Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens

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In vitro cytokine profiles of peripheral blood mononuclear cells (PBMC) from pigs with postweaning multisystemic wasting syndrome (PMWS) and healthy pigs were determined in response to recall viral antigens (porcine circovirus type 2; PCV2), mitogens (phytohaemagglutinin) or superantigens (staphylococcal enterotoxin B). PBMC from PMWS-affected pigs, in contrast to those from healthy pigs, responded to recall PCV2 antigen by releasing IL-10 and IFN-γ, but they were less able or even unable to produce IL-4, IL-2 or IFN-γ upon challenge with mitogen or superantigen. Moreover, only PCV2 had the ability to downregulate or suppress the release of IL-4 and IL-2 from PBMC from both healthy and diseased animals, and to stimulate the production of pro-inflammatory cytokines (IL-1β, IL-8). In conclusion, the immune system cells of PMWS pigs have a diminished ability to perform their immunological functions upon viral or immunostimulatory molecules. In addition, PCV2 can alter the functionality of PBMC in both healthy and PMWS pigs.

Porcine circovirus (PCV) is a small non-enveloped single-stranded DNA virus belonging to the family Circoviridae. To date, two types of porcine circoviruses have been identified: the non-pathogenic porcine circovirus type 1 (PCV1), originally isolated from the porcine kidney cell line PK15 (Tischer et al., 1982), and porcine circovirus type 2 (PCV2), currently recognized as the agent of the postweaning multisystemic wasting syndrome (PMWS) of pigs (Allan et al., 1998; Ellis et al., 1998). This syndrome was first identified in western Canada in 1991 (Clark, 1997; Harding, 1997) and, since then, it has been reported worldwide (Allan et al., 1998; Choi et al., 2000; Kiupel et al., 1998; Segalès et al., 1997). PMWS is characterized by progressive weight loss, generalized enlargement of lymph nodes, dyspnoea and, in some cases, pallor, jaundice and diarrhoea. The main histopathological findings are lymphocyte depletion, interstitial pneumonia and hepatitis (Allan et al., 1998; Rosell et al., 1999). PCV2 genome or antigens have been detected in a wide number of cell types from PMWS pigs, but most commonly in macrophages/monocytes and other cells of this lineage. Also, T- and B-lymphocytes of naturally diseased pigs have been found to be infected (Kiupel et al., 1999; Rosell et al., 1999; Shibahara et al., 2000). Field cases of PMWS present a severe and generalized cellular depletion in lymphoid tissues. Some reports show that PMWS-affected pigs have lymphopenia and have decreased proportions of CD4+, CD8+ and IgM+ blood lymphocyte subsets (Darwich et al., 2002; Segalès et al., 2001). However, it remains unknown whether or not the functional capabilities of lymphocytes of PMWS pigs are affected.

Previous studies on cytokine mRNA expression patterns of PCV2-infected pigs (Darwich et al., 2003) showed an increased level of IL-10 mRNA expression in thymus and decreased levels of IL-4 and IFN-γ mRNA in other lymphoid organs. However, the abovementioned report did not test actual cytokine release or determine the effect of PCV2 upon the immune cells. The study presented in this paper determined the cytokine profiles of PMWS-affected and healthy pigs when their peripheral blood mononuclear cells (PBMC) were challenged in vitro with PCV2, with or without co-stimulation with T-cell mitogens or superantigens.

Donor animals were 11 conventional 8-week-old pigs free of porcine reproductive and respiratory syndrome (PRRSV), pseudorabies virus (PRV), porcine parvovirus, swine influenza and Mycoplasma hyopneumoniae as determined...
by serology. All pigs were obtained from a farm experiencing
a PMWS outbreak. Six of them had clinical signs of PMWS
(less than 15 days of clinical evolution) and were PCV2-
positive as detected by PCR and in situ hybridization. The
other five animals were healthy PCV2-negative pigs as
detected by both techniques in both blood and lymph nodes.
Later, the pigs were sacrificed and the diagnoses were
confirmed by histopathological examinations and detection
of the PCV2 genome in tissues by in situ hybridization.
Blood samples were collected by jugular venipuncture in
heparinized tubes and PBMC were separated by density
gradient centrifugation using Histopaque-1.077 (Sigma).
Washed cells were resuspended in RPMI-1640 supplemen-
ted with 10% foetal calf serum (Invitrogen), 1 mM non-
essential amino acids (Invitrogen), 1 mM sodium pyruvate
(Invitrogen), 5 mM 2-mercaptoethanol (Sigma), 50,000 IU
penicillin 1 1.1 (Invitrogen), 50 mg streptomycin 1 1.1
(Invitrogen) and 50 mg gentamicin 1 1.1 (Sigma) and
dispensed in 96-well plates (5 105 cells per well).
Differential counts using standard rapid stains for mono-
cytes and lymphocytes were performed to determine the
relative proportion of these cell types in the samples.

In the first experiment, cells were stimulated for 24 h (37°C,
5 % CO2) with a PCV2 strain recovered in swine kidney
(SK) cells from experimentally infected pig tissues (Rovira et al.,
2002) at an m.o.i. of 0.1, phytohaemagglutinin (PHA)
(10 mg ml-1), Aujeszky's disease virus at an m.o.i. of 0.1
(unspecific viral stimulation) or were mock-stimulated
with SK cell culture supernatants. Cultures were done in
duplicate and supernatants corresponding to the same
animal and stimulus were mixed and stored at -80°C until
examined by ELISA.

In the second experiment, PBMC were cultured first in the
presence of PCV2 (m.o.i. of 0.1) for 24 or 48 h and then
were stimulated with PHA (10 mg ml-1) or staphylococcal
enterotoxin B (SEB) (1 mg ml-1). As positive controls,
PCV2-uninfected cultures were exposed to PHA or SEB at
the same doses as described above. Mock-stimulated
cultures (as in the first experiment) were also included.
Supernatants were recovered after 24 h of incubation with
SEB or PHA and stored at -80°C until subsequent analysis.
Cultures were done in duplicate and supernatants corre-
sponding to the same animal and stimulus were mixed for
the analysis.

Supernatants from PBMC cultures were examined by means
of capture ELISAs prepared with monoclonal antibodies
available commercially (Swine IFN-γ, IL-10, IL-2, IL-4
CytoSets and Swine IL-1β and IL-8 Immunoassay kits;
Biosource Europe, Nivelles, Belgium). The cut-off point of
each ELISA was calculated as the average optical density
of negative controls plus three standard deviations. Levels
of IFN-γ, IL-1β, IL-2, IL-4, IL-8 and IL-10 in the examined
supernatants were determined using the regression line
constructed with the optical densities of the cytokine
standards provided by the manufacturer.

All statistics were performed using STATSDIRECT and EPI-
INFO V.6.01. One-way analysis of variance with the Tukey–
Kramer multiple comparison method was also used to
compare cytokine level production and healthy/PMWS-
animal groups. The results with P values ≤0.05 were
considered statistically significant.

Differential counts showed that, in PMWS pigs, the
proportion of monocytes in PBMC was below 9.5 %
(mean, 5.7%; standard deviation, 2.1%; range, 0–9.5%).
In healthy animals, monocytes accounted for 1.4–2.8% of
total mononuclear cells (mean, 2.1%; standard deviation,
0.6%).

In the first experiment, both PCV2-positive and -negative pigs
produced high levels of IL-1β and IL-8 in response to the
virus (Fig. 1). In contrast, stimulation with the virus
induced only IFN-γ and IL-10 in cells from PMWS pigs (5/6
positive animals for IFN-γ; 7–49 pg ml-1; 3/6 positive
animals for IL-10; 3–102 pg ml-1). With respect to IL-4,
PBMCS from diseased pigs failed to release this cytokine
when stimulated with PHA or PCV2. All five healthy pigs
produced IL-4 upon stimulation with the mitogen but, as
expected, did not with PCV2 (Fig. 1).

In the second experiment, previous infection of cell cultures
with PCV2 had significant effects on the release of cytokines
after stimulation with PHA or SEB. Thus, PCV2-infected
cultures subsequently stimulated with PHA or SEB
produced lower levels of IFN-γ and IL-2 compared to the
uninfected cultures, regardless of whether the cells came
from PMWS or healthy pigs (P<0.05). In addition to this,
uninfected cultures from PMWS animals produced lower
amounts of IL-2 after PHA or SEB stimulation [48 and 72 h
post-infection (p.i.)]. The same was observed for IFN-γ after

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**Fig. 1.** Cytokine production in cell culture supernatants of
PBMCs from PMWS and healthy pigs after 24 h of stimulation
with PCV2 or T-cell mitogen (PHA), as determined by ELISA.
Unstimulated control cultures are not represented since no
spontaneous release of the examined cytokines was detected in
any of them.
SEB challenge at 72 h p.i. \((P<0.05)\) (Fig. 2). For IL-4, addition of PCV2 only prevented the release of this cytokine in both PMWS and healthy pigs at any of the tested times.

Interestingly, uninfected cultures stimulated with PHA showed different behaviour depending on whether they came from healthy or diseased animals. This T-cell mitogen induced IL-4 secretion in healthy pigs, 4/5 at 48 h p.i. (23–115 pg ml\(^{-1}\)) and 3/5 at 72 h p.i. (53–119 pg ml\(^{-1}\)). In contrast, supernatants from PMWS pigs were negative for IL-4 at 24 and 48 h p.i. and only two animals were positive at 72 h (40–112 pg ml\(^{-1}\)) (Fig. 2). With respect to IL-10, at 48 h p.i., four PMWS pigs showed a response to the virus (22–44 pg ml\(^{-1}\)), while only one healthy animal produced IL-10 (Fig. 2).

The evaluation of IL-1\(\beta\) levels showed that uninfected cultures from PMWS pigs had an extremely reduced ability to produce this cytokine upon stimulation with PHA or SEB, in comparison to healthy animals (Fig. 3). In PCV2-infected cultures, no differences were seen between groups at 48 h upon PHA or SEB challenge. However, at 72 h of incubation, cell culture supernatants from healthy animals had higher levels of IL-1\(\beta\) after the addition of the different stimuli, whereas in samples from PMWS pigs the level of IL-1\(\beta\) remained as low as at 48 h (Fig. 3). Finally in the case of IL-8, PHA and PCV2 induced the production of high amounts of this cytokine in both groups. Notwithstanding, PMWS pigs whose cells were stimulated with SEB were poor IL-8 producers compared to the controls (Fig. 3).

One of the most debated concepts concerning PMWS is the role of the immune system in the pathogenesis of this syndrome. Several authors have pointed out that immunosuppression is one of the main features of the disease. However, to date, there is no report showing that the immune system cells of affected pigs have a diminished ability to perform their immunological functions, and all evidence refers only to the fact that wasted pigs have severe lymphocyte depletions. In this paper we give evidence that PCV2 not only has the ability on its own to suppress the \textit{in vitro} release of some cytokines in both healthy and diseased pigs, but also is able to stimulate the production of pro-inflammatory cytokines. In addition, we also show that cells from PMWS-affected pigs have a particular response, in the sense that they are less able to produce IL-2, IL-4 and IFN-\(\gamma\) upon mitogen or superantigen challenges.

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**Fig. 2.** Levels of cytokine production in supernatants from PBMCs from PMWS and healthy pigs cultured in the presence or absence of recall PCV2 viral antigen and subsequently challenged with PHA or SEB stimuli, as determined by ELISA at 48 and 72 h post-incubation (PI).
Different hypotheses can be formulated from these observations. One theory could be that the infection acts upon regulatory or suppressor mechanisms of the immune system. It is known that persistent antigenic stimulation in vivo, for example as happens in a systemic infection with the lymphocytic choriomeningitis virus in mice, can induce the generation of anergic CD4^+ or CD8^+ T-cells (Mueller et al., 1989; Zajac et al., 1998). These anergic or unresponsive cells are unable to proliferate upon antigen stimulation and fail to develop cytolytic effector functions. However, they have the capacity to produce high levels of IL-10 (Buer et al., 1998). It has been suggested that T-cells rendered anergic in vivo could act as regulatory cells, influencing neighbouring immune responses through the release of IL-10 (Buer et al., 1998). We recently reported the presence of high IL-10 mRNA levels in the thymus in PMWS-affected pigs (Darwich et al., 2003), associated with a massive destruction of the organ structure and an evident thymocyte depletion. In this report, we showed that most cultures from PMWS pigs produced IL-10 after stimulation with recall viral antigen. This fact and the inability to produce IL-2 or IL-4 could be signs of the activity of regulatory or suppressing cells.

A different explanation for the specific changes observed in our PMWS pigs is based on the kinetics of depletion for the different mononuclear cell subsets during the course of PCV2 infection. As is already known, PMWS is accompanied by intense lymphopenia (Darwich et al., 2002; Segalés et al., 2001; Shibahara et al., 2000). Interestingly, Nielsen et al. (2003) recently reported that depletion affects all lymphocyte subsets and also natural killer (NK) cells. The collapse of CD3 T-cells occurs around 14 days p.i. but remnants of CD8^+ and γδ cells persist until 21 days p.i.. NK cells virtually disappear at this later time. Blood samples were taken from our PMWS pigs at approximately 14 days p.i., and thus the inability of PBMC to produce IL-2 or IL-4 in response to recall viral antigens may well reflect that memory/effector cells (corresponding to the CD4/CD8 double-positive subset) had virtually disappeared. On the other hand, since some levels of IFN-γ and IL-10 were released in response to the virus, it is tempting to think of NK and γδ subsets as responsible for this.

With respect to the IL-1β and IL-8 results, it is evident that the virus acts by stimulating the inflammatory response. This fact could explain the typical granulomatous lesions observed in PCV2-infected pigs. This mechanism can contribute to the attraction of macrophages to the infection sites, thus enhancing the chances of the virus finding new target cells. However, Kim and Chae (2003) reported that IL-8 mRNA was not detected in macrophages from injured tissues from pigs with PMWS. This discrepancy can be explained by the nature of IL-8 expression as an early inflammatory phenomenon. As we have shown previously, IL-8 mRNA expression is high when the amount of PCV2 is low and lesions are slight (early phases of infection) but is low in more advanced cases that have more severe lesions (Darwich et al., 2003).

On the other hand, the data for IL-1β revealed an interesting fact in cultures from PMWS pigs. While stimulation of cell cultures with PCV2, PHA or SEB for 24 h did not show differences between both groups with respect to IL-1β, when cultures were first infected with PCV2 and then, after 24 or 48 h of infection, stimulated with PHA or SEB, cells from PMWS pigs produced considerably lower amounts of this cytokine compared to healthy animals. This fact became clearer as the period of infection increased. With the present data, it is difficult to interpret this; however, we consider that it might reflect a regulatory response.

Taking all these observations together, it seems clear that the capabilities of PBMC from PMWS pigs to respond to recall viral antigens, mitogens or superantigens are clearly altered, or even non-existent, compared to a normal profile. Considering that extensive lymphocyte depletion is the main feature of this syndrome, we opine that the study of the immune response to PCV2 in asymptomatic and diseased pigs will be one of the keys to understanding PMWS. However, the fact that remains a paradox is, if PCV2 is able to alter the immune responses of PMWS in all the examined pigs, why do only some of them develop the disease. In our
opinion, this could be the result of individual factors, such as the genetic background of the immune response that counteracts PCV2, the need for some coadjuvant phenomenon or even of the timing and amount of virus received.

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