Research paper

Development of cell-mediated immunity to porcine circovirus type 2 (PCV2) in caesarean-derived, colostrum-deprived piglets

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ABSTRACT

The interaction between porcine circovirus type 2 (PCV2) and the pig immune system has been suggested to be a determinant event for the pathogenesis of postweaning multisystemic wasting syndrome (PMWS). To gain insight into the host immune mechanisms developed upon PCV2 infection, early innate and adaptive immune responses were examined in 1-week-old, caesarean-derived, colostrum-deprived piglets using a subclinical infection model of PCV2 in combination with lipopolysaccharide (LPS) as a potential immunostimulation factor. The use of LPS did not show any significant effect on the course of PCV2 infection, nor did in the evolution of the immunological parameters evaluated. *Ex vivo* responses were detected as early as 1 day post-infection (PI) and consisted of an elevation of the plasmatic levels of interleukin (IL)-8 in PCV2-inoculated pigs followed by an increase on plasmatic IFN-α at day 5 PI. Regarding IL-10, only one PCV2-inoculated pig was positive (day 7 PI); this pig was the only one in which viremia persisted until the end of the study. *In vitro* cytokine determination showed that, regardless of the treatment administrated to the pigs, an IL-10 release was observed when peripheral blood mononuclear cells (PBMC) cultures were stimulated with PCV2. Seroconvertion to PCV2 measured by an immunoperoxidase monolayer assay (IPMA) occurred between 7 and 14 days PI, whereas neutralizing antibodies (NA) did not appear until day 29 PI. PCV2 DNA was first detected in serum at day 7 PI, reaching the peak of viremia between days 14 and 21 PI, followed by a drop in viral load that was found coincident with the appearance of PCV2-specific IFN-γ-secreting cells (PCV2-IFN-γ-SC) and NA. Results from the present work suggest that viral clearance might be mediated by the development of PCV2-IFN-γ-SC in contribution to the PCV2-specific NA.

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1. Introduction

Porcine circovirus type 2 (PCV2) is the causative agent of the postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that affects nursery and fattening pigs (Segalés et al., 2005). This syndrome is characterized by a progressive loss of weight, and secondary or opportunistic infections are reported to be common (Carrasco et al., 2000; Clark, 1997). The main histological lesions consist of extensive lymphocyte depletion together with histiocytic and/or multinucleate giant cells infiltration (Clark, 1997; Rosell et al., 1999). Thus, the whole picture of the disease is highly suggestive of an acquired immunodeficiency.

Several studies have suggested the interaction between PCV2 and the immune system as a key event in the pathogenesis of PMWS. PCV2 infects monocytic-lineage cells (Gilpin et al., 2003; Rosell et al., 1999; Vincent et al., 2003), and its long-lasting persistence within macrophages and dendritic cells (DC) has been suggested as a potential mechanism of dissemination for PCV2 throughout the body (Vincent et al., 2003). In addition, PCV2 impairs the ability of peripheral blood mononuclear cells (PBMC) to respond to mitogens (Darwich et al., 2003a) and viral DNA has been shown to block the activity of natural interferon-producing cells (NIPC) to produce IFN-α, which in turns affects the ability of these cells to mediate antiviral responses upon the infection (Vincent et al., 2007). Besides, PCV2 has been demonstrated to induce IL-10 secretion in *in vitro* cultured PBMC (Darwich et al., 2003a; Kekarainen et al., 2008), leading to down-regulation of other cytokines produced during recall antigen responses (Kekarainen et al., 2008). The authors of these latter studies suggested the involvement of IL-10 in the suppressed Th_1 responses observed during the course of PMWS. This hypothesis is also supported by other works performed on blood samples of PCV2-experimentally inoculated pigs, in which the elevation of IL-10 in plasma was correlated with either development of PMWS (Stevenson et al., 2006) or increased PCV2 load in serum (Darwich et al., 2007). Altogether, these data suggest that the mechanisms used by PCV2 to counteract the host immune defences are likely to lie under the immunomodulatory activity of its viral components.

Most PCV2 infections are subclinical and different factors have been postulated as potential triggers for the development of the disease. Experimentally, the most successful models used to reproduce PMWS include co-inoculation with porcine parvovirus (Allan et al., 1999) or the use of substances modulating the immune system such as keyhole limpet haemocyanin in incomplete Freund's adjuvant (Krakowka et al., 2001). Occasionally, PMWS has been also reproduced using PCV2 alone (Allan et al., 1999; Kennedy et al., 2000). Although none of these models has been consistently and repeatedly demonstrated in conventional pigs, evidences point towards an alteration of the immune response – induced by PCV2 itself or by other yet unknown factors – as the triggering factor for PMWS development.

Lipopolysaccharide (LPS) has been used in previous works aimed to reproduce viral-induced multifactorial respiratory disease, by being inoculated in pigs infected with either porcine respiratory and reproductive syndrome virus (PRRSV) or porcine respiratory coronavirus (PRCV) (Van Gucht et al., 2003, 2006). Results from those studies demonstrated that the inmunomodulatory effect of LPS, seen as an overexpression of certain pro-inflammatory cytokines, was found to correlate with clinical disease. In addition, another study performed by Chang et al. (2006b) demonstrated that LPS was able to *in vitro* induce upregulation of PCV2 replication in swine pulmonary macrophages.

The aim of the present work was to gain insight into the immune response generated upon PCV2 infection, by studying the innate and adaptive immune responses in experimentally inoculated pigs, either using PCV2 alone or in combination with LPS as a potential immunostimulant. The parameters evaluated were used to set a reference pattern to be later compared with the events taking place in the course of PMWS.

2. Materials and methods

2.1. Experimental design

All details on source and housing of animals as well as the experimental conditions have been previously described (Fernandes et al., 2007). Briefly, fifty-four 1-week-old colostrum-deprived, caesarean-derived (CDCD) piglets were included in this study. Pigs were randomly distributed into four groups, namely A ($n = 10$), B ($n = 8$), C ($n = 18$) and D ($n = 18$). Group A pigs were kept as uninoculated controls; group B animals were intraperitoneally inoculated with 50 μg/kg of LPS from *Salmonella typhimurium* (Sigma–Aldrich, L7261); group C pigs were inoculated with $10^{5.2}$ $TCID_{50}$ of the Burgos strain of PCV2 (1 ml orally and 1 ml nasally) produced in PK-15 cells, and group D pigs received simultaneously PCV2 and LPS at the doses stated above. In order to study the events taking place at the early stages of infection, thirty-two piglets were sequentially necropsied within the first 8 days of the experiment. The remaining pigs were followed up throughout the experimental period, being bled at days 0, 7, 14, 21 and 29 post-inoculation (PI) for serum and PBMC collection. During the experimental period, clinical signs were monitored daily and pigs were weighted three times a week until day 29 PI (euthanasia). Table 1 summarizes the experimental design.

Animal care activities and study procedures were conducted in accordance with the guidelines of the Good Experimental Practices (GEP), under the approval of the Ethical and Animal Welfare Committee of the *Universitat Autònoma de Barcelona*

Table 1
Summary of the experimental design.

Group	Inoculum	Dose	Days post-infection								
			Number of piglets sampled per day								
			0	1	2	5	7	8	14	21	29
A	MEM[a]	2 mL	6	1	1	1	4	1	4	4	4
B	LPS	50 μg/kg	4	1	1	1	4	1	4	4	4
C	PCV2	$10^{5.5}$ $TCID_{50}$	6	3	3	3	6	3	6	6	6
D	PCV2 + LPS	B + C	6	3	3	3	6	3	6	6	6
Total number of piglets			22	8	8	8	20	8	20	20	20

[a] Minimum Essential Medium.

2.2. Virological and serological studies

Viral load was determined in serum by means of a quantitative Taqman PCR (Q-PCR) (Olvera et al., 2004) and in tissues using an *in situ* hybridisation (ISH) procedure (Rosell et al., 1999).

Antibody-mediated responses to PCV2 were assessed by an immunoperoxidase monolayer assay (IPMA) and a viral neutralization test as described elsewhere (Fort et al., 2007).

2.3. Ex vivo cytokine profile determination

Levels of IFN-α, IL-10, IL-1β, IL-8 and TNF-α in plasma samples were examined by means of capture ELISAs developed using commercially available antibodies (anti-IFN-α antibodies from PBL Biomedical Laboratories, Piscataway, NJ, USA; anti-IL-1β, anti-IL-8 and anti-TNF-α from R&D Systems, Spain; and IL-10 from Biosource, Spain). For each ELISA, the cut-off value was calculated as the average of the optical density of negative controls plus three standard deviations. Cytokine concentrations were determined using a regression line built up with the optical densities of the cytokine standards used in each test.

2.4. In vitro cytokine profile determination

PBMC were separated from whole blood by gradient density centrifugation using Histopaque 1.077 (Sigma–Aldrich). Cells were cultured for 20 h at 37 $^\circ$C in 5% CO_2 (5×10^5 cells/well) in presence of either PCV2 (m.o.i. of 0.01 $TCID_{50}$/cell), phytohaemagglutinin (PHA) (10 μg/ml), or were mock stimulated with cell culture supernatants of uninfected PK-15 cells. Cultures were done at least in triplicate and supernatants corresponding to the same animal and stimulus were mixed for the analysis. Capture ELISAs for IFN-γ (BD, Madrid, Spain), IL-2, IL-4 and IL-10 (Biosource, Spain) were performed and cytokine concentrations were calculated as explained above.

Frequencies of PCV2-specific IFN-γ-secreting cells (PCV2-IFN-γ-SC) in PBMC were determined by ELISPOT at 7, 14, 21 and 29 days PI, by using commercial mAbs (Swine IFN-γ Cytosets kits, Biosource, Spain) following a previously described protocol (Díaz and Mateu, 2005). Briefly, 96-well flat bottom plates (Costar 3590, Corning, USA) were coated overnight with anti-IFN-γ antibody at 8.3 μg/ml in carbonate–bicarbonate buffer (pH 9.6). Plates were washed three times with phosphate-buffered saline (PBS) and blocked with PBS containing 10% of foetal bovine serum (FBS) for 1 h at 37 $^\circ$C. After removing the blocking solution, 100 μl containing 5×10^5 PBMC were dispensed per well and stimulated with either PCV2 (m.o.i. = 0.01), PHA (10 μg/ml) or mock stimulated for 20 h at 37aC in presence of 5% CO_2. Cells were removed and wells were then incubated 1 hour at 37 $^\circ$C with 50 μl of the biotinylated antibody at 2.5 μg/ml in PBS containing 0.05% of Tween 20 and 0.5% of bovine serum albumin (PBS-T-BSA). After three washings with PBS-T, plates were incubated 1 h with Streptavidin-HRP (Biosource, Spain) at 0.5 μg/ml in PBS-T-BSA, and finally, insoluble TMB blue (Calbiochem, Spain) was used to reveal the reaction. PCV2-IFN-γ-SC were calculated by subtracting the number of spots counted in mock-stimulated wells from PCV2-stimulated ones. Results were expressed as numbers of IFN-γ-SC per million PBMC.

2.5. Leukocyte subsets determination

PBMC subsets were phenotypically characterized by flow-cytometry for CD3$^+$ (BD #559582), CD4$^+$ (BD #12516), CD8$^+$ (Southern Biotech #4520-09), CD21$^+$ (Southern Biotech #4530-02) and SWC3/CD172a (Southern Biotech #4525-09) cells. Fifty microliters of a cell suspension adjusted at 4×10^6 cells per ml in flow-cytometry medium (FCM; PBS containing 0.1% BSA) were placed into a 96-well plate and incubated for 30 min with 50 μl of each mAb, followed by two washes with FCM. Except for CD21$^+$, which was already conjugated with FITC, and CD8$^+$ and SWC3$^+$ with PE, a rabbit F(ab)2 anti-mouse Ig-FITC (Dako, Denmark) was used as secondary antibody. Finally, PMBC were washed twice and fixed in FMC containing 0.3% paraformaldehyde. The analysis was performed using an EPICS_XL MCL flow cytometer (Beckman-Coulter, USA). Irrelevant isotype-matched antibodies were used as background controls.

2.6. Statistical analysis

Statistical analyses to compare means of the different parameters among groups were performed using the GLM procedure (SAS 9.1 software, SAS Institute Inc., Cary, NC, USA). When no differences attributed to the effect of LPS were detected, data were analysed considering PCV2 inoculation as the only classificatory variable, being groups A + B considered as control group and groups C + D as PCV2-inoculated one. Significance level (α) was set at 0.05

3. Results

3.1. Clinical and pathological outcomes

Details on clinical and pathological data are found in Fernandes et al. (2007). At the end of the study (day 29 PI), PCV2-inoculated groups showed lower mean body weight (6.6 ± 2.3 kg in group C and 6.6 ± 2.7 kg in D) compared to control ones (8.0 ± 1.72 kg in group A and 7.5 ± 1.6 kg in B) ($p < 0.05$) However, none of the pigs developed clinical signs compatible with PMWS throughout the whole study. One day after inoculation, all pigs receiving LPS (groups B and D) had significantly higher rectal body temperatures compared to groups A and C (39.2 ± 0.9 vs. 38.4 ± 0.4; $p < 0.001$); from then onwards, no differences in rectal temperatures were observed among groups. Regarding pathological studies, animals euthanized within the first 8 days PI had no detectable virus in their tissues by ISH. In contrast, from the 20 piglets necropsied at the end of the study (day 29 PI), 9/12 PCV2-inoculated animals showed presence of mild PCV2-like lesions in lymphoid tissues together with low to moderate amounts of PCV2 DNA in those lesions as determined by ISH (5/6 in group C and 4/6 in group D).
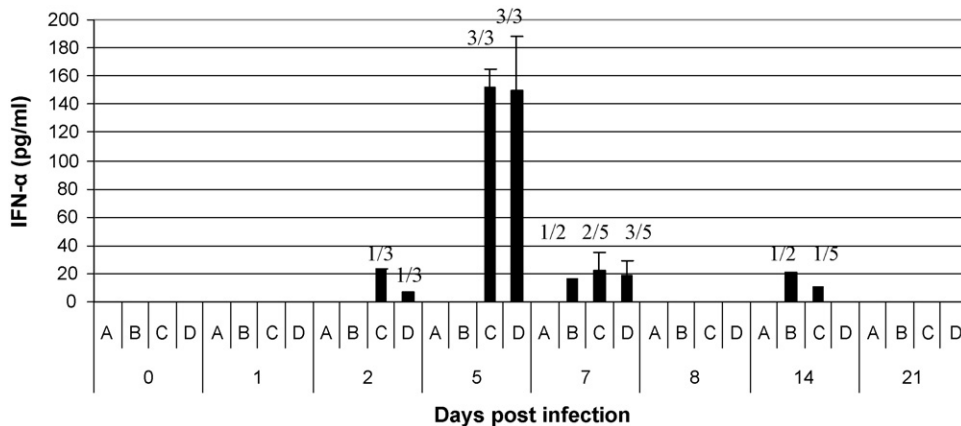
Fig. 1. Levels of IFN-α (pg/ml) detected in plasma, expressed as mean ± S.D. of positive pigs in each experimental group (A–D), from 0 to 29 days PI. The number of positive pigs at each time point is indicated.

3.2. Viremia and humoral response to PCV2

Results of the Q-PCR showed that PCV2 DNA firstly appeared in sera of PCV2-inoculated pigs by day 7 PI, with increasing titres until day 14 PI in group C ($6.4 \times 10^5 \pm 1.3 \times 10^5$ viral copies/ml) and day 21 PI in group D ($1.3 \times 10^6 \pm 2.8 \times 10^5$ viral copies/ml) ($p > 0.05$). PCV2-uninoculated pigs remained free of virus all along the study.

Development of humoral response started in PCV2-inoculated pigs (groups C and D) with the appearance of IPMA-detectable antibodies between 7 and 14 days PI, with increasing titres until day 29 PI (mean IPMA titre: $10.3 \pm 1.2 \log_2$). Seroconversion for NA occurred between 21 and 29 days PI, with a mean titre of $5.0 \pm 1.1 \log_2$. No differences between groups C and D were noticed regarding IPMA or NA titres.

3.3. Ex vivo cytokine responses

All PCV2-inoculated piglets had a transient increase in plasma levels of IL-8 (184.8 ± 37.5 pg/ml) by day 1 PI, whereas uninoculated controls remained negative. At the time IL-8 faded out, levels of IFN-α started to be detectable in serum of PCV2-inoculated pigs. Thus, at day 2 PI, one animal in group C and one in D were positive for this cytokine, and, by day 5 PI, sera from all PCV2-inoculated pigs had detectable IFN-α (3/3 in group C, 151 ± 12.7 pg/ml and 3/3 in group D, 149.7 ± 39.1 pg/ml). Later on, positive results were only sporadically detected (Fig. 1).

Regarding IL-10 detection in serum, only one animal from group C was positive (day 7 PI; 154 pg/ml). For IL-1β and TNF-α, most piglets were negative and these cytokines were only detected sporadically regardless of the PCV2 inoculation status (data not shown).

3.4. In vitro cytokine responses

After *in vitro* treatment of PBMC with PCV2, no induction on IL-2, IL-4 or IFN-γ was observed. On the other hand, the levels of IL-10 detected in supernatants of PBMC stimulated with PCV2 were significantly higher than those detected in mock-stimulated cultures ($p < 0.0001$).

Indeed, the IL-10 release induced by the virus itself was observed independently of the administrated treatment with no significant differences, although individual results showed certain variation within the same group along the study. Thus, in group A, 9/10 pigs were detected positive (range: 17.2–273.9 pg/ml), 8/8 in group B (range: 19.3–194.4 pg/ml), 16/18 in group C (range: 13.1–298.3 pg/ml), and 15/18 in group D (range: 36.3–282.4 pg/ml).

Development of PCV2-specific IFN-γ-SC was only observed in PCV2-inoculated groups (C and D) and started between 14 and 21 days PI. Thus, at day 21 PI, 4/5 pigs in group C and 3/4 in group D were positive with mean
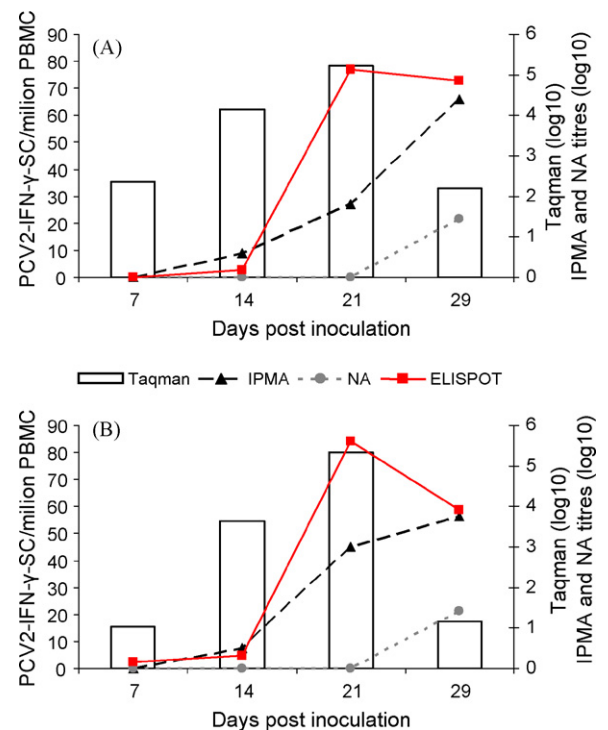

Fig. 2. Mean values evolution of PCV2-IFN-γ-secreting cells (PCV2-IFN-γ-SC), IPMA antibodies, neutralizing antibodies (NA) and viremia (Q-PCR) of PCV2-inoculated groups C (A) and D (B), from 7 to 29 days PI.

frequencies of 77 ± 30 and 84 ± 49 PCV2-IFN-γ-SC per million of PBMC ($p > 0.05$), respectively. At day 29 PI, those values were 73 ± 30 and 59 ± 49 ($p > 0.05$). None of the pigs from groups A and B had positive results in the IFN-γ ELISPOT during the study. Fig. 2 summarizes the results for the development of IFN-γ-SC, PCV2 antibodies and viremia in PCV2-inoculated groups (C and D).

3.5. Leukocyte subsets determination

In the flow-cytometry analyses, most changes in the relative proportions of cell subsets were sporadic and transient and could not be attributed to treatments received. However, PCV2-infected pigs showed a decrease in the relative proportions of CD4$^+$SwC3$^+$ cells (0.3 ± 0.2 vs. 1.1 ± 0.4; $p = 0.004$) as well as CD4$^+$CD8$^+$ lymphocytes (3.5 ± 0.8 vs. 5.9 ± 1.9; $p = 0.005$) at days 14 and 21 PI, respectively compared to PCV2-free pigs. In addition, a downshift in CD21$^+$ cells was observed in PCV2-inoculated groups compared to controls at same days (1.5 ± 0.7 vs. 3.3 ± 1.0; $p = 0.003$ and 2.1 ± 0.7 vs. 3.0 ± 0.5; $p = 0.056$, respectively). By day 29 PI those differences disappeared.

4. Discussion

Pathogenesis of PMWS is not fully understood yet and there is not a single parameter or group of parameters to be used as reliable predictors for the development of the disease. Previous reports suggested that the complex host–virus interaction and the subsequent immune response generated might be critical determinants for the understanding of the disease (Meerts et al., 2006; Stevenson et al., 2006; Vincent et al., 2007). In the present study we tried to characterize some of the parameters of the innate and adaptive immune responses against PCV2 in animals that withstand the infection without developing clinical disease.

To date, although reproduction of PMWS has been achieved using several experimental models (Tomás et al., 2008), none of them has been demonstrated to be consistently repeatable in conventional pigs. Among them, the use of immunostimulants and co-infections following PCV2 infection is apparently the most successful strategy to experimentally reproduce clinical disease, suggesting the activation of the immune system as a potential triggering factor of PMWS (Tomás et al., 2008). In the present study, the effect of immunostimulation on the course of PCV2 infection was evaluated in 1-week-old CDCD piglets by injecting them with LPS, simultaneously to PCV2-oronasal inoculation. Although LPS was claimed to have a positive effect on PCV2 replication in pulmonary macrophages (Chang et al., 2006b), our data showed no significant effect of LPS on the evolution of PCV2 infection (Fernandes et al., 2007) and the immunological parameters evaluated throughout the experiment. These results might suggest that the LPS-induced viral replication reported in vitro does not apparently occur in vivo, or not extensively enough to trigger PMWS under the present experimental conditions. Nevertheless, further studies such as the local effect of LPS on PCV2 target tissues should be performed to conclude that, since no significant induction of plasmatic

pro-inflammatory cytokines attributed to LPS administration were detect.

Regarding the innate immune responses against PCV2, the earliest event detected following PCV2 infection was an increase in the plasma levels of IL-8 (day 1 PI). The ability of PCV2 to induce IL-8 production in porcine alveolar macrophages or PBMC has been previously reported (Chang et al., 2006a; Darwich et al., 2003a) and agrees with the inflammatory nature of the lesions usually seen in PCV2-infected animals. In the present study, other pro-inflammatory cytokines such as IL-1β or TNF-α were not detected in serum with a consistent pattern. However, the lack of detection of these cytokines in plasma does not exclude them from playing a role in early phases of the infection, since they could have been acting locally at the site of viral replication without reaching levels high enough to be detected in serum. In contrast, a clear IFN-α response was observed in PCV2-inoculated pigs at day 5 PI. IFN-α is considered a crucial cytokine for the host antiviral defences, being involved not only in innate responses but also in regulating the adaptive immunity generated upon viral infections. Indeed, the ability to counter IFN-α-mediated responses through different pathways, and thereby interfere with the immune mechanisms of the host, has been reported for several viruses (Garcia-Sastre and Biron, 2006). In the case of PCV2, recent studies showed that PCV2 or PCV2-CpG motifs may inhibit or induce IFN-α responses (Vincent et al., 2005; Wikstrom et al., 2007) depending on the cell subset studied and on the structure of CpGs. Vincent et al. (2007) reported that PCV2 was able to block IFN-α induction in NIPCs, suggesting the inmunomodulatory activity of PCV2 as a key event in the pathogenesis of PMWS. In this respect, those animals withstanding the infection without developing clinical disease should be able to counteract the inhibitory activity of PCV2, probably by means of strong innate responses. In the present study, the substantial amounts of IFN-α detected in plasma of all PCV2-inoculated pigs at early stage of infection indicated that development of an early innate response against the virus was generated. The fact that no PCV2 genome neither PCV2-associated lesions were found in tissues of the early necropsied pigs might be attributed to the ability of PCV2 to persist in monocytic-lineage cells without active replication (Vincent et al., 2003), and therefore being undetectable by a standard HIS technique. These results, together with the fact that PCV2 is known to mediate inhibition of NIPC responsiveness, suggest that the balance between the host ability to mount a proper innate antiviral response and the virus ability to dampen it might be determinant for the infection evolution and triggering of the disease.

Elevated levels of IL-10 in plasma were detected only in one PCV2-inoculated pig, at day 7 PI. In contrast, in vitro results showed an induction of IL-10 release in response to PCV2 stimulation of PBMC, regardless of the treatment administrated to the pigs. The involvement of IL-10 in the pathogenesis of PMWS has been suggested in several studies. Darwich et al. (2003b) found an overexpression of IL-10 mRNA in thymus of PMWS-affected pigs in correlation with lymphoid lesions. Also, cytokine profile

evaluation of blood samples from PCV2-experimentally inoculated pigs showed an association between elevated plasma levels of IL-10 and development of PMWS (Stevenson et al., 2006). *In vitro*, PCV2 has been demonstrated to induce IL-10 secretion in PBMC cultures, which, in turns, lead to repression of other cytokines (Kekarainen et al., 2008). Altogether, these data suggest that the ability of PCV2 to induce IL-10 might contribute to the immunosuppressive status observed in the course of PMWS. In our study, none of the pigs developed clinical disease and viremia decreased by day 29 in all pigs but one, in which viremia persisted until the end of the study (data not shown). Interestingly, this pig was the only one with detectable IL-10 in serum and also with one of the lowest IFN-α response generated. These results suggest that some pigs – those controlling the progression of the infection – might counteract the ability of PCV2 to induce an IL-10 release, most probably by means of strong IFN-α responses.

Regarding adaptive responses, IPMA antibodies to PCV2 appeared between 7 and 14 days PI, whereas NA appeared 7–14 days later. Inefficient or delayed development of antibody-mediated immunity to PCV2 has been previously correlated with high level of PCV2 replication and the outcome of clinical disease (Fort et al., 2007; Meerts et al., 2006; Okuda et al., 2003). However, certain delay on the neutralizing response has been also observed in PCV2 subclinically infected pigs in which PMWS was not developed (Fort et al., 2007).

The cytometric analysis indicated that infected pigs suffered a downshift in CD4$^+$SwC3$^+$, CD4$^+$CD8$^+$ and CD21$^+$ cells at 14 and 21 days PI, and these values returned to normality by the end of the study. Changes in PBMC subsets in PCV2-infected pigs that do not developed the disease have been previously reported (Darwich et al., 2004; Nielsen et al., 2003) and differ from those ones observed in PMWS-affected ones (Nielsen et al., 2003); whereas leukopenia observed in pigs infected subclinically seems to be transient, leukopenia in PMWS-affected ones appears earlier and is stronger, being correlated with the appearance of clinical disease.

The role of specific cellular defence mechanisms in providing protection against PMWS has not been elucidated yet. Herein, we describe for the first time the development of IFN-γ-SC in response to PCV2 infection. IFN-γ is considered to be a key cytokine for Th1 polarisation; by controlling the differentiation of naïve CD4 T cells into Th1 effectors, this cytokine mediates cellular immunity against viral infections. In the present study, development of IFN-γ-SC in PCV2 subclinically infected animals started between days 14 and 21 PI and was coincidental with the decrease of viral load in blood. Our results suggest that cell-mediated response, measurable as IFN-γ-SC, might also contribute, together with development of PCV2 NA, to the clearance of PCV2.

In conclusion, the results of the present study suggest that in PCV2-infected pigs, an early innate IFN-α response, together with the development of PCV2-IFN-γ-SC and NA may be adequate predictors of the evolution of PCV2 infection and should be explored in experiments aimed to elucidate the mechanisms lying behind the different clinical outcomes of PCV2 infections in pigs.

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