A POSSIBLE ROLE FOR REGULATORY T CELLS IN THE PROGRESSION OF JOHNE'S DISEASE

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ABSTRACT

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Johne's disease, or paratuberculosis, is a chronic wasting disease that is caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Johne's disease affects wild and domestic ruminants including livestock species such as cows. Due to factors including losses in milk production and early culling of infected animals, Johne's disease represents a major financial burden, estimated at between $250 million and $1.5 billion annually for the U.S. dairy industry alone. MAP is an obligate intracellular pathogen that is transmitted through the fecal-oral route. MAP infects the host by invading the ileum of the small intestine through M cells lining the lumen, following which it colonizes tissue-resident macrophages thereby establishing persistent infection. The immune response to MAP infection is characterized by a shift from a productive Th1 immune response to an unproductive Th2 response. As a chronic condition, MAP infection may result in the development of a regulatory T cell (Treg) population within infected animals, and these Tregs may play a role in the Th1-to-Th2 immune shift observed in animals progressing from subclinical to clinical disease. Alternatively, Tregs may be critical for controlling chronic inflammation, and their loss may result in severe widespread inflammation that results in damage to host tissues and progression into clinical disease.

In this project, we sought to investigate the relationship between MAP infection and Treg activity. First, we utilized a monocyte-derived macrophage (MDM) model of MAP infection in an effort to induce the development of a Treg phenotype in naïve T cells from cows with Johne's disease. Second, we developed a method to expand the relative abundance of Tregs present in peripheral blood mononuclear cell (PBMC) populations from MAP-infected cows. Following this, expanded Tregs were used in functional assays to determine if they dampened Th1 immune responses to stimulation of

PBMCs with live MAP. Finally, we developed a scheme to classify lesions from cows with Johne's disease according to histopathology and abundance of MAP within ileal and mesenteric lymph tissues. Based on this system, we graded lesions from cows with Johne's disease and measured several variables, including relative Treg abundance, within these lesions as compared to healthy control tissues.

Although a Treg phenotype did not develop in naïve T cells cultured with MAP-infected MDMs, we observed a state of T cell unresponsiveness in naïve T cells from cows with clinical disease, and a state of reduced responsiveness in naïve T cells from cows with subclinical disease, as compared to responses from control cows. In our second set of experiments, we found that we were able to successfully expand bovine Tregs, and although these expanded Tregs are not MAP-specific, they are functional and do suppress Th1 immune activity generally. Within infected tissues we found that cows with clinical disease can likely be categorized further into early and late clinical disease, based on overall animal health and lesion severity. Further, we observed that Treg abundance decreases with increasing lesion severity in both the ileum and mesenteric lymph nodes. Additionally, within the ileum it was observed that the expression of many immune genes was elevated in mild lesions but was unchanged or reduced in severe lesions, whereas within the mesenteric lymph nodes the expression of many immune genes increased with increasing lesion severity.

Altogether, our results suggest that T cell (and thus, Treg) unresponsiveness may be responsible for driving the immune shift seen in cows progressing from subclinical to clinical Johne's disease. The ability to expand non-specific Tregs from bovine peripheral blood is a novel development in bovine immunology and will be useful for future researchers. Finally, our data suggests that a robust $CD4^+$ T cell and inflammatory response to MAP occurs in the ileum of infected cows, followed by the development of T cell unresponsiveness that likely contributes to a loss in control of MAP infection. This in turn would allow progression of Johne's disease from early to late clinical disease and ultimately the demise of the infected host.

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KEY TO ABBREVIATONS

AFB: Acid-fast bacilli

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

CD8A: Cluster of differentiation 8 alpha chain

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

FOXP3: Forkhead box protein 3

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GATA3: GATA-binding protein 3

GITR: Glucocorticoid-induced TNFR-related protein

H&E: Hematoxylin and eosin

IFNG: Interferon gamma

IL1A: Interleukin 1 alpha

IL10: Interleukin 10

IL12P40: Interleukin 12 subunit p40

IL17A: Interleukin 17 alpha

IL2: Interleukin 2

IL23: Interleukin 23

IL4: Interleukin 4

IL6: Interleukin 6

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

MDM: Monocyte-derived macrophage

PBMC: Peripheral blood mononuclear cell

PFN1: Perforin 1

PPIA: Peptidylprolyl isomerase A

RAR: Retinoic acid receptor

RORC: RAR-related orphan receptor C

TBP: TATA-binding protein

TBX21: T-box transcription factor 21

TCD: T cell receptor delta chain

TGFB1: Transforming growth factor beta 1

TNFA: Tumor necrosis factor alpha

WC1: Workshop cluster 1

Chapter 1: Introduction and literature review

1.1 Introduction to Johne's disease

Johne's disease, or paratuberculosis, was first identified by Heinrich Johne in 1905. Johne's disease is a chronic wasting disease primarily affecting ruminants, notably including many livestock species such as cattle and sheep. The disease is characterized by persistent diarrhea, progressive wasting, and premature death of infected animals that progress to clinical disease [2]. Caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), Johne's disease has a long subclinical stage of infection during which animals show little or no signs of disease and intermittently shed MAP in their feces. This stage of disease frequently lasts for 2-5 years [3], allowing an undetected yet significant spread of MAP throughout a herd.

Johne's disease has a worldwide occurrence [4], with only Sweden and certain states in Australia proven free of disease [5]. In the United States, it is estimated that roughly 8% of beef cattle herds and 40-68% of dairy herds contain some prevalence of MAP [3, 6]. Outside the United States, estimates of herd-level prevalence of Johne's disease vary widely, with estimates as low as 7.9% in Belgium [7] and as high as 71% in the Netherlands [8]. Estimates of within-herd prevalence also vary widely, ranging from 1.5% - 42% [9, 10]. Critically, Johne's test-positive cows are reported to produce 4-20% less milk than test-negative herdmates [11, 12]. Taken together, the significant prevalence of Johne's disease within dairy herds combined with reduced milk production and early culling of infected animals results in a substantial economic burden. Annual losses, to the U.S. dairy industry alone, due to Johne's disease are estimated at between $250 million and $1.5 billion annually [13].

Despite a significant increase in farmers' awareness of Johne's disease [3], effective control of MAP has proven difficult. The two major diagnostic tests used to track Johne's disease are a serum/milk

ELISA and fecal culture. While fecal culture remains the gold standard in Johne's diagnostic testing, with a specificity of close to 100% [14], the test can take up to 6 weeks until positive culture results and its sensitivity is only estimated to be 30-50% [15]. Johne's ELISA testing, while very fast compared to fecal culture (~24 hr. turnaround), has a slightly lower specificity (95-99%) and a reduced, variable sensitivity depending on fecal shedding. In animals not shedding MAP in their feces, around 15% of infected animals may be detected, whereas those animals with positive fecal culture results are detected by serum ELISA roughly 50% of the time [16]. Poor diagnostic sensitivity is a key to the "iceberg effect" hypothesis, first put forth by Whitlock and Buergelt, which suggests that for every animal with signs of clinical disease, another 15-20 animals are silently infected [2]. Taken together, this information demonstrates a clear need to further our understanding of Johne's disease and its causative agent, MAP, with an ultimate goal of aiding in control and prevention of disease.

1.2 MAP and the bovine immune system during early infection

Mycobacterium avium subspecies *paratuberculosis* is a gram-positive bacterium of the *Mycobacterium* genus. This genus also includes the causative agents of tuberculosis and leprosy, demonstrating a close genetic similarity between MAP and other virulent *Mycobacteria*. As with most *Mycobacteria*, MAP's thick, waxy cell wall gives it many unique characteristics, the most notably of which being its acid-fast property – MAP resists de-colorization by acidified alcohol following staining with, e.g., carbol fuchsin. Additionally, when compared to most other bacteria, the mycobacterial cell wall gives MAP increased resistance to chemical [17] and physical damage [18, 19], as well as conferring on it a strong hydrophobicity [20]. MAP can be distinguished from other *Mycobacteria* in certain ways, as well. MAP is unable to produce mycobactin, an essential iron-sequestering compound critical to extracellular growth of *Mycobacteria*. In addition, MAP is the slowest-growing of all cultivatable

Mycobacteria [21]. Finally, MAP carries a genetically unique marker known as Insertion Element 900 (IS900) [22], and this marker is key for genetic detection of MAP.

Characteristically, MAP has been broadly classified into two major categories based on pigmentation and growth rate. Type I, or Sheep ("S") type MAP, has a yellow pigmentation and grows very slowly (>16 weeks of incubation time to detection). Type II, or Cattle type MAP, has no pigmentation and grows relatively quickly by comparison (6 to 12 weeks of incubation time to detection) [23]. Different isolates of MAP are not distinguishable from one another by a wide variety of traditional methods including chromatography, serology and antimicrobial susceptibility (antibiotic resistance) [24, 25], making distinction of different strains difficult. Insertion Sequence 900 (IS900) restriction fragment length polymorphism (RFLP) analysis has been used to discriminate between Type I and Type II MAP strains [26]. It has been shown that Type I MAP organisms survive within bovine monocyte-derived macrophages more readily than do Type II organisms [27]. Due to the significance of the bovine monocyte-derived macrophage model in the experiments contained within this study, a bovine MAP isolate was used throughout all experimental infections discussed herein.

MAP is transmitted between animals primarily through the fecal-oral route, although *in utero* transmission is known to occur, and ingestion of infected colostrum also results in significantly greater chances of developing disease later in life [28]. MAP invades the small intestine through microfold (M) cells as well as differentiated epithelial cells [29, 30]. Fibronectin attachment protein is activated upon passage through the digestive system, promoting fibronectin-based attachment of MAP to the luminal surface of M cells, thus promoting preferential uptake of MAP via these cells [31, 32]. Following uptake, these intestinal cells subsequently transfer MAP to the submucosa [33], whereupon MAP colonizes tissue-resident macrophages within the ileum of the small intestine [34, 35]. MAP is an obligate intracellular pathogen that primarily infects macrophages. Extremely slow-growing, MAP can remain within host macrophages in a state of homeostasis with little response from the host immune system for

years before eliciting a robust immune response; the ability of the host to detect and kill intracellular MAP is critical to controlling and ideally eliminating the infection. During the early, subclinical stage of infection, MAP induces development of ileal lesions characterized by the presence of macrophages, $CD4^+$ T cells, $CD8^+$ T cells, γδ T cells, epithelioid cells, and occasional multinucleated giant cells [36, 37]. It has been suggested that γδ T cells in particular are important both in proper granuloma development as well as in controlling dissemination of MAP to other organs [38]. Production of TNFA by macrophages, as well as by γδ T cells and $CD4^+$ T cells, is a key component of the pro-inflammatory response (defined within this study as expression of pro-inflammatory cytokines including IL1A, TNFA, and in some cases IL6, and does not include allergy-induced inflammation (Type I hypersensitivity) and is important in inducing granuloma formation [39]. Infected macrophages also produce IL10 (possibly induced by MAP directly [40]), however, and this may dampen $CD4^+$ T cell-driven IFNG responses critical to priming newly recruited macrophages for resistance to persistent infection [41] , ultimately leading to increased dissemination of MAP within the host.

Whereas macrophages readily degrade most phagocytosed bacteria via development of a phagolysosome, in MAP-infected cells, maturation of the phagolysosome is arrested prior to fusion of the phagosome with the lysosome [42]. It is known that only viable MAP is capable of blocking phagosome-lysosome fusion, demonstrating that this is an active process [43, 44]. SapM, a Mycobacterial lipid phosphatase, actively eliminates phosphatidylinositol 3-phosphate from the phagosome, and this lipid is an essential component of phagolysosome fusion [43]. A mechanism by which MAP inhibits phagosome maturation involves the integration of mycobacterial lipids into the phagosome membrane [45], a process which has been shown to impair phagosome fusion [46]. Additionally, is has been shown that the closely-related *Mycobacterium tuberculosis* secretes a protein, protein tyrosine phosphatase, shown to inhibit phagosome acidification [47]. Finally, MAP promotes retention of Rab5 and subsequent inhibition of early endosomal autoantigen 1, inhibiting the

phagosome's ability to develop bactericidal compartments [48]. Clearly, the wide variety of mechanisms by which MAP is capable of inhibiting phagolysosome maturation and subsequent bacterial killing demonstrates how high a priority this is for MAP's intracellular survival. Altogether, these actions result in MAP not being degraded within the macrophage, leading to reduced antigen presentation and subsequently a reduced adaptive immune response. This evasion mechanism results in a greatly delayed initiation of the adaptive immune response and establishment of a reservoir of MAP within the host.

Ultimately some MAP does get degraded and eventually an adaptive immune response is initiated. Upon recognition of the pathogen, the bovine immune system responds in a manner typically characterized by a cytotoxic and pro-inflammatory Th1 profile. Initially, classically-activated macrophages produce interleukin (IL) 1, which aids in initiation of inflammatory responses and recruitment of immunocompetent cells, and IL12, which helps promote Th1 polarization of antigen-primed $CD4^+$ helper T cells [35]. In turn, MAP-responsive Th1 helper T cells begin to secrete various pro-inflammatory cytokines including interferon gamma (IFNG), tumor necrosis factor alpha (TNFα), and IL2 (a T cell growth factor)[35]. These factors, particularly IFNG, are critical for priming naïve macrophages newly recruited to sites of infection; macrophages primed with IFNG are able to destroy MAP and present antigens to T cells, which in turn can suppress the growth of MAP. Indeed, in MAP-infected goats it has been shown that neutralization of $CD14^+$ monocyte-derived IL10 results in enhanced IFNG production in peripheral blood mononuclear cells [49], further suggesting a key role for monocytes and monocyte-derived macrophages in shaping the early immune response to MAP.

Gamma Delta (γδ) T cells are unique in that they do not require interaction with a MHC-peptide complex for activation [50], and they may play an important role in the initiation of adaptive immune responses to MAP due to the minimal antigen presentation that occurs during this early stage. Indeed, in humans it has been shown that γδ T cells produce roughly twice as much IFN γ as $CD4^+$ T cells in response to monocytes infected with *M. tuberculosis*, suggesting a critical role for these cells in

recognizing mycobacterial antigens and activating macrophages during early infection. Finally, there is strong evidence for cytotoxic γδ T cell activity [51], providing yet another significant means by which these cells may play an important role in helping control MAP infection. A distinction should also be made between emerging subsets of γδ T cells, namely between workshop cluster 1 (WC1)-positive and $WC1^-$ γδ T cells. Generally speaking, $WC1^+$ γδ T cells are known to be inflammatory in nature and are important mediators of cell-mediated (Th1) immunity, whereas $WC1^-$ γδ T cells are thought to be regulatory in nature [52, 53]. To date, there is mounting evidence that γδ T cells do play some role in bovine immune responses to MAP [54-58], but subpopulations of γδ T cells based on WC1 expression, in the context of Johne's disease, are just now garnering attention. In bovine tuberculosis, however, it has been shown that *in vivo* depletion of $WC1^+$ results in significantly reduced levels of IFNG and *M. bovis*-specific IgG2 antibodies, and significantly increased levels of IL4 [59], suggesting that $WC1^+$ γδ T cells are key cells in promoting a Th1 immune response. Due to what appears to be highly opposed functions of these very plastic cells depending on WC1 expression, a better understanding of these subpopulations, especially in the ileum, is warranted and indeed needed. Finally, recent evidence suggests that γδ T cells may be a key regulatory cell population within the bovine periphery [60], suggesting an as-yet only minimally explored possible function for these unique cells.

Although priming of newly recruited macrophages may result in reduced spread of MAP to these new potential host cells, macrophages already infected with MAP do not benefit from these cytokines and as such serve as a reservoir for MAP within the animal. Cytotoxic T cell activity during early infection is presumably critical in inhibiting the spread of MAP, as these cells possess the ability to directly lyse persistently infected macrophages. Further, it has been shown in both human tuberculosis [61] and leprosy [62] (two diseases with similar etiology to Johne's disease) that a strong cytotoxic T cell response during early infection is important in reducing the severity of disease, presumably through the destruction of persistently infected macrophages.

Beyond the immune cells discussed above, one significant possibility is that innate lymphoid cells (ILCs) play a role in early immune responses to MAP. Unfortunately, to date there has been very little (if any) research done on these cells in relation to MAP infection. Still, these cells represent a very logical possibility as a key effector population in response to early MAP infection. Innate lymphoid cells lack antigen-specific receptors and instead respond to more general signals such as alarmins [63, 64]. Due to the slow development of adaptive immunity in response to infection with MAP, innate lymphoid cells may fill a critical role in helping control the spread of MAP during early infection. Further, innate lymphoid cells can be divided into three broad categories, and these three subsets of ILCs show cytokine secretion profiles consistent with Th1 (ILC1), Th2 (ILC2), or Th17 (ILC3; Th17 responses are herein defined as expression of Th17-associated genes including IL17A, IL23, and RORC) responses [65]. Thus, depending on the particular subset of ILC activated, these cells may contribute substantially to the shaping of early immune responses to MAP. ILC1 cells are of particular interest as they respond to IL12 in part by producing IFNG [66], and they express TBX21, a key Th1 helper T cell transcription factor [67]. Overall, innate lymphoid cells are known to be particularly prevalent at mucosal surfaces and have been shown to be important in contributing to resistance against bacterial infections as well as promote both inflammation and tissue repair [68], all factors important in the pathogenesis of MAP infection seen during Johne's disease. In summary, innate lymphoid cells possess many characteristics that suggest they may play a significant role in shaping early immune responses to MAP infection, but research in this area is sorely lacking. Although the role of innate lymphoid cells during Johne's disease is beyond the scope of this research project, this field represents a major gap in Johne's disease research and presents a major opportunity for future researchers.

1.3 Changing immune responses: Progression of Johne's disease and the case for regulatory T cells

During the early portion of the subclinical stage of Johne's disease, the adaptive immune response primarily consists of a cell-mediated Th1 response, as discussed above. Over time, however, seroconversion occurs and the Th1 response wanes in favor of a Th2 (humoral) immune response [35, 69-71]. In response to infection with MAP, macrophages begin to secrete IL10 as opposed to the more beneficial IL12 [35]. Macrophage-produced IL10 may help drive the Th1-to-Th2 conversion by promoting the Th2 immune response, and it also serves to dampen production of important Th1 cytokines, most notably IFNG. This change is not linear and absolute, however, as the primary antibody response to MAP infection following the initial IgM response is production of anti-MAP IgG1 antibodies, which requires CD4+ or γδ T-produced IFNG [72]. Both IgG1 and IgG2 antibody responses may be detected at this stage of infection [2], as the animal progresses toward clinical disease. Due to the intracellular nature of MAP, however, the antibody-mediated response is ineffective at controlling the infection. The loss of critical immune responses leads to accelerated spread of MAP both within the ileum and to secondary sites of infection including mesenteric lymph nodes. It should also be noted that there is evidence demonstrating losses in both Type 1 (defined herein as expression of traditional Th1 immune genes as well as Th1-promoting factors derived from other immune cells (besides helper T cells) such as macrophages) and Type 2 immune responses (defined herein as expression of traditional Th2 immune genes as well as Th2-promoting factors derived from other immune cells (besides helper T cells) including macrophages) during late clinical disease, suggesting that the shift in immune function may be more profound than simply changing from Type 1 to Type 2. It is currently unknown precisely why this shift occurs [69], although one possibility involves regulatory T cells.

Induced regulatory T cells (Tregs) are characterized by the expression of surface markers CD4 (Helper T cell co-receptor), CD25 (IL2 receptor alpha chain), and the transcription factor FOXP3, the master regulator of Treg activity. While many activated T cells express CD25, Tregs express this receptor constitutively. They are known to exert their effects through the production of IL10 and transforming

growth factor beta (TGFB), and it has been demonstrated that they exert suppressive effects on Th1 cytokine expression [73, 74]. Evidence suggests that a population of Tregs may develop in response to chronic, low-level stimulation with MAP antigens and that these Tregs subsequently function to limit effector T cell responses to MAP [75]. Although Thymic-derived Tregs generally function to control autoimmunity, the possibility that Tregs develop in response to MAP antigens is not without precedent. Numerous other studies have shown that induced Tregs may develop in response to many infectious agents including *M. tuberculosis* and *M. leprae* [76-82].

A significant body of work implicates Tregs in Johne's disease directly. Our group and others have previously shown that removal of either $CD4^+$ or $CD25^+$ peripheral blood mononuclear cells (PBMCs) from MAP-infected cattle prior to MAP antigen stimulation results in enhanced IFNG, and reduced IL10 mRNA production by those PBMC populations following stimulation with MAP antigens [75, 83]. It has also been demonstrated that antibody-mediated neutralization of IL10 in PBMCs from MAP-infected cows results in an increase in recall responses to MAP, such as production of IFNG [83]. Further, it has been shown that addition of anti-IL10 neutralizing antibody to MAP-stimulated PBMC cultures results in a significant increase in the relative mRNA expression of IFNG, IL12P35, IL12P40, IL1A, and IL1B, relative to cells stimulated with MAP only [75]. Recent evidence has demonstrated that there is a significantly greater proportion of $CD4^+CD25^+FOXP3^+$ Tregs in the periphery of cows with Johne's disease compared to healthy cohorts [84], and perhaps most critical of all is research showing a significantly increased abundance of Tregs in the ileum of cows with subclinical Johne's disease as compared to healthy controls [75]. As the ileum is the primary site of infection, this is a critical piece of data suggesting that not only does the abundance of Tregs increase during certain stages of Johne's disease, but also that they are found in increased numbers in key regions of MAP infection, where the major immune response is occurring.

There are two key effects Tregs may have in the context of the progression of Johne's disease. First, MAP antigen-reactive Tregs may develop as a result of the immune microenvironment present within the ileum of MAP-infected cows. It is well-documented that failure to properly co-stimulate T cells combined with chronic antigen stimulation can result in the induction of a Treg phenotype [85-87]. Importantly, both chronic antigen stimulation and MAP infection of host macrophages resulting in decreased CD40:CD40 ligand interaction is seen in the course of MAP infection [88, 89], creating an environment of ineffective T cell co-stimulation. If Tregs were to develop under these conditions, they would likely function to limit effector T cell responses to MAP. As a result of this 'Treg bloom' and the large increase in expression of IL10 and TGFB that would accompany it, host immunity against MAP, in particular Th1 immune responses such as IFNG, would be reduced substantially. This is turn would result in reduced priming of newly-recruited macrophages, reduced levels of direct killing of intracellular MAP, and decreased rates of cytotoxic T cell-mediated killing of chronically MAP-infected macrophages. Altogether, control of the infection would be greatly diminished and the reproduction and spread of MAP within the host ileum would increase dramatically, leading to development of clinical disease. This idea is not without precedent, as it has been shown that the presence of Tregs can directly enhance survival of pathogens within the host [90, 91].

A second possibility is that Tregs do not serve to limit effector T cell responses to MAP, but rather that they are required to control chronic inflammation. As Johne's disease is a chronic inflammatory disease [32] with slow progression, it is understandable that severe damage to the ileum can occur over the course of disease. It is possible that Tregs are critical mediators of this inflammation by producing IL10 in regions of inflammation. IL10 is recognized as one of the most important and potent anti-inflammatory cytokines (an anti-inflammatory response is defined herein as expression of genes known to inhibit inflammation including IL10 and TGFB), as it is both produced and received by many innate and adaptive immune cells and [92]. Thus, the effects of a population of IL10-producing

Tregs may be wide-ranging and critical to preventing runaway inflammation within the ileum. In this situation, Tregs would be beneficial to the host, helping to control inflammation and preserve the integrity and functionality of the ileum while the adaptive immune system works to eliminate the infection.

In the first possibility outlined above, the development of Tregs would precede and ultimately induce the development of clinical Johne's disease through immunomodulation and the down-regulation of Th1 immune responses necessary to control an infection with MAP. The situation proposed in the second option would essentially be the opposite of the first. Tregs would either migrate to the ileum in response to the infection, or already-present tissue-resident Tregs would be the main effectors. These Tregs would be responsible for controlling chronic inflammation (through the production of IL10); it would be the loss of this Treg activity that would result in the development of clinical Johne's disease. Clearly, evidence strongly suggests that there are more Tregs in cows with Johne's disease as compared to healthy controls [75, 83, 84], but the two scenarios outlined here demonstrate a major knowledge gap within this field.

1.4 Means of Treg function: T cell unresponsiveness and M2 macrophages

Although there is significant evidence for a role of Tregs in the progression of Johne's disease, it is not yet known whether this is a result of increasing or decreasing Treg activity. In the case of increasing activity, the development of Tregs would likely precede progression toward clinical disease, whereas in the case of decreasing Treg activity, the loss of Treg abundance or function would precede or accompany the development of clinical disease. One possible reason for a loss of Treg abundance or function is the development of T cell unresponsiveness. A lack of T cell response can come in several degrees of severity: hyporesponsiveness, unresponsiveness, or anergy. Responsiveness in this work is

defined as a significant increase in expression of immune genes studied in response to stimulus, whereas unresponsiveness is defined as a significantly reduced expression of immune genes studied altogether in comparison to healthy control cows, and hyporesponsiveness is considered to be when cells respond to stimulus specifically, but still exhibit overall reduced expression of most genes studied as compared to what is seen in healthy control cows. Previous work has provided strong evidence for T cell hyporesponsiveness [89] or T cell anergy [51, 93] in T cells taken directly from MAP-infected ileal tissues, and our group recently published strong evidence of T cell unresponsiveness within PBMC populations from cows with clinical Johne's disease [94]. When examining the potential nature of unresponsiveness, one must also consider the distinction between general unresponsiveness, or immunosuppression, and antigen-specific tolerance. Indeed, if T cell unresponsiveness is present in the immune response of cows infected with MAP, making this distinction is key. Tregs are widely recognized as cells that induce unresponsiveness in other T cells [95-97], especially in cases of auto- and alloantigens, but also in cases of chronic bacterial or viral infectious disease [98], including mycobacterial diseases such as leprosy [99]. Therefore, one distinct possibility is that Tregs are inducing antigen-specific T cell unresponsiveness in other T cells during subclinical Johne's disease, allowing progression to clinical disease.

Alternatively, it may be that Tregs are as susceptible to T cell unresponsiveness as are most other T cells. In this situation, it is probable that a cell type other than Tregs is inducing this unresponsiveness. The likely cell responsible for such a shift is the macrophage, as macrophages, being the primary hosts of MAP, are a key cell in Johne's disease. There are two main directions in which macrophages can polarize, M1 (or classically-activated) and M2 (alternatively-activated). M1 macrophages display behavior traditionally associated with macrophages: they are highly bactericidal, produce IL12 in response to infection, and promote pro-inflammatory responses. M2 macrophages, on the other hand, are generally accepted as anti-inflammatory, produce IL10, and are involved in tissue

remodeling [100, 101]. Clearly, M1 macrophages are beneficial in controlling intracellular infections whereas M2 macrophages are not. Of great interest to this issue, it has been shown that advanced stage mycobacterial infection is often associated with M2 macrophages serving in immunosuppressive capacities [102]. Further, it has been shown that IL10 is important in maintaining T cell hyporesponsiveness in certain parasitic infections [103]. Bringing this issue full circle, then, is evidence that IL12 is a key factor in overcoming T cell unresponsiveness in mycobacterial disease [104]. Finally, the mycobacterial protein product DnaK has been shown to polarize macrophages to an M2-like phenotype in cases of human tuberculosis [103], suggesting a possible virulence mechanism of pathogenic mycobacteria. As mentioned previously, macrophages tend to produce IL10 in response to infection with MAP, as opposed to the more beneficial IL12. Altogether, this information provides substantial evidence that macrophages may adopt an M2 phenotype in response to MAP infection, possibly as a result of MAP virulence factors, and promote the development of T cell unresponsiveness, including in Tregs. This would then serve to shut off the critical control mechanism that Tregs may play in controlling inflammation during Johne's disease.

1.5 Concluding remarks

Johne's disease presents a significant burden for the worldwide dairy industry but also to any ruminants raised as livestock. Beyond the economic impacts, Johne's disease presents a significant malady to ruminants as well as several non-ruminant species [105-108] including primates [109]. The chronic wasting nature of the disease likely results in long-term suffering of infected animals as clinical disease runs its course. To date, vaccine development has yielded some mild successes. The commercially-available vaccine, Gudair®, is the most successful such example. In one trial [110], vaccination of sheep prior to exposure to MAP (using an established sheep infection model) reduced

infection rates from 72% to 24%. Although this is a significant difference, with only 1/3 as many vaccinated animals contracting MAP, 24% is still a far cry from truly effective vaccination. Further, vaccination is capable of reducing Johne's disease-related mortality by up to 90% [111], but current vaccines are unable to completely prevent the spread of MAP or completely eradicate the disease [112, 113]. It has been shown that there are significant differences between vaccinated animals that did not contract disease, as compared to those that did, in several different cell subsets including $CD4^+$ T cells, B cells, and γδ T cells [110]. Altogether, despite modest vaccine success, true prevention of infection and control of the spread of MAP has yet to be attained, and it seems that differential immune responses from one animal to another are a major culprit for these shortcomings. Clearly, a more detailed understanding of immune responses to challenge with MAP is needed to advance prevention and control strategies.

One would be remiss to omit any mention of the possible relationship between Johne's disease and Crohn's disease. Altogether, the association between the two conditions remains largely observational – the symptoms and pathology of both conditions are highly similar. Association between presence of MAP in the human gut and development of Crohn's disease is much more ambiguous, however. Several papers implicate MAP as a causative factor in the development of Crohn's disease [114-116], including evidence demonstrating a therapeutic benefit of anti-MAP drug therapy for Crohn's patients [117], isolation of MAP strains from Crohn's patients [118], and the presence of MAP antigen-reactive $CD4^+$ T cells in the ileum of individuals with Crohn's disease. There is also a substantial body of evidence refuting MAP as an agent of development of Crohn's disease [119-121]. If the link between MAP and Crohn's disease is established, however, then the significance of MAP (which can be found viable in pasteurized milk) for human health will be enormous.

Clearly, understanding MAP, how it interacts with the mammalian immune system is critical, both for ruminants and other mammals including humans. MAP is largely ubiquitous on farms and in the

environment, and thanks to widespread consumption of dairy milk, within the human population as well. As discussed above, gaining a better understanding of how Tregs and T cell unresponsiveness (amongst other factors) relate to Johne's disease is critical for future efforts to understand and control MAP infection and spread. Ultimately, the knowledge gained through this research will serve to help minimize the negative impact of MAP infection both economically and on quality of life. Altogether, based on the information reviewed in this chapter, we hypothesize that a population of regulatory T cells develops in response to persistent infection with MAP, subsequently limiting pro-inflammatory and Type 1 immune responses to MAP. Further, we propose that it is either the presence or absence of Tregs that leads to the progression of Johne's disease.

Chapter 2: Induction of regulatory T cell phenotype in naïve T cells

2.1 Rationale, significance, and methodology

For better or worse, regulatory T cells appear to be present in increased numbers in cows with Johne's disease when compared to healthy controls [75, 84, 89, 122]. Although some research suggests the subclinical stage of Johne's disease as the key period during which more Tregs are present (Figure 1) [75], most research does not pinpoint a specific stage of disease during which Tregs are more abundant or active [84, 89, 122]. There is mounting evidence that factors besides Treg activity are important in the immune response to MAP, including various degrees of T cell unresponsiveness, specifically including hyporesponsiveness [89] or anergy [51, 93]. However, due to the strong evidence of Treg activity in chronic disease, including in response to closely-related *Mycobacteria* including *M. tuberculosis* [81, 82] and *M. leprae* [80], there are ample reasons to investigate Treg activity in the context of MAP infection.

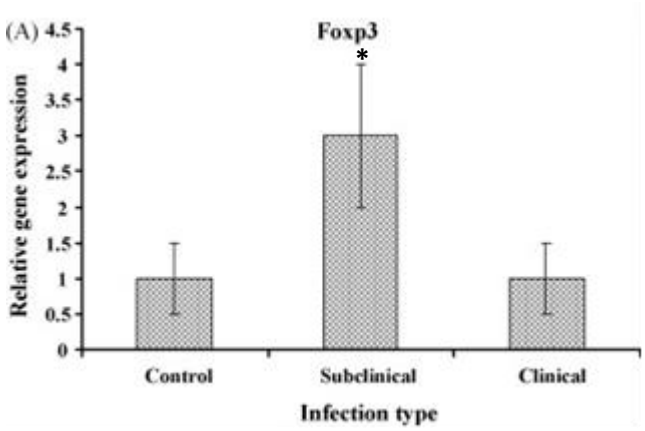


Figure 1: Relative FOXP3 mRNA abundance in ilea from cows in different stages of Johne's disease. Ilea from cows with subclinical disease show significantly greater expression of FOXP3 mRNA than ilea from cows with clinical disease or uninfected controls. *p < 0.05. Borrowed from *de Almeida, D.E., C.J. Colvin, and P.M. Coussens, Antigen-specific regulatory T cells in bovine paratuberculosis. Vet Immunol Immunopathol, 2008.* ***125****(3-4): p. 234-45. doi: 10.1016/j.vetimm.2008.05.019.*

Despite the previous work mentioned above, there are still significant gaps in research relating to Tregs and Johne's disease. Specifically, there is very little research into whether or not the presence

of MAP can induce the development of a Treg phenotype in naïve T cells. A key reason to suspect that this occurs is that it has been shown that a lack of adequate co-stimulation combined with chronic antigen stimulation can result in the development of a Treg phenotype in various other disease models [85-87]. Critically, the immune environment of Johne's disease contains both of these conditions: a decreased degree of CD40:CD40L interaction [88], as well as chronic stimulation of T cells with MAP antigens [89].

Additionally, although FOXP3 mRNA expression has been examined in ilea from cows in different stages of disease (Figure 1) [75], no work has been done specifically examining naïve T cell responses to Treg-inducing conditions, and it has not been done using cows within different stages of disease. The purpose of this study, then, was to investigate whether or not a Treg phenotype can be induced in naïve T cells from cows with both subclinical and clinical Johne's disease. If Tregs are identified as being functional in response to MAP antigens, it may provide a novel avenue on which future researchers can focus development of drugs and vaccines to help prevent, control, or eliminate Johne's disease. We hypothesized that $CD4^{+}CD25^{-}$ PBMCs from cows with subclinical disease would develop a Treg phenotype (considered herein as a significant increase in expression of *FOXP3* as well as increases in *IL10* and/or *TGFB1*) in response to culture with MAP-infected MDMs. Alternatively, the null hypothesis is that CD4+CD25- PBMCs from cows with subclinical disease do not develop a Treg phenotype in response to culture with MAP-infected MDMs.

Whereas most research to date has focused on a simple stimulation of blood, PBMCs, or lesion-derived lymphocytes with live MAP or MAP antigens, the critical feature of the system presented here is that macrophages are infected with MAP and subsequently present MAP antigens to $CD4^{+}CD25^{-}$ T cells in the context of the MHC II – antigen complex. This design is key to testing the interaction between $CD4^{+}CD25^{-}$ T cells and MAP-infected macrophages, as previous research strongly suggests this as necessary to the development of a Treg phenotype. The overall design was as follows: Initially, blood

was drawn from study animals. Following a three-day incubation to allow maturation of PBMCs into macrophages, macrophages were subsequently infected with nothing or with MAP for a total of 24 hours. After this, blood was drawn from the same animals a second time, PBMCs were once again isolated, and fluorescent cell sorting was utilized to select the $CD4^{+}CD25^{-}$ T cell population. These cells were then cultured separately with both the nil- and MAP-infected macrophages for 18 hours. Following this, non-adherent cells were collected and RNA was isolated for cDNA conversion and qPCR analysis.

2.2 Materials and methods

2.2.1 Animal cell donors and Johne's disease testing

Nineteen adult Holstein cows (age > 2 years) were used in the current study. The cows were housed and maintained in a typical commercial two-milking-per-day dairy operation. Johne's disease status was verified by serum ELISA (positive result indicated by OD ≥ 1.0) and fecal PCR using insertion sequence 900 (IS900). Diagnostic tests were performed by commercial testing firm Antel Biosystems, 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.

2.2.2 Isolation of peripheral blood mononuclear cells

PBMCs were isolated from blood samples aseptically drawn into acid-citrate-dextrose (ACD) Vacutainer Blood Collection Tubes (Becton Dickinson catalog #364606) by Percoll gradient density centrifugation (density =1.084 g/mL). Blood samples were centrifuged for 20 min at 2200 rpm at room temperature. Buffy coats were removed and added to 50-mL conical tubes containing 10 mL Percoll

overlayed with 20 mL phosphate buffered saline (PBS), followed by centrifugation at 1380 rpm for 41 min at room temperature. PBMCs were removed from the Percoll/PBS interface and washed twice with 50 mL PBS followed by centrifugation (5 min, 1800 rpm, room temperature). Cells were finally suspended in PBS at approximately $2x10^7$ PBMCs / mL for counting and use in subsequent experimentation.

2.2.3 Generation of monocyte-derived macrophages and MAP infection

Approximately $3.33x10^6$ PBMCs were plated per well in 24-well plates with Roswell Park Memorial Institute (RPMI) complete media (RPMI plus 10% fetal bovine serum, 1% penicillin/ streptomycin, and 1% fungizone, pH 7.4) and allowed to settle and adhere for 4 hours. Non-adherent cells were washed away with 3 washes of warm PBS, and adherent monocytes ($5x10^5$ per well on average) were allowed to differentiate into monocyte-derived macrophages (MDMs) for 3 days at 38°C with 5% CO_2, as described elsewhere [123]. Monocyte-derived macrophages were then infected with live MAP (American Type Culture Collection Strain #19698) at a multiplicity of infection (MOI) of 20 bacteria per macrophage. This strain of MAP has previously been shown to infect 40-80% of bovine MDMs [118, 124]. Further, this strain of MAP is known to be unaffected by penicillin and streptomycin (Sreevatsan S, personal communication), and as such antibiotics were not removed from cultures prior to infection. As an added benefit, this further minimized the risk of unwanted contamination. Infection was allowed to progress for 4 hours at which time supernatant was removed and MDMs were washed 3 times with warm PBS. Processing of phagocytosed MAP was allowed to proceed for an additional 20 hours.

2.2.4 Antibodies and cell sorting

$5x10^7$ PBMCs were labeled with mouse monoclonal antibodies raised against bovine CD4 (isotype IgG1, Washington State University monoclonal antibody center (WSUMAC) catalog #BOV2012, clone CACT138) and bovine CD25 (isotype IgG3, WSUMAC catalog #BOV2076, clone LCTB2A) at a dilution of 10 µg antibody per $5x10^7$ PBMCs in 500 µL staining buffer (PBS with 2% horse serum, 10% ACD, and 0.09% sodium azide) for 30 min at 4°C in the dark. 4 mL washing buffer (PBS with 10% ACD and 0.09% sodium azide) was added to each sample and centrifuged for 5 min at 1500 rpm and 4°C. Cells were then incubated with secondary antibodies raised against mouse IgG1 (goat anti-mouse, Tri-Color, Life Technologies SKU# M32006) and mouse IgG3 (goat anti-mouse, Alexa Fluor® 488, Life Technologies SKU# A-21151) for 30 min in 500 µL staining buffer at 4°C in the dark. 4mL washing buffer was added to each sample and centrifuged for 5 min at 1500 rpm and 4°C. Cells were diluted to a final concentration of $5x10^6$ PBMCs/mL in Hank's balanced salt solution (HBSS) and kept at 4°C in the dark until cells were sorted. Cell sorting was performed with a Becton Dickinson Influx Cell Sorter. Cells were first gated on lymphocytes, and within the lymphocyte population both $CD4^+CD25^-$ lymphocytes and $CD4^+CD25^+$ lymphocytes were collected in separate tubes. For each cow, $5x10^5$ $CD4^+CD25^-$ lymphocytes and $7.5x10^5$ $CD4^+CD25^+$ lymphocytes were collected for use in cell culture.

2.2.5 Identification of subclinical infected, T cell-responsive cows

Subclinical infected animals were diagnosed using a modified Bovigam assay in which *M. bovis* antigens are replaced with MAP antigens (purified protein derivative of Johnin; PPDj) and IFNG release in response to these antigens is measured by ELISA. To demonstrate responsiveness of cows' T cells to MAP antigens, an anti-IL10 neutralizing antibody (Thermo Scientific, clone CC320, catalog #MA5-16619) was used in decreasing concentrations in the modified Bovigam assay. In addition to nil negative control

stimulation, pokeweed mitogen (PWM) positive control stimulation, and MAP antigens alone, blood was incubated with MAP antigens and the anti-IL10 antibody at either 1:400, 1:2400, or 1:14,400 dilution.

2.2.6 $CD4^+CD25^-$ T cell stimulation

$CD4^+CD25^-$ lymphocytes ($2.4x10^5$) were co-cultured with 4-day-old nil- or MAP-infected MDMs in RPMI complete media for 18 h in 24-well plates at 38°C and 5% CO_2. Non-adherent cells were collected from the culture and plates were washed twice with warm PBS to remove remaining non-adherent cells. Non-adherent cells were then centrifuged for 5 min at 1800 rpm and 4°C, and RNA was isolated using a Qiagen RNeasy Plus Mini Kit (catalog #74134) according to manufacturer's instructions. RNA quality was assessed in all cases using an Agilent 2100 Bioanalyzer. A minimum RNA Integrity Number (RIN) of 7.5 was consistently observed and considered acceptable for downstream analyses.

2.2.7 cDNA conversion and quantitative real-time polymerase chain reaction

200 ng of RNA was reverse-transcribed into copy 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 (qPCR) was performed using Applied Biosystems 7000 and 7500 Real-Time PCR systems in duplicate 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 1.

2.2.8 qPCR data interpretation and statistical analysis

Each gene was calibrated against an internal control (*GAPDH*; primer validation performed prior to qPCR for genes of interest. Primer validation was performed by obtaining Ct values for different proposed control genes across all experimental conditions tested. No significant differences (n=3) between experimental conditions were seen in Ct values for GAPDH; as such this was chosen as an internal control), followed by comparing samples from test-positive cows against pooled averages from test-negative cows for each particular culture condition. The linear mixed model used in this study included fixed effects of Johne's disease status (uninfected, subclinical and clinical), presence or absence of MAP in macrophages, and their interaction, and random effects of cow nested within disease status level. This is equivalent to a split-plot design with disease status being the between plot factor and MAP infection being the within plot factor. The interaction test between disease status and MAP infection was performed first, and if it failed to reject the null hypothesis (i.e. no evidence of interaction) a main effect test of disease status or MAP infection was performed. Alternatively, if the interaction test was significant, simple effect comparisons of MAP within disease status was performed. This sequential procedure is typical of analysis of factorial experiments and type I error rate is controlled at the nominal level. Moreover, the correct elicitation of random effect and the use of the linear mixed model guarantees good statistical properties of all tests, as demonstrated elsewhere [125]. $p < 0.05$ was considered significant.

Primers used in quantitative reverse-transcriptase RT-PCR			
Gene name	**Forward primer**	**Reverse primer**	**Chapters Used**
CD4	ACGTCAAGAGCCTGTCACTG	TGCTGGCTCTGGGAGATAGT	3
CD8A	ACGTGTCTGCAAATGTCCCA	CTCTGAAAGGTTGGGCTTGC	3
CTLA4	CTGTGCTGGGACCTACATGG	TTCCTCTGGAGGTGCCAATG	1,2
FOXP3	CACAACCTGAGCCTGACAA	TCTTGCGGAACTCAAACTCATC	1,2,3
GAPDH	AGGAGCACGAGAGGAAGAGT	TTCTCAGTGTGGCGGAGATG	1,2
GATA3	AGGTACGTCCTGTGCAAACT	AGACAGGGTCTCCATTGGCA	1,2,3
GITR	CCTGTTTCCCGGAAACAAGAC	CCAGAGAGAGGATGACGATGGT	1,2
IFNG	TCTGGTTCTTATGGCCAGGG	GAAGAGAGGCCCACCCTTAG	1,2,3

Table 1: qPCR primers. GAPDH was used as an internal control in Chapters 1-2; TATA-binding protein (TBP) and peptidylprolyl isomerase A (PPIA) were used as internal controls in Chapter 3.

Table 1 (cont'd)

IL1A	TTGGTGCACATGGCAAGTG	GCACAGTCAAGGCTATTTTTCCA	1,2,3
IL10	GCTGTATCCACTTGCCAACC	ATCCAGCAGAGACTGGGTCA	1,2,3
IL12P40	CACCCCGCATTCCTACTTCT	TGGCATGTGACTTTGGCTGA	1,2
IL17A	CATCTCACAGCGAGCACAAG	CCACCAGACTCAGAAGCAGT	1,2,3
IL2	CAAGCTCTACGGGGAACACA	TAGCGTTAACCTTGGGCACG	1,2
IL23	AGCTCTCACAGCAACTCTGC	TGTCCCATTGGTAGGTGTGC	1,2
IL4	GGCGGACTTGACAGGAATCT	TTGTGCTCGTCTTGGCTTCA	1,2
IL6	GGCTCCCATGATTGTGGTAGTT	GCCCAGTGGACAGGTTTCTG	1,2,3
PFN1	CATGGACCACGGTCTCTTGAA	GGTGAGGCAAGCATTTGACC	1,2
PPIA	GCAAGCACGTGGTACTTTGG	TTGCTGGTCTTGCCATTCCT	3
RORC	GAGTTCGCTAAGAGGCTCCC	TCCATGGCTCCTGCTTTGAG	1,2
TBP	ACAGAGAACACCAGAGCGTC	CAGCACTGCCCGTATAGCA	3
TBX21	ACCACCTGTTGTGGTCCAAG	ATCCGGTAATGGCTGTTGGG	1,2,3
TCD	ACAAGCAAACCTGGCACTCT	TGCCATCGGTTTTGGAGTTG	3
TGFB1	CGAGCCCTGGACACCAACTAC	CCGGAAGTCAATGTAGAGCTGA	1,2,3
TNFA	TCTACCAGGGAGGAGTCTTCCA	GTCCGGCAGGTTGATCTCA	1,2,3
WC1	CACTTCGGAGCAGGATCAGG	GGTGGGACTCCTTTCCTGTG	3

2.3 Results

2.3.1 Identification of cows with MAP-responsive T cells

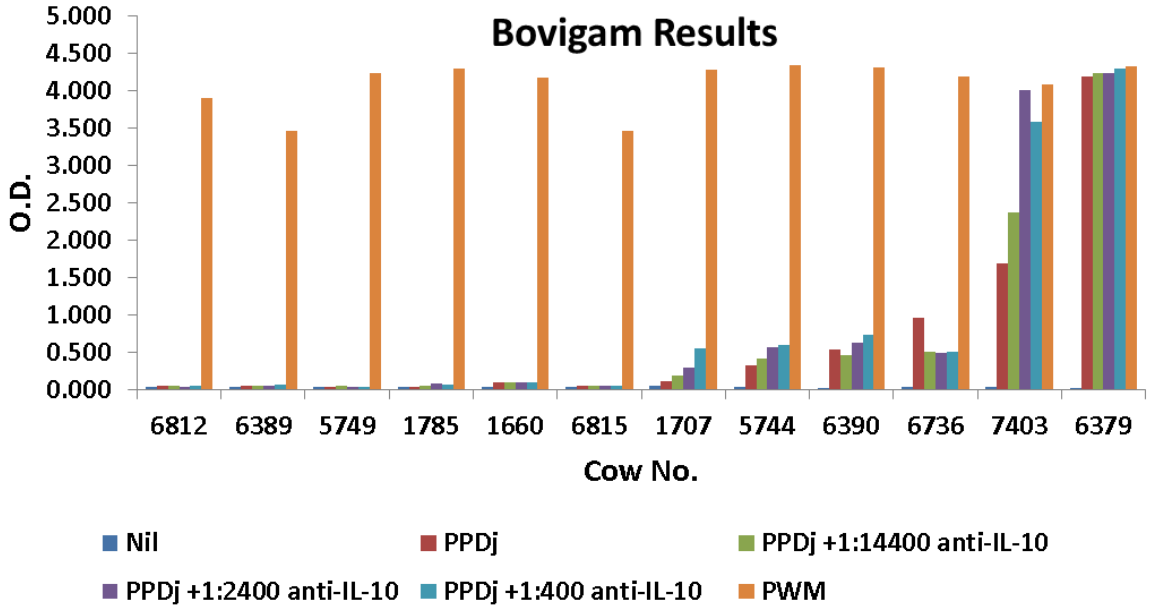


Figure 2: Modified Bovigam results. All twelve cows tested originally tested positive for Johne's disease by both Johne's ELISA and fecal PCR. Of those twelve cows, six showed some response to MAP antigens; five cows showed a dose-dependent response. The six remaining cows showed no discernible response to MAP antigens. An optical density (O.D.) > 0.250 was considered positive.

Twelve cows were identified as being infected with MAP as measured by Johne's fecal PCR and Johne's ELISA, as described above. Blood from these twelve cows was stimulated with nil stimulation, MAP antigens, and an anti-IL10 neutralizing antibody in varying concentrations (Figure 2). Of the twelve cows tested, five registered a positive test result (O.D. > 0.250) when stimulated with PPDj alone. As IL10 has been shown to inhibit PBMC responses to MAP antigens [75, 83], indeed the addition of anti-IL10 neutralizing antibody revealed one additional cow with T cells responsive to MAP antigens. Of the six responsive cows, five showed a dose-dependent response to the anti-IL10 neutralizing antibody, suggesting that IL10 is present in the macrophages of some cows with Johne's disease, and that it is inhibiting Th1 immune responses to MAP antigens. Significantly, the other six cows tested showed no response to MAP antigens under any experimental condition tested, suggesting that Th1 cells from another, distinct group of cows with Johne's disease are unresponsive to MAP antigens. Cows whose blood showed no response to MAP antigens, but who were ELISA and fecal positive, were considered to

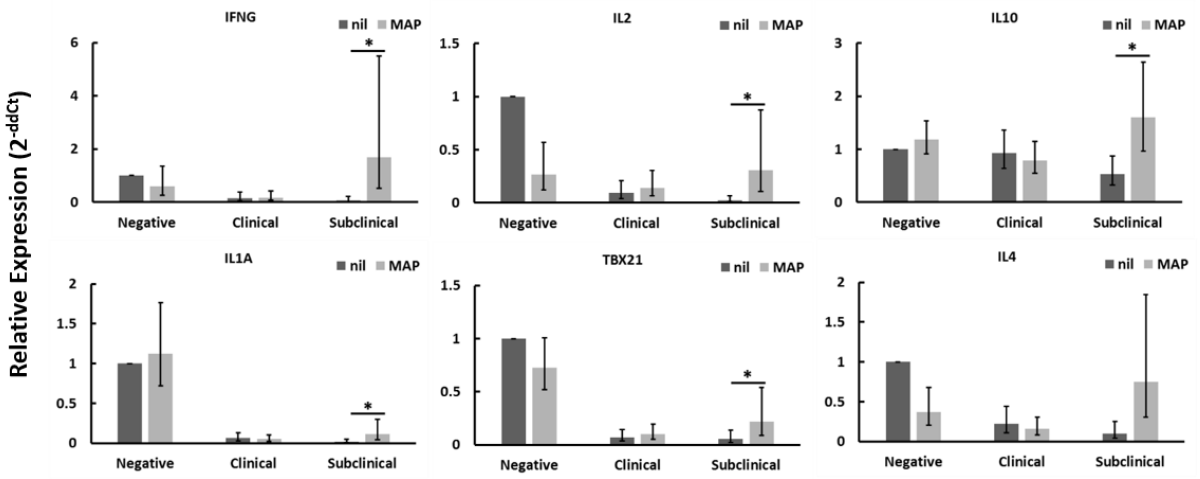


Figure 3: Relative mRNA abundance of select genes in naïve T cells cultured with nil- or MAP-infected MDMs. Naïve ($CD4^+CD25^-$) PBMCs were cultured with 4-day old MDMs that were either nil- or MAP-infected. Non-adherent cells were collected following 18-hour incubation. Subsequent RNA extraction and qPCR results are shown. Subclinical infected animals showed up-regulation of a variety of immune genes including those encoding both Th1 (*IFNG*, *IL1A*, *TBX21*) and Th2 (*IL10*, *IL4*) cytokines, whereas clinical infected animals showed no significant changes. n=3-8/group, *p<0.05. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

have clinical Johne's disease and those whose blood showed antigen-specific responses to MAP antigens were considered to have subclinical disease.

2.3.2 $CD4^+CD25^-$ T cells from cows with subclinical disease respond to MAP-infected macrophages

Naïve ($CD4^+CD25^-$) T cells from Johne's test-negative control cows showed a significant reduction in *TGFB1* mRNA expression when cultured with MAP-infected MDMs (data not shown) as compared to nil-infected MDMs, but no other significant changes in expression of genes tested were seen. When cultured in contact with MAP-infected MDMs, naïve T cells from cows with clinical Johne's disease did not exhibit significant changes in mRNA expression of any gene tested, relative to cells in contact with nil-infected MDMs.

Of the three groups of cows studied, only naïve T cells from subclinical Johne's cows showed substantial responses to MAP-infected MDMs when compared to responses to nil-infected (uninfected) MDMs. There was a significant increase in mRNAs encoding *IFNG*, *IL2*, *IL10*, *IL1A*, and *TBX21* (Figure 3), with a near-significant increase seen in *IL4* (Figure 3) and *TNFA* (data not shown) mRNAs. Of these seven genes, four (*IFNG*, *IL1A*, *TBX21*, *TNFA*) are characteristic of a Th1 response and two are characteristic of a Th2 response (*IL4*, *IL10*). These data suggest that naïve $CD4^+CD25^-$ T cells from cows with subclinical Johne's disease respond to MAP antigen presentation on MDMs with a primarily Th1-biased phenotype. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

2.3.3 $CD4^+CD25^-$ T cells from cows with clinical disease are unresponsive and those from cows with subclinical disease show signs of reduced responsiveness

When disregarding nil or MAP infection of autologous MDMs, the relative mRNA abundance of

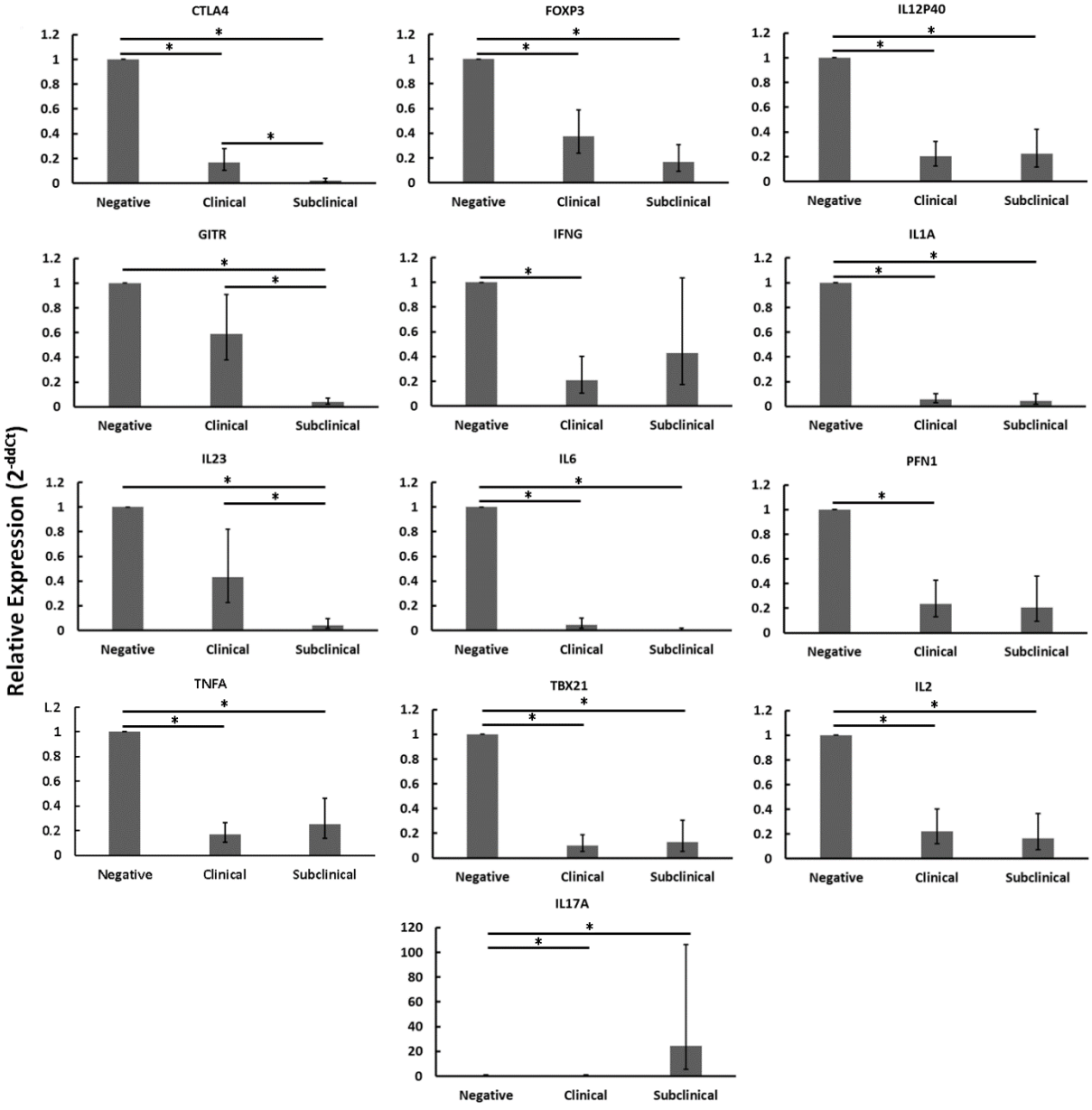


Figure 4: Relative mRNA abundance of select genes in naïve T cells cultured with nil- or MAP-infected MDMs, disregarding infection status of MDMs (nil- and MAP-infected MDMs were pooled in this analysis). Naïve ($CD4^+CD25^-$) PBMCs were cultured with 4-day old MDMs that were either nil- or MAP-infected. Non-adherent cells were collected following 18-hour incubation. Subsequent RNA extraction and qPCR results are shown. Both clinical and subclinical infected animals showed either unchanged expression (data not shown) or down-regulation of a variety of immune genes including those encoding Th1, Th2, and Treg proteins. n=3-8/group, *p<0.05. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

numerous immune genes was significantly reduced in $CD4^+CD25^-$ T cells from cows with clinical disease as compared to healthy controls (Figure 4: *CTLA4*, *FOXP3*, *IFNG*, *IL12P40*, *IL1A*, *IL2*, *IL6*, *PFN1*, *TBX21*, *TNFA*). Similarly, the relative mRNA abundance of several genes was significantly reduced in $CD4^+CD25^-$ T cells of cows with subclinical disease as compared to healthy controls (Figure 4: *CTLA4*, *FOXP3*, *GITR*, *IL12P40*, *IL1A*, *IL2*, *IL23*, *IL6*, *TBX21*, *TNFA*). In both cases, down-regulated genes represented a mix of immune phenotypes. While the mRNA encoding the hallmark Th17 cytokine, IL17, was significantly up-regulated in $CD4^+CD25^-$ T cells from cows with subclinical disease as compared to both negative control cows and cows with clinical disease, no other genes were significantly up-regulated in either of the groups with Johne's disease as compared to negative controls. When combined with the generally unchanged expression of most mRNAs studied in $CD4^+CD25^-$ T cells from cows with clinical disease stimulated with MAP-infected MDMs as compared to nil-infected MDMs, it appears probable that $CD4^+CD25^-$ T cells from cows with clinical disease are unresponsive. The fact that mRNA abundance of nearly all genes studied was reduced in $CD4^+CD25^-$ T cells from cows with subclinical disease as compared to healthy controls, combined with the significant increases in various responses to MAP-infected MDMs as compared to nil-infected MDMs in these same animals, suggests that cells from cows with subclinical disease, while still responsive to MAP antigens, are substantially less active than T cells from healthy control cows. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

2.4 Discussion

Although most evidence suggests that Tregs do not play a role in establishing an infection with MAP in cows [126], numerous studies have suggested Tregs as a likely effector of the progression of Johne's disease [75, 84, 89], possibly playing a key role in facilitating or triggering the shift between Th1

and Th2 immune responses seen during the course of Johne's disease [75, 84]. Research has shown that both ineffective co-stimulation and chronic antigen stimulation are conditions encountered during the course of Johne's disease [35, 88], and both of these conditions can promote the development of Tregs [85-87]. It has also been shown that removal of either $CD4^+$ or $CD25^+$ PBMC populations from MAP-infected cows prior to MAP antigen stimulation results in a decrease in *IL10* mRNA expression and an increase in *IFNG* mRNA expression. Taken together, these data suggest that a population of Tregs may be present and responsive to MAP antigens in cows with Johne's disease.

The design of this experiment was critical to testing the hypothesis that $CD4^+CD25^-$ T cells from cows with subclinical disease do or do not develop a Treg phenotype when brought into contact with autologous MAP-infected macrophages. We anticipated this might happen based on the idea that Tregs develop during subclinical disease, limit Th1 effector immune responses to MAP, and promote the transition to a Th2 immune response and ultimately progression of Johne's disease. As $CD4^+$ T cells are traditional αβ T cells, they are MHC II restricted and as such the natural host of MAP, the macrophage, was utilized to present MAP antigens on MHC II molecules to peripheral $CD4^+CD25^-$ T cells. Ultimately, we rejected our hypothesis and the null hypothesis was accepted – CD4+CD25- PBMCs from subclinical infected cows did not develop a Treg phenotype in response to culture with MAP-infected MDMs. Additionally, we observed unexpected results. When $CD4^+CD25^-$ T cells from cows with both clinical and subclinical disease were brought into contact with MDMs, expression of most genes was significantly reduced as compared to values seen in cells from healthy controls (when disregarding MAP infection status of macrophages), with the exception of *IL17A*, which was expressed significantly more in $CD4^+CD25^-$ T cells from cows with subclinical disease (Figure 4).

Of equal interest, then, was the observation that $CD4^+CD25^-$ T cells from cows with clinical disease did not significantly respond to MAP-infected macrophages for any gene tested, when compared to nil-infected controls. $CD4^+CD25^-$ T cells from cows with subclinical disease did respond

significantly to MAP-infected MDMs, despite having overall reduced expression of most genes tested compared to uninfected control cows. In fact, these cells responded in a mixed Th1/Th2 manner, with mRNAs encoding the Th1-oriented *IFNG*, *IL1A*, and *TBX21* being significantly upregulated, and mRNAs encoding the Th2-oriented *IL10* and *IL4* being upregulated. Curiously, with the exception of an up-regulation of *IL10* mRNA (which can be either or both Th2 and/or Treg derived), there was no substantial evidence to suggest the induction of a Treg phenotype for any research group studied.

Altogether, our data suggests that $CD4^+CD25^-$ T cells from cows with clinical disease are unresponsive, as they show significantly reduced mRNA expression of all genes studied. $CD4^+CD25^-$ T cells from cows with subclinical respond to MAP antigens in a mixed Th1/Th2 manner, although they too show generally reduced expression of all immune genes studied (exception: *IL17A*) when compared to $CD4^+CD25^-$ T cells from healthy controls. Taken together, this data suggests that $CD4^+CD25^-$ T cells from cows with clinical disease are unresponsive, and that $CD4^+CD25^-$ T cells from cows with subclinical disease show signs of significant hyporesponsiveness. In regard to our original hypothesis, this work suggests that the development of T cell unresponsiveness, rather than the development of a MAP antigen-reactive Treg population, may be the driving factor in the progression of Johne's disease from subclinical to clinical disease. It is also important to note that definitively proving the notion of unresponsiveness is difficult. Reduced gene expression can be due to a variety of reasons; unresponsiveness to antigenic stimulation is only one such possible reason. As such, while it is readily possible to observe responses to stimulation, the absence of a response is not necessarily indicative of unresponsiveness generally. One possible alternative is that the cells being studied do in fact respond, but not via the expression of the chosen genes studied. Of the thousands of genes present within every cell, we only studied the expression of 18. Altogether, we cannot definitively claim to have observed unresponsiveness, but we believe that there is strong evidence of this phenomenon based on the selection of highly-relevant immune genes investigated in this study.

Finally, the fact that PBMCs from five of the six cows showing a response to MAP antigens did so in an anti-IL10 dose-dependent manner suggests that there is a population of cells dampening Th1 immune responses to MAP within the peripheral blood of cows with subclinical disease. As our data demonstrates that there is no real evidence for the induction of a Treg phenotype in $CD4^{+}CD25^{-}$ T cells from cows with subclinical disease, future work should investigate whether other cell types or cytokines are necessary for the differentiation of Tregs. As we know from previous work [84] that there are more Tregs in the periphery of cows with Johne's disease as compared to healthy controls, it is likely that additional factor are required to drive their differentiation.

Future research should also see these experiments repeated, but with an effective positive control, such as PWM, to determine whether the different cell populations respond to any other forms of antigenic stimulation. Additionally, it will be important to determine the precise nature of the observed T cell unresponsiveness, as it may be a more specific form of unresponsiveness, such as T cell exhaustion or T cell anergy. Portions adapted from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

Chapter 3: Expansion of regulatory T cells and their effects on perpipheral blood mononuclear cell responses to MAP stimulation

3.1 Rationale, significance, and methodology

Although there is sufficient evidence established to implicate regulatory T cells in Johne's disease [75, 84, 89, 122], little work has been done from a functional perspective. In one functional study, addition of a neutralizing anti-IL10 antibody caused a significant increase in IFNG protein levels in whole blood from cows with subclinical Johne's disease following stimulation with purified protein derivative of johnin (PPDj), in a dose-dependent fashion [83]. This effect was not seen in blood from uninfected control cows. This data suggests that there is a Th1 MAP-antigen reactive population of cells within the periphery of cows with subclinical Johne's disease, and that the response of these cells is being suppressed by IL10. The question of what cell population is producing this IL10 is pertinent; fortunately, another study has investigated this and narrowed down candidate cell types [75]. In a cell depletion experiment, various cell populations were removed from whole PBMC populations from cows with Johne's disease, prior to stimulation with MAP antigens. As shown in Figure 5, the removal of either the $CD25^+$ or $CD4^+$ cell populations resulted in a significant decrease in *IL10* mRNA expression and a significant increase in *IFNG* mRNA expression. This suggests that a cell population expressing CD4 and CD25 is functional in response to MAP antigens, and that it suppresses IFNG expression perhaps through the production of IL10. Importantly, Tregs constitutively express both CD25 and CD4, implicating Tregs as one possible culprit for the observed changes. Although that study does effectively eliminate some cell populations, including γδ T cells, $CD8^+$ T cells, and monocytes, as being the likely cells responsible for the effects seen, it does not distinguish between different $CD4^+CD25^+$ cell populations. Thus, additional investigation is needed to determine if the effects seen are Treg-specific.

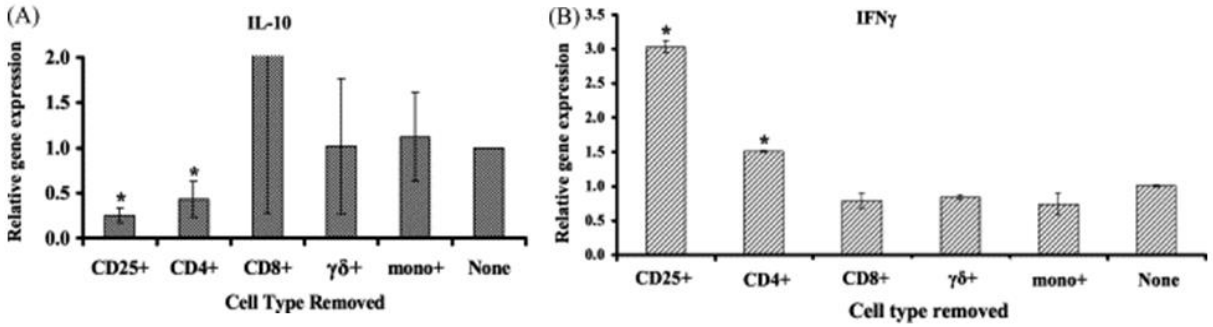


Figure 5: Q-RT-PCR analysis of IL10 mRNA and IFNG mRNA abundance when particular cell types are removed from total PBMCs (none) prior to stimulation with MAP-antigens. Cells were removed by magnetic cell sorting using specific antibodies to cell surface antigens. Remaining cells were stimulated with MAP-antigens for 6 h, collected in Trizol and processed as described [1]. Data shows that removal of $CD4^+$ or $CD25^+$ cells prior to stimulation reduces *IL10* mRNA expression and increases *IFNG* mRNA expression significantly (*$p < 0.05$). Borrowed from *de Almeida, D.E., C.J. Colvin, and P.M. Coussens, Antigen-specific regulatory T cells in bovine paratuberculosis. Vet Immunol Immunopathol, 2008.* ***125****(3-4): p. 234-45. doi: 10.1016/j.vetimm.2008.05.019.*

In any investigation into Treg activity, one major obstacle is that Tregs represent a very small percentage of immune cells; in the case of the bovine periphery, this has frequently been observed to be less than 1% of PBMCs (unpublished results). Further, within the context of the entire Treg population, only a small fraction of the Tregs would likely be reactive to any one particular antigen or microbe, such as MAP. On the other hand, Tregs not reactive to MAP may still play a role as most induced Tregs function in a manner that is not antigen-reactive and may exert their effects simply due to proximity to an infection site. Taken together, these two considerations result in a very simple problem: the proportion of Tregs within a PBMC population is so small that any Treg-specific effects within an experimental system would likely not be seen, as they may be washed out due the large number of other immune cells present (including nonspecific Tregs), some of which would also likely be MAP-reactive (within cows with Johne's disease). To mitigate these issues, expanding the relative abundance of Tregs within a PBMC population is prudent. The ability to expand Tregs in humans is well-documented [127-130], but to our knowledge has not been attempted in the bovine system.

Considering the above, a major goal of this study was to develop a method to expand the relative abundance of Tregs in bovine PBMC populations. Upon successful establishment of this system, the expanded Tregs could be used in add-back experiments in which their ability to affect autologous PBMC responses to stimulation with live MAP can be monitored. The overall design of the study was to first expand Tregs by drawing blood, isolating PBMCs and subsequently generating MDMs and infecting them with MAP. Following this, blood was drawn a second time from the same animals, PBMCs were isolated, and fluorescent cell sorting was used to isolate $CD4^{+}CD25^{+}$ PBMCs. These cells were subsequently cultured for 8 days with the MAP-infected macrophages in a culture containing an enrichment cocktail consisting of IL2, TGFB1, and rapamycin. Following the expansion of Tregs, blood was drawn a third time, PBMCs were isolated, and subsequently cultured with or without live MAP and with or without expanded autologous Tregs. Altogether, we hypothesized that Tregs would limit Th1 effector immune responses to MAP in PBMCs from MAP-infected cows. The null hypothesis is that Tregs do not limit Th1 effector immune responses to MAP in PBMCs from MAP-infected cows.

3.2 Materials and methods

3.2.1 Animal cell donors and Johne's disease testing

Nineteen adult Holstein cows (age > 2 years) were used in the current study. The cows were housed and maintained in a typical commercial two-milking-per-day dairy operation. Johne's disease status was verified by serum ELISA (positive result indicated by OD ≥ 1.0) and fecal PCR using insertion sequence 900 (IS900). Diagnostic tests were performed by commercial testing firm Antel Biosystems, 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. Subclinical infected animals were diagnosed using a modified Bovigam assay in which *M. bovis* antigens are replaced with MAP antigens (purified

protein derivative of johnin: PPDj; National Animal Disease Center, Ames, Iowa) and IFNG release in response to these antigens is measured by ELISA. All protocols were reviewed and approved by the Michigan State University Animal Use and Care Committee.

3.2.2 Isolation of peripheral blood mononuclear cells

PBMCs were isolated from blood samples aseptically drawn into acid-citrate-dextrose (ACD) Vacutainer Blood Collection Tubes (Becton Dickinson catalog #364606) by Percoll gradient density centrifugation (density =1.084 g/mL). Blood samples were centrifuged for 20 min at 2200 rpm at room temperature. Buffy coats were removed and added to 50-mL conical tubes containing 10 mL Percoll overlayed with 20 mL phosphate buffered saline (PBS), followed by centrifugation at 1380 rpm for 41 min at room temperature. PBMCs were removed from the Percoll/PBS interface and washed twice with 50 mL PBS followed by centrifugation (5 min, 1800 rpm, room temperature). Cells were finally suspended in PBS at approximately $2x10^7$ PBMCs / mL for counting and use in subsequent experimentation.

3.2.3 Generation of monocyte-derived macrophages and MAP infection

Approximately $3.33x10^6$ PBMCs were plated per well in 24-well plates with Roswell Park Memorial Institute (RPMI) complete media (RPMI plus 10% fetal bovine serum, 1% penicillin/ streptomycin, and 1% fungizone, pH 7.4) and allowed to settle and adhere for 4 hours. Non-adherent cells were washed away with 3 washes of warm PBS, and adherent monocytes ($5x10^5$ per well on average) were allowed to differentiate into monocyte-derived macrophages (MDMs) for 3 days at 38°C with 5% CO_2, as described previously [123]. Monocyte-derived macrophages were then infected with

live MAP (American Type Culture Collection Strain #19698) at a multiplicity of infection (MOI) of 20 bacteria per macrophage. This strain of MAP has previously been shown to infect 40-80% of bovine MDMs [118, 124]. Further, this strain of MAP is known to be unaffected by penicillin and streptomycin (Sreevatsan S, personal communication), and as such antibiotics were not removed from cultures prior to infection. As an added benefit, this further minimized the risk of unwanted contamination. Infection was allowed to progress for 4 hours at which time supernatant was removed and MDMs were washed 3 times with warm PBS. Processing of phagocytosed MAP was allowed to proceed for an additional 20 hours.

3.2.4 Antibodies and cell sorting

$5x10^7$ PBMCs were labeled with mouse monoclonal antibodies raised against bovine CD4 (isotype IgG1, Washington State University monoclonal antibody center (WSUMAC) catalog #BOV2012, clone CACT138) and bovine CD25 (isotype IgG3, WSUMAC catalog #BOV2076, clone LCTB2A) at a dilution of 10 µg antibody per $5x10^7$ PBMCs in 500 µL staining buffer (PBS with 2% horse serum, 10% ACD, and 0.09% sodium azide) for 30 min at 4°C in the dark. 4 mL washing buffer (PBS with 10% ACD and 0.09% sodium azide) was added to each sample and centrifuged for 5 min at 1500 rpm and 4°C. Cells were then incubated with secondary antibodies raised against mouse IgG1 (goat anti-mouse, Tri-Color, Life Technologies SKU# M32006) and mouse IgG3 (goat anti-mouse, Alexa Fluor® 488, Life Technologies SKU# A-21151) for 30 min in 500 µL staining buffer at 4°C in the dark. 4mL washing buffer was added to each sample and centrifuged for 5 min at 1500 rpm and 4°C. Cells were diluted to a final concentration of $5x10^6$ PBMCs/mL in Hank's balanced salt solution (HBSS) and kept at 4°C in the dark until cells were sorted. Cell sorting was performed with a Becton Dickinson Influx Cell Sorter. Cells were first gated on lymphocytes, and within the lymphocyte population both $CD4^+CD25^-$ lymphocytes and $CD4^+CD25^+$ lymphocytes were collected in separate tubes. For each cow, $5x10^5$ $CD4^+CD25^-$ lymphocytes and $7.5x10^5$

$CD4^+CD25^+$ lymphocytes were collected for use in cell culture. A representative flow cytometry cell sorting scatter plot is shown in Figure 6.

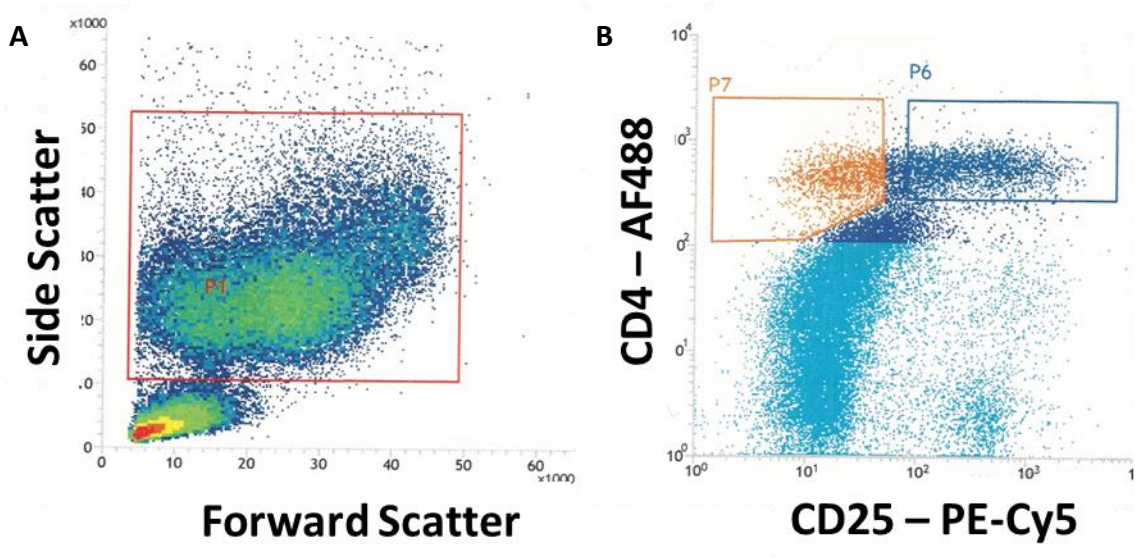


Figure 6: Representative flow cytometry cell sorting scatter plot. Based on forward scatter (size) and side scatter (granularity), debris and polymorphonuclear leukocytes (PML) were excluded while peripheral blood mononuclear cells were selected using a Becton Dickinson Influx Cell Sorter (A). CD4+CD25+ PBMCs (gate "P6") were then selected based on fluorescent antibody labeling (B).

3.2.5 $CD4^+CD25^+$ T cell enrichment

$3x10^5$ $CD4^+CD25^+$ lymphocytes were cultured with 4-day-old MDMs that had been infected with MAP for 24 hours in RPMI complete media plus enrichment cocktail (1 nM rapamycin (Sigma-Aldrich catalog #R03950-1MG), 5 ng/mL recombinant bovine interleukin 2 (Kingfisher Biotech, inc. catalog #RP0026B-005), and 2.5 ng/mL purified human transforming growth factor beta 1 (BD Biosciences catalog #354039)) for 8 days at 38°C and 5% CO_2, with enriched media being refreshed every 72 h. The procedure is diagrammed in Figure 7.

3.2.6 Co-culture of enriched $CD4^+CD25^+$ lymphocytes with autologous PBMCs and live MAP

Autologous PBMCs were freshly isolated and $7.5x10^5$ PBMCs were combined with or without live MAP (MOI=2.5) and with or without $3.75x10^5$ enriched $CD4^+CD25^+$ T cells. Cultures incubated for 18 hours at 38°C and 5% CO_2 at which point they were collected and RNA was isolated using a Qiagen RNeasy Plus Mini Kit (catalog #74134) according to manufacturer's instructions. RNA quality was assessed in all cases using an Agilent 2100 Bioanalyzer. A minimum RNA Integrity Number (RIN) of 7.5 was consistently observed and considered acceptable for downstream analyses.

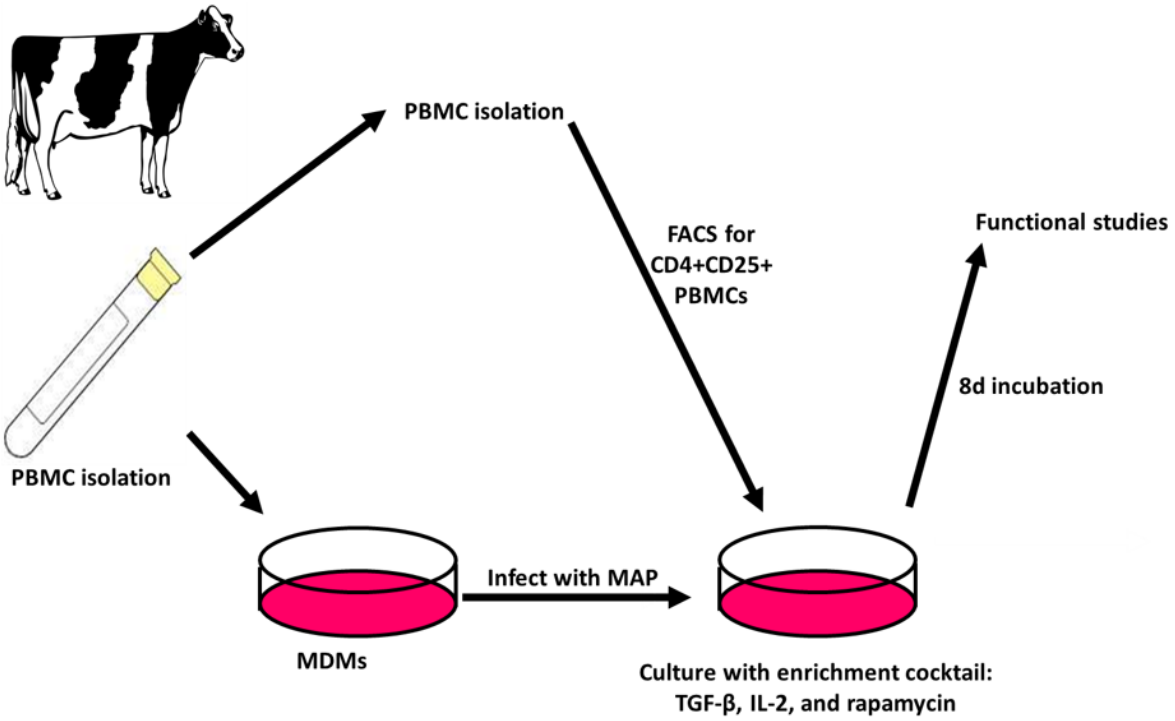


Figure 7: $CD4^+CD25^+$ lymphocyte expansion. PBMCs are isolated and used to generate MDMs by allowing adherence of monocytes to culture dishes followed by washing away of non-adherent cells. The adherent monocytes were allowed to mature into MDMs for 4 days followed by 24 hour infection with MAP. After 3 days, autologous PBMCs are again isolated and FACS is used to select $CD4^+CD25^+$ lymphocytes. The cells are then combined with MAP-infected MDMs and cultured for 8 days with an enrichment cocktail, after which time the expanded $CD4^+CD25^+$ lymphocytes are ready for use in functional studies. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

3.2.7 Flow cytometry and data analysis

Peripheral blood mononuclear cells were labeled with anti-CD4 (WSUMAB catalog #BOV2012) and anti-CD25 (WSUMAB catalog #BOV2076) primary antibodies and subsequently labeled with Tri-Color (Life Technologies SKU #M32006) and Alexa Fluor® 488 (Life Technologies SKU #A-21151) as described previously. The eBiosciences FOXP3 fixation/permeabilization buffer was used to permeabilize cells according to manufacturer's instructions, followed by 45 min incubation at 4°C in the dark with eBiosciences anti-mouse FOXP3-RPE antibody (clone FJK-16s). Rat IgG2a-RPE isotype was used as a control. Using a BD FACSCalibur Flow Cytometer, 50,000 events were collected and analyzed by using a subsequent gating strategy targeting lymphocytes, followed by $CD4^+$ lymphocytes, then $CD25^{hi}$ $CD4^+$ lymphocytes, and finally $FOXP3^+CD25^{hi}CD4^+$ lymphocytes. These cells were then compared to the total number of lymphocytes present to determine the relative abundance of Tregs within a population.

3.2.8 cDNA conversion and quantitative real-time polymerase chain reaction

200 ng of RNA was reverse-transcribed into copy 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 (qPCR) was performed using Applied Biosystems 7000 and 7500 Real-Time PCR systems in duplicate 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 1.

3.2.9 qPCR data interpretation and statistical analysis

Each gene was calibrated against an internal control (*GAPDH*; primer validation performed prior to qPCR for genes of interest. Primer validation was performed by obtaining Ct values for different proposed control genes across all experimental conditions tested. No significant differences (n=3)

between experimental conditions were seen in Ct values for *GAPDH*; as such this was chosen as an internal control), followed by comparing samples from test-positive cows against pooled averages from test-negative cows for each particular culture condition. The mixed model analysis used in this study included fixed effects of Johne's disease status, presence or absence of live MAP bacteria, and presence or absence of expanded autologous Tregs as well as all possible two-way and three-way interactions, and random effects of cow within disease status. For the statistical testing, we followed a similar

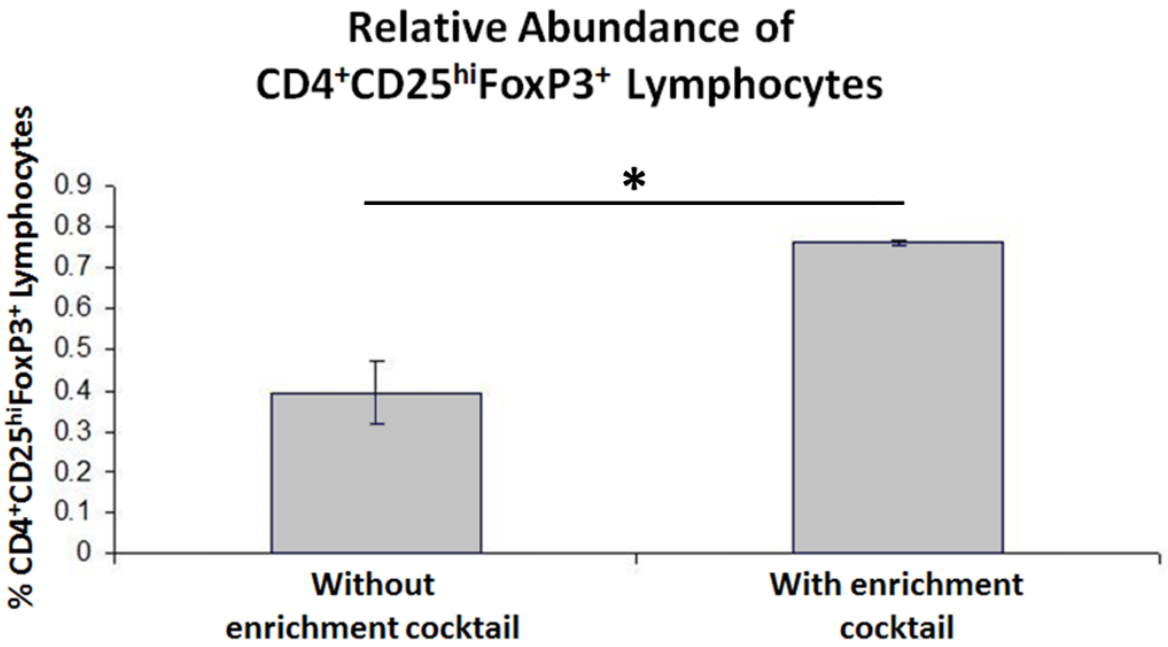


Figure 8: Results of CD4⁺CD25⁺ lymphocyte expansion. Three-color flow cytometry was used to measure relative Treg abundance. A successive gating strategy was used to select CD4⁺CD25^{hi}FOXP3⁺ lymphocytes. n=4, *p<0.05. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

hierarchical test as described in Chapter 2: the three way interaction was tested first, followed by lower order interactions and main effects, as long as the higher order interaction was not significant. Specifying the correct random effects and performing the described hierarchical testing provided appropriate control of false positives as demonstrated elsewhere [125]. $p < 0.05$ was considered significant. The mixed model analysis used in this study included fixed effects of Johne's disease status, presence or absence of live MAP bacteria, and presence or absence of expanded autologous Tregs as

well as all possible two-way and three-way interactions, and random effects of cow within disease status. For the statistical testing, we followed a similar hierarchical test as described in Chapter 2: the three way interaction was tested first, followed by lower order interactions and main effects, as long as the higher order interaction was not significant. Specifying the correct random effects and performing the described hierarchical testing provided appropriate control of false positives as demonstrated elsewhere [125]. $p < 0.05$ was considered significant.

3.3 Results

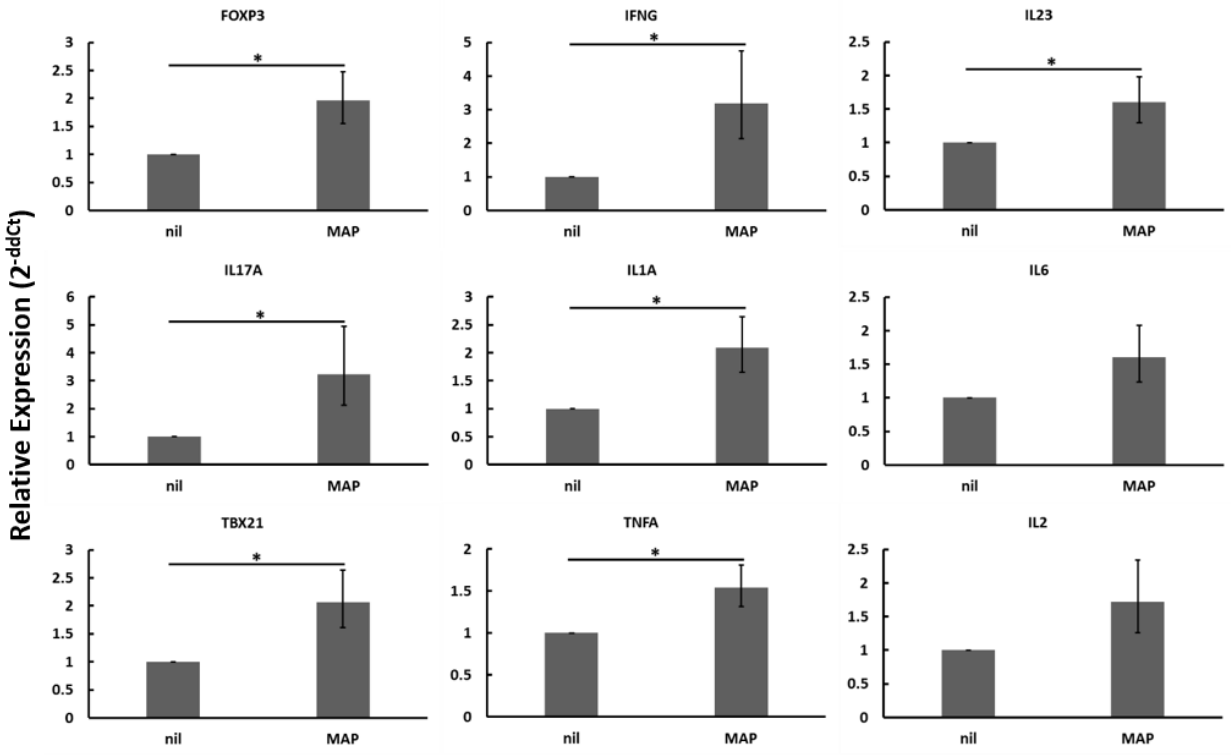


Figure 9: Effects of MAP on pooled PBMCs. When disregarding Johne's test status or the addition of expanded Tregs (these differing culture conditions were pooled in this analysis), the addition of live MAP resulted in an increase in the relative mRNA abundance of several genes including the Treg-associated FOXP3. mRNAs encoding numerous Th1- (*IFNG*, *IL1A*, *TBX21*, *TNFA*) and Th17-associated (*IL23*, *IL17A*, *IL6*) proteins were significantly or near-significantly up-regulated. n=3-8/group, *p<0.05. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

3.3.1 Expansion of $CD4^+CD25^+$ T cell populations results in an increase in relative Treg abundance in peripheral blood T cell populations from cows with Johne's disease

When the Treg-containing fraction of PBMCs ($CD4^+CD25^+$ lymphocytes) from cows with Johne's disease (serum ELISA-positive) was cultured with MAP-infected MDMs in the presence of the Treg stimulation cocktail (RPMI complete media supplemented with IL2, TGFB1, and rapamycin; Figure 7), the relative abundance of Tregs increased significantly, as compared to the same culture conditions without the stimulation cocktail present (Figure 8). Tregs were defined as $CD4^+CD25^{hi}FOXP3^+$ lymphocytes. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

3.3.2 MAP Stimulation results in a Th1 response by PBMCs

Following Treg expansion (Figure 7), autologous PBMCs from negative control animals and animals in different stages of Johne's disease were cultured with or without expanded Tregs and with or

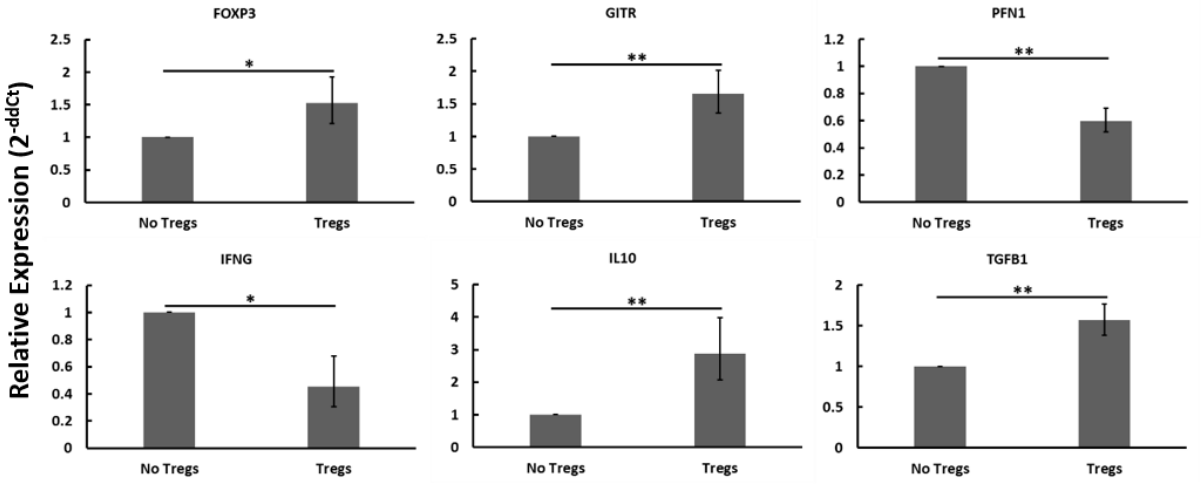


Figure 10: Relative mRNA abundance of select genes considering the effect of adding expanded Tregs to heterogeneous PBMC cell cultures. When disregarding Johne's test status and nil v MAP stimulation (these conditions were pooled in this analysis), the addition of expanded Tregs resulted in a significant increase in mRNA expression of several Treg-associated genes including *FOXP3*, *GITR*, *IL10*, and *TGFB1*. It also resulted in a significant decrease in two Th1 cytokines, *IFNG* and *PFN1*. n=3-8/group, *p<0.05. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi:*

without live MAP. While we hoped to see Treg-mediated suppression of PBMC responses to MAP as compared to nil stimulation within either the subclinical or clinical infected cow populations, no significant three-way interactions were observed (data not shown). When disregarding Johne's test status and presence or absence of Tregs, live MAP stimulation resulted in up-regulation of mRNA encoding many genes in the PBMC populations (Figure 9). Overall, up-regulation of both a Th1-like response (*IFNG*, *IL1A*, *TBX21*, *TNFA*) and a Th17-like response (*IL17A*, *IL23*, *IL6*) was observed; the Th1-like response would be anticipated in negative control cows and cows with subclinical Johne's disease. It is possible that an unresponsive state of PBMCs from cows with clinical disease resulted in an overall lack of response that may have otherwise dampened the observed Th1- and Th17-like responses upon pooling the test groups as done in the analysis represented in Figure 9. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

3.3.3 Expanded Tregs may not be MAP-reactive

Autologous PBMCs were cultured with or without expanded Tregs and with or without live MAP. When disregarding Johne's test status and the presence or absence of live MAP, the addition of Tregs resulted in notable changes to the gene expression profile that are consistent with Treg function and activity (Figure 10). Namely, the relative abundance of mRNAs encoding Treg-associated genes (*FOXP3*, *GITR*, *IL10*, *TGFB1*) all increased, with only *FOXP3* not reaching significance (p=0.0764). Further, and critical to the argument in favor of the functionality of the expanded Tregs, the relative mRNA abundance of Th1-associated factors *PFN1* (which encodes perforin, a protein key to cytotoxic T cell cytolytic function) and *IFNG* decreased when Tregs were added to the cultures. Due to the apparent presence and activity of the expanded Tregs, while considering the lack of significant interaction when specifically examining different test statuses or nil versus MAP stimulation, it seems probable that the

expanded Tregs may not all be MAP-reactive, despite the intentions of the experimental design of the Treg enrichment procedure. Borrowed from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

3.4 Discussion

The initial goal of this study was to expand the relative abundance of regulatory T cells within a PBMC population. Specifically, we hoped to expand the relative abundance of MAP antigen-reactive Tregs, although an expansion of non-MAP-reactive Tregs was a possibility as well. In the future, an additional experimental group is needed to determine whether or not MAP-infected MDMs are necessary to expand the Treg population. Our results suggest that these cells were unnecessary, but this is speculative in the absence of another study group lacking MAP-infected MDMs. As Tregs are typically anergic [131-133], devising a means by which Tregs can be made to proliferate was critical. Our assay created two generally favorable conditions for Treg development, reduced co-stimulation between T cells and macrophages due to MAP infection of macrophages [88] and chronic antigen stimulation [89]; both of these factors are known to create an environment conducive to the development of Tregs [85-87]. Second, and most critically, the assay was developed to induce proliferation of the normally-anergic Tregs. This was accomplished by adapting a system shown to greatly expand relative Treg abundance in human cord $CD4^+$ cells [127]. As shown in Figure 7, this process involved the addition of three exogenous factors (IL2, TGFB1, and rapamycin) to an 8-day cell culture. This assay successfully expanded the relative abundance of Tregs within a $CD4^+CD25^+$ T cell population from cows with Johne's disease, as shown in Figure 8. Due to the design of this Treg expansion assay, we anticipated that the expanded Tregs may be MAP antigen-reactive. This in turn led us to use the expanded Tregs in functional assays.

PBMCs were taken from three different groups of cows (healthy controls, subclinically infected and clinically infected) and cultured them with or without expanded Tregs and with or without live MAP.

Initially we anticipated that the addition of autologous Tregs would suppress PBMC-derived Th1 immune responses to MAP stimulation in PBMCs from cows with clinical Johne's disease. Unexpectedly, we observed no such suppression, although in retrospect these results are in line with results seen in Chapter 2. In that study, it was found that $CD4^{+}CD25^{-}$ T cells from cows with clinical disease are unresponsive; therefore, it is likely that Tregs taken from these same animals would not suppress immune responses to MAP. Within cells from clinical animals, expanded Tregs failed to inhibit any immune responses to MAP (data not shown).

Results were more surprising in the group of subclinically-infected animals. In Chapter 2, we showed that there were small but significant responses to MAP antigens in $CD4^{+}CD25^{-}$ PBMCs. Based on this, in retrospect we would anticipate finding Treg-mediated suppression of PBMC responses to MAP stimulation in PBMCs taken from subclinically-infected animals. However, similar to what was seen in cells from our clinically-infected animals, we did not see any evidence of Treg-mediated suppression to MAP stimulation in PBMCs from cows with subclinical Johne's disease. One possible reason for the lack of response within this group was the small sample size (n = 3). Altogether, no significant 3-way interactions (interactions comparing Johne's disease status, presence or absence of autologous expanded Tregs, and presence or absence of live Tregs) were observed for any gene studied; a larger sample size may be necessary to reach significance in such an experiment when working with an outbred species. Due to this lack of significance, the data was re-analyzed by collapsing the smaller data sets into larger, more generic groups, whereupon significance was observed in many instances. This again suggests that larger sample sizes may be beneficial for reaching significance with increasingly specific study conditions.

When looking at one-way interactions (in which we examined e.g. nil vs. MAP stimulation, completely disregarding Johne's disease test status and presence or absence of Tregs), significance was observed in many instances. When considering only nil vs. MAP stimulation, in particular, evidence suggested that altogether bovine PBMCs tend to respond to live MAP stimulation with a mixed Th1/Th17 phenotype. We observed significant increases in the mRNA abundance of Th1-associated genes including *IFNG*, *IL1A*, *TBX21*, and *TNFA*, and significant increases in the mRNA abundance of Th17-associated genes including *IL6*, *IL17A*, and *IL23* (Figure 9). Altogether this data suggests that PBMCs from cows, disregarding Johne's disease test status, respond to MAP in a Th1/Th17 manner. Although the Th1 response is widely recognized as the appropriate response for controlling MAP infection [1, 69, 134], Th17 responses have been reported in several instances as well [135, 136], although this may be indicative of an inappropriate immune response. Of particular interest to the study of Tregs in the context of Johne's disease is the fact that *FOXP3* mRNA abundance increased significantly in PBMCs stimulated with MAP as compared to those challenged with nil stimulation, possibly suggesting a role for MAP in inducing expression of *FOXP3* mRNA in bovine PBMCs.

Some of the most interesting results were seen when we compared the presence of expanded regulatory T cells to the absence of expanded Tregs, when disregarding Johne's disease status or nil or MAP stimulation. Under these circumstances, we found that several Treg-associated genes (*FOXP3*, *GITR*, *IL10*, and *TGFB1*) were up-regulated when expanded Tregs were added to autologous PBMC cultures, compared to PBMCs alone. On the one hand, at a basic level this was expected and confirms our flow cytometric findings (Figure 8) that the Treg expansion assay did indeed expand relative Treg abundance, it also suggests that the expanded Tregs may not be MAP-reactive as originally anticipated. Intriguingly, whereas *FOXP3* mRNA was up-regulated both when PBMCs were stimulated with MAP and when expanded Tregs were added to PBMC cultures, *IL10* and *TGFB1* mRNA was only up-regulated when expanded Tregs were added to the cultures. A possible reason for this is that MAP may be

important in inducing FOXP3 expression and subsequent generation of Tregs, but that these Tregs subsequently do not specifically respond to MAP via production of IL10 or TGFB1. Most strongly suggesting that MAP is not important in the expansion of specifically MAP-reactive Tregs is the fact that had MAP been important in this process, then there would not have been up-regulation of Treg-associated genes in cultures containing cells from cows without Johne's disease and that this up-regulation would have only been seen in cultures from cows with Johne's disease. In fact, however, we only observed significant increases in Treg-associated genes when data from all three disease status groups were pooled together. Altogether, our data suggests that our Treg expansion procedure did not expand MAP antigen-reactive Tregs specifically, but rather it is possible that the stimulation cocktail alone was sufficient to induce the expansion of non-MAP-reactive Tregs in bovine $CD4^{+}CD25^{+}$ T cell populations. It has been shown that memory Tregs proliferate less well [137] and therefore in this context it is not entirely surprising that a MAP-reactive suppressive effect was not observed. Although this study does not strongly implicate Tregs in the progression Johne's disease, additional work performed at the site of infection (the ileum) will be more telling about the true nature of Treg function in the context of Johne's disease. Altogether, we cannot effectively accept or reject either our hypothesis or our null hypothesis. Based on the results we found, we surmise that additional controls are necessary to more effectively test our hypotheses. In particular, a lack of MAP-infected macrophages (or macrophages altogether) would serve to determine if presentation of MAP antigens makes any difference in development of Tregs. A pokeweed mitogen control would also be of great benefit as it would demonstrate whether or not Tregs are expanded in the presence of mitogen, generally. In addition, greater animal numbers are needed to increase the power of the study and subsequently determine if Johne's disease test status and presence or absence of live MAP does in fact affect the ability of Tregs to mediate PBMC responses to MAP stimulation.

A significant silver lining found within the results of this study is that our data suggests that although expanded Tregs may not be MAP-reactive, they do appear to be functional regardless of the presence or absence of MAP. When considering only the presence or absence of expanded Tregs, we observed a decrease in the relative mRNA abundance of two critical Th1-related genes, *IFNG* and *PFN1*, in the presence of expanded Tregs. It is well-known that Tregs exert immunosuppressive effects on Th1 cytokine expression [73, 74], and based on this information we feel that the data presented here demonstrates that the expanded Tregs from this study are both non-antigen-reactive and are functional. Although these expanded Tregs appear to be constitutively functional *in vitro*, whether or not they require antigen stimulation *in vivo* has yet to be investigated. Additional studies should be performed with the aim of increasing sample size in an effort to reach significance within the specific research groups. Additionally, the method shown to expand regulatory T cell abundance in this study should be investigated further, with a possible aim of using them in an *in vivo* model of MAP infection. Most importantly of all, it will be critical to examine the abundance and functionality of Tregs within ileal and mesenteric lymph node lesions caused by MAP infection. If Tregs are found, it will be critical to determine what if any function they serve within lesions. Adapted from *Roussey, JA, Steibel JP, and Coussens PM (2014) Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis. Front. Vet. Sci. 1:20. doi: 10.3389/fvets.2014.00020.*

Chapter 4: Regulatory T cells and immune function in tissues from MAP-infected cows

4.1 Rationale, significance, and methodology

In the previous two chapters, our studies focused on peripheral regulatory T cells and peripheral T cells generally. Although a great deal of insight was gleaned into peripheral T cell activity and responses to stimulation with MAP or MAP-infected macrophages, the most effective understanding of disease generally occurs through the detailed study of active sites of MAP infection. In the case of Johne's disease, this means the primary site of infection, the ileum of the small intestine, and draining mesenteric lymph nodes, which are the secondary sites of infection. In this chapter, we discuss a detailed study of tissue lesions within these organs taken from cows with clinical Johne's disease of varying severity, as well as from healthy control cows.

Upon progression of clinical disease, infected animals exhibit characteristic symptoms of Johne's disease including increasingly severe diarrhea, progressive emaciation, and premature death [2]. At the organ level, progressively worsening inflammation is seen at sites of infection, particularly within the ileum [2, 138, 139]. Within infected tissues, the presence of large numbers of infected macrophages leads to widespread inflammation, possibly caused by production of IL1 by macrophages in regions of infection [123, 140]. As has been discussed in previous chapters, there is substantial evidence suggesting a possible role for regulatory T cells in the progression of Johne's disease. As shown in Figure 1, a significantly increased abundance of *FOXP3* mRNA is seen in ilea from cows with subclinical Johne's disease, as compared to both healthy controls cows and cows with clinical disease [75]. Infection with *M. tuberculosis* causes a disease with similar pathology to Johne's disease (tuberculosis), and an increased abundance of Tregs has also been shown in the lungs of human tuberculosis patients [141, 142]. These studies and others provide evidence suggesting a possible role for Tregs in the progression of Johne's disease at the site of active infection.

There are two distinct possibilities regarding what role Tregs may play in disease progression. The first possibility, which was originally suspected upon initiation of this research project, is that regulatory T cells develop during the course of Johne's disease (due to Treg-inducing conditions that exist during Johne's disease, as discussed in previous chapters [85-89]) and function to limit effector T cell responses to MAP. In this scenario, Tregs inhibit appropriate Th1-type immune responses necessary to control the infection and ultimately lead to increased dissemination of MAP within the host and widespread disease. A second possibility is that Tregs are beneficial to controlling damage to the host. During subclinical disease, Tregs may serve to limit unchecked inflammation that occurs in response to chronic MAP infection within ileal tissues. Over time, the Treg response may wane and allow the inflammatory response to predominate. In this situation, it is possible that Tregs are necessary to maintain homeostasis and thus the loss of Tregs or Treg activity would accompany development of clinical disease (due to the now-unchecked inflammation). Therefore, in this scenario a lack of Treg abundance and/or function in animals with severe disease would be anticipated. Recent work from our group has shown strong evidence for T cell unresponsiveness in PBMC populations from cows with clinical disease [94]. Other groups have provided evidence suggesting T cell hyporesponsiveness [89] or even anergy [93] in cows with clinical Johne's disease. Thus, it is possible that Treg unresponsiveness, rather than Treg activity, is at least in part responsible for the progression from subclinical to clinical Johne's disease.

It is important to point out that samples were taken at numerous locations within each tissue examined, to increase our ability to examine differences in lesions both between different cows and between different lesions within each cow. Tissues were collected from cows with Johne's disease and from healthy control cows. Specifically, for each cow, samples were taken from the terminal 40 cm of the ileum as well as from several ileum-associated mesenteric lymph nodes. Samples were taken in

triplicate from each location. One sample was processed for lesion grading analysis, one for immunofluorescence, and one for RNA extraction.

Altogether, this study serves to investigate the relationship between regulatory T cell presence or absence, and activity or inactivity, in tissue lesions caused by MAP infection. Specifically, it is of great importance to gain insights into whether Tregs are helpful or harmful in regard to Johne's disease. Tregs may develop during subclinical disease, inhibit Th1 immune responses necessary to control MAP infection, and subsequently result in widespread dissemination of MAP and progression to clinical disease. On the other hand, Treg activity may be necessary to limit chronic inflammation characteristic of Johne's disease, and over time Tregs become unresponsive and fail to effectively control the inflammation. This study aims to determine which of these two scenarios is more likely through a comprehensive classification of lesions in cows with Johne's disease, characterization of Tregs in tissue lesions caused by MAP infection, and investigation of the possibility of T cell unresponsiveness in MAP-infected tissues. Within this context, relationships between lesion severity, bacterial burden, and Treg abundance will be investigated. As an added layer of analysis, the expression of several key immune genes will be studied within these lesions. Considering these points, we hypothesized that there were two key possibilities for the role of regulatory T cells in the progression of Johne's disease in the context of 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 maintaining 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 are playing a role in the progression of disease; the null hypothesis is that Tregs do not play a role in the progression of Johne's disease.

4.2 Materials and methods

4.2.1 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 ≥ 1.0) and fecal PCR using insertion sequence 900 (IS900). Diagnostic tests were performed by commercial testing firm Antel Biosystems, 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.

4.2.2 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 N_2. These samples were stored at -80°C until used in downstream immunofluorescence applications. The third replicates were flash frozen in liquid N_2 directly for use in RNA extraction, cDNA conversion, and quantitative real-time polymerase chain reaction (qPCR).

4.2.3 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 2. Representative images for the H&E grading scheme are shown in Figure 11, and representative images for the AFB grading scheme are shown in Figure 12.

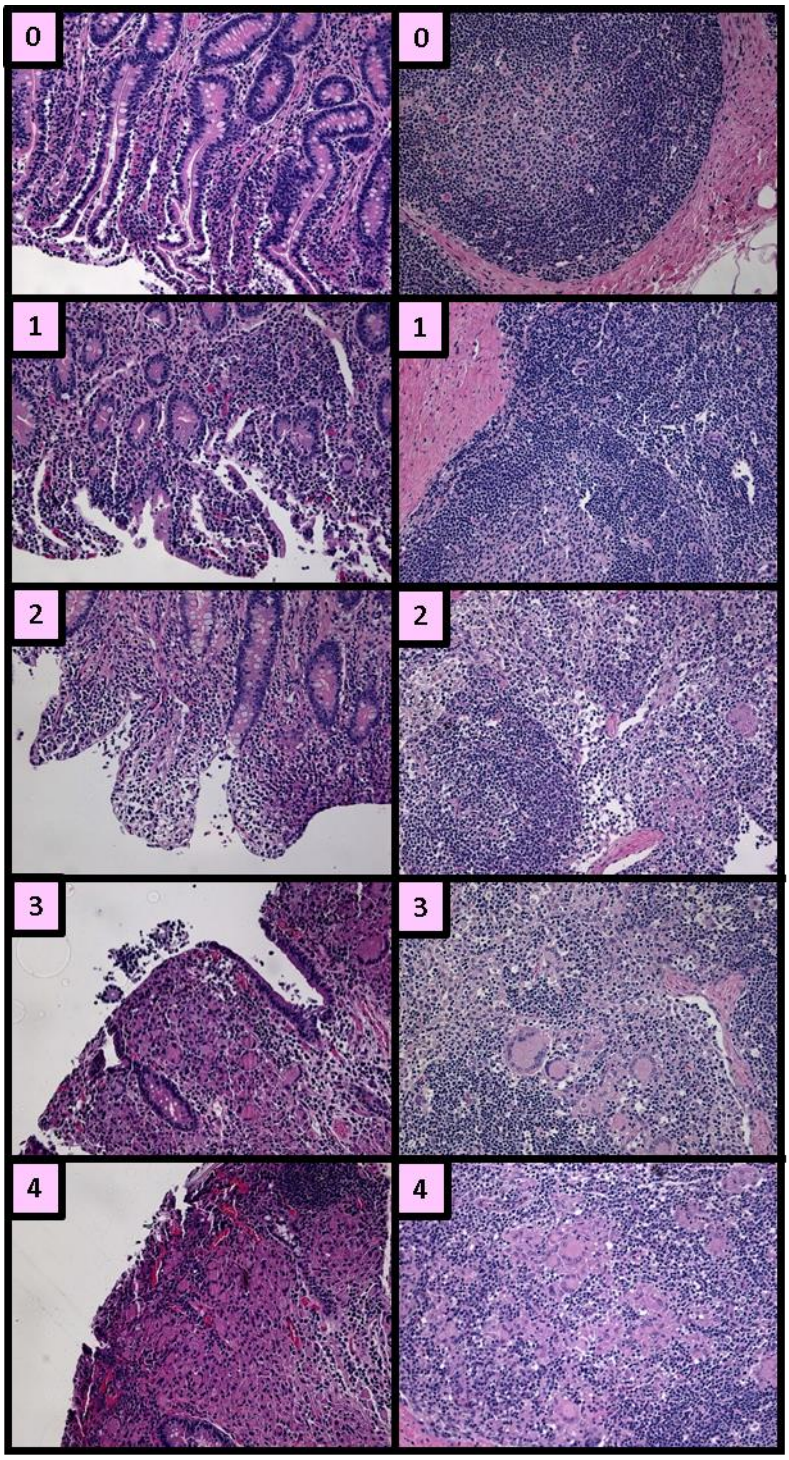


Figure 11: Lesion classification system (H&E). Hematoxylin and eosin staining was used to visualize tissue histology for ileal (left) and mesenteric lymph (right) tissues. Images are arranged from top to bottom, demonstrating progressively increasing lesion severity ranging from grade 0 (top) to grade 4 (bottom). Lesion severity was assessed based on overall tissue architecture, relative amount of inflammatory cell infiltrate, and distribution of inflammatory infiltrate.

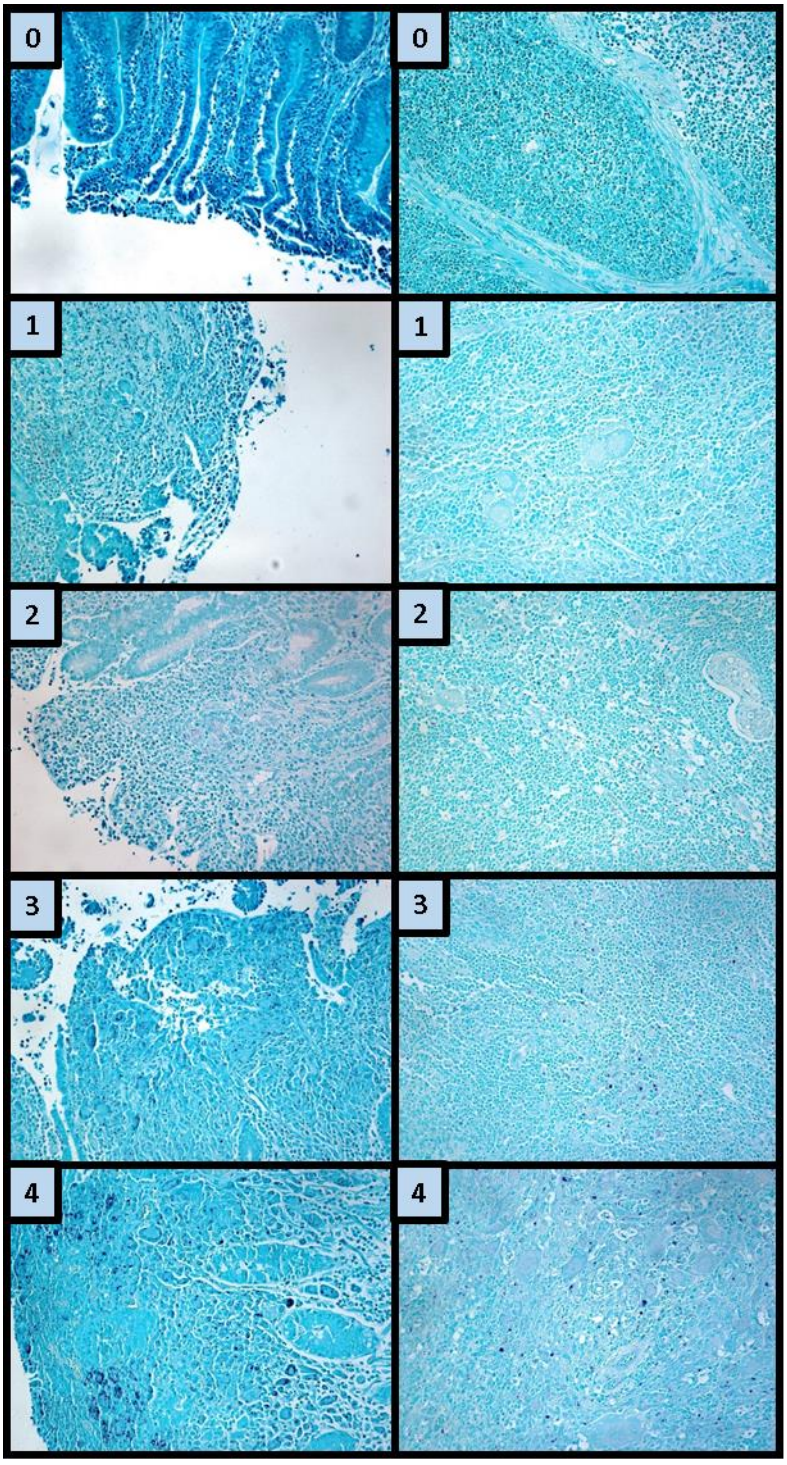


Figure 12: Lesion classification system (AFB). Acid-fast bacilli staining was used to assess relative MAP burden within ileal (left) and mesenteric lymph (right) tissues. Images are arranged from top to bottom, demonstrating increasingly greater MAP burden ranging from grade 0 (top) to grade 4 (bottom). 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).

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.	Typically localized to paracortex and/or medullary areas.	Minor disruption of tissue architecture possibly including 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 lamina propria.	As above but more widespread.	Expansion of villus lamina propria.	Mild expansion of medulla and paracortex.	Occasional macrophages contain AFB, typically 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. Extensively-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.

Table 2: 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.

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 with results from grade 1 lesions caused by MAP infection in the cows with Johne's disease. 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 2 for details on how scores were assessed for each individual category.

4.2.4 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.

4.2.5 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 1.

4.2.6 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 [143]. 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. Homogeneity of variance was assessed prior to t-test

analysis; t-testing was performed accordingly. An unpaired one-way student's t-test was used to assess significance; $p < 0.05$ was considered significant.

4.3 Results

4.3.1 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 3. For control cows, 25 of 40 ileal samples were considered to have a histological grade 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 grade 0 ileal sections were used in qPCR analysis; the 25 grade 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 grade of 1 from control cows and were excluded from subsequent analysis in the same manner.

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.

Lesion Grading 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 3: 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 grades 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.

Of the 100 ileal samples taken from cows with Johne's disease, 93/100 samples received a histological grade of 2 or greater, suggesting significant histological changes as a result of infection with MAP. The presence of MAP bacilli was found in all samples, both from ilea and mesenteric lymph nodes, from cows with Johne's disease. As detailed in Table 2, 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. These 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 grade of 2.2. Late clinical animals had mostly grade 3 and 4 lesions (a combined 49 of 50 samples) for an average grade 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 grade of 2.79. Late clinical animals had mostly grade 4 lesions (a combined 13 of 14 samples) for an average grade of 3.92.

For AFB scoring, within the ilea, early clinical animals had mostly grade 1 and 2 bacterial burdens (a combined 40 of 50 samples) for an average grade of 1.72. Late clinical animals had mostly grade 3 and 4 bacterial burdens (a combined 47 of 50 samples) for an average grade 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 grade of 1.29. Late clinical animals had a wide distribution of bacterial burdens for an average grade of 2.67 (Figure 13).

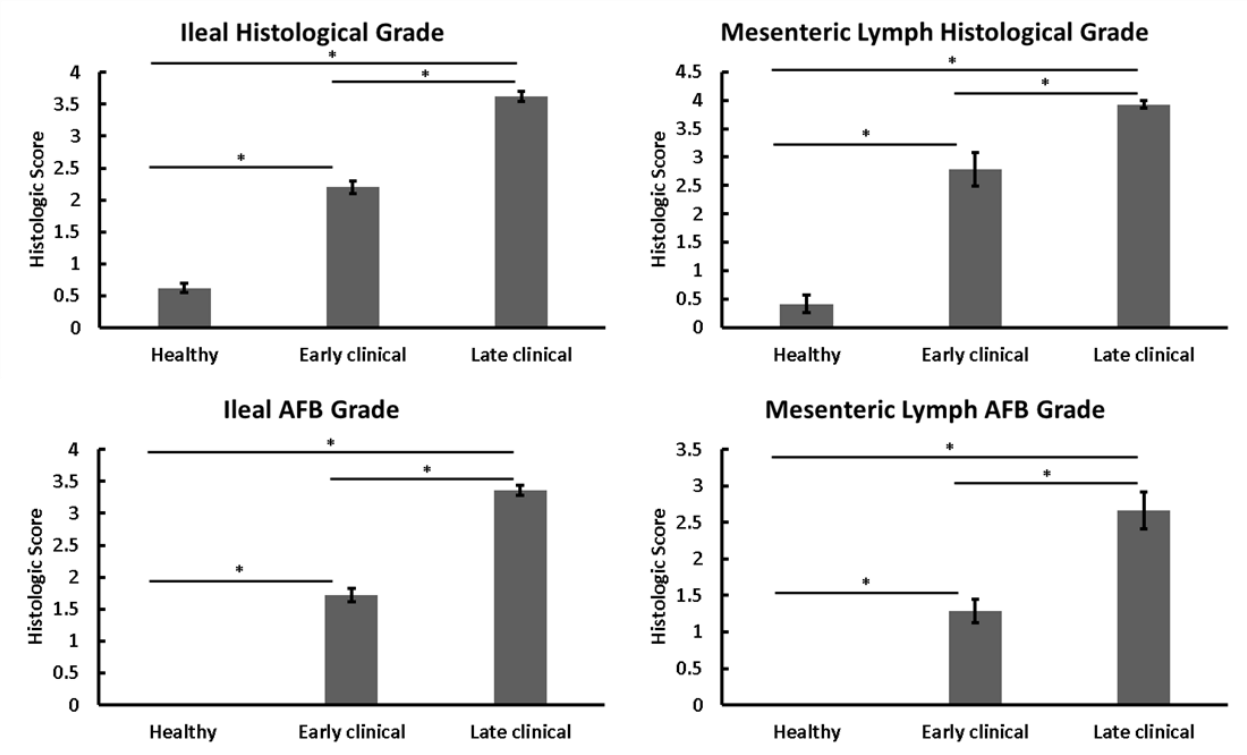


Figure 13: Lesion Classification System Grade Summary. Animals were divided into category based on being negative for Johne's disease (healthy), testing positive for clinical disease but not showing major disease symptoms (early clinical), or testing positive for clinical disease and showing significant symptoms of end-stage Johne's disease (late clinical).

Within the ileum, there was a strong correlation ($r^2 = 0.7971$) between H&E grade and bacterial burden (Figure 14). 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 13).

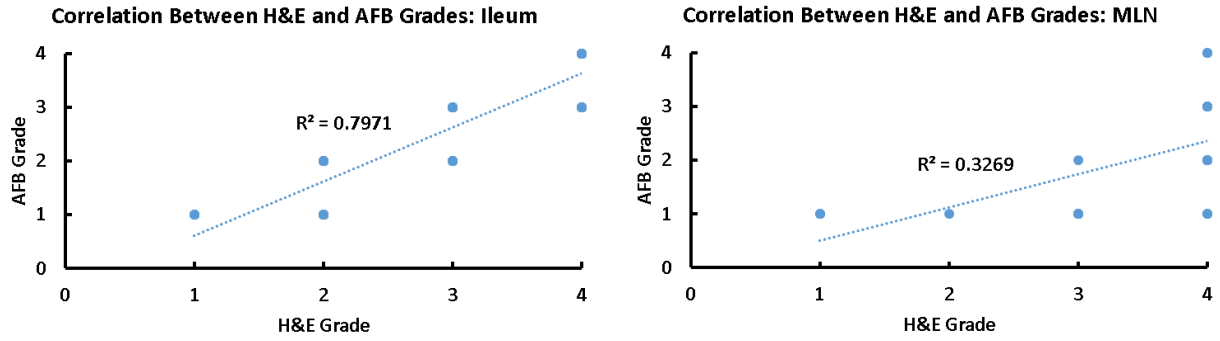


Figure 14: H&E and AFB Score Correlation. For cows with Johne's disease, H&E and AFB grades were paired and the coefficients of determination were calculated. A strong correlation was seen in ileal grades ($r^2 = 0.7971$). A weak correlation was seen in mesenteric lymph node grades ($r^2 = 0.3269$).

4.3.2 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 15, and quantified results are shown in Figure 16. 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 *TGFB1* was found in grade 4 lesions (Figure 17), when compared to tissues from healthy control cows, demonstrating a decrease in regulatory/Th2 gene expression in the most severe ileal lesions.

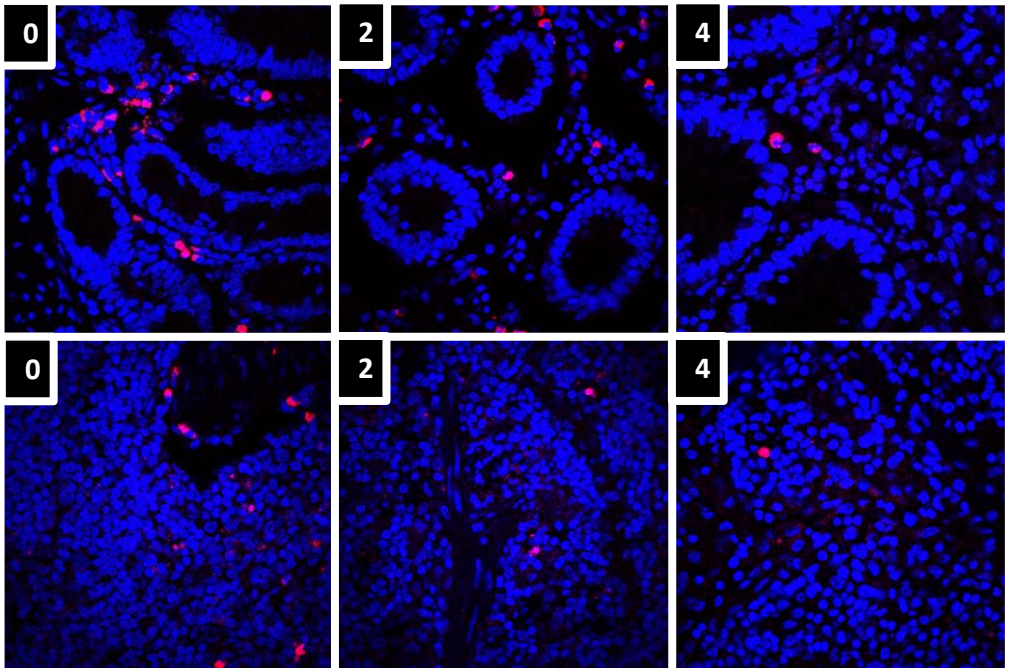


Figure 15: Regulatory T cell abundance representative images. Regulatory T cell abundance was measured by confocal microscopy, with FOXP3+ cells stained red against a blue nuclear counterstain. In both the ileum and the mesenteric lymph nodes, Treg abundance tended to decrease with increasing lesion severity. (Top) Representative Treg abundance in the ileum. (Bottom) Representative Treg abundance in the mesenteric lymph node.

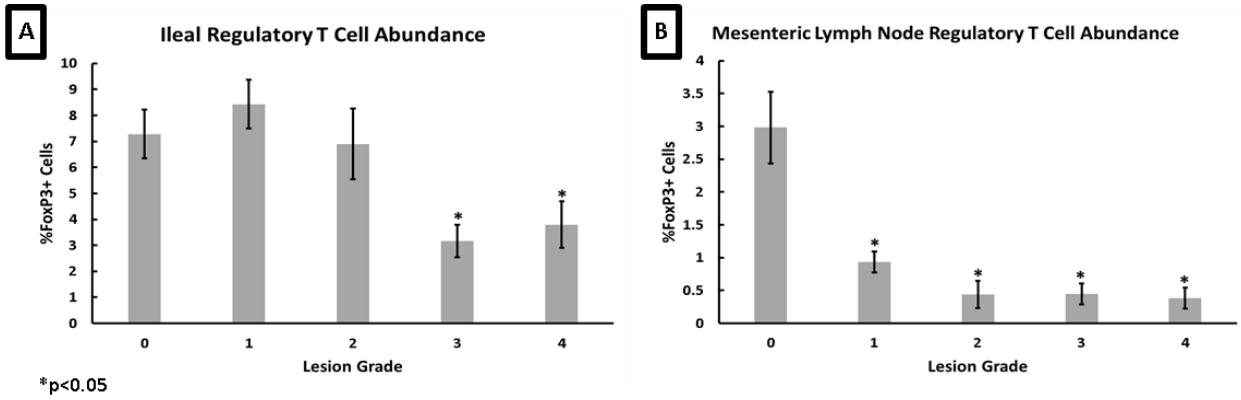


Figure 16: Regulatory T cell abundance. Regulatory T cell abundance was measured by comparing the number of FOXP3+ cells to the total number of eukaryotic cell nuclei present (%FOXP3^{+}/total) for each of the five lesion grades, within both the ileum (A) and mesenteric lymph nodes (B). Ten fields were measured for each of five tissue sections per lesion grade. *p<0.05

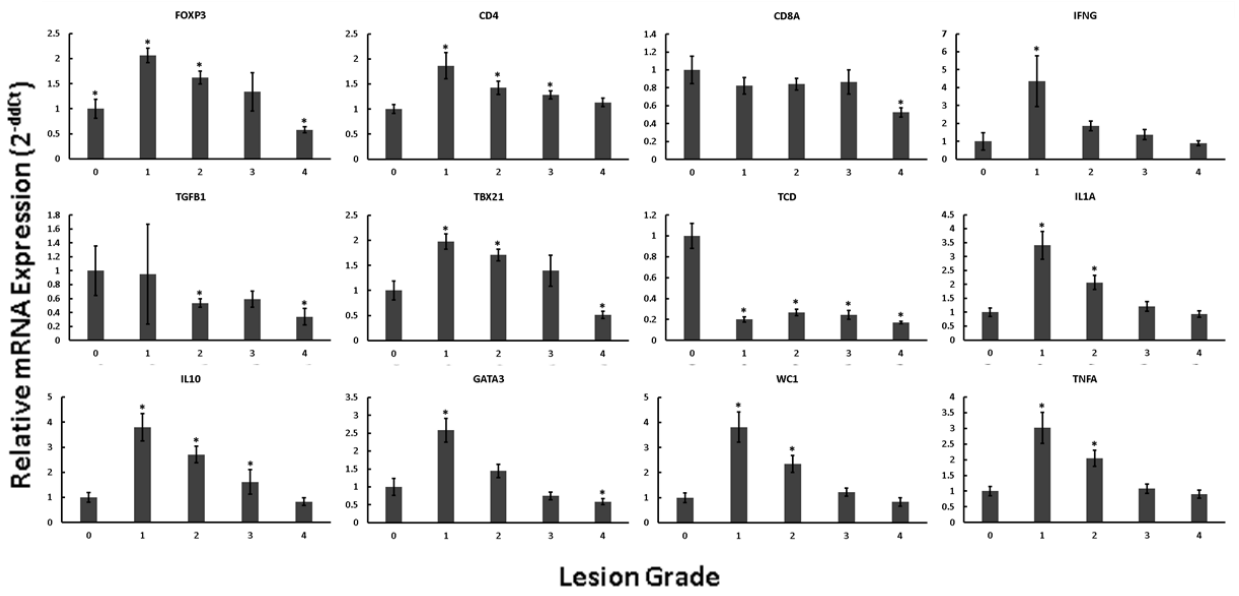


Figure 17: Relative ileal gene expression. Tissues were collected from healthy control cows and cows with Johne's disease. Lesion grades were assessed via the lesion classification system presented within this paper. Snap-frozen tissue sections were processed for RNA extraction, cDNA conversion, and qPCR for a variety of immune-related genes, and these data were separated based on lesion grade. Grades 1-4 lesions were compared to grade 0 lesions. n = 9-35 per grade,*$p < 0.05$.

Although this data suggests a decrease in Treg activity and function in grade 4 lesions, it must be noted that IL10 in particular is produced 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 18), 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.

4.3.3 Of the αβ 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 αβ 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 *CD8A* was not upregulated at any lesion severity, when compared to tissues from healthy controls.

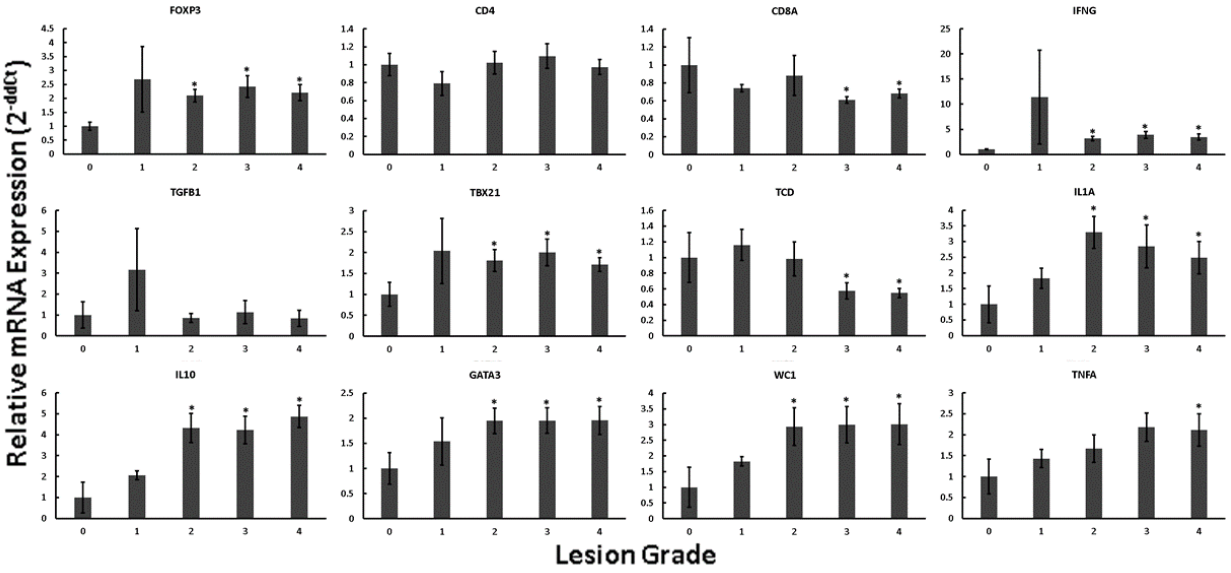


Figure 18: Relative mesenteric lymph node gene expression. Tissues were collected from healthy control cows and cows with Johne's disease. Lesion grades were assessed via the lesion classification system presented within this paper. Snap-frozen tissue sections were processed for RNA extraction, cDNA conversion, and qPCR for a variety of immune-related genes, and these data were separated based on lesion grade. Grades 1-4 lesions were compared to grade 0 lesions. n = 4-9 per grade,*p< 0.05.

CD4 mRNA abundance was unchanged in grade 4 lesions, whereas *CD8A* 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 non-significance 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 non-significance 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

CD8A 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.

4.3.4 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 γδ 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 γδ T cell co-receptor typically representative of an inflammatory population of cells [144, 145], 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.

4.3.5 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 ($p > 0.05$, Appendix A). Relative mRNA abundance of *IL6* tended to increase in grade 2 lymph node lesions, but this result also failed to reach significance ($p > 0.05$, Appendix A).

4.4 Discussion

Although regulatory T cells have been suggested as a significant contributor to the progression of Johne's disease [75, 84, 89], 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 [84], but also an increased relative abundance of *FOXP3* mRNA in the ileum of subclinical infected cows [75]. 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 IFNG, perhaps due to Treg-produced IL10. 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 [146]. 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.

Of the four cows with Johne's disease, we observed that two animals were in a later stage of clinical disease than the other two. The two animals in late clinical disease were euthanized due to extreme infirmity (including difficulty standing or eating), whereas the other two acted relatively normal despite marked weight loss and persistent diarrhea, at the time of death. There was a significant difference in average lesion severity between these two groups of animals (Figure 13) as well as between both groups and the healthy control cows ($*p < 0.02$ in all comparisons). In fact, cows with late clinical disease had an average ileal combined grade (H&E plus AFB grades, averaged) of 3.525 compared to an average combined grade of 1.8625 in ileal lesions from cows with early clinical disease. In the mesenteric lymph nodes, cows with late clinical disease had an average combined grade of 3.275, compared to an average combined grade of 1.9375 in lymph lesions from cows with early clinical

disease. Thus, this data suggests that there is a wide range of pathological severity seen in infected tissues from cows with clinical Johne's disease, and that perhaps clinical disease can be further distinguished into two sub-stages, being early clinical disease and late clinical disease. However, as all four of these animals had similar diagnostic test scores that are indicative of clinical disease, distinguishing these two populations may prove difficult prior to histopathological analysis. Indeed, it may be possible to combine lesion grades of 1-2 and lesion grades of 3-4, resulting in a condensed grade 0-1-2 lesion grading scheme, as suggested by one contributor to this work (Dodd Sledge, personal communication). This possible alternative perspective may more readily allow distinction between cows with early clinical as compared to late clinical disease, based on the results of this study.

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 15-16). 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 grades 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 grade 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 IL10 during Johne's disease. Relative expression of another Treg-associated factor, *TGFB1*, was unchanged or significantly reduced in all lesions relative to control, suggesting that TGFB is unlikely to play a significant role in Treg responses to MAP.

Although determining the effect(s), if any, of Tregs on the progression of Johne's disease was the primary goal of this study, an additional, major component was to assess the overall immune profile in regions of MAP infection according to lesion severity, and this was accomplished through qPCR on all lesion samples obtained. Within the ileum (Figure 17), some unique trends were observed. First, relative *CD4* mRNA abundance increased significantly in lesions of grades 1-3, when compared to grade 0 (healthy control) lesions, whereas neither *CD8A* 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 lesions of grades 1-3.

There is mounting evidence that γδ T cells play a significant role in the immune response to MAP infection [54-58]. Our results show that in MAP-infected ileum, regardless of lesion score (1-4), the relative mRNA abundance encoding the delta chain of the γδ 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 γδ T cells may be reduced in the MAP-infected ileum, the expression of *WC1* in the remaining γδ T cells is much higher than in uninfected control tissues. Therefore, it is likely that $WC1^{+}$, rather than $WC1^{-}$ γδ T cells are the dominant subset of γδ 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 [94] 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., lesions of grades 3-4). Results of this study suggest that this is not the case, or at least that IL1A and TNFA 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 18), 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 mixed Th1/Th2/Treg, prolonged 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 *CD8A* 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 grades 3-4 lesions while *WC1* expression was significantly increased, again suggesting that $WC1^+$ γδ T cells are the most important γδ T cell subset in immune responses to MAP infection.

Altogether, the results from this study build upon the results of our other recent work (discussed in Chapter 2) [94]. 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 (Appendix A) to what was observed with *IL1A* and *TNFA* expression (an increase in expression in mild lesions with unchanged expression in severe lesions; Figure 17), thus it is unlikely that the Th17 response is driving chronic inflammation. Rather, it may be that loss of 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. 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 light of what this study has revealed, however, we in part accept our 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. We reject the null hypothesis that Tregs do not play a role in the progression of Johne's disease.

Chapter 5: Concluding Remarks

5.1 Focus of the Research

The overall goal of this research project was to determine what role if any regulatory T cells play in the progression of Johne's disease. As detailed previously, there is ample evidence, both in the study of Johne's disease and elsewhere, to suggest that regulatory T cells play some role in the immune response to MAP. Specifically, work has shown that there are increased levels of Tregs in the periphery of cows with Johne's disease as compared to the periphery of healthy control cows, and that there is increased expression of *FOXP3* mRNA in ileal tissues from cows with subclinical Johne's disease, as compared to ileal tissues from either healthy control cows or cows with clinical Johne's disease. With this in mind, we sought to investigate Tregs taken from cows with Johne's disease, specifically looking at first peripheral Tregs, and second, lesion-associated Tregs within the primary (ileum) and secondary (mesenteric lymph node) sites of MAP infection.

Despite evidence clearly demonstrating the presence of increased levels of Tregs in cows with Johne's disease, little work has been done investigating the factors driving the development of Tregs, as well as the actual functionality of these Tregs, in the context of Johne's disease. Considering these apparent research gaps, we identified three objectives for this study altogether:

1) Determine if MAP-infected macrophages can induce the development of Tregs from naïve T cells taken from cows with Johne's disease.
2) Elucidate the effects of Tregs on peripheral blood mononuclear cell responses to stimulation with live MAP.
3) Characterize immune cell profiles in regions of MAP infection in ilea and mesenteric lymph nodes.

Within the context of these three objectives, various obstacles needed to be overcome, and a great deal of work was needed prior to addressing the questions at the core of each objective. As such, significant additional information related to Johne's disease, the histopathology of MAP infection, and bovine Tregs both in the context of Johne's disease and generally, was gained during the course of the study. The purpose of this chapter is to briefly summarize our results, tie together discussions from each of the previous chapters, and draw overall conclusions gleaned from this research project. Finally, we will also touch on shortcomings in the research and suggest critical future research topics needed to further this line of study.

5.2 Induction of regulatory T cell phenotype in naïve T cells

In our first set of experiments, we focused on the development of Tregs. Specifically, we sought to determine if MAP-infected monocyte-derived macrophages were sufficient to induce the development of a regulatory T cell phenotype in naïve T cells taken from cows in differing stages of Johne's disease. The experimental design was based on the fact that two factors shown to induce the development of Tregs, ineffective co-stimulation between T cells and macrophages and chronic antigen stimulation, are present during Johne's disease. We originally hypothesized that, in cells from subclinical infected cows, a Treg phenotype would develop in naïve T cells exposed to MAP-infected MDMs, when compared to cells from cows with clinical disease or from healthy controls. This hypothesis was based on the idea that Tregs develop during subclinical Johne's disease and lead to progression into clinical disease due to a Treg-mediated shift from a Th1 to a Th2 immune response. Unexpectedly, our data showed that MAP-infected MDMs alone are not sufficient for inducing a Treg phenotype in naïve T cells from cows with John's disease. Importantly, however, our data did provide novel insights into another possible mechanism for the shift into clinical disease: T cell unresponsiveness.

One possible shortcoming is that our method of selecting naïve T cells only focused on sorting $CD4^+CD25^-$ lymphocytes; as such it is possible that this cell population may have also included un-activated effector T cells and memory T cells and these cells may have mitigated the effects of our experimental setup. Still, we believe that our design was robust enough and that our cell populations were sufficiently pure for the target cell population, and that our hypothesis was simply incorrect. Despite this, our data yielded very interesting (if unexpected) results. Of the 18 genes examined, the expression of every gene was significantly reduced in $CD4^+CD25^-$ lymphocytes from cows with clinical disease, and expression of all but two genes was significantly reduced in the same cells from cows with subclinical disease, when compared to cells from healthy control cows. Critically, however, we found that within the $CD4^+CD25^-$ lymphocyte population from subclinical infected cows, there was a significant response to MAP-infected MDMs as compared to uninfected MDMs within the same study group. This response was characterized by a mixed Th1/Th2 phenotype, suggesting that, despite overall significantly reduced expression of most immune genes when compared to cells from healthy control cows, $CD4^+CD25^-$ lymphocytes respond to MAP-infected MDMs whereas cells from clinical infected cows do not. Altogether, these results suggest that $CD4^+CD25^-$ T cells from cows with clinical Johne's disease are unresponsive, and similar cells from cows with subclinical Johne's disease are hyporesponsive, as they have reduced but still significant responses to MAP antigens. The overall reduced gene expression when compared to healthy control cows suggests that chronic MAP infection may lead to some form of T cell unresponsiveness in $CD4^+$ T cells in cows with Johne's disease. It may be possible that the observed reduced responsiveness may be due to T cells becoming trapped in the mesenteric lymph nodes of clinically infected cows (see below; section 5.4) and thus failing to travel through the periphery to sites of infection; thus, future researchers may hypothesize that this is indeed the case and will subsequently develop experiments to test this.

5.3 Expansion of regulatory T cells and their effects on peripheral blood mononuclear cell responses to MAP stimulation

In our second study, we sought to examine the functional nature of Tregs in cows with Johne's disease. Specifically, we wanted to see what if any effect $CD4^+CD25^+$ Tregs would have on PBMC responses to stimulation with live MAP in PBMCs from cows with both subclinical and clinical disease. Our hypothesis was that Tregs would inhibit Th1 immune responses from PBMCs to stimulation with live MAP, in PBMCs from cows with both subclinical and clinical disease. This was based on evidence demonstrating the presence of a functional, $CD4^+CD25^+$, IL10-producing population of cells in cows with subclinical Johne's disease, and the notion that these Tregs would persist throughout clinical disease, resulting in persistently-dampened Th1 immune responses to MAP. One unique aspect of our experimental design involved increasing and expanding the relative abundance of Tregs within a PBMC population, based on methods used in humans, in an effort to best draw out potential effects of Tregs amongst other potential cell populations. As there is no unique combination of surface markers to specifically define Tregs, we selected $CD4^+CD25^+$ lymphocytes as our Treg-containing population. We used a unique cocktail of factors combined with MAP-infected macrophage stimulation (as detailed in Chapter 3) to expand the relative abundance of Tregs within that population, although one potential drawback of course is the presence of effector T cells within our Treg-containing population.

Altogether, we did not find any significant 3-way interactions (that is, interactions examining cows of different Johne's disease test status, the presence or absence of Tregs, and the presence or absence of live MAP). This may have been due to two compounding factors: relatively low cow numbers (n = 3-8/group) and the fact that cows represent an outbred population with wider genetic variability than model organisms such as mice. As such, we examined the data from a broader context in which we collapsed the study groups into broader categories, such as simply presence or absence of expanded Tregs, regardless of Johne's test status or presence of MAP. In this particular instance, we found that the

addition of expanded Tregs to cultures resulted in a significant increase in the relative mRNA abundance of *FOXP3*, the key Treg transcription factor. We also observed a significant increase in *IL10* mRNA abundance, and significant decreases in *IFNG* and *PFN1* mRNA abundance, suggesting that the expanded $CD4^+CD25^+$ T cell population is secreting IL10, and that this IL10 is functional in reducing Th1 immune responses. Critically, these Tregs do not appear to be responsive to MAP specifically. When we re-analyzed the data examining only the presence or absence of live MAP, regardless of Johne's disease test status or presence or absence of Tregs, we found that PBMCs respond to MAP in a mixed Th1/Th17 manner. While a Th1 response is typically considered an effective response to infection with MAP, a Th17 response may be indicative of an errant inflammatory response. Altogether, these results were largely inconclusive, although greater cow numbers may reveal significant changes not seen here. Future researchers should aim to use increased cow numbers in similar experiments in an effort to better elucidate significant results. Importantly, however, our results did demonstrate that it is possible to expand a functional, non-specific Treg population, and that these Tregs are capable of dampening Th1 gene expression. This result will prove useful to future researchers looking to investigate Treg activity in the bovine system. Looking forward, one may hypothesize that Tregs are not antigen reactive yet would still potentially inhibit Type 1 immune activity in infected tissues; thus, addition of expanded Tregs to MAP-infected tissues (such as in a mouse model) would be an interesting way to test such a hypothesis.

5.4 Regulatory T cells and immune function in tissues from MAP-infected cows

Our final study shifted focus from the periphery to the primary (ileum) and secondary (draining mesenteric lymph node) sites of MAP infection, to investigate Treg presence (or absence) and function within MAP-infected tissues. Based on established information as well as results obtained in our first and second studies, we proposed that Tregs played one of two distinct roles in the progression of

Johne's disease. First, our primary hypothesis was that Tregs develop during subclinical disease, due to conditions outlined in section 5.1 above, and that these Tregs subsequently promote a shift from a productive Th1 response to an unproductive Th2 immune response. This shift in turn would result in a loss of effective control of the infection, and ultimately development of clinical disease due to increased dissemination of MAP. An alternative hypothesis we put forth, largely due to results seen in our first two studies, was that Tregs are present during subclinical disease and function to limit chronic inflammation that may occur due to the large macrophage influx into MAP-infected tissues, and the continued stimulation of these and other immune cells with MAP antigens. At some point, these Tregs would be reduced in number and/or functionality, thereby disrupting tissue homeostasis and allowing unchecked inflammation to cause severe tissue damage that is characteristic of cows with clinical Johne's disease.

To structure this study more effectively, we initially sought to categorize MAP-infected lesions based on tissue histopathology and MAP bacterial burden. We found that the cows we studied, despite all showing signs and diagnostic test results of cows with clinical disease, could be further subdivided into early clinical and late clinical disease. Cows with late clinical disease were extremely weak, barely able to eat or stand, and had primarily lesions of grades 3-4, whereas cows with early clinical disease were much more spry and healthy despite chronic diarrhea and weight loss, and had primarily lesions of grades 1-2 within their tissues. Regardless of that interesting observation, we found that as lesion severity increased, the relative abundance of Tregs (based on FOXP3 protein expression) decreased, initially lending support to the notion that Tregs are lost, rather than induced, during the progression into clinical Johne's disease. However, we also found, in the ileum, that the expression of two key inflammatory genes, *IL1A* and *TNFA*, increased in grades 1 and 2 lesions but then decreased in grade 4 lesions, as compared to healthy control tissues. This would suggest that the loss of Tregs is not contributing to an increase in chronic inflammation, as inflammatory gene expression is being reduced in the most severe tissue lesions. We also found that the expression of several other immune genes

increased in lesions of grades 1-2, and decreased in grade 4 lesions, suggesting a widespread, mixed Th1/Th2 immune response in mild lesions that is lost in the most severe lesions, and that this response is mediated primarily by $CD4^+$ T cells and $WC1^+$ γδ T cells. Within the lymph node, on the other hand, expression of many immune genes was elevated throughout lesions of grades 2-4, suggesting a persistent immune response. Future studies should focus on examining protein expression of numerous immune cell types in lesions from cows with Johne's disease including CD4, CD8, and γδ T cells, as well as inflammatory cells, particularly macrophages. Altogether, our second hypothesis (in which a loss of Treg abundance and activity promotes progression into clinical disease) seems most likely, although our data suggests that this does not tell the entire story; future work is needed to pinpoint the source of inflammation seen in severe lesions. One possible hypothesis that may explain the stark difference in gene expression observed between the ileum and lymph nodes is that immune cells are becoming trapped within the lymph nodes and failing to home to the primary site of infection. This would result in an accumulation of immune activity in the lymph nodes and possibly the eventual development of T cell unresponsiveness in the ileum (Figure 19). Future research should also investigate this possibility.

5.5 Conclusions

As these studies progressed, we encountered unique data and results that, while unexpected, led us to novel insights into the bovine immune response during the progression of Johne's disease. Perhaps most importantly, however, is the fact that results from our various studies all seem to fit together and provide an effective explanation for what may be occurring during Johne's disease, at least in part. Generally, our overall hypothesis was that Tregs develop during subclinical Johne's disease and function to limit effector Th1 immune responses to MAP, ultimately allowing dissemination of MAP within the host and subsequent progression into clinical disease. Although our results differed from our

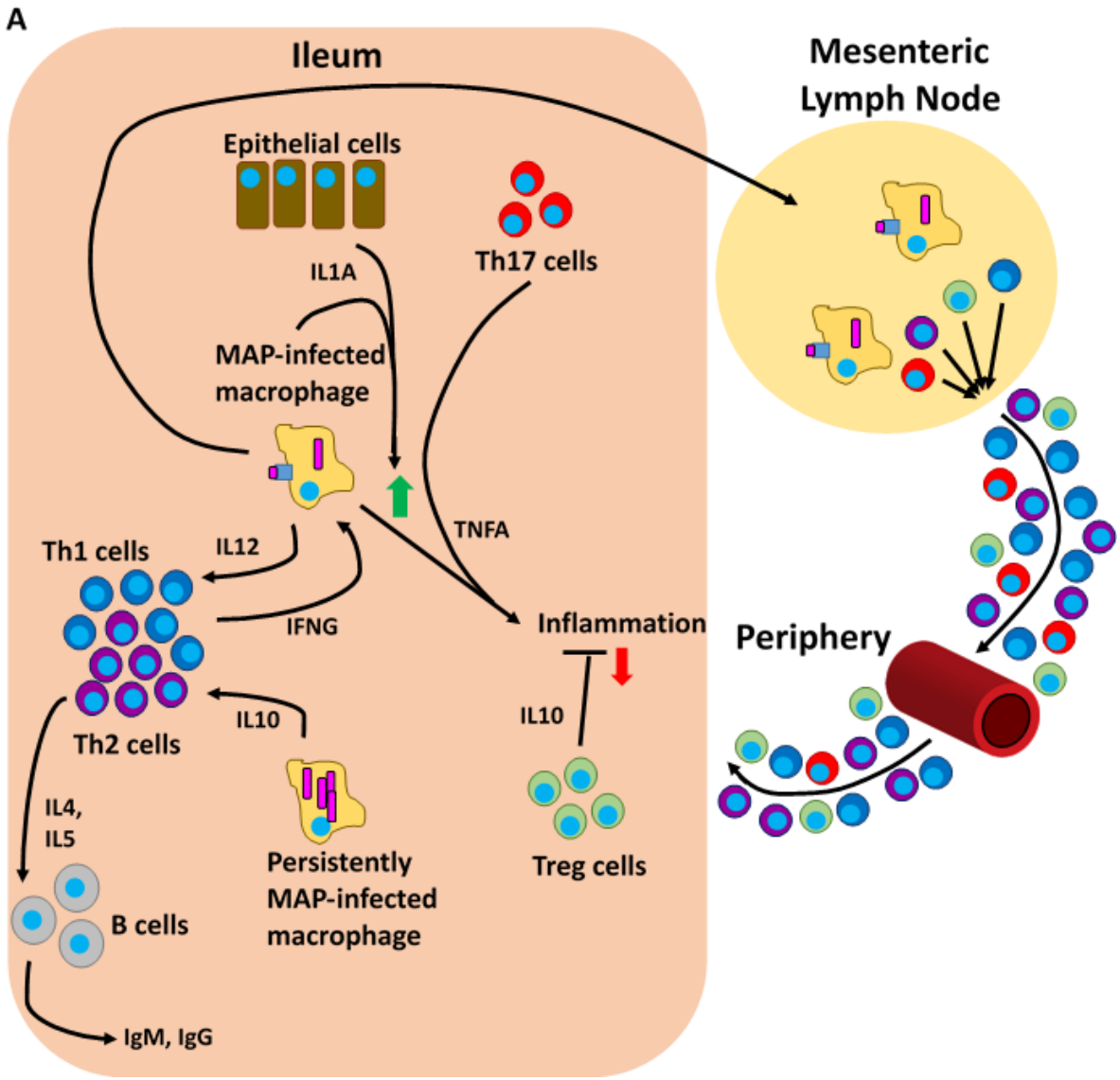
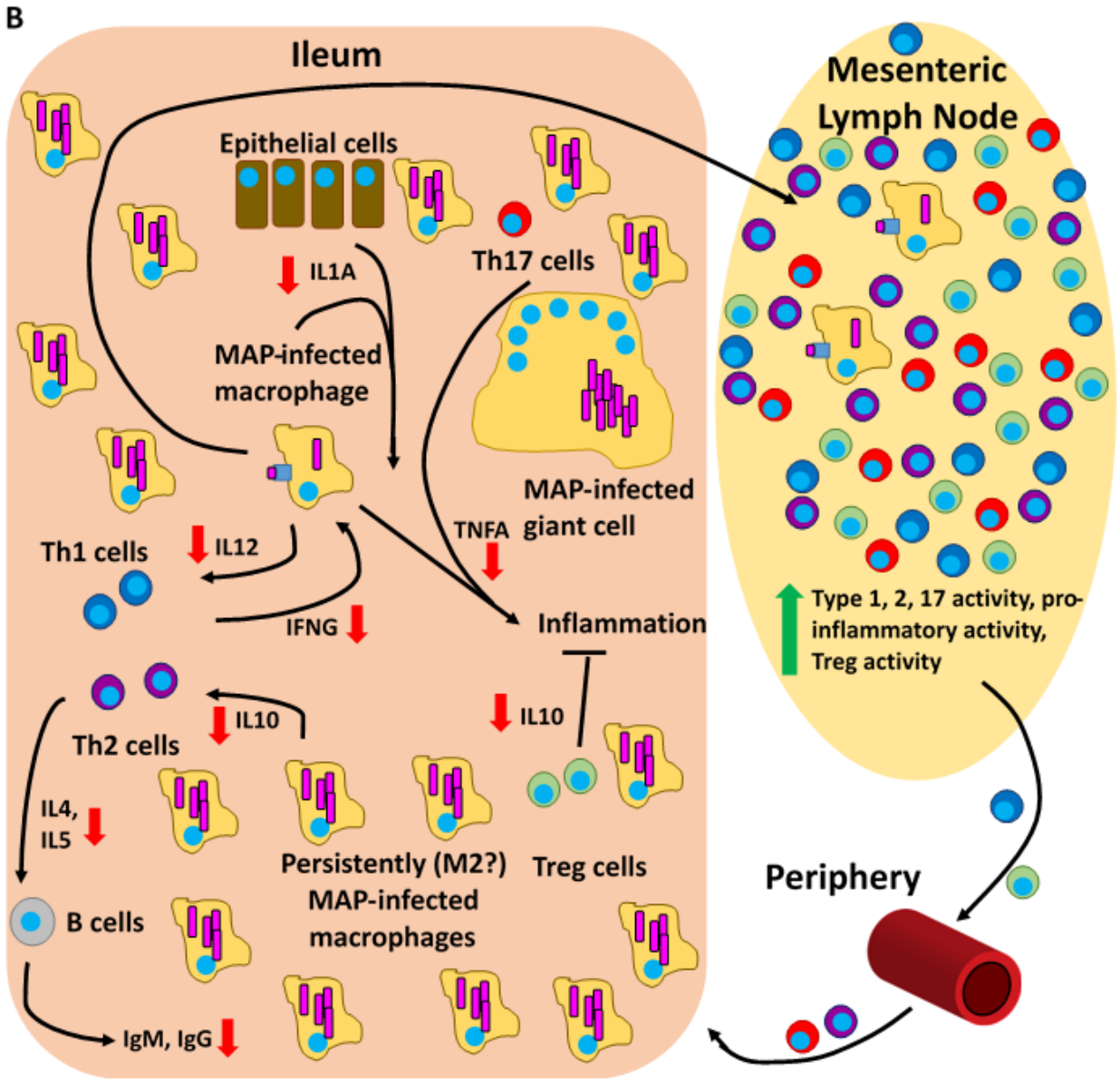


Figure 19: Possible Model for Immune Response Shift in Regions of MAP Infection. In subclinical disease (A), a dynamic immune response occurs in regions of MAP infection. Some macrophages successfully degrade MAP, some do not. This results in production of both IL12 and IL10, priming development of both Th1 and Th2 immune cells. Th1 cells produce IFNG and promote killing of MAP in newly infected macrophages. Th2 cells promote humoral immunity. Th17 cells are present, and along with macrophages and epithelial cells, promote inflammatory responses. Tregs are present and serve to limit inflammation, thereby maintaining homeostasis in the ileum while the immune system mounts a long-term response against MAP. Immune cells are actively recruited from the mesenteric lymph nodes to regions of ileal infection. In clinical disease (B), immune activity in the ileum is minimal. There is a large presence of macrophages and multinucleated giant cells. The macrophages are possibly polarized increasingly toward a Type 2 phenotype. T cells may become trapped in the lymph node, resulting in swollen nodes and apparent local T cell unresponsiveness both in the periphery and in the ileum, as well as enhanced immune activity in the lymph node. Consequently, Treg abundance is also diminished in the ileum, and homeostasis is lost. In the lymph node, expression of numerous immune genes remains elevated due to increased accumulation of lymphocytes.

Figure 19 (cont'd)



expectations, as the study progressed, we realized that our data was pointing toward the idea of T cell unresponsiveness, rather than Treg activity, as being a possible reason for the progression of Johne's disease, lending support to a paradigm that has been studied little in the context of this chronic condition. In the periphery, we found consistently reduced gene expression of many immune genes in cells from cows with subclinical and clinical disease. A key distinction, however, was that those cells

from cows with subclinical disease did still show some small yet significant responses to stimulation with MAP-infected macrophages, whereas those from cows with clinical disease did not. This suggested that cows with subclinical disease had hyporesponsive T cells and that cows with clinical disease had unresponsive T cells. We also found that Tregs do not respond to MAP specifically, further opening the possibility that something other than Tregs is resulting in the progression of Johne's disease. Ultimately, these results open a major avenue of research for future investigators. Future work should focus on determining if the development of T cell unresponsiveness is temporal, through an experimental infection model of MAP infection. Second, the nature of the observed T cell unresponsiveness should be investigated to determine if general unresponsiveness, anergy, or T cell exhaustion specifically are the mechanism by which unresponsiveness develops. Last, but perhaps most significant in the context of animal welfare and production, is the implication that the results of this project have for vaccine development. If Treg activity is important in the control of MAP-induced inflammation, then a vaccine that promotes the perpetuation of Treg activity at sites of infection may provide a simple yet effective means of limiting disease-induced morbidity and mortality. Such a vaccine, while perhaps not effective at preventing initial infection, would likely reduce fecal shedding (due to increased control of the pathogen within the host) and therefore reduce horizontal transmission. The combination of increased animal welfare and reduced spread of disease would result in a powerful tool in the fight to mitigate the effects of, and control the spread of, Johne's disease in dairy herds worldwide.

When we looked within MAP-infected tissues, we found that Treg abundance and activity decreases with increasing lesion severity, but that the loss of Treg activity does not correspond to an increase in inflammatory gene expression. Rather, the loss of Treg activity occurs in line with a loss in the expression of numerous other immune factors including Th1, Th2, and inflammatory genes. These results lend support to the notion that T cell unresponsiveness occurs during the progression of Johne's disease. As mentioned above, future work would greatly benefit this line of study if a temporal study

was initiated in which T cell unresponsiveness is investigated throughout the course of the development of Johne's disease, both in the periphery and within the tissues. Clearly, observing the abundance and activity of various immune cell types within the ileum and lymph nodes of cows as they progress through Johne's disease would be immensely beneficial. This of course would require a very invasive study utilizing numerous biopsies on each animal over time, and as such would be an elaborate investigation. Further investigation into unresponsiveness in other immune cells including B cells and macrophages would greatly aid in gaining a better overall understanding of immune unresponsiveness in the context of Johne's disease. Finally, it would be of great benefit to investigate whether MAP is actively inducing T cell unresponsiveness, and whether or not macrophage polarization may play an additional role in allowing the spread of MAP and loss of control of the infection.

Altogether, our study supports the hypothesis that a loss of Treg abundance and activity promotes the progression from subclinical to clinical Johne's disease, as well as progression from early to late stage clinical disease. Although we demonstrated that Tregs can in fact result in dampened Th1 immune responses, this response does not seem to be specific to situations of MAP infection. Tregs may merely be developing unresponsiveness throughout the course of disease, as we suspect is occurring with other types of T cells, although a temporal study would be necessary to verify this. Our study therefore suggests that T cell unresponsiveness, rather than Treg activity, is the major factor driving the progression of Johne's disease from subclinical to clinical disease.

APPENDIX

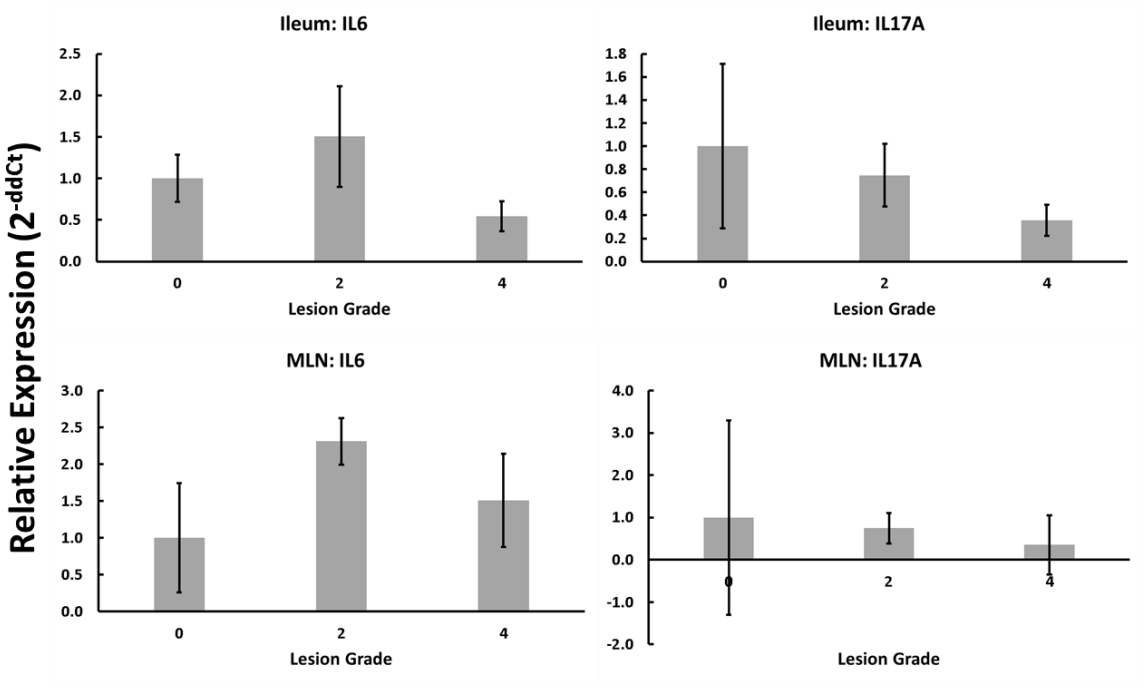


Figure 20: Relative expression of *IL6* and *IL17A* in tissue lesions caused by MAP infection. A subset of samples (ileum: n = 6 per lesion grade, MLN: n = 4 per lesion grade) was used to measure relative expression of *IL6* and *IL17A* in the ileum and mesenteric lymph nodes.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Coussens, P.M., *Model for immune responses to Mycobacterium avium subspecies paratuberculosis in cattle.* Infect Immun, 2004. **72**(6): p. 3089-96.

2. Whitlock, R.H. and C. Buergelt, *Preclinical and clinical manifestations of paratuberculosis (including pathology).* Vet Clin North Am Food Anim Pract, 1996. **12**(2): p. 345-56.

3. USDA-APHIS:VS, C., *Johne's Disease on U.S. Dairies, 1991-2007*. 2008.

4. Good, M., et al., *Prevalence and distribution of paratuberculosis (Johne's disease) in cattle herds in Ireland.* Ir Vet J, 2009. **62**(9): p. 597-606.

5. Collins, M.T.a.M., E. *Johne's Information Center*. 2010 2011 [cited 2015 2 February]; Available from: www.johnes.org.

6. Yakes, B.J., et al., *Detection of Mycobacterium avium subsp. paratuberculosis by a sonicate immunoassay based on surface-enhanced Raman scattering.* Clin Vaccine Immunol, 2008. **15**(2): p. 227-34.

7. Boelaert, F., et al., *Prevalence of paratuberculosis (Johne's disease) in the Belgian cattle population.* Vet Microbiol, 2000. **77**(3-4): p. 269-81.

8. Muskens, J., et al., *Prevalence and regional distribution of paratuberculosis in dairy herds in The Netherlands.* Vet Microbiol, 2000. **77**(3-4): p. 253-61.

9. Smith, R.L., et al., *Environmental contamination with Mycobacterium avium subsp. paratuberculosis in endemically infected dairy herds.* Prev Vet Med, 2011. **102**(1): p. 1-9.

10. Pradhan, A.K., et al., *Dynamics of endemic infectious diseases of animal and human importance on three dairy herds in the northeastern United States.* J Dairy Sci, 2009. **92**(4): p. 1811-25.

11. Buergelt, C.D. and J.R. Duncan, *Age and milk production data of cattle culled from a dairy herd with paratuberculosis.* J Am Vet Med Assoc, 1978. **173**(5 Pt 1): p. 478-80.

12. Lombard, J.E., et al., *Risk of removal and effects on milk production associated with paratuberculosis status in dairy cows.* J Am Vet Med Assoc, 2005. **227**(12): p. 1975-81.

13. Jones, R.L., *Review of the economic impact of Johne's disease in the United States*, in *Johne's Disease. Current Trends in Research, Diagnosis, and Management.*, Wood, Editor. 1989, A.R.M.a.P.R. p. 46-50.

14. Chiodini, R.J., H.J. Van Kruiningen, and R.S. Merkal, *Ruminant paratuberculosis (Johne's disease): the current status and future prospects.* Cornell Vet, 1984. **74**(3): p. 218-62.

15. McNab, W.B., et al., *An evaluation of selected screening tests for bovine paratuberculosis.* Can J Vet Res, 1991. **55**(3): p. 252-9.

16. Sockett, D.C., et al., *Evaluation of four serological tests for bovine paratuberculosis.* J Clin Microbiol, 1992. **30**(5): p. 1134-9.

17. Whan, L.B., et al., *Bactericidal effect of chlorine on Mycobacterium paratuberculosis in drinking water.* Lett Appl Microbiol, 2001. **33**(3): p. 227-31.

18. Grant, I.R., H.J. Ball, and M.T. Rowe, *Effect of high-temperature, short-time (HTST) pasteurization on milk containing low numbers of Mycobacterium paratuberculosis.* Lett Appl Microbiol, 1998. **26**(2): p. 166-70.

19. Grant, I.R., et al., *Effect of commercial-scale high-temperature, short-time pasteurization on the viability of Mycobacterium paratuberculosis in naturally infected cows' milk.* Appl Environ Microbiol, 2002. **68**(2): p. 602-7.

20. Primm, T.P., C.A. Lucero, and J.O. Falkinham, 3rd, *Health impacts of environmental mycobacteria.* Clin Microbiol Rev, 2004. **17**(1): p. 98-106.

21. Lambrecht, R.S., J.F. Carriere, and M.T. Collins, *A model for analyzing growth kinetics of a slowly growing Mycobacterium sp.* Appl Environ Microbiol, 1988. **54**(4): p. 910-6.

22. Green, E.P., et al., *Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of Mycobacterium paratuberculosis.* Nucleic Acids Res, 1989. **17**(22): p. 9063-73.

23. Stevenson, K., et al., *Molecular characterization of pigmented and nonpigmented isolates of Mycobacterium avium subsp. paratuberculosis.* J Clin Microbiol, 2002. **40**(5): p. 1798-804.

24. Chiodini, R.J. and H.J. Van Kruiningen, *Characterization of Mycobacterium paratuberculosis of bovine, caprine, and ovine origin by gas-liquid chromatographic analysis of fatty acids in whole-cell extracts.* Am J Vet Res, 1985. **46**(9): p. 1980-9.

25. Chiodini, R.J., *Biochemical characteristics of various strains of Mycobacterium paratuberculosis.* Am J Vet Res, 1986. **47**(7): p. 1442-5.

26. Pavlik, I., et al., *Standardisation of restriction fragment length polymorphism analysis for Mycobacterium avium subspecies paratuberculosis.* J Microbiol Methods, 1999. **38**(1-2): p. 155-67.

27. Janagama, H.K., et al., *Cytokine responses of bovine macrophages to diverse clinical Mycobacterium avium subspecies paratuberculosis strains.* BMC Microbiol, 2006. **6**: p. 10.

28. Nielsen, S.S., H. Bjerre, and N. Toft, *Colostrum and milk as risk factors for infection with Mycobacterium avium subspecies paratuberculosis in dairy cattle.* J Dairy Sci, 2008. **91**(12): p. 4610-5.

29. Momotani, E., et al., *Role of M cells and macrophages in the entrance of Mycobacterium paratuberculosis into domes of ileal Peyer's patches in calves.* Vet Pathol, 1988. **25**(2): p. 131-7.

30. Pott, J., et al., *Internalization-dependent recognition of Mycobacterium avium ssp. paratuberculosis by intestinal epithelial cells.* Cell Microbiol, 2009. **11**(12): p. 1802-15.

31. Bannantine, J.P. and L.E. Bermudez, *No holes barred: invasion of the intestinal mucosa by Mycobacterium avium subsp. paratuberculosis.* Infect Immun, 2013. **81**(11): p. 3960-5.

32. Sigurethardottir, O.G., M. Valheim, and C.M. Press, *Establishment of Mycobacterium avium subsp. paratuberculosis infection in the intestine of ruminants.* Adv Drug Deliv Rev, 2004. **56**(6): p. 819-34.

33. Lamont, E.A., et al., *Infection with Mycobacterium avium subsp. paratuberculosis results in rapid interleukin-1beta release and macrophage transepithelial migration.* Infect Immun, 2012. **80**(9): p. 3225-35.

34. Tessema, M.Z., et al., *How does Mycobacterium avium subsp. paratuberculosis resist intracellular degradation?* Vet Q, 2001. **23**(4): p. 153-62.

35. Coussens, P.M., *Mycobacterium paratuberculosis and the bovine immune system.* Anim Health Res Rev, 2001. **2**(2): p. 141-61.

36. Corpa, J.M., et al., *Classification of lesions observed in natural cases of paratuberculosis in goats.* J Comp Pathol, 2000. **122**(4): p. 255-65.

37. Begara-McGorum, I., et al., *Early immunopathological events in experimental ovine paratuberculosis.* Vet Immunol Immunopathol, 1998. **63**(3): p. 265-87.

38. Tanaka, S., et al., *Reduced formation of granulomata in gamma(delta) T cell knockout BALB/c mice inoculated with Mycobacterium avium subsp. paratuberculosis.* Vet Pathol, 2000. **37**(5): p. 415-21.

39. Kaneko, H., et al., *Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice.* Lab Invest, 1999. **79**(4): p. 379-86.

40. Kaufmann, S.H. and C.H. Ladel, *Role of T cell subsets in immunity against intracellular bacteria: experimental infections of knock-out mice with Listeria monocytogenes and Mycobacterium bovis BCG.* Immunobiology, 1994. **191**(4-5): p. 509-19.

41. Jacobs, M., et al., *Increased resistance to mycobacterial infection in the absence of interleukin-10.* Immunology, 2000. **100**(4): p. 494-501.

42. Ullrich, H.J., W.L. Beatty, and D.G. Russell, *Direct delivery of procathepsin D to phagosomes: implications for phagosome biogenesis and parasitism by Mycobacterium.* Eur J Cell Biol, 1999. **78**(10): p. 739-48.

43. Brumell, J.H. and M.A. Scidmore, *Manipulation of rab GTPase function by intracellular bacterial pathogens.* Microbiol Mol Biol Rev, 2007. **71**(4): p. 636-52.

44. Cheville, N.F., et al., *Intracellular trafficking of Mycobacterium avium ss. paratuberculosis in macrophages.* Dtsch Tierarztl Wochenschr, 2001. **108**(6): p. 236-43.

45. Karakousis, P.C., W.R. Bishai, and S.E. Dorman, *Mycobacterium tuberculosis cell envelope lipids and the host immune response.* Cell Microbiol, 2004. **6**(2): p. 105-16.

46. Goren, M.B., A.E. Vatter, and J. Fiscus, *Polyanionic agents do not inhibit phagosome-lysosome fusion in cultured macrophages.* J Leukoc Biol, 1987. **41**(2): p. 122-9.

47. Wong, D., et al., *Mycobacterium tuberculosis protein tyrosine phosphatase (PtpA) excludes host vacuolar-H+-ATPase to inhibit phagosome acidification.* Proc Natl Acad Sci U S A, 2011. **108**(48): p. 19371-6.

48. Fratti, R.A., et al., *Role of phosphatidylinositol 3-kinase and Rab5 effectors in phagosomal biogenesis and mycobacterial phagosome maturation arrest.* J Cell Biol, 2001. **154**(3): p. 631-44.

49. Lybeck, K.R., A.K. Storset, and I. Olsen, *Neutralization of interleukin-10 from CD14(+) monocytes enhances gamma interferon production in peripheral blood mononuclear cells from Mycobacterium avium subsp. paratuberculosis-infected goats.* Clin Vaccine Immunol, 2009. **16**(7): p. 1003-11.

50. Kabelitz, D., A. Glatzel, and D. Wesch, *Antigen recognition by human gammadelta T lymphocytes.* Int Arch Allergy Immunol, 2000. **122**(1): p. 1-7.

51. Chiodini, R.J.a.D., W.C., *The cellular immunology of bovine paratuberculosis: the predominant response is mediated by cytotoxic gamma/delta T lymphocytes which prevent CD4+ activity.* Microbial pathogenesis, 1992. **13**(6): p. 447-63.

52. Hedges, J.F., et al., *Differential mRNA expression in circulating gammadelta T lymphocyte subsets defines unique tissue-specific functions.* J Leukoc Biol, 2003. **73**(2): p. 306-14.

53. Meissner, N., et al., *Serial analysis of gene expression in circulating gamma delta T cell subsets defines distinct immunoregulatory phenotypes and unexpected gene expression profiles.* J Immunol, 2003. **170**(1): p. 356-64.

54. Stabel, J.R., et al., *Disparate host immunity to Mycobacterium avium subsp. paratuberculosis antigens in calves inoculated with M. avium subsp. paratuberculosis, M. avium subsp. avium, M. kansasii, and M. bovis.* Clin Vaccine Immunol, 2013. **20**(6): p. 848-57.

55. Plattner, B.L., et al., *T lymphocyte responses during early enteric Mycobacterium avium subspecies paratuberculosis infection in cattle.* Vet Immunol Immunopathol, 2014. **157**(1-2): p. 12-9.

56. Plattner, B.L., E.L. Huffman, and J.M. Hostetter, *Gamma-delta T-cell responses during subcutaneous Mycobacterium avium subspecies paratuberculosis challenge in sensitized or naive calves using matrix biopolymers.* Vet Pathol, 2013. **50**(4): p. 630-7.

57. Sohal, J.S., et al., *Immunology of mycobacterial infections: with special reference to Mycobacterium avium subspecies paratuberculosis.* Immunobiology, 2008. **213**(7): p. 585-98.

58. Badi, F.A., A.I. Haroon, and A.M. Alluwaimi, *The gammadelta cells as marker of non-seroconverted cattle naturally infected with Mycobacterium avium subspecies paratuberculosis.* Res Vet Sci, 2010. **88**(1): p. 72-6.

59. McGill, J.L., et al., *The role of gamma delta T cells in immunity to Mycobacterium bovis infection in cattle.* Vet Immunol Immunopathol, 2014. **159**(3-4): p. 133-43.

60. Guzman, E., et al., *Bovine gammadelta T cells are a major regulatory T cell subset.* J Immunol, 2014. **193**(1): p. 208-22.

61. Pathan, A.A., et al., *High frequencies of circulating IFN-gamma-secreting CD8 cytotoxic T cells specific for a novel MHC class I-restricted Mycobacterium tuberculosis epitope in M. tuberculosis-infected subjects without disease.* Eur J Immunol, 2000. **30**(9): p. 2713-21.

62. Sasiain, M.C., et al., *T-cell-mediated cytotoxicity against Mycobacterium antigen-pulsed autologous macrophages in leprosy patients.* Infect Immun, 1992. **60**(8): p. 3389-95.

63. McKenzie, A.N., H. Spits, and G. Eberl, *Innate lymphoid cells in inflammation and immunity.* Immunity, 2014. **41**(3): p. 366-74.

64. Diefenbach, A., M. Colonna, and S. Koyasu, *Development, differentiation, and diversity of innate lymphoid cells.* Immunity, 2014. **41**(3): p. 354-65.

65. Cortez, V.S., M.L. Robinette, and M. Colonna, *Innate lymphoid cells: new insights into function and development.* Curr Opin Immunol, 2015. **32C**: p. 71-77.

66. Fuchs, A., et al., *Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-gamma-producing cells.* Immunity, 2013. **38**(4): p. 769-81.

67. Klose, C.S., et al., *Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages.* Cell, 2014. **157**(2): p. 340-56.

68. Tait Wojno, E.D. and D. Artis, *Innate lymphoid cells: balancing immunity, inflammation, and tissue repair in the intestine.* Cell Host Microbe, 2012. **12**(4): p. 445-57.

69. Stabel, J.R., *Transitions in immune responses to Mycobacterium paratuberculosis.* Vet Microbiol, 2000. **77**(3-4): p. 465-73.

70. Coussens, P.M., et al., *Cytokine gene expression in peripheral blood mononuclear cells and tissues of cattle infected with Mycobacterium avium subsp. paratuberculosis: evidence for an inherent proinflammatory gene expression pattern.* Infect Immun, 2004. **72**(3): p. 1409-22.

71. Harris, N.B. and R.G. Barletta, *Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine.* Clin Microbiol Rev, 2001. **14**(3): p. 489-512.

72. Abbas, B. and H.P. Riemann, *IgG, IgM and IgA in the serum of cattle naturally infected with Mycobacterium paratuberculosis.* Comp Immunol Microbiol Infect Dis, 1988. **11**(3-4): p. 171-5.

73. Uhlig, H.H., et al., *Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis.* J Immunol, 2006. **177**(9): p. 5852-60.

74. Baecher-Allan, C., V. Viglietta, and D.A. Hafler, *Human CD4+CD25+ regulatory T cells.* Semin Immunol, 2004. **16**(2): p. 89-98.

75. de Almeida, D.E., C.J. Colvin, and P.M. Coussens, *Antigen-specific regulatory T cells in bovine paratuberculosis.* Vet Immunol Immunopathol, 2008. **125**(3-4): p. 234-45.

76. Belkaid, Y. and B.T. Rouse, *Natural regulatory T cells in infectious disease.* Nat Immunol, 2005. **6**(4): p. 353-60.

77. Belkaid, Y., *Role of Foxp3-positive regulatory T cells during infection.* Eur J Immunol, 2008. **38**(4): p. 918-21.

78. Raghavan, S., E. Suri-Payer, and J. Holmgren, *Antigen-specific in vitro suppression of murine Helicobacter pylori-reactive immunopathological T cells by CD4CD25 regulatory T cells.* Scand J Immunol, 2004. **60**(1-2): p. 82-8.

79. Geffner, L., et al., *CD4(+) CD25(high) forkhead box protein 3(+) regulatory T lymphocytes suppress interferon-gamma and CD107 expression in CD4(+) and CD8(+) T cells from tuberculous pleural effusions.* Clin Exp Immunol, 2014. **175**(2): p. 235-45.

80. Ottenhoff, T.H., et al., *Cloned suppressor T cells from a lepromatous leprosy patient suppress Mycobacterium leprae reactive helper T cells.* Nature, 1986. **322**(6078): p. 462-4.

81. Wergeland, I., J. Assmus, and A.M. Dyrhol-Riise, *T regulatory cells and immune activation in Mycobacterium tuberculosis infection and the effect of preventive therapy.* Scand J Immunol, 2011. **73**(3): p. 234-42.

82. Ribeiro-Rodrigues, R., et al., *A role for CD4+CD25+ T cells in regulation of the immune response during human tuberculosis.* Clin Exp Immunol, 2006. **144**(1): p. 25-34.

83. Buza, J.J., et al., *Neutralization of interleukin-10 significantly enhances gamma interferon expression in peripheral blood by stimulation with Johnin purified protein derivative and by infection with Mycobacterium avium subsp. paratuberculosis in experimentally infected cattle with paratuberculosis.* Infect Immun, 2004. **72**(4): p. 2425-8.

84. Coussens, P.M., et al., *Regulatory T cells in cattle and their potential role in bovine paratuberculosis.* Comp Immunol Microbiol Infect Dis, 2012. **35**(3): p. 233-9.

85. Mills, K.H. and P. McGuirk, *Antigen-specific regulatory T cells--their induction and role in infection.* Semin Immunol, 2004. **16**(2): p. 107-17.

86. Bluestone, J.A. and A.K. Abbas, *Natural versus adaptive regulatory T cells.* Nat Rev Immunol, 2003. **3**(3): p. 253-7.

87. Ferrer, I.R., et al., *Antigen-specific induced Foxp3+ regulatory T cells are generated following CD40/CD154 blockade.* Proc Natl Acad Sci U S A, 2011. **108**(51): p. 20701-6.

88. Sommer, S., et al., *Mycobacterium avium subspecies paratuberculosis suppresses expression of IL-12p40 and iNOS genes induced by signalling through CD40 in bovine monocyte-derived macrophages.* Vet Immunol Immunopathol, 2009. **128**(1-3): p. 44-52.

89. Weiss, D.J., O.A. Evanson, and C.D. Souza, *Mucosal immune response in cattle with subclinical Johne's disease.* Vet Pathol, 2006. **43**(2): p. 127-35.

90. Sanchez, A.M. and Y. Yang, *The role of natural regulatory T cells in infection.* Immunol Res, 2011. **49**(1-3): p. 124-34.

91. Gravano, D.M. and D.A. Vignali, *The battle against immunopathology: infectious tolerance mediated by regulatory T cells.* Cell Mol Life Sci, 2012. **69**(12): p. 1997-2008.

92. Saxena, A., et al., *Interleukin-10 paradox: A potent immunoregulatory cytokine that has been difficult to harness for immunotherapy.* Cytokine, 2014.

93. Begg, D.J., et al., *Does a Th1 over Th2 dominancy really exist in the early stages of Mycobacterium avium subspecies paratuberculosis infections?* Immunobiology, 2011. **216**(7): p. 840-6.

94. Roussey, J.A., J.P. Steibel, and P.M. Coussens, *Regulatory T cell activity and signs of T cell unresponsiveness in bovine paratuberculosis.* Front Vet Sci, 2014. **1**(20).

95. Wood, K.J., A. Bushell, and N.D. Jones, *Immunologic unresponsiveness to alloantigen in vivo: a role for regulatory T cells.* Immunol Rev, 2011. **241**(1): p. 119-32.

96. Chatenoud, L. and J.F. Bach, *Resetting the functional capacity of regulatory T cells: a novel immunotherapeutic strategy to promote immune tolerance.* Expert Opin Biol Ther, 2005. **5 Suppl 1**: p. S73-81.

97. Ferrer, I.R., et al., *Induction of transplantation tolerance through regulatory cells: from mice to men.* Immunol Rev, 2014. **258**(1): p. 102-16.

98. Cools, N., et al., *Regulatory T cells and human disease.* Clin Dev Immunol, 2007. **2007**: p. 89195.

99. Nath, I., C. Saini, and V.L. Valluri, *Immunology of leprosy and diagnostic challenges.* Clin Dermatol, 2015. **33**(1): p. 90-8.

100. Labonte, A.C., A.C. Tosello-Trampont, and Y.S. Hahn, *The role of macrophage polarization in infectious and inflammatory diseases.* Mol Cells, 2014. **37**(4): p. 275-85.

101. Liu, Y.C., et al., *Macrophage polarization in inflammatory diseases.* Int J Biol Sci, 2014. **10**(5): p. 520-9.

102. Mosmann, T.R. and R.L. Coffman, *TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties.* Annu Rev Immunol, 1989. **7**: p. 145-73.

103. Prendergast, C.T., et al., *CD4+ T cell hypo-responsiveness after repeated exposure to Schistosoma mansoni larvae is dependent upon interleukin-10.* Infect Immun, 2015.

104. de Jong, R., et al., *IL-2 and IL-12 act in synergy to overcome antigen-specific T cell unresponsiveness in mycobacterial disease.* J Immunol, 1997. **159**(2): p. 786-93.

105. Greig, A., et al., *Epidemiological study of paratuberculosis in wild rabbits in Scotland.* J Clin Microbiol, 1999. **37**(6): p. 1746-51.

106. Motiwala, A.S., et al., *Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis isolates recovered from wild animal species.* J Clin Microbiol, 2004. **42**(4): p. 1703-12.

107. Stevenson, K., et al., *Occurrence of Mycobacterium avium subspecies paratuberculosis across host species and European countries with evidence for transmission between wildlife and domestic ruminants.* BMC Microbiol, 2009. **9**: p. 212.

108. Beard, P.M., et al., *Paratuberculosis infection of nonruminant wildlife in Scotland.* J Clin Microbiol, 2001. **39**(4): p. 1517-21.

109. Munster, P., et al., *Distribution of Mycobacterium avium ssp. paratuberculosis in a German zoological garden determined by IS900 semi-nested and quantitative real-time PCR.* Vet Microbiol, 2013. **163**(1-2): p. 116-23.

110. de Silva, K., et al., *CD4(+) T-cells, gammadelta T-cells and B-cells are associated with lack of vaccine protection in Mycobacterium avium subspecies paratuberculosis infection.* Vaccine, 2015. **33**(1): p. 149-55.

111. Reddacliff, L., et al., *Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks.* Vet Microbiol, 2006. **115**(1-3): p. 77-90.

112. Rosseels, V. and K. Huygen, *Vaccination against paratuberculosis.* Expert Rev Vaccines, 2008. **7**(6): p. 817-32.

113. Bastida, F. and R.A. Juste, *Paratuberculosis control: a review with a focus on vaccination.* J Immune Based Ther Vaccines, 2011. **9**: p. 8.

114. Singh, A.V., et al., *Presence and characterization of Mycobacterium avium subspecies paratuberculosis from clinical and suspected cases of Crohn's disease and in the healthy human population in India.* Int J Infect Dis, 2008. **12**(2): p. 190-7.

115. Hermon-Taylor, J., *Protagonist. Mycobacterium avium subspecies paratuberculosis is a cause of Crohn's disease.* Gut, 2001. **49**(6): p. 755-6.

116. Uzoigwe, J.C., M.L. Khaitsa, and P.S. Gibbs, *Epidemiological evidence for Mycobacterium avium subspecies paratuberculosis as a cause of Crohn's disease.* Epidemiol Infect, 2007. **135**(7): p. 1057-68.

117. Hermon-Taylor, J., *Treatment with drugs active against Mycobacterium avium subspecies paratuberculosis can heal Crohn's disease: more evidence for a neglected public health tragedy.* Dig Liver Dis, 2002. **34**(1): p. 9-12.

118. Kabara, E., et al., *A large-scale study of differential gene expression in monocyte-derived macrophages infected with several strains of Mycobacterium avium subspecies paratuberculosis.* Brief Funct Genomics, 2010. **9**(3): p. 220-37.

119. Qual, D.A., et al., *Lack of association between the occurrence of Crohn's disease and occupational exposure to dairy and beef cattle herds infected with Mycobacterium avium subspecies paratuberculosis.* J Dairy Sci, 2010. **93**(6): p. 2371-6.

120. Lozano-Leon, A., M. Barreiro-de Acosta, and J.E. Dominguez-Munoz, *Absence of Mycobacterium avium subspecies paratuberculosis in Crohn's disease patients.* Inflamm Bowel Dis, 2006. **12**(12): p. 1190-2.

121. Quirke, P., *Antagonist. Mycobacterium avium subspecies paratuberculosis is a cause of Crohn's disease.* Gut, 2001. **49**(6): p. 757-60.

122. Valheim, M., et al., *Localisation of CD25+ cells and MHCII+ cells in lymph nodes draining Mycobacterium avium subsp. paratuberculosis vaccination granuloma and the presence of a systemic immune response.* Res Vet Sci, 2002. **73**(1): p. 77-85.

123. Chiang, S.K., et al., *Relationship between Mycobacterium avium subspecies paratuberculosis, IL-1alpha, and TRAF1 in primary bovine monocyte-derived macrophages.* Vet Immunol Immunopathol, 2007. **116**(3-4): p. 131-44.

124. Murphy, J.T., et al., *Gene expression profiling of monocyte-derived macrophages following infection with Mycobacterium avium subspecies avium and Mycobacterium avium subspecies paratuberculosis.* Physiol Genomics, 2006. **28**(1): p. 67-75.

125. Steibel, J.P., et al., *A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data.* Genomics, 2009. **94**(2): p. 146-52.

126. Park, K.T., et al., *Deletion of relA abrogates the capacity of Mycobacterium avium paratuberculosis to establish an infection in calves.* Front Cell Infect Microbiol, 2014. **4**: p. 64.

127. Asanuma, S., et al., *Expansion of CD4(+)CD25 (+) regulatory T cells from cord blood CD4(+) cells using the common gamma-chain cytokines (IL-2 and IL-15) and rapamycin.* Ann Hematol, 2011. **90**(6): p. 617-24.

128. Godfrey, W.R., et al., *In vitro-expanded human CD4(+)CD25(+) T-regulatory cells can markedly inhibit allogeneic dendritic cell-stimulated MLR cultures.* Blood, 2004. **104**(2): p. 453-61.

129. Li, L., et al., *CD4+CD25+ regulatory T-cell lines from human cord blood have functional and molecular properties of T-cell anergy.* Blood, 2005. **106**(9): p. 3068-73.

130. Tanaka, J., et al., *Expansion of natural killer cell receptor (CD94/NKG2A)-expressing cytolytic CD8 T cells and CD4+CD25+ regulatory T cells from the same cord blood unit.* Exp Hematol, 2007. **35**(10): p. 1562-6.

131. Bacchetta, R., S. Gregori, and M.G. Roncarolo, *CD4+ regulatory T cells: mechanisms of induction and effector function.* Autoimmun Rev, 2005. **4**(8): p. 491-6.

132. Belkaid, Y., *Regulatory T cells and infection: a dangerous necessity.* Nat Rev Immunol, 2007. **7**(11): p. 875-88.

133. Fehervari, Z. and S. Sakaguchi, *Development and function of CD25+CD4+ regulatory T cells.* Curr Opin Immunol, 2004. **16**(2): p. 203-8.

134. Hostetter, J.M., et al., *Cytokine effects on maturation of the phagosomes containing Mycobacteria avium subspecies paratuberculosis in J774 cells.* FEMS Immunol Med Microbiol, 2002. **34**(2): p. 127-34.

135. Robinson, M.W., et al., *Immunoregulatory cytokines are associated with protection from immunopathology following Mycobacterium avium subspecies paratuberculosis infection in red deer.* Infect Immun, 2011. **79**(5): p. 2089-97.

136. Faisal, S.M., et al., *Evaluation of a Salmonella vectored vaccine expressing Mycobacterium avium subsp. paratuberculosis antigens against challenge in a goat model.* PLoS One, 2013. **8**(8): p. e70171.

137. Hoffmann, P., et al., *Only the CD45RA+ subpopulation of CD4+CD25high T cells gives rise to homogeneous regulatory T-cell lines upon in vitro expansion.* Blood, 2006. **108**(13): p. 4260-7.

138. Hines, M.E., 2nd, J.M. Kreeger, and A.J. Herron, *Mycobacterial infections of animals: pathology and pathogenesis.* Lab Anim Sci, 1995. **45**(4): p. 334-51.

139. Burrells, C., et al., *A study of immunological responses of sheep clinically-affected with paratuberculosis (Johne's disease). The relationship of blood, mesenteric lymph node and intestinal lymphocyte responses to gross and microscopic pathology.* Vet Immunol Immunopathol, 1998. **66**(3-4): p. 343-58.

140. Aho, A.D., A.M. McNulty, and P.M. Coussens, *Enhanced expression of interleukin-1alpha and tumor necrosis factor receptor-associated protein 1 in ileal tissues of cattle infected with Mycobacterium avium subsp. paratuberculosis.* Infect Immun, 2003. **71**(11): p. 6479-86.

141. Welsh, K.J., et al., *Immunopathology of postprimary tuberculosis: increased T-regulatory cells and DEC-205-positive foamy macrophages in cavitary lesions.* Clin Dev Immunol, 2011. **2011**: p. 307631.

142. Scott-Browne, J.P., et al., *Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis.* J Exp Med, 2007. **204**(9): p. 2159-69.

143. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

144. Rogers, A.N., et al., *Gammadelta T cell function varies with the expressed WC1 coreceptor.* J Immunol, 2005. **174**(6): p. 3386-93.

145. Chen, C., et al., *Antigenic basis of diversity in the gammadelta T cell co-receptor WC1 family.* Mol Immunol, 2009. **46**(13): p. 2565-75.

146. Dennis, M.M., L.A. Reddacliff, and R.J. Whittington, *Longitudinal study of clinicopathological features of Johne's disease in sheep naturally exposed to Mycobacterium avium subspecies paratuberculosis.* Vet Pathol, 2011. **48**(3): p. 565-75.