Interleukin-17A as a Biomarker for Bovine Tuberculosis

W. Ray Waters,⁎ Mayara F. Maggioli,⁎ Mitchell V. Palmer,⁎ Tyler C. Thacker,⁎ Jodi L. McGill,⁎ H. Martin Vordermeier,⁎ Linda Berney-Meyer,⁎ William R. Jacobs, Jr,⁎ Michelle H. Larsen⁎

National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa, USA; Iowa State University, College of Veterinary Medicine, Ames, Iowa, USA; Department of Diagnostic Medicine and Pathobiology, College of Veterinary Medicine, Kansas State University, Manhattan, Kansas, USA; TB Research Group, Animal and Plant Health Agency, Addlestone, United Kingdom; Albert Einstein College of Medicine, Department of Microbiology and Immunology, Bronx, New York, USA.

T helper 17 (Th17)-associated cytokines are integral to the immune responses to tuberculosis, initiating both protective and harmful inflammatory responses. The aim of the present study was to evaluate applied aspects of interleukin-17 (IL-17) biology in the context of Mycobacterium bovis infection of cattle. Using transcriptome sequencing (RNA-Seq), numerous Th17-associated cytokine genes (including IL-17A, IL-17F, IL-22, IL-19, and IL-27) were upregulated >9-fold in response to purified protein derivative stimulation of peripheral blood mononuclear cells from experimentally M. bovis-infected cattle. Protective vaccines elicited IL-17A, IL-17F, IL-22, and IL-27 responses. Reduced IL-17A responses by vaccine recipients, compared to nonvaccinated animals, at 2.5 weeks after M. bovis challenge correlated with reduced disease burdens. Additionally, IL-17A and interferon gamma (IFN-γ) responses were highly correlated and exhibited similar diagnostic capacities. The present findings support the use of Th17-associated cytokines as biomarkers of infection and protection in the immune responses to bovine tuberculosis.
sponses were directly compared to IFN-γ responses, given the widespread use of IGRAs for TB diagnosis, vaccine, and pathogenicity studies.

**MATERIALS AND METHODS**

**Study overview and aerosol infection with Mycobacterium bovis.** Samples from four independent studies were included, i.e., one study that compared the virulence of two field strains of _M. bovis_ in calves, two vaccine efficacy studies, and a _M. bovis_ infection-only study (for RNA-Seq samples). An overview of the studies, including treatment groups, challenge strains and doses, and timing of treatments, is provided in Table 1. Two strains of _M. bovis_ were used for challenge inocula in the various studies, i.e., 95-1315 (Michigan white-tailed deer isolate) (16) and 10-7428 (Colorado Holstein isolate) (17). Challenge inocula from frozen stocks were prepared in Middlebrook 7H9 liquid medium (Becton Dickinson, Franklin Lakes, NJ) supplemented with 10% oleic acid-albumin-dextrose complex (OADC) plus 0.05% Tween 80 (Sigma, St. Louis, MO), following standard techniques (18). Holstein steers were obtained from TB-free herds in Iowa and housed in a biosafety level 3 (BSL3) facility at the National Animal Disease Center (Ames, IA), according to institutional biosafety and animal care and use committee guidelines and oversight (i.e., formal review and approval of studies). For experimental infection, Holstein steers received virulent _M. bovis_ by aerosol, as described previously (19). Strict biosafety protocols were followed to protect personnel from exposure to _M. bovis_ throughout the study, including BSL3 containment upon initiation of _M. bovis_ challenge in animal rooms and standard BSL3 laboratory practices for handling of _M. bovis_ cultures and samples from _M. bovis_-infected animals.

**Vaccine efficacy studies.** Two independent vaccine efficacy studies were performed. The age of calves at vaccination and the vaccine and challenge intervals are provided in Table 1. Briefly, calves were vaccinated subcutaneously at 2 to 3 weeks of age, challenged with virulent _M. bovis_ at ~4 months of age, and euthanized at ~8 months of age. In the 2007 study (20), the vaccine treatments were as follows: no vaccination (n = 11), 10^6 CFU _M. bovis_ bacillus Calmette-Guerin (BCG) Danish (n = 11), and 10^6 CFU _M. bovis_ Rv Aerged DRD1 (n = 10). In the 2014 study, the vaccine treatment groups were as follows: no vaccination (n = 10), 10^6 CFU _M. bovis_ BCG Danish (n = 9), and 10^6 CFU (total dose) of a cocktail of four BCG Danish deletion strains, i.e., BCG Δfdr8, BCG ΔleuCD Δpsk16, BCG ΔmmaA4 (21), and BCG ΔmetA (22) (n = 10). All four BCG Danish deletion derivatives (Δfdr8, ΔleuCD Δpsk16, ΔmetA, and ΔmmaA4) are more attenuated and safer than the parent BCG strain in immunocompromised mice (21; L. Berney-Meyer, M. Larsen, and W. R. Jacobs, unpublished data). In immunocompetent mice, the BCG deletion derivatives Δfdr8, ΔmmaA4, and Δpsk16 each result in enhanced mycobacterial immunogenicity through enhanced cross-presentation of mycobacterial antigens (Δfdr8), cytokine modulation (ΔmmaA4), and biofilm formation (Δpsk16), compared to the parental BCG (21; Berney-Meyer et al., unpublished). BCG mutants, such as these, may also be used as vaccine vectors to promote epitope-specific responses (e.g., BCG Δpsk12 for enhanced CD8 responses) (23).

**Assessment of mycobacterial lesions and colonization.** All calves were euthanized ~4 to 4.5 months (Table 1) after challenge, by intravenous administration of sodium pentobarbital. Tissues were examined for gross lesions and processed for microscopic analysis and isolation of _M. bovis_. Tissues collected included lung, liver, and mandibular, parotid, median retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric lymph nodes. Lymph nodes were sectioned at 0.5- to 1.0-cm intervals and examined. Each lung lobe was sectioned at 0.5- to 1.0-cm intervals and examined separately. Lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semiquantitative gross pathology scoring system adapted from the report by Vordermeier et al. (24). Head and abdominal lymph nodes were not included in the pathology scoring analysis because the route (aerosol) and duration (4 to 4.5 months) of experimental infection resulted in lesions focused primarily in the lungs and lung-associated lymph nodes. Lung lobes (left cranial, left caudal, right cranial, right caudal, and lingular) were examined and scored (0-4) for lesions, with 0 denoting absence of lesions and 4 representing severe congestion, edema, and infiltration of macrophages and lymphocytes.

**TABLE 1 Experimental design and efficacy parameters**

| Study and group | Vaccination | M. bovis challenge | Pathology score | Culture (log CFU/g) |
|-----------------|-------------|-------------------|-----------------|-------------------|
|                 | Age (wk)   | Interval (mo)      | Strain          | Dose (CFU)        | Interval (mo) | (mean ± SEM) | (mean ± SEM) |
| Comparative virulence study | NA | NA | NA | NA | 0 ± 0 | 0 ± 0 |
| No infection (n = 7) | NA | NA | NA | NA | 0 ± 0 | 0 ± 0 |
| M. bovis strain 95-1315 infection (n = 8) | 95-1315 | 10^4 | 4 | 16.4 ± 2.5^c | 3.25 ± 0.33^c |
| M. bovis strain 10-7428 infection (n = 8) | 10-7428 | 10^4 | 4 | 20.5 ± 1.3^c | 3.53 ± 0.20^c |
| Vaccine efficacy study from 2014 | NA | 10-7428 | 600 | 4.5 | 12.0 ± 1.6 | 3.86 ± 0.28 |
| No vaccination (n = 10) | NA | 10-7428 | 600 | 4.5 | 0.6 ± 0.4^d | 0.34 ± 0.34^d |
| BCG vaccination (n = 9) | 3 | 3.5 | 10-7428 | 600 | 4.5 | 0.9 ± 0.4^d | 1.74 ± 0.72^d |
| BCG mutant vaccination (n = 10) | 3 | 3.5 | 10-7428 | 600 | 4.5 | 14.1 ± 1.4 | 3.95 ± 0.13 |
| Vaccine efficacy study from 2007 | NA | 95-1315 | 10^3 | 4.5 | 3.4 ± 0.9^e | 2.59 ± 0.42^e |
| No vaccination (n = 11) | NA | 95-1315 | 10^3 | 4.5 | 6.0 ± 1.8^f | 2.62 ± 0.59^f |
| BCG vaccination (n = 11) | 2 | 3 | 95-1315 | 10^3 | 4.5 | 11.7 ± 1.6 | 4.17 ± 0.28 |
| M. bovis ΔRD1 vaccination (n = 10) | 2 | 3 | 95-1315 | 10^3 | 4.5 | 11.7 ± 1.6 | 4.17 ± 0.28 |
| RNA-Seq study | NA | 95-1315 | 8 × 10^9 | 11 | All had lesions | All were culture positive |

^a^ Age of vaccination and interval between vaccination and aerosol challenge with virulent _M. bovis_.

^b^ Strain and dose of virulent strain administered by aerosol and interval between challenge and necropsy.

^c^ Total gross pathology scores, which include scores for tracheobronchial and mediastinal (i.e., pulmonary) lymph nodes and lung lobes.

^d^ _M. bovis_ CFU per gram of tracheobronchial lymph node.

^e^ See reference 37 for additional details of this study.

^f^ NA, not applicable (i.e., animals were not vaccinated or challenged).

^g^ Differences from the noninfected or nonvaccinated group within each study (P ≤ 0.05, ANOVA followed by Tukey’s multiple-comparison test).

^h^ See reference 20 for additional details of this study.

^i^ Pathology scoring and quantitative culture were not applied; however, all animals had tuberculous lesions and _M. bovis_ was isolated from each animal.
craniol, right caudal, middle, and accessory) were individually assessed with the following scoring system: 0, no visible lesions; 1, no external gross lesions but lesions seen after slicing; 2, <5 gross lesions <10 mm in diameter; 3, >5 gross lesions <10 mm in diameter; 4, >1 distinct gross lesion >10 mm in diameter; 5, gross coalescing lesions. Scoring of lymph node pathology was based on the following 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 total gross pathology scores (mean ± standard error of the mean [SEM]), including scores for each lung lobe as well as the tracheobronchial and mediastinal lymph nodes.

Tissues collected for microscopic analysis were fixed by immersion in 10% neutral buffered formalin. For microscopic examination, formalin-fixed tissues were processed with standard paraffin-embedding techniques, cut in 5-μm sections, and stained with hematoxylin and eosin. Adjacent sections from samples containing casonoecectric granulomata, suggesting tuberculosis, were stained with the Ziehl-Neelsen technique. Whole-blood stimulation. Duplicate 250-μl heparinized whole-blood aliquots were distributed in 96-well plates with RPMI 1640 medium (Sigma) and lysed with 150 g/ml RLT (Qiagen, Valencia, CA), according to the manufacturer’s directions. Replicate wells were incubated at 39°C for 8 weeks at 37°C for determination of log_{10} CFU per gram of tissue. Is6110 real-time PCR, as described by Thacker et al. (27), was used to confirm that colonies were M. bovis.

**Whole-blood stimulation.** Duplicate 250-μl heparinized whole-blood aliquots were distributed in 96-well plates with RPMI 1640 medium (Sigma) alone, 1 μg/ml recombinant early secretory antigenic target 6 (rESAT-6):culture filtrate protein 10 (rCFP10) (a gift from Chris Minion, Iowa State University), 1 μg/ml each of recombinant Ag85A (rAg85A) and recombinant TB10.4 (rTB10.4) (Lionex Diagnostics and Therapeutics GmbH, Braunschweig, Germany), 10 μg/ml M. bovis PPD (CSL; Pri-onics Ag), or 1 μg/ml pokeweed mitogen (PWM) (Sigma) and were incubated at 39°C in 5% CO₂ for 18 h for cytokine analysis by enzyme-linked immunosorbent assay (ELISA). The normal body temperature of cattle (Bos taurus) is 39°C and incubation of human blood at 39°C, rather than 37°C, augments cytokine responses (28).

**Cell culture.** Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation of peripheral blooduffy coat fractions collected into 2× acid-citrate-dextrose, as described previously (12). PBMCs were seeded into 96-well round-bottom microtiter plates (Falcon; Becton-Dickinson, Lincoln Park, NJ) at 1×10⁵ cells in a total volume of 200 μl of complete RPMI 1640 (RPMI 1640 with 10% [vol/vol] fetal bovine serum [FBS] [Atlanta Biologics, Lawrenceville, GA], 2 mM l-glutamine, 25 mM HEPES buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 1% nonessential amino acids, 2% essential amino acids, 1% sodium pyruvate, and 50 μM 2-mercaptoethanol [all reagents in complete RPMI 1640 medium from Sigma except for FBS]). Wells contained medium alone (nonstimulated), 1 μg/ml each of rAg85A and rTB10.4 (Lionex Diagnostics and Therapeutics GmbH), 1 μg/ml rESAT-6:CFP10, or 10 μg/ml M. bovis PPD (Prionics Ag). Cultures were incubated at 39°C in 5% CO₂ for 16 h for cytokine mRNA analysis in cell lysates or protein analysis in supernatants.

**RNA isolation and analysis of cytokine gene expression by real-time PCR.** Isolation and reverse transcription of RNA in PBMCs were performed as described previously (29). Briefly, PBMCs were harvested by centrifugation and lysed with 150 μl/well buffer RLT (Qiagen, Valencia, CA), according to the manufacturer’s directions. Replicate wells were combined, and samples were stored at −80°C. RNA was isolated using an RNAeasy minikit (Qiagen), according to the manufacturer’s directions, and was eluted from the column with 50 μl RNase-free water (Ambion, Austin, TX). Contaminating DNA was enzymatically removed by treating

---

**TABLE 2 Cytokine primers/probes for RT-qPCR identification**

| Cytokine      | Entrez Gene ID | Assay ID   |
|---------------|---------------|------------|
| IL-17A        | 282863        | B03210252_m1 |
| IL-17F        | 506030        | B04309062_m1 |
| IL-22         | 507778        | B03261459_m1 |
| IL-23p19 subunit | 511022     | B04284624_m1 |
| IL-27         | 649227        | B04298832_m1 |
| IFN-γ         | 281237        | B03212723_m1 |

- **Entrez Gene** ([http://www.ncbi.nlm.nih.gov/gene](http://www.ncbi.nlm.nih.gov/gene)) identification (ID) number.
- **Applied Biosystems product number.**

---

**TABLE 3 Histological evaluation of lesion severity in 2014 vaccine efficacy study**

| Treatment group          | No. of granulomas (mean ± SEM) |
|--------------------------|--------------------------------|
|                          | Stage I | Stage II | Stage III | Stage IV | Total (stages I–IV) |
| Nonvaccinated (n = 10)   | 12.2 ± 2.5 | 2.9 ± 1.0 | 4.3 ± 1.2 | 4.0 ± 1.3 | 23.4 ± 4.4 |
| BCG-vaccinated (n = 9)   | 1.9 ± 1.2b | 1.3 ± 0.9b | 0.1 ± 0.1b | 0 ± 0b | 3.3 ± 1.9b |
| BCG mutant-vaccinated (n = 10) | 1.1 ± 0.6b | 0.8 ± 0.5b | 0.8 ± 0.7b | 0.1 ± 0.1b | 2.8 ± 1.7b |

- **Microscopic tuberculous lesions were staged (stage 1 to IV) as described by Wangoo et al. (25).** Data are presented as the mean ± SEM of the sum of the number of granulomas representing each stage observed in mediastinal and tracheobronchial lymph nodes and lung histological sections.
- **Value differs from that for nonvaccinated animals (same stage or total) (P < 0.05, ANOVA followed by Tukey’s multiple-comparison test).**

---

**TABLE 4 Th17-associated genes upregulated >9-fold in response to M. bovis PPD after M. bovis infection**

| Gene | Fold change | Rank |
|------|-------------|------|
| IL-22 | 91,019  | 3    |
| LIF  | 6,162     | 10   |
| LTA  | 2,652     | 14   |
| IL-19 | 2,149    | 17   |
| IL-17A | 1,229   | 20   |
| IL-17F | 328     | 29   |
| TNF  | 89        | 47   |
| TBX21 | 56        | 66   |
| IL-27 | 51        | 71   |
| IRF4 | 20         | 120  |
| IFN-γ | 13,147   | 7    |

- **The Th17 global transcriptional regulatory network was defined by Ciofani et al. (39).** LIF, leukemia inhibitory factor; LTA, lymphotixin α; IRF4, interferon regulatory factor 4.
- **Genes were differentially expressed (upregulated >9-fold) in response to M. bovis PPD stimulation after versus before M. bovis infection.**
- **IFN-γ was used for comparison.”**

---

Links and references are cited in the text for further reading.
FIG 1 Antigen-specific gene expression of Th17-associated cytokines in response to vaccination and subsequent challenge with virulent M. bovis. Relative gene expression levels were calculated using the $2^{-\Delta \Delta Ct}$ method, using nonstimulated cells as the calibrator and eukaryotic 18S rRNA as the endogenous control. Data are presented as individual animal responses to PPD (A), rAg85A-rTB10.4 (B), or rESAT-6:CFP10 (C) in nonvaccinated animals and vaccinated animals, at the indicated time points. Responses did not differ ($P > 0.05$) between animals vaccinated with BCG mutants versus BCG; thus, these two groups were pooled as vaccinated. #, responses differ between nonvaccinated animals and vaccinated animals for the respective cytokine ($P < 0.05$, Student’s t test, using $\Delta \Delta Ct$ values for comparisons).
RNA with DNA-free (Ambion). One microgram of RNA was reverse transcribed in a 50-μl reaction mixture using SuperScript II (Invitrogen, Carlsbad, CA) with 0.5 μg of oligo(dT)12-18 and 40 units of RNaseOut (Invitrogen), according to the manufacturer’s directions. Samples were heated to 70°C for 5 min and then reverse transcribed at 42°C for 60 min. The resulting cDNA was stored at −80°C until used in real-time PCR assays. Real-time PCR assays were performed using a TaqMan gene expression assay kit (Applied Biosystems, Foster City, CA), according to the manufacturer’s directions. Applied Biosystems primers and probes for cytokine genes are presented in Table 2, and amplification conditions were established according to the manufacturer’s directions. Reactions were performed on an Applied Biosystems 7300 real-time PCR system (Life Technologies, Grand Island, NY). Relative gene expression was expressed as \(2^{-\Delta\Delta CT}\) (30), with eukaryotic 18S rRNA (catalogue no. 4333760; Applied Biosystems) as the endogenous control, and the medium-only (i.e., no stimulation) sample from each animal was used as the calibrator for evaluation of PBMC responses.

**RNA-Seq analysis.** Whole blood was collected from six *M. bovis*-infected calves prior to and 9 weeks after challenge (Table 1). PBMCs were isolated and stimulated with PPD as described previously (29). According to the manufacturer’s directions, RNA was isolated from stimulated PBMCs using an RNeasy Maxi kit (Qiagen) and treated with DNase (DNA-free; Ambion). RNA was concentrated using 30K Microcon centrifugal filter devices (Millipore). Samples were quantitated, and the RNA integrity was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies), according to the manufacturer’s directions. All samples had a RNA integrity number greater than 7.0. Each sample (3.3 μg RNA) was randomly added to one of two pooled samples for each time point. Three time points were chosen for sequencing, i.e., prior to infection, 1 month postinfection, and 2 months postinfection. Pooled samples were sequenced at the Iowa State University DNA Sequencing Facility. Each pool was sequenced on an Illumina Genome Analyzer II, using a 75-base run. Sequences were analyzed using FastQC (version 0.10.0) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), and low-quality reads were trimmed with PRINSEQ-lite (31). Reads were aligned to the Ensembl Btau 4.0 version of the cattle genome. Reads mapping to identified genes were counted using HTseq (version 0.5.3p3) (32). The counts per gene for each sample were collated using a database developed in-house using MySQL. Genes that had combined expression of <20 reads were removed from further analysis. Differentially expressed genes were identified using EdgeR (version 2.8) (33–35). A total of 348 genes with adjusted \(P\) values of \(<0.01\) were considered significant using tag-wise dispersion. Data are presented as genes upregulated (i.e., >9-fold) after *M. bovis* infection versus before *M. bovis* infection. Gene expression did not differ (\(<0.01\)) for 1 month versus 2 months postinfection; therefore, postinfection data were analyzed as a single data point.

**IFN-γ and IL-17A ELISAs.** IFN-γ and IL-17A concentrations in stimulated plasma from whole blood or supernatants from PBMC cultures were determined using commercial ELISA-based kits (Bovigam [Prionics Ag] and bovine IL-17A ELISA VetSet [Kingfisher Biotech Inc., Saint Paul, MN]), according to the manufacturers’ instructions. Absorbance values for standards (recombinant bovine IFN-γ [Endogen, Rockford, IL] and recombinant bovine IL-17A [Kingfisher Biotech Inc.]) and test samples were determined at 450 nm using an ELISA plate reader (Molecular Devices, Menlo Park, CA). Duplicate samples for individual treatments were analyzed, and both IL-17A and IFN-γ data are presented as nanograms of protein per milliliter of plasma.

**IL-17 ELISPOT assay.** The protocol for the IL-17A enzyme-linked immunosorbent spot (ELISPOT) assay was as described previously (36). Briefly, 2 × 10^6^ PBMCs were added to polystyrene dihydrofuro 96-well assay plates (Millipore, Watford, United Kingdom) that had been coated with anti-bovine IL-17A polyclonal antibodies (5 μg/ml; Kingfisher Biotech) and were incubated in the presence or absence of 10 μg/ml *M. bovis* PPD, 3 μg/ml rAg65A-rTB10.4, or 3 μg/ml rESAT-6-CFP10. Plates were incubated for 18 h, washed, and incubated for 2 h with biotinylated anti-bovine IL-17A protein per milliliter of plasma.

**FIG 2** IL-17A responses (protein) to *M. bovis* infection of cattle. Treatment groups included noninfected (\(n = 7\)), strain 95-1315-infected (white-tailed deer *M. bovis* isolate; \(n = 8\)), and strain 10-7428-infected (Holstein *M. bovis* isolate; \(n = 8\)) calves, with the experimental design described in Table 1. Whole blood was collected into heparinized tubes and stimulated with 1 μg/ml rESAT-6-CFP10 (A), 20 μg/ml *M. avium* PPD (Lelystad; Prionics Ag) (B), 20 μg/ml *M. bovis* PPD (Lelystad; Prionics Ag) (C), or medium alone (no stimulation) for 16 h at 39°C. Plasma was harvested for IL-17A analysis by ELISA (bovine IL-17A ELISA VetSet; Kingfisher Biotech). Data (mean ± SEM) are presented as the change in nanograms per milliliter (i.e., antigen stimulation minus medium alone) for each treatment group at the indicated time points relative to challenge. a to c, different letters indicate that responses differ for the given time point (\(P < 0.05\), ANOVA followed by Tukey’s multiple-comparison test).
TABLE 5 Diagnostic capacity 8 weeks after M. bovis challenge (M. bovis PPD minus M. avium PPD)

| Treatment group | IL-17A | IFN-γ |
|-----------------|--------|-------|
|                 | CSL^a  | Lelystad^b | CSL | Lelystad |
|                  | ΔOD (mean ± SEM) | No. positive | ΔOD (mean ± SEM) | No. positive | ΔOD (mean ± SEM) | No. positive |
| No infection (n = 7) | 0.1 ± 0.04 | 2 | −0.1 ± 0.11 | 0 | 0.07 ± 0.01 | 1 | −0.1 ± 0.07 | 0 |
| M. bovis 95-1315 infection (n = 8) | 0.6 ± 0.07^c | 8 | 0.7 ± 0.12 | 8 | 1.0 ± 0.12 | 7 | 1.7 ± 0.20 | 8 |
| M. bovis 10-7428 infection (n = 8) | 0.6 ± 0.15^c | 8 | 0.7 ± 0.17 | 8 | 0.7 ± 0.30^c | 8 | 1.4 ± 0.30^c | 8 |

^a Data are presented as mean ± SEM of changes in optical densities (ODs) (i.e., M. bovis PPD minus M. avium PPD) and number positive (i.e., change in optical density of >0.1).
^b Sources of PPD.
^c Differs from the response by the noninfected group (P < 0.05, ANOVA followed by Tukey’s multiple-comparison test).

RESULTS
Characterization of tuberculous lesions and M. bovis colonization in vaccine efficacy trials. In the comparative virulence study, the levels of M. bovis colonization, distributions of lesions, and severity of gross and microscopic lesions were similar (P > 0.05) for the M. bovis strain 95-1315- and 10-7428-challenged groups (Table 1) (37). Tuberculous lesions were not detected and M. bovis was not isolated from any of the animals in the noninfected control group. Similarly, infection of 6-month-old Holstein steers for samples used in the RNA-Seq study (Table 1) resulted in tuberculous lesions and M. bovis colonization typical of aerosol M. bovis infection, as described by Palmer et al. (19). Samples from these studies were used to evaluate IL-17A responses as a biomarker of M. bovis infection in cattle.

Vaccination of neonatal calves with BCG, M. bovis ΔRD1, or BCG mutants (i.e., the Δdrb8, ΔlevCD Δpks16, ΔmetaA, and ΔmmmaA4 mutants) resulted in significant (P < 0.05) protection against challenge with virulent M. bovis, as determined by assessment of gross pathology and M. bovis colonization (Table 1) (20), as well as microscopic staging of lesions (Table 3). For TB, organ weight is often associated with the degree of lesion severity. Lung-associated lymph nodes (i.e., tracheobronchial and mediastinal lymph nodes) from vaccine recipients weighed less (P < 0.05) than the respective lymph nodes from nonvaccinated animals (data not shown). Lesion severity and M. bovis colonization did not differ (P > 0.05) between vaccinated groups (i.e., BCG versus M. bovis ΔRD1 or BCG versus BCG mutants), compared within the two vaccine efficacy trials (Tables 1 and 3) (20). Samples from these studies were used to evaluate IL-17A responses as a correlate of protection.

RNA-Seq analysis and gene expression of Th17-associated cytokines. Before and after M. bovis challenge, PBMCs were stimulated with 1 μg/ml M. bovis PPD for 16 h and mRNA was isolated for whole-transcriptome sequencing. Compared to preinfection, 200 genes were >9-fold upregulated and 142 genes were >9-fold downregulated in response to PPD after M. bovis infection. Upregulated genes included numerous genes related to T cell function, especially Th1-related (i.e., IFN-γ, lymphoxygenin α, TNF-α, IL-12Rβ2, and IL-12Rα) and Th17-related (i.e., IL-17A, IL-17F, IL-22, and IL-19) cytokine genes and IL-27, a cytokine that limits IL-17 responses through inhibition of the transcription factor retinoic acid receptor (RAR)-related orphan receptor γ (RORγ) (38). Upregulated cytokine and transcriptional regulatory network genes associated with in vitro differentiation of Th17 cells, as defined by Ciofani et al. (39), are presented in Table 4. As measured by RT-qPCR, BCG vaccination elicited Th17-associated cytokine responses to PPD (Fig. 1A) and rAg85A-rTB10.4 (Fig. 1B). At 2.5 weeks after M. bovis challenge, IL-17A, IL-17F, and IL-27 responses by nonvaccinated animals to PPD (Fig. 1A), rAg85A-rTB10 (Fig. 1B), and rESAT-6:CFP10 (Fig. 1C) exceeded (P < 0.05) the respective responses by vaccinated animals; however, IL-22 responses by nonvaccinated animals and vaccine recipients did not differ at that time point. At 8 weeks after challenge, IL-22 responses to M. bovis antigens by vaccinated and nonvaccinated animals did not differ, with one exception (i.e., responses to rAg85A-rTB10) (Fig. 1B). IL-17A gene expression and protein (ELISA) responses were correlated (Spearman’s ρ = 0.60, with analysis including rESAT-6:CFP10, rAg85A-rTB10.4, and PPD stimulation; data not shown). IL-23p19 was also evaluated (data not shown); however, antigen-specific changes in gene expression were not detected for this cytokine, which is associated with expansion of Th17 responses.

IL-17A responses to M. bovis infection and comparisons with IFN-γ responses. Experimental infection of cattle with M. bovis strain 95-1315 or 10-7428 elicited IL-17A responses to M. bovis antigens (Fig. 2). The response to M. bovis PPD by strain 10-7428-infected calves exceeded (P < 0.05) the response by 95-1315-infected calves at 2 weeks after challenge, whereas responses to rESAT-6:CFP10 and M. bovis PPD by 95-1315-infected calves exceeded (P < 0.05) the responses by 10-7428-infected calves at 8 weeks after challenge. As with IFN-γ responses (40), IL-17A responses to M. bovis PPD generally exceeded the responses to M. avium PPD (Fig. 2), and the responses to PPDs from two commercial sources were similar (M. avium PPD, CSL versus Lelystad, Spearman’s ρ = 0.69; M. bovis PPD, CSL versus Lelystad, ρ = 0.82). Using a standard diagnostic algorithm of the response to M. bovis PPD minus the response to M. avium PPD, IL-17A responses were comparable to IFN-γ responses (Table 5). Considering all in vitro treatments (i.e., medium alone, PWM, rESAT-6:CFP10, M. bovis PPDs, and M. avium PPDs), IL-17A and IFN-γ responses were highly correlated (ρ = 0.74).
IL-17A responses to vaccination and subsequent challenge with virulent M. bovis. Vaccination with BCG or BCG mutants elicited IL-17A responses to PPD and rAg85A-rTB10.4 (Fig. 3). As expected, responses were not detected after stimulation with rESAT-6:CFP10 (i.e., antigens encoded within the RD1 region of virulent M. bovis and absent in BCG) prior to challenge with virulent M. bovis (Fig. 3C). At 2.5 weeks after M. bovis challenge, IL-17A and IFN-γ responses to rESAT-6:CFP10 and PPD increased dramatically in nonvaccinated calves, greatly exceeding (P < 0.05) the respective responses in vaccinated animals (Fig. 3). As in the comparative virulence study, IL-17A and IFN-γ responses were highly correlated (Spearman’s ρ = 0.63 to 0.78, de-
Responses to PPD were elicited by vaccination with either M. bovis Ravenel ARD1 or BCG in the 2007 vaccine efficacy study (see Fig. S2 in the supplemental material). Also, at 3.5 weeks after challenge, responses to rESAT-6:CFP10 and PPD by nonvaccinated animals exceeded (P < 0.05) the respective responses by both BCG- and M. bovis Ravenel ARD1-vaccinated animals. It should be noted that levels of IL-17A were ~5-fold lower in PBMC culture supernatants from the 2007 study, compared to stimulated plasma from whole blood from the 2014 study; however, direct comparison of samples collected at the same time point (i.e., in the comparative virulence study) demonstrated that the responses determined using the two culture methods were correlated (ρ = 0.6) (see Fig. S3 in the supplemental material). Together, these findings demonstrate that protective TB vaccines elicit IL-17A responses, and these responses are dampened at 2.5 weeks after infection, compared to responses by nonvaccinated animals, likely coincident with reduced antigen loads (41) associated with protective vaccination.

Correlations with protection. BCG, BCG mutants, and M. bovis ARD1 vaccines elicited IL-17A responses to PPD and/or rAg85A-rTB10.4, as detected 11 to 12 weeks after vaccination and prior to challenge (Fig. 3 and 5; see also Fig. S1 and S2 in the supplemental material). In the 2014 efficacy study, vaccination with BCG mutants and BCG afforded exquisite protection, with 2- to 3.5-\log_{10} reductions in mycobacterial colonization and greatly reduced lesion severity (Table 1) at necropsy 4.5 months after challenge. Lower (P < 0.05) IL-17A and IFN-γ responses to PPD and rESAT-6:CFP10 at 2.5 weeks after infection were associated with no detectable M. bovis by quantitative culture (Fig. 6A) and low pathology scores (Fig. 7A) at necropsy 4.5 months after challenge. Significantly lower (P < 0.05) IFN-γ responses, but not IL-17A responses, at 10 weeks after infection were also associated with no detectable M. bovis by quantitative culture (Fig. 6B) and low pathology scores (Fig. 7B) at necropsy 4.5 months after challenge. In general, lesion severity and mycobacterial burdens in both the vaccinated and nonvaccinated groups were greater in the 2007 efficacy study than in the 2014 efficacy study, possibly due to a slightly higher M. bovis challenge dose (Table 1). In the 2007 vaccine efficacy study, greater (P < 0.05) IL-17A responses at 8 weeks after M. bovis challenge were positively associated with lesion severity (Fig. 8A) and mycobacterial burdens (Fig. 8B) determined at necropsy 4.5 months after challenge. Furthermore, greater (P < 0.05) IL-17A responses were associated with increased (P = 0.076) lesion severity among vaccinated animals (Fig. 8C) but not mycobacterial burdens (Fig. 8D) determined at necropsy 4.5 months after challenge.

DISCUSSION

Significant IL-17 responses are elicited by M. tuberculosis infection of mice (42) and humans (43, 44), as well as M. bovis infection of cattle (7, 11). With M. tuberculosis infection of mice, early expression of IL-17 in response to vaccination is required for the rapid accumulation of protective memory cells in the lungs (45). Re-
sponding quickly upon aerosol challenge with M. tuberculosis, IL-17-producing cells recruit other effector cells that limit pathogen growth (42). PPD-specific IL-17 responses to BCG vaccination are also associated with reduced disease burdens upon subsequent M. tuberculosis infection of cynomolgus macaques (46). With BCG plus virus-vectored Ag85A vaccination of cattle, vaccine-elicited IL-17 mRNA responses to Ag85A stimulation at 10 weeks after vaccination correlated with reduced TB-associated pathology (11). Rizzi et al. (15) also demonstrated that IL-17 mRNA responses in cattle vaccinated with a BCG strain overexpressing Ag85B correlated with reduced lesion severity after experimental M. bovis infection. Using RNA-Seq analysis followed by RT-qPCR analysis, Bhuju et al. (47) demonstrated that IL-22 responses to PPD after vaccination correlated with protection in cattle. The present findings support and extend those prior studies, demonstrating that protective bovine TB vaccines elicited IL-17F and IL-27 mRNA responses, IL-17A, IL-17F, and IL-27 responses at 2.5 weeks after M. bovis infection were reduced in vaccinated an-

FIG 5 Dampening of IL-17A responses (ELISPOT assay) to M. bovis infection with prior BCG vaccination. Treatment groups included nonvaccinated animals (n = 10) and vaccinated animals (n = 19). The vaccinated group consisted of animals vaccinated with BCG (n = 9) or BCG mutants (i.e., BCG ΔΔβ8, BCG ΔΔetCD ΔΔpsk16, BCG ΔΔmetA, and BCG ΔΔmmaA4; n = 10). Virulent M. bovis strain 10-7428 was administered by aerosol to all calves 3.5 months after vaccination, and calves were euthanized 4.5 months after M. bovis challenge (Table 1). Whole blood from all calves was collected into heparinized tubes at 2.5 and 10 weeks after challenge and stimulated with 1 μg/ml rESAT-6-CFP10 or medium alone (no stimulation) for 16 h at 39°C, and plasma was harvested for IFN-γ and IL-17A analyses using commercial ELISA kits (Bovigam [Prionics Ag] and bovine IL-17A ELISA VetSet [Kingfisher Biotech]). Mycobacterial burdens were determined by serial dilution culture of entire tracheobronchial lymph node homogenates and are presented as culture-forming units per gram of tissue. Groups were split based on mycobacterial burdens of 0 CFU/g (n = 14) or >0 CFU/g (n = 15). All 14 animals in the 0-CFU/g group were vaccinated animals. In the >0-CFU/g group, 5/15 animals were vaccinated animals and 10/15 were nonvaccinated animals. Data (mean ± SEM) are presented as IFN-γ (left) and IL-17A (right) responses (changes in nanograms per milliliter, i.e., antigen stimulation minus medium alone) to rESAT-6-CFP10 at 3 weeks after M. bovis challenge (A) and 10 weeks after M. bovis challenge (B), as related to mycobacterial burdens determined at necropsy 4.5 months after challenge. Similar results were obtained for responses to M. bovis PPD (data not shown). Student’s t test P values are provided in the upper left corner of each graph.

FIG 6 Association of ESAT-6-CFP10-specific IFN-γ and IL-17A responses with mycobacterial burdens in the 2014 vaccine efficacy study. Treatment groups included nonvaccinated animals, BCG-vaccinated animals, and animals vaccinated with BCG mutants (i.e., BCG ΔΔβ8, BCG ΔΔetCD ΔΔpsk16, BCG ΔΔmetA, and BCG ΔΔmmaA4). Virulent M. bovis strain 10-7428 was administered by aerosol to all calves 3.5 months after vaccination, and calves were euthanized 4.5 months after M. bovis challenge (Table 1). Whole blood from all calves was collected into heparinized tubes at 2.5 and 10 weeks after challenge and stimulated with 1 μg/ml rESAT-6-CFP10 or medium alone (no stimulation) for 16 h at 39°C, and plasma was harvested for IFN-γ and IL-17A analyses using commercial ELISA kits (Bovigam [Prionics Ag] and bovine IL-17A ELISA VetSet [Kingfisher Biotech]). Mycobacterial burdens were determined by serial dilution culture of entire tracheobronchial lymph node homogenates and are presented as culture-forming units per gram of tissue. Groups were split based on mycobacterial burdens of 0 CFU/g (n = 14) or >0 CFU/g (n = 15). All 14 animals in the 0-CFU/g group were vaccinated animals. In the >0-CFU/g group, 5/15 animals were vaccinated animals and 10/15 were nonvaccinated animals. Data (mean ± SEM) are presented as IFN-γ (left) and IL-17A (right) responses (changes in nanograms per milliliter, i.e., antigen stimulation minus medium alone) to rESAT-6-CFP10 at 3 weeks after M. bovis challenge (A) and 10 weeks after M. bovis challenge (B), as related to mycobacterial burdens determined at necropsy 4.5 months after challenge. Similar results were obtained for responses to M. bovis PPD (data not shown). Student’s t test P values are provided in the upper left corner of each graph.
In prior studies, we demonstrated, with samples from a limited number of animals, that CD4+ and γδ+ T cells from *M. bovis*-infected cattle produced IL-17A in response to *M. bovis* PPD or rESAT-6:CFP10 (36). PPD-specific IL-17 mRNA responses at 60 and 90 days after experimental *M. bovis* infection correlated with the presence of gross tuberculous lesions at necropsy 4 months after challenge (7). Using laser capture microdissection followed by qPCR, Aranday-Cortes et al. demonstrated increased IL-17A and IL-22 expression within tuberculous granulomas versus nonaffected tissues from experimentally infected cattle, particularly in more advanced lesions (50). In the present study, *M. bovis* infection also elicited IL-17A protein responses that correlated with infection, similar to IFN-γ responses (Table 3). Infection also elicited IL-17F, IL-22, and IL-27 responses. IL-27 is a known inhibitor of Th17 responses in mice and humans (51, 52); however, both IL-27 (44) and IL-17 (41, 43) are associated with active disease in *M. tuberculosis* infections in humans. Thus, perhaps it is not too surprising that IL-27 and Th17 cytokine responses followed similar kinetics. Antigen-specific IL-23p19 mRNA expression was not detectable in response to either infection or vaccination, possibly due to the duration of culture, poorly represented dendritic cell and macrophage populations within PBMCs, or the lack of “danger signals” (e.g., Toll-like receptors or nucleotide-binding oligomerization domain-like signals) within the antigen preparations. Similarly, Blanco et al. (7) did not detect IL-12p35 expression with similar protocols. In contrast to IL-17A, IL-17F, and IL-27, IL-22 responses were not different between vaccinated animals and nonvaccinated animals at 2.5 weeks after *M. bovis* infection, suggesting that this cytokine is less affected by antigen loads or that vaccine-elicited IL-22 responses are more durable than other Th17-associated cytokine responses. Together, these findings indicate that IL-17A and potentially other Th17-associated cytokines, such as IL-17F, IL-22, and IL-27, may prove useful as biomarkers for *M. bovis* infections in cattle.

The Th17 lineage is known for its plasticity and instability, that is, IL-17 expression may cease over time (53). Th17 cells can start expressing cytokines typical of other T helper subsets as a result of a nonresolving immune response (switch to a Th1 phenotype), chronic inflammation or autoimmunity (switch to a T regulatory 1 [Tr1] phenotype), *Nippostrongylus brasiliensis* infection (switch to a Th2 or Tr1 phenotype), or *Staphylococcus aureus* infection (switch to a Tr1 phenotype), thereby providing a mechanism to contribute to resolution of inflammation (53). With aerosol BCG infection of mice, IL-17A produced by Vγ4+ and Vγ6+ γδ T cells is necessary for appropriate maturation of granulomas (54), and early IL-17 produced by γδ T cells occurs prior to αβ+ T cell priming, thus biasing the ensuing adaptive response (55). However, excessive IL-17 responses may be detrimental; repeated BCG vaccination of *M. tuberculosis*-infected mice exacerbated inflammation due to infection, and this exaggerated response was not detected in mice treated with anti-IL-17 blocking antibody or in IL-23p19-deficient mice, demonstrating the IL-17 dependence of the damaging response (56). Also, treatment regimens that block IL-17 responses (e.g., RORγ inhibitors to promote a more favorable IL-17/IFN-γ balance via inhibition of IL-17 production) are being considered for inclusion in treatment regimens for *M. tuberculosis* infections in humans (57). Together, these findings suggest that the timing and amounts of IL-17 production in response to TB infections are critical for the balance between responses that support control of the bacilli and detrimental inflammatory re-

---

**FIG 7** Association of ESAT-6:CFP10-specific IFN-γ and IL-17A responses with lesion severity (i.e., gross pathology scores) in the 2014 vaccine efficacy study. Treatment groups included nonvaccinated animals, BCG-vaccinated animals, and animals vaccinated with BCG mutants (i.e., BCG Δfob8, BCG ΔmetA, and BCG ΔmmaA4). Virulent *M. bovis* strain 10-7428 was administered by aerosol to all calves 3.5 months after vaccination, and calves were euthanized 4.5 months after challenge (A) and 10 weeks after infection (B), as indicated by ELISAs or ELISPOT assays correlated with IL-17A and IFN-γ expression (left) and IL-17A responses (changes in nanograms per milliliter, all vaccinated animals), 1 to 5 (n = 7; all vaccinated animals), or 6 to 22 (n = 10; all nonvaccinated animals). Data (mean ± SEM) are presented as IFN-γ (left) and IL-17A (right) responses (changes in nanograms per milliliter, i.e., antigen stimulation minus medium alone) to rESAT-6:CFP10 at 3 weeks after *M. bovis* challenge (A) and 10 weeks after *M. bovis* challenge (B), as related to gross pathology scores determined at necropsy 4.5 months after challenge. Similar results were obtained for responses to *M. bovis* PPD (data not shown). *, response differs from the other responses in the graph (P < 0.05, ANOVA followed by Tukey's multiple-comparison test).

Animals versus nonvaccinated animals, IL-17A protein levels measured by ELISAs or ELISPOT assays correlated with IL-17A mRNA responses in both the kinetics and levels of responses, and higher IL-17A and IFN-γ levels at 2.5 weeks after *M. bovis* infection correlated with increased lesion severity and mycobacterial burdens upon postmortem inspection 4 months after challenge. Evaluating a *M. bovis* Δmce2 deletion mutant, Blanco et al. (14) also demonstrated reduced IL-17 mRNA responses in vaccinated animals versus nonvaccinated animals after *M. bovis* challenge; however, the responses differed 160 days after challenge, and time points earlier than 80 days postchallenge were not evaluated. In the present study, diminished IL-17-related cytokine and IFN-γ responses early after infection likely result from reduced antigen loads at the site of aerosol-delivered *M. bovis* infection, due to protective responses initiated by vaccination; however, further studies are required to confirm this notion. Also, as with IFN-γ and delayed-type hypersensitivity (DTH) responses (48, 49), it is likely that many TB vaccines will induce IL-17 responses but not all IL-17-inducing vaccines will be protective.
responsive. Given the plasticity of the responses, Th17 cells may transdifferentiate into phenotypes not expressing IL-17 or Th17-associated cytokines. In future studies, it will be critical to evaluate the responses at sites of *M. bovis* infection and over the course of infection, to account for local environmental factors associated with chronic inflammation that affect the plasticity of Th17 responses. Studies utilizing mycobacterial antigens or live bacteria in subcutaneous biopolymers as *in vivo* models of granuloma formation and maturation (58) may provide additional insights into the kinetics of Th17 responses. In conclusion, the present findings support the use of IL-17-associated cytokines as biomarkers of infection and protection in the immune responses to bovine tuberculosis.

**ACKNOWLEDGMENTS**

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

**FUNDING INFORMATION**

Agriculture and Food Research Initiative provided funding to W. Ray Waters and Mitchell V. Palmer under grant number 2011-67015-30736. This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2011-67015-30736 from the USDA National Institute of Food and Agriculture to W. Ray Waters and Mitchell V. Palmer. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**REFERENCES**

1. Smith NH, Hewinson RG, Kremer K, Brosch R, Gordon SV. 2009. Myths and misconceptions: the origin and evolution of Mycobacterium tuberculosis. Nat Rev Microbiol 7:537-544. doi:10.1038/nrmicro2165.
2. Müller B, Dürr S, Alonso S, Hattendorf J, Laisse CJ, Parsons SD, van Helden PD, Zinsstag J. 2013. Zoonotic Mycobacterium bovis-induced tuberculosis in humans. Emerg Infect Dis 19:989-990. http://dx.doi.org/10.3201/eid1906.120543.
3. Palmer MV. 2013. Mycobacterium bovis: characteristics of wildlife reservoir hosts. Transbound Emerg Dis 60(Suppl 1):S1-S13.
4. Schiller I, Oesch B, Vordermeier HM, Palmer MV, Harris BN, Orloski KA, Buddle BM, Thacker TG, Lyashchenko KP, Waters WR. 2010. Bovine tuberculosis: a review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transbound Emerg Dis 57:205-220.
5. Walzl G, Ronacher K, Hanekom W, Scriba TJ, Zumla A. 2011. Immunological biomarkers of tuberculosis. Nat Rev Immunol 11:343-354. http://dx.doi.org/10.1038/nri2960.
6. Salgame P, Geadas C, Collins L, Jones-López E, Ellner JJ. 2015. Latent tuberculosis infection: revisiting and revising concepts. Tuberculosis (Edinb) 95:373-384. http://dx.doi.org/10.1016/j.tube.2015.04.003.
7. Blanco FC, Blanco MV, Meikle V, Garbaccio S, Vagnoni L, Forrellad M, Klepp L, Cataldi AA, Bigi F. 2011. Increased IL-17 expression is associated with pathology in a bovine model of tuberculosis. Tuberculosis (Edinb) 91:57-63. http://dx.doi.org/10.1016/j.tube.2011.10.007.
8. Goosen WJ, Cooper D, Miller MA, van Helden PD, Parsons SD. 2015. IP-10 is a sensitive biomarker of antigen recognition in whole blood stimulation assays used for the diagnosis of Mycobacterium bovis infection in African buffaloes (*Syncerus caffer*). Clin Vaccine Immunol 22:974-978. http://dx.doi.org/10.1128/CVI.00324-15.
23. Jones GJ, Pirson C, Hewinson RG, Vordermeier HM. 2010. Simultaneous measurement of antigen-stimulated interleukin-1β and gamma interferon production enhances test sensitivity for the detection of Mycobacterium bovis infection in cattle. Clin Vaccine Immunol 17:1946–1951. http://dx.doi.org/10.1128/CVI.00377-10.

21. Rhode DG, Steinbach S, Dean GS, Villarreal-Ramos B, Whelan AO, Pirson C, Jones GJ, Clifford D, Vordermeier HM. 2014. Use of antigen-specific interleukin-2 to differentiate between cattle vaccinated with Mycobacterium bovis BCG and cattle infected with M. bovis. Clin Vaccine Immunol 21:39–45. http://dx.doi.org/10.1128/CVI.00223-14.

16. Vordermeier HM, Villarreal-Ramos B, Cockle PJ, McAulay M, Rhodes SG, Thacker TJ, Gilbert SC, McShane HP, Hill AV, Xing Z, Hewinson RG. 2009. Viral booster vaccines improve Mycobacterium bovis BCG-induced protection against bovine tuberculosis. Infect Immun 77:3364–3373. http://dx.doi.org/10.1128/IAI.00287-09. (Erratum, 79:2134, 2011.)

12. Waters WR, Palmer MV, Whipple DL, Carlson MP, Nonnecke BJ. 2007. Diagnostic implications of antigen-induced IFN-γ, nitric oxide, and TNF-α production by blood mononuclear cells from Mycobacterium bovis-infected cattle. Clin Diagn Lab Immunol 10:960–965.

11. Waters WR, Thacker TC, Nonnecke BJ, Palmer MV, Schiller I, Oesch B, Vordermeier HM, Silva E, Estes DM. 2012. Evaluation of gamma interferon (IFN-γ)-induced protein 10 responses for detection of infection with Mycobacterium bovis: comparisons to IFN-γ responses. Clin Vaccine Immunol 19:346–351. http://dx.doi.org/10.1128/CVI.00567-11.

14. Blanco FC, Blanco MV, Garbaccio S, Meikle V, Gravissaco MJ, Montenegro V, Alfonseca E, Singh M, Barandiaran S, Canal A, Vagnoni L, Biermann K, Jacobs WR. 2010. Simultaneous detection of double deletion mutant protects cattle against challenge with virulent M. bovis. Tuberculosis (Edinb) 93:363–372. http://dx.doi.org/10.1016/j.tube.2013.02.004.

13. Rizzi C, Blanco MV, Blanco FC, Soria M, Gravissaco MJ, Montenegro V, Vagnoni L, Buddle B, Garbaccio S, Delgado LS, Leal KS, Cataldi AA, Dellogastino OA, Bigi V. 2012. Vaccination with a BCG strain overexpressing Ag85b protein protects cattle against Mycobacterium bovis challenge. PLoS One 7e51396. http://dx.doi.org/10.1371/journal.pone.0051396.

12. Schmitt SM, Fitzgerald SD, Cooley TM, Bruning-Fann CS, Sullivan L, Barry D, Carlson T, Minnis RB, Payeur JB, Sikarskie J. 1997. Bovine tuberculosis in free-ranging white-tailed deer from Michigan. J Wildl Dis 33:749–758. http://dx.doi.org/10.7589/0090-3558-33.4.749.

9. Francisco TJ, Orloski KA, Roberts NJ. 2014. Investigation of a Mycobacterium tuberculosis outbreak in cattle at a Colorado dairy in 2010. J Vet Med Assoc 244:805–812. http://dx.doi.org/10.2460/jvma.244.7.805.

8. Larsen MH, Biermann K, Jacobs WR. 2007. Laboratory maintenance of Mycobacterium tuberculosis. Curr Protoc Microbiol 10:1A.1.

7. Palmer MV, Waters WR, Whipple DL. 2002. Aerosol delivery of virulent Mycobacterium bovis to cattle. Tuberculosis (Edinb) 82:275–282. http://dx.doi.org/10.1079/095852502320846815.

6. Waters WR, Palmer MV, Nonnecke BJ, Thacker TC, Scherer CF, Estes DM, Hewinson RG, Vordermeier HM, Barnes SW, Federe GC, Walker DM, Hewinson RG, Vordermeier M. 2013. Efficacy and immunogenicity of M. bovis BCG-induced protein 10 and IFN-γ responses to Mycobacterium tuberculosis antigens. Clin Vaccine Immunol 18:1150–1156. http://dx.doi.org/10.1128/CVI.00051-11.

5. Thacker TC, Palmer MV, Waters WR. 2007. Associations between cytokine gene expression and pathology in Mycobacterium bovis infected cattle. Vet Immunol Immunopathol 119:204–213. http://dx.doi.org/10.1016/j.vetimm.2007.05.009.

4. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods 25:402–408. http://dx.doi.org/10.1016/S1046-2023(01)00499-X.

3. Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27:863–864. http://dx.doi.org/10.1093/bioinformatics/btr026.

2. Anders S, Pyl PT, Huber W. 2015. HTSeq: a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169. http://dx.doi.org/10.1093/bioinformatics/btu638.

1. Robinson MD, Smyth GK. 2007. Moderated statistical tests for assessing differences in tag abundance. Bioinformatics 23:2881–2887. http://dx.doi.org/10.1093/bioinformatics/btm453.

Robinson MD, Smyth GK. 2008. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. Biostatistics 9:321–332.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: A Bioconduc- tor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. http://dx.doi.org/10.1093/bioinformatics/btp616.

McGill JL, Sacco RE, Baldwin CL, Telfer JC, Palmer MV, Waters WR. 2014. Specific recognition of mycobacterial protein and peptide antigens by γδ T cell subsets following infection with virulent Mycobacterium bovis. J Immunol 192:2756–2769. http://dx.doi.org/10.4049/jimmunol.1300526.

Robinson MD, Schmieder R, Joint E, Smyth GK. 2014. Use of Biotools for differential expression analysis of digital gene expression data. Bioinformatics 30:160–167. http://dx.doi.org/10.1093/bioinformatics/btu456.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: A Bioconduc- tor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. http://dx.doi.org/10.1093/bioinformatics/btp616.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: A Bioconduc- tor package for differential expression analysis of digital gene expression data. Bioinformatics 26:139–140. http://dx.doi.org/10.1093/bioinformatics/btp616.

Diveu C, McGeachie MJ, Boniface K, Stumhofer JS, Dathe M, Joyce- shikh B, Chen Y, Tato CM, McClanahan TK, de Waal Malefyt R, Hunter CA, Cui DJ, Kastelein RA. 2009. IL-27 blocks RORγT expression to inhibit lineage commitment of Th17 cells. J Immunol 182:5748–5756. http://dx.doi.org/10.4049/jimmunol.0801162.

Giofani M, Madar A, Galan C, Sellars M, Mace K, Pauli F, Agarwal A, Huang W, Parkurst CN, Muratet M, Newberry KM, Meadows S, Maggiolini MP, Greenwald R, Esfandiar J, Lyashchenko KP, Palmer MV. 2014. Virulence of two strains of Mycobacterium bovis in cattle following aerosol infection. J Comp Pathol 151:410–419. http://dx.doi.org/10.1016/j.jcpa.2014.08.007.

Bass KE, Nonnecke BJ, Palmer MV, Thacker TC, Hardeger R, Schrøeder B, Raebert AJ, Waters WR. 2013. Clinical and diagnostic development of a gamma interferon release assay for use in bovine tuberculosis control programs. Clin Vaccine Immunol 20:1827–1835. http://dx.doi.org/10.1128/CVI.01109-14.

Bass KE, Nonnecke BJ, Palmer MV, Thacker TC, Hardeger R, Schrøeder B, Raebert AJ, Waters WR. 2013. Clinical and diagnostic development of a gamma interferon release assay for use in bovine tuberculosis control programs. Clin Vaccine Immunol 20:1827–1835. http://dx.doi.org/10.1128/CVI.01109-14.

Ciofani M, Aabye KE, Nonnecke BJ, Palmer MV, Thacker TC, Hardegger R, Schr- eder B, Raebert AJ, Waters WR. 2013. Clinical and diagnostic development of a gamma interferon release assay for use in bovine tuberculosis control programs. Clin Vaccine Immunol 20:1827–1835. http://dx.doi.org/10.1128/CVI.01109-14.
42. Khader SA, Cooper AM. 2008. IL-23 and IL-17 in tuberculosis. Cytokine 41:79–83. http://dx.doi.org/10.1016/j.cyt.2007.11.022.

43. Jurado JO, Pasquelinelli V, Alvarez IB, Peña D, Rovetta AI, Tatesosian NL, Romeo HE, Musella RM, Palmero D, Chuluayn HE, García VE. 2012. IL-17 and IFN-γ expression in lymphocytes from patients with active tuberculosis correlates with the severity of the disease. J Leukoc Biol 91:991–1002. http://dx.doi.org/10.1189/jlb.12111619.

44. Torrado E, Fountain JJ, Liao M, Tighe M, Reiley WW, Lai RP, Meintjes G, Pearl JE, Chen X, Zak DE, Thompson EG, Aderem A, Ghilardi N, Solache A, McKinstry KK, Strutt TM, Wilkinson RJ, Swain SL, Cooper AM. 2015. Interleukin 27R regulates CD4+ T cell phenotype and impacts protective immunity during Mycobacterium tuberculosis infection. J Exp Med 212:1449–1463. http://dx.doi.org/10.1084/jem.20141520.

45. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, Cilley GE, Shen F, Eaton SM, Gaffen SL, Swain SL, Locksley RM, Haynes I, Randall TD, Cooper AM. 2007. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol 8:369–377. http://dx.doi.org/10.1038/ni1449.

46. Wareham AS, Tree JA, Marsh PD, Butcher PD, Dennis M, Sharpe SA. 2014. Evidence for a role for interleukin-17, Th17 cells and iron homeostasis in protective immunity against tuberculosis in cynomolgus macaques. PLoS One 9:e88149. http://dx.doi.org/10.1371/journal.pone.0088149.

47. Bhaju S, Aranday-Cortes E, Villarreal-Ramos B, Xing Z, Singh M, Vordermeier HM. 2012. Global gene transcription analysis in vaccinated cattle revealed a dominant role of IL-22 for protection against bovine tuberculosis. PLoS Pathog 8:e1003077. http://dx.doi.org/10.1371/journal.ppat.1003077.

48. Black GF, Weir RE, Floyd S, Bliss L, Warndorff DK, Crampin AC, Ngwira B, Sichili I, Nazareth B, Blackwell JM, Branson K, Chaguluka SD, Donovan L, Jarman E, King E, Fine PE, Dockrell HM. 2002. CO2-induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: two randomised controlled studies. Lancet 359:1393–1401. http://dx.doi.org/10.1016/S0140-6736(02)08353-8.

49. Waters WR, Palmer MV, Buddle BM, Vordermeier HM. 2012. Bovine tuberculosis vaccine research: historical perspectives and recent advances. Vaccine 30:2611–2622. http://dx.doi.org/10.1016/j.vaccine.2012.02.018.

50. Aranday-Cortes E, Hogarth PJ, Kavelh DA, Whelan AO, Villarreal-Ramos B, Lalvani A, Vordermeier HM. 2012. Transcriptional profiling of disease-induced host responses in bovine tuberculosis and the identification of potential diagnostic biomarkers. PLoS One 7:e30626. http://dx.doi.org/10.1371/journal.pone.0030626.

51. Batten M, Li J, Yi S, Klijavin NM, Danilenko DM, Lucas S, Lee J, de Sauvage FJ, Ghilardi N. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat Immunol 7:929–936. http://dx.doi.org/10.1038/ni11375.

52. Liu H, Rohowsky-Kochan C. 2011. Interleukin-27-mediated suppression of human Th17 cells is associated with activation of STAT1 and suppressor of cytokine signaling protein 1. J Interferon Cytokine Res 31:459–469. http://dx.doi.org/10.1089/jir.2010.0115.

53. Gagliani N, Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, de Zoete MR, Licona-Limón P, Paiva RS, Ching T, Weaver C, Zi X, Pan X, Fan R, Garmire LX, Cotton MJ, Drier Y, Bernstein B, Geginat J, Stockinger B, Esplugues E, Huber S, Flavell RA. 2015. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. Nature 523:221–225. http://dx.doi.org/10.1038/nature14452.

54. Okamoto Yoshida Y, Unemura M, Yahagi A, O’Brien RL, Ikuta K, Kishihara K, Hara H, Nakae S, Iwakura Y, Matsuzaki G. 2010. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. J Immunol 184:4414–4422. http://dx.doi.org/10.4049/jimmunol.0903332.

55. Lockhart E, Green AM, Flynn JL. 2006. IL-17 production is dominated by γδ T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177:4662–4669. http://dx.doi.org/10.4049/jimmunol.177.7.4662.

56. Cruz A, Fraga AG, Fountain JJ, Rangel-Moreno J, Torrado E, Saraiva M, Pereira DR, Randall TD, Pedrosa J, Cooper AM, Castro AG. 2010. Pathological role of interleukin 17 in mice subjected to repeated BCG vaccination after infection with Mycobacterium tuberculosis. J Exp Med 207:1609–1616. http://dx.doi.org/10.1084/jem.20100265.

57. Baures PW. 2012. Is RORγt a therapeutic target for treating Mycobacterium tuberculosis infections? Tuberculosis (Edinb) 92:95–99. http://dx.doi.org/10.1016/j.tube.2011.11.009.

58. Plattner BL, Huffman EL, Hostetter JM. 2013. Gamma-delta T-cell responses during subcutaneous Mycobacterium avium subspecies paratuberculosis challenge in sensitized or naïve calves using matrix biopolymers. Vet Pathol 50:630–637. http://dx.doi.org/10.1177/0300985812463404.