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Porcine circovirus 2 infection induces IFNβ expression through increased expression of genes involved in RIG-I and IRF7 signaling pathways

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ABSTRACT

Porcine circovirus-associated disease (PCVAD), caused by porcine circovirus 2 (PCV2), is characterized by a highly variable pathogenesis that is manifested by various disease syndromes and includes immune evasion. Hence, even though PCVAD is effectively controlled by vaccination, pigs and farms remain infected so that continued vaccination is necessary to control disease. We investigated the molecular interactions of PCV2 and its permissive VR1BL host cell for gene expression signatures that could provide insight into mechanisms leading towards disease. Molecular pathways involved in the innate immune response to PCV2 infection were examined to identify changes in gene expression associated with productive infection of VR1BL cells. RNA profiling from infected and uninfected cells showed that 139 genes were induced by infection and 43 genes were down-regulated, using a p value < 0.05 and an absolute fold-change difference > 2. A strong type 1 interferon response, including an increase in genes involved in the RIG-I/MDA5 pathway and downstream interferon induced genes, was observed. Key regulators involved in PCV2 infection were identified as IFNβ, DDX58 (RIG-I), and IRF7. PCV2 infection induces a strong interferon response which unexpectedly facilitates viral gene expression, perhaps due to the presence of an interferon-sensitive response element in the viral promoter. The findings suggest that PCV2 interventions that attenuate type 1 interferon responses at the cellular level might enhance immunity and eliminate persistent infection.

1. Introduction

Porcine circovirus associated disease (PCVAD) is caused by porcine circovirus 2 (PCV2), a small circular single-stranded DNA virus belonging to the family *Circoviridae,* genus *Circovirus* (Allan and Ellis, 2000). The pathogenesis of PCV2 infection is highly variable, as observed by the various disease syndromes associated with PCV2 infection. Clinical signs of disease depend upon the syndrome and include lymphocyte depletion, high serum levels of PCV2 DNA, and antigen-positive cells (Allan and Ellis, 2000; Chae, 2004; Opriessnig et al., 2007). Vaccination of pigs across the United States beginning in 2006 effectively controlled PCVAD and reduced the level of virus in serum, but did not eliminate infection. Since PCV2 infection in the absence of disease is widespread (Dvorak et al., 2016; Fort et al., 2008; Opriessnig et al., 2010, 2008b; Patterson et al., 2011; Puvanendiran et al., 2011), a better understanding of the PCV2-host cell interaction is needed to explain maintenance of infection in immune pigs.

PCV2 disease is characterized by lymphocyte depletion and infiltration by histiocytes suggestive of the induction of an inflammatory response (Darwich and Mateu, 2012; Kekarainen et al., 2010; Rosell et al., 1999; Segales et al., 2005). Virus has been observed in macrophage and monocyte lineage cells (especially alveolar macrophage and dendritic cells) as a persistent infection, but efficient viral replication does not occur, suggesting they are not the primary targets for infection (Darwich et al., 2004; Gilpin et al., 2003; Vincent et al., 2003). However, persistent PCV2 infection in these cells does induce an altered cytokine response to immune stimulation, an effect on antigen presentation, and ultimately suppresses the innate immune defense functions, thus increasing susceptibility to disease (Darwich et al., 2003a, b; Kekarainen et al., 2008a, b; Núñez et al., 2003; Vincent et al., 2007). Given the knowledge that immune stimulation (such as the presence of a secondary agent) is necessary for PCVAD to occur in vivo, it follows that this altered innate immune function observed in persistently infected cells is one of the steps involved in induction of PCVAD (Ellis et al., 1999; Krakowka et al., 2001, 2000).

Molecular and cellular studies of PCV2 in cell culture are challenging due to the inability to clearly identify permissive cell types that maintain infection in pigs, and low infection and viral replication rates

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Table 1
Primer sequences used for RT-qPCR to confirm RNA-seq gene expression.

Name	Gene	Primer Sequence 5'-3' (F and R)	RNA-seq P-value	RNA-seq fold change (mean RPKM)	RT-qPCR fold change (avg Ct)
Myxovirus (influenza virus) resistance 2	MX2	CAGAGGCAGCGGAATCAT AGCTCCACTTTGCGGTAGC	0.02	(14.87)**	(28.0)**
Galectin 9	LGALS9	CATTGCCTTCCACTTCAACC TCACTGTCACCTGGAACTGC	0.00	(16.78)**	(26.8)**
DExD/H-box helicase 60	DDX60	TGAAGGTGAAACCACAAAGG CTTGCCAACTCCTCAAATCC	0.01	(8.09)**	(29.6)**
Interferon beta 1	IFNB1	TCTCCACCACAGCTCTTTCC CTTCTGACATGCCAAATTGC	0.01	(2.67)**	(29.8)**
Interferon induced protein with tetratricopeptide repeats 3	IFIT3	TGAGAAGGAACTGGGCTACC TGGTCCATTTCCTCAGTGC	0.00	19.54	100.63
C-C motif chemokine ligand 20	CCL20	TATCATGGGCTTCACACAGC CTGTGGATCTGCACACAGG	0.01	14.46	20.97
Poly(ADP-ribose) polymerase family member 12	PARP12	CAGACACCGGCTTTAAGAGG CGTGCAGGTAAAGAGGTTCC	0.00	7.67	17.28
DExH-box helicase 58	DHX58 (LGP2)	TCATGTCACCCCAGAACTCC GGTGGATTTTGTCCATGAGC	0.00	5.23	7.27
Interferon induced with helicase C domain 1	IFIH1 (MDA5)	TGCACAAAGGCTTAGATTTGC AAGATCGGGAAATGTGATGG	0.00	4.87	7.23
Eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	GACAAGGTGCCTTTTCATCC TGTTTCTTTGGCTGTCAATCC	0.02	3.21	2.80
Endothelial cell specific molecule 1	ESM1	GGAGGATGATTTTGGTGACG ACTGCACAGCTGCAAGTCTGC	0.04	3.17	3.17
DNA fragmentation factor subunit beta	DFFB	AGAGAAGAAGCGCACAGTGG CAGCTTGAGGTTGTCCAAGG	0.76	1.10	− 1.05
Ribosomal protein S16	RPS16	GGAACCTGTTCTGCTTTTGG GTGACCACGACCCTTCACC	0.25	− 1.61	− 1.01
Spleen associated tyrosine kinase	SYK	TATTTGCAGGATCGGTTTCC CCCTATGGTGATTCCAGTGC	0.03	− 1.75	− 1.28
Peptidylprolyl isomerase A (Cyclophilin A)	PPIA	CCGTCGATGGCGAGCCC CCCGTATGCTTCAGGATAAAA	0.07	1.04	− 1.01*

* PPIA qPCR fold change is a ratio of infected/uninfected, without normalization.
** Fold-change could not be calculated since uninfected samples were negative by RNA-seq (mean RPKM < 0.3) and by RT-qPCR (mean Ct > 34). Thus, mean RPKM or Avg Ct value is shown for the infected sample.

in commonly used epithelial cell culture systems (Dvorak et al., 2013b; Gilpin et al., 2003). By contrast, a mesenchymal-like cell line, VR1BL, supports efficient infection and replication at high multiplicities of infection (MOI) (Dvorak et al., 2013b). Interestingly, high levels of infection in VR1BL cells result in a cytopathic effect mimicking PCVAD, whereas low MOI infection produces a persistent, noncytopathic infection similar to persistent, nonclinical infections in swine farms (Dvorak et al., 2013b). Infection of VR1BL cells induces viral replication in the majority of the cells, decreasing the background noise of uninfected cells and allowing for more sensitive detection of changes in gene expression. Thus, PCV2 infection of VR1BL cells can be used as a model system to examine molecular mechanisms of host-pathogen interactions leading towards PCVAD.

In this study, we examined the gene expression signature involved in the innate cellular defense following PCV2 infection of VR1BL cells as a model for cellular responses leading to PCVAD. We identified a strong type 1 interferon (IFN) response mainly using the RIG-I/MDA5 antiviral response pathway with the key regulators identified as IFNβ and IRF7.

2. Materials and methods

2.1. Cells and viral infection

VR1BL cells were infected with PCV2b (GenBank ID JF290418) at an MOI of 1 for 4 days as previously described, which leads to approximately 70% infection (Dvorak et al., 2013b). Control cells (mock infection) were treated the same, but without virus. Three individual infections and control treatments were performed and were used as independent replicates for RNA-sequencing (RNA-seq).

2.2. Transcriptome sequencing and analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) following manufacturer's instructions. Genomic DNA was removed using two treatments of the TURBO DNA-free Kit (Thermo Fischer Scientific, Waltham, MA) and confirmed by PCR. RNA was quantified using a NanoDrop spectrophotometer (Thermo Fischer Scientific, Waltham, MA), and analyzed for RNA integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). High quality RNA (RNA integrity number (RIN) > 9) was sent to the University of Minnesota Genomics Center for library preparation and RNA-seq. Briefly, libraries were prepared by isolating and purifying mRNA using the mRNA-Seq sample preparation kit (Illumina, Inc., San Diego, CA) and fragmented to approximately 200 bp fragments. First and second strand cDNA was synthesized, followed by end repair adapter ligation, purification, and then multiplexed. A total of 6 independent libraries were subjected to high throughput sequencing in a single lane of a flow cell using an Illumina Genome Analyzer GAIIx (Illumina, Inc., San Diego, CA). Illumina fastq data files were analyzed using CLC genomics workbench version 6.0.5 (QIAGEN, Redwood City, CA) using the reference database Ensembl Sscrofa 10.2 (Ensembl release 89, www.ensembl.org/). In addition, unmapped reads were screened against the RefSeq viral genome database and Mycoplasma spp (*M. fermentans, M. hyorhinis, M. hyopneumoniae, M. bovis, M. agalactiae* and *Acholeplama laidlawii*) (O'Leary et al., 2016). Fold-change values were calculated as a ratio of average RPKMs of infected over uninfected and ratios below 1 transformed by calculating -1/fold change. Differentially expressed genes were further analyzed and figures were designed using Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, CA) to identify affected pathways (Krämer et al., 2014). The RNA-seq data sets are available in the Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE112156 (Edgar et al., 2002).