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Tuberculosis vaccination sequence effect on protection in wild boar

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ABSTRACT

The Eurasian wild boar (*Sus scrofa*) is a reservoir for tuberculosis (TB) in which vaccination is a valuable tool for control. We evaluated the protection and immune response achieved by homologous and heterologous regimes administering BCG and heat-inactivated *Mycobacterium bovis* (IV). Twenty-one wild boar piglets were randomly allocated in five groups: Control, homologous BCG, homologous IV, heterologous IV-BCG, heterologous BCG-IV. Significant 67% and 66% total lesion score reductions were detected in homologous IV (IVx2) and heterologous IV-BCG groups when compared with Control group ($F_{4,16} = 6.393$, $p = 0.003$; Bonferroni $_{\text{Control vs IVx2}}$ $p = 0.026$, Tukey $_{\text{Control vs IV-BCG}}$ $p = 0.021$). No significant differences were found for homologous BCG (although a 48% reduction in total lesion score was recorded) and BCG-IV (3% reduction). Heterologous regimes did not improve protection over homologous regimes in the wild boar model and showed variable results from no protection to similar protection as homologous regimes. Therefore, homologous regimes remain the best option to vaccinate wild boar against TB. Moreover, vaccine sequence dramatically influenced the outcome underlining the relevance of studying the effects of prior sensitization in the outcome of vaccination.

1. Introduction

TB is a major concern to both human and animal health worldwide. Animal TB produces economic losses in the livestock industry, has zoonotic potential and is an issue in wildlife conservation [1]. Furthermore, the scenario of animal TB is complex because, although cattle are the key domestic host and the main TB control target, other livestock and several wild hosts do contribute to *Mycobacterium tuberculosis* complex (MTC) maintenance [2].

In wildlife, vaccination is perceived as a feasible disease control option for cost effective and long-term TB control [3]. Thus, vaccination is studied worldwide in several reservoir host models in laboratory and field trials [4–6] using the live attenuated Bacille Calmette Guerin (BCG). BCG does not provide sterilizing immunity and has shown variable efficacy in cattle and wildlife [7–9]; therefore, improved vaccines or alternative immunization strategies such as prime-boost regimes that provide better protection are being investigated [10].

Heterologous prime-boost vaccination consists of priming the immune system against target antigen/s with one formulation and subsequently boosting with the same antigen/s using a different type of vaccine [11,12]. This strategy aims to achieve an additive or synergistic effect that strengthens vaccine-induced protection or expands duration of immunity and is perceived as a potential strategy to control tuberculosis (TB) by immunization. In fact, the cellular response generated is stated to be stronger, broader and more durable than the one achieved by homologous vaccination regimes [13,14]. This approach can be useful to control intracellular pathogens, as those causing TB, that require powerful T cell responses [11].

In Spain, the native Eurasian wild boar (*Sus scrofa*) is considered the main wild reservoir for MTC [15], displaying a high TB prevalence (63%) and an increasing trend (e.g. in Ciudad Real province, Spain, from 2000 to 2012 [16]), posing an evident risk to cattle [17]. In this context, wild boar vaccination represents a valuable tool that could be implemented in an integrated strategy for TB control in Mediterranean

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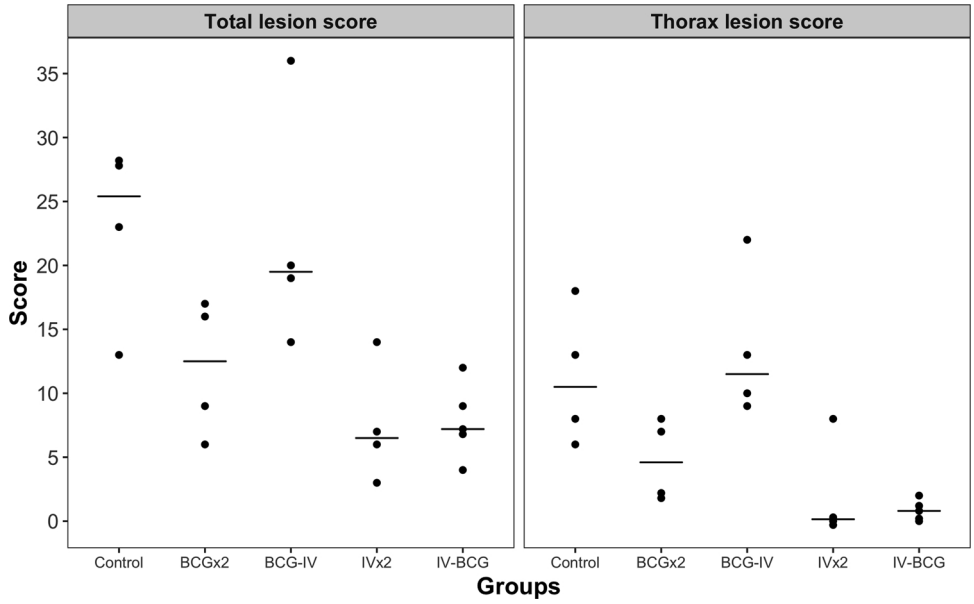

Fig. 1. Individual total (left panel) and thorax lesion score (right panel) values by group. Solid lines represent median values for each group.

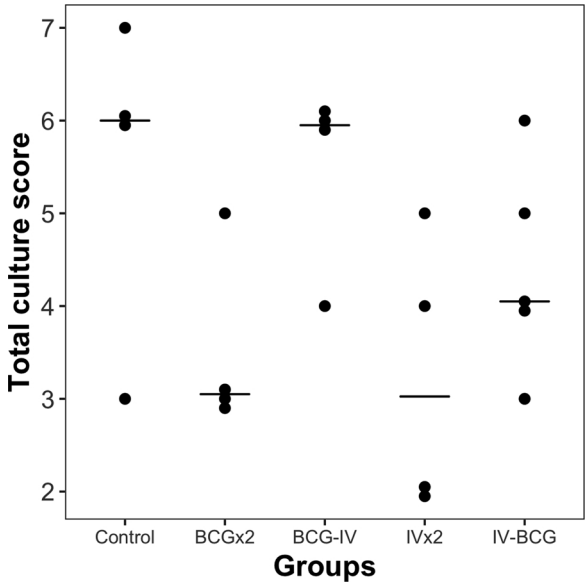

Fig. 2. Individual total culture score values by group. Solid lines represent median values by group.

Iberia.

Consequently, laboratory trials with BCG [18] and a recently developed heat-inactivated *Mycobacterium bovis* vaccine (*M. bovis* inactivated vaccine, IV) have been conducted showing comparable levels of protection of both vaccines in wild boar [19]. Homologous re-vaccination (administering more than one dose of the same vaccine) with both BCG [20] and IV [21] resulted in increased protection against *M. bovis* challenge as compared to single dose strategies [19] i.e. homologous vaccination works in the wild boar model. Moreover, recent field trials evidenced a positive effect of homologous prime-boost strategies for parenteral IV and oral IV, but not for oral BCG [9,22].

The aim of this study is to evaluate the protection and the immune response achieved by heterologous regimes compared to homologous regimes administering BCG and IV by the oral route in wild boar under the hypothesis that heterologous prime-boost strategies will be more protective than homologous prime-boost ones [12].

2. Material and methods

2.1. Ethics statement

The protocol was approved by the Committee on the Ethics of Animal Experiments of the Regional Agriculture Authority (Diputación Foral de Bizkaia, Permit Number: JAVACON-2013/6329-BFA). Handling procedures and sampling frequency were designed to minimize stress and health risks according to European (Directive 63/2010) and Spanish legislation (Royal Decree 53/2013 and Act 6/2013).

2.2. Animals and experimental design

Twenty-one 3 to 4 month-old male wild boar piglets were purchased from a commercial farm known to be free of mycobacterial infections and tested on arrival by bPPD ELISA [23] to confirm absence of antibodies against *M. bovis*, yielding a fully negative result.

During the experiment wild boar were housed in the Biosafety Level 3 containment of the Basque Institute for Agricultural Research and Development (NEIKER-Tecnalia) with *ad libitum* food and water and monitored daily. The piglets were randomly allocated in five groups: Control (n = 4), homologous BCG (n = 4; BCGx2), homologous IV (n = 4; IVx2), heterologous IV-BCG (n = 5), heterologous BCG-IV (n = 4).

The wild boar vaccination schedule applied was the same used in previous experiments [18–20]. Briefly, piglets were vaccinated orally with the first dose at T_0, revaccinated one month later on T_1 (day 31), challenged 4 months later on T_2 (day 125), sampled on T_3 (day 183) and necropsied on T_4 (day 238). During the first four handlings restraint was not longer than 10 min and anaesthesia was not required. Vaccines were delivered orally by means of a syringe. Challenge involved the oropharyngeal administration of 2 ml of a suspension containing 10^5 colony-forming units (CFU) of an *M. bovis* field strain (spoligotype SB0339, www.mbovis.org). At the end of the experiment (fifth handling), animals were anesthetized with the protocol described by Barasona et al. ([24]; intramuscular injection of tiletamine-zolazepam, 3 mg/kg, and medetomidine, 0.05 mg/kg) and euthanized by the use of the captive bolt method.

Table 1
Description of culture results in each individual by tissue and by experimental group (Control, BCGx2, BCG-IV, IVx2, IV-BCG). Tissues are further grouped and categorized as cephalic, thoracic and abdominal to show differences in regional involvement. Positive culture results appear in grey and negative culture results appear in white.

Group	Id	Tonsils	Mandibular LN	Parotid LN	Retropharingeal LN	Tracheobronchial LN	Mediastinic LN	Lung	Ileocecal Valve – Mesenteric LN pool
Control	1	■	■		■	■		■	■
	2	■	■			■	■	■	■
	3	■	■	■	■	■	■	■	
	4	■	■		■	■			
BCGx2	5		■			■	■		
	6	■	■			■			
	7		■			■		■	
	8	■	■			■			■
BCG-IV	9		■	■	■	■	■		■
	10	■	■	■	■		■	■	
	11	■	■	■	■	■			
	12	■	■			■			■
IVx2	13	■	■		■	■	■		
	14	■	■	■	■				
	15	■	■						
	16	■	■						
IV-BCG	17	■	■		■	■	■		■
	18	■	■		■	■	■	■	
	19	■	■						■
	20	■	■			■			■
	21	■	■	■	■				
Regional involvement		Cephalic				Thoracic			Abdominal

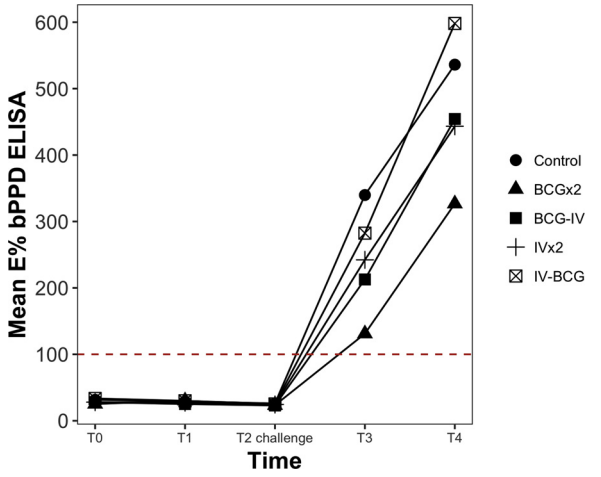

Fig. 3. Mean antibody response (mean E%) of the bPPD ELISA by group for each sampling time point. Horizontal dashed line stands for cut off level (negative < 100 E%≤ positive).

2.3. Vaccines

The two vaccines involved in the experiment were BCG (*M.bovis* BCG Danish reference strain, CCUG 27,863) and IV (obtained from a *M. bovis* field isolate spoligotype SB0339, Neiker 1403). The vaccines were prepared as described for previous experiments [18,19]. Single BCG and IV doses contained 10^6 and 10^7 CFU, respectively, in 2 ml. The Control group received 2 ml PBS. BCG was freshly prepared (< 24 h) and stored in refrigeration (4 °C) until delivery. Both vaccines and PBS were placed in the syringe just prior administration and delivered in the back of the mouth to ensure swallowing and avoid spillage.

2.4. Sample collection and necropsy

Blood samples were collected without anticoagulant for serum preparation to conduct bPPD ELISA, Complement component C3 ELISA and the cytokine array; in lithium heparin to conduct interferon-gamma (IFN-γ) release assays (IGRA) and in EDTA to perform the peripheral blood mononuclear cells (PBMC) RNA PCR.

A thorough post-mortem examination was conducted to assess visible TB-compatible lesions by carefully inspecting selected tissues after slicing them into 1–2 mm thick pieces. Organs inspected included lymph nodes (mandibular, parotid, retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric, ileocecal and inguinal), oropharyngeal tonsils and visceral organs (lung considering each lobe separately, spleen, liver and kidneys). Scoring of TB-compatible lesions was based on lesion distribution in the inspected organs and lesion severity (categorized from 0 to 4 according to the presence, size and number of lesions) following the method described in Ballesteros et al. [18].

2.5. Microbiology

Samples for culture were immediately processed. Tonsils, mandibular lymph nodes (LN), parotid LN, retropharyngeal LN, tracheobronchial LN, mediastinic LN, lung and a pool of ileocoecal valve and mesenteric LN were cultured after tissue homogeneization and isolates were spoligotyped in order to confirm the strain following the procedure in Garrido et al. ([19]). A culture score was calculated (number of samples yielding a positive result of the total number of cultured samples, score range 0–8) as previously described [19].

2.6. Serology

2.6.1. Antibody response to bPPD

Serum samples (T_0 to T_4) were tested for immunoglobulin G (IgG) against purified protein derivative (PPD) from *M. bovis* (bPPD; CZ Veterinaria, Porriño, Spain) using IgG antibodies (Bethyl, Inc., Montgomery, TX) as a conjugate by means of an in-house ELISA (protocol described in Boadella et al., [23]). Sample results were expressed as an ELISA percentage (E%) that was calculated using the formula: Sample E% = [(mean sample OD/ 2 x mean negative control OD) x100]. Samples with E% values ≥100 were considered positive.

2.6.2. Complement component C3 determination

For the quantitative determination of pig C3 protein concentration (μg/ml) in serum samples at T_0, T_2 and T_4, a sandwich ELISA was used (Pig Complement C3 ELISA kit, CUSABIO, Wuhan, China). Serum samples and standards were analysed following the manufacturer's instructions. Data were linearized by a standard curve and regression analysis was used to determine sample C3 concentrations.

2.6.3. Cytokine determination

The cytokine concentration in pooled sera was determined at T_0, T_2 and T_4 using the Quantibody porcine cytokine array (RayBiotech Inc, Norcross, GA, USA), an array- based multiplex ELISA system for the simultaneous quantitative measurement of multiple cytokines, following the recommendations of the manufacturer. The signals were visualized using a Gene Pix 4100A laser scanner (Molecular Devices,

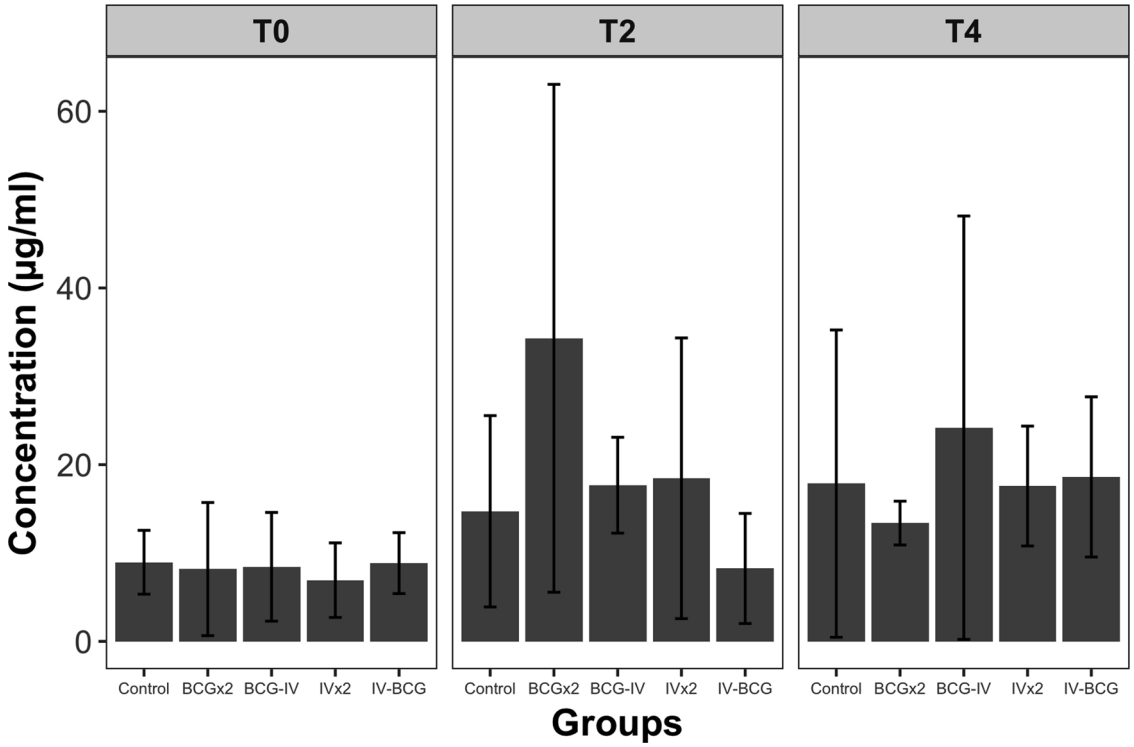

Fig. 4. Serum C3 concentration (µg/ml) by group at T_0 (basal), T_2 (after completing vaccination regime) and T_4 (at necropsy, ca. 4 months after challenge).

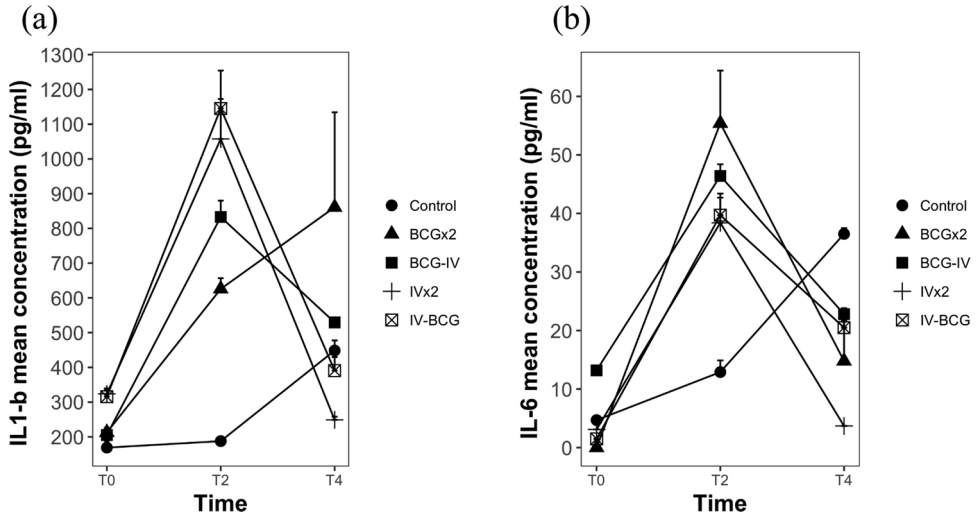

Fig. 5. (a) Mean IL-1B and (b) mean IL-6 concentration in serum (pg/ml) before vaccination (T_0), after full vaccination regime (T_2) and by end of the experiment (T_4) for each group. Error bars represent standard deviation of spot values for each cytokine in each well (sample).

Sunnyvale, CA, USA) and data were extracted by GenePix Pro 6 software (Molecular Devices). Finally, the quantitative data analysis was performed using the Quantibody Q-Analyzer software (RayBiotech Inc). Cytokine concentration was expressed in pg/ml.

2.7. IGRA

At T_2, T_3 and T_4, blood samples were collected into tubes with lithium-heparin. Within 8 h of collection, stimulation of whole blood with PBS (nil control) and the avian and bovine purified protein derivative (PDD) (CZ Veterinaria, Porriño, Spain) was performed as described for other species [25]. The detection of IFN- γ in the supernatant was performed using a quantitative ELISA (IFN gamma Porcine ELISA Kit, Thermo Fisher Scientific, Madrid, Spain), according to the manufacturer's recommendations.

2.8. RNA isolation and real time RT-PCR

Total RNA was extracted from wild boar PBMC using ARNzol spin kit (Real, Durviz, Spain) following manufacturer's recommendations. RNA was used for quantitative real- time RT-PCR analysis of mRNA levels of selected genes in individual samples. Selected genes were complement component 3 (C3), interleukin-1beta (IL-1B) and methylmalonyl CoA mutase (MUT). Real-time RT-PCR was performed with gene-specific primers (C3, SsC3-L: acaaattgacccagcgtagg and SsC3-R: gcacgtccttgctgtactga; IL-1B, SsIL1beta-L: ccaaagagggacatg gagaa and SsIL1beta-R: ttatatcttggcggcctttg; MUT, Ss MUT-L: gtttgccaacggtgaaaagt and SsMUT-R: aatgagcttcaaggcagcat) using the Quantitech SYBR Green

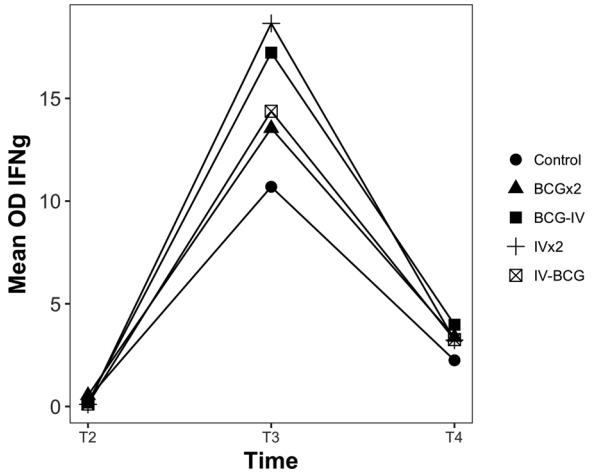

Fig. 6. Mean optical density (OD) readings of the interferon-gamma (IFN-γ) release assay at T_2 (after full vaccination regime), T_3 (2 months post challenge) and T_4 (ca. 4 months post challenge) by group.

RT PCR kit and the Rotor GENE-Q (Qiagen Inc. Valencia, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of RT-PCR reaction to ensure that only one amplicon was formed and that the amplicon denatured consistently at the same temperature range for every sample [26]. All reactions were performed in duplicate. The mRNA values were normalized against *Sus scrofa* cyclophilin (SsCyclophilin-L: agcactggggagaaaggatt and SsCyclophilin-R: cttggcagtgcaaatgaaaa), using the genNorm ddCT method [27]. The normalized expression was calculated at each time point and the mean of replicate values was used to compare data between vaccinated and control groups.

2.9. Statistical analyses

Variables that met normality and homoscedasticity assumptions (tested by Shapiro and Levene´s test respectively) were: total lesion score, culture score, serology values (%E); in them comparisons between groups were conducted using ANOVA and subsequent Bonferroni posthoc. Thorax lesion score, IFN- γ optical densities (OD), C3 concentration and gene expression results met the homoscedasticity assumption but are not normally distributed, thus comparisons between groups were conducted using Kruskal-Wallis and subsequent Dunn's test of multiple comparisons using rank sums. Correlation among serology (E%) and total lesion score values were performed using Pearson´s rank test while correlation among IFN-γ OD, C3 concentration and gene expression with total lesion score values were performed using Spearman´s rank test. Significance was fixed at $p < 0.05$. Analyses were carried out in the R statistical package (R Development Core Team) using the ggplot2 library to obtain the figures [28].

3. Results

3.1. Clinical signs

No clinical signs were observed in any of the wild boar after vaccination or challenge.

3.2. Pathology and M. bovis isolation

All wild boar developed TB-compatible lesions, and infection by challenge strain was confirmed by re-isolation from tissues and spoligotyping (SB0339). Fig. 1 presents individual lesion score values and median by group for total lesion score and thorax lesion score. Fig. 2 presents individual culture score values and median by group. Table 1 provides additional detail on positive culture distribution.

Considering the homologous vaccinates, wild boar of the IVx2 and BCGx2 groups showed total lesion score reductions of 67% and 48% and thorax lesion score reductions of 82% and 58%, respectively, as compared to controls. Heterologous vaccinates showed variable results. Wild boar of the IV-BCG group showed reduced total and thorax lesion score (66% and 93% respectively) when compared to controls while BCG-IV vaccinates had negligible reduction in total lesion score and

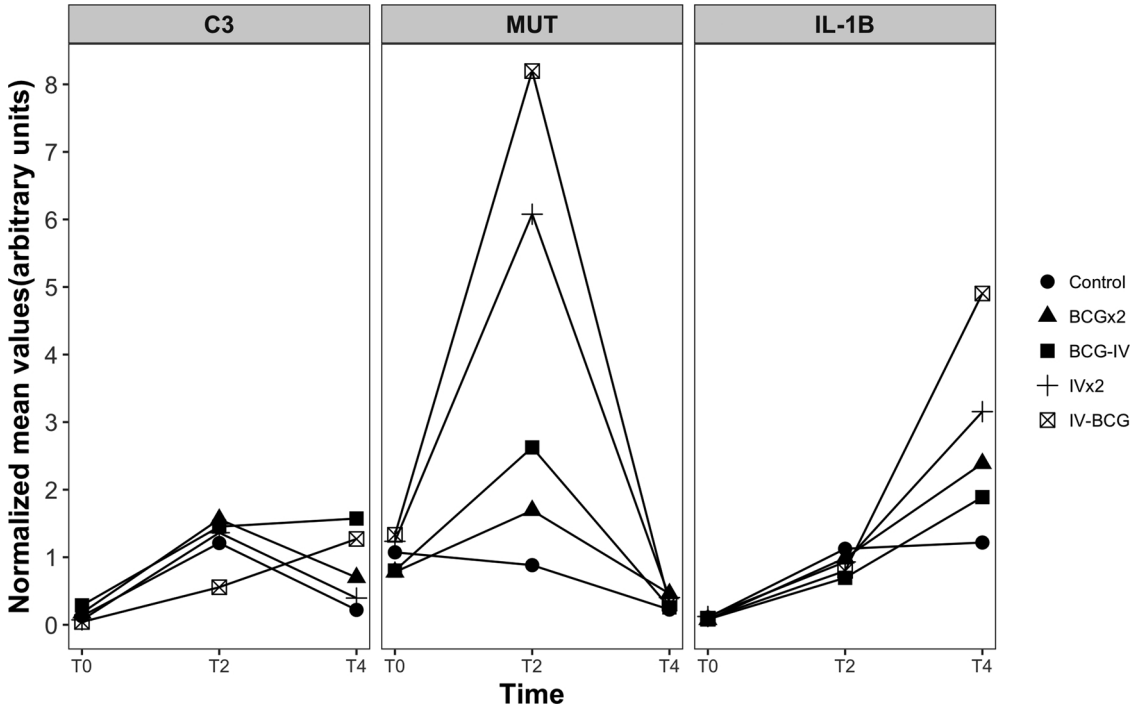

Fig. 7. Quantitative C3, MUT and IL-1B gene expression analysis in PMBC using qRT-PCR. Mean normalized mRNA levels of C3 (left panel), MUT (middle panel) and IL-1B (right panel) before vaccination (T_0), after full vaccination regime (T_2) and by end of the experiment (T_4) for each group.

worse performance than controls in thorax lesion score (3% reduction and 20% increase, respectively).

This was confirmed by the detection of significant differences among groups in the total lesion score ($F_{4,16} = 6.393$, $p = 0.003$). Post hoc tests revealed differences only among Control group and IVx2 and IV-BCG (Bonferroni $p = 0.026$ and $p = 0.021$ respectively) and between BCG-IV group and IVx2 and IV-BCG (Bonferroni $p = 0.038$ and $p = 0.030$ respectively).

Differences among groups in thorax lesion score were also significant (Kruskal-Wallis chi-squared = 14.68, df = 4, $p = 0.005413$) and followed the same pattern affecting Control group versus IVx2 and IV-BCG (Dunn $p = 0.0099$ and $p = 0.0063$ respectively) and BCG-IV versus IVx2 and IV-BCG (Dunn $p = 0.0022$ and $p = 0.0012$ respectively).

Regarding lesion distribution, four individuals presented localized lesions circumscribed to the head and neck region (no thoracic or abdominal lesions, thus no generalization). These individuals belonged to groups IVx2 (3/4) and IV-BCG (1/5). Subsequent culture confirmed contained infection (negative thoracic cultures) in the three individuals belonging to the IVx2 group. Differences of total culture score with controls were not significant ($F_{4,16} = 2.7$, $p > 0.05$).

3.3. Serological analysis

3.3.1. Antibody response to bPPD

Antibody levels against bPPD are represented in Fig. 3. Antibody levels remained negative until challenge (T_0-T_2), thus oral vaccination did not generate antibody response. Positive responses started to be detected at T_3 and increased at T_4. Response to challenge generating detectable antibodies did not occur in all animals (at T_3 one individual of each group and at T_4 one individual of the BCGx2 group had not seroconverted, respectively). Differences between groups were not significant at any time point ($p > 0.05$), further information on mean E % and standard deviation values by group for each time point is available in SM Table 1. However, individuals of the Control group and the IV-BCG group had the highest responses, while those of the BCGx2 group had the lowest responses. No correlation between antibody response at T_4 and lesion or culture scores was found ($p > 0.05$).

3.3.2. Complement component C3

Serum C3 protein levels are shown in Fig. 4. No significant differences were detected among groups for any time point ($p > 0.05$), due to high individual variability. Serum C3 protein levels were significantly higher at T_2 and T_4 than in T_0 (Kruskal-Wallis chi-squared = 11.995, df = 2, $p = 0.002485$; Dunn $p = 0.0078$ and $p = 0.0004$ respectively). No significant correlation between C3 in serum and lesion or culture scores was detected ($p > 0.05$).

3.3.3. Cytokines in serum

As Fig. 5a and b show for IL-1B and IL-6 respectively, the levels of these cytokines at T_0 were similar among groups, rose at T_2 in vaccinated animals and decreased post-infection, at T_4, except for the Control group for both cytokines and BCGx2 for IL-1B.

3.4. IGRA

No response to bPPD stimulation was detected at T_2. An IFN-γ peak appeared at T_3 in all groups (mean OD ± SE ranging from 18.7 ± 4.5 in IV−IV group to 10.7 ± 1.8 in controls) and a sharp decline (mean) at necropsy time (T_4) as shown in Fig. 6. All PBS controls yielded consistently low results (mean OD ± SE, 0.12 ± 0.01). Mean OD and standard deviation values by group for each time point are shown on SM Table 2. Differences among groups were not significant at any time ($p > 0.05$). No correlation with lesion or culture score was evidenced ($p > 0.05$).

3.5. Gene expression

The C3, MUT and IL-1B mRNA levels were analysed in PBMC at T_0 (before vaccination), T_2 (after full vaccination regime), and T_4 (at the end of the experiment ca. 4 months after challenge). Fig. 7 shows normalized mRNA values for C3 (left panel), MUT (middle panel) and IL-1B (right panel) by group for each time point. Mean normalized values (arbitrary units) and standard deviation for C3, MUT, IL-1B genes by group for each time point are displayed in SM Table 3.

C3 expression levels significantly increased at T_2 and at T_4 (mostly driven by heterologous vaccination groups) compared to T_0 (Kruskal-Wallis chi-squared = 25.5436, df = 2, $p = 0.000$, Dunn $p = 0.000$ and $p = 0.000$ respectively). Significant difference among groups was detected at T_4 (Kruskal-Wallis chi-squared = 11.5413, df = 4, $p = 0.02$) and the Dunn test was significant for pair comparisons involving heterologous regimes versus Controls and IVx2 (for BCG-IV, $p = 0.0037$ and $p = 0.0230$ respectively; for IV-BCG, $p = 0.0035$ and $p = 0.0243$ respectively). No correlation with lesion or culture score was evidenced ($p > 0.05$).

MUT expression rose significantly after vaccination (at T_2; Kruskal-Wallis chi-squared = 35.0347, df = 2, $p = 0.000$, Dunn $p = 0.000$ for both T_0 and T_4) and was of similar magnitude among BCG primed groups and IV primed groups, respectively. Significant differences among groups were found at T_2 (Kruskal-Wallis chi-squared = 11.3756, df = 4, $p = 0.02$). Dunn posthoc analysis revealed significantly higher MUT expression levels in the IVx2 group compared to Controls ($p = 0.0071$) and in the IV-BCG group as compared to Controls ($p = 0.0021$) and to the BCGx2 group ($p = 0.0175$). MUT gene expression levels at T_2 negatively correlated with total lesion score ($r_s = -0.499$, $p = 0.021$).

Regarding IL-1B, expression levels increased significantly towards the end of the experiment (Kruskal-Wallis chi-squared = 40.3924, df = 2, $p = 0.000$, significant for pair comparisons T_2 against T_0 and T_4, $p = 0.000$ and $p = 0.014$ respectively, and T_4 against T_0, $p = 0.000$). No significant differences among groups were detected ($p > 0.05$) although gene expression levels rose at T_4 in vaccinated groups and more intensely in IV primed groups. No significant correlations were found with lesion or culture score ($p > 0.05$).

4. Discussion

Contrary to our expectations, heterologous prime-boost strategies were not more protective than homologous prime-boost ones, at least with this specific experimental design. The results suggest that IV effectively primes the protective immune response boosted by IV or BCG. By contrast, we did not found a significant effect in BCG followed by BCG (despite the substantial reduction in total lesion score, ca. 50%, and the cumulative evidence of the protection conferred by BCG in the wild boar model, [18,20] or IV (no evidence of protection at all). In fact, heterologous regimes showed variable results ranging from no protection, in the case of BCG-IV, to similar protection as homologous regimes, in the case of IV-BCG. The protection obtained by IV-BCG is not greater than the conferred by homologous prime-boost regimes (logistically simpler to conduct, especially if IV is used) hence, in practical terms, homologous regimes are the best option for vaccination of wild boar (and pigs) against TB.

The significant reduction in lung lesions and overall dissemination seen in the IVx2 and IV- BCG groups (Fig. 1) was further highlighted by culture results (Fig. 2) and is likely to translate into decreased *M. bovis* shedding and transmission. However, the only three wild boar without lesion generalization and thoracic infection belonged to the homologous IVx2 group, suggesting that homologous prime-boosting with this heat-inactivated vaccine is a good choice for TB control in wild boar [21]. Oral delivery of IV and BCG and their combinations did not induce antibodies or IFN-γ response (Figs. 3 and 6) in agreement with results of previous studies [18–21]. Thus, both oral BCG and oral IV

vaccination, either with homologous or heterologous regimes, are compatible with TB bPPD ELISA and IGRA diagnostic, as vaccination does not incur in false positives.

The results of this study support the model for the protective mechanism elicited by IV through the activation of innate immune responses [21,29,30]. The upregulation of C3 and proinflammatory cytokines IL-1B and IL-6 in response to vaccination, in agreement with previous studies with the IV in wild boar, deer and zebrafish [19,21,29–31], support a role for C3 in the immune response to this vaccine. Moreover, complement components and proinflammatory cytokines have been identified to be involved in innate immune response to limit mycobacterial infection in vertebrate hosts including humans [19,21,29,32–34].

The implication of the obtained results in control strategies in real-life settings is that with current results, the logistical implications of implementing heterologous IV-BCG regime outweigh the benefits. Nonetheless, heterologous strategies would be probably easier to carry out in farm-like settings (where vaccine access is controlled and ensured, timespans are adjustable and individuals are identified) than in free-ranging wild boar populations (in which, despite the feasibility of deploying two types of vaccine baits, is not possible to ensure 100% compliance with an heterologous prime-boost regime). Moreover, these results also provide an additional explanation of the lack of detectable vaccine impact in a recent field trial in sites where 20–40% of wild boar accessed both vaccines [9]. It was not possible to assess the prime-boost sequence in the field trial but unintended priming with BCG or with other mycobacteria may explain the low efficacy of vaccination in those sites ([9], labelled in the study as “Natural sites”).

In the wild boar model homologous boosting with BCG does not ablate protection conferred by priming [20] but enhances protection as in the deer and possum models [35,36]. This contrasts with the bovine model in which BCG boosting has been contraindicated [37], although once vaccine-derived immunity has waned revaccination with BCG restores protection [38]. Regarding heterologous prime boosting, we found differences in protection due to sensitization sequence in our study, however the overall influence of the order of prime–boost administration remains unclear. Some authors have found that is not critical in terms of achieved protection [13,39] while others found that depending on the administration order some combinations fail to confer protection at all [40,41] as in the BCG-IV group of this experiment. These differences are probably due to the heterogeneity in vaccine types, challenge strains (*M. bovis* or *M. tuberculosis*), doses, administration and challenge routes, readouts and animal models used.

One hypothesis for the differences in protection found in our study is that the interval between prime and boost must be long enough to allow the primer to induce a response before the booster is administered [14]. The influence of timing between prime and boost has already been demonstrated in a homologous BCG vaccination study in the deer model [36] and, perhaps, the optimization of protocols is an issue to address in future experiments.

Another hypothesis for this particular vaccine failure is the interference of protection mechanisms. Empirical evidence in human and animal trials [42,43] suggests that pre-existing responses to mycobacterial antigens can interfere with protection of subsequent vaccination by blocking and/or masking mechanisms [44,45]. This sensitization is attributed to contact with environmental bacteria, but environments with high circulation of *M. tuberculosis* or *M. bovis* or even BCG vaccination (if later boosting is intended) could have a sensitizing effect as well [46]. While is often assumed that this interference affects live vaccines due to restricted persistence or replication and not to non-replicating vaccines [45] we hypothesize it could also affect protective mechanisms induced by inactivated vaccines. This interference has important implications for mycobacterial vaccination and may explain cases where no protection is obtained underlining the need to consider very early vaccination and, if possible, testing for mycobacterial sensitization before vaccinating. In this regard, is important to consider the particularities of detecting sensitization by traditional techniques when contact occurs by the oral route. In cattle *M. bovis* challenge by the oral route led to a late, less uniform and IFN-γ production and even seronegativization [47]; and non-detectable IGRA and ELISA responses to oral BCG and IV have been evidenced in several species as opposed to other administration routes [18,31,48,49]. While this is an advantage in terms of diagnostic interference it evidences the aforementioned particularities of oral route sensitization.

5. Conclusions

Heterologous regimes involving BCG and IV do not provide improved protection in the wild boar model as compared to homologous regimes. Moreover, our results indicate that when different vaccine products are administered vaccine sequence dramatically influences the outcome with differences in protection ranging from no protection at all to consistent significant reductions in scores and organic dissemination (similar to homologous regimes). Hence, in practical terms, homologous regimes are the best option for vaccination of wild boar (and pigs) against TB. These results also underline the relevance of studying the effects of sensitization in the outcome of vaccination.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.cimid.2019.101329.

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