Short communication

Cytokine mRNA expression profiles in peripheral blood mononuclear cells from piglets experimentally co-infected with porcine reproductive and respiratory syndrome virus and porcine circovirus type 2

Kai-Chuang Shi\textsuperscript{a,b}, Xin Guo\textsuperscript{a}, Xin-Na Ge\textsuperscript{a}, Qi Liu\textsuperscript{b}, Han-Chun Yang\textsuperscript{a,*}

\textsuperscript{a} Key Laboratory of Zoonosis of Ministry of Agriculture, College of Veterinary Medicine and State Key Laboratory of Agrobiotechnology, China Agricultural University, No. 2 Yuanmingyuan West Road, Beijing 100193, China
\textsuperscript{b} Guangxi Center for Animal Disease Control and Prevention, No. 51 Youai North Road, Nanning 530001, China

\textbf{ARTICLE INFO}

Article history:
Received 20 February 2009
Received in revised form 2 July 2009
Accepted 31 July 2009

Keywords:
Porcine reproductive and respiratory syndrome virus (PRRSV)
Porcine circovirus type 2 (PCV2)
Co-infection
Cytokine
Immunomodulation
Immunosuppression

\textbf{ABSTRACT}

Porcine reproductive and respiratory syndrome virus (PRRSV) and porcine circovirus type 2 (PCV2) significantly impact the swine industry worldwide. Co-infections with these viruses are common and several lines of evidence suggest that both PRRSV and PCV2 modify host immune responses that facilitate infection. This study examined cytokine mRNA expression profiles of peripheral blood mononuclear cells (PBMCs) from piglets experimentally co-infected with PRRSV and PCV2 to define the influence of co-infection on host immunity. PBMCs from infected and control piglets were stimulated with concanavalin A and the IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-\(\gamma\) and TNF-\(\alpha\) mRNA levels were determined by quantitative reverse transcription-polymerase chain reaction (RT-PCR). PBMCs from PRRSV/PCV2 co-infected piglets had significantly reduced IL-2, IL-4, IL-6, IL-12p40 and IFN-\(\gamma\) and significantly increased TNF-\(\alpha\) mRNA levels compared to those of the piglets infected with either PRRSV or PCV2 alone. The IL-10 mRNA levels in all virus-infected groups were significantly up-regulated early during infection. These results suggested that co-infection synergistically suppresses T helper 1 (Th1)-type and Th2-type cytokine production by PBMCs, indicating that co-infection likely compromises cell-mediated and humoral immune responses resulting in increased severity of the diseases in piglets.

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) can cause porcine reproductive and respiratory syndrome (PRRS) characterized by reproductive disorders in pregnant sows and gilts or by respiratory problems in pigs of all ages (Rossow, 1998). Porcine circovirus type 2 (PCV2) can cause postweaning multisystemic wasting syndrome (PMWS) characterized by weight loss, jaundice, generalized lymphadenopathy, interstitial pneumonia and nephritis (Allan and Ellis, 2000). These diseases currently affect the swine industry worldwide.

Cytokine or cytokine mRNA levels in the PRRSV-infected pigs have been measured to study immune mechanisms affected by PRRSV infection that may facilitate host immune evasion. The PRRSV-infected pigs had up-regulated IL-6, IL-10 and IFN-\(\gamma\) mRNA in peripheral blood mononuclear cells (PBMCs), up-regulated IL-10 and IFN-\(\gamma\) mRNA in bronchoalveolar lavage cells (BALCs), up-regulated IL-6, IL-10, IL-12 and TNF-\(\alpha\) mRNA in pulmonary alveolar macrophages (PAMs) and elevated IL-6 and IFN-\(\gamma\) in serum (Asai et al., 1999; Johnsen et al., 2002; Feng et al., 2003; Suradhat and Thanawongnuwech, 2003; Thanawongnuwech et al., 2004; Wesley et al., 2006). In addition,
IL-10, IL-12, IFN-γ and TNF-α mRNA were detected in the lungs of pigs infected with PRRSV (Choi et al., 2002; Chung and Chae, 2003). However, the PRRSV-infected piglets showed diminished IFN-α levels in serum, undetectable or minimally increased proinflammatory cytokines in bronchoalveolar lavage (BAL) fluids and minimal changes in the IL-2, IL-4 and IL-12 mRNA or protein levels in either PBMCs or BALCs and developed slow and weak anti-PRRSV-specific IFN-γ-secreting PBMCs (Johnsen et al., 2002; Murtaugh et al., 2002; Feng et al., 2003; Díaz et al., 2005; Mateu and Díaz, 2008). These changes in cytokine production may contribute to poor innate immune responses as well as slow developing and ineffective adaptive immune responses in PRRSV-infected pigs.

PBMCs harvested from pigs subsequent to PCV2 infection produced low levels of IL-10 and IFN-γ following challenge with PCV2 in vitro but no IL-2, IL-4 or IFN-γ after mitogen stimulation (Darwich et al., 2003b). The PMWS-affected pigs over-expressed IL-10 and IFN-γ mRNA in the thymus and tonsils, respectively, and had significantly reduced IL-2, IL-4, IL-10, IL-12p40 and IFN-γ mRNA levels in other lymphoid tissues (Darwich et al., 2003a). PMWS was also associated with significant IL-10 increases (accompanied by slight increases in IL-6, IFN-γ and TNF-α) and a decrease in IL-2 and IL-4 mRNA levels in white blood cells as well as slight decreases in IL-4 and IFN-γ in PBMCs (Sipos et al., 2004). Furthermore, subclinically PCV2-infected pigs or pigs developing severe PMWS showed a significant increase in IL-10 levels in serum (Stevenson et al., 2006; Darwich et al., 2008) further suggesting that PCV2 could alter immune responses in the infected pigs.

Since PRRSV and PCV2 co-infections are common in pigs (Grau-Roma and Segalés, 2007) and immunosuppression and immunodeficiency are associated with animals presenting with either PRRS or PMWS (Murtaugh et al., 2002; Segalés et al., 2004), the study described here monitored the cytokine mRNA expression profiles of PBMCs isolated from piglets experimentally co-infected with PRRSV and PCV2 by quantitative RT-PCR to define the influence of co-infection upon host immunity.

2. Materials and methods

2.1. Viruses and animals

The virus strains and animals used in this study have been previously described (Shi et al., 2008). The PRRSV HB-2 (sh)/2002 strain (North American genotype) was used to infect piglets following three passages through porcine alveolar macrophages (AMs) using viral inoculums of 10^4–8.5 TCID₅₀/ml. The PCV2 BJ-HB strain was passaged in PK-15 cells and supernatants of tissue homogenates from piglets at a titer of 10^8–22 TCID₅₀/ml prepared as previously described (Shi et al., 2008) were used to inoculate piglets.

Twenty healthy, weaned, 6-week-old, Large White-Dutch Landrace crossbred piglets were confirmed to be free of PRRSV and PCV2 prior to the infection studies by enzyme-linked immunosorbent assay (ELISA) and RT-PCR or PCR. PRRSV-specific antibodies were detected using the IDEXX Labs Inc. (Westbrook, ME, U.S.A.). ELISA kit and PCV2-specific antibodies were detected using the Ingezim Circovirus IgG/IgM kit (Ingenasa, Spain). PRRSV and PCV2 nucleic acids in serum were detected by RT-PCR or PCR. In addition, RT-PCR, PCR or serology were used to confirm that the animals were negative for classical swine fever virus (CSFV), pseudorabies virus (PRV), porcine parvovirus (PPV), swine influenza virus (SIV) and Mycoplasma hyopneumoniae infections.

2.2. Experimental design

Briefly, piglets were randomly divided into four groups (five piglets per group) and inoculated intranasally as described previously (Shi et al., 2008). Each piglet in the control group was inoculated with 5 ml of RPMI-1640 medium. Each piglet in the PRRSV- or PCV2-infected group was inoculated with 5 ml each virus and each piglet in the PRRSV/PCV2 co-infected group was inoculated with 5 ml of PRRSV and 5 ml of PCV2.

Blood samples were collected for virological and serological examination on days 0 (before inoculation), 3, 7, 10, 14, 21, 28, 35, 42 and 49 post-infection (PI). EDTA-stabilized blood samples were collected for PBMC isolation which were then used to establish cytokine mRNA expression profiles on days 0, 7, 14, 21, 28 and 42 PI. For each piglet, body temperatures were measured and clinical signs were recorded daily during the experiment. The experiment was terminated on day 50 PI and all piglets were sacrificed, autopsied and the severity of gross lesions recorded.

2.3. Virology and serology

Serum viral loads were measured by quantitative RT-PCR, and antibodies against PRRSV and PCV2 were detected by ELISA and indirect immunofluorescence assay (IFA), respectively, as previously described (Shi et al., 2008).

2.4. Quantification of cytokine mRNA by quantitative RT-PCR

Ten milliliters of EDTA-stabilized blood were collected from each piglet. PBMCs isolated by density gradient centrifugation using Histopaque-1.077 (Sigma, St. Louis, MO) were resuspended in RPMI-1640 complete medium (Innogenetix, Carlsbad, CA) at a concentration of 5 × 10⁶cells/ml, plated in 24-well culture plates (1 ml/well) and stimulated with concanavalin A (Con A, Sigma) (final concentration, 10 μg/ml). After a 24 h incubation at 37 °C with 5% CO₂, PBMCs were harvested for the measurement of cytokine mRNA expression levels using oligonucleotide primers (Table 1) designed to amplify porcine cytokine genes (i.e., IL-2, IL-4, IL-6, IL-10, IL-12p40, IFN-γ and TNF-α) and β-actin (as an internal control) by quantitative RT-PCR following the methods described by Spagnuolo-Weaver et al. (1999) with minor modifications. Total RNA was extracted from approximately 5 × 10⁶ PBMCs using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Contaminating genomic DNA was removed using the RNase-Free DNase Set (Qiagen). Total RNA from each sample was eluted in 60 μl of RNase-free water. Total RNA was reverse-transcribed in the presence of Oli-
go(dT)₁₈ using AMV reverse transcriptase (Promega, Madison, WI). PCR detection of cytokine cDNA was performed using a continuous fluorescence detector (DNA Engine Opticon™ 2, MJ Research, Waltham, MA) with the SYBR® Green Realtime PCR Master Mix (Toyobo, Osaka, Japan). Raw data were normalized against the values obtained for β-actin and the cytokine mRNA measurements were expressed as the ratio of cytokine to β-actin mRNA expression. To compare mRNA levels between experimental groups, the relative values of each cytokine were expressed as the fold-increase of the virus-infected groups over the control group.

3. Results

3.1. Clinical and gross pathological observations

Assessment of the clinical signs and gross lesions found in the experimentally infected piglets was carried out as described previously (Shi et al., 2008). Briefly, piglets in the PRRSV group showed pronounced febrile responses (over 40.0 °C) and loss of appetite intermittently during the first 3 weeks PI and developed respiratory complications which included tachypnea, dyspnoea, coughing and sneezing beginning on days 10–12 PI and lasting for approximately 2 weeks. At necropsy, all piglets had enlarged and/or hemorrhagic lymph nodes, and two piglets displayed gastric ulceration. In addition, the lungs of two piglets failed to collapse and had focal and diffuse areas that were tan to purple in color. Piglets in the PCV2 group also showed inappetence, febrile responses and mild tachypnea during the first 2 weeks PI. Three piglets had diarrhea on day 3 PI, which lasted for approximately 1 week. Necropsy revealed enlargement of one or more lymph node, including some with hemorphages. All piglets in the PRRSV/PCV2 group showed febrile responses, severe respiratory distress, and enlarged and hemorrhagic lymph nodes. Three piglets had consolidated lungs that did not collapse and gastric ulcerations. These results showed that the clinical signs and gross lesions in the PRRSV/PCV2 group were more severe than those observed in the PRRSV or PCV2 groups.

3.2. Virus and antibody detection

The kinetics of serum viral loads and PRRSV- and PCV2-specific antibodies in infected piglets have been described previously (Shi et al., 2008). Briefly, all PRRSV inoculated piglets showed a significant increase in PRRSV loads and the viral loads in the PRRSV/PCV2 group were significantly higher than those in the PRRSV group from day 7 PI onwards. Piglets inoculated with PCV2 also exhibited a dramatic increase in PCV2 loads and the viral loads in the PRRSV/PCV2 group were significantly higher than those in the PCV2 group from day 21 PI onwards. These data demonstrated that co-infection with PRRSV and PCV2 resulted in an increased and prolonged presence of serum viral loads.

PRRSV-specific antibodies were detected in one piglet in the PRRSV group on day 7 PI and all piglets in the PRRSV and PRRSV/PCV2 groups seroconverted by day 10 PI. However, piglets in the PRRSV/PCV2 group generated significantly lower levels of anti-PRRSV antibodies on days 21, 28, 35 and 49 PI. PCV2-specific antibodies were detected in three piglets in the PCV2 group on day 10 PI, and the other two piglets seroconverted by day 14 PI. However, antibodies to PCV2 in the PRRSV/PCV2 group were detected for the first time on day 14 PI in two of the five piglets, and were detected on day 21 PI in the remaining three piglets. The levels of anti-PCV2 antibodies were lower in the PRRSV/PCV2 group than those in the PCV2 group during the experiment and were significantly lower on days 10, 14 and 28 PI. These data demonstrated

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide sequences (5’ → 3’)</th>
<th>Product (bp)</th>
<th>Annealing temp a (°C)</th>
<th>Fluorescent acquisition (°C)</th>
<th>Product Tm (°C)</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2</td>
<td>F: GATTACAGCTGCTTTTGAARc: GTGAGTAGATGCTTTTGAAC</td>
<td>338</td>
<td>54.0</td>
<td>80.0</td>
<td>83.0</td>
<td>X56750</td>
</tr>
<tr>
<td>IL-4</td>
<td>F: ATCCAACCCCTGTCCTGCARc: TCCCTGAATGCTCCCTCA</td>
<td>265</td>
<td>57.0</td>
<td>86.0</td>
<td>89.5</td>
<td>X68330</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: CAGGAACCCAGTATGAACARc: CTGACAGGCTCAGACATT</td>
<td>446</td>
<td>56.0</td>
<td>85.0</td>
<td>88.7</td>
<td>NM214399</td>
</tr>
<tr>
<td>IL-10</td>
<td>F: GCTACACCTCCACGCGCARc: CTCCCTACATCATGCGACAC</td>
<td>446</td>
<td>60.0</td>
<td>86.0</td>
<td>89.3</td>
<td>L20001</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>F: GATGCCTGCCAGTACCAARC: TCCAGCAGACCACTTAAGT</td>
<td>377</td>
<td>59.0</td>
<td>86.0</td>
<td>90.0</td>
<td>U08317</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>F: CGCCGCTGCTGTTCTTGCARc: GATGCGTCTGATGTCCTT</td>
<td>380</td>
<td>50.0</td>
<td>80.0</td>
<td>83.6</td>
<td>X53085</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: CGTTTAGCCAAATCAGCCACRc: TGCCGAGTCCAGGAAAGTCCA</td>
<td>402</td>
<td>62.0</td>
<td>88.0</td>
<td>91.0</td>
<td>X54859</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: CTGCATCTTCAGGATCTGARc: GCCGATCTGATGATCTTCA</td>
<td>547</td>
<td>59.0</td>
<td>85.0</td>
<td>88.5</td>
<td>U07786</td>
</tr>
</tbody>
</table>

a temp: temperature.  
b F: forward.  
c R: reverse.
that co-infection with PRRSV and PCV2 resulted in the slower generation and lower levels of antibodies against PRRSV and PCV2.

3.3. Cytokine mRNA expression profiles in PBMCs

Since disease severity was greatest in the co-infected piglets compared to the piglets infected with either PRRSV or PCV2, the cytokine profiles were defined for each infected group (and the uninfected control group). The PBMC cytokine mRNA expression profiles from piglets after infection are shown in Fig. 1. Paralleling disease presentation, the piglets co-infected with PRRSV/PCV2 had the most significant changes in cytokine mRNA expression. Of the seven cytokines examined, the IL-2, IL-4, IL-6, IL-12p40 and IFN-γ mRNA levels were all lower than the levels observed in PBMCs harvested from PRRSV- or PCV2-infected piglets and in the case of IL-2 and IL-4, the levels were significantly lower than the mRNA levels detected in the controls (Fig. 1A and B).

The IL-10 mRNA levels were increased in all virus-infected groups and PBMCs from the PRRSV group had significantly up-regulated IL-10 mRNA levels at all time points examined while the PCV2 and PRRSV/PCV2 groups showed significant up-regulation on days 7, 14 and 21 PI compared to that of the control group. The IL-10 mRNA levels in the PRRSV/PCV2 group were significantly lower than those of the PRRSV group on days 14, 21 and 28 PI and were slightly (but not significantly) higher than those of the PCV2 group at all time points (Fig. 1D).

PBMCs from the PRRSV group had a slightly (but not significantly) higher TNF-α mRNA expression than that of the control group at all time points examined. The TNF-α mRNA levels in the PCV2 group showed significant up-regulation from day 14 PI onwards and the levels in the PRRSV/PCV2 group were significantly up-regulated at all time points examined compared to those of the control group. Surprisingly, the TNF-α mRNA levels in the PRRSV/PCV2 co-infected group were higher than those of the singly infected groups at all time points examined and were significantly higher than the levels observed in the PRRSV group and the PCV2 group on days 14, 21 and 28 PI and days 21 and 28 PI, respectively (Fig. 1G).

4. Discussion

Cytokines play a crucial role in the induction and modulation of the immunological processes. PBMCs isolated from the PRRSV/PCV2 co-infected piglets or from the PRRSV- or PCV2-infected piglets were stimulated in vitro with Con A and their cytokine mRNA expression profiles were determined to categorize the host immune responses following viral infections. As a result, the PRRSV- and PCV2-infected piglets had increased IL-2, IL-4, IL-10, IL-12p40, IFN-γ and TNF-α gene expression profiles similar to those described previously (López Fuentes et al., 1999; Feng et al., 2003; Suradhat and Thanawongnuwech, 2003; Darwich et al., 2003a; Sipos et al., 2004). Interestingly, the IL-2, IL-4, IL-6, IL-12p40 and IFN-γ mRNA levels in the PRRSV/PCV2 co-infected piglets were significantly lower than those observed in the PRRSV- or PCV2-infected piglets. Reduction in Th1-type cytokines (e.g., IL-2, IL-12p40 and IFN-γ) that contribute to the development of cellular immunity and Th2-type cytokines (e.g., IL-4 and IL-6) that are associated with humoral immune responses was shown to be significantly diminished following PRRSV/PCV2 co-infection compared to levels associated with either PRRSV or PCV2 infection, suggesting that immune suppression or altered immune responses following co-infection could be responsible for increased disease severity. Particularly, suppression of IL-2 and IL-4, which are important for primary T-cell responses and B-cell activation, respectively, could result in prolonged immunosuppression following co-infection with PRRSV/PCV2. It is likely that the significant suppression in cytokine production associated with co-infection observed in this study contributed to lower antibody responses, increased levels of viraemia and more intense disease severity compared to infections caused by either PRRSV or PCV2.

IL-10 has potent immunosuppressive properties that include suppressing immune responses. PRRSV-induced IL-10 contributed to the significantly reduced IFN-γ and TNF-α expression by T cells (Charerntantanakul et al., 2006) and induction of IL-10 production may be one of the strategies used by PRRSV to modulate the host’s immune responses thereby contributing to the unique clinical picture observed following PRRSV infection (Suradhat and Thanawongnuwech, 2003). Kekarainen et al. (2008) reported that PCV2-induced IL-10 participated in the down-regulation of the responses to a recall antigen by inhibiting IFN-γ, IFN-α and IL-12 production and Darwich et al. (2008) showed that in subclinically PCV2-infected pigs a transient IL-10 response correlated with the viraemic phase of infection. Based on these previous observations, the increased IL-10 production observed during the early stages of PRRSV or PCV2 infection in the present study may serve to enhance viral survival and disease severity by suppressing the induction of protective immunity. We hypothesized that IL-10 mRNA levels would be highest in the co-infected piglets since IL-10 mRNA levels were elevated in the PRRSV- and PCV2-infected pigs (Feng et al., 2003; Sipos et al., 2004). However, IL-10 mRNA levels in the co-infected piglets were significantly lower than those of the PRRSV-infected piglets and slightly higher than those observed in the PCV2-infected piglets suggesting that other mediators (or the interaction thereof) are likely responsible for the immunosuppression observed in the co-infected piglets.

IFN-γ has potent antiviral properties that contribute to the control of acute viral infections and is an important mediator of cellular responses. The elevated IFN-γ mRNA levels observed in PBMCs from all PRRSV-infected groups in this study were not surprising since IFN-γ plays a key role in cell-mediated immune responses shown to inhibit PRRSV replication in vitro (Bautista and Molitor, 1999). Significant IFN-γ mRNA down-regulation following PRRSV/PCV2 co-infection might be indicative of suppressed Th1 responses facilitating viral persistence and delayed viral clearance. Conversely, IFN-γ has been reported to enhance PCV2 infection and replication in vitro (Meerts et al., 2005), therefore the significant increase in IFN-γ gene expression in the PCV2-infected and PRRSV/
Fig. 1. Cytokine mRNA expression profiles in PBMCs from the PRRSV- and/or PCV2-infected piglets. The expression levels of cytokine mRNA in PBMCs were analyzed by quantitative RT-PCR. The relative levels of IL-2 (A), IL-4 (B), IL-6 (C), IL-10 (D), IL-12p40 (E), IFN-γ (F) and TNF-α (G) mRNA expression are expressed as the mean fold-increase of the virus-infected groups compared to those of the control group; error bars represent standard deviations. Data were analyzed using the analysis of variance (ANOVA) and comparisons of means were conducted using Duncan’s multiple range test. Data with different letters (a–d) indicate significant differences between groups ($P < 0.05$).
PCV2 co-infected piglets might contribute to the high viral loads in the serum of these piglets at the end of the experiment described here.

TNF-α is a pleiotropic cytokine that plays important roles in the induction and regulation of inflammatory responses. The TNF-α mRNA levels in the PRRSV/PCV2 co-infected piglets were significantly higher than the levels observed in either PRRSV- or PCV2-infected piglets. Some studies have suggested that the excessive TNF-α production in the lungs was associated with respiratory disease severity induced by PRRSV–lipopolysaccharide inoculation or by PRRSV and M. hyopneumoniae co-infection in piglets (Van Gucht et al., 2003; Thanawongnuwech et al., 2004). Moreover, it has been demonstrated that the potentiation of PPV in P2R-mediated PMWS was associated with the excessive production of TNF-α (Kim et al., 2006). Therefore, significantly increased TNF-α mRNA levels might be responsible for increased disease severity associated with PRRSV/PCV2 co-infection.

In conclusion, PRRSV/PCV2 co-infection produced significant alterations in cytokine mRNA expression in PBMCs resulting in immunomodulation that may contribute to the prolonged viral persistence and increased disease severity associated with PRRSV and PCV2 co-infection.

Acknowledgements

This work was supported by National Natural Science Funds for Distinguished Young Scholar (30825031) from National Natural Science Foundation of China, and National Key Basic Research Plan Grant (2005CB523204) and National Key Technology R&D Program of China (Grant No. 2006BAD06A03) from the Chinese Ministry of Science and Technology, and the Program for Cheung Kong Scholars and Innovative Research Team in University of China (No. IRT0866).

References