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Short Communication

Transient correlation between viremia levels and IL-10 expression in pigs subclinically infected with porcine circovirus type 2 (PCV2)

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Abstract

Immunological impairment by porcine circovirus type 2 (PCV2) infection is well documented in pigs suffering from postweaning multisystemic wasting syndrome. Nonetheless, little is known about immune status of pigs that remain PCV2 subclinically infected. Thus, seven pigs successfully infected in an experimental inoculation and without developing disease and nine control non-inoculated pigs were examined. Serological, virological and immunological determinations were done throughout ten weeks post-infection (PI). At week 3 PI, inoculated animals presented the peak of viremia and produced higher levels of IL-10 than the controls; correlation between viral load and IL-10 amounts was observed ($p < 0.05$). Also, the ratio IgM/IgG suffered a shift skewing IgM production towards an IgG response. By 10 weeks PI, levels of IL-10 disappeared and the viremia decreased. In summary, subclinically PCV2-infected pigs developed a transient PCV2-specific IL-10 response during the viremic phase of infection which coincided with the inversion of the IgM/IgG ratio.

Keywords: Porcine circovirus type 2 (PCV2); Subclinical infection; IL-10; IgM/IgG ratio; PCV2 viremia

Postweaning multisystemic wasting syndrome (PMWS) was first described in Western Canada by mid 1990s (Harding and Clark, 1997). This syndrome is caused by porcine circovirus type 2 (PCV2), a member of the family *Circoviridae* (Allan et al., 1998; Ellis et al., 1998). The disease mainly appears in six to 12-weeks-old pigs and is characterized by a progressive lost of weight and a generalized lymphocyte depletion (Allan and Ellis, 2000; Darwich et al., 2002). Some authors have suggested that PMWS is an immunosuppressive disease (Darwich et al., 2004; Segalés et al., 2004).

Although most if not all herds are seropositive to PCV2, the incidence of disease ranges from 5% to 20% of the total number of infected pigs in those PMWS affected farms (Segalés and Domingo, 2002). The fact that almost all animals are infected by PCV2 in some life point but only some of them develop the disease remains as one of the most important key points to be solved for PMWS. The aim of the present study was to evaluate immune parameters of PCV2 subclinically infected pigs and to correlate those results with the virological and clinical course of the infection.

The experimental design included 16 conventional pigs from a conventional 50-sow, farrow-to-finish operation, free of the most common swine pathogens including porcine parvovirus, porcine reproductive and respiratory syndrome virus, Aujeszky's disease virus, swine influenza virus, transmissible gastroenteritis coronavirus, porcine respiratory coronavirus, *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, *Bordetella bronchiseptica* and swine erysipelas. Moreover, PMWS-like clinical signs were never observed on the farm and, although the farm was known to be enzootically infected by PCV2, all piglets had no detect-

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able PCV2 maternal antibody titres at the moment of their housing, assessed by immunoperoxidase monolayer assay (IPMA) technique (Rodriguez-Arrioja et al., 2000). Pigs were distributed in two groups: group A with PCV2 experimentally inoculated pigs ($n = 7$), and group B with healthy non-inoculated pigs ($n = 9$). Group A pigs were inoculated with 2.5 ml of the PCV2 strain Burgos ($10^{4.7}$ $TCID_{50}$/ml, intranasal) while control group received the same volume of sterile saline as placebo. To obtain the viral inoculum, swine kidney (SK) cells were inoculated with a tissue homogenate prepared from tissues (lymph nodes, spleen, liver and kidney) of a PMWS affected pig. After two serial passages, culture supernatants were recovered, clarified by centrifugation at 650*g*, and further ultracentrifuged at 100.000*g*. The viral pellet was resuspended in DMEM, titrated in non-infected SK cells, aliquoted and frozen at −80 °C until used. The inoculum was shown to be free of PRRSV, PPV, and PCV1, by PCR. Detection of total anti-PCV2 antibodies was weekly monitored by means of IPMA technique. Virus-specific IgG and IgM antibodies were detected by Ig capture ELISAs (Ingezim PCV IgG® and Ingezim PCV IgM®, Ingenasa, Madrid, Spain) as manufacturer's recommendations.

Clinical monitoring was done daily and the rectal temperature was taken every 2 days until day 23 post-inoculation (PI) and thereafter at weekly intervals. Viremia was evaluated at days 7, 14, 21, 49 and 69 post-infection (PI) by using a real time (Taq-Man) PCR as described in Resendes et al. (2004). Viral shedding was also examined in nasal exudates and faecal samples those same days. Pigs were euthanized at day 69 PI (19 weeks of age) with an overdose of intravenous pentobarbital, according to guidelines of animal care committee of the Autonomous University of Barcelona. Pigs were necropsied and studied histopathologically; PCV2 nucleic acid presence in formalin-fixed, paraffin-embedded tissues was screened by an *in situ* hybridization technique (Rosell et al., 1999).

Heparinized blood samples were collected at days 21 PI (considered as early stage of infection) and 69 PI (considered as late stage of infection) and peripheral blood mononuclear cells (PBMC) were separated from blood by gradient density centrifugation with Histopaque® 1.077 (Sigma, Alcobendas, Spain). The phenotypic analysis of PBMC was done by flow cytometry (FCM) using appropriated monoclonal antibodies in the EPICS XL-MLC cytometer (Coulter) as described by Darwich et al. (2002). *In vitro* culture of PBMC at days 7, 21 and 69 PI were done in a complete RPMI-1640 growth medium supplemented with 10% foetal calf serum (Invitrogen, Prat de Llobregat, Spain) (Darwich et al., 2003b). Cells were dispensed in 96-well plates at a density of 5×10^5 cells per well. After that, PBMC were stimulated with PCV2 (Burgos strain) at multiplicity of infection (m.o.i.) of 0.1 for 24 h at 37 °C in a 5% CO_2 atmosphere. PHA (10 μg/ml) stimulation was used as a positive control and Aujeszky's disease virus (m.o.i. of 0.1) as an unspecific viral stimulation. Mock-stimulated cells were included as background control. Cultures were done by triplicate and stored at −80 °C until ELISA cytokine determinations (Swine IL-2, IL-4, IL-10 and IFN-γ CytoSets, Biosource Europa, Nivelles, Belgium) (Darwich et al., 2003b).

Comparisons of the relative proportions of PBMC subsets were done using the non-parametric Mann–Whitney test. The Spearman Test was applied to determine correlations between non-parametric data. Inference statistics were performed using the Statsdirect (v.2.4.3) and GraphPad Prism® (v 4.0) software.

None of the PCV2 inoculated pigs developed PMWS in the course of the study. The only clinical evidence of the viral infection was a slight intermittent fever (40.5–41.5 °C) between days 16 and 19 PI, which coincided with the onset of viremia. All pigs in group A had seroconverted by week 3 PI [mean total IPMA antibody titre ($\log_{10}$) 2.60 ± 0.41] and reached a mean IPMA titre ($\log_{10}$) of 4.14 ± 0.45 by week 10 PI. Specific IgG seroconversion occurred from days 21 to 49 PI. IgG detection was maintained until the end of the experimental period, with increasing optical density mean until 49 days PI and slight decrease by 69 days PI (Fig. 1). PCV2 IgM seroconversion occurred early, mainly between 7 and 21 days PI. The highest optical density mean of IgM occurred at 21 days PI (Fig. 1). None of the non-inoculated animals seroconverted to PCV2.

In PCV2-inoculated pigs, the onset of viremia was detected at day 7 PI reaching a peak at day 21 PI ($10^{3.3} \pm 1.4$ $\log_{10}$ viral particles/ml) and slowly decreasing until day 69 PI when none of the animals was viremic. Regarding nasal shedding of PCV2, at day 7 PI six out of seven pigs of group A were positive and by day 14 PI all of them were shedding the virus in nasal secretions. A similar pattern was observed for faecal excretion of virus that started at day 7 PI (4 of 7 pigs) and reached a maximum by day 21 (7 of 7 pigs). By day 69 PI, three of the inoculated animals still were shedding PCV2 in faeces.

Relative proportions of lymphocyte subsets of peripheral blood were examined at days 21 and 69 PI. No differences were seen at day 21 PI among experimental groups. At day 69 PI, although PCV2 inoculated pigs showed a significant decrease in the proportion of IgM+ cells ($p < 0.05$) compared to non-inoculated ones the proportion of these cells were very close to the values obtained by day 21 PI. All results are summarized in Table 1.

The *in vitro* stimulation of PBMC with PCV2 did not induce significant levels of IFN-gamma or IL-4 in any of the groups. In contrast, at 3 weeks PI, inoculated animals produced higher amounts of IL-10 (5 pigs produce detectable levels out of 7 pigs, 117 ± 51 pg/ml) compared to control animals (6 out of 9 pigs, 40 ± 33 pg/ml; $p < 0.05$). At that time (day 21 PI), the viral load in blood as determined by Taq-Man PCR correlated with the production of IL-10 upon PCV2 recall stimulation ($r = 0.85$, $p = 0.02$) (Fig. 2). IL-2 was only produced by PBMC of two out of the nine uninfected animals, 73 pg/ml and 22 pg/ml, respectively, being these last values very close to the detection limit of

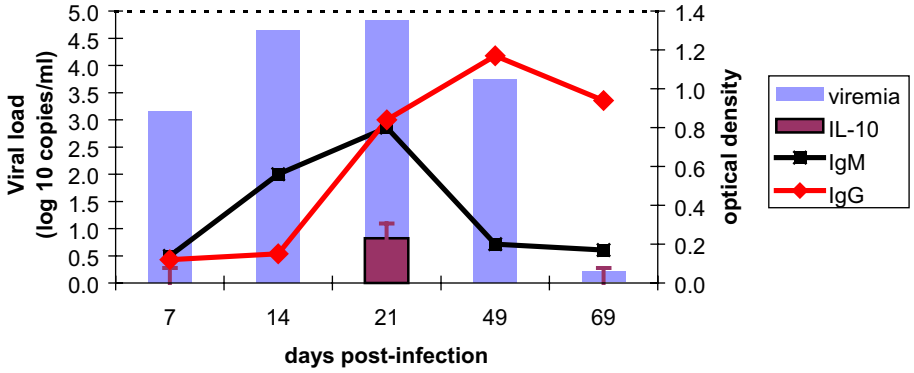

Fig. 1. Serum antibodies (IgM and IgG) and IL-10 levels in front of the viremia evolution in subclinically PCV2-infected pigs.

Table 1
Relative proportions of blood lymphocyte subsets and cytokine concentrations of IL-10 in blood mononuclear cell culture supernatants after *in vitro* stimulation with porcine circovirus type 2

Group (*n*)	Percentage of positive cells (mean ± standard deviation)				Cytokine concentration (pg/ml)
	CD8+	CD4+	CD4+/CD8+	IgM+	IL-10[a]
Early stage of infection (3 weeks post-infection)					
CONTROL (9)	22.1 ± 3.9	17.6 ± 2.7	8.4 ± 6.1	6.0 ± 1.9	40 ± 33[a]
PCV2 (7)	15.7 ± 2.8	15.4 ± 0.9	4.6 ± 1.2	4.8 ± 0.7	117 ± 51[a]
Late stage of infection (10 weeks post-infection)					
CONTROL (9)	17.2 ± 3.8	21.5 ± 4.2	6.3 ± 1.2	7.6 ± 2.1[a]	0
PCV2 (7)	14.5 ± 6.3	19.4 ± 4.5	6.2 ± 1.9	4.7 ± 1.4[a]	0

[a] Significant differences between groups ($p < 0.05$).

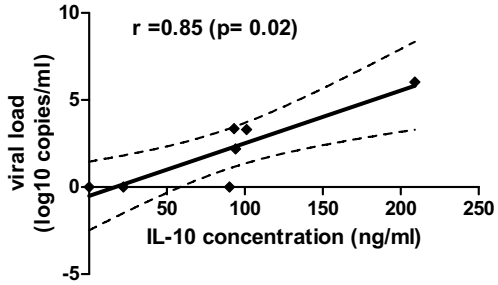

Fig. 2. Correlation plot between levels of IL-10 and the PCV2 load in blood of PCV2 subclinically infected pigs at 3 weeks PI. The Spearman correlation test was used to evaluate the *r* and *p* (two-tailed) values. Errors bars (95% of confidence interval) are represented in discontinuous line.

the ELISA. By 10 weeks PI, no IL-10 or IL-2 production was detected in none of the groups.

The mechanisms involved in the development of PMWS are still unclear. Previous reports indicated that a severe alteration of the immune system and the immune response may take place during the course of the disease (Darwich et al., 2003a; Nielsen et al., 2003) but little is known about the immunological course of PCV2 subclinically infected pigs. In this work, we describe some immune features of a PCV2 experimental and subclinical infection to have an overall view for further and more specific immune assays.

As shown in the present report, inoculation with PCV2 alone allowed the establishment of a subclinical infection, and pigs were able to shed virus up to 10 weeks PI. It is reported that PCV2 circulates in the swine population and it is excreted through the nasal, bronchial, tonsilar, faecal and urine routes for weeks (Krakowka et al., 2000; Caprioli et al., 2006) without being necessarily viremic nor diseased. The control of clinical signs does not necessary involves the eradication of infection, thus, although animals remained subclinical they continued shedding virus all along the study indicating an immune impairment to eliminate the infection.

On the other hand, none of the animals developed PMWS and viremia levels remained low compared to the previous data for natural cases of severe PMWS but similar to those animals presenting only mild lesions (Olvera et al., 2004). On the other hand, the pattern of seroconversion in PCV2 asymptomatic pigs occurs later (21 days PI) compared to other common viral infections (7–14 weeks PI) (Diaz et al., 2005). Although the increase of virus-specific IgG was delayed, these antibodies seemed to be disease protective since pigs developing PMWS apparently present an altered seroconversion pattern (Rovira et al., 2002; Okuda et al., 2003). However, further immunological determinations should be done to confirm this observation.

At 21 days PI, when viremia levels peaked, no alterations were seen in the relative proportions of the different lymphocyte subsets examined but virus-specific IL-10 responses of the PCV2-infected animals were higher than those of controls. IL-10 levels correlated with the viral load

in blood at the early phase of infection and to the maximum IgM plasma detection. This event also concurred with the peak of faecal shedding of virus. As shown before in clinically diseased animals (Stevenson et al., 2006), IL-10 seems to play an important role in the development of PMWS. Results of the present study suggest that recovering of PCV2-infection is related to the ceasement of virus-specific IL-10 responses. In contrast, other studies showed that in diseased animals, this cytokine still predominates at later stages (Darwich et al., 2004). However, it is unclear whether IL-10 production is a consequence of viral replication or if it is the triggering event that allows the virus to replicate more efficiently.

Regarding to IFN-gamma production, asymptomatic and healthy pigs did not show differences in the secretion levels found in PBMC culture supernatants. In the course of a viral infection an increase of IFN-gamma levels is expected to occur as result of T cell-mediated immune response. However, for subclinically infected inoculated pigs this was not observed. A possible reason for this lack of IFN-gamma production could be the induction of IL-10 responses in the experimental pigs. This cytokine may have a inhibitory or regulatory effect on the production of TH1 cytokines. This is in contrast to previous observations in diseased animals for which the levels of IFN-gamma were notably higher (Darwich et al., 2003b). Whether this increase of IFN-gamma could be indicative or not of the stage of development of the disease remains unclear. On the other hand, no IL-2 production was detected in supernatants of PCV2-infected pigs, indicating a specific impairment of this cytokine in infected pigs. The slight detection of IL-2 in supernatants of only two control animals can be considered as an unspecific stimulation of these samples. Moreover, no IL-4 production was observed in PBMC cultures of subclinically pigs, presenting the same pattern of clinically PMWS affected pigs (Sipos et al., 2004). Previous data have shown that in vitro co-cultures of PBMC and PCV2 induced an impairment in the secretion of IL-2 and IL-4 cytokines, even after PHA stimulation (Darwich et al., 2003b), indicating some type of down-regulation induced by PCV2. Thus, the IL-4 down-shift should be considered an effect of the PCV2 infection but it does not seem to be involved in the development of PMWS.

Cellular phenotyping did not reveal any relevant information, as no statistical differences were observed among animal groups and lymphocyte subsets, with the exception of the IgM cell proportions at 69 days PI. If we deepen into this difference, we observe that it cannot be attributable to the decrease of this cellular subset which seemed to remain time-constant in the PCV2 infected group, but with a slight increase in the healthy one. Based on these results, alterations in the relative proportion of lymphocyte subsets cannot be stated as a evident trait of this subclinical PCV2 infection.

The results presented in this study indicate that subclinically PCV2-infected pigs develop a transient IL-10 PCV2-specific response during the viremic phase of infection that disappears later on and coincides with the inversion of the IgM/IgG ratio. In addition, subclinical PCV2-infection seems not to alter significantly the relative proportion of lymphocyte subsets. However, these are preliminary results in a small population sample that need further assessment and confirmation.

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