UNIVERSITY OF CALGARY

Profiling immune responses in dairy calves experimentally infected with *Mycobacterium avium* subsp. *paratuberculosis* (*Map*)

by

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A THESIS

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Abstract

Johne’s disease (JD) is a debilitating chronic enteritis in ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Map*). JD causes substantial losses to the dairy industry due to reduced milk production, fertility rates and increased culling. The control of JD is complicated by the difficult diagnosis of the disease in its early stages and low sensitivity of the current diagnostics. As *Map* eventually survives in the host tissue despite of aggressive immune response, our aim was to profile early *Map* - induced immune responses in dairy calves infected with a high or low dose of *Map* for a 6-month period. The circulating T-lymphocytes were profiled by their phenotypic markers and signature cytokines. Regulatory T cell markers (Foxp3, IL-10 and TGF-β) were all upregulated during the first 2 months after infection, and a shift to Th1 response with robust IFN-γ production was observed, which lasted until the end of the study. Th2 response (IL-4) was very weak during the course of this study. Upregulation of CCR9 after 6 months of infection was also observed. In conclusion, regulatory T cell response that occurs early after *Map* exposure is vigorous and infective dose dependent but short lived; it is then replaced by a strong Th1 response and interferon-gamma production, characteristic of subclinical JD.
Acknowledgments:

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<thead>
<tr>
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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Foxp3</td>
<td>Forkhead box P3</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>iTreg</td>
<td>Induced regulatory T-cells</td>
</tr>
<tr>
<td>Th1</td>
<td>Helper T cell 1</td>
</tr>
<tr>
<td>Th2</td>
<td>Helper T cell 2</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
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<td>IgG</td>
<td>Immunoglobulin type G</td>
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<tr>
<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
</tr>
<tr>
<td>CCR9</td>
<td>Chemokine receptor type 9</td>
</tr>
<tr>
<td>CCL25</td>
<td>Chemokine (C-C motif) ligand 25</td>
</tr>
<tr>
<td>MADCAM-1</td>
<td>Mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>JD</td>
<td>Johne’s disease</td>
</tr>
<tr>
<td>LAM</td>
<td>Liboarabinomannan</td>
</tr>
<tr>
<td>Man</td>
<td>Mannose</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like receptors</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme - linked immunosorbent assay</td>
</tr>
<tr>
<td>IS900</td>
<td>Insertion sequence 900</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>PBMCs</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>LCM</td>
<td>Leukocyte complete media</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>I</td>
<td>Ionomycin</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette – Guerin</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>RPE</td>
<td>R-Phycoerythrin</td>
</tr>
<tr>
<td>APC</td>
<td>Alexa Fluor® Cy7</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbezidine</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>H$_2$SO$_4$</td>
<td>Sulfuric acid</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Δ ct</td>
<td>delta cycle threshold</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>AFB</td>
<td>Acid fast bacilli</td>
</tr>
<tr>
<td>HEPES</td>
<td>Hydroxyethyl - Piperazineethanesulfonic acid</td>
</tr>
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</table>
CHAPTER 1: INTRODUCTION

1.1 Johne’s disease (JD) in dairy calves

Johne’s disease (JD) is debilitating fatal chronic enteritis in ruminants caused by *M. avium* subsp. *paratuberculosis* (*Map*). JD has a substantial economic impact on dairy industry worldwide, causing major losses due to premature culling and reduced milk production. JD is characterized by a long incubation period and subclinical stage; it usually takes more than 3 years before the animal starts a typical clinical stage eventually leading to chronic diarrhea, emaciation, debilitation and death since there is no cure for this disease. Control of JD is problematic, as current diagnostics have low sensitivity and specificity, making the early diagnosis of *Map* a big challenge especially in the early stages of the disease in which the animal is infectious without being identified as *Map* positive. The prevalence of JD is very high in North America [1], costing the Canadian dairy industry $15 to $90 million annually as estimated by a previous study [2]. Sero-prevalence reported in Canadian dairy cattle ranges at the herd level from 10% in Ontario to 40% in Alberta [3]. An Alberta study with fecal samples suggests that the true herd-level prevalence ranges from 28 to 57%, estimated to be the highest in the country [4]. The characteristic long incubation period and subclinical stage make it challenging to diagnose this infection early enough to allow effective test-and-cull control strategies [3].

Neonatal animals are commonly believed to be most susceptible to infection [5]. Animals get infected usually via fecal-oral route through contaminated milk, colostrum, water or feed, but also the placental (vertical) route has been found in sheep [6] and cattle [7]. During natural *Map* infection, neonatal calves are infected either through ingestion of fecal material, milk or colostrum contaminated with *Map* [8].
JD pathology is classified into four main stages: silent infection with no symptoms or *Map* shedding, subclinical infection (carrier/infective stage) with no symptoms however decreased milk production and low fertility rates might occur with possible fecal shedding and positive fecal culture and/or serum ELISA, the clinical stage with diarrhea and weight loss as typical clinical symptoms, severe fecal shedding and positive diagnostic test results (fecal culture, IS900 PCR and serology) [9] and the advanced clinical stage in which the animals become lethargic, very weak and emaciated with persistent diarrhea and positive diagnostic test results. Intermandibular edema is characteristic for this stage due to malabsorption of nutrients from the inflamed intestinal mucosa leading to hypoproteinemia [10]. There are other classifications of JD according to the histopathological picture of tissue lesions as described in detail later.

While the mechanisms and causes behind the switches in disease stage are unclear, the onset of clinical stage could be accelerated by many stress factors such as viral infections, transport, lactation and parturition [11].

The mechanisms responsible for *Map* survival, persistence and intestinal invasion are also not clear. More efforts are needed to develop effective control strategies, which is not possible without a better understanding of the early *Map*-host interaction and pathogenesis of JD.

### 1.2 Mucosal immunity in neonatal calves

The adaptive immune system in newborn calves is relatively immature compared to adult cattle [12]. Various types of adaptive immune cells are present in their gut mucosa but they are mostly naïve. Specific adaptive immune reactivity improves through their life by exposure to various antigens in the environment or by vaccination. Newborn calves have generally smaller number of circulating blood lymphocytes than mature cattle, therefore they have lower levels of circulating
antibodies [13]. Increased numbers of innate effector cells (NK cells and WC1+γδ T-cells) in young calves may compensate for their immature adaptive system as these innate immune cells are more frequent and highly functioning (interaction with DCs) in neonatal cattle than adult animals [12].

The relative percentage of T-cells expressing γδ-TCR (γδ T-cells) are abundant in young cattle (before 6 month of age), they represent about 80% from all CD3+ lymphocytes in the circulation [14].

Previous studies done on young dairy calves have shown that there are age dependent changes in the composition and frequency of mucosal myeloid dendritic cells, CD4+, CD8+ and γδ T cells within epithelium and lamina propria of the small intestine. Populations of the previously mentioned cells were significantly increased with age as shown by Fries et al. [15] when weaned calves (6month old) were compared with newborn calves (2 to 3 weeks old). It was suggested that calves are more susceptible to get infected with Map at a young age of up to 6 month of age than older calves due to underdeveloped mucosal immune system [16]. Older animals (>10 months) seem to be less susceptible to develop JD [5], therefore the present studies focus on animals at a young age.

Other studies were done to explore the phenotype and functions of bovine regulatory cells. In 2007 [17] and 2008 [18] Seo et al. have found that bovine Foxp3+ cells have functional suppressor activity against CD4+ CD25+ T-cells in response to staphylococcal superantigen. However later in 2009, Hoek at al. [19] have demonstrated that CD4+ Foxp3+ T cells have a limited role in immune homeostasis and regulation in cattle, unlike their counterparts in humans and rodents, their findings were based on bovine in vitro co-culture suppression assays.
Furthermore, Chiodini and Davis et al. have also demonstrated in similar study that CD14$^+$ monocytes and γδ T-cells have a potential regulatory role and are able to produce IL-10 [20, 21] particularly in Map infection as Weiss et al. also demonstrated in their study on bovine macrophages [22, 23]. Additionally, DCs are also considered a significant source of IL-10 [22, 24].

1.3 Map infection; the pathogen

Map is an acid fast bacillus, and a member of Mycobacteriaceae family which is characterized by their lipid rich cell wall consisting mainly of mycolic acids with lipoarabinomannan (LAM) as one of the major antigens. It belongs to the Mycobacterium Avium Complex (MAC), with the M. avium subsp. hominissuis, M. avium subsp. avium (MAV) and M. avium subsp. silvaticum as other important members of the complex. Map is the subspecies characterized by slowest rate of growth [25].

Map is a facultative intracellular pathogen which means that it cannot survive outside the host cells. It multiplies and survives inside the host macrophages leading to granuloma formation and lymphocyte infiltration of intestinal mucosa, all playing a significant role in disease progression [26].

Bacteriological diagnosis of Map could be done by conventional culture method on solid or liquid media followed by Ziehl-Neelsen (ZN) staining of acid fast bacilli (AFB) and it is useful for diagnosis and categorization of an animal as a fecal shedder (low, moderate or severe). However, this method is slow and has poor sensitivity, especially in early stages when a rapid decision could be useful in control strategies [27].

Fecal shedding usually occurs before detectable antibody response arise [28]. It can be investigated by fecal culture followed by Map specific PCR (IS900) which is considered a gold
standard for *Map* diagnosis (100% specificity) and the best confirmatory test of positive culture result (growth of characteristic mycobacteria’s colonies with acid fast staining), serology and IFN-γ assay [9]. However, fecal shedding usually occurs in later stages of infection and thus considered an imperfect test. Apart from the intermittent and low level shedding in the subclinical stages; positive results might also occur due to the passive shedding of *Map* (bacteria just pass through the gut without establishing infection) [29]. Direct fecal PCR is an alternative method to detect fecal shedding and might detect dead and non-culturable *Map* bacteria with an equal sensitivity to fecal culture [29]. On the other hand, indirect detection of infection by measuring *Map* specific antibodies in serum and milk by ELISA, also has low sensitivity due to delayed humoral immune response and antibody production [27, 29] at this stage of the infection. In silent infection, the screening tests like Johnin skin testing and IFN-γ ELISA could be used to detect cellular immune response. A major disadvantage of those methods is the cross reactivity of *Map* antigens with other environmental mycobacteria *spp.* due to the low specificity of those tests [30, 31].

Another disadvantage of IFN-γ ELISA test is the challenge of getting fresh blood samples from the farm to the laboratory within the recommended time period (8 h) in order to preserve the necessary viability of the blood cells.

**1.4 Pathogenesis and host immune response to *Map***

In *Map* infection, the mycobacteria enter the small intestinal mucosa in the ileum and invade the Peyer’s patches via M-cells [32, 33], the subepithelial macrophages in Peyer’s patches engulf *Map* bacilli in order to destroy them. However for some reasons that will be described later, the macrophages usually fail to kill the mycobacteria which survive and multiply inside the unactivated macrophages that turn into a protective shelter and a supplier of essential growth
elements for *Map*. Then, the multiplying *Map* bacilli eventually kill the cells and spread to infect other cells in the gut establishing a persistent infection and subsequent chronic granulomatous enteritis [34] which is the hallmark of JD.

The pathology caused by *Map* results microscopically in severe transmural inflammation of lamina propria, intestinal lymphadenopathy, lymphangitis, granuloma formation and infiltration with Langhans giant cells, lymphocytes and macrophages [25, 35]. Acid fast bacilli can be found in the microscopic lesions as described by Dennis et al. [36].

Macroscopic lesions include thickening, corrugation, hyperemia and focal necrosis associated with ulceration and haemorrhages of the intestinal mucosa. Those lesions are observed mostly in the ileocaecal junction and adjoining segment of ileum and caecum, with enlargement of regional lymph nodes (especially mesenteric LNs), then diffuse inflammation of the colon at the clinical stage of the disease [35].

The histopathological lesions of JD have been classified by Dennis et al. [36] based on the number of bacilli in the granulomas as either multibacillary or paucibacillary or as mild, moderate and marked, depending on severity. They have been also classified according to the extent of granulomas as focal, multifocal and diffuse [35]. Lesions have been also classified by Balseiro *et al.* [37] as tuberceloid or lepromatous based on proportion of lymphocytes, macrophages and acid fast bacilli infiltrating the lesions. A single classification that is able to include all the different types of the lesions is difficult to achieve, as the lesions are usually attributed by many factors such as different stage of the disease, different strains of the organism, and the immunological or genetic status of the host [35, 38].
Map antigens as pathogen associated molecular patterns (PAMPs), most importantly Man-LAM (Mannose-capped lipoarabinomannan), are recognized by the pattern recognition receptors (PRRs) expressed on DCs, and other antigen presenting cells. Main PRRs involved in this process are TLR2, TLR4, TLR9, NOD2, mannose receptors, Dectin-1 and DC-SIGN (as C-type Lectin receptors) [39, 40]. Activated DCs transmigrate to mesenteric lymph nodes where they process and present Map antigens to naïve T-cells; that interaction induces T-cell polarization into various T-cell subsets including the activation of γδ T-cells [41]. Proper mechanisms of antigen presentation are critical at this stage for developing an effective immune response against Map. However, Map has been shown to be able to interfere with these mechanisms as described in more detail below. Cytokines like IFN-γ and chemokines are produced by the activated cells for further leukocyte recruitment, induction of phagocytosis and intracellular bacterial killing mechanisms of the phagosome. Tissue injury occurs due to the activation of macrophages and aggressive production of reactive oxygen species (ROS), proteolytic enzymes and other inflammatory mediators [19].

Dendritic cells (DCs) are involved in the immune response against mycobacterial infections as one of the main antigen presenting cells. Their main function is to engulf the pathogen, then combine its foreign antigen with the MHCII receptors on their surface in order to present them to the naive T-cells in the secondary lymphoid organs such as Peyer’s patches. Therefore DCs play a critical role as key link between innate and adaptive immune response [15] as they induce naïve T-cells to undergo polarization into variable T-cell phenotypes according to the type of cytokine released from different types of immune cells that activates specific signaling pathways and transcription factors. Naïve T-cells undergo polarization into various T-cell phenotypes according to the type of signals (cytokines) they receive from other cells of the immune system.
Treg cells are activated by TGF-β and secrete IL-10 and TGF-β to down-regulate other T-cell phenotypes (Fig.1). Each T-cell phenotype has a specific role in the immune system. In this study, we focused on CD4+ T-cells that have two subsets T helper-1 (Th1) cells, T-helper-2 (Th2) and iTregs. Th1 cells release - and are activated by - IFN-γ as a pro-inflammatory cytokine, and have as a main function to activate the cell mediated immune response and induce MHC-II expression on APCs for proper antigen presentation. IFN-γ also activates intracellular bacterial killing mechanisms in the macrophages. T helper-2 (Th2) cells release IL-4, IL-5, IL-6, and are required for the activation of B-lymphocytes to differentiate into plasma cells and produce immunoglobulins responsible for the humoral immune response. Regulatory T-cells are now a well recognized critical component of the mammalian immune system [20], Tregs are either naturally occurring (thymic-derived) or antigen induced; we focused on the induced (iTreg) subset in this study.

Treg cells that are antigen induced (iTreg), with CD4+CD25+/−Foxp3+ phenotype, have as a main function to maintain immune homeostasis through the production of anti-inflammatory cytokines (IL-10 and TGF- β), they provide tolerance to self-antigens and modulation of host immune response during infection in order to avoid overstimulation and excessive production of pro-inflammatory cytokines, inflammatory mediators and chemokines that could harm the host cells and tissues causing self induced immune-pathology. Treg cells also express CTLA-4 [42], a co-signaling molecule, and CD28 antagonist. Moreover, they release anti-inflammatory cytokines TGF-β (Th3 cells) and IL-10 (Tr1 cells) [43, 44], while their key activator is TGF- β. Treg cells express Forkhead box P3 (Foxp3) transcription factor as a unique marker [45, 46].
The immune response against *Map* is similar to other mycobacterial infections. Cows usually mount a cellular immune response with the activation of many cells, especially T-lymphocytes and the production of cytokines, most importantly IFN-γ to activate macrophages and their bacterial killing mechanisms. Previous studies demonstrated that *Map* infection is accompanied by an early pro-inflammatory Th1 response in the subclinical stage, followed by a predominant but ineffective antibody-mediated Th2 response as the disease progresses [41, 48] to the clinical stage. The induction of a cellular immune response is considered a key step in creating an effective immune response against *Map* and controlling the infection without developing clinical signs, but instead the animal remains in the subclinical stage as a carrier for life [48]. When the cellular immune response cannot control the infection, the host immune system shifts to a humoral response and, as a result, antibody production and eventually the development of the
clinical picture of JD with increased bacterial shedding and progression of the disease into later stages [49, 50].

Several mechanisms have been suggested regarding Map intracellular survival and virulence mechanisms such as Map’s ability to cause inadequate phagosome-lysosome fusion and abrogation of oxygen radical production by the infected cells [51, 52], thus interfering with phagosome maturation and limiting efficient bacterial killing and antigen presentation to other immune effective cells specially T-cells. Therefore, Map is eventually capable of evading host’s first line of defense. Furthermore, it has been demonstrated that Map interferes with the IFN-γ induced signaling in the monocytes [53]. Mycobacteria produce catalase and peroxidase that makes it resistant to most phagocytes and their oxidative killing mechanisms [54, 55]. As another potential virulence mechanism, Map was described to cause repression or subversion of the antigen presenting mechanisms by MHC-II on DCs and other antigen presenting cells by signaling through DC-SIGN receptors and overproduction of IL-10 [56]. Map also prevents apoptosis in infected macrophages [57] and reduces the activation of macrophages by T-cells via CD40 receptor [58], therefore interfering with proper antigen presentation required for effective immune response. Together these suppressing mechanisms of early immune responses are believed to lead to a favorable environment for Map to survive in the host tissues.

Human studies have shown that in mycobacterial infections there is an early activation of γδ T-cells directly by mycobacterial superantigens via interaction with V regions of the TCR that do not require specific antigen recognition by V-J region of the MHC [59]. Activation of γδ T-cells requires less antigen threshold than αβ T-cells and is independent of co-stimulation with CD28 [60]. Thereby, γδ T-cells produce robust IFN-γ and TNF-α response [61] to activate phagosome-lysosome fusion in the macrophages [62], but disease outcomes in terms of pathogenesis and
clinical picture are dependent on many factors affecting the host immune response to mycobacteria.

The outcome of mycobacterial infection indeed greatly depends on a variety of genetic factors and the immune background [63]. Host immune response can either control mycobacterial infection without developing any clinical symptoms, leading to a latent infection in which the mycobacteria stay dormant inside the macrophages [64], or the immune system fails to control the infection which sends the host on a path to develop clinical disease. The disease susceptible host (with genetic or immune defects) might develop a clinical disease with granulomatous formations and production of proinflammatory cytokines with all its pathological consequences [41].

During the clinical stage of JD, there is a severe depression of the circulating CD4⁺ T-cell response that becomes unresponsive (antigen specific anergy) to further stimulation by Map antigens [65] accompanied typically by increased anti-Map serum IgG levels [17]. The specific role of Treg cells in this type of infection and whether it has a role in early suppression of effective immune response against Map that might contribute to the survival of Map inside the host, causing all the previously mentioned consequences and pathology of JD, is not clear. Early activation of regulatory mechanisms with the production of anti-inflammatory cytokines, which are mainly IL-10 and TGF-β, could be playing an important role in the attenuation of macrophages and their intracellular bacterial killing mechanisms through insufficient production of IFN-γ [66]. As IFN-γ is a key cytokine for the activation of macrophages, any factor that interferes with sufficient IFN-γ production and/ or signaling could contribute to Map survival inside the host [67, 68].
The intestinal mucosa is the first and largest body surface to interact with exogenous ingested pathogens. Therefore, numerous unconventional T-cells continuously reside in the lamina propria and intestinal epithelium to take up foreign antigens, then undergo activation independently of antigen presenting cells in the secondary lymphoid organs [69].

Despite this fact, the vast majority of activated T-lymphocytes in the gut primed against a particular antigen are actually recruited into the intestinal mucosa subsequent to their activation in the secondary lymphoid organs and mesenteric lymph nodes. Antigen induced T-lymphocytes circulate in the blood, then home into the intestinal mucosa where inflammation takes place. They express special gut homing molecules allowing them to interact and transmigrate across the intestinal capillary endothelium [69].

The potential role of gut homing receptors after Map infection is not known and they might be playing an important role in the intestinal pathogenesis occurring after Map infection. Two gut homing receptors present on the circulating T-lymphocytes are of particular interest: the CC-chemokine receptor 9 (CCR9) and α4β7-integrin. Their ligands are CC-chemokine ligand 25 (CCL25), and mucosal vascular cell-adhesion molecule 1 (MADCAM1) respectively (Fig. 2). CCL25 and MADCAM1 are expressed preferably in the small bowels’ vasculature to recruit T-lymphocytes by enabling transmigration across the intestinal capillary endothelium in order to be activated via antigen presenting cells of the Peyer’s patches and mesenteric lymph nodes [69]. In Map infected cattle, the expression of adhesion molecules like LFA-1, CD26L and LPAM-1(α4β7-integrin) on cells from their milk and blood became higher upon parturition and lactation [70]. Crohn’s disease is a multi-factorial chronic intestinal inflammation in humans, and it shares lots of similarities with Johne’s disease in terms of clinical picture, intestinal lesions and pathogenesis. Human studies have shown that the expressions of gut homing receptors are
altered in circulating memory T-cells in Inflammatory Bowel Disease (IBD) patients compared to healthy controls. The resulting disturbed leukocyte recruitment into intestine is believed to be essential to initiate and maintain inflammatory bowel disease (IBD) in humans [71].

Figure 2: Recruitment of T-lymphocytes into the intestinal mucosa (modified from Agace et al. 2008) [72] depicting circulating T-lymphocyte bearing CCR9 and α4β7-integrin as gut homing receptors and CCL25 and MADCAM-1 as their ligands on the high endothelial venules of intestinal mucosa.
1.5 *Map* and Crohn’s disease (CD); a zoonotic potential

Crohn’s disease is multifactorial, involving the interaction between genetic, environmental triggers and immune-mediated tissue injury. Environmental triggers include infectious agents and diet [73]. The role of *Map* as a causative agent of CD and the association of the organism with CD has been longstanding topics of controversy. Recent meta-analyses demonstrate that the association of *Map* with Crohn's disease in humans is specific and undeniable, although a causal role has not yet been demonstrated [74], neither has the transmission from animal to human been proven.

*Map* could be transmitted to humans through contaminated milk, milk products or water, thus is being considered a concern for the food industry [25], [75] although it is not proven to cause a disease in the normal population.

The genetically very closely related *M. avium subsp. hominissuis* cannot cause a disease in healthy human host (opportunistic infections) but can in immune-compromised hosts such as AIDS patients [25], or in hosts with genetic defects especially in the genes responsible for the killing and clearance of intracellular pathogens like ATG16L1 or NOD2 gene, implicated in Crohn’s disease [64], [76]. There are multiple similarities between CD and JD, both diseases cause non-caseating chronic granulomatous intestinal inflammation and primarily target the ileum.

While the debate about the role of *Map* in CD is unresolved, several studies have provided indications to support such a hypothesis. *Map* and *Map* DNA (IS900) were isolated from CD patients’ blood [77], feces [78] and resected gut tissues more than from healthy controls [64], and the genotyping of the isolates revealed high genetic similarity to bovine isolates [79]. Also, *Map* specific humoral immune responses were detected in the serum of CD patients [80], [81].
Furthermore, some cases of CD were improved after treatment with antibiotics against intracellular pathogens using a triple therapy consisting of rifabutin, clarithromycin, and clofazimine [82, 83].

The spectrum of clinical and pathological manifestations that occur in CD are similar to JD. Similarities appear in the cytokine profile and correspond with a persistent Th1 driven cell mediated response and the common granulomas in both disease. All this together has leads to the speculation that perhaps a more effective therapeutic strategy for CD will be achieved by targeting both mycobacteria and inflammation [84].

1.6 Rationale:

It has been demonstrated in previous studies that early cellular and humoral immune responses specific to Map infection could be profiled using T-cell activation markers [48, 85, 86].

T-lymphocytes are involved in the early immune events that occur after infection with Map and likely determine whether infection proceeds to clinical disease. The breakdown of gut homeostasis activates T-lymphocyte reaction against antigens from external pathogens and un-tolerizable antigens (self or gut microbiota), leading to the recirculation of memory T-lymphocytes in the blood stream. In addition to the scientific prospective, blood collection is a low invasive procedure and requires a fairly easy process to test for markers in the laboratory.

We had a unique opportunity to benefit from an experimental infection trial with Map in dairy calves to study immune cell profiles in a model of inflammatory bowel disease with a known causative agent. In this trial, nineteen calves were infected with a virulent Map strain at young age (1-6 months old). Nine calves received a relatively high bacterial inoculum dose of $10^9$ CFU
and ten calves received a relatively low dose of $10^7$ CFU (lowest confirmed and consistent infectious dose for young calves).

Therefore; Th1, Th2 and Treg populations were profiled using their signature cytokines, surface and intracellular molecules as markers at different time points after infection. We analyzed circulating T-lymphocytes as they are directly linked and influenced by local gut inflammation. The expression of Foxp3 (Treg cells) and gut homing receptors at this early stage of the disease are not clear, in the presented study we focused on those markers expressed by the circulating T-lymphocytes. Hereby we focus on three populations of T-lymphocytes (Th1, Th2 and iTreg). The subsets of T-lymphocytes which are defined by their released signature cytokine were examined in peripheral blood mononuclear cells (PBMCs) from Map infected cattle. Three major T cell subsets were studied: T helper 1 (Th1; secreting IFN-γ), T helper 2 (Th2; secreting IL-4), and regulatory T cells (Treg; secreting TGF-β1 and IL-10 and expressing Foxp3 as unique transcription factor).
Hypothesis

We hypothesize that factors like the age of the calf, *Map* infection dose or time point after infection will have a direct effect on one or more of the following parameters:

1) Altered cytokine profiles as a result of early imbalance between Th1/Th2/Treg in the circulation during subclinical period of *Map* infection.

2) Early up-regulation of Treg cells (expressing Foxp3), with overproduction of anti-inflammatory cytokines IL-10 and TGF-β.

3) Expression of gut homing receptors (CCR9 and β7-integrin) on circulating memory T cells after *Map* infection.

Objectives

1) Determine the onset of T-cell responses to *Map* in the circulation and to profile them longitudinally over 6 months for better understanding of the early *Map* – host interaction and immune pathogenesis in a natural host of *Map* (cows).

2) Determine the effect of two different inoculum doses on the predominating T-cell phenotype, and to find whether there is a time dependent effect on those T-cell phenotypes.
CHAPTER 2: MATERIALS AND METHODS

2.1 Experimental design of the *Map* infection trial

Animals:

Twenty five male Holstein-Friesian calves were obtained from 10 dairy herds suspected to be free of Johne’s disease or have a low prevalence as intra-uterine infection is a potential route of *Map* transmission, this equates to a closed herd with no history of Johne’s disease in the previous 5 years, with a negative pooled fecal testing on all animals older than 5 years of age and a herd seroprevalence lower than 5% (IDEXX Paratuberculosis Ab Test, Westbrook, ME, US). For this purpose, pooled fecal samples were processed (decontaminated for culture and incubated in TREK ESP® Diagnostic systems, Ohio, US), and then confirmed of being *Map* negative with conventional IS900 PCR [87]. During calving, contact of the calves with the dam environment was avoided at all the times, additional serum and fecal testing was done for the dams soon after calving or within 2 weeks of calving and all tested negative.

Feeding, weaning and housing:

The calves were fed colostrum that was collected from seronegative farms and gamma irradiated (Co-60 source, McMaster Nuclear Reactor, Hamilton, Ontario, Canada). Pails containing 20L of colostrum were ensured to receive a minimum dose of 10 kGy per pail. The calves were fed 6 L of colostrum in the first 12 hours after birth. Then they were fed calf milk replacer until 2 weeks of age. Later, calf starter and high quality hay were given to the calves. Weaning of the calves was at about 2 month of age.

The calves were then randomized between the age groups. The calves were individually housed in a biosecurity level 2 large animal research facility operated by the Faculty of Veterinary
Medicine, U of C (UCVM), with highly trained personnel to take care of the animals on a daily basis.

An animal care protocol (M09083) was approved by the Health Sciences Animal Care Committee at the University of Calgary. All experimental work was conducted in accordance with federal and provincial legislation and regulations.

*Map* inoculum: A virulent cattle type *Map* field strain isolated from a clinical case C69 (identical to the reference strain K10 as demonstrated by IS900-RFLP, and recommended by expert panel [88]). The inoculum was tested for contamination using Gram stain, blood agar, RT-PCR and High Resolution Melt Analysis (HRMA) then the inoculum’s dose was adjusted with quantitative real-time PCR, using a standard curve of *Mycobacterium smegmatis*’ 16S gene (*M. smegmatis* has faster growth and doesn’t form clumps as opposed to *Map*). The *Map* inoculum was mixed with a small volume of culture broth, and then used to orally inoculate the calves at 3 different ages (2 weeks, 3 and 6 months at the time of oral inoculation with *Map*) with a relatively high (5x10⁹ CFU) or low (5x10⁷ CFU) bacterial dose on 2 consecutive days. The calves were induced to suckle from a syringe containing the mixture.

Nine calves in total were inoculated with high dose *Map*, and 10 calves with low dose *Map*. The calves were assigned to age and dose groups as follows; six month old group (5 calves received high dose *Map* and 5 calves received low dose *Map*), three month old group (2 calves received high dose *Map* and 3 calves received low dose *Map*), two weeks old group (2 calves received high dose and 2 calves received low dose *Map*).

The *Map* inoculum was prepared in a 50 ml volume of culture broth for the high dose infection group and 100 fold dilution of the inoculum was prepared for the low dose infection group. The inoculum was given to the calves orally, at the back of their tongue using syringes.
Six healthy uninfected controls of the same age were included in the trial (age matched with all of our Map inoculated calves at time of sampling).

Sampling:

Two 10 ml heparinized blood collection tubes were collected from each calf in the 3 age groups (2 weeks, 3 month and 6 month old) at 6 time points (pre-infection, 1, 2, 3 and 6 months after infection), and the age matched controls at each corresponding age.

2.2 Peripheral blood mononuclear cell (PBMC) separation and ex-vivo culture

PBMC separation: The Buffy Coat was taken after centrifugation of the whole blood (10 ml in BD vacutainer tube containing sodium heparin, BD, catalogue # 367874) at 1200 g for 20 min without using the brake function on the centrifuge, and diluted with the same volume of Dulbecco’s Phosphate Buffered Saline (DPBS, BioWittaker, VWR). The diluted blood solution was uploaded on top of Ficoll-histopaque (Sigma Aldrich, St Louis, Missouri, USA, catalogue # 10771). Tubes were then centrifuged at 750 g for 30 min without using the break function on the centrifuge. The mononuclear cells rich middle layer was taken (opaque thin layer between plasma and histopaque) and then washed with DPBS by centrifugation at 1000 g for 10 min. Red Blood Cells (RBCs) were lysed with 5 ml RBCs lysis buffer (Sigma Aldrich) for 15 min at 37 °C followed by washing with DPBS as described above. PBMCs were resuspended in 10 ml LCM (leukocyte complete media, DMEM base, Gibco Life Technologies Inc., Ontario, Canada). For the preparation of 500 ml LCM, about 70 ml of DMEM was removed and replaced by 0.5 ml mercaptoethanol, 5 ml L-glutamine, 5 ml sodium pyruvate, 5 ml Penicillin-Streptomycin (10,000 U/ml), 5 ml HEPES and 50 ml heat-inactivated bovine serum (Gibco Life Technologies). Trypan blue dye (Invitrogen, Burlington, Ontario, Canada) was used for discriminating live cells.
from dead cells, the numbers of live cells were counted and their concentration was calculated by using Countess® automated cell counter (Invitrogen).

*Ex vivo* culture:

Cells were placed in 12 well plates (VWR International, Mississauga, ON, Canada) at a concentration of $2 \times 10^6$ live cells/ml and each sample was incubated with different stimulants: un-stimulated, Phorbol Myristate Acetate (5 ng/ml) + Ionomycin (5 nM) (PMA+I) (BD Bioscience, Mississauga, Ontario, Canada) as a non specific stimulant for cytokine production, and avian purified protein derivative (PPD) as a Map-specific antigen stimulant (50 µl/ml, Prionics, Switzerland), and then the culture plates were incubated for 48 hrs (37°C / CO₂ incubator). Another 2 wells were used for shorter incubation time (18 hours for FACS analysis) in presence or absence of PMA+I at the same concentration to the 48 hrs-culture followed by addition of 1.3 µl Golgi stop solution (BD Bioscience) for the last 16 hrs of culture to accumulate the cytokines inside the cells.

Supernatants were collected after 48 hrs for cytokine analysis by ELISA. Cultured cells from the same wells were harvested and then mixed with 1ml Trizol (Invitrogen) after complete removal of the culture media. All samples were kept in -80°C until further analysis.

2. 3 Cell staining and *Fluorescence-activated cell sorting (FACS)* analysis

Cell staining:

The reactivity of all conjugated monoclonal antibodies was confirmed for bovine samples and purchased from AbD SeroTech, NC, US. The PBMCs cultured for 18 hrs were stained with anti-human CD3-Pacific Blue (Catalogue # MCA1477PB) as a pan T-cell marker, anti-bovine CD4-
FITC (Catalogue # MCA1653F) as a marker for T-helper subset. Intracellular expressions of Foxp3, IFN-gamma and IL-10 were also measured by FACS with anti-human Foxp3-APC (Catalogue # HCA116A647) as a unique marker for regulatory T cells, anti-bovine IFN-γ RPE (Catalogue # MCA1783PE) and anti-bovine IL-10 biotin conjugated (Catalogue # MCA2111B ). Cells harvested after 18 hrs culture were washed with 1 ml DPBS and then collected by centrifugation at 1000 g for 10 min, and 250 µl Foxp3 fixation buffer (eBioscience, San Diego, US) was added to each tube. Tubes were incubated for 30 min at 8°C. 1ml permeabilization/wash buffer (eBioscience) was added before the tubes were centrifuged at 1000 g for 10 min. Supernatant was discarded and the tubes were vortexed to resuspend the cells. An antibody cocktail consisting of rat anti-human CD3-Pacific Blue (0.1 mg/ml), mouse anti-bovine CD4-FITC (0.1 mg/ml), mouse anti-bovine IL-10 biotin (1.0 mg/ml), mouse anti-bovine IFN-γ RPE (1.0 mg/ml), and human anti-bovine Foxp3-APC (0.05 mg/ml) was added in the dilution recommended by the manufacturer to all tubes (10 µl of each conjugated antibody per 10⁶ live cells in 100 µl, except for the anti-bovine IL-10 of which only 1 µl was added in µl per 10⁶ live cell in 100 µl). None of the conjugated antibodies were added to the negative control cells (un-stained cells).

As recommended by the manufacturer of antibodies, cells were incubated with the antibody cocktail for 30 min at room temperature (RT), and then washed with 1 ml DPBS. 10 µl of 100 fold diluted PE-Cy7 conjugated Streptavidin (eBioscience) was added to the tubes and incubated for 10 min and then washed with 1ml DPBS. Samples were then kept in 0.5 ml PBS with 4% formaldehyde at 5-8 °C until FACS analysis. As an optimization step to exclude non-specific binding and false positive signals, iso-type control was prepared using iso-type antibodies conjugated with the same fluorochromes used for this experiment. Color compensation for
A multicolor examination was done every time immediately before the FACS analysis. A compensation particles set (BD Bioscience, catalogue # 01111142) was used for color compensation to establish an accurate correction of the spectral overlapping. First, negative beads (20 µl) were added in all the 5 tubes, and then 20 µl of the mouse/rat beads were added in each tube according to the species the antibody was generated. Antibodies (3.25µl each) were then added to each corresponding tube. Tubes were vortexed and incubated for 5 min in the dark at RT, and then 0.5 ml PBS/formaldehyde 4% was added to all compensation tubes.

The stained cells were examined by FACS Aria III flowcytometer (BD biosciences) to enumerate helper T cell subsets. After acquisition, the flow data were re-analysed using FlowJo software (Treestar, San Jose USA). The percentage of the total lymphocyte was determined for each sample according to forward and side scatter of the spectrum; smaller cells with less complexity are in the lower left side of the dot plot (Fig. 3). Then, the percentage of CD3+ and CD4+ within the total lymphocyte population was gated by plotting CD3+ cells stained with Pacific Blue (X-axis) versus CD4+ cells stained with FITC (Y-axis), followed by adjusting the gating window on the upper most distinct cell population on the right side of the dot plot for double positive population CD3+ CD4+. The same strategy was followed for detecting other CD4+ T-cell populations by plotting CD4+ cells stained with FITC versus either Foxp3+ cells stained with APC to define the Treg population or IFN-γ+ cells to detect Th1 population. Treg cells expressing Foxp3+ IL-10+ were also gated by plotting Foxp3+ cells stained with APC versus PE-Cy7 stained IL-10+ cells. The distinct population on the upper right quarter of the dot plot was always considered our double positive population (Fig. 3).
Figure 3: Gating strategy for FACS interpretation illustrated by a specific example. (A) Percentage of the total lymphocyte (B) Percentage of CD3\(^+\) and CD4\(^+\) within the total lymphocyte population (C) Foxp3\(^+\)IL-10\(^+\) cell population (Treg cells).

2.4 Sandwich ELISA for cytokine detection

Sandwich ELISA method was used to assess cytokine secretion pattern of PBMCs in the response to *Map*-specific and non-specific antigens. For bovine IL-10, a monoclonal non-conjugated mouse anti-bovine IL-10 (AbD SeroTec) antibody was used to capture IL-10 in the supernatant, biotin-conjugated mouse anti-bovine IL-10 (AbD SeroTec) was used for the detection of captured IL-10 molecules and recombinant Equine IL-10 (R&D Systems Minneapolis, USA) was the IL-10 standard. Bovine IFN-\(\gamma\) ELISA kit from R&D Systems and bovine TGF-\(\beta\) kit (Genorise Scientific Inc., Paoli, PA, USA) were used following the manufacturer’s instructions for each. The ELISAs were performed on culture supernatants to measure cytokine levels released by T-lymphocytes upon the stimulation.
Bovine IL-10 ELISA:

A sandwich ELISA was designed for the measurement of bovine IL-10 in the supernatant. Bovine IL-10 was captured using a monoclonal non-conjugated mouse anti-bovine IL-10 (AbD Serotec, catalogue # MCA2110). Biotin-conjugated mouse anti-bovine IL-10 (AbD Serotec, catalogue # MCA2111B) was used for the detection of captured IL-10 molecules. Recombinant Equine IL-10 (R&D Systems, Minneapolis, MN, USA) was used as a standard as it crosses react with bovine IL-10, and because recombinant bovine IL-10 was not commercially available. Dulbecco’s Phosphate Buffered Saline (DPBS, BioWittaker, VWR International, Mississauga, ON, Canada) with 0.05% Tween 20 was used for washing the plate, and PBS with 5% Tween 20 was used as blocking buffer. Horseradish Peroxidase (HRP) conjugated Streptavidin (R&D Systems) was diluted in the blocking buffer for detection. For color development, enzyme substrate reagents (R&D Systems) were used per manufacturer’s instruction. Stop solution consisting of 1% H$_2$SO$_4$ (R&D systems) was used to stop the reaction.

Capture IL-10 antibody (non-conjugated) was diluted in DPBS (5 µg/ml), 50 µl of the diluted antibody were placed per well in a flat bottom 96-well plate (BD Bioscience). The plates were covered and incubated overnight at room temperature (RT) and washed 3 times with wash buffer (200 µl/well). Ultra block buffer (AbD Serotec, catalogue # BUF033A) was used to reduce non-specific binding and false positive signals, (50 µl/well) were added and plates were incubated for 1h at RT followed by washing plates3 times with wash buffer. Supernatant samples and standards were added (50 µl/well) without dilution in duplicates. IL-10 standard was prepared by 2 fold serial dilutions for 7 points using the DMEM culture base as diluent. The standard concentration started at 20.000 pg/ml (highest point) to 312 .5 pg/ml (lowest point). DMEM culture base was used as blank for subtraction of the background. Plates were incubated for 1h at
RT and washed 3 times with wash buffer. Detection IL-10 antibody conjugated with Biotin (AbD SeroTec) was diluted with detection buffer at concentration of 2 µg /ml. Antibody solution was added to all wells (50 µl/well) and incubated for 1h at RT. Plates were then washed 3 times with wash buffer. HRP- streptavidin was diluted with blocking buffer (1/200) and added to all wells. Plates were incubated for 20 min at RT and then washed 3 times with wash buffer. Color was developed with addition of 50 µl enzyme substrate (Reagent A (H2O2) + Reagent B (TMB-tetramethylbenzidine) at 1:1 ratio) for 10 min at RT, followed by 50 µl of 1% H2SO4 to stop the reaction, then plates were measured using a plate reader (Perkin Elmer, Santa Clara, CA, US) at wavelength 450 nm within 20 min. Corrections were done by subtraction of the blank’s absorbance value. A seven points’ standard curve was generated using log 2 concentrations of the standard. We had an exponential/log shaped standard curve in all of our experiments (Fig. 4-A), we calculated the Log2 for each standard concentration to generate a linear standard curve (Fig. 4-B). Next, actual IL-10 concentrations were back-calculated and plotted on a scatter plot. All the calculations and data analysis were done using the Microsoft Office Excel 2007. Figure 4-B displays the typical standard curve of IL-10 ELISAs.
Figure 4: IL-10 standard curves generated by plotting absorbance versus A) IL-10 pg/ml, and B) log 2 IL-10 concentrations on a scatter plot.

Bovine IFN-γ ELISA:

Bovine IFN-γ was measured in the supernatant of cultured PBMCs using Bovine IFN-γ ELISA kit (R&D Systems) following the manufacturer’s instructions. A standard curve was generated by plotting the absorbance versus concentrations on a scatter plot. Figure 5 displays the typical standard curve of IFN-γ ELISAs.
Bovine TGF-β1 ELISA:

Bovine TGF-β1 was measured in the supernatant of cultured PBMCs using Bovine TGF-β1 ELISA kit (Genorise Scientific Inc., Paoli, PA, USA) following the manufacturer’s instructions. Standard curve was generated by plotting the Xs (concentration) versus Ys (absorbance) on a scatter plot. Figure 6 displays the typical standard curve of TGF-beta1 ELISAs.
2.5 Quantitative real-time PCR for relative gene expression analysis

The expression of all our candidate genes were measured in the age-matched non-infected controls relative to GAPDH as a reference gene ($\Delta ct = ct_{\text{candidate gene}} - ct_{\text{GAPDH}}$), then we calculated the relative expression of each candidate gene in each infected calf versus the age matched control using the $\Delta\Delta ct$ method ($\Delta\Delta ct = \Delta ct_{\text{infected calf}} - \Delta ct_{\text{control calf}}$), followed by a fold change calculation ($2^{-\Delta\Delta ct}$).

RNA extraction from PBMCs and genomic DNA elimination:

mRNA was extracted from PBMCs that had been stored in Trizol at -80°C using the Trizol RNA extraction method (Invitrogen, Life Technologies, Carlsbad, CA, USA), followed by further purification using the RNeasy® Mini kit (Qiagen, Mississauga, ON, Canada, catalogue # 74104), following the manufacturer’s instructions. Briefly, cultured PBMCs were homogenized in 1 ml Trizol by vigorous shaking and vortexing for 3 min. Homogenized samples were incubated for 5 min at RT, and then 200 µl chloroform was added and samples were vigorously shaken for 20 sec, then incubated for 3 min at RT. Tubes were spun at 12,000 g for 15 min at 5°C. The upper aqueous phase was transferred to fresh RNase-free tubes. To precipitate the RNA, 0.5 ml isopropyl alcohol was added to the aqueous phase and incubated for 10 min at RT, and then the tubes were spun at 12,000 g for 10 min at 5°C. The supernatant was discarded and 0.5 ml ethanol (99-100%) was added to each sample.

After adding ethanol, the samples were transferred to RNeasy spin columns (Qiagen) and spun for 30 sec at 12,000 g. Next, the flow-through was discarded from the collection tube. To wash the column, 350 µl RW1 buffer (provided with the kit) was added and spun for 30 sec at 12,000
g then the flow-through was discarded. For genomic DNA elimination, the RNase-free DNase set® (Qiagen, catalogue # 79254) was used. DNase was reconstituted with 550 µl of RNase-free water as described by the manufacturer, then aliquoted in small tubes and stored at -20°C. For each sample, 10 µl of the reconstituted DNase was added to 70 µl RDD buffer provided with the kit. The diluted DNase was added and incubated for 15 min at RT. To wash the column, 350 µl RW1 buffer provided with the RNeasy® Mini kit was added and spun for 30 sec at 12,000 g then the flow-through was discarded. The membrane was washed with 500 µl RPE buffer (mixed with 100% ethanol as described by the kit manufacture) and spun for 2 min at 12,000 g to dry the column. The collection tubes with the flow-through were discarded, and new collection tubes were used for the elution of RNA. RNase-free water was added (30-50 µl) to the column (after placing it in a new collection tube), incubated for 5 minutes at RT and then spun for 2 min at 12,000 g to elute the RNA. The RNA tubes were placed immediately on ice and stored at -80 °C.

A Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the Agilent 2100 Bio-analyzer (Agilent Technologies Inc., Mississauga, ON, Canada) were used to determine the quality, integrity and yield of the extracted RNA (Fig. 7).
Figure 7: RNA analysis of one sample by the Bio-analyzer microfluidics chip technology to assess the integrity, yield and quality of the extracted RNA. In the presented example, the RNA concentration was 79 ng/μl, rRNA ratio [28s/18s] was 1.6, and RNA Integrity Number (RIN) was 9.

Reverse transcription reaction:

Reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, catalogue # 205313) following the manufacturer’s instructions. RNA concentration was adjusted to 500 ng /RT reaction in all of the samples on ice. gDNA Wipe-out buffer was added to each RNA sample diluted in RNase-free water to reach a total volume of 28 μl/gDNA elimination reaction. Samples were incubated for 2 min at 42°C then immediately returned to ice. The reverse transcription mix was prepared on ice (2μl of the reverse transcriptase enzyme + 4μl of RT buffer + 2μl of RT primer mix + the whole 28 μl of the previous reaction containing the RNA), in a total reaction volume of 40 μl. Tubes were incubated for 15 min at 42°C, then for 3 min at 95°C to inactivate the RT enzyme. The cDNA was stored in -20°C for future use.
Relative Quantitative Real-time PCR (q-RT PCR):

qPCR was performed on the cDNA using SYBR green in a QuantiTect SYBR Green kit for two step RT-PCR (Qiagen, catalogue # 204143). PCR tubes containing SYBR green master-mix (25 μl), ≤500 ng cDNA and primer pairs (final primer concentration was 0.3 μM per primer, MgCl2 concentration was 2.5 μM as recommended by the kit manufacturer) were prepared in a total reaction volume of 50 μl. The amplification reaction was performed in a Bio-Rad CFX 96 real-time PCR detection system (BioRad, Mississauga, ON, Canada) (Fig. 8). The sets of bovine primer pairs for IL-10, IL-4, IFN-γ [89], Foxp3 [18], β7-integrin [90], CCR9 [91] and GAPDH [92] were synthesized in the core facility lab at the University of Calgary. GAPDH primers were used to detect the reference gene. All of the previous primers were matched to the bovine genome for each corresponding gene using NCBI-primer blast website (URL). Amplification products were visualized on 1% agarose gels and melt peaks were assessed using the Bio-Rad CFX96 real-time PCR detection system.
Figure 8: (A) Amplification cycles, (B) melt curves and (C) melt peaks for the eight genes including the reference gene GAPDH, using Bio-Rad CFX96 real-time PCR detection system.

Statistical analyses of the data:

The maximum number of calves that could be used in the experiment was 25 calves. We were not able to calculate the power for this study based on previous similar studies, because no similar studies have been published. Additionally, this kind of studies on large animal models usually have the same issue of a relatively low sample size compared to those studies done on small laboratory animals. Moreover, the sample size of each age group was very low; therefore we could not always perform statistical analyses on this small sample size to study effect of age at time of inoculation on the outcomes. Data were pooled from the 3 age groups together under each of the dose groups. Finally, we had two larger dose groups; 9 calves in total received HD
Map at 3 different ages, and 10 calves in total received LD at 3 different ages. Six un-inoculated calves served as negative controls. We also had to pool the data obtained from the 6 age-matched controls at different time points to age match with our infected animals at a given time point after infection. Statistical analyses for all the data were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Grouped analyses were chosen to create graphs showing means and SEMs for each group (HD, LD and controls) at a given time point. The means of cytokine concentrations, Treg percentages, and fold changes in the expression of candidate genes were compared for each dose group with the age-matched control group at each time point. Two-way analysis of variance (ANOVA) for repeated measures was done, followed by a Bonferroni post-hoc test to determine whether differences in the previously mentioned means between the 3 groups at the different time points were statistically significant.
CHAPTER 3: RESULTS

3.1 Helper T-cell phenotyping by flow cytometry

We tested the expression of Foxp3 and IL-10 as intracellular markers for Treg cells to study their percentage in the total T-lymphocytes population. The percentage of total T-lymphocytes ranged from 58-68%. We also studied the Th1 cells expressing CD4 as a surface marker and IFN-γ as a characteristic intracellular cytokine.

Most of the calves infected with Map showed higher percentage of IL-10+ Treg cells (Foxp3 expression) than age matched controls and the same calves at pre-infection, data are shown on individual bases in figures 9-12 to appreciate differences. The percentages of IL-10+ Treg cells in Map infected calves remained higher than age matched controls in the first three months after infection; the Treg percentage in both HD and LD calves was significantly higher than age matched controls in the first and the second months after infection as data from all calves are summarized in figure 13. Generally by 6 months after infection, Treg cell percentage returned back to control level in the entire group of Map infected calves. CD4+ IFN-γ+ (Th1 cells) were slightly upregulated after 6 months of infection in some of the Map infected calves (Th1 percentage was 6-8%) than age matched controls (Th1 percentage was 2-3.5%), with respectively Th1 percentage at 6-8% and 2-3.5% the data were not statistically significant from the un-infected controls. We noticed as shown later with other methods, that the IFN-γ response was generally antigen-specific (avian PPD).
Figure 9: Treg percentage at 1 month after infection detected by flow cytometry; (A) 2 age matched un-infected calves, (B) 2 LD infected calves, (C) Treg percentage in the 2 HD infected calves. Double positive population for (IL-10 and Foxp3) is shown by arrows on the right upper window on the graph.
Figure 10: Foxp3$^+$ expression measured by flow cytometry. (A) Example presented is of animal at 2 months after infection, (B) Age matched control calf. Arrows are pointing to Foxp3$^+$ population.

Figure 11: Histograms showing the difference in IL-10$^+$ expression (right side peak) by intracellular staining.
Figure 12: Treg % by individual calf at the 5 time points. HD infected calves are shown in black lines, LD infected calves are shown in grey lines. (A) Three age matched controls, (B) 4 calves infected at 2 weeks old, (C) 5 calves infected at 3 month old, and (D) 4 calves at 6 month old respectively. PI: pre-infection.
Figure 13: Treg cell percentage detected by flow cytometry at the different time points. Data are presented as means ± SEM. Significant differences from the age matched controls within given time points are presented by asterisks (**, P<0.01; ***, P<0.001)
3.2 Supernatant cytokine assessment by ELISA

IL-10 was measured in the PBMCs culture supernatant and it was upregulated in most of the infected calves compared to the uninfected controls of the same age in the first 2 months after infection, after which it returned to control level after 6 months. Data for individual calves are presented in figures 14 to 17 to appreciate variations between calves and trendiness of IL-10 production. IL-10 in the supernatant was significantly upregulated in the group received HD Map in the first and second months after infection using both stimulants. IL-10 was also significantly upregulated in the LD infected group but only in the first month infection in the avian PPD stimulated samples. All the data for IL-10 in the supernatants are summarized in figure 18. The findings were similar using both types of stimulants (avian PPD and PMA+I), which indicates that the IL-10 response was not antigen specific.

TGF-β measured in the PBMCs culture supernatant had a similar profile as IL-10; it was generally up regulated early after infection, and it was significantly higher than age matched controls in the HD Map-infected calves one month after infection, after which TGF-β declined back to control level or even lower than controls in some calves 6 months after infection (Fig. 20; A).

INF-γ was also measured in the PBMCs culture supernatant and it was only detected in the samples stimulated with specific antigen (Avian PPD), IFN-γ production increased progressively with time in the Map-infected calves (HD and LD) compared to age matched controls. Data for individual calves are presented in figure 19 to appreciate variations between calves and trendiness of IFN-γ production. Control level was close to zero at all time points (data not shown).
All the data for IFN-γ in the supernatants are summarized in figure 20; B. There was significant difference between HD versus LD in the IFN-γ production in the PBMCs culture supernatant (ELISA) 6 months after infection in the PPD stimulated samples (P<0.001). Generally, cytokine production was very weak in the un-stimulated samples.

Figure 14: IL-10 response in HD Map-infected calves at 6 month of age. (A) PMA+I, and (B) avian PPD
Figure 15: IL-10 response in LD *Map*-infected calves at 6 month of age, (PMA+I).

Figure 16: IL-10 response in HD *Map*-infected calves at 2 weeks of age and at 3 month of age, (PMA+I).
Figure 17: IL-10 response in LD *Map*-infected calves at 2 weeks of age and at 3 month of age, (PMA+I)
Figure 18: IL-10 in the PBMCs culture supernatant stimulated with: (A) PMA+I, (B) avian PPD and measured by ELISA at the different time points; summarizing data in figures 14-17. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (*,P<0.05;**,P<0.01;***,P<0.001)
Figure 19: (A) IFN-γ response in the 9 calves inoculated with high dose Map at different age. (B) IFN-γ response in the 10 calves inoculated with low dose Map at different age. Avian PPD stimulated. Control level was close to zero at all time points (data not shown).
Figure 20: (A) TGF-β and (B) IFN-γ in the PBMCs culture supernatant measured by ELISA at the different time points. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (*, P<0.05; **, P<0.01; ***, P<0.001)
3.3 RT-qPCR and gene expression analysis

Almost all of the candidate genes were differentially expressed in the Map-infected calves compared to the controls for at least one of the time point after infection, except b7-integrin that had similar expression as age matched controls in all the samples taken at the different time points of this study.

IL-10, FoxP3 and TGF-β genes were upregulated early after infection, then declined after 6 months. At the first month after infection, IL-10 and Foxp3 genes were significantly upregulated in both dose groups inoculated with Map at three different ages (Figs. 21 and 22). There were significant differences between HD versus LD in the expression of IL-10 and TGF-β genes, at the first month after infection (P<0.01, P<0.001 respectively).

The IFN-γ gene on the other hand, had a different profile as it was upregulated significantly with time starting from the second month after infection and up in the HD infected group (P<0.001), and in the third and sixth months after infection in the LD group (P<0.05) (Fig. 23). The IFN-γ response was stronger with avian PPD stimulation, while IL-4 and TGF-β responses were only noticed in the avian PPD stimulated samples.

The CCR9 gene was also upregulated after Map infection and it was significantly expressed 6 months after infection but only in the HD infection group (Fig. 24).

The B7- intergrin gene was highly expressed in all of our subjects including controls (expression level was usually very close to the reference gene GAPDH) and there were no significant differences between the inoculated animals versus age matched controls in the expression of this gene at the entire length of the study (data not shown).
TGF-β as a critical cytokine for the activation and maintenance of Tregs had similar profile as IL-10 and Foxp3; it was significant up regulated at the first, second and third months after infection in the HD group (Fig. 25).

IL-4 was expressed slightly higher than controls in just a few numbers of calves 2 months after infection and up, but the data was not statistically significant at any of the time points we tested in this study (Fig. 26). Gene expression of all candidate genes was similar to control in the un-stimulated samples.
Figure 21: Foxp3 gene expression in cultured PBMCs stimulated with (A) PMA+I, and (B) avian PPD. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (*, P<0.05).
Figure 22: IL-10 gene expression in cultured PBMCs stimulated with (A) PMA+I, and (B) avian PPD. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (**, P<0.01; ***, P<0.001)
Figure 23: IFN-γ gene expression in cultured PBMCs stimulated with (A) PMA+I, and (B) avian PPD. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (*, P<0.05; **, P<0.01; ***, P<0.001)
Figure 24: CCR9 gene expression in cultured PBMCs stimulated with (A) PMA+I, and (B) avian PPD. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (*, P<0.05)
Figure 25: TGF-β gene expression in cultured PBMCs stimulated with avian PPD. Data are presented as means ± SEM. Significant differences from age matched controls within given time points are presented by asterisks (*, P<0.05; **, P<0.01; ***, P<0.001)

Figure 26: IL-4 gene expression in cultured PBMCs stimulated with avian PPD. Data is presented as means ± SEM.
CHAPTER 4: DISCUSSION

4.1 Data summary and discussion:

A *Map* infection trial in dairy calves enabled us to observe and profile the time course progression of cell mediated immune responses and associated infection markers in the circulating T-lymphocytes. In this study, T-lymphocytes markers such as cytokines (IL-10, IL-4, IFN-γ and TGF-β), Foxp3 as a unique Treg cell marker and adhesion molecules (CCR9 and β7-integrin) in peripheral blood were investigated at the early subclinical stages of the disease. Moreover, the use of 2 different *Map* infection doses allowed us to study the effect of infection dosage on the above mentioned markers.

Our first objective was to determine the onset of T cell responses to *Map* in the circulation and to profile them longitudinally over 6 months. We were able to observe and profile an early Treg response to *Map* that preceded other T cell responses (Th1 and Th2). The Foxp3^+^Treg cells were the earliest T cell population to predominate in the circulation after *Map* infection. The percentage of Foxp3^+^ IL-10^+^ regulatory T-cells detected by FACS assay was upregulated significantly in the infected calves compared to age matched controls early after *Map* infection then declined back to control level after 6 months. We were also able to sort and identify the Treg population using their unique Foxp3 marker and IL-10 with the gating strategy that was described earlier in details (pages 29-30). In the pilot study for this project, we encountered some challenges in sorting out the Foxp3^+^ cells using flow cytometry; we therefore decided to stain intracellular IL-10 for better detection of this population. However in some of the calves, the level of IL-10 expression detected by the flow cytometry did not always match the Foxp3 expression (figure 10), suggesting that the Treg cells were not the only source of IL-10 as observed in previous studies [20, 21]. IL-10, TGF-β and Foxp3 as markers for Treg cells had
similar profiles using ELISA or qPCR. Both assays showed upregulation in the Map-infected calves compared to the age matched controls in the first 2-3 months after infection, followed by a decline back to control level by 6 mo after infection in most of the calves. A stronger Treg response was observed in the HD infected calves compared to the low dose infected calves. IL-10 was generally upregulated in the first 2-3 months after infection as detected by the three methods (ELISA, flow-cytometry and qPCR) used for this study. IL-10 upregulation in response to Map antigen was also observed in many previous studies [65, 67, 68, 93, 94]. All these studies concluded that IL-10 is produced by PBMCs from Map naturally infected cows following Map specific antigen stimulation and that CD4⁺ CD25⁺ cells are mostly responsible for this IL-10 production as their removal by magnetic cell sorting results in depletion of IL-10 and enhancement of IFN-γ production [68]. Elevated expression of Foxp3 was noticed after stimulation of human PBMCs with M. tuberculosis antigen [95]. Furthermore, some studies have shown that neutralization of IL-10 in johnin PPD stimulated whole blood using monoclonal antibodies enhances the expression of IFN-γ in both Map and M. bovis infections in cattle [23, 96, 97] and that IL-10 in cattle is capable of suppressing the functions of all CD4⁺αβ TCR⁺ T cells and decreasing local concentration of IFN-γ [66]. Since in those studies the cows were naturally infected with Map, including cows with clinical JD [93]; so it was not possible to know the exact time of infection, age of the animal at time of infection or the infection stage in order to specifically study the immune responses at the early stages of the infection. Our second objective was to determine the effect of two different inoculum doses on the predominating T-cell phenotype. We observed animals that received HD Map had stronger, more significant and earlier Treg cell activation (upregulated Foxp3, IL-10 and TGF-β) than the animals received LD Map. Similarly, stronger responses in the HD inoculated animals were noticed at 6 months after
infection for the IFN-\(\gamma\) production and CCR9 expression. As mentioned before; significant differences were found between HD versus LD in the expression of IL-10, TGF-\(\beta\) genes at some time points during this study. Such effects of Map inoculum dose on mounting cellular immune response to Map, Foxp3 expression, and CCR9 were not investigated before. It has been demonstrated that both IL-10 and TGF-\(\beta\) as anti-inflammatory cytokines actively downregulate and interfere with the production of IFN-\(\gamma\); the critical cytokine in controlling mycobacterial infections [98, 99]. Furthermore, this too early production of regulatory cytokines IL-10 and TGF-\(\beta\) might be responsible for the early attenuation of effective immune response required for the clearance of Map. Therefore, we aimed in this study to evaluate and profile the effect of challenging young calves with Map on the cytokine pattern of circulating T-lymphocytes at various time points. This early upregulation of Treg cells after Mycobacterium infection was also observed in a recently published study in which pathogen specific Treg cells expanded early after Mycobacterium tuberculosis infection in mice, followed by a decline of this Treg cell population after 3 weeks of initial infection [100]. Previous studies have described the potential role of Fop3\(^+\) Treg cells in the suppression of effective immune response against various chronic and persistent infections. In some chronic infections, the immune system fails to maintain the equilibrium and fine balance between aggressive anti-pathogen immune response and homeostatic protective mechanisms, through over activation of Treg cells with excessive regulatory immune response that allows the pathogen to multiply inside the host with persistent infection [101]. This imbalance between anti-pathogen (effector T cells) and homeostatic (Treg cells) mechanisms occurs in parasitic infection with Schistosomia, Leishmania, Trypanosoma, Plasmodium (malaria) in mice and human, viral infections as HSV, HCV, HBV, CMV, fungal infections as in Candida albicans and bacterial infections as in H. pylori and Mycobacterium...
tuberculosis (reviewed in [101]). In human pulmonary T.B., the frequency of Treg cells is significantly higher in the peripheral blood of those patients compared to healthy controls [102]. In experimental infection of mice with the above mentioned pathogens; the authors have observed that removal of Treg cells generally enhanced microbial control by the effector cells in vitro [101]. Conversely, addition of Treg cells back to cultures significantly suppresses antigen specific IFN-γ production by effector T cells [102], as we also observed opposite time profile of Foxp3+ cells and IFN-γ production. Th1 response and IFN-γ production were dramatically suppressed in mice following experimental infection with a hypervirulent strain of Mycobacterium tuberculosis, this suppression was correlated with rapid emergence of IL-10 producing Foxp3+ cells [103].

In mouse model; Scott et al. have observed an expansion in number and function of Foxp3+ Treg cells in the lung of T.B. infected mice [104], they have demonstrated that Treg cell – depleted mice have less bacterial burden in their lungs. In persistent infections - an action induced by the organism for survival [105] – pathogens use their own antigens to indirectly suppress effector T cells through aberrant activation of APCs or modulation of their functions to induce the differentiation of Naïve T cells into Treg cells; a previous study showed that signaling through DC-SIGN, which is an important receptor for Mycobacterium tuberculosis on tissue macrophages and DCs [56] promotes the differentiation of DCs into a tolerogenic phenotype which may explain the expansion of Treg cells during tuberculosis [106]. Kursar et al. have demonstrated that Treg cells producing IL-10 interfere with efficient clearance of Mycobacterium tuberculosis from the lung and spleen of infected mice [107], however this suppression of immune response was not accompanied by neither general increase of IL-10 expression nor by higher number of CD4+ T cells that produce IL-10.
One of our main objectives was to find time dependent effect on the predominating T cell phenotype; we observed an increasing profile of IFN-γ (Th1), a declining profile of Foxp3+, IL-10, TGF-β (Tregs), and a borderline IL-4 (Th2) profile within the same 6 mo length of this study. Our findings regarding the opposite profiles of IL-10 and TGF-β on one hand versus IFN-γ on the other hand; are generally in agreement with previous reports of inhibitory effects of IL-10 and TGF-β on the production of sufficient IFN-γ to kill Map. We also expected similar profiles of IL-10 and TGF-β since both cytokines act synergistically to downregulate IFN-γ production by T cells [67], the potentiating effect of adding exogenous TGF-β on IL-10 production was previously determined [67] and [99], as TGF-β is a critical and key cytokine for the activation and maintenance of the iTreg cells [47]. It is assumed that if the three cytokines (IL-10, TGF-β and IFN-γ) are upregulated in Map infected animals, that this will eventually lead to less-effective killing of Map in these animals [67].

As the infection progressed, Th1 activity progressively increased as indicated by IFN-γ that was detected in the samples stimulated with the specific antigen avian PPD in both ELISA and qPCR assays. It was significantly up-regulated in the infected calves compared to the controls from 2 up to 6 mo after infection. The up-regulation of IFN-γ in response to Map infection after stimulation with PPD has been shown by several studies [48, 49, 67] and despite of relatively low sensitivity of IFN-γ test (41%) specially in herds with mixed infections (tuberculosis and paratuberculosis) [108, 109] due to the cross reacting mycobacterial antigens; this assay still has higher sensitivity than serological tests and it might be used in Map diagnostics as a potential indicator of animals in the subclinical stage [27]. Other studies were also able to assess immune response in experimentally Map infected calves [85, 86]; they noticed upregulation of CD4+ IFN-γ+ cells, CD26 and CD45RO in the early subclinical stage indicating activation of memory
T-cells. Bovine tuberculosis is also characterized by increased IFN-γ production by PBMCs, as shown by Rhodes et al. [110] when they measured IFN-γ produced from PBMCs of *M. bovis* infected calves and stimulated them with PPD or BCG antigens in an ex-vivo culture. All the previously mentioned factors suggest that IFN-γ test is not the best method to specifically identify *Map* infection but instead might be used generally as a biomarker for mycobacterial infection [111].

Th1 cells and γδ T cells are significant sources of IFN-γ in cattle as described by Brown and Estes in 1997 [112]. The overproduction of IFN-γ from Th1 cells is also a main characteristic of Crohn’s disease in humans [113, 114]. The overproduction of IFN-γ however dosen’t mean an effective/ protective immune response to Mycobacterial infection, as Kursar et al. have found that protective CD4⁺ T cell response was not correlated with an increase in IFN-γ expression level or in the cells producing IFN-γ [107].

Overall, the measured responses in our presented study were stronger in animals infected with high dose *Map* than in the low dose infected animals.

Meanwhile, IL-4 expression was similar to age matched controls in most of the infected calves during the 6 month length of the study, indicating weak activity of the Th2 subset at this early stage of the disease that was also demonstrated before by [115] and [48]. The few calves showed slight up-regulation of IL-4 had received high dose *Map* and they had also detectable antibody responses in their serum and various intestinal lesion observed at necropsy one and half years after infection when tissue lesions were scored [116]. In the same infection trial, most high dose infected animals had strong *Map* specific antibody response (positive serology), earlier shedding and higher scores for tissue lesions induced by *Map* (gross and microscopy) [116]. The immune
response against *Map*, tissue lesions and disease outcome seems to greatly depend on two main factors; the initial or cumulative dose of *Map* that the animal gets from surrounding environment along with the animal genetic/immune predisposition [63] as explained earlier in the introduction.

We observed a coincident decline in TGF-β, foxp3 and IL-10 6 mo after initial infection, this time dependant decrease of TGF-β might indicate impaired generation and function of Treg cells as a crucial cell population to maintain immune tolerance. Our data indicates that there is a decrease in Treg cell population 6 mo after infection which will eventually lead to the development of chronic intestinal inflammation and collateral tissue injury with the disease progress, there is a similar consequence of inflammation process that occurs in CD due to impaired generation and function of Treg cells with the predominance of proinflammatory cytokines like IL-17 [117] and IFN-γ [118] in those patients. The robust Th1 response with over production of pro-inflammatory cytokine IFN-γ that we noticed in the peripheral blood after 6 mo are also characteristic profiles in the peripheral blood of both CD and ulcerative colitis patients [118]. Furthermore, JD and CD are both characterized by diffuse granulomatous lesions as result of increased activity of Th1 cells and macrophages leading to development of a delayed-type hypersensitivity reaction and granuloma formation [118]. All of the previously mentioned similarities in the immune profile of *Map*-infected calves and chronic colitis in IBD could give us the implication that that cows infected with *Map* might be used in the future as models for studying T-cell driven immune–pathogenesis of the intestine that occur in IBD.

Most of cytokine responses observed in this study were antigen specific responses except IL-10, suggesting that IFN-γ, TGF-β and IL-4 eventually need T cell polarization in response to antigen
recognition, while IL-10 is produced earlier and un-specifically from innate immune cells (monocytes and DCs) or γδ T cells [20, 21].

We hypothesized the increased expression of the gut homing receptors on circulating T cells after Map infection. The CCR9 was significantly up-regulated in the high dose Map infected group, which could be a potential index for inflammation onset and established infection. In 2004, Harp et al. [70, 119] explored adhesion molecules in Map-infected cattle and the authors have found that expression of LFA-1, CD26L and LPAM-1(α4β7-integrin) on T cells from milk and blood increased upon parturition and lactation, but they did not study the expression of CCR9 as an important gut homing receptor. The CCR9 over-expression is also a characteristic of chronic colitis in IBD patients and it’s antagonists are considered for future treatment strategies [120]. Our findings regarding Foxp3 longitudinal profiling by both flowcytometry and gene expression assays, apart from CCR9 gene expression early after Map infection, are novel and unique in the field of Johne’s disease immune pathogenesis and Map – host interaction.

4.2 Limitations and drawbacks:

First and biggest limitation of our study was the sample size of calves infected with each of the Map doses within the 2 weeks and the 3 months age groups; we could only include 4 calves which had received a high or low dose from calves infected at 2 weeks of age and 4 calves which had received a high dose or low dose from the calves infected at three months of age. Therefore, it was not possible to do statistics on just 2 animals to determine and conclude an age dependent immune response to Map. To overcome this issue, we pooled calves from the 3 age groups together in each of the dose groups, finally we had 9 calves in total received HD Map at 3 different ages and 10 calves in total received LD at 3 different ages. We also had to pool the data obtained from the 6 age matched controls at different time points to age match with our infected
animals at a given time point after infection and then we performed the ANOVA repeated measures followed by Bonferroni post-hoc test to find significant differences between each dose group versus age matched controls with age variability within a given time points. We assumed that errors due to age differences abolish each other when we compared the infected (mixed age) versus non infected calves (mixed age). We also assumed that the age variability will not make a big difference since all of our subjects were all within the first year of age, and that the largest difference in response to *Map* infection would be due to the inoculum dose and time after infection.

Other important challenges were transporting the blood samples from the barn into the laboratory at an optimal and stable temperature and within the recommended time for good cell viability (8 h) and at the specified time points of infection. In our case, working with calves in a distant facility meant a restricted accessibility for sampling in contrast to a typical laboratory setting when using small murine models. The high costs and efforts associated with this kind of infection trial in calves as big animal model is also a limitation such as; the cost of suitable well prepared barn with partitions to separate each animal in the trial, animal care, feeding, animal transportation with precautions taken to avoid contact with contaminated environment, preventive measures taken to avoid trans/auto infection, sampling, sample transportation and trained persons to take care of all the previous tasks are all considerable complicating factors.

The total time of our follow up was only 6 months which is generally short term and not long enough to determine the disease outcome and how the immune response looks like when the animal shifts from a subclinical to a clinical case. No cell sorting was done before the ELISA and qPCR assays, so it was hard to tell which specific population was the source of our upregulated cytokines and CCR9 expression.
4.3 Conclusions:

Our findings suggest that T-lymphocytes are involved in the early immune events that occur after *Map* infection that induce time dependent shifts between variable T-cell subsets Th1/Th2/Treg. An early Treg response to *Map* exposure was found and followed by a progressive and robust Th1 response in young calves. After *Map* exposure, the regulatory T cell response is vigorous and infective dose dependent but short lived, this might be responsible for onset of the intestinal inflammation which is typical for JD and most apparent and described in the later stages of the infection.

Decreased secretion of TGF-β in PBMC of *Map* infected calves 6 months after infection suggests a cause for reduced generation and/or impaired function of Treg cells in those calves during the infection progress. The Treg cell response is then replaced by a predominant Th1 response as is characteristic of the subclinical stage of Johne’s disease.

Our observations in comparison with other commonly used immunological and microbiological tests could be used in the future for better interpretation of current diagnostic tests and the development of improved diagnostic and control strategies. Two sets of markers in the blood can be considered in the future for following up *Map* infection and pathogenesis and as indicators of intestinal infection with *Map* and/or inflammation; cytokine profile of circulating T-lymphocytes that can reflect the infection status of the host whether in the early subclinical (Treg) or late subclinical (Th1) stages, and the gut homing receptor CC-Chemokine receptor 9 (CCR9) expressed on circulating T-lymphocytes that could potentially indicate the onset of intestinal inflammation.

This study represents a helpful tool and a step forward to understanding the immune-pathogenesis driven by intestinal infection with *Map* in its natural host. It provides new potential
infection biomarkers that can be used to detect early immune response against Map during the subclinical stage. This is a critical characteristic of novel diagnostics to prevent the further spread of this disease within and between herds, as current diagnostic tests fail at this stage to detect the infection.

**4.4 Future directions:**

Further longitudinal studies on experimentally Map infected calves are needed in the future for a better identification of the cell population responsible for our findings, specially phenotypes and actual function of bovine regulatory cells as well as other immune cells producing IL-10 such as γδ T-cells and monocytes as suggested by previous studies [19-21].

Treg cells and anti-inflammatory cytokines as IL-10 could be the best targets for future control of Map infection; suppression of that regulatory population – using an adjuvant in a vaccine for example - can induce more effective immune response against Map.

Th17 cells are a main component of the proinflammatory cytokine response occurring in Crohn’s disease causing immune – pathogenesis of the intestine [114, 121] and it was found to be activated after vaccination against bovine T.B. [122, 123], future studies of bovine Th17 and their role in Map infection are also needed.

JD is characterized by a very long subclinical stage; a longer term follow up of cell mediated immune response after Map exposure is required for better profiling of the transition stage from subclinical to clinical case which might also help in determination and/or prediction of the disease outcome.
Future RNA sequencing and microarray studies are needed after cell sorting of various cell populations in blood and/ or tissue of Map infected host. RNA microarrays were done to the animals in this trial to study the differentially expressed genes in whole blood after Map infection (J. David et al., unpublished data). The local immune responses in tissue samples after necropsy or by using an intestinal loop model might be interesting also to look at.

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