Porcine circovirus type 2 is the aetiological agent of post-weaning multisystemic wasting syndrome (PMWS) (Allan et al., 1998). There is increasing evidence that PMWS-affected pigs are more prone to develop concomitant infectious diseases, a fact suggesting that PMWS is actually an immunosuppressive disease (Ellis et al., 2004; Segalés et al., 2005).

The immune response seems to play a major role in the outcome of PCV2 infections. The studies carried out on natural cases of PMWS show that affected pigs suffer extensive lymphoid lesions (lymphocyte depletion and granulomatous inflammation) and altered cytokine expression patterns in peripheral blood mononuclear cells (PBMCs) and lymphoid organs. In PBMCs, mitogen-induced gamma interferon (IFN), interleukin (IL)-10, IL-2 and IL-4 secretion are decreased in the presence of PCV2. Also, in vitro, PCV2 itself seems to stimulate the production of IL-10 (Darwich et al., 2003a). Ex vivo, PCV2-infected pigs have increased IL-10 mRNA expression in the thymus (Darwich et al., 2003b). Also, elevated systemic IL-10 mRNA levels have been reported in PMWS-affected pigs (Sipos et al., 2004); however, contradictory results on IL-10 mRNA levels have been described elsewhere (Sipos et al., 2005). In a recent report, serum IL-10 was associated with PCV2-infected pigs that subsequently developed PMWS (Stevenson et al., 2006).

Non-replicating PCV2 can persist in dendritic cells (DCs) without affecting myeloid DC differentiation or antigen uptake and presentation (Vincent et al., 2005). However, in myeloid and plasmacytoid DCs (the latter also known as natural interferon producing cells or NIPCs), the presence of PCV2 leads to the inhibition of virus or cytosine phosphorothioate-guanine oligodeoxynucleotides (CpG-ODNs) induced IFN-α and tumour necrosis factor alpha release (Vincent et al., 2005, 2007). However, the mechanism by which PCV2 is able to alter normal immune responses is largely unknown. In this study, the role of PCV2 and the closely related, non-pathogenic porcine circovirus type 1 (PCV1) on the modulation of the immune system was investigated during recall antigen responses against pseudorabies (Aujeszky’s disease) virus (PRV), a well known viral infection model.

Two groups of three Landrace crossed with Duroc pigs free of PRV and PCV2 infections were included in the study. Animals in one group (n=5) were immunized against PRV at 10 and 13 weeks of age with a commercial attenuated live gE− PRV vaccine (Syvayesky-2, Laboratorios SYVA). The other group (n=5) consisted of non-vaccinated animals. Blood samples were collected 5 weeks after the last immunization (at 18 weeks of age) and PBMCs were separated by gradient density centrifugation. The PRV vaccine strain was propagated in PK-15 cells and the cell culture supernatants were titrated and inactivated with UV light for 30 min. Inactivated PRV with a titre of 5×10⁶ TCID₅₀ ml⁻¹ was used for stimulation of PBMCs at a concentration equivalent to an m.o.i. of 0.1 as determined before inactivation. The PR-15 cell line was checked to be free of PCV1 and PCV2 by PCR before being used. PCV1 (isolate 3384) and PCV2 (isolate Burgos) were produced in PK-15 cells as described previously (McNeilly et al., 2001). Virus stocks were prepared by collecting cell culture
supernatants from infected cells and a mock preparation was produced similarly from uninfected cultures.

Supernatants were examined for IFN-γ, IFN-α, IL-10, IL-2 and IL-12 by capture ELISAs. The reagents for IFN-γ, IFN-α, IL-10 and IL-2 ELISAs were from Biosource and for IL-12 from R&D Systems. Anti-IFN-α monoclonal antibodies (K9 and K17) and an IFN-α recombinant protein (PBL Biomedical laboratories) were used in ELISA as described previously (Guzylack-Piriou et al., 2004). The role of IL-10 was assessed by adding a neutralizing IL-10 antibody (2 µg ml⁻¹, clone 148801; R&D systems).

In a second set of experiments, antigen presenting cells were examined for their ability to produce IL-10 upon circovirus stimulation. The adherent PBMC fraction was separated from the non-adherent subpopulation by adhesion to plastic cell culture flasks. The SwC3⁺ fraction of PBMCs (namely monocytes, macrophages and DCs) was purified from healthy pigs using an anti-SwC3 porcine pan-myeloid cell marker (CD172; monoclonal antibody BL1H7) and magnetic sorting (MACS; Miltenyi Biotech) as described previously (Guzylack-Piriou et al., 2004). Purity achieved for that cell subpopulation was ≥95 % as determined by flow cytometry. SwC3⁺ cells were cultured with PCV2 as explained for PBMCs. A final experiment was carried out by stimulating bone marrow-derived dendritic cells (BMDCs) with PCV2 as above. BMDCs were generated from bone marrow precursors as described previously (Carrasco et al., 2001).

All experiments were done in triplicate. One-way analysis of variance (ANOVA) was used to determine statistical differences between data from virus-stimulated cells and P<0.05 was considered significant.

After stimulation with PRV alone, high amounts of IFN-γ were detected in cell culture supernatants from PRV-immunized animals (Fig. 1a). In contrast, none of the cultures had detectable levels of IFN-γ upon stimulation with PCV1 or PCV2. A significant inhibition (77 %; P<0.01) of the IFN-γ response against PRV in immunized pigs was observed in cells co-stimulated with PCV2 and PRV but not with PCV1, although some reduction was observed. Infectivity of PCV2 was not a requirement for this phenomenon, since similar results were obtained when UV-inactivated PCV2, or PCV2 neutralized with the neutralizing antibody F190 were used (data not shown).

IFN-α is considered to play a major role in antiviral defence. Thus, we addressed whether PCV2 could modulate IFN-α responses in PRV-vaccinated animals. In vitro PRV stimulation induced IFN-α responses in both PRV-immunized and naive pigs, although levels of this cytokine in PBMC culture supernatants were significantly higher in PRV-immunized pigs (164 ± 18 U ml⁻¹) compared with PRV-naïve pigs (37 ± 38 U ml⁻¹) (P<0.01) (Fig. 1b). Co-stimulation of PBMCs from naïve animals with PRV and PCV1 or PCV2 resulted in significantly (P<0.01) more IFN-α production compared with the results obtained with PRV alone (147 ± 66 U ml⁻¹ for PCV1 plus PRV; 119 ± 65 U ml⁻¹ for PCV2 plus PRV and 37 ± 38 U ml⁻¹ for PRV alone). Conversely, for PRV-immunized pigs, co-stimulation with PRV and PCV2 resulted in a significant (P<0.01) inhibition of IFN-α production (84 ± 13 U ml⁻¹). This effect was not evident (P>0.05) when co-stimulating with PCV1 (144 ± 21 U ml⁻¹). Thus, PCV2 significantly inhibits IFN-α secretion during PRV-recall responses but induces IFN-α secretion in PRV-naïve pigs.

The ability of PBMCs to secrete IL-10 in response to circoviruses was examined. PCV2 induced a substantial IL-10 production in PBMCs from all pigs (non-vaccinated: 108 ± 43 pg ml⁻¹ and vaccinated: 342 ± 89 pg ml⁻¹), whereas stimulation with PCV1 alone induced very low or no IL-10 at all (P<0.01). However, co-stimulation with PRV and PCV1 resulted in increased IL-10 levels (over 100 pg ml⁻¹ in both PRV-vaccinated and non-vaccinated pigs) compared with the effect of PCV1 or PRV alone (Fig. 1c). To determine if infectivity of circoviruses was a requirement for the induction of IL-10 production in

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PBMCs, PCV1 and PCV2 UV-inactivated preparations, and antibody-neutralized PCV2 were used. These treatments did not significantly change IL-10 production by PBMCs (data not shown).

To test if the previously observed downregulation of PRV-specific IFN-\(\gamma\) responses was due to the PCV2-induced IL-10, PBMCs from immunized animals were cultured in the absence or presence of a neutralizing anti-IL-10 antibody. Neutralization of IL-10 in the cultures was confirmed by IL-10 ELISA. When PRV was used as the only stimulus, addition of the anti-IL-10 antibody did not have any effect on the IFN-\(\gamma\) levels in culture supernatants of PBMCs from immunized pigs (Fig. 2a). When PBMCs from PRV-immunized animals were co-stimulated with PCV2 and PRV, the addition of the IL-10 neutralizing antibody to the cultures partially restored PRV-specific IFN-\(\gamma\) production. A similar effect was seen for PCV1 (Fig. 2a).

To examine if PCV2-induced IL-10 affected IL-2 or IL-12 production, we analysed the IL-2 and IL-12 responses in the presence of a neutralizing IL-10 antibody. PRV-induced IL-2 secretion was unaffected by the presence of the neutralizing IL-10 antibody (Fig. 2b). Co-stimulation of PRV with either PCV1 or PCV2 reduced IL-2 on average by 50 and 80%, respectively. Addition of the neutralizing IL-10 antibody to the PBMC cultures did not restore IL-2 levels (Fig. 2b). Next, we measured IL-12 levels. In the absence of IL-10 neutralizing antibody, IL-12 levels were 128 ± 55 pg ml\(^{-1}\) for PRV, 155 ± 25 pg ml\(^{-1}\) for PCV1 plus PRV and 185 ± 25 pg ml\(^{-1}\) for PCV2 plus PRV. The addition of the IL-10 neutralizing antibody produced a twofold increase in IL-12 levels for PRV, a fivefold increase for PCV1 and a 52-fold increase when PRV and PCV2 were simultaneously used as co-stimuli (Fig. 2c). Taken together, these results indicated that PCV2-induced IL-10 in PBMCs strongly inhibits the production of IFN-\(\gamma\) and IL-12 but not IL-2.

To gain insight into the cell subtype responsible for IL-10 secretion, adherent and non-adherent cells from PBMCs were cultivated with PCV2. The major source of IL-10 was the adherent cell subset, while the non-adherent one, mostly monocyte-free, produced very low, if any, amounts of IL-10 (Fig. 3a) \((P<0.05)\). The SwC3\(^+\) cells from blood also produced IL-10 when PCV2 was added to the cultures (Fig. 3b). Negligible IL-10 levels were obtained when PCV1 or mock were used.

To determine whether DCs were involved in the IL-10-dependent downregulation by PCV2, BMDCs were used. Fig. 3(c) shows that PCV2 was able to downregulate IFN-\(\alpha\) secretion in BMDCs by 2.2 times \((180 ± 32 \text{ U ml}^{-1}\) for mock plus PRV and 83 ± 7.5 U ml\(^{-1}\) for PCV2 together with PRV; \(P<0.001)\), whereas PCV1 had no significant effect. This was similar to what has been observed in PBMCs from PRV-immunized animals (Fig. 1a). Additionally, IL-10 was induced when PCV2 was present in the cultures; in fact, presence of PRV together with PCV2 significantly increased IL-10 secretion \((P<0.001)\) (Fig. 3d). Finally, IL-12 detection was significantly higher \((P<0.001)\) after infection of BMDC cultures with PCV2 as compared with PCV1- or mock-infected samples (Fig. 3e).

Previous studies (Darwich et al., 2003a, b; Hasslung et al., 2005; Stevenson et al., 2006) have shown that PCV2 may substantially alter unspecific cytokine responses and suggested that IL-10 might be one of the mechanisms by which these alterations take place. Based on these previous ex vivo and in vitro studies, the role of PCV2-induced IL-10 in immune disorders in PMWS could not be precisely determined. Our results show that PCV2-induced IL-10 participates in the downregulation of the responses to a recall antigen through the inhibition of IFN-\(\gamma\), IFN-\(\alpha\) and IL-12. In addition, PCV2 was able to inhibit IL-2 through an IL-10-independent mechanism. Surprisingly, we observed a variation in IFN-\(\alpha\) responses by PBMCs upon PCV2 stimulation depending on whether cells came from PRV-immunized or naïve pigs. While PCV2 inhibited IFN-\(\alpha\) release in response of PRV-vaccinated pigs against recall
antigens, PCV2-stimulated IFN-γ secretion in cells from naïve animals. The reason for this is unclear but the finding suggests that PCV2 also affects the cross-talk between memory/effector T-cells, as IFN-γ-producing cells (monocytes, macrophages and DCs) lack memory capabilities.

The IL-10-mediated suppression of T-cell activity is well documented for some human and animal viruses (Cacciarelli et al., 1996; Clerici et al., 1996; Rico et al., 2001; Brady et al., 2003; Brooks et al., 2006; Dolganiuc et al., 2006; Marin-Serrano et al., 2006; Orsilles et al., 2006) and IL-10 has also been suggested as the cause of immunosuppression leading to viral persistence (Brooks et al., 2006). There is evidence that IL-10 may contribute to the development of PMWS. Thus, Stevenson et al. (2006) reported that PMWS pigs showed elevated levels of IL-10 in serum and Darwich et al. (2008) showed that, in subclinically PCV2-infected pigs, a transient IL-10 response correlates with the viraemic phase of infection. Our in vitro results show that PCV2 induces IL-10, which in turn partially suppresses the IFN-γ secretion stimulated by the PRV recall antigen. This clearly indicates that PCV2 may substantially modify recall responses through IL-10 induction. Interestingly, stimulation of PBMCs with the non-pathogenic PCV1 did not result in a considerable IL-10 production, although some (statistically not significant) downregulation of IFN-γ was observed.

In humans, IL-10 has been shown to inhibit Th1-driven proliferation by suppressing the production of IL-2 and IL-12 (Taga et al., 1993), both being potent stimulators of IFN-γ production. Our results show that IL-2 release was inhibited by both PCV1 and PCV2, although the stronger effect was observed with PCV2. However, in this case, neutralization of IL-10 did not restore IL-2, suggesting that PCV2 might have mechanisms other than IL-10 induction to downregulate recall responses. In contrast, neutralization of IL-10 led to a substantial increase of IL-12. As a consequence of IL-10 induction, PCV2 infection could result in a loss of antigen-specific responses as reported in the case of human T-cell leukemia virus type 1 infection (Yarchoan et al., 1986; Suzuki et al., 1999) and make pigs more susceptible to other pathogens. Opportunistic or secondary infections are thought to be common in natural cases of PMWS (Ellis et al., 2004; Segalés et al., 2005).
PCV2 is known to inhibit IFN-γ production in NIPC cells after stimulation with several viruses (PRV, transmissible gastroenteritis virus and classical swine fever virus), and toll-like receptor (TLR) 7 and TLR9 agonists (Vincent et al., 2007). Recently, the inhibitory effect was shown to be mediated by PCV2 DNA without evidence of virus replication in NIPC cells (Vincent et al., 2005, 2007). However, non-pathogenic PCV1 DNA did not inhibit NIPC functionality (Vincent et al., 2007). In the present study, PCV2-induced IL-10 did not require the infectivity of the virus. This could point out the importance of viral DNA in the regulation of host responses as shown by others (Vincent et al., 2005, 2007), and also suggests that interactions of viral and host proteins are probably not disturbed upon loss of infectivity. The cells responsible for IL-10 production were shown to be SwC3+ cells (monocytes, macrophages and dendritic cells), suggesting the additional role of PCV2-induced IL-10 in counteracting the activation of these cells.

In summary, we have shown that PCV2, and to a lesser extent PCV1, may inhibit IFN-γ, IFN-α and IL-12 secretion upon a recall stimulation of PBMCs. We have also shown that this inhibition is not caused by PCV2 replication and we have provided clear evidence for IL-10 involvement in the central role of a downregulatory mechanism. The source of IL-10 was the circulating mononuclear cells. These results show the potential of PCV2 to strongly suppress Th1 responses and add a basis for understanding the pathogenic mechanism of this virus. Moreover, our experiments suggest that PCV2 has the potential to strongly inhibit recall responses and, although our work was only done in vitro, this opens the question of the impact that PCV2 infections may have in the control through vaccination of certain diseases such as pseudorabies.

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