Seroconversion against antigen MPB83 in badgers (Meles meles) vaccinated with multiple doses of BCG strain Sofia

Kevina Mcgill, Tara Fitzsimons, Anthony Duignan, Leigh Corner, Sandrine Lesellier, Eamonn Gormley

Research in Veterinary Science 149 (2022) 119–124

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Seroconversion against antigen MPB83 in badgers (*Meles meles*) vaccinated with multiple doses of BCG strain Sofia

Kevina McGill [a], Tara Fitzsimons [a], Anthony Duignan [b], Leigh Corner [a], Sandrine Lesellier [c], Eamonn Gormley [a,*]

[a] School of Veterinary Medicine, University College Dublin (UCD), Dublin 4, Ireland
[b] Department of Agriculture, Food and the Marine, Dublin, Ireland
[c] Laboratoire de la Rage et de la Faune Sauvage de Nancy (LRFSN), Technopole Agricole et Vétérinaire, 54220, Malzéville, France

ARTICLE INFO

Keywords:
Badgers
BCG
MPB83
Tuberculosis
Serology
DPP VetTB assay

ABSTRACT

Serological diagnosis of *Mycobacterium bovis* infection in badgers (*Meles meles*) has relied primarily on antibody recognition of MPB83, a sero-dominant antigen of *M. bovis*. Most vaccine studies in badgers to date have used the Bacille Calmette-Guerin (BCG) Danish strain, a low producer of MPB83. Due to a supply shortage of the BCG Danish strain, the BCG Sofia SL222 strain has been considered as an alternative vaccine. This strain is a high producer of MPB83 raising the possibility that vaccinated animals will test sero-positive in diagnostic assays that use this antigen. In this study we vaccinated a group of eleven badgers with BCG Sofia SL222 by injection via the intramuscular route and a booster vaccine dose was similarly delivered at 12 weeks and 64 weeks. Primary vaccination did not result in measured detection of antibodies against MPB83 in any badger during the first twelve weeks using serum or whole blood tested by the Dual Path Platform (DPP) VetTB, however, MPB83 antibodies were detected in a semi-quantitative ELISA assay. Following delivery of booster BCG at 12 weeks and 64 weeks, antibody responses against MPB83 were recorded in badgers using whole blood and serum on DPP VetTB and by ELISA. At all time points, vaccination was also associated with the in vitro production of gamma interferon (IFN-γ) following stimulation of lymphocytes with bovine and avian tuberculin (PPD) but not with MPB83 or *M. bovis* specific antigen CFP-10. The results indicate that serological diagnosis of tuberculosis using tests that target MPB83 may be compromised if badgers are repeatedly vaccinated with BCG Sofia.

1. Introduction

It is well established in Ireland and the UK that badgers infected with *Mycobacterium bovis* are involved in the epidemiology of tuberculosis in cattle herds (Clifton-Hadley et al., 1995; Corner et al., 2011; Ni Bhuachalla et al., 2015). In the past two decades a considerable body of research work has been carried out to develop a greater understanding of the disease in badgers and to address how non-lethal interventions such as BCG vaccination can help to reduce the transmission rate of infection within the badger population. The BCG vaccine has been administered to badgers via a variety of routes, including subcutaneous, intramuscular and mucosal (conjunctival and oral) and delivery by these routes has induced significant protection against experimental endobronchial challenge or against natural infection with *M. bovis* (Balseiro et al., 2020; Corner et al., 2008; Gormley et al., 2021; Gormley et al., 2017; Lesellier et al., 2011). Until recently the BCG Danish strain 1331, manufactured in Denmark, was the only commercially licensed BCG vaccine for use in the European Union and the vaccine of choice for delivery to badgers in Ireland and UK. However, due to a shortage of supply of this vaccine strain, the BCG Sofia SL222 vaccine strain, derived from the Russian BCG master stock strain (Stefanova et al., 2003), has been used as an alternative vaccine strain in Ireland and the UK.

One of the goals of any vaccination programme involving wildlife is to have a concomitant diagnostic testing strategy to rapidly identify those animals among a vaccinated population that have become infected with *M. bovis* (Chambers, 2013). In this context, diagnostic assays adapted for badgers based on cell-mediated immune responses (e.g., lymphocyte transformation assays, ELISPOT IFN-γ assays) to specific antigens of *M. bovis* are not ideal as they require specialised laboratory expertise and equipment (Dalley et al., 1999; Dalley et al., 2008;

* Corresponding author.
E-mail addresses: kevina.mcgill@ucd.ie (K. McGill), tara.fitzsimons@ucd.ie (T. Fitzsimons), Anthony.Duignan@agriculture.gov.ie (A. Duignan), leigh.corner@ucd.ie (L. Corner), Sandrine.lesellier@anses.fr (S. Lesellier), egormley@ucd.ie (E. Gormley).

https://doi.org/10.1016/j.rvsc.2022.06.011
Received 18 January 2022; Received in revised form 15 June 2022; Accepted 18 June 2022
Available online 23 June 2022

Lesellier et al., 2006). Instead, serological tests that can be applied under field conditions offer an attractive alternative despite well-recognised limitations in test performance characteristics, particularly with levels of diagnostic sensitivity (test positives are biased towards advanced disease cases). The *M. bovis* pathogen is a high producer of the sero-dominant antigen MPB83, and this has facilitated the development of serological tests targeting this antigen to identify infected animals (Chambers, 2013; Wiker, 2009). In contrast, the BCG Danish vaccine is a low producer of MPB83 and serological analysis of blood from badgers vaccinated with the BCG Danish have shown that badgers do not respond with detectable antibody levels against antigen MPB83 (Corner et al., 2008; Gormley et al., 2017; Greenwald et al., 2003; Lesellier et al., 2008). Replacing the BCG Danish strain with BCG Sofia therefore raises the possibility that BCG Sofia vaccinated animals will test positive in diagnostic assays that target MPB83.

A number of serological assays have been developed commercially to diagnose tuberculosis in badgers. The lateral flow assay, known as the BrockTB Stat-Pak® (Chembio Diagnostic Systems Inc., Medford, NY, USA) was the first available serological test validated for use in badgers (Chambers et al., 2010; Greenwald et al., 2003). This assay detected the presence of IgG and IgM antibodies to MPB83, also ESAT-6 / CFP-10 fusion protein and TBF10. The sensitivity of the BrockTB Stat-Pak was estimated at 53%, with a specificity of 95% (Greenwald et al., 2003) with higher detection rates in badgers with advanced disease (Chambers et al., 2008). In recent years, the DPP VetTB assay has replaced the BrockTB StatPak for use with badgers (Ashford et al., 2020). This lateral flow test detects IgG antibodies when they bind to antigen MPB83 and ESAT-6/CFP-10 fusion protein embedded on two test lines. The performance of the DPP VetTB assay has been evaluated using sera from confirmed naturally infected *M. bovis* badgers and TB-free badgers, and showed a sensitivity ranging from of 60% - 67% (at a specificity of 95%) depending on the precision of the method used to determine a positive test result (Ashford et al., 2020). An independent evaluation of test performance was carried out in Northern Ireland as part of 'Test and Vaccinate or Remove (TVR)' badger research intervention project (Courcier et al., 2020). This study employed a relative gold standard (IFN-γ response and culture of *M. bovis*) and the reported estimates of sensitivity were 50% and 42% in serum samples and whole blood respectively, and specificity estimates of 95% and 89% respectively. A follow up Bayesian latent class analysis of data from the same study estimated the DPP VetTB with serum to have a sensitivity of 58% and specificity of 97%. Intriguingly, the DPP VetTB when used in the field with whole blood showed a higher sensitivity (70%) but similar specificity (97%), whereas serum had a much higher sensitivity (83%) compared with the field DPP VetTB test (43%) when badgers were vaccinated with the BCG Sofia strain (Arnold et al., 2021).

With commencement of badger vaccination in Ireland with the BCG Sofia strain, a question was raised as to whether vaccination with this strain would result in detectable serological responses against the MPB83 antigen, thus compromising the test performance of the DPP VetTB assay following vaccination. The principle objective of this study was to vaccinate and boost a group of captive badgers by the intramuscular route with BCG Sofia strain to measure serological and cell-mediated immune responses over the course of 18 months post-vaccination. The primary outcome measures were detection of antibody responses in vaccinated badgers using the VetTB-DPP assay and a semi-quantitative ELISA assay. The CMI responses were also measured using an IFN-γ ELISPOT assay with peripheral blood mononuclear cells (PBMC) stimulated with purified protein derivative of tuberculin antigens PPD-bovine (PPD-Bov), PPD-avian (PPD-Av), also with single antigens MPB83 and *M. bovis* specific antigen CFP-10.

2. Methods

2.1. Study design and statistical analysis

All work with badgers was carried out under a licence (AE19113 / P010 / 2017–2019) issued by the Irish Health Products Regulatory Authority (HPRA) and ethical approval was obtained from the UCD animal research ethics committee (AREC-17-28-Gormley). The methodology used to determine the sample size was based on calculations to estimate the sample size needed to determine 'freedom from disease' within animal populations as described by Cameron and Baldock (Cameron and Baldock, 1998). The rationale is that we assumed a priori that our target badger population is free of TB disease. In an *M. bovis* infected population, badgers will generate antibodies against MPB70/MPB83 that are detected using the BrockTB Stat-Pak assay with a test performance of sensitivity (50%) and specificity (98%). Experimental challenge studies of non-vaccinated badgers with *M. bovis* have shown that up to 80% of animals sero-convert within six weeks of challenge (Lesellier et al., 2009). We used this information to calculate the samples size necessary to determine if a positive serology test result in a TB free badger is due to vaccination with BCG Sofia or is a false positive result related to performance characteristics of the test. For convenience, a calculator based on this formula is available at <https://epitools.ausvet.com.au>. The inputs included the sensitivity (50%) and specificity (98%) of the serology test and the hypothetical seroconversion rate to be detected (ie. 80% seroconversion). The size of the population sampled was based on a projected 1000 badgers that may be vaccinated annually. The calculation uses a hypergeometrical method for small populations, with a threshold population size of 10,000 badgers. The output generated a sample size of 10 badgers with a cut-off of one test positive badger; representing the minimum sample size and cut-off limit for the number of seroconverted badgers required to achieve the specified type I (0.05) and type II errors (0.05) for the given population size, hypothetical seroconversion rate and test performance (sensitivity / specificity). If a random sample of 10 badgers were taken from a population of 1000 vaccinated (and TB free) animals and more than one seroconversion event was recorded, this would indicate that seroconversion may have resulted from vaccination. The corollary is that if a random sample of 10 animals is taken from a population of 1000 badgers and 1 or fewer animals seroconvert, the test result is likely to be a false positive, based on performance characteristics of the test.

In the IFN-γ ELISPOT assay, the responses to stimulation of blood lymphocytes with PPD-Bov and PPD-Av were analysed by Student's unpaired *t*-test using GraphPad Prism software Version 6.0b.

2.2. Handling of captive badgers

The eleven badgers enrolled in the study (six females, five males, minimum age $>$ 2 years based on tooth wear) were captured in cages from wild populations at setts that were historically free of tuberculosis. They were allocated to pens based on their social group of origin. The badgers were maintained in outdoor pens, in groups of 2–4 based on their social group of origin, each pen with an area of ~200 m^2, with natural earthen floors covered in grass, shrubs and other natural plant species. In each pen were two bespoke wooden setts that each contained an inner nest box layered with fresh straw. The badgers were fed proprietary dog biscuits, raw peanuts and had access to natural food sources in their earthen environment. Water was provided *ad libidum*. The background immune responses were assessed at two time points prior to the commencement of the study by lymphocyte transformation assay and the badger IFN-γ ELISPOT assay using PPD-Bov, PPD-Av and *M. bovis* specific antigen CFP-10 (see Section 2.6). Prior to blood sampling and vaccination, the badgers were anaesthetised with ketamine hydrochloride (10 mg/kg) and medetomidine hydrochloride (0.1 mg/kg, Domitor®, Pfizer) co-administered by intramuscular injection. Eleven badgers were vaccinated with BCG Sofia by the intramuscular

route on Day 0 of the study. The badgers were examined for clinical signs (palpation at injection site and monitoring for signs of adverse reactions) and blood samples taken at week 0, 4, 12, 16, 20, 64, 68 and 74 weeks post - primary vaccination. One badger died at week 16. Badgers were boosted at week 12 post- primary vaccination and re-vaccinated at week 64 (ie., 52 weeks after booster vaccination). Blood was collected by jugular venopuncture into vacutainer tubes containing lithium-heparin and into Serum Collection Tubes (Becton Dickinson, Plymouth UK).

2.3. Vaccination of badgers with BCG Sofia SL222

The BCG Sofia SL222 vaccine was sourced from Intervax Ltd., Canada. On the day of vaccination, the supplied diluent (1 ml) was added to the freeze-dried BCG contents in the ampoule. The suspension was shaken for 5 s to disperse large particulates and drawn into a 2 ml syringe fixed with a 20 g × 1.0″ hypodermic needle (Kendall monoject). A patch of hair was shaved in the shoulder region of the sedated badger and vaccine was delivered by intramuscular injection into the shoulder muscle below the neck. The inoculated vaccine dose (approximately 1–5 × 10^6 cfu / ml) was determined post vaccination by culture of 10-fold serial dilutions of residual suspension on modified Middlebrook 7H11 agar medium (Becton Dickinson, France). The protocol was similar for delivery of the booster vaccine and re-vaccination, with injection into the opposite shoulder on each occasion.

2.4. DPP VetTB assay

The DPP VetTB assay was performed as described by the manufacturer (DPP® VetTB Assay for Cervids, Chembio Diagnostic Systems Inc., Medford, NY, USA). Briefly, a serum sample a volume of 30 µl was added to the test well (well 1) and results were recorded 15 min after addition of buffer (4 drops) to the buffer well (well 2). When testing whole blood, 10 µl of blood and 3 drops of buffer were used. The test results were interpreted 'by eye' (based on visualisation of visible test lines by the test operator) and recorded as positive or negative.

2.5. MPB83 / MPB70 ELISA test

The ELISA test on badgers was carried out using the Idexx ELISA kit for bovine TB (Idexx Laboratories, Inc., Westbrook, ME) according to the manufacturer's instructions. In this kit the ELISA plates are pre-coated with mycobacterial antigens MPB83 and MPB70. The test was modified by using a secondary anti-badger IgG CF2-HRP monoclonal antibody coupled to horseradish peroxidase (HRP) for the detection of badger antibody in the serum samples (Animal Plant and Health Agency, United Kingdom).). Briefly, serum samples were diluted (1/100) in Idexx sample diluent containing extract of *Mycobacterium phlei* (Allied Monitor, Fayette, MO). Diluted samples (100 µl /well in duplicate) were then added to the ELISA plate together with plate positive and negative controls provided by Idexx. The plates were incubated at room temperature for 60 min. After five washes with 200 µl per well of wash buffer (supplied with the kit), the CF2-HRP monoclonal antibody (Animal Plant and Health Agency, United Kingdom) was diluted to a working concentration of 1µg/ml in conjugate diluent, and 100 µl were added to each well. The plate was incubated in a dark incubator at 24 °C for 30 min. After five washes with 200 µl of wash buffer 100 µl of TMB substrate was added to each well. Following a 15-min incubation at 24 °C, colour development in the wells was stopped by the addition of 50 µl stop solution (0.5 M H_2SO_4). The Optical Density (OD) values were read at 450 nm on a Versamax microplate reader (Molecular Devices LLC, San Jose, CA) and the data points were plotted as OD units (no predetermined cut-off point was used as a measure of diagnostic performance).

2.6. The IFN-γ ELISPOT assay

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood and the badger IFN-γ ELISPOT assay was conducted as described previously (Corner et al., 2010; Lesellier et al., 2006). The 96-well ELISPOT plates (Millipore multi-screen, Millipore Ltd., UK) were coated with capture mAb 10H6—C1 (APHA, UK) at 10 µg/ml in carbonate/bicarbonate buffer, pH 9.6 overnight at 4 °C. Excess capture antibody was removed and the plates were blocked by the addition 200 µl of culture medium (RPMI, supplemented with 5% foetal calf serum, (Invitrogen Life Technologies), non-essential amino acids, 5 × 10^{-5} M β-mercaptoethanol (Invitrogen Life Technologies) and 100 U penicillin/100 µg streptomycin per ml (Invitrogen Life Technologies) per wells and incubated at 37 °C for two hours. Badger lymphocytes were isolated from the heparinised whole blood and 100 µl of cells re-suspended in culture medium at 2 × 10^6 cells/ml. For each animal, 100 µl of PBMCs (at final concentration of 2 × 10^5 cells/ml) were incubated for 18 h at 37 °C and 5% CO_2 with 100 µl of each of the following antigens: PPD-Bov (final conc. 30 µg/ml, Prionics Lelystad BV, Lelystad, The Netherlands), PPD-Av (final conc. 30 µg/ml, Prionics Lelystad BV, Lelystad, The Netherlands), MPB83 (final conc. 5 µg/ml, APHA UK), CFP10 (final conc. 5 µg/ml, Lionex, GmbH). The mitogen ConA (final conc. 5.0 µg/ml, Sigma Ltd., UK) diluted in supplemented RPMI was used as a positive control. Negative cell controls were incubated in culture medium without antigen. The liquid was discarded and the plates washed twice in distilled water and three washes with buffer (PBS, 0.05% Tween-20). 100 µl of biotin labelled mAb 11B9 was added to each well (final concentration of 0.2.5 µg/ml per well) and the plates incubated at 37 °C for 1 h. The plates were washed four times with wash buffer and 100 µl of Streptavidin-alkaline-phosphatase (Mabtech AB, Hamburg, Germany) diluted 1/4000 in PBS, 0.05% Tween-20, 0.1% albumin, and incubated at 37 °C for 1 h. The plates were washed with wash buffer and 100 µl of BCIP/NBT (bromo-4-chloro-3-indolyl phosphate - Nitro blue tetrazolium, Sigma-Aldrich) substrate was added to each well; the reaction was stopped after 10–20 min by copious washing with water. The plates were air-dried and spots counted using an automated AID ELISPOT reader (Autoimmun Diagnostika AID GmbH, Strassberg, Germany). The net Spot Forming Units (SFU of antigen-stimulated minus negative control cells) were calculated per million cells for each antigen.

3. Results

3.1. Clinical examination of vaccinated badgers

Eleven badgers were vaccinated with BCG Sofia by the intramuscular route on Day 0 of the study. The badgers received a booster vaccination at 12 weeks and were subsequently re-vaccinated at 64 weeks (i.e. one year following the booster vaccine). Throughout the study, the general health of all badgers was monitored daily to detect evidence of any adverse behaviour resulting from vaccination. The body weights of the badgers decreased from the time of vaccination in November (mean 13.5 kg ± sd 1.7) and were lowest at the 12-week booster BCG time-point in March (mean 8.6 kg ± sd 0.6). Thereafter they gained weight until week 64 (mean 10.5 kg ± sd 0.7) when their weight started to decrease again (week 74: mean 9.1 kg ± sd 0.6). No clinical signs related to vaccination were detected in any badger throughout the course of the study. One badger died at week 16: a detailed post-mortem examination followed by histopathology and microbiological cultures did not reveal any specific cause of death or otherwise any cause that might be associated with vaccination.

3.2. IgG responses to vaccination with BCG Sofia

The DPP VetTB tests were carried out using whole blood and serum samples at each blood sampling time-point on the 10 badgers enrolled

for the duration of the study (see Section 2.2). The DPP VetTB result was determined by visual inspection of the test cartridge and results were subjectively categorised as weak or strong positives depending on the intensity of the reaction at the MPB83 antigen line 1 (no reaction was detected at the ESAT-6/CFP-10 fusion protein antigen line 2 throughout the study, consistent with the tuberculosis - free status of the animals). The pre-vaccination IgG responses to MPB83 recorded on day 0 were negative for all whole blood and serum samples (Fig. 1). The primary vaccination with BCG Sofia did not result in any positive DPP VetTB tests during the first twelve weeks of the study (Fig. 1). Following delivery of a booster dose of BCG at week 12, an antibody response to MPB83 was recorded by the DPP VetTB assay in serum of 7/9 badgers and 4/9 whole blood samples tested at week 16. At week 20, 6/10 serum samples were positive on the DPP VetTB assay though only one was classified as strong positive. In contrast, all of the whole blood samples were negative on the DPP VetTB assay at this time point. At 64 weeks post-vaccination, all whole blood samples were negative and all but one badger serum sample was also negative on DPP VetTB. Four weeks and ten weeks after the revaccination with BCG, i.e. at 68 and 74 weeks, all of the badgers were strong seropositive on DPP VetTB using serum. In the whole blood test, all of the badgers were strong seropositive on DPP VetTB at week 68. However, six weeks later (week 74), only three of the whole blood samples tested positive.

The IgG levels in serum samples were also determined in a semi-quantitative ELISA assay (Fig. 2). Following the primary vaccination IgG was detected with a rapid elevation in levels observed after the 12-weeks boost. At the time of re-vaccination at 64 weeks, the levels had dropped but remained above the baseline levels measured prior to vaccination. Re-vaccination at 64 weeks resulted in a rapid increase in IgG followed by a decrease at the 74-week endpoint of the study.

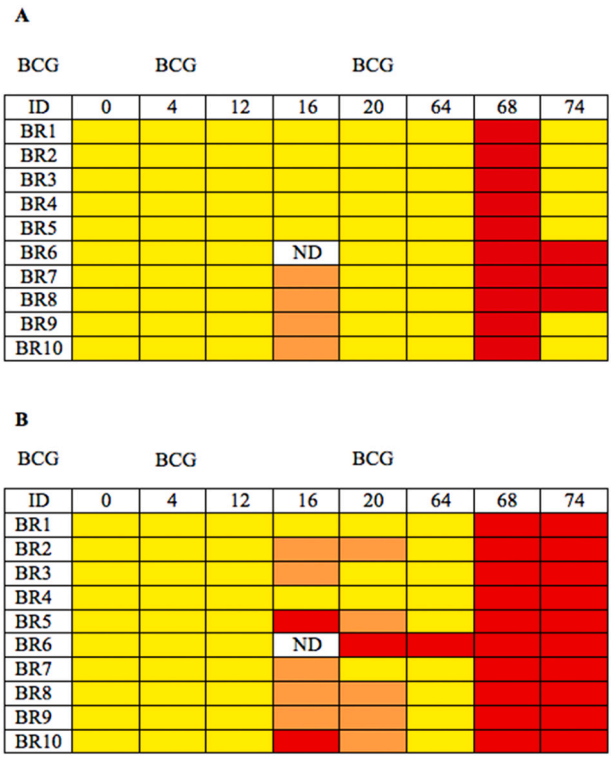

Fig. 1. Serological responses of badgers (BR1–10) when whole blood (A) or serum (B) was tested with DPP VetTB. Results were classified by visual analysis as negative (yellow), weak positive (orange) and strong positive (red). BCG Sofia was delivered to badgers at time points indicated (weeks) immediately following collection of blood. ND; not determined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

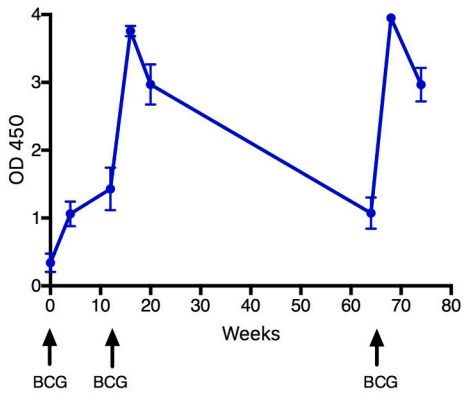

Fig. 2. Serological responses of badgers to antigen MPB83/MPB70 measured by ELISA. Results are expressed as mean optical density (OD) ± SEM. BCG Sofia was delivered to badgers at time points indicated.

3.3. IFN-γ ELISPOT responses of badgers following BCG vaccination

The IFN-γ responses in PBMC of all badgers were monitored in vitro by the badger IFN-γ ELISPOT assay using PPD-Bov, PPD-Av, MPB83 and CFP-10 as stimulating antigens (Fig. 3). The CFP-10 antigen was included as a potential marker of *M. bovis* infection. Following vaccination with BCG Sofia at day 0, antigen specific IFN-γ responses were detected with PPD-Bov and PPD-Av antigens at the week 4 blood sampling point. At week 12, the responses to PPD-Bov appeared to increase over the PPD-Av responses but the difference was not statistically significant ($p > 0.05$). Following the booster vaccination at week 12, the responses to PPD-Bov exceeded the PPD-Av responses at week 16 and week 20 ($p = 0.006$). There was a relatively small increase in the responses to MPB83 following the BCG boost at week 12. By week 64 (point of re-vaccination), the antigen specific production of IFN-γ had declined to the baseline levels measured at day 0. However, following the re-vaccination at week 64 there was a rapid increase in IFN-γ responses to PPD-Bov and PPD-Av, and a lower but detectable increase in responses to MPB83. By the end of the study at week 74, there was a decline in the responses to these antigens though the responses to PPD-Bov remained significantly higher than responses to PPD-Av ($p = 0.005$). Throughout the study, the responses to the *M. bovis* specific antigen CFP-10 remained at baseline levels.

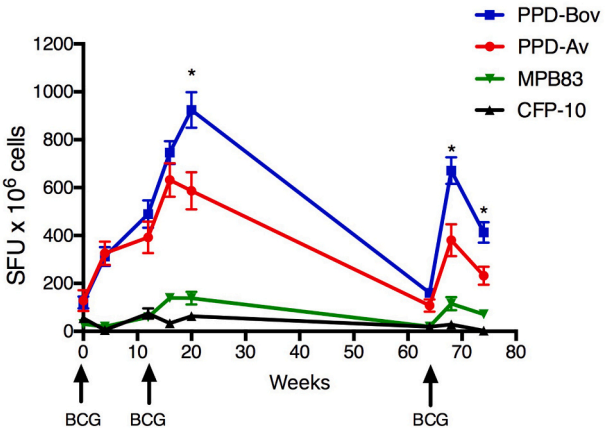

Fig. 3. IFN-γ ELISPOT analysis of responses of badgers vaccinated with BCG Sofia at times points indicated. PBMC were stimulated with antigens, PPD-Bov, PPD-Av, MPB83 and CFP-10. Data points are expressed as group mean ± SEM. * denotes statistically significant ($p < 0.05$) difference in responses to stimulation of PBMC with PPD-Bov and PPD-Av.

4. Discussion

The BCG vaccine is currently the only licensed vaccine for use in humans and it has been adapted for use in a variety of livestock and wild animal species (Buddle et al., 2018). With a global supply shortage of the BCG Danish strain impacting on the continuation of vaccine studies in Ireland; the BCG Sofia strain has been sourced as a potential replacement. This strain, derived from BCG-Russia, belongs to the MPB83 high producing family of BCG strains, raising a key question as to whether the test performance of the DPP VetTB assay would be compromised following vaccination with this strain. In addition to delivery of a primary high-dose of BCG Sofia to badgers (1–5 $\times$ 10^6 cfu / ml), the design of the present study included a twelve-week booster vaccination, as this is the approximate time point used in previous experimental models to infect vaccinated animals with *M. bovis* (Balseiro et al., 2020; Chambers et al., 2017; Corner et al., 2008; Murphy et al., 2014). An annual boost with the same dose was also incorporated to simulate a potential strategy of annual re-vaccination programmes in wild badger populations. The results showed that primary vaccination with a single dose of BCG Sofia did not generate any test positive responses in the DPP VetTB assay when serum or whole blood was tested. Following booster vaccination, more animals tested positive in serum than with whole blood and the duration of positive responses was also longer when measured in serum. The apparent higher test sensitivity when using serum is consistent with a Bayesian latent class analysis of the results from the TVR study in Northern Ireland that showed a higher test sensitivity using serum for diagnosis of *M. bovis* infected badgers when badgers were vaccinated with BCG Sofia strain during the final year of that study (Arnold et al., 2021). The biological explanation for this difference between whole blood and serum remains unknown. However, as highlighted in the Northern Ireland study (Courcier et al., 2020), the smaller volume of whole blood used in the assay (10 μl cf. 30 μl of serum) may have contributed to lower test sensitivity, resulting in lower Relative light Units (RLU) for samples sourced from the same animal (Ashford et al., 2020). When using the more sensitive Idexx ELISA platform, antibodies were detected in badgers after primary vaccination, and throughout the period of study. Semi-quantitative measurement of IgG in the ELISA indicated a strong anamnestic antibody response following boosting with BCG and the IgG levels remained above baseline at all time points post-vaccination. The Idexx ELISA detects antibodies to two serodominant antigens, MPB83 and MPB70, which may partly explain the apparent increased test sensitivity of the ELISA assay compared with the DPP VetTB test (Waters et al., 2011). We cannot rule out, however, if differences in temporal expression in vivo of MPB83 and MPB70 may lead to preferential detection of these antigens in the ELISA assay at any time point post-vaccination.

The vaccination of the badgers was also associated with the in vitro production of IFN-γ in response to PBMC stimulation with PPD-Bov and PPD-Av tuberculin in the IFN-γ ELISPOT assay. Throughout the post-vaccination phase of the study the responses to PPD-Bov exceeded that of PPD-Av. It is likely that the responses to PPD-Av arise from frequent exposure to environmental mycobacterial antigens that are cross reactive with *M. avium*, and/or in the case of this study, shared epitopes with the BCG vaccine. The dominant responses to PPD-Bov antigens have been observed previously in wild badgers vaccinated with BCG (Southey et al., 2001).

The weakly detectable responses to MPB83 in the IFN-γ ELISPOT assay showed that it was not a dominant CMI response biomarker for vaccination and the absence of a response to CFP-10 was consistent with the TB free status of the badgers (Lesellier, 2018). The co-secreted antigen ESAT-6 was not used as the response to this antigen has been shown to be non- detectable in TB infected badgers (Lesellier, 2018; Lesellier et al., 2008). The study of the kinetics of the IFN-γ responses to *M. bovis* BCG vaccination in badgers has previously shown a lower magnitude response following re-vaccination with a lower dose BCG, suggestive of a dose-dependent response (Lesellier et al., 2006). In this study the IFN-γ response following BCG re-vaccination was also of lower magnitude and of shorter duration than that observed following the initial BCG vaccination. Given that the vaccine delivery dose was similar, this might reflect a more general decreased response to re-vaccination where the cell-mediated response is controlled so that the antigenic response of effector cells does not surpass a threshold level necessary to control the infection.

Tuberculosis in many species including cattle and badgers induces a spectrum of immunological responses where the balance of the host immune response shifts from a predominantly CMI Th1 IFN-γ response during the early stages of *M. bovis* infection towards a Th2 antibody-based response as the disease progresses (Lesellier et al., 2009; Ritacco et al., 1991; Welsh et al., 2005). It was noted in the current BCG vaccine-only study that the kinetic profile of the IgG response to MPB83 measured by ELISA was very similar to the IFN-γ response profile against PPD-Bov and PPD-Av. This may reflect a high dose response against BCG delivered directly into the muscle of the host. It has previously reported from experimental infections of cattle that antibody responses to MPB83 were detected early post-infection (4 weeks) and appeared to be influenced by route of infection (Waters et al., 2006).

The finding that badgers produced anti-MPB83 antibodies detectable by ELISA after primary vaccination and thereafter detectable by the DPP VetTB tests and ELISA in response to boosting with the BCG Sofia strain is potentially significant. It indicates that diagnosis of infection based on antibody recognition of MPB83 alone might not be accurate if badgers receive more than a single dose of BCG Sofia: the anamnestic antibody response to MPB83 would render it unlikely in most cases to distinguish BCG-vaccinated from *M. bovis* infected badgers. Identification of the infection status of a vaccinated badger using DPP VetTB would then rely on a positive test result on line 2 containing the ESAT-6/CFP-10 fusion protein. However, the study by Courcier et al., in Northern Ireland showed that diagnostic sensitivity was significantly higher when using line 1 compared with line 2 (Courcier et al., 2020). That study also reported that parallel interpretation of line 1 and line 2 did not improve the sensitivity or specificity of the test result when compared with line 1 alone.

The absence of antibody responses to MPB83 following primary vaccination using DPP VetTB with whole blood or serum samples should also be treated with caution as these experimental animals are living in a controlled and enriched environment, and receiving sufficient food conducive to maintaining good levels of health. In addition the age range of the badgers enrolled in this study was estimated between 2 and 5 years, when they are likely to be at their healthiest. In their natural environment, badgers of different age groups ranging from cubs to old badgers are subject to many stressors including periodic scarcity of food and other resources, all of which could influence the quality of the immune responses. During the period of this study, the weight fluctuations of the badgers were consistent with seasonal changes in weight observed in free-living badgers (data not shown). Nevertheless, it cannot be taken for granted that all TB free wild badgers, across the full spectrum of ages, that receive a single BCG dose would be test negative with DPP VetTB in the first weeks following vaccination. We can therefore conclude from the results of this study that vaccination of badgers with BCG Sofia has the potential to cause seroconversion against MPB83 and the risk of this happening is increased with delivery of additional doses within the time frame of twelve months. From the perspective of a vaccine strategy, it may be that tuberculosis free badgers only receive a single dose delivery of BCG Sofia as part of any vaccination programme in order to minimise the likelihood of testing positive when using the DPP VetTB assay. This may depend on the duration of protective immunity generated by vaccination with BCG Sofia, which is currently unknown. However, a single dose would also reduce the need to sedate and collect blood samples from follow up captures of badgers; it would also serve to reduce stress on badgers and improve animal welfare.

Funding

The study was funded by the Department of Agriculture, Food & the Marine (Grant No. UCD/R9762-2017/019).

CRediT authorship contribution statement

Kevina McGill: Methodology, Investigation, Data curation, Writing – review & editing, Project administration. **Tara Fitzsimons:** Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Anthony Duignan:** Methodology, Investigation, Writing – review & editing. **Leigh Corner:** Conceptualization, Writing – review & editing. **Sandrine Lesellier:** Conceptualization, Writing – review & editing. **Eamonn Gormley:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

We would like to thank Pat Rooney (DAFM) for his expertise and assistance with working with the badgers. We acknowledge the support of staff at Longtown research farm (DAFM) for all their efforts in maintaining the BROC facility and facilitating the research work. We thank Dipesh Dave (APHA, Weybridge UK) and Paul Anderson (APHA, Weybridge UK) for their expertise and help with setting up the ELISA test. We also thank Tracy Clegg (CVERA, UCD) for statistical support.

References

Arnold, M.E., Courcier, E.A., Stringer, L.A., McCormick, C.M., Pascual-Linaza, A.V., Collins, S.F., Trimble, N.A., Ford, T., Thompson, S., Corbett, D., Menzies, F.D., 2021. A Bayesian analysis of a test and vaccinate or remove study to control bovine tuberculosis in badgers (*Meles meles*). PLoS One 16, e0246141.

Ashford, R.T., Anderson, P., Waring, L., Dave, D., Smith, F., Delahay, R.J., Gormley, E., Chambers, M.A., Sawyer, J., Lesellier, S., 2020. Evaluation of the dual path platform (DPP) VetTB assay for the detection of *Mycobacterium bovis* infection in badgers. Prev. Vet. Med. 180, 105005.

Balseiro, A., Prieto, J.M., Alvarez, V., Lesellier, S., Dave, D., Salguero, F.J., Sevilla, I.A., Infantes-Lorenzo, J.A., Garrido, J.M., Adriaensen, H., Juste, R.A., Barral, M., 2020. Protective effect of Oral BCG and inactivated *Mycobacterium bovis* vaccines in European badgers (*Meles meles*) experimentally infected with *M. bovis*. Front. Vet. Sci. 7, 41.

Buddle, B.M., Vordermeier, H.M., Chambers, M.A., de Klerk-Lorist, L.M., 2018. Efficacy and safety of BCG vaccine for control of tuberculosis in domestic livestock and wildlife. Front. Vet. Sci. 5, 259.

Cameron, A.R., Baldock, F.C., 1998. A new probability formula for surveys to substantiate freedom from disease. Prev. Vet. Med. 34, 1–17.

Chambers, M.A., 2013. Review of the diagnosis of tuberculosis in non-bovid wildlife species using immunological methods–an update of published work since 2009. Transbound. Emerg. Dis. 60 (Suppl. 1), 14–27.

Chambers, M.A., Crawshaw, T., Waterhouse, S., Delahay, R., Hewinson, R.G., Lyashchenko, K.P., 2008. Validation of the BrockTB stat-pak assay for detection of tuberculosis in Eurasian badgers (*Meles meles*) and influence of disease severity on diagnostic accuracy. J. Clin. Microbiol. 46, 1498–1500.

Chambers, M.A., Lyashchenko, K.P., Greenwald, R., Esfandiari, J., James, E., Barker, L., Jones, J., Watkins, G., Rolfe, S., 2010. Evaluation of a rapid serological test for the determination of *Mycobacterium bovis* infection in badgers (*Meles meles*) found dead. Clin. Vaccine Immunol. 17, 408–411.

Chambers, M.A., Aldwell, F., Williams, G.A., Palmer, S., Gowtage, S., Ashford, R., Dalley, D.J., Dave, D., Weyer, U., Salguero, F.J., Nunez, A., Nadian, A.K., Crawshaw, T., Corner, L.A., Lesellier, S., 2017. The effect of Oral vaccination with *Mycobacterium bovis* BCG on the development of tuberculosis in captive European badgers (*Meles meles*). Front. Cell. Infect. Microbiol. 7, 6.

Clifton-Hadley, R.S., Wilesmith, J.W., Richards, M.S., Upton, P., Johnston, S., 1995. The occurrence of *Mycobacterium bovis* infection in cattle in and around an area subject to extensive badger (*Meles meles*) control. Epidemiol. Infect. 114, 179–193.

Corner, L.A., Costello, E., Lesellier, S., O'Meara, D., Gormley, E., 2008. Vaccination of European badgers (*Meles meles*) with BCG by the subcutaneous and mucosal routes induces protective immunity against endobronchial challenge with *Mycobacterium bovis*. Tuberculosis (Edinb) 88, 601–609.

Corner, L.A., Costello, E., O'Meara, D., Lesellier, S., Aldwell, F.E., Singh, M., Hewinson, R. G., Chambers, M.A., Gormley, E., 2010. Oral vaccination of badgers (*Meles meles*) with BCG and protective immunity against endobronchial challenge with *Mycobacterium bovis*. Vaccine 28, 6265–6272.

Corner, L.A., Murphy, D., Gormley, E., 2011. *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*): the disease, pathogenesis, epidemiology and control. J. Comp. Pathol. 144, 1–24.

Courcier, E.A., Pascual-Linaza, A.V., Arnold, M.E., McCormick, C.M., Corbett, D.M., O'Hagan, M.J.H., Collins, S.F., Trimble, N.A., McGeown, C.F., McHugh, G.E., McBride, K.R., McNair, J., Thompson, S., Patterson, I.A.P., Menzies, F.D., 2020. Evaluating the application of the dual path platform VetTB test for badgers (*Meles meles*) in the test and vaccinate or remove (TVR) wildlife research intervention project in Northern Ireland. Res. Vet. Sci. 130, 170–178.

Dalley, D., Chambers, M.A., Cockle, P., Pressling, W., Gavier-Widen, D., Hewinson, R.G., 1999. A lymphocyte transformation assay for the detection of *Mycobacterium bovis* infection in the Eurasian badger (*Meles meles*). Vet. Immunol. Immunopathol. 70, 85–94.

Dalley, D., Dave, D., Lesellier, S., Palmer, S., Crawshaw, T., Hewinson, R.G., Chambers, M., 2008. Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). Tuberculosis (Edinb) 88, 235–243.

Gormley, E., Ni Bhuachalla, D., O'Keeffe, J., Murphy, D., Aldwell, F.E., Fitzsimons, T., Stanley, P., Tratalos, J.A., McGrath, G., Fogarty, N., Kenny, K., More, S.J., Messam, L.L., Corner, L.A., 2017. Oral vaccination of free-living badgers (*Meles meles*) with Bacille Calmette Guerin (BCG) vaccine confers protection against tuberculosis. PLoS One 12, e0168851.

Gormley, E., Ni Bhuachalla, D., Fitzsimons, T., O'Keeffe, J., McGrath, G., Madden, J.M., Fogarty, N., Kenny, K., Messam, L.L.M., Murphy, D., Corner, L.A.L., 2021. Protective immunity against tuberculosis in a free-living badger population vaccinated orally with *Mycobacterium bovis* Bacille Calmette-Guerin. Transbound. Emerg. Dis 1–10. https://doi.org/10.1111/tbed.14254.

Greenwald, R., Esfandiari, J., Lesellier, S., Houghton, R., Pollock, J., Aagaard, C., Andersen, P., Hewinson, R.G., Chambers, M., Lyashchenko, K., 2003. Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multiantigen test formats. Diagn. Microbiol. Infect. Dis. 46, 197–203.

Lesellier, S., 2018. Immunological responses of European badgers (*Meles Meles*) to infection with *Mycobacterium bovis*. Comp. Immunol. Microbiol. Infect. Dis. 61, 9–15.

Lesellier, S., Palmer, S., Dalley, D.J., Dave, D., Johnson, L., Hewinson, R.G., Chambers, M.A., 2006. The safety and immunogenicity of Bacillus Calmette-Guerin (BCG) vaccine in European badgers (*Meles meles*). Vet. Immunol. Immunopathol. 112, 24–37.

Lesellier, S., Corner, L., Costello, E., Sleeman, P., Lyashchenko, K., Greenwald, R., Esfandiari, J., Singh, M., Hewinson, R.G., Chambers, M., Gormley, E., 2008. Antigen specific immunological responses of badgers (*Meles meles*) experimentally infected with *Mycobacterium bovis*. Vet. Immunol. Immunopathol. 122, 35–45.

Lesellier, S., Corner, L., Costello, E., Lyashchenko, K., Greenwald, R., Esfandiari, J., Singh, M., Hewinson, R.G., Chambers, M., Gormley, E., 2009. Immunological responses and protective immunity in BCG vaccinated badgers following endobronchial infection with *Mycobacterium bovis*. Vaccine 27, 402–409.

Lesellier, S., Palmer, S., Gowtage-Sequiera, S., Ashford, R., Dalley, D., Dave, D., Weyer, U., Salguero, F.J., Nunez, A., Crawshaw, T., Corner, L.A., Hewinson, R.G., Chambers, M.A., 2011. Protection of Eurasian badgers (*Meles meles*) from tuberculosis after intra-muscular vaccination with different doses of BCG. Vaccine 29, 3782–3790.

Murphy, D., Costello, E., Aldwell, F.E., Lesellier, S., Chambers, M.A., Fitzsimons, T., Corner, L.A., Gormley, E., 2014. Oral vaccination of badgers (*Meles meles*) against tuberculosis: comparison of the protection generated by BCG vaccine strains Pasteur and Danish. Vet. J. 200, 362–367.

Ni Bhuachalla, D., Corner, L.A., More, S.J., Gormley, E., 2015. The role of badgers in the epidemiology of *Mycobacterium bovis* infection (tuberculosis) in cattle in the United Kingdom and the Republic of Ireland: current perspectives on control strategies. Vet. Med. (Auckl) 6, 27–38.

Ritacco, V., Lopez, B., De Kantor, I.N., Barrera, L., Errico, F., Nader, A., 1991. Reciprocal cellular and humoral immune responses in bovine tuberculosis. Res. Vet. Sci. 50, 365–367.

Southey, A., Sleeman, D.P., Lloyd, K., Dalley, D., Chambers, M.A., Hewinson, R.G., Gormley, E., 2001. Immunological responses of Eurasian badgers (*Meles meles*) vaccinated with *Mycobacterium bovis* BCG (bacillus calmette guerin). Vet. Immunol. Immunopathol. 79, 197–207.

Stefanova, T., Chouchkova, M., Hinds, J., Butcher, P.D., Inwald, J., Dale, J., Palmer, S., Hewinson, R.G., Gordon, S.V., 2003. Genetic composition of *Mycobacterium bovis* BCG substrain Sofia. J. Clin. Microbiol. 41, 5349.

Waters, W.R., Palmer, M.V., Thacker, T.C., Bannantine, J.P., Vordermeier, H.M., Hewinson, R.G., Greenwald, R., Esfandiari, J., McNair, J., Pollock, J.M., Andersen, P., Lyashchenko, K.P., 2006. Early antibody responses to experimental *Mycobacterium bovis* infection of cattle. Clin. Vacc. Immunol. 13, 648–654.

Waters, W.R., Buddle, B.M., Vordermeier, H.M., Gormley, E., Palmer, M.V., Thacker, T. C., Bannantine, J.P., Stabel, J.R., Linscott, R., Martel, E., Milian, F., Foshaug, W., Lawrence, J.C., 2011. Development and evaluation of an enzyme-linked immunosorbent assay for use in the detection of bovine tuberculosis in cattle. Clin. Vacc. Immunol. 18, 1882–1888.

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

Wiker, H.G., 2009. MPB70 and MPB83–major antigens of *Mycobacterium bovis*. Scand. J. Immunol. 69, 492–499.