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Efficacy and immunogenicity of *Mycobacterium bovis* $\Delta RD1$ against aerosol *M. bovis* infection in neonatal calves

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Abstract

An attenuated *Mycobacterium bovis* *RD1* deletion ($\Delta RD1$) mutant of the Ravenel strain was constructed, characterized, and sequenced. This *M. bovis* $\Delta RD1$ vaccine strain administered to calves at two weeks of age provided similar efficacy as *M. bovis* bacillus Calmette Guérin (BCG) against low dose, aerosol challenge with virulent *M. bovis* at 3.5 months of age. Approximately 4.5 months after challenge, both $\Delta RD1$ - and BCG-vaccinates had reduced tuberculosis (TB)-associated pathology in lungs and lung-associated lymph nodes and *M. bovis* colonization of tracheobronchial lymph nodes as compared to non-vaccinates. Mean central memory responses elicited by either $\Delta RD1$ or BCG prior to challenge correlated with reduced pathology and bacterial colonization. Neither $\Delta RD1$ or BCG elicited IFN- γ responses to rESAT-6:CFP-10 prior to challenge, an emerging tool for modern TB surveillance programs. The $\Delta RD1$ strain may prove useful for bovine TB vaccine programs, particularly if additional mutations are included to improve safety and immunogenicity.

Keywords

Tuberculosis; Live bacterial vaccine; RD1; BCG; Neonatal calf; Aerosol Inoculation

1. Introduction

Mycobacterium bovis, a member of the *M. tuberculosis* complex, has a wide host range as compared to other species in this disease complex, is infectious to humans, and is the species

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most often isolated from tuberculous cattle. Control of *M. bovis* in cattle is particularly difficult due to wildlife reservoirs such as white-tailed deer, European badgers, and brush-tailed possums [1,2]. While the mainstay of bovine TB control has been abattoir inspection plus targeted test / cull campaigns, vaccines are now being considered as an additional tool for control, both in cattle and wildlife [3,4]. The first known tuberculosis (TB) vaccine purposefully administered to cattle was *M. bovis* bacillus Calmette Guérin (BCG), circa 1911 [5]. The efficacy of BCG, however, varies under both experimental and field conditions [6]. Other vaccine approaches have also been evaluated, with promising results in certain instances [7]. For example, heterologous prime / boost strategies combining subunit vaccines with BCG are particularly promising [8,9,10]. An attenuated live vaccine with more consistent efficacy and/or improved safety as compared to BCG should prove invaluable as a bovine TB vaccine, either alone or in combination with subunit vaccines.

Development of efficient tools for the genetic manipulation of *Mycobacteria spp.* has enabled the targeted discovery of highly immunogenic attenuated live strains of *M. tuberculosis* and *M. bovis* (reviewed in [11]). This approach is particularly appealing for the development of effective TB vaccines considering the relative success of BCG (a naturally attenuated live mutant), the complex interplay of the host / *Mycobacterium* interaction requiring potent immune activation, and the ability to target regions that positively alter the ensuing response. A key component of an attenuated live vaccine strategy for bovine TB will be the capability to differentiate infected from vaccinated animals (i.e., DIVA approach). An emerging strategy for TB ante-mortem testing programs is the use of a plasma-based IFN- γ assay (Prionics AG, Schlieren, Switzerland) of whole blood samples stimulated with early secretory antigenic target-6 kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10) antigens, either as a peptide cocktail or recombinant protein(s) [12,13]. ESAT-6 and CFP-10 are co-secreted by *M. tuberculosis* complex mycobacteria [14] and are potent inducers of Th-1 cytokines [15]. *esxA* and *esxB* genes encode CFP-10 and ESAT-6, respectively, and are located in the region of difference 1 (*RD1*), present in virulent *M. tuberculosis* complex genomes but absent in BCG and most other non-tuberculous mycobacteria [16-19]. CFP-10 and ESAT-6 are critical for tuberculosis pathogenesis [20]. The introduction of *RD1* to BCG partially restores virulence of BCG [19,21]. The deletion of the *RD1* region from virulent *M. bovis* and *M. tuberculosis* results in attenuation [22,23]. Although an *M. bovis* *RD1* knockout ($\Delta RD1$) has been evaluated as a vaccine approach for mice to protect against *M. tuberculosis* challenge, it has not been evaluated for immunogenicity or efficacy with cattle against *M. bovis* infection.

Our long-term goal is to develop a live attenuated DIVA vaccine with improved efficacy and safety as compared to BCG for use in cattle and associated wildlife hosts. Currently, ESAT-6 and CFP-10 are leading candidates for modern bovine TB diagnostic assays; thus, a $\Delta RD1$ vaccine is a logical selection for evaluation. The selection of *M. bovis*, as compared to *M. tuberculosis*, as the parent strain for production of the *RD1* deletion mutant was based on the antigens that might differ between *M. tuberculosis* and *M. bovis*. The *M. bovis* Ravenel strain was selected for construction of the *RD1* deletion as it is well characterized as being highly virulent in mice [24] and is more easily transduced than the previously sequenced *M. bovis* AF2122/97 strain. DNA from the parental *M. bovis* Ravenel strain was sequenced and compared to other tubercle bacilli. The resulting *M. bovis* Ravenel $\Delta RD1$ mutant was evaluated for safety by examining its attenuation in immunocompromised mice. Lastly, the immunogenicity and efficacy of the *M. bovis* Ravenel $\Delta RD1$ mutant was evaluated and compared to that of *M. bovis* BCG Danish strain in calves.

2. Materials and Methods

2.1 Strains, Growth Media and and Strain Constructions

The strains used in this study are listed in Table 1. The *M. bovis* 95-1315 challenge strain was prepared as previously described [25]. The *M. bovis* Ravenel and *M. tuberculosis* strains, were grown in Middlebrook 7H9 medium supplemented with 10% OADC enrichment, 0.5% Glycerol and 0.05% Tween-80 and the *M. smegmatis* mc²155 strain was grown in the same medium for all genetic manipulations.

Specialized transduction was performed to generate an *RD1* null deletion in *M. bovis* Ravenel. Allelic exchange substrates (AES) and knockout phages were constructed according to Bardarov et al [26]. Primers used to PCR amplify the upstream and downstream homologous sequences of *RD1* are presented in Supplemental Table 1 and the PCR products were cloned into pCR2.1 (TA cloning kit, Stratagene). The sequences were verified and the inserts then cloned into pYUB854 [26] using unique primer-derived restriction sites. The resulting AES plasmid was cloned into shuttle phasmid phAE159 [26], which resulted in the generation of specialized transducing phage phAE343. High titers of the phage phAE343 were grown on *M. smegmatis* mc²155 and then used to transduce *M. bovis* Ravenel. The *RD1* deletion of mc²5391 was confirmed by both PCR and Southern analysis and was used in the SCID mouse experiment. In order to eventually construct an unmarked *RD1* deletion of the *M. bovis* Ravenel, the *RD1* flanks were cloned into pHSU4 (T. Hsu and W.R. Jacobs, Jr, unpublished data) a vector with a *sacB* and *hyg* (hygromycin) cassette flanked by gamma delta resolvase sites. The resulting specialized transducing phage phAE344 was used to transduce *M. bovis* Ravenel and mc²5401, a $\Delta RD1::hyg sacB$ transductant, was confirmed by Southern analysis and was used in the cattle vaccination studies.

2.2 Whole Genomic sequencing of *M. bovis* Ravenel

Genomic DNA from the *M. bovis* Ravenel strain was prepared for sequencing using standard protocols from the manufacturer (Illumina, San Diego, CA). In brief, DNA was nebulized, end-repaired, ligated to adapter oligos and 130-150 bp fragments were isolated by size selection. DNA fragments were amplified by single molecule PCR on the Illumina cluster station and then sequencing by synthesis was performed through 36 cycles. The sequencing images were analyzed using the Solexa pipeline v. 0.2.2.6 software and the resulting 9.1 million reads were aligned to the *M. bovis* AF2122/97 [27] or the *M. bovis* BCG Pasteur 1173P2 sequence using SOAP software [28]. Ninety-nine percent of the genome could be aligned to reads to a depth of ten reads or greater where reads were aligned to the genome with less than or equal to five mismatches. Nucleotide changes from the reference sequence were called where at least six separate reads called the alternate letter, and where the sum of the Solexa quality scores for the alternate letter was five-fold greater than the sum of the Solexa quality score for the reference letter. A new reference sequence was generated incorporating these changes and the reads were then aligned with SOAP against the edited genomic sequence. This process of alignment, detection of polymorphisms, and sequence correction was iterated nine times.

Solexa reads were aligned to reference sequences consisting of transcripts plus 100 bp of flanking genomic sequence upstream and downstream. SNPs were called by the criteria above, and new reference sequence generated to reflect the changes. Nucleotides that did not have a Solexa quality score sum of at least 120 were deemed not confirmable, and were recorded as "N". The resulting transcript sequence with *M. bovis* Ravenel SNPs was aligned by BLASTX [36] to *M. bovis* BCG Pasteur proteins (collected from the bovis Pasteur page of TubercuList (<http://genolist.pasteur.fr/BCGList/>). 3414 (87%) of the *M. bovis* Ravenel translated sequences were perfect matches to the BCG reference. 370 of the *M. bovis* Ravenel translated sequences have amino acid differences compared to corresponding *M. bovis* BCG Pasteur proteins. All

translated protein sequences that contain non-determinable amino acids (secondary to non-determinable nucleotides) are listed in the supplementary tables 2 and 3 and contain an “X” at the position of the unknown amino acid.

In order to gain insight into potential evolutionary relationships between the strains studied and the possible role of SNPs in observed phenotypes, the SNPs were analyzed that resulted in amino acid substitutions, deletions and gene truncations between the previously sequenced *M. bovis* AF2122/97 and *M. bovis* Ravenel. As a representative set, 115 of the 233 SNPs that resulted in differences in the proteins coded for by annotated genes were analyzed. Of the 115 SNPs characterized, nearly half of the *M. bovis* Ravenel sequences had the consensus residue for the *M. tuberculosis* (MTB) complex.

2.3. Animals, vaccination, and challenge procedures

For mouse infections, 6 to 9 week old female SCID mice (National Cancer Institute) were infected intravenously into the tail vein with 2×10^6 CFU of bacteria in 0.2 mL PBS. For each strain, ten mice were infected and 24 hours post infection, three mice were sacrificed to confirm the dose administered and the remaining seven mice were followed for survival.

Thirty-two newborn Holstein bull calves were obtained from a TB-free herd in Sioux Center, IA and housed in a biosafety level-3 (BSL-3) facility at the National Animal Disease Center, Ames, Iowa according to Institutional Biosafety and Animal Care and Use Committee guidelines. Vaccine treatment groups included: no vaccination (n = 11), 10^6 cfu *M. bovis* BCG-Danish (n = 11), and 1×10^6 cfu *M. bovis* Ravenel $\Delta RD1$ (n = 10). Vaccines were administered subcutaneously at 2 wks of age. M. J. Brennan, United States Food and Drug Administration, Bethesda, MD kindly provided the BCG-Danish vaccine. The strain of *M. bovis* used for the challenge inoculum {95-1315, USDA, Animal Plant and Health Inspection Service (APHIS) designation} was originally isolated from a white-tailed deer in Michigan, USA and prepared as described previously [29].

Challenge inoculum of $\sim 10^3$ cfu in 2 ml of phosphate-buffered saline solution (PBS, 0.01 M, pH 7.2) was administered to calves at ~ 3.5 months of age by aerosol inoculation [25]. Briefly, inoculum was delivered to restrained calves by nebulization into a mask (Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. Upon inspiration, inoculum was inhaled through a one-way valve into the mask and directly into the lungs via the nostrils. The process continued until the inoculum, a 1 ml PBS wash of the inoculum tube, and an additional 2 ml PBS were delivered, a process taking ~ 10 min. Strict BL-3 safety protocols were followed to protect personnel from exposure to *M. bovis*.

2.2. Vaccine efficacy evaluation: bacterial recovery, mean disease score, histopathology, and lung radiographic analysis

All calves were euthanized by intravenous administration of sodium pentobarbital at approximately 4.5 months after challenge (~ 8 months of age). Tissues were collected and processed for characterization of gross lesions, the isolation of *M. bovis* and microscopic analysis included: palatine tonsil; lung; liver; spleen; and mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, mesenteric, and prescapular lymph nodes. Lymph nodes were sectioned at 0.5 cm intervals and examined. Following radiography, each lung lobe was sectioned at 0.5 – 1.0 cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semi-quantitative gross pathology scoring system adapted from Vordermeier *et al*, 2002 [30]. Lung lobes (left cranial, left caudal, right cranial, right caudal, middle and accessory) were individually scored based upon the following scoring system: 0 = no visible lesions; 1 = no external gross lesions, but lesions seen upon slicing; 2 = < 5 gross lesions of < 10 mm in diameter; 3 = > 5 gross

lesions of < 10 mm in diameter; 4 = >1 distinct gross lesion of > 10 mm in diameter; 5 = gross coalescing lesions. Cumulative mean scores were then calculated for each entire lung. Lymph node pathology was based on the following scoring system: 0 = no necrosis or visible lesions; 1 = small focus (1 to 2 mm in diameter); 2 = several small foci; 3 = extensive necrosis. Gross pathology data are presented as mean ($\pm$ standard error) disease score for mediastinal lymph node, tracheobronchial lymph node and lung.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed by standard paraffin-embedment techniques, cut in 5 μ m sections and stained with hematoxylin and eosin. Adjacent sections from samples containing caseonecrotic granulomata suggestive of tuberculosis were excised and stained by the Ziehl-Neelsen technique for identification of acid-fast bacteria. Microscopic tuberculous lesions were staged (I-IV) as described [31]. Data are presented as total and mean number of granulomas observed in each histologic lesion stage (i.e. I – IV) for lung and mediastinal lymph node sections.

For quantitative assessment of mycobacterial burden, left tracheobronchial lymph nodes were removed, examined for gross lesions, weighed, and entire lymph nodes (other than a small ~ 1 gram section for histology) homogenized in phenol red nutrient broth using a blender (Oster, Shelton, CT). Logarithmic dilutions (10^0 - 10^{-9}) of homogenates in PBS were plated in 100 μ l aliquots plated on Middlebrook 7H11 selective agar plates (Becton Dickinson) and incubated for 8 weeks at 37°C. Data are presented as mean ($\pm$ standard error) cfu per gram of tissue.

To provide an additional measure of the extent of lung lesions, lung lobes were removed at necropsy and individually radiographed using a MinXray machine (Model HF-100, Diagnostic Imaging, Rapid City, SD) with 3M Asymetrix Detail Screens and Ultimate 2000 film (3M Animal Care Products, St. Paul, MN) as described [9,31]. Lesions were identified on digital images of scanned radiographs, outlined and measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD) software (Figure 1). Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Using combined data from each lung lobe, results for individual animals are presented as the mean ($\pm$ standard error) percentage of affected lung.

2.4. Mononuclear cell culture and IFN- γ ELISA

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of peripheral blood buffy coat fractions collected into 2X acid citrate dextrose. Individual wells of 96-well round-bottom microtiter plates (Falcon, Becton-Dickinson; Lincoln Park, New Jersey) were seeded with 5×10^5 PBMC in a total volume of 200 μ l per well. Medium was RPMI 1640 (GIBCO, Grand Island, New York) supplemented with 2 mM L-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1% non-essential amino acids (Sigma), 2% essential amino acids (Sigma), 1% sodium pyruvate (Sigma), 50 mM 2-mercaptoethanol (Sigma), and 10% (v/v) fetal bovine sera (FBS). Wells contained medium plus 10 μ g/ml *M. bovis* PPD (Prionics AG, Schlieren, Switzerland), 1 μ g/ml rESAT-6:CFP-10, 1 μ g/ml pokeweed mitogen (PWM, as an indicator of cell functioning capacity, data not shown), or medium alone (no stimulation). After incubation for 48h at 39°C in 5% CO₂, culture supernatants were harvested and stored at -80° C until thawed for analysis by an ELISA kit (Bovigam, Prionics AG). Concentrations of IFN- γ in test samples were determined by comparing absorbances of test samples with absorbances of standards within a linear curve fit. Mean IFN- γ concentrations (ng/ml) produced in 48-hr cultures in response to antigen or mitogen minus concentrations in non-stimulated cultures (i.e., Δ IFN- γ) are presented.

2.5. IFN- γ ELISPOT assays

Cultured ELISPOT assay was based on a previously described method [12,32]. Short-term T-cell lines were generated by stimulating 2×10^6 /ml PBMC with a cocktail of recombinant Ag85A (1 μ g/ml), TB10.4 (1 μ g/ml), and PPD-B (5 μ g/ml). 10 U/ml of recombinant human IL-2 (Sigma, Poole, UK) was added to the PBMC cultures at days 5 and 8. Fresh media without IL-2 was added to the cultures at days 10 and 12. On day 13 of culture, 2×10^4 of cultured PBMC/well were added to polyvinylidene difluoride (PVDF) anti-bovine IFN- γ mAb (MABTECH, bovine/ovine/equine IFN- γ ELISPOT kit, Stockholm, Sweden) -coated 96-well assay plates (Millipore, Watford, UK) and incubated in the presence of antigen presenting cells (APC, see below for APC preparation methods) and 5 μ g/ml (per individual peptide) of a cocktail of 13 Ag85A derived synthetic peptides [33] and 14 TB10.4 derived peptides (20mers, overlapping by 12 residues), or medium alone. ELISPOT culture plates were incubated for 20h at 39°C/5% CO₂ and numbers of spot forming cells (SFC) were determined following the MABTECH kit instructions. For APC preparation, ELISPOT plates were coated at 4°C overnight with an anti-bovine IFN- γ from MABTECH (MABTECH) according to the kit instructions, followed by a blocking step (10% FCS/ RPMI 1640 media, 2h, 39°C/5% CO₂). Autologous APCs were prepared by incubating 2×10^5 freshly isolated PBMC (50 μ l volume, contains at least 10^4 adherent cells at a saturating level for response capability) in complete medium at 39°C/5% CO₂ for 90 min on coated and blocked ELISPOT plates. Non-adherent cells were discarded and the adherent cells (APCs) washed twice with warm RPMI 1640 media. Fresh complete media containing antigen and short-term line cells were then incubated as described above.

2.6. Skin test procedures

Three days prior to necropsy, calves received 0.1 ml (100 μ g) of *M. bovis* PPD and 0.1 ml (40 μ g) of *M. avium* PPD intradermally at separate clipped sites in the mid-cervical region according to guidelines described in USDA, APHIS circular 91-45-01 for the comparative cervical test. Skin thickness was measured with calipers prior to PPD administration and 72 hrs after injection. Balanced PPD's were obtained from the Brucella and Mycobacterial Reagents section of National Veterinary Services Laboratory, Ames, IA.

2.7. Dye tracking and Flow cytometry

Isolated PBMC cultures were prepared as described for IFN- γ analysis, stained with PKH67, and then incubated for 6 d. Staining of PBMC with PKH67 was performed according to manufacturer instructions (Sigma) and as described [34]. Briefly, 2×10^7 PBMC were centrifuged (10 min, 400 $\times$ g), supernatants aspirated, and cells resuspended in 1 ml of diluent provided in the PKH67 kit. Cells in diluent were added to 1 ml of PKH67 green fluorescent dye (2 μ M) and incubated 5 min followed by a 1 min incubation with 2 ml of fetal bovine sera (FBS) to adsorb the excess dye and stop further dye uptake by cells. Phenotype analysis of PBMC was performed as described previously [34]. Briefly, cells were harvested and incubated with 1 μ g primary monoclonal antibodies/ 10^6 cells (mAb's; CACT116A, CD25; GC50A1, CD4; BAQ111A, CD8; CACT61A, $\gamma\delta$ TCR; TH14B, MHC class II; all obtained from VMRD, Pullman WA) at room temperature for 15 min. Cells were then washed and stained with isotype appropriate goat anti-mouse phycoerythrin- (Southern Biotechnology Associates, Birmingham, AL), allophycocyanin- (Caltag Laboratories, Burlingame, CA), or Peridinin Chlorophyll Protein- (Becton Dickinson) conjugated secondary antibodies or hCTLA4/Ig-PE for CD80/86 (Ancel, Bayport, MN) at room temperature for 15 min. Four-color flow cytometric analyses was performed using a Becton Dickinson LSR flow cytometer. Data were analyzed with FlowJo software (Tree Star Inc., San Carlos, CA) and presented as the mean ($\pm$ standard error) percent positive cells or geometric mean fluorescence intensity (mfi) for each marker.

2.8. Statistics

Data were assessed for normality prior to statistical analysis and analyzed as a split-plot with repeated measures ANOVA using Statview software (version 5.0, SAS Institute, Inc., Cary, NC). Fisher's protected-LSD test was applied when effects ($P < 0.05$) were detected. For antibody data, one-way ANOVA and pairwise Holm-Sidak test were used for normally distributed data and Kruskal-Wallis / Dunn's method non-normally distributed data using SigmaStat software (version 3.0, Systat Software Inc., San Jose, CA). Cultured ELISPOT data were analysed by 1-way ANOVA (two-paired), with Tukey post-test analysis, with unpaired t-tests, linear regression analysis, or Spearman Rank correlation analysis using GraphPad Instat v3 (San Diego, CA).

3. Results and Discussion

3.1. Comparison of the sequence of *M. bovis* Ravenel genome to other tubercle bacilli

To extend the development of *M. bovis*-based vaccines in animals, the parental *M. bovis* Ravenel strain was sequenced to: (1) facilitate regulatory approval, (2) assure quality control for vaccine production and (3) compare with other mycobacterial strains. From SNP analysis (Supplementary Tables 2 and 3), it appears that *M. bovis* Ravenel is less diverged from the ancestral strain than *M. bovis* AF2122/97. Of particular interest were the identification of potential antigenic differences between *M. bovis* Ravenel and other sequenced *M. bovis* strains. Apparent deletions in the *M. bovis* Ravenel sequence were noted where coverage compared to the reference *M. bovis* AF2122/97 [27] sequence or *M. bovis* BCG Pasteur [35] was not apparent, and where flanking reads bridged the deletion. These deletions were between bases 209133-209222, 1782571-1783373, 3392498-3392536 and 4334012-4336417. Other regions with little to no coverage that may be deletions or rearrangements were detected, but the exact nature of these genomic differences was not clear.

Among the 54 SNPs between *M. bovis* AF2122/97 and *M. bovis* Ravenel in which Ravenel codes for the MTB complex consensus residue were differences in genes involved in carbohydrate utilization, including *aceE*, *glpK*, *pflA*, *pykA*, and *ugpB*. These changes may allow *M. bovis* Ravenel to grow on glycerinated media in the absence of pyruvate, as is true of *M. bovis* BCG and *M. tuberculosis* strains but not of *M. bovis* AF2122/97 [37]. In several genes involved in sulfate assimilation and metabolism such as *atsA*, *cysK2*, *cysN*, *cysW*, *metK*, *sseA*, and *sseB*, *M. bovis* Ravenel bears the MTB complex consensus residue, this suggests that unlike AF2122/97, it may require more sulfur beyond that available as sulfur-containing amino acids in the host. Additionally, a SNP in *recC* may contribute to differences in recombination between Ravenel and other members of the MTB complex and AF2122/97.

Consensus residues in several proteins involved in RNA metabolism such as those coded for by *rhlE*, *rnhB*, and *rphA* and extracytoplasmic function sigma factor gene *sigM* may lead to important regulatory differences between *M. bovis* Ravenel and other MTB complex members and *M. bovis* AF2122/97. It is noteworthy that *sigM* has been shown to regulate several ESAT-6 homologs; although a *sigM* mutant of *M. tuberculosis* CDC1551 is not attenuated in mice this may be due to its low basal expression [38]. Of potential importance to the relative pathogenicity of the strains, *M. bovis* Ravenel shares the MTB complex consensus for *mntH*, which codes for a manganese transport system that is similar to the NRAMPs (Natural resistance-associated macrophage proteins) [39]. Although several studies have failed to demonstrate a role for *mntH* in virulence [40,41], it is possible that the strain or animal model chosen masked an effect. The presence of MTB complex consensus residues at several positions in members of the NADH dehydrogenase complexes may also contribute to Ravenel's virulence (42).

3.2. *M. bovis* Ravenel $\Delta RD1$ is attenuated in mice

Previous studies have shown that genes in the *M. tuberculosis* *RD1* region are required for full virulence in SCID mice and mediate lung epithelial necrosis [22]. Since *M. bovis* is generally more virulent in mouse strains than *M. tuberculosis*, we compared the virulence of the *RD1* deletion of *M. bovis* Ravenel to the *RD1* deletion of three *M. tuberculosis* strains (Fig. 1). The parental strains and the *RD1* deletion mutants were all administered intravenously into Balb/c SCID mice for evaluation of pathogenicity as measured by survival. *M. bovis* Ravenel was as virulent as *M. tuberculosis* Erdman and more virulent than *M. tuberculosis* CDC1551 and H37Rv (Fig. 1). For all four strains, the *RD1* deletion was highly attenuated and the *M. bovis* Ravenel $\Delta RD1$ was slightly more virulent than the *M. tuberculosis* $\Delta RD1$ strains (Fig. 1).

3.3. Vaccine efficacy

Variables used to evaluate vaccine efficacy included: gross pathology (i.e., mean disease scores, Fig. 2), radiograph morphometry (Fig. 3), histopathology (Tables 2 and 3), and mycobacterial culture (Fig. 4). BCG- and $\Delta RD1$ -vaccinates had lower gross pathology scores as compared to non-vaccinates (Fig. 2, $P < 0.05$ except $P = 0.08$ for $\Delta RD1$ - versus non-vaccinates, lungs). Mean disease scores did not differ statistically between BCG- and $\Delta RD1$ -vaccinates. Lesion scoring by histology corroborated gross lesion scores (Tables 2 and 3). Likewise, the percent of affected lung area (i.e., radiograph morphometry data, Fig. 3) did not differ between BCG- and $\Delta RD1$ -vaccinates; and, both vaccine groups had lower ($P < 0.05$) percent affected lung area as compared to non-vaccinates. For TB, organ weight is often positively associated with the degree of pathological changes. Lung-associated lymph nodes (i.e., tracheobronchial and mediastinal lymph nodes) from BCG- and $\Delta RD1$ -vaccinates weighed less ($P < 0.05$) than respective lymph nodes from non-vaccinates (data not shown).

Despite considerable variability within the two vaccine groups, mean colonization of tracheobronchial lymph nodes was lower ($P < 0.05$) in BCG- and $\Delta RD1$ -vaccinates as compared to non-vaccinates (Fig. 4). *M. bovis* was cultured from mediastinal lymph nodes from all non-vaccinates (i.e., 11 / 11), all but one BCG-vaccinate (i.e., 10/11) and all but one $\Delta RD1$ -vaccinate (i.e., 9/10). With lungs, *M. bovis* was cultured from 6 of 11 BCG-vaccinates, 7 of 10 $\Delta RD1$ -vaccinates and 10 of 11 non-vaccinates. Additionally, *M. bovis* was isolated from one mesenteric, one hepatic, and one prescapular lymph node from a non-, BCG-, $\Delta RD1$ -vaccinate, respectively.

3.4. Immune responses to vaccination

IFN- γ responses to *M. bovis* PPD by BCG- and $\Delta RD1$ -vaccinates exceeded ($P < 0.05$) that of non-vaccinates ~3 months after vaccination and prior to challenge (Fig. 5A). By 30d after challenge, IFN- γ responses to *M. bovis* PPD by non-vaccinates exceeded ($P < 0.05$) that of BCG- and $\Delta RD1$ -vaccinates. Responses to *M. bovis* PPD by BCG- and $\Delta RD1$ -vaccinates did not appreciably change upon challenge. As expected, IFN- γ responses to rESAT-6:CFP-10 were not detected for any of the groups prior to challenge (Fig. 5B). After challenge, IFN- γ responses to rESAT-6:CFP-10 by non-vaccinates exceeded ($P < 0.05$) that of BCG- and $\Delta RD1$ -vaccinates confirming earlier findings suggesting ESAT-6 IFN- γ responses post-challenge as markers of disease progression and severity [30].

Using an assay to detect memory responses [32,43], IFN- γ ELISPOT responses to Ag85A and TB10.4 by BCG- and $\Delta RD1$ -vaccinates exceeded ($P < 0.05$) that of non-vaccinates ~2.5 months after vaccination and prior to challenge (Fig. 6). Results were similar to those detected with the standard ELISA for IFN- γ within PPD-stimulated supernatants (Fig. 5A). IFN- γ ELISPOT responses to Ag85A and TB10.4 were negatively correlated to the bacterial loads in the tracheobronchial lymph node (Fig. 7A, Spearman $r = -0.8195$, 95% CI: -0.9323 to -0.5608, $p < 0.0001$) and extent of gross pathology (data not shown, Spearman $r = -0.5514$,

95% CI: -0.8149 to -0.09893, $p = 0.017$); indicating that these responses are a positive surrogate/correlate for protection. Supporting this interpretation are results shown in Fig. 7B which indicated that vaccinated and protected calves from both vaccination groups, defined as calves without gross pathology, or reduced gross pathology compared to unvaccinated animals (defined by a pathology score < 7 which represented a natural separation in the data considering the distribution) presented with significantly higher cultured ELISPOT responses than unprotected ones (Fig. 7B, $p = 0.007$). Despite the strong association between protection and central memory responses, this parameter cannot be used as a general correlate to define the protection status of individual vaccinated animals. However, when responses within a group of vaccinated animals are considered, strong mean central memory responses/group are indicative of vaccination success.

Concerning skin test, the average change in skin thickness to *M. bovis* and *M. avium* PPD was 26 mm and 4 mm, respectively and regardless of vaccine status. The skin test response was not affected by vaccine treatment ($P > 0.05$, Supplemental Table 4).

Immediately prior to challenge, PPD-specific expansion and activation of CD4⁺ cells was detected in PBMC cultures from BCG- and $\Delta RD1$ -vaccinates as indicated by increased ($P < 0.05$) percentages of CD4⁺,CD25⁺(activated) cells; and CD4⁺,PKH67^{lo} (proliferative) cells (Table 4A). In response to PPD, MHC-II and CD80/86 expression was also increased on PBMC from BCG- and $\Delta RD1$ -vaccinates, a further indicator of activation. As with IFN- γ responses to PPD (Fig. 5), proliferative and activation responses by $\Delta RD1$ -vaccinates generally exceeded ($P < 0.05$) those of BCG-vaccinates (Table 4A). Significant PPD-specific proliferative responses were not detected for CD8⁺ or $\gamma\delta$ TCR⁺ cells (data not shown).

One month after challenge, immune responses by non-vaccinates exceeded ($P < 0.05$) respective responses prior to challenge (comparison of Table 4A to 4B), indicating a PPD-specific response to infection. As with IFN- γ responses to PPD (Fig. 5), proliferative and activation responses by BCG- and $\Delta RD1$ -vaccinates did not change significantly ($P > 0.05$) upon challenge with virulent *M. bovis* (comparison of Table 4A to 4B). Similar to previous findings [31], reduced in vitro recall responses after challenge were associated with effective vaccines (Table 4B).

4. Conclusions

Comparison of the sequence of the *M. bovis* Ravenel strain with the previously sequenced *M. bovis* AF2122/97 strain revealed that at many SNPs, *M. bovis* Ravenel has the MTB complex consensus residue, suggesting that the *RD1* deletion mutant of Ravenel is an attractive substrate for vaccine development. Key findings from the calf vaccine trial were: (1) vaccination of neonatal calves with $\Delta RD1$ provided equivalent efficacy as did BCG against aerosol challenge with virulent *M. bovis*, (2) mean central memory responses elicited by either $\Delta RD1$ or BCG prior to challenge correlated with reduced pathology and bacterial colonization, (3) vaccination with either $\Delta RD1$ or BCG did not interfere with an IFN- γ -based TB assay using ESAT-6 and CFP-10 antigens, and (4) reduced in vitro recall responses after challenge were associated with effective $\Delta RD1$ and BCG vaccines. The $\Delta RD1$ strain may prove useful as an alternative to *M. bovis* BCG for bovine TB vaccine programs, particularly if additional mutations are included for improved safety and immunogenicity. For future work, the inclusion of mutations that have been shown to evade host immunity such as pro-apoptotic [44] or IL-12 suppressing mutations [45] will enhance the usefulness of these vaccine candidates thus engendering vaccines that will limit colonization more dramatically than those observed for BCG and $\Delta RD1$. Future studies are scheduled to evaluate the effect of parent strain (i.e., *M. tuberculosis* versus *M. bovis*) and incorporation of additional deletions in the $\Delta RD1$ strain(s) to improve efficacy and/or safety. In the present study, we demonstrate that $\Delta RD1$ provides similar efficacy and immune

responses, as does BCG; thus, $\Delta RD1$ will be an appropriate comparator for later studies with multi-deletion $\Delta RD1$ vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. McNair J, Welsh MD, Pollock JM. The immunology of bovine tuberculosis and progression toward improved disease control strategies. *Vaccine* 2007;25(30):5504–5511. [PubMed: 17363118]
2. Palmer MV. Tuberculosis: a reemerging disease at the interface of domestic animals and wildlife. *Curr Top Microbiol Immunol* 2007;315:195–215. [PubMed: 17848066]
3. Cross ML, Aldwell F. Oral vaccination against bovine tuberculosis with *Mycobacterium bovis* BCG. *Expert Rev Vaccines* 2007;6(3):323–331. [PubMed: 17542748]
4. Krebs, JR. Bovine Tuberculosis in Cattle and Badgers. London, Ministry of Agriculture, Fisheries and Food Publications; London, UK: 1997.
5. Calmette A, Guérin C. Recherches expérimentales sur la défense de l'organisme contre l'infection tuberculeuse. *Ann Inst Pasteur (Paris)* 1911;25:625–641.
6. Buddle BM. Vaccination of cattle against *Mycobacterium bovis*. *Tuberculosis (Edinb)* 2001;81(12):125–32. [PubMed: 11463233]
7. Hope JC, Vordermeier HM. Vaccines for bovine tuberculosis: current views and future prospects. *Expert Rev Vaccines* 2005;4(6):891–903. [PubMed: 16372884]
8. Maue AC, Waters WR, Palmer MV, Nonnecke BJ, Minion FC, Brown WC, Norimine J, Foote MR, Scherer CF, Estes DM. An ESAT-6:CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis*. *Vaccine* 2007;25(24):4735–4746. [PubMed: 17499400]
9. Maue AC, Waters WR, Palmer MV, Whipple DL, Minion FC, Brown WC, Estes DM. CD80 and CD86, but not CD154, augment DNA vaccine-induced protection in experimental bovine tuberculosis. *Vaccine* 2004;23(6):769–779. [PubMed: 15542201]
10. Vordermeier M, Hewinson RG. Development of cattle TB vaccines in the UK. *Vet Immunol Immunopathol* 2006;112(12):38–48. [PubMed: 16682085]
11. Sambandamurthy VK, Jacobs WR Jr. Live attenuated mutants of *Mycobacterium tuberculosis* as candidate vaccines against tuberculosis. *Microbes Infect* 2005;7(56):955–61. [PubMed: 15914065]
12. Vordermeier HM, Whelan A, Cockle PJ, Farrant L, Palmer N, Hewinson RG. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. *Clin Diagn Lab Immunol* 2001;8:571–578. [PubMed: 11329460]
13. Waters WR, Nonnecke BJ, Palmer MV, Robbe-Austermann S, Bannantine JP, Stabel JR, Whipple DL, Payeur JB, Estes DM, Pitzer JE, Minion FC. Use of recombinant ESAT-6:CFP-10 fusion protein for differentiation of infections of cattle by *Mycobacterium bovis* and by *M. avium subsp. avium* and *M. avium subsp. paratuberculosis*. *Clin Diagn Lab Immunol* 2004;11(4):729–735. [PubMed: 15242948]
14. Renshaw PS, Panagiotidou P, Whelan A, Gordon SV, Hewinson RG, Williamson RA, Carr MD. Conclusive evidence that the major T-cell antigens of the *Mycobacterium tuberculosis* complex ESAT-6 and CFP-10 form a tight, 1:1 complex and characterization of the structural properties of ESAT-6, CFP-10, and the ESAT-6:CFP-10 complex. Implications for pathogenesis and virulence. *J Biol Chem* 2002;277:21598–21603. [PubMed: 11940590]

15. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. *Lancet* 2000;356(9235):1099–1104. [PubMed: 11009160]
16. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, Small PM. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science* 1999;284:1520–1523. [PubMed: 10348738]
17. Gey Van Pittius NC, Gamielien J, Hide W, Brown GD, Siezen RJ, Beyers AD. The ESAT-6 gene cluster of *Mycobacterium tuberculosis* and other high G+C Gram-positive bacteria. *Genome Biol* 2001;2:44–48.
18. Mahairas GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. *J Bacteriol* 1996;178:1274–1282. [PubMed: 8631702]
19. Pym AS, Brodin P, Brosch R, Huerre M, Cole ST. Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Mol Microbiol* 2002;46(3):709–717. [PubMed: 12410828]
20. Brodin P, de Jonge MI, Majlessi L, Leclerc C, Nilges M, Cole ST, Brosch R. Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of *Mycobacterium tuberculosis*, reveals key residues involved in secretion, complex formation, virulence, and immunogenicity. *J Biol Chem* 2005;280(40):33953–33959. [PubMed: 16048998]
21. Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, et al. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *Nat Med* 2003;9(5):533–539. [PubMed: 12692540]
22. Hsu T, Hingley-Wilson SM, Chen B, Chen M, Dai AZ, Morin PM, et al. The primary mechanism of attenuation of bacillus Calmette-Guerin is a loss of secreted lytic function required for invasion of lung interstitial tissue. *Proc Natl Acad Sci USA* 2003;100(21):12420–12425. [PubMed: 14557547]
23. Lewis KN, Liao R, Guinn KM, Hickey MJ, Smith S, Behr MA, Sherman DR. Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guerin attenuation. *J Infect Dis* 2003;187(1):117–23. [PubMed: 12508154]
24. Dunn PL, North RJ. Virulence ranking of some *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains according to their ability to multiply in the lungs, induce lung pathology, and cause mortality in mice. *Infect Immun* 1995;63:3428–3437. [PubMed: 7642273]
25. Palmer MV, Waters WR, Whipple DL. Aerosol delivery of virulent *Mycobacterium bovis* to cattle. *Tuberculosis (Edinb)* 2002;82(6):275–282. [PubMed: 12623270]
26. Bardarov S, Bardarov S Jr, Pavelka MS Jr, Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, Jacobs WR Jr. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis*, *M. bovis* BCG and *M. smegmatis*. *Microbiology* 2002;148:3007–3017. [PubMed: 12368434]
27. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, Pryor M, Duthoy S, Grondin S, Lacroix C, Monsempe C, Simon S, Harris B, Atkin R, Doggett J, Mayes R, Keating L, Wheeler PR, Parkhill J, Barrell BG, Cole ST, Gordon SV, Hewinson RG. The complete genome sequence of *Mycobacterium bovis*. *Proc Natl Acad Sci USA* 2003;100:7877–7882. [PubMed: 12788972]
28. Li R, Li Y, Kristiansen K, Wang J. SOAP: short oligonucleotide alignment program. *Bioinformatics* 2008;24:713–714. [PubMed: 18227114]
29. Palmer MV, Waters WR, Whipple DL. Aerosol exposure of white-tailed deer (*Odocoileus virginianus*) to *Mycobacterium bovis*. *J Wildlife Dis* 2003;39:817–823.
30. Vordermeier HM, Chambers MA, Cockle PJ, Whelan AO, Simmons J, Hewinson RG. Correlation of ESAT-6 specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infect Immun* 2002;70(6):3026–3032. [PubMed: 12010994]
31. Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Capinos Scherer CF, Estes DM, Jacobs WR, Glatman-Freedman A, Larsen ML. Failure of a *Mycobacterium tuberculosis* RD1 × panCD double deletion mutant in a neonatal aerosol *M. bovis* challenge model: comparisons to responses elicited by *M. bovis* bacilli Calmette Guérin. *Vaccine* 2007;25:7832–7840. [PubMed: 17931755]

32. Whelan AO, Wright DC, Chambers MA, Singh M, Hewinson RG, Vordermeier HM. Evidence for enhanced central memory priming by live *Mycobacterium bovis* BCG vaccine in comparison with killed BCG formulations. *Vaccine* 2008;26(2):166–173. [PubMed: 18055073]
33. Vordermeier HM, Rhodes SG, Dean G, Goonetilleke N, Huygen K, Hill AV, et al. Cellular immune responses induced in cattle by heterologous prime-boost vaccination using recombinant viruses and bacille Calmette-Guerin. *Immunol* 2004;112(3):461–470.
34. Waters WR, Rahner TE, Palmer MV, Cheng D, Nonnecke BJ, Whipple DL. Expression of L-Selectin (CD62L), CD44, and CD25 on activated bovine T cells. *Infect Immun* 2003;71(1):317–326. [PubMed: 12496181]
35. Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, Dos Santos S, Duthoy S, Lacroix C, Garcia-Pelayo C, Inwald JK, Golby P, Garcia JN, Hewinson RG, Behr MA, Quail MA, Churcher C, Barrell BG, Parkhill J, Cole ST. Genome plasticity of BCG and impact on vaccine efficacy. *Proc Natl Acad Sci USA* 2007;104:5596–5601. [PubMed: 17372194]
36. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 1997;25:3389–3402. [PubMed: 9254694]
37. Keating LA, Wheeler PR, Mansoor H, Inwald JK, Dale J, Hewinson RG, Gordon SV. The pyruvate requirement of some members of the *Mycobacterium tuberculosis* complex is due to an inactive pyruvate kinase: implications for in vivo growth. *Mol Microbiol* 2005;56:163–174. [PubMed: 15773987]
38. Agarwal N, Woolwine SC, Tyagi S, Bishai WR. Characterization of the *Mycobacterium tuberculosis* sigma factor SigM by assessment of virulence and identification of SigM-dependent genes. *Infect Immun* 2007;75:452–461. [PubMed: 17088352]
39. Bellamy R. The natural resistance-associated macrophage protein and susceptibility to intracellular pathogens. *Microbes Infect* 1999;1:23–27. [PubMed: 10594973]
40. Boechat N, Lagier-Roger B, Petit S, Bordat Y, Rauzier J, Hance AJ, Gicquel B, Reyat JM. Disruption of the gene homologous to mammalian Nramp1 in *Mycobacterium tuberculosis* does not affect virulence in mice. *Infect Immun* 2002;70:4124–4131. [PubMed: 12117920]
41. Domenech P, Pym AS, Cellier M, Barry CE 3rd, Cole ST. Inactivation of the *Mycobacterium tuberculosis* Nramp orthologue (mntH) does not affect virulence in a mouse model of tuberculosis. *FEMS Microbiol Lett* 2002;207:81–86. [PubMed: 11886755]
42. Velmurugan K, Chen B, Miller JL, Azogue S, Gurses S, Hsu T, Glickman M, Jacobs WR Jr, Porcelli SA, Briken V. *Mycobacterium tuberculosis* nuoG is a virulence gene that inhibits apoptosis of infected host cells. *PLoS Pathog* 2007;3:e110. [PubMed: 17658950]
43. Vordermeier HM, Huygen K, Singh M, Hewinson RG, Xing Z. Immune responses induced in cattle by vaccination with a recombinant adenovirus expressing Mycobacterial antigen 85A and *Mycobacterium bovis* BCG. *Infect Immun* 2006;74:1416–1418. [PubMed: 16428796]
44. Hinchey J, Lee S, Jeon B, Basaraba RJ, Venkataswamy M, Chen B, Chan J, Braunstein M, Orme IM, Derrick S, Morris S, Jacobs WR, Porcelli SA. Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. *J Clin Invest* 2007;117:2279–2288. [PubMed: 17671656]
45. Dao D, Sweeney K, Hsu T, Gurucha SS, Nascimento I, Roshevsky D, Besra GS, Chan J, Porcelli SA, Jacobs WR. Mycolic acid modification by the mmaA4 gene of *M. tuberculosis* modulates IL-12 production. *PLoS Pathog* 2008;4:e1000081. [PubMed: 18535659]
46. Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR Jr. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 1990;4:1911–1919. [PubMed: 2082148]
47. Ravenel MP. The Comparative Virulence of the Tubercle Bacillus from Human and Bovine Sources. *Lancet* 1901;2:349–356.
48. Schmitt SM, Fitzgerald SD, Cooley TM, Bruning-Fann CS, Sullivan L, Berry D, et al. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. *J Wildl Dis* 1997;33(4):749–758. [PubMed: 9391958]

Fig. 1. Attenuation of *M. bovis* and *M. tuberculosis* $\Delta RD1$ strains in SCID mice

Balb/c SCID mice (ten per group) were infected intravenously with 2×10^6 CFU of bacteria in 0.2 mL. For each strain, ten mice were infected and 24 hours post infection, three mice were sacrificed to confirm the dose administered and the remaining seven mice were followed for survival. Data represent the number of mice surviving at each time point. Groups are plotted as: wild-type *M. bovis* Ravenel, filled diamond; *M. bovis* Ravenel $\Delta RD1$ (mc²5391), open diamond; wild-type *M. tuberculosis* CDC1551, filled square; *M. tuberculosis* CDC1551 $\Delta RD1$ (mc²5342), open square; wild-type *M. tuberculosis* Erdman, filled triangle; *M. tuberculosis* Erdman $\Delta RD1$ (mc²5341), open triangle; wild-type *M. tuberculosis* H37Rv, filled circle; and *M. tuberculosis* H37Rv $\Delta RD1$ (mc²5340), open circle.

Fig. 2. Gross pathology

At necropsy, tracheobronchial lymph nodes, mediastinal lymph nodes, and lungs were evaluated for lesions based upon a scoring system adapted from Vordermeier et al., 2002 [30]. Values represent mean ($\pm$ standard error) mean disease scores ($n = 11$, non-vaccinates; $n = 11$, BCG-vaccinates; $n = 10$, *M. bovis* Δ RD1-vaccinates).

*Differs from controls, $P < 0.05$.

+Differs from controls, $P = 0.08$ and BCG-vaccinates, $P < 0.05$.

Fig. 3. Radiographic morphometry

Lung lobes were removed at necropsy and individually radiographed. Lesions were identified on digital images of scanned radiographs, outlined, and measured (Fig. 1). Affected area was divided by total lung area then multiplied by 100 to determine percent affected lung. Values represent mean ($\pm$ standard error) percent affected lung (n = 11, non-vaccinates; n = 11, BCG-vaccinates; n = 10, *M. bovis* Δ RD1-vaccinates).

*Differs from controls, $P < 0.05$.

Fig. 4. Quantitative culture

To eliminate bias based on organ sampling site, entire tracheobronchial lymph nodes were homogenized and cultured for *M. bovis*. Values represent mean ($\pm$ standard error) log₁₀ cfu / g of tissue (n = 11, non-vaccinates; n = 11, BCG-vaccinates; n = 10, *M. bovis* ΔRD1-vaccinates).

*Differs from controls, P < 0.05.

Fig. 5. Interferon- γ responses

Blood mononuclear cells were isolated from cattle at indicated time points relative to challenge with virulent *M. bovis* and cultured with 10 μ g/ml *M. bovis* PPD (Prionics AG) (panel A), 1 μ g/ml rESAT-6:CFP-10 (panel B), or medium alone (no stimulation) for 48 hrs. Supernatants were harvested and IFN- γ concentrations determined by ELISA (Bovigam, Prionics, Ag).

Values represent mean ($\pm$ standard error) responses to antigen minus the response to media alone ($n = 11$, non-vaccinates; $n = 11$, BCG-vaccinates; $n = 10$, *M. bovis* Δ RD1-vaccinates).

^{a-c} Differing superscripts at individual time points indicate that the responses differ, $P < 0.05$.

Fig. 6. Memory responses post-vaccination

Cultured ELISPOT analysis was performed ~2.5 months after vaccination and prior to challenge with virulent *M. bovis*. Short-term T-cell lines were generated by stimulating PBMC with a cocktail of recombinant Ag85A (1 µg/ml), TB10.4 (1 µg/ml), and PPD-B (5 µg/ml) for 13 days followed by transfer to ELISPOT plates with APC and addition of a cocktail of 13 Ag85A derived synthetic peptides and 14 TB10.4 derived peptides (20mers, overlapping by 12 residues), or medium alone. Results are expressed as mean spot forming cells (SFC)/million cells ($\pm$ standard deviation).

Fig. 7. Correlation of memory responses ~2.5 months after vaccination (cultured ELISPOT assay) with disease-outcome (bacterial colonization and pathology scores) after infection

A. Responses of individual animals in relation to bacterial loads in the tracheobronchial lymph nodes (Log_{10} CFU/g tissue) are compared to cultured ELISPOT responses. For ELISPOT responses, short-term T-cell lines were generated by stimulating PBMC with a cocktail of recombinant Ag85A (1 $\mu\text{g}/\text{ml}$), TB10.4 (1 $\mu\text{g}/\text{ml}$), and PPD-B (5 $\mu\text{g}/\text{ml}$) for 13 days followed by transfer to ELISPOT plates with APC and addition of a cocktail of 13 Ag85A derived synthetic peptides and 14 TB10.4 derived peptides (20mers, overlapping by 12 residues), or medium alone. Spearman Rank $r = -0.8195$ (95% CI: -0.9323 to -0.5608), $p < 0.0001$. Linear regression analysis: $r^2 = 0.5348$ ($p = 0.0006$, broken line indicates 95% confidence intervals).

B. Memory responses in relation to disease severity (pathology scores). Responses of vaccine (BCG, or Δ RD1, pathology score ≤ 7 , $n = 10$)-protected animals, against animals that were not protected (i.e., unvaccinated controls, and those BCG or Δ RD1 vaccinates not protected, pathology scores > 7 , $n = 9$). Cumulative pathology scores were determined by addition of scores from individual lung lobes, tracheobronchial lymph node, and mediastinal lymph node scores. Results are expressed as mean spot forming cells (SFC)/million cells ($\pm$ standard deviation).

Table 1**Strains Used in this study**

Strain	Genotype	How constructed	Source or Reference
<i>M. smegmatis</i> mc ² 155	<i>ept</i>	NA	Snapper et al., 1990 [46]
<i>M. bovis</i> Ravenel	Wild-type	Gift from John Chan who acquired the strain ATCC (TMC 401)	American Type Culture Collection, Ravenel 2001 [47]
mc ² 5391	$\Delta RD1::\gamma\delta res\ hyg\ \gamma\delta res$	Specialized transduction of <i>M. bovis</i> Ravenel using phAE343	This work
mc ² 5401	$\Delta RD1::\gamma\delta res\ hyg\ sacB\ \gamma\delta res$	Specialized transduction of <i>M. bovis</i> Ravenel using phAE344	This work
<i>M. bovis</i> 93-1315		NA	Schmitt et al, 1997 [48]
<i>M. tuberculosis</i> CDC1551	Wild-type		
mc ² 5342	$\Delta RD1::\gamma\delta res\ hyg\ \gamma\delta res$	Specialized transductant of <i>M. tuberculosis</i> CDC1551 using phAE343	This work
<i>M. tuberculosis</i> Erdman	Wild-type		
mc ² 5341	$\Delta RD1::\gamma\delta res\ hyg\ \gamma\delta res$	Specialized transductant of <i>M. tuberculosis</i> Erdman using phAE343	This work
<i>M. tuberculosis</i> H37Rv	Wild-type		
mc ² 5340	$\Delta RD1::\gamma\delta res\ hyg\ \gamma\delta res$	Specialized transductant of <i>M. tuberculosis</i> H37Rv using phAE343	This work

Table 2
Histologic evaluation of lung-associated lymph node (i.e., mediastinal)

Treatment	Histologic Stage ^a				Total (I-IV) ^b
	I	II	III	IV	
Controls (n = 11)	10.8 (3.8)	1.9 (0.9)	4.9 (1.2)	5.2 (0.9)	22.8 (4.6)
BCG (n = 11)	5.0 (1.3)	1.2 (0.5)	4.6 (1.2)	0.6 (0.4) [‡]	11.5 (2.7) [‡]
$\Delta RD1$ (n = 10)	5.3 (2.1)	1.9 (1.1)	1.4 (0.7) [‡]	1.8 (1.0) [‡]	10.4 (4.2) [¶]

^aMicroscopic tuberculous lesions were staged (I-IV) as described [31]. Disease severity progresses from stage I to IV. The number of granulomas in each histologic stage per section of mediastinal lymph node was enumerated. Similar results were also detected with tracheobronchial lymph nodes (data not shown).

^bMean ($\pm$ standard error) number of granulomas (i.e., regardless of stage) detected on a section of mediastinal lymph node for each vaccine treatment.

[‡]P $\leq$ 0.05 as compared to controls

[¶]P $\leq$ 0.1 as compared to controls

Table 3

Histologic evaluation of lung

Treatment	Histologic Stage ^a				
	I	II	III	IV	Total (I-IV) ^b
Controls (n = 11)	3.0 (1.4)	0.9 (0.5)	3.1 (1.1)	1.7 (0.7)	8.7 (3.2)
BCG (n = 11)	0.1 (0.1) [†]	0.0 (0.0) [¶]	0.5 (0.3) [†]	0.1 (0.1) [†]	0.6 (0.4) [†]
ΔRD1 (n = 10)	0.9 (0.6)	0.3 (0.2)	0.6 (0.3) [†]	0.4 (0.2) [¶]	2.2 (0.9) [¶]

^aMicroscopic tuberculous lesions were staged (I-IV) as described [31]. Disease severity progresses from stage I to IV. The number of granulomas in each histologic stage per section of lung was enumerated.

^bMean number of granulomas (i.e., regardless of stage) detected on a section of lung for each vaccine treatment.

[†]P ≤ 0.05 as compared to controls

[¶]P ≤ 0.1 as compared to controls

Table 4
Alterations in PBMC composition upon in vitro antigen stimulation

A. Three months after vaccination, prior to challenge with virulent <i>M. bovis</i>					
Vaccine Group	CD4+	CD4+,CD25+	CD4+,PKH67 ^{lo}	CD80/86 mfi	MHC-II mfi
Non-vaccinated	-0.9 (0.3) ^a	0.1 (0.5) ^a	0.0 (0.3) ^a	-2.7 (1.7) ^a	-0.4 (1.5) ^a
BCG-vaccinated	8.9 (2.8) ^b	6.7 (0.8) ^b	9.8 (2.6) ^b	11.9 (3.1) ^b	16.1 (5.8) ^b
ΔRD1-vaccinated	22.8 (5.6) ^c	14.3 (2.4) ^c	24.0 (6.0) ^c	17.7 (3.9) ^b	27.5 (5.2) ^c
B. One month after challenge with virulent <i>M. bovis</i>					
Vaccine Group	CD4+	CD4+,CD25+	CD4+,PKH67 ^{lo}	CD80/86 mfi	MHC-II mfi
Non-vaccinated	36.4 (6.2) ^{a†}	18.5 (3.1) [‡]	36.4 (5.9) ^{a†}	17.6 (4.2) [‡]	38.2 (4.0) ^{a†}
BCG-vaccinated	9.4 (2.3) ^b	11.5 (1.6)	10.1 (2.3) ^b	15.9 (4.1)	13.2 (8.6) ^b
ΔRD1-vaccinated	17.3 (4.4) ^b	17.3 (2.8)	19.0 (4.5) ^b	21.0 (4.6)	19.4 (6.3) ^b

PBMC were cultured with 10 µg/ml *M. bovis* PPD or medium alone for 6d and analyzed by flow cytometry. Data are presented as the difference in either percent positive cells or mean fluorescence intensity (mfi) in PBMC cultures from PPD-stimulated cultures minus the respective levels in non-stimulated cultures [i.e., Δmean (± standard error), n = 11, 11, and 10 for non-, BCG-, and ΔRD1-vaccinates].

^{a-c} Differing superscripts for each phenotype indicate that the responses differ between vaccine groups (i.e., vertical comparisons within each table), P < 0.05.

[‡] Differs (P < 0.05) between the two time-points within each vaccine group (i.e., comparisons between Table 4A and 4B).