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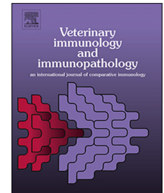
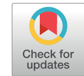


Research paper

Characterisation of peripheral blood mononuclear cell populations in periparturient dairy cows that develop metritis



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ABSTRACT

Bacterial contamination of the uterus following calving is ubiquitous in the dairy cow, 40% of cows develop postpartum uterine infection, including metritis. While predisposing factors like twinning and dystocia are associated with metritis, it is unclear why some cows remain healthy following calving and others develop uterine infection, negatively impacting animal health, milk production and economic return. Here, we profiled peripheral blood mononuclear cells of cows before calving and during postpartum metritis. We hypothesized that peripheral blood mononuclear cell function and proportions would be altered during the prepartum period in cows that develop postpartum metritis. Using flow cytometry we observed reduced proportions of peripheral $CD3^+/CD4^+$, $CD4^+/CD62L^+$, $FOXP3^+$ and $CD21^+$ populations from −10 to 40 days relative to calving associated with metritis, while the proportion of peripheral $CD3^+/CD4^+$ lymphocytes were specifically reduced in the prepartum period before the onset of metritis. Peripheral blood mononuclear cells from cows with metritis had a perturbed capacity to secrete IL-1β or IFNγ in response to in vitro stimulus; cells collected during the prepartum period from cows that would go on to develop metritis failed to increase IL-1β secretion in response to stimulation, while IFNγ secretion was altered at calving and postpartum in cows with metritis compared to healthy herd mates. No effect of metritis was observed in the capacity of cows to mount a humoral immune response to antigen administered on the day of calving. The studies discussed here suggest that while minor changes to the prepartum immune system are observed in cows that develop metritis, changes observed in the postpartum period are more prevalent and likely a consequences of disease and not causative. Future studies to modulate the prepartum immune system may help to limit postpartum metritis.

1. Introduction

Bacterial contamination of the uterus following calving is ubiquitous in the dairy cow (Griffin et al., 1974; Moore et al., 2017; Sheldon et al., 2002). While the majority of cows will be able to clear pathogenic bacteria, approximately 40% of all cows will develop postpartum metritis (Gilbert et al., 2005; Ribeiro et al., 2013; Sheldon et al., 2009). Why some cows in a herd develop metritis and others clear pathogenic bacteria is unclear.

Uterine infection, defined here as metritis or endometritis, is the result of pathogenic bacterial infection of the uterus during the first 3 weeks after calving. Resulting infection has significant negative impacts on the health and productivity of the dairy cow. It is estimated that uterine infection costs the US dairy industry in excess of $650 million per year due to reduced milk production, treatment costs and negative reproductive consequences (Drillich et al., 2001; Sheldon et al., 2009). Cows that resolve uterine infection display persistent reproductive deficiencies with a reduced calving rate, delayed conception and increased days open (Borsberry and Dobson, 1989; Gilbert et al., 2005; LeBlanc et al., 2002; Ribeiro et al., 2013). Negative reproductive consequences of uterine disease force producers to maintain increased replacement animals in the herd, further inflating economic and environmental impacts.

A number of risk factors have been associated with the development of metritis in the dairy cow including, dystocia, retained fetal membranes, twining and ketosis (Bruun et al., 2002; Dohmen et al., 2000; Potter et al., 2010). Many of these risk factors are associated with compromising the normal protective mucosa of the endometrium, leaving the stroma of the endometrium vulnerable to pathogenic bacterial infection. A number of studies have demonstrated the importance of bacterial strain in the development of metritis, with *Escherichia coli*, *Fusobacterium necrophorum* and *Trueperella pyogenes* being causative pathogens of disease, while it is interesting to note that non-pathogenic bacterial populations are present in the uterus of healthy cows during gestation and up to 7 weeks postpartum (Gilbert and Santos, 2016; Griffin et al., 1974; Moore et al., 2017; Sheldon et al., 2010). It has been

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surmised that postpartum negative energy balance plays a role in the predisposition of uterine disease in the dairy cow, potentially by compromising metabolically expensive immune function (Kvidera et al., 2017; Swangchan-Uthai et al., 2013). Little is known about the role of peripheral blood mononuclear cells in the development of uterine disease in the cow. While uterine disease can be treated with systemic antibiotics, treatment does not improve reproductive performance after resolution of disease (Drillich et al., 2001; Haimerl and Heuwieser, 2014). This is also the case with newly developed vaccines targeted to uterine disease causing bacteria (Machado et al., 2014). These observation may suggest that there are inherent difference in immune function of cows that develop uterine disease.

The postpartum innate immune system of the dairy cow has been studied extensively, and data suggests that the functionality of the innate immune response, particularly neutrophil function, during the postpartum period is associated with the development of uterine disease (LeBlanc, 2012; Martinez et al., 2012; Pinedo et al., 2013). Indeed, the innate immune function of the endometrium itself has also been shown to be perturbed during uterine infection and may play a role in uterine disease onset (Herath et al., 2006; Turner et al., 2016). Cows with active uterine infection have alterations in the proportions of peripheral lymphocyte populations postpartum (Hine et al., 2011). Rodent models of systemic immune deficiencies indicate susceptibility to infection, especially in severe scenarios of immune cell depletion, including irradiation, the severe combined immunodeficiency (**SCID**) mouse and nude mouse(Dickerson et al., 1983; Miller et al., 1960; Teles et al., 1997). Indeed, micronutrient deficiencies reduce immune competence in cows leading to increased disease susceptibility (reviewed in (Spears, 2000)). Here, we asked specifically whether populations and functionality of peripheral blood immune cells are altered in cows before and during metritis in the dairy cow. We hypothesized that functionality and proportional populations of specific peripheral blood immune cells are altered in the dairy cow prior to the onset of metritis. We profiled the periparturient proportions of specific peripheral blood lymphocyte populations by flow cytometry, including T-helper and T-cytotoxic cells, B cells, gamma delta (γ**δ**) cells (which are abundant in cattle) and forkhead box P3 (**FOXP3**) positive cells thought to have an immune regulatory function (although this is now challenged in cattle (Hoek et al., 2009)) (Mackay and Hein, 1989). We then assessed the functional capacity of peripheral blood mononuclear cells (**PBMCs**) to secrete immune modulating cytokines interleukin (**IL**)-1β and interferon gamma (**IFN**γ), and finally the ability of the cow to mount a humoral immune response to an inert antigen administered at the time of calving (reflecting the normal timing of uterine exposure to disease causing pathogens). We propose that these three factors contribute to the overall immune status of the cow and the capacity for defense from uterine pathogens. Therapeutic modulation of the immune system during the prepartum period may aid in reducing the incidence or severity of uterine disease in the dairy cow and circumvent the negative consequences of pathology.

2. Materials and methods

All reagents were acquired from Fisher Scientific (Waltham, MA) unless otherwise stated.

2.1. Animal use and clinical diagnosis of uterine disease

Holstein cows were housed at the University of Florida dairy research unit. All procedures were approved by the University of Florida Institutional Animal Care and Use Committee. The University of Florida herd is free of USDA APHIS notifiable diseases and conditions. A total of 12 cows were used throughout the entire experimental period starting 10 days prior to the expected calving date through 40 days in milk (**DIM**). Blood was collected every other day prior to calving and then every fifth day during the postpartum period via the coccygeal vein into EDTA vacutainers. Blood was processed according to the desired analysis platform outlined below. Uterine health of all cows was evaluated by in-house veterinary staff after examining vaginal discharge on day 3, 7, 10 and 21 postpartum according to Sheldon et al. (Sheldon et al., 2009). Rectal temperature was recorded on days of blood collection. Cows with uterine disease were classified as grade I or II metritis defined by the presence of purulent uterine discharge, without any systemic signs of ill-health, and without fever (> 39.5 °C). Cows categorized as grade III or IV metritis were excluded as they routinely receive antibiotic treatment. All cows were monitored for other clinical diseases including mastitis, lameness, ketosis and displaced abomasum. In the absence of other clinical disease during the postpartum period, cows were described as healthy or metritis and all samples retrospectively categorized. All cows were managed as a single group and those with metritis were not treated. Prepartum cows were fed once daily and postpartum cows were fed twice daily. Feed was supplied ad libitum as a total mixed ration. A total of seven healthy and five metritis cows were used throughout the experimental period.

Milk parameters (yield and components) were collected by the AfiLab milk analyzer (Kibbutz Afikim, Isreal) at each milking and compiled using AfiFarm software. Cow weight was collected daily as cows left the milking parlor.

2.2. Peripheral blood mononuclear cell isolation

Whole blood was collected from Holstein cows via the coccygeal vein into EDTA vacutainers (Becton Dickson, Franklin Lakes NJ) and centrifuged at 400 × *g* for 10 min at room temperature. The buffy coat containing PBMCs was aspirated and washed again in PBS by centrifugation at 400 × *g* for 10 min at room temperature. Cell suspensions were layered above Ficoll 1.078 g/ml Premium (GE Lifesciences, Pittsburgh PA) for density separation centrifugation at 400 × *g* for 30 min at room temperature. The cell layer was removed and washed in PBS at 400 × *g* for 10 min at room temperature. Washes were repeated at 300 × *g*, 200 × *g*, and 100 × *g* for 10 min at room temperature to remove platelets. Cells were either cultured for cell stimulation assays or used immediately for flow cytometry analysis (see below for details).

2.3. Flow cytometric analysis of peripheral blood lymphocyte populations

Freshly isolated PBMCs were resuspended at 1.5×10^6 cells/ml in cold PBS. Cells were aliquoted into FACS tubes in 200 µl volumes and pelleted by centrifugation at 400 × *g* for 5 min. For cell surface markers, cells were resuspended in staining buffer containing PBS/1% fetal calf serum and directly conjugated antibody (see Table 1 for antibodies and dilutions). Cells were incubated on ice for 60 min in the dark. Cells were washed three times in cold PBS by centrifugation at 400*g* for 5 min. For intracellular FOXP3, cells were fixed and permeabilized after washing using the commercial Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, Waltham MA). Following permeabilization cells were incubated with directly conjugated antibody for 60 min

Table 1
Antibodies used for flow cytometric analysis of peripheral blood lymphocyte populations.

Target molecule	Antibody clone	Fluorochrome	Dilution
CD3	MM1A (WSU)	Alexfluor-488	1:200
CD4	CACT138A (WSU)	Alexfluor-647	1:200
CD8α	CACT80C (WSU)	R-phycoerythrin	1:200
CD21	GB25A (WSU)	Alexfluor-488	1:800
CD62L	IVA94 (ThermoFisher)	Alexfluor-488	1:200
FOXP3	FOX5A (WSU)	R-phycoerythrin	1:100
γδ TCR (WC+)	ILA29 (WSU)	R-phycoerythrin	1:50

CD, cluster of differentiation. FOXP3, forkhead box P3. TCR, T-cell receptor. WSU, Washington State University, Monoclonal Antibody Center.

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