

Tea tree oil prevents mastitis-associated inflammation in lipopolysaccharide-stimulated bovine mammary epithelial cells

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Abstract

Background

Effective prevention and treatment of cow mastitis can provide a good guarantee for the healthy growth of cows and the qualified production of dairy products. The main purpose of this study was to explore the effect of tea tree oil on lipopolysaccharide (LPS) -induced mastitis in dairy cows, and the key gene in LPS-stimulated bovine mammary epithelial cells (BMECs) was identified.

Results

In this study, a model of mastitis induced by LPS was constructed, to which tea tree oil and LPS were added. The protective effects of tea tree oil on LPS-induced mastitis in BMECs were verified by the CCK-8 method, flow cytometry, real-time fluorescence quantitative detection, ELISA and other methods. The results showed that LPS at a concentration of 200 µg/ml could reduce the proliferative activity of the cells, induce a high proportion of apoptosis, and promote the expression of TNF-α, IL-6 and STAT1. Upon addition of tea tree oil, the proportion of apoptosis was reduced, and the expression of NF-κB, MAPK and caspase-3 was inhibited. Mammary epithelial cells were compared under control and LPS-treatment conditions and analyzed by second-generation sequencing. A total of 1270 mRNAs were identified as differentially expressed, of which 787 genes were upregulated and 483 were downregulated. These differentially expressed genes include TNF - α, IL6, STAT1, mapk4, etc. H&E staining and immunohistochemistry were used to verify the function of candidate genes. TNF-α and IL6 were observed to play important roles in mediating the preventive effect of tea tree oil on mastitis in LPS-stimulated bovine mammary epithelial cells.

Conclusions

The results showed that tea tree oil had a protective effect against LPS-induced mastitis. TNF - α and IL6 may be the marker genes of LPS-induced mastitis which provided a theoretical basis and experimental support for further research to determine new strategies for the prevention and treatment of mastitis and improvement of milk quality.

Introduction

Cow mastitis affects the health of cows(1). Human consumption of this kind of milk affects human health(2). The cause of mastitis in dairy cows is highly complex, but the most important cause is pathogenic microorganisms(3). There are more than 20 kinds of common pathogenic bacteria, including *Staphylococcus aureus*, *Streptococcus agalactis*, *Streptococcus lactis* and *Escherichia coli*. lipopolysaccharide (LPS) is one of the main components of the cell wall of Gram-negative bacteria, such as *Escherichia coli* (*E. coli*)(4). In dairy cow mastitis caused by *E. coli*, the acute clinical indicators caused by LPS are closely related to the enzyme activity and acute phase protein in milk(5, 6). Most cow mastitis is caused by pathogenic bacteria and can be treated with antibiotics(7). However, with the increasingly

prominent problem of drug resistance, how to effectively prevent and treat cow mastitis and find new antibacterial substances has become a heavily research topic in drug development. It is important to find an effective substitute for antibiotics for the prevention and treatment of cow mastitis. Tea tree oil (TTO) is the essential oil from several plants of *Melaleuca* L. The main one is *M. alternifolia*(8); It is also called *M. alternifolia* oil. TTO is a common ingredient in many over-the-counter health products and cosmetics. With the vigorous development of the natural and alternative medicine industry, an increasing number of people are using products containing tea tree oil(9). TTO has a broad antibacterial spectrum and strong antibacterial activity(10). Therefore, TTO is used to treat related diseases caused by fungi, bacteria or viruses. TTO is also expected to be developed into a natural antibacterial agent that can alleviate the global "antimicrobial crisis". Therefore, it is of great significance to study the molecular basis of tea tree oil. The purpose of this study was to explore the protective effect of tea tree oil against LPS-induced mastitis and to explore the feasibility of replacing antibiotics with tea tree oil in the treatment of mastitis.

Omics analysis is an important component of systems biology, which has developed rapidly in recent years(11). Omics analysis plays an important role not only in the field of human medicine but also in food and animal husbandry research(12). The change in generation after generation of omics technology enables people to explore the micro world and analyze the mystery of life at the molecular level. In research on cow mastitis, previous biotechnology has been unable to meet the needs of researchers because of its limitations(13). The emergence of histochemistry technology has allowed the analysis of the complex pathogenesis of mastitis in dairy cows from multiple perspectives followed by the screening out effective biological markers for timely and accurate prevention(14). Concurrently, this approach can provide precise targets for the research and development of related therapeutic drugs, ultimately achieving the expected combined results of prevention and treatment(15). In the omics study of mastitis, miRNA expression in bovine tissues was first confirmed in 2007(16). Naeem et al. detected changes in 14 miRNAs in breast tissue 12 h after infection with *S. lactis*, and they compared these results with findings obtained using a gene expression chip(17). Bioinformatic analysis revealed changes in miRNA target genes and related cellular pathways. Compared with normal tissues, the expression levels of miR-15b, miR-16a, miR-21, miR-145 and miR-181a were down-regulated, and only miR-223 was up-regulated. The downregulation of mir-16a was related to some interleukins (IL-6, IL-8 and IL-10). However, few reports have investigated the transcriptome sequencing of LPS-stimulated bovine mammary epithelial cells. Therefore, this research starts with transcriptome technology to analyze LPS-induced mastitis, identify key candidate genes, and carry out a series of functional verifications with the hope of providing a research basis for follow-up research on dairy safety.

Materials And Methods

Culture of bovine mammary epithelial cells

The bovine mammary epithelial cells (BMECs) were cultured in DMEM/F12 medium. The complete medium consisted of 90% DMEM/F12 medium and 10% fetal bovine serum. The BMECs were cultured at

37°C in a 5% CO₂ incubator, and the medium was changed every 48 h. The cells were digested with 0.25% trypsin for passaging, and the growth of cells was observed under an inverted microscope(18).
Construction Of The Lipopolysaccharide-stimulated BMEC Mastitis Model

CCK-8 detection of cell proliferation activity induced by LPS: The density of BMECs was adjusted to 1×10^4 and inoculated into a 96-well culture plate. After 24 h of culture, the culture medium was discarded, and medium containing LPS at a concentration of 50, 100, 200, 500 and 1000 µg/ml was added to the culture dish. In addition, the control group (without LPS) and the blank control group (only culture medium without cells) were established. After 4, 8, 12 and 24 h of culture, the cell proliferation activity was detected according to the CCK-8 kit procedure.

Detection of the apoptosis rate induced by LPS by flow cytometry: BMECs were inoculated into a 6-well culture plate. After 24 h of culture, the medium was discarded. The cells were washed and collected with PBS, and the cell concentration was adjusted with buffer to 1×10^6 / 100 µL / test. Then, 5 µL annexin V-FITC and 5 µL PI were added, and the apoptosis of the cells was detected using 400 µL buffer after 20 min at room temperature in the dark.

Effect of tea tree oil on the dairy cow mastitis model induced by LPS

Effect of tea tree oil on the apoptosis rate in the mastitis model by flow cytometry: Cells were inoculated into a 6-well culture plate, cultured for 24 h, and the medium was discarded. LPS and tea tree oil at different concentrations were added to the mixed culture. Annexin V-FITC and PI were added for the detection of apoptosis.

Detection of the expression of inflammatory factors and apoptotic factors by qPCR: Total RNA was extracted using the TRIzol method. According to the procedure supplied with the reverse transcription kit, RNA was reverse transcribed into cDNA, and the gene detection was performed by qPCR using the real-time fluorescent kit. The expression of each gene was compared with β-actin as the internal reference, and the PCR data were analyzed by the $2^{-\Delta\Delta C_t}$ method (Table 1).

Table 1
Primer sequences for the real-time polymerase chain
reaction

Gene name	Primer sequences(5'-3')
β-actin	Forward: ctctgcttgctgatccacatctg
	Reverse: ctacaccaacacggtgctgtc
TNF-α	Forward: aagcctcaagtaacaagccggtag
	Reverse: tcacaccgttgccatgag
IL-6	Forward: gaccagcagtgggtctgatcaagc
	Reverse: ccgaagctctcattaagcgcatgg
STAT1	Forward: ttcaacattctgggcactca
	Reverse: atcaccacgacgggtagaga

Table 2
Pretreatment results of sequencing data quality

Sample	Raw_reads	Raw_bases	Clean_reads	Clean_bases	Valid_bases	Q30GC
Control-1	55.89M	8.38G	54.92M	7.87G	93.91%	94.67%
Control-2	42.20M	6.33G	41.46M	5.95G	94.03%	94.59%
Control-3	50.36M	7.55G	49.65M	7.17G	94.95%	94.70%
LPS-1	55.44M	8.32G	54.64M	7.85G	94.42%	94.70%
LPS-2	48.63M	7.29G	47.92M	6.89G	94.41%	94.56%
LPS-3	50.42M	7.56G	49.69M	7.11G	94.06%	94.73%

Table 3
Distribution statistics of fpkm value of genes

Sample	Median	Mean	3rd_Qu	Max	Sd	Sum
LPS-1	1.257	18.037	10.989	4228.29	88.006	383749.433
LPS-2	1.250	17.696	10.972	4094.32	85.822	376491.190
LPS-3	1.281	17.724	11.030	4103.98	86.040	377102.881
Control-1	1.13097	18.990	11.420	3030.33	92.6130	403993.012
Control-2	1.145	18.600	11.381	2831.02	89.415	395729.721
Control-3	1.143	18.359	11.341	2779.9	88.448	390597.831
A. BLANK; B. 200 µg/mL LPS; C. 0.0002%TTO + LPS; D. 0.0004%TTO + LPS;						
E. 0.0006%TTO + LPS; F. 0.0008%TTO + LPS; G. 0.001%TTO + LPS; H. 0.002%TTO + LPS;						
I. 0.004%TTO + LPS; J. 0.006%TTO + LPS; K. 0.008%TTO + LPS; L. 0.01%TTO + LPS.						

Influence of the expression of inflammatory protein and apoptotic protein by ELISA: After washing with PBS, RIPA lysate was added to the cell lysate. According to the operation steps supplied with the ELISA kit, inflammatory related bovine nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK) and apoptosis-related caspase-3 were detected.

Analysis Of Transcriptome Sequencing

Computer sequencing: After total RNA was extracted and digested with DNase, eukaryotic mRNA was enriched with magnetic beads with oligo (DT). Using the interrupted mRNA as template, a strand of cDNA was synthesized with six-base random primers. Double-stranded cDNA was synthesized using the two-strand synthesis system and purified. The purified double-stranded cDNA was repaired followed by poly-(A) addition and sequencing. After the library construction passed the quality inspection with the Agilent 2100 Bioanalyzer, it was sequenced with the Illumina hiseqtm 2500 sequencer(19).

Data preprocessing and quality control: The raw data generated by high-throughput sequencing was in the FASTQ format sequence. To obtain high-quality reads that could be used for subsequent analysis, we first used NGS QC Toolkit software to conduct quality control and remove joints. Using bowtie2, clean reads were compared to the study species, and the situation of the samples was evaluated by the genome and gene comparison rate.

Gene quantification, differential gene screening, functional enrichment and cluster analysis: The results of the comparison between clean reads and the reference genome were stored in a binary file (BAM file). The genes were quantified to obtain the FPKM value using cufflinks. When calculating the difference in gene expression, we used Htseq-count software to determine the number of gene reads in each sample.

The estimate SizeFactors function in the DESeq R package was used to standardize the data, and the nbinomTest function was used to calculate the p-value and fold change values in the difference comparison. Differentially expressed genes with a P value less than 0.05 were selected, and GO and KEGG enrichment analysis of differentially expressed genes were carried out to determine the main biological functions or pathways affected by different genes.

HE Staining Of Cells

After dewaxing, hydration and staining, the cells were differentiated with 80% ethanol according to the color. A drop of neutral gum was dripped into the center of the paraffin section, and then the cells were sealed with a cover glass and observed and photographed with a microscope.

Immunohistochemistry

After heating the cells, the sections were dewaxed in water and then immersed in distilled water for 5 min for antigen repair. After PBS washing 3 times for 3 min, the samples were sealed. The cells were incubated with primary and secondary antibodies and washed with PBS 5 min x 3 times. DAB working solution was added to the test drop, and the samples were sealed for microscopic observation after re-staining.

Statistical analysis

Real-time fluorescent quantitative PCR results were determined using the $2^{-\Delta\Delta Ct}$ method. One-way ANOVA and SPSS 19.0 were used to analyze the differences among the treatment groups, and the results are expressed as the mean \pm standard deviation. * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$.

Results

CCK-8 detection of cell proliferation activity induced by LPS

As shown in the Fig. 1, the activity of the cells began to decline to varying degrees in the 100 $\mu\text{g/ml}$ LPS for 12 h. As the activity of cells induced by LPS of 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ was too low, we chose 100 $\mu\text{g/ml}$ culture for 12 h as the best treatment condition.

Apoptosis Of LPS-induced BMECS

In the Fig. 2A, approximately only 4.44% early apoptosis and late apoptosis were observed without LPS. In the Fig. 2B, upon addition of 100 $\mu\text{g/ml}$ LPS, the whole image shifted to the right, and approximately 7.48% (Early apoptosis 2.73 + Late apoptosis 4.75) apoptosis occurred. The apoptosis effect was not obvious. When 200 $\mu\text{g/ml}$ LPS was added to group C (Fig. 2C), the whole image of group C showed obvious clustering, and approximately 49.12% of the cells showed early and late apoptosis, which was suitable for the mastitis model in the follow-up experiment (Fig. 2).

Effect Of Tea Tree Oil On LPS-induced BMECs

The blank control group A (Fig. 3A) showed apoptosis of normal growth mammary epithelial cells without any treatment. The proportion of living cells was 92.10%, the proportion of early apoptotic cells was 2.08%, and the proportion of late apoptotic cells was 4.44%. In group B, mammary epithelial cells treated with 200 µg/ml LPS treatment showed apoptosis. Among these cells, the proportion of living cells was 50.66%, the proportion of early apoptotic cells was 45.70%, and the proportion of late apoptotic cells was 3.42%. After adding different concentrations of TTO to C-L (Fig. 3C- Fig. 3L), Group D (Fig. 3D), E (Fig. 3E), and F (Fig. 3F) achieved some protective effects, especially group E. The proportion of living cells, early apoptotic cells and late apoptotic cells was 71.95%, 22.15% and 5.11%, respectively.

Effect of tea tree oil on inflammatory factors and apoptotic factors in the LPS-induced mastitis model

TNF-α and IL-6 are inflammatory factors related to the inflammatory response(20). STAT1 is related to apoptosis(21). Compared with the blank group, TNF-α and IL-6 were expressed more than 15 times higher in response to LPS treatment at a concentration of 200 µg/ml. Additionally, STAT1 expression increased nearly 6 times after addition of TTO at 0.0004%, 0.0006% and 0.0008%, respectively. The expression of TNF-α and IL-6 decreased with the increase in TTO concentration. The decrease in TNF-α expression was more obvious. The expression of IL-6 was significantly different. The expression of STAT1 increased slightly upon addition of 0.0004% TTO. The expression level with 0.0006% and 0.0008% was lower than in the LPS group. The expression level was lowest with a 0.0006% concentration of TTO (Fig. 4).

Expression Of Inflammatory And Apoptotic Proteins

The experimental results are shown in Fig. 5. After addition of 200 µg/ml LPS, the LPS group showed significantly increased protein expression of NF-κB, MAPK and caspase-3. The protein expression levels of NF-κB, MAPK and caspase-3 were significantly reduced in the experimental TTO groups.

Transcriptome Analysis Of Data Output Statistics

Through the Illumina platform, a large number of samples were sequenced. Considering the impact of the data error rate on the results, we used trimmatomatic software to preprocess the quality of the original data and to generate a statistical summary of the number of reads in the whole quality control process.

Analysis Of The Gene Expression Level

In the transcriptome sequencing analysis, the protein coding gene expression level was estimated by counting the sequencing sequence (reads) located in the region of the protein-coding genome or exon of the protein-coding gene. In addition to the true expression level of protein-coding genes, the read count was also positively related to the length and sequencing depth of the protein-coding genes. The Fpkm method was used to calculate the expression of protein-coding genes, which is the number of fragments from a protein coding gene per thousand base length in every million fragments. Fpkm is one of the most commonly used methods to estimate the expression level of protein-coding genes.

Horizontal Box Plot Of Gene Expression

The box Whistler plot is a method to describe data using five statistics (minimum, first quartile (25%), median (50%), third quartile (75%) and maximum) in data. This plot can also roughly assess the symmetry, distribution dispersion and other information in the data. Figure 6 shows that the degree of symmetry and dispersion was good.

Correlation Test Between Samples

The correlation of protein-coding gene expression among samples is an important index to test the reliability of the experiment and the rationale for the sample selection. The closer the correlation coefficient is to one, the higher the similarity is between the expression patterns. Figure 8 shows that the similarity of the LPS group was close to one. The similarity of the control group was close to 1 (Fig. 7).

Principal Component Analysis

Principal component analysis (PCA) was used to investigate the distribution of samples, to explore the relationship between samples or to verify the experimental design. PCA can show the relationship between samples from different dimensions. The LPS group and control group shown in the PCA diagram are close to each other. The data are very good (Fig. 8).

Differential Gene Screening

The number of counts of each sample gene was standardized using DESeq software. Nb (negative binomial distribution test) was used to test the significance of the read difference. Finally, the differentially expressed protein-coding genes were screened according to the test results showing multiple and significant differences. The condition used to screen differences was $p < 0.05$ and that to screen multiple differences was more than 2. A total of 1270 mRNAs were identified as differentially expressed, of which 787 genes were upregulated and 483 genes were downregulated. The differentially expressed genes include TNF - α , IL6, STAT1, mapk4, etc. Among these genes, TNF- α and IL6 were significantly up-regulated. The difference multiples were 4.41 and 6.28 times, respectively (Fig. 9, Table S1).

GO Analysis Of Differentially Expressed Genes

After identifying differentially expressed genes by GO analysis, we analyzed the enrichment of differentially expressed genes and described their functions. GO annotation of differentially expressed mRNA was used to identify participation in biological process, molecular function, cellular component and signal pathway in the cell before and after LPS treatment. The GO annotation results indicated that the differentially expressed mRNA might participate in biological adhesion, biological regulation, cell killing, cellular component organization or biogenesis, cellular process, developmental process, growth, immune system process, negative regulation of biological process, positive regulation of biological process, cell junction, among others (Fig. 10).

KEGG Analysis Of Differentially Expressed Genes

The KEGG database was used to perform pathway analysis of differentially expressed protein-coding genes, and the hypergeometric distribution test was used to calculate the significance of differential gene enrichment in each pathway entry. The top 20 were obtained by KEGG enrichment analysis. The differentially expressed mRNA might participate in TNF signaling pathway, Rheumatoid arthritis, Inflammatory, Staphylococcus aureus infection, Systemic lupus erythematosus, Graft-versus-host disease, Allograft rejection, Intestinal immune network for IgA production, Type I diabetes mellitus, Herpes simplex infection, Toll-like receptor signaling pathway, NF-kappa B signaling pathway, among others (Fig. 11).

Gene Function Research

To verify the accuracy of the screening, we verified the function of some genes. In this study, the functions of TNF- α and IL6 were verified by treating mammary epithelial cells with LPS and TTO + LPS. Compared with Blank, the cells treated with LPS showed a heightened degree of apoptosis. However, the TTO + LPS group inhibited this state (Fig. 12A). In the immunohistochemical experiment, the cells treated with LPS also showed heightened degree of TNF- α and IL6 expression. The expression of TNF- α and IL6 was significantly in TTO + LPS group (Fig. 12B and 12C). Results showed TNF- α and IL6 were observed to play important roles in mediating the preventive effect of tea tree oil on mastitis in LPS-stimulated bovine mammary epithelial cells (Fig. 12).

Discussion

LPS is a macromolecular structural component on the outer membrane of Gram-negative bacteria(22). LPS is released after bacteria die and dissolves or destroys bacterial cells via artificial methods(23). The toxic component of LPS is mainly lipid a, which can trigger the immune response in mammalian cells and lead to the release of pro-inflammatory factors. The whole process of mastitis can be reproduced using an LPS-induced mastitis model(23, 24). In this experiment, when the mastitis model was constructed, the proliferation activity of the cells was enhanced following stimulation with LPS (50 $\mu\text{g/ml}$). When the concentration was more than 100 $\mu\text{g/ml}$, the activity decreased. This result is consistent with previous studies. In addition, LPS shows dual effects. Stimulation with a low concentration of LPS can improve immune system activity and enhance the proliferation activity of original cells, while a high concentration of LPS can lead to a serious inflammatory reaction followed by apoptosis.

Tea tree oil has a good inhibitory effect on *Escherichia coli* bacteria and endotoxins(25). Gustafson et al. have found that tea tree oil can promote autolysis of *Escherichia coli*(26). These researchers also showed that tea tree oil has a significant inhibitory effect on LPS-induced inflammation. Therefore, tea tree oil can also play a positive role in protection against cow mastitis. In the present analysis, flow cytometry results showed that the proportion of normal living cells infected by LPS increased significantly after addition of

tea tree oil at an appropriate concentration. The proportion of early apoptosis, late apoptosis and dead cells decreased. In addition, the immune response of cells to infection was mainly manifested by the release of cytokines. When LPS induces breast acute inflammation, the cells will produce TNF- α , IL-6 and STAT1(27). TNF- α is the main cytokine in the early stage of infection, which is closely related to endotoxin shock in *E. coli* mastitis and has a chemotactic effect on neutrophils(28). IL-6 is a pleiotropic cytokine that can mediate many immune and inflammatory reactions(29). The results showed that tea tree oil could inhibit the expression of TNF- α and IL-6 induced by LPS with superior inhibition of TNF- α . STAT1 can promote apoptosis, inhibit cell growth and differentiation, and play an important role in inhibiting the occurrence and development of tumors. Thus, the appropriate concentration of tea tree oil can inhibit the expression of STAT1 to a certain extent. It is possible that when LPS stimulates cells, the body's immunity will be improved. The expression level (TNF- α , IL-6 and STAT1) of related factors also increased. However, the expression level of these factors was down regulated when TTO treated the cells.

Previous studies have shown that inflammatory cytokines are mainly produced by activation of the NF- κ B and MAPK signaling pathway, while apoptosis-promoting factors are mainly produced by activation of the caspase-3 pathway(30, 31). To further explore the mechanism of tea tree oil inhibition of the production of inflammatory cytokines and pro-apoptotic factors, we detected the expression of NF- κ B, MAPK and caspase-3 in response to tea tree oil. According to the ELISA results, the expression of NF- κ B, MAPK and caspase-3 was high in LPS-infected mammary epithelial cells and decreased significantly after addition of tea tree oil. The above results showed that tea tree oil at an appropriate concentration could inhibit the production of NF- κ B, MAPK and caspase-3 and prevent overexpression of TNF- α , IL-6 and STAT1 to protect against the mastitis induced by LPS. Therefore, it is speculated that tea tree oil can inhibit activation of the NF- κ B, MAPK and caspase-3 signaling pathway in the body. Xiu et al. used *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* to infect mammary epithelial cells of dairy cows, and they sequenced the transcriptome of the infected cells using the Solexa system(32). GO analysis showed that the differentially expressed genes in the three groups were concentrated in cell metabolism, apoptosis and embryonic development. Cluster analysis of homologous proteins showed that they were involved in translation, ribosome biosynthesis and repair. KEGG analysis showed that the three pathways were enriched in the oxidative phosphorylation pathway, nod-like receptor pathway and apoptosis pathway, respectively. Wang et al. sequenced the mammary gland tissue of healthy cattle and *S. aureus* mastitis cattle using the paired end technology of the Illumina system, and they screened 1352 differentially expressed genes. Among them, ITGB6, MYD88, ADA, ACKR1, and TNFRSF1B were closely related to *S. aureus* mastitis, which could be used as candidate genes for *S. aureus* mastitis resistance(33). In addition, the CCL5, coec2, LTF, CD46 and Ncf1 genes exhibited complex alternative splicing in the infected breast tissue. Wang et al. sequenced the transcriptome of mammary epithelial tissue induced by s56, s178 and S36 *S. aureus*, and they screened 1720, 427 and 219 differentially expressed genes, respectively(34). GO and pathway analysis showed that these genes were significantly involved in the inflammatory response, metabolic transformation, cell proliferation and apoptosis signaling pathways. IL-1 α , TNF, efnb1, IL-8 and EGR1 were upregulated. These studies provide a reference for breast milk mastitis-related gene transcription, post-transcriptional regulation and the host

cell immune response to pathogens. These findings are consistent with the differentially expressed genes determined in this study. These new potential genes can be used as biological markers for the diagnosis and prevention of recessive and clinical mastitis in dairy cows. In addition, our preliminary identification of these gene functions may help to elucidate the molecular mechanism of LPS-induced mastitis at the overall level of the gene network.

Conclusions

Mastitis severely affects milk production and milk quality in dairy cattle. The results showed that tea tree oil had a protective effect against LPS-induced mastitis. The difference of TNF- α and IL-6 expression was significant in LPS-induced BMECs. They were also observed to play important roles in mediating the preventive effect of tea tree oil on mastitis in LPS-stimulated BMECs. They provide the laboratory research basis for prevention and treatment of mastitis in cows.

Abbreviations

LPS: Lipopolysaccharide; BMECs: Bovine mammary epithelial cells; NF-Kb: Nuclear factor kappa B; MAPK: mitogen-activated protein kinase; E. coli: Escherichia coli; TTO: Tea tree oil; TNF- α : Tumour necrosis factor alpha-like; STAT1: Signal transducer and activator of transcription 1; IL-6: Interleukin 6; IL-8: Interleukin 8; IL-10: Interleukin 10; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; CCK-8: Cell Counting Kit-8; PCA: Principal component analysis

Declarations

Acknowledgements

Not applicable.

Authors' Contributions

Zhi Chen, Jingpeng Zhou and Yi Zhang contributed equally to this work. Zhi Chen and Zhangping Yang conceived and designed the experiments. Zhi Chen, Yi Zhang, Jingpeng Zhou, Xiaolong Wang performed the experiments. Zhi Chen and Deming Gou analyzed the data. Zhi Chen, Juan J. Loo wrote the paper.

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Availability of data and materials

Not applicable.

Ethics statement

The animal use protocol was approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Yang Zhou University, Yang Zhou, China.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing financial interest.

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Figures

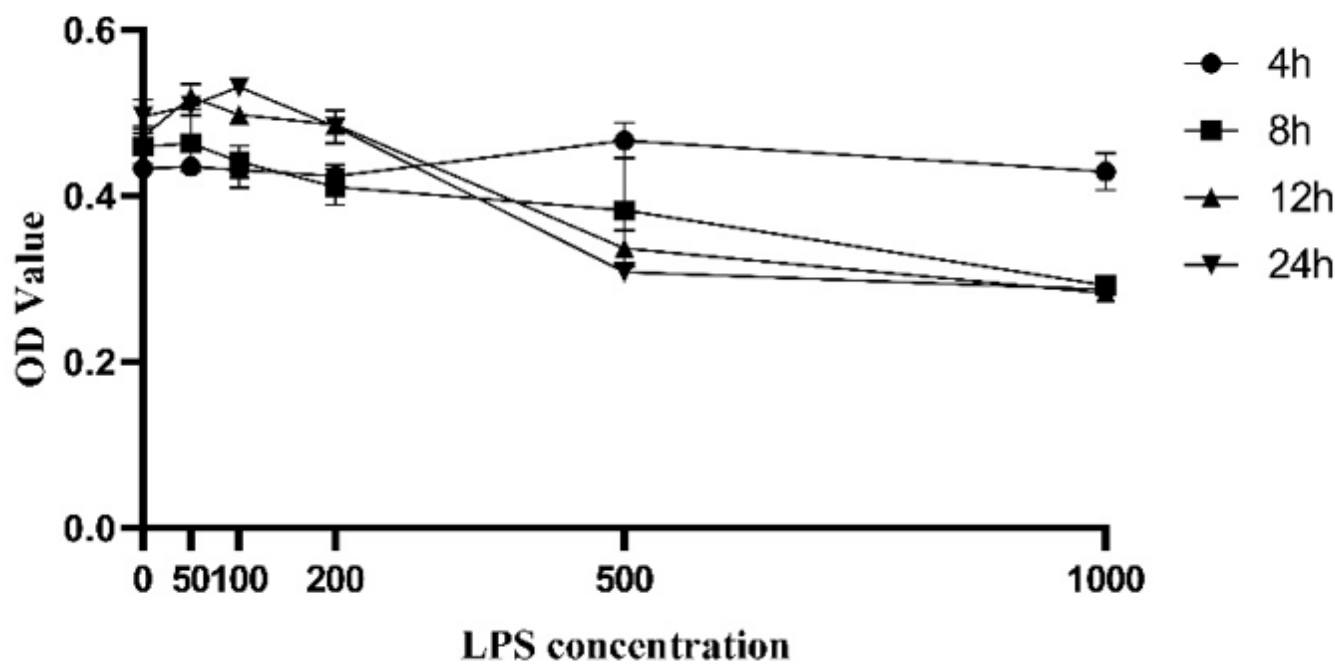


Figure 1
Cell proliferation activity induced by LPS in different concentrations and times

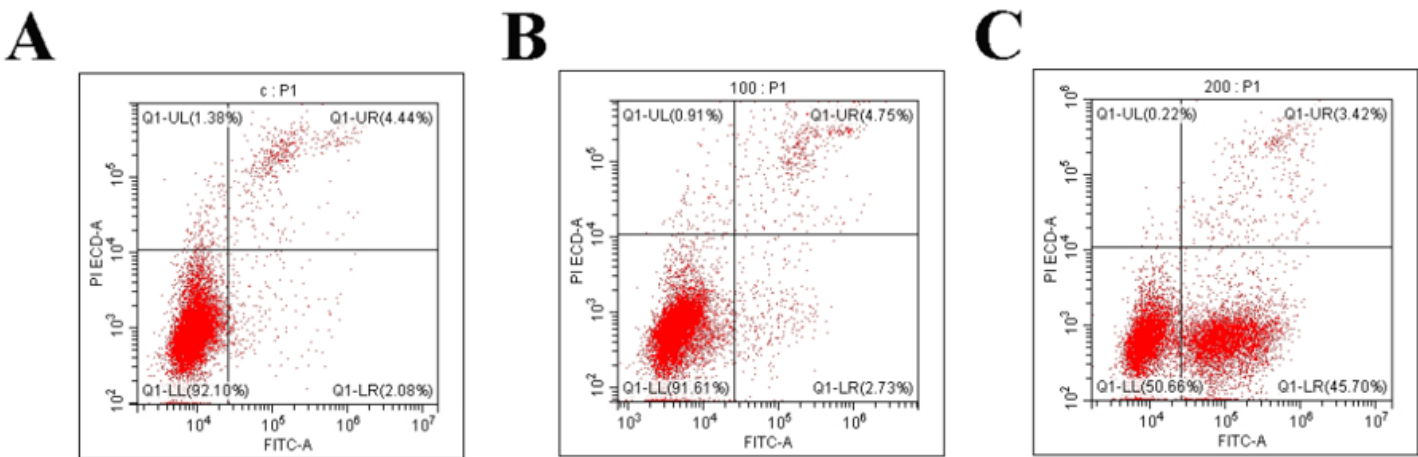


Figure 2
Effect of LPS-induced apoptosis by flow cytometry. A: BLANK; B: 100 µg/ml LPS; C: 200 µg/ml LPS

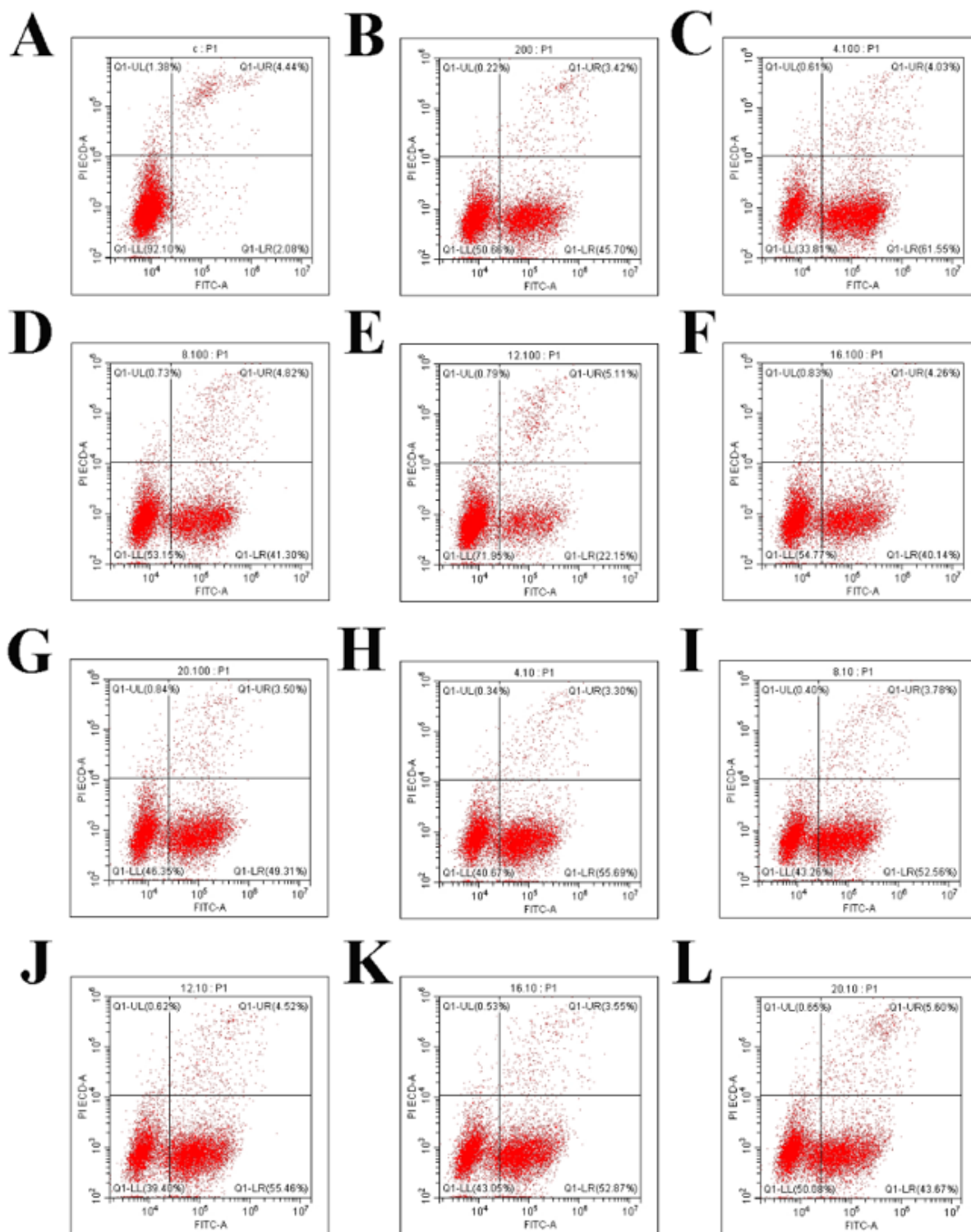


Figure 3

Effect of different concentrations of TTO on apoptosis in the mastitis model flow cytometry. A. BLANK; B. 200 μ g/mL LPS; C. 0.0002%TTO+LPS; D. 0.0004%TTO+LPS; E. 0.0006%TTO+LPS; F. 0.0008%TTO+LPS; G. 0.001%TTO+LPS; H. 0.002%TTO+LPS; I. 0.004%TTO+LPS; J. 0.006%TTO+LPS; K. 0.008%TTO+LPS; L. 0.01%TTO+LPS.

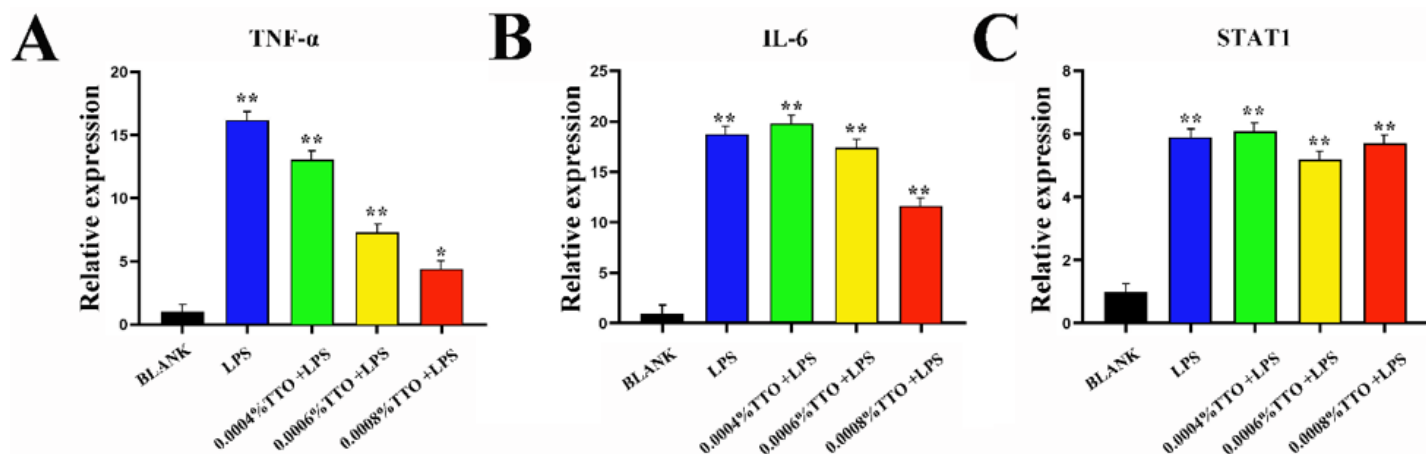


Figure 4

Effect of TTO on the expression level of genes

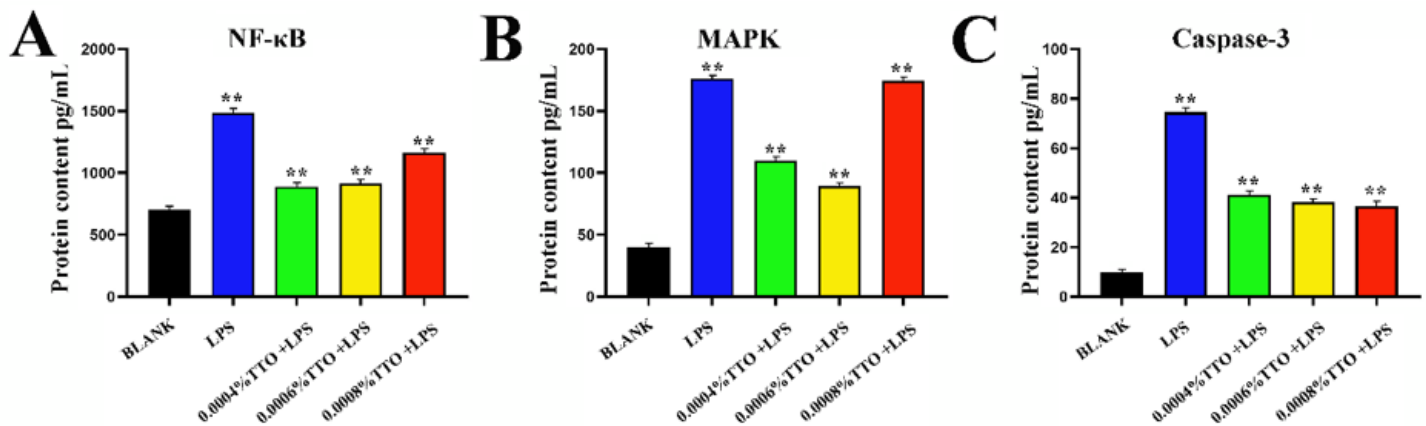


Figure 5

Effect of TTO on the protein expression level of genes

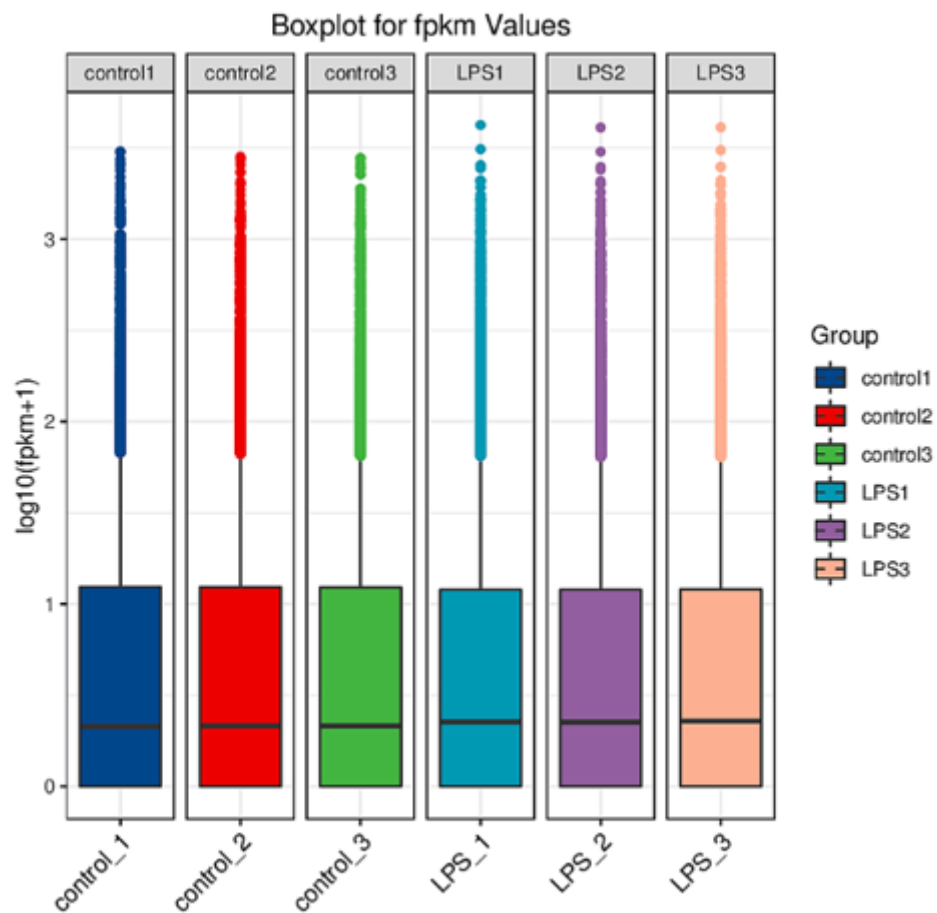


Figure 6

Box line diagram in control and LPS group The abscissa is the sample name and the ordinate is log10 (fpkm + 1).

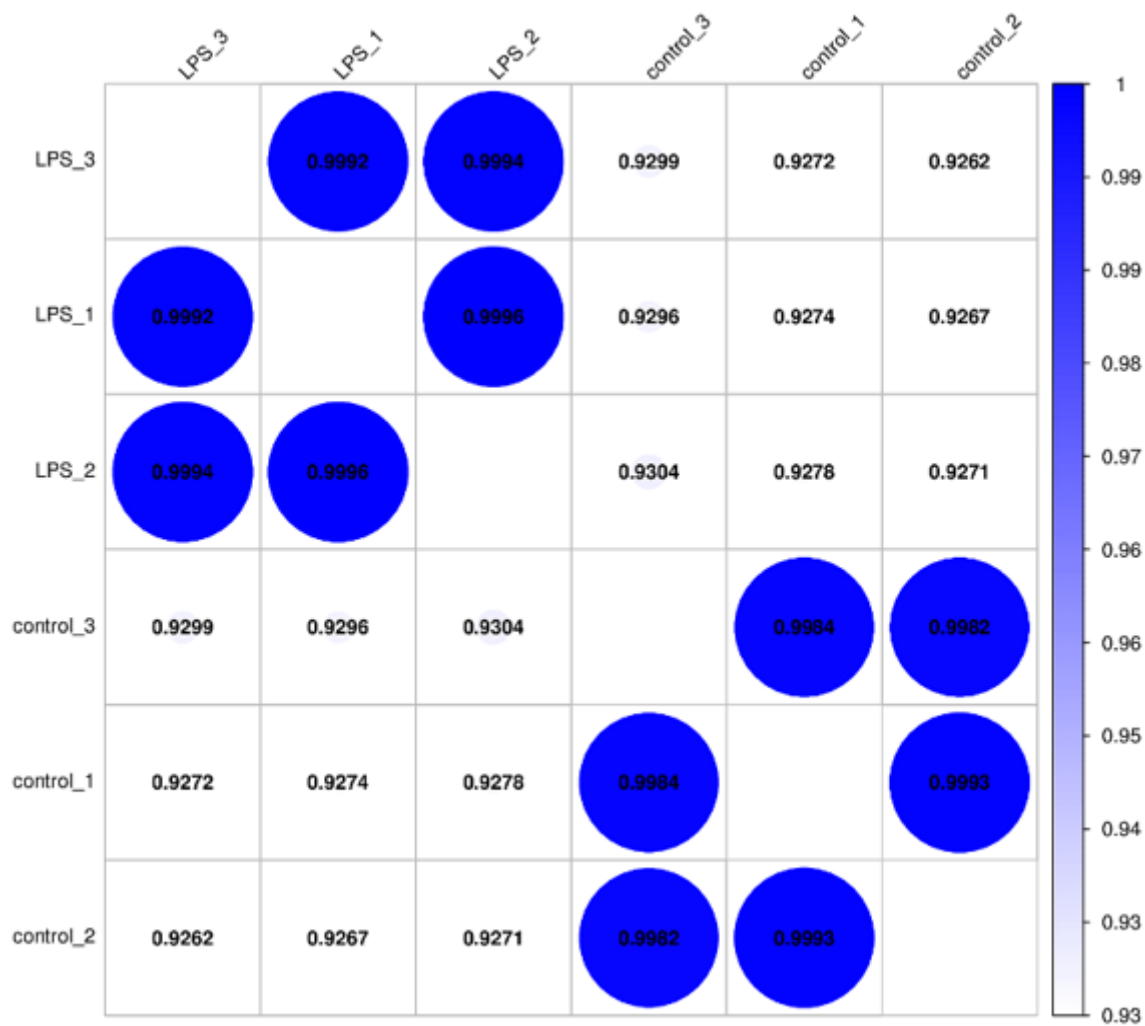


Figure 7

Thermal diagram of the correlation coefficient between samples The abscissa represents the name of the sample, and the ordinate represents the name of the corresponding sample. The color represents the size of the correlation coefficient.

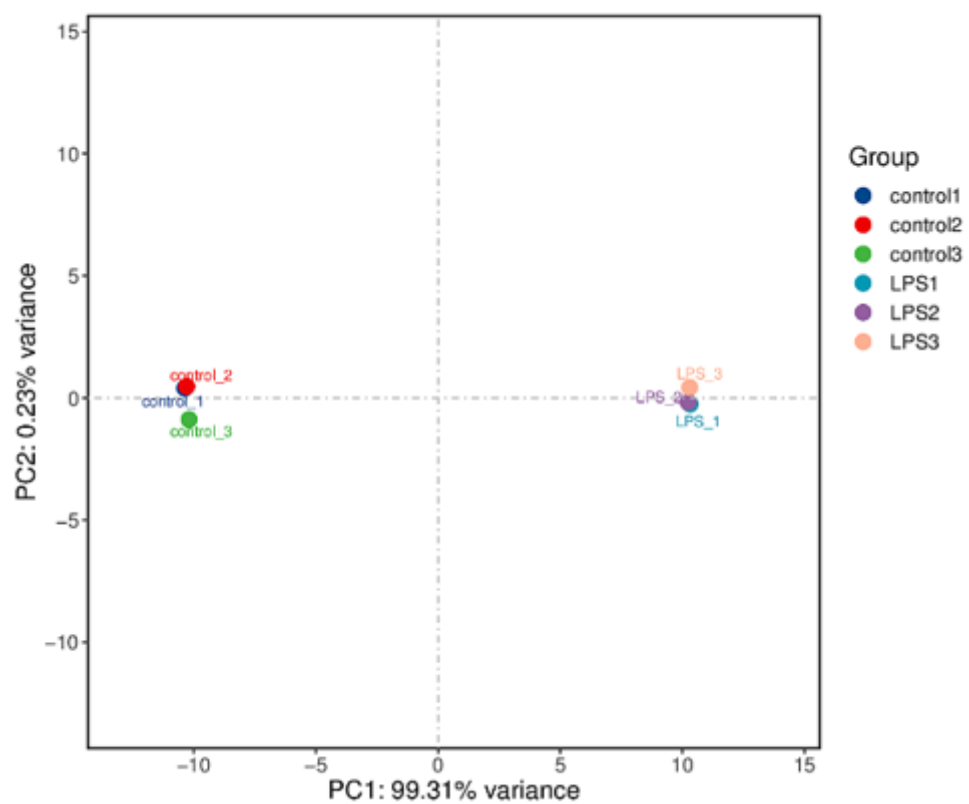


Figure 8

PCA diagram in control and LPS group

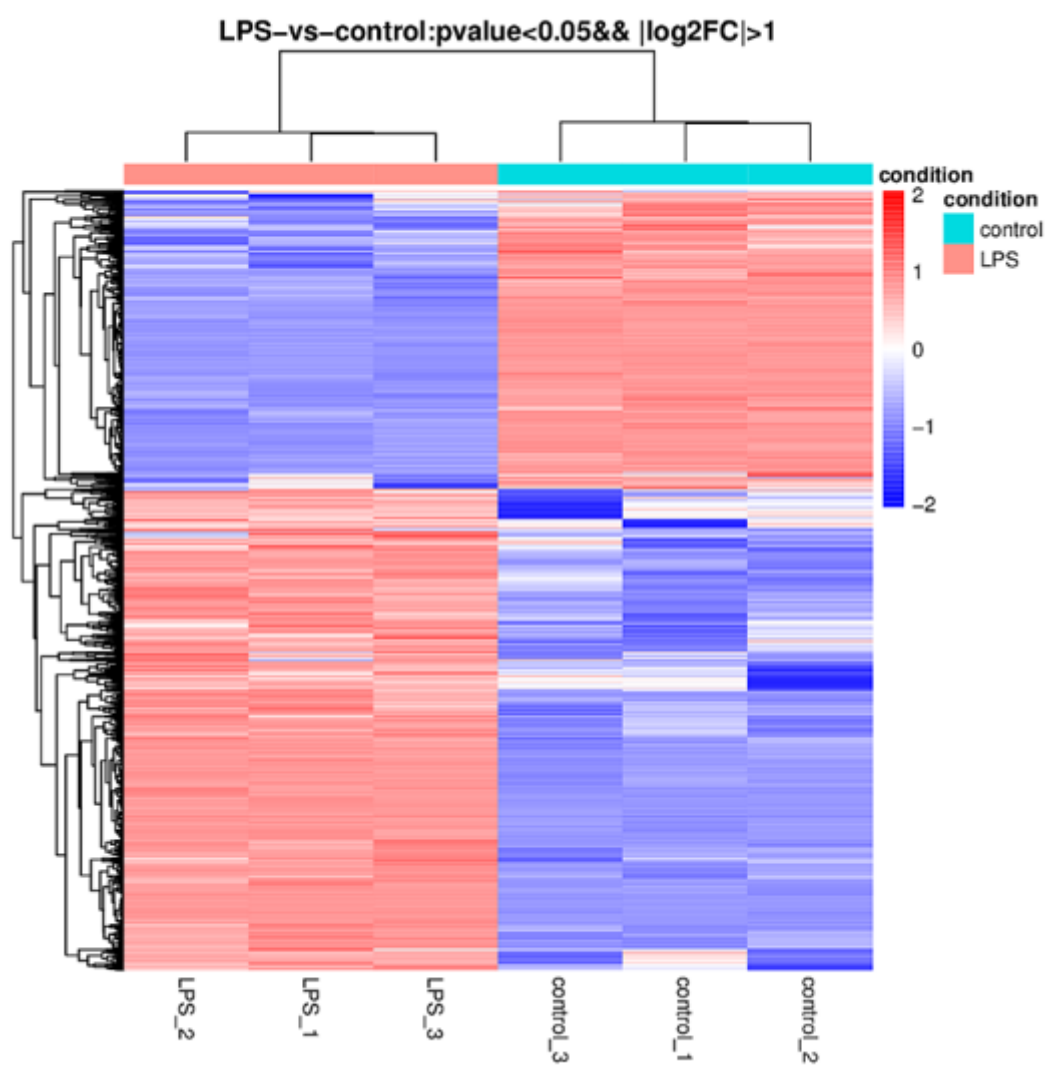


Figure 9

Screening for mRNAs with differential expression in the LPS-induced BMECs

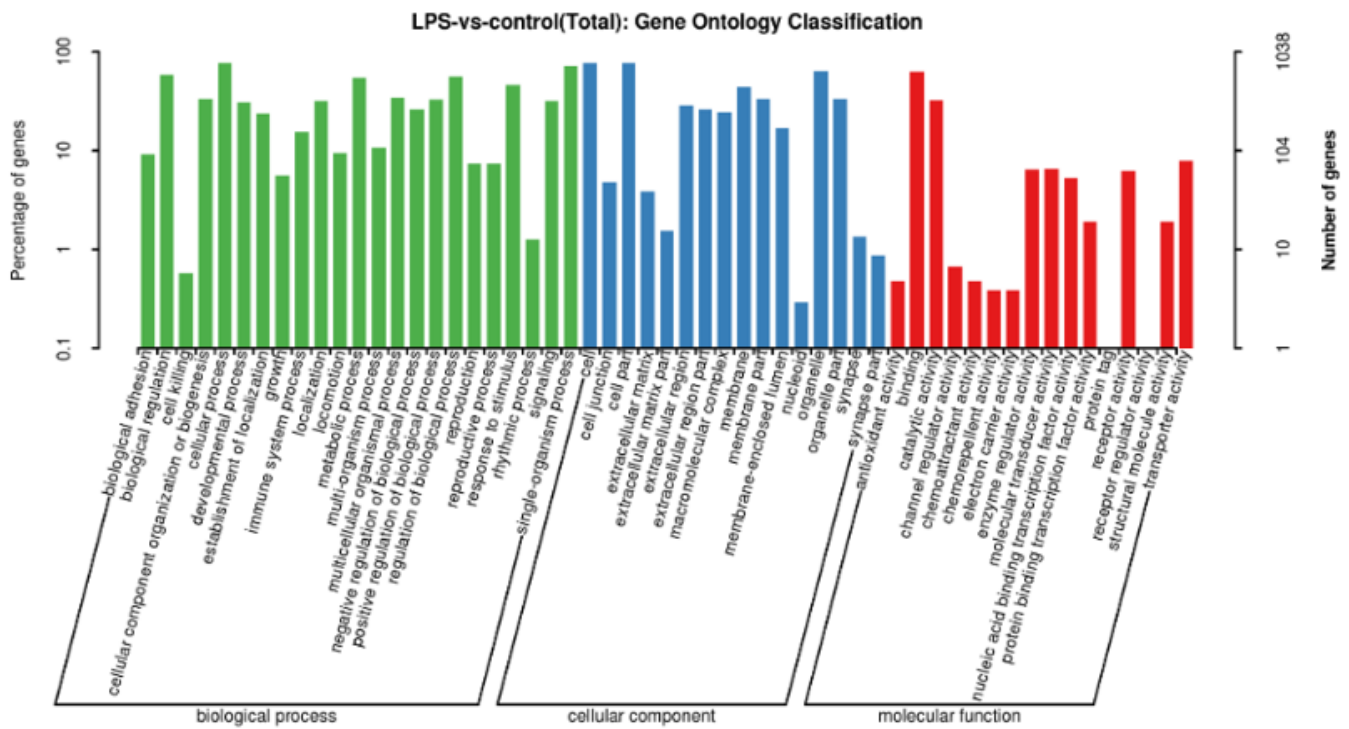


Figure 10

GO enrichment. Horizontal axis is the GO entry name and the vertical axis is the $-\log_{10}$ p-value.

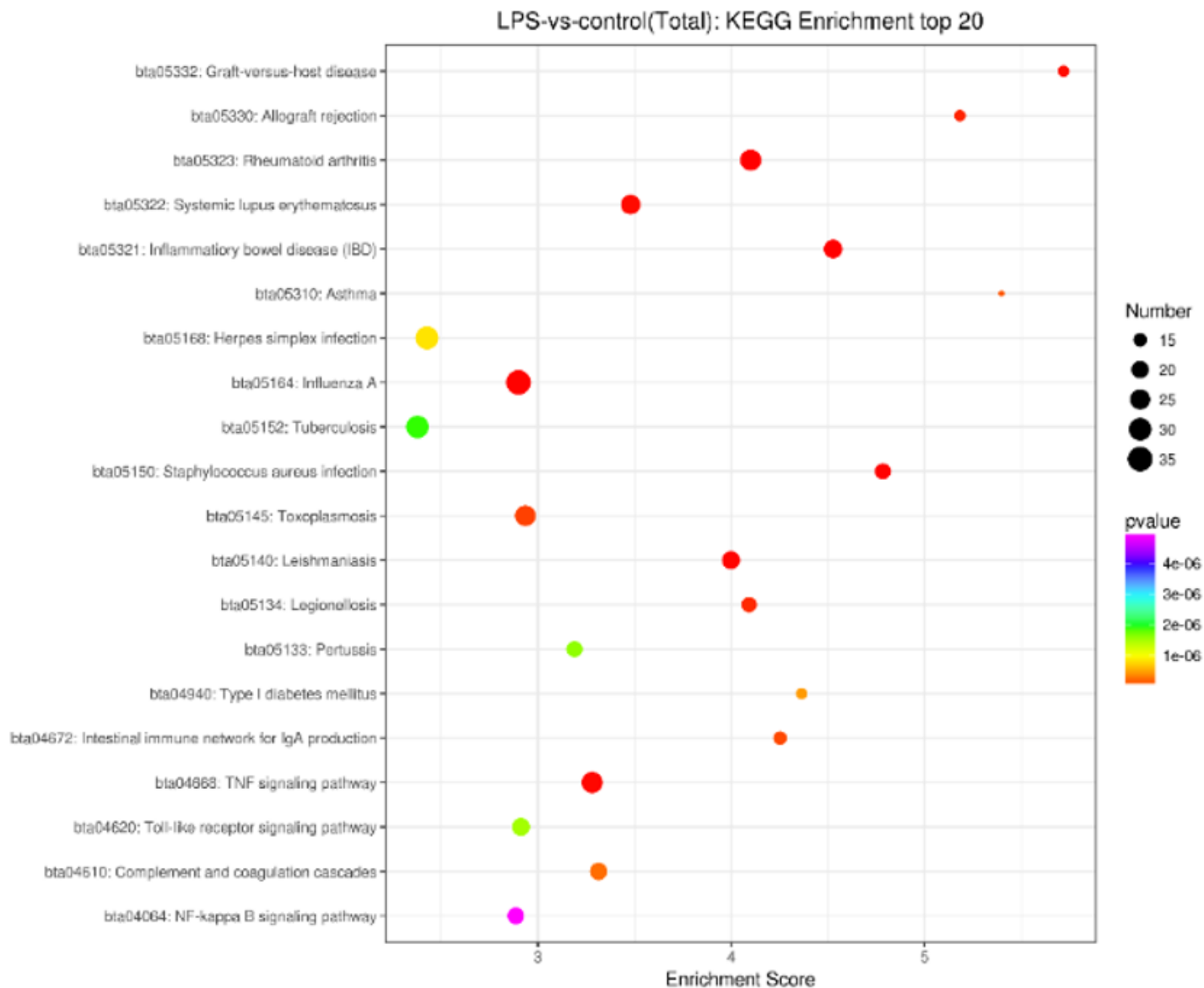


Figure 11

KEGG enrichment, top 20 genes

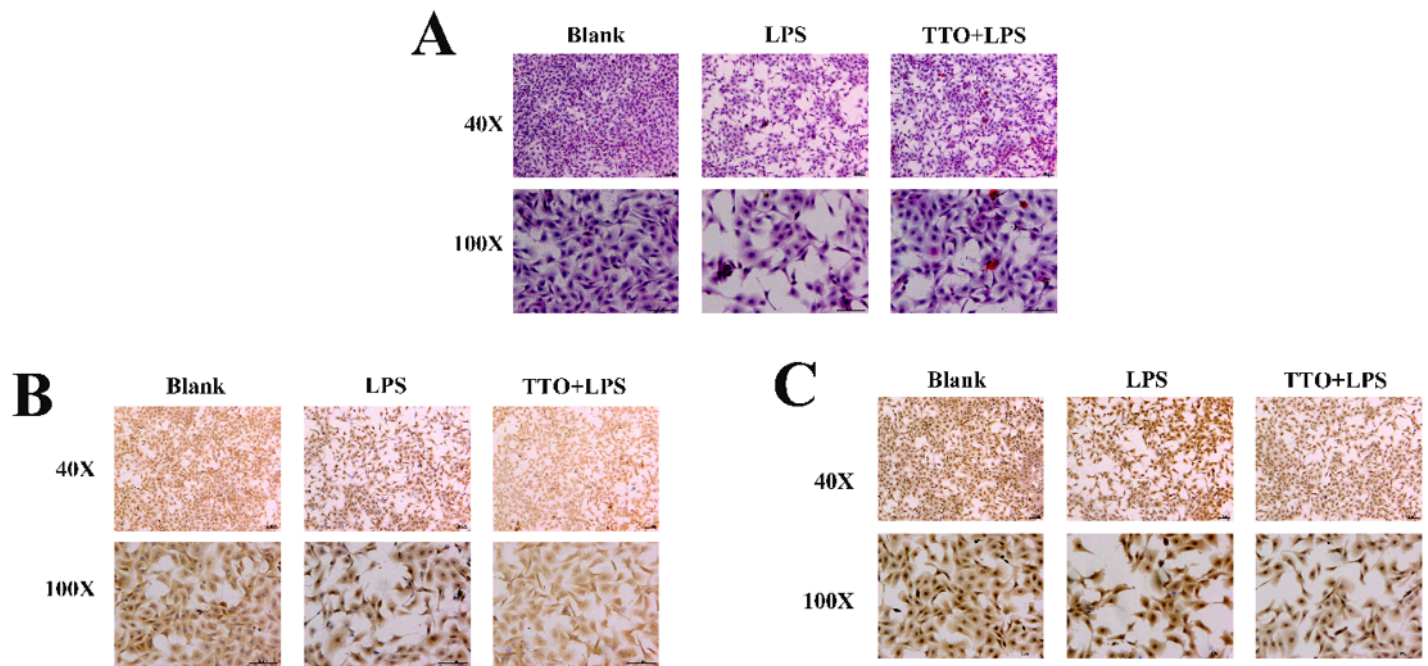


Figure 12

TNF- α and IL6 function research. A. HE staining of BMECs; B. TNF - α protein immunohistochemical of BMECS; C. IL-4 protein immunohistochemical of BMECS.

Supplementary Files

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