Disparate Host Immunity to *Mycobacterium avium* subsp. *paratuberculosis* Antigens in Calves Inoculated with *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium*, *M. kansasii*, and *M. bovis*

J. R. Stabel, W. R. Waters, J. P. Bannantine, M. V. Palmer

USDA-ARS, National Animal Disease Center, Ames, Iowa, USA

The cross-reactivity of mycobacterial antigens in immune-based diagnostic assays has been a major concern and a criticism of the current tests that are used for the detection of paratuberculosis. In the present study, *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins were evaluated for antigenic specificity compared to a whole-cell sonicate preparation (MPS). Measures of cell-mediated immunity to *M. avium* subsp. *paratuberculosis* antigens were compared in calves inoculated with live *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium* (*M. avium*), *Mycobacterium kansasii*, or *Mycobacterium bovis*. Gamma interferon (IFN-γ) responses to MPS were observed in all calves that were exposed to mycobacteria compared to control calves at 4 months postinfection. Pooled recombinant *M. avium* subsp. *paratuberculosis* proteins also elicited nonspecific IFN-γ responses in inoculated calves, with the exception of calves infected with *M. bovis*. *M. avium* subsp. *paratuberculosis* proteins failed to elicit antigen-specific responses for the majority of immune measures; however, the expression of CD25 and CD26 was upregulated on CD4, CD8, gamma/delta (γδ) T, and B cells for the calves that were inoculated with either *M. avium* subsp. *paratuberculosis* or *M. avium* after antigen stimulation of the cells. Stimulation with MPS also resulted in the increased expression of CD26 on CD45RO$^+$ CD25$^+$ T cells from calves inoculated with *M. avium* subsp. *paratuberculosis* and *M. avium*. Although recombinant proteins failed to elicit specific responses for the calves inoculated with *M. avium* subsp. *paratuberculosis*, the differences in immune responses to *M. avium* subsp. *paratuberculosis* antigens were dependent upon mycobacterial exposure. The results demonstrated a close alignment in immune responses between calves inoculated with *M. avium* subsp. *paratuberculosis* and those inoculated with *M. avium* that were somewhat disparate from the responses in calves infected with *M. bovis*, suggesting that the biology of mycobacterial infection plays an important role in diagnosis.

A major struggle in the field diagnosis of paratuberculosis has been the cross-reactivity of serologic diagnostic tests with other mycobacteria. Of particular concern is the potential for false-positive identification of *Mycobacterium avium* subsp. *paratuberculosis* infection in animals that are either infected or have been exposed to *M. avium* subsp. *avium* (*M. avium*) or other environmental mycobacteria when using serum-based paratuberculosis diagnostic tests (1). Genomic studies have demonstrated a 98 to 99% homology between *M. avium* subsp. *paratuberculosis* and *M. avium*, making it difficult to identify antigens that will distinguish between exposures or infections with the 2 subspecies in a specific and sensitive manner (2, 3). The presence of *M. avium* in a wide breadth of environmental sources, such as water, soil, biofilms, and plants, as well as the contamination of feedstuffs and bedding by birds, makes cross-reactivity with current diagnostics for *M. avium* subsp. *paratuberculosis* a staunch reality (4, 5).

In recent years, the reemergence of *M. bovis* infection in U.S. dairy herds has contributed to concerns about the cross-reactivity of *M. avium* subsp. *paratuberculosis* diagnostics in animals that are infected with or exposed to *M. bovis*. The development of new serodiagnostic tests based upon *M. bovis* antigens has improved the ability to distinguish between animals that have been either vaccinated or infected with *M. avium* subsp. *paratuberculosis* from those with bovine tuberculosis (6, 7, 8, 9, 10, 11). *Mycobacterium kansasii* infections occur rarely in cattle, and cross-reactivity might be more of a concern with a bovine tuberculosis diagnosis; however, reports suggest that *M. kansasii* might share some epitopes with *M. avium* subsp. *paratuberculosis*, thereby confounding the diagnostic tests for paratuberculosis (12, 13, 14, 15, 16).

Although numerous studies have evaluated *M. avium* subsp. *paratuberculosis* recombinant proteins as potential diagnostic tools, the ability of *M. avium* subsp. *paratuberculosis* proteins to discriminate between different mycobacterial infections has not been adequately addressed (17, 18). This dearth of information is particularly evident for measures of cell-mediated immunity in animals exposed to different mycobacteria, as most studies report only serologic data based upon antigen-specific antibody responses. The present study is the first of its kind to compare immune responses to *M. avium* subsp. *paratuberculosis* antigens in calves infected with either live *M. avium* subsp. *paratuberculosis*, *M. avium*, *M. bovis*, or *M. kansasii*. Recombinant *M. avium* subsp. *paratuberculosis* proteins were compared to a whole-cell sonicate preparation of *M. avium* subsp. *paratuberculosis* (MPS) as antigens, focusing on assays to measure cell-mediated immune function, since these responses occur early in infection.

TABLE 1 *Mycobacterium avium* subsp. *paratuberculosis* recombinant proteins used in this study

GenBank gene no.	*M. avium* subsp. *paratuberculosis* protein characteristics			Identity (%)[a] to:		
	Protein name	Size (kDa)	Predicted function	*M. avium*[b]	*M. kansasii*[c]	*M. bovis*[d]
2720779	MAP0900	29.6	Hypothetical protein	93	62	59
2719512	MAP1087	15.4	ABC transporter permease	45	16	15
2719085	MAP1203	49.6	Hypothetical protein	95	77	79
2720812	MAP1204	25.4	Invasion protein (NlpC/P60 family)	100	81	78
2720835	MAP1272c	33.4	Invasion protein (NlpC/P60 family)	87	65	54
2719950	MAP2077c	11.1	STAS domain-containing protein	97	61	21
2718170	MAP4327c	18.5	Putative membrane protein	No ID[e]	35	36

[a] Identity determined based on the global pairwise alignment tool EMBOSS Needle (available from EMBL-EBI).
[b] Alignment to corresponding *M. avium* subsp. *avium* ATCC 25291 sequences.
[c] Alignment to corresponding *M. kansasii* ATCC 12478 sequences.
[d] Alignment to corresponding *M. bovis* AF2122/97 sequences.
[e] No identity.

MATERIALS AND METHODS

Animals. Dairy calves were infected via aerosol or intratonsillar routes as described previously (12, 19, 20, 21). Briefly, castrated Holstein-Friesian bull calves (4 to 6 months old) received either 10^8 CFU *M. avium* subsp. *paratuberculosis* strain 167 (a clinical bovine isolate), 10^9 CFU *M. avium* subsp. *avium* strain TMC 702 (a chicken isolate) (*M. avium*), or 10^8 CFU *M. kansasii* strain 03-6931 (a bovine isolate) (*M. kansasii*) by direct intratonsillar instillations of sedated calves ($n = 5$ per group), with inoculum delivered in two equal doses 2 weeks apart, or a single-dose aerosol inoculation of 10^4 CFU *M. bovis* strain 95-1315 (*M. bovis*) (a Michigan deer isolate, $n = 7$). The decision to use a larger dose of inoculum for the nontuberculous *Mycobacteria* spp. than for *M. bovis* was based upon prior studies (12, 17, 18) and the relatively low virulence of these species. A group of 8 age-, breed-, and gender-matched calves were also included as noninfected calves (controls).

All calves were housed in a biosafety level 3 (BL-3) facility according to the institutional guidelines and the approved animal care and use protocols. BL-3 procedures were followed for the *M. bovis* group of calves, whereas BL-2 procedures were followed for all other groups. Mycobacterial culture and the enumeration of challenge inoculum, postmortem procedures, and histopathology were as described previously (19, 21) and standard techniques were used. For calves inoculated with *M. bovis*, gross lesions typical of *M. bovis* infection were observed in the lungs and pulmonary lymph nodes from all calves (see Table S1 in the supplemental material). As described previously (12, 19, 20), gross lesions were not detected in noninfected control or *M. avium*-, *M. avium* subsp. *paratuberculosis*-, or *M. kansasii*-inoculated calves; however, these groups of calves each developed cell-mediated immune responses as measured by a comparative cervical skin test (22). All procedures performed on the animals were approved by the Institutional Animal Care and Use Committee (National Animal Disease Center [NADC], Ames, IA).

Blood collection and culture conditions. Whole blood was collected into heparinized Vacutainer tubes prior to the inoculation of calves (day 0), and monthly thereafter. Antigens used in the gamma interferon (IFN-γ) assay were medium only (nonstimulated [NS]), concanavalin A (ConA) (10 μg/ml; Sigma), pokeweed mitogen (PWM) (10 μg/ml; Sigma), and whole-cell sonicates of *M. avium* subsp. *paratuberculosis* (MPS) (clinical cow 167, 10 μg/ml; NADC), *M. avium* (MavS) (10 μg/ml; NADC), *M. kansasii* (MkS) (10 μg/ml; NADC), and *M. bovis* (MboS) (10 μg/ml; NADC). In addition, 7 *M. avium* subsp. *paratuberculosis* proteins (MAP0900, MAP1087, MAP1203, MAP1204, MAP1272c, MAP2077c, and MAP4327c [Table 1]) were arrayed in 3 cocktails containing 3 of the 7 proteins as follows: cocktail 1, MAP1087, MAP1272c, and MAP2077c; cocktail 2, MAP0900, MAP1203, and MAP1272c; cocktail 3, MAP1204, MAP1272c, and MAP4327. The selection of the *M. avium* subsp. *paratuberculosis* proteins used in the present study was based upon their immunogenic potential as defined by prior testing that was performed for IFN-γ

and antibody responses in cattle. Pools were randomly arrayed with 3 proteins each, except for the presence of MAP1272c in each array. The protein MAP1272c was present in each of the 3 pools because of its level of antigenicity and its potential as a specific *M. avium* subsp. *paratuberculosis* antigen, as described recently (23). Pools of proteins, rather than individual proteins, were used as antigen preparations, as immune responses to individual antigens might be too selective, resulting in false-negative results in some animals.

The 7 annotated coding sequences of the proteins were selected from a battery of >600 recombinant proteins amplified from *M. avium* subsp. *paratuberculosis* strain K-10 genomic DNA. The methods for the expression and purification of *M. avium* subsp. *paratuberculosis* recombinant proteins are described in detail (3). Briefly, maltose binding protein-*M. avium* subsp. *paratuberculosis* fusion proteins were obtained by overexpression in *Escherichia coli* by the induction with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Proteins were purified via affinity chromatography using an amylose resin (New England BioLabs, Ipswich, MA) and then pooled and dialyzed in 1 liter of phosphate-buffered saline (PBS) (140 mM NaCl, 10 mM NaHPO$_4$, 2.7 mM KCl, and 2 mM KH$_2$PO$_4$ [pH 7.2]) at 4°C for 4 h with 3 total exchanges. Confirmation of the expression was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels stained with GelCode blue (Thermo Scientific).

Mitogens and antigens were added to 1 ml of heparinized whole blood and incubated at 39°C in 5% CO$_2$ under a humidified atmosphere for 18 h. The assay protocol was modified by increasing the incubation temperature to 39°C, which is the body temperature of a cow, as is performed on all cellular assays within our laboratory (J. R. Stabel, unpublished data). The addition of each protein cocktail to whole blood yielded a final concentration of 3 μg/ml, with each protein being equally represented. The final concentration of each mitogen and whole-cell sonicate preparation in whole blood was 10 μg/ml. Following incubation, the plates were centrifuged at 1,300 × g for 5 min, and plasma was collected and stored at −20°C until analyzed.

Prior to termination of the study at 5 months postinoculation, blood was collected from the jugular vein in 2× acid-citrate-dextrose (ACD) (1:10). Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat fractions of blood. PBMCs were resuspended in complete medium (RPMI 1640 [Gibco, Grand Island, NY] with 10% fetal calf serum [Atlanta Biologicals, Atlanta, GA], 100 U of penicillin G sodium [Gibco] per ml, 100 μg of streptomycin sulfate [Gibco] per ml, 0.25 μg of amphotericin B [Gibco] per ml, and 2 mM L-glutamine [Gibco]). Cells were cultured at 2.0×10^6/ml in replicate 48-well flat-bottomed plates (Corning Incorporated, Corning, NY) at 39°C in 5% CO$_2$ under a humidified atmosphere. Cell viability was determined to be >95% by the use of propidium iodide exclusion. Duplicate wells were set up for each animal for each *in vitro* treatment. *In vitro* treatments consisted of nonstimulated (medium only), concanavalin A (ConA) (10 μg/ml; Sigma Chemical Co.,

St. Louis, MO), and a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (10 µg/ml). After 24 h, one set of plates were removed and centrifuged at $400 \times g$ for 5 min. Supernatants were removed without disturbing the cells in culture and were stored at $-20°C$ prior to cytokine measurement. The replicate set of plates was incubated for 6 days and cells were harvested for flow cytometric analyses.

Cytokine analyses. Bovine IFN-γ was measured in plasma using the Bovigam test kit (Prionics, La Vista, NE) as described by the manufacturer. Bovine IFN-γ levels measured in plasma (whole-blood assay) were expressed as absorbance units of each stimulated blood sample minus the absorbance measured for the corresponding nonstimulated sample within each animal (e.g., $Abs_{450nm}MPS - Abs_{450nm}NS$). The remaining cytokines, interleukin-10 (IL-10), IL-4, and IL-12, were measured on cell supernatants. Bovine IL-10 was quantified by coating MaxiSorp microtiter plates (Nunc, Rochester, NY) with mouse anti-bovine IL-10 in coating buffer (15 mM sodium carbonate, 34 mM sodium bicarbonate [pH 9.6]; 100 µl per well at 2 µg/ml) (MCA2110; Serotec, Raleigh, NC) overnight at room temperature (RT). Plates were washed 5 times with PBS containing 1% Tween 80 (washing buffer). The samples and serial 2-fold dilutions of bovine IL-10 standard (0.3125 to 20 ng/ml) (generous gift from Jayne Hope, Compton, United Kingdom) were added to the duplicate wells and incubated at RT for 1 h. Plates were then washed 5 times with washing buffer before incubating with the detection antibody, mouse anti-bovine IL-10-biotin (MCA2111B; Serotec). Plates were washed 5 times with washing buffer, 100 µl of avidin-horseradish peroxidase (HRP) conjugate (diluted 1:800) (Pharmingen, San Diego, CA) was added to each well, and the plates were incubated for 45 min at RT. After another wash cycle, plates were incubated with substrate solution (40 mM ABTS [2,2′-azino-bis(3-ethylbenzthiazolinesulfonic acid)]) in citrate buffer (United States Biological, Swampscott, MA). Color development was quantified after 30 min by measuring absorbance at 405 nm with a Wallac Victor 1420 multilabel counter enzyme-linked immunosorbent assay (ELISA) plate reader (PerkinElmer, Gaithersburg, MD). Bovine IL-12 was measured following a protocol similar to that with IL-10, using a mouse anti-bovine IL-12 antibody (MCA1782EL; Serotec) as the capture antibody and a mouse anti-bovine IL-12-biotin antibody (MCA2173B; Serotec) as the detection antibody. Serial 2-fold dilutions of bovine IL-12 standard (1.56 to 100 ng/ml; Kingfisher Biotech) were used for the quantification of IL-12 in the samples. IL-4 was determined using mouse anti-bovine IL-4 (CC313; Serotec) and mouse anti-bovine IL-4-biotin (CC314; Serotec) for capture and detection, respectively, along with serial dilutions of bovine IL-4 standard (31.25 to 2,000 pg/ml, PBP006; Serotec) for quantification. As for IL-10, measurements of IL-12 and IL-4 in cell supernatants were performed at 405 nm on the ELISA plate reader.

Flow cytometric analysis. Briefly, plates that were cultured for 6 days were centrifuged at 1,500 rpm for 5 min and the supernatant was decanted. Cells were gently resuspended in 300 µl of PBS (0.15 M [pH 7.4]). In 96-well round-bottom plates (Corning Incorporated, Corning, NY), 50 µl of the cell suspension was added to wells containing 50 µl of primary monoclonal antibody to CD4, CD8, gamma/delta (γδ), CD25, CD26, CD45RO, and CD335 T-cell markers, as well as markers for B cells and monocytes-macrophages (Table 2). All wells received 10 µg/ml of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) to differentiate live from dead cells and to allow gating on viable cells. Cells were then incubated at 4°C for 30 min. After incubation, plates were centrifuged at $330 \times g$ for 2 min at 4°C and the supernatant was decanted. One hundred microliters of secondary antibody cocktail consisting of fluorescein-conjugated anti-mouse IgM (SouthernBiotech, Birmingham, AL), R-phycoerythrin-conjugated goat F(ab)$_2$ anti-mouse IgG2a (SouthernBiotech, Birmingham, AL), and peridinin chlorophyll protein complex-conjugated rat anti-mouse IgG1 (Becton, Dickinson, San Jose, CA) diluted 1:312, 1:625, and 1:42, respectively, in PBS with 1% fetal calf serum and 0.04% sodium azide was added to designated wells and the plate was centrifuged again at $330 \times g$ for 2 min at 4°C. The cells were then suspended in 200 µl of BD FACS Lyse (BD Biosciences, San Jose, CA) for immediate flow cytometric anal-

TABLE 2 Primary antibodies[a]

Antigen	MAb[b] clone	Isotype	Working MAb concn[c] (µg/ml)	Specificity
CD4	GC50A1	IgM	14	T-helper cells
CD8	BAQ111A	IgM	14	T-cytotoxic/suppressor cells
CD25	CACT116A	IgG1	15	IL-2 receptors
	CACT108A	IgG2a	15	IL-2 receptors
	LCTB2A	IgG3	15	IL-2 receptors
CD26	CACT114A	IgG2b	15	Activation marker
CD45RO	GC42A1	IgG1	10	Memory/activation marker
N12	CACT61A	IgM	14	γδ cell receptors
B cell	BAQ155	IgG1	7	Total B cells

[a] VMRD Inc. (Pullman, WA).
[b] MAb, monoclonal antibody.
[c] Diluted in PBS with 1% fetal calf serum and 0.04% sodium azide.

ysis. Samples were evaluated using 30,000 events per sample using a FACScan flow cytometer (CellQuest Software; Becton, Dickinson). Analysis was conducted by gating on mononuclear cells based on forward and side scatter characteristics (FlowJo; Tree Star, Inc., San Carlos, CA).

Statistical analyses. Cytokine secretion and the percentages of each cell population were analyzed by using the PROC Mixed analysis of SAS (PROC MIXED in SAS PC Windows version 9.1.3 software). Values were reported as least square mean $\pm$ the standard error of the mean unless noted otherwise. When significant effects ($P < 0.05$) owing to infection or *in vitro* treatment were detected, a means comparison was conducted using the Tukey-Kramer *post hoc* test.

RESULTS

Whole-blood IFN-γ responses to mitogens or whole-cell sonicate preparations in calves inoculated with *M. avium* subsp. *paratuberculosis*, *M. avium*, *M. kansasii*, and *M. bovis* are presented in Fig. 1. The mitogens ConA and PWM, which were utilized to generate nonspecific IFN-γ responses, demonstrated similar reactivities for all infected calves, with the exception of calves inoculated with *M. bovis*, who demonstrated more robust ($P < 0.05$) responses to both mitogens (Fig. 1A). Antigen-specific responses to whole-cell sonicates aligned themselves somewhat with the respective calf infection, as significant ($P < 0.05$) responses to MavS were observed only in calves inoculated with *M. avium*. In addition, responses to MkS and MboS ($P < 0.05$) also aligned themselves to the respective infection in calves, but calves infected with *M. bovis* also responded ($P < 0.05$) very robustly to MkS. In contrast, IFN-γ responses to MPS were significantly ($P < 0.05$) greater for all infected calves, regardless of the inoculum used, than those with control calves, demonstrating no specificity in response (Fig. 1B). Pooled *M. avium* subsp. *paratuberculosis* proteins also did not demonstrate specificity as stimulators of IFN-γ responses in *M. avium* subsp. *paratuberculosis*-infected calves (Fig. 1B) and were ineffective as specific antigens for the majority of assays measured in this study (data not shown). However, IFN-γ responses to the *M. avium* subsp. *paratuberculosis* protein pools were consistently ($P < 0.05$) lower for calves inoculated with *M. bovis* than for those inoculated with *M. avium* subsp. *paratuberculosis*, indicating that further refinement of *M. avium* subsp. *paratuberculosis* protein pools might lead to their potential utility as tools to discriminate
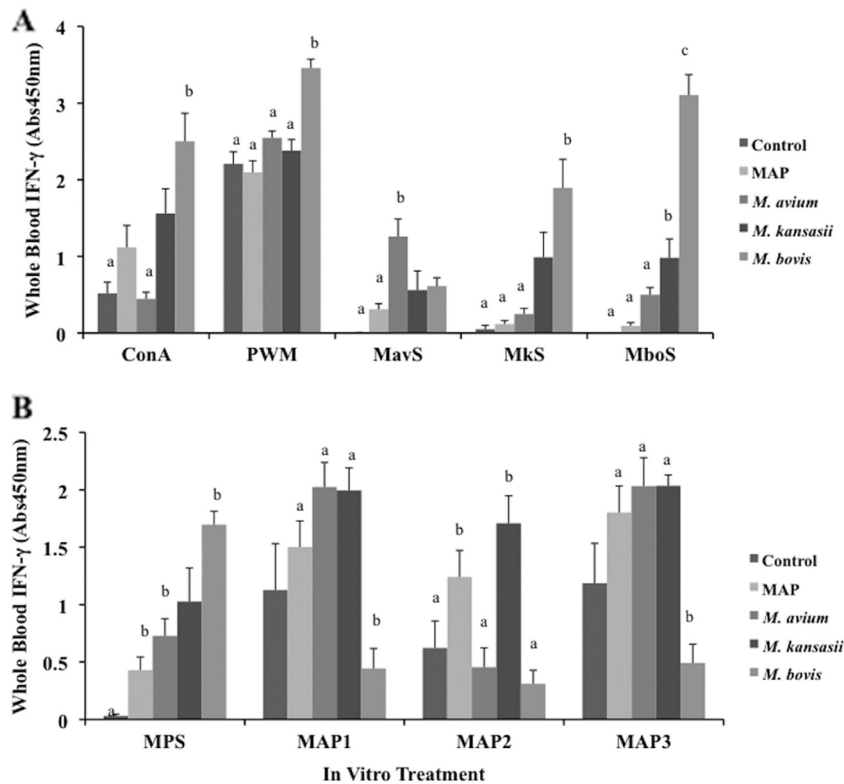
FIG 1 Secretion of gamma interferon (IFN-γ; Abs450nm) in a whole-blood assay by control noninfected calves (control) or calves infected with live *Mycobacterium avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium* (*M. avium*), *M. kansasii*, or *M. bovis*. (A) Whole blood was incubated for 18 h with concanavalin A (ConA), pokeweed mitogen (PWM), or whole-cell sonicates of *M. avium* subsp. *avium* (MavS), *M. kansasii* (MkS), or *M. bovis* (MboS). (B) Whole blood was incubated with a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (MPS) or pools of recombinant *M. avium* subsp. *paratuberculosis* proteins (MAP1, MAP2, MAP3). Data are expressed as means ± standard errors of the means (SEM). Significant ($P < 0.05$) differences between the infection groups and within each *in vitro* treatment are represented by different letters.

between *M. avium* subsp. *paratuberculosis* and *M. bovis* infections in the field (Fig. 1B).

To identify additional immunologic biomarkers for infection that might distinguish between *M. avium* subsp. *paratuberculosis* and other mycobacteria, further determination of the IL-4, IL-10, and IL-12 levels in supernatants from cultured PBMCs was performed. Although not a major focus of the current study, it was interesting to note that ConA stimulation resulted in measurable differences in the host responses to mycobacterial infection, including lower ($P < 0.05$) IL-12 in culture supernatants in calves exposed to *M. avium* and *M. bovis* than in the other treatment groups (Fig. 2A) and higher ($P < 0.05$) levels of IL-4 secretion for calves exposed to *M. bovis* (Fig. 2B). Although infected calves did not yield significant responses to MPS, calves inoculated with *M. avium* subsp. *paratuberculosis* tended ($P < 0.10$) to have higher antigen-specific IL-4 responses. Stimulation of cells with MPS resulted in increased ($P < 0.05$) IL-10 secretion and decreased ($P < 0.05$) IL-12 secretion in *M. bovis*-infected calves than in other infected calves (Fig. 2C).

An increase ($P < 0.05$) in the percentage of CD4 T cells was observed for calves inoculated with *M. avium* subsp. *paratuberculosis* after the stimulation of PBMCs with MPS, and a concomitant decrease ($P < 0.05$) was noted for calves exposed to *M. bovis* (Fig. 3A). The expression of CD25 on CD4$^+$ T cells was significantly ($P < 0.05$) upregulated on cells from *M. avium* subsp. *paratuberculosis*-, *M. avium*-, and *M. bovis*-infected calves, with

the highest expression in *M. bovis*-infected calves (Fig. 3B). A downregulation in the percentage of CD4$^+$ CD26$^+$ T cells was paralleled ($P < 0.05$) in these calf groups. The expression of both CD25 and CD26 on CD4$^+$ T cells was only significant ($P < 0.05$) for the *M. avium* subsp. *paratuberculosis*- and *M. avium*-infected calves.

The percentage of total CD8$^+$ T cells was marginally increased ($P < 0.10$) in calves exposed to *M. avium* subsp. *paratuberculosis* and those exposed to *M. avium* but was significantly ($P < 0.05$) lower in the calves exposed to *M. bovis* after stimulation of the cells with MPS (Fig. 3B), and reductions ($P < 0.05$) in the subpopulations of CD8$^+$ CD25$^+$ and CD8$^+$ CD26$^+$ T cells were also noted in the calves exposed to *M. bovis*. Interestingly, a highly significant ($P < 0.05$) upregulation of CD26 expression on CD8 T cells was observed for the *M. avium* subsp. *paratuberculosis*- and *M. avium*-infected calves in this study. A similar pattern of expression for these markers was noted for γδ T cells, but the upregulation of expression noted for the *M. avium* subsp. *paratuberculosis*- and *M. avium*-infected calves was concomitant with a significant ($P < 0.05$) decrease for the *M. bovis*-infected calves (Fig. 4A). Results for MPS-stimulated γδ T cells also followed this pattern of expression as defined by the infection group, with *M. avium* subsp. *paratuberculosis*, *M. avium*, and *M. kansasii* infections invoking increased ($P < 0.05$) numbers of γδ T cells and *M. bovis* infection effecting a decrease in this population of T cells.

Neither the total B cell number nor the B cell-CD25 cell ratio
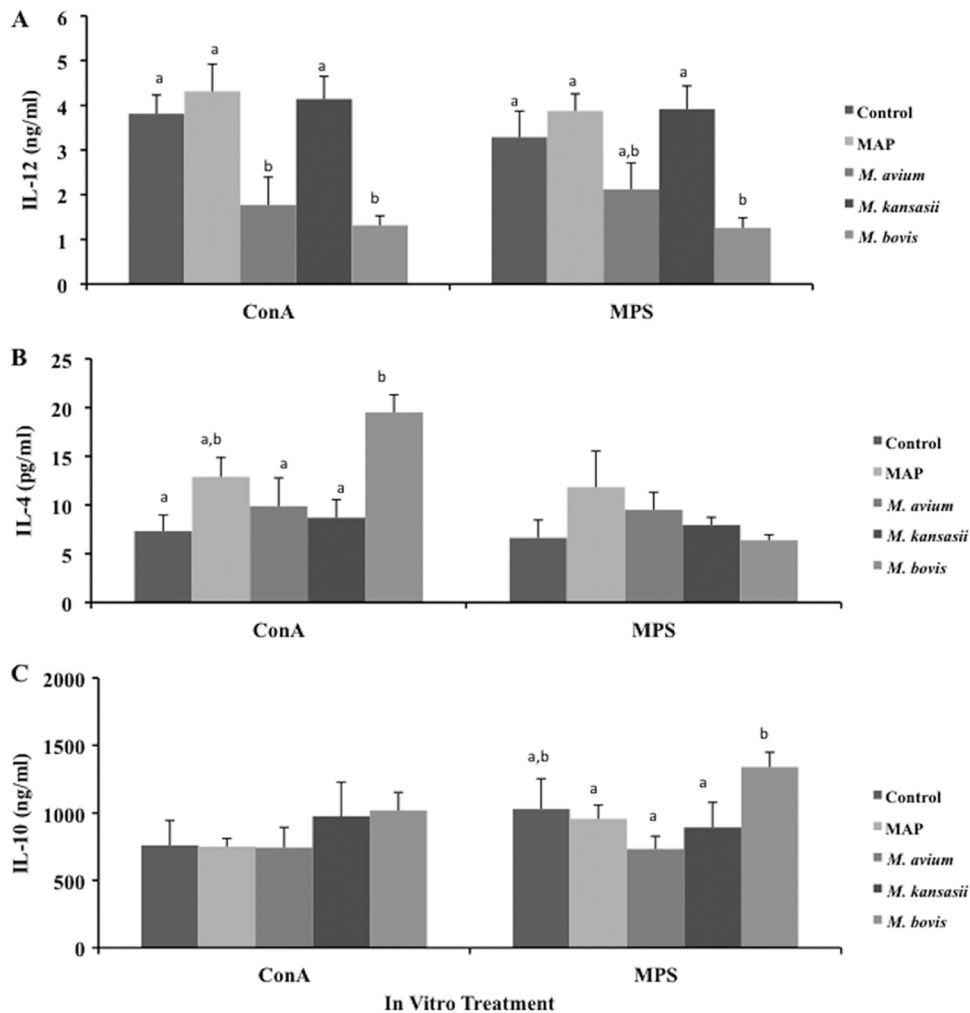
FIG 2 Secretion of interleukin-12 (IL-12) (A), IL-4 (B), and IL-10 (C) by PBMCs isolated from control noninfected calves (control) or calves infected with live *Mycobacterium avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium* (*M. avium*), *M. kansasii*, or *M. bovis*. PBMCs were incubated for 24 h with concanavalin A (ConA) or a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (MPS). Data are expressed as means ± SEM. Significant differences ($P < 0.05$) between the infection groups and within each *in vitro* treatment are represented by different letters.

was markedly affected by infection status of the calves, but significantly ($P < 0.05$) increased numbers of CD26$^+$ B-cell subpopulations were observed for the calves exposed to *M. avium* subsp. *paratuberculosis* and those exposed to *M. avium* after the stimulation of total PBMCs with MPS (Fig. 4B).

Antigen-specific upregulation of CD45RO$^+$ cells in total PBMCs was noted for the calves in this study exposed to *M. avium* subsp. *paratuberculosis* and those exposed to *M. avium* (Fig. 5). Further, stimulation with the *M. avium* subsp. *paratuberculosis* protein cocktails (MAP1, MAP2, and MAP3) specifically upregulated ($P < 0.05$) the expression of CD45RO for the *M. avium* subsp. *paratuberculosis*-infected calves compared to the *M. bovis*-infected calves (Fig. 5A). Further delineation of CD45RO$^+$ CD25$^+$ T cells demonstrated an increased expression of CD26 on CD4 ($P < 0.05$), CD8 ($P < 0.01$), and γδ ($P < 0.01$) T cells for the *M. avium* subsp. *paratuberculosis*- and *M. avium*-infected calves (Fig. 5B). Interestingly, CD26 expression decreased ($P < 0.01$) for the *M. bovis*-infected calves for all 3 subpopulations of T cells, especially γδ T cells.

DISCUSSION

The results of the present study demonstrate that measurable immune responses to *M. avium* subsp. *paratuberculosis* antigens can occur in cattle that are infected with other mycobacterial pathogens. The discovery of novel antigens or cocktails of antigens that can discriminate *M. avium* subsp. *paratuberculosis* infection from exposure to or infection with other mycobacteria is critical to the control and management of this disease. Presently, commercial serodiagnostic tests for paratuberculosis are reliant upon crude cell lysates as the capture antigens in their immunoassays; this leads to potential specificity issues since many proteins are conserved across mycobacterial species.

Previously, it was shown by our laboratory that recombinant *M. avium* subsp. *paratuberculosis* proteins could be used to detect *M. avium* subsp. *paratuberculosis*-specific antibodies in the serum of naturally infected cattle, with moderate-to-negligible reactivity to serum from *M. avium*- and *M. bovis*-infected cattle (3). However, little work has been performed to evaluate the utility of re-
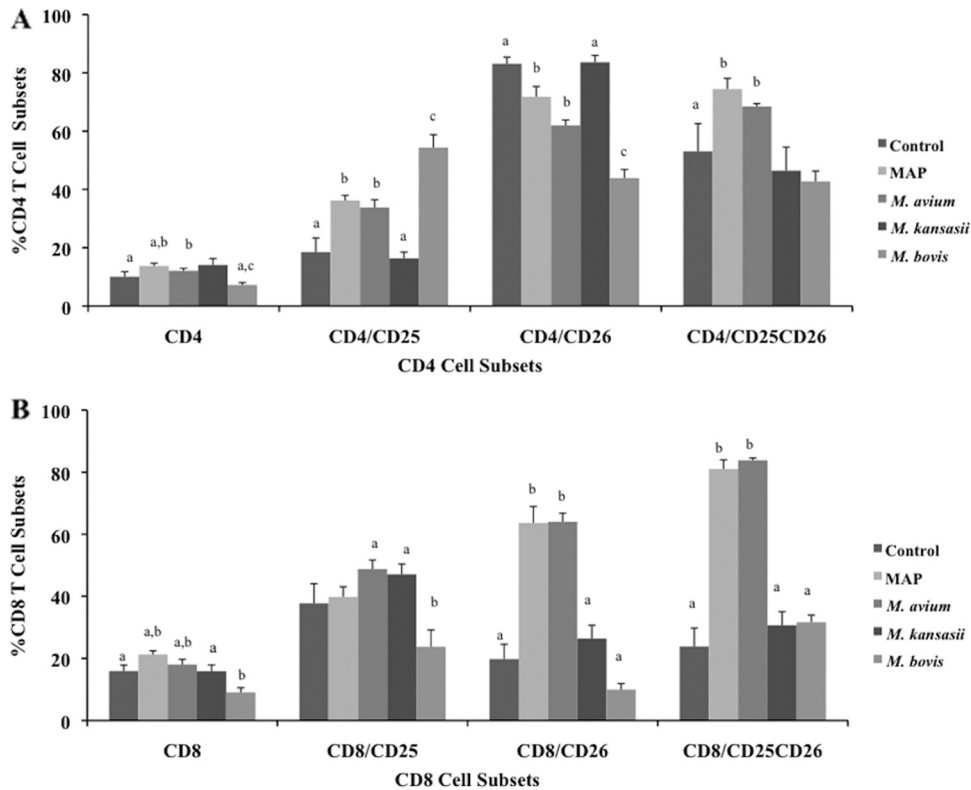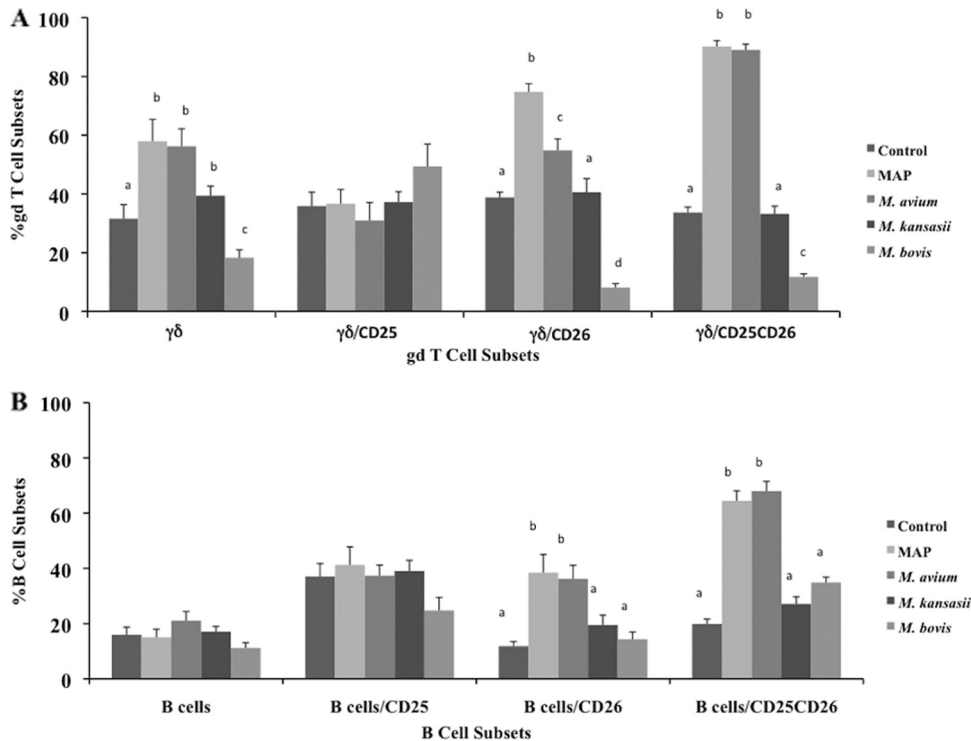
FIG 3 Percentages of CD4 (A) and CD8 (B) T cells from PBMCs isolated from control noninfected calves (control) or calves infected with live *Mycobacterium avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium* (*M. avium*), *M. kansasii*, or *M. bovis*. Cells are expressed as CD4 or CD8 populations with coexpression of CD25 and CD26 subpopulations, after stimulation with a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (MPS). Data are expressed as means ± SEM. Significant differences (*P* < 0.05) between the groups are represented by different letters.

combinant *M. avium* subsp. *paratuberculosis* proteins as antigens in cell-mediated immune function assays. Recently, an evaluation of 14 recombinant proteins selected from three major groups, latency proteins, secreted proteins, and proteins of the early secretory antigenic target 6 (ESAT-6), an important group of antigenic proteins in *M. bovis* infections, was performed using the whole-blood IFN-γ assay platform in dairy cattle (17). All proteins tested demonstrated greater specificity than did johnin purified protein derivative (PPD) in the IFN-γ assay, but efficacy for the diagnosis of paratuberculosis was best for the latency protein group, with higher immunogenicity and specificity observed in that group. An additional comprehensive search for *M. avium* subsp. *paratuberculosis* proteins with diagnostic potential conducted by aligning mass spectrophotometric analyses of *M. avium* subsp. *paratuberculosis*, *M. avium*, and *M. bovis* PPDs demonstrated that up to 73% of proteins were shared between the 3 PPD preparations (24). Due to the high degree of homology between the *M. avium* subsp. *paratuberculosis* and *M. avium* PPDs, only one unique protein (MAP1718c) was identified. MAP1718c did not demonstrate any specificity in either the lymphocyte stimulation test or serum antibody ELISA; however, MAP1138, a conserved protein present in all 3 PPDs, was able to differentiate between noninfected and *M. avium* subsp. *paratuberculosis* shedders in the serum antibody test. A more recent study evaluated the diagnostic potential of 54 *M. avium* subsp. *paratuberculosis* proteins in a macroarray, blotting against serum samples from healthy animals, animals with paratuberculosis, and animals that were experimentally infected with

M. bovis (25). Based upon differential spot intensities between the infection groups, 7 *M. avium* subsp. *paratuberculosis* proteins (MAP0038, MAP0210c, MAP1272, MAP1692c, MAP0209, MAP2020, and MAP2513) were selected for further study. Blotting against a cocktail of the 7 *M. avium* subsp. *paratuberculosis* proteins resulted in a sensitivity of detection of 72% (18/25) for cows with paratuberculosis, compared to no reactivity with serum from control cows (*n* = 26) and low reactivity with serum from *M. bovis* cows (3/17). In contrast, ELISA reactivity using *M. avium* strain 18 protoplasmic antigen (PPA-3) demonstrated a comparable sensitivity of detection to the antigen cocktail, but it had much lower specificity.

Proteins selected for use in the present study had been demonstrated previously to induce antigen-specific IFN-γ responses in cattle (see Fig. S1 in the supplemental material) and, as such, were considered suitable for evaluating them as specific mediators of cell-mediated immunity. However, the results were disappointing, yielding responses that clearly showed a lack of specificity across infection groups for most parameters that were measured. Responses to the whole-cell sonicate preparations of *M. avium* subsp. *paratuberculosis* (MPS) in the IFN-γ assay were also observed for all infection groups. This is not surprising considering that any mycobacterial whole-cell lysate would contain proteins that would be conserved across species and potentially contain antigenic components that could elicit a response. However, the robustness of the response noted in the calves infected with *M. bovis* compared to calves infected with either *M. avium* subsp.
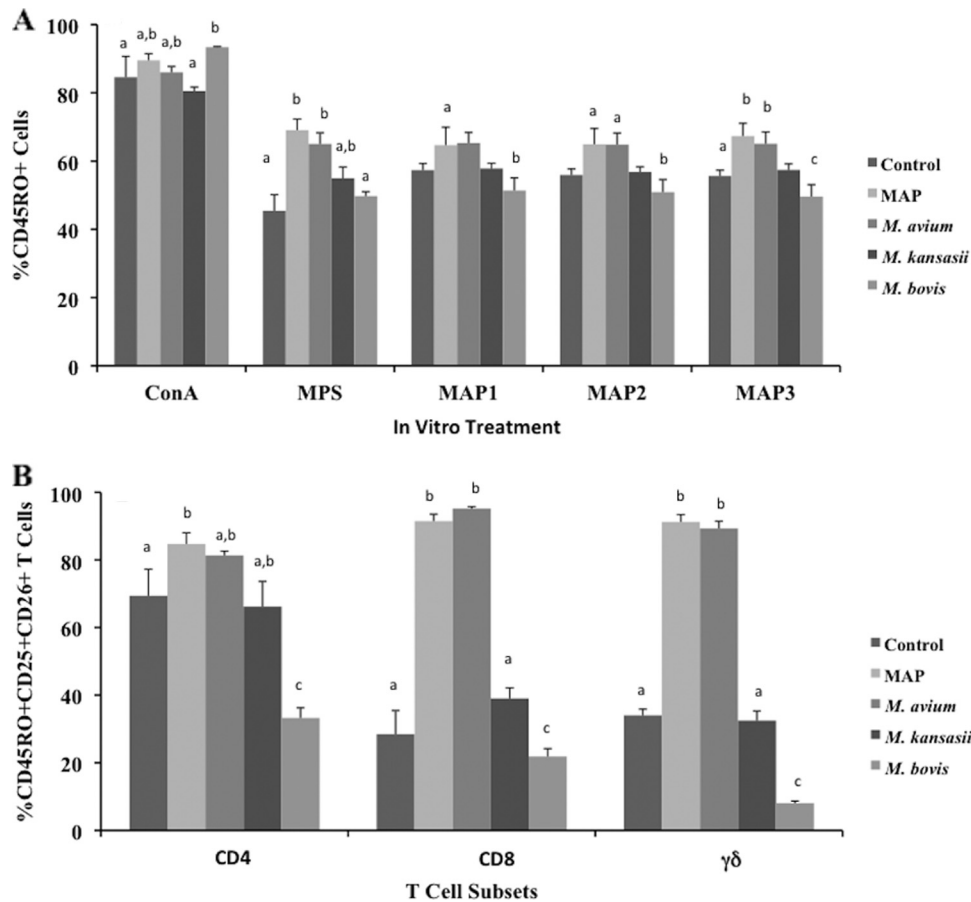
FIG 4 Percentages of γδ (gd) T cells (A) and B cells (B) from PBMCs isolated from control noninfected calves (control) or calves infected with live *Mycobacterium avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium* (*M. avium*), *M. kansasii*, or *M. bovis*. Cells are expressed as total γδ T-cell or B-cell populations with coexpression of CD25 and CD26, after stimulation with a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (MPS). Data are expressed as means ± SEM. Significant differences (*P* < 0.05) between groups are represented by different letters.

paratuberculosis or *M. avium* was unforeseen. In fact, the calves infected with *M. bovis* had greater IFN-γ responses overall than those in the other infected groups, with higher responses to both ConA and PWM mitogens, and the most robust responses to their corresponding antigen preparation (MboS) of any infection group. Nonspecific responses in the IFN-γ assay and the comparative cervical skin test have been reported previously for calves infected with *M. avium* subsp. *paratuberculosis*, *M. avium*, and *M. bovis* (12). Responses to *M. avium* subsp. *paratuberculosis*, *M. avium*, and *M. bovis* PPDs were noted in both tests for all infected calves, confirming the lack of specificity of these reagents; however, the responses of the *M. bovis*-infected calves were always more robust, especially to the *M. bovis* PPD. Interestingly, the *M. avium* subsp. *paratuberculosis* protein cocktails used in the present study did not elicit specific IFN-γ responses in the calves infected with *M. avium* subsp. *paratuberculosis*, but they did result in a consistent decreased response for the calves infected with *M. bovis*. This differential response might allow for discrimination between the *M. bovis*-infected calves and those in other infection groups, indicating a potential use in the field to distinguish *M. bovis* from *M. avium* subsp. *paratuberculosis* infections.

Measurement of other secreted cytokines after stimulation of the cells with *M. avium* subsp. *paratuberculosis* protein cocktails or MPS did not demonstrate any specificity of response across infection groups. Infection of calves with *M. bovis* did result in the increased secretion of IL-4 and decreased secretion of IL-12 after stimulation of cells with ConA. This is interesting, since this finding does not correspond with a strong Th1-mediated immunity that is suggested by the IFN-γ responses. Concomitant IL-4 and

IFN-γ responses have been described previously for cattle infected with *M. bovis* after the stimulation of cells with an *M. bovis* PPD; however, IFN-γ responses were sustained over 20 weeks of infection and IL-4 responses were more phasic and short-lived, peaking at 8 weeks postinfection (26). It was suggested that the delay in IL-4 secretion might be part of an anti-inflammatory response invoked by the presence of high levels of IFN-γ.

Previously, we observed that CD25 and CD26 expression was upregulated on T and B cells within 1 month after experimental infection of calves with *M. avium* subsp. *paratuberculosis* (27, 28). Expression levels of both of these activation markers were also increased on CD4, CD8, and γδ T cells after stimulation of PBMCs from *M. bovis*-infected cattle with either a recombinant early secretory antigenic target 6 and culture filtrate protein 10 fusion protein (rESAT-6:CFP-10) or *M. bovis* PPD (29). In the present study, CD25 and CD26 expression on CD4, CD8, and γδ T-cell subpopulations was increased by mycobacterial infection after the stimulation of cells with MPS. However, CD26 expression on CD8 and γδ T cells was more discrete for *M. avium* subsp. *paratuberculosis*-infected and *M. avium*-infected calves, and when cell subsets were further defined by CD25 expression, there was a definitive association with T- and B-cell populations in the *M. avium* subsp. *paratuberculosis*- and *M. avium*-infected calves that was not observed for the *M. kansasii*- or *M. bovis*-infected calves. Although the stratification between the infection groups suggests a possible utility for these cell markers to discriminate between *M. avium* subsp. *paratuberculosis* and *M. bovis* infections, understanding the biological meaning of these two markers is more intriguing. Both CD25 (IL-2 receptor) and CD26 are activation markers present

FIG 5 Percentage of CD45RO cells from PBMCs isolated from control noninfected calves (control) or calves infected with live *Mycobacterium avium* subsp. *paratuberculosis*, *M. avium* subsp. *avium* (*M. avium*), *M. kansasii*, or *M. bovis*. Cells are expressed as the total CD45RO population (A), after stimulation with either concanavalin A (ConA), a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (MPS), or pools of recombinant *M. avium* subsp. *paratuberculosis* proteins (MAP1, MAP2, MAP3). Subpopulation (%) of CD45RO$^+$ CD25$^+$ CD26$^+$ within CD4, CD8, and γδ T cells (B) after stimulation with a whole-cell sonicate of *M. avium* subsp. *paratuberculosis* (MPS). Data are expressed as the mean ± SEM. Significant differences ($P < 0.05$) between groups are represented by different letters.

primarily on T cells but also on other cell populations (30). CD26 also serves as a maturation marker for T cells, and its expression is highly correlated with Th1-mediated immunity and inflammation (29, 30). Further, CD26 is linked with CD45 on the surface of T cells, resulting in the induction of T-cell signal transduction pathways (28, 31). The expression of CD26 appears to be preferentially upregulated on CD4$^+$ CD45RO$^+$ memory T cells (32). In *M. avium* subsp. *paratuberculosis* infection, it has been shown previously that CD4$^+$ T cells with a memory phenotype (CD45RO$^+$) and expressing both CD25 and CD26 were the primary cell population that responded to antigen stimulation in calves within 6 months of oral inoculation with *M. avium* subsp. *paratuberculosis* (33). After 18 months of infection, a shift to a CD8$^+$ CD45RO$^+$ memory cell population expressing CD25 and CD26 occurred that was suggestive of a change in cell phenotype as the infection progressed. Although the infection period in the current study was relatively short (4 months), antigen recall responses as defined by increases in the CD45RO$^+$ population, as well as coexpression of CD26 on CD45RO$^+$ CD25$^+$ CD8 and γδ T subsets, were observed only for the calves exposed to *M. avium* subsp. *paratuberculosis* or *M. avium*. In contrast to previous reports, little effect of infection was noted on the expression of CD26 on the CD4$^+$ memory phenotype.

The lack of or decreased CD26 expression on CD45RO$^+$ T cells

in the calves infected with *M. bovis* is not easy to explain, as there should have been enough antigenic cross-reactivity to invoke some level of response. However, a previous study reported a lack of expression of CD26 on CD4$^+$ CD45RO$^+$ and CD8$^+$ CD45RO$^+$ cell populations from *M. bovis*-infected cattle after stimulation of cells with *M. bovis* antigens, suggesting that the effect is a factor of infection rather than being due to the antigen preparation that was used to stimulate the cells (29). One possible explanation for a lack of CD26 expression might be related to the role that CD26 plays in the migration of T cells during infection. Increased numbers of CD26$^+$ T cells have been reported at sites of inflammation for a number of disorders, including autoimmune disorders, such as rheumatoid arthritis, Grave's disease, and multiple sclerosis, as well as infectious diseases, such as leishmaniasis (34, 35). It might be plausible that infections, such as that with *M. avium* subsp. *paratuberculosis*, that cause unrestrained inflammation in gut tissues invoke a greater degree of CD26 expression so that cells can migrate to those sites. In contrast, CD26 expression in either *M. bovis* or *M. tuberculosis* infections might be less likely because infections are sequestered within granulomatous lesions in the lungs, trapping sensitized lymphocytes (36). This explanation might be consistent for the influences of infection noted on γδ T-cell populations in the present study as well,

since γδ T cells play a role in the migration of cells to areas of inflammation (37, 38).

In summary, the whole-cell preparation of *M. avium* subsp. *paratuberculosis* antigen elicited nonspecific responses for all measured cytokines, regardless of the infection group. Using cocktails of *M. avium* subsp. *paratuberculosis* recombinant proteins to stimulate the cells largely resulted in nonspecific responses, except for the differences noted between other infection groups and *M. bovis*-infected calves. Infection of calves with *M. avium* subsp. *paratuberculosis* or *M. avium* invoked antigen-specific increases in γδ T cells, but γδ T cells declined in *M. bovis* infection. Novel information included the differential expression of CD26 on CD45RO⁺ T-cell subpopulations in calves infected with *M. avium* subsp. *paratuberculosis* and *M. avium* compared to *M. bovis*-infected calves, suggesting distinct differences in the antigen recall responses in the disease states.

ACKNOWLEDGMENTS

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