The contribution of IFN gamma, IL-10, and TGF beta in *Mycobacterium paratuberculosis*
infection

by

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For the Major Program

This dissertation is dedicated to:

My Father: Subhi Mohammad Khalifeh

My Mother: Salam Serree AL-Bustami

My Wife: Rula Ahmed Eid AbuJaradeh

My Son: Omar Mohammad Khalifeh

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ABSTRACT

The purpose of this work was to investigate the interaction of the pro-inflammatory cytokine, IFN- γ , and the anti-inflammatory cytokines, TGF- β and IL-10, in the subclinical and clinical stages of Johne's disease. A retrospective quantitative analysis of IFN- γ , IL-10 and TGF- β mRNA gene expression in tissues such as ileum, ileal-cecal junction, ileal-cecal node, and mesenteric lymph node obtained from cows naturally infected with *Mycobacterium paratuberculosis* and healthy non-infected control cows revealed an upregulation of IL-10 and TGF- β in tissues obtained from clinically infected animals but not in tissues obtained from healthy or subclinically infected cows. Analysis of IFN- γ mRNA gene expression in these tissues showed that expression of this cytokine is upregulated only in subclinical cows. We further evaluated the effects of exogenous IFN- γ , IL-10 and TGF- β in an extended culture system (7-12 days) using unfractionated PBMC cultures from naturally infected and healthy non-infected cows after infection with live *M. paratuberculosis*. We determined that the presence of IL-10 and TGF- β in culture supernatants was correlated with decreased ability of monocyte-derived macrophages in the unfractionated cell cultures to kill *M. paratuberculosis* in vitro, with a resultant decrease in IFN- γ production in these cultures. IL-10 and TGF- β were upregulated in response to in vitro infection in cell cultures from infected animals regardless of their disease status. Prior to in vitro infection with *M. paratuberculosis* TGF- β levels were lower in subclinically infected animals compared to clinically infected or healthy animals. After in vitro infection, TGF- β levels in culture supernatants of subclinically infected animals were similar to that of healthy animals. This is the first study to investigate the interactive roles of IFN- γ , IL-10 and TGF- β in natural and experimental *M. paratuberculosis* infections. The data presented here suggest an important regulatory role of anti-inflammatory cytokines IL-10 and TGF- β on IFN- γ production and the ability of macrophages to kill *M. paratuberculosis* in vitro.

INTRODUCTION

The immune system does not always succeed in recognizing and destroying all of the organisms that are present in any given infection. Normally the function of the macrophages is to ingest and destroy bacteria. When mycobacteria infect the body, they can evade the defenses of the host immune system by hiding inside macrophages (2, 7, 10, 11). In early stages of mycobacterial infection, the cell-mediated immunity is activated and successful in containing but not eliminating the infection (22). This is represented by the increased presence of pro-inflammatory cytokines in the contained stage of the disease. When mycobacterial infection progresses into clinical stage of the disease, the ability to mount an effective cell-mediated immunity appears suppressed as indicated by the decreased production and detection of pro-inflammatory cytokines such as IFN- γ (22). The reason(s) for suppression of the cell-mediated immune response is not completely understood. However, the increased presence of anti-inflammatory cytokines such as IL-10 and TGF- β as the infection progresses to a more clinical stage suggests that it might be an active process orchestrated by these cytokines (1, 8, 14, 18, 25, 26).

There is a great body of evidence that increased expression of IL-10 and TGF- β and decreased expression of IFN- γ may be involved in the transition of mycobacterial infections such as tuberculosis or leprosy from contained states to more clinical states. For instance, the involvement of IFN- γ in tuberculosis is well studied and evidence suggests that this cytokine is the most important pro-inflammatory cytokine in defense against *M. tuberculosis* (3, 5, 6, 17). Addition of recombinant IFN- γ to macrophage cell cultures enhanced the ability of these cells to limit *M. tuberculosis* and *M. bovis* growth (6, 17). Conversely, infection of IFN- γ knockout mice with a sublethal dose of *M. bovis* or *M. tuberculosis* lead to an increase in mortality and increased bacterial recovery from spleen, liver and lung (3, 5). In addition, measurement of IFN- γ production in response to in vitro mycobacterial antigen stimulation is used as an indicator of *M. tuberculosis* infection (23). Similar to tuberculosis, cytokine

involvement in leprosy indicates that the contained and localized infection which is known as the tuberculoid form of disease is mediated by a Th-1 type of response represented by IFN- γ production (1, 15, 25).

The presence of the anti-inflammatory cytokines that counteract, inhibit and manipulate the effects of pro-inflammatory cytokines might be one mechanism for intracellular survival of mycobacteria. Evidence obtained from studies with IL-10 transgenic mice and IL-10 knockout mice indicates that IL-10 is often associated with immune suppression in mycobacterial infection and with the enhanced replication of mycobacteria (13). Similarly, TGF- β has also been shown experimentally to enhance the intracellular growth of *M. tuberculosis* in human monocytes (9). The interaction between IL-10 and TGF- β in *M. tuberculosis* infection appears to be synergistic and effective in downregulation of *M. tuberculosis* induced T cell production of IFN- γ (16). These two cytokines also were found to be upregulated in advanced stage of leprosy (i.e., lepromatous leprosy) (1, 8, 14, 18, 25, 26).

Similar to other mycobacterial infections, *M. paratuberculosis* taken up by macrophages can evade the aggressive host immune reaction generated inside these cells. This bacterium causes chronic enteric disease characterized by intermittent diarrhea, weight loss and eventually animal death. The disease progresses from a contained stage that is referred to as a subclinical infection where animals are asymptomatic and shed few or no bacteria in their feces to a more advanced clinical stage. With progression to the clinical stage of disease, the bacterial shedding increases and clinical signs begin to appear. The transition of the disease from the subclinical stage to the clinical stage reflects an ongoing host immune response struggle.

Cytokine expression in the two stages of disease is typified by the presence of high levels of IFN- γ in the contained stage of disease, representing an activation of the host's cell-mediated immune response, and a decreased expression and production of this cytokine in the

advanced clinical stage. Animals in the excretory subclinical stage are reported to have high IFN- γ locally at the site of infection in tissues such as ileum and mesenteric lymph nodes (21) and produce high amounts of IFN- γ in peripheral blood mononuclear cell (PBMC) cultures stimulated with *M. paratuberculosis* antigens (19, 20). This response is downregulated when animals progress to the clinical stage of the disease where other proinflammatory cytokines such as IL-1 β and IL-6 become elevated (12, 20). This suggests that an immunomodulatory response alters an effective cell-mediated immunity represented by the presence of IFN- γ in the subclinical animals. Recently, two reports correlated the presence of IL-10 with the clinical stage of *M. paratuberculosis* infection. The first study reported that stimulation of PBMC obtained from two clinically infected cows with ConA upregulated the gene expression of IL-10 more than cells obtained from healthy and subclinically infected cows (4). Another reported that after stimulation of monocyte-derived macrophage cultures obtained from two non-infected cows with live *M. paratuberculosis*, IL-10 gene expression was significantly elevated at all time points (i.e., 6h, 24h and 72h) compared to non-stimulated cell culture (24). In regards to TGF- β , no report addresses the role of this important immunoregulatory cytokine in bovine *M. paratuberculosis* infection.

In this study, we investigated the involvement of IL-10 and TGF- β in Johne's disease. Similar to other mycobacterial infections, IL-10 and TGF- β were found to negatively control *M. paratuberculosis* infection. We found that exogenous addition of these two anti-inflammatory cytokines downregulated the ability of monocyte-derived macrophages in the extended (7-12 days) unfractionated PBMC culture to kill live *M. paratuberculosis* in vitro. Moreover, these cytokines resulted in a reduction in IFN- γ production in all groups (i.e., healthy and naturally infected groups) with more pronounced inhibitory effects noted in cell cultures from subclinically infected animals. We also found that these two cytokines were specifically induced in PBMC cultures obtained only from infected animals regardless of their disease status after stimulation with live *M. paratuberculosis*. However, before stimulation with live *M. paratuberculosis*, PBMC from subclinically infected animals

produced less TGF- β when incubated in control medium than cells from clinically infected and healthy animals. After in vitro infection, TGF- β production increased in cell cultures of subclinically infected animals and became similar to the levels of production in cell cultures of healthy animals. This study also determined that these anti-inflammatory cytokines were expressed at higher levels in tissues from clinically infected cows compared to tissues from subclinically infected or healthy cows. In contrast, the gene expression of the pro-inflammatory cytokine, IFN- γ , was higher in tissues from subclinically infected animals compared to healthy or clinically infected animal tissues.

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CHAPTER ONE: LITERATURE REVIEW

Introduction to Johne's Disease

Johne's disease is a chronic enteric disease characterized by intermittent diarrhea, weight loss and death. The causative agent of Johne's disease is *Mycobacterium paratuberculosis*. This organism has been shown to infect a wide variety of animals including cattle, sheep, goats, camels, bison, and deer (100, 151). Typically, cattle first encounter the infection during the first twelve months of life (93). It is likely that the infection occurs through the ingestion of milk, feed or water that has been contaminated by manure containing *M. paratuberculosis* (27, 157, 160). Up to ten percent of calves are infected with *M. paratuberculosis* through vertical transmission from the dam to the fetus (161). The primary site of infection for *M. paratuberculosis* is the intestine; thus, fecal material is the most common vehicle for disease transmission and ingestion of contaminated fecal material is considered the common means for *M. paratuberculosis* infection. Milk from infected dams is another source of *M. paratuberculosis* infection (157, 160). The duration that the calves spend with infected dams as well as the frequency of nursing increases the probability for young animals to become infected (157). In addition to ingestion of the bacteria that are excreted into the mother's milk, calves may also become infected through fecal contamination present on the teats (157).

The excretion of *M. paratuberculosis* in the milk of infected cows increases as the infection progresses (160). At early stages of the disease when fecal shedding was light only 3% of milk samples and 6% of supramammary lymph node samples were positive for *M. paratuberculosis* (160). When the disease state and *M. paratuberculosis* shedding in feces was intermediate, the percentage of milk samples that were positive for *M. paratuberculosis* increased to 11% of (i.e., one sample out of 9 tested milk samples) and 22% of supramammary lymph node samples (i.e., two samples out of 9 tested samples) (160). In animals that were heavy shedders, the percentage of milk and supramammary lymph node

samples that were positive for *M. paratuberculosis* increased to 19% (i.e., 7 samples out of 37 tested samples) and 46% (i.e., 18 samples out of 39 tested samples), respectively (160). Therefore, the risk of calf infection increased if they were fed milk of heavily infected dams.

Johne's disease typically progresses through three distinct stages (Table 1) (27). The division of these stages depends upon the existence of clinical signs and the correlation of these signs with fecal bacterial shedding. Accordingly, the first and the second stages are considered subclinical stages where animals are asymptomatic. Animals may remain in these stages without developing clinical disease or might progress into showing clinical signs after 3-4 years of infection. During the first subclinical stage, the infectious process develops without detectable fecal shedding followed by the subclinical intermittent excretory phase (stage II). The third or the clinical excretory stage is characterized by progressive symptoms associated with chronic shedding of high levels of bacteria in the feces and severe diarrhea with concomitant weight loss.

Table 1. Stages of Johne's disease.

Stage	Fecal shedding	Clinical signs	Animal Age
Stage I	light	None	Calves, heifers and adults
Stage II	Intermediate	None	Adults (3-4 years)
Stage III	Heavy	Present	Adults older than 4 years

Johne's disease in the adult cows is characterized by persistent diarrhea, emaciation, normal or increased appetite, absence of fecal blood or mucus and non-responsiveness to standard antibiotic treatment (188). Other clinical manifestations that occur in advanced cases, sometimes referred to as stage IV, include lethargy, weakness and submandibular edema (188). Submandibular edema, also known as bottle jaw, is a typical clinical sign of an advanced Johne's disease. It occurs as a result of decreased intestinal function with loss of

albumin (i.e., protein-losing enteropathy), and edema formation secondary to hypoproteinemia. Dependence upon these clinical signs alone for diagnostic purposes will not determine the extent of *M. paratuberculosis* infection in the herd. Due to the difficulties in detecting animals within the long incubation period (i.e., stage I and II), there is a great underestimation of the numbers of infected animals identified within any given herd. However, diagnosis of a single clinical case may be an indication of a serious herd problem and represent the “tip of the iceberg” on that farm (188).

There are many available tests to detect animals with Johne's disease. These tests include detection of the bacterium by culture or PCR, or detection of either humoral or cell-mediated immune response (CMI). The most sensitive and accurate method for Johne's disease diagnosis is to detect the bacterium that causes the infection. There are three ways to detect infection through identification of the microorganism. Two of these methods focus on isolation of the entire organism: standard culture and BACTEC radiometric culture. Standard conventional culture methods using Herrold's egg based medium (HEYM) may take 2 – 3 months before a colony is visible on the surface of the medium (28). BACTEC culture methods take advantage of the speed and sensitivity of the radioisotope-based detection and growth of *M. paratuberculosis* can easily be noted within 2 – 4 weeks (28, 92). It basically correlates the amount of CO₂ release in the vial containing *M. paratuberculosis* to a statistically calculated growth index (92). Compared to the standard culture methods which could detect as low as 15 -50 organisms per gram of feces (28, 131, 139), the BACTEC culture method is more sensitive and permits the detection of as few as 3 organisms per gram of feces (28). The BACTEC culture method is also less likely to be influenced by bacterial clumping (28). Moreover, the contamination rate reported in the BACTEC radiometric culture is as low as 3.95% when the concentration of the fecal specimen is applied by filtration (28). This is half the contamination rate reported with conventional standard culture on HEYM (28). However, *M. paratuberculosis* growth confirmation is required with BACTEC by Ziehl-Neelsen stain, since the colonies cannot be visually identified as they are

on HEYM and often contaminating microorganisms may cause false positive readings. Unfortunately, most infected animals are asymptomatic and either do not continuously shed *M. paratuberculosis* or shed the bacterium in undetectable numbers. Even when the number and the frequency of *M. paratuberculosis* shedding increases in clinical stage, it is still impractical to use bacterial isolation as a diagnostic tool due to the slow growing nature of *M. paratuberculosis*.

The third diagnostic method that depends upon bacterial detection is fast, but is relatively less sensitive than culture methods. This test depends on the detection of *M. paratuberculosis* specific genes through PCR. The major limitation with this detection method is to find the gene or genes that are specific to *M. paratuberculosis* and are not shared with *M. avium* or other closely related environmental mycobacteria. The most routinely utilized gene sequence to detect *M. paratuberculosis* infection is IS900. This insertion sequence is present in multiple copies with approximately 10 –20 genomic copies in all tested *M. paratuberculosis* strains but absent in most *M. avium* genomic DNA (177). Sequences related to IS900 have recently been isolated from some *M. avium* and other mycobacterial strains. For example, clinical strains of *M. avium* isolated from HIV patients were positive for IS900 (115). Moreover, recent studies reported the presence of sequences that have 71 - 79% homology to *M. paratuberculosis* IS900 in *M. scrofulaceum* and *M. marinum* (85). A single IS900 copy was also found in *M. cookii* that carries up to 94% homology to *M. paratuberculosis* IS900 (47).

Despite the limitation of IS900 specificity, several studies reported an improvement in both sensitivity and specificity using IS900 PCR for *M. paratuberculosis* detection. For example, the use of real time PCR to detect *M. paratuberculosis* IS900 not only quantitated *M. paratuberculosis* but also obtained results highly comparable in its sensitivity to fecal culture results (50). When the real time PCR method for IS900 was tested for its specificity and compared with fecal culture of 41 specimens, only one sample tested positive with the real-

time PCR and not with fecal culture. This suggests either a low number of bacteria that were not viable and therefore couldn't be detected by culture but could be detected by PCR; or that the real time PCR was detecting IS900 from a closely related environmental mycobacterium. Consequently, the need to find more specific genes to confirm the presence of *M. paratuberculosis* is still a necessity. Recently, twenty-seven new coding sequences from *M. paratuberculosis* have been identified via total genome sequencing, compared with *M. avium* and were shown to be specific for *M. paratuberculosis* (8). These sequences might be further used to improve the sensitivity and specificity of PCR as a molecular diagnostic tool.

In ruminants, *M. paratuberculosis* infection leads to the development of both cellular and humoral immunity. During the subclinical stage of Johne's disease, IFN- γ secretion by peripheral blood mononuclear cells (PBMC) is elevated in response to *M. paratuberculosis* infection (148, 158). Therefore, an IFN- γ ELISA has been developed to aid in the detection of animals at this critical stage of the disease. Later, after animals progress to the clinical stage, the cell-mediated immunity begins to decrease and humoral immunity increases. Animals in the clinical stage have elevated serum antibodies that are specific to infection with *M. paratuberculosis* (27). This humoral immune response is not helpful in protecting animals but has a great benefit in confirming the presence of *M. paratuberculosis* infection. The antibody response becomes stronger as animals progress further in the clinical stage of the disease and shed large numbers of bacteria in their feces, while animals in the subclinical stage of disease have negligible antibody levels. For example, only 15% of cows identified as low *M. paratuberculosis* shedders (i.e., subclinical Johne's cows) tested positive for *M. paratuberculosis*-specific antibodies by ELISA versus 87% detected positive among the high shedders (i.e., clinical cows) (36, 159). Therefore, using antibody detection as a diagnostic tool is limited primarily to animals that are in the clinical stage of disease and cannot be employed to identify infected animals in the subclinical stage of the disease. In other words, when testing herds for Johne's disease, tests for IFN- γ and antibody response should be done in parallel in order to detect the animals that are in the subclinical stage and in the clinical

stage, respectively. For instance among 16 cows that were identified as low shedders, the IFN- γ assay detected 8 cows as positive producers of IFN- γ while *M. paratuberculosis*-specific antibody ELISA detected another 4 cows (36). In animals with clinical stage of the disease and high bacterial shedding, 2 out of 6 cows tested were positively detected by IFN- γ assay and the others were positive based upon the presence of antibody (36).

Other tests used to evaluate CMI are based on measurements of lymphocyte proliferation or migration. Peripheral blood mononuclear cells (PBMC) were obtained from *M. paratuberculosis*-infected animals and stimulated with concanavalin A (Con A) or with either *M. paratuberculosis* or *M. bovis* purified protein derivatives (88). PBMC cultures from the infected animals that were stimulated with Con A had lower proliferation capacity than cell cultures obtained from non-infected animals, while stimulation with PPD induced higher proliferative responses in the infected animals. In addition, placing peripheral blood mononuclear cells in agar plates then stimulating them with johnin resulted in an inhibition in the ability of cells obtained from infected animals to migrate in agar (10). However, neither assay has been adopted as a tool of routine diagnosis of Johne's disease cases. Both assays involve the isolation of leukocytes from peripheral blood and require long incubation periods (3-7 days) (88). In addition, IFN- γ measurement correlates well with the results of lymphocyte proliferation and migration assays, requires a shorter incubation period and is less labor intensive. Therefore, the IFN- γ assay is more preferable than lymphocyte proliferation and migration assays.

The long incubation period, difficulty in early detection, lack of an effective vaccine and the poor understanding of the pathogenesis of this disease makes it very difficult to develop an effective control program and thus exacerbates the economic impact of this disease. Control programs mainly suggest culling the animals that show clinical signs (135). Although this might lead to the eradication of infected animals from the herd in the long run, it has a great economic impact on the herd productivity. It also increases the overall cull rate and reduces

the culling of other less productive animals because it forces the owner to cull valuable animals with great productivity or outstanding desirable genetics because they become infected or were exposed to *M. paratuberculosis* (77). Other economic losses are mainly due to the decreased weight and decreased milk production, which may reach as high as 25% over lactation in infected animals (77).

In addition to the direct economic impact of Johne's disease on the animal industry, some evidence suggests that *M. paratuberculosis* is a potential agent in a chronic enteric disease in humans, known as Crohn's disease (CD). This disorder shares some clinical symptoms with Johne's disease. Both are chronic granulomatous diseases affecting gut nutrient absorption and fluid retention, leading to diarrhea and continuous weight loss (163). Although the causative agent has not been specified in CD, some studies have isolated *M. paratuberculosis* from tissues of infected individuals. Isolation of *M. paratuberculosis* from some CD patients was first reported in 1984 (163). Recently, using improved culture techniques and IS900 PCR on centrifugal pellets obtained from surgically resected guts from Crohn's disease patients, 20% of the biopsies were positives for *M. paratuberculosis* (142). Another study showed detection of *M. paratuberculosis* in 72% of intestinal biopsy samples from CD by employing an in situ hybridization method using the IS900 probe (76). The same study concluded that infection might be caused by cell wall-deficient *M. paratuberculosis* since no bacteria were detected by Ziehl-Neelsen staining. Another study also correlated *M. paratuberculosis* with CD by demonstrating the presence of *M. paratuberculosis* in the centrifugal pellets of breast milk from 2 mothers with Crohn's disease (116). However, there is still a strong debate concerning the causative agent of CD as other bacterial and viral pathogens have been implicated in this disorder (132, 175). Nevertheless, the possible association of *M. paratuberculosis* with CD is another reason to develop mandatory programs to control Johne's disease.

The key element to control Johne's disease is mainly achieved through herd management. Testing animals provides information about the presence, extent of infection and the number of infected animals in the herd. Once the extent of the infection is identified, appropriate management must follow to prevent bacterial exposure of susceptible newborn calves (135). The main control element is to prevent oral manure contamination by infected adults to calves (135). The control strategies to achieve this goal vary according to each farm situation. Housing young calves in separate facilities from adult cattle and then following strict sanitation procedures to ensure this separation is a key in any successful control program (135). It is also helpful to feed calves milk obtained only from Johne's negative cows, use milk replacer or pasteurize the dam's milk prior to consumption (135). Another important point is to reduce the total farm exposure level to *M. paratuberculosis* by removing the bacteria from the environment and reducing the number of infected animals that are shedding the bacteria, with the most severely infected cows to be culled first (135). In the end it is important to be aware that complete eradication of Johne's disease from an infected herd is likely to take many years and also needs to be applied in a mandatory rather than through voluntary control programs.

Immunology of the Gastrointestinal Tract in Health and Disease

The mucosal immune system at the gastrointestinal tract is functionally divided into inductive and effector sites (101). An inductive site is where the antigen uptake and processing occurs. The main inductive site in the gastrointestinal tract is the Peyer's patches in the ileum (101). Peyer's patches are large aggregates of lymphoid tissue which lie beneath the interior surface of the intestine. The aggregates are organized clusters of antigen presenting cells (APC), mainly macrophages and dendritic cells, overlying B cell follicles and interfollicular regions enriched in T lymphocytes (82, 101). This lymphoid dome is covered with specialized epithelial cells called M cells (82). These cells translocate antigens found in the intestinal lumen to the Peyer's patches where they are ingested by the APCs,

then processed and presented to the lymphocytes. This antigen exposure at the inductive site is very important for the education of the immune system to its surrounding environment.

Presentation of the antigen to CD4+ T cells leads to their differentiation into functionally distinct subsets of cells. The type of cells generated controls the balance between antigen responsiveness and antigen unresponsiveness (118). CD4+ T cells at these inductive sites can differentiate into T helper 1 (Th-1) cells that secrete cytokines such as IFN- γ and TNF- α ; T helper 2 (Th-2) cells that secrete IL-4, IL-5, IL-9, IL-10 and IL-13; T helper 3 (Th-3 cells) that mainly produce IL-10 and TGF- β , or regulatory T cells (Tr cells) that mainly produce IL-10 (66, 133, 184, 185). Once these cells differentiate into effector T cells they migrate out to the regional lymph nodes then into the peripheral blood and later home to mucosal tissues (i.e., effector sites) (81, 82, 101). These T cells and their associated pro-inflammatory or anti-inflammatory cytokines play an important role in maintaining healthy and protective mucosal surfaces (152, 153).

Due to the abundant antigen load, exposure of the lymphoid aggregates in the Peyer's patches to most antigens results in a state of immunological unresponsiveness (152). However, selected antigens can provoke an immune response that will result in effective cell-mediated and/or antibody-mediated immune responses (15, 133). The site of entrance and the dose of the antigen, the manner in which the antigen is presented to APCs and the cytokines present at the time of T cell – APC interaction determine the outcome of T cell activation and differentiation (82, 133, 152). For instance, a very low continuous doses of antigen or a very high single dose of antigen leads to a state of tolerance to that particular antigen (Th-3 or Tr cells development). A low level of antigen presentation on the surface of APCs causes activation of T cells and differentiation that bias toward Th-2 cells while a high level of antigens presentation at the surface of APCs causes differentiation to Th-1 cells. Alternatively, a high dose of antigen processed and presented by enterocytes expressing

MHC II can lead to a state of tolerance. This is mainly due to the inability of these cells to provide the appropriate co-stimulatory signal required for generating strong T cell activation.

T helper cell differentiation is also affected by the presence of cytokines and chemokines. Cytokines such as IL-12 and IL-18, for example, direct cell differentiation towards Th-1 cells (9), whereas IL-4 and IL-13 induce Th-2 differentiation (106, 123). TGF- β and IL-10 are involved in a positive feed back mechanism that results in the up-regulation of Th-3 and Tr cell activation (66, 143). Chemokines such as I-TAC (IFN-inducible T cell alpha-chemoattractant), Mig (monokine induced by IFN- γ), and IP10 (IFN-inducible protein 10) are expressed on Th-1 cells and favor Th-1 recruitment and down regulate Th-2 recruitment though emphasize Th-1 polarization (97).

Other factors involved in the commitment of T cell differentiation include co-stimulatory factors and transcriptional factors present at the time of T cell differentiation as well as the type of antigen presenting cells. For example the co-stimulatory molecules such as OX40 on APCs and OX40L on T cells prime T cells towards Th-2 development (56, 123). The inducible costimulator (ICOS) expression on APCs and ICOSL on T cells leads to the production of IL-10 which mainly induces Th-2 development (105, 190). Alternatively, CD40/CD40L interaction primes T cells bearing CD40L towards Th-1 development (17, 124). The interaction of CD4⁺ T cells with immature DC seems to preferentially induce Tr, CD8 α ⁻ DC interaction with CD4⁺ T cells induces Th-2 differentiation, while interactions with CD8 α ⁺ DC produce cytokines such as IL-12 that lead to Th-1 differentiation (49, 90, 102). Finally, the activation of transcription factors such as T-bet and STAT-4 direct T cells differentiation toward Th-1 cells (110) while GATA-3, c-maf and NFATc1 have been shown to induce IL-4 production by T cells and, therefore, favor Th-2 development (84, 195, 199). Differentiation of T cells depends upon the combination of the factors mentioned above. The resultant population of T cells generated controls the balance between activation of a protective immune response and the development of tolerance.

On the other hand, disturbance of Th-1 and Th-2 homeostatic balance at the mucosal surfaces of the gastrointestinal tract may lead to the development of immunological disorder. The uncontrolled action of both Th-1 and Th-2 cells contribute to the induction of intestinal inflammation. However, the uncontrolled action of these cells may be regulated by cytokines produced from Tr and Th-3 cells (14). Intrarectal administration of oxazolone to SJL/J mice (i.e., a Th-2 mediated colitis model) caused a rapid onset (i.e., 2 days) of colitis marked by weight loss and diarrhea and leading to death of 50% of the mice by day 4 (14). When T cells extracted from the inflamed lamina propria were cultured in vitro without stimulation, they produced 10-fold higher IL-4 production and 15-fold higher TGF β production than cells isolated from non-inflamed lamina propria mononuclear cell culture (LPMC) (14). Mice administered anti-IL-4 antibody quickly regained the lost weight and were near baseline weight within 4 days of oxazolone/ antibody administration (14). In contrast, mice administered anti-TGF- β antibody developed a severe colitis that involved the entire length of the colon and was associated with severe weight loss (14). The immune response manifestation and the histopathological changes observed in this experiment were similar to that reported in ulcerative colitis patients (14).

Colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) administration is Th-1-mediated (87) colitis and similar to the oxazolone colitis model (Th-2-mediated colitis) TGF- β is responsible for the decrease in disease and lesion manifestation (119). Mice fed haptenized colonic proteins (HCP) before rectal administration of TNBS generated mucosal T cells producing high TGF- β (Th-3 cells) and Th-2 like cytokines (Tr cells) that caused abrogation of Th-1 cytokines such as IFN- γ and IL-12 and consequently led to suppression of TNBS induced colitis (87). Similarly, colitis induced by TNBS was prevented by administration of TGF- β plasmid intranasally to mice (87). This administration of TGF- β 1 caused an increase in serum TGF- β and an increase in the presence of TGF- β DNA detected by PCR in intestinal mucosal tissues. In addition, stimulation of LPMCs isolated from mice given TNBS via the rectum and intranasally administered TGF- β plasmid produced

decreased levels of IFN- γ and IL-1, high amounts of TGF- β and a striking increase in IL-10 production compared to mice not administered TGF- β plasmid (87).

Involvement of Th-1 cells in mucosal inflammation in Crohn's disease (CD) is well established (58, 127). Aberrant mucosal Th-1 cell activation and production of IL-12, the major Th-1 driving factor was found to be associated with Crohn's disease (11, 127). CD4⁺ cells isolated from the lamina propria of CD patients display a Th-1 cytokine pattern where they are able to produce 12 times more IFN- γ , 2 times more TNF- α , and 3 times less IL-4 than cells isolated from healthy individuals (103). In addition, expression of the co-stimulatory molecule, B7-2, was upregulated on lamina proprial mononuclear cells isolated from CD patients (121). The high level of this co-stimulatory molecule on lamina propria monocytes may be a factor in up-regulating T cell activation in the LP (121).

In contrast to Crohn's disease, ulcerative colitis predominantly involves upregulation of cytokines such as IL-4, IL-5 and IL-10 suggesting Th-2-mediated colitis (59, 120). In oxazolone colitis, high levels of IL-4 and IL-5 and low to normal levels of IFN- γ production support the theory that an uncontrolled Th-2 response may lead to a severe colitis that is characterized by the rapid onset of inflammation progressing to either complete recovery or death (14). Thus, it is different from Th-1-driven TNBS colitis in mice that develops more slowly and tends to persist in surviving mice (14).

T helper Cells and Their key Regulatory Cytokines

Th-1 cells

Th-1 is the key regulator of type I immunity and IFN- γ is the chief cytokine that orchestrates most of Th-1 activities. IFN- γ upregulates MHC class II expression on macrophages (29, 42). Therefore, IFN- γ boosts the effectiveness of T cell-macrophage interactions. IFN- γ

upregulates the production of cytotoxic reactive oxygen and nitrogen intermediates from macrophages, and as a result increases the intracellular killing of microorganisms (140). There is a positive correlation between the upregulation of ROI and NOI in macrophages and the reduction of intracellular survival of pathogens in macrophages. For example, the survival of *Toxoplasma gondii* in macrophages was significantly decreased after treatment of macrophages with IFN- γ (111). The decreased survival was correlated with an upregulation of ROI and NOI production in these cells (111). IFN- γ also stimulates cytotoxic T lymphocyte and NK cell generation in addition to positively regulating Th-1 differentiation.

In addition, IFN- γ accumulation induces the four cardinal signs of inflammation. IFN- γ induces inflammation by upregulation of adhesion molecule expression on endothelial cells (82, 83, 91). IFN- γ also causes vascular smooth muscle relaxation and endothelial retraction (70, 154), therefore, increasing the permeability of the epithelial layer (125). Thus, these effects on endothelial tissue could provoke the blood vessels to pass leukocytes, plasma proteins and inflammatory mediators to the site of inflammation where excess IFN- γ is produced. As a result swelling, pain, erythema and heat generation will be prominent at the site where IFN- γ is upregulated. These signs of inflammation are helpful in protection against many diseases but if left unchecked may lead to vigorous chronic inflammatory response as in the case for CD or JD.

Th-2 cells

Induction of Th-2 promotes humoral immunity including a strong antibody response. Th-2 differentiation also induces mast cells and eosinophilic growth and differentiation factors (98), inhibition of several macrophage functions such as nitric oxide and reactive oxygen intermediates production (3, 12, 13, 61), and down-regulation of the Th-1 response. The Th-2 regulatory cytokines include IL-4, IL-5, and IL-10 (51, 109). IL-4 is critical in driving Th-0 towards Th-2 differentiation (68). It also specifically inhibits IFN- γ -production from Th-1

cells (128) and promotes the proliferation of activated human B lymphocytes (26) as well as the secretion of immunoglobulins such as IgG1, IgG4 or IgE (60, 146). IL-5 is associated mainly with eosinophilic inflammation (182) but also acts as a late factor in B cell differentiation causing an enhancement of IgM, IgG or IgA production (138).

IL-10 is considered an immunosuppressive cytokine that can be produced by several cell types including activated Th-2 cells, B cells, and macrophages (37, 38). LPS-induced production of IL-10 from monocytes and macrophages is enhanced in the presence of TNF- α and suppressed in the presence of IL-4, IL-10, IFN- γ and TGF- β (25, 38, 130, 183). The production of IL-10 in PHA-activated T cells (i.e., CD4⁺ and CD8⁺ T cells) was enhanced in the presence of IL-12 (34). Stimulation of pure CD4⁺ and CD8⁺ T cells isolated from PBMC by positive selection with various concentrations of IL-12 and in the presence of PHA resulted in at least a 3-fold increase in the amount of IL-10 produced (34). This study suggests that IL-12 and IL-10 are key regulatory cytokines that manipulate the outcome of T helper cell activation. IL-12 produced from monocytes might play an early role in the immune response by generating and activating Th-1 responses, but may later stimulate the production of IL-10. This is also supported in the study of cytokine production kinetics in human monocyte cell cultures after LPS stimulation (38). Following mitogen activation of PBMC, pro-inflammatory cytokines such as TNF- α , IFN- γ and IL-12 were upregulated in early stages of culture (i.e., 4-8h) followed by detection of IL-10 after 7 hours of incubation with a peak production of IL-10 at 24-48h (38).

IL-10 is also produced by both Ly-1⁺ B-1 cells and Ly-1⁻ conventional B cells but B-1 cells are more extensive producers of IL-10 (62, 122). In mice, this cytokine positively regulates the growth and development of the B-1 cell lineage, (78) and, interestingly, rIL-12 stimulates B-1a cells (CD5⁺ B cells) in splenocyte cell cultures to express high levels of IL-10 mRNA (178). IL-10 interrupts the expansion of memory B cells in the secondary lymphoid organs germinal center (GC) (24, 197). The absence of CD40L stimulation and the presence of IL-

IL-10 drives B cell development and differentiation in the GC towards plasma cells while the absence of IL-10 leads to the development of memory B cells (24, 197).

IL-10 is considered as an important immune regulatory cytokine that controls the shift of the immune response from a pro-inflammatory to an anti-inflammatory response. IL-10 decreases the production of a broad spectrum of pro-inflammatory cytokines such as IL-1, IL-6, GM-CSF, G-CSF, M-CSF, MIP-1 and -2, TNF- α , RANTES and IL-12 from monocytes (40). IL-10 specifically inhibits IFN- γ synthesis by T cells (53) and NK cells (75). It also can inhibit the microbicidal activity as well as nitric oxide production of IFN γ treated macrophages that is essential for the control of intracellular pathogens such as listeria, leishmania and mycobacteria (61). In contrast to the inhibitory effect of IL-10 on monocyte/macrophage activities, this cytokine upregulates the phagocytic ability of these cells through an increase in the expression of FcR's such as CD16 and CD64 (162).

T regulatory cells

IL-10 induces the differentiation of regulatory T cells (66, 67). It inhibits the synthesis of CD80, CD86 and ICAM-1 which decreases the strength of interactions between APC's and T cells leading to weak stimulatory effects for T cell activation (20, 45, 189). In a situation like this where effective APC's are absent, IL-10 can extend its inhibitory effects on T cells causing a suppression of IL-2 and TNF- α production from CD4⁺ cells (39, 41). In mice and humans, such T cells are designated T regulatory cells (Tr1) (66). These anergic T cells are unresponsive to antigen stimulation so they do not proliferate and produce low quantities of cytokines (i.e., IL-5, IFN- γ , TGF- β and TNF- α) compared to undifferentiated Th-0 cells (67). These cells are also different from Th-0 in their ability to produce very high levels of IL-10, low quantities of IL-2 and no IL-4 (66).

Th-3 cells

Regulatory T cells generated as a result of oral tolerance produce high levels of TGF- β and variable amounts of IL-4 and IL-10 upon activation (22). These cells are recognized as Th-3 cells (184, 185). While the induction of Th-3 is favored in a Th-2 environment, Th-1 conditions inhibit their induction. Therefore, conditions that favor high IL-4, IL-10, TGF- β production and/or low IFN- γ production greatly enhance TGF- β production from Th-3 cells (184, 185).

The most recent study evaluating the effects of TGF- β effect on T cells demonstrated that the effect on T helper cells is dependent upon T cell differentiation and activation conditions (64). Briefly, in the case of suboptimal antigen stimulation of T cells, the presence of TGF- β resulted in the inhibition of T cell activation and proliferation. In contrast, with optimal antigen activation, exposure of cells to TGF- β before they differentiate into either Th-1 or Th-2 causes a halt in their activation and results in the development of cells carrying neither Th-1 or Th-2 phenotype properties but still able to produce IL-2 (64). If T cells receive proper activation signals before getting exposed to TGF- β they differentiate normally towards either Th-1 or Th-2 (64, 136). If TGF- β is present with these fully differentiated cells along with IL-2, clonal expansion will occur. The inhibitory effect of TGF- β in the early stages of cell activation is more prominent for Th-2. IL-4 could not reverse the inhibitory effects of TGF- β but the development of Th-1 cells was possible with the addition of IFN- γ (96, 156).

Therefore, the inhibitory effect of TGF- β on Th-1 differentiation could be overcome in the presence of IFN- γ (136). Despite this effect, TGF- β was still able to inhibit the production of cytokines, specifically IFN- γ from these fully differentiated Th-1 cells (87, 99). In contrast, cytokine production from fully differentiated Th-2 cells is not inhibited by TGF- β (99, 136). The inhibitory effect of TGF- β on both resting Th-1 and Th-2 is due to high expression of

TGF- β receptors on their surfaces (31). In contrast, T cell activation causes a down regulation of TGF- β RII expression, which can be restored in the presence of IL-10 (31).

In addition to Th-3 cells, tissue macrophages (e.g., alveolar) and peripheral monocytes produce TGF- β (6). Similar to T helper cells, TGF- β also has both inhibitory and stimulatory effects on macrophage activities. Low TGF- β concentrations induce chemotaxis of human peripheral blood monocytes to the site of inflammation while high TGF- β concentrations activate these monocytes and causes increased expression of TNF- α and IL-1 β mRNA (21, 180, 181). TGF- β also activates the phagocytic ability of monocyte/macrophage through upregulation of CD16 expression on their surface (187). The effect of TGF- β on tissue macrophages is mainly suppressive. This includes increased expression of IL-1 receptor antagonist (170), and limited ROI and RNI production from macrophages activated with LPS or IFN- γ (12).

Cytokines Involved in Defense and Pathology of Mycobacterial Infection

Mycobacterium tuberculosis* and *M. bovis

Tuberculosis primarily affects the lower respiratory system and is characterized by a chronic productive cough, low-grade fever, night sweats, and weight loss. Mycobacteria enter the lung alveoli by airborne transmission. They resist destruction by alveolar macrophages and multiply, forming the primary lesion. Exposure to mycobacteria may not establish disease because of the complete and immediate destruction of bacteria by host innate immunity (173).

Recognition of mycobacteria by phagocytes is an important step in disease progression and bacterial destruction. Elevated levels of circulating factors such as plasma LPS-binding protein has been noted in patients with active tuberculosis (80). This resulted in enhanced

responsiveness of macrophages to LPS and lipoarabinomannan (LAM), a major mycobacterial cell wall component which resembles LPS (52). Through toll-like receptors (TLR 2 and TLR 4), which are an important mediator of microbial recognition by macrophages and dendritic cells (179), *M. tuberculosis* cell lysate or soluble cell wall lipoprotein components can induce IL-12 production (16). Failure to destroy the bacteria after the initial exposure by a component of innate immunity results in chronic infection. This infection will then be either contained (150) through development of a protective immune response (i.e., latent infection) or proceed into active tuberculosis within one to three years (155). Then the fate of the latent infection is either recovery or reactivation and development of tuberculosis.

Replication of bacteria inside the macrophage results in production of cytokines that initiate and activate the inflammatory response to mycobacterial infection. These cytokines mediate the cross-talk between macrophages and lymphocytes and result in granuloma formation that consists of macrophages, T cells, and granulocytes (141). Although mycobacteria continue to survive within the granuloma (i.e., tuberculous lesion), the granuloma formation in tuberculosis is essential in containing or segregating the infection from spreading to more healthy tissues (46). When the equilibrium between the host immunity and the bacteria within the tuberculous lesion weakens, release of the mycobacteria from the granuloma will result in either more granulomatous lesions or more commonly severe disseminated uncontained infection (46, 141).

Pro-inflammatory cytokines produced in response to mycobacteria are often associated with the contained form of infection. For example, infection of APC with mycobacteria or stimulation of APC with mycobacterial products induces TNF- α production (69, 172). TNF- α production regulates several factors involved in containing the mycobacterial infection such as granuloma formation and macrophage activation (86, 169). In addition, IL-1 is spontaneously released from bronchoalveolar lavage cells obtained from the infected lung of

TB patients (94). Studies with IL-1 knockout mice or IL-1R type I deficient mice indicated that the absence of IL-1 as well as its effect (i.e., IL-1R deficiency) causes an abrogation of granuloma formation allowing *M. tuberculosis* to replicate (79, 192). Almost 65% of the mice in the experiment with IL-1 receptor deficiency died, and the remaining animals become severely ill (79, 192). Another important immune regulatory cytokine involved in defense against *M. tuberculosis* is IL-12. Interleukin-12 is found to be elevated in the granulomas and in the lung infiltrates of tuberculosis patients (18). The absence of bioactive IL-12 resulted in the inability of IL-12 knockout mice to control bacterial growth in the lung, spleen and liver (30). The absence of this cytokine is also associated with a reduction of mRNA expression for both IFN- γ and TNF- α and subsequent amelioration of macrophage activity represented by the reduction of mRNA expression of inducible nitric oxide synthase (30).

The involvement of IFN- γ in tuberculosis is well studied and evidence suggests that this cytokine is the most important pro-inflammatory cytokine in defense against *M. tuberculosis*. Recombinant IFN- γ addition to macrophage cell cultures enhanced the ability of these cells to limit *M. tuberculosis* and *M. bovis* growth (54, 134). Continuous infusion of rIFN- γ to BALB/c mice via osmotic micropumps leads to an increase in their resistance to a lethal dose of *M. tuberculosis* as well as to decreased bacterial growth in spleen, liver and lung (43). Conversely, infection of IFN- γ knockout mice with a sublethal dose of *M. bovis* or *M. tuberculosis* lead to an increase in mortality and increased bacterial numbers recovered from spleen, liver and lung (29, 35). Measurement of IFN- γ production in response to in vitro mycobacterial antigen stimulation is also used as an indicator of *M. tuberculosis* infection (174). For example, whole blood cultures obtained from individuals with positive tuberculin skin test stimulation with PPD results in higher levels of IFN- γ than stimulation of blood cultures obtained from individuals with negative tuberculin skin test. This selective production of IFN- γ is present only if cells are stimulated with mycobacteria purified protein derivative, not when cells were stimulated with mycobacterium whole cell sonicate. The

latter stimulation induces IFN- γ production in both tuberculin skin test positive and negative individuals.

A more recent study indicated that *M. tuberculosis* has developed a mechanism by which it can block the effects of IFN- γ (164). This inhibition is mediated by interfering with the IFN- γ signaling pathway. One important step in IFN- γ signaling is the translocation of STAT1 homodimers into the nucleus (63). This translocated dimer interacts with the basal transcriptional apparatus in the nucleus through the CBP/P300 family of transcriptional coactivators which are essential for effective IFN- γ signaling (196). The presence of *M. tuberculosis* disrupts this interaction and causes further disruption through a reduction in the ability of IFN- γ treated macrophages to kill other intracellular organisms such as *Toxoplasma gondii* (164). The presence of IFN- γ in tuberculosis patients indicates a struggle between the immune system and the mycobacteria. The result is either a chronic latent infection or reactivation of infection along with a failure of the immune system and, thus, disease progression. Disruption of IFN- γ signaling may explain why the elevated IFN- γ production in *M. tuberculosis* does not clear the infection. Perhaps the inhibition of macrophage function due to reduced response to IFN- γ is one mechanism by which mycobacteria avoid destruction (55).

The survival of mycobacteria in macrophages likely involves more than one factor. The presence of the anti-inflammatory cytokines that counteract, inhibit and manipulate the effect of pro-inflammatory cytokines might be one mechanism for intracellular survival. Infection of monocytes isolated from human PBMC with *M. tuberculosis* induces the production of IL-12, TNF- α and IL-10 (57, 145). The early induction of IL-10 (3-6h) does not affect the ability of monocytes to co-express and release IL-12 since neutralization by addition of anti-IL-10 monoclonal antibody to the culture does not affect IL-12 production (57). Conversely, excess levels of IL-10 present before and during the first few hours of tuberculosis infection decreased IL-12 production and delayed its expression for 6 hours (57). These data suggest

that in the very early phase of monocyte exposure to *M. tuberculosis* infection and when the bacterial load is low, IL-12 production is optimal and is not affected by IL-10 co-induction. Later, when endogenous IL-10 expression is upregulated it causes the inhibition of IL-12 production with the most pronounced effect on the newly recruited monocytes. However, the recruitment of monocytes is accompanied by the recruitment of T cells. The presence of T cells could overcome the inhibitory effect of IL-10 on IL-12 production through IFN- γ release as IFN- γ dramatically enhances IL-12 production in monocyte cultures infected with *M. tuberculosis* (57).

Evidence obtained from studies with IL-10 transgenic mice and IL-10 knockout mice indicates that IL-10 is often associated with immune suppression in mycobacterial infection and with the replication of mycobacteria. One study showed that intravenous injection of IL-10 transgenic mice with Calmette-Guerin bacillus (BCG) resulted in dissemination of the bacteria throughout the body that persisted over the course of the experiment (12 weeks) (112). Interestingly, the excess IL-10 present in transgenic mice did not interfere with the ability of splenocytes to produce IFN- γ in response to BCG stimulation (112). Thus, it might be that an imbalance favoring IL-10 over IFN- γ production during mycobacterial infection results in reduced macrophage activation and subsequent decreased destruction of mycobacteria. In IL-10 knockout mice, infection with BCG resulted in a lower burden of bacteria in spleen and liver in the first two weeks of the experiment (113). Macrophages isolated from the peritoneum of these mice produced increased levels of inflammatory mediators such as NO, prostaglandin and TNF- α compared to wild type mice. When IL-10 was added to the cell cultures the increased production of these mediators was clearly reversed. The limited resistance to mycobacteria infection in the IL-10 knockout mice during early infection (first two weeks) suggests that other factors such as TGF- β or IL-4 might play a similar role to IL-10.

TGF β is upregulated during tuberculosis and at the site of active disease (166). Moreover, neutralization of TGF- β with anti-TGF antibody or with natural TGF- β inhibitors (e.g., decorin and latency-associated peptide (LAP)) is reported to improve T cell responses in PBMC cultures obtained from patients with pulmonary tuberculosis (71). PBMC culture from patients with active tuberculosis spontaneously produced high levels of TGF- β (167). Moreover, tissues from patients with active tuberculosis stained for intracellular TGF- β revealed that this cytokine was mainly present in Langhans giant cells and to a lesser extent in the epithelioid cells present in the granulomas (167). A comparison of alveolar macrophage and monocyte cell cultures after LPS, PMA and Con-A stimulation revealed that alveolar macrophages have a reduced ability to produce TGF- β (168). This suggests that TGF- β present within the granulomas may be a result of recruitment and activation of monocytes which originating from the blood.

TGF- β has also been shown experimentally to enhance the intracellular growth of *M. tuberculosis* in human monocytes (73). After infection of monocytes with *M. tuberculosis*, production of TGF- β in cell culture supernatant was low in the first 24 hours of incubation (<50 pg/ml) then increased up to 8-fold after 4 days in culture (73). This increased production of TGF- β was accompanied by an inability of monocytes in culture to control growth of *M. tuberculosis*. Neutralization of TGF- β resulted in a 3-fold reduction of the intracellular replication of *M. tuberculosis* by day 7 (73). Incubation of monocytes with 10 ng/ml of rTGF- β followed by infection with *M. tuberculosis* resulted in at least 3-fold increase in the number of CFU recovered from the cell lysate after 7 days in culture (i.e., 32×10^4 CFU/ml of cell lysate obtained from cells cultured with medium control compared to 112×10^4 CFU/ml in cells treated with rTGF- β). In this same study, addition of TNF- α to monocyte cultures after *M. tuberculosis* infection caused a 4-fold reduction in the CFU obtained from the cell lysate between day 4 and 7 (73). When TGF- β was co-cultured with TNF- α it reversed this effect resulting in a 2.9 fold increase in viable bacteria recovered (73).

Similar experimental results were obtained when infected monocytes were cultured with IFN- γ alone or in combination with TGF- β (73).

Finally, the interaction between IL-10 and TGF- β in *M. tuberculosis* infection appears to be synergistic (126). One study demonstrated that upregulation of TGF- β specifically upregulated IL-10 production and that these two cytokines overlap with each other to potentiate their downregulatory effect on *M. tuberculosis*-induced T cell production of IFN- γ (126). An interaction between TGF- β and IL-4 also occurs, but interestingly the presence of both cytokines directs the development of T cells towards a Th-1 profile of response (i.e., CD8+ type I cells) (48). TGF- β alone in the absence of IL-4 suppresses the development of CD8+ type I cells leading to the reduced production of IFN- γ (48). When both IL-4 and TGF- β were present in the lymph node cell culture of the CD8+ T cells, a strong Th-1 response characterized by high production of IFN- γ was noted (48). In tuberculosis, the role of IL-4 is in great dispute with suggestions ranging from the implication that this cytokine is involved in disease manifestation to the suggestion that its association with the tuberculosis is only a consequence of infection rather than a causative factor (173).

Mycobacterium leprae

Leprosy is an infection of the skin, peripheral nerves, and mucous membranes, progressing to lesions of hypopigmentation, and loss of sensation particularly in the cooler areas of the body (129). As in tuberculosis, the spectrum of leprosy pathogenesis ranges from localized tuberculoid, paucibacillary leprosy with strong cell-mediated immunity to disseminated lepromatous multibacillary leprosy, with loss of specific cell-mediated immunity (137). Cytokine involvement in leprosy is quite similar to tuberculosis. Briefly, the protective immunity that contains and localizes the disease in the tuberculoid form of leprosy is mediated by a Th-1 type of response represented by IFN- γ production (5, 117, 193).

Cytokines such as TGF- β , IL-10 and IL-4 are mainly associated with lepromatous leprosy and disseminated disease (5, 65, 114, 144, 193, 194).

Mycobacterium avium* and *Mycobacterium intracellulare

Mycobacterium avium Complex (MAC) disease is among the most common bacterial infections in people with AIDS. MAC bacteria were identified in the blood of 43% of people within two years of diagnosis with AIDS (2). MAC is the term for two related bacteria: *Mycobacterium avium* and *Mycobacterium intracellulare* (MAI) (19). The most common infection symptoms in MAI infection are persistent fevers plus night sweats, loss of appetite, weight loss, tiredness or worsening diarrhea (2).

Cytokine involvement in MAI infection follows a similar pattern of other mycobacterial infections including *M. tuberculosis*. Macrophages activated with IFN- γ or TNF- α can restrict *M. avium* growth (4). PBMC from healthy people that are exposed to MAC infection (i.e., positive skin test to *M. avium*) secreted increased levels of IFN- γ in their cell culture supernatants while PBMC cultures from HIV patients with active MAC infection produced high levels of IL-10 and low levels of IL-12, IFN- γ and TNF- α (176). Similar to other mycobacterial infections, TGF- β has an immunosuppressive role in *M. avium* infection. Mice injected weekly with 1 μ g of rTGF- β intraperitoneally and intravenously infected with 10^5 CFU of *M. avium* had a 3 log increase in *M. avium* recovered from the spleen after 80 days of infection compared to mice not treated with TGF- β (44). Treatment of mice receiving rTGF- β with an anti- TGF- β monoclonal antibody lead to a decrease in the numbers of CFU recovered from these mice (i.e., 0.7-0.9 log decrease at day 80) compared to mice received rTGF- β alone (44).

In summary, the involvement of anti-inflammatory and pro-inflammatory cytokines in immunity or the pathology of mycobacterial infection appears to involve a wide network of

cytokines. The effect of each cytokine alone might imply effects ranging from increased protection to increased susceptibility to mycobacterial infection. Yet the interaction of these cytokines during infection may yield similar, different or amplified results depending upon how they regulate one another.

The Immune Response to *Mycobacterium Paratuberculosis* Infection

Mycobacterium paratuberculosis the causative agent for Johne's disease generally colonizes in the lower part of the small intestine (108). Within a few hours after *M. paratuberculosis* ingestion, the bacteria cross M cells lining the Peyer's patches and can be detected in the subepithelial macrophages (108). The precise mechanism of *M. paratuberculosis* entry into macrophages is still unknown. Generally, mycobacteria could enter macrophages through TLR-2 which provoke cell activation and production of TNF- α (107, 165, 171). Complement receptors could also mediate an opsonic and non-opsonic phagocytosis (72) and activation or inhibition of macrophages activities after phagocytosis depending upon the complement receptor involved. For example, antigen uptake through CR3 is not associated with a respiratory burst and results in decreased IL-12 production by macrophages (104, 191). Uptake through the mannose receptor also facilitated phagocytosis of mycobacteria but did not elicit macrophage activation (e.g., phagosome maturation) (7). Therefore, the selective entry into macrophages that mycobacteria may create an environment that does not trigger macrophage defense mechanisms such as oxidative burst.

In response to this infection the host immune response initiates a series of immune defense mechanisms to deal with the *M. paratuberculosis* infection. The host's immune system responds to *M. paratuberculosis* infection by recruiting more macrophages to the site of infection as the number of macrophages present in the ileum of naturally infected cows was reported to be higher than non-infected control animals, with neutrophils being the predominant phagocytic cells in the latter group (95). Based upon information from other

mycobacterial diseases, it has been suggested that the host's immune system responds to *M. paratuberculosis* infection by recruiting more macrophages and lymphocytes to the site as well as by activation of immune cells such as gamma delta T cells, CD4⁺ T cells and cytolytic CD8⁺ T cells (32). Infiltration of infected tissues with lymphocytes and macrophages leads to thickening of the intestine and the mucosal surface becomes corrugated in appearance over time. Thus, the ileum loses its thin and pliable appearance due to the influx of numerous lymphocytes and macrophages (23, 32). The draining lymph nodes such as the mesenteric and ileocecal lymph nodes will become enlarged. Lesions in paratuberculosis infection are mainly due to accumulation of macrophages population in the intestinal wall (23, 95).

During this ongoing interaction between host immunity and the *M. paratuberculosis* infected macrophages, a deviation from the proper immune response against this intracellular infection might arise and disrupt the host's attempts to contain the disease. This might lead to progressive failure of the macrophages ability to kill the bacteria, increase the ability of *M. paratuberculosis* to replicate and eventually result in death of the macrophages.

Similar to other mycobacterial infections, *M. paratuberculosis* is an intracellular pathogen that once phagocytized by macrophages can evade the aggressive host immune reaction generated inside these cells. *M. paratuberculosis* resides inside phagosomes that retain features of the early phagosomal compartment. Experimental infection of the J774 mouse macrophages cell line with *M. paratuberculosis* has shown a decrease in phagosomal acidification (23, 74, 89). Macrophage activation with recombinant IFN- γ and LPS lead to increased acidification and maturation of phagosomes containing *M. paratuberculosis* (74). There was a significant decrease in the percentage of *M. paratuberculosis* surviving in the macrophage cell line treated with IFN- γ and LPS compared to non-treated cells (74). In addition, the colonization of *M. paratuberculosis* in the late phagosomal cell compartments after treatment with IFN- γ and LPS increased the bacterial exposure to more hostile

environment present in these late phagosomes (74). This suggests that activated macrophages are more effective in *M. paratuberculosis* killing than monocytes or inactive macrophages. Moreover, this result provides further evidence of the importance of IFN- γ in macrophage activation and maturation. In addition, treatment of *M. paratuberculosis*-infected bovine monocytes with IFN- γ increased the release of nitric oxide by macrophages (198).

The stages of Johne's disease reflect an ongoing struggle of host immunity. The induction of IFN- γ that is usually present in the contained stage of disease represents an activation of the host cell-mediated immune response. Animals in the excretory subclinical stage are reported to have high IFN- γ locally at the site of infection in tissues such as ileum and mesenteric lymph nodes (158). IFN- γ gene expression in the clinically infected cows is reported to be higher than in the control non-infected animals (95) but the highest gene expression of this cytokine was reported in tissues obtained from subclinical animals (158). Gene expression of other proinflammatory cytokines such as IL-1 α , IL-1 β and IL-6 was elevated in *M. paratuberculosis*-infected animals with clinical disease compared to control animals (95). In sheep, TNF- α , IL-1 β and IL-6 gene expression were also elevated in the intestinal tissues obtained from animals in the multibacillary stage of the disease (1). Nevertheless, TNF- α gene expression in cattle intestinal tissues was not different between *M. paratuberculosis* infected and non-infected animal groups (95). Moreover, PBMC cultures from subclinical cows stimulated with *M. paratuberculosis* antigens produced higher amounts of IFN- γ compared to clinical and healthy cows (148, 149). In addition, in vitro production of TNF- α from PBMC after stimulation with *M. paratuberculosis* whole cell sonicate followed the same pattern of IFN- γ production where it was noted to be elevated in the subclinical and not in the clinically infected or control groups (149). In one study, TNF- α had a dual effect on *M. paratuberculosis* survival where stimulation of a mouse macrophage cell line with high doses of rTNF- α (i.e., 4000 IU/ml) suppressed the intracellular survival of *M. paratuberculosis* yet moderate doses (i.e., 10-1000 IU/ml) resulted in increased survival and replication of *M. paratuberculosis* within cells (147). This information indicates that

macrophages at the site of infection are active (i.e., high IL-1 β , IL-6 production) but it seems that the interaction with the surrounding immune cells may be impaired. This is demonstrated by the decrease in IFN- γ gene expression noted after animals progresses from subclinical to the clinical stage of disease.

Most of the other information about the interaction between *M. paratuberculosis* and the immune system is speculative and mainly based on information derived from other mycobacterial diseases. There is strong evidence that the cell mediated immune response represented by high IFN- γ is fully functional in the subclinical stage of the disease (148, 149, 158). This response is down-regulated when animals progress to the clinical stage of the disease when other proinflammatory cytokines such as IL-1 β and IL-6 become elevated (95, 149). This may suggest that an immune modulatory response surmounts the effective cell-mediated immunity represented by the presence of IFN- γ presence in the subclinical animals. In other mycobacterial diseases such as *M. tuberculosis* infection, both TGF- β and IL-10 facilitated the suppression of mononuclear cell function and decreased IFN- γ expression (126). Recently, two new reports correlated IL-10 presence with clinical stage of *M. paratuberculosis* infection. The first study reported that stimulation of PBMC cultures obtained from two clinical Johne's cows with Con A upregulated the gene expression of IL-10 more than in cells from non-infected and subclinical Johne's cows (33). The other study reported that after stimulation of PBMC cultures obtained from two non-infected cows with live *M. paratuberculosis*, IL-10 gene expression was significantly elevated at all time points (i.e., 6h, 24h and 72h) (186). In regards to TGF- β , no single report addresses the role of this important immune regulatory cytokine in bovine *M. paratuberculosis* infection.

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CHAPTER TWO. UPREGULATION OF TGF BETA AND IL-10 IN COWS WITH CLINICAL JOHNE'S DISEASE

A paper prepared for submission to *Veterinary Immunology and Immunopathology*

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Abstract

Johne's disease is an important disease that results in great economic losses for both dairy and beef production worldwide. The disease progresses through distinct stages including a subclinical stage and a clinical stage that is characterized by progressive symptoms associated with chronic shedding of *Mycobacterium paratuberculosis* in the feces along with severe diarrhea and concomitant weight loss. The host immune response to *M. paratuberculosis* is paradoxical in that there is a strong cell-mediated immune response during the subclinical stage and a strong humoral response during the clinical stage of the disease. It is possible that immune modulation of the cell-mediated immune response in paratuberculosis may play an important role in disease progression. We hypothesized that the clinical stage of Johne's disease is mediated by the presence of cytokines such as transforming growth factor-beta (TGF- β) and interleukin-10 (IL-10) that may be accompanied by the downregulation of IFN- γ gene expression. In the present study, tissue samples were collected from the ileum, ileal-cecal junction, ileal-cecal lymph node, and mesenteric lymph nodes of healthy, subclinically or clinically infected cows. The gene expression of TGF- β , IL-10, and IFN- γ mRNA in these tissues was determined by quantitative competitive RT-PCR. The results show that TGF- β and IL-10 mRNA levels are higher in cows that have progressed to the clinical stage of disease compared to subclinically infected or healthy cows. However, IFN- γ expression was higher in subclinically infected cows. A change in the balance of cytokines at the site of infection may significantly contribute to the ability of the host to control *M. paratuberculosis* infection.

Introduction

M. paratuberculosis is the causative agent of Johne's disease, a chronic enteritis characterized by intermittent diarrhea, weight loss, and eventual death. Animals become infected at an early age through ingestion of contaminated feces and milk; however, fetal infection is also possible in dams with advanced disease (Cocito et al. 1994; Sweeney et al. 1992a; Sweeney et al. 1992b). Clinical disease generally does not become apparent for 3 to 5 years post-exposure; however, significant economic losses may be incurred due to a decrease in milk production and reproductive problems, leading to premature culling of infected animals from the herd (Hutchinson 1996).

As in other mycobacterial infections, a cell-mediated immune response is vigorously active during the early stages of disease. This type of immunity limits the disease progression and is characterized by elevated antigen-specific IFN- γ production (Stabel 1996; Stabel 2000). Animals in the subclinical stage of disease generally shed low numbers of bacteria in their feces and have low antibody titers to *M. paratuberculosis*. As infection progresses to a more clinical state, bacterial shedding in the feces is increased, serum antibody titers increase and IFN- γ production declines (Stabel 1996).

It is unclear what mechanism(s) initiate the progression from subclinical to clinical stage of disease. It is probable that activation of an immune response that antagonizes the cell-mediated response is responsible for the change in the ability of the host to contain the infection. Immunomodulatory cytokines (e.g., TGF- β and IL-10) have been identified in the late stages of mycobacterial infection and are associated with increased intracellular replication of mycobacteria (Flesch et al. 1994). It has been further demonstrated that these two cytokines actively interfere with IFN- γ production, an essential factor for containment of infection (Othieno et al. 1999).

Although the effects of natural or experimental *M. paratuberculosis* infection on IFN- γ production at different stages has been addressed in several studies (Stabel 1996; Stabel 2000; Sweeney et al. 1998), there is no published information to date on the expression of IL-10 and TGF- β in cattle infected with *M. paratuberculosis*. We hypothesize that the decrease in IFN- γ production in the clinical stage of Johne's disease is accompanied by an increase in the production of the regulatory cytokines, IL-10 and TGF- β . In the present study, we performed competitive RT-PCR for quantitation of cDNA levels of IFN- γ , IL-10 and TGF- β at the site of infection (i.e., ileal-cecal node, mesenteric lymph node, ileum, and ileal-cecal junction) in subclinically and clinically infected cows and compared this to expression of these cytokine genes in healthy, non-infected cows.

Materials and Methods

Tissue collection and preparation: The number of tissues obtained from Holsteins cows within each group is presented in Table 1. The non-infected control cows (n=7) were characterized by repeated negative fecal cultures performed quarterly over a 3-5 year period. In addition, these animals were negative on any serologic assays (i.e., production of antibody specific for *M. paratuberculosis* and IFN- γ) performed during that period. Positive cows were classified as clinically infected on the basis of high level of fecal *M. paratuberculosis* shedding (> 100 CFU/g) and clinical signs of disease such as intermittent diarrhea, rapid weight loss, and inappetence in the period immediately prior to obtaining the tissue samples while Subclinically infected animals shed low numbers of *M. paratuberculosis* (< 10 CFU/g feces). Prior to necropsy, animals were sedated with an intravenous injection of xylazine (0.1 mg/kg) and euthanized with an overdose of pentobarbital. Ileal samples of all cattle were collected at necropsy by removing the last 6 inches of the distal ileum proximal to the ileocecal junction. Other tissue samples, ileocecal junction, ileocecal lymph node, and mesenteric lymph node were also collected at necropsy from each cow. Tissues were immediately snap frozen in liquid nitrogen and then stored at -80 °C until analyzed. Ileal

tissues from clinically infected cows stained positive by Ziehl-Neelsen stain indicating the presence of high numbers of acid-fast bacilli within the cytoplasm of macrophages. The macrophages were present in the lamina propria and submucosa most frequently. Tissues from subclinically infected cows had very few acid-fast bacteria and non-infected control cows were negative for organisms in their tissues.

RNA Extraction and Competitive RT-PCR: Total RNA was extracted from each tissue cell lysate preparation using the TRIzol method (Gibco, Grand Island, NY). RNA was dissolved in diethyl pyrocarbonate (DEPC) (Sigma, St. Louis, MO) treated sterile water and quantitated at A260 nm with a spectrophotometer. The quality of extracted RNA was also assessed by running the RNA on 1% native agarose gel. For cDNA synthesis from RNA, 6 µg of total RNA was added to a final volume of 60 µl mixed with 3 µl of poly dT primer (pDT₁₅) at 0.5 µg/µl (Roche diagnostics, Mannheim, Germany), and heated at 72°C for 10 min then snap cooled on ice. The reverse transcriptase reaction was performed by adding 5 µl of RNase inhibitor at 40 U/ µl (Roche), 7.5 µl of 10 mM PCR nucleotide mix (dNTP) (Roche), 5 µl of 20U/ µl Molony murine leukemia virus reverse transcriptase (M-MuLV RT), 15 µl of the supplied 5X M-MuLV RT cDNA synthesis buffer (Roche) and 54.5 µl of DEPC treated water. Reaction tubes were then heated at 45°C for 1 hr followed by heating at 95°C for 5 min to inactivate the reverse transcription enzyme and stored at -20°C until used for quantitative analysis.

Bovine IL-10, IFN-γ, and TGF-β mimic cDNA fragments were produced by construction of composite primers designed to amplify a specific sequence on these cytokines as well as a short sequence of cDNA specific to the mimic fragments (Figure 1). The mimic fragment is an exogenous template that is used as an internal standard in PCR. It shares the same target primer sequence but has a completely different intervening sequence. The exogenous templates (i.e., the mimic fragments) were produced via amplification of a heterologous DNA fragment from Clontech (Palo Alto, CA) with each cytokine mimic primer (Figure 1

right) by 35 cycles of denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min and extension at 72°C for 1 min, followed by 1 cycle of extension at 72°C for 10 min in a Perkin-Elmer Cetus thermal cycler (Norwalk, CT). Concentration of the constructed mimic for each cytokine was determined by spectrophotometric absorption at 260 nm.

Primer pairs for IFN- γ and TGF- β were selected from previous reports (Cerretti et al. 1986; Plath et al. 1997). IL-10 primers were selected in house by MacVector (Genetics Computer Group, Inc, Madison, WI). IFN- γ target product size was 270 bp and the corresponding mimic product size was 409 bp, TGF- β target product size was 223 bp and the corresponding mimic product size was 439 bp; IL-10 PCR product was 288 bp and the corresponding mimic product size was 135 bp. A second round of PCR was performed using cytokine target primers (Figure 1 left). Each reaction contained a fixed target concentration (1 μ g) and the mimic fragment concentration was serially diluted (1:10) starting at 1 pg/ μ l - 10^{-5} pg/ μ l for the IFN- γ mimic, 50 pg - 5×10^{-3} pg/ μ l for the TGF- β mimic and 0.05 pg/ μ l - 5×10^{-6} pg/ μ l for the IL-10 mimic. Each PCR reaction contained 10 μ l of 10X ampliTaq gold PCR buffer II (Applied Biosystems, Foster city, CA), 15 μ l of 25 mM MgCl₂ solution (Applied Biosystems), 2 μ l of 10 mM PCR nucleotide Mix (dNTP) (i.e., 0.4 mM per reaction) and 0.5 mM of upstream and down stream primers for a given cytokine (IDT, Coralville, IA) (Figure 1) and 1 μ l of 5 U/ μ l ampliTaq gold enzyme (Applied biosystems). Thermocycler conditions for IL-10 or IFN- γ was 3 min hot start at 95°C followed by 42 cycles of denaturation at 95°C for 1 min, primer annealing at 57°C for 1 min and extension at 72°C for 1 min, followed by 1 cycle of extension at 72°C for 10 min. TGF- β PCR cycles also began with a hot start (2 min at 94°C) followed by 50 cycles of denaturation at 94°C for 1 min, primer annealing at 61°C for 1 min and extension at 72°C for 1 min, followed by 1 cycle of extension at 72°C for 10 min.

Analysis of RT-PCR products: Following amplification, products were separated on 1.5% ultrapure agarose (Gibco) in TBE buffer (Invitrogen, Carlsbad, CA), stained with ethidium

bromide and photographed under UV with a Gel-Doc system (Bio-Rad, Hercules, CA) (Figure 2). The digitized image was analyzed using an image analysis software program (multi-analyst version 1.0.2, Bio-Rad). The fluorescence of the target and the mimic bands was measured in each lane. For each point, the log of the target fluorescence area/mimic fluorescence area ratio was plotted against the logarithmic value for the concentration of each mimic added to the sample. The amount of target cytokine was calculated by determining the X-intercept for the point of the standard curve where the ratio of target to mimic equal to one.

Statistical analysis. Results were compared by using ANOVA, and significant differences between means were tested by Fisher protected least significant difference test using the Statview software package (Graphpad software, San Diego, CA). For all tests and comparisons only P value less than 0.05 were considered significant.

Results and Discussion

In the present investigation, cows with clinical Johne's disease had increased ($P < 0.05$) IL-10 cytokine gene expression locally at the site of infection in all tissues tested (Figure 3A). The TGF- β gene expression in the ileum of the clinically infected animals was similar to subclinically infected animals but was significantly higher ($P < 0.05$) than the TGF- β gene expression in the ileum of healthy animals (Figure 3B). In other tissues, TGF- β gene expression in clinically infected cows was significantly higher ($P < 0.05$) than the levels of expression present in healthy and subclinically infected cows (Figure 3B). In contrast, cows with subclinical infection had significantly higher ($P < 0.05$) IFN- γ gene expression in all tissues compared to clinically infected animals (Figure 3C). The IFN- γ gene expression in healthy animal tissues was significantly lower than the levels of expression in the subclinically infected cows except in the ileal-cecal lymph node where levels of gene expression was similar for these two groups. Expression of IFN- γ in tissues from healthy

cows was similar to the levels of expression in tissues from clinically infected animal except in the ileal-cecal lymph node where it was significantly higher ($P < 0.05$) than the IFN- γ gene expression of the clinically infected cows. The latter results are in agreement with a previous study which also showed that IFN- γ expression was elevated in ileal tissues and mesenteric lymph nodes of subclinically infected (asymptomatic) cows (Sweeney et al. 1998).

Generally, regulatory cytokines such as TGF- β and IL-10 play an active role in macrophage deactivation and interfere in macrophage antigen-presenting ability (de Vries 1995; Lang et al. 2002; Tomioka et al. 2000; Tsunawaki et al. 1988). In addition, these cytokines also drive T cell development towards Th-2, Th-3, or T regulatory (Tr1) cells, with a corresponding increase in antibody production and a concomitant downregulation of the Th-1 response (Asseman et al. 1999; Groux et al. 1997; Groux et al. 1999; Seder et al. 1998). In this study, the decreased IFN- γ gene expression in tissues obtained from cows with clinical paratuberculosis infection compared to subclinically infected cows (Figure 3A) suggests a weakened cell-mediated immune response. Moreover, the increased expression of TGF- β and IL-10 noted in clinical cows may be indicative of a mechanism causing the shift in T helper response from the strong Th-1 type (cell-mediated immunity) observed in subclinical cows towards a Th-2 type response observed in clinically infected animals.

A previous study which reported the reduced expression of IFN- γ in tissues of clinically infected cows also demonstrated that IL-4 gene expression in these cows was not different from the subclinical or healthy group (Sweeney et al. 1998). This information suggests that the down regulation of IFN- γ gene expression was not solely a result of a shift to Th-2 type responses. The increased gene expression of IL-10 and TGF- β noted in this study instead suggests that cell types such as Tr-1 or Th-3 cells may be involved (Groux et al. 1997; Othieno et al. 1999).

Although not documented in cattle, Tr-1 cells in mice and humans are anergic T cells that

produce high levels of IL-10, low levels of IL-2 and undetectable levels of IL-4 (Groux et al. 1997). Another subset of anergic cells referred to as Th-3 cells were also shown to produce high levels of TGF- β and variable amounts of IL-4 and IL-10 upon activation (Chen et al. 1994). Moreover, conditions which favor high IL-4, IL-10, or TGF- β production and/or low IFN- γ production greatly enhance TGF- β production from Th-3 cells (Seder et al. 1998). Therefore, one possible mechanism involved in the downregulation of IFN- γ in cows with clinical Johne's disease is the activation of Tr-1 cells producing high amounts of IL-10 with the subsequent activation of TGF- β production by Th-3 cells.

The role of IL-10 and TGF- β in the pathogenesis of other mycobacterial diseases has been investigated in several studies. For example, increased IL-10 levels in the sera of leprosy patients as well as increased IL-10 mRNA in lepromatous lesions has been observed (Yamamura et al. 1991). IL-10 transgenic mice had a high mycobacterial burden compared to wild-type mice (Murray et al. 1997) while IL-10 knockout mice showed an increased antimicrobial immunity and lower mycobacterial burden compared to wild-type mice (Murray et al. 1999). In addition, IL-10 was found to inhibit mycobacterial killing initiated by the presence of IFN- γ in infected macrophage cell cultures (Flesch et al. 1994).

TGF- β has also been demonstrated to have effects on mycobacterial infections other than *M. paratuberculosis*. Elevated expression of TGF- β has been noted in human patients with tuberculosis and incubation of macrophages with *M. avium* antigens, *M. tuberculosis* PPD, LAM or lipoglycan induced TGF- β production (Bermudez 1993; Dahl et al. 1996; Hirsch et al. 1994; Toossi et al. 1995). In addition, inhibition of TGF- β with anti-TGF- β antibodies increased the ability of *M. avium*-infected macrophages to kill the bacteria after stimulation with TNF- α and IFN- γ (Bermudez 1993). This study demonstrated that IFN- γ induced mycobactericidal activity required depletion of TGF- β prior to macrophage infection. Therefore, IL-10 and TGF- β appear to have powerful inhibitory activities that may abrogate the ability of IFN- γ to induce microbicidal activity of macrophages (Hirsch et al. 1994;

Roach et al. 2001) in addition to their inhibitory effects on IFN- γ production by T cells (Othieno et al. 1999).

In conclusion, containment of infection during the subclinical phase is achieved through the upregulation of cell-mediated immune responses that increase the microbicidal activity of macrophages and is characterized by high IFN- γ expression. The results of this study demonstrate that animals in the clinical stage of Johne's disease have decreased levels of IFN- γ expression and an upregulation of TGF- β and IL-10. This suggests that the downregulation of IFN- γ at the site of *M. paratuberculosis* infection in cattle may be a direct result of regulatory cytokines, IL-10 and TGF- β . Taking this data together we proposed a model (Figure 4) to illustrate the gene expression of IFN- γ , IL-10 and TGF- β during Johne's disease progression. In chronic paratuberculosis, it is very likely that this aberrant balance of cytokine gene expression might contribute to the progression of *M. paratuberculosis* infection from the contained subclinical stage of disease to a more clinical state (Figure 4).

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Table 1. Number of tissues analyzed by RT-PCR from a total of 7 healthy cows, 7 subclinically infected cows and 9 clinically infected cows.

	Ileum	IC junction	IC node	MLN
Healthy	7	7	5	5
Subclinical	6	6	6	7
Clinical	9	9	9	9

Target primers		Composite primers

IL-10	5'- TAC TGT TGA CCC AGT CTC TGC TGG -3'	IL-10	5'- TAC TGT TGA CCC AGT CTC TGC TGG GCT GGA TGA TTG ATG CAG ACA GC -3'
TGF β	5'-GGA CCT GGG CTG GAA GTG -3	TGF β	5'-GGA CCT GGG CTG GAA GTG GTT CTC CAA AAT GGC TCG TGA C -3'
IFN γ	5'- AAT GCA AGT AGC CCA GAT G -3'	IFN γ	5'- AAT GCA AGT AGC CCA GAT GGC TGG ATG ATT GAT GCA GAC AGC -3'

IL-10	5'- CAC TCA TGG CTT TGT AGA CAC CC -3'	IL-10	5'- CAC TCA TGG CTT TGT AGA CAC CCA ATC TGT AGG GCT AGG CAA GTG C -3'
TGF β	5- CTG CTC CAC CTT GGG CTT -3'	TGF β	5'- CTG CTC CAC CTT GGG CTT TGG GGA GCT TTG AGA TTG CTG-3'
IFN γ	5'- GAT CTG CAG ATC ATC CAC CGG -3'	IFN γ	5'- GAT CTG CAG ATC ATC CAC CGG TGG TTT ACA TAC TCT GGA GCA GGC -3'

Figure 1. Various target primer sequences used to amplify IL-10, IFN γ , and TGF β cDNA (left) as well as their constructed mimic DNA fragment (right). Composite primer sequences used to compare IL-10, IFN γ and TGF β mimic DNA fragments. Notice that the composite primers share the same primer sequence of the target (red part of the arrow)

Figure 2. Demonstration of electrophoretic separation of RT-cPCR products in 1.5% TBE agarose gels stained with ethidium bromide. IFN- γ PCR product size is 270 bp and the mimic product size is 409 bp, TGF- β PCR product size is 223 bp and the mimic product size is 439 bp, IL-10 PCR product is 288 bp and its mimic product size is 135 bp. Serial ten fold dilution of each mimic were add (left to right) along with fixed concentrations of the target cDNA (1 $\mu\text{g}/\mu\text{l}$) to each PCR reaction. Concentration for IFN- γ mimic starts at 1 $\text{pg}/\mu\text{l}$, TGF- β starts at 50 $\text{pg}/\mu\text{l}$ and for IL-10 starts at 0.05 $\text{pg}/\mu\text{l}$.

A: IL-10

B: TGF- β C: IFN- γ

Figure 3. Expression of cytokine-specific gene in tissues recovered from healthy or *M. paratuberculosis* infected cattle. Cytokine concentrations from the total target cDNA added in each reaction. Following quantitation of PCR products, log target fluorescence /mimic fluorescence ratio was calculated and plotted against the log mimic concentration. The intercept X represents the target concentration for each cytokine (A) IFN- γ , (B) IL-10, (C) TGF- β (different letters represent a statistical difference ($P < 0.05$) when comparing production of each cytokine within a given tissue). The number of tissues analyzed is represented in table 1.

Figure 4. The hypothetical expression of IL-10, IFN γ and TGF β at the site of infection (i.e., ileum) during progression of *M. paratuberculosis* infection from asymptomatic subclinical state to full-blown clinical disease.

CHAPTER THREE. EFFECTS OF IFN- γ , IL-10 AND TGF- β ON SURVIVAL OF *MYCOBACTERIUM PARATUBERCULOSIS* IN PERIPHERAL BLOOD MONONUCLEAR CELL CULTURES

A paper prepared for submission to *Infection and Immunity*

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Abstract

The presence of IFN- γ is critical in containing *Mycobacterium paratuberculosis* infection. However, the contribution of other immunoregulatory cytokines such as IL-10 and TGF- β in Johne's disease is not investigated yet. In this study we examined the effects of in vivo and in vitro infection with *M. paratuberculosis* on the production of IFN- γ , IL-10 and TGF- β by peripheral blood mononuclear cells (PBMC). We also examined the effects of IFN- γ , IL-10 and TGF- β on *M. paratuberculosis* survival in the cell cultures. PBMC obtained from naturally infected cows, regardless of their disease status, specifically upregulated IL-10 and TGF- β in culture supernatants in response to stimulation with live *M. paratuberculosis*. Non-stimulated PBMC recovered from subclinically infected animals secreted the lowest levels of TGF- β but after stimulation with live *M. paratuberculosis*, TGF- β levels in the culture supernatant increased to levels similar to that produced by PBMC from healthy animals. Compared to healthy cows, naturally infected animals had higher numbers of viable *M. paratuberculosis* recovered from their culture after in vitro infection with *M. paratuberculosis*. The addition of exogenous IL-10 and TGF- β to PBMC recovered from healthy cows inhibited the bactericidal activity of these cells as evidenced by the increased number of viable *M. paratuberculosis* present in the culture compared to cell cultures containing medium alone. These data suggest an important immune regulatory role of IL-10 and TGF- β during infection with *M. paratuberculosis* which may be directly related to their effects on macrophage activation and killing of *M. paratuberculosis*.

Introduction

Johne's disease typically progresses through three distinct stages (2). The division of these stages depends upon the existence of clinical signs and the correlation of these signs with the fecal shedding of *M. paratuberculosis*. Accordingly, the first and the second stages are considered subclinical stages where animals are asymptomatic. Animals may remain in these stages without developing clinical disease or might progress to the clinical stage after 3-4 years of infection. During the first subclinical stage, the infection persists without the ability to detect shedding of *M. paratuberculosis* in the feces followed by the subclinical intermittent excretory phase. The third or the clinical excretory stage is characterized by progressive symptoms associated with persistent shedding of high numbers of bacteria in the feces and severe diarrhea with concomitant weight loss. The stages of Johne's disease reflect an ongoing struggle within the host animal's immune system. Animals in the excretory subclinical stage are reported to have increased IFN- γ expression locally at the site of infection (19) as well as higher IFN- γ production in culture supernatants after stimulation of peripheral blood mononuclear cell culture with *M. paratuberculosis* antigens (18). As *M. paratuberculosis*-infected animals progress to a more clinical state, local and peripheral IFN- γ production declines (16, 18, 19).

Following ingestion of *M. paratuberculosis* by the neonatal calf, the bacteria cross M cells lining the Peyer's patches and can be then engulfed by resident macrophages in the distal ileum where the bacteria can survive and replicate (11). It has been suggested that the host's immune system responds to *M. paratuberculosis* infection by recruiting more macrophages and lymphocytes to the site as well as by activation of immune cells such as $\gamma\delta$ T cells, CD4⁺ T cells, and cytolytic CD8⁺ T cells (4, 10). These cells could operate directly on the infected macrophages through cell to cell contact or/and indirectly through production of cytokines such as IFN- γ , TNF- α , and IL-6 which cause macrophage activation and further activation of

T cells (10). Therefore, it is important for macrophages to remain in contact with the proper immune cells and their products to overcome this type of infection.

It has been hypothesized that during the interaction between host immunity and *M. paratuberculosis*, a deviation from the proper immune response arises and disrupts the ability of the host to contain the disease. Although a mechanism has not been identified, the decrease in IFN- γ noted during the clinical stage signals a disruption in the effector phase of host immunity. Exogenous IFN- γ has been shown to stimulate monocytes to kill *M. paratuberculosis* (23). In other mycobacterial diseases, such as tuberculosis, a similar upregulation of IFN- γ production during the contained stage of the disease has been reported (8, 15). With regards to macrophage activation, addition of recombinant human IFN- γ to monocyte cell cultures enhanced the ability of these cells to limit growth of *M. tuberculosis* and *M. bovis* (8, 15). Conversely, infection of IFN- γ knockout mice with a sublethal dose of *M. bovis* or *M. tuberculosis* resulted in increased mortality and increased bacterial numbers recovered from spleen, liver, and lung (3, 6).

When *M. tuberculosis* infection progresses into an advanced stage (disseminated tuberculosis) a decrease in IFN- γ production occurs and a new profile of cytokines dominated by the anti-inflammatory cytokines TGF- β and IL-10 emerges. Following mycobacterial infection, evidence obtained from studies with IL-10 transgenic mice and IL-10 knockout mice indicates that IL-10 is often associated with immune suppression in and with the increased replication of mycobacteria (12, 13). TGF- β has also been shown experimentally to enhance the intracellular growth of *M. tuberculosis* in human monocytes (9). After infection of monocytes with *M. tuberculosis*, production of TGF- β in cell culture supernatants was low in the first 24 hrs of incubation (<50 pg/ml) then increased up to 8-fold after 4 days in culture (9). This increased production of TGF- β was accompanied by decreased ability of monocytes in culture to control growth of *M. tuberculosis*.

Recently, we reported that the immunomodulatory cytokines, TGF- β and IL-10, were upregulated in the tissues of cows with clinical Johne's disease (manuscript in preparation). The presence of these anti-inflammatory cytokines that modulate the effects of pro-inflammatory cytokines such as IFN- γ might be one mechanism that enhances the survival of mycobacteria within the host. In the present study, we investigated the effects of in vitro infection with *M. paratuberculosis* on the production of IFN- γ , IL-10, and TGF- β production by PBMC isolated from healthy and naturally infected cows. We also investigated the effects of exogenous IFN- γ , IL-10, and TGF- β stimulation on the ability of monocyte-derived macrophages in unfractionated cell cultures to kill *M. paratuberculosis*. These studies indicate that IL-10 and TGF- β downregulate the bactericidal activity of monocyte-derived macrophages.

Materials and Methods

Animals. The animals used in this experiment were placed in three groups consisting of five non-infected healthy cows, four cows naturally infected with *M. paratuberculosis* but asymptomatic (i.e., subclinical), and five cows with the clinical form of Johne's disease. Infection was monitored bacteriologically for the fecal shedding of *M. paratuberculosis* using standard culture methods previously described (17). By definition, clinical animals were shedding more than 100 colony forming units (CFU) per gram of feces and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/g of feces. All animals were housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities and all animal related procedures were approved by the IACUC (National Animal Disease Center, Ames, Iowa).

Blood collection, culturing conditions and sample collection. Blood was collected from the jugular vein in 2X acid-citrate-dextrose (ACD) (1:10). Peripheral blood mononuclear cells (PBMC) were isolated from the buffy-coat fractions of the peripheral blood as

previously described (1). PBMC were resuspended in RPMI-1640 (Gibco, Grand Island, NY) containing 10% FCS (Atlanta Biologics, Atlanta, GA), 100 units/ml penicillin G sodium (Gibco) 100 µg/ml streptomycin sulfate (Gibco), 0.25 µg/ml amphotericin B (Gibco), and 2 mM L-glutamine (Gibco). Cells were cultured at 2×10^6 /ml in 24-well flat-bottomed plates (Nunc, Life Technologies) for 6 days at 39°C in 5% CO₂ in a humidified atmosphere to allow monocytes in the unfractionated PBMC cultures to develop into macrophages. The plates were then centrifuged at 400 x g for 2 min and the supernatants were removed without disturbing the cells in culture. Cells in duplicate wells were cultured with fresh medium containing 100 ng/ml human IL-10 (200-10, Peprotech, Rocky Hill, N.J.), 100 ng/ml bovine IFN-γ (generously donated by Novartis Animal Health, Basel, Switzerland), 10 ng/ml human TGF β (100-21R, Peprotech), 100 ng/ml IL-10 plus 10 ng/ml TGF-β or left without cytokine stimulation. Cultured cells were incubated overnight (18 hrs) then infected the next day with *M. paratuberculosis*, strain 19698 (NADC, Ames, IA) at a ratio of 10 bacteria per one adherent PBMC (24). Replicate wells of the in vitro infected and non-infected cell cultures were also concurrently treated with 1 µg/ml of LPS (*Escherichia coli* O11:B4-W; Sigma) as a non-specific stimulator of macrophages in the study. To assess cytokine production, cell culture supernatants were collected at 8 hrs, 3 days, or 6 days after in vitro infection and stored at -20°C prior to cytokine measurement. For cultures that had been infected with *M. paratuberculosis*, cells that were left in each well after the collection of culture supernatant were lysed and washed several times with distilled H₂O, left overnight at room temperature (RT) and cultured on Herrold's egg yolk medium (HEYM).

Bacteria. *M. paratuberculosis* strain 19698 (NADC) was grown in Middle-brook 7H9 broth (pH 6.0) supplemented with oleic acid albumin dextrose complex (OADC, Becton Dickinson Microbiology). The bacteria were harvested, washed three times with phosphate-buffered saline (PBS pH 7.4; 0.15M), resuspended in PBS to a final concentration of 10^9 /ml as determined by the absorbance at 540 nm. Bacterial stocks were then frozen in PBS at - 80°C until used in the experiments. Prior to in vitro infection, frozen bacterial stocks were thawed

and clumps were dispersed by brief sonication at 25 W for 40 sec with a Tekmar sonic disturber (Lorton, VA). The frozen bacterial stocks were monitored for viable counts by culturing on HEYM. Viable counts in stocks were reduced after thawing and sonication to approximately 1×10^8 CFU/ml.

Assessment of cell phenotypes in the unfractionated PBMC before cytokine treatments as well as prior to in vitro stimulation with live *M. paratuberculosis*. To assess the cell phenotypes present in the unfractionated PBMC, flow cytometric analysis of the cultured, unfractionated PBMC was performed after 6 and 7 days in culture. Cells at each time point were stained with monoclonal antibodies (mAb) for different cell surface molecules. A single color flow cytometric analysis of T cells for the expression of CD4 (CACT138A), CD8 α (CACT80A), or $\gamma\delta$ TCR-N24 (GB21A) (VMRD, Pullman, WA) was performed. Briefly, 50 μ l of anti-CD4, CD8, or $\gamma\delta$ cell surface markers were incubated for 15 min in room temperature (RT) with 100 μ l of 2×10^6 cells/ml at the working concentration of 7, 10, or 7 μ g/ml, respectively. Cells were then washed once by centrifugation at 400 x g for 2 min. Bound markers were visualized by incubating cells for 15 min with 50 μ l of phycoerythrin-(PE) conjugated goat anti-mouse IgG1 (1:1000) (Southern Biotechnology Associates, Inc., Birmingham, AL) for the mouse anti-bovine CD4 marker, anti-IgG2b (1:1000) for the mouse anti-bovine $\gamma\delta$ marker, and anti-IgM (1:2000) for the mouse anti-bovine CD8 marker (Southern Biotechnology Associates, Inc., Birmingham, Ala). Cells were then washed and incubated for 5 min with DAPI (4',6-diamidino-2-phenylindole) (Sigma, St. Louis, Mo) to gate out the dead cells then washed and resuspended in FACS buffer (PBS, 1% FCS and 0.1 % sodium azide).

For analysis of B cells and MHC II bearing cells, a two-color flow cytometric analysis was performed. Briefly, 100 μ l of cell cultures at 2×10^6 cells/ml were incubated for 15 min at RT with 50 μ l of B cell marker BAQ155A (7 μ g/ml) (VMRD) and 50 μ l of MHC II marker CH34A (3.5 μ g/ml) (VMRD). Cell surface markers were then visualized by incubating cells

for 15 min with 50 µl of goat anti-mouse immunoglobulin IgG2a PE conjugated (1:500) to detect bound anti-bovine MHC II marker and 50 µl of goat anti-mouse immunoglobulin IgG1 (FITC) conjugated (1:250) to detect the bound anti-bovine B cell marker. Cells were then washed and resuspended in FACS buffer. To gate out the dead cells from the flow analysis, cells were incubated with DAPI at 1:1000 (10 mg/ml) (Sigma, St. Louis, MO) before the final wash.

Data from 5000 events per sample were acquired (Cell quest software; BD Biosciences, San Jose, CA) using flow cytometry (BD LSR, BD Biosciences). For all analyses, only mononuclear cells that were DAPI negative were gated and analyzed for phenotypic marker expression by Flowjo software (Tree Star Inc, San Carlos, CA).

Assessment of *M. paratuberculosis* survival in the extended un-fractionated PBMC cultures. The number of viable *M. paratuberculosis* in each cell culture lysate and its corresponding cell culture supernatant from each treatment were separately cultured on HEYM medium. Growth was measured by counting the CFU following serial 10-fold dilutions on duplicate HEYM slants for each treated well. HEYM slants were incubated at 39°C for 12 weeks. CFU recovered from the cell culture lysates were added to the number of CFU recovered from the corresponding cell culture supernatants and represented the surviving *M. paratuberculosis* after each treatment. The number of CFU from the cell culture supernatants alone represented *M. paratuberculosis* that was either not phagocytized originally or was phagocytized and released by macrophages after cell death.

Measurement of IFN- γ , IL-10, and TGF- β production in cell culture supernatant by ELISA. IFN- γ was measured using Bovigam bovine IFN- γ test kit (CSL Veterinary, Parkville, Victoria, Australia) as described by the manufacturer. For the detection of bovine IL-10, purified rat anti-human IL-10 capture mAb (JES3-19F1) (PharMingen, San Diego, CA) and biotinylated rat anti-human IL-10 detection mAb (JES3-12G8) (PharMingen) were

used. MaxiSorp microtiter plates (NUNC, Rochester, NY) were coated with the capture mAb (100 μ l per well at 2 μ g/ml) overnight at 4°C. Plates were then blocked with 1 % gelatin in PBS for 2 hrs at RT. Plates were then washed 4 times with 1% Tween 80 in PBS washing buffer. The diluted samples (1: 1.5 in RPMI) and serial 2-fold dilutions of rhIL-10 standard (starting at 20 ng/ml) were added in duplicate and incubated overnight at 4°C. Plates were then washed 5 times with washing buffer before incubating with the detection biotinylated anti-human IL-10 mAb (100 μ l at 1 μ g/ml) for 1 hour at RT. Plates were washed 5 times with washing buffer and 100 μ l of avidin – horseradish peroxidase (HRP) conjugate (1:2000) (PharMingen) was added to each well and incubated for 30 min at RT. Following another wash cycle, wells were incubated with substrate solution (40 mM ABTS (2,2' –azino-di-ethylbenzthiozoline-6-sulfonic acid) in citrate buffer, pH 4.0) and color development quantified after 10 min by measuring absorbance at 405 nm with a Wallac Victor 1420 multi-label counter ELISA plate reader (PerkinElmer, Gaithersburg, MD).

TGF- β was detected by coating MaxiSorp microtiter plates (NUNC) with 100 μ l of anti-human TGF- β capture mAb (MAB240) (R&D system, Minneapolis, MN) at 2 μ g/ml and then using biotinylated rat anti-human TGF- β mAb (BAF240) (R&D system) at 100 ng/ml as the detection mAb. The plates with the capture mAb were incubated overnight at RT then washed 4 times with 0.5% Tween 20 in PBS and blocked with 5% Tween 20, 5% sucrose and 0.05% NaN₃ in PBS (i.e., blocking buffer) for 2 hrs at RT. Diluted samples (1 : 6) or the rhTGF β standards (5 ng/ml to 0.078 ng/ml diluted in RPMI) (Peprotech) were acidified to pH 2.0 with 1 N HCL for 10 min at RT to activate the latent TGF- β then samples were neutralized to pH 7.2 – 7.6 by 1.2 N NaOH/ 0.5 M HEPES. Activated samples were then added to plates coated with the TGF- β capture mAb within 5 min and incubated for 2 hrs at RT. Plates were then washed 4 times with washing buffer followed by incubation with TGF- β detection mAb for 2 hrs at RT. Plates were then washed 4 times and incubated with 100 μ l of strepavidin - HRP (1:2000) (PharMingen) followed by ABTS substrate solution addition as described above for the IL-10 ELISA. The concentration of cytokines for each of the

assays was estimated from the standard curve generated using recombinant bovine IFN- γ (Novartis Animal Health), rhIL-10 (PharMingen), and rhTGF- β (Peprotech).

Statistical analysis. Results were compared by using 2 way ANOVA, and significant differences among means were tested by Fisher protected least significant difference test using the Statview software package (Graphpad software, San Diego, C A). For all tests and comparisons only P values less than 0.05 were considered significant.

Results

Mononuclear cell profile in unfractionated cell culture. After six days in culture, most of the adherent cells in culture assessed by light microscopy were stellate in shape, consistent with typical macrophage morphology (Figure 1). This suggested the generation of monocyte-derived macrophages in the extended unfractionated PBMC culture. Flow cytometric analysis of the cellular phenotypes in the unfractionated PBMC cultures were noted to be different between animal groups (Table 1 & 2). The subclinically infected cows had more ($P < 0.05$) $\gamma\delta$ T cells in their unfractionated cell cultures than healthy and clinically infected cows after 7 days in culture. MHC II⁺ B cells⁺ and MHC II⁺ B cells⁺ were higher ($P < 0.05$) in the clinical group compared to healthy or subclinically infected group after 6 days in culture and on day 7 after the addition of cytokines in the fresh complete medium (Table 1 & 2).

Effect of infectious status of cows and in vitro infection of PBMC cultures on IFN- γ IL-10, TGF- β . Effects of animal infection status (i.e., healthy, subclinical or clinical) and in vitro infection with *M. paratuberculosis* on the secretion of IFN- γ , IL-10, or TGF- β by PBMC is presented in Tables 3, 4, and 5, respectively. In non-infected cell cultures isolated from clinical cows, IFN- γ production significantly ($P < 0.05$) decreased with time (Table 3). Infected animals (subclinical or clinical) had significantly higher ($P < 0.05$) IFN- γ production in non-infected culture supernatants compared to healthy non-infected controls. In contrast,

after in vitro infection with *M. paratuberculosis*, IFN- γ production remained fairly constant at all time points in PBMC cultures of subclinically and clinically infected cows. In PBMC cultures of healthy animals, the in vitro infection resulted in a continuous accumulation of IFN- γ production with time ($P < 0.05$).

The in vitro production of IL-10 by PBMC from clinically infected and healthy animals decreased ($P < 0.05$) with time (8 vs 6 days) while it remained fairly constant in cell cultures of the subclinically infected animals at all time points tested in this experiment (Table 4). After in vitro infection with live *M. paratuberculosis*, there was an upregulation ($P < 0.05$) of IL-10 production in the cultures containing cells recovered from naturally infected animals but not in cultures containing cells isolated from the healthy animals (Table 4).

The level of TGF- β production increased ($P < 0.05$) with time (8 hrs vs. 6 days) in cell cultures obtained from healthy cows in the absence of *M. paratuberculosis* (Table 5). Compared to healthy and clinical animals at day 6, TGF- β levels were significantly ($P < 0.05$) lower in non-infected cell culture supernatants of cows in the subclinical stage of the disease. Following the in vitro infection with *M. paratuberculosis*, there was a resultant upregulation ($P < 0.05$) of TGF- β production for both subclinical and clinical cows (Table 5), with levels of TGF- β produced by clinical cows becoming similar to levels produced by healthy cows in non-infected cultures.

The effect of LPS on TGF- β , IL-10 and IFN- γ . Cells in the unfractionated PBMC cultures were inherently able to produce IFN- γ when non-specifically stimulated with LPS (Figure 2). While the addition of *M. paratuberculosis* to cell culture caused an upregulation of TGF- β and IL-10 in the naturally infected animals, addition of LPS as a non-specific stimulator of monocyte-derived macrophages in culture did not affect TGF- β (Figure 2C) or IL-10 (Figure 2B) production but resulted in a significant ($P < 0.05$) boost in IFN- γ production in cell

cultures from all animal groups (Figure 2A). The effect of co-stimulation with LPS and live *M. paratuberculosis* on cytokine production was not additive (data not shown).

Effects of exogenous cytokines on *M. paratuberculosis* survival. Regardless of disease status, PBMC from naturally infected animals had decreased ($P < 0.05$) capacity to kill *M. paratuberculosis* in vitro by day 6 (Figure 3). There was no significant difference between the number of CFU recovered from subclinical animals and clinical animals (data not shown). Pretreatment with exogenous IL-10 or TGF- β or a combination of both for 18 hrs before infection of cultured PBMC isolated from healthy animals with live *M. paratuberculosis* resulted in higher ($P < 0.05$) numbers of CFU recovered when compared to medium control cultures (no added cytokines) control cultures 6 days after in vitro infection (Figure 4). Pretreatment with IFN- γ had no significant effect on the number of viable *M. paratuberculosis* recovered from cell culture (Figure 4) although a trend for decreased number of recovered bacteria was observed.

Interaction of exogenous cytokines and infection on IFN- γ , IL-10 and TGF- β production. Pretreatment of PBMC with exogenous TGF- β significantly increased ($P < 0.05$) endogenous IL-10 production in all groups (Figure 5). This effect was noted regardless of period of incubation, LPS stimulation or in vitro infection with live *M. paratuberculosis*. Addition of exogenous IL-10 and TGF- β to PBMC cultures had significant ($P < 0.05$) downregulatory effects on IFN- γ production (Figure 6). Production of IFN- γ was significantly ($P < 0.05$) reduced by the addition of TGF- β alone or in combination with IL-10 to PBMC regardless of animal group (Figure 6A). In vitro infection with *M. paratuberculosis* resulted in an overall increase in IFN- γ production compared to noninfected PBMC cultures regardless of cytokine treatment or animal groups (Figure 6B). Similar to noninfected cell cultures, the addition of anti-inflammatory cytokines, IL-10 and TGF- β , to infected cell culture resulted in a significant ($P < 0.05$) abrogation of IFN- γ production. The inhibitory effects of these anti-inflammatory cytokines on IFN- γ production was most noticeable ($P < 0.05$) in cell cultures

obtained from subclinically infected animals, whereas, cells isolated from clinically infected cows were the least ($P < 0.05$) affected (Figure 6).

Discussion

Previous studies have reported significant effects of IFN- γ , on the ability of monocytes to kill mycobacteria, including *M. paratuberculosis* (8, 15, 23). In the present study, we evaluated the effect of IFN γ , IL-10 or TGF- β , on the survival of *M. paratuberculosis* in monocyte-derived macrophages present in unfractionated PBMC cultures recovered from cows naturally infected with *M. paratuberculosis* as well as healthy animals. Relative to non-infected cultures, in vitro challenge of cell cultures with live *M. paratuberculosis* resulted in enhanced production of IFN- γ in cell cultures derived from clinically infected and healthy animals but not in cultures derived from the subclinically infected group. These data differ from what was previously reported for IFN- γ production where the amounts of IFN- γ after stimulation with either *M. paratuberculosis* whole cell sonicate or live *M. paratuberculosis* was higher in PBMC cultures from subclinically infected cows compared to cultures from healthy or clinical cows (16, 18). However, the culture conditions in the current study were quite different as we used an extended (i.e., 7-12 days) cell culture system and previous studies utilized freshly isolated PBMC.

The difference in IFN- γ production might indicate that after development of monocytes into macrophages, antigen presentation as well as cytokine production from cultured macrophages and lymphocytes favors a Th-1 response as represented by increased IFN- γ production. Before in vitro infection with *M. paratuberculosis*, IFN- γ production remained low in the supernatants obtained from PBMC culture of healthy animals. In the subclinically infected animals production of IFN- γ remained consistent across time while in cell cultures obtained from clinically infected cows, it decreased with time. However, the production of IFN- γ across time in culture on average was higher in the supernatant of cells from naturally

infected animals than healthy animals. After infection with *M. paratuberculosis* the production of IFN- γ in PBMC cultures from healthy animals increased with time while in subclinically infected animals the amount of IFN- γ produced did not change over time. In cell cultures derived from clinically infected animals, IFN- γ was upregulated and remained constant across time. These changes may imply that the control mechanism(s) for IFN- γ production are different in the three groups and it seemed that PBMC from clinically infected animals were generating some type of downregulatory mechanism that led to decreased IFN- γ production over time. However, in the presence of a stimulus like LPS, IFN- γ production was enhanced. In subclinically infected animals, the low TGF- β presence in cell culture before in vitro infection with *M. paratuberculosis* correlate with the consistent and high production of IFN- γ in these animals. Nevertheless, after in vitro infection, IL-10 amounts increased and possibly resulted in the inhibition of further IFN- γ production. In the clinically infected animals, the regulation of IFN- γ production might involve different factors such as cells survival and phenotypes present.

The differences between cultured cells from subclinically and clinically infected animals included a higher percentage of $\gamma\delta$ T cells in cultures of subclinical animals while higher percentage of B cells and MHC II⁺ B cell in cultures of clinically infected animals were noted. The higher percentage of these cells may have affected the cytokine production in this study. Therefore, it might be informative to investigate the role of these cells in the pathogenesis of *M. paratuberculosis* infection and whether these cells are the source of the inhibitory cytokines, IL-10 and TGF- β .

In this study, extended culture of unfractionated PBMC specifically upregulated IL-10 and TGF- β production after stimulation with live *M. paratuberculosis* but not in response to non-specific stimulation with LPS. Interestingly, the ability of *M. paratuberculosis* to upregulate IL-10 and TGF- β was noted in animals naturally infected with *M. paratuberculosis* regardless of the disease state but not from cells recovered from healthy animals. Therefore,

in the absence of IL-10 and TGF- β upregulation, cells from all groups regardless of their infection status were capable to produce IFN- γ (e.g., LPS stimulation). This is also demonstrated after the addition of exogenous cytokines to the unfractionated PBMC culture. The interaction of TGF- β , IL-10, and IFN- γ confirmed that the anti-inflammatory cytokines (i.e., TGF- β and IL-10) downregulate IFN- γ production in cell culture and that TGF- β potentiated IL-10 production. Similarly, TGF- β has been shown to upregulate IL-10 production in *M. tuberculosis* infected cell cultures (14). TGF- β and IL-10 were found to synergistically down-regulate IFN- γ production by T cell stimulated with *M. tuberculosis* PPD (14).

This is the first published observation to report significant effects of *M. paratuberculosis* infection on the production of the regulatory cytokine, TGF- β and IL-10. Results noted in the present study are similar to results documented for other mycobacterial diseases such as tuberculosis (9, 21). Tuberculosis patients with advanced disease (i.e., disseminated tuberculosis) had high TGF- β as well as IL-10 production compared to patients in the early stage (contained tuberculosis) of infection (7, 14, 20). In addition, mycobacteria in vitro infection of cell cultures from tuberculosis patients further increased TGF- β and IL-10 production (14). Regarding IL-10, one study recently reported higher IL-10 gene expression in PBMC isolated from cows with clinical paratuberculosis compared to cells from healthy animals (5). In addition, upregulation of IL-10 gene expression was also noted in monocyte-derived macrophages obtained from healthy animals after stimulation with live *M. paratuberculosis* (22).

The decreased bactericidal activity of PBMC recovered from naturally infected animals correlated with the increased production of TGF- β and IL-10 in these cell cultures. In support to this observation, it was determined that the addition of these cytokines to cell cultures of healthy animals also increased the number of surviving *M. paratuberculosis* recovered compared to control cell cultures. These results are in agreement with previous reports on the

effects of TGF- β and IL-10 on the survival of *M. tuberculosis*. For example, incubation of monocyte cell cultures from healthy individuals with 10 ng/ml of rTGF- β prior to infection with *M. tuberculosis* resulted in 3-fold increase in the number of CFU recovered from cell culture lysates (9). A singular effect of IL-10 on mycobacterial survival was also demonstrated in a study in which intravenous inoculation of IL-10 transgenic mice with Calmette-Guerin bacillus (BCG) resulted in dissemination of the bacteria throughout the body for the course of the study (12 weeks) (12)

Addition of exogenous IFN- γ to monocyte-derived macrophages in the unfractionated PBMC culture did not decrease the number of viable bacteria recovered from cell culture after in vitro infection. This is different from what was previously reported where monocyte cell culture stimulated with IFN- γ resulted in decreased *M. paratuberculosis* viability (23). Therefore, it might be that under our culture conditions, stimulation of the monocyte-derived macrophages in the presence of T cells in the unfractionated cell culture left macrophages refractory to further IFN- γ stimulation probably through the production of inhibitory cytokines such as IL-10 and TGF- β .

In summary, IL-10 and TGF- β had inhibitory roles on the destruction of intracellular *M. paratuberculosis*, perhaps through their effects on IFN- γ production. Therefore, upregulation of IL-10 and TGF- β even in the presence of high IFN- γ production (e.g., cells from naturally infected cows) likely resulted in less effective killing of the *M. paratuberculosis* in these animals compared to healthy animals which. Taken together, data from this study supports a model in which the progression of Johne's disease from a subclinical stage to a more clinical stage is the result of an imbalance of cytokine production favoring TGF- β and IL-10 over IFN- γ production. This imbalance might be due to the difference in the type of specific cells activated at each disease stage.

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Table. 1. Phenotypic analysis of bovine PBMC after 6 days in culture.

Cell types*	Healthy	Subclinical	Clinical
$\gamma\delta$ T cells	$16.28 \pm 1.07^\dagger$	16.85 ± 5.35	14.90 ± 0.3
CD4 ⁺ cells	28.15 ± 2.96	36.85 ± 3.35	24.95 ± 6.15
CD8 α ⁺ cells	23.60 ± 2.5	22.00 ± 1.10	18.95 ± 0.15
MHC II ⁺ B cell ⁺	$17.55^a \pm 3.15$	$10.13^a \pm 4.38$	$31.60^b \pm 6.30$
MHC II ⁻ B cell ⁺	7.54 ± 2.46	5.73 ± 1.74	7.10 ± 1.79
MHC II ⁺ B cell ⁻	$3.34^a \pm 0.76$	$4.11^a \pm 0.15$	$15.35^b \pm 0.55$

Different letters in rows represent a statistically significant difference ($P < 0.05$)

* cells were analyzed by flow cytometry as described in material and methods, 5000 cells were analyzed for each sample.

† results are expressed as the mean $\pm$ SEM for 4 healthy animals, 2 subclinically infected, and 2 clinically infected animals.

Table. 2. Phenotypic analysis of bovine PBMC after 7 days[†] in culture.

Cell types*	Healthy	Subclinical	Clinical
$\gamma\delta$ T cells	20.09 ^a $\pm$ 6.81	27.25 ^a $\pm$ 0.25	14.80 ^b $\pm$ 1.00
CD4 ⁺ cells	28.23 $\pm$ 2.71	33.70 $\pm$ 9.70	18.00 $\pm$ 6.30
CD8 α ⁺ cells	24.63 $\pm$ 0.84	19.65 $\pm$ 4.85	10.72 $\pm$ 7.18
MHC II ⁺ B cell ⁺	14.38 ^a $\pm$ 2.22	14.82 ^a $\pm$ 4.98	36.45 ^b $\pm$ 1.95
MHC II ⁻ B cell ⁺	17.88 $\pm$ 5.18	7.51 $\pm$ 1.05	9.39 $\pm$ 4.91
MHC II ⁺ B cell ⁻	4.82 ^a $\pm$ 2.12	5.05 ^a $\pm$ 3.06	15.30 ^b $\pm$ 3.00

Different letters in rows represent a statistical significance ($P < 0.05$)

‡ 18 hours after the replacement of the supernatant in culture with fresh medium and before the addition of *M. paratuberculosis* to the in vitro cultures.

* cells were analyzed by flow cytometry as described in Material and Methods, 5000 cells were analyzed for each sample.

† results are expressed as the mean $\pm$ SEM for 4 healthy animals, 2 subclinically infected, and 2 clinically infected animals.

Table 3. IFN- γ production in cell culture supernatants ^X from non-infected healthy cows and cows with subclinical or clinical Johne's disease.

	<i>M. paratuberculosis</i> infection	8 hours	3 days	6 days	Average
Healthy ^Y	No	0.40^{**} $\pm$ 0.17	0.06[*] $\pm$ 0.04	0.89[†] $\pm$ 0.49	0.45^a $\pm$ 0.19
	Yes	0.92[*] $\pm$ 0.38	4.85[*] $\pm$ 2.00	8.73[†] $\pm$ 2.74	4.83^c $\pm$ 1.40
Subclinical ^Y	No	3.20[*] $\pm$ 1.03	1.44[*] $\pm$ 0.87	2.36[*] $\pm$ 1.66	2.33^{bd} $\pm$ 0.68
	Yes	2.96[*] $\pm$ 0.26	3.36[*] $\pm$ 0.88	4.05[*] $\pm$ 2.00	3.45^{bc} $\pm$ 0.68
Clinical ^Y	No	2.91[*] $\pm$ 0.32	1.16[†] $\pm$ 0.34	0.72[†] $\pm$ 0.45	1.60^d $\pm$ 0.32
	Yes	5.26[*] $\pm$ 0.73	6.79[*] $\pm$ 1.49	4.84[*] $\pm$ 1.73	5.63^c $\pm$ 0.77

Different letters in columns represent a statistically significant difference (P< 0.05)

Different symbols in rows represent statistically significant difference (P< 0.05)

X PBMC culture medium was replaced with complete medium on day 6. After 18 hours (day 7), cells in culture were stimulated with live *M. paratuberculosis* at 10:1 bacteria to adherent cell ratio. Culture supernatants were collected at 8 hours, 3 days, and 6 days after in vitro infection and analyzed by ELISA. The average represents the concentration $\pm$ SEM of IFN- γ produced (ng/ml) at all time points in the media control supernatants with or without in vitro infection with *M. paratuberculosis*.

Y Number of animals in each group was five healthy cows, four subclinically infected cows and five clinically infected cows

Table 4. IL-10 production in cell culture supernatants^x from non-infected healthy cows and cows with subclinical or clinical Johne's disease.

	<i>M. paratuberculosis</i> infection	8 hours	3 days	6 days	Average
Healthy ^y	No	4.72* ± 0.76	3.24* [†] ± 0.78	2.28 [†] ± 0.22	3.41 ^a ± 0.45
	Yes	6.38* ± 1.5	5.00* ± 1.63	3.79* ± 0.61	5.06 ^{ac} ± 0.79
Subclinical ^y	No	4.59* ± 1.09	5.37* ± 0.92	3.30* ± 0.79	4.42 ^a ± 0.55
	Yes	13.76* ± 4.31	5.54* ± 0.50	8.18* ± 2.51	9.16 ^b ± 1.83
Clinical ^y	No	7.44* ± 2.067	4.33* [†] ± 0.75	2.51 [†] ± 0.44	4.76 ^a ± 0.88
	Yes	10.51* ± 2.01	10.33* ± 3.14	4.6* ± 1.23	8.48 ^{bc} ± 1.42

Different letters in columns represent a statistically significant difference (P< 0.05)

Different symbols in rows represent statistically significant difference (P< 0.05)

X PBMC culture medium was replaced with complete medium on day 6. After 18 hours (day 7), cells in culture were stimulated with live *M. paratuberculosis* at 10:1 bacteria to adherent cell ratio. Culture supernatants were collected at 8 hours, 3 days, and 6 days after in vitro infection and analyzed by sandwich ELISA using anti-human IL-10 monoclonal antibodies. The average represents the concentration ± SEM of IL-10 produced (ng/ml) at all time points in the media control supernatants with or without in vitro infection with *M. paratuberculosis*

Y Number of animals in each group was five healthy cows, four subclinically infected cows and five clinically infected cows

Table 5. TGF- β production in cell culture supernatants^X from non-infected healthy cows and cows with subclinical or clinical Johne's disease.

	<i>M. paratuberculosis</i> infection	8 hours	3 days	6 days	Average
Healthy	No	7.18* $\pm$ 1.124	9.97^{††} $\pm$ 2.2	14.51[†] $\pm$ 3.10	11.42^a $\pm$ 1.74
	Yes	11.82* $\pm$ 2.12	13.79* $\pm$ 3.02	15.41* $\pm$ 2.51	13.68^{ac} $\pm$ 1.48
Subclinical	No	6.77* $\pm$ 0.58	7.27* $\pm$ 1.64	7.64* $\pm$ 1.40	7.22^b $\pm$ 0.68
	Yes	10.39* $\pm$ 0.53	13.78[†] $\pm$ 1.24	12.3^{††} $\pm$ 1.22	12.15^a $\pm$ 0.69
Clinical	No	8.90* $\pm$ 1.16	11.62* $\pm$ 1.63	12.86* $\pm$ 1.66	11.13^a $\pm$ 0.92
	Yes	14.57* $\pm$ 3.01	16.09* $\pm$ 2.54	21.41* $\pm$ 1.63	17.35^c $\pm$ 1.53

Different letters in columns represent a statistically significant difference (P< 0.05)

Different symbols in rows represent statistically significant difference (P< 0.05)

X PBMC culture medium was replaced with complete medium on day 6. After 18 hours (day 7), cells in culture were stimulated with live *M. paratuberculosis* at 10:1 bacteria to adherent cell ratio. Culture supernatants were collected at 8 hours, 3 days, and 6 days after in vitro infection and analyzed by ELISA. The average represents the concentration $\pm$ SEM of TGF- β produced (ng/ml) at all time points in the media control supernatants with or without in vitro infection with *M. paratuberculosis*.

Y Number of animals in each group was five healthy cows, four subclinically infected cows and five clinically infected cows

A

B

Figure 1. Photomicrograph depicting the morphological appearance of peripheral blood mononuclear cells (PBMC) in all experimental groups following in vitro culture. (A) PBMC 2 hours after isolation from the buffy-coat; (B) Unfractionated PBMC after 6 days in culture and before supernatants replacement with fresh complete medium containing different cytokine treatments. The stellate morphology of cells in culture indicates the development of monocyte-derived macrophages.

A: IFN- γ

B: IL-10

C: TGF- β

Figure 2. Cytokines production in the unfractionated peripheral blood cell culture of healthy and naturally *M. paratuberculosis* infected cows after LPS (*E. coli* O11:B4) stimulation. (A) IFN γ , (B) IL-10 and (C) TGF β production in cell culture supernatants from non-infected healthy cows and cows with subclinical and clinical Johne's disease. PBMC culture media were replaced with complete medium at day 6. After 18 hours (day 7), cells in culture were stimulated with LPS (1 $\mu\text{g/ml}$). Culture supernatants were collected at 8 hours, 3 days, and 6 days after LPS stimulation and analyzed by ELISA. Data represent the concentration mean ($\pm$ SEM) of each cytokine produced at all time points in the supernatants of healthy (n=5), subclinically infected (n=4), or clinically infected cows (n=5) cell culture with or without in LPS stimulation. Different letters represent a statistical significance ($P < 0.05$).

Figure 3. Bactericidal activity of the unfractionated peripheral blood mononuclear cell culture recovered from healthy cows (n=5) or cows naturally infected with *M. paratuberculosis* (n=9). PBMC culture medium was replaced with complete medium at day 6 and 18 hours later (day 7), cells in culture were infected with *M. paratuberculosis* at 10:1 bacteria to adherent cell ratio. Data shown are the number of viable bacteria represented by the number of the colony forming unit (CFU) $\pm$ SEM recovered from cell culture lysates and supernatants 6 days after in the vitro infection with *M. paratuberculosis*. Different letters represent statistically significant difference ($P < 0.05$).

Figure 4. The effect of exogenous cytokine stimulation on the bactericidal activity of the unfractionated peripheral blood mononuclear cell (PBMC) culture from healthy noninfected cows (n=5). After 6 days, PBMC culture supernatants were replaced with fresh medium containing IFN γ (100 ng/ml), IL-10 (100 ng/ml), TGF β (10 ng/ml), TGF β (10 ng/ml) + IL-10 β (100 ng/ml) or left without stimulation (medium control). Eighteen hours later, cultures were infected with live *M. paratuberculosis* (10:1 bacteria to adherent cell ratio). Data shown are the number of viable bacteria represented by the number of the colony forming unit (CFU) $\pm$ SEM recovered from cell culture lysates and supernatants 6 days after in vitro infection with *M. paratuberculosis*. Different letters represent a statistical significance ($P < 0.05$).

Figure 5. The overall effect of exogenous rTGF- β on endogenous IL-10 production in cell culture supernatant. PBMC culture medium were replaced at day 6 with complete medium containing exogenous rTGF- β (10 ng/ml) or left without stimulation (medium control). Culture supernatants were collected 24 hours, 4 days, or 7 days after cytokine stimulation and analyzed by ELISA. Data represents the compression of all values regardless of time, LPS stimulation, or *M. paratuberculosis* infection and is presented as the concentration $\pm$ SEM. Different letters represent a statistical significance ($P < 0.05$).

A: Without *M. paratuberculosis* in vitro infection

B: With *M. paratuberculosis* in vitro infection

Figure 6. The inhibitory effect of exogenous anti-inflammatory cytokines (IL-10 and TGF β) on IFN γ production. PBMC culture media were replaced at day 6 with fresh complete medium containing, IL-10 (100 ng/ml), TGF β (10 ng/ml), TGF β (10 ng/ml) + IL-10 β (100 ng/ml) or left without stimulation (medium control). Cells in culture were infected on day 7 with *M. paratuberculosis* (A) or left without infection (B) Culture supernatants were collected 8 hours, 3 days, or 6 days after in vitro infection and analyzed by ELISA. Data represents the compression of all values regardless of time and presented, as the concentration $\pm$ SEM. Number of animals in each group was five healthy cows, four subclinically infected cows and five clinically infected cows. Different letters represent a statistical significance comparing the anti-inflammatory cytokine stimulation with medium control in all experimental groups ($P < 0.05$).

GENERAL CONCLUSIONS

The susceptibility and resistance to *M. paratuberculosis* infection and disease progression represents a struggle between the bacteria and host immunity. The increased IFN- γ production noted locally at the site of infection in the subclinical animals or as a result of in vitro infection with *M. paratuberculosis* represents an attempt by the host immune system to contain the infection. In this work, we proposed that cell-mediated immunity characterized by high IFN- γ production noted in the subclinical stage of Johne's disease is downregulated by the induction of cytokines such as IL-10 and TGF- β . The downregulation of cell-mediated immunity contributes to disease progression from the subclinical stage to clinical stage.

To evaluate this hypothesis, we analyzed the gene expression of these cytokines locally at the site of infection as well as the draining lymph nodes of these sites. In chapter 2, tissue such from ileum, ileal-cecal junction, ileal-cecal lymph node and mesenteric lymph node were collected from healthy animals as well as from animals at subclinical and clinical stages of the disease. The gene expression for IFN- γ , IL-10, and TGF- β were assessed by RT-PCR in all animal groups. We found an increased expression of TGF- β and IL-10 specific mRNA in the tissues of animals in the clinical stage of the disease and an upregulation of IFN- γ gene expression in the subclinical stage of the disease. We then evaluated the effect of these cytokines on the ability of the monocyte-derived macrophages in unfractionated PBMC cultures to limit *M. paratuberculosis* survival. The profile of cytokine production in each group after in vitro infection was also evaluated. The results of these in vitro experiments revealed that animals at the subclinical stage of the disease had lower TGF- β production compared to healthy and clinically infected animals. After exposure to live *M. paratuberculosis* there was an upregulation of this cytokine only in the naturally infected animals and not in healthy animals. IL-10 production was also upregulated in infected animals after in vitro infection. Therefore, the upregulation of these cytokines that we noted

locally at the site of infection in the clinical animals might be a result of infection with *M. paratuberculosis*.

In healthy animals, in vitro infection with *M. paratuberculosis* resulted in increased production of this cytokine at all time points tested. In contrast, IFN- γ production by PBMC from clinically infected cows was high after 8 hours of *M. paratuberculosis* infection then decreased with time. This might be due to the increased production of IL-10 and TGF- β reported in this group after the in vitro infection with live *M. paratuberculosis*. In the subclinical group, the IFN- γ production remained constant after the in vitro infection with live *M. paratuberculosis* across the time points tested in this study. Therefore, cells obtained from all animals either healthy or naturally infected animals are capable of producing IFN- γ after exposure to *M. paratuberculosis* but the dynamics of the IFN gamma response appear to be affected by the relative amount of the IL-10 or TGF- β .

In addition to the differences in the cytokine production noted in this study, cell phenotype was also noted to be different among groups. Before exposure of cells in culture to *M. paratuberculosis*, clinically infected animals had a higher percentage of B cells and MHC II B cells. The higher percentage of these cells may have affected the cytokine production in this study. Therefore, it might be informative to investigate the role of these cells in the pathogenesis of *M. paratuberculosis* infection and whether these cells are the source of the inhibitory cytokines, IL-10 and TGF- β .

Finally, in healthy animals, the correlation of the increased IFN- γ production and the steady IL-10 and TGF- β production across time with *M. paratuberculosis* survival in the cell culture implies that such conditions favor increased bacterial killing by monocyte-derived macrophages in the unfractionated PBMC culture. In contrast, the culture conditions in both subclinical and clinically infected animals on average favor an increase IL-10 and TGF- β production after *M. paratuberculosis* in vitro infection. Therefore, even with the presence of

IFN- γ the bacterial survival in these cultures were high compared to healthy animals. This indicates that upregulation of IL-10 and TGF- β even with the presence of high IFN- γ production results in less effective killing of the *M. paratuberculosis*. We also tested this hypothesis through the addition of IL-10 and TGF- β to the healthy animals cell culture and found that these cytokines inhibited the bactericidal activity as well as had a down regulatory effect on the IFN- γ production in these cultures. Therefore, the results from these studies indicate that anti-inflammatory cytokines, IL-10 and TGF- β are induced and play a key role in the regulation of host immunity during *M. paratuberculosis* infection.

APPENDIX: CD5 EXPRESSION ON B CELLS

Peripheral blood mononuclear cells (PBMC) from animals in the clinical stage of Johne's disease contain a high percentage of B cells (4). In this study we examined the level of CD5 expression on B cells with the understanding that this may be indicative of B1 cells. Flow cytometric analysis was performed on PBMC culture in the presence or absence of *M. paratuberculosis* and the key regulatory cytokines such as IFN- γ , IL-10, and TGF- β . We reported the presence of two populations of CD5⁺ B cells, one population CD5^{bright} and the other population CD5^{dim} (Figure 1). We also found a correlation between the percentage of CD5^{bright} B cells and the disease stage of *M. paratuberculosis* infection (Figure 2). A previous study reported an increase in the number of B cells in clinically infected animals (4). These B cells did not proliferate in response to antigen-specific stimulation with *M. paratuberculosis* whole cell sonicate (WCS) but proliferated after the addition of ConA to the culture medium. In contrast, B cells obtained from subclinically infected cows demonstrated a normal antigen-specific proliferative response to *M. paratuberculosis* WCS (4). In present study, we demonstrated that 78% of the B cells in the clinically infected animals were CD5^{bright}. However, the percentage of CD5^{bright} B cells in PBMC obtained from animals with subclinical Johne's disease did not exceed 47% of the total B cells and an even lower percentage noted for PBMC from healthy animals (34%). Culturing PBMC from healthy, subclinical, or clinical Johne's cows resulted in downregulation of CD5 expression on B cells in all groups (Figure 3A). Culturing PBMC with live *M. paratuberculosis* (Figure 3B) increased the percentage of the CD5^{bright} B cells in cultures from subclinically infected cows but not in cultures from healthy cows or clinically infected cows. Activation of cells in culture with cytokines such as IFN γ , IL-10, or TGF β slightly increased the percentage of the CD5^{bright} B cells in cultures compared to cells cultured in medium alone (Figure 3A). It should be noted that these changes were only observed in cells recovered from subclinically infected animals and not those from healthy or clinically infected animals.

Bovine leukemia virus (BLV) infection in cows is known to be associated with an increased population of CD5⁺ B cells (2). In our study, the increased percentage of CD5^{bright} B cells after cytokine treatment or after in vitro infection with live *M. paratuberculosis* was not affected by the presence or absence of BLV infection. Cells from both BLV positive and BLV negative cows with subclinical disease had a significantly increased percentage of CD5^{bright} B cells (Table 1). None of the clinically infected cows tested negative for BLV; therefore, it is difficult to speculate if the high percentage of CD5⁺ B cells found in the clinical animals is a result of *M. paratuberculosis*, BLV, or both infections. Taking that into consideration, we hypothesize that there might be a relation between the presence of CD5^{bright} B cells and the progression of Johne's disease. Whether this correlation relates to a cause or effect relative to clinical disease is unknown, but it is tempting to speculate that a ubiquitous agent such as BLV may hasten the progression of disease in *M. paratuberculosis* infection from subclinical infection to clinical disease.

Materials and Methods

Animals. PBMC were collected from 9 healthy cows, 17 cows naturally infected with *M. paratuberculosis* but asymptomatic (i.e., subclinical), and 9 cows with clinical Johne's disease. The Holstein dairy cows used in this experiment were monitored by frequent testing for the fecal shedding of *M. paratuberculosis* using standard culture methods as previously described (3). By definition, clinical animals were shedding more than 100 colony forming units (CFU) per gram of feces and presented with weight loss and intermittent diarrhea. Subclinically infected cows were shedding less than 10 CFU/g of feces and were otherwise asymptomatic. All animals in the experiment were approved by the IACUC and housed in American Association for Accreditation of Laboratory Animal Care-accredited facilities (National Animal Disease Center, Ames, Iowa).

Blood collection, culturing conditions and sample collection. Blood was collected from the jugular vein into 2X acid-citrate-dextrose (ACD) (1:10). Peripheral blood mononuclear cells (PBMC) were isolated from the buffy-coat fractions of the peripheral blood as previously described (1). PBMC were re-suspended in RPMI-1640 (Gibco, Grand Island, NY) containing 10% FCS (Atlanta Biologics, Atlanta, GA.), 100 units/ml penicillin G sodium (Gibco) 100 µg/ml streptomycin sulfate (Gibco), 0.25 µg/ml amphotericin B (Gibco), and 2 mM L-glutamine (Gibco). Cells were cultured at 2×10^6 /ml in 24-well flat-bottomed plates (Nunc, Life Technologies) at 39°C in 5% CO₂ in a humidified atmosphere. Cells in culture were stimulated with 100 ng/ml human IL-10 (200-10, Peprotech, Rocky Hill, N.J.), 100 ng/ml bovine IFN-γ (generously donated by Novartis Animal Health, Basel, Switzerland), 10 ng/ml human TGF β (100-21R, Peprotech), 100 ng/ml IL-10 plus 10 ng/ml TGF-β or left without cytokine stimulation. PBMC were in vitro infected with live *M. paratuberculosis*, strain 19698 (NADC, Ames, IA) at a ratio of 10 bacteria per cell. Cells were collected for flow cytometric analysis at 8, 24, and 72 hours after cytokine treatments and in vitro infection with *M. paratuberculosis*.

Bacteria. *M. paratuberculosis* strain 19698 (NADC) was grown in Middle-brook 7H9 broth (pH 6.0) supplemented with oleic acid albumin dextrose complex (OADC, Becton Dickinson Microbiology). The bacteria were harvested, washed three times with phosphate buffer saline (PBS), resuspended in PBS to a final concentration of 10^9 /ml as determined by the absorbance at 540 nm. Bacterial stocks were then frozen in PBS at -80°C until used in the experiments. Prior to in vitro infection, frozen bacterial stocks were thawed and clumps were dispersed by brief sonication at 25 W for 40 sec with a Tekmar sonic disturber. The frozen bacterial stocks were monitored for viable count by culturing on HEYM. Viable counts in stocks were reduced after thawing and sonication to approximately around 10^8 CFU/ml.

Assessment of expression of CD5⁺ cell surface marker on B cells. For analysis of the expression of CD5 on B cells, two-color flow cytometry protocol was used. Briefly, 100 µl

of cell cultures at 2×10^6 cells/ml were incubated for 20 min at RT with 50 μ l of a monoclonal antibody specific for a B cell marker (BAQ155A 7 μ g/ml, VMRD) and 50 μ l of a monoclonal antibody specific for CD5 (B29A 7 μ g/ml, VMRD). Cell surface markers were then visualized by incubating cells for 20 min with 50 μ l of phycoerythrin-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted at 1:500 (vol/vol) to detect cells labeled with anti-CD5 marker and 50 μ l of PerCP conjugated rat anti-mouse IgG1 (Becton Dickinson Immunocytometry Systems, San Jose, CA) diluted at 1:25 (vol/vol) to detect cells labeled with anti-B cell monoclonal antibody. Cells were then washed and resuspended in FACS buffer (PBS, 1% FCS, 0.01% sodium azide) containing DAPI stain at 1:1000 (10 mg/ml) (Sigma, St. Louis, MO) in order to eliminate dead cells from the analysis. Cells were then washed and resuspended in FACS buffer and analyzed the same day by flow cytometry. Data from 5000 events that were DAPI negative (i.e., live cells) per sample were acquired (Cell quest software; BD Biosciences, San Jose, CA) using a FACSCAN flow cytometer (BD, LSR). Data were analyzed using Flowjo software (Tree Star Inc, San Carlos, CA).

Statistical analysis. Results were compared by using ANOVA, and significant differences between means were tested by Fisher protected least significant difference test using the Statview software package (Graphpad software, San Diego, C. A.). For all tests and comparisons only P value less than 0.05 were considered significant.

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A: Healthy cow

B: Subclinical cow

C: Clinical cow

Figure 1. Flow cytometric analysis of CD5⁺ B cells in PBMC cultures obtained from a representative healthy cow after 24 hours in vitro culture (A), subclinically infected cow (B), clinically infected cow (C). The B cells were examined for CD5 expression using two-color flow cytometry. Cells were reacted with a monoclonal antibody (BAQ155A) that defines bovine B cells and monoclonal antibody (B29A), which identifies CD5. Three populations of B cells were noted: CD5⁻ B cells (gate 1), CD5^{dim} B cells (gate 2) and CD5^{bright} B cells (gate 3) that had the same level of CD5 expression as observed for the distinct non B cell CD5 + population.

Figure 2. Peripheral blood mononuclear cells from healthy (n=9), subclinical (n=17) or clinical infected cows (n=9) were isolated, stained and analyzed by flow cytometry. Data represent the percentage of CD5^{bright} B cells from the total B cell population ($\pm$ SEM). Different letters represent a statistical difference ($P < 0.05$).

A: Without *M. paratuberculosis* infection

B: With *M. paratuberculosis* infection

Figure 3. The percentage of CD5^{bright} B cells from the total B cell population in PBMC cultures obtained from healthy, subclinical or clinical infected cows. Cells were cultured for in medium alone (no cytokine stimulation), IFN γ (100 ng/ml), IL-10 (100 ng/ml), TGF β (10 ng/ml), or the combination of TGF β (10 ng/ml) + IL-10 (100 ng/ml) (A). In addition to these cytokines, cells in culture were also infected with live *M. paratuberculosis* at 10:1 bacteria to cell ratio (B). Cells were collected for flow analysis at 8, 24 and 72 hours after cytokine treatments or in vitro infection. Data represent the average percentage of CD5^{bright} B cells at all time points $\pm$ SEM.

Table 1. Percentages of CD5^{bright} expressed on B cells obtained from subclinically infected cows (n=17) that were seropositive (n=7) for bovine leukemia virus (BLV) or were found to be seronegative (n=10) for BLV infection.

BLV status	Medium control	In vitro infected with <i>M. paratuberculosis</i>
Negative	31.0 ^a ± 2.02	46.4 ^b ± 3.06
Positive	38.1 ^a ± 0.71	52.0 ^b ± 1.72

Cells in culture were infected with *M. paratuberculosis* at 10:1 bacteria to cell ratio or were not infected. Data represents the average for all the time points tested in this study (8 hours, 3 days and 6 days).