Construction and analysis for dys-regulated lncRNAs and mRNAs in LPS-induced porcine PBMCs

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Abstract
Long non-coding RNAs (lncRNAs) are emerging as key regulators in inflammation. However, their functions and profiles in LPS-induced inflammation in pigs are largely unknown. In this study, we profiled global lncRNA and mRNA expression changes in PBMCs treated with LPS using the lncRNA-seq technique. In total 43 differentially expressed (DE) lncRNAs and 1082 DE mRNAs were identified in porcine PBMCs after LPS stimulation. Functional enrichment analysis on DE mRNAs indicated these genes were involved in inflammation-related signaling pathways, including cytokine–cytokine receptor interaction, TNF-α, Nf-κB, Jak-STAT and TLR signaling pathways. In addition, co-expression network and function analysis identified the potential lncRNAs related to inflammatory response and immune response. The expressions of eight lncRNAs (ENSSSCT00000045208, ENSSSCT00000051636, ENSSSCT00000049770, ENSSSCT0000005966, ENSSSCT00000047491, ENSSSCT00000049750, ENSSSCT00000054262 and ENSSSCT00000044651) were validated in the LPS-treated PBMCs by quantitative real-time PCR (qRT-PCR). In LPS-challenged piglets, we identified that expression of three lncRNAs (ENSSSCT00000051636, ENSSSCT00000049770, and ENSSSCT00000047491) was significantly up-regulated in liver, spleen and jejunum tissues after LPS challenge, which indicated that these lncRNAs might be important regulators for inflammation. This study provides the first lncRNA and mRNA transcriptomic landscape of LPS-mediated changes in porcine PBMCs, which might provide potential insights into lncRNAs involved in regulating inflammation in pigs.

Keywords
Inflammation, lipopolysaccharide, lncRNA, mRNA, peripheral blood mononuclear cells, pig

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Introduction
Gram-negative bacteria cause a serious disease of pigs that is a major threat to swine production industries in China.¹ Endotoxin/LPS, which originates from the cell envelope of Gram-negative bacteria, stimulates macrophage/monocyte cells to produce pro-inflammatory cytokines (e.g., TNF-α, IL-1β and IL-6).² In inflammatory processes, LPS induces TLR4 signaling with activation of the TLR4/NF-kB pathway and the NLR family protein 3 (NLRP3) inflammasome.³,⁴ However, considering the complexity in LPS-induced inflammatory responses, other levels of regulation may also be involved.

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides, which have been implicated in diverse functions, including transcriptional regulation in cis or trans, organization of nuclear domains, and regulation of proteins or RNA molecules.⁵ LncRNAs are emerging as key regulators in inflammation. For example, lncRNA-HOTAIR is up-regulated in cardiomyocytes...
of LPS-induced sepsis mice and induces TNF-α production through NF-κB activation.⁶ LncRNA THRIL is over-expressed in the LPS-stimulated cells, and aggravates LPS-induced injury possibly via sponging miR-34a.⁷ LncRNA MALAT1 is induced by IL-6 in LPS-treated cardiomyocytes and its over-expression can enhance TNF-α expression via activation of SAA3.⁸ The LPS-induced lncRNA Mirt2 functions as a repressor of LPS-induced inflammatory injury in ATDC5 chondrocytes by inhibiting the NF-κB and Notch signaling pathways.⁹ However, the definition of functional lncRNAs in pigs is still limited, partly due to their low sequence conservation and lack of identified shared properties across species.¹⁰

Considering the role of lncRNAs in the inflammatory response, the present study was designed to discover and explore the lncRNA expression profile of porcine PBMCs in response to LPS. To date, there has been no systematic attempt to identify the lncRNAs whose expression is changed after the induction of the innate immune response in pigs. This analysis of lncRNA expression changes in porcine PBMCs after LPS stimulation would contribute to the current knowledge of lncRNA functions in Gram-negative bacterial infection disease pathogenesis in pigs.

**Material and methods**

**Cell isolation, culture and stimulation**

The PBMCs from the blood of the Duroc × Landrace × Large White (DLW) crossbred piglets (~15 kg, ~8 wk old) were isolated by Ficoll-Hypaque density gradient centrifugation at 25°C.¹² The PBMCs were cultured in RPMI 1640 medium (Gibco, Australia) supplemented with 10% heat-inactivated FBS (Gibco, Australia), 2 mmol/l L-glutamine, 100 U/ml penicillin and streptomycin (Gibco) at 37°C under 5% CO₂. LPS (Escherichia coli serotype 026: B6, Sigma Chemical, St Louis, MO, USA) was dissolved (10 μg/ml) in saline solution. The PBMCs were cultured at a concentration of 1 × 10⁶/ml per well of the 6-well plate, and were treated with LPS (the final concentration was 1 μg/ml) for 4, 8, 12 and 24 h. Cells were further centrifuged for 10 min at 3500 g and harvested for RNA extraction.

**Animals and tissues collection**

A total of 42 (7.1 ± 0.9 kg) weaned piglets (DLW) were randomly divided into seven treatments (six pigs per treatment). The piglets were injected with 100 μg/kg body mass LPS, and slaughtered at 0 h (before LPS challenge), 1, 2, 4, 8, 12 and 24 h (after LPS challenge). The various tissues samples (skeletal muscle, heart, liver, spleen, lung, kidney, jejunum, stomach, brain and thymus) were dissected and snap-frozen in liquid nitrogen. All animal procedures were conducted according to the guidelines for experimental animals established by the Hubei Province, China Biological Studies Animal Care and Use Committee and approved by Wuhan Polytechnic University (Wuhan, China).

**Quantitative RT-PCR**

Total RNA was isolated from cell and tissues samples using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA synthesis and quantitative real-time PCR (qRT-PCR) were carried out as previously described.¹³ Expression of mRNA and lncRNA was analyzed using the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal normalization control. All data were analyzed using the 2^ΔΔCT method.¹⁴ Sequences of specific primers are shown in Table S1 in the supplemental materials.

**Cytokine TNF-α measurement**

The concentration of TNF-α in the supernatants of PBMCs was measured using commercially available porcine ELISA kits (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer’s instruction.

**High-throughput sequencing**

RNA quality was examined by gel electrophoresis, and only paired RNA with high quality was used for lncRNA-seq. LncRNA-seq libraries were prepared according to the manufacturer’s instructions and then applied to sequencing on Illumina HiSeq 3000 in Shanghai Genery Co. Ltd (Shanghai, China). The original reads were harvested from the Illumina HiSeq sequencer. 3’ adaptor-trimming and low-quality removal was performed with Trim Galore software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), after which the resulting clean reads were used for lncRNA analysis. Clean reads were aligned to the pig reference genome (Sscrofa11.1) using STAR software (https://github.com/alexdobin/STAR). All the data are available in the Sequence Read Archive database under the accession number RPJNA656175.

**LncRNA and mRNA differential expression analysis**

To determine lncRNA and mRNA differential expression between the LPS-stimulated and unstimulated
groups, the expression of each transcript was normalized to the total number of reads in the samples using the following formula: \( \text{FPKM} = \frac{\text{total fragments}}{(\text{mapped reads (millions)} / C2 \times \text{exon length (KB)})} \). The fold change in lncRNA or mRNA reads was presented as \( \log_2 \) transformation using the following formula: Fold change = \( \log_2 (\text{LPS/Control}) \). An adjusted \( P \) value less than 0.05 was considered significant. The 2-fold change criterion was chosen. Genome distribution of differentially expressed (DE) lncRNAs and mRNAs was illustrated with Circos (http://circos.ca/).

**Functional enrichment analysis**

Gene ontology (GO) enrichment and KEGG analysis of DE mRNA was performed with DAVID 6.8 database (https://david.ncifcrf.gov/). Protein–protein interaction networks between DE mRNA were analyzed by Ingenuity Pathway Analysis (IPA).

**Target gene prediction and co-expression network construction**

The predicted potential target genes whose loci were within a 10-kb window upstream or downstream of the lncRNA were considered cis-regulated genes. To determine the trans-regulated genes of the DE lncRNAs, the lncRNA and mRNA co-expression analysis was performed using the Pearson correlation coefficient (PCC) method. The PCC was \( \geq 0.9 \) The common genes between the potential targets of DE lncRNAs and DE mRNAs were analyzed using Venn analysis.

The network of coding–non-coding co-expressed genes was constructed with the biological functions to recognize the novel and significant lncRNA. Correlations between lncRNAs and their corresponding mRNAs were calculated with Pearson’s correlation \( (|\text{correlation}| \geq 0.9) \) and were used to draw the co-expression network through Cytoscape v 3.7.1.

**Statistical analyses**

The data are shown as means ± SD. Differences were tested using ANOVA and the Student’s paired \( t \)-test. The level of significance was set at \( P < 0.05 \) for all data analysis.

**Results**

**The expression of TNF-α, IL-1β and IL-6 in LPS-induced PBMCs**

To identify principal LPS-responsive lncRNAs, PBMCs isolated from the whole blood of the three healthy pigs were stimulated with LPS for 4, 8, 12 and 24 h to induce inflammatory response. The mRNA of TNF-α increased significantly in porcine PMBCs within 12 h and peaked at 4 h after LPS stimulation (Figure 1a). IL-6 and IL-1β were significantly increased at each time point and peaked at 8 h and 4 h, respectively (Figure 1b, c). We also applied ELISA to determine TNF-α protein level in the cell culture supernatant. The concentration of TNF-α was obviously increased by LPS stimulation compared with the control at each time point (Figure 1d), and it was undetected in controls at 12 and 24 h. These results suggested an acute inflammation was induced by LPS stimulation in PBMCs. Then, the RNA samples isolated from PBMCs treated for 8 h were used for further high-throughput sequencing.

**Characters of lncRNA-seq**

After quality control, a total of 92,550,258, 93,643,366, 107,683,222, 125,384,254, 121,886,474 and 223,904,698 clean reads with greater than 94.36% of Q30 were obtained in L1, L2, L3, N1, N2 and N3 libraries (Table 1). Among them, a total of 97.60% (L1), 98.20% (L2), 97.90% (L3), 97.30% (N1), 97.50% (N2) and 96.99% (N3) reads from the six libraries were mapped to the pig reference genome (Sus scrofa 11.1).

**LncRNA and mRNA profile changes in response to LPS stimulation**

The whole expression feature of transcripts is shown in Figure 2a. The expression level of the transcripts in the control was slightly higher than that in LPS-treated PBMCs. A total of 27,430 mRNAs and 1074 lncRNAs were obtained from our six libraries. Of the 43 DE lncRNAs, 31 were significantly up-regulated and 12 were significantly down-regulated in LPS-stimulated PBMCs compared with the control (Figure 2b, c, e and Table S2). Of the 1082 DE mRNAs, 636 were significantly up-regulated and 446 were significantly down-regulated in PBMCs after LPS stimulation (Figure 2b, d and Table S3).

We next predicted the cis- and trans-target mRNA of the DE lncRNAs and compared these predictions with our mRNA sequencing results (Table S4, S5). As shown in Figure 2f, 621 target mRNAs of up-regulated lncRNAs and 564 targets of down-regulated lncRNAs showed up-regulated expression, while 419 target mRNAs of up-regulated lncRNAs and 387 targets of down-regulated lncRNAs showed down-regulated expression during LPS stimulation.

**Basic characteristics of lncRNAs in the PBMCs**

The basic characteristics of all DE lncRNAs and DE mRNAs in PBMCs, which were widely distributed in all chromosomes except the Y, were shown in the Circos plot (Figure 3a). Next, we classified the PBMC lncRNAs into five categories according to the
genomic loci of their neighboring genes (Figure 3b). Although 10% of lncRNAs were not successfully categorized, the well-annotated lncRNAs were classified into the following categories: intergenic (22%), antisense (9%), intronic (57%) and bidirectional (2%), and sense (0%).

Function analysis of DE mRNAs

The functional enrichment analysis of the 636 significantly up-regulated genes was performed. Our GO analysis included three parts: biological process (BP), cellular component (CC) and molecular function (MF). The top 15 GO enrichment for BP for the up-regulated mRNAs are illustrated in Figure 4a, including inflammatory response, immune response, positive regulation of inflammatory response and chemokine-mediated signaling pathway. The significantly enriched GO terms for CC and MF were identified, such as extracellular space, external side of plasma membrane, integral component of plasma membrane, etc.

Table 1. Basic data of sequencing in LPS- stimulated and unstimulated PBMCs.

| Terms                | L1 library   | L2 library   | L3 library   | N1 library   | N2 library   | N3 library   |
|----------------------|--------------|--------------|--------------|--------------|--------------|--------------|
| Raw reads            | 93,474,028   | 94,531,006   | 108,795,010  | 126,394,540  | 123,017,688  | 225,897,868  |
| Clean reads          | 92,550,258   | 93,643,366   | 107,683,222  | 125,384,254  | 121,886,474  | 223,904,698  |
| Clean reads rate, %  | 99.01        | 99.06        | 98.98        | 99.2         | 99.08        | 99.12        |
| Clean Q30 bases rate, %    | 95.11        | 95.16        | 95.16        | 94.36        | 94.41        | 94.36        |
| Mapped reads         | 89,943,602   | 91,841,971   | 105,013,431  | 121,730,896  | 118,421,869  | 215,902,508  |
| Mapped rate, %       | 97.60        | 98.20        | 97.90        | 97.30        | 97.50        | 96.99        |

L1, L2 and L3 represent three experimental libraries (LPS-stimulated PBMCs); N1, N2 and N3 represent three control libraries (unstimulated PBMCs).
Figure 2. Expression profiling changes of mRNA and lncRNA in LPS-stimulated and -unstimulated PBMCs. (a) The relative expression level of the transcripts in the control and LPS groups. (b) Histogram showing the number of up- and down-regulated lncRNAs and mRNAs in the LPS group compared with the control. (c) Volcano plot indicating up- and down-regulated lncRNAs in LPS group when compared with the control. Red represents up-regulation and green represent down-regulation. (d) Volcano plot indicating up- and down-regulated mRNA in LPS group when compared with the control. (e) Heat map of lncRNA showing hierarchical clustering of altered lncRNAs in six groups. (f) Venn diagram showing the overlapping number of targeted mRNAs in up-regulated lncRNAs, targeted mRNAs in down-regulated lncRNAs, up-regulated mRNAs, and down-regulated mRNAs.
membrane, cytoplasm, IκB/NF-κB complex, cytokine activity, chemokine activity and growth factor activity.

The 446 significantly down-regulated genes were also selected to carry out the functional enrichment analysis. Twenty-four GO terms, including protein localization to plasma membrane, regulation of G-protein coupled receptor protein signaling pathway, cell surface, cell–cell junction, and kinase activity, are shown in Figure 4b.

Meanwhile, KEGG results from the significantly up- and down-regulated genes indicated that the top 30 significantly signaling pathways were enriched (Figure 4c), such as cytokine–cytokine receptor interaction, TNF-α signaling pathway, NF-κB signaling pathway, Jak-STAT signaling pathway, chemokine signaling pathway and TLR signaling pathway.

In addition, the interaction network between proteins was elucidated using IPA. The inflammatory immune network is shown in Figure 4d. Seven candidate genes (NTRK1, S100A8, S100A9, TNIP1, TNFAIP3, TAX1BP1 and NOD2) were screened out as Hub genes.

**Function analysis of DE lncRNA and the lncRNA-mRNA co-expression network**

A total of 1053 potential target genes of DE lncRNAs were selected to carry out functional enrichment analysis. Our results showed that the top 20 GO enrichment for BP focused on immune response, inflammatory response, neutrophil chemotaxis, positive regulation of inflammatory response, chemokine-mediated signaling pathway, positive regulation of ERK1 and ERK2 cascade, lymphocyte chemotaxis, positive regulation of NF-κB import into nucleus, positive regulation of IL-6 production, necroptotic signaling pathway, monocyte chemotaxis, cell chemotaxis, positive regulation of NF-κB transcription factor activity, negative regulation of IL-10 production and LPS-mediated signaling pathway, etc., (Figure 5a). Meanwhile, the KEGG results from these target genes of DE lncRNAs were mainly involved in immune response (Figure 5b).

To identify the key lncRNAs related to the regulation of inflammatory response and immune response, 54 DE mRNAs associated with these two biological processes and 31 DE lncRNAs targeting them were chosen to build the mRNA–lncRNA co-expression network. The co-expression network comprised 1241 connections, and each lncRNA might correlate with multiple mRNAs (Figure 5c and Table S6). More importantly, a total of 29 lncRNAs were found to be co-expressed with chemokines (CCL2, CCL3L1, CCL11, CCL17, CCL20, CCL22, CXCL2, CXCL8, CXCL10 and CXCL13) and cytokines (IL-1A, IL-6, IL-7, IL-10, IL-12B, IL-13, IL-18, IL-19, IL-20, IL23A, IL-27).

**qRT-PCR validation of DE lncRNAs in PBMC**

We focused on the known lncRNAs and successfully designed nine DE lncRNA primer pairs for qRT-PCR...
validation. We identified the nine DE lncRNAs expressions in porcine PBMCs at 4, 8, 12 and 24 h after LPS treatment. The results are shown in Figure 6. Four lncRNAs were significantly up-regulated by LPS treatment at all time points, including lncRNA ENSSSCT00000045208, lncRNA ENSSSCT00000051636, lncRNA ENSSSCT00000049770 and lncRNA ENSSSCT00000050966. The expression of lncRNA ENSSSCT00000047491 was significantly up-regulated by LPS treatment at 4, 8 and 12 h, and lncRNA ENSSSCT00000049750 expression was significantly increased at 4 and 8 h. After 8 h of LPS stimulation, the expression of lncRNA ENSSSCT00000054262 was significantly up-regulated and reached the peak at 24 h. LncRNA ENSSSCT00000054262 was significantly increased only at 4 h after LPS stimulation. However, there was no change in lncRNA ENSSSCT00000059583 expression following LPS treatment.

Figure 4. GO term, KEGG pathway and proteins network analysis of DE mRNAs. (a) GO analysis of up-regulated mRNAs. (b) GO analysis of down-regulated mRNAs. The GO terms ranked by P value. (c) KEGG pathway enrichment analysis of DE mRNAs. The top 30 enriched KEGG pathways ranked by P value. (d) The inflammatory immune network between proteins was produced using IPA.
Three IncRNAs (ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636) expressions were increased more than 2.5-fold in LPS-treated PBMCs at 4 h. Therefore, we further identified the expression changes of the three IncRNAs in various tissues of the piglets challenged with LPS at different time points.

Expression of IncRNAs (ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636) in various tissues of piglet challenge with LPS

As shown in Figure 7a, in the liver tissue, IncRNA ENSSSCT00000047491 was dramatically up-regulated at least 4-fold by LPS from 2 h to 24 h, while the up-regulation (> 9-fold) of IncRNA ENSSSCT00000051636 was observed after LPS challenge for 8 h. LncRNA ENSSSCT00000049770 was dramatically (4 to 12-fold) up-regulated within 8 h but it was down-regulated at 24 h.

As shown in Figure 7b, in the jejunum tissue, lncRNA ENSSSCT00000049770 was increased significantly at least 1.9-fold from 1 h until 24 h after LPS challenge, while the expression of lncRNA ENSSSCT00000051636 was significantly increased by > 2-fold from 1 h to 4 h. The expression of IncRNA ENSSSCT00000047491 was significantly up-regulated by LPS challenge at 4, 12 and 24 h.

As shown in Figure 7c, in the spleen tissue, the expression of the three IncRNAs was significantly
increased within 8 h and reached the peak at 2 h, but their expression was decreased at 24 h.

As shown in Figure 7d, in the thymus tissue, the expression of the three lncRNAs showed no significant difference.

**Tissue expression pattern analysis of IncRNA ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636**

We further detected the differences of the three lncRNAs (ENSSSCT00000047491, ENSSSCT00000049770 and ENSSSCT00000051636) expression levels in various piglet tissues by qRT-PCR. The results showed that the three lncRNAs were expressed in all the 10 tissues: skeletal muscle, heart, liver, spleen, lung, kidney, stomach, brain and thymus. We also found that the expression levels of IncRNA ENSSSCT00000047491 and ENSSSCT00000051636 were higher in liver and spleen than in other tissues, while the expression level of IncRNA ENSSSCT00000049770 was higher in spleen, lung and jejunum than in other tissues (Figure 8).

**Discussion**

Accumulating evidence has indicated that lncRNAs play roles in immune/inflammatory processes. The molecular mechanisms of associated lncRNA responsible for the LPS-induced inflammation in PBMCs remain largely undefined. In human PBMCs, Zhang et al. applied a microarray platform to profile global lncRNA and mRNA expression changes in response to LPS, and identified 846 DE lncRNAs (596 up-regulated and 250 down-regulated) and 1351 DE mRNAs (802 up-regulated and 549 down-regulated), respectively. However, to date there has been no systematic attempt to identify LPS-associated lncRNAs in porcine PBMCs.

In this study, we provide the first lncRNA and mRNA transcriptomic landscape of LPS-mediated changes in porcine PBMCs. Using a sequencing approach, we identified 43 DE lncRNAs and 1082 DE mRNAs, suggesting more mRNAs than lncRNAs were dys-regulated in response to LPS. In our recent study, we applied small RNA sequencing to investigated
miRNA expression changes in porcine PBMCs in response to LPS and identified only 15 DE miRNAs. Therefore, we thought LPS might have more influence on protein-coding RNA than non-coding RNA in porcine PBMCs. In addition, we found that there were no DE IncRNAs and mRNAs distributed on chromosome Y. The reason was that the porcine PBMCs were collected from female piglets for sequencing.
It is well recognized that a series of genes is involved in LPS-induced inflammation. In this study, the DE mRNAs functional enrichment results showed that these genes were related to some biological processes, including inflammatory response, immune response, cytokine activity, chemokine activity, cytokine–cytokine receptor interaction, TNF-α, NF-κB, JAK-STAT, NLR and TLR signaling pathways, which were closely associated with LPS-induced inflammation. Through the IPA network analysis, we identified some genes that might play key roles in LPS-induced inflammation, including NTRK1, S100A8, S100A9, TNFAIP3, TNIP1, TAX1BP1 and NOD2. As a high-affinity receptor for nerve growth factor (NGF), NTRK1 is expressed on various structural and hematopoietic cells including basophils and eosinophils. IL-13 can confer epithelial cell responsiveness to NGF by regulating NTRK1 levels in a transcriptional and epigenetic mechanism, and this process likely contributes to allergic inflammation. Calcium-binding proteins S100A8 and S100A9 have been identified as important DAMPs and recognized by TLR4 on monocytes, which function as an innate amplifier of infection, autoimmunity, and cancer. The TNFAIP3 gene encodes the ubiquitin-modifying enzyme A20, that restricts NF-κB-dependent signaling and prevents inflammation via its deubiquitinase activity. TNIP1 is increasingly being recognized as a key anti-inflammatory protein by negatively regulating TANK-binding kinase 1 (TBK1), receptor-interacting serine/threonine kinase 1 (RIP1 or RIPK1), and IL-1 receptor-associated kinase 1 (IRAK1). TAX1BP1 is a negative regulator of NF-κB activation induced by TNF-α and IL-1β. It inhibits RIP1 and TRAF6 polyubiquitination and recruits A20 to these molecules in order to influence NF-κB activation. NOD2 is a macrophage-specific protein containing two CARD domains and can directly bind bacterial LPS and subsequently act as an activator of NF-κB via the association of the CARD domains with Rip2/RICK/CARD1A.

Figure 8. Expression pattern of the three IncRNAs (ENSSSCT0000004749, ENSSSCT00000049770 and ENSSSCT00000051636) in porcine various tissues. The data represent the mean ± SD; n = 2.
LncRNAs can be categorized into five broad subcategories: antisense, sense, intergenic, intronic, and bidirectional.39 Approximately half of lncRNAs in porcine PBMCs belonged to the intronic subcategory, which describes lncRNAs that are located within protein-coding genes and could regulate functional gene expression. Based on their mode of action on gene expression, lncRNAs can be classified as either cis- or trans-acting. Cis-acting lncRNAs affect the expression of genes located near their site of transcription on the same chromosome. Trans-acting lncRNAs can control gene expression at independent loci on other chromosomes.40 We predicted the cis and trans potential targets of DE lncRNAs and compared these with our mRNAs sequencing results. The DE lncRNA-associated DE mRNAs were further analyzed for GO category and KEGG pathway annotation to investigate the potential regulatory roles of LPS-mediated DE lncRNAs. Bio-informatics analysis of DE lncRNAs target genes showed that these genes played important roles in immune response, inflammation, positive regulation of ERK1 and ERK2 cascade, positive regulation of NF-κB import into nucleus, positive regulation of IL-6 production and immune cell chemotaxis. ERK1 and ERK2 were reported to be required for LPS-induced production of cytokines and chemokines by macrophages.41 KEGG analysis also indicated that the DE lncRNAs were predominantly associated with the regulation of multiple inflammatory-associated genes. In addition, the lncRNA-mRNA co-expression analysis showed revealed that 29 DE lncRNAs targeted CCL and CXCL chemokines, indicating that these lncRNAs might participate in immune cell chemotaxis. Consequently, our results provide new evidence that lncRNAs are involved in LPS-induced inflammation in porcine PBMCs.

To confirm the statistical significance of our findings, we performed qRT-PCR analysis of the relevant lncRNAs. We found expression of three lncRNAs (ENSSSCT0000004749, ENSSSCT00000049770 and ENSSSCT0000051636) was increased more than 2.5-fold after LPS stimulation in porcine PBMCs. Then, we further confirmed the expression changes of the three lncRNAs in liver, spleen, jejunum, and thymus of the piglets challenged with LPS at times (0, 1, 2, 4, 8, 12 and 24 h). Previous studies have demonstrated LPS challenge can induce severe inflammation in the piglet model, causing liver injury, intestinal damage and histological changes of spleen.42,46 In liver tissues, lncRNA ENSSSCT00000047491 expression increased approximately 4- to 33-fold, lncRNA ENSSSCT00000049770 increased 4- to 12-fold and lncRNA ENSSSCT0000051636 increased 9- to 64-fold in response to LPS challenge. In the spleen and jejunum, LPS challenge induced an increase in expression of these lncRNAs with a maximum response of 4-fold increase. However, in the thymus, LPS challenge had no effect on the expression of the three lncRNAs. In addition, we performed the tissues expression pattern analysis for these three lncRNAs. Although the three lncRNAs displayed different tissue expression patterns in the piglets, they all showed abundant expression in liver, spleen, jejunum and thymus. Therefore, we thought that the three lncRNAs may play a vital role in liver inflammation in pigs. We predicted the cis-target mRNAs of the three lncRNAs and compared these predictions with co-expression analysis results. As shown in Figure S1, the potential cis-target genes of those lncRNAs included BIRC5, TK1, PGSI, TMEM233, AFMID, SYNGR2, KREMEN2, PRSS33, PKMYT1, FLYWCH1, HCFC1R1, TNFRSF1A2, PAQR4, CLDN6, THOC6, SRRM2, PRSS21, FLYWCH2, CLDN9, BICDL2, PRSS41 and ELOB. Only the FLYWCH2 gene was also co-expressed with lncRNA ENSSSCT00000049770. However, there is scarce specific information on the expression and function of FLYWCH2 gene in inflammation.

In the current study, we have provided the changes of lncRNA expression in porcine PBMCs after LPS stimulation, which provided a novel foundation for improving our understanding of the association between PBMC lncRNA homeostasis and inflammatory response in pigs. Further investigations are still required to evaluate the biological functions of these identified lncRNAs and these signaling pathways with regard to their roles in immunity and disease.

Declaration of conflicting interests
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Supplemental material
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