Veterinary Immunology and Immunopathology 180 (2016) 34–39

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

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

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ARTICLE INFO

Article history:
Received 3 June 2016
Received in revised form 17 August 2016
Accepted 31 August 2016

Keywords:
Cytokine
Giant cell
Granuloma
Mycobacterium bovis
RNAScope®

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.

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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

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http://dx.doi.org/10.1016/j.vetimm.2016.08.015
0165-2427/Published by Elsevier B.V.

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 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 (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-μm thick, cut from formalin-fixed, paraffin embedded tissues were heated for 60 min at 60 °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 (ddH_2O), followed by immersion in pretreatment 2 (antigen retrieval citrate buffer) for 15 min at 100–104 °C (boiling). Slides were washed in ddH_2O and pretreatment 3 (protease) applied for 30 min at 40 °C. Slides were washed in ddH_2O and target or control probes applied with incubation at 40 °C for 2 h 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 40 °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 60 °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*

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