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## First data on Eurasian wild boar response to oral immunization with BCG and challenge with a *Mycobacterium bovis* field strain

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### ABSTRACT

The Eurasian wild boar (*Sus scrofa*) is considered a reservoir for bovine tuberculosis (bTB) caused by *Mycobacterium bovis* and closely related members of the *Mycobacterium tuberculosis* complex in south-central Spain. The vaccination of wildlife with BCG offers an alternative to culling and to movement restriction for the control of bTB among wildlife reservoirs. In this study, we hypothesized that oral BCG immunization of wild boar would affect the expression of immunoregulatory genes and confer protection against *M. bovis*. Three groups were used to describe the infection, pathological findings and gene expression profiles in wild boar: BCG-vaccinated and *M. bovis*-challenged (vaccinated challenged group;  $N=6$ ), non-vaccinated and *M. bovis*-challenged (non-vaccinated challenged group;  $N=4$ ), and non-vaccinated and mock-infected (control group;  $N=2$ ) animals. *M. bovis* was isolated from 50% (3/6) and 75% (3/4) of vaccinated challenged and non-vaccinated challenged animals, respectively. All four wild boar from the non-vaccinated challenged group developed bTB-compatible lesions 114 days after challenge. In contrast, only 50% of vaccinated challenged wild boar developed lesions. The PBMC mRNA levels of IL4, RANTES, C3, IFN- $\gamma$  and methylmalonyl-CoA mutase (MUT) were analyzed at several days post-vaccination (dpi). When vaccinated challenged animals were compared to controls, all five genes were significantly upregulated at the time of *M. bovis* infection at 186 dpi but IFN- $\gamma$  levels were also upregulated at 11 and 46 dpi. The C3 and MUT mRNA levels were higher at 46 dpi, and 11 and 186 dpi, respectively, in vaccinated protected wild boar when compared to non-vaccinated challenged animals. At the end of the experiment (300 dpi), the mRNA levels of selected genes were lower in non-vaccinated challenged animals when compared to control wild boar. Exposing wild boar to a dose of  $10^4$  cfu of *M. bovis* by the oropharyngeal route is an adequate protocol to produce an infection model in this species. Our results suggested that oral BCG immunization of wild boar results in the upregulation of immunoregulatory genes that may be associated with protective response to *M. bovis* infection in this species. More studies on vaccine efficacy, delivery, and safety will be needed to confirm if oral vaccination with BCG could be used in bTB control programs for reducing *M. bovis* infection and clinical disease in wild boar.

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### 1. Introduction

The European wild boar (*Sus scrofa*) is increasingly relevant as a host for several pathogenic mycobacteria [1,2]. In the Mediterranean habitats of central and southern Spain, characterized by an artificial wildlife management and overabundant wild ungulate populations, the wild boar is considered a reservoir for bovine tuberculosis (bTB) caused by *Mycobacterium bovis* and

closely related members of the *Mycobacterium tuberculosis* complex [3].

*M. bovis* bacilli Calmette–Guerin (BCG) is currently the only available commercial vaccine against tuberculosis [4]. The vaccination of wildlife with BCG offers an alternative for bTB control by culling or by movement restriction of wildlife reservoirs [2,5].

Oral delivery of lipid-formulated BCG to calves was performed by Buddle et al. [6] to test immune responses and protection against challenge with virulent *M. bovis*. The level of protection was similar to that induced by subcutaneous BCG vaccination. This oral bait has also successfully induced protection against *M. bovis* and *M. tuberculosis* infection in laboratory mice [7]. BCG vaccination

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of wildlife reservoirs of bovine tuberculosis is being extensively researched in many countries, including New Zealand, United Kingdom, Ireland, United States and South Africa [8-13]. Oral BCG vaccination against bTB has been proven to reduce tuberculosis lesions in cattle and wildlife species such as badgers [14], brush-tail possums (*Trichosurus vulpecula*) [8,15,16] and white-tailed deer (*Odocoileus virginianus*) [9,17], even if protection from infection is incomplete [4]. However, whether wild boar vaccinated with BCG are protected against bTB is unknown. Recently, the expression of immunoregulatory genes was characterized in wild boar vaccinated with parenteral BCG [18]. These experiments demonstrated BCG-specific responses of genes in vaccinated animals that were different from those observed in naturally *M. bovis*-infected wild boar. This may be used to monitor BCG vaccination during experimental vaccine and challenge experiments. Also recently, experimental infection of wild boar with *M. avium avium* (MAA) provided evidence of an appropriate route (the oropharyngeal one) and doses for challenge experiments, and the analysis of immunoregulatory gene expression allowed detecting wild boar response to MAA (Garrido et al., unpublished results).

In parallel, we developed oral baits suitable to deliver vaccines such as BCG to free-living wild boar, and developed techniques to improve specificity and uptake rate in overabundant wild boar populations [19,20]. These baits could be used for the oral immunization of 2-4-month-old wild boar piglets [20]. However, trials involving vaccination with BCG and challenge for the control of bTB are needed to fully address the efficacy of these oral vaccines.

This is the first report on experimental infections of wild boar with *M. bovis*, and also the first attempt to study whether wild boar vaccinated with BCG are protected against bTB. However, several experiments have already been documented in its domestic relative, the pig. For example, a pig model for *M. tuberculosis* complex infection in humans using *M. bovis* was developed [21]. Recently, pigs were infected with both *M. bovis* and MAA in a study on skin-test responsiveness [22]. Several other experimental mycobacterial infections of pigs are found in the literature (e.g. [23-25]).

The aim of this study was to describe the infection, the pathology, and the gene expression profile of three groups of animals: (1) BCG-vaccinated and *M. bovis*-challenged (vaccinated challenged group), (2) non-vaccinated and *M. bovis*-challenged (non-vaccinated challenged group) and (3) non-vaccinated and mock-infected wild boar (control group). Based on prior findings,

we hypothesized that BCG vaccination would affect the expression of immunoregulatory genes and confer protection against bTB.

## 2. Materials and methods

### 2.1. Animals

We obtained twelve 3-4-month-old female wild boar piglets from a commercial source. Wild boar from this site were known to be free of mycobacterial lesions at slaughter. Furthermore, a sample of 50 animals from the same source yielded negative *M. bovis* ELISA test [26] results. The wild boar were taken to class III bio-containment facilities where they had *ad libitum* food and water. Animals were allowed 1 week of acclimatization before the start of the experiment. All experiments were carried out following European, National and Regional Law and Ethics Committee regulations.

### 2.2. BCG vaccine preparation

Oral baits for BCG immunization were prepared as described previously [20]. The *M. bovis* BCG Danish reference strain (CCUG 27863) was purchased from the Culture Collection, University of Göteborg, Sweden. It was cultured on Coletsos medium (Biomerieux, France), an egg yolk medium without glycerol, and colonies from the slant were scraped and transferred to a sterile tube containing 8-10 glass beads. The suspension was mixed in a vortex for a few seconds and sterile distilled water was added. Then it was allowed to settle for 5 min. The supernatant was adjusted with water to turbidity equal to 1.0 McFarland standard. To calculate the amount of BCG colony forming units (cfu) per bait, dilutions from  $10^{-1}$  to  $10^{-6}$  were prepared and 0.150 ml from each dilution were cultured in Coletsos medium in duplicate. The tubes were cultured at 37 °C and cfu readings were taken after 5 weeks. Each bait contained 0.150 ml of the 1.0 McFarland standard.

### 2.3. Preparation of challenge inocula

A clinical *M. bovis* isolate was cultured from a wild boar captured in the Monte de El Pardo Natural Park. The animal was randomly hunted in 2006 by the staff of the Park during active surveillance. Necropsy was performed by veterinarians in the field. A tissue pool was homogenized with sterile distilled water and decontam-

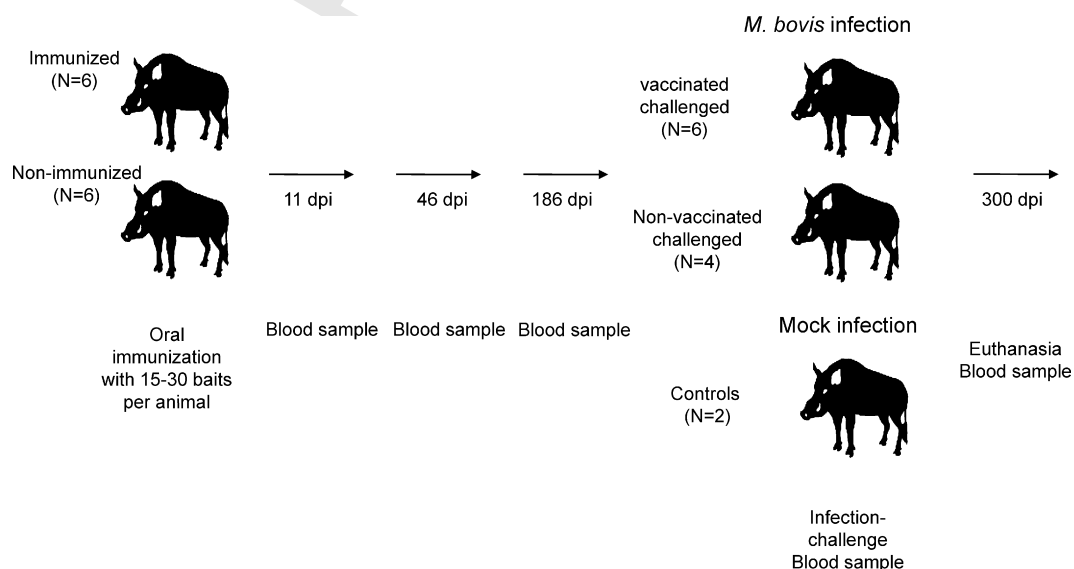


Fig. 1. Experimental design.

inated with 0.35% hexadecylpyridinium chloride for 30 min [27], centrifuged at 3500 rpm (1068 × g) for 30 min and cultured onto Coletsos and 0.2% (w/v) pyruvate-enriched Löwenstein-Jensen media at 37 °C (bioMérieux España and Biomedics, Madrid, Spain). The isolate was identified as *M. bovis* and characterized by spoligotyping as described [28], showing the profile SB0339 according to the *M. bovis* Spoligotype Database website ([www.mbovis.org](http://www.mbovis.org)). As described above, the isolate was subcultured in Coletsos and the *M. bovis* cfu's was determined as described in Section 2.2.

#### 2.4. Experimental design

The experimental design is summarized in Fig. 1. Twelve wild boar were randomly assigned to either the vaccinated group (N=6) or the non-vaccinated group (N=6). Vaccination took place in autumn 2007, with 15–30 oral baits containing  $1 \times 10^5$  cfu per bait. Non-vaccinated animals received placebo baits containing PBS. Wild boar were physically restrained by two caretakers. Baits were introduced one by one into the mouth of each wild boar, confirming that they were chewed and the capsule containing the liquid (either the vaccine or the placebo) opened in the oral cavity. Blood samples were collected from vaccinated and non-vaccinated animals at 11, 46 and 186 days post-vaccination (dpi). At 186 dpi, 10 animals (6 vaccinated and 4 non-vaccinated) were challenged with *M. bovis* (vaccinated challenged and non-vaccinated challenged groups, respectively) and 2 non-vaccinated animals were left uninfected (control group). Challenged animals were anaesthetized with 50 mg of a 1:1 solution of zolacepam and tiletamine (Zoletil 100, Virbac, 06511 Carros Cedex, France) by the intramuscular route. Five milliliters of mycobacterial suspension were administered by the oropharyngeal route by emptying a needleless syringe onto the back of the tongue. Three final doses of *M. bovis* suspension were used per animal,  $10^2$  cfu,  $10^4$  cfu and  $10^6$  cfu. Of the vaccinated challenged group, two wild boar were used for each dose. Of the non-vaccinated challenged group, one wild boar received  $10^2$  cfu, one  $10^4$  cfu, and two  $10^6$  cfu.

All wild boar were euthanized and blood samples were taken at 300 dpi, 114 days after challenge. At the postmortem a wide range of tissues were examined for gross and histopathological lesions of tuberculosis and cultured for mycobacteria.

#### 2.5. Necropsy, sample collection and histopathology

Wild boar were anaesthetized by intramuscular injection of zoletil as described in Section 2.4, and euthanized by captive bolt. A thorough postmortem examination was done to detect the presence of macroscopic lesions. Tissue specimens were collected and processed individually for histopathology and were frozen at  $-80$  °C for culture and mRNA isolation. The tissues collected were as follows: head (oropharyngeal tonsil and mandibular, parotid, and retropharyngeal lymph nodes); thorax (lung and tracheobronchial and mediastinal lymph nodes); abdomen (spleen, liver, kidney, ileocaecal valve, and mesenteric and hepatic lymph nodes). Lymph nodes and tonsils were serially sliced into 1–2 mm thick slices and carefully inspected for visible TB-compatible lesions and sampled for histopathology and culture. Individual tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin-eosin (HE) by use of standard procedures. An additional section of those tissues with lesions indicative of tuberculosis were stained by Ziehl-Neelsen (Z-N) staining procedure to detect the presence of acid-fast organisms. Microscopic lesions were classified as previously described for naturally *M. bovis*-infected wild boar [29].

We defined a bTB lesion score for wild boar, based on lesion distribution and lesion intensity (Table 1). We also defined a culture score for *M. bovis* infection in wild boar, as the number of

**Table 1**

Definitions of severity scores for gross lesions compatible with bovine tuberculosis in wild boar.

Tissue	Score	Definition
Lymph nodes, tonsil	0	No visible lesion
	1	1–2 small (<1 cm) caseous foci ("A" lesion)
	2	Several foci A but no B lesions
	3	Several foci. At least one lesion >1 cm ("B" lesion)
	4	Diffusely affected
Lung (per lobe)	0	No visible lesion
	1	Few "A" lesions scattered throughout lobe
	2	Numerous or Clustered "A" lesions with some coalescence of foci
	3	"A" lesions densely clustered throughout lobe
	4	At least one "B" lesion
5	Two or more "B" lesions	
Visceral organs and pleura	0	No visible lesion
	1	1–2 mm foci scattered throughout organ
	2	5–10 mm diameter clusters of 1–2 mm foci or single focus >1 cm diameter

lymph node or organ samples yielding a *M. bovis* isolate, of the total number of culture attempts (N=17 samples cultured per wild boar; score range 0–17).

#### 2.6. Selection of immunoregulatory genes

Immunoregulatory genes were selected based on their putative role during mycobacterial infection and their association with *M. bovis* infection in European wild boar [18,30–32]. The genes analyzed herein included interferon gamma (IFN-gamma), Regulated on Activation, Normal T Expressed and Secreted cytokine (RANTES), also known as CCL5, complement component 3 (C3), interleukin 4 (IL-4) and methylmalonyl-CoA mutase (MUT).

#### 2.7. Real-time RT-PCR analysis

Blood samples were collected at 11, 46, 186 and 300 dpi (Fig. 1). Total RNA was extracted from peripheral blood mononuclear cells (PBMC) using TriReagent (Sigma, Madrid, Spain) following manufacturer's recommendations. The RNA yield and quality were assessed using the Experion Bioanalyzer (Bio-Rad, Madrid, Spain). Gene-specific oligonucleotide primers were designed and used for quantitative real-time RT-PCR (qRT-PCR) (Table 2). The 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 the reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently in the same temperature range for every sample [33]. All reactions were performed in triplicate. Oligonucleotide primers were used to amplify the cyclophilin gene transcript as a control gene to normalize expression data [18]. 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. The mRNA values were normalized against *S. scrofa* cyclophilin (Genbank accession number AY008846) gene expression using the  $2^{-\Delta\Delta Ct}$  method [34]. The normalized relative expression was calculated at each time point and the mean of triplicate values was used to compare data of vaccinated (vaccinated challenged

**Table 2**  
Genes and primers used for real-time RT-PCR analysis.

Gene	Genbank accession number	Primer sequence (5'–3')	Size of PCR product (bp)
RANTES	AJ583704	SsRANTES-L tgctgctttgcctacagcta SsRANTES-R ggcggttctttctggtgata	233
IFN-gamma	DQ913893	SsIFNg-L ttcagctttgcgtgactttg SsIFNg-R tcctttgaatggcctggtta	246
IL-4	NM.214123	SsIL4-L tctcacctccaactgatcc SsIL4-R aaggttctcttccctcgt	201
C3	NM.214009	SsC3-L acaaattgaccagcgtagg SsC3-R gcagctcttctgtactga	246
Cyclophilin	AY008846	SsCyclophilin-L agcactggggagaaaggatt SsCyclophilin-R cttggcagtgcacaaatgaaaa	235
MUT	AJ277795	MUTF: tcctttcacaccatgtgc MUTR: agtactgtggaagagagc	250–288

group) vs. non-vaccinated (non-vaccinated challenged and control groups), vaccinated protected (vaccinated challenged group, no lesions and no positive culture) vs. vaccinated infected (vaccinated challenged group, lesion and/or culture positive) and *M. bovis*-infected (non-vaccinated challenged group) vs. non-vaccinated and mock-infected animals (control group) by analysis of variance (ANOVA) followed by a series of Tukey's post hoc tests for pair comparisons ( $p = 0.05$ ).

### 3. Results

#### 3.1. Clinical signs and *M. bovis* isolation

No clinical signs were observed in any of the wild boar after BCG vaccination. No clinical signs were observed after challenge in 11 of 12 wild boar included in the experiment. Only wild boar #3 from the non-vaccinated group challenged with  $10^6$  cfu showed weakness and a poor general condition for the last month of the experiment.

*M. bovis* was isolated from three of four non-vaccinated challenged animals and from three of six vaccinated challenged animals. The culture score was lower in the vaccinated challenged wild boar than in the non-vaccinated challenged ones (Fig. 2). No mycobacterial isolates were obtained from the two controls (Table 3). Molecular typing confirmed that all isolates belonged to the field strain used for challenge and not to the BCG vaccine strain.

#### 3.2. Pathology

All four non-vaccinated challenged wild boar developed bTB-compatible lesions 114 days after challenge. In contrast, only three of six vaccinated challenged wild boar developed lesions. Neither of the two controls showed lesions. The lesion score was lower in the vaccinated challenged wild boar than in the non-vaccinated challenged animals (Fig. 2). No lesions were observed in the control animals. The difference in lesion prevalence (Fisher's test,  $p = 0.17$ ) was not significant.

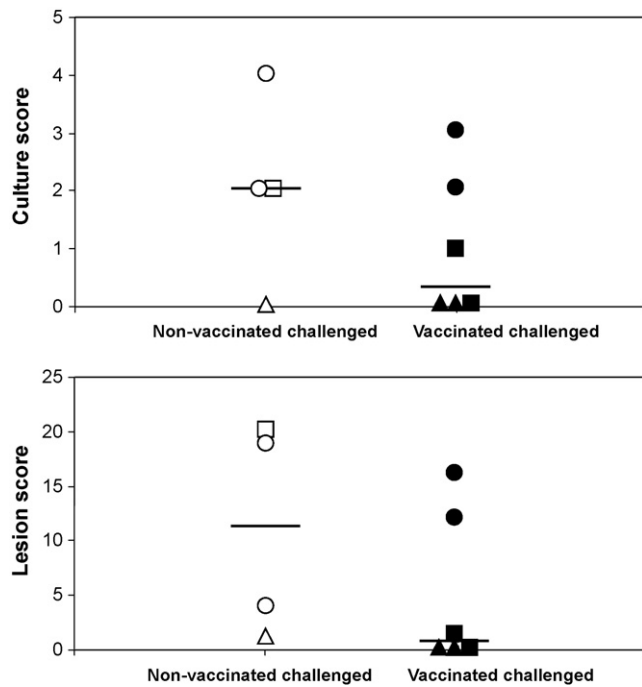
At necropsy, two of four non-vaccinated challenged wild boar showed severe tuberculous lesions. Wild boar #3 (exposed to the highest dose, of  $10^6$  cfu) was the only one with clinical signs, and had abdominal and thoracic organs affected. The lung had consolidated areas and numerous miliary necrotic foci in the accessory lobe. Both tracheobronchial lymph nodes (tbLNs) and the mediastinal LN were enlarged. These and the right mandibular (m) LN contained scattered small (1–2 mm) necrotic foci. Wild boar #4 (exposed to the medium dose, of  $10^4$  cfu) had severe lung lesions, with 13 large tubercles (0.5–2 cm) affecting four different lobes. One necrotic focus smaller than 1 mm was observed in the L tbLN of this animal. In contrast, non-vaccinated challenged wild boar #1 and #2, exposed to  $10^2$  cfu and  $10^6$  cfu, only showed tonsil and tbLN or mLN lesions, respectively (Table 3).

At histopathology, the non-vaccinated challenged group showed a wide range of bTB-compatible lesions. Non-vaccinated challenged wild boar #3 and #4 showed epithelioid granulomas

**Table 3**  
Experimental group, visible lesions of tuberculosis, presence of acid-fast bacilli, and culture results for 12 wild boar used in the infection experiments.

Anim. #	Experimental group	Challenge (cfu, oropharyngeal)	Visible lesions	Acid-fast bacilli	Lesion score	Culture	Culture score
1	Non-vaccinated challenged	$10^2$	Tonsil	–	1	–	0
2	Non-vaccinated challenged	$10^6$	L and R mLN	–	4	Tons, L mLN	2
3	Non-vaccinated challenged	$10^6$	L&R mLN, L and R tbLN, mediastinal LN, hepatic LN, lung, spleen	Lung+	19	Hepatic LN; mLN; tbLN; Lung	4
4	Non-vaccinated challenged	$10^4$	L tbLN, ung (4)	–	20	Lung, L tbLN	2
5	Vaccinated challenged	$10^2$	–	–	0	–	0
6	Vaccinated challenged	$10^2$	–	–	0	–	0
7	Vaccinated challenged	$10^4$	–	–	0	–	0
8	Vaccinated challenged	$10^4$	L mLN	–	1	L mLN	1
9	Vaccinated challenged	$10^6$	L and R mLN, L and R parotid LN, L and R lateral retropharyngeal LN, L tbLN	–	12	Tons, L mLN	2
10	Vaccinated challenged	$10^6$	L and R mLN, L and R parotid LN, L tbLN, lung	Lung + Tonsil +	16	Tons, L mLN; L tbLN	3
11	Control	–	–	–	0	–	0
12	Control	–	–	–	0	–	0

Anim. #, animal number; cfu, colony forming units; L, left; R, right; tb, tracheobronchial; m, mandibular; LN, lymph node.



**Fig. 2.** Culture scores (upper graph) and lesion scores (lower graph) for the non-vaccinated challenged wild boar ( $N=4$ , open symbols) and the vaccinated challenged wild boar ( $N=6$ , solid symbols). The shape of each plot represents the challenge dose: triangles  $10^2$  cfu, squares  $10^4$  cfu, circles  $10^6$  cfu. The solid lines show the median values.

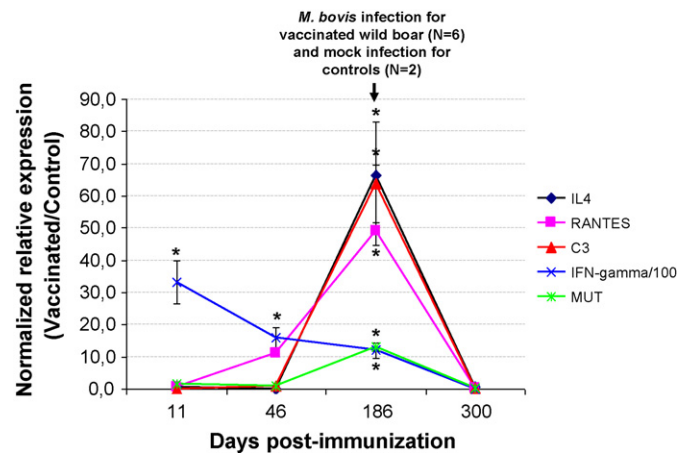
with or without variably mineralized central necrosis. In addition, non-vaccinated challenged wild boar #3 showed low numbers of acid-fast bacilli in the Ziehl-Neelsen stains of the lung. Wild boar #1 had one single tubercle smaller than 1 mm diameter and with a necrotic mineralized core in the tonsil, and one initial granuloma composed of macrophages and lymphocytes in the left tbLN. Wild boar #2 had several confluent granulomas in the mLNs, some of them with central necrosis and only limited calcification.

Among the six vaccinated challenged wild boar, the three animals with visible bTB-compatible lesions corresponded with the two that received the highest challenge dose ( $10^6$  cfu) and one that received an intermediate dose ( $10^4$  cfu). The lesion observed in the latter was minimal, consisting of one single 3 mm diameter necrotic focus in the left mandibular LN. Both vaccinated wild boar challenged with  $10^6$  cfu developed severe lesions: Wild boar #9 showed numerous bTB-compatible lesions in head and neck LNs and a single small focus in the left tbLN. No lung lesions were however detected in this animal. Wild boar #10 showed lesions in both mLN, the lung was severely affected with miliary lesions, and scarce acid-fast organisms were observed in Z-N stained tonsil and lung tissue.

### 3.3. Gene expression analysis

The mRNA levels of IL4, RANTES, C3, IFN-gamma and MUT were analyzed in PBMC using qRT-PCR. The qRT-PCR data revealed that all five genes were significantly upregulated at the time of *M. bovis* infection (186 dpi) in vaccinated animals as compared to non-vaccinated ones (Fig. 3). IFN-gamma levels were also significantly upregulated at 11 and 46 dpi (Fig. 3). At the end of the experiment at 300 dpi the mRNA levels for all genes were similar between vaccinated challenged and control wild boar (Fig. 3).

The mRNA levels of IL4, RANTES, C3, IFN-gamma and MUT were also compared between three vaccinated protected and three vaccinated infected animals. Significant upregulation was observed in



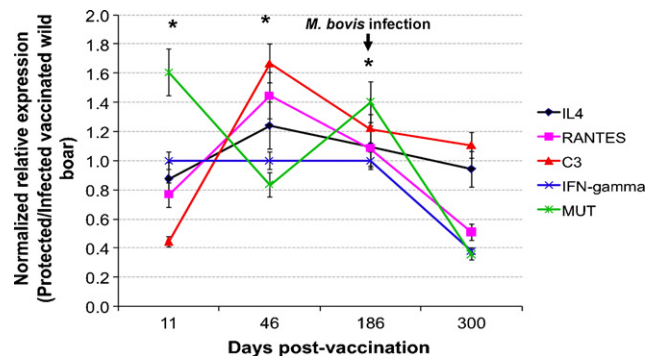
**Fig. 3.** Quantitative IL4, RANTES, C3, IFN-gamma and MUT gene expression analysis in PBMC of orally vaccinated European wild boar and controls using qRT-PCR. Results were recorded as the ratio of vaccinated to control animals after normalization for cyclophilin gene expression using the  $2^{-\Delta\Delta Ct}$  method for each of the four time points (11, 46, 186 and 300 dpi). In all cases, the mean of triplicate values was used to compare data by ANOVA followed by a series of Tukey's post hoc tests for pair comparisons between vaccinated and control animals ( $*p < 0.05$ ). Values are shown as average  $\pm$  SD.  $N=6$  for each group at time points 11, 46 and 186 dpi. At 300 dpi,  $N=6$  for vaccinated wild boar and  $N=2$  for controls.

vaccinated protected animals for C3 at 46 dpi and for MUT at 11 and 186 dpi (Fig. 4).

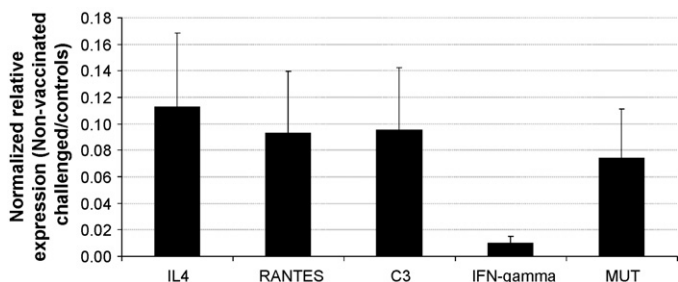
Finally at the end of the experiment (300 dpi), the IL4, RANTES, C3, IFN-gamma and MUT mRNA levels were compared between non-vaccinated challenged and control animals. In all cases, significantly lower mRNA levels were observed in non-vaccinated challenged animals (Fig. 5).

## 4. Discussion

To the best of our knowledge this is the first report on experimental infection of wild boar with *M. bovis*. This study describes a preliminary infection model with a small sample size. Data from this work will allow expanding the study to more animals using an appropriate challenge dose. The results provide valuable information on the response of this wildlife bTB reservoir host to oral BCG immunization. Furthermore, this study provides the first data



**Fig. 4.** Quantitative IL4, RANTES, C3, IFN-gamma and MUT gene expression analysis in PBMC of orally vaccinated European wild boar using qRT-PCR. Results were recorded as the ratio of vaccinated protected to vaccinated infected animals after normalization for cyclophilin gene expression using the  $2^{-\Delta\Delta Ct}$  method for each of the four time points (11, 46, 186 and 300 dpi). In all cases, the mean of triplicate values was used to compare data by ANOVA followed by a series of Tukey's post hoc tests for pair comparisons between vaccinated protected and vaccinated infected animals ( $*p < 0.05$ ). In all cases, the mean of triplicate values was used and data was compared by Student's *t*-test ( $*p < 0.05$ ). Values are shown as average  $\pm$  SD.  $N=3$  for each group.



**Fig. 5.** Quantitative IL4, RANTES, C3, IFN-gamma and MUT gene expression analysis in PBMC of European wild boar using qRT-PCR. Results were recorded as the ratio of non-vaccinated challenged ( $N=4$ ) to controls ( $N=2$ ) after normalization for cyclophilin gene expression using the  $2^{-\Delta\Delta Ct}$  method at the end of the experiment (300 dpi). In all cases, the mean of triplicate values was used to compare data by ANOVA followed by a series of Tukey's post hoc tests for pair comparisons between non-vaccinated challenged and control animals. All comparisons were statistically significant ( $p < 0.05$ ). Values are shown as average + SD.

on the differential expression of five immunoregulatory genes in response to oral BCG and to *M. bovis* infection, suggesting that some of these genes may be associated with a protective response to *M. bovis* infection in wild boar.

All four non-vaccinated challenged wild boar developed bTB-compatible lesions after exposure to  $10^2$  cfu to  $10^6$  cfu of *M. bovis* field strain. However, infection was not confirmed by isolation in the wild boar exposed to the lowest dose. Two wild boar exposed to high doses ( $>10^2$  cfu) developed lung lesions. Mild lung lesions are often observed in naturally *M. bovis*-infected wild boar [29]. However, severe generalized lesions such as those found in the non-vaccinated challenged wild boar exposed to  $10^6$  cfu are less frequently observed in the field [35]. Of the three wild boar with culture confirmed *M. bovis* infection, one had only lung and tLN lesions. This lesion distribution is rather infrequent in natural infections, where mandibular LN lesions are the most frequently observed visible lesions. However, lung lesions can be missed in the field since only a piece of the lung is usually collected and examined [29]. Despite this exception, we consider that exposing wild boar to a dose of  $10^4$  cfu of *M. bovis* by the oropharyngeal route is an adequate protocol to produce an infection in this species. The oropharyngeal route used might account in part for the variable levels of disease induced by the same challenge dose. In future studies, it would be important to describe the pathology of wild boar repeatedly exposed to variable doses, as compared to the single dose challenge used in this study. Also, a longer experimental time would allow knowing if the initial tonsil and mLN lesions eventually develop into generalized bTB. We infer that experimental infections of Eurasian wild boar with *M. bovis* need at least 114 days post-infection, and suggest that slightly longer periods are desirable.

The relatively short experimental time was probably the explanation to the observation that none of the two control wild boar became infected, despite sharing the same box with four infected wild boar (non-vaccinated challenged group), one of them with severe lung lesions. Alternatively, this observation would suggest a limited ability of wild boar to transmit *M. bovis* to individuals of the same species. Data on the time needed for *M. bovis* transmission are scarce. In one experiment by Little et al. [36] calves exposed to diseased badgers became infected mostly within 6 months of exposure. In experiments with cattle, in-contact animals often remained not infected even after 1 year [37].

In wild boar, gene expression has been shown to be affected by *M. bovis* infection [30–32,38,39]. Some of these genes such as C3 and MUT were shown to correlate with resistance to *M. bovis* infection in wild boar [3,32] while others such as IL4, RANTES, C3 and IFN-gamma were upregulated in response to parenteral BCG immu-

nization [18]. Despite the fact that pooling data across animals with different *M. bovis* challenge dose is a limitation of the analysis, the results of gene expression reported herein confirmed previous findings in wild boar after parenteral BCG vaccination and in naturally *M. bovis*-infected animals. The analysis of mRNA levels in PBMC of infected and uninfected wild boar showed downregulation of IL4, RANTES, C3, IFN-gamma and MUT gene expression in infected animals, a result that agreed with previous findings in naturally *M. bovis*-infected animals [3,18,30–32,38] and in experimentally *M. avium avium*-infected animals (Garrido et al., unpublished results). The immune response of BCG orally vaccinated wild boar was very similar to that observed after parenteral BCG vaccination [18], a result that suggested the possibility of using IL4, RANTES, C3, IFN-gamma and MUT gene expression to evaluate wild boar response to vaccination. Furthermore, as in previous reports [3,30–32,38], the comparison between vaccinated protected and vaccinated infected wild boar suggested that C3 and MUT expression levels correlated with protection at least at some time points after BCG oral immunization. Taken together, these results showed at the molecular level that wild boar respond to oral and parenteral BCG vaccination with similar gene expression profiles and provided additional evidences that C3 and MUT expression correlate with protection to *M. bovis* infection in this species.

Apparently, animals exposed to the highest challenge dose of  $10^6$  cfu developed bTB after BCG vaccination. However, vaccinated wild boar that were challenged with  $10^2$  cfu to  $10^4$  cfu remained either uninfected or developed only a limited infection. This result suggested that a single oral BCG vaccination might protect wild boar from infection by a virulent *M. bovis* field strain. However, if the infection challenge is too high, the host is probably unable to respond adequately and develops clinical bTB. This result is relevant from a wildlife management point of view. For instance, low dose exposures are expected if transmission takes place by social interactions among wild boar or by direct or indirect contact with other *M. bovis*-infected hosts. In contrast, exposure of wild boar to a very high dose could occur by contact with gutpiles or by carrion consumption. This suggests that controlling these sources of massive exposure to *M. bovis* is important for bTB control in wild boar habitats.

In conclusion, exposing wild boar to a dose of  $10^4$  cfu of *M. bovis* by the oropharyngeal route is an adequate protocol to produce an infection model in this species. The results reported herein suggest that oral BCG vaccination of wild boar may be useful for reducing *M. bovis* infection and clinical disease. At the molecular level, oral BCG immunization of wild boar results in the upregulation of immunoregulatory genes that may be associated with protective response to *M. bovis* infection in this species. More studies on vaccine efficacy, with larger sample sizes; on vaccine delivery success under real field conditions; and on vaccine safety regarding wild boar and other accidental hosts as well as BCG excretion after vaccination, will be needed to confirm if oral vaccination with BCG could be used to immunize free ranging wild boar against *M. bovis*.

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