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RESEARCH ARTICLE

Thymic stromal lymphopoietin suppresses markers of neuroinflammation and the JAK2/STAT5 pathway in activated microglia

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ABSTRACT. Thymic stromal lymphopoietin (TSLP) is highly expressed in the central nervous system in response to inflammation, but its exact function remains unclear. In this study, we used a model of LPS-stimulated microglia to investigate the direct impact of TSLP on microglial activation and the underlying mechanism. We measured oxidative stress, expression of microglial activation markers, and inflammatory indexes. The results show that TSLP treatment increased the expression of TSLP receptors and reduced LPS-induced oxidative stress, inflammation, and the expression of M1-type markers in microglia. Interestingly, TSLP treatment also influenced the differentiation of microglia towards the M2 type, suppressing LPS-induced activation, mediated by the JAK2/STAT5 pathway. Moreover, TSLP also promoted the expression of macrophage markers in the absence of LPS. These findings support the hypothesis that TSLP plays a role in reducing neuroinflammation by blocking the JAK2/STAT5 pathway induced by LPS, thus indicating a regulatory role in the central nervous system. Targeting this cytokine might provide a novel strategy for controlling an inflammatory response in the central nervous system.

Key words: TSLP, microglia, JAK2/STAT5 pathway, neuroinflammation

Microglia play a crucial role in regulating the inflammatory response and promoting brain tissue repair after damage. These cells are involved in the formation of brain tissue and maintaining a healthy neurological environment [1]. Following brain injury, specific signals cause microglia to activate and differentiate into two types: M1 and M2. The M1 type is characterized by phagocytosis, high nitric oxide synthase activity, and the production of pro-inflammatory molecules [2, 3]. These processes can have detrimental effects on neuronal function and worsen tissue damage. In contrast, the M2 type is associated with tissue regeneration and repair. It is characterized by increased expression of anti-inflammatory factors that promote brain remodelling and repair [3]. Studies have shown that shifting microglia towards the M2 phenotype and suppressing the M1 phenotype may represent potential therapeutic strategies for conditions such as LPS-induced septic encephalopathy [4].

Thymic stromal lymphopoietin (TSLP) is a cytokine that has recently attracted attention due to its association with allergies and cancer [5]. TSLP has been found in thymic stromal cell lines [6]. There are two isoforms of human TSLP, long-chain TSLP and short-chain TSLP, which are encoded by separate promoters. Only the long-chain TSLP is known to interact with its receptor, TSLPR, and its expression is induced in response to inflammation [7, 8]. TSLPR consists of the IL-7 receptor alpha chain and the TSLPR alpha chain. The affinity of the IL-7 receptor for TSLP depends on the presence of TSLPR [9, 10].

Experimental evidence has shown that TSLP is expressed in the healthy central nervous system and upregulated in degenerative conditions affecting myelination. It has also been reported that spinal cord microglia express TSLPR dimers [11]. However, the exact function of long-chain TSLP in septic encephalopathy is still unclear. This experimental study aimed to evaluate the direct impact of long-chain TSLP on LPS-stimulated microglial activation and the underlying mechanisms by examining oxidative stress, markers of microglial activation, and inflammatory markers.

MATERIALS AND METHODS

Reagents

For TSLP, 100 µL of cytokine lysis solution and 100 µL of PBS buffer containing 5% anginose were added to TSLP (PEPRO Tech, USA). For LPS, 10 mL of sterile PBS was used to dissolve 10 mg of LPS powder (L4391,

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SIGMA, USA). The dissolved LPS solution was then filtered through a 0.22-μm bacterial filter and stored at -20 °C in a refrigerator.

Cell culture

The BV-2 cell line was purchased from Wuhan Huarner Biotechnology Company. The cells were cultured in a high sugar medium (Gibco, Life Technologies, USA) supplemented with 10% inactivated foetal bovine serum (Gibco, Life Technologies, USA) and 1% penicillin/streptomycin mixed antibiotics (Gibco, Life Technologies, USA). The cell culture was maintained in a humidified incubator with 5% CO_2.

ROS assay

BV-2 cells were seeded in 24-well plates at a density of 1×10^4/mL and allowed to grow for 24 hours. The cells were then stimulated with LPS (10 ng/mL), TSLP (10 ng/mL), or a combination of LPS and TSLP at the same concentrations for 24 hours. The ROS Assay Kit (Solebro, China) was used according to the provided instructions. The intensity of fluorescence was measured using an electron microscope (Olympus, Japan).

Enzyme-linked immunosorbent assay

BV-2 cells were inoculated onto 6-well plates at a density of 1×10^5/mL. After 24 hours, the cells were stimulated with the same concentrations of LPS and TSLP. The pro-inflammatory factors, IL-6 and TNF-α, in the cell supernatant were measured using an ELISA kit (Neobioscience, China) following the provided instructions.

Flow cytometry

BV-2 cells were inoculated onto 6-well plates at a density of 1×10^5/mL. The cells were treated as mentioned previously. After stimulation, the cells were collected in flow tubes and incubated at 4°C in a light-protected environment for 30 minutes with CD86 and CD206 antibodies (Biolegend, USA).

Immunofluorescence

BV-2 cells (4,000/mL) were plated on coverslips placed in 24-well plates. The cells were fixed with 4% paraformaldehyde for 20 minutes and then washed three times with PBS, each for 5 minutes. The cells were permeabilized with 0.5% Triton X-100 for 10 minutes and washed with PBS for 30 minutes at room temperature. Next, the cells were incubated with primary antibody overnight at 4°C and then with a fluorescent secondary antibody in the dark at room temperature for an additional hour.

Real-time PCR

Total RNA was extracted using TRIzol (Takara, Japan) according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (Takara, Japan). Quantitative real-time PCR was performed using cDNA and TB Green premix Ex Taq II (Takara, Japan). The cycle threshold (CT) value was determined as the number of cycles required for the fluorescent signal to cross the threshold. The delta-CT value was calculated as the difference in CT values between the target gene and the reference gene (actin). The fold difference in gene expression was determined using the delta-delta-CT equation. Primer sequences were as follows: IL-6, sense, 5′-GAGGATAC CACTCC CAACAGACC-3′ and antisense, 5′- AAGTGCATC ATCGTTGTTCATACA -3′; TNF-α, sense, 5′-GGTGCCTATGTCTCAGCCTCTT -3′ and antisense, 5′- GCTCCTCCACTTGGTGGTTT -3′; INOS, sense, 5′- CAAGCACATTTGGGAATGGAGA-3 and antisense, 5′- CAGAACTGAGGGTACATGCTGGAG-3; TSLPR, sense, 5′-CGTGGGATGCTATTGACTT GG-3 and antisense, 5′-TGTGACGGATACAGCGA ACC-3; IL-7R, sense, 5′-TCGAAACTCCAGAACCCA AGAAT-3 and antisense, 5′-GACTAGGCCATACGAC AGGTTTA-3; β-actin, sense, 5′-TGTCCACCTTCC AGCAGATGT-3 and antisense, 5′-AGCTCAGT AACAGTCCGCCTAGA-3.

Western blot analysis

Total protein was extracted from BV-2 cells using a commercially available kit (Biyuntian, China). The protein concentration was determined using the bicinchoninic acid protein test kit (PhD, China). The target proteins were separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis and subsequently transferred to nitrocellulose membranes. The membranes were then incubated at room temperature in a blocking solution containing 5% skimmed milk for one hour. After cleaning, the membranes were placed in a refrigerator and incubated with diluted primary antibodies overnight at 4°C. The membrane was incubated with a secondary antibody (1:50,000) at room temperature for one hour, followed by three washes with TBST buffer. The immunoreactive bands were photographed and visualized using the EC3 Imaging System (UVP Inc, USA). The primary antibodies used included rabbit anti-phosphorylated JAK2 (Abcam, UK; 1:5000), rabbit anti-JAK2 (Affinity, USA; 1:2,000), rabbit anti-STAT5 (Affinity, USA; 1:2000), and rabbit anti-actin (Affinity, USA; 1:2,000 and Abcam, U; 1:6,000).

Statistical analysis

One-way analysis of variance (ANOVA) was employed to evaluate group differences, and post hoc tests were conducted to investigate the differences between multiple groups in pairwise comparisons. GraphPad Prism 9.1 software was utilized for all statistical analyses and graph plotting. The data were presented as mean ± standard error. P values less than 0.05 were considered statistically significant.

RESULTS

Effect of TSLP on TSLPR expression by activated microglia

By binding to both TSLPR and IL-7Rα concurrently, TSLP induces dimerization of TSLPR and generates

high-affinity binding sites [12]. To investigate the expression of these receptors on microglia, BV-2 cells were treated with LPS (10 ng/mL), TSLP (10 ng/mL), or a combination of both LPS and TSLP for 24 hours. Real-time quantitative PCR was then performed to investigate receptor expression. The results demonstrated the expression of both TSLPR and IL-7Rα on microglia. Moreover, simultaneous induction of LPS and TSLP resulted in a significant increase in TSLPR transcript level (*figure 1A, B*).

Subsequently, TSLPR immunofluorescence experiments revealed that both LPS and TSLP significantly enhanced the fluorescence intensity of TSLPR on microglia (*figure 1C, D*). These findings suggest that TSLP can bind to its receptor TSLPR and trigger subsequent reactions under LPS-induced inflammatory conditions.

Effect of TSLP on oxidative stress and inflammatory response in activated microglia

The initiation and progression of the inflammatory response is characterised by oxidative damage and changes in inflammatory markers. As an initial step, reactive oxygen species (ROS) were identified in microglia using the DCFH method. As depicted in *figure 2A, B*, the fluorescence intensity of ROS was found to be low in the control and TSLP groups, indicating a low concentration of ROS. In contrast, fluorescence intensity of ROS significantly increased in the LPS group compared to the control group, indicating a higher concentration of ROS. Notably, in the LPS+TSLP group, the fluorescence intensity of ROS was significantly suppressed compared to the LPS group, suggesting a decrease in ROS concentration.

Expression levels of nitric oxide synthase (INOS), as well as the pro-inflammatory factors, TNF-α and IL-6, were shown to be significantly higher in the LPS group compared to the control group, according to further analysis using real-time PCR (*figure 2C-E*) and ELISA on the collected cell supernatants (*figure 2F, G*). On the other hand, in the LPS+TSLP group, the expression levels were significantly lower. These findings indicate that TSLP may have the potential to mitigate the inflammatory response caused by LPS-induced microglial damage (*figure 2C-G*).

Effect of TSLP on the expression of M1/M2-type markers by microglia

Microglia were divided into four groups: a control group, LPS-treated group, TSLP-treated group, and LPS and TSLP co-treated group. The microglia were then labelled with the M1-type cell membrane marker, CD86, and M2-type cell membrane marker, CD206 (*figure 3A*). The results obtained from flow cytometry analysis showed a significant increase in the expression of the M1-type marker CD86 in both the LPS and TSLP groups, compared to the control group. However, there was a slight decrease in the expression of CD86 in the

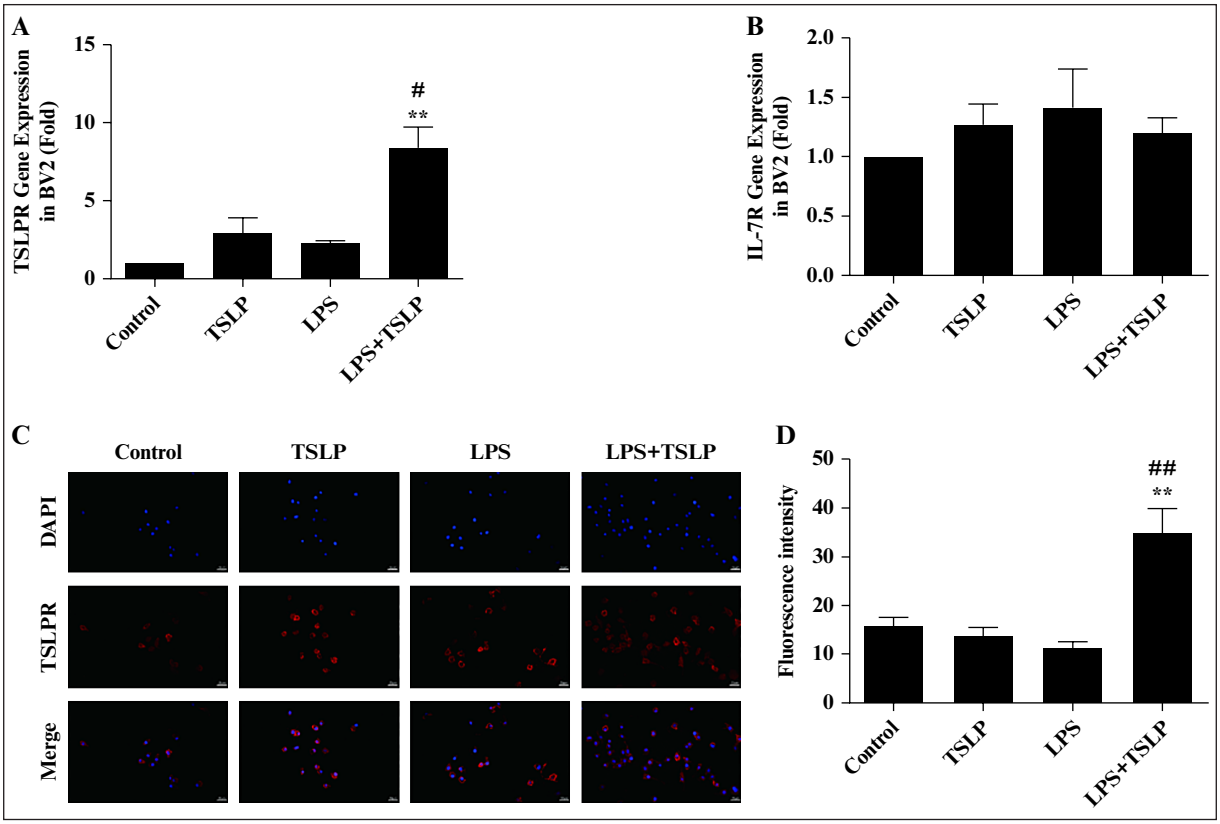

Figure 1

Effect of TSLP on TSLPR gene and protein expression by activated microglia. **A, B**) RT-PCR showing the level of TSLPR (n = 3) (**A**) and IL-7R (n = 3) (B) mRNA. **C, D**) Immunofluorescence analysis showing TSLPR protein expression (n = 3) (**C**) and quantification of the fluorescence (**D**). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ when compared to the control group; #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ when compared to the LPS group.

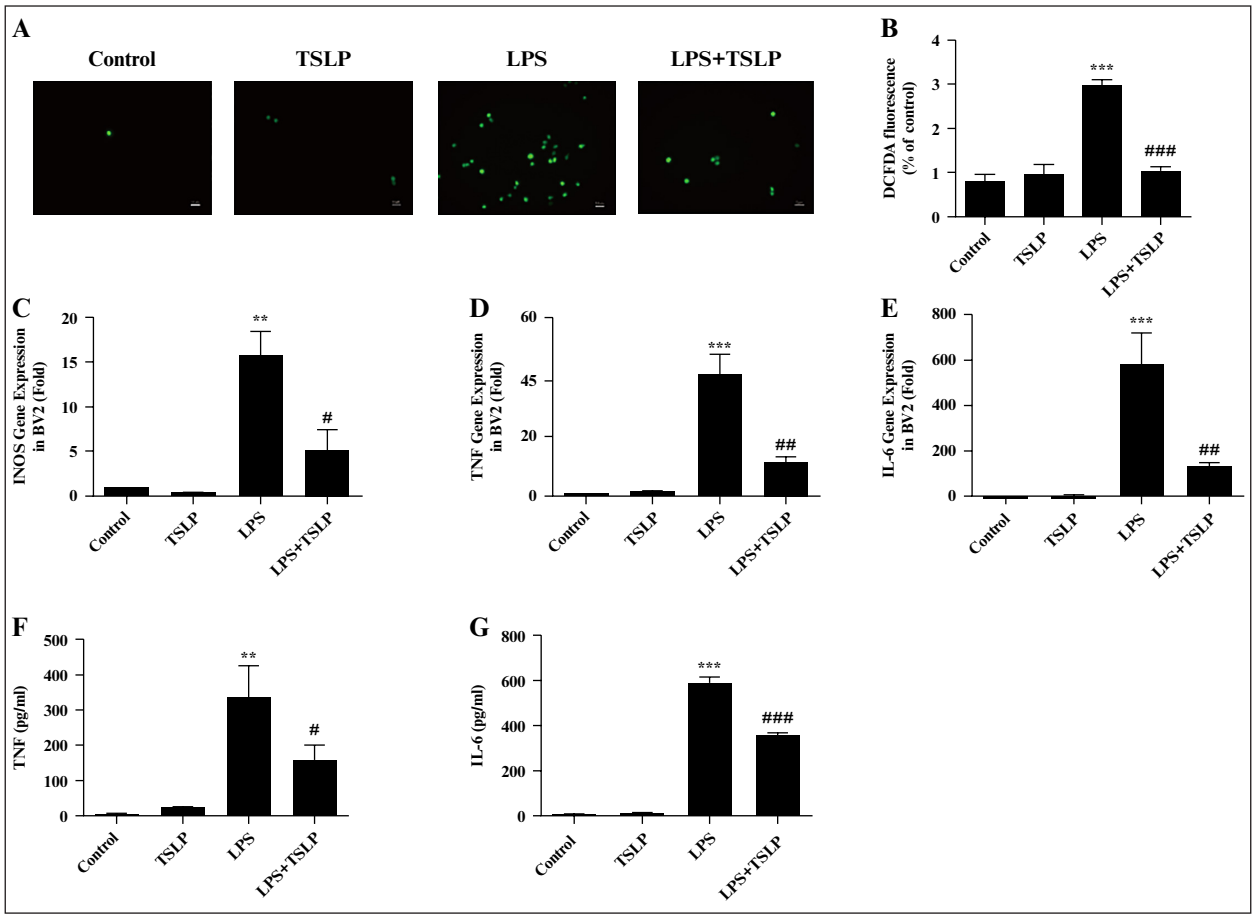

Figure 2

Effect of TSLP on LPS-induced oxidative stress and inflammatory response in microglia. A, B) Visualization of ROS using a fluorogram (20x magnification) (**A**) and statistical analysis of ROS fluorescence intensity ($n = 3$) (**B**). **C-E**) Quantification of RT-PCR for INOS (**C**), TNF-α (**D**) and IL-6 (**E**) mRNA ($n = 3$). **F, G**) Quantification of ELISA for TNF-α (**F**) and IL-6 (**G**) ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$ when compared with control group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ when compared with the LPS group.

LPS+TSLP group when compared to either the LPS or TSLP group (*figure 3B*). Additionally, stimulation with LPS+TSLP promoted the differentiation of cells into the M2 type, as observed by a higher level of expression of the M2-type marker CD206, compared to the LPS group (*figure 3C*).

Effect of TSLP on activation of the JAK/STAT pathway and expression of pro-inflammatory markers by activated microglia

To gain a comprehensive understanding of the potential molecular mechanism of inhibition of microglia inflammation by TSLP, an extensive literature analysis was previously performed revealing that the JAK2/STAT5 pathway plays a critical role in promoting inflammatory responses during microglia activation in the central nervous system (CNS) [13, 14]. To identify changes in the JAK2/STAT5 pathway in response to TSLP and/or LPS, we performed western blot analysis in order to examine the phosphorylation of JAK2 and STAT5 proteins. The results showed that the phosphorylation levels of p-JAK2 and p-STAT5 were significantly higher in the LPS group compared to the control group, but these levels were reduced in the presence of TSLP (*figure 4 A-E*). These findings suggest that TSLP likely inhibits the JAK2/STAT5 signalling pathway, leading to a reduction in LPS-induced neuroinflammation.

Previous research has shown that the JAK2 inhibitor, casein AG490, can mitigate damage caused by LPS in microglia. Based on this, we compared the mRNA of inflammatory factors induced by LPS in the presence of TSLP or AG490 (*figure 4F-H*). The results revealed that following LPS exposure, AG490 reduced the mRNA level of LPS-induced pro-inflammatory factors IL-6 and TNF-α by 70% and 64%, respectively. Similarly, TSLP also reduced these levels by 63% and 54%, respectively, following LPS activation.

Taken together, these findings suggest that TSLP may exert its effects through inhibition of the JAK2 pathway.

DISCUSSION

In this study, we investigated the potential mechanism of TSLP, a cytokine, in supressing LPS-induced neuroinflammation. Our findings indicate that microglia express the receptor for TSLP, suggesting that TSLP can directly interact with microglia through its receptor and thus influence the pathophysiological responses of microglia. Furthermore, we observed that the JAK2/STAT5 signalling pathway is involved in the function of TSLP, which acts to inhibit LPS-induced microglial inflammation.

Neuroinflammation plays a significant role in the pathophysiology and progression of neurodegenerative

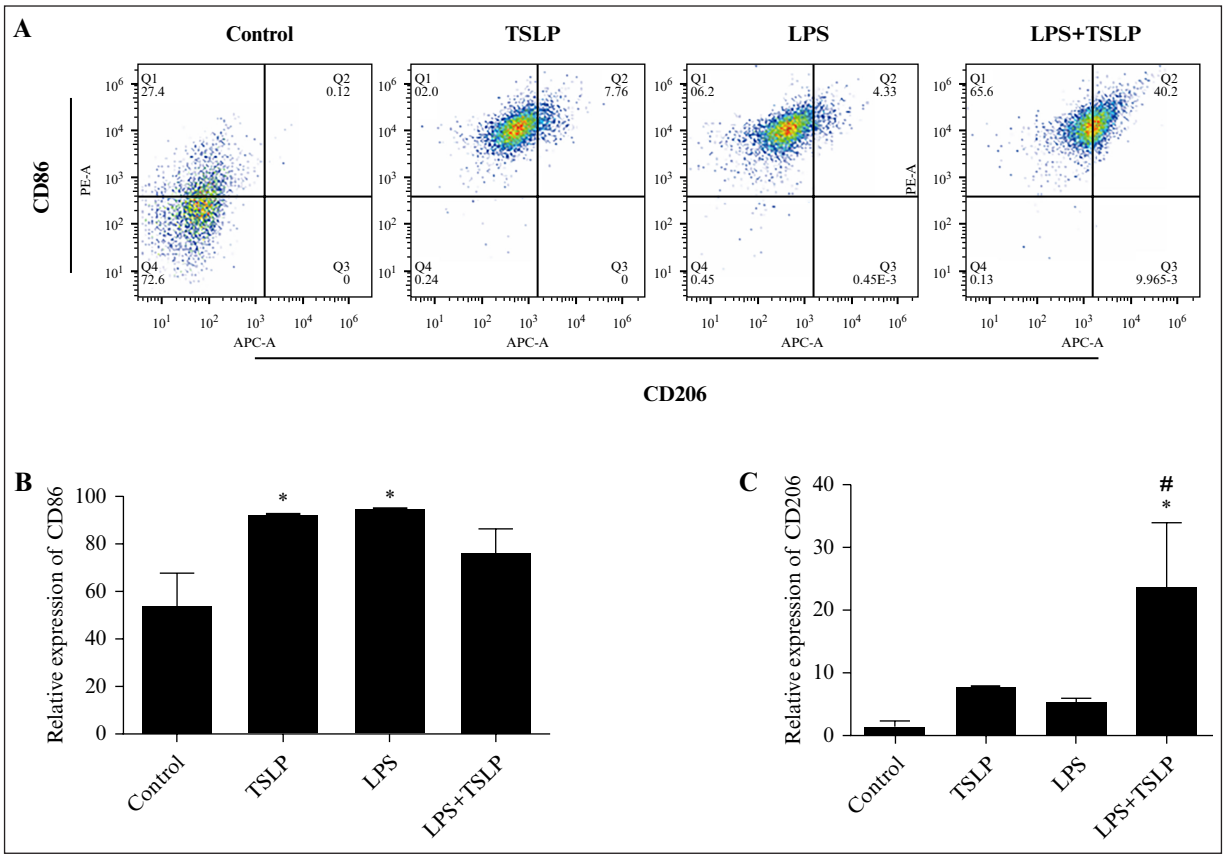

Figure 3

Effect of TSLP stimulation on the activation of microglia cells. **A**) Flow analysis plots (n = 3). **B**) Statistical analysis of the expression level of the M1 type marker, CD86. **C**) Statistical analysis of the expression level of the M2 type marker, CD206. *p<0.05 when compared to the control group; #p < 0.05 when compared to the LPS group.

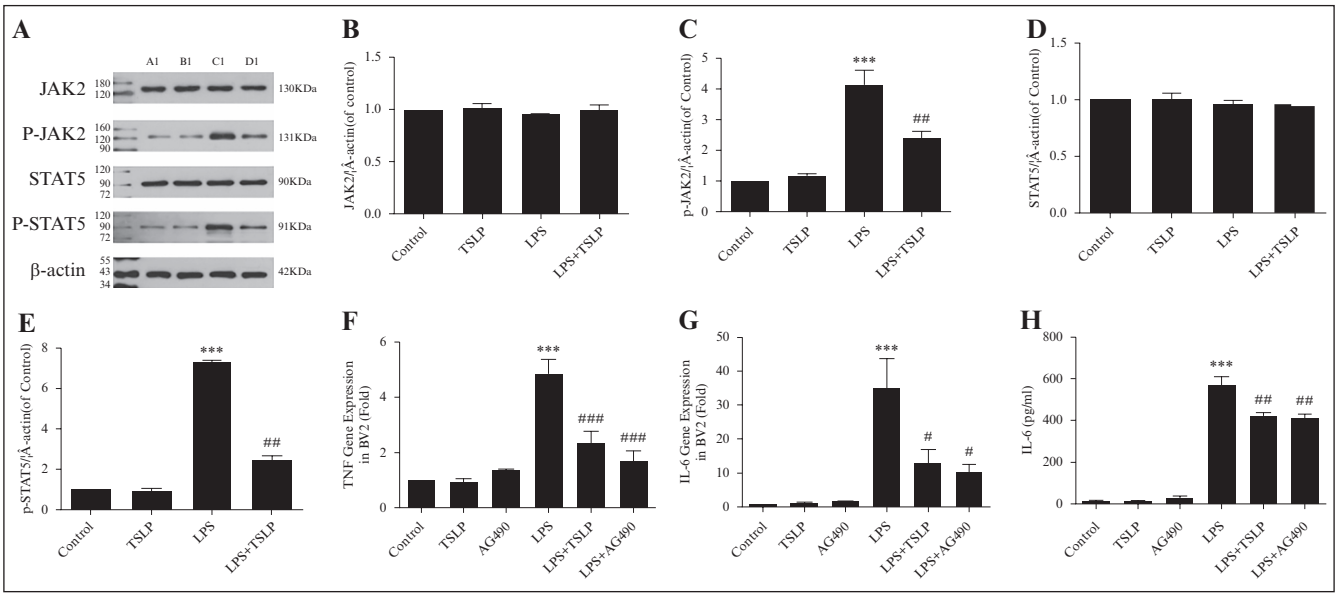

Figure 4

A-E) The effect of TSLP on activation of the JAK/STAT pathway in LPS-induced microglia: protein expression of phosphorylated (p-JAK2 and p-STAT5) and non-phosphorylated JAK2 and STAT5 (n = 3) via western blot (**A**), and quantification of the corresponding JAK2, p-JAK2, STAT5, and p-STAT5 protein expression levels (n = 3) (**B-E**). **F-H**) Effect of TSLP and AG490 on LPS-induced TNF-α (F) and IL-6 (**G**) mRNA (n = 3) as well as IL-6 protein expression (**H**) (n = 3). ***p < 0.001 when compared with the LPS group; #p < 0.05, ##p < 0.01, and ###p < 0.001 when compared with the LPS group.

diseases such as Alzheimer's disease, Parkinson's disease, frontotemporal dementia, and amyotrophic lateral sclerosis [13]. Peripheral administration of LPS has been shown to activate astrocytes and microglia, leading to the expression of pro-inflammatory cytokines, such as COX-2 and iNOS, in the brain [14-17]. Moreover, microglia-mediated inflammatory reactions are primarily responsible for the neuroinflammation that occurs

in the central nervous system [18]. Multiple peripheral treatments with LPS have been shown to induce the M1 phenotype of microglia, characterized by high levels of inflammatory mediators such as TNF and ROS [1, 13]. Lastly, LPS treatment of murine microglia BV-2 cells has also been shown to induce M1 polarization and the production of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α [19, 20].

The available pharmacological options for reducing microglial inflammation have demonstrated only modest benefits, highlighting the urgent need for novel active compounds that can effectively treat neuroinflammation. TSLP, originally discovered in airway epithelial cells, is known to be crucial for type 2 inflammation associated with asthma. However, there is increasing evidence that TSLP also has non-type 2 inflammatory effects in the airway, such as promoting neutrophilic airway inflammation through the activation of dendritic cells [21, 22]. In the context of LPS-induced inflammation, TSLP has been shown to exacerbate LPS-induced acute lung injury [23] and septic inflammation [24]. While TSLP is primarily associated with promoting allergy and inflammatory diseases, controversial reports suggest it has antimicrobial effects [25] as well as a potential to enhance survival and reduce inflammation in mice with cecal ligation and puncture-induced sepsis [26]. CNS epithelial cells have also been shown to produce TSLP [11]. However, the precise role of TSLP in the CNS is rarely discussed.

In our study of how TSLP might impact microglial activation and neuroinflammation, our data led us to the initial conclusion that LPS treatment increases TSLPR gene and protein expression in microglia. Han *et al*. demonstrated that macrophages produce TSLP after being exposed to LPS [24]. However, TSLP level in BV-2 cells was not assessed in this investigation, and it is not clear whether TSLP is generated by microglia themselves or solely supplied by epithelial cells of the CNS. However, by modulating microglial polarization, our study has identified TSLP as a target for therapy of neuroinflammation. Our subsequent results showed that TSLP prevented M1-type microglia activation and promoted differentiation into the anti-inflammatory M2 type. As a result, the expression of inflammatory markers, INOS, TNF-α, and IL-6, was down-regulated.

Through various mechanisms, including the JAK/STAT pathway, which interprets signals from several cytokines and regulates inflammation, microglia may polarize towards the M1 phenotype [27, 28]. The JAK2/STAT5 pathway may therefore be responsible for the inhibitory effect of TSLP on neuroinflammation, as evidenced by the significant decrease in p-JAK2 and p-STAT5 phosphorylation levels after TSLP treatment. Moreover, our data demonstrate the role of the JAK/STAT pathway in the inhibition of M1-type microglial activation which is also inhibited by TSLP. Previous research has shown that TSLP binds to IL-7 receptor α, to activate the JAK/STAT pathway, which in turn induces transcription of target genes and initiates tightly controlled type-2 immune responses [29, 30].

In vivo studies are needed to confirm the role of TSLP in neuroinflammation, and further research is required to fully understand the molecular processes and functions. However, our research has contributed to a better understanding of the role of TSLP in neuroinflammatory effects.

CONCLUSION

In summary, TSLP treatment was found to mitigate oxidative stress and inflammation induced by LPS, as well as reduce the expression of M1-type markers in microglia. Moreover, TSLP was shown to enhance TSLP receptor expression, which in turn may influence the differentiation of microglia towards the M2 type, and further suppress LPS-induced activation of the JAK2/STAT5 pathway in microglia. However, it is worth noting that TSLP also promoted the expression of macrophage markers in the absence of LPS. These findings provide evidence supporting the notion that TSLP can alleviate neuroinflammation and inhibit the JAK2/STAT5 pathway triggered by LPS. Consequently, this sheds light on the regulatory role of TSLP in the central nervous system and may contribute to a novel approach to managing inflammatory responses in the central nervous system.

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Author contributions. ZQ and ZS were involved in the study's conception and design. ZQ and CN collected the samples, organized the database, and conducted the statistical analysis. ZQ, ZM, and XY wrote the initial draft of the manuscript. All authors participated in the revision of the manuscript, and have read and approved the final version for submission.

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