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### *Vaccination of cattle with a high dose of BCG vaccine 3 weeks after experimental infection with Mycobacterium bovis increased the inflammatory response, but not tuberculous pathology*

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# Accepted Manuscript

Vaccination of cattle with a high dose of BCG vaccine 3 weeks after experimental infection with *Mycobacterium bovis* increased the inflammatory response, but not tuberculous pathology

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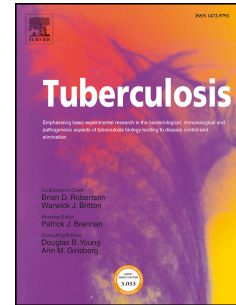
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1 **Vaccination of cattle with a high dose of BCG vaccine 3 weeks after experimental**  
2 **infection with *Mycobacterium bovis* increased the inflammatory response, but not**  
3 **tuberculous pathology**

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18 Running title: Effect of BCG vaccination post-*M. bovis* challenge

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24

25 **Summary**

26 A study was undertaken to determine whether BCG vaccination of cattle post-  
27 challenge could have an effect on a very early *Mycobacterium bovis* infection. Three  
28 groups of calves (n=12/group) were challenged endobronchially with *M. bovis* and  
29 slaughtered 13 weeks later to examine for tuberculous lesions. One group had been  
30 vaccinated prophylactically with BCG Danish vaccine 21 weeks prior to challenge; a  
31 second group was vaccinated with a 4-fold higher dose of BCG Danish 3 weeks post-  
32 challenge and the third group, remained non-vaccinated. Vaccination prior to challenge  
33 induced only minimal protection with just a significant reduction in the lymph node  
34 lesion scores. Compared to the non-vaccinated group, BCG vaccination post-challenge  
35 produced no reduction in gross pathology and histopathology, but did result in significant  
36 increases in mRNA expression of pro-inflammatory mediators (IFN- $\gamma$ , IL-12p40, IL-17A,  
37 IRF-5, CXCL9, CXCL10, iNOs, and TNF- $\alpha$ ) in the pulmonary lymph nodes. Although  
38 there was no significant differences in the gross pathology and histopathology between  
39 the post-challenge BCG and non-vaccinated groups, the enhanced pro-inflammatory  
40 immune responses observed in the post-challenge BCG group suggest caution in the use  
41 of high doses of BCG where there is a possibility that cattle may be infected with *M.*  
42 *bovis* prior to vaccination.

43 **KEY WORDS:** Bovine tuberculosis; *Mycobacterium bovis*; cattle, vaccination; BCG;  
44 vaccine dose

45

46

47

48 **1. Introduction**

49 Bovine tuberculosis (TB) caused predominantly by *Mycobacterium bovis* poses  
50 significant economic hardship to livestock farmers as well as constituting a public health  
51 problem. It is estimated that >50 million cattle worldwide are infected with *M. bovis*,  
52 costing US\$3 billion annually [1]. Although, the implementation of “test and slaughter”  
53 control programmes has resulted in bovine TB being eradicated from a number of  
54 countries [2], these measures have been less effective in countries which have wildlife  
55 reservoirs of *M. bovis* infection or where these programmes are not economically or  
56 socially acceptable. There is renewed interest in the use of TB vaccines for cattle  
57 stemming from the realisation of the financial impact of bovine TB on animal health and  
58 trade and also due to the difficulty of controlling the disease. Currently, there are no TB  
59 vaccines licenced for use in cattle, although the human TB vaccine, bacille Calmette-  
60 Guérin *M. bovis* (BCG) vaccine has been shown to induce significant levels of protection  
61 in cattle in experimental challenge and field trials (reviewed in [1,3]).

62 The major caveats which have restricted BCG being used in cattle until now have  
63 been that vaccination sensitises animals to respond in routine TB diagnostic tests,  
64 particularly in the first year after vaccination [4,5] and protection may not be complete  
65 [1,3]. Research has recently shown that the problem of BCG vaccination compromising  
66 conventional bovine TB diagnostic tests can be overcome by using tests which  
67 differentiate infected from vaccinated animals (DIVA tests), utilising specific  
68 mycobacterial antigens which are expressed by *M. bovis*, but not by BCG [6,7].  
69 Secondly, protection can be enhanced by revaccinating with BCG when immunity has  
70 waned [8] or priming cattle with BCG and boosting with a sub-unit vaccine [9].  
71 Registration of BCG vaccine for cattle will require extensive testing in the field as well as  
72 an assurance of safety for use of BCG vaccine in cattle, including the effect of  
73 vaccination of animals with a pre-existing *M. bovis* infection.

74 The effect of administration of mycobacterial preparations on an existing *M. bovis*  
75 infection in cattle is not documented, although insights can be gained from studies in  
76 humans and small animal models. Studies by Koch in the late 19<sup>th</sup> century revealed that  
77 immunisation of humans with a strong immunogen such as “old tuberculin”, a glycerin  
78 filtrate of cultures of the tubercle bacillus, resulted in the exacerbation of the disease

79 leading to severe toxicities and worsening of the disease, a reaction now known as the  
80 “Koch phenomenon” [10]. Further, it is established that vaccination of humans or small  
81 animal models with BCG does not have a therapeutic effect on an existing *M.*  
82 *tuberculosis* infection [11,12], but a question remains whether BCG vaccine, could  
83 exacerbate an existing mycobacterial infection. It has been proposed that BCG  
84 vaccination of immunocompetent *M. tuberculosis*-infected individuals may result in  
85 increased reactogenicity and morbidity in latently-infected persons (Koch phenomenon)  
86 [13,14].

87 A dose of lyophilised BCG Danish vaccine, equivalent to five human doses (1-4 X  
88  $10^6$  colony forming units, CFU), has commonly been used in TB vaccine efficacy trials  
89 for cattle, [9,15], although a 10-fold lower dose is still protective [16]. The aim of the  
90 current study was principally to test for the safety of administering a relatively high dose  
91 of BCG to cattle with a pre-existing *M. bovis* infection and a 4-fold variation in viable  
92 bacilli can be contained in a commercial human BCG vaccine dose (BCG Danish, Statens  
93 Serum institute, Copenhagen, Denmark). One group of cattle were vaccinated with the  
94 standard cattle dose of BCG at 21 weeks prior to challenge with *M. bovis* and a second  
95 group were vaccinated with a 4-fold higher dose of BCG vaccine at 3 weeks after  
96 challenge.

97

98

## 99 2. Materials & methods

100

### 101 2.1. Animals

102 Thirty-six Friesian-cross, male-castrated calves, 5-6 months old were obtained from  
103 herds which were accredited as TB-free for the previous 5 years and from an area of New  
104 Zealand where both farmed and feral animals were free of TB. Prior to the studies, the  
105 cattle tested negative for bovine TB in the whole blood IFN- $\gamma$  test. The cattle were grazed  
106 on pasture in a biocontainment unit.

107

### 108 2.2 . Bacterial strains and vaccines

109 The lyophilised *M. bovis* BCG Danish 1331 vaccine (Statens Serum Institute,  
110 Copenhagen, Denmark) formulated for humans was utilised to vaccinate the calves. *M.*  
111 *bovis* WAg202, originally isolated from a tuberculous possum in New Zealand, was used  
112 as the challenge strain and had been used in previous vaccination/challenge studies in  
113 cattle [17,18]. Bacteria were grown to mid-log phase in Tween albumin broth (Dubos  
114 broth base, Difco Laboratories, Detroit, Mich.) supplemented with 0.006% (vol/vol)  
115 alkalinised oleic acid, 0.5% (wt/vol) albumin fraction V and 0.25% (wt/vol) glucose.  
116 Dilutions were made in Tween albumin broth to obtain the dose for inoculation. The  
117 number of CFU inoculated was determined retrospectively by plating 10-fold dilutions on  
118 Middlebrook 7H11 (Difco) supplemented with 0.5% (wt/vol) albumin, 0.2% (wt/vol)  
119 glucose and 1% (wt/vol) sodium pyruvate.

120

### 121 2.3 Vaccination and *M. bovis* challenge

122 The calves were divided into three groups, each containing 12 calves using a  
123 randomised stratified sampling system so that all groups contained animals with a similar  
124 distribution of IFN- $\gamma$  responses to avian purified protein derivative (PPD; prepared from a  
125 *M. avium* culture) in the weeks prior to the start of the study. Calves from one group  
126 (BCG-vaccinated group) were each vaccinated subcutaneously in the left side of the neck  
127 with 0.5 ml of BCG vaccine (equivalent to  $1.5 \times 10^6$  CFU/dose, with the other two  
128 groups remaining non-vaccinated. Each vial of BCG vaccine was reconstituted in 1 ml  
129 Sauton medium (Statens Serum Institute) and contained an estimated  $2-8 \times 10^6$  CFU/vial,  
130 with retrospective culturing providing a count of  $3 \times 10^6$  CFU/vial. All three groups of

131 calves were challenged endobronchially with  $5 \times 10^3$  CFU of virulent *M. bovis* as  
132 previously described [17] at 21 weeks after vaccination. Three weeks after the *M. bovis*  
133 challenge, calves in one of the previously non-vaccinated groups (Post-challenge BCG  
134 group) were each vaccinated subcutaneously in the left side of the neck with 2 ml of BCG  
135 vaccine ( $6 \times 10^6$  CFU; contents of two vaccine vials) The remaining group was named  
136 the Non-vaccinated group.

137

#### 138 2.4. Necropsy procedure

139 All cattle were killed 13 weeks after challenge. Procedures for identifying  
140 macroscopic tuberculous lesions and processing for histopathology have been described  
141 previously [16]. A lung lesion score was calculated by counting the total number of  
142 lesions and applying a score as follows: 0, no lesions; 1, 1-9 lesions; 2, 10-29 lesions; 3,  
143 30-99 lesions; 4, 100-199 lesions; 5,  $\geq 200$  lesions. A total lymph node lesion score per  
144 animal was calculated by pooling scores for four major pulmonary lymph nodes (left  
145 bronchial and right bronchial/tracheobronchial and anterior and posterior mediastinal).  
146 Scores for individual lymph nodes were: 0, no lesions; 1, 1-19 small lesions (1-3 mm  
147 diameter); 2,  $\geq 20$  small lesions or medium size lesion (4-6 mm diameter); 3, large lesion  
148 ( $>6$  mm). Samples from four pulmonary lymph nodes were collected from all of the  
149 animals for histology and bacterial culture. Additional samples were collected from any  
150 tuberculous-like lesions observed in lungs, other lymph nodes or organs. For histological  
151 examination, sections were stained with hematoxylin and eosin. Scoring of  
152 histopathological lesions for the four pulmonary lymph nodes was based on the scale of  
153 stage I to IV granulomas as described by Wangoo et al. [19]. Briefly, stage I granulomas  
154 were composed of accumulations of epithelioid macrophages with low numbers of  
155 lymphocytes, neutrophils and Langhans multinucleated giant cells and there was an  
156 absence of necrosis. Stage II granulomas were similar to stage I granulomas but also had  
157 central infiltrates of neutrophils and lymphocytes and necrosis could be present. Stage III  
158 granulomas exhibited complete fibrous encapsulation and significant necrosis and  
159 mineralisation could be present. Stage IV granulomas were characterised by multiple  
160 coalescing caseo-necrotic granulomas with multicentric necrosis and mineralisation. The  
161 percentage of the granulomas classified as Stage I, II, III or IV was calculated from the  
162 total number of granulomas for each group. Scoring of gross and histopathological  
163 lesions was undertaken blinded for animal number and treatment groups. For bacterial



164 culture, tissue samples (2-3 g) were homogenised in a Tenbroeck grinder (Wheaton,  
165 Millville N.J.), decontaminated in 0.75% cetylpyridinium chloride for 1 h, centrifuged at  
166 3500 g for 20 min (included in the decontamination time) and processed for isolation of  
167 mycobacteria as described previously [17]. The CFU/g for each of the four pulmonary  
168 lymph nodes was determined and when no *M. bovis* was isolated from a sample, a value  
169 of half the minimal count was applied (5 CFU/sample) as not all the sample was cultured.  
170 The value for each animal was the mean of the  $\log_{10}$  CFU/g of tissue for the four lymph  
171 nodes and the mean for the group calculated from these values. To measure cytokine  
172 mRNA expression, a small tissue sample from the left bronchial lymph node was  
173 collected from each animal and stored in RNAlater® (Life Technologies, USA). In the  
174 absence of a lesion in this lymph node, but if a lesion was identified in another pulmonary  
175 lymph node, the other lymph node was selected, otherwise a sample from the non-  
176 lesioned left bronchial node was selected.

177

#### 178 2.5. *IFN- $\gamma$ assay*

179 Heparinised blood samples were collected from the calves at regular intervals to  
180 analyse cellular immune responses. Blood samples (1.5 ml) were dispersed into wells of a  
181 24-well plate and preservative-free bovine PPD prepared from a *M. bovis* culture or avian  
182 PPD (24  $\mu$ g/ml final concentration; Prionics, Schlieren-Zurich, Switzerland) or  
183 phosphate-buffered saline (PBS, negative control) was added. Blood cultures were set up  
184 within 6 hours following blood collection. After incubation at 37°C for 24 h, the plasma  
185 supernatants were harvested and their IFN- $\gamma$  levels measured using a sandwich ELISA kit  
186 (Mabtech, Sweden). Results were reported as optical density units 450 nm (OD<sub>450</sub>) for  
187 bovine or avian PPD minus OD<sub>450</sub> for PBS.

188

#### 189 2.6. *Tuberculin skin test*

190 The comparative cervical tuberculin skin test was undertaken at 10 weeks post-  
191 challenge. For this test, cattle were inoculated intradermally with 0.1 ml volumes  
192 containing either 3,000 IU of bovine PPD or 2,500 international units (IU) of avian PPD  
193 (Prionics, Lelystad, The Netherlands) at separate sites on the right side of the neck. The  
194 skin-fold thickness was measured with callipers prior to injection and 72 h after injection  
195 of the PPDs.

196

197 2.7. *Reverse-transcription and qPCR*

198 RNA was extracted from lymph node samples, purified and transcribed to cDNA as  
199 previously described [20]. All cDNA samples were stored at  $-20^{\circ}\text{C}$  until the qRT-  
200 PCR was undertaken. The primer sequences for IFN- $\gamma$ , IL-10, IL-12p40, IL-17A,  
201 interferon releasing factor-5 (IRF-5), iNOs and TNF- $\alpha$  were described by Shu et al. [21].  
202 The forward and reverse primers for IL-2 were AACGGTGCACCTACTTCAAGCTCT  
203 and TAGCGTTAACCTTGGGCGCGTAAA, respectively, while the corresponding  
204 primers for CXCL9 were ACTGGAGTTCAAGGAGTTCCAGCA and  
205 TCTCACAAGAAGGGCTTGGAGCAA and those for CXCL10 were  
206 TCCTCGAACACGGAAAGAGGCATA and AGCTGATATGGTGACTGGCTTGGT.  
207 For the qRT-PCR analysis, 10  $\mu\text{l}$  of SyBr®Premix Ex Taq™ II master mixture (Takara  
208 Bio Inc., Japan), 2  $\mu\text{l}$  of template cDNA and 1  $\mu\text{l}$  of 5  $\mu\text{M}$  of each gene-specific primers  
209 were combined in a 20  $\mu\text{l}$  reaction mixture in duplicate. The amplification was performed  
210 in a Rotor-Gene 6000 machine (Corbett Research, Australia). The cycle number at which  
211 the various transcripts became detectable was referred to as the threshold cycle (Ct) and  
212 data were analysed using Rotor-gene 6000 series software 7.0. The average Ct value of  
213 duplicates was used for calculation of the relative fold changes using the  $\Delta\Delta\text{Ct}$  method  
214 [22]. A previous study showed that the Ct values of the PCR with three house-keeping  
215 genes, GAPDH,  $\beta$ -actin and U1 were consistent within each gene and U1 showed the  
216 lowest Ct value [21]. We used U1 as the house keeping gene for normalisation and the  
217  $\Delta\text{Ct}$  from a pool of non-lesioned, prescapular lymph nodes from *M. bovis*-infected cattle  
218 sourced from a previous study [21] was used as the calibrator to generate  $\Delta\Delta\text{Ct}$ .

219

220 2.8. *Statistical analyses*

221 For analysis of IFN- $\gamma$  responses a mixed effects model was applied to natural log-  
222 transformed IFN- $\gamma$  responses; time, group and their interaction were fixed effects, and  
223 animal and challenge (an indication variable for identifying before or after challenge)  
224 were random effects. The Kruskal-Wallis test with multiple comparisons was used for  
225 analysing lesion scores and qPCR data. Multiple comparisons of the different groups  
226 were performed with a p-value adjusted by the 'BH' method [23]. These analyses were  
227 undertaken using the R packages 'nlme', 'lme4' and 'predictmeans' in R 3.2.0 [24]. The  
228  $\chi^2$  test was used for comparing the distribution of the different granulomas stages for each  
229 group. Fisher's Exact test was used for comparing the proportion of animals with lung or

230 lymph node lesions. For the remaining data, statistical analyses were undertaken using  
231 Minitab 16. The mean skin test values, numbers of lesioned lymph nodes/animal and *M.*  
232 *bovis* culture positive lymph nodes/animal as well as the mean log<sub>10</sub> CFU/g from lymph  
233 nodes were compared using ANOVA with Tukey's multiple comparisons. Statistical  
234 significance was denoted when  $P < 0.05$ .  
235

### 236 3. Results

237

#### 238 3.1. Pathological and microbiological findings following *M. bovis* challenge

239 Vaccination of calves with BCG prior to *M. bovis* challenge (BCG-vaccinated  
240 group) produced a significant degree of protection against the challenge in one gross  
241 pathology parameter, with a lower median lymph node lesion score in the BCG-  
242 vaccinated group compared to those for the Non-vaccinated group ( $P < 0.05$ , Figure 1A).  
243 In addition, BCG vaccination prior to challenge resulted significant reductions in the  
244 proportions of animals with lymph node and lung lesions, lower median lymph node and  
245 lung lesion scores and lower mean number of lesioned lymph nodes per animal compared  
246 to those for the Post-challenge BCG group (Table 1 and Figure 1A and B;  $P < 0.05$ ).  
247 There were no significant differences between the gross pathology parameters for the  
248 Post-challenge BCG and Non-vaccinated groups. The lesions were typical of those for  
249 bovine TB with multiple small (1-3 mm in diameter) calcified lesions in the lung and  
250 variable sized calcified lesions in the pulmonary lymph nodes (1-20 mm in diameter).  
251 The number of animals in the BCG-vaccinated, Post-challenge BCG and Non-vaccinated  
252 groups with gross tuberculous lesions were 7, 12 and 10, respectively. No gross  
253 tuberculous lesions were observed outside of the pulmonary cavity.

254 Following histopathological examination, a comparison of the relative distribution  
255 of granuloma developmental stages was undertaken. This analysis revealed that the  
256 distribution of granuloma stages was significantly unequal between the three groups  
257 (Figure 2;  $P = 0.0145$ ,  $\chi^2$  test). This was characterized by higher percentages of the most  
258 severe lesions (Stage IV) in the Post-challenge BCG and Non-vaccinated groups, and  
259 lower proportions of the less severe Stage 2 granulomata, compared to those for the  
260 BCG-vaccinated group of calves. The BCG-vaccinated and Non-vaccinated groups of  
261 animals had significantly lower mean numbers of pulmonary lymph nodes culture  
262 positive for *M. bovis* and lower mean  $\log_{10}$  CFU of *M. bovis*/g of pulmonary lymph node  
263 than those for the Post-challenge BCG group ( $P < 0.05$ , Table 1). No significant  
264 differences were detected between the BCG-vaccinated and Non-vaccinated groups.

265 No vaccination site reactions were observed following BCG vaccination in the  
266 Post-challenge BCG group.

267

#### 268 3.2. *IFN- $\gamma$* responses after vaccination and challenge

269 The kinetics of T cell responses to *M. bovis* antigens were determined by measuring  
270 the release of IFN- $\gamma$  from whole blood stimulated with bovine PPD (Figure 3A).  
271 Vaccination with BCG at the commencement of the study resulted in a significant  
272 increase in antigen-specific IFN- $\gamma$  responses at 3, 6, 8, 10, 12 and 21 weeks after  
273 vaccination compared to the Non-vaccinated group ( $P < 0.05$ ). Following challenge with  
274 *M. bovis* at 21 weeks post-vaccination, the mean IFN- $\gamma$  responses for all groups  
275 increased, with the mean responses for the BCG-vaccinated and Post-challenge BCG  
276 groups significantly greater than that for the Non-vaccinated group at 3 weeks post-  
277 challenge ( $P < 0.05$ ). The mean IFN- $\gamma$  response for the Post-challenge BCG group was  
278 not boosted following vaccination with BCG at 3 weeks after challenge.

279 Although all the groups had a similar distribution of IFN- $\gamma$  responses to avian PPD  
280 at 3 weeks prior to the start of the study, the mean responses for the Post-challenge BCG  
281 group were greater than those for the Non-vaccinated group at six of the seven time-  
282 points prior to challenge, although none of these differences were statistically significant  
283 (Figure 3B). In the period prior to challenge, there was a cumulative increase in the IFN- $\gamma$   
284 responses to both avian and bovine PPD in the Post-challenge BCG and Non-vaccinated  
285 groups which suggested exposure to environmental mycobacteria with the animals  
286 grazing on pasture.

287

### 288 3.3. Skin test responses after challenge

289 At 10 weeks after challenge with *M. bovis*, all animals with the exception of one  
290 animal from the Non-vaccinated group showed an increase in the skin fold thickness of  $>$   
291 1 mm at 72 hours following injection of bovine PPD. The only significant difference  
292 between the mean skin test responses was that the mean bovine PPD response for the  
293 Post-challenge BCG group (23.3 mm increase in skin fold thickness) was greater than  
294 that for the Non-vaccinated group (mean of 16.0 mm; Table 2;  $P < 0.05$ ).

295

### 296 3.4. mRNA expression of immune mediators from pulmonary lymph nodes post- 297 challenge

298 Tissue samples were collected from a pulmonary lymph node from each animal  
299 following slaughter of the animals 13 weeks after challenge to measure mRNA  
300 expression of immune mediators by qRT-PCR. Samples were preferentially selected from  
301 the left bronchial lymph node. Comparisons between the mean responses of immune

302 mediators are shown in Figure 4. The mean gene expression for IFN- $\gamma$ , IRF-5, IL-12p40,  
303 IL-17A, iNOs, CXCL9, CXCL10 and TNF- $\alpha$  were significantly greater for the Post-  
304 challenge BCG group than those for the Non-vaccinated group ( $P < 0.05$ ). In addition, the  
305 mean mRNA expression for IFN- $\gamma$ , CXCL9 and CXCL10 were significantly greater for  
306 the Post-challenge BCG group than those for the BCG-vaccinated group ( $P < 0.05$ ). No  
307 significant differences were detected between the groups for IL-2 and IL-10 mRNA  
308 expression, or between the BCG-vaccinated and Non-vaccinated groups for any of the  
309 immune mediators.

310

311

#### 312 4. Discussion

313 There is increasing interest in the use of BCG vaccine to protect cattle against  
314 bovine TB, although it is recognized that similar to the situation in humans, BCG does  
315 not provide complete protection against TB at a population or individual animal level. In  
316 this study, a very stringent test was chosen to answer the question whether vaccinating  
317 infected cattle with BCG would modulate disease outcome. Cattle were vaccinated with a  
318 high dose of BCG only 3 weeks after a relatively high dose experimental challenge with  
319 *M. bovis*. Three weeks post-challenge is considered as an early stage of a *M. bovis*  
320 infection for cattle [25]. The dose of lyophilised BCG Danish vaccine most commonly  
321 administered subcutaneously to cattle in TB vaccine efficacy trials has been 0.05 to 0.5  
322 ml ( $1-4 \times 10^5$  to  $1-4 \times 10^6$  CFU/dose) [8,9,16] and for the current study, the 0.5 ml dose  
323 was chosen for immunisation prior to challenge. In the current study, this dose of BCG  
324 administered prior to challenge induced only minimal protection against TB with a  
325 significant lower median lymph node lesion score compared to the Non-vaccinated  
326 group. Marked variations in the efficacy of BCG vaccination have been previously  
327 reported for protection of cattle against experimental challenge with *M. bovis*, varying  
328 from a significant reduction in a single pathological or microbiological disease parameter  
329 [18] to a reduction in up to six parameters in a subsequent study [16]. The reasons for this  
330 variation are not clear, although prior sensitisation to environmental mycobacteria has  
331 been considered as a possible explanation for poor responses to BCG vaccination in cattle  
332 [26].

333 A 4-fold higher dose of BCG was chosen to vaccinate a group of previously non-  
334 vaccinated calves (Post-challenge BCG group) at 3 weeks post-challenge to test for safety  
335 due to the potential variation in the bacterial count that may be present in commercial  
336 BCG vaccines.. There was no significant difference in the gross pathology for the Post-  
337 challenge BCG and Non-vaccinated groups and both of these groups had a high  
338 percentage of the more advanced Stage IV granulomata compared to the pre-challenge  
339 BCG group. However, results from the mRNA expression of immune mediators indicated  
340 that there was a more severe inflammatory response at the site of infection in the  
341 pulmonary lymph nodes of the Post-challenge group compared to that for the Non-  
342 vaccinated group. qRT-PCR measurement of mRNA expression for eight of the 10  
343 immune mediators from pulmonary lymph node tissues of the Post-challenge BCG group

344 was significantly greater than those for the Non-vaccinated group. Although, the colony  
345 counts of *M. bovis* in these lymph nodes were significant greater for the Post-challenge  
346 BCG group compared to that for Non-vaccinated group, the bacterial culture method did  
347 not allow virulent *M. bovis* to be differentiated from BCG. It is possible that BCG bacilli  
348 may have colonised the pulmonary lymph nodes in the Post-challenge BCG animals,  
349 contributing to the higher *M. bovis* counts.

350 Despite the limited availability of safety data for BCG vaccination of humans in  
351 high burden settings, no serious effects were reported following primary vaccination of  
352 tuberculin skin test positive persons in a large Indian trial [11]. Furthermore, BCG  
353 revaccination of latently infected adults with prior infant BCG vaccination was also  
354 shown to be safe and reactogenicity similar to that for primary BCG vaccination [27].  
355 However, there are major differences in these trials compared to the current study. In the  
356 human TB trials, the infections were only defined as possible *M. tuberculosis* infections  
357 based on tuberculin skin test reactivity with the likelihood of non-specific mycobacterial  
358 or latent *M. tuberculosis* infections. In contrast, the *M. bovis* infection in the cattle  
359 resulted in a rapid development of tuberculous lesions in the non-vaccinated animals. It  
360 also needs to be stressed that the post-challenge vaccination took place very early after a  
361 severe *M. bovis* challenge at the height of the development of anti-tuberculous, cellular  
362 immune responses. Despite these severe experimental conditions, the post-challenge  
363 BCG did not lead to significant increase in gross and microscopic pathology.

364 A study in deer demonstrated that subcutaneous vaccination with  $5 \times 10^4$  and  $5 \times$   
365  $10^7$  CFU of BCG Pasteur induced comparable levels of protection against infection and  
366 disease following intratracheal challenge with *M. bovis*, [28]. In contrast, vaccination  
367 with a higher dose of  $5 \times 10^8$  CFU of BCG Pasteur did not induce protection and evoked  
368 immune responses with a bias towards Type 2 rather than Type 1 reactivity. In the  
369 current study, there was no boosting of the whole blood antigen-specific IFN- $\gamma$  responses  
370 following BCG vaccination for the Post-challenge BCG group. This may have been in  
371 part due to the enhanced reactivity to avian PPD antigens for this group in the period  
372 prior to challenge, resulting in a marked increase immediately post-challenge, masking  
373 any subsequent increase in the immune response following BCG vaccination. In  
374 comparison, vaccination with BCG prior to challenge (BCG-vaccinated group) using a 4-  
375 fold lower dose was shown to induce a sustained increase in the antigen-specific IFN- $\gamma$



376 response in the period, 3 to 21 weeks post-vaccination. The stronger tuberculin skin test  
377 response observed in the Post-challenge BCG group compared to that for the Non-  
378 vaccinated group was indicative of a stronger inflammatory response, possibly as a  
379 consequence of an enhanced reactogenicity following BCG vaccination post-challenge.

380 Studies in mice have provided information on possible detrimental effects of  
381 administering BCG following infection with *M. tuberculosis*. Although, BCG vaccination  
382 of mice prior to challenge with *M. tuberculosis* was protective, BCG vaccination of  
383 already infected mice did not improve the course of infection and repeated revaccination  
384 resulted in an exacerbation of the granulomatous response [12,29]. One of these studies  
385 showed that the increase in the lung tissue damage was associated with an increase in IL-  
386 17, TNF- $\alpha$ , IL-6 and MIP-2 expression and influx of granulocytes/neutrophils [12]. A  
387 pathological role for IL-17 was indicated as this response was abrogated in mice deficient  
388 in the gene encoding IL-23p19 or in the presence of IL-17 blocking antibody. In a further  
389 study, a single subcutaneous administration of live BCG to mice infected with *M.*  
390 *tuberculosis* increased antigen-specific T-cell proliferation and induced larger  
391 tuberculous lung granulomas, but did not induce a reduction in the bacterial load [30].  
392 The authors suggested that an increased production of TNF- $\alpha$  resulting from vaccination  
393 post-challenge contributed to the increased inflammation in the lungs and accelerated  
394 death.

395 It has been reported that following a mycobacterial infection, an equilibrium is  
396 established between mycobacteria and the host through the interaction of mycobacteria  
397 and macrophages in granulomas, maintained by the release of immune mediators [31].  
398 Vaccination after challenge may disturb this equilibrium causing a heightened immune  
399 response in the lesions, particularly when the vaccine is administered at the height of anti-  
400 *M. bovis* effector immune response. Administration of a high dose BCG vaccine to calves  
401 only 3 weeks after the *M. bovis* challenge induced a pro-inflammatory immune response  
402 in the pulmonary lymph nodes at 13 weeks post-challenge. There was a significantly  
403 higher expression of IFN- $\gamma$ , IRF-5, IL-12p40, IL-17A, , iNOs, CXCL9, CXCL10 and  
404 TNF- $\alpha$  compared to that for the Non-vaccinated group, although only the responses to  
405 IFN- $\gamma$ , CXCL9 and CXCL10 were also higher in this group compared to the animals  
406 vaccinated with BCG before challenge. The sequence of events is likely to have been  
407 initiated by the induction of IRF-5, a “master regulator” of the pro-inflammatory

408 cytokines, which up-regulates expression of IL-6, IL-12, IL-17, IL-23, TNF- $\alpha$ , CXCL10  
409 and type 1 IFNs [32]. Subsequent production of IFN- $\gamma$  induces the production of  
410 chemokines, CXCL9 and CXCL10, attracting more T lymphocytes and monocytes into  
411 the granulomas [33,34]. Expression of IL-10, the anti-inflammatory cytokine which  
412 inhibits the activity of Th1 cells, NK cells and macrophages [35], was not significantly  
413 increased in the pulmonary lymph nodes of the Post-challenge BCG group. Although,  
414 pro-inflammatory cytokines play an important role in control of mycobacterial infections,  
415 the timing and balance of the cytokines will influence whether these responses support  
416 control of infection versus detrimental inflammatory responses..

417

418 **5. Conclusion**

419 A very stringent test was used to determine the effect of administering BCG  
420 vaccine post-challenge, with cattle vaccinated with a high dose of BCG only 3 weeks  
421 after experimental infection with *M. bovis*. Compared to the Non-vaccinated group,  
422 vaccination with BCG post-challenge did not lead to protection or, alternatively, to a  
423 significant increase in gross and histo-pathology, although there was an up-regulation of  
424 an array of pro-inflammatory immune mediators from pulmonary lymph node tissues  
425 samples. The strong systemic IFN- $\gamma$  responses to avian PPD observed in the Post-  
426 challenge BCG group prior to challenge may have contributed to the enhanced pro-  
427 inflammatory immune responses in the pulmonary lymph nodes of these animals  
428 following challenge and BCG vaccination. However, it does suggest caution in the use of  
429 high doses of BCG vaccine for cattle, where there is a possibility that animals may be  
430 infected with *M. bovis* prior to vaccination.

431

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436 **Author's contributions**

437 Study conception and design: BMB, NAP, AH, RGH, H MV, DNW; Data  
438 acquisition, analysis and interpretation: BMB, DS, NAP, SS, AH, DNW; Drafting and  
439 revising the manuscript: BMB, DS, NAP, SS, AH, H MV, DNW; Final approval: BMB,  
440 DS, NAP, SS, AH, RGH, H MV, DNW.

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446

447

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545

546 **Table 1.** Gross pathological and microbiological findings after *Mycobacterium bovis*  
 547 challenge.

Group	Proportion with		Mean $\pm$ SEM	Mean $\pm$ SEM no.	Mean $\pm$ SEM
	PLN lesions	Lung lesions	no. of lesioned PLNs/animal	of <i>M. bovis</i> positive PLNs/animal	$\log_{10}$ CFU of <i>M. bovis</i> /g of PLN
BCG- vaccinated	4/12*	6/12 <sup>†</sup>	0.67 <sup>†</sup> ( $\pm$ 0.33)	1.97 <sup>†</sup> ( $\pm$ 0.29)	1.23 <sup>†</sup> ( $\pm$ 0.38)
Post-challenge BCG	11/12	11/12	2.25 ( $\pm$ 0.35)	3.17 ( $\pm$ 0.30)	2.34 ( $\pm$ 0.48)
Non- vaccinated	10/12	7/12	1.5 ( $\pm$ 0.34)	1.75 <sup>†</sup> ( $\pm$ 0.35)	1.42 <sup>†</sup> ( $\pm$ 0.46)

548 PLN Pulmonary lymph node (pulmonary lymph nodes were the only lymph nodes with  
 549 gross tuberculous lesions)\* Significantly less than those for the Post-challenge BCG and  
 550 Non-vaccinated groups ( $P < 0.05$ )

551 <sup>†</sup> Significantly less than that for the Post-challenge BCG group ( $P < 0.05$ )

552



553 **Table 2.** Mean ( $\pm$  SEM) skin test responses for cattle at 10 weeks after *Mycobacterium*  
 554 *bovis* challenge.

Group	Bovine PPD	Avian PPD
BCG-vaccinated	20.0 ( $\pm$ 2.4)	6.2 ( $\pm$ 1.1)
Post-challenge BCG	23.3* ( $\pm$ 1.7)	6.3 ( $\pm$ 0.8)
Non-vaccinated	16.0 ( $\pm$ 1.9)	4.7 ( $\pm$ 0.8)

555 \* Significantly greater than that for the Non-vaccinated group ( $P < 0.05$ )

556

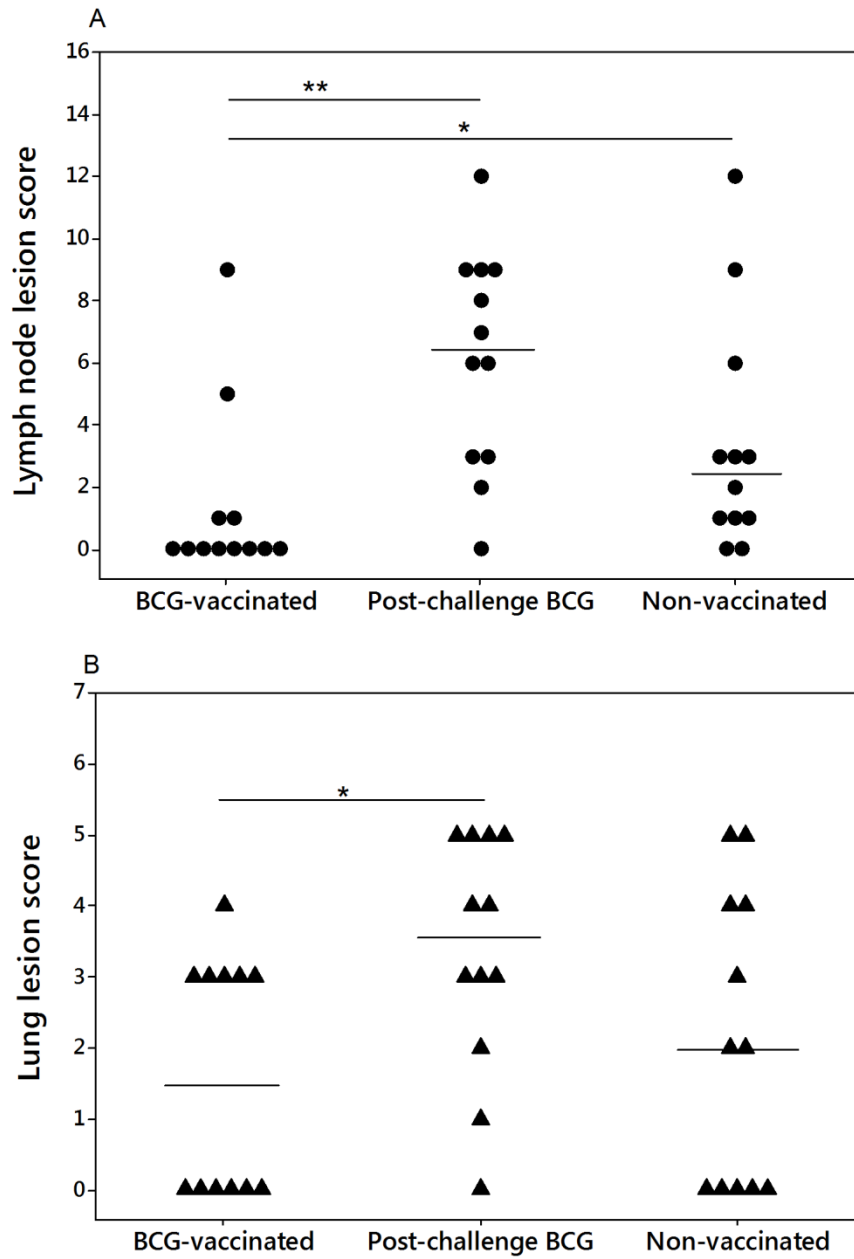
557 **Figure 1.** Lesion scores from the lymph nodes (A) and lung (B) for the BCG-vaccinated  
558 group (n=12); Post-challenge BCG group (n=12) and the Non-vaccinated group (n=12)  
559 after the *M. bovis* challenge. Total lymph node lesion score per animal: score for  
560 individual node: 0, no lesions; 1, 1-19 small lesions (1-3 mm diameter); 2,  $\geq 20$  small  
561 lesions or medium size lesion (4-6 mm diameter); 3, large lesion ( $>6$  mm diameter), total  
562 lesion scores for four pulmonary lymph nodes pooled. Lung lesion score: 0, no lesions; 1,  
563 1-9 lesions; 2, 10-29 lesions; 3, 30-99 lesions; 4, 100-199 lesions; 5,  $\geq 200$  lesions.  
564 Median indicated by horizontal line. Significant difference between groups, \*  $P < 0.05$ ,  
565 \*\*  $P < 0.01$ .

566 **Figure 2.** Percentages of the different granuloma stages in pulmonary lymph nodes of the  
567 BCG-vaccinated, Post-challenge BCG and Non-vaccinated groups. The histopathological  
568 granulomata stages (I, II, III and IV) are described in the Material and Methods. In total,  
569 383, 952 and 391 granulomata were included in the analysis from BCG vaccinated, Post-  
570 challenge BCG treated and Non- vaccinated animals, respectively.

571 **Figure 3.** Mean IFN- $\gamma$  responses following vaccination with BCG and *M. bovis*  
572 challenge. Figure 3 shows mean IFN- $\gamma$  responses to bovine PPD (A) and avian PPD (B)  
573 from blood cultures reported as optical density units 450 nm (OD<sub>450</sub>). BCG-vaccinated  
574 group (◆, n=12); Post-challenge BCG group (■, n=12) and the Non-vaccinated group  
575 (◇, n=12). Arrow V1 (Week 0) indicates vaccination for the BCG-vaccinated group;  
576 arrow C (Week 21) indicates *M. bovis* challenge for all groups; arrow V2 (Week 24)  
577 indicates vaccination for Post-challenge BCG group. Error bar represents SEM. Group  
578 mean was significant difference to that for the Non-vaccinated group was indicated by \*,  
579  $P < 0.05$ , with analyses performed on natural log-transformed data.

580 **Figure 4.** Relative mRNA expression of IFN- $\gamma$ , IL-12p40, IL-2, CXCL9, IL-10, IRF-5,  
581 IL-17A, TNF- $\alpha$ , IL-10, CXCL10 and iNOs from pulmonary lymph nodes of the BCG-  
582 vaccinated group (BCG, n=12); Post-challenge BCG group (PC-BCG, n=12) and the  
583 Non-vaccinated group (NV, n=12). Target Ct values were normalised to U1 and a pool of  
584 non-lesioned prescapular lymph nodes was used as calibrator. The results were presented  
585 as relative fold change of mRNA in a box and whisker plot, with median shown as a  
586 horizontal line. Significant difference between groups, \*  $P < 0.05$ , \*\*  $P < 0.01$ .

587

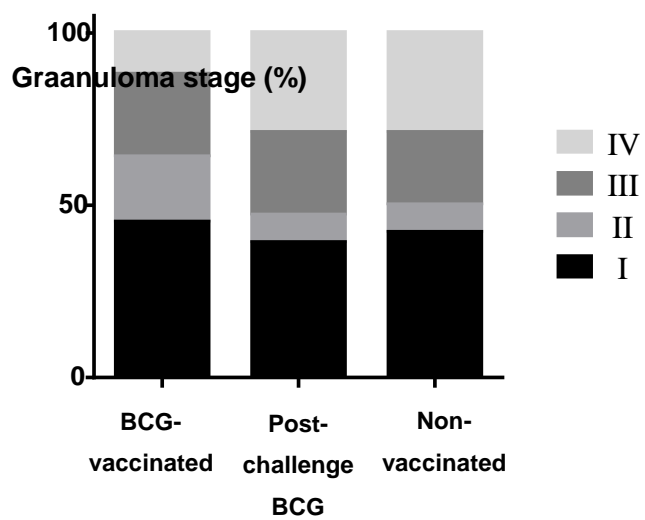
588 **Figure 1.**

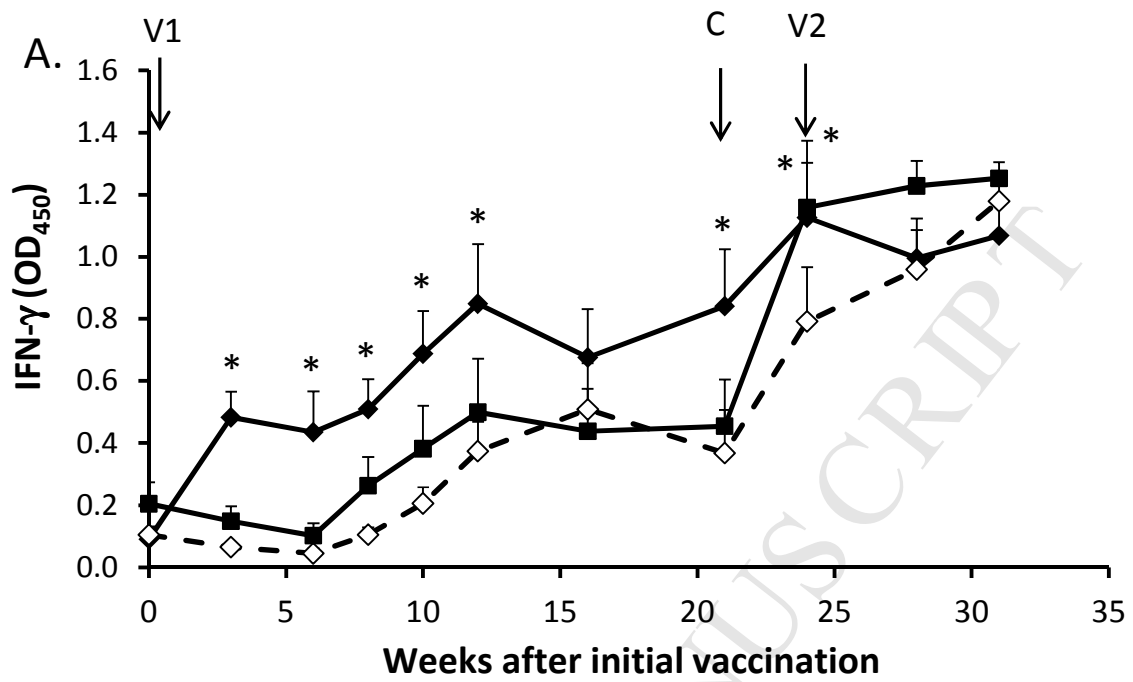
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590 **Figure 2.**

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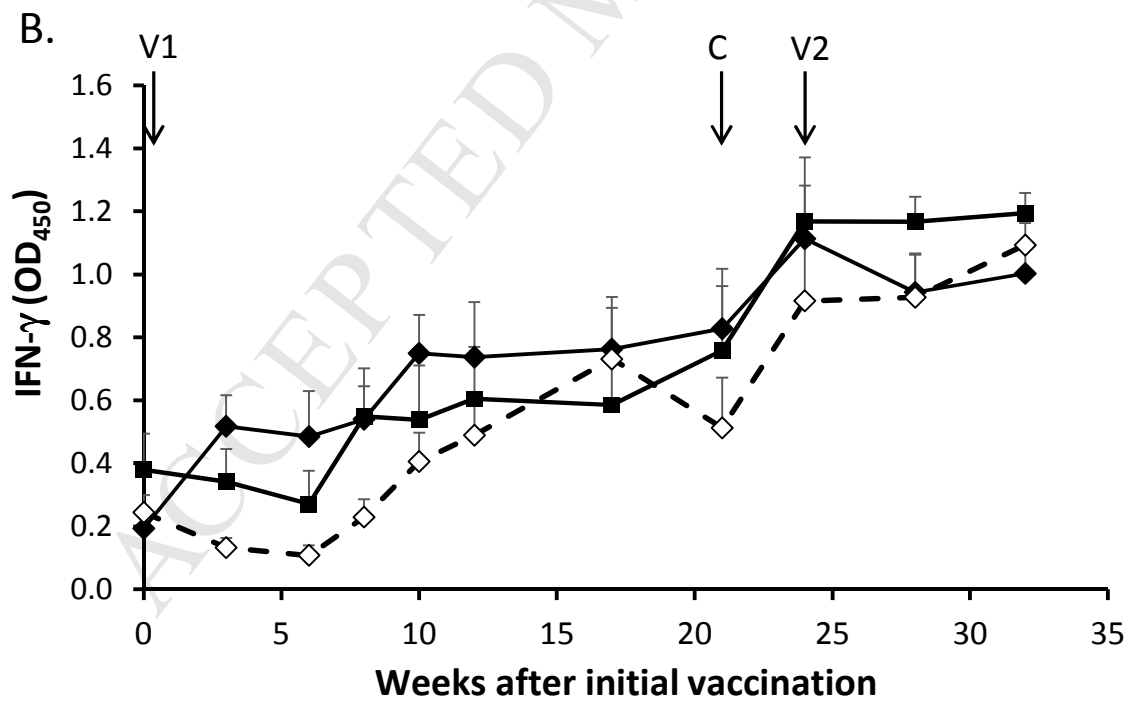
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593 **Figure 3.**

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597

598 **Figure 4.**

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