Interleukin-17A as a Biomarker for Bovine Tuberculosis

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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.

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TABLE 1 Experimental design and efficacy parameters

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<th>Study and group</th>
<th>Vaccination&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M. bovis challenge&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pathology score&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Culture&lt;sup&gt;d&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>Age (wk)</td>
<td>Interval (mo)</td>
<td>Strain</td>
<td>Dose (CFU)</td>
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<td>M. bovis strain 95-1315 infection (n = 8)</td>
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<td>Vaccine efficacy study from 2014</td>
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<td>3.5</td>
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<td>600</td>
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<td>BCG mutant vaccination (n = 10)</td>
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<td>95-1315</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.5</td>
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<td>BCG vaccination (n = 11)</td>
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<td>3</td>
<td>95-1315</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td>M. bovis ΔRD1 vaccination (n = 10)</td>
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<td>3</td>
<td>95-1315</td>
<td>10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>RNA-Seq study&lt;sup&gt;i&lt;/sup&gt;</td>
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<tr>
<td>M. bovis strain 95-1315 infection (n = 6)</td>
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<td></td>
<td>8 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>11</td>
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<sup>a</sup> Age of vaccination and interval between vaccination and aerosol challenge with virulent M. bovis.

<sup>b</sup> Strain and dose of virulent strain administered by aerosol and interval between challenge and necropsy.

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

<sup>d</sup> M. bovis CFU per gram of tracheobronchial lymph node.

<sup>e</sup> See reference 37 for additional details of this study.

<sup>f</sup> NA, not applicable (i.e., animals were not vaccinated or challenged).

<sup>g</sup> Differs from the noninfected or nonvaccinated group within each study (P < 0.05, ANOVA followed by Tukey’s multiple-comparison test).

<sup>h</sup> See reference 20 for additional details of this study.

<sup>i</sup> Pathology scoring and quantitative culture were not applied; however, all animals had tuberculous lesions and M. bovis was isolated from each animal.

Responses 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 70-7428 (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 and challenge were as follows: no vaccination (n = 11), 10<sup>6</sup> CFU M. bovis bacillus Calmette-Guerin (BCG) Danish (n = 11), and 10<sup>6</sup> CFU M. bovis Ravenel ΔRD1 (n = 10). In the 2014 study, the vaccine treatment groups were as follows: no vaccination (n = 10), 10<sup>6</sup> CFU M. bovis BCG Danish (n = 9), and 10<sup>6</sup> CFU (total dose) of a cocktail of four BCG Danish deletion strains, i.e., BCG Δfdr8, BCG ΔleuCDΔpks16, BCG ΔmmaA4 (21), and BCG ΔmetA (22) (n = 10). All four BCG Danish deletion derivatives (Δfdr8, ΔleuCDΔpks16, ΔmetA, and ΔmmaA4) are more attenuated and safer than the parental BCG strain in immunocompromised mice (21; Lerner-Beyer, M. Larsen, and W. R. Jacobs, unpublished data). In immunocompetent mice, the BCG deletions Δfdr8, ΔmmaA4, and Δpks16 each result in enhanced mycobacterial immunogenicity through enhanced cross-presentation of mycobacterial antigens (Δfdr8), cytokine modulation (ΔmmaA4), and biofilm formation (Δpks16), compared to the parental BCG (21; Lerner-Beyer et al., unpublished). BCG mutants, such as these, may also be used as vaccine vectors to promote epitope-specific responses (e.g., BCG Δpks12 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 included lung, liver, and mandibular, parotid, medial retropharyngeal, mediastinal, tracheobronchial, hepatic, and mesenteric lymph nodes. Lymph nodes were sectioned at 0.5-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
cervical, 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, 5–10 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 caseonecrotic granuloma, suggesting tuberculosis, were stained with the Ziehl-Neelsen technique suggesting tuberculosis, were stained with the Ziehl-Neelsen technique for identification of acid-fast bacteria. Microscopic tuberculous lesions were staged (stage I to IV) based on a scoring system developed by Wangoo et al. (25). Data are presented as the mean pathology scores (mean ± standard error of the mean [SEM]), including scores for each lung lobe as well as the tracheobronchial and mediastinal lymph nodes.

Quantitative assessments of mycobacterial burdens were evaluated as described previously (26). Briefly, tracheobronchial lymph node samples were removed, examined for gross lesions, and weighed, and entire lymph nodes (other than an ~1-g section for histological assessment) were homogenized in phenol red nutrient broth using a blender (Oster, Shelton, CT). Logarithmic dilutions (10^6 to 10^-4) of homogenates in phosphate-buffered saline (PBS) were plated in 100-μl aliquots on Middlebrook 7H11 selective agar plates (Becton Dickinson) and incubated 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 (CFP10) (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 (SSI; Prionics 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 × 10^6 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 18 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 30 μl RNase-free water (Ambion, Austin, TX). Contaminating DNA was enzymatically removed by treating

### Table 2: Cytokine primers/probes for RT-qPCR identification

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Entrez Gene ID</th>
<th>Assay ID</th>
</tr>
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<tr>
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<td>B03210252_m1</td>
</tr>
<tr>
<td>IL-17F</td>
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<td>IL-27</td>
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<tr>
<td>IFN-γ</td>
<td>281237</td>
<td>B03212723_m1</td>
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### Table 3: Histological evaluation of lesion severity in 2014 vaccine efficacy study

<table>
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<tr>
<th>Treatment group</th>
<th>No. of granulomas (mean ± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonvaccinated</td>
<td>Stage I: 12.2 ± 2.5; Stage II: 2.9 ± 1.0; Stage III: 4.3 ± 1.2; Stage IV: 4.0 ± 1.3; Total (stages I–IV): 23.4 ± 4.4</td>
</tr>
<tr>
<td>BCG-vaccinated</td>
<td>Stage I: 1.9 ± 1.2b; Stage II: 1.3 ± 0.9b; Stage III: 0.1 ± 0.1b; Stage IV: 0 ± 0b; Total (stages I–IV): 3.3 ± 1.9b</td>
</tr>
<tr>
<td>BCG mutant-vaccinated</td>
<td>Stage I: 1.1 ± 0.6b; Stage II: 0.8 ± 0.5b; Stage III: 0.8 ± 0.7b; Stage IV: 0.1 ± 0.1b; Total (stages I–IV): 2.8 ± 1.7b</td>
</tr>
</tbody>
</table>

* Values differ from that for nonvaccinated animals (same stage or total) (P < 0.05, ANOVA followed by Tukey’s multiple-comparison test).
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 $2^{-\Delta\Delta C_T}$ 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 $\Delta\Delta C_T$ 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^(-ΔΔCt) (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 RNAeasy Maxi kit (Qiagen) and treated with DNase (DNAfree; 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 polyvinylidene difluoride 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 rAg85A–rTB10.4, or 3 μg/ml rESAT-6CFP10. 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-6CFP10 (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).
detected and developed using a Vectastain ABC-AP standard kit and a Vector blue alkaline phosphatase substrate kit (both from Vector Laboratories, Burlingame, CA), according to the manufacturer’s instructions. Plates were read and analyzed using a standard ELLISPOT reader (Cellular Technology).

**Statistical analysis.** Data were analyzed by analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test. Student’s t test, or Spearman’s correlation, using commercially available software (Prism 6.0c; GraphPAD Software, La Jolla, CA). Comparisons with P values of <0.05 were considered significant.

**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 10-7428-infected calves exceeded (P < 0.05) the response by 95-1315-infected calves at 2 weeks after challenge, whereas responses to PPD by strain 95-1315-infected calves at 2 weeks after challenge. As with IFN-γ responses to PPD by strain 95-1315-infected calves at 2 weeks after challenge, whereas responses to PPD by strain 95-1315-infected calves exceeded (P < 0.05) the response by 10-7428-infected calves at 8 weeks after challenge. IL-17A responses to *M. bovis* antigens by vaccinated and nonvaccinated animals did not differ, with one exception (i.e., responses to 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-rTB10.4 (Fig. 1B). IL-17A gene expression and protein (ELISA) responses were correlated (Spearman’s r = 0.60, with analysis including rESAT-6-CFP10, rAg85A-rTB10.4, and PPD stimulations; 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 r = 0.69; M. bovis PPD, CSL versus Lelystad, r = 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 (r = 0.74).

### Table 5 Diagnostic capacity 8 weeks after *M. bovis* challenge (*M. bovis* PPD minus *M. avium* PPD)

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<th>Treatment group</th>
<th>IL-17A</th>
<th>IFN-γ</th>
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<td></td>
<td>CSL&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Lelystad&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ΔOD&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No. positive</td>
<td>ΔOD&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>No infection (n = 7)</td>
<td>0.1 ± 0.04</td>
<td>2</td>
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<tr>
<td><em>M. bovis</em> 95-1315 infection (n = 8)</td>
<td>0.6 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td><em>M. bovis</em> 10-7428 infection (n = 8)</td>
<td>0.6 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8</td>
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</tbody>
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<sup>a</sup> 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).

<sup>b</sup> Sources of PPD.

<sup>c</sup> Differs from the response by the noninfected group (P < 0.05, ANOVA followed by Tukey’s multiple-comparison test).
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 *p* = 0.63 to 0.78, de-
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-\( \gamma \) 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-\( \gamma \) 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-
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-
responses. 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.

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FIG 8 Correlation of ESAT-6:CFP10-specific IL-17A responses at 8 weeks after M. bovis challenge with lesion severity and mycobacterial burden in the 2007 vaccine efficacy study. Treatment groups included nonvaccinated, BCG-vaccinated, and M. bovis ARD1-vaccinated animals. Virulent M. bovis strain 95-1315 was administered by aerosol to all calves 3 months after vaccination, and calves were euthanized 4.5 months after M. bovis challenge (Table 1). Eight weeks after M. bovis challenge, PBMCs (2 × 10^7 PBMCs/well) were stimulated with 1 μg/ml rESAT-6:CFP10 or medium alone at 39°C for 16 h, and supernatants were harvested for IL-17A analysis by ELISA (bovine IL-17A ELISA VetSet; Kingfisher Biotech). For evaluation of gross pathology, lungs and lymph nodes (mediastinal and tracheobronchial) were evaluated using a semiquantitative scoring system (24). Mycobacterial burdens were determined by culture of a series of dilutions of entire tracheobronchial lymph node homogenates and are presented as CFU per gram of tissue (26). Data (mean ± SEM) are presented as changes in nanograms per milliliter (i.e., antigen stimulation minus medium alone) for each treatment group based on gross pathology scores (A and C) or mycobacterial burdens (B and D). Student’s t test P values are provided in the upper left corner of each graph.


