# Measuring Bovine γδ T Cell Function at the Site of *Mycobacterium bovis* Infection

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# Abstract

The causative agent of tuberculosis (TB) in cattle is *Mycobacterium bovis (M. bovis).* γδ T cells are a unique subset of nonconventional T cells that play major roles in both the innate and adaptive arms of the immune system. Bovine γδ T cells have the capacity for multiple immune functions during infection with *M. bovis.* However, the alternative functions of γδ T cells as well as the responses of γδ T cells *in vivo* at the site of infection remain unclear.

To identify novel functions for γδ T cells in response to *M. bovis* infections, RNA sequencing and transcriptomics analysis was completed on peripheral blood γδ T cells isolated from virulent *M. bovis*-infected cattle. Differentially expressed genes were confirmed with real-time PCR. In an attempt to model *in vivo* cell-to-cell interactions at the site of infection, γδ T cells were also isolated from naïve and *M. bovis*-infected calves and co-cultured with autologous, BCG-infected, monocyte-derived macrophages. γδ T cell chemokine and cytokine expression was analyzed via ELISA and real-time PCR. The characteristic lesions of bovine tuberculosis are well-organized pulmonary granulomas. To determine the relevance of the RNA-sequencing and *in vitro* co-culture results to *in vivo* infection, tissue samples from granulomatous lesions in the lungs and mediastinal lymph nodes of virulent *M. bovis*-infected cattle were collected 3 months after infection. mRNA transcripts for γδ T cells expression of-- IFN-γ, IL-17, IL-10, IL-22, and CCL2 were microscopically evaluated within the granulomas using an *in situ* hybridization system, RNAScope (Advanced Cell Diagnostics Inc.).

Co-culture experiments and transcriptomics analysis revealed increased expression of chemokines and various cytokines by γδ T cells responding to *M. bovis* infection. The novel *in situ* hybridization assay revealed that cytokine expression by γδ T cells varied within the lesions, with significant levels of CCL2 and IFN-γ, and low expression of IL-10, IL-22, and IL-17 *in situ*

at this time-point after infection. Co-culture experiments also revealed that γδ T cells from virulent *M. bovis*-infected cattle have the capacity to directly impact the viability of *M. bovis in vitro*. Our results suggest that γδ T cells accumulate within the granulomas, and influence host immunity to *M. bovis* by secretion of cytokines and chemokines, and direct cytotoxicity, in response to infected macrophages.

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# Chapter 1 - General Introduction

## Introduction

Tuberculosis (TB) is among the most important infectious diseases worldwide. In 2015 1.8 million people died from this disease (WHO, 2017). *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 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 loses each year. Eradication attempts have been successful in some countries; however, the broad host range and low infective dose of BTB make worldwide eradication difficult. BTB parallels human TB in regards to disease pathogenesis and development of innate and adaptive immune responses, making the bovine an excellent model to study human disease.

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 increase in the periphery of patients with active TB, and are among the first cells recruited to the site of infection in cattle, suggesting they play a critical role in early immunity to TB. However, the frequency of these cells in humans and mice is very low compared to ruminants, making cattle an ideal model to elucidate the role of γδ T cells in the immune response to infection. γδ T cells share several adaptive immune characteristics with their α/β T cell counterparts, such as interferon gamma (IFN-γ) production and clonal expansion; and such responses have been well described in the context of human and bovine TB. However, γδ T cells are shown to have the capacity for a wide variety of additional functions, such as chemokine production, direct cytotoxicity, immunoregulation, and immune cell cross-talk. The occurrence and biological significance of these functions, particularly during TB infection, is

poorly understood. Further, although significant numbers of γδ T cells accumulate at the site of *M. bovis* and *Mycobacterium tuberculosis* (*M.tb)* infection, particularly during the early stages of disease, their role at the site of infection remains poorly defined.

## Literature Review

### *Mycobacterium bovis*

Mycobacteria are slow-growing, aerobic, gram positive, rod-shaped bacteria. Unlike other gram positive bacteria, mycobacteria do not retain the gram stain due to the unique structure of the cell wall. Mycobacteria are therefore identified as being acid-fast and are detected using the Ziehl-Nielsen stain (Ulrichs et al., 2005). *M. bovis* is one of eight members in the Mtbc. The Mtbc includes: *Mycobacterium tuberculosis*, *Mycobacterium bannettii*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium pinnipedii*, *Mycobacterium mungi*, *Mycobacterium caprae*, and *Mycobacterium microti* (Thoen et al., 2014). The Mycobacterium genus includes several species of TB causing agents; however, not all mycobacteria cause TB disease. Members of the Mtbc are characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA sequences; however, they differ in their hosts, phenotypes, and pathogenicity (Brosch et al., 2002). Members of the Mtbc cause TB in a wide range of hosts. *M. tb*, *M. africanum,* and *M. canettii* primarily infect humans, *M. mungi* infects mongooses, *M. pinnipedii* infects pinnipeds (seals and sea lions), *M. microti* infects voles, and *M. bovis* infects cattle and goats. Many other wildlife species (deer, badgers, foxes, wild ruminants, etc.) can be infected by a number of these mycobacterial strains as well (Thoen et al., 2014). *M. bovis* is responsible for causing TB primarily in cattle, but is also capable of causing disease in most other mammals, including humans. *M. tb* is the pathogen that most commonly causes TB

in humans; however, symptoms of *M. bovis* and *M. tb* infection are indistinguishable (Thoen et al., 2014).

## Epidemiology of BTB in humans and animals

One third of the world's population is infected with TB, making it one of the leading causes of infectious disease-related deaths world-wide (Thoen et al., 2014). In the early 1990's the World Health Organization (WHO) declared TB to be a global emergency due to the development of antibiotic resistant strains of M. tb (Luca and Mihaescu, 2013; WHO, 1994). TB is responsible for killing approximately 1.7 million people per year; with the majority of those deaths attributed to infection with *M. tb*. 95% of TB cases occur in developing countries, especially in populations where HIV/AIDS is endemic (Thoen et al., 2014).

The most common route of *M. bovis* infection for humans is through ingestion of contaminated foods, such as unpasteurized milk and cheese (Thoen et al., 2014). It is currently stated by the Centers for Disease Control and Prevention (CDC) that *M. bovis* accounts for less than 2% of human TB cases; however, there is growing evidence that suggests the *M. bovis* burden in humans as the cause of TB is significantly underestimated (Thoen et al., 2010; Olea-Popelka et al., 2016). Clinically distinguishing a *M. bovis* from a *M. tb* infection is difficult without the proper diagnostic tools, and the most commonly used assays for detecting TB infection are not able to differentiate between *M. bovis* and *M. tb*, leading to misdiagnosis and under-reporting of actual *M. bovis* cases (Thoen et al., 2010; Olea-Popelka et al., 2016). Further, reports of *M. bovis* cases are likely skewed due to more available data from high-income countries where TB prevalence is low. It is estimated that 3 million cases of TB, caused by either *M. tb* or *M. bovis*, are missed each year (Olea-Popelka et al., 2016). In the U.S., *M. bovis*

is estimated to account for 1.5% of TB cases; however, in areas with large populations of foreign-born people (especially Hispanic), there continues to be a disproportionately higher burden of TB (Rodwell et al., 2008; CDC, 2015). It is estimated that there are 9 million people infected with TB each year, and even if a small percentage of those cases are due to *M. bovis*, that results in a substantial absolute number of people infected and killed by *M. bovis* infection each year (Müller et al., 2013; Olea-Popelka et al., 2016). While cases of BTB are rare in the United States, many other countries have seen a continual increase in disease incidence over the past 20 years (Witchell et al., 2010; CDC, 2012). In 2015 there were an estimated 149,000 new cases of human zoonotic TB globally, with Africa carrying the heaviest burden with over 76,000 incidences followed by South-east Asia with more than 47,000 cases (WHO 2016).

BTB continues to have a significant impact on the agricultural industry world-wide, infecting over 50 million cattle and resulting in a $3 billion loss to the industry each year due to culling, testing, and control in animals (Palmer et al., 2007). *M. bovis* is easily spread, and its primary route of infection for cattle in a natural setting is by inhalation of aerosolized droplets; however, *M. bovis* infection can also occur via ingestion of contaminated food or water, or experimentally by intravenous, intranasal, intra-tracheal, or intra-tonsillar injection (Harris et al., 2009, Palmer et al., 2002; Neil et al., 1994). Efforts to eradicate BTB have been successful in some countries, such as the United States, where the prevalence is limited only to sporadic outbreaks. However, eradication in other developed countries, such as the United Kingdom (UK) and New Zealand, has been largely unsuccessful due to wildlife reservoirs of *M. bovis* as well as imports received from countries with endemic TB. The UK has seen an overall long-term upward trend in incidence rates of TB in cattle, specifically herds in England and Wales, since 1996 (Animal and Plant Health Agency, 2017). The Department for Environment, Food,

and Rural Affairs and Animal and Plant Health Agency reported in 2016 that approximately 80,000 reactor animals were slaughtered in Great Britain, England, Scotland, and Wales, which is a drastic increase from the 45,000 animals slaughtered in 2006.

The wildlife reservoirs for BTB vary depending on geographical region, making universal control strategies difficult to implement. Historically, control of BTB has depended on the detection of infected animals and the subsequent slaughter (Aranaz et al., 2006). Test and cull methods have been implemented in cattle herds with varying results. Culling large groups of animals is very costly to producers, especially those in third-world countries, leading to refusal of participation (Thoen et al., 2014). This method was also ineffective in areas where a wildlife reservoir of BTB was present, causing a continual re-infection of herds (Buddle et al., 2002).

## BCG and TB Vaccine Development

TB has proved to be challenging to control due to its unique and complex nature. There is currently only one vaccine licensed for use against TB in humans, the Bacille Calmette-Guèrin (BCG) vaccine. BCG is a live attenuated form of *M. bovis*, isolated by Albert Calmette and Camille Guerin in 1919 (Lee et al., 2004). Studies have shown that BCG has variable efficacy, ranging from 0-80% against the pulmonary forms of the disease (Widdison et al., 2006). Despite its controversial effectiveness, the *M. bovis* BCG vaccine is the most used vaccine world-wide (McShane, 2011; Ottenhoff and Kaufmann, 2012). The BCG vaccine offers enhanced protection when used in infants compared to use in adults, and thus it is commonly administered to children in countries with a high risk for TB infection (Lee et al., 2004). Due to the low prevalence of disease, and the questionable efficacy, individuals in the United States are not vaccinated for TB unless they are deemed to be at high risk for contracting the disease (Thoen et al., 2014).

Since TB was declared to be a global emergency, funding for research towards developing a more efficacious vaccine has been increasing. In humans, several new vaccines against TB have been developed, and are currently in human clinical trials (Reviewed in Ottenhoff and Kaufmann, 2012; Rowland and McShane, 2011), including: sub-unit vaccines that use non-replicating viral vectors or recombinant proteins combined with adjuvants in order to boost the BCG response; live vaccines that use variations of attenuation of *M. tb* with hopes to completely replace the current BCG vaccine; and creation of recombinant BCG that genetically introduces *M. tb*-specific antigens that are not naturally present in BCG (Abel et al., 2010; Brodin et al., 2004; McShane et al., 2004; Pym et al., 2003; Scriba et al., 2010; Sun et al., 2009; Sweeney et al., 2011; Tullius et al., 2008). The aim of these new vaccine platforms is to be more immunogenic and induce long lasting protection while remaining safe and providing protection against various TB strains.

There is currently no vaccine available for use against BTB infection in cattle. BCG vaccination in cattle demonstrates similarly variable efficacy as in humans receiving BCG, with some field trials reporting efficacy as high as 80%, and others ranging as low as 0% protection with minimal to no benefit (Haring et al., 1930; Watson, 1928; Buddle, 2001; Buddle et al., 1995; Buddle et al., 1995; and reviewed in Waters et al., 2012). However, further implementation of BCG as a vaccine in cattle is complicated by the need to differentiate naturally infected from *M. bovis* BCG vaccinated animals. Recently, several diagnostic tests have been developed for differentiating between BCG and virulent *M. bovis*-infected cattle, some of which rely on detecting antigens that are present in virulent *M. bovis* but are absent from *M. bovis* BCG such as early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) (Andersen et al., 2000).

It is believed that development of a more efficacious vaccine and enhanced diagnostic tools could make eradication of TB possible for both humans and animals. However, in order to develop a more effective vaccine or improved diagnostic tools, there needs to be a more detailed understanding of the immunological factors of this disease (Thacker et al., 2007). Using human patients to study TB immunity poses many challenges, so multiple animal models have been developed to allow for adequate sampling and testing after BCG vaccination or TB infection (Lee et al., 2004). Given the many similarities in disease pathogenesis and immunity, the bovine model of TB is amongst the best for testing novel vaccine candidates and for identifying immunologic correlates of protection from disease.

## Immune Response to *M. bovis*

*M. bovis* is an obligate intracellular pathogen that initially infects the host's macrophages and other mononuclear phagocytes (Meade et al., 2006). There are multiple factors that affect the susceptibility of the host to infection with *M. bovis*, such as the route of exposure, the dose of bacteria, and the virulence of the bacterial strain involved. For cattle in a natural setting, once the bacilli enter the nasal cavity after inhalation, the bacteria must then pass through the mucus and epithelial cilia associated with the upper respiratory tract. The bacilli then enter the terminal bronchioles of the lungs where they are phagocytosed by resident alveolar macrophages. The bacilli are able to enter the cell by binding to cell surface molecules such as complement receptors, mannose receptors, and Fc receptors present on macrophages (Ernst, 1998; Cambi et al., 2005; Greenberg, 1999). Next, the bacilli enter the phagosome of the macrophage which is ultimately how the bacilli are able to protect themselves from immunologic defenses in the serum. The mycobacteria inhibit phagosome fusion with the lysosome, therefore hindering the

macrophage's ability to form a phagolysosome where acidification would normally occur to destroy the invading bacteria (Hart et al., 1987). This mycobacterial escape mechanism involves the intercalation of mycobacterial membrane lipids with endosomal membranes, leading to the phagosome arrest in maturation. After the infected macrophage undergoes apoptosis, the intracellular bacilli are released from the cell where they can then spread throughout the body via the blood stream or lymphatics system (Thoen et al., 2014). The mycobacteria can then take up residence in nearly any part of the body; however, the most common areas of dissemination include the lungs, kidneys, brain, bones, and lymph nodes. It is in these areas that the body attempts to overcome the virulence mechanisms of the mycobacteria and confine the bacilli from further spread by formation of granulomas.

Mycobacteria have several factors such as complex lipids and proteins contained in the cell wall and cytoplasm that contribute to their virulence (Thoen et al., 2014). *M. bovis* BCG attenuation can be attributed to the loss of the region of difference 1 (RD1) gene region which encodes ESAT-6 which participates in phagosome lysis and forms a complex with CFP-10 (Pym et al., 2003; Guo et al., 2012). These antigens are recognized by T cells during natural and experimental mycobacterial infection in humans and cattle (Ravn et al., 1999; Aagaard et al., 2010). While there are numerous virulence factors, other important *M. bovis* proteins that contribute to pathogenesis include: antigen 85 complex (A, B, and C) which may contribute to altered phagocytosis and participate in cell mediated immunity development, and exported repetitive protein P36 (Erp) which is involved in intracellular replication in macrophages (Armitige et al., 2000; Thoen et al., 2014).

The characteristic lesion of TB disease in animals and humans is the granuloma. Granulomas are the body's attempt to localize the invading bacteria in order to allow immune

cells to arrive and destroy the bacilli. A granuloma results from the accumulation of cells around a foci of infected cells; however, development of granulomas can cause severe tissue damage, even to the extent of a loss of function (Guirado and Schlesinger, 2013; Widdison et al., 2009). Depending upon the route of infection, TB granulomas can appear almost anywhere throughout the body, but are commonly found in the lungs, or the lymph nodes associated with the head and thoracic regions (Whipple et al., 1996). Characteristic TB granulomas contain a caseonecrotic core that is surrounded by various immune cells. The most common cells involved in granuloma formation are epithelioid or foamy macrophages, Langerhans-type multinucleated giant cells, T and B lymphocytes, fibroblasts, and neutrophils (Palmer et al., 2007).

TB granulomas are dynamic lesions that follow an orderly progression throughout the disease stages, and therefore progression of disease is assessed by the pathology of the granulomas (Palmer et al., 2007). There are four stages of granulomas with Stage I being the earliest stage with accumulation of some innate immune cells such as epithelioid macrophages, lymphocytes, and neutrophils, and no encapsulation or necrosis present. In Stage II of granuloma formation the innate immune cells are still present and a thin capsule of fibrous connective tissue begins to form. In Stage III there is full fibrous encapsulation and the beginning formation of a necrotic center surrounded by a zone of epithelioid macrophages, multinucleated giant cells, and lymphocytes. The final Stage IV granulomas are characterized by complete encapsulation by a thick layer of fibrous material, areas of caseous necrosis, and mineralization (Thoen et al., 2014; Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). In most immunocompetent individuals, the granulomas are able to successfully stop the spread of the mycobacteria leading to the latent form of infection. However, in

immunocompromised situations, the immune system is not able to contain the replication and spread of the bacteria, leading to active TB disease (Palmer et al., 2007).

Host-pathogen interactions within the granuloma over the course of TB infection lead to changes in the bacilli, the phenotypes of immune cells, and the levels of immune mediators that are produced. These complex immunological interactions that contribute to the control or the exacerbation of TB progression allow for the formation of many varying granuloma structures between individuals and even within a single host (Flynn et al., 2011; Mattila et al., 2013). Normal granuloma formation is characterized by multinucleated giant cells surrounded by macrophages and lymphocytes, and fibroblasts forming a wall around the lesion. Neutrophils and γδ T cells are amongst the first immune cells to arrive at the site of infection, and have been found to localize around the periphery of the granuloma (Borregaard, 2010; Palmer et al., 2007; Cassidy et al., 1998). Studies using mice have found that at this stage, γδ T cells are a major source of the pro-inflammatory cytokine, IL-17, which leads to neutrophil recruitment (Lockhart et al., 2006; Umemura et al., 2007; Guirado and Schlesinger, 2013). Granulomas typically display high monocyte and lymphocyte turn-over rates, making cell recruitment crucial to mycobacterial control (Fenton and Vermeulen, 1996). The accumulation of macrophages and dendritic cells ultimately initiates the adaptive immune response, and this cell mediated immunity (CMI) typically occurs around three weeks post initial infection. $CD4^+$ and $CD8^+$ T cells also accumulate at the site of infection and produce inflammatory cytokines and release cytotoxic molecules to aid in mycobacterial destruction and containment. If the bacterial load is large, necrosis and caseum begin to develop, and are indicative of disease progression and poor disease outcome (Guirado and Schlesinger, 2013). Bacilli are unable to proliferate within the granuloma due to the acidic pH, low oxygen availability, and the presence of toxic fatty acids.

An adequate CMI response will successfully stop infection, and the granuloma will heal, leaving only small calcified lesions. An inadequate CMI response leads to the escape of infected macrophages from the granuloma, spreading infection throughout the body. The center of the granuloma then liquefies, providing an ideal environment for the mycobacteria to thrive (Fenton and Vermeulen, 1996). To date, the dynamics of the immune response within the developing granulomas is still poorly understood, especially during the initial stages of development, and it remains unclear as to how granuloma development ultimately influences disease outcome.

It is well accepted that protective immunity to mycobacterial infections is reliant on interactions between macrophages and T cells (Palmer et al., 2007; Kaufmann, 2006). Macrophages are the preferred host cell for mycobacteria, and these cells have important effector functions that aid in the control and destruction of the invading bacteria. The initial stages of granuloma formation are dependent upon the production of tumor necrosis factor (TNF-α) by infected macrophages. This TNF-α signaling is crucial in maintaining chemokine concentrations in order for immune cell recruitment (Algood et al, 2005; Kindler et al., 1989; Roach et al., 2002). Aldwell et al. found that in a range of animal hosts, naïve macrophages are cable of preventing *M. bovis* BCG growth, while T cell activation and freshly recruited macrophages are capable of arresting the growth of virulent *M. bovis* (Aldwell et al., 2001). Macrophages are capable of mycobacterial inhibition and killing by induction of phagosome acidification, apoptosis, autophagy, and production of reactive oxygen intermediates (ROI) such as $H_2O_2$ and $O_2$ and reactive nitrogen intermediates (RNI) such as NO and $NO_2$-, all of which aid in the control of TB infection (Fenton and Vermeulen, 1996; Chan et al., 1992; Gutierrez et al., 2004). However, despite the many effector mechanisms possessed by macrophages, mycobacteria possess evasion techniques such as inhibiting phagosome-lysosome fusion in order to escape

acidic environments inside the phagolysosome, which in some cases allows for uncontrolled intracellular bacterial growth (Meena and Rajni, 2010; Ferrari et al., 1999; Xu et al., 1994).

Similar to macrophages, dendritic cells (DC) are also important phagocytes that can ingest and harbor mycobacteria, and are commonly found at the mucosal surfaces where mycobacterial infections typically occur (Banchereau et al., 2000; Henderson et al., 1997). Macrophages and DCs produce chemokines (IL-8, CCL2, and CCL5) during initial infection in order to recruit inflammatory cells to the site of infection. These antigen presenting cells (APC) also produce a wide array of cytokines in response to infection such as: TNF-α, IL-1β, IL-6, IL-10, IL-12, IFN-γ, and TGF-β that help to further shape the immune response (Fenton and Vermeulen, 1996; Blanchard et al., 1991; Johansson et al., 2001; Denis, 1991). However, similar to macrophages, DC can also become infected by mycobacteria, and mycobacterial virulence factors are capable of altering normal DC function hindering these cells' immune inducing abilities (Hanekom et al., 2003; Johansson et al., 2001; Denis and Buddle, 2007).

To date, there are no reliable correlates of protection for TB. However, research has shown that a Th1 type response is critical for the control of the initial mycobacterial infection. A Th2 response is more often observed during the late stages of infection, likely to limit the amount of inflammation in order to minimize tissue damage. Th1-type responses are characterized by a specific cytokine profile such as IFN-γ and TNF-α, while Th2-type responses are characterized by IL-4 and IL-10 secretion. Specifically, IFN-γ has been described as a critical mediator for protection against TB infection (Lee et al., 2004). IFN-γ works with TNF-α to activate macrophages which promotes nitic oxide synthase production which participates in mycobacterial killing (Flesch and Kaufmann, 1991; Saito and Nakano, 1996). It has been well established that $CD4^+$ T cells produce significant quantities of IFN-γ, and are required for host

survival during chronic and acute stages of mycobacterial infection (Caruso et al., 1999; Scanga et al., 2000). One study found that $CD4^{-/-}$ T cell mice are unable to control mycobacterial burden and succumb quickly to infection; however these mice are able to survive twice as long as mice that are unable to produce IFN-γ (Caruso et al., 1999; Flynn et al., 1993). These results emphasize the fact that IFN-γ production from cell types other than $CD4^+$ T cells is not sufficient alone for control of mycobacterial infection. Another study utilizing a murine adoptive transfer model also found that $CD4^+$ T cell IFN-γ production was necessary for optimal long-term TB disease control and that IFN-γ was required to initiate a robust $CD8^+$ T cell response (Green et al., 2003). In contrast, it has also been shown that over production of IFN-γ by $CD4^+$ T cells during *M. tb* infection can negatively impact the host and lead to exacerbated pathology (Sakai et al., 2016). $CD4^+$ T cells have been shown to carry out other functions during mycobacterial infections such as IL-21 production which suggests cytotoxic capabilities; however, these roles are less vital to host survival during TB infection when compared to IFN-γ production (Waters et al., 2011). $CD4^+$ T cells, though necessary for host survival against mycobacterial infection, are not sufficient alone to control TB disease.

Other innate immune cells, such as natural killer (NK) cells and neutrophils, have been shown to play a role in the innate response to *M. bovis* infection. NK cells are a significant population in young calves, and their frequency declines with age, suggesting they have an important role in the innate immune response of young animals that have yet to develop sufficient adaptive immunity (Hope et al., 2002). NK cells have been shown to specifically target infected cells that display little to no MHC I molecules on their surface (Moretta et al., 2002). In response to *M.bovis* or BCG infection, bovine NK cells provide the initial sources of IFN-γ and reduce BCG proliferation in infected macrophages (Portevin and Young, 2013; Denis

et al., 2007; Endsley et al., 2006). NK cells are also a source of perforin and granulysin that aid in the killing of infected cells; however, these cells are not efficient at directly killing bacteria (Fenton and Vermeulen, 1996; Waters et al., 2011).

Neutrophils are short-lived, professional phagocytes that make up a significant population in the peripheral blood, and are among the first inflammatory cells to arrive at the site of TB infection (Pedrosa et al., 2000; Borregaard, 2010; Reviewed in Nathan, 2006). The exact role of neutrophils during mycobacterial infection is still not completely understood, but recent evidence suggests that these cells are capable of a wide array of functions from antigen presentation, to cross-talk with other immune cells (Sandilands et al., 2005; Morel et al., 2008). Neutrophils are known to play a role in TB infection by producing a diverse array of antimicrobial molecules; however, the role neutrophils play in *M. bovis* killing is controversial. Some studies have shown that neutrophils are capable of eliminating intracellular *M. tb,* while other studies in humans, mice, and cattle have found that neutrophils are unable to eliminate the mycobacteria (González-Cano et al., 2010; Berry et al., 2010; Pedrosa et al., 2000). Neutrophils likely play an important role in accurate early granuloma formation, and are capable of initiating the innate and adaptive immune responses by producing pro-inflammatory cytokines and chemokines such as TNF-α and CXCL10 (Seiler et al., 2003; Wang et al., 2013).

Studies have found B lymphocytes to be involved in TB granuloma maintenance in some species; however, only a small percentage of cattle infected with TB exhibit a measurable antibody response (Gonzalez-Juarrero et al., 2001; Tsai et al., 2006). In mice, B cells account for 1–10% of the leukocytes present in the granuloma; however, absence of B cells in mice has also shown no effect on TB progression in the chronic phase of disease (Tsai et al., 2006; Turner et al., 2001). In one study using a macaque *M. tb* B cell depletion model, they found that B cells

can modulate the local granulomatous response during acute infection; however, there was no difference in the pathology, disease progression, and clinical outcome between the treated and untreated macaques (Phuah et al., 2016). In some cases IgG1 has been associated with lesion development; however, it is well accepted that the immune response to intracellular mycobacterial infection in all species is predominantly a cell mediated response, therefore making antibody responses unimportant in terms of protective immunity (McNair et al., 2007; Pollock and Neill, 2002).

$CD8^+$ T cells, often referred to as cytotoxic T lymphocytes (CTL), are also capable of producing IFN-γ, but only in small quantities compared to $CD4^+$ T cells. CTLs are more commonly known for their cytotoxic activities during a TB infection. CTLs produce granulysin, which lyses infected macrophages (Buddle et al., 2002). One study using *M. bovis*-infected cattle found that the growth of *M. bovis* within macrophages was inhibited when the cells were cultured with *M. bovis* specific $CD8^+$ T cells (Skinner et al., 2003). Cytotoxic T lymphocytes also have the ability to kill human and mouse macrophages infected with *M. tb* (Lalvani et al., 1998; Skinner et al., 1997). The role of $CD8^+$ T cells in cattle during *M. bovis* infection is not well understood; however one study showed that $CD8^+$ T cells contribute to the IFN-γ response, but also found that these cells may contribute to immunopathology of bovine TB (Villarreal-Ramos et al., 2003).

## γδ T Cells

γδ T cells were discovered in 1985, and have been found in the circulation and tissues of every vertebrate examined thus far (Rhodes et al., 2001, Hayday 2000). Similar to αβ T cells, γδ T cells are produced in the bone marrow and travel to the thymus where differentiation occurs.

These T cells can be found in circulation and are also commonly found in tissues, such as the dermis, intestine, lung, and uterus (Vantourout and Hayday, 2013). γδ T cells are a unique subset of $CD3^+$ T cells whose functions are still not completely understood. These T cells have been found to play an important role in the innate immune system by acting as immune-surveillance cells, and are also capable of adaptive functions, such as cytotoxicity, and are therefore described as bridging the innate and adaptive arms of the immune system (Born et al., 2006; Vantourout and Hayday, 2013). γδ T cells comprise only a small portion of the circulating lymphocytes in humans and mice, 5-10%, but are much more abundant in ruminant species, accounting for up to 70% of the circulating lymphocytes in calves and 10-20% in adult animals (Kabelitz, 2011, Jutila et al., 2008). The large proportion of γδ T cells in young ruminants suggests that these cells play an important role in innate immunity prior to development of robust adaptive immunity, and also makes cattle an exceptional model for studying the role that these cells play during TB infection.

Bovine γδ T cells can be divided into sub-populations depending on their expression of Workshop Cluster 1 (WC1). WC1 is a transmembrane glycoprotein and a member of the scavenger receptor cysteine rich family which is found to be expressed uniquely on the surface of γδ T cells in cattle but not humans or mice (Rogers et al., 2005; Pillai et al., 2007). However, two human gene sequences have been identified that are 85% homologous with the bovine WC1 sequence (Wijngaard et al., 1994). The γδ T cells primarily present in circulation are characterized as $WC1^+$ $CD2^-$ $CD4^-$ $CD8^-$, while the γδ T cells primarily found in the tissues are $WC1^-$ $CD2^+$ $CD8^+$ (Rhodes et al., 2001; Machugh et al., 1997; Wijngaard et al., 1994).

There are 13 different WC1 genes that can be used to categorize WC1-expressing γδ T cells. Differential expression of these genes on γδ T cells can be used to divide $WC1^+$ cells into

the subpopulations, WC1.1, WC1.2, and WC1.3. WC1.1 and WC1.2 are expressed on exclusive subpopulations of γδ T cells, while WC1.3 is expressed on a small portion of $WC1.1^+$ γδ T cells (Chen et al., 2009; Rogers et al., 2006; Wijngaard et al., 1994). These subpopulations of γδ T cells have recently been described as having distinct immune functions. $WC1.1^+$ γδ T cells produce IFN-γ in response to stimulation and infection, and the $WC1.2^+$ γδ T cells produce little IFN-γ and are thought to play more of a regulatory role (Rogers et al., 2005; Hoek et al., 2009; Wang et al., 2011). Although studies have found these subpopulations to have distinct roles in modulating γδ T cells responses, the biological significance of these subsets is not well understood (Rogers et al., 2005).

The way in which γδ T cells are activated through their T cell receptor (TCR) and co-receptors differs significantly from that of αβ T cells (Chien and Konigshofer, 2007). Unlike αβ T cells, γδ T cells do not require antigen processing and presentation via major histocompatibility complex (MHC) I or II (Chien et al., 1996). However, recognition of antigen by γδ T cells via their TCR does appear to require the presence of antigen presenting cells (i.e. monocytes, macrophages or dendritic cells) and presentation of antigen. To date, most antigen presenting molecules for γδ T cells remain poorly defined; however, γδ T cells have been shown to be capable of responding to antigen presented on CD1, which is a MHC-like surface molecule that processes and presents non-peptide antigens to other T-lymphocytes (Beckman et al., 1994; Van Rhijn et al., 2006), and recent reports in humans suggest that γδ T cells may respond to pyrophosphate antigens presented by the butyrophilin 3 receptor, a member of the immunoglobulin superfamily (Harly et al., 2012; Palakodeti et al., 2012). γδ T cells from humans and cattle have also been shown to respond innately through the recognition of danger associated molecular patterns (DAMP) or pathogen associated molecular patterns (PAMP),

which leads to activation and initial chemokine and cytokine production (Hedges et al., 2005; Schwacha et al., 2013). γδ T cells are unique in the fact that they are capable of responding to antigens via their TCR and multiple pattern recognition receptors (PRR); however, co-stimulatory and co-receptors for γδ T cells are still not well understood. Human, mouse, and bovine γδ T cells have all been shown to express PRRs such as toll-like receptors (TLRs), and bovine γδ T cells are able to respond to bacterial TLR agonists by use of TLR 2 and TLR 4 (Hedges et al., 2005; Jutila et al., 2008; Wesch et al., 2011). WC1 has also been found to participate in antigen recognition and γδ T cell activation, behaving as a PRR, similar to that of a TLR, and serves as a co-receptor on γδ T cells, similar to CD4 or CD8 (Baldwin et al., 2014; Wang et al., 2011).

After antigen recognition and activation, γδ T cells have been shown to behave as antigen presenting cells to other immune cells leading to initiation of the adaptive immune response. Studies have found that activated bovine γδ T cells express high levels of MHC class II and have the capacity to directly induce $CD4^+$ T cell proliferation (Collins et al., 1998; Toka et al., 2011). Similar antigen presenting characteristics of γδ T cells have been described in humans and mice as well (Brandes et al., 2005; Cheng et al., 2008). It is currently unknown if γδ T cell antigen presentation occurs *in vivo,* and the possible biological significance of antigen presentation by γδ T cells during mycobacterial infections needs to be further elucidated.

## γδ T cell Responses to Mycobacteria

The important role for γδ T cells in response to TB infection was first hypothesized after it was noticed that patients with active pulmonary TB had significantly increased proportions of peripheral blood γδ T cells (Ito et al., 1992). In cattle, γδ T cells are known to undergo dynamic

changes in circulation following *M. bovis* infection and BCG vaccination. After vaccination with BCG or infection with *M. bovis,* an increase in the population of circulating γδ T cells can been seen with an increase in expression of CD25, a T cell activation marker (Smyth et al., 2001; Buza et al., 2009). However, this increase is only after an initial decrease in frequency, likely due to γδ T cells traveling out of the periphery to the infected tissues (Pollock et al., 1996).

Studies conducted *in vitro* have found that γδ T cells from naïve and *M. bovis*-infected cattle proliferate and produce cytokines in response to stimulation with different mycobacterial antigens (Smyth et al., 2001). Similar results have also been demonstrated in mice and human models of *M. tb* (Welsh et al., 2002; Balaji and Boom, 1998). There are currently only a few defined antigens of γδ T cells; however, these T cells have been shown to be capable of recognizing small phosphate molecules, peptides, and fully intact protein antigens (Hayday, 2000; Tanaka et al., 1995 & 1994; Vantourout and Hayday, 2013). Bovine γδ T cells are capable of responding directly to several mycobacterial proteins such as *M. bovis* purified protein derivative (PPD), Ag85, ESAT6, MPB83, and hsp16.1 (Rhodes et al., 2001), as well as the non-protein antigens mycolyl-arabinogalactan-peptidoglycan (mAGP) and lipoarabinomannan (LAM) (McGill et al., 2014). Human γδ T cells are unique from bovine γδ T cells in the fact that they respond to the non-peptide mycobacterial antigen isopentenyl pyrophosphate (IPP), which is a metabolite found in prokaryotic and eukaryotic cells, and hydroxymethyl-but-2-enyl-pyrophosphate (HMBPP), which is an intermediate in the alternative pathway of cholesterol synthesis that is used by numerous bacterial species (Tanaka et al., 1995; Morita et al., 2007). Other mycobacterial antigens that initiate γδ T cell activation in humans, mice, and primates include mycobacterial heat shock protein and non-protein phosphoantigens (Born et al., 1990;

Chen, 2013; Haregewoin et al., 1989; Morita et al., 1995), with more continually being described (Xi et al., 2013).

During the early immune response to TB infection, γδ T cells are an important source of cytokines and chemokines which aid in the recruitment of other immune cells to the site of infection. γδ T cells produce significant amounts of IFN-γ, similar to that of $CD4^+$ T cells, in response to mycobacterial antigens, and there is increasing evidence that these cells contribute to immunity and possess unique immunological functions (Lee et al., 2004). In a study using *M. bovis*-infected calves depleted of γδ T cells, there was a decrease in the production of IFN-γ, an increase in the production of IL-4, and a lack of specific IgG2 antibodies, suggesting that γδ T cell cytokine production plays a role in the shaping of adaptive immunity to a Th1 response (Kennedy, 2002). Another study utilizing SCID-bo mice found that bovine γδ T cells produced IL-2, IL-10, IL-15, and IFN-γ in response to *M. bovis* infection (Alvarez et al., 2009). It has also been documented that γδ T cells in humans with *M. tb*, mice challenged with BCG, and cattle infected with *M. bovis* produce significant amounts of IL-17 during early mycobacterial infection (Cowan et al., 2013; Jurado et al., 2012; Lockhart et al., 2006; Umemera et al., 2007; Aranday-Cortes et al., 2013; Vordermeier et al., 2009). The exact role of IL-17 remains unclear; however, these studies suggest a possible role for this cytokine in granuloma formation and initiation of a Th1 response. In studies with non-human primates and cattle, γδ T cell secretion of IL-22 was directly correlated with IL-17 secretion, and these cytokines were seen in early lesions with diminishing results over the course of infection (Aranday-Cortes et al., 2013; Yao et al., 2010).

γδ T cells in mice and cattle have been found to accumulate in the lungs and associated lymph nodes after infection and vaccination with BTB, and are one of the first cells to arrive at the site of infection (Price et al., 2010; Doherty et al., 1996; Dieli et al., 2003). γδ T cells are

often seen localizing to the lymphoid mantle, surrounding the periphery of the lesion (Cassidy et al., 1998). One study using low dose *M. tb* and *M. bovis* BCG-infected mice depleted of γδ T cells reported irregular granuloma formation, suggesting a crucial role for these cells in the recruitment of immune cells and granuloma development (D'Souza et al., 1997; Ladel et al., 1995). However, there have been conflicting results on γδ T cell movement to and from granulomas throughout the progression of TB disease. Some groups have found that γδ T cell accumulation within granulomas was greatest during early infection with a decrease in number of γδ T cells in the developing granuloma throughout disease progression, while Wangoo et al. found γδ T cells to be more abundant within advanced granulomas during the later stages of disease (Cassidy et al., 1998; Palmer et al., 2007; Wangoo et al., 2005; Aranday-Cortes et al., 2013). It is clear that γδ T cells accumulate to the site of TB infection; however, the kinetics of their response within the granuloma throughout infection have yet to be well characterized.

γδ T cells have been found to have cytotoxic capabilities during early TB infection. γδ T cells express the natural killer (NK) receptor, NKG2D, that allows these cells to have innate cytolytic functions (Steinle et al., 2001). However, these T cells are capable of both innate and acquired antigen-specific cytotoxicity (Olin et al., 2005). In human *M. tb* models, γδ T cells produce granulysin and perforin, which are able to directly kill bacilli or inhibit their growth (Stenger et al., 1998). Bovine γδ T cell clones have been shown to express Fas-ligand at the mRNA level which suggests the potential for cytotoxic activity through Fas-Fas ligand interactions (Hirano et al., 1998). γδ T cells in cattle are able to use their cytotoxic properties to kill macrophages that are infected with *M. bovis* (Skinner et al., 2003). One study from mice found that BCG-infected γδ T cells were able to kill infected and uninfected macrophages (Dieli et al., 2003). Another study found that γδ T cells can be stimulated by infected macrophages to

acquire cytotoxic activity, which resulted in macrophage death (Carding and Egan, 2000). However, these cytotoxic capabilities have been negatively correlated with disease progression, and are likely not adequate alone to control mycobacterial infection (De La Barrera et al., 2003).

Cross-talk between γδ T cells and DC has been identified in mice and humans (Conti et al., 2004; Devilder et al., 2006; Martino et al., 2007). It has been well documented that γδ T cells and DC exert regulatory influences on one another (Born et al., 2006). *M. tb* and BCG infection in humans has been shown to impair DC maturation as a mechanism to escape immune detection; however, γδ T cells are capable of reversing this effect on the DC (Dulphy et al., 2007; Martino et al., 2007; Meraviglia et al., 2010). There is increasing evidence that this cross-talk also occurs in cattle. One study showed that cross-talk between bovine γδ T cells and *M. bovis*-infected DC is contact dependent, and resulted in increased expression of CD25 and MHC II (Price and Hope, 2009). Cross-talk between γδ T cells and DC may play a role in initiating the adaptive immune response during TB infection; however much remains to be elucidated in the bovine model.

Bovine γδ T cells have been shown to have regulatory functions, with the capacity to secrete the anti-inflammatory cytokine IL-10; to proliferate in response to IL-10, IL-4, and TGF-β; and to suppress antigen-specific and non-specific proliferation of both CD4+ and CD8+ T cells (Guzman et al., 2014; Hoek et al., 2009). Some studies have found that depletion of γδ T cells from PBMC cultures resulted in increased antigen-specific proliferation and cytokine production in *ex vivo* cultures of T cells (Rhodes et al., 2001; Brown et al., 1994; Graaf et al., 1998). Thus, γδ T cell regulatory cytokine production could potentially play a role during TB infection; however, this alternative function has not been characterized in the context of TB infection.

The ability of γδ T cells to produce chemokines continues to be debated, and it is thought that γδ T cells chemokine production in the blood may differ from that at the site of TB infection. In a study utilizing *M. bovis*-infected SCID-bo mice depleted of γδ T cells, there was a significant reduction in the production of the chemokines CXCL10 and CCL2 in the serum; however, this study concluded that although the γδ T cells did produce several cytokines they were not the predominant source of chemokine production (Alvarez et al., 2009). In contrast to those findings, other studies evaluating bovine γδ T cell gene expression have found these cells to express high levels of CCL2, CXCL1, CXCL2, CXCL6, and CXCL10 (Hedges et al., 2003; Lahmers et al., 2006; McGill et al., 2013). Chemokine production by CD4+ and CD8+ T cells in response to TB has not been well characterized, and may be a unique contribution by γδ T cells to the immune response during mycobacterial infection. However, it is clear that further investigation is essential to evaluate the role of γδ T cells in chemokine production and immune cell recruitment during BTB infection.

## Purpose

γδ T cells are a unique subset of T cells whose immunologic functions bridge the gap between the innate and adaptive immune responses. These cells have been identified in every vertebrate examined, and are often referred to as immune sentinel cells. γδ T cells are capable of recognizing antigens through their T cell receptor, similar to that of their α/β counterparts; however, γδ T cells also respond independently of their T cell receptor by use of their various pattern recognition receptors. Several characteristics of γδ T cells, such as IFN-γ production, have been well described; however, *in vitro*, γδ T cells are capable of a wide array of immune

functions. To date, it is unclear if γδ T cells participate in these alternative functions *in vivo*; and the biological significance of these alternative roles during disease needs to be further examined.

γδ T cells are found in large numbers in the circulation of young ruminants compared to humans and mice. These cells and are also known to respond robustly to TB infection, and respond specifically to numerous protein and non-protein mycobacterial antigens. Cattle are a natural host for TB infection, therefore making the bovine model ideal for evaluating γδ T cell function during TB infection. The goal of this research was to expand upon current knowledge by further elucidating the alternative roles of γδ T cells in the immune response to *M. bovis*, and further defining the role of γδ T cells at the site of infection. The knowledge gained from our studies contributes to the understanding of basic γδ T cell biology and the immune response to TB infection in both humans and animals.

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# Chapter 2 - Measuring Bovine γδ T Cell Function at the Site of *Mycobacterium bovis* Infection

**A paper waiting review by co-authors for submission**

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## Abstract

Bovine γδ T cells are amongst the first cells to accumulate at the site of infection in the lungs, and are known to contribute to the immune response to *M. bovis* infection by secreting inflammatory cytokines such as IFN-γ. However, their specific role *in vivo,* particularly at the site of infection remains unclear. γδ T cells have the capacity for a broad array of immune functions, and the importance of these alternative functions in immunity to *M. bovis* has not been determined. In this study, we used transcriptomics analysis, an *in situ* hybridization assay, and a novel, macrophage/γδ T cell co-culture system to further elucidate the role of γδ T cells in the immune response to *M. bovis* infection. Transcriptomics analysis revealed that γδ T cells upregulated expression of a number of novel genes in response to *M. bovis* antigen, including IL-15RA, SOCS1, NOS2 and TNF. *In situ* within late stage granulomas, as expected, a significant frequency

of γδ T cells expressed IFN-γ; however, we observed little expression of IL-10, IL-22, or IL-17. Interestingly, we observed for the first time, robust expression of CCL2 by γδ T cells accumulating in chronic TB granulomas. Results from our macrophage/γδ T cell co-culture system suggest that γδ T cells secrete multiple cytokines and chemokines, including CCL4, CXCL10 and CCL8 in response to *M. bovis* BCG-infected macrophages and, in comparing γδ T cell responses from uninfected calves with those from virulent *M. bovis*-infected animals, their expression profile may change over the course of disease. Consistent with previous reports of direct cytotoxicity by γδ T cells responding to *M. bovis*, we also found that γδ T cells from virulent *M. bovis*-infected animals, but not naïve animals, had the capacity to significantly impact *M. bovis* BCG viability in our co-culture systems. Together, our results suggest that γδ T cells accumulate within the granulomas and influence host immunity to *M. bovis* by secretion of cytokines and chemokines, and by direct cytotoxic responses.

Key words: *Mycobacterium bovis*, γδ T cell, granuloma, bovine

## Introduction

Tuberculosis (TB) is among the most important infectious diseases worldwide. In 2015 1.8 million people died from this disease (WHO, 2017). *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), and was tested in cattle before being administered to humans; 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 et al., 2014; Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). Importantly, simple formation of a granuloma is not sufficient to control or eliminate the disease, and 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, including the cells and cytokine production necessary for formation and maintenance of an effective granuloma.

γδ T cells are a unique subset of $CD3^+$ T cells that possess functions that are 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, and are generally recognized to be critical as 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., 2003). These cells are also one of the first cells to arrive at the site of infection (Doherty et al., 1996). These cells have also 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), and mice and rodents depleted of γδ T cells show alterations in granuloma architecture

with increases in neutrophil infiltration and necrosis (Smith et al., 1991), suggesting 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), but less is known about their capacity to secrete IFN-γ *in vivo,* particularly at the site of infection; and their ability 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 and 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 to assess the expression of multiple cytokines by γδ T cells accumulating in the chronic, granulomatous lesions of cattle infected with virulent *M. bovis*; and developed a novel, *in vitro* macrophage/ γδ T cell co-culture system that 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 was 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, as well as aid in developing effective ways in which to manipulate protective responses to TB in both humans and animals.

# Materials and Methods

*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. (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.

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.

*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, USA). 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 2mM L-glutamine, 1% antibiotic-antimycotic solution, 1% nonessential amino acids, 2% essential amino acids, 1% sodium pyruvate, 50μM 2-mercaptoethanol (ME), and 10% fetal bovine serum (FBS).

*γδ RNA Sequencing*

PBMCs from 5 *M. bovis*-infected animals were collected, and stimulated with either purified protein derivative of bovine tuberculin (PPD-b) (Prionics AG, Schlieren, Switzerland) at 200 U/mL or cRPMI for 18 hours. γδ T cells were then sorted to >90% purity by magnetic activated cell sorting (MACS) according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). γδ T cell RNA was extracted using Trizol Reagent (Invitrogen, Life Technologies) according to manufacturer's instructions. Samples were sent to The University of Kansas Center for Molecular Analysis of Disease Pathways Genome Sequencing Core to be processed. 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 Next Generation Sequncer.

Further assistance with analysis was provided by The Bioinformatics Center at Kansas State University. 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 sequences, and Perl script was written and used to remove the over-represented reads. Differential expression analysis was performed using RNA Sequencing by Expectation Maximization (RSEM) and empirical Bayes sequencing (EBSeq) software (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).

*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 sectioned from formalin-fixed, paraffin embedded tissues from animals 3 months post-infection. The tissue sample slides were baked for 1 hour at 60°C in a HybEZ ™ hybridization oven (Advanced Cell Diagnostics). Tissues were then de-paraffinized in xylene followed by rehydration in ethanol and dried at room temperature (RT) for 5 minutes. Slides were treated with an endogenous peroxidase block for 10 minutes at RT. Slides were rinsed in double distilled water (ddH2O), and immersed in an antigen retrieval citrate buffer, for 22 minutes at boiling (210°). Slides were washed in dd$H_20$ followed by an ethanol rinse and allowed to air dry. A hydrophobic barrier was created around the tissue sections and the slides were then allowed to dry at RT overnight.

The following day, slides were incubated with a protease for 30 minutes at 40°C in the HybEZ oven. Slides were then washed in ddH2O, and target or control probes applied, and incubated at 40°C for 2 hours. *Bos taurus*-specific probe combinations (Adanved Cell Diagnostics) were used; γδ T cell TCR (Cat. No. 407481-C2), IFN-γ (Cat. No. 315581), IL-10 (Cat. No. 420941), IL-17A (Cat. No. 406601), IL-22 (Cat. No. 420931) and CCL2 (Cat. No. 14314A). The positive control probe consisted of a proprietary 2-plex probe for *Bos taurus* cyclophilin B (Cat. No. 319451-C2), while the negative control probe targeted dapB of *Bacillus subtilis* strain SMY (Cat. No. 320751). The slides were then rinsed in wash buffer (Advanced

Cell Diagnostics) for 2 minutes. Signal amplification reagents 1 through 6 were serially applied for 30 minutes, 15 minutes, 30 minutes, 15 minutes, 30 minutes and 15 minutes, respectively, with a 2 minute rinse in wash buffer between each amplification reagent. Incubations with amplifier reagents 1 through 4 were done in the HybEZ oven at 40°C, while incubations with amplifier reagents 5 and 6 were done at RT. Slides were then incubated with a Red A and B mixture for 30 minutes at RT, followed by a rinse in wash buffer. Slides were incubated in a Green A and B mixture and RT for 10 minutes, and washed in dd$H_20$. Slides were immersed in Gill's hematoxylin for 30 seconds at RT, washed in ddH20 and then briefly immersed into ammonia, followed by a wash in ddH20. Finally, slides were dried for 15 minutes at 60°C, and cover-slipped using mounting media (EcoMount, Biocare Medical, Concord, CA, USA).

In order to quantify the amount of cytokine or chemokine being expressed by γδ T cells, 10 representative images at 100X magnification from 5 *M. bovis*-infected calves were taken around the periphery of each late-stage granuloma. Granulomas were determined to be late-stage, meaning stage III or stage IV, based on descriptions by previous groups (Thoen et al., 2014; Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). Stage III and IV granulomas are described to have full fibrous encapsulation, and a necrotic center surrounded by a zone of epithelioid macrophages, multinucleated giant cells, and lymphocytes (Thoen et al., 2014; Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). The images were then used to quantify the number of the cells that were expressing the cytokine or chemokine of interest, and what percentage of those cells were γδ T cells.

*γδ T cell and Monocyte Sorting and co-cultures*

Monocytes and γδ T cells were enriched from PBMCs using Magnetic Activated Cell Sorting (MACS) according to the manufacturer's instructions. Briefly, PBMCs were re-

suspended at $10^7$ cells/mL in MACS buffer (0.5% BSA, 2mM EDTA in PBS) and labeled with 10µg/mL mouse anti-bovine CD14 (Clone CAM36A) or 10µg/mL mouse anti-bovine γδ T cell receptor (Clone GB21A), both from Washington State Monoclonal Antibody Center (Pullman, WA, USA), for 20 minutes at 4°C. Monocytes and γδ T cells were washed then labeled with anti-mouse IgG1 or IgG2a+b Microbeads (Miltenyi Biotech) respectively. $CD14^+$ and γδ T cell populations were purified over magnetic columns by positive selection.

Isolated monocytes were allowed to differentiate into macrophages by plating at $5x10^5$ monocytes per well in 24 well plates and cultured in cRPMI media with GM-CSF (Kingfischer Biotech, St. Paul, MN, USA) at 4ng/mL for 7 days at 37°C in 5% $CO_2$, and the culture media was changed every 3 days (Werling et al., 2004). After 7 days of culture, monocyte-derived macrophages (MDM) were cultured in media alone or infected with BCG Danish strain 1331 at a multiplicity of infection (MOI) of either 1:1 or 10:1 for 4 hours in cRPMI without antibiotics and antimycotics at 37°C in 5% $CO_2$. After infection, BCG-infected or uninfected macrophages were cultured either alone or with $2.5x10^6$ autologous γδ T cells for 24 and 72 hours.

*BCG culture*

*BCG Danish* strain 1331 was cultured in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase) enrichment (BD Biosciences), and 0.05% Tween 80 (Sigma Aldrich) (7H9-OADC-T). Optical density (OD) was measured with a SmartSpec™ 3000 spectrophotometer (Bio Rad, Hercules, CA, USA).

*BCG viability.*

BCG viability assay was performed as previously described (Baquero and Plattner, 2016). Briefly, after 72 hours, MDM cell cultures were collected in 7H9-OADC-T media and

frozen at -80°C until ready for analysis. After thawing, cells were vortexed vigorously for 10 seconds and centrifuged at 400 x g for 2 minutes. Pellets were re-suspended in 7H9-OADC-T and incubated in 24-well plates for 24 h at 37°C in 5% $CO_2$. Contents of each well were centrifuged at 4200 x g for 10 minutes and pellets were re-suspended in 50 µL of sterile saline solution in 2 mL conical tubes. 1 µL of fluorescein diacetate (FDA) (Sigma Aldrich) at a concentration of 2 mg/mL was added to each tube. After 30 min of incubation at 37°C, samples were analyzed by flow cytometry using a BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, New Jersey, USA). Standardization of the procedure and determination of gates was generated from known proportions of live and heat-killed BCG (Figure S2).

*Cytokine Profile Secretion*

Cell culture supernatants were collected after 72 hours of incubation in co-cultures, and stored at -80°C until thawed for ELISA analysis. Commercial bovine VetSet™ ELISA kits (Kingfisher Biotech, Saint Paul, MN, USA) were used to quantify bovine IFN-γ (detection range 0.125-8 ng/mL ), IL-17 (detection range 0.188-12 ng/mL), and CXCL10 (detection range 0.188-12 ng/mL) according to manufacturer's instructions. Optical density was measured using an Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA).

*Real-time PCR*

Total RNA was extracted using the RNeasy Mini RNA Isolation Kit (Qiagen, Germantown, MD, USA), according to manufacturer's instructions. Contaminating genomic DNA was removed using RNase-Free DNase digestion set (Qiagen), according to manufacturer's instructions. The RNA concentration in each sample was measured by using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total eluted RNA was reverse

transcribed into cDNA using Random Primers and Superscript III Reverse Transcriptase per the manufacturer's instructions (Invitrogen, Life Technologies).

Quantitative real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). Forward and Reverse primers are listed in Table 1. Reactions were performed on Mx2005P qPCR System (Agilent Technologies). The following amplification conditions were used: 2 minutes at 50°, 10 minutes at 95°, 40 cycles of 15 seconds at 95°, and 1 minute at 60°, followed by a dissociation step, 15 seconds at 95°, 1 minute at 60°, 15 seconds at 95°, and 15 seconds at 60°. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method with RPS9 as the reference housekeeping gene.

*Statistical Analysis*

ΔCt values were used in the statistical analysis of relative gene expression. ΔΔCt values were transformed ($2^{-\Delta\Delta Ct}$) and are shown as expression relative to uninfected control samples, as appropriate. Data were analyzed using a paired one-way analysis of variance (ANOVA) and Tukey's multiple comparisons test using Prism v7.0 (GraphPad Software, La Jolla, CA, USA). The mean and standard error of the mean (SEM) were calculated in experiments containing multiple data points. A $P$ value of $\leq 0.05$ was considered statistically significant.

## Results

*RNA Sequencing of M. bovis-specific γδ T cells.*

PBMC were prepared from the peripheral blood of 5 virulent *M. bovis*-infected calves and were stimulated *in vitro* for 18 hours with *M. bovis* PPD-b, or remained unstimulated. γδ T cells were then purified and mRNA was isolated for whole-transcriptome RNA sequencing. Utilizing RNA sequencing allowed us to conduct an in-depth molecular analysis to identify

possible novel functions for γδ T cells that are responding to *M. bovis* infection that may contribute to their role in establishing and maintaining maturing granuloma lesions. Our transcriptomics analysis revealed a range of 299-2,240 genes that were > 2-fold differentially expressed (DE) between the unstimulated and PPD-b-stimulated γδ T cells for all five animals. Within those genes, a range of 169-1,514 genes were down-regulated, and a range of 130-1,437 genes were up-regulated. Of all of the genes that were DE, 132 of those genes were common between all animals. Sixty of the common DE genes were up-regulated, 60 genes were down-regulated, and 12 genes had mixed regulation between animals. Importantly, both IFN-γ and IL-17A were amongst the genes identified as being significantly upregulated in response to PPD-b. We and others have previously demonstrated that γδ T cells produce both cytokines in response to mycobacterial antigens (Lee et al., 2004; Smyth et al., 2001; Rhoades et al., 2001; Lockhart et al., 2006; McGill et al., 2014), therefore corroborating these previous results and confirming that the results of our RNASeq analysis were in agreement with previously published observations.

In order to aid in the understanding of uncommon or unknown DE genes, we selected the 132 genes that were commonly DE between the stimulated and unstimulated γδ T cells from all five calves, and subjected them to a pathways analysis (Figure 1) where the most significant canonical pathways were revealed. A closer look revealed that genes encoding for chemokines, regulatory cytokines, and cytotoxic factors, such as CXCL10, TNF, SOCS1 and Granzyme B represented a significant proportion of the genes involved in the immune system related pathways (Table 2). The next most significantly represented pathway was G protein signaling which included genes such as: adrenomedullin (ADM), endothelin β receptor (EDNRB), and platelet-activating factor receptor (PTAFR). Other genes relating to the signal transduciton pathway such as, apolipoprotein E (APOE), inhibin beta A chain (INHBA), and cytokine

inducible SH2 containing protein (CISH) also comprised a significant portion of the pathways identified (Table 3). The RNA sequencing results were validated using qRT-PCR (Table 4).

*Evaluation of γδ T cell cytokine and chemokine production in situ in response to M. bovis infection.*

Our transcriptomics analysis gave us a better understanding of the *M. bovis*-specific response of peripheral γδ T cells; however, systemic immune responses are often not an accurate measure of the response at the site of BTB infection. In order to compare the peripheral and localized responses, as well as to gain better understanding of cytokine and chemokine production by γδ T cells at the site of *M. bovis* infection, tissue samples from the lungs and mediastinal lymph nodes were collected from 5 Holstein calves, at 3 months post infection with virulent *M. bovis*. The tissue samples were evaluated by RNAScope, an *in situ* hybridization assay that allows for the detection of target RNA within intact cells, and is much more sensitive and specific than previous *in situ* assays. Proprietary probes were used to stain for mRNA transcripts of the γδ TCR, IFN-γ, IL-10, IL-17, IL-22 and CCL2 (Figure 2). All granulomas were determined to be late-stage based on exhibition of necrotic centers with numerous lymphocytes, including γδ T cells, surrounding the periphery (Thoen et al., 2014; Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). Ten representative images at 100X magnification were taken around the periphery of each late-stage granuloma and were used to quantify instances of co-expression between γδ T cells and the cytokine/chemokine of interest (Table 5).

*In vitro,* γδ T cells are a significant source of IFN-γ, which is thought to contribute to Th1 polarization during TB infection (Lee et al., 2004). To our surprise, however, fewer than 10% of

the cells in the lungs expressing transcripts for IFN-γ at this time point were γδ T cells (Figure 2C and Table 5). In contrast, γδ T cells comprised greater than 20% of the cells expressing IFN-γ within the lymph nodes of these same animals. Interestingly, we observed that γδ T cell expression of all cytokines/chemokines examined was higher within the lymph node lesions compared to lesions in the lungs. CCL2 expression is known to be upregulated in TB granulomas, and has been associated with increased disease severity in both humans and animals, although the source of CCL2 within the granuloma has not been defined (Hasan et al., 2010; Mastroianni et al., 1998; Alvarez et al., 2009). We observed that γδ T cells comprise a significant proportion of the cells expressing transcripts for CCL2 within both the lungs and the lymph nodes of infected animals (Figure 2E, Table 5). Expression of IL-10, IL-17, and IL-22 is also upregulated during TB, and γδ T cells have been implicated as a significant source of these cytokines at the site of infection (Steinbach et al., 2016; Aranday-Cortes et al., 2013; Palmer et al., 2015). However, in contrast to published reports, we found that fewer than 20% of cells producing IL-10 or IL-17 were γδ T cells (Figure 2D and F and Table 5), and we observed no γδ T cells expressing IL-22 in the tissues at this stage of infection (data not shown).

*Cytokine expression during initial interactions between γδ T cells and BCG-infected macrophages.*

One of the challenges associated with measuring the early immune response to TB at the site of pulmonary infection in experimental models such as cattle and non-human primates, is the difficulty locating and isolating granulomas prior to the development of grossly apparent lesions, which are often undetectable until 2-4 weeks after infection (Palmer et al., 2007). Further, *in situ* analysis of tissues collected at the time of necropsy in a chronically infected animal allows for an

assessment of only a single time point after infection, and does not allow for functional analysis of viable immune cells that accumulate at the site of infection. *M. bovis* is primarily transmitted to cattle via the aerosol route (Neill et al., 1994). Therefore, the first cells likely to encounter the infection are macrophages residing in the lungs and upper respiratory tract, followed by other lung-resident sentinel cells. γδ T cells represent a significant proportion of the sentinel cells lining the respiratory mucosa (Reviewed in Vantourout and Hayday, 2013). In order to gain insight into γδ T cell functions during the early stages of infection, our next objective was to develop an *in vitro* model that allowed us to assess the initial interactions that may occur between *M. bovis*-infected macrophages and sentinel γδ T cells. Therefore, we developed a γδ T cell and MDM co-culture system, using γδ T cells from *M. bovis*-infected or *M. bovis*-naïve animals, cultured with autologous *M. bovis* BCG-infected MDM. Peripheral blood monocytes were isolated from naïve or virulent *M. bovis*-infected calves, and cultured for 7 days with GM-CSF to generate MDM (Werling et al., 2004). On day 7, MDM were infected at 1 or 10 MOI with *M. bovis* BCG Danish Strain 1331. 4 hours later, autologous γδ T cells were added at a ratio of 1:5 with infected monocytes. Cells were then collected for qPCR analysis, and in parallel cultures, cell culture supernatants were preserved for measurements of cytokine secretion by ELISA. We measured expression of IL-17 and IFN-γ, cytokines known to be upregulated by γδ T cells responding to *M. bovis* infection (Alvarez et al., 2009; Lockhart et al., 2006; Aranday-Cortes et al., 2013), and utilized the results from our *M. bovis*-specific γδ T cell RNA sequencing data in order to decide which biological factors would be most appropriate to assess in our co-culture experiments. As seen in Figure 3, co-cultured γδ T cells and MDM from virulent *M. bovis*-infected animals expressed higher levels of IFN-γ and IL-1β than did γδ T cells and MDM from *M. bovis*-naïve animals, regardless of MOI. Inter-animal variability was a complication in

our co-culture experiments; however, general trends were observed. Co-cultured cells from naïve animals tended to express increased levels of IL-10, IL-22, and TNF-α compared to co-cultured cells from infected animals. In the naïve animals, a lower MOI correlated with increased expression of IL-22 and TNF-α, while a MOI of 10 was correlated with increased IL-10 expression (Figure 3). Interestingly, although expression of IL-17 was increased, no difference was detected between γδ T cell/MDM co-cultures from naïve compared to *M. bovis*-infected animals. IL-17 is hypothesized to be an innate response by γδ T cells (Lockhart et al., 2006; Reviewed in Chien et al., 2013), and can also be produced by activated macrophages (Barin et al., 2012), therefore, it's possible that IL-17 production is a component of both the early and chronic immune response to TB. BCG-infected MDM cultured in the absence of γδ T cells also expressed some IL-10, IL-22, and TNF-α, and significant levels of IL-1β, IL-17, and IFN-γ (Supplemental Figure 1).

*Chemokine expression during initial interactions between γδ T cells and BCG-infected MDM.*

γδ T cells are thought to contribute to the establishment and maintenance of well-organized granulomas (D'Souza et al., 1997; Plattner et al., 2009), and the results of our RNA sequencing analysis suggested that γδ T cells express transcripts for a number of chemokines that may play a role in recruiting other immune cells to the site of infection. Therefore, we used qPCR to measure expression of CCL1, CCL2, CCL4, CCL8, CCL24 and CXCL10 in our BCG-infected γδ T cell/MDM co-cultures. As seen in Figure 4, co-cultures from virulent *M .bovis*-infected animals tended to express increased levels of CCL2, CCL8, and CXCL10 compared to co-cultures from naïve animals. CCL2 and CCL8 expression were greatest when MDM were infected at an MOI of 1, while CXCL10 expression was greatest at the higher MOI (Figure 4).

Co-cultures from naïve animals tended to express increased levels of CCL1 and CCL24 compared to *M. bovis*-infected animals. CCL4 expression was significantly increased for co-cultures from both naïve and infected animals at an MOI of 10; however, co-cultured cells from *M. bovis* infected animals expressed more CCL4 than the naïve animals at the lower MOI. BCG-infected MDM cultured in the absence of γδ T cells also upregulated expression for CCL2, CCL4, CCL8, CCL1, and CXCL10, but not CCL24 (Supplemental Figure 2).

*Cytokine and chemokine production during initial interactions between γδ T cells and BCG-infected MDM.*

To confirm the results of our qPCR analysis, commercial ELISA kits were used to measure the concentration of selected cytokines and chemokines in cell culture supernatants isolated from γδ T cell/MDM co-cultures after 72 hours. Consistent with our qPCR analysis, co-culturing γδ T cells from *M. bovis*-infected animals in direct contact with BCG-infected MDM resulted in increased production of IFN-γ, IL-17, and CXCL10 compared to BCG-infected MDM cultured alone (Figure 5). A higher MOI induced increased production of IFN-γ and CXCL10 in γδ T cell/MDM co-cultures from virulent *M. bovis*-infected animals, while the MOI did not seem to have an effect on IL-17 production. No significant IFN-γ, IL-17 or CXCL10 production was detected in cell culture supernatants from naïve animal co-cultures.

*Expression of cytotoxic factors during initial interactions between γδ T cells and BCG-infected MDM.*

γδ T cells have been found to have cytotoxic capabilities during early TB infection. One group found that γδ T cells in cattle are able to directly kill macrophages that are infected with

*M. bovis* (Skinner et al., 2003). In support of this previous report, our transcriptomics analysis also revealed that γδ T cells upregulate expression of both Granzyme B and nitric oxide synthase (NOS2) in response to stimulation with PPD-b. Therefore, we evaluated expression of the cytotoxic factors granzyme B, NOS2 and granulysin in our γδ T cell/MDM co-cultures. As seen in Figure 6, co-cultures from *M. bovis*-infected animals tended to express higher levels of granulysin and granzyme B at an MOI of 10 compared to the lower MOI as well as compared to the co-cultured cells from naïve animals at either MOI. NOS2 was more highly expressed by infected animals at the lower MOI with little to no difference between groups at the higher MOI. BCG-infected MDM cultured in the absence of γδ T cells expressed granulysin and NOS2 but not granzyme B (Supplemental Figure 3).

*BCG viability within co-culture systems.*

Given that both our transcriptomics analysis of γδ T cells, and PCR analysis of our co-cultures suggested increased expression of various cytotoxic factors, we next measured the functional cytotoxic capacity of γδ T cells interacting with MDM by analyzing BCG viability within our co-culture systems. Cell cultures were collected, stained with FDA, and analyzed by flow cytometry to quantify amounts of live BCG present using a protocol adapted from Baquero and Plattner (2016). As seen in Figure 7, BCG viability was significantly reduced in γδ T cell/MDM co-cultures established from virulent *M. bovis*-infected calves, compared to co-cultures established from *M. bovis*-naïve animals. This reduction was most apparent in cultures infected with the higher MOI. Together, our results suggest that in addition to a role in recruitment of immune cells to developing granulomas, that γδ T cells may contribute to the control of *M. bovis* infection through their ability to directly impact bacterial viability.

# Discussion

γδ T cells are thought to play an important role during *M. bovis* infection in cattle primarily through their production of IFN-γ, which is critical for promoting the development of a strong Th1 immune response (Lee et al., 2004). However, in addition to production of IFN-γ, γδ T cells have the capacity for a variety of other innate and adaptive immune functions, such as regulatory cytokine production and chemokine production (Guzman et al., 2014; Hoek et al., 2009; Lahmers et al., 2006; McGill et al., 2013). As a significant population in the respiratory mucosa, it is highly likely that γδ T cells participate in early, local immunity to *M. bovis* infection; however, their specific role at the site of infection, particularly with regard to these so-called alternative functions, has not been well characterized. In the present study, we utilized transcriptomics analysis to further appreciate the breadth of the γδ T cell response to *M. bovis* infection, and an *in situ* hybridization assay to assess the local γδ T cell response *in vivo* during the chronic stage of BTB infection. We also developed a novel γδ T cell/MDM co-culture system to model interactions that may occur between γδ T cells and *M. bovis*-infected macrophages in the early stages of aerosol TB infection.

To the best of our knowledge, we are the first to perform transcriptomics analysis on γδ T cells responding to *M. bovis* infection. Through this approach, we identified thousands of genes that were DE between unstimulated and PPD-b stimulated peripheral γδ T cells, many of which have not been previously described, thus providing significant insight into the diverse functions of these cells during *Mycobacterium* infection. Of particular interest, we observed upregulation of a number of cytokine genes, some of which were expected (IFN-γ, IL-17A, IL-10) and some that have not been previously described. IL-1β was identified as being significantly upregulated by *M. bovis*-specific γδ T cells in our transcriptomics analysis, and was also up-regulated in our

γδ T cell/macrophage co-cultures. IL-1β has been found to play an important role in the anti-mycobacterial response by aiding in macrophage destruction of mycobacteria (Zhou et al., 2016; Master et al., 2008); however, its expression by γδ T cells has not been previously characterized during *M. bovis* infection. In both mice and humans, IL-1β, in synergy with IL-23 has been shown to play a critical role in the production of IL-17 by γδ T cells (Sutton et al., 2009). In mice, the absence of IL-1 type I receptor results in failure to secrete IL-17 in response to IL-23 or TLR agonists (Reviewed in Sutton et al., 2012). γδ T cells express the IL-1 receptor; therefore, one could speculate that IL-1β secretion by γδ T cells may act to enhance IL-17 production during the early stages of infection.

TNF-α was also identified in our transcriptomics analysis as a gene that was upregulated by γδ T cells in response to *M. bovis* antigen. Interestingly, in our *in vitro* co-culture assay, TNF-α appeared to be more predominantly expressed by γδ T cells isolated from naïve animals compared to γδ T cells from *M. bovis*-infected animals. The initial stages of granuloma formation are dependent upon the production of TNF-α, as its signaling is crucial in maintaining chemokine concentrations to mediate early immune cell recruitment (Algood et al, 2005; Kindler et al., 1989; Roach et al., 2002). Human γδ T cells have been shown to produce TNF-α in response to *M. tb* infection (Tsukaguchi et al., 1999); however the expression of TNF-α by bovine γδ T cells responding to *M. bovis* infection has not been previously described. Its significant upregulation in our *in vitro* co-culture system, particularly in co-cultures from naïve animals, supports an important role for this cytokine during the early stages of infection.

Chemokine gradients are critical for the recruitment of other immune cells to the site of infection. It is hypothesized that the chemokine response by infected cells during the initial infection are crucial for the control of the invading mycobacteria, whereas the chemokine

response during chronic infection may aid in granuloma formation and maintenance, in attempt to physically wall-off the pathogens (Aranday-Cortes et al., 2013). Consistent with a role for γδ T cells in both controlling infection at the early stages of disease, as well as in promoting the formation and maintenance of a well-organized granuloma, our sequencing results and co-culture system allowed us to identify several novel chemokines that were upregulated by γδ T cells responding to *M. bovis* antigen, including CCL4, CCL8 and CXCL10. CCL4, also known as macrophage inflammatory protein-1β (MIP-1β), is commonly produced by T cells and is involved in inflammatory functions (Rollins, 1997). CCL4 production in the context of TB has been previously described (Sutherland et al., 2016), however not in the bovine model and not by γδ T cells. We observed increased CCL4 expression in the co-cultures from both *M. bovis*-infected and naïve animals, suggesting a potential role for this chemokine throughout BTB disease progression. CCL8, also known as monocyte chemoattractant protein 2 (MCP-2), is chemotactic for monocytes, lymphocytes, basophils, and eosinophils (Rollins, 1997). Consistent with our results in BTB, γδ T cells have been previously shown to express CCL8 in response to *Anaplasma marginale* infection (Lahmers et al., 2006). Our findings suggest γδ T cell production of CCL8 may be an important mediator in cell recruitment during chronic BTB infection. CXCL10 was identified as differentially upregulated in our transcriptomics analysis, and was up-regulated in co-cultures from *M. bovis*-infected animals compared to *M. bovis*-naïve animals. CXCL10, also known as interferon gamma-induced protein 10 (IP-10), is chemotactic for monocytes, NK cells, and T cells (Rollins, 1997). CXCL10 is known to be increased in the stage I and stage IV granulomas in cattle infected with *M. bovis*, however the source of CXCL10 has not been identified (Aranday-Cortes et al., 2013; Palmer et al., 2015). Our results suggest that γδ T cells may be one important source of CXCL10 at the site of infection.

Interestingly, through our *in situ* analysis, we identified CCL2 as a chemokine that was highly expressed by γδ T cells at the site of *M. bovis* infection; although we did not observe significant CCL2 expression in our *in vitro* co-cultures, nor was this chemokine identified as significantly DE in our transcriptomics analysis. CCL2 expression is significantly upregulated in *M. tb* granulomas, and has been associated with increased disease severity in both humans and animals, although the source of CCL2 within the granulomas was not been defined in these previous reports (Hasan et al., 2010; Mastroianni et al., 1998; Alvarez et al., 2009). In the report by Alvarez *et al.*, depletion of γδ T cells from *M. bovis*-infected mice resulted in a significant reduction in CCL2 expression; however, the authors concluded that γδ T cells themselves were not a significant source of the chemokine, but indirectly contributed to its production. Our results are in direct contrast to this report, as we identified bovine γδ T cells as major producers of CCL2 at the site of infection in the context of chronic *M. bovis* infection. The reasons for this disparity are unclear; however, our results rely on the more physiologic model of *M. bovis* infection in cattle, while the results by Alvarez *et al.* employed a murine model of BTB infection. The increased expression of CCL2 by γδ T cells at the site of infection may support effective granuloma formation, leukocyte recruitment, or bacterial containment within the granuloma.

γδ T cells from non-human primates are a significant source of IL-22 during *M. tb* infection, both in the peripheral blood and at the site of infection (Yao et al., 2010). In agreement, Steinbach *et al.* recently described bovine γδ T cells from the periphery as being major producers of IL-22 during infection with *M. bovis* (Steinbach et al., 2016). Consistent with these previous reports, we measured significant expression of IL-22 in our γδ T cell/macrophage co-cultures; in contrast, however, we observed no expression of IL-22 by γδ T

cell within late-stage *M. bovis* granulomas. This is in agreement with Aranday-Cortes *et al.*, who observed a decrease in IL-22 levels by qPCR in late-stage granulomas from *M. bovis*-infected cattle (Aranday-Cortes et al., 2013; Steinbach et al., 2016), and Palmer *et al.* who observed very low levels of expression of IL-22 by RNAScope in any stage of lesion collected at 150 days post *M. bovis* infection (Palmer et al., 2016). In human patients chronically infected with *M. tb*, cellular immune responses become depressed (Zhang et al., 1995); therefore, it may not be surprising that decreased levels of IL-22 were found at the later stages of infection. The disparities we observed in both CCL2 and IL-22 expression between our *in vitro* re-stimulation assay and our *in situ* analysis confirms previous reports describing critical differences between the systemic and mucosal immune responses during TB (Brighenti and Andersson, 2012), and underlines the importance of analyzing the local immune response rather than relying solely on *in vitro* assays with peripheral blood populations.

After discovering multiple cytotoxicity genes upregulated in our γδ T cell RNA sequencing results, we evaluated the expression of cytotoxic genes in our co-culture systems. In agreement with our RNASeq analysis, we measured increased expression of NOS2, granzyme B, and granulysin in our co-cultures from both *M. bovis*-infected and naïve animals. Granulysin expression in our co-culture system was expected, as a bovine homologue of granulysin has been previously identified, and production of granulysin by γδ T cells has been previously described in the context of human TB (Dieli et al., 2003; Endsley et al., 2004). Until recently, NOS2 expression has been primarily attributed to myeloid cells, and the production of iNOS has been well documented in human and murine macrophages in response to TB and BCG infection (Sciorati et al., 1999; Douguet et al., 2016; Saito and Nakano, 1996). However, recent studies have also established that lymphoid cells have the capacity to express NOS2 (Yang et al., 2013;

Obermajer et al., 2013), and a report in mice has shown that NOS2 expression has a significant impact on the viability and proliferative capacity of γδ T cells *in vivo* (Douguet et al., 2016). To our knowledge, ours is the first report describing NOS2 expression by γδ T cells in cattle, and the first to demonstrate this expression in the context of BTB. Importantly, in addition to expressing molecules associated with cytotoxicity, we determined that γδ T cells had the functional capacity to eliminate *M. bovis*-infected cells in our *in vitro* co-culture system. Although the exact mechanisms of cytotoxicity are unclear, we determined here that γδ T cells from *M. bovis*-infected animals were able to significantly reduce the viability of BCG in our γδ T cells/macrophage co-cultures compared to γδ T cells from naïve animals. This result is in agreement with a previous report by Martino *et al.*, showing that BCG-infected human monocyte-derived dendritic cells induce the development of a functionally cytotoxic central memory Vγ9Vδ2 T cell population that is highly efficient at killing infected monocytes *in vitro* (Martino et al., 2007), as well as a report by Skinner *et al.* demonstrating that, *in vitro*, bovine γδ T cells can directly kill macrophages that are infected with *M. bovis* (Skinner et al., 2003). *In vivo*, γδ T cells from non-human primates have been shown to express both perforin and granulysin (Chen et al., 2013). The capacity of bovine γδ T cells for cytotoxicity at the site of *M. bovis* infection is unknown and will be the subject of future research in our laboratory.

Measuring the function of immune cells within developing TB granulomas remains challenging, primarily due to technical difficulties in locating lesions during the early stages of infection, and in isolating viable immune cells from lesions during the later stages of infection. However, utilizing RNAScope allowed us to look at mRNA expression of cytokines and chemokines by γδ T cells directly at the site of late BTB infection. Consistent with our own previous work (McGill et al., 2014), and reports from others (Wangoo et al., 2005; Aranday-

Cortes et al., 2013), we observed γδ T cells accumulating in the lymphoid mantle surrounding the periphery of late-stage lesions. In our RNAscope analysis, γδ T cells comprised a significant portion of the immune cells expressing IFN-γ within lymph node granulomas, although this proportion was much reduced within lung granulomas. Overall, we noted that the expression of inflammatory cytokines was reduced within lung granulomas compared to lymph node granulomas from the same animal. This difference between tissue cytokine expression is in agreement with recent results by Palmer *et al.* who observed significant differences in inflammatory cytokine expression between lung and lymph node lesions, as well as differences between lesions located in the caudal mediastinal vs. tracheobronchial lymph nodes (Palmer et al., 2016). Although the biological significance of these findings is unclear, it suggests that anatomic location has a significant impact on the host immune response to *M. bovis.* We were surprised to observe relatively few cells, γδ T cells or otherwise, expressing transcripts for any of the inflammatory cytokines that we measured. However, our findings are in agreement with another recent report by Palmer *et al.*, which used RNAscope to quantify overall expression of inflammatory cytokines, including IFN-γ, within virulent *M. bovis* granulomas at various stages of development (Palmer et al., 2015). In this study, Palmer *et al.* observed no significant expression of IL-10 and only low levels of IL-17, while the most highly expressed transcripts included IFN-γ and the chemokines CXCL9 and CXCL10. It is important to recall that *in situ* analyses such as those performed using RNAScope are limited to only a single time point, often belying the complex and dynamic interactions that are occurring during an active *M. bovis* infection.

Previous studies have used *in vitro* co-culture systems to study interactions between bovine or human γδ T cells with DC (Price and Hope, 2009; von Lilienfeld-Toal et al., 2005). In

these previous reports, it was found that there was an increase in IFN-γ production when γδ T cells were cultured in direct contact with infected DC, which is consistent with our findings. A recent report by Baquero and Plattner (2016) also used a similar co-culture system to assess interactions between γδ T cells and macrophages infected with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Similar to our own co-culture findings, culturing bovine γδ T cells with *Map*-infected MDM resulted in increased production of IFN-γ, IL-17A, and nitrites (Baquero and Plattner, 2016), suggesting that γδ T cells have the capacity to enhance the immune response to both tuberculous and non-tuberculous *Mycobacterium*.

Significant inter-animal variability was observed during this study. This variability has been described previously and can likely be attributed to the fact that cattle are not an inbred species (Svendsen and Hansen, 1999). It has been shown that individuals respond differently to TB infection due to genetic predisposition. Genetic differences between breeds have been found to play a role in breed susceptibility to BTB (Vordermeier et al., 2012). Further work has identified genetic factors that influence susceptibility of cattle to BTB (Driscoll et al., 2011). Another study identified a genetic marker that is associated with a decreased reaction of cattle to the tuberculin test, making these animals less likely to test positive for BTB (Amos et al., 2013). Continued work to better understand individual animal variability is important as it contributes to our understanding of immunity and disease resistance during TB infection.

In summary, our findings show that γδ T cells are extremely dynamic and are capable of producing a number of different cytokines and chemokines, suggesting an important role for these cells throughout disease progression at the site of *M. bovis* infection. Although peripheral γδ T cells were initially used for RNAseq, the results allowed us to identify novel immune factors that could potentially play a role at the site of *M. bovis* infection. Utilizing RNAScope

allowed us to look directly at the site of infection; however, it is important to note that the tissue samples collected for this study depict only a brief snap-shot of γδ T cell function within granulomas during chronic infection. Future studies should be aimed further describing γδ T cell functions at the site of infection over the full course of *M. bovis* infection. Moreover, our unique co-culture approach to mock initial interactions of γδ T cells with infected macrophages at the site of infection gave us insight into γδ T cell expression of immune factors during the early stages of granuloma development. Future studies utilizing trans-well systems or blocking antibodies will allow for a better understanding of γδ T cell interactions with innate immune cells. Taken together, our findings strongly support the hypothesis that γδ T cells play a dynamic role in immune cell recruitment and granuloma maintenance in response to BTB infection, thus contributing to control of the disease and promoting a positive disease outcome.

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## Conflict of Interests

The authors declare no conflict of interests.

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Table 1. *Primers used for qPCR*

| Gene | Accession number | Strand | Sequence (5'-3') | Reference |
|---|---|---|---|---|
| RPS9 | NM_001101152.1 | Forward<br>Reverse | CGCCTCGACCCAGAGCTGAAG<br>CCTCCAGACCTCACGTTTGTTCC | (Sacco et al., 2012) |
| IFN-γ | NM_174086.1 | Forward<br>Reverse | AGAATCTCTTTCGAGGCCGGAG<br>TATTGCAGGCAGGAGGACCATTAC | (Sacco et al., 2012) |
| IL-17 | NM_001008412 | Forward<br>Reverse | CACAGCATGTGAGGGTCAAC<br>GGTGGAGCGCTTGTGATAAT | (Thacker et al., 2009) |
| IL-10 | NM_174088.1 | Forward<br>Reverse | TTACCTGGAGGAGGTGATG<br>GTTCACGTGCTCCTTGATG | (Sacco et al., 2012) |
| TNF-α | NM_173966.3 | Forward<br>Reverse | CGGGGTAATCGGCCCCCAGA<br>GGCAGCCTTGGCCCCTGAAG | (Sacco et al., 2012) |
| IL-22 | NM_001098379 | Forward<br>Reverse | GAGGTGCTGTTCCCCCAAT<br>GAAGGGCACCACCTTTTC | (Rainard et al., 2013) |
| IL-1β | X54796 | Forward<br>Reverse | ATGGGTGTTCTGCATGAG<br>AAGGCCACAGGAATCTTG | (Sacco et al., 2012) |
| CCL1 | XM_002695632.3 | Forward<br>Reverse | CCTCTGCCAGTGAAAGGAAA<br>GGAAGAAGGACTGGTGTGAAG | |
| CCL2 | NM_174006.2 | Forward<br>Reverse | CGCCTGCTGCTATACATTCA<br>GCTCAAGGCTTTGGAGTTTG | |
| CCL4 | NM_001075147.2 | Forward<br>Reverse | ATGGCTGCCTTCTGTTCTC<br>GGAATCTTCCGCAGAGTGTAA | |
| CCL8 | NM_174007.1 | Forward<br>Reverse | GCTCAGCCAGATTCAGTTTCT<br>GGTGATTCTCGTGTAGCTGTC | |
| CCL24 | NM_001046596.2 | Forward<br>Reverse | TCCAACTCACAGGTTGCATAA<br>GCCTCACTACAAGACCAGAAG | |
| CXCL10 | NM_001046551.2 | Forward<br>Reverse | ACACCGAGGCACTACGTTCT<br>TAAGCCCAGAGCTGGAAAGA | |
| GNLY | NM_001075143.1 | Forward<br>Reverse | CTGCTGCTCCAAGGAGAAGA<br>GCAGTGGAGGGAGTTTGGT | (Endsley et at., 2004) |
| GZMB | NM_174296.2 | Forward<br>Reverse | TATGCCCTCTACAGACAATCTA<br>CTTGGATCTCCAGCACATATC | |
| NOS2 | NM_001076799.1 | Forward<br>Reverse | GGGAGATTGGAGGGAGATTA<br>TTGGTAGCAGGTCAAGTAAAG | |

The Integrated DNA Technologies PrimerQuest Tool was used to create custom primers that have not been previously published.

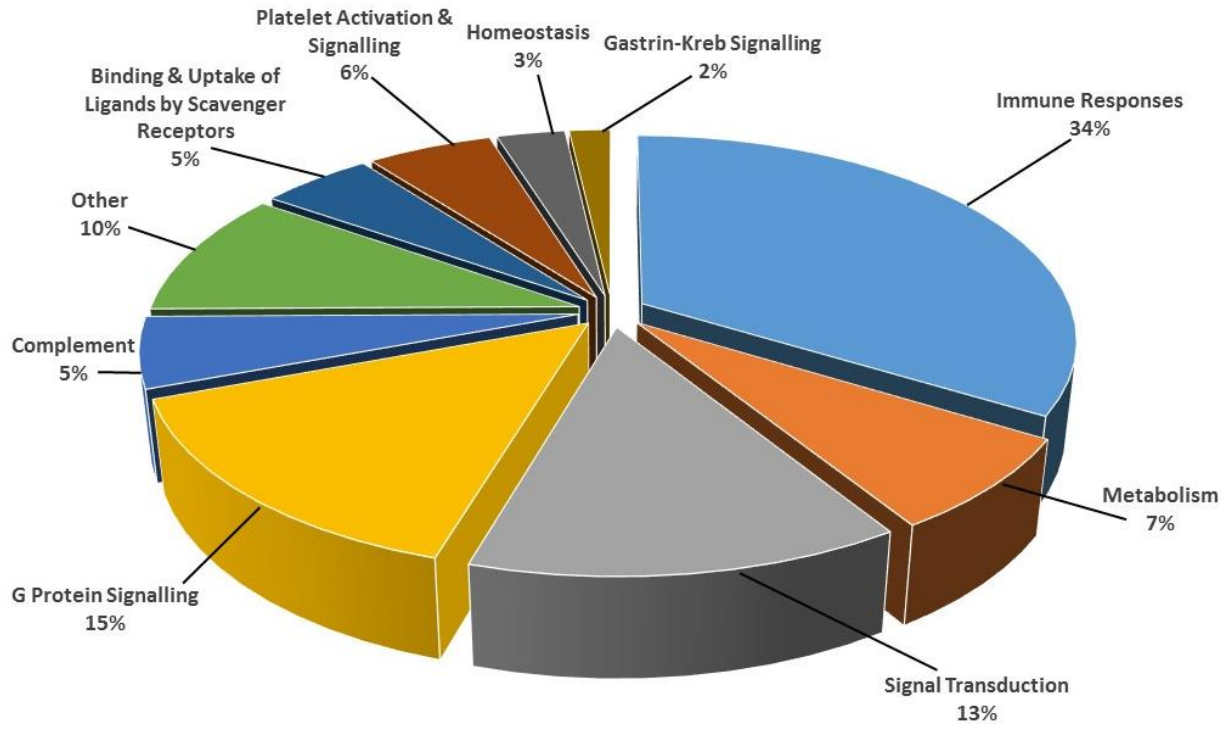



Figure 1. *Pathways related to the immune response are most significantly modulated in M. bovis-specific γδ T cells.* PBMCs were collected from calves (n=5) infected with virulent *M. bovis* and stimulated over-night with PPD-b or culture media alone. γδ T cells were isolated by magnetic separation and RNA was extracted and subjected to transcriptomics analysis. Differential gene expression values were calculated for each gene as the fold change of stimulated γδ T cells over mock treated γδ T cells for each animal. Genes commonly expressed between all five calves were subjected to a pathways analysis to reveal the most significant canonical pathways being represented by RNA sequencing results.

Table 2. *RNA sequencing genes relating to the immune system commonly expressed between M. bovis-infected calves.*

| Gene | Fold Change | Function |
|---|---|---|
| *IFN-γ* | 99646.37 (± 69587.71) | Inflammatory cytokine |
| *IL-17A* | 88228.55 (± 52531.11) | Pro-inflammatory cytokine |
| *CXCL10* | 80726.06 (± 31605.93) | Chemoattractant |
| *GZMB* | 27526.51 (± 20565.8) | Cytotoxicity |
| *IL-15RA* | 6464.67 (± 4269.81) | Proliferation |
| *SOCS1* | 67.1 (± 19.08) | Negative feedback cytokine signaling |
| *IL-1β* | 26.11 (± 6.21) | Inflammatory cytokine |
| *NOS2* | 25.99 (± 11.85) | Antimicrobial |
| *TNF* | 13.1 (± 3.16) | Inflammatory cytokine |
| *CCL2* | 1.06 (± 0.1) | Immune cell recruitment |

Values are the mean ± SEM.

Table 3. *RNA sequencing genes relating to various pathways commonly expressed between all M. bovis-infected calves*.

| Pathway | Gene | Name | Fold Change |
|---|---|---|---|
| G Protein Signaling | *CXCL10* | Interferon gamma induced protein 10 | 80726.06 ± 31605.93 |
| | *LOC504773* | Regakine-1 | 6.87E-02 ± 3.74E-02 |
| | *PTAFR* | Platelet-activating factor receptor | 0.014483 ± 0.009 |
| | *EDNRB* | Endothelin B receptor | 0.001417 ± 0.001 |
| | *ADM* | ADM Adrenomedullin-11-26 Proadrenomedullin N-20 terminal peptide | 7285.566 ± 4096.943 |
| Metabolism | *APOE* | Apolipoprotein E | 3.01E-02 ± 1.95E-02 |
| | *STXBP1* | Syntaxin-binding protein 1 | 5310.156 ± 2220.274 |
| | *HMOX1* | Heme oxygenase 1 | 2.25E-02 ± 1.91E-02 |
| | *CD36* | Platelet glycoprotein 4 | 5.18E-05 ± 1.14E-05 |
| | *HBA* | Hemoglobin subunit alpha | 19829.58 ± 7702.572 |
| | *INHBA* | Inhibin beta A chain | 7737.023 ± 8867.887 |
| Signal Transduction | *TNF* | Tumor necrosis factor | 13.0989 ± 3.161 |
| | *CISH* | Cytokine-inducible SH2-containing protein | 10123.22093 ± 8427.815 |
| | *ISG15* | Ubiquitin-like protein ISG15 | 34.81213 ± 12.267 |
| | *TYROBP* | TYRO protein tyrosine kinase binding protein | 0.044947 ± 0.016 |

Values are the mean ± SEM.

Table 4. *Confirming RNA sequencing with qPCR.*

| Gene | RNA Sequencing | RT-qPCR |
|---|---|---|
| *IFN-g* | 99646.37 (± 69587.71) | 2145.75 (± 1994.26) |
| *IL-17* | 88228.55 (± 52531.11) | 1546.28 (± 1343.04) |
| *TNF-a* | 13.1 (± 3.16) | 523.49 (± 493.45) |
| *CCL2* | 1.06 (± 0.1) | 100.79 (±68.69) |
| *CXCL10* | 80726.06 (± 31605.93) | 247.42 (± 196.03) |
| *NOS2* | 25.99 (± 11.85) | 87.38 (± 57.96) |
| *SOCS1* | 67.11 (± 19.08) | 24.16 (± 9.03) |
| *Granzyme B* | 27526.51 (± 20565.78) | 29.8 (± 20.77) |

γδ T cells from calves that were subjected to RNA sequencing were also analyzed by qPCR to confirm sequencing results. Values indicate average fold change ± SEM in gene expression between unstimulated and PPD-b stimulated γδ T cells isolated from *M. bovis*-infected calves (n=5).

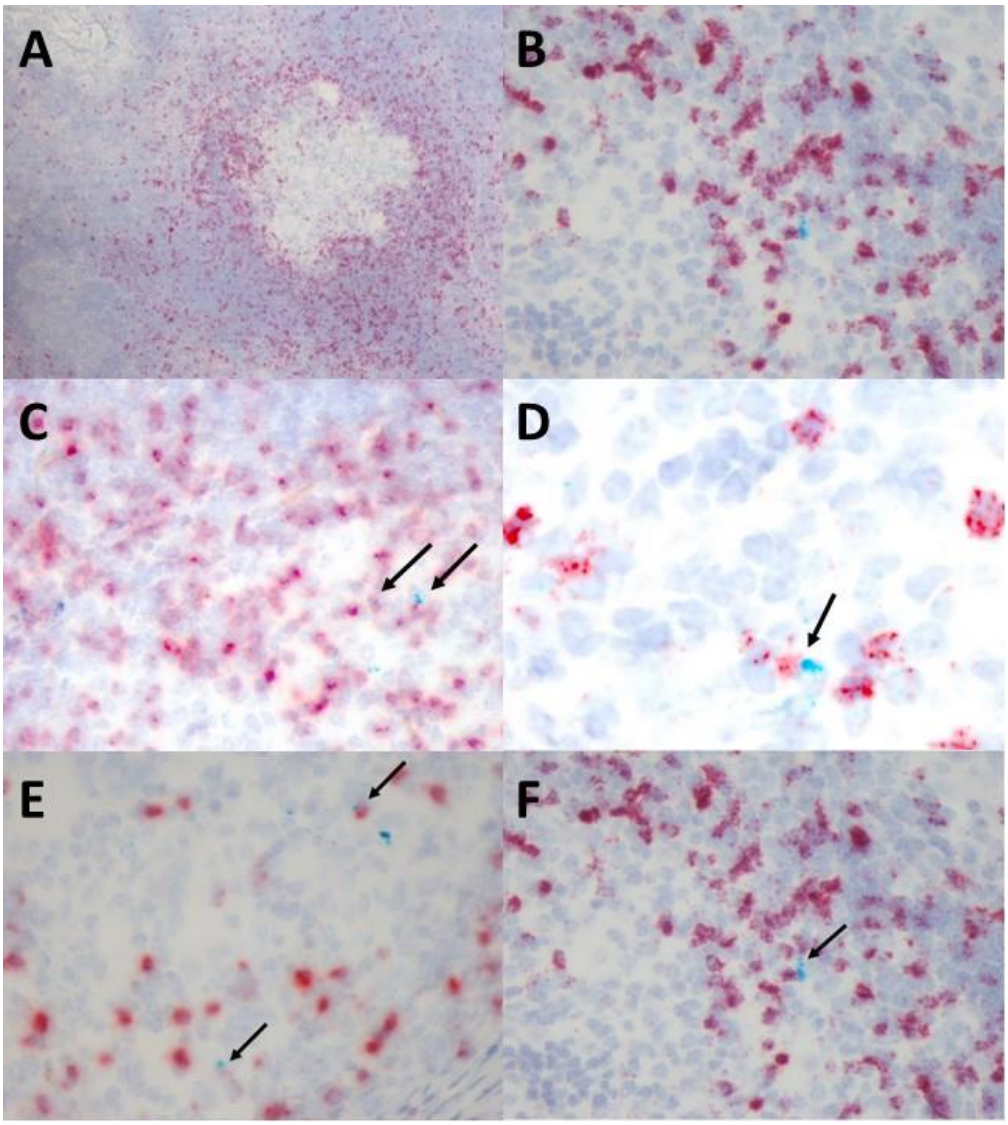



Figure 2. *γδ T cells express various cytokines and chemokines within late-stage granulomas.* Tissue samples from granulomatous lesions in the lungs and mediastinal lymph nodes were harvested from calves (n=5) 3 months post-infection with virulent *M. bovis*. Tissue sections were preserved onto slides by formalin fixation and paraffin embedding. RNAScope was used for *in situ* analysis of mRNA transcripts of various cytokines/chemokines (green) and the γδ TCR (red). Characteristic TB granuloma at 10X magnification (A) and at 40X magnification (B) within the lymph node of a chronically infected animal. Arrows indicate instances of γδ T cell and IFN-γ (C) IL-10 (D) CCL2 (E) IL-17 (F) co-expression.

Table 5. *Quantitative measure of mRNA labeling using RNAScope for various cytokines and chemokines expressed by γδ T cells within bovine pulmonary tuberculoid granulomas.*

| | Lung (n=5) | Lymph Node (n=5) |
|---|---|---|
| IFN-γ | 6.94% (± 0.04%) | 22.82% (± 0.09%) |
| IL-10 | 4.68% (± 0.03%) | 16.45% (± 0.01%) |
| IL-17 | 12.00% (± 0.08%) | 12.87% (± 0.08%) |
| CCL2 | 38.22% (±0.09%) **(n=4) | 21.37% (± 0.06%) |

Tissue samples from granulomatous lesions in the lungs and mediastinal lymph nodes were harvested from calves (n=5) 3 months post-infection with virulent *M. bovis*. Tissue sections were preserved onto slides by formalin fixation and paraffin embedding. RNAScope was used for *in situ* analysis of mRNA transcripts of various cytokines/chemokines and the γδ TCR. Ten representative images at 100X magnification from around the periphery of the granuloma were used for quantification. Values represent the percentage of γδ T cells expressing the cytokine or chemokine of interest out of the total number of cells expressing the cytokine or chemokine of interest ± SEM.

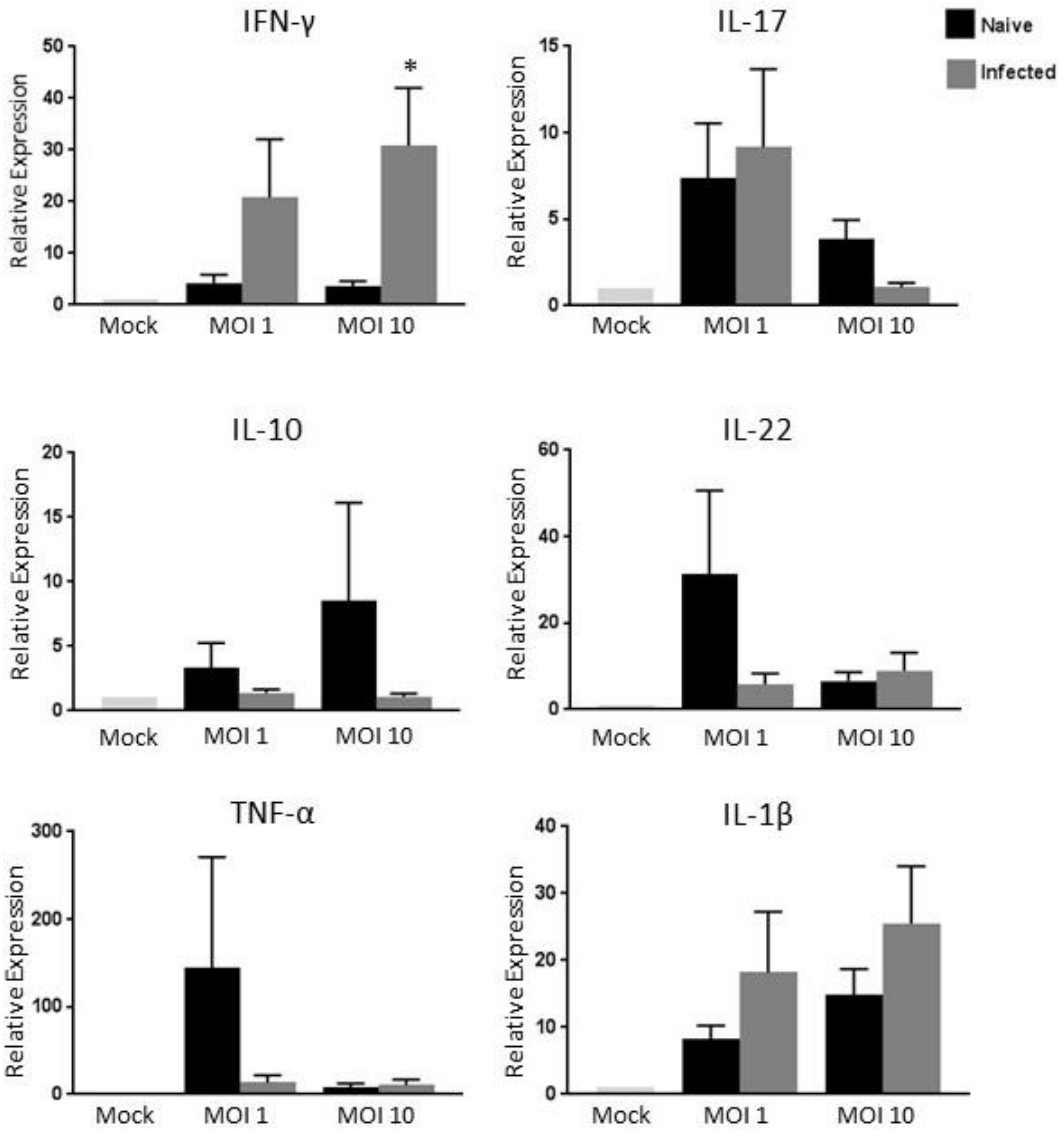



Figure 3. *Cytokine expression in γδ T cell/MDM co-cultures from naïve and M. bovis-infected calves stimulated with BCG.* MDM and autologous γδ T cells isolated from *M. bovis*-infected or naïve animals were cultured together for 24 hours after a 4 hour infection with BCG at an MOI of 1:1 or 10:1. RNA was extracted and reverse transcribed into cDNA and qPCR was performed for various inflammatory, anti-inflammatory, and regulatory cytokines. Results were normalized to the housekeeping gene RPS-9, and expressed relative to uninfected γδ/ MDM co-culture (mock) samples. Data represent means ± SEM (n=19 for naïve group and n=10 for infected group) (* $P \leq 0.05$; ** $P \leq 0.01$; ANOVA).

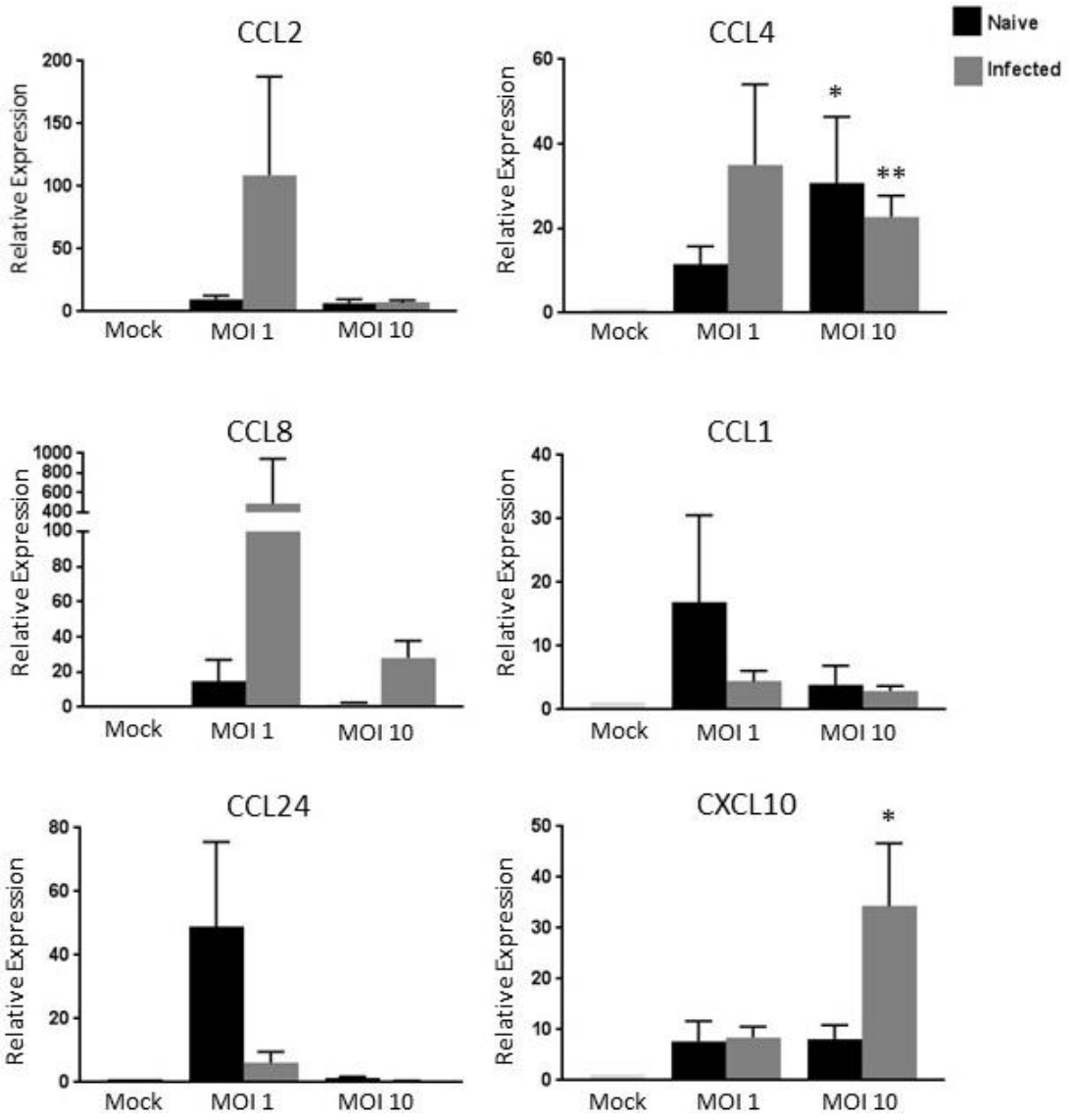



Figure 4. *Chemokine expression in γδ T cell/MDM co-cultures from naïve and M. bovis-infected calves stimulated with BCG.* MDM and autologous γδ T cells isolated from *M. bovis*-infected or naïve animals were cultured together for 24 hours after a 4 hour infection with BCG at an MOI of 1:1 or 10:1. RNA was extracted and reverse transcribed into cDNA and qPCR was performed on various chemokines. Results were normalized to the housekeeping gene RPS-9, and expressed relative to uninfected γδ/ MDM co-culture (mock) samples. Data represent means ± SEM (n=19 for naïve group and n=10 for infected group) (* $P \leq 0.05$; ** $P \leq 0.01$; ANOVA).

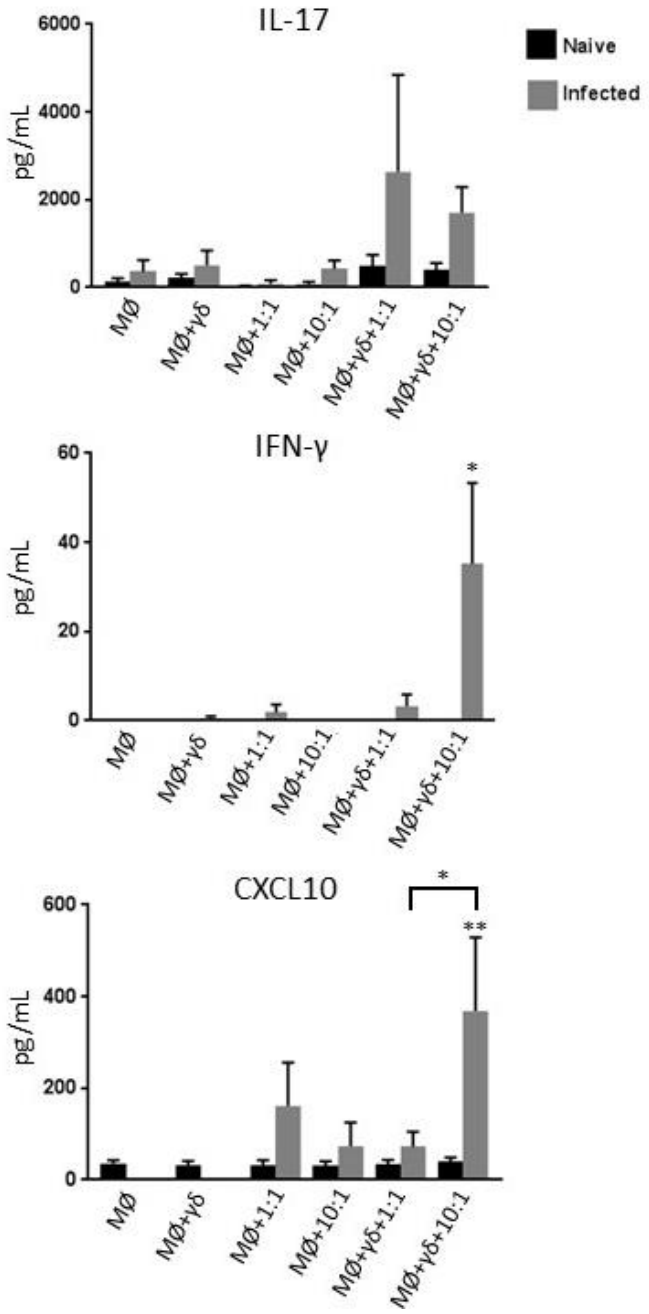



Figure 5. *γδ T cells from M. bovis-infected calves are the main source of IL-17, IFN-γ and CXCL10 when in contact with BCG-infected MDM.* Commercial ELISA kits were used to measure IL-17A, IFN-γ, and CXCL10 from the supernatants of uninfected and BCG-infected co-cultures of MDM in direct contact with autologous γδ T cells. Data represent mean ± SEM (n=19 for naïve group and n=10 for infected group) (* $P \leq 0.05$; ** $P \leq 0.01$; ANOVA).

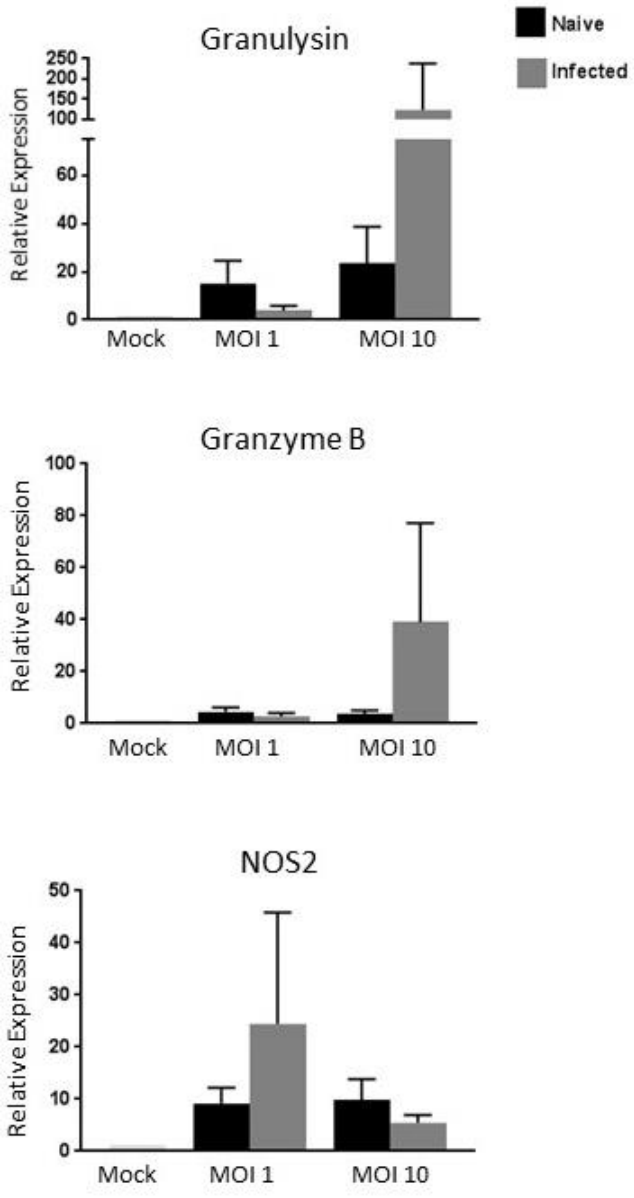



Figure 6. *Cytotoxic factors expressed in γδ T cell/MDM co-cultures from naïve and M. bovis-infected calves stimulated with BCG.* MDM and autologous γδ T cells isolated from *M. bovis*-infected or naïve animals were cultured together for 24 hours after a 4 hour infection with BCG at an MOI of 1:1 or 10:1. RNA was extracted and reverse transcribed into cDNA and qPCR was performed on various cytolytic factors. Results were normalized to the housekeeping gene RPS-9, and expressed relative to uninfected γδ/ MDM co-culture (mock) samples. Data represent means ± SEM (n=19 for naïve group and n=10 for infected group).

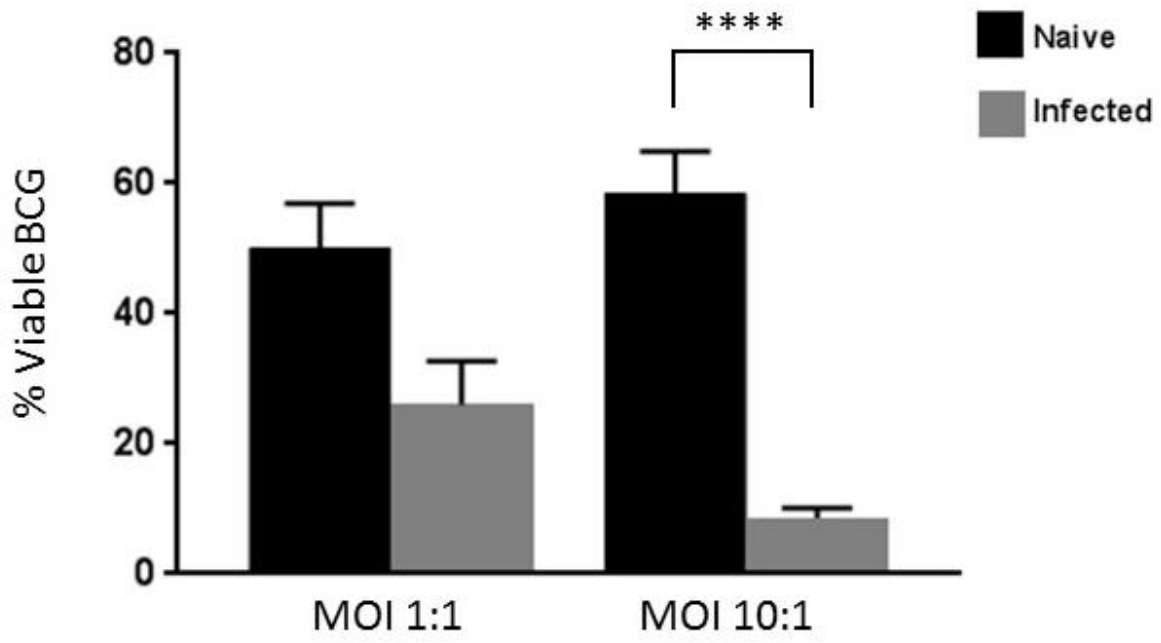
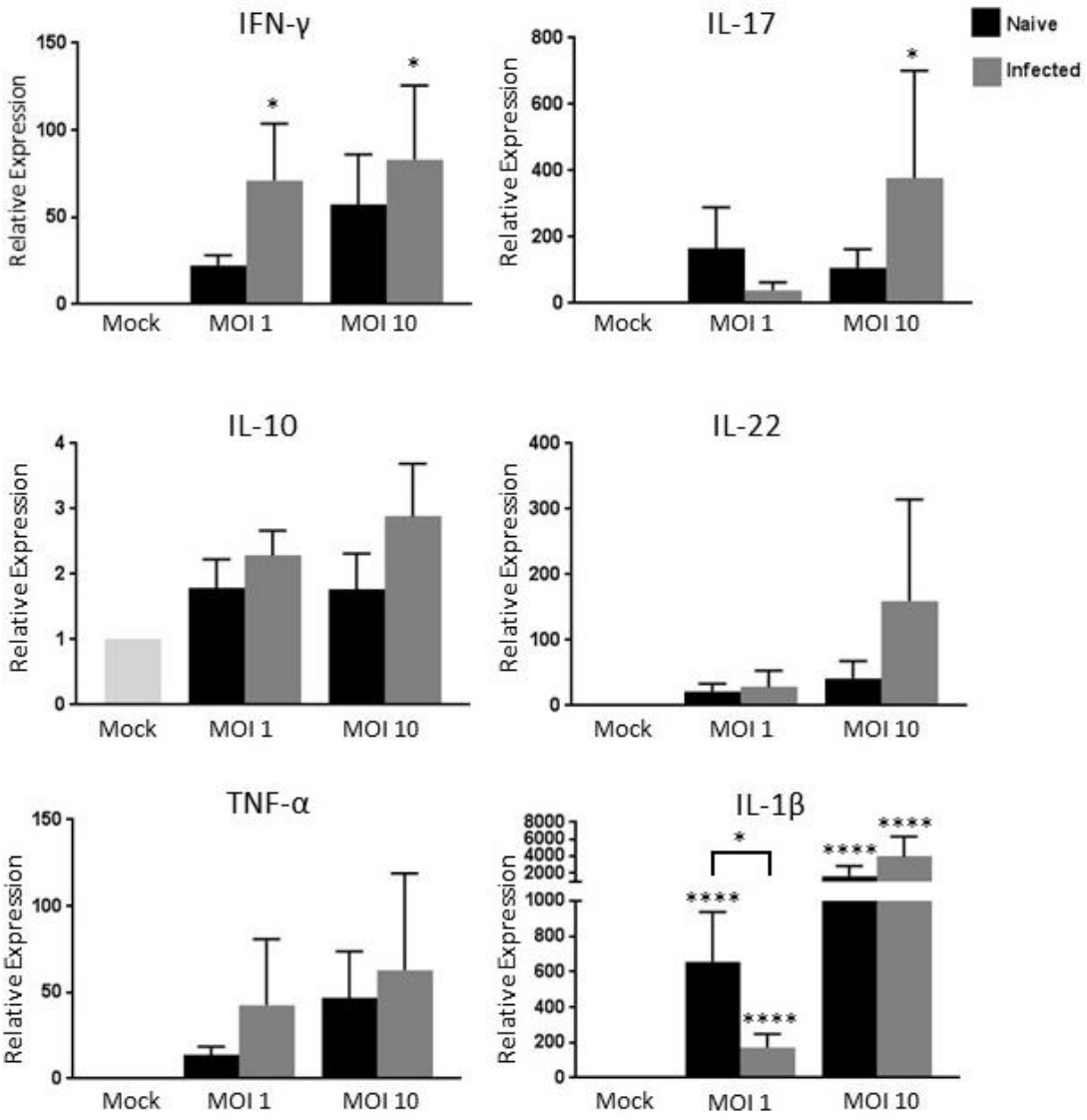
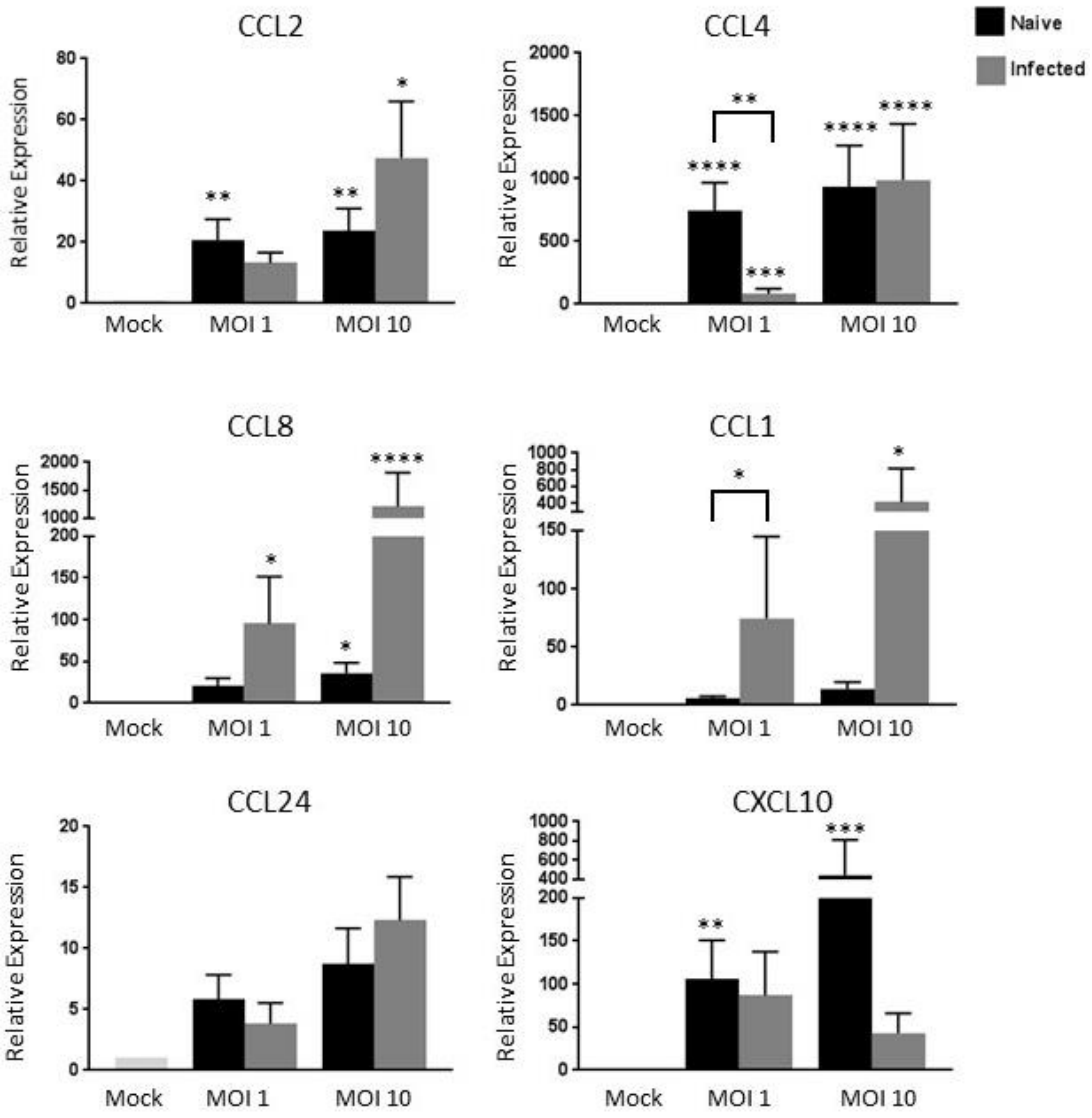


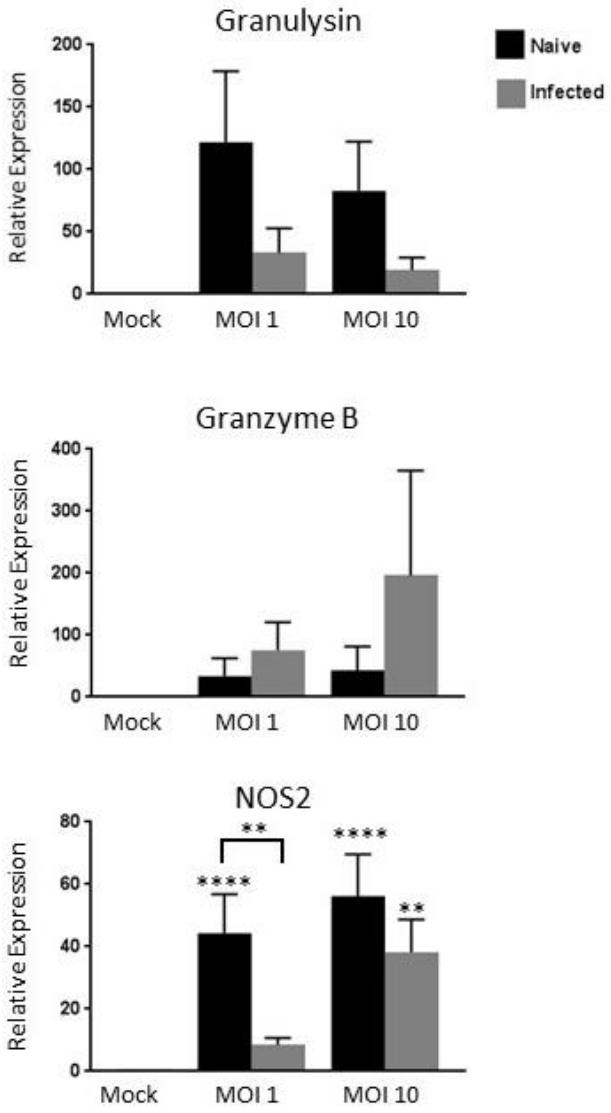

Figure 7. *BCG viability is reduced when cultured with cells from M. bovis-infected animals.* Cells were collected 72 hours after infection with BCG and frozen until ready for analysis. After thawing, cells were re-suspended in 7H9-OADC-T and incubated in for 24 h. Cells were centrifuged and re-suspended in saline solution, and 1 μL of FDA at a concentration of 2 mg/mL was added to each tube. After 30 min of incubation at 37°C, samples were analyzed by flow cytometry. Data represent means ± SEM (n=19 for naïve group and n=8 for infected group) (**** $P \leq 0.0001$; ANOVA).



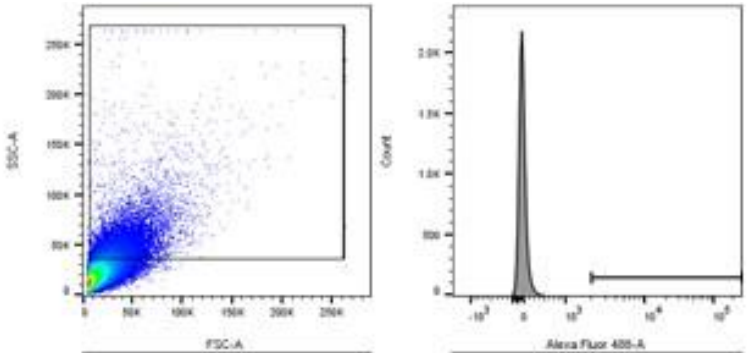

Supplemental Figure 1. *Cytokine expression by MDM from naïve and M. bovis-infected calves cultured alone or with BCG.* MDM isolated from *M. bovis*-infected or naïve animals were cultured together for 24 hours after a 4 hour infection with BCG at an MOI of 1:1 or 10:1. RNA was extracted and reverse transcribed into cDNA and qPCR was performed on various inflammatory, anti-inflammatory, and regulatory cytokines. Results were normalized to the housekeeping gene RPS-9, and expressed relative to uninfected MDM culture (mock) samples. Data represent means ± SEM (n=19 for naïve group and n=10 for infected group) (* $P \leq 0.05$; **** $P \leq 0.0001$; ANOVA).



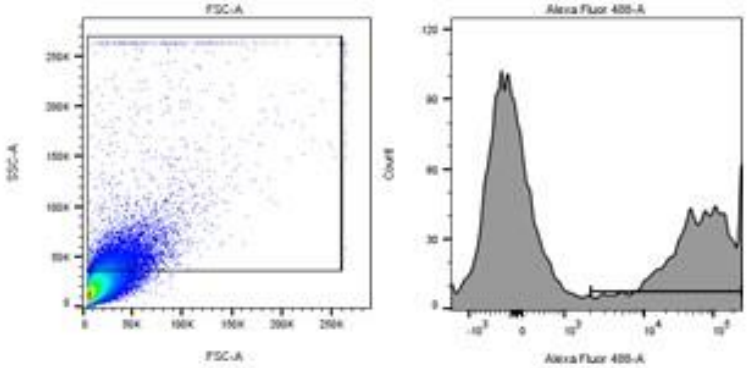

Supplemental Figure 2. *Chemokine expression by MDM from naïve and M. bovis-infected calves cultured alone or with BCG.* MDM isolated from *M. bovis*-infected or naïve animals were cultured together for 24 hours after a 4 hour infection with BCG at an MOI of 1:1 or 10:1. RNA was extracted and reverse transcribed into cDNA and qPCR was performed on various chemokines. Results were normalized to the housekeeping gene RPS-9, and expressed relative to uninfected MDM culture (mock) samples. Data represent means ± SEM (n=19 for naïve group and n=10 for infected group) (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ANOVA).



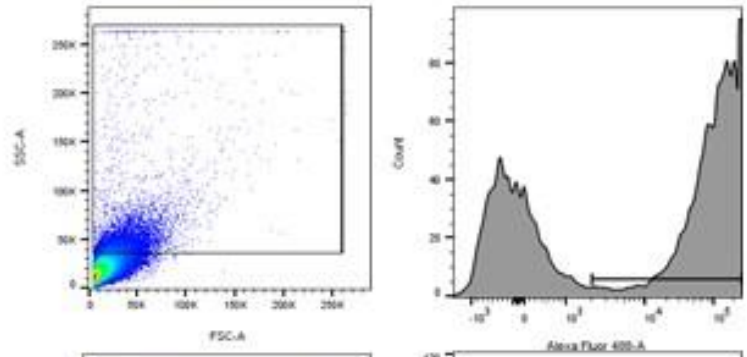

Supplemental figure 3. *Cytotoxic factors expressed by MDM from naïve and M. bovis-infected calves cultured alone or with BCG.* MDM isolated from *M. bovis*-infected or naïve animals were cultured for 24 hours after a 4 hour infection with BCG at an MOI of 1:1 or 10:1. RNA was extracted and reverse transcribed into cDNA and qPCR was performed on various cytolytic factors. Results were normalized to the housekeeping gene RPS-9, and expressed relative to uninfected MDM culture (mock) samples. Data represent means ± SEM (n=19 for naïve group and n=10 for infected group) (* $P \leq 0.05$; ** $P \leq 0.01$; **** $P \leq 0.0001$; ANOVA).



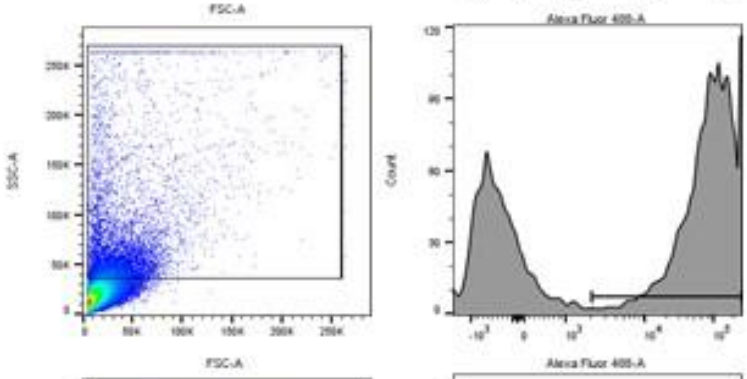

0% Live BCG



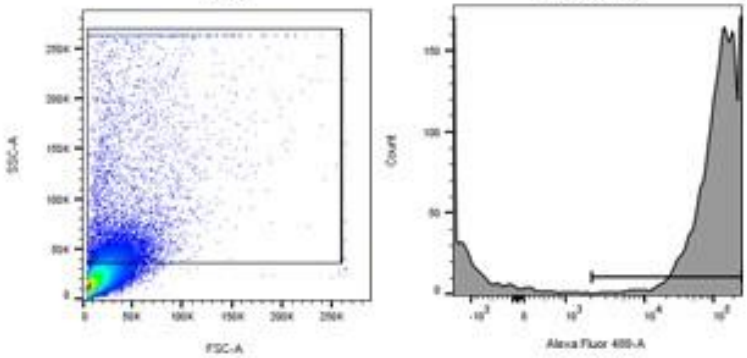

25% Live BCG




50% Live BCG




75% Live BCG




100% Live BCG

Supplemental figure 4. *BCG viability assessed with FDA using known proportions of live and heat-killed bacteria.* Optical density was measured with a SmartSpec™ 3000 spectrophotometer (Bio Rad, Hercules, CA, USA). BCG suspension with a concentration of 1.5 x $10^7$ bacteria/mL was heat-killed using a water bath incubator at 80°C for 30 minutes. Bacteria were then centrifuged at 2000 rpm for 5 minutes. Supernatants were discarded and the pellet was re-suspended in a sterile saline solution to get a concentration of 1 x $10^7$ bacteria/100 µl. Five different proportions of live and heat-killed bacteria (100:0, 75:25, 50:50, 25:75 and 0:100) were mixed to have a total of 1 x $10^7$ bacteria/tube. 1 µL of FDA (Sigma Aldrich) at a concentration of 2 mg/mL was added to each tube, and incubated at 37°C for 30 minutes in 5% $CO_2$. Samples were analyzed by flow cytometry using a BD LSRFortessa X-20 (BD Biosciences, Franklin Lakes, New Jersey, USA), and gating was determined based on the known proportion of live, dead, and unstained-live BCG.

# Chapter 3 - Conclusions

## General Discussion

The aim of the current study was to identify novel functions of γδ T cells at the site of *M. bovis* infection. It has been well established that γδ T cells play a role in BTB infection, and that the response of peripheral blood γδ T cells is not necessarily an accurate representation of their response at the site of infection (Gideon et al., 2015). To date, the specific role γδ T cells play *in vivo* is not well understood; however, it has been shown that these cells accumulate at the site of infection and, in murine models, are necessary for proper granuloma development; suggesting an important role for these cells in shaping the immune response against BTB infection (D'Souza et al., 1997; Ladel et al., 1995).

As a unique approach to identifying novel functions of γδ T cells, we utilized RNA sequencing to conduct an in-depth molecular analysis of the response of *M. bovis*-specific γδ T cells. We found a significant number of immune-related genes to be differentially expressed by γδ T cells responding to *M. bovis* antigen. Some of the cytokines and chemokines identified were in-line with previous research such as IFN-γ, CCL2, IL-10, and IL-17 (Lee et al., 2004; Kennedy, 2002; Alvarez et al., 2009; Lockhart et al., 2006; Guzman et al., 2014), while others had not been characterized in this context such as SOCS1, IL-15RA, IL-1β, NOS2, TNF-α, CCL8, and CCL24. Our transcriptomics analysis also revealed γδ T cell expression of the cytotoxic factors nitric oxide synthase and granzyme B. When we assessed γδ T cell cytotoxic activity in our γδ T cell/MDM co-cultures, we found a significant reduction in BCG viability in co-cultures established from *M. bovis*-infected animals, but not naïve animals. Cytotoxic activities of γδ T cells has been previously described; however, the importance of these cytotoxic

functions in response to *M. bovis* infection is not well understood (Hirano et al., 1998; Skinner et al., 2003; Dieli et al., 2003; Egan and Carding, 2000; De La Barrera et al., 2003).

Granulomas are characteristic of TB disease and aid in the containment of mycobacterial spread; however, they are not sufficient alone to control the disease. The immune response within the granuloma, and the signals required to establish and maintain an effective granuloma structure, are not well understood; therefore, we sought to elucidate the role of γδ T cells in these events. Utilizing an *in situ* hybridization assay allowed us to directly examine γδ T cell mRNA expression within the granulomas of calves chronically infected with *M. bovis*. Consistent with previous findings (Palmer et al., 2007; Cassidy et al., 1998), we observed a significant population of γδ T cells in the lymphoid mantle surrounding the periphery of the granulomas. We determined that γδ T cells comprised a significant population expressing CCL2 and IFN-γ within the lesions, which has not been previously described. However, γδ T cell IFN-γ expression was significantly lower in the lung lesions compared to the lymph node lesions. We observed few γδ T cells expressing IL-17 or IL-10 at this time point of infection, and detected no γδ T cells expressing IL-22. Expression of these cytokines by γδ T cells responding to *M. bovis* infection has been described (Aranday-Cortes et al., 2013; Steinbach et al., 2016; Palmer et al., 2015). However, it is likely that the role of γδ T cells changes as disease progresses, and it may be possible that γδ T cells express IL-17, IL-10 and IL-22 more prominently during early infection, or possibly, that γδ T cells do not contribute significantly to local expression of these cytokines during infection. Limited availability of tissue sections hindered our ability to look at additional cytokines and chemokines identified by our RNA sequencing in this study, but will be the subject of future research for our group.

After identifying novel immune genes in our sequencing data and looking at the γδ T cell response *in vivo* to chronic *M. bovis* infection, we next sought to explore the γδ T cell response during early *M. bovis* infection. In order to do so, we created a novel *in vitro* co-culture system in which γδ T cells from *M. bovis*-infected or *M. bovis*-naïve calves are in direct contact with autologous BCG-infected MDM in an attempt to mock the initial interactions that may occur between these cells at the site of infection, prior to granuloma development. Similar co-culture systems have been utilized by other groups to study γδ T cells interactions with innate immune cells (Baquero and Plattner, 2016; von Lilienfeld-Toal et al., 2005); however, ours is the first to be used in the context of BCG-infected macrophages and is a novel approach to identifying functions of γδ T cells at the site of initial mycobacterial infection.

Consistent with our sequencing results, co-cultured γδ T cells and MDM from naïve and *M. bovis*-infected animals expressed various cytokines, chemokines, and cytotoxic factors. We detected expression of IL-1β and TNF-α by both γδ T cells and macrophages, and identified expression of several novel chemokines (CCL4, CCL8, & CCL24), giving further insight into the role of γδ T cells in immune cell recruitment during initial formation of granulomas. Expression of the cytotoxic factors granzyme B, NOS2, and granulysin were also analyzed in our co-culture system. BCG viability within the co-culture system was significantly reduced in cultures from *M. bovis*-infected animals compared to naïve animals. Similar to our results, γδ T cells in cattle have been shown to directly kill macrophages that are infected with *M. bovis* (Skinner et al., 2003); although, it is likely that these cytotoxic activities are not sufficient alone for the control of BTB.

Our findings show that γδ T cells are capable of a wide array of functions when responding to mycobacterial infection. These cells are important for recruiting other immune

cells to the site of infection by chemokine secretion, helping to shape the immune response by secretion of various inflammatory and regulatory cytokines, and interacting with other innate immune cells throughout disease progression. This work contributes to the understanding of bovine γδ T cell function in response to *M. bovis* infection. An enhanced understanding of the immune response to TB is essential in the effort to create more efficacious vaccines and diagnostic tests for animals and humans.

## Recommendations for future research

Our research is aimed at elucidating the role of γδ T cells at the site of *M. bovis* infection. We have shown that γδ T cells are capable of a wide array of functions in response to *M. bovis* infection, and identified functions for these cells that have not been well characterized. We have identified a number of novel genes expressed by γδ T cells responding both *in vivo* and *in vitro* to *M. bovis* infection. Future work to expand upon these initial findings is expected to contribute to a more complete understanding of γδ T cell function in the context of BTB infection.

Our transcriptomics analysis and co-culture experiments showed that γδ T cells have the capacity to produce numerous cytokines and chemokines within granulomas, thus contributing to the recruitment of immune cells to the site of infection and shaping the outcome of disease progression. One approach to develop a more complete understanding of the dynamic role that γδ T cells play at the site of infection throughout TB disease progression would be a time-course study in which RNAScope could be used to analyze γδ T cell co-expression of various cytokines and chemokines in all the different stages of granulomas. This would allow for a more complete understanding of how γδ T cell function changes as the immune response develops after mycobacterial infection. It would also allow us to evaluate γδ T cell expression of the

chemokines we identified in the current study, which would give further insight into the role of these cells in local immunity. A recent study by Palmer et al., utilized RNAScope as a way to analyze cytokine expression within pulmonary granulomas in cattle and found increased expression of IFN-γ, TNF-α, IL-16, CXCL10, and CXCL9; however, expression of cytokines and chemokines by γδ T cells was not specifically evaluated (Palmer et al., 2015).

TB granulomas take time to develop and it is difficult to grossly identify lesions in the very early stage of infection. Therefore, our co-culture system provided an *in vitro* model to gain insight into γδ T cell function during early interactions with mycobacteria-infected macrophages, similar to what may happen at the initial site of infection. γδ T cells are often referred to as immune sentinel cells, and are prominent at mucosal surfaces such as the lungs and respiratory tract. Therefore, γδ T cells are likely to be one of the first cells to encounter an infected macrophage following an aerosol TB infection (Reviewed in Vantourout and Hayday, 2013). We analyzed γδ T cells and MDM together in our co-culture system, as initial interactions between these two populations is likely critical for dictating subsequent events in the immune response to TB; however, in order to gain a better understanding of the individual contributions of γδ T cells or macrophages, these cells could be sorted back out into two separate populations to be analyzed. Sorting the two cell types back out of culture could be completed by magnetic separation or by FACS sorting. Another way in which the two cell types could be co-cultured and sorted back out would be the use of a trans-well system. This system could be utilized to differentiate between contact-dependent and soluble interactions between γδ T cells and infected macrophages. A study similar to this has been completed by Baquero and Plattner where, similar to our co-culture results, they found that when they cultured bovine γδ T cells with *Mycobacterium avium* subspecies *paratuberculosis* (*Map)*-infected MDM, there was increased

IFN-γ, IL-17A, and nitrite secretion (Baquero and Plattner, 2016). Also, expanding upon our work with more ELISAs on the cytokines, chemokines, and cytotoxic factors would be useful when comparing mRNA expression to actual protein secretion.

The ability of bovine γδ T cells to behave in a cytotoxic manner towards *M. bovis*-infected macrophages has been previously described (Skinner et al., 2003); however the cytotoxic function of these cells is not well understood. γδ T cell expression of cytotoxic factors was identified in our transcriptomics analysis and in our co-culture systems. We also found that BCG viability was reduced in our co-cultures; however, further work to evaluate the exact mechanisms of cytotoxicity by γδ T cells would provide further insight into the complex role these cells play during early interactions with infected cells. Also, the capacity of bovine γδ T cells for cytotoxicity at the site *M. bovis* infection is unknown and should be the subject of further investigation, potentially by RNAScope, to further address the functional cytotoxic capabilities of these cells.

There are multiple subsets of γδ T cells in cattle, and they are hypothesized to possess unique biological functions, although there is little known about the contribution of the individual populations during infection. $WC1^+$ γδ T cells are the predominant γδ T cell population present in circulation, while $WC1^-$ γδ T cells are more prominent in the tissues (Rhodes et al., 2001; Machugh et al., 1997; Wijngaard et al., 1994). A logical next step would be to elucidate the role of these individual subsets in response to *M. bovis* infection, as opposed to analyzing the response of the γδ T cell population as a whole. During *Map* infection, $WC1^+$ and $WC1^-$ subsets have been reported to play differing roles in granuloma formation, with $WC1^+$ γδ T cells accumulating after antigenic priming and likely functioning as early effector or memory cells, and $WC1^-$ γδ T cells having an enhanced ability to respond innately to live

mycobacteria (Plattner et al., 2009). These subsets may influence the protective immune response to mycobacterial infection in cattle.

Since γδ T cells were first discovered in 1985, significant efforts have gone into understanding the role of these unique T cells (Weintraub and Hedrick, 1995), but many questions still remain. The quest to understand the importance of these cells in the immune system is far from complete and research seems to raise as many questions as it answers. This study contributes to our understanding of basic γδ T cell biology and provides critical information regarding the role of γδ T cells in immunity to TB.

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