

Aberystwyth University

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

Buddle, Bryce M.; Shu, Dairu; Parlane, Natalie A.; Subharat, Supatsak; Heiser, Axel; Hewinson, R. Glyn; Vordermeier, H. Martin; Wedlock, D. Neil

Published in:
Tuberculosis

DOI:
[10.1016/j.tube.2016.05.004](https://doi.org/10.1016/j.tube.2016.05.004)

Publication date:
2016

Citation for published version (APA):

Buddle, B. M., Shu, D., Parlane, N. A., Subharat, S., Heiser, A., Hewinson, R. G., Vordermeier, H. M., & Wedlock, D. N. (2016). 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. *Tuberculosis*, 99, 120-127. <https://doi.org/10.1016/j.tube.2016.05.004>

Document License CC BY-NC-ND

General rights

Copyright and moral rights for the publications made accessible in the Aberystwyth Research Portal (the Institutional Repository) are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the Aberystwyth Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the Aberystwyth Research Portal

Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

tel: +44 1970 62 2400
email: is@aber.ac.uk

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

Bryce M. Buddle, Dairu Shu, Natalie A. Parlane, Supatsak Subharat, Axel Heiser, R. Glyn Hewinson, H. Martin Vordermeier, D. Neil Wedlock

PII: S1472-9792(16)30109-3

DOI: [10.1016/j.tube.2016.05.004](https://doi.org/10.1016/j.tube.2016.05.004)

Reference: YTUBE 1459

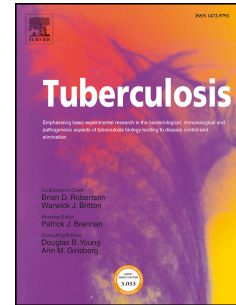
To appear in: *Tuberculosis*

Received Date: 18 March 2016

Accepted Date: 22 May 2016

Please cite this article as: Buddle BM, Shu D, Parlane NA, Subharat S, Heiser A, Hewinson RG, Vordermeier HM, Wedlock DN, 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, *Tuberculosis* (2016), doi: 10.1016/j.tube.2016.05.004.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



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**

4

5

6 Bryce M. Buddle^{a*}, Dairu Shu^a, Natalie A. Parlane^a, Supatsak Subharat^a, Axel Heiser^a, R.
7 Glyn Hewinson^b, H. Martin Vordermeier^b and D. Neil Wedlock^a

8 ^a AgResearch, Hopkirk Research Institute, Grasslands Research Centre, Palmerston North
9 4442, New Zealand

10 ^b TB Research Group, Animal and Plant Health Agency, New Haw, Addlestone, Surrey
11 KT15 3NB, United Kingdom

12

13

14 * Author for correspondence. AgResearch, Hopkirk Research Institute, Grasslands
15 Research Centre, Private Bag 11008, Palmerston North 4442, New Zealand. Tel.: +64 6
16 3518679.

17

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

19 E-mail addresses: bryce.buddle@agresearch.co.nz (B.M. Buddle),
20 dairu.shu@agresearch.co.nz (D.Shu), natalie.parlane@agresearch.co.nz (N.A. Parlane),
21 art.subharat@agresearch.co.nz (S. Subharat), axel.heiser@agresearch.co.nz (A. Heiser),
22 glyn.hewinson@apha.gsi.gov.uk (R.G. Hewinson), martin.vordermeier@apha.gsi.gov.uk
23 H.M. Vordermeier, neil.wedlock@agresearch.co.nz (D.N. Wedlock)

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- γ , IL-12p40, IL-17A,
37 IRF-5, CXCL9, CXCL10, iNOs, and TNF- α) 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 19th 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- γ 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- γ 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 1.5×10^6 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 3×10^6 CFU/vial. All three groups of

131 calves were challenged endobronchially with 5×10^3 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 (6×10^6 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, ≥ 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, ≥ 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 log₁₀ 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-γ* 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 µg/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-γ levels measured using a sandwich ELISA kit
186 (Mabtech, Sweden). Results were reported as optical density units 450 nm (OD₄₅₀) for
187 bovine or avian PPD minus OD₄₅₀ 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°C until the qRT-
200 PCR was undertaken. The primer sequences for IFN- γ , IL-10, IL-12p40, IL-17A,
201 interferon releasing factor-5 (IRF-5), iNOs and TNF- α 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 μl of SyBr®Premix Ex Taq™ II master mixture (Takara
208 Bio Inc., Japan), 2 μl of template cDNA and 1 μl of 5 μM of each gene-specific primers
209 were combined in a 20 μl 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, β -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 ΔCt 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- γ responses a mixed effects model was applied to natural log-
222 transformed IFN- γ 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 χ^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 log₁₀ 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; $P = 0.0145$, χ^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- γ* 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- γ 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- γ 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- γ 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- γ 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- γ 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- γ
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- γ , IRF-5, IL-12p40,
303 IL-17A, iNOs, CXCL9, CXCL10 and TNF- α 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- γ , 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 5×10^4 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 5×10^8 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- γ 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- γ

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- α , 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- α 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- γ , IRF-5, IL-12p40, IL-17A, , iNOs, CXCL9, CXCL10 and
404 TNF- α compared to that for the Non-vaccinated group, although only the responses to
405 IFN- γ , 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- α , CXCL10
409 and type 1 IFNs [32]. Subsequent production of IFN- γ 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- γ 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

432 **Acknowledgements**

433 The authors thank Allison McCarthy, Tania Wilson, Keith Hamel, Gary Yates and
434 Melissa Surrey for excellent technical assistance and Dr Dongwen Luo for help with
435 statistical analyses.

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.

441 **Funding:** The study was funded by the New Zealand Ministry of Business, Innovation
442 and Employment and Department of Environment, Food and Rural Affairs (UK).

443 **Competing interests:** The authors declare that no competing interests exist.

444 **Ethical approval:** All animal procedures were approved by an independent animal
445 ethics committee from the AgResearch Grasslands Research Centre.

446

447

448 **References**

- 449 1. Waters WR, Palmer MV, Buddle BM, Vordermeier HM. Bovine tuberculosis
450 vaccine research: Historical perspectives and recent advances. *Vaccine* 2012;30:2611-22.
- 451 2. Cousins DV. *Mycobacterium bovis* infection and control in domestic livestock. *Rev*
452 *Sci Tech Off Int Epiz* 2001;20:71-85.
- 453 3. Buddle BM, Parlane NA, Wedlock DN, Heiser A. Overview of vaccination trials
454 for control of tuberculosis in cattle, wildlife and humans. *Transbound Emerg Dis*
455 2013;60(Suppl.1):136-46.
- 456 4. Berggren SA. Field experiment with BCG vaccine in Malawi. *Br Vet J*
457 1981;137:88-94.
- 458 5. Whelan AO, Coad M, Upadhyay BL, Clifford DJ, Hewinson RG, Vordermeier
459 HM. Lack of correlation between BCG-induced skin test reactivity and protective
460 immunity in cattle. *Vaccine* 2011;29:5453-8.
- 461 6. Whelan AO, Clifford D, Upadhyay B, Bredon EL, McNair J, Hewinson RG, et al.
462 Development of a skin test for bovine tuberculosis for differentiating infected from
463 vaccinated animals. *J Clin Microbiol* 2010;48:3176-81.
- 464 7. Vordermeier M, Gordon SV, Hewinson RG. *Mycobacterium bovis* antigens for the
465 differential diagnosis of vaccinated and infected cattle. *Vet Microbiol* 2011;151:8-13.
- 466 8. Parlane NA, Shu D, Subharat S, Wedlock DN, Rehm BHA, de Lisle GW, et al.
467 Revaccination of cattle with Bacille Calmette-Guérin two years after first vaccination
468 when immunity has waned, boosted protection against challenge with *Mycobacterium*
469 *bovis*. *PLOS ONE* 2014;9:e106519.
- 470 9. Vordermeier HM, Villaraeal-Ramos B, Cockle PJ, McAulay M, Rhodes SG,
471 Thacker T, et al. Viral booster vaccines improve *Mycobacterium bovis* BCG-induced
472 protection against bovine tuberculosis. *Infect. Immun.* 2009;77:3364-73.
- 473 10. Koch R. Weitere. Mitteilungen uber ein Heilmittel gegen Tuberkulose. *Dtsch Med*
474 *Wochenschr* 1890;16:1029-32.
- 475 11. Baily GV. Tuberculosis prevention trial: Madras. *Indian J Med Res*
476 1980;72(Suppl.):1-74.
- 477 12. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, et al.
478 Pathological role of interleukin-17 in mice subjected to repeated BCG vaccination after
479 infection with *Mycobacterium tuberculosis*. *J Exp Med* 2010;207:1609-16.

- 480 13. Rook GA, al Attiyah R, Filley E. New insights into immunopathology of
481 tuberculosis. *Pathobiology* 1991;59:148-52.
- 482 14. Rook GA, Stanford JL. The Koch phenomenon and the immunopathology of
483 tuberculosis. *Curr Top Microbiol Immunol* 1996;215:239-62.
- 484 15. Ameni GG, Vordermeier HM, Aseffa AA, Young DB, Hewinson RG. Field
485 evaluation of the efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin against
486 bovine tuberculosis in neonatal calves. *Clin Vaccine Immunol* 2010;17:1533-8.
- 487 16. Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., Wedlock, D.N. 2013.
488 Subcutaneous administration of a 10-fold lower dose of a human tuberculosis vaccine,
489 bacille Calmette-Guérin Danish compared to a standard cattle dose induced similar levels
490 of protection against bovine tuberculosis and responses in the tuberculin intradermal test.
491 *Clin. Vaccine Immunol.* 20: 1559-1562.
- 492 17. Buddle BM, de Lisle GW, Pfeffer A, Aldwell FE. Immunological responses and
493 protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG.
494 *Vaccine* 1995;13:1123-30.
- 495 18. Wedlock DN, Denis M, Skinner MA, Koach J, de Lisle GW, Vordermeier HM, et
496 al. Vaccination of cattle with a CpG oligodeoxynucleotide-formulated mycobacterial
497 protein vaccine and *Mycobacterium bovis* BCG induces levels of protection against
498 bovine tuberculosis superior to those induced by vaccination with BCG alone. *Infect*
499 *Immun* 2005;73:3540-6.
- 500 19. Wangoo A, Johnson L, Gough J, Ackbar R, Inglut S, Hicks D, et al. Advanced
501 granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with
502 increased expression of type I procollagen, gammadelta (WC1+) T cells and CD 68+
503 cells. *J Comp Pathol* 2005;133:223-34.
- 504 20. Shu D, Subharat S, Wedlock DN, Luo D, de Lisle GW, Buddle BM. Diverse
505 cytokine profile from mesenteric lymph node cells of cull cows severely affected with
506 Johne's disease. *Clin Vaccine Immunol* 2011;18:1467-76.
- 507 21. Shu D, Heiser A, Wedlock DN, Luo D, de Lisle GW, Buddle BM. Comparison of
508 gene expression of immune mediators in cattle lung and pulmonary lymph node
509 granulomas from cattle experimentally infected with *Mycobacterium bovis*. *Vet Immunol*
510 *Immunopathol* 2014;160:81-9.
- 511 22. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T)
512 method. *Nat Protoc* 2008;3:1101-8.

- 513 23. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and
514 powerful approach to multiple testing. *J. Royal Stat. Soc. Series B* 1995;57:289-300.
- 515 24. Pinheiro JD, Bates S, DebRoy, Sarkar D, the R Development Core Team. 2013.
516 nlme: Linear and Nonlinear Mixed Effects Models. R package version 2013;3:1-107.
- 517 25. Cassidy JP, Bryson TD, Pollock JM, Forster F, Evans RT, Neill SD. Early lesion
518 formation in cattle experimentally infected with *Mycobacterium bovis*. *J Comp Pathol*
519 1998;119:27-44.
- 520 26. Buddle BM, Wards BJ, Aldwell FE, Collins DM, de Lisle GW. Influence of
521 sensitisation to environmental mycobacteria on subsequent vaccination against bovine
522 tuberculosis. *Vaccine* 2002;20:1126-33.
- 523 27. Hatherill M, Geldenhuys H, Pienaar B, Suliman S, Chheng P, Debanne SM, et al.
524 Safety and reactogenicity of BCG revaccination with isoniazid. *Vaccine* 2014;32:3982-8.
- 525 28. Griffin JFT, Mackintosh CG, Slobbe L, Thomson AJ, Buchan GS. Vaccine
526 protocols to optimise the protective efficacy of BCG. *Tubercle Lung Dis* 1999;79:135-43.
- 527 29. Turner J, Rhoades ER, Ken M, Belisle JT, Frank AA, Orme IM. Effective pre-
528 exposure tuberculosis vaccines fail to protect when given in an immunotherapeutic mode.
529 *Infect Immun* 2000;68:1706-9.
- 530 30. Moreira AL, Tsenova L, Aman MH, Bekker L-G, Freeman S, Mangaliso B, et al.
531 2002. Mycobacterial antigens exacerbate disease manifestations in *Mycobacterium*
532 *tuberculosis*-infected mice. *Infect Immun* 2002;70:2100-7.
- 533 31. Ernst JD. The immunological life cycle of tuberculosis. *Nat Rev Immunol*
534 2012;12:581-91.
- 535 32. Cham CM, Ko K, Niewold TB. Interferon regulatory factor 5 in the pathogenesis of
536 systemic lupus erythematosus. *Clin Dev Immunol* 2012;2012:ID780436,1-11.
- 537 33. Aranday-Cortes E, Bull NC, Villarreal-Ramos B, Gough J, Hicks D, Ortiz-Peláez
538 A, et al. Upregulation of IL-17, CXCL9 and CXCL10 in early-stage granulomas induced
539 by *Mycobacterium bovis* in cattle. *Transbound Emerg Dis* 2013;60:525-37.
- 540 34. Pak-Wittel MA, Yang L, Riverbark JG, Yokoyama WM. Interferon- γ mediates
541 chemokine-dependent recruitment of natural killer cells during viral infection. *Proc Natl*
542 *Acad Sci USA*. 2013;110:E50-9.
- 543 35. Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to
544 infection. *J Immunol* 2008;180:5771-7.

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 [†]	0.67 [†] (\pm 0.33)	1.97 [†] (\pm 0.29)	1.23 [†] (\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 [†] (\pm 0.35)	1.42 [†] (\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 [†] 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

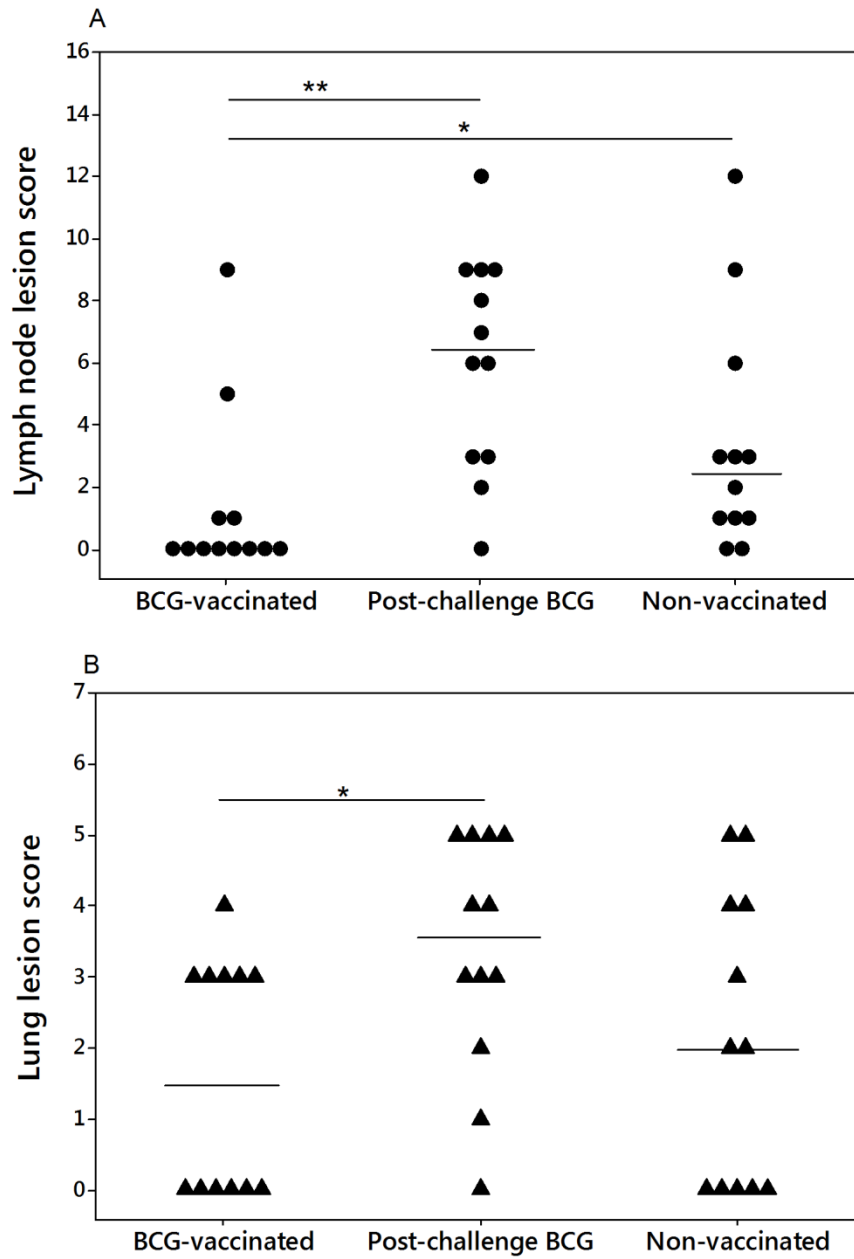
557 **Figure 1.** Lesion scores from the lymph nodes (A) and lung (B) for the BCG-vaccinated
558 group (n=12); Post-challenge BCG group (n=12) and the Non-vaccinated group (n=12)
559 after the *M. bovis* challenge. Total lymph node lesion score per animal: score for
560 individual node: 0, no lesions; 1, 1-19 small lesions (1-3 mm diameter); 2, ≥ 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, ≥ 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- γ responses following vaccination with BCG and *M. bovis*
572 challenge. Figure 3 shows mean IFN- γ responses to bovine PPD (A) and avian PPD (B)
573 from blood cultures reported as optical density units 450 nm (OD₄₅₀). 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- γ , IL-12p40, IL-2, CXCL9, IL-10, IRF-5,
581 IL-17A, TNF- α , 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

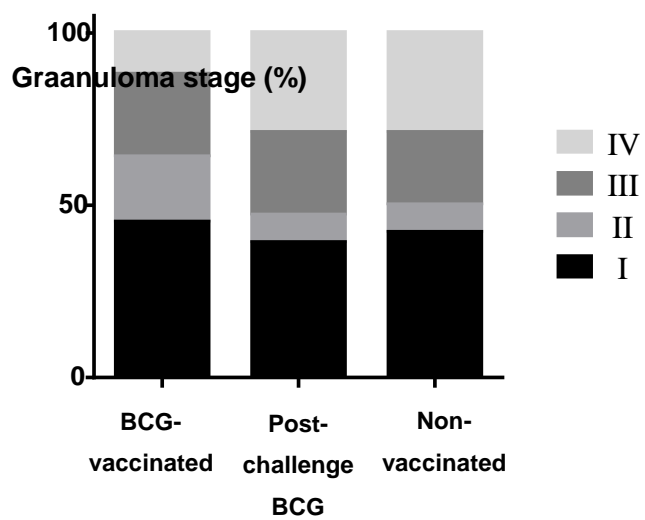
588 **Figure 1.**

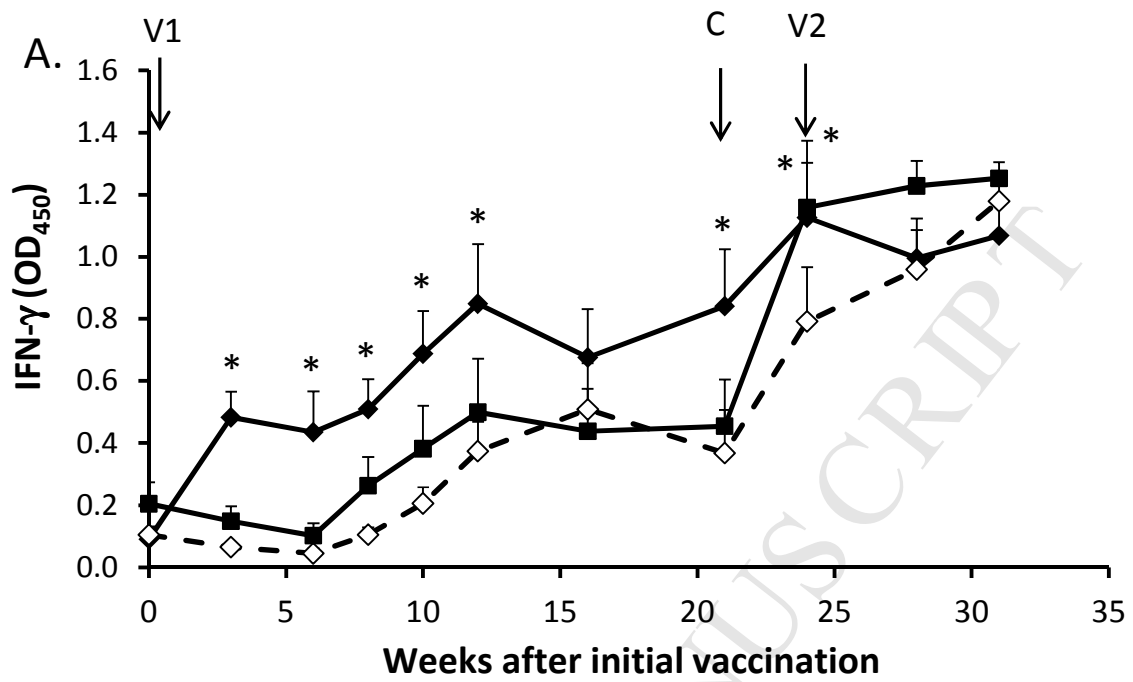
589

590 **Figure 2.**

591

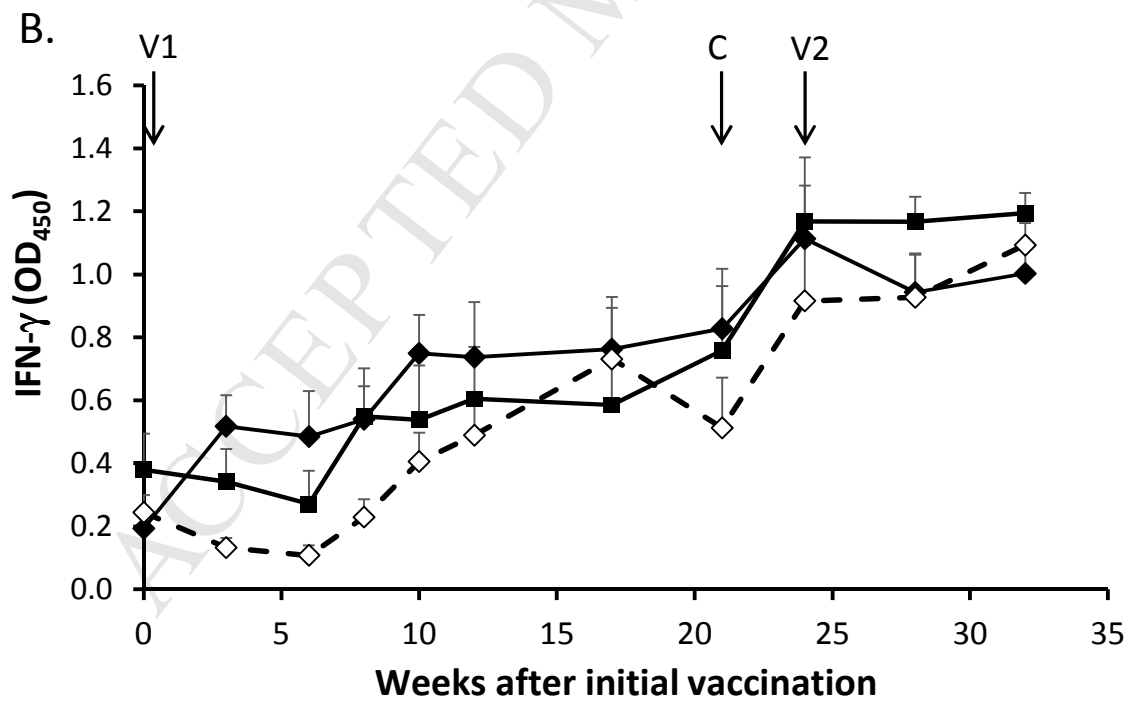
592



593 **Figure 3.**

594

595



596

597

598 **Figure 4.**

599

600

