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Diverse Cytokine Profile from Mesenteric Lymph Node Cells of Cull Cows Severely Affected with Johne's Disease▽

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***Mycobacterium avium* subsp. *paratuberculosis*, the causative agent of Johne's disease, is able to dampen or distort immune responses at the mucosal sites and coexist with a massive infiltration of immune cells in the gastrointestinal tract. Knowledge of the mechanism by which *M. avium* subsp. *paratuberculosis* subverts the immune response at the mucosal level in cattle is important for the development of improved disease control strategies, including new vaccines and diagnostic tests. In this study, 38 cull cows from herds infected with *M. avium* subsp. *paratuberculosis* were divided into four groups, based on *M. avium* subsp. *paratuberculosis* culture from gut tissues and histopathological lesion scores. Cytokine gene expression and secretion from *M. avium* subsp. *paratuberculosis* sonicate-stimulated peripheral blood mononuclear cell (PBMC) and mesenteric lymph node (MLN) cultures of the animals were compared. Antigen stimulation of MLN cells from the severely lesioned group resulted in significant upregulation of the mRNA expression of five cytokines, gamma interferon (IFN-γ), interleukin-10 (IL-10), IL-13, IL-17A, and tumor necrosis factor alpha (TNF-α), which have a diverse range of functions, while there was no significant upregulation of these cytokines by the other groups. There were major differences between the responses of the PBMC and MLN cultures, with higher levels of secreted IFN-γ released from the MLN cultures and, conversely, higher levels of IL-10 released from the PBMC cultures. The upregulation of all five cytokines from cells at the site of infection in the severely lesioned animals suggested a dysregulated immune response, contributing to a failure to clear infection in this group of animals.**

Johne's disease caused by *Mycobacterium avium* subsp. *paratuberculosis* is a chronic enteric disease of cattle and other ruminants (45) and causes major economic losses in many countries. There is evidence for an association (rather than a causal relationship) between *M. avium* subsp. *paratuberculosis* and Crohn's disease in humans (27). Cattle usually become infected as young calves either via the oral route or *in utero*, and infection persists in a subclinical state for 2 or more years. After ingestion, the bacteria are endocytosed by M cells in the Peyer's patches of the ileum and are subsequently phagocytosed by macrophages in the intestinal lamina propria and submucosa. Lesions are prominent in the ileum, particularly the ileocecal valve region as well as the associated lymph nodes, and the mucosal tissue damage results primarily from severe immune pathology and chronic inflammation. In the late stage of the disease, large numbers of acid-fast bacilli (AFB) are found in lesions (3, 6).

An understanding of the immune responses associated with the progression of the disease and the interaction between host and pathogen underpins many of the outcomes required for improving disease control. Most of our current knowledge on the interaction between *M. avium* subsp. *paratuberculosis* and immune cells, such as T cells, B cells, and macrophages, has relied on the study of peripheral blood cells (1, 44). However, this does not address antigen-specific immunological events at the site of infection in the gastrointestinal tract. There are substantial differences in the development of a mucosal immune response compared to that at systemic locations such as the blood, and the study of immune responses in the blood of diseased animals may provide incomplete and sometimes erroneous information (43). Mucosal immune responses are tightly controlled by a network of cells and mediators, which ensures that immune responses do not lead to uncontrolled inflammatory responses with associated mucosal tissue damage (24, 26). *M. avium* subsp. *paratuberculosis* may ensure its survival in the animal by dampening or distorting the immune response at the mucosal level by interfering with regulatory networks.

Various immune effector cells and signals direct the immune response to a pathogen, and cytokines are critically important in this signaling cascade. The balance of the different cytokines will influence the outcome of the immune response. The current study has a particular emphasis on antigen-specific responses in both blood and mucosal sites and has focused on five cytokines, gamma interferon (IFN-γ), interleukin-10 (IL-10), IL-13, IL-17A, and tumor necrosis factor alpha (TNF-α), which contribute to a diverse range of immune responses. IFN-γ is crucial for the activation of macrophages and clearance of mycobacterial infections (15). Mice and humans with a deficiency in the ability to produce or respond to IFN-γ are very susceptible to mycobacterial infections such as tuberculosis (8, 20). IL-17A is critical for enhancement of memory responses to mycobacterial infections, and after challenge with

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TABLE 1. Scoring system for histopathology of lesions in the distal ileum, ileocecal valve, and mesenteric and ileocecal lymph nodes of cull cows naturally infected with *M. avium* subsp. *paratuberculosis*

Parameter[b]	Tissue[a]	Score	Description of histopathological changes
Villus	DI, ICV	0	No abnormality
		1	Mild blunting of villi
		2	Moderate blunting and fusion of villi
		3	Marked blunting and fusion of villi
Cellular infiltration	DI, ICV	0	No cellular infiltration
		1	Occasional scattered cellular infiltration
		2	Moderate cellular infiltration
		3	Severe and extensive cellular infiltration
Granuloma	DI, ICV, MLN, ICLN	0	No lesion
		1	Small focal granulomas or scattered giant cells
		2	Multifocal granulomas in some areas of tissue
		3	Diffused granulomas in large areas of tissue
No. of AFB* per macrophage	DI, ICV, MLN, ICLN	0	No AFB
		1	<10 AFB
		2	10 to 50 AFB
		3	>50 AFB

[a] DI, distal ileum; ICV, ileocecal valve, MLN, mesenteric lymph node; ICLN, ileocecal lymph node.
[b] AFB*, acid-fast bacillus stained with the Ziehl-Neelsen (ZN) method.

M. tuberculosis, IL-17-inducible chemokine expression mediates the recruitment of Th1 lymphocytes to lung tissue (28). TNF-α has an important role in granuloma formation, assisting in localizing mycobacterial infections (30). In contrast, IL-10 is considered to have an inhibitory effect on the killing of mycobacteria and suppresses T cell functions (21, 42). IL-13 is a Th2 cytokine, with suppressive and anti-inflammatory properties (13), and both IL-4 and IL-13 have been shown to inhibit autophagy (18). Autophagy is an important pathway for inhibiting the intracellular survival of mycobacteria.

The animals used for the current study were cull cows from dairy herds which had a history of Johne's disease. The aim was to measure cytokine responses in animals which had various severities of histopathology in the gut tissues. Cytokine responses in immune cells from the blood and mesenteric lymph nodes (MLN) were measured, with a particular emphasis on immune responses from cell cultures stimulated with *M. avium* subsp. *paratuberculosis* antigens to determine possible mechanisms by which *M. avium* subsp. *paratuberculosis* evades protective host defenses.

MATERIALS AND METHODS

Animals. The 38 cull cows were purchased from seven dairy herds in the lower North Island, New Zealand, which had a history of Johne's disease. These dairy herds remained outdoors all year and were on a pasture-based diet. These animals were culled because of poor milk production, not in-calf, or had a positive serological response to *M. avium* subsp. *paratuberculosis*. The cows which were negative for *M. avium* subsp. *paratuberculosis* serology were randomly selected from the cull cow populations. The breeds of the cows were Friesian, Jersey, and Friesian/Jersey cross, and their mean age was 5.3 years. All procedures performed on the animals were approved by the Institutional Animal Ethics Committee (AgResearch Grasslands, Palmerston North, New Zealand).

Sample collection. Prior to slaughter of the cows, 40 ml of blood was collected into heparinized tubes (BD Vacutainer, Becton Dickinson, NJ) for peripheral blood mononuclear cell (PBMC) culture. Five milliliters of blood was collected for serum separation, and sera were stored at −20°C. Following slaughter, a sample of MLN (1 cm^3) draining the ileum was collected for cell culture studies. Samples of MLN, ileocecal lymph nodes, distal ileum (50 cm from the ileocecal valve), and ileocecal valves were collected for *M. avium* subsp. *paratuberculosis* culture and histopathological assessment.

***M. avium* subsp. *paratuberculosis* culture.** For the *M. avium* subsp. *paratuberculosis* culture using tissue samples, approximately 1 g of tissue was homogenized in 20 ml of sterile water in a Stomacher (Colworth; Seaward, Norfolk, United Kingdom). The tissue homogenate was filtered through sterile cheesecloth and decontaminated with an equal volume of 0.75% cetylpyridinium chloride (CPC) for 40 min. The decontaminated tissue homogenate was centrifuged at 3,500 × *g* for 20 min. The pellet was resuspended in 1 ml of sterile water, and 0.5 ml was inoculated into a Bactec 12B vial, supplemented with the antibiotic cocktail PANTA (Becton Dickinson, Sparks, MD), 0.8 ml of sterile egg yolk, and mycobactin (Allied Monitor, Fayette, MO). The inoculated vials were incubated at 37°C and read at weekly intervals for the presence of $^{14}CO_2$, and the growth indices were recorded. Vials registering a positive growth index (GI; >15) were examined for AFB using Ziehl-Neelsen (ZN) staining and also inoculated onto blood agar for the presence of microbial contamination. *M. avium* subsp. *paratuberculosis* was identified on the basis of the presence of slow-growing clumps of AFB that were mycobactin dependent. A cow was classified as *M. avium* subsp. *paratuberculosis* infected if *M. avium* subsp. *paratuberculosis* was cultured from one or more tissue samples. The group of cows classified as noninfected were chosen as matched controls from the same herds, and it is possible that they may have previously been infected or had a very low level of infection. Fecal samples were not collected for *M. avium* subsp. *paratuberculosis* culture, as culture for *M. avium* subsp. *paratuberculosis* from tissues was considered more definitive.

Histology. Tissue samples for histology were fixed in 10% buffered formalin, and 4-μm sections were stained with hematoxylin and eosin (H&E) and ZN stain. Histopathological changes from sections of distal ileum and ileocecal valve were scored based on the condition of villi, cellular infiltrates in the lamina propria, the presence and severity of granulomatous lesions, and the presence of AFB, while sections of the mesenteric and ileocecal lymph nodes were scored on the presence and severity of granulomatous lesions and the presence of AFB (Table 1). This scoring system was adapted from those described by Clarke and Little (7) and Clark et al. (5). Each parameter was scored separately from 0 to 3. Scores for the four parameters (villi, cellular infiltration, granulomas, and AFB) were pooled to give a score for an individual intestine sample, and scores for the two intestinal samples were added together to provide a combined intestinal tissue score (maximum score of 24). Similarly, scores for the two parameters (granulomas and AFB) for the two lymph node samples were added together for a combined lymph node score (maximum score of 12), and a total lesion score was obtained by combining the intestinal and lymph node scores (maximum score of 36). The scoring of the sections was undertaken blindly by one person.

Serology. Serological responses to *M. avium* subsp. *paratuberculosis* were measured using the Pourquier enzyme-linked immunosorbent assay (ELISA; IDEXX Laboratories), as described by the manufacturer. Results were expressed by an equation, sample/positive-control (S/P) percentage = 100 × [sample optical density at 450 nm (OD_{450}) − negative-control OD_{450}]/[positive-control OD_{450} − negative-control OD_{450}], and with a positive cutoff of ≥55%, as specified by the manufacturer.

Preparation of cell cultures. PBMCs were prepared using Lymphoprep (Axis-Shield, Norway), according to the manufacturer's instructions. The cells were

resuspended in culture medium, RPMI 1640 supplemented with 25 mM HEPES, 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U penicillin, 100 μg streptomycin, and 2.5 μg amphotericin B per ml (RPMI 1640-10). For preparation of cells from the MLN, the MLN sample was washed 3 times in a wash buffer, GKN (10 mM phosphate-buffered saline [PBS], pH 7.2, supplemented with 2 g glucose, 0.1 g each of penicillin and streptomycin, 2.5 mg of amphotericin B, and 10 mg phenol red per liter) after fat tissue had been removed. The sample was cut into small pieces and gently pressed through a metal mesh into a petri dish containing about 10 ml of GKN. The cell suspension was transferred from the dish, passed through a strainer (70-μ nylon; BD Falcon) into a 50-ml centrifuge tube, and centrifuged at 300 × *g* for 10 min. The supernatant was discarded, and cells were washed twice before resuspension in RPMI 1640-10.

Antigen preparation and cell culture stimulation. *M. avium* subsp. *paratuberculosis* strain 84/989, originally isolated from an infected cow, was used for the antigen preparation and was propagated as described previously (32). The culture of 10^8 bacilli per ml was sonicated after 2 washes in PBS and four cycles of freeze-thaw. Sonication was undertaken to disrupt the cell structure of the mycobacteria to enhance immunogenicity and was performed on ice using Sonics VCX750 (Sonics & Material, Inc.) at 30% output for 40 cycles of 20 s on and 20 s off. Aliquots of the *M. avium* subsp. *paratuberculosis* sonicate were stored at −80°C until use. Two-milliliter aliquots of suspensions of PBMCs or MLN cells in RPMI 1640-10 at a concentration of 2×10^6 cells per ml were dispensed into wells of 24-well plates (Becton Dickinson). *M. avium* subsp. *paratuberculosis* sonicate (equivalent concentration of 10^6 bacilli per ml), pokeweed mitogen (10 μg per ml), or PBS (nonstimulated) was added to each well. Previous studies had determined the optimal concentration of the *M. avium* subsp. *paratuberculosis* sonicate for cell culture studies (data not shown). Two replicate 24-well plates were set up for each of the two cell types (PBMCs and MLN cells), with one set cultured overnight (20 h) and the other for 4 days. Culture supernatant was collected at 20 h (day 1) and day 4 for cytokine production. Cells were harvested at 20 h for quantitative reverse transcription PCR (qRT-PCR).

IFN-γ and IL-10 ELISA. The release of IFN-γ in cell supernatants was measured by ELISA using the Bovigam test kit for bovine IFN-γ (Prionics, Switzerland). The amount of IFN-γ was calculated against a standard curve prepared with recombinant IFN-γ. IFN-γ levels in the supernatants were measured from both 1- and 4-day cultures. Two time points were used, as levels had reached the maximum detectable levels from some samples of the 4-day cultures. For IL-10, data were presented as absorbance (OD_{450}) values, as no bovine IL-10 protein was available to produce a standard curve. IL-10 released from cell cultures was measured using an IL-10 ELISA (14). Briefly, plates were coated with mouse anti-bovine IL-10 (MCA 2110; Serotec), samples were added, and IL-10 was detected using biotin-labeled anti-bovine IL-10 (MCA 2111B; Serotec), followed by addition of streptavidin-horseradish peroxidase. IL-10 levels in the supernatants were measured only from the 4-day cultures.

Measurement of mRNA expression from cultured cells. RNA was extracted from cultured PBMCs and MLN cells (2 ml at 2×10^6 cells per ml) using the TRIzol reagent (Invitrogen, Carlsbad, CA). The amount of total RNA was spectrophotometrically determined at 260 nm using the NanoDrop 1000 machine (Thermo Scientific, Wilmington, DE). RNA samples were frozen at −80°C until converted to cDNA. Total RNA extracted from cells was purified using DNase I treatment prior to cDNA synthesis. Briefly, 2 μg total RNA, 1 μl 10× DNase I buffer (200 mM Tris-HCl [pH 8.4], 500 mM KCl, 20 mM $MgCl_2$), 1 μl amplification-grade DNase I (1 unit/μl) (Invitrogen), and diethyl pyrocarbonate (DEPC)-treated water (Invitrogen, Carlsbad, CA) were combined and made up to 10 μl. The RNA was incubated for 15 min at room temperature and inactivated by adding 1 μl of 25 mM EDTA and heating for 10 min at 65°C. Purified RNA (2 μg) was then reverse transcribed to cDNA. Briefly, 2 μg of purified RNA was added to a 12-μl reaction mixture consisting of 0.5 μg oligo$(dT)_{20}$ primer (Invitrogen) and DEPC-treated water. The reaction mixture was incubated at 65°C for 10 min and then quickly chilled on ice. To the reaction mixture, 4 μl of 5× first-strand buffer (Roche Diagnostics, Manheim, Germany), 2 μl of dNTP mix (Invitrogen), 0.5 μl of Protector RNase inhibitor (40 unit/μl) (Roche Diagnostics), and 0.5 μl of transcriptor reverse transcriptase (20 unit/μl) (Roche Diagnostics) were added for a total volume of 20 μl. The reaction mixture was incubated at 55°C for 30 min and heated to 80°C for 5 min. Sterile double-distilled water was added to bring the concentration of cDNA to an equivalent of 20 ng/μl. All cDNA samples were stored at −20°C until qRT-PCR analyses were performed.

Quantitative PCR. For qRT-PCR analysis, 10 μl of Sybr Premix Ex *Taq* II master mixture (Takara Bio Inc., Japan), 1 μl of template cDNA, and 5 μM each of gene-specific primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β-actin, U1 (17), IFN-γ, IL-10, IL-13, IL-17A, and TNF-α (primer sequences listed in Table 2) were combined in a 20-μl reaction mixture. The amplification was performed in a Rotor Gene 6000 machine (Corbett Research, Australia). Thermal cycling consisted of initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s. The melting temperature (T_m) of the PCR product was determined by melting curve analysis performed by heating the PCR product from 65°C to 90°C and monitoring fluorescence change every 0.1°C. The relative fold changes in cultured PBMCs and MLN cells were calculated using the comparative threshold cycle (*CT*) method (33), taking into account the mean PCR amplification efficiency obtained from the Rotor Gene 6000 software (Corbett Research, Australia). PCR on each cDNA sample from *M. avium* subsp. *paratuberculosis*-stimulated and -nonstimulated MLN cells and PBMCs was routinely conducted with three reference genes, GAPDH, β-actin, and U1. We analyzed the *CT* values obtained with the three reference genes to determine the most stable values (smallest difference in *CT*) between *M. avium* subsp. *paratuberculosis* sonicate-stimulated and -nonstimulated cells. For each comparison (*M. avium* subsp. *paratuberculosis* sonicate-stimulated versus -nonstimulated MLN cells and PBMCs from each animal), the reference gene that gave the most stable value was used to normalize the data sets. The mean Δ*CT* value of the equivalent nonstimulated cultured cells was used as the calibrator to generate ΔΔ*CT*.

TABLE 2. Sequences of primers used for qRT-PCR of bovine cytokines

Gene	Primer	Sequence
GAPDH	Forward	CACCATCTTCCAGGAGCGAG
	Reverse	CCAGCATCACCCCACTTGAT
β-actin	Forward	CGCCATGGATGATGATATTGC
	Reverse	AAGCCGGCCTTGCACAT
U1	Forward	CCATGATCACGAAGGTGGTTT
	Reverse	ATGCAGTCGAGTTTCCCACAT
IFN-γ	Forward	TGATTCAAATTCCGGTGGATG
	Reverse	TTCATTGATGGCTTTGCGC
IL-10	Forward	TGCTGGATGACTTTAAGGGTTACC
	Reverse	TCATTTCCGACAAGGCTTGG
IL-13	Forward	AGAACCAGAAGGTGCCGCT
	Reverse	GGTTGAGGCTCCACACCATG
IL-17A	Forward	AACATCGTTAACCGGAGCAC
	Reverse	GGTGGAGCGCTTGTGATAAT
TNF-α	Forward	TCCATCAACAGCCCTCTGGT
	Reverse	TGAGGCTTGAGAAGAGGACCTGA

Statistical analysis. Data for cytokines, IFN-γ, and IL-10 released from cell cultures were square root transformed for statistical analysis. A mixed model with random effects of cattle and tissue nested within cattle was used to detect differences between groups, between *M. avium* subsp. *paratuberculosis* sonicate-stimulated and -nonstimulated controls for each group, and between PBMC and MLN cell cultures (R package 'nlme' version 3.1-101). Individual comparisons were subsequently made using a least significant difference (LSD) form of the *t* test by REML (29). A *P* value of <0.05 denoted statistical significance. Correlations between histopathological scores, IFN-γ and IL-10 released from *M. avium* subsp. *paratuberculosis* sonicate-stimulated cultures (square root transformed), or serological responses (S/P percentages) were undertaken using Pearson's correlation.

Statistical analysis of quantitative PCR data was performed using Minitab v.15 (Minitab Inc., PA) on the ΔΔ*CT* values. Prior to data analysis, ΔΔ*CT* values for each cultured cell type and infection group were tested for normality using the Anderson-Darling test. For normally distributed data, ΔΔ*CT* values were computed for the mean and 95% confidence interval. For nonnormally distributed data, ΔΔ*CT* values were computed for the Wilcoxon median and 95% confidence interval. Relative fold change was computed by mean PCR amplification efficiency raised to the power of the ΔΔ*CT* value. A value of relative fold change with a low 95% confidence interval of >1 denoted significant upregulation, whereas relative fold change with a high 95% confidence interval of <1 denoted

TABLE 3. Classification of Johne's disease in cull cows based on culture of *M. avium* subsp. *paratuberculosis* from gut tissue, followed by histopathological scores

Classification	No. of animals	*M. avium* subsp. *paratuberculosis*		Histopathology scores[a]
		Culture	Serology	
Control	8	−	−	≤8
Nonlesioned/*M. avium* subsp. *paratuberculosis* infected	8	+	−	≤8
Moderately lesioned	9	+	+	9 to 27
Severely lesioned	13	+	+	≥28

[a] Based on sections from the distal ileum, ileocecal valve, and mesenteric and ileocecal lymph nodes.

significant downregulation. One-way analysis of variance was used to compare $\Delta\Delta CT$ values between groups, followed by a Fisher's LSD multiple-comparisons test as a posttest to compare paired sets of data when the data were normally distributed. Alternatively, for nonnormal distributed data, a Kruskal-Wallis nonparametric test was used, followed by a Dunn's multiple-comparisons test as a posttest. A P value of <0.05 denoted statistical significance.

RESULTS

Disease classification. The cows were divided into four groups based on the culture of *M. avium* subsp. *paratuberculosis* and total histopathological lesion scores (Table 3). The four groups consisted of (i) control (culture negative for *M. avium* subsp. *paratuberculosis*); (ii) nonlesioned/*M. avium* subsp. *paratuberculosis* infected; (iii) moderately lesioned; and (iv) severely lesioned. The control and nonlesioned/*M. avium* subsp. *paratuberculosis*-infected groups had histopathological scores of ≤8 with no lesions pathognomonic for Johne's disease (no granulomas or AFB observed). The moderately and severely lesioned groups had scores of 9 to 27 and 29 to 36, respectively. AFB were observed in sections of the tissues of all animals in the moderately and severely lesioned groups. Comparisons of the lesion scores for the intestinal, lymph node, and combined tissues are shown in Table 4. All animals in the moderately and severely lesioned groups were positive in the Pourquier serological test, while none were positive in the other two groups (Table 4). There was no correlation between the histopathological scores for the moderately and severely lesioned animals and their serological responses. The control and nonlesioned/*M. avium* subsp. *paratuberculosis*-infected animals were not included in this analysis, as they did not have specific *M. avium* subsp. *paratuberculosis* lesions and had minimal serological reactivity. *M. avium* subsp. *paratuberculosis* was cultured from all four tissues sites for all of the moderately and severely lesioned animals, while the proportion of *M. avium* subsp. *paratuberculosis*-culture positives for the different tissues in the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group was 8/8 for MLN, 5/8 for ileocecal lymph node, and 3/8 for both distal ileum and ileocecal valve samples. Further evidence of a low level of infection in the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group was indicated from no AFB having been observed in histological sections from these animals. No *M. avium* subsp. *paratuberculosis* was cultured from tissues of the control group. Animals in the moderately and severely lesioned groups had bouts of scouring and were generally in poor condition, particularly those in the severely lesioned group. The mucosa of the distal ileum was thickened with convoluted folds for animals in these two groups and a number of these animals had swollen MLNs. Animals in the control and nonlesioned/*M. avium* subsp. *paratuberculosis*-infected groups generally had normal body condition scores and feces, as well as no gross pathology attributable to Johne's disease.

Secreted IFN-γ and IL-10 released from *M. avium* subsp. *paratuberculosis* sonicate-stimulated cell cultures. IFN-γ levels released from cultured MLN cells and PBMCs with or without *M. avium* subsp. *paratuberculosis* sonicate stimulation are shown in Fig. 1. Combining results from the four groups, the MLN cultures had significantly higher levels of secreted IFN-γ than those for the PBMC cultures ($P < 0.001$). Significant differences between *M. avium* subsp. *paratuberculosis* sonicate-stimulated and -nonstimulated cultures were observed for the 1- and 4-day MLN cultures from the moderately and severely lesioned groups and for the 1- and 4-day PBMC cultures from the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected and severely lesioned groups ($P < 0.05$) (Fig. 1). In comparisons between the animal groups, the *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN cultures from the moderately and severely lesioned groups released significantly larger amounts of IFN-γ than those from the control and nonlesioned/*M. avium* subsp. *paratuberculosis*-infected groups for both 1- and 4-day cultures ($P < 0.05$). The *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN cultures from the severely lesioned group released significantly higher levels of IFN-γ compared to those released from the moderately lesioned group, but only for the 1-day cultures ($P < 0.05$). For the *M. avium* subsp. *paratuberculosis* sonicate-stimulated PBMC cultures, significantly higher levels of IFN-γ were released from the severely lesioned group compared to those

TABLE 4. Serological responses and lesion scores of cull cows infected with *M. avium* subsp. *paratuberculosis*

Group	Median (range) of serological response (S/P %)[a]	Lesion score (range)[b]		
		Distal ileum and ileocecal valve	Mesenteric and ileocecal lymph nodes	Combined tissues
Control	1.3 (0.4–32.1)	3.5 (2–7)	0 (0–0)	3.5 (2–7)
Nonlesioned/*M. avium* subsp. *paratuberculosis* infected	0.3 (0–4.2)	4.5 (2–8)	0 (0–0)	4.5 (2–8)
Moderately lesioned	243.6 (139.8–290.6)	15 (10–19)	5 (3–10)	20 (13–27)
Severely lesioned	259.5 (180.4–338.0)	22 (20–24)	12 (8–12)	34 (29–36)

[a] S/P percentage = 100 × [sample optical density at 450 nm (OD_{450}) − negative-control OD_{450}]/[positive-control OD_{450} − negative-control OD_{450}].
[b] Maximum score of 24 for distal ileum and ileocecal valve, 12 for mesenteric and ileocecal lymph nodes, and 36 for the combination of the four tissues.

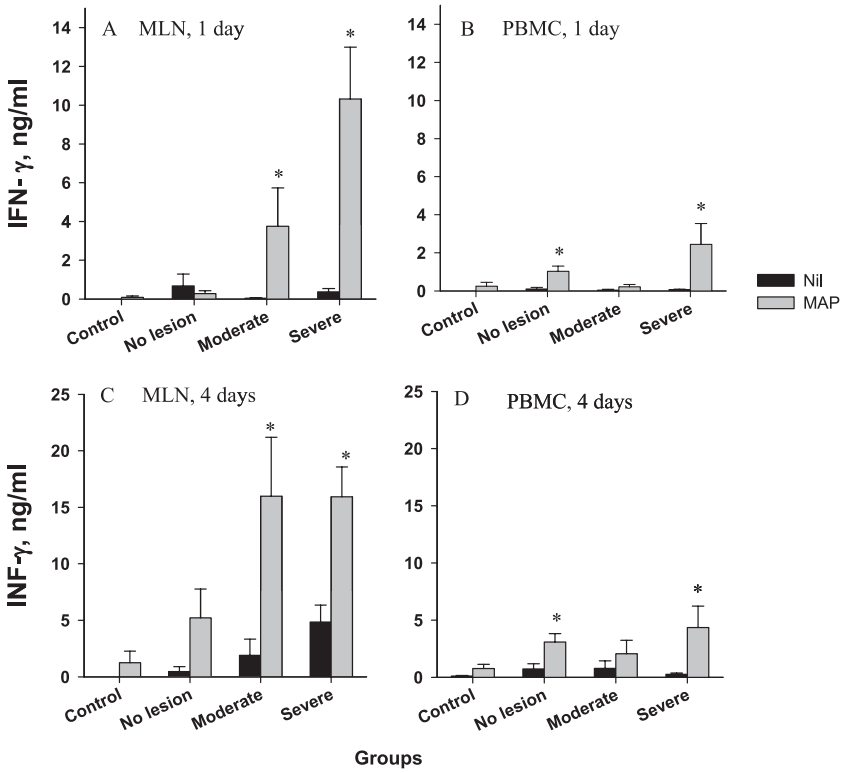

FIG. 1. IFN-γ released from cultures of MLN cells (A and C) and PBMCs (B and D) from control ($n = 8$), nonlesioned/*M. avium* subsp. *paratuberculosis*-infected ($n = 8$), moderately lesioned ($n = 9$), and severely lesioned ($n = 13$) groups. Cultures were stimulated with *M. avium* subsp. *paratuberculosis* sonicate (MAP) for 1 day (A and B) or 4 days (C and D) and compared to nonstimulated (Nil) cultures. Data are presented as mean concentrations (ng/ml) ± SE. Significant differences between *M. avium* subsp. *paratuberculosis* sonicate-stimulated and -nonstimulated cultures are denoted with asterisks ($P < 0.05$).

released from the control group for both 1- and 4-day cultures ($P < 0.05$). The only between-group significant difference for nonstimulated cultures was higher levels for 4-day MLN cultures from the severely lesioned group compared to those for the control group ($P < 0.05$).

Overall, secreted IL-10 levels released from PBMC cultures were significantly higher than those released from MLN cultures ($P < 0.001$) (Fig. 2). For the MLN cultures, significantly higher levels of IL-10 were released from *M. avium* subsp. *paratuberculosis* sonicate-stimulated cultures compared to those released from the nonstimulated ones for the moderately and severely lesioned groups and for the PBMC cultures of the severely lesioned group ($P < 0.05$). For comparisons between the animal groups, the *M. avium* subsp. *paratuberculosis* soni-

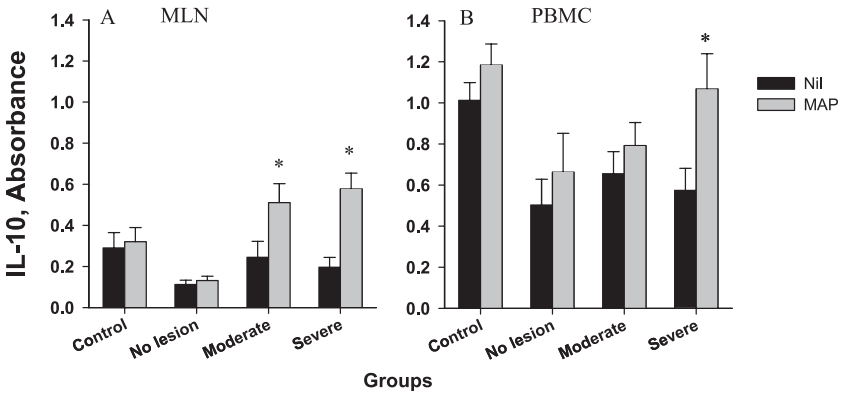

FIG. 2. IL-10 released from cultures of MLN cells (A) and PBMCs (B) from control ($n = 8$), nonlesioned/*M. avium* subsp. *paratuberculosis*-infected ($n = 8$), moderately lesioned ($n = 9$), and severely lesioned ($n = 13$) groups. Cultures were stimulated with *M. avium* subsp. *paratuberculosis* sonicate for 4 days and compared to nonstimulated cultures. Data are presented as mean absorbance levels (OD_{450}) ± SE. Significant differences between *M. avium* subsp. *paratuberculosis* sonicate-stimulated and -nonstimulated cultures are denoted with asterisks ($P < 0.05$).

cate-stimulated MLN cultures from the control and moderately and severely lesioned groups released significantly higher levels than those released from the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group, and the levels for severely lesioned group were higher than those for the control group ($P < 0.05$). For the *M. avium* subsp. *paratuberculosis* sonicate-stimulated PBMC cultures, the control and severely lesioned groups released significantly higher levels than those released from the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group, and the levels for the control group were also higher than those for the moderately lesioned group ($P < 0.05$). For the nonstimulated MLN cultures, the control group released significantly higher IL-10 levels than those released from the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group, and for the nonstimulated PBMC cultures, higher levels of IL-10 were released from those for the control group compared to those for the three other groups ($P < 0.05$).

Positive correlations were observed between the histopathological scores and levels of secreted IFN-γ released from MLN cultures stimulated with *M. avium* subsp. *paratuberculosis* sonicate from 1-day ($r = 0.621$, $P < 0.001$) and 4-day cultures ($r = 0.563$, $P < 0.001$). A positive correlation was also observed between the histopathological scores and levels of secreted IL-10 from the 4-day *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN cultures ($r = 0.595$, $P < 0.001$). In contrast, significant correlations were not observed between the histopathological scores and secreted IFN-γ or IL-10 from PBMC cultures. A positive correlation was observed between the secreted IFN-γ and IL-10 from the 4-day *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN cultures ($r = 0.386$, $P < 0.05$).

mRNA expression for cytokine genes. Relative fold changes in mRNA expression of five cytokine genes, IFN-γ, IL-10, IL-13, IL-17A, and TNF-α, were measured for *M. avium* subsp. *paratuberculosis* sonicate-stimulated cultures of MLN cells and PBMCs relative to those of the nonstimulated cultures (Fig. 3). For the MLN cultures, significant upregulation of mRNA expression as a result of *M. avium* subsp. *paratuberculosis* sonicate stimulation was observed only for the severely lesioned group, and this was shown for all five cytokines ($P < 0.05$). The relative fold change was most marked for IFN-γ in particularly the severely lesioned group, where there was a mean 40-fold increase in mRNA expression for *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN cultures compared to that for the nonstimulated cells. For the PBMC cultures, significant upregulation of mRNA expression as a result of *M. avium* subsp. *paratuberculosis* sonicate stimulation was observed only for IFN-γ and IL-17A from the control and moderately and severely lesioned groups, and downregulation of IL-10 mRNA expression was observed only for the moderately lesioned group ($P < 0.05$). Significant differences between the four animal groups were observed for the mean relative fold changes for IFN-γ from the MLN cultures, where the severely lesioned group values were higher than those of the control ($P < 0.01$), nonlesioned/*M. avium* subsp. *paratuberculosis*-infected ($P < 0.01$), and moderately lesioned ($P < 0.05$) groups. In addition, the mean relative fold changes for IL-13 and TNF-α from the MLN cultures were higher for the severely lesioned group than those for the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group ($P < 0.05$ and $P < 0.01$, respectively). No significant differences were observed between the animal groups for the PBMC cultures.

DISCUSSION

This study has demonstrated that there are numerous differences between the responses of immune cells obtained from blood and those obtained from a site of the infection (MLN), and the immune responses of the *M. avium* subsp. *paratuberculosis*-infected animals varied with the severity of histopathological lesions in the gastrointestinal tract. To assess how the gut immune responses impacted disease pathogenesis, the infected animals were divided into groups based on their histopathological scores. A division into three disease categories, nonlesioned/*M. avium* subsp. *paratuberculosis* infected, moderately lesioned, and severely lesioned, proved to be very useful, as there were clear differences between these disease groups in terms of their cytokine and serological responses. Positive serological responses were strongly associated with the presence of histopathological lesions pathognomonic for Johne's disease, such as granulomas without necrosis and AFB. The majority of previous studies have classified different disease categories in Johne's disease of cattle principally based on a division into subclinical and clinical states (10, 35). This classification may be less reliable when studying the disease in cull cows, for which their general condition is poor, and a classification based on histopathological scores from a site of infection may be more meaningful. Clinical condition (body scores) and scouring of the animals on a pasture diet were not reliable indicators of severe histopathological lesions related to Johne's disease in the current study (data not shown). Interestingly, 50% of the cull cows which were negative for *M. avium* subsp. *paratuberculosis* serology and selected randomly from the cull cows populations were culture positive for *M. avium* subsp. *paratuberculosis*, suggesting a high level of infection in these herds. There is a possibility that the control animals may have been previously infected or had an undetectable level of infection; however, these animals had minimal antigen-specific immune responses. These animals were matched with the animals in the other groups coming from the same herds. They either were relatively resistant to infection or had not been exposed.

M. avium subsp. *paratuberculosis* sonicate stimulation of MLN cells from cows in the severely lesioned group resulted in significant upregulation of the mRNA expression of all five selected cytokines, IFN-γ, IL-10, IL-13, IL-17A, and TNF-α, while no significant changes in these cytokines were observed for the other groups. These cytokines were chosen on the basis that they contributed to a diverse range of immune responses, and upregulation of all five cytokines from cells from a site of infection in the severely lesioned animals suggested a dysregulated immune response. IFN-γ, IL-17A, and TNF-α are important in the control of mycobacterial infections and are Th1-type cytokines, while IL-13 is a Th2-type cytokine and IL-10 downregulates Th1-type immune responses. The enhanced cytokine responses from MLN cultures of the severely lesioned group compared to those from the PBMCs or from the other groups may have been influenced by the antigen-presenting cells being preprimed by *M. avium* subsp. *paratuberculosis* antigens from the natural infection as well as sequestration of sensitized lymphocytes to the site of infection. The cytokine

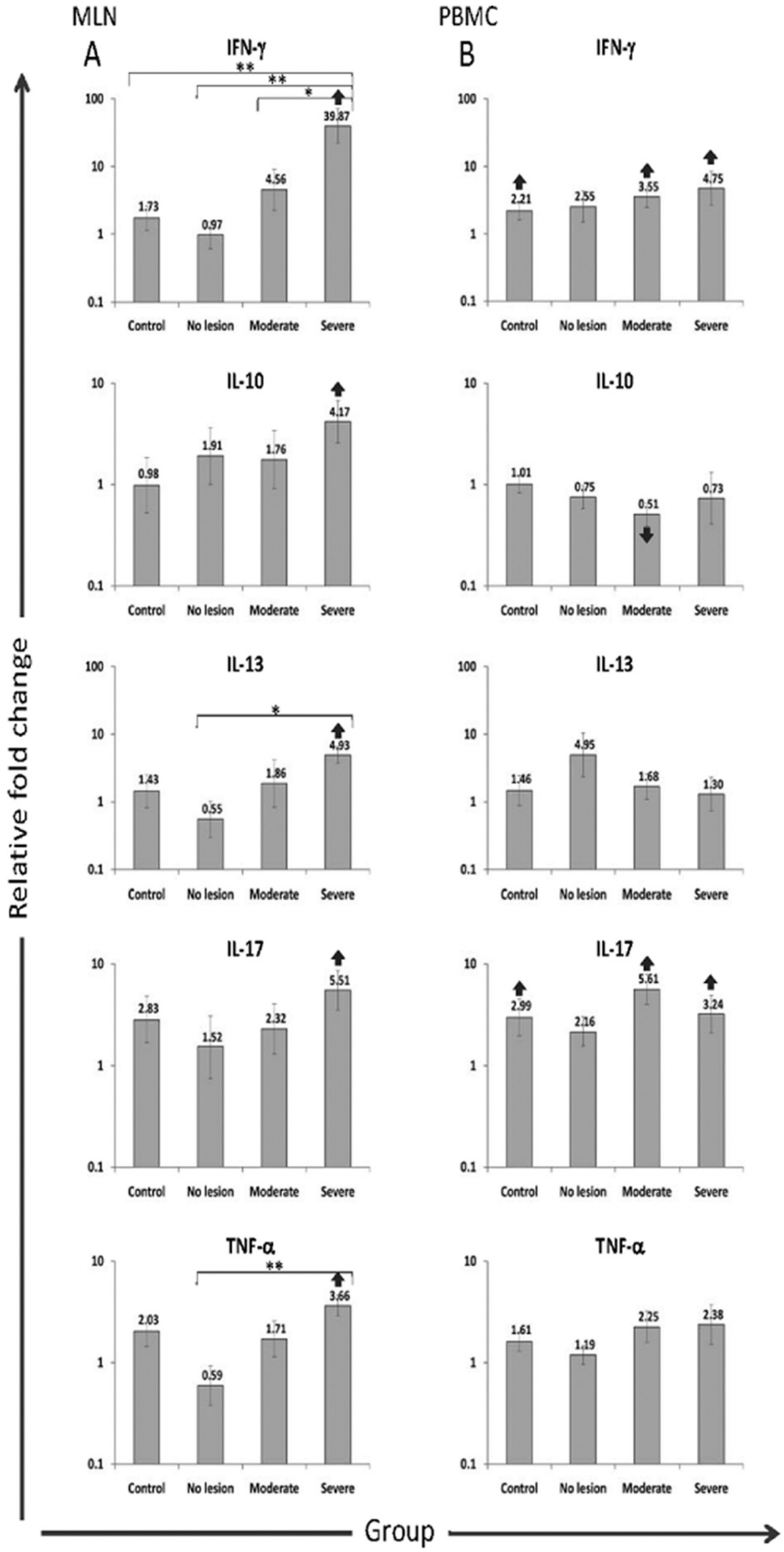

FIG. 3. Cytokine mRNA expression for IFN-γ, IL-10, IL-13, IL-17A, and TNF-α in cultures of MLN cells (A) and PBMCs (B) from control ($n = 8$), nonlesioned/*M. avium* subsp. *paratuberculosis*-infected ($n = 8$), moderately lesioned ($n = 9$), and severely lesioned ($n = 13$) groups. Cells were stimulated with *M. avium* subsp. *paratuberculosis* sonicate for 20 h. The levels of mRNA were normalized to the most stable reference gene among GAPDH, β-actin, and U1. Nonstimulated cells were used as the calibrators to generate fold change values using the $\Delta\Delta CT$ method. The results were presented as mean relative fold changes ± SE. Significant upregulation (↑) or downregulation (↓) relative to nonstimulated values ($P < 0.05$) is shown. To compare groups, significant differences between groups were denoted. *, $P < 0.05$; **, $P < 0.01$.

gene expression in the *M. avium* subsp. *paratuberculosis* sonicate-stimulated PBMC cultures produced a different pattern with upregulation of IFN-γ and IL-17A in the control and moderately and severely lesioned groups.

The marked upregulation of the five cytokines in the MLN cultures from the severely lesioned group may have been related to a higher proportion of sensitized lymphocytes in these tissues as well as possible interactions between many of the cytokines. IL-17 is produced primarily by Th-17 and γδ T cells, and secretion of IL-17 by γδ T cells has been shown to enhance IFN-γ release by T cells (41), and similarly, *in vitro* studies have shown that recombinant TNF-α enhanced antigen-specific IFN-γ mRNA expression in guinea pig cell cultures (4). Conversely, IL-10 can have a direct effect on monocyte-macrophages, limiting the production of proinflammatory cytokines, and can act directly on $CD4^+$ T cells, inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5, and TNF-α (9). The cytokine profile may also be influenced by the virulence of the mycobacteria. Virulent strains of *M. tuberculosis* preferentially upregulated the production of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13), while nonvirulent strains induced a Th1-type response (16, 25, 37). Thus, in a typically Th1 environment of the granuloma, localized secretion of IL-4 and Il-13 could impair the response of infected macrophages to IFN-γ.

For the severely lesioned group, the release of secreted IFN-γ and IL-10 from *M. avium* subsp. *paratuberculosis*-stimulated MLN cultures correlated well with mRNA expression of these cytokines in the same cultures. The induction of IFN-γ mRNA expression from the *M. avium* subsp. *paratuberculosis*-stimulated MLN cultures was particularly marked with a 40-fold increase compared to that of nonstimulated cultures. The results for the moderately lesioned group followed a similar trend for IFN-γ, with a significant release of secreted IFN-γ together with a 5-fold upregulation in IFN-γ mRNA expression, but the significant release of IL-10 from the MLN cultures was not matched with a marked increase in mRNA expression. Another instance in which secreted and mRNA cytokine expression did not match was when a significant level of IL-10 was released from *M. avium* subsp. *paratuberculosis*-stimulated PBMCs cultures of the severely lesioned group, while there was no apparent IL-10 mRNA expression from these cultures. A possible explanation was that there is a variation in the optimal times to measure mRNA expression for different cytokines, and Weiss et al. (42) found that the optimal time for measurement of IL-10 mRNA was 2 h after culture, while our measurements for all cytokines were undertaken at 20 h.

There were major differences in the type of cytokine response for the *M. avium* subsp. *paratuberculosis*-stimulated cultures from the MLN cells and PBMCs. Overall, the secreted IFN-γ responses in the MLN cultures were greater, while the secreted IL-10 responses in PBMC cultures were greater. This is likely to be a consequence of different proportions of antigen-presenting cells in the two cell populations. The MLN cell preparation will contain dendritic cells, and it has been reported that these cells predominantly produce IL-12 upon stimulation with mycobacteria, promoting Th1 lymphocytes to induce IFN-γ (19, 23). In contrast, monocytes and monocyte-derived macrophages from PBMCs produce IL-10 responses and little IL-12 (42). Other differences between the MLN and PBMC cultures included the significant release of secreted IFN-γ and IL-10 from the MLN cultures of the moderately lesioned group, while for the PBMC cultures, there was a significant release of secreted IFN-γ from those of the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected and -lesioned group and a significant release of secreted IL-10 from those of the control group. These differences contributed to the finding that there were positive correlations between the histopathological scores and levels of IFN-γ and IL-10 released from the MLN cultures stimulated with *M. avium* subsp. *paratuberculosis* sonicate but not between those released from the equivalent PMBC cultures.

Flow cytometry studies of $CD25^+$ expression on T lymphocytes (marker of activation) from the MLN and PBMC cultures supported findings from the cytokine analyses. $CD25^+$ expression on T lymphocytes was significantly increased in *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN cultures for the three *M. avium* subsp. *paratuberculosis*-infected groups compared to that for the control group, while for the *M. avium* subsp. *paratuberculosis* sonicate-stimulated PBMC cultures, the only significant difference was increased $CD25^+$ expression for the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group compared to that for the control group ($P < 0.05$) (D. Shu, unpublished observations). The latter finding concurred with the increased release of secreted IFN-γ in the PBMC cultures of the nonlesioned/*M. avium* subsp. *paratuberculosis*-infected group following *M. avium* subsp. *paratuberculosis* sonicate stimulation.

In contrast to results found in many other studies, the highest levels of IFN-γ released from *M. avium* subsp. *paratuberculosis* sonicate-stimulated MLN and PBMC cultures were from those with the most severe lesions. However, the higher levels of IL-10 released from cell cultures of the severely lesioned group concurred with results from other studies. The paradigm for immune responses to *M. avium* subsp. *paratuberculosis* in cattle is that Th1-mediated responses predominate in the early stage of infection, with a switch to Th2-mediated responses as the disease progresses (10, 12). A number of studies found that IFN-γ responses in PBMC cultures stimulated with *M. avium* subsp. *paratuberculosis* were higher in subclinically affected cattle than in clinically affected ones (34, 36). Studies of gut tissues have generally involved direct RT-PCR on tissues, and again, higher IFN-γ mRNA expression has been found from ileum and gut lymph node tissues of subclinically affected animals compared to those from clinically affected animals (22, 39). Higher levels of IL-10 have been observed in *M. avium* subsp. *paratuberculosis*-stimulated PBMCs or by RT-PCR on gut tissues of clinically affected animals compared to those of subclinically affected animals (22). Few Johne's disease studies have reported on comparisons of the presence of immune responses with histopathological lesions. One exception was a study by Tanaka et al. (40) in which they divided groups of *M. avium* subsp. *paratuberculosis*-infected cattle into those with tuberculoid-type lesions and those with lepromatous-type lesions, although none had clinical signs of disease. Higher levels of IL-10 and IL-4 mRNA expression were found by direct RT-PCR of ileal lymph nodes with lepromatous lesions, while IFN-γ and TNF-α levels were not significantly different. Our group of nonlesioned/*M. avium*

subsp. *paratuberculosis*-infected animals was probably at an earlier stage of disease than those classified as tuberculoid or paucibacillary, as no lesions or AFB were observed in these animals.

The upregulation of a wide range of cytokines has also been observed in response to other mycobacterial infections, particularly in clinical cases. Human clinical tuberculosis cases could be differentiated from controls by the significant release of secreted IFN-γ, IL-10, IL-13, IL-17, and TNF-α from whole-blood cultures stimulated with purified protein derivative (PPD), while latent tuberculosis cases could be differentiated from controls by the significant release of secreted IFN-γ, IL-13, and IL-17 (38). IL-17 mRNA expression was significantly upregulated in jejunal lymph node tissues of deer severely affected with Johne's disease compared to those with minimal disease or controls (31).

The upregulation of a diverse range of cytokines in groups with the most severe pathology, including cytokines associated with both Th1- and Th2-type immune responses as well as inhibitory cytokines such as IL-10, suggested that this complex immune response may not be effective for the control of the disease. The findings of this study did not follow the paradigm of a distinct shift from a Th1-type immune response to a Th2-type response as the disease progressed. In *M. avium* subsp. *paratuberculosis*-infected cattle, there can be a gradual transition between the Th1 and Th2 immune responses in the more severe forms of the disease (11), and with time, our severely lesioned animals may have developed more of a bias to a Th2 immune response. Begg et al. (2) found that this pattern of transition to a Th2 response varied dramatically between individuals and was not observed at all in some. The cows in the current study were representative of cull dairy cows in New Zealand, were sourced from different farms, and included animals of mixed breeds. There are unknown factors which may have contributed to the lack of a defined cytokine profile in the animals, including the genetics of the animals, bacterial load, coinfections with other pathogens, and environmental stressors. This mixed profile of Th1- and Th2-type cytokines and a strong induction of IL-10 in the severely lesioned animals have the capacity to downregulate the effective killing of *M. avium* subsp. *paratuberculosis* and could help to explain how *M. avium* subsp. *paratuberculosis* may evade effective host immune responses.

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