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**Immune B cell responsiveness to single-dose intradermal vaccination against *Mycoplasma hyopneumoniae***

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**Abstract**

*Mycoplasma hyopneumoniae* is a major pathogen affecting pig herds and vaccination is the most utilized approach, despite providing partial protection. Age at vaccination, the delivery route, and vaccination protocol can influence vaccine efficacy. The influence of age and the presence of maternally-derived antibodies at vaccination on single-dose needle-less intradermal (ID) administration of an inactivated bacterin-based vaccine (Porcilis® M Hyo ID Once) were assessed in conventional pigs under field conditions. The induction of IgA+ and IgG+ B cell responses and the expression of the activation markers *TLR2*, *TLR7*, *CCR9*, and *CCR10* were determined in PBMC. Vaccination at 4 weeks efficiently elicited an anamnestic antibody response associated with *TLR2* and *TLR7* upregulation. Although animals vaccinated at 1 week did not show seroconversion and a recall response upon infection, the responsiveness of *Mycoplasma*-recalled IgA+ B cells suggests the activation of mucosal immune cells after vaccination and infection. Vaccination at 1 week induced *TLR2*, *TLR7*, and *CCR9* upregulation, suggesting the potential for systemic and local activation of immune cell trafficking between blood and target tissues. Vaccination at 4 weeks induced a *CCR10* increase, suggesting that recalled IgA+ and IgG+ B cells can display an activated status upon infection. The antibody response after *Mycoplasma* infection in 4-week-old ID-vaccinated pigs was associated with *TLR2* and *CCR10* increases, confirming the potential use of this vaccination schedule for the safe and efficient delivery of single-dose *M. hyopneumoniae* vaccines. ID vaccination, especially at 4 weeks, was associated with a great degree of protection against enzootic pneumonia (EP)-like lung lesions.

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**Keywords**: *Mycoplasma hyopneumoniae*, IgA+ B lymphocytes, intradermal (ID) vaccination, field infection, age.

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## 1. Introduction

*Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) is one of the major pathogens causing respiratory diseases in pigs and is a primary agent of enzootic pneumonia (EP). EP is also responsible for increased susceptibility to infections by other bacterial (e.g., *Haemophilus parasuis, Streptococcus suis*, and *Actinobacillus pleuropneumoniae*) and viral agents [e.g., porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), and swine influenza virus (SIV)], which can lead to the development of porcine respiratory disease complex (PRDC) (Maes et al., 2017).

Among measures used to reduce the impact of *M. hyopneumoniae* infection and dissemination, vaccination is the most widely employed, even though only partial protection has been demonstrated both experimentally and under field conditions (Marchioro et al., 2013; Martelli et al., 2014; Maes et al., 2017; Matthijs et al., 2019a,b). Commercial vaccines consist of adjuvanted inactivated whole-cell bacterins based on different *M. hyopneumoniae* strains. Different protocols for vaccine administration have been studied and applied in the field to obtain better protection and health status (Maes et al., 2017). However, the induction of effective immune responses and the factors sustaining protection and limiting tissue lesions are not completely understood.

Age at vaccination is an important factor, influencing the onset of antibody and cellular immune responses, and several vaccines are administered as a single dose or double dose, including a second booster injection; however, other factors may contribute to successful outcomes (Maes et al., 2017; Pieters and Sibila, 2017). Besides the more conventional intramuscular (IM) route, which is effective for inducing immunity and reducing clinical signs and lesions, intradermal (ID) delivery proved to be effective and promising for triggering the immune response at both the systemic and local levels (Thacker et al., 2000; Marchioro et al., 2014; Martelli et al. 2014). The ID route using a needle-less device has been studied also for other major pig pathogens [e.g., PRRSV, SIV, Aujeszky's disease virus (ADV), foot-and-mouth disease virus (FMDV)], as an improved delivery method for efficient

antigen presentation to dendritic cells (DC) in the dermis and activation of adaptive immune B and T cells that are able to respond to further pathogen exposure (Martelli et al., 2009; Borghetti et al., 2010; Ferrari et al., 2013; Le Luduec et al., 2016; Borggren et al., 2016; Magiri et al., 2018; Hwang et al., 2019). Another important issue in evaluating the suitable timing for vaccination in newborn piglets is the potential positive or negative effects of maternally-derived immunity (MDI) in terms of antibodies and lymphocyte subsets derived from the colostrum and milk (Poonsuk and Zimmerman, 2017).

Regarding immune responsiveness to *M. hyopneumoniae* in piglets, passively-acquired antibodies can interfere with the onset and development of piglet antibody immunity (Bandrick et al., 2014a) or have almost no effect on vaccine-primed and anamnestic responses in vaccinated piglets (Martelli et al., 2006). Other studies have demonstrated that the transfer of maternal immunity reduces infection rates in weaned piglets (Sibila et al., 2008) and that vaccination of newborn piglets can be efficacious in the presence of high maternal antibody titers (Wilson et al., 2013). The transfer of functional T cells from immunized sows to offspring can contribute to specific antigen stimulation, also influencing the neonatal immune response, and is not dampened by maternal immunity (Bandrick et al., 2008, 2014a,b). However, the role of B lymphocytes in transferred immunity to piglets is still unclear (Poonsuk and Zimmerman, 2017). It is known that passively-transferred maternal IgA and IgG possess high specificity due to the increased immunological experience of the sows boosted by several adaptive immune responses to specific antigens (Butler et al., 2017). Regarding *M. hyopneumoniae* infections, secretory IgA (sIgA) and IgA+ B cells/plasma cells seem to play pivotal roles in the respiratory tract and lungs (Thacker et al., 2000; Sarradell et al., 2003). Therefore, circulating IgA+ cells, comprehending pre-plasma cells (PPC)/plasmablasts (immature plasma cells) and memory B cells (MBC), are important antigen-specific cells to monitor in that they undergo activation upon stimulation and mature into plasma cells in mucosal tissues (Brandtzaeg and Johansen, 2005; Braun et al., 2017, Butler and Sinkora, 2017).

Among the receptors expressed by immune cells, Toll-like receptors (TLR) are fundamental receptors which recognize conserved molecular patterns expressed on different pathogens (pathogen-associated molecular patterns, PAMP) and can trigger both early innate and adaptive immune responses (Uenishi and Shinkai, 2009). In fact, in addition to T-cell-dependent stimulation, B cell activation can occur when their TLR are upregulated. In particular, TLR2 and TLR7 are strong B cell activators in both naïve and experienced B cells, with the ability to stimulate proliferation (Braun et al., 2017). Very scant data are available on B cell TLR-mediated immunomodulation in response to *M. hyopneumoniae* stimulation in the case of a vaccine antigen or a virulent strain. It was demonstrated that IgA secretion is augmented in response to TLR2 and TLR4 in a mouse infection model (Li et al., 2019) and the upregulation of these receptors is associated with less severe pig lung lesions (Borjigin et al., 2017). C-C chemokine receptors (CCR) are also widely expressed immune receptors mainly involved in cell migration and functional differentiation driven by chemokines in tissues under several physiological and pathological conditions such as inflammation and infections. As observed in humans, fractions of porcine IgA+ B plasmablasts express CCR9 and CCR10, which play important roles in IgA B lymphocyte mucosal homing and trafficking, by interacting with CCL25 and CCL28 chemokines, respectively. CCR9 is reported to be mainly involved in gut homing, and at lower extent in the upper respiratory tract (Meurens et al., 2006; Berri et al., 2014). However, a recent study highlights its strong upregulation in peripheral blood mononuclear cells (PBMC) upon immunization with an H1N1 influenza virus vaccine in pigs (Liu et al., 2019). CCR10 and CCL28 are involved in the recruitment into several mucosal tissues, including the respiratory tract, intestine and mammary gland. This reflects the recirculation of activated cells among different compartments such as the gut and the lungs (defined as gut-lung axis or upper aero-digestive tract) in the common mucosal immune system, displaying the overlapping of homing markers (Bourges et al., 2004; Meurens et al., 2006; Perdijk et al., 2018).

The present study aimed at assessing the influence of age (1 week vs. 4 weeks) and the presence of maternally-derived antibodies at vaccination on a single-dose *M. hyopneumoniae* needle-less ID vaccination in conventional piglets under field conditions. In particular, the effects of vaccination on eliciting and sustaining circulating IgA+ and IgG+ B cell responses and modulating gene expression of the activation markers *TLR2*, *TLR7*, *CCR9*, and *CCR10* in PBMC were evaluated. These immunological parameters were evaluated in association with EP-like lung lesions (i.e. lesions mainly characterized by cranioventral pulmonary consolidation due to bronchopneumonia) attributable to *M. hyopneumoniae* infection detected at the slaughterhouse to assess the effects of various vaccination protocols against *M. hyopneumoniae* natural infection. Also, pleuritis lesions were assessed at the slaughterhouse.

## 2. Materials and methods

### *2.1. Animals and sample collection*

The study involved conventional healthy pigs born to sows vaccinated against *M. hyopneumoniae* as young piglets and boosted before the first mating in a herd in Italy. The pigs were individually ear-tagged, randomly enrolled at birth, and divided into three groups (N = 100/group) according to the age at vaccination against *M. hyopneumoniae* or no vaccination. Animals were kept in the same housing/husbandry field conditions for the whole duration of the study period. Specifically, pigs were kept in the same barns, with the same ventilation system, in the same period of the year, at the same pig density, according to the regulations on animal welfare. For ID vaccination, pigs were vaccinated at 1 week of age (V1wID group) or at 4 weeks of age (V4wID pigs) with Porcilis® M Hyo ID Once (0.2 mL; MSD-Animal Health, Whitehouse Station, NJ, USA) composed of inactivated *M. hyopneumoniae* strain 11 adjuvanted with DL-α-tocopherol acetate using a needle-less IDAL® injector (MSD-Animal Health) on the left side of the neck. The non-vaccinated control group (NV) was administered adjuvant only using the same device. Piglets were weaned at 4 weeks of age. The

health status of sows and pigs was assessed throughout the study period, from birth to 24 weeks of age. PRRSV and PCV2 infections were excluded by quantitative PCR (RT-qPCR/qPCR, respectively) performed at each time-point on blood samples, according to previously established protocols (Martelli et al., 2013). Li-heparinized blood samples were collected from 10 pigs/group at 0, 4, 12, 16, and 20 weeks post-vaccination (PV) (corresponding to 1, 5, 13, 17, and 21 weeks of age for the V1wID group and to 4, 8, 16, 20, and 24 weeks of age for the V4wID group). Serum samples were collected and stored at −20 °C until *M. hyopneumoniae* ELISA were performed.

Animal housing, husbandry, treatments and sample collection of the present study were carried out following national ethics and good clinical practice (GCP) guidelines in animal treatment and welfare, including the EU Directive 2010/63/EC and institutional guidelines. The study was approved by the Ethics Committee of University of Parma, Parma (Italy).

*2.2. Lung lesion evaluation*

All pigs enrolled in the study were evaluated at the slaughterhouse (9 months of age on average) to determine lung lesions attributable to *M. hyopneumoniae* infection. Lung lesions were assessed and quantified using the SPES (slaughterhouse pleuritis evaluation system) grid for pleuritis and the Madec's grid for EP-like lesions mainly attributed to *M. hyopneumoniae* infection, according to Merialdi and coll. (2012). Briefly, pleuritis lesions were scored by using the SPES grid which has five values (from 0 to 4) based on the extension and location of pleural adherences. The SPES mean value (i.e. the sum of each lung score/number of scored lungs) was calculated. Bronchopneumonic lesions, suggestive of EP, were scored by using the Madec's grid in which each lung lobe is scored from 0 to 4, to a maximum possible value of 28. The EP-like lesion mean value (i.e. the sum of single lung EP-like lung score/number of scored lungs) was calculated.

*2.3. ELISA for M. hyopneumoniae antibody quantification in serum*

The serological responses to *M. hyopneumoniae* vaccination and field infection were measured by an indirect ELISA using the commercially available ID Screen® Kit (IDvet, Grabels, France) for the detection of anti-*M. hyopneumoniae* antibodies. Results are expressed as sample-to-positive (S/P) ratios, and samples with S/P > 0.4 are considered positive according to the manufacturer's instructions. The sample absorbance was read by using a VICTOR$^{3}$™ multilabel plate reader (PerkinElmer, Shelton, CT, USA). The presence of serum antibodies to *M. hyopneumoniae* is an indicator of exposure to the agent.

*2.4. Isolation of porcine PBMC*

Porcine PBMC were isolated by Histopaque-1077® density gradient centrifugation (Sigma, St. Louis, MO, USA) as previously described (Ferrari et al., 2013). Cells were washed twice with sterile PBS + 1% heat inactivated (hi) fetal bovine serum (FBS), suspended in complete RPMI-1640 (cRPMI-1640) + 10% DMSO + 40% FBS (Gibco, Carlsbad, CA, USA), frozen at −80 °C, and stored in liquid nitrogen. Thawed cells were washed twice with cRPMI-1640 + 10% hi-FBS, resuspended in the same medium, and counted/checked for viability by Trypan blue (Sigma) using a TC20™ Automated Cell Counter (BioRad, Hercules, CA, USA).

*2.5. Flow cytometry quantification of IgA+ and IgG+ B cells upon M. hyopneumoniae stimulation*

Detection of B cells expressing surface IgA or IgG upon in vitro stimulation with *M. hyopneumoniae* was assessed by flow cytometry. PBMC ($3 \times 10^6$ PBMC/well) were incubated in 24-well culture plates with purified *M. hyopneumoniae* (strain 11, batch BMHY0025) kindly provided by Raes M., MSD-AH, Boxmeer, the Netherlands), at R = 100 bacterins/cells (Trueeb et al., 2020), or without antigen (unstimulated controls) in cRPMI-1640 + 10% hi-FBS for 44 h at 37 °C, 5% $CO_2$. Polyclonal stimulation of B cells with the B-Poly-S™ reagent (Cellular Technology Ltd, OH, USA) was performed for positive controls. After stimulation, samples were counted with Trypan blue and

processed for flow cytometry staining. PBMC were first washed in PBS and stained using the LIVE/DEAD™ Fixable Far Red Dead Cell Stain Kit (Invitrogen-ThermoFisher Scientific, Paisley, UK) in the dark according to the manufacturer's recommendations. Cells were then stained with mouse anti-pig IgA (clone K611B4; $IgG_1$, BioRad) or mouse anti-pig IgG (clone F007-1241; $IgG_1$, BioRad) antibodies (Ab) for 15 min at room temperature in the dark and then with a secondary goat anti-mouse-$IgG_1$-FITC Ab (1070-02; Southern Biotech, Birmingham, AL, USA) for 15 min in the dark on ice. Samples were fixed (15 min, room temperature, in the dark) and permeabilized (50 min, room temperature, in the dark) using Leucoperm™ Kit Solutions (BioRad). Intracellular staining of CD79α during permeabilization was performed using a mouse anti-human CD79α-PE cross-reactive antibody (clone HM57, $IgG_1$, BioRad; Faldyna et al., 2007). Unstained PBMC or PBMC incubated with secondary Ab were used as negative controls. FMO controls were also performed for each staining combination. The analysis was performed using a Cytomics FC500 flow cytometer and CXP software (Beckman-Coulter, Indianapolis, IN, USA) based on doublet discrimination, live cells, and lymphocyte gating after the acquisition of at least 40,000 cell events (Fig. 1S). Washes after each surface staining step were performed with 2 mL of PBS + 1% hi-FBS, except for the step before/after LIVE/DEAD™ staining, in which PBS was used, according to the manufacturer's recommendations. Differences between *M. hyopneumoniae*-stimulated and unstimulated cells were determined and expressed as percentages.

*2.6. Total RNA extraction and reverse transcription (RT)*

RNA isolation and processing, and quantitative real-time PCR were performed as described by Borghetti et al. (2013), with minor modifications. Briefly, RNA extraction was performed using TRI-reagent (Ambion-Life Technologies, Grand Island, NY, USA), and purity and concentration were assessed using a BioSpectrometer® (Eppendorf AG, Hamburg, Germany). RNA integrity and quality were assessed using an Agilent Bioanalyzer 2100 and RNA 6000 Labchip Kit (Agilent Technologies,

Santa Clara, CA, USA). RNA samples were stored at −80 °C until used for RT. RNA samples were DNase-treated (Sigma) and 1 µg/20 µL was reverse-transcribed using a High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). RT was performed using a StepOne Thermocycler (Applied Biosystems, StepOne v.2.3), according to the manufacturer's instructions, under the following thermal conditions: 10 min at 25 °C and 120 min at 37 °C, followed by 5 min at 85 °C. The cDNA samples were stored at −20 °C.

*2.7. Quantification of immune marker gene expression by real-time PCR*

cDNA samples (20 ng) were used as templates for real-time quantitative PCR (qPCR) using a StepOne Thermocycler and StepOne Software v.2.3 for analyses (Applied Biosystems). The cDNA (20 ng/20 µL) was amplified in triplicate using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) along with specific primer sets reported in Table 2. The primers were based on published gene sequences (Collado-Romero et al., 2010; Ondrackova et al., 2013; Zhang et al., 2013) or designed using Primer Express™ software v.3.0 and were purchased from Eurofins MWG Operon (Ebersberg, Germany). The reference gene *GAPDH* was selected among others [β-actin (Meissonnier et al., 2008), *RPL19* (Kiros et al., 2011), and *RPL32* (Royaee et al., 2004)] (Table 1) as an endogenous control according to minimal intra-/inter-assay variation and previous results (Facci et al., 2011, Borghetti et al., 2013, Ferrari et al., 2013).

Samples were kept at 95 °C for 20 s and then subjected to 40 cycles consisting of a denaturation step at 95 °C for 3 s followed by an annealing/extension step at 60 °C for 30 s. Fluorescence due to SYBR Green incorporation was acquired at the end of each extension step. No-template controls (NTC) and no-RT controls were included as negative controls. A melting curve was generated at the end of the amplification cycles. NTC were assumed to be negative and reliable if the quantification cycle (Cq) was ≥35. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001; Schmittgen and

Livak, 2008; Shi et al., 2010, Borghetti et al., 2013), with the expression of each immune marker normalized against the level of GAPDH cDNA and expressed as a relative quantity (RQ).

*2.8. Statistical analysis*

Statistical analyses were performed using Analysis of Variance (ANOVA) with group, sampling time, and interaction between group and sampling time as fixed factors. Lung lesion scores were evaluated using a Kruskal–Wallis Chi-squared test, after confirming the non-normal distribution of the data. Differences among groups and over time were considered significant when $p < 0.05$. Statistical analyses were carried out using SPSS v.26.0 (IBM SPSS Statistics, NY, USA). Experimental data are presented as means ± standard deviation.

**3. Results**

*3.1. Lung lesion scores at the slaughterhouse*

According to the SPES grid scores (pleurisy), there were no significant differences in lung lesion scores depending on the vaccination schedule (single-shot vaccine administration at 1 week or 4 weeks of age). Scores did not differ significantly between the NV controls and ID-vaccinated piglets (data not shown).

Conversely, vaccine administration significantly influenced the Madec's grid score ($p < 0.05$), as determined by EP-like lung lesions mainly attributable to *M. hyopneumoniae* infection. Specifically, 25.3% of 91 V1wID pigs, 18.4% of 92 V4wID pigs, and 79.0% of 86 NV pigs had such lesions. Scores differed significantly between ID-vaccinated piglets and NV controls ($p < 0.05$) (Fig. 1).

*3.2. M. hyopneumoniae antibody response in serum*

In one-week-old vaccinated pigs, *M. hyopneumoniae* serum antibodies were detected at vaccination and decreased during the following 12 weeks PV. Thereafter, S/P ratios increased and remained close to the cut-off value (0.4) for positivity (Fig. 2A).

Four-week-old vaccinated animals (Fig. 2B) had no antibodies at vaccination and no significant increase after 4 weeks PV. Significant increases were observed from 12 to 20 weeks PV. The V4wID group showed higher levels of antibodies (max. mean S/P = 1.49) than those in the control groups (max mean S/P = 0.67) during this period (Fig. 2B). Moreover, antibody levels in the V4wID group were significantly higher than those in controls at 16–20 weeks PV ($p < 0.05$).

Non-vaccinated animals showed detectable levels at 1 week of age (0 weeks PV for the V1wID group), followed by a decrease and then a gradual increase up to 20 weeks PV (Fig. 2A). Non-vaccinated animals showed seroconversion, with levels increasing significantly from 4 weeks PV to 20 weeks PV ($p < 0.05$) also in pigs vaccinated at 4 weeks of age (i.e., at 8, 16, 20, and 24 weeks of age) (Fig. 2B). Seroconversion in non-vaccinated animals and the significant anamnestic antibody response in the ID-vaccinated group indicate that a *M. hyopneumoniae* natural infection occurred.

### *3.3. M. hyopneumoniae B cell responsiveness in PBMC*

The transfer and/or the development of B cell immunity against *Mycoplasma* was studied by quantifying circulating *Mycoplasma*-recalled IgA+ and IgG+ B cells in PBMC.

The levels of IgA+ and IgG+ B cells showed comparable courses in pigs vaccinated at 1 week of age (Fig. 3A, B) and at 4 weeks of age (Fig. 3C, D); however, values differed substantially between groups.

The V1wID group (vaccinated at 1 week of age) and unvaccinated pigs sampled at 1 week of age showed detectable levels of both IgA+ and IgG+ B cells. Such cell fractions declined earlier in non-vaccinated animals (at 4 weeks PV) ($p < 0.05$), were maintained at very low levels until 16 weeks PV, and then increased significantly at 20 weeks PV ($p < 0.05$).

The levels of IgA+ and IgG+ B cells in ID-vaccinated pigs decreased at 12 weeks PV ($p < 0.05$), with subsequent increases at 16 weeks PV compared to those in controls.

In pigs vaccinated at 4 weeks of age, the levels of IgA+ and IgG+ B cells were very low and comparable to those in non-vaccinated animals at the time of vaccination. Thereafter, they increased significantly in the V4wID group ($p < 0.05$). From 12 weeks PV, the levels of both cell fractions increased significantly in all groups, and values in the ID-vaccinated group were higher than those in controls at 20 weeks PV ($p < 0.05$) (Fig. 3).

*3.4. Gene expression of Toll-like receptors and chemokine receptors*

Gene expression levels of *TLR2* and *TLR7* were quantified by real-time qPCR in PBMC of *M. hyopneumoniae*-vaccinated and non-vaccinated animals.

Vaccination at both 1 week and at 4 weeks of age induced increased levels of *TLR2* at 4 weeks PV ($p < 0.05$). Subsequently, significantly higher levels were detected at 16 weeks PV in the vaccinated group than in controls ($p < 0.05$) (Fig. 4A, B).

*TLR7* expression levels increased in pigs vaccinated at 1 week of age (V1wID), showing a peak response at 12 weeks PV ($p < 0.05$) (Fig. 5A). Non-vaccinated animals showed a delayed increase at 16 weeks PV ($p < 0.05$)

Similarly, pigs vaccinated at 4 weeks of age (V4wID) showed increased *TLR7* levels at 12 weeks PV ($p < 0.05$), similar to non-vaccinated animals (Fig. 5B).

*CCR9* gene expression increased significantly after vaccination in both age groups and returned to basal levels at 12 weeks PV ($p < 0.05$) (Fig. 6A, B). The upregulation of the receptor was also observed upon infection at 16 weeks PV in vaccinated animals. No significant modulation was detected in non-vaccinated pigs, except for an increase at 8 weeks of age ($p < 0.05$) (Fig. 6A, B).

*CCR10* gene expression was comparable in animals vaccinated at 1 week of age and controls at the time of vaccination and then declined by 12 weeks PV in both groups ($p < 0.05$) (Fig. 7A). A more

gradual decline was observed in non-vaccinated animals than in vaccinated animals ($p < 0.05$). Levels were very low or negligible from 12 to 20 weeks PV (Fig. 7A).

Conversely, animals vaccinated at 4 weeks of age showed a gradual increase in *CCR10* expression after vaccination and maintained higher values than those in controls during infection ($p < 0.05$) (Fig. 7B).

## 4. Discussion

The present study aimed to evaluate the effect of intradermal (ID) vaccination against *M. hyopneumoniae* in 1-week-old or 4-week-old vaccinated conventional pigs in field conditions.

In addition to the antibody response in serum, *Mycoplasma*-induced B cell immunity was evaluated in terms of circulating IgA+ and IgG+ B cell responses and markers of immune responsiveness (*TLR2*, *TLR7*, *CCR9*, and *CCR10*) in PBMC.

These parameters were evaluated in association with the potential protective effect of vaccination against *M. hyopneumoniae* natural infection determined by the reduction of EP-like lung lesions and pleuritis at the slaughterhouse.

As quantified by ELISA, ID vaccination at 1 week of age did not induce a significant primary humoral response, and the anamnestic response observed after 12 weeks PV (13 weeks of age) was very low and comparable to that observed in non-vaccinated controls. This suggests that interference by MDI influenced the response to vaccination, as most piglets did not show seroconversion and did not respond to infection. This is in accordance with previous results obtained by Bandrick and coll. (2014), who showed that for piglets vaccinated at 1 week of age, there was no significant response to immunization. Conversely, the serum antibody course in animals vaccinated at 4 weeks of age indicates a significant recall response when infection occurred. Therefore, we can assume that under the conditions of this field study, MDI interfered with the immune response of piglets.

Regarding the *M. hyopneumoniae* humoral response, total antibodies detectable in serum are not predictive of protection against this pathogen and are not correlated with the severity of lung lesions; in addition, the actual levels in serum can be biased by false-negative results (Maes et al., 2017; Matthijs et al., 2019). Therefore, the identification and modulation of *in vitro* Ig-expressing B cells recalled by *Mycoplasma* may provide an effective indicator of the ongoing B cell responsiveness to vaccination and infection.

In our study, the different outcomes of the *in vitro* re-stimulation of PBMC with the whole inactivated homologous *Mycoplasma* strain used in the vaccine (strain 11) suggest that piglets vaccinated at 1 week of age have *M. hyopneumoniae*-specific B cells expressing surface IgA and IgG at vaccination, which may survive and re-circulate in the blood during the following 12 weeks. Therefore, vaccination at 1 week of age sustained IgA+ B cells (at 4 weeks PV) already present in the blood of piglets at vaccination due to the transfer of maternal immunity and the induction of B cell immunity in piglets in the ID-vaccinated group, allowing an earlier but low/suboptimal anamnestic response during natural infection after 12 weeks PV.

Vaccination at 4 weeks of age induced both primary and anamnestic responses in vaccinated pigs. The secondary response, after infection, was stronger than that in controls. Similar results were obtained for IgG+ B cells in animals subjected to the two different vaccine inoculation schedules. The presence of such IgA+ B cells would support the hypothesis of maternal transfer by the colostrum and milk of antigen-specific adaptive immune cells. These cells can undergo phenotypic and functional maturation to plasma cells that are able to secrete IgA in mucosal tissues, including the lung and respiratory tract, where *Mycoplasma* interacts with epithelial cells and can induce severe lesions. However, these B cells may also include the progeny of transferred maternal cells, in accordance with Bianchi and coll. (1999).

In fact, IgA+ B cells in the peripheral blood include immature PPC and circulating MBC, which can be re-activated by antigens. The transfer of B and T cells and, specifically, the transfer of antigen-

specific immune cells through the colostrum and milk has been demonstrated in mice, pigs, and humans (Arvola et al., 2000; Tuaillon et al., 2009; Peroni et al., 2013; Ogawa et al., 2016; Poonsuk and Zimmerman, 2017; Langel et al., 2019; Kick et al., 2020). This is thought to contribute to early responses to infections in newborns and may have helped to prime the immune response in ID-vaccinated piglets to efficiently cope with later *Mycoplasma* infection in our study. Furthermore, given the specificity of passively transferred IgA, it should reflect the mucosal immune experience of the mother (Butler and Sinkora, 2017) and hence is of great importance for the early immune status and further immune development of the piglets.

The decline of IgA+ B cells in non-vaccinated piglets at 4 weeks PI (5 weeks of age) indicates the reduction of passively-acquired cells in the absence of *in vivo* stimulation.

Memory IgA+ and IgG+ cells were mainly involved in the observed responses in vaccinated pigs upon *M. hyopneumoniae* infection after 13 weeks of age, whereas the primary response was induced in non-vaccinated animals. These immune subsets are likely responsible for the secretion of IgG and IgA (in the airway-associated lymphoid tissue and in the respiratory tract parenchyma), which were found in bronchoalveolar lavage fluid (BALF) of IM- and ID-vaccinated animals (Marchioro et al., 2013; Martelli et al., 2014) and in serum or respiratory tissues of infected pigs (Li et al., 2019).

*TLR2* expression in PBMC increased after vaccination and natural exposure/infection, independently of age at vaccination, with comparable changes in IgA+ and IgG+ B cells. This suggests the involvement of this receptor on B cells responsible for the *M. hyopneumoniae* memory B cell response. Indeed, B cells can rapidly upregulate this receptor and can be subjected to clonal expansion and differentiation into antibody-secreting and memory cells (Braun et al., 2017). Additionally, the modulation of *TLR7* suggests that this receptor is involved in the regulation of B cell response during *M. hyopneumoniae* infection. The earlier peak in the *TLR7* response in animals vaccinated at 1 week of age may have contributed to a more efficient response during the early phase of infection. The lower but not significantly different peak response in ID-vaccinated pigs compared

to controls suggests that this pathogen recognition receptor has a less pronounced role in the response to *M. hyopneumoniae* infection.

IgM+ and IgG+ B cells can be effectively activated by TLR2, TLR7, and TLR9 ligands to proliferate and express activation markers (Braun et al., 2017). In particular, in addition to B cells, other cell types, such as classical DC (cDC), plasmacytoid DC (pDC), and monocytes may have been involved in TLR7 activation early upon infection, in accordance with previous reports (Braun et al., 2017; Li et al., 2019).

Consistent with the results by Meurens and coll. (2006), we found lower expression levels of *CCR10* than *CCR9*. Despite the higher expression levels of *CCR9* on T lymphocytes than on B lymphocytes (Meurens et al., 2006), under our field conditions, the course of *CCR9* expression appeared to be concomitant with IgA+ and IgG+ B cell modulation induced by *M. hyopneumoniae* stimulation *in vivo* (vaccination and infection) and the *in vitro* antigen recall of recently activated and memory B cells. Therefore, we can hypothesize that CCR9 is involved in the response of antigen-specific immune cells and may be also upregulated on T cells. It has been demonstrated that T cell-specific responses [lymphoproliferation and delayed-type hypersensitivity (DTH)] can be transferred to offspring and can facilitate the piglet response to *Mycoplasma* infection (Bandrick et al., 2008, 2014a,b). More specifically, T helper-1 cell-derived cytokines can activate B cells and macrophage functions, while IFN-γ secreting cells (Martelli et al., 2014) and T helper 17 lymphocytes can favor pathogen clearance and IgA-mediated tissue protection (Matthijs et al., 2019a,b).

We detected the upregulation of *CCR10* in both vaccinated and non-vaccinated groups at 1 week of age, and this marker was associated with significant levels of IgA+ B lymphocytes at the same age. It is known that CCL28, the CCR10 ligand, is upregulated in the mammary parenchyma during lactation and is possibly involved in the recruitment of IgA-secreting cells in maternal secretions and the transfer of passive immunity to piglets, in which specialized cells can be recruited to several mucosal tissues, including the respiratory tract (Bourges et al., 2004; Wilson and Butcher, 2004;

Meurens et al., 2006; Berri et al., 2014). For this reason, the transfer of *M. hyopneumoniae* immunity may have been sustained and therefore, may have played a beneficial effect upon vaccination in pigs vaccinated at 1 week of age. However, *CCR10* levels were not modulated by infection, suggesting that the recruitment and homing of B cells is likely to be less efficient in animals vaccinated at 1 week than at 4 weeks of age.

Pigs vaccinated at 4 weeks of age showed the upregulation of *CCR10* after vaccination and especially after natural infection. *CCR10* in PBMC is mainly expressed by recently activated B cells expressing IgA and IgM (Meurens et al., 2006, Berri et al., 2014), therefore this suggests a differential activation status of IgA+ B cells detected by flow cytometry between vaccinated and control groups. *CCR10* and especially *CCR9* were found to be strongly upregulated upon nasal vaccination of 6-week-old pigs using an inactivated H1N1 influenza virus vaccine, and associated with clinical protection, suggesting the homing of B cells producing specific antibodies in the respiratory tract (Liu et al., 2019). In our study conditions, 4-week-old ID-vaccinated animals, which showed a much more pronounced anamnestic response upon infection, had higher levels of both *CCR9* and *CCR10*.

Regarding the lung lesions observed, needle-less ID vaccination (especially at 4 weeks of age) was associated with a lower lung lesion score compared to no vaccination. This was observed under both vaccination conditions tested (1 week and 4 weeks of age), thus supporting the efficacy of *M. hyopneumoniae* ID vaccination. Other pathogens may have had a role in the onset and degree of the lung lesions. EP-like lesions are not pathognomonic (Merialdi, et al 2012) but are highly suggestive of *M. hyopneumoniae* infection, and the significant reduction of lung lesions in vaccinated animals compared to unvaccinated controls makes us reasonably believe that the contribution of other pathogens, if present, was marginal. The association between the efficacy of *M. hyopneumoniae* vaccination and the reduction of EP-like lesions is very well documented in literature (Kyriakis et al., 2001; Tassis et al., 2012; Wilson et al., 2012; Hillen et al., 2014; Cvjetković et al., 2018).

## 5. Conclusions

The evaluation of the impact of early vaccination of piglets, at 1 week of age, in comparison with vaccination at 4 weeks of age showed that the anamnestic response upon natural infection was influenced in terms of a substantially lower total antibody response in the blood.

Vaccination at 4 weeks of age proved to be efficient in eliciting the anamnestic response, which was associated with the upregulation of *TLR2* and *TLR7* in ID-vaccinated pigs. Although the serological profile in animals vaccinated at 1 week of age did not highlight any significant seroconversion or recall response upon infection, the responsiveness of IgA+ B cells indicate the activation of mucosal immune cells after vaccination and infection. These cells are precursors of plasma cells which secrete antibodies in tissues, and IgA+ cells are fundamental for pathogen elimination in various tissues of the respiratory tract where *Mycoplasma* interacts with and enters the organism through epithelial cells. Also the vaccination of 1-week-old animals induced the upregulation of mucosal markers (*TLR2*, *TLR7*, and *CCR9*), indicating the potential for the systemic and local activation of immune cells trafficking between the circulation and target tissues.

Vaccination at 4 weeks of age increased the levels of *CCR10*, suggesting that recalled IgA+ and IgG+ B cells can display an activated status upon infection. Intradermal vaccination using a needle-less device effectively induced an anamnestic response, as determined by total serum antibodies after *Mycoplasma* infection in 4-week-old vaccinated pigs, and this response was associated with changes in *TLR2* and *CCR10* levels in PBMC, thus confirming the potential use of needle-less ID vaccination for the safe and efficient delivery of single-dose vaccines against *M. hyopneumoniae*. The immunomodulation of B cells and several B cell-associated markers was also associated with less severe EP-like lesions, indicating a high degree of protection against *M. hyopneumoniae*-related lesions after natural infection.

## Competing Interests

None of the authors has any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the present paper, therefore the authors declare that they have no competing interests.

**Authors' contributions**

Conceptualization, methodology, supervision, writing – original draft preparation, visualization, PM, LF, and PB; investigation, data acquisition and curation: PM, LF, PB, MA, RS, VC, and EDA.; writing – review and editing, PM, LF, PB, EDA, MA, RS, and VC; project administration and funding acquisition, PM. All authors read and approved the manuscript.

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**Table 1**

Target genes and primer details for SYBR Green quantitative PCR detection of immune markers. The *GAPDH* gene was selected as endogenous control gene.

| Target gene | GenBank accession nr | Primer sequence (base number) | Concentration (nM) | Efficiency (%) | Slope | $r^2$ | Amplicon length (bp) |
|---|---|---|---|---|---|---|---|
| Porcine *TLR2* (Zhang et al., 2013) | GU138028 | for 5'-TGCTGGAACCCATCGAGAA-3' (19)<br>rev 5'-AGGTAGGTCCTGGTGTTCACTATCTT-3' (26) | 300 | 110.4 | –3.07 | 0.99 | 79 |
| Porcine *TLR7* (Collado-Romero et al., 2010) | NM_001097434 | for 5'-TCAGTCAACCGCAAGTTCTG-3' (20)<br>rev 5'-GATGGATCTGTAGGGGAGCA-3' (20) | 300 | 104.6 | –3.22 | 0.99 | 105 |
| Porcine *CCR9* (Ondrackova et al., 2013) | NM_001001624 | for 5'-CCACAGAAGCCGCAAGTCTGATGC-3' (23)<br>rev 5'-TGGCTTGCAAACTGCCTGACATGGT-3' (25) | 200 | 104.9 | –3.21 | 0.99 | 139 |
| Porcine *CCR10* (Ondrackova et al., 2013) | NM_001044563 | for 5'-CTGCAGCTGCCCTACAGTCTCCG-3' (23)<br>rev 5'-AGATCCTTGCGCTTGCTGGCACG-3' (23) | 300 | 98.8 | –3.35 | 0.91 | 95 |
| Porcine *GAPDH* (Primer Express) | NM_001206359 | for 5'-GGTGAAGGTCGGAGTGAACG-3' (20)<br>rev 5'-GCCAGAGTTAAAAGCAGCCCT-3' (21) | 300 | 102.0 | –3.27 | 0.99 | 70 |
| Porcine *β-actin* (Meissonnier et al., 2008) | AY550069 | for 5'-TCATCACCATCGGCAACG-3' (18)<br>rev 5'-TTCCTGATGTCCACGTCGC-3' (19) | 300 | 100.5 | –3.30 | 0.99 | 133 |
| Porcine *RPL19* (Kiros et al., 2011) | AF435591 | for 5'-AACTCCCGTCAGCAGATCC-3' (19)<br>rev 5'-ACTACCCTTCCGCTTACCG-3' (19) | 200 | 99.6 | –3.32 | 0.99 | 147 |
| Porcine *RPL32* (Royaee et al., 2004) | NM_001001636 | for 5'-AGGAAGAGACGTTGTGAGCAA-3' (21)<br>rev 5'-CGGAAGTTTCTGGTACACAATGTAA-3' (25) | 300 | 106.3 | –3.18 | 0.98 | 94 |

bp: base pairs; for: forward primer; rev: reverse primer. *TLR*: *Toll-like receptor*; *CCR*: C-C *chemokine receptor*; *GAPDH*: *glyceraldehyde 3-phosphate dehydrogenase*; *RPL*: *ribosomal protein L*.

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**Figure captions**

**Fig. 1S.** Gating strategy for flow cytometry analysis of porcine circulating CD79+IgA+ and CD79+IgG+ B lymphocytes upon in vitro stimulation with *M. hyopneumoniae*. Cells were gated for singlets, and then for lymphocytes. Dead cells were excluded by selecting low LIVE/DEAD™ Fixable Far Red fluorescent cells. Analysis was performed after acquisition of at least 40 000 live cells.

**Fig. 1.** Lung score determined by using the Madec's grid in lungs of pigs at slaughterhouse after natural infection by *M. hyopneumoniae* occurred. The V1wID group was needle-less intradermally vaccinated at 1 week of age while the V4wID group was vaccinated at 4 weeks of age. The asterisk indicates a significant difference compared to the non-vaccinated (NV) control group.

**Fig. 2.** *M. hyopneumoniae* antibody response in serum of needle-less ID-vaccinated pigs against *M. hyopneumoniae* and in non-vaccinated control pigs (NV, non-vaccinated). The V1wID group was vaccinated at 1 week of age (**A**) while the V4wID group was vaccinated at 4 weeks of age (**B**). PV: post-vaccination. S/P cut-off value for positivity = 0.4. Asterisks indicate significant difference compared to the non-vaccinated control group.

**Fig. 3.** Percentage levels of CD79α+IgA+ (**A**, **C**) and CD79α+IgG+ (**B**, **D**) B cells in *Mycoplasma*-stimulated PBMC of ID-vaccinated pigs and in non-vaccinated control pigs (NV, non-vaccinated). The V1wID group was vaccinated at 1 week of age while the V4wID group was vaccinated at 4 weeks of age. PV: post-vaccination. Asterisks indicate significant difference compared to the non-vaccinated control group.

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**Fig. 4.** Relative gene expression levels of *TLR2* in PBMC of ID-vaccinated pigs against *M. hyopneumoniae* and in non-vaccinated control pigs (NV, non-vaccinated) exposed to *M. hyopneumoniae* natural infection. Data were obtained by RT-qPCR ($2^{-\Delta\Delta Cq}$ method) and shown as mean RQ values ± standard deviation. (**A**) V1wID group: vaccinated at 1 week of age; (**B**) V4wID group: vaccinated at 4 weeks of age. PV: post-vaccination. Asterisks indicate significant difference compared to the non-vaccinated control group

**Fig. 5.** Relative gene expression levels of *TLR7* in PBMC of ID-vaccinated pigs against *M. hyopneumoniae* and in non-vaccinated control pigs (NV, non-vaccinated) exposed to *M. hyopneumoniae* natural infection. Data were obtained by RT-qPCR ($2^{-\Delta\Delta Cq}$ method) and shown as mean RQ values ± standard deviation. (**A**) V1wID group: vaccinated at 1 week of age; (**B**) V4wID group: vaccinated at 4 weeks of age. PV: post-vaccination. Asterisks indicate significant difference compared to the non-vaccinated control group.

**Fig. 6.** Relative gene expression levels of *CCR9* in PBMC of ID-vaccinated pigs against *M. hyopneumoniae* and in non-vaccinated control pigs (NV, non-vaccinated) exposed to *M. hyopneumoniae* natural infection. Data were obtained by RT-qPCR ($2^{-\Delta\Delta Cq}$ method) and shown as mean RQ values ± standard deviation. (**A**) V1wID group: vaccinated at 1 week of age; (**B**) V4wID group: vaccinated at 4 weeks of age. PV: post-vaccination. Asterisks indicate significant difference compared to the non-vaccinated control group.

**Fig. 7.** Relative gene expression levels of *CCR10* in PBMC of ID-vaccinated pigs against *M. hyopneumoniae* and in non-vaccinated control pigs (NV, non-vaccinated) exposed to *M. hyopneumoniae* natural infection. Data were obtained by RT-qPCR ($2^{-\Delta\Delta Cq}$ method) and shown as mean RQ values ± standard deviation. (**A**) V1wID group: vaccinated at 1 week of age; (**B**) V4wID

group: vaccinated at 4 weeks of age. PV: post-vaccination. Asterisks indicate significant difference compared to the non-vaccinated control group.

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# Immune B cell responsiveness to single-dose intradermal vaccination against *Mycoplasma hyopneumoniae*

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**Highlights**

**1.** ID vaccination at 4 weeks elicited a humoral response associated with *TLR2* and *TLR7* up-regulation.

2. Vaccination at 4 weeks induced *Mycoplasma*-recalled IgA+ and IgG+ B cells, and *CCR10* up-regulation.

3. ID vaccination at 1 week stimulated IgA+ B cells and *TLR2*, *TLR7*, and *CCR9* increases.

4. ID vaccination was associated with a great protection against EP-like lung lesions.

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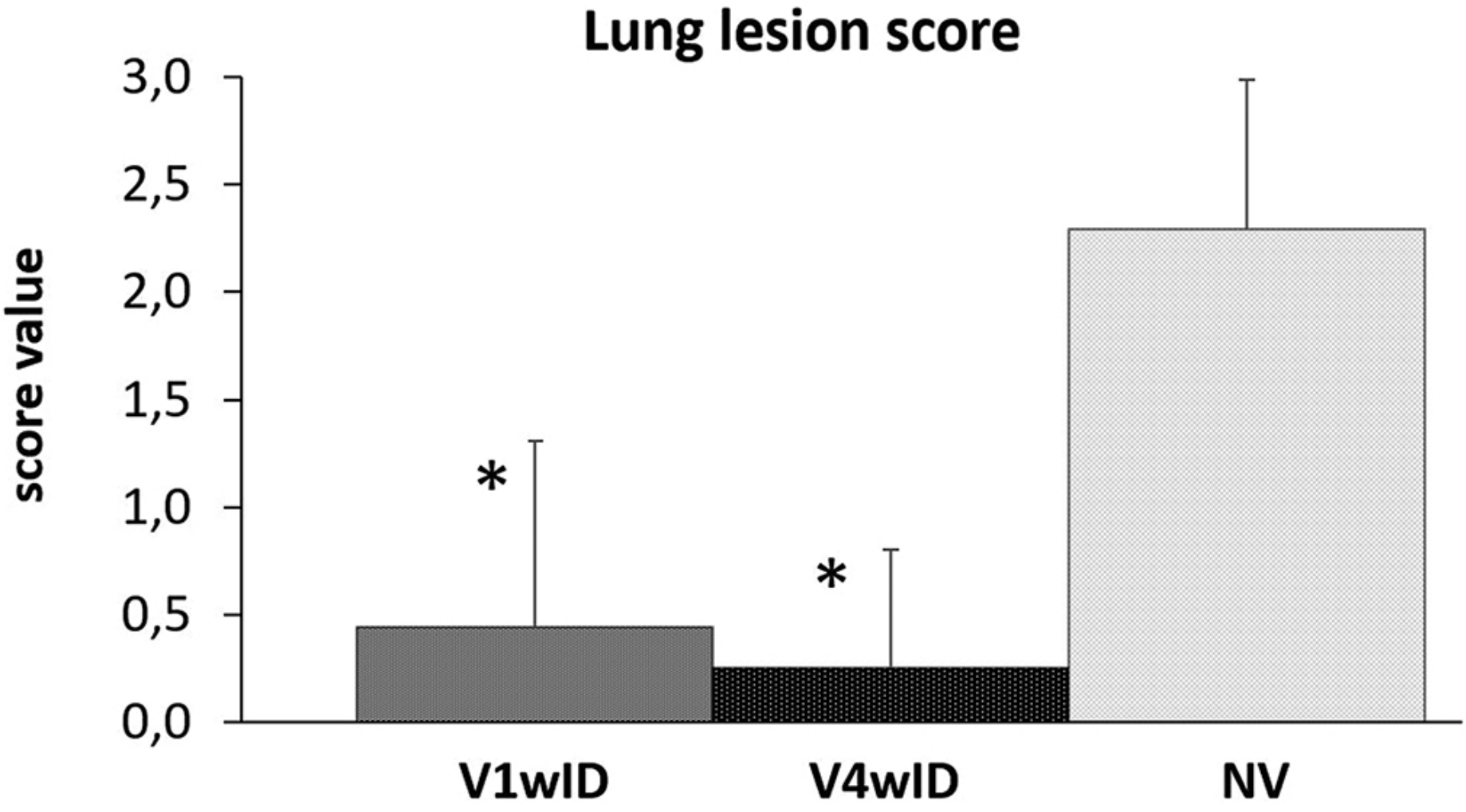
Lung lesion score
score value
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
*
V1wID
V4wID
NV

Figure 1

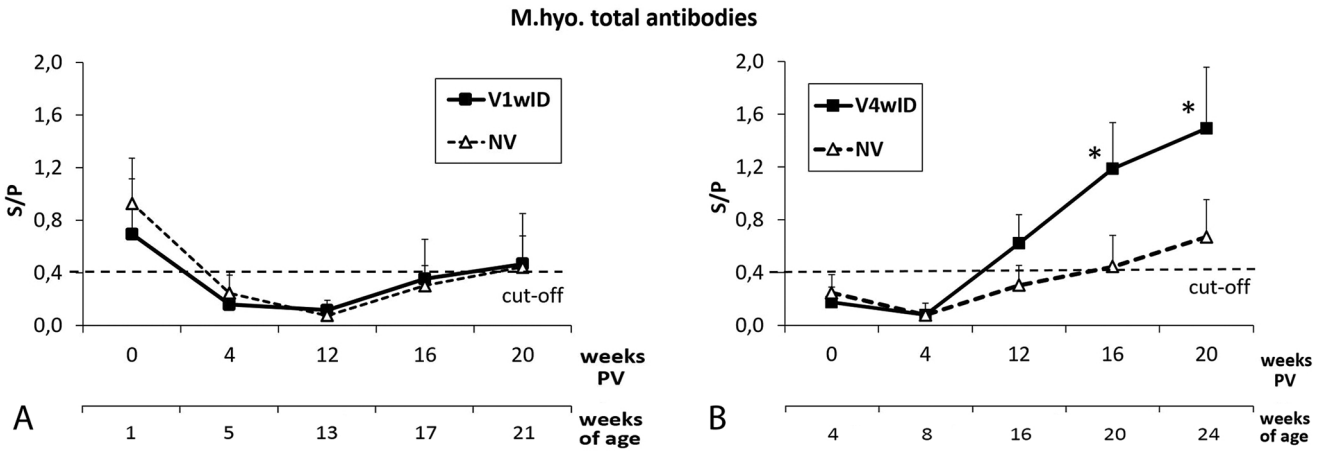
M.hyo. total antibodies
2,0
1,6
1,2
0,8
0,4
0,0
S/P
V1wID
NV
cut-off
0
4
12
16
20
weeks PV
A
1
5
13
17
21
weeks of age
V4wID
NV
*
*
cut-off
B
4
8
16
20
24
weeks of age

Figure 2

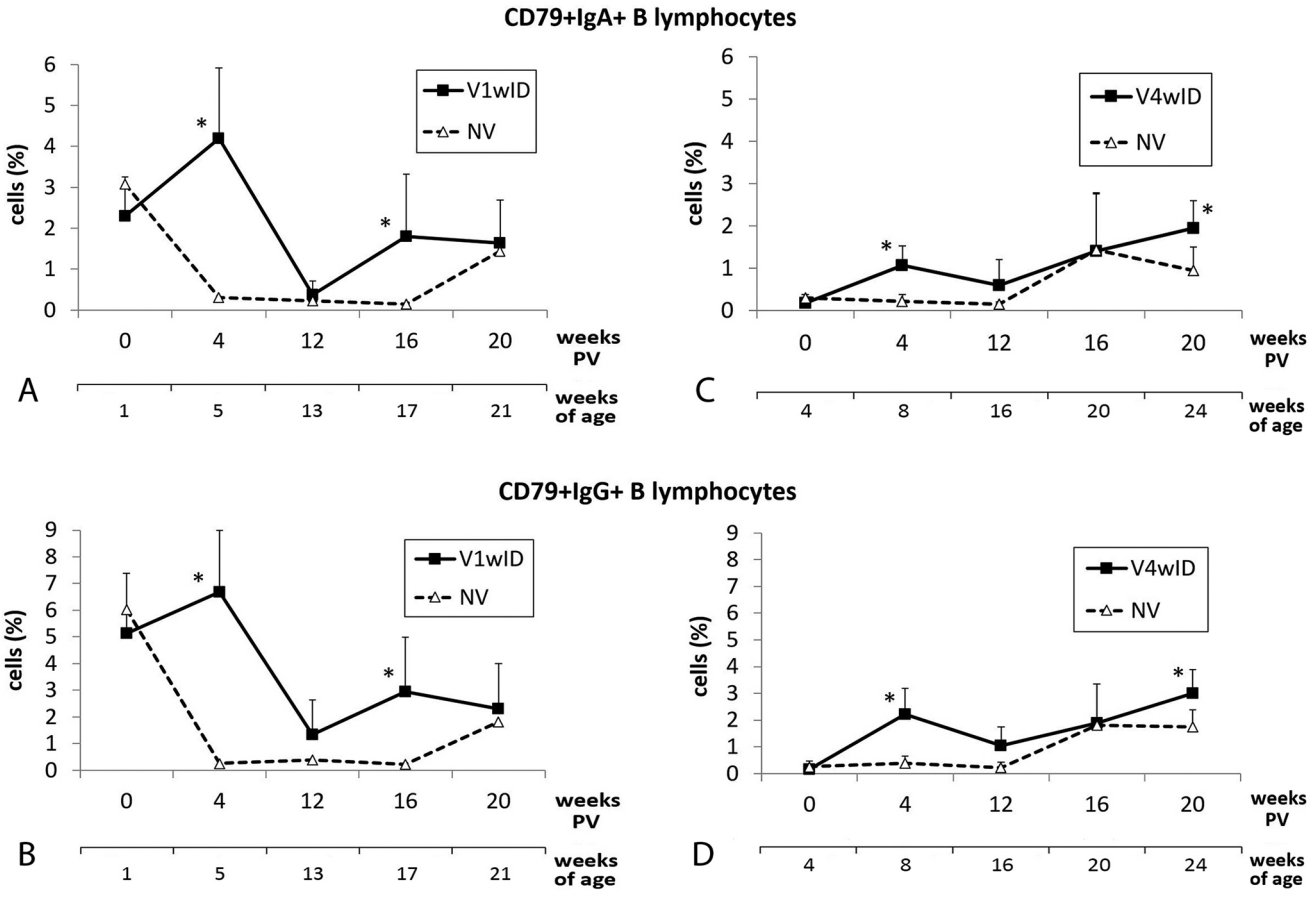
CD79+IgA+ B lymphocytes
cells (%)
V1wID
NV
weeks PV
weeks of age
A
V4wID
C
CD79+IgG+ B lymphocytes
B
D

Figure 3

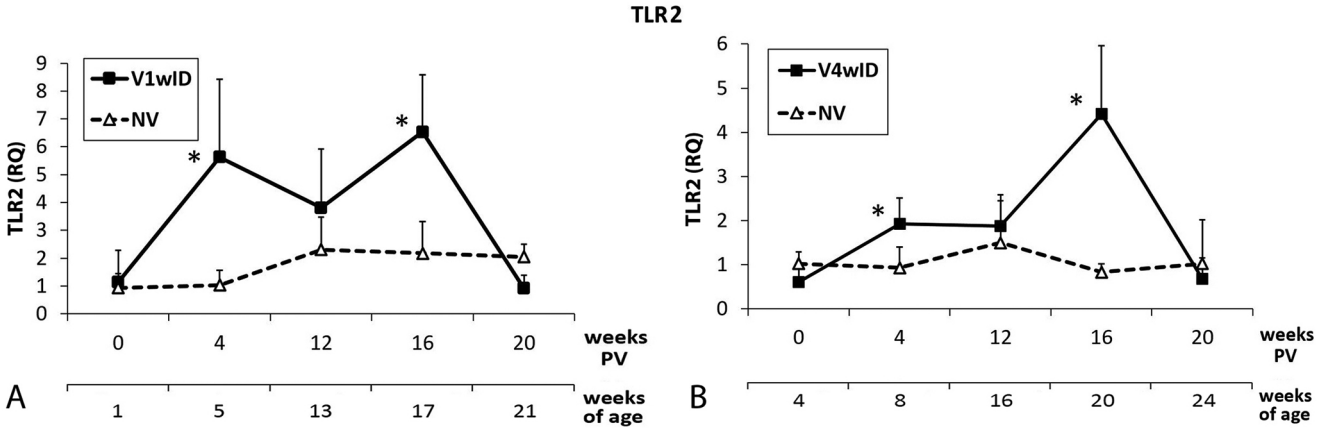
TLR2
V1wID
NV
TLR2 (RQ)
0
1
2
3
4
5
6
7
8
9
*
*
0
4
12
16
20
weeks PV
A
1
5
13
17
21
weeks of age
V4wID
NV
TLR2 (RQ)
0
1
2
3
4
5
6
*
*
0
4
12
16
20
weeks PV
B
4
8
16
20
24
weeks of age

Figure 4

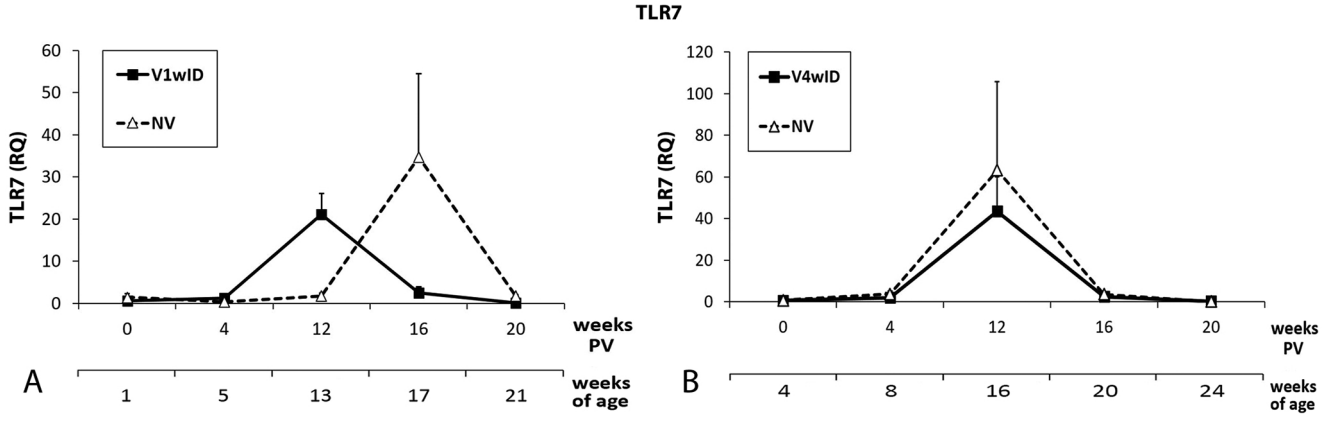
TLR7
TLR7 (RQ)
V1wID
NV
0
10
20
30
40
50
60
0
4
12
16
20
weeks PV
A
1
5
13
17
21
weeks of age
TLR7 (RQ)
V4wID
NV
0
20
40
60
80
100
120
0
4
12
16
20
weeks PV
B
4
8
16
20
24
weeks of age

Figure 5

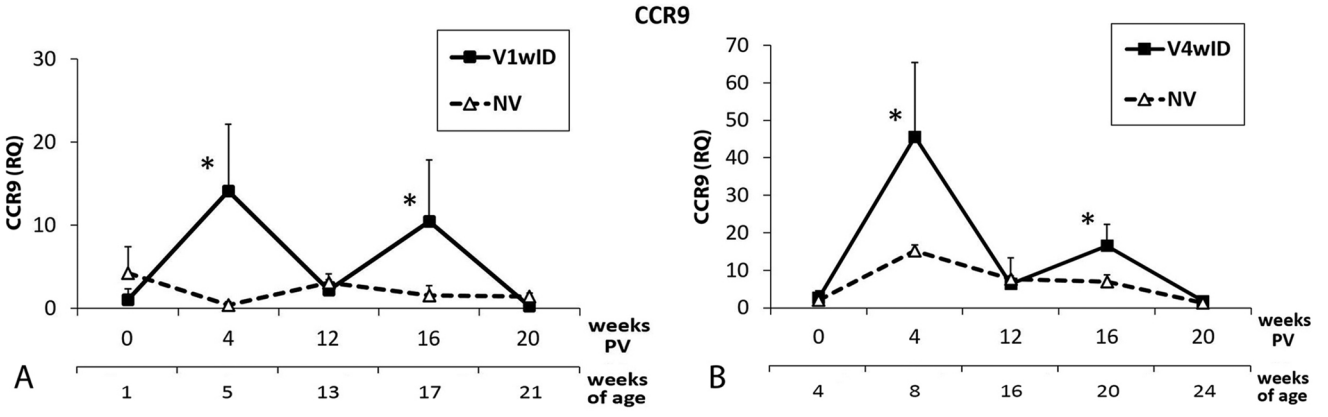
CCR9
V1wID
NV
CCR9 (RQ)
30
20
10
0
*
*
0
4
12
16
20
weeks PV
A
1
5
13
17
21
weeks of age
V4wID
NV
CCR9 (RQ)
70
60
50
40
30
20
10
0
*
*
0
4
12
16
20
weeks PV
B
4
8
16
20
24
weeks of age

Figure 6

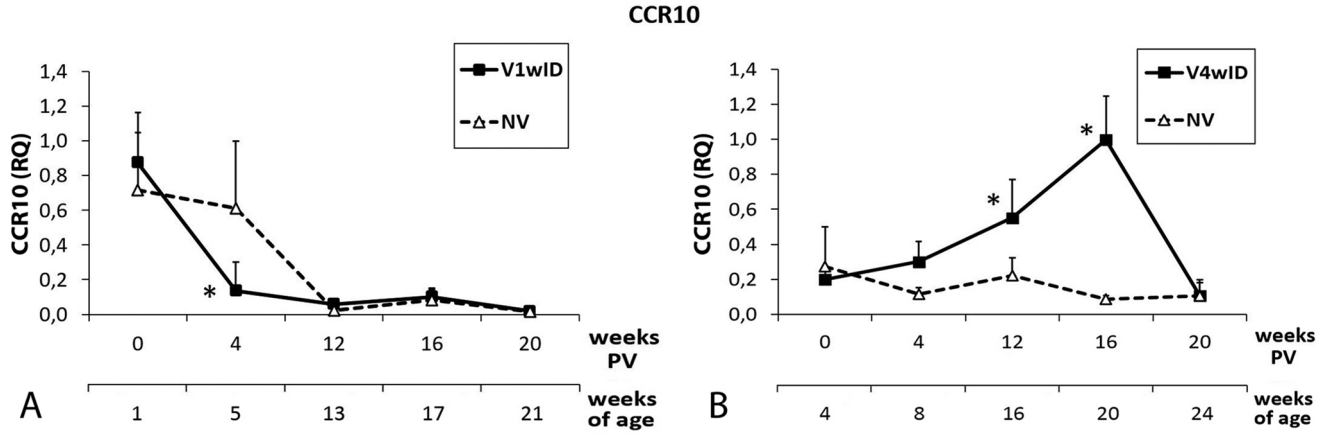
CCR10
V1wID
NV
CCR10 (RQ)
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
*
0
4
12
16
20
weeks PV
A
1
5
13
17
21
weeks of age
V4wID
NV
CCR10 (RQ)
*
*
0
4
12
16
20
weeks PV
B
4
8
16
20
24
weeks of age

Figure 7