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Expression of immunoregulatory genes in peripheral blood mononuclear cells of European wild boar immunized with BCG

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20 **Abbreviations:** PBMC, peripheral blood mononuclear cells; Tb, tuberculous.

Keywords: wild boar, porcine, BCG, immunization, tuberculosis, signature, genomics, real-time RT-PCR.

25

Abstract

The objective of this study was to analyze the expression of immunoregulatory genes in European wild boar (*Sus scrofa*) immunized with BCG. Eighteen immunoregulatory genes were selected for expression analysis based on their role in host immune response during tuberculosis and/or for their association with resistance to bovine tuberculosis in European wild boar populations. Initially, mRNA levels were analyzed by quantitative real-time reverse transcription PCR (qRT-PCR) in spleen samples from *Mycobacterium bovis* infected (N=18) and uninfected (N=22) European wild boar. Statistical analysis of qRT-PCR data revealed that four genes, complement component C3, IFN-gamma, IL-4 and RANTES were downregulated in infected animals ($P<0.05$). These genes were selected for analysis of mRNA levels in peripheral blood mononuclear cells (PBMC) from seven wild boar experimentally immunized with BCG and seven non-immunized controls. Blood was collected at 0, 5, 13 and 25 weeks post-immunization (wpi). The mRNA levels of IFN-gamma and C3 showed a peak (>15 fold increase) at 5 wpi, whereas transcripts for RANTES and IL-4 showed a peak (>2 fold increase) at 13 wpi in BCG-immunized animals when compared to non-immunized controls. The pattern of expression of these genes over the time provides the first description of BCG specific immune response in European wild boar. These results provide new insights into the molecular basis of wild boar response to *M. bovis* infection and BCG vaccination and may be used to monitor BCG vaccination in this species.

1. Introduction

The *Mycobacterium bovis* and closely related mycobacteria of the *Mycobacterium tuberculosis* complex that cause bovine tuberculosis (bTB) have an extensive host range and may cause zoonotic TB (de Lisle et al., 2002; Gortazar et al., 2005; Neill et al., 2005). Despite efforts to eradicate bTB (Pollock et al., 2006), the disease has not been eradicated from many countries (Pavlik, 2006), partly because of the reservoir of *M. bovis* in wildlife (Corner, 2006; Naranjo et al., 2008). One potential tool for the management of disease in wildlife populations and to limit the spread of infection is vaccination (Buddle et al., 2006). The European wild boar (*Sus scrofa*) is a major reservoir of *M. bovis* in some regions of Spain and vaccination of wild boar might prove necessary for the control of bTB in this country (Buddle et al., 2000; Naranjo et al., 2008).

During mycobacterial infection, a dynamic cross-talk occurs between the host and the pathogen, in which they reciprocally influence their gene expression profiles (Gordon et al., 2001). The study of gene expression profiles in tissues from infected wild boar may be crucial for the understanding of the regulation of anti-mycobacterial immunity in this wildlife reservoir species. Recently, we demonstrated differential gene expression in tonsils and mandibular lymph nodes of infected and uninfected European wild boar exposed to natural *M. bovis* infection and identified possible correlates of resistance to bTB in this species (Naranjo et al., 2006; Naranjo et al., 2007a; Naranjo et al., 2007b). Herein, we first analyzed the expression of immunoregulatory genes in spleen samples from naturally *M. bovis*-infected and uninfected European wild boar to select host immunoregulatory genes differentially regulated in response to bTB in this species. Genes significantly downregulated in infected animals were selected for qRT-PCR analysis of mRNA levels in wild boar immunized with BCG. This study provides the

first description of the BCG specific response in wild boar that may provide insights into the molecular basis of wild boar response to *M. bovis* infection and BCG vaccination and may be used to monitor BCG vaccination in this species.

5 **2. Materials and Methods**

2.1. Wild boar and tissue samples

Spleen fragments of approximately 2 cm³ were collected *post mortem* from adult ($\geq$ 1 year old) hunter-harvested European wild boar and stored in liquid nitrogen for RNA extraction. Animals were sampled in hunting estates from Castilla-La Mancha, Spain.

10 Wild boars were classified as infected or uninfected based on the presence or absence, respectively of the following criteria: (1) the presence of lesions characteristic of bTB and (2) the identification of positive mycobacterial cultures and determination of *M. bovis* spoligotypes. According to these criteria, 18 wild boars were infected with *M. bovis* and 22 were used as uninfected controls.

15 **2.2. Selection of immunoregulatory genes**

Immunoregulatory genes (Table 1) were selected based on their putative role during mycobacterial infection and/or for their association with resistance to bTB in European wild boar (Naranjo et al., 2006). The genes analyzed herein included Interferon gamma (IFN-gamma), Regulated on Activation, Normal T Expressed and Secreted cytokine, 20 also known as CCL5 (RANTES), superoxide dismutase-Mn type (SOD), vascular cell adhesion molecule 1 (VCAM-1), monocyte chemoattractant protein 1 (MCP-1), apolipoprotein A-I (APOA1), CXCL8 or interleukin 8 (IL-8), tumour necrosis factors alpha (TNF-alpha), interleukin-1 beta (IL-1 beta), methylmalonyl Coenzyme A mutase (MUT), complement component 3 (C3), interleukin-12 (IL-12), secreted 25 phosphoprotein 1 (SPP-1), granulocyte-macrophage colony-stimulating factor (GM-

CSF), interleukin 4 (IL-4), fatty acid translocase/CD36 (CD36), interleukin 12 (IL-12), and vitamin D receptor (VDR).

2.3. Immunization with BCG

5 Fourteen wild boar (8 males and 6 females, <1 year old and 7-11 Kg weight at the beginning of the experiment) were captured in a bTB-free hunting state. Wild boars were placed together in an outdoor paddock, identified with an ear tag and allowed to acclimatise for one week. Seven animals were immunized at week 0 with a single intra dermal dose of 0.1 ml containing 0.075 mg of BCG Danish strain (Statens Serum
10 Institut, Copenhagen, Denmark). Control wild boars were not immunized. Blood was collected at 0, 5, 13 and 25 weeks post-immunization (wpi). Blood was obtained by puncture of the right cranial vena cava and was collected in 10 ml tubes with EDTA as anti-coagulant. Blood (10 ml) was diluted 1:1 (vol:vol) with PBS (Sigma) and the mononuclear fraction containing PBMC was obtained by density gradient centrifugation
15 on Lymphoprep (Axis-Shield, Oslo, Norway).

At week 25, animals were euthanized by the captive bolt method (Chambers & Grandin, 2001), following European, National and University of Castilla-La Mancha Ethics Committee regulations. Wild boars were subjected to detailed *post-mortem* examination. In each animal, the bronchial, mediastinal, submandibular,
20 retropharyngeal, mesenteric and hepatic lymph nodes and lungs were examined macroscopically for bTB lesions and cultured on stonebrinks and Lowenstein-Jensen media at 37 °C for 8 weeks to detect mycobacteria of the *M. tuberculosis* complex (Costello et al., 1998). These analyses showed that the animals did not present bTB-compatible lesions and mycobacteria were not isolated from examined tissues, thus
25 demonstrating that the animals were bTB free at the end of the experiment.

2.4. qRT-PCR analysis

Total RNA was extracted from PBMC using the RNeasy kit (Qiagen, Izasa, Madrid, España) following manufacturers` recommendations. Total RNA from spleen samples was extracted using TRI-reagent (Sigma, Madrid, Spain) following manufacturers` recommendations. The RNA yield and quality were assessed using the Experion Bioanalyzer (Bio-Rad, Madrid, Spain). Gene-specific oligonucleotide primers were designed using Primer3 software (Rozen and Skaletsky, 2000), synthesized (Bonsai Technologies, Madrid, Spain) and used for qRT-PCR (Table 1). qRT-PCR was performed in 25 µl reaction volumes with 12.5 µl SYBR Green iScript® (Bio-Rad). Amplification conditions consisted of 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 55 °C for 60 s. A dissociation curve was run at the end of qRT-PCR reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample (Ririe et al., 1997). All reactions were performed in duplicate. Oligonucleotide primers were used to amplify the cyclophilin gene transcript as a control gene to normalize expression data (Meadus, 2003). Control reactions were performed using the same procedures, but without RT to control for DNA contamination in the RNA preparations and without RNA added to control contamination of the PCR reaction. Results were recorded as relative gene expression changes after normalizing for cyclophilin gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the relative abundance of each mRNA transcript was calculated as the ratio of the value from BCG-vaccinated animals to the value from controls after cyclophilin correction for each of the four time points, producing a fold change value. In all cases, the mean of the duplicate values was used and data for vaccinated/control samples were compared using the Student`s t-test (P=0.05).

2.5. Determination of serum IFN-gamma levels

Blood was collected from wild boar and serum separated and stored at -20 °C. The serum concentration of IFN-gamma was determined using a quantitative ELISA (Pierce Endogen, Rockford, IL, USA) following manufacturer's recommendations.

5 **3. Results and Discussion**

The objective of the experiments described herein was to test whether vaccination of wild boar with BCG induces immunization-specific changes in gene expression. In addition, we wanted to investigate time-dependent gene expression changes over the period of immunization.

10 Prior to the analysis of the gene expression profiles in animals vaccinated with BCG, a study was designed to examine immunoregulatory gene expression profile in spleen samples from naturally *M. bovis*-infected and uninfected wild boar to find genes that are significantly downregulated in response to infection and thus are potentially being manipulated by the pathogen to establish infection. The expression of these genes in
15 wild boar vaccinated with BCG may reflect BCG-specific changes in gene expression that are different from those observed in animals naturally infected with *M. bovis* and could be used to monitor the immune response in BCG-vaccinated wild boar.

The work with wildlife species is difficult but allows the analysis of gene expression in naturally infected individuals (Naranjo et al., 2006). One disadvantage of working with
20 wild boar is that fresh blood is not always available in hunted animals. Therefore, we considered the spleen as the tissue sample of choice for the study of gene expression profile in response to mycobacterial infection in hunter-killed wild boar. In fact, it has been reported that the expression of cytokine genes such as IFN-gamma is similarly elevated after mycobacterial infection in PBMC and spleen cells of animal models of
25 TB (Kawahara et al., 2002).

Statistical analysis of the normalized gene expression data in naturally *M. bovis* infected and uninfected wild boar revealed that 4 of 18 genes analyzed, C3, IFN-gamma, IL-4 and RANTES were downregulated in *M. bovis*-infected wild boar ($P < 0.05$) (Fig. 1). The
5 genes differentially downregulated in infected wild boar were selected to investigate the host response to BCG immunization. Vaccination with BCG did not produce adverse reactions in wild boar as it has been reported in humans and domestic and wild animals (Murphy et al., 2008). Total RNA was extracted from PBMC of seven BCG-immunized and seven non-immunized control animals and used for transcriptional profiling. The
10 mRNA transcripts for IFN-gamma and C3 were significantly upregulated at 5 wpi only with over 15-fold increase in immunized animals (Fig. 2). In contrast, the mRNA transcripts for IL-4 and RANTES were significantly upregulated in BCG-immunized wild boar with over 2-fold increase at 13 wpi only (Fig. 2).

IFN-gamma is known to play a major role in macrophage activation and inflammation
15 (Koul et al., 2004). The changes in IFN-gamma gene expression in BCG-vaccinated animals (Fig. 2) were opposite to those observed in naturally *M. bovis*-infected versus uninfected wild boar (Fig. 1). However, these results are congruent with the reported increase in IFN-gamma expression during early stages of mycobacterial infection in humans, laboratory and farm animals and cultured macrophages (McMurray et al.,
20 2005) and after BCG vaccination of guinea pigs (Tree et al., 2006). Moreover, serum IFN-gamma concentrations were consistent with mRNA levels in naturally *M. bovis*-infected and BCG-vaccinated wild boar (Table 2).

IL-4 is a key regulator of humoral and adaptive immunity and inhibits the production of proinflammatory cytokines (Howard and Paul, 1982). RANTES is a proinflammatory
25 chemokine that plays an active role in recruiting leukocytes into inflammatory sites and also induces the proliferation and activation of certain natural-killer (NK) cells to form

CHAK (CC-Chemokine-activated killer) cells (Maghazachi et al., 1996). RANTES is induced by *M. tuberculosis* in mouse and human macrophages (Ehrt et al., 2001; Ragno et al., 2001) and in mouse lungs after aerogenic infection (Keller et al., 2006). RANTES is also weakly induced in BCG vaccinated guinea pigs (Tree et al., 2006). The overexpression of IL-4 and RANTES in BCG-vaccinated wild boar may result from an adjuvant effect of the mycobacteria in the BCG vaccine that causes a rapid entry of macrophages, lymphocytes, granulocytes, and probably dendritic cells into local sites of antigen deposition (Sugisaki et al., 1998).

Although it is generally recognized that humoral immunity is not important for the control of TB (Raja, 2004), IL-4 induced antibody response against *M. bovis* may be important for bTB control in wild boar and may be used for disease surveillance and treatment monitoring (Lyashchenko et al., 2007; Lyashchenko et al., 2006; Naranjo et al., 2007a; Waters et al., 2006). In contrast, the increase in IL-4 levels in humans correlates with disease severity (Seah and Rook, 2001), a fact that may underline the existence of host-specific responses to mycobacterial infection that vary between species (Naranjo et al., 2007a).

This study describes the first attempt to use real-time qRT-PCR to investigate the host response to BCG immunization in wild boar. The balance between host genes upregulated and downregulated in response to mycobacterial infection may be key determinants for disease progression. The results reported herein provide new insights on the expression of immunoregulatory genes that are integral to the host response to *M. bovis* in wild boar.

The vaccination of wild boar with BCG offers an alternative for control of bTB (Buddle et al., 2000). However, several issues need to be addressed before introducing wild boar vaccination into practice. These issues include the design of oral immunization

formulations and strategies and the assessment of possible vaccination drawbacks such as environmental impact and potential interference with tuberculin skin testing in cattle and farmed deer. Furthermore, whether wild boars vaccinated with BCG are protected against bTB is unknown and falls outside the scope of this paper. However, gene expression profile in vaccinated animals showed BCG-specific responses that are different from those observed in naturally *M. bovis*-infected wild boar and may be used to monitor BCG vaccination during experimental vaccine studies in this species.

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15

Legends to Figures

- Figure 1. Quantitative gene expression analysis in spleen samples of *M. bovis*-infected and uninfected European wild boar using qRT-PCR analysis. Three experimental replicates were done for each analysis with similar results. Amplification efficiencies were validated and normalized against *S. scrofa* cyclophilin (GenBank accession number AY008846). Bars show uninfected to infected average $\pm$ S.D. mRNA ratios. Asterisks (*) indicate significant differences in mRNA levels between uninfected and infected animals ($P < 0.05$, Student's *t*-test).
- Figure 2. Quantitative C3, IFN-gamma, IL-4 and RANTES gene expression analysis in PBMC of European wild boars immunized with BCG and controls using qRT-PCR. Results were recorded as the ratio of immunized to non-immunized after normalisation for cyclophilin gene expression using the $2^{-\Delta\Delta C_t}$ method for each of the four time points (0, 5, 13 and 25 wpi). In all cases, the mean of duplicate values was used and data was compared between immunized and control animals by Student's *t*-test (* $P < 0.05$). Circles show average $\pm$ SD.

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13, 648-654.
- 15

Figure 1

Figure 2

Table 1. Genes and oligonucleotides used for real time RT-PCR analysis

Gene description	Accession number	Name and Sequence of primers	Size of product
IL-1beta	NM_214055	SsIL1beta-L ccaaagagggacatggagaa SsIL1beta-R ttatatcttggcggccttg	160
RANTES	AJ583704	SsRANTES-L tgctgcttgcctacagcta SsRANTES-R ggcgggtcttctggtgata	233
TNFalfa	X54859	SsTNFalfa-L ccaccaacgtttcctcact SsTNFalfa-R ccaaaatagacctgccaga	247
IL-8	AB057440	SsIL8-L tggcagtttctgctttct SsIL8-R cagtgggtccactctcaat	154
MCP-1	NM_214214	SsMCP1-L tctccagtcacctgctgcta SsMCP1-R tccagtggttatggagtc	185
IL-12	L35765	SsIL12-L cctccaaactagcgacctca SsIL12-R ctgagatgggtccaggtggtt	150
IFNgamma	DQ913893	SsIFNg-L ttcagcttgcgtgacttg SsIFNg-R tectttgaatggcctggta	246
IL-4	NM_214123	SsIL4-L tctcacctcccaactgatcc SsIL4-R aaggtttccttctccgtcgt	201
IL-2	NM_213861	SsIL2-L caaacggtgcacctactca SsIL2-R cctgcttgggcatgtaaaat	156
GM-CSF	DQ108393	SsGMCSF-L gcagcctcactaggetcaag SsGMCSF-R caaaggggatggtgaaaaga	156
CD36	DQ192230	SsCD36-L cggttgcaggtcaacctatt SsCD36-R tgatttctaccaggccaagg	184
MUT	NM_214405	SsMUT-L gtttgccaacggtgaaaagt SsMUT-R aatgagcttcaaggcagcat	155
C3	NM_214009	SsC3-L acaaattgaccagcgtagg	246

		SsC3-R gcacgtccttgctgtactga	
SPP1	NM_214023	SsSPP1-L cttggacagccaagagaagg SsSPP1-R tggctgactttgggatttc	165
APO A1	NM_214398	SsAPOa1-L cgatcaaagacagtggcaga SsAPOa1-R gctgcaccttcttcacc	234
Mn-SOD	X64057	SsSOD-L cgctgaaaaagggatgatt SsSOD-R agcggtaactctccttga	197
VCAM-1	NM_213891	SsVCAM1-L atccaagctgtccaaaaga SsVCAM1-R ggcctgtggatggatatg	189
VDR	EF405627	SsVDR-L acctggatctgagcgaagaa SsVDR-R gatggcacttgacttcagca	194
Cyclophilin	AY008846	SsCyclophilin-L agcactggggagaaaggatt SsCyclophilin-R cttggcagtgcaaatgaaaa	235

Table 2. IFN-gamma serum levels in wild boar uninfected and naturally infected with *M. bovis* and in BCG-immunized and control animals.

IFN-gamma serum levels in wild boar (ng/ml)			
Uninfected and naturally infected with <i>M. bovis</i>			
	Mean ± SD		
Infected	2.7 ± 1.0		
Uninfected	3.0 ± 1.5		
BCG-immunized and controls			
	5 wpi	13 wpi	25 wpi
	Mean ± SD	Mean ± SD	Mean ± SD
Immunized	3.2 ± 0.7	1.8 ± 0.4	1.5 ± 0.2
Control	2.7 ± 0.6	1.8 ± 0.5	1.8 ± 1.0

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The concentration of serum IFN-gamma (ng/ml) was determined by a quantitative ELISA (Pierce Endogen, Rockford, IL, USA) following manufacturer's recommendations.

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