Modeling the initial host immunopathologic response to *Mycobacterium avium* subspecies *paratuberculosis* infection in young cattle

By

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This dissertation is dedicated to:

Rhoda
Mason
Mischa
Malia
# TABLE OF CONTENTS

## ACKNOWLEDGEMENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>v</td>
</tr>
</tbody>
</table>

## GENERAL INTRODUCTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Statement of the Problem</td>
<td>1</td>
</tr>
<tr>
<td>Specific Aims</td>
<td>5</td>
</tr>
<tr>
<td>Dissertation Organization</td>
<td>5</td>
</tr>
<tr>
<td>References</td>
<td>7</td>
</tr>
</tbody>
</table>

## CHAPTER 1. LITERATURE REVIEW

<table>
<thead>
<tr>
<th>Subsection</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunology of <em>Map</em> infection in cattle</td>
<td>10</td>
</tr>
<tr>
<td>Modeling systems to study mycobacterial infections</td>
<td>35</td>
</tr>
<tr>
<td>References</td>
<td>38</td>
</tr>
<tr>
<td>Table 1 Features of major subsets of human and bovine $\gamma\delta$ T cells</td>
<td>52</td>
</tr>
</tbody>
</table>

## CHAPTER 2. GAMMA-DELTA ($\gamma\delta$) T CELLS ARE DIFFERENTIALLY ASSOCIATED WITH GRANULOMA DEVELOPMENT AND ORGANIZATION IN A BOVINE MODEL OF MYCOBACTERIAL DISEASE

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>53</td>
</tr>
<tr>
<td>Introduction</td>
<td>54</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>56</td>
</tr>
<tr>
<td>Results</td>
<td>61</td>
</tr>
<tr>
<td>Discussion</td>
<td>64</td>
</tr>
<tr>
<td>References</td>
<td>68</td>
</tr>
<tr>
<td>Figures</td>
<td>74</td>
</tr>
</tbody>
</table>

## CHAPTER 3. EVALUATION OF THE INITIAL HOST IMMUNE RESPONSE TO *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* INFECTION IN SENSITIZED OR NAÏVE CALVES USING MATRIX BIOPOLYMERS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>80</td>
</tr>
<tr>
<td>Introduction</td>
<td>81</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>84</td>
</tr>
<tr>
<td>Results</td>
<td>89</td>
</tr>
<tr>
<td>Discussion</td>
<td>94</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>98</td>
</tr>
<tr>
<td>References</td>
<td>99</td>
</tr>
<tr>
<td>Figures</td>
<td>104</td>
</tr>
</tbody>
</table>
CHAPTER 4. DIRECT INOCULATION OF MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS INTO ILEOCECAL PEYER’S PATCHES RESULTS IN COLONIZATION OF THE INTESTINE IN A CALF MODEL  
Abstract 111  
Introduction 112  
Materials and Methods 114  
Results 119  
Discussion and Conclusions 123  
Acknowledgements 127  
References 129  
Tables 132  
Figures 135

CHAPTER 5. GENERAL CONCLUSIONS 139  
Gamma-delta (γδ) T cells are differentially associated with granuloma development and organization in a bovine model of mycobacterial disease 140  
Evaluation of the initial host response to Mycobacterium avium subspecies paratuberculosis infection in sensitized and naïve calves using matrix biopolymers 141  
Direct inoculation of Mycobacterium avium subspecies paratuberculosis into ileocecal peyer’s patches results in colonization of the intestine in a calf model 142  
Recommendations for future research 145  
References 152
ACKNOWLEDGEMENTS

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The adventure continues.
GENERAL INTRODUCTION

Statement of the Problem

*Mycobacterium avium* subspecies *paratuberculosis* (*Map*) is the causative agent of paratuberculosis (Johne’s disease), a disease that is widely disseminated in domestic and free-range ruminants around the globe. The worldwide prevalence is unknown, but in United States dairy herds has been estimated to be as high as 68% [1]. *Map* infection is usually acquired by neonatal calves during the first few days of life, yet development of clinical disease generally is not evident for several years due to a prolonged asymptomatic subclinical infection period. This is a significant research problem to overcome when studying pathogenesis of *Map*, and thus far models to effectively study host immunopathology during the span from the initial infection to clinical disease have been elusive. To address this challenge, a considerable amount of research has been directed at this disease, yet the pathogenesis of early and subclinical *Map* infection remains a key scientific knowledge gap. The following dissertation project is directed towards understanding the immunopathology of early *Map* infection in young calves, and how the initial events following *Map* infection may relate to host mechanisms of resistance or progression of this devastating disease.

**Immunopathology of Map infection**

The obligate intracellular bacterium *Map* is a pathogen of macrophages. The virulence of *Map* (and other pathogenic mycobacteria) is largely considered to be due to its 1) evasion of macrophage-mediated bacterial killing and 2) attenuation of antigen
presentation to the immune system [2]. In light of this, most research efforts have focused on these two aspects of the host innate immune response. Due to challenges associated with studying host anti-mycobacterial immunopathology during early *Map* infection *in vivo*, the main descriptions of innate immunology relevant to *Map* infection have been derived from experimental *in vitro* macrophage cell culture systems [3].

Evasion of macrophage killing has been experimentally shown to occur primarily by prevention of macrophage activation as well as blockage of phagosome acidification and maturation [4]. It has been shown that macrophages possess the vast arsenal for killing of invading mycobacterial organisms, yet not all *Map* are killed and at least a subset of bacteria are able to persist and proliferate within subepithelial macrophages of the intestine [5]. Classical macrophage activation with killing of ingested bacteria is vital for host resistance to *Map* infection, and this is presumably mediated by interferon gamma (IFNγ) secreting CD4+ T cells [6].

Traditionally, macrophage function during *Map* infection has been most widely studied, but more recent efforts have revealed that development of an efficacious adaptive immune response against *Map* is also profoundly influenced by the dendritic cell (DC). DCs have been recently described in cattle where as a pool of professional antigen presenting cells (APC) they are, as in humans, thought to play an essential role during initiation of host T cell immunity [7, 8]. In humans, development of effective cell-mediated immunity is dependent on DCs [9, 10], and modulation of DC function by mycobacteria has been shown to inhibit this process [11]. The complete role of DCs in cattle continues to be elucidated; however *Map* has been recently shown to influence bovine DC maturation status and ultimately development of the adaptive immune
response [12, 13]. Further understanding the role of these important cells will be helpful for fully developing an inclusive model for understanding the pathogenesis of Map.

The influence of gamma-delta (γδ) T cells in host response to Map

The role of minor lymphocyte classes (natural killer (NK) and γδ T cells) during the initial anti-mycobacterial immune response has recently received significant research attention. This is because data from in vitro human studies has suggested that these cells can modulate macrophage killing or DC function either directly or via cytokine secretion which may ultimately lead to alteration of the adaptive immune response [14, 15]. This is a significant observation which may have profound implications in bovine immunology because compared to humans or mice, young cattle have significant numbers of γδ T cells circulating in their peripheral blood and tissues [16]. Correlation between human and animal model data is incomplete, but human and bovine γδ T cells have been shown to share some functional features. Further, the in vivo responses of γδ cells have yet to be fully explored in any species, and the young calf is an ideal model to employ in efforts to understand their effects during the anti-mycobacterial immune response [17].

Ultimately it seems that interaction between invading mycobacterial pathogen and host cells at the infection site determines the course and outcome of infection [18, 19]. The probability of successful control of Map infection is greatly enhanced by three specific features of the host response: 1) efficient bacterial killing by immune cells at the infection site, 2) initiation of efficient antigen presentation at the infection site which
eventually determines 3) development of a systemic adaptive T helper 1 (Th1) immune response.

**Modeling the early host immune response to *Map* in calves**

Key knowledge gaps identified by *Map* researchers are due in part to difficulty associated with successfully modeling early *Map* infection in cattle [20, 21]. The most relevant data on host-pathogen interaction is best acquired using the natural host in research trials, but use of ruminants in conventional model systems for *Map* research is limited by the time required to maintain animals in containment facilities before they develop disease. An *in vivo* bovine model which successfully recapitulates all phases of the infection has not yet been described and so in order to fully elucidate the immunopathology of the infection site and during subclinical *Map* infection in calves there is significant need for improved model efficacy [3]. Though a number of novel experimental model systems have recently been published [20, 22-24], additional work is needed. Focusing our efforts on the localized infection site immune response to *Map* and how this influences the subsequent disease process is expected to shed considerable light on the pathogenesis of *Map* infection in ruminants.

This literature review will focus on components of both the innate and adaptive arms of the host immune response, with an emphasis on the immunobiology of the γδ T cell and how the interactions of these components may result in containment or progression of *Map* infection in the young bovine calf.
Specific Aims

The primary goal of these studies was to understand the initial immunopathologic events occurring at the *Map* infection site in calves, and how these events may ultimately result in containment of infection, development of subclinical infection or ultimately allow progression to chronic end-stage wasting syndrome of clinical Johne’s disease. To address this goal, we first adapted a young calf model of subcutaneous *Map* infection [25, 26] in order to evaluate the cellular and molecular events at *Map* infection sites in cattle. Our central hypothesis for this set of studies was that γδ T lymphocyte subsets play a central role in shaping the immune response at early *Map* infection sites in young calves. To address this hypothesis, the following specific aims were set forth: 1) investigate recruitment of γδ T cell subsets to *Map* infection sites in context of morphologic lesion development (Chapter 2) and 2) understand the functional role of T lymphocytes including γδ T cell subsets recruited to *Map* infection sites (Chapter 3). Finally, we were interested in the correlation of our observations from the subcutaneous *Map* model with the development of intestinal *Map* disease in calves. As a result of the initial work, our final specific aim was 3) to develop an intestinal model for studying subclinical *Map* infection thereby allowing testing of findings of the earlier specific aims in the gastrointestinal tract of cattle (Chapter 4).

Dissertation organization

This dissertation is organized in the alternative format with the first chapter being a general introduction and literature review (Chapter 1). This is then followed by three individual manuscripts (Chapter 2-4), followed by general conclusions with references
cited at the end of each chapter. Two manuscripts have been published in the

*International Journal of Experimental Pathology* (Chapter 2) and *Veterinary Pathology* (Chapter 4). The third manuscript has been prepared for submission to *Veterinary Pathology* (Chapter 3).
References


CHAPTER 1: LITERATURE REVIEW

Immunology of Map infection in cattle

It is generally accepted that Map infection occurs primarily during the perinatal period when calves ingest an infectious dose of live bacteria from feces or colostrum. The organism is likely endocytosed by microfold cells (M cells) of the dome epithelium or the epithelial cells themselves [1-3] overlying ileal Peyer’s patches and subsequently delivered to subepithelial macrophages and dendritic cells [4]. Classically, there is development of an initial T helper type 1 (Th1) immune response mediated by interferon gamma (IFNγ)-secreting CD4+ T cells. Effective Th1 polarization of the host immune response is considered vital for controlling mycobacterial infections [5], and detection of Map-specific IFNγ has been successfully used diagnostically during natural Map infections of cattle [6]. Following initial infection, a lengthy (months to several years) subclinical period occurs prior to development of fatal clinical disease in a subset of animals. In these individuals, clinical disease is characterized by development of progressive granulomatous enteritis, diarrhea and wasting which eventually leads to death of the host. Transition to clinical disease is immunologically associated with a loss of Th1 dominance [7] and development of T helper 2 (Th2) dominance. The Th2 response is characterized by high levels of interleukin (IL)-4 and IL-5-induced Map-specific immunoglobulin production, which serves as the basis for commercially available serum diagnostic assays during clinical phase of disease. Accurate diagnosis during latent
disease is at best challenging [8], and typically requires the use of multiple testing strategies that can be difficult to interpret [9].

**Macrophages**

The role of macrophages during anti-mycobacterial immunity can be divided into two consecutive phases of the host immune response. During the acute response, macrophages are recruited to the *Map* infection site where they participate in phagocytosis and bacterial killing and as professional antigen presenting cells (APCs) they are capable of stimulating the adaptive immune response. The acute phase of the inflammatory response ends with either resolution of the infection or progression to the chronic inflammatory phase which is characterized by macrophage-rich cellular infiltrates (granulomatous inflammation) at the infection site.

*Map* is primarily an infection of macrophages. Experimental infection models in calves have not resulted in consistent colonization by *Map* of the distal small intestine as observed during late subclinical and clinical stages of natural disease [10]. As a consequence, studies evaluating *in vivo* macrophage function during *Map* infection are scarce [11-14] and most data regarding the early post-infection period of cattle are derived from experimental *in vitro* studies [15]. The intracellular survivability of *Map* (and other pathogenic mycobacteria) appears to be largely due to its 1) evasion of macrophage-mediated bacterial killing and 2) attenuation of antigen presentation to the immune system [16]. Avoidance of macrophage killing has been experimentally shown to occur by prevention of macrophage activation and by blockage of phagosome acidification [17], which provides an environment for pathogen survival and potentially
intracellular growth [18]. Macrophages possess the vast mechanistic arsenal for killing invading mycobacterial organisms [19] and many Map-infected animals appear to eliminate the infection. However, not all invading organisms are killed and so a subset of Map persist and proliferate within subepithelial macrophages of the intestine and this ultimately results in disease progression through the subclinical and clinical phases [20]. The in vivo mechanisms surrounding these transitions have not been fully examined. It is however clear from in vitro studies that classical macrophage activation mediated primarily by IFNγ-secreting CD4+ T cells plays a key role in destruction of the ingested bacteria [21].

In the event that the acute response is incapable of effectively resolving the infection, a transition to chronic inflammation occurs. Chronic granulomatous inflammation lies at the center of mycobacteria-associated immunopathology and is considered to play an integral role in mycobacterial containment [22, 23]. In fact, granuloma morphology in humans and animals has been strongly associated with disease progression and outcome [24, 25]. On one hand, morphologically diffuse lesions (granulomatous inflammation) are associated with Th2 dominance and typified by clinical intestinal Map infection of cattle. These are considered to be dysfunctional lesions that fail to control pathogen proliferation. Morphologically, these lesions are dense sheets of macrophages and lymphocytes lacking distinct organization or stratification of the inflammatory cells. There are generally large numbers of viable mycobacteria (multi-bacillary disease) present in diffuse granulomatous lesions, signifying the inability of the host to effectively limit mycobacterial viability and enhancing the potential for widespread dissemination.

In contrast to diffuse lesions is the discrete caseating granuloma, which is associated with
Th1 dominance and considered the hallmark histologic feature of human and bovine tuberculosis where pathogen proliferation and spread is limited and disease is controlled. These lesions are highly organized aggregates of macrophages with central regions of caseous necrosis and mineralization. The macrophages are cuffed by lymphocytes and the discrete nodules are typically encapsulated and separated from adjacent tissue by distinct bands of mature fibrous connective tissue. These lesions are accompanied by low numbers of viable mycobacteria (pauci-bacillary disease), potentially signifying the effectiveness of the host to limit bacterial survival and replication. Discrete granulomas are thought to contribute to host protection, most importantly by limiting dissemination of bacteria and restriction of tissue damage by shielding surrounding tissue from the chronic on-going inflammatory process [22]. Granuloma morphology and its potential role during mycobacterial disease has been well-studied in humans [26, 27] and cattle [28-30]. These studies have revealed that many other factors (lymphocytes, cytokines) also greatly influence the formation and maintenance of granuloma morphology and some of these factors are further discussed below.

**Dendritic cells**

Though macrophage function during *Map* infection is most widely studied, more recent research efforts have revealed that development of an efficacious adaptive immune response against *Map* is profoundly influenced by the dendritic cell (DC). Most commonly studied in humans and mice [31], DCs have been more recently described in cattle where as a pool of professional APCs they are also thought to play an essential role during initiation of host T cell immunity [32, 33]. DCs are derived from bone marrow
precursors, and exist in an immature state throughout the body. Most notably DCs are present within organized (Peyer’s patches) and unorganized (lamina propria of intestine) lymphoid tissue at mucosal sites where they serve as sentinels against incoming pathogens. Immature resident DCs have high antigen-uptake capabilities and are thought to be the primary recipient of antigen from M cells of the distal small intestine. Following antigen uptake by pathogen recognition receptors (PRRs), DC’s undergo dramatic phenotypic and functional changes that are characteristic of the maturation process [33]. Mature DCs have reduced phagocytic activity but now the cells upregulate chemokines (CCR7) that aid in migration from Peyer’s patches and into the draining lymph nodes where they present processed antigen to naïve T lymphocytes via major histocompatibility complex (MHC) molecules. By this mechanism, naïve T cells are primed and effector T cells are induced to differentiate; thus the development of effective T cell-mediated immunity is heavily dependent on DCs [34, 35].

In mice and humans, DCs function as initial host cells for mycobacteria and play an important role during mobilization of immunity during the early infection period [36]. Similar results have also been found in cattle, where the initial Th1 priming is dependent on DCs because DCs are thought to be a primary source of the Th1-promoting cytokine IL-12 [37]. Following infection with *Mycobacterium bovis* (*M bovis*) or Bacillus Calmette-Guerin (BCG), bovine DCs contain greater numbers of bacteria compared to macrophages, and they stimulate more effective proliferation and IFN\(\gamma\) secretion by autologous CD4+ and CD8+ T lymphocytes. Consistent with *M tuberculosis*-infected human and murine DCs, bovine DCs infected with *M bovis* in this study consistently upregulated CD40, CD80 and MHC molecules and the magnitude of these effects
appears to vary proportionally with the virulence of the infecting agent [37]. Not all DC-mycobacteria interactions lead to strong enhancement of an effective cell mediated immune response however. DCs of human or murine origin [38] and bovine origin [37] have also been shown to secrete other cytokines (such as IL-10) following mycobacterial infection, and this may serve a regulatory role, or to dampen the pro-inflammatory response. For example, infection with *M leprae* has been shown to inhibit or suppress activation and maturation of human DC relative to *M tuberculosis* and *M bovis* [39]. In this same study, the authors reported that *M leprae*-infected DCs induced mixed Th1 and Th2 immune responses, and they concluded that this effect was probably an important determinant of the dramatic anergy of *M leprae*-specific cell-mediated immunity in human lepromatous patients [39].

These are important observations that are uniquely relevant for *Map* because the progressive stages of bovine Johne’s disease share some histologic and immunologic features with *M leprae*-induced disease in human beings. Though the target organ differs, late *Map* infection in cattle is characterized by a lack of *Map*-specific T cell-mediated immune response coupled with development of diffuse granulomatous intestinal inflammation. In a mouse model, the fibronectin attachment protein of *Map* has been shown to modulate the adaptive immune response to *Map* infection by inducing maturation and activation of DCs which then drives Th1 polarization [40]. The *in vitro* data from this study would suggest that *Map* is capable of driving effective Th1 polarized immune responses *in vivo*; however other reports do not support this hypothesis. It was shown by Lei et al that bovine DCs harvested from subcutaneous diffuse granulomatous *Map*-induced lesions had significantly reduced expression of CD40, CD80 and CD86
genes compared to DCs harvested from distinct granulomas induced in the same calves by *Map* vaccine. Further, DCs from *Map* granulomas in this study also displayed reduced ability to induce autologous CD4+ T cell proliferation [41]. Reduced phenotypic and functional maturation of monocyte-derived bovine DCs following *Map* infection was also demonstrated in an *in vitro* system [42]. Contrasting with the mouse model studies, results from these calf studies suggest that *Map* induces limited phenotypic and functional maturation of bovine DCs. The role of DCs during *Map* infection of cattle is only beginning to be explored yet it seems evident that further defining their function is key to developing a model for understanding the pathogenesis of *Map*.

**Lymphocytes**

Two distinct lineages of CD3+ lymphocytes expressing a heterodimeric T cell receptor (TCR) exist in most species. The dominant CD3+ lymphocyte population of most species expresses α and β TCR chains while a smaller population (except in neonatal ruminants) expresses γ and δ TCR chains. As they relate to pathogenesis of mycobacterial infections, αβ T cell biology is well understood and will be briefly reviewed; this will be followed by a more extensive comparative review of the less well-understood and more recently discovered γδ T cells of humans, mice and cattle.

**αβ T lymphocytes**

The role of major lymphocyte subsets (αβ T lymphocytes) during mycobacterial diseases in humans and animals is well described. αβ T lymphocytes can be traditionally...
assigned into two T helper (Th) subsets based on their distinct patterns of cytokine secretion in response to antigenic stimulation. Th1 cells are characterized by secretion of IL-2 and IFNγ but not IL-4 or IL-5 while Th2 cells are characterized by secretion of IL-4 and IL-5 but not IL-2 or IFNγ [43]. Th1 cells tend to be mediators of macrophage activation and delayed-type (T cell mediated) hypersensitivity, whereas Th2 cells promote immunoglobulin production by B cells and immediate hypersensitivity reactions. Initially it was described that cytokine secretion patterns were mutually exclusive in that cytokines such as IL-12 promoted Th1 development while suppressing Th2 development and likewise IL-13 promoted Th2 development at the expense of the Th1 response. In the time since the initial descriptions, variations have been described in a variety of natural disease conditions that defy the classically described Th1-Th2 axis [44]; however this traditional dichotomous immunologic model persists in the literature [43]. The host defense against mycobacterial agents also continues to be largely defined in this way so the terminology is utilized in this review.

In humans infected with *M tuberculosis*, activation of Th1 CD4+ lymphocytes with IFNγ production and macrophage activation are considered crucial for protection [45]. Similarly, Th2 predominance with IL-4 production is associated with progressive or disseminated disease and the immunologic balance measured by Th1/Th2 ratio is directly correlated with the favorability of disease outcome [46]. As for many mycobacterial diseases (such as *M leprae* in humans), *Map* infection of cattle is associated with an initial Th1 mediated cellular response with *Map*-specific IFNγ production. At some point during the subclinical phase of the infection, loss of systemic and local cell-mediated immune responses [7, 47] correlates with development of a strong Th2 response.
characterized by high levels of *Map*-specific immunoglobulin [48] and development of clinical disease. The cellular and molecular events surrounding and modulating these transitions during pathogenesis of *Map* infection in cattle remain incompletely characterized.

\textbf{γδ T lymphocytes}

In this section, the known immunobiology of γδ T cells in humans and cattle will be described, compared and contrasted. The data described herein are also summarized in table form (Table 1, Features of major subsets of human and bovine γδ T cells). The γδ TCR was serendipitously discovered and first described in humans in 1986 [49], and a few years later was first described in cattle [50]. The γδ T cells have thus been less well studied compared to their well-known αβ counterparts. However, since their discovery the immunobiology of γδ T cells has been most studied in humans and mice and the data have indicated that these cells have a variety of functions during both innate and adaptive immunity. To date, demonstrated or proposed functions of γδ T cells include granuloma formation or maintenance, direct cellular cytotoxicity, cytokine and chemokine secretion, modulation of macrophage function, modulation of T lymphocyte function, modulation of DC function, phagocytosis with antigen presentation/processing capability and immune surveillance or regulation. Regardless of some key differences between species, a significant amount of recent data indicates several common themes of γδ T cell immunobiology that span the species barrier and thus there are substantial generalizations
which can be made regarding the function of these unique cells during infectious diseases or inflammatory processes.

**Specialized anatomic distribution and phenotype:** Within lymphoid tissues, \(\gamma\delta\) T cells are considered to be a minor T lymphocyte population compared to the major \(\alpha\beta\) T lymphocyte population. In contrast, \(\gamma\delta\) T cells are enriched in many organs containing epithelia or at mucosal surfaces such as intestine, lung, skin or reproductive tract which seems to indicate a role for these cells during immune surveillance and antigen sampling as mucosal surfaces are constantly confronted with potential pathogens [51, 52]. In addition to epithelial sites, \(\gamma\delta\) T cells are also well represented among peripheral blood mononuclear cells. In humans and mice \(\gamma\delta\) T cells can represent 1-10% of circulating T lymphocytes in adults [53], but notably in ruminants this can be as high as 40% in young calves but generally diminishes to 10-25% as the animals reach adulthood [54].

The genetic arrangement of the human \(\gamma\delta\) TCR has been studied. As for \(\alpha\) and \(\beta\) genes, \(\gamma\) and \(\delta\) genes have variable (V), joining (J) and constant (C) regions. The \(\delta\) and \(\beta\) genes also contain diversity (D) segments whereas \(\alpha\) and \(\gamma\) genes do not [55]. In humans, the ability to distinguish \(\gamma\delta\) T cell subsets based on surface marker expression has not been reported. Rather, \(\gamma\delta\) T cell subsets of humans are defined by their \(\gamma\) and \(\delta\) gene segment usage and it has been noted that \(\gamma\delta\) T cells from different anatomic sites show preferential V gene expression suggesting that human \(\gamma\delta\) T cell subsets have distinct functional roles [56]. For example, the two major \(\gamma\delta\) T cell subsets in humans are \(V\delta1+\) and \(V\delta2+\) cells and distinctions in their distribution and function have been observed. \(V\delta2+\) cells predominate in peripheral blood circulation and have been shown to
significantly expand during a variety of infectious diseases including mycobacterial diseases [57]. In contrast, the Vδ1+ subset is less frequently observed in the blood, but are the majority subset in tissues and they appear to have cytolytic properties and secrete Th1 cytokines upon activation [58, 59]. Specific known functions of γδ T cell subsets are further discussed in following sections of this dissertation. Expression of other molecules (CD2, CD5, CD4, CD6, and CD8) on γδ T cells has also been described in humans, mice and cattle: key features in humans and bovine cells are summarized in table 1. There is considerable interspecies variability suggesting that these molecules are less useful for defining functionally distinct subsets and there is currently no species-wide γδ T cell-specific marker [60, 61].

The γδ TCR of cattle has also been cloned and characterized, but little is known about how preferential gene segment usage correlates with tissue distribution or functionality in this species [62]. Cell surface expression of the cysteine-rich scavenger receptor molecule workshop cluster 1 (WC1) is specific for γδ T cells in ruminants; however both WC1- and WC1+ subsets of γδ T cells exist. While the WC1 molecule has been most widely used to define bovine γδ T cell subsets, other molecules (GD3.1, GD3.5, GD3.8) have been described [63, 64], but their significance or function remains unknown. Homologous WC1-like genes have been found in sheep, goats, horses, mice, pigs and humans; however gene expression of WC1 appears to be limited to ruminants [65]. Based on their expression of the WC1 molecule, bovine γδ T cells are subdivided into two categories. The larger of these subsets has the phenotype WC1-CD2+CD3+ and is found primarily within splenic red pulp and the intestinal tract while the second subset
has the phenotype WC1+CD2-CD3+ is found predominantly in peripheral blood where they can constitute between 10-25% of the peripheral blood lymphocytes in adults and up to 40% or more in young calves [61]. As in humans, it is thought that both phenotypes represent functionally distinct γδ T cell subsets that preferentially home to different tissue localizations [61]. Though this concept in cattle has not been completely explored in vivo, Jutila et al have demonstrated that subsets of bovine γδ T cells differentially express genes associated with proliferation and inflammation indicating distinct functional differences [63, 64].

There is compelling evidence to support the hypothesis that γδ T cells are innate cells during host defense. The fact that γδ T cells are preferentially found at mucosal surfaces seems to indicate a role during immune surveillance and antigen sampling [60]. Two additional features fundamentally distinguish between αβ and γδ T cells. First, in contrast to conventional αβ T cells, it is recognized that most γδ T cells do not express the CD4 or CD8 accessory molecules and thus there is no MHC class I or MHC class II restriction. Second, γδ T cells do not recognize ligands processed from complex antigens by professional APCs but instead see unconventional antigens such as phosphorylated microbial metabolites or lipid [66].

**Ligands:** The nature of ligands or potential ligands for γδ T cell subsets of humans or animals is poorly characterized, yet it is clear that these cells are capable of recognizing a broad range of molecules. Responses of γδ T cells to mycobacteria have been described for several years [67] and both protein [68, 69] and nonprotein [70, 71] antigens have been shown to induce γδ T cell responses. In humans, the majority of studies have
examined reactive patterns of the Vδ2+ subset of γδ T cells, which are unique to primates and the dominant subset present in human peripheral blood. Vδ2+ cells recognize low molecular weight non-peptide phosphate-containing metabolites produced by a variety of bacterial pathogens including mycobacteria [72]. Variations or other important ligands for Vδ2+ cells also include microbial byproducts such as negatively charged alkyl phosphate antigens [73] and positively charged alkylamine antigens [74]. It is notable that many of the putative microbial ligands described for human γδ T cells have autologous counterparts or endogenous metabolites of the mevalonate pathway (present in all higher eukaryotes and some bacteria) which are upregulated during periods of cellular stress suggesting that γδ T cells also function during non-infectious processes [75]. Specific ligands for human Vδ1+ cells are even less well described. Spada et al reported that Vδ1+ cells directly recognized CD1c molecules, and recognition was TCR-dependent but did not depend on presence of foreign lipid or glycolipid antigens [59]. The authors observed that CD1c is expressed by DCs in granulomas induced by M leprae infection [76] and hypothesized that Vδ1+ cells could be thus activated during initial mycobacterial infection. Specific ligands for Vδ1+ cells should be further investigated given the distinct tissue distribution and functions that have thus far been described for this subset of γδ T cells.

As in humans, γδ T cell ligands in cattle are not clearly defined and the data have been conflicting. In cattle, the majority of studies have examined the reactive patterns of WC1+ cells, which represent the subset with greatest ease of access because they are dominant in the peripheral blood of young calves. WC1+ cells from M bovis-infected
calves respond to both protein and nonprotein antigens of *M. bovis* and in one study the authors concluded that mycobacterial proteins including the novel early secretory antigenic target of *M. tuberculosis* (ESAT-6) were dominant [77]. The dominant human Vδ2+ cell phosphoantigen ligand isopentenyl pyrophosphate (IPP) induced proliferation of WC1+ cells from *M. bovis*-infected calves [77], but this antigen was not recognized by naïve bovine γδ T cells [78]. Vesosky et al also showed that WC1+ cells from healthy calves did respond to stimulation with live mycobacteria, mycobacterial cell wall and mycobacterial culture filtrate proteins [78]. This study further demonstrated that proteolytic digestion of the mycobacterial cell wall component did not affect the WC1+ cellular response, suggesting that naïve WC1+ cells recognize a proteolytically-resistant component of mycobacterial peptidoglycan. In both humans and cattle, the interactions surrounding γδ T cell activation have largely been considered to be TCR-dependent but MHC-independent [71] although TCR-independent activation has also been shown [79]. Recent work has also demonstrated that purified human and bovine γδ T cells can be directly activated by pathogen-associated molecular patterns (PAMPs) in the absence of antigen presenting cells [80], which may have significant implications for the innate role of γδ T cells. In summary, though the specific ligands for bovine γδ T cells remain to be characterized, it is clear that these cells have the capacity to respond to mycobacterial antigens in both naïve and infected individuals.

**Importance of IL-2:** The role of the T cell growth factor IL-2 in T cell proliferation is well known. The initial encounter with specific antigen along with the appropriate co-stimulatory signals (CD28 of T cell binding B7 of APC) induces the synthesis of IL-2
and the $\alpha$ chain of the IL-2 receptor (CD25). Subsequent binding of IL-2 to its high-affinity receptor then triggers progression through the cell cycle, proliferation and differentiation of naïve T cells [55]. Distinct from their $\alpha\beta$ T cell counterparts, it has been known that $\gamma\delta$ T cells produce minimal amounts of IL-2 upon activation, and thus the proliferative response of human $\gamma\delta$ T cells after antigenic stimulation is dependent on CD4+ T cell secretion of IL-2 [81]. In cattle, the WC1+ subset of cells requires the addition of exogenous IL-2 for proliferation in response to *Theileria annulata*-infected monocytes, and stimulation with IL-2 alone induced a limited proliferative response [82]. However, WC1+ cells of *M bovis*-infected calves showed strong proliferation and upregulation of CD25 but minimal IFN$\gamma$ production after stimulation with *M bovis* sonic extract and IL-2 or with IL-2 alone [77, 83]. IL-2 is thus considered a required secondary signal for activation of $\gamma\delta$ T cells which ultimately drives them to proliferation.

**Effects on granuloma formation, maintenance:** The importance of the granuloma during pathogenesis of mycobacterial disease was previously reviewed in this dissertation. The role of $\gamma\delta$ T cells in controlling elimination or progression of mycobacterial disease by mediating the formation, generation and maintenance of the mycobacterial granulomas has been explored. Few studies have investigated $\gamma\delta$ T cells in human granulomatous lesions, and results have conflicted as one study reporting no increase [84] and another studied reporting elevated $\gamma\delta$ T cells [85] within human lesions of sarcoidosis. Mice generally are not considered appropriate models to study granuloma morphology because it is well recognized that mice lack both the highly stratified structures and the presence of central caseation necrosis that are prominent features in
human granulomas [86]. However, reduced granuloma formation has been demonstrated in a γδ TCR knockout mouse model of Map infection [87]. In cattle, γδ T cells have been evaluated in the context of mycobacterial granulomas; however the data here has also been somewhat conflicting. Wangoo et al showed that late-stage lymph node granulomas from M bovis-infected calves had significantly greater numbers of WC1+ T cells compared to early stage lesions, and that the WC1+ cells were spatially distributed at the peripheral zone near the fibrotic capsule of late stage highly organized granulomas [88]. In a separate study however, this group could not confirm distinct spatial relationships of the γδ T cells within the granulomatous lesions [89]. In contrast, Palmer et al demonstrated that the number of CD8+ T cells and WC1+ cells was high during early stage M bovis-induced lymph node granulomas but diminished over time as the granulomas became progressively morphologically advanced. The data from this study suggested that loss of CD8+ and WC1+ cells during late stages correlates with failure of the immune system to control infection [30]. Bovine γδ T cells have also been observed infiltrating the non-progressive diffuse granulomatous lesions induced by subcutaneous Map inoculation in a calf model [90], but more studies are necessary to fully examine the role of γδ T cell subsets during lesion development.

**Immediate effector function: cytotoxicity:** The described effector functions for subsets of γδ T cells are many. Activated human Vδ2+ γδ T cells have broad cytotoxic activity mediated by different pathways. In one study, Vδ2+ cells were able to directly lyse infected macrophages and reduce intracellular bacterial numbers by the Fas/Fas ligand pathway [91]. Dieli et al showed that generation of perforin and granzyme by
Vδ2+ cells also was able to reduce the viability of both extracellular and intracellular *M. tuberculosis* [92, 93]. A study that specifically examined both major subsets of human γδ T cells demonstrated broad *in vitro* cytotoxicity by Vδ2+ cells, but importantly observed that Vδ1+ cells also exhibited this capacity [94]. In cattle, cytotoxicity mediated by bovine natural killer (NK) cells reduces intracellular viability of *M. bovis* [95]; however the evidence for γδ T cell-mediated cytotoxicity is less clear. It has been suggested that cytotoxicity is a feature of bovine γδ T cells during bovine paratuberculosis [96, 97]. However, other reports show that bovine peripheral blood derived and antigen-stimulated γδ T cells (WC1 phenotype not reported, but likely predominantly of the WC1+ subset) were unable to mediate upregulation of nitric oxide production and bacterial killing by *Map*-infected macrophages [98]. Recent data showed that WC1- cells were capable of expressing the natural cytotoxicity receptor CD335 (Nkp46) while producing the pro-inflammatory cytokine IFNγ *in vitro* [99]. Additional experiments should be performed in order to further clarify and define the functional cytotoxicity of γδ T cell subsets in humans and cattle.

**Immediate effector function: cytokine secretion:** It has been known for several years that a key mechanism by which T lymphocytes respond to infectious agents and mediate immune functions is secretion of specific patterns of cytokines. Upon recognition of their ligands, γδ T cells are able to generate a range of pro-inflammatory cytokines or antimicrobial peptides [100] and by this mechanism are thought to provide an initial barrier until antigen-specific αβ T cells have been expanded. Cytokine production by γδ T cell subsets has been analyzed at the gene and protein levels in
humans and cattle. Microarray analysis of stimulated human Vδ2+ cells has shown upregulation of pro-inflammatory genes such as tumor necrosis factor alpha (TNFα), IFNγ, macrophage-colony stimulating factor, IL-17 and IL-21 [101, 102]; however secretion of some of these cytokine proteins by stimulated Vδ2+ cells has not been confirmed. Initial studies in humans showed that peripheral blood-derived γδ T cells rapidly expand and produce IFNγ in response to nonpeptide phosphate antigens [103]. Wang et al demonstrated that human Vδ2+ cells generate IFNγ and TNFα as early as 2 hours following exposure to the live bacterial product iso-butylamine. An interesting observation in this study was that production of cytokines was cyclic and limited to periods of direct contact with live bacteria, suggesting that γδ T cells focus their resources at the infection site [104]. Vδ2+ production of IFNγ and TNFα was also confirmed by Wesch et al [105]. In contrast, Vδ2+ cells from human peripheral blood can be driven towards IL-4 production under specific culture conditions [105]. Depending on the physiologic or pathologic context, subsets of murine γδ T cells have also been shown to produce Th2 cytokines [106]. Still other studies have attributed the production of keratinocyte growth factor or connective tissue growth factor to γδ T cells, thus suggesting more specialized tissue repair functions to these cells [66]. Microarray data for human Vδ1+ cells suggests that when stimulated, these cells upregulate cytokine genes that are considered important during regulatory functions such as IL-10 and IL-11 [101, 102], but secretion of these cytokine proteins has not been confirmed. Even though Vδ1+ cells have been demonstrated in mycobacteria-induced lesions in humans [67],
specific functional roles for this subset remain unclear and this is an area that needs further investigation.

In cattle, evidence for cytokine secretion patterns by \( \gamma \delta \) T cell subsets is less clear and the data are often conflicting, which mostly likely reflects numerous experimental systems that are used. In a fetal bovine-severe combined immunodeficient (SCID-bo) xenochimeric mouse model, WC1+ cells did not produce significant IFN\( \gamma \), but were shown to be involved in recruitment of other cells to mycobacterial infection sites, though this role was incompletely characterized [107]. Depletion of WC1+ \( \gamma \delta \) T cells from \( M \) bovis-infected calves was correlated with increased antigen-specific IL-4, reduced innate IFN\( \gamma \), and reduced IgG2 antibody, which has been specifically associated with Th1 cytokines in cattle [108]. These results support the hypothesis that WC1+ cells have a role in directing the Th1 bias of the immune response. WC1+ cells from \( M \) bovis-infected calves are induced to proliferate strongly \textit{in vitro} by both ultrasonicated extracts of \( M \) bovis cultures and by crude culture filtrates of \( M \) bovis but produce minimal amounts of IFN\( \gamma \), compared to CD4+ T cells from the same animals [83]. From this study, the authors suggested that WC1+ cells represent the major population of mycobacterially-responsive bovine \( \gamma \delta \) T cells, though this role remains to be precisely defined. Vesosky et al demonstrated that while proliferation of bovine \( \gamma \delta \) T cells from healthy cattle could be induced by a variety of mycobacterial antigens, the requirements for IFN\( \gamma \) production by these cells was more stringent. Specifically, purified WC1+ cells produced significant amount of IFN\( \gamma \) in response to a non-protein component of mycobacterial cell wall antigen only when antigen-presenting cells and exogenous IL-2
were added to the cultures [78]. Though it has been suggested by many, no published studies document the production of IL-4 or other Th2-like cytokines from bovine γδ T cells, and bovine WC1+ cells are generally viewed as pro-inflammatory due to their production of IFNγ. As is true for Vδ1+ cells in humans, specific functional roles for WC1- cells of cattle have not been described. Direct response to PAMPs in the absence of APCs has been described for both human and bovine γδ T cells (discussed previously), and this is an area where further studies are necessary in order to clarify the role of these cells during the anti-mycobacterial immune response, particularly in healthy calves.

The role of γδ T cells in inducing Th1 immune responses has received the most attention; however more recently γδ T cells have been shown to also play a role during the Th17 response. Th17 responses are defined by the production of IL-17, and are thought to play a critical role in inflammatory pathology (specifically neutrophil recruitment) or autoimmune diseases and appear to be particularly important at mucosal surfaces [109]. Production of IL-17 has been demonstrated by naïve γδ T cells in mice [110], and it has been proposed that γδ T cells initiate Th17 response by up-regulation of IL-6 and IL-8, which in turn enhances neutrophil chemotaxis during early bacterial infections [111]. Sutton et al recently demonstrated that murine γδ T cells express the IL-23 receptor, the transcription factor RORγT, IL-17, IL-21 and IL-22 in response to IL-1β and IL-23 (all features of Th17 response), and that their cytokine production is independent of γδ TCR ligation [112]. This raises yet another question regarding the importance of and possible redundancy of the γδ TCR in mice. Yet another intriguing possibility for this novel γδ T cell role during the early immunopathology of
mycobacterial disease has been proposed by Yoshida et al, when they recently reported that IL-17 is essential for granuloma formation in mice [113]. IL-17 production by γδ T cells has also recently been confirmed in humans [114], but has not yet been reported in other animal model systems and should be investigated specifically in the bovine model.

**Specific effects on other cell types:** Given the patterns of antigen recognition by γδ T cells, and the fact that they reside preferentially in mucosal surfaces lined by lymphoid tissue, it seems logical to assume that γδ T cells potentially exert influence on the development of the immune response. Specific interactions between γδ T cells and other immune cells have recently been investigated. The ability of γδ T cells to innately produce IFNγ during mycobacterial infection is particularly interesting in context of mycobacterial diseases. As a major product of Th1 cells that further skews the immune response toward a Th1 phenotype, this has been postulated to be a mechanism that would account for initial Th1 phenotype priming and overall Th1 bias of the immune response [115]. It has been proposed that early IFNγ production at the site of infection could stimulate killing of bacteria by macrophages [116] and enhance antigen presentation by stimulation of infection site dendritic cells to mature and migrate to draining lymph nodes thus initiating adaptive T cell immunity [117]. The specific effects of human and bovine γδ T cells on mycobacteria-infected macrophages have briefly been explored and were previously discussed in the γδ T cell-mediated cytotoxicity section of this dissertation. The data are conflicting and this phenomenon should be further investigated, particularly in cattle. The influence of γδ T cells on DC function has been more extensively explored. The importance of the effective T cell-mediated immune response during mycobacterial
infection simply cannot be overlooked, and the development of pathogen-specific IFNγ-producing CD4+ T cells seems to be directly influenced by innate immune activation. As the primary antigen presenting cells of the innate immune system, DCs are thus considered to be primary determinants of the efficacy of the T cell mediated immune response. It is known that compared to antigen alone, the addition of exogenous IFNγ enhances the maturation of human DCs in vitro and the potency of the ensuing Th1 immune response [118]. In light of data regarding the functional capacity of γδ T cells and DCs in separate studies, the dynamics of γδ T cell-DC interactions have been more closely examined [72]. Enhanced DC maturation has been documented in vitro following activation of phosphoantigen-specific Vδ2+ [119] and CD1c-restricted Vδ1+ [120] human γδ T cell subsets. Upon γδ T cell activation, there was increased expression of CD86 on co-cultured DCs and enhanced IL-12 production by the DCs which ultimately resulted in improved priming of downstream T cell responses. It is well known that immature DCs are induced to maturation by microbial antigens interacting with cell surface Toll-like receptors. Leslie et al elegantly demonstrated that efficient Th1 polarization of naïve CD4+ T cells is induced by CD1-restricted Vδ1+ cell signals, and in contrast DCs matured by microbial stimuli alone (no Vδ1+ cells) resulted in “exhausted” DCs that were unable to induce efficient Th1 polarization [120]. These are important observations for several reasons. As previously mentioned the Vδ1+ subset of γδ T cells in humans are found predominantly in tissues and mucosal sites which makes these cells a prime candidate for affecting the adaptive immune response from the initial mycobacterial infection site. The response described by Leslie et al was found to be
partially mediated by the cytokines TNFα and IFNγ in a non antigen-specific manner [120]. Thus, unlike other innate cells which could potentially provide the initial cytokine signaling to induce maturation of DCs at infection sites, Vδ1+ cells are uniquely positioned to function in this role due to its tissue distribution. It has also been shown that fully mature DCs stimulate γδ T cells for sustained innate immune responses [119], and this interaction is thought to occur at sites of infection or in secondary lymphoid organs [117]. Other research groups have documented similar interactions of Vδ2+ cells of humans [121-123] or murine γδ T cells [124] with DCs, and this appears to involve both cytokine- and cell contact-dependent mechanisms [117]. Still another mechanism by which γδ T cells influence the adaptive immune response is when DCs acquire, process and present fragments or residual particulate antigen following lysis of infected cells or direct pathogen killing by cytotoxic γδ T cells [117].

In contrast to the many research reports investigating the interactions of human γδ T cells with DCs, studies of the cellular interactions between bovine γδ T cells and DCs are few. An early study demonstrated that Theileria annulata-infected monocytes induce bovine WC1+ cell proliferation in the presence of exogenous IL-2 [82]. In the only report to examine both γδ T cell subsets of cattle, this group also showed that neither WC1- nor WC1+ cells from cattle were stimulated to proliferate in response to allogeneic DCs [125]. These results contrast to CD4+ and CD8+ T cells from these calves, which were strongly induced to proliferate by DCs in this system. Recent work by Price et al, examined reciprocal interactions between monocyte-derived DCs and WC1+ cells from M bovis-infected calves in an in vitro co-culture system [126]. In this study it was
demonstrated that WC1+ cells upregulated surface expression of MHC class II and CD25 (IL-2 receptor) and generated significantly greater amounts of IFNγ when co-cultured with DC, and that the DCs reciprocally produced significantly greater amounts of IL-12 when co-cultured with WC1+ cells. It was also shown that IFNγ secretion by WC1+ cells in this system was abrogated when the cells were separated, indicating that this phenomenon is cell-contact dependent [126]. These results further suggest that in cattle, γδ T cells may play a role in the activation of MHC class II-restricted αβ T cells, and that γδ T cells are able to provide the initial IFNγ burst that is required for full maturation of DCs. It is important to observe that the cells used in this experiment were from M. bovis-infected animals. Thus, while these observations may help to explain enhanced protection observed following BCG vaccination of neonatal calves and humans compared to adults [127], the ability of innate γδ T cells to function in this manner in a bovine system remains unknown. The mechanisms by which γδ T cell subsets differentially mature DCs continues to be explored, and in vivo animal modeling systems for examining these interactions would be highly advantageous.

**Memory function:** The capability of primate γδ T cells to mount a memory response after microbial infection has also been demonstrated [128]. In a macaque model of tuberculosis, the characteristic features of memory T cell response were demonstrated which included antigen-specific persistence and the rapid and prolonged recall response upon re-infection. Interestingly in this study, the expansion of Vδ2+ cells in the peripheral blood compartment was associated with clearance of detectable bacteremia,
which supports the hypothesis that antigen-specific \( \gamma\delta \) T cells play an important role in overall immune competence [129].

**Other functions of \( \gamma\delta \) T cells:** Additional roles for \( \gamma\delta \) T cells have been described in a variety of experimental systems, which further challenge the paradigms regarding these unique cells. The ability of \( \gamma\delta \) T cells to directly present antigen to \( \alpha\beta \) T cells was first demonstrated in cattle [130] and pigs [131]. Evaluation of gene expression and protein expression using CTLA-4 antibody, Collins et al first demonstrated that B7 molecules were widely expressed on the surface of \( \gamma\delta \) T cells. They next showed that antigen-primed but not freshly isolated WC1+ cells directly induced significant CD4+ T cell proliferation, suggesting that this function is acquired during \( \gamma\delta \) T cell maturation, activation or differentiation [130]. Similar results have been demonstrated in human V\( \delta \)2+ and murine \( \gamma\delta \) T cells [132]. Rapid and transient expression of the lymph node homing chemokine CCR7 was demonstrated on human \( \gamma\delta \) T cells after ligation of the \( \gamma\delta \) TCR [133]. The investigators then stimulated V\( \delta \)2+ cells with the ligand isopentenyl pyrophosphate (IPP) and observed that V\( \delta \)2+ cells expressed a repertoire of antigen presentation and co-stimulatory molecules indistinguishable from lipopolysaccharide (LPS)-stimulated DCs [134]. The antigen-stimulated V\( \delta \)2+ cells were capable of processing and presenting soluble protein antigen and inducing proliferation and differentiation of autologous naïve CD4+ and CD8+ T cells, confirming the ability of these cells to acquire antigen presenting capabilities [134]. Brandes et al also demonstrated the antigen cross-presenting capabilities of human V\( \delta \)2+ cells by showing that these cells could process soluble extra-cellular microbial or tumor antigens in the
context of MHC class I molecules to effector CD8+ T cells [135]. Importantly, it was shown that the γδ T cells were more efficient at antigen cross-presentation than monocyte derived DCs, but the lack of antigen presentation by resting γδ T cells also suggests that γδ T cells cannot serve as substitutes for DCs [134]. The authors noted that it is uncertain where in the body antigen presentation by γδ T cells occurs, and they speculated that a likely location could be at the site of microbial encounter (infection site) in peripheral tissues [135]. The importance of γδ T cells among other innate lymphocyte populations is further highlighted as Wu et al recently reported that human γδ T cells but not NK cells were capable of professional antigen-presentation functions [136]. Further exploration of these functions of γδ T cells in the context of anti-mycobacterial immunity in many different animal species are necessary, and development of animal models whereby the in vivo infection site responses of γδ T cells and their influence on development of the immune response would be advantageous.

**Modeling systems to study mycobacterial infections**

In the previous discussion of the host immune response to mycobacterial pathogens, several key areas regarding γδ T cell immunobiology were identified that need further attention. Appropriate selection of animal models used to study various features of immunobiology or pathology should be carefully made. In the case of γδ T cells, interspecies differences clearly exist between humans and various animal models; however significant similarities between humans and cattle warrant further evaluation of this
particular animal model. Limitations such as difficulty obtaining appropriate tissues exist for many species, thus the study of human and cattle γδ T cell biology has been thus far largely limited to in vitro evaluation of peripheral blood-derived γδ T cells and the lack of studies evaluating other γδ T cells subsets (Vδ1+ in humans, WC1- in cattle) is reflected in the literature. This could represent a significant knowledge gap, because in both species, the minor γδ T cell subsets have been shown to have powerful local and downstream effector functions. Thus, there is a pressing need for animal modeling systems which allow not only evaluation of multiple tissue-specific γδ T cell subsets, but also the ability to examine these cells in the context of infection site-specific immunopathology. Unlike mice, cattle display a remarkable similarity with humans regarding immunopathology of mycobacterial diseases [137], and this coupled with readily-available γδ T cells make young calves an excellent model choice to study the pathogenesis of mycobacterial disease.

One of the most significant challenges associated with study of Map infection in cattle is the evolving immunopathologic events in infected individuals that transition in vivo from early infection into a lengthy subclinical infection and eventually in some animals to severe chronic progressive wasting and eventual death of the host. To address these significant knowledge gaps, there has recently been significant interest in the development of appropriate models to study the pathogenesis of early Map infection in cattle [10, 138]. The host immune response to Map infection is typically studied locally in ileal tissue or systemically using peripheral blood cells during the late subclinical to clinical stages of Map infection [4, 139, 140], but few studies have evaluated the local
response during early or subclinical infection [13, 14]. These studies have revealed significant information about the immunopathology at the *Map* infection site, but more studies are needed.

Because the ultimate progression of mycobacterial disease is strongly influenced or determined by the events occurring at the level of the infection site [86], and recognizing the need for models of *Map* infection, we set out to design a series of studies to evaluate γδ T cell subset responses at *Map* infection sites which would ultimately lead to expanding our understanding of how these cells influence and shape the subsequent local and systemic immuno-pathologic response to *Map* infection, and how this may correlate to clearance, containment or progression of this disease in cattle.
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Table 1. Features of major subsets of human and bovine γδ T cells

<table>
<thead>
<tr>
<th>Tissue Distribution</th>
<th>Human Vδ1+</th>
<th>Bovine WC1-</th>
<th>Human Vδ2+</th>
<th>Bovine WC1+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue Distribution</td>
<td>most in spleen, epithelial tissues</td>
<td>most in spleen, epithelial tissues</td>
<td>most in peripheral blood</td>
<td>most in peripheral blood</td>
</tr>
<tr>
<td>CD3</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
<td>positive</td>
</tr>
<tr>
<td>WC1</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>positive</td>
</tr>
<tr>
<td>CD2</td>
<td>unknown</td>
<td>most positive</td>
<td>positive</td>
<td>most negative</td>
</tr>
<tr>
<td>CD4</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
<td>negative</td>
</tr>
<tr>
<td>CD8</td>
<td>most negative</td>
<td>50% of splenic cells are CD8-</td>
<td>most negative</td>
<td>most negative</td>
</tr>
<tr>
<td>Ligands</td>
<td>microbial lipids or glycolipids?</td>
<td>unknown</td>
<td>protein, non-protein antigens, phosphoantigens, alkylamines, stress-induced molecules, PAMPs</td>
<td>protein, non-protein antigen of <em>M. bovis</em></td>
</tr>
<tr>
<td>Known biological functions</td>
<td>expand in blood during HIV, herpes and malaria infection</td>
<td>migrate to early inflammation sites</td>
<td>expand in blood during <em>M. tuberculosis</em> infection</td>
<td>expand in blood during <em>M. bovis</em> infection</td>
</tr>
<tr>
<td>Cytokines produced</td>
<td>IL10, IL11 (gene-expression only)</td>
<td>unknown</td>
<td>IFNγ, TNFα, IL17, CCR7, IL4</td>
<td>IFNγ</td>
</tr>
<tr>
<td>Effects on DCs</td>
<td>induce maturation</td>
<td>unknown</td>
<td>induce maturation</td>
<td>induce maturation</td>
</tr>
<tr>
<td>Effects on other T cells</td>
<td>unknown</td>
<td>unknown</td>
<td>present antigen to CD4+ and CD8+ cells</td>
<td>present antigen to CD4+ cells</td>
</tr>
<tr>
<td>Response to Mycobacteria</td>
<td><em>M. leprae</em></td>
<td>unknown</td>
<td><em>M. bovis, M. tuberculosis</em></td>
<td><em>M. bovis, M. avium paratuberculosis</em></td>
</tr>
</tbody>
</table>
CHAPTER 2: GAMMA-DELTA (γδ) T CELL SUBSETS ARE DIFFERENTIALLY ASSOCIATED WITH GRANULOMA DEVELOPMENT AND ORGANIZATION IN A BOVINE MODEL OF MYCOBACTERIAL DISEASE

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Abstract

The characteristic lesion in bovine tuberculosis is well-organized respiratory granulomas. This is typically associated with a strong T-helper 1 (Th1) biased cell-mediated immune response and eventual containment of the infection. In bovine paratuberculosis, the classic lesion is unorganized granulomatous intestinal inflammation. Clinical paratuberculosis is associated with a T-helper 2 (Th2) biased humoral immune response and eventual death due to inability of the host to contain the infection. Recent reports have suggested that gamma-delta (γδ) T cells play a significant role in granuloma development and/or maintenance during initial stages of infection and may influence the
subsequent adaptive immune response. The objective of this study was to use an *in vivo* bovine model to evaluate $\gamma\delta$ T cells during the early host immune response to mycobacterial infection. We used immunofluorescent staining, hyperspectral microscopy, and computerized assisted morphometry to evaluate staining and distribution of $\gamma\delta$ T cells during development of organized and unorganized granulomas. Our data suggests that bovine $\gamma\delta$ T cell subsets are differentially recruited to early infection sites, and may be instrumental during the initial anti-mycobacterial host immune response as well as for granuloma organization.

**Key words:** bovine, gamma-delta T cells, granuloma, mycobacteria, paratuberculosis

**Introduction**

The role of major T lymphocyte subsets is well documented in both tuberculous and non-tuberculous mycobacterial disease [1, 2]; however, recent studies in humans [3, 4], non-human primates [5] and cattle [6-9] have provided evidence that minor T cell populations including $\gamma\delta$ T cells are also involved. It is thought that $\gamma\delta$ T cells play an early role during infection and provide a link between the innate and adaptive immune responses [10, 11]. It is known that $\gamma\delta$ T cells differ widely between species and interspecies correlation of the data has not been reported; however, human and bovine $\gamma\delta$ T cells share some notable features including subset specific tissue distribution [12, 13] and proposed function [14, 15]. Human $\gamma\delta$ T cell subsets are distinguished by germ-line gene segment usage and are thought to represent a heterogeneous population of cells
capable of serving immediate effector, tissue homing, recall or even memory functions [3, 16]. Bovine γδ T cell subsets are differentiated by a unique workshop cluster 1 (WC1) molecule which is a member of the scavenger receptor cysteine rich family and is expressed on the surface of 50-90% of peripheral blood γδ T cells [17]. Distinct isoforms of the WC1 molecule have been characterized [18-20], yet its exact function remains unknown. Work by Jutila and colleagues has suggested that bovine WC1+ γδ T cells have inflammatory characteristics while WC1- cells have a regulatory function [21, 22]. A role for γδ T cells during antimycobacterial immunity is implied as WC1+ γδ T cells from either infected or uninfected cattle proliferate and produce interferon (IFN)-γ when stimulated with M. bovis antigens [23, 24]. The importance of WC1+ γδ T cells is further supported by studies showing modulation of the immune response in calves depleted of WC1+ γδ T cells [25]. Evidence that γδ T cells play a significant role at initial infection sites is less clear, but these cells are observed within mycobacteria-induced lesions of humans and cattle [16, 26, 27] and in lymph nodes draining mycobacterial infection sites of cattle [9, 28, 29]. A role for γδ T cells during granuloma development in mice or as early effectors during mycobacterial infection of humans and macaques is supported by recent reports [5, 30-32].

It is generally accepted that progression of mycobacterial disease is influenced at the level of the infection site and that survival of the host depends on the ability to limit mycobacterial proliferation through effective granuloma formation [33]. Well organized lesions correlate with longer host survival while unorganized lesions are associated with a poorer prognosis, and this suggests that the local host tissue response is indicative of a
The patient’s likely response to therapy and long-term survival [2, 34, 35]. A variety of in vivo and in vitro animal model systems have been utilized to further characterize the complex immunopathology of the infection site with variable success. The murine model has limitations for understanding granuloma development and progression in response to mycobacterial infection [33]. The bovine is an attractive alternative model system [36], and cattle are natural hosts of tuberculous and non-tuberculous mycobacterial infections.

The goal of the current study was to evaluate γδ T cell subsets in mycobacterial granulomas to better understand the early role of these cells in host defense against infection. A bovine subcutaneous model was employed to address two fundamental questions concerning the relationship of γδ T cells to the developing granuloma during mycobacterial infection. First, we sought to identify differences in γδ T cell recruitment into initial stages of organized vs. unorganized granulomas. Second, we asked if the ability of an animal to initially generate an organized granuloma to Mycobacterium avium subspecies paratuberculosis (Map) antigens (killed bacterin vaccine) would translate into the development of an organized granuloma at a subsequent focus of Map infection. Our data support the initial hypothesis that γδ T cell subsets are differentially associated with organized and unorganized granulomatous lesions in the bovine. Data from these experiments suggest that bovine γδ T cell subsets have different immune roles, and may be central regulators of both local and systemic antimycobacterial immune responses.

Materials and methods

Animals and care. Four- to six-week-old castrated male Holstein calves were used in the following experiments and were housed in isolation at the Iowa State University
College of Veterinary Medicine biosafety level II animal care facility. A total of nine calves were used for this project and were maintained three at a time for handling purposes. Animals were acquired from the Iowa State University dairy research farm (Ankeny, IA), a herd certified free of Map infection. All live animal-related protocols were approved by the Committee on Animal Care and Use at Iowa State University.

**Vaccine.** *M. avium* subspecies *paratuberculosis* bacterin (Mycopar®, Fort Dodge Animal Health, Fort Dodge, IA) was used in this experiment. Mycopar is a commercially available whole-cell bacterin containing inactivated *Mycobacterium paratuberculosis* in oil. Mycopar is known to generate a variably sized granuloma *in vivo*, and as such was used in these experiments as an example of a highly organized (tuberculoid-like) granuloma allowable in biosafety level II facilities.

**Bacterial inoculum and infection.** The Map strain 19698 was obtained from the American Type Culture Collection (Manassas, VA) and maintained in Middlebrook 7H9 broth supplemented with mycobactin J. Logarithmic growth-phase bacteria were washed and resuspended in sterile saline for inoculation. Bacterial concentration was determined by measuring absorbance at 540nm, comparing the absorbance optical density to the standard curve, and adjusted to a final concentration of 1 X 10^9 CFU/mL in 0.9% sterile saline solution. The Map inoculum used in these studies was shown to have greater than 90% viability via fluorescein diacetate (FDA) staining and flow cytometry analysis prior to inoculation. In addition, challenge inocula were confirmed negative for contaminants by streaking onto sheep blood agar plates 24 hours prior to injection.

**Monoclonal antibodies and antigens.** The following mouse anti-bovine monoclonal antibodies were purchased from VMRD (Pullman, WA) and used to characterize γδ T
cells by multi-color fluorescent immunohistochemistry: anti-γδ TCR (GB21A), anti-WC1(n1) (B7A1). Alexa-fluor 555-conjugated goat anti-mouse IgG2b and Alexa-fluor 488-conjugated goat anti-mouse IgM (Invitrogen Molecular Probes, Carlsbad CA) were used as secondary antibody conjugates. DAPI nucleic acid stain (Invitrogen Molecular Probes) was used to stain nuclei.

**Experimental Design.** For these experiments, a localized *Map* infection system developed in our laboratory was used with a few modifications [37, 38]. Calves were inoculated on day 0 of the experiment with either *Map* vaccine (n=6) or sterile saline (n=3) in the following manner: Two hundred fifty (250) μL was administered subcutaneously in three locations separated by 8-10cm in the left cervical region. A single vaccine injection site nodule was surgically removed from each calf at three post-inoculation time points designated early (7-10 days), middle (30 days) and late (greater than 60 days).

Calves were then inoculated with live *Map* sixty days following Mycopar or sham saline vaccination. Each calf was subcutaneously inoculated with 5 X 10^8 live *Map* in 500μL sterile saline in three locations separated by 8-10cm in the right cervical region. A *Map* infection site nodule was surgically removed from each calf at three post-inoculation time points designated early (7-10 days), middle (30 days) and late (greater than 60 days). All tissues were fixed for histopathology in 10% neutral-buffered formalin or snap-frozen for immunofluorescence in liquid Tissue-Tek Cryo-OCT compound (Fisher Scientific, Pittsburgh, PA) and stored at -80°C.

**Scoring of hematoxylin and eosin stained lesions.** Formalin-fixed, paraffin-embedded vaccine and infection site lesions were sectioned (4μm) and stained with
hematoxylin and eosin (HE) for histologic evaluation and lesion scoring. HE stained vaccine and infection site lesions were individually scored using a scoring system adapted from previous studies [9, 37, 39]. Scores of 0-3 were assigned for each parameter to reflect the degree and organization within each lesion. A score of 0 indicates absence of the parameter from the lesion (normal), while a score of 3 reflects both a significant presence (most severe) and a high degree of organization of the parameter within the lesion. The following parameters were evaluated: fibrous connective tissue, macrophages, polymorphonuclear cells (neutrophils and eosinophils), small mononuclear cells (lymphocytes and plasma cells), multinucleated giant cells, necrosis, and mineralization. Individual parameter scores were summated, and a single collective score was assigned for each lesion which reflected the histological organization and stage of development of each lesion. Negative control sites (saline) lacked significant inflammatory components.

**Immunofluorescent tissue staining.** OCT frozen sections were cut at 4μm and fixed in 100% ice-cold ethanol. Sections were washed once with phosphate-buffered saline (0.01 M PBS, pH 7.2), blocked for 30 minutes with 10% normal goat serum, then stained at 4°C overnight with a cocktail containing monoclonal antibodies for γδ TCR (1/10000) and WC1 (1/1000). Slides were then washed twice with sterile PBS and stained for 45 minutes at 20°C sequentially with appropriate secondary conjugates (AF555 goat anti-mouse IgG2b and AF488 goat anti-mouse IgM, 1/1000), prior to nuclei staining with DAPI (1/30000). Following staining, slides were washed twice with sterile PBS, then mounted with anti-fade medium (4% n-propyl gallate (Sigma Aldrich, St. Louis, MO) in 90% glycerol and 10% PBS) and cover-slipped.
Immunofluorescent hyperspectral microscopy. Fluorescence image data was collected using an Optical Insights hyperspectral microscopy system composed of the Optical Insights (MAG Biosystems, Tucson, AZ) instrument attached to an inverted Nikon TE2000 microscope (Nikon, Melville, NY) equipped with a Photometrics Cascade 512B digital camera and Melange® software (Molecular Devices, Sunnyvale, CA). All images were captured at 200X magnification (20X objective). Fluorescence data were first collected from single-color (AF488, AF555 or DAPI) stained slides, and individual fluorescent spectra were defined. Sample slides stained with all three fluorophores were then imaged. Raw data was corrected for tissue autofluorescence, and hyperspectral unmixing was performed to extract individual spectra based on the previously defined parameters for each fluorescent spectra.

Computer-assisted morphometric analysis of immunofluorescent stained slides. Image analysis was performed using Metamorph 7.0 software (Molecular Devices). Briefly, multicolor images were separated based on their unmixed and corrected spectral profiles. A visual threshold was applied to individual spectral profiles, and object definition was defined in pixels based on the scorer’s (BLP) visual interpretation of a single positive cell. Data from individual images was then collected and stored as the following parameters: total cell count (DAPI positive nuclei per image), total γδ TCR positive cells (AF555-expressing cells) and total WC1 positive cells (AF488-expressing cells per image).

Statistical analysis. Statistical analysis was performed using JMP 7.0.2 Statistical Discovery system (SAS Institute, Cary, NC). Data are presented as the mean value ± standard error of the mean except where stated otherwise. Student’s t-test and one-way
analysis of variance were used for the statistical analysis. Group mean differences were considered significant if the p value was <0.05.

Results

**Differential histopathology of subcutaneous granulomas.** To generate unorganized and organized granulomas we inoculated either live *Map* organisms or *Map* vaccine subcutaneously in calves. *Map* inoculation site nodules reached a maximal size of 20-25mm diameter approximately 7-10 days post injection and gradually resolved. *Map* vaccine site nodules reached a maximal size of 30-50mm diameter approximately 20-30 days post injection and generally remained grossly unchanged in size for the study duration (data not shown). Histopathology observed at *Map* inoculation sites was similar to intestinal lesions of cattle with naturally occurring cases of *Map* infection (Johne’s disease) and were characterized by an unorganized accumulation of macrophages with fewer lymphocytes and plasma cells. The lesions did not exhibit significant histologic progression over time (Fig. 1A), and at later stages diffuse granulomatous lesions lacked fibrous tissue encapsulation, central necrosis and mineralization (Fig. 1B). Initial lesions at *Map* vaccine sites were similar to infection sites; however in contrast to *Map* infection sites, they became highly organized over time (Fig. 1A, *p<0.0023*). Features of later stage granulomas included central coagulative to lytic cellular necrosis with variable degrees of mineralization surrounded by a dense rim of macrophages, giant cells and aggregates of lymphocytes and plasma cells. The entire nodular structure was eventually rimmed by a band of mature dense fibrous tissue (Fig. 1C).
\(\gamma\delta\) T cell subsets in unorganized and highly organized granulomas. We compared \(\gamma\delta\) T cell subsets during developing stages of highly organized and unorganized granulomatous lesions using immunofluorescence staining and computer assisted morphometry. In unorganized lesions, the total number of recruited \(\gamma\delta\) T cells was significantly greater in late stages compared to early stages (Fig. 2A, \(*p=0.0002\)). Compared to unorganized lesions, highly organized granulomas contained significantly higher numbers of WC1+ cells during initial stages (Fig. 2B, \(*p=0.0062\)) and WC1- cells during later stages (Fig. 2C, **\(p=0.0017\)).

Spatial organization of \(\gamma\delta\) T cell subsets in granulomas. We evaluated spatial orientation and arrangement of \(\gamma\delta\) T cell subsets within developing granuloma subtypes. Early stages of all lesions lacked significant histologic organization, and both \(\gamma\delta\) subsets were distributed evenly and diffusely throughout these sections. While the distribution of \(\gamma\delta\) T cell subsets remained diffuse over time in unorganized lesions, a distinct subset-specific stratification pattern was observed as granulomas exhibited progressive histologic organization. Specifically, the WC1+ subset was restricted to the outer margins of the granuloma near the fibrous border (Fig. 3A-D) while WC1- cells were heavily concentrated near central regions of the granuloma adjacent to the area of central cavitation and necrosis (Fig. 3A, E-G).

Histology of Map infection sites in naïve and vaccinated calves. We next set out to answer the question “if calves initially produce a well-organized granuloma to Map vaccine, will they subsequently generate a highly organized granuloma to Map infection at a distant subcutaneous site?” We compared the histology of early and late Map
infection sites in naïve calves to those in previously vaccinated calves. Regardless of the vaccination history of the animals, granulomas at *Map* infection sites did not achieve the same level of organization as the vaccine sites (Fig 1A). Nevertheless, we did find that at later time points, *Map* infection sites in vaccinated calves had significantly higher histologic scores compared to *Map* infection sites in naïve calves (Figs. 4A, *p=0.0449*).

We examined the lesions to determine which parameter(s) of our scoring rubric (macrophages, lymphocytes, giant cells, fibrous tissue, necrosis and mineralization) accounted for differences observed. Late stage *Map* infection sites of vaccinated calves contained prominent large Langhans-type multinucleated giant cells (Fig. 4B arrowheads), mildly increased fibrosis (Fig. 4B, arrow) and random small foci of necrosis. These parameters were not significant morphologic features of *Map* infection sites at any time point in naïve animals.

**γδ T cell subsets in Map infection sites of naïve and vaccinated calves.**

Considering the effect of vaccination on *Map* granuloma morphology we were interested to know if vaccination influenced recruitment of γδ T cell subsets to *Map* infection sites. We found the percent of γδ T cells to be significantly greater in late stage lesions compared to early lesions in naïve calves (Fig 5A, *p=0.0043*) but not in vaccinates. When compared to vaccinated calves, the WC1- subset was more heavily recruited to early *Map* infection sites of naïve calves (Fig. 5C, *p<0.05*), but the percent of WC1-cells decreased over time. Though the WC1+ subset represented the minority of γδ T cells at early infection sites in naïve calves, the percent of WC1+ cells increased significantly by the late stages of the infection sites (Fig. 5B, **p<0.05**).
Discussion

Initial cellular interactions at mycobacterial infection sites are considered critical for generating effective and sustained protective immunity [40], and an immunoregulatory role for $\gamma \delta$ T lymphocytes in this process in humans and cattle has been proposed [41-43]. Infection site immunopathology is difficult to investigate, and low numbers of $\gamma \delta$ T cells in most species is an additional challenge to overcome for $\gamma \delta$ T cell studies. As a result, $\gamma \delta$ T cell studies have largely been limited to in vitro work using peripheral blood $\gamma \delta$ T cells (mostly WC1+ in cattle and V$\delta$2 in humans) and have not examined $\gamma \delta$ T cell subsets found in lower numbers in peripheral blood (bovine WC1- and human V$\delta$1). For example, bovine $\gamma \delta$ T cells have been examined within mycobacterial granulomas, but these studies examined only WC1+ $\gamma \delta$ T cells within granulomas of lymph nodes draining infection sites [9, 28, 29]. We thus set out to use an in vivo system to build upon the knowledge gained from in vitro (peripheral blood) experiments. Our study is unique because it describes the in vivo recruitment of both WC1+ and WC1- $\gamma \delta$ T cell subsets to mycobacterial infection sites in cattle with correlation to the morphology of developing granulomas. Using this model, we have previously described the histology of granulomas during generation and maintenance stages at subcutaneous infection sites by inducing granulomas on near opposite ends of the morphologic spectrum. Specifically, Map vaccine induced well-organized granulomas resembling tuberculoid type while live Map induced unorganized granulomas resembling lepromatous type [37, 38]. Further, we have shown a diminished phenotypic and functional maturation status of dendritic cell populations harvested from unorganized granulomas compared with those of well
organized granulomas [37]. It has been shown that human γδ T cells are able to enhance dendritic cell maturation and thus effect initiation of the adaptive immune response [44-46], though γδ T cell-dendritic cell interaction has not been fully investigated in the bovine.

In the present study, we have identified unique recruitment patterns of γδ T cell subsets that correlate to the degree of granuloma organization and provide evidence to suggest that previous antigen exposure influences recruitment of γδ T cells to an infection site. Lesions that progressed to highly organized granulomas in our study contained significantly more WC1+ γδ T cells at initial stages and significantly more WC1- γδ T cells during later stages. In contrast, lesions without progressive organization during the study demonstrated an inverse recruitment pattern of γδ T cells where WC1- cells predominated initially and WC1+ cells were found at elevated numbers at later stages. Progressive organization of the granuloma correlated to a distinct stratification pattern of γδ T cell subsets where WC1+ cells localized to marginal (peripheral) regions and WC1- cells accumulated adjacent to necrotic (central) regions. Finally, we observed that WC1- γδ T cells are transiently and preferentially recruited to early mycobacterial infection sites in naïve calves but not vaccinated calves. To our knowledge this has not been previously described, but the observation is interesting as one feature of the tissue subset of human γδ T cells (Vδ1 cells compared to Vδ2 cells) is that they also seem to be preferentially recruited to local mycobacterial or viral infections [26, 47].

These findings suggest that subsets of γδ T cells play unique roles during the anti-mycobacterial immune response. Specifically, WC1- γδ T cells appear to have an
enhanced ability to respond innately to live mycobacteria while WC1+ cells are best recruited after antigenic priming and we hypothesize that WC1+ cells function as early effector or memory cells during the immune response. A shift in the predominance of WC1+ to WC1- cells over time correlates to the transition from unorganized to a highly organized granuloma morphology. We believe this finding supports the hypothesis that \( \gamma\delta \) T cell subsets play a role in granuloma organization and/or maintenance during pathogenesis of mycobacterial disease, though further studies are needed for confirmation.

The ability to form organized granulomas to vaccine did not translate to an ability to generate highly organized granulomas to subsequent Map challenge. However, a significant difference in morphology of granulomas induced at Map infection sites in vaccinates but not naïve calves was the prominence of Langhans-type multinucleated giant cells. Giant cells are observed in a number of neoplastic or chronic inflammatory conditions of humans and animals, though few studies have examined inflammatory giant cells during mycobacterial infections [48, 49]. A recent in vitro study reported that human macrophages responding to virulent mycobacteria (\( M \) tuberculosi) formed larger multinucleated cells with reduced phagocytic ability and elevated antigen presentation capacity compared to macrophages responding to avirulent mycobacteria (\( M \) avium). The authors noted that these giant cells resembled mature dendritic cells as a population of cells seemingly well-suited for enhanced antigen presentation and hypothesized an association with a stronger local host immune response [48]. The presence of Langhans-type giant cells within Map vaccination sites and live Map infection sites of vaccinated but not naïve calves in our study is in vivo evidence to support the hypothesis that
Langhans-type giant cells are indicative of the strength and possibly effectiveness of the local immune response, though to our knowledge this effect has not been definitively shown in vivo during the developing mycobacteria-induced lesions in a species other than bovine.

We recognize that anatomic and immunologic variables existing between our model (skin) and the natural infection site (intestine) may alter the host immune response at these locations. In addition, we know that vaccine adjuvant further influences the local immune response, and thus we do not attribute the final consequences of the infection site granuloma in our model to any one component of the host immune response described in this work. However, we believe this study demonstrates important features of γδ T cell recruitment within unorganized and highly organized granulomas at sites of mycobacterial-induced inflammation. We are now working toward understanding the functions of these γδ subsets and how they influence the induction of either protective or non-protective immune responses to mycobacterial diseases.
References


Figure 1 Histology of unorganized and highly organized granulomas. Panel (A) summarizes lesion scores for infection sites (gray bars) and vaccination sites (black bars). Late stage vaccine sites scored significantly higher than all other sites (*p<0.0023). Late stage live *Map* infection sites (B) were unorganized sheets of macrophages and lymphocytes without central necrosis or peripheral fibrosis. Late stage vaccine sites (C) were highly organized with macrophages and lymphocytes oriented around central lakes of necrotic debris (arrows) with mineral. Granulomas were encircled by a prominent fibrous capsule (arrowheads). Bar=200μm.
Figure 2 γδ T cell subsets in poorly organizing and highly organizing granulomas.

In panel A, the percent of γδ T cells recruited to unorganized lesions was higher in late lesions compared to early lesions (*p=0.0002). Panels B and C show that in comparison to unorganized sites, lesions that became highly organized were dominated initially by WC1+ γδ T cells (*p=0.0062) and during later stages by WC1- γδ T cells (**p=0.0017).
Figure 3 Spatial organization of γδ T cell subsets by immunofluorescent staining.

Sections from highly organized granulomas were examined for γδ T cell subset organization revealed distinct stratification patterns. Sections were taken from near the fibrous margin (panels B-D) or from near the necrotic center (panels E-G) as indicated by
the boxes in panel 3A. The $\gamma\delta$ TCR is labeled in red (panels B and E), WC1 is labeled in green (panels C and F) and nuclei are labeled blue in the overlay images (panels D and G). WC1+ $\gamma\delta$ T cells (red and green overlay) are limited to outer margins along the fibrous capsule while the lesion centers contain almost predominantly WC1- $\gamma\delta$ T cells (single red staining). Bar = 200$\mu$m
Figure 4 Histology of *Map* infection sites in naïve and vaccinated calves. Panel (A) shows lesion score summary; late stage lesions scored significantly higher in vaccinates compared to naïve calves (*p*=0.0449). Late stage *Map* infection site lesions in naïve calves were characterized by diffuse granulomatous inflammation (see Fig 1B) while *Map* infection sites in previously vaccinated calves (panel B) scored higher due to increased fibrous tissue (arrow) and prominent large multinucleated Langhans-type giant cells (arrow heads). Bar=100µm
Figure 5 γδ T cell subsets in *Map* infection sites of naïve and vaccinated calves. The percent of γδ T cells was significantly higher in late lesions compared to early lesions in naïve calves (panel A, *p=0.0043) but not vaccinates. In panels B and C, WC1-γδ T cells were recruited more heavily to infection sites of naïve calves compared to vaccinates (*p<0.05) and the number of WC1+ γδ T cells significantly increased over time in infection sites of naïve calves but not in vaccinates (**p<0.05).
CHAPTER 3: EVALUATION OF THE INITIAL HOST IMMUNE RESPONSE TO MYCOBACTERIUM AVIUM SUBSPECIES PARATUBERCULOSIS INFECTION IN SENSITIZED OR NAÏVE CALVES USING MATRIX BIOPOLYMERS

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Abstract

We have developed a model to explore the early immune response to Mycobacterium avium subspecies paratuberculosis (Map) in the bovine calf using a gel matrix biopolymer. This system utilized subcutaneous placement of a liquid biopolymer containing live Map that rapidly polymerizes and retains recruited cellular infiltrates and soluble mediators. After 48 hours the matrix is removed and recruited cells and cytokines can be measured by histopathology, multi-parameter flow cytometry, enzyme-linked immunosorbent assay (ELISA), and Luminex bead-based immunoassays. A focus of this study was to evaluate recruitment patterns of gamma-delta (γδ) and CD4 T cell subsets to the mycobacterial infection site, local cytokine generation, and to ultimately determine how these cells might influence the development of the adaptive immune response. Our
results show distinct cellular and cytokine profiles in the gels recovered from animals that are naïve versus calves previously sensitized to *Map* antigen. In addition, our data supports our hypothesis that bovine γδ T cell subsets are differentially recruited to early mycobacterial infection sites with respect to prior exposure of the host to *Map* antigens. We also found that different bovine γδ T cell subsets display distinct patterns of cytokine secretion. Our results provide unique insight into initial host responses at mycobacterial infection sites that may promote novel diagnostic strategies.

**Key words:** bovine, γδ T cells, interferon γ, mycobacteria, paratuberculosis

**Introduction**

The host immunologic response during *Map* infection in cattle is not completely understood. Transmission of *Map* occurs most commonly via the fecal-oral route when young calves ingest an infectious dose of live *Map* shed in the feces of infected herdmates. An initial T helper type 1 (Th1) immune response is mediated by IFNγ secreting CD4+ T cells. A lengthy subclinical period ranging from months to several years follows, during which time there is generally absence of clinical signs, histologic or gross lesions, bacterial shedding and definitive cellular or humoral evidence of an individual’s infection status [1, 2]. Development of clinical disease is generally associated with a loss of CD4+ T cell-mediated immunity, development of T helper type 2 (Th2) immune response characterized by high levels of *Map*-specific antibody along with development of granulomatous intestinal lesions, clinical signs of wasting, diarrhea and eventual death of the host [3]. One of the significant challenges to studying this
disease in cattle is effectively modeling the host immunologic responses to Map infection within the context of each disease stage over the course of transition from initial Map infection to clinical disease. Specifically, the inability to identify Map-infected animals during early stages of disease [4] has significantly impeded examination of the early host response to infection. Review of the current literature reveals these scientific knowledge gaps and emphasizes the fact that appropriate models of disease are necessary at all stages of Map infection in order to fully elucidate the evolution of this disease [5] and potentially enhance the development of future novel diagnostic strategies.

Our research group is interested in understanding how the initial immune response to Map infection influences development of the subsequent adaptive response and disease outcome. In particular, we are interested in the role that γδ T cells may play during this process. It is well documented that young calves have large numbers of γδ T cells in the peripheral blood compartment, though specific functions of these cells have not yet been clearly defined [6]. Additional details of roles for γδ T cells in many species continue to emerge, and though some functions are shared across species, distinctions are also apparent. These cells have been shown to be important effectors or regulators of the immune response during mycobacterial or other infections of mucosal sites of mice [7-9], humans [10, 11] and cattle [12, 13]. More specifically in cattle, γδ T cells have been demonstrated within mycobacterial-induced lesions [14] and within lymph nodes draining mycobacterial infection sites [15-17].

Distinct subsets of bovine γδ T cells have been described [18, 19], and these subsets are largely defined by their expression of the unique workshop cluster 1 molecule (WC1) as well as CD2 and CD8 expression [20]. Unique roles of both WC1+ [12, 21-25] and...
WC1- [26, 27] subsets have been reported, but their functional capacity has not been completely characterized. In light of these observations, we have previously utilized the subcutaneous model to investigate the host immunopathologic response to Map infection [28-30]. Though not a natural route of infection or tissue target, diffuse granulomatous lesions induced by subcutaneous Map injection share characteristics of the small intestinal lesions observed during early clinical Map infection of cattle. An advantage of this model is that following subcutaneous inoculation, surgically-accessible subcutaneous lesions develop rapidly, tend to persist and can thus be monitored over time. We have previously demonstrated differential recruitment patterns of γδ T cell subsets into the mycobacterial infection sites of young calves [30], leading us to hypothesize that these subsets had distinct functional roles at the infection site.

Based on the observations from our previous work, one of the goals of this study was to expand the subcutaneous model such that we were able to collect immune cells recruited to Map infection sites from live calves for dynamic laboratory analysis, adding to our image-based studies within developing granulomas over time. We adapted a technique developed by Russell et al [31], which utilizes Matrigel matrix biopolymer as a medium to facilitate collection and extraction of the cells recruited to Map infection sites. Utilizing this mechanism, we were able to address a second goal of this study, which was to investigate the differential functional capacity of bovine γδ T cell subsets recruited to Map infection sites over time. Results from these experiments are further evidence to support the hypothesis that bovine γδ T cell subsets have distinct roles during antimycobacterial immunity, and they may be key regulators of both local and systemic immunologic responses. Finally, further characterization of these distinct roles may lead
to development of translational endeavors including enhancement of novel diagnostic testing strategies.

Materials and methods

Animals and experimental design. Twelve (12) five to six week old castrated male Holstein calves were acquired from the Iowa State University dairy research farm (Ames, IA), a herd certified free of *Map* infection. Calves were individually housed in biosafety level II animal care facility during the study and were maintained four at a time for logistical purposes. All animal protocols were approved prior to the study by the Committee on Animal Care and Use at Iowa State University. Animals were divided randomly into the following groups: noninfected/naive (n=4), *Map* infected (n=4) and *Map* vaccinated (n=4). Animals were administered live *Map* (1mL in sterile 0.9% isotonic saline), *Map* vaccine (0.5mL Mycopar®, Fort Dodge Animal Health Company, Fort Dodge IA) or sham saline (1 mL sterile 0.9% isotonic saline) in the subcutaneous space of the left cervical region on post infection day (PID) 0. In order to evaluate the systemic immune response development at *Map* infection sites, calves were subsequently challenged (PID 7, PID 15 and PID 30) with live *Map*, *Map* antigen (whole cell sonicate, WCS) or saline in BD Matrigel™ in the subcutaneous space (right cervical or paralumbar fossa). These Matrigel challenge sites were harvested approximately 48 hours after injection either surgically (PID 7 and PID 15) or during necropsy (PID 30).

Vaccine. *M. avium* subspecies *paratuberculosis* bacterin (Mycopar®, Fort Dodge Animal Health Company, Fort Dodge IA) was used in this experiment. Mycopar® is a whole-cell bacterin containing inactivated *Mycobacterium paratuberculosis* in oil.
**Generation of *Map* antigen (whole cell sonicate, WCS).** Late growth phase live *Map* K10 bacteria suspended in approximately 250 mL Middlebrook 7H9 broth supplemented with mycobactin J were collected from culture stock in our laboratory. Bacteria were pelleted by centrifugation at 3500 G for 20 minutes, washed twice with cold phosphate buffered saline (PBS) and pooled into approximately 10 mL cold PBS and sonicated on ice with a probe sonicator. Sonication consisted of three cycles of 10 minute bursts (18 watts) with 10 minute chilling periods between sonication steps. Debris is removed by centrifugation at 12000 G for 5 minutes and supernatants were then harvested and stored at -20°C. Protein concentration was determined using the Pierce BCA protein assay (Pierce Biotechnology, Rockford, IL). Approximately 250µL whole cell sonicate (WCS) was streaked onto blood agar and incubated for 24 hours at 37°C to check for contamination prior to use.

**Bacterial inoculum, infection and challenge.** The *Map* strain K10 was obtained from the National Animal Disease Center (Ames, IA) and maintained in Middlebrook 7H9 broth supplemented with mycobactin J. Logarithmic growth-phase bacteria were washed and resuspended in sterile saline for inoculation or challenge. The *Map* inoculum used in these studies was shown to have greater than 90% viability via fluorescein diacetate (FDA) staining and flow cytometry analysis prior to inoculation. In addition, challenge inocula were confirmed negative for contaminants by streaking onto sheep blood agar plates 24 hours prior to injection. Bacterial concentration was determined by measuring absorbance at 540 nm, comparing the absorbance optical density to the standard curve, and adjusted to appropriate final concentration. For initial infection, bacteria were diluted to a final concentration of 1 X 10⁹ CFU/mL in 0.9% isotonic saline.
solution. For challenge, bacteria were diluted to a final concentration of $1 \times 10^6$ CFU/mL in 0.9% isotonic sterile saline.

**Matrigel™.** BD Matrigel Basement Membrane Matrix (Matrigel™, BD Biosciences, Bedford, MA) is a commercially available purified solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm mouse sarcoma. Matrigel™ (hereafter referred to as Matrigel) was stored at -20°C and thawed at 4°C by placing on ice 24 hours prior to use. Immediately prior to challenge, live *Map* ($1 \times 10^5$ CFU), *Map* antigen (WCS, 5µg) or an equal volume of sterile saline was added to 500µL aliquots of thawed Matrigel.

**Collection and processing challenge sites for flow cytometry or culture.** At PID 7 and PID 15, animals were heavily sedated (0.1 mg/kg xylazine IM followed by 2.0mg/kg ketamine IV), placed in lateral recumbency and the surgical sites were steriley prepared for surgical removal of Matrigel plug sites. At PID 30, calves were euthanized with an intravascular overdose of pentobarbital sodium (Beuthanasia-D®, Intervet/Schering Plough Animal Health Company). A single cutaneous incision was placed overlying the challenge site and the Matrigel material was visually identified in the subcutaneous space, removed and placed into 5mL sterile isotonic saline and transported on ice to the laboratory for evaluation. In live animals, skin incisions were then sutured closed and the calves were allowed to recover. Cells harvested directly from the Matrigel *Map* inoculation sites were transported on ice to the laboratory for flow cytometry and/or culture. Total cell number was determined using a Beckman Coulter counter (Beckman Coulter Inc, Miami, FL). Cells were then resuspended in either sterile PBS (for cell surface marker staining and flow cytometry) or complete RPMI (for cell culture) at a
final concentration of $1 \times 10^6$ cells per 100μL media. PBMC were collected by jugular
venipuncture, the buffy coat was separated from the whole blood, remaining erythrocytes
were lysed and PBMC were washed twice with PBS. Gamma-delta ($\gamma\delta$) T cell subsets
were sorted from PBMC using autoMACS beads technology on autoMACS cell
separation column per the manufacturer’s instructions (MiltenyiBiotec Inc, Auburn, CA).
Briefly, workshop cluster 1 (WC1) expressing $\gamma\delta$ T cells were positively selected from
PBMC using mouse anti-bovine WC1(n1) IgM primary antibody (B7A1, VMRD Inc,
Pullman, WA, USA) at a dilution of 1:50 followed by rat anti-mouse IgM microbeads at
dilution 1:5 (MiltenyiBiotec). Following the initial sort, $\gamma\delta$ T cells were positively
selected from the previously sorted WC1- subpopulation using mouse anti-bovine delta
chain $\gamma\delta$ T cell receptor (GB21A, VMRD) at a dilution of 1:50 followed by rat anti-
mouse IgG2a+b microbeads (MiltenyiBiotec) at dilution of 1:5. Following the second
sort using autoMACS cell separation column, two distinct populations of $\gamma\delta$ T cells were
achieved: $\gamma\delta$ TCR+/WC1 and $\gamma\delta$ TCR+/WC1+. Prior to culture, the purity of these
populations was verified using two color flow cytometry to be greater than 85% (data not
shown).

Monoclonal antibodies and antigens. The following mouse anti-bovine monoclonal
antibodies were used to characterize T cell phenotype by multi-color flow cytometry:
anti-$\gamma\delta$ TCR (GB21A, VMRD, Pullman WA, USA), anti-WC1(n1) (B7A1, VMRD), anti-
CD4 (CACT138A, VMRD) and anti-IFN$\gamma$ (MCA1964, Serotec, Raleigh NC). Secondary
antibodies (all from Invitrogen Molecular Probes, Carlsbad CA) included Alexa-fluor
488-conjugated goat anti-mouse IgG2b ($\gamma\delta$ TCR), phycoerythrin (PE)-conjugated goat
anti-mouse IgM (WC1), PE-conjugated goat anti-mouse IgG1 (CD4), and Alexa-fluor 700-conjugated goat anti-mouse IgG1 (IFNγ). For intracellular cytokine staining, surface markers were stained as described above and cells were fixed in 2% paraformaldehyde for 20 minutes. The cells were then permeabilized using permawash buffer (0.1% saponin, 0.1% sodium azide in 1X phosphate buffered saline) followed by addition of primary and secondary antibodies. Cells were stored in 2% paraformaldehyde solution prior to flow cytometry.

**Flow cytometric data collection and analysis.** Flow cytometric data were collected using a FACSCanto flow cytometer (Becton Dickinson Biosciences, San Jose, CA), and FlowJo cell analysis software (Tree Star Inc, San Carlos, CA) was used to analyze data.

**Cell culture.** Total recruited Matrigel site cells or purified γδ T cell subsets were suspended in complete RPMI 1640 complete medium (diluted or concentrated to a final concentration of 1x10⁶ cells/well) in 96-well plates. Where appropriate, antigen was added prior to culture for 6 days at 37°C. Whole cell sonicate was used at a final in-well concentration of 5μg/mL, and recombinant human interleukin-2 (rhIL2, Peprotech Inc, Rocky Hill NJ) to a final concentration of 1ng/mL.

**Luminex® Immunoassay.** Gel or culture supernatants were incubated with agitation for 2 hours at room temperature, then overnight at 2-8°C with mouse anti-bovine IFNγ-coupled Luminex® beads. After addition of detection antibodies (biotin-labeled mouse anti-bovine IFNγ; streptavidin-PE), mean fluorescence intensities of individual samples (in duplicate) were compared to standard curve to determine cytokine concentrations.
**Whole blood IFNγ assay.** IFNγ ELISA assay was performed according to the manufacturer’s instruction (*Mycobacterium bovis* gamma interferon test kit for cattle, Bovigam®, Prionics USA, Inc, LaVista, NE). Briefly, whole blood was collected into heparinized tubes, and aliquoted to 1mL/well in 6 well plates. In place of the kit-supplied *M bovis* antigen, *Map* K10 strain WCS antigen (10µg/well) was added to appropriate wells and samples were incubated at 37°C. After 24 hours, the supernatant was harvested from each well and stored at -80°C until analysis. Optical density (OD) ratios were derived by dividing the OD (450nm) of the sample by the OD (450nm) of the positive control, provided in the kit by the manufacturer.

**Delayed-type hypersensitivity reaction.** DTH was assessed by measuring the skin pinch thickness 72 (+/- 6) hours following intradermal injection of 0.1mL of purified protein derivative (Johnin PPD=4.6mg/mL, National Veterinary Services Laboratories, Ames IA).

**Statistical analysis.** Statistical analysis was performed using JMP 8.0.2 (SAS Institute, Cary, NC). Data are presented as the mean values ± standard error of the mean. Student’s t-test and one-way analysis of variance (ANOVA) were used for the statistical analysis, unless otherwise specifically stated. Group mean differences were considered significant if the p value was <0.05, and highly significant if the p value was <0.01.

**Results**

**Model description.** Following initial *Map* infection or vaccination, we evaluated the systemic and localized host immune responses to *Map* at three time points (early, middle, late). Systemic immune responses were measured using two methods: 1) the peripheral
blood leukocyte-generated *Map*-specific interferon gamma (IFNγ) assay, and 2) the *Map* purified protein derivative (PPD)-induced delayed-type hypersensitivity reaction (DTH).

The local host immune response was measured at Matrigel only or Matrigel with *Map* infection sites in calves at three time points (early, middle and late). Also, at each Matrigel only or Matrigel with *Map* infection site, we evaluated gross and histologic changes, measured quantity and phenotype of recruited cells and quantified secreted cytokines at each time point.

**Systemic immune response.** First we measured *Map*-specific IFNγ production by peripheral blood leukocytes at each time point and the data are shown in figure 1a. As expected, minimal IFNγ was generated initially and differences were not observed between groups prior to *Map* challenge or vaccination. Compared to pre-challenge, only vaccinates generated significantly more *Map*-specific IFNγ at the late (PID 30) time point (Fig. 1a, *p=0.0103). In addition to *Map*-specific IFNγ, we also measured systemic immune response at PID 30 by delayed type hypersensitivity (DTH) reaction using the skin test, which is commonly utilized for evaluation of the cell-mediated response to mycobacterial infections in humans and animals. The data are shown in figure 1b, and using the cross-sectional skin thickness measured at PID 30 we were able to distinguish calves based on their treatment group (Fig. 1b, *p<0.01* for each group).

**Local (infection site) immune response.** Grossly subtle but digitally palpable soft tissue swelling was detected at Matrigel *Map* infection sites in *Map*-sensitized animals (*Map* infected or *Map* vaccinated) but not naïve calves at all time points. A significant benefit of Matrigel is that the inoculation site can be evaluated histologically and infiltrating cells are easily recovered after surgical removal of the Matrigel plug. In
figure 2, representative photographs of the gross and histologic appearance of a Matrigel only site (Fig. 2a, b) and Matrigel Map infection site (Fig. 2c, d) is shown. At Matrigel only sites (Fig. 2a, b) very few leukocytes were recruited while at Matrigel Map infection sites, large numbers of leukocytes were recruited and these cells were intermixed with fibrin (Fig. 2d). For each time point, we used a Beckman-Coulter cell counter to quantify total cells recruited to each site in naïve and Map sensitized calves, and this data is summarized in figure 3. More cells were recruited to Matrigel sites at the late time point (PID 30) compared to the early time (PID 7) point in all calves (Fig. 3a, *p<0.0001). At the early time point, more cells were recruited to Matrigel sites in Map sensitized calves compared to naïve calves (Fig. 3b, #p=0.0507). At the late time point, no difference was observed between total cells recruited to Matrigel sites of Map sensitized or naïve calves (data not shown).

**Phenotype of cells recruited to Matrigel Map infection sites.** In order to evaluate the dynamic T cell response at Map infection sites in this model, we used flow cytometry to compare the phenotypic recruitment patterns of T cells recruited to Matrigel with Map sites at each time point. For this study, we focused specifically on CD4 and γδ T cells, and the data are shown in figure 4. γδ T cells were significantly recruited to early Matrigel Map sites of all calves (naïve, infected and vaccinated) compared to late Matrigel Map sites, though it was statistically significant only in naïve calves and vaccinates (Fig. 4a, p<0.0033). In contrast to the recruitment dynamics of γδ T cells, significant numbers of CD4+ T cells were recruited only to late Matrigel Map infection sites in vaccinated calves but not Map infected or naïve calves (Fig. 4b, p=0.0427).
**IFNγ production at Matrigel Map infection sites.** As an additional measure of the local immune response, we measured IFNγ protein secretion into Matrigel alone or Matrigel Map infection sites in all calves. Compared to late time points (Fig. 5b), we detected low amounts of secreted IFNγ within Matrigel only or Matrigel Map infection sites at early time points (Fig. 5a, notice y axis scale difference between panels a, b). Compared to Matrigel only sites, higher IFNγ was detected in Matrigel Map infection sites in Map infected calves at the early time point (Fig. 5a, p=0.0339, Wilcoxon/Kruskal-Wallis test), and in Map vaccinated calves at the late time point (Fig. 5b, p<0.0339).

**IFNγ production by γδ T cell subsets.** Based on γδ T cell infiltration and IFNγ secretion profiles, we used two experimental techniques to investigate the hypothesis that bovine γδ T cells recruited to early Map infection sites are capable of secreting significant amounts of IFNγ. First, we collected total recruited leukocytes from Matrigel Map infection sites for culture. After 6 days culture with WCS-stimulation, we used flow cytometry to label both the surface γδ T cell receptor (γδTCR) and intracellular IFNγ. The results of this flow cytometric study are depicted graphically in figure 6. The percent of γδTCR expressing cells (as a percent of total live cells) following culture (Fig. 6a) was similar to the percentage of γδTCR expressing cells prior to culture and harvested directly from Matrigel Map infection sites (shown in Fig. 4a) at all time points. The percentage of γδTCR expressing cells correlated well with the percentage of γδTCR expressing cells that also expressed IFNγ (Fig. 6b, γδTCR+IFNγ+). Following culture and WCS-stimulation, there is a significantly higher percentage of γδTCR+IFNγ+ cells from
Matrigel Map infection sites at early time points compared to later time points (Fig. 6b, *p=0.0087). Even though most γδTCR+ cells after culture produced IFNγ, we found that the majority of IFNγ produced by cells recruited to both Matrigel alone and Matrigel Map infection sites after culture was by non-γδTCR expressing cells (Fig. 6c, γδTCR-IFNγ+ cells). Further phenotypic information about this particular cell population was not specifically investigated in this study; however likely candidates include αβ T cells, natural killer (NK) cells or NK T cells.

Because cattle have large numbers of γδ T cells in peripheral blood, we also collected peripheral blood γδ T cells from our calves and sorted these into subpopulations based on their expression of the surface marker workshop cluster 1 (WC1). We thus obtained two distinct populations of peripheral blood γδ T cells defined by their surface phenotype: WC1+γδTCR+ and WC1-γδTCR+. At each time point, we cultured peripheral blood-derived γδ T cell subsets to measure their ability to secrete IFNγ as a response to secondary stimulation with media alone, media with rhIL2, or Map antigen (WCS) and rhIL2. Without the addition of rhIL2 to cell cultures in our system, we were unable to detect responses in purified populations of γδ T cells (data not shown). Regardless of time (PID 0 vs. PID 30) or prior Map antigen sensitization status (naïve vs. sensitized), the WC1-γδTCR+ enriched cell populations demonstrated the greatest capacity for IFNγ generation (Fig. 7a, b). In particular, we found that the WC1-γδTCR+ enriched cell population from naïve calves generated significantly greater amount of IFNγ compared to WC1+γδTCR+ enriched cell population from the same calves (Fig. 7a, *p=0.0153). Only after antigen sensitization of the animals were the WC1+γδTCR+ enriched cell
population of cells able to produce IFNγ (Fig. 7b). We found that the ability to generate IFNγ by both subsets of peripheral blood-derived γδ T cells was rhIL2 dependent. It is clear that WC1-γδTCR+ cells are able to generate IFNγ in a Map antigen independent manner; however the role for Map antigen during stimulation of IFNγ production by WC1+γδTCR+ cells is less clear.

**Discussion**

Much effort has been focused on study of the bovine immune response during subclinical or clinical Map infection by evaluation of lymph node cells, intestinal mucosal (lamina propria) cells or peripheral blood cells [32-34], yet the ability to investigate immunologic responses at the initial Map infection site has thus far been elusive [35, 36]. This distinct gap in understanding the early immune response to Map infection in cattle emphasizes the need for models by which factors that shape the early response to Map and development of Map-associated disease can be clarified. The goal of the current study was to further evaluate the early host response to subcutaneous Map infection in calves to facilitate identification of novel features of the response that may be useful for development of new diagnostic strategies.

A limitation to the subcutaneous granuloma model is the difficulty of efficient collection of recruited cells for *ex vivo* analysis without excessive manipulation of the tissue which may induce alterations of cellular phenotype or function. Adaptation of this model by inclusion of Matrigel greatly facilitated collection of recruited cells for *ex vivo* laboratory analysis; however because Matrigel is a product of murine sarcoma, we observed the development of significant immunologic responses to the Matrigel vehicle
in *Map*-naïve calves upon repeated Matrigel exposure. We observed the recruitment of minimal inflammatory cells in response to Matrigel without antigen (Fig. 2a, b). At the early time point, more total leukocytes were recruited to Matrigel sites in *Map* antigen sensitized calves compared to naïve calves (Fig. 3b); however at subsequent Matrigel injections we observed the recruitment of significant inflammatory cellular infiltrates in all animals (Fig. 3a). This impeded interpretation of the immunologic data at late time points because were unable to differentiate *Map*-specific from Matrigel-specific immunologic responses in some calves (most prominent in total recruited cell number). Thus, total recruited cells to Matrigel *Map* sites may be useful for determining *Map* exposure in naïve calves. Regardless, we were able to demonstrate the development of distinct systemic immunologic responses in *Map*-infected and *Map*-vaccinated calves in this study using the DTH and IFNγ tests (Fig. 1).

Consistent with the current immunologic pattern of mycobacterial diseases, *Map* sensitization in our experimental system resulted in the development of an IFNγ-secreting memory CD4+ T cell-mediated response. This was detected in our system by whole blood leukocyte IFNγ assay, DTH responses (Fig. 1), as well as by recruitment of increased CD4+ T cells to Matrigel *Map* sites at late time points (Fig. 4b) in this system. Interestingly, recruitment of γδ T cells has an opposing pattern to that of CD4+ T cells in our study. Previous work in our laboratory in which immuno-fluorescence microscopy was utilized to investigate the involvement of bovine γδ T cell subsets in *Map*-induced granulomatous lesions over time suggested differential roles for γδ T cell subsets at *Map* infection sites [30]. Based on this work, our hypothesis for the current study was that the
WC1-γδTCR+ subset was recruited heavily to initial Map infection sites of naïve calves where they were able to influence the development of the subsequent immune response specifically via IFNγ secretion. In the prior study, we also showed that WC1+γδ T cells were recruited more heavily to Map infection sites following antigenic priming and this shaped our new hypothesis for the current study that these cells function primarily as early effector or memory cells during the anti-mycobacterial immune response in calves.

For the present study we were limited to two simultaneous labels per flow cytometric assay and future work needs to be done to confirm the recruitment patterns of specific γδ T cell subsets (WC1+ vs. WC1-). We were able to show that peripheral blood derived γδ T cell subsets of cattle display a striking differential ability to generate IFNγ during the course of infection. This evidence suggests that, at least for the WC1- subset, ability to generate IFNγ is not dependent on antigen as WC1-γδTCR+ cells from naïve and antigen-sensitized calves consistently generated large amounts of IFNγ in an IL2-dependent but antigen-independent manner. Our data also suggests that WC1+γδTCR+ cells have moderate ability to generate IFNγ, but this was observed only when the cells originated from calves previously sensitized to Map antigen (already primed) and not from naïve calves.

The activation of bovine γδ T cells has been recently described in two distinct stages. It has been known for some time that γδ T cells are non-major histocompatibility (MHC) restricted, and capable of responding to pathogen-associated molecular patterns (PAMPs). The subtle responses by γδ T cells to PAMPs has been termed antigenic-independent priming, and primed cells are in fact defined by upregulation of interleukin
2-receptor α (IL2Rα) [37, 38]. Primed γδ T cells have been shown to robustly produce cytokines in response to secondary signals such as IL2 or specific antigen [37, 39]. It was recently demonstrated that naive lymphatic-derived γδ but not αβ T cells from calves with enteric salmonellosis were primed early during infection as shown by upregulation of both the IL2-Rα gene and IL2-Rα protein on the cell surface. In that study evidence of γδ T cell proliferation or pro-inflammatory cytokine secretion was not reported, but the authors concluded that both events were likely to occur secondary to downstream signaling effects [40]. In our study, these distinct stages (priming vs. secondary activation) were not clearly defined; however our data offers further support to the hypothesis that γδ T cell subsets are capable of significant IFNγ generation at *Map* infection sites. In the previous models, other investigators have hypothesized that the localized response by γδ T cells may be sufficient to control infection and that primed γδ T cells would then return to a resting state [37].

Further work in our laboratory will be directed toward more clearly understanding the role of γδ T cells in our system. Reports in humans and mice have shown that γδ T cell subsets are capable of modulating the adaptive immune response [41-47]. We are also interested in understanding how bovine γδ T cell subsets initially recruited to *Map* infection sites may function locally in control or progression of disease, and how these cells may influence the eventual development of the adaptive immune response to *Map* in cattle.

We recognize that the subsets of γδ T cells in humans and animals continue to be characterized and their functions clarified, and a definitive functional link between γδ T
cells of peripheral blood and those of various mucosal sites has been elusive. However, we believe that the subcutaneous model is useful for defining initial features of the host response and this should prove beneficial for improvement of current diagnostic tests or development of novel diagnostic strategies during early *Map* infection in cattle.

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Systemic immune responses in naïve, infected, and vaccinated calves.

Compared to pre-challenge times (PID 0), significantly greater amount of Map-specific IFNγ was generated by whole blood in vaccinates but not Map infected or naïve calves at the late time point (Fig. 1a, PID 30, *p=0.0103). Delayed type hypersensitivity reaction was able to distinguish all groups (naïve, Map infected, Map vaccinated) at PID 30 (Fig. 1b, p<0.01 between all groups).
Figure 2 Subcutaneous space from *Map* antigen sensitized calves showing gross (Fig. 2a, c) and histologic (Fig. 2b, d, HE stain) appearance of Matrigel injection sites. In Matrigel only sites (Fig. 2a, b), few leukocytes were recruited after 48 hours, though erythrocytes were present (Fig. 2b, arrows). When $10^5$ live *Map* were added to the Matrigel (Fig. 2c, d), large numbers of leukocytes were intermixed with fibrin at the injection site.
Figure 3 Total recruited leukocytes to Matrigel sites in all calves. Significantly more cells were recruited to Matrigel sites at the late time point compared to the early time point (Fig. 3a, *p<0.0001). At the early time point, more cells were recruited to Matrigel sites in Map sensitized calves compared to naïve calves (Fig. 3b, #p=0.0507).
Figure 4 Recruitment of γδ T cells and CD4+ T cells to Matrigel Map infection sites in naïve, infected and vaccinated calves over time. Compared to CD4+ T cells, γδ T cells were recruited to Matrigel Map infection sites in naïve and vaccinated calves at early time points (Fig. 4a, p<0.0033). CD4+ T cells were recruited to Matrigel Map infection sites only in vaccinated calves at late time points (Fig. 4b, p=0.0427). Letters indicate statistically significant differences between groups.
Figure 5 Secreted IFNγ within Matrigel alone or Matrigel Map infection sites in naïve, infected and vaccinated calves. At early time points (panel a), Matrigel Map infection sites contained greater amounts of secreted IFNγ compared to Matrigel only sites; however this was statistically significant only in Map infected calves (Fig. 5a, p=0.0339, Wilcoxon/Kruskal-Wallis test). At late time points (panel b), significantly greater levels of IFNγ were secreted at Matrigel Map infection sites in vaccinated calves compared to the other groups (Fig. 5b, p=0.0339). At early time points, significantly less IFNγ protein was detectable compared to late time points; note the scale difference between panel a and panel b.
**Figure 6** IFNγ is produced by cells recruited to early Matrigel sites compared to later time points, regardless of previous antigen sensitization status of the animal, dual color flow cytometry data. After culture, significantly higher percentage of cells expresses γδTCR in early (22.7%) Matrigel Map infection sites compared to late (3.9%) Matrigel Map infection sites (Fig. 6a, *p=0.005). More γδ T cells from early (13%) Matrigel Map infection sites produce IFNγ compared to the late (1.3%) time point (Fig. 6b, *p=0.0087). After culture, the majority of IFNγ is produced by non-γδ T cells from early Matrigel alone sites (Fig. 6c, 38.5%, *p=0.0026) and Matrigel Map infection sites (Fig. 6c, 8.7%, **p=0.0119) compared to late time points (Fig. 6c, 8.7%, 9.4%, respectively).
Figure 7 Differential IFNγ production by IL2-stimulated bovine peripheral blood derived γδ T cell subsets. Non-stimulated (media) cells of both γδ T cell subsets at all times generated minimal IFNγ. In naïve calves (Fig. 7a, PID 0), cultured enriched WC1-γδTCR+ cells secreted significantly higher amounts of IFNγ after stimulation with recombinant human interleukin 2 (rhIL2) compared to culture enriched WC1+γδTCR+ cells from the same calves (Fig. 7a, *p=0.0153). In sensitized calves (Fig. 7b, PID 30), cultured enriched WC1-γδTCR+ cells stimulated with rhIL2 secrete higher amounts of IFNγ compared to cells stimulated with media alone (Fig. 7b, *p=0.0084).
CHAPTER 4: DIRECT INOCULATION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* INTO ILEOCECAL PEYER’S PATCHES RESULTS IN COLONIZATION OF THE INTESTINE IN A CALF MODEL

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Abstract

The objective of this study was to develop an intestinal model of *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) infection in the calf for evaluation of mucosal pathology, and local and systemic immunologic responses. We inoculated *Map* into Peyer’s patches of young calves using a right flank surgical approach in standing calves to exteriorize the ileocecal junction. Inoculum dosages ranging from $10^3$ to $10^9$ colony forming units of strain K10 *Map* were injected through the serosal surface into Peyer’s patches of the distal ileum near the ileocecal valve. Fecal samples were collected for
culture from each calf weekly until termination of the study. Calves were necropsied at 07, 30, 60, and 90 days post infection when inoculation sites, lymph nodes, spleen, and peripheral blood were collected for evaluation. Ileocecal lymph nodes (ICLN) were consistently colonized by Map in the $10^5$-$10^9$ groups. The ileocecal valve was also colonized in $10^7$ and $10^9$ group animals. This correlated with fecal culture results as infected calves intermittently shed Map in their feces throughout the study. We identified granulomatous lesions with giant cells and acid-fast bacilli at the ileocecal junction, ileocecal lymph nodes, and lamina propria of high dose animals ($10^7$ and $10^9$) from each time point. Flow cytometry was used to detect antigen-specific production of IFN$\gamma$ and IL-4 locally (ICLN) and systemically (PBMC) which defined distinct immunologic profiles in low dose and high dose calves. This study demonstrates intestinal Map infection via Peyer’s patch inoculation, a novel model with many shared features of natural Map infection.

**Key words:** bovine, immunology, intestinal, mycobacteria, paratuberculosis

**Introduction**

*Mycobacterium avium* subspecies *paratuberculosis* (Map) is the causative agent of ruminant paratuberculosis (Johne’s disease), a disease widely distributed in domestic and free-range ruminants throughout the world. The prevalence is unknown in many parts of the world, but in the United States it has been recently estimated to affect as high as 68% of dairy herds [1]. Economic losses result directly from reduced milk yield, premature
culling, and reduced slaughter value, and indirectly as shedding adults serve as a source of infection for susceptible herd mates and young calves [2].

A considerable amount of research using a variety of experimental model systems has been devoted to this disease in recent years, yet the pathogenesis of intestinal Map infection remains incompletely understood. It is generally accepted that infection occurs primarily during the neonatal period when calves ingest an infectious dose of Map primarily from feces or colostrum. The organism is likely endocytosed by epithelial M cells overlying ileal Peyer’s patches, and subsequently phagocytosed by subepithelial macrophages and dendritic cells [3]. A major challenge for studying the disease is the lengthy subclinical infection period of Map (from 2 to 5 years) and the difficulty associated with accurate identification of infected animals during this period. A number of recent studies in cattle have documented infection by tissue culture, fecal culture, and/or polymerase-chain reaction following oral inoculation [4-12] or intra-tonsillar inoculation [11]; however intestinal histopathologic lesions containing acid-fast bacteria are not a consistent feature of experimental infection.

In a recent review of experimental model systems for studying Map infection, Hines et al. [13] articulated pressing knowledge gaps regarding Map infection which include the following questions. How does early tissue colonization relate to protection or lack of protection? Is there correlation between tissue colonization and fecal shedding? What is the optimal-but-practical number of necropsy tissues to collect for culture and histopathology?

Given the lengthy time course and cost associated with studying natural disease and in view of recent recommendations for studying Map infection, our objective for the current
study was to develop a short-term experimental calf model in order to adequately address these unanswered questions relevant to the immunopathogenesis of intestinal *Map* infection. With the rationale that submucosal Peyer’s patches of the distal ileum are the initial site of *Map* infection in the calf, our hypothesis was that direct delivery of an infectious dose of live *Map* into this anatomic location is sufficient to induce rapid intestinal infection with pathologic and immunologic similarity to naturally occurring disease. We set out to establish intestinal *Map* infection by injecting bacteria directly into the proposed site of natural infection in young calves, and we thus designed the experiment to test a range of appropriate inoculation dosages. Our overall study goals were two-fold. First, we utilized bacteriology and histopathology to determine the optimal *Map* dosage that results in intestinal colonization after direct inoculation. Second, we evaluated key T-helper cytokine expression patterns (interferon γ and interleukin-4) both locally and systemically to characterize the progression of local and systemic immune responses in infected calves.

Materials and methods

**Experimental design and animals.** The experimental design is outlined in table 1. Briefly, forty (40) five week old castrated male dairy breed calves (Holstein, Jersey, and Brown Swiss) were acquired for the study from various local private dairy herds. All herds were actively participating in the voluntary Johne’s disease certification program, and have not had positive cases for at least 2 years. Animals were housed within the biosafety level 2 large animal housing facilities on the campus of Fort Dodge Animal Health in Fort Dodge, IA for the duration of this study. Calves were housed in groups of
7-9 animals based on randomly assigned Map dosage. Control calves were housed separate from infected calves and their feces. Live Map was prepared in colony forming units (cfu) per dose including: $10^3$ (n=7), $10^5$ (n=7), $10^7$ (n=9), $10^9$ (n=9), and uninfected negative control (n=8). Each group was separately housed, but for logistical purposes the two low dose groups ($10^3$ and $10^5$ cfu) were infected simultaneously and the two high dose groups ($10^7$ and $10^9$ cfu) were infected simultaneously. For each group, individual calves were randomly selected for euthanasia at the following time points (post infection day, PID): PID 07 (n=1-2), PID 30 (n=2), PID 60 (n=2), and PID 90 (n=2-3). A single uninfected control animal was sacrificed at each time point for low dose and high dose groups. All live animal protocols were approved prior to the experiment by the internal Institutional Committee on Animal Care and Use (IACUC) at the Fort Dodge Animal Health Company.

**Bacterial inoculum preparation.** The Map strain K10 was obtained from the American Type Culture Collection (Manassas, VA), and maintained in Middlebrook 7H9 broth supplemented with mycobactin J. Logarithmic growth-phase bacteria were washed and suspended in sterile saline. Bacterial concentration was determined by measuring absorbance at 540 nm and comparing the optical density to a standard curve generated in our laboratory. By dilution or concentration, inocula were adjusted to the appropriate final concentration of $10^3$-$10^9$ cfu/250 µL in 0.9% sterile saline solution. The inoculum used in these studies was shown to have greater than 90% viability using fluorescein diacetate (FDA) staining with analysis by flow cytometry prior to inoculation. Challenge inocula were confirmed to be acid fast by Ziehl-Nielsen (ZN) staining and negative for contaminants by streaking onto sheep blood agar plates 24 hours prior to injection.
**Animal inoculation.** Animals were inoculated via direct subserosal injection into the Peyer’s patch regions in the distal ileum adjacent to the ileocecal valve. Briefly, a right-sided approach to the ileocecal valve was utilized through a 4-5cm vertical incision in the paralumbar fossa using local anesthesia in standing calves. Once access to the peritoneum was achieved, the cecum and distal ileum were isolated and exteriorized. The anti-mesenteric Peyer’s patches of the distal ileum were visualized and 250 µL containing the appropriate dosage of live *Map* K10 was injected. The intestine was then replaced into the abdominal cavity and the body wall was sutured using a standard three-layer closure. Sterile instrumentation and surgical techniques were used, and perioperative systemic antibiotics were not administered.

**Bacterial fecal and tissue cultures.** Following inoculation, fecal samples were collected per rectum from each calf (PID 01-02), and then weekly for the duration of the study. Fecal samples and tissue samples collected at necropsy were cultured using the BACTEC MGIT 960 system (BD Biosciences, Sparks, MD, USA). All samples positive by the BACTEC culture system were subsequently subjected to PCR for confirmation by identification of the unique *Map* sequence IS900. Within the text of this manuscript, culture positive status is only used to indicate fecal or tissue samples that were positive by both the BACTEC culture system and confirmatory PCR testing.

**Polymerase chain reaction.** Tissue and fecal samples identified as positive using BACTEC liquid culture system were verified as *Map* using real time polymerase chain reaction (PCR) to detect the *Map*-specific sequence IS900. Genomic DNA was extracted from egg yolk media-grown bacterial cultures using a beadbeater (4500rpm for 5 minutes), high-speed centrifugation (16000g for 10 minutes) followed by alcohol
precipitation of the bacterial nucleic acid. Commercially available oligonucleotide primers were derived from the IS900 DNA insertion sequence unique to *Map* (forward 5’-CCGCTAAATGAGAGATGGGATTGG-3’ and reverse 3’-ATTCAACTCCAGCAGCGCGCCTCG-3’, Integrated DNA Technologies, Coralville, IA). A commercially available IS900 fluorescent probe (IDT, Coralville, IA) was used for detection.

**Tissue and sample collection.** At each time point small groups of 5-6 calves were humanely euthanized, and the following tissue samples were collected during a complete necropsy examination: ileocecal valve (ICV), ileocecal lymph node (ICLN), mesenteric lymph node, pre-scapular lymph node, liver, spleen, and ileum. All tissues were aseptically collected and placed either into sterile containers for mycobacterial culture or into 10% neutral buffered formalin for histopathologic evaluation. An additional 4-5 gram section of ileocecal lymph node, mesenteric lymph node, and pre-scapular lymph node was placed into sterile phosphate buffered saline (PBS) with antibiotic and antimycotic agents (penicillin, streptomycin, amphotericin B), and transported to the laboratory for lymph node cell collection, cell culture, and flow cytometric evaluation. Thirty milliliters (mL) of peripheral blood from each calf was obtained by jugular venipuncture, and aseptically collected into 2X acid citrate dextrose anticoagulant (2X ACD) for peripheral blood mononuclear cell (PBMC) culture, antigen stimulation, and flow cytometric analysis.

**Histopathology and acid-fast staining.** Formalin-fixed paraffin-embedded tissues were sectioned (4µm) and stained with hematoxylin and eosin (HE) for histopathologic evaluation and ZN acid fast staining per standard laboratory protocols. All tissue sections
were examined thoroughly for evidence of mycobacterial infection both by identification of appropriate lesions (granulomatous inflammation) and presence of acid-fast bacteria.

**Tissue processing for flow cytometry.** For PBMC’s, the buffy coat was separated from whole blood, the remaining erythrocytes were lysed, and the PBMC were washed with PBS as previously described [14]. For lymph node-derived lymphocytes, fresh ICLN sections were minced, filtered through 7µm mesh filter, and washed twice with PBS. All cells were counted using a Becton-Dickinson (BD) Coulter counter (Becton-Dickinson, Franklin Lakes, NJ), and diluted to 5 x 10^6 cells/ml in culture medium.

**Multi-parameter flow cytometry.** PBMC and lymphocytes from ICLN of each animal were activated as previously described [15], with minor alterations using Map K-10 whole cell sonicate (WCS, 20 µg/ml final dilution) as recall antigen. Six fluorophores were used to simultaneously identify T lymphocyte subsets (CD4, CD8, and γδ), the activation marker CD25 (IL-2α receptor), and the intracellular cytokines interferon gamma (IFNγ) and interleukin-4 (IL-4) as previously described [14].

**Flow cytometric data collection and analysis.** Flow cytometric data were collected using a FACSCanto flow cytometer (Becton Dickinson Biosciences, San Jose, CA), and FlowJo cell analysis software (Tree Star Inc, San Carlos, CA) was used to analyze data. Expression index values were calculated to reflect the % gated positive events and mean fluorescence intensity (MFI) for each parameter from antigen stimulated samples as described earlier [14]. Index values for each parameter were then normalized to stimulated cells of the same tissue harvested from noninfected (negative control) calves at the same time point. Using normalized values, Map-specific immune response locally (ICLN) was compared to the systemic (PBMC) response at each time by comparing
expression of CD25 and cytokine (IFNγ and IL-4) production by T cell subsets (CD4, CD8, and γδ).

**Statistical analysis.** Statistical analysis was performed using JMP 8.0 (SAS Institute, Cary, NC). Data are presented as the mean values ± standard error of the mean. Student's t-test and one-way analysis of variance were used for the statistical analysis. Group mean differences were considered significant if the p value was <0.05, and highly significant if the p value was <0.01. Because multi-parameter flow cytometry data from low and high dose groups were collected on different days, it was not statistically valid to compare the two groups to each other due to significant daily variation inherent in these assays. A single non-infected control animal was analyzed for each *Map* dosage group each day, so values for infected vs. non-infected animals could not be statistically compared. Instead, animals were grouped into low dose (10³ and 10⁵ cfu) and high dose (10⁷ and 10⁹ cfu), and the data was normalized by subtracting values for the control animals from the values for the infected animals prior to statistical analysis. We then compared local (ICLN) to systemic (PBMC) cellular immune responses within each T cell subset and dosage group each day after *Map* infection.

**Results**

**Post-surgical health status.** Calves demonstrated minimal post-surgical effects. Incisions healed normally and minimal soft tissue swelling of the incision site occurred in some calves, but these tissue changes resolved during healing. No calf developed clinical signs of disease attributable to intestinal *Map* infection (diarrhea, weight loss or inappetence) during the study.
**Gross pathology.** Gross lesions uncommonly observed during the necropsy procedures were limited to fibrous adhesions between the omentum and the body wall at the surgical site.

**Histopathology.** We examined ileocecal valve, ileum, spleen, liver, and lymph nodes from control and infected calves at each time point to identify significant microscopic pathology. Tissue histopathology results are summarized by inoculation dose group (low dose=10^3 and 10^5 cfu, and high dose=10^7 and 10^9 cfu), and by day (PID 07-90). Significant histopathologic lesions were not identified in uninfected or low dose calves at any time point during the study. Mild lymphofollicular hyperplasia of ileal Peyer’s patches and lymph nodes as well as multifocal ileocecal crypt abscesses were present; however Map was not identified by culture or acid-fast staining in these calves.

In contrast, the high dose group calves had significant morphologic changes. High dose calves had consistent granulomatous lesions in which acid-fast organisms were identified in the intestinal mucosa and draining lymph node. As early as PID 07, intestinal tissues harvested from high dose calves had mucosal and submucosal macrophage aggregates containing acid-fast bacteria. In one calf (#917) a focal submucosal pyogranuloma with central necrosis and large numbers of intracellular and extracellular acid-fast bacteria occurred at the site of Map injection. At PID 07, multifocal and coalescing inflammatory cellular aggregates composed of macrophages, epithelioid macrophages, and occasional Langhans-type multinucleated giant cells were present within the intestinal submucosa (Fig. 1a) and ZN staining showed intralesional acid-fast bacteria (Fig. 1b). By PID 60, inflammatory cells also extended into the lamina propria of villous tips of infected animals (Fig. 2a), and ZN staining revealed myriad
intracellular and extracellular acid-fast positive short bacterial rods in these areas (Fig. 2b). Low numbers of intracytoplasmic acid-fast organisms were also identified within macrophages in the draining (ileocecal) lymph node at PID 07, 30, and 90 (not shown).

**Bacteriology.** We collected a complete set of tissues during necropsy, and feces weekly from each calf for *Map* culture and PCR to determine tissue colonization and fecal shedding. *Map* culture was performed using the BACTEC system with IS900 PCR as a confirmatory test. Tissue culture results were grouped by inoculation dose (low dose=10^3 and 10^5 cfu, and high dose=10^7 and 10^9 cfu), and by time after inoculation (PID 07-90); the data are summarized in table 2. All tissue and fecal samples from noninfected control calves were *Map* culture negative. *Map* cultures from low dose calf tissues were sporadically positive while fecal cultures from this group were consistently negative throughout the study. In contrast to low dose calves, live *Map* was detected in one or more tissues harvested at necropsy from all high dose calves (18/18). A majority of calves in the high dose group had multiple tissues culture positive for *Map* (13/18). Of high dose calf tissues, the ileocecal lymph node (16/18) and ileocecal valve (14/18) were most frequently *Map* culture positive while the pre-scapular lymph node (4/18) and spleen (4/18) were less frequently *Map* culture positive. High dose calves also displayed intermittent *Map* shedding in fecal samples. We recognize that a small percentage of the inoculum may have been inadvertently deposited into the intestinal lumen or the peritoneal cavity during intestinal *Map* inoculation. As a result, culture positive feces acquired from experimentally infected animals during the first 2 days after inoculation (6/32) were interpreted to represent inoculum flow-through rather than active fecal shedding, and were thus excluded from the final analysis.
**Map-specific cell mediated immune responses (multi-parameter flow cytometry).**

The mean percentages of lymphocyte subsets (CD4, CD8, γδ, and non-T cells) within unstimulated PBMC and ICLN cells from uninfected and *Map*-infected calves are shown in table 3. The lymphocyte subset profiles of each tissue at 6 days incubation were similar between infected and non-infected animals. The major difference was the γδ T cell population was high in PBMC but low in ICLN. To evaluate *Map*-specific immune responses, we used immunofluorescence and flow cytometry to compare lymphocyte activation and cytokine secretion from PBMC (systemic) and ICLN (local) cells during recall assays. Flow cytometry data were grouped by challenge dose (low, high), and evaluated within each day (PID 30-90). In low dose calves, significant differences between local and systemic immune responses were limited to PID 60 and PID 90 (Fig. 3). Compared to PBMC, lymphocyte subsets from ICLN generated significantly greater amounts of *Map*-specific cytokines IFNγ (CD8 T cells at PID 60, Fig 3b; CD4 and γδ T cells at PID 90, Fig. 3e) and IL-4 (CD4 and γδ T cells at PID 90, Fig. 3f). Overall in low dose calves, systemic immune responses to intestinal *Map* infection detected in PBMC remained significantly lower than local immune responses detected in ICLN throughout the study.

Significant differences between the local and systemic immune responses were detected as early as PID30 in high dose calves (Fig. 4). Increased generation of *Map*-specific IFNγ was detected in ICLN cells (CD4 T cells, Fig. 4b) at PID 30. Though not statistically significant, ICLN CD8 T cells tended to generate more IFNγ and IL-4 at PID 30 compared to PBMC CD8 T cells (Fig. 4b, c). Peripheral blood-derived CD4 T cells
tended to generate elevated IFNγ and IL-4 at PID 60 and PID 90 compared to ICLN cells (Fig. 4e, f, h, i). At PID 90, peripheral blood CD8 T cells generated significantly more IL-4 than ICLN CD8 T cells (Fig. 4i). Overall in high dose calves (in contrast to low dose animals), systemic responses to intestinal Map infection were detected in PBMC during later time points (PID 60, PID 90) and did not remain significantly lower than local immune responses detected in ICLN.

**Discussion and conclusions**

Difficulty in controlling Map-related disease in ruminants throughout the world is related to poor sensitivity and specificity of available diagnostic assays and the lack of completely protective vaccines [16]. Progress in improving diagnostic assays and vaccines is greatly hindered by challenges associated with the study of subclinical disease, when infected animals lack clinical signs, typical intestinal pathology, and definitive cellular or humoral evidence of their infection status [2, 16]. This gap in understanding the early immune response to Map infection in cattle underscores the need to develop models by which factors that shape the early response to Map and development of Map-associated disease can be clarified.

A wide range of oral dosages (10^3-2.5x10^10 live Map organisms) has been used in ruminant model studies with variable success [13, 17], and Hines et al recently suggested 10^9 live Map organisms be administered on each of two consecutive days for experimental studies in cattle [13]. We have used a novel approach to directly inoculate live Map into Peyer’s patches of the ileocecal valve to successfully induce intestinal Map infection in young calves in a dose-dependent manner. Unlike oral or intra-tonsillar
infection models [5, 13, 17], high dose calves consistently and rapidly developed characteristic intestinal lesions with intral esional acid-fast mycobacteria throughout the mucosa, ileal Peyer’s patches, and draining lymph nodes. In addition, Map was cultured from tissues of high dose animals, and these calves intermittently shed live Map in their feces. Importantly, the morphologic and bacteriologic features identified in high dose calves in this study that mimic aspects of natural infection in the bovine have not been collectively demonstrated in an experimental Map intestinal infection model.

Dose-dependent responses (histology and bacteriology) are evident in our study. We were unable to histologically demonstrate intestinal infection in low dose animals though up to 50% of these calves were tissue culture positive for Map at necropsy. Mild intestinal inflammation and lymphofollicular hyperplasia of Peyer’s patches and draining lymph nodes were observed in low dose calves, but were not significantly different from control calves.

The host immune response to Map infection has been studied locally during the clinical stages of Map infection or systemically using peripheral blood cells [3, 18, 19], but few studies have evaluated the local response during early or subclinical infection [20, 21]. The classic immunologic paradigm associated with mycobacterial disease is biphasic, and characterized by an initial T-helper 1 (Th1) response. Effective Th1 polarization mediated by IFNγ is considered to be integral for controlling mycobacterial infections [22], and detection of Map-specific IFNγ has been successfully utilized diagnostically during early stages of mycobacterial disease including Map infection in cattle [23]. Clinical Map infection in cattle is associated with a strong T-helper 2 (Th2) polarized response characterized by dominance of Th2 cytokines. IL-4 and IL-5 play
roles in activation and proliferation of B lymphocytes, and subsequent immunoglobulin production which is prominent during clinical disease, and serves as the basis for commercially-available serum antibody diagnostic assays. The shift from Th1 to Th2 dominance of Map-associated disease has been well documented, yet the classic Th1/Th2 paradigm may not be completely accurate in the bovine, and based on the work of several laboratories, a more complex interplay seems to be a more likely scenario [18, 24].

In the current study distinct immunologic profiles were evident in the low and high dose groups. In general, Map-specific immune responses were strongest in the draining lymph nodes for the study duration in low dose Map-infected calves. In the high dose Map-infected animals the local immune response dominated initially, but diminished in relation to the systemic response as the study progressed. In both dose groups, we observed mixed Th1-like and Th2-like responses that were characterized by the production of both IFNγ and IL-4 from multiple lymphocyte subsets. This response was identified sooner following inoculation in the high dose group. Interpretation of the immunologic data is limited because of animal numbers and statistical power in this proof of principal study. However we are intrigued by the dose-dependent responses observed in the draining lymph node and systemic circulation in our system. Because they coincide temporally with tissue colonization, development of intestinal lesions, and active fecal shedding in high dose but not low dose calves, we believe the data provide preliminary clues regarding the evolution of the immunopathologic response during early intestinal Map infection in calves. Given the complexity of host immune responses to mycobacterial pathogens [24, 25], our model affords a unique opportunity to analyze the
interplay of specific cellular subsets and cytokines occurring at the infection site and systemically during the initial stages of intestinal infection.

Other models have been successfully developed to study intestinal *Map* infection in cattle, and these systems offer invaluable opportunities to study the immunopathogenesis of *Map*-associated disease. A ligated intestinal loop model with direct luminal *Map* inoculation has been used to elucidate mechanisms of initial intestinal invasion by *Map* and local host transcriptional events [12, 21]. Use of this model appears limited to the very early stages (hours) of infection, and the procedure requires prolonged surgical anesthesia of experimental animals. A report by Allen et al describes the development of an ileal cannulation model in cattle to study the local and systemic immune response to *Map* in an oral challenge system [20]. Rare *Map*-infected macrophages were observed in the intestinal propria within 24 hours after oral infection, and *Map* DNA was identified using PCR in sequentially biopsied intestinal tissues; however significant differences in the activation status of intraepithelial lymphocytes of infected vs. uninfected animals were not demonstrated in the study [20]. The characteristic *Map*-induced lesions, acid-fast organisms, or active fecal shedding of *Map* have not been observed in either the ligated intestinal loop or ileal cannulation models. Regardless, a clear advantage of intestinal loop, ileal cannulation, and oral infection models is the ability to potentially investigate interactions of live *Map* with intestinal epithelia and/or M cells, which are proposed to play a significant but incompletely explored role in the pathogenesis of natural infection [26]. A unique and significant advantage of the experimental system reported here is that we have described a method to accelerate the pathogenesis of intestinal *Map* infection via direct delivery of a known infectious dose to the ileocecal
valve, the proposed natural infection site in young calves. An objective of the current study was to develop a model resulting in consistent intestinal *Map* infection, and though this was successfully achieved, we did not observe classic lesions or clinical signs of naturally-occurring disease in cattle such as diffuse granulomatous enteritis leading to chronic diarrhea and emaciation. Future work focusing on the disease progression of intestinal *Map* infection using this model should be performed; however a reliable model to study lesion development and immune responses during establishment of initial intestinal infection and progression of disease is important. Given the current challenges associated with efficacious vaccination and accurate diagnosis of the subclinically infected animal, use of this model’s unique features including granulomatous intestinal inflammation and intermittent fecal *Map* shedding is expected to prove useful for evaluation of novel vaccine candidates and/or diagnostic approaches in subclinically infected animals.

In conclusion, we have developed a reproducible model of intestinal *Map* infection in calves which shares important microbiologic, pathologic and immunologic features of natural *Map* infection in cattle, all within a short time period. We believe the model has significant potential for not only expanding our understanding of the pathogenesis of *Map*-induced disease but also for identification of factors that influence the development of protective immune responses to infection.

**Acknowledgements**

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Wonderlich, the veterinary staff, and the many animal caretakers of the Fort Dodge Animal Health company for expert animal care and assistance during surgical and necropsy procedures.
References


10. Uzonna, J.E., P. Chilton, R.H. Whitlock, P.L. Habecker, P. Scott, and R.W. Sweeney, Efficacy of commercial and field-strain Mycobacterium


### Table 1 Experimental Design

<table>
<thead>
<tr>
<th>Dosage Group</th>
<th>Map dose (cfu)</th>
<th>Collection day</th>
<th>No. of animals</th>
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<td>Low</td>
<td>$1 \times 10^3$</td>
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<td></td>
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<td>2</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>PID 30</td>
<td>1</td>
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<td>$1 \times 10^9$</td>
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cfu = colony forming units; PID = post-infection day
Table 2 Tissue and fecal culture summary

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</tr>
<tr>
<td></td>
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Sample positivity was defined by BACTEC liquid culture system with confirmation by *Map*-specific IS900 sequence PCR.

Low dose group = 10³ cfu and 10⁵ cfu; High dose group = 10⁷ cfu and 10⁹ cfu; PID = post-infection day
Table 3 Summary of % cell types in lymph nodes or peripheral blood

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>CD4+</th>
<th>CD8+</th>
<th>γδ+</th>
<th>Non-T</th>
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<tr>
<td>Control</td>
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<td>26.2</td>
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<td>Map infected</td>
<td>PBMC</td>
<td>29.7</td>
<td>23.3</td>
<td>37.7</td>
<td>7.4</td>
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</table>

Mean percentages of lymphocyte subsets (CD4+, CD8+, γδ+ and non-T cells) within unstimulated PBMC and ICLN cells harvested from uninfected control and Map infected calves are shown.

ICLN = ileocecral lymph node; PBMC = peripheral blood mononuclear cells
Figure 1 Small intestine, *Map*-infected calves, PID 07. Panel 1a is an HE stained low magnification view of ileum near ileocecal valve where the submucosa is focally expanded by well demarcated aggregate of macrophages, multinucleated giant cells and fewer lymphocytes or neutrophils (arrows). Panel 1b is a ZN acid-fast stained higher magnification view demonstrating the presence of acid fast bacilli in the submucosal granuloma. A large cluster of bacteria is present within the cytoplasm of a giant cell (arrow); smaller aggregates of bacteria are present within macrophages (arrowheads).

Figure 2 Small intestine, *Map*-infected calves, PID 60. Panel 2a is an HE stained section of ileum near the ileocecal valve demonstrating the presence of epithelioid macrophages and Langhans-type multinucleated giant cells in a microvillus (arrow).
Panel 2b is a ZN acid-fast stained section showing intracellular and extracellular acid fast bacilli (arrow and inset) of a *Map*-infected calf at PID 60.
**Figure 3 Immune responses in low dose *Map*-infected calves.** Flow cytometric detection of local (ICLN) vs. systemic (PBMC) immune responses in low dose calves are shown. Net expression indices (mean +/- standard error of mean) of CD25, IFNγ, and IL-4 of ICLN and PBMC’s from the low dose *Map*-infected calves are shown for each T cell subset. EI=expression index, ICLN=ileocecal lymph node, PBMC=peripheral blood mononuclear cells, LPC=lymphocytes. Significantly higher responses within the same T cell subset are indicated *p<0.05 and **p<0.01.
Figure 4 Immune responses in high dose *Map*-infected calves. Flow cytometric detection of local (ICLN) vs. systemic (PBMC) immune responses in high dose calves are shown. Net expression indices (mean +/- standard error of mean) of CD25, IFNγ, and IL-4 of ICLN and PBMC’s from the low dose *Map*-infected calves are shown for each T cell subset. EI=expression index, ICLN=ileocecal lymph node, PBMC=peripheral blood mononuclear cells, LPC=lymphocytes. Significantly higher responses within the same T cell subset are indicated *p<0.05.
The central hypothesis for the set of studies discussed in this thesis dissertation has been that γδ T cells play a significant and central role at Map infection sites in young calves. A longer-term goal of this work is to investigate and relate how these initial cellular and molecular events shape the ensuing adaptive immune response during Map infection, and ultimately how this may influence clearance, containment or progression of the disease. A lack of data regarding systemic or local tissue immunopathology during early and subclinical disease periods underscores the challenges associated with accurate identification of subclinically infected individuals, and this ultimately affects our ability to control spread of this devastating disease.

The young calf is the model of choice to investigate this hypothesis, because they have abundant γδ T cells, are natural hosts for Map infection, and are normally infected with Map during the perinatal period. Data generated from these studies supports our central hypothesis in two ways, namely that γδ T cell subsets 1) are differentially recruited to initial subcutaneous Map infection sites, and 2) have differential function at these sites. In addition, we are interested in applying these observations to the intestine to more fully understand the immunopathology of Map during natural disease, and we have made significant progress towards this goal by successfully developing an in vivo intestinal model of Map that mimics several features of natural disease.
Gamma-delta (γδ) T cell subsets are differentially associated with granuloma development and organization in a bovine model of mycobacterial disease

In Chapter 2, we set out to answer key questions regarding the role of bovine γδ T cell subsets during granuloma formation or maintenance during initial stages of Map infection, using a subcutaneous model developed by Simutis et al [1]. Though a number of reports have suggested distinct roles for human γδ T cell subsets during the anti-mycobacterial immune response, a distinct role for γδ T cell subsets in the calf has not previously been investigated. Considering the human and murine data in the literature, the hypothesis for this study was that bovine γδ T cell subsets, defined by their surface expression of the WC1 molecule, are differentially associated with the development of organized or unorganized granulomatous lesions at mycobacterial infection sites. Compared to unorganized Th2-like granulomatous lesions, highly organized Th1-like granulomas contained significantly more WC1+ cells during early stages and significantly more WC1- cells as the lesions became highly stratified and organized. Similar to a prior report by Wangoo et al [2], we demonstrated that as lesions stratify and organize over time, distinct spatial orientation of γδ T cell subsets becomes evident. Specifically, the WC1+ cells were restricted to the outer margins of the granuloma near the fibrous capsule while the WC1- cells were heavily concentrated near central regions of the granuloma adjacent to area of central caseation necrosis. Finally, our data suggested distinct functional roles for γδ T cell subsets because we found that WC1-subset cells were preferentially recruited to early Map infection sites of naïve calves
while the WC1+ subset cells were more heavily recruited to early \textit{Map} infection sites of antigen-sensitized (vaccinated) calves.

The study contributes towards understanding the immunopathology of the \textit{Map} infection site in calves, suggests that $\gamma\delta$ T cell subsets are involved with granuloma formation, and supports the hypothesis that $\gamma\delta$ T cell subsets play distinct functional roles during the initial anti-mycobacterial host immune response, though these distinct roles remain to be completely defined.

\textbf{Evaluation of the initial host response to \textit{Mycobacterium avium} subspecies \textit{paratuberculosis} infection in sensitized and naïve calves using matrix biopolymers}

In Chapter 3, we sought to expand our observations of the previous study by evaluating recruitment patterns of $\gamma\delta$ and CD4+ T cells and cytokine production at the mycobacterial infection site of naïve or antigen-sensitized calves. Our hypothesis for this study was that the differential recruitment of $\gamma\delta$ T cell subsets (and CD4+ T cells) to early \textit{Map} infection sites reflects their distinct functional roles during the developing immune response. In the previous study, we were able to evaluate $\gamma\delta$ T cell subsets within mycobacteria-induced lesions over time, but in that model the cells were difficult to recover for further phenotypic or functional analysis. A goal for the second study was to modify the model so that recruited cells could be harvested and further evaluated \textit{ex vivo}, and this goal was successfully met using the Matrigel biopolymer matrix. A strong CD4+ T cell-mediated systemic immune response to \textit{Map} vaccine [3] was also evidenced during this study by CD4+ T cell recruitment to late Matrigel \textit{Map} infection sites of
vaccinated but not naïve calves. In contrast, $\gamma\delta$ T cells were recruited to early but not late Matrigel Map infection sites of all calves, supporting the hypothesis that $\gamma\delta$ T cells have distinct roles during the early host immune response. Others have evaluated responses of bovine peripheral blood-derived $\gamma\delta$ T cells to mycobacterial antigens [4, 5]; our data expands these data by suggesting that in vivo $\gamma\delta$ T cell recruitment to early Map infection sites is independent of previous Map antigen sensitization of calves. We also found that $\gamma\delta$ T cells harvested from Matrigel Map infection sites produced significantly more IFN$\gamma$ at early compared to later times after infection. We then demonstrated that bovine $\gamma\delta$ T cell subsets have differential ability to generate IFN$\gamma$ based on their expression of the WC1+ molecule. Specifically WC1- but not WC1+ cells of naïve calves produced significant amounts of IFN$\gamma$ after stimulation ex vivo, but this is apparently not antigen-specific. In contrast, WC1+ cells showed ability to generate IFN$\gamma$ only if the host had previously been sensitized to Map antigen. In summary for Chapter 3, our data supports the original hypothesis that bovine $\gamma\delta$ T cells are not only differentially recruited to early Map infection sites, but also that these cells differentially secrete cytokines. Given these features, our data supports the hypothesis that bovine $\gamma\delta$ T cell subsets have distinct functions during different phases (innate response vs. memory response) of the antmycobacterial immune response.

Direct inoculation of Mycobacterium avium subspecies paratuberculosis into ileocecal Peyer’s patches results in colonization of the intestine in a calf model
Considering our data from the prior two studies that suggests distinct functional roles for bovine γδ T cell subsets at early Map infection sites, the goal for project described in Chapter 4 was to develop a model where Map could be identified within granulomatous intestinal lesions in the young calf. With the rationale that the submucosal Peyer’s patches of the distal small intestine are the initial site of Map infection during natural disease of the calf, our hypothesis for this study was that direct delivery of an infectious dose of live Map into this location will result in rapid intestinal infection with pathologic and immunologic similarity to natural disease. The development of Map-induced early intestinal lesions within current modeling systems has not been described [6] and as a result we are unable to directly test our infection-site hypotheses outlined in this dissertation. We thus set out to develop a novel short-term experimental model in order to adequately address the unanswered questions relevant to immunopathogenesis of early intestinal Map infection. Using this experimental inoculation model, we were successfully able to establish intestinal Map infection in calves. This was verified by histology with Ziehl-Nielsen acid-fast staining, tissue and fecal culture and real-time polymerase chain reactions. In addition, we evaluated Map-specific cell mediated immune response during early intestinal Map infection and found distinct differences between the local (ileocecal lymph node cells) and systemic (peripheral blood cells) response. Specifically, we identified mixed Th1-like (IFNγ) and Th2-like (IL-4) responses of T cell subsets (CD4+, CD8+ and γδ T cells) occurring first in the ileocecal lymph node cells followed by similar responses in the peripheral blood cells of infected calves. We described dose-dependent responses to intestinal Map infection both locally and systemically in this system. Further, we showed that these immune responses
correlate temporally with the development of histologic intestinal lesions as well as the onset of intermittent fecal shedding of *Map*. Obvious limitations of our model system exist, and the most prominent drawback is that it lacks the ability to investigate the role of the intestinal epithelial barrier during intestinal *Map* infection. Regardless, use of our model provides for the first time a unique opportunity to analyze the development of the host immune response (including specific cellular subsets and cytokines) at the *Map* infection site in the distal small intestine during initial stages of the disease. In summary, the results of these studies represent a significant step forward for understanding the pathogenesis of *Map* infection in cattle, particularly during the early and subclinical infection periods of disease which thus far has proved elusive in *Map* research.
Recommendations for Future Research

Mycobacterial diseases remain a major threat to animal and human health. The epidemiologic range and incidence of *Map* and *Map*-associated disease of ruminants (Johne’s disease) continue to expand in the US and around the globe [7]. The long asymptomatic subclinical infection period of this disease is a major contributing factor for continued knowledge gaps regarding the immunopathologic events and the evolving host immune response. Understanding how the host immune system responds both locally and systemically during initial *Map* infection and how these initial events influence the ultimate disease outcome are expected to profoundly enhance our progress towards development of more successful diagnostic or vaccination strategies.

In the previous dissertation, we have approached these problems by utilizing a variety of unique animal modeling systems in the calf to answer important questions regarding the early *Map* infection-site immunopathologic response. Our observations during these projects have supported our hypotheses that similar to humans, bovine γδ T cells readily respond to mycobacterial infection *in vivo*. Based on these data, our ongoing hypothesis is that γδ T cells of calves have subset-specific responses to *Map* infection, and that these cells profoundly influence (at different phases) the ensuing anti-mycobacterial immune response. Extending from this work are several distinct but overlapping avenues along which future effort should be directed.
Defining bovine γδ T cell subset ligands

We defined distinct patterns of recruitment of WC1+ and WC1- γδ T cell subsets to Map infection sites in naïve vs. antigen sensitized calves. We also defined distinct in vivo and ex vivo responses by WC1+ and WC1- cells at Map infection sites. While these data support our initial hypotheses, additional work to clarify the ligands recognized by γδ T cell subsets and the mechanisms by which responses are induced would be significant. It appears that distinct differences between human and bovine γδ T cells, such as the failure of bovine naïve WC1+ cells to recognize the prototypical human Vδ2+ ligand phosphoantigens [5], would preclude the use of cattle as an appropriate model for human γδ T cell immunobiology. However, ligands for human γδ T cells continue to be described, and specific ligands for bovine γδ T cells have only briefly been investigated. Our data coupled with the known tissue distribution of bovine γδ T cell subsets [8] would support a hypothesis that WC1- cells function predominantly in immune surveillance and innate responses while the WC1+ cells function more in memory recall responses during subsequent infections. To investigate this hypothesis, it will be important to characterize each subset of bovine γδ T cells by screening for reactivity of the cells toward potential ligands at varying stages of disease.

Defining the cytotoxicity of bovine γδ T cell subsets

In humans, broad cytotoxicity has been reported for both Vδ1+ and Vδ2+ cells [9] and these mechanisms can be mediated by either the Fas/Fas ligand pathway [10] or the perforin/granzyme pathway [11]. The cytotoxicity of γδ T cell subsets in cattle has not
been fully characterized, though a natural cytotoxicity receptor (NKp46) is expressed on the surface of WC1- cells during specific in vitro conditions [12]. Based on our data that WC1- cells of cattle are preferentially recruited to Map infection sites of naïve calves, in vivo cytotoxic activity against mycobacteria by WC1- cells seems to be a reasonable hypothesis. Testing this hypothesis by demonstrating in vivo cytotoxicity of WC1- cells at Map infection sites in calves is important, and could potentially be a mechanism to target in order to reduce the viability of extracellular and intracellular mycobacteria. This mechanism could also be important for limiting progression of Map during the very initial stages of infection.

**Defining interactions of bovine γδ T cell subsets with macrophages**

We have shown that γδ T cells are rapidly recruited to early Map infection sites of cattle. Though we have documented that specific subsets of these cells display distinct recruitment patterns (WC1- in naïve calves, WC1+ in Map antigen-sensitized calves), we have observed that both subsets have ability to generate IFNγ, albeit in distinctly different scenarios. Specifically, WC1- cells from both naïve and antigen-sensitized calves generate significant amounts of IFNγ while WC1+ cells from antigen-sensitized calves but not naïve calves generate increased IFNγ. Given this data, further investigation of the interactions of WC1- cells with Map-infected macrophages at the initial infection site is important. Simutis et al previously showed the failure of peripheral blood-derived γδ T cells from sensitized cattle to induce macrophage killing of Map in a co-culture system [13]. In that study however, the WC1 phenotype of the cells was not reported and
because they were harvested from peripheral blood compartment of young calves, it is reasonable to assume that most were WC1+. It would be very interesting to use a similar co-culture system to investigate the potential of purified γδ T cell subsets from both naïve and antigen-sensitized animals to affect macrophage killing of Map. The Matrigel system outlined in this dissertation could also potentially be adapted to investigate the role of γδ T cell subsets to induce macrophage killing of Map at Matrigel Map infection sites in vivo.

**Defining interactions of bovine γδ T cell subsets with DCs**

The key to clearance or at least containment of mycobacterial infection appears to be the development of an appropriate CD4+ T cell mediated response. It has recently become clear that the DC is the single most important cell for antigen presentation to naïve T cells, thus DCs play a primary role for initiation of effective cell-mediated immunity. For these reasons, the role that γδ T cells play in enhancing DC maturation and antigen presentation could potentially be a mechanism for stimulation of more efficacious responses. As previously mentioned, the high prevalence of γδ T cells in young individuals has been proposed as a mechanism mediating improved vaccine efficacy compared to adults [14]. The mechanistic interactions between γδ T cell subsets of cattle with DCs should be investigated. Data from our lab has demonstrated that DCs harvested from Map infection sites of calves are phenotypically and functionally impaired [15]. Enhancing initial antigen presentation by stimulating infection site DCs using activated γδ T cells during Map infection could improve DC maturation, antigen
presentation and ultimately enhance development of protective CD4+ T cell mediated immunity. Several approaches could be utilized to test this hypothesis. First, a co-culture system could be developed to evaluate in vitro the ability of γδ T cell subsets to effect the maturation of Map-infected DCs. If this could be further clarified, the Matrigel system could then be adapted to test similar hypotheses in vivo. Ultimately, understanding the γδ T cell-DC interactions and the effects on development of the downstream adaptive immunity could potentially have profound effects on future vaccine development for a variety of intracellular infections of humans and animals [16].

**Investigation of IL-17 production by bovine γδ T cell subsets**

The role of IL-17 during inflammatory pathology and autoimmune diseases at mucosal sites is well described in mice and humans [17]. Similar data in cattle has not been described. Given the profound effects of IL-17 on these processes, it seems reasonable to hypothesize that like humans and mice, bovine γδ T cell subsets also produce IL-17 in certain scenarios and this should be investigated. Studies using either ex vivo culture systems or the in vivo Matrigel Map infection system could be utilized to investigate this hypothesis. It would be intriguing to evaluate the potential role for γδ T cells and the IL-17 axis during key transition points of intestinal Map infection (ie, development of lesions and establishment of an infection nidus or during the subclinical to clinical phase transition), as strong Th17 responses have been described during chronic inflammatory bowel disease of humans [18].
Investigation of bovine $\gamma\delta$ T cell subsets to process and present antigen in vivo

Over ten years ago it was demonstrated that antigens can be taken up by bovine WC1+$\gamma\delta$ T cells and presented to CD4+ T cells [19], and more recently it has been shown that human V$\delta$2+$\gamma\delta$ T cells have similar capacity [20, 21]. It has been hypothesized that antigen presentation by cattle and human $\gamma\delta$ T cells is restricted to inflammatory settings because resting $\gamma\delta$ T cells of both species lack antigen presentation capabilities [21]. We have demonstrated that $\gamma\delta$ T cell subsets differentially respond to mycobacteria by migrating to and generating cytokines at early *Map* infection sites in the calf, yet the ongoing hypothesis that $\gamma\delta$ T cell subsets influence the adaptive immune response by modulation of DCs or $\alpha\beta$ T cells remains to be fully explored. The ability to investigate functionality of $\gamma\delta$ T cell subsets in vivo at the initial mycobacteria-induced infection and inflammation site represents a unique opportunity to explore the full biological potential of these cells, and use of this model to study these interactions should be pursued.

Translation of bovine $\gamma\delta$ T cell function to the intestinal *Map* model

Ultimately how the data presented in this thesis dissertation relate to Johne’s disease in cattle will depend on elucidation of $\gamma\delta$ T cell-mediated immune mechanisms at the level of the intestine. The calf model of localized subcutaneous *Map* infection has distinct advantages for in vivo study of $\gamma\delta$ T cell immunobiology and as such provides a much needed “window to an infection site,” yet in order to understand the full biologic significance of these findings as they relate to Johne’s disease, an intestinal model should prove valuable. As a result, the development of a model which results in consistent
intestinal colonization by Map (described in Chapter 4), represents a significant step forward. Filling significant knowledge gaps regarding the immunopathology of early and subclinical Map infection in cattle greatly enhances our ability to address the considerable challenges associated with prevention and diagnosis of Johne’s disease within the cattle industry. For example, a major factor leading to spread of Map within and between herds is the inability to accurately diagnose subclinically-infected animals [22]. Understanding the “immunologic footprint” during early vs. subclinical vs. clinical Johne’s disease, for example, allows for the improvement of current tests or the development of new diagnostic testing strategies for this disease at various stages of disease.

In conclusion, future studies should thus focus on two distinct but interconnected areas: 1) understanding the in vivo role of γδ T cells in shaping the cell-mediated immune response to Map, and 2) applying these data to the intestinal immunopathology of Map infection. Taken together, these studies are expected to continue to shed considerable light on the pathogenesis of Map infection in cattle.
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