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Original Article

The potential mechanisms of dehydrocostus lactone in treating the inflammatory processes in lipopolysaccharide-activated microglial cells: a transcriptome analysis

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Abstract: Objective: Microglial activation can cause the loss of neuronal cells after brain ischemia and trauma. Dehydrocostus lactone (DDL), a natural sesquiterpene lactone, has anti-inflammatory properties and can cross the blood-brain barrier. The aim of this study was to evaluate the anti-inflammatory effects of DDL on lipopolysaccharide (LPS)-activated microglial cells, thus confirming the use of DDL to treat brain inflammation. Methods: We used LPS to induce inflammation in mouse BV2 microglial cells and treated the LPS-activated microglial cells with DDL. The proinflammatory cytokine levels in the culture media were tested with ELISAs. The transcriptome of the cells was analyzed with a Clariom S microarray. Results: The inhibition of IL1β, IL6, and TNFα released by DDL was significant in the BV2 microglial cells. DDL downregulated the LPS-induced inflammation-related gene expression. DDL regulated the immune process and the production of the key proinflammatory cytokines in the inflammation-related signaling pathways. Conclusion: DDL can inhibit the immune process and microglial cell activation and may be a candidate drug for the treatment of brain inflammation-related diseases.

Keywords: Dehydrocostus lactone, microglial cells, anti-inflammatory

Introduction

Brain ischemia and trauma can induce the release of cytokines, including inflammatory cytokines, that trigger neuronal apoptosis and thereby lead to secondary brain injury [1]. Approximately 795,000 new or recurrent cases of stroke occur worldwide each year. Stroke is a brain disease, the main clinical manifestations of which are cerebral ischemia and bleeding injuries [2-4]. Many factors contribute to stroke-induced brain injury, including inflammation, neuronal apoptosis, excitotoxicity, and oxidative damage [5]. Neuronal glial cells are the first neuronal cells activated in the pathogenesis of cerebral and cardiac ischemia. Glial cells can secrete various inflammatory cytokines. The activation of glial cells and inflammatory processes are important for the progression of atherosclerosis [5, 6]. Decreasing or preventing the activation of neuronal glial cells and the release of proinflammatory cytokines has become an important treatment strategy for brain injury.

In this study, we investigate a new, potential approach for inhibiting glial activity in brain injury. Dehydrocostus lactone (DDL), a natural sesquiterpene lactone product, is derived from the root of *Saussurea lappa. S. lappa* has various biological activities, such as hepatoprotective activity [7] and anti-inflammatory activity [8, 9], in various types of cells. Moreover, intraperitoneally injected DDL can cross the blood-brain barrier in animals [10]. These observations suggest that DDL has the potential to inhibit the

activation of early neuronal inflammation in the CNS in clinical application.

It has been reported that more than 10% of the glial population is comprised of microglia cells that reside in the CNS. Microglia are in a resting state under normal conditions and exhibit a highly branching phenotype that helps them respond rapidly to injury. Microglia are primarily activators that participate in the early response to neuronal damage and brain disease. Activated microglial cells migrate to damaged sites via chemotaxis [11]. The sustained exposure of neurons to inflammatory cytokines released by microglia can induce neuronal dysfunction and apoptosis [12, 13]. Thus, microglial cells are a common target and focus of research on early brain inflammation. BV2 microglial cells are widely used as an *in vitro* cell model to investigate inflammation in brain diseases [14]. Lipopolysaccharide (LPS) can activate microglial cells *in vitro* and induce them to secrete various neuroinflammatory factors that have been shown to lead to neuronal loss *in vivo* [15]. LPS-activated BV2 microglial cells can serve as a good model for investigating the anti-inflammatory activity of DDL and the underlying mechanism.

To analyze the effects of DDL on brain cells and evaluate its potential application to the treatment of brain injury and neuroinflammation, we used LPS to create inflammatory microglial cells and DDL to treat inflammatory cells *in vitro*. An enzyme-linked immunosorbent assay (ELISA) was applied to confirm the inhibitory effect of DDL on the release of inflammatory cytokines. A transcriptome analysis was then applied to investigate the expressions of the genes in microglial cells with or without inflammation and in those treated with DDL. The identification of the differentially expressed genes (DEGs) between the inflammatory microglial cells treated with DDL and the untreated microglial cells is important for evaluating whether DDL can interfere with the activation of inflammatory processes and, if so, the underlying molecular mechanism. The systematic investigation of the expression patterns of thousands of genes among the control, inflammatory, and medical treatment conditions using gene expression profiling might provide meaningful knowledge of the gene expression response to inflammation or medication, in addition to determining whether DDL can inhibit the early events of neuroinflammation.

Materials and methods

Cell culture and treatment

BV2 cells were obtained from ATCC (Manassas, VA, USA) and maintained in DMEM cell culture medium (Gibco, Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA). A total of 5×10^5 BV2 cells were cultured on a 35 mm cell-culture plate. After 24 h, the medium was replaced with a fresh medium for treatment. The BV2 cells in the activated-inflammation group were treated with 1 μg/ml LPS (Sigma-Aldrich, Germany) for 24 h. The BV2 cells in the DDL (Sigma-Aldrich, Germany) anti-inflammation group were treated with 4 μM DDL for 30 min and then LPS for another 24 h. The cells in the control group were maintained without any treatment.

Enzyme-linked immunosorbent assay

The cell culture media of the three groups were collected. The levels of the inflammatory cytokines in the media were determined using ELISAs. ELISA kits (R&D Systems, Minneapolis, MN) were used to quantify the IL-1β, IL-6, and TNF-α levels in accordance with the manufacturer's directions. The absorbance at 450 nm, which reflects the concentration of cytokines, was determined with a quick microplate reader (Bio-Rad, Hercules, CA).

RNA isolation and Clariom S microarray

Total RNA was extracted using RNAiso Plus (Takara Biomedical Technology Co., Ltd., Beijing, China) and a QIAGEN RNeasy Mini kit (QIAGEN, Hilden, Germany). The cells were completely lysed using 1 ml of RNAiso Plus reagent. The RNA was extracted with chloroform in accordance with the manufacturer's directions. Three independent replicates, one each from the DDL anti-inflammation group, the LPS-stimulated group, and the control group were analyzed. The RNA integrity was assessed using standard denaturing agarose gel electrophoresis.

Transcriptome profiling assays were performed using the Clariom S mouse assay microarray

Table 1. The primers used in the real-time PCR experiments

Gene	Forward primer	Reverse primer
Xaf1	GCAGACCAAGGAAAGCCAACA	CCTGCCCTTTGCACTCAGACA
Wfdc17	GCCACAGTCTTGTTTCTGGTG	TTTGCAGACATGACCACAGCT
Slfn4	GAGCCCTCTGTTCAAGTCAAG	ACCAGTTCAGCGTAGTCCGT
Rgs1	TCTCCATGCCAAGGTTGAAC	GGGATCATCGATCTCAGGTATG
Hmgn5	ACTGTCTGCTATGCCTGTGC	TTTCCATGTTGCATTCGTCT
Cxcl2	ACCAACCACCAGGCTACAGG	GCTTCAGGGTCAAGGCAAAC
Bst2	AGTCACGAAGCTGAACCAGG	TGACACTTTGAGCACCAGTAGG
Sh3bgrl	AGAATCGGAAGTGGATGAGAG	TTGAACCAGGTGGTGCTGTC
Saa3	CCTGGGCTGCTAAAGTCATC	CACTCATTGGCAAACTGGTC
Rhob	ATCCTTATGTGCTTCTCGGTG	GGGCAGAAGTGCTTTACCTC
Rasgrp3	CTCTAAGTGGGTCCAGTTGATG	AGAATGGGTGTCTTTGAGTCG
Omd	ATAGAACTCAACGTTGGACAC	TACTGTGTATACGAGGGAAGC
Junb	ATGGAACAGCCTTTCTATCACG	GTGGGTTTCAGGAGTTTGTAGTC
Usp38	GCTCATACACAAAGGGAAGC	AAGGCTTATGTGAGGACTGAAC
Tubb4b	TGGAGCGCATCAACGTGTAC	CCTTTCTCACAACGTCCAACAC
Tlr13	AGACGCCTTCACTCCTCTAATC	TGTGGTCCAAGTACATGATGC
Gapdh	TGGTGAAGGTCGGTGTGAAC	GCTCCTGGAAGATGGTGATGG

platform (ThermoFisher Scientific, MA, USA). The gene expression profiles and the quality control were processed with Transcriptome Analysis Console software (ThermoFisher Scientific).

Bioinformatics analysis

In the DEG analysis, the genes with a normalized-expression |log2-fold change| ≥1.5 and P≤0.05 between the LPS-stimulated BV2 cells and the control BV2 cells or between the DDL-treated anti-inflammatory BV2 cells and the LPS-stimulated BV2 cells were considered DEGs.

For the expression cluster analysis, the average value of the three repetitions of each group was used to perform the gene cluster analysis using the K-means clustering algorithm. The typical pattern of the analysis is shown for the results.

For the gene ontology (GO) analysis of the DEGs, the DEGs were uploaded to the DAVID database, and the top-level ontology terms were listed.

For the Metascape analysis, the DEGs were uploaded to the online database. The top biological processes with significant *P* values are listed, reflecting the functional characteristics of the DEGs [16].

The STRING database was used to analyze the protein-protein interaction (PPI) networks of the DEGs. The interaction relationships of all the neighboring nodes in the network were calculated and visualized using Cytoscape software [17].

Real-time PCR

For the quantification of the DEGs, a SuperReal PreMix Plus kit (Tiangen, China) was applied to determine the mRNA levels of the DEGs according to the manufacturer's instructions. The ABI PRISM 7300 system (Applied Biosystems, Thermo Fisher Scientific, USA) was used to determine the amplification and melting curves. The Ct values were determined using the ABI system. The 2-ΔΔCt analysis method was used to determine the relative expression level of each target gene for each sample compared to the control group sample. The sequences of the primers used are shown in **Table 1**.

Statistical analysis

The data were analyzed and are expressed as the mean ± standard error of the mean (SEM). The comparisons between two groups were analyzed with an unpaired t test with GraphPad Prism software (version 7.0). P≤0.05 was considered statistically significant.

Results

DDL inhibits the production of inflammatory cytokines by BV2 cells

The chemical structure of DDL, a sesquiterpene lactone compound, includes a lactone ring and is shown in **Figure 1A**. The immune-activated glia can release several key proinflammatory factors, including IL-6, TNF-α, and IL-1β [18-20], which are associated with neuroinflammation in the CNS and regulate the activation of neurons and astrocytes [21, 22]. To confirm the inhibitory effect of DDL on the activation of microglial cells, the levels of proinflammatory cytokines, including IL-1β, TNF-α, and IL-6, released by the BV2 microglial cells in

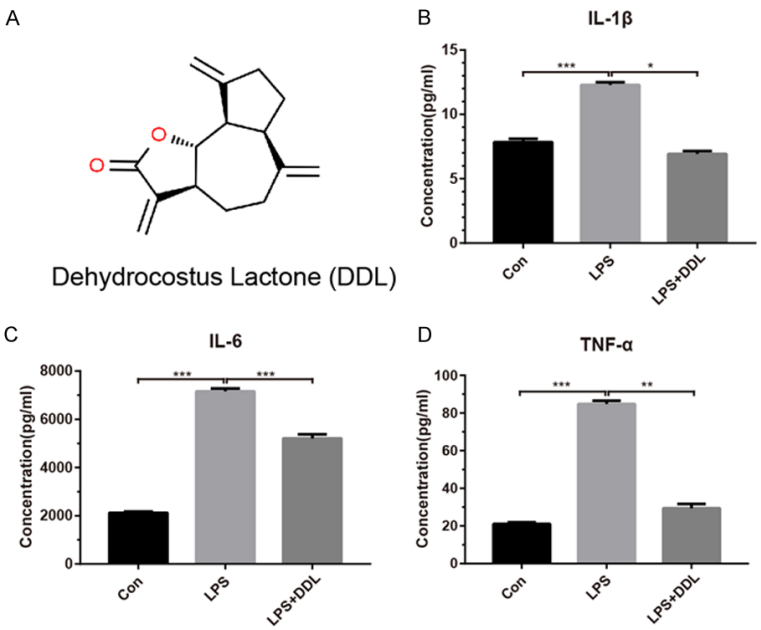

Figure 1. DDL suppresses the release of proinflammatory cytokines from LPS-stimulated microglial cells. (A) Chemical structure of DDL. (B-D) Bar chart of the mean concentrations of IL1β (B), IL6 (C), and TNFα (D) in the culture media of the control, LPS-stimulated microglial cells and DDL-treated anti-inflammatory-microglial cells (*: P<0.05; **: P<0.01; ***: P<0.001).

the treatment groups were determined using ELISA. As shown in **Figure 1B-D**, compared to the control BV2 cells, the LPS-stimulated BV2 cells secreted higher levels of IL-1β (corresponding to a 1.5-fold increase), IL-6 (3.5-fold increase), and TNFα (4.5-fold increase) into the culture media. The DDL significantly inhibited the production of proinflammatory cytokines through the activated BV2 cells. The IL-1β and TNFα levels almost returned to their control levels in the DDL-treated activated microglial cells. These results indicate that DDL can efficiently inhibit the release of proinflammatory cytokines from activated BV2 cells, demonstrating that DDL has an anti-inflammatory effect on the early phase of the microglia activation.

Identification of the DEGs

To study the potential mechanism by which DDL inhibits the LPS-stimulated inflammatory process in BV2 cells, we analyzed the transcripts of the LPS-stimulated BV2 cells and the DDL-treated anti-inflammatory BV2 cells with a Clariom S mouse assay. According to the criterion of a ≥1.5-fold change in expression, compared to the control cells, the LPS-stimulated BV2 cells had 84 upregulated transcripts and 66 downregulated transcripts (**Figure 2A**). Compared to the LPS-stimulated BV2 cells, the DDL-treated anti-inflammatory cells had 215 upregulated transcripts and 150 downregulated transcripts (**Figure 2B**).

To further investigate the transcript patterns, the DEGs were classified into four clusters, as shown in **Figure 2C**. Cluster 1 and cluster 2 comprise the down regulated DEGs in the LPS-stimulated BV2 cells. Cluster 3 and cluster 4 consist of the upregulated DEGs in the LPS-stimulated BV2 cells. Most of the transcripts, including Il1a, Il1b, Il6, Cxcl10, Irf7, Isg15, Gvin1, Slfn4 and Xaf1, are involved in the inflammatory response in the microglial cells (**Figure 2D**). We also confirmed the expression change of most of the DEGs with real time PCR, as shown in **Figure 3**. These results indicate that DDL can regulate the expressions of the inflammatory-activation and development-related transcripts in the BV2 microglial cells.

Process and biological mechanism enrichment analysis

To assess the influence of DDL and LPS on the biological functions of the BV2 microglial cells, a GO analysis was used to examine the genes upregulated in the LPS-stimulated cells relative to the control cells and the genes downregulated in the DDL-treated anti-inflammatory cells relative to the LPS-stimulated cells. As shown in **Figure 4A**, the top three enriched GO terms in the biological process (BP) category of the genes with upregulated expressions in the LPS-stimulated cells relative to the control cells included the immune response (P=3.4E-21), the immune system process (P=9.68E-20), and the defense response (P=8.26E-19). The top three enriched GO terms in the molecular function (MF) category included cytokine receptor binding (P=0.000000142), cytokine activity (P=0.00000052), and receptor binding (P=0.0000175). The top three enriched GO terms

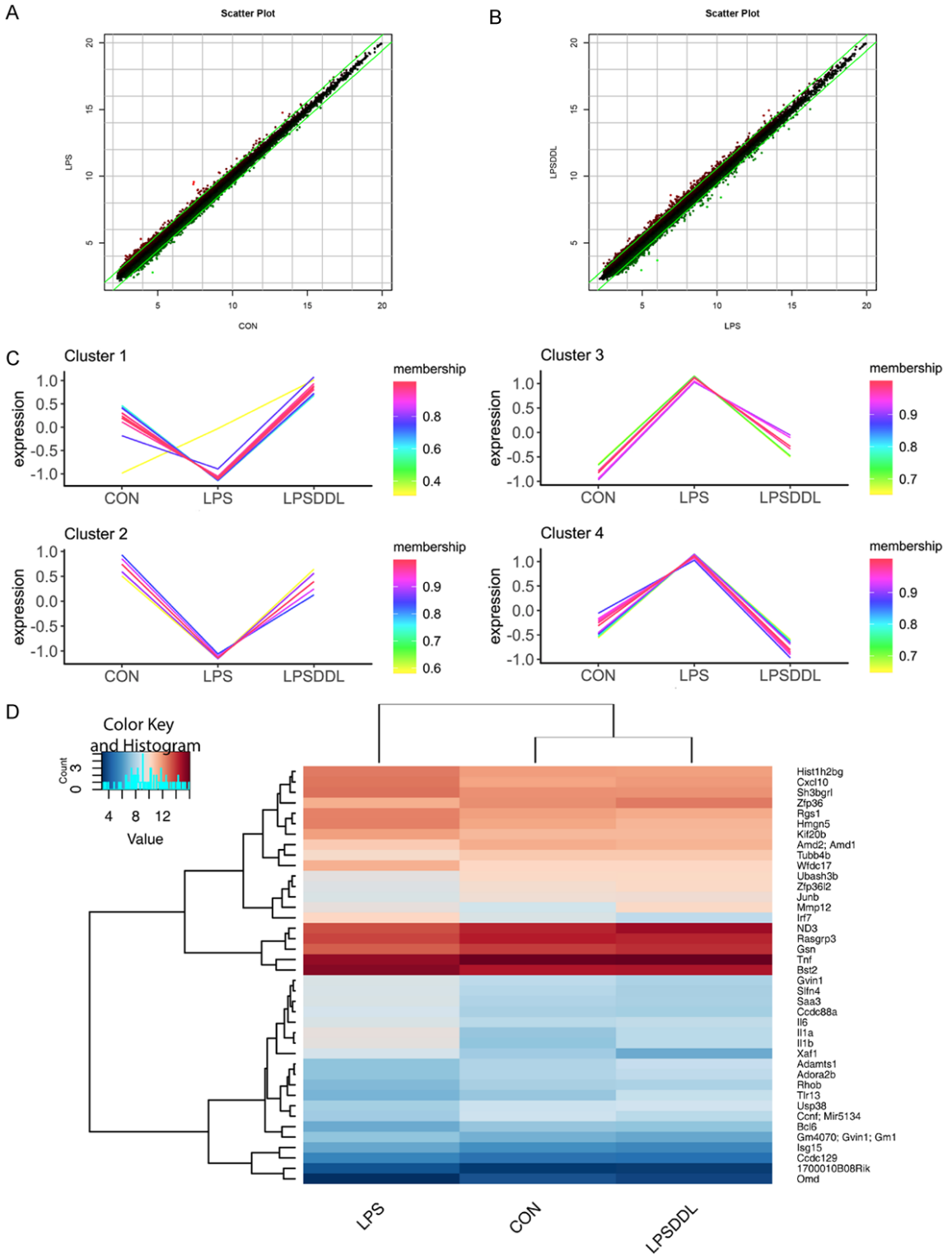

Figure 2. Microarray analyses of the DEGs. DEG analysis of the LPS-upregulated and LPS plus DDL-downregulated transcripts in the BV2 microglial cells are shown. (A, B) Scatter plot showing the variation in LPS-stimulated BV2 cells (A) and DDL-treated anti-inflammatory microglial cells (B). (C) An expression cluster analysis showing the overall pattern of the DEGs. (D) Hierarchical clustering analysis of the DEGs.

in the cellular component (CC) category included extracellular space (P=6.18E-09), cell surface (P=0.000000764), and the intrinsic component of the plasma membrane (P=

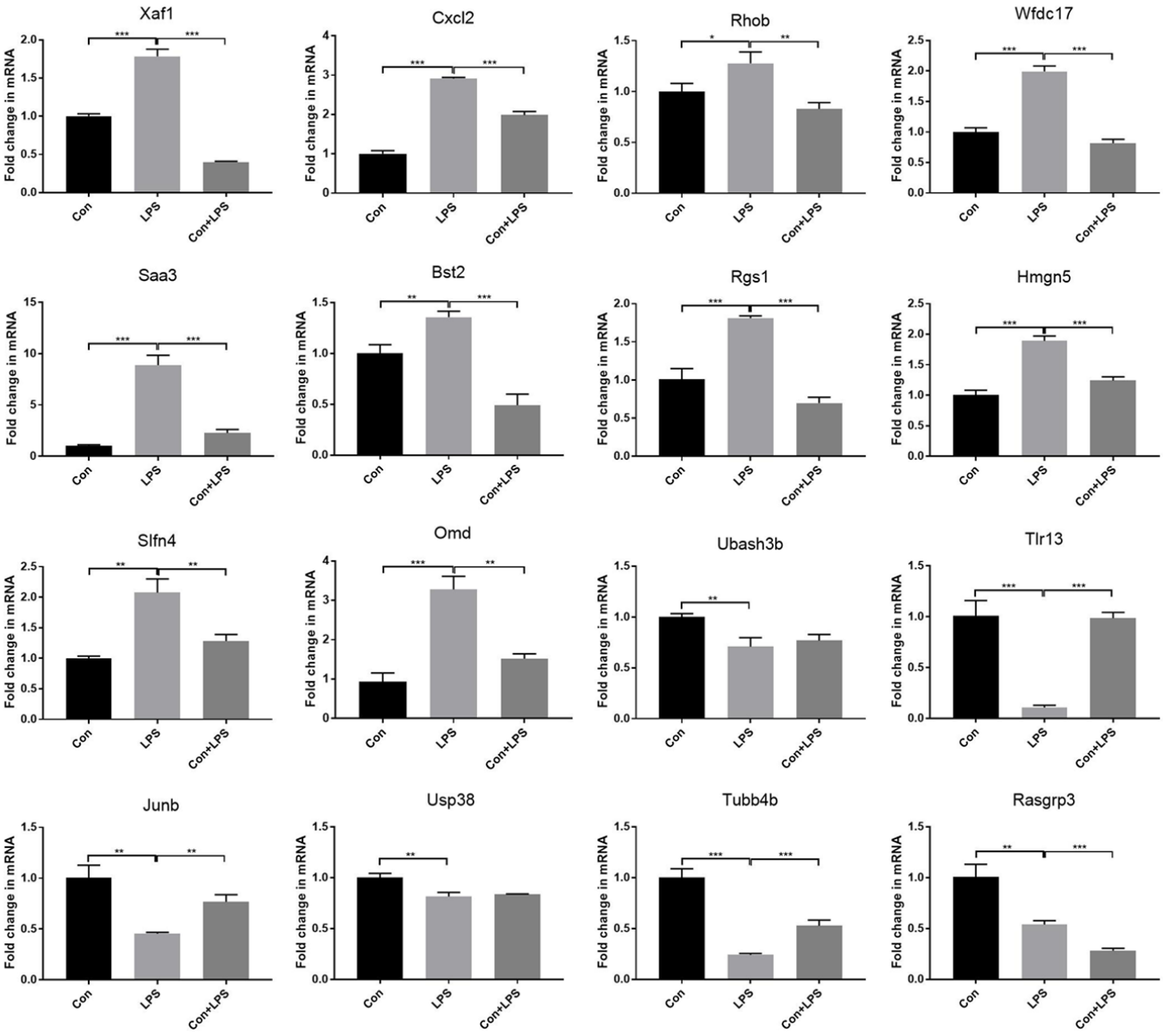
Xaf1
Cxcl2
Rhob
Wfdc17
Saa3
Bst2
Rgs1
Hmgn5
Slfn4
Omd
Ubash3b
Tlr13
Junb
Usp38
Tubb4b
Rasgrp3
Fold change in mRNA
Con
LPS
Con+LPS

Figure 3. Real-time PCR analyses of the DEGs.

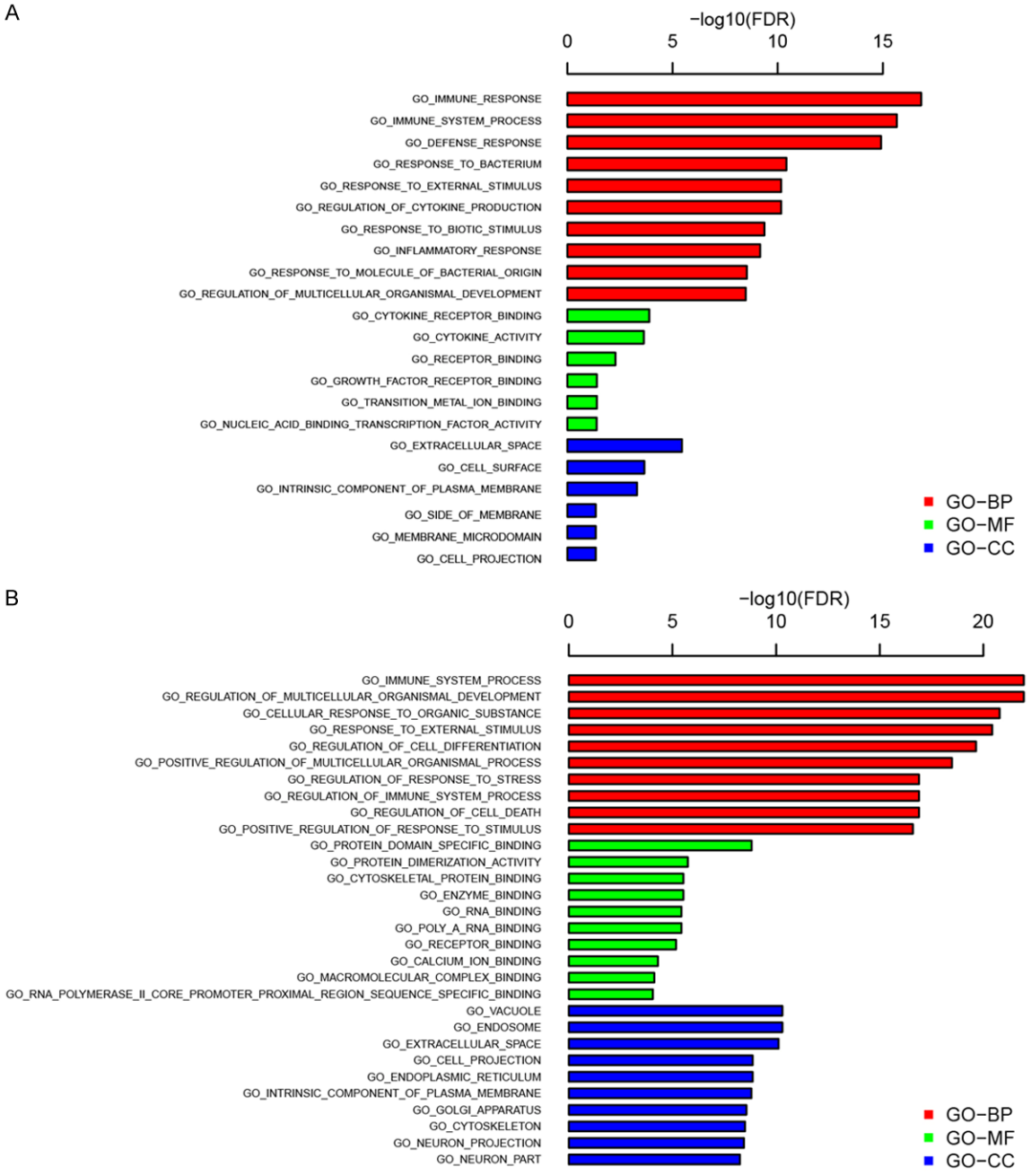

Figure 4. GO analyses of the DEGs. (A) Upregulated DEGs in LPS-activated microglial cells and (B) downregulated DEGs in DDL-treated anti-inflammatory microglial cells.

0.00000251). For the genes with downregulated expressions in the DDL-treated anti-inflammatory cells relative to the LPS-stimulated cells (**Figure 4B**), the top three enriched GO terms in the BP category included the immune system process (P=2.52E-26), the regulation of multicellular organismal development (P=5.06E-26), and the cellular response to organic substances (P=1.09E-24). The top three enriched GO terms in the MF category included cytokine receptor binding (P=1.68E-12), cytokine activity (P=4.05E-09), and receptor binding (P=1.14E-08). The top three enriched GO terms in the CC category included vacuole (P=1.28E-13), endosome (P=1.73E-13), and extracellular space (P=3.92E-13). These GO analysis results

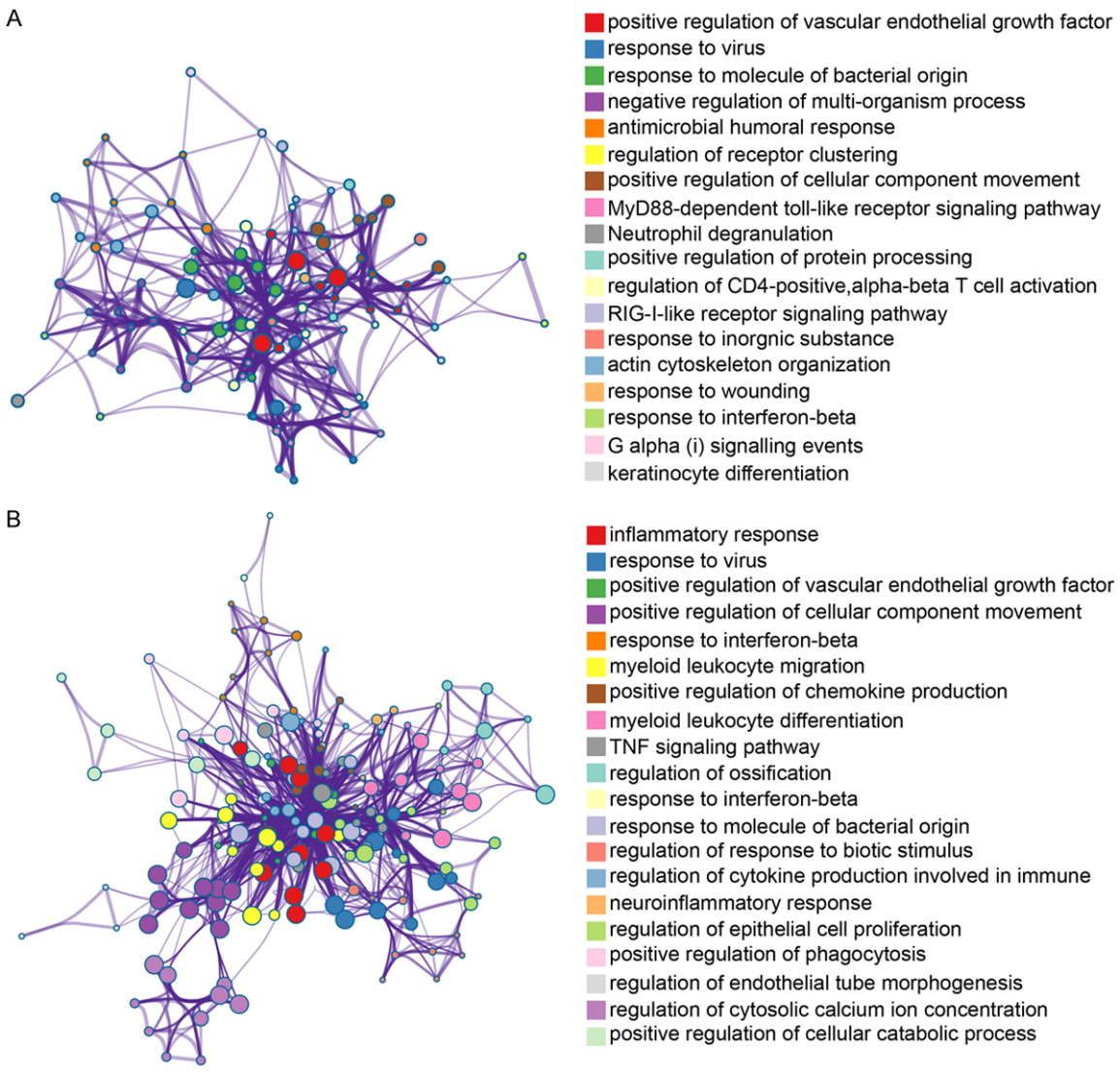

Figure 5. Metascape analysis of the DEGs. A. LPS-stimulated BV2 cells vs. control cells. B. DDL-treated anti-inflammatory BV2 cells vs. LPS-stimulated cells.

suggest that exposing BV2 cells to LPS induces the immune processes, the inflammatory biological processes, and the phagocytosis-related processes. DDL can downregulate the LPS-induced immune response in the microglia cells and inhibit the phagocytosis process.

To identify the potential biological mechanism of the DEGs, we performed a Metascape analysis to identify the biological pathways and protein complexes at the OMIC level. The DEGs between the LPS-stimulated cells and control cells were enriched in the inflammatory responses, such as the response to viruses, the response to molecules of bacterial origin, and the antimicrobial humoral response. They were also enriched in the regulation of receptor clustering and actin cytoskeleton organization, which are associated with microglial phagocytosis (**Figure 5A**). Furthermore, the genes differentially expressed between the LPS-stimulated cells and the DDL-treated anti-inflammatory cells were enriched in the inflammatory response, the response to viruses, and the positive regulation of phagocytosis (**Figure 5B**). These results show that DDL can inhibit inflammatory activation, the inflammatory response, and the phagocytosis function of microglial cells.

Pathway enrichment analysis

To analyze the cell signaling pathways involved in the regulation of the LPS or DDL response,

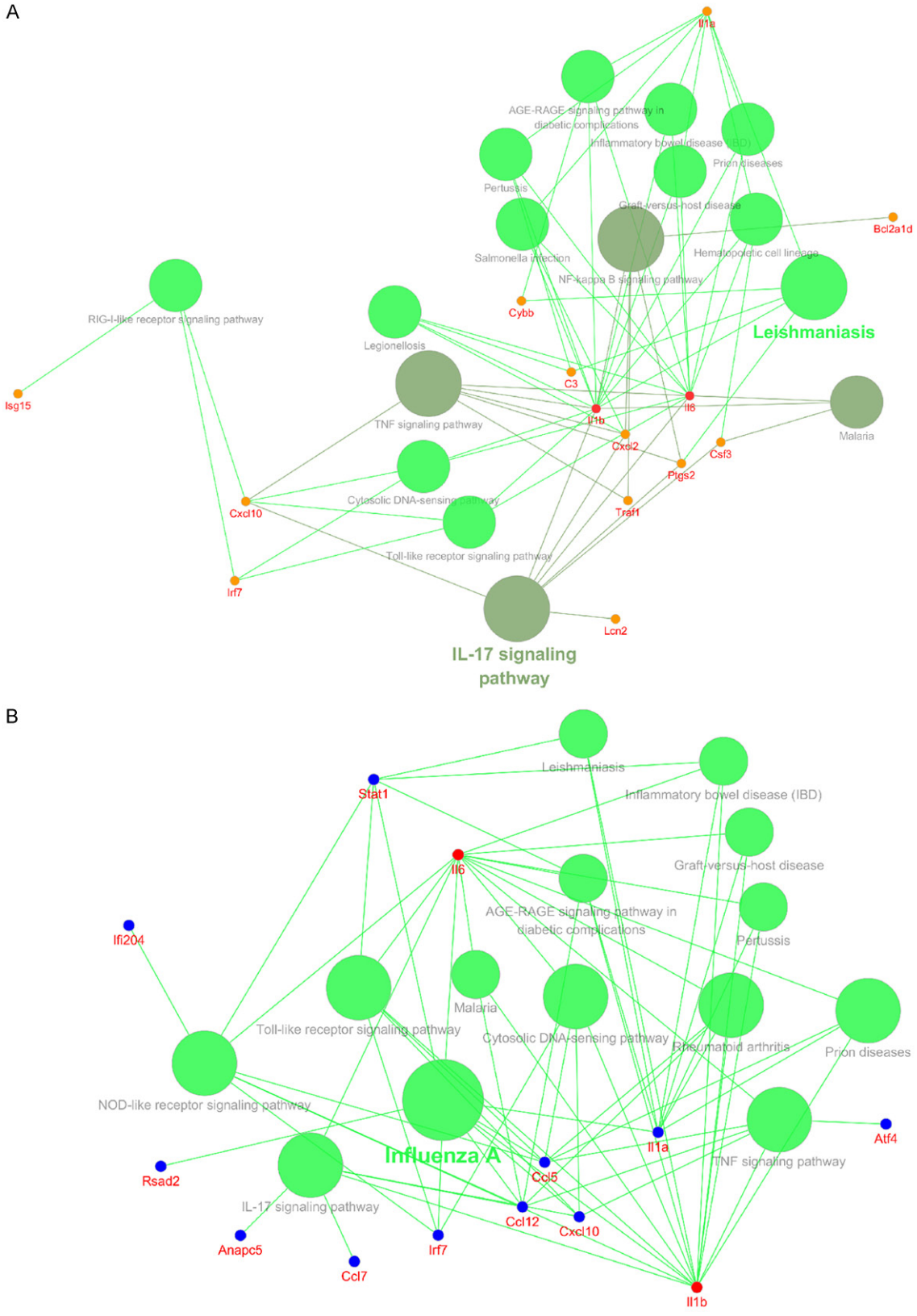

Figure 6. Coexpression network analysis of the DEGs. (A) Upregulated DEGs in the LPS-activated microglial cells and (B) downregulated DEGs in the DDL-treated anti-inflammatory microglial cells. The intensity of the green color in the signaling pathways indicates the *P* value; dark green indicates small *P* values.

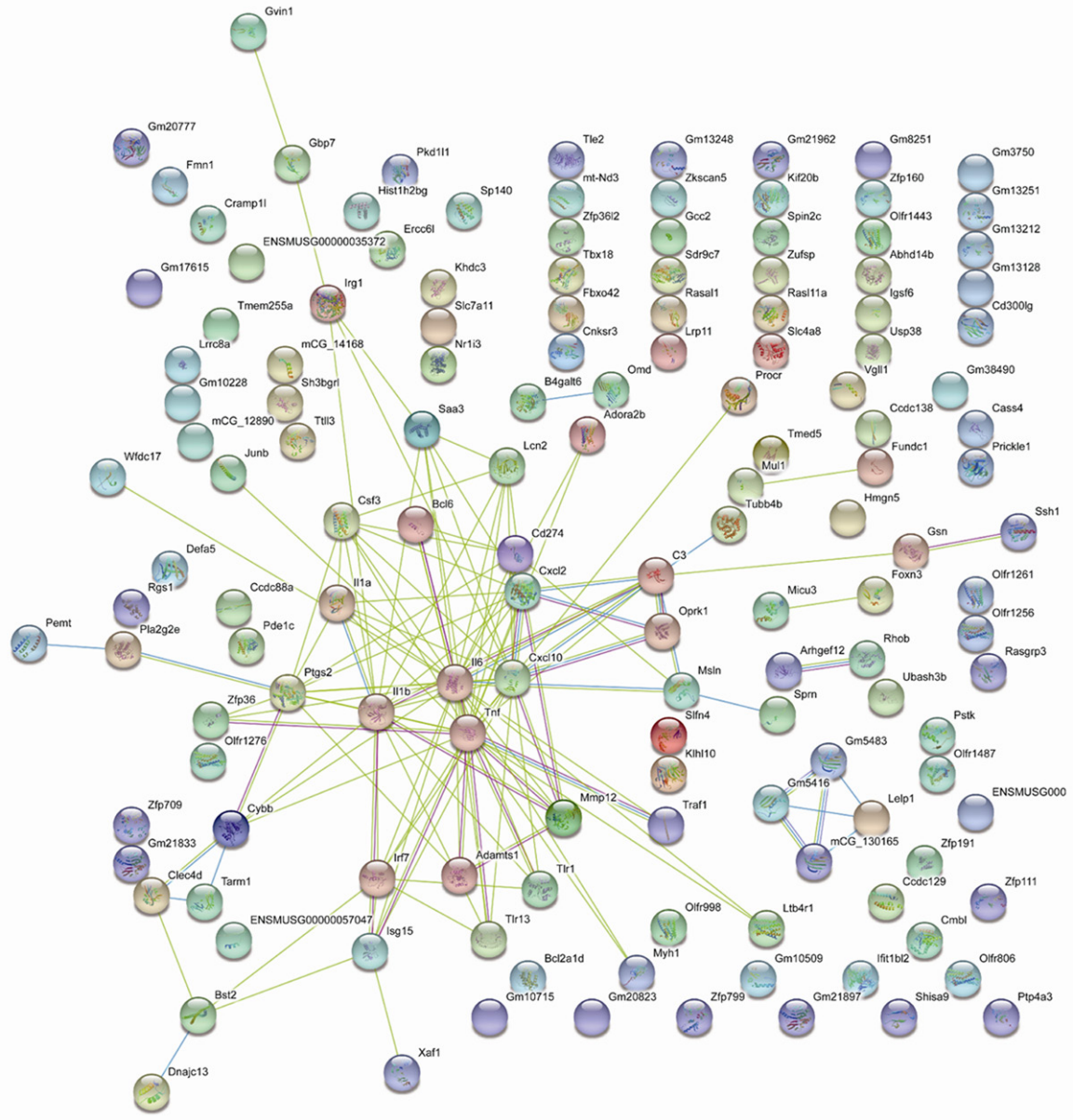

Figure 7. PPI network analysis of the DEGs between the LPS-stimulated BV2 cells and the control cells.

the genes with upregulated expressions in the LPS-stimulated cells relative to the control cells and the genes with downregulated expressions in the DDL-treated anti-inflammatory cells relative to the LPS-stimulated cells were added to the gene network map. The genes with upregulated expression in the LPS-stimulated cells relative to the control cells are involved in the TNF signaling pathway, *Salmonella* infection, the IL-17 signaling pathway, the toll-like receptor signaling pathway, inflammatory bowel disease, and graft-versus-host disease (**Figure 6A**). The genes with downregulated expressions in the DDL-treated anti-inflammatory cells relative to the LPS-stimulated cells are involved in similar immune-related signaling pathways and include the chemokines Ccl5, Ccl7, Ccl12, and Cxcl10 (**Figure 6B**). IL-1β and IL-6 play key roles in these pathways, and Atf4 is involved in the inhibition of inflammation by DDL. These results show that DDL may downregulate the immune-related pathways and that proinflammatory cytokines and chemokines play a significant role in these signaling pathways.

PPI network analysis

To elucidate the correlations among the DEGs and further investigate their roles, the STRING

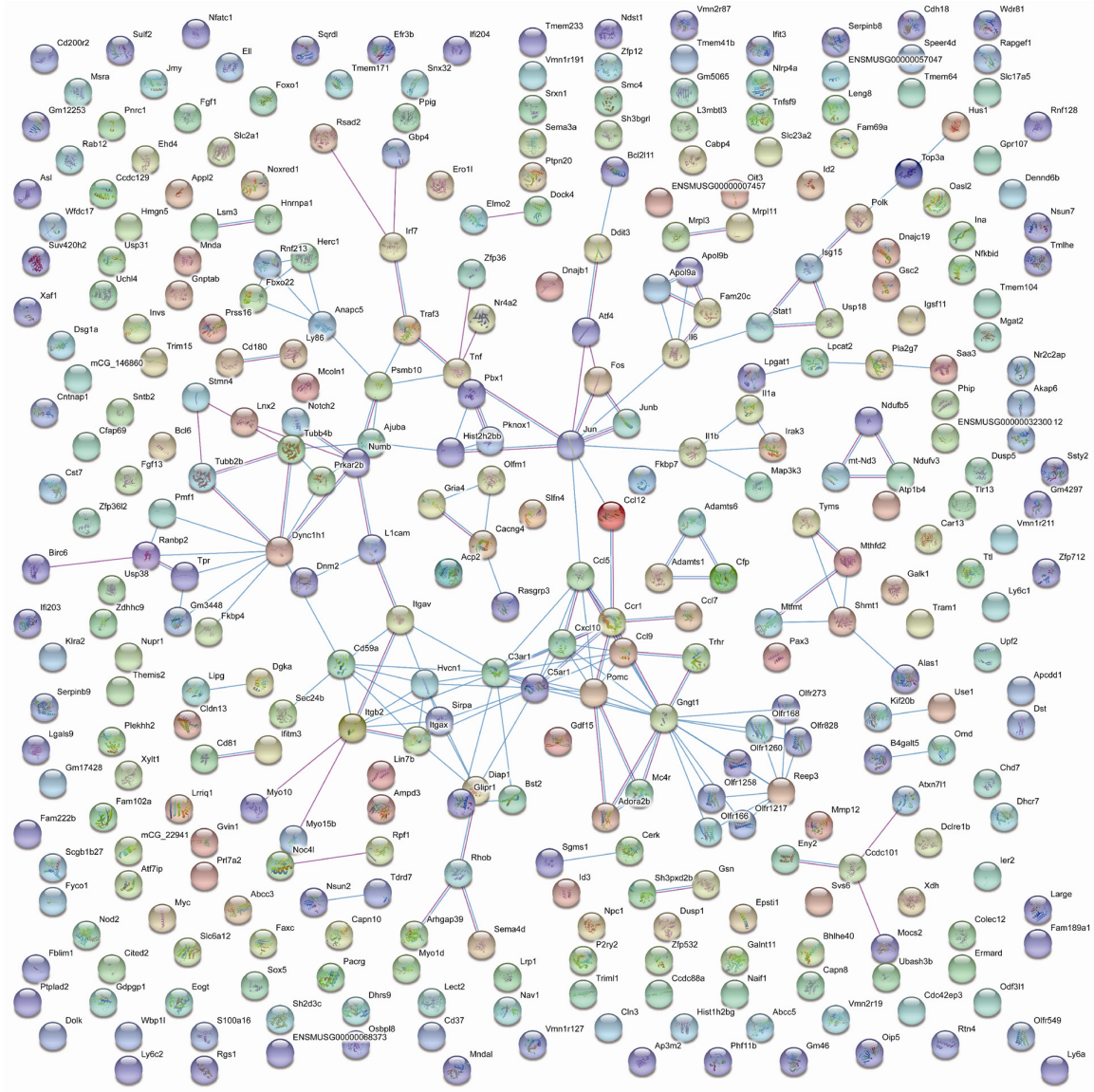

Figure 8. PPI network analysis of the DEGs between the DDL-treated anti-inflammatory and the LPS-stimulated BV2 cells.

PPI database was used to construct a PPI network. A total of 134 PPI relationships were identified among the DEGs between the LPS-stimulated cells and the control cells (**Figure 7**). The genes Il1a, Il6, Cxcl10, Cxcl2, Tnf, and Ptgs2 showed strong associations with other genes and were considered key regulators. Among the DEGs between the LPS-stimulated cells and the DDL-treated anti-inflammatory cells, Cxcl10, C5ar1, C3ar1, Gngt1, Jun, Ccl5, and Itgb2 were the main nodes in the 195 PPIs (**Figure 8**). The PPI analysis indicated that LPS upregulates the expression of genes involved in the initiation and promotion of inflammation, while DDL regulates the cell cytoskeleton and the phagocytic receptors of microglia to inhibit the phagocytosis of neuronal cells in pathological conditions.

Discussion

DDL has been reported to exert anti-inflammatory effects in various cell types and penetrate the blood-brain barrier, suggesting its therapeutic potential in treating CNS disease [5, 23]. In the present study, treatment with DDL led to downregulated expressions of the inflammation-related and chemotaxis-related genes and

attenuated the LPS-induced production of proinflammatory cytokines in BV2 microglial cells.

The initial events of a brain injury, such as stroke, can cause the activation of microglial cells, which can phagocytose neuronal cells and cause subsequent neurological impairment during the pathological process. IL-1β, IL6, and TNF-α are proinflammatory cytokines released by activated microglia and are highly expressed in the plasma of patients with brain injury [24-26]. Furthermore, the production of IL6, IL1β, and TNF-α is increased in neuronal degenerative diseases [12]. IL-1β, IL6, and TNF-α have multiple roles in brain injury, such as regulating systemic host responses to disease, altering neuronal and vessel endothelial cells, and promoting the destruction of the blood-brain barrier and brain edema. DDL may be applied to treat several brain diseases associated with increased levels proinflammatory cytokines. Furthermore, it may be applied to protect against glial cell activation-induced central neurodegenerative diseases and attenuate the damage that proinflammatory cytokines cause to neurons.

The potential mechanisms by which DDL suppresses inflammation include the inhibition of canonical inflammatory pathway initiation and the upregulation of phagocytic receptors. The main inflammation-related pathways, including the nuclear factor-κB (NfκB) signaling pathway, the TNF signaling pathway, and the IL17 signaling pathway, are involved in the LPS-induced inflammatory process. In the present study, treatment with DDL led to the downregulated expression of Atf4 and the regulation of the TNF signaling pathway in activated microglial cells. Atf4, a nuclear transcription factor, can promote the activation of the NfκB signaling pathway, initiate and activate the transcription of Il6 to trigger the neuroinflammatory response, and lead to the apoptosis of neuronal cells [27, 28]. Suppression of brain injury-initiated neuroinflammatory response-related pathways and key nuclear factors is the potential pharmacological function of DDL in CNS disease.

Activated microglial cells are chemotactic; via chemotaxis, they migrate to damaged sites and phagocytose abnormal neurons and harmful substances. DDL inhibits the expression of chemokines such as Ccl5, Ccl7, Ccl12, and Cxcl10 in LPS-stimulated microglial cells. These chemokines are reported to induce chemotaxis in a variety of cell types, including microglia and monocytes [29, 30]. In the ischemic brain, the chemokine Cxcl10 is highly expressed to recruit leukocytes and activate astrocytes [31]. Cxcl10 and its response receptor Cxcr3 regulate microglial migration [32]. The inhibition of chemokines and receptors can suppress the recruitment of activated microglial cells and inflammatory cells to the brain injury region. Thus, DDL inhibition of chemokine expression may play a role in inhibiting secondary inflammatory injury.

Early inhibition of the activation and the inflammatory response of microglial cells is necessary for the recovery from a brain injury clinically. This study shows that DDL exhibits excellent anti-inflammatory initiation and progression in the CNS. The intravenous injection of DDL may be useful to block the activation of microglial cells during the acute phase after brain injury.

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Disclosure of conflict of interest

None.

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