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Regulatory T cells and immune profiling in johne's disease lesions

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ABSTRACT

Johne's disease, caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic wasting disease of ruminants. Hallmark symptoms of clinical Johne's disease include diarrhea, progressive weight loss, and premature death; symptoms due largely to chronic inflammation in the small intestine. MAP colonizes resident macrophages within the ileum of the small intestine, subsequently establishing a persistent infection in the host. It has been proposed that regulatory T cells may play a role in the progression of Johne's disease, either through promotion of tolerance to MAP or via a loss in homeostasis that subsequently allows widespread inflammation. In this report, we evaluated the presence of Tregs, as well as other immune parameters, in the ileum and draining lymph nodes of MAP associated lesions. A lesion classification scheme was developed to categorize severity of MAP-induced lesions within infected tissues and subsequently regulatory T cell presence and overall immune activity were assessed corresponding to lesions of varying severity, in comparison to tissues from healthy control animals. Our results revealed a relationship between animal health and overall lesion severity within the infected tissues, as well as a relationship between bacterial burden and severity of pathology. Regulatory T cell abundance was shown to decrease with increasing lesion severity. Within the ileum, the expression of many Th1, Th2, and Treg-associated genes increased in mild lesions and decreased in severe lesions, whereas in the lymph nodes the expression of these genes tended to increase with increasing lesion severity. Based on our results, we conclude that a local loss of T cell (including Treg) activity occurs within severe ileal lesions associated with MAP, resulting in a loss of homeostasis that ultimately leads to the progression of clinical Johne's disease.

1. Introduction

Johne's disease, or paratuberculosis, is a chronic enteritis affecting ruminants worldwide, with a significant impact on certain livestock, including cattle and sheep. Johne's disease is caused by infection with the intracellular *Mycobacterium avium* subspecies *paratuberculosis* (MAP). During the asymptomatic and subclinical stages of disease, which typically lasts 2–5 years (USDA-APHIS:VS, 2016), infected animals intermittently shed MAP in their feces, despite showing little to no sign of infection. This leads to a widespread disease incidence, with an estimated 40–68% of dairy herds being infected (USDA-APHIS:VS, 2016; Yakes et al., 2008). The cost of Johne's disease, in the form of reduced milk production and premature culling, has been estimated to be at least $250 million and as high as $1.5 billion annually for the U.S. dairy industry (Jones, 1989; Ott et al., 1999). At the animal level, Johne's disease is characterized by increasingly severe diarrhea, progressive emaciation, and premature death (Whitlock and Buergelt, 1996). At the organ level, progressively worsening inflammation is seen in the ileum and in draining lymph nodes (Burrells et al., 1998; Hines et al., 1995; Whitlock and Buergelt, 1996).

Immunologically, MAP-infected cattle initially develop a productive Th1 immune response to MAP, characterized by both pro-inflammatory and cytotoxic immune activity (Coussens, 2001, 2004; Stabel, 2000). As Johne's disease progresses toward clinical disease, the peripheral Th1 response becomes diminished and is largely replaced by a Th2 (humoral) immune response (Coussens, 2001; Coussens et al., 2004; Harris and Barletta, 2001; Stabel, 2000). The Th2 response is dominated by production of anti-MAP anti-

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bodies ineffective at controlling intracellular infections. This shift in predominate immune response correlates with reduced control of infection, increased dissemination of the bacterium, and ultimately progression of clinical Johne's disease. Within infected tissues, the presence of large numbers of infected macrophages leads to widespread inflammation, possibly caused by production of IL-1 (Aho et al., 2003).

Although reasons for this immune shift are unknown (Stabel, 2000), one possibility is that a population of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) develops in response to chronic, low-level stimulation with MAP antigens and that these Tregs limit effector cell responses to MAP antigens (de Almeida et al., 2008). Tregs exert immunomodulatory effects through production of interleukin 10 (IL-10) and transforming growth factor beta (TGF-β). Tregs may develop in environments of ineffective T cell co-stimulation and chronic low-level antigen stimulation (Bluestone and Abbas, 2003; Ferrer et al., 2011; Mills and McGuirk, 2004), and both of these conditions have been shown to exist during Johne's disease (Sommer et al., 2009; Weiss et al., 2006). Indeed, Tregs have been shown to develop in response to numerous other chronic infectious agents including *M. tuberculosis* (Belkaid, 2008; Belkaid and Rouse, 2005; Geffner et al., 2014; Raghavan et al., 2004). Further, recent work by our group has shown an increased abundance of FoxP3 mRNA in the ileum of subclinical infected cows (de Almeida et al., 2008). It has also been shown that MAP-infected cows contain more Tregs in peripheral blood as compared to healthy controls (Coussens et al., 2012). These studies amongst others provide evidence for the presence of Tregs in cows with Johne's disease.

Although much research has centered around the possibility of Tregs resulting in diminished Th1 responses to MAP (Coussens, 2004; Coussens et al., 2012; de Almeida et al., 2008) (and hence reduced immune control of the pathogen), another possibility is that Tregs help to limit unchecked inflammation within infected tissues in subclinical infection, but this response wanes over time in some animals, allowing the inflammatory response to predominate. In this scenario, Tregs would be necessary to maintain homeostasis and one could hypothesize that loss of Tregs would accompany development of clinical disease. If this is the case, a lack of Treg (and possibly T cell, generally) abundance and/or function in animals with more severe disease would be expected. In fact, recent work from our group (Roussey et al., 2014) has shown strong evidence for T cell unresponsiveness in PBMC populations from cows with clinical Johne's disease. There is mounting evidence further suggesting T cell hyporesponsiveness (Weiss et al., 2006) and/or anergy (Begg et al., 2011) within T cell populations taken from infected ileal tissue.

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

2. Materials and methods

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 $OD \geq 1.0$) 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. 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 h 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).

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 1. Some tissue sections from healthy control cows received a grade of 1, although this was not from MAP infection. These tissues were subsequently excluded from future analyses (such as qPCR) to avoid any potential interference from non-MAP related intestinal issues. For H&E scores, the criteria considered were total epitheliod macrophages, distribution of inflammation, and integrity of tissue architecture. For AFB scores, both the overall abundance and density of individual pockets of MAP were considered. Refer to Table 1 for details on how scores were assessed for each individual category.

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