• Basic Research •

Anti-inflammatory mechanisms of *Hedysarum polybotrys* polysaccharide in endotoxin-induced uveitis: insights into candidate genes and pathways

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Abstract

● **AIM:** To identify key genes and inflammatory signaling pathways involved in the anti-inflammatory effects of *Hedysarum polybotrys* polysaccharide (HPS) in a rat model of endotoxin-induced uveitis (EIU).

● **METHODS:** EIU was induced in Wistar rats through subcutaneous injection of lipopolysaccharide (LPS, 200 μg) and the rats were then randomly assigned to EIU group (n=5) and the HPS intervention group (n=5). HPS (400 mg/kg, intraperitoneally) or its carrier was administered 24h and 1h prior to EIU induction. Eyes were examined and enucleated 24h post-induction, and total RNA was extracted from the iris-ciliary body. Gene expression microarrays were used to identify differentially expressed genes (DEGs), followed by bioinformatics analyses, including gene ontology (GO) and pathway analysis. Key findings were not experimentally validated at the mRNA or protein level.

● **RESULTS:** A total of 322 DEGs were identified, comprising 254 mRNA and 68 lncRNA genes. GO analysis revealed significant functional categories, including response to LPS. Pathway analysis identified key signaling pathways involved in uveitis, such as cytokine-cytokine receptor interactions. Notably, 16 mRNA and 7 lncRNA DEGs emerged as central nodes in the gene correlation network.

● **CONCLUSION:** HPS exerts its anti-inflammatory effects through coordinated signaling pathways, offering insights into potential therapeutic targets for managing uveitis.

● **KEYWORDS:** differentially expressed genes; *Hedysarum polybotrys* polysaccharide; endotoxin-induced uveitis; lncRNA; gene expression microarray

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INTRODUCTION

Uveitis is a common ocular inflammatory disease, with acute anterior uveitis (AAU) being its most prevalent subtype[1-2]. AAU often presents acutely with severe symptoms and high recurrence. If left untreated or inadequately managed, it may lead to irreversible visual impairment[3]. Current treatments rely on glucocorticoids and immunosuppressants, which are effective but limited by side effects and relapse upon withdrawal.

Toll-like receptor 4 (TLR4) is present in human uveal tissue, especially in resident antigen-presenting cells, suggesting a role in ocular immunity[4]. In an endotoxin-induced animal model of AAU, Chen *et al*[5] observed morphological changes and activation of macrophages in the iris and ciliary body, alongside upregulated TLR4 expression. Similarly, Wang *et al*[6] found that TLR4-deficient mice showed reduced peritoneal macrophage activation upon lipopolysaccharide (LPS) stimulation, linked to impaired nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling, highlighting TLR4's key role in inflammation.

Studies on cytokine secretion patterns during endotoxin-induced uveitis (EIU) have revealed early peaks of pro-inflammatory factors such as tumor necrosis factor (TNF)-α, followed by increased levels of the anti-inflammatory cytokine interleukin (IL)-10, mirroring the immunological profile

Table 1 The characteristics of the purified *Hedysarum polybotrys* polysaccharide

Molecular weights, Da	Monosaccharid proportion, %				
	Rhamnose	Arabinose	Xylose	Glucose	Galactose
4.1×10^3–1.2×10^3	1.31	26.49	17.51	43.69	11.01

observed in human AAU[7-8]. Notably, uveitis could not be induced in TLR4 knockout mice. These animals also exhibited altered cytokine secretion patterns, further underscoring the importance of TLR4 signaling in EIU pathogenesis[7].

Hedysarum polybotrys polysaccharide (HPS), the main bioactive component of *Hedysarum polybotrys* Hand.-Mazz (Fabaceae), has attracted interest for its immunomodulatory properties. Prior research demonstrated that HPS can improve gastrointestinal immune balance and protect against gastric ulcers by suppressing TNF-α and IL-6[9-10]. Our previous work showed that intraperitoneal administration of HPS significantly reduced intraocular inflammation in EIU rats. This was accompanied by decreased expression of TLR4, MyD88, and NF-κB p65 in the iris-ciliary body, suggesting that HPS may act by modulating the TLR4/NF-κB signaling pathway[11].

While high-throughput RNA expression profiling has traditionally focused on protein-coding genes, recent studies emphasize the biological significance of non-coding RNAs. Although only a small fraction of the genome encodes proteins, approximately 75% of it is transcribed into RNA[12]. Long non-coding RNAs (lncRNAs), typically exceeding 200 nucleotides, are a major class of these transcripts. Unlike conserved small RNAs such as snoRNAs and microRNAs[13-14], lncRNAs often lack evolutionary conservation. Nevertheless, they are increasingly recognized for their diverse regulatory roles in gene expression[15]. These functions span DNA, RNA, protein, and microRNA interactions, and thousands of lncRNAs have now been experimentally validated[16-19]. They have been implicated in a broad spectrum of diseases, including cancers, cardiovascular disorders, and neurodegenerative conditions[20-23].

Based on these findings, we used genome-wide RNA microarrays to profile mRNAs and lncRNAs in the iris-ciliary body of EIU rats pretreated with HPS. We aimed to identify key differentially expressed genes (DEGs) and their functional networks through gene ontology (GO), pathway enrichment, and co-expression analyses, to reveal the molecular mechanisms of HPS in uveitis.

MATERIALS AND METHODS

Ethical Approval All experimental protocols and procedures were ethically reviewed and approved by the Ethics Review Board of Capital Medical University and Peking University Third Hospital (Approval No.201445; No.A20240175), ensuring compliance with established ethical guidelines for animal experimentation. The study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals Ten male specific-pathogen-free (SPF) Wistar rats weighing between 180 and 200 g were procured from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). These rats were maintained under standardized laboratory conditions, with a temperature of 22°C and a 12-h light/dark cycle, ensuring optimal environmental conditions. Adequate food and water were provided ad libitum throughout the experimental period.

Experimental Groups All animals were randomly assigned to one of two experimental groups: EIU group and the HPS intervention group, with each group comprising 5 rats. Rats in the HPS group received intraperitoneal injections of 2 mL of a 400 mg/kg HPS solution, as previously reported[11], kindly provided by the Lanzhou University Pharmacology Lab (Table 1, for the characteristics of the HPS). Conversely, rats in the EIU group were administered equivalent volumes of sterile saline. After a 23-h interval, rats in the HPS group received a second intraperitoneal injection of 2 mL of the 400 mg/kg HPS solution, while those in the EIU group received sterile saline injections. Subsequently, all animals received a subcutaneous injection of 200 μg of Vibrio cholerae LPS (Lanzhou Biological Product Research), dissolved in 100 μL of sterile saline, 1h following the second injection[5].

Clinical Evaluation Twenty-four hours post-LPS challenge, rat eyes underwent examination using a slit-lamp. The severity of inflammation was assessed utilizing the grading system outlined in Table 2, with evaluations conducted by an investigator blinded to the experimental conditions[24]. Subsequently, rats were sacrificed, and both eyes were promptly enucleated under a surgical microscope to facilitate the extraction of total RNA from the iris-ciliary body complexes.

Total RNA Extraction and Microarray Assay Total RNA extraction from the iris-ciliary body complexes was performed using Trizol reagent (Invitrogen, USA), following the manufacturer's protocol, with subsequent assessment of its integrity *via* standard denaturing agarose gel electrophoresis. The extracted total RNA was then reverse-transcribed into double-stranded cDNA, which was further synthesized into cRNA and labeled with fluorescent dyes (Cy5 and Cy3-dCTP) utilizing the CapitalBio cRNA amplification and labeling kit (CapitalBio, Beijing, China). Subsequently, the labeled cRNAs derived from both lncRNAs and mRNAs were purified and

Table 2 Scoring system for clinical evaluation of uveitis

Clinical signs	Grade of uveitis (score)
Iris hyperemia	
Absent	0
Mild	1
Moderate	2
Severe	3
Pupil	
Normal	0
Miosed	1
Exudate in anterior chamber	
Absent	0
Small	1
Large	2
Hypopyon	
Absent	0
Present	1
Maximum possible score	7

hybridized onto the CapitalBio Tachnology Rat LncRNA Array V1 (CapitalBio, Beijing, China). Following hybridization, the microarray images were scanned using an Agilent microarray scanner, and subsequently gridded and analyzed employing Agilent Feature Extraction software. Raw data were then summarized and normalized using GeneSpring software (Agilent).

Statistical Analysis Differential expression analysis of lncRNAs and mRNAs between the HPS group and the EIU group was conducted using a paired *t*-test and fold change filtering, with statistical significance defined as $P<0.05$ and fold change greater than 1.5. False discovery rate (FDR) adjustment using the Benjamini-Hochberg procedure was applied to correct the *P*-values[25-27], ensuring a minimized error rate. The smaller the FDR value, the smaller the error rate of the *P* value[28].

GO analysis was employed to elucidate the genetic regulatory networks associated with DEGs, categorizing them into hierarchical classes based on molecular function, biological process, and cellular component aspects[29-30]. Additionally, pathway analysis was performed utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to identify significant pathways enriched among the DEGs.

To elucidate interactions among differentially expressed lncRNAs and mRNAs, a co-expression network was constructed based on correlation analysis of these transcripts[31]. Correlation coefficients greater than 0.99 or less than -0.99, along with a $P<0.05$, were considered significant. The number of genes surrounding each other was analyzed to identify genes with the highest connectivity or "degree" within the network. Cytoscape software (The Cytoscape Consortium, San Diego, CA, USA) was employed for network visualization, with yellow dots representing lncRNAs, green dots representing mRNAs, and the size of each dot indicating the degree of connectivity. Positive and negative correlations were denoted by red and blue lines, respectively.

Furthermore, the transcription factor prediction tool (TRANSFAC) was utilized to identify and match binding sites within the 2000 bp upstream and 500 bp downstream regions of the start site of each lncRNA, providing insights into potential regulatory mechanisms.

Table 3 Inflammation scores of the rats in the two groups 24h after LPS injection mean±SD

Group	Eye (*n*)	Inflammation score	*P*
EIU	10	6.40±0.66	
HPS	10	2.00±0.78	<0.001

LPS: Lipopolysaccharide; EIU: Endotoxin-induced uveitis; HPS: *Hedysarum polybotrys* polysaccharide.

RESULTS

Clinical Evaluation of Intraocular Inflammation Slit lamp microscopy observation results revealed signs of ocular inflammation in both experimental groups following LPS injection. At 6h post-LPS injection, rats in both groups exhibited conjunctival edema, ciliary hyperemia, and iris vasodilatation. However, only rats in the EIU group displayed flare and cells in the anterior chamber. Between 12 and 16h post-injection, fibrinous exudation was evident in the pupils of rats in the EIU control group, accompanied by flare and a substantial presence of cells in the anterior chamber. Conversely, minimal changes were observed in the HPS group during this timeframe.

Ocular inflammation in the anterior segment peaked at 22h–24h post-LPS injection in both groups. Rats in the EIU group displayed severe iris vasodilatation and notable signs of ocular inflammation, including iris posterior synechiae, pupil occlusion, and fibrinous membrane formation (Figure 1A). In contrast, rats in the HPS group exhibited only occasional small amounts of exudate on the pupillary margin (Figure 1B).

The EIU clinical scores of both groups 24h post-LPS injection are summarized in Table 3. Notably, pretreatment with HPS significantly attenuated intraocular inflammation in rats compared to the EIU group ($P<0.01$).

mRNA and lncRNA Expression Profile in the EIU Rat Model After HPS Treatment DEGs were obtained by comparing the HPS group and the EIU group with 5 rats in each group, and the screening was performed based on the criteria of FC>1.5 and $P<0.05$. The *P* value was obtained by paired *t*-test. The greater the FC value, the greater the difference between the two samples, and the smaller the *P*-value, the higher the reliability of the differential expression of the gene. A total of 254 differentially expressed mRNA genes were identified, with 122 genes up-regulated and 132 genes down-regulated. Additionally, 68 differentially expressed lncRNA genes were identified, with 50 up-regulated and 18

Table 4 Predicted transcripts factors

Probe name	Matrix identifier	Position (strand)	Core match	Matrix match	Sequence [always the (+)- strand is shown]	Factor name
NONRATT023434	V$SOX9_B1	331 (+)	1	0.997	acagaACAATggtt	SOX-9
gi|672020349|ref|XR_601309.1|	V$PAX6_01	16 (-)	0.925	0.802	gtcccttgataCGTCAatgca	Pax-6
NONRATT018715	V$VBP_01	409 (+)	0.982	0.988	gTTACAtaat	VBP
gi|82830445|ref|NR_002597.1|	V$RFX1_02	470 (+)	0.982	0.971	ccgttgccataGAAACcg	RFX1
gi|672014901|ref|XR_599244.1|	V$NFKB_Q6	66 (+)	1	0.967	tgGGGAAattccaa	NF-kappaB
NONRATT029264	V$ER_Q6	170 (-)	1	0.965	atgGGTCAgggtctcctaa	ER
gi|672031375|ref|XR_598519.1|	V$EVI1_04	158 (-)	0.842	0.829	tATTTTattttattt	Evi-1

down-regulated (Figure 2). Validation of a subset of these DEGs using quantitative polymerase chain reaction (qPCR) would strengthen confidence in these findings.

GO analysis revealed the enrichment of various terms targeted by the differentially expressed mRNAs, including response to LPS, response to bacterial molecules, chemokine activity, lymphocyte migration, cytokine activity, and leukocyte chemotaxis, among others (Figure 3).

KEGG pathway analysis highlighted the involvement of differentially expressed mRNA genes in various signaling pathways, including cytokine-cytokine receptor interactions, Toll-like receptor (TLR) signaling, inflammation mediated by chemokine and cytokine signaling, TNF signaling, and rheumatoid arthritis pathways, among others. The top 30 enriched KEGG pathways are depicted in Figure 4.

Through the co-expression analysis of DEGs (Figure 5), the genes with the highest correlation in the network were selected, which included 16 mRNA genes and 7 lncRNA genes. The 16 mRNA genes were Lrr1, Cep55, MGC114483, Olr1736, Mms22l, Csf1, Fam111a, Olr848, Kcnk13, RGD1559536, Olr711, A_64_P116352, A_64_P125210, A_64_P129841, LOC102552945, and RGD1563136. The 7 lncRNA genes were NONRATT023434, gi|672020349|ref|XR_601309.1|, NON-RATT018715, gi|82830445|ref|NR_002597.1|, gi|672014901|ref|XR_599244.1|, NONRATT029264, and gi|672031375|ref|XR_598519.1| (Table 4).

DISCUSSION

This study elucidated the mRNA and lncRNA expression patterns in the EIU rat model following HPS treatment, revealing significant alterations in expression profiles post-intervention. Notably, HPS pretreatment resulted in a significant reduction in EIU inflammation. A total of 254 differentially expressed mRNAs were identified, with 122 up-regulated and 132 down-regulated genes. Additionally, 68 lncRNA genes were screened, with 50 up-regulated and 18 down-regulated.

GO analysis highlighted the enrichment of various terms targeted by the differentially expressed mRNAs, including response to LPS, response to molecule of bacterial origin, chemokine activity, chemokine receptor binding, lymphocyte migration, positive regulation of leukocyte migration, cytokine activity, lymphocyte chemotaxis, and leukocyte migration. KEGG pathway analysis indicated the involvement of the DEGs in pathways such as cytokine-cytokine receptor interaction, TLR signaling pathway, chemokine and cytokine signaling pathway, TNF pathway, and rheumatoid arthritis.

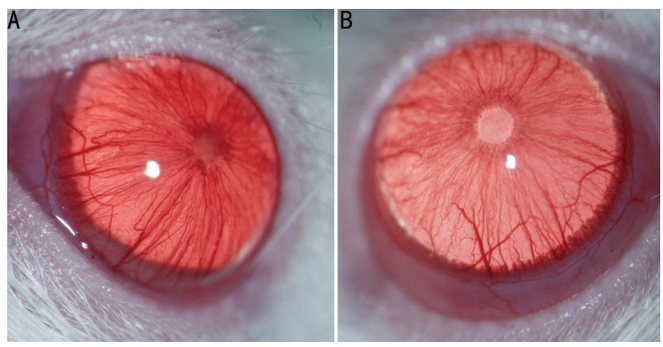

Figure 1 Representative slit-lamp photographs illustrating anterior segment inflammation in rats at 24h after lipopolysaccharide (LPS) injection A: Pupil occlusion and fibrous membrane were observed in the endotoxin-induced uveitis group at 24h after LPS injection; B: Only blood vessel dilatation in the iris could be found in the *Hedysarum polybotrys* polysaccharide intervention group at 24h after LPS injection.

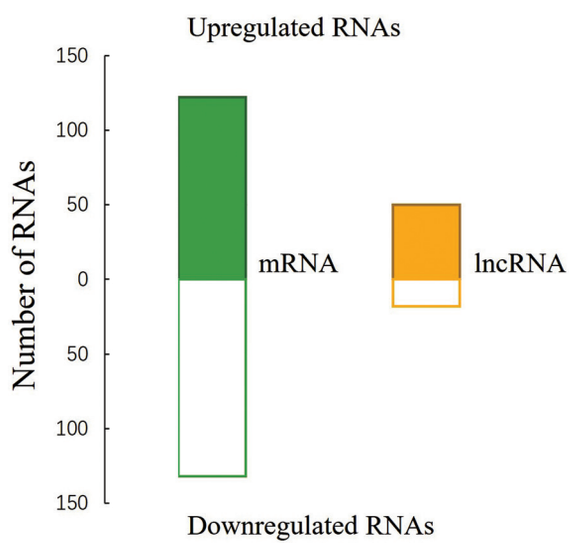

Figure 2 A total of 254 differentially expressed mRNA genes were identified, with 122 genes being up-regulated and 132 genes down-regulated Additionally, 68 lncRNA genes were screened, with 50 up-regulated and 18 down-regulated.

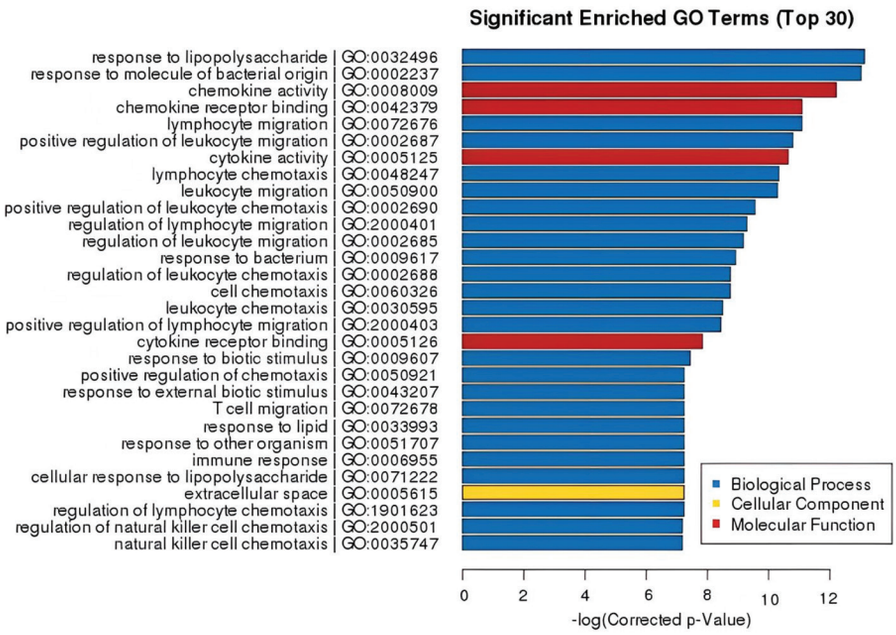

Figure 3 Top 30 gene ontology terms for the differentially expressed RNAs The abscissa represents -LgP, and the ordinate represents gene function. The gene ontology enrichment analysis provided a controlled vocabulary to describe the differentially expressed RNAs. The ontology covered three domains: biological process, cellular component, and molecular function.

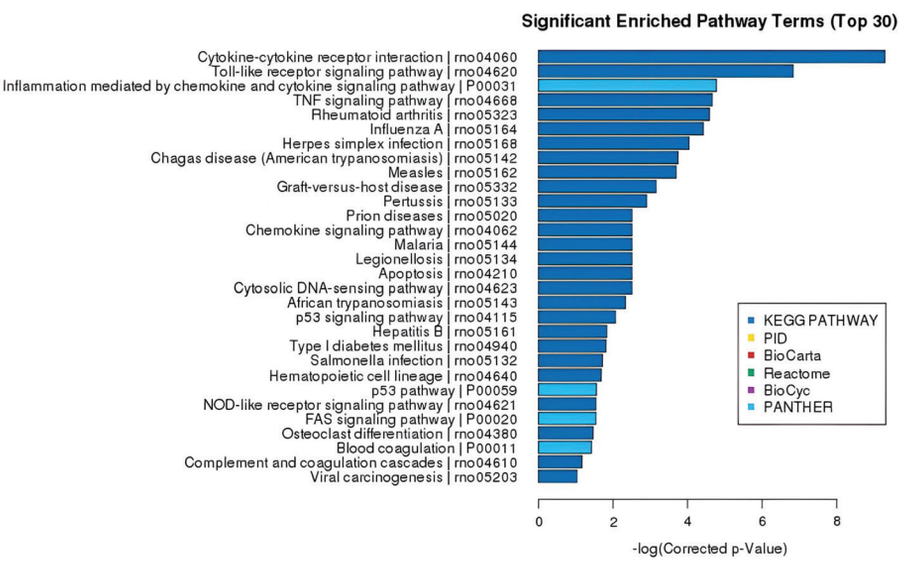

Figure 4 Kyoto encyclopedia of genes and genomes pathways analysis Top 30 pathways for the differences in mRNA genes co-expressed in EIU group and HPS group. The abscissa represents -LgP, and the ordinate represents gene pathways.

LPS is a critical component of the cell wall of Gram-negative bacteria. Upon recognition by macrophages through the TLR4/myeloid differentiation factor-2 (MD-2) receptor complex on the cell membrane surface, it triggers the body's innate immune response[3]. Initially, LPS binds to the LPS-binding protein (LBP) in the serum, followed by the interaction of cluster of differentiation 14 (CD14) molecules on the cell surface, forming the LPS-LBP-CD14 complex. This complex then activates TLR4 signaling. Subsequently, LPS within the complex undergoes depolymerization and interacts with TLR4, leading to TLR4 dimerization. Additionally, MD-2 binds to the extracellular domain of TLR4, further facilitating LPS signaling[32]. Our previous studies have indicated that HPS can inhibit the expression of TLR4 and MD-2 in the iris and ciliary body of rats with EIU, suggesting a close association between HPS's anti-inflammatory effect and the TLR4 signaling pathway[11].

Cytokines and chemokines play crucial roles in controlling inflammation and are implicated in the pathogenesis of various types of uveitis[33-38]. Consequently, they have emerged as major therapeutic targets. For instance, biological therapies targeting TNF-α have been effectively employed in autoimmune inflammatory diseases like Behcet's disease, owing to significantly elevated TNF-α levels in the aqueous

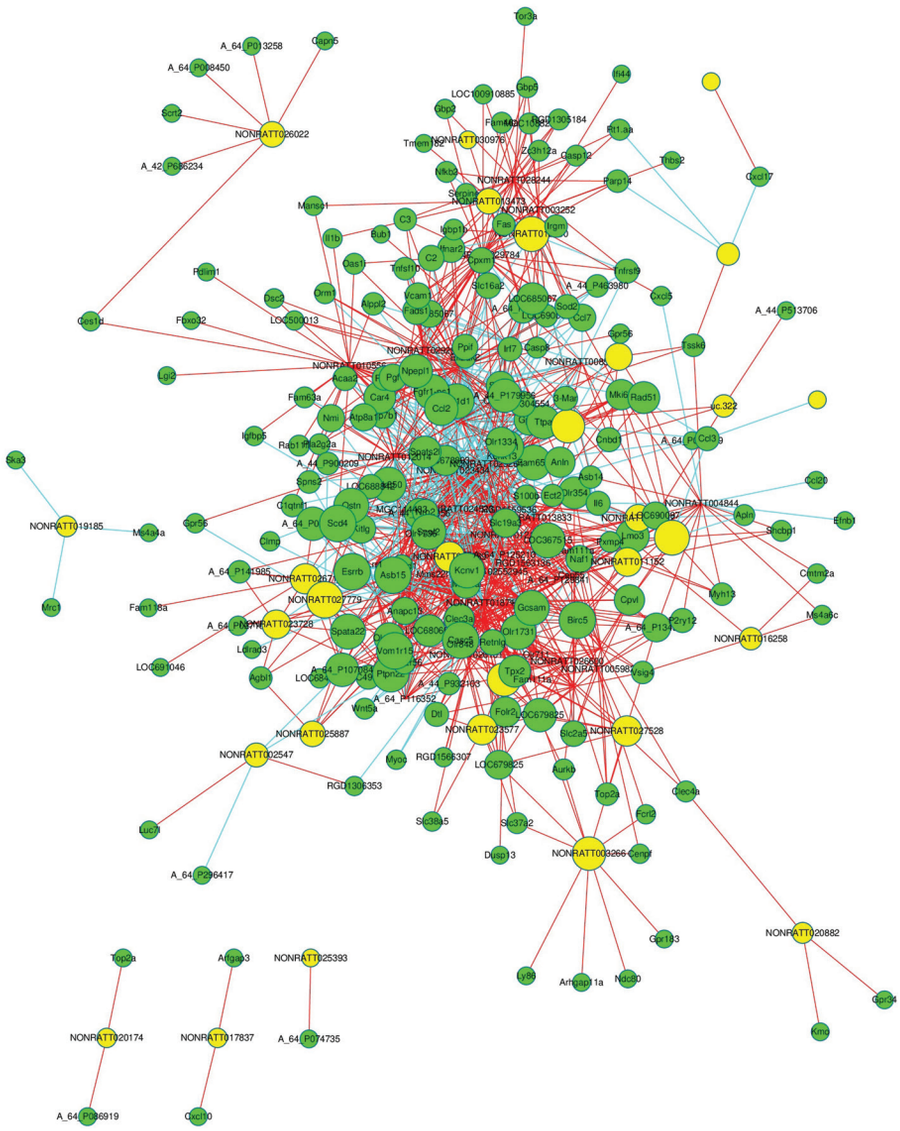

Figure 5 The gene correlation network diagram Yellow circles represent lncRNA, green circles represent mRNA, and the size of the circle corresponds to the degree of the gene in the network (the number of neighbors). Red lines represent positive correlation, while blue lines represent negative correlation.

humor of patients with Behçet's disease compared to other forms of uveitis[39-40]. Chemokines, multifunctional regulatory factors, modulate leukocyte accumulation in inflammatory tissues, promote inflammation, and enhance immune responses. Classified into four subfamilies (CXC, CC, C, and CX3C) based on the arrangement of their conserved N-terminal cysteine (Cys), chemokines exert specialized functions by binding to different members of the G protein-coupled receptor family[41-42]. Numerous chemokines have been implicated in the activation of immature dendritic cells, B lymphocytes, CD4-positive, and CD8-positive T cells, linking them to various autoimmune diseases, including autoimmune uveitis[43-45]. Studies have identified specific associations, such as chemokine (C-C motif) ligand 20 (CCL20) with human leukocyte antigen (HLA)-B27-related AAU and C-X-C motif chemokine ligand 13 (CXCL13) with Vogt-Koyanagi-Harada disease[46].

Through co-expression analysis of DEGs, key genes within the core of the co-expression network were identified, often playing pivotal roles in disease pathogenesis. In this study, 16 mRNA genes and 7 lncRNA genes were pinpointed. Notably, the leucine-rich repeat protein 1 (LRR1) gene, exhibiting the highest correlation strength, was significantly up-regulated in the HPS group. The protein en-coded by LRR1 contains leucine-rich repeats and interacts specifically with TNFRSF9/4-1BB, a member of the tumor necrosis factor receptor superfamily. Studies have indicated that LRR1 overexpression might inhibit NF-κB activation through TNFRSF9 or tumor necrosis factor receptor-related factor 2 (TRAF2), suggesting LRR1 as a negative regulator of the TNFRSF9-mediated signaling pathway[47].

Another core gene identified, colony-stimulating factor 1 (CSF1), was down-regulated in the HPS group. CSF1 is a cytokine

associated with monocyte/macrophage differentiation[48-49]. Studies by Hamilton and Achuthan[50] have demonstrated that CSF1 regulates the quantity and function of myeloid cells during inflammation, indicating its potential therapeutic relevance in autoimmune and inflammatory diseases. Additionally, the expression of CSF1 has been linked to various pathological processes, including pigmented villous nodular synovitis, autophagy, and neutrophil infiltration inflammation[51-53].

Ling *et al*[54] demonstrated that endotoxin tolerance induced by low-dose LPS pretreatment exerts a protective effect on EIU in rats. In the study, CSF1 and NF-κB were significantly downregulated, whereas LRR1 was upregulated in endotoxin tolerance in uveitis. Our findings reveal similar expression changes in CSF1 and LRR1 with HPS treatment, highlighting the importance of these genes in the anti-inflammatory response. These parallels underscore the potential of LRR1 and CSF1 as therapeutic targets for modulating inflammatory responses in uveitis. Further research is warranted to elucidate the precise mechanisms underlying their roles in uveitis pathogenesis and to explore their therapeutic implications.

Since the functions of the identified lncRNAs in this study remain elusive, we employed the TRANSFAC online database to predict their functions based on their genomic location and sequence similarities with mRNAs. When a lncRNA and its related mRNA are closely located on the chromosome or share sequence similarities, it suggests a potential regulatory relationship between the two RNAs[55]. One of the lncRNAs identified in our study, gi|672014901|ref|XR_599244.1|, was found to be highly correlated with NF-κB in function prediction. NF-κB p65 is increasingly recognized for its pivotal role in regulating various pro-inflammatory genes implicated in diverse inflammatory diseases[56]. Notably, the activation of NF-κB p65 is crucial in both EIU and autoimmune-induced uveitis[6,57]. This finding suggests that the lncRNA gi|672014901|ref|XR_599244.1| may play a regulatory role in the NF-κB signaling pathway, potentially influencing the expression of pro-inflammatory genes involved in uveitis pathogenesis. Further research is warranted to elucidate the precise mechanisms by which this lncRNA modulates NF-κB activity and its implications for uveitis.

While our study identified altered lncRNAs and mRNAs potentially involved in EIU inflammation and the anti-inflammatory effects of HPS, several limitations should be noted. First, analyses were performed on whole iris/ciliary body tissues, making it difficult to attribute gene expression changes to specific cell types. Single-cell analyses or cell-sorting approaches are needed to clarify the cellular sources and targets of HPS. Second, although microarray and bioinformatic analyses were used, experimental validation (*e.g.*, qPCR or Western blot) was not performed, which is essential to confirm key gene expression changes. Third, the functional roles of identified lncRNAs remain speculative, as they were predicted solely through computational methods; loss- and gain-of-function studies are required to elucidate their mechanisms. Fourth, while TLR4/NF-κB signaling may mediate HPS effects, this was not experimentally demonstrated; mechanistic studies such as pathway inhibition and reporter assays are warranted. Additionally, the small sample size (n=5 per group) may limit statistical power and generalizability, and interspecies differences between Wistar rats and humans should be considered. Future studies could incorporate genetically tractable models, such as zebrafish[58], to better approximate human uveitis.

Despite these limitations, our findings provide a foundation for exploring HPS as a therapeutic candidate and highlight molecular targets for translational research. Further validation and mechanistic studies are needed to determine whether the identified mRNAs and lncRNAs are conserved in humans and to clarify their roles in uveitis pathogenesis. Specifically, our data suggest that HPS may exert anti-inflammatory effects by upregulating LRR1, downregulating CSF1, and modulating NF-κB signaling through lncRNA regulation. Comprehensive screening also identified several candidate anti-inflammatory target genes, providing preliminary insights into the molecular mechanisms of HPS.

In conclusion, this study suggests that HPS may exhibit anti-inflammatory effects in EIU by modulating multiple signaling pathways associated with inflammation and immunity. Further functional validation and larger sample sizes are required to substantiate these findings and fully elucidate the molecular mechanisms underlying HPS's therapeutic effects.

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