Genomic expression profiling in lymph nodes with lymphoid depletion from porcine circovirus 2-infected pigs

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Porcine circovirus type 2 (PCV2) is the main causative agent of porcine circovirus-associated disease, such as post-weaning multisystemic wasting syndrome, which involves lymphocyte depletion. However, little is known about the molecular mechanisms of lymphoid depletion. To gain insight into the interaction between virus and host cells, microarrays were used to analyse changes in genomic expression in lymph nodes following PCV2 infection of pigs, together with negative controls. Total RNA was subjected to microarray analysis with an Affymetrix Porcine Genome Array GeneChip. Of the 23 256 pig genes arrayed on a chip, 160 genes showed altered expression after infection (upregulated, 64; downregulated, 96). The altered genomic expression of 18 selected genes was confirmed by quantitative real-time PCR. The expression changes of numerous genes involved in innate immune defence (TLR1, CD14 and CD180), immunosuppressed responses (FGL2 and GPNMB), pro-inflammatory signals (galectin-3) and fasting processes (ANGPTL-4) indicate that PCV2 has developed an intricate mechanism to cause immunosuppression, inflammatory cell infiltration and weight loss in pigs. The results of this study provide a basis for understanding the molecular pathogenesis of PCV2 infection.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is an unenveloped, single-stranded circular DNA virus and is the main causative agent of porcine circovirus-associated disease (PCVAD) such as post-weaning multisystemic wasting syndrome (PMWS) (Allan & Ellis, 2000; Chae, 2005). The characteristic clinical symptoms of PMWS include progressive weight loss, enlarged lymph nodes and dyspnoea with high mortality. The hallmark histopathological lesions are one or both of lymphoid depletion and granulomatous inflammation, in the lymphoid tissues (Chae, 2004). PCVAD can be subclinical or may include one or more clinical manifestations such as multisystemic wasting disease with weight loss, high mortality, respiratory signs, enteric signs and porcine dermatitis and nephropathy syndrome (PDNS) (Chae, 2005; Opiressnig et al., 2007).

Specific pathogen-free (SPF) pigs infected with cloned genomic PCV2 DNA developed PCVAD with lymphoid lesions (Fenaux et al., 2002). In a study conducted by Fenaux et al. (2002), evidence of wasting was not observed. However, the PCV2 infectious DNA clone research clearly established PCV2 as the cause of the hallmark lymphoid lesions (Fenaux et al., 2002). These pathological hallmarks also suggest potential immunosuppression of affected animals (Segalès et al., 2005).

DNA microarray technology, in combination with bioinformatics, has proved to be a very efficient high-throughput tool and offers great advantages in the study of the genomic expression profiles of cells (Solis et al., 2006). High-density gene arrays have been used to evaluate the status of host gene expression following infection by viruses such as dengue virus (Fink et al., 2007), measles virus (Sato et al., 2008) and classical swine fever virus (Shi et al., 2009).

Although the role of PCV2 as a major aetiological agent in PCVAD is well established (Chae, 2004, 2005), little is known about the genes that are involved in lymphoid depletion triggered by PCV2 infection. In the previous study, monocyte chemotactant protein-1 and macrophage inflammatory protein-1 from granulomatous inflammation of lymph nodes were expressed in vivo (Kim & Chae, 2008).
Cytokine mRNA expression was profiled in lymph nodes of pigs affected by PMWS (Darwich et al., 2003). The global transcriptome profile was done in Caesarean-derived, colostrum-deprived piglets experimentally infected with PCV2 (Fernandes et al., 2009). However, all those results were limited to known cytokines and chemokines.

The objective of this study was to analyse genomic expression profiles involved in the molecular mechanisms of lymphoid depletion in the lymph nodes of experimentally virus-infected pigs using microarrays.

**RESULTS**

**PCV2-induced alteration of porcine genomic expression in lymphocyte-depleted lymph nodes**

Microscopic examination revealed moderate depletion of lymphocytes and infiltration of histiocytic cells into the lymph nodes of infected animals. No microscopic lesions were observed in control pigs. The pools of the lymph nodes (superficial inguinal, mesenteric and mediastinal) were analysed for gene expression profiling. By analysing the genomic expression of PCV2-infected pigs with lymphoid depletion using Porcine Genome Array GeneChips (Affymetrix), expression changes were identified for 160 genes in all three pigs (P < 0.05; unpaired t-test). Of all genes present in the array, 160 (0.7 %) were confirmed as having at least 1.5-fold-altered expression, with 64 (40 %) being upregulated and 96 (60 %) downregulated. These 160 genes were classified into 19 biological-process- and 16 molecular-function groups by PANTHER gene group analysis (http://david.abcc.ncifcrf.gov/home.jsp). There was no functional annotation for 58 genes. Among the biological-process groups, the signal transduction (7/160; 4.4 %), mRNA transcription regulation (5/160; 3.2 %), mRNA transcription (4/160; 2.5 %), protein metabolism (3/160; 1.9 %) and oxidative phosphorylation (3/160; 1.9 %) categories contained a high number of genes (Fig. 1a). Among the biological molecular functional groups, the extracellular matrix (6/160; 3.8 %), nucleic acid-binding (5/160; 3.2 %), receptor (5/160; 3.2 %), signalling molecule (4/160; 2.5 %) and KRAB-box transcription factor (3/160; 1.9 %) categories had a high number of genes (Fig. 1b). The microarray dataset of 45 differentially expressed genes (DEGs) showing their functional annotations and changes in expression level are summarized in Supplementary Table S1 (available in JGV Online).

**Validation of microarray data by real-time RT-PCR**

To validate the result of the microarray analysis, 18 genes with different levels of altered expression were selected for confirmation by quantitative real-time RT-PCR (qPCR) (Table 1). The altered expression of 14 genes among the 18 genes identified by microarray was highly consistent with the results of qPCR. The genes were: galectin-3/lectin, galactoside-binding, soluble, 3 (LGALS3), glycoprotein (transmembrane) nmb (GPNMB), intelectin 2 (ITLN2), glutamate–ammonia ligase/glutamine synthetase (GLUL), CD14, Toll-like receptor (TLR1), chemokine (C–C motif)
Table 1. Validation of microarray data by real-time RT-PCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence 5′–3′</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microarray</td>
<td>Real-time RT-PCR</td>
</tr>
<tr>
<td>LGALS3</td>
<td>F: TCCTGGCACCTACCCTGGGC 5.149</td>
<td>5.82</td>
</tr>
<tr>
<td></td>
<td>R: CTTGGCTGTGCAAGGAGGGG</td>
<td></td>
</tr>
<tr>
<td>GPNMB</td>
<td>F: CAGGGAGACATCCCCACCGA 3.861</td>
<td>5.94</td>
</tr>
<tr>
<td></td>
<td>R: AAGGGTGCTCGTGAGGGCA</td>
<td></td>
</tr>
<tr>
<td>ITLN2</td>
<td>F: GGAAACGGATAGGAAACAGGT 2.133</td>
<td>6.02</td>
</tr>
<tr>
<td></td>
<td>R: GGGCTCCAGCTCCCACCAA</td>
<td></td>
</tr>
<tr>
<td>GLUL</td>
<td>F: GGGGCAAATGCGGAGGTC 2.105</td>
<td>1.95</td>
</tr>
<tr>
<td></td>
<td>R: GCAGCCGCGACCATTCGAGT</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>F: TCCGGGCTCTCACAACCCAGTG 1.885</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>R: GTGTCCTACACCCCGGCTTG</td>
<td></td>
</tr>
<tr>
<td>TLR1</td>
<td>F: ACAGACGCCAGCAGGACAAGA 1.798</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>R: GTGCCCAATGACGCTCGGT</td>
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</tr>
<tr>
<td>CCR1</td>
<td>F: TGCTGTCGCCCGCCTCCACT 1.681</td>
<td>3.78</td>
</tr>
<tr>
<td></td>
<td>R: GAGGCAAGCCAGCAGCCCAAG</td>
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</tr>
<tr>
<td>ANGPTL4</td>
<td>F: ACCTGGCGGGGCCGCTATTA 1.607</td>
<td>3.18</td>
</tr>
<tr>
<td></td>
<td>R: CTGGCAAGGGAGGAGGGG</td>
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<tr>
<td>EDN1</td>
<td>F: CGGCTGCGAGGATTCAGGGA 1.591</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>R: GGCGGAGGTGCAAGAGGTG</td>
<td></td>
</tr>
<tr>
<td>CD180</td>
<td>F: CTCGGAGCACACACCCACCT 1.584</td>
<td>1.93</td>
</tr>
<tr>
<td></td>
<td>R: ATGCCCAAGCAAGCAGGCCC</td>
<td></td>
</tr>
<tr>
<td>SPDYA</td>
<td>F: GACCCGGATGTGGAGGGAGG 1.582</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>R: GTCCGGGGGGGATTTGACCT</td>
<td></td>
</tr>
<tr>
<td>FGL2</td>
<td>F: TGGCTGAGCTGCCTGGCTCT 1.576</td>
<td>1.97</td>
</tr>
<tr>
<td></td>
<td>R: ACCTGGTAGGGACACCCGCGG</td>
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<tr>
<td>PDXK</td>
<td>F: ACCACAGGGCTAGCGGCA 1.507</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>R: TGCTGTCCTTACGCTGGGCA</td>
<td></td>
</tr>
<tr>
<td>Mucosal vascular addressin cell adhesion molecule</td>
<td>F: TGGCCTCTCCTGCTTTCCCTC 0.572</td>
<td>1.34</td>
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<tr>
<td>Oestrogen receptor 1</td>
<td>R: CATCTGCCAGGAGGGCACTTG</td>
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<tr>
<td>Interleukin-2 receptor, alpha</td>
<td>F: TGAAGGCGCCCGCAGCTCAAG</td>
<td>0.552</td>
</tr>
<tr>
<td>PRP3 pre-mRNA processing factor 3 homologue</td>
<td>F: CATGGCACTCTGCCCTCCCGA 0.550</td>
<td>1.42</td>
</tr>
<tr>
<td>GZMH</td>
<td>R: CCTGGGCTGCTTGCAAGGGG 0.365</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>F: CCAAGGAGCAGTGGAAGGGG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GCAGCCGCTGTAATAGGCGG</td>
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</table>

receptor 1 (CCR1), angiopoietin-like 4 (ANGPTL4), endothelin 1 (EDN1), CD180, speedy homologue A (Drosophila) (SPDYA), fibrinogen-like 2 (FGL2), pyridoxal (pyridoxine, vitamin B6) kinase (PDXK) and granzyme H (GZMH). Several genes involving the innate immune defences (TLR1 and TLR4), immunosuppression (FGL2 and GPNMB), pro-inflammatory signalling (galectin-3) and weight loss (ANGPTL 4) were upregulated.

**DISCUSSION**

Lymph nodes were selected for microarray analysis because it is well known that PCV2 replicates in the lymph nodes during natural and experimental infection (Allan & Ellis, 2000; Kim et al., 2003), and causes the hallmark lymphoid lesions (Chae, 2004; Fenaux et al., 2002). It would be interesting to know which genes show changed expression levels in lymphocyte-depleted lymph nodes caused by PCV2.

By cDNA differential display PCR, several porcine genes were found to be upregulated in lymph node tissue. The transcriptional profile of mesenteric lymph node and lung in subclinically infected pigs was demonstrated by Fernandes et al. (2009). This exploratory study showed that most upregulated genes were related to the immune response genes, such as cytokines and MHC-binding molecules, in the PCV2-infected group.
Table 1 shows many of the DEGs found in lymph nodes with lymphoid depletion from PCV2-infected pigs in this study. Based on the results of this study DEGs were categorized into four groups by function: (i) innate immune defences (TLR1, CD14 and CD180), (ii) immunosuppression (FGL2 and GPNMB), (iii) pro-inflammatory signals (galectin-3) and (iv) weight loss (ANGPTL4).

Toll-like receptors (TLRs) are essential sensors of microbial infection that are involved in the recognition of a variety of microbial products (Janeway & Medzhitov, 2002; Kawai & Akira, 2006; Takeda et al., 2003). Each TLR is associated with specific ligands and response patterns (Trinchieri & Sher, 2007). TLR7 recognizes guanosine- and uridine-rich ssRNA to detect ssRNA viruses, such as influenza virus and vesicular stomatitis virus (Lund et al., 2004). TLR9 senses dsDNA viruses, such as herpes simplex viruses 1 and 2 and murine cytomegalovirus (Krug et al., 2004a, b; Lund et al., 2003). Following PCV2 infection, differential gene expression of TLRs was also found: increased expression of TLR1, TLR4 cofactor CD14 and TLR4 homologue CD180. These data show that TLR1 and TLR4 are involved in PCV2 infection.

Fibrinogen-like protein 2 (FGL2), also known as fibroleukin prothrombinase, has been demonstrated to be involved in the pathogenesis of diseases including viral-induced fulminant hepatitis and Th1 cytokine-induced fetal loss syndrome, in which fibrin deposition is a prominent feature (Clark et al., 1998, 2001; Ding et al., 1997). The coagulatory activity of FGL2 was first described in a murine fulminant hepatitis model (Levy & Abecassis, 1989; Parr et al., 1995) and FGL2 prothrombinase was detected in activated reticuloendothelial cells (Ding et al., 1997, 1998; Fung et al., 1991). A marked increase in FGL2 mRNA expression in immunosuppressive CD8αα intraepithelial lymphocytes has been reported (Denning et al., 2007). Soluble FGL2 exerts immunosuppressive effects on T-cell proliferation and DC maturation (Chan et al., 2003). The immunosuppressive activity of FGL2 occurs through binding to specific receptors FcγRIIB and FcγRIII on antigen-presenting cells via a caspase-dependent apoptosis mechanism (Liu et al., 2008). Characteristic microscopic lesions of lymphocyte depletion suggest potential immunosuppression of affected animals (Segalés et al., 2005). In this study, lymphoid depletion was observed in the lymph nodes of PCV2-infected pigs. FGL2 has important implications for the pathogenesis of immunosuppression in PCV2 infection.

Galectins are members of a large, growing family of animal lectins that are highly conserved throughout animal evolution (Cooper & Barondes, 1999; Liu, 2000; Rabinovich, 1999). Galectin-3 has been proposed to be a powerful pro-inflammatory signal. It has been reported to activate NADPH oxidase (Karlsson et al., 1998), stimulate superoxide production by neutrophils (Yamaoka et al., 1995), potentiate lipopolysaccharide-induced interleukin-1 production and promote monocyte chemotaxis (Jeng et al., 1994; Sano et al., 2000). Studies of galectin-3-deficient knockout mice have provided significant support for a pro-inflammatory role for this lectin (Colnot et al., 1998; Hsu et al., 2000). After intraperitoneal challenge, galectin-3-deficient mice had fewer inflammatory cells (Hsu et al., 2000). In this study, galectin-3 was upregulated. Based on the hallmark histopathological lesions of infiltrating monocytic cells, galectin-3 has an important role in attracting inflammatory cells to PCV2-infected lymph nodes.

ANGPTL4, a recently discovered adipokine (Kersten et al., 2000; Kim et al., 1999), was originally identified as the target gene of peroxisome proliferator-activated receptors (PPAR) including both insulin-sensitizing PPARγ and lipid-lowering PPARα (Mandard et al., 2004; Yoon et al., 2000). A variety of hormonal factors produced by adipose tissue can greatly affect organ function, especially at the metabolic and immunological levels (Ahima & Flier, 2000). These so-called adipocytokines or adipokines are now known to affect diverse biological processes, ranging from energy intake, insulin sensitivity and hepatic glucose production, to reproductive and immunological function (Berg et al., 2001; Hotamisligil et al., 1993; Steppan et al., 2001; Zhang et al., 1994). A relatively poorly characterized adipocytokine that may be involved in regulation of plasma lipid metabolism is the fasting-induced adipose factor (FIAF), also known as PPAR angiopoietin-related protein, angiopoietin-like protein 4, or hepatic fibrinogen/angiopoietin-related protein (Kersten et al., 2000; Kim et al., 2000; Yoon et al., 2000; Yoshida et al., 2002). Transgenic mice with ANGPTL4 overexpression have an approximately 50% reduction in adipose tissue weight, partly by stimulating fatty-acid oxidation and uncoupling in fat (Mandard et al., 2006). PCV2-infected SPF pigs showed no clinical signs or weight loss in this study. However, considering that PMWS is clinically characterized by a progressive loss of weight in pigs, ANGPTL4 should be confirmed in PMWS-affected pigs in further studies.

In conclusion, the present study has described a complete transcriptional response to lymphoid depletion observed in the lymph nodes of pigs infected with PCV2. Microarray analysis of PCV2-infected lymph nodes showed that expression of 160 genes was altered. This was confirmed for 18 genes by real-time RT-PCR. This work has established a comprehensive differential transcriptional profile of PCV2-infected lymph nodes. Further functional study of the altered expression of genes such as ANGPTL4, FGL2 and galectin-3 may facilitate understanding the pathogenesis and pathways of host cells to PCV2 infection.

**METHODS**

**Experimental infection of pigs.** Eight SPF swine of 4 weeks of age were randomly assigned into two rooms of four animals each. Prior to inoculation, the SPF pigs were negative for antibodies and antigens to PCV, porcine reproductive and respiratory syndrome virus, porcine parvovirus and swine influenza. Pigs in group 1 were uninoculated and served as a negative control. Pigs in group 2 were inoculated with 2 ml of...
Korean isolate PCV2a, PCK01-2 (10^5 TCID_{50} ml^{-1}) virus (1 ml intranasally and 1 ml intramuscularly). Based on the observations of lymphoid lesions in pigs experimentally infected with PCV2, the pigs were euthanized 21 days post-infection. The lymph nodes (superficial inguinal, mesenteric and mediastinal) were collected for microarray analysis. Three pigs in the PCV2-infected group were chosen on the basis of the presence of lymphoid depletion in lymph nodes. The study protocol was approved by the NVRQS Animal Care Committee.

**RNA isolation and gene expression profiling.** In the present study, we performed global gene expression analyses using GeneChip Porcine Genome arrays (Affymetrix). The sample preparation was performed according to the instructions and recommendations provided by the manufacturer. Total RNA was isolated from lymph node pools (superficial inguinal, mesenteric and mediastinal) of each of three pigs using RNeasy Mini kit columns (Qiagen) as described by the manufacturer. RNA quality was assessed using an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Chip (Agilent Technologies), and the quantity determined using an ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA (6 µg from each sample) was converted to double-stranded cDNA using oligo(dT) primers incorporating a T7 promoter. Amplified RNA (cRNA) was generated from the double-stranded cDNA template though an *in vitro* transcription reaction and purified with the Affymetrix sample cleanup module as recommended by the manufacturer’s protocol (Sample cleanup module; Affymetrix, http://www.affymetrix.com). The amplified RNA was fragmented using 8 µl of 5× fragmentation buffer. Fragmentation was checked by gel electrophoresis (1% agarose gel stained with ethidium bromide) and hybridized to the GeneChip containing >22,500 probe sets, as described in the GeneChip Expression Analysis Technical Manual (Affymetrix; www.affymetrix.com/support/technical/manuals.auxx). After hybridization, the chips were stained and washed in a GeneChip Fluidics Station 450 (Affymetrix) and scanned using a GeneChip Array Scanner 3000 7G (Affymetrix).

**Data analysis.** Image data were extracted for the scanned Porcine Genome arrays using AFFYMETRIX COMMAND CONSOLE 1.1. The raw .cel files generated by the above procedure recorded expression intensity data and were used for the next step. For the normalization, AFFYMETRIX EXPRESSION CONSOLE 1.1 was used to implement an algorithm from Affymetrix Microarray Suite (mas5) (Pepper et al., 2007). To reduce noise for the significance analysis, probe sets were filtered by the MAS5 detection call. To determine whether genes were differentially expressed between the two groups, an unpaired *t*-test was performed on the MAS5 expression values and the genes with values of *P*<0.05 were extracted. Highly expressed genes that showed ≥ 1.5-fold differences comparing the signal value of the control and PCV2-infected groups were selected for the further study. The web-based tool DAVID (the Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2009) was used to perform the biological interpretation of the differentially expressed genes. These genes were then classified based on the gene function information in gene ontology, PANTHER ontology database (http://david.abcc.ncifcrf.gov/home.jsp).

**Quantitative real-time RT-PCR for confirmation.** Microarray results were validated by qPCR of some of the genes showing altered expression by using SYBR green-based detection (Invitrogen) with a LightCycler 480 (Roche). All primers were synthesized based on the sequences of the corresponding porcine mRNAs in GenBank (Table 1). qPCRs were carried out in triplicate to guarantee the reproducibility of amplification of the cDNA sample from each animal. Changes in gene expression revealed by qPCR were calculated by the 2^(-ΔΔCT) method (Livak & Schmittgen, 2001). The normalization of the expression level of our genes of interest was done by dividing their relative expression level by the relative expression level of glyceraldehyde-3-phosphate dehydrogenase, a housekeeping gene.

**ACKNOWLEDGEMENTS**

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