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The role of gamma delta T cells in immunity to *Mycobacterium bovis* infection in cattle

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ABSTRACT

Accumulating evidence suggests that γδ T cells play a critical role in the early response to *Mycobacterium bovis* and may be key in bridging innate and adaptive immunity following infection. In vitro, γδ T cells proliferate and produce robust amounts of IFNγ in response to complex, protein and non-protein mycobacterial antigens including *M. bovis* purified protein derivative (PPD), heat shock proteins and cell wall components such as mycolylarabinogalactan peptidoglycan (mAGP). Vaccination with Bacille Calumette-Guerin (BCG), as well as infection with virulent *M. bovis*, induces an increase in the frequency and activation of $WC1^+$ γδ T cells circulating in the blood. Gamma delta T cells are rapidly recruited to the lungs and draining lymph nodes following BCG vaccination, and accumulate in developing lesions early following *M. bovis* infection. In Severe Combined Immuno-deficient (SCID)-bo mice, depletion of γδ T cells prior to *M. bovis* infection results in impaired granuloma formation, suggesting a role for γδ T cells in immune cell recruitment and lesion development. In vivo depletion of $WC1^+$ γδ T cells from calves prior to *M. bovis* infection results in significantly reduced levels of *M. bovis* specific IgG2 and IFNγ, and increased IL-4 production compared to non-depleted control animals, suggesting that γδ T cells may also play a role in shaping the character of the adaptive *M. bovis* specific immune response. Whereas it is clear that γδ T cells are responding during *M. bovis* infection, much remains to be understood about their function in vivo and their ability to shape the innate and adaptive immune responses. This review focuses on recent advances in our understanding of γδ T cell biology with a particular emphasis on the immune response of γδ T cells in cattle during *M. bovis* infection.

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1. Introduction

Bovine tuberculosis (TB) is the cause of significant economic hardship to the livestock industry, as well as a risk to human health. *Mycobacterium bovis*, the causative agent of bovine TB and a member of the *M. tuberculosis* complex, is capable of causing disease in humans and infecting a wide

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array of wildlife species. Understanding immunity to *M. bovis* is a continuing challenge and one that is of interest to the fields of human and animal medicine alike. Recently, there is increasing attention to the role of γδ T cells in immunity to mycobacterium. A unique subset of $CD3^+$ cells, γδ T cells share important characteristics of both the innate and adaptive arms of the immune response. γδ T cells play a pivotal role in the immune response to mycobacterium infection in mice and humans, and recent and accumulating evidence has shown that γδ T cells are also an important component of the immune response to *M. bovis* infection in cattle. This review will highlight the current advances in our understanding of bovine γδ T cell biology and the role of γδ T cells in the immune response to *M. bovis* infection in cattle.

2. Bovine γδ T cells

Gamma delta T cells have been identified in all vertebrate species examined thus far, including humans, mice and non-human primates (Hayday, 2000). The frequency of γδ T cells circulating in humans and mice is rare, generally representing 5–10% of the circulating peripheral lymphocyte population (Kabelitz, 2011). In contrast, γδ T cells are significantly more abundant in ruminant species, where they constitute up to 70% of the circulating peripheral blood lymphocytes in very young animals (Hein and Mackay, 1991; Jutila et al., 2008). As the animal ages, the frequency of γδ T cells declines to an average of 10–20% of circulating lymphocytes in the adult bovine. The increased frequency of γδ T cells, particularly in young animals, suggests a critical role for these cells in the immune system of the ruminant.

2.1. Functional characteristics of γδ T cells in cattle

Gamma delta T cells share functions of both the innate and adaptive response and have been proposed to play a key role in bridging the two arms of the immune system. Functions for γδ T cells that have been well described include inflammatory chemokine and cytokine production (Bonneville et al., 2010; Brown et al., 1994; Collins et al., 1996; Fikri et al., 2001; Hayday, 2000; Jutila et al., 2008), as well as direct cytotoxicity (Brown et al., 1994; Chiodini and Davis, 1992; Daubenberger et al., 1999; Lundberg and Splitter, 2000; Skinner et al., 2003). Gamma delta T cells may also play a role as regulatory cells, as they have been demonstrated to suppress CD4 T cell proliferation and produce IL-10 and TGF-β (Chiodini and Davis, 1992; Hoek et al., 2009; Rhodes et al., 2001).

In addition to cytokine production, γδ T cells can also fulfill the innate function of antigen presentation. First demonstrated in the bovine, activated γδ T cells have the capacity to upregulate MHC II and CD80 and CD86, and directly induce CD4 T cell proliferation (Collins et al., 1998; Toka et al., 2011). Subsequently, the same has been shown for human (Brandes et al., 2009, 2005) and murine (Cheng et al., 2008) γδ T cells. Currently, the physiologic role of antigen presentation by γδ T cells in vivo remains unknown.

2.2. Phenotypic and functional subsets of bovine γδ T cells

Bovine γδ T cells are divided into sub-populations based upon their expression of Workshop Cluster 1 (WC1) or lack thereof (Clevers et al., 1990; Machugh et al., 1997; Mackay et al., 1989; Morrison and Davis, 1991). $WC1^+$ γδ T cells are $CD2^-CD4^-CD8^-$ and are the predominant γδ T cell subset in circulation. $WC1^{neg}$ γδ T cells are most numerous in tissues such as the spleen, intestinal mucosa and mesenteric lymph nodes (LN), (Machugh et al., 1997; Wijngaard et al., 1994; Wilson et al., 1999). The majority of $WC1^{neg}$ γδ T cells express $CD2^+CD8^+$.

There are 13 WC1 genes (Chen et al., 2012) and differential expression of these gene products can be used to divide $WC1^+$ γδ T cells into several serologically defined subpopulations: $WC1.1^+$, $WC1.2^+$ and $WC1.3^+$ (Chen et al., 2009; Rogers et al., 2006). With the exception of less well-represented $WC1.1^+/WC1.2^+$ double positive subset, WC1.1 and WC1.2 are expressed by discrete subpopulations, while WC1.3 is expressed by a subset of the $WC1.1^+$ γδ population (Chen et al., 2009; Rogers et al., 2006).

In addition to differences in tissue distribution, there are clearly functional differences between $WC1^+$ and $WC1^{neg}$ γδ T cells. Studies examining $WC1^{neg}$ responses following antigenic stimulation or infection are lacking; however, reports that have analyzed $WC1^{neg}$ gene expression in a resting animal suggest an immune surveillance role, with expression of genes promoting tissue quiescence and apoptosis (Graff et al., 2006; Hedges et al., 2003; Meissner et al., 2003). This result is consistent with their likely role as sentinel mucosal cells. $WC1^+$ γδ T cells are clearly more transcriptionally active, as well as more proliferative and pro-inflammatory (Blumerman et al., 2007a,b; Graff et al., 2006; Hedges et al., 2003; Meissner et al., 2003).

Recently, it has become clear that differential expression of the WC1 gene can be ascribed to distinct immune function. $WC1.1^+$ γδ T cells are more pro-inflammatory and more able to produce IFNγ in response to mitogen stimulation (Rogers et al., 2005a,b) and to experimental infection (Rogers et al., 2005a,b; Wang et al., 2011). In contrast, the $WC1.2^+$ γδ T cell subset produces little IFNγ in response to mitogen stimulation, and has been described to play a regulatory role in the resting bovine (Hoek et al., 2009). However, $WC1.2^+$ γδ T cells do produce IFNγ in response to the bovine rickettsial pathogen *Anaplasma marginales* (Lahmers et al., 2006), suggesting this subset may have some functional plasticity.

In addition to delineating the subsets, it is likely that WC1 also plays a functional role on γδ T cells. A recent report by Wang et al. demonstrated that WC1 participates directly in antigen recognition by γδ T cells (Wang et al., 2011). Gamma delta T cells respond specifically to *Leptospira*, and this response is contained within the $WC1.1^+$ population of γδ T cells (Rogers et al., 2005a,b). RNA interference-induced downregulation of 3 of the 13 WC1 gene products, specifically those encoding for WC1.1, significantly reduced γδ T cell proliferation and IFNγ production in response to *Leptospira* antigen (Wang et al., 2011). From these results, it is thought that WC1 acts as

a pattern recognition receptor on γδ T cells, similar to a Toll Like Receptor (TLR).

3. *Mycobacterium bovis* infection

M. bovis is a chronic infectious disease of cattle and a wide range of wildlife species including white-tailed deer (Schmitt et al., 1997), Eurasian badgers (Murhead and Burns, 1974) and brushtail possums (Coleman et al., 2006). The livestock industry loses an estimated $3 billion annually to *M. bovis* through the loss of animals to culling and the costs associated with disease testing and control (Waters et al., 2012a). *M. bovis* also infects humans and, prior to the implementation of pasteurization, was a significant cause of disease in developed countries (Roswurm and Ranney, 1973). Today, *M. bovis* continues to cause disease in humans in developing countries and is of increasing concern to communities living at the human–animal interface and to those populations with high incidence of HIV/AIDS (reviewed in Michel et al., 2010). Disease pathogenesis and the ensuing immune response in cattle infected with *M. bovis* reflect many of the characteristics of *M. tuberculosis* infection in humans (reviewed in Buddle et al., 2005; Endsley et al., 2009; Waters et al., 2011); as such, cattle represent an excellent model for understanding human disease.

BCG is an attenuated form of *M. bovis* that is currently the only available licensed vaccine against TB in humans and is widely used in a number of vaccine regimens and experimental trials in cattle and wildlife. BCG is also commonly used in most murine models of TB. Although it is clear that currently, the most effective vaccine strategies to induce protective immunity to TB include BCG (Buddle et al., 2011; Waters et al., 2012a), the efficacy of vaccination in humans (Brewer and Colditz, 1995; Colditz et al., 1995; Fine, 1995), cattle and wildlife reservoirs (Buddle et al., 2011; Waters et al., 2012a) is variable and there is a great need for improved vaccination regimens and novel vaccine platforms.

Following infection, innate immune responses are important for recruiting immune cells and establishing early lesion formation; however, it is important to note that these responses do little to limit infection and it is the adaptive cell-mediated immune response that is essential for controlling the disease. Antigen-specific CD4 T cells are crucial to the antibacterial response (Pollock et al., 2001, 2005); although, CD8 T cells also contribute (Skinner et al., 2003; Smith et al., 1999; Villarreal-Ramos et al., 2003). Key soluble mediators against *M. bovis* are the Th1 cytokines IFNγ and TNF (Buddle et al., 2005; Pollock et al., 2005; Vordermeier et al., 2002; Waters et al., 2003), but additional factors likely participate in disease protection including IL-17 (Aranday-Cortes et al., 2012; Blanco et al., 2011), granulysin (Endsley et al., 2004), nitric oxide (Waters et al., 2003), IP-10 (Alvarez et al., 2009; Waters et al., 2012b) and IL-2 (Buddle et al., 2003). *M. bovis* infection and a detailed description of the anti-microbial immune response in cattle have been the subject of several recent and excellent reviews (McNair et al., 2007; Pollock et al., 2001, 2005; Siddiqui et al., 2012; Waters et al., 2011). As such, this document focuses primarily on the role of γδ T cells in the bovine immune response to *M. bovis*.

4. Gamma delta T cells respond to *M. bovis* infection in vivo

4.1. Gamma delta T cells undergo dynamic changes in circulation following M. bovis challenge

Early studies of calves challenged with virulent *M. bovis* reported dynamic changes in the frequency of circulating γδ T cell populations over the course of infection (Cassidy et al., 1998; Pollock et al., 1996). Pollock et al. described a significant drop in the circulating γδ T cell population in the first few days post infection (Pollock et al., 1996). The authors hypothesized this reduction was due to increased migration of γδ T cells out of the blood to the site of infection. Following the initial decrease, $WC1^+$ γδ T cells subsequently expand in frequency in circulation and express increased levels of CD25, the high affinity IL-2R and a marker of T cell activation, indicating the cells were undergoing an active response to infection (Pollock et al., 1996). Recent work by Buza et al. described a similar expansion of active γδ T cells in the blood of calves following BCG vaccination, and a concomitant increase in serum IFNγ levels (Buza et al., 2009).

4.2. γδ T cells localize to the site of M. bovis infection

$WC1^+$ γδ T cells are amongst the first cells to accumulate at sites of *M. bovis* infection, suggesting they may play an important role during early granuloma formation. In *M. bovis* infected cattle, Doherty et al. demonstrated that γδ T cells preceded even neutrophils at sites of skin delayed type hypersensitivity reactions, forming perivascular aggregates as early 6–24 h post-injection (Doherty et al., 1996). By 72 h post-injection, however, γδ T cells were only a minor cellular component compared to infiltrating macrophages and CD4 and CD8 T cells. Cassidy et al. went on to examine lymphocyte infiltration in early tuberculous lesions in the lungs and LN of *M. bovis* infected cattle (Cassidy et al., 1998). $WC1^+$ γδ T cells were observed at increased numbers in lung lesions as early as 7 days post infection and remained increased through day 21 post infection, primarily localizing to the lymphoid mantle at the periphery of the lesions. After day 21, $WC1^+$ cells were rare and only randomly distributed around sites of necrosis. In agreement, Palmer et al. reported high numbers of γδ T cells in early stage LN granulomas, but reduced numbers as the lesions matured, although the expression of WC1 by the infiltrating populations was not examined (Palmer et al., 2007). Following intranasal BCG vaccination, $WC1^+$ γδ T cells infiltrate the lungs and LN of the head as early as 7 days post vaccination (Price et al., 2010). Similar to the results observed in cattle, inoculation of mice with BCG also results in increased localization of γδ T cells to the lungs and pulmonary LN (Dieli et al., 2003). Importantly, the infiltrating γδ T cells are activated, produce robust levels of IFNγ and are cytotoxic against infected target cells.

Although the evidence for γδ T cell accumulation at the site of infection is strong, the kinetics of the response

continues to be controversial. In contrast to the observations of γδ T cells early during lesion formation, some studies have reported increased accumulation of γδ T cells in late stage, rather than early stage granulomas. Wangoo et al. described few γδ T cells in stage I/II lesions, but increased accumulation of WC1$^+$ γδ T cells near the periphery of the fibrotic capsule in stage III and IV granulomas (Wangoo et al., 2005). Aranday-Cortes et al. observed a similar pattern with a dense distribution of γδ T cells in the outer layers of late stage lesions (Aranday-Cortes et al., 2012). Currently, the reasons for these disparate results remain unclear; however, it is likely that the virulence of the *M. bovis* strain used, age and genetics of the animals and timing of necropsies all effect the kinetics and nature of γδ T cell accumulation.

4.3. The role of WC1 in the γδ T cell response to M. bovis infection

The majority of studies concerning γδ T cells and *M. bovis* infection have focused on the responses of the WC1$^+$ γδ T cell population as a whole. Recently, however, it has become evident that differential expression of the WC1 genes can be ascribed to unique immunologic functions (Hoek et al., 2009; Rogers et al., 2005a; Wang et al., 2011). In the field of *M. bovis* infection, the role of the individual WC1$^+$ subsets is just beginning to be examined. Recent work by Price *et al.* has demonstrated that γδ T cells localize to sites of infection following intranasal vaccination with BCG, and that the infiltrating γδ T cell population is positive for WC1.1 (Price et al., 2010). On day 7 post-intranasal BCG vaccination, the frequency of bovine WC1.1$^+$ γδ T cells increased significantly in all lobes of the lungs, the pharyngeal tonsils and the LN of the head, while the frequency of WC1.2$^+$ γδ T cells infiltrating these areas did not change. Not only does this reinforce a role for γδ T cells in the early immune response to mycobacterium, it also suggests the WC1.1$^+$ subset specifically may be responding to *M. bovis*. This would be particularly interesting given the propensity of the WC1.1$^+$ subset to produce more IFNγ and the knowledge that WC1.1$^+$ cells are also the critical γδ T cell subpopulation responding to *Leptospira* (Rogers et al., 2005a,b).

4.4. Gamma delta T cells and granuloma formation during M. bovis infection

Given the reports demonstrating an early accumulation of γδ T cells at sites of *M. bovis* infection, several studies have attempted to establish a function for γδ T cells in granuloma formation. Although high-dose *M. tuberculosis* infection is rapidly lethal (D'Souza et al., 1997), γδ T-cell deficient mice are able to control BCG (Ladel et al., 1995) and low-dose *M. tuberculosis* infection (D'Souza et al., 1997), but exhibit significantly larger and less-organized granulomas in both cases. Gamma delta T-cell deficient mice infected with *Map* exhibit significantly fewer granulomas compared to γδ T-cell sufficient animals (Stabel and Ackermann, 2002). In an approach to analyze bovine γδ T cells, Smith et al. utilized severe combined immune-deficient (SCID) mice transplanted with fetal bovine tissue to create xenochimeric SCID-bo mice with an intact bovine immune system (Smith et al., 1999). Depletion of WC1$^+$ γδ T cells from SCID-bo mice prior to *M. bovis* infection resulted in significantly altered architecture in the developing granuloma with increased neutrophil infiltration and coalescing areas of caseous and liquefactive necrosis. Further, the mice depleted of WC1$^+$ cells exhibited similar levels of mycobacterial colonization, but significantly increased levels of mortality compared to non-depleted controls (Smith et al., 1999). From these results, it appears that the role of γδ T cells in early lesions may not be for direct control of infection, but for the establishment of an appropriate and thus, more effective immune response. In a model of bovine paratuberculosis, Plattner et al. (2009) demonstrated that the recruitment pattern of γδ T cells could be correlated to the degree of granuloma organization. In animals with highly organized granulomas, γδ T cells infiltrated lesions early, peaking at 7–10 days post infection, with WC1$^+$ cells localized to the outer margins, near the fibrous border. In animals with poorly organized granulomas, γδ T cell infiltration peaked at >60 days post infection and was unorganized and diffuse (Plattner et al., 2009). In a separate report, using Matrigel technology, Plattner et al. (2012) went on to demonstrate that the γδ T cells infiltrating the site of *Map* infection produced high levels of IFNγ, further supporting the hypothesis that γδ T cell infiltration and cytokine production is important in the establishment of early immunity against mycobacterium.

In a direct approach to study the effects of γδ T cells on mycobacterium infection in the natural host, Kennedy et al. (2002) depleted WC1$^+$ γδ T cells from calves prior to *M. bovis* challenge. WC1$^+$ γδ T cell depletion had no significant effects on the formation of tuberculous lesions, nor did it alter bacterial burden. However, calves depleted of WC1$^+$ γδ T cells did exhibit an altered immune phenotype with a significant reduction in early IFNγ production and PBMC proliferative responses, reduced production of the Th1-associated IgG2 antibody isotype and significantly enhanced production of the Th2-associated cytokine IL-4 (Kennedy et al., 2002). Interestingly, this result mirrors that observed following depletion of γδ T cells from calves infected with the viral pathogen BRSV (Taylor et al., 1995; Thomas et al., 1996), suggesting the role for γδ T cells in shaping early immunity may extend to responses against other intracellular pathogens. Given the results from SCID-bo mice (Smith et al., 1999) and γδ T cell deficient mice (D'Souza et al., 1997; Ladel et al., 1995), the failure of Kennedy et al. to observe changes in lung histopathology following WC1$^+$ γδ T cell depletion is surprising. Due to limitations of the antibody depletion approach, the authors found it necessary to increase the dose of *M. bovis* inoculum and reduce the length of the study prior to necropsy to only 5 weeks post infection (Kennedy et al., 2002). Thus, it is possible that depletion of γδ T cells may have an effect on tissue pathology or long-term immunity during a more physiologic *M. bovis* infection that was not appreciated under the described challenge conditions. Regardless, the increased bias towards a Th2 type response indicates a pivotal role for γδ T cells in the formation of appropriate anti-bacterial immunity.

The impaired immune response observed in their absence, and the propensity of γδ T cells to accumulate at lesion sites early in the response to *M. bovis* implicates γδ T cells in initial chemokine production and immune cell recruitment. Utilizing the SCID-bo mouse model, Alvarez et al. demonstrated that depletion of $WC1^+$ γδ T cells led to significant reduction in serum levels of IP-10 (CXCL10), CCL2 and IL-12p70 (Alvarez et al., 2009). In this report, bovine γδ T cells produced several cytokines, including IL-2, IL-10, IL-15 and IFNγ, but were not a significant source of chemokines including IP-10, CCL2, CCL3 or XCL1. From these results, γδ T cells may not be a primary source of chemokines during *M. bovis* infection, but rather, may contribute indirectly to immune cell recruitment. The capacity of γδ T cells to produce chemokines remains controversial however, as contradictory reports have demonstrated gene expression of several chemokines including CCL2, CCL8, CXCL1, CXCL2, CXCL6 by bovine γδ T cells (Hedges et al., 2003; Lahmers et al., 2006; Meissner et al., 2003) and work from our own laboratory has demonstrated robust production of CCL2, CCL3 and GM-CSF by message and protein (McGill et al., 2012a). Importantly, the capacity and nature of chemokine expression by γδ T cells from the blood may differ significantly from that of γδ T cells at the site of infection and important future work should be aimed at identifying the contribution of γδ T cells in the tissues during early granuloma formation.

5. Gamma delta T cells respond to *M. bovis* infection in vitro

5.1. Gamma delta T cells proliferate and produce cytokines in response to mycobacterial antigens

Following the observation that γδ T cells undergo significant expansion in the blood of mycobacterium infected mice (Janis et al., 1989) and human patients (Meraviglia et al., 2011), several in vitro studies described the capacity of human γδ T cells to proliferate and produce cytokines in response to killed *M. tuberculosis* bacilli (Kabelitz et al., 1991), live *M. tuberculosis* infected monocytes (Boom et al., 1992; Havlir et al., 1991) and *M. tuberculosis* infected alveolar macrophages (Havlir et al., 1991). Subsequently, many of the antigens driving murine and human γδ T cell activation during mycobacterial infection were identified and include protein antigens such as mycobacterial heat shock protein (Born et al., 1990; Haregewoin et al., 1989) and non-protein phosphoantigens (Fournie and Bonneville, 1996; Morita et al., 1995). Phosphoantigens, recognized primarily by the Vγ9Vδ2 subset, are low molecular weight phosphorylated metabolites of isoprenoid synthesis that include (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate and isopentynyl pyrophosphate (IPP). These compounds are produced by many types of protozoa and bacteria, including *M. tuberculosis* and *M. bovis*. Phosphoantigens induce robust proliferation and cytokine responses by human and non-human primate γδ T cells (Chen, 2011; Chen and Letvin, 2003).

As with mice and humans, it was initially in vivo evidence that indicated bovine γδ T cells were responding to *M. bovis* infection. Although γδ T cells clearly expanded in the blood of *M. bovis* infected animals and accumulated at sites of infection, it was not known if γδ T cells were responding specifically and directly to *M. bovis*, or they were reacting innately to the increased inflammatory signals associated with infection and lesion formation. Thus, a series of studies focused on the ability of γδ T cells to respond to *M. bovis* in vitro and on identifying the specific mycobacterial antigens triggering these responses. Smyth et al. provided some of the first evidence that γδ T cells respond directly to mycobacterial antigens alone. Gamma delta T cells from control and *M. bovis* infected animals upregulated CD25 and proliferated extensively when stimulated in mixed PBMC cultures with *M. bovis* sonic extract (MBSE) or *M. bovis* culture filtrate (CF) (Smyth et al., 2001). A similar result was observed when γδ T cells from infected animals, but not control animals, were purified and cultured with MBSE or CF, indicating that at least a portion of the γδ T cell response in infected animals was specific (Smyth et al., 2001). Interestingly, when compared to CD4 T cells, γδ T cells purified from *M. bovis* infected animals produced significantly reduced levels of IFNγ, suggesting they may have additional requirements for proliferation vs. cytokine production (Smyth et al., 2001). Rhodes et al. further refined our knowledge of mycobacterial γδ T cell antigens in the bovine, demonstrating that γδ T cells purified from *M. bovis* infected animals proliferated extensively in response to the complex mycobacterial antigen PPD, but also the protein antigens Ag85 and ESAT6. Gamma delta T cells in this study also exhibited minor responsiveness to the protein antigens MPB83, MPB70 and hsp16.1 (Rhodes et al., 2001). In contrast to the report by Smyth et al., Rhodes et al. observed that γδ T cells produced significant amounts of IFNγ in response to antigen stimulation. Interestingly, γδ T cells also demonstrated robust production of TGF-β in response to mycobacterial antigens, and their depletion from mixed PBMC cultures resulted in increased PBMC proliferation, suggesting γδ T cells may also play a role in modulating the responses of other lymphocyte subsets (Rhodes et al., 2001).

Gamma delta T cells from mice and humans respond to both protein and non-protein phosphoantigens of *M. tuberculosis*. Although the ligands are not as clearly defined, the same also appears true for bovine γδ T cells responding to *M. bovis*. Welsh et al. demonstrated that while proteinase-K treatment of MBSE ablates the ability of CD4 T cells to respond, γδ T cells retain their ability to proliferate and produce IFNγ (Welsh et al., 2002), thus confirming bovine γδ T cells also have the capacity to respond to both the protein and non-protein components of mycobacterial antigens. In Welsh's study, bovine γδ T cells also responded to IPP and monomethyl phosphate (Welsh et al., 2002), both potent phosphoantigens for human γδ T cells; however, we and others have not been able to confirm this result using bovine γδ T cells (Vesosky et al., 2004, and McGill et al., unpublished observations).

Although bovine γδ T cells respond specifically to *M. bovis* antigens, several studies have also reported non-specific responses by γδ T cells from healthy animals, particularly in response to crude and complex mycobacterial antigens such as PPD and MBSE. Vesosky et al. specifically examined the γδ T cell response from naïve

calves to mycobacterial antigens. In agreement with previous studies, γδ T cells from healthy animals proliferated and produced IFNγ following in vitro stimulation with complex antigens including live *M. bovis*, mycobacterial crude cell wall extract and mycobacterial CF (Vesosky et al., 2004). Further analysis of the γδ T cell response to *M. bovis* cell wall identified the primary antigenic component as mycolylaribinogalactan peptidoglycan (mAGP), which is both proteolytically-resistant and non-sodium dodecyl sulfate-soluble. Interestingly, although our laboratory has confirmed that γδ T cells from virulent *M. bovis* infected animals respond strongly to mAGP, we did not observe this response by γδ T cells from uninfected control animals (McGill et al., 2012b). At this time, the receptors mediating γδ T cell recognition of mycobacterial antigens, both specifically and non-specifically, remain unknown; however, bovine γδ T cells possess several receptors capable of recognizing bacterial ligands including pattern recognition receptors such as TLR-2 and -4 and NLR (Hedges et al., 2005; Kerns et al., 2009) – all of which are capable of activating bovine γδ T cells independent of additional TCR signals – as well as WC1 and the TCR itself. Future work should be aimed at a specific description of the mycobacterial ligands recognized by bovine γδ T cells and more clearly elucidating the mechanisms governing this recognition and responsiveness.

5.2. The role of γδ T cell cytotoxicity in controlling *M. bovis* infection

In addition to cytokine production and immune cell recruitment, γδ T cells are also potently cytotoxic and may contribute to the immune response against mycobacterium by directly killing the bacilli, killing infected cells or inhibiting bacterial growth. Cytotoxic γδ T cells accumulate in the lungs of BCG-infected mice as early as day 7 post infection and demonstrate robust killing of BCG-infected peritoneal macrophages (Dieli et al., 2003). Human Vγ9Vδ2 T cells express potent levels of the cytotoxic molecules granulysin and perforin—both known to affect the viability of intracellular and extracellular *M. tuberculosis* (Stenger et al., 1998). Vγ9Vδ2 T cells isolated from the blood of infected patients demonstrate direct granule-dependent cytotoxicity against macrophages infected with live *M.tuberculosis* (Dieli et al., 2000, 2001). Although it is likely that γδ T cell cytotoxicity is an important component of the anti-microbial immune response, increases in CTL activity, particularly that mediated by γδ T cells, has been negatively correlated with disease progression and tuberculosis severity (De La Barrera et al., 2003; Ordway et al., 2005), suggesting that increased cytotoxic γδ T cell responses may be involved in a compensatory, but ineffective, anti-mycobacterial response.

M. bovis infection results in an increase in cytotoxic T lymphocytes circulating in the blood as early as 4 weeks post infection (Skinner et al., 2003). Whereas the majority of circulating CTLs are $CD8^+$ T cells, cytotoxic $WC1^+$ γδ T cells also contribute significantly to the response (Skinner et al., 2003; Stenger et al., 1998). Like human γδ T cells, bovine γδ T cells express potent levels of the cytotoxic molecules perforin and granulysin (Alvarez et al., 2009; Blumerman et al., 2006; Endsley et al., 2004; Guzman et al., 2012). Cytotoxic $WC1^+$ γδ T cells from infected animals inhibit bacterial growth in macrophage cultures infected with *M. bovis*, and demonstrate robust specific lysis of *M. bovis*-infected target cells (Skinner et al., 2003). BCG vaccination also induces an increase in $WC1^+$ γδ T cells in the blood with the capacity to lyze autologous *M. bovis* infected monocytes (Olin et al., 2005). Given the early accumulation of γδ T cells at the site of *M. bovis* infection, it is plausible that γδ T cell cytotoxicity may help contain bacterial growth prior to the development of adaptive immune response; however, additional work is required to understand the contribution of γδ T cell cytotoxicity to the immune response in vivo during *M. bovis* infection.

6. Cross-talk between γδ T cells and dendritic cells (DC)

Following BCG vaccination, bovine γδ T cells infiltrate the lungs and lymphoid tissues of the head, where they co-localize with DC (Price et al., 2010), suggesting potential interactions between DC and activated γδ T cells. Indeed, reciprocal cross-talk between γδ T cells and DC has been clearly demonstrated in mice and humans in several experimental settings (Conti et al., 2005; Devilder et al., 2006; Fang et al., 2010; Ismaili et al., 2002; Leslie et al., 2002; Martino et al., 2005) and, more relevant here, in response to mycobacterium (Dieli et al., 2004; Martino et al., 2007; Meraviglia et al., 2010). *M. tuberculosis* has been reported to impair DC maturation, resulting in altered DC migration and antigen presentation (Dulphy et al., 2007; Martino et al., 2005), reduced IL-12 secretion and an inhibited ability to prime antigen-specific T cell responses (Wolf et al., 2007). However, incubation with human $V\gamma9V\delta2^+$ T cells is able to correct this defect and induce full maturation of immature *M. tuberculosis*- (Meraviglia et al., 2010) or BCG- (Martino et al., 2007) infected DC, including a significant enhancement in DC secretion of IL-12p70. In turn, a co-incubation with DC contributes to stronger and more sustained activation of the γδ T cells (Devilder et al., 2006), indicating an important role for these reciprocal interactions in the anti-mycobacterial immune response. A murine model demonstrated a similar functional relationship between $V\gamma1^+$ T cells and BCG-infected DC, with enhanced γδ T cell IFNγ production and increased DC IL-12 production (Dieli et al., 2004).

Recently, there is accumulating evidence that DC-γδ T cell cross-talk also occurs in cattle and may be critical to the induction of adaptive immune responses. Correlatively, γδ T cells produce significantly enhanced amounts of IFNγ when cultured in the presence of IL-12 and IL-18, cytokines that are produced primarily by antigen presenting cells (Price et al., 2007). More directly, Price and Hope demonstrated that in vitro co-incubation of $WC1^+$ γδ T cells with *M. bovis*-infected DC induced significantly enhanced expression of MHC II and CD25, as well as increased secretion of IFNγ by the γδ T cells (Price and Hope, 2009). In turn, DC produced enhanced levels of biologically active IL-12 when incubated with γδ T cells. Although soluble cytokines likely contributed to this reciprocal cross-talk, trans-well experiments revealed the interaction between DC and γδ T

cells was contact dependent (Price and Hope, 2009). As further evidence that DC- γδ T cell cross-talk may contribute to immune responses in vivo, WC1$^+$ γδ T cells express several receptors for inflammatory chemokines and migrate specifically towards *M. bovis* infected DC when cultured together in vitro (Guzman et al., 2012).

7. Th17 responses and γδ T cells during *M. bovis* infection

Recently, increasing attention has turned to understanding the role of IL-17 in immune pathology and protection during *Mycobacterium spp.* infections. IL-17 responses increase significantly in humans infected with *M. tuberculosis* (Cowan et al., 2012; Jurado et al., 2012), in mice challenged with BCG (Lockhart et al., 2006; Umemura et al., 2007) and in cattle infected with *M. bovis* (Aranday-Cortes et al., 2012; Vordermeier et al., 2009). Currently, however, the role of IL-17 in the immune response to mycobacterial infections remains unclear. Mouse studies suggest that IL-17 is critical for granuloma formation (Okamoto Yoshida et al., 2010); however, the absence of IL-17 has only minor effects on the anti-mycobacterial immune response (Khader et al., 2005; Umemura et al., 2007), and excessive IL-17 responses are associated with exacerbated inflammation and immunopathology (Basile et al., 2011; Cooper, 2009; Cruz et al., 2010). In mice and cattle, vaccination-induced IL-17 responses appear to only modestly correlate to a reduction in disease severity (Desel et al., 2011; Rizzi et al., 2012; Vordermeier et al., 2009).

In mice infected with TB, the primary IL-17 producing cells are γδ T cells rather than CD4 T cells (Lockhart et al., 2006; Umemura et al., 2007). Indeed, even in naïve animals, Sutton et al. reported that γδ T cells exposed to IL-1β and IL-23 exhibit several features of the Th17 profile including expression of the IL-23 receptor and the transcription factor RORγT, and production of IL-17, IL-21, and IL-22 (Sutton et al., 2009). Lockhart *et al.* reported that during murine BCG infection, γδ T cells are the principle producers of IL-17 in the lungs. In particular, γδ T cell IL-17 production precedes the initiation of adaptive immunity and is important for establishing early inflammatory events (Lockhart et al., 2006). Okamoto Yoshida et al. recently confirmed these findings, reporting that aerosol BCG infection induces an accumulation of IL-17 producing Vγ4 and Vγ6 γδ T cells in the lungs that are critical for appropriate granuloma formation (Okamoto Yoshida et al., 2010). Recent reports have further suggested that this early IL-17 production is necessary for driving an effective Th1 immune response and robust IFNγ production following BCG infection (Gopal et al., 2012).

Human Vγ9Vδ2 T cells can be induced to secrete IL-17 and IL-22 by culture with IL-1β, TGF- β and IL-23 (Ness-Schwickerath et al., 2010) and emerging evidence suggests a physiologic role for this phenotype during infection. Gamma delta T cells from *M. tuberculosis* infected humans produce IL-17 in response to stimulation with heat-treated mycobacterial antigens, and IL-17 producing γδ T cells are present at significantly increased proportions in patients with active pulmonary TB compared to healthy donors (Peng et al., 2008). IL-22 expression can be directly correlated with IL-17 expression, and in a study of non-human primates, IL-22 producing γδ T cells expanded in the blood and lymphoid tissue following *M. tuberculosis* infection (Yao et al., 2010). Importantly, IL-22 producing T cells were also abundant in tuberculous granulomas in the lungs.

In cattle, although *M. bovis* infection elicits an IL-17 response, the identity of the IL-17 producing populations remains unclear. Aranday-Cortez et al. recently demonstrated robust IL-17 and IL-22 expression within tuberculous lesions, with expression being most significant in early lesions and declining as the granuloma matured (Aranday-Cortes et al., 2012). This time course would correlate with the appearance of γδ T cells in early lesions as reported by some (Cassidy et al., 1998; Dieli et al., 2003; Palmer et al., 2007; Price et al., 2010); however, in their model, Aranday-Cortez et al. observed little γδ T cell accumulation in stage I/II granulomas. As bovine reagents for IL-17 and the associated cytokines becoming increasingly available, it will be important to more closely analyze the Th17 response by lymphocyte populations in infected cattle, and to further elucidate the role of IL-17 in the immune response to *M. bovis*.

8. Summary and conclusions

Recent studies have provided compelling evidence for the role of bovine γδ T cells in the immune response to *M. bovis*. Research in cattle can be limited by availability of reagents and the many challenges associated with studying outbred populations; however, workers in the field of bovine γδ T cell biology have made many exciting and impressive advancements in our understanding of γδ T cells and their role in the immune system of ruminants, particularly in response to *M. bovis* infection. Through this research, it has become clear that γδ T cells proliferate and produce cytokines in vitro in specific and direct response to complex, protein and non-protein antigens of *M. bovis*. In vivo, γδ T cells undergo robust activation and expansion following BCG vaccination or virulent *M. bovis* infection, and rapidly home to sites of infection. Therein, they likely produce chemokines to mediate immune cell recruitment and granuloma formation, and produce Th1 cytokines to promote the development of an effective adaptive immune response.

As our knowledge of γδ T cells responses to *M. bovis* continues to grow, a number of opportunities for study remain. Many of the *M. bovis* antigens, both protein and non-protein, and the specific receptors mediating their recognition are unclear. γδ T cells recognize ligands through PRR, WC1 and the TCR itself, but our knowledge of how these individual signals are integrated into the γδ response as a whole is incomplete.

Many exciting questions also remain concerning the role for γδ T cells in the *M. bovis* response in vivo. Although γδ T cells home to sites of lesion formation, a detailed profile of the receptors mediating this infiltration, and the chemokines and cytokines they produce upon arrival are lacking. In addition to their classical role in IFNγ production, γδ T cells have been suggested to produce IL-17, process and present antigens and kill *M. bovis* infected

macrophages; however, the physiologic relevance of these functions remains to be proven. Finally, there is emerging evidence that bovine γδ T cells interact with DC to enhance activation and cytokine production by both cell types. Currently, however, it remains unknown if these interactions occur in vivo and how they may shape the developing *M. bovis*-specific immune response. The tools and reagents available for addressing these in-depth questions in cattle, as well as other species, continue to improve and hold great promise for our future understanding of the immune response to *M. bovis*.

In conclusion, although we continue to rapidly expand our knowledge of γδ T cells and their response to *M. bovis* infection in cattle, additional research is required in order to fully delineate the functions of this unique cell type in innate and adaptive immunity. It is imperative that we pursue this understanding in order to effectively harness the γδ T cell response through novel treatment and vaccination strategies.

Conflict of interest

The authors declare no conflict of interest.

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