1 **Full title:**

2 Differential cell composition and cytokine expression within lymph node granulomas
3 from BCG vaccinated and non-vaccinated cattle experimentally infected with
4 *Mycobacterium bovis*

5 **Short title:**

6 Cell types and cytokines in BCG and *M. bovis* infected cattle

7 **Authors:**

8 Francisco J. Salguero^{1,2*}, Sophie Gibson^{1,2}, Waldo Garcia-Jimenez², Julie Gough¹, Tony
9 S. Strickland¹, Hans M. Vordermeier¹, Bernardo Villarreal-Ramos¹.

10

11 ¹ TB Research Group. Department of Bacteriology. Animal Health and Veterinary
12 Laboratories Agency, APHA-Weybridge, New Haw, Addlestone, Surrey, United
13 Kingdom

14 ² Department of Pathology and Infectious Diseases. School of Veterinary Medicine.
15 University of Surrey. Guildford, Surrey, United Kingdom

16

17 ***Corresponding author:**

18 Francisco J. Salguero
19 Department of Pathology and Infectious Diseases. School of Veterinary Medicine.
20 University of Surrey. Guildford, Surrey, United Kingdom

21 Tel: +44 (0) 1483 689872

22 Fax: +44 (0) 1483 686711

23 E-mail: f.salguero@bodes@surrey.ac.uk

24 Summary

25 Cattle vaccination against bovine tuberculosis (bTB) has been proposed as a
26 supplementary method to help control the incidences of this disease. Bacillus
27 Calmette-Guérin (BCG) is currently the only viable candidate vaccine for immunisation
28 of cattle against bTB, caused by *Mycobacterium bovis* (*M. bovis*).

29 In an attempt to characterise the differences in the immune response following *M.*
30 *bovis* infection between BCG vaccinated and non-vaccinated animals, a combination of
31 gross pathology, histopathology and immunohistochemical (IHC) analyses was used.
32 BCG vaccination was found to significantly reduce the number of gross and
33 microscopic lesions present within the lungs and lymph nodes. Additionally, the
34 microscopically visible bacterial load of stage III and IV granulomas was reduced.

35 IHC using cell surfaces markers revealed the number of CD68+ (macrophages), CD3+
36 (T-lymphocytes) and WC1+ cells ($\gamma\delta$ T-cells) to be significantly reduced in lymph node
37 granulomas of BCG vaccinated animals, when compared to non-vaccinated animals. B
38 lymphocytes (CD79a+) were significantly increased in BCG-vaccinated cattle for
39 granulomas at stages II, III and IV. IHC staining for iNOS showed a higher expression in
40 granulomas from BCG vaccinated animals compared to non-vaccinated animals for all
41 stages, being statistically significant in stages I and IV. TGF β expression decreased
42 alongside the granuloma development in non-vaccinated animals, whereas BCG
43 vaccinated animals showed a slight increase alongside lesion progression. IHC analysis
44 of the cytokines IFN- γ and TNF α demonstrated significantly increased expression
45 within the lymph node granulomas of BCG vaccinated cattle. This is suggestive of a
46 protective role for IFN γ and TNF α in the response to *M. bovis* infection.

Findings shown in this study suggest that the use of BCG vaccine, can reduce the number and severity of lesions , induce a different phenotypic response and increase the local expression of key cytokines related to protection.

Keywords

Bovine tuberculosis, BCG, immunohistochemistry, cytokine, immune response.

Introduction

Bovine tuberculosis (bTB), caused by infection with *Mycobacterium bovis*, is a disease of international importance, with an estimated 50 million cattle infected worldwide, at an annual cost of US\$3 billion (Buddle et al., 2011; Waters et al., 2012). Despite the application of the test and slaughter policy, the incidence of bTB in GB has increased steadily since the 1980s and this is thought to be partly due to the existence of wildlife reservoirs (Vordermeier et al., 2006; Donnelly and Woodroffe 2015). It is thought that, in the presence of a wildlife reservoir, the test and slaughter policy will not be sufficient to stem the increase in the incidence of bTB and cattle vaccination has been proposed as a supplementary measure to reduce the duration of time that herds are

66 under restrictions and the number of animals removed during testing (EFSA, 2013).
67 Furthermore, a vaccination strategy would also benefit developing countries, where a
68 ‘test and slaughter’ approach is not feasible to be implemented (Vordermeier et al.,
69 2002).

70 However, vaccination is not currently used as part of the European bovine TB control
71 program. The most important fact against the use of cattle vaccination is the
72 interference of BCG with the specificity of the tuberculin skin test (Whelan et al.,
73 2011), which is the cornerstone of current surveillance and eradication strategies
74 (Conlan et al., 2012). However, the development of diagnostic tests that can
75 Differentiate Infected from Vaccinated Animals (DIVA) (Vordermeier et al., 2011; Jones
76 et al., 2012) could open up the use of BCG as an important tool to complement current
77 control programs.

78 Reports of the use of BCG vaccination in experimentally infected cattle show that the
79 vaccine is effective in reducing the number and severity of lesions and also the
80 bacterial count (Johnson et al., 2006). Absolute protection is not conferred; the
81 infecting *M. bovis* can often be detected in the lymph nodes draining the site of entry,
82 however, secondary spread is slowed, or even prevented (Buddle et al., 2006; Ly et al.,
83 2007). Recent field trials and experiments under natural transmission conditions have
84 also reported its effectiveness, although with variable levels of protection (Suazo et al.,
85 2003; Ameni et al., 2010; Lopez-Valencia et al., 2010).

86 The evaluation of bTB lesions using a combination of gross pathology, light microscopy
87 and immunohistochemical markers is useful to discriminate granuloma stages as

88 indicators for the progress of infection and determine important aspects like vaccine
89 efficacy and disease severity (Johnson et al., 2006). The main objective of this study
90 was to characterise the immune response (differential granuloma stages and cytokine
91 expression) within lymph node granulomas from BCG vaccinated and experimentally
92 infected cattle with *M. bovis*.

93

94

95 **Materials and Methods**

96 **Ethical statement**

97 This study has been carried out in accordance with the UK Animal (Scientific
98 Procedures) Act 1986, under appropriate personal and project licences. The protocol
99 used was approved by the APHA Ethics Review Process Committee.

100 **Experimental Infection and post-mortem examination**

101 Ten male Holstein-Friesian calves aged between 5 and 7 months were vaccinated
102 subcutaneously with 1×10^6 cfu of Danish BCG (group BCG). A further ten animals were
103 used as the non-vaccinated group. Twelve weeks later all 20 animals were challenged
104 with 2000 cfu of *M. bovis* AF 2122/97 via endotracheal instillation. All cattle were
105 obtained from herds of high health status originating from bTB-free areas and after
106 infection with *M. bovis* were housed in BSL3 accommodation. Animals were
107 euthanised using a captive bolt gun 13 weeks following infection and post-mortem
108 examination was performed immediately by a veterinary pathologist. Lungs were

109 examined for lesions externally and internally by slicing at 0.5-1 cm intervals. Lymph
110 nodes of the head and neck region: lateral and medial retropharyngeal, submandibular
111 and parotid lymph nodes; and the bronchial and mediastinal lymphocentres : cranial
112 tracheobronchial, left and right bronchial (tracheobronchial lymph nodes within the
113 bifurcation), cranial and caudal and mediastinal lymph nodes were incised to inspect
114 for lesions. Scores were assigned based on a previously scoring scheme reported in
115 Vordermeier et al., 2002. Briefly, lung lobes (left apical, left cardiac, left diaphragmatic,
116 right apical, right cardiac, right diaphragmatic, and right accessory lobes) were
117 examined individually, and the following scoring system was applied: 0= no visible
118 lesions; 1= no gross lesions but lesions apparent on slicing; 2= <5 gross lesions of <10
119 mm in diameter; 3= >6 gross lesions of <10 mm in diameter or a single distinct gross
120 lesion of >10 mm in diameter; 4= >1 distinct gross lesion of >10 mm in diameter; 5=
121 gross coalescing lesions. The scores of the individual lobes were added up to calculate
122 the lung score. For lymph nodes, the severity of the observed gross pathology in
123 individual lymph nodes was scored applying the following scoring system: 0= no
124 necrosis or visible lesions; 1= small focus (1 to 2 mm in diameter); 2= several small foci
125 or necrotic area of at least 5 by 5 mm; 3= extensive necrosis. Individual lymph node
126 scores were added up to calculate the lymph node score. Both lung and lymph nodes
127 pathology scores were added to determine the total pathology score per animal.
128 Additionally, samples of lymph nodes were taken for bacterial culture. Sections of the
129 cranial and caudal mediastinal, left bronchial (tracheobronchial lymph node in the
130 tracheal bifurcation), cranial tracheobronchial and left parotid lymph nodes were fixed
131 in buffered formalin for further processing, as described below. The left parotid lymph

node served as a control tissue as no lesions would be expected here because of the infection route, based on previous studies by this group (Aranday-Cortes et al., 2013).

Histopathology

Sections of lymph nodes were stained with haematoxylin and eosin (H&E) to be examined under light microscope. Granuloma were classified in four stages (Wangoo et al., 2005). Briefly, Stage I (Initial), formed by irregular unencapsulated clusters of epithelioid macrophages, with interspersed lymphocytes and small numbers of neutrophils. Stage II (Solid), composed primarily of epithelioid macrophages and partly or completely enclosed by a thin capsule. Minimal necrotic areas were sometimes present. Stage III (Minimal necrosis), at this stage the granulomas were fully encapsulated, with central necrotic areas, which were mainly caseous and sometimes slightly mineralized. Epithelioid macrophages admixed with multinucleated giant cells (MNGCs) surrounded the necrotic areas. A peripheral zone of macrophages mixed with clusters of lymphocytes and scattered neutrophils extended to the fibrous capsule. Stage IV (Necrosis and mineralization) were characterize by large, irregular, multicentric and thickly encapsulated granulomas. Prominent caseous necrosis with extensive islands of mineralization occupying the greater part of the lesion. Epithelioid macrophages together with MNGCs surrounded the necrosis, with particularly dense clusters of lymphocytes near the peripheral fibrotic capsule.

In addition, the number of MNGCs in each lesion were scored between 0 (no MNGCs) and 2 (many MNGCs). Ziehl-Neelsen stain (ZN) was used to determine the microscopically visible number of acid fast bacilli (AFBs) present in each granuloma.

154 Immunohistochemistry

155 The IHC procedures are summarised in Table 1. Formalin fixed samples lymph nodes
156 were cut in 4 µm sections, dewaxed and rehydrated at room temperature, and placed
157 in a fresh solution of 3% hydrogen peroxide in methanol for 15 minutes to block
158 endogenous peroxidase activity. Samples were then washed in tap water. These stages
159 were carried out using a Shandon Varistain 24-4® (Shandon Scientific, Runcorn, UK).
160 Epitope demasking was performed with different methods, according to the different
161 primary antibodies used, as detailed in Table 1. Samples were either subjected to heat
162 digestion, using a Thermotissuewave microwave (Shandon Scientific), or enzyme
163 digestion, using a solution of 0.5% trypsin/0.5% chemotrypsin (Sigma, Poole, UK)
164 adjusted to pH 7.8 with 0.1M sodium hydroxide. Running tap water was then used to
165 wash the tissue sections and the slides were then mounted onto Shandon
166 coverplates™ and loaded into Sequenza® trays (Shandon Scientific). The samples were
167 washed with either tris-buffered saline (TBS) (0.85% NaCl, 0.0605% tris, adjusted to pH
168 6.7 using 1M HCl) or TBS with 0.05% tween-20 (TBST) (Sigma), as detailed in Table 1.
169 Goat normal serum (190 µl) was then added to the slide at a 1:66 dilution in TBS or
170 TBST (Table 1) as blocking agent. After twenty minutes, 190 µL of the primary antibody
171 was added. Appropriate controls were included in each IHC run. These included
172 sequential sections with an isotype control for each primary antibody, and the
173 omission of the primary antibody. The incubation time varied for each primary
174 antibody used (Table 1). After two washes with TBS or TBST buffer, 190 µL of
175 biotinylated link antibody and link block (normal goat serum) were added, followed by
176 two further buffer washes, 30 minutes later. Primary and secondary antibody binding

177 was amplified using avidin–biotin–peroxidase conjugate (ABC elite; Vector
178 Laboratories) and visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB).
179 Unbound conjugate was removed prior to DAB application with two buffer washes.
180 Slides were then washed in purified water, removed from the coverplates and placed
181 in a rack. Samples were rinsed with tap water for five minutes, before being placed in
182 Mayers Haematoxylin counterstain, followed by a further wash in tap water. Finally,
183 sections were dehydrated, cleared and mounted for analysis.

184 **Image analysis**

185 Immunolabelled sections were analysed using light microscopy and digital image
186 analysis (Nikon NIS Br, Nikon, Japan). All granulomas were examined with the x40
187 objective to give a final magnification of x400, to ascertain the percentage of
188 immunostained cells in the lesion. The whole area of the granuloma was selected as
189 Region of Interest (ROI) and the area with immunohistochemical positive reaction
190 within the ROI was calculated by the software after setting the thresholds. The results
191 as expressed as the percentage of positively immunolabeled area within the total area
192 of the granuloma. The total number of analysed granulomas was up to 219 in the BCG
193 group and up to 687 for the non-vaccinated group. Necrotic or mineralised areas were
194 not included in the analysis, as described previously (Aranday-Cortes et al., 2013).

195 **Statistics**

196 χ -square analysis was applied to analyse the trend in granuloma classification
197 (histopathology) comparison of BCG and NC group.

198 The results of immunohistochemical analysis are expressed as mean $\pm$ standard
199 deviation (S.D). The mean results between groups for each granuloma stage were
200 analysed by comparing means assuming unequal variance using the t-test with Welch
201 approximation. Differences were considered significant at $P < 0.05$.

202 All the analyses were conducted using Graph Pad Prism 4.0 (San Diego, CA, U.S.A.) and
203 STATA10 (College Station, TX: StataCorp LP).

204 **Results**

205 **Gross pathology**

206 Gross lesions were present in the lymph nodes and the lungs of 9/10 and 8/10 non-
207 vaccinated animals respectively and in the lymph nodes and the lungs of 3/10 and 4/10
208 BCG vaccinated animals respectively (Fig. 1). Lesions were either solitary or coalescing
209 areas of caseous necrosis. Both, BCG vaccinated and non vaccinated animals, had
210 lesions of an identical appearance, although granulomas in vaccinated animals were
211 generally smaller and isolated. Fig. 1 demonstrates that BCG vaccinated cattle had a
212 lower average gross pathology score for both the lung and lymph nodes compared
213 with non-vaccinated animals. One BCG vaccinated animal displayed lesions similar in
214 severity to the majority of non-vaccinated cattle. Interestingly, one non-vaccinated
215 animal had no visible lesions.

216 **Histopathology**

217 As shown in Fig. 2, there are statistically significantly fewer granulomas present in BCG
218 vaccinated animals compared to non-vaccinated lymph nodes. Using a system

219 previously developed (Wangoo et al., 2005) to grade the stage of development of the
220 granuloma it was shown that for BCG vaccinated animals, the majority of granulomas
221 were stage I. These appeared as small, irregular clusters of predominantly epithelioid
222 macrophages, with occasional MNGCs, lymphocytes and neutrophils. In contrast, non-
223 vaccinated animals displayed mostly stage IV granulomas, which are large and
224 multicentric, with prominent necrosis and mineralisation and are surrounded by a
225 thick fibrous capsule. Additionally, it was seen that stage IV granulomas were often
226 surrounded by small stage I and II 'satellite granulomas'. These were numerous in non-
227 vaccinated animals but were uncommon in BCG vaccinated cattle, where the majority
228 of granulomas were isolated. The granulomas of both vaccinated and non-vaccinated
229 cattle appeared similar microscopically, although MNGCs appeared to be increased in
230 non-vaccinated animals.

231 Four animals exhibited no microscopic lesions: three from BCG group and 1 from the
232 NC group. In contrast, one BCG vaccinated animal appeared unprotected and showed
233 many granulomas of all stages, histologically appearing similar to many of the non-
234 vaccinated cattle. This animal contributed the majority of stage III and IV granulomas
235 present in BCG vaccinated cattle.

236 Examination of ZN stained sections revealed that statistically significantly fewer AFBs
237 were present within each stage of granuloma for BCG vaccinated cattle in comparison
238 to non-vaccinated, as shown in Fig. 3. AFBs were also more numerous in stage III and
239 IV granulomas than stages I and II and were most commonly present within areas of
240 necrosis, although were also located intracellularly within epithelioid macrophages and
241 MNGCs (Fig. 3).

242 Immunohistochemistry

243 *Macrophages (CD68) and iNOS+ cells*

244 Immunohistochemistry for CD68 indicates the location of macrophages (including
245 epithelioid macrophages and MNGCs). Within lymph node sections, BCG vaccinated
246 animals showed a statistically significant reduction in the mean number of CD68⁺ cells
247 for all stages of granuloma when compared to non-vaccinated animals (Fig. 4).

248 Distribution of macrophages within lesions was similar between the BCG vaccinated
249 and non-vaccinated groups. Macrophages were dispersed throughout stage I and II
250 granulomas, however, were predominantly situated around the necrotic centre of
251 stage III and IV lesions. BCG vaccinated cattle displayed fewer MNGCs than non-
252 vaccinated animals upon IHC (Fig. 4).

253 Immunohistochemical staining for iNOS showed a higher mean percentage staining in
254 the granulomas of BCG vaccinated animals compared to non-vaccinated cattle for all
255 granuloma stages, being statistically significant in stages I and IV.

256 Positive staining for iNOS was present in all stages of granulomas in both groups.
257 Stages I and II showed scattered staining throughout and stage III and IV displayed a
258 rim of positive macrophages, epithelioid and MNGCs surrounding the necrotic centres
259 (Fig. 5).

260 *T lymphocytes (CD3)*

261 For each stage, a statistically significant reduction in the mean number of CD3⁺ cells
262 was noted in BCG vaccinated animals compared to non-vaccinated, as shown in Fig. 6.

263 Distribution of CD3+ cells was similar for both groups of animals; in stages I and II CD3+
264 cells were dispersed throughout the granuloma, but were predominantly situated
265 peripherally around the fibrous capsule in stage III and IV granulomas (Fig. 6).

266 *$\gamma\delta$ T cells (WC1)*

267 Immunohistochemistry for WC1 shows the location of a subset of bovine $\gamma\delta$ T cells,
268 which for all granulomas examined formed a small proportion of the total cells. As
269 shown in Fig. 7, there was a statistically significant reduction in the mean number of $\gamma\delta$
270 T cells for BCG vaccinated cattle in comparison to non-vaccinated for each stage. For
271 both groups independently, the percentage of WC1+ cells was similar when comparing
272 each stage of granuloma development. WC1+ cells were scattered throughout stage I
273 and II granulomas, but were present peripherally around the edge of the fibrous
274 capsule in stages III and IV (Fig. 7).

275 *B lymphocytes (CD79a)*

276 Immunohistochemical staining for CD79a showed the location of B lymphocytes within
277 the lesions. For granuloma stages II, III and IV, BCG vaccinated animals had a
278 statistically significantly higher mean number of positively stained cells compared to
279 non-vaccinated animals. Stage I granulomas of both groups had similar mean
280 percentage of CD79a positive cells. It was also noted that BCG vaccinated animals
281 showed an increase in the mean percentage of B cells through stages I to III, whereas
282 non-vaccinated animals show a decrease in the mean percentage of positively stained
283 cells between the same stages (Fig. 8).

284 Distribution of B lymphocytes in both BCG vaccinated and non-vaccinated animals was
285 identical for both groups of animals; stage I and II granulomas showed scattered

286 positive staining throughout the lesion, whereas for stage III and IV granulomas
287 staining was peripheral. Additionally, stage III and IV granulomas were frequently
288 surrounded by clusters of B cells situated outside the fibrous capsule (Fig. 8D).

289 *IFN γ*

290 Immunohistochemical staining for IFN γ showed a statistically significantly higher mean
291 percentage staining in the granulomas of BCG vaccinated animals compared to non-
292 vaccinated cattle for all stages (Fig. 9). For both groups of animals, there was an
293 increase in the percentage of cells expressing IFN γ between stages I to IV.

294 Staining was most prominently associated with lymphocytes, with occasional
295 macrophages also showing positivity. For both, BCG vaccinated and non-vaccinated
296 cattle, stage III and IV granulomas showed most of cells expressing IFN γ at the
297 periphery of the granulomas, whereas stage I and II showed staining dispersed
298 throughout the lesion.

299 *TNF α*

300 Immunohistochemistry for TNF α shows a statistically significantly higher mean
301 percentage of staining in BCG vaccinated animals compared to non-vaccinated for
302 stages I, II, and III. The mean percentage of positive staining was not statistically
303 significantly different between stage IV granulomas of both groups. Additionally, the
304 expression of TNF α was shown to increase between stages I to IV for BCG and non-
305 vaccinated animals (Fig. 10). TNF α expression was mostly associated with
306 macrophages, including epithelioid and MNGCs as shown in Fig. 10. The higher
307 numbers of MNGCs in stage IV granulomas of non-vaccinated animals could be the

308 cause as the expression of TNF α was very similar to that observed in stage IV
309 granulomas of BCG vaccinated animals. Positive staining was dispersed throughout
310 stage I and II granulomas and mostly located around the necrotic centres of stage III
311 and IV granulomas for both BCG and non-vaccinated groups.

312 *TGF β*

313 Immunohistochemistry for TGF β showed that for granuloma stages I, II and III, BCG
314 vaccinated animals displayed a lower mean percentage of positive staining; although
315 this difference was only statistically significant when comparing stage I granulomas of
316 the two groups (Fig. 11). Non-vaccinated animals showed a pattern of decreased TGF β
317 expression as the granulomas developed between stages I and IV, whereas BCG
318 vaccinated animals showed a slight increase with lesion progression. TGF β was
319 expressed in all type of macrophages throughout all the layers of the granulomas (Fig.
320 11).

321

322 **Discussion**

323 Eradication of bTB has proven to be difficult in some areas of UK using test and
324 slaughter strategies, and other approaches such as cattle vaccination are being
325 proposed. Candidate vaccines need to be tested in order to know important aspects
326 like safety and efficacy. The evaluation of bTB lesions using a combination of gross
327 pathology, histopathology and immunohistochemical markers is useful to determine
328 these important aspects (Johnson et al., 2006). In this study, we have characterised the

329 immune response (differential cellular composition and cytokine expression in
330 different granuloma stages) within lymph node granulomas from cattle vaccinated
331 with BCG and non-vaccinated animals, experimentally infected with *M. bovis*.

332 BCG vaccination does confer a significant degree of protection against experimental *M.*
333 *bovis* infection in cattle (Vordermeier et al., 2002; Buddle et al., 2005). BCG vaccinated
334 cattle exhibited a lower mean gross pathology score for both lungs and lymph nodes
335 compared to non-vaccinated animals following *M. bovis* infection. This was supported
336 by the detection of significantly fewer granulomas in the tissues of BCG vaccinated
337 animals compared with the non-vaccinated animals by examination of H&E stained
338 sections. Three of the ten BCG vaccinated cattle showed no detectable lesions thirteen
339 weeks post infection. Granulomas from the majority of BCG vaccinated animals
340 showed significantly fewer AFBs and fewer MNGCs for all stages of granulomas,
341 suggestive of a lower bacterial load and greater ability to control infection.

342 One BCG-vaccinated animal showed no protection from *M. bovis* infection, exhibiting a
343 high gross pathology score and multiple stage III and IV granulomas. This animal also
344 showed similar numbers of AFBs to non-vaccinated animals. One non-vaccinated
345 animal demonstrated no granulomas upon gross or microscopic examination.

346 BCG vaccinated cattle displayed predominantly stage I and II granulomas 13 weeks
347 post *M. bovis* infection. These early stage granulomas are considered protective to the
348 animal, in that they contain and control the replication of the bacteria, without causing
349 the excess tissue destruction associated with stage III and IV lesions. This idea is
350 supported by the observation that fewer AFBs were present in stage I and II

351 granulomas of both groups. Together this substantiates the theory that the smaller
352 size of these lesions facilitates macrophage-lymphocyte interactions and leads to the
353 increased production of cytokines such as IFN γ , which induce mycobactericidal
354 pathways within macrophages (Saunders and Cooper, 2000; Cassidy, 2006; Palmer et
355 al., 2007).

356 IHC revealed there to be a statistically smaller percentage of macrophages (CD68+)
357 within all stages of granuloma for BCG vaccinated cattle compared to non-vaccinated
358 animals.. Possible reasons for this finding are that fewer MNGCs were present within
359 granulomas of BCG vaccinated animals which, because of their large size, contribute a
360 larger amount towards the total percentage area (Johnson et al., 2006). MNGCs cells
361 are formed by the fusion of macrophages in response to the intracellular persistence
362 of the pathogen, therefore, because of the lower bacterial count of the majority of
363 BCG vaccinated *M. bovis* induced granulomas, fewer macrophages will have been
364 stimulated to differentiate into MNGCs (Quinn and Schepetkin, 2009). Studies carried
365 out in other species have found that the higher number of MNGCs in granulomas of
366 stages III and IV is consistent with a reduced ability of the animal to contain the
367 infection (García-Jiménez et al., 2013a, García-Jiménez et al., 2013b). In these cases,
368 the presence of fewer MNGCs in granulomas of BCG vaccinated animals could be
369 considered a sign of protection.

370 . The majority of BCG vaccinated granulomas were of stages I and II and did not
371 progress to the more advanced stages III and IV. In this sense, it is possible to speculate
372 that despite the smaller percentage of macrophages present within these lesions,

373 interactions with other cells were more efficient than in non-vaccinated animals, which
374 overall resulted in a lower bacterial count and minimal tissue damage.

375 Statistically significantly fewer CD3+ cells were detected in the granulomas of BCG
376 vaccinated cattle compared to non-vaccinated animals after *M. bovis* infection. These
377 results are surprising since CD4+ cells (a CD3 subset) are recognised to have an
378 important role in the production of IFN γ , which was found to be elevated in BCG
379 vaccinated animals in this study (Pollock et al., 1996). The importance of CD4+ cells in
380 BCG mediated protection is well documented; vaccination in mice has been shown to
381 induce a persistent mucosal population of multifunctional CD4+ T effector memory
382 cells, which expand following *M. bovis* infection (Kaveh et al., 2011). Future work could
383 use IHC to assess the relative contributions of CD4+ and CD8+ T lymphocytes in the
384 BCG mediated protection against *M. bovis* infection, to ascertain reasons for the
385 reduction in CD3+ cells demonstrated here.

386 The findings of this study show that 13 weeks following *M. bovis* infection significantly
387 fewer WC1+ $\gamma\delta$ T cells are present in the granulomas of BCG vaccinated cattle
388 compared to non-vaccinated animals. This corroborates findings of a similar study
389 which used IHC to quantify expression of WC1+ cells eighteen weeks post-infection
390 (Johnson et al., 2006). Other studies in mice, cattle and humans have demonstrated an
391 increase in WC1+ $\gamma\delta$ T cells within peripheral blood or lung mononuclear cells in
392 response to BCG vaccination and following infection, these innate immune effectors
393 quickly migrate to the tissue (Pollock et al., 1996; Dieli et al., 2003; Mazzola et al.,
394 2007; Buza et al., 2009; Kaveh et al., 2011). In particular, cells expressing the WC1.1+
395 isoform accumulate in the respiratory tract and pharyngeal tonsils of BCG vaccinated

calves when compared to non-vaccinated animals (Price et al., 2010). These cells secrete high levels of IFN γ which may partially explain the robust IFN γ response as detected by IHC in vaccinated cattle in this study. Differences in the findings between studies are most likely explained by the different time points at which measurements were taken. Because the immune response to *M. bovis* infection is highly dynamic and because $\gamma\delta$ T cells are implicated in the innate immune response, it would be likely that the time of sampling post infection would influence the results (Wangoo et al., 2005).

A statistically significant increased number of B lymphocytes was present in stage II, III and IV granulomas within the lymph node of BCG vaccinated cattle when compared to non-vaccinated animals. BCG vaccinated animals showed a general trend of increasing numbers of B cells as the lesion developed. Prominent clusters of B lymphocytes were observed surrounding later stage granulomas of both vaccinated and non-vaccinated animals, as has been observed before in cattle (Aranday-Cortes et al., 2013) and other species including human (Ulrichs et al., 2004; García-Jiménez et al., 2012). It has been suggested that these clusters resemble active follicles, as found in secondary lymphoid organs. These have been shown in humans to contain antigen presenting cells and B cells at different stages of differentiation, surrounded by CD4+ and CD8+ lymphocytes. These structures are suggested to be instrumental in directing a lasting, co-ordinated response to the mycobacterium (Ulrichs et al., 2004).

IFN γ expression within lymph node granulomas of BCG vaccinated animals was significantly higher than for non-vaccinated animals. Furthermore, animals exhibiting the fewest and least advanced granulomas expressed the most IFN γ . These results are supportive of the hypothesis that IFN γ has a protective role in the response to *M. bovis*

419 infection, as reported in others studies (Welsh et al., 2005; Johnson et al., 2006;
420 Boddu-Jasmine et al., 2008; Aguilar León et al., 2009).

421 The results presented here potentially indicate that the CD3⁺ cells recruited to the
422 lymph node following infection of BCG vaccinated animals produced higher quantities
423 of IFN γ than CD3⁺ cells within the granulomas of non-vaccinated cattle. This idea is
424 supported by the identification of multi-cytokine producing CD4⁺ lymphocytes induced
425 by BCG vaccination, which produce higher amounts of IFN γ than single cytokine
426 expressing cells (Kaveh et al., 2011; Whelan et al., 2011). Additionally, natural killer
427 cells are innate immune effectors that produce large amounts of IFN γ when stimulated
428 with mycobacterial antigens (Olsen et al., 2005). Following BCG vaccination, these
429 cells expand in both the lymph nodes and peripheral blood and so IHC using markers
430 against CD335 (expressed on the surface of NK cells) could be used to assess the *in situ*
431 contribution of these cells to BCG induced protective immunity (Siddiqui et al., 2012).

432 Similarly to IFN γ , TNF α showed statistically increased expression in the BCG vaccinated
433 animals compared to non-vaccinated for granuloma stages I, II and III when examined
434 using IHC. This cytokine is a multi-functional cytokine primarily involved in the initial
435 stages of innate immunity, which could explain the higher expression in early stages
436 granulomas (Roach et al., 2002). TNF α has also an essential role in the induction of the
437 chemokines and the recruitment of macrophages and T cells required to clear the
438 intracellular bacterial infections and maintaining the microenvironment of protective
439 granulomas (Roach et al., 2002; Boddu-Jasmine et al., 2008), as could be occurring in
440 BCG vaccinated animals in our study.

441 TGF β expression was shown to be downregulated in BCG vaccinated animals compared
442 to non-vaccinated for granuloma stages I, II, and III, although was only statistically
443 significant when comparing stage I granulomas. Given the role of this cytokine in
444 suppressing IFN γ and iNOS production, this result is in agreement with our other
445 findings (Barnes et al., 1994).

446 An important role of TGF β within the granuloma is the increased production of type 1
447 collagen (Quaglino Jr et al., 1991). The slight increase of TGF β expression between
448 stages I to IV in BCG vaccinated animals could be associated to the formation of the
449 fibrous capsule that surrounds more advanced granulomas.

450 iNOS catalyses the production of nitric oxide, which inactivates infecting mycobacteria
451 through the production of highly reactive nitrogen intermediates. The increased
452 presence of iNOS and therefore nitric oxide, seems likely to have contributed to the
453 much reduced bacterial count within lymph nodes of the BCG vaccinated cattle.
454 Increased expression of iNOS is corroborated by the higher levels of IFN γ detected
455 within the granulomas of BCG vaccinated cattle, since Th1 cytokines are important in
456 promoting mycobactericidal pathways (Palmer et al., 2007).

457 The increased expression of B lymphocytes within the granulomas coupled with high
458 IFN γ production throughout the course of the experiment imply that a Th0 response
459 (characterised by aspects of both Th1 and Th2 immune responses) has developed in
460 the BCG vaccinated animals following *M. bovis* infection. This is further supported by
461 the increased detection of TGF β in advanced lesions of BCG vaccinated cattle. In
462 addition, other studies have found vaccinated animals to maintain higher levels of the

463 anti-inflammatory cytokines IL4 and IL10 in comparison with unvaccinated cattle
464 (Widdison et al., 2006). Human data also support this idea, in that BCG vaccination has
465 been shown to stimulate both Th1 and Th2 responses, although interestingly, Th2
466 responses are not maintained (Djuardi et al., 2012). This is believed to be an attempt
467 by the host to reduce damage inflicted by high levels of pro-inflammatory cytokines
468 (Blanco et al., 2009).

469 **Conclusions**

470 Findings shown in this study suggest that the use of BCG as a vaccine, even though it
471 does not provide 100% protection from disease, reduces the number and severity of
472 lesions and the number of ZN visible bacteria.

473 The lower number of inflammatory cells but on the other hand the higher efficiency in
474 the expression of beneficial cytokines with bactericidal effect by this cells, seems to be
475 the reason of protective induced granulomas in BCG vaccinated animals in our study.

476

477 **Conflict of interest statement**

478 The authors declare to have no conflicts of interest.

479 **Acknowledgments**

480 This study was supported by Defra (grant SE3226), the European Community's Seventh
481 Framework Programme (FP7/2007-2013) under grant agreement n° 228394 (NADIR)
482 and the University of Surrey FHMS.

483 W.L. García-Jiménez acknowledges the Junta de Extremadura and the European Social
484 Fund for his research contract (PO14022).

485 **References**

486 Aguilar León, D., M. J. Zumárraga, R. Jiménez Oropeza, A. K. Gioffré, A. Bernardelli, H.
487 Orozco Estévez, A. A. Cataldi and R. Hernández Pando, 2009: *Mycobacterium*
488 *bovis* with different genotypes and from different hosts induce dissimilar
489 immunopathological lesions in a mouse model of tuberculosis. *Clin. Exp.*
490 *Immunol.* 157, 139-147.

491 Ameni, G., M. Vordermeier, A. Aseffa, D. B. Young and R. G. Hewinson, 2010: Field
492 evaluation of the efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin
493 against bovine tuberculosis in neonatal calves in Ethiopia. *Clinical and Vaccine*
494 *Immunology.* 17, 1533-1538.

495 Aranday-Cortes, E., N. C. Bull, B. Villarreal-Ramos, J. Gough, D. Hicks, A. Ortiz-Peláez, H.
496 M. Vordermeier and F. J. Salguero, 2013: Upregulation of IL-17A, CXCL9 and
497 CXCL10 in Early-Stage Granulomas Induced by *Mycobacterium bovis* in Cattle.
498 *Transboundary and Emerging Diseases.* 60, 525-537.

499 Barnes, P. F., R. L. Modlin and J. J. Ellner, 1994: T-cell responses and cytokines. In: B.R.,
500 B. (ed.), *Tuberculosis: Pathogenesis, Protection and Control.* pp. 417-435.
501 American Society for Microbiology Washington D.C.

502 Blanco, F. C., P. Schierloh, M. V. Bianco, K. Caimi, V. Meikle, A. E. Alito, A. A. Cataldi, M.
503 D. C. Sasiain and F. Bigi, 2009: Study of the immunological profile towards
504 *Mycobacterium bovis* antigens in naturally infected cattle. *Microbiol. Immunol.*
505 53, 460-467.

- 506 Boddue-Jasmine, H. C., J. Witchell, M. Vordermeier, A. Wangoo and M. Goyal, 2008:
507 Cytokine mRNA expression in cattle infected with different dosages of
508 *Mycobacterium bovis*. *Tuberculosis*. 88, 610-615.
- 509 Buddle, B. M., D. N. Wedlock and M. Denis, 2006: Progress in the development of
510 tuberculosis vaccines for cattle and wildlife. *Vet. Microbiol.* 112, 191-200.
- 511 Buddle, B. M., D. N. Wedlock, M. Denis and M. A. Skinner, 2005: Identification of
512 immune response correlates for protection against bovine tuberculosis. *Vet.*
513 *Immunol. Immunopathol.* 108, 45-51.
- 514 Buddle, B. M., D. N. Wedlock, M. Denis, H. M. Vordermeier and R. G. Hewinson, 2011:
515 Update on vaccination of cattle and wildlife populations against tuberculosis.
516 *Vet. Microbiol.* 151, 14-22.
- 517 Buza, J., T. Kiros, A. Zerihun, I. Abraham and G. Ameni, 2009: Vaccination of calves with
518 *Mycobacterium bovis* Bacilli Calmete Guerin (BCG) induced rapid increase in the
519 proportion of peripheral blood gamma delta T cells. *Vet. Immunol.*
520 *Immunopathol.* 130, 251-255.
- 521 Cassidy, J. P., 2006: The pathogenesis and pathology of bovine tuberculosis with
522 insights from studies of tuberculosis in humans and laboratory animal models.
523 *Vet. Microbiol.* 112, 151-161.
- 524 Conlan, A. J. K., T. J. McKinley, K. Karolemeas, E. B. Pollock, A. V. Goodchild, A. P.
525 Mitchell, C. P. D. Birch, R. S. Clifton-Hadley and J. L. N. Wood, 2012: Estimating
526 the Hidden Burden of Bovine Tuberculosis in Great Britain. *PLoS Computational*
527 *Biology*. 8.

- 528 Dieli, F., J. Ivanyi, P. Marsh, A. Williams, I. Naylor, G. Sireci, N. Caccamo, C. Di Sano and
529 A. Salerno, 2003: Characterization of lung gamma delta T cells following
530 intranasal infection with *Mycobacterium bovis* bacillus Calmette-Guérin. *J.*
531 *Immunol.* 170, 463-469.
- 532 Djuardi, Y., E. Sartono, H. Wibowo, T. Supali and M. Yazdanbakhsh, 2012: A
533 longitudinal study of BCG vaccination in early childhood: The development of
534 innate and adaptive immune responses. *PLoS ONE*. 5.
- 535 Donnelly, C. A. and Woodroffe, R, 2015. Bovine tuberculosis: Badger cull targets
536 unlikely to reduce TB. *Nature* 526(7575), 640.
- 537 EFSA, 2013: Scientific Opinion on field trials for bovine tuberculosis vaccination. In:
538 Journal, E. (ed.), *EFSA Journal* 11: 3475.
- 539 García-Jiménez, W. L., J. M. Benítez-Medina, P. Fernández-Llario, J. A. Abecia, A.
540 García-Sánchez, R. Martínez, D. Risco, A. Ortiz-Peláez, F. J. Salguero, N. H.
541 Smith, L. Gómez and J. Hermoso de Mendoza, 2013a: Comparative Pathology of
542 the Natural infections by *Mycobacterium bovis* and by *Mycobacterium caprae*
543 in Wild Boar (*Sus scrofa*). *Transboundary and Emerging Diseases*. 60, 102-109.
- 544 García-Jiménez, W. L., P. Fernández-Llario, L. Gómez, J. M. Benítez-Medina, A. García-
545 Sánchez, R. Martínez, D. Risco, J. Gough, A. Ortiz-Peláez, N. H. Smith, J. H. d.
546 Mendoza and F. J. Salguero, 2012: Histological and immunohistochemical
547 characterisation of *Mycobacterium bovis* induced granulomas in naturally
548 infected Fallow deer (*Dama dama*). *Vet. Immunol. Immunopathol.* 149, 66-75.
- 549 García-Jiménez, W. L., F. J. Salguero, P. Fernández-Llario, R. Martínez, D. Risco, J.
550 Gough, A. Ortiz-Peláez, J. Hermoso-de-Mendoza and L. Gómez, 2013b:

- 551 Immunopathology of granulomas produced by *Mycobacterium bovis* in
552 naturally infected wild boar. *Vet. Immunol. Immunopathol.* 156, 54-63.
- 553 Johnson, L., J. Gough, Y. Spencer, G. Hewinson, M. Vordermeier and A. Wangoo, 2006:
554 Immunohistochemical markers augment evaluation of vaccine efficacy and
555 disease severity in bacillus Calmette-Guerin (BCG) vaccinated cattle challenged
556 with *Mycobacterium bovis*. *Vet. Immunol. Immunopathol.* 111, 219-229.
- 557 Jones, G. J., A. Whelan, D. Clifford, M. Coad and H. M. Vordermeier, 2012: Improved
558 skin test for differential diagnosis of bovine tuberculosis by the addition of
559 Rv3020c-derived peptides. *Clinical and Vaccine Immunology.* 19, 620-622.
- 560 Kaveh, D. A., V. S. Bachy, R. G. Hewinson and P. J. Hogarth, 2011: Systemic BCG
561 immunization induces persistent lung mucosal multifunctional CD4 TEM cells
562 which expand following virulent mycobacterial challenge. *PLoS ONE.* 6.
- 563 Lopez-Valencia, G., T. Renteria-Evangelista, J. d. J. Williams, A. Licea-Navarro, A. D. I.
564 Mora-Valle and G. Medina-Basulto, 2010: Field evaluation of the protective
565 efficacy of *Mycobacterium bovis* BCG vaccine against bovine tuberculosis. *Res.*
566 *Vet. Sci.* 88, 44-49.
- 567 Ly, L. H., M. I. Russell and D. N. McMurray, 2007: Microdissection of the cytokine
568 milieu of pulmonary granulomas from tuberculous guinea pigs. *Cellular*
569 *Microbiology.* 9, 1127-1136.
- 570 Mazzola, T. N., M. T. N. Da Silva, Y. M. F. Moreno, S. C. B. S. Lima, E. F. Carniel, A. M.
571 Morcillo, M. A. R. G. M. Antonio, M. L. Zanolli, A. A. Netto, M. H. Blotta, I. Raw
572 and M. M. S. Vilela, 2007: Robust gamma delta T cell expansion in infants
573 immunized at birth with BCG vaccine. *Vaccine.* 25, 6313-6320.

- 574 Olsen, I., P. Boysen, S. Kulberg, J. C. Hope, G. Jungersen and A. K. Storset, 2005: Bovine
575 NK cells can produce gamma interferon in response to the secreted
576 mycobacterial proteins ESAT-6 and MPP14 but not in response to MPB70.
577 *Infect. Immun.* 73, 5628-5635.
- 578 Palmer, M. V., W. R. Waters and T. C. Thacker, 2007: Lesion development and
579 immunohistochemical changes in granulomas from cattle experimentally
580 infected with *Mycobacterium bovis*. *Vet. Pathol.* 44, 863-874.
- 581 Pollock, J. M., D. A. Pollock, D. G. Campbell, R. M. Girvin, A. D. Crockard, S. D. Neill and
582 D. P. Mackie, 1996: Dynamic changes in circulating and antigen-responsive T-
583 cell subpopulations post-*Mycobacterium bovis* infection in cattle. *Immunology*.
584 87, 236-241.
- 585 Price, S., M. Davies, B. Villarreal-Ramos and J. Hope, 2010: Differential distribution of
586 WC1 + gammadelta TCR + T lymphocyte subsets within lymphoid tissues of the
587 head and respiratory tract and effects of intranasal *M. bovis* BCG vaccination.
588 *Vet. Immunol. Immunopathol.* 136, 133-137.
- 589 Quaglini Jr, D., L. B. Nanney, J. A. Ditesheim and J. M. Davidson, 1991: Transforming
590 growth factor-beta stimulates wound healing and modulates extracellular
591 matrix gene expression in pig skin: Incisional wound model. *J. Invest. Dermatol.*
592 97, 34-42.
- 593 Quinn, M. T. and I. A. Schepetkin, 2009: Role of NADPH oxidase in formation and
594 function of multinucleated giant cells. *Journal of Innate Immunity.* 1, 509-526.
- 595 Roach, D. R., A. G. D. Bean, C. Demangel, M. P. France, H. Briscoe and W. J. Britton,
596 2002: TNF regulates chemokine induction essential for cell recruitment,

- 597 granuloma formation, and clearance of mycobacterial infection. *J. Immunol.*
598 168, 4620-4627.
- 599 Saunders, B. M. and A. M. Cooper, 2000: Restraining mycobacteria: Role of granulomas
600 in mycobacterial infections. *Immunol. Cell Biol.* 78, 334-341.
- 601 Siddiqui, N., S. Price and J. Hope, 2012: BCG vaccination of neonatal calves: Potential
602 roles for innate immune cells in the induction of protective immunity. *Comp.*
603 *Immunol. Microbiol. Infect. Dis.* 35, 219-226.
- 604 Suazo, F. M., A. M. A. Escalera and R. M. G. Torres, 2003: A review of *M. bovis* BCG
605 protection against TB in cattle and other animals species. *Prev. Vet. Med.* 58, 1-
606 13.
- 607 Ulrichs, T., G. A. Kosmiadi, V. Trusov, S. Jörg, L. Pradl, M. Titukhina, V. Mishenko, N.
608 Gushina and S. H. E. Kaufmann, 2004: Human tuberculous granulomas induce
609 peripheral lymphoid follicle-like structures to orchestrate local host defence in
610 the lung. *J. Pathol.* 204, 217-228.
- 611 Vordermeier, H. M., M. A. Chambers, B. M. Buddle, J. M. Pollock and R. G. Hewinson,
612 2006: Progress in the development of vaccines and diagnostic reagents to
613 control tuberculosis in cattle. *Vet. J.* 171, 229-244.
- 614 Vordermeier, H. M., M. A. Chambers, P. J. Cockle, A. O. Whelan, J. Simmons and R. G.
615 Hewinson, 2002: Correlation of ESAT-6-specific gamma interferon production
616 with pathology in cattle following *Mycobacterium bovis* BCG vaccination against
617 experimental bovine tuberculosis. *Infect. Immun.* 70, 3026-3032.

618 Vordermeier, M., S. V. Gordon and R. G. Hewinson, 2011: *Mycobacterium bovis*
619 antigens for the differential diagnosis of vaccinated and infected cattle. *Vet.*
620 *Microbiol.* 151, 8-13.

621 Wangoo, A., L. Johnson, J. Gough, R. Ackbar, S. Inglut, D. Hicks, Y. Spencer, G.
622 Hewinson and M. Vordermeier, 2005: Advanced granulomatous lesions in
623 *Mycobacterium bovis*-infected cattle are associated with increased expression
624 of type I procollagen, (WC1+) T cells and CD 68+ cells. *J. Comp. Pathol.* 133,
625 223-234.

626 Waters, W. R., M. V. Palmer, B. M. Buddle and H. M. Vordermeier, 2012: Bovine
627 tuberculosis vaccine research: Historical perspectives and recent advances.
628 *Vaccine.* 30, 2611-2622.

629 Welsh, M. D., R. T. Cunningham, D. M. Corbett, R. M. Girvin, J. McNair, R. A. Skuce, D.
630 G. Bryson and J. M. Pollock, 2005: Influence of pathological progression on the
631 balance between cellular and humoral immune responses in bovine
632 tuberculosis. *Immunology.* 114, 101-111.

633 Whelan, A. O., M. Coad, B. L. Upadhyay, D. J. Clifford, R. G. Hewinson and H. M.
634 Vordermeier, 2011: Lack of correlation between BCG-induced tuberculin skin
635 test sensitisation and protective immunity in cattle. *Vaccine.* 29, 5453-5458.

636 Widdison, S., L. J. Schreuder, B. Villarreal-Ramos, C. J. Howard, M. Watson and T. J.
637 Coffey, 2006: Cytokine expression profiles of bovine lymph nodes: Effects of
638 *Mycobacterium bovis* infection and bacille Calmette-Guérin vaccination. *Clin.*
639 *Exp. Immunol.* 144, 281-289.

640

641 **Figure captions**

642

643 **Fig. 1. BCG confers protection against *M. bovis* induced pathology.** Pathology scores
644 for lungs (closed symbols) and lymph nodes (open symbols). Scores for non-vaccinated
645 animals (diamonds) are higher than for BCG-vaccinated cattle (circles). Bars indicate the
646 mean for each group. * = $P < 0.05$

647 **Fig. 2. Protection induced by BCG correlates with the number and severity of**
648 **granuloma development.** Lymph node slides were analysed for the presence of
649 granulomas, which were classified according to the system developed by Wangoo et
650 al., 2005 as described in materials and methods. Statistically significantly fewer
651 granulomas ($P < 0.05$) were present in lymph nodes of BCG vaccinated animals
652 compared to non-vaccinated. * = $P < 0.05$

653 **Fig. 3. Acid fast bacilli (AFBs) accumulate in late stage granulomas.** Acid fast bacilli
654 (AFB) were counted as described in material and methods in the different types of
655 granulomas. AFB were found in greater number in non-vaccinated (black bars) than in
656 BCG-vaccinated animals (white bars). Error bars show standard deviation * = $P < 0.05$.
657 ZN staining of a MNGC from the lymph node of a BCG-vaccinated animal (A) and the
658 necrotic centre of a stage IV granuloma from a non-vaccinated (B) animal.

659 **Fig. 4. Macrophages (CD68+).** Mean percentage of positive immunolabelling within
660 granulomas for CD68 within the lymph nodes for non-vaccinated and BCG vaccinated
661 animals. Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for CD68 in
662 lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Numerous

663 CD68+ macrophages and MNGCs can be seen in stage II granulomas (A,C) and
664 surrounding necrotic centres in stage IV granulomas (n; B,D).

665 **Fig. 5. iNOS.** Mean percentage of positive immunolabelling within granulomas for
666 iNOS in non-vaccinated and BCG vaccinated animals for each stage of granuloma
667 within the lymph node. Immunolabelling for iNOS in the lymph nodes of non-
668 vaccinated (A,B), and BCG vaccinated(C,D) animals. Scattered epithelioid and MNGCs
669 were positively stained in stage I granulomas (A,C) and a rim of positively stained cells
670 were observed surrounding the necrotic centres (n) in stage IV granulomas (B,D).

671 **Fig. 6. CD3 (T cells).** Mean percentage of positive immunolabelling within granulomas
672 for CD3 (T lymphocytes) in non-vaccinated and BCG vaccinated animals for each stage
673 of granuloma within the lymph node. Results are expressed as means $\pm$ SD. * $P<0.05$.
674 Immunolabelling for CD3 in the lymph nodes of non-vaccinated (A,B), and BCG
675 vaccinated (C,D) animals. Numerous CD3+ T cells were positively stained scattered in
676 stage II granulomas (A,C). Abundant scattered T cells were also observed towards the
677 periphery of stage IV granulomas (B,D). n = necrotic centres.

678 **Fig. 7. WC1 ($\gamma\delta$ T cells).** Mean percentage of positive immunolabelling within
679 granulomas for WC1 ($\gamma\delta$ T cells) in non-vaccinated and BCG vaccinated animals for
680 each stage of granuloma within the lymph node. Results are expressed as means $\pm$ SD.
681 * $P<0.05$. Immunolabelling for WC1 in the lymph nodes of non-vaccinated (A,B), and
682 BCG vaccinated (C,D) animals. Few WC1+ cells were positively stained scattered in
683 stage I and II granulomas (A,C). The number of WC1+ cells in BCG stage I and II (C) is
684 very low in comparison to NC animals (A). Abundant scattered WC1+ cells were also

685 observed towards the periphery of stage IV granulomas in non-vaccinated animals (C)
686 compared to very few in BCG group (D). n = necrotic centres.

687 **Fig. 8. B cells (CD79+).** Mean percentage of positive immunolabelling within
688 granulomas for CD79a (B cells) within the lymph node of non-vaccinated and BCG
689 vaccinated animals for each stage of granulomas development within the lymph node.
690 Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for CD79 in the lymph
691 nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Abundant B cells
692 (CD79+) were positively stained scattered in stage II granulomas (A,C) and at the
693 periphery of stage IV granulomas (B,D). Clusters of B cells can be observed outside
694 stage IV granulomas (D). n = necrotic centres.

695 **Fig. 9. IFN- γ .** Mean percentage of positive immunolabelling per granuloma for
696 IFN γ within the lymph node of non-vaccinated and BCG vaccinated animals for each
697 stage of granuloma development. Results are expressed as means $\pm$ SD. * $P < 0.05$.
698 Numerous cells were positively stained for IFN γ in stage I (A,C) and through all the
699 layers of stage IV granulomas (B,D). The expression of IFN γ was significantly higher in
700 BCG vaccinated animals (C,D) than in non-vaccinated (A,B) n = necrotic centres.

701 **Fig. 10. TNF- α .** Mean percentage of positive immunolabelling within granulomas for
702 TNF α within the lymph node of non-vaccinated and BCG vaccinated animals for each
703 stage of granuloma development within the lymph node. Results are expressed as
704 means $\pm$ SD. * $P < 0.05$. Immunolabelling for TNF α in the lymph nodes of non-vaccinated
705 (A,B), and BCG vaccinated (C,D) animals. Numerous macrophages were positively
706 stained for TNF α in stage II (A,C) and at the periphery of stage IV granulomas (B,D). The

707 expression of TNF α was significantly higher in BCG vaccinated animals (C,D) than in
708 non-vaccinated (B,D) n = necrotic centres.

709 **Fig. 11. TGF- β .** Mean percentage of positive immunolabelling within granulomas for
710 TGF β within the lymph node of non-vaccinated and BCG vaccinated animals for each
711 stage of granuloma development within the lymph node. Results are expressed as
712 means $\pm$ SD. * P <0.05. Immunolabelling for TGF β in the lymph nodes of non-vaccinated
713 (A,B), and BCG vaccinated (C,D) animals. Numerous macrophages were positively
714 stained for TGF β in stage I and II (A,C) and throughout all layers of stage IV granulomas
715 (B,D). The expression of TNF α was significantly higher in BCG vaccinated animals (C,D)
716 than in non-vaccinated (B,D) n = necrotic centres.

717

Table 1: Immune markers used for immunohistochemistry.

Primary antibody	Antibody type	Dilution	Supplier	Epitope demasking method	Link antibody (dilution)	Buffer
CD3	Rabbit v Human CD3 (Polyclonal)	1/500	Dako, A0452 (Ely, UK)	Trypsin/Chymotrypsin ^a	Goat v Rabbit (1/1000)	TBS
CD79a	Mouse v Human CD79a (Monoclonal)	1/100	Dako M7051 (Ely, UK)	Microwave, 3x 6mins at 100 °C, in citric acid buffer, pH 6	Goat v Mouse (1/200)	TBS
CD68	Mouse v Human CD68 (Monoclonal)	1/50	Dako M0718 (Ely, UK)	Trypsin/Chymotrypsin	Goat v Mouse (1/200)	TBS
WC1	Mouse v Bovine WC1 (Monoclonal)	1/100	Serotec MCA 8385 (Oxford, UK)	Trypsin/Chymotrypsin	Goat v Mouse (1/200)	TBST
IFN-γ	Mouse v Bovine IFN- γ (Monoclonal)	1/250	Serotec CC330 (Oxford, UK)	Microwave, 3x 6mins at 100 °C, in citric acid buffer, pH 6	Goat v Mouse (1/200)	TBS
TNF-α	Mouse v Bovine TNF α (Monoclonal)	1/400	Serotec MCA Ab/15-3 (Oxford, UK)	Trypsin/Chymotrypsin	Goat v Mouse (1/200)	TBST
TGF-β	Rabbit v Human TGF β 1 (Polyclonal)	1/250	Santa Cruz SC-146 (Santa-Cruz, USA)	Microwave, 3x 6mins at 100 °C, in citric acid buffer, pH 6	Goat v Rabbit (1/1000)	TBS
iNOS	Rabbit v Mouse iNOS (Polyclonal)	1/500	Millepore 06-573 (Billerica, USA)	Microwave, high pH buffer, 2x5mins at 100°C	Goat v Rabbit (1/7500)	TBST

^aTrypsin/Chemotrypsin was prepared as follows: 0.5g of trypsin, 0.5g of chemotrypsin and 1g of CaCl₂ were dissolved in 1L of distilled water and the resulting solution titrated to pH 7.8 using 0.1M sodium hydroxide solution.

Gross pathology score

Fig. 1. BCG confers protection against *M. bovis* induced pathology. Pathology scores for lungs (closed symbols) and lymph nodes (open symbols). Scores for non-vaccinated animals (diamonds) are higher than for BCG-vaccinated cattle (circles). Bars indicate the mean for each group. * = $P < 0.05$

101x58mm (300 x 300 DPI)

Total number of granulomas in thoracic LNs

Fig. 2. Protection induced by BCG correlates with the number and severity of granuloma development. Lymph node slides were analysed for the presence of granulomas, which were classified according to the system developed by Wangoo et al., 2005 as described in materials and methods. Statistically significantly fewer granulomas ($P < 0.05$) were present in lymph nodes of BCG vaccinated animals compared to non-vaccinated. * = $P < 0.05$

101x51mm (300 x 300 DPI)

AFBs in Lymph nodes

Fig. 3. Acid fast bacilli (AFBs) accumulate in late stage granulomas. Acid fast bacilli (AFB) were counted as described in material and methods in the different types of granulomas . AFB were found in greater number in non-vaccinated (black bars) than in BCG-vaccinated animals (white bars). Error bars show standard deviation * = P<0.05. ZN staining of a MNGC from the lymph node of a BCG-vaccinated animal (A) and the necrotic centre of a stage IV granuloma from a non-vaccinated (B) animal.

101x91mm (300 x 300 DPI)

Macrophages (CD68+)

Fig. 4. Macrophages (CD68+). Mean percentage of positive immunolabelling within granulomas for CD68 within the lymph nodes for non-vaccinated and BCG vaccinated animals. Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for CD68 in lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Numerous CD68+ macrophages and MNGCs can be seen in stage II granulomas (A,C) and surrounding necrotic centres in stage IV granulomas (n; B,D).

101x138mm (300 x 300 DPI)

Fig. 5. iNOS. Mean percentage of positive immunolabelling within granulomas for iNOS in non-vaccinated and BCG vaccinated animals for each stage of granuloma within the lymph node. Immunolabelling for iNOS in the lymph nodes of non-vaccinated (A,B), and BCG vaccinated(C,D) animals. Scattered epithelioid and MNGCs were positively stained in stage I granulomas (A,C) and a rim of positively stained cells were observed surrounding the necrotic centres (n) in stage IV granulomas (B,D).

101x138mm (300 x 300 DPI)

T cells (CD3+)

Fig. 6. CD3 (T cells). Mean percentage of positive immunolabelling within granulomas for CD3 (T lymphocytes) in non-vaccinated and BCG vaccinated animals for each stage of granuloma within the lymph node. Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for CD3 in the lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Numerous CD3+ T cells were positively stained scattered in stage II granulomas (A,C). Abundant scattered T cells were also observed towards the periphery of stage IV granulomas (B,D). n = necrotic centres.

101x138mm (300 x 300 DPI)

Fig 7. WC1 ($\gamma\delta$ T cells). Mean percentage of positive immunolabelling within granulomas for WC1 ($\gamma\delta$ T cells) in non-vaccinated and BCG vaccinated animals for each stage of granuloma within the lymph node. Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for WC1 in the lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Few WC1+ cells were positively stained scattered in stage I and II granulomas (A,C). The number of WC1+ cells in BCG stage I and II (C) is very low in comparison to NC animals (A). Abundant scattered WC1+ cells were also observed towards the periphery of stage IV granulomas in non-vaccinated animals (C) compared to very few in BCG group (D). n = necrotic centres.

101x137mm (300 x 300 DPI)

Fig. 8. B cells (CD79+). Mean percentage of positive immunolabelling within granulomas for CD79a (B cells) within the lymph node of non-vaccinated and BCG vaccinated animals for each stage of granulomas development within the lymph node. Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for CD79 in the lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Abundant B cells (CD79+) were positively stained scattered in stage II granulomas (A,C) and at the periphery of stage IV granulomas (B,D). Clusters of B cells can be observed outside stage IV granulomas (D). n = necrotic centres.

101x137mm (300 x 300 DPI)

Fig. 9. IFN-gamma. Mean percentage of positive immunolabelling per granuloma for IFN γ within the lymph node of non-vaccinated and BCG vaccinated animals for each stage of granuloma development. Results are expressed as means $\pm$ SD. *P<0.05. Numerous cells were positively stained for IFN γ in stage I (A,C) and through all the layers of stage IV granulomas (B,D). The expression of IFN γ was significantly higher in BCG vaccinated animals (C,D) than in non-vaccinated (A,B) n = necrotic centres.

101x135mm (300 x 300 DPI)

Fig. 10. TNF- α . Mean percentage of positive immunolabelling within granulomas for TNF α within the lymph node of non-vaccinated and BCG vaccinated animals for each stage of granuloma development within the lymph node. Results are expressed as means $\pm$ SD. * $P < 0.05$. Immunolabelling for TNF α in the lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Numerous macrophages were positively stained for TNF α in stage II (A,C) and at the periphery of stage IV granulomas (B,D). The expression of TNF α was significantly higher in BCG vaccinated animals (C,D) than in non-vaccinated (B,D) n = necrotic centres.

101x137mm (300 x 300 DPI)

Fig. 11. TGF-β. Mean percentage of positive immunolabelling within granulomas for TGFβ within the lymph node of non-vaccinated and BCG vaccinated animals for each stage of granuloma development within the lymph node. Results are expressed as means ± SD. *P<0.05. Immunolabelling for TGFβ in the lymph nodes of non-vaccinated (A,B), and BCG vaccinated (C,D) animals. Numerous macrophages were positively stained for TGFβ in stage I and II (A,C) and throughout all layers of stage IV granulomas (B,D). The expression of TNFα was significantly higher in BCG vaccinated animals (C,D) than in non-vaccinated (B,D) n = necrotic centres.

101x137mm (300 x 300 DPI)