

RESEARCH ARTICLE

Transcriptomic basis of neutrophil ratio variation induced by poly I:C stimulation in porcine peripheral blood

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Abstract Neutrophils are vital components of defense mechanisms against invading pathogens and are closely linked with the individual antiviral capacity of pigs and other mammals. Neutrophilia is a well-known clinical characteristic of viral and bacterial infections. Using Affymetrix porcine genome microarrays, we investigated the gene expression profiles associated with neutrophil variation in porcine peripheral blood before and after polyriboinosinic-polyribocytidylic acid stimulation. Transcriptomic analysis showed 796 differentially expressed genes (DEGs) in extreme response (ER) pigs and 192 DEGs in moderate response (MR) pigs. Most DEGs were related to immune responses, included *MXD1*, *CXCR4*, *CREG1*, *MyD88*, *CD14*, *TLR2*, *TLR4*, *IRF3* and *IRF7*. Gene ontology analysis indicated that the DEGs of both ER and MR pigs were involved in common biological processes, such as cell proliferation, growth regulation, immune response, inflammatory response and cell activation. The ER and MR groups also showed differences in DEGs involved in biological processes. DEGs involved in cell division and cell cycle were specifically found in the ER pigs, whereas DEGs involved in cell migration were specifically found in the MR pigs. The study provides a basic understanding of the molecular basis for the antiviral capacity of pigs and other mammals.

Keywords neutrophil, peripheral blood, pig, poly I:C, transcriptome

1 Introduction

The pig industry is still facing the threat of infectious diseases at present, which have impacted seriously on

animal health and brought huge economic losses. Therefore, genetic improvement of the disease resistance of pigs should be enhanced. Some phenotypic immune traits have been used to identify disease resistance^[1], therefore, analysis of the transcriptome and the phenotypic immune traits to identify individual difference in pigs could be used to investigate the genetic basis of antiviral capacity and disease resistance.

Polymorphonuclear neutrophilic granulocytes, also known as PMNs or neutrophils, are the most abundant population of white blood cells. Neutrophils are characterized by a multi-lobed nucleus and numerous cytoplasmic granules^[2]. Neutrophils, which are derived from stem cells in the bone marrow, proliferate and differentiate into mature neutrophils equipped with many antimicrobial granules^[3]. Neutrophils provide effective defense mechanisms by performing phagocytosis, producing reactive oxygen intermediates and releasing antimicrobial granules^[4]. Under normal conditions, most neutrophils are localized in the bone marrow. Once infection occurs, neutrophils rapidly mobilize into the blood, and migrate to infection sites to fight against bacterial and fungal pathogens^[2,5]. Consequently, chronic neutrophilia is mainly associated with autoimmune disease and immunodeficiency syndromes including X-linked agammaglobulinemia, hyper IgM syndrome, common variable immunodeficiency and IgA deficiency^[6,7]. However, neutrophilia is also a well-known clinical characteristic of infections (e.g., bacteria, fungi, parasites and viruses), stress, acute inflammation (e.g., from asthma, burns, glomerulonephritis and gout), poisoning (e.g., arsphenamine, camphor, insect venoms, mercury, phenacetin, pyrogallol, quinidine and turpentine), acute hemorrhage (e.g., in intracranial, joint, peritoneal, and pleural cavities), and neoplasms and blood malignancies (e.g., chronic myelocytic leukemia, myelofibrosis, myeloid metaplasia and polycythemia vera)^[8–15]. Therefore, neutrophils, the

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key components of innate immunity and the first line of defense against invading pathogens, can be a representative immune trait in the study of disease resistance. Several studies have indicated that neutrophils increase during infection and inflammation in pigs^[16–18], but the molecular mechanism by which genes regulate this response remains unclear.

Polyriboinosinic-polyribocytidylic acid (poly I:C), a synthetic analog of double-stranded RNA (dsRNA) viruses, can trigger immune responses similar to those activated by real viral dsRNAs^[19–21]. Thus, poly I:C is commonly used to simulate viral infections for scientific research on the immune system and antiviral capacity^[22–24].

Previous studies have reported that poly I:C induces neutrophilia in mice and fish. The numbers of neutrophils detected was significantly increased in the lung and liver of mice by treatment with poly I:C^[25] and the neutrophil count in blood was significantly increased in fish after poly I:C stimulation^[26,27]. Despite its frequent use, detailed studies of neutrophil responses and transcriptomic alterations in response to poly I:C administration in pigs are lacking. In the present study, we employed poly I:C to simulate viral infection in pigs, and investigated the neutrophil responses and transcriptomic alterations in porcine peripheral blood with large and small variations of neutrophil ratio before and after poly I:C stimulation. The aim of this study was to gain insights into the transcriptome basis of varied neutrophil ratio in pigs and enrich understanding of the molecular basis for the antiviral capacity of pigs.

2 Materials and methods

2.1 Animals and stimulation

The experimental animals comprised 393 pigs from a Duroc-Erhualian F₂ population, including 216 females and 177 males, which were derived from 51 F₁ and 26 F₀ parents. The F₂ offspring were the F₁ sow's first litters and the pedigree was recorded in detail. All piglets were bred under the same forage and feeding management conditions. At the age of 35 days, the piglets were intravenously injected with poly I:C (CAS Registry Number 24939-03-5, Hangzhou Meiya Pharmacy Co., Ltd., No. 85, Sandun Road, Gongshu District, Hangzhou, Zhejiang, China) at a dose of 0.5 mg·kg⁻¹. Blood samples were separately collected from each individual by jugular venipuncture on day 33 as unstimulated controls and at 4 h after poly I:C stimulation on day 35^[22]. All disposals of the experimental animals were approved by the Scientific Ethic Committee of Huazhong Agriculture University (HZAUSW-2013-014).

Neutrophil counts and ratios were measured with a hematology analyzer (MEK-8222K 22, Nihon Kohden). In

most of the experimental pigs, neutrophil ratios in the whole blood increased after poly I:C stimulation. Three pigs with the maximum increases in neutrophil ratios were selected as extreme response (ER) pigs and another three pigs with the minimum increases in neutrophil ratios were considered as moderate response (MR) pigs.

2.2 RNA extraction and microarray hybridization

Microarray hybridization was conducted with total RNA from the six selected pigs. Total RNA was extracted from peripheral blood samples using TRizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and treated with RNase-free DNase I (MBI Fermentas, Thermo Fisher Scientific, Waltham, MA, USA). The 12 RNA samples, for microarray hybridization were extracted from the selected piglets on days 33 and 35. The gene expression in each sample was identified by Porcine Genome Arrays (Affymetrix, Santa Clara, CA, USA). RNA labeling and hybridization were performed by a commercial Affymetrix array service (GeneTech (Shanghai) Co., Ltd., Shanghai, China).

2.3 Microarray data analysis and functional annotation

Microarray data were deposited in the ArrayExpress repository (www.ebi.ac.uk/arrayexpress) and can be accessed with the accession number E-MTAB-4928^[28]. All raw microarray data sets (CEL files) were normalized by the Robust Multichip Average method of the Bioconductor AFFY package^[29] in the R environment. Differentially expressed genes (DEGs) were identified using the tool Linear Models For Microarray Data (LIMMA)^[30] with an empirical Bayes method. The DEGs were considered statistically significant for $P < 0.05$ and an absolute value of $\log_{2}FC > 1.0$. Two-way hierarchical clustering analysis was performed with all the identified DEGs.

Probe sets on the Affymetrix Porcine GeneChip were annotated with the annotation file obtained from Affymetrix (www.affymetrix.com/support/technical/annotationfilesmain.affx). Lists of DEGs were submitted to the DAVID Functional Annotation Bioinformatics Microarray Analysis website v6.7^[31] to annotate gene functions, including Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. In the functional enrichment analysis, $P < 0.05$ were considered statistically significant. The lists of Affymetrix probe sets were also uploaded into VENNY^[32] to illuminate the relationships of all DEGs.

2.4 Quantitative Real Time Polymerase Chain Reaction for validation of DEGs

The results of microarray analysis were validated via quantitative Real Time Polymerase Chain Reaction

(qRT-PCR). The qRT-PCR reaction system included 1/80 μg RNA from each sample, 0.4 μL primers, 2 μL template cDNA and 7.2 μL H_2O . The qRT-PCR reaction conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 20 s, annealing at 60°C for 20 s and 39 cycles of 72°C for 15 s. The primers were designed with OLIGO 7 software (Molecular Biology Insights, Colorado Springs, CO, USA), and the list of primers for qRT-PCR is shown in Table S1. *GAPDH* was selected as the housekeeping gene for normalization. Relative quantification analysis was performed through calculations using the $2^{-\Delta\Delta C_T}$ value method^[33]. Comparisons between qRT-PCR data sets were conducted by Student's *t*-test. Statistical significance was considered at $P < 0.05$.

3 Results

3.1 Phenotypic properties of neutrophil ratio

Most of the neutrophil ratios for all 393 pigs, with two exceptions, clearly increased after poly I:C stimulation. With the two tails of normal distribution as the basis for selection, three pigs with the maximum increases in neutrophil ratio were selected as the ER group and another three pigs with the minimum increases in neutrophil ratio were selected as the MR group. The difference in the average values between the two groups was statistically significant ($P < 0.01$) before and after poly I:C stimulation. The mean increase in neutrophil ratio was 51.33% (SD = 6.33) in the ER group and 13.03% (SD = 1.89) in the MR group (Fig. 1). The maximum difference in neutrophil ratio in the ER pigs (58.4%) was over 5-fold greater than the

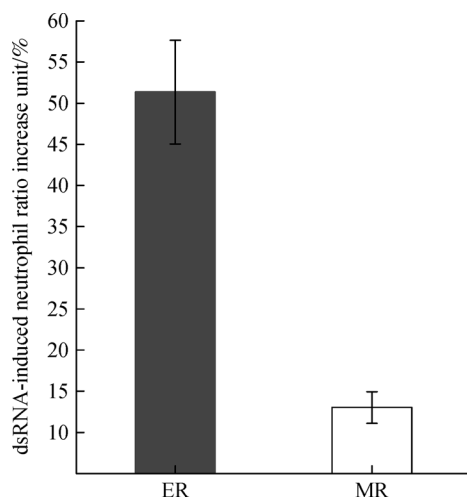


Fig. 1 Increase in neutrophil ratio induced by polyribinosinic-polyribocytidylic acid. The black and white bars indicate the average increase in neutrophil ratio in the extreme and moderate response pigs (ER and MR) after poly I:C stimulation, respectively. The difference between two groups of pigs was significant ($P < 0.01$).

minimum difference in the MR pigs (11.4%) before and after treatments. These results indicated that the increase in neutrophil ratio induced by dsRNA (poly I:C) was highly variable in the experimental pig population.

3.2 Differentially expressed genes identification

DEGs after poly I:C treatment in each group were detected using LIMMA. After poly I:C stimulation, 796 DEGs were identified in the ER pigs ($P < 0.05$) (Table S2), whereas 192 DEGs were identified in the MR pigs ($P < 0.05$), with the fold changes ranging from -3.19 to 4.24 and -3.34 to 3.01 , respectively (Table S3). Up to 561 genes were downregulated and the remaining 235 genes were upregulated among the DEGs in the ER group. The expression of 132 genes decreased after stimulation and the expression of 60 genes increased in the MR group. According to the results, the number of DEGs was higher in the ER group than the MR group, and both ER and MR piglets had more downregulated genes than upregulated genes.

3.3 Transcriptome clustering and Venn diagram for differentially expressed genes

An unsupervised two-way hierarchical clustering analysis was performed to understand further the transcriptional effect of the poly I:C treatment in all samples. The results showed that all the samples could be clearly classified into one of two major clusters: poly I:C treatment and non-treatment clusters (Fig. 2).

A Venn diagram was generated to display the overlapping or unique members of DEGs between the classifiers. As shown in Fig. 3, the MR and ER groups shared 34 upregulated DEGs and 40 downregulated DEGs in common. Notable upregulated DEGs in the two groups included *CSF1*, *CXCL16*, *C3*, *PTK2B*, *STOM* and *IL1RN*, and the downregulated DEGs in the two groups included *CCL8* and *PIK3R1*. These genes are associated with immune response, which suggested some common immune responses in the ER and MR pigs had been activated by poly I:C stimulation. The Venn diagram (Fig. 3) also showed differences between MR and ER pigs. In the ER group 199 DEGs were upregulated and 521 DEGs were downregulated. In the MR pigs, 26 upregulated DEGs and 90 downregulated DEGs did not overlap with other categories. The results indicated that more genes were downregulated in response to poly I:C stimulation. Two genes, *CD14* and block of proliferation 1 (*BOPI*), were upregulated in the ER group but downregulated in the MR group. *CD14* and *BOPI* respectively have important roles in the cell recruitment and cell cycle. Therefore, we speculated that the different expression of the two genes could indicate that the neutrophil ratio in the ER and MR pigs responded differently of poly I:C stimulation.

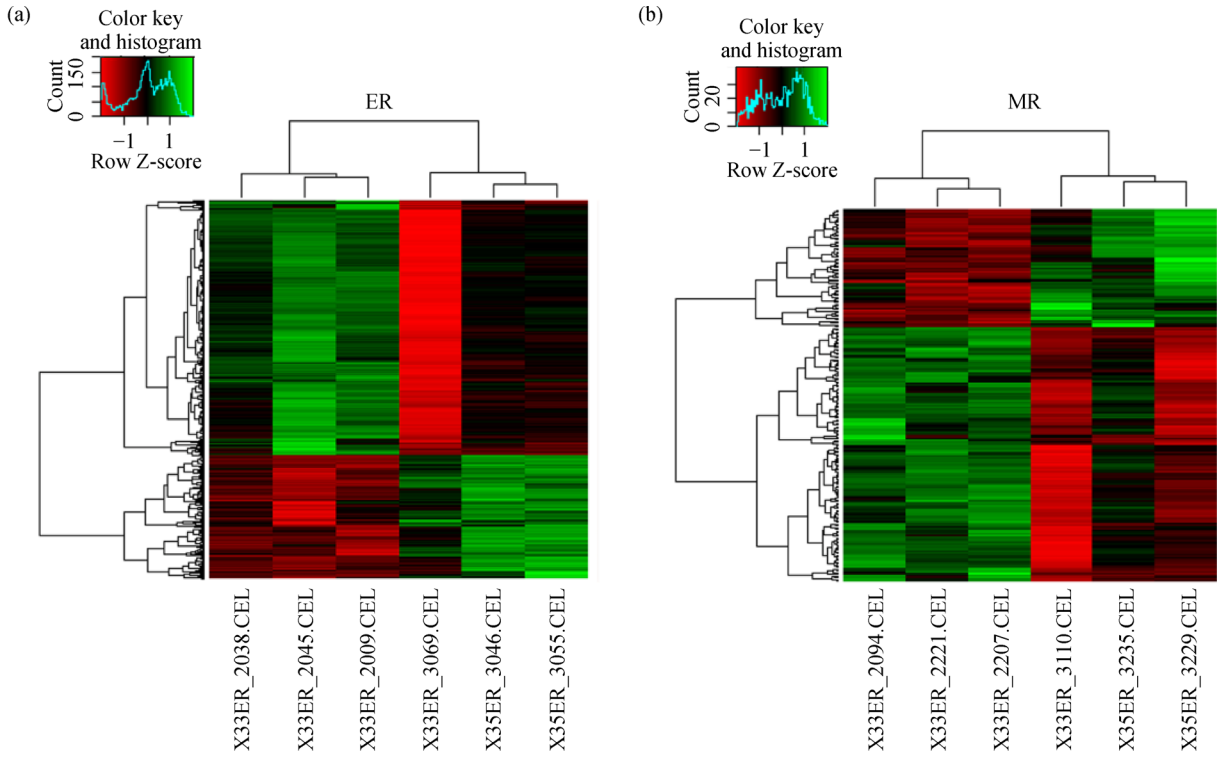


Fig. 2 Hierarchical cluster analysis of all samples. (a) Cluster of unstimulated and poly I:C-stimulated conditions in extreme response (ER) pigs; (b) cluster of unstimulated and poly I:C-stimulated conditions in the moderate response (MR) pigs. The figure depicts high (red) and low (green) relative levels of gene expression. Mean-centered gene expression ratios are depicted by a log₂ pseudocolor scale (ratio fold change indicated); gray denotes poorly measured data.

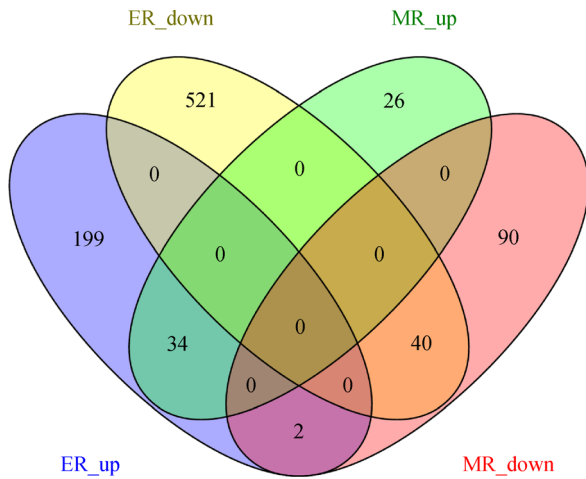


Fig. 3 Venn diagram of differentially expressed genes in the extreme and moderate response pigs (ER and MR)

3.4 Validation of microarray data by qRT-PCR analyses

Some genes were selected for qRT-PCR analysis with the same RNA samples used for Affymetrix microarray hybridization to validate the reliability of the microarray data. The selected genes included *CD14*, *CXCR4*, *STAT5A*, *STOM*, *TLR4* and *HOPX* in the ER group and *CD14*,

HOPX, *STOM*, *CXCL16*, *BOP1*, cellular repressor of E1A-stimulated genes (*CREG1*), *C3*, *PTK2B*, *RETN*, and *PIK3R1* in the MR group. The results of qRT-PCR analysis (Fig. 4) indicated that all detected genes had the same expression trends as in the microarray analysis. In the ER group, only *CD14* did not reach a significant level in qRT-PCR. In the MR group, eight genes were significant by qRT-PCR except for *CD14* and *HOPX*. To summarize, the qRT-PCR results validated the reliability of the microarray data.

3.5 Comparative functional annotation of differentially expressed genes

GO annotation provides ontologies for biological processes, cellular components and molecular functions by DAVID. The GO annotation showed that the ER group had more terms than the MR group regardless of biological processes, cellular components and molecular functions, which indicated that the ER pigs reacted more intensely than the MR pigs to poly I:C treatment. Furthermore, the number of terms for biological processes in both groups was more than that for cellular components and molecular functions.

GO annotation showed that the ER group had 113 terms associated with biological processes, whereas the MR

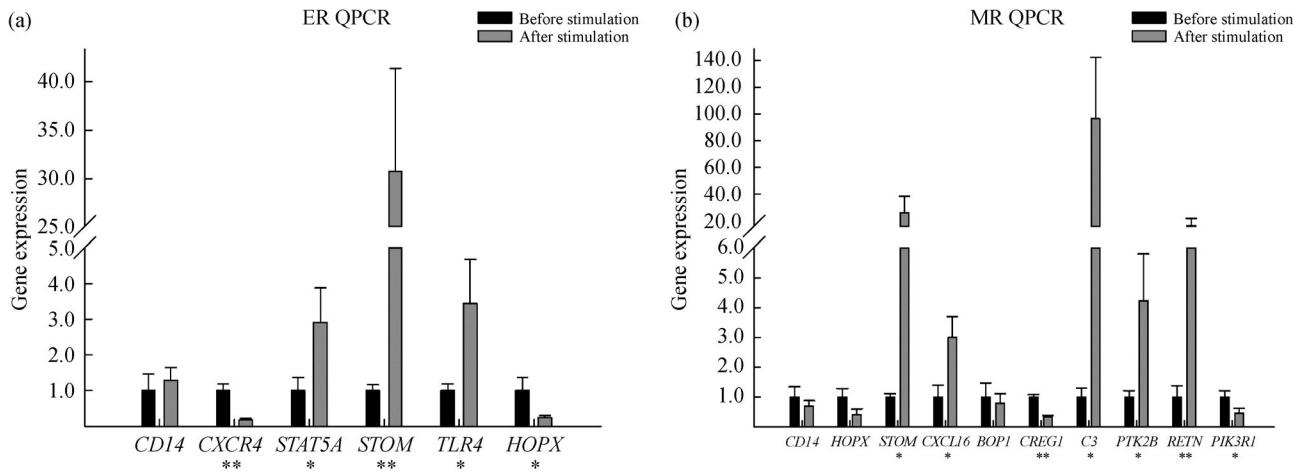


Fig. 4 Validation of microarray data by real-time qRT-PCR analyses. (a) Six genes were selected for qRT-PCR experiments in extreme response (ER) pigs; (b) expression of ten genes in moderate response (MR) pigs was measured by qRT-PCR experiments. The black and gray bars show gene expression before and after poly I:C stimulation. * denotes statistically significant differences ($P < 0.05$).

group only had 20. This suggests that the dsRNA-induced responses were highly variable between the ER and MR groups. The two groups only had nine terms of biological processes in common. These terms included cell proliferation, growth regulation, immune response and inflammatory response. Given the common terms of the biological processes, DEGs were more involved in the ER group than in the MR group.

In terms of biological processes, the DEGs of both the ER and MR groups participated in cell proliferation, growth regulation, immune response and inflammatory response, but more DEGs in the ER group were involved in exocytosis, negative regulation of cell death, cell division and M phase of the mitotic cell cycle (Fig. 5a). These findings revealed that, regardless of neutrophil ratio, the immune response was activated via common and essential biological processes at an early stage of viral invasion.

We further annotated biological processes associated with the DEGs according to the Venn diagram categories (Table S4) to compare the differences between the MR and ER groups. The terms were considered significant when $P < 0.05$ and if the count of DEGs was more than five. We found that the 199 upregulated DEGs in the ER group were involved in biological processes related to immune response, inflammatory response, regulation of innate immune response, cell activation, regulation of cell death and negative regulation of cell death. The 26 upregulated DEGs in the MR group, on the other hand, were only related to peptide cross-linking and transforming growth factor beta receptor signaling pathway. Furthermore, the 521 downregulated DEGs in the ER group were mainly involved in the biological processes of cell division, M phase of mitotic cell cycle, nuclear division, organelle fission and chromosome segregation, whereas the 90 downregulated DEGs in the MR group were related to

responses to oxygen levels, macromolecule catabolic process and protein catabolic process. From these findings, we inferred that the predominance of downregulation may be mostly attributed to increased neutrophil ratio induced by poly I:C, and that some upregulated DEGs may be involved in normal immune response after poly I:C stimulation. In the ER group, the biological processes related to regulation of cell death and negative regulation of apoptosis were found simultaneously, which suggests that the neutrophils increased after poly I:C stimulation.

DEGs of the MR group were associated with terms for cellular components, such as intracellular organelle lumen, membrane-enclosed lumen, organelle lumen and nuclear lumen. DEGs of the MR group were also associated with terms for cellular components, such as extracellular space, extracellular region part, and extracellular region (Fig. 5b). The two groups had no common terms for molecular functions. Compared with the MR group, the ER group presented more genes involved in kinase binding, protein kinase binding, unfolded protein binding and inorganic cationtransmembrane transporter activity (Fig. 5c).

The DEGs were further classified according to the KEGG pathway annotation (Fig. 5d). No common pathways were identified between the ER and MR groups, suggesting that the ER and MR pigs had very different responses to poly I:C stimulation. The DEGs in the ER group were involved in the chemokine signaling pathway, oxidative phosphorylation, insulin signaling pathway and toll-like receptor signaling pathway. The MR group had only a few DEGs related to pathway terms, such as the complement and coagulation cascades and lysosome and fatty acid elongation in the mitochondria. On the basis of these differential annotation results, we hypothesized that the ER pigs exhibit less immune capacity against viral infections than the MR pigs.

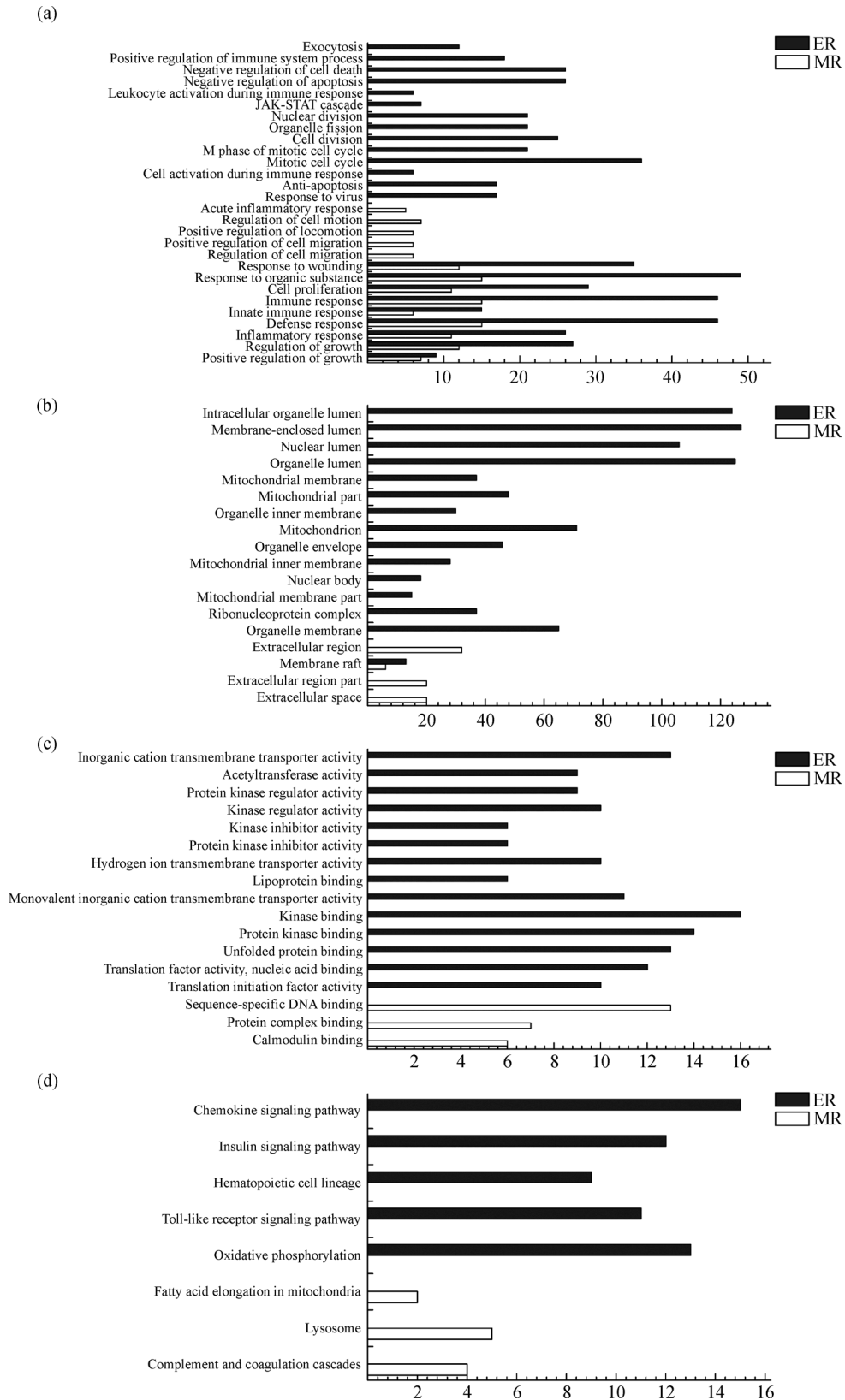


Fig. 5 Comparative functional annotation of differentially expressed genes (DEGs). DEGs were grouped according to their putative functions. (a) Comparison of distribution of the DEGs by Gene Ontology (GO) biological process induced by poly I:C treatment in the extreme and moderate response pigs (ER and MR); (b) comparison of distribution of the DEGs by GO cellular component induced by poly I:C treatment in the ER and MR pigs; (c) comparative GO molecular function distribution of the DEGs following poly I:C treatment of the ER and MR pigs; (d) comparative KEGG pathway annotation distribution of the DEGs following poly I:C treatment in the ER and MR pigs. The black and white bars indicate the number of altered genes in the ER and MR groups, respectively.

4 Discussion

4.1 Common biological processes found in extreme and moderate response pigs

According to functional analysis, the extreme response (ER) and moderate response (MR) pigs shared some common biological processes, such as cell proliferation, immune response, inflammatory response, innate immune response, and growth regulation. However, the members of these biological processes in the two groups were different. The results showed that the innate immune responses in both the ER and MR groups can be activated rapidly.

The ER group had 31 DEGs (10 upregulated and 21 downregulated) involved in the biological process of cell proliferation. The important upregulated DEGs were *MXD1*, *ISG20*, *IRF2*, *BOP1*. *MXD1* mediates cellular proliferation and granulocyte differentiation^[34,35]. *ISG20* is an interferon-inducible exonuclease gene that prevents the replication of several RNA viruses^[36]. *IRF2*, an interferon regulatory factor, has a critical role in mediating gene expression, immune responses and cell growth^[37,38]. The regulation of cell growth is often tightly linked to cell proliferation in mammals^[34]. *BOP1*, a conserved nucleolar protein, reportedly blocks cell cycle progression^[39,40]. *CXCR4* is a key regulator of neutrophil counts in the bone marrow under infection or stress conditions. Several studies found that neutrophil counts in the bone marrow of *CXCR4*-deficient mice decrease while neutrophil numbers in the blood markedly increase^[41–43]. The study indicates that *CXCR4*, by interacting with *CXCL12* signaling, has a key role in controlling neutrophil homeostasis and therefore negatively regulates their release from the bone marrow^[5]. In the ER pigs, *CXCR4* was one of the downregulated DEGs involved in cell proliferation. Our results are consistent with previous studies that suggested *CXCR4* negatively regulates neutrophil circulation from the bone marrow to the blood during viral infection. In the MR pigs, we detected six upregulated genes and five downregulated genes involved in cell proliferation. The upregulated genes, which were also found in ER pigs, included *ERG*, *PTK2B*, colony stimulating factor-1 (*CSF1*), *BOP1*, *BCAT1*, and *GPC4*. The downregulated genes were *CREG1*, *S100B*, *PELO*, *NPY*, and *TGFBI*, which were specifically expressed in the MR pigs. *CSF-1* controls the production, differentiation and function of macrophages, and greatly influences the innate immune response to external infections^[44]. *CREG1* promotes cellular proliferation and inhibits cell differentiation and reportedly promotes the proliferation and migration of human umbilical vein endothelial cells^[45].

Mature neutrophils reside in the bone marrow and when stimulated by inflammatory cytokines, they are acutely released from the bone marrow reserve, resulting in a dramatic rise in circulating neutrophil numbers in the blood^[46]. The release of neutrophils from the bone marrow

therefore has a critical role in their movement to sites of inflammation^[47]. A total of 26 and 11 DEGs related to inflammatory responses were found in the ER and MR groups, respectively. *TLR2*, *TLR4*, myeloid differentiation primary response 88 (*MyD88*), and *CD14* were upregulated in the ER group. Toll-like receptors (TLRs) are essential in the recognition of and defense against invading microorganisms during the inflammatory response by recognizing pathogen-associated molecular patterns^[48,49]. *TLR2* and *TLR4* are expressed by a variety of immune cell types, including neutrophils^[50]. *TLR2* and *TLR4* have also been reported to recognize and bind numerous exogenous and endogenous ligands from inflamed tissue^[51]. The activated TLRs recruit cytosolic adaptors, including *MyD88*, which can trigger downstream signaling pathways promoting the production of inflammatory cytokines and chemokines^[52]. An earlier study also suggested that *TLR4* in blood induces thrombocytes to bind to neutrophils, which activates neutrophils and releases neutrophil extracellular traps^[53]. *CD14* can be expressed by neutrophils and is crucial to the circulation of neutrophils^[54]. The neutrophil *CD14* participates in acute inflammatory response^[55]. Neutrophils can respond to bacterial lipopolysaccharides via *CD14* with *TLR4* and *MD-2*^[56]. However, the absence of *CD14* can induce hypoinflammation and insufficient immune cell recruitment^[57]. In the MR group *CD14* was downregulated, and *TLR2*, *TLR4* and *MyD88* were not detected. The findings may explain why the neutrophil ratio was lower in the MR group than in the ER group.

4.2 Specific biological processes found in extreme and moderate response pigs

In total, 104 specific biological processes were identified in the ER pigs, whereas only 11 were detected in the MR pigs. These specific biological processes in the ER and MR groups can also provide clues about the large difference between their neutrophil ratios.

In the ER group, a large proportion of specific biological processes, including cell division, mitotic cell cycle, cell cycle phase, cell cycle, cell cycle process, M phase of mitotic cell cycle and M phase, were related to the cell cycle. On average, about 36 DEGs were involved in these biological processes. The primary DEGs were *SETD8*, *MAD2L2*, *CETN3*, *SMC1A*, *NUF2*, *PDS5B*, *CDC10*, *NUSAP1*, *CD2AP*, *CCNG1*, *CCNB2*, *SMC3*, *SMC4*, *NEK9*, *POLS*, *NSL1*, *NCAPG*, *HELLS* and *TIPIN*. Cell division is the basis for individual growth and reproduction in multicellular organisms^[58]. It occurs as part of a complete cell cycle, which is a highly integrated process involving physical division and DNA replication^[59].

Although the specific biological processes in the MR pigs were far fewer than those in the ER pigs, they were associated with cell migration, including regulation of cell migration, positive regulation of cell migration, regulation

of cell motion and positive regulation of locomotion. On average, just over six DEGs were involved in these biological processes, and the upregulated genes included *ICAMI*, *CSF1*, *CXCL16*, *PTK2B*, *EDN1*, among others. Cell migration is critically important not only in the development of multicellular organisms, but also in immune responses^[60]. Immune cell migration is crucial in delivering protective defense responses to damaged tissues^[61]. For example, leukocytes rapidly migrate into infection areas to perform immune functions during inflammatory response^[62]. The specific biological processes in the MR and ER pigs can provide a partial explanation for neutrophil variation.

4.3 Special pathways found in extreme and moderate response pigs

Neutrophils are one of the major phagocytes that differentiate between pathogens and innate cells by TLR signaling^[63]. TLRs can recognize pathogen-associated molecular patterns by recruiting specific adaptor molecules. Therefore, TLR signaling plays crucial roles in many aspects of innate immunity^[49]. The TLR signaling pathway was the special pathway found in the ER pigs. The primary DEGs in ER pigs included *TLR2*, *TLR4*, *MyD88*, interferon regulatory factor-3 (*IRF3*), interferon regulatory factor-7 (*IRF7*), and *CD14*, all of which were upregulated. The cell-surface TLRs, *TLR2* and *TLR4*, can recognize microbial membrane lipids and lipoproteins^[64]. MyD88 is an adapter protein linking TLRs and interleukin-1 receptors (IL-1Rs) to activate the downstream molecules^[65]. The transcription factors *IRF3* and *IRF7* have pivotal roles in inducing the activation of the IFNA genes, which strengthen the development of the antiviral response in the host^[66,67]. *CD14* and TLRs, are essential in recognizing bacterial envelope components from Gram-negative and Gram-positive bacteria, mycobacteria and other bacteria^[68]. Recent studies have identified that these two important receptors play the same role in antiviral response, such as the response to respiratory syncytial virus in humans^[69].

The chemokine signaling pathway is a specific pathway in the ER group. Chemokines are important in a broad array of biological responses, such as cell migration and immune and inflammatory responses^[70]. Neutrophils are not only chemokine producers but are also the primary targets for chemokines^[71]. Once having migrated to the sites of inflammation and injury, neutrophils increase in number to produce chemokines^[71,72] and these neutrophil-derived chemokines coordinate the innate and adaptive immune responses^[73]. In the ER pigs, several identified DEGs including *CCL8*, *CCR1*, *CXCR4*, *CXCL16*, and *PTK2B*, are involved in the chemokine signaling pathway.

During phagocytosis, neutrophils exhibit degranulation and release a series of lysosomal enzymes. The lysosome pathway was identified as one of specific pathways in the

MR group, suggesting that neutrophils are involved in the immune response in the MR pigs. The DEG members of this pathway included *TCIRG1*, *ASAH1*, *PPT1*, *IDS* and *GNS* in the MR group.

5 Conclusions

This study investigated the transcriptome profiles of neutrophil variation in peripheral blood of pigs using the Affymetrix porcine genome microarray. Analyses of DEGs, biological process and pathways revealed that many DEGs were involved in the immune defense responses of both ER and MR pigs, although the two groups showed different immune responses after dsRNA stimulation. These DEGs could be used as promising candidate gene targets for disease resistance in pig breeding. In the future we will identify the function of important DEGs in other pig breeds. Overall, our studies provide a deeper understanding of the molecular basis of genetic resistance in pigs.

Supplementary materials The online version of this article at <http://dx.doi.org/10.15302/J-FASE-2017162> contains supplementary materials (Tables S1–S4).

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Compliance with ethics guidelines Haiyan Wang, Qiaoxia Zhang, Lilin Yin, Xiangdong Liu, Shuhong Zhao, Mengjin Zhu, and Changchun Li declare that they have no conflicts of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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