1 Multinucleated giant cell cytokine expression in pulmonary granulomas of cattle experimentally
2 infected with *Mycobacterium bovis*.

3
4 Mitchell V. Palmer ^{a,*}, Tyler C. Thacker ^a, W. Ray Waters ^a

5
6 ^a Infectious Bacterial Diseases of Livestock Unit, National Animal Disease Center, Agricultural
7 Research Service, United States Department of Agriculture, Ames, IA, United States

8
9
10
11
12
13
14
15
16 *Corresponding author:

17 National Animal Disease Center

18 1920 Dayton Avenue

19 Ames, IA 50010

20 Tele: +1-515-337-7474

21 Fax: +1-515-337-7458

22 Email: mitchell.palmer@ars.usda.gov

Abstract

Regardless of host, pathogenic mycobacteria of the *Mycobacterium tuberculosis* complex such as *Mycobacterium bovis*, induce a characteristic lesion known as a *granuloma*, *tubercle* or *tuberculoid granuloma*. Granulomas represent a distinct host response to chronic antigenic stimuli, such as foreign bodies, certain bacterial components, or persistent pathogens such as *M. bovis*. Granulomas are composed of specific cell types including epithelioid macrophages, lymphocytes and a morphologically distinctive cell type, the multinucleated giant cell. Multinucleated giant cells are formed by the fusion of multiple macrophages; however, their function remains unclear. In humans, giant cells in tuberculous granulomas have been shown to express various cytokines, chemokines and enzymes important to the formation and maintenance of the granuloma. The objective of this study was to quantitatively assess multinucleated giant cell cytokine expression in bovine tuberculoid granulomas; focusing on cytokines of suspected relevance to bovine tuberculosis. Using calves experimentally infected with *M. bovis*, *in situ* cytokine expression was quantitatively assessed using RNAScope® for the following cytokines TNF- α , IFN- γ , TGF- β , IL-17A and IL-10. Multinucleated giant cells in bovine tuberculoid granulomas expressed all examined cytokines to varying degrees, with differential expression of TGF- β , IL-17A and IL-10 in giant cells from early versus late stage granulomas. There was a modest, positive correlation between the level of cytokine expression and cell size or number of nuclei. These results suggest that multinucleated giant cells are active participants within bovine tuberculoid granulomas, contributing to the cytokine milieu necessary to form and maintain granulomas.

Keywords: cytokine; giant cell; granuloma; *Mycobacterium bovis*; RNAScope®

1. Introduction

Bacteria of the genus *Mycobacterium* are Gram-positive, acid-fast organisms that include several major human and animal pathogens (Sizemore et al., 2015). Although human tuberculosis is generally caused by *Mycobacterium tuberculosis*, indistinguishable disease can be caused by the zoonotic agent *Mycobacterium bovis* (Langer and LoBue, 2014). The range of susceptible hosts to *M. bovis* is extremely broad and includes humans, domestic and wild ruminants, swine and carnivores. The hallmark lesion of tuberculosis, regardless of host or tissue, is the *granuloma*. Granulomas are the host response to contain or eliminate the inciting agent, and can be seen with various bacterial, fungal and protozoal diseases, as well as several diseases of unknown etiology (Kumar et al., 2013). When a granuloma is the result of infection with mycobacteria belonging to the *M. tuberculosis* complex (i.e., *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. caprae*, *M. canetti*, *M. pinnipedii*, *M. mungi*) (Rodriguez-Campos et al., 2014) it may also be referred to as a *tubercle* or *tuberculoid granuloma* (Myers et al., 2012).

The granuloma is a distinctive morphological lesion associated with chronic inflammation, in which the predominant cell type is a modified epithelial-like (epithelioid) macrophage. Other cell types commonly found within granulomas are lymphocytes and multinucleated giant cells (MGCs) (Ackermann, 2012). Multinucleated giant cells are formed through fusion of multiple macrophages (Cheville, 1999) and can be seen in granulomas induced by mycobacterial and non-mycobacterial stimuli, including foreign bodies. These morphologically distinct cells may be classified by the arrangement of nuclei. Multiple nuclei centrally aggregated or distributed haphazardly, are known as foreign body type MGCs. Nuclei may also be arranged in an arcuate or semicircular arrangement at the periphery of the cytoplasm, as is the case in Langhans type MGCs (Okamoto et al., 2003). It is not known if there are

functional differences between the two types of MGCs. Other multinucleated cells, distinct from those found in granulomas, are found in both physiological (e.g., osteoclasts) and pathological (e.g., viral-induced syncytia) conditions (Sacco et al., 2012; Vignery, 2008).

The process of MGC formation is poorly understood; however, several factors appear necessary. Development of MGCs requires chronic antigenic stimulation, such as that generated by foreign bodies, poorly digestible pathogen factors (e.g. muramyl dipeptide, liporabinomannan), or persistent pathogens such as mycobacteria (Ackermann, 2012; Okamoto et al., 2003). This requirement is illustrated by the fact that MGC formation is triggered by virulent, but not avirulent mycobacteria (Lay et al., 2007), likely due to the fact that virulent and avirulent mycobacteria differ in cell wall components and secreted products (Torrelles et al., 2011). Also requisite is the presence of IFN- γ , critical in driving MGC formation (Zhu and Friedland, 2006).

The function of MGCs in tuberculoid granulomas is also poorly understood; however their absence may signal an ineffective immune response such as that seen with the lepromatous (i.e., multibacillary) form of leprosy where MGCs are uncommon (Kaplan et al., 1983). *In vitro* studies of granulomas from human tuberculosis patients have demonstrated that MGCs are capable of producing various cytokines (e.g., IL-10, IFN- γ , TNF- α , TGF- β) chemokines (e.g., CXCL10, CCL2) and enzymes (e.g., matrix metalloproteinases) (Mustafa et al., 2006; Zhu and Friedland, 2006). Compared to epithelioid macrophages, which share a common origin with MGCs, giant cells express more anti-inflammatory cytokines (i.e. TGF- β) and have higher antigen load and reduced mycobactericidal abilities (Mustafa et al., 2008). *In vitro* studies with human cells show MGCs lose the ability to mediate bacterial uptake, but retain antigen presentation capabilities (Lay et al., 2007). Little is known of the cytokine expression of MGCs

in bovine granulomas induced by *M. bovis* infection. The objective of this study is to document and quantify the *in situ* gene expression of various cytokines of known or implied relevance to bovine tuberculosis by these morphologically unique cells.

2. Materials and Methods

2.1. Mycobacterium bovis aerosol challenge

Inoculum preparation and details of experimental aerosol infection of calves with virulent *M. bovis* have been described in detail previously (Palmer et al., 2015) (Waters et al., 2014). The calf aerosol model results in lesions similar to naturally infected cattle in both character and distribution, including the appearance of MGCs (Palmer et al., 2002). Briefly, Holstein calves (9 months of age, castrated males) were infected with virulent *M. bovis* by nebulization of inoculum into a mask (Equine AeroMask®, Trudell Medical International, London, ON, Canada) covering the nostrils and mouth. Calves received either 10⁴ colony-forming units (cfu) of *M. bovis* 95-1315 (n = 8) or 10⁴ cfu *M. bovis* 10-7428 (n = 8) by aerosol as described (Palmer et al., 2002). Both are field strains of *M. bovis* and have been shown to be equally virulent in the calf aerosol model (Waters et al., 2014). All experimental procedures involving animals were performed according to NADC Institutional Biosafety and Animal Care and Use Committee guidelines.

2.2. Sample collection and lesion staging

As per the calf aerosol model (Palmer et al., 2002), all calves were euthanized 150 days after challenge by intravenous administration of sodium pentobarbital. Tissues were examined for gross lesions and processed for microscopic analysis and isolation of *M. bovis* by bacteriological culture as described (Waters et al., 2014). The lesion severity findings and

bacteriological culture results for all tissues examined are reported in detail elsewhere (Waters et al., 2014). In brief, aerosol inoculations with either isolate of *M. bovis* resulted in indistinguishable levels of *M. bovis* colonization, lesion severity, lesion distribution and immune response (Waters et al., 2014). As the results were indistinguishable between isolates, all inoculated calves were considered as a single group for this analysis. Although lesions were consistently present in the tracheobronchial and mediastinal lymph nodes, the *in situ* cytokine expression reported herein, was limited to lung, where lesions were present in one or more lobes in all inoculated calves. Samples were fixed by immersion in 10% neutral buffered formalin and processed by standard paraffin-embedment techniques, cut in 5 µm sections and stained with hematoxylin and eosin (HE). Adjacent sections from samples containing caseonecrotic granulomas suggestive of tuberculosis were stained by the Ziehl-Neelsen technique for visualization of acid-fast bacteria (AFB). Numerous adjacent unstained sections were used for *in situ* cytokine expression analysis.

Not every tissue section processed for microscopic analysis contained lesions; however, one to three slides with tuberculoid granulomas were analyzed from each of the 16 animals. For each slide, all granulomas were staged (stages I-IV) according to criteria adapted from that described previously (Palmer et al., 2007; Rhoades et al., 1997; Wangoo et al., 2005). Briefly, these stages are defined as follows: initial (stage I), solid (stage II), necrotic (stage III) and necrotic and mineralized (stage IV). For each cytokine investigated a minimum of 50 granulomas were examined and all MGCs present were assessed for cytokine gene expression.

2.3 RNA chromogenic in situ hybridization (ISH)

Visualization of mRNA transcripts for the following cytokines; TNF- α , TGF- β , IFN- γ , IL-17A, IL-10 and mycobacterial 23s ribosomal RNA was done according to manufacturer's instructions for RNAScope® 2.0 (Advanced Cell Diagnostics, Hayward, CA, USA)(Tubbs et al., 2013; Wang et al., 2012). *Bos taurus*-specific proprietary probe combinations were used for each of the cytokines described. RNAScope® has been shown to be capable of single mRNA molecule detection (Wang et al., 2012). Briefly, sections 5- μ m thick, cut from formalin-fixed, paraffin embedded tissues were heated for 60 min at 60⁰ C in a HybEZ™ hybridization oven (Advanced Cell Diagnostics). Tissues were deparaffinized in xylene followed by rehydration in an ethanol series and air-dried for 5 min. Tissue sections were incubated with pretreatment 1 solution (endogenous peroxidase block) for 10 min at room temperature (RT). Slides were rinsed by immersion in double distilled water (ddH₂O), followed by immersion in pretreatment 2 (antigen retrieval citrate buffer) for 15 min at 100-104⁰ C (boiling). Slides were washed in ddH₂O and pretreatment 3 (protease) applied for 30 min at 40⁰ C. Slides were washed in ddH₂O and target or control probes applied with incubation at 40⁰ C for 2 hours followed by a rinse in wash buffer (Advanced Cell Diagnostics) for 2 min at RT. Signal amplification reagents 1 through 6 were serially applied for 30 min, 15 min 30 min, 15 min, 30 min and 15 min, respectively. Slides were rinsed in wash buffer for 2 min between amplification reagents. Incubations with amplifier reagents 1 through 4 were done at 40⁰ C, while incubations with amplifier reagents 5 and 6 were done at RT. Positive signal was visualized using Fast Red chromogenic substrate with a Gill's hematoxylin counterstain. Slides were then dehydrated through an ethanol series to xylene. After drying 15 min at 60⁰ C, slides were coverslipped using mounting media (EcoMount, Biocare Medical, Concord, CA, USA). The positive control probe

consisted of a proprietary probe for *Bos taurus cyclophilin B* (PPIB), while the negative control probe targeted *dapB* of *Bacillus subtilis*.

2.4 Morphometry

Images of all MGCs present on all slides were captured with a SPOT™ RT CCD digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and light microscope (Nikon Eclipse E800: Nikon Instruments, Inc., Tokyo, Japan). For all MGCs, mRNA transcripts (dots) and number of nuclei were enumerated and cell size (μm^2) calculated using Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA).

2.5 Statistical Analysis

Mean values of signal (dots)/MGC were analyzed using one-way analysis of variance (GraphPad Prism 6.0, GraphPad Software, San Diego, CA, USA). Differences between means were compared using Tukey's multiple comparison test. Pearson correlation coefficients (r) were calculated for gene expression to cell size and number of nuclei. Student's t-test was used to compare gene expression between early and late stage granulomas. Effects with a p -value < 0.05 were considered significant.

3. Results

The majority of granulomas were categorized as either stages I or IV. Few stage III granulomas and even fewer numbers of stage II granulomas were identified. For analysis purposes, stage II granulomas were not included and stages III and IV were combined, resulting in an analysis of MGCs found in early stage (I) or late stage (III and IV combined) granulomas,

as done previously (Palmer et al., 2015). A total of 879 MGCs were analyzed; 235 of which were found in early stage granulomas, while 644 were found in late stage granulomas. Of 879 MGCs, 5 were of the foreign body type, while 874 were of the Langhans type. Due to the low number of foreign body type cells they were excluded from the analysis. The mean number of nuclei was 13 ± 0.3 (SEM) and the average size was 9274 ± 183 (SEM) μm^2 .

Labeled mRNA transcripts were represented as punctate dots of Fast Red chromogenic substrate. The size of a single dot varied from approximately 0.2 to 1.5 μm . The number of dots within a single MGC varied from a single dot (Fig 1) to >100 dots (Fig 2). Each dot represented a single transcript (Wang et al., 2012) allowing quantitative analysis.

For analysis of each cytokine, adjacent 5 μm sections were used such that only a single cytokine was analyzed on each slide. Adjacent sections cut from the paraffin block represented spatially different and distinct parts of the same sample, with each slide differing in the numbers of granulomas and MGCs. As a consequence, the number of MGCs evaluated for each cytokine was different; nevertheless, all MGCs on all slides were analyzed. The number of MGCs demonstrating cytokine mRNA labeling/number of MGCs examined were as follows: TNF- α , 85/86 (99%); IFN- γ , 180/198 (91%); TGF- β , 184/184 (100%); IL-10, 181/191 (95%); IL-17A, 150/156 (96%); mycobacterial 23s rRNA, 55/64(84%).

Gene expression, to varying degrees, was detected for all cytokines examined (Fig 3). Compared to IFN- γ and IL-10, other cytokines (TNF- α , IL-17A and TGF- β) showed significantly increased levels of expression ($p < 0.05$), with significantly greater TGF- β expression than all other cytokines ($p < 0.05$). Both IL-17A and IL-10 were expressed to a significantly greater degree ($p < 0.05$) in MGCs found in early stage granulomas than those of late stage granulomas, while TGF- β was expressed at higher levels ($p < 0.05$) in MGCs found in

late stage granulomas (Fig 4). Differences in expression of TNF- α and IFN- γ between early and last stage granulomas were not significant.

In vitro studies with human cells suggest that functional classes of MGCs may exist and differentiation of classes may be made by the number of nuclei present (Lay et al., 2007). In the present study, correlation analysis revealed moderate, but significant, positive correlations between MGC cell size or nuclear number with gene expression of all cytokines examined (Table 1). Further examination revealed that when granulomas were characterized as either early or late stage, similar positive correlations were observed, with a few exceptions. A weaker, non-significant positive correlation was seen between nuclear number and IFN- γ gene expression in early stage granulomas (Table 1), while the correlation of TNF- α expression and cell size or nuclear number was stronger in early stage granulomas compared to late stage granulomas (Table 1). To further examine effect of cell size on cytokine expression, the density (cytokine expression/ μm^2) was calculated for each cytokine. Cytokine density results (not shown) mirrored cytokine/cell results and did not reveal additional differences.

The number of AFB in bovine tuberculoid granulomas is generally low; with only a portion of that number found within MGCs (Aranday-Cortes et al., 2012). Indeed, it is common to see bovine tuberculoid granulomas with few or no AFB, as in this study. When present in early stage granulomas, AFB were seen within macrophages or MGCs, in contrast to late stage granulomas where AFB were seen almost exclusively in the necrotic caseum. Due to the paucity of AFB in this study and the fact that visualization through acid-fast staining techniques only reveals metabolically active bacilli with an intact cell wall (Chandrasekhar and Ratnam, 1992); we used an RNAScope® probe to mycobacterial 23s rRNA to visualize bacillary gene expression within MGCs as a surrogate means of quantifying bacterial burden (Fig 5). No

significant differences were noted in mycobacterial rRNA probe labeling between early and late stage granulomas. Likewise, no correlations between mycobacterial rRNA probe labeling and MGC size or nuclear number was seen (Table 1).

4. Discussion

The present study quantitatively documents *in situ* expression of various cytokines by MGCs found within pulmonary granulomas of cattle experimentally infected with *M. bovis*. To date there has been little information on the precise function of these morphologically distinct cells that are a common feature of bovine tuberculoid granulomas of all stages (Wangoo et al., 2005). Some insight is gained from previous studies using immunohistochemistry (IHC), which demonstrated the presence of TGF- β (Aranday-Cortes et al., 2012; Wangoo et al., 2005) and TNF- α (Aranday-Cortes et al., 2012) in MGCs found in lymph node granulomas of various stages from cattle experimentally infected with *M. bovis*. Additionally, inducible nitric oxide synthase (iNOS) and the macrophage lysosomal glycoprotein, CD68, have also been demonstrated in MGCs associated with granulomas of both bovine tuberculosis and paratuberculosis (Ackermann et al., 1994; Aranday-Cortes et al., 2012; Palmer et al., 2007; Wangoo et al., 2005).

With human cells, *in vitro* studies reveal that TNF- α and IFN- γ are responsible for granuloma formation and enhance the bactericidal activity of both monocytes and macrophages; moreover, IFN- γ may be essential to drive MGC formation (Zhu and Friedland, 2006). These results are in keeping with the present study documenting both IFN- γ and TNF- α expression in MGCs found in both early and late stage granulomas.

In lymph node biopsies of human tuberculosis patients, a greater number of MGCs express IL-10 and TGF- β than express TNF- γ or IFN- γ , with some MGCs expressing multiple cytokines (Mustafa et al., 2006). In the current study, dual or triple RNAScope® labeling was not done; therefore, demonstration of multiple cytokine expression by a single MGC was not possible. Nevertheless, in the present study, expression of IL-10, TGF- β , TNF- α , IFN- γ and IL-17A by MGCs was documented. Moreover, differential expression by MGCs found in early vs late stage granulomas was observed for IL-10, TGF- β and IL-17A.

IL-17A has been shown to play a role in the immunopathology of tuberculosis and other chronic diseases (Reynolds et al., 2010). IL-17A is produced by T-cells, a subset of which, are known as Th17 cells; however, its production by MGCs has not been reported previously. Using qPCR, IL-17A has been detected in bovine tuberculoid lymph node and pulmonary granulomas (Aranday-Cortes et al., 2012; Shu et al., 2014) but the source of IL-17A was not determined. Similar to findings in the present study, decreased expression was noted in late stage granulomas compared to early stage granulomas (Aranday-Cortes et al., 2012). The function of IL-17A in bovine tuberculosis is unclear; however, in peripheral blood cells of cattle experimentally infected with *M. bovis*, antigen specific *in vitro* expression of IL-17A has been correlated to both increased disease severity (Blanco et al., 2011) and vaccine induced protection (Vordermeier et al., 2009; Waters et al., 2015).

IL-10 is an important anti-inflammatory cytokine controlling innate and adaptive immune responses to tuberculous mycobacteria (Etna et al., 2014). The principal function of IL-10 is to deactivate macrophages, resulting in diminished Th1 cytokine production, as well as decreased production of reactive nitrogen and oxygen species (Beamer et al., 2008). An important source of IL-10 is the specialized T cell, known as a T-regulatory (Treg) cell (Belkaid, 2007). Treg cells

and their products are important in modulating immune responses, striving to maintain a balance between effective pathogen clearance and host tissue damage. In the present study, expression of IL-10 by MGCs in early stage granulomas may suggest that MGCs are an early source of IL-10; regulating granulomatous responses until Treg cells infiltrate into the developing granuloma.

Among other functions, TGF- β is a potent stimulator of collagen production. Late stage bovine tuberculoid granulomas are often surrounded by a substantial fibrous capsule composed of collagen fibers and extracellular matrix. Not surprisingly, the number of TGF- β positive cells has been shown to be more numerous in late stage granulomas (Wangoo et al., 2005). The present finding of greater TGF- β expression in late stage granulomas suggests that MGCs may be one of the sources of TGF- β responsible for fibrous capsule formation. TGF- β may also play an anti-inflammatory role, downregulating a Th1 immune response. The presence of higher levels of TGF- β expression in MGCs of late stage granulomas is consistent with the paradigm of an early Th1 response that modulates to a Th2 dominated response as tuberculosis progresses (Sander et al., 1995; Rhodes and Graham, 2002). It must be remembered that TGF- β is synthesized as a precursor protein and must be modified before secretion as an active protein (Khalil, 1999). Thus analysis of TGF- β mRNA may not necessarily reflect the levels of biologically active TGF- β .

An *in vitro* model of human granulomas suggests that MGCs with <7 nuclei behave differently and have different capabilities than MGCs with >15 nuclei (Lay et al., 2007). Exploring the possibility that MGC size or nuclear number may also influence function, the present study showed a moderate positive correlation between cell size and nuclear number with gene expression for all cytokines. The significance of this finding remains unclear.

In conclusion, the present study documents *in situ* expression of multiple cytokines by MGCs found within pulmonary granulomas of cattle experimentally infected with *M. bovis*. Indeed, 96% of the MGCs seen expressed one of the cytokines examined, suggesting that MGCs are not only morphologically distinct cells, but also active participants within granulomas, contributing to the cytokine milieu necessary to form and maintain granulomas.

Acknowledgments

The authors thank A. Lasley, S. Zimmerman, H. Trenkamp and the animal care staff at NADC for excellent technical support and animal care. USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this manuscript is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. Research funds were provided by the US Department of Agriculture, Agricultural Research Service Project (CRIS# 3625-32000-104).

Figure Legends

Fig. 1. Photomicrograph of stage IV pulmonary granuloma of calf experimentally infected with *M. bovis*. Note MGC with labeling of single mRNA transcript for bovine IL-10 (arrow). RNAScope® *in situ* hybridization method (ISH) (X400).

Fig. 2. Photomicrograph of stage IV pulmonary granuloma of calf experimentally infected with *M. bovis*. Note MGC (asterisk) with labeling of numerous mRNA transcripts for bovine TGF- β . RNAScope® ISH method (X400).

Fig. 3. Quantitative assessment of cytokine gene expression of MGCs using the RNAScope® ISH method. Values represent mean $\pm$ SEM mRNA transcripts/MGC in pulmonary granulomas of all stages (early and late) from calves experimentally infected with *M. bovis*. * Significantly greater ($p < 0.05$) than values measured for IFN- γ and IL-10. ** Significantly greater ($p < 0.05$) than values measured for all other cytokines examined.

Fig. 4. Quantitative assessment of cytokine gene expression of MGCs as measured with RNAScope® ISH method. Values represent mean $\pm$ SEM mRNA transcripts/MGC in pulmonary granulomas of either early (stage I) or late stages (stages III and IV) from calves experimentally infected with *M. bovis*, * $p < 0.05$.

340 Fig. 5. Photomicrograph of stage IV pulmonary granuloma of calf experimentally infected with
341 *M. bovis*. Note MGC (asterisk) with labeling of numerous mRNA transcripts for mycobacterial
342 23s rRNA. RNAScope® ISH method (X400).
343
344

Table 1. Pearson correlation coefficients (r) for cell size (μm^2) or number (#) of nuclei/MGC with gene expression of individual cytokines. Values in bold, $p < 0.05$.

	# Nuclei	TNF- α	IFN- γ	TGF- β	IL-17A	IL-10	23s rRNA
All granulomas							
Cell size	0.536	0.532	0.427	0.662	0.635	0.547	-0.006
# Nuclei		0.440	0.249	0.402	0.420	0.426	0.003
Early stage granulomas							
Cell size	0.556	0.645	0.370	0.695	0.628	0.602	0.249
# Nuclei		0.510	0.194	0.494	0.270	0.376	0.242
Late stage granulomas							
Cell size	0.496	0.333	0.457	0.727	0.654	0.425	-0.005
# Nuclei		0.285	0.300	0.424	0.424	0.353	0.014

References

- Ackermann, M., 2012. Inflammation and Healing. In: Zachary, J.F., McGavin, M.D. (Eds.) Pathologic Basis of Veterinary Disease. Elsevier, St. Louis, MO, pp. 119-131.
- Ackermann, M.R., DeBey, B.M., Stabel, T.J., Gold, J.H., Register, K.B., Meehan, J.T., 1994. Distribution of anti-CD68 (EBM11) immunoreactivity in formalin-fixed, paraffin-embedded bovine tissues. Vet. Pathol. 31, 340-348.
- Aranday-Cortes, E., Bull, N.C., Villarreal-Ramos, B., Gough, J., Hicks, D., Ortiz-Pelaez, A., Vordermeier, H.M., Salguero, F.J., 2012. Upregulation of IL-17A, CXCL9 and CXCL10 in early-stage granulomas induced by *Mycobacterium bovis* in cattle. Transbound. Emerg. Dis. 60, 525-537.
- Beamer, G.L., Flaherty, D.K., Assogba, B.D., Stromberg, P., Gonzalez-Juarrero, M., de Waal Malefyt, R., Vesosky, B., Turner, J., 2008. Interleukin-10 promotes *Mycobacterium tuberculosis* disease progression in CBA/J mice. J. Immunol. 181, 5545-5550.
- Belkaid, Y., 2007. Regulatory T cells and infection: a dangerous necessity. Nat. Rev. Immunol. 7, 875-888.
- Blanco, F.C., Bianco, M.V., Meikle, V., Garbaccio, S., Vagnoni, L., Forrellad, M., Klepp, L.I., Cataldi, A.A., Bigi, F., 2011. Increased IL-17 expression is associated with pathology in a bovine model of tuberculosis. Tuberculosis (Edinb) 91, 57-63.
- Chandrasekhar, S., Ratnam, S., 1992. Studies on cell-wall deficient non-acid fast variants of *Mycobacterium tuberculosis*. Tuber. Lung Dis. 73, 273-279.
- Cheville, N.F., 1999. Healing and Chronic Inflammation, 2nd Edition. Iowa State University Press, Ames, Iowa, 135-152 pp.

377 Etna, M.P., Giacomini, E., Severa, M., Coccia, E.M., 2014. Pro- and anti-inflammatory
378 cytokines in TB: A two-edged sword in TB pathogenesis. *Semin. Immunol.* 26, 543-551.

379 Kaplan, G., Van Voorhis, W.C., Sarno, E.N., Nogueira, N., Cohn, Z.A., 1983. The cutaneous
380 infiltrates of leprosy. A transmission electron microscopy study. *J. Exp. Med.* 158, 1145-
381 1159.

382 Khalil, N., 1999. TGF-beta: from latent to active. *Microb. and Infect.* 1, 1255-1263.

383 Kumar, S.N., Prasad, T.S., Narayan, P.A., Muruganandhan, J., 2013. Granuloma with Langhans
384 giant cells: An overview. *J. Oral Maxillofac. Pathol.* 17, 420-423.

385 Langer, A., LoBue, P.A., 2014. Public health significance of zoonotic tuberculosis in animals
386 and humans. In: Thoen, C.O., Steele, J.H., Kaneene, J.B. (Eds.) *Zoonotic Tuberculosis:
387 Mycobacterium bovis and other Pathogenic Mycobacteria*. Wiley Blackwell, Ames, Iowa,
388 pp. 21-34.

389 Lay, G., Poquet, Y., Salek-Peyron, P., Puissegur, M.P., Botanch, C., Bon, H., Levillain, F.,
390 Duteyrat, J.L., Emile, J.F., Altare, F., 2007. Langhans giant cells from *M. tuberculosis*-
391 induced human granulomas cannot mediate mycobacterial uptake. *J. Pathol.* 211, 76-85.

392 Mustafa, T., Mogga, S.J., Mfinanga, S.G., Morkve, O., Sviland, L., 2006. Immunohistochemical
393 analysis of cytokines and apoptosis in tuberculous lymphadenitis. *Immunology* 117, 454-
394 462.

395 Mustafa, T., Wiker, H.G., Morkve, O., Sviland, L., 2008. Differential expression of
396 mycobacterial antigen MPT64, apoptosis and inflammatory markers in multinucleated
397 giant cells and epithelioid cells in granulomas caused by *Mycobacterium tuberculosis*.
398 *Virchows Arch.* 452, 449-456.

399 Myers, R.K., McGavin, M.D., Zachary, J.F., 2012. Cellular adaptations, injury, and death:
 400 Morphologic, biochemical, and genetic bases. In: Zachary, J., F., McGavin, M.D. (Eds.)
 401 Pathologic Basis of Veterinary Disease. Elsevier Mosby, St. Louis, MO, pp. 122-124.
 402 Okamoto, H., Mizuno, K., Horio, T., 2003. Langhans-type and foreign-body-type multinucleated
 403 giant cells in cutaneous lesions of sarcoidosis. Acta. Derm. Venereol. 83, 171-174.
 404 Palmer, M.V., Thacker, T.C., Waters, W.R., 2015. Analysis of cytokine gene expression using a
 405 novel chromogenic in-situ hybridization method in pulmonary granulomas of cattle
 406 infected experimentally by aerosolized *Mycobacterium bovis*. J. Comp. Pathol. 153, 150-
 407 159.
 408 Palmer, M.V., Waters, W.R., Thacker, T.C., 2007. Lesion development and
 409 immunohistochemical changes in granulomas from cattle experimentally infected with
 410 *Mycobacterium bovis*. Vet. Pathol. 44, 863-874.
 411 Palmer, M.V., Waters, W.R., Whipple, D.L., 2002. Aerosol delivery of virulent *Mycobacterium*
 412 *bovis* to cattle. Tuberculosis (Edinb) 82, 275-282.
 413 Reynolds, J.M., Angkasekwinai, P., Dong, C., 2010. IL-17 family member cytokines: regulation
 414 and function in innate immunity. Cytokine Growth Factor Rev. 21, 413-423.
 415 Rhoades, E.R., Frank, A.A., Orme, I.M., 1997. Progression of chronic pulmonary tuberculosis in
 416 mice aerogenically infected with virulent *Mycobacterium tuberculosis*. Tuber. Lung Dis.
 417 78, 57-66.
 418 Rhodes, S.G., Graham, S.P., 2002. Is timing important for cytokine polarization?. Trends in
 419 Immunol. 23, 246-249.

420 Rodriguez-Campos, S., Smith, N.H., Boniotti, M.B., Aranaz, A., 2014. Overview and phylogeny
 421 of *Mycobacterium tuberculosis* complex organisms: Implications for diagnostics and
 422 legislation of bovine tuberculosis. Res. Vet. Sci. 97, S5-S19.

423 Sacco, R.E., McGill, J.L., Palmer, M.V., Lippolis, J.D., Reinhardt, T.A., Nonnecke, B.J., 2012.
 424 Neonatal calf infection with respiratory syncytial virus: drawing parallels to the disease in
 425 human infants. Viruses 4, 3731-3753.

426 Sander, B., Skansen-Saphir, U., Damm, O., Hakansson, L., Andersson, J., 1995. Sequential
 427 production of Th1 and Th2 cytokines in response to live bacillus Calmette-Guerin.
 428 Immunology 86, 512-518.

429 Shu, D., Heiser, A., Wedlock, D.N., Luo, D., de Lisle, G.W., Buddle, B.M., 2014. Comparison
 430 of gene expression of immune mediators in lung and pulmonary lymph node granulomas
 431 from cattle experimentally infected with *Mycobacterium bovis*. Vet. Immunol.
 432 Immunopathol. 160, 81-89.

433 Sizemore, C., Lacourciere, K., Parker, T., 2015. The Many Hosts of Mycobacteria: An
 434 Interdisciplinary Approach to Understanding Mycobacterial Diseases. In: Mukundan, H.,
 435 Chambers, M.A., Waters, R.W., Larsen, M.H. (Eds.) Tuberculosis, Leprosy and
 436 Mycobacterial Diseases of Man and Animals. CABI, Boston, MA, pp. xv-xvii.

437 Torrelles, J.B., Sieling, P.A., Arcos, J., Knaup, R., Bartling, C., Rajaram, M.V., Stenger, S.,
 438 Modlin, R.L., Schlesinger, L.S., 2011. Structural differences in lipomannans from
 439 pathogenic and nonpathogenic mycobacteria that impact CD1b-restricted T cell responses.
 440 J. Biol. Chem. 286, 35438-35446.

441 Tubbs, R.R., Wang, H., Wang, Z., Minca, E.C., Portier, B.P., Gruver, A.M., Lanigan, C., Luo, Y.,
 442 Cook, J.R., Ma, X.J., 2013. Ultrasensitive RNA in situ hybridization for detection of

restricted clonal expression of low-abundance immunoglobulin light chain mRNA in B-cell lymphoproliferative disorders. *Am. J. Clin. Pathol.* 140, 736-746.

Vignery, A., 2008. Macrophage Fusion: Molecular Mechanisms. In: Chen, E.H. (Ed.) *Cell Fusion: Overviews and Methods*. Humana Press, Totowa, New Jersey, pp. 149-164.

Vordermeier, H.M., Villarreal-Ramos, B., Cockle, P.J., McAulay, M., Rhodes, S.G., Thacker, T., Gilbert, S.C., McShane, H., Hill, A.V., Xing, Z., Hewinson, R.G., 2009. Viral booster vaccines improve *Mycobacterium bovis* BCG-induced protection against bovine tuberculosis. *Infect. Immun.* 77, 3364-3373.

Wang, F., Flanagan, J., Su, N., Wang, L.C., Bui, S., Nielson, A., Wu, X., Vo, H.T., Ma, X.J., Luo, Y., 2012. RNAscope[®]: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *J. Mol. Diag.* 14, 22-29.

Wangoo, A., Johnson, L., Gough, J., Ackbar, R., Inglut, S., Hicks, D., Spencer, Y., Hewinson, G., Vordermeier, M., 2005. Advanced granulomatous lesions in *Mycobacterium bovis*-infected cattle are associated with increased expression of type I procollagen, gamma delta (WC1+) T cells and CD 68+ cells. *J. Comp. Pathol.* 133, 223-234.

Waters, W.R., Maggioli, M.F., Palmer, M.V., Thacker, T.C., McGill, J.L., Vordermeier, H.M., Berney-Meyer, L., Jacobs, W.R., Larsen, M.H., 2015. Interleukin 17A as a biomarker for bovine tuberculosis. *Clin. Vaccine. Immunol.* 23, 168-180.

Waters, W.R., Thacker, T.C., Nelson, J.T., DiCarlo, D.M., Maggioli, M.F., Greenwald, R., Esfandiari, J., Lyashchenko, K.P., Palmer, M.V., 2014. Virulence of two strains of *Mycobacterium bovis* in cattle following aerosol infection. *J. Comp. Pathol.* 151, 410-419.

Zhu, X.W., Friedland, J.S., 2006. Multinucleate giant cells and the control of chemokine secretion in response to *Mycobacterium tuberculosis*. *Clin. Immunol.* 120, 10-20.

466

467 Figure 1

468

469 Figure 2

470

471 Figure 3

Figure 4

474

475 Figure 5