TLR2/MyD88/NF-κB signalling pathway regulates IL-8 production in porcine alveolar macrophages infected with porcine circovirus 2

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Porcine circovirus 2 (PCV2) is the primary cause of post-weaning multisystemic wasting syndrome, in which it stimulates a strong IL-8 response that is associated with chronic inflammation as well as lesions in the lymphoid organs. However, the mechanism underlying PCV2-induced IL-8 production is still unclear. In the present study, we demonstrated that increased IL-8 expression during PCV2 infection depends on Toll-like receptor (TLR2), but not TLR4 or TLR9 signalling pathways in porcine alveolar macrophages. Moreover, we found that impairment of the MyD88/NF-κB signalling pathway by MyD88 knockdown or NF-κB inhibitors markedly decreased PCV2-induced IL-8 secretion. These results suggest that PCV2 induces IL-8 secretion via the TLR2/MyD88/NF-κB signalling pathway. Therefore, it is important to elucidate the molecular mechanisms of the PCV2-induced inflammatory response.

INTRODUCTION

Porcine circovirus 2 (PCV2) belongs to the genus Circovirus of the family Circoviridae (Marks et al., 2010), and is known to cause post-weaning multisystemic wasting syndrome (PMWS) in pigs (Chae, 2005), primarily at 5–16 weeks (Marks et al., 2010). The clinical features of PMWS include progressive weight loss, lymphadenopathy, jaundice and respiratory disease (Chae, 2005), accompanied by increased litter mortality that is significantly related to sow PCV2 status (Calsamiglia et al., 2007).

PCV2 attacks the porcine monocytes/macrophages (Choi & Chae, 2000), and activates a strong inflammatory response. Interstitial pneumonia is frequently observed in pigs suffering from naturally acquired PMWS or experimental PCV2 infection (Chae & Choi, 2011; Darwich et al., 2003a). It has been reported that PCV2 infection triggers IL-8, IL-1β and TNF-α expression in porcine alveolar macrophages (PAMs), monocyte-derived dendritic cells, and PBMCs (Darwich et al., 2003b; Li et al., 2013; Mavrommatis et al., 2014). Moreover, upregulated mRNA expression of IL-1α and IL-8 was found in an in vivo model of PCV2-associated respiratory disease (Chae & Choi, 2011). These findings indicate that IL-8 plays an important role in the PCV2-induced inflammatory response. IL-8, also known as CXCL8, is produced in abundance by a variety of cell types, including monocytes/macrophages, in response to inflammatory stimuli such as IL-1, TNF-α and LPS (Baggiolini & Clark-Lewis, 1992). It facilitates the recruitment of inflammatory cells from the peripheral blood to the inflammatory sites, and contributes to pathogen clearance (Baggiolini & Clark-Lewis, 1992; Hoffmann et al., 2002). It is also known that PCV2 infection results in IL-8 production both in vivo and in vitro. However, there is remarkable variation in the extent and duration of IL-8 production associated with the occurrence of PMWS and the viral burden (Borghetti et al., 2013). The molecular mechanism that regulates PCV2-induced IL-8 expression remains unknown.

Toll-like receptors (TLRs), a well-characterized class of pattern recognition receptors, are essential for triggering the innate immune response by sensing pathogen-associated molecular patterns. Activated TLRs stimulate the production of multiple inflammatory cytokines via NF-κB, and dysregulated TLR-mediated responses can lead to acute and chronic inflammation (Kawai & Akira, 2010). Levels of IL-8 are barely detectable in normal cells; however, IL-8 is strongly induced by the NF-κB pathway via relieving the repression of the IL-8 promoter (Hoffmann et al., 2002). Therefore, we hypothesized that increased IL-8 expression during PCV2 infection is regulated through TLR signalling, possibly the MyD88-dependent pathway, which is universally targeted by TLRs to activate NF-κB transcription (Akira et al., 2006). TLR2, TLR4 and TLR9 are associated with infections caused by DNA viruses (Rathinam & Fitzgerald, 2011). Therefore, we focused on the roles of TLR2, TLR4 and TLR9 in PCV2-induced IL-8 production in PAMs. In this study, we demonstrated...
that TLR2 is indispensable for increased IL-8 production in PCV2 infection in vitro. Furthermore, PCV2-induced IL-8 secretion depends on the TLR2/MyD88/NF-κB signalling pathway.

RESULTS

PCV2 infection triggers IL-8 secretion in PAMs

The experiments were performed in PAMs, which are a major PCV2-targeted cell line. PAMs were infected with PCV2 strain TJ1 at an m.o.i. of 0.5, and the viral antigens were detected by immunoblotting using the anti-Cap antibody. As shown in Fig. 1(a), PCV2 capsid protein (Cap) was specifically detectable at 6 and 24 h post-infection (p.i.). The expression levels of Cap increased at 24 h p.i. compared with that at 6 h p.i. (Fig. 1b), which indicated that the amount of antigen increased with incubation time. Previous studies have reported that PCV2 infection induces porcine IL-8 expression both in vivo and in vitro (Chae & Choi, 2011; Darwich et al., 2003; Li et al., 2013; Mavrommatis et al., 2014). To confirm these findings, IL-8 secretion from PCV2-infected PAMs was measured at the protein level by ELISA. As expected, PCV2 infection induced secretion of IL-8 from PAMs (Fig. 1c). To rule out the possibility that LPS contamination (Ketchum & Novitsky, 2000) in the virus stocks induced IL-8 secretion, we measured the levels of LPS in the viral inoculum by the Limulus assay. The analysis revealed that the viral inoculum contained undetectable levels of LPS (<0.03 U ml⁻¹).

TLR2 is required for the induction of IL-8 in PCV2-infected PAMs

TLRs are key components involved in the induction of the innate immune response against pathogens. PAMs express multiple innate immune receptors, including TLR2, TLR4 and TLR9, that participate in the inflammatory response to different DNA viruses (Rathinam & Fitzgerald, 2011). This prompted us to investigate whether these receptors are important for the IL-8 response to PCV2. As shown in Fig. 2, expression levels of TLR2, TLR4 and TLR9 mRNA in PCV2-infected PAMs increased significantly in a time-dependent manner. To investigate the role of these TLRs in PCV2-induced IL-8 production, we synthesized specific small interfering RNA (siRNA) constructs targeting TLR2, TLR4 and TLR9. The knockdown efficiency of siRNA against TLR2, TLR4 or TLR9 was confirmed by real-time reverse transcription (RT)-PCR. As shown in Fig. 3(a), expression of TLR2, TLR4 and TLR9 mRNA decreased significantly in siTLR2-, siTLR4- and siTLR9-transfected cells, respectively, compared with the negative control siRNA (siNegative)-transfected cells. Furthermore, PAMs were pre-transfected with specific siRNAs or siNegative followed by PCV2 infection. As shown in Fig. 3(b), TLR2 knockdown significantly reduced IL-8 secretion in PCV2-infected cells compared with the cells transfected with siNegative. Interestingly, TLR4 or TLR9 knockdown had no effects on PCV2-induced IL-8 secretion. We confirmed that TLR2, TLR4 and TLR9 signalling was indeed impaired by the knockdown treatments using the known ligands [TLR2 agonist Pam3CysSerLys4 (Pam3Cys), TLR4 agonist LPS and TLR9 agonist CpG-ODN]. As shown in Fig. 3(b), Pam3Cys, LPS and CpG-ODN were capable of inducing IL-8 in PAMs. These results suggest that TLR2 mediated PCV2-induced IL-8 production.

NF-κB activation is involved in PCV2-induced IL-8 secretion

Previous studies have demonstrated that PCV2 can activate NF-κB signalling (Duan et al., 2014; Lv et al., 2013; Zhang
Therefore, we assessed phosphorylation of IkBα or p65 during the course of PCV2 infection. PAMs infected with PCV2 for 6 or 24 h were harvested for Western blot analysis using phospho-specific antibody for phosphorylated (p)-IkB-α or p-p65. As expected, phosphorylation of IkB-α and p65 was detectable at 6 h after PCV2 infection, whereas IkB-α phosphorylation increased at 24 h p.i. (Fig. 4 a, b). To determine whether activation of NF-κB is essential for PCV2-induced IL-8 production,
PAMs were pretreated with DMSO, BAY11-7082 or ammonium pyrrolidine dithiocarbamate (PDTC) for 1 h, and then infected with PCV2. As shown in Fig. 4(c), both BAY11-7082 and PDTC significantly suppressed production of IL-8 in PCV2-infected PAMs. The blocked NF-κB activation pathway strongly inhibited PCV2-induced IL-8 mRNA expression (Fig. 4d). The data suggested that NF-κB activation is involved in IL-8 production induced by PCV2 infection.

**MyD88 is required in PCV2-induced IL-8 secretion**

It has been reported that levels of MyD88 are significantly upregulated in PCV2-infected lymphocytes (Duan et al., 2014). To investigate whether PCV2-induced IL-8 is mediated by MyD88, PAMs were pre-transfected with siRNA targeting MyD88 (siMyD88) or siNegative following PCV2 infection. The cells were harvested for immunoblotting to analyse the expression of MyD88 at 24 h p.i. The cell culture supernatants were collected and analysed by ELISA (c). Total cells were harvested and analysed for IL-8 mRNA expression by real-time RT-PCR (d). PCV2-triggered IL-8 release was markedly reduced by the inhibitors compared with the DMSO-treated cells (c). Similar results were obtained at the mRNA level (d). ***P<0.001. Results are representative of three independent experiments and are presented as mean ± SE. Statistical analysis was performed using ANOVA.
and rely on the efficiency of the innate immune response (Borghetti et al., 2013). As a macrophage-derived mediator of angiogenesis, IL-8 contributes to persistent angiogenesis, which commonly causes chronic inflammatory disorders (Koch et al., 1992). Thus, regulation of IL-8 plays a crucial role in inflammatory lesions during PCV2 infection. This study aimed to decipher the underlying mechanisms that regulate PCV2-induced IL-8 production; we found that TLR2 signalling is important for the secretion of IL-8.

Innate immune response is the first line of defence against invading pathogens in the host. TLRs are critical sensors of microbial infection as well as in the recognition of a variety of microbial products (Kawai & Akira, 2010). In the present study, we focused on TLR2, TLR4 and TLR9, which are closely related to DNA viral infections (Rathinam & Fitzgerald, 2011). The data indicated that PCV2 infection upregulated expression of TLR2, TLR4 and TLR9 mRNA in PAMs. Similar results were reported previously in PCV2-infected lymphocytes (Duan et al., 2014). The TLR-mediated response is essential for activation of an adaptive immune response via the production of various cytokines and chemokines (Akira et al., 2006; Kawai & Akira, 2010). Among the three TLRs, we found that only TLR2 knockdown inhibited IL-8 secretion in the infected PAMs, indicating TLR2 as a key receptor mediating PCV2-induced IL-8 production.

TLR2 is believed to favour signalling that produces inflammatory cytokines, but not type I IFN in macrophages and dendritic cells (Kawai & Akira, 2010). Various studies have reported that the TLR2 signalling pathway during an inflammatory response to a viral infection regulates the production of cytokines. Rotavirus NSP4 triggers the secretion of pro-inflammatory cytokines from macrophages via TLR2 (Ge et al., 2013). Hepatitis C virus induces inflammation and an immune response through a TLR2-dependent pathway (Brown et al., 2010). TLR2 expression is upregulated in response to human immunodeficiency virus (HIV)-1, which in turn influences the inflammatory response during the infection (Hernandez et al., 2012). In addition, a hepatitis E virus-induced inflammatory response via TLR2, TLR3 and TLR4 results in robust induction of inflammatory cytokines and chemokines, including IL-8 (Devhare et al., 2013).

NF-κB is a pivotal downstream signalling molecule in the TLR2/MyD88 pathway, and stimulation of NF-κB transcriptional activity is required for induction of IL-8 in most cell types (Hoffmann et al., 2002). NF-κB phosphorylation and nuclear translocation after PCV2 infection have been previously reported (Duan et al., 2014; Lv et al., 2013; Zhang et al., 2013). Thus, we hypothesized that PCV2 induced the production of IL-8 via NF-κB activation downstream of the TLR2 signalling pathway. To address this, we examined the protein levels of IL-8 in PAMs treated with BAY11-7082 or PDTC, which are inhibitors of IκB phosphorylation, as well as in cells with knockdown of MyD88, which is a major adaptor shared by the

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**DISCUSSION**

Upregulated expression of PCV2-induced cytokines contributes to multisystemic inflammatory lesions, including hepatitis, dermatitis, enteritis, pneumonia and lymphadenitis (Chae, 2005). IL-8, a major neutrophil chemotactic factor, is an important participant in the migration of specific inflammatory cells from the peripheral blood to the inflammatory site during an innate immune response (Kunkel et al., 1991). In this study, we found that PCV2 activated IL-8 expression in PAMs, which is consistent with previous reports that IL-8 is upregulated both *in vivo* (Chae & Choi, 2011; Darwich et al., 2003a, b) and *in vitro* (Li et al., 2013; Mavrommatis et al., 2014). Expression levels of IL-8 vary with PCV2 infection status and rely on the efficiency of the innate immune response (Borghetti et al., 2013). As a macrophage-derived mediator of angiogenesis, IL-8 contributes to persistent angiogenesis, which commonly causes chronic inflammatory disorders (Koch et al., 1992). Thus, regulation of IL-8 plays a crucial role in inflammatory lesions during PCV2 infection. This study aimed to decipher the underlying mechanisms that regulate PCV2-induced IL-8 production; we found that TLR2 signalling is important for the secretion of IL-8.

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On: Tue, 16 Oct 2018 08:07:32

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449
TLR family with the exception of TLR3 (Takeda & Akira, 2004). NF-κB is a heterodimeric (p65/p50) transcription factor in the canonical pathway, normally sequestered by IκBz within the cytoplasm as a latent complex. Stimulus-induced phosphorylation of IκBz leads to its degradation by a proteasome, resulting in dissociation and nuclear translocation of NF-κB, which is critical for DNA binding and further induction of inflammatory cytokine gene transcription (Gilmore, 2006). In line with the hypothesis, impairment of IκBz phosphorylation or absence of MyD88 signalling considerably reduced IL-8 production in the infected cells. PCV2 infection is not the only case of viral invasion involving IL-8 regulated by NF-κB activation.

For instance, human cytomegalovirus gene UL76-generated IL-8 expression in response to DNA damage is NF-κB-dependent (Costa et al., 2013; Zhang et al., 2013). In addition, NF-κB and C/EBP-like cis-elements regulate IL-8 secretion induced by hepatitis B virus X protein (Mahe et al., 1991). However, the viral elements that affect IL-8 induction via the TLR2/MyD88/NF-κB pathway during PCV2 infection remain unclear. Further studies are required to elucidate the related molecular events.

In summary, this study provides evidence that increased expression of IL-8 in PAMs trigged by PCV2 infection results from activation of the TLR2/MyD88/NF-κB signalling pathways. This finding contributes towards understanding the mechanism underlying the IL-8-mediated response associated with the chronic inflammatory nature of PMWS.

METHODS

Cells and virus. PCV-free PK-15 cells were used for PCV2 multiplications. PK-15 cells obtained from ATCC were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HyClone), 1% non-essential amino acids (Gibco), and 200 U ml⁻¹ (each) penicillin and streptomycin. PAMs were obtained from post-mortem lung lavage (Wensvoort et al., 1991) of healthy 8-week-old specific pathogen free pigs that were free of PCV2, porcine reproductive and respiratory syndrome virus, classical swine fever virus, pseudorabies virus, porcine parvovirus, swine influenza virus and Mycoplasma hyopneumoniae infections. The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids, and 200 U ml⁻¹ (each) penicillin and streptomycin.

The PCV2 strain TJ1 (GenBank ID: KC751546) was originally isolated from the lung of a pig with naturally occurring PMWS in Tianjin, China, and propagated in a PK-15 cell monolayer. The PCV2-TJ1 stock titres were 1 × 10⁵ TCID₅₀ ml⁻¹ determined by titration of the PK-15 cells using an immunofluorescence assay as previously described (Zhu et al., 2007). LPS contaminations were determined by the Limulus assay.

Reagents and antibodies. NF-κB inhibitors BAY11-7082 and PDTC were purchased from Sigma-Aldrich. BAY11-7082 was dissolved in DMSO prior to use. PEM from Escherichia coli serotype 0111:B4 was obtained from Sigma-Aldrich. Synthetic bacterial-like lipopeptide Pam3Cys was obtained from EMCO Microcollections. Cysteine-phosphate-guanine (Cpg) oligodeoxynucleotides (ODN) 2007 were purchased from InviroGen. An ELISA kit for porcine IL-8 was purchased from R&D Systems. TRIZol and RNase-free DNase were purchased from Invitrogen Life Technology and Promega, respectively. Perfect Real-time SYBR Premix Ex Taq was obtained from TaKaRa Bio. Antibodies against p-IκBz, IκBz, p-p65 and p65 were purchased from Cell Signaling Technology. Anti-MyD88 was obtained from Abcam, and anti-β-actin was obtained from Sigma-Aldrich. Anti-PCV2 Cap polyclonal antibody was produced from BALB/c mice immunized with recombinant Cap of PCV2 strain TJ1.

RNA extraction and real-time RT-PCR. PAMs were infected with PCV2 at an m.o.i. of 0.5 for 1, 3, 6 and 24 h. Total RNA was extracted from PCV2-infected cells using the TRizol reagent, and 1 μg of RNA was used for cDNA synthesis using Moloney murine leukemia virus (Promega) to examine expression of TLR2, TLR4 and TLR9. Real-time PCR was performed using specific primers for TLR2 (Duan et al., 2014) (sense 5'-TCA TCT CCC AAA TCT GCG CCT-3', antisense 5'-GGC TGA TGT TCT GAA TTG ACCTG-3'), TLR4 (Duan et al., 2014) (sense 5'-CCG TCA TTA GTG CGT CCT-3', antisense 5'-TTG CAG CCC ACA AAA AGCA-3'), TLR9 (Duan et al., 2014) (sense 5'-AGC CTC AAC CTG TCC TTC AAT TACC-3', antisense 5'-CTG AGC GAG CCG AAG ATGC-3'), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fu et al., 2012) (sense 5'-CCT TGC CTT CCA CTG CCA AC-3', antisense 5'-GAC GCC TGC TTC ACC TTCT-3') and the Real-time SYBR master mix kit (Takara Bio) in accordance with the manufacturer’s protocol for the ABI7900 real-time PCR system. IL-8 mRNA from PAMs pretreated with DMSO, BAY11-7082 (1 μM) or PDTC (100 μM) for 1 h followed by 24 h of PCV2 infection at an m.o.i. of 0.5 was analysed by real-time RT-PCR using specific primers for IL-8 (Arce et al., 2010) (sense 5'-TTC GAT GCC AGT GCA TAA ATA-3', antisense 5'-CTG TAC AAC CTT CTG CAC CCA-3'). Gene expression was normalized to GAPDH (internal reference) and presented as relative fold change compared with the mock-infected control.

siRNA and cell transfection. The siRNAs targeting the porcine TLR2 (Bi et al., 2014) (sense 5'-GCC CUCU CCA CAC UUU ATT-3', antisense 5'-UAA AGU GUG UAG GAA GGG CTT-3'), TLR4 (Bi et al., 2014) (sense 5'-CAG UAC UCC UGU UCA AUA ATT-3', antisense 5'-UUA UAG ACC AGG AUU CUC GTT-3'), TLR9 (Bi et al., 2014) (sense 5'-CCU UCA AUU ACC ACA AGA ATT-3', antisense 5'-UUUC UUG UGG UAA UUG AAG GTT-3'), MyD88 (Qianjun Zhou et al., 2010) (sense 5'-AUG CCU GCG CAU UUU GAU GTT-3', antisense 5'-CAU CAA AUC GCC GAC GCA UTT-3') and negative control (Zhou et al., 2010) (sense 5'-CUG CCC CAG CCA UAU CCA GTT-3', antisense 5'-CUG CAU GCC GGA GCA CTA GAA-3') were synthesized by GenePharma. Transient transfection of siRNA was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The amount of siRNA used for transfection was optimized in preliminary experiments and no significant cellular toxicity was observed. Cells were harvested 24 h post-transfection for TLR2, TLR4 and TLR9 mRNA analysis by real-time PCR or for MyD88 expression by Western blotting.

Inhibition of signal transduction pathway. PAMs were pretreated with DMSO, BAY11-7082 (1 μM) or PDTC (100 μM) for 1 h, and then infected with PCV2 at an m.o.i. of 0.5. The cells and the supernatants were harvested after 24 h for analysis of IL-8 mRNA and protein by real-time RT-PCR and ELISA, respectively.

Western blot analysis. PAMs were infected with PCV2 at an m.o.i. of 0.5 for 6 or 24 h. Total cell extracts were prepared in NP-40 lysis buffer supplemented with protease and phosphatase inhibitors. The cell lysates were separated by 12% SDS-PAGE and transferred onto the nitrocellulose membranes. After blocking with 5% (w/v) non-fat dried milk in PBS, membranes were incubated with primary antibodies against Cap, p-IκBz, IκBz, p-p65, p65, MyD88 or β-actin. Membranes were then washed and incubated with appropriate secondary antibodies and HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG, followed by visualized by enhanced chemiluminescence (ECL) reagents supplied by Amersham.
secondary antibodies. Proteins were visualized using the ECL reagent (Amersham Pharmacia Biotech, UK) according to the manufacturer’s instructions.

**IL-8 ELISA.** The levels of secreted IL-8 in cell culture supernatants were measured using a commercially available porcine IL-8 ELISA kit (R&D Systems) according to the manufacturer’s instructions.

**Statistical analysis.** All experiments were performed with at least three independent replicates. Statistical analysis was performed using GraphPad Prism, and differences in data were evaluated by Student’s t-test. A P value of <0.05 was considered statistically significant.

**ACKNOWLEDGEMENTS**

This study was supported by National Science Grant of China (no. 31402087) and projects in the National Science & the President Funding of Tianjin Academy of Agricultural Sciences (no. 13004).

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