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Evaluating the cytokine profile of the WC1$^+$ γδ T cell subset in the ileum of cattle with the subclinical and clinical forms of MAP infection

S.M. Albarrak[a,*], W.R. Waters[b], J.R. Stabel[b], J.M. Hostetter[a]

[a] Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, 1600 S 16th St, Ames, IA, 50011, USA
[b] USDA-ARS, National Animal Disease, 1920 Dayton Ave, Ames, IA, 50010, USA

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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 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 non-WC1$^+$ 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.

1. 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 (Guzman et al., 2012; Plattner and Hostetter, 2011). 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 (Wilson et al., 1996). 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 (Herzig et al., 2010; Machugh et al., 1997; Wijngaard et al., 1992). The WC1$^-$ subset predominates in tissues such as spleen and intestine whereas the majority of γδ T cells in peripheral blood are WC1$^+$ (Machugh et al., 1997). Given their localization to mucosal tissues, an immune surveillance role has been suggested for the WC1$^-$ subset (Hedges et al., 2003; Meissner et al., 2003). Compared to the WC1$^-$ subset, the WC1$^+$ subset has been shown to be more active with regard to proliferation and proinflammatory cytokine production (Hedges et al., 2003; Meissner et al., 2003; Rogers et al., 2005).

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 (Charavaryamath et al., 2013; Plattner et al., 2013). 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 (Stabel, 2000, 2006). 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 (Albarrak et al., 2017). 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 (Khalifeh and Stabel, 2004b). 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 (Coussens et al., 2004; Khalifeh and Stabel, 2004b; Lee et al., 2001; Sweeney et al.,

* Corresponding author.
E-mail addresses: albarrak@iastate.edu (S.M. Albarrak), Ray.Waters@ars.usda.gov (W.R. Waters), Judy.Stabel@ars.usda.gov (J.R. Stabel), jesseh@iastate.edu (J.M. Hostetter).

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1998; Tanaka et al., 2005). 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 (Clarke, 1997). 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) (Albarrak et al., 2017). 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 WC1$^-$ subset because its characterization requires a probe for γδ TCR, WC1 and cytokine of interest. Three colour 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 (Kaufmann and Dorhoi, 2016). Our key finding in the present study was a significant increase in the frequency of ileal WC1$^+$ γδ T cells expressing IL-10 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.

2. Materials and methods

2.1. 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 (Albarrak et al., 2017; Khalifeh and Stabel, 2004b). 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. Naturally infected cows were routinely observed (quarterly at least) for weight loss by visual observation. Weight loss due to clinical disease equate to a reduction in body score from ≥3.0 down to 1.5. Control or non-infected cows were obtained from accredited JD free herds and repeatedly tested negative by fecal culture and serological assays.

2.2. 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.

2.3. mRNA detection by in situ mRNA 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). 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 h followed by a deparaffinization step and a protease treatment to uncover RNA. 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)-based Green (cytokines) and alkaline phosphatase (AP)-Fast Red (WC1) chromogenic stains (catalogue No: 320701, Advanced Cell Diagnostic, CA, USA). 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 400× magnification.

2.4. Image analysis

In each slide, six 400× magnification non-overlapping fields were photographed. In each field, cells with positive signals in the lamina propria or in the epithelium were counted. Cells counting was done manually by the same observer using the micro imaging software cellSens (Olympus Corporation, PA, USA). Cells labeled with red and green signals located in or near the nucleus are WC1$^+$ cells expressing the cytokine of interest whereas cells labeled with only green signals are non-WC1$^+$ cells expressing the cytokine of interest.

2.5. 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 between groups' means.

3. 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 (Chiodini et al., 1984; Clarke, 1997). Using HE stained ileal sections and serial sections stained with ZN, we defined the presence or absence of granulomatous inflammation and acid-fast organisms 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 lamina propria and submucosa by sheets of macrophages with abundant eosinophilic to granular cytoplasm (Fig. 1A) (Hostetter et al., 2005; Tanaka et al., 2005). Acid fast staining demonstrated that most macrophages in the granulomatous lesions in the clinical group contained large numbers of acid-fast 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 lamina propria (Fig. 1C). Granulomatous lesions were not detected in the ileum of the remaining subclinical animals. Acid fast staining did not demonstrate acid-fast organisms in the ileum from any of the subclinically infected cattle (Fig. 1D). PCR/culture was not performed directly on the formalin-fixed sections. However, upon necropsy, MAP was isolated from the distal-ileum of the clinical animals and only one of the subclinical animals (the subclinical animal with pathological lesions). Ileum from control cattle did not have any detectable granulomatous lesions or acid fast bacilli. These findings demonstrate that, as expected, cattle in the clinical group all had progressed to multibacillary disease with