Regulatory T Cells and Immune Profiling in Johne's Disease Lesions

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Abstract

Johne's disease, caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic wasting disease of ruminants. Hallmark symptoms of clinical Johne's disease include diarrhea, progressive weight loss, and premature death; symptoms due largely to chronic inflammation in the small intestine. MAP colonizes resident macrophages within the ileum of the small intestine, subsequently establishing a persistent infection in the host. It has been proposed that regulatory T cells may play a role in the progression of Johne's disease, either through promotion of tolerance to MAP or via a loss in homeostasis that subsequently allows widespread inflammation. In this report, we

evaluated the presence of Tregs, as well as other immune parameters, in the ileum and draining lymph nodes of MAP associated lesions. A lesion classification scheme was developed to categorize severity of MAP-induced lesions within infected tissues and subsequently regulatory T cell presence and overall immune activity were assessed corresponding to lesions of varying severity, in comparison to tissues from healthy control animals. Our results revealed a relationship between animal health and overall lesion severity within the infected tissues, as well as a relationship between bacterial burden and severity of pathology. Regulatory T cell abundance was shown to decrease with increasing lesion severity. Within the ileum, the expression of many Th1, Th2, and Treg-associated genes increased in mild lesions and decreased in severe lesions, whereas in the lymph nodes the expression of these genes tended to increase with increasing lesion severity. Based on our results, we conclude that a local loss of T cell (including Treg) activity occurs within severe ileal lesions associated with MAP, resulting in a loss of homeostasis that ultimately leads to the progression of clinical Johne's disease.

Keywords

Paratuberculosis, Treg, pathology, bovine, immunology

Introduction

Johne's disease, or paratuberculosis, is a chronic enteritis affecting ruminants worldwide, with a significant impact on certain livestock, including cattle and sheep. Johne's disease is caused by infection with the intracellular *Mycobacterium avium* subspecies *paratuberculosis* (MAP). During the asymptomatic and subclinical stages of disease, which typically lasts 2-5 years [32], infected animals intermittently shed MAP in their feces, despite showing little to no sign of infection. This leads to a widespread disease incidence, with an estimated 40-68% of dairy herds being infected [32, 35]. The cost of Johne's disease, in the form of reduced milk production and premature culling, has been estimated to be at least \$250 million and as high as \$1.5 billion annually for the U.S. dairy industry [19, 22]. At the animal level,

Johne's disease is characterized by increasingly severe diarrhea, progressive emaciation, and premature death [34]. At the organ level, progressively worsening inflammation is seen in the ileum and in draining lymph nodes [7, 18, 34].

Immunologically, MAP-infected cattle initially develop a productive Th1 immune response to MAP, characterized by both pro-inflammatory and cytotoxic immune activity [9, 10, 30]. As Johne's disease progresses toward clinical disease, the peripheral Th1 response becomes diminished and is largely replaced by a Th2 (humoral) immune response [9, 12, 17, 30]. The Th2 response is dominated by production of anti-MAP antibodies ineffective at controlling intracellular infections. This shift in predominate immune response correlates with reduced control of infection, increased dissemination of the bacterium, and ultimately progression of clinical Johne's disease. Within infected tissues, the presence of large numbers of infected macrophages leads to widespread inflammation, possibly caused by production of IL-1 [1].

Although reasons for this immune shift are unknown [30], one possibility is that a population of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) develops in response to chronic, low-level stimulation with MAP antigens and that these Tregs limit effector cell responses to MAP antigens [13]. Tregs exert immunomodulatory effects through production of interleukin 10 (IL-10) and transforming growth factor beta (TGF- β). Tregs may develop in environments of ineffective T cell co-stimulation and chronic low-level antigen stimulation [6, 15, 21], and both of these conditions have been shown to exist during Johne's disease [29, 33]. Indeed, Tregs have been shown to develop in response to numerous other chronic infectious agents including *M. tuberculosis* [4, 5, 16, 25]. Further, recent work by our group has shown an increased abundance of FoxP3 mRNA in the ileum of subclinical infected cows [13]. It has also been shown that MAP-infected cows contain more Tregs in peripheral blood as compared to healthy controls [11]. These studies amongst others provide evidence for the presence of Tregs in cows with Johne's disease.

Although much research has centered around the possibility of Tregs resulting in diminished Th1 responses to MAP [10, 11, 13] (and hence reduced immune control of the pathogen), another possibility is that Tregs help to limit unchecked inflammation within infected tissues in subclinical infection, but this response wanes over time in some animals, allowing the inflammatory response to predominate. In this scenario, Tregs would be necessary to maintain homeostasis and one could hypothesize that loss of Tregs would accompany development of clinical disease. If this is the case, a lack of Treg (and possibly T cell, generally) abundance and/or function in animals with more severe disease would be expected. In fact, recent work from our group [27] has shown strong evidence for T cell unresponsiveness in PBMC populations from cows with clinical Johne's disease. There is mounting evidence further suggesting T cell hyporesponsiveness[33]and/or anergy[3] within T cell populations taken from infected ileal tissue.

The present study aims to more completely classify the severity of lesions in ileal and lymph tissues of cows with Johne's disease based on tissue inflammation and bacterial burden. Upon establishment of this lesion classification system, further investigation has sought to identify a relationship between degree of inflammation, bacterial burden, and Treg abundance within these tissues. Finally, an analysis of the expression of several key immune and inflammatory genes has been performed to add a third layer to this analysis. These experiments will also serve to investigate the possibility of T cell unresponsiveness within MAP-infected tissues. Altogether, we hypothesize that Tregs are playing one of two possible roles within MAP-infected tissues. First, it may be that Tregs develop and limit effector immune responses to MAP, eventually allowing unabated bacterial growth and subsequent development of clinical disease. Alternatively, Tregs may be necessary to help maintain homeostasis in subclinical disease, and the loss of Tregs or Treg activity may lead to unchecked inflammation and development of clinical disease. In either case, we suggest that Tregs play a role in the progression of Johne's disease.

Materials and Methods

1. Animals and Johne's disease testing

Four adult Holstein cows (age >2 years) were used in the current study. The cows were purchased from a commercial dairy operation and subsequently housed at the Michigan State University Veterinary Research Farm. Animals were euthanized due to severe illness (n=2) by a licensed veterinarian using a lethal dose of pentobarbital (100 mg/Kg), or prior to slaughter using a captive bolt system at the MSU Meat Laboratory. Samples from three healthy control animals were obtained at the MSU meat laboratory abattoir as per the facility's schedule and animal availability. All diagnostic testing was performed prior to purchase from the dairy operation, and again at the time of euthanasia. Johne's disease status was verified by serum ELISA (positive result indicated by OD $\geq$ 1.0) and fecal PCR using insertion sequence 900 (IS900). Diagnostic tests were performed by commercial testing firm AntelBiosystems, Inc. (Northstar Cooperative, Lansing, MI 48910). ELISA readings were taken at least twice for each animal over a 3-month period to confirm disease status. All protocols were reviewed and approved by the Michigan State University Animal Use and Care Committee.

2. Tissue collection and preservation

Following euthanasia, the gastrointestinal tract was removed and the terminal 40 cm of the ileum was located and removed, including the ileocecal valve. Ileum-associated mesenteric lymph nodes were identified and 3-4 nodes were removed for each animal. Small sections of spleen and liver were also taken from each animal. All tissues were removed, washed with PBS, and placed on ice. Cork punches (8-10 mm diameter) were used to collect individual samples. For each animal, samples were collected in triplicate from each of 20-30 locations within the ileum, 5-10 locations in the lymph nodes, and 3-5 locations in the spleen and liver. Triplicate samples were preserved in three different ways for differing uses. First, one replicate was preserved in fresh 4% paraformaldehyde in phosphate-buffered saline

(PBS) for 24 hours followed by three one-hour washes in PBS. These samples were subsequently embedded in paraffin and processed for hematoxylin and eosin (H&E) staining and acid fast bacilli (AFB) staining. H&E and AFB staining was performed at the MSU Histopathology lab. The second replicate was preserved and washed in the same manner as the first, and subsequently embedded in Tissue-Tek® Optimum Cutting Temperature (O.C.T.) compound followed by slow freezing with liquid N₂. These samples were stored at -80°C until used in downstream immunofluorescence applications. The third replicates were flash frozen in liquid N₂ directly for use in RNA extraction, cDNA conversion, and quantitative real-time polymerase chain reaction (qPCR).

3. Lesion Grading

All lesion grades were assessed using a Leica bright light microscope at 100-400x magnification, according to the lesion classification system shown in Table 1. Some tissue sections from healthy control cows received a grade of 1, although this was not from MAP infection. These tissues were subsequently excluded from future analyses (such as qPCR) to avoid any potential interference from non-MAP related intestinal issues. For H&E scores, the criteria considered were total epithelioid macrophages, distribution of inflammation, and integrity of tissue architecture. For AFB scores, both the overall abundance and density of individual pockets of MAP were considered. Refer to Table 1 for details on how scores were assessed for each individual category.

4. Slide preparation, immunofluorescence, and image analysis

O.C.T.-embedded tissues were warmed to -25°C in a cryostat microtome. 5 µm sections were cut and applied to positively charged microscope slides. Slides were stored at -80°C until labeling. Slides were fixed in -20°C acetone for 10 minutes and subsequently allowed to air dry for 30 min. Slides were then blocked with 10% goat serum in Tris-buffered saline (TBS, pH 7.6) for 1 hr at room temperature. Subsequently, primary antibody (eBioscience rat anti-mouse FoxP3 clone FJK-16s, catalog #14-5773-80) was added in labeling buffer (1% goat serum in TBS) at a dilution of 1:50 and incubated in the dark

overnight at 4°C. Slides were washed in TBS 3 times for 3 min per wash. Secondary antibody (Life Technologies goat-anti-rat IgG-AF633, catalog #A-21050) was added in labeling buffer at a 1:300 dilution and incubated in the dark for 1 hr at room temperature. Slides were washed 3 times for 3 min per wash in TBS. NucBlue® ReadyProbes® was used to stain nuclei by diluting 3 drops per 1 mL of labeling buffer, and samples were incubated in the dark for 15 min at room temperature. Slides were washed 3 times for 3 min per wash. Finally, Prolong® Antifade mounting media was used to mount coverslips on the slides. Slides were stored in the dark at 4°C for no more than 1 hr before imaging on an Olympus Fluoview FV1000 laser scanning confocal microscope. Five tissue sections were imaged for each lesion score; within each tissue section, ten fields were imaged and averaged to obtain a value for that section. To avoid bias, tissue sections were chosen semi-randomly (i.e. muscularis and serosa in the ileum was avoided) by only viewing cell nuclei to select fields followed by dual-color image acquisition. ImageJ (NIH) was used to quantify total cell nuclei and total FoxP3+ cells. The ratio of FoxP3+ cells to total cell nuclei was used to quantify the relative abundance of FoxP3+ cells (Tregs) within each tissue section. All images were assigned a random identification prior to cell counting to ensure total blinding in analysis.

5. cDNA Conversion and quantitative real-time polymerase chain reaction

500 ng of RNA was reverse-transcribed into complimentary DNA (cDNA) using the Applied Biosystems high-capacity cDNA reverse transcription kit (Life Technologies catalog #4387406) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction was performed using Applied Biosystems 7000 and 7500 Real-Time PCR systems in triplicate for each sample using Power SYBR Green master mix (Life Technologies catalog #4367659), with 20 µL reaction volumes. Transcripts examined and primer sets used are summarized in Table 3.

6. qPCR data interpretation and statistical analysis

Each gene was calibrated against two internal controls (TATA binding protein (TBP) and peptidylprolyl isomerase A (PPIA)), followed by comparing values from lesions with grades 1-4 against those from grade 0 (control) scores, using the 2^{-ddCt} method as described elsewhere [20]. Control genes were selected by assessing Ct values for a panel of possible control genes for all experimental conditions tested (lesion grades 0-4). No significant differences were observed (n=10 per organ) for TBP or PPIA; as such these were chosen as internal control genes. An unpaired student's t-test was used to assess significance; $p < 0.05$ was considered significant.

Results

1. Classification of Lesions from cows with Johne's disease based on histological analysis and relative quantification of bacterial burden.

40 total tissue sections were collected from the ilea of 3 negative control cows and 100 total tissue sections were collected from the ilea of 4 cows with Johne's disease. 12 total tissue sections were collected from the mesenteric lymph nodes of 3 negative control cows and 29 total tissue sections were collected from the mesenteric lymph nodes of 4 cows with Johne's disease. Results are summarized in Table 2. For control cows, 25 of 40 ileal samples were considered to have a histological score of 1 despite showing zero evidence of Johne's disease or any presence of MAP. This is likely due to typical wear-and-tear seen within the small intestine during the life of an animal due to exposure to a wide variety of food and non-food materials as well as immune responses to a variety of microorganisms that the host is sure to encounter during the course of its life. The remaining 15 score 0 ileal sections were used in qPCR analysis; the 25 score 1 samples were excluded from the analysis entirely as the study was only interested in histopathology associated with MAP infection. 5 of 12 lymph node samples had a score of 1 from control cows and were also excluded from subsequent analysis.

Within tissues of healthy animals, there was a notable lack of epithelioid macrophages and inflammation. Tissue architecture was good. There were numerous thin, densely packed villi protruding into the lumen of the small intestine, along with a generally compact, well-organized mucosal layer. Within the lymph nodes, T and B cell zones were dense with minimal macrophage infiltrate; in particular B cell follicles were well-formed and found to consist almost exclusively of lymphocytes (presumably B cells). Altogether, as expected these tissue sections (minus those that were previously excluded) represented examples of healthy ileal and lymph node tissues within the bovine host.

Of the 100 ileal samples taken from cows with Johne's disease, 93/100 samples received a histological score of 2 or greater, suggesting significant histological changes as a result of infection with MAP. MAP bacilli were found in all samples, both from ilea and mesenteric lymph nodes, from cows with Johne's disease. As detailed in Table 1, ileal lesions with mild grades (1 and 2) generally showed mild to moderate mucosal thickening, somewhat inflamed villi, individual or small groups of epithelioid macrophages, and single or small dense clumps of MAP bacilli. Infected macrophages were rarely seen in defined granulomas; generally the cells were seen to be closer to the luminal side of the mucosa without granulomatous interference. In more severe ileal lesions (grades 3 and 4), villous atrophy was significant as was mucosal thickening, epithelioid macrophages were found in large aggregates or confluent sheets with frequent giant cells, and MAP was found in widespread, consistently dense intracellular clumps with occasional extracellular bacilli present.

Within lymph nodes, tissue sections with mild grades (1 and 2) showed some evidence of increased macrophage infiltrate within the paracortical area, some disruption of germinal center integrity, and occasional macrophages containing one to a few MAP bacilli. Within more severe lesions (grades 3 and 4), distinction between macrophage, T cell, and B cell zones was blurred substantially due to severe infiltration of epithelioid macrophages, frequently found containing dense clumps of MAP. All four cows with Johne's disease tested positive on both a serum ELISA and a fecal PCR (as described in

Methods), but of the four cows with Johne's disease, two (6211 and 6222: henceforth referred to as 'late clinical') had to be euthanized due to extreme weakness (unable to eat or walk) and very poor body condition. The other two animals (6651 and 1688: henceforth referred to as 'early clinical') were noticeably underweight but still in relatively good health at time of death. For H&E scoring, within the ilea, early clinical animals had mostly grade 2 and 3 lesions (a combined 41 of 50 samples) for an average score of 2.2. Late clinical animals had mostly grade 3 and 4 lesions (a combined 49 of 50 samples) for an average score of 3.62. Within the lymph nodes, early clinical animals had mostly grade 3 and 4 lesions (a combined 10 of 14 samples) for an average score of 2.79. Late clinical animals had mostly grade 4 lesions (a combined 13 of 14 samples) for an average score of 3.92.

For AFB scoring, with the ilea, early clinical animals had mostly grade 1 and 2 bacterial burdens (a combined 40 of 50 samples) for an average score of 1.72. Late clinical animals had mostly grade 3 and 4 bacterial burdens (a combined 47 of 50 samples) for an average score of 3.36. Within the lymph nodes, early clinical animals had mostly grade 1 and 2 bacterial burdens (a combined 13 of 14 samples) for an average score of 1.29. Late clinical animals had a wide distribution of bacterial burdens for an average score of 2.67 (Figure 3). Within the ileum, there was a strong correlation ($r^2 = 0.7971$) between H&E grade and bacterial burden (Figure 4). There was a highly significant difference between the three study groups (healthy, early clinical, and late clinical) both histologically and in terms of bacterial burden, in both ileal and mesenteric lymph tissues (Figure 3).

2. Regulatory T cell abundance decreases with increasing lesion severity.

In both the ileum and mesenteric lymph nodes, our data demonstrated that there is a highly significant decrease in relative Treg abundance in both the ileum (for lesions of grades 3 and 4) and the mesenteric lymph nodes (for lesions of grades 1-4) when compared to healthy control tissues (grade 0 lesions). Representative images are shown in Figure 5, and quantified results are shown in Figure 6. In

the ileum, interestingly, relative FOXP3 and IL10 mRNA abundance increased in less severe lesions (FOXP3: grades 1-2, IL10: grades 1-3) yet significantly decreased mRNA abundance of FOXP3, IL10, and TGF β 1 was found in grade 4 lesions (Figure 7), when compared to tissues from healthy control cows, demonstrating a decrease in regulatory/Th2 gene expression in the most severe ileal lesions. Although this data suggests a decrease in Treg activity and function in grade 4 lesions, it must be noted that IL10 in particular is expressed by cells other than Tregs, including Th2 helper T cells and macrophages. Thus, IL10 gene expression is reduced altogether in grade 4 lesions, regardless of source. Within mesenteric lymph nodes (Figure 8), a significant increase in FOXP3 mRNA abundance was observed in lesions of grades 2-4. The mRNA abundance of IL10 also increased in lesions of grades 2-4 in mesenteric lymph nodes, although whether this is the result of Treg activity remains unclear.

3. Of the $\alpha\beta$ T cell subsets examined, CD4⁺ T cells are the primary responders in low grade ileal lesions, demonstrating a mixed Th1/Th2 profile.

Upon examination of two major $\alpha\beta$ T cell subsets (CD4⁺ and CD8⁺ T cells), our data showed that expression of CD4 mRNA was significantly upregulated in lesions of grades 1-3 in the ileum whereas expression of CD8 was not upregulated at any lesion severity, when compared to tissues from healthy controls. CD4 mRNA abundance was unchanged in grade 4 lesions, whereas CD8 mRNA abundance was significantly decreased in grade 4 lesions. Interestingly, the expression of both the Th1-driving transcription factor TBX21, and the Th2-driving transcription factor GATA3, was significantly increased in grade 1 lesions, and significantly decreased in grade 4 lesions. The mRNA expression of the Th1-associated IFNG was significantly increased in grade 1 lesions, and reduced to levels not significantly different from control tissues in lesions of grades 2-4. As mentioned previously, the mRNA abundance of the Th2/Treg-associated IL10 was significantly increased in lesions of grades 1-3, and reduced to levels similar to controls in grade 4 lesions. Altogether this data is suggestive of an initial CD4 T cell, Th1/Th2-mediated response to MAP infection in low grade lesions (grades 1-2) that is no longer present in severe

lesions (grades 3-4). Within the mesenteric lymph nodes, there was no change in CD4 mRNA expression at any lesion severity, while CD8 expression was significantly reduced in lesions of grades 3-4. Differing from trends seen in the ileum, expression of TBX21 and GATA3 mRNA was significantly increased in lesions of grades 2-4 in the lymph nodes. Similarly, expression of both IFNG and IL10 was significantly increased in lesions of grades 2-4. This data is suggestive of a mixed Th1/Th2(Treg) immune profile in the lymph nodes that does not develop until lesion severity increases to grade 2 or above.

4. T cell receptor delta (TCD) mRNA abundance decreases, but WC1 mRNA abundance increases, in cows with Johne's disease.

In the ileum, the relative abundance of TCD mRNA(encoding the delta chain of the $\gamma\delta$ T cell receptor) was significantly decreased in all lesion grades from cows with Johne's disease (grades 1-4) when compared to healthy control (grade 0) tissues. The relative mRNA abundance of WC1, a co-receptor often associated with IFN γ -producing $\gamma\delta$ T cells[8, 26], is significantly increased in lesions of grades 1-2, despite the reduction in overall TCD abundance. Within the lymph nodes, relative TCD mRNA abundance decreased significantly in lesions of grades 3-4, but the relative WC1 mRNA abundance increased significantly in lesions of grades 2-4.

5. Two key inflammatory cytokines, IL1A and TNFA, have increased relative mRNA abundance in lesions of grades 1-2 in the ileum.

Following the pattern seen with numerous other factors including WC1, TBX21, and GATA3, relative IL1A and TNFA mRNA abundance increases in lesions of grades 1-2 in the ileum and is unchanged in lesions of grades 3-4, when compared to healthy control tissues. Within the mesenteric lymph nodes, again similar to the pattern seen with WC1, TBX21, and GATA3, relative mRNA abundance of both IL1A and TNFA is significantly increased in grade 4 lesions, with IL1A mRNA being significantly increased in lesions of grades 2-3 as well. A subset of tissue samples were examined for relative

expression of IL6 and IL17A, two cytokines with inflammatory potential. Relative mRNA abundance of IL6 increased in grade 2 ileal lesions and relative mRNA abundance of both cytokines decreased in grade 4 ileal lesions, although these results did not reach significance ($p > 0.05$, data not shown). Relative mRNA abundance of IL6 tended to increase in grade 2 lymph node lesions, but this result also failed to reach significance ($p > 0.05$, data not shown).

Discussion

Although regulatory T cells have been suggested as a significant contributor to the progression of Johne's disease [11, 13, 33], the nature of their contribution, if any, is unclear. Indeed, previous work by our group has shown not only an increased frequency of Tregs in the peripheral blood of cows with Johne's disease as compared to healthy controls [11], but also an increased relative abundance of FOXP3 mRNA in the ileum of subclinical infected cows [13]. We have proposed two possible scenarios for a role for Tregs. First, it may be that Tregs develop over time as subclinical Johne's disease progresses and function to limit effector immune responses to MAP. In particular, in this scenario Tregs would inhibit Th1 responses to MAP, most notably production of IFN γ , perhaps due to Treg-produced IL-10. In this scenario, Tregs would allow outgrowth of MAP and lead to clinical disease. On the other hand, it is also conceivable that Tregs play a beneficial role in tissues of animals with subclinical disease, serving to limit unchecked inflammation that may be observed during chronic disease due to the large influx of inflammatory cells (macrophages) observed during Johne's disease. In this case, Tregs would prevent the spiral toward clinical disease as long as they were present. Over time, this Treg population could begin to wane, disrupting homeostasis and allowing inflammation to predominate. Thus, in one scenario it is the development of Tregs that accompanies development of clinical disease, whereas in the second

situation it is actually the loss of Treg presence and/or function that accompanies the development of clinical disease. This study helped to investigate these two possibilities.

The first objective of this study was to develop a system for classifying lesions in the ilea and mesenteric lymph nodes of cows with Johne's disease. The classification system was adapted from a system used to classify MAP-related lesions in sheep [14]. We measured both overall condition of the tissues based on hematoxylin-eosin histology as well as bacterial burden, based on acid-fast staining. Following development of the lesion classification system, seven cows were euthanized, four with clinical Johne's disease and three healthy controls. From these animals, 140 ileal samples (40 control, 100 diseased) and 40 mesenteric lymph node samples (12 control, 28 diseased) were collected. Additional samples from the same regions were collected and processed for RNA extraction as well as for fluorescent confocal microscopy. Altogether this allowed for a detailed, layered analysis of lesions of varying severity from cows with Johne's disease as compared to tissues from healthy cows.

Upon establishment of the lesion classification system and subsequent grading of all collected ileum and mesenteric lymph node samples, a representative selection of these tissues was processed for confocal microscopic imaging of FoxP3 protein expression (Figures 5-6). In the ileum, we found a significant reduction in the relative abundance of FoxP3⁺ cells in lesions of grades 3-4 as compared to healthy control tissues (grade 0 lesions) and lesions of grades 1-2 from infected cows ($p < 0.05$ in all cases). Grade 1 lesions tended to have a higher number of FoxP3⁺ cells than grade 0 and grade 2 lesions, though this difference was not statistically significant ($p = 0.2$ in both cases). In the mesenteric lymph node, we found a significant reduction in FoxP3⁺ cells within lesions of grades 1-4 compared to healthy control tissues ($p < 0.05$ in all cases). These results suggest that of the two possible ways in which Tregs may affect the progression of Johne's disease, it is most likely that the loss of Tregs within lesions of MAP infection leads to a loss of homeostasis and a subsequently impaired ability to limit chronic inflammation. In contrast to what was seen with FoxP3 cell numbers, relative FOXP3 mRNA abundance

was significantly increased in ileal lesions of grades 1-2, relative to grade 0 lesions. This suggests that while the number of FoxP3 expressing cells was not significantly different, cells in lesions of grade 1-2 express more FOXP3 mRNA than those in grade 0 lesions. Relative FOXP3 mRNA abundance was significantly reduced in grade 4 ileal lesions, supporting the notion that a loss of Tregs accompanies development and/or progression of clinical disease. This did not appear to be true in the lymph nodes, as relative FOXP3 mRNA abundance was significantly increased in lesions of grades 2-4, while the numbers of FoxP3⁺ cells were lower in all lesions relative to those of grade 0 lesions. This suggests that Tregs in the lymph nodes are expressing higher levels of FOXP3 mRNA despite a reduction in the percentage of FoxP3⁺ cells. Finally, the relative mRNA abundance of the Treg- (and Th2-) associated gene IL10 was significantly increased in ileal lesions of grades 1-3 and lymph node lesions of grades 2-4. When considered in the context of the observed relative Treg abundance (as measured by %FoxP3⁺ cells) and mRNA expression, it seems likely that cells other than Tregs are producing IL-10 during Johne's disease. Relative expression of another Treg-associated factor, TGFβ1, was unchanged or significantly reduced in all lesions relative to control, suggesting that TGFβ is unlikely to play a significant role in Treg responses to MAP. Although beyond the scope of this study, more detailed analysis at the cellular level should be performed in future studies to further validate the tissue-level results seen here. Critically, such studies should examine protein, as opposed to gene, expression, to demonstrate genuine expression of the molecules whose genes are discussed throughout this manuscript.

Although determining the effect(s), if any, of Tregs on the progression of Johne's disease was a primary goal of this study, an additional, major component was to assess the overall immune profile at the gene expression level in regions of MAP infection according to lesion severity, and this was accomplished through qPCR on all lesion samples obtained. Within the ileum (Figure 7), some unique trends were observed. First, relative CD4 mRNA abundance increased significantly in grades 1-3 lesions, when compared to grade 0 (healthy control) lesions, whereas neither CD8 nor TCD (γδ T cell receptor)

expression increased at any lesion grade, suggesting that CD4 T cells are one of the key responder cells in regions of MAP infection. Within the CD4⁺ T cell population, expression of both the Th1-driving transcription factor TBX21 and the Th2-driving transcription factor GATA3 were significantly upregulated in grade 1 lesions, suggesting that the CD4⁺ T cell population responds to early MAP infection with a mixed Th1/Th2 response. This is further supported by results showing a significant increase in the relative mRNA abundance of the Th1-associated IFNG in grade 1 lesions, and the Th2/Treg-associated IL10, in grades 1-3 lesions.

There is mounting evidence that $\gamma\delta$ T cells play a significant role in the immune response to MAP infection [2, 23, 24, 28, 31]. Our results show that, in MAP-infected ileum regardless of lesion score (1-4), the relative mRNA abundance encoding the delta chain of the $\gamma\delta$ T cell receptor is expressed at approximately 20% of the levels seen in uninfected control tissues. On the other hand, the relative mRNA abundance of the WC1 co-receptor is significantly up-regulated by roughly 400% in grade 1 lesions, and roughly 200% in grade 2 lesions, as compared to uninfected control tissues. These results suggest that although the abundance of $\gamma\delta$ T cells may be reduced in the MAP-infected ileum, the proportion of the remaining $\gamma\delta$ T cells that are WC1⁺ is much higher than in uninfected control tissues. Therefore, it is likely that WC1⁺, rather than WC1⁻ $\gamma\delta$ T cells are the dominant subset of $\gamma\delta$ T cells that may play a role in immune responses to MAP infection.

One final trend observed within the ileum, which was observed in 9 of the 12 genes studied, is that there was a significant increase in relative mRNA abundance in grade 1 lesions, followed by a trend toward reduced gene expression as lesion severity increased. This trend was observed in two major pro-inflammatory genes, IL1A and TNFA, which was unexpected. Although our previous research [27] has suggested T cell unresponsiveness as a possible factor contributing to the progression of Johne's disease, we anticipated seeing T cell unresponsiveness lead to loss of T cell (including Treg) function and reduced regulation of inflammation, ultimately leading to increased levels of inflammatory genes in

more severe lesions (e.g., grades 3-4 lesions). Results of this study suggest that this is not the case, or at least that IL-1 α and TNF α are not major effector cytokines in this situation. Considering this data in the context of the large macrophage infiltration seen in MAP-infected tissues, it is possible that macrophages may be shifting to an M2 phenotype, perhaps following prolonged infection with MAP. Future research should investigate this possibility.

Within mesenteric lymph nodes (Figure 8), we observed increased expression of several immune genes (FOXP3, IFNG, TBX21, IL1A, IL10, GATA3, WC1, TNFA) that persisted even in grade 4 lesions, suggesting that in this secondary site of infection there is a prolonged mixed Th1/Th2/Treg, immune response to MAP, unlike in the ileum, where immune responses are consistently unchanged or reduced in grade 4 lesions. Finally, the relative mRNA abundance of TCD and CD8 was reduced in grades 3-4 lesions, whereas CD4 expression was unchanged, suggesting that CD4 T cells play a more prominent role in immune responses to MAP in the mesenteric lymph nodes, similar to what was observed in the ileum. Similar to what was seen in the ileum, TCD expression was significantly reduced in grade 3-4 lesions while WC1 expression was significantly increased, again suggesting that WC1⁺ $\gamma\delta$ T cells are possibly the most important $\gamma\delta$ T cell subset in immune responses to MAP infection.

Altogether, the results from this study build upon the results of our other recent work [27]. In that study, we suggested that T cell unresponsiveness, rather than Treg activity, was responsible for the progression of Johne's disease. Our results, especially within the ileum, support this theory, as we did not observe an increase in the relative expression of any of the genes studied, in grade 4 lesions. In fact, 50% of the genes studied showed a significant reduction in expression within grade 4 lesions. Additionally, a brief examination of IL6 and IL17A mRNA abundance showed similar trends (data not shown) to what was observed with IL1A and TNFA expression (an increase in expression in mild lesions with unchanged expression in severe lesions; Figure 7), thus it is unlikely that the Th17 response is driving chronic inflammation. Rather, it may be that loss of local T cell (including Treg) activity may lead

to reduced control of MAP proliferation, which in turn may lead to increased macrophage recruitment, and possible M2 polarization of persistently-infected macrophages. This would account for the large degree of macrophages present within grade 4 lesions despite not seeing an increase in inflammatory gene expression.

Although speculative, one possible explanation for the stark differences seen in gene expression between the ileum and the mesenteric lymph node is that, in cows with increasingly progressed Johne's disease, T cells are failing to effectively home to the ileum and thus are accumulating within the mesenteric lymph nodes. This would explain unchanged or reduced expression of all genes studied in the ileum, as well as the increased gene expression observed within the lymph nodes. This potential explanation would also explain the apparent T cell unresponsiveness observed in our previous work with peripheral blood mononuclear cells [27]. Clearly, further research is needed to better understand the relationship between Tregs, T cell unresponsiveness, and macrophage activation, in the context of Johne's disease. In sum, we in part accept our initial hypothesis. Our results suggest that Tregs help maintain homeostasis in mild lesions and their subsequent loss, in combination with local T cell unresponsiveness, promotes progression of lesion severity and by extension the progression of Johne's disease.

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514 **Tables**

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Lesion Classification System						
	Histopathology					Acid-fast bacilli abundance and distribution
	Total epithelioid macrophages	Distribution of inflammation		Integrity of tissue architecture		
Grade			Ileum	Mesenteric lymph node	Ileum	Mesenteric lymph node
0	None	N/A	N/A	N/A	N/A	None
1	Scattered individual cells or small clusters.	Typically found within Peyer’s patches and/or villus lamina propria (LP).	Typically localized to paracortex and/or medullary areas.	Minor disruption of tissue architecture, may include expansion of villi.	Minor disruption of tissue architecture; robust germinal centers.	Very infrequent even in presence of inflammatory cells; bacilli typically found isolated with one per macrophage.
2	Regular but inconsistent small groups.	As above but with more extensive spread in LP.	As above but more widespread.	Expansion of villus lamina propria.	Mild expansion of medulla and paracortex.	Few macrophages contain AFB, often with 1-10 per macrophage. Increased abundance of MAP-containing macrophages.
3	Many small to large groups that are beginning to coalesce. Some giant cells likely present.	As above but more widespread and with involvement of the mucosa.	Significant presence in cortex and lymphoid follicles	Significant disruption and effacement of tissue architecture including loss of crypts and villi.	Significant disruption of tissue architecture including inflamed, abnormal medulla. Fewer/ damaged lymphoid follicles.	Increased frequency of macrophages, often found in aggregates, frequently containing greater than 10 MAP per macrophage, including macrophages markedly distended with MAP. Moderate to widespread occurrence of macrophages containing at least some MAP.
4	Densely coalesced aggregates spanning large regions of tissue. Numerous giant cells likely present.	As above but denser and more widespread, possibly with involvement of submucosa.	As above but more widespread.	Severe disruption of boundaries between lamina propria, mucosa, and submucosa. Near-total villus atrophy.	Severe disruption of architecture including effacement of trabeculae. Few if any recognizable lymphoid follicles. Very inflamed cortex.	Macrophages frequently contain many bacilli (>10) potentially with occasional extracellular AFB present. MAP-containing macrophages may be found in large aggregates and/or distributed over large areas.

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Table 1: Lesion classification system. Histopathology was graded based on the number and location of total epithelioid macrophages, distribution of inflammation, and changes to tissue architecture. Bacterial burden was graded based on abundance, distribution, and density of acid-fast bacilli.

Lesion Scoring Summary														
Ileum														
	C1		C2		C3		6651		1688		6211		6222	
Grade	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB
0	5	15	5	15	5	10	0	0	0	0	0	0	0	0
1	10	0	10	0	5	0	5	20	2	4	0	0	0	0
2	0	0	0	0	0	0	15	0	13	16	1	3	0	0
3	0	0	0	0	0	0	0	0	13	10	12	19	5	7
4	0	0	0	0	0	0	0	0	2	0	17	8	15	13
Total	15	15	15	15	10	10	20	20	30	30	30	30	20	20
Average	0.67	0	0.67	0	0.5	0	1.75	1	2.5	2.2	3.53	3.17	3.75	3.65
Mesenteric Lymph Node														
	C1		C2		C3		6651		1688		6211		6222	
Grade	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB	H&E	AFB
0	2	4	1	4	4	4	0	0	0	0	0	0	0	0
1	2	0	3	0	0	0	2	4	1	7	0	2	0	0
2	0	0	0	0	0	0	1	1	0	1	0	1	0	3
3	0	0	0	0	0	0	2	0	4	1	1	4	0	2
4	0	0	0	0	0	0	0	0	4	0	9	3	4	0
Total	4	4	4	4	4	4	5	5	9	9	10	10	4	5
Average	0.5	0	0.75	0	0	0	2	1.2	3.22	1.33	3.9	2.8	4	2.4

Table 2: Lesion classification system scoring summary. Randomly selected samples from both ilea and draining mesenteric lymph nodes were taken from 3 control cows (C1 → C3) as well as 4 cows with Johne's disease (6651, 1688, 6211, 6222). Lesion scores were obtained by applying the lesion classification system (Table 1) to each individual tissue section using both a hematoxylin and eosin (H&E) stain and an acid fast bacilli (AFB) stain.

Primers used in quantitative reverse-transcriptase RT-PCR		
Gene name	Forward primer	Reverse primer
CD4	ACGTCAAGAGCCTGTCACTG	TGCTGGCTCTGGGAGATAGT
CD8A	ACGTGTCTGCAAATGTCCCA	CTCTGAAAGGTTGGGCTTGC
FOXP3	CACAACCTGAGCCTGACAA	TCTTGCGGAACCTCAAACATCATC
GATA3	AGGTACGTCCTGTGCAAAC	AGACAGGGTCTCCATTGGCA
IFNG	TCTGGTTCTTATGGCCAGGG	GAAGAGAGGCCACCCTTAG
IL1A	TTGGTGACATGGCAAGTG	GCACAGTCAAGGCTATTTTTCCA
IL17A	CATCTCACAGCGAGCACAAG	CCACCAGACTCAGAAGCAGT
IL6	GGCTCCCATGATTGTGGTAGTT	GCCCAGTGGACAGGTTTCTG
IL10	GCTGTATCCACTTGCCAACC	ATCCAGCAGAGACTGGGTCA
PPIA	GCAAGCACGTGGTACTTTGG	TTGCTGGTCTTGCCATTCCT
TBP	ACAGAGAACACCAGAGCGTC	CAGCACTGCCCCGTATAGCA
TBX21	ACCACCTGTTGTGGTCCAAG	ATCCGGTAATGGCTGTTGGG
TCD	ACAAGCAAACCTGGCACTCT	TGCCATCGGTTTTGGAGTTG
TGFB1	CGAGCCCTGGACACCAACTAC	CCGGAAGTCAATGTAGAGCTGA
TNFA	TCTACCAGGGAGGAGTCTTCCA	GTCCGGCAGGTTGATCTCA
WC1	CACTTCGGAGCAGGATCAGG	GGTGGGACTCCTTTCCTGTG

Table 3: qPCR primers. TATA-binding protein (TBP) and peptidylprolyl isomerase A (PPIA) were used as internal controls.

Figure Legends

Figure 1: Lesion classification system

Hematoxylin and eosin staining was used to visualize tissue histology for ileal (left) and mesenteric lymph (right) tissues. Images are arranged from left to right, demonstrating progressively increasing lesion severity ranging from grade 0 (top) to grade 4 (bottom). Lesion severity was assessed based on overall tissue architecture, relative amount of inflammatory cell infiltrate, and distribution of inflammatory infiltrate.

Figure 2: Lesion classification system

Acid-fast bacilli staining was used to assess relative MAP burden within ileal (A→E) and mesenteric lymph (F→J) tissues. Images are arranged from left to right, demonstrating increasingly greater MAP burden ranging from grade 0 (A,F) to grade 4 (E,J). The range of MAP burden is relative to each individual organ, as the overall magnitude of burden is greater at the primary (ileum) site of infection as compared to the secondary sites (lymph nodes).

Figure 3: Lesion Classification System Score Summary.

Animals were divided into category based on being negative for Johne's disease (healthy), testing positive for clinical disease but not showing major disease symptoms (early clinical), or testing positive for clinical disease and showing significant symptoms of end-stage Johne's disease (late clinical).

Figure 4: H&E and AFB Score Correlation

For cows with Johne's disease, H&E and AFB grades were paired and the coefficients of determination were calculated. A strong correlation was seen in ileal grades ($r^2 = 0.7971$). No correlation was seen in mesenteric lymph node grades ($r^2 = 0.3269$).

Figure 5: Regulatory T cell abundance

Regulatory T cell abundance was measured by confocal microscopy, with FoxP3+ cells stained red against a blue nuclear counterstain. In both the ileum and the mesenteric lymph nodes, Treg abundance tended to decrease with increasing lesion severity. (top) Representative Treg abundance in the ileum. (bottom) Representative Treg abundance in the mesenteric lymph node.

Figure 6: Regulatory T Cell abundance

Regulatory T cell abundance was measured by comparing the number of FoxP3+ cells to the total number of eukaryotic cell nuclei present (%FoxP3+/total) for each of the five lesion grades, within both the ileum (A) and mesenteric lymph nodes (B). Ten fields were measured for each of five tissue sections per lesion grade. * $p < 0.05$.

Figure 7: Relative ileal gene expression.

Tissues were collected from healthy control cows and cows with Johne's disease. Lesion grades were assessed via the lesion classification system presented within this paper. Snap-frozen tissue sections were processed for RNA extraction, cDNA conversion, and qPCR for a variety of immune-related genes, and these data were separated based on lesion grade. Grade 1-4 lesions were compared to grade 0 lesions. $n = 9-35$ per grade, * $p < 0.05$.

Figure 8: Relative mesenteric lymph node gene expression.

Tissues were collected from healthy control cows and cows with Johne's disease. Lesion grades were assessed via the lesion classification system presented within this paper. Snap-frozen tissue sections were processed for RNA extraction, cDNA conversion, and qPCR for a variety of immune-related genes, and these data were separated based on lesion grade. Grade 1-4 lesions were compared to grade 0 lesions. $n = 4-9$ per grade, * $p < 0.05$.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8