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Research Article

The autoimmune encephalitis-related cytokine TSLP in the brain primes neuroinflammation by activating the JAK2-NLRP3 axis

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Summary

NLRP3 inflammasome hyperactivation contributes to neuroinflammation in autoimmune disorders, but the underlying regulatory mechanism remains to be elucidated. We demonstrate that compared with wild-type (WT) mice, mice lacking thymic stromal lymphopoietin (TSLP) receptor (TSLPR) (*Tslpr⁻/⁻* mice) exhibit a significantly decreased experimental autoimmune encephalomyelitis (EAE) score, reduced CD4⁺ T cell infiltration, and restored myelin basic protein (MBP) expression in the brain after EAE induction by myelin oligodendrocyte glycoprotein$_{35–55}$ (MOG$_{35–55}$). TSLPR signals through Janus kinase (JAK)2, but not JAK1 or JAK3, to induce NLRP3 expression, and *Tslpr⁻/⁻* mice with EAE show decreased JAK2 phosphorylation and NLRP3 expression in the brain. JAK2 inhibition by ruxolitinib mimicked loss of TSLPR function *in vivo* and further decreased TSLP expression in the EAE mouse brain. The NLRP3 inhibitor MCC950 decreased CD4⁺ T cell infiltration, restored MBP expression, and decreased IL-1β and TSLP levels, verifying the pro-inflammatory role of NLRP3. *In vitro* experiments using BV-2 murine microglia revealed that TSLP directly induced NLRP3 expression, phosphorylation of JAK2 but not JAK1 or JAK3, and IL-1β release, which were markedly inhibited by ruxolitinib. Furthermore, EAE induction led to an increase in the Th17 cell number, a decrease in the regulatory T (Treg) cell number in the blood, and an increase in the expression of the cytokine IL-17A in the WT mouse brain, which was drastically reversed in *Tslpr⁻/⁻* mice. In addition, ruxolitinib suppressed the increase in IL-17A expression in the EAE mouse brain. These findings identify TSLP as a prospective target for treating JAK2-NLRP3 axis-associated autoimmune inflammatory disorders.

Keywords: autoimmune encephalitis, thymic stromal lymphopoietin receptor, NLRP3, JAK, T cells

Abbreviations: NLRP3, nucleotide-binding domain, leucine-rich repeat containing protein family, pyrin domain containing 3; TSLPR, thymic stromal lymphopoietin; JAK, Janus Kinase; CNS, central nervous system; EAE, experimentally atoimmune encephalomyelitis; STAT, signal transducer and activator of transcription; UVB, ultraviolet radiation b.

Introduction

Experimental autoimmune encephalomyelitis (EAE), which is mediated by myelin-specific autoreactive T helper cells, is a classical animal model of autoimmune encephalitis, which includes multiple sclerosis (MS), a disease characterized by typical demyelination and neurodegeneration-associated symptoms [1]. Although drugs targeting several immunological pathways have shown beneficial effects in patients with demyelinating diseases, no cure is currently available [2].

Nucleotide-binding domain, leucine-rich repeat containing protein family, pyrin domain containing 3 (NLRP3), and the interleukin (IL)-1β pathway have been shown to be crucial for the development of EAE by participating in neuroinflammation [3, 4]. NLRP3 is activated in two ways: by stimulation of pathogen recognition receptors that activate nuclear factor κB (NF-κB) pathways and by stimuli such as lysosomal rupture, adenosine 5′-triphosphate (ATP), and reactive oxygen species (ROS) [5]. Activation of the NLRP3

inflammasome leads to the production of cleaved caspase-1, which cleaves proIL-1β/pro-IL-18 into mature IL-1β/IL-18 and induces massive cell pyroptosis-related cytokine release and systemic inflammation [6]. The potential mechanisms by which NLRP3 causes neuroinflammation include mediation of Th1 and Th17 responses [7] and induction of chemotactic immune cell migration to the central nervous system (CNS) [8]. NLRP3-dependent IL-1β maturation has been detected in the lesions and cerebrospinal fluid (CSF) of MS patients [9] and correlates strongly with cortical lesion load [10]. Conversely, mice lacking IL-1 or with impaired IL-1 signalling show accelerated EAE development and worse EAE severity [11]. There are only a few available IL-1-targeting drugs for the treatment of autoimmune disorders [12, 13]. Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling is critical for T helper cell polarization and autoimmune neuroinflammation [14, 15]. In fact, several JAK inhibitors targeting a specific JAK or a wide range of JAKs have

been used over the past decades as promising alternatives to traditional disease-modifying antirheumatic drugs for the treatment of several autoimmune diseases [16, 17]. Moreover, JAK mediates IL-4 receptor signals to prime Th2 response-dominant symptoms, such as itch in atopic dermatitis [18], in which cytokine IL-4 production is closely regulated by the function of thymic stromal lymphopoietin (TSLP) [19]. Despite the high expression of TSLP and its role in JAK-STAT signalling, it is unclear whether TSLP functions via NLRP3 to induce autoimmune inflammation and if so, whether JAK is involved in this process.

TSLP is an IL-7-related cytokine that acts on cells of various lineages, including macrophages, dendritic cells (DCs), and T cells [20]. By promoting the expression of major histocompatibility complex (MHC)-II and co-stimulatory molecules such as CD40, CD80, and CD86 and the production of chemokines, TSLP strongly enhances DC maturation and function [21]. In fact, MS and EAE have been reported to be associated with single-nucleotide polymorphisms (SNPs) in the IL-7Rα gene [22]. Binding of TSLP to the TSLP receptor (TSLPR) initiates intracellular JAK/STAT signalling to induce the production of IL-2, TNF, and IL-6 to potentiate inflammatory responses. Targeting these cytokines has been shown to be effective in alleviating EAE and other autoimmune diseases [23–26]. It was reported that directly blocking JAK/STAT signalling pathways with tofacitinib inhibits NLRP3 inflammasome and IL-1β production in neutrophils [27]. However, whether TSLPR signalling is capable of controlling the initiation of inflammation in EAE remains unclear.

In this study, we show that $Tslpr^{-/-}$ mice presented reduced myelin oligodendrocyte glycoprotein peptide (MOG_{35-55})-induced EAE severity due to decreased phosphorylation of JAK2 and expression of NLRP3 and suppression of the Th17 response. Inhibition of JAK by ruxolitinib mimicked the effects of TSLPR deficiency. In addition, ruxolitinib and the NLRP3 inhibitor MCC950 both reduced inflammatory cell and CD4+ cell infiltration, decreased IL-1β and TSLP levels, and restored myelin expression in the brain tissues of EAE mice. Furthermore, an increased Th17 response was accompanied by a decrease in the number of regulatory T (Treg) cells in blood, while TSLPR deficiency and ruxolitinib reversed this phenomenon after EAE induction. These findings reveal that TSLP plays an essential role in the positive regulation of JAK2-NLRP3 axis-driven neuroinflammation in autoimmune disorders.

Materials and methods

EAE induction and scoring

$Tslpr^{-/-}$ mice and wild-type (WT) mice were bred under specific pathogen-free (SPF) conditions. $Tslpr^{-/-}$ mice were purchased from Shanghai Biomodel Organism Science & Technology Development Co., Ltd. Female $Tslpr^{-/-}$ and WT mice (10–12 weeks old) were immunized subcutaneously with MOG_{35-55} (Beyotime, China) and 4 mg/ml heat-inactivated *Mycobacterium tuberculosis* H37Ra (BD) on Day 1 to induce EAE as described previously [28]. Pertussis toxin (200 ng/mouse; List Biological Laboratories Inc.) was injected intraperitoneally on Day 0 and Day 2. The mice were sacrificed on Day 15, and brain tissues were collected for western blotting and immunohistochemistry. EAE-induced paralysis in mice was scored as follows: 0, no disease; 1, tail weakness; 2, paraparesis; 3, paraplegia; 4, paraplegia with forelimb

weakness; and 5, morbidity or death. To evaluate the contribution of the NLRP3 inflammasome and JAK to CNS inflammation, 50 mg/kg MCC950 (dissolved in DMSO) was injected intraperitoneally [29] or ruxolitinib (90 mg/kg/day) was administered orally [30] after MOG_{35-55} immunization.

CNS inflammation

To assess inflammatory infiltrates, brain tissues were harvested, fixed in 4% formalin, and stored at room temperature. The brain tissues were subjected to histological analysis, including HE staining, staining for NLRP3 and CD4 staining, and Luxol Fast Blue (LFB) (Servicebio, China) staining, to assess inflammatory cell infiltration, the number of CD4+ T cells, and the myelin sheath.

Flow cytometry

Mouse blood was collected from the retro-orbital plexus in sodium citrate anticoagulant tubes. Peripheral blood mononuclear cells (PBMCs) were isolated with Mouse 1× Lymphocyte Separation Medium (DAKEWE, China) according to the manufacturer's instructions. The cells were collected, and the following monoclonal antibodies were used for flow cytometry analysis (Beckman Coulter): mouse CD4 (RM4-5, Biolegend) and CD25 (3C7, Biolegend). For intracellular staining, the cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin (PS, Gibco) and then stimulated for 4 h with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and brefeldin A (1 µg/ml, Merck). The cells were fixed with BD Cytofix/Cytoperm (BD), and the cell membrane was permeabilized with BD Perm/Wash buffer (BD) according to the manufacturer's instructions. For staining of the nuclear factor Foxp3, the cells were stained using a Foxp3 staining buffer set (eBioscience) according to the manufacturer's instructions.

Cell stimulation

BV-2 mouse microglia (ATCC) were cultured in DMEM supplemented with 10% FBS and 1% PS. Confluent cells were pretreated for 30 min with 7.5 nM MCC950 (Selleck, USA) [29] or for 4 h with 10 nM ruxolitinib (MCE, USA) [31] before exposure to 10 ng/ml TSLP (Peprotech, USA) for an additional 24 h [32].

Western blot analysis

NLRP3 expression (CST, USA), JAK1 phosphorylation (ABSIN, China), JAK2 phosphorylation (CST), JAK3 phosphorylation (CST), basic myelin protein expression (MBP), β-actin expression, GAPDH expression, and β-tubulin expression (Servicebio, China) in mouse brain tissues and BV-2 cell lysates were assessed by western blotting as previously described [33].

ELISA

IL-1β (Biolegend), TSLP (Biolegend), and IL-17A (Biolegend) levels in mouse brain tissue homogenates or the BV-2 cell supernatant were evaluated by ELISA according to the manufacturer's instructions.

Statistical analysis

Unpaired two-tailed Student's *t*-test was used to statistically analyse all data (GraphPad Prism version 5.0; GraphPad Software).

$P < 0.05$ was considered statistically significant. The data are expressed as the mean ± standard error of the mean (SEM).

Results

$Tslpr^{-/-}$ mice with EAE show decreased neuroinflammation

Mice treated with MOG_{35-55} developed EAE, presenting with tail weakness, limb numbness, and paralysis, which resembled the symptoms of humans with MS [34]. Because androgens have been reported to be protective in EAE [35], we administered MOG_{35-55} to female C57BL/6 mice to construct an EAE model (Fig. 1A). To investigate the role of TSLPR in CNS autoimmunity, the clinical EAE scores of $Tslpr^{-/-}$ and $Tslpr^{+/+}$ mice were determined. After EAE induction, $Tslpr^{+/+}$ mice developed typical monophasic EAE symptoms, manifested by ascending paralysis, 10–12 days after MOG_{35-55} immunization (Fig. 1B). In contrast, $Tslpr^{-/-}$ mice showed delayed onset of paralysis at 11–13 days and less severe symptoms than $Tslpr^{+/+}$ mice. However, the EAE scores of both groups peaked on Day 15 (Fig. 1B). In addition, immunohistochemical analysis of brain tissues from $Tslpr^{+/+}$ and $Tslpr^{-/-}$ mice showed that on Day 15 after EAE induction, there were significantly fewer infiltrating CD4+ lymphocytes in the brain tissues of $Tslpr^{-/-}$ mice than in those of $Tslpr^{+/+}$ mice (Fig. 1C). Importantly, the western blot results revealed that the expression of myelin basic protein (MBP) was reduced in $Tslpr^{+/+}$ mice injected with MOG_{35-55} compared to those not injected with MOG_{35-55}. However, compared with $Tslpr^{+/+}$ mice with EAE, $Tslpr^{-/-}$ mice with EAE showed restored expression of MBP (Fig. 1D).

TSLPR signals via JAK2 and NLRP3

The development of EAE requires NLRP3 [8]. As presented above, TSLPR deficiency results in reduced CD4+ T lymphocyte infiltration during EAE development. Thus, we examined whether TSLPR signalling in EAE requires NLRP3 inflammasome activation. The results showed that the phosphorylation of JAK2 and expression of NLRP3 were increased in MOG_{35-55}-treated mice compared to control mice (Fig. 2A). No significant difference was observed in the phosphorylation of JAK1 or JAK3 (Fig. 2B). Immunohistochemical analysis of brain tissues from $Tslpr^{+/+}$ and $Tslpr^{-/-}$ mice showed that the number of NLRP3+ cells in the brain was markedly increased in $Tslpr^{+/+}$ mice injected with MOG_{35-55} compared to those not injected with MOG_{35-55}, while a significant reduction in the number of NLRP3+ cells in the brain tissue was observed in $Tslpr^{-/-}$ mice compared to $Tslpr^{+/+}$ mice after EAE induction (Fig. 2C).
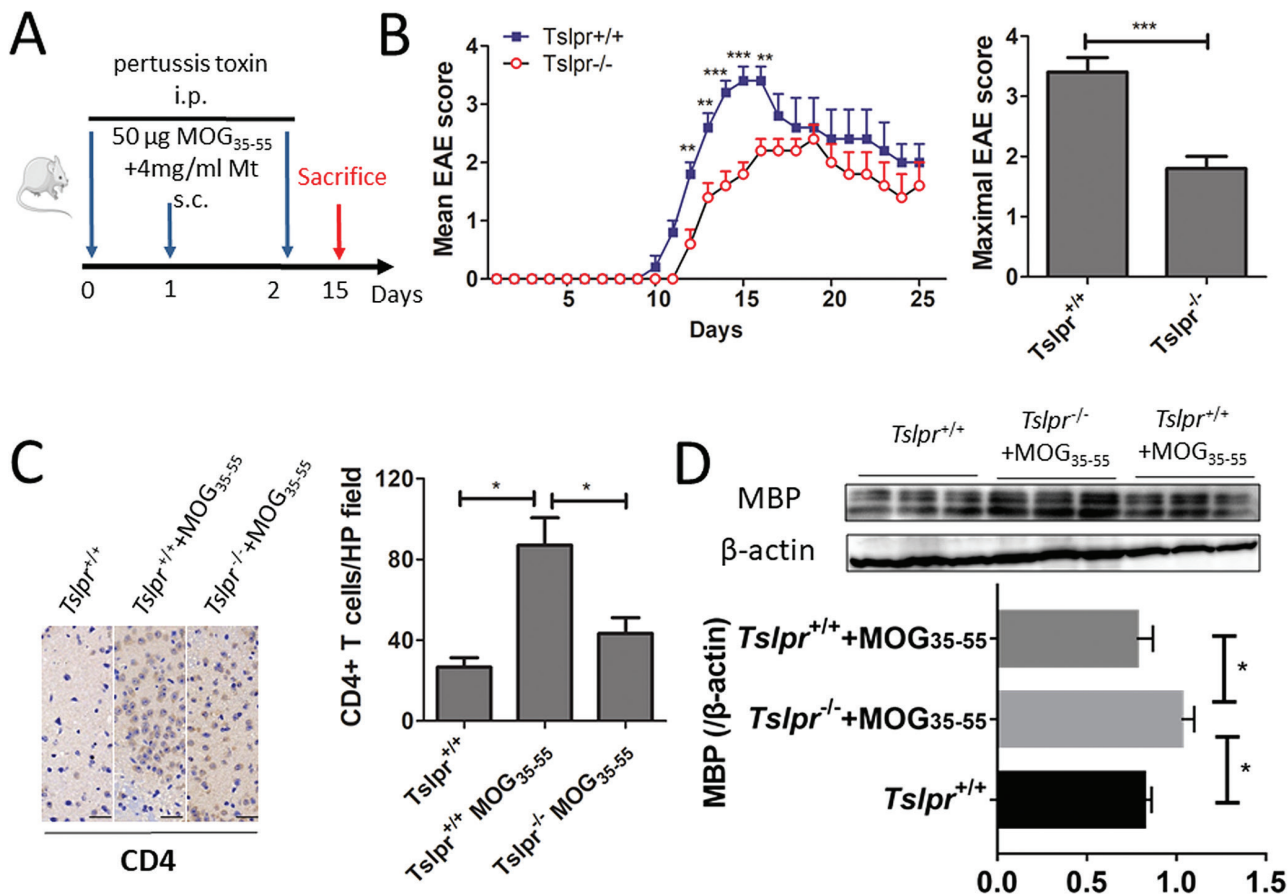

Fig. 1. TSLPR deficiency alleviates neuroinflammation in an EAE model. (A) Schematic diagram of the EAE induction protocol by subcutaneous injection of MOG_{35-55} in mice. (B) The EAE score of $Tslpr^{-/-}$ and $Tslpr^{+/+}$ mice ($n = 5$ each group). (C) CD4 staining in the brain tissues of $Tslpr^{-/-}$ and $Tslpr^{+/+}$ mice after EAE induction ($n = 3$ each group). (D) Expression of MBP in the mouse brain ($n = 3$ each group). The data are presented as the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Scale bar, 50 μm.
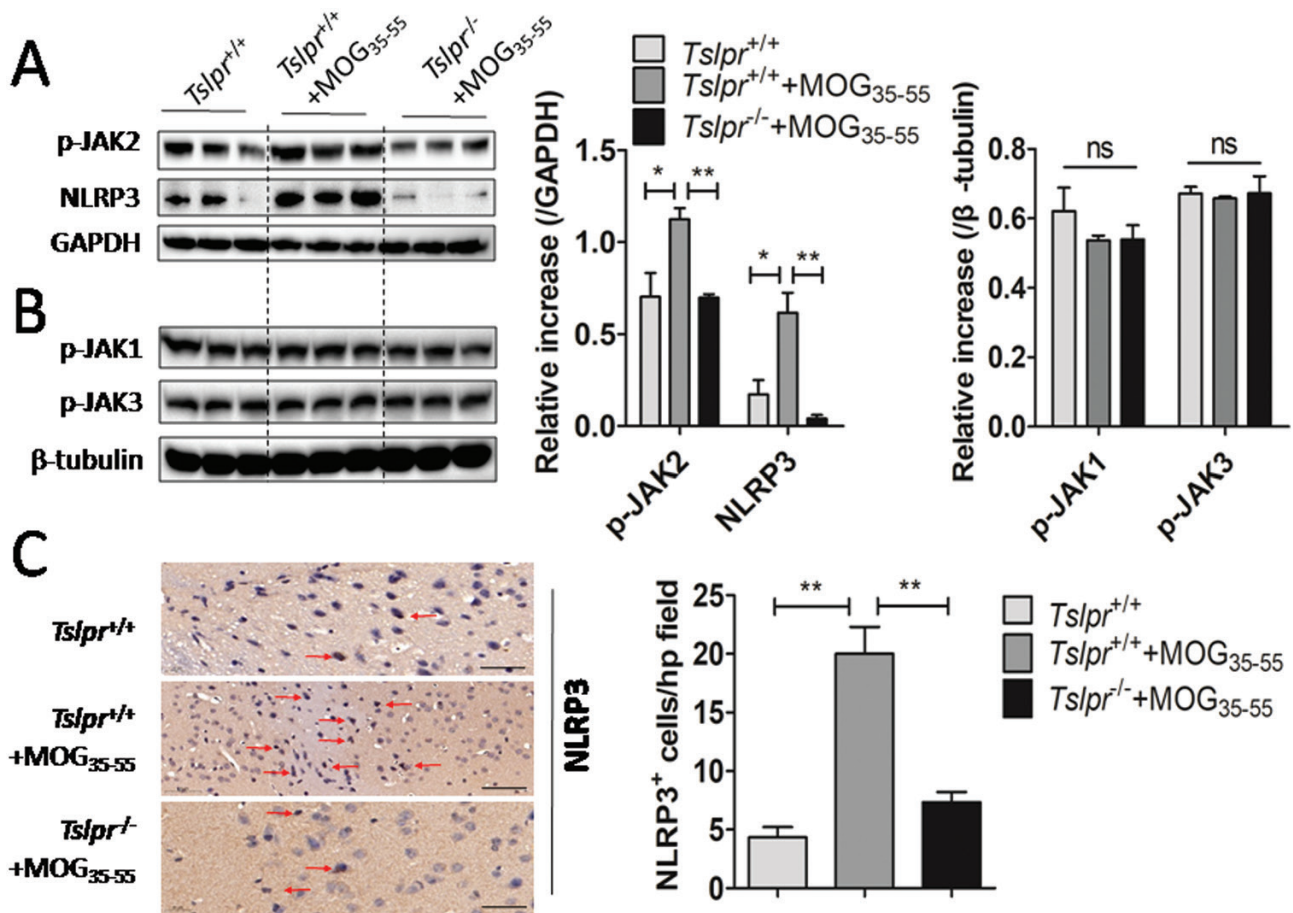
Fig. 2. TSLPR signalling activates JAK2 and NLRP3 in EAE. (A) Expression of NLRP3 and phosphorylation of JAK2 in the brain tissues of *Tslpr⁻/⁻* and *Tslpr⁺/⁺* mice after EAE induction (*n* = 3 each group). (B) Phosphorylation of JAK1 and JAK3 in brain tissues of *Tslpr⁻/⁻* and *Tslpr⁺/⁺* mice after EAE induction (*n* = 3 each group). (C) NLRP3 staining in brain tissue (*n* = 3 each group). The red arrows indicate NLRP3⁺ cells. The data are presented as the mean ± SEM (*n* = 3, *P < 0.05, **P < 0.05). Scale bar, 50 μm.

NLRP3 is involved in JAK2-associated neuroinflammation

Binding of TSLP to TSLPR activates JAK, specifically JAK1 and JAK2 but not JAK3, in primary T cells [33, 36]. To further explore the role of JAK2 in mediating neuroinflammation, we applied a JAK inhibitor, specifically a selective and orally bioavailable JAK1/2 inhibitor widely used for the treatment of myelofibrosis, to block JAK signalling. Oral administration of ruxolitinib to EAE mice resulted in significant reductions in inflammatory cell in filtration, as determined by HE staining (Fig. 3A); CD4⁺ T cell in filtration (Fig. 3B); restoration of the myelin sheath, as determined by LFB staining (Fig. 3C); and MBP expression, as indicated by western blotting (Fig. 3D). The expression of NLRP3 in the brain was decreased in EAE mice treated with the JAK inhibitor ruxolitinib compared with those not treated with the JAK inhibitor (Fig. 3D). ELISA showed that JAK inhibition reduced IL-1β levels in the brain, further confirming NLRP3 hyporeactivity (Fig. 3E). ELISA also showed that TSLP levels in the brain were reduced after in JAK inhibitor-treated EAE mice compared to EAE mice not treated with the JAK inhibitor (Fig. 3F). Together, our data demonstrate that JAK2 mediates neuroinflammation via NLRP3.

NLRP3 inhibition alleviates neuroinflammation

To evaluate the role of NLRP3 in neuroinflammation in EAE, MCC950, a potent and selective small-molecule inhibitor of NLRP3, was used to block canonical and non-canonical NLRP3 activation [29]. Intraperitoneal injection of MCC950 into EAE mice resulted in significant reductions in inflammatory cell infiltration, as determined by HE staining (Fig. 4A); CD4⁺ cell infiltration (Fig. 4B); restoration of the myelin sheath, as determined by LFB staining (Fig. 4C); and MBP expression, as determined by western blotting (Fig. 4D). The levels of IL-1β (Fig. 4E) and TSLP (Fig. 4F) in the brain were also reduced in NLRP3 inhibitor-treated EAE mice compared to EAE mice not treated with the NLRP3 inhibitor.

TSLP induces NLRP3 expression in a JAK-dependent manner *in vitro*

Microglia are the principal immune cells of the brain and have been identified as risk factors for neurodegenerative disease [37, 38]. To study whether TSLP induces NLRP3 expression and cytokine release, we used BV-2 cells, commonly employed mouse microglia, and found that NLRP3 expression was increased in BV-2 cells stimulated with TSLP compared to in BV-2 cells in the control group (Fig. 5A). To verify whether the expression of NLRP3 depends on JAK signalling, as seen in the *in vivo* experiments, we first evaluated JAK1, JAK2, and JAK3 phosphorylation in BV-2 cells. The results showed that TSLP stimulation led to increased JAK2 phosphorylation but not JAK1 or JAK3 phosphorylation (Fig. 5B). An increase in NLRP3 expression led to substantial release of IL-1β,

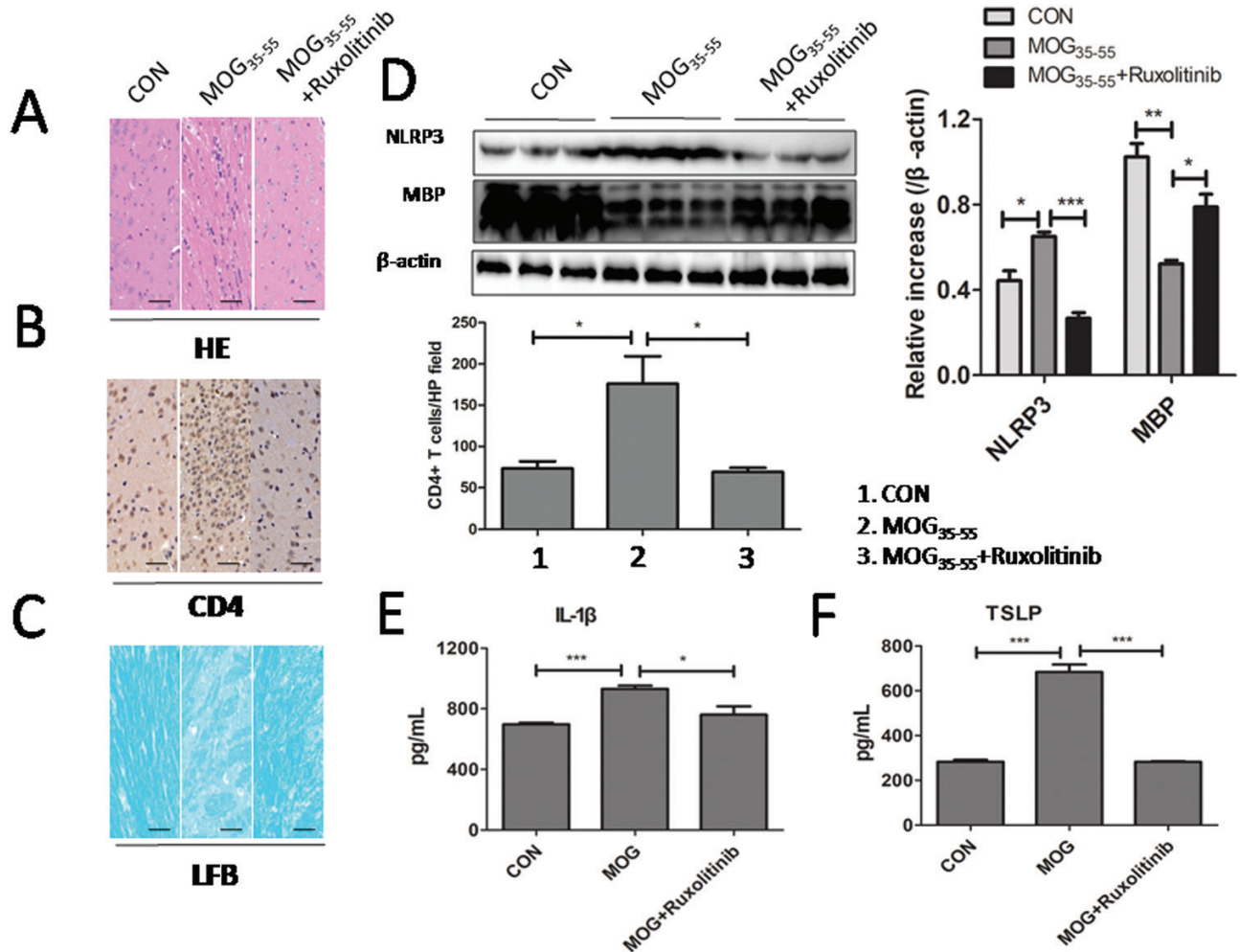**Fig. 3.** MOG_{35-55}-induced neuroinflammation depends on JAK2 signalling. (A) HE staining of the mouse brain after induction of EAE in the absence or presence of ruxolitinib. (B) CD4 staining in the mouse brain ($n = 3$ each group). (C) LFB staining of mouse brain. (D) Expression of NLRP3 and MBP in the mouse brain ($n = 3$ each group). (E) Analysis of IL-1β levels in the mouse brain ($n = 4$ each group). (F) Analysis of TSLP levels in the mouse brain ($n = 4$ each group). The data are presented as the mean ± SEM and were obtained from a high-power field (HPF) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Scale bar, 50 μm.

which was significantly reduced when cells were pretreated with MCC950 to inhibit NLRP3 (Fig. 5C). Further results revealed that ruxolitinib suppressed TSLP-induced NLRP3 expression (Fig. 5D). This suppression of NLRP3 expression was accompanied by decreased release of IL-1β (Fig. 5E).

TSLP is involved in Th17 immune response in EAE

An extensive Th17 immune response contributes to neuroinflammation in EAE [39], while induction of Treg cells ameliorates EAE [40]. We thus analysed Th17 cells and Treg cells in peripheral blood by flow cytometry. The number of Th17 ($CD4^+IL-17A^+$) cells was increased in mice immunized with MOG_{35-55} compared to control mice (Fig. 6A). In contrast, the number of Treg ($CD4^+CD25^+Foxp3^+$) cells in the blood was deceased after EAE induction (Fig. 6B). However, a significant decrease in the number of Th17 cells was observed in $Tslpr^{-/-}$ mice compared to WT mice after EAE induction. Notably, this decrease in the number of Th17 cells was accompanied by an increase in the number of Treg cells in the blood (Fig. 6A and B). The expression of the cytokine IL-17A in the mouse brain was evaluated. As anticipated, IL-17A expression was increased in mice with EAE compared to control

WT mice, while TSLPR deficiency led to a decrease in the expression of IL-17A by nearly half (Fig. 6C). Furthermore, we observed that inhibition of JAK2 by ruxolitinib significantly decreased IL-17A levels in the brains of WT mice after EAE induction (Fig. 6D).

Discussion

Activation of NLRP3 is a tightly regulated process and a key step in autoimmunity in the CNS. In this study, we report three novel findings that broaden our understanding of NLRP3-associated neuroinflammation. First, by using $Tslpr^{-/-}$ mice, we demonstrated that TSLP signalling regulates neuroinflammation and paralysis in mice. Second, we showed that TSLPR signals via JAK2 to activate NLRP3-associated neuroinflammation in the context of EAE. Third, we found that manipulation of the Th17/Treg balance is involved in TSLP-induced neuroinflammation. Taken together, the findings of the current study identify novel functions of TSLP-dominant JAK pathways upstream of NLRP3, which represent promising targets for the treatment of autoimmune disorders.
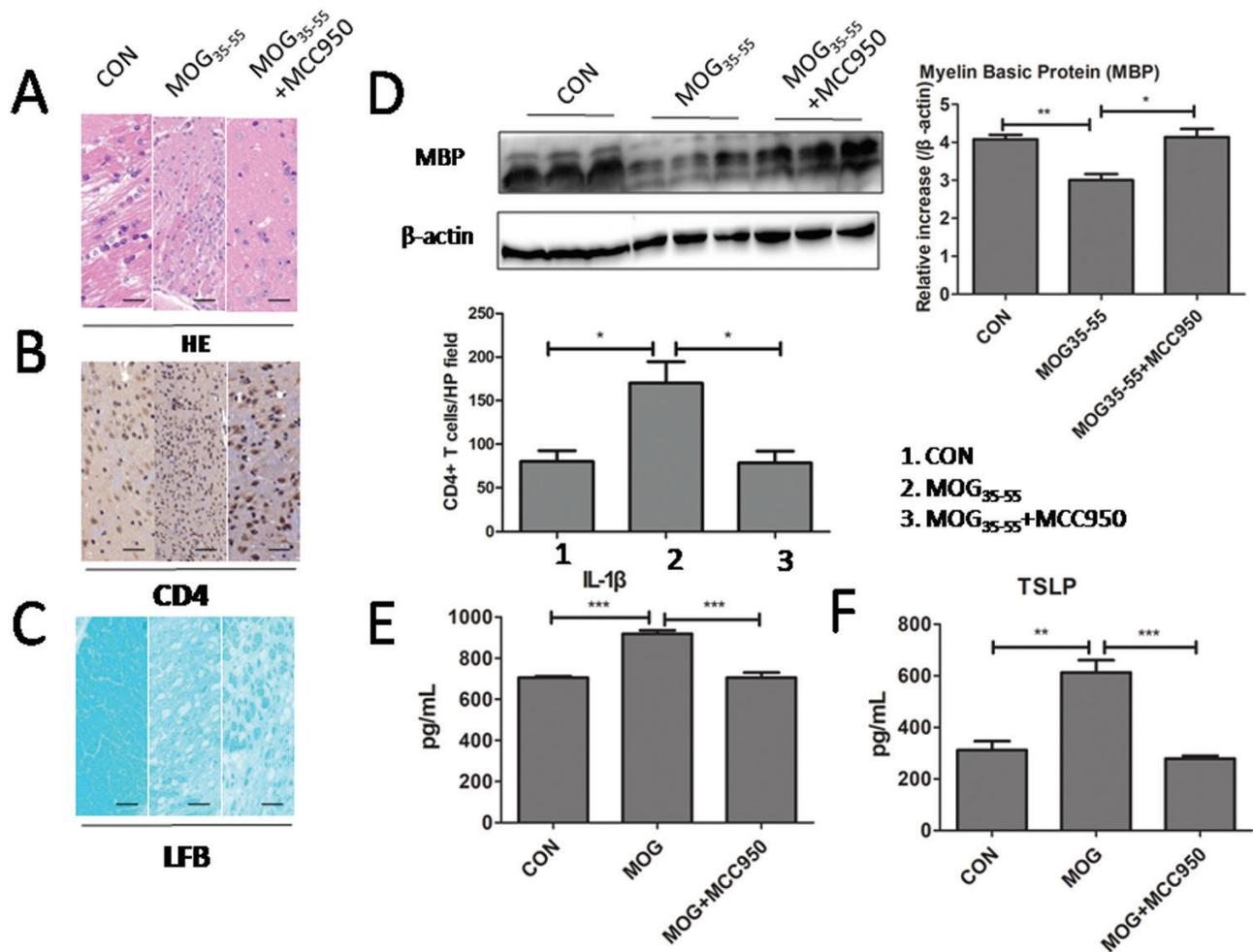
Fig. 4. NLRP3 regulates neuroinflammation in EAE. (A) HE staining of the mouse brain after induction of EAE in the absence or presence of MCC950. (B) CD4 staining in the mouse brain ($n = 3$ each group). (C) LFB staining of the mouse brain. (D) Expression of MBP in the mouse brain ($n = 3$ each group). (E) Analysis of IL-1β levels in the mouse brain ($n = 4$ each group). (F) Analysis of TSLP levels in the mouse brain ($n = 4$ each group). The data are presented as the mean ± SEM and were obtained from a HPF (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Scale bar, 50 μm.

Generally, the Th17 response is believed to cause neuronal death or an inflammatory response in autoimmune disorders [39, 41]. In addition, recent studies have shown that type 3 innate lymphoid cells (ILC3s) produce IL-17 in autoimmune disorders, such as ankylosing spondylitis [42], and can maintain neuroinflammation by supporting T cell survival [43]. In this study, we found that BV-2 also produced IL-17A after TSLP stimulation (data not shown). Thus, both neuroinflammation and demyelination are largely believed to be mediated by the adaptive immune system via Th17 cells and by the innate immune system via ILC3- and even microglia-related responses. Based on the data of the current and previous studies showing a critical role for TSLP in DCs [21, 44] and ILC3 function [45], we speculate that microglia and ILC3s, in addition to Th17 cells, play important roles in promoting autoimmune inflammation-associated demyelination. The precise role of TSLP in IL-17A+ cell-mediated neuroinflammation and demyelination needs to be further investigated.

Despite previous findings that Treg cell formation requires TSLP [46, 47], we found that compared to EAE induction, which led to a decrease in the number of Treg cells, TSLPR deficiency led to an increase in the number of Treg cells in the blood, which is consistent with the reduction in Treg function

observed in MS in a previous study [48]. Our data suggest that TSLP-producing cells are a more general means by which immune responses are facilitated, as they suppress the Treg cell response. In addition, these changes in the number of Treg cells in the blood after deletion of *Tslpr* correlate with alleviation of neuroinflammation and restoration of myelin expression, which is in agreement with a previous study showing that EAE symptoms are ameliorated in *Tslp*−/− mice [49]. In fact, the epithelial cell-derived cytokines TSLP, GM-CSF, and IL-25 have been shown to be master initiators of type 3 inflammation via their effects on a variety of cells, including Th17 cells, ILC3, and mast cells [50–52]. These cytokines are believed to rapidly bind to membrane receptors to generate innate immune responses and therefore prime adaptive immune cells. Strikingly, two recent studies have demonstrated that TSLP directly activates neurons [32, 53]. Our current data showing that TSLP is highly regulated in the brain in the context of EAE are consistent with the findings of a previous study [54] and further demonstrate that TSLPR signalling activates NLRP3-mediated inflammation through phosphorylation of JAK2 in response to MOG35−55 administration. Thus, we speculate that the cytokine TSLP may act as a master regulator of neuroinflammation in immune cells in the brain.
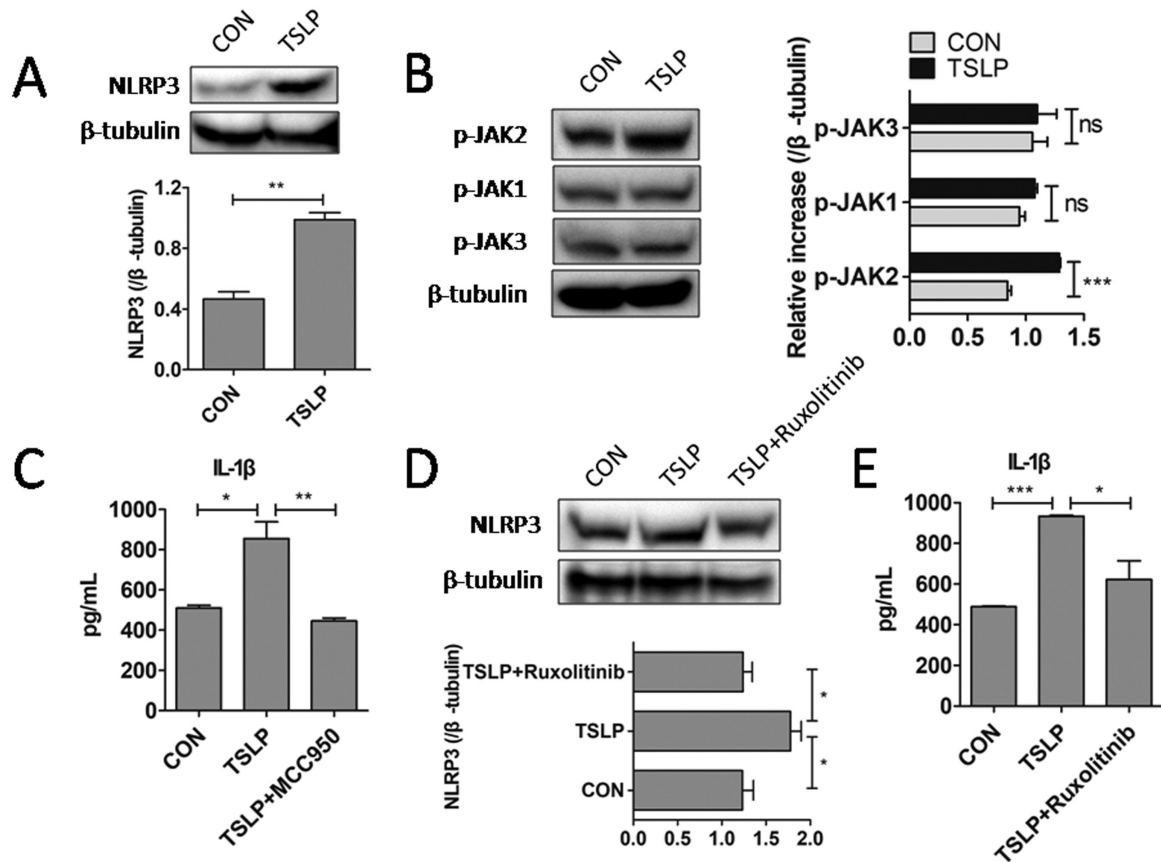
Fig. 5. TSLP directly induces NLRP3 expression via JAK. BV-2 cells were cultured in RPMI 1640, pretreated with or without MCC950 (7.5 nM) for 30 min or ruxolitinib (10 nM) for 4 h and stimulated with TSLP (10 ng/ml) for 24 h. (A) Expression of NLRP3 in BV-2 cells in the presence or absence of TSLP. (B) Phosphorylation of JAK1, JAK2, and JAK3 in BV-2 cells stimulated with TSLP. (C) Analysis of IL-1β levels in the supernatant of BV-2 cells pretreated with MCC950 and stimulated with TSLP (n = 3 in each group). (D) Expression of NLRP3 in BV-2 cells pretreated with ruxolitinib and stimulated with TSLP. (E) Analysis of IL-1β levels in the supernatant of BV-2 cells pretreated with ruxolitinib and stimulated with TSLP (n = 3 in each group). For western blotting data, the results of one of three experiments are presented. The data are presented as the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

In immune cells, cytokine signalling by the JAK-STAT pathway causes transcriptional changes to promote cellular activation. However, although JAK inhibitors have been reported as alternative immunotherapies in patients with autoimmune disorders such as neuromyelitis optica [55], our data indicated that application of the JAK1/2 inhibitor ruxolitinib failed to induce typical neuroinflammation following MOG$_{35-55}$ injection, as observed in the EAE mouse brain. Also, this decrease in neuroinflammation after treatment with ruxolitinib was accompanied by restored expression of MBP. Thus, we predict that alterations in classic JAK-mediated NLRP3 inflammasome activation, especially decreased IL-17A levels, as a reflection of the Th17 immune response, are sufficient to explain neuroinflammation and demyelination. One previous study showed that JAK1 mediates sensory neuronal responsiveness, which can be enhanced by cytokines such as IL-4 [18]. Our data are consistent with this study and further demonstrate that JAKs have novel functions in neurons and regulate the myelination/demyelination balance, at least through NLRP3-mediated pathways. However, we note that this alteration in MBP expression does not exclude the role of JAK2 or other pathways in modulating the transcription or posttranscriptional modification of MBP within the CNS. Future studies are required to better understand how changes in the JAK-STAT pathway impact myelin expression and neuroinflammation in autoimmune disorders.

Clinical application of ruxolitinib, a non-selective JAK1/2 inhibitor, has been reported to alleviate neurologic disability in neuromyelitis optica [55]. In fact, other JAK inhibitors, such as tofacitinib and baricitinib, have shown significant clinical efficacy in autoimmune disorders in clinical trials [56, 57]. Previously, changes in neuroinflammation following JAK inhibition were attributed to the anti-inflammatory role of the Th17 response [41]. Recently, a study demonstrated that transient receptor potential (TRP) plays a critical role in EAE by mediating axonal and neuronal degeneration [58] and that the JAK-STAT pathway determines TRP expression [59, 60], which indicates the involvement of TRP in JAK pathway-mediated neuroinflammation. Based on studies by our group and others, we speculate that the amelioration of neuroinflammation in EAE mice treated with ruxolitinib may be mediated, at least in part, by disruption of these signals in the CNS and that such treatments may alleviate neuroinflammation in autoimmune disorders. Strikingly, recent studies have provided experimental and clinical evidence for the efficacy of evobrutinib, the first Bruton's tyrosine kinase (BTK)-inhibiting molecule to be developed, and reported that impairment of encephalitogenic T cells reduces disease severity in clinical and mouse models of MS [61, 62]. Given that our current study demonstrates a direct role for JAK in neuroinflammation, whether blockade of a combination of TSLP, JAK, and BTK can synergistically alleviate
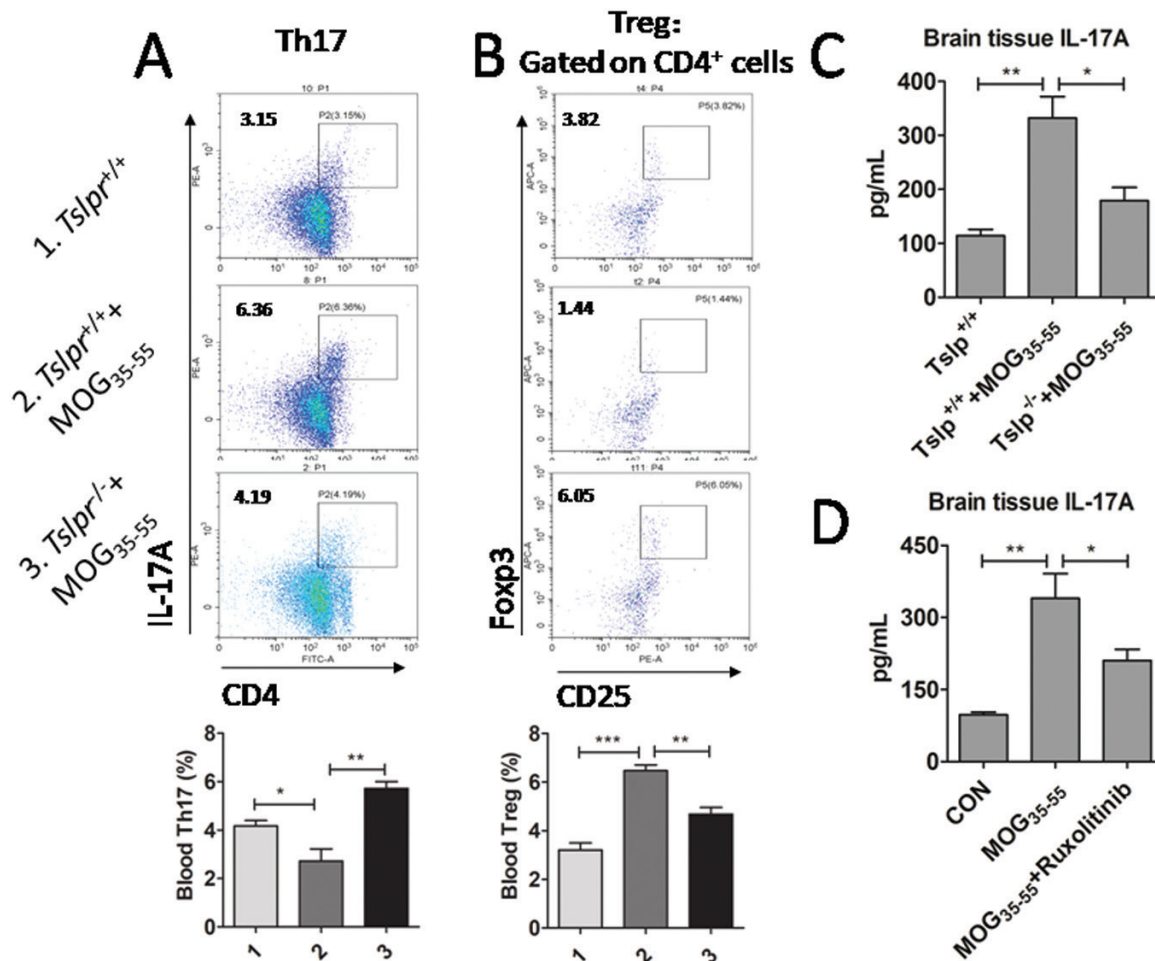
Fig. 6. TSLP mediates the MOG_{35-55}-induced Th17 immune response. (A) Flow cytometry analysis of Th17 ($CD4^+IL-17A^+$) cells in the blood. (B) Flow cytometry analysis of Treg ($CD4^+CD25^+Foxp3^+$) cells in the blood. (C) Analysis of IL-17A levels in the brains of WT and $Tslpr^{-/-}$ mice after induction of EAE by ELISA ($n = 4$ in each group). (D) Analysis of IL-17A levels in the brains of WT mice treated with or without ruxolitinib after induction of EAE by ELISA ($n = 4$ in each group). The data are presented as the mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

neuroinflammation in autoimmune disorders such as MS needs to be further investigated.

In conclusion, our data establish and highlight the capability of TSLPR-JAK signalling inhibition to control disease-driving neuroinflammation associated with NLRP3 in inflammatory demyelination in the CNS. This was specifically demonstrated by using ruxolitinib, a non-selective JAK inhibitor that has been tested in clinical trials of several autoimmune diseases, but the immunological effects of this inhibitor may be similar to those of other JAK inhibitors in clinical development. Thus, the mechanistic data provided here will be instrumental in facilitating how this molecule is integrated into current treatments for autoimmune disorders.

Funding

Conflict of interest

All authors declare that they have no competing interests.

Author contributions

X.Y., J.L., J.W., and Y.C. performed the experiments, data acquisition, analysis and interpretation and drafting the article. F.C. analysed the data and critically revised the article. L.W. designed the project and critically revised the article for the important intellectual content and final approval of the version to be published. All authors provided important review of the manuscript.

Ethical approval

This study was approved by the institutional research ethics committee of Nanjing Medical University affiliated Nanjing Brain Hospital.

Data availability

The data sets used and/or analysed in the present study are available from the corresponding author on reasonable request.

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