RESEARCHREPORT
SWINE HEALTH

Title: Gene expression by PRRSV-infected macrophages – **NPB #98-056**

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I. ABSTRACT:

The detailed mechanism(s) by which porcine reproductive and respiratory syndrome virus (PRRSV) impairs alveolar Mø homeostasis and function remains to be elucidated. We used differential display reverse-transcription PCR (DDRT-PCR) to identify molecular genetic changes within PRRSV-infected Mø over a 24 h post infection period. From over 4,000 DDRT-PCR amplicons examined, 19 porcine-derived DDRT-PCR products induced by PRRSV were identified and cloned. Northern blot analysis confirmed that four gene transcripts were induced during PRRSV infection. PRRSV attachment and penetration alone did not induce these gene transcripts. DNA sequence revealed that one PRRSV-induced expression sequence tag (EST) encoded porcine *Mx*1, while the remaining 3 clones represented novel ESTs. A full-length cDNA clone for EST G3V16 was obtained from a porcine blood cDNA library. Sequence data suggests that it encodes an ubiquitin-specific protease (UBP) that regulates protein trafficking and degradation. In pigs infected *in vivo*, upregulated transcript levels were observed for *Mx*1 and *Ubp* in lung and tonsils, and for *Mx*1 in tracheobronchial lymph node (TBLN). These tissues correspond to sites for PRRSV persistence, suggesting that the *Mx*1 and *Ubp* genes may play important roles in clinical disease during PRRSV infection.

II. INTRODUCTION:

Porcine reproductive and respiratory syndrome (PRRS) is prevalent in Europe, North America, and Asia, and leads to significant economic losses in swine industry. PRRS virus (PRRSV), the causative agent, was identified in 1991 in the Netherlands and in 1992 in the United States. PRRSV infection presents as reproductive failures through premature farrowing and/or interstitial pneumonia characterized by alveolar wall

thickening with macrophage (Mø) and necrotic cell debris. PRRSV is a small enveloped RNA virus of the family *Arteriviridae*, order *Nidovirales*, and contains an approximately 15 kb positive strand RNA genome. PRRSV structural proteins encoded from open reading frames (ORF) 2 to 7 were identified as glycoprotein $(GP)_2$ (29-30 kD), GP_3 (45-50 kD), GP_4 (31-35 kD), major envelop protein (E; 24-26 kD), a viral membrane protein (M; 18-19 kD), and a nucleocapsid (N; 15 kD). The E protein is strongly cytotoxic via induction of apoptosis *in vitro*. As yet, the functions for GP_2, GP_3, and GP_4 have not been elucidated.

Arteriviridae replicate primarily within Mø, and porcine alveolar Mø are a primary target cell for PRRSV replication *in vivo*. PRRSV replication in alveolar Mø is associated with cytopathic effects. PRRSV infection decreases alveolar Mø release of superoxide anion and the number of alveolar Mø in the lung. It is presumed that altered alveolar Mø function is linked to the apparent increased incidence of pulmonary bacterial co-infections in PRRSV-infected herds. The high incidence of respiratory microbial co-infection in chronically infected herds suggests that PRRSV interferes with host Mø activities used to clear respiratory pathogens. However, the molecular pathways by which PRRSV infection disrupts normal Mø homeostasis have not been elucidated.

Once an intracellular pathogen such as a virus invades a host cell, the interactions become physiological and biochemical as well as immunological. Pathogens that replicate within host cells usurp host biological processes for their own benefit. In response, the host cell manipulates gene expression in order to inhibit those pathways or processes required or induced by the pathogen. Viruses in particular subvert host cell metabolism in such a way that viral components can be synthesized via host cell pathways to initiate viral replication. Viral particles, viral components, and virus-induced cellular factors all have the potential to alter host cell gene expression. Molecular genetics and cell biology can be implemented to define the specific host cell molecules and cellular components with which virus-encoded molecules interact.

Differential display reverse transcription polymerase chain reaction (DDRT-PCR) is a powerful approach used to directly compare gene expression between cells or tissues at specific physiological states. DDRT-PCR provides an unbiased mRNA fingerprint for direct observation of cDNAs PCR amplified to different levels reflective of relative transcript levels within a total RNA sample. By using a reverse transcription/PCR primer that anchors on the nucleotide just 5′ of the poly(A) tail, DDRT-PCR specifically reverse transcribes only a subset of the mRNAs. PCR is then performed using the 3′-anchor primer and an arbitrary 5′ primer to amplify 100-400 fragments of the cDNAs generated by reverse transcription. Recently, DDRT-PCR has been used to describe host cell genetic responses to infection by pseudorabies virus, cytomagolovirus, HIV, herpes simplex virus and rhabdovirus. *We hypothesized that PRRSV infection alters alveolar Mø homeostatic gene expression, leading to compromised host defenses in the lungs of infected animals*. The effect of PRRSV infection on alveolar Mø gene expression was therefore observed by monitoring changes in gene expression using DDRT-PCR. We identified 19 porcine Mø-derived DDRT-PCR amplicons induced during a 24 h *in vitro* infection period. Many of these transcripts appear to encode previously unknown gene products. Further, we confirmed that four of these genes are induced during PRRSV infection, and are induced *in vivo* in tissues where

PRRSV persistently resides, suggesting that they may provide insight for understanding host cell molecular responses during PRRSV pathogenesis.

III. OBJECTIVES:

Macrophages in the lungs form the first line of defense against microbial respiratory pathogens, coordinating both cellular and humoral immune responses. In that PRRSV replicates within alveolar macrophages, this suggests that alteration of host macrophage defenses during PRRSV infection offers an advantage to respiratory bacterial pathogens which infected animals subsequently encounter. ***The overall objective of this research program is to understand the molecular basis by which PRRSV infection is associated with respiratory co-infections***. Hence, we have examined the relationship between PRRSV infection and alveolar macrophage immune function by characterizing those genes altered as a consequence of PRRSV infection. These genes provide insight into possible virulence mechanisms and potential targets for therapeutic interventions. Further we will place these immune response genes on the porcine genome map in order to permit future studies for linkage with disease resistance and selection of improved seed stock.

IV. PROCEDURES:

Cells, viruses, and pigs. Six- to eight-week old pigs were selected from healthy and PRRSV-negative pig populations. Alveolar Mø were collected by lung lavage. Lungs were washed 2-4 times with phosphate-buffered saline (PBS, pH 7.2). Each wash was centrifuged at 1200 rpm at 4°C for 10 min. Cell pellets were mixed, washed again in PBS, and then resuspended in 20-50 ml of RPMI-1640. Mø were incubated overnight at 37°C, 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 0.1 mM nonessential amino acids, 25 mM HEPES, and antibiotics.

ATCC PRRSV strain VR2332 (passage 9, 5 x 10^6 PFU/ml) and CL2621 cell culture supernatant were obtained from Dr. K. S. Faaberg (University of Minnesota). PRRSV suspension (m.o.i. = 0.1) or medium was inoculated after washing Mø monolayer. For UV inactivation, PRRSV stock placed in a 10 cm-diameter petri dish was irradiated using an UV-Crosslinker with 120 μJ/cm^2 for 15 min. Pseudorabies virus (PRV) strain 086 was used to infect porcine alveolar Mø (m.o.i. = 0.1) *in vitro*.

For *in vivo* infection, six-week-old pigs obtained from a PRRSV seronegative farm were infected intranasally with 10^5 $TCID_{50}$ of PRRSV strain VR2332 or PBS as a control. Serum samples were collected at day 0, 2, 5, 7, 10, and 14 post-infection, and stored at -80°C (data not shown). Tissues were collected at 14 days post-infection and immediately placed into TRIzol (Life Technologies, Grand Island, NY) reagent and frozen in dry ice/ethanol. All tissues were stored at -80°C until used.

Total cellular RNA isolation and Northern blot analysis. Total cellular RNA was extracted from alveolar Mø cultures and tissues using TRIzol Reagent according to the manufacturer's protocol. RNA integrity was evaluated on 1% agarose gels with formaldehyde (0.4 M) after staining with ethidium bromide. For DDRT-PCR analyses, trace genomic DNA contamination was removed with MessageClean (GenHunter Corp., Nashville, TN) before performing reverse transcription. For Northern blots, total

cellular RNAs (10 μg per lane) were fractionated on 1% agarose-0.4 M formaldehyde gels, transferred to nylon membranes, and cross-linked using a UV-Crosslinker. The cDNA probe was labeled by random primer labeling (Life Technologies) following the manufacturer's protocol. Hybridization was carried out at 42°C in 10 ml of solution containing 5 x SSPE, 50% formamide, 0.5% SDS, 5 x Denhardt's reagent, and 100 μg/ml sonicated salmon sperm DNA overnight. The hybridized membrane was washed twice with 2 x SSC/0.1% SDS for 15 min at room temperature, followed by 0.1 x SSC/0.1% SDS at 55°C for 20 min. Blots were exposed to film overnight at -80°C or quantitated by phosphorimagery (Molecular Dynamics, Sunnyvale, CA).

Differential display assays. First-strand cDNAs (20 μl) were synthesized for each RNA sample separately using one of three H-T_{11}M anchor primers (where M is G, A, or C, GenHunter Corp.), 0.2-0.4 μg total cellular RNA, 4 μl 5 x RT buffer, 20 μM dNTPs, and 200 U of Superscript II reverse transcriptase (Life Technologies) at 42°C for 1 h. PCR reactions (10 μl) were performed using the RNAimage Kit (GenHunter) and contained 1 x PCR buffer, 2 μM dNTPs, 0.2 μM 5′ H-AP primer/3′ H-T_{11}M anchored primer, 0.15 μl [α-^{33}P] dATP (2500 Ci/mM, Amersham), 1 μl reverse transcription product, and 1 U AmpliTaq DNA polymerase (Perkin Elmer). The PCR cycling profile was 94°C for 2 min, [94°C for 30 sec, 40°C for 2 min, 72°C for 30 sec] for 40 cycles, then 72°C for 5 min. Denatured DDRT-PCR products were loaded onto a 6% denaturing polyacrylamide DNA sequencing gel, and run for 3.5 h. The gel was blotted onto filter paper, dried under vacuum on a gel dryer at 80°C for 1 h, and then exposed to film at room temperature for 16-24 h. Amplicon intensities were compared visually for each infection time across duplicate samples, and differentially expressed amplicons were prepared. Briefly, bands were excised from acrylamide gels, placed in 100 μl of dH_2O for 10 min, and then boiled for 15 min. DDRT-PCR products were collected by centrifuging for 2 min and stored at -20˚C. Reamplified cDNAs were purified from the 2% agarose gel using the QIAEX II kit (Qiagen Corp., Chatsworth, CA), and stored at -20°C for cloning and hybridizing analysis.

DDRT-PCR amplicon cloning and sequencing. Reamplified DDRT-PCR products were ligated into the *Eco*RV site of pBluescript SKII (Stratagene) using a modified T-A cloning approach and recombinant clones were identified by colony PCR. Briefly, cleaved vector was purified by GENECLEAN (Bio 101, Inc. Vista, CA), and a 3′-thymidine residue was added with AmpliTaq DNA polymerase (Perkin Elmer) and dTTP at 72°C for 2 h. Reaction mixtures were treated by T4 DNA ligase at 15°C overnight, and non-ligated vector was purified from 1% of agarose gel. DDRT-PCR amplicons were ligated with T overhang vectors at 15˚C overnight, transfected into XL1-blue cells (Stratagene). Plasmid DNA from clones with insert was prepared by miniprep (Qiagen). DNA sequencing was performed on an Applied Biosystem 377 Automatic DNA sequencer (Perkin Elmer) in the Advanced Genetic Analysis Center, College of Veterinary Medicine, University of Minnesota. Sequences were analyzed by a BLAST search (NCBI, NIH). The accession numbers are: AF102503 for clone A5V12, AF102504 for clone G3V16, AF102505 for clone G2V12, AF102506 for clone G12V24 and AF134195 for porcine *Ubp*.

Reverse transcription PCR assay. Reverse transcription (20 µl) was performed as above described using total cellular RNA (2 µg). The reaction was stopped by heating to 70°C for 10 min, and RT products were treated with RNase H for 20 min at 37°C. PCR reactions (25 µl) were performed with RT product (1 µl), 10 x PCR buffer, 25 µM dNTPs, 0.2 µM each of 5′ primer and 3′ primers, and 1 U of AmpliTaq DNA polymerase. The primer pairs used were: *Mx*1: 5′ primer GCTTGAGTGCTGTGGTTG/3′ primer GGACTTGGCAGTTCTGTGGAG; *Ubp*: 5′ primer AGGGGCCAAGCTCATGTGAC/3′ primer GTGGCCAGCATACCATCTCC. Primer sequences and PCR profile for porcine hypoxanthine phosphoribosyltransferase (HPRT) have been described. Each cDNA was amplified for 14 cycles and 17 cycles. Amplicons were analyzed by Southern blot hybridization against DDRT-PCR probes. Signals were quantified by phosphorimagery.

Isolation of cDNA clones. A pig cDNA library (kindly provided by Dr. C. W. Beattie, University of Minnesota) prepared from peripheral blood cells and cloned in Uni-ZAP XR Vector (Stratagene) was screened with DDRT-PCR clone G3V16. The probe was labeled with [α–^{32}P] dATP by the random priming (Life Technologies), and hybridization was performed as described for Northern blots. A total of 1 x 10^6 phage plaques were screened using G3V16 cDNA, and a single positive clone was identified. By sequence analysis, the clone identified by G3V16 probe was found to contain a full-length coding sequence.

V. RESULTS:

PRRSV replication and altered gene expression in alveolar Mø. Alveolar Mø were infected using PRRSV strain VR2332 (m.o.i. = 0.1) *in vitro*. CPE was not observed until 16 h post infection and was less than 10% at 24 h post infection. At 72 h post infection, CPE was more than 70%. In order to identify differentially expressed mRNAs during PRRSV infection, we collected total cellular RNA from mock- and PRRSV-infected porcine alveolar Mø at 4, 12, 16, and 24 h post infection. PRRSV genome replication was confirmed via RT-PCR detection of accumulation for ORF 7 sequences of PRRSV genomic RNA. PRRSV ORF 7 transcript levels increased with time, demonstrating active viral genomic replication in alveolar Mø.

Mø mRNAs differentially expressed during PRRSV infection were detected by DDRT-PCR comparison against mock-infected alveolar Mø at various times post infection. For each DDRT-PCR primer pair, duplicate RNA samples from each time point were reverse-transcribed, PCR amplified, and fractionated on adjacent lanes to ensure DDRT-PCR accuracy and reproducibility. Using 16 of the possible upstream various septamer H-AP primers (GenHunter), over 4000 DDRT-PCR products were visually compared for band intensity. Twenty DDRT-PCR products that were reproducibly induced (> 2-fold difference compared to mock-infected cultures) in both DDRT-PCR reactions for a given RNA sample during a 24 h PRRSV infection *in vitro*. All differentially expressed DDRT-PCR products were extracted from acrylamide gels, reamplified, and cloned into pBluescript SKII. DDRT-PCR amplicons that showed altered levels in only one of two identical samples were not considered further.

Amplicon sequence analysis. To determine whether DDRT-PCR clones were derived from porcine cellular genes or from the PRRSV genome, all 20 PRRSV-induced

DDRT-PCR amplicons were sequenced. One DDRT-PCR clone encoded a portion of the PRRSV ORF2 (data not shown) and was removed from further study. Only 5 of the remaining 19 PRRSV-induced transcripts matched previous Genbank submissions. Clone G12V24 represented the porcine *Mx*1 cDNA as determined by 99% nucleotide sequence similarity. *Mx*1 is a previously described interferon-inducible protein with allelic association to viral resistance/susceptibility phenotypes in mice.

Confirmation of DDRT-PCR results. All 19 PRRSV-induced porcine Mø DDRT-PCR clones were screened by Northern blot analysis against total cellular RNA from mock- and PRRSV-infected porcine alveolar Mø. Three clones gave no signal on Northern blot analysis, suggesting that they were either cloning artifacts or represented sequences expressed at levels too low to detect by this technique. An additional 12 PRRSV-induced DDRT-PCR clones showed less than 2-fold induced expression on Northern blot and their expression was not further investigated. The four remaining DDRT-PCR clones were confirmed by Northern blot analysis to be induced by PRRSV infection. All 4 transcripts were induced by PRRSV infection, exhibited distinct expression levels, and were of different sizes.

Temporal accumulation of the molecular markers identified by DDRT-PCR during PRRSV infection was determined. Total cellular RNA was isolated from alveolar Mø from 0 to 36 h after treatment with medium alone, conditioned medium from CL2621 cells used to generate infectious PRRSV preparations, UV-irradiated PRRSV, and PRRSV. Transcripts detected by DDRT-PCR clones A5V12, G3V16, G2V12, and G12V24 increased concomitant with PRRSV replication. Transcripts detected by A5V12, G2V12, and G3V16 were not detected until 16 h post infection, whereas G12V24 transcripts were induced as early as 8 h post infection. Gene transcripts were not induced in medium control cultures or in Mø treated with CL2621 cell-conditioned medium. Importantly, Mø infected with UV-irradiated PRRSV, which can bind to and penetrate Mø but not replicate, did not express detectable levels for any of the transcripts examined. Together, these data indicate that active PRRSV genomic replication within Mø is required for induction of gene expression for these selected amplicons.

Common molecular responses of porcine alveolar Mø. To determine whether the DDRT-PCR clones identified and characterized from PRRSV-infected Mø were specific to this pathogen or instead result from a general viral response molecular program, transcript expression was determined for porcine alveolar Mø infected with pseudorabies virus (PRV) *in vitro*. As was observed for PRRSV, all 4 transcripts were induced by PRV infection (Fig. 4).

Identification of a full-length cDNA clone. To further characterize PRRSV-induced porcine Mø genes, a porcine peripheral blood cell cDNA library was screened using clone G3V16 as a probe. A phage clone was isolated via plaque lift hybridization and contained an insert (1.7 kb) of approximately the same size as the mRNA, suggesting that it contained a full-length cDNA. DNA sequence determination (Accession number AF134195) of the isolated cDNA clone confirmed that it contained a full-length coding sequence, which included a 3′ untranslated region (UTR, 572bp), coding sequence (966 bp), and a 5′ UTR (172bp). Deduced amino acid sequence

identified a conserved Cys domain (block entry, BL00972A) and a His domain (block entry, BL00972D), which are thought to act as active sites for ubiquitin-specific proteases (UBPs). Further, a Genbank data search and analyses by Blast (NCBI, NIH) and FEX (Find exon program, Sanger Center, UK) indicated that a putative human homologue is located at chromosome 22q11.2. The putative human UBP has eight ORFs derived from approximately15 kb of genomic DNA. The amino acid similarity and identity of porcine UBP with the putative human UBP were 81% and 75%, respectively. These results indicate that porcine UBP is a novel member of an UBP superfamily, and suggest that intracellular protein trafficking, turnover or degradation may be altered during PRRSV infection of porcine alveolar Mø. The identity for PRRSV-induced EST G2V12 was recently established to be an RNA helicase gene (RHIV-1) that regulates RNA secondary structure.

Tissue specific expression in *in vivo* PRRSV-infected animals. In order to determine whether expression of DDRT-PCR products identified *in vitro* reflected events during PRRSV infection *in vivo*, we examined tissue-specific expression of DDRT-PCR amplicons in PRRSV-infected pigs. Whole tissues were collected at 14 days post infection from 2 PRRSV-infected pigs and 2 mock-infected pigs. Quantitative RT-PCR demonstrated the presence of PRRSV genomic RNA in lungs, lymph nodes, and tonsils. Tissue RNAs from identically treated animals were pooled to minimize animal-to-animal variation, and RT-PCR was performed for 14 and 17 cycles. PCR amplicon levels for each tissue sample were normalized to HPRT amplicon levels, and normalized values for each tissue were compared between mock- and PRRSV-infected animal. Porcine *Ubp* transcripts were greatly upregulated during PRRSV infection in the lungs (4.5-fold) and tonsils (11.4-fold), but were reduced 30% in TBLN from PRRSV-infected animals. In contrast, *Mx*1 transcripts were greatly induced in all three tissues (Fig. 7). Taken together, these data show (1) constitutive expression for these genes *in vivo*; (2) tissue-specific regulation of gene expression; and (3) PRRSV-induced upregulation of transcript levels in tissues where PRRSV is persistent.

Immediate or future benefit to pork producers. The host cell gene targets identified here provide the first description of PRRSV disruption of gene expression. Two targets in particular, UBP and RHIV-1, suggest that the virus uses host cell genes for its own benefit. Hence, we can propose experiments to test this hypothesis, and, if true, we can propose therapeutic interferences that disrupt viral replication. Further, it may be possible to identify porcine alleles for these genes that provide little or no benefit to the virus, thereby imbuing the animal with an increased resistance to PRRSV.