Cytokine expression, glucocorticoid and growth hormone changes after porcine reproductive and respiratory syndrome virus (PRRSV-1) infection in vaccinated and unvaccinated naturally exposed pigs

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\textbf{A B S T R A C T}

The objective of this paper was to study the changes of some cytokines and neuroendocrine hormones in vaccinated and unvaccinated pigs that were naturally infected by a PRRSV-1 (porcine reproductive and respiratory syndrome virus) heterologous field strain. We analyzed gene expression of pro-inflammatory (TNF-\( \alpha \), IL-1\( \beta \), MCP-1, IL-6), pro-immune (IFN-\( \gamma \)) and anti-inflammatory cytokines (IL-10) in PBMC, as well as hormonal (GH and cortisol) levels in blood samples of pigs obtained in a field trial previously reported [Martelli P, Gozio S, Ferrari L, Rosina S, De Angelis E, Quintavalla C, et al. Efficacy of a modified-live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: clinical protection and cell-mediated immunity. Vaccine 2009;27:3788–99]. All vaccinated pigs showed an increase in pro-inflammatory and pro-immune cytokine gene expression with respect to controls and a prompt increase in GH that could be consistently associated with pro-inflammatory cytokines in sustaining innate immunity; moreover, the higher levels of cortisol indicates the activation of the hypothalamus–pituitary–adrenal (HPA) axis response. In contrast, unvaccinated pigs showed down-regulation of the cortisol and GH responses, and the pro-inflammatory and pro-immune cytokines remained at a basal or low level, with an increase of TNF-\( \alpha \) and IL-6 in association with a higher level of IL-10 in the late phase of natural infection. The associated trends of pro-inflammatory and anti-inflammatory cytokines together with the cortisol level demonstrate that a previous vaccination promotes an early immune responsiveness in pigs and a more efficient control of inflammation in the late phase of infection with a heterologous PRRSV isolate; both events could sustain clinical protection.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important diseases in the world because of its high prevalence and clinical impact on swine herds, manifested as reproductive failure and respiratory disease. PRRS virus has a complex interaction with the immune system by replication in and negative modulation of innate tissue cells such as macrophages, monocytes and dendritic cells. In contrast to other viral infections, PRRSV...
causes an acute infection, with viremia lasting approximately 1 month, followed by a phase of chronic persistence in lymphoid tissues up to several months. The mechanisms by which PRRSV escapes the immune system and by which the immune response is not triggered to give effective clearance remain partially unknown. During PRRSV infection, the humoral and cell-mediated immune responses develop but their specific role in protection and in eliminating the virus is still conflicting [1,2]. A supported hypothesis is that infection of permissive immune cells, monocytes/macrophages and dendritic cells, does not prime efficient pathogen recognition, with a consequent down-regulation of inflammatory cytokines and a weak innate immune response that compromises the following onset and development of the antigen-specific adaptive immune response [3–7].

Moreover, despite the observation that exposure to the virus induces protective immunity against re-exposure to an homologous virus [8–10], infected pigs can become re-infected and clinically affected when exposed to a heterologous PRRSV isolate. At present, it is not possible to predict the degree of protection provided by individual PRRSV strains that have genetic similarity; it has been shown that variable levels of protection may be achieved in modified live vaccine (MLV)-vaccinated pigs after infection with unrelated PRRSV strains [10–16], but the role played by the innate and/or adaptive immune response in this protection is still unclear [17].

Furthermore, although important information about the immune response of pigs to experimental infection by American and European genotype strains have been obtained [18–21], few studies have been carried out on natural infection and the related respiratory disease. In the present study, pigs were vaccinated with an attenuated European-type vaccine (DV strain, Lelystad cluster) and naturally exposed, under farm conditions, to an Italian wild-type strain of PRRSV-1. As previously described [15], the conditions of this infection model following natural exposure to a heterologous PRRSV-1 isolate allowed the assessment of vaccination efficacy in terms of clinical protection because the PRRS-related disease occurred with overt clinical abnormalities.

For a successful resolution of infection, efficient activation of innate/inflammatory and acquired immunity is required to block pathogen replication and invasion, as well as to promote tissue clearance of the pathogens and/or infected cells. The most prominent pro-inflammatory cytokines (interleukin (IL)-1, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1)), are produced early after pathogen recognition and play a pivotal role in eliciting the innate response as well as in priming and coordinating the adaptive immune response. However, if production is impaired, the innate response will be delayed and inefficient in clearing the pathogen. On the other hand, if production is not dampened and controlled by feed-back mechanisms, the persistence of cytokines will increase tissue damage and worsen the severity of the disease. Thus, qualitative and quantitative, time-related changes in cytokine production during infection can be studied as functional markers of protective immunity and/or of the outcome of the disease [3,6,22–25].

In fact, during infection, the host’s defensive response is modulated by an integrated response, involving a bi-directional communication between the immune and the neuroendocrine systems; pro-inflammatory cytokines and hormones are the effectors of this coordinated and controlled cross-talk, which potentiates innate immunity, controls potential harmful effects due to uncontrolled inflammation and permits the return to a homeostatic state, therefore playing a pivotal role in the efficiency of the immune response against infectious agents. Recently, a lot of knowledge has been obtained on the molecular signals orchestrating this integrated adaptive response; this information has permitted investigators to focus on the systemic mediators that drive the efficiency of the response and also the signalling alterations and control pathway dysfunctions that may be involved in the persistence and/or over-expression of inflammation and consequent tissue damage, thereby influencing the clinical outcome of the disease [26–28]. Indeed, neuroendocrine regulatory factors, such as glucocorticoids and somatotropic hormones, are known to play a major role in the development of the immune system, in the modulation of the innate immune response during infection and in the return to the homeostatic state after the clearance of the pathogen [29,30]. In a previous study we analyzed the immunological response and the clinical evolution of a naturally occurring infection by a PRRSV-1 field strain in vaccinated and unvaccinated pigs. The obtained results showed that vaccination with a MLV conferred clinical protection and that the overall vaccine efficacy was 0.72. The present study aims at investigating, in the same blood samples, some additional parameters (cytokines, cortisol and GH) as indicators of the integrated adaptive neuroendocrine-immune response. These associated parameters could provide new insight to monitor the development and the role of the innate immune response in PRRSV infected animals in combination with the outcomes of the disease.

2. Materials and methods

2.1. Animals and experimental design

The protocol was described in details in a previous paper [15]. Briefly, a total of 30, 4-week-old, conventional pigs were purchased from a PRRSV-free, Mycoplasma hypopneumoniae-free farm. Pigs were randomly divided into three groups (designated IM, ID and C groups) and conventionally housed in an isolation barn located away from the farm of origin (site 2 unit). Different groups were housed in different rooms.

After 1 week of acclimatization (5 weeks of age), the pigs of IM (n = 10 pigs) and ID (n = 10 pigs) groups were vaccinated with Porcilis® PRRS at a dose of 103.5 TCID50 per pig via the intramuscular (2 ml) or intradermal route (0.2 ml), respectively. Intradermal vaccination was performed by using a needle-less vaccinator (I.D.A.L.® Intervet BV, Boxmeer, The Netherlands). Pigs from group C (n = 10 pigs) were not vaccinated and served as controls. The PRRSV vaccine used was the attenuated European virus strain DV (Porcilis® PRRS, Intervet BV, Boxmeer, The Netherlands). The vaccine was suspended in a tocopheryl acetate-
containing aqueous adjuvant (Diluvac Forte®, Intervet BV). A strict control group with no vaccination and challenge is missing because this is impossible to be achieved in a field setting.

Forty-five days post-vaccination (PV), IM, ID and C groups (30 pigs) were moved to the site 3 unit, which housed a conventional, continuous-flow herd, to be naturally exposed to field pathogens. The experimental pigs were commingled with 30 resident animals and housed in the same pen for the duration of the experiment. During the post-exposure period (PE), blood samples were collected on day 0 (45 days PV), 7, 14, 21, 28 and 34 PE for the quantification of pro-inflammatory and anti-inflammatory cytokine gene expression and hormone (GH, cortisol) plasmatic levels.

Pigs were monitored daily for general and respiratory clinical signs throughout the post-exposure period (35 days). Pigs were evaluated in a blind manner by visual inspection for a period of at least 15 min and scores were given for general clinical signs (rectal temperatures, appetite and level of consciousness/lethargy) and respiratory signs as previously described in details [15].

The “recipient-site 3” herd was positive for porcine circovirus type 2 (PCV2) and free from post-weaning multisystematic wasting syndrome and porcine circovirus associated disease (PCVD). There was a history of respiratory problems caused by PRRSV, along with the most common bacteria. In the 2 weeks preceding the exposure of experimental pigs, in animals of the recipient herds (resident pigs) that were affected by acute respiratory disease and subsequently died, microbiological, pathological and serological investigations were carried out. PRRSV was identified by polymerase chain reaction (PCR) in the pneumatic lungs and blood of dead pigs, and the virus was isolated from all samples. Pasteurella multocida and Strep-tococcus spp. were also isolated from pneumatic lungs. Moreover, blood samples were taken from 20 pigs that had recovered from respiratory disease (convalescent samples) to evaluate seroconversion to the most common agents of respiratory disease. Seroconversion was shown for PRRSV (ELISA kit, IDEXX Laboratories Inc., Westbrook, ME, USA) and M. hyopneumoniae but not for Actinobacillus pleuropneumoniae and Aujeszky’s disease. Low titers of antibodies to swine influenza virus (SIV) at hemagglutination inhibition were obtained from some samples and were inconclusive. No lesions referable to PCVD were detected from pathological investigations.

The isolated PRRSV (05R1421) from the resident pigs and from the naturally exposed experimental animals belongs to the Italian cluster of the PRRSV-1. The nucleotide sequence of the open reading frame 5 from strain 05R1421 is 84% identical to that of the DV strain (vaccine strain) [15].

2.2. Cytokine gene expression

Cytokine (TNF-α, IL-6, IFN-γ, IL-1β, IL-10, MCP-1) gene expression was evaluated in PBMC as follows. PBMC were separated by density gradient centrifugation using Histopaque-1077® solution (Sigma–Aldrich®, St. Luis, MO, USA) and washed twice with phosphate-buffered saline (PBS). RNA was extracted by TRI Reagent® solution (Applied Biosystems-Ambion®, Foster City, CA, USA). Briefly, 1 ml of TRI Reagent® solution was added to 1 x 10^7 PBMC and RNA was extracted according to the manufacturer’s instructions.

RNA quantification was carried out by using a spectrophotometer (GeneQuant pro®, Amersham Pharmacia Biotech-GE Healthcare Life Sciences, Little Chalfont, UK).

Reverse transcription (RT) was carried out with the Ready-To-Go™ You-Prime First-Strand Beads kit (Amersham Pharmacia Biotech-GE Healthcare Life Sciences, Little Chalfont, UK) as described by the manufacturer. Two micrograms of total RNA were used in the RT. Aliquots (5 μl) from the generated cDNA were used for the subsequent PCR amplification in the reaction buffer containing 1.5 μl of MgCl₂ (50 mM), 1 μl of dNTPs (12.5 mM) and 1 μl of Taq DNA polymerase (1 μg/μl), to a final volume of 50 μl. Amplification was carried out for 27 cycles, when the reaction was in the middle of the linear range (before reaching the amplification plateau). Each cycle consisted of denaturation at 94 °C for 1 min, annealing at a specific temperature for each primer set for 1 min, extension at 72 °C for 1 min; at the end of the 27th cycle, an additional extension was carried out for 5 min. The primer sequences, annealing temperatures and amplification product weights (base pairs, bp) are summarized in Table 1. The amplified PCR products were visualized after electrophoresis on a 2% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen Corp., Carlsbad, CA, USA). The density of each band was quantified by densitometric analysis by using Scion Image software (Scion Capture Driver 1.2 for Image-Pro Plus; Scion, MA). Ribosomal 18S RNA was used as an internal standard according to manufacturer’s instructions (QuantumRNA™ 18S Internal Standards Kit, Ambion Inc., Austin, TX, USA). The values are shown as ratio of the band intensity of each cytokine normalized with the corresponding ribosomal 18S and expressed as arbitrary units (A.U.).

2.3. Hormonal analysis

2.3.1. GH assay

Plasma samples were assayed for GH by ELISA as previously described [31] and modified for porcine GH (pGH). Briefly, 40 μl of unknown or standard samples (ranging from 39 to 5000 pg of pGH) were incubated with 100 μl of anti-pGH antiserum (1:300,000). After incubation for 48 h at 4 °C, 10 ng/well of biotinyl-pGH were added, followed by incubation for 2 h at 4 °C. Extravidin conjugated with peroxidase (1:2000) was then added in 100 μl of assay buffer, followed by incubation for 1 h; subsequently, 100 μl of ABTS (2,2’-azinobis(3-ethylbenzothiazolinesulfonic acid)/H₂O₂ were added, and the reaction was stopped after 25 min. The absorbance was measured at 405 nm using a Spectra Max Microplate Reader. ED₅₀, ED₇₅ and ED₁₀ were 0.039, 0.166 and 5 ng/well, respectively. The intra- and inter-assay coefficients of variation were 5.3% and 7.8%, respectively.

pGH (AFP10864B) and anti-pGH antiserum (AFP5672099) were provided by Dr. A. F. Parlow (National Hormone and Pituitary Program, Harbor-University of California-Los Angeles Medical Center, La Jolla, CA).
Table 1
Oligonucleotide sequences designed for the detection of different porcine cytokines, with the respective annealing temperatures and amplified products (bp).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Primer set</th>
<th>Annealing temperature (°C)</th>
<th>Amplified product (bp)</th>
</tr>
</thead>
</table>
| IL-1β [14] | F: 5′-ACA GGG GAC TTG AAG AGA G-3′  
R: 5′-CTC TCT GAG TGCT ATG T-3′ | 54.5                        | 285                    |
| IL-6     | F: 5′-ATG AAC TCC CTC TCC ACA AGC-3′  
R: 5′-TGG CTT TGT CAG GAT TCT TCT-3′ | 56.0                        | 493                    |
| IL-10    | F: 5′-AGC CAG CAT TAA GTC TGA GAA-3′  
R: 5′-CCT CTC TTG CAG CTT AA-3′ | 56.0                        | 394                    |
| TNF-α [56] | F: 5′-CCA CCA ACG TTT TCC TCA CT-3′  
R: 5′-AAT AAA GGG ATG GAC AGG GG-3′ | 57.3                        | 351                    |
| IFN-γ    | F: 5′-CTC TCC CAA ACA ATG AGT TAT ACA-3′  
R: 5′-GCT CTC TGG CCT TGG AA-3′ | 55.0                        | 503                    |
| MCP-1 [21] | F: 5′-ATC CTC CAG CAT GAA GGT-3′  
R: 5′-GGA AAT GAA TTG TCT TCA GAT-3′ | 53.7                        | 354                    |

2.3.2. Cortisol assay

The concentration of plasma cortisol was determined by a validated radioimmunoassay, as previously described [32]. Cortisol, H3-labeled cortisol and anti-cortisol antiserum were purchased from Sigma–Aldrich (St. Louis, MO, USA). The intra- and inter-assay coefficients of variation were 7.4% and 9.5%, respectively.

2.4. Statistics

Group comparison for hormonal and cytokine data at each time point was performed by Analysis of Variance and Dunnett’s Two-Sided Multiple-Comparison Test With Control. Comparison of data at different time points within the same group was performed by Repeated Measures Analysis of Variance (full model) and Dunnett’s Two-Sided Multiple-Comparison Test. p-Values less than an alpha of 0.05 (probability of Type I Error) were considered significant throughout this study. Statistical analysis was carried out using the SPSS System for Windows, Version 14.0.

The Pearson’s correlation coefficient was used to determine the degree of association between pairs of variables. The statistical significance of the correlation was determined by the F test, using the statistical package NCSS 2007.

3. Results

3.1. Cytokine gene expression

The results of cytokine gene expression are shown in Table 2 where the statistically significant differences at the comparison between time-points within the same group are reported. The statistically significant differences between groups are shown in Figs. 1–6. The comparison between the vaccinated groups (IM and ID) did not show any statistically significant difference for any of the cytokines at any of the considered times. On the contrary, significant differences were recorded when vaccinated groups were compared with controls (unvaccinated pigs). It is worth remembering that, as briefly reported above and detailed in a previous paper [15], experimental pigs became naturally infected by the PRRSV field isolate, starting from day 4 PE. From day 11 to day 14 all pigs were viremic at PCR.

IFN-γ gene expression in PBMC (Table 2) showed a slight increase during the first week PE in all groups. Then, IFN-γ rose significantly (p < 0.05) only in the vaccinated groups, reaching the highest levels at 14 and 21 days PE; after the third week, IFN-γ in vaccinated pigs fell to basal levels on day 28 PE. In contrast, in unvaccinated pigs, IFN-γ gene expression did not show any significant changes during the whole period, however, an increasing trend was observed from the third week PE. IFN-γ gene expression was significantly higher (p < 0.05) at 14 and 21 days PE in the vaccinated pigs and at 28 and 34 days in the controls (Fig. 1).

IL-6 gene expression (Table 2) increased significantly (p < 0.05) in all groups during the first week. In both vaccinated groups, IL-6 gene expression showed a further significant increase at 14 days PE, and thereafter a decrease to basal levels at 21 up to 34 days PE. At 14 days PE the levels of this cytokine in both vaccinated pigs were significantly higher compared with controls (Fig. 2). In unvaccinated animals, IL-6 showed a significant transient increase in the first week PE, then it decreased up to day 21. After 21 days PE, IL-6 gene expression began to significantly rise and continued up to day 34 PE. In this time period, the higher level of IL-6 in controls was statistically significant compared with vaccinated pigs (Fig. 2).

In the vaccinated groups, both IL-1β and MCP-1 gene expression (Table 2) showed a significant increase at 7 days PE, when the pigs started to become infected by the field PRRSV, with a decline to a basal level at 14 and 21 days PE and 1–2 weeks post-infection, respectively. In control pigs, no changes in IL-1β and MCP-1 expression were observed over time, with the exception of a significant increase in MCP-1 on day 34 (3 weeks post-infection) compared with vaccinated pigs. At 7 days PE, IL-1β gene expression in both vaccinated groups was significantly higher as compared to controls (Fig. 3). MCP-1 gene expression was significantly higher (p < 0.05) at 7 and 14 days PE in vaccinated pigs and at 34 days in controls (Fig. 4).

TNF-α gene expression (Table 2) in PBMC from vaccinated animals showed a significant increase at 14 days PE,
Different superscript letters indicate a statistical difference (p < 0.05) among time points within the same group.

Values are expressed as mean ± S.D. of the cytokine/18S gene expression ratio (arbitrary units). Asterisk (*) indicates a statistically significant difference (p < 0.05) between vaccinated (groups IM and ID) and unvaccinated pigs.

and a declining trend 21 days PE, reaching basal levels at 28 and 34 days PE. In unvaccinated pigs, TNF-α expression significantly increased on day 21 PE and remained at significantly higher levels until the end of the experiment, compared with vaccinated animals (Fig. 5).

IL-10 levels (Table 2) slightly increased up to 14 days PE in all groups; in vaccinated animals, IL-10 gene expression showed a prompt and marked decrease from 14 days PE to the end of the observations. Conversely, unvaccinated pigs maintained a significantly higher level with respect to vaccinated pigs from 21 to 34 days PE (Fig. 6).

The study of the statistical correlations showed that in unvaccinated pigs IL-6 and TNF-α are positively correlated with clinical signs as reported by Martelli et al. [15] with a p value of 0.01 and 0.03, respectively. The r values are 0.42
Fig. 2. Course of IL-6 gene expression in PBMC of PRRSV-vaccinated (ID and IM groups) and unvaccinated (C group) pigs in the post-exposure period. Data are shown as ratios of IL-6/18S rRNA gene expression, arbitrary units (A.U.). Asterisk (*) indicates a statistically significant difference ($p<0.05$) between vaccinated groups IM and ID) and unvaccinated pigs.

Fig. 3. Course of IL-1β gene expression in PBMC of PRRSV-vaccinated (ID and IM groups) and unvaccinated (C group) pigs in the post-exposure period. Data are shown as ratios of IL-1β/18S rRNA gene expression, arbitrary units (A.U.). Asterisk (*) indicates a statistically significant difference ($p<0.05$) between vaccinated (groups IM and ID) and unvaccinated pigs.

Fig. 4. Course of MCP-1 gene expression in PBMC of PRRSV-vaccinated (ID and IM groups) and unvaccinated (C group) pigs in the post-exposure period. Data are shown as ratios of MCP-1/18S rRNA gene expression, arbitrary units (A.U.). Asterisk (*) indicates a statistically significant difference ($p<0.05$) between vaccinated (groups IM and ID) and unvaccinated pigs.
Fig. 5. Course of TNF-α gene expression in PBMC of PRRSV-vaccinated (ID and IM groups) and unvaccinated (C group) pigs in the post-exposure period. Data are shown as ratios of TNF-α/18S rRNA gene expression, arbitrary units (A.U.). Asterisk (*) indicates a statistically significant difference (p < 0.05) between vaccinated (groups IM and ID) and unvaccinated pigs.

Fig. 6. Course of IL-10 gene expression in PBMC of PRRSV-vaccinated (ID and IM groups) and unvaccinated (C group) pigs in the post-exposure period. Data are shown as ratios of IL-10/18S rRNA gene expression, arbitrary units (A.U.). Asterisk (*) indicates a statistically significant difference (p < 0.05) between vaccinated (groups IM and ID) and unvaccinated pigs.

and 0.37 for IL-6 and TNF-α, respectively. No statistically significant correlations were detected in vaccinated pigs.

3.2. Hormonal changes

The results regarding hormone plasma levels are shown in Table 2 where the statistically significant differences at the comparison between time-points within the same group are reported. The statistically significant differences between groups are shown in Figs. 7 and 8.

In the first week of exposure, the plasmatic level of cortisol fell in unvaccinated pigs whereas remained at significantly higher levels in vaccinated animals (Fig. 7). On day 14 PE, plasmatic cortisol levels reached basal levels in all considered animals and maintained those levels up to the end of the experimental period.

The GH plasmatic trend showed significant differences between vaccinated and unvaccinated groups in the PE period (Fig. 8). On day 7 PE, plasmatic GH significantly increased (p < 0.05) in vaccinated pigs (both groups, IM and ID), with statistically significant differences observed at the comparison with unvaccinated pigs. Thereafter, GH levels decreased to the basal level at 21 days PE. In unvaccinated pigs, GH showed a slight transient increase at 14 days PE.

In vaccinated pigs the levels of GH are statistically correlated (p < 0.01) with the levels of gene expression of IFN-γ (r = IM: 0.33; ID: 0.38), IL-6 (r = IM: 0.46; ID: 0.63), TNF-α (r = IM: 0.36; ID: 0.52), IL-1β (r = IM: 0.49; ID: 0.54), and MCP-1 (r = IM: 0.52; ID: 0.65).

Moreover, the levels of IL-10 in vaccinated pigs, belonging to both groups, were positively correlated with the cortisol course (p < 0.001) (r = IM: 0.31; ID: 0.53). No statistical correlations between cytokines and hormones were detected in control animals. Conversely, the levels of cortisol is negatively correlated (p < 0.001; r = −0.56) with clinical signs measured as reported by Martelli et al. [15].

4. Discussion

As a previous paper [15] is the underlying study for this follow-up research, to make reading easier the major results are briefly summarized. After exposure, accord-
Fig. 7. Course of cortisol plasma levels in PRRSV-vaccinated (ID and IM groups) and control (C group) pigs in the post-exposure period. Data shown as mean values ± S.D. Asterisk (*) indicates a statistically significant difference (p < 0.05) between vaccinated (groups IM and ID) and unvaccinated pigs.

Fig. 8. Course of growth hormone (GH) plasma levels in PRRSV-vaccinated (ID and IM groups) and control (C group) pigs in the post-exposure period. Data shown as mean values ± S.D. Asterisk (*) indicates a statistically significant difference (p < 0.05) between vaccinated (groups IM and ID) and unvaccinated pigs.

According to PCR results, PRRSV infection first occurred on day 4 PE, and between days 7 and 14, all of the experimental pigs (vaccinated by both routes of administration and unvaccinated) were infected. Moreover, gross pathology and microbiological investigations were performed on two experimental pigs that died, and \( P. \text{ multocida} \) was isolated from pneumatic lungs. Nested-PCR and immunofluorescence assay for \( M. \text{ hyopneumoniae} \) gave consistently negative results. SIV was not isolated from pneumatic lungs. No seroconversion to \( A. \text{ pleuropneumoniae} \), \( M. \text{ hyopneumoniae} \), Aujeszky's disease virus and SIV was detected at any considered time. At vaccination and on days 0 and 21 PE, all serum samples were negative for the PCV2 genome by quantitative PCR. At the end of the study (day 34 PE), 1 pig (IM group) out of 27 had a viral load in serum of \( 3.45 \log_{10} \) [15]. These results confirm that PRRSV and \( P. \text{ multocida} \) were involved in the outcome of the observed disease and do not allow to completely exclude the effect of other significant concurrent/secondary sub-lethal bacterial infections in a proportion of these pigs that may have altered some of the later responses. It is well known that in field the existence of whatever other pathological conditions could affect the results presented in this study. Therefore, whatever other potential infection under the conditions of this field experiment should have affected all treatment groups (vaccinated and unvaccinated animals) so that, if significant differences were obtained, these must be mainly attributed to vaccination. The clinical evaluation recorded statistically significant differences between vaccinated and controls, clearly supporting that vaccination conferred clinical protection (vaccine efficacy of approximately 70%) as detailed in a previous report [15]. Furthermore, in accordance with previous reports [14,15], the results of this study show that the immune parameters are not affected by the route of administration of the vaccine, as intradermally vaccinated pigs did not show any differences in terms of cytokine and hormonal trends compared to intramuscularly injected animals.

The study investigated the induction of some pro-inflammatory (IL-1\( \beta \), TNF-\( \alpha \), IL-6, MCP-1), pro-immune (IFN-\( \gamma \)) and anti-inflammatory (IL-10) cytokines in blood leucocytes from MLV-vaccinated pigs and unvaccinated pigs naturally infected with a PRRSV-1 field strain. Several studies have analyzed cytokine gene expression in vivo and in vitro and/or cytokine secretion in the blood or bronchoalveolar lavage (BAL) fluid during experimental infection by PRRSV [5,33–35]. However, the results were not conclusive and were occasionally contrasting.
The present study evaluated gene expression of systemic cytokines in PBMC with the objective of assessing cytokine temporal patterns. Many immune cell subpopulations are represented in PBMC (i.e. monocytes, dendritic cells, innate and acquired cytotoxic cells, regulatory cells) and their proportion and functional status are expression of immune cell recruitment, migration and activation against pathogens. We therefore used PBMC as a biological sample and gene expression as a parameter to functionally measure inflammatory and immune cell reactivity during PRRSV infection. The Authors considered that gene expression in PBMC could also be a suitable parameter for achieving a more stable measurement of cytokine production for the evaluation of immune cell activation over time. Indeed, the determination of cytokine protein levels in serum could be erratic and less informative under the conditions of this experiment, namely natural infection. Moreover, cytokine levels in serum are not strictly related to the levels produced locally in the target organs (e.g. BAL fluid) [3,33].

Furthermore, to our knowledge, this is the first study evaluating cytokine profiles in relation to the time-related changes of some immune-modulating hormones, namely cortisol and GH.

The time-related changes of pro-inflammatory and pro-immune cytokine expression in vivo demonstrate that natural infection by PRRSV does not activate an early and efficient inflammatory and innate immune response, which agrees with other laboratory trials that showed how PRRSV infection alters the immune response, mainly inducing down-regulation of pro-inflammatory and pro-immune cytokines [3,4,20,33].

IFN-γ production is a fundamental event for the effectiveness of innate and acquired immunity against viruses; innate [natural killer (NK) cells and γ/δ T lymphocytes] and acquired (helper and cytotoxic T lymphocytes) immune cells produce IFN-γ as the pivotal paracrine and autocrine signal for their activation [25,36]. IFN-γ gene expression did not show any significant increase after field PRRSV exposure in unvaccinated pigs. This impaired cell-mediated immune response was confirmed by the late changes in IFN-γ secreting cells (SC), natural and MHC-specific cytotoxic cells during PRRSV infection occurring at 21 days PE [15].

It is well-known that PRRSV infection can induce a systemic production of IFN-γ, characterized by individual variability in terms of intensity and occurrence, coupled with extremely variable efficiency of the innate and adaptive immune response that sustains viral clearance [1,2,37].

Under the conditions of this experiment, in unvaccinated pigs infected by PRRSV-1, the levels of pro-inflammatory and pro-immune cytokines significantly increased only when infection was established by 2 weeks; conversely, the expression of these cytokines in PBMC of vaccinated pigs was higher as soon as the first week post-infection. These different trends sustain the prompt immune reactivity of systemic inflammatory and immune cells in vaccinated animals, and the impaired, delayed reaction in controls coupled with the occurrence of more severe clinical signs, namely a higher proportion of febrile pigs associated with lethargy and anorexia, as reported in the previous paper [15].

We observed that PRRSV natural infection does not induce variations in systemic TNF-α gene expression up to 1–2 weeks post-infection in unvaccinated pigs. It is interesting to note that TNF-α gene expression was low in the first 2 weeks of infection when the health status of the infected control pigs was impaired by fever and general clinical abnormalities [15]. This result is in accordance with that by Thanawongnuwech et al. [5] whose study assessed cytokine gene expression in pulmonary alveolar macrophages; moreover, Van Reeth et al. [33] detected minimal TNF-α levels at 10 days post-infection when the maximal pathology associated with PRRSV infection occurs. Some mechanisms have been hypothesized to explain this feature: PRRSV strongly inhibits TNF-α production and conversely it is well-known that increased TNF-α levels can play a pivotal role in viral clearance by inflammatory cells in the respiratory tract [5]. It is known that TNF-α has a pivotal role in innate immune response efficiency, showing antiviral effect, activation and differentiation of the monocyte/macrophage lineage, and activation of NK cells to produce IFN-γ [25].

Taken together, we can speculate that the lack of TNF-α gene expression in PBMC indicates a reduced reactivity of systemic inflammatory and innate cells, which can also negatively affect their efficiency when recruited in the respiratory tract. The evidence of higher TNF-α gene expression in PBMC of vaccinated infected pigs demonstrates that the reactivity of these cells to produce this cytokine has been primed and, at the time of infection, they are able to play a more efficient functional role in clinical protection.

The results of this trial show a significant, prompt increase of IL-1β soon after infection in vaccinated piglets and no modification in its gene expression in unvaccinated pigs. In the unvaccinated pigs, IL-1β gene expression in PBMC did not show any changes during the whole post-exposure period. Van Reeth and Nauwynck [3] reported an early increase of IL-1β after experimental challenge with PRRSV in the BAL fluid; the authors hypothesized that this increase can be related to systemic clinical signs (i.e. fever). Contrarily, our results obtained from a different biological sample (systemic PBMC vs. BAL fluid) lead to a different interpretation in terms of functional meaning: in our case, cytokine gene expression in PBMC has to be evaluated as a marker of efficiency of innate cell reactivity rather than as an indicator of severity of local and systemic abnormalities.

Our data confirm that clinical signs in unvaccinated pigs occur without early signals of inflammatory and innate reactivity (i.e. IL-1β, TNF-α and MCP-1), even in the severely affected animals. Conversely, the patterns of the mentioned cytokines in vaccinated pigs indicate that, although infection occurs with the same timing as that of unvaccinated pigs, the vaccinated pigs are able to show and sustain more efficient reactivity of innate immunity within the first week post-infection, even in the case of infection by a genetically different, heterologous field isolate (85% of homology). This difference in immune reactivity in vaccinated pigs parallels with a less severe outcome of the disease in comparison with controls.
The results of IL-6 gene expression are more difficult to interpret. It is likely that the changes of IL-6 activity should be functionally evaluated in terms of their occurrence in the early (acute) or late (chronic) phase of inflammation, taking into account the complex and modulating role exerted during the immune response and IL-6’s interplay with other pro-immune factors, namely neuro-immune hormones. At the beginning of the acute inflammatory phase, IL-6 mediates the acute phase reaction (APR) and modulates the over activity of inflammation; it is well-known that IL-6 is also critical in controlling the extent of local and systemic acute inflammation, particularly in decreasing the levels of pro-inflammatory cytokines by exerting a protective effect against potential damage and promoting anti-inflammatory activity [38]. However, if its levels persist and chronic inflammation occurs, IL-6 can have a detrimental effect by favouring the recruitment, proliferation and survival of mononuclear cells at the site of injury [39]. We detected low IL-6 levels of gene expression in unvaccinated pigs exposed to the PRRSV field isolate in the first 2 weeks post-infection in accordance with the results by Thanawongnuwech et al. [5], who demonstrated increased levels of IL-6 only after 28 dpi, with no increased levels of IL-1β. Conversely, in vaccinated pigs, higher levels of IL-6 were observed only during the first week post-infection, followed by a prompt and long-lasting significant decrease. Moreover, the observed early increase of IL-1β preceding the increase of IL-6 in vaccinated pigs is expected because the former cytokine is a strong inducer of IL-6 production during viral infection [40]. Thus, in this paracrine interaction, an increase in both IL-1β and IL-6 can sustain, along with TNF-α, a more efficient innate response. In contrast, in unvaccinated pigs considered in this trial, the late occurrence and persistence of IL-6 gene expression in PBMC could impair activation of the adaptive immune response by further inhibition of Th1 differentiation [41] and could drive chronicization of the inflammation [38], with a strong influence on the outcome of the observed disease in unvaccinated pigs. Moreover, the positive statistically significant correlation between IL-6 and TNF-α with clinical signs in controls [15] corroborate this last assumption.

MCP-1 is a chemokine involved in the onset of inflammation; it mediates mainly the chemotaxis of monocytes and also of memory T lymphocytes and NK cells [42]. MCP-1 and its receptor play an important role in the T cell-mediated immune response against intracellular pathogens by controlling leukocyte recruitment and clearance of pathogens [43–45]. Its production is induced by IL-6, TNF-α and IFN-γ [38,42].

The pattern of MCP-1 in the present study was characterized by an increase and subsequent decrease in vaccinated pigs during the first week of infection, which indicates, similarly to the other pro-inflammatory cytokine pattern, namely IL-1β and TNF-α, that an early danger signal was produced and mediated prompt activation of an inflammatory/innate response. On the contrary, in unvaccinated pigs, the increase of MCP-1 was observed later with respect to infection (4 weeks); the difference in its level, compared with those of vaccinated animals, differs significantly and coincides with a higher level of IL-6, a strong inducer of MCP-1 production. The combination of these two cytokines in PBMC is consistent with a delayed recruitment of monocytes and with a chronicization of inflammation [38]. It is well-known that under several conditions, high and persistent levels of MCP-1 are involved in many autoimmune- or infection-mediated inflammatory diseases associated with monocyte recruitment with uncontrolled inflammatory damage [42].

During infection, activation of the HPA axis is one of the major neuroendocrine features of APR. Indeed, pro-inflammatory cytokine (IL-1, TNF-α, IL-6) production, which sustains the APR, is tightly controlled by intrinsic anti-inflammatory mechanisms and also by the stimulation of the HPA and the release of cortisol by adrenal glands [26,46]. It is well known that the increase of glucocorticoid (GC) plasmatic levels suppresses the further release of pro-inflammatory cytokines, preventing long-term and/or over activation of inflammation.

Interestingly, PRRSV infection down-regulates the cortisol response; our results on plasmatic levels of cortisol are in accordance with those by Sutherland et al. [47], who observed that plasma cortisol, considered an indicator of HPA axis activation, was suppressed during PRRSV infection. Our results confirm and further characterize the above feature: we demonstrated that, in unvaccinated pigs, cortisol down-regulation is associated with no increase of pro-inflammatory cytokines which are the major systemic signals for HPA activation during the acute phase reaction [48]. Interestingly, in vaccinated animals cortisol maintained statistically significant higher levels than in unvaccinated animals in the first week post-infection.

At present, a potential direct or indirect mechanism by which PRRSV infection can alter HPA activation and GC production is unknown and warrants further study.

If GC have a central role in the control of local and systemic inflammatory responses during and after pathogen challenge and in the prevention of excessive inflammation, it is not surprising that a failure of GC production or tissue resistance to their inhibitory effects may result in uncontrolled inflammation and tissue damage, and thus increased susceptibility to and severity of the related disease, influencing survival [26,27,30,49,50]. We can hypothesize that in unvaccinated pigs the down-regulation of cortisol production in the early phase of infection may have negatively influenced HPA responsiveness so that in the subsequent late phase cortisol did not increase to counterbalance the rise of pro-inflammatory cytokines (i.e. TNF-α, IL-6 and MCP-1). In unvaccinated animals, the long-term maintenance of higher levels of TNF-α and IL-6 after infection in association with low levels of cortisol could be consistent with a long-lasting inflammatory status and lack of control of the disease, thereby influencing the overt clinical signs observed in the unvaccinated animals.

IL-6 is also a key cytokine in the neuro-immune communication during infection. It has important effects on the production of corticotrophin-releasing hormone by the hypothalamus and the secretion of adrenocorticotropic hormone, prolactin and GH from the pituitary gland [51,52]. In vaccinated pigs, we detected increased levels of IL-6 gene expression statistically correlated with increases of GH and IFN-γ; in this context, IL-6 could act positively on innate immunity. High and early IL-6 levels are consistent
with an APR, whereas prolonged production of IL-6 and the related deregulation of the HPA axis have been considered an important pathway affecting health during inflammation, chronic stress, aging and metabolic disease [38,53]; therefore, the persistence of this cytokine can be a valuable marker for ongoing infections [54].

Furthermore, this is the first study considering the GH course during PRRSV natural infection. It is well known that pro-inflammatory cytokines (i.e. IL-1 and TNF-α) have a direct effect on pituitary GH secretion during the acute phase response and several studies have suggested that this hormone plays an important role in the improvement of innate immune responses during infection [30]. The infection in unvaccinated pigs did not significantly increase plasmatic GH, indicating inefficient pituitary activation. Contrarily, both after vaccination with an MLV (data not shown) and, in vaccinated animals, after infection with PRRSV under field conditions, an early and prompt rise of GH was clearly detected. In a previous study, we demonstrated that vaccination against PRRSV increases plasmatic GH regardless of a weaning-dependent increase in plasmatic cortisol [55].

The rise of plasmatic GH during infection could be a reliable indicator of immune responsiveness in vaccinated pigs; GH has a positive effect on the development and maturation of lymphoid cells and on cytokine secretion in primary lymphoid organs such as thymus, and promotes the efflux of mature lymphoid cells in the blood and the migration in secondary lymphoid organs and target tissues. Moreover, GH improves the efficiency of innate immune cell activity, mainly NK cells [39,56,57]. Recently in pig, Thacker et al. [58] showed that plasmid-mediated supplementation of growth hormone-releasing hormone before vaccination may enhance protection against M. hyopneumoniae pneumonia and reduces the clinical outcome of the disease.

Indeed, there is evidence that IL-6 may regulate GH secretion [59] and, in turn, GH increases IL-6 secretion [60] and directly affects the innate immune response [61]. Since a paracrine influence exists between GH and IL-6, under a stress condition such as immune challenge, GH appears fundamental to increasing the levels of these cytokines for prompt imprinting of an innate response to pathogens.

IL-10, mainly produced by Th2 and regulatory T lymphocytes, but also by activated macrophages, is a crucial cytokine in controlling the extent of the immune response and preventing immune-mediated tissue damage. Conversely, early over-expression and/or persistence of IL-10 might prevent or delay the clearance of pathogens, thereby resulting in persistent infection [62]. In the present study, high levels of IL-10 occurred in vaccinated and unvaccinated pigs from the beginning of PRRSV infection; this is consistent with the delay of the acquired response in the first 2–3 weeks post-infection and consequently of the inefficient viral clearance during the period of observation [15]. Some studies indicate that enhanced in vivo and in vitro IL-10 expression in different permissive or unpermissive immune cells might be an important mechanism for modulating the host’s immune response during experimental infection by PRRSV and for playing an essential role in a virus evading the immune response, which prevents the development of protective immunity [7,12,63,64].

Similar to Thanawongnuwech et al. [5], we found high levels of IL-10 gene expression during the whole period of exposure, but compared with vaccinated pigs, unvaccinated pigs showed significantly higher levels of IL-10 gene expression during the last 2 weeks post-exposure.

In the model proposed in this field study, the evidence for differences of IL-10 gene expression in PBMC between vaccinated and unvaccinated pigs, with higher levels of this cytokine in the latter animals, can parallel with the severity of the clinical scores we observed, and conversely with the protection induced by vaccination. In contrast, the statistically correlated trends between IL-10 and cortisol sustain a more balanced control of inflammation in the early phase of the disease. It can be hypothesized that the difference in the immune response and, likely, in the severity of the disease between vaccinated and control groups depends mainly on the quality of the innate immune response, which was evaluated as a time-related, pro-inflammatory cytokine and anti-inflammatory balance. In our study, vaccinated animals, in contrast to unvaccinated pigs, showed a prompt increase of TNF-α gene expression balanced by high levels of IL-10 and a rapid decrease of both cytokines after infection with a heterologous PRRSV-1 field isolate. Interestingly, other Authors, although using other approaches, have suggested that the quality of the cytokine pattern induced by a given strain is important for modulating the entire immune response against PRRSV. In agreement with this, the present study and a previous paper [15], Díaz et al. [65] found that strains inducing TNF-α and IL-10 simultaneously yielded a more sustained cell-mediated immune response, measured as IFN-γ SC.

The early and prompt increase after PRRSV infection, and the subsequent decrease to basal levels within 1–2 weeks, of IL-1β, IL-6, TNF-α, MCP-1 and IFN-γ in vaccinated animals is a further, clear indication of both a more efficient responsiveness of peripheral innate immune cells (i.e. monocytes, NK cells) in the early phase of infection and a more efficient control of inflammation in a later phase. Both aspects coincides with the clinical protection (70%) induced by vaccination as previously reported by Martelli et al. [15] in the same experimental animals.

On the contrary, unvaccinated animals subsequently infected with a heterologous PRRSV-1 strain show an inefficient and delayed immune response; furthermore, long-term maintenance of TNF-α, IL-6 and IL-10 levels and deregulation of cortisol production could be markers of uncontrolled inflammatory damage and could functionally explain the severity of clinical signs.

Thus, the associated trends of pro-inflammatory and anti-inflammatory cytokines together with the cortisol level could provide indicators to study the progression of the persistent PRRSV infection and its severity.

**Conflict of interest statement**

None of the authors of this paper has a financial or personal relationship with other people or organisations that
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