Research paper

T and B cell activation profiles from cows with and without Johne's disease in response to *in vitro* stimulation with *Mycobacterium avium* subspecies *paratuberculosis*

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ABSTRACT

Johne's disease (JD) is a chronic wasting disease of ruminants caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (MAP). JD is particularly problematic on US dairy farms: estimates show that over 50% of farms are MAP-contaminated and as many as 91% of dairy herds could be infected. Although estimates vary widely, JD may cost the dairy industry between $200 million and $1.5 billion every year. One major obstacle to JD management is that JD is difficult to detect in many animals, in part due to the variable immunity against MAP demonstrated by JD+ cattle. To characterize the diversity of immune responses against MAP, peripheral blood mononuclear cells from 154 JD test negative and 96 JD test positive cows from the same dairy herds were stimulated with MAP *in vitro*. The activation of CD4+, CD8+ and γδ T cells and surface IgM+ B cells was measured using flow cytometry. CD4 + CD45R0+ T cells, γδ + MHCII+ and γδ + MHCII- T cells and SIgM+ B cells from JD test positive cows all exhibited increased proportions expressing CD25 after MAP stimulation, while CD8+ T cells did not demonstrate increased CD25 expression in response to MAP.

1. Introduction

Johne's disease (JD) is a chronic intestinal infection in ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (Coussens, 2001) with both subclinical and clinical stages of infection. Subclinical JD is characterized by intermittent shedding of MAP in the feces, a predominant cell-mediated immune response against MAP, and low-grade intestinal inflammation. Clinical JD is characterized by high fecal shedding of MAP, a predominantly humoral immune response against MAP, and high-grade intestinal inflammation (Coussens, 2004). This causes persistant diarrhea and subsequent wasting, leading to the infected animal's death (Koets et al., 2015). JD is of particular concern to the dairy industry in the US: an estimated 91% of dairy farms are contaminated with MAP (Lombard et al., 2013) and the economic loss due to JD may be anywhere from $200 million to $1.5 billion annually (Garcia and Shalloo, 2015).

Controlling the transmission of MAP within and between dairy herds remains difficult. A major hindrance is the difficulty in detecting MAP in infected cattle. Common diagnostic tests include detecting MAP shed in the feces via culture or PCR and detecting anti-MAP antibodies present in the serum or milk of infected cows. However, all common JD diagnostic tests are limited in their sensitivity. Tests to measure MAP shed in the feces have documented sensitivities ranging from 23%–60%, while tests measuring anti-MAP antibodies vary widely from 7%–94% sensitivity, depending on cattle being in subclinical or clinical stages of JD (Britton et al., 2016). A major limitation of these diagnostics is their dependence on the immune response of infected cattle to MAP; cattle with less advanced infection typically only shed MAP intermittently (Coussens, 2001) and may not produce anti-MAP antibodies. Only 33% of cattle that will eventually seroconvert to test positive are detectable at 2 years of age, while 94% of those cattle will test seropositive at 5 years of age (Nielsen et al., 2013).

Complicating efforts to improve diagnostics is the well-documented diversity in cow susceptibility and response to MAP infection. Variability has been observed in freshly isolated peripheral blood mononuclear cells (PBMCs) from cows with subclinical and clinical JD (Coussens et al., 2002); in PBMCs isolated from JD test positive cows after *in vitro* MAP stimulation (Coussens et al., 2003); and in PBMCs isolated from JD test positive Holstein or JD test positive Jersey cows (Verschoor et al., 2010). Diverse cytokine profiles have also been observed in cattle with subclinical and clinical JD (Coussens et al., 2004; Khalifeh and Stabel, 2004). This diversity in immunity against MAP is

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likely caused by a variety of factors, including host and MAP genetics, as well as the exposure dose and age of infection (Koets et al., 2015). Understanding the nature of the underlying causes for this diversity is important for improved control of JD in US dairy herds through the improvement of diagnostic testing and reduction of between- and within-herd transmission of MAP.

In this study, we characterized the diversity of T cell activation profiles from a large cohort of adult JD test negative (hereafter referred to as JD-) and JD test positive (hereafter referred to as JD+) cows in response to *in vitro* MAP stimulation. The ultimate goal of this project is to investigate the genetics of resistance or susceptibility to JD and in this manuscript we present results of the immune phenotyping portion of the project. PBMCs from JD- and JD+ cows were cultured with MAP, and T and B cell activation was characterized using four-color flow cytometry. CD4+, CD8+, γδ T cell and B cell activation was characterized using the marker CD25, the a chain of the IL2 receptor. We focused on surveying both T and B cell profiles because of previous research demonstrating immune activation profiles that are important for MAP infection control or JD progression. CD4+ T cell activity is critical: the progression to clinical JD is often attributed to a combination of a shift to Th2-dominated immunity and CD4+ T cell exhaustion (Koets et al., 2015; Okagawa et al., 2015). This shift is also associated with the production of systemic anti-MAP antibodies (Koets et al., 2015). In contrast, CD8+ T cell activation in response to MAP is delayed and less robust in comparison to CD4+ T cell acivation (Allen et al., 2011; Koo et al., 2004). Although less well-characterized than CD4+ or CD8+ ab T cells, γδ T cells have been shown to mediate granuloma formation and cytoxocity during MAP infection, as well as play an immunomodulatory role against CD4+ T cells (Plattner and Hostetter, 2011). γδ T cells may also play a critical role during early infection (Krueger et al., 2016). Differential responses between JD- and JD+ cows to *in vitro* MAP stimulation were then assessed.

2. Materials and methods

2.1. Animals, sample collection, and diagnostics

Animals enrolled in the current study were all mature Holtsein cows from eight different commercial Michigan dairies. Farms were selected based on voluntary enrollment from interested producers concerned about JD within their herds. Six farms independently tested for JD and two did not. Environmental contamination by MAP was established at each farm by sampling from the barn floor of each group. Ten ~100 g samples were collected with clean gloves from approximately equidistant locations around each group, placed into 700 mL Whirl-Pak bags (Nasco) and mixed well. Bags were submitted for diagnostic testing by mParaTeq fecal PCR assay (AntelBio®). All farms were confirmed to be contaminated by MAP. The prevalence of MAP contamination across groups ranged from 43% to 100% of total environmental samples from any particular farm.

Whole blood (30 mL) was collected by coccygeal venipuncture into Vacutainer tubes containing the anticoagulant ACD solution A (Becton Dickinson) using 20 G double-sided needles. Individual fecal samples (~100 g) were collected into 700 mL Whirl Pak bags. Serum was collected for subsequent diagnostic testing. Animals were considered to be JD test positive (JD+) if at least one of the three following tests was positive: milk ELISA, serum ELISA, and/or mParaTeq fecal PCR. Animals were considered JD test negative only when all available results (milk ELISA, serum ELISA and mParaTeq fecal PCR) were diagnosed negative (JD-). While it is possible that JD- cattle included in this study were persistantly infected with MAP and will seroconvert in the future, for the purposes of this manuscript JD+ denotes test positive animals and JD- denotes test negative animals.

Farm records were obtained from producers for all animals to monitor age, stage of lactation (days in milk (DIM)), and any health issues. In particular, serum ELISAs to test for bovine leukemia virus (BLV) infection were conducted in parallel with JD serum ELISA testing.

All animal use was reviewed and approved by the Michigan State University Institutional Animal Care and Use Committee (approval #09/15-134-00).

2.2. Cell isolation, culture, and stimulation

PBMCs were purified by Percoll density centrifugation. Two ACD-anticoagulated Vacutainer tubes of blood per cow were centrifuged at $930 \times g$ for 20 min at 22 °C in either a Jouan CR422 (M4 rotor) or Beckman Allegra 6R (GH-3.8 rotor). Buffy coats were carefully removed and applied to Percoll (GE Healthcare) density gradients (1.084 g/mL) and centrifuged at $360 \times g$ for 40 min at 22 °C to exclude erythrocytes and granulocytes. PBMC layers were then transferred to new 15 mL conical tubes and washed with 1X phosphate-buffered saline (PBS), pelleted at $600 \times g$ for 8 min at 22 °C twice, then resuspended in 2 mL 1X PBS. Cell counts were determined microscopically using a hemacytometer.

Table 1
Immune stains used to phenotype stimulated PBMCs in Johne's-positive or –negative cows.

Stain 1

Protein Marker	Cell Type	Isotype	Antibody Clone	Primary dilution	Secondary Antibody	Secondary dilution
CD45R0	Memory cells	IgG3	ILA116A	1:100	Goat anti-mouse IgG3 Alexa Fluor 488-conjugate (Life Technologies)	1:1000
CD4	Helper T cells	IgG2a	IL11A	1:100	Goat anti-mouse IgG2a PE-conjugate (Life Technologies)	1:200
CD8	Cytotoxic T cells	IgM	BAQ111A	1:100	Rat anti-mouse IgM PE-Cy7-conjugate (Affymetrix eBioscience)	1:200
CD25	IL-2 receptor; marker of activation	IgG1	CACT116A	1:100	Goat anti-mouse IgG1 Alexa Fluor 647-conjugate (Life Technologies)	1:1000

Stain 2

Protein Marker	Cell Type	Isotype	Antibody Clone	Primary dilution	Secondary Antibody	Secondary dilution
CD25	IL-2 receptor; marker of activation	IgG3	LCTB2A	1:100	Goat anti-mouse IgG3 Alexa Fluor 488-conjugate (Life Technologies)	1:1000
MHCII	Antigen-presenting cells	IgG2a	TH16A	1:100	Goat anti-mouse IgG2a PE-conjugate (Life Technologies)	1:200
SIgM	B cells	IgG1	BIG73A	1:100	Rat anti-mouse IgG1 PE-Cy7-conjugate (Biolegend)	1:200
TCR1-N24 (δ chain)	γδ T cells	IgG2b	GB21A	1:100	Goat anti-mouse IgG2b Alexa Fluor 647-conjugate (Life Technologies)	1:1000