Viral Booster Vaccines Improve *Mycobacterium bovis* BCG-Induced Protection against Bovine Tuberculosis[▽]

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Previous work with small-animal laboratory models of tuberculosis has shown that vaccination strategies based on heterologous prime-boost protocols using *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) to prime and modified vaccinia virus Ankara strain (MVA85A) or recombinant attenuated adenoviruses (Ad85A) expressing the mycobacterial antigen Ag85A to boost may increase the protective efficacy of BCG. Here we report the first efficacy data on using these vaccines in cattle, a natural target species of tuberculous infection. Protection was determined by measuring development of disease as an end point after *M. bovis* challenge. Either Ad85A or MVA85A boosting resulted in protection superior to that given by BCG alone: boosting BCG with MVA85A or Ad85A induced significant reduction in pathology in four/eight parameters assessed, while BCG vaccination alone did so in only one parameter studied. Protection was particularly evident in the lungs of vaccinated animals (median lung scores for naïve and BCG-, BCG/MVA85A-, and BCG/Ad85A-vaccinated animals were 10.5, 5, 2.5, and 0, respectively). The bacterial loads in lymph node tissues were also reduced after viral boosting of BCG-vaccinated calves compared to those in BCG-only-vaccinated animals. Analysis of vaccine-induced immunity identified memory responses measured by cultured enzyme-linked immunospot assay as well as in vitro interleukin-17 production as predictors of vaccination success, as both responses, measured before challenge, correlated positively with the degree of protection. Therefore, this study provides evidence of improved protection against tuberculosis by viral booster vaccination in a natural target species and has prioritized potential correlates of vaccine efficacy for further evaluation. These findings also have implications for human tuberculosis vaccine development.

More than 50 million cattle are infected with *Mycobacterium bovis*, resulting in economic losses of approximately $3 billion annually (34). Over the last two decades, in Great Britain, failure of the (tuberculin) test-and-slaughter strategy has resulted in a dramatic rise in the incidence of tuberculosis (TB) in cattle (19). The urgent need for new and improved cattle vaccines and diagnostic reagents has been acknowledged by the British government, and development of a cattle vaccine is a research priority. As cattle can be considered a large-animal model for human TB vaccination, experiments with cattle will also be of direct interest to human TB vaccine development programs (15).

M. bovis bacillus Calmette-Guérin (BCG) is associated with variable efficacy both in humans and in cattle, and a major research effort has been directed at improving both the efficacy and reliability of BCG (10, 15). Heterologous prime-boost strategies based on the combination of BCG with either DNA vaccines, proteins, or live attenuated viruses have been developed to improve the efficacy of BCG vaccination against TB (9,

22, 23, 33, 43). In particular, boosting with recombinant attenuated viruses such as modified vaccinia virus Ankara (MVA) expressing the mycobacterial protective antigen Ag85A (Rv3804c), designated MVA85A, in conjunction with BCG priming has shown promise in animal models (13, 22, 46, 47). Its immunogenicity and safety in humans have been documented in both United Kingdom and African volunteers, and this vaccine is now being tested in extended phase II clinical trials in Africa (2, 14, 24).

Another promising viral vaccine vector system is based on recombinant adenoviruses. Such adenovirus vaccines are good inducers of TH1 immunity and have been shown to induce robust levels of antigen release important for long-term memory formation. Further, because they can be constructed to be replication deficient, viral infection is self limited (48). Recently, such a replication-deficient recombinant virus based on adenovirus type 5 (Ad5) and also expressing Ag85A (Ad85A) has been developed and extensively evaluated (31, 40). When administered systemically or mucosally to vaccinate mice, it imparted significant levels of protection against pulmonary *M. tuberculosis* infection (31, 40). In previous studies of cattle, we have demonstrated that both MVA85A (39) and Ad85A (38) induced strong cellular immunity and, in particular, were able to significantly and strongly boost in vitro gamma interferon (IFN-γ) responses to Ag85A in animals primed with BCG (38,

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39). Based on these promising results, we investigated if these recombinant viral vaccines, when used to boost BCG-induced immunity, were able to improve the protection imparted by BCG against *M. bovis* infection in this natural target species of TB. The results of this study demonstrated that both viral vectors were able to markedly enhance BCG-induced protection. Furthermore, analysis of vaccine-induced immune responses enabled us to identify memory responses measured by cultured enzyme-linked immunospot (ELISPOT) assay as well as strong interleukin-17 (IL-17) responses in vitro as promising predictors of vaccination success.

MATERIALS AND METHODS

Recombinant viruses. Recombinant MVA85A was prepared as described earlier (21). The recombinant adenovirus expressing Ag85A (Ad85A) used in this study was prepared as described by Wang et al. (40). For vaccinations, MVA85A and Ad85A were diluted in phosphate-buffered saline (PBS).

Vaccination and infection schedules. Animal experiments were undertaken under a license granted by the United Kingdom Home Office that was obtained after approval by the local ethical review committee. Four groups of 10 calves each (ca.-6-month-old Friesians obtained from herds free of bovine TB and selected on the basis of absence of in vitro IFN-γ responses to bovine and avian tuberculin) were subjected to the following immunization schedules: (i) BCG SSI (Staten Serum Institute, Copenhagen, Denmark) at week 0 (abbreviated as BCG in text and illustrations); (ii) BCG at week 0, followed by Ad85A at week 8 ("BA" in text and illustrations); (iii) BCG at week 0, followed by MVA85A at week 8 ("BM" in text and illustrations); (iv) no vaccination (controls; "C" in illustrations). Injections, doses, and routes were as follows: BCG, subcutaneous injection in the side of the neck of 10^6 CFU suspended in 1 ml of PBS; Ad85A, intradermal injection in the side of the neck, 2×10^9 PFU suspended in 0.5 ml PBS; MVA85A, intradermal injection in the side of the neck, 10^9 PFU. Fourteen weeks after the priming vaccinations, animals were infected with an *M. bovis* field strain from Great Britain (AF 2122/97) by intratracheal instillation of circa 2×10^3 CFU as described previously (8, 37). At week 27, single intradermal comparative cervical tuberculin tests were performed as specified in the EEC Directive 80/219EEC, amending Directive 64/422/EEC, Annex B. The infection and protection statuses of the animals were established at postmortem and microbiological examinations (see below) performed 28 weeks postinfection.

Postmortem examination. At week 28, the calves were euthanized and postmortem examinations were performed as described by Vordermeier et al. (37). The personnel performing the postmortem examinations and scoring tissues were blinded to the vaccination statuses of the animals under examination. Lungs were first examined externally for lesions and then sliced into 0.5- to 1-cm-thick slices, which were individually examined for lesions. In addition, lymph nodes of the head and pulmonary regions were removed and weighed. Tissues were sliced into thin sections (1 to 2 mm thick) and examined for the presence of visible lesions. Tissue samples, removed from the central parts of the lymph nodes, were taken for *M. bovis* culture and for histopathological examination (Ziehl-Neelsen staining for acid-fast bacilli and hematoxylin-eosin staining). The samples taken for culture were weighed to allow an estimation of the bacterial burden per lymph node. These samples were homogenized and plated onto 7H10 agar plates (see below). The severity of the gross pathological changes was scored by using the systems described below (20, 37).

(i) Lungs. Lung lobes (left apical, left cardiac, left diaphragmatic, right apical, right cardiac, right diaphragmatic, and right accessory) were examined individually. For each lobe, the following scoring system was applied: 0, no visible lesions; 1, no gross lesions but lesions apparent upon slicing; 2, <5 gross lesions with diameters of <10 mm; 3, >6 gross lesions with diameters of <10 mm or a single distinct gross lesion with a diameter of >10 mm; 4, >1 distinct gross lesion with diameters of >10 mm; 5, gross coalescing lesions. The scores of the individual lobes were added up to calculate the lung score.

(ii) Lymph nodes. The severity of the observed gross pathology in individual lymph nodes was scored using the following system: 0, no necrosis or visible lesions; 1, small focus (1 to 2 mm in diameter); 2, several small foci or a necrotic area of at least 5 by 5 mm; 3, multiple necrotic areas of at least 5 by 5 mm distributed throughout the node or one necrotic area affecting >5% of the node. Individual lymph node scores were added up to calculate the lymph node score. Both lymph node and lung pathology scores were added to determine the total pathology score per animal. All scoring was performed by the same operator for all animals to ensure scoring consistency.

Bacterial enumeration. Tissue sections were collected randomly postmortem from different parts of the lymph nodes and were individually homogenized in 5 ml of sterile distilled water by using a stomacher macerator system. Both weight of lymph nodes and weight of tissue sections were recorded, which allowed the calculation of bacterial loads/g lymph node (CFU/g). Results from all lymph nodes were thus determined and combined as bacterial loads/animal (CFU/g total pulmonary lymph node tissue). Viable counts were performed on serial dilutions of the macerate in water containing 0.05% (vol/vol) Tween 80 to maintain dispersion. Suspensions were plated onto 7H10 agar containing sodium pyruvate (4.16 mg/ml) and 10% (vol/vol) Middlebrook OADC (oleic acid, albumin, dextrose, catalase) enrichment. The detection limit was 90 CFU/g lymph node tissue.

Antigens. Bovine (purified protein derivative B [PPD-B]) and avian (PPD-A) tuberculin were obtained from the Tuberculin Production Unit at the Veterinary Laboratories Agency-Weybridge and used in culture at 10 μg/ml. Recombinant Ag85A (rAg85A) was obtained from Lionex Ltd. (Braunschweig, Germany) and used at 5 or 10 μg/ml. Staphylococcal enterotoxin B (Sigma-Aldrich, Poole, United Kingdom) was used as a positive control at 1 μg/ml.

Whole-blood IFN-γ assay (Bovigam assay). Blood samples were collected from vaccinated or *M. bovis*-infected cattle into heparinized Vacutainers (Becton Dickinson, Oxford, United Kingdom). The Bovigam assay was performed according to the manufacturer's instructions (Prionics, Schlieren, Switzerland). Briefly, whole-blood cultures (0.2 ml) were performed in 96-well tissue culture plates. Cultures contained 0.10 ml heparinized blood and 0.1 ml RPMI 1640 (no antigen), tuberculin, or recombinant antigens. After 24 h of culture at 37°C, plasma supernatants (100 μl/well) were transferred to a 96-well plate by using a sterile pipette and stored at −20°C until tested by IFN-γ enzyme-linked immunosorbent assay (ELISA). Bovigam IFN-γ ELISA was performed according to the supplier's instructions. Color change in the ELISA reaction was measured as optical density at 450 nm (OD_{450}) (37).

IFN-γ ELISPOT assay and cultured ELISPOT analysis. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Histopaque-1077 (Sigma-Aldrich) gradient centrifugation and cultured in tissue culture medium (RPMI 1640 [Sigma-Aldrich] supplemented with 10% fetal calf serum [Sigma-Aldrich], nonessential amino acids [Sigma-Aldrich], 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate). Cultured ELISPOT assays were performed as follows (38, 45). PBMC were stimulated in 24-well plates (2×10^6 PBMC/ml, 1-ml aliquots) with rAg85 protein at 5 μg/ml. PBMC cultures were fed on days 5 and 8 with recombinant human IL-2 (to a final concentration of 10 U/ml) (Sigma). On days 10 and 12, half of the supernatant was removed and replaced with tissue culture medium (see above) without IL-2. Cells were washed four times on day 13 by centrifugation and counted, and 1×10^4 to 2×10^4 cells were added to wells of ELISPOT plates and cultured together with a cocktail of immunodominant Ag85A peptides (for details of peptides used, see references 38 and 39) for 24 h. Prior to the addition of cells, ELISPOT plates (Immobilon-P polyvinylidene difluoride membranes; Millipore, Molsheim, France) were prepared by coating them overnight at 4°C with a bovine IFN-γ specific monoclonal antibody (Mabtech, Stockholm, Sweden). Unbound antibody was then removed by washing, and the wells were blocked with 10% fetal calf serum in RPMI 1640 medium. After 24 h of incubation in the presence of cells, spots were developed with a biotinylated bovine IFN-γ-specific monoclonal antibody (Mabtech) followed by alkaline phosphatase-conjugated streptavidin (Mabtech). The spots were visualized with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium substrate (Sigma-Aldrich).

Cytokine quantitative RT-PCR. For cytokine quantitative reverse transcriptase PCR (RT-PCR) (28, 29, 35, 42), PBMC were resuspended at 10^6/ml in tissue culture medium (see above for details) and incubated overnight in the presence or absence of rAg85A or PPD-B (all at 10 μg/ml) in 1-ml volumes in 48-well tissue culture plates (Life Technologies, United Kingdom). On the following day, the plates were centrifuged (300 × g, 10 min at room temperature) and the supernatant was removed. Three hundred fifty microliters/well lysis buffer (RLT [Qiagen], supplemented with 10 μl/ml 2-mercaptoethanol) was then added, and the cell lysates were stored at −80°C.

RNA was prepared from PBMC lysates by using the MagNApure LC RNA isolation kit (high performance) and the MagNApure extraction robot (Roche Diagnostics GmbH, Germany). RNA preparations were finally treated with Turbo DNase (Ambion [Europe] Ltd., Huntingdon, United Kingdom) for 30 min at 37°C.

PCR of the RNA samples was carried out using the Quantifast Sybr-Green RT-PCR kit (Qiagen) in 96-well PCR plates (ABgene Thermofast plates and ABgene ultraclear cap strips) on a Stratagene MX3000P PCR machine. Cycling conditions of an initial activation step of 10 min at 95°C followed by 45 cycles of

95°C for 15 s and 60°C for 60 s were employed. Data were analyzed using Stratagene MxPro version 3. Cytokine primers (IFN-γ, IL-4, tumor necrosis factor alpha [TNF-α], IL-10, IL-17, FoxP3, and glyceraldehyde-3-phosphate dehydrogenase [1–4]) were manufactured by MWG-Biotech (Ebersberg, Germany). Each RNA sample was run in duplicate and against an RT control (no RT enzyme), and primer-only controls were included on every plate. Efficiency curves for all primers were checked and were comparable. PCR products were checked using melt curves and by running samples on 3% agarose gels. Increases in antigen-stimulated cytokine RNA expression were calculated for each animal based upon unstimulated PBMC cytokine expression and glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis. Statistical analysis was performed using Instat v3.0a (GraphPad, San Diego, CA). Differences in the degree of pathology were compared by employing the Kruskal-Wallis test with Dunn's multiple comparison test (pathology scores), analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test (numbers of infected lymph nodes and lung lobes), or Fisher's exact test (proportion of animals with lesions). Bacterial loads were compared by ANOVA and Tukey-Kramer multiple comparison test on log_{10}-transformed CFU data. Correlations between cultured ELISPOT assay responses and the degree of pathology or bacterial load were assessed by linear regression analysis. IFN-γ ELISA results were compared by ANOVA and Tukey-Kramer multiple comparison test. Cytokine RT-PCR and cultured ELISPOT assay results between vaccinated/protected (vaccine success) animals and controls and vaccinated/unprotected (vaccine failure) animals were assessed by using the t test with Welch's correction for unequal variances.

RESULTS

IFN-γ responses after vaccination and challenge. Four groups of 10 calves each were vaccinated on the following schedules: BCG, BCG followed by MVA85A (BM), BCG followed by Ad85A (BA), or no vaccination. PPD-B- and Ag85A-induced in vitro IFN-γ responses were determined regularly, and the results are shown in Fig. 1A (PPD-B) and B (Ag85A). Following BCG vaccination, significant PPD-B-specific IFN-γ responses were induced in whole-blood assays as early as week 2 after BCG priming; this response was maintained at a significantly elevated level until week 8 ($P < 0.05$ at weeks 2 to 8 compared to unvaccinated controls). As no statistically significant differences in the responses between the three vaccinated groups were observed, we present in Fig. 1 the combined responses of all 30 BCG-vaccinated animals until viral boosting at week 8. Viral boosting at week 8 resulted in a sharp and significant increase in responses to PPD-B in the BM and BA groups at week 9 ($P < 0.05$ compared to control group and BCG vaccination), which then sharply declined in these two groups in the following weeks, although they remained significantly higher than responses in control and BCG groups ($P < 0.05$) until challenge at week 14. Following *M. bovis* challenge at week 14, a rapid increase in PPD-B-specific responses could be observed in all four groups, which were maintained at high levels throughout the rest of the experiment (Fig. 1A). Apart from significantly greater responses in the BA group than in the control group at week 16 ($P < 0.05$), no other significant differences were observed in the postchallenge PPD-B responses between groups and sampling time points.

Figure 1B depicts responses observed after in vitro stimulation with Ag85A protein, which is the antigen expressed by the viral subunit vaccines. Low responses to Ag85A were induced after BCG vaccination, with the strongest responses observed 2 weeks post-BCG vaccination. However, following administration of the viral boost at week 8, strong and consistent boosting responses were observed 1 week after boosting (week 9) in both Ad85A- and MVA85A-boosted animals (Fig. 1B),
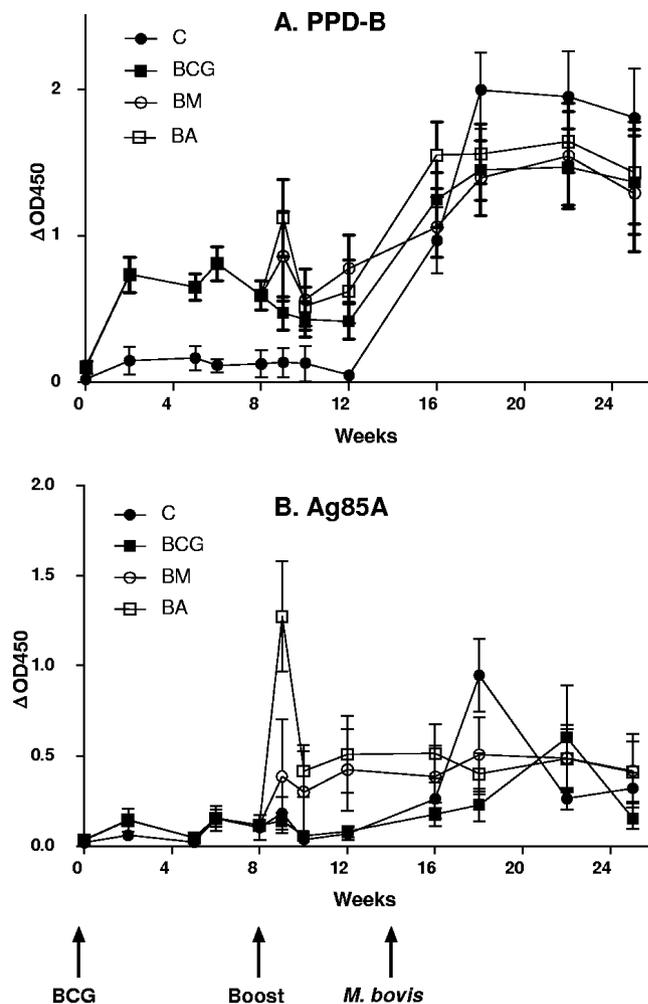

FIG. 1. In vitro IFN-γ production following vaccination and after challenge. (A) Responses following PPD-B stimulation of whole-blood cultures. (B) Responses following Ag85A stimulation. Arrows indicate times of vaccination with BCG, boosting with MVA85A and Ad85A (Boost), and infection with *M. bovis*. Results are expressed as mean $OD_{450} \pm$ standard error of the mean/group with medium-only values subtracted (ΔOD_{450}). C, unvaccinated controls; BA, BCG-Ad85A group; BM, BCG-MVA85A group.

thus confirming the observations made after stimulation with tuberculin (Fig. 1A). Although not reaching statistical significance at the $P = 0.05$ level, the boosting effect of Ad85A was considerably stronger than that observed after MVA85A vaccination (1.155 ± 0.286 OD_{450} units compared to 0.439 ± 0.294 OD_{450} units), and responses to Ag85A in these two groups were significantly stronger than responses in control and BCG groups at week 9 ($P < 0.05$). As expected from the PPD-B responses, Ag85A IFN-γ levels in the BA and BM groups decreased from week 10 onwards relative to the levels at week 9; nevertheless, they remained elevated compared with those in BCG and control groups until challenge at week 14 (Fig. 1B). Following *M. bovis* infection at week 14, Ag85A-specific IFN-γ levels remained high in the BA and BM groups throughout the remainder of the experiment. Interestingly, infection boosted Ag85A responses in BCG-vaccinated calves slowly, with a peak reached at week 22, while IFN-γ levels

TABLE 1. Visible pathology observed in vaccinated and control cattle[d]

Group	No. of animals with VL/total no. of animals[a]	No. of animals with VL in LN/total no. of animals[a]	Mean no. of LN with VL (95% CI)[b]	Median LN score (range)[c]	No. of animals with VL in lungs/total no. of animals	Mean no. of lobes with VL (95% CI)[b]	Median lung score (range)[c]	Median total score (range)[c]
Control	10/10	9/10	2.6 (1.2, 3.9)	7.5 (0–14)	10/10	2.9 (1.6, 4.2)	10.5 (5–35)	19.5 (5–35)
BCG	9/10	7/10	1.4 (0.6, 2.4)	2.5 (0–9)	7/10	1.2 (0.5, 1.9)*	5 (0–10)	6 (0–18)
BM	6/10	6/10	1.5 (0.4, 2.6)	3.5 (0–11)	5/10*	0.7 (0.1, 1.3)***	2.5 (0–9)**	7.5 (0–16)*
BA	6/10	6/10	1.2 (0.2, 2.2)	2 (0–12)	4/10*	0.6 (0, 1.2)***	0 (0–7)***	3 (0–17)**

[a] Fisher's exact test.
[b] ANOVA with Tukey-Kramer multiple comparison test.
[c] Kruskal-Wallis test with Dunn's multiple comparison test.
[d] Shown are results of pathological changes found at postmortem examination performed 14 weeks postchallenge. Lung, lymph node, and total pathology scores were established using the scoring system described by Vordermeier et al. (37), as modified by Lyashchenko et al. (20). LN, lymph node(s); VL, visible lesions; CI, confidence interval. Significance is shown as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

increased quickly from background levels in unvaccinated controls, peaking at week 18 and then gradually declining (Fig. 1B). Responses of the BCG group toward both antigens remained at preboost levels (Fig. 1A and B).

As previously published data demonstrated that heterologous prime-boost approaches with BCG and recombinant viruses induced tuberculin skin test reactivity (4, 38, 39), no skin test was performed before challenge. However, immediately before postmortem examination, all animals were tuberculin skin tested using the comparative intradermal test at week 27. Regardless of vaccination status, all were positive by the tuberculin test (data not shown).

Pathological and microbiological findings after *M. bovis* infection. No adverse reactions were reported after BCG or viral vaccinations. In addition, no patent clinical signs of TB were seen at any time after *M. bovis* challenge, as the model is designed to resemble the disease presentation found in Britain. Fourteen weeks after the intratracheal challenge with virulent *M. bovis*, cattle were killed and postmortem examinations were performed to determine the effects of the various vaccination regimens on the development of gross pathology. Pathological changes in all animals irrespective of vaccination status were restricted to the lymph nodes of the thoracic cavity and lungs. The results of the postmortem examinations were described by measuring eight individual parameters of disease presentation and are tabulated in Table 1, which shows the proportions of animals presenting lung and lymph node lesions and numbers of lymph nodes and lung nodes affected, as well as lymph node, lung, and total pathology scores describing pathology severity. While all three vaccine protocols (BCG, BM, and BA) reduced pathology in the lymph nodes compared to that in control animals as judged by the proportion of animals with lymph node lesions, the numbers of lesioned lymph nodes, and lymph node pathology severity scores, none of these parameters reached statistical significance at the $P < 0.05$ level (Table 1). Further, the three vaccination protocols induced similar levels of protection in the lymph nodes. In contrast, statistically significant protection ($P < 0.05$) was observed when lung parameters were considered (Table 1). BCG vaccination alone reduced the numbers of infected lobes significantly ($P < 0.05$) from 2.9 to 1.2/animal. Both BA and BM reduced these values even further (to 0.6 and 0.7 lobes/animal, respectively), which is reflected by the higher level of statistical significance observed ($P < 0.001$). While this was the only parameter that was

statistically significantly reduced after BCG vaccination, BA and BM vaccination protocols also reduced the proportion of animals with lung lesions ($P < 0.05$) and the disease severity measured by lung scores (median scores: C, 10.5; BCG, 5; BM, 2.5; BA, 0; $P < 0.01$ for BA and BM [Table 1]). In addition, the total pathology scores, which take into account disease severity in lungs and lymph nodes, were also significantly reduced after BA and BM vaccination compared to those for control animals ($P < 0.01$ and 0.05, respectively [Table 1 and Fig. 2]). The total pathology scores were lowest in the BA group relative to BM animals, and yet these differences were not statistically significant. While total pathology scores were also reduced after BCG vaccination, this reduction did not reach statistical significance at the 5% level (Table 1; Fig. 2). Thus, the BA and BM heterologous prime-boosting protocols resulted in statistically significant reduction in pathology in four/eight parameters, while BCG vaccination reduced pathology significantly in only one parameter. Most importantly, while BCG vaccination resulted in only one animal without detectable gross pathology, both BA and BM resulted in eight such animals (four in each group; $P = 0.019$ when BM and BA groups are compared to BCG and control groups). In conclusion, therefore, both BA and BM improved the protective efficacy of BCG, resulting in lower visible pathology and an increased proportion of animals
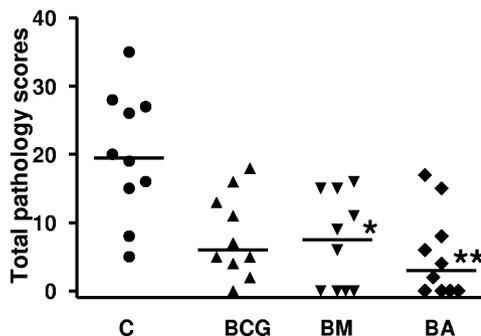

FIG. 2. Protective efficacy as measured by gross pathology. Total pathology scores were calculated as described earlier (20, 37), and scores for individual calves were plotted. Horizontal lines indicate median values. Significance was determined by the Kruskal-Wallis test with Dunn's multiple comparison test: *, $P < 0.05$; **, $P < 0.01$. C, unvaccinated controls; BA, BCG-Ad85A group; BM, BCG-MVA85A group.

TABLE 2. Microbiological evaluation of bacterial loads[a]

Group	Bacterial load, mean log CFU/g (95% CI)	Mean log reduction (95% CI)
Control	3.487 (2.693, 4.280)	NA
BCG	2.930 (2.144, 3.715)	−0.556 (−1.342, 0.229)
BM	2.313 (1.165, 3.461)	−1.173 (−2.321, −0.025)
BA	1.757 (0.701, 2.813)*	−1.729 (−2.785, −0.673)*

[a] *, $P < 0.05$, ANOVA and Tukey-Kramer multiple comparison test. NA, not applicable; CI, confidence interval.

without visible lesions, with BA apparently providing the best overall level of protection.

Lymph node tissue samples taken during the postmortem examination were processed and cultured for the presence of *M. bovis*. The results in Table 2 detail the bacterial load/animal expressed as $\log_{10}$ CFU/g lymph node tissue. All vaccination schedules (BCG, BA, and BM) resulted in a reduction of bacterial loads, with both viral vector boosting protocols resulting in lower bacillary loads than those with BCG vaccination (Table 2, $\log_{10}$ CFU reduction: B, −0.556; BM, −1.173; BA, −1.729), although only the bacillary counts found in the BA group were statistically significantly lower than those in the control group CFU (ANOVA, $P < 0.05$, Table 2). Therefore, the overall differences in bacterial burden are in agreement with the relative levels of protective efficacy (Tables 1 and 2). As observed in previous publications (for example, references 3, 4, 7, and 37), bacteria could be isolated from a proportion of animals (three/nine) that displayed no visible pathology.

Memory responses as measured by cultured ELISPOT assays are predictors of vaccine efficacy. To define potential

predictors of vaccine efficacy, we investigated a subset of five randomly selected animals per treatment group in greater detail. As we had recently shown that memory responses measured by the cultured ELISPOT system correlated with vaccine efficacy after vaccination of cattle with BCG or an attenuated *M. bovis* vaccine (41), we used this assay in the first set of experiments to determine if a similar positive relationship with protection and cultured ELISPOT assay responses could be found in this experiment. Blood samples were drawn from these animals before *M. bovis* challenge (at week 14 of the experiment), PBMC were prepared, and cultured IFN-γ ELISPOT assays were performed to establish if the level of long-lived memory cells correlated with protection. Cultured ELISPOT assay responses thus determined were correlated with bacterial load or pathology scores. Figure 3A represents the linear regression analysis of cultured ELISPOT assay responses and bacterial loads in tissues. We demonstrated a significant direct inverse linear relationship between the mean cultured ELISPOT assay responses/group and bacterial load ($r^2 = 0.987$, $P = 0.007$), i.e., the number of cultured SFC directly correlated with protection. This was in contrast to ex vivo effector ELISPOT assay responses, which at the same time point were low and did not correlate significantly with bacterial loads ($P = 0.299$ [data not shown]). When protection was described as reduction in total pathology scores, we also found a significant correlation between cultured ELISPOT assay responses and vaccine-induced reduction in pathology (Fig. 3B, $r^2 = 0.917$, $P = 0.042$).

Cross-sectional analysis of memory and cytokine responses. Having established that cultured ELISPOT assay responses
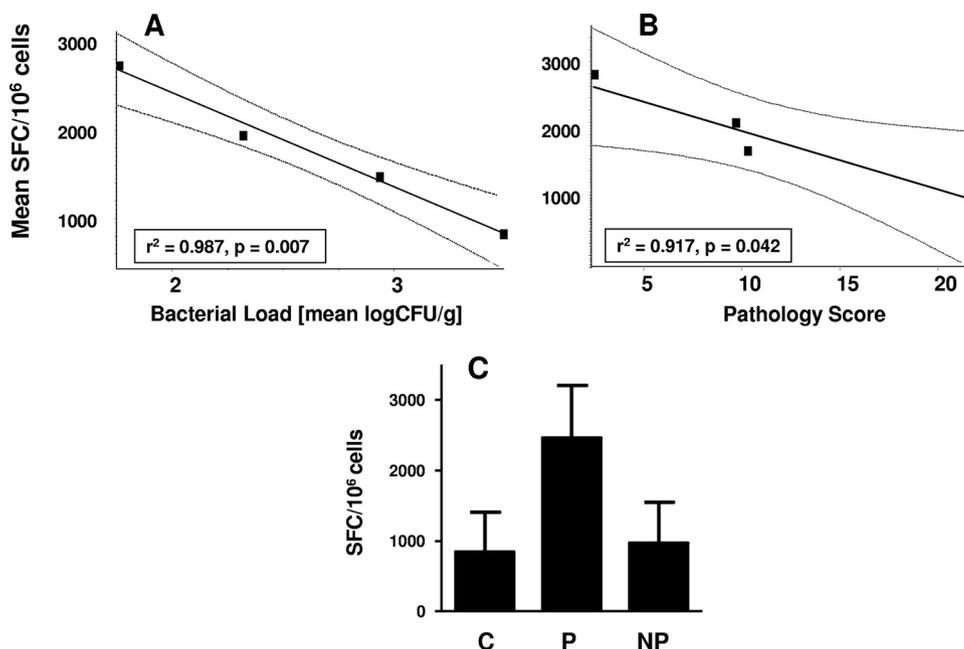

FIG. 3. Correlation of memory responses with vaccination outcome. Memory cell responses were determined by cultured IFN-γ ELISPOT assay; results of ELISPOT analysis are expressed as mean spot-forming cells (SFC)/million cells. Cultured ELISPOT assays were performed before *M. bovis* infection at week 14 postvaccination, and bacterial loads were determined from tissues collected at postmortem examinations (week 28 postvaccination). (A) Correlation of cultured ELISPOT assay responses with bacterial loads. (B) Correlation of cultured ELISPOT assay responses with pathology scores. Solid lines in panels A and B indicate linear regression; dashed lines indicate 95% confidence intervals. (C) Cultured ELISPOT assay responses in relation to vaccine success (P, vaccinated/protected) or failure (NP, vaccinated/not protected). C, unvaccinated controls.
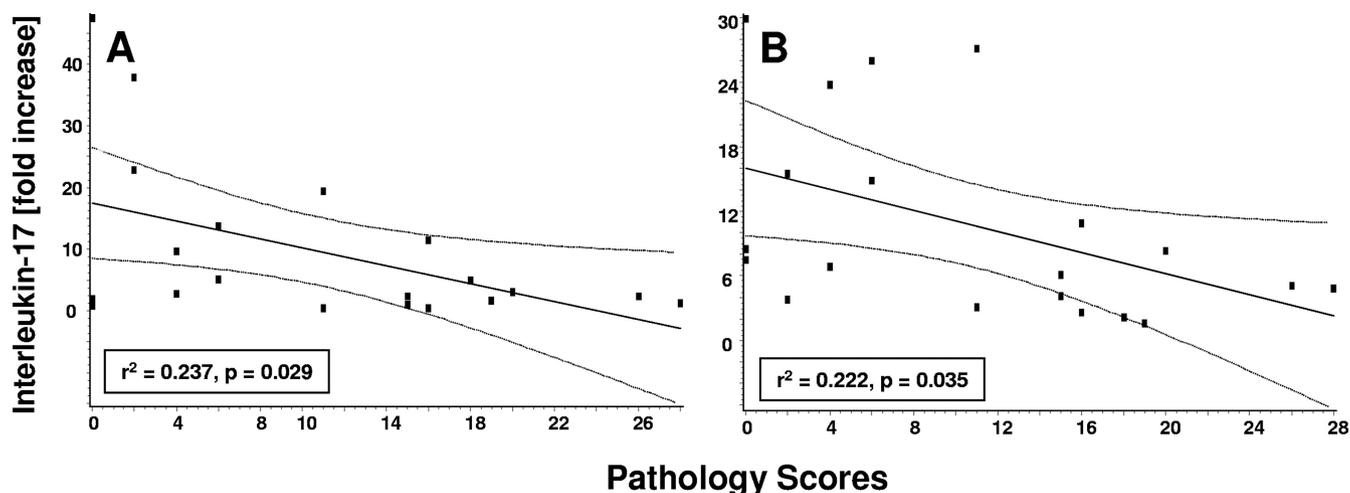
FIG. 4. Association of IL-17 expression in vitro after Ag85A stimulation prior to *M. bovis* challenge (A) (week 10) and following challenge (B) (week 27) stimulation postvaccination (week 10) and postinfection (week 27). Results are expressed as mean increases in IL-17 expression of individual calves in relation to the corresponding pathology scores. Results of linear regression analysis are indicated. Solid lines indicate linear regression; dashed lines indicate the 95% confidence intervals.

measured at the time of challenge correlated with protection when pathology or bacterial loads were compared between different treatment groups, we next set out to determine whether we could gain insights into the differences of immune responses between individual calves in relation to their individual protection status. The results shown in Table 1 and Fig. 2 indicated that vaccinated animals across the three vaccine groups (BCG, BA, and BM) can be grouped into calves that were protected, i.e., had no visible pathology or had reduced pathology compared to unvaccinated controls, or vaccinated animals that were not protected, i.e., presented with pathology scores akin to those of unvaccinated animals. We therefore performed a cross-sectional study by dividing the subgroups of 20 animals described in the previous paragraph into unvaccinated control animals and either vaccinated/protected animals ("vaccine success") or vaccinated/unprotected animals ("vaccine failure"). The division into these two groups was based on the total pathology scores observed in the same subset of unvaccinated control calves that were assessed in the previous section, with protection defined as animals with scores below the minimum score of 15 found in these five control animals (median, 20; minimum, 15; maximum, 28). Applying these criteria, 11 animals were defined as protected with pathology scores in this subset of animals equal to or lower than 11 (median total pathology score, 4; range, 0 to 11), and four were defined as not protected with scores of >15 (median pathology score, 16; range, 15 to 18).

First, we assessed cultured ELISPOT assay responses of protected and unprotected calves. Confirming data presented in the previous section on the correlation between ELISPOT assay responses and protection in animals within groups, we were able to show that the vaccinated/protected animals presented with higher ELISPOT assay responses after vaccination than those of the vaccinated/unprotected calves whose responses were akin to the responses of unvaccinated controls (Fig. 3C).

We next extended our analysis to other immunological pa-

rameters and examined the expression of a range of cytokines characteristic of different T-cell populations by quantitative RT-PCR (IL-4, IL-10, TNF-α, and IL-17) as well as FoxP3. IFN-γ expression was also determined by RT-PCR and compared to IFN-γ ELISA results to validate the RT-PCR analysis. In these experiments we used the same animals as those described in the previous sections for cultured ELISPOT assay analysis. Blood samples were tested after vaccination but before challenge (week 10) and compared with results obtained from samples taken before postmortem examinations at week 27. PBMC were stimulated with Ag85A protein and PPD-B, and results for IL-17 expression are shown in Fig. 4 in relation to the pathology found postmortem. Both before infection (week 10 [Fig. 4A]) and at the end of the experiment 13 weeks after challenge (week 27 [Fig. 4B]), we found a weak yet statistically significant negative correlation ($P = 0.029$ and 0.035, respectively) with in vitro IL-17 expression after Ag85A stimulation and disease severity (pathology scores), although no such correlation was found after PPD-B stimulation (data not shown). Extending our analysis, these animals were next grouped as described above into groups where vaccination was successful (vaccinated/protected) or where it failed (vaccinated/unprotected). Interestingly, the Ag85A- and PPD-B-induced IL-17 expression was higher after vaccination (week 10) in vaccinated/protected animals than in unprotected and unvaccinated controls (Fig. 5A and B, $P < 0.05$), an effect mirrored by IFN-γ responses (Fig. 5C and D, $P < 0.01$ and 0.05, respectively). Thus, both high IL-17 and IFN-γ responses in vaccinated animals prior to challenge predicted the outcome of subsequent challenge. Further, Ag85A-induced in vitro IL-17 responses postchallenge, measured immediately prior to postmortem examination at week 27, remained high in vaccinated/protected animals compared to responses in animals where vaccination failed or in animals that were not vaccinated (Fig. 5A, $P < 0.01$). This was not the case for IL-17 responses to the complex antigen mixture PPD-B, which at this time point were comparable at this time among all three groups of
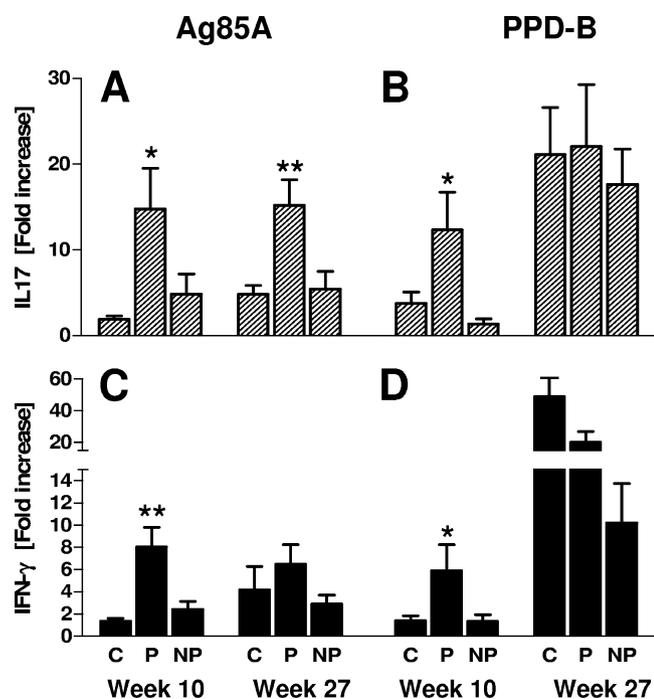
FIG. 5. IL-17 (A and B) and IFN-γ (C and D) expression in vitro after Ag85A (A and C) and PPD-B (B and D) stimulation postvaccination (week 10) and postinfection (week 27). Results are expressed as mean increases (±standard errors of the means) in expression with data normalized to the mean expression in unvaccinated and unchallenged animals. C, unvaccinated controls; P, vaccinated/protected calves; NP, vaccinated/nonprotected calves. *, $P < 0.05$, and **, $P < 0.01$, compared to C and NP groups (t test with Welch correction).

animals (Fig. 5B). IFN-γ responses postchallenge, whether to Ag85A or to PPD-B, were comparable among the three groups regardless of protection status (Fig. 5C and D) and could not be used as a prognostic marker for vaccination outcome. The pattern of IFN-γ responses established by PCR mirrored closely ELISA responses measured at the same time points and thus was consistent with the PCR expression analysis (for example, Ag85A responses measured by ELISA at week 10 were as follows: mean OD_{450}s for control, vaccinated/protected, and vaccinated/unprotected animals were 0.065, 0.461, and 0.177, respectively).

In addition, although not statistically significant ($P < 0.1$), there was a trend for in vitro Ag85A-specific TNF-α responses to be slightly up-modulated in the vaccinated/protected animals compared to vaccinated/nonprotected animals at both week 10 and week 27 (data not shown). IL-10, IL-4, or FoxP3 responses were not different between the groups of animals (data not shown).

DISCUSSION

In this paper we demonstrate that boosting BCG with MVA85A or Ad85A enhanced protection against bovine TB compared to that given by BCG vaccination alone. While this has been demonstrated in some animal models of TB before (13, 22, 31, 40, 46), this is the first report to confirm these preclinical findings in a natural target species of TB infection.

The design of the present study of cattle was based on recent experiments from our laboratory where we reported that boosting BCG with MVA85A or Ad85A resulted in stronger peak ex vivo IFN-γ responses, a wider Ag85A-specific epitope repertoire (39), and stronger memory responses characterized by cultured ELISPOT assays (38). As in the current study, in these previous studies we observed that ex vivo responses specific for Ag85A were boosted rapidly following viral vaccination with a sharp peak observable between 1 and 2 weeks postboost. These peaks of effector ex vivo responses then declined quickly but remained higher than prevaccination levels. Interestingly, high numbers of cultured ELISPOT cells were maintained up to the experimental *M. bovis* infection (see below for further discussion). Since human phase I and II clinical trials have confirmed the safety and immunogenicity of MVA85A (2, 14, 24); Ad85A is about to enter a phase I human trial (Z. Xing, personal communication); and another adenovirus vector, Ad35 encoding Ag85A, is in human trials at the moment, our data have considerable relevance not only for cattle TB vaccination but also for human vaccine development. Cattle should be considered a useful additional preclinical model to test human TB vaccines. The advantages of the bovine model include the ability to utilize large group sizes of age-, sex-, and breed-matched individuals; the ability to conduct experiments in neonates; lower financial costs; and fewer ethical considerations than with primate models (5, 15).

Preexisting antivector antibody responses could negatively impact on vaccine efficacy, and one advantage of using recombinant viral vectors in cattle based on human viruses could be that it is less likely that antivector specific preexisting immunity exists in animals. For example, preexisting antibody prevalence in cattle of antibodies against human Ad5, the type of adenovirus used in our study, was evaluated in the study by Gogev et al. (12), who found a very low prevalence and concluded that the efficacy of vaccination using recombinant human Ad5 is unlikely to be dampened by preexisting neutralizing antibodies. Indeed, human Ad5 has been used to successfully deliver, for example, foot-and-mouth disease virus antigens to cattle (25, 27).

Both BCG and viral booster vaccinations were given in this study via parenteral (subcutaneous and intradermal) routes. Recent data for mice suggested that MVA85A, when given intranasally, could induce protection comparable to that induced by intradermal vaccination (13), while Ad85A delivered by the intranasal route increased protection remarkably compared to that given by intramuscular delivery (31, 40). The difference in the efficacy of parenteral boosting between the murine and bovine models highlights the importance of evaluating vaccine candidates in multiple species. Parenteral boosting of BCG-primed guinea pigs with Ad85A also significantly improved their survival after *M. tuberculosis* challenge (Z. Xing, personal communication). Notwithstanding, we have shown in a previous study that intranasal vaccination with Ad85A in cattle is effective in boosting systemic BCG vaccination (38), and it will be of interest in future experiments to determine if this and other mucosal routes will further improve the protection imparted by heterologous prime-boost scenarios based on vaccination with BCG and viral vectors.

By comparing responses between groups and by cross-sectional analysis of responses from those animals in which vaccination was successful (vaccinated/protected) compared to

animals in which vaccination failed (vaccinated/unprotected), we were able to search for potential predictors of protection. Such predictors or surrogates of protection would greatly facilitate vaccine development as they would allow the estimation of vaccine efficacy without the need to infect animals with *M. bovis*.

In this report, as in a previous one (41), we found that responses measured by cultured ELISPOT assays are potential predictors of vaccine success. Correlations of protection with memory responses measured by this method have been described in humans, for example, for malaria vaccine (16). In contrast to ex vivo ELISA or ELISPOT assay responses, which measure effector T-cell responses, cultured ELISPOT analysis probes memory, and in particular, central memory T-cell responses in humans (11) characterized by expression of CCR7, thus allowing circulation through peripheral lymphatic organs, although one cannot formally rule out that proliferating effector cells can also contribute to this response. In this study, we have not phenotyped these memory populations, a task which will be done in follow-up analysis once more bovine-specific reagents like CCR7-specific antibodies become available. However, preliminary results in our laboratory suggest that both $CD4^+$ $CD45RO^+$ $CD62L$ high- and low-expressing cells contribute to these memory responses and that therefore both effector and central memory responses are likely to be assessed by cultured ELISPOT analysis in cattle (H. M. Vordermeier and S. G. Rhodes, unpublished data).

Comparison of immune responses in protected and unprotected animals also confirmed that IFN-γ induction postvaccination is correlated with vaccine success (reviewed in references 6 and 36) and that antigen-specific IFN-γ induction is mirrored by antigen-specific IL-17 production, which therefore is also associated with protective immunity. Interestingly, vaccine antigen-induced IL-17 is also a prognostic marker of vaccine success postchallenge, whereas PPD-B or vaccine antigen-specific (Ag85A) IFN-γ is not. This may be because IL-17 responses in our study also correlated inversely with disease severity as defined by pathology scores (Fig. 4). Thus, a biomarker signature that combines high PPD-B and protective antigen-specific (Ag85A) IL-17 and IFN-γ responses prior to challenge and high Ag85A-specific IL-17 responses after challenge could be used to define vaccination success. This analysis also highlights the need to use defined antigens rather than PPD-B to assess vaccine-induced responses postchallenge, as responses to other antigens that are present in PPD-B and were induced after challenge are likely to mask protective immune responses.

Recently, the role of IL-17-producing cells in TB has been studied in an experimental mouse model described by Khader et al. (17). While these studies did not demonstrate a role for IL-17 in primary TB infections, in vaccinated animals the absence of IL-17-producing memory cells resulted in the loss of TH1 responses and protection (17). Thus, TH1 and TH17 responses seem to cross-regulate each other and are both important for protective antituberculous responses (17, 18). In contrast, in another recent study, increased IL-17 production postvaccination in mice vaccinated with BCG and MVA or DNA vaccines expressing Ag85A was associated with decreased protection (30), and the role of IL-17 in the murine system needs to be studied further. However, our results with

this natural target species of TB are consistent with the result of Khader et al. (17). Nevertheless, the IL-17-producing cells in cattle need to be determined, as apart from TH17 cells a range of other cell types have been described as IL-17 producers in humans and murine models. We also observed a minor increase in the expression of TNF-α after Ag85A stimulation both postvaccination and postchallenge in the vaccinated/protected animals compared to that in controls and nonprotected/vaccinated calves. It will be of interest in further studies to establish whether cells producing both IFN-γ and TNF-α (and other cytokines) simultaneously can be induced following vaccination with these viral vaccines, as recent studies have associated vaccine success in small-animal models with the increased presence of so-called polyfunctional T cells that produce, for example, IL-2, IFN-γ, and TNF-α (32). Such cells have also been induced in humans after boosting BCG vaccination with MVA85A (1). In contrast to other cattle studies, we did not find that expression of IL-4 (44), IL-10 (35), or FoxP3 (42), the latter two being potential T-regulatory-cell markers, correlated in this study with either pathology, vaccine success, or vaccine failure. The high-IFN-γ responses to PPD-B and Ag85A observed postchallenge are in contrast to IFN-γ responses to antigens that are not components of the vaccines (viruses or BCG) such as the RD1 region antigens ESAT-6 and CFP-10 (26). Responses to these antigens are lower postchallenge in the protected animals than in control cattle (37). These antigens, reflective of bacterial loads (20), are therefore markers of disease severity and inverse correlates of protection (33, 37). Confirming these earlier reports, ESAT-6 and CFP-10 responses were also lower postchallenge in the vaccinated groups used in this study (data not shown).

In summary, we have demonstrated improved protective efficacy for the first time in a natural host of TB after viral boosting of BCG and find that Ag85A is a protective antigen in cows. Furthermore, we have highlighted potential predictors of vaccine-induced protection based on cultured ELISPOT assay responses and a biosignature of in vitro IL-17 and IFN-γ production. Further studies will establish the optimal boosting dose and route for use of these promising viral vectors.

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ERRATUM

Viral Booster Vaccines Improve *Mycobacterium bovis* BCG-Induced Protection against Bovine Tuberculosis

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