Comparative Transcriptomic Analysis of Porcine Peripheral Blood Reveals Differentially Expressed Genes from the Cytokine-cytokine Receptor Interaction Pathway Related to Health Status

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3 **Pathway Related to Health Status**
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Abstract: While some research has looked into the host genetic response in pigs challenged with specific viruses or bacteria, few studies have explored the expression changes of transcripts in the peripheral blood of sick pigs which may be infected with multiple pathogens on farms. In this study, the architecture of the peripheral blood transcriptome of 64 Duroc sired commercial pigs, including 18 healthy animals at entry to a growing facility (set as a control) and 23 pairs of samples from healthy and sick pen mates, was generated using RNA-Seq technology. In total, 246 differentially expressed genes were identified to be specific to the sick animals. Functional enrichment analysis for those genes revealed that the over-represented gene ontology terms for the biological processes category were exclusively immune activity related. The cytokine-cytokine receptor interaction pathway was significantly enriched. Nine functional genes from this pathway encoding members (as well as their receptors) of the interleukins, chemokines, tumor necrosis factors, colony stimulating factors, activins and interferons exhibited significant transcriptional alteration in sick animals. Our results suggest a subset of novel marker genes which may be useful candidate genes in the evaluation and prediction of health status in pigs under commercial production conditions.

Key words: RNA-Seq, transcriptome, porcine, peripheral blood, cytokine

Introduction

Pig (*Sus scrofa*) is one of the most important domestic animals due to the popularity of pork products in many markets, especially Europe, North America and China. Infectious disease is a significant problem for pig production and is the cause of very large economic losses. For example, Streptococcosis, one of the leading infectious diseases in the swine industry is caused by *Streptococcus suis*, a member of a family of pathogenic gram-positive bacteria responsible for meningitis, septicemia, and arthritis in both pig and human (Feng et al. 2014; Varela et al. 2013). Glasser's disease, one of the bacterial diseases with highest economic impact in nursery piglets, is caused by *Haemophilus parasuis*, which causes fibrinous polyserositis in pig and is present in all the major swine-raising countries (Correa-Fiz et al. 2016; Macedo et al. 2015). *Brachyspira spp.* associated with diarrhoeic pigs (Barth et al. 2012) remains a significant pathogen in contemporary swine production systems. Viruses, often in combination with bacterial pathogens (Zimmerman et al. 1997), are also responsible for significant economic losses to the swine industry worldwide. In the US, porcine reproductive and respiratory syndrome virus (PRRSV) alone costs swine producers millions of dollars per year (Brito et al., 2014). In China, the annually economic loss caused by infectious disease in the pig industry is estimated to be RMB 40 billion (equivalent to > \$6Bn) (Zhao et al. 2012). The main approaches for prevention and control of diseases include vaccination and medication, elimination of sick animals, the maintenance of high levels of bio-security, and all-in-all-out production. Early recognition of pigs that may be infected by pathogens

can not only provide more effective therapeutic options for these pigs, but also provide better opportunities for preventive interventions in the whole herd. Therefore, establishing efficient prediction and diagnostic systems based on new traits or indicators such as the activity of immune response genes is a major opportunity for improving pig production.

It is known that multiple pathogens, i.e. bacteria, viruses, fungi, parasites and/or their components, are capable of triggering inflammatory and immune responses as part of defense against infectious agents. These processes result in changes in the transcriptional and translational profile of peripheral blood. Alterations in the serum level of cytokines, the key mediators of inflammation and immune-mediated diseases, have been found to be closely associated with various diseases. For example, significantly elevated serum levels of cytokines were observed in bovine with clinical and subclinical mastitis (including interleukin (IL)-1, IL-2, IL-6, and tumor necrosis factor-alpha) (Sadek et al. 2017) as well as in rabbits infected with *Mycobacterium tuberculosis* (including vascular endothelial growth factor, platelet derived growth factor, IL-17A, and IL-18) (Dehnad et al. 2016). Significant down-regulation of serum IL-7 was identified in acute human cholangitis (Suwa et al. 2017), and substantial increase in plasma IL-6 level was observed in mice infected with influenza A virus (Chockalingam et al. 2016) as well as in human patients infected with *Trypanosoma brucei rhodesiense* (Kato et al. 2015). In fact, IL-6 has also been identified as a biomarker for ischemic stroke (Doll et al. 2014), differential diagnosis of pancreatic cancer (Shaw et al. 2014), and congenital heart

disease (Nassef et al. 2014). All of these findings indicated that specific indices of biomarkers in peripheral blood might be promising for the prognosis of disease.

RNA-Seq has been used as an effective method for exploring pathology-associated transcriptional changes in peripheral blood in many species, such as human (Liu et al. 2014), cattle (McLoughlin et al. 2014), and swine (Koltjes et al. 2015; Hu et al. 2016). It has also enabled detailed analysis of the host transcriptome for *Salmonella* (Kommadath et al. 2014) and *Streptococcus suis* (Wu et al. 2014) infections in pig. However, it has not yet been used to explore such alterations in peripheral blood of pigs in commercial conditions where multiple pathogens may be present. Here, we used RNA-seq to generate whole blood transcriptome profiles from commercial pigs, with the aim of identifying differentially expressed genes associated with health status. We anticipate that this study can provide more information about host-pathogen interactions in the pig.

Materials and Methods

Ethics statement

Animals used for the study were from the Drumloche research facility managed by Sunhaven Farms, Alberta, Canada. All pigs were raised in a typical intensive system and fed *ad libitum* with a cereal-based commercial diet without the use of antibiotics. All animals were cared for in accordance with the “Guide to the Care and Use of Experimental Animals” (<http://www.ccac.ca>) and all procedures were approved by the Livestock Care Committee of the University of Alberta (AUP00000166).

Sample collection and animal grouping

94 A total of 2,100 pigs were housed in 100 pens in the growing facility which was part of
95 a three-site production system with relatively good health and performance. A low level
96 of clinical disease was associated with the presence of *Streptococcus suis*, *Brachyspira*
97 spp and Glasser's Disease (*Haemophilus parasuis*) based on routine veterinary
98 diagnostics at the unit prior to the study. From each sampled animal, approximately 3-4
99 mL of whole blood was collected from the jugular vein into TempusTM Blood RNA tubes
100 (processed according to manufacturer's instructions) and stored at -20°C until analyzed.
101 Blood samples were collected randomly from 2 pigs per pen over a three-day period
102 when the animals were transferred from the sow-nursery barn to the grow-finish barn.
103 These samples were classified as the **entry** group. Eighteen samples were subsequently
104 selected from these entry samples to match the pens sampled for sick and healthy pairs
105 (below). Pigs in the grow-finish barn were carefully monitored for sickness in order to
106 reduce the impact of infectious disease and to maintain good animal care. Animals
107 observed by staff or veterinarians as showing overt signs of illness were isolated, treated,
108 and tagged to ensure that they were not subsequently marketed as being raised without
109 antibiotics. Examples of sickness included scours, bloody or watery stool, and/or
110 "poor-doers" and were generally in line with the sub-clinical disease status of the barn.
111 Blood samples collected from these treated animals were classified as the **sick** group. At
112 the same time, a blood sample was taken from another individual in the same pen
113 showing no sign of any illness, which was classified as the **healthy** group. Twenty-three
114 samples were collected from the sick and healthy groups (for a total of 46 samples).

These samples were collected over a period of 71 days, with the first samples collected 25 days after entry. Altogether, the peripheral blood transcriptome was evaluated for the complete set of 64 samples (18, 23, and 23 from the entry, sick and healthy group, respectively) using an RNA-Seq approach.

Library construction and Illumina sequencing

Total RNA was isolated from blood samples using the Total RNA Purification Kit (Norgen, Biotek Corp.) following the manufacturer's protocol. An in-solution DNase I digestion step was employed to remove contaminant DNA as recommended. RNA was then recovered by the RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) and quantified using the Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA). The purity and integrity of total RNA were assessed with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). RNA samples with integrity number (RIN) larger than 6.0 (range 6.0 to 8.7, mean 7.76) were then subjected to a globin depletion protocol to remove the majority of globin transcripts (Choi *et al.* 2014). The RIN values after globin depletion ranged from 5.2 to 8.2 (mean 6.65). mRNA libraries were then constructed using the TruSeq RNA Sample Preparation Kit v2 (Illumina, Inc., San Diego, USA) according to the TrueSeq protocol. The quality and quantity of the mRNA libraries were confirmed using the Agilent Bioanalyzer 2100 and Qubit 3.0 Fluorometer (Life technologies). Eight individual libraries with different indexes were pooled together and sequenced in one lane using an Illumina HiSeq2000 instrument with 100 bp paired-end reads. Real-time analysis and base calling were performed using the HiSeq Control

136 Software, version 1.4.8 (Illumina).

137 **Mapping, assembling and annotation of sequences**

138 Briefly, Tophat software (version 2.0.2) with default settings was employed using the
139 pig genome (Sscrofa10.2) and the annotation database Ensembl Genes 67
140 (<http://www.ensembl.org./info/data/ftp/index.html>) as references. The number of reads
141 uniquely mapped to each gene was determined using Htseq-count (v0.5.3.p3). Reads not
142 mapped uniquely were discarded. The number of reads overlapping the genomic span of
143 each Ensembl gene was normalized to counts per million (CPM). Genes expressed at
144 very low levels were removed by keeping only those genes that achieved CPM above 1 in
145 at least 24 samples. Trimmed mean of M-values (TMM) normalization was applied to the
146 dataset to account for compositional differences between the libraries.

147 **Differential gene expression analysis**

148 The Bioconductor (version 2.18.0) package edgeR (version 3.0.8) within the R
149 (version 2.15.2) statistical programming language was employed to identify differentially
150 expressed genes (DEGs) between groups with p -value < 0.001 and false discovery rate
151 (FDR) < 0.01 . An exact test with a negative binomial distribution was used for
152 differential expression testing. Functional analysis for DEGs was conducted with
153 Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al.
154 2009). The human orthologs of the corresponding porcine genes were used in the analysis
155 to take advantage of the more complete gene ontology (GO) annotation available for
156 human genes. Data were analyzed with the “Functional Annotation Chart” tool using

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways or the Biological Process Gene Ontology FAT set term. Enrichment of GO terms was measured by Expression Analysis Systematic Explorer (EASE). The cutoff criterion was an EASE score *p*-value of less than 0.01.

Validation of DEGs by quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed for validating the expression data identified by RNA-Seq. Nine genes included up-regulated ILs (*IL18* and *IL22RA2*), chemokines (*CCL8* and *CCR1*), tumor necrosis factors (*TNFSF13B* and *REL1*), colony stimulating factors (*CSF3R*), activins (*ACVR1B*) and interferons (*IFNGR1*), and one significantly down-regulated *CCL8* in sick pigs. Twelve RNA samples (six samples from the healthy and sick groups, respectively) depleted of globin transcripts were used for qRT-PCR. The CPM values for those samples were within the range of average CPM $\pm$ SD for the selected nine genes. The same 12 RNA samples before globin depletion were also used for qRT-PCR to evaluate the effects of globin depletion on the transcriptional profile. RNA samples were synthesized to first-strand cDNA using SuperScript II RT (Invitrogen). *β -actin* was used as the internal reference gene. Primers used in this study are shown in Table 1. qRT-PCR in a 10 μ L reaction volume with 0.1 μ M of primers was performed in triplicate for each reaction using KAPA SYBR FAST qPCR Kits (Kapa Biosystems). The PCR conditions were as follows: 95°C 2 min for initial denaturation, 40 cycles of denaturation at 95°C for 30 sec and 60°C for 30 sec. The melt analysis curve was observed to confirm the amplification of single specific PCR fragments. Gene expression

data was normalized to the *β-actin* gene and relative to the samples from the healthy group.

Data Availability

The supplemental file S1 contains the raw count data of all genes for 64 samples. The supplemental file set S2 is the CPM file of transcripts used for the identification of DEGs. The supplemental files S3 and S4 comprise the differentially expressed transcripts, 517 significant DEGs (compared between the healthy group and the entry group) and 1096 DEGs (compared between the sick group and the entry group), respectively. The data from this study were deposited in NCBI's Sequence Read Archive under the accession number GSE96640.

Results

Characterization of pig peripheral blood transcriptome

The sequencing depths achieved for the libraries from the 64 blood samples ranged from 14.91 to 27.06 million reads (mean 24.73 ± 4.22 million reads), of which an average of 84.22% mapped to the reference pig genome assembly Sscrofa 10.2. Percentage of mapped reads observed per individual ranged from 82.60% to 86.30%. The average percentage of reads mapped to the pig genome was similar to the results of a previous study (90%) using peripheral blood as the RNA source (Kommadath et al. 2014).

Similar numbers of genes, 16,120, 16,052 and 16,134 genes, were expressed in the entry, healthy and sick groups respectively. Since the blood transcriptome has a high

complement of globin RNA that can potentially saturate next-generation sequencing platforms, a globin reduction (GR) protocol was employed to remove the majority of globin transcripts. After GR depletion, the proportion of total reads mapping to globin transcripts (HBA, HBB) ranged from 0.045% to 10.06%, with an average of only 2.64%. This is in line with the results of Choi et al. (2014) who reported a proportion of 8.9% post-GR. The globin genes were removed from the gene expression matrix prior to normalization and further analysis.

Identification of differentially expressed genes (DEGs)

A set of 11,666 genes expressed in porcine peripheral blood was obtained and used to perform the differential expression analysis using a cut-off of p value < 0.001 and FDR < 0.01 . In the present study, animals in the entry group were essentially of the same age, while pigs in the sick and healthy groups were distributed in different stages of growth development. The maximum age difference among animals within the sick/healthy group was almost three months. In order to eliminate the expression difference caused by the discrepancy in age, we obtained DEGs by making comparisons first between the entry and healthy group, and then between the entry and sick group for the remaining genes. The DEGs associated with growth and age were therefore filtered or removed and in this way, the remaining DEGs were more likely to reflect transcription alteration as a result of changes in health status rather than responses to growth development.

Transcriptome comparison between animals from the healthy and the entry group resulted in 517 significant DEGs (supplemental file S3). Of which, 127 were up-regulated

in the healthy group while 390 DEGs were down-regulated. The fold changes for those genes ranged from 1.43 to 10.94. When considering a more conservative change, only **64 DEGs** had fold changes higher than 2.0-fold or lower than -2.0-fold. Fourteen of them were up-regulated and 50 genes down-regulated in the healthy group. Regarding gene annotation, 57 genes were annotated, accounting for 89.06% of the significant DEGs.

When animals from the sick group were compared to individuals in the entry group, a total of 1096 DEGs were identified (supplemental file S4). Of which, 812 DEGs were significantly up-regulated in the sick group and the remaining 284 genes down-regulated in the same group. The fold changes for those DEGs ranged from 1.40 to 61.32. Further filtering with fold change higher than 2.0 resulted in **280 DEGs** with 223 of them up-regulated in the sick group and 57 DEGs down-regulated in the same group. 82.50% of these significant DEGs were successfully annotated with 181 unigenes up-regulated in the sick group and 50 genes down-regulated.

Comparison of the 64 and 280 significant DEGs revealed 34 common genes. We removed them and obtained the remaining 246 gene transcripts significantly altered in animals from the sick group, which were considered to be the unique genes responding to the alteration of health status rather than being related to growth development.

Functional annotation of DEGs

Gene ontology (GO) information was obtained for 185 of the 246 health-associated DEGs and possible functions and pathways were analyzed using DAVID. A large proportion of the annotated genes (62/185) were categorized into GO biological process

240 of the immune system process. They were involved in GO terms such as antigen
 241 processing and presentation, immune response and macrophage activation. In the
 242 biological processes category, the significantly enriched groups were exclusively immune
 243 activity related. Of the 185 genes 17.3% were annotated to response to wounding ($p =$
 244 $1.1\text{E-}13$, $\text{FDR} = 1.8\text{E-}10$), 13.5% to inflammatory response ($p = 6.4\text{E-}13$, $\text{FDR} =$
 245 $1.1\text{E-}09$), 16.8% to defense response ($p = 2.8\text{E-}11$, $\text{FDR} = 4.7\text{E-}08$) and 16.8% to
 246 immune response ($p = 4.6\text{E-}10$, $\text{FDR} = 7.7\text{E-}07$). The fold enrichment for these four GO
 247 terms was 5.1, 6.5, 4.2 and 3.8, respectively. In the molecular functions category, the two
 248 significant GO terms were cytokine binding ($p = 3.5\text{E-}08$, $\text{FDR} = 4.8\text{E-}05$) and cytokine
 249 receptor activity ($p = 3.2\text{E-}05$, $\text{FDR} = 4.5\text{E-}02$) with fold enrichment of 9.8 and 11.3,
 250 respectively. In the cellular components category, 39.5% and 23.8% of the genes were in
 251 the two abundant GO groups, plasma membrane and extracellular region, respectively.
 252 One up-regulated KEGG pathway, cytokine-cytokine receptor interaction pathway ($p =$
 253 $4.8\text{E-}09$, $\text{FDR} = 5.0\text{E-}06$) was differentially modulated. A total of 20 (10.8%) DEGs
 254 (*IL1A*, *IL18*, *IL1RAP*, *IL18RAP*, *IL1R2*, *IL22RA2*, *CCL23*, *CCL8*, *CCR1*, *CCR2*, *XCRI*,
 255 *TNFSF13B*, *TNFRSF21*, *TNFRSF8*, *RELT*, *CSF2RA*, *CSF2RB*, *CSF3R*, *ACVR1B* and
 256 *IFNGRI*) were included in this pathway (Table 2). Fifteen of the twenty genes (75%)
 257 were also found in the challenge studies with PRRSV (Kommadath et al 2017) and
 258 *Salmonella* (Kommadath et al. 2014).

259 Besides the 34 common genes, the healthy group yielded much fewer unique DEGs
 260 (30). Twenty four of these 30 were successfully annotated in DAVID. According to the

GO ontology, these transcriptionally altered genes participated mainly in biological processes of growth development including developmental maturation, cell maturation, myeloid cell differentiation, erythrocyte maturation and development, and collagen catabolic and metabolic process ($p < 0.05$).

Hierarchical clustering analysis was applied to compare the expression patterns of the 246 DEGs in healthy and sick animals (Figure 1). The expression patterns of these 246 genes can be divided into two clusters. Most of the animals could be differentiated on the basis of their general health category (sick or healthy), suggesting that the transcriptome clustering reconfirmed the status of the animals.

Gene expression validation of the nine DEGs by qRT-PCR

qRT-PCR analysis confirmed the direction of expression level changes detected by RNA-Seq (Figure 2). High consistency (Pearson's correlation coefficient $r > 0.950$, $p < 0.001$) was found between the RNA-Seq and qRT-PCR data for the transcription levels of the nine tested transcripts. The same magnitude of differential expression was obtained using cDNA from RNA samples before and after globin depletion in qRT-PCR, which confirmed that no bias was introduced by the globin depletion protocol employed before the construction of the mRNA library.

Discussion

Our results identified 246 gene transcripts in sick animals which corresponded to their different physical condition from the healthy pigs. In this study, the specific causes of sickness were undefined, which could be different types of immunogens/pathogens or

282 other conditions capable of triggering the production of cytokines. As expected, the
283 cytokine-cytokine receptor interaction pathway was significantly activated in the sick
284 animals. The 20 significant DEGs detected in this pathway may be a set of genes relating
285 to an early general response to the stimuli. Of which, the 9 genes (identified by RNA-seq
286 and confirmed by qRT-PCR) that also overlapped with DEGs detected both after specific
287 bacteria (*Salmonella*) and virus (PRRSV) challenge may be important candidate genes
288 reflecting the responses of sick animals to the exogenous and/or endogenous pathogens
289 that caused the alteration of the health status. They may also be potential targets for
290 selection for reduced susceptibility in breeding programs.

291 Transcriptional expression of *IL-18* was significantly up-regulated in sick pigs in the
292 present study. IL-18, produced mainly by monocytes/macrophages in response to stimuli
293 of viral/bacterial origin (Boraschi and Dinarello 2006), is one member of the IL-1 family
294 of cytokines, which are linked closely to the innate immune response and as the first line
295 of host defense against stress-induced acute and chronic inflammation (Dinarello 2009).
296 It plays key roles in inflammatory and immune responses and has significant influence on
297 the pathogenesis of diseases (Lukens et al. 2012). Up-regulated serum IL-18 level was
298 also reported in sepsis (Feng et al. 2016), asthma (Xu et al. 2017), and viral myocarditis
299 (Wang et al. 2016) and was reported to be involved in intestinal inflammation (Sivakumar
300 et al. 2002; Siegmund 2010) and colitis (Kanai et al. 2001; Ishikura et al. 2003).
301 Circulating IL-18 level has been described as a potential biomarker of cardiovascular
302 death (Blankenberg et al. 2002), radiation exposure (Ha et al. 2014), and pancreatic

adenocarcinoma (Usul Afsar et al. 2017). The significant difference in *IL18* gene expression observed here was therefore likely to be one of the effects of the innate immune response initiated by host-pathogen interaction. Consistent with this was the concomitant over-expression detected in sick animals of *IL-18RAP* mRNA, which encodes the beta chain of the IL-18 receptor required for signal transduction initiated by IL-18.

Another interleukin transcript that exhibited significant up-regulation in sick animals was *IL-22RA2*, which was also one of the top-ranking DEGs based on its fold-change in expression (14.06). IL-22RA2 is associated with antimicrobial defense, protection against injury and regeneration (Huber et al. 2012). Increased expression of IL-22RA2 was reported in gastric adenocarcinoma (Yang et al. 2016) and inflammatory bowel disease (Pelczar et al. 2016). Up-regulated transcriptional expression of *IL-22RA2* was also documented in the case of hepatitis C virus infection (Sertorio et al. 2015). Recently, increased urinary IL-22RA2 level was considered to be a potential marker for renal involvement in patients with systemic lupus erythematosus (Yang et al. 2014). Known as IL-22 binding protein, IL-22RA2 functions to antagonize IL-22 signaling (Laaksonen et al. 2014). No significant change of *IL-22* mRNA expression was detected in sick pigs, however, the over-expressed *IL-22RA2* transcript may lead to less availability of IL-22. Previous findings demonstrated that IL-22 may be pathogenic rather than protective depending on the target tissue and the cytokine milieu (Ciccia et al. 2014), suggesting that the imbalanced *IL-22/IL-22RA2* may act in a protective fashion to limit the

324 deleterious effects caused by IL-22 during the ongoing inflammatory process.

325 Significantly altered transcriptional expression of *CCR1* (chemokine C-C motif
326 receptor 1) and *CCL8* (chemokine C-C motif ligand 8) were observed in sick pigs.
327 Elevated expression of CCR1 has been found to be associated with a variety of
328 inflammatory conditions (Gao et al. 2000; Rottman et al. 2000; Trebst et al. 2002; John et
329 al. 2005; Miller et al. 2006; Grunin et al. 2012). Significant increased expression of
330 *CCR1*, which encodes the receptor for CCL23 (chemokine C-C motif ligand 23) (Chou et
331 al. 2002), was correlated with significant up-regulation of *CCL23* transcripts in sick
332 animals. *CCL8* was the only chemokine member significantly down-regulated in sick
333 pigs. CCL8 activates various immune cells, including mast cells, eosinophils, basophils,
334 monocytes, T cells, and NK cells (Proost et al. 1996). It has also been demonstrated to
335 possess anti-tumor (Struyf et al. 2009), anti-inflammatory, and anti-tumor metastatic
336 effects (Hiwatashi et al. 2011). A recent study revealed that CCL8 was a direct target of
337 the transcriptional repressor B lymphocyte-induced maturation protein 1 (BLIMP1)
338 which mediated transcriptional repression in macrophages (Severa et al. 2014). Since
339 multiple pathogens have the ability to induce BLIMP1 and in this way evade or suppress
340 innate immunity, the much lower expression level of *CCL8* in sick pigs may be a sign of
341 successful establishment of infection. This result suggests that CCL8 may serve as a
342 target for immunomodulation by pathogens to facilitate survival in the host.

343 In the sick pigs, *TNFSF13B* (tumor necrosis factor superfamily, member 13b) was
344 significantly up-regulated with a fold change of 2.53. TNFSF13B, also known as B cell

activating factor, is an immunostimulant and a central cytokine for B-cell survival, proliferation, maturation and immunoglobulin secretion (Wiens and Glenney 2011). Elevated serum level of TNFSF13B has been reported to be associated with autoimmune diseases (Lee et al. 2016; Lin et al. 2016) as well as in acute lymphoblastic leukemia (Sun et al. 2016). It was also suggested to be an additional biomarker for rejection in kidney and heart transplantation (Pongpirul et al. 2016) (Sarmiento et al. 2016). Another member that belongs to the TNF (tumor necrosis factor) receptor superfamily, *RELT* (receptor expressed in lymphoid tissues), showed significantly up-regulated expression in sick pigs. *RELT* can induce apoptosis and activate the proliferation of T cells (Sica et al. 2001). The serum level of autoantibody to *RELT* has diagnostic value in breast cancer (Zhong et al. 2008). In the present study, the significant up-regulated expression of *TNFSF13B* and *RELT* were indicative of the stimulated immune response in the sick animals.

The expression of three other cytokine receptors, *CSF3R* (colony stimulating factor 3 receptor), *ACVR1B* (activin A receptor, type IB), and *IFNGR1* (interferon gamma receptor 1), also showed significant increase in sick pigs. They encode receptors for granulocyte colony stimulating factors, activins, and the ligand-binding chain of gamma interferon receptor, respectively. Higher expression of *CSF3R* was associated with rectal cancer (Yang et al. 2005). Elevated expression of *ACVR1B* was reported in serous ovarian cancer (Dean et al. 2017). The increased transcriptional expression of *IFNGR1* was documented in several infection cases (Tang et al. 2015; Dettleff et al. 2017). The observed up-regulation of all these transcripts may be the result of host-pathogen

interactions in the sick pigs, however, further investigation is needed to help understand the dynamic regulation network.

It should be mentioned that the study consisted of a field experiment which was far less predictable than a more standardized infection model. However, it has the benefit of reflecting the situation in commercial production. There were substantial variations in health status between animals in the study. The nine DEGs that were identified and validated in this study and two *in vivo* experimental *Salmonella* and PRRSV infection models helped to reinforce our suggestion that these genes may act as valuable biomarkers for monitoring infections in pig. Nevertheless, it is also important to recognize that only results from the transcription level were obtained. Further research measuring the concentration-time profiles of cytokines in the serum of sick pigs are needed to determine the response of these cytokines. Moreover, studies at the translational level might also contribute to the verification of results from the present study.

Conclusions

We explored the transcriptional profile of the peripheral blood in pigs of different health status using RNA-Seq. Genes participating in a variety of local and systemic immune and inflammatory responses in many physiological processes were identified as differentially expressed in the sick pigs. These changes were consistent across a relatively large number of sick pigs collected across time and may therefore represent a robust set of genes associated with early infection. The DEGs highlighted in this work, especially

those encoding cytokines and their receptors, were also identified in *Salmonella* and PRRSV infection models in pigs. We suggested that these cytokines genes are directly involved in peripheral regulation of immune responses in the pig. Further studies are needed to better comprehend their contribution to the maintenance of health status.

Conflict of interest

The authors declare that there is no conflict of interest associated with this publication.

Author contributions

G.P., P.S., and L.L.G. conceived and designed the research. M.H.Y. and Y.M. performed the experiments while M.H.Y. was a visiting scholar in the University of Alberta sponsored by China Scholarship Council. M.H.Y. and H.B. analyzed the data. M.H.Y. wrote the original manuscript. All authors reviewed and approved the final manuscript.

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Table 1 Primer sequences used in qRT-PCR for the validation of DEGs

Gene	Sequence (5' to 3')
<i>IL-18</i>	Forward: TTAAAGGAAATGAGTCCTCCTGA Reverse: CCACATTCATCCTTTTCTTTCA
<i>IL22RA2</i>	Forward: TGGGAAACAAAAATCGATCC Reverse: CTGCGGGCTCCTTCATATAC
<i>CCL8</i>	Forward: GCCAGATTCAGTCTCCATCC Reverse: TGGAATTCTGGACCCACTTC
<i>CCR1</i>	Forward: CTGGACCTGGCCATACAAGT Reverse: AGAAGCCAGCAGAGAGTTCTG
<i>TNFSF13B</i>	Forward: GGGGCATCTCATACAGAGGA Reverse: CGTCTCCATCCCGTGATATT
<i>RELT</i>	Forward: GCTCCCGCTGTAGTCAGAAG Reverse: CAGAACTCGTCCGCTGCT
<i>CSF3R</i>	Forward: CTCTGGTCCAGGCCTATGTG Reverse: AAGAGGGGCTGAGTGGAGTC
<i>IFNGR1</i>	Forward: ATAGTGGCCTTGTGGAATCG Reverse: CCTCGGATTCTGCTGTGACT
<i>ACVR1B</i>	Forward: GGGAACCAAACGATACATGG Reverse: ACATACGACCTTCCGCATCT
<i>β-actin</i>	Forward: CACGCCATCCTGCGTCTGGA Reverse: AGCACCGTGTTGGCGTAGAG

Table 2 Significant DEGs encoding cytokines and their receptors

Cytokine	Gene symbol	Gene description	Ensembl gene No.	Fold change	logFC	logCPM
IL	<i>IL-1A</i>	interleukin 1, alpha	ENSSSCG00000008090	2.35	-1.23	2.94
	<i>IL-18*</i>	interleukin 18 (interferon-gamma-inducing factor)	ENSSSCG00000015037	3.73	-1.90	7.57
	<i>IL-1RAP*</i>	interleukin 1 receptor accessory protein	ENSSSCG00000021206	2.87	-1.52	3.76
	<i>IL-18RAP*</i>	interleukin 18 receptor accessory protein	ENSSSCG00000008157	2.63	-1.39	6.42
	<i>IL-1R2*</i>	interleukin 1 receptor, type II	ENSSSCG00000008163	7.15	-2.84	3.89
	<i>IL-22RA2*</i>	interleukin 22 receptor, alpha 2	ENSSSCG00000004158	14.06	-3.81	4.67
Chemokine	<i>CCL23</i>	chemokine (C-C motif) ligand 23	ENSSSCG00000017702	2.39	-1.26	9.63
	<i>CCL8*</i>	chemokine (C-C motif) ligand 8	ENSSSCG00000017721	2.59	1.37	1.94
	<i>CCR1*</i>	chemokine (C-C motif) receptor 1	ENSSSCG00000011322	2.37	-1.24	9.12
	<i>CCR2*</i>	chemokine (C-C motif) receptor 2	ENSSSCG00000024270	2.01	-1.01	3.53
	<i>XCR1</i>	chemokine (C motif) receptor 1	ENSSSCG00000026707	2.08	-1.06	2.67
TNF	<i>TNFSF13B*</i>	tumor necrosis factor superfamily, member 13b	ENSSSCG00000009542	2.53	-1.34	6.12
	<i>TNFRSF21</i>	tumor necrosis factor receptor superfamily, member 21	ENSSSCG00000001727	2.31	-1.21	5.81
	<i>TNFRSF8</i>	tumor necrosis factor receptor superfamily, member 8	ENSSSCG00000003437	2.63	-1.39	1.23
	<i>RELT*</i>	receptor expressed in lymphoid tissues	ENSSSCG00000014825	2.47	-1.31	6.51
CSF	<i>CSF2RA*</i>	colony stimulating factor 2 receptor, alpha, low-affinity	ENSSSCG00000030088	2.06	-1.04	6.78
	<i>CSF2RB*</i>	colony stimulating factor 2 receptor, beta, low-affinity	ENSSSCG00000000136	2.06	-1.04	8.66
	<i>CSF3R*</i>	colony stimulating factor 3 receptor	ENSSSCG00000025795	2.66	-1.41	8.17
Actvin	<i>ACVR1B*</i>	activin A receptor, type IB	ENSSSCG00000000233	5.79	-2.53	3.97
IFN	<i>IFNGR1*</i>	interferon gamma receptor 1	ENSSSCG00000004156	2.44	-1.29	7.58

* Fifteen genes found in the challenge studies with PRRSV and *Salmonella*.

Figure 1 Heatmap showing expression data for 246 differentially expressed genes. Rows represent individual samples from healthy and sick animals; columns indicate genes with significant expression differences between the two groups.

Figure 2 Expression of nine significant DEGs detected by RNA-Seq and validated by qRT-PCR. Results from RNA-Seq are shown by line graphs on the top and values are shown on the right Y-axis as log₂ (normalized reads). Results from qRT-PCR are shown by bar graphs on the bottom and values are shown on the left Y-axis as relative expression level. A,B and a,b – indicate significant difference in the relative expression of genes detected via qRT-PCR at $p < 0.05$ using cDNA from RNA samples before and after globin reduction (GR), respectively. C and c indicate significant difference in the expression of mRNA detected from RNA-Seq at $p < 0.001$ and FDR < 0.01 . Data are presented as Mean $\pm$ SE.