Analysis of transcriptomic changes in bovine endometrial stromal cells treated with lipopolysaccharide

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Abstract

Background: Endometritis adversely affects the ability of cattle to reproduce, and significantly reduces milk production. Consequently, it has great influence on the economic value of dairy cows. The endometrium is mainly composed of epithelial and stromal cells and they produce the first immune response to invading pathogens. Epithelial cells are the first cellular barrier through which bacteria enter the uterine endometrium. However, most of the epithelial cells are disrupted and stromal cells are exposed to an inflammatory environment when endometritis occurs, especially postpartum. A loss of the protective epithelium allows bacteria or toxins to access the underlying stromal cells. The activation of Toll-like receptor（TLRs）on stromal cells induces increased production of cytokines. Understanding the genome-wide characterization of the bovine endometritis will be beneficial for prevention and treatment of endometritis. In this study, whole-transcriptomic gene changes in bovine stromal cells treated with LPS were compared with those treated with PBS (control group) and were analyzed by RNA sequencing (RNA-seq). This was done in a cell culture model in vitro.

Results: Compared with the control group, a total of 366 differentially expressed genes (DEGs) were identified in LPS-induced group (234 upregulated and 132 downregulated genes), with an adjusted *P*-value＜0.05 by DESeq. Gene ontology (GO) enrichment analysis revealed DEGs were most enriched in lymphocyte activation, interleukin-1 receptor binding, regulation of cell activation, and lymphocyte-activated interleukin-12 production. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed DEGs were most enriched in TNF signaling pathway, Toll-like receptor signaling pathway, cytokine-cytokine receptor interaction, nucleotide-binding oligomerization domain-like (NOD-like) receptor signaling pathway, NF-κB signaling pathway, and chemokine signaling pathway.

Conclusion: The results of this study unraveled bovine endometrial stromal cells affected with LPS transcriptome profile alterations，which may have a significant effect on the treatment inflammation by comprehending molecular mechanisms and authenticating unique genes related to endometritis.

Background

Endometritis severely affects the ability of cattle to reproduce, compromises animal welfare, and significantly reduces milk production [1-3]. Bacterial infection is the most prevalent element of bovine endometritis, especially, *Escherichia coli*. Lipopolysaccharide (LPS) is a vital constituent of the outer membrane of gram-negative bacteria which can mimic characteristics of an actual gram-negative bacterial infection [4].

The endometrium is mainly composed of epithelial cells and stromal cells and they produce the first immune response to the invading pathogens [5]. After parturition, the epithelial layer is damaged and invading pathogens access the stroma [6-9]. Once the stroma is colonized with bacteria, *T. pyogenes* infection and endometritis appear to persist [9, 10]. The ability of T. pyogenes to cause pathology in the denuded endometrium is similar to the discovery of pathogenic *E. coli* adapted to the bovine

endometrium [11]. In addition, previous study showed also LPS treated bovine endometrial explants in vitro, which resulted in luminal epithelial cells shedding and damage. A loss of the protective epithelium allows bacteria or toxins to access the underlying stromal cells. The activation of TLRs on stromal cells induces increased production of cytokines [12]. The stromal cells are more sensitive than epithelial cells to pyolysin-mediated cytolysis and more accessible to the vascular system and mononuclear cells, thus, the effect of stromal cytokines is more significant [3, 13-15]. Moreover, stromal cells outnumber epithelial cells and secrete soluble growth factors as much as epithelial cells in order to increase immunological function of uterus endometrium [7, 14-16]. So stromal cells play a significant role in initiating bovine endometritis response.

There are lots of study about bovine endometrial stromal cells. The researchers evaluated the effects of the LPS and pro-inflammatory mediators (IL-1β and TNFα) on bovine endometrial epithelial (bEEL) and stromal (bCSC) cell lines by detecting gene expression and production of cytokine and eicosanoid biosynthesis pathway. Result showed bEEL and bCSC are brilliant models *in vitro* for intrauterine environment studies [17, 18]. Other study used primary isolated endometrial epithelial and stromal cells and ex vivo organ culture (EVOCs) as well-characterized models of endometritis to explore cellular pathways that modulate inflammatory responses within the endometrial tissue. Result showed that manipulating the mevalonate pathway modulates innate immunity in endometrial cells and tissue [19]. Another study explored the role of TLR3 and retinoic acid-inducible gene I (*RIG-I*) in the innate immune response to bovine herpesvirus-4 (BoHV-4) and ovine viral diarrhea virus (BVDV), and viralpathogen-associated molecular patterns (PAMPs). Result showed that stromal cells responded to viable viruses by inducing inflammatory cytokines, and endometrial cells could check and respond to virus, and their PAMPs, through TLR3 and *RIG-I* [20].

Genome-wide characterization is crucial for understanding development and pathophysiology of bovine endometritis, which will be beneficial for prevention and treatment of endometritis. Previous studies on RNA-seq analysis in mixed bovine epithelial and stromal endometrial cells treated with LPS showed 20 differentially-expressed miRNAs and 108 DEGS, as well as enriched 118 GO and 66 KEGG pathways, which provided an understanding which effect of miRNA in bovine endometritis [21]. Another study on RNA-seq analysis of change in miRNA and mRNA expression in 15 cows at 7 and 21 days postpartum showed 4197 DEGs in healthy cows, while only 31 DEGs in cows with cytological endometritis from proinflammatory to hyperplasia stages [22]. Other studies have been performed on RNA-seq analysis of effects of LPS on whole transcriptome of bovine endometrial epithelial cells with focus on genes involved in embryo-maternal interactions. They identified 2,035 and 2,073 DEGs in control cells and cells treated with LPS [23]. However, comprehensive transcriptomic analysis of response of bovine endometrial stromal cells to LPS by RNA-seq remains to be elucidated.

In this study, genome-wide gene expression profiles in primary bovine endometrial stromal cells treated with LPS were examined using RNA-Seq, and the results were confirmed by real-time quantifitative reverse-transcriptase polymerase chain reaction (qRT-PCR). Based on these data, the functions and pathways involved in the bovine endometritis, especially postpartum in the stromal cells were

characterized. The bioinformatics analysis confirmed that some of the expressed genes in our study have been shown to play important roles in bovine endometritis, especially postpartum, thus supporting the reliability of our data.

Results

Immunofluorescence analysis

Stromal cells exhibited flat, fibroblast-like morphology (Additional file 1: Figure S1). Endometrial stromal cells were estimated by immunostaining for vimentin (stromal cells marker). Stromal cells expression was vimentin-positive and cytokeratin-18 negative (Additional file 2: Figure S2). Cells morphology and the positive staining for vimentin confirmed that the cultured cells were indeed stromal cells.

Quality control of the RNA sequence

The RIN values of RNA samples including LPS1, LPS2 , LPS3, PBS1, PBS2 and PBS3 were 9.8, 10, 9.9, 10, 10 and 9.8 respectively (Additional file 3: Figure S3). And the results of RNA specific agarose electrophoresis showed that the three bands were 28s, 18S and 5S respectively. The brightness of the three bands decreases gradually, the brightest in 28s and the darkest in 5S. The width of 28s band is twice as wide as that of 18S (Additional file 4: Figure S4). These indicated RNA quality was fine. We established three RNA libraries for control group and three RNA libraries for LPS-induced group. After filtering the low-quality reads, the average number of clean reads was 42,381,010 (92.67%) and 44,167,270 (92.77%) for the control group and LPS-induced group, respectively (Table 1). The clean reads were used for the following analyses and most of them (> 93.99%) were mapped to the *Bos taurus* genome (Table 2).

Identification of the source of variance in the expressed transcripts by principal component analysis (PCA)

The purpose of PCA analysis is to account for the origin of variance in our data. We performed PCA analysis with two elements: PC1 and PC2. PC1 was 55% and PC2 was 42% (Additional file 5: Figure S5). PCA analysis can cluster similar samples together, the closer the distance, the higher the similarity between samples. This indicated that these data could be used further.

Differential gene expression

There were a total of 366 DEGs (composed of 234 upregulated and 132 downregulated DEGs) identified in LPS-induced group compared to control group. Some of these genes are related to inflammation, such as inflammatory cytokines including *IL1A*, *IL2RB*, and *IL6*, increased 3.16–20.53, 15.88 fold, respectively. Cytokines including *CCL2*, *CCL5*, *CXCL8*, and *CXCL2*, increased 16.91, 10.48, 3.39, 2.14 fold respectively. Antimicrobial factor *DEFB10* increased 2.79 fold (Figure 1 and Additional file 6: Table S1)

The heatmap of DEGs

Heatmap analysis of DEGs identified genes of expression levels with high correlation among samples. Some of these genes may be involved in biological processes, metabolic processes, or signaling pathways (Figure 2).

GO enrichment of DEGs

GO enrichment was used to characterize DEGs. DEGs were enriched in immunity-related GO terms, such as leucocyte-mediated immunity, intracellular signal transduction, IL-1 receptor binding, IL-1β secretion, and response to cytokines. There were 638 GO terms, including 574 biological process (BP), 21 cellular component (CC), and 43 molecular function (MF). GO enrichment results for all DEGs are illustrated (Additional file 7, Table S2), and the top 20 GO terms with the most significant enrichment were selected (Figure 3).

KEGG enrichment analysis

The purpose of KEGG enrichment analysis is to appraise pathways that are vital in inflammation in endometrial stromal cells. The results of the analysis showed that inflammation pathways in endometrial stromal cells are mainly centered on TNF signaling pathway, complement and coagulation cascades, cytokine-cytokine receptor interaction, IL-17 signaling pathway, chemokine signaling pathway, nucleotide-binding oligomerization domain-like (NOD-like) receptor signaling pathway, NF-κB signaling pathway, and so on. KEGG enrichment results of DEGs are illustrated (Additional file 8, Table S3). The top 20 KEGG terms with the most significant enrichment were selected (Figure 4).

Confirmation of DEGs by quantitative reverse-transcription PCR (qRT-PCR)

We selected ten DEGs which included seven upregulated genes, *NFKBIZ, NFKBIA, CCL2, C3, CSF3, GRO1, STEAP4* and three downregulated gene, *L1CAM*, *CD8A, SBSPON,* and confirmed them by real time qRT-PCR (as shown in Figure 5). The expression level of the ten DEGs were found to be similar with RNA-seq results.

Discussion

In this study, we described whole transcriptomic genes changes in bovine endometrial stromal cells treated with LPS compared with control group by RNA-seq.

We chose P6 stromal cells. Previous study using P5 bovine endometrial epithelial cells to investigate effects of LPS on whole transcriptome with a special focus on genes involved in embryo-maternal interactions [23]. And transcriptome profiling analysis of on P6 bovine vaginal epithelial cell response to an isolated lactobacillus strain [24]. Another study using P6 beard dermal papilla cells to determine whether transiently transfected androgen receptors and Hic-5/ARA55 interact [25]. Another work has showed the negative effects of cell passaging on mRNA expression and the secretion rate of some pro-inflammatory factors in human gingival fibroblasts [26]. However, some study indicated that the mRNA expression of some genes in primary bovine oviductal epithelial cells (BOEC) is different from P0 to P3 in

vitro. Such as the transcription rate of MUC4 and encoded proteins of IL8. But, there are also mRNA expression of some genes not affected by culture passaging, such as *OXCT2, PGE2, PTGS2* and *IL8* mRNA [27]. Likewise, BOEC incubated with *T. pyogenes* showed not only different mRNA expression of some genes, but also similar mRNA expression in P0 and P3 compared with the controls (without *T. pyogenes*) [28]. Cells in this study of P1 to P3 were pure, but number were not enough. So we think P6 cell expression profile is not exactly the same as P1, but P6 cells still represent most of the characteristics of primary stromal cells.

The main function of serum is to provide basic nutrients in vitro cell culture. We use 15% serum instead of 10% in order to provide enough nutrition for cells to grow better. Such as culturing primary bovine vaginal epithelial cells and bovine endometrial luminal epithelial cells in vitro with 15% FBS [24, 29].

LPS binds to lipoprotein binding protein (LBP), which presents it to CD14 receptor. This induces CD14 to present LPS-LBP complex to MD-2 (myeloid differentiation factor 2) [30, 31]. This, in turn, promotes dimerization of Toll-like receptor 4 (TLR4)/MD-2, activating two downstream signaling pathways including MyD88-dependent pathway (MyD88-) and TRIF-dependent pathway. The former triggers NF-κB and mitogen-activated protein kinase (MAP kinase) signaling and induces inflammatory cytokines, and the latter causes the induction of type I interferons (IFN) through interferon regulatory factor 3 (IRF3) activation and inflammatory cytokines through NF-κB activation [30]. Our data presents evidence for the activation of the MyD88-independent pathway as LPS upregulated inflammatory cytokines, such as *IL6, IL1A, CXCL8* and activated NF-κB pathway (Additional file 6: Table S1 and Additional file 8: Table S3). Bovine endometrial epithelial and stromal cells express the TLR4/CD14/MD2 receptor complex. We found increased expression levels of CD14 but constant levels of TLR4 or MyD88 in our study (Additional file 6: Table S1), consistent with previous studies that exposed mixed bovine epithelial and stromal endometrial cells to LPS [32]. In addition, LPS upregulated TLR4 only in bovine endometrial epithelial cells treated with 1 μg/mL LPS for 6 h and 12h [33].

After the membrane surface receptor is recognized, intracellular inflammation is recognized and intracellular signaling cascades are activated by LPS, which is mediated by innate pattern recognition receptors (PRR) including TLR, retinoic acid-inducible gene-I-like (RIG-I-like) receptors, NOD-like receptors, and C-type lectin receptors [34]. These lead to the expression of inflammatory mediators and contribute to the clearance of pathogens [35]. Our data further confirm these findings because LPS triggers NOD-like receptor, TLR, and C-type lectin receptor signaling pathway. (Additional file 8: Table S3). In addition, our RNA-seq results also demonstrate the upregulation of expression of inflammatory and chemotactic cytokines related genes, such as *IL6, IL1A, IL2RB, CCL2, CCL5, CXCL5, CXCL8, CXCL2, CX3CL1*, and *CCL20* (Additional file 6: Table S1). This is also consistent with previous studies that mRNA of proinflammatory cytokines *IL1A, IL1B, IL6, TNF*, and expression of chemokines *IL8* and *CXCL5* increased in endometrial epithelial cells during the estrous cycle and subclinical or clinical endometritis [36, 37]. Similarly, stromal cells responded to viable viruses by inducing inflammatory cytokines and chemokines [20]. Antiviral defense of endometrial stromal cells requires coordinated recognition of PAMPs, initially via TLR3 and later via inducible RIG-I [20]. On the other hand, activation of PRR also triggers many signal

transduction pathways through one or more of the IRF family of transcription factors, leading to the expression of IFNs [38]. LPS upregulated the expression IFN-stimulated genes (ISG) including *RSAD2*, *MX2*, *OAS1Y*, *ISG15*, and *BST2* in mixed epithelial and stromal cells [39]. Our results show that LPS upregulated the expression of *ISG15* and *IRF1*, 3.71 fold and 2.36 fold, respectively (Additional file 6: Table S1).

Local immune responses are activated which result in expression of proinflammatory cytokines when the uterus is exposed to bacteria. This is followed by production of antimicrobial peptides (AMP) and acute phase proteins by epithelial and innate immune cells [40]. Many of AMP genes are expressed, including lingual antimicrobial peptide (*LAP*), tracheal antimicrobial peptide (*TAP*), and some β-defensins [38, 40, 41] [35, 54, 55]. Some studies have identified an increase in expression levels of TAP, LAP, DEFB1, and DEFB5 in endometrium of cows with serious inflammation or following LPS treatment *in vitro* [42-44]. Other studies showed LAP, TAP, neutrophil β-defensins (*BNBD4, DEFB5*) were all upregulated in bovine epithelial cells but not stromal cells with LPS [3, 5]. In this study, we found *DEFB10* was upregulated 2.79 fold in bovine stromal cells with LPS (Additional file 6: Table S1).

Cascade reaction of complement signals also plays a key role in immune defense, and complement activation lead to opsonization of pathogens and their removal by phagocytes as well as cell lysis [45]. Our results showed some complement genes upregulated, such as *C3*, *C1S*, *C1R*, *CF1*, and *C4A* (Additional file 6: Table S1). LPS can change expression level of many genes involved in cell adhesion, such as *VCAM1*, *SELP*, *CADM2*, *HEPACAM*, and *SDK2* (Additional file 6: Table S1). Leucocytes attach to cell adhesion molecules (CAM) on endothelial cells and are involved in inflammation and immune function [46]. Each CAM has an inherent effect on immune response process, such as vascular cell adhesion molecule-1 (VCAM-1), which promotes firm binding of T cells and induces trans-migration [47].

In our next study, we will continue to analyze and validate immune-related proteins and KEGG signaling pathways by western blot and compare genes and proteins in stromal cells treated with LPS *in vitro* with stromal cells from bovine endometritis with subclinical or clinical disease. These can better elucidate characteristics of bovine endometritis.

Conclusions

RNA-seq showed many important immune-related genes and signaling pathways in bovine endometrial stromal cells in LPS-induced group as compared to the control group, which may be involved in the pathological process of bovine endometritis, especially postpartum and deserve further study and discussion. Our analysis paves the way for the future elucidation of molecular mechanism of microbial invasion and host cell response.

Methods

Bovine endometrial stromal cell isolation and primary culture

Whole stromal cells were isolated from bovine endometrium as described before with some changes [48, 49]. All experimental procedures were approved by Institutional Animal Use Committee of Henan Agricultural University (approval number. 2005-0026). Bovine uteri, stored on ice, were transported to to the laboratory from a local abattoir. The endometrium was cut into strips and placed in serum-free DMEM/F12 (Gibco, Grand Island, NY, USA) containing 50 IU/mL penicillin, 50 μg/mL streptomycin, and 2.5 μg/mL amphotericin B. Then, endometrial strips were cut into about 1 mm^3 fragments and placed in phosphate-buffered saline (PBS). They were then digested with 30 mL sterile digestive solution, composed of 60 mg trypsin III (Roche, Lewes, UK), 60 mg collagenase II (Sigma, Poole, UK), and 12 μL deoxyribonuclease I (DNase I, Sigma) in 120 mL PBS at 37°C for about 1 h. The cell suspension was filtered with a 40 μm mesh and the filtrate was resuspended with 2 mL DMEM/F-12 containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). After washing the cells five times with DMEM/F-12 containing 10% FBS, the filtrate was centrifuged at 100 rpm for 10 min at room temperature (RT) [11]. The cells were resuspended in DMEM/F-12 containing 15% FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 100 U/mL amphotericin B. Cells were seeded in 25 cm^2 culture flask at a density of 2×10^5 cells/mL, and were incubated at 37°C with 5% CO_2. To obtain stromal cells and remove epithelial cells populations, the cell suspension was removed 18 h after plating, which allowed selective attachment of stromal cells. The medium was changed every two days. Cells were passaged with 0.25% trypsin-EDTA until the cells reached ~80% confluence. Cell morphology was recorded and photographed under an inverted microscope.

Cells treatment with LPS

Stromal cells from passage 6 (P6) were seeded in 25 cm^2 culture flask at a density of 2×10^5 cells/mL. The cells were incubated at 37°C with 5% CO_2. Once the cells reached 80% confluence, the medium was removed and cells were washed with PBS. We divided the cells from an individual cow into two groups of three duplicates each. DMEM/F12 containing 15% FBS and PBS (control) was added to one group and DMEM/F12 containing 15% FBS and 0.5 μg/mL ultrapure LPS obtained from a pathogenic E. coli strain (serotype 055:B5, Sigma-Aldrich, Madison, USA, L2880) was added to the other group and incubated for 12 h each.

RNA extraction, quantification, and qualification

Total RNA from stromal cells was extracted by RNAisio Plus (Takara, Dalian, Liaoning, China). Concentration and quality of RNA were estimated with NanoDrop spectrophotometer (Thermo Scientific) and integrity of RNA was verified with 1% agarose gel. RNA quality was determined using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA).

Library preparation and transcriptome sequencing

Input material of 3 μg RNA was used for RNA sample preparation. Sequencing libraries were generated using the TruSeq RNA Library Preparation Kit v2 (Illumina, San Diego, CA, USA). In summary, poly-A

containing mRNA was enriched by oligo-dT magnetic beads and fragmentation was completed by divalent cations under elevated temperature in an Illumina proprietary fragmentation buffer. Random oligonucleotides and SuperScript II were used for synthesizing first strand cDNA. Second strand cDNA was synthesized. Remaining overhangs were converted into blunt ends by exonuclease/polymerase. Before hybridization, 3' ends of the DNA fragments was adenylated and Illumina paired-end (PE) adapter oligonucleotides were ligated. The amplified library fragments were enriched using Illumina PCR primer cocktail in a 15-cycle PCR reaction, then the library size was selected at 300-400 bp fragments. AMPure XP system was used to purify PCR products and Bioanalyzer 2100 system (Agilent, Santa, Clara, CA, USA)) was used to detect library size. Finally, library sequencing of PEs was accomplished with Next-Generation Sequencing (NGS) on Illumina HiSeq platform.

Quality control

The raw data of FASTQ was generated by the software of the sequencing platform, Illumina HiSeq. Raw data of each sample was counted separately, including sample names, percentage of ambiguous base, Q20, and Q30 sequencing data along with some connectors and low-quality reads. These sequences may cause interference to subsequent data analysis, so sequencing data need to be further filtered. The criteria for data filtering included: 1) Cutadapt is used to remove 3' end connectors, and the removed parts have at least 10 bp overlap with known connectors, allowing 20% base mismatch; 2) removal of reads with average mass fraction lower than Q20.

Mapping reads to the reference genome

The reference genome used was of *Bos taurus*. ARS-UCD1.2.dna.toplevel.fa. were downloaded from Ensembl genome browser 95. The clean reads were mapped to the reference genome with HISA2 (http://ccb.jhu.edu/software/hisat2/index.shtml).

Gene expression level quantification

The reads of each gene was counted by HTSeq and normalized by fragments per kilobase of transcript sequence per millions base pairs sequ.enced (FPKM). FPKM was then calculated [50].

Analysis of DEGs

Genes that were differentially expressed in two groups (three biological replicates per group) were screened with DESeq. The screening conditions for differentially expressed genes were: |log2FoldChange| > 1, significance of false discovery rate (FDR) <0.05. FDR is the adjusted *P*-value.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of DEGs

We performed GO enrichment analysis for function annotation and KEGG enrichment analysis for signaling pathway annotation on DEGs [51, 52]. GO and KEGG enrichment analyses were completed by

the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.7 with threshold of FDR <0.05 [53].

qRT-PCR

The genes for qPCR used to confirm the results by NGS were randomly selected [54].

The expression profiles of randomly selected 10 differentially expressed genes were validated using SYBR Green based qRT-PCR using sequence specific primers (Additional file 9: Table S4) designed using the online primer design tool (http://frodo.wi.mit.edu/primer3/). The cDNA samples were synthesized by reverse transcription of equal amount of total RNA from stromal cells treated LPS (experimental group) and PBS (control group), three duplicates each. respectively, using cDNA synthesis kit (Takara, Dalian, Liaoning, China) according to the manufacturer's instructions. The reverse transcription was first performed in 10 μl reaction volume to remove the genomic DNA reaction containing RNA samples, 1μL; 5×gDNA Eraser Buffer, 2μL; gDNA Eraser, 1 μL, dd H_2O, 6 μL. Reaction conditions included 42°C for 2 min and 4°C 1 min, which followed 20 μl reaction volume reaction containing 10 μL reaction volume of the previous step, 1μL Prime Script RT Enzyme Mix, 1μL Prime Mix, 4 μL 5×Prime Script Buffer, 4 μL dd H_2O. Reaction conditions included 37°C for 15 min, 85°C 5 s, 4°C 1 min. Primer specificity was tested by first performing a conventional PCR and confirmed by the presence of a single melting curve during qRT-PCR. Serial dilutions(1:10, 1:20, 1:50, 1:200) were made from a pool of cDNA and calibration curves were performed for each gene. Afterwards, The qRT-PCR was then performed in 20 μl reaction volume containing TB Green Premix ExTaq II, 10 μL (Takara, Dalian, Liaoning, China); the cDNA samples, 1μL; the specific forward and reverse primer, 1μL (respectively); and dd H_2O, 7 μL in the 0.1 mL white PCR 8-strip Tubes (NEST Biotechnology, Wuxi, China) with CFX96 real-time PCR detection system (Bio-Rad, Munich, Germany). Reaction conditions included pre-degeneration at 95°C for 30 s, followed by 40 cycles of degeneration at 95°C for 5 s, and annealing at 60°C for 30 s. At the end of each PCR reaction, the specificity the amplification was confirmed by evaluating the dissociation curve. The abundance of each transcript in each sample was determined using a comparative threshold cycle comparative Ct ($2^{-\Delta\Delta CT}$) method as described previously[55]. The data obtained from qRT-PCR was analyzed after the Ct value of the target genes was normalized with the Ct value of Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*. The qRT-PCR was performed in three biological. The Student's t-test or the least significant difference test procedure was employed to detect the mRNA expression differences between the samples. Differences with $p < 0.05$ were considered as significant. Statistical significance of the data was determined by a Student's t-test carried out with SPSS (PASW Statistics for Windows, Version 18.0, Chicago: SPSS Inc., USA).

List Of Abbreviations

LPS: Lipopolysaccharide; DEGs: Differentially expressed genes; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPARs peroxisome proliferator-activated receptors; RNA-Seq: High-throughput mRNA sequencing; PCA: Principal component analysis; LBP: lipoprotein binding protein;

PRRs: Innate pattern recognition receptors; AMP: Antimicrobial peptides; *LAP*: Lingual antimicrobial peptide; *TAP*: tracheal antimicrobial peptide; MD-2: Myeloid differentiation factor 2; FDR: False discovery rate; IRF3: interferon regulatory factor 3; MAP kinase: Mitogen-activated protein kinase; ISG: IFN-stimulated genes; GAPDH: Glyceraldehyde 3-phosphate-dehydrogenase; STEAP4: Six-transmembrane epithelial antigen of prostate 4; CAM: Cell adhesion molecules; VCAM-1: Vascular cell adhesion molecule-1; HA: hyaluronan; ECM: extracellular matrix; HAS2: hyaluronan synthase 2

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by Institutional Animal Use Committee of Henan Agricultural University (approval number. 2005-0026) and Beijing Association for Science and Technology (approval SYXK [Beijing] 2007-0023).

Consent for publication

Not applicable.

Availability of data and materials

The sequence data of this study have been deposited into Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra, accession number PRJNA574911). The datasets supporting the conclusions of this article are included in this article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XW, CT and XD designed the study and revised manuscript. XD, HL, LD, WH, ZP, CY, and DY performed the study. XD, HL, LD, CT and XW analyzed the data. XD, HL and LD wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Data filtering statistics after Illumina sequencing

Sample	Raw Reads	Clean Reads No.	Clean Data (bp)	Clean Reads %	Q20 (%)	Q30 (%)
PBS1	47469396	44046138	6650966838	92.78	96.08	91.45
PBS2	46293936	42854784	6471072384	92.57	96.49	92.24
PBS3	43441712	40242110	6076558610	92.63	96.25	91.79
LPS1	42929910	39806986	6010854886	92.72	96.19	91.59
LPS2	50813700	47094016	7111196416	92.67	96.16	91.6
LPS3	49082750	45600808	6885722008	92.9	96.42	91.9

N %: The percentage of fuzzy bases

Q20: The percentage of bases with a Phred value > 20

Q30: The percentage of bases with a Phred value > 30

Table 2 Summary of clean reads mapped to the reference genome

e	Clean_Reads	Total_Mapped	Multiple_Mapped	Uniquely_Mapped
	44046138	41598276 (94.44%)	2394502 (5.76%)	39203774 (94.24%)
	42854784	39556838 (92.30%)	2206194 (5.58%)	37350644 (94.42%)
	40242110	37935794 (94.27%)	2240965 (5.91%)	35694829 (94.09%)
	39806986	37586818 (94.42%)	2233726 (5.94%)	35353092 (94.06%)
	47094016	44530730 (94.56%)	2676831 (6.01%)	41853899 (93.99%)
	45600808	43007562 (94.31%)	2461139 (5.72%)	40546423 (94.28%)

Clean Reads: The total number of sequences used for alignment

Total Mapped: The total number of sequences identical to the reference genome in clean reads，percentage is total mapped / clean reads

Multiple Mapped: The total number of sequences aligned to multiple locations, percentage is multiple mapped / total mapped

Uniquely Mapped：The total number of sequences aligned to only one location, percentage is uniquely mapped / total mapped

Additional Files

Additional file 1: Figure S1. Morphological characteristics of P6 endometrial stromal cell cells. Stromal cells were fibroblast-like and enlarged at the time of confluence after which they overgrew in multiple layers. (Scale bars =100um).

Additional file 2: Figure S2. Immunofluorescence staining shows positive expression of vimentin expression (green) and negative expression of cytokeratin-18 in endometrial stromal cell (Scale bars =50um).

Additional file 3: Figure S3. The RIN values of RNA including LPS 1, LPS 2, LPS 3, PBS 1, PBS 2, PBS 3.

Additional file 4: Figure S4. RNA quality image. M: DNA Marker; 1: PBS 1; 2: PBS 2; 3: PBS 3; 4: LPS 1; 5: LPS 2; 6: LPS 3.

Additional file 5: Figure S5. PCA analysis of the expressed transcripts. The X-axis is the first principal component, and the Y-axis is the second principal component. Different shapes represent different

samples, and different colors represent different groups. (A: control group; B: LPS-induced group).

Additional file 6: Table S1. DEGs in the bovine endometrial stromal cells were treated with LPS compared with PBS (control). The upregulated genes are shown in purple; the downregulated genes are shown in blue. Inf: infinite.

Additional file 7: Table S2. The significant GO terms of DEGs.

Additional file 8: Table S3. KEGG enrichment analysis of DEGs.

Additional file 9: Table S4. QRT-PCR primers used in the study.

Figures

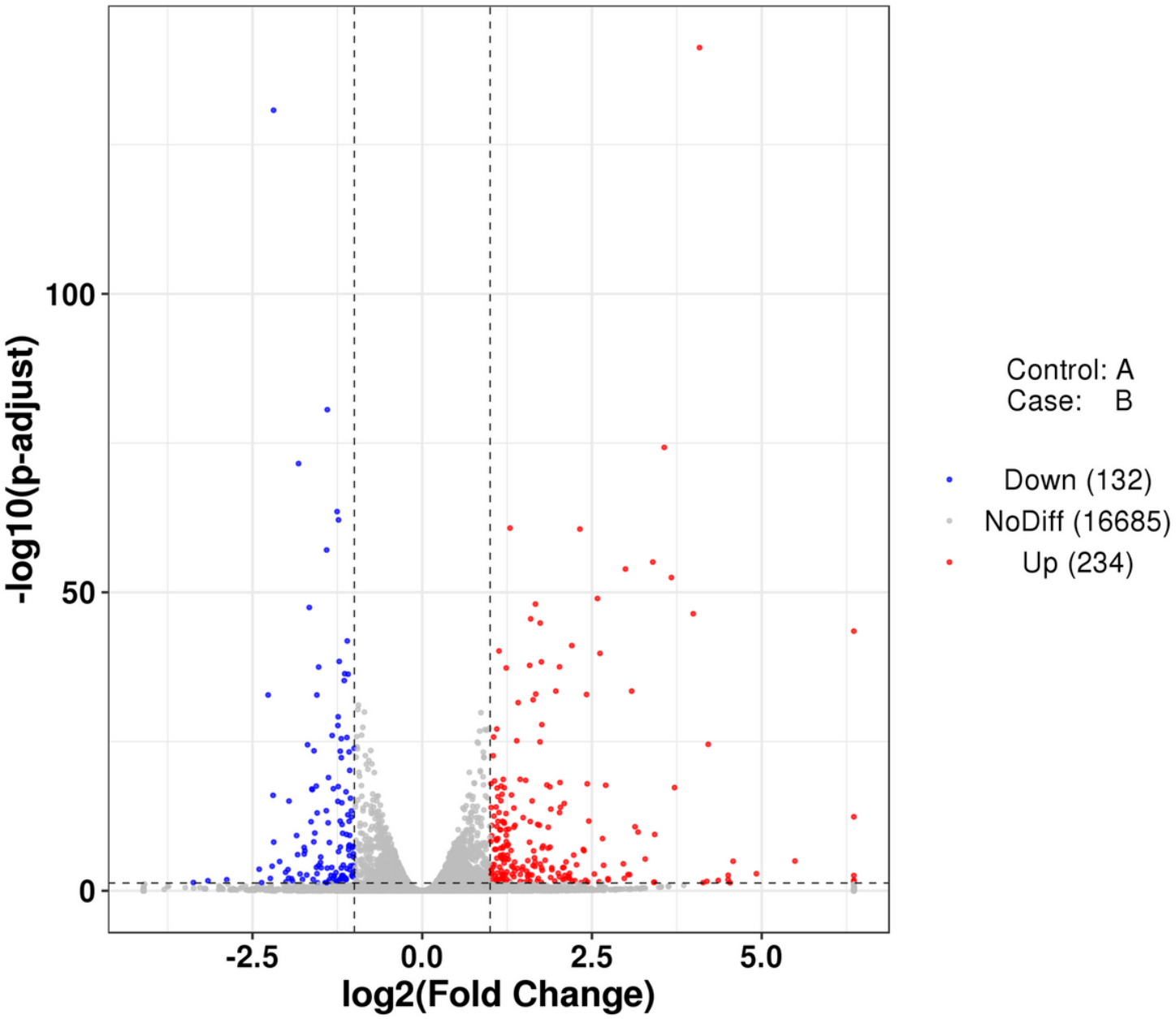

Figure 1

Volcano map of DEGs. The two vertical dotted lines are the threshold of differential expression. The horizontal dotted line is the threshold FDR of 0.05. Upregulated and downregulated genes are shown as a red and blue dots, and gray dots represents non-significantly differentially expressed genes. (A: control group; B: LPS-induced group)

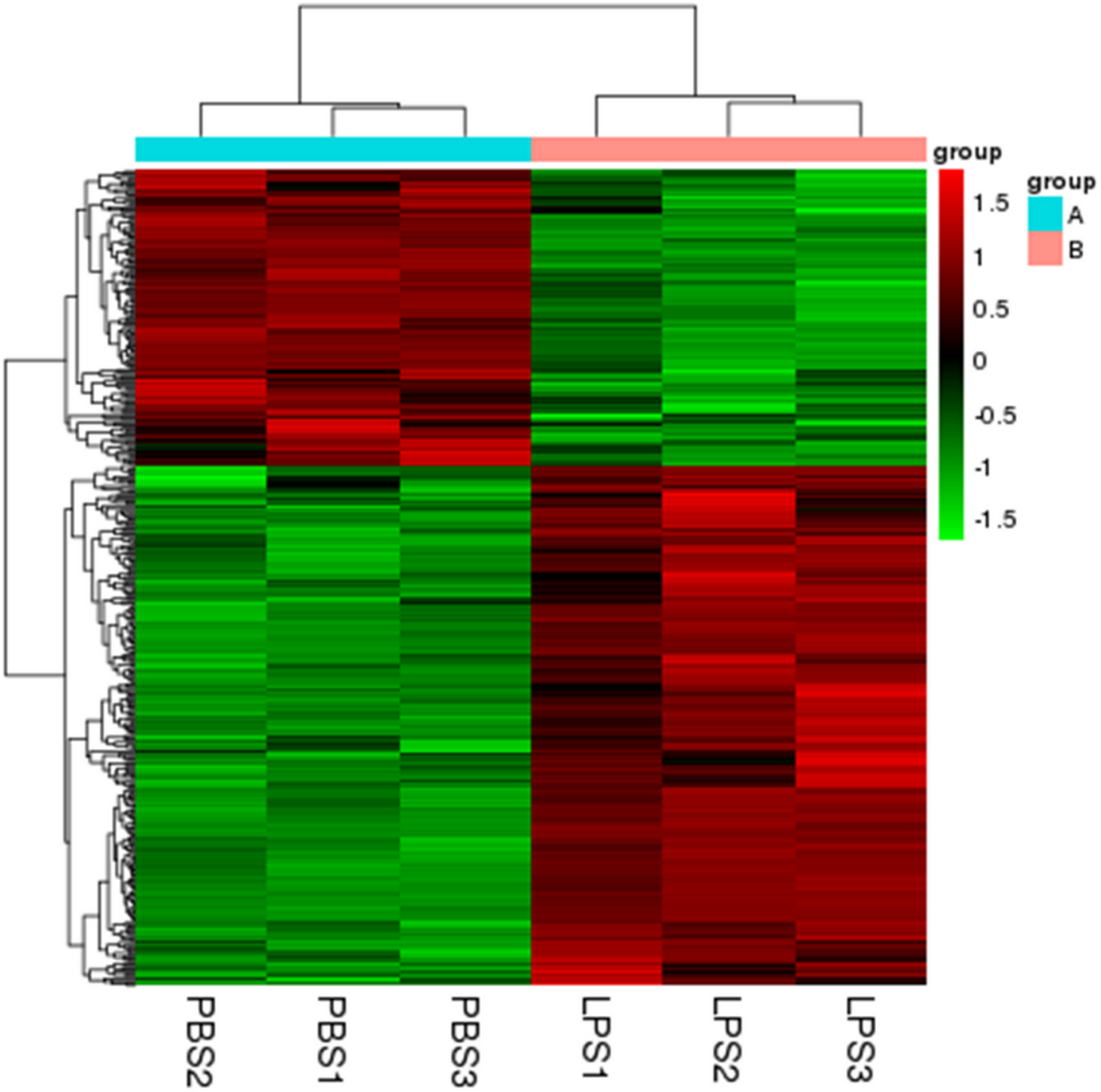

Figure 2

Heatmap analysis of DEGs. The horizontal lines represent genes, and each column is a sample. Red represents high-expression genes and green represents low-expression genes. The X-axis is the sample number and the Y-axis is the DEGs.

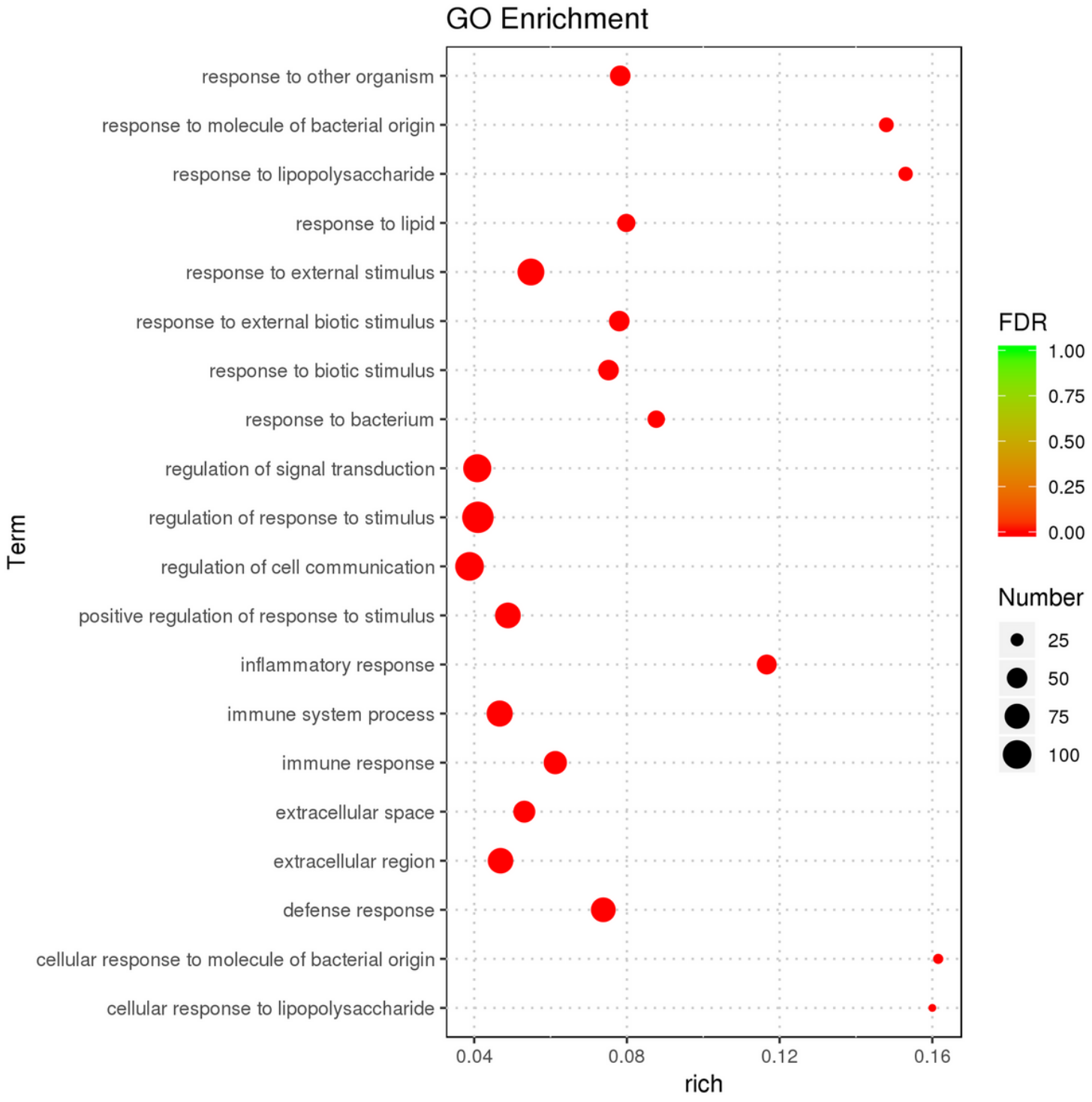

Figure 3

GO enrichment analysis displaying the first 20 GO terms with the most significant enrichment.

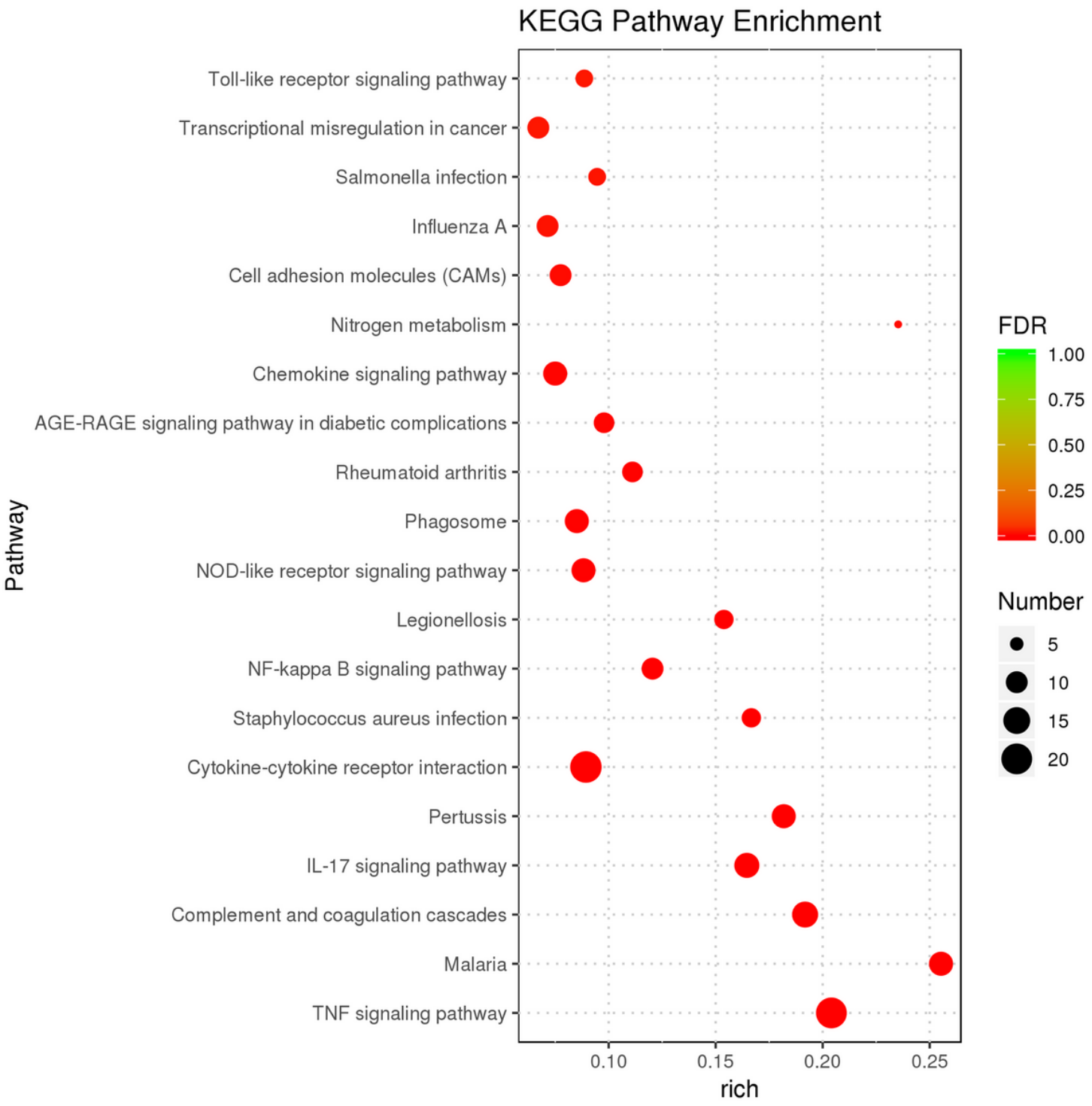

Figure 4

KEGG enrichment analysis displaying the first 20 KEGG terms with the most significant enrichment.

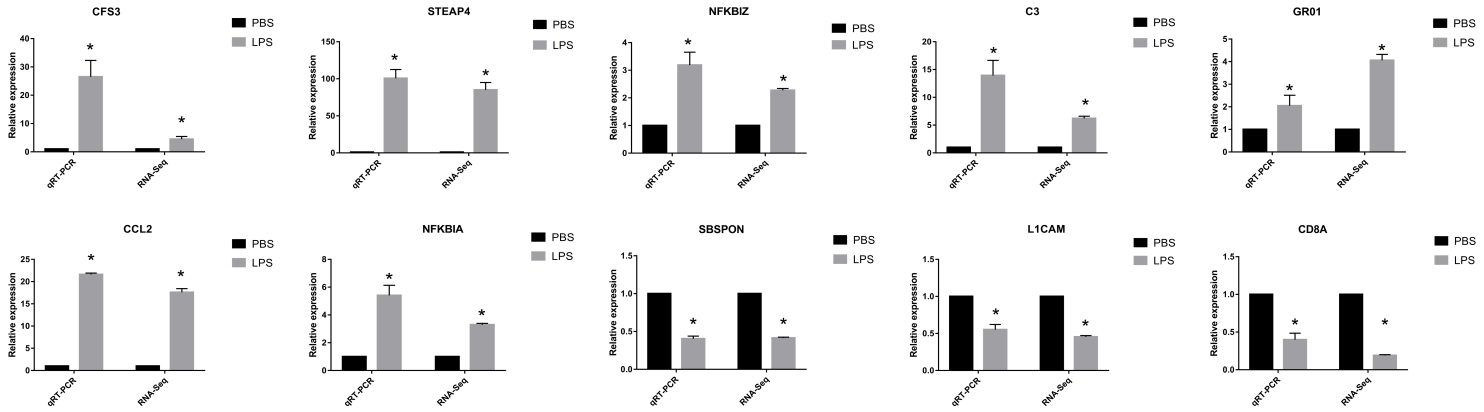

Figure 5

QRT-PCR verified features of DEGs by RNA-seq. The relative expression level of target mRNAs was calculated using the 2- ΔΔCt method and expressed relative to the value in the control group. Results were displayed in mean ± SEM (n = 3). Log2 fold change was the ratio of average log2 folds between groups.

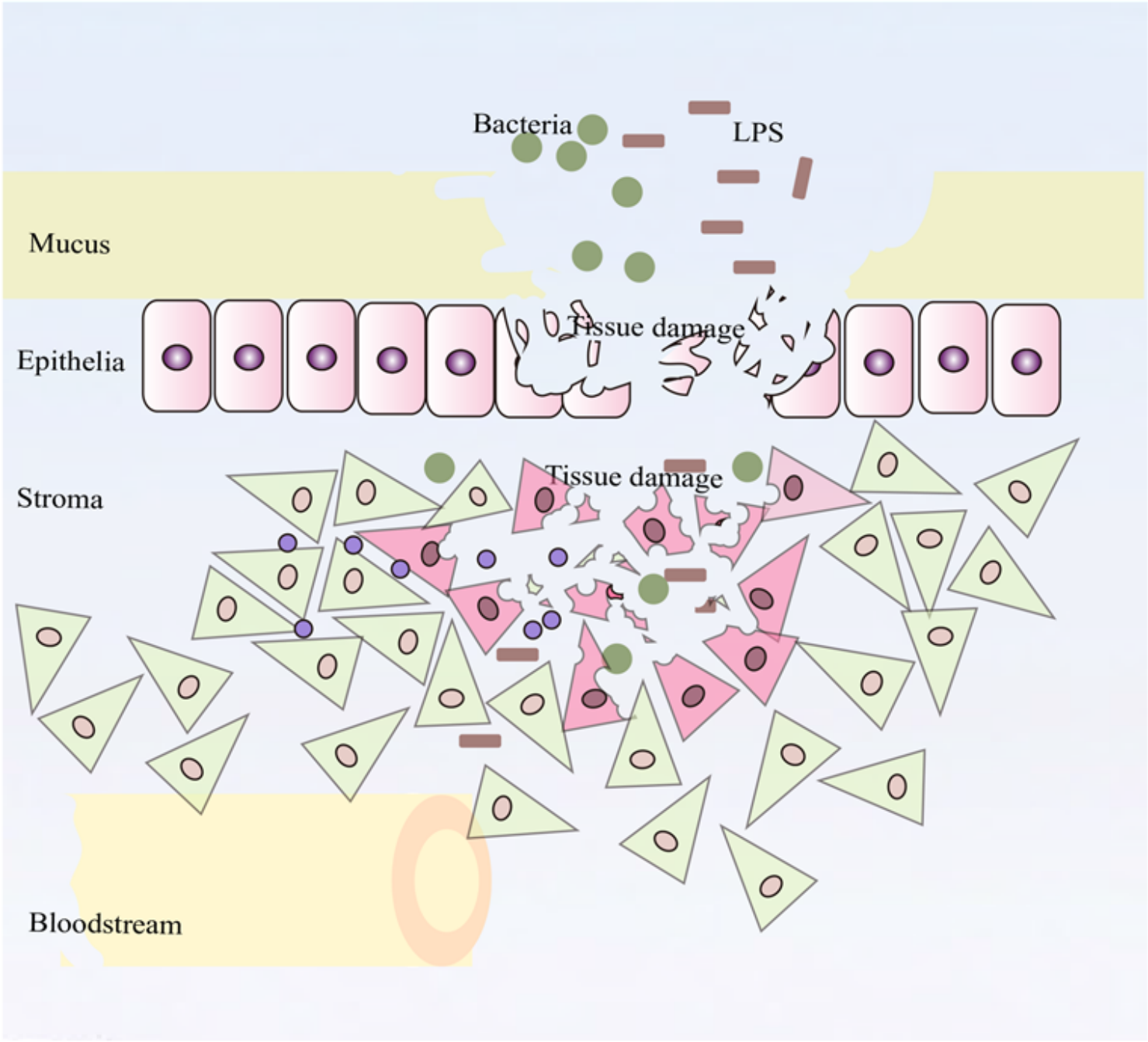

Figure 6

Stromal cells were exposed to an inflammatory environment when epithelial cells were disrupted.

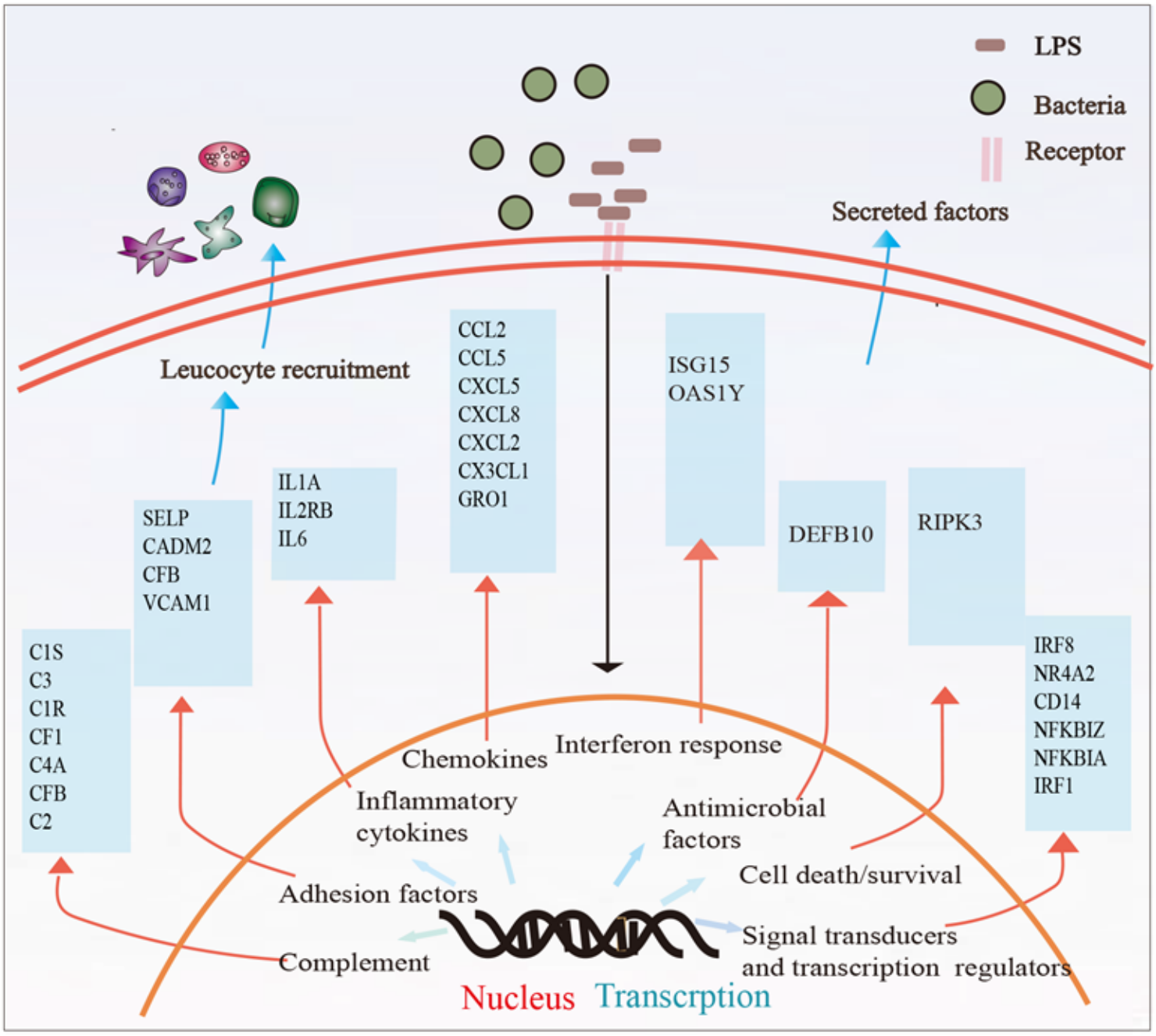

Figure 7

Summary of the immune response in bovine endometrial stromal cells exposed to bacterial LPS. LPS treatment for 12 h changed the mRNA expression of many genes involved inflammatory and innate immune response.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile5.pdf

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