Identifying putative candidate genes and pathways involved in immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) infection


*Animal Parasitic Diseases Laboratory, ANRI, ARS, USDA, BARC-East, Beltsville, MD 20705, USA. †Department of Animal Science, Michigan State University, East Lansing, MI, USA. ‡Department of Fisheries and Wildlife, Michigan State University, East Lansing, MI, USA. §Animal Science Department, University of Nebraska, Lincoln, NE, USA.

Summary

Differences in gene expression were compared between RNAs from lungs of high (HR) and low (LR) porcine reproductive and respiratory syndrome virus (PRRSV) burden pigs using the swine protein–annotated long oligonucleotide microarray, the Pigoligoarray. Pathway analyses were carried out to determine biological processes, pathways and networks that differ between the LR and HR responses. Differences existed between HR and LR pigs for 16 signalling pathways (P < 0.01/ log (P-value) >1.96). Top canonical pathways included acute phase response signalling, crosstalk between dendritic cells and natural killer cells and tight junction signalling, with numerous immune response genes that were upregulated (SOCS1, SOD2, RBP4, HLA-B, HLA-G, PPP2R1A and TAP1) or downregulated (IL18, TF, C4BPA, C1QA, C1QB and TYROBP). One mechanism, regulation of complement activation, may have been blocked in HR (PRRSV-susceptible) pigs and could account for the poor clearance of PRRSV by infected macrophages. Multiple inhibiting signals may have prevented effective immune responses in susceptible HR pigs, although some protective genes were upregulated in these pigs. It is likely that in HR pigs, expression of genes associated with protection was delayed, so that the immune response was not stimulated early; thus, PRRSV infection prevented protective immune responses.

Keywords complement, genetic resistance, immune pathways, pigoligoarray, porcine reproductive and respiratory syndrome, porcine reproductive and respiratory syndrome susceptibility.

Porcine reproductive and respiratory syndrome (PRRS) is a major swine disease that costs US swine producers approximately $700 million annually (Neumann et al. 2005). PRRS virus (PRRSV)-infected pigs are susceptible to pneumonia and reproductive losses (Lowe et al. 2005; Cho & Dee 2006). The PRRS threat has been expanded by reports of ‘Pig High Fever Disease’, a highly pathogenic pig disease in China for which PRRSV has been identified as the single most prominent virus (Tian et al. 2007; Zhou & Yang 2010). With principal component analyses of phenotypic data collected from Hampshire-Duroc cross and NE Index line pigs infected with PRRSV, other researchers have identified low (LR) pigs, with low viremia, greater weight gain and few lung lesions, and high (HR) pigs, with high viremia, low/no weight gain, and many lung lesions (Petry et al. 2007). Genetic control of anti-PRRS responses has been reviewed (Lewis et al. 2007; Lunney & Chen 2010), and microarrays have been used to identify genes and pathways involved in controlling response to PRRSV infection (Bates et al. 2008; Genini et al. 2008). Based on a control reference design, Bates et al. (2008) utilized pig NRSP8-Qiagen arrays, with 12 500 long oligo probes, to assess gene expression of RNA extracted from lung and bronchial lymph node (BLN) tissue of HR and LR PRRSV burden pigs (Petry et al. 2005, 2007).

This manuscript expands on previous studies and uses the same panel of samples (Petry et al. 2005, 2007; Bates...
expression data were submitted to GEO (GSE25120) and Alexa Fluor analysed separately for each tissue using MAANOVA (Cui et al. 2005) after spot alignment. Normalized (Yang et al. 2002) and labelled with Alexa Fluor/C210 dyes (Invitrogen). A microarray reference design was applied, hybridizing four randomly selected samples from each group (LR and HR) of lung and BLN (16 arrays in total). A common pig reference sample was generated using RNA isolated from brain, liver, lung, Mesenteric LN, spleen and testis of uninfected animals. Fluorescent images were detected by an Axon GenePix 4000B scanner (Molecular Devices), and fluorescence intensity data were collected using GENEXP Pro 6 software (Molecular Devices) after spot alignment. Normalized (Yang et al. 2005) expression data were submitted to GEO (GSE25120) and analysed separately for each tissue using MAANOVA (Cui et al. 2005) from Bioconductor (Gentleman et al. 2004) in R (http://www.r-project.org/). The linear model fitted to normalized log-intensity data included fixed effects of dye, array, reference sample and response group (HR/LR). The use of a fixed effect model is justified for a common reference design, because it is equivalent to a log-ratio model (Kerr 2003; Muller & Hannappel 2003; Hsiao et al. 2006). QPCR was used to validate the microarray. Seven candidate genes were selected for microarray confirmation based on a combination of their expression (Table 1) and additional functions including cell proliferation, migration and differentiation (Muller & Hannappel 2003; Hsiao et al. 2006). QPCR was used to validate the microarray. Seven candidate genes were selected for microarray confirmation based on a combination of their expression (Table 1) and the availability of probes and primers at the Porcine Immunology and Nutrition database (Dawson et al. 2005; http://www.ars.usda.gov/Services/docs.htm?docid=6065). Synthesis of cDNA was performed using SuperScript Reverse Transcriptase (Invitrogen) and oligo dT with 5 µg of total RNA. QPCR amplification reactions were carried out using the Brilliant kit (Stratagene) and ABI Prism 7500 Sequence Detector System (Applied Biosystems) as previously described (Dawson et al. 2005). RPL32 was used as a reference gene. After statistical analysis, the agreement between significance [P < 0.05] and direction of fold change was observed for CD163, RBP4 and IL18. A further two candidate genes, namely SOCS1 and STAT3, show a very similar FC value; however, the difference in gene expression was not significant. TAP1 and TNF may be false positives for the microarray. In conclusion, two of seven candidate genes were not confirmed by QPCR validation, which results in FDR = 30%. The correlation between microarray and QPCR for estimated FC and log-FC is 0.89 and 0.67 after excluding the most significant gene RBP4 (which can arguably be considered an influential point). These results indicate that the overall measure of expression obtained with the

Table 1 Candidate genes involved in swine lung responses to porcine reproductive and respiratory syndrome virus (PRRSV) infection.

<table>
<thead>
<tr>
<th>HGN</th>
<th>Fold change</th>
<th>P-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBP4*</td>
<td>3.1328</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>C1QA</td>
<td>0.2934</td>
<td>&lt;0.0001</td>
<td>&lt;0.0722</td>
</tr>
<tr>
<td>TF</td>
<td>0.4087</td>
<td>&lt;0.0001</td>
<td>&lt;0.0774</td>
</tr>
<tr>
<td>PPP2R1A</td>
<td>1.6174</td>
<td>&lt;0.0006</td>
<td>&lt;0.1520</td>
</tr>
<tr>
<td>TAP1*</td>
<td>1.6607</td>
<td>&lt;0.0006</td>
<td>&lt;0.1520</td>
</tr>
<tr>
<td>HLA-B</td>
<td>1.6488</td>
<td>&lt;0.0007</td>
<td>&lt;0.1595</td>
</tr>
<tr>
<td>SOCS1*</td>
<td>1.5795</td>
<td>&lt;0.0008</td>
<td>&lt;0.1686</td>
</tr>
<tr>
<td>IL18*</td>
<td>0.5598</td>
<td>&lt;0.0009</td>
<td>&lt;0.1761</td>
</tr>
<tr>
<td>HLA-G</td>
<td>1.5420</td>
<td>&lt;0.0009</td>
<td>&lt;0.1778</td>
</tr>
<tr>
<td>TNF*</td>
<td>1.3267</td>
<td>&lt;0.0011</td>
<td>&lt;0.1839</td>
</tr>
<tr>
<td>C1QB</td>
<td>0.4130</td>
<td>&lt;0.0013</td>
<td>&lt;0.1937</td>
</tr>
<tr>
<td>PK3C2A</td>
<td>0.8657</td>
<td>&lt;0.0015</td>
<td>&lt;0.2072</td>
</tr>
<tr>
<td>C4BPA</td>
<td>0.6775</td>
<td>&lt;0.0020</td>
<td>&lt;0.2303</td>
</tr>
<tr>
<td>SOD2</td>
<td>1.5888</td>
<td>&lt;0.0025</td>
<td>&lt;0.2442</td>
</tr>
<tr>
<td>TYROBP</td>
<td>0.6604</td>
<td>&lt;0.0029</td>
<td>&lt;0.2482</td>
</tr>
<tr>
<td>TMSB4X</td>
<td>0.4967</td>
<td>&lt;0.0031</td>
<td>&lt;0.2503</td>
</tr>
<tr>
<td>CEBPD</td>
<td>1.5229</td>
<td>&lt;0.0035</td>
<td>&lt;0.2581</td>
</tr>
<tr>
<td>CD163*</td>
<td>1.4678</td>
<td>&lt;0.0045</td>
<td>&lt;0.2781</td>
</tr>
<tr>
<td>STAT3*</td>
<td>1.3818</td>
<td>&lt;0.0055</td>
<td>&lt;0.2988</td>
</tr>
<tr>
<td>TXNIP</td>
<td>1.8532</td>
<td>&lt;0.0067</td>
<td>&lt;0.3118</td>
</tr>
</tbody>
</table>

Genes were identified after statistical analysis of microarray experiments comparing gene expression between HR and LR PRRSV burden pigs. Genes are ordered based on q-value, with the double line marking the q < 0.25 cut-off.

*Candidate genes used for microarray validation.
microarray has high positive correlation with the reference technique (QPCR).

IPA analyses identified 16 signalling pathways (data not shown) \( P < 0.01 / \log (P\text{-value}) > 1.96 \) and 24 additional pathways \( P < 0.05 / \log (P\text{-value}) > 1.31 \) (Table S2). The top five canonical pathways were acute phase response signalling, graft-versus-host disease signalling, crosstalk between dendritic cells and natural killer cells, tight junction signalling and IL-9 signalling. Important immune response-associated genes were upregulated (SOCS1, SOD2, RBP4, HLA-B, HLA-G, PPP2R1A, TAP1) or downregulated (IL18, TF, C4BPA, C1QA, C1QB, TYROBP) (Fig. 1). Two other DE genes, TNF (FC = +1.3) and PIK3C2A (FC = 1.2), were widely distributed in these pathways.

Innate defence via the complement (C) system plays an important role in protecting against virus infection. Macrophages infected with PRRSV are protected against antibody-dependent C-mediated cell lysis both in vivo and in vitro (Coster et al. 2006). Protection may be due to viral proteins not being incorporated into the plasma membrane of the infected macrophages, which masks the infected cells from recognition by antibodies and porcine C. Both the classical pathway and the lectin C pathways were significantly affected, with downregulated C1QA, C1QB, and C4BPA expression in HR pigs (Fig. 1a); C1QA and C1QB are critical for the formation of the membrane attack complex activated through C4 (Duvall et al. 2010), whereas C4BPA is expected to decrease activation of C4B. Another mechanism, regulation of C activation, may have been blocked in HR (PRRSV-susceptible) pigs, and this accounts for the poor removal of PRRSV-infected macrophages via C in these pigs.

![Figure 1](image-url)

_Figure 1_ Summary of highly significant \( (P < 0.01) \) IPA canonical pathways identified in anti-PRRS responses in lungs of LR and HR pigs. (a) IPA pathways/networks based on TNF regulation and showing the influence of these networks on differential complement (C) gene expression. (b) IPA pathways/networks including the main suppressors (TYROBP, SOCS1, RBP4 and PIK3C2A) of immune pathway interactions. (c) Combined IPA pathways/networks for PPP2R1A, an important negative regulator of gene expression. For a, b and c, the inhibited (downregulated) signals of HR pigs compared with those of LR pigs are highlighted in green; upregulated genes are in red. The intensity of shading is proportional to the fold difference in gene expression. The solid lines between genes represent known direct interactions, and dashed lines represent indirect interactions; ‘’\( T \)’’ indicates negative regulation and ‘’\( + \)’’ positive regulation.

© 2011 USDA ARS, Animal Genetics © 2011 Stichting International Foundation for Animal Genetics, 43, 328–332
SOCS1 participates in the inhibition of interferon (IFN)-mediated antiviral and anti-proliferative activities through JAK/STAT signalling (Song & Shuai 1998) and negatively regulates innate immune responses through a RIG-I/IFNAR1-dependent pathway during influenza infections (Pothlichet et al. 2008). A relationship between SOCS1 and PRRSV infection has not been previously reported. Recent research has shown that pro-inflammatory cytokine proteins, interleukin 1 beta (IL-1β), IL-6 and tumour necrosis factor-alpha (TNF-α), are poorly expressed during acute phase response of experimental PRRSV infection (Gómez-Laguna et al. 2010; Lunney et al. 2010). Because SOCS1 is involved in acute phase response signalling, its upregulation in susceptible pigs may be responsible for immune inhibitions of pro-inflammatory cytokines. After PRRSV infection, inhibition of STAT signalling by SOCS1 was further strengthened by the downregulation of TYROBP in HR pigs (Fig. 1b). Importantly, both NF-κB and ERK signalling were also negatively regulated by the synergism of SOCS1 and TYROBP in HR pigs. Thus, the higher level of SOCS1 and downregulation of TYROBP correlate with delayed immune responses during PRRSV infection for HR pigs.

Another DE gene, PPP2R1A, is upregulated in HR pigs and acts as a critical suppressor in AMPK signalling (Wu et al. 2007) to decrease phosphorylation of P38 MAPK (Prickett & Brautigan 2007) and to affect CTLA4 signalling in cytotoxic T lymphocytes by inhibiting AKT (Rudd et al. 2009). It may also suppress the production of nitric oxide and reactive oxygen species in macrophages (Forman et al. 1998). IPA network analysis showed that many molecules downstream of PPP2R1A-MAPK were downregulated during PRRSV infection in HR pigs (Fig. 1c). Downregulation of PIK3C2A played overlapping roles with PPP2R1A. SOCS1 and TYROBP in host immune inhibition. An upregulated gene, RBP4, is a well-known negative regulator of PIK3C2A (Yang et al. 2005) and ERK signalling (Ost et al. 2007) (Fig. 1a).

Innate responses of the lung comprise the initial defence against PRRSV, as confirmed previously by examinations of gene expression in porcine lung and porcine alveolar macrophages using microarrays (Bates et al. 2008; Genini et al. 2008). Our work has identified many more immune-related DE genes with a wider functional spectrum as compared to previous findings. This is likely because the 20k Pigoligo-array has a wider coverage than the earlier pig arrays, particularly of those genes involved in stress and disease responses (Steibel et al. 2009). Multiple inhibitory signals in susceptible HR pigs were identified. There were stronger immune responses in HR pigs at 14 dpi (Petry et al. 2007), and some protective genes were upregulated in these pigs, e.g. SOD2, TAP1, HLA-B and TNF. The upregulation of SOD2 is consistent with previous proteomics data in pulmonary alveolar macrophages (Zhang et al. 2009). It is likely that protective gene expression in the HR pigs was too late; thus, the immune response was not stimulated early enough to send positive signals for a protective immune response. By this stage, PRRSV infection had already turned on too many negative immune regulators in these PRRS-susceptible pigs.

Acknowledgements

The authors thank S. Abrams and A. Tietgens for technical assistance with this project. This work was supported by USDA ARS project funds, by USDA NRI PRRS CAP Grant 2004-35605-14197 and USDA NIFA PRRS CAP Grant 2008-55620-19132. H. Chen was supported by a China Scholarship Council grant for his PhD research in the United States.

References


**Supporting information**

Additional supporting information may be found in the online version of this article.  
*Table S1* Differentially expressed genes in lungs of PRRSV-infected pigs.  
*Table S2* List of the significant IPA canonical pathways identified in lungs of PRRSV-infected pigs.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.