

RESEARCH ARTICLE

Role of IL-9 and IL-10 in the pathogenesis of chronic spontaneous urticaria through the JAK/STAT signalling pathway

Hua Feng^{1,2} | Jiangao Feng² | Zhongwei Zhang² | Qunying Xu² | Min Hu² | Yongning Wu^{1,3,4}  | Yuanan Lu⁵

¹State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, China

²School of Public Health, Nanchang University, Nanchang, China

³NHC Key Laboratory of Food Safety Risk Assessment, Beijing, China

⁴China National Center for Food Safety Risk Assessment, Beijing, China

⁵Environmental Health Laboratory, Department of Public Health Sciences, University of Hawaii, Honolulu, HI, USA

Correspondence

Yongning Wu, State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330000, China.
Email: wuyongning@cfsa.net.cn

Yuanan Lu, Environmental Health Laboratory, Department of Public Health Sciences, University of Hawaii, Honolulu, HI 96822, USA.
Email: yuanan@hawaii.edu

This study investigated the role of interleukin (IL)-9 and IL-10 in the pathogenesis of chronic spontaneous urticaria (CSU). Autologous serum skin test and histamine release test were performed in CSU patients and normal subjects. Kunming mice were used to develop a mouse model for CSU. We induced IL-9 overexpression, IL-10 overexpression, and JAK/STAT pathway inhibition as well as a combination of all three conditions in CSU and control mice. Eosinophils in the skin tissues, inflammatory cytokine expression, and distribution of T lymphocyte subsets in peripheral blood of mice were detected. Expression patterns of IL-9, IL-10, STAT3, JAK2, and INF- γ in clinical samples and mice were detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and western blot analysis. The positive rate of autologous serum skin test and the histamine release rate of CSU patients, compared with normal subjects, were apparently elevated. Compared with controls, mice with CSU experienced longer duration and higher frequency of pruritus and demonstrated enhanced levels of CD8⁺, the ratio of CD4⁺/CD8⁺, number of eosinophils, and inflammatory cytokine expression in serum as well as activated JAK/STAT signalling pathway; at the same time, levels of CD4⁺ and INF- γ were reduced. This trend was found in CSU mice overexpressing IL-9 and IL-10 when compared with the CSU mice without treatment. In contrast, JAK/STAT inhibition reversed the above trend. Overall, our study suggests that IL-9 and IL-10 contribute to CSU development via activation of the JAK/STAT signalling pathway.

KEYWORDS

chronic spontaneous urticaria, inflammation, interleukin-9, interleukin-10, JAK/STAT signalling pathway

1 | INTRODUCTION

Chronic urticaria (CU), defined as urticaria that has been continuously or intermittently existing for more than 6 weeks, has an estimated prevalence in the general population of 0.5% to 5% and an incidence of 1.4% per year.¹ Patients where no underlying cause for CU was identified were diagnosed as chronic idiopathic urticaria (mainly used

in North America) or chronic spontaneous urticaria (CSU).² CSU is caused by degranulation of basophils and/or cutaneous mast cells, which results in effective vasoactive mediators released to induce vasodilatation and increased capillary permeability, leading to erythema and wheal formation.³ Wheal is an elementary dermatological lesion, characterized by itchiness and the presences of central oedema in various sizes, with a reflex erythema encircling and ephemeral

nature; the skin usually returns to its normal aspect in 1 to 24 hours.⁴ CSU is classified according to the duration of evolution, into acute (less than 6 wk) or chronic (over 6 wk).⁵

Activation of basophils or mast cells leading to histamine release is particularly specific to CSU and defines this autoimmune subgroup.⁶ Mast cells typically act as effectors in IgE-mediated allergic or anti-parasitic responses and carry out their role in inflammation and innate immunity by releasing a large amount of inflammatory mediators.^{7,8} These mediators include compounds such as histamine and mast cell-specific proteases, which are prestored in cytoplasmic secretory granules and newly synthesized lipid mediators including leukotrienes, prostaglandins, and a variety of cytokines, chemokines, and growth factors.⁹ CSU displays both autoimmune and allergic disease characteristics, which are most commonly caused by an imbalance between cytokines and T lymphocyte subgroups.¹⁰

IL-10 has a pivotal role in the development and maintenance of immune tolerance and homeostasis.¹¹ IL-10 is released by numerous cell types, including T helper cells, monocytes, macrophages, dendritic cells, B cells, cytotoxic T cells, NK cells, mast cells, and granulocytes.¹² An IL-10 deficiency is associated with the development of a number of autoimmune diseases.^{13,14} IL-9 is one member of the four-helix bundle cytokine family.¹⁵ Initially identified as a T cell and mast cell growth factor, IL-9 has also been seen for a long time as a crucial mediator of allergic inflammation.^{16,17} Cytokines such as IL-6 exert effects through the transmembrane receptor gp130 to activate the JAK/STAT signalling pathway.¹⁸ The JAK/STAT signalling pathway is crucial in the process of inflammation after activation by upstream inflammatory factors including growth factors and interleukins.¹⁹ The correlation between interleukins, the JAK/STAT signalling pathway, and CSU is rarely studied. In this study, we investigated the role of IL-9/IL-10 in CSU through the JAK/STAT signalling pathway with the hope of finding out novel therapeutic targets for CSU.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

All patients were informed of the sample collection and signed the informed consent. This study was approved by the ethics committee of Nanchang University.

2.2 | Study subjects

During January 2013 and October 2016, a total of 148 patients (78 males and 70 females) diagnosed by the First Affiliated Hospital of Nanchang University with recurrent CSU and no obvious cause of disease were selected for this study. The age ranged from 10 to 75 years old, with an average age of 42.56 ± 18.57 years, and the course of disease ranged from 4 months to 6 years with an average course of disease 28.55 ± 12.97 months. Patients meeting the following

requirements were included: Patients who display of wheals in various sizes along with the occurrence of pruritus or angioedema with attacks of wheals of no less than two times a week for at least 6 weeks (a small number of CSU patients presented intermittent attacks); patients with CSU in active phase; and patients with no disease of vital organs such as heart, lung, kidney, or brain or disease that would interfere with the study results. Any patients with the following conditions were excluded: patients with chronic inducible urticarias; patients younger than 10 years or older than 75 years; patients with serious disease of heart, lung, kidney, or brain; patients who are pregnant or lactating; and patients in the process of treatment.²

A total of 148 normal subjects (76 males and 72 females) were selected as the normal control group, who were confirmed healthy at the First Affiliated Hospital of Nanchang University. The age ranged from 20 to 78 years old, with an average age of 43.85 ± 20.21 years old. All subjects were without systemic diseases, anaphylactic diseases, endocrine diseases, or diseases on vital organs such as heart, lung, kidney, or brain or short-term local or systemic infectious diseases.

2.3 | Establishment of CSU mouse model

In total, 80 Kunming mice (J018, Nanjing Better Biotechnology Co, Ltd, Nanjing, Jiangsu, China) weighing 18 to 22 g were included in this study. From these mice, 72 were taken to establish a CSU mouse model, while the remaining mice served as normal controls. Mice for model establishment (the CSU group) were subcutaneously injected with 0.1 mL of 10 mg/mL histamine in the abdomen, while control group mice were injected with 1% Evans blue saline at 4 mL/kg in the caudal vein. The mice were then kept under observation for latency of scratching, number of times of scratching (pruritus signs: scratching head with the forepaw, scratching body with the hind paw, and biting all parts of the body with the mouth), and duration of pruritus. The CSU mouse model was successfully established if the number of times of scratching and the duration of scratching were increased compared with those in the normal control group.²⁰ Mice where CSU was successfully induced were divided into a blank group without injection, negative control (NC) group injected with NC plasmid, IL-9 overexpression group injected with IL-9 overexpression plasmid, IL-10 overexpression group injected with IL-10 overexpression plasmid, AG490 group injected with 20 μ mol/L of JAK/STAT signalling pathway inhibitor AG490, IL-9 overexpression + IL-10 overexpression + AG490 group injected with IL-9 overexpression, IL-10 overexpression, and 20 μ mol/L of JAK/STAT pathway inhibitor AG490. All plasmids were purchased from Vigene Bioscience Company (Shandong Vigene Bioscience Co, Ltd, Jinan, Shandong, China), and the JAK/STAT pathway inhibitor AG490 was purchased from Thermo Fisher (Thermo Fisher Scientific [China] Co, Ltd, Shanghai, China). After injection, the number of scratching times and duration of pruritus of mice in each group were observed. After successful injection, mice were euthanized for collection of blood and skin with wheals or roseolas, which were preserved at -80°C .

2.4 | Autologous serum skin test

A total of 5 mL of peripheral venous blood collected from the patients was transferred into sterile plain tubes and allowed to stand for 30 minutes at room temperature. The blood was then centrifuged for 15 minutes at 3000 r/min, and the serum was separated under sterile conditions. A total of 50 μ L of serum was intracutaneously injected into the flexor surface of the forearm. Then intracutaneous injection of the same amount of saline was performed in the normal group. The two sites of injection were kept at least 5 cm apart. After 30 minutes, if a wheal reaction was observed on the injection site and the wheal was larger than 2 mm in parameter, the test was considered positive. The remaining serum was cryopreserved for further use.

2.5 | Enzyme-linked immunosorbent assay

A total of 75 μ L of serum to be tested or control serum were respectively mixed with 75 μ L of cell suspension of mast cells. The mixture was then incubated for 20 minutes at 37°C and centrifuged for 15 minutes at 3000 r/min. The supernatant was preserved to detect the histamine release rate of the two groups (the above tests all used double tubes) using enzyme-linked immunosorbent assay (ELISA).

Samples were acylated and diluted by a commercial kit (ZK-G7274, Shenzhen Ziker Biological Technology Co, Ltd, Shenzhen, Guangdong, China) in accordance with the manufacturer's instructions; the volume tested was 50 μ L. A total of 50 μ L of monoclonal antibody containing antihistamine drugs was added into each well of the incubator plate and further incubated for 3 hours at room temperature with the mixture of 50 μ L of enzyme conjugate and 50 μ L of histamine antiserum. Then, 200 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) acylated solution was added and incubated for 20 minutes. Finally, 100 μ L of TMB solution was used to terminate the reaction, and the optical density (OD) value was detected on a Microplate Reader at 450 nm. The histamine contents in these tubes were measured by using the standard curve ($X = OD \times 50/m$). The final results were presented as the histamine release rate. Spontaneous histamine release rate = spontaneous release of histamine/total histamine content $\times 100\%$; histamine release rate of specimens = histamine content in specimens/total histamine content $\times 100\%$. The levels of IL-2 (hj-C14129, Hong Ju Biotechnology Co, Ltd, Shanghai, China), IL-4 (hj-C14083, Hong Ju Biotechnology Co, Ltd, Shanghai, China), and IFN- γ (hj-C14119, Hong Ju Biotechnology Co, Ltd, Shanghai, China) in serum of mice were detected using ELISA.

2.6 | Haematoxylin-eosin staining

The mouse skin tissues were fixed for 24 hours in 4% paraformaldehyde and then dehydrated in 80%, 90%, and 100% graded ethanol and *N*-butanol, wax embedded at 60°C, and sliced into 5- μ m pieces. After being incubated for 1 hour at 60°C, tissues were dewaxed with xylene. After hydration, the prepared slices were stained with

haematoxylin (Solarbio Life Sciences Co, Ltd, Beijing, China) for 2 minutes, differentiated with 1% hydrochloric acid-ethanol for 10 seconds, and then stained by eosin solution for 1 minute. After dehydration in graded ethanol and clearing with xylene, the tissues were mounted using neutral balsam. Then, the slices were placed under a light microscope ($\times 400$; Shanghai Bingyu Optics Co, Ltd, Shanghai, China) to observe eosinophils. Eosinophils displayed an oval shape with a comparatively large size and the cytoplasm in pink and nucleus in dark blue. Five fields of view were randomly selected in each slice to take a photo for counting, and the average number was taken.

2.7 | Flow cytometry

A total of 100 μ L of mice peripheral blood was incubated with 10 μ L of antibody for 20 minutes at room temperature without light exposure. Then, red blood cells were lysed using red blood cell lysate for 10 minutes. Next, cells were centrifuged for 5 minutes at 1500 r/min, washed with 2 mL of phosphate-buffered saline (PBS), and resuspended. The flow cytometer (Attune NxT, Thermo Fisher, MA, USA) was used to detect the proportion of CD3⁺, CD4⁺, CD8⁺, and CD4⁺/CD8⁺ in peripheral blood of mice.

2.8 | Reverse transcription quantitative polymerase chain reaction

An Ultrapure RNA Extraction Kit (D203-01, Beijing GenStar Biotechnology Co, Ltd, Beijing, China) was used to extract total RNA. IL-9, IL-10, STAT3, JAK, INF- γ , and GAPDH primers were designed by Takara (Takara Biomedical Technology [Beijing] Co, Ltd, Beijing, China) (Table 1). The reverse transcription was performed in accordance with the instruction for Taq Man Micro RNA Assays Reverse Transcription Primer (4 366 596, Thermo Scientific, Waltham, MA, USA). The RT-qPCR was performed with the SYBR Premix ExTaq TMII kit (RR820A, Guangzhou Xingzhi Biotechnology Company, Guangzhou, Guangdong, China). RT-qPCR was completed in an ABI PRISM 7300 system (Prism 7300, Shanghai Kunke Instrument & Equipment Co, Ltd, Shanghai, China). GAPDH (abs830032, Shanghai Absin Bioscience Inc, Shanghai, China) was the internal reference for IL-9, IL-10, STAT3, JAK, and INF- γ . The ratio of target gene expression between the CSU group and the normal group was represented by $2^{-\Delta\Delta Ct}$ in the formula: $\Delta\Delta Ct = \Delta Ct_{CSU \text{ group}} - \Delta Ct_{normal \text{ group}}$ in which $\Delta Ct = Ct_{mRNA} - Ct_{GAPDH}$.

2.9 | Western blot analysis

The skin tissue collected from mice was lysed with 1 mL of tissue lysis buffer (C1051, Guangzhou Whiga Biotechnology Co, Ltd, Guangzhou, Guangdong, China) for 30 minutes at 4°C and then centrifuged at 12 000 r/min for 20 minutes at 4°C. The supernatant was kept to detect the protein concentration of every specimen with the

TABLE 1 RT-qPCR primer sequence

Gene	Sequence
IL-9	F: 5'-CCAGCTTCCAAGTGCCACTGC-3' R: 5'-TGCATGGTGGTATTGGTCATCTG-3'
IL-10	F: 5'-ATGCTGCCTGCTCTTACTGACTG-3' R: 5'-CAGGACTACAAGGACGACGATGAC-3'
STAT3	F: 5'-TTGCCAGTTGTGGTGATC-3' R: 5'-AGAC CCAGAAGGAGCCGC-3'
JAK2	F: 5'-GCAAAGGTAACCTCGGGAGTGT-3' R: 5'-AGTCTCGGAGGTGCTCTTCAG-3'
INF- γ	F: 5'-TGAACGCTACACACTGCATCTTGG-3' R: 5'-CGACTCCTTTCCGCTTCCTGAG-3'
GAPDH	F: 5'-CATGGCCTTCCGTGTTCTTA-3' R: 5'-CCTGCTTACCACCTTCTTGAT-3'

Abbreviations: F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-10, interleukin-10; IL-9, interleukin-9; INF- γ , interferon- γ ; JAK 2, Janus kinase 2; R, reverse; RT-qPCR, reverse transcription quantitative polymerase chain reaction; STAT3, signal transducers and activators of transcription 3.

bicinchoninic acid assay kit (20201ES76, Shanghai Yeasen Biotechnology Co, Ltd, Shanghai, China). After electrophoretic separation, proteins were transferred onto a nitrocellulose membrane and blocked in 5% skimmed milk powder at 4°C overnight. Diluted rabbit antimouse polyclonal antibodies to IL-9 (500-P59, 1:500), IL-10 (500-P60, 1:1000), STAT3 (ab5073, 1:1000), phosphorylated (p)-STAT3 (ab30647, 1:500), p-JAK2 (ab32101, 1:1000), JAK2 (ab39636, 1:500), and INF- γ (ab102049, 1:400) were added to the nitrocellulose membrane for overnight incubation. All primary antibodies were purchased from Abcam (Cambridge, UK) except IL-9 and IL-10 (PeproTech Inc, Rocky Hill, NJ, USA). The membrane was then incubated with secondary horseradish peroxidase-labelled goat antirabbit antibody to IgG (1:1000, Wuhan Boster Biological Technology Co, Ltd, Wuhan, Hubei, China) for 1 hour at 37°C. Then, the membrane was dipped into electrochemiluminescence reaction liquid (Pierce, Waltham, MA, USA) for 1 minute at room temperature. After exposure in a darkroom, development, and fixing, the membrane was observed. GAPDH was used as the internal reference, and the ratio of grey value of the target band to the reference band was considered to be the relative expression of proteins.

2.10 | Statistical analysis

All data were processed using the SPSS 21.0 statistical software (IBM Corp, Armonk, NY, USA). The measurement data were expressed as mean $\pm$ SD. The independent sample *t* test was used for comparisons between two groups. One-way analysis of variance was used for comparisons between multigroups. Enumeration data were expressed as percentage and analysed with chi-squared test. *P* < .05 indicated that the difference was of statistical significance.

TABLE 2 Autologous serum skin test

Item	Normal Group	CSU Group	<i>P</i>
Positive rate (%)	0	45.93	<.001
Diameter of wheal (mm)	0	1.98 $\pm$ 0.21	<.001
Duration of wheal (h)	0	1.47 $\pm$ 0.51	<.001
Number of wheal	0	3.05 $\pm$ 0.31	<.001

Abbreviation: CU, chronic urticaria.

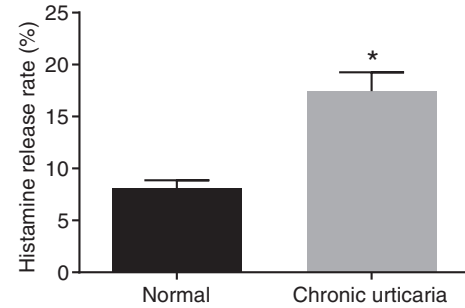


FIGURE 1 Histamine release rate of normal subjects and patients with CSU. * represents *P* < .05 when compared with the normal group

3 | RESULTS

3.1 | The results of the autologous serum skin test

According to the autologous serum skin test, among the 148 CSU patients, 68 cases (45.95%) were confirmed to be positive. The number of wheals was 3.05 $\pm$ 0.31 with diameter of 1.98 $\pm$ 0.21 mm and duration of 1.47 $\pm$ 0.51 hour. All 148 subjects in the normal group were confirmed as negative (Table 2).

3.2 | Histamine release rate is elevated in the patients with CSU

An ELISA test was used to measure histamine release from human mast cells (Figure 1). The histamine release rate in the normal group was negative. However, 91 positive cases of histamine release induced by human skin mast cells activated by serum and 57 negative cases were observed among CSU patients. The histamine release rate from the CSU group was significantly higher than the normal group (*P* < .05).

3.3 | Expression of IL-9, IL-10, STAT3, and JAK is increased while the expression of INF- γ is decreased in the skin tissues of the CSU group

We performed RT-qPCR in order to compare the mRNA expression of IL-9, IL-10, STAT3, and JAK2 in skin tissues of the CSU group with

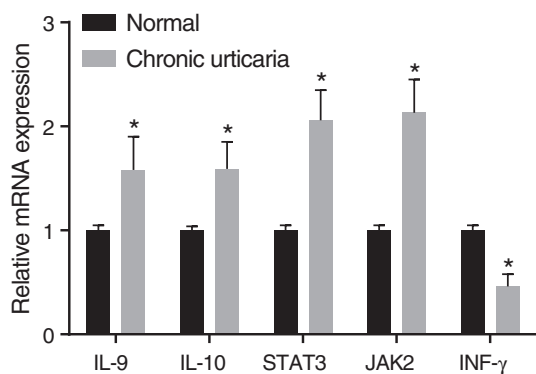


FIGURE 2 The mRNA expression of related genes in skin tissues of patients with CSU and normal subjects. * represents $P < .05$ compared with the normal group. IL-10, interleukin-10; IL-9, interleukin-9; INF-γ, interferon-γ; JAK, Janus kinase; STAT3, signal transducers and activators of transcription 3

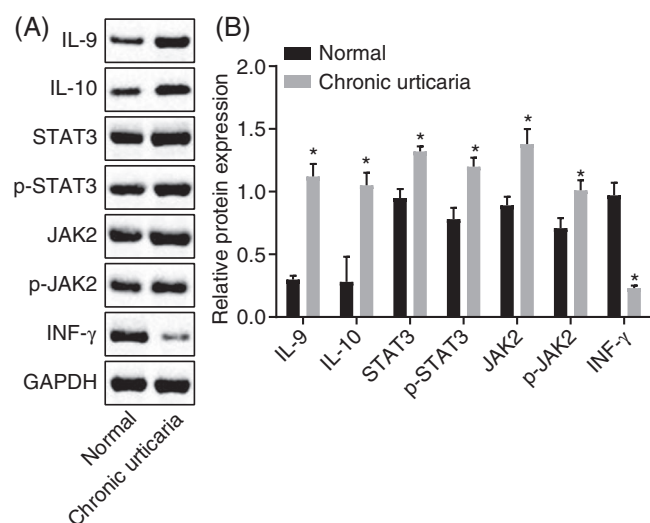


FIGURE 3 The protein expression of IL-9, IL-10, STAT3, JAK2, and INF-γ as well as the extent of STAT3 and JAK2 phosphorylation in the skin tissues of patients with CSU and normal subjects. A, Protein expression of IL-9, IL-10, STAT3, JAK2, and INF-γ as well as the extents of STAT3 and JAK2 phosphorylation, normalized to GAPDH. B, The statistical analysis of panel A; * represents $P < .05$ when compared with the normal group. IL-10, interleukin-10; IL-9, interleukin-9; INF-γ, interferon-γ; JAK2, Janus kinase 2; p-JAK2, phosphorylated-Janus kinase 2; p-STAT3, phosphorylated-signal transducers and activators of transcription 3; STAT3, signal transducers and activators of transcription 3

normal controls. Our results indicate that the mRNA expression of these target genes was significantly increased; in contrast, the expression of INF-γ was significantly decreased (all $P < .05$; Figure 2). Western blot analysis shows that, compared with the normal group, the protein expression of IL-9, IL-10, STAT3, and JAK2 in skin tissues of CSU patients was significantly increased while that of INF-γ was decreased. In addition, the extent of STAT3 and JAK2

TABLE 3 Frequency and duration of pruritus in the CSU and normal group

Item	Normal Group	CSU Group	P
Frequency of pruritus (times)	0	68.24 ± 6.87	<.001
Duration of pruritus (min)	0	25.66 ± 4.59	<.001

Abbreviation: CU, chronic urticaria.

phosphorylation was also increased in CSU patients over controls (all $P < .05$; Figure 3A,B). Taken together, these data suggest that overexpression of IL-9, IL-10, STAT3, and JAK could promote CSU progression.

3.4 | CSU mice show more signs of pruritus

The results from the established CSU mice exhibited that in the CSU group, the frequency of pruritus was 68.24 ± 6.87 times, and the duration of pruritus was 25.66 ± 4.59 minutes, while the normal group showed no sign of pruritus ($P < .05$). The mice used for establishing models were all qualified with a success rate of 100% (Table 3).

3.5 | Overexpression of IL-9 and IL-10 increases the frequency and duration of pruritus

After injection with our panel of plasmids, mice were observed for the frequency and duration of pruritus (Table 4A,B). The pruritus frequency and duration of mice in all other groups, compared with the normal group, were increased ($P < .05$). The pruritus frequency and duration in the NC group and IL-9 overexpression + IL-10 overexpression + AG490 group, compared with the blank group, exhibited no major change ($P > .05$), while those of IL-9 overexpression group and IL-10 overexpression group were all elevated ($P < .05$). These results indicate that overexpression of IL-9 and IL-10 could promote the frequency and duration of pruritus.

3.6 | Overexpression of IL-9 and IL-10 leads to an increase in the number of eosinophils

We performed HE staining on skin lesions from mice treated with our overexpression panel (Figure 4). Compared with the normal group, the number of eosinophils in skin tissues of mice in all other groups was significantly increased (all $P < .05$). IL-9 or IL-10 overexpression alone significantly increased the number of eosinophils compared with the control group ($P < .05$). In contrast, AG490 treatment significantly decreased eosinophil numbers ($P < .05$). Interestingly, compared with the blank and NC control groups, the combination of IL-9 overexpression + IL-10 overexpression + AG490

showed no significant change. Overall, these results indicate that overexpression of IL-9 or IL-10 cytokines elevates the number of eosinophils in tissues.

TABLE 4 Frequency and duration of pruritus in each group

Group	Pruritus Frequency, times	Pruritus Duration, min
Normal	0	0
Blank	64.25 ± 6.37 ^a	23.12 ± 3.18 ^a
NC	65.33 ± 6.88 ^a	21.84 ± 3.56 ^a
IL-9 overexpression	86.75 ± 8.84 ^{a,b}	31.84 ± 3.56 ^{a,b}
IL-10 overexpression	87.17 ± 8.89 ^{a,b}	29.12 ± 3.18 ^{a,b}
AG490	41.17 ± 4.09 ^{a,b}	12.16 ± 1.22 ^{a,b}
IL-9 overexpression + IL-10 overexpression + AG490	44.92 ± 6.73 ^a	13.89 ± 2.61 ^a

Abbreviations: IL, interleukin; NC, negative control.

^a*P* < .05 compared with the normal group.

^b*P* < .05 compared with the blank group.

3.7 | Overexpression of IL-9 and IL-10 promotes inflammatory cytokine expression

The serum of mice in the remaining experimental groups, compared with the normal group, showed significantly elevated expression of IL-2, IL-4, and IFN- γ in ELISA assay (*P* < .05) (Figure 5). The expression of IL-2, IL-4, and IFN- γ in the serum of mice in the NC and IL-9 overexpression + IL-10 overexpression + AG490 groups, compared with the blank group, exhibited no apparent difference (*P* > .05), while that in the IL-9 overexpression group and IL-10 overexpression group was increased and reduced in the AG490 group (*P* < .05). High levels of IL-9 and IL-10 appear to enhance the inflammatory cytokine environment.

3.8 | IL-9 and IL-10 increase the proportion of CD8⁺ and CD4⁺/CD8⁺ but decreased the proportion of CD4⁺

T cell subsets were detected by flow cytometry (Figure 6). The number of CD3⁺ in skin tissues of mice in each group exhibited no remarkable differences (*P* > .05). The proportion of CD4⁺ in the serum of

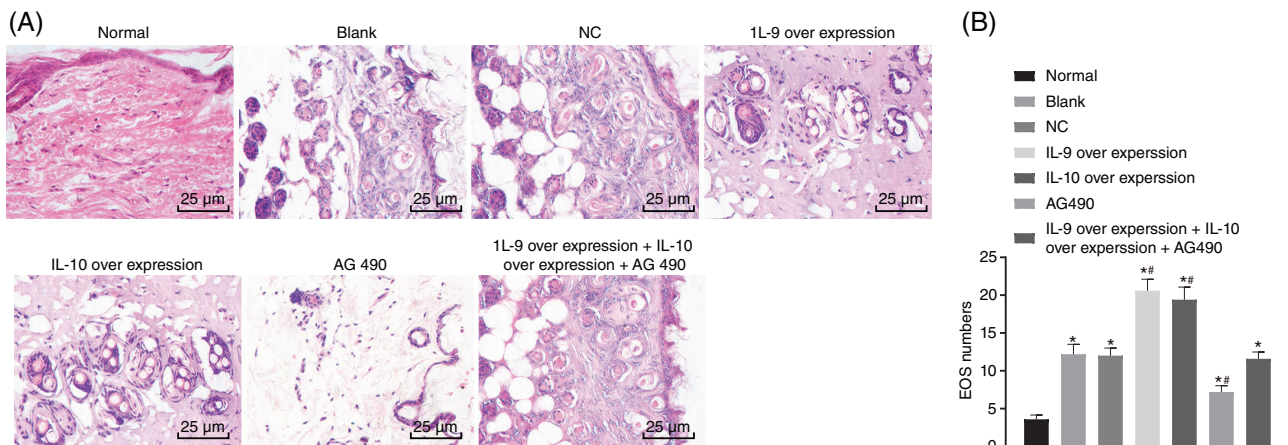


FIGURE 4 IL-9 or IL-10 overexpression aggravates CSU in mice. The CSU mice were injected with IL-9, IL-10, AG490, or all three combined. A, HE staining of skin tissues of mice. B, The number of eosinophils in each group. * represents *P* < .05 in comparison with the normal group; # represents *P* < .05 in comparison with the blank group. IL, interleukin; NC, negative control

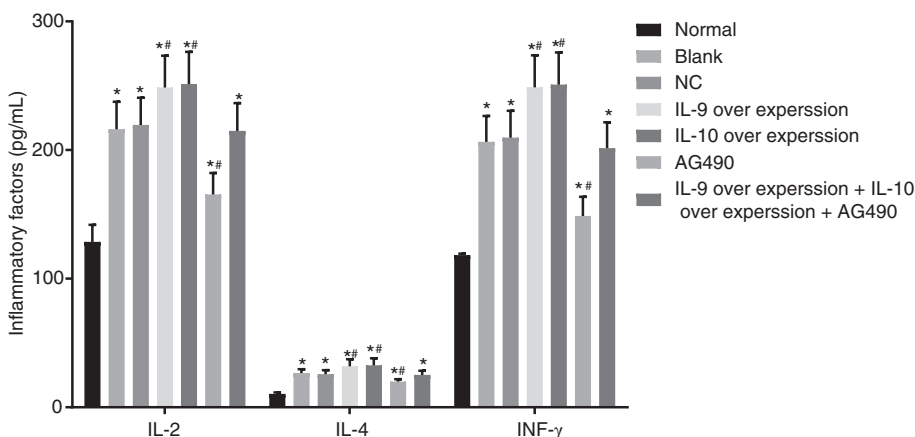


FIGURE 5 IL-9 or IL-10 overexpression contributes to elevated IL-2, IL-4, and IFN- γ expression in the serum of mice in each group. The CSU mice were injected with IL-9, IL-10, AG490 respectively, or all three combined. * represents *P* < .05 compared with the normal group; # represents *P* < .05 compared with the blank group. IL, interleukin; NC, negative control

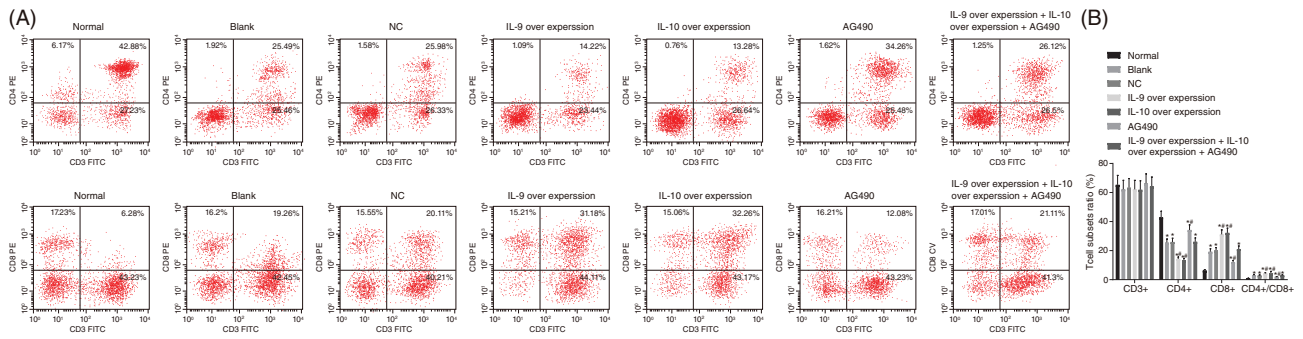


FIGURE 6 IL-9 or IL-10 overexpression leads to enhanced numbers of CD8⁺ T cells and the ratio of CD4⁺/CD8⁺. The CSU mice were injected with IL-9, IL-10, and AG490 all alone or combined. A, Percent distribution detected by flow cytometry. B, The statistical analysis of panel A. * represents $P < .05$ compared with the normal group; # represents $P < .05$ compared with the blank group. IL-10, interleukin-10; IL-9, interleukin-9; INF- γ , interferon- γ ; JAK2, Janus kinase 2; STAT3, signal transducers and activators of transcription 3

TABLE 5 Comparison of the T cell subsets in serum of mice in each group

Group	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD4 ⁺ /CD8 ⁺
Normal	65.28 $\pm$ 6.54	42.88 $\pm$ 4.31	6.28 $\pm$ 0.63	1.03 $\pm$ 0.10
Blank	62.19 $\pm$ 6.22	25.49 $\pm$ 2.55 ^a	19.26 $\pm$ 2.01 ^a	3.07 $\pm$ 0.44 ^a
NC	63.29 $\pm$ 6.35	25.98 $\pm$ 2.61 ^a	20.11 $\pm$ 2.01 ^a	2.93 $\pm$ 0.45 ^a
IL-9 overexpression	62.18 $\pm$ 6.24	14.22 $\pm$ 1.44 ^{a,b}	31.18 $\pm$ 3.12 ^{a,b}	4.36 $\pm$ 0.44 ^{a,b}
IL-10 overexpression	61.88 $\pm$ 6.21	13.28 $\pm$ 1.36 ^{a,b}	32.26 $\pm$ 3.22 ^{a,b}	4.32 $\pm$ 0.45 ^{a,b}
AG490	66.27 $\pm$ 6.61	34.26 $\pm$ 3.43 ^{a,b}	12.08 $\pm$ 1.21 ^{a,b}	2.00 $\pm$ 0.09 ^{a,b}
IL-9 overexpression + IL-10 overexpression + AG490	64.26 $\pm$ 6.44	26.12 $\pm$ 2.62 ^a	21.11 $\pm$ 2.11 ^a	3.00 $\pm$ 0.42 ^a

Abbreviations: IL, interleukin; NC, negative control.

^a $P < .05$ compared with the normal group.

^b $P < .05$ compared with the blank group.

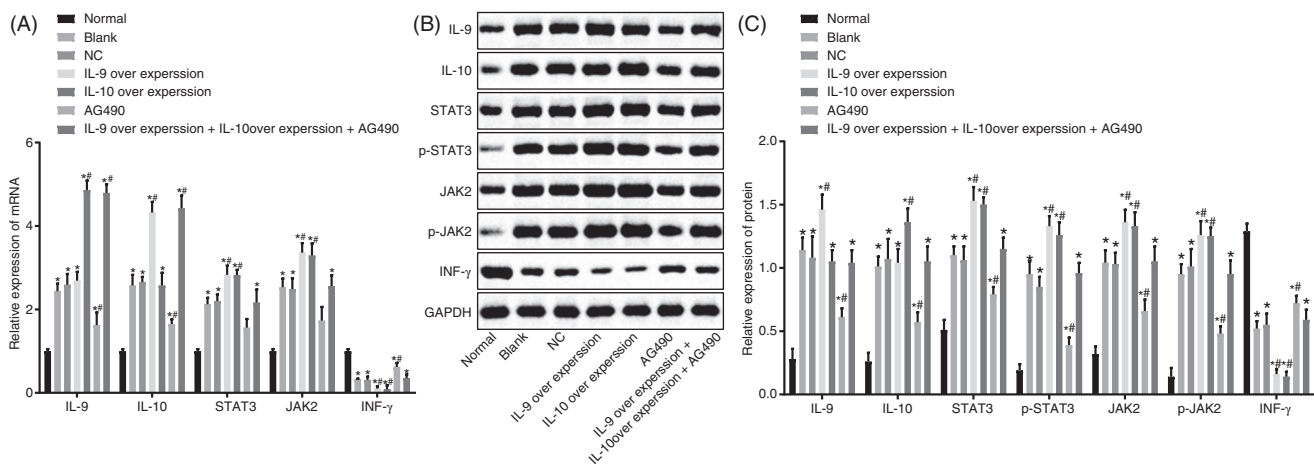


FIGURE 7 IL-9 or IL-10 overexpression induces the activation of the JAK/STAT signalling pathway. The CSU mice were injected with IL-9, IL-10, and AG490 all alone or combined. A, Percent distribution detected by flow cytometry. B, The mRNA expression of IL-9, IL-10, STAT3, JAK2, and INF- γ determined by RT-qPCR. C, The protein expression of IL-9, IL-10, STAT3, JAK2, and INF- γ as well as the extents of STAT3 and JAK2 phosphorylation normalized to GAPDH measured by western blot analysis. * represents $P < .05$ when compared with the normal group; # represents $P < .05$ compared with the blank group. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-10, interleukin-10; IL-9, interleukin-9; INF- γ , interferon- γ ; JAK 2, Janus kinase 2; p-STAT3, phosphorylated-signal transducers and activators of transcription 3; STAT3, signal transducers and activators of transcription 3

mice in other groups, in comparison with the normal control group, was significantly decreased while the proportion of CD8⁺ and CD4⁺/CD8⁺ was increased ($P < .05$). The proportion of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ in the serum of mice in the NC group and the IL-9 overexpression + IL-10 overexpression + AG490 group, compared with the blank group, showed no significant difference ($P > .05$); the proportion of CD4⁺ in the IL-9 overexpression group and IL-10 overexpression group was reduced, while that of the CD8⁺ and CD4⁺/CD8⁺ was increased. In the AG490 group, the proportion of CD4⁺ was increased, while the proportion of CD8⁺ and CD4⁺/CD8⁺ was diminished ($P < .05$; Table 5). Overexpression of IL-9 and IL-10 induced the proportion of CD8⁺ and CD4⁺/CD8⁺ but reduced the proportion of CD4⁺ via the JAK/STAT signalling pathway.

3.9 | Downregulation of IL-9 and IL-10 inhibits the JAK/STAT signalling pathway in CSU

Finally, the mRNA and protein expression of IL-9, IL-10, STAT3, and JAK2 and the extent of STAT3 and JAK2 phosphorylation in skin tissues of mice were evaluated by RT-qPCR (Figure 7A) and western blot analysis (Figure 7B,C). The mRNA and protein expression of IL-9, IL-10, STAT3, JAK2, and the extent of STAT3 and JAK2 phosphorylation were significantly upregulated, while the expression of INF- γ was downregulated in the blank group, NC group, IL-9 overexpression group, IL-10 overexpression group, AG490 group, and IL-9 overexpression + IL-10 overexpression + AG490 group, compared with the normal group ($P < .05$). The expression of IL-9, STAT3, and JAK2 in the IL-9 overexpression group, compared with the blank and NC groups, was increased, the expression of INF- γ was reduced ($P < .05$), and IL-10 expression showed no significant difference ($P > .05$). In the IL-10 overexpression group, compared with the blank and NC groups, the expression of IL-10, STAT3, JAK2, and the extent of STAT3 and JAK2 phosphorylation was elevated. However, the expression of INF- γ was drastically downregulated ($P < .05$), while IL-9 expression indicated no significant difference ($P > .05$). The expression of IL-9, IL-10, STAT3, and JAK2 and the extent of STAT3 and JAK2 phosphorylation in the AG490 group were evidently downregulated, and the expression of INF- γ was potentiated ($P < .05$). The indexes of IL-9 overexpression + IL-10 overexpression + AG490 group presented no difference compared with the blank and NC groups ($P > .05$). Overall, downregulation of IL-9 and IL-10 suppressed CSU progression upon inhibiting the JAK/STAT signalling pathway.

4 | DISCUSSION

CSU is a skin disorder characterized by the daily or almost daily occurrence of wheals and itching lasting for at least 6 weeks.²¹ The activation of cutaneous mast cells has been established as the underlying mechanism for this allergic reaction.²² Inflammation is well recognized to be an active factor in CSU and is induced by a complex interaction of pro-inflammatory mediators, cytokines, and adhesion molecules

that regulate specific progression of cellular infiltration and vasoactivity. The result of this process is the transformation into lymphocyte and granulocytes-mediated hypersensitivity reaction, defined as urticarial wheals.²³ This study explored the effect of IL-9 and IL-10 in CSU via the JAK/STAT3 signalling pathway.

Our results show that the expression of IL-9 and IL-10 cytokines in the CSU group was significantly higher than in the normal group. IL-9 was originally considered a Th2-cytokine but is now ascribed to the Th9 specialized subset of CD4⁺ T helper cells.^{24,25} IL-9 plays an important role in human and murine atopic diseases,^{26,27} antitumour immunity,²⁸ and autoimmune inflammation.^{29,30} IL-9 is a pleiotropic cytokine, producing direct or indirect effects on diverse cell types.³¹ For example, it enhances the production of IgE from B cells³² and is a potent growth factor promoting the proliferation and differentiation of mast cells.^{33,34} In a previous study, IL-9 was identified as a major source in allergic pulmonary inflammation in mice by inducing mast cell accumulation and activation.³¹ CSU patients display significantly increased numbers of Th9 cells in peripheral blood, though not as high as in acute urticaria, yet still indicating a high expression of IL-9 in peripheral blood because of the positive correlation between Th9 and IL-9.³⁵ IL-10 is another inflammatory cytokine mainly produced by Treg cells. It has been suggested that IL-10 levels are increased in order to control inflammation in CSU patients.³⁶⁻³⁸

We have also discovered that in the IL-9 and IL-10 overexpression groups, the number of eosinophils and the level of inflammatory factors in serum have profoundly increased, indicating their decisive role in CSU. Eosinophils produce a range of cytokines, including IL-3, IL-5, and GM-CSF.^{39,40} Previously, eosinophils were reported to increase in asthma as along with inflammatory Th2 cytokines such as IL-4 and IL-13, thus contributing to asthmatic symptoms.⁴¹ IFN- γ , an inflammatory factor, is important in the immune system owing to its capacity to suppress viral replication and its immunostimulatory and immunomodulatory effects.⁴² IFN- γ also enhances recruitment of lymphocyte and prolonged activation in tissues.⁴³

In this study, the mRNA and protein expression of STAT3 and JAK was significantly increased in the IL-9 and IL-10 overexpression groups but decreased in the AG490 group. JAK/STAT is a common signalling pathway shared by many cytokines and growth factors.⁴⁴ This notion supports the crucial and complicated role of this pathway in the biology of haematopoietic cells, oncogenesis, and therapy for some blood malignancies.⁴⁵ Specifically, the IL-6R/JAK/STAT3 pathway principally makes contribution to the pathogenesis and multistep transformation process of multiple myeloma, making its therapeutic targeting an attractive strategy for durable remissions in patients with multiple myeloma.⁴⁶⁻⁴⁸ The JAK/STAT signalling pathway, especially JAK2/STAT3, can be triggered by IL-6 and other cytokines, exerting their effects via the transmembrane receptor gp130, and once triggered, STAT3 positively regulates some pro-inflammatory and prosurvival gene signatures.¹⁸ Activated JAK receptor tyrosine kinase after phosphorylated can phosphorylate STATs to form dimers, which are translocated to the nucleus to bind with nuclear DNA, regulating downstream inflammatory genes.¹⁹ The JAK/STAT signalling pathway also interacts with other signalling

pathways such as MAPK and NF- κ B to enhance the expression of inflammatory genes.⁴⁹⁻⁵¹

5 | CONCLUSION

To sum up, we propose that upregulation of IL-9/10 accelerates the development of CSU while the inhibition of JAK/STAT signalling pathway can effectively suppress the development of CSU. These new insights into the pathogenesis of CSU can contribute to further development of more effective therapies for this allergic disease. Further clinical trials would be needed to validate the research results.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

H.F., J.F., and J.F. designed the study. Q.X. and M.H. collated the data, carried out data analyses, and produced the initial draft of the manuscript. Y.L., Y.W., and H.F. contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

ORCID

Yongning Wu  <https://orcid.org/0000-0002-9337-5324>

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