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Research paper

Measuring bovine γδ T cell function at the site of *Mycobacterium bovis* infection

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ABSTRACT

Bovine γδ T cells are amongst the first cells to accumulate at the site of *Mycobacterium bovis* infection; however, their role in the developing lesion remains unclear. We utilized transcriptomics analysis, *in situ* hybridization, and a macrophage/γδ T cell co-culture system to elucidate the role of γδ T cells in local immunity to *M. bovis* infection. Transcriptomics analysis revealed that γδ T cells upregulated expression of several novel, immune-associated genes in response to stimulation with *M. bovis* antigen. BCG-infected macrophage/γδ T cell co-cultures confirmed the results of our RNAseq analysis, and revealed that γδ T cells from *M. bovis*-infected animals had a significant impact on bacterial viability. Analysis of γδ T cells within late-stage *M. bovis* granulomas revealed significant expression of IFN-γ and CCL2, but not IL-10, IL-22, or IL-17. Our results suggest γδ T cells influence local immunity to *M. bovis* through cytokine secretion and direct effects on bacterial burden.

1. Introduction

Tuberculosis (TB) is among the most important infectious diseases worldwide. In 2015 1.8 million people died from this disease (World Health Organization, 2016). *Mycobacterium bovis (M. bovis)* is a member of the *Mycobacterium tuberculosis* complex (Mtbc), and is the causative agent of TB in cattle (BTB). *M. bovis* is an aerobic pathogen capable of causing zoonosis in most mammals, including humans. This disease has a significant detrimental impact on the livestock industry; costing billions of dollars in losses each year due to disease testing and control efforts (Waters et al., 2012). Eradication attempts have been successful in some countries; however, the broad host range and low infective dose of BTB makes worldwide eradication difficult.

Cattle are a natural host for *M. bovis*, and BTB parallels human TB in several aspects of disease pathogenesis and the development of innate and adaptive immune responses (Van Rhijn et al., 2008; Waters et al., 2011). Historically, the study of bovine and human TB has been closely intertwined, and our understanding of disease in animals has been instrumental in our understanding of that in humans. For example, the vaccine strain that is widely administered to infants and people at high risk for TB is actually *M. bovis* Bacille Calmette Guerin (BCG). This vaccine was tested in cattle before being administered to humans. Similarly, IFN-γ release assays were first implemented in the bovine TB eradication program, and are now widely used in human diagnostics. Thus, the study of virulent *M. bovis* infection in cattle represents an excellent model for understanding *Mycobacterium tuberculosis* (*M. tb)* infection in humans, and for testing novel vaccine strategies and therapeutics (Waters et al., 2012).

Granulomas are characteristic of TB infections, and are the body's attempt to protect the host by containing the invading mycobacteria. They are an organized structure of immune cells that form around the invading bacterium and are comprised of macrophages, neutrophils and lymphocytes. The structures undergo a process of ordered maturation during the course of disease, and can be staged (I–IV) based upon cellular composition and amount of fibrosis and necrosis (Thoen and Steele, 2017; Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). Importantly, simple formation of a granuloma is not sufficient alone to control or eliminate the disease. The ability of the host to establish well-organized granulomas, with an appropriate balance of pro- and anti-inflammatory immune responses is crucial to controlling the infection (Flynn et al., 2011; Gideon et al., 2015). Despite the importance of the granuloma structure in dictating the outcome of infection, we understand very little about the dynamics of the immune response at the site of infection. Specifically, the cells and cytokine production necessary for formation and maintenance of an effective granuloma.

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γδ T cells are a unique subset of $CD3^+$ T cells that possess functions characteristic of both innate and adaptive immunity, and are therefore thought to bridge the two arms of the immune system. γδ T cells constitute a significant proportion of the immune cells found in the mucosal and epithelial surfaces of the respiratory tract. These cells are generally recognized to be critical in the first line of defense against invading pathogens and in shaping the downstream adaptive immune response (Hayday, 2000). However, the frequency of γδ T cells circulating in mice, humans, and non-human primates is low, representing 1–5% of the circulating peripheral lymphocyte population (Kabelitz, 2011), making it difficult to experimentally dissect the role of the γδ T cells in the immune response. In contrast, γδ T cells circulate at significantly increased frequencies in ruminant species, where they constitute 30–60% of the peripheral blood lymphocytes in young animals (Hein and Mackay, 1991; Jutila et al., 2008). The increased incidence of these cells in blood makes the bovine an excellent model for studying γδ T cells and for understanding their role in innate and adaptive immunity.

γδ T cells in mice and cattle accumulate in the lungs and lung-associated lymph nodes after either *M. bovis* infection or BCG vaccination administered via respiratory routes (Price et al., 2010; Dieli et al., 2003a). These cells are also among the first cells to arrive at the site of infection (Doherty et al., 1996). γδ T cells have been shown to accumulate within all stages of lesions in cattle infected with *M. bovis*, and are often found localizing to the lymphoid mantle surrounding the periphery of the lesions (Cassidy et al., 1998). Mice deficient in γδ T cells develop large and poorly organized granulomas during *M. tb* infection (D'Souza et al., 1997). Similarly, mice and rodents depleted of γδ T cells show alterations in granuloma architecture with increases in neutrophil infiltration and necrosis (Smith et al., 1999). These findings suggest that γδ T cells may be an important source of cytokines and chemokines which aid in the recruitment of other immune cells to the site of infection. *In vitro,* γδ T cells have been shown to produce significant amounts of IFN-γ, similar to that of $CD4^+$ T cells, in response to mycobacterial antigens (Lee et al., 2004). However, less is known about their capacity to secrete IFN-γ *in vivo*, particularly at the site of infection. The ability of these cells to secrete chemotactic molecules or other immune factors in response to *M. bovis* infection is not well defined. Therefore, in this study, we used RNASeq analysis to further define the *M. bovis*-specific γδ T cell response. This approach allowed us to identify previously unrecognized immunologic factors that may contribute to the γδ T cell's capacity to establish and maintain granuloma structures *in vivo*. To correlate the *in vitro* responses measured by our RNASeq analysis with those that occur *in vivo* at the site of infection, we also used *in situ* hybridization. This allowed us to assess the expression of multiple cytokines by γδ T cells accumulating in the chronic, granulomatous lesions of cattle infected with virulent *M. bovis*. We also developed a novel, *in vitro* macrophage/γδ T cell co-culture system. This system allowed us to model the interactions that may occur in the lungs between tissue-resident γδ T cells and *M. bovis*-infected macrophages in the early stages of BTB infection. Our hypothesis is that γδ T cells influence immune cell recruitment and granuloma formation, and shape the adaptive *M. bovis*-specific immune response by producing inflammatory and regulatory cytokines and chemokines at the site of *M. bovis* infection. Determining the role that γδ T cells play in the localized immune response to *M. bovis* infection is expected to further our understanding of basic γδ T cell biology. Our findings may also aid in developing effective ways in which to manipulate protective responses to TB in both humans and animals.

2. Materials and methods

2.1. Animals

Tissues samples were collected from animals used in a previous study (Waters et al., 2014). Briefly, 23 Holstein steers approximately 6 months of age were obtained from a tuberculosis-free herd in Sioux Center, Iowa and housed in a biosafety level-3 (BSL-3) facility at the National Animal Disease Center (NADC), Ames, Iowa, USA, according to Institutional Biosafety and Animal Care and Use Committee guidelines. Treatment groups consisted of non-infected steers (n = 7) and animals receiving 10^4 colony forming units (cfu) of *M. bovis* 95–1315 (n = 8) or 10^4 cfu *M. bovis* 10–7428 (n = 8) by aerosol as described by Palmer et al. (Palmer et al., 2002).

Animals used for co-culture experiments were 10 Holstein steer calves that were housed at the NADC in Ames IA. Calves were experimentally infected with 10^4 cfu of virulent *M. bovis* 10–7428 as above and peripheral blood was collected at ~12 weeks after challenge. Prior to sample collection, the calves were confirmed BTB positive by skin test and whole blood IFN-γ release assay. Blood samples were also obtained from 19 Holstein steer calves maintained in an *M. bovis*-free herd housed at the Kansas State University Dairy Facility in Manhattan, KS. Animals from both herds were age, breed, and sex matched in order to minimize possible herd-to-herd variation.

All animal procedures were conducted in strict accordance with federal and institutional guidelines and were approved by the NADC Institutional Animal Care and Use Committee or the Kansas State University Institutional Animal Care and Use Committee.

2.2. Preparation of PBMCs

Peripheral blood was collected from the jugular vein into 2X acid citrate dextrose. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coat fractions and overlaid onto Histopaque 1077 (Sigma Aldrich, St. Louis, MO). Contaminating red blood cells were removed using a hypotonic lysis. Cells were washed and re-suspended in complete RPMI (cRPMI) composed of RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 1% nonessential amino acids, 2% essential amino acids, 1% sodium pyruvate, 50 μM 2-mercaptoethanol (ME), and 10% fetal bovine serum (FBS).

2.3. γδ RNA sequencing

PBMCs from 5 *M. bovis*-infected animals were collected, and stimulated samples were cultured with purified protein derivative of bovine tuberculin (PPD-b) (Prionics AG, Schlieren, Switzerland) at 200 U/mL in cRPMI. Unstimulated control samples were cultured with cRPMI only for 18 h. γδ T cells were then sorted (as described in Section 2.5) to > 90% purity by magnetic activated cell sorting (MACS) according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). γδ T cell RNA was extracted using Trizol Reagent (Invitrogen, Life Technologies) according to manufacturer's instructions. Sample quality and quantity was confirmed by Tape Station analysis and Qubit quantification, and then Truseq RNA Libraries prepared per manufacturer's instructions. The libraries were sequenced on an Illumina HiSeq 2500 System.

Sequencing reads were aligned to the most recently annotated version of the bovine genome (Bos_taurus_UMD_3.1.1). Single end reads were obtained and FastQC was ran to identify over-represented reads. Differential expression analysis was performed using RNA Sequencing by Expectation Maximization (RSEM) and empirical Bayes sequencing (EBSeq) (Li and Dewey, 2011; Leng et al., 2013).

A list of commonly differentially expressed genes resulting from the RNA sequencing analysis was submitted to Ingenuity Pathway Analysis (IPA; Ingenuity Systems, USA) in order to identify the most significant canonical pathways (Breuer et al., 2013).

2.4. RNA scope

Visualization of γδ T cells, cytokine, and chemokine mRNA transcripts was done according to manufacturer's instructions for RNAScope 2.0 (Advanced Cell Diagnostics, Hayward, CA, USA). Samples were