Trained immunity is not universal: oral heat-inactivated Mycobacterium bovis confers no protection against the non-enveloped Porcine Circovirus 2

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Abstract

Background

Trained immunity, the enhanced response of innate cells leading to an improved innate immune response, and antibodies against the glycan galactose-α-1,3-galactose (α-Gal), produced by animals unable to synthesize α-Gal epitopes, have been suggested to provide the host certain advantage in infections with enveloped viruses. Conversely, the evidence of protection against non-enveloped viruses attributed to the referred mechanisms remains scarce. Aiming to evaluate whether a heat-inactivated *Mycobacterium bovis* (HIMB) immunostimulant, which had proven to protect against related and non-related pathogens, confers an advantage against non-enveloped viruses, we performed an immunization and challenge experiment with porcine circovirus 2 (PCV-2) in swine. Sixteen piglets were randomly assigned to the immunized group (n = 8), which received two oral doses of HIMB with an interval of three weeks, or to the control group (n = 8). All animals were infected by intranasal inoculation with PCV-2 21 days later and euthanized at day 21 post-challenge.

Results

No differences in body weight and body temperature, viremia and viral burden in target tissues, antibody production and histopathological changes in target tissues were observed between the immunized and the control group. Overall, oral immunization with HIMB did not protect pigs against PCV-2 infection.

Conclusions

Our study suggests that HIMB confers no advantage against pathogens lacking α-Gal, mainly non-enveloped viruses such as PCV-2, in α-Gal-producing hosts, such as the swine.

Background

The term “trained immunity” was proposed by Netea et al. (2011) [1] to refer to an adaptative response elicited by the innate immune system towards a subsequent encounter not only with the same pathogen but also with non-related ones. Trained immunity or “innate immune memory” occurs in a T and B cell-independent and non-specific manner and, thus, the innate immunity may provide cross-protection against a wide range of pathogens even in the absence of an adaptative response [2]. Trained immunity involves epigenetic modifications and metabolic changes functioning interdependently within innate cells, mainly natural killer (NK) cells and monocytes/macrophages [3–5].

The Bacille Calmette-Guérin (BCG) vaccine, a live attenuated *Mycobacterium bovis* strain, beyond being the only commercialized vaccine to prevent human tuberculosis, has emerged as one of the most widely explored inducers of trained immunity [6]. Indeed, numerous *in vitro* and *in vivo* experiences have demonstrated that BCG boosts proinflammatory cytokines production in NK cells and
monocytes/macrophages upon restimulation with non-related microorganisms, thus leading to non-specific and adaptative-independent protection in the host [6].

Since 2020, worldwide labelled as the year of the pandemic of coronavirus disease 19 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the scientific interest regarding the role of “trained immunity” in viral infections has risen (over 60 entries per year in 2020, 2021 and 2022 versus 20 or less in previous years in the Scopus database). Nevertheless, the protection against viral infection attributed to trained immunity is still unclear (Table 1). Preliminary experiences of immunization with BCG or β-glucans evidenced protection against diverse viruses. Further assessment of the effect of both immunostimulants in viral infections confirmed the reduction in viral burden, mortality, and morbidity, as well as a shift in the cytokine profile (Table 1). However, certain experimental studies reported failure of the heterologous protection expected from trained immunity inducers. For instance, no protective effect of prior BCG vaccination was observed regarding influenza infection [7], and no protective effect of β-glucans in zebrafish infected with spring viremia carp virus [8] nor in turbots infected with viral hemorrhagic septicemia virus [9]. In addition, BCG does not reduce lesions, morbidity, mortality or viral burden in the SARS-CoV-2 infection model in mice [10] or primates [11]. Most of the literature refers to a beneficial effect of trained immunity against viruses that are enveloped, while evidence of protection against non-enveloped viruses is limited (for non-enveloped viruses see: [12–18]).

Primates, including humans, as well as fish, amphibians, reptiles, and birds, evolved with the inability to synthesize the glycan galactose-α-1,3-galactose (α-Gal). Therefore, they naturally produce antibodies in response to this modification present in glycoproteins, glycolipids and other biomolecules present in other species’ cells, including gastrointestinal microbiota, and viruses [19,20]. Thus, antibodies against α-Gal have been suggested to confer non-specific protection against enveloped viruses containing α-Gal epitopes in its glycoproteins [19,21,22]. Anti-α-Gal antibodies bound to viruses may activate the complement system for virus lysis or uptake by dendritic cells and macrophages and be presented to T cells to trigger adaptative responses [19]. Trained immunity has been also proposed to be associated with α-Gal [22,23]. Accordingly, several experimental studies have demonstrated that anti-α-Gal antibodies contribute to neutralization of enveloped viruses [24,25]; thus, efficacy of enveloped virus vaccines can be increased by expression of α-Gal epitopes in the viral surface [26–29].

Although it has been more extensively explored in rodents [30–32] and humans [14,15,33], the novel phenomenon of training the innate immunity arouses growing interest in domestic animals (reviewed in Angulo and Angulo, 2022). In particular, piglets that had been administered *Saccharomyces cerevisae* β-glucan orally prior to being challenged with the enveloped swine influenza virus displayed milder clinical signs and lung lesions, as well as higher interferon (IFN)-γ and nitric oxide (NO) levels [35]. Likewise, priming with β-glucan impaired cell invasion and replication of the enveloped African Swine Fever virus and boosted IFNα and interleukin (IL)-6 levels in porcine alveolar macrophages [36]. Recently, Byrne et al. (2020) [37] provided the first evidence of trained immunity induced by BCG in pigs, as they observed that stimulating porcine monocytes with BCG *in vitro* enhanced IL-1β and tumor necrosis factor (TNF)-α gene expression and protein production upon LPS re-stimulation. In line with this finding, pigs
that had received an immunostimulant composed of heat-inactivated *M. bovis* (HIMB) via the oral route prior to being challenged with *Salmonella enterica* serovar Choleraesuis displayed increased proinflammatory cytokines production and antioxidant activity, together with reduced pulmonary lesions, compared with non-immunized pigs [38]. Moreover, HIMB also elicits partial immunity against protozoal [39] and arthropod (ticks; [40] infections in different animal models, although its cross-protection against viruses has not been evaluated to date.

Porcine circovirus 2 (PCV-2), a small non-enveloped virus, is considered one of the most relevant pathogens that affect swine worldwide, mainly due to the substantial economic losses it causes to the pig industry [41]. PCV-2 infection has been associated with various disease syndromes in both domestic pigs and wild boar, such as PCV-2-systemic disease (PCV-2-SD, formerly known as postweaning multisystemic wasting syndrome), PCV-2-reproductive disease, PCV-2-subclinical infection and porcine dermatitis and nephropathy syndrome (PDNS). Importantly, PCV-2-SD affected pigs suffer from immunosuppression [41–44]. Currently, all commercialized PCV-2 vaccines are only indicated for the parenteral route [41], which is a limitation to target wild boar and free-range pigs [45,46]. Consequently, although several attempts to develop oral vaccines composed of recombinant bacteria [47,48] or yeasts [49–52] expressing PCV-2 Cap protein have revealed promising results, there is still a long way to go before achieving an orally delivered preparation that is feasible to protect swine against PCV-2.

Thus, the objective of the present study was to evaluate the effect of the oral HIMB immunostimulant in the porcine model of infection with the non-enveloped virus PCV-2.

**Results**

**Clinical signs**

No clinical signs were observed throughout the experiment. No significant effect of the immunization was observed on either body temperature (Figure 2A) or weight gain (Figure 2B).

**PCV-2 load**

Viremia and viral load were estimated weekly by detecting PCV-2 DNA load in blood and the progression was similar in both groups: steadily increased after infection (viremia: HIMB = 3/8, control = 3/8; viral load: HIMB = 2.97 x 10^4 copies/ml, control = 3.38 x 10^4 copies/ml), reaching a peak 14 dpi (viremia: HIMB = 8/8, control = 8/8; viral load: HIMB = 5.75 x 10^4 copies/ml, control = 5.59 x 10^4 copies/ml), and then decreased until the end of the experiment (viremia: HIMB = 3/8, control = 5/8; viral load: HIMB = 2.20 x 10^4 copies/ml, control = 2.40 x 10^4 copies/ml) (Figure 3A). No differences in percentage of viremic animals and mean viral loads between groups were detected at any timepoint. PCV-2 antigen in target tissues was evaluated after necropsy, detecting low amount of antigen in both groups (mean total score: HIMB = 0.0892; control = 0.0892). Although the immunized group showed an apparently higher proportion of animals with one or more PCV-2-immunolabeled tissues (HIMB: 4/8 (50%), control: 3/8 (37.5%)), the differences between groups were not significant (\(P = 0.72\)).
**Humoral response**

Humoral response was measured through the production of anti-PCV-2 antibodies. As expected, antibody titers started raising upon infection, reaching maximum levels twenty-one days after infection (Figure 3B). No differences in antibody S/P ratios between groups were detected. No anti-P22 antibodies were detected.

**Pathological findings**

To assess protection against PCV-2, macroscopic and microscopic lesions were examined at the end of the experiment. All individuals showed a good general condition at necropsy. Among the macroscopic lesions, the main findings were moderate congestion and lymphadenomegaly in several LNs, especially in the mandibular, tracheobronquial, mediastinal, mesenteric and inguinal ones (Figure 4A). The proportion of animals with lymphadenomegaly in the pulmonary LNs (tracheobronquial and mediastinal) was significantly higher in the HIMB group (7/8, 87.5%) compared with the control group (1/8, 12.5%) (P<0.04). Microscopically, mandibular and pulmonary LNs presented mild lymphocyte depletion and low number of histiocytes and multinucleated giant cells infiltrate (Figure 4B, and Figures 5A, B), occasionally associated to low amount of PCV-2 antigen (Figure 4B inset and Figures 5A, B). Scarce presence of PCV-2 antigen also was observed in the tonsil of some animals, along with light infiltrating of histiocytes and multinucleated giant cells, and lymphocyte depletion (Figure 5C). Moreover, mild to moderate interstitial pneumonia was frequently observed in both groups, composed by infiltrate of mononuclear cells in the pulmonary parenchymal (Figures 4C, D and Figure 5D). In addition, one individual from each group showed mild interstitial nephritis (data not shown). The appearance and severity of microscopic lesions was similar between groups and no significant differences were detected (Figure 5).

**α-Gal content in PCV-2**

The presence of α-Gal in PCV-2 was assessed by a direct ELISA of the infective viral inoculum, with an uninfected PK15 cell culture as control. As expected for non-enveloped viruses such as PCV-2, α-Gal content was lower in purified viruses (0.20 ng/μg) than in cultured cells from an α-Gal producing animal such as the pig (0.43 ng/μg).

**Discussion**

Stimulating the immune system with trained immunity inducers, such as mycobacterial or fungal derived compounds, constitutes a strategy to elicit non-specific protection against a wide range of pathogens, including certain viruses [5]. However, oral immunization with HIMB, an immunostimulant that had previously demonstrated protective effect attributed to trained immunity in heterologous infections [38, 39, 61] failed to exert protective effects in the PCV-2 infection model.

A paramount concern about PCV-2-SD in the pig industry is the detriment in the average daily weigh gain [62]. In the present study, oral HIMB immunization did not influence weigh gain or body temperature in
pigs infected with PCV-2. Although [63] did not observe a beneficial effect on growth performance after immunization, most field trials showed better weight gain in pigs vaccinated with a commercial vaccine [64, 65].

Other hallmarks of PCV-2-SD in pigs are lymphocyte depletion together with granulomatous inflammation of lymphoid tissues and interstitial pneumonia [66]. In agreement with previous studies [57, 67, 68], interstitial pneumonia and enlarged LNs were our predominant findings, with occasional presence of lymphocyte depletion and histiocytic infiltrate with multinucleated giant cells, pigs orally immunized with HIMB not showing lower lesion scores the compared to the control animals. The capacity of commercialized vaccines for alleviating pathology in naturally PCV-2 infected pigs has been widely demonstrated in the field [69, 70], and the beneficial effect has also been evident under experimental conditions. For instance, Seo et al. (2014) [71] observed lower lymphoid depletion and granulomatous replacement in pigs vaccinated with either Fostera PCV/Suvaxyn PCV-2 one dose (Zoetis), Circovac (Merial), Circoflex (Boehringer Ingelheim Vetmedica) and Porcilis PCV (Merck, Sharp and Dohme Animal Health) and infected with PCV-2 compared with non-vaccinated pigs.

Oral immunization with HIMB did not reduce PCV-2 load in pig, illustrated by viremia and viral load in tissues, and did not stimulate a specific humoral response. Conversely, the efficacy of commercialized specific vaccines to reduce viremia and viral burden in tissues in pigs infected with PCV-2, associated to a strong antibody response, has been confirmed under experimental and field conditions [63–66, 71, 72].

The present study challenges the prevailing paradigm of trained immunity being universal and suggests that it might not be extendable to all pathogens, for instance, non-enveloped viruses such as PCV-2. Similarly, the lack of effect of β-glucan or BCG in experimental or natural infections with enveloped viruses had been previously reported [7–11, 73, 74] However, to the best of our knowledge, only a few studies have explored the protective efficacy of the compounds of interest against non-enveloped viruses [12–18]. Xue et al. (2017) [16] assessed the effect of Astragalus spp. polysaccharides on PCV-2 infection in an in vitro model using porcine PK15 cells and in an in vivo model using mice, and concluded that Astragalus spp. polysaccharide suppressed PCV-2 infection by inhibiting endoplasmic reticulum stress. Still, the latter results should be addressed carefully and further research is needed to extrapolate them to PCV-2 infection in its natural host, the pig.

Ultimately, we confirmed that, as expected from a non-enveloped virus [19, 21], PCV-2 does not contain α-Gal. Unlike for enveloped viruses (because the carbohydrate chains of the envelope glycoproteins are acquired from the host golgi apparatus during viral replication), activation of the innate components, such as the complement system and antigen presenting cells, mediated by natural anti-α-Gal antibodies [19, 21] would not contribute in the host immunity against non-enveloped viruses including PCV-2. Furthermore, non-specific immune antibody-mediated and non-antibody-mediated mechanisms in response to α-Gal may not be activated in α-Gal-positive hosts.

Conclusions
In conclusion, our results demonstrate that oral immunization with HIMB does not protect swine against challenge with PCV-2, a non-enveloped DNA virus. Considering the absence of α-Gal in PCV-2, our study suggests that HIMB confers no advantage against pathogens lacking α-Gal in hosts producing this molecule.

Methods

Animals and experimental design

Sixteen 21 days-old Landrace x Large White hybrid female piglets with homogeneous weights and tested as PCV-2 PCR negative with low anti-PCV2 antibody titers were obtained from a commercial pig production farm. Animals were housed in class III biocontainment animal facilities (BSL-3) located at VISAVET Health Surveillance Centre (Madrid, Spain) for acclimatization one week before the experiment. All animals received continuous access to water, non-medicated pig feed and veterinary monitorization. Individuals were randomly assigned to either the immunized group (n=8), which received two oral doses of HIMB with an interval of three weeks (-42 and -21 days pre-infection), or the control group (n=8), which received PBS instead of immunostimulant. Twenty-one days after the second immunization dose (day 0), all animals from both groups were infected by intranasal inoculation with $10^5$ TCID$_{50}$ of PCV-2 per animal (Figure 1). All pigs were confirmed as PCV-2 PCR negative and PCV-2 ELISA negative before challenge. Animals were handled on 0, 7, 14 and 21 days post-infection (dpi) for blood sampling, rectal temperature and weight measurements.

All piglets were euthanized 21 dpi by captive bolt after sedation with xylazine (Xilagesic 2%, Laboratories Calier, Barcelona, Spain) and were subjected to necropsy to assess PCV-2 gross lesions in palatine tonsil, as well as mandibular, tracheobronchial and mediastinal lymph nodes (LNs), lung (cranial and caudal lobes) and kidney. Typical PCV-2 gross lesions such as lymphadenomegaly, interstitial pneumonia and nephritis were evaluated by experienced pathologists. Lesion severity was graded with a lesion score (0 = absent, 1 = mild, 2 = moderate or 3 = severe).

Samples from palatine tonsil, lung (cranial and caudal lobes), as well as mandibular, tracheobronchial and mediastinal LNs were fixed by immersion in 10% neutral buffered formalin during 24 hours at room temperature (RT), dehydrated in a graded series of ethanol, immersed in xylol, and embedded in paraffin wax using an automatic processor for further histopathology and immunohistochemical studies.

Heat-inactivated Mycobacterium bovis (HIMB) immunostimulant

The oral immunostimulant consisted of 2 mL of sterile PBS containing approximately $10^7$ heat-inactivated colony forming units (CFU)/mL of a field isolate (Strain 1403; spoligotype SB0339) originally obtained from a naturally infected wild boar. HIMB was prepared at Neiker-Tecnicalia (Derio, Spain) following the protocol described by Garrido et al. (2011) [53], except for an extended inactivation step at 83 °C for 45 minutes (min). Bacterial concentration was determined prior to inactivation by measuring Turbidity in a VITEK® DensiCHEK® (BioMerieux) and by plating a serially diluted aliquot onto agar-
solidified Middlebrook 7H9 with glycerol (0.2% v/v) and OADC (10% v/v) (Becton Dickinson, Franklin Lakes, NJ, USA).

**Porcine circovirus 2**

PCV-2 genotype b (Sp-10-7-54-13 strain at 14th passage) was used as the inoculum [54]. This strain was isolated from the lymphoid tissues of a field case of PCV-2-SD in 2006 in Spain. For this study, the PCV-2 strain was propagated in PCV-free PK15 cells in minimal essential medium (MEM) [55] to a titer of $10^5$ TCID$_{50}$/mL.

**Humoral response**

Blood was extracted through puncture of the sinus ophthalmicus at intervals of one week upon infection. Blood samples were centrifuged at 400 g for 10 min to extract sera which was further stored at – 20 °C.

Sera were tested in duplicate by means of Ingezim CIRCO IgG ELISA (R.11.PCV.K1; Eurofins Technologies, SA; Madrid, Spain) to detect antibodies against PCV-2 VP2 protein, following manufacturer’s instructions.

Serum samples were also analyzed by ELISA to detect antibodies against *Mycobacterium tuberculosis* complex using P22 as antigen in an indirect in-house ELISA previously described by Thomas et al. (2019) [56]. The estimated sensitivity and specificity of this ELISA in swine was 84.1% and 98.4%, respectively.

**Detection of porcine circovirus 2 infection by real time quantitative PCR (qPCR)**

DNA was extracted from 200 μL of serum by using the MagMAX™ Pathogen RNA/DNA Kit (Thermo Fischer Scientific Baltics. Vilnius, Lithuania) following the manufacturer’s instructions. The DNA obtained was suspended in 90 μL of elution solution. The commercial QPCR kit VetMAX™ Porcine PCV2 Quant Kit (Applied Biosystems, Lisseu, France) was used to detect and quantify PCV-2 DNA in serum samples. PCV-2 qPCR results were expressed as PCV-2 copies/mL of serum. A cutoff value of $10^4$ PCV-2 DNA copies/mL was considered the quantification limit of the PCV-2 qPCR. Viremia represented the proportion of positive animals (quantifiable and non-quantifiable) per group, and viral load represented the mean of the quantifiable individuals per group.

**Detection of porcine circovirus 2 infection by immunohistochemistry**

Immunohistochemistry was performed to detect PCV-2 as previously described in Chianini et al. (2003) [57]. Briefly, tissue sections were deparaffinized with xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by incubation with hydrogen peroxide 3% in distilled water for 30 min. Afterwards, antigen retrieval was performed using 0.1% Protease XIV (Sigma, ref. P5147) in PBS for 8 min at 37°C. A monoclonal PCV-2 antibody (Ingenasa, M.11.PCV.I36A9) able to recognize the Cap protein was used at 1/1000 dilution in 0.1 M Tris-buffered saline and incubated overnight at 4-8°C. Dako EnVision System-HRP Labelled Polymer Anti-Mouse (Dako, ref. K4001) was
used as secondary antibody and incubated during 60 min at RT. Sections were finally incubated in diaminobenzidine (DAB)–hydrogen peroxide solution for 10 min, counterstained with Harris’ haematoxylin, dehydrated and coverslipped.

Sections from a previous case of PCV-2-SD diagnosed in Spain were used as positive control and tissues from pigs from an experimental farm with no history of PCV-2 infection as negative controls.

To evaluate the number of immunolabeled cells, the sections were randomly chosen and blinded to which group was being analyzed and counted by an experienced pathologist (J.S.). PCV-2 antigen detection by immunohistochemistry was graded by score as low (1), moderate (2) and intense (3) amount of antigen.

**Histopathology**

Sections of tissue samples embedded in paraffin wax were cut at 3 µm and stained with hematoxylin and eosin (HE) for further histological examinations. The presence of lymphocyte depletion, infiltrating histiocytic and multinucleated giant cells in LNs and tonsils, the lymph-epithelium necrosis in tonsils, as well as the interstitial pneumonia, type-II pneumocyte hyperplasia and alveolar edema in lungs were graded as absent or 0% (0), mild or 1% to 30% affected (1), moderate or 30% to 70% affected (2), and severe or 70% to 100% affected (3). The referred pathological findings were independently examined by two blinded and experienced observers (E.F.C. and M.A.R.).

**α-Gal content in PCV-2**

The PCV-2 infective inoculum and an uninfected PK15 cell culture were tested with in-house direct ELISA to detect α-Gal, following the protocol described by Villar et al. (2021) [58] with slight modifications. First, protein concentration in the inoculum and in the cells was determined with the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), following manufacturer’s instructions. Thereafter, 96-well plates were coated with four serial dilutions of protein (from 0.1 to 0.00001 µg/µL per well) from the viral inoculum and the PK15 cells in duplicate, as well as five serial dilutions of α-Gal (from $2 \times 10^{-7}$ to $1.25 \times 10^{-8}$ µg/µL per well), in carbonate/bicarbonate buffer and incubated overnight at 4°C. Wells were subsequently washed with PBS solution containing 0.05% Tween-20 (PBST) (Tween 20; Sigma-Aldrich Quimica S.A., Madrid, Spain) three times and blocked with 5% skimmed milk powder solution in PBS (SM) for 1 h at RT. Then, wells were emptied and the anti-α-Gal epitope monoclonal antibody (M86; Enzo Life Sciences) was added at 1:100 dilution in PBS and incubated for 1 h at 37°C. After three washes with PBST, goat anti-mouse IgM (µ-chain specific) peroxidase-conjugated antibody (Sigma-Aldrich Quimica S.A.) was added at 1:2000 dilution in PBS. After three washes with PBST, 100 µL of 3,3’,5,5’-Tetramethylbenzidine (Promega, Madison, WI, USA) were incubated for 20 min in the dark at RT. The colorimetric reaction was stopped with H$_2$SO$_4$ 3N and the optical density (OD) was measured at 450 nm with a Multiskan FC ELISA reader (Thermo Fisher Scientific). The average value of the blanks (uncoated wells; n = 2) was subtracted from all reads and the mean value of the replicates (wells coated with the same sample at the same protein dilution; n = 2) was calculated. α-Gal content (ng/µg) in each sample (PCV2 inoculum and uninfected PK15 cells) was calculated from an α-Gal standard curve.
**Statistical analysis**

Statistical analysis was performed using R version 4.0.3 (R Core Team, 2020) using packages lme4 [59] and ggplot2 [60]. All data were assessed to calculate mean ± standard deviation (SD) or error (SE) values. The variables viremia, viral burden, antibody titers, increments in weight gain and increments in body temperature were fitted with linear mixed-effects models including the categorical variables “group” and “day” as fixed factors with interaction, as well as “individual” as random factor [response variable ~ group * day + (1 | individual)]. Differences in macroscopic and microscopic lesion scores between groups were tested through Mann-Whitney U test. *P* values ≤ 0.05 were considered statistically significant with a confidence level (CL) of 95%.

**Declarations**

**Ethics approval**

Handling of the animals and sampling was performed according to European (Council Directive 86/609) and Spanish Legislation (RD53/2013) and approved by the Ethics Committee (Complutense University of Madrid) and the Regional Agriculture Authority (Comunidad de Madrid; permit number: PROEX: 66/16).

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

**Funding**

The present study has been funded by project MYCOTRAINING SBPLY/19/180501/000174 (Junta de Castilla-La Mancha, Spain, and EU-FEDER). E. Ferreras-Colino (2020/3836) and R. Vaz-Rodrigues (2022/20675) were supported by the predoctoral contract from Universidad de Castilla-La Mancha (UCLM), co-financed by the European Social Fund (ESF). Marinela Contreras was supported by the Ministerio de Ciencia, Innovación y Universidades, Spain, grant IJC2020-042710-I.

**Author’s contributions**

CG, MAR, JF, JMG, LD: conceived, designed and supervised the study. JMG: prepared the immunostimulant. MS, JS: prepared the viral inoculum and performed PCR and IHQ to detect PCV2. JAB, MM, FC, MAR: performed the necropsies, evaluated macroscopic lesions and collected the samples. MAR: performed ELISA to detect anti-PCV2 antibodies. EFC and MAR: evaluated microscopic lesions. EFC and MC: performed ELISA to detect anti-gal antibodies. EFC and RVR: performed the statistical analyses and
graphic visualization. EFC: prepared the first manuscript draft. All authors read and approved the final manuscript.

**Acknowledgements**

The authors would like to thank the staff of VISAVET and IREC for their technical assistance, as well as to Mónica Pérez, Anna Llorens and Eva Huerta at IRTA-CReSA for their technical assistance on immunohistochemistry, virology and qPCR, respectively.

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Figures
**Figure 1**

**Experimental design.** Sixteen 21 days-old Landrace x Large White hybrid female piglets were randomly assigned to the immunized group (n=8), which received two oral doses of HIMB with an interval of 3 weeks, or the control group (n=8), which received PBS instead of the immunostimulant. Twenty-one days after the second immunization dose, all the animals of both groups were infected by intranasal inoculation with $10^5$ TCID$_{50}$/pig of PCV-2. Twenty-one days after infection, all animals were euthanized and necropsied.
Figure 2

**Temperature and body weight increments.** Mean ± SE of body temperature (A) and body weight (B) in immunized and control groups of animals infected with PCV-2.

Figure 3

**Viral burden and antibody S/P ratio of PCV-2.** 

A) PCV-2 DNA copies/mL of serum by individuals from immunized and control groups. A cutoff value of $10^4$ PCV-2 DNA copies/mL (black dashed line) was considered the quantification limit of the PCV-2 qPCR. 

B) Mean ± SE of anti-PCV2 antibody S/P ratio by group at each time point after PCV2 infection in immunized and control groups. An S/P ratio of 0.29 (black dashed line) or greater was considered seropositive for anti-PCV-2 antibodies.
Figure 4

**PCV-2 lesions at 21 days post-infection.** PCV-2-infected pigs from both groups showed similar lesions, presenting a moderate congestion and lymphadenomegaly, e.g. mediastinal lymph node (LN) (A). Mild infiltrating of histiocytes and multinucleated giant cells was observed in the mandibular and pulmonary LNs of some animals from both groups (B), associated to the presence of PCV-2 antigen (B, inset). Both, immunized and control groups, also presented mild to moderate interstitial pneumonia (C), mainly characterized by mononuclear leukocyte infiltrates in the pulmonary parenchyma (D). Hematoxylin and eosin stain.
Score of histopathological findings and antigen detection associated to PCV-2 infection. Mean of the score for each microscopic lesion and PCV-2 antigen detection in mandibular (A) and pulmonary (tracheobronchial and mediastinal) lymph nodes (B), tonsil (C) and lung (D). Lesions were graded by score as absent or 0% (0), mild or 1% to 30% of the parenchyma affected (1), moderate or 30% to 70% affected (2), and severe or 70% to 100% affected (3). PCV-2 antigen detection by immunohistochemistry was graded by score as low (1), moderate (2) and intense (3) amount of antigen.