ANIMAL GENETICS Immunogenetics, Molecular Genetics and Functional Genomics

SHORT COMMUNICATION doi:10.1111/j.1365-2052.2011.02251.x

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

M. Wysocki*,[1], H. Chen*,[2], J. P. Steibel[†,‡], D. Kuhar*, D. Petry[§,3], J. Bates[§], R. Johnson[§], C. W. Ernst[†] and J. K. Lunney*

*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

Address for correspondence

J. K. Lunney, APDL, ANRI, ARS, USDA, Building 1040, Room 103, BARC-East, Beltsville, MD 20705, USA.
E-mail: Joan.Lunney@ars.usda.gov

Present addresses:
[1]Lehrstuhl für Tierzucht, TUM, Munich, Germany.
[2]School of Animal Science and Nutritional Engineering, Wuhan Polytechnic University, Wuhan, China.
[3]Triumph Foods, LLC, Saint Joseph, MO, USA.

Accepted for publication 2 May 2011

et al. 2008) and summarizes the results of microarray experiments using the new Pigoligoarray (http://www.pigoligoarray.org; Steibel *et al.* 2009). RNA prepared from LR and HR lung and BLN Hampshire-Duroc cross pigs 14 d post-PRRSV infection (Petry *et al.* 2005, 2007; Bates *et al.* 2008) was tested for differences in gene expression using the Pigoligoarray. Total RNA was converted to aRNA using Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion Inc.) and labelled with Alexa Fluor® 555 and Alexa Fluor® 647 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 GENEPIX® 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; Smyth 2004). Considering the array effect as random allows recovery of inter-array information and increased power (Kerr 2003), at the expense of increasing the computational burden. Keeping the array as a fixed effect facilitates the implementation of permutation testing (Cui *et al.* 2005); a moderated *F*-test was computed, and significance was assessed using sample permutation with the array set as a fixed effect. Correction for multiple tests consisted of false discovery rate (FDR, Storey 2002) calculation as implemented in the q-value package of Bioconductor.

To declare differentially expressed (DE) genes, differences in gene expression measures between HR and LR PRRSV were statistically evaluated in BLN and lung. Significant differential expression was found in lung RNAs. To explore the expression pattern, the cut-off of FDR = 20% ($q \leq 0.2$ and $P \leq 0.0029$) was applied, yielding a final list of 156 DE genes (Table S1) that were analysed through the use of IPA (Ingenuity Pathways Analysis software, Ingenuity® Systems, http://www.ingenuity.com). Such a relatively high FDR was used to increase the number of genes that could be subjected to pathway analysis. Table 1 provides detailed information about the top DE candidate genes of HR pigs compared to LR pigs. Additionally, the use of the threshold $q = <0.3$ revealed an additional five candidate genes that are likely to play an important role in PRRSV infection, namely *CEBPD*, *CD163*, *TMSB4X*, *TXNIP* and *STAT3*. For example, *TMSB4X*, previously identified by Bates *et al.* (2008), is involved in resistance to apoptosis and has many 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 Dystem (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.

HGNC	Fold change	*P*-value	*q*-value
*RBP4**	3.1328	<0.0001	<0.0001
C1QA	0.2934	<0.0001	<0.0722
TF	0.4087	<0.0001	<0.0774
PPP2R1A	1.6174	<0.0006	<0.1520
*TAP1**	1.6607	<0.0006	<0.1520
HLA-B	1.6488	<0.0007	<0.1595
*SOCS1**	1.5795	<0.0008	<0.1686
*IL18**	0.5598	<0.0009	<0.1761
HLA-G	1.5420	<0.0009	<0.1778
*TNF**	1.3267	<0.0011	<0.1839
C1QB	0.4130	<0.0013	<0.1937
PIK3C2A	0.8657	<0.0015	<0.2072
C4BPA	0.6775	<0.0020	<0.2303
SOD2	1.5888	<0.0025	<0.2442
TYROBP	0.6604	<0.0029	<0.2482
TMSB4X	0.4967	<0.0031	<0.2503
CEBPD	1.5229	<0.0035	<0.2581
*CD163**	1.4678	<0.0045	<0.2781
*STAT3**	1.3818	<0.0055	<0.2988
TXNIP	1.8532	<0.0067	<0.3118

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*-value) >1.96] and 24 additional pathways [$P < 0.05$/−log (*P*-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* (Costers *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.

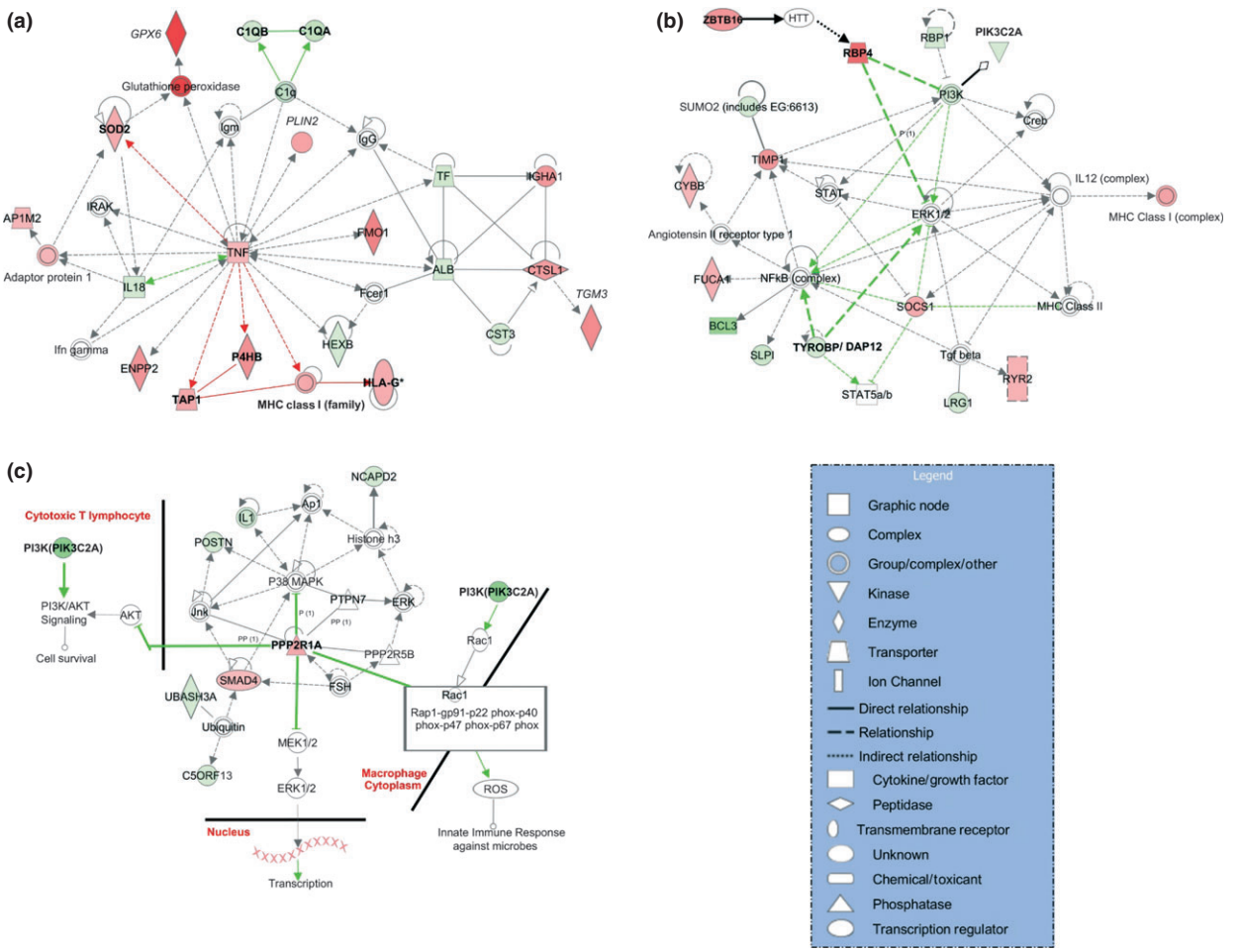

Figure 1 Summary of highly significant ($P < 0.01$) IPA canonical pathways identified in anti-PRRS responses in lungs of LR and HR pigs. (a) IPA pathways/networks based on TNF regulation and showing the influence of these networks on differential complement (C') gene expression. (b) IPA pathways/networks 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.

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 proinflammatory cytokine proteins, interleukin 1beta (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 CAP2 Grant 2008-55620-19132. H. Chen was supported by a China Scholarship Council grant for his PhD research in the United States.

References

Bates J.S., Petry D.B., Eudy J., Bough L. & Johnson R.K. (2008) Differential expression in lung and bronchial lymph node of pigs with high and low responses to infection with porcine reproductive and respiratory syndrome virus. *Journal of Animal Science* **86,** 3279–89.

Cho J.G. & Dee S.A. (2006) Porcine reproductive and respiratory syndrome. *Theriogenology* **66,** 655–62.

Costers S., Delputte P.L. & Nauwynck H.J. (2006) Porcine reproductive and respiratory syndrome virus-infected alveolar macrophages contain no detectable levels of viral proteins in their plasma membrane and are protected against antibody dependent, complement-mediated cell lysis. *Journal of General Virology* **87,** 2341–51.

Cui X.G., Hwang J.T.G., Qiu J., Blades N.J. & Churchill G.A. (2005) Improved statistical tests for differential gene expression by shrinking variance components estimates. *Biostatistics* **6,** 59–75.

Dawson H.D., Beshah E., Nishi S. *et al.* (2005) Localized multigene expression patterns support an evolving Th1/Th2-like paradigm in response to infections with Toxoplasma gondii and Ascaris suum. *Infection and Immunity* **73,** 1116–28.

Duvall M.R., Hwang H.Y. & Boackle R.J. (2010) Specific inhibition of the classical complement pathway with an engineered single-chain Fv to C1q globular heads decreases complement activation by apoptotic cells. *Immunobiology* **215,** 395–405.

Forman H.J., Zhou H., Gozal E. & Torres M. (1998) Modulation of the alveolar macrophage superoxide production by protein phosphorylation. *Environmental Health Perspectives* **106**(Suppl 5), 1185–90.

Genini S., Delputte P.L., Malinverni R., Cecere M., Stella A., Nauwynck H.J. & Giuffra E. (2008) Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. *Journal of General Virology* **89,** 2550–64.

Gentleman R.C., Carey V.J., Bates D.M. *et al.* (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology* **5,** R80.

Gómez-Laguna J., Salguero F.J., Pallarés F.J., Fernández de Marco M., Barranco I., Cerón J.J., Martínez-Subiela S., Van Reeth K. & Carrasco L. (2010) Acute phase response in porcine reproductive and respiratory syndrome virus infection. *Comparative Immunology, Microbiology and Infectious Diseases* **33,** e51–8.

Hsiao H., Wang W., Chen P. & Su Y. (2006) Overexpression of thymosin β-4 renders SW480 colon carcinoma cells more resistant to apoptosis triggered by FasL and two topoisomerse II inhibitors via downregulating Fas and upregulating Survivin expression, respectively. *Carcinogenisis* **27,** 936–44.

Kerr M.K. (2003) Linear models for microarray data analysis: Hidden similarities and differences. *Journal of Computational Biology* **10,** 891–901.

Lewis C.R., Ait-Ali T., Clapperton M., Archibald A.L. & Bishop S. (2007) Genetic perspectives on host responses to porcine reproductive and respiratory syndrome (PRRS). *Viral Immunology* **20,** 343–58.

Lowe J.F., Husmann R., Firkins L.D., Zuckermann F.A. & Goldberg T.L. (2005) Correlation of cellular immunity to PRRS virus and clinical disease during outbreaks of PRRS in commercial swine herds. *Journal of the American Veterinary Medical Association* **226,** 1707–11.

Lunney J.K. & Chen H. (2010) Genetic control of porcine reproductive and respiratory syndrome virus responses. *virus Research,* **154,** 161–9.

Lunney J.K., Fritz E.R., Reecy J.M., Kuhar D., Prucnal E., Molina R., Christopher-Hennings J., Zimmerman J. & Rowland R.R.R. (2010) Interleukin-8, interleukin-1β and interferon-γ levels are linked to PRRS virus clearance. *Viral Immunology* **23,** 127–34.

Muller C.T.H. & Hannappel E. (2003) Reduction of thymosin β4 and actin in HL60 cells during apoptosis is preceded by a decrease of their mRNAs. *Molecular and Cellular Biology* **250,** 179–88.

Neumann E.J., Kliebenstein J.B., Johnson C.D., Mabry J.W., Bush E.J., Seitzinger A.H., Green A.L. & Zimmerman J.J. (2005) Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. *Journal of the American Veterinary Medical Association* **227,** 385–92.

Ost A., Danielsson A., Lidén M., Eriksson U., Nystrom F.H. & Strålfors P. (2007) Retinol-binding protein-4 attenuates insulin-induced phosphorylation of IRS1 and ERK1/2 in primary human adipocytes. *FASEB Journal* **21,** 3696–704.

Petry D.B., Holl J.W., Weber J.S., Doster A.R., Osorio F.A. & Johnson R.K. (2005) Biological responses to porcine respiratory and reproductive syndrome virus in pigs of two genetic populations. *Journal of Animal Science* **83,** 1494–502.

Petry D.B., Lunney J., Boyd P., Kuhar D., Blankenship E. & Johnson R.K. (2007) Differential immunity in pigs with high and low responses to porcine reproductive and respiratory syndrome virus infection. *Journal of Animal Science* **85,** 2075–92.

Pothlichet J., Chignard M. & Si-Tahar M. (2008) Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by *SOCS1* and *SOCS3* through a RIG-I/IFNAR1-dependent pathway. *Journal of Immunology* **180,** 2034–8.

Prickett T.D. & Brautigan D.L. (2007) Cytokine activation of p38 mitogen-activated protein kinase and apoptosis is opposed by alpha-4 targeting of protein phosphatase 2A for site-specific dephosphorylation of MEK3. *Molecular and Cellular Biology* **27,** 4217–27.

Rudd C.E., Taylor A. & Schneider H. (2009) CD28 and CTLA-4 coreceptor expression and signal transduction. *Immunological Reviews* **229,** 12–26.

Smyth G.K. (2004) Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statistical Applications in Genetics and Molecular Biology* **3,** Article3.

Song M.M. & Shuai K. (1998) The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. *Journal of Biological Chemistry* **273,** 35056–62.

Steibel J.P., Wysocki M., Lunney J.K., Ramos A.M., Hu Z.L., Rothschild M.F. & Ernst C.W. (2009) Assessment of the swine protein-annotated oligonucleotide microarray. *Animal Genetics* **40,** 883–893.

Storey J.D. (2002) A direct approach to false discovery rates. *Journal of the Royal Statistical Society Series B-Statistical Methodology* **64,** 479–498.

Tian K., Yu X., Zhao T. *et al.* (2007) Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS ONE,* **2,** e526.

Wu Y., Song P., Xu J., Zhang M. & Zou M.H. (2007) Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase. *Journal of Biological Chemistry* **282,** 9777–88.

Yang Q., Graham T.E., Mody N., Preitner F., Peroni O.D., Zabolotny J.M., Kotani K., Quadro L. & Kahn B.B. (2005) Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* **436,** 356–62.

Zhang H., Guo X., Ge X., Chen Y., Sun Q. & Yang H. (2009) Changes in the cellular proteins of pulmonary alveolar macrophage infected with porcine reproductive and respiratory syndrome virus by proteomics analysis. *Journal of Proteome Research* **8,** 3091–7.

Zhou L. & Yang H. (2010) Porcine reproductive and respiratory syndrome in China. *Virus Research* **154,** 31–7.

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.