

Goats Primed with *Mycobacterium bovis* BCG and Boosted with a Recombinant Adenovirus Expressing Ag85A Show Enhanced Protection against Tuberculosis

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This is the first efficacy study using the experimental goat model, a natural host of tuberculosis (TB), to evaluate the efficacy of heterologous *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) prime followed by boosting with a replication-deficient adenovirus expressing the antigen Ag85A (AdAg85A). Three experimental groups of 11 goat kids each were used: BCG vaccinated, BCG vaccinated and AdAg85A boosted, and nonvaccinated. Twenty-two goat kids were vaccinated with $\sim 5 \times 10^5$ CFU of BCG (week 0), and 11 of them were boosted at week 8 with 10^9 PFU of AdAg85A. At week 14, all goats were challenged by the endobronchial route with $\sim 1.5 \times 10^3$ CFU of *Mycobacterium caprae*. The animals were euthanized at week 28. Cellular and humoral immunity induced by vaccination and *M. caprae* infection was measured throughout the study. After challenge BCG-AdAg85A-vaccinated animals exhibited reduced pathology compared to BCG-vaccinated animals in lungs and in pulmonary lymph nodes. There were significant reductions in bacterial load in both groups of vaccinated goats, but the reduction was more pronounced in prime-boosted animals. Antigen-specific gamma interferon (IFN- γ) and humoral responses were identified as prognostic biomarkers of vaccination outcome depending on their correlation with pathological and bacteriological results. As far as we know, this is the first report using multidetector computed tomography (MDCT) to measure vaccine efficacy against pulmonary TB in an animal model. The use in vaccine trials of animals that are natural hosts of TB may improve research into human TB vaccines.

Tuberculosis (TB) in goats can be caused by *Mycobacterium caprae* or *Mycobacterium bovis*, which are both members of the *Mycobacterium tuberculosis* complex (MTBC) (2) and are endemic throughout the Iberian Peninsula (14, 26). Apart from being a reservoir of TB for animals, infected goats present a zoonotic risk for human infections (11, 18, 23, 25).

Eradication of TB infection from domestic animals seems to be unachievable in areas where wildlife is endemically infected with *M. bovis* (14, 26), and vaccination against *M. bovis* infection in domestic and wild animals has become a field of intensive research (10, 19, 35). *M. tuberculosis* bacillus Calmette-Guérin (BCG) is the basis of most new vaccine strategies assayed in animals, and there is a large body of knowledge regarding its use against *M. tuberculosis* in humans. In this regard, BCG shows protection against severe and disseminated forms of childhood TB, but protection against pulmonary TB in adults is limited (3). BCG has been shown to confer a degree of protection in cattle challenged with *Mycobacterium bovis* (4, 17, 32). Attempts to increase this protection by boosting with BCG in BCG-primed calves have failed (6). Therefore, in recent years, new vaccination regimens to improve upon BCG vaccination have been developed. Heterologous prime-boost strategies have been established as promising approaches combining BCG priming with virus-vectored subunit vaccine boosting. These strategies have been applied to humans (20, 38) and cattle (34, 35). In this regard, a molecular construct based on a recombinant replication-deficient human type 5 adenovirus expressing the MTBC protein Ag85A (AdAg85A) was developed and assessed as a booster vaccine in BCG-primed mice

(36) and cattle (35). In both cases, this approach provided greater protection than BCG alone.

Neither BCG vaccination against TB nor heterologous prime-boost vaccination regimens have yet been assessed as a prophylactic treatment against TB in goats. In a study carried out in goats experimentally infected with *M. caprae*, we observed that, a short time after infection (14 weeks), the majority of goats had developed typical caseous necrotizing granulomas, often with liquefactive necrosis and cavitory lung lesions (22), features similar to those of active TB in humans (13). In this study, we assessed quantitative methods for measuring pathological changes after *M. caprae* infection.

Attempts to improve the semiquantitative scoring systems used until now, employing image technologies and quantitative reading of macroscopic lesions, have been carried out recently. Magnetic resonance imaging (MRI) has been used in macaques experimentally infected with *M. tuberculosis* for scoring pathological changes in a precise and quantitative way (29, 30), and volumetric computed tomography (CT) has been applied for scanning the thoraxes of humans in TB vaccine clinical trials (27). We have

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also shown that multidetector computed tomography (MDCT) represents a rigorous quantitative method to measure the magnitude of lung TB lesions in goats experimentally infected with *M. caprae* (22).

The aim of the present work was to compare relative levels of protection of goats vaccinated with BCG, alone or in combination with AdAg85A, against *M. caprae*. MDCT was used for assessing the magnitude of pulmonary pathological changes, and commonly used immunological tests were performed to determine immunological responses to vaccination and challenge.

MATERIALS AND METHODS

Experimental animals. A total of 33 3-month-old Murciano-Granadina female goats obtained from an officially certified TB-free flock were used in the study. All of them were reconfirmed negative for TB by the single intradermal comparative cervical tuberculin (SICCT) test and a gamma interferon (IFN- γ) assay (Bovigam, Prionics, Schlieren, Switzerland). Also, the goats were seronegative for paratuberculosis (Paratub.Serum-S; Institut Pourquier, Montpellier, France).

All experimental procedures were approved by the Animal Welfare Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya and were in agreement with European Union laws for protection of experimental animals.

Vaccination and challenge procedures. Goats were divided randomly in three experimental groups of 11 animals each. The first group (BCG group) was vaccinated with BCG at week 0; a second group (BCG-Ad group) was vaccinated at the same time with BCG and boosted at week 8 with a recombinant adenovirus expressing Ag85A (AdAg85A); a third group of unvaccinated goats was kept as a control group. The BCG Pasteur strain (ATCC accession no. 35734) was subcultured in Middlebrook 7H9 media (BD Diagnostics, Sparks, MD). For immunization, BCG growth was diluted to 5×10^5 CFU by suspension in 0.5 ml of phosphate-buffered saline (PBS) and injected subcutaneously into the right axilla. The AdAg85A inoculum was prepared at 10^9 PFU in 0.5 ml of PBS without adjuvant, as previously described (36), and injected intramuscularly into the left brachiocephalic muscle. Rectal temperatures were recorded before and at 6, 24, 48, and 72 h after boosting with AdAg85A.

At week 14, goats were anesthetized with 4 to 6 mg/kg of body weight of propofol (Propofol Lipuro) and 0.2 mg/kg of midazolam (Dormicum) administered intravenously and then challenged with an *M. caprae* field strain from Catalonia (SB0416; www.mbovis.org) by endobronchial inoculation of 1.5×10^5 CFU suspended in 0.5 ml of PBS; the dose of inoculum was confirmed by plating dilutions on Middlebrook 7H11 medium (BD Diagnostics, Sparks, MD) as described previously (22).

Antigens and peptides. Tuberculin of *M. bovis* (PPD-B) and *M. avium* (PPD-A) were obtained from CZ Veterinaria (Porriño, Galicia, Spain). Immunodominant MTBC peptide cocktail ESAT-6/CFP-10 (E/C) and Rv3615c (ESAT-6 system 1 substrate protein C) were received from the Animal Health and Veterinary Laboratories Agency (Weybridge, United Kingdom) and were synthesized as described previously (31, 33). Recombinant MTBC-specific antigens ESAT-6, Ag85A, and MPB83 were obtained from Lionex (Braunschweig, Germany). Phytohemagglutinin (PHA) obtained from Sigma-Aldrich (Steinheim, Germany) was used as a positive control of blood stimulation assays.

Skin test. The SICCT test was performed in all goats at week 26 (2 weeks before sacrifice) by inoculating 0.1 ml of both PPD-B and PPD-A into the left and the right sides of the neck, respectively. The preinoculation skin fold thickness was recorded before PPD injection, and the skin fold thickness was measured again after 72 h. The goats were considered positive if the increase of skin fold thickness after PPD-B application was greater than 2 mm and greater than the increase at the site of PPD-A application.

Whole-blood IFN- γ assay. The progression of the infection was followed with the IFN- γ assay by collecting whole-blood samples from the jugular vein in heparinized blood tubes. Whole blood was stimulated in

96-well cell culture plates with the following final concentrations of stimuli: 10 μ g/ml of PPD-B and PHA and 5 μ g/ml of Ag85A, ESAT-6, E/C, and Rv3615c. PBS was added to cultures used as nonstimulated controls. Plasma supernatants were collected after 24 h of culture at 37°C and 5% CO₂ and were stored at -20°C; samples were thawed just before performing the Bovigam IFN- γ enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. ELISA results were obtained as optical density determined at 450 nm (OD₄₅₀). The specific reaction was expressed as Δ OD₄₅₀ (OD₄₅₀ of antigen-stimulated sample minus OD₄₅₀ of nonstimulated control). A sample was classified as a positive reactor to any antigen when specific Δ OD₄₅₀ was higher than 0.05. The assay was performed every 2 weeks throughout the experiment using PPD-B, ESAT-6, and Ag85A as stimuli. Peptide cocktails were also included at weeks 14, 16, 20, 24, and 28.

Serology. The presence of antibodies to mycobacterial antigens was analyzed in plasma samples using an IgG indirect ELISA as described previously (22). Flat-bottom 96-well plates (Nunc Multisorp; Thermo Fisher Scientific, Roskilde, Denmark) were coated with MPB83 (1 μ g/ml) and Ag85A (2 μ g/ml) separately. The antigens were diluted in carbonate/bicarbonate buffer and incubated overnight at 4°C. Plates were washed five times with PBS containing 0.05% Tween 20 (PBS-T20) and then incubated for 45 min at 37°C with PBS-T20 with 0.5% casein (blocking solution). After a washing, goat sera were added in duplicate (at 1/200 dilution in PBS-T20 with 1% casein) and incubated for 1 h at 37°C. Plates were washed again, and a mixture of protein A (50 ng/ml) and protein G (100 ng/ml), conjugated with peroxidase (Sigma-Aldrich, Steinheim, Germany) was added to each well. Plates were then read in a spectrophotometer, and Δ OD₄₅₀ was calculated as sample OD₄₅₀ minus background OD₄₅₀ (unspecific absorbance in wells where antigen had not been added). A sample was classified as positive when the Δ OD₄₅₀ was higher than the cutoff point, calculated as the mean of background OD₄₅₀ plus 3 standard deviations (SD). Samples were analyzed at weeks 0, 14, and 28 for the MPB83 ELISA and at weeks 0, 4, and 8 and every 2 weeks thereafter for the Ag85A-ELISA.

Postmortem examination. All goats were euthanized at week 28 (14 weeks postinfection) by intravenous sodium pentobarbital overdose and carefully examined in order to evaluate the extension of tuberculous lesions in lungs and respiratory lymph nodes (LN). TB lesions in nonrespiratory organs were also recorded.

(i) Lungs. Lung gross lesions were first evaluated by palpation and visual examination of each lobe. Then whole lungs were fixed with 10% buffered formalin by pouring the fixative into the trachea while sustaining it in a vertical position. After that, the trachea was tied, and whole lungs were immersed in a container with formalin. After complete fixation, lungs were scanned with a high-resolution 64-slice multidetector computed tomography (MDCT) scan (Brilliance CT, 64 channels; Philips Medical Systems, Cleveland, OH), and sequential slices were analyzed on a workstation (Aquarius Station; TeraRecon, Foster City, CA) as described previously (22), allowing calculation of the total volume of granulomatous lesions and the whole lung volume.

(ii) Lymph nodes. The diameters of gross lesions in pulmonary (caudal and cranial mediastinal, right and left tracheobronchial) and right and left retropharyngeal LN were also recorded by direct observation after slicing. The degree of pathology in LN was determined by adding together the approximated volumes of granulomas in respiratory LN of each animal. The volume was calculated as $4/3 \times \pi \times r^3$ (where r is lesion radius) considering the sphere-like morphology of the lesions found. The measurement of gross lesions was performed by the same pathologist in order to ensure the same criterion for all samples.

Bacterial count. After pathological evaluation, the whole pulmonary and retropharyngeal LN were homogenized in 10 ml of sterile distilled water in a Masticator (IUL Instruments, Barcelona, Catalonia, Spain). The homogenate was decontaminated with a final concentration of 0.35% (wt/vol) hexadecylpyridinium chloride (HPC) (9) for 15 min in continuous shaking, after which it was centrifuged at $2,471 \times g$

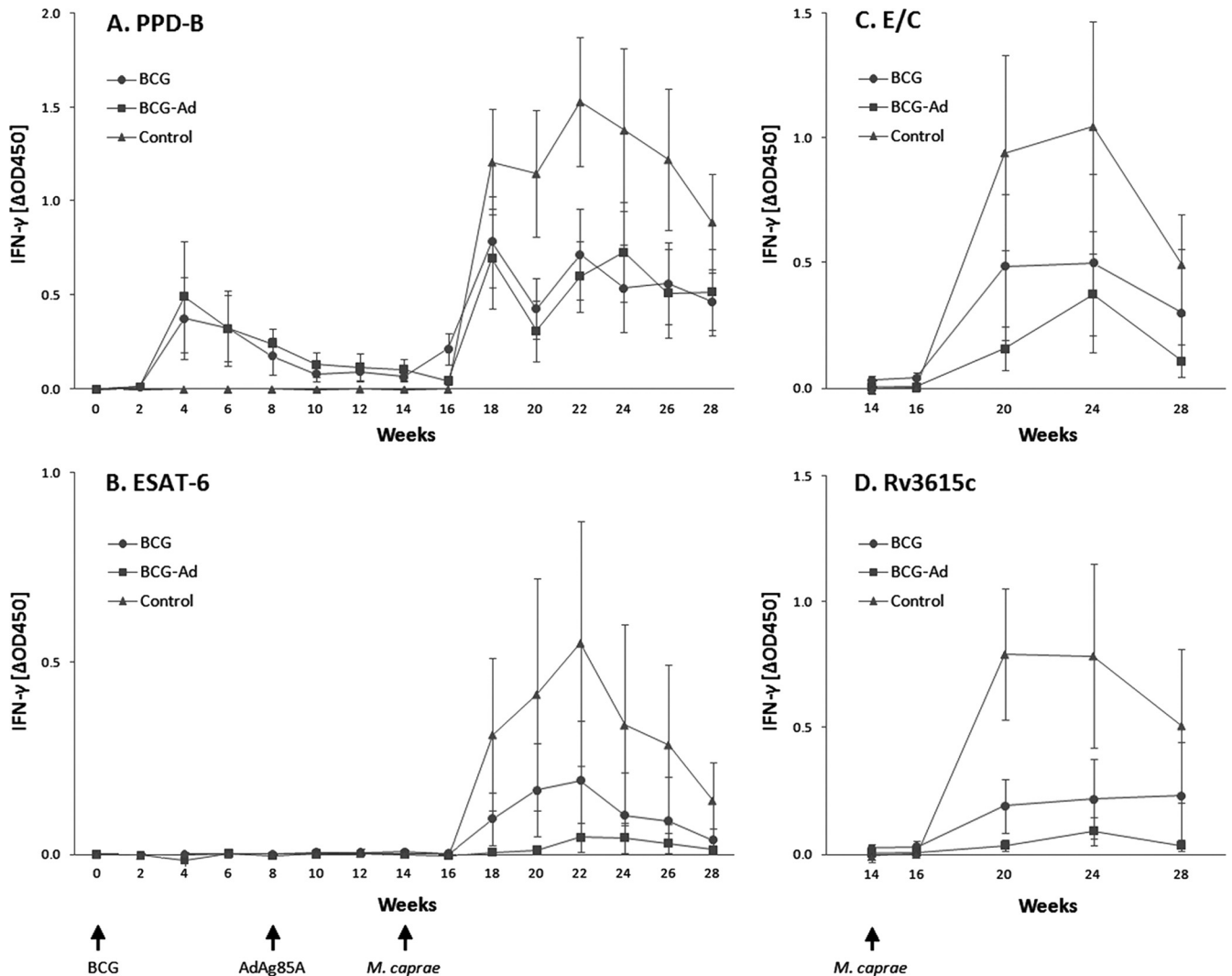


FIG 1 Antigen-specific IFN- γ responses in goats after vaccination and *M. caprae* challenge. Whole blood was stimulated *in vitro* with PPD-B (A), ESAT-6 (B), E/C (C), and Rv3615c (D). Results are expressed as means of $\Delta OD_{450} \pm 95\%$ CIs.

for 30 min. The supernatant was discarded, and the pellet was resuspended in 10 ml of PBS containing 0.05% Tween 80. The viable bacterial enumeration was determined by plating 0.1 ml of serial dilutions of LN homogenates on Middlebrook 7H11 agar plates. The inoculated plates were incubated at 37°C for 28 days. After that, the total count of CFU of each LN was calculated.

Data analysis. Differences in antigen-specific IFN- γ responses, as well as Ag85A-specific IgG responses, among treatment groups were compared by using the nonparametric Kruskal-Wallis test with the *post hoc* Mann-Whitney test, whereas one-way analysis of variance (ANOVA) with the Student Newman-Keuls (SNK) multiple comparison test was applied to compare SICCT results and MPB83-specific IgG responses among treatment groups. Rectal temperature changes after AdAg85A vaccination were also compared by one-way ANOVA with the *post hoc* SNK test. All postmortem data (pathological parameters and \log_{10} -transformed bacterial counts) were compared by the Kruskal-Wallis test with the *post hoc* Mann-Whitney test. Correlations between immune responses and post-mortem parameters were assessed by employing the nonparametric Spearman rank test. Data analysis was performed using SPSS for Windows statistical package, version 17.0 (IBM Inc., Chicago, IL).

RESULTS

Effect of different vaccination regimens on cell-mediated antigen-specific responses. The release of IFN- γ after *ex vivo* stimulation of whole blood with PPD-B, Ag85A, and ESAT-6 was measured by ELISA every 2 weeks throughout the course of the experiment, whereas responses to ESAT-6/CFP-10 (E/C) or Rv3615c peptide cocktails were measured at weeks 14, 16, 20, 24, and 28 (Fig. 1; data for Ag85A are not shown). Ag85A-specific IFN- γ responses were not detectable in any of the groups and at any time point during the study. BCG vaccination induced an increase in mean IFN- γ responses to PPD-B in vaccinated groups, with a peak at week 4 followed by a progressive decrease until week 12, at which point responses were still significantly higher than unvaccinated control group responses ($P < 0.001$). After *M. caprae* challenge (at week 14), all goats showed a considerable increase in the levels of IFN- γ to PPD-B beginning at week 18. The two vaccinated groups responded with similar intensities, which were considerably lower than that for the unvaccinated control

TABLE 1 Numbers of goats of each treatment group positive in the IFN- γ assay (Bovigam) using different antigens

Group (n)	Antigen	No. of goats positive at wk:				
		14 ^a	16	20	24	28
BCG (11)	PPD-B	6	9	8	9	11
	ESAT-6	0	0	4	2	2
	E/C	0	0	7	8	5
	Rv3615c	0	0	5	4	5
	E/C + Rv3615c	0	0	9	9	9
BCG-Ad (11)	PPD-B	5	3	9	11	11
	ESAT-6	0	0	1	2	1
	E/C	0	0	8	10	6
	Rv3615c	0	0	2	5	3
	E/C + Rv3615c	0	0	10	11	8
Control (11)	PPD-B	0	0	11	11	11
	ESAT-6	0	0	7	4	7
	E/C	0	0	10	9	10
	Rv3615c	0	0	11	9	9
	E/C + Rv3615c	0	0	11	9	10

^a Prior to challenge with *M. caprae*.

group at weeks 18 to 22 and 26 (Fig. 1A). Responses to ESAT-6 after challenge with *M. caprae* were slightly lower in AdAg85A-boostered goats (BCG-Ad group) than in goats vaccinated with BCG alone (BCG group), although this difference was statistically significant only at week 26 ($P < 0.05$). As occurred with PPD-B, responses to ESAT-6 in unvaccinated goats were also stronger than those in the vaccinated groups (Fig. 1B); they were significantly higher than those in the BCG-Ad group at weeks 18 to 28 and in the BCG group at weeks 18 to 26 ($P < 0.05$). The peptide cocktails E/C and Rv3615c induced an ESAT-6-like pattern of IFN- γ responses (Fig. 1C and D, respectively), with higher responses in the control group than in vaccinated groups ($P < 0.05$ at weeks 20 and 28 for E/C and at weeks 20, 24, and 28 for R3615c). The results of the IFN- γ assay at weeks 14, 16, 20, 24, and 28 (positive or negative) using PPD-B, ESAT-6, E/C, and Rv3615c as diagnostic reagents are shown in Table 1. The highest and the lowest numbers of positive animals were found when using PPD-B and ESAT-6, respectively. However, if results of E/C and Rv3615c are combined, the number of animals scoring positive in the IFN- γ assay was similar to results with PPD-B from weeks 20 to 28. Moreover, 11 out of 22 BCG-vaccinated goats were positive prior to *M. caprae* challenge (week 14) when PPD-B was used, whereas all of them were negative when ESAT-6 was used.

At week 26 all goats were positive by the SICCT test (individual data not shown). There were minor differences among groups in the mean increase in skin fold thickness 72 h after the application of PPD-B and PPD-A: 19.3 mm (17.6 to 21 mm, 95% confidence interval [CI]) and 9.7 mm (8 to 11.3 mm, 95% CI), respectively, for the BCG group, 17.3 mm (15.7 to 19 mm, 95% CI) and 8.7 mm (6.9 to 10.4 mm, 95% CI), respectively, for the BCG-Ad group, and 21.3 mm (19.4 to 23.2 mm, 95% CI) and 10.9 mm (9 to 12.7 mm, 95% CI), respectively, for the control group. The PPD-B-induced increase in skin fold thickness in the BCG-Ad group was significantly lower than that obtained in the control group ($P < 0.05$).

Humoral responses to MPB83 and Ag85A. All goats were seronegative by the MPB83 indirect ELISA prior to vaccination

(week 0), whereas two goats of the BCG group seroconverted at week 14 (just before challenge). At week 28 (2 weeks after the SICCT test), all goats were seropositive (data not shown). At this time point, the mean ΔOD_{450} found in vaccinated groups was significantly lower than that in the control group ($P < 0.05$; individual data not shown), with mean ΔOD_{450} s of 1.614 (0.895 to 2.334, 95% CI) for the BCG group, 1.502 (0.932 to 2.071, 95% CI) for the BCG-Ad group, and 2.644 (2.105 to 3.183, 95% CI) for the control group. Thus, the overall levels of MPB83-specific antibody responses showed a temporal pattern similar to that seen with *M. caprae* antigen-specific T cell responses (Fig. 1).

The IgG responses to Ag85A were determined at weeks 0, 4, and 8 and then every 2 weeks (Fig. 2). Goats in the control group and BCG group were seronegative at all time points during the experiment. Goats in the BCG-Ad group were also negative prior to AdAg85A inoculation. Two weeks after that, the mean values of ΔOD_{450} reached a peak, followed by a progressive decrease until week 28, when the boost effect of the SICCT test (applied at week 26) dramatically raised again the mean value of ΔOD_{450} . These values of ΔOD_{450} were significantly higher than values for the other groups ($P < 0.001$).

Clinical, pathological, and bacteriological findings. A mild but not statistically significant increase of mean rectal temperature was detected at 6 h after BCG inoculation (0.31°C [0.13 to 0.49°C, 95% CI]), followed by normalization at 24 h and subsequent time points (data not shown). After AdAg85A inoculation, a significant change in mean rectal temperature was observed at 24 h in comparison to the mean basal temperature (+1.47°C [1.11 to 1.84°C, 95% CI]; $P < 0.001$), which returned to normal at 48 h. Inoculation with *M. caprae* produced only minimal clinical signs. Occasional coughing was observed from week 20 (6 weeks after challenge) until the end of the experiment in 8 out of 11 goats of the BCG group, 7 out of 11 goats of BCG-Ad group, and 9 out of 11 unvaccinated goats. The main weight gains from challenge (week 14) to the end of the experiment (week 28) were 6.5 kg (5.4 to 7.7 kg, 95% CI) in the BCG group, 6.5 kg (6.0 to 7.0 kg, 95% CI) in the BCG-Ad group, and 6.4 kg (5.2 to 7.5 kg, 95% CI) in the control group. Therefore,

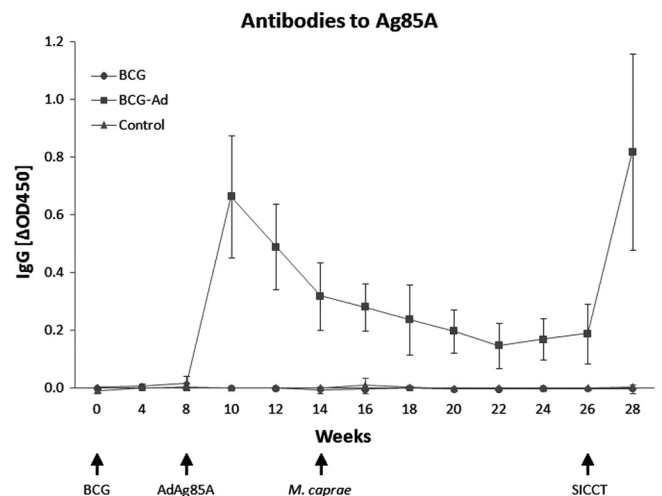


FIG 2 Temporal kinetics of goat IgG responses to Ag85A protein. Results are expressed as means of $\Delta OD_{450} \pm 95\%$ CIs.

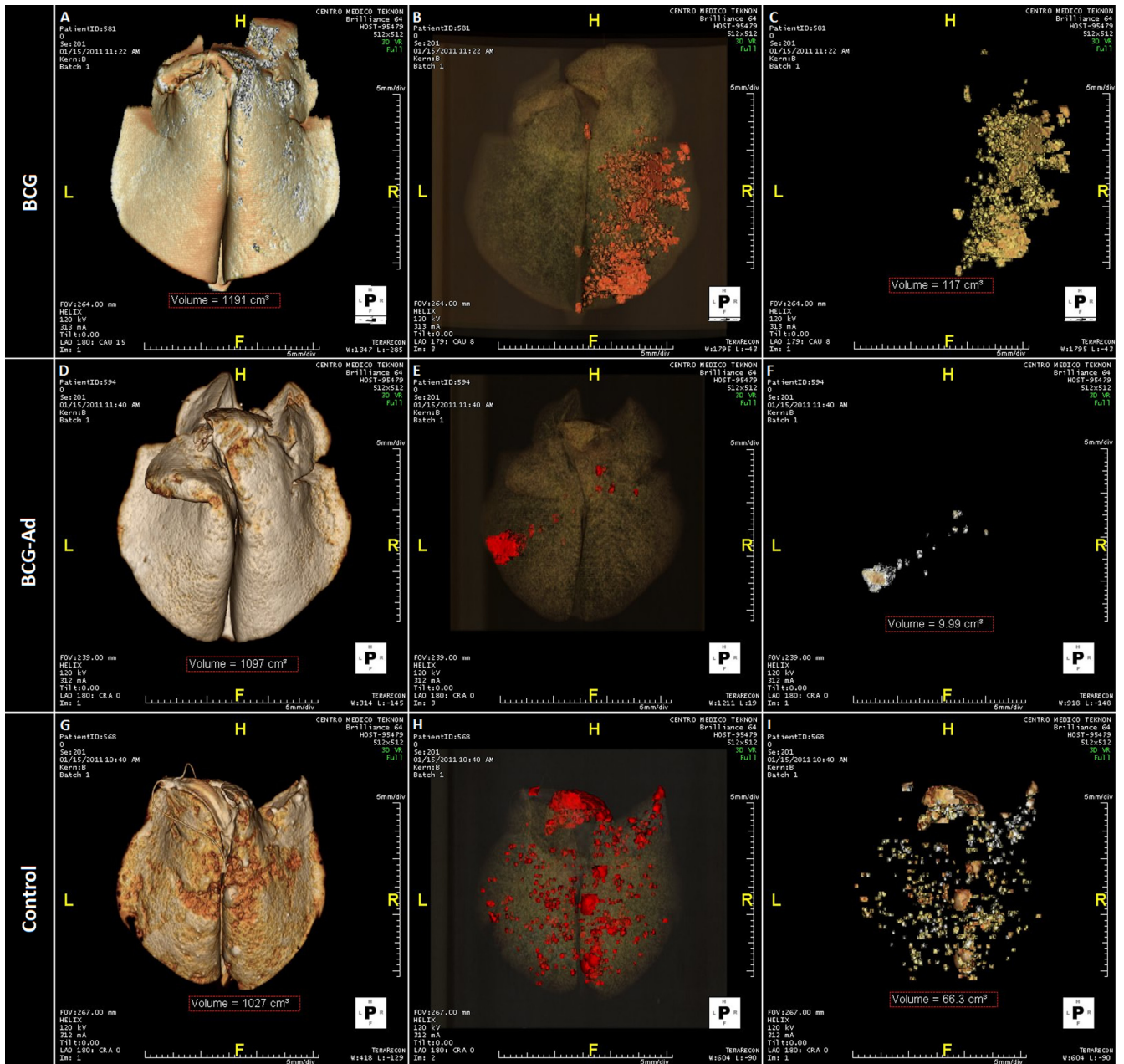


FIG 3 Multidetector computed tomography analysis of gross lung lesions. Representative lungs from goats in treatment groups BCG (A to C), BCG-AdAg85A prime-boost (D to F), and control (G to I) are shown. (A, D, and G) Three-dimensional reconstructions of the whole lung. (B, E, and H) Volume-rendering images of the whole lungs with the different tissue densities discriminated by color: water in gray, air in black, and TB lesions in red. Total volumes of the lungs are shown in red dashed boxes. (C, F, and I) Volume-rendering images of the affected lungs showing only lesions. Total volumes of the affected lungs are shown in red dashed boxes. Lung orientation is indicated as follows: H, head; F, foot; L, left; R, right.

there was no difference in weight gain during the challenge period among the three groups.

At necropsy, lungs and respiratory lymph nodes were processed separately. Pathological findings were restricted to the thoracic cavity in vaccinated animals, whereas the disease was more disseminated in 4 out of 11 control goats, which presented TB extrathoracic lesions in retropharyngeal LN, mesenteric LN, or spleen.

The mean volumes of TB lesions in formalin-fixed lungs were compared among groups by MDCT. All goats showed

granulomatous necrotizing lesions in lung parenchyma and in lymph nodes, and although none of the lungs was completely free of TB lesions, AdAg85A-boosted goats showed much less gross lung lesions than goats belonging to the BCG group and unvaccinated group (group-representative MDCT lung images are shown in Fig. 3). As shown in Table 2, the volume of TB lung lesions in the BCG-Ad group was very much lower than in the BCG group ($P < 0.005$) and in the control group ($P < 0.001$). Also, the percentage of lung involvement (volume of TB lesions relative to the volume of the whole lung) was much

TABLE 2 Pathological and bacteriological findings at postmortem in vaccinated and control goats^a

Group	Lungs			LN	
	Vol of VL (cm ³)	Vol of VL/lung vol (%)	No. of lobes with VL ^b	Vol of VL (cm ³)	Bacterial load (log CFU)
BCG	80 (44–157)	8.1 (4–13.5)	2/7 (1–3/7)	0.2 (0–4.1)*	3.7 (2.6–4.3)*
BCG-Ad	4 (0–22)**	0.5 (0.1–1.8)**	1.9/7 (1.4–2.5/7)	0.1 (0–0.2)**	3.2 (2.3–3.9)**
Control	36 (27–94)	3.8 (2.4–8.1)	3.4/7 (2.3–4.4/7)	6.1 (2.3–30.1)	4.5 (4.3–4.9)

^a The volumes of visible lung lesions were calculated using multidetector computed tomography and those for lymph nodes (LN) by direct visual observation. Values are medians; interquartile ranges are in parentheses. Significant differences between groups are shown as follows: *, $P < 0.005$; **, $P < 0.001$ (Kruskal-Wallis test with the *post hoc* Mann-Whitney test).

^b Values are means.

lower in the BCG-Ad group than in the BCG group ($P < 0.005$) and control group ($P < 0.001$). Individual data and median values of the volumes of TB lesions are plotted in Fig. 4A. The number of affected lung lobes (of a total of 7 in goats) has been used frequently to assess the spread of the infection within the lung. The extents of scoring for lung lobe involvement were similar for both vaccinated groups and lower than that for the control unvaccinated group, where TB lesions were more widespread (Table 2). Gross lesions in pulmonary and retropharyngeal LN were also analyzed by measuring the volume of TB lesions by direct visual assessment. In the BCG-Ad group there was a strong reduction of mean volume of TB lesions in comparison to the unvaccinated control group ($P < 0.001$), and the volume of lesions was also smaller in the BCG group than in the control group ($P < 0.005$), whereas the difference between both vaccinated groups was not statistically significant (Fig. 4B).

In order to investigate the effect of the different vaccination protocols, the mycobacterial burden in respiratory LN was also assessed. Median values of total burden of mycobacteria (expressed as log CFU/pulmonary LN) of each group are shown in Table 2. The amounts of mycobacteria recovered in respiratory LN were significantly smaller in both BCG and BCG-Ad groups than in the control group ($P < 0.005$ and $P < 0.001$, respectively) (Fig. 4C).

Immunological responses as predictors of vaccine efficacy and disease status. Immunological parameters were correlated with pathological and bacteriological parameters in order to assess their predictive value as biomarkers of vaccine efficacy or disease status of individual goats.

Antigen-specific IFN- γ responses were compared with pathological and bacteriological parameters (volume of visible lesions [VL] and log₁₀ CFU in LN, respectively) at weeks 20, 24, and 28 (Table 3). Significant positive correlations between IFN- γ to all antigens (PPD-B, ESAT-6, E/C, and Rv3615c) and the two postmortem parameters were found only at week 20, whereas inconsistent and less-significant correlations were found at subsequent time points.

Humoral responses were also compared with postmortem parameters. There was a significant inverse correlation between Ag85A-induced IgG responses at weeks 10 to 28 and the total volume of VL found postmortem ($P < 0.005$, data not shown), whereas the observed negative correlations between the bacterial counts in LN and Ag85A-specific IgG responses were statistically significant only at weeks 10, 12, 16, 18, 20, 24, and 26 ($P < 0.05$; data not shown). However, at week 28 (just prior to sacrifice) we found a significant positive correlation between MPB83-induced IgG responses and both total volume of VL (Spearman $\rho = 0.312$; $P < 0.05$) and bacterial counts (Spearman $\rho = 0.389$; $P < 0.05$).

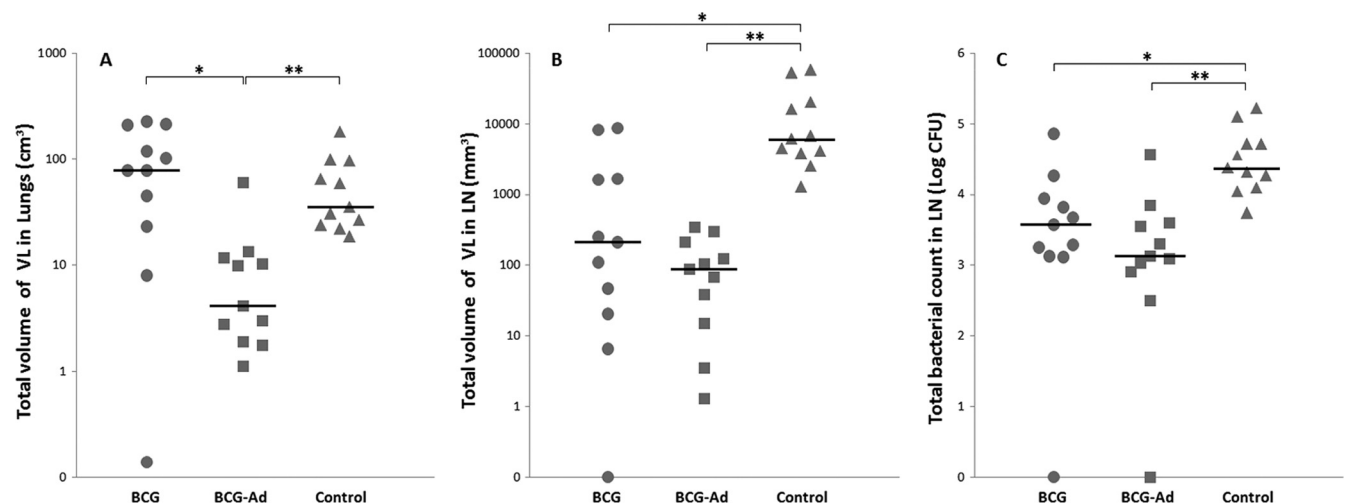


FIG 4 Postmortem results for individual goats for the three treatment groups. (A and B) Total volume of VL in lungs and LN. (C) Total bacterial counts in the pulmonary LN expressed as log₁₀-transformed CFU. ●, BCG group; ■, BCG-AdAg85A prime-boosted group; ▲, unvaccinated control group. Horizontal lines indicate median values. Significance was determined by Kruskal-Wallis test with the *post hoc* Mann-Whitney test (*, $P < 0.005$; **, $P < 0.001$).

TABLE 3 Correlation of antigen-specific IFN- γ released in whole blood as measured by ELISA (ΔOD_{450}) with postmortem parameters volume of VL and \log_{10} -transformed bacterial counts^a

Antigen	Wk 20		Wk 24		Wk 28	
	Vol of VL	Log CFU	Vol of VL	Log CFU	Vol of VL	Log CFU
PPD-B	0.461**	0.533**	0.211	0.310*	0.148	0.133
ESAT-6	0.388*	0.363*	0.181	0.212	0.380*	0.361*
E/C ^b	0.451**	0.554***	0.303*	0.368*	0.077	0.014
Rv3615c	0.446**	0.561***	0.202	0.319*	0.401*	0.487**

^a Tabulated are results of the Spearman rank test. Significance is shown as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

^b E/C, ESAT-6/CFP-10.

DISCUSSION

Research in vaccines against human TB also benefits from large-animal models based on natural TB hosts where histopathological features are similar to those in human disease (39). We have reported recently the development of a TB infection model in goats and found the induction of TB lesions in all the infected animals after inoculating *M. caprae* by the endobronchial route (22). In the present work we have used this model for testing a candidate human and cattle vaccine against TB, based on BCG priming and subsequent boost with an adenoviral vector expressing the immunogenic antigen Ag85A (AdAg85A), followed by challenge with *M. caprae*. To our knowledge, this is the first attempt to assess the safety and efficacy of a prophylactic vaccination strategy against TB in goats.

The rationale for this strategy is based on previous studies in cattle showing only partial efficacy of BCG or adenovirus-vectored vaccines alone but improved results when a combined prime-boost strategy was used. In cattle, homologous prime-boost vaccination strategies failed to confer protective immunity against *M. bovis*, either by using BCG-BCG or AdAg85A-AdAg85A (6, 34). In contrast, heterologous BCG-AdAg85A prime-boost procedures showed strong improvement in protection, compared to vaccination with BCG alone (28, 35, 36).

In our goat model, BCG vaccination followed by AdAg85A boosted protection against a challenge with *M. caprae*, leading to a reduction of lung and lymph node lesions and reduction of mycobacterial burden in respiratory lymph nodes in the vaccinated group in comparison to control nonvaccinated goats.

The strong reduction of pathological and bacteriological parameters observed in the BCG-Ad group is in agreement with the results obtained previously in mice (28) and, particularly, with those reported in a recent study in calves challenged with *M. bovis* (34, 35). In the latter study, with a design analogous to that of the present study, 4 out of 10 BCG-vaccinated and AdAg85A-boosted calves did not show visible pulmonary lesions at the necropsy. The main difference between the two studies is that we did not find any goat free of VL. This could be due to the use of a highly sensitive, quantitative method for evaluation of pulmonary lesions (MDCT), which allowed us to find TB lesions in all goats, in some cases even smaller than 1 mm³; such lesions would be difficult to observe by direct macroscopic evaluation. However, another possible explanation for this difference in pathological expression of TB between both experiments could be that different species of host and mycobacteria were used and that there was faster progression of TB infection in goats than in cattle, as has been postulated recently (22).

Unexpectedly, the mean volume of lung lesions of the BCG

group, measured by MDCT, was slightly higher than that for the control group (without statistically significant differences). This result contrasts with that obtained in calf and badger vaccination studies, where a significant reduction of pulmonary pathology score was reported in BCG (Pasteur strain)-vaccinated and *M. bovis*-challenged animals (5, 10, 19, 37). Nevertheless, the different scoring system used in our study precludes us from making firm conclusions about a different response to BCG vaccination in goats and other species. In cattle experiments, the size and distribution of gross lesions were integrated in a semiquantitative pathology scoring, whereas we have quantified lung lesion volumes and have evaluated extension to the different lung lobes and extrapulmonary involvement separately.

Considering the intrapulmonary distribution of lesions, measured as number of affected lobes, we observed better containment of internal dissemination of infection within the lung in the two vaccinated groups than in the unvaccinated goats. Also, both gross lesions and bacterial burden in pulmonary drainage lymph nodes were significantly higher in the control group, corroborating the greater disease severity in untreated goats. In addition, extrathoracic TB lesions were found only in unvaccinated goats (4 out of 11), showing that vaccination mitigates hematogenous dissemination of mycobacteria. This finding is consistent with the widely reported capacity of BCG to prevent extrapulmonary TB in children (8, 24).

With regard to the relationship between cell-mediated immunity and vaccination outcome, we found strong positive correlations between postmortem parameters and IFN- γ produced in peripheral blood effector T cells in response to MTBC antigens at week 20 (6 weeks postinfection). However, very weak or no correlations were found at subsequent time points. It is well known that IFN- γ is a necessary Th1 cytokine for host defense against mycobacterial infection (15), although its production alone is insufficient for a protective response to TB (16). Thus, paradoxically, IFN- γ response is also a biomarker of protective immunity failure (reviewed in reference 1). In fact, a constant high secretion of IFN- γ by ESAT-6-specific T cells is associated with uncontrolled mycobacterial replication, and it is considered a predictor of active TB in humans (1) and cattle (32).

By contrast, we observed a decrease of both single ESAT-6- and E/C cocktail-specific IFN- γ responses at 14 weeks postinfection in all treatment groups. This IFN- γ kinetics is not in accordance with those observed in calf experiments with similar infective doses of *M. bovis* (32, 35). Notwithstanding, in our study the IFN- γ decrease does not seem to be related to the control of infection, but it might be an immune response failure or even an early anergic process. Therefore, the use of antigen-specific IFN- γ as a prognos-

tic biomarker of control/severity of the infection must be employed with caution. It may be useful to determine vaccine success in the early stages of infection (<8 to 10 weeks), but it is inconclusive with regard to infection status of an animal at subsequent weeks.

Ag85A-specific IFN- γ production was not detected during the experiment. However, we found indirect correlations of Ag85A-specific humoral responses with postmortem parameters prior to challenge and after the SICCT booster effect. The kinetics of antibody responses to Ag85A confirmed the successful recognition of this antigen by the goat immune system and its usefulness as a biomarker of Ag85A expression.

We have confirmed the usefulness of DIVA candidate peptides as novel diagnostic reagents for use in goats. None of the vaccinated goats produced detectable IFN- γ to the DIVA antigens prior to challenge, whereas half of the vaccinated goats were positive in the bovine tuberculin-based IFN- γ assay, an assay that is being applied in some bovine TB eradication programs (12). The usefulness of whole-blood IFN- γ release assays (IGRAs) using ESAT-6 and CFP-10 to distinguish infected from vaccinated individuals has been reported previously for humans and cattle (7, 21). In goats infected with *M. caprae*, we found that the tuberculin-based IFN- γ assay and DIVA-based IFN- γ assays, where results for E/C and Rv3615c IFN- γ assays were taken together, had similar sensitivities. In this sense, the capacity of these antigen-specific assays to increase the detection of infected animals when they are used together was as previously reported for cattle (31) and goats (22).

Vaccination is the best strategy to control TB in humans and may be a valid approach in domestic and wild animal species when eradication is not feasible. Employing for the first time a goat model of TB using *M. caprae* infection, the goat being a natural host of this organism, we have demonstrated enhanced protection after boosting BCG-primed goats with an adenoviral vector expressing the antigen Ag85A. Furthermore, we have identified specific IgG response to be an immunomarker of protection and disease severity useful for monitoring vaccine efficacy. We believe that our current study serves as an important step for investigating immune mechanisms of protection in the goat model of TB.

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B.P.D.V. and M.D. conceived and designed the experiments, analyzed the data, and drafted the manuscript. M.D., M.N., and S.L.-S. were responsible for the necropsy and pathological records; B.P.D.V. and F.X.A. performed the immunological and bacteriological assays; N.R. performed the MDCT and analyzed resulting data; B.V.-R., M.S., Z.X., and H.M.V. contributed substantially in scientific discussion of the study design and results.

We declare that we have no competing interests.

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