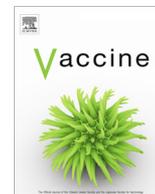




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Evaluating the sensitivity of the bovine BCG challenge model using a prime boost Ad85A vaccine regimen

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ABSTRACT

In the absence of biomarkers of protective immunity, newly developed vaccines against bovine tuberculosis need to be evaluated in virulent *Mycobacterium bovis* challenge experiments, which require the use of expensive and highly in demand Biological Safety Level 3 (BSL3) animal facilities. The recently developed bovine BCG challenge model offers a cheaper and faster way to test new vaccine candidates and additionally reduces the severity of the challenge compared to virulent *M. bovis* challenge in line with the remit of the NC3Rs. In this work we sought to establish the sensitivity of the BCG challenge model by testing a prime boost vaccine regimen that previously increased protection over BCG alone against *M. bovis* challenge. All animals, except the control group, were vaccinated subcutaneously with BCG Danish, and half of those were then boosted with a recombinant adenoviral vector expressing Antigen 85A, Ad85A. All animals were challenged with BCG Tokyo into the prescapular lymph node and the bacterial load within the lymph nodes was established. All vaccinated animals, independent of the vaccination regimen, cleared BCG significantly faster from the lymph node than control animals, suggesting a protective effect. There was however, no difference between the BCG and the BCG-Ad85A regimens. Additionally, we analysed humoral and cellular immune responses taken prior to challenge for possible predictors of protection. Cultured ELISpot identified significantly higher IFN- γ responses in protected vaccinated animals, relative to controls, but not in unprotected vaccinated animals. Furthermore, a trend for protected animals to produce more IFN- γ by quantitative PCR and intracellular staining was observed. Thus, this model can also be an attractive alternative to *M. bovis* challenge models for the discovery of protective biomarkers.

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1. Introduction

Mycobacterium bovis is an intracellular bacterium that predominantly causes tuberculosis in cows (bTB), but can also infect humans [1,2]. Human tuberculosis caused by *M. bovis* is recognised by the WHO as one of several neglected zoonotic diseases, mainly affecting low and middle income countries [3,4]. In the UK, bTB causes animal welfare and productivity issues to the farming industry with a cost to the government of approximately £100 million a year [5]. Currently, there is no vaccine licensed for use in cattle. Experimentally, however, Bacillus Calmette-Guérin (BCG), a live attenuated *M. bovis* strain that is routinely used to vaccinate

humans against *Mycobacterium tuberculosis*, has shown to confer partial protection in cattle, similar to humans [6–8]. A more efficacious vaccine along with current control measures could help to eradicate bTB from the UK [9] and reduce the burden of bTB in countries in which other control measures, such as test and slaughter, are not used due to cost or societal reasons. Vaccine development, however, is hindered by the lack of biomarkers of protective immunity. Therefore, vaccine candidates have to be tested in expensive and lengthy pathogenic *M. bovis* challenge experiments which require BSL3 facilities which are not widely available (Table 1). Methodologies which allow for an easier, faster and cheaper way to evaluate the efficacy of newly developed vaccine candidates are urgently needed. We recently developed a bovine BCG challenge model that has the potential to be used as a gating system to preselect vaccine candidates [10]. In the BCG challenge model, animals are challenged into the prescapular

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Table 1

Comparison of experimental parameters of the *M. bovis* challenge and BCG challenge model. BSL3 requires animals to be housed in high containment facilities which are in high demand and very expensive, in contrast to less stringent requirements for BSL2 facilities.

| | <i>M. bovis</i> challenge | BCG challenge |
|-------------------------------|--------------------------------------------------------------------------------------------------|--------------------------------------------------|
| Biological safety level (BSL) | BSL 3 | BSL 2 |
| Duration of experiment | 28 weeks | 15 weeks |
| Challenge | <i>in vivo</i> endobronchial virulent <i>M. bovis</i> | <i>in vivo</i> intranodal non virulent BCG |
| Protection read out | Total pathology Histopathology Bacterial burden in lung and lymph nodes (CFU counts) | Bacterial burden in lymph node (CFU counts) |

lymph node with a non-virulent BCG Tokyo strain, obviating the need for expensive BSL3 containment facilities and reducing the challenge phase from over six to three weeks. Protected animals exhibit an immune response capable of rapid removal of mycobacteria from the lymph node, whereas unprotected animals are less able to control BCG replication. The number of BCG bacilli in the lymph nodes of protected animals is therefore expected to be lower than in lymph nodes of unprotected animals. Only vaccines providing protection greater than that conferred by BCG in the BCG challenge model would have to be tested in *M. bovis* challenge experiments, which would reduce the amount of animals challenged with virulent *M. bovis*. The BCG challenge model thus represents both refinement and reduction to the currently used *M. bovis* challenge model (Table 1), consistent with the principles of the 3Rs in research using animals [11]. The aim of this work was to evaluate the sensitivity of the BCG challenge model by testing its ability to differentiate between different vaccine regimens. To do so we tested a previously developed heterologous prime boost vaccine regimen, where animals were initially vaccinated with BCG and then boosted with a recombinant adenovirus type 5 expressing Ag85A (Ad85A) [12,13]. This vaccination regimen significantly increased protection over that of BCG vaccination alone based on histopathological evaluation of numbers and severity of granulomas [14]. Furthermore, we were interested to determine whether the BCG challenge model would confirm previously identified predictors of protection in *M. bovis* challenge experiments such as antigen specific T cell memory response and antigen specific gene expression in peripheral blood mononuclear cells (PBMC). We additionally analysed IgG titres in *ex vivo* serum and T cell memory response before and after challenge in effector CD45RO⁺CD62L^{lo} and central memory cells CD45RO⁺CD62L^{hi} as potential novel predictors of protection.

2. Material and methods

2.1. Animals and experimental set up

Experiments were carried out according to the UK Animal (Scientific Procedures) Act 1986 under project license PPL70/7737. The study protocol was approved by the APHA Animal Use Ethics Committee (UK Home Office PCD number 70/6905). Thirty six Holstein-Friesian crosses of between 6 and 10 months of age were sourced from an officially bovine TB free herd. Animals were selected based on their lack of *in vitro* IFN- γ response to *M. avium* purified protein derivative (PPD-A) or to PPD from *M. bovis* (PPD-B). Cattle were randomly divided into two groups of 14 animals each – BCG and BCG-Ad85A group respectively – and one group of 8 animals – control group. At week 12 one animal of the BCG group developed

pneumonia and had to be culled with no other animal of any of the groups being affected.

2.2. Mycobacteria

BCG SSI, for vaccination, and BCG Tokyo, for challenge, were grown to mid-log phase in 7H9 medium supplemented with OADC and 0.05% Tween 80 and frozen in aliquots at -80°C until used. Mycobacteria were thawed and diluted or concentrated to the appropriate density in 7H9 medium (see below).

2.3. Vaccination, boost and challenge

Fig. 1 depicts the experimental schedule starting with week zero, when animals of the BCG and BCG-Ad85A group were vaccinated subcutaneously into the left prescapular area of the neck with 2×10^6 colony forming units (CFU) BCG SSI Danish 1331. Eight weeks later animals of the BCG-Ad85A group received an intradermal booster of 10^9 plaque forming units (PFU) Ad85A [15]. At week 12 all animals were challenged intranodally with 2×10^7 CFU BCG Tokyo into the left and right prescapular lymph node. Three weeks later cattle were euthanized (week 15) and the prescapular, submandibular and popliteal lymph nodes were harvested. Detailed description of intranodal challenge, including a video, can be found in Villarreal-Ramos et al. [10].

2.4. Post mortem examination and bacterial culture

As previously described lymph nodes were cleared from ligaments and fat, dipped in 70% EtOH, cut into small pieces and then macerated with 7 ml HBSS in a stomacher (Seward) for 2 min. The macerate was stored in 1 ml aliquots at -80°C . For each animal a 1 ml aliquot was thawed and a 10 fold serial dilution in 7H9 media was prepared from the macerates and plated on modified 7H11 Agar plates. In addition, bacterial load was determined using the liquid culture BD BACTECTM MGITTM system. For that, 1 ml of macerate was decontaminated using the BD BBLTM MycoPrepTM system before 500 μl of ten times diluted macerate were added to the BD BACTECTM MGITTM mycobacterial growth indicator tubes. Colony counts, obtained after incubating plates for 6 weeks at 37°C , were then compared to time to positivity (TTP), the read out of the BACTEC liquid culture.

2.5. Sampling and PBMC isolation

Whole blood was collected from the jugular vein at weeks 0, 4, 6 and 8 before boost and then every week after boost (Fig. 1) and PBMC were isolated as follows. Heparinised whole blood was diluted 1 in 2 with HBSS buffer (Gibco Life Technologies, UK) and 30 ml of the dilution was overlaid on 15 ml histopaque-1077 (Sigma, UK) in a 50 ml falcon tube and centrifuged for 45 min at 800g with no break. Using a 3 ml pastette the cell interphase was collected and washed twice in HBSS before cells were frozen in FCS containing 10% DMSO and stored in liquid nitrogen until further use.

2.6. Whole blood IFN- γ ELISA

At the time points described above, triplicates of whole blood, 250 μl each, were incubated with antigens or media only (negative control); bovine tuberculin purified protein derivative (PPD-B) 1 in 100 final dilution from the stock solution provided by the manufacturer (Prionics, Switzerland); recombinant Ag85A 5 $\mu\text{g}/\text{ml}$ final concentration (Lionex, Germany); BCG Tokyo 10^6 CFU/ml final concentration; poke weed mitogen (PWM, positive control) 10 $\mu\text{g}/\text{ml}$ final concentration (Sigma, UK) at 37°C and 5% CO_2 . After 16 h

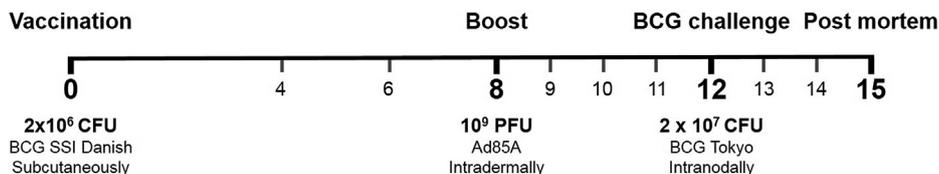


Fig. 1. Experimental schedule. Out of 36 animals, 28 were vaccinated with BCG Danish at week 0 (BCG only). 8 weeks later 14 of the vaccinated animals were boosted with Ad85A (BCG-Ad85A) and 12 weeks after initial vaccination all animals of the three groups (control, BCG only and BCG-Ad85A) were challenged intranodally with BCG Tokyo. One animal of the BCG only group had to be euthanised due to an unrelated pneumonia infection. At week 15, animals were euthanised and prescapular, submandibular and popliteal lymph nodes were harvested (post mortem). Blood samples were collected at all time points indicated and always before further treatment was performed.

100 μ l supernatant was harvested from each well and stored at -20°C . Once samples of all weeks had been collected IFN- γ expression was measured using a BOVIGAMTM TB Kit (Prionics, Switzerland) as per manufacturer's instructions.

2.7. Cultured elispot

Cultured Elispot was carried out as described previously with the following modifications [12]. Cryopreserved PBMC collected at week 10 were stimulated with recombinant Ag85A at 5 $\mu\text{g}/\text{ml}$ for 13 days. On day 13 all cells were washed and resuspended in cell culture medium (RPMI 1640 containing 2 mM GlutaMax, 25 mM HEPES, 0.1 mM NEAA, 5×10^{-5} M β -mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (all from Gibco Life Technologies, UK)). Additionally, freshly isolated autologous CD14⁺ cells were used as antigen presenting cells (APC) and plated out at 2×10^3 APC/well on a pre-coated ELISPOT plate. CD14⁺ cells were isolated using magnetic beads coated with an antibody to human CD14 that crossreacts with bovine CD14 cells (MACS, Miltenyi Biotec, UK). 2×10^4 PBMC were added to each well and the cell mix was stimulated with either PPD-B (10 $\mu\text{g}/\text{ml}$), Ag85A (5 $\mu\text{g}/\text{ml}$), PWM (10 $\mu\text{g}/\text{ml}$) or RPMI for 24 h. Staining and calculation of spot forming units was performed as previously described [12].

2.8. Real time PCR

To measure antigen specific gene expression 2×10^6 PBMC were stimulated overnight (o/n) with PPD-B (10 $\mu\text{g}/\text{ml}$), Ag85A (5 $\mu\text{g}/\text{ml}$) or media. PBMC were then lysed in lysis buffer containing TCEP (Qiagen) and kept at -80°C until further use. RNA of PBMC was extracted using the Qiagen RNeasy Minikit according to manufacturer's protocol and RNA quantity was measured using a NanoDropTM 1000 spectrometer (Thermo Fisher, UK). RNA was transcribed into cDNA using the InvitrogenTM SuperScriptTM VIL0TM kit (Sigma) and qPCR was performed using TaqMan[®] Assay (Thermo Fisher Scientific). Gene expression is presented as fold change, calculated using the $2^{-\Delta\Delta\text{CT}}$ method [16] and normalised against the geometrical mean of three reference genes (GAPDH, SDHA and YWAHZ).

2.9. IgG ELISA

We optimised the evaluation of mycobacterial specific IgG by ELISA as follows. 96 well polysorp plates (Nunc-ImmunoTM Micro-WellTM 96 well solid plates) were coated with 100 μ l of 10 $\mu\text{g}/\text{ml}$ PPD-B or 1 $\mu\text{g}/\text{ml}$ Ag85A in 0.1 M NaHCO₃ and incubated o/n at 4°C . The following day wells were washed three times with 200 μ l PBS containing 0.1% Tween20 (PBTween) and then incubated with 200 μ l blocking buffer consisting of PBS and 3% bovine serum albumin (BSA, SIGMA, UK) for one hour at room temperature (RT). Subsequently, blocking buffer was discarded and serum samples were diluted 1 in 50 in blocking buffer and incubated for one hour at RT. After four washes with PBTween, 100 μ l of mouse antibody

to bovine IgG conjugated to horse radish peroxidase (IgG-HRP) at a concentration of 1 $\mu\text{g}/\text{ml}$ using PPD-B and 0.25 $\mu\text{g}/\text{ml}$ using Ag85A as the coating antigen- (clone IL-A2, MCA2439P, BioRad, UK) was added to the wells and plates were incubated for 1 h at RT, followed by four washes with PBTween. Finally 100 μ l TMB substrate (Sigma-Aldrich, UK) was added to the wells and the ELISA was developed for 10 min at RT. The addition of 50 μ l 1 M sulphuric acid to each well stopped the reaction and the plate was read at 450 nm absorbance.

2.10. PBMC culture and intracellular flow staining

Frozen PBMC were thawed, resuspended in tissue culture medium (TCM, RPMI 1640 containing 2 mM GlutaMax, 25 mM HEPES, 0.1 mM NEAA, 5×10^{-5} M β -mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin [all from Gibco Life Technologies, UK] and 10% fetal calf serum [Sigma-Aldrich, UK]) and incubated for 2 h at 37°C in an atmosphere of 95% humidity and 5% CO₂ (all following incubation steps were performed under the same conditions). Subsequently cells were diluted in TCM at a concentration of $2 \times 10^6/\text{ml}$ and transferred to a 12 well plate (2 ml/well). To analyse antigen specific memory response, cells were cultured for 6 h with 2 ml of TCM containing either PPD-B (1 in 100), PWM (10 $\mu\text{g}/\text{ml}$) or TCM only in the presence of 10 $\mu\text{g}/\text{ml}$ Brefeldin A (Biolegend, UK). After 16 h culture, cells were washed and resuspended in FACS buffer (HBBS containing 0.5% BSA and sodium azide) and stained for cell surface markers either with a primary antibody followed by a secondary fluorescently labelled antibody – CD45RO (IL-A116, IgG3) followed by rat anti mouse IgG3-BV421 (Biolegend, UK), CD62L (CC32, IgG1) followed by rat anti mouse IgG1-PerCP/Cy5.5 (Biolegend, UK and $\gamma\delta$ (GB21A, IgG2b, Kingfisher) followed by goat anti mouse IgG2b-PE-Cy7 (Abcam, UK) – or with a directly labelled antibody – CD4-APC/FireTM 750 (custom conjugated Biolegend, UK), CD8-AF647 (BioRad, UK). Additionally, cells were stained with Zombie AquaTM (Biolegend, UK) to discriminate live from dead cells. For intracellular staining (ICS), cells were treated with Cytofix/Cytoperm (BD Biosciences, UK) according to manufacturer's instruction. Cells were then incubated with an antibody to IL-2 [17] followed by staining with a secondary polyclonal goat anti-human IgG F(ab')₂-FITC (BioRad, UK) and anti-bovine IFN- γ -PE antibody (clone CC302, BioRad, UK). A mix of single stained beads and single stained cells, unstimulated samples and FMO controls were used as controls and to calculate compensation. Up to 600,000 total events per sample were acquired on a LSRFortessaTM analyser (BD) and the data was analysed using FlowJo[®] software (version 10, Tree Star Inc.). An example of the gating strategy used is provided in [supplementary material Fig. 1](#).

2.11. Statistics

Statistical analysis of data and preparation of graphs was performed using GraphPad Prism7. If not mentioned otherwise one

or two-way ANOVA with Tukey's multiple comparison test was performed on datasets.

3. Results

3.1. Vaccination, boost and challenge induce antigen specific immune responses

To demonstrate an immunogenic effect of vaccination, boost and challenge, antigen specific IFN- γ expression in whole blood induced by o/n stimulation with either bovine PPD-B, or Ag85A was analysed, using a commercially available ELISA (BOVIGAM, Prionics). PPD-B specific IFN- γ expression in whole blood was measured at week 0 (prior to vaccination), 4, 6 and 8 after vaccination, and every week after boost (week 9 to week 15, Fig. 2A). No antigen specific IFN- γ was detected in any animal of the three groups prior to vaccination. Four weeks later IFN- γ expression had increased significantly in both vaccinated groups compared to the control group (BCG-Ad85A $P < 0.0001$; BCG $P = 0.005$). At week six IFN- γ expression decreased, followed by a slight increase at week eight. Boosting animals of the BCG-Ad85A group with Ad85A at week 8 resulted in increased IFN- γ expression in this group at week 9 ($P = 0.002$). One week later (week 10) expression levels declined to the same level as seen in the BCG only group. After challenge (week 13) IFN- γ expression increased significantly in both vaccinated groups compared to the control group ($P < 0.0001$) and in the control group compared to week 12 ($P = 0.0004$), followed by a decline in vaccinates and boosted animals one week later but not in control animals. To determine the effect of the intradermal boost, Ag85A specific IFN- γ expression

was measured immediately before boost and then all weeks thereafter (week 9 to 15, Fig. 2B). Ag85A specific IFN- γ expression significantly increased in the BCG-Ad85A group one week after boost ($P = 0.022$ difference to control group, week 9) declining immediately after (week 10) to background level. There was no change in expression detectable in the BCG only and control group. After intranodal challenge (week 13) IFN- γ expression increased both in the BCG and BCG-Ad85A ($P < 0.001$ and $P < 0.01$ respectively). The graph additionally suggests an increase of IFN- γ secretion by stimulated whole blood cells in the control group (not significant). However, it is worth mentioning that this is due to one control animal expressing unusual amounts of Ag85A specific IFN- γ , both weeks after challenge (weeks 13 and 14). Two weeks after challenge, IFN- γ secretion by stimulated whole blood cells declined rapidly in all groups.

To evaluate humoral responses, an optimised PPD-B and Ag85A ELISA was used. IgG titres were determined before vaccination and at weeks 4 and 8 after vaccination, at week 12 after boost and week 14 after challenge (Fig. 2C and D). No increase of PPD-B specific IgG was detected after vaccination (week 4, Fig. 2C), however, intranodal challenge with BCG Tokyo resulted in rising titres in both vaccinated groups and to a lesser extent in the control group (week 14, vaccinates versus controls $P < 0.01$, Fig. 2C). Ag85A specific IgG titres remained stable after vaccination but increased strongly after boosting with Ad85A in the BCG-Ad85A group as measured in sera at week 12 before challenge (BCG-Ad85A versus BCG only and controls $P < 0.0001$, Fig. 2D). Intranodal challenge with BCG did not alter IgG titres in any of the three groups and titres in the BCG-Ad85A group stayed significantly higher compared to BCG only and controls ($P < 0.0001$, Fig. 2D).

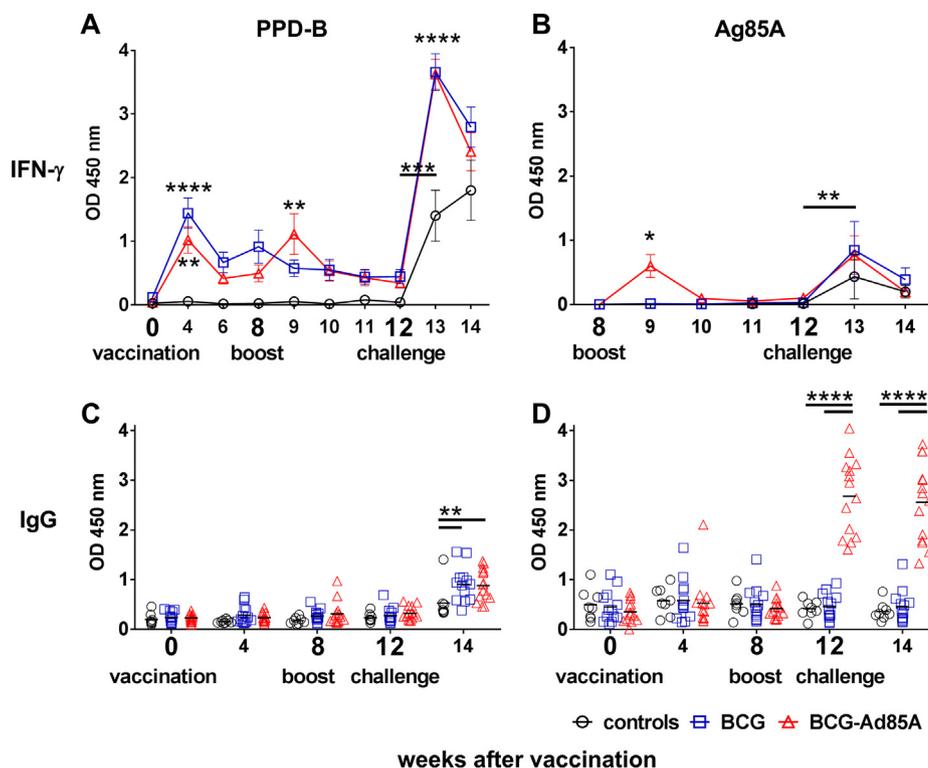


Fig. 2. Antigen specific immune response induced by vaccination, boost and challenge. Whole blood was stimulated with either PPD-B (A) or Ag85A (B) for 16 h before supernatant was harvested and IFN- γ expression analysed using a commercial ELISA (Bovigam). Shown is the optical density (OD) at 450 nm of the group mean \pm SEM after subtraction of media only control (A and B). Additionally, occurrence of antigen specific IgG against either PPD-B (C) or Ag85A (D) was determined in *ex vivo* serum. Controls $n = 8$ (\circ), BCG only vaccinates $n = 13$ (\square), Ad85A boosted animals $n = 14$ (\triangle). Two-way-ANOVA with Tukey's multiple comparison. Controls versus BCG-Ad85A and BCG only at week 4 **** $P < 0.0001$, ** $P = 0.005$ respectively and week 13 **** $P < 0.0001$; Controls versus BCG-Ad85A at week nine ** $P = 0.002$, Controls at week 13 compared to week 12 **** $P = 0.0004$ (A); BCG-Ad85A versus control group at week 9 * $P = 0.03$; BCG and BCG-Ad85A at week 13 compared to week 12 ** $P < 0.001$ and * $P < 0.01$ respectively (B); Controls versus BCG-Ad85A and BCG only at week 14 ** $P < 0.01$ (C); BCG-Ad85A versus BCG only and control group at week 12 and 14 **** $P < 0.0001$ (D).

3.2. BCG vaccination alone, and in combination with an Ad85A boost, increased protection against intranodal challenge significantly over unvaccinated controls

In the bovine BCG challenge model improved protection is defined as the ability of animals to induce more effective clearance of BCG from the injected prescapular lymph node compared to unvaccinated animals. In this study, vaccination with BCG or prime boost with BCG-Ad85A resulted in significantly lower numbers of CFU recovered from lymph nodes compared to control animals (One-way ANOVA with Tukey's multiple comparison test, *** $P = 0.0001$; **** $P < 0.0001$, mean difference log 1.121 and 0.99, Fig. 3A). There was no significant difference detectable between the two different vaccination regimens. In addition to the conventional method of plating out lymph node macerate on solid media (Fig. 3A) CFUs were determined using BD BACTEC™ MGIT™ liquid culture, which provided results up to 4 weeks earlier. CFUs measured by liquid culture were congruent with the results obtained by plating on solid media (Pearson $r = -0.94$; $p < 0.0001$, Fig. 3B). Additionally, we investigated dissemination of BCG within the lymphatic system by measuring bacterial load in the popliteal and submandibular lymph nodes, hypothesising that there would be more dissemination in unprotected and control animals compared to protected animals. BCG Tokyo was detected in popliteal and submandibular lymph nodes of 3 out of 8 controls and in none of the vaccinated animals.

3.3. Possible predictors of protection

A major limitation in developing new vaccines against bovine and human TB is the lack of predictors of protection. In this work samples were analysed for four possible predictors of protection in protected and unprotected animals: Antigen specific memory response measured by cultured IFN- γ ELISPOT, differential gene expression of IL-22, IL-17 and IFN- γ in PBMC, intracellular IL-2 and IFN- γ expression by CD4⁺ memory cells and IgG antibody titres in *ex vivo* serum. Protected animals were defined as BCG vaccinates with CFU counts lower than the animal presenting the lowest CFU counts in the unvaccinated control group and all BCG vaccinated animals with CFU counts equal to or higher than those animals that were defined as unprotected. According to this criterium 6 animals were unprotected, 4 animals from the BCG group and 2 animals from the BCG-Ad85A group, and all the remaining vaccinated animals were protected (Fig. 3A).

Memory response measured by cultured ELISPOT. Differences in memory response, measured through antigen specific IFN- γ expression by individual T cells before challenge, have previously been shown to correlate with protection in pathogenic *M. bovis* challenge experiments [12]. Here, we investigated whether this was also the case in the BCG challenge model. Analysing samples taken 10 weeks after initial vaccination and 2 weeks after boost of the BCG-Ad85A group a significant difference in numbers of antigen specific T cells between the protected group and the control group, both against PPD-B and Ag85A (PPD-B $P = 0.0047$, Ag85A $P = 0.0003$) but not compared to the unprotected group (Fig. 4A) was measured.

Antigen specific gene expression. We further analysed antigen specific IFN- γ , IL-17 and IL-22 expression in PBMC, taken 10 weeks after vaccination and 2 weeks after boost, of all unprotected animals, six randomly chosen protected animals and four control animals. There was no significant difference in expression for any of the three genes evaluated, independent of the antigen used. However, there was a clear trend for control animals and unprotected animals to express less antigen specific IFN- γ (Fig. 4B and C).

IgG ELISA. Additionally humoral responses were evaluated for possible differences in IgG titres between protected and unprotected animals (Fig. 4D and E). Antigen specific IgG concentration did not increase over background levels (week 0) prior to boost and challenge. After challenge, however, PPD-B specific IgG levels increased in all three groups, albeit to a much lesser extent in the control group (Fig. 4D). An increase in Ag85A specific IgG was detected in sera of all animals of the BCG-Ad85A group after boost, independent of their protection status. IgG levels did not change after challenge (Fig. 4E).

CD4 T cell central and effector memory response. We sought to investigate if there were differences in central and effector memory responses in protected compared to unprotected animals. To do so we initially measured antigen specific intracellular IFN- γ and IL-2 expression in stimulated and unstimulated PBMC in the four most protected and four unprotected animals from the BCG only group and four randomly chosen control animals before and after challenge (week 10 and 14 respectively, see Fig. 3A). An exemplary gating strategy is provided in [supplementary material Fig. 2](#). We compared percentages of total CD4⁺ IFN- γ , IL-2 single and double producers (Fig. 5A and D) and analysed IFN- γ and IL-2 expression by effector CD45RO⁺CD62L^{lo} and central memory cells CD45RO⁺CD62L^{hi} (Fig. 5B, C, E and F). Two unprotected animals produced very high amounts of IL-2 and IFN- γ both before

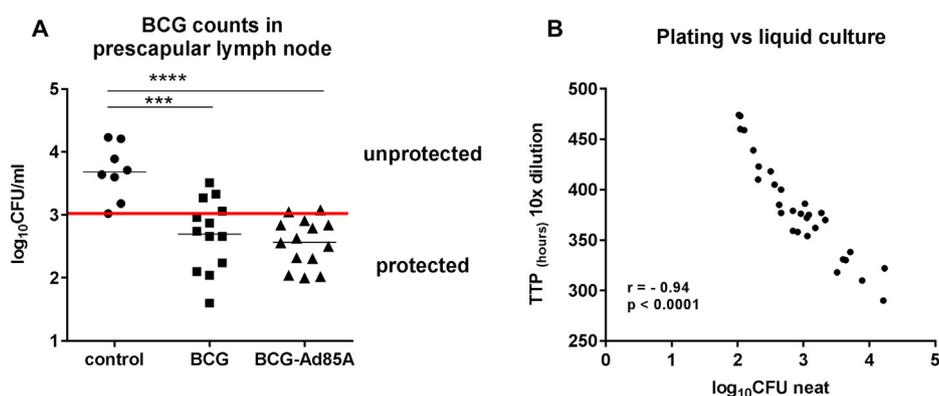


Fig. 3. Level of protection determined by CFU counts in macerate of prescapular lymph nodes. Neat lymph node macerate was plated on 7H11 agar plates and examined for colonies 6 weeks later. Animals were divided into protected and unprotected, defining all animals with CFUs lower than the control animal with the lowest CFU counts as protected and all animals equal or above as unprotected (red line). *** $P = 0.0001$; **** $P < 0.0001$, one-way ANOVA with Tukey's multiple comparison test (A). Additionally, liquid culture of one in ten diluted macerate was performed using the BD BACTEC™ MGIT™ system. Time to positivity (TTP) of liquid culture inversely correlated with CFU counts on solid media, Pearson $R = -0.94$; $P < 0.0001$ (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

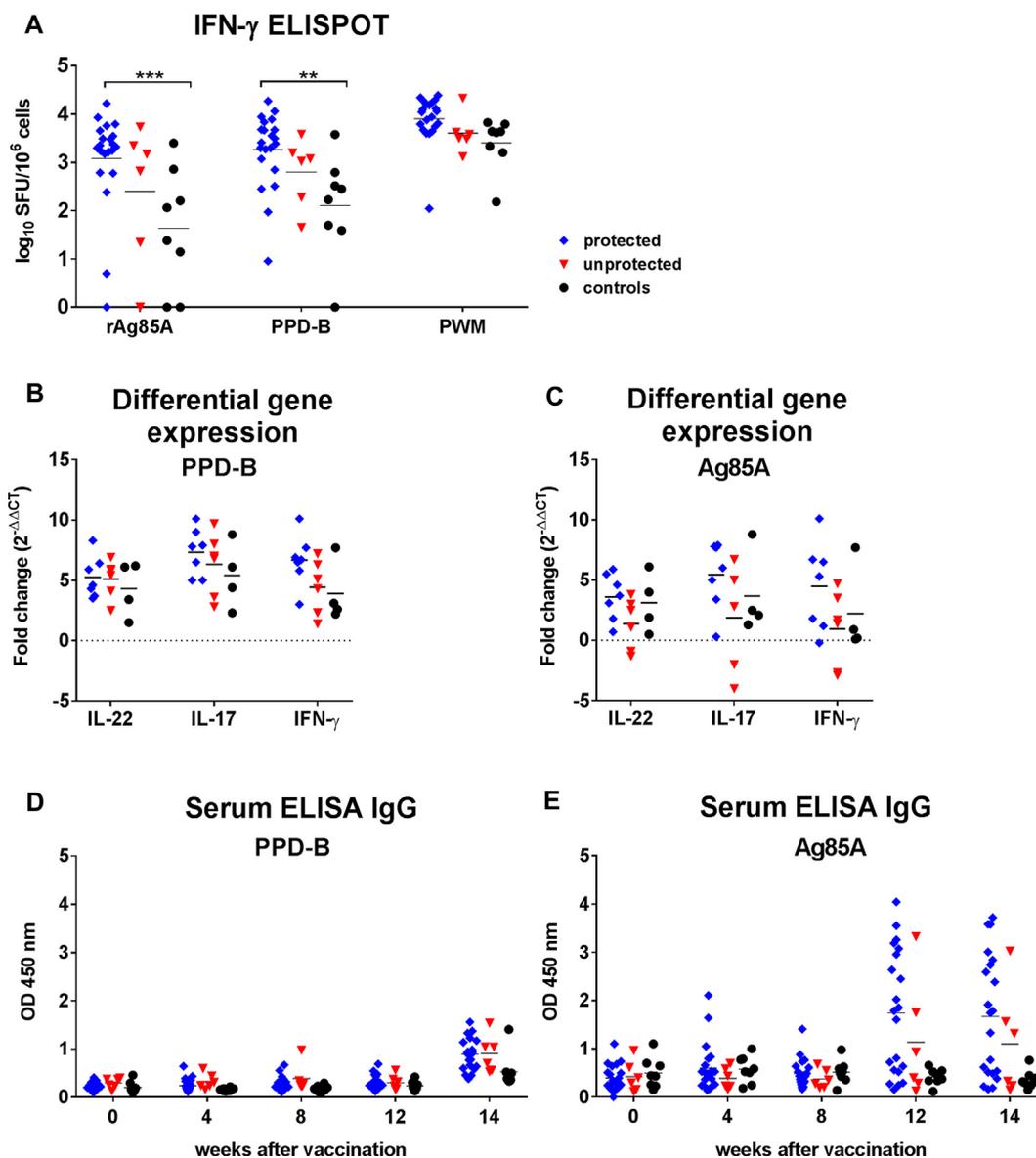


Fig. 4. Predictors of protection. Cultured ELISPOT, RT-qPCR and serum ELISA were performed as described in material and methods using either rAg85A or PPD-B as the stimulating antigen and PWM as a positive control in the ELISPOT. For the ELISPOT all animals were analysed and spot forming units were calculated according to Vordermeier et al. 2009 and are presented as $\log_{10}\text{SFU}/10^6$ cells. Two-way-ANOVA with Tukey's multiple comparison test *** $P = 0.0003$, ** $P = 0.0047$ (A). Differential gene expression was compared between 4 control animals, 6 unprotected (due to alignment of data points it may appear that there are only 5 unprotected animals in B and C) and 7 randomly chosen protected animals (B and C). Serum ELISA was performed for all animals. Controls (●), unprotected (▼), protected (◆).

and after challenge. There was a trend for vaccinated animals, both protected and unprotected, to have more IFN- γ and IL-2 single producer and especially more double producers. CD8 and $\gamma\delta$ T cells did not express any IL-2 and very little IFN- γ , with no difference between the three groups (data not shown). In general, there was an increase of IFN- γ and IL-2 single and double producers within the CD4⁺, TCM and TEM cell populations after challenge (week 14) compared to before challenge (week 10) with no significant difference between protected, unprotected and control animals. Given there was no significant difference between the most and least protected animals we did not proceed with testing further animals.

4. Discussion

The ability of a vaccine to confer protection is often determined through challenge studies with the pathogen of interest. In the case

of human TB, there is no experimental model for *M. tuberculosis* human challenge studies, however, challenge models to evaluate the efficacy of vaccine candidates prior to evaluation in clinical trials using BCG as a surrogate challenge are being evaluated [18–20]. In cattle, unlike in humans, it is possible to carry out challenge studies with *M. bovis* [21]. Those studies, however, are very costly due to their long duration and necessity of housing animals in BSL3 facilities which are not widely available. Methods allowing for an initial screening of vaccine candidates would help in the selection of the most promising candidates to be tested in *M. bovis* challenge studies and as a consequence reduce the number of vaccine evaluation trials. The recently developed bovine BCG challenge model would offer a fast and comparatively cheap way to pre-select vaccine candidates. The aim of this work was to further determine the potential utility of this model as a gating strategy for the selection of vaccine candidates prior to evaluation in *M. bovis* challenge experiments. No statistically significant difference in bacterial counts were found between the BCG-vaccinated and BCG-Ad85A

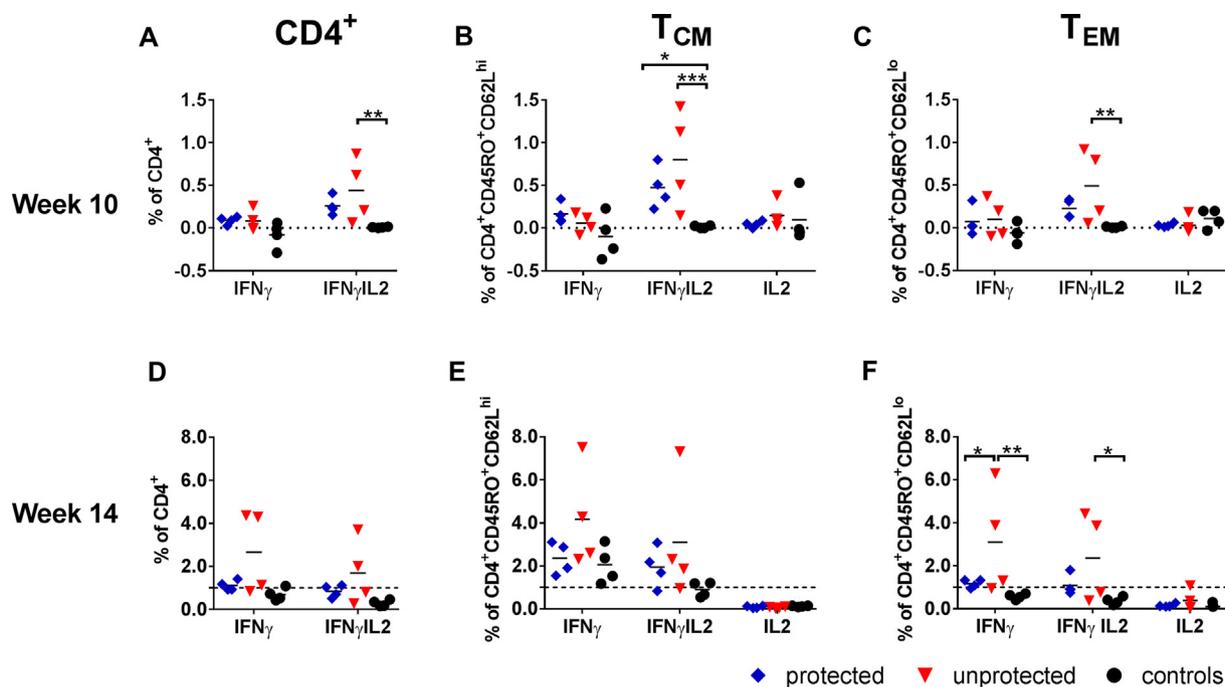


Fig. 5. Antigen specific memory response measured in $CD4^+$ and effector $CD45RO^+CD62L^{lo}$ and central $CD45RO^+CD62L^{hi}$ memory cells. $IFN-\gamma$ and IL-2 expression was measured in the four most protected, four unprotected animals from the BCG only group and four randomly chosen control animals. A and D show the percentage of $CD4^+$ cells producing either $IFN-\gamma$ or $IFN-\gamma$ and IL-2. B and E show percentage of $CD45RO^+CD62L^{hi}$ $IFN-\gamma/IL-2$ single and double producers, C and F show percentage of $CD45RO^+CD62L^{lo}$ $IFN-\gamma/IL-2$ single and double producers. Results are presented as stimulated minus unstimulated PBMC. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. D – F dotted line at 1%. Controls (●), unprotected (▼), protected (◆).

vaccinated and boosted groups. The bacterial load in prescapular lymph nodes of both vaccinated groups was reduced by around 1 log CFU/ml compared to animals in the control group. Whilst this may be considered a narrow window for the distinction of the level of protection conferred by two different vaccination regimens, it is important to highlight a few points. BCG-Ad85A was shown to confer a significantly greater degree of protection than BCG alone in a vaccination and *M. bovis* challenge trial at the histopathological [14] but not gross pathology level [12,14]. The BCG challenge model cannot evaluate pathology to the same degree as possible in *M. bovis* challenge experiments since BCG being less virulent than *M. bovis* does not induce comparable immunopathology and therefore can only provide indicators of the ability of individuals to contain/kill mycobacteria. Dividing animals into protected and unprotected, as described above, indicated that in the BCG group there were four unprotected animals, out of a total of 13 animals in the group, whilst in the BCG-Ad85A group there were two unprotected animals, out of a total of 14 animals. So even though there was no significant difference detectable between the two vaccination regimens, the data suggests a slightly better protection of BCG-Ad85A compared to BCG alone. There is a need to develop vaccines that confer substantially better protection than the currently experimentally used BCG and the BCG challenge model may be able to show these larger differences.

Previously, dissemination of BCG from the injected prescapular lymph node to popliteal and submandibular lymph nodes was measured as an indicator of the ability of the individual to contain/kill mycobacteria [10]. Similar to previous studies, BCG was found in submandibular and popliteal lymph nodes of a few control animals but not in lymph nodes other than the injected lymph node in the vaccinated groups.

Transcription of $IFN-\gamma$ in antigen stimulated PBMC and memory responses, as measured by cultured $IFN-\gamma$ ELISPOT, have been shown to correlate with protection in *M. bovis* challenge experiments [12]. Therefore we sought to analyse if this was also the case

in the BCG challenge model. For this analysis, animals were divided into protected and unprotected, as described above, resulting in two out of 14 unprotected in the BCG-Ad85A and four out of 13 unprotected in the BCG only group. In both, cultured ELISPOT and quantitative PCR, a trend for less $IFN-\gamma$ expression by T cells in the unprotected group compared to the protected group, similar to the control group was measured in samples taken 10 weeks after initial vaccination and 2 weeks after animals of the BCG-Ad85A group had received the boost. Even though there was no significant difference in $IFN-\gamma$ expression between protected and unprotected animals in the cultured Elispot, protected but not unprotected, animals produced significantly larger amounts of $IFN-\gamma$ compared to controls. We additionally analysed whether there were differences in numbers of central and effector memory T cells and the amount of $IFN-\gamma/IL-2$ they produced. Initially we analysed our most protected and least protected animals with the idea to include all animals should there be significant differences between the most protected and unprotected animals. However, the data did not suggest that we would find more differences. In summary there is a trend for protected animals to produce more $IFN-\gamma$ as measured in all our assays. The analysis for predictors of protection is limited by the number of animals that are classified as protected and unprotected, in this study the proportion of protected animals was larger than anticipated from previous studies and therefore, future studies may require a larger number of animals to enable identification of significant predictors of protection.

Experiments carried out in the 1980s had indicated that antibodies were not necessarily involved in the protection against mycobacteria. However, there was a degree of disagreement between different experiments [22]. More recent experiments [23] have indicated that antibodies could play a role in protection against mycobacteria and therefore, it was of interest to analyse the extent to which the different vaccination regimens induced humoral response in order to determine whether the induction of antibodies could be used as a marker of protection. We did

not observe any differences in the induction of humoral responses against PPD-B between the two vaccination regimens. Although antibodies to Ag85A were induced by the BCG-Ad85A vaccination and boosting regimen, no correlation between level of antibodies and degree of protection were observed. Further experiments will need to be carried out to determine the extent to which this observation can be applied to the bovine TB model.

In summary the bovine BCG challenge model offers a faster, cheaper way to evaluate newly developed vaccines against bovine tuberculosis, in particular to gate out vaccines that are less effective than BCG, and also reduces the severity of challenge compared to *M. bovis* challenge. Additionally, protection in the current BCG challenge experiments correlated with predictors of protection found in *M. bovis* challenge experiments and might thus be a useful model for research on biomarkers of protection.

5. Contribution

Lucia Biffar designed, performed and analysed the experiments and wrote the paper. Laura Blunt, William Atkins, Paul Anderson and Tom Holder were involved in collecting samples and acquiring data. Zhou Xing provided the Ad85A virus for the boost. Bernardo Villarreal-Ramos, Martin Vordermeier and Helen McShane were involved in obtaining funding, designing the project, contributed to experimental planning and reviewed/edited the paper. All authors approved the final version of the submitted manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.11.005>.

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