

Inhibition of JAK/STAT pathway restrains TSLP-activated dendritic cells mediated inflammatory T helper type 2 cell response in allergic rhinitis

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Abstract Thymic stromal lymphopoietin (TSLP) has recently been implicated as a key molecule for initiating allergic rhinitis (AR) at the cell-dendritic cell (DC) interface. Previous studies demonstrated that TSLP activated DCs to express more OX40 ligand (OX40L), which is associated with the initiation of T helper type 2 (Th2) cell responses. STAT phosphorylation has been reported to be promoted by TSLP. Thus, we investigated if the JAK/STAT pathway inhibitor CYT387 could affect TSLP-DC-mediated Th2 cell response in naive T cell and AR mice model. Western blot showed that the levels of phosphorylated JAK1, JAK2, STAT1, STAT3, and STAT5 were increased in TSLP-DCs, which can be offset by CYT387. Flow cytometry indicated that CYT387 had obviously down-regulated the surface maturation co-stimulatory molecules (CD11c, CD80, CD86, and MHCII) in DCs, which were increased by TSLP. Moreover, CYT387 markedly reduced the ability of TSLP-DCs to promote the differentiation of naive CD4⁺ T cells into IL-4-expressing Th2 cells. The histological examination showed that the

CYT387-treated group showed less epithelial disruption, epithelial cell proliferation, and reduced eosinophil infiltration compared with AR group. Western blot and RT-PCR demonstrated that the expression of OX40L was increased in AR mice, but that it was decreased by CYT387. Furthermore, CYT387 treatment resulted in the reduction of IL-4 and IL-5 expression and increased IFN- γ level in AR mice, which was consistent with the levels of intracellular cytokine in Th2 cell. In conclusion, we suggest that blocking the JAK/STAT pathway restrains inflammatory Th2 cell response induced by TSLP-DCs in AR.

Keywords Allergic rhinitis · JAK/STAT · Dendritic cells · OX40L

Introduction

Allergic rhinitis (AR) belongs to one of the chronic inflammatory upper airway diseases characterized by intense eosinophil infiltration, mucus hypersecretion, and airway remodeling, which is orchestrated mainly by antigen-specific T helper type 2 (Th2) cells and their cytokines including IL-4, IL-5, and IL-10 [1]. It has been estimated that about 10–30% adults and 40% of children suffer from painful AR [2]. For clinical therapy, although drugs including intranasal steroids, antihistamines and anti-leukotrienes, are able to alleviate the symptoms of AR, they cannot modulate the pathophysiological basis of hypersensitivity and scarcely aim at the early stage of AR cascade reaction [3]. Therefore, it is necessary to focus on allergy upstream regulatory factors of AR to look for their regulatory mechanism and thus aim to restrain AR at an early stage.

The cytokine thymic stromal lymphopoietin (TSLP) has been linked to multiple human allergic inflammatory

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diseases, such as asthma [4, 5], atopic dermatitis [6], and AR [7, 8]. Dendritic cells (DCs) are important immune cells and critical for the upstream initiation of immune responses relying on their “professional” function of antigen uptake and presentation [9]. In AR, TSLP plays important roles in the activation and migration of DCs [10]. A previous study has indicated that TSLP-activated DCs drive allergen-specific naive CD⁴⁺ T cells to expand and differentiate into inflammatory Th2 cells, thereby leading to the induction of allergic inflammation [11].

OX40 ligand (OX40L), a member of the TNF superfamily expressed by DCs, has been demonstrated to regulate DC-T cell interaction [12] and function as a molecular switch in TSLP-activated DC-mediated initiation of Th2 cell responses [12–15]. Meanwhile, the expression of OX40L in DCs can be increased by the stimulation of TSLP [11]. In addition, recombinant OX40L gene-transfected TSLP-DCs strongly prime naive CD⁴⁺ T cells to produce IL-4, IL-5, IL-13, and TNF- α , but not IL-10 [11]. The above findings reveal that OX40L is the original trigger of TSLP-DCs to induce the generation of inflammatory Th2 cells, which acts in the early stage of AR cascade reaction. Given all that, the regulatory mechanism of TSLP on OX40L expression in DCs is still unknown, which may be helpful in looking for the upstream target of AR.

Through binding with TSLP receptor (TSLPR) on the surface of DCs [16–18], TSLP can activate multiple signal transduction pathways [19, 20]. Previously, studies have shown that stimulation of the IL7R/TSLPR complex by TSLP induces the phosphorylation and activation of Janus kinases (JAKs). Activated JAKs regulate the activity of signal transducers and activators of transcription (STAT) factors including STAT1, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [21]. Moreover, earlier studies demonstrated that TSLP induces STAT3 and STAT5 phosphorylation, resulting in transcription of the STAT-responsive genes [22, 23]. However, whether the JAK/STAT pathway involved in the regulation of TSLP on OX40L expression in DCs and the process of TSLP-DCs induced inflammatory Th2 cell response have not been investigated.

Here, we used JAKs inhibitor to block the JAK/STAT pathway in TSLP-activated DCs and OVA-induced AR mice model. This strategy allows us to determine the roles of the JAK/STAT pathway in TSLP-induced OX40L expression in DCs, DC maturation, and inflammatory Th2 cell response in AR.

Materials and methods

DC isolation

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of

Institutional Animals of The Fourth Military Medical University. DCs were acquired as described previously [24], with minor modifications [25]. Briefly, mice femoral and tibial bone marrow cells were first flushed and passed through a nylon mesh. Red blood cells were lysed by ACK lysis buffer. Then, 1×10^6 cells were placed in 24-well plates in 1 ml RPMI-1640 medium containing 2 mM L-glutamine, 10% (v/v) heat-inactivated FBS, nonessential amino acid, 1 mM sodium pyruvate, rmGM-CSF (20 ng/ml), rmIL-4 (20 ng/ml), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C, in 5% CO₂. On the third day, 1 ml complete RPMI-1640 medium containing rmGM-CSF and rmIL-4 was added into the wells upto 2 ml. After cultures for 7 days, non-adherent cells (immature DCs) were collected and used for the following experiments. Flow cytometry (Becton Dickinson, Mountain View, USA) was performed when CD11c⁺ cells reached 80% to identify the surface molecules/markers of DCs. Then, DCs were allowed to receive TSLP (15 ng/ml) stimulation or TSLP plus CYT387 co-stimulation for 48 h.

Scanning electron microscopy (SEM)

DCs obtained after 12 h of culture at 37 °C were centrifuged and fixed on sterile poly-L-lysine glass coverslips. Cells were then fixed in 0.1 M phosphate buffer (pH 7.2) with 1% glutaraldehyde, 4% paraformaldehyde, and 1% osmium tetroxide. Cells were then dehydrated in graded ethanol baths, dried to critical point using carbon dioxide, and coated by gold sputtering. They were examined with a scanning electron microscope (Carl Zeiss S.A.S., Le Pecq, France).

Western blot

Cell lysates were prepared with Triton X-100 lysis buffer. After centrifugation, protein concentrations were determined by BCA method as per the standard protocol. Equal amounts of cell proteins (typically 40 μ g) were separated by 10% SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) and then electrophoretically transferred onto a PVDF membrane. After blocking for 1 h, primary antibodies against phospho-JAK1 (1:1500 dilution), JAK1 (1:2000), phospho-JAK2 (1:1000), JAK2 (1:2000), phospho-STAT1 (1:1000), STAT1 (1:2000), phospho-STAT3 (1:1500), STAT3 (1:2000), phospho-STAT5 (1:1000), STAT5 (1:1500), and anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA) were incubated with membranes at 4 °C overnight. Secondary antibodies conjugated with horseradish peroxidase were reacted with the membranes at 1:2000 for 1 h at room temperature followed by enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ). The intensity of bands was measured using Image Quant software.

Flow cytometry

The expression of surface molecules on DCs was determined by flow cytometry as described previously [25]. Cells were harvested and resuspended in ice-cold FACS washing buffer (0.1% sodium azide and 2% FBS in PBS). Cells were then stained with FITC-conjugated anti-MHC class II (5 µg/ml), anti-CD80 (5 µg/ml), and anti-CD86 (5 µg/ml) with PE-conjugated anti-CD11c (5 µg/ml, final concentration) antibody for 30 min at 4°C in the dark. Stained cells were washed and analyzed by flow cytometry. The data were collected for 1×10^4 cells per sample.

For intracytoplasmic staining of interferon (IFN)- γ and IL-4, CD4⁺ T cells were co-cultured with DC cells, and the cells were treated with 15 ng/ml TSLP and then stimulated with or without 100 nM CYT387. Cells were harvested and fixed with freshly prepared ice-cold 4% paraformaldehyde (Merck, Darmstadt, Germany) for 15 min, and permeabilized with 0.1% saponin in PBS containing 1% FCS for 3 min at 4°C. Subsequently, cells were stained for 15 min using an FITC-conjugated anti-IFN- γ and a PE-conjugated anti-IL-4 antibody (BD Biosciences), washed twice and analyzed by flow cytometry.

Quantitative real-time PCR

Real-time PCR was performed with specific primers and probes [using TaqMan Gene Expression Assays and TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) in a QPCR System (Mx3005P; Stratagene, La Jolla, CA)]. The ID for murine OX40L was Mm00442039. The results were analyzed by the comparative threshold cycle (Ct) method [26] and normalized by GAPDH.

ELISA assay

DCs were washed twice and co-cultured with 2×10^4 freshly purified allogeneic naive CD4⁺ T cells (DC/T ratio, 1:5) in round-bottomed 96-well culture plates for 7 days. The levels of IL-4, IL-10, and IFN- γ in T cell media were measured with an ELISA kit after the co-culture of DCs and T cells. The frequency of autoreactive T cells producing IFN- γ or IL-4 was determined by the enzyme-linked immunospot (ELISPOT) technique.

AR mice model and CYT387 treatment

For induction of AR, 6- to 8-week-old BALB/c mice were sensitized intraperitoneally on day 0 with 0.5 ml of ovalbumin (OVA; 10 mg of OVA, grade V; Sigma, St Louis, Mo) emulsified in 2 mg of AL(OH)₃ (OVA/alum) or as a

control with PBS in alum, followed from day 14 onward by three-times weekly nasal challenges for 3 weeks with 10 µl of OVA (1 mg/ml) in each nostril. CYT387-treated mice including AR and normal mice were sensitized intraperitoneally with CYT387 (30 mg/kg). All experimental mice were killed 24 h after the last nasal application.

Histological examination

For histological examination of the nasal mucosa, the head was fixed in 10% buffered formalin solution 24 h after the last intranasal antigen challenge. Then the head was severed between the upper and lower jaws and the facial skin stripped. The tip of the nose area was decalcified in Plank-Rychlo solution and embedded in paraffin. Frontal sections of the nasal tissues were stained with hematoxylin and eosin.

Statistical analysis

All data are expressed as mean $\pm$ SD. The significance of differences between groups was analyzed by Student's *t* test using GraphPad Prism 5 (La Jolla, CA). Statistical comparisons of different groups were performed using ANOVA. Differences were considered statistically significant at $P < 0.05$.

Results

The expression of phosphorylated JAK/STAT in TSLP-dendritic cells

When cultured in the presence of GM-CSF and IL-4 for 6 days, monocytes differentiated into large, non-adherent veiled cells with a typical immature DC morphology (Fig. 1a). Using SEM, we identified morphological characteristics of mature dendritic cells such as stellate appearance and numerous very thin protrusions, among large macrophage-like cells with larger flat pseudopods and cells of an intermediate more immature phenotype (Fig. 1b). Moreover, flow cytometry assays detected positive expression of cell surface markers, including CD80, CD86, CD11c, and MHCII molecules (Fig. 1c).

To determine the physiological role of the JAK/STAT signaling pathway in TSLP-DCs, we performed in vitro experiments in which we blocked phosphorylation of JAK/STAT pathway protein in DCs with CYT387 (JAK2 inhibitor). Western blot showed that the protein levels of p-JAK1, p-JAK2, p-STAT1, p-STAT3, and p-STAT5 were markedly increased by TSLP in DCs (Fig. 1d). However, this up-regulation of protein expression was inhibited by CYT387. These results indicate that the JAK/STAT signaling pathway may be involved in the activation of TSLP-DCs.

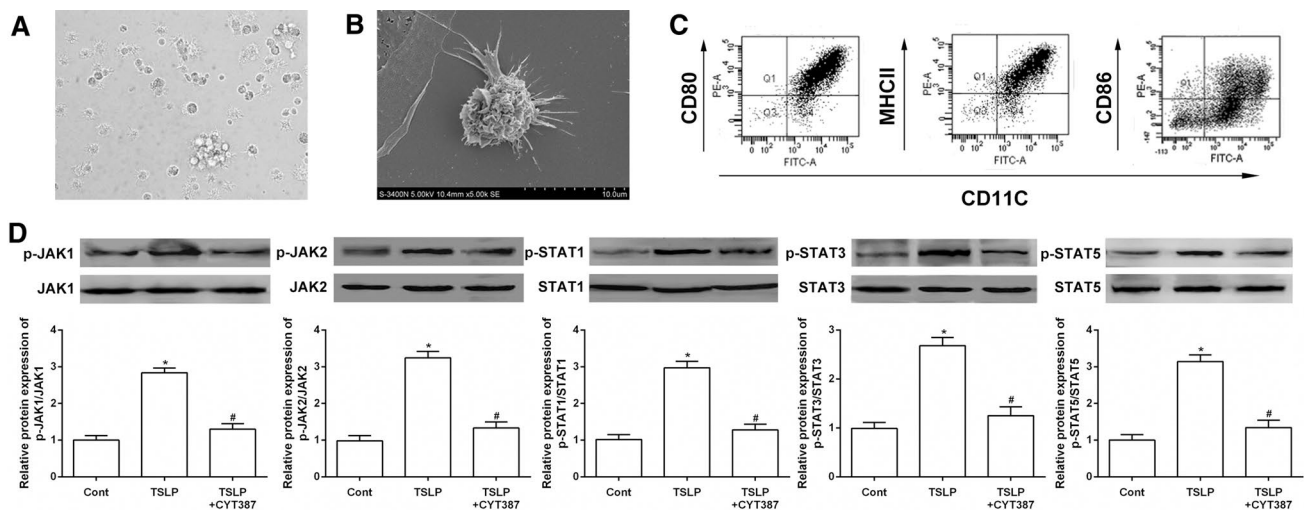


Fig. 1 Morphological identification of DCs and protein expression of JAK/STAT in TSLP-induced dendritic cells. **a** Morphological identification of DCs shown by microscope. **b** Morphological identification of DCs shown by scanning microscopy. **c** Expression of cell surface

markers MHCII, CD80, and CD86 was detected by flow cytometry analysis. **d** Protein expression of JAK/STAT was detected in TSLP-DCs by Western blot analysis. ($n=3/\text{group}$, $*P<0.05$ vs. control group, $\#P<0.05$ vs. TSLP group)

Blockading the JAK/STAT signaling pathway leads to a down modulation of OX40L in TSLP-dendritic cells

The direct effects of the JAK/STAT signaling pathway on the maturation of dendritic cells, which link the innate and adaptive immunity, have not been investigated. Thus, we then investigated whether the JAK/STAT signaling pathway can trigger the maturation of TSLP-DCs. DCs were incubated overnight with TSLP, CYT387, or PBS and the expression of MHC-II and the co-stimulatory molecules CD11c, CD80, and CD86 was assessed by flow-cytometry. TSLP had obviously up-regulated co-stimulatory molecules including CD11c, CD80, CD86, and MHCII in DCs compared with the control group (Fig. 2a). However, the CYT387 inhibited this effect of TSLP. In addition, the expression of OX40L was increased by TSLP in DCs; similarly, this effect of TSLP was reversed by the JAK/STAT pathway inhibitor CYT387 (Fig. 2b). Collectively, these findings suggest that blockading the JAK/STAT signaling pathway might influence the maturation of co-stimulatory molecules of DCs and lead to a down modulation of OX40L.

Blockading the JAK/STAT signaling pathway inhibits the inflammatory Th2 cell response

A previous study has demonstrated that TSLP-activated DCs favor the differentiation of naive CD4^+ T lymphocytes into Th2 cells [12]. Following these findings, the impact

of JAK/STAT inhibition on the ability of DCs to promote Th2-programming was explored. Therefore, TSLP-DCs were co-incubated with naive CD4^+ T cells in the presence of CYT387. Notably, CYT387 markedly reduced the capability of TSLP-DCs to promote the differentiation of naive CD4^+ T cells into IL-4-expressing Th2 cells. In contrast, the ability of TSLP-DCs to polarize naive CD4^+ T cells into IFN- γ -producing Th1 cells was not modulated by CYT387 (Fig. 3a). Moreover, an ELISA assay demonstrated that the production of IL-4, IL-5, and IFN- γ was markedly up-regulated in the supernatants of CD4^+ T cells that were co-cultured with DCs treated by TSLP. But the levels of these cytokines were down-regulated by CYT387 (Fig. 3b). These results provide further evidence that JAK/STAT inhibition impairs mature DC-mediated polarization of naive CD4^+ T cells into Th2 cells.

Blockading the JAK/STAT signaling pathway affects Th2 responses through modulating DC function in allergic rhinitis mice

To determine whether the JAK/STAT signaling pathway was involved in the inflammatory responses of the nasal turbinate mucosa from allergic rhinitis mice, histological examination of tissues from AR mice and normal controls was carried out (Fig. 4a). The results showed that obvious epithelial disruption, epithelial cell proliferation, mucosa edema, and infiltration of inflammatory cell including eosinophils were observed in the nasal mucosa of AR mice. Notably, the CYT387-treated group showed less epithelial

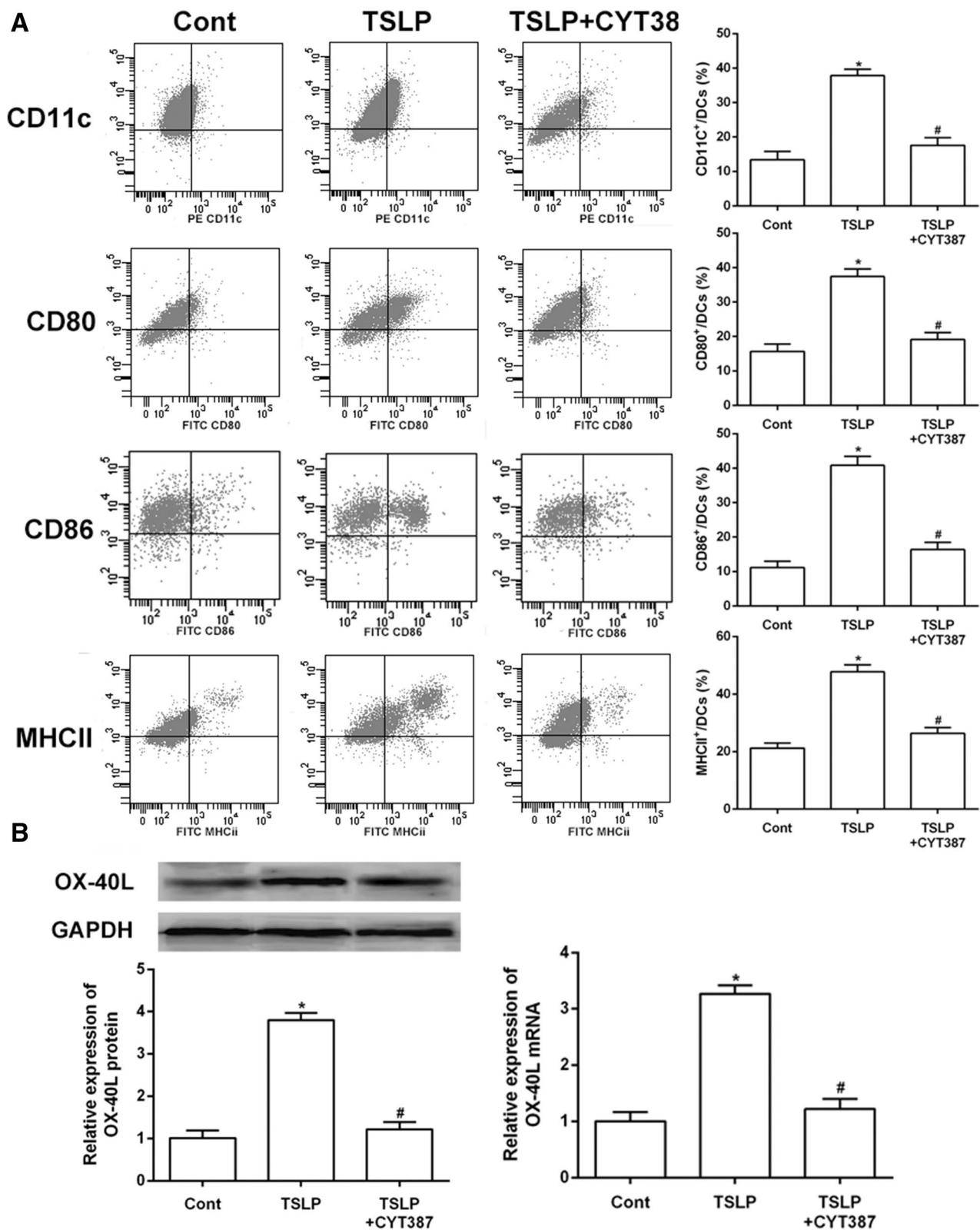


Fig. 2 JAK/STAT pathway inhibitor leads to a down modulation of OX40L in TSLP-induced dendritic cells. DCs were pretreated with 15 ng/ml TSLP and then stimulated with or without 100 nM CYT387·2HCl for 48 h. **a** Surface expression of CD80, CD86, MHC

class II, and CD11c was analyzed by flow cytometry. **b** Protein and mRNA expression of OX40L were indicated by Western blot and RT-PCR analysis, respectively. ($n=3$ /group, $*P<0.05$ vs. control group, $#P<0.05$ vs. TSLP group)

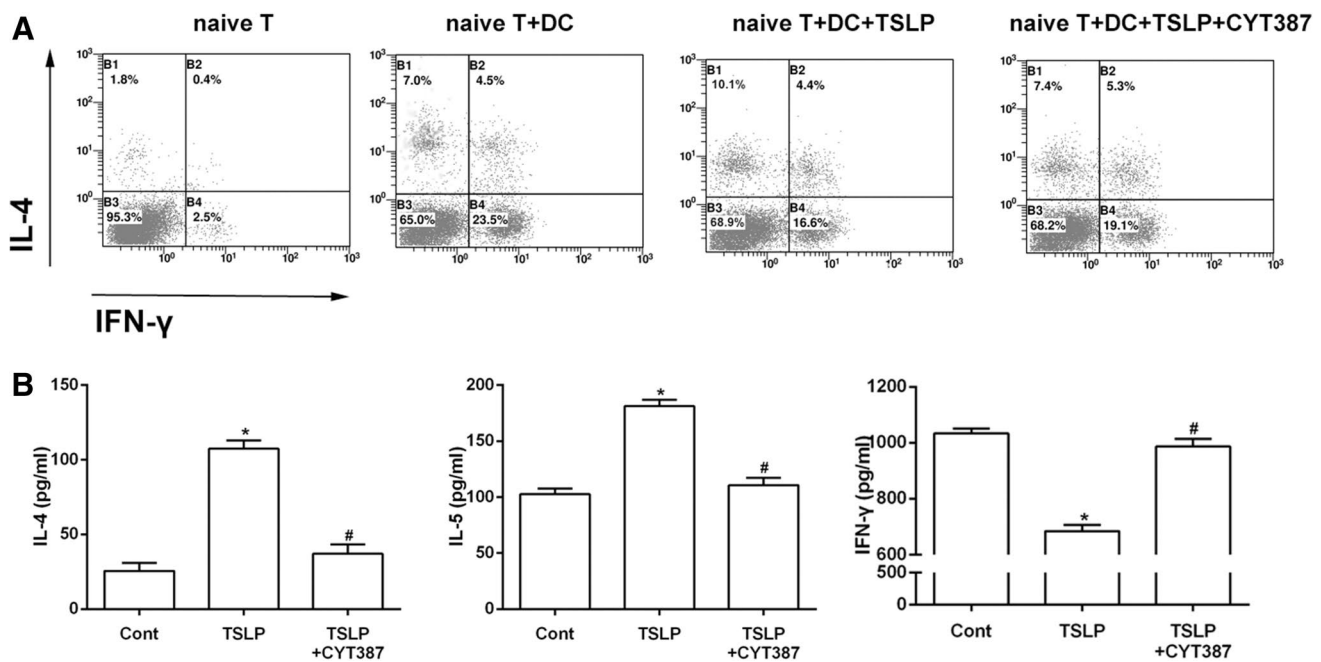


Fig. 3 JAK/STAT pathway inhibitor suppresses the inflammatory Th2 cell response. **a** DCs were maintained in the presence or absence of TSLP and CYT387 for 12 h, washed, and subsequently co-cultured with naive CD4⁺ T lymphocytes in the presence of LPS. After 8 days,

the percentage of IFN-γ and IL-4-producing CD4⁺ T cells was determined by flow cytometry. **b** The cytokine levels in supernatants were determined by ELISA. ($n=3/\text{group}$, * $P<0.05$ vs. control group, # $P<0.05$ vs. TSLP group)

disruption, epithelial cell proliferation and mucosa edema and reduced eosinophil infiltration compared with AR group. Moreover, significant elevation of eosinophil infiltration was found in AR group compared with normal control group (Fig. 4a), and the CYT387-treated AR group showed less eosinophil infiltration than AR group, indicating that CYT387 treatment reduced eosinophil infiltration in AR mice.

In addition, Western blot and RT-PCR demonstrated that the expression of OX40L was increased in AR mice, but that was decreased by CYT387 treatment (Fig. 4b). The OVA-induced AR mice exhibited enhanced IL-4 and IL-5 expression and decreased IFN-γ expression compared with normal control mice by ELISA assay (Fig. 4c). However, CYT387 treatment resulted in the reduction of IL-4 and IL-5 expression and increased IFN-γ level in AR groups compared with the untreated AR group.

To assess the effector Th phenotype, we analyzed the cytokine profile. We then determined the effect of CYT387 on the Th1/Th2 responses in AR mice by IFN-γ and IL-4 ELISPOT assays (Fig. 4d). AR results in the generation of effector T cells producing high levels of Th2-type cytokine (IL-4) and low levels of Th1-type cytokine (IFN-γ). In contrast, T cells from CYT387-treated AR mice produced a lower level of Th2 and higher level of Th1 cytokine.

Discussion

Allergic rhinitis (AR) is one of the most common allergic diseases in China, affecting 10.4% of the children and 11.4% of the adults [27]. AR is a respiratory disease of the upper airways characterized by high concentrations of serum allergen-specific IgE, infiltration of inflammatory cells in the nasal mucosa, and the release of several inflammatory cytokines. Thus, it is very important to explore the pathogenesis of allergic rhinitis. Previous studies have suggested that the pathogenesis of allergic diseases is mainly associated with the imbalance of Th1/Th2 cells [28]. In addition, the cytokine TSLP expression was augmented in the nasal mucosa of allergic rhinitis patients, and TSLP production correlated well with the severity of the disease and the number of eosinophils [7, 29]. DCs are specialized antigen-presenting cells that are strategically located in the skin and the mucosal system. The maturation status of DCs is believed to play a decisive role in the stimulation of immune T-cell responses [30]. DCs induce interferon (IFN)-γ-secreting Th1 cells with high cytotoxicity in response to viral and intracellular bacterial infection, whereas the same cells induce Th2 cells that secretes interleukin (IL)-4, IL-5, and IL-13, which are required for induction of IgE, hypersecretion of mucus, and recruitment of eosinophils in response to parasite infection [31].

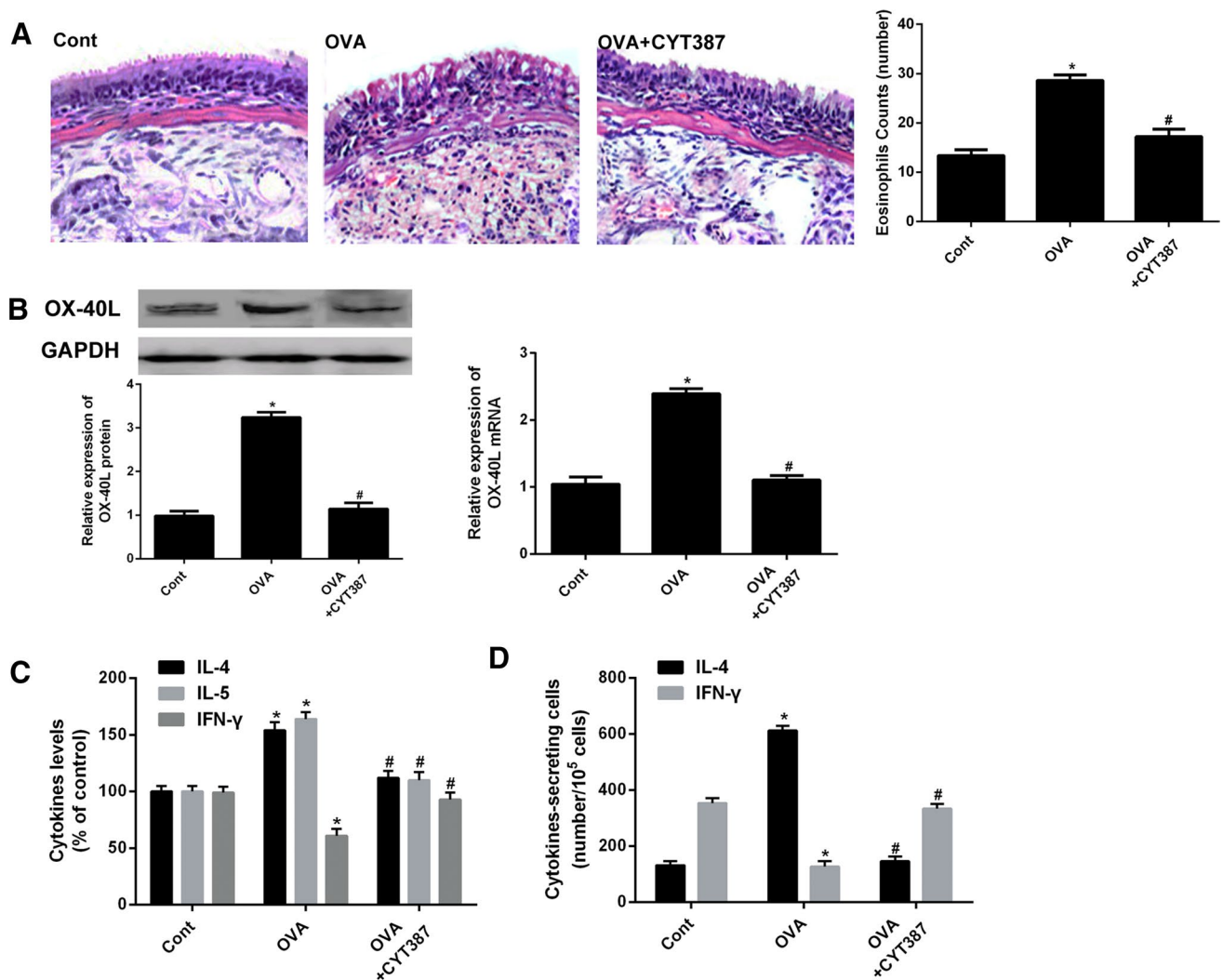


Fig. 4 JAK/STAT pathway inhibitor affects Th2 responses by modulating DC function in AR mice. **a** Histopathological characteristics in control, AR and CYT387-treated AR groups. **b** Protein and mRNA expression of OX40L in nasal mucosa of AR mice were detected by Western blot and RT-PCR, respectively. **c** The cytokine levels in nasal

mucosa of AR mice were determined by ELISA. **d** The number of T cells producing IFN- γ or IL-4 in nasal mucosa of AR mice was determined by ELISPOT. ($n=3$ /group, * $P<0.05$ vs. control group, # $P<0.05$ vs. TSLP group)

TSLP has been reported to be a key cytokine that initiates DC-mediated Th2 immune response in the allergic cascade [13], but the mechanism is unclear.

A previous study has reported that TSLP induced phosphorylation of JAK1 and JAK2 in myeloid DCs. Consequently, TSLP induced phosphorylation of STAT1, STAT3, STAT4, STAT5, and STAT6 [32]. Thus, we suspect that the JAK/STAT pathway is involved in the process of TSLP-activated DCs. TSLP-induced myeloid cell proliferation is preceded by phosphorylation of STAT-5 [33]. In our study, the results showed that phosphorylation of JAK1, JAK2, STAT1, STAT3, and STAT5 was markedly increased by TSLP in DCs, which indicates that JAK/STAT signaling pathway may involve in the activation of TSLP-DCs.

Previous reports showed that the change in antigen uptake can be used to monitor DC maturation, since DCs exhibit a gradual loss of antigen uptake capacity when undergoing development from immaturity to maturity [34]. Up-regulation of some critical cellular molecules is observed in mature DCs, including cytokines, and co-stimulatory and MHC class I and II molecules, which is essential for the activation of T cells [35]. DC maturation process is characterized by the production of cytokines and by increased expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules (CD11c, CD80, CD86) [36]. CD80 and CD86, members of the B7 family, are expressed by DCs on the cellular surface. Recently, a study reported that CD80 and

CD86 regulate mechanical interactions of T-cells with antigen-presenting DCs [37]. Blocking CD80/86 with specific antibodies reduces the intercellular interactions and inhibits T cell functional activation, thereby suggesting that CD80 and CD86 play crucial roles in the initiation of immune response [38]. Our results indicate that TSLP significantly increases the expression of MHC II and co-stimulatory molecules on the surfaces of DCs. However, this increase was inhibited by CYT387 (JAK/STAT pathway inhibitor), which indicated that the JAK/STAT pathway plays key roles in TSLP-DC maturation.

OX40L is associated with mature DC-driven Th2 polarization of naive CD4⁺ T cells by TSLP [14], which has been demonstrated to be the molecule downstream of TSLP-DCs that induces naive CD4⁺ T cells to differentiate into inflammatory Th2 cells [11]. Our study suggested that TSLP up-regulated OX40L expression in DCs and this up-regulation was restrained by CYT387.

TSLP-primed DC mainly promotes Th2 polarization. When TSLP-DC is used to stimulate naive allogeneic CD4⁺ T cells *in vitro*, they induce a unique type of Th2 cell that produces the classic Th2 cytokines IL-4, IL-5, and IL-13 and large amounts of TNF but little or no IL-10 [39]. As described above, IL-4 is a key cytokine produced by immune cells and has a crucial role in the development of Th2 responses. Similarly, IFN- γ is produced by Th1 cells [40]. A previous study has demonstrated that TSLP-stimulated DCs favor the differentiation of naive CD4⁺ T lymphocytes into Th2 cells [12]. In our study, the ability of TSLP-DCs to polarize naive CD4⁺ T cells into IL-4-producing Th2 cells was down-regulated by CYT387. Moreover, the increased levels of IL-4, IL-5, and down-regulated levels of IFN- γ were inhibited by CYT387. Thus, our results indicated that JAK/STAT inhibitor may impair mature DC-mediated polarization of naive CD4⁺ T cells into Th2 cells.

To evaluate whether these effects of CYT387 existed in an allergic situation *in vivo*, we utilized AR mice models. The CYT387-treated group showed less epithelial disruption, epithelial cell proliferation and mucosa edema, and reduced eosinophil infiltration compared with AR group. AR results in the generation of effector T cells producing high levels of Th2-type cytokine (IL-4) and low levels of Th1-type cytokine (IFN- γ). These results are consistent with previous studies that investigated the effects of JAK inhibition on TSLP-DCs [32]. In addition, CYT387 treatment decreased the secretion of IL-4, IL-5, and increased IFN- γ secretion in AR groups compared with the untreated AR group. In a word, we demonstrated that blockading the JAK/STAT signaling pathway suppresses Th2 responses by modulating DC function in the AR mice.

In summary, we explored the effects of the JAK/STAT pathway in allergic rhinitis by using CYT387 in TSLP-DCs

and OVA-induced AR mice. The results showed that CYT387 inhibited the maturation of DCs induced by TSLP. Furthermore, the JAK/STAT inhibitor was observed to decrease the expression of OX40L and alleviate the Th2 response in TSLP-DCs and OVA-induced AR mice. These characteristics make JAK/STAT an excellent target for clinical development with a view to treating allergic rhinitis.

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Compliance with ethical standards

Conflict of interest None.

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