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Comparative genomics and proteomics to study tissue-specific response and function in natural *Mycobacterium bovis* infections

Victoria Naranjo[1], Christian Gortazar[1], Margarita Villar[2] and José de la Fuente[1,3]*

[1]*Instituto de Investigación en Recursos Cinegéticos IREC (CSIC-UCLM-JCCM), Ronda de Toledo s/n, 13071 Ciudad Real, Spain,* [2]*Instituto de Tecnologías Química y Medioambiental ITQUIMA, University of Castilla La Mancha, Campus Universitario s/n, 13071 Ciudad Real, Spain, and* [3]*Center for Veterinary Health Sciences, Department of Veterinary Pathobiology, Oklahoma State University, Stillwater, OK 74078, USA*

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Abstract

Bovine tuberculosis (bTB) is an established zoonotic disease which affects cattle and wildlife worldwide and new strategies are required to control and eradicate the disease. The European wild boar (*Sus scrofa*) is a major reservoir of bTB in Spain. The objective of this paper was to review tissue-specific response and function of mandibular lymph nodes (MLN) and oropharyngeal tonsils (OT) in European wild boar naturally infected with *Mycobacterium bovis*. Genomics and proteomics data were used to compare differential gene expression and global protein patterns in OT and MLN of *M. bovis*-infected and uninfected European wild boar and the results were analyzed considering previous reports of experimental infections in laboratory and domestic animals. The results showed tissue-specific differences in OT and MLN in response to *M. bovis* infection. Tissue-specific differences in gene expression and protein profiles suggested different functions for OT and MLN during mycobacterial infection and provided information to characterize the pathobiology of *M. bovis* infection in European wild boar with important implications for the control of bTB in Spain. The characterization of molecular events in tissues that play different roles during mycobacterial infection in naturally infected individuals may be relevant to understand the pathobiology of *M. bovis* infection and to design effective strategies for the control of bTB in wildlife reservoirs.

Keywords: bovine tuberculosis, wild boar, mycobacteria, Iberian red deer, vaccine

Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*Mycobacterium tuberculosis* complex), is an established zoonotic disease which affects cattle and wildlife worldwide (Buddle *et al.*, 2006). Several wildlife species are naturally infected with *M. bovis*, but European wild boar (*Sus scrofa*) is a major reservoir host in Spain (Gortazar *et al.*, 2005; Vicente *et al.*, 2006). In this species, most tuberculous lesions are located in the mandibular lymph nodes (MLN) and oropharyngeal tonsils (OT) and disseminated lesions are occasionally observed in the liver, spleen, lungs and other organs (Gortazar *et al.*, 2003; Martín-Hernando *et al.*, 2007). The presence of common *M. bovis* genotypes in both cattle and wild boar suggests a threat of interspecies transmission of the pathogen (Gortazar *et al.*, 2005).

In European wild boar as in other species, genetic factors have been associated with resistance to bTB (Acevedo-Whitehouse *et al.*, 2005; Naranjo *et al.*, 2006a, b). However, limited information is available on the protective response of this and other wildlife reservoirs to mycobacterial infection. Recent studies in cultured cells and laboratory or domestic animals using functional genomic analysis have provided data toward understanding the mechanisms involved in host–pathogen

*Corresponding author. E-mail: jose_delafuente@yahoo.com or jose.de_la_fuente@okstate.edu

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interactions and host cell responses to mycobacteria (reviewed by Koul *et al.*, 2004). However, little is known about the immune response and the genes differentially expressed in naturally infected animal populations (Naranjo *et al.*, 2006a, 2007; Thacker *et al.*, 2006). Analysis of genes differentially expressed in tissue biopsies collected from infected and uninfected individuals exposed to mycobacteria in endemic areas would contribute to the understanding of the cellular response to natural mycobacterial exposure (Naranjo *et al.*, 2006a; Orlova *et al.*, 2006). These results provide information on genes that may correlate with resistance to tuberculosis and/or contribute to the development of protective responses in wildlife reservoirs of infection.

In this paper we review the application of genomic and proteomic analyses to the study of tissue-specific response and function in natural wild boar *M. bovis* infections and the results were analyzed considering previous reports of experimental infections in laboratory and domestic animals. For this study, we used the comparative analysis between MLN and OT, which may have different exposure to mycobacteria in wild boar, a natural reservoir of infection. These analyses may impact on the understanding of the pathobiology of *M. bovis* infection and the control of bTB.

Methodological approach

The wild boar data reviewed herein was obtained from the genomic and proteomic analyses in OT and MLN of *M. bovis*-infected and uninfected European wild boar (Naranjo *et al.*, 2006a, 2007). The analysis of differential gene expression and global protein patterns in OT and MLN of *M. bovis*-infected (N=4) and uninfected (N=3) European wild boar was done by suppression-subtractive hybridization (SSH) and two-dimensional gel electrophoresis followed by matrix-assisted laser desorption/ionization–time of flight mass spectrometry, respectively (Naranjo *et al.*, 2006a, 2007). Infected animals were diagnosed by pathology, culture and spoligotyping of mycobacteria (Naranjo *et al.*, 2006a). The extremely detailed necropsies performed, in parallel with the complete histopathology and the immediate culture of fresh lymphoid and non-lymphoid tissues warrant an optimal sensitivity to detect mycobacterial infections in mammals that almost rules out infection in the uninfected animals (Naranjo *et al.*, 2006a). Some of the differentially expressed genes and regulated proteins were validated by quantitative real-time RT–PCR, immunohistochemistry and protein level determinations in 15–90 samples from infected and uninfected individuals (Naranjo *et al.*, 2006a, 2007; Vidal *et al.*, 2006). Protein ontology was determined using protein reference databases (http://www.hprd.org and http://www.proteinlounge.com). Results were compared between *M. bovis*-infected and uninfected European wild boar and between OT and MLN.

Comparison of the differential gene expression profiles in MLN and OT of *M. bovis*-infected and uninfected European wild boar

The genomic analysis resulted in 270 and 199 genes differentially regulated in MLN and OT, respectively. These genes affected cellular functions such as cell structure, protein and DNA/RNA metabolism, signal transduction, cell–cell interactions, transport, growth, inflammation/stress, immune response and enzymatic processes (Fig. 1A). However, most of the differentially expressed genes identified were of unknown function (Fig. 1A).

Differences were observed in the genes differentially expressed in infected and uninfected animals. The MLN and OT of infected wild boar showed characteristics commonly found during mycobacterial infection such as the inhibition of phagosomal maturation and/or endocytosis and the expression of osteopontin (SPP1), which correlates with pathologic processes (Naranjo *et al.*, 2006a). On the other hand, in MLN and OT of uninfected animals common differentially expressed genes included the vitamin D receptor (VDR), complement component C3 (C3) and genes involved in Th1 response, antigen presentation and prevention of apoptosis, which may correlate with protective responses to mycobacterial infection in this species (Naranjo *et al.*, 2006a, b).

The results of the differential gene expression analysis were also different between MLN and OT. Immune response genes were differentially expressed predominantly in MLN (Fig. 1A). Evidence of apoptosis in infected animals was found in OT only, whereas correlates of Th2 responses such as arginase I (ARGI) were found exclusively in MLN (Fig. 1B). Cell structure, protein metabolism and signal transduction mechanisms were predominantly affected in MLN of infected boar (Fig. 1B). Although stress/inflammatory responses were characterized in MLN and OT of wild boar infected with *M. bovis* (Fig. 1B), the heat shock protein (HSP) response was activated in OT while the acute phase protein (APP) response was increased in MLN of infected animals (Naranjo *et al.*, 2007).

Comparison of the protein profiles in MLN and OT of *M. bovis*-infected and uninfected European wild boar

Proteomic analysis of MLN and OT identified differentially regulated stress/inflammatory proteins as the main difference between uninfected and *M. bovis*-infected boar. These proteins included annexin V, albumin and apolipoprotein A1 (ApoA1), which appeared down-regulated, whereas manganese superoxide dismutase (MnSOD) was up-regulated in MLN of infected boar (Naranjo *et al.*, 2007). In OT, NADP-dependent isocitrate dehydrogenase (IDP), two isoforms of creatine kinase (CK) and MHC class II (MHC II) were up-regulated in infected animals

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(Naranjo *et al.*, 2007). In accordance with genomic analysis, evidence of APP response and organ damage was found in MLN while MHC II up-regulation in OT of infected animals suggested macrophage activation in this tissue. The analysis of serum proteins demonstrated higher levels of MnSOD and serum amyloid A (SAA) in infected boar and ApoA1, IgG and C3 in uninfected animals (Naranjo *et al.*, 2006a, 2007; Vidal *et al.*, 2006).

Similarities and differences in the response to mycobacterial infection between naturally infected European wild boar and experimentally infected laboratory and domestic animals

In our studies, we hypothesized that gene and protein response to mycobacterial infection may be different between experimentally infected laboratory and domestic animals and naturally infected wildlife species. The analysis of gene and protein profiles in MLN and OT of European wild boar naturally infected with *M. bovis* did result in some unique findings in these species. The differences in the response to mycobacterial infection in European wild boar when compared to experimental infections in laboratory and domestic animals may have resulted from variations in the methodological approach (global expression analysis versus SSH), or from the use of different mycobacterial species/strains, tissues (isolated bone marrow-derived macrophages and peripheral blood monocytes versus MLN and OT) and stage and condition of infection (experimental versus natural infections). Different gene expression profiles have been reported between different mycobacterial strains (Blumenthal *et al.*, 2005; Wedlock *et al.*, 2006), infected cell types (Danelishvili *et al.*, 2003) and time points after infection (Meade *et al.*, 2006). However, the results of our studies also suggest that the response in European wild boar and other wildlife species to natural mycobacterial infection could differ from cell responses reported previously in experimental studies. These results also suggest that the selection of biological samples may be crucial to identify biomarkers and correlates of pathology and protection in tuberculosis (Orlova *et al.*, 2006).

Some of the differentially expressed genes that were identified in European wild boar were reported previously in other organisms. For example, the lysosomal-associated protein, which is involved in phagosomal maturation and is negatively regulated by pathogenic mycobacteria in human and mouse macrophages (Clemens and Horwitz, 1995; Koul *et al.*, 2004) was differentially expressed in European wild boar. The ubiquitin system, involved in protein degradation and regulation of the cell cycle, was differentially expressed in OT of European wild boar as reported previously in human (Chaussabel *et al.*, 2003; Wang *et al.*, 2003) and cattle (Weiss *et al.*, 2004) macrophages. Other genes, such as those encoding MHC II, vascular cell adhesion molecule 1 (VCAM-1), chemokine receptor 4 (CXCR4), VDR and C3, were differentially expressed in European wild boar and in *M. tuberculosis*-infected mouse and human macrophages and/or bovine peripheral blood mononuclear cells (Ragno *et al.*, 2001; Chaussabel *et al.*, 2003; Velasco-Velazquez *et al.*, 2003; Keller *et al.*, 2004; Selvaraj *et al.*, 2004; Kim *et al.*, 2006; Meade *et al.*, 2006), although contradictory results have been reported for VCAM-1 and CXCR4 expression between different organisms (Ragno *et al.*, 2001; Shalekoff *et al.*, 2001; Chaussabel *et al.*, 2003; Keller *et al.*, 2004; Naranjo *et al.*, 2006a).

The stress/inflammatory responses to *M. bovis* characterized in European wild boar, such as those mediated by annexin, SAA, ARGI, SPP1, MnSOD and MHC II, have been previously described in macrophages during *in vitro* and *in vivo* experimental infections with mycobacteria (Nau *et al.*, 1999; Aggeli *et al.*, 2000; Mishra *et al.*, 2000; Ragno *et al.*, 2001; Koguchi *et al.*, 2003; Keller *et al.*, 2004, 2006; Koul *et al.*, 2004; Meijer *et al.*, 2005). However, other stress/inflammatory response molecules identified in European wild boar such as ApoA1, HSPs, CK and IDP constitute new findings.

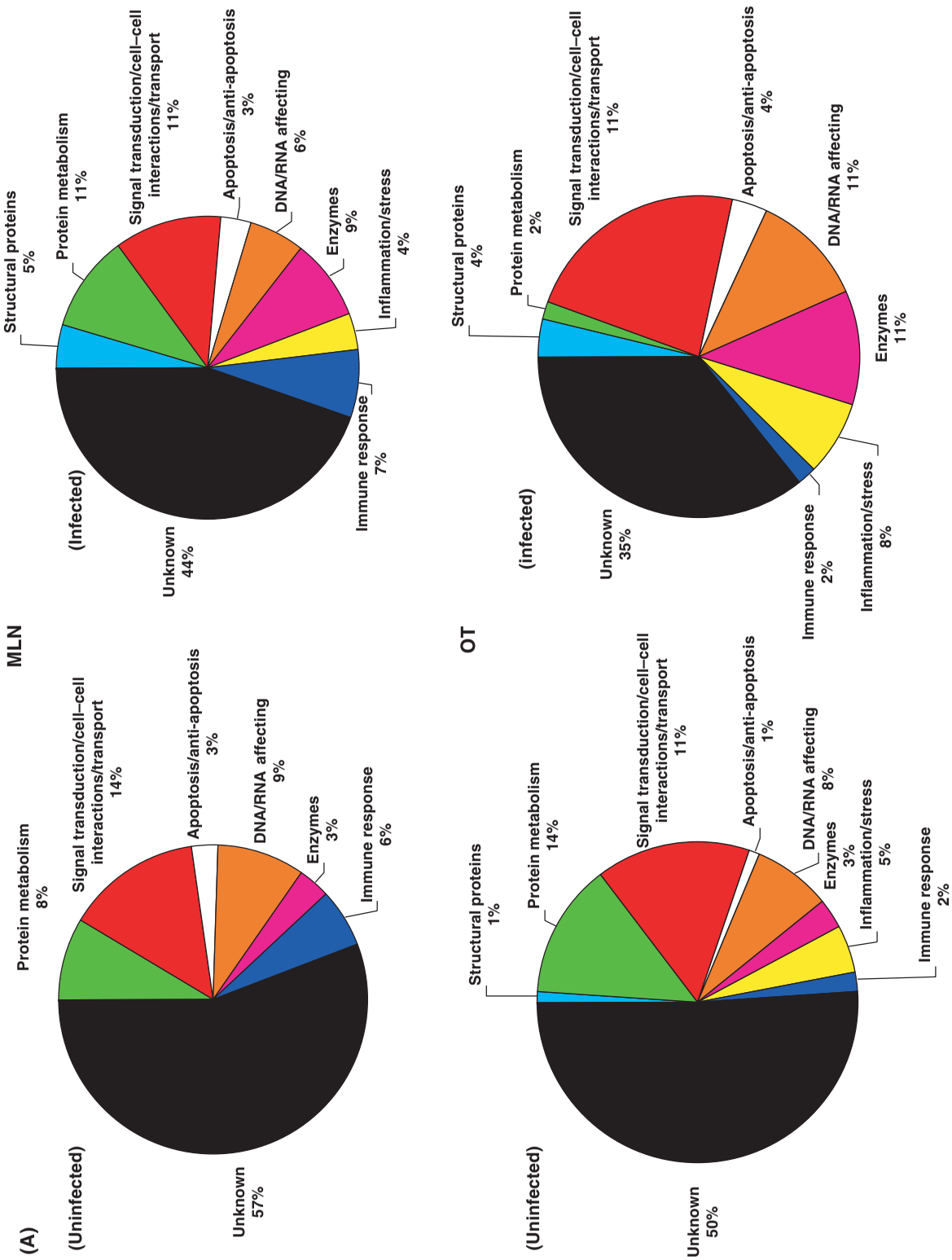
Pathobiology of *M. bovis* infection in European wild boar

The results of genomic and proteomic analyses may reflect differences in the role of MLN and OT during infection and progression of bTB in European wild boar. The OT may be one of the infection routes in boar, in which mycobacteria apparently enter through the respiratory and digestive routes (Vicente *et al.*, 2006). The OT may act as the first barrier for infection and the heat shock response in this tissue may be induced to protect boar from environmental mycobacteria. The finding of apoptosis in the OT of infected animals is in agreement with the hypothesis that mycobacteria prevent apoptosis in the early phase of infection to allow for efficient bacterial replication, but they may induce or are unable to prevent cell death in later phase when other organs such as MLN become infected, which would contribute to their systemic dissemination via infection of immune cells (Koul *et al.*, 2004).

The MLN are the most affected tissue by the formation of granulomatous lesions during progression of bTB in wild boar (Gortazar *et al.*, 2003; Martín-Hernando *et al.*, 2007). The APP response in MLN of infected animals may reflect tissue injury in the granulomas. These results are in agreement with histopathology findings which suggest that the up-regulation of stress/inflammatory responses in MLN may contribute to organ damage in *M. bovis*-infected animals, resulting in frequent large granulomatous lesions in more than one anatomical region among juvenile wild boar (Martín-Hernando *et al.*, 2007).

The inflammatory/stress responses in MLN and OT of *M. bovis*-infected wild boar suggest active bTB in these

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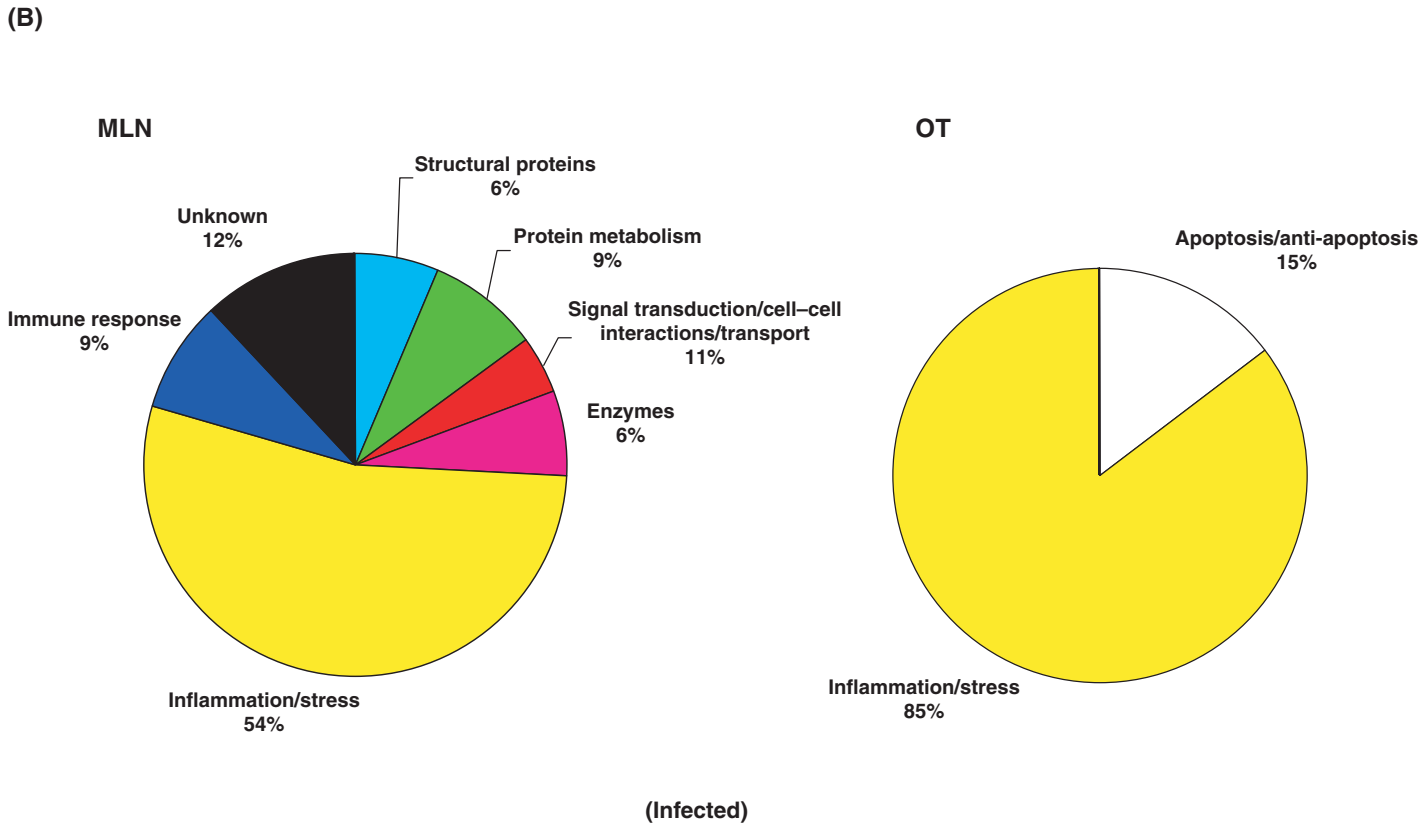


Fig. 1. Protein ontology assignments of (**A**) unique and (**B**) redundant (not unique) genes differentially expressed in MLN and OT of uninfected and *M. bovis*-infected European wild boar. The analysis of differential gene expression in OT and MLN of *M. bovis*-infected and uninfected European wild boar was done by SSH (Naranjo *et al.*, 2006a, 2007). Protein ontology was determined using protein reference databases (http://www.hprd.org and http://www.proteinlounge.com).

animals. These findings, together with the histopathological characteristics of the tuberculous reaction and the associated tissue damage in various organs, indicate that at least those wild boar with large lesions and generalized infections have the potential to excrete *M. bovis* by several routes, which reinforces the suggestion that wild boar is a true bTB reservoir in Spain (Martín-Hernando *et al.*, 2007).

Implications for the control of bTB in Spain

The genes differentially expressed and the proteins up-regulated in *M. bovis*-infected wild boar likely reflect cell responses to mycobacterial infection. If proven specific for *M. bovis* infection, some of these markers could be used for the development of assays for the identification of infected animals (Vidal *et al.*, 2006).

However, the genes differentially expressed and the proteins up-regulated in uninfected wild boar may represent down-regulation due to mycobacterial infection in infected animals and/or up-regulation in uninfected individuals as a result of individual resistance and/or protective response to infection. Some of the genes differentially expressed in uninfected animals, such as C3, methylmalonyl-CoA mutase (MUT) and certain MHC alleles, have been associated with resistance to bTB in European wild boar (Acevedo-Whitehouse *et al.*, 2005; Naranjo *et al.*, 2006a, b). Genetic polymorphisms in these loci may be identified and correlated with resistance to bTB, thus providing a tool for the selective genetic improvement of wild boar and domestic pig populations when animal translocations and introductions are approved by regulatory authorities. Additionally, the genes differentially expressed and the proteins up-regulated in uninfected wild boar may be used as biomarkers of protective response against *M. bovis* infection in experimental trials to identify and characterize new vaccine candidates and vaccination strategies in this species (Buddle *et al.*, 2005).

Mycobacterial infections induce antibody production but the profile of immunoglobulin (Ig) expression in *M. bovis*-infected animals is poorly understood (Waters *et al.*, 2006). Experiments in wild boar provided evidence of Ig differential expression in OT and MLN of *M. bovis*-infected and uninfected wild boar (Naranjo *et al.*, 2006a) and in mesenteric lymph nodes of Iberian red deer infected with *M. bovis* (I. G. Fernández de Mera *et al.*, 2007 submitted for publication). Serum IgG levels were also increased in uninfected boar (Vidal *et al.*, 2006). These results suggest that antibody responses against *M. bovis* may be important in natural infections of wildlife species and may be used for bTB surveillance and treatment monitoring (Lyashchenko *et al.*, 2006; Waters *et al.*, 2006).

Finally, the results discussed here may be relevant for vaccination strategies against bTB. In addition to systemic immune response activation, pre-exposure vaccines may be developed to target tonsil protective response to prevent infection by the pathogen. Post-exposure therapeutic vaccines may need to stimulate immune response at the lymph node to limit the multiplication, dissemination and reactivation of mycobacteria. Oral vaccines for the control of bTB in wildlife may be developed with these criteria to prevent *M. bovis* infection and transmission (Cross *et al.*, 2007).

Conclusions

In summary, we have focused our review on research on the characterization of gene expression and protein profiles in naturally infected tissues of wildlife reservoirs of bTB, instead of using experimental infections in cultured cells or laboratory and domestic animals. The results reviewed herein show similarities and differences between naturally *M. bovis*-infected tissues of wildlife reservoir species and those reported in cell culture or laboratory and domestic animals. Very little information is available on the response of wildlife species to mycobacterial infection (Lyashchenko *et al.*, 2006; Naranjo *et al.*, 2006a, b, 2007; Thacker *et al.*, 2006; Vidal *et al.*, 2006) although we can anticipate that this information will increase in the near future (Ballesteros *et al.*, 2007). Despite difficulties associated with work with naturally infected wildlife species such as the number of animals available, the potential presence of other infectious agents and the differences in the stage of infection between individuals, the results discussed in this review support the importance of studying naturally infected tissues in wildlife species. The understanding of the response to natural *M. bovis* infection may be critical to the development of effective strategies for the control of bTB. In particular, the response of tissues that play different roles during mycobacterial infection may be relevant to understand the pathobiology of *M. bovis* infection and to design effective vaccines for the control of bTB in wildlife reservoirs.

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