RESEARCH REPORT
SWINE HEALTH

**Title:** Global Gene Expression Profiling of PRRSV-infected Alveolar Macrophages - **NPB #06-122**

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## II. Industry Summary:

This study examined the effect of porcine reproductive and respiratory virus (PRRSV) on how genes are expressed in porcine alveolar macrophages (PAMs). PAMs were chosen for this study because they are the primary targets of infection by PRRSV. Serial analysis of gene expression (SAGE) was used here because it allowed us to look at most of the genes expressed in these cells. We determined the normal levels that genes are expressed in normal, non-infected PAMs and then compared this to the gene expression levels in PRRSV-infected PAMs at several time points after infection. It is well established that many pathogens cause changes in expression of specific genes that act to protect the host and clear the infection. This type of response was not seen in these cells. There was surprisingly little in the way of a protective response. Of particular interest was the minimal expression of genes that are involved in attracting other immune cells to the area of the infection. Additionally, there was no response by genes that cause inflammation. This is the first comprehensive study to show the actual breadth of inhibition of an immune response in PAMs by PRRSV. However, the results have also given us tantalizing clues to the mechanism(s) behind this inhibition. There are specific cellular proteins that control the expression of the protective genes and future studies will look at how the virus may be inhibiting their function. This may possibly lead to a means for a producer to intervene that may act to limit or end an active PRRSV infection by restoring the animal's natural protective mechanisms.

## III. Scientific Abstract:

Porcine reproductive and respiratory syndrome virus (PRRSV) is a major pathogen of swine worldwide and causes considerable economic loss. The primary target of infection is the porcine alveolar macrophage (PAM). Infection of PAMs by PRRSV causes significant changes in their function by mechanisms that are not understood. Serial Analysis of Gene Expression (SAGE) was used to examine the global expression of genes in PRRSV-infected PAM. Total cellular RNA was prepared from *in vitro* mock-infected and PRRSV strain VR-2332-infected PAMs at 0, 6, 12, 16 and 24 hours after infection. Each SAGE library was sequenced to obtain >95,000 tags per time point. The sequences were processed to account for sequencing error before generating

tag:count databases. Examination of the SAGE data indicated that there were changes in gene expression occurring in the PRRSV-infected PAM over time post-infection. More than 400 unique tags with significantly altered expression levels were identified ($p<0.01$ with Bonferroni correction). The validity and kinetics of expression of genes identified by SAGE were confirmed using real-time RT-PCR. The most striking finding was that the expression of most of the genes identified in this study that are involved in the innate immune response (including IL-8, CCL4, TNF-$\alpha$, and IL-1$\beta$, genes whose expression is known to be altered in response to other viral pathogens) showed little or no change or declined in transcript abundance following infection. Others showing little change or declined in transcript numbers included CCL3, macrophage inhibitory protein 3 (MIP3), and CXCL2. These data indicated that no innate immune response was initiated following infection. In addition, the enzyme arginase showed a significant, short-lived increase in transcript numbers at 6 hours PI. The increased expression of arginase indicated a possible inhibition of a pro-inflammatory response in the PAMs by inhibition of inducible nitric oxide synthase (iNOS) activity by competition for the common arginine substrate.

**IV. Introduction:**

The intracellular changes that occur following infection by a virus are, for the most part, poorly understood. It is known that viruses hijack the biosynthetic, metabolic and signaling machinery of the cell for their own ends. Viral proteins interact with specific cellular components to alter the function of these pathways and even alter gene expression in the host cell to bring about successful replication and production of progeny virus. The cell has a number of innate mechanisms for detecting the diversion of these functions and will initiate events to inhibit viral replication or to kill itself in an attempt to stop the infection. These events, and how effective they are, have a profound effect on the events that follow. These include the ability to respond to and end the infection at the cellular or organismal level and whether pathological changes occur that may, in severe cases, lead to death.

It is well established that PRRSV establishes an active infection in PAMs. PAMs are also the site of chronic, low level viral replication for a considerable period of time following the acute phase of the disease (Molitor et al., 1997). Replication of PRRSV in PAMs causes cytopathic effect (apoptosis), both in vitro and in vivo (Rossow, 1998). In addition, cell functions such as phagocytosis and superoxide anion production are inhibited (Thanawongnuwech, et al., 1997; Chiou, et al., 2000). Apoptosis reduces the number of PAMs in an infected lung, but this does not appear to explain totally the reduced ability to respond to the virus or to secondary pathogens. It is believed that PRRSV interference with PAM function is a contributing factor to the inability to clear respiratory pathogens (Thanawongnuwech, et al., 1997). Macrophages are important regulators of the immune response and dysfunction would have a profound effect on the clearance of the pathogen and outcome of the disease. Macrophages possess a number of important immunoregulatory functions that include phagocytosis, antigen presentation and production of cytokines and chemokines. Inhibition of these functions by PRRSV would have an immunosuppressive effect at the earliest level of infection (Lopez-Fuertes, et al., 2000).

A primary function of the PAM is to combat bacterial insults within the terminal airway. Reports have shown that pigs infected with PRRSV have a higher rate of concurrent or secondary bacterial infections (Van Reeth and Adair, 1997; Zimmerman et al., 2006). This has led investigators to examine the effect of PRRSV infection on bacterial killing by PAM (Chiou et al., 2000a; Solano et al., 1997; Thanawongnuwech et al., 1997, 1998). *In vitro* infection of PAMs by PRRSV reduced the bactericidal ability from 69.3% to 61.0% ($p<0.1$) at 24 hr post-infection (Thanawongnuwech et al., 1997). Furthermore, these studies reported that there were significant decreases in the production of superoxide anion and myeloperoxidase hydrogen peroxide halide product, both of which contribute to the ability of PAM to kill bacteria (Chiou et al., 2000a). Another report by (Oleksiewicz and Nielsen, 1999) demonstrated that PRRSV infection resulted in cytotoxicity to PAMs that led to a 40% reduction in the uptake of *Escherichia coli*. The specific mechanism(s) by which PRRSV infection causes altered PAM function is unknown.

One study attempted to better understand the altered gene expression of PAM upon infection by PRRSV by using differential display reverse-transcription PCR (DDRT-PCR) to identify host cell genes responding to PRRSV infection of PAM over a 24 hour period (Zhang et al., 1999). The result was the identification of four

genes that specifically responded to PRRSV infection and were also induced *in vivo* in tissues where PRRSV persistently resides. Of the four genes identified, three of these genes have been identified and the fourth remains a novel EST. The three genes identified are Mx1 (myxovirus resistance), UBP (ubiquitin protease), and RHIV-1 (RNA helicase). Presumably there are more, yet to be identified, genes that differentially respond to PRRSV infection. Recently it was reported that PRRSV infection results in lowered PAM-induced mRNA expression of the pro-inflammatory response cytokines TNF-a, IL-1a, and MIP-1b (Lopez Fuertes et al., 1999). However, conflicting reports have shown that TNF-a, IL-8, IFN-a, and IL-1b are not significantly altered by PRRSV infection (Ait-Ali et al., 2007; Buddaert et al., 1998; Choi et al., 2002; Thanawongnuwech et al., 2001; Zhang et al., 1999). Thus, it remains unclear which macrophage genes PRRSV affects upon infection.

The study described here is the first in depth, global analysis of changes in transcript abundance following PRRSV infection. The data obtained from this study allowed for a thorough examination of the effect of the virus on PAM function and has provided insights into how the virus inhibits cellular functions and prevents a strong, specific and sterilizing immune response.

## V. Objectives:

1) Construct and sequence SAGE libraries made from mRNA derived from noninfected and PRRSV-infected PAMs. Determine normal gene expression levels in noninfected PAMs and compare to infected PAMs.
2) Identify genes with altered expression levels in PRRSV-infected PAMs that interfere with the function of these cells and/or the formation of a protective immune response.
3) Identify gene expression changes that may allow viral persistence.
4) Identify gene products that may be exploited as early diagnostic markers or vaccination targets.

## VI. Material and Methods:

*Cells and Virus*

PAMs were harvested from three clinically healthy, PRRS-negative gilts 6-8 weeks of age. Animals were humanely euthanized, following animal care and use protocols, and PAMs were harvested under aseptic conditions. Viability of PAMs was determined by trypan blue dye exclusion. PAMs were tested by PCR for porcine circovirus and *Mycoplasma* spp (Opriessnig et al., 2003; Stakenborg et al., 2006) and found to be free of both. Aliquots of PAM were frozen and stored in liquid nitrogen. Typical yields were $10^8$-$10^9$ PAM with >95% viability. Immediately prior to use, PAMs were thawed and cultured at 37°C, 5% $CO_2$ in Dulbecco's Modified Eagles Media with 5% fetal bovine serum (FBS; Gibco-Invitrogen, Carlsbad, CA) and 1% antibiotic/antimyotic (Gibco-Invitrogen) for 2 hours.

PRRSV strain VR-2332 (Allende et al., 2000) stock was propagated in MARC-145 cells (Kim et al., 1993) and stored frozen at -80°C until use.

*Infection, RNA isolation and SAGE library construction*

PAMs isolated from three pigs were maintained separately. All three sets of PAMs were treated identically. After establishing the PAMs in culture, the cells were infected with PRRSV strain VR-2332. To achieve a near synchronous infection, flasks containing adherent PAMs were infected at a multiplicity of infection (MOI) of 10 in chilled media and incubated at 4°C for 1 hour to allow for virus binding, but not entry into the cell. After 1 hour, pre-warmed media was added and the cells placed at 37°C, 5% $CO_2$ until collected for RNA isolation. Total cellular RNA was prepared from each PRRSV-infected PAM flask at 0, 6, 12, 16 or 24 hours post-infection. Mock-infected PAMs were collected at 0 and 24 hours. Total cellular RNA was purified using the Qiagen RNeasy minikit according to the manufacturers instructions.

SAGE libraries were constructed as described previously (Velculescu et al., 1995) using *Nla* III as the anchoring enzyme. Each library was made from pooled equimolar amounts of total RNA from each pig at each time point. The SAGE libraries provided the population means of the gene expression levels for each time point. SAGE clones were amplified and sequenced using a high-throughput sequencing pipeline with an ABI 3730 automated sequencer and ABI chemistry (Applied Biosystems Inc., Foster City, CA). The SAGE libraries with tag counts were submitted to GenBank GEO and have the accession number GSE10346.

The database of tags derived from the raw sequence data was analyzed to identity the transcripts from which tags were derived as well as their relative abundance. Tag sequences were corrected for sequencing errors using R and sagenhaft (Beissbarth et al., 2004). The libraries were normalized to total tags. Relative abundance was calculated based upon the number of times a tag was represented in a given SAGE library (Madden et al., 2000). Tags were mapped to transcripts and genes by exact regular expression matching to sequences in GenBank, Harvard Gene Index, Pig Expression Database (Japan), and the USMARC EST databases. Multidimensional statistical tests: Audic and Claverie pairwise test; the Fisher's exact test; Greller and Tobin test; the R test and pairwise and general Chi-square tests (Romualdi et al., 2003) were applied to determine which changes in tag abundance were significant. SAGE libraries were compared with each other to identify common or differential patterns of expression. Attention was given to those transcripts where expression changes may affect PAM function; particularly in regard to innate immunity, antigen presentation, and intra- and extracellular signaling.

*Real-time RT-PCR validation*

Validation of the results and corroboration of the altered gene expression levels were analyzed by real-time reverse transcription-PCR (RT-PCR). Real-time RT-PCR was done in 25 µl reaction volumes using the SuperScript III Platinum SYBR green One Step qRT-PCR kit (Invitrogen) according to the suppliers specifications. The primer sets used for this analysis are shown in Table 1. All primers were used at 200 nM. PCR cycling conditions were 95°C for 15 minutes followed by 40 cycles of 94°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds using an Opticon 2 fluorescent thermocycler (BioRad, Inc., Hercules, CA). Final analysis of amplification products was done by melt curve where the PCR reactions were heated from 50 to 94°C at a rate of 0.5°C/second. For real-time PCR and SAGE tag count comparisons, β2-microglobulin (β2m) served as the internal control where the number of SAGE tags for β2m derived from this library and the amplification curve from real-time PCR were considered equal. Relative expression levels for the other genes were calculated by the method of Liu and Saint (2002).

**VII. Results:**

**Objective 1:** Construct and sequence SAGE libraries made from mRNA derived from noninfected and PRRSV-infected PAMs. Determine normal gene expression levels in noninfected PAMs and compare to infected PAMs.

Total cellular RNA was prepared from *in vitro* PRRSV-infected PAMs at 0, 6, 12, 16 and 24 hours after infection, and mock-infected PAMs at 0 and 24 hours. SAGE libraries were constructed from the 0 hour mock-infected and the 6, 12, 16 and 24 hours PRRSV-infected cells. The libraries were subsequently sequenced to approximately 100,000 tags each, with the exception of the 24 hour infected library which was sequenced to nearly 200,000 tags (Table 2). All told, the five SAGE libraries yielded 643,255 sequenced tags, which were used to generate a database representing genes expressed in mock and PRRSV-infected PAM. Examination of the SAGE data indicated that there were major changes in gene expression occurring in the PRRSV-infected PAMs based on more than 400 unique tags with significantly altered expression levels being identified ($p<0.01$ with Bonferroni correction). The derived catalog of expressed genes represents a first attempt to generate comprehensive analysis of the PRRSV-infected PAM expression profile. The wealth of information obtained allows detection of genes involved in normal porcine alveolar macrophage physiology, as well as genes whose expression is altered by PRRSV infection.

The validity and kinetics of expression of genes of interest were evaluated using real-time RT-PCR (Fig. 1A). Several housekeeping genes were assayed for inclusion in this analysis as internal controls. In this study, β2-microglobulin was found to have the most stable expression level across all the times tested (Figure 1A) and was used as the internal control in all real-time assays. Real-time PCR amplification of transcripts known to have altered expression levels following PRRSV infection was done to confirm that the infection had proceeded as expected (Figure 1A). The transcripts encoding the proteins Mx1 and rHIV (a RNA helicase) showed 6.4- and 4.8-fold increases at 24 hours PI, respectively. These transcripts were induced between 0 and 12 h post-

PRRSV-infection as previously described by Zhang et al. (1999) (Zhang et al., 1999). The pro-inflammatory proteins IL-1α and CCL4 (macrophage inflammatory protein, MIP-1β) declined in transcript abundance in accordance with the findings of Lopez-Fuertes et al. (2000). All four transcripts showed a high degree of correlation between real-time PCR and SAGE. The tags for the Mx1 transcript were identified in the 16 and 24 hour infected SAGE libraries but at low level and subsequently were found to be negligible following normalization of the SAGE data.

Analysis of the database revealed the presence of the tag derived from the RNAs produced by the infecting PRRSV strain. PRRSV produces 3' co-terminal subgenomic RNAs, thus all viral RNAs will have the same tag. The viral tag, not detected in the mock-infected library, was first present at 6 hours PI and increased in number to its highest point at 12 hours after which it declined (Table 1). These data were validated by real-time PCR (data not shown). The numbers given in Table 1 are tags sequenced/library and percent of the total tags. This number represents total viral RNA in the infected cells and not solely genomic RNA. There is a significant level of viral RNAs present in the infected cells at 12 hours post-infection, where viral RNAs accounted for almost 10% of all polyadenylated RNA in these cells. The amount of viral RNA declined at 24 h hours post-infection to a level approximately 25% of that at 12 hours. It is not known why this occurred, whether there is a decline in RNA replication/transcription, degradation of viral RNAs, release of virus from the cells or a combination of all three.

Additional transcripts validated by real-time PCR included several chemokines and cytokines (Figure 1B). The changes in levels of the transcripts encoding CCL3 (MIP1-α), AMCF1 (IL-8 precursor), and IL-1β were found to be very close to that indicated by SAGE with all declining as the infection progressed. Interestingly, the transcript encoding IL-6 does not contain a tag (no *Nla* III restriction site in the transcript), therefore, it was not detected by SAGE. Real-time PCR revealed a sharp decline in IL-6 transcripts during the infection.

**Objective 2:** Identify genes with altered expression levels in PRRSV-infected PAMs that interfere with the function of these cells and/or the formation of a protective immune response.

Additional transcripts of interest were validated with real-time PCR (Figure 1B). These consisted of chemokine and cytokine-encoding transcripts as well as representative transcripts showing significant differences from the non-infected control PAMs. In sum, a total of 13 tags derived from chemokine or cytokine transcripts were identified (Table 2). There was a general decline in numbers of the cytokine and chemokine transcripts, indicating that there was no induction of expression that would be expected in an innate immune response. SAGE showed greater variation in transcript numbers than did rt-PCR, particularly later in infection, for AMCF1, and IL-1α and IL-1β. Nine of the 13 transcripts declined during infection or showed no change. The remaining four transcripts, RANTES, MIF1, MCP3 and AMCF2, showed increases during the infection. MCP3 and AMCF2 showed increases only late in the infection process. SAGE also showed increases in transcript numbers at 24 hours that was not reflected by rt-PCR. The cause and meaning of the late differences were unclear, but is is apparent that the lack of response by these genes would have an impact on the immune response to the virus.

An interesting finding was the sharp increase in arginase transcript levels at 6 hours PI, with the rt-PCR and SAGE data mirroring each other almost exactly. In addition, a total of four different tags were found that corresponded to arginase transcripts with all showing the same increased level of expression at 6 hours PI (Table 2). The primer set used to validate the arginase transcript levels amplified sequences found within the coding sequences of the transcript, thus, the real-time PCR results were from amplification of sequences from all four transcripts. The four different tags corresponding to transcripts encoding the same protein were most likely a result of alternate 3' ends or intron splicing. The 6 hour PI time point examined here represents only a 'snap-shot' of the cells at that time. It is not known what the actual extent and highest level of the expression of arginase is in these cells beyond what was illustrated here. It is possible that the increased expression of arginase might have a negative impact on the expression and function of iNOS further inhibiting an inflammatory response.

Overall, the results obtained here indicated that there was no overt innate immune response nor was there an obvious inflammatory response taking place in the infected PAMs. The signaling normally observed in virus-infected cells, both intra- and extra-cellular, was not seen here.

**Objectives 3 and 4:** Identify gene expression changes that may allow viral persistence.
Identify gene products that may be exploited as early diagnostic markers or vaccination targets.

Nothing specifically relating to these 2 objectives has been characterized. There are a number of tags that have yet to be associated with specific transcripts and something related to these objectives may yet be found. This will be dependent on further work in porcine genomics, which at this time, is progressing. The absence of an innate immune response would allow the virus to become established in the PAMs. In addition, there is no sign of apoptosis being initiated these cells during the times studies here. One area of note that we have not investigated at this time is the apparent general decline in transcripts encoding MHC molecules. Both MHC class 1 and class II molecules appear to be down-regulated. This may apply to viral persistence if signaling by MHC is diminished or eliminated.

**VIII: Discussion:**

The ability of an animal to respond to specific foreign characteristics or patterns of pathogens is an important aspect of innate immunity. This is the first, generally rapid step in the response to the invasion of a pathogen and the beginning of a protective, sterilizing immune response. It is important that this response begin quickly and in strong enough fashion to stop the spread of the invader and terminate the infection. In many cases, the pathogen possesses the ability to inhibit or thwart the immediate innate immune response, thus giving it an early advantage. Deciphering the mechanisms of disruption of the innate immune response is the focus of considerable research and is beginning to provide answers into the myriad of different means pathogens employ to achieve this. This was also the focus here, to discern how PRRSV inhibits the innate immune response in infected PAMs. This study generated SAGE libraries that defined the transcriptional profiles of non-infected and PRRSV-infected PAMs that has provided insight into the extent of the negative effect by the virus on gene expression that is necessary for a strong immune response. This work has resulted in the characterization of macrophage transcript abundance in normal cells as well as gene expression changes that occurred with progressive PRRSV replication. The virus-specific gene expression changes that were found provided intriguing clues to possible mechanisms behind immune suppression and the lack of a strong innate and adaptive immune response.

Previous work by others has demonstrated that there are gene expression differences between noninfected and PRRSV-infected PAMs. In this study, greater than 400 significant changes in gene expression levels were identified. However, a most important aspect of this study was not in what was altered, but rather what was not. Of particular interest was the apparent lack of any overt innate immune response in the PRRSV-infected PAMs. This was borne out by the lack of increased transcription of chemokine and cytokine genes that are commonly observed increased in infections with other pathogens. These included CCL3, CCL4, TNF-$\alpha$, type 1 interferons, and a number of pro-inflammatory chemokines and cytokines.

In the initial stage of a PRRSV infection, the primary target cells of the virus are PAMs. It is as yet unknown what signals are necessary to call the immune system into action. The most likely candidates are cytokines and particularly those that initiate an immigration and activation of leukocytes. Sprenger et al. ( ) have shown that influenza A virus selectively induces mononuclear leukocyte-attracting chemokines and suppresses neutrophil-attracting chemokines. In this study, we have shown that PPRSV does not activate an alveolar macrophage pro-inflammatory response and at the same time suppresses IFN production and apoptotic pathways. No activation of the PAMs was indicated by an increase in IL-1$\alpha$, IL-6, or IL-8. Knoetig et al. (1999) has shown that IL-1 is released from CSFV-infected macrophages. IL-8 is, for example, an important chemo-attractant for immune cells, while IL-1 and IL-6 prime B- and T-cell responses against infected cells. The chemokines CCL3 (MIP-1$\alpha$), CCL4 (MIP-1$\beta$), RANTES, MCP1, MCP3, and MIP3 are important chemo-attractants and mediators of virus-induced inflammation *in vivo*. Most of these genes showed no or only late

increases in transcription during the course of this study. The immediate activation of the proinflammatory response seen at 6 h p.i with increased IL-8 and was transient and probably due to virus binding. This response was not maintained as the secondary increase after 24 h p.i. was seen in non-infected cells as well. Overall these increases were insignificant in terms of change of transcript abundance. Cytokine mRNAs have a short half-life after synthesis and the rapid reduction in mRNA levels a few hours after addition of virus suggests rapid intracellular cytokine mRNA degradation. Virus replication and protein synthesis has been shown to commence 10–15 h p.i. {Snijder, 1998}(although SAGE showed an increase in viral RNAs at 6 hours PI), suggesting that any increase in cytokine mRNA levels after 24 h p.i. was due to the presence of replicating virus. Similarly, we showed no activation of NF-kB, indicating a lack of transcriptional activation of these genes. The transcription factor NF-κB is a central regulator of the expression of these pro-inflammatory genes and many viruses manipulate the NF-κB pathway, resulting in suppression of antiviral responses or prevention of apoptosis (Hiscott 2001 J Clin Invest).

The finding of a sharp spike in transcription of the arginase gene at 6 hours pi may give a clue to the early events following infection that inhibit an innate immune response. Arginase competes with nitric oxide synthases (NOS) for the substrate arginine for the production of nitric oxide (NO). NO is an important early signal in many pathophysiologic processes. Early inhibition of its production would have an impact on downstream events. Increased expression of arginase has been shown to modulate NO production in macrophages and impact the downstream immune response (Chang et al., 1998; Bansal and Ochoa, 2003; Johann, et al, 2007).

Further study of the genes, their transcript abundance, protein level, and protein function will enhance our understanding of the interaction of PRRSV with the porcine macrophage. Possible outcomes may include identification of virulence mechanisms, development of next generation diagnostic assays and more rational vaccine design to more effectively limit viral replication and spread.

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**Table 1. Sequencing completed**

| Library (hours PI) | 0 | 6 | 12 | 16 | 24 |
|---|---|---|---|---|---|
| **Tags sequenced** | 111,214 | 96,968 | 103,662 | 134,990 | 196,421 |
| **Unique tags** | 24,356 | 29,520 | 32,683 | 31,419 | 37,370 |
| **PRRSV tag (CGGCCGAAAT)** | 0 | 255 (0.27%) | 9500 (9.2%) | 6902 (5.1%) | 3632 (1.8%) |

**Table 2. Changes in Gene Expression Profiles in PRRSV-infected PAMs**

| | | Normalized SAGE tags[1] (Time Post-Infection) | | | | |
|---|---|---|---|---|---|---|
| **Transcript** | **rt-PCR** | **Mock** | **6** | **12** | **16** | **24** |
| CCL3 (MIP1α) | decline | 191 | 21 | 3 | 0 | 21 |
| CCL4 (MIP1β) | decline | 419 | 68 | 25 | 19 | 16 |
| RANTES | nd[2] | 7 | 7 | 17 | 23 | 13 |
| MIF1 | nd | 8 | 1 | 25 | 35 | 32 |
| MIP3 | nd | 196 | 78 | 14 | 19 | 11 |
| MCP1 | nd | 1 | 1 | 1 | 4 | 31 |
| MCP3 | nd | 0 | 0 | 4 | 1 | 22 |
| CXCL2 | decline[3] | 1569 | 659 | 335 | 215 | 155 |
| GM-CSF | nd | 4 | 3 | 1 | 0 | 4 |
| TNFα | | 49 | 3 | 1 | 0 | 0 |
| IL-1α | decline | 152 | 31 | 23 | 16 | 40 |
| IL-1β | decline | 283 | 103 | 31 | 52 | 163 |
| IL-6 | decline | | | no tag in transcript | | |
| IL-8 (AMCF1) | 6 hr | 353 | 807 | 370 | 202 | 449 |
| (2 tags) | increase[4] | 8 | 172 | 121 | 55 | 17 |
| AMCF2 | 6 hr | 0 | 88 | 31 | 13 | 80 |
| | increase[3,4] | | | | | |
| arginase | 6 hr | 227 | 915 | 169 | 141 | 103 |
| | increase[4] | 37 | 131 | 21 | 18 | 24 |
| (4 tags) | | 1 | 88 | 17 | 4 | 1 |
| | | 14 | 84 | 31 | 5 | 3 |
| A20 | nd | 11 | 12 | 6 | 1 | 4 |
| IκKα | nd | 4 | 7 | 3 | 2 | 1 |
| IκBα | nd | 205 | 72 | 42 | 11 | 26 |
| c-Jun | nd | 8 | 3 | 0 | 1 | 5 |
| c-Fos | nd | 134 | 9 | 11 | 18 | 9 |
| JunB | nd | 101 | 6 | 11 | 17 | 33 |
| JunD | nd | 13 | 1 | 0 | 4 | 9 |
| ICAM1 | nd | 55 | 43 | 59 | 32 | 11 |
| ICAM2 | nd | 4 | 15 | 6 | 7 | 6 |
| VCAM1 | nd | 26 | 13 | 31 | 36 | 11 |
| integrin β1 | nd | 37 | 24 | 18 | 22 | 19 |
| integrin β2 | nd | 83 | 31 | 51 | 98 | 61 |
| lamin B1 | nd | 12 | 13 | 14 | 22 | 12 |
| TLR4[5] | nd | 24 | 22 | 14 | 4 | 15 |
| CD163 | nd | 32 | 3 | 0 | 3 | 5 |

[1]normalized to 100,000 tags
[2]not done
[3]analyzed cells from one pig only
[4]increase seen only at 6 hours PI
[5]toll-like receptor 4

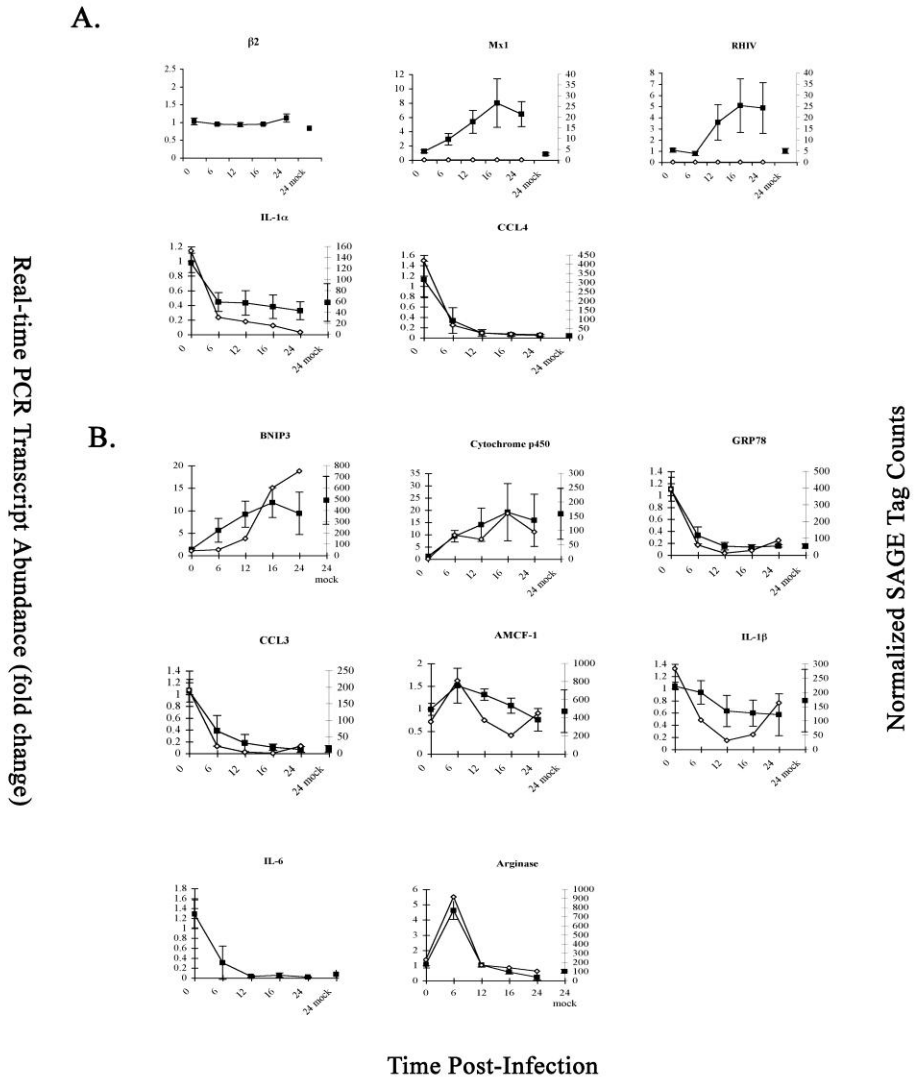


Fig. 1

**Figure 1. Real-time RT-PCR validation of SAGE results**

Validation by real-time RT-PCR on RNA from the *in vitro* PRRSV-infected PAM samples used in the SAGE analysis of selected transcripts showing differential expression. Real-time RT-PCR transcript abundance results (filled squares; left *y* axis) and SAGE tag counts normalized to total tags per library (open diamonds; right *y* axis) are shown for transcripts: Cytochrome P450 (TC281236), heat shock protein chaperone GRP78/BiP (GRP78; BW956325), BCl-2 family member (BNIP3; AK240169), alveolar macrophage-derived chemotactic factor-I/interleukin-8 precursor (AMCF-1; NM_213867), interleukin-1α (IL-1α; NM_214029), interleukin-1β (IL-1β; NM_214055), C-C chemokine ligand 3/macrophage inflammatory protein-1α (CCL3; NM_001009579) ,C-C chemokine ligand 4/macrophage inflammatory protein-1β (CCL4; TC288791). The '24 mock' result shows the real-time RT-PCR fold change in transcript abundance as a result of being in cell culture.