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Research paper

Mycobacterium avium sp. *paratuberculosis* (MAP) induces IL-17a production in bovine peripheral blood mononuclear cells (PBMCs) and enhances IL-23R expression in-vivo and in-vitro

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ABSTRACT

Johne's disease (JD) is a chronic inflammatory gastrointestinal disease of ruminants caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Control of JD is difficult largely due to insensitive diagnostic tools, a long subclinical stage of infection, and lack of effective vaccines. Correlates of protection are lacking in model systems of JD and the sources of inflammation due to JD are not well characterized. Commonly studied immune responses, such as the Th1/Th2 paradigm, do not adequately explain host responses to MAP. A potential role for non-classical immune responses to MAP, such as that mediated by Th17 cells, has been suggested. Indeed, MAP antigens induce mRNAs encoding the cytokines IL-23 and IL-17a in bovine peripheral blood mononuclear cells (PBMCs). IL-23 and IL-17a production have both been associated with Th17-like immune responses. Th17 cells are also defined by surface expression of the IL-23 receptor (IL-23R). To determine the relative prevalence of potential Th17 cells in PBMCs from MAP test positive and MAP test negative cows, PBMCs were isolated and analyzed by immunostaining and flow cytometry. Fresh PBMCs from MAP test positive cows (n = 12) contained a significantly higher proportion of IL-23R positive cells in populations of CD4+, CD8+, and Yδ + T cells than in cells from MAP test negative cows (n = 12; $p < 0.05$). Treatment with MAP antigens increased the percentage of all T cell subsets with surface expression of IL-23R when compared to untreated (n = 12; $p < 0.05$) cells. ELISA results for IL-17a secretion revealed a higher concentration of IL-17a secreted from PBMCs treated with MAP antigen (n = 20) than from PBMCs not treated with MAP antigens (n = 20) ($p < 0.001$), regardless of the JD test status of source cows. Also, we observed a moderate negative correlation between JD diagnostic scores for JD + cows and plasma IL-17a concentration (n = 42; $r = -0.437$; p-value < 0.004). Plasma with low and mid JD- scores (n = 31; n = 9; $0.1 \leq X < 0.3$) had significantly more IL-17a when compared to plasma with high JD- scores (n = 10; $0.3 \leq X < 0.46$; p-values < 0.05). Similarly, plasma with low JD + score values ($0.55 \leq X < 1.0$; n = 9) had significantly more IL-17a when compared to plasma with high JD + score values ($X \geq 2.0$; n = 21; $p < 0.05$). Overall, plasma from JD + cows ($0.55 < X \leq 2.86$; n = 41) had significantly less IL-17a than plasma from JD- cows ($0 < X \leq 0.46$; n = 70). Our data suggests that Th17-like cells may indeed play a role in early immune responses to MAP infection and development or control of JD.

1. Introduction

Johne's disease (JD) is a growing concern in both animal welfare and the dairy industry. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent behind JD (American Society for Microbiology, 1923; Coussens, 2001; Ignatov et al., 2012; Dudemaine et al., 2014). MAP infects the ileum of cattle, inducing inflammation and disruption of the intestinal lining, subsequently reducing the ability of clinically infected animals to absorb nutrients, and causing chronic incurable diarrhea. Clinical JD thus leads to reduced production, culling, and/or premature death. According to the 2007 National Animal Health Monitoring System (NAHMS; APHIS, 2007), the percentage of dairy operations infected with MAP in the US was approximately 68% and may now be as high as 91% (Lombard et al., 2013). JD economically represents a $200 million to $1.5 billion annual loss to the US dairy industry (Garcia and Shalloo, 2015). Cows infected with MAP can take years to present clinical signs of disease and often demonstrate a wide range of immune responses to MAP, within the same animal over time and across different animals within a herd (Frie et al., 2017). In cattle, vaccination against MAP can limit presentation of clinical

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symptoms, but does not prevent infection or shedding of infectious bacteria in feces (Stabel et al., 2012). Lack of knowledge regarding correlates of protection against MAP, the sources of inflammation leading to clinical disease, genetics of resistance or susceptibility to MAP infection, and a lack of definitive diagnostics all combine to result in perpetuation of infection at a herd level.

In general, later stages of JD appear to coincide with a classical Th2-like immune response with production of readily detectable antibodies against MAP antigens (Ganusov et al., 2015; Koets et al., 2015). Defining immune responses early in infection has been more difficult, although there is general agreement that a proinflammatory response is both appropriate and present in many infected animals. Sources of proinflammatory activity previously identified during early MAP infections include both classical Th1-like T cell responses and γδ T cell activity, both leading to production of IFNγ and TNFα (Begg et al., 2011; Dudemaine et al., 2014; Koets et al., 2002; Roussey et al., 2014). Dogma suggests that the classical Th1-like response declines over time with a concomitant increase in the classical Th2-like response (Ganusov et al., 2015; Roussey et al., 2014, 2016). This would suggest that the abundance of Th1-relatyed cytokines, including IFNγ and TNFα would also decline. Indeed, at sites of later-stage MAP infection the abundance of these two cytokines is not necessarily consistent with the degree of inflammation observed in infected tissues (Roussey et al., 2016; Khalifeh and Stabel, 2004; Coussens, 2004).

Two alternative candidate cytokines for inflammation characteristic of clinical JD are IL-1α and IL-1β. Expression of mRNAs encoding both IL-1α and IL-1β, as well as the respective proteins, are dramatically increased in ileal tissues of cows during late stages of Johne's disease (Aho et al., 2003; Chiang et al., 2007). Another, as yet unexplored, set of proinflammatory mediators that may play a role in MAP infections are IL-17a from Th17 cells and IL-23, which promotes a Th17 phenotype in responsive T cells, typically expressing the IL-23 receptor (IL-23R) (Ahern et al., 2010). In support of this notion, other Mycobacterium such as *M. tuberculosis and M. bovis*, are known to stimulate Th17 cytokines (Khader et al., 2011; Steinbach et al., 2016). Furthermore, mRNAs encoding cytokines that direct T-cell differentiation to a proinflammatory Th17-like phenotype are upregulated in PBMCs exposed to MAP in culture, including IL-6, IL-1, IL-23, and IL-17a (Roussey et al., 2014). This is also true in MAP-infected monocyte-derived macrophages (MDMs) (primarily IL-1β, IL6, and IL-23) (Dudemaine et al., 2014). These same cytokine mRNAs, particularly IL-17a and IL-6, are upregulated in early stage MAP-infected lesions (Grade 1) (Roussey et al., 2016). The Th17 signature cytokine IL-17a mRNA expression is upregulated in naïve helper T cells (CD4+ CD25-) from JD positive cows when these cells are co-cultured with autologous MAP-infected MDMs (Roussey et al., 2014). The relative abundance of IL-23R expressing T cells in MAP infected cows has not been previously reported.

In this study we began to examine the potential role of a Th17 response in MAP-infected cows as one explanation for inflammation in the ileum. We began by comparing IL-17a secretion levels from MAP-treated PBMCs derived from JD ELISA test-positive (JD+) and JD ELISA test-negative (JD-) cows by IL-17a ELISA analysis of culture supernatants. In addition to IL-17a, we began to define T-cell subtypes from JD + and JD- PBMCs by their corresponding T-cell surface markers (CD8+, CD4+, TCR1+) and their potential as Th17-like cells via co-expression of surface IL-23 receptor (IL-23R), an indicator of IL-23 responsive cells that could become Th17 cells. Lastly, in light of the known positive correlations between fecal shedding of MAP bacteria, stage of infection, and Johne's serum-ELISA score (Collins et al., 2005; Magombedze et al., 2017), we wished to determine if IL-17a levels in plasma might be correlated with Il-17a and IL-23R expression, as seen with *M. bovis* (Waters et al., 2016). Although our data do not support a direct correlation, we did observe consistent differences in mean plasma IL-17a levels in cows with differing JD serum-ELISA scores.

2. Materials and methods

2.1. Study animals

In this study we used mature Holstein cows with a similar lactation number (2nd–3rd lactation). JD + cows naturally infected with MAP and with a positive Johne's test result (serum ELISA) were readily obtained from commercial Michigan dairies. Farms for our studies were selected based on previous voluntary enrollment in JD studies. MAP environmental contamination had been assessed on each farm via fecal sampling from the barn floor for MAP (by fecal PCR) as described previously (Frie et al., 2017). The prevalence of MAP contamination in JD+ farm environments ranged from 43% to 100% of total samples. JD- cows were sourced from the Michigan State University Dairy Cattle Research and Teaching Center based on an extremely low herd prevalence of MAP infection combined with MAP-negative environmental testing results.

2.2. Preparation of PBMCs, MDMs, and treatments

Whole blood (30 mL) was collected by coccygeal venipuncture into 10 ml Vacutainer tubes containing the anticoagulant acid citrate dextrose (ACD) using 21 gauge (ga) double-sided needles. PBMCs were isolated from whole blood using a standard Percoll gradient centrifugation protocol (Frie et al., 2017). Plasma was saved for further JD diagnostic testing, as well as for assessment of circulating cytokine levels as described below. PBMCs were then plated at a density of 2.5×10^6 in 48-well flat-bottom plates, stimulated (see below), and cultured for 18 h in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2% Fungizone at 39 °C and 5% CO2. For cell stimulation, separate cultures of PBMCs were treated with 25 μg/mL PWM, a general T-cell stimulant, as a positive control. Test cultures received either 10 μg/mL purified protein derivative of Johne's (PPDj) or intact MAP at a multiplicity of infection (MOI) of 2. After incubation, cultures were transferred to 96-well round-bottom plates and centrifuged at 600X*g* for 5 min at 4 °C. Supernatants were saved at −80 °C for ELISA analysis and cells were resuspended in cold PBS for staining and flow cytometry analysis.

MDMs were generated by plating 2.5×10^6 PBMCs in 48-well flat-bottom plates and allowing monocytes to adhere to the plate surface for 4 h. After 4 h non-adherent cells were washed away with three warm (38 °C) PBS rinses. For MDM experiments, adherent monocytes were cultured as described above for PBMCs and allowed to differentiate into macrophages for 5 days. After differentiation, MDMs were treated with MAP at a MOI of 20 for 4 h (T1; Fig. 1) to allow phagocytosis of the bacteria for T1 and as part of treatment 3 (T3; Fig. 1). All remaining extracellular MAP was rinsed away with four warm PBS rinses prior to the addition of 2.5×10^6 PBMCs (Fig. 1). After a 20 -h incubation of MDM and PBMCs, MAP was added at a MOI of 2 (T2) against the total amount of cells in culture for T2 and the second exposure in T3 (Fig. 1). After an additional 18 -hs in culture, cells were spun down at 600 X G for 5 min at 4 °C (Murphy et al., 2007; Kabara et al., 2010). Supernatants were saved at −80 °C for IL-17a ELISA. All treatments (T) in this experiment were run concurrently.

2.3. Cell surface staining and flow cytometry

For staining of primary cells, PBMCs were washed in PBS and pelleted at 600 ×g for 5 min at 4 °C and then resuspended in primary antibody cocktail diluted in sterile First Wash Buffer (1X PBS with 10% acid citrate dextrose, 2% heat-inactivated horse serum (Gibco), 0.09% sodium azide) (Table 1). Plates were incubated at 4 °C for 30 min, washed, and pelleted. Supernatants were aspirated from the wells and cells were resuspended in secondary antibody cocktail diluted in sterile First Wash Buffer (Table 2). Incubation of cells with secondary antibody for 30 min at 4 °C was followed by another wash. Cells were then

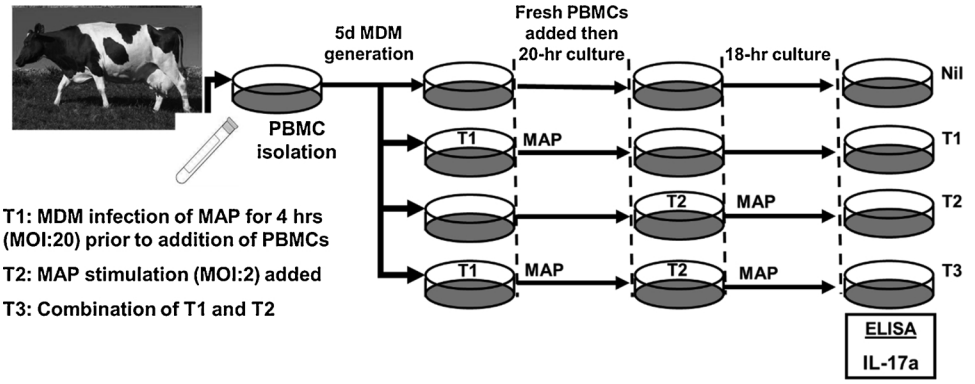

Fig. 1. Experimental set-up for analysis effects of MDMs on IL-17a production by PBMCs during MAP stimulation. MDMs co-cultured with autologous PBMCs and their respective treatments. Treatment 1 (T1) is defined as an initial infection of MDMs with MAP (MOI: 20), 20 h prior to the addition of PBMCs. Treatment 2 (T2) is defined as an uninfected MDM culture with a stimulation of MAP (MOI: 2) for 18 h after the initial 20 h and addition of PBMCs. Treatment 3 (T3) is defined as the combination of T1 and T2. Supernatants were collected after a total of 38 h in culture for IL-17a ELISA.

Table 1
Primary antibodies. Immuno-staining of T-cell specific surface markers and Th17 signature receptors and cytokines.

Antibody	Dilution	Company	Specificity	Clone	Isotype
CD4	1:100	WSU	Bovine	CACT138A	IgG1
CD8	1:100	WSU	Bovine	BAQ111A	IgM
TCR1	1:100	WSU	Bovine	GB21A	IgG2b
IL-23R	1:100	Sigma	Human	3D7	IgG2ak

Table 2
Secondary antibodies. Immuno-staining of T-cell specific surface markers and Th17 signature receptors and cytokines.

Antibody	Dilution	Company	Specificity	Clone
FITC	1:1000	eBioscience	α ms-IgG2b	m2b-25 g4
R-PE	1:200	Invitrogen	α ms-IgG2a	γ2a
PE-Cy7	1:200	eBioscience	α ms-IgM	m1-14d12
AF647	1:1000	Invitrogen	α ms-IgG1	γ1

pelleted, resuspended and fixed in 1X PBS containing 4% paraformaldehyde for 10 min at 4 °C. Fixed cells were washed, pelleted, resuspended in Second Wash Buffer (90% 1X PBS, 10% acid citrate dextrose, 0.09% sodium azide), and analyzed by flow cytometry (Accuri C6, Becton Dickinson USA, New Jersey). If immediate analysis was unable to be performed, plates were briefly stored at 4 °C. PBMCs were determined using forward and side scatter with log scaling on both the x and y-axis. Primary gating regards all events outside of the indicated PBMC gate as cellular debris (Fig. 4**A**).

After an 18 h culture with stimulants as described, PBMCs from JD + and JD- cows were incubated with CD4, CD8, TCR1, and IL-23R primary antibodies (Table 1) to measure Th17-like expression of IL-23R on the cell surface of helper T cells, killer T cells, and γδ T cells, respectively. Expression of IL-23R was used as an indication of how T cells are responding to natural MAP infection. Mean relative percent (MRP) of cells with surface markers was determined by positive gating strategies (Fig. 4**A**) using FCS Express 4 analytical software. Briefly, MRP of T cell subtype surface markers within gated PBMCs were analyzed by log side scatter on the y-axis and fluorescence height on the x-axis. IL-23R was observed within each of the T cell subtype gates using the same x and y-axis strategy (Fig. 4**A**). Unstained (without antibodies), negative primary control (secondary antibodies only), negative IL-23R (all primary antibodies except IL-23R and all secondary antibodies), and compensation (isolated primary antibody with the corresponding secondary antibody) controls were also included on each plate.

2.4. MAP culture

Isolated MAP cultures (American Type Culture Collection Strain #19698 (Murphy et al., 2007; Kabara et al., 2010; Roussey et al., 2014,) and K10 (Li et al., 2005)) were grown in Middlebrook 7H9 media with 10% oleic acid dextrose catalase (OADC) with 0.2% Mycobactin J supplementation. MAP cultures were maintained at 38 °C and kept in log phase until use. MAP was purified from cultures essentially as described previously (Janagama et al., 2006). Briefly, once cultures reached an OD 600 nm of 0.6, MAP was removed from culture media by centrifugation. Pellets were resuspended and rinsed three times with warm PBS using repeated cycles through a 25 g syringe to reduce MAP cell clumps. MAP cell concentrations were estimated as described previously (Janagama et al., 2006) where an OD 600 nm of 0.3 is set approximately equal to 109 bacteria/ml. Potential contamination of MAP cultures was monitored bi-weekly by inoculation of brain-heart infusion media with MAP culture aliquots and incubation for 72 h at 37oC. In all cases where not specifically mentioned, such as MAP K10, the MAP strain used was strain ATCC #19698.

2.5. ELISA and statistics

Secreted IL- 17a in culture supernatants was analyzed via ELISA (KingFisher Biotechnology) with a sample dilution of 1:2 using a capture antibody coating concentration of 2.5 μg/mL and detection antibody at 0.1 μg/mL. Plate construction and sample analysis were performed as recommended by the manufacturer with overnight incubation at 4 °C. Plasma samples were diluted 1:2 and analyzed for circulating IL-17a using pre-made plates and solutions (Sigma) following the manufacturer's recommended protocol.

All ELISA analytics were performed at 450 nm using a Molecular Devices Spectra Max M5 and SoftMax Pro 6.5.1 analytical software. Cytokine levels were analyzed using Kruskal-Wallis and Dunn's multiple comparison tests and two-way ANOVA with Tukey's multiple comparison test when applicable. Flow Cytometry was analyzed by one-way ANOVA and Dunnett's multiple comparison test or the non-parametric alternative Kruskal-Wallis and Dunn's multiple comparison test when applicable. Correlations were analyzed using Pearson's correlation coefficient. Cell surface staining between JD+ and JD- cows and between different treatments was compared using Student's t-tests. Statistical significance was set at $p \leq 0.05$ ($\alpha = 0.05$; chance of making a Type I error). Data were normalized when necessary using Log10. Experiments were performed using different sets of cows with a n determined by a power of 0.8 ($\beta = 0.2$; chance of making a type 2 error). GPower statistical software was used in each experiment to determine n using preliminary results and the above parameters. Due to biological variations of the cows in this study extreme outliers were assessed in each experiment set by Grubb's test and only the one most extreme within any group was removed ($\alpha = 0.05$) and would account for any differences of n within each experiment. All statistics other than power analysis were computed using Prism GraphPad statistical analysis software.

2.6. Johne's disease diagnosis

JD status was determined following manufacturer's protocol and guidelines of commercially available IDEXX ELISA for serum samples from cows. These assays were conducted by Northstar Cooperative Laboratories (Grand Ledge, Michigan). All Associated numbers and coordinating JD status ranges are predetermined by the manufacturer of the assay.

3. Results

3.1. Effect of MAP-infected MDMs on IL-17a secretion in PBMC cultures from JD- cows

Previous analysis of mRNA encoding IL-17a demonstrated that PBMCs from JD+ and JD- cows showed no difference between each other in the expression of IL-17a mRNA after 18 h of treatment with live MAP (Roussey et al., 2014). JD+ and JD- cultures both upregulated IL-17a mRNA expression when comparing MAP-stimulated cultures to unstimulated cultures. We wanted to replicate the MAP stimulated PBMCs to obtain an IL-17a protein secretion baseline. Considering that mRNA encoding IL-17a showed no difference in expression levels between PBMCs from JD+ and JD- cows, only PBMCs from JD- cows were analyzed to determine if MAP stimulated PBMCs were able to upregulate IL-17a secretion. In this study, we included MAP-infected MDMs or uninfected MDMs in co-culture with their autologous PBMCs to determine if the MDMs (plus or minus MAP) had an influence on IL-17a secretion from JD- PBMCs (Fig. 1). All cultures contained MDMs with autologous PBMCs that were added following differentiation and initial treatment with MAP or Nil for 20 h (Fig. 1). Treatment 1 (T1) is defined as having MAP-infected MDMs prior to the addition of PBMCs and without an additional MAP stimulation at the 20 h mark of the 38 h in culture (Fig. 1). Treatment 2 (T2) is defined as a coculture of uninfected MDMs and PBMCs with a MAP stimulation only after 20 h in culture together (Fig. 1). After a total of 38 h in culture, supernatants were analyzed by IL-17a ELISA as described in Materials and Methods. Our rationale was that a MAP-infected APC would better stimulate PBMCs to secrete IL-17a than only an exogenous or late MAP stimulation given that the difference of IL-23 mRNA production in MAP-infected MDMs from different time points IL-23 (Dudemaine et al., 2014). Kruskal-Wallace and Dunn's multiple comparison testing concluded that the cultures with MAP-infected MDMs (T1 and T3; n = 5/group) produced significantly more IL-17a than control cultures (Nil; uninfected MDMs/autologous PBMCs without MAP stimulation (Fig. 1); n = 5; p-value < 0.05 and p-value < 0.10 respectively; Fig. 2). Treatment 2 (T2) also appeared to increase IL-17a secretion, but this difference compared to the Nil samples was not statistically significantly in this model (n = 5 p-value = 0.11) (Fig. 2). All treatments (T1, T2, and T3) were statistically similar to one another (Fig. 2). All five cows enrolled in this preliminary experimentation are included in the analysis.

3.2. Secretion of IL-17a by PBMCs from JD+ and JD- cows stimulated with M. paratuberculosis (MAP)

To follow up on potential IL-17a secretion from JD- and JD+ cows, PBMCs from both disease statuses were cultured with two different strains of live MAP, purified protein derivative of Johne's (PPDj), and PWM as a general T-cell stimulant. Since JD- cultures showed no differences in IL-17a production when cultured without MAP-infected macrophages as mentioned earlier, additional JD- (n = 10) and JD+ (n = 10) PBMCs were cultured without MDMs and stimulated directly with either PWM, PPDj, MAP, or left unstimulated (Nil) (Fig. 3**A**). Indeed, we observed no significant differences in secreted IL-17a levels between cells from JD+ and JD- cows in any of the stimulated groups (PWM, PPDj, MAP) (Fig. 3**B**). However, regardless of the JD-status of the cow, live-MAP stimulation from either strain significantly

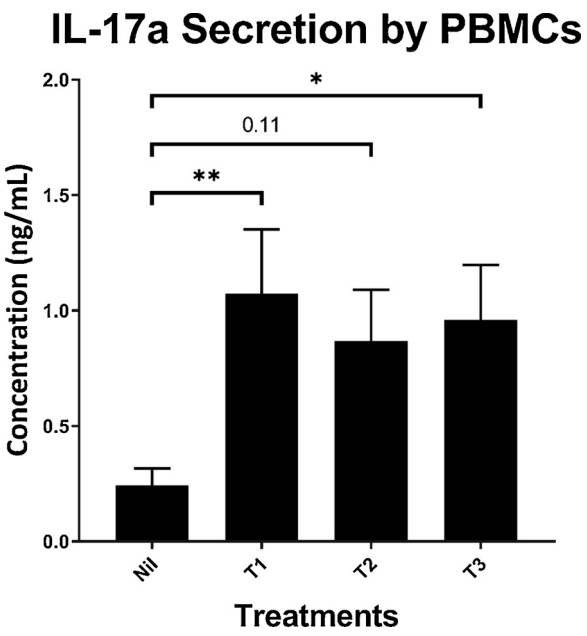

Fig. 2. Effect of MAP-infected MDMs on IL-17a secretion in PBMC cultures from JD- cows. IL-17a ELISA of supernatants from cocultures of MDMs with autologous PBMCs after 38 h total culture with their respective treatments (Fig. 1). n = 5/treatment. Analyzed by Kruskal-Wallace and Dunn's multiple comparison test. * = p-value < 0.10. ** = p-value < 0.05. Error bars = SEM. Grubb's Test = no outliers found in any group.

upregulated IL-17a secretion when compared to Nil cultures (MAP #19698 (n = 10/JD status) or MAP K10 (n = 5/JD status) (Fig. 3**B**; Two-way ANOVA and Tukey's multiple comparison test: Table 3). PPDj was not as strong of a stimulate and although it seemed to increase IL-17a when compared to Nil, it was not significant. MAP-K10 was added as an additional variable during the last set of cows as an added confidence to the MAP Strain #19698 commonly used in our lab. Each treatment had one extreme outlier as indicated by Grubb's test and is not included in this analysis.

3.3. IL-23 receptor (IL23R) expression on T cells from Johne's-test positive (JD+) and test negative cows (JD-)

Sensitivity to IL-23, is one factor that can also lead to a Th17 phenotype in T cells and is dependent upon expression of IL-23R on the surface of T cells. Therefore, we sought to study the basal expression of IL-23R on the surface of T cells from JD+ cows (n = 12) and compared this to expression levels on similar cells from JD- cows (n = 12). To further characterize which T cells were expressing IL-23R, we also stained for lineage surface markers CD4, CD8, and TCR1 ($\gamma\delta$ T cells) (Fig. 4**A**). Cells were classified as either IL-23R negative, having low expression of IL-23R (IL-23R^{Low}), or having a high expression of IL-23R (IL-23R^{High}). Unstimulated PBMCs from JD + cows showed a significantly higher mean relative percent (MRP) of IL-23R^{Low} expressing CD4+, CD8+, and TCR1+ cells than those from JD- cows (Fig. 4**B**; p-values < 0.01). The mean relative percent (MRP) of IL-23R^{High} cells in PBMCs from JD + cows was also higher than in cells from JD- cows for all T cell subsets (CD4+, CD8+, and TCR1+) (p < 0.05, p < 0.05, and p < 0.05 respectively) (Fig. 4**C**). Following an 18 -h stimulation with live-MAP or PPDj, the MRP of JD- cells with surface expression of IL-23R^{Low} was significantly higher compared to unstimulated T cell subsets from JD- cows (Fig. 4**D**; unpaired t-tests; p-values < 0.01, 0.1, and 0.01 for CD4+, $\gamma\delta$, and CD8+ respectively). The MRP for untreated JD+ cells are included in Fig. 4**D** for reference. No differences were seen with the expression IL-23R^{High} on T cell subsets from MAP-stimulated JD- PBMCs, nor was IL-23R^{Low} or IL-23R^{High} on T cell

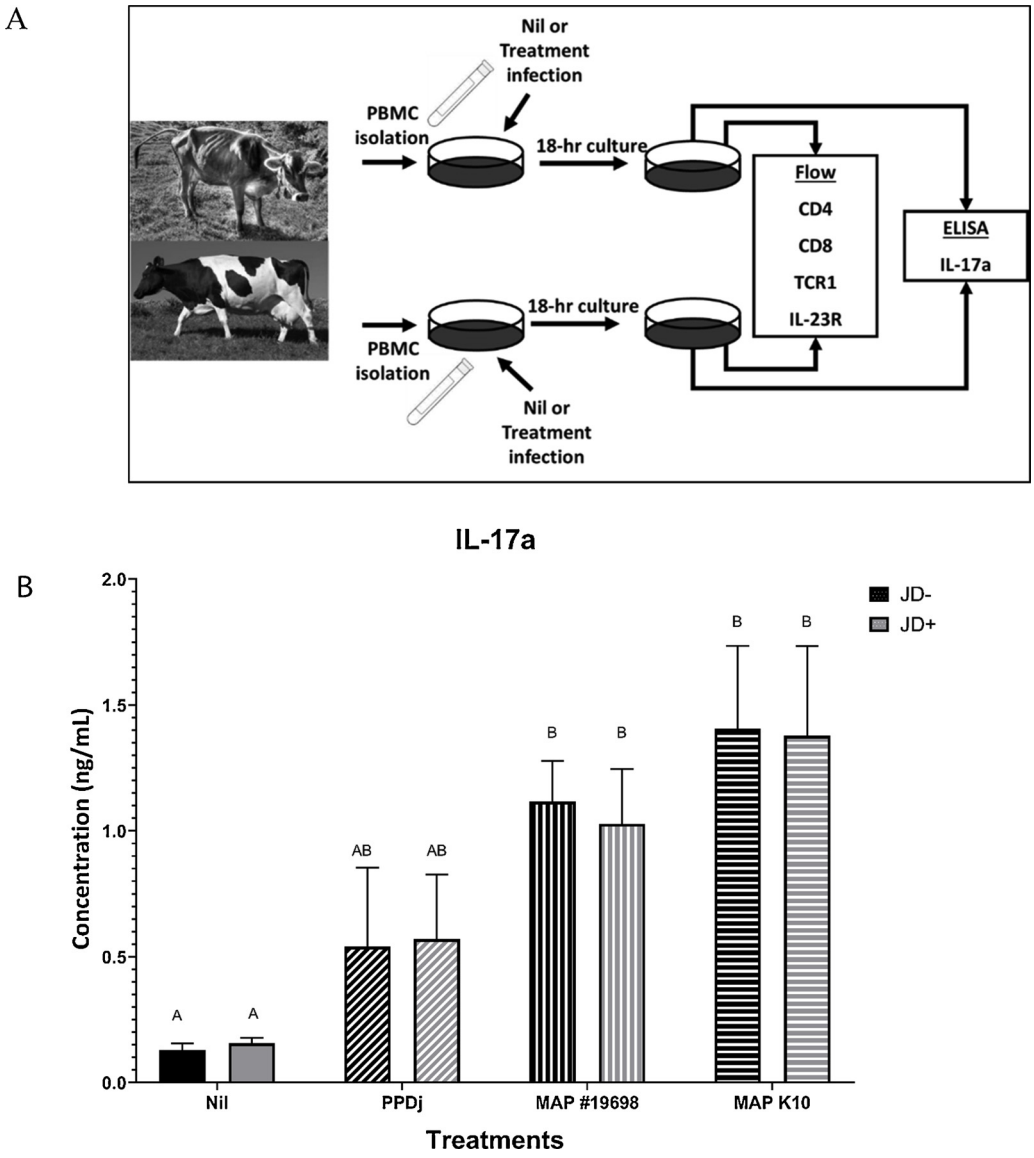

Fig. 3. A: Experimental set-up for analysis of PBMCs from JD+ and JD- cows stimulated with *M. paratuberculosis* (MAP). PBMCs from JD+ and JD- cows were cultured and left untreated or treated with PWM (25 μg/mL), purified protein derivative of Johne's (PPDj; 10 μg/mL), or MAP (MOI: 2) for 18 h. After incubation supernatants were collected and froze at −80 °C for later IL-17a ELISA analysis. PBMCs were stained for surface markers (CD4, CD8, TCR1, and IL-23R) analyzed using flow cytometry (Tables 1 and 2).
B: Secretion of IL-17a by PBMCs from JD+ and JD- cows stimulated with *M. paratuberculosis* (MAP). IL-17a ELISA of PBMC supernatants after 18 h stimulation with their respective treatments. Different letters indicate significant differences (Table 3); p-values by two-way ANOVA and Tukey's multiple comparison test. Error bars = SEM.

subsets from MAP-stimulated JD+ PBMCs when compared to their respective unstimulated groups.

3.4. Johne's-test positive cows (JD+) have less circulating IL-17a than Johne's-test negative cows (JD-)

MAP was able to generally stimulate production of IL-17a in PBMCs and increased the MRP of cells expressing IL-23R in PBMCs from JD- cows. Next, we wished to study the potential role MAP infection might play in circulating IL-17a levels in serum or plasma. To accomplish this, we first divided plasma samples from previously tested cows into groups based on Johne's ELISA OD scores. The diagnostic IDEXX MAP Antibody ELISA used to analyze plasma samples suggests that a S/P score (referred as OD in this paper) greater than 0.55 is considered a JD positive sample, samples scoring under 0.45 OD are considered negative, and OD values in-between are labeled as suspect. Cows with ELISA OD scores above 2.00 would be considered to be highly positive based on previous reports (Collins et al., 2005; Magombedze et al., 2017). We thus chose to initially study IL-17a levels in plasma from a group of cows with ELISA OD scores above 2.00 (highly JD+; n = 19), a group of cows from the same herd with an ELISA-value less than 0.03 (very low JD-; n = 18), and a third group consisting of JD- cows from a herd lacking any recent JD prevalence and an environment free of MAP (JD naïve cows; n = 20). We observed no significant difference in circulating IL-17a levels between JD- cows from either herd. However, cows with a JD+ ELISA OD score greater than 2.0 had a lower amount of circulating IL-17a than JD- cows from the same herd (Kruskal-Wallis and Dunn's multiple comparison test; p-value = 0.10) and significantly less than the JD- cows from the JD naïve herd (p-value < 0.05) (Fig. 5).

In order to refine our analysis and to gain a clearer picture of MAP effects on circulating IL-17a levels, we added more groups to the study, based on JD ELISA OD scores (regardless of originating farm). Groups were determined with the forethought of the previous OD score and stage of infection correlation (Collins et al., 2005; Magombedze et al., 2017). The resulting groups were: 1) low JD- (OD < 0.2; n = 50), 2) mid JD- (0.2 < OD < 0.3; n = 8), 3) high JD- (0.3 < OD < 0.46; n = 10), 4) low JD+ (0.55 < OD < 1.0; n = 9), and 5) mid JD+ (1.0 < OD < 2.0; n = 11) and high JD+ (OD > 2.00; n = 18) for a total of 6 groups (3 in each JD status group). Indeed JD- cows generally had significantly higher IL-17a in circulation than JD+ cows (Mann-Whitney; p-value < 0.05) (Fig. 6). However, Welch's ANOVA analysis revealed significant differences amongst the groups (p-value < 0.01) (Fig. 6). Using Games-Howell's multiple comparisons test, plasma from low JD- and mid JD- cows had significantly higher levels of IL-17a than plasma from high JD- cows (p-values = 0.07) (Fig. 6). Within the JD+

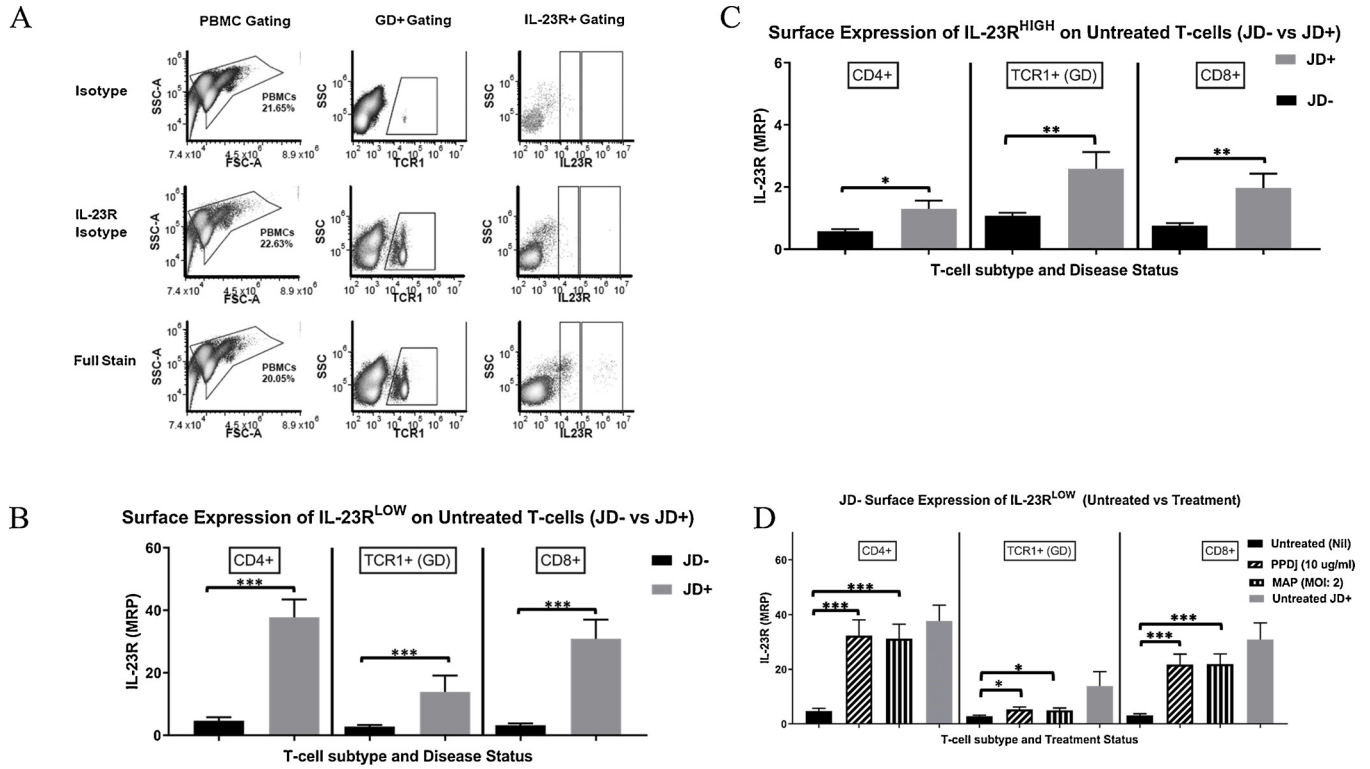

Fig. 4. A: Flow cytometry gating strategy example. Strategy includes no stain control (not shown), negative primary control (secondary only), and a negative IL-23R control (all other 1° Ab and all 2° Ab). This same strategy is used for gating of the other T cell subtypes and IL-23R. The IL-23R gating strategy within the T cell sub type populations uses the left gate as IL-23R^{Low} and the right gate as IL-23R^{High}. All events outside of the PBMC gate are considered debris.
B: Untreated JD+ vs. JD- IL-23R^{Low}. The mean relative percent (MRP) of T cell subtypes (CD4, GD, CD8) expressing a low level of IL-23R (IL-23R^{Low}) in unstimulated PBMCs from JD+ (n = 12, 12, 11 respectively) and JD- (n = 11, 12, 11 respectively) cows.* = p-value < 0.1. ** = p-value < 0.05. *** = p-value < 0.01. Error bars = SEM. Student *t*-test was used when all assumptions were met; Welch's *t*-test was used when samples passed normality testing but had unequal variance; Mann-Whitney test was used when normality of the sample was not met in one sample or both.
C: Untreated JD+ vs. JD- IL-23R^{High}. The mean relative percent (MRP) of T cell subtypes (CD4, GD, CD8) expressing a high level of IL-23R (IL-23R^{High}) in unstimulated PBMCs from JD+ (n = 12, 12, 11 respectively) and JD- (n = 11, 12, 12 respectively) cows. * = p-value < 0.1. ** = p-value < 0.05. *** = p-value < 0.01. Error bars = SEM. Student *t*-test was used when all assumptions were met; Welch's *t*-test was used when samples passed normality testing but had unequal variance; Mann-Whitney test was used when normality of the sample was not met in one sample or both.
D: Surface expression of IL-23R^{Low} on JD- T **cells after stimulation with MAP antigen** The mean relative percent (MRP) of T cell subtypes (CD4, GD, CD8) expressing a low level of IL-23R (IL-23R^{Low}) in stimulated PBMCs from JD- (n = 12/treatment/group respectively) compared to their respective unstimulated controls (n = 11, 12, 11 respectively). Untreated JD+ T cells (grey) is included as a visual reference for comparison only. * = p-value < 0.1. ** = p-value < 0.05. *** = p-value < 0.01. Error bars = SEM. Student *t*-test was used when all assumptions were met; Welch's *t*-test was used when samples passed normality testing but had unequal variance; Mann-Whitney test was used when normality of the sample was not met in one sample or both.

Table 3
2-Way ANOVA using Tukey's Multiple Comparison Test to compare all the means to each other. Reporting p-values.

Tukey's Multiple Comparison Test	p-value
JD- Nil V JD- MAP	0.025
JD- Nil V JD- MAP-K10	0.014
JD- Nil V JD+ MAP	0.056
JD- Nil V JD+ MAP-K10	0.018
JD+ Nil V JD+ MAP	0.070
JD+ Nil V JD+ MAP-K10	0.022
JD+ Nil V JD- MAP	0.032
JD+ Nil V JD- MAP-K10	0.018

group, cows with low JD+ OD scores had significantly more IL-17a in plasma than samples from the high JD+ cows (p-value < 0.01). High JD- scored samples include those from "JD suspect" cows, as defined above. The high JD- revealed significantly less IL-17a than low JD+ (p-value < 0.05). The low JD- also had significantly more IL-17a compared to high JD+ (p-value < 0.05).

To further analyze the trend seen within the disease groups, we conducted a correlation study between JD plasma ELISA OD scores and the amount of IL-17a within the sample. Circulating IL-17a levels were found to be moderately negatively correlated with JD+ cow OD scores as they increase (Pearson score (r) = −0.437; p-value < 0.004) (Fig. 7). No correlation was seen within the JD- group (not shown).

4. Discussion

Th17-like cells may be of potential importance in development of, or host response to Johne's disease (Roussey et al., 2014, 2016). The precise role of Th17 responses in Johne's disease are not clear at present and the existing literature can be conflicting. For example, IL-17a plays a protective role in some inflammatory diseases (Maxwell et al., 2015; Lee et al., 2015) and in mycobacterial infections when expressed early following infection (Palmer et al., 2016; Khader et al., 2011). However, chronic inflammation, a distinct feature of MAP infection, can also be induced by IL-17a (Leppkes et al., 2009) and by the Th17 promoting cytokine IL-23 (Neurath, 2007, 2019).

Promotion of a Th17-like phenotype over other T-cell phenotypes can be influenced by the immediate cytokine environment with IL-1β, IL-6, IL-23, and TGF-β all promoting a Th17-like phenotype in responsive T cells (Passos et al., 2010; Neurath, 2007; Santarlasci et al., 2009; Nyirenda et al., 2011). MAP is known to upregulate expression of

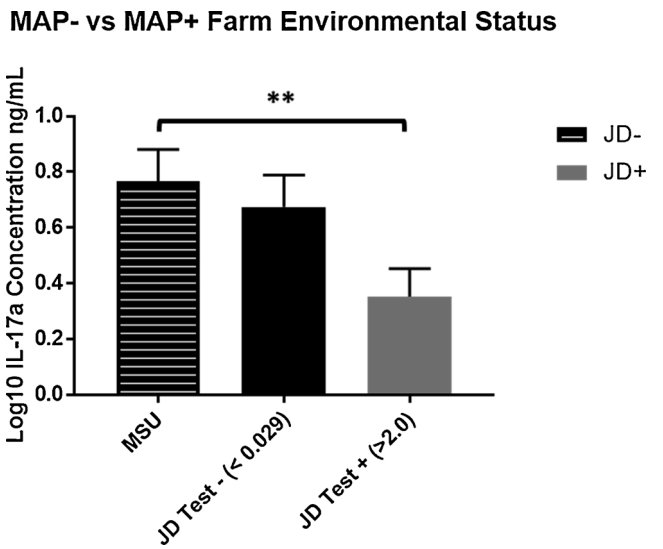

Fig. 5. Plasma IL-17a levels of cows based on environmental MAP status of farm and JD status. IL-17a concentration (pg/mL) in plasma by ELISA. JD+ cows (grey; n = 19) are from the same environmentally MAP+ commercial farm as the JD- cows (solid black; n = 18). JD- cows from an environmentally MAP- farm (black and white horizontal stripe; n = 18) are from the Michigan State University Dairy Cattle Research and Teaching Center. ** = p-value < 0.05. Error bars = SEM. Samples were Log_{10} transformed then Kruskal-Wallis and Dunn's multiple comparison were used for statistical analysis.

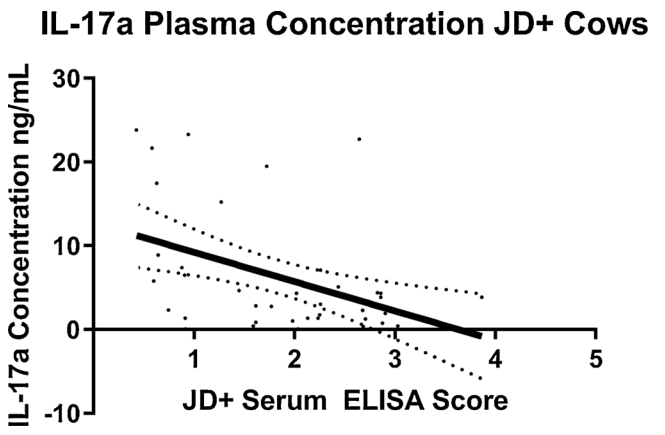

Fig. 7. Correlation of JD+ IDEXX ELISA score and IL-17a plasma ELISA concentration. Linear regression and correlation analysis. Pearson score (r) = −0.44. p-value < 0.004. n = 42.

mRNAs encoding IL-6, IL-23, IL-1β, and TGF-β as early as 1-h post infection in MAP-infected MDMs (Dudemaine et al., 2014), suggesting that the local cytokine environment near sites of MAP infection would tend to promote Th17 T cell development. One untested possibility is that IL-17a is helpful during early MAP infection, but failure to tightly regulated expression of IL-17a or IL-23 could lead to inflammation characteristic of clinical JD.

In this study, we sought to determine if the major cytokine product of Th17-like T cells, IL-17a was associated with MAP infection and Johne's disease. Previous work from our laboratory suggested that PBMCs from both MAP-positive (JD+) and MAP-negative (JD-) cows upregulated mRNA encoding IL-17a when exposed to MAP-infected MDMs (Roussey et al., 2014). These studies support the notion that MAP might be a general stimulator of IL-17a mRNA expression from PBMCs. To extend these observations to IL-17a protein secretion, we focused on co-cultures of MDMs (from JD- cows) and autologous PBMCs in the presence of different MAP treatments (Fig. 1). All treatments with MAP were able to significantly increase IL-17a production from PBMCs when compared to samples not exposed to MAP. A lack of differences between treatments indicated that prior exposure of PBMCs to MAP-infected MDMs was not required for live MAP bacteria to stimulate IL-17a secretion (Fig. 2). We therefore simplified our model to treatment of PBMCs with MAP only, without co-culture with autologous MDMs (Fig. 3A).

Data presented in this report demonstrate that MAP is indeed capable of increasing IL-17a levels produced by PBMCs. This effect is not dependent on the disease status of PBMC source cows, nor on the specific strain of MAP used. Our results thus suggest that MAP acts as a general and stimulator of IL-17a secretion from PBMCs. As to the possible mechanisms of MAP-induced IL-17a secretion, there is the possibility that monocytes or B cells in PBMCs are taking up MAP and presenting it to T cells. MAP may also be inducing IL-17a secretion more directly via a mechanism similar to Toll-like receptor (TLR) signaling. Experiments with purified populations of T cells and TLR blocking reagents will help to define these mechanisms. In either case, our results suggest that IL-17a may be part of an innate immune response to MAP.

T cells known to produce IL-17a, including during mycobacterial infections, are CD4+, CD8+, and γδ T cells (Roussey et al., 2014; Srenathan et al., 2016; Steinbach et al., 2016). Of note, γδ T cells (including IL-23R+ γδ T cells) can produce IL-17a independent of IL-23 and are an innate source of IL-17a (Lee et al., 2015; Zeng et al., 2012). However, long term expression of IL-17a in all cells studied to date requires IL-23 acting through IL-23R (Khader et al., 2011). Increased production of IL-23 has been observed in MDM cells infected with not only MAP (Dudemaine et al., 2014) but *Mycobacterium avium* isolates as well (Agdestein et al., 2014). Thus, macrophages could act as one source of IL-23 that would help promote IL-17a production in local regions of MAP infection associated with Johne's disease. Although not in vivo, previous work in our laboratory noted that mRNA encoding IL-

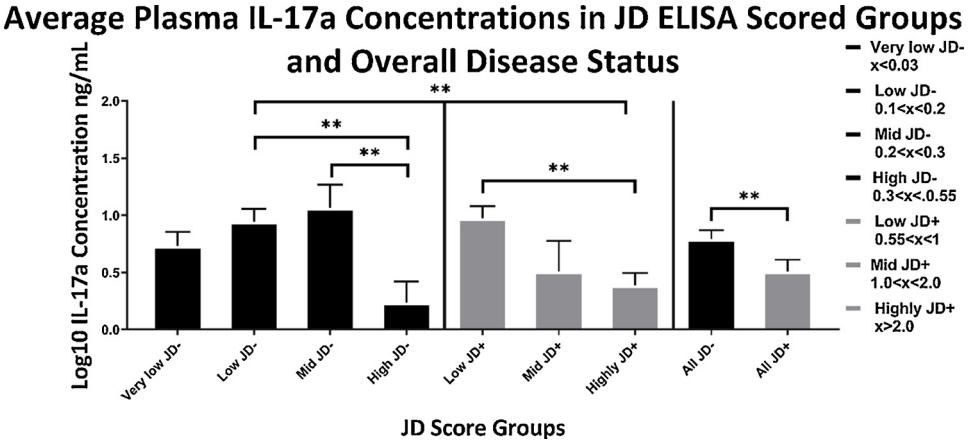

Fig. 6. Plasma IL-17a levels of cows based on IDEXX Johne's ELISA score. IL-17a concentrations (pg/mL) circulating in the plasma from the periphery of by ELISA. Low JD- (x < 0.2; n = 50). Mid JD- (0.2 < x0.3; n = 8). High JD- (0.3 < x < 0.55; n = 10). Low JD+ (0.55 < x < 1.0; n = 9). Mid JD+ (1.0 < x < 2.0; n = 11). High JD+ (x > 2.0; n = 18). Overall JD- cows (n = 68). Overall JD + cows (n = 40). * = p-value < 0.1. ** = p-value < 0.05. *** = p-value < 0.01. Error bars = SEM. Cow *n* is based on available stocked plasma samples. Welch's ANOVA and Games-Howell's multiple comparisons test were used in the observation of score groups. Mann-Whitney test was used between overall JD- and JD+ groups.

23 was upregulated in PBMCs stimulated with MAP relative to appropriate control cells (Roussey et al., 2014). Since mRNA increases do not always translate into enhanced protein production, additional experiments examining IL-23 and IL-17a secretion seem warranted.

In the current study, we demonstrated that the mean relative percent (MRP) of CD4+, CD8+ and TCR1+ ($\gamma\delta$) T cells expressing IL-23R^{Low} and IL-23R^{High} was greater in untreated PBMCs from JD+ cows than in untreated PBMCs from JD- cows (Fig. 4B & C). This novel data suggests that natural MAP infection causes circulating T cells to upregulate IL-23R expression. The significant increase of IL-23R on T cells from JD+ cows may suggest that JD+ T cells would be more responsive to IL-23. We also found that MAP antigen stimulation enhanced the MRP of IL-23R^{Low} T cells in PBMCs from JD- cows up to levels observed in circulating cells from JD+ cows. Thus providing evidence that MAP can indeed upregulate IL-23R expression on T cells. Although it did not appear that differences in IL-23R expression translated into more IL-17a production by JD+ PBMCs in our limited studies, examination of MAP-induced lesions may be more informative. It is unclear at this point what the difference is between cells with IL-23R^{High} and IL-23R^{Low}, but the level of IL-23R expression may be related to their specific function, as noted in other studies (Liang et al., 2013; Sivanesan et al., 2016). In addition, longer time studies and IL-23R expression as well as studies using recombinant IL-23 to stimulate cells from JD- and JD+ cows with subsequent measure of IL-17a secretion should be informative.

We lastly sought to understand the significance of IL-17a in plasma as it pertains to Johne's disease status. Overall, the mean IL-17a level in plasma from JD+ cows is significantly less than in plasma from JD- cows. Also, IL-17a levels have a moderate negative correlation with increasing JD+ MAP ELISA scores (Fig. 7). This correlation is also observed in the different JD+ disease score groups (Fig. 6). Roussey et al. (2014) demonstrated that CD4+ T cells from subclinical cows responded to MAP-infected macrophages in part by upregulating IL-17a mRNA expression, however, CD4+ T cells from clinical cows did not. This was further demonstrated in ileal tissues in which increasing MAP-burdens in lesions lead to decreasing levels of mRNA encoding IL-17a (Roussey et al., 2016). Thus, our results would appear to be entirely consistent with other reports. Dudemaine et al. (2014), demonstrated that cows with double MAP positive scores (fecal and serum) had significantly more IL-17a in plasma, while cows with only single MAP-positive scores (fecal) showed significantly less IL-17a than double negatives and double positives. Typically, the very late stages of Johne's disease are accompanied by a loss in both Th1 and Th2 responses. With this in mind, it is possible that the double positive JD cows studied by Dudemaine et al. (2014) represent an earlier infection stage than the single positives. Future studies will focus on which cells are responsible for either an innate or acquired Th17-like response to MAP, including potential MAP responding T cells and the potential role of epithelial cells, particularly with regard to IL-23 production. Studies within lesions from infected cows should also prove valuable in discerning the role of Th17 cells in MAP infections and Johne's disease.

To our knowledge, this study is one of the first to look at IL-23R as an expression marker in natural infection and in response to MAP antigens in culture. Previous studies have concluded that Th17 related mRNAs encoding IL-23 and IL-17a are increased in PBMCs and MDMs treated with MAP (Roussey et al., 2014; Dudemaine et al., 2014). Our data now confirm that IL-17a protein is indeed upregulated in cells from both JD- and JD+ cows in response to MAP antigen stimulation. Although IL-17a does not seem to be a good potential indicator of JD disease status for serum based diagnostic tools, it does add a potential layer of difference between subclinical cows (MAP infected) and cows who are clinically diagnosed. A major limitation of our work thus far is not identifying the mechanism of MAP-induced IL-17a secretion in cells from JD- and JD+ cows. Another unanswered question is the effect that IL-23 will have on cells from JD+ versus JD- cows, where our data suggests significant differences in IL-23R expression. Finally, it will be of interest to translate findings presented in this report to tissues with defined lesion scores from MAP infected cows.

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