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Porcine circovirus type 2 (PCV2) viral components immunomodulate recall antigen responses

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Abstract

Porcine circovirus type 2 (PCV2) is a single-stranded circular DNA virus infecting domestic pigs worldwide. Interaction of this virus with the immune system apparently modulates the immune response of the host. In the present study, the implication of different components of PCV2 in the modulation of the immune response of the host were investigated by using PCV2 viral-like particles (VLPs) and 16 novel oligodeoxyribonucleotides containing CpG motifs (CpG-ODNs) based on the PCV2 genomic sequence. The role of these viral components was studied by evaluating the cytokine profiles (IFN-α, IFN-γ, IL-10, IL-2 and IL-12) on porcine peripheral mononuclear cell (PBMC) and bone marrow-derived dendritic cell (BMDC) cultures. Also, the effect of PCV2 and its elements were examined in recall antigen (pseudorabies virus, PRV) responses. While PCV2 was a potent inducer of IL-10 by PBMCs, such effect was not observed using CpG-ODNs or VLPs. However, IFN-γ and IL-2 production by recall antigen was repressed in presence of PCV2 and most of the studied CpG-ODNs. VLPs did not have such repressive effect. In BMDC cultures, PCV2 and most of CpG-ODNs were able to inhibit IFN-α secretion induced by PRV. Interestingly, CpG-ODNs with inhibitory effect were located within the PCV2 Rep gene. Additionally, PCV2 virus was a very strong IL-12 inducer in BMDC cultures. Whereas, IFN-α modulation on BMDC after PCV2 VLP treatment was neglectable, PCV2 VLPs were potent IL-12 inducers. Our data shows that PCV2 viral elements can distinctly regulate cytokine production depending on the cell population studied. Thus, the final immune response upon PCV2 infection seems to depend on the fine balance between the regulatory elements present in viral DNA and structural protein within the host immune system.

Keywords: Porcine circovirus type 2 (PCV2); Postweaning multisystemic wasting syndrome (PMWS); Virus-like particles (VLPs); Immune modulation; CpG oligodeoxyribonucleotides (CpG-ODNs); Recall antigen responses

1. Introduction

Porcine circovirus type 2 (PCV2) is a single-stranded circular DNA virus infecting domestic pigs worldwide. The clinical course of PCV2 infection range from subclinical to a number of PCV2 associated syndromes, collectively named porcine circovirus diseases (PCVDs); postweaning multisystemic wasting syndrome (PMWS) is the most economically important PCVD (Segalés et al., 2005).

PMWS affected pigs suffer a deep alteration of the immune system, including a depletion of T and B

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lymphocytes (Darwich et al., 2002; Nielsen et al., 2003), increased numbers of cells of the monocyte/macrophage lineage in tissues (Chianini et al., 2003) and an altered pattern of cytokine responses (Darwich et al., 2003). Furthermore, PCV2 itself stimulates the production of IL-10 in peripheral blood mononuclear cells (PBMCs) (Darwich et al., 2003). These evidences, together with the fact that the experimental reproduction of PMWS usually requires co-factors such as other viruses or substances affecting the immune system (Allan et al., 1999; Harms et al., 2001; Krakowka et al., 2000, 2001; Rovira et al., 2002), suggest that PMWS pathogenesis is strongly related to the events taking place in the immune response to PCV2 (Krakowka et al., 2001).

Unmethylated CpG motifs, present in bacterial and some viral genomes, are recognized by the immune system as danger signals. Recently, it was shown that PCV2 viral DNA can inhibit the capacity of stimulated plasmacytoid dendritic cells, also called natural interferon producing cells (NIPC), to produce IFN-α (Vincent et al., 2007). On the other hand, in PBMC cultures, PCV2 DNA was demonstrated to induce INF-α, indicating the role of different cell subpopulations in cytokine production (Wikström et al., 2007). Some CpG motif containing sequences from PCV2 genome are known to modulate IFN-α production by porcine PBMCs and it has been suggested that the IFN-α stimulatory or inhibitory effect is dependent on the secondary structure of CpG containing oligodeoxyribonucleotides (CpG-ODN) (Hasslung et al., 2003; Wikström et al., 2007).

The role of PCV2 capsid protein (Cap) in the immune responses against PCV2 is poorly understood. However, it is known that the protection induced in pigs by vaccination with viral-like particles (VLPs) can be efficient enough to avoid PCV2 replication upon a challenge (Blanchard et al., 2003). These and other results (Fan et al., 2007) indicate that viral capsids alone participate in the immune response to PCV2 and can be one of the key elements to induce protection against PCV2.

Until now, the roles of the different PCV2 viral elements in immune responses have not been examined in detail. Thus, in this study, the role of PCV2 components in immune modulation was investigated by using on one side 16 novel CpG-ODNs based on the PCV2 genomic sequence and, on the other side, by means of baculovirus-expressed VLPs. These studies were performed using pseudorabies virus (PRV) immunized pigs, testing the ability of these different PCV2 elements to induce or inhibit cytokine responses in both PBMCs (recall responses) and dendritic cells (DC) (innate responses).

2. Material and methods

2.1. Oligodeoxynucleotides (CpG-ODNs)

PCV2 genome (GenBank accession number: AF201310) was screened for CG dinucleotides using cpgreport-program (http://bioweb.pasteur.fr). Sixteen CpG-ODNs located throughout the PCV2 genome were selected for synthesis. Previously published CpG-ODNs shown to regulate IFN-α were used as controls (Hasslung et al., 2003). Desalted oligodeoxynucleotides with phosphodiester backbone were purchased from Bonsai Technologies (Barcelona, Spain). CpG-ODNs were diluted in endotoxin free TE buffer (10 mM Tris, pH 7.0 and 1 mM EDTA) at a concentration of 0.5 mol/ml.

2.2. Animals and immunizations

Landrace × Duroc pigs ($n = 5$) were immunized against PRV at 10 and 13 weeks of age with a commercial attenuated live gE^- PRV vaccine (Syvayesky-2, Laboratorios SYVA, León, Spain). Blood samples for PBMC separation were collected 5 weeks after the last immunization against PRV. The animals were shown to be healthy and free of PRV, PCVs and other major swine viral and bacterial infections.

2.3. Viruses and virus-like particles

The gE^- PRV vaccine strain (Syvayesky-2, Laboratorios SYVA, León, Spain) was propagated in PCV1/PCV2 free (as determined by PCR) PK-15 cells. Cell culture supernatants from infected cells were titrated and inactivated by treatment with ultraviolet (UV) light for 30 min. The inactivated PRV was used for stimulation of PBMCs and DCs at a concentration equivalent to a multiplicity of infection (MOI) of 0.1 as determined before inactivation. PCV2 (Burgos isolate) virus was produced in the same PK-15 cell line as previously described (McNeilly et al., 2001). Virus stocks were prepared by collecting cell culture supernatants from infected cells and a mock preparation (MP) was produced similarly from uninfected cultures. PCV2 stocks with an infectious titre of $10^{4.4}$ $TCID_{50}$/ml were used. Baculovirus recombinant PCV2 VLPs were a generous gift from Merial SAS (Lyon, France) and used at concentrations of 0.5 and 2 μg/ml (Misinzo et al., 2005).

2.4. Cell cultures

PCV1/PCV2 free PK15 cells were propagated in DMEM with 10% foetal calf serum (FBS), 2 mM L-

glutamine (Sigma, Madrid, Spain) and 1% penicillin/streptomycin.

PBMCs were separated from blood by a gradient density centrifugation using Histopaque 1.077 (Sigma, Madrid, Spain) and were cultivated in RPMI-1640 supplemented with 5% FBS, 10 mM HEPES, 2 mM L-glutamine, 1% penicillin and streptomycin, and 0.05 mM 2-mercaptoethanol. For *in vitro* PBMC stimulation, CpG-ODNs were diluted at 6 nmol/ml in RPMI-1640 without serum. Lipofectamine (Invitrogen, Frederick, MD) was diluted 10 μg/ml in plain RPMI-1640 and incubated at room temperature for 60 min. Equal volumes of diluted CpG-ODNs and lipofectamine were mixed and further incubated for 15 min; then 100 μl of the mixture were added to PBMC cultures. For the stimulation, 5×10^5 PBMCs per well were cultured in flat-bottom 96-well plates with CpG-ODNs, PCV2 or the MP for 12 h at 37 °C and 5% CO_2. UV-inactivated PRV was subsequently added and incubation continued for another 24 h. Three replicas of each culture were done and cells from at least three animals were used. The cell culture supernatants from triplicates were pooled, aliquoted and stored at −80 °C for further cytokine analysis.

Bone marrow haematopoietic cells (BMHCs) were isolated from sterna or femora of SPF pigs as previously described (Summerfield and McCullough, 1997). BMHCs were cultured in RPMI-1640, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml polymyxin B (Sigma) and penicillin/streptomycin. Bone marrow-derived dendritic cells (BMDCs) were generated from BMHCs in 8–9 day culture with 100 ng/ml of recombinant porcine granulocyte-macrophage colony-stimulating factor (GM-CSF) as previously described (Carrasco et al., 2001). After derivation, GM-CSF enriched medium was removed by centrifugation. A total of $0.8–1 \times 10^6$ BMDC per well were cultured in flat-bottom 24-well plates at 37 °C and 5% CO_2 with PCV2 or CpG fragments in RPMI-1640 supplemented with 5% FBS, 10 mM HEPES, 2 mM L-glutamine, 1% penicillin and streptomycin, and 0.05 mM 2-mercaptoethanol. All selected PCV2 CPG-ODNs were used at a concentration of 30 μg/ml. UV-inactivated PRV (MOI 0.1) was subsequently added and incubation continued for another 16 h. All above described experiments were performed in triplicates using cells from at least three different animals.

2.5. Cytokine detection and reagents

The IFN-γ, IL-2 and IL-12 reagents for ELISA were purchased from Biosource (Nivelles, Belgium). Anti-IFN-α monoclonal antibodies (K9 and K17) and an IFN-α recombinant protein (PBL Biomedical Laboratories, Piscataway, NJ) were used in ELISA as described (Guzylack-Piriou et al., 2004). TMB (3,3′,5,5′-tetramethylbenzidine) was from Sigma.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine statistical differences between data from stimulated cells and $p < 0.05$ was considered significant.

3. Results

3.1. Design and biological characterization of CPG-ODNs sequences

Computer-assisted scanning of the PCV2 genome detected 63 CG dinucleotides. For further detailed analysis, 16 CpG-ODNs located throughout the PCV2 genome were chosen (Fig. 1A and Table 1). Eight of the selected CpG-ODNs contained more than one CpG (Table 1). One CpG-ODN was situated in the untranslated region of the genome, eight in the Rep-and seven in the Cap-encoding regions (Table 1). CpG-ODNs Nos. 2–4, 13, 15, 17 and 21 contained palindromic sequences. Initially, the 16 synthetic CpG-ODNs were tested for their ability to induce IFN-α production in PBMC cultures. Fourteen CpG-ODNs induced comparable levels of IFN-α while two (CpG-ODNs Nos. 4 and 16) did not induce IFN-α production by PBMCs (Fig. 1B).

3.2. Cytokine modulation by PCV2 CpG-ODNs in PBMCs

To examine if PCV2 and sequences derived from its genome induced the release of cytokines by PBMCs, cell cultures were treated with PCV2 or CpG-ODNs preparations and levels of IL-10, IL-2 and IFN-γ were determined in cell culture supernatants. CpG-ODNs did not induce IL-10, IL-2 or IFN-γ. In contrast, PCV2 alone resulted in a high production of IL-10 (232 pg/ml ± 118) but no IFN-γ and scarce amounts of IFN-α (8 U/ml ± 6) and IL-2 (22 pg/ml ± 2).

Next, the effects of CpG-ODNs were examined in the recall antigen responses to PRV. Cytokine secretion by PBMCs from PRV-immunized animals treated with PCV2 or CpG-ODNs and subsequently stimulated with PRV was determined. On one hand, PCV2 significantly ($p < 0.05$) down-regulated PRV-induced IFN-γ and IL-2

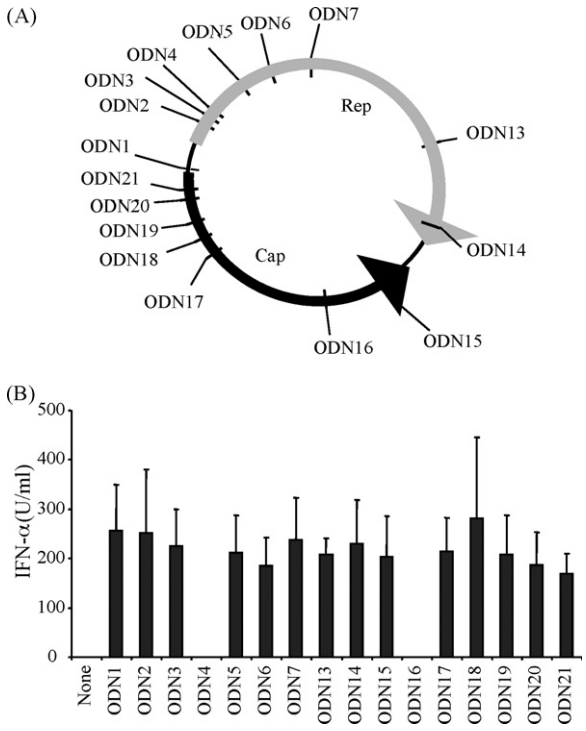

Fig. 1. (A) Location of studied CpG-ODNs in the PCV2 genome. Cap: viral structural capsid protein; Rep: replication associated protein. (B) IFN-α production by PBMCs upon no or CpG-ODNs treatment. The values are mean ± S.D. of three replicas using PBMCs from three different pigs. Similar results were obtained in two independent experiments.

release in recall antigen responses (Fig. 2B and C). On the other hand, PRV-induced IFN-γ and IL-2 production was clearly inhibited by most CpG-ODNs ($p < 0.05$) except by CpG-ODNs 4 and 16, which were the non-IFN-α producers. CpG-ODNs did not have any significant effect on the PRV-induced IFN-α or sporadically increased its production (Fig. 2A–C). These data indicated that cytokine production by PBMCs differs depending on whether the whole PCV2 particle or just the DNA (CpG-ODNs) is added to the culture.

3.3. Cytokine modulation by PCV2 CpG-ODNs in BMDC

Porcine myeloid BMDCs were generated *in vitro* and PCV2-derived CpG-ODNs were tested to characterize whether they modulate cytokine secretion in *in vitro* responses against PRV. All BMDCs were phenotypically characterized for CD45, SwC3, SLA II DR, CD163, CD3 and CD4 and were shown to be mostly composed of $CD45^+/SwC3^+/SLA\ II\ DR^+/CD163^{low}/CD3^-/CD4^-$ cells.

BMDCs stimulated with inactivated PRV produced on average 335.4 pg/ml of IFN-α. When CpGs were used as co-stimuli, in most cases (11 out of 16) a decrease in the levels of IFN-α was observed, although this effect was statistically significant only for CpG No. 18, which was able to decrease IFN-α production by 16 times ($p < 0.05$) as compared with PRV alone. In contrast, four CpG-ODNs (Nos. 15–17 and 21) had no effect on IFN-α at all (Fig. 3A). Interestingly, all CpG-ODNs that produced a decrease of IFN-α levels were located within the Rep gene, while the ones having no effect on IFN-α production were located within the Cap gene (Fig. 1A).

IL-12 has been widely accepted as an important regulator of T helper-1 cell (Th1) responses and is

Table 1
Tested CpG-ODN sequences derived from PCV2 genome

CpG-ODN	Sequence 5′–3′	Region in PCV2 genome
CpG-ODN1	TGCGCTGTAAGTATTACCGGCGC	Non-translated region
CpG-ODN2	GGGTGTTCACGCTGAATAAT	Rep
CpG-ODN3	CTTCCGAAGACGAGCGCAAG	Rep
CpG-ODN4	AGAAAATACGGGAGCTTCC	Rep
CpG-ODN5	GGGGTTCGCTAATTTTGTGA	Rep
CpG-ODN6	TCGAGAAAGCGAAAGGAACT	Rep
CpG-ODN7	CAGGGACAACGGAGTGACCT	Rep
CpG-ODN13	CTGTGTGATCGGTATCCATT	Rep
CpG-ODN14	GGGGCCAGTTCGTCACCCTTTCC	Rep
CpG-ODN15	GTTTTCGAAGGCAGTGCCGA	Cap
CpG-ODN16	GGGTAAAGTACCGGGAGTGGTAG	Cap
CpG-ODN17	CCAGGAGGGCGTTCTGACTG	Cap
CpG-ODN18	GGTGCGGGAGAGGCGGGTG	Cap
CpG-ODN19	CAGCGGTAACGGTGGCGGGG	Cap
CpG-ODN20	AGATGGCTGCGGGGGCGGTG	Cap
CpG-ODN21	CGTCTGCGGAAACGCCTCCT	Cap

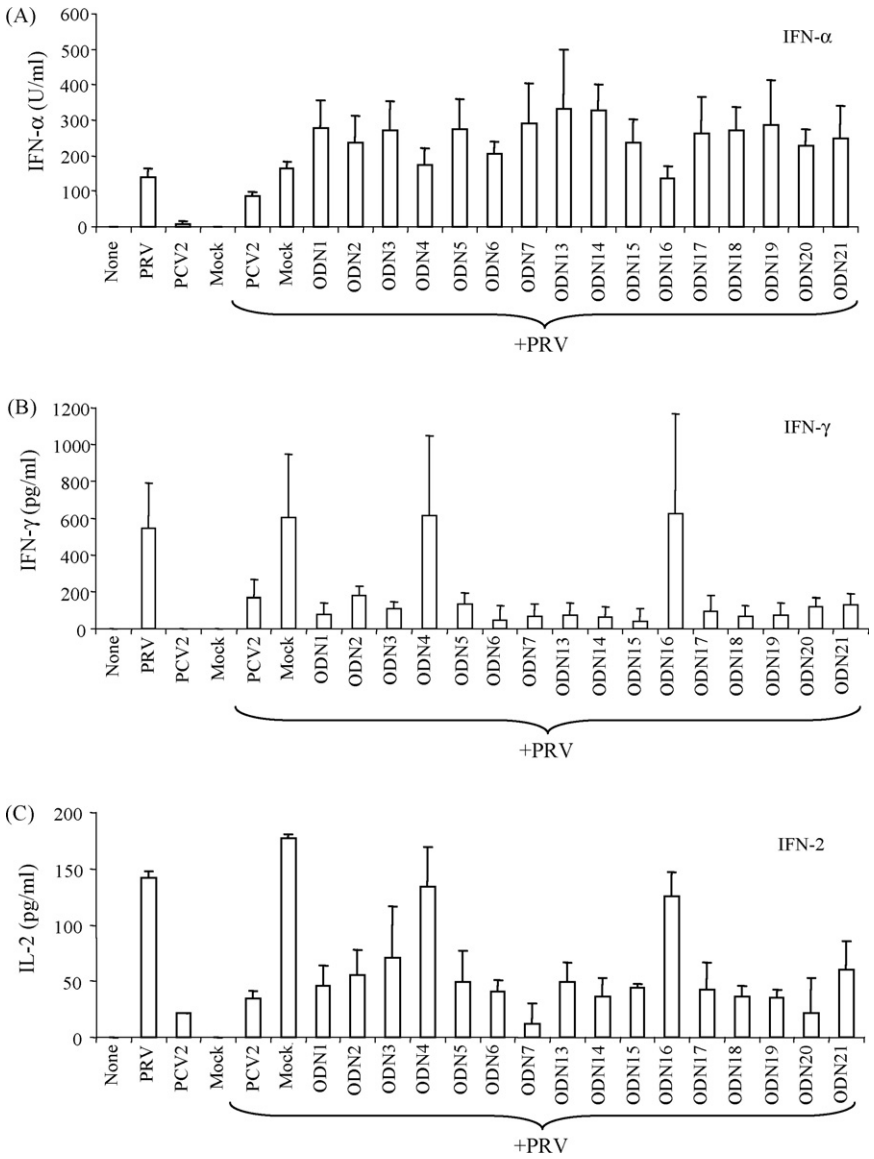

Fig. 2. Production of IFN-α (A), IFN-γ (B) and IL-2 (C) after stimulation of PBMCs with medium, PRV, PCV2 or mock. PBMCs stimulated with CpGs, PCV2 or mock and subsequently infected with PRV is indicated. The values are mean ± S.D. of three replicas using PBMCs from three different pigs. Similar results were obtained in two independent experiments.

predominantly produced by DCs, monocytes and macrophages. We thus tested whether CpG-ODNs were able to modulate IL-12 secretion by BMDCs. Fig. 3B shows that the whole PCV2 virion is a very strong IL-12 inducer whereas PRV alone or in combination with any of the PCV2-derived CpG-ODNs did not induce such IL-12 levels, indicating that IL-12 induction might be triggered by the PCV2 proteins or by other DNA segments not included in the set of CpG-ODNs selected in this study.

3.4. Cytokine modulation by PCV2 VLP on PBMCs and BMDCs

The role of the structural (Cap) PCV2 protein was studied with regards to its ability to modulate the immune response. For this purpose, baculovirus-expressed capsid proteins known to form VLPs were used to treat PBMC and BMDC cultures. VLPs themselves did not induce IFN-α, IL-10, IFN-γ or IL-2 in PBMCs nor influenced the PRV-induced IFN-α,

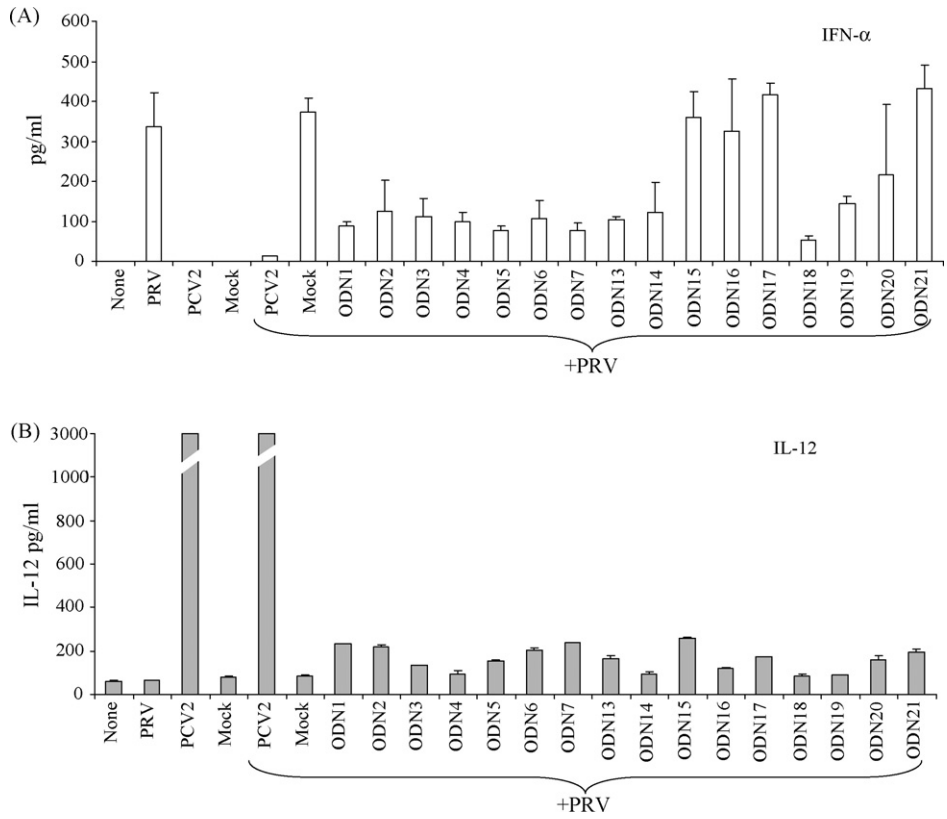

Fig. 3. BMDC IFN-α (A) and IL12 (B) secretion after PRV infection and stimulation with medium, CpGs, Mock and PCV2. The values are mean ± S.D. of three replicas. Similar results were obtained in three independent experiments using cells from three different animals.

IFN-γ or IL-2 production (Fig. 4) in contrast to what was shown with PCV2 and certain CpG-ODNs (Fig. 1).

In line with the above results, VLPs (at 2 or 0.5 μg/ml) did not alter the IFN-α responses of BDMCs against PRV antigen (Fig. 5A). PCV2 VLPs were by themselves potent IL-12 inducers regardless of whether PRV was added or not to the cultures (Fig. 5B); however, levels of IL-12 induced by VLPs were lower than those induced by the whole PCV2 virion (Fig. 3B). IL-12 secretion by VLPs was significantly enhanced as compared with no stimulus or PRV alone, particularly at the highest concentration tested ($p < 0.001$). There was a dose–response effect when the two different concentrations of PCV2 VLPs were used.

4. Discussion

PCV2 has been shown to down-regulate the immune response (Darwich et al., 2003; Vincent et al., 2003, 2005) although the precise mechanisms by which it does so are not fully clear yet. Moreover, the viral components responsible for this down-regulation have not been elucidated to date. Earlier reports have shown that full-length PCV2 DNA induces IFN-α in PBMCs and suppresses this cytokine production in DCs (Vincent et al., 2007; Wikström et al., 2007). However, based on ours and other earlier data, PCV2 DNA contains both IFN-α inhibitory and activating sequences (Hasslung et al., 2003). In PBMCs, most of the studied PCV2 CpG-ODN sequences act as potent IFN-α inducers and CpG-ODNs containing palindromic sequences have been shown to be the most potent IFN-α inducers (Wikström et al., 2007). However, the results of the present paper suggest that this is not an absolute requirement since IFN-α induction took place as well when CpG-ODNs without palindromic sequences were used.

It is worth to note that variations in the IFN-α induction by phosphodiester CpG-ODNs might be also due to different sequences at the ends of CpG-ODNs and/or to the lipofectin treatment. Both factors influence the stability and delivery of CpG-ODNs into endosomes (Honda, 2005; Wikström et al., 2007). In DCs, our results indicated that certain PCV2-derived CpG sequences were able to induce similar immunomodulatory effects than the whole PCV2 particle by decreasing IFN-α secretion after PRV stimulation. In

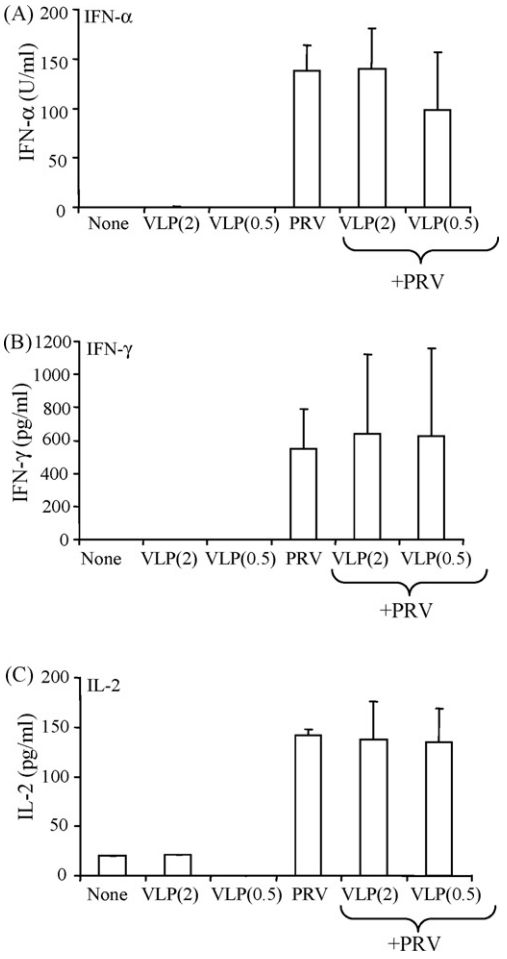

Fig. 4. Production of IFN-α (A), IFN-γ (B) and IL-2 (C) after stimulation of PBMCs with medium, 2 μg/ml PCV2 VLP (2), 0.5 μg/ml PCV2 VLP (0.5) and PRV. PBMCs stimulated with PCV2 VLPs and subsequently infected with PRV are indicated. The values are mean ± S.D. of three replicas using PBMCs from three different pigs. Similar results were obtained in two independent experiments.

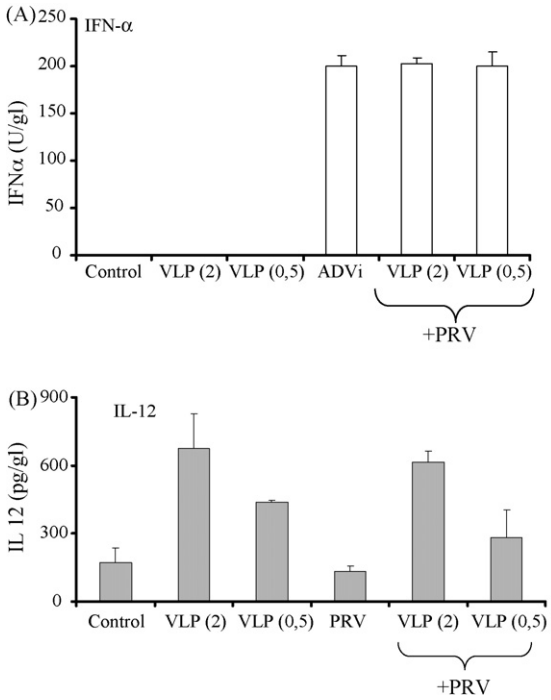

Fig. 5. Production of IFN-α (A) and IL-12 (B) after stimulation of BMDC with medium (none), 2 μg/ml PCV2 VLP (2), 0.5 μg/ml PCV2 VLP (0.5) in presence or absence of PRV. The values are mean ± S.D. of three replicas using BMDC from three different pigs. Similar results were obtained in two independent experiments.

particular, there was one CpG-ODN located in the Cap gene that was able to decrease IFN-α secretion by 16 times but, in general, it seemed that potential regions for IFN-α down-regulation were mainly located in the Rep region of the PCV2 genome. In contrast, the addition of CpGs to PBMCs stimulated with recall PRV antigens had no effect on IFN-α production. The fact that CpG-ODNs produced different effects upon IFN-α in recall antigen responses of PBMCs on one hand and on BMDC (which lack memory cells) on the other, could be attributed to the complexity of the interactions taking place in recall responses. Thus, logical hypothesis would be that either the process of antigen presentation to T cells by itself or the cross talk between memory T cells and antigen presenting cells (APC) can counteract the inhibitory effect of CpGs upon IFN-α release by APC. The causes of this different response should be elucidated in the future to precisely assess the impact of the presence PCV2 DNA in pigs clinically or subclinically infected by PCV2 upon: (a) their ability to develop efficient memory responses; (b) the development of specific responses when the immune system encounters a pathogen for the first time.

The influence of PCV2 DNA on secretion of other key regulatory cytokines has been assessed for the first time in the current work. Interestingly, whereas PCV2 was a potent IL-10 inducer, this cytokine was not produced by any of the examined CpG-ODNs. Therefore, these data suggests that either other DNA sequences (including the whole PCV2 DNA sequence) and/or viral proteins are responsible for IL-10 induction. On the other hand, PCV2 and the IFN-α inducing CpG-ODNs resulted in decreased levels of IFN-γ and IL-2 in antigen recall responses. Thus, a similar cytokine inhibition was obtained by using the whole virus or its sequences. However, in the case of PCV2, this is likely to be through IL-10 induction and CpG-ODN via another TLR9 signalling pathway. TLR9 is a key receptor in the activation of innate immunity by

bacterial and viral DNA containing unmethylated CpG motifs (Bauer et al., 2001; Latz et al., 2004).

Contrary to the PCV2 virus, the baculovirus expressed capsid protein did not affect production of IFN-α, IFN-γ, IL-2 and IL-10. While PCV2 and its DNA inhibit most of the cytokine responses, VLPs seem to be a good immunogen able to generate protective immunity against PCV2 infection *in vivo* (Blanchard et al., 2003; Fan et al., 2007). Thus, it was expected that VLPs would not interfere with the cytokine induction caused by PRV or induce IL-10. However, PCV2 and VLPs but not CpGs effectively activated BMDCs to produce IL-12. These results strongly suggest that the interaction between the PCV2 capsid protein with some unknown cellular receptor could be responsible for this IL-12 secretion. This cytokine plays a central role in the innate response against viral infections and is involved in the regulation of adaptive immune responses favouring the differentiation of Th1 cells. Therefore, these results might be an indication of the mechanism induced by VLPs in immunized animals and they are consistent with the protection induced by the VLPs (Blanchard et al., 2003; Fan et al., 2007). Understanding of how PCV2 DNA sequences and the capsid protein are able to modulate the immune response may also give clues about how PCV2 may favour opportunistic or secondary pathogens.

In summary, the data presented here indicate that different PCV2 viral elements can distinctly regulate cytokine production and this also depends on the cell population studied. In addition, the effect of this regulation can be different as well if the innate or the memory response is examined.

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References

Allan, G.M., Kennedy, S., McNeilly, F., Foster, J.C., Ellis, J.A., Krakowka, S.J., Meehan, B.M., Adair, B.M., 1999. Experimental reproduction of severe wasting disease by co-infection of pigs with porcine circovirus and porcine parvovirus. J. Comp. Pathol. 121, 1–11.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U.S.A. 98, 9237–9242.

Blanchard, P., Mahe, D., Cariolet, R., Keranflec'h, A., Baudouard, M.A., Cordioli, P., Albina, E., Jestin, A., 2003. Protection of swine against post-weaning multisystemic wasting syndrome (PMWS) by porcine circovirus type 2 (PCV2) proteins. Vaccine 21, 4565–4575.

Carrasco, C.P., Rigden, R.C., Schaffner, R., Gerber, H., Neuhaus, V., Inumaru, S., Takamatsu, H., Bertoni, G., McCullough, K.C., Summerfield, A., 2001. Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology 104, 175–184.

Chianini, F., Majo, N., Segales, J., Dominguez, J., Domingo, M., 2003. Immunohistochemical characterisation of PCV2 associate lesions in lymphoid and non-lymphoid tissues of pigs with natural post-weaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. 94, 63–75.

Darwich, L., Segales, J., Domingo, M., Mateu, E., 2002. Changes in CD4(+), CD8(+), CD4(+) CD8(+), and immunoglobulin M-positive peripheral blood mononuclear cells of postweaning multisystemic wasting syndrome-affected pigs and age-matched uninfected wasted and healthy pigs correlate with lesions and porcine circovirus type 2 load in lymphoid tissues. Clin. Diagn. Lab. Immunol. 9, 236–242.

Darwich, L., Balasch, M., Plana-Duran, J., Segales, J., Domingo, M., Mateu, E., 2003. Cytokine profiles of peripheral blood mononuclear cells from pigs with postweaning multisystemic wasting syndrome in response to mitogen, superantigen or recall viral antigens. J. Gen. Virol. 84, 3453–3457.

Fan, H., Ju, C., Tong, T., Huang, H., Lv, J., Chen, H., 2007. Immunogenicity of empty capsids of porcine circovius type 2 produced in insect cells. Vet. Res. Commun. 31, 487–496.

Guzylack-Piriou, L., Balmelli, C., McCullough, K.C., Summerfield, A., 2004. Type-A CpG oligonucleotides activate exclusively porcine natural interferon-producing cells to secrete interferon-, tumour necrosis factor-α and interleukin-12. Immunology 112, 28–37.

Harms, P.A., Sorden, S.D., Halbur, P.G., Bolin, S.R., Lager, K.M., Morozov, I., Paul, P.S., 2001. Experimental reproduction of severe disease in CD/CD pigs concurrently infected with type 2 porcine circovirus and porcine reproductive and respiratory syndrome virus. Vet. Pathol. 38, 528–539.

Hasslung, F.C., Berg, M., Allan, G.M., Meehan, B.M., McNeilly, F., Fossum, C., 2003. Identification of a sequence from the genome of porcine circovirus type 2 with an inhibitory effect on IFN-alpha production by porcine PBMCs. J. Gen. Virol. 84, 2937–2945.

Honda, K., 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. Nature 434, 1035–1040.

Krakowka, S., Ellis, J.A., Meehan, B., Kennedy, S., McNeilly, F., Allan, G., 2000. Viral wasting syndrome of swine: experimental reproduction of postweaning multisystemic wasting syndrome in gnotobiotic swine by coinfection with porcine circovirus 2 and porcine parvovirus. Vet. Pathol. 37, 254–263.

Krakowka, S., Ellis, J.A., McNeilly, F., Ringler, S., Rings, D.M., Allan, G., 2001. Activation of the immune system is the pivotal event in the production of wasting disease in pigs infected with porcine circovirus-2 (PCV-2). Vet. Pathol. 38, 31–42.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K.A., Monks, B.G., Knetter, C.F., Lien, E., Nilsen, N.J., Espevik, T., Golenbock,

D.T., 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat. Immunol. 5, 190–198.

McNeilly, F., McNair, I., Mackie, D.P., Meehan, B.M., Kennedy, S., Moffett, D., Ellis, J., Krakowka, S., Allan, G.M., 2001. Production, characterisation and applications of monoclonal antibodies to porcine circovirus 2. Arch. Virol. 146, 909–922.

Misinzo, G., Meerts, P., Bublot, M., Mast, J., Weingartl, H.M., Nauwynck, H.J., 2005. Binding and entry characteristics of porcine circovirus 2 in cells of the porcine monocytic line 3D4/31. J. Gen. Virol. 86, 2057–2068.

Nielsen, J., Vincent, I.E., Botner, A., Ladekaer-Mikkelsen, A.S., Allan, G., Summerfield, A., McCullough, K.C., 2003. Association of lymphopenia with porcine circovirus type 2 induced postweaning multisystemic wasting syndrome (PMWS). Vet. Immunol. Immunopathol. 92, 97–111.

Rovira, A., Balasch, M., Segales, J., Garcia, L., Plana-Duran, J., Rosell, C., Ellerbrok, H., Mankertz, A., Domingo, M., 2002. Experimental inoculation of conventional pigs with porcine reproductive and respiratory syndrome virus and porcine circovirus 2. J. Virol. 76, 3232–3239.

Segalés, J., Allan, G.M., Domingo, M., 2005. Porcine circovirus diseases. Anim. Health Res. Rev. 6, 119–142.

Summerfield, A., McCullough, K.C., 1997. Porcine bone marrow myeloid cells: phenotype and adhesion molecule expression. J. Leukoc. Biol. 62, 176–185.

Vincent, I.E., Carrasco, C.P., Herrmann, B., Meehan, B.M., Allan, G.M., Summerfield, A., McCullough, K.C., 2003. Dendritic cells harbor infectious porcine circovirus type 2 in the absence of apparent cell modulation or replication of the virus. J. Virol. 77, 13288–13300.

Vincent, I.E., Carrasco, C.P., Guzylack-Piriou, L., Herrmann, B., McNeilly, F., Allan, G.M., Summerfield, A., McCullough, K.C., 2005. Subset-dependent modulation of dendritic cell activity by circovirus type 2. Immunology 115, 388–398.

Vincent, I.E., Balmelli, C., Meehan, B., Allan, G., Summerfield, A., McCullough, K.C., 2007. Silencing of natural interferon producing cell activation by porcine circovirus type 2 DNA. Immunology 120, 47–56.

Wikström, F.H., Meehan, B.M., Berg, M., Timmusk, S., Elving, J., Fuxler, L., Magnusson, M., Allan, G.M., McNeilly, F., Fossum, C., 2007. Structure-dependent modulation of alpha interferon production by porcine circovirus 2 oligodeoxyribonucleotide and CpG DNAs in porcine peripheral blood mononuclear cells. J. Virol. 81, 4919–4927.