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JAK/STAT5 signaling pathway inhibitor ruxolitinib reduces airway inflammation of neutrophilic asthma in mice model

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of JAK/STAT signaling pathway inhibitor Ruxolitinib in neutrophilic airway inflammation and its possible immunological mechanism.

MATERIALS AND METHODS: A total of 60 female C57BL/6 mice were randomly divided into neutrophilic asthma (NA) group, Ruxolitinib-treated (Ruxo) group and control (Con) group. Mice in NA and Ruxo groups were sensitized with ovalbumin (OVA) and excited to establish mice models of asthma. Bronchoalveolar lavage fluid (BALF) was collected at 24 h after the last atomization, and the total number of karyocyte and the percentages of sorted cells were detected. The activity of interleukin-17 (IL-17) in BALF was detected by enzyme-linked immunosorbent assay (ELISA). Lung tissue was separated and subjected to hematoxylin-eosin (HE) staining, and the pathological changes of lung tissue were observed under an optical microscope. The proportion of T helper 17 (Th17) cells in the lung was detected by flow cytometry (FCM). After successful modeling of NA mice, immunomagnetic bead purified mouse splenic cluster of differentiation 4+(CD4+) T was treated with IL-7 and Ruxolitinib, and the proportion of differentiated Th17 cells to CD4+ T cells and Ki-67, B-cell lymphoma 2 (Bcl-2) and activated Caspase3 expressions in Th17 cells were detected via FCM.

RESULTS: Compared with those in NA group, the number of karyocytes and the percentages of neutrophils (NEU) and eosinophils (EOS) in BALF in Ruxo group were significantly reduced. The pathological changes of lung tissue in Ruxo group were overtly less than those in NA group. In comparison with NA group, Ruxo group had decreased IL-17A level in BALF and reduced proportion of Th17 cells in lung tissue. In in vitro experiment, compared with those in Con group, decreased percentages of Ki-67 and Bcl-2 proteins and increased percentage of Caspase3 in Th17 cells were found in Ruxo group.

CONCLUSIONS: Ruxolitinib may suppress the survival of Th17 cells by inhibiting the JAK/STAT5 signaling pathway and regulate the anti-apoptosis proteins Bcl-2 and Caspase3, thus promoting the increase of Thl7 cells entering the apoptotic pathway and reducing airway inflammation in NA mice.

Key Words:
Ruxolitinib, Neutrophilic asthma, Th17, Inflammation, Apoptosis.

Introduction

Asthma is the most common chronic airway disease in childhood, which seriously affects children's physical and mental health. Currently, it is considered that asthma is a chronic inflammatory disease of the airway, which involves a variety of cells (such as airway inflammatory cells and structural cells) and cellular components[1], and is accompanied by hyper-responsiveness of the airway[2]. With the progress of the research, it has been recognized that there are different subtypes of airway inflammation in asthma. These subtypes can be divided into eosinophilic asthma (EA) and non-eosinophilic asthma (NEA)[3,4], in which neutrophilic asthma (NA) accounts for more than half of NEA[5]. At present, inhalation of glucocorticoids and long-acting β2 receptor agonists is mainly used in the treatment of bronchial asthma. Glucocorticoids have anti-inflammatory effects, which can inhibit the function of most inflammatory cells. Although glucocorticoids are effective in controlling the condition of most patients with mild and moderate asthma, poor curative effect is still found in 10-20% of patients after inhalation of glucocorticoids[6,7]. Glucocorticoid-resistant asthma is only found in a small number of patients, but it takes up half of the medical resources[8]. Current studies[9,10] have found that most of these patients have NA, whose symptoms can only be controlled by inhaling a large dose of glucocorticoids.

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However, inhalation of larger doses of glucocorticoids in the whole body is sure to cause certain side effects. Researchers have tried many new treatment methods, but none of them have been successfully applied in clinical practice so far. To find new methods, the pathogenesis of NA needs to be further explored, and targeted therapies for the cause of disease may be feasible methods.

The pathogenesis of NA is complex and not yet fully understood. Studies have found that T helper 17 (Th17) cells in NA mice mediate the occurrence of neutrophilic airway inflammation in NA mice through the secretion of interleukin-17A (IL-17A) cytokines[11]. Sustained survival of Th17 cells is one of the important mechanisms leading to the predominance of Th17 cells in NA mice[12]. In addition, studies have shown that IL-6 and transforming growth factor beta (TGF-β) can differentiate Th0 cells into Th17 cells by activating the Janus kinase 2/signal transducers and activators of transcription 3 (JAK2/STAT3) signaling pathway, up-regulating the transcription factor RORrt[13]. Therefore, targeted treatment for Th17 cells has also become a hotspot of current research. JAK/STAT signaling pathway is involved in the regulation of Th17 cell differentiation, survival and proliferation. In this study, mice models of NA were established to explore the role of JAK/STAT signaling pathway inhibitor Ruxolitinib in neutrophilic airway inflammation and the possible immunological mechanism, so as to find new ideas for the early intervention of NA.

Materials and Methods

Experimental Animals and Grouping

C57BL/6 mice (weighing 18-22 g and aged 6-8 weeks old) were purchased from Jining Medical College Laboratory Animal Center and randomly divided into 3 groups: neutrophilic asthma group (NA group, n=20), Ruxolitinib intervention group (Ruxo group, n=20) and normal control group (Con group, n=20). These mice were raised in separate cages. This investigation was approved by the Animal Ethics Committee of Jining Medical College Animal Center.

Establishment of Mice Models of Asthma[14]

Mouse sensitization: mice were sensitized via airway instillation of ovalbumin [OVA (100 μg)] and lipopolysaccharide [LPS (0.1 μg)] for one time on day 0, day 6 and day 13 of the experiment, respectively; there were 3 times of sensitization in total. Mouse excitation: from day 21 of the experiment, the sensitized mice were placed in a self-made mouse aerosol inhalation box and excited by atomization inhalation of 1% OVA solution. The atomization lasted for 1 h per day for 7 consecutive days. Mice in Ruxo group were intragastrically given Ruxolitinib (180 mg/kg) at 30 min before atomization, once a day, while mice in NA group were intragastrically given the same amount of normal saline at 30 min before atomization. Mice in Con group received no treatment.

Bronchoalveolar Lavage Fluid (BALF) Collection

After 24 h of the last atomization excitation, mice were anesthetized with 1% phenobarbital sodium and then euthanized. After disinfecting with 75% alcohol, the trachea was fully exposed, and a 22G vein remaining needle was used for endotracheal intubation. After the needle entered in about 0.5 cm, the wick-in-needle was pulled out, and a surgical suture was passed under the trachea to fix the remaining needle. Then, 0.5 mL precooled sterile phosphate-buffered saline (PBS) were used for lavage for 2 times, and lavage fluid was slowly pumped back while gently squeezing the mouse chest. BALF with a recovery rate of 80% or above was deemed as qualified BALF. After lavage, BALF was immediately placed on ice, re-suspended and mixed. 10 μL BALF were taken and placed in a cell counting chamber to count the total number of cells. The remaining BALF was centrifuged at 4°C, cell precipitation was re-suspended with an appropriate amount of PBS, and cell smear was prepared. Cell smear was fixed with 95% ethanol for 1 min and then subjected to Diff-Quik staining. 400 karyocytes were counted under a microscope. The percentages of neutrophils (NEU), eosinophils (EOS) and mononuclear-macrophages (MO) were calculated.

Hematoxylin-Eosin (HE) Staining of Pulmonary Histopathologic Sections

Mice that did not receive bronchoalveolar lavage were taken from each group. Their left lungs were taken and placed in pre-configured 4% polyformaldehyde solution to fix for 24 h. After that, lungs were dehydrated with gradient ethanol, subjected to xylene transparency, dipped in wax, embedded in paraffin, and cut into 5 μm slices. Last, slices were subjected to HE staining, dried, mounted with neutral resin and observed under a light microscope.

Detection of the Expression of Th17 Cells Through Flow Cytometry (FCM)

Lungs of mice were taken to make single-cell suspension, and 1 μL Golgi Plug, 1 μg/mL ionomycin and 50 ng/mL propylene glycol methyl ether acetate (PMA) were added in proportion and cultivated under conditions of sterile cell culture for 5 h. Then, cells were collected, severally added with 1.25 μL Percp-anti-mouse cluster of differentiation 4 (CD4) antibody, and incubated for 30 min. Each tube was added with 500 μL CytofixTM Fixation Buffer, and incubated at room temperature for 20 min in a dark place. After that, 1.25 μL pseudomonas exotoxin (PE)-labeled anti-mouse IL-17 monoclonal antibody was added, followed by incubation in the dark for 30 min. Last, samples were loaded on a machine for detection within 24 h.

Measurement of IL-17 Cytokine Level in BALF via Enzyme-Linked Immunosorbent Assay (ELISA)

An appropriate amount of BALF was taken from mice in each group, and the level of IL-17 cytokine in BALF of mice was measured according to the procedure of ELISA instruction.

Separation of Mononuclear Cells in Mouse spleen

After mice in NA group received the last atomization for 24 h, mice were sacrificed and their spleens were collected under aseptic conditions. Spleens were placed on a 300 mesh metal screen for grinding and filtration, and spleen cell suspension was collected in a 15 mL centrifuge tube. Then, an appropriate amount of red cell lysis solution was added to the tube and centrifuged, and the supernatant was discarded. After that, cells were re-suspended with cold phosphate-buffered saline (PBS), and the cell concentration was adjusted to 1x108/mL. Last, 20 μL cell suspension were taken and mixed with an equal volume of 0.2% trypan blue, in which cell viability was > 95% detected via trypan blue exclusion assay, and cell suspension was reserved for use.

Immuno-Magnetic Bead Separation and Culture of Splenic CD4+T Cells in Mice

The appropriate amount of magnetic beads (200 μL/107 cells) was taken and added with the same volume of cold PBS. An appropriate amount of mouse splenic mononuclear cells was taken, added with an appropriate amount of antibody (20 μL/107 cells) and inactivated fetal bovine serum (20 μL/107 cells), incubated at 4°C for 20 min. After that, centrifugation was carried out, the supernatant was discarded, and cold PBS (800 μL/107 cells) was added to re-suspend cells. Then, re-suspended cells were added with magnetic beads and placed still at room temperature for 15 min. After that, the above tube was put into a sorter and placed still for 2 min. Next, the supernatant (namely, purified CD4+T cells) was sucked into another 15 mL centrifuge tube. The detected purity was greater than 90%. On day 1 of the culture, each well was coated with Anti-CD3 (2 μg/mL) and anti-CD28 (2 μg/mL), and added with anti-INF (5 μg/L) and anti-IL-4 (5 μg/mL) to prepare basal culture medium. Mouse splenic CD4+T cells were isolated and purified, and according to the above procedures, inoculated on a 24-well plate at 1.5×106/mL per well. After that, ruxolitinib and IL-7 were added for culture and observation, respectively.

Detection of Ki-67 or Caspase3 or B-Cell Lymphoma 2 (Bcl-2) Expression in Th17 Cells by FCM

Firstly, living lymphocytes were circled according to the lateral and forward divergence angles. Then, CD4 was used as gating, CD4+ cells were used as helper T lymphocytes, immunoglobulin G1 (IgG1) was used as isotype control, and CD4+IL-17A fluorescence-positive cells were used as Th17 cells. After that, the expressions of protein fluorescence-positive cell mass of Ki-67, Caspase3 and Bcl-2 in Th17 cells were detected, respectively.

Statistical Analysis

Measurement data were expressed as mean ± standard error of mean (`X ± SEM). Statistical Product and Service Solutions 19.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical processing of experimental data. For data consistent with normal distribution and homogeneity of variance, analysis of variance was adopted for comparison among multiple groups, and t-test was used for comparison between two groups. For data not consistent with normal distribution and homogeneity of variance, rank-sum test (Mann-Whitney U-test) was used for comparison between two groups. $p<0.05$ suggested that the difference was statistically significant.

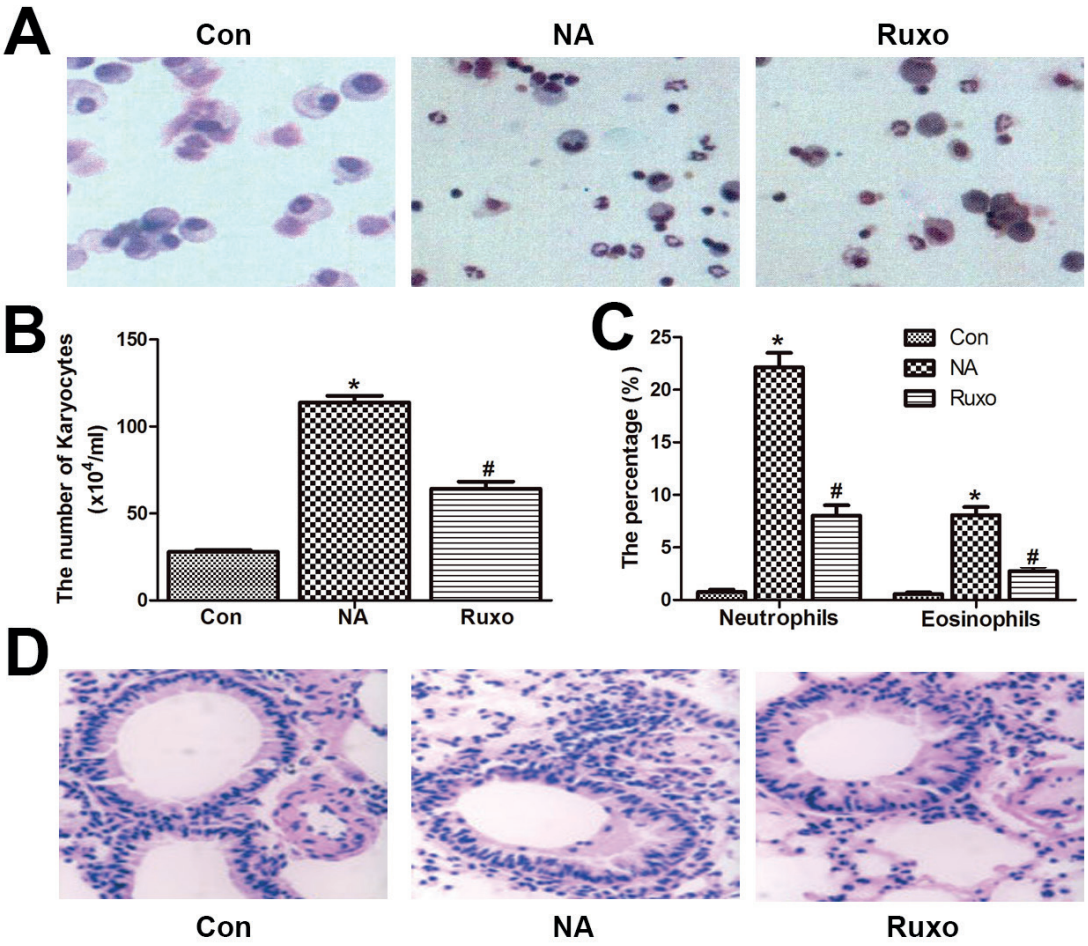

Figure 1. Ruxolitinib reduced the inflammatory cells and alleviated the histopathological inflammatory manifestations in NA mice. ***(A)*** The representative images of Diff-Quick staining of cells in BALF. ***(B)*** Analysis of a total number of karyocytes in BALF. ***(C)*** Analysis of the percentage of neutrophils and eosinophils in BALF. ***(D)*** The representative images of hematoxylin and eosin (HE) staining of lung tissue. $^{*}p<0.05$ vs. Con group, $^{\#}p<0.05$ vs. NA group.

Results

Ruxolitinib was Able to Reduce the Total Number of Cells and the Percentages of NEU and EOS in BALF in NA Mice

Compared with those in Con group, the total number of karyocyte and the percentages of NEU and EOS in mouse BALF in NA group were significantly increased. In comparison with NA group, Ruxo group had significantly lowered total number of karyocyte and percentages of NEU and EOS in mouse BALF; the differences were statistically significant. However, the total number of karyocyte and the percentages of NEU and EOS in mouse BALF in Ruxo group were still higher than those in Con group,; the differences showed statistical significance (Figure 1A-B).

Pathological Changes in Mouse Lung Tissue After Ruxolitinib Intervention

The results of mouse lung histopathological section showed that in Con group, the bronchial lumen and alveolar structure of mice were intact, no infiltration of inflammatory cells was found, and airway epithelial cells were arranged neatly. In NA group, structural disorder of lung tissue, widened alveolar septa, ruptured alveolar walls, and inflammatory cell infiltration around the airway and in interstitial tissue were found, in which infiltration was mainly dominated by NEU infiltration. In Ruxo group, inflammatory cell infiltration around the bronchus was reduced compared with that in NA group, the bronchial structure was relatively intact, the wall of the bronchial epithelium was thicker, and infiltration of NEU, lymphocytes and mast cells was observed in the submucosa and alve-

olar interstitium. The infiltration in Ruxo group was less than that in NA group (Figure 1C).

Decrease of the Proportion of Mouse Lung Thl7 Cells After Ruxolitinib Intervention

The results of FCM revealed that in comparison with Con group, NA group had an overtly increased proportion of Thl7 cells in mouse lung, and the difference was statistically significant. Compared with that in NA group, the proportion of mouse lung Thl7 cells in Ruxo group was decreased evidently, and the difference was of statistical significance. However, the proportion of Thl7 cells in the lungs of mice in Ruxo group was still higher than that in Con group, and the difference showed statistical significance (Figure 2A-B).

Reduction of IL-17 Level in Mouse BALF After Ruxolitinib Intervention

The results of ELISA showed that the content of IL-17 in mouse BALF in NA group was significantly higher than that in Con group; the difference was statistically significant. Compared with that in NA group, the content of IL-17 in mouse BALF was decreased clearly in Ruxo group; the difference showed statistical significance. However, the level of IL-17 in mouse BALF in Ruxo group was still higher than that in Con group; the difference was of statistical significance (Figure 2C).

Decline of Ki-67 Expression in Thl7 Cells of NA Mice After Ruxolitinib Intervention

CD4+T cells were separated from the spleen of NA mice by magnetic beads and incubated with T-cell receptor (TCR) irritant (anti-CD3/CD28) and IL-7 (10 ng/mL) or Ruxolitinib for 72 h, followed by FCM, and the results indicated that the positive rate of Ki-67 expression in Th17 cells in Ruxo group was significantly reduced compared that in Con group; the difference had statistical significance (Figure 3).

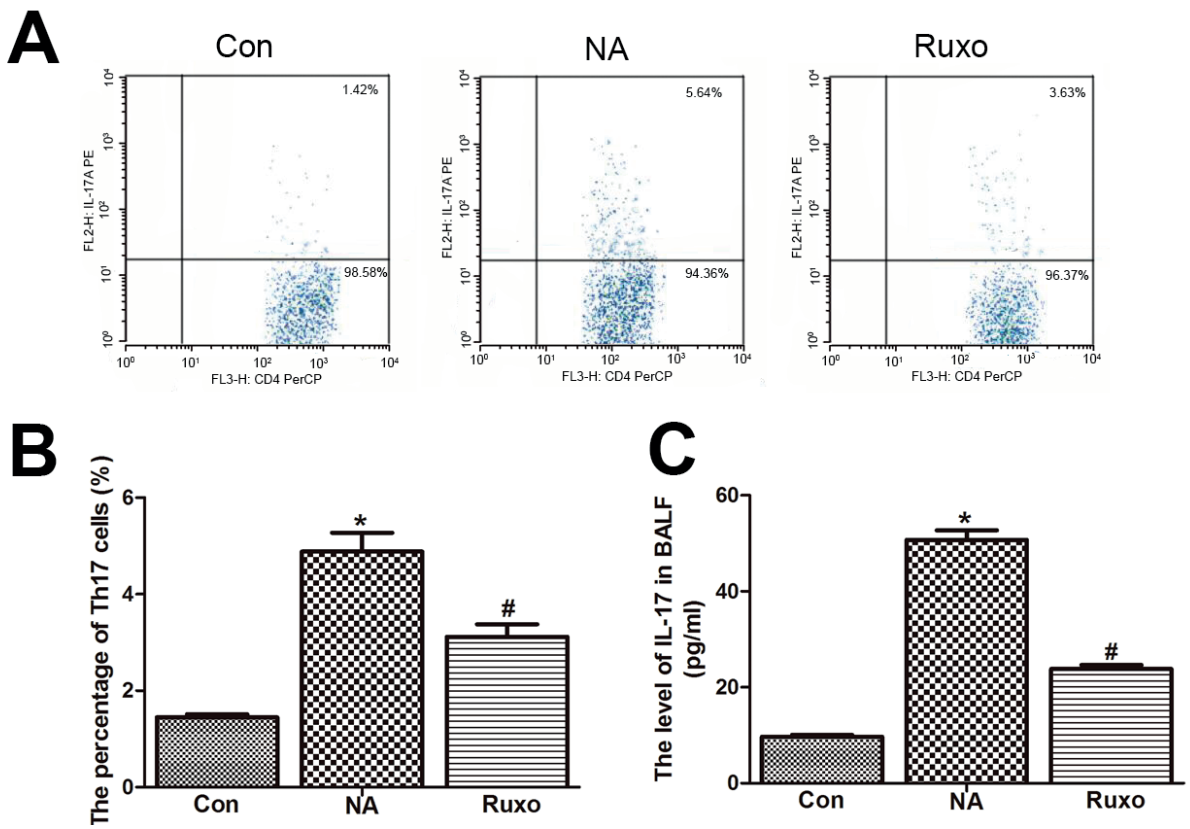

Figure 2. Ruxolitinib decreased of the proportion of mouse lung Thl7 cells and reduce IL-17 level in mouse BALF. (A) Comparison of the expression of Thl7 cells in lung of mice in each group by flow cytometry. (B) Analysis of Thl7 cells in lung of mice in each group. (C) Analysis of the level of IL-17 by ELISA. *p<0.05 vs. Con group, #p<0.05 vs. NA group.

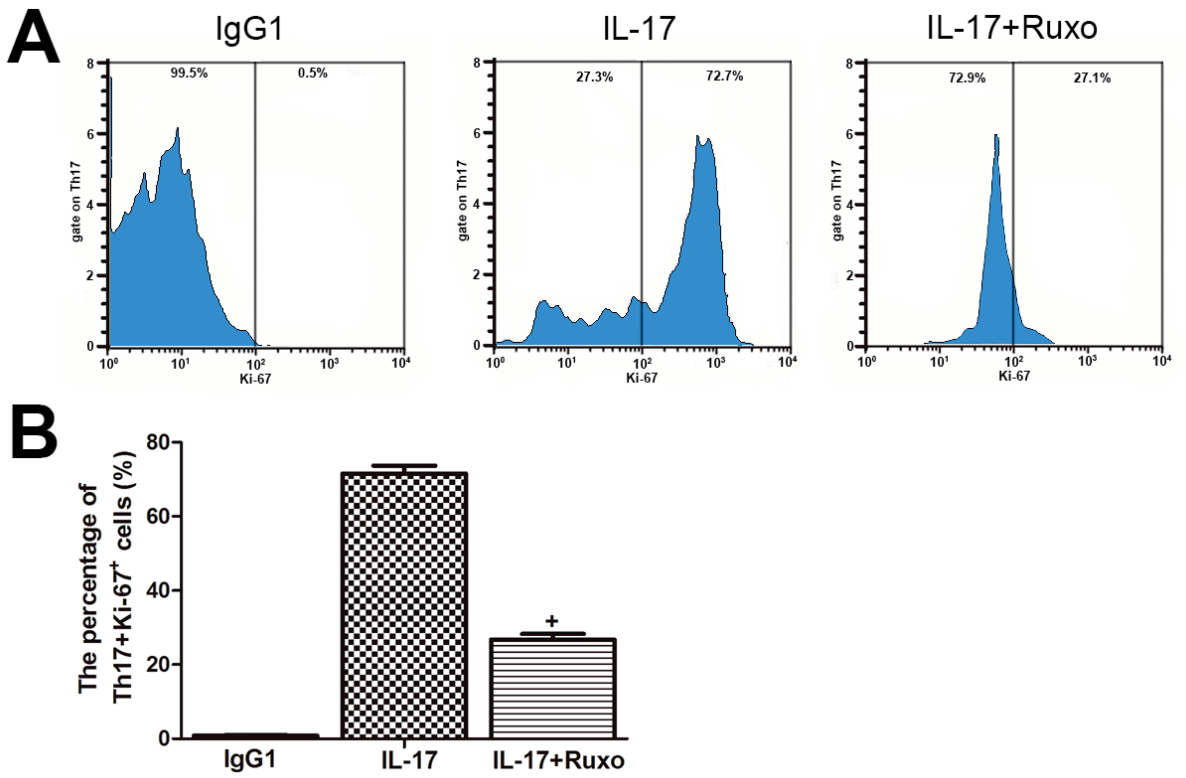

Figure 3. Ruxolitinib declined of Ki-67 expression in Thl7 cells of NA mice. ***(A)*** The percentage of Th17+Ki-67+ cells in different groups by flow cytometry. ***(B)*** Analysis of the percentage of Th17+Ki-67+ cells in different groups. + $p<0.05$ vs. IL-17 group.

Decrease of Bcl-2 Expression in Thl7 Cells of NA Mice After Ruxolitinib Intervention

CD4+T cells were separated from the spleen of NA mice by magnetic beads, incubated with TCR irritant (anti-CD3/CD28) and IL-7 (10 ng/mL) or Ruxolitinib for 72 h, and then subjected to FCM. The results indicated that Ruxo group had a significantly lowered positive rate of Bcl-2 expression in Th17 cells in comparison with Con group; the difference was statistically significant (Figure 4A-C).

Increase of Caspase3 Expression in Thl7 cells of NA Mice After Ruxolitinib Intervention

CD4+T cells were separated from the spleen of NA mice by magnetic beads and incubated with TCR irritant (anti-CD3/CD28) and IL-7 (10 ng/mL) or Ruxolitinib for 72 h, followed by FCM; the results indicated that the positive rate of Caspase3 expression in Th17 cells in Ruxo group was overtly elevated compared with that in Con group; the difference showed statistical significance (Figure 4B-D).

Discussion

JAK/STAT signaling pathway is an important pathway for signal transduction of various growth factors and cytokines. JAK/STAT pathway plays important roles in regulating proliferation, differentiation and survival of immune cells in innate and adaptive immune diseases[15,16]. In addition, most inflammatory factors and cytokines play roles through this pathway, so JAK/STAT pathway is closely related to autoimmune and immunodeficiency diseases[17]. Ruxolitinib is a selective JAK1/2 amino acid protein kinase inhibitor that acts by blocking JAK signal transduction and STAT pathway. Ruxolitinib has been approved for application in the treatment of diseases of blood system such as primary myelofibrosis in the United States and the European Union, but its use for NA has not been reported[17-19]. In view of the above experience, this study used Ruxolitinib to intervene in Th17 cell-mediated NA mice to explore its impact on neutrophilic airway inflammation. The results showed that Ruxoliti-

nib intervention in NA mice in the excitation phase could reduce the infiltration of airway inflammatory cells in asthmatic mice, and decrease the percentages of NEU and EOS. The pathology of lung tissue was also improved. After Ruxolitinib intervention, the infiltration of NEU, lymphocytes and mast cells around the bronchioles was significantly reduced, suggesting that Ruxolitinib can reduce neutrophilic airway inflammation in NA mice. Th17 cells are new helper T-cells mainly secreting IL-17, which are involved in the pathogeneses of various chronic inflammatory diseases[20], autoimmune diseases[21], and tumors[22]. More and more evidence have shown that Th17 cells participate in the pathogenesis of NA. Th17 cells primarily induce chronic airway inflammation and airway hyper-responsiveness through IL-17. Therefore, targeted treatment for IL-17/Th17 cells has become a hotspot of research so far. Some researchers have used GSI (γ secretase inhibitor) to intervene in asthmatic mice, to decrease acute airway inflammation in mice by reducing the number of Th17 cells. In this work, the proportion of Th17 cells in the lung was detected, and the results suggested that the percentage of Th17 cells to CD4+T cells was decreased, and the concentration of IL-17 in BALF was lowered in intervention group. Therefore, the results suggest that in animal experiments, Ruxolitinib

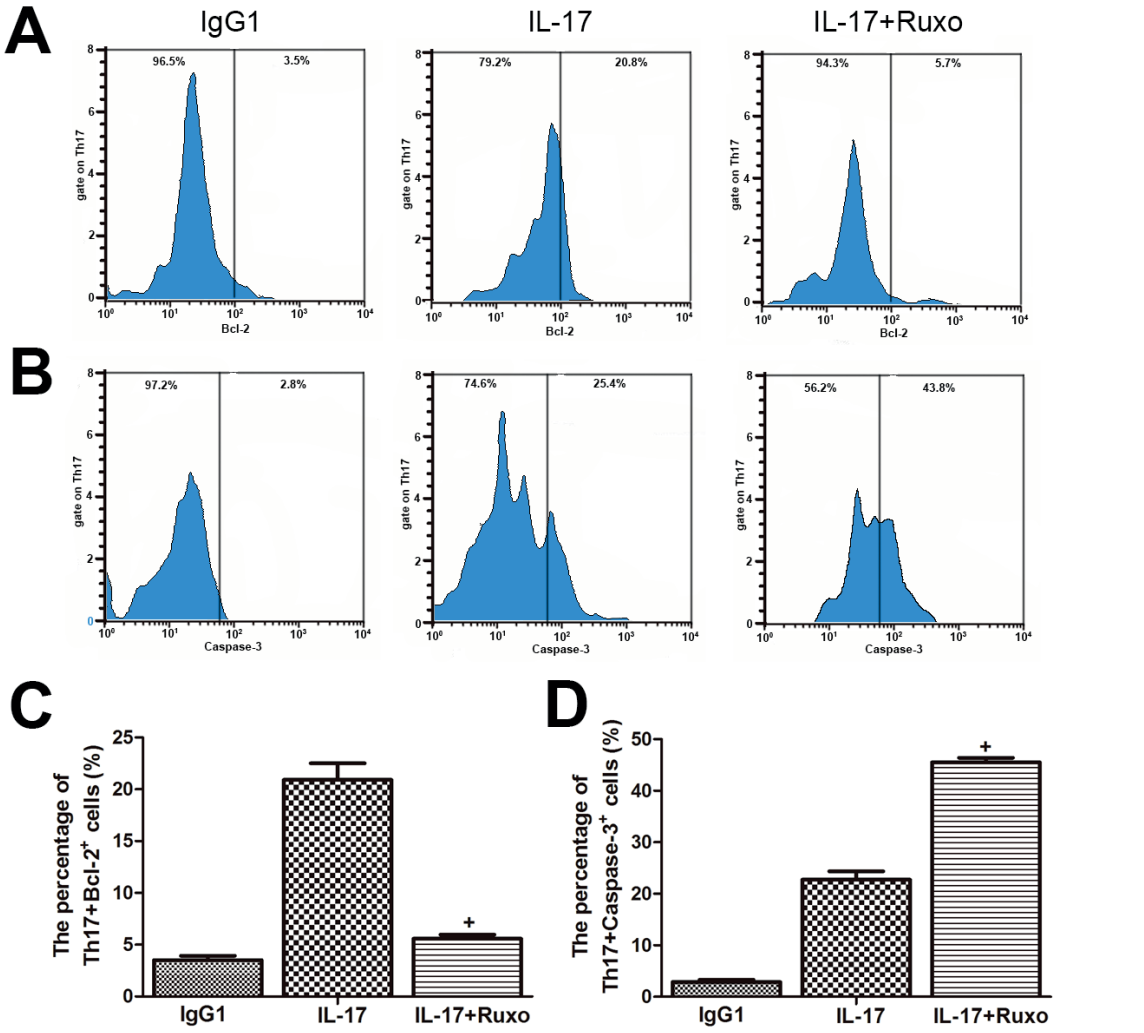

Figure 4. Ruxolitinib decreased he expression of Bcl-2 and increased the expression of Caspase3 in Thl7 cells of NA mice. ***(A)*** The percentage of Th17+Bcl-2+ cells in different groups by flow cytometry. ***(B)*** The percentage of Th17+Caspase-3+ cells in different groups by flow cytometry. ***(C)*** Analysis of the percentage of Th17+Bcl-2+ cells in different groups. ***(D)*** Analysis of the percentage of Th17+Caspase-3+ cells in different groups. + $p<0.05$ vs. IL-17 group.

can potentially reduce airway neutrophilic inflammation in asthma by down-regulating the number of Th17 cells and its major inflammatory factor IL-17. JAK/STAT signaling pathway participates in regulating the differentiation, survival and proliferation of Th17 cells. Moreover, IL-6, IL-23 and other cytokines induce the differentiation of initial CD4+T cells into Th17 cells by activating JAK/STAT3 and JAK/STAT4 signaling pathways[23,24]. Some scholars pointed out in the study of animal models of experimental autoimmune encephalomyelitis (EAE) that JAK1/JAK2 inhibitors can inhibit the differentiation of pathogenic Th17 cells by inhibiting the activation of JAK/STAT signaling pathway, so as to achieve the target of treating EAE mice[25]. Asakawa et al[26] showed that a new JAK/STAT inhibitor can suppress the phosphorylation of JAK/STATs, thus inhibiting the differentiation of effect or T-cells (Th1, Th2 and Th17) and promoting Treg differentiation, so that symptoms of mice models of collagen-induced arthritis are improved. However, the relationships between suppression of JAK/STAT signaling pathway and Th17 cell survival and apoptosis have rarely been reported. In this study, Ruxolitinib was co-cultured with CD4+T cells of NA mice, and downstream proteins of JAK/STAT5 signaling pathway were detected. Ki-67 is a proliferation-related antigen, suggesting that the survival of Th17 cells in NA mice is prolonged. After Ruxolitinib intervention, the expression of ki-67 was decreased in Th17 cells, suggesting that Ruxolitinib can inhibit the survival of Th17 cells by inhibiting the JAK/STAT5 signaling pathway and Ruxolitinib has certain anti-inflammatory effects. Subsequently, the expressions of Bcl-2 and activated Caspase3 in Th17 cells were detected in this research; the results showed that the expression of Bcl-2 in Th17 cells was decreased after Ruxolitinib intervention, suggesting that anti-apoptosis ability of Th17 cells in asthmatic mice is reduced after Ruxolitinib intervention, inducing the apoptosis of Th17 cells. Moreover, the expression of activated Caspase3 in Th17 cells in asthmatic mice was elevated after Ruxolitinib intervention, indicating that more Th17 cells enter the apoptotic pathway. The above results suggest that Ruxolitinib can inhibit the JAK/STAT5 signaling pathway, down-regulate the anti-apoptosis protein Bcl-2 and promote the activation of Caspase 3, thereby increasing the number of Thl7 cells entering the apoptotic pathway, and leading to the decrease of the number of Thl7 cells.

Conclusions

Ruxolitinib may suppress the survival of Th17 cells by inhibiting the JAK/STAT5 signaling pathway and regulate the anti-apoptosis proteins Bcl-2 and Caspase3, thus promoting the increase of Thl7 cells entering the apoptotic pathway and reducing airway inflammation in NA mice, which may be a new and potential therapeutic method for NA.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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