2018

Investigating immunological responses of WC1+ gamma delta T cells in cattle naturally infected with mycobacterium avium subspecies paratuberculosis (MAP)

Saleh Mohammed Albarrak

Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd

Part of the Allergy and Immunology Commons, Immunology and Infectious Disease Commons, and the Medical Immunology Commons

Recommended Citation

Albarrak, Saleh Mohammed, "Investigating immunological responses of WC1+ gamma delta T cells in cattle naturally infected with mycobacterium avium subspecies paratuberculosis (MAP)" (2018). Graduate Theses and Dissertations. 16305.
https://lib.dr.iastate.edu/etd/16305

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
Investigating immunological responses of WC1+ gamma delta T cells in cattle naturally infected with *mycobacterium avium subspecies paratuberculosis* (MAP)

by

Saleh M. Albarrak

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Immunobiology

Program of Study Committee:
Jesse M. Hostetter, Co-Major Professor
Ray W. Waters, Co-Major Professor
Douglas E. Jones
Judith R. Stabel
Brett A. Sponseller

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2018

Copyright © Saleh M. Albarrak, 2018. All rights reserved.
DEDICATION

This dissertation is dedicated to my parents, my wife, my daughters and my relatives and friends.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>CHAPTER 1. GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Statement of the Problem</td>
<td>1</td>
</tr>
<tr>
<td>Central Hypothesis &amp; Specific Aims</td>
<td>2</td>
</tr>
<tr>
<td>Dissertation Organization</td>
<td>2</td>
</tr>
<tr>
<td>Literature Review</td>
<td>3</td>
</tr>
<tr>
<td>References</td>
<td>19</td>
</tr>
</tbody>
</table>

| CHAPTER 2. WC1+ γδ T CELLS FROM CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS RESPOND DIFFERENTIALLY TO STIMULATION WITH PPD-J | 30   |
| Abstract                                                               | 30   |
| Introduction                                                           | 31   |
| Materials and Methods                                                  | 33   |
| Results & Discussion                                                  | 41   |
| References                                                             | 47   |

| CHAPTER 3. DIFFERENTIAL CYTOKINE EXPRESSION BY WC1+ γδ T CELLS AND TOTAL MUCOSAL CELLS IN THE ILEUM OF CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS | 58   |
| Abstract                                                               | 58   |
| Introduction                                                           | 59   |
| Materials and Methods                                                  | 61   |
| Results & Discussion                                                  | 63   |
| References                                                             | 69   |
CHAPTER 4. WC1+ γδ T CELLS DEPLETION ALTERS CYTOKINE PRODUCTION BY PBMCs OBTAINED FROM CATTLE WITH THE SUBCLINICAL FORM OF MAP INFECTION.........................................................76
Abstract ..................................................................................................................76
Introduction .............................................................................................................77
Materials and Methods ..........................................................................................79
Results & Discussion ..............................................................................................82
References ...............................................................................................................87

CHAPTER 5. GENERAL CONCLUSIONS.................................................................96
Summary ..................................................................................................................96
Directions for Future Studies ................................................................................100
References ..............................................................................................................102
LIST OF FIGURES

CHAPTER 1. GENERAL INTRODUCTION

Figure 1  Schematic representation of the WC1 molecule indicating antibody binding sites ................................................................. 28
Figure 2  WC1+ γδ T cells links the innate and adaptive arms of the immune system ............................................................. 29

CHAPTER 2. WC1+ γδ T CELLS FROM CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS RESPOND DIFFERENTIALLY TO STIMULATION WITH PPD-J

Figure 1  Percentages of γδ T cells within (non-stimulated) peripheral blood lymphocytes ............................................................... 53
Figure 2  WC1+ γδ T cell subsets frequencies in the intestinal mucosa of control, subclinical and clinically infected cattle ................................................. 54
Figure 3  γδ T cells proliferative responses to MAP complex Ag, PPD-J .......... 55
Figure 4  γδ T cells IFN-γ response to MAP complex Ag, PPD-J ..................... 56
Figure 5  γδ T cells IL-10 response to MAP complex Ag, PPD-J .................... 57

CHAPTER 3. DIFFERENTIAL CYTOKINE EXPRESSION BY WC1+ γδ T CELLS AND TOTAL MUCOSAL CELLS IN THE ILEUM OF CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS

Figure 1  Photomicrographs of serial sections of bovine ileum stained with either HE for histopathological evaluation or ZN for MAP detection............. 73
Figure 2  Photomicrographs of bovine ileal tissue sections in which WC1, IL-10 and TGF-β mRNA messages are detected by in situ mRNA hybridization ..... 74
Figure 3  Differences in regulatory cytokine gene expression in ileal tissues from non-infected, subclinical and clinically infected cattle ......................... 75
Figure 4  Differences in proinflammatory cytokine gene expression in ileal tissues from non-infected, subclinical and clinically infected cattle ..................... 75

CHAPTER 4. WC1+ γδ T CELLS DEPLETION ALTERS CYTOKINE PRODUCTION BY PBMCs OBTAINED FROM CATTLE WITH THE SUBCLINICAL FORM OF MAP INFECTION

Figure 1  IFN-γ, IL-12, IL-4 and IL-10 production by PBMCs cultures stimulated for 24h with PWM, PPD-J or medium only ........................................ 92
Figure 2  Detection of intracellular IFN-γ in CD4+ T cells ............................... 93
Figure 3 Detection of intracellular IFN-γ in PBMCs cultures depleted of total γδ T cells (Δ γδ T cells) or WC1+ γδ T cells (Δ WC1+ γδ T cells) before stimulation for 24h with PPD-J or medium only ......................................................... 94

Figure 4 IFN-γ, IL-12, IL-4 and IL-10 production by PBMCs cultures depleted of total γδ T cells (Δ γδ T cells) or WC1+ γδ T cells (Δ WC1+ γδ T cells) before stimulation for 24h with PPD-J ......................................................... 95
LIST OF TABLES

CHAPTER 2. WC1+ γδ T CELLS FROM CATTLE NATURALLY INFECTED WITH Mycobacterium Avium Subsp. Paratuberculosis Respond Differentially to Stimulation with PPD-J

Table 1 Antibodies used in Immunofluorescence (IF) and flow cytometry (F) staining ........................................................................................................................................ 52

CHAPTER 4. WC1+ γδ T Cells Depletion Alters Cytokine Production by PBMCs Obtained from Cattle with the Subclinical Form of MAP Infection

Table 3 Antibodies used in cell sorting and flow cytometry staining ................... 91
ACKNOWLEDGMENTS

I would like to express my most profound gratitude to Dr. Jesse Hostetter, my mentor, for his patience, encouragement, and immense knowledge. I am very thankful for the unlimited academic and emotional support that Dr. Hostetter has given me throughout my journey to finish this thesis. I am very grateful to Dr. Ray Waters, my co-major professor, who helped join the Hostetter’s lab, which I could not imagine a better place to do my PhD work. This thesis could not be done without the help of Dr. Judy Stabel and her lab members who provided me with the samples whenever needed, I can not thank them enough, and their help is much appreciated. Thanks are extended to the rest of my committee members Dr. Douglas Jones and Dr. Brett Sponseller for their guidance and the support that they were always willing to provide.

I would like to express my sincere thanks to Tracy Lindquist, Mayara Maggioli, Jodi McGill, Kevin Esch, Taylor Wherry, Shawn Rigby and Christine Deal for their technical support and assistance. Thanks are extended to the current and past members of the Vet Path and the Immunobiology programs for the support and for the wonderful time I had here at ISU.
ABSTRACT

In these studies, we used non-infected cattle and cattle naturally infected with MAP that were either in the subclinical or clinical stage of infection to test the hypothesis that infection status influences WC1⁺ γδ T cells frequency, proliferation, and cytokine production. We found no significant differences between subclinical and clinical cattle with regard to WC1⁺ γδ T cells frequency in peripheral blood or in the ileum, a primary site for MAP infection. In PBMCs taken from MAP-infected cattle, WC1⁺ γδ T cells responded differentially to stimulation with PPD-J. In the clinical group, WC1⁺ γδ T cells failed to proliferate and the WC1.1 subset did not produce IFN-γ or IL-10 suggesting unresponsiveness. We evaluated the cytokine profile (mRNA) of the WC1⁺ γδ T cell subset in the ileum. Our data indicate a significant increase in the numbers of WC1⁺ γδ T cells expressing IL-10 in ileal tissues obtained from clinical cattle compared to subclinical and non-infected cattle. Expression of IFN-γ, TNF-α, IL-17 and TGF-β by the WC1⁺ γδ T cell subset in the ileum was comparable among the examined groups. We used subclinical infected cattle to evaluate the impact of WC1⁺ γδ T cell depletion on cytokine production by PBMCs stimulated ex vivo with PPD-J. Independent of antigen (PPD), depletion of WC1⁺ γδ T cells resulted in a significant decrease in IL-4 secretion and a significant increase in IL-10 secretion suggesting a modulatory role for the WC1⁺ subset in this system. The present dissertation supports our hypothesis that MAP infection status influences immunological responses of WC1⁺ γδ T cells in peripheral blood and at the sites of MAP infection. Our data suggest that the WC1⁺ γδ T cell subset may contribute to the immune responses that control MAP infection during the
subclinical stage and those that promote disease progression during the terminal stages of MAP infection.
CHAPTER 1: GENERAL INTRODUCTION

Statement of the Problem

The incidence of *Mycobacterium avium subsp. paratuberculosis* (MAP) infection continues to expand worldwide resulting in a considerable economic loss to beef and dairy industries. An early study estimated that MAP infection costs the US dairy industry between US$ 200 – US$ 250 million annually [1]. Control of MAP infection has been a challenge, in part due to the incomplete understanding of the host response to this infection. In cattle, previous studies have suggested a protective role for the WC1$^+$ $\gamma\delta$ T cell subset in the host response to mycobacterial infections, specifically to infection with *M. bovis* [2]. Our lab and others have demonstrated the responsiveness of WC1$^+$ $\gamma\delta$ T cells to MAP infection using experimental *in vivo* and *in vitro* infection systems with a primary focus on IFN-$\gamma$ production [3-5]. Studies examining immunological responses of the WC1$^+$ $\gamma\delta$ T cell subsets in cattle naturally infected with MAP are lacking. One interesting and challenging feature of MAP infection in cattle is the presence of subclinical and clinical stages which have been shown to associate with different immune responses [6]. It is not clear whether the responses of WC1$^+$ $\gamma\delta$ T cells differ with the stage of MAP infection. Thus, the primary goal of this thesis was to evaluate WC1$^+$ $\gamma\delta$ T cells frequency, immunological responses and functions (cytokine production) in cattle naturally infected with MAP to better understand how this T lymphocyte subset may influence the responses that control MAP infection and those that promote disease progression.
Central Hypothesis & Specific Aims

Three groups of cattle were used for the studies described in this thesis that included non-infected, subclinical and clinically infected cattle with MAP. In the subclinical group, MAP shedding was < 10 CFU/g of feces and animals were asymptomatic. In the clinical group, MAP shedding was > 100 CFU/g of feces and animals demonstrated weight loss and intermittent diarrhea. The infected groups were naturally infected. Our central hypothesis was that frequency, immunological responses and functions of WC1+ γδ T cells differ with the stage of infection (subclinical vs. clinical). To address this hypothesis, we developed the following specific aims: 1) determine the frequency of WC1+ γδ T cell subsets in peripheral blood and at the primary site of MAP infection, the distal-ileum. 2) Examine the immunological responses of WC1+ γδ T cell subsets in PBMCs stimulated ex-vivo with MAP antigens. 3) Evaluate the cytokine profile of the WC1+ γδ T cell subset at the sites of MAP infection (distal-ileum). We were further interested in defining the influence that the WC1+ γδ T cell subset may have on the host response controlling MAP infection during the subclinical stage. Thus, our final aim was 4) to examine cytokine production by PBMCs obtained from subclinically infected cattle and stimulated with MAP antigens in presence or absence of the WC1+ γδ T cell subset.

Organization of the Dissertation

The dissertation is composed of five chapters and organized in the alternative journal paper format with the first chapter being introduction and literature review. The next chapters are three individual manuscripts (chapters 2-4) with references cited at the
end of each chapter, followed by general conclusions (Chapter 5). One manuscript has been published in the *Journal of Veterinary Immunology and Immunopathology* (Chapter 2). Another manuscript is currently under review by the *Journal of Veterinary Immunology and Immunopathology* (Chapter 3). The third manuscript has been prepared for submission to the *Journal of Comparative Immunology, Microbiology and Infectious Diseases*.

**Literature Review**

**WC1+ gamma-delta (γδ) T cells: a possible role in the host response to**

*Mycobacterium avium subsp. paratuberculosis* (MAP) infection in cattle

1. Introduction

Understanding the host immune response to MAP infection at the cellular level is essential for overcoming the existing challenges regarding prevention and control of this infection. Gamma-delta (γδ) T cells, a subset of CD3+ T lymphocytes, have been an attractive research subject in studying mycobacterial infections. The growing interest in studying γδ T cells is attributed to their diverse functions that link the innate to adaptive arms of the immune system. This review will highlight the potential role(s) that WC1+ γδ T cells may play during mycobacterial infections in cattle with a particular reference to MAP infection.
2. Bovine γδ T cells

γδ T cell numbers within circulating lymphocytes is generally higher in ruminants than many domestic mammalian species. In young calves, more than 60% of circulating lymphocytes are γδ TCR+ [7, 8]. The percentage of circulating γδ T cells decreases to 8-18% in adult bovine which is still high compared to 2-5% in adult humans [8, 9]. This suggests that γδ T cells might be of particular importance to the functions of the bovine immune system, especially in young calves. Bovine γδ T cells are differentiated based on the surface expression of the workshop cluster 1 (WC1) molecule into two major subsets, WC1+ CD8neg and WC1neg CD8+ [10, 11]. The WC1+ subset represents the majority of γδ T cells in peripheral blood whereas the WC1neg subset dominates in tissues such as spleen and intestine [10]. Differential expression of genes encoding the WC1 molecule has been used to further divide the WC1+ γδ T cell subset into three serological subpopulations, WC1.1, WC1.2 and WC1.3. WC1.1 and WC1.2 are distinct subsets whereas the WC1.3 subset is a small subpopulation within the WC1.1 subset [12, 13].

Sequencing of the δ TCR loci revealed that the δ TCR chain is highly diverse in cattle compared to human and mice [14-16]. In cattle, there are 56 genes coding for the variable region (V) of the δTCR chain compared to 3 and 6 genes in human and mice, respectively [14]. This large expansion in the Vδ TCR genes in cattle suggests that γδ T cells might be more essential to the functions of the bovine immune system than to the functions of the human and mouse immune systems. While all combinations of Vδ TCR genes are used by both WC1+ and WCneg γδ T cell subsets, the WC1+ subset expresses only two combinations of the γTCR chain either Vγ3-C5 or Vγ7-C5 [17, 18]. The
WC1$^{\text{neg}}$ subset expresses all combinations of variable and constant genes in the $\gamma$TCR loci.

3. WC1 molecule

The WC1 molecule, a transmembrane glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR) super family (CD163), is uniquely expressed by $\gamma\delta$ T cells in many species including ruminants, pigs and poultry [19, 20]. In cattle, the WC1 molecule is encoded by at least 13 genes [15]. Each WC1 gene codes for a cytoplasmic tail, a transmembrane sequence and 11 extracellular SRCR domains (Fig. 1) [21]. The most distal SRCR domain, domain 1, has the greatest sequence variation among WC1 gene products and is thought to be determinant for antigen recognition by the WC1 receptor [21]. IL-A29 is a monoclonal antibody (mAb) that binds to the majority of WC1 gene products [13, 22]. Other mAbs have been shown to recognize subpopulations of the WC1$^+$ $\gamma\delta$ T cell subset (Fig. 1) [13].

The functions of the WC1 molecule remain to be fully elucidated. It has been suggested that the WC1 receptor provides costimulatory signals for the $\gamma\delta$ TCR similar to CD4 and CD8 molecules. This was supported by the presence of several tyrosine-based motifs on the cytoplasmic tail of the WC1 molecule [23]. A role as a pattern recognition receptor (PRR) has been also suggested. This was supported by the finding that the WC1.1 and WC1.2 subsets share the same $\gamma\delta$ TCR restrictions but respond differentially to different antigens [18, 24, 25]. Wang et al. demonstrated a role for the WC1 receptor in antigen recognition as down-regulating expression of 3 out of 13 WC1 genes using RNA interference significantly reduced $\gamma\delta$ T cell responses to stimulation with *Leptospira*. 
antigens [26]. It has been hypothesized that the WC1 molecule binds first to antigens and presents them to the γδ TCR which results in γδ TCRs recruitment to the immunological synapse [8]. Crosslinking of WC1 molecules did not activate γδ T cells, whereas cross-linking of WC1 with γδ TCR enhanced γδ T cell activation [23, 27].

4. Functional characteristics of bovine γδ T cells

γδ T cells have diverse immunological functions of innate or adaptive nature and thus have been termed as non-conventional T cells. In contrast to αβ T cells, γδ T cells are MHC-independent and can recognize and respond to protein and non-protein antigens, pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) [28, 29]. γδ T cells have been shown to recognize and respond to cells expressing stress-inducible antigens. MIC, a stress-inducible molecule, has been shown to activate WC1+ γδ T cells through its interaction with the NKG2D receptor [30]. Through the expression of granulysin and perforin, γδ T cells may play a cytotoxic role similar to natural killer cells (NK cells) [31, 32]. Activated bovine γδ T cells can upregulate expression of molecules such as MHC-II, B7-1, and B7-2 and induce CD4+ T cells proliferation meeting the innate function of antigen presentation [33, 34].

Following infection or vaccination, γδ T cells may 1) use their innate feature and produce cytokines that may influence the outcome of the adaptive response, and 2) become memory cells and provide a stronger and more robust response upon antigen reexposure. Few studies have examined the ability of γδ T cells to mount memory responses. A study by Lahmers et al. demonstrated that WC1+ γδ T cells isolated from calves vaccinated with major surface protein 2 (Msp2) of A. marginale responded
specifically to stimulation with Msp2 in vitro [25]. Interestingly, some of the Msp2-specific WC1\(^+\) \(\gamma\delta\) TCR\(^+\) clones were peptide-specific. These responses were MHC-independent, but antigen presentation by professional APCs was required. WC1\(^+\) \(\gamma\delta\) T cells proliferated and produced IFN-\(\gamma\) in recall responses to in vitro stimulation with *Leptospira* antigens following in vivo priming with a *Leptospira* vaccine [35, 36]. WC1\(^+\) \(\gamma\delta\) T cells from cattle vaccinated with killed *Leptospira borgpetersenii* have been shown to express markers of central memory such as CD62\(_L^{\text{high}}\) and CD45RO\(_{\text{int}}\) [36].

\(\gamma\delta\) T cells contribute to regulation of the bovine immune system. Hoek et al. demonstrated that bovine CD4\(^+\) CD25\(^{\text{high}}\) Foxp3\(^+\) T cells (Treg) were not suppressive in vitro. Instead, regulatory functions appeared to be mediated by the WC1\(^+\) \(\gamma\delta\) T cell subset [37]. Guzman et al. have recently demonstrated that bovine \(\gamma\delta\) T cells were able to suppress proliferation and IFN-\(\gamma\) production by CD4\(^+\) and CD8\(^+\) T cells and that the suppressive \(\gamma\delta\) T cells were present in both WC1\(^+\) and WC1\(^{-}\) populations [38].

5. Functional subsets of bovine \(\gamma\delta\) T cells

Besides the differences in their anatomic distribution, functional differences between \(\gamma\delta\) T cell subsets have been reported. Because of their high frequency in peripheral blood, the vast majority of studies examining bovine \(\gamma\delta\) T cells have focused on the WC1\(^+\) \(\gamma\delta\) T cell subset. Gene expression analysis has suggested an immune surveillance role for the WC1\(^{-}\) subset with genes promoting apoptosis and tissue homeostasis being elevated [39, 40]. This observation is consistent with their localization to mucosal tissues such as the intestine. In contrast to the WC1\(^{-}\) subset, the WC1\(^+\) \(\gamma\delta\) T
cell subset has been shown to be more active with regard to proliferation and proinflammatory cytokines production [39, 40].

It is evident that WC1+ γδ T cells reaction to stimulation with antigens varies based on the expressed WC1 gene [24]. WC1.1+ and WC1.2+ γδ T cell subsets proliferate to mitogen stimulation with a bias towards the responses by the WC1.2 subset [41]. Proliferation and IFN-γ production by γδ T cells to stimulation with Leptospira antigens were mainly mediated by the WC1.1 subset [41]. The WC1.2+ γδ T cell subset has been shown to proliferate and produce IFN-γ in response to stimulation with Anaplasma marginales antigens [25]. Both WC1.1 and WC1.2 subsets have been shown to proliferate and produce IFN-γ in response to experimental M. bovis infection [42]. There are gaps in the current knowledge regarding immunological responses of γδ T cell subsets to MAP infection and whether these responses change over the course of MAP infection.

6. The host’s response to MAP infection

MAP infection (Johne’s disease) is a chronic and progressive enteric disease that affects ruminants worldwide and causes considerable economic loss to producers [43, 44]. Due to the incomplete understanding of the host response to MAP infection, efforts to control and eliminate the disease have been unsuccessful. In bovine, appearance of clinical signs is typically preceded by a prolonged subclinical phase that typically lasts 2-5 years [6]. The subclinical stage usually associates with a Th1 type immune response characterized by secretion of proinflammatory mediators such as IFN-γ, TNF-α, IL-2 and IL-12 [45-47]. The Th1 response is essential for controlling MAP replication and diseases progression [48]. This response typically diminishes as infected animals
progress to the clinical disease. The clinical stage of MAP infection is typically associated with a prominent MAP-specific Th2 type antibody response (IgG1) that has been shown to correlate with MAP shedding and development of granulomatus lesions in the small intestine; consistently in the ileum and ileocecal valve [6, 49, 50]. Recent studies have questioned a distinct shift in the host response to Map infection from Th1 to Th2. A study by Vazquez et al. has indicated a lack of clear polarization to Th1/Th2 immune responses in cattle naturally infected with MAP [51]. Moreover, IFN-γ and antibody responses were detected early in sheep experimentally infected with Map and were present in both paucibacillary and multibacillary forms [52]. Together, these studies suggested that the host response to MAP infection is complex and that humoral and cellular immunity may play a role during both early and late stages of MAP infection.

Progression to the clinical form of MAP infection has been hypothesized to be facilitated by development of regulatory responses that neutralize the effects of the Th1 response [48]. Previous studies have reported differences in the cytokine profile between the subclinical and clinical stages of MAP infection. In the ileum, a primary site for MAP infection, IFN-γ expression has been shown to be higher in subclinical cattle, whereas clinical cattle expressed higher levels of IL-10, TGF-β and IL-4 [46, 53]. Whether the cytokine profile of WC1+ γδ T cells differs with the stage of MAP infection is unknown.

7. WC1+ γδ T cells contribute to the host’s response to MAP infection

There is a growing body of literature suggesting a protective role for the WC1+ γδ T cell subset in the host response to mycobacterial infections in cattle. Besides being an early and significant source of IFN-γ, WC1+ γδ T cells have been shown to promote
development of the effective Th1 response and contribute to granulomas formation and maintenance [3, 4, 54, 55]. In contrast to the WC1\(^+\) subset, the role of the WC1\(^{-}\) subset in the host response to mycobacterial infections is less studied. However, it has recently been shown that the WC1\(^{-}\) subset is capable of producing IFN-\(\gamma\) in response to experimental MAP infection both \textit{in vivo} and \textit{in vitro} [4, 56].

Bovine \(\gamma\delta\) T cells have been shown to undergo dynamic changes in circulation following mycobacterial infections. The frequency of WC1\(^+\) \(\gamma\delta\) T cells in peripheral blood has been shown to increase in calves following vaccination with BCG. Interestingly, the IFN-\(\gamma\) levels in the BCG vaccinates related to the numbers of WC1\(^+\) \(\gamma\delta\) T cells and not CD4\(^+\) or CD8\(^+\) T cells [57]. A study by Badi et al. reported a significant increase in the frequency of WC1\(^+\) \(\gamma\delta\) T cells in blood and milk of cattle subclinically infected with MAP compared to their non-infected counterparts [58]. \(\gamma\delta\) T cell percentages in peripheral blood have been shown to increase in calves following infection with MAP compared to infection with \textit{M. bovis} [59]. Together these studies suggested a role for the WC1\(^+\) \(\gamma\delta\) T cell subset in the host response to mycobacterial infections.

7.1 WC1\(^+\) \(\gamma\delta\) T cells recognize various mycobacterial antigens

Bovine \(\gamma\delta\) T cells have been shown to recognize and respond to various mycobacterial antigens. Welsh et al. demonstrated responsiveness of WC1\(^+\) \(\gamma\delta\) T cells to mycobacterial protein and nonprotein antigens with responses to protein antigens being predominant [60]. WC1\(^+\) \(\gamma\delta\) T cells have also been shown to proliferate in response to stimulation with prenyl pyrophosphates such as isopentenyl pyrophosphate (IPP) (IPP can be secreted by mycobacteria) [60]. WC1\(^+\) \(\gamma\delta\) T cells from \textit{M. bovis} infected cattle
proliferated and produced IFN-γ in recall responses to stimulation with protein and non-protein antigens of *M. bovis* [42]. These antigens included the wall components mycolylarabinogalactan-peptidoglycan (mAGP) and lipoarabinomannan (LAM). In this study, the WC1⁺ γδ T cells responding to *in vitro* stimulation with *M. bovis* antigens were a mixture of WC1.1 and WC1.2 subpopulations [42]. The same study demonstrated that γδ T cells purified from peripheral blood (the majority are WC1+) are capable of responding to a peptide cocktail derived from ESAT6: CFP10 protein. These responses were MHC II-independent but required direct contact with APCs. Identification of bovine γδ T cells cognate antigens, receptors and mechanisms involved in γδ T cells activation could assist in development of strategies to target γδ T cells by future vaccines.

7.2 Cytokines involved in WC1⁺ γδ T cell responses to mycobacterial infections

The host response to invading pathogens and the outcome of infections rely on cytokines produced by various cell types including WC1⁺ γδ T cells. Besides cytokines production, WC1⁺ γδ T cells augment cytokine production by other cell types. One example is the enhancement in IL-12 production by *M. bovis*-infected dendritic cells (DCs) cocultured with the WC1⁺ γδ T cell subset [61]. Bovine WC1⁺ γδ T cells produce cytokines including IFN-γ, TNF-α, IL-17, IL-10, and TGF-β, which represent different immune responses [38, 42, 62, 63]. Below cytokines produced by WC1⁺ γδ T cells with known relevance to the pathogenesis of MAP infection is discussed.
7.2.1 Interferon gamma (IFN-γ)

IFN-γ is essential for protection against intracellular pathogens including mycobacteria [64]. IFN-γ activates macrophages to kill intracellular MAP and further derives development of the Th1 immune response [65]. Mice lacking the IFN-γ gene have been shown to be highly susceptible to infection with intracellular pathogens [66]. Humans expressing mutated IFN-γ receptor chains exhibit high susceptibility to infection with non-pathogenic species of mycobacteria [67]. In the bovine, WC1⁺ γδ T cells have been shown to be a significant source of IFN-γ. CD4⁺ and WC1⁺ γδ T cells were the major IFN-γ producers in antigen-stimulated PBMCs of cattle vaccinated against Leptospira [68]. WC1⁺ γδ T cells proliferating in response to stimulation with Leptospira antigens were shown to express IFN-γ at comparable levels to CD4⁺ T cells [69].

IFN-γ production by γδ T cells is critical in the sense that the γδ T cell response can precede and may influence initiation of the adaptive response. Bovine γδ T cells, isolated from healthy cattle, produced a significant amount of IFN-γ in response to stimulation with live mycobacteria and mycobacterial products [70]. WC1⁺ γδ T cells have been shown to be the major source of IFN-γ during the first month after cattle vaccination against serovar Hardjo strains of Leptospira [35, 71]. Following in vitro stimulation with Leptospira antigens, WC1⁺ γδ T cells specifically proliferated and produced IFN-γ hours to days before CD4⁺ T cells [36]. Bovine γδ T cells have been shown to infiltrate MAP-infection sites earlier than CD4⁺ T cells. Interestingly, our lab has previously shown that the early infiltration of γδ T cells to MAP infection sites associated with a significant increase in IFN-γ production at these infection sites [4]. Baquero et al. recently reported that both WC1⁺ and WC1neg γδ T cell subsets produced
IFN-γ and induced a significant reduction in MAP viability when cocultured with MAP-infected monocytes-derived macrophages (MDMs) [5, 56]. As mentioned earlier, WC1⁺ γδ T cells from cattle experimentally infected with *M. bovis* mounted a specific IFN-γ response following ex-vivo stimulation with protein and non-protein antigens of *M bovis*. IFN-γ production by WC1⁺ γδ T cells was mediated by both WC1.1 and WC1.2 subpopulations [42].

7.2.2. Interleukin-10 (IL-10)

IL-10 is an inhibitory cytokine that regulates the host’s innate and adaptive responses [72]. IL-10 favors MAP replication and disease progression through macrophages deactivation and suppression of Th-1 responses [73, 74]. A study by Hoek et al. reported that bovine CD4⁺ CD25<sup>high</sup> Foxp3⁺ T cells (Treg) were not suppressive in vitro, and that the Treg activities in the bovine system were mediated by the WC1⁺ γδ T cell subset [37]. Another study by Guzman et al. suggested a major regulatory role for γδ T cells in cattle. Besides proliferation to stimulation with IL-10, bovine γδ T cells produced IL-10 and suppressed antigen-specific and non-specific responses of CD4⁺ and CD8⁺ T cells. The suppressive effect of γδ T cells was inhibited by addition of anti-IL-10 to stimulated cultures. In this study, suppressive γδ T cells were shown to be present in both WC1<sup>neg</sup> and WC1⁺ γδ T cell subsets [38]. Progression to the clinical form of MAP infection has been hypothesized to be facilitated by increased expression of regulatory cytokines such as IL-10 and TGF-β [53]. Whether WC1⁺ γδ T cells promote suppression of Th1 responses during late stages of MAP infection by producing regulatory cytokines such as IL-10 is unknown.
7.2.3. Interleukin-17 (IL-17)

Studies examining IL-17 production by γδ T cell subsets in cattle naturally infected with MAP have not been reported. IL-17 responses have been shown to increase significantly in *M. tuberculosis*-infected human, BCG challenged mice, *M. bovis* infected cattle, and MAP infected cattle [42, 75-78]. The role of IL-17 in protective immunity against mycobacterial infections remains to be fully understood. IL-17 has been shown to contribute to the host’s protective immune response by inducing expression of essential cytokines such as IFN-γ as well as adhesion molecules required for cells recruitment and granuloma formation such as ICAM-1 and LFA-1 [75, 79]. Impaired granulomas formation has been reported in IL-17 deficient mice challenged with BCG [79]. IL-17A receptor deficient mice were shown to be defective in long-term control of *M. tuberculosis* [80]. However, excessive IL-17 responses have been shown to associate with increased lesion severity and high bacterial load [81-83].

γδ T cells have been shown to be a primary source of IL-17 in TB-infected mice [75, 84]. Steinbach et al. have recently demonstrated that CD4+ T cells and γδ T cells (the majority are assumed to be WC1+) are the primary producers of IL-17 in cattle naturally infected with my *M. bovis* [62]. Peripheral (WC1+) γδ T cells from cattle experimentally infected with *M. bovis* produced IL-17 specifically in response to in vitro stimulation with *M. bovis* antigens. In this study McGill et al. have demonstrated that CD4+ T cells were the major source of IL-17 [42]. Examining systemic and local expression of IL-17 by different lymphocyte subsets in cattle naturally infected with MAP is necessary for understanding the role of this cytokine during MAP infection.
7.3 Infiltrating the sites of MAP infection

Studies examining the frequency of γδ T cell subsets at the sites of MAP infection in naturally infected cattle are lacking. Previous studies have demonstrated bovine γδ T cells recruitment to the infection sites (granulomas) in animals with mycobacterial infection. However, the majority of these studies have focused on γδ T cells or the WC1+ population as a whole. As described earlier, immunological responses of WC1+ γδ T cells vary based on the expressed WC1 gene [41]. WC1+ γδ T cells have been shown to infiltrate the lungs and lymphoid tissues of the respiratory tract shortly after calves are vaccinated with BCG. These infiltrating γδ T cells expressed the WC1.1 isoform [85]. A study by McGill et al. reported a significant infiltration of WC1+ γδ T cells to lung lesions of cattle experimentally infected with *M. bovis*. In this study, the majority of infiltrating WC1+ γδ T cells were WC1.2+ [42]. Using a subcutaneous Matrigel as a vehicle containing live MAP, Plattner et al. demonstrated the early recruitment of γδ T cells to Matrigel/MAP sites. Interestingly, recruited γδ T cells were activated and produced IFN-γ [4]. Charavaryamath et al. experimentally infected newborn calves by inoculated surgically isolated ileal segments with MAP, and MAP was detected at the infection sites nine months post infection. The study has reported a significant increase in the numbers of CD8+ and γδ T cells in the lamina propria along with a significant increase in secretion of MAP-specific IFN-γ and TNF-α by the lamina propria leukocytes [86].
7.4 Granuloma formation

A role for γδ T cells in granulomas formation and maintenance has been suggested. Following MAP infection, mice lacking γδ T cells exhibited a significant reduction in granuloma formation compared to mice with γδ T cells [87]. Mice deficient for γδ T cells successfully controlled infection with BCG and a low dose of *M. tuberculosis* [88, 89]. However, granulomatous lesions in these mice were larger and less organized. Smith et al. used a fetal bovine-severe combined immunodeficient (SCID-bo) xenochimeric mouse model to evaluate the protective role of WC1⁺ γδ T cells in the host response to *M. bovis* [90]. In this study, the bacterial load was similar between nontreated bo-mice and bo-mice depleted of WC1⁺ γδ T cells by being treated with an antibody specific for the WC1 receptor (ILA29). However, WC1⁺ γδ T cells depleted bo-mice exhibited altered granuloma architecture characterized by presence of areas of caseous and liquefactive necrosis along with increased infiltration of neutrophils. Our lab previously demonstrated an association between the degree of granuloma organization and the recruitment timing of the WC1⁺ γδ T cell subset in calves. In well-organized granulomas induced by killed MAP bacterin (Mycopar), WC1⁺ γδ T cells infiltrated earlier; however, over time, the WC1⁺ γδ T cell subset became dominant as granulomas matured. The reverse sequence was observed in disorganized granulomas induced by live MAP [3]. Palmer et al. evaluated the potential role of γδ T cells in granuloma development in calves experimentally infected with *M. bovis* [91]. Although the study did not examine WC1 expression on infiltrating γδ T cells, Palmer et al. demonstrated that the numbers of CD8⁺ T cells and γδ T cells were highest in early stage granulomas, but reduced as granulomas matured. The study suggested that the failure of the immune response to
control the infection might be attributed to the loss of these T cell subsets [91]. Together, these studies suggested a role for bovine γδ T cells in directing granuloma formation during mycobacterial infections.

7.5 WC1⁺ γδ T cells may promote the Th1 response

Many studies have suggested that WC1⁺ γδ T cells may play a role in the formation of appropriate immune responses against intracellular pathogens (Fig 2) [92]. WC1⁺ γδ T cells are among the first responders to mycobacterial infections [4, 93], and thus, the cytokines they produce may drive the host response towards a predominant Th-1 or Th-2 phenotype. WC1⁺ γδ T cells have been shown to secrete large quantities of IFN-γ in response to stimulation with IL-12 and IL-18 [94]. Kennedy et al. examined the effects of WC1⁺ γδ T cells on the host’s response to infection with *M. bovis* [54]. In this study, calves were depleted of WC1⁺ γδ T cells before being challenged with *M. bovis*. Although depletion of WC1⁺ γδ T cells had no significant impact on disease progression, calves depleted of WC1⁺ γδ T cells exhibited a reduction in IFN-γ and IgG2 responses along with a notable increase in IL-4 and IgG1 responses, which suggested altered immune phenotype. Interestingly, similar observations were reported in calves depleted of WC1⁺ γδ T cells and infected with bovine respiratory syncytial virus (BRSV) [95, 96]. SCID-bovine mice depleted of WC1⁺ γδ T cells before being infected with *M. bovis* exhibited significantly lower levels of circulating IL-12 compared to their non-depleted counterparts [32]. Together, these in vivo studies suggested a role for the WC1⁺ γδ T cell subset in manipulating the host immune responses to infections.
WC1⁺ γδ T cells may influence the host responses to mycobacterial infections through interaction with dendritic cells (DCs). WC1⁺ γδ T cells produce IFN-γ and are well placed to interact with DCs in blood and in peripheral tissues. Following vaccination of calves with BCG, WC1⁺ γδ T cells have been shown to migrate to the lungs and respiratory lymph nodes and selectively cluster with DCs suggesting crosstalk between DCs and WC1⁺ γδ T cells in vivo [85, 97]. When co-cultured with M. bovis-infected DCs, bovine WC1⁺ γδ T cells expressed higher levels of CD25 and IFN-γ and DCs had increased IL-12 production suggesting reciprocal interactions between DCs and WC1⁺ γδ T cells [61]. These interactions have been shown to be contact-dependent [61]. Baquero et al. have recently demonstrated that WC1⁺ γδ T cells induce maturation of MAP infected DCs as defined by their reduced phagocytic ability [98]. This is important when taken into consideration that DCs are the most potent T cells activators and the most efficient APCs in priming naïve αβ T cells.

8. Conclusion

Further research is needed to fill the gaps in the current knowledge regarding the role(s) of γδ T cells in the host response to MAP infection. These gaps included defining γδ T cells cognate antigens, receptors, mechanisms of activation and migration, and functional responses at the sites of MAP infection. Identifying γδ T cells ligands and discovering the best way to prime and maintain a good γδ T cells memory population is essential for targeting γδ T cells by future vaccines. Previous studies have demonstrated the early recruitment of activated γδ T cells to the sites of MAP infection and suggested that γδ T cells may influence the host specific (adaptive) response to MAP infection.
However, these studies have looked at γδ T cells or the WC1⁺ population as a whole with a primary focus on IFNγ production. Studies examining frequency, immunological responses and functions of γδ T cell subsets in cattle naturally infected with MAP are lacking. One important question that warrants answering is whether γδ T cell subsets frequency, immunological responses and functions change over the course of natural MAP infection.

Exciting advancements have been made in exploring the role of bovine γδ T cells in the host response to mycobacterial infections, particularly in response to infection with *M. bovis*. However, MAP and *M. bovis* are two different pathogens causing two separate diseases, and thus, differences in γδ T cells responses to these two granulomatous infections may exist. Studying γδ T cells responses and functions in cattle naturally infected with MAP is essential for a complete understanding of the host response to MAP infection and for incorporating the γδ T cells to novel treatment and vaccine strategies.

**References**


Figure 1. Schematic representation of the WC1 molecule indicating antibody binding sites (source: Guzman et al. 2012)
Figure 2. WC1+ γδ T cells links the innate and adaptive arms of the immune system (adapted from Baldwin et al. 2014).
CHAPTER 2: WC1+ γδ T CELLS FROM CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS RESPOND DIFFERENTIALLY TO STIMULATION WITH PPD-J

Modified from a manuscript published in the *Journal of Veterinary Immunology and Immunopathology*

*Vet Immunol and Immunopathol* (2017); 190:57-64.

Albarrak SM¹, Waters WR², Stabel JR², Hostetter JM¹

From the Department of Veterinary Pathology ¹ and the Department of Veterinary Microbiology and Preventive Medicine ², College of Veterinary Medicine, Iowa State University, 1600 S 16th St, Ames, IA 50011

**Abstract**

A role for γδ T cells in protection against mycobacterial infections including Johne’s disease (JD) has been suggested. In neonatal calves where the risk to infection with *Mycobacterium avium subsp. paratuberculosis* (MAP) is high, the majority of circulating CD3⁺ lymphocytes are γδ TCR⁺. Bovine γδ T cells are divided into two major subsets based on the surface expression of workshop cluster 1 (WC1). The WC1⁺ subset, the predominant subset in periphery, is further divided into WC1.1⁺ and WC1.2⁺ subpopulations. The ability of γδ T cells to produce IFN-γ prior to CD4⁺ αβ T cell activation could be crucial to the outcome of MAP infection. In the current study, cattle were naturally infected with MAP and were classified as either in the subclinical or clinical stage of infection. Compared to the control non-infected group, γδ T cell frequency in circulating lymphocytes was significantly lower in the clinical group. The observed decline in frequency was restricted to the WC1.2⁺ subset, and was not
associated with preferential migration to infection sites (distal-ileum). \( \gamma \delta \) T cells proliferated significantly in recall responses to stimulation with purified protein derivative from MAP (PPD-J) only in subclinically infected cattle. These responses were a heterogeneous mixture of WC1.1 and WC1.2 subsets. Proliferation and IFN-\( \gamma \) production by the WC1.1\( ^+ \) \( \gamma \delta \) T cell subset was significantly higher in the subclinical group compared to the control and clinical groups. Our data indicates differences in MAP-specific ex-vivo responses of peripheral WC1\( ^+ \) \( \gamma \delta \) T cells of cattle with the subclinical or clinical form of JD.

**Introduction**

*Mycobacterium avium subsp. paratuberculosis* (MAP) is the causative agent of Johne’s disease (JD), a debilitating chronic granulomatous enteritis affecting ruminants worldwide [1-4]. JD is a serious economic concern to dairy and beef industries due to reduced productivity and early culling of infected animals [5]. Calves typically become infected early in life, mainly through the fecal-oral route [6]. One hallmark of JD is the lengthy subclinical phase during which infection is controlled by a dominant cell mediated (Th1) immune response. Usually, progression to the clinical form of JD is associated with development of an ineffective humoral response (Th2) [7]. Mechanisms regulating this shift in the immune response during the course of MAP infection remain to be elucidated.

The role of gamma delta (\( \gamma \delta \)) T cells in the pathogenesis of mycobacterial infections is receiving a growing interest due to their diverse functions spanning the innate to adaptive immunity. In calves, \( \gamma \delta \) T cells circulate in high numbers and are the
dominant CD3+ lymphocyte subset [8-11]. Bovine γδ T cells are differentiated into two
phenotypically distinct subsets based upon the surface expression of a workshop cluster 1
(WC1) co-receptor [12]. The WC1- subset represents the majority of γδ T cells in organs
such as spleen and intestine, while the WC1+ subset is primarily found in peripheral
blood [13]. The WC1 receptor is encoded by a complex gene structure consisting of at
least 13 genes [14, 15]. Differential expression of the WC1 gene is used to further divide
the WC1+ γδ T cell subset into three serological subpopulations, WC1.1, WC1.2 and
WC1.3 [16]. While WC1.1 and WC1.2 are nonoverlapping subpopulations, a small
portion of WC1.1+ γδ T cells express the WC1.3 isoform.

It is evident that the responses of WC1+ γδ T cells to stimulation with antigens
vary based on the expressed WC1 gene. γδ T cells responding to stimulation with
Leptospira antigens are WC1.1+ [17]. WC1.2+ γδ T cells proliferate and produce IFN-γ in
response to stimulation with antigens from Anaplasma marginale [18]. Both subsets have
been shown to respond to stimulation with antigens from M. bovis [11]. Although the
exact function of the WC1+ molecule remains unknown, recent reports suggest that WC1
molecules act as pattern recognition receptors (PRRs) on γδ T cells, similar to TLRs [19].

WC1+ γδ T cells are suggested to play a significant role in granuloma
development and/or maintenance early during mycobacterial infections and may
influence the initiation of the adaptive immune response. The γδ T cells infiltrating lung
granulomas in bovine tuberculosis are of WC1+ phenotype [20, 21]. Another in vivo
study done in our lab showed that WC1+ γδ T cells accumulate in MAP-induced
granulomas [22]. Recently, McGill and colleagues have demonstrated that the γδ T cell
response specific to Mycobacterium bovis is composed of a heterogeneous mix of WC1-
expressing populations and that both WC1.1+ and WC1.2+ subsets responded to in vitro stimulation with mycobacterial antigens and accumulated at the site of infection [11].

The goal of the current study was to evaluate the immunological functions of WC1+ γδ T cell subsets in cattle naturally infected with MAP to better understand the role of these cells in host defense during natural MAP infection. We evaluated WC1+ γδ T cells in subclinical and clinically infected cattle to address two questions concerning the relationship of WC1+ γδ T cell subsets to shifts in immune responses and progression to the clinical disease. First, we sought to identify differences in frequency of WC1+ γδ T cell subsets within circulating lymphocytes and in the distal-ileum mucosa in subclinical and clinically infected cattle. Second, we asked if the WC1+ γδ T cell subsets would differentially respond to ex vivo stimulation with purified protein derivative from MAP (PPD-J). Our data support the hypothesis that WC1+ γδ T cells promote protective immunity during the subclinical phase of MAP infection. Our results demonstrated that WC1.1+ γδ T cells proliferated and produced IFN-γ in recall responses to ex vivo stimulation with PPD-J and that these responses were detected only in the subclinical animals. The data presented in this study suggests that the WC1.1+ γδ T cell subset represents an early source of IFN-γ during MAP infection and may serve as a marker of protection for future MAP vaccines to target.

Materials and Methods

Animals

All animal procedures in this study were approved by the IACUC of the National Animal Disease Center (Ames, Iowa). Treatment groups (4-5 animals/group) consisted
of adult Holstein dairy cows with subclinical MAP infection (average age: 7.2 years), clinical MAP infection (average age: 7.0 years) and non-infected animals (average age: 4.2 years). The infected groups were naturally infected with MAP. In the clinical group, MAP shedding exceeded 100 CFU/g of feces and animals demonstrated weight loss and intermittent diarrhea. In the subclinical group, MAP shedding was less than 10 CFU/g of feces and animals were asymptomatic. Control non-infected cows were acquired from accredited Johne’s disease-free herds and repeatedly tested negative by fecal culture and serological assays.

Antibodies
Primary and secondary antibodies used for cell phenotyping by flow cytometry and fluorescent microscopy are listed in Table 1.

Preparation of PBMC
Blood samples were collected from the jugular vein into 60 cc syringes containing 3-4 ml 0.5M EDTA, pH 8.0 (Life Technologies, Grand Island, NY, USA). EDTA was replaced by Acid Citrate Dextrose (ACD) when assessing cytokine production. PBMCs were purified from buffy coat fractions by density centrifugation. Contaminated RBCs were removed by osmotic lysis. Cells were washed with Alsever’s solution (Sigma, St. Louis, MO, USA) and resuspended in complete RPMI (Life Technologies) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 2% essential amino acids, 1% sodium pyruvate, 50 mM 2-ME, penicillin–streptomycin (100 units/ml
penicillin and 0.1 mg/ml streptomycin), and 10% (v/v) FBS (all obtained from Life Technologies).

PBMC proliferation assay

For PBMCs proliferation assays, cells were labeled with 1.25 μM carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) and then cultured for 6 days at 37°C (5% CO₂) at a density of 2.5 × 10⁵ cells/well in 96-well plates (Sigma). Cells were stimulated in triplicate wells with PPD-J (10 µg/ml, USDA, Ames, IA, USA). Con A (Concanavalin A, Sigma, 2 µg/ml) was used as a positive control and medium alone was used as a negative control for cells stimulation. Results were corrected for background proliferation by subtracting the percentage of cells that divided in non-stimulated cultures.

Cytokine secretion assay

For intracellular staining of IFN-γ and interleukin-10 (IL-10), PBMC were isolated as described above. Cells were cultured for 24 hours (IFN-γ) or 6 days (IL-10) at 37°C at a density of 5 × 10⁵ cells/well in 96-well plates. Cells were stimulated in triplicate wells with PPD-J (10 µg/ml). Pokeweed mitogen (2 µg/ml) and medium only (non-stimulated) were used as positive and negative controls, respectively. Brefeldin A (10 µg/ml, Sigma), ionomycin (500 ng/ml, Sigma) and phorbol 12-myristate 13-acetate (PMA) (50 ng/ml, Sigma) were added to cultures for the last 3 hours of incubation.
Staining for flow cytometry

Staining was performed in wells of 96-well plates (Sigma). For surface staining, cells were resuspended in FACS buffer (PBS with 2% FCS and 0.02% (w/v) sodium azide) and incubated for 20 min on ice with primary Abs (2 µg /ml of each). Cells were washed once and incubated for 20 min on ice with secondary Abs (0.2 µg /ml). PBMCs were then washed and fixed with 2% paraformaldehyde fixation buffer. For intracellular staining cells were resuspended in a permeabilization buffer (FACS buffer containing 0.5% (w/v) saponin) containing anti-bovine IFN-γ (10 ug/ml,) and anti-bovine IL-10 (2 ug/ml,) (all obtained from AbD Serotec, Raleigh, NC, USA) and incubated for 30 mins at 4°C. Cells were washed once with the permeabilizing buffer and incubated for 30 mins at 4°C with secondary Abs (0.2 µg /ml). Cells were then washed and fixed with a stabilizing fixative solution (BD Biosciences, San Jose, CA, USA).

Immunofluorescence staining of tissue sections

For immunofluorescence staining, archive tissues from non-infected, subclinical and clinically infected cattle were used. Tissues were selected from animals that met the grouping criteria described in section 2.1. Samples of distal-ileum were cut into 6-µm sections and fixed in 50% acetone / 50% methanol. Blocking and staining were carried out at RT in a humidified chamber. Briefly, tissue sections were blocked with goat serum (Thermo Fisher Scientific, Waltham, MA USA) for 30 mins. The tissue sections were then incubated with primary Abs for 90 mins followed by 3 washes (3 mins each). The first wash was with 0.05 M Tris buffer followed by washing with 0.05 M Tris buffer containing 0.2% Tween-20 and 0.9% NaCl. The third wash was with 0.05 M Tris buffer.
Tissue sections were incubated with secondary Abs (1.5 ug/ml) for 1 hour, washed as described earlier and mounted with ProLong® Gold Antifade Reagent with DAPI (Life technologies). Anti γδ TCR, WC1 and WC1.2 antibodies were used at a dilution of 1:1000 (0.05 M Tris buffer) while anti WC1.1 was used at a dilution of 1:50. For information about the Abs, please refer to table 1. To quantify the number of γδ T cell subsets, the number of each subset present in a single highly-magnified field was counted based upon the surface expression of γδ TCR, WC1, WC1.1 and WC1.2 molecules. Six high-magnification fields were counted for each animal, and a total of 5-7 animals per group were examined.

Statistics

JMP pro 12 (SAS institute, Cary, NC) software was used for statistical analysis. All data are shown as means ± SEM. ANOVA with Tukey’s HSD and Wilcoxon Each Pair were used to determine the significance of differences between group means.

Results

WC1⁺ γδ T cell subset frequencies within circulating lymphocytes

We set out to determine the frequency of γδ T cells within circulating lymphocytes. We isolated PBMCs from non-infected, subclinical and clinically infected cattle, labeled for CD4 and the γδ TCR and measured percentages of these cells via flow cytometry. We found that while the frequency of CD4⁺ T cells was comparable between groups, the percentage of total γδ T cell in peripheral lymphocytes was lower in infected animals and this was significant in the clinical group (P = 0.042) (Fig. 1B). Since the
WC1+ γδ T cell subset represents the majority of circulating γδ T cells, we tested whether the lower γδ T cell percentages observed in the clinical group was restricted to a specific WC1+ subpopulation. To test this hypothesis, we specifically stained for WC1.1 and WC1.2 molecules to differentiate between these two major WC1+ subpopulations using flow cytometry (Fig 1A). We found that in the subclinical and clinical groups, the percentages of circulating WC1.2+ γδ T cells were significantly lower compared to that of the control group (P < 0.01) (Fig. 1D). In all the examined groups, around 25% of circulating γδ T cells expressed the WC1.1 serotype. In the control group, 50% of circulating γδ T cells were WC1.2+ compared to 25-30% in the infected groups (Fig. 1C and D).

WC1+ γδ T-cell subset frequency in distal-ileum

We next determined the frequency of γδ T cell subsets in the small intestine. We examined the ileum, as this is a primary target tissue of MAP infection and lesion development in cattle (Clarke 1997). We measured the number of γδ T cells within WC1 subsets in the distal-ileum from non-infected, subclinical and clinically infected cattle by immunofluorescence. Frozen distal-ileum sections were stained with Abs specific to the γδ TCR, WC1 (total), WC1.1 and WC1.2 surface antigens (Fig. 2A). As expected, in non-infected and clinically infected cattle, γδ T cells in the distal-ileum were predominantly WC1+ and were frequently observed near or within the mucosal epithelium (P < 0.02) (Fig. 2A and B). In subclinically infected cattle, the number of WC1− γδ T cells was elevated over the WC1+ subset, but did not reach statistical significance (P = 0.09). WC1+ γδ T cells localized to the lamina propria and their numbers were similar among
the groups (Fig. 2A and B). Similarly, there were no significant differences in the total numbers of the WC1+ subsets (subpopulations) among control, subclinical or clinically infected cattle. (Fig. 2A and C). Although there was a trend for the WC1.2 to be increased over the WC1.1 in all groups, this was significant only in the clinical group (P = 0.027) (Fig. 2C). Our data indicates no significant differences among the examined groups with regard to the frequency of γδ T cell subsets in the ileum.

PPD-J specific proliferation of WC1+ γδ T cells

Bovine WC1+ γδ T cell subsets from M. bovis infected cattle have been reported to proliferate to stimulation with protein and nonprotein mycobacterial antigens (McGill, Sacco et al. 2014). We next set out to characterize the capacity of WC1+ γδ T cell subsets from cattle naturally infected with MAP to respond to in vitro stimulation with MAP antigens (i.e., PPD-J). We hypothesized that the proliferative response of WC1+ γδ T cell subsets in the clinical animals would be impaired. To test this hypothesis, PBMCs collected from non-infected, subclinical and clinically infected cattle were labeled with CFSE, and cultured in the presence or absence of PPD-J. On day 6, cultures were analyzed by flow cytometry for cells co-expressing γδ TCR and either WC1.1 or WC1.2 that had proliferated in response to Ag, as measured by CFSE dilution. Results were gated on total live lymphocytes, and total cells expressing the γδ TCR. WC1.1+ or WC1.2+ subpopulations were gated on the total γδ TCR+ population. Total peripheral γδ T cells from cattle naturally infected with MAP proliferated specifically in response to PPD-J and this response was restricted only to the subclinical group (P < 0.001) (Fig. 3B). The impairment in γδ T cells proliferation in the clinical group was MAP-specific as
γδ T cells from these animals proliferated to stimulation with Con A (Fig. 3E). In the subclinical group, both WC1.1+ and WC1.2+ subsets proliferated equally in response to stimulation with PPD-J suggesting a heterogeneous response (P < 0.001) (Fig. 3C and D). Our data indicates that WC1-expressing cells in peripheral blood of cattle with subclinical JD proliferate in response to one or more PPD-J antigens.

IFN-γ and IL-10 production by WC1+ γδ T-cell subsets in response to stimulation with PPD-J

A recent report has shown that both WC1.1+ and WC1.2+ γδ T cell subsets from *M. bovis* infected cattle produce IFN-γ to stimulation with *M. bovis*-specific antigens (McGill, Sacco et al. 2014). We hypothesized that the function (cytokine production) of WC1+ γδ T cell subsets may differ based on the infection status of the animal and that these cells may produce higher levels of IFN-γ during the subclinical stage and higher levels of IL-10 during the clinical stage of infection. To test this hypothesis, PBMCs isolated from control, subclinical or clinically infected animals were cultured with and without PPD-J for 24 hours (IFN-γ) or 6 days (IL-10). By titration in our lab, we determined that these incubation periods were optimal for generation of each cytokine. Specific production of IFN-γ and IL-10 by WC1+ γδ T cell subsets was evaluated using flow cytometry. As shown in Fig 4B, there was a strong trend for the percentages of IFN-γ+ γδ T cells to be higher in the infected groups compared to the control group but was not quite statistically significant (P = 0.06). Intracellular IFN-γ production by total γδ T cells was comparable between the subclinical and clinically infected animals. Interestingly, the percentages of PPD-J specific IFN-γ+ WC1.1+ γδ T cells were
significantly higher in subclinical cattle compared to non-infected (P = 0.008) and clinically infected cattle (P = 0.006) (Fig 4C). The IFN-γ response of WC1+ γδ T cell subset in the subclinical group was a heterogeneous mix of WC1.1 and WC1.2 subpopulations (Fig 4C and D). Within the clinical group, the mean percentage of WC1.2+ cells expressing IFN-γ was significantly higher than the mean percentage of WC1.1+ IFN-γ+ cells (P = 0.04) (Fig 4C and D). There was a strong trend for the PPD-J specific IFN-γ response by WC1.2+ γδ T cells to be higher in the clinical group compared to the control group but was not statistically significant (P = 0.07) (Fig 4D). Our data indicates that bovine γδ TCR+ WC1+ subsets from cattle with JD differentially express IFN-γ in response to PPD-J. This could be because of memory responses as demonstrated for *Leptospira* [23], or non-specifically by PPD-J antigens as seen of bovine tuberculosis [24].

We found no significant difference between the three groups in the percentages of IL-10+ γδ T cells (Fig 5B). Although there were trends for IL-10 production by WC1.1+ and WC1.2+ γδ T cell subsets to be lower in the clinical group compared to the subclinical group, this did not reach statistical significance (Fig 5C and D).

**Discussion**

The goal of this study was to identify differences in γδ T cell phenotype and function in cattle with subclinical and clinical forms of MAP infection. Our main findings in this study were that γδ T cell percentages within circulating lymphocytes were significantly lower in cattle with the clinical form of MAP infection compared to that of non-infected cattle. This decrease in the percentages of circulating γδ T cells in the
clinical group was not associated with preferential migration of γδ T cells to the site of infection (ileum). Furthermore, γδ T cells obtained from clinically infected cattle failed to proliferate when stimulated with PPD-J \textit{ex vivo}. Interestingly, in contrast to subclinically infected cattle where the WC1.1 subset proliferated and produced IFN-γ in response to \textit{in vitro} stimulation with PPD-J, this subset was unresponsive to stimulation with PPD-J in clinically infected cattle.

We know that γδ T cells contribute to protective immunity early during infection likely by producing proinflammatory mediators such as IFN-γ [25]. Depletion of WC1⁺ γδ T cells prior to infection of calves with \textit{M. bovis} resulted in reduced IFN-γ and enhanced IL-4 production suggesting that γδ T cells may promote cell mediated immunity during mycobacterial infection [4]. Our results indicate that γδ T cell percentages within circulating lymphocytes are lower in clinically infected animals. This is likely due to a decrease in γδ T cell numbers rather than an increase in the numbers of other lymphocytes such as αβ T cells. This is supported by peripheral CD4⁺ T cells remaining at a similar frequency among non-infected subclinical and clinically infected cattle. However, we do not exclude the age effect on this observation, as the averages of ages in the infected groups were higher compared to the control group. Badi et al. have examined peripheral γδ T cells frequency in clinically infected and non-infected cattle with similar ages and reported no significant differences [26]. In the periphery, bovine γδ T cells are predominantly WC1⁺. Our data indicates that within the WC1⁺ γδ T cell subset, WC1.2 subset was significantly reduced in infected animals regardless the stage of infection. It is difficult to draw conclusions because of the limited number of animals
used in this study and the difference in age average between non-infected and infected animals; these observations warrant further investigation.

One possible explanation for the decrease in the frequency of peripheral γδ T cells in the clinical group is preferential migration to infection sites in response to progression to multibacillary disease. Our results show that in the distal ileum, a consistent site for MAP infection, the frequency of WC1−, total WC1+, WC1.1+ and WC1.2+ were comparable among clinical, subclinical and non-infected cattle with WC1.2+ γδ T cells being the predominant WC1+ subset. Thus in this study decreased peripheral γδ T cells does not appear to be the result of increased recruitment to the ileum. It will be interesting to determine if the decline in γδ T cells observed in the clinical group is restricted to cells that are MAP antigen specific. Reduction in peripheral total CD3+ lymphocytes has been reported in humans with active pulmonary tuberculosis [27]. Koets et al. reported that the frequency of CD4+ T cells was lower in cattle clinically infected with MAP compared to cattle with the subclinical stage of MAP infection [28]. Apoptotic death of CD4+ αβ and/or γδ T-cells has been described for experimental *M. bovis* infection [29-31]. Among other factors, increased production of TGFβ, a cytokine known to be elevated during the clinical stage of MAP infection, can promote apoptotic deletion of T cells [32].

In the clinical group, γδ T cells failed to proliferate to *in vitro* stimulation with PPD-J. It is well established that progression to the clinical stage of MAP infection associates with a decrease or complete anergy of MAP-specific cell-mediated immune responses [33]. Loss of T cell effector functions or T cell exhaustion has been associated with several chronic infectious diseases including mycobacterial infections [34-38]. MAP
has been shown to infect and interfere with DC maturation [39, 40]. Semi-mature DCs are known to favor the development of anergic T cell responses [41]. T cell exhaustion that develops late during MAP infection is in part mediated by increased expression of inhibitory molecules such as programmed death-1 (PD1) and lymphocyte activation gene 3 (LAG-3) [42]. Our results support the hypothesis that progression to the clinical stage of JD is in part mediated by diminished protective responses of both CD4+ αβ and γδ T-cells.

Our finding that the recall IFN-γ response of WC1+ γδ T cells in the subclinical group was a heterogeneous mix of WC1.1+ and WC1.2+ positive cells corresponds with the reported IFN-γ response of this subset in cattle experimentally infected with M. bovis [11]. Interestingly, the WC1+ γδ T cell IFN-γ response in the clinical group was mainly mediated by the WC1.2 subset. Although the WC1.1 subset was present at similar frequency within circulating γδ T cells among the subclinical and clinical group, WC1.1+ cells showed a complete MAP-specific unresponsiveness in clinically infected cattle. Decreases in circulating γδ T cells combined with diminished antigen-specific recall responses of WC1.1+ cells in MAP infected cattle may serve as biomarkers for progression from subclinical to clinical disease. The timing of these changes with fecal shedding will need to be further explored. The WC1+ γδ T cell response may differ with different pathogens. While WC1.1+ cells are the critical γδ T cell subpopulation responding to Leptospira, γδ T cells responding to bovine rickettsial pathogen Anaplasma marginale are shown to be WC1.2+ [17, 18]. Both WC1.1 and WC1.2 subsets proliferated and produced IFN-γ in cattle infected with M. bovis [11]. The differential response of WC1+ γδ T cell to different pathogens has been attributed to the role of WC1
molecules in antigen recognition since WC1.1 and WC1.2 subsets share the same γδ TCR usage restriction [43].

Our data indicate no significant differences among clinical, subclinical and non-infected cattle with regard to the percentages of IL-10+ γδ T cells in PBMC cultures stimulated with PPD-J. IL-10 production by γδ T cells in the control group is largely attributed to one control animal. In this animal, IL-10 production by γδ T cells was mainly mediated by the WC1.1 subset. In infected groups, the IL-10 response by WC1+ γδ T cells was mediated by both WC1.1 and WC1.2 subpopulations. In the bovine model, γδ T cells have been shown to modulate and suppress CD4+ T cells proliferation and cytokine production in PBMCs cultures stimulated ex vivo [44, 45]. Regulatory γδ T cells are shown to be present in both WC1neg and WC1+ subsets [46, 47]. In the subclinical group, WC1.1 and WC1.2 subsets produced both IL-10 and IFNγ in response to stimulation with PPD-J suggesting functional plasticity.

The role of IL-10 during MAP infection is less clear and needs to be elucidated. While some studies reported no distinction in IL-10 production among non-infected and MAP infected animals, others reported elevated IL-10 production late during MAP infection. In cattle, Khalifeh et al. found that IL-10 production by PBMCs was elevated during the clinical stage of MAP infection [48]. Using quantitative PCR, Coussens et al. showed no significant differences in IL-10 expression in PBMCs stimulated with MAP among non-infected, subclinical and clinically infected cattle [49]. IL-10 production by PBMCs stimulated in vitro with MAP was similar among paucibacillary, multibacillary and infected sheep with no lesions [50]. In sheep infected with MAP, peripheral blood IFN-γ and IL-10 responses were elevated at 4 months post infection and the IL-10
response remained elevated until the trial termination [51]. Following *in vitro* stimulation with MAP, IL-10 gene expression was elevated in PBMCs of cattle subclinically infected with MAP [49]. Our observations may support these data and suggest that the early host response to MAP infection consists of mixed inflammatory response to control infection and suppressive response to minimize tissue damage.

In summary, we report that the frequencies of circulating γδ T cells were significantly reduced in cattle with the clinical form of MAP infection. We observed no significant differences in the percentages of circulating γδ T cells between noninfected cattle and cattle subclinically infected with MAP. In the infected groups, the frequencies of WC1.2+ γδ T cell subset within circulating γδ T cells were significantly reduced regardless of the infection stage. These observations did not associate with increased infiltration of γδ T cells to MAP infection sites (distal-ileum). In the subclinical group, γδ T cells proliferated specifically in response to ex-vivo stimulation with PPD-J, a response that was a heterogeneous mix of WC1.1 and WC1.2 subsets. These proliferative responses were not detected in cattle clinically infected with MAP. Our data show that progression to the clinical stage of MAP infection associated with a significant decrease in γδ T cells representation within circulating lymphocytes as well as impaired γδ T cell proliferative response to ex-vivo stimulation with MAP-specific antigen, PPD-J. It is interesting that the WC1.1+ γδ T cell subset proliferated and produced IFN-γ in response to stimulation with PPD-J in subclinically infected cattle while this subset exhibited unresponsiveness in clinically infected cattle. Our data support the hypothesis that in PBMCs cultures of cattle subclinically or clinically infected with MAP, WC1+ γδ T cells respond differentially to stimulation with one or more of MAP antigens.
Acknowledgments

We thank Dr. Shawn Rigby and Dr. Tracy Lindquist for their technical support and assistance. This work was funded by Iowa State University CVM seed grants.

References


Table 1 Antibodies used in Immunofluorescence (IF) and Flow cytometry (F) staining

<table>
<thead>
<tr>
<th>Primary</th>
<th>Catalog Number</th>
<th>Isotype</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>γδ TCR &lt;sup&gt;a&lt;/sup&gt;</td>
<td>GB21A</td>
<td>IgG2b</td>
<td>Goat anti-mouse IgG2b AF488 &lt;sup&gt;c&lt;/sup&gt; (IF and F) /PE &lt;sup&gt;c&lt;/sup&gt; (F)</td>
</tr>
<tr>
<td>WC1 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>ILA29</td>
<td>IgG1</td>
<td>Goat anti-mouse IgG1 AF594 &lt;sup&gt;c&lt;/sup&gt; (IF)</td>
</tr>
<tr>
<td>WC1.1 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>BAG25A</td>
<td>IgM</td>
<td>Goat anti-mouse IgM AF647 &lt;sup&gt;c&lt;/sup&gt; (F)/AF594 (IF)</td>
</tr>
<tr>
<td>WC1.2 &lt;sup&gt;a&lt;/sup&gt;</td>
<td>CACTB32A</td>
<td>IgG1</td>
<td>Goat anti-mouse IgG1PE-cy5.5 &lt;sup&gt;c&lt;/sup&gt; (F)/AF488 &lt;sup&gt;c&lt;/sup&gt; (IF)</td>
</tr>
<tr>
<td>CD4 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1653GA</td>
<td>IgG2a</td>
<td>Goat anti-mouse IgG2a AF680 &lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>IFNγ &lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA1783PE</td>
<td>IgG1</td>
<td>Direct conjugate (PE)</td>
</tr>
<tr>
<td>IL-10 &lt;sup&gt;b&lt;/sup&gt;</td>
<td>MCA2111B</td>
<td>IgG1 (Biotin)</td>
<td>Streptavidin-PE/CY7 &lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Monoclonal Antibody Center, Pullman, WA, USA.
<sup>b</sup> AbD SeroTech, Raleigh, NC, USA.
<sup>c</sup> Life Technologies, Grand Island, NY, USA.
<sup>d</sup> SouthernBiotech, Birmingham, Alabama, USA.
Fig. 1. Percentages of γδ T cells within (non-stimulated) peripheral blood lymphocytes. (A) Representative flow plots from a clinical animal are shown for total γδ TCR+ T cells, WC1.1+ or WC1.2+ γδ TCR+ T cells. WC1.1+ & WC1.2+ subsets are gated on the total γδ TCR+ population. (B) Frequency (percentages) of total γδ TCR+ T cells within non-stimulated peripheral blood lymphocytes. (C) Frequency (percentages) of WC1.1+ γδ T cells within non-stimulated peripheral γδ T cells. (D) Frequency (percentages) of WC1.2+ γδ T cells within non-stimulated peripheral γδ T cells. (E) Frequency (percentages) of CD4+ T cells within non-stimulated peripheral blood lymphocytes. The data were collected from non-infected (n = 5), subclinical (n = 5) and clinically infected animals (n = 5). Data are presented as mean ± SEM. * P ≤ 0.05 and ** P ≤ 0.01.
Fig. 2. WC1+ γδ T cell subsets frequencies in the intestinal mucosa of control, subclinical and clinically infected cattle. Frozen distal-ileum tissue sections were analyzed by immunofluorescence for surface expression of γδ TCR, WC1 (total), WC1.1 and WC1.2 molecules. (A: Left image) A representative image for a field stained for nuclei (Blue), γδ TCR (Green) and WC1 molecule (Red). (A: Right image) A representative image for a field stained for nuclei (Blue) and WC1.1 (Red) and WC1.2 molecule (Green). (B and C) The number of each γδ T cell subset/high-magnification field (40X) was counted, 6 fields from each animal were examined to determine the predominant γδ T cell subset(s) in intestinal mucosa of the distal-ileum of non-infected, subclinical and clinically infected cattle. n = 5-7 animals/group. Data are presented as mean ± SEM. * P ≤ 0.05, ** P ≤ 0.01.
Fig. 3. γδ T cells proliferative responses to MAP complex Ag, PPD-J. Isolated PBMCs were stained with CFSE prior to culture for 6 days in the presence or absence of PPD-J. (A) Representative CFSE profiles from a subclinical animal, gated on total live cells and total γδ T cells. The percentage of total γδ T cells (B), WC1.1+ (C) and WC1.2+ γδ T cells (D) that divided in response to stimulation with PPD-J was measured by CFSE dilution. (E) The percentage of total γδ T cells that divided in response to stimulation with Con-A. Cells were assayed in triplicates. Responses were normalized by subtracting the background response (medium alone). n = 5 animals/group. Data are presented as mean ± SEM. *** P ≤ 0.001.
Fig. 4. γδ T cells IFN-γ response to MAP complex Ag, PPD-J. Isolated PBMCs were cultured for 24 h with or without PPD-J. (A) Representative flow plots from a subclinical animal gated on total live cells, total γδ T cells and WC1.1+ γδ T cells. The percentage of total γδ T cells (B), WC1.1+ (C) and WC1.2+ γδ T cells (D) that produced IFN-γ in response to stimulation with PPD-J was measured using flow cytometry. Cells were assayed in triplicates and PPD-J-specific responses were normalized by subtracting the background response (medium alone). n = 4 animals/group. NS: P > 0.05. ** P ≤ 0.01. Data are presented as mean ± SEM.
Fig. 5. γδ T cells IL-10 response to MAP complex Ag, PPD-J. Isolated PBMCs were cultured for 6 days with or without PPD-J. (A) Representative flow plots from a subclinical animal gated on total live cells and total γδ T cells. The percentage of total γδ T cells (B), WC1.1+ (C) and WC1.2+ γδ T cells (D) that produced IL-10 in response to stimulation with PPD-J was measured using flow cytometry. Cells were assayed in triplicates and PPD-J-specific responses were normalized by subtracting the background response (medium alone). n = 4 animals/group. NS: P > 0.05. Data are presented as mean ± SEM.
CHAPTER 3: DIFFERENTIAL CYTOKINE EXPRESSION BY WC1+ γδ T CELLS AND TOTAL MUCOSAL CELLS IN THE ILEUM OF CATTLE NATURALLY INFECTED WITH MYCOBACTERIUM AVIUM SUBSP. PARATUBERCULOSIS

Modified from a manuscript submitted to the Journal of Veterinary Immunology and Immunopathology

Albarrak SM¹, Waters WR², Stabel JR², Hostetter JM¹

From the Department of Veterinary Pathology ¹ and the Department of Veterinary Microbiology and Preventive Medicine ², College of Veterinary Medicine, Iowa State University, 1600 S 16th St, Ames, IA 50011

Abstract

In the present study, we evaluated expression of IFN-γ, IL-17, TNF-α, IL-10 and TGF-β by mucosal cells, including WC1+ γδ T cells, in ileal tissues taken from non-infected cattle and cattle naturally infected with Mycobacterium avium subsp paratuberculosis (MAP). Infected cattle were either in the subclinical or clinical stage of infection. We hypothesized that the cytokine profile of the WC1+ γδ T cell subset would be different between subclinical and clinical cattle. Our data indicate a significant increase in the numbers of mucosal cells, including WC1+ γδ T cells, expressing IL-10 in clinical cattle compared to subclinical and non-infected cattle. We observed a significant increase in TGF-β expression by total mucosal cells in clinically infected cattle. Expression of IFN-γ, IL-17 and TNF-α in mucosal cells, including the WC1+ γδ T cell
subset, was identified in all examined groups. However, our data indicate that the stage of infection did not significantly influence expression of these proinflammatory cytokines. This study demonstrates changes in the cytokine mRNA expression profile of mucosal cells in the ileum, and specifically WC1⁺ γδ T cells, as cattle progress to the clinical disease. The change is characterized by an increase in expression of anti-inflammatory cytokines.

**Introduction**

There has been a growing interest in defining the role(s) that γδ T cells may play during mycobacterial infections. This is because γδ T cells link the innate and adaptive arms of the immune system by providing a diverse set of immunological functions including cytokine production, antigen presentation and cytotoxic activities [1, 2]. In young calves where the risk of infection with *Mycobacterium avium subsp paratuberculosis* (MAP) is high, γδ T cells represent the majority of peripheral CD3⁺ lymphocytes [3]. Based on surface expression of workshop cluster 1 (WC1) molecule, a transmembrane glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR) super family (CD163), bovine γδ T cells are broadly differentiated into two major subsets [4-6]. The WC1neg subset predominates in tissues such as spleen and intestine whereas the majority of γδ T cells in peripheral blood are WC1⁺ [5]. Given their localization to mucosal tissues, an immune surveillance role has been suggested for the WC1neg subset [7, 8]. Compared to the WC1neg subset, the WC1⁺ subset has been shown to be more active with regard to proliferation and proinflammatory cytokine production [7-9].
The host response to invading pathogens and the infection outcome relies on cytokines produced by recruited immune cells including WC1⁺ γδ T cells. Bovine γδ T cells are early responders to MAP infection, and their contribution to the host response includes cytokine production [10, 11]. The contribution of the WC1⁺ γδ subset in the immune response to MAP infection is not completely understood. Studies evaluating cytokines generated in ex vivo MAP stimulated total peripheral blood mononuclear cells (PBMC) have reported differences in the cytokine profiles between the subclinical and clinical stages of MAP infection [12, 13]. We recently demonstrated differences between subclinical and clinical cattle with regard to proliferation and IFN-γ production by the WC1⁺ γδ T cell subset in ex vivo MAP-derived purified protein derivative (PPD-J) stimulated PBMCs [14]. Progression to the clinical stage of MAP infection has been hypothesized to be facilitated by increased expression of regulatory cytokines that antagonize the effective Th-1 mediated responses [15]. Many studies have evaluated the cytokine expression profiles within the tissue at sites of MAP infection and reported differences between the subclinical and clinical stages of MAP infection [15-19]. However, none of these studies have examined the cell phenotype, and thus, contribution of the individual cell subsets like the WC1⁺ γδ T cell subset to cytokine production in the ileum during MAP infection is unknown.

The ileum and ileocecal valve are considered consistent locations for MAP infection and granuloma development in cattle naturally infected with MAP [20]. In a recent study, we reported no significant differences in the frequency of WC1⁺ γδ T cells in ileal tissues taken from non-infected cattle and cattle subclinically or clinically infected with MAP (natural infection) [14]. As a follow up to our previous study, the goal of the
current study was to better define the cytokine profiles within the ileum of MAP infected cattle in the subclinical or clinical stage of disease. In this study we evaluated total mucosal cells and WC1+ γδ T cells in the ileum. We did not include the WC1neg subset because its characterization requires a probe for γδ TCR, WC1 and cytokine of interest. Three colors labeling can get more complex. We hypothesized that in the distal-ileum, cytokine expression by the WC1+ γδ T cell subset would be different between cattle in the subclinical and clinical stages of MAP infection. In this study, we examined the expression of cytokines with known or implied relevance to the pathogenesis of MAP infection. The selected cytokines included IFN-γ, IL-17, TNF-α, IL-10, and TGF-β, which represent a diverse range of immune responses and important mediators of mycobacterial infection [21]. Our key finding in the present study was a significant increase in the frequency of ileal mucosal cells expressing IL-10, including the WC1+ γδ T cell subset, in cattle with the clinical form of MAP infection. IL-10 expression occurred in cattle with advanced granulomatous inflammation and very high intracellular MAP bacterial burdens.

**Materials and Methods**

**Animals and tissues**

Archived formalin-fixed, paraffin-embedded (FFPE) tissues (distal-ileum) obtained from adult Holstein dairy cows ranged in age from 5 to 10 years were used in the present study. These animals were part of a herd of cattle maintained by the National Animal Disease Center in Ames IA. Animals were divided into 3 groups consisting of 3 non-infected cows, and 6 infected cows. 3 of the infected cows were in the subclinical
stage and 3 were in the clinical stage of infection. All the infected animals were naturally infected with MAP and were classified into the subclinical and clinical categories based on fecal shedding, determined by fecal culture, and clinical signs [14, 15]. The subclinical cows were asymptomatic, and their fecal shedding was < 10 CFU/g of feces. Clinical cows shed > 100 CFU/g of feces and had clinical signs including weight loss and intermittent diarrhea. Control or non-infected cows were obtained from accredited JD free herds and repeatedly tested negative by fecal culture and serological assays.

**Histopathology and Acid-Fast Staining**

FFPE tissues were cut into 5 μm sections and mounted on Superfrost® Plus slides (Fisher Scientific, PA, USA). Tissues sections were stained with hematoxylin and eosin (H&E) for histopathologic evaluation using standard laboratory protocols. Serial sections of all tissues were examined for the presence of mycobacterial bacilli by Ziehl–Neelsen acid-fast staining.

**Probes for in situ hybridization**

The RNA probes specific for bovine WC1 (Cat No: 445931-C2), IFN-γ (Cat No: 315581), TNF-α (Cat No: 316151), IL-17A (Cat No: 406601), IL-10 (Cat No: 420941) and TGF-β (Cat No: 42721) were all obtained from ACD (Advanced Cell Diagnostics, Hayward, CA, USA).

mRNA detection by in situ mRNA hybridization

FFPE RNAscope 2-plex Assay kit (Advanced Cell Diagnostics, Hayward, CA, USA) was used for the in situ detection of mRNA transcripts coding for the WC1
molecule, inflammatory and regulatory cytokines according to the manufacturer’s instructions. Briefly, formalin-fixed paraffin-embedded (FFPE) ileal tissues were cut into 5 μm sections and mounted on Superfrost® Plus slides (Fisher Scientific, PA, USA). Slides were baked at 60°C for 1 hour followed by a deparaffinization step and a protease treatment to permeabilize the tissue. Hybridization of the target mRNAs with the probes was done at 40°C for 2 h followed by multiple washing and amplification steps. The target mRNA was detected with horseradish peroxidase (HRP) (cytokines) and Fast Red (WC1) chromogenic stains. Hematoxylin (American Master Tech, CA, USA) was used for nuclei counterstaining. Positive staining was indicated by green and red dots in the cytoplasm. Actin-β was used as a positive control while DapB, a house keeping soil bacterial gene, was used as a negative control. The stained slides were examined with a brightfield microscope at 400X magnification. Cells with positive signals in a 400X field were manually counted. Six 400X fields were counted on each slide.

Statistics

Statistical analysis was performed using JMP Pro 12 (SAS Institute, NC, USA). All data are shown as means ± SEM. Wilcoxon Each Pair test and ANOVA with Tukey's HSD were used to determine the significance of the differences among the groups.

Results and Discussion

Development of multibacillary granulomatous inflammation in the ileum is a hallmark microscopic finding in cattle that progress to the clinical stages of MAP infection [20, 22]. Using HE stained ileal sections and serial sections stained with ZN, we
defined the presence or absence of granulomatous inflammation and intracellular MAP for all the cattle in this study. All the clinical animals used in the current study had a diffuse type of granulomatous inflammation characterized by extensive infiltration of the lumina propria and submucosa by sheets of macrophages with abundant eosinophilic to granular cytoplasm (Fig 1A) [18, 23]. Acid fast staining demonstrated that most macrophages in the granulomatous lesions in the clinical group contained large numbers of MAP bacilli (Fig 1B). In the subclinical group, one animal had mild granulomatous inflammation with low numbers of individual macrophages and giant cells in the distal villus lumina propria (Fig 1C). Granulomatous lesions were not detected in the ileum of the remaining subclinical animals. Acid fast staining did not demonstrate MAP in the ileum from any of the subclinically infected cattle (Fig 1D). Likewise, ileum from control cattle did not have any detectable granulomatous lesions or acid fast bacilli. These finding demonstrate that, as expected, cattle in the clinical group all had progressed to multibacillary disease with advanced granulomatous lesions in the ileum. In contrast, MAP bacilli were not detected in the ileal sections of the subclinical group where granulomatous lesions were minimal.

We next evaluated the cytokine expression profiles in the tissue microenvironment of the distal ileal mucosa; a site where MAP infection has been reported to be most consistent [20, 22]. Our approach was to use a relatively new in situ hybridization technique called RNAscope. The RNAscope technique simultaneously amplifies the target-specific signals and reduces background from nonspecific hybridization which makes it more sensitive and specific than previous in situ mRNA hybridization (ISH) techniques. The signals are detected by microscopy, and a benefit of
this approach is that the cellular source of the signal can be determined by morphology or by cell marker labeling. We evaluated expression for each of the target cytokines in total mucosal cells using probes specific for each cytokine. We evaluated cytokine expression in WC1⁺ γδ T cells by using probes for each cytokine in combination with a probe for WC1 mRNA and identified colocalized signals (Fig 2). Each cytokine was evaluated in the distal ileum of non-infected cattle and cattle naturally infected with MAP that were either in the subclinical or clinical stage of infection.

The most significant overall change in cytokine expression was in IL-10 and TGF-β expression. Our data indicate a significant increase in the number of WC1⁺ γδ T cells (p < 0.01) as well as the number of total mucosal cells (p < 0.05) expressing IL-10 in sections of ileum from clinically infected cattle compared to ileal tissues taken from subclinically infected and non-infected cattle (Fig 3A and Fig 3B). We observed a significant (p < 0.05) increase in the frequency of total mucosal cells expressing TGF-β in the clinical group compared to the subclinical and control groups (Fig 3B). Much of this signal was identified in cells with a macrophage type morphology (Fig 2D). The number of WC1⁺ γδ T cells expressing TGF-β trended higher in clinically infected cattle but did not reach statistical significance (Fig 3A).

In this study the increase in ileal expression of IL-10 and TGF-β in clinical cattle is consistent with previous studies indicating increased expression of these two cytokines in ileal tissues taken from cattle clinically infected with MAP [15, 18]. Our data further demonstrate that the WC1⁺ γδ T cells are among the mucosal cells that increase IL-10 expression in the clinical group. IL-10 and TGF-β are well-characterized anti-
inflammatory cytokines and thought to contribute to the progression of MAP infection from subclinical to clinical stage through dysregulation of Th1 responses and suppression of macrophages activation [15, 24, 25]. MAP has been shown to induce expression of these regulatory cytokines, likely as a strategy to persist and modulate inflammatory responses [26, 27]. MAP replication has been shown to positively correlate with IL-10 and TGF-β expression at the sites of MAP infection [27, 28]. Addition of exogenous IL-10 and TGF-β has been shown to favor MAP replication in bovine monocytes [24]. In vitro studies have suggested a regulatory role for the WC1⁺ γδ T cell subset in the bovine [29]. Bovine γδ T cells have been shown to proliferate and produce IL-10 in response to stimulation with IL-10 and TGF-β, which had a suppressive effect on specific and non-specific responses of αβ T cells [30]. This effect of IL-10 production from WC1⁺ γδ T cells in the local tissue response to MAP infection needs to be further evaluated. We hypothesized that IL-10 production by WC1⁺ γδ T cells promotes the anti-inflammatory microenvironment of the ileum in clinical cattle.

Expression of IFN-γ, IL-17 and TNF-α by the ileal mucosal cells, including WC1⁺ γδ T cells, was identified in all animals regardless of infection status. To the best of our knowledge, this is the first report to look specifically at expression of these proinflammatory cytokines by the WC1⁺ γδ T cell subset in ileum of naturally infected cattle. While trends were present, especially for TNF-α, we observed no significant differences among the three groups with regard to expression of these proinflammatory cytokines by the WC1⁺ γδ T cell subset or total mucosal cells (Fig 4A & 4B). WC1⁺ γδ T cells have been shown capable of expressing TNF-α, a cytokine essential for cells recruitment and granuloma formation [31, 32]. Using conventional ISH, Lee et al
examined TNF-α expression (mRNA) in ileal tissues taken from non-infected cattle and cattle clinically infected with MAP (naturally infection) and reported no significant differences in the number of cells expressing TNF-α between the clinical and control groups. However, the study did not define the phenotype of the TNF-α⁺ cells [17]. Our data indicate that the WC1⁺ γδ T cell subset is a source of TNF-α in the ileum of cattle. Our observation that TNF-α expression was increased in clinical cattle suggests that TNF-α may contribute to the immunopathology of MAP infection.

IFN-γ expression was similar in total mucosal cells in the ileum of infected and non-infected cattle. We demonstrate that ileal WC1⁺ γδ T cells express IFN-γ, but this does not significantly change with infection status. IFN-γ is essential for the immune defense against intracellular pathogens including mycobacteria [33]. There is some disparity in reports on expression of IFN-γ transcript in cattle naturally infected with MAP. Lee et al. reported a significant increase in the number of cells expressing the IFN-γ transcript in ileal tissues of cattle clinically infected with MAP compared to non-infected cattle [17]. A study by Sweeney et al indicated a significant increase in IFN-γ transcript expression in ileal tissues of subclinically infected cattle compared to clinically infected cattle with similar expression between non-infected and clinical cattle [19]. Fernandez et al. have recently shown a significant increase in the number of cells expressing IFN-γ in ileal tissues with focal granulomatous lesions compared to tissues with diffused granulomatous lesions. In regions of tissue without granulomas, Fernandez et al. found no differences in IFN-γ expression in the lamina propria and lymphoid tissues, among non-infected cattle and cattle with granulomatous inflammation [34].
Bovine γδ T cells have been shown to produce IL-17 in response to infection with *M. bovis* [35-37]. To the best of our knowledge, the level of IL-17 expression in the ileum of MAP-infected cattle has not been reported. We detected low numbers of mucosal cells, including WC1⁺ γδ T cells, expressing IL-17 in the ileal mucosa of all cattle regardless of infection status. Expression did not significantly change with the stage of MAP infection. It is possible that changes in IL-17 expression in the ileum are not associated with progression of MAP infection in cattle. However, Dudemaine et al. have shown that in ELISA positive cattle there is an increase in levels of circulating IL-17, which may suggest differences in the local and systemic immune responses to MAP [38].

In summary, we have examined the cytokine profile of WC1⁺ γδ T cells and total mucosal cells in sections of ileum from non-infected cattle, and naturally infected cattle in the subclinical or clinical stage of MAP infection. We demonstrate a significant increase in the number of mucosal cells, including WC1⁺ γδ T cells, expressing IL-10 in clinically infected cattle. In addition, we demonstrate increased expression of TGF-β in total mucosal cells in cattle in the clinical stage of disease. The expression of the cytokines was in a local environment of advanced granulomatous inflammation with heavy intracellular MAP burden. We report expression of IFN-γ, IL-17, TNF-α, in total mucosal cells and in the WC1⁺ γδ T cell subset. Infection status or stage of infection did not significantly influence expression of these proinflammatory cytokines in the ileum. These data demonstrates changes in the cytokine mRNA expression profile of mucosal cells in the ileum, and specifically the WC1⁺ γδ T cell subset, as cattle progress to the
clinical stage of disease. This change is characterized by an increase in expression of anti-inflammatory cytokines.

Acknowledgement

This study was funded by Iowa State University CVM seed grants.

References


35. Steinbach, S., H.M. Vordermeier, and G.J. Jones, CD4+ and gammadelta T Cells are the main Producers of IL-22 and IL-17A in Lymphocytes from *Mycobacterium bovis*-infected Cattle. Sci Rep, 2016. 6: p. 29990.


**Fig 1.** Photomicrographs of serial sections of bovine ileum stained with either HE for histopathological evaluation or ZN for MAP detection (magnification = 200X). (A) HE stain - Diffuse granulomatous inflammation in the ileal lamina propria of a clinically infected animal. (B) ZN stain – serial section of massive aggregation of macrophages heavily infected with MAP in the ileal lamina propria of a clinically infected animal. (C) HE stain - There is minimal granulomatous inflammation in the lamina propria of a subclinically infected cow with low numbers of isolated macrophages. Arrows demonstrate isolated macrophages in the lamina propria of this animal (D) ZN stain serial section of C – there are no acid fast bacilli in the lamina propria.
**Fig 2.** Photomicrographs of bovine ileal tissue sections in which WC1, IL-10 and TGF-β mRNA messages are detected by in situ mRNA hybridization (magnification = 1000X). Cytokines mRNA are stained with HRP (Green) and WC1 mRNA is stained with Fast Red chromogenic stain (Red). (A) A subclinically-infected cow examined for IL-10. (B) A clinically infected cow examined for IL-10. (C) A subclinically infected cow examined for TGF-β. (D) A clinically infected cow examined for TGF-β. Arrows are pointed on cells with dual staining.
Fig 3. Differences in regulatory cytokine gene expression in ileal tissues from non-infected, subclinical and clinically infected cattle. (A) Mean cell counts of WC1+ γδ T cells expressing IL-10 or TGF-β. (B) Mean cell counts of total mucosal cells expressing IL-10 or TGF-β. The number of cells with positive signals present in a single 400X magnification field was counted. Six 400X fields were counted for each animal, and a total of 3 animals per group were examined. Data are shown as means ± SEM. NS: p > 0.05. * p ≤ 0.05. ** p ≤ 0.01.

Fig 4. Differences in proinflammatory cytokine gene expression in ileal tissues from non-infected, subclinical and clinically infected cattle. (A) Mean cell counts of WC1+ γδ T cells expressing IFN-γ, IL-17A or TNF-α. (B) Mean cell counts of total mucosal cells expressing IFN-γ, IL-17A or TNF-α. The number of cells with positive signals present in a single 400X magnification field was counted. Six 400X fields were counted for each animal, and a total of 3 animals per group were examined. Data are shown as means ± SEM. NS: p > 0.05.
CHAPTER 4: WC1+ γδ T CELLS DEPLETION ALTERS CYTOKINE PRODUCTION BY PBMCs OBTAINED FROM CATTLE WITH THE SUBCLINICAL FORM OF MAP INFECTION

Modified from a manuscript to be submitted to the Journal of Comparative Immunology, Microbiology and Infectious Diseases

Albarrak SM¹, Waters WR², Stabel JR², Hostetter JM¹

From the Department of Veterinary Pathology ¹ and the Department of Veterinary Microbiology and Preventive Medicine ², College of Veterinary Medicine, Iowa State University, 1600 S 16th St, Ames, IA 50011

Abstract

Previous in vivo cell depletion studies have suggested a role for the WC1+ γδ T cell subset in modulating the host immune responses to infections. In the current study, we used cattle naturally infected with Mycobacterium avium subsp. paratuberculosis (MAP) and were in the subclinical stage of the infection to examine the impact of WC1+ γδ T cell depletion on production of IFN-γ, IL-12, IL-4, and IL-10 by PBMCs stimulated ex vivo with MAP-derived purified protein derivative (PPD-J). With the exception of IL-12, our data indicate that PBMCs taken from subclinical cattle used in the present study were hyporesponsive to stimulation with PPD-J with regard to IFN-γ, IL-4, and IL-10 production. Our key findings in this study were that depletion of the WC1+ subset did not affect IFN-γ and IL-12, but did lead to a significant reduction in IL-4 secretion and a significant increase in IL-10 secretion. The observed changes in IL-4 and IL-10 secretion were not dependent on the presence of PPD-J. Our data demonstrate that depletion of the
WC1⁺ γδ T cell subset resulted in altered cytokine production by PBMCs obtained from cattle with the subclinical form of MAP infection and suggest a modulatory role for the WC1⁺ subset in this system.

**Introduction**

Bovine Gamma-delta (γδ) T cells respond to mycobacterial infections including infection with *Mycobacterium avium subsp. paratuberculosis* (MAP) [1-3]. There is a growing interest in studying γδ T cells because of their diverse functions linking the innate to adaptive arms of the immune system. These functions include cytokine production, immune regulation, cytotoxic activities and antigen presentation [4, 5]. Compared to humans and mice, γδ T cells circulate in higher frequency in ruminants [6]. In young calves, where the risk of MAP infection is high, > 40% of circulating lymphocytes are γδ TCR⁺ [6, 7]. The frequency of γδ T cells in the peripheral blood decreases as calves age.

Surface expression of workshop cluster 1 (WC1), a transmembrane glycoprotein belonging to the scavenger receptor cysteine-rich (SRCR) superfamily (CD163), has been used to divide bovine γδ T cells into two major subsets, WC1neg and WC1⁺ [8-10]. The WC1neg subset is prominent in tissues such as spleen and intestine, whereas the WC1⁺ subset represents the majority of γδ T cells in peripheral blood. (Machugh et al., 1997). An immune surveillance role has been described for the WC1neg subset which corresponds to their anatomic distribution [11, 12]. In contrast to the WC1neg subset, the WC1⁺ subset has been shown to be more proliferative and more proinflammatory [11, 12].
Many studies have suggested a role for the WC1⁺ γδ T cell subset in modulating the host’s immune responses to infections. Bovine WC1⁺ γδ T cells are among the first responders to mycobacterial infections, and thus the cytokines they produce may drive the host immune response toward a predominant Th-1 or Th-2 immune phenotype [3, 13]. Previous reports have suggested a role for the WC1⁺ γδ T cell subset in directing early immunity towards the formation of Th1 responses. Calves depleted of the WC1⁺ γδ T cell subset before challenged with M. bovis exhibited a reduction in IFN-γ and IgG2 responses along with a notable increase in IL-4 and IgG1 responses suggesting altered immune phenotype [14]. Similar observations were reported in calves depleted of WC1⁺ γδ T cells and infected with the bovine respiratory syncytial virus (BRSV) [15]. An in vitro study by Price et al. demonstrated the ability of WC1⁺ γδ T cells to enhance IL-12 production by dendritic cells infected with M. bovis [16].

A regulatory role has also been suggested for the WC1⁺ subset as some in vitro studies have reported enhanced proliferation and cytokine production by αβ T cells in PBMCs depleted of WC1⁺ γδ T cells [17, 18]. The contribution of the WC1⁺ γδ T cell subset to the host’s response to MAP infection is less defined. Thus, the goal of the current study was to examine whether depletion of the WC1⁺ γδ T cell subset would impact cytokine production in cattle in the subclinical stage of MAP infection. We chose subclinical cattle (natural infection) because these animals were in the stage of disease where the immune response was controlling MAP replication and disease progression had not occurred. We specifically hypothesized that the WC1⁺ γδ T cell subset influences IFN-γ production by CD4⁺ T cells. We evaluated changes in IFN-γ, IL-12, IL-4 and IL-10 production by PBMCs stimulated with PPD-J in presence or absence of total γδ T cells.
or just the WC1+$\gamma$$\delta$ T cell subset. Our key findings in the present study were $\gamma$$\delta$ T cells depletion in PBMCs from subclinically infected cattle did not affect IFN-$\gamma$, but did lead to a significant reduction in IL-4 secretion and a significant increase in IL-10 secretion. The observed alterations in IL-4 and IL-10 secretion were independent of PPD-J presence.

**Materials and Methods**

**Animals**

Six adult Holstein dairy cows (age average = 8.2 years) naturally infected with MAP and were in the subclinical stage of MAP infection were used in the current study. MAP shedding was less than 10 CFU/g of feces and animals were asymptomatic. All animal procedures in this study were approved by the IACUC of the National Animal Disease Center, Ames, Iowa.

**Antibodies**

Primary and secondary antibodies used for cell phenotyping and sorting are listed in Table 1.

**Preparation of PBMCs**

Blood samples were collected from the jugular vein into 60 cc syringes containing Acid Citrate Dextrose (ACD) as an anticoagulant. PBMCs were purified from buffy coat fractions by density centrifugation, and contaminated RBCs were removed by osmotic lysis. Cells were washed with Alsever’s solution (Sigma, St. Louis, MO, USA) and
resuspended in complete RPMI (Gibco, Thermo Fisher Scientific, MA USA) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 2% essential amino acids, 1% sodium pyruvate, 50 mM 2-ME, penicillin-streptomycin (100 units/ml penicillin and 0.1 mg/ml streptomycin), and 10% (v/v) FBS (all obtained from Life Technologies, CA USA).

Cytokine secretion assay

Cells were seeded at a density of 3 × 10^5 cells/well in 96-well plates. Cells were then cultured at 37°C (5% CO2) for 24 hours in presence or absence of PPD-Jhonin (10 µg/ml). Pokeweed mitogen (2 µg/ml) was used as a positive control stimulus. Brefeldin A (10 µg/ml final concentration, Sigma, MO USA) was added to cultures after the first 4 hours of stimulation.

γδ T cells depletion

Depletion of total γδ T cells or the WC1+ γδ T cell subset was performed using magnetic activated cell sorting (MACS) isolation. Briefly, PBMCs were resuspended in MACS running buffer (2mM EDTA, 0.5% BSA in PBS) containing anti-bovine γδ TCR (GB21A) or anti-bovine WC1 antibody (ILA29) at a concentration of 10 µg/ml and incubated for 25 minutes at 4°C at a density of 10^7 cells/ml. Cells were washed once and incubated for 15 minutes at 4°C with microbeads specific for mouse IgG2a+b (γδ TCR) or mouse IgG1 (WC1) (Miltenyi Biotec, CA USA). Cell sorting was performed using a Miltenyi Biotec AutoMACS Pro magnetic cell separation instrument.
Flow cytometry

Cells staining was performed in 96-well plates. For surface labeling, cells were resuspended in FACS buffer (PBS with 2% FCS and 0.02% (w/v) sodium azide) and incubated for 20 minutes at 4°C with 2 ug/ml primary antibodies (100 ml/well). Cells were washed once and resuspended with FACS buffer containing secondary antibodies (0.5 ug/ml, 100 ml/well) and incubated for 20 minutes at 4°C. Cells were washed once and fixed in BD FACS Lysing solution (BD Bioscience, CA USA). For intracellular staining (IFN-γ), fixed cells were resuspended in FACS buffer containing 0.5% (w/v) saponin (FACS-SAP) and incubated for 20 minutes at 4°C. Cells were then incubated for 30 minutes at 4°C with PE-conjugated anti-bovine IFN-γ antibody (AbD SeroTec, NC, USA) diluted 1:100 with FACS-SAP buffer (50 ml/well). Cells were washed once and analyzed by flow cytometry.

Luminex assay

Supernatants were incubated with agitation for 30 mins at RT with Luminex beads conjugated with mouse antibodies specific for bovine IFN-γ, IL-4, and IL-10 (all obtained from Bio-Rad, CA, USA). Detection antibodies (biotinylated mouse anti-bovine IFN-γ, IL-4, and IL-10; streptavidin-PE, all obtained from Bio-Rad, CA, USA), were added and mean fluorescence intensities of individual samples were converted to concentrations by comparing to standard curves.
ELISA

Concentrations of IL-12 protein in the culture supernatants were quantified by a commercial ELISA kit (ABclonal Technology, MA USA) according to the manufacturer’s instructions.

Statistics

Statistical analysis was performed using JMP Pro 12 (SAS Institute, NC, USA). All data are shown as mean ± SEM. ANOVA with Tukey’s HSD was used to determine the significance of the differences between the treatments’ means.

Results and Discussion

The current study aimed to examine the impact of WC1⁺γδ T cells depletion on cytokine production by PBMCs taken from subclinical cattle and stimulated ex vivo with MAP specific antigens (PPD-J). In this study, we evaluate PBMCs for production of cytokines representing diverse immune responses with known relevance to the pathogenesis of MAP infection. Examined cytokines included IFN-γ, IL-12, IL-4, and IL-10.

We initially evaluated total antigen (PPD-J) stimulated PBMCs in subclinical cattle. with the exception of IL-12, our results indicate that PBMCs from the subclinical cattle were hyporesponse to ex vivo stimulation with PPD-J with regard to IFN-γ, IL-4, and IL-10 production (Fig 1 and 2). Pokeweed mitogen led to strong production of these cytokines indicating that PBMCs from subclinical cattle did retain the ability to produce these cytokines. In addition, the PPD-J used in this study elicited strong IFN-γ responses
in PBMCs taken from calves vaccinated with Mycopar (data not shown). This observation is consistent with previous studies indicating a decrease in expression of these cytokines by MAP stimulated PBMCs taken from subclinical cattle. Using qRT PCR, Coussens et al. reported similar changes in expression of IFN-γ, IL-4, and IL-10 transcripts in subclinical cattle [19]. T cell exhaustion (dysfunction) has been described for many chronic infections including, MAP infection [20-22]. Okagawa et al. recently reported low IFN-γ responses in PBMCs following stimulation with PPD-J in cattle subclinically infected with MAP (Okagawa et al., 2015). The authors suggested that the increase in expression of inhibitory molecules such as PD-1 and LAG-3 contributed to the dysregulation in MAP-specific Th1 responses during the late subclinical stage. A study by Weiss et al. demonstrated that lymphocytes isolated from the ileum of subclinically infected cattle were hyporesponsive to stimulation with MAP, concanavalin A and pokeweed (Weiss et al., 2006). We observed a significant increase (p ≤ 0.01) in IL-12 secretion by PPD-J stimulated PBMCs compared to PBMCs stimulated with medium only (Fig 1). This increase in IL-12 secretion is likely to be mediated by intact innate immune responses, especially those from antigen presenting cells [23].

Using flow cytometric analysis, we demonstrated that CD4+ T cells failed to produce IFN-γ in PBMCs taken from subclinical cattle and stimulated ex-vivo with PPD-J (Fig 2). In PBMCs taken from cattle vaccinated against FMD, Guzman et al. demonstrated that the WC1+ γδ T cell subset had a suppressive effect on IFN-γ production by αβ T cells following in vitro stimulation with the vaccine antigen [17]. We hypothesized that depletion of the WC1+ γδ T cell subset would partially restore IFN-γ production by total PBMCs and specifically in CD4+ T cells. We next depleted the WC1+
γδ T cell subset from PBMCs derived from subclinically infected cattle. γδ T cells frequency in PBMCs before and after depletion are shown in Fig 3B. We used intracellular IFN-γ staining and flow cytometry to evaluate IFN-γ production in total PBMCs and specifically in CD4+ T cells. Depletion of total γδ T cells or just the WC1+ γδ T cell subset before PBMC stimulation with PPD-J had no significant effect on IFN-γ production (intracellular IFN-γ) by total PBMCs or CD4+ T cells (Fig 3A and 3B). Our observation that IFN-γ production by total PBMCs and specifically the CD4+ T cells remained unchanged in response to depletion of WC1+ γδ T cells suggests that the WC1+ subset might not be suppressive in PBMCs from subclinically infected cattle.

We measured IFN-γ, IL-12, IL-4 and IL-10 secretion in the supernatants of γδ T cell depleted and non-depleted PBMCs. Our results indicate that depletion of total γδ T cells or just the WC1+ subset before PBMC stimulation resulted in a significant reduction in IL-4 secretion (p ≤ 0.001) (Fig 4A). The decrease in the IL-4 secretion was not dependent on the PPD-J stimulation (Fig 4A). Using IL-4 knockout mice, Sugawara et al. demonstrated the requirement of IL-4 for controlling murine tuberculosis as IL-4 deficient mice exhibited larger granulomas along with higher bacterial load [24]. In contrast, the role of IL-4 in the host immune response controlling MAP infection in subclinical cattle has not been defined. Dudemaine et al. found no differences in the levels of circulating IL-4 between non-infected and subclinical cattle [25]. To the best of our knowledge; this is the first report to evaluate the effect of γδ T cells depletion on IL-4 production by PBMCs from MAP-infected cattle. Our observation differs from an early study demonstrating that in vivo depletion of WC1+ γδ T cells before calves infection with M. bovis increases circulating IL-4 [14]. However, this study is different from our
study with regard to the infectious agent, infection mode, and animals ages. The observed decrease in IL-4 secretion in γδ T cell depleted PBMCs suggests that γδ T cells, specifically the WC1+ subset, may contribute to IL-4 production or regulation. Bovine WC1+ γδ T cells have shown capable of producing IL-4 [26, 27]. Baquero et al. have recently reported a significant increase in IL-4 secretion in WC1+ γδ T cell cultures from non-infected cattle following stimulation with live MAP. Interestingly, when WC1+ γδ T cells were cocultured with monocyte-derived macrophages (MDMs), IL-4 secretion was increased regardless of MAP presence [27]. It is also possible that depletion of the γδ T cells, including the WC1+ subset, negatively impacted IL-4 production by other cell types including CD4+ T cells. Some murine γδ T cell subsets have been shown to regulate IL-4 production and generation of IgE antibodies [28]. Our data suggest that bovine γδ T cells, specifically the WC1+ γδ T cell subset, might be involved in regulating IL-4 production in PBMCs from cattle subclinically infected with MAP. Further work is needed to define the effects of γδ T cell subsets on the MAP-specific IL-4 response.

Our data demonstrate a significant increase in IL-10 secretion in PBMCs depleted of γδ T cells or the WC1+ γδ T cell subset compared to non-depleted PBMCs (p < 0.01) (Fig 4B). This increase in the IL-10 secretion was not MAP-specific as it was observed in both PPD-J stimulated and non-stimulated PBMCs cultures (Fig 4B). This observation is interesting when taken into consideration that bovine WC1+ γδ T cells have been shown to produce IL-10 and have a regulatory role in vitro [17, 18]. The exact mechanism by which depletion of γδ T cells, including the WC1+ γδ T cell subset, enhances IL-10 production in PBMCs cultures remains to be elucidated. However, IL-10, a potent
inhibitory cytokine, can be produced by various cell types in PBMCs including different T cell subsets, monocytes, and B cells [29-31].

Our data indicate that depletion of total γδ T cells or the WC1⁺ subset had no significant impact on IL-12 secretion by PBMCs stimulated with PPD-J (Fig 4D). While PPD-J stimulation led to a significant increase in IL-12 production, depleted PBMCs secreted similar levels of IL-12 regardless of PPD-J presence. This observation suggests that depletion of total γδ T cells, specifically the WC1⁺ subset, resulted in an increase in IL-12 secretion in non stimulated PBMCs. Bovine WC1⁺ γδ T cells have been shown to enhance IL-12 production by dendritic cells infected with M. bovis [16]. IL-12 is involved in the generation of Th1 responses through its induction of IFN-γ production; a cytokine that upregulates IL-12 expression through a feedback mechanism [32, 33]. The suppressed MAP-specific IFN-γ response observed in our study might explain the absence of differences in IL-12 secretion between γδ T cells-depleted and non-depleted PBMCs stimulated with PPD-J. In the present study, γδ T cells failed to produce IFN-γ in response to stimulation with mitogens like pokeweed (data not shown). It is possible that IL-12 was secreted at its basal level as IL-12 production was not negatively impacted by the increase in IL-10 secretion in γδ T cells-depleted PBMCs [34].

In summary, we used cattle naturally infected with MAP and were in the subclinical stage of infection to examine the impact of WC1⁺ γδ T cells depletion on cytokine production by PBMCs stimulated ex vivo with MAP antigens. PBMCs from the animals used in the current study exhibited hyporesponsiveness to ex vivo stimulation with PPD-J with regard to the production of IFN-γ, IL-4, and IL-10. In contrast, PPD-J stimulation led to a significant increase in IL-12 production. Independently of PPD-j
presence, depletion of total γδ T cells, specifically the WC1⁺ γδ T cell subset, resulted in a significant decrease in IL-4 secretion and a significant increase in IL-10 secretion. No significant changes were observed in IFN-γ and IL-12 production by PBMCs stimulated in presence or absence of the WC1⁺ subset. The data presented in this study suggest a role for the WC1⁺ γδ T cell subset in modulating cytokine production by PBMCs taken from cattle subclinically infected with MAP. Since the animals used in the present study were hyporesponsive to stimulation with MAP antigens, the possibility that the WC1⁺ γδ T cell subset modulates MAP-specific responses warrants further investigation.

Acknowledgment

We thank Dr. Tracy Lindquist for her assistance with the Luminex assay. This work was funded by Iowa State University CVM seed grants, Iowa livestock health advisory council and Iowa veterinary medical association.

References


27. Baquero, M.M. and B.L. Plattner, Bovine WC1(+) gammadelta T lymphocytes modify monocyte-derived macrophage responses during early *Mycobacterium*


34. Rahim, S.S., et al., Interleukin-10 (IL-10) mediated suppression of IL-12 production in RAW 264.7 cells also involves c-rel transcription factor. Immunology, 2005. 114(3): p. 313-21.
Table 1. Antibodies used in cells sorting and flow cytometry staining.

<table>
<thead>
<tr>
<th>Primary</th>
<th>Catalog Number</th>
<th>Isotype</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>γδ TCR</td>
<td>GB21A</td>
<td>IgG2b</td>
<td>Goat anti-mouse IgG2b AF647</td>
</tr>
<tr>
<td>WC1</td>
<td>ILA29</td>
<td>IgG1</td>
<td>Goat anti-mouse IgG1 AF488</td>
</tr>
<tr>
<td>CD4</td>
<td>MCA1653GA</td>
<td>IgG2a</td>
<td>Goat anti-mouse IgG2a AF680</td>
</tr>
<tr>
<td>IFNγ</td>
<td>MCA1783PE</td>
<td>IgG1</td>
<td>Direct conjugate (PE)</td>
</tr>
</tbody>
</table>

a Monoclonal Antibody Center, Pullman, WA, USA.
b AbD SeroTech, Raleigh, NC, USA
c Life Technologies, Grand Island, NY, USA.
Fig 1. IFN-γ, IL-12, IL-4 and IL-10 production by PBMCs cultures stimulated for 24h with PWM, PPD-J or medium only. The data were collected from six subclinically infected cattle. Data are presented as mean ± SEM. * P ≤ 0.05.
Fig 2. Detection of intracellular IFN-γ in CD4+ T cells. (A) Representative flow plots from a subclinical animal gated on CD4+ T cells and IFN-γ. (B) The percentage of CD4+ T cells that produced IFN-γ in response to 24h stimulation with PWM, PPD-J or medium only was measured using flow cytometry. The data were collected from six subclinically infected cattle. * P < 0.05. Data are presented as mean ± SEM.
Fig 3. Detection of intracellular IFN-γ in PBMCs cultures depleted of total γδ T cells (Δ γδ T cells) or WC1+ γδ T cells (Δ WC1+ γδ T cells) before stimulation for 24h with PPD-J or medium only. (A) Representative flow plots from a subclinical animal gated on total live cells and total γδ T cells. (B) The frequency of γδ T cells within PBMCs with no cell depletion, γδ TCR+ cells depletion or WC1+ cells depletion. The percentage of PBMCs (C) and CD4+ T cells (D) that produced IFN-γ in response to 24h stimulation with PPD-J or medium only was measured using flow cytometry. The data were collected from six subclinically infected cattle. Data are presented as mean ± SEM.
Fig 4. IFN-γ, IL-12, IL-4 and IL-10 production by PBMCs cultures depleted of total γδ T cells (Δ γδ T cells) or WC1+ γδ T cells (Δ WC1+ γδ T cells) before stimulation for 24h with PPD-J. The data were collected from six subclinically infected cattle. Data are presented as mean ± SEM. ** P ≤ 0.01 and *** P ≤ 0.001.
CHAPTER 5: GENERAL CONCLUSIONS

Summary

The central hypothesis of the studies discussed in this dissertation has been that in cattle naturally infected with MAP, WC1+ γδ T cell frequency, immunological responses and functions (cytokine production) differ with the stage of infection. A longer-term goal was to investigate how the WC1+ γδ T cell subset relates to the immune responses that control MAP infection or those that facilitate disease progression. Defining systemic and local responses of WC1+ γδ T cells at different stages of MAP infection is essential for a complete understanding of host-MAP interactions and for overcoming challenges regarding control and prevention of JD. In these studies, we used non-infected cattle and cattle naturally infected with MAP, either with subclinical or clinical infection.

In the first study, we compared the three groups with regard to WC1+ γδ T cells frequency both in peripheral blood and at the primary site of MAP infection, the ileum. We have also examined immunological responses of WC1+ γδ T cells (proliferation and cytokine production) in PBMCs stimulated ex vivo with MAP antigens (PPD-J). Our first hypothesis was that progression to the clinical form of MAP infection would associate with a significant decrease in WC1+ γδ T cell representation within circulating lymphocytes as a result of increased infiltration to MAP infection sites. Our data indicate that the frequency of γδ T cells within PBMCs was lower in the infected animals compared to that of the non-infected animals and this was significant in the clinical group. The frequency of the WC1.2+ γδ T cell subset within circulating γδ T cells was significantly reduced in the infected groups compared to the control group regardless of
the infection status. These observations did not associate with increased infiltration of γδ T cell subsets to MAP infection sites (ileum).

Chronic infections with high bacterial load typically associate with dysregulated immune responses, thus we hypothesized that cattle with the clinical form of MAP infection would have weak or impaired γδ T cell proliferative responses in PBMCs stimulated *ex vivo* with MAP antigens. We were able to show that in PBMCs, γδ T cells proliferated specifically in response to stimulation with PPD-J. However, these proliferative responses were detected only in the subclinical group. The MAP-specific proliferative response of WC1⁺ γδ T cells was a heterogeneous mix of the WC1.1 and WC1.2 subsets. Our data suggested that progression to the clinical stage of MAP infection associated with a significant decrease in γδ T cells frequency in peripheral blood along with impaired γδ T cell proliferative responses to *ex vivo* stimulation with MAP-specific antigens.

We hypothesized that IFN-γ and IL-10 production by WC1⁺ γδ T cell subsets in PBMCs stimulated with MAP antigens would be influenced by the infection status. We expected these cells to express more IFN-γ in the subclinical group and more IL-10 in the clinical group. Our data indicate no significant differences in the frequency of IFN-γ⁺ γδ T cells in PPD-J stimulated PBMCs between subclinically and clinically infected cattle. However, there was a significant increase in the frequency of WC1.1⁺ γδ T cells expressing IFN-γ in the subclinical group compared to the clinical and control groups. Our data demonstrate that WC1⁺ γδ T cells in the infected groups differentially expressed IFN-γ. While the WC1.2 subset produced IFN-γ in all the the infected groups, the WC1.1 subset produced IFN-γ in the subclinical group only. The unresponsiveness of the WC1.1
subset observed in the clinical group may serve as a biomarker for disease progression in MAP infected cattle. We found no significant differences among the groups with regard to IL-10 production by the WC1+ γδ T cell subsets in PBMCs stimulated with PPD-J.

Data generated from this study supported our hypothesis that WC1+ γδ T cells from cattle with JD respond differentially to stimulation with MAP antigens and suggest that WC1+ γδ T cells may contribute to the immune response that controls MAP infection during the subclinical stage of the disease.

In the second study, we used archived ileal tissues to examine the WC1+ γδ T cell subset for expression of a set of regulatory and proinflammatory cytokines with known relevance to the pathogenesis of MAP infection. Our approach was to use a novel in situ mRNA hybridization assay (ISH) known as RNAscope. By using probes specific for the WC1, IFN-γ, IL-17, TNF-α, IL-10 and TGF-β transcripts, we were able to define the cytokine profile of the WC1+ subset by simultaneously staining transcripts coding for the WC1 molecule and one of the cytokines of interest. With respect to regulatory cytokines, our data indicate a significant increase in the numbers of WC1+ γδ T cells expressing IL-10 in clinical cattle compared to subclinical and non-infected cattle. TGF-β expression by the WC1+ subset was elevated in clinical cattle but was not of statistical significance. We found no significant differences among the groups with regard to IFN-γ, IL-17 and TNF-α expression by WC1+ γδ T cells. Our results suggest that the cytokine profile of the WC1+ subset differs with the stage of MAP infection. Given the inhibitory role of IL-10, our data suggest that the WC1+ γδ T cell subset may promote MAP proliferation and disease progression during the terminal stages of MAP infection. Data generated from studies 1 & 2 demonstrate differences between subclinical and clinical cattle and support
our central hypothesis in two ways, namely that WC1$^+$ γδ T cells from MAP-infected cattle 1) respond differentially to stimulation with MAP-specific antigens, and 2) their functions at MAP infection sites differ with the stage of infection.

Several in vivo studies have suggested a modulatory role for the WC1$^+$ γδ T cell subset. Thus in our third study, we used cattle subclinically infected with MAP to examine the impact of WC1$^+$ γδ T cell depletion on PBMCs responses to stimulation with MAP antigens. We were interested in measuring changes in IFN-γ, IL-12, IL-4 and IL-10 production by PBMCs stimulated with PPD-J in presence or absence of the WC1$^+$ subset. Our data indicate that depletion of the WC1$^+$ γδ T cell subset before PBMC stimulation with PPD-J had no significant effect on the percentages of cells stained for cytoplasmic IFN-γ as measured by flow cytometry. Our data indicate a significant increase in IL-10 secretion and a significant decrease in IL-4 secretion in the supernatants of PBMCs depleted of the WC1$^+$ subset compared to PBMCs with no cell depletion. The effect of WC1$^+$ γδ T cell depletion on IL-10 and IL-4 secretion was independent of PPD-J presence. We observed no significant differences between WC1$^+$ γδ T cells depleted and non-depleted PBMCs with regard to IFN-γ and IL-12 secretion. Our results demonstrate that depletion of the WC1$^+$ γδ T cell subset altered cytokine production by PBMCs obtained from cattle subclinically infected with MAP. Changes were observed in Th2 cytokines and were independent of presence or absence of MAP-specific antigens.

We have made some progress towards understanding the role of the WC1$^+$ γδ T cell subset during MAP infection through the assessment of their ex vivo responses and in vivo functions (cytokine production) using naturally infected cattle at two different stages.
of the disease. The generated data fill some of the gaps in the current knowledge regarding the role of this T cell subset in the host response to MAP infection.

**Directions For Future Studies**

In the previous thesis, we evaluated WC1⁺ γδ T cells frequency, immunological responses and functions in cattle naturally infected with MAP. Our observations suggest differences between subclinical and clinical cattle with regard to immunological responses and functions of the WC1⁺ γδ T cell subset both in peripheral blood and at the sites of MAP infection. Based on these data, our ongoing hypothesis is that WC1⁺ γδ T cells may have an impact on the outcome of MAP infection. This work can be extended in several pathways in which future efforts should be directed.

In chapter 2, we demonstrated that WC1⁺ γδ T cells from cattle subclinically infected with MAP proliferate and produce IFN-γ specifically in response to *ex vivo* stimulation with MAP antigens. In this study, cells were stimulated with a MAP-specific complex-antigen, PPD-J. Compared to humans where γδ T cell ligands are extensively studied, bovine γδ T cells ligands are less defined. Differences between human and bovine γδ T cells may exist with regard to antigen recognition. Bovine γδ T cells (the majority are WC1⁺) failed to respond to phosphoantigens recognized by human Vδ2⁺ γδ T cells [1]. Identification of bovine γδ T cells cognate antigens, receptors and mechanisms of activation is critical for appropriate targeting of γδ T cells by future vaccines.

In chapter 2, we examined the frequency of the WC1⁺ γδ T cell subsets in ileal tissues from non-infected cattle, subclinical cattle and cattle with the clinical form of
MAP infection. Our data indicate no significant differences in the numbers of WC1+ γδ T cells infiltrating MAP infection sites between the three groups. This study can be extended to describe WC1+ γδ T cells colocalization with other cell types including dendritic cells (DCs) and macrophages. WC1.1+ γδ T cells have been shown to be selectively recruited to the lungs and respiratory lymph nodes following BCG vaccination. Interestingly, these cells have been shown to cluster with DCs suggesting direct interactions between WC1+ γδ T cells and DCs in vivo [2]. Defining WC1+ γδ T cell interactions with other cell types at the sites of MAP infection is essential for understanding the role of the WC1+ γδ T cell subset in the host response to MAP infection.

In chapter 3, we evaluated WC1+ γδ T cells in the ileum for expression of a set of proinflammatory and regulatory cytokines. In this study, we used a small number of animals due to the high cost of the RNAscope reagents and our limited research budget. This study needs to be extended to incorporate a larger number of animals and examine different parts of the intestines and their associated lymph nodes. Such a study should not be limited to the cytokine profile but evaluate WC1+ γδ T cells for chemokine expression as WC1+ γδ T cells were shown to be involved in chemotaxis of αβ T cells [3]. In our study, we could not differentiate between the WC1+ γδ T cell subsets through the staining of the WC1 transcript. However, this can be achieved by performing both immunofluorescence and fluorescent RNAscope staining on the same slide. Differentiating between WC1+ γδ T cell subsets is essential as WC1+ γδ T cells function varies with the expressed WC1 isoform [4]. Our data indicate a significant increase in the number of WC1+ γδ T cells expressing IL-10 in ileal tissues from clinical cattle and
suggest that the WC1⁺ γδ T cell subset may play a regulatory role during the clinical stage of MAP infection. The possibility that these cells exhibit inhibitory effects in vivo remains to be investigated.

In chapter 4, we used cattle subclinically infected with MAP to examine the impact of WC1⁺ γδ T cells depletion on cytokine production by PBMCs in response to ex vivo stimulation with MAP antigens. Our results indicate that depletion of the WC1⁺ γδ T cell subset modulates cytokine production by PBMCs independently of antigen stimulation. Since PBMCs obtained form the animals used in this study were hyporesponsive to stimulation with PPD-J, the possibility that WC1⁺ γδ T cells depletion impacts MAP-specific responses remains to be investigated. This study needs to be repeated in calves with active MAP specific responses (Vaccinated or experimentally infected). Due to the high frequency of γδ T cells in peripheral blood of young calves, the effect of WC1⁺ γδ T cells depletion on the host response to MAP infection can be examined at the subset level (WC1.1 vs. WC1.2).

References


4. Baldwin, C.L. and J.C. Telfer, The bovine model for elucidating the role of
gammadelta T cells in controlling infectious diseases of importance to cattle and