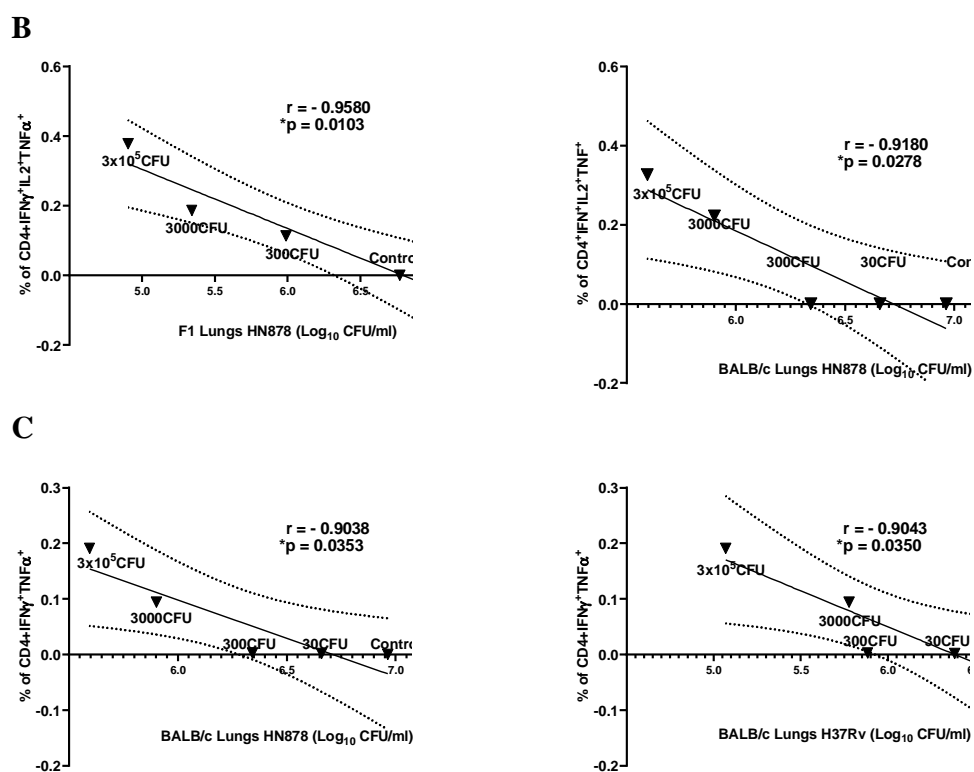
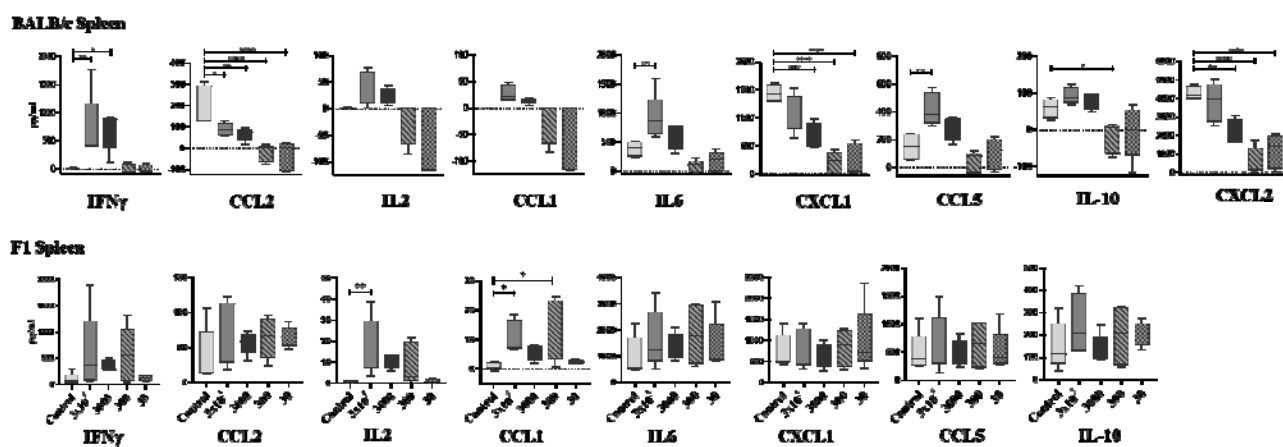
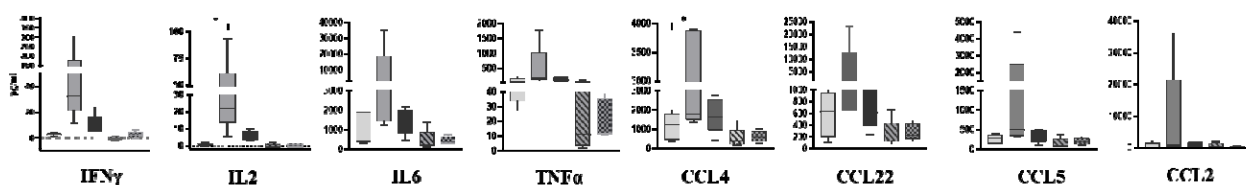


Figure 3

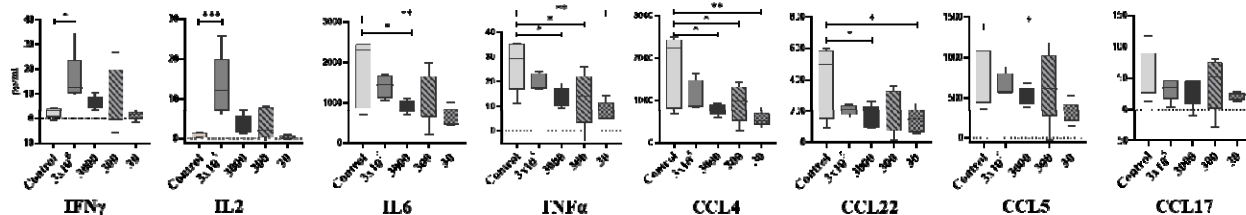
A



**BALB/c Lungs**



**F1 Lungs**



B

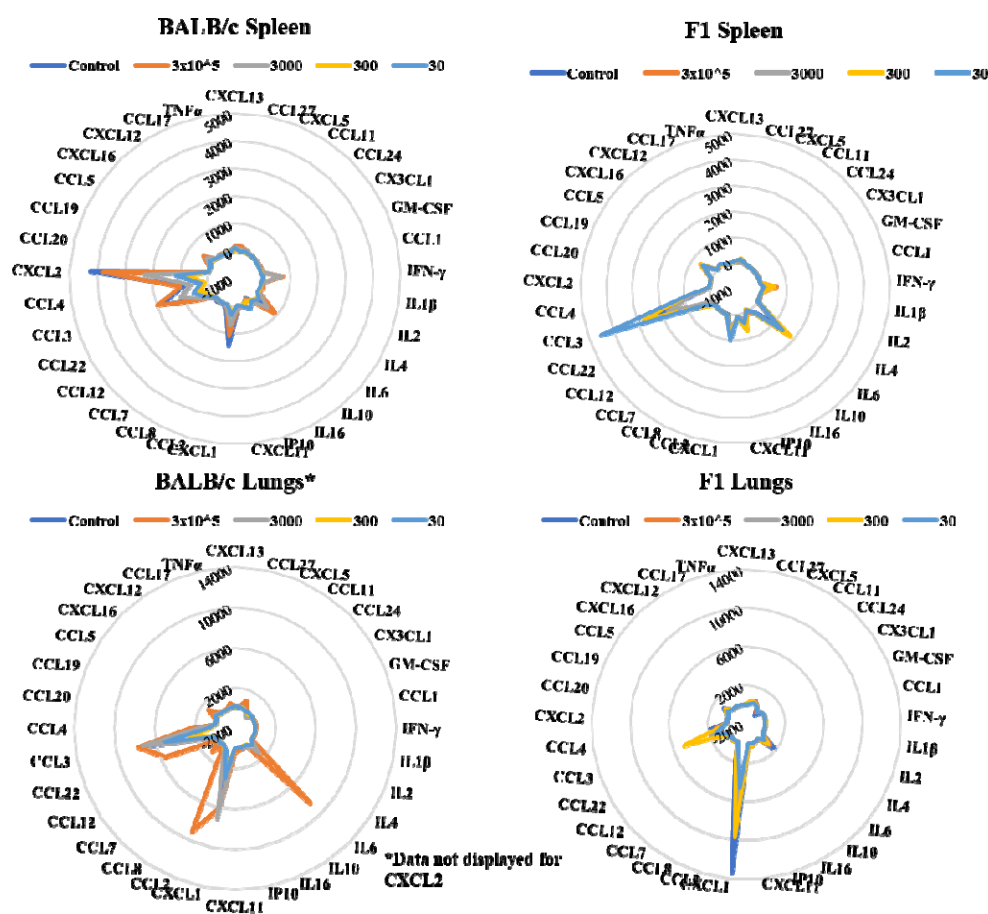


Figure 4

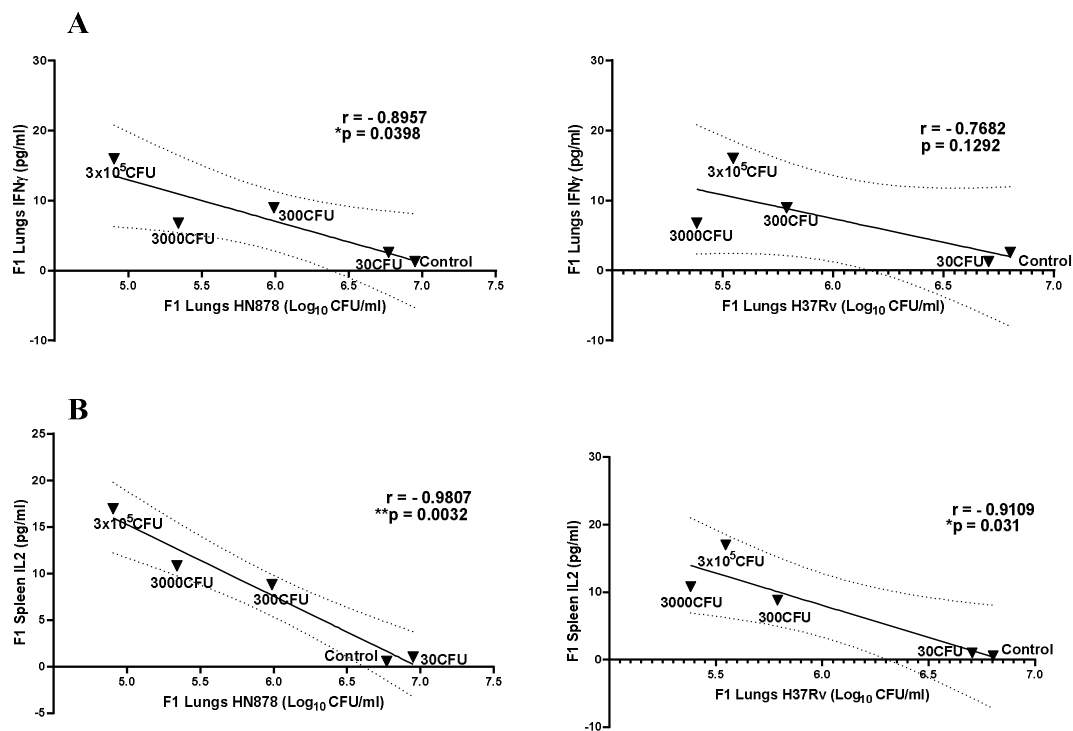


Figure 5