ORF2 protein of porcine circovirus type 2 promotes phagocytic activity of porcine macrophages by inhibiting proteasomal degradation of complement component 1, q subcomponent binding protein (C1QBP) through physical interaction

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Defining how each ORF of porcine circovirus type 2 (PCV2) manipulates the host immune system may be helpful to understand the disease progression of post-weaning multisystemic wasting syndrome. In this study, we demonstrated a direct interaction between the PCV2 ORF2 and complement component 1, q subcomponent binding protein (C1QBP) within the cytoplasm of host macrophages. The physical interaction between PCV2 ORF2 and C1QBP inhibited ubiquitin-mediated proteasomal degradation of C1QBP in macrophages. Increased stability of C1QBP by the interaction with PCV2 ORF2 further enhanced the phagocytic activity of porcine macrophages through the phosphoinositol 3-kinase signalling pathway. This may explain the molecular basis of how PCV2 ORF2 enhances the phagocytic activity of host macrophages.

Porcine circovirus type 2 (PCV2) is non-enveloped, icosahedral, small DNA virus with a circular ssDNA genome (Segalles et al., 2013). PCV2 infection often impairs host immunity, with the subsequent development of multifactorial post-weaning multisystemic wasting syndrome (PMWS) due to secondary infections (Tischer et al., 1995; Allan et al., 1999; Allan & Ellis, 2000; Segalles et al., 2005; Opriessnig et al., 2007). Among the four major ORFs of PCV2, ORF1, which is also termed the ‘rep’ gene, encodes the viral replicase and ORF2 (the ‘cap’ gene) encodes the capsid protein (Mankertz et al., 1998; Nawagitgul et al., 2000). ORF3 and ORF4 encode non-structural genes that may modulate the phenotype of host cells (Liu et al., 2005, 2006, 2007; He et al., 2013).

How each ORF of PCV2 modulates the activity of host immune cells is crucial to understanding the pathogenesis of PMWS. The interaction between PCV2 ORF2 and complement component 1, q subcomponent binding protein (C1QBP) was reported in a yeast two-hybrid system (Finsterbusch et al., 2009). C1QBP is a ubiquitously expressed cellular protein involved in various inflammatory responses including coagulation, chemotaxis, phagocytosis and the production of reactive oxygen species (Guan et al., 1991; Goodman & Tenner, 1992; Peerschke et al., 1993; Ghebrehiwet et al., 1995, 2001).

To investigate the biological consequences of the interaction between PCV2 ORF2 and pig C1QBP, we first cloned and determined the full-length pig C1QBP cDNA sequence (details available in Supplementary methods available in the online Supplementary Material). Pig C1QBP cDNA (GenBank accession no. LN835347) contained an ORF (846 bp) encoding 281 aa with a conserved C1q binding site among mammalian species (Fig. S1a, b). Pig C1qbp mRNA was detected in various tissues, indicating the multicellular functions of C1QBP in pigs (Fig. S1c). The results from Western blotting and flow cytometry analyses demonstrated that an anti-human C1QBP antibody (Santa Cruz Biotechnology) recognized a pig C1QBP protein expressed in PK15 cells (pig epithelial cells) and 3D4/31 cells (pig macrophages; Fig. S2a–c). Moreover, immunohistochemistry analysis revealed that C1QBP expression was widely diffused in spleen tissue (Fig. S2d).

Next, we confirmed the interaction between PCV2 ORF2 and pig C1QBP by co-immunoprecipitation (co-IP) and confocal microscopy (Supplementary methods). Cell lysates from Flag-tagged ORF2 protein (ORF2-Flag)-expressing 3D4/31 cells (pig macrophages) or 3D4/31

Supplementary methods and two figures are available with the online Supplementary Material.
Fig. 1. PCV2 ORF2, but not other ORF proteins, specifically interact with C1QBP in host macrophages. (a) Co-IP of PCV2 ORF2 with C1QBP. Cell lysates from ORF2–Flag-expressing 3D4/31 cells or 3D4/31 cells expressing empty vector (mock) were immunoprecipitated (IP) using anti-C1QBP antibody, followed by Western blotting (WB) using relevant antibodies. Input, total cell lysates. (b) Confocal microscopy analyses indicating co-localization of PCV2 ORF2 and C1QBP within the
cytoplasm of 3D4/31 cells. ORF2–EGFP-expressing 3D4/31 cells (ORF2, green fluorescence) were fixed and stained with anti-C1QBP antibody followed by Alexa Fluor 594 anti-rabbit IgG antibody (C1QBP, red fluorescence). DAPI staining (blue) was performed to visualize the nuclei. (c) C1QBP protein expression level in empty-vector transfectants (mock) and PCV2 ORF1-, ORF2-, ORF3- or ORF4-expressing 3D4/31 cells. Total cell lysates from each transfectant were analysed by Western blotting using anti-C1QBP antibody. All results are shown as means ± SEM. ***P < 0.001 (Student’s t-test). (d) C1qbp mRNA expression level in mock and the ORF-expressing 3D4/31 cells. Total RNA was isolated from each transfectant and used as template for real-time PCR. Glyceraldehyde 3-phosphate dehydrogenase was used as the internal control. (e) Co-localization of PCV2 ORF2 and C1QBP within the cytoplasm of PCV2-infected macrophages. PCV2-infected macrophages were harvested at 48 h after infection, and the cells were fixed and stained with the appropriate antibodies. DAPI staining was performed to visualize nuclei. (f) C1QBP expression pattern in PCV2-infected 3D4/31 cells or uninfected cells at 48 h after infection. Total cell lysates from each infected cell were analysed by Western blotting using anti-C1QBP or anti-β-actin antibodies. **P < 0.01 (Student’s t-test).

cells expressing empty vector (mock) were subjected to IP using anti-C1QBP antibody. After IP, immunoprecipitates were Western blotted using anti-Flag antibody. C1QBP was co-immunoprecipitated with PCV2 ORF2 (anti-Flag) (Fig. 1a, upper panel), whereas no interaction was detected between mock and C1QBP (Fig. 1a, upper panel). Western blotting using anti-C1QBP antibody indicated that the same amounts of C1QBP were present in total cell lysates (Fig. 1a, middle panel). Equivalent protein from both samples was verified by Western blotting using anti-β-actin antibody (Fig. 1a, lower panel). In addition, EGFP-tagged ORF2 protein (ORF2–EGFP) was expressed in 3D4/31 cells to determine the subcellular localization of the interaction between C1QBP and PCV2 ORF2. The results indicated that C1QBP and ORF2–EGFP were expressed mainly in the cytoplasm (Fig. 1b). The combined results indicated an interaction of PCV2 ORF2 and C1QBP within the cytoplasm of host cells. Real-time PCR and Western blotting were performed to investigate C1QBP mRNA and protein expression, respectively, in 3D4/31 cells that expressed each PCV2 ORF protein or mock-transfected cells. C1QBP protein was markedly increased in only PCV2 ORF2 transfectants and not in cells expressing ORF1, ORF3 or ORF4 (Fig. 1c), whereas the level of C1QBP mRNA was similar in cells expressing each ORF (Fig. 1d). These results may indicate that the increased level of C1QBP protein in PCV2 ORF2 transfectants was not a result of altered mRNA transcription. Furthermore, C1QBP protein expression was measured in PCV2-infected 3D4/31 cells or uninfected 3D4/31 cells by Western blotting. Within 48 h of infection, PCV2 ORF2 and C1QBP were co-localized within the cytoplasm of host cells (Fig. 1e), and C1QBP protein was significantly increased in cell extracts of PCV2-infected cells but not in uninfected cells (Fig. 1f).

The stability of the C1QBP protein in PCV2 ORF2 transfectants was compared with empty-vector transfectants (mock) following treatment with cycloheximide (CHX) to investigate whether the interaction between PCV2 ORF2 and C1QBP stabilized C1QBP expression in host cells. The C1QBP protein in PCV2 ORF2 transfectants degraded much slower compared with mock-transfected cells (Fig. 2a). This result could be interpreted as indicating that the physical interaction between PCV2 ORF2 and C1QBP leads to increased half-life of C1QBP protein by delaying the degradation of C1QBP in host cells. To investigate the cellular degradation mechanism involved in the regulation of the half-life of C1QBP, cells were treated with either the lysosomal inhibitor chloroquine (CQ) or the proteasomal inhibitor MG132, and stability of the C1QBP protein was determined in 3D4/31 cells. The results clearly showed that chloroquine treatment could not rescue degradation of C1QBP (Fig. 2b), whereas MG132 treatment partially rescued C1QBP from degradation in 3D4/31 cells compared with untreated cells (Fig. 2c). This result was consistent with the idea that the physical interaction between PCV2 ORF2 and C1QBP protects C1QBP protein from proteasome-dependent degradation. To prove this possibility, the ubiquitination assay was performed in the absence or presence of MG132 to determine whether the direct binding of PCV2 ORF2 to C1QBP inhibited ubiquitination of C1QBP. First, exogenous HA-tagged ubiquitin (HA–Ub) was expressed in mock-transfected cells or PCV2 ORF2 transfectants. Each cell lysate was then subjected to IP using anti-C1QBP antibody followed by Western blotting using anti-HA antibody. Decreased C1QBP ubiquitination was observed in PCV2 ORF2 transfectants (Fig. 2d, upper panel, lanes 4 and 6) compared with mock-transfected cells (Fig. 2d, upper panel, lanes 3 and 5) in the absence or presence of MG132. These data clearly indicated that the interaction between PCV2 ORF2 and C1QBP increases the stability of C1QBP by inhibiting ubiquitin-mediated proteasomal degradation.

Previous observations showed that C1QBP signalling enhances phagocytic activity in macrophages (Bobak et al., 1987, 1988). Therefore, we next examined phagocytic activity in 3D4/31 cells expressed each PCV2 ORF protein (ORF1, ORF2, ORF3 or ORF4) or empty vector (mock). The percentage of phagocytic cells (cells with fluorescence bead uptake/total cells) increased by as much as twofold in PCV2 ORF2 transfectants compared with cells expressing ORF1, ORF3 or ORF4, or mock-transfected cells (Fig. 3a, left panel). We also measured the relative phagocytic activity by sorting fluorescence bead uptake in cells based on the number of beads engulfed. The relative phagocytic activity increased significantly compared with that of other ORF-expressing cells or mock-transfected cells (Fig. 3a, right panel). To confirm these results, the
Fig. 2. Binding PCV2 ORF2 to C1QBP increases the stability of C1QBP by inhibition of the ubiquitin-mediated proteasome-dependent degradation pathway. (a) Mock and PCV2 ORF2-expressing 3D4/31 cells were treated with CHX (100 μg ml⁻¹) at the indicated times and C1QBP expression levels were determined from total cell lysates by Western blotting using anti-C1QBP or anti-β-actin antibodies. All results are shown as means ± SEM. *P<0.05 (Student's t-test). (b) 3D4/31 cells were untreated or treated with CQ for the indicated times and C1QBP expression levels were determined from total cell lysates by Western blotting using anti-C1QBP or anti-β-actin antibodies.

(a) CHX treatment
- Mock
- ORF2

(b) CQ treatment
- Untreated
- CQ

(c) MG132 treatment
- Untreated
- MG132

(d) IP and WB
- Mock ORF2
- Mock ORF2
- Mock ORF2
- Mock ORF2
- Mock ORF2

Interaction between PCV2 ORF2 and host C1QBP.

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Western blotting with anti-C1QBP or anti-β-actin antibodies. (–), No treatment; (+), treatment with CQ. (c) 3D4/31 cells were untreated or treated with the proteasome inhibitor MG-132 for the indicated times and C1QBP expression levels were determined from total cell lysates by Western blotting with anti-C1QBP or anti-β-actin antibodies. (–), No treatment; (+), treatment with MG-132. *P < 0.05. (d) Ubiquitination assays of C1QBP in mock and PCV2 ORF2-expressing 3D4/31 cells transfected with haemagglutinin–ubiquitin (HA–Ub). Cells were treated with MG-132, harvested and resuspended in lysis buffer. The upper panel shows IP of total cell lysates using anti-C1QBP antibody and Western blotting using anti-HA antibody. The middle panels show Western blotting of total cell lysates with anti-HA antibody or anti-C1QBP antibody. The lower panel shows Western blotting of total cell lysates with anti-β-actin antibody. Input denotes total cell lysates.
percentage of phagocytic cells and the relative phagocytic activity were measured in PCV2-infected 3D4/31 cells or uninfected cells. The results clearly showed that both the percentage of phagocytic cells and the relative phagocytic activity were increased in PCV2-infected cells compared with uninfected cells (Fig. 3b).

Fig. 3. Increased stability of C1QBP by the interaction with PCV2 ORF2 enhances the phagocytic activity of host macrophages via the phosphoinositide 3-kinase signalling pathway. (a, b, d, f) Cells were cultured with green fluorescence beads for 4 h and harvested. Bead uptake was measured by flow cytometry. (a) Left panel: the percentage of phagocytic cells (cells with fluorescence bead uptake/total cells) from mock and 3D4/31 cells expressing each PCV2 ORF (ORF1, ORF2, ORF3 or ORF4). Right panel: the relative phagocytic activity from mock and each PCV2 ORF transfectant. Number of beads per cell indicates the number of beads engulfed by each phagocytic cell. (b) Left panel: the percentage of phagocytic cells from PCV2-infected 3D4/31 cells and uninfected cells. Right panel: the relative phagocytic activity from PCV2-infected 3D4/31 cells and uninfected cells. (c) Mock or PCV2 ORF2-expressing 3D4/31 cells were untreated or treated with LY294002 (50 μM) for 1 h, harvested and analysed by Western blotting using the relevant antibody. pAKT, phosphorylated AKT. (d) Percentage of phagocytic cells from mock or PCV2 ORF2 expressing 3D4/31 cells that were untreated or treated with LY294002 (50 μM). (e) Mock or PCV2 ORF2-expressing 3D4/31 cells were transduced with control or shC1QBP lentiviral particles. After transfection, the cells were analysed by Western blotting using the relevant antibody. (f) Percentage of phagocytic cells from mock or PCV2 ORF2-expressing 3D4/31 cells that were transfected with control plasmid or shC1QBP plasmid. (g) ELISAs were performed to detect IL-6 and IL-8 secreted by mock or PCV2 ORF2-expressing 3D4/31 cells. All results are shown as means±SEM. *P<0.05; **P<0.01 (Student's t-test).
increased expression of pAKT relative to that of mock-transfected cells, whereas no change was detected in the levels of total AKT in both cells (Fig. 3c). This result was consistent with the view that increased PI3K activity through C1QB P signalling enhances the phagocytic activity of PCV2 ORF2 transfectants. To confirm this result, ORF2- or mock-transfected cells were treated with the PI3K inhibitor LY294002 and the percentage of phagocytic cells was determined. LY294002 treatment decreased the level of pAKT in both ORF2 and mock (Fig. 3c). In addition, the percentage of phagocytic cells was markedly decreased in LY294002-treated cells but not in untreated cells (Fig. 3d). Next, we inhibited C1QB P expression in ORF2- or mock-transfected cells using small hairpin RNA (shRNA) and performed the same sets of experiments. The results clearly indicated that the enhanced expression of pAKT in ORF2-transfected cells and the enhanced phagocytic activity of ORF2-transfected cells were eliminated by the inhibition of C1QB P (Fig. 3e, f). These results indicated that increased phagocytic activity in PCV2 ORF2 transfectants is dependent on PI3K activity through C1QB P signalling. Finally, we examined IL-6 and IL-8 expression patterns to confirm whether the increased phagocytic activity by PCV2 ORF2 increased pro-inflammatory cytokine expression in macrophages. The results demonstrated that PCV2 ORF2 expression leads to increased secretion of IL-6 and IL-8 in macrophages (Fig. 3g).

The fine molecular mechanism of disease progress after PCV2 infection is not fully understood. However, the fact that severe inflammation occurs during the early stage of PCV2 infection is key in the development of more-severe clinical symptoms, because if initial inflammation is not alleviated, it often leads to chronic inflammatory responses that deplete immune cells within the secondary lymphoid tissues of infected pigs (Darwich et al., 2003; Dvorak et al., 2013). We propose a novel role of PCV2 ORF2 in modulating the phagocytic activity of host macrophages through the C1QB P signalling pathway. Finally, enhanced phagocytic activity of macrophages may cause more-severe inflammation during the early stage of PCV2 infection. These results may add additional knowledge to our understanding of the molecular basis of how PCV2 modulates the activity of host immune cells after infection.

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References


