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ORIGINAL RESEARCH

Relationship Between the Expression of LC3 (Microtubule-Associated Protein 1A/1B-Light Chain 3) in Nasal Mucosa and Serum IL-5 and IL-4 Concentrations in Allergic Rhinitis Mice

Hui Pi[1,2,*], Ming-Fang Xu[3,*], Hai-Tao Lu[3]

[1]Yangtze University Health Science Center, Jingzhou, 434020, People's Republic of China; [2]Department of Otolaryngology, GongAn County People's Hospital, Jingzhou, 434300, People's Republic of China; [3]Department of Otolaryngology, Jingzhou Central Hospital, Jingzhou, 434020, People's Republic of China

*These authors contributed equally to this work

Correspondence: Hai-Tao Lu, Department of Otolaryngology, Jingzhou Central Hospital, No. 26 Chuyuan Avenue, Jingzhou District, Jingzhou, 434020, People's Republic of China, Tel +86 18107167033, Email haitaolu02@126.com

Objective: To investigate the expression and correlation of autophagy-related microtubule-associated protein 1 light chain 3 beta LC3 and interleukin-5 IL-5 in allergic rhinitis AR.

Methods: Fifty-six 7-week-old BALC/C mice were randomly divided into experimental group (n = 56) and control group (n = 8). The experimental group used Dermatophagoides farinae (Der f) for AR modeling, and control group used PBS solution. As the experimental group sampled at 6 time points, and 8 mice were sacrificed each time, while the control group was sacrificed 24 hours after the last dose. The contents of serum IL-4, IL-5, and dust mite specific IgE HDM-sIgE in mice were detected by enzyme-linked immunosorbent assay ELISA, and the morphological changes of nasal mucosa were detected by a hematoxylin-eosin H&E staining. The expression of LC3 in mouse nasal mucosa was detected by immunohistochemical staining. Spearman correlation coefficient was used to assess the relationship between LC3 and IL-5 levels.

Results: In AR mice modelled with dust mites, the serum levels of IL-4 and HDM-sIgE increased gradually, and the serum IL-5 concentration had a peak at the early intraperitoneal administration stage similar to that at the end of modelling. The LC3 level in nasal mucosa of AR mice modelled with dust mites increased gradually in the early stages, but stabilized in the later stages. The expression of LC3 level in nasal mucosa was a positively correlated ration between serum IL-5 level in AR mice.

Conclusion: In the early stage of AR mice, the level of nasal mucosal autophagy and serum IL-5 levels were significantly increased and correlated, suggesting that nasal mucosal autophagy played a promoting role in the early stage of AR.

Keywords: allergic rhinitis, animal model, autophagy IL-5

Introduction

Allergic rhinitis (AR) is a common disease in the Department of Otolaryngology. It is generally considered to be an allergic disease of nasal mucosa mediated by IgE, which is caused by inhalation allergens entering the nasal mucosa of the sensitized individuals, causing degranulation of mast cells, involvement of numerous inflammatory cytokines (such as IL-4 and IL-5).[1] The prevalence of AR is on the rise in all parts of China.[2] AR is affected by both genetic and environmental factors, and the prevalence of AR in each region is closely related to the level of local economic development and the prevalence of asthma AS.[3,4] The prevalence of AR in economically developed cities was higher than that in economically underdeveloped rural areas. The prevalence of AS was also higher in areas with high AR prevalence. AR prevalence in other countries has similar characteristics to China.[5] Due to their contempt for their own disease and an easy access to over-The-counter drugs, AR patients are prone to delayed medication, non-prescribed

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medication, and irregular medication. Therefore, patients often suffer from recurrent nasal symptoms and develop even into AS, which seriously affects their quality of life and increases their economic burden of patients and the country.

In the past, Th1/Th2 immune imbalance dominated the research on the pathogenesis of AR. With the in-depth research, people gradually improved their understanding of the pathogenesis of AR. In recent years, the role of autophagy in AR has become one of the research hotspots. Autophagy is a cell degradation pathway by which eukaryotes utilize cytoplasmic contents. Under physiological conditions, the cells show a stable and low autophagy level, and the autophagy level is rapidly up-regulated under stress.[6] The role of autophagy in AR is still under exploration. It is currently believed that autophagy can participate in AR process from airway response, immune response, chronic inflammatory response[7] and airway remodeling[8] by participating in endogenous antigen presentation, regulating T cell development and activation, participating in airway epithelial injury and promoting nasal mucosa fibrosis. Autophagy is divided into macroautophagy, microautophagy and autophagy mediated by molecular chaperones. We often refer to autophagy as macroautophagy. Commonly used markers to detect macroautophagy include autophagosome markers, lysosome markers and autophagy substrate P62.[9] Autophagosome markers are some genes involved in encoding proteins in the process of autophagosome formation and phagocytosis, called autophagy-related protein (Atg), among which LC3 is a key upstream signal known to be positively correlated with autophagosome formation.[10] LC3 (Microtubule-associated protein 1A/1B-light chain 3), a mammalian homologue of yeast Apg8p, is a soluble protein prevalent in mammalian tissues and cultured cells.[11] LC3 has two forms: Cytoplasmic LC3-I and phosphatidyl ethanolamine bind LC3-II. LC3-II is the first recognized mammalian protein that is specifically associated with autophagosome membrane, and its amount is positively correlated with the degree of autophagosome formation.[11] The level of autophagy in nasal epithelial cells of AR patients was up-regulated, and high levels of autophagosomes and autophagy markers (LC3) were expressed, which were associated with airway remodeling.[8] Previous studies have found that the nasal epithelial cells of AR patients[12] and nasal mucosa of AR mice[13] express high levels of LC3. However, how the autophagy level of mouse nasal mucosa changes during the whole process of allergic mouse model is still unknown.

The article will discuss the change of nasal mucosa autophagy level and its relationship with IL-5 level in allergic mouse model.

Materials and Methods

Experimental Animals

Fifty-six 7-week-old healthy female BALB/C mice were the subjects used in the research, weighing about 17–21 g, were selected as the research objects. There was no significant difference in body weight between groups. All mice were fed the national standard chow with 5 mice per cage. Mice were fed with free diet and water, natural light, room temperature 20–26°C, and humidity 40–50% for 2 weeks. Animal handling and experimental procedures were in accordance with the National Code for Laboratory animals and all approved by the Ethics committee.

Molding Method

Prepare 1mg/mL Der f solution (Zhejiang Wowu Biotechnology Co., LTD), 4% aluminum hydroxide gel (Shaanxi Zhonghui Hecai Biomedical Technology Co., LTD) and ultrapure water. A 0.25 mg/mL mixture of dust mite solution and aluminum hydroxide gel was prepared (ie, 75ul of 4% aluminum hydroxide gel was added to every 25ug of 1mg/mL dust mite solution). The first dose was recorded as day 0 (day 0). On days 0, 7, and 14, the experimental group was intraperitoneally injected with 0.1mL of dust mite mixture, and on days 21–27, 20μL of dust mite solution was given intranasally for 7 consecutive days. In the control group, 0.1mL PBS solution was given intraperitoneally on days 0, 7, and 14, and 20ul PBS solution was given intranasally on days.[14]

Sample Collection

The mice killed on day 1 were labeled as experimental group 1 (day 0 group), the mice killed on day 8 were labeled as experimental group 2 (day 7 group), the mice killed on day 15 were labeled as experimental group 3 (day 14 group), the mice killed on day 22 were labeled as experimental group 4 (day 21 group), the mice killed on day 25 were labeled as

experimental group 5 (day 24 group), and the mice killed on day 28 were labeled as experimental group 6 (day 27 group). Mice in the control group were sacrificed on day 28. The venous blood was collected from the eyeballs of the mice. After staining at room temperature, for two hours, the serum was separated by high-speed centrifuge (3500r, centrifuged at 4°C for 25min), and the separated serum was stored in the refrigerator at −20°C. The expression of cytokines in serum was detected by ELISA. After the nasal skin of mice was separated, the two sides of the nasal bone were cut with a knife, and the nasal bone was lifted up to expose the turbinate and nasal septum. The nasal septum of mice was separated, and the mucous membrane of the turbinate terminal behind the nasal septum was taken as far as possible. After removal, the mucous membrane was soaked in 4% formaldehyde solution to fix the label and stored in a refrigerator at 4°C. The nasal mucosa of 5 mice in each group was randomly selected to observe the pathological changes of the nasal mucosa by H&E staining. The nasal mucosa of 5 mice in each group was randomly selected for immunohistochemical staining to detect the expression level of LC3.

Detection Method

Behavioral Evaluation Criteria[13]

Morphological Observation of Nasal Mucosa

Mouse nasal mucosa samples were fixed with 4% formaldehyde, dehydrated, transparent, and dipped in wax to make tissue paraffin blocks. Paraffin sections were stained with hematoxylin and eosin. Hematoxylin made the nucleus purplish blue, and eosin made the cytoplasm and extracellular matrix light red. The infiltration of inflammatory cells in the upper and submucosa of mouse nasal mucosa was observed under optical microscope.

Serum Cytokine Detection

The levels of serum cytokines (IL-5, IL-4, HDM-sIgE) were determined by double antibody sandwich method after thawing.

Observation of Nasal Mucosa by Histochemical Staining

After the paraffin block is sliced, the slices are baked in the oven at 60 °C for 2 hours, and then the slices are dewaxed and hydrated. Firstly, the citrate repair solution with PH=6 0.1 mmol/L is preheated for 5 minutes, and then the hydrated sections are placed in the repair solution. Then, the citrate repair solution containing slices was heated in the microwave oven at 100 heat for 3 minutes until slightly boiling, then changed to 50 heat for 7 minutes, and finally cooled naturally for 30 minutes. After antigen repair, the tissue was blocked with 3% H_2O_2 solution and incubated for 5 minutes at room temperature. The sections were rinsed 3 times for 5 minutes on a shaker in a tissue chemistry box with PBS solution. About 100ul of diluted primary antibody (LC3 working concentration 1:200, Shanghai biyuntian) was added to the tissue of each section to completely cover the tissue, and the solution was incubated at 37 °C for 30 minutes. Then rinsed 3 times and soakinse 3 times with PBS solution, 5 minutes each time. Then, 100ul of secondary antibody (HRP labeled) was added to the tissue of each section to completely cover the tissue, and the solution was incubated at 37 °C for 20 minutes. Rinse and soak in the PBS solution 3 times, 5 minutes each time. Add 100ul of DAB solution to the tissue of each section, incubate at room temperature for 8 minutes to develop color, rinse with tap water and counterstain with hematoxylin, and then seal slices with neutral gum after dehydration. After drying, they were observed under a light microscope, and pictures were taken and analyzed.

The evaluation criteria

The distribution and positive expression of LC3 (mainly brown and tan) in mouse nasal mucosa were observed under a light microscope. Pictures were taken under a field of 200 times, and 5–10 fields were randomly selected for each section. Image Pro Plus 6.0 software was used to analyze the average optical density value of each section, namely the positive ratio of LC3.

Statistical Analysis Method

The measurement data of normality were expressed as $\bar{x} \pm s$, and comparison among groups was analyzed by one-way analysis of variance. The constituent ratio and enumeration data of each group were expressed as frequency and

Table 1 Behavioral Scoring Criteria for Allergic Rhinitis in Mice

Score	Sneeze (One/30Min)	Running Nose	Friction Nose
0	0	Not have	Not have
1	1–3	Flow reach the anterior naris	Scratch a few times
2	4–10	Flow over the anterior naris	Frequent scratching nose
3	>11	Flow all over the face	Cannot stop scratching face

percentage (%) and analyzed by chi-square (χ^2) test. Spearman's method was used for correlation analysis. GraphPad Prism 8.0.1 software was used to analyze the data of each group, and $P \leq 0.05$ was used to indicate statistical significance.

Results

Evaluation of Behavior

Under the same conditions of gender, age, feeding environment and administration method, the mice were observed for 30 minutes on the 27th day after the end of administration. According to the behavioral scoring standard (Table 1), the score of mice in experimental group 6 (day 27 group) was 5.83 ± 0.72, and the score of mice in the control group was 1.13 ± 0.83. The symptoms of sneezing, nose rubbing, and runny nose in day 27 group were more obvious than those in control group, and the differences were statistically significant ($P < 0.05$).

Morphological Changes of Nasal Mucosa

The mice in the experimental group and the control group were sacrificed on the 28th day after the end of administration. When the nasal mucosa of the mice was taken, macroscopic observation showed that the nasal mucosa of the mice in day 27 group was swollen and the turbinate was hyperplastic, while the nasal mucosa of the mice in the control group was light red with no swelling and the turbinate was not large. After the H&E staining, electron microscopy showed that the epithelial layer of nasal mucosa of mice in day 27 group was significantly thickened, with the disorderly arrangement of epithelial cells, thickened basement membrane, increased glands, and obvious lymphocyte infiltration. However, the nasal mucosa of the control group showed no obvious epithelial thickening and hyper glandular phenomenon (Figures 1 and 2).

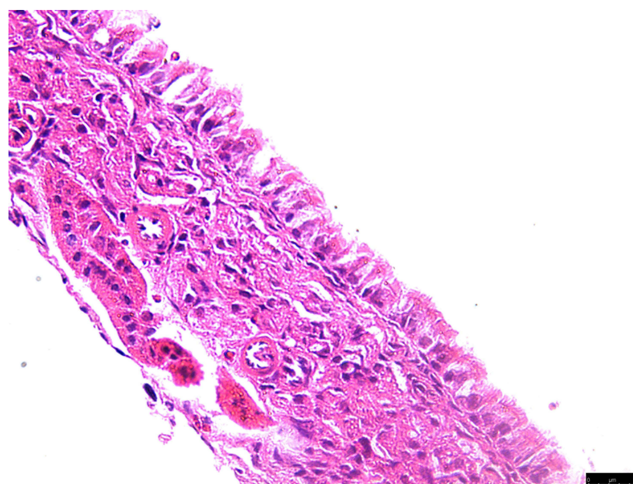

Figure 1 H&E staining results of the control group, viewed under a 400X microscope.

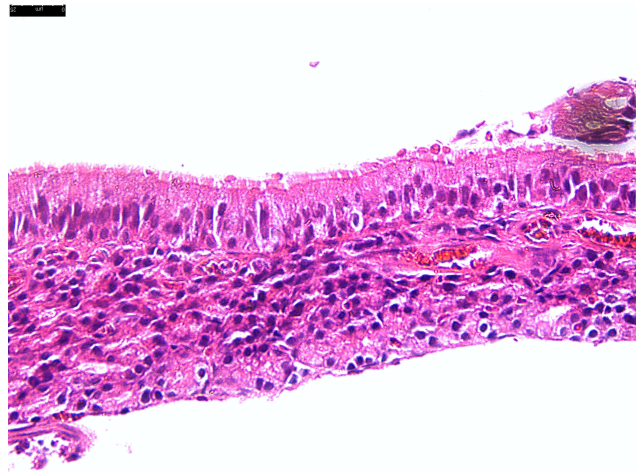

Figure 2 H&E staining results of the experimental group, viewed under a 400X microscope.

Serum Cytokine Assay Results

Expression of IL-5 in Serum of Mice in Each Group

The serum IL-5 concentration in day 0 group was 34.56 ± 4.40 pg/mL, day 7 group was 28.67 ± 5.34 pg/mL, day 14 group was 40.30 ± 9.75 pg/mL, and day 21 group was 33.75 ± 8.82 pg/mL. The concentration of day 24 group was 44.70 ± 12.66 pg/mL, day 27 group was 50.46 ± 12.24 pg/mL, and the control group was 32.28 ± 5.12 pg/mL. Compared with the control group, the content of IL-5 in day 24 group and day 27 group was significantly increased ($P < 0.05$). Comparison between experimental groups showed that the serum IL-5 concentration of allergic rhinitis mice in the process of modeling reached a peak after the third intraperitoneal administration, and there was no significant difference in IL-5 content between day 27 group and day 14 group ($P > 0.05$), and the serum IL-5 content gradually increased after continuous nasal stimulation (Figure 3).

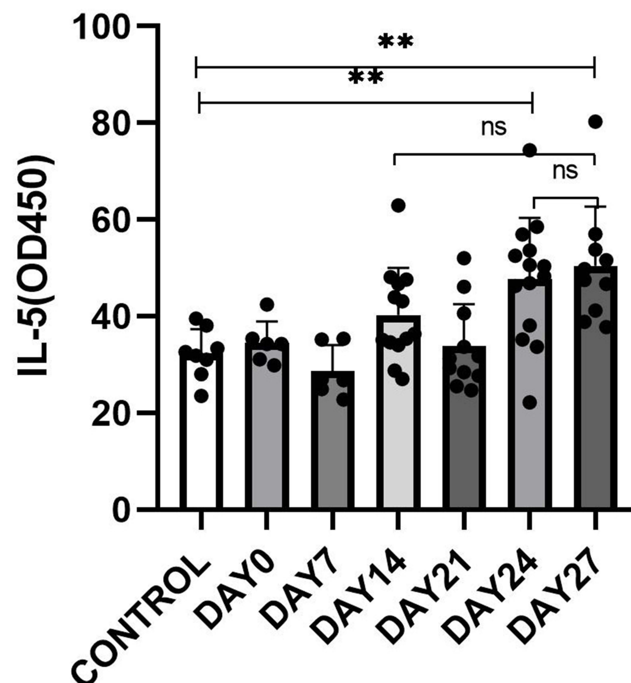

Figure 3 Comparison of serum IL-5 expression levels between experimental mice and control mice, **P < 0.01.

Expression of IL-4 in Serum of Mice in Each Group

The serum IL-4 concentration in day 0 group was 170.11 ± 24.60 pg/mL, day 7 group group was 129.69 ± 15.43 pg/mL, day 14 group was 170.50 ± 17.18 pg/mL, and day 21 group was 162.72 ± 41.90 pg/mL. The concentration of day 24 group was 208.26 ± 45.06 pg/mL, the concentration of day 27 group was 214.20 ± 49.62 pg/mL, and the concentration of control group was 168.33 ± 10.54 pg/mL. Compared with the control group, the concentration of IL-4 in day 24 and day 27 groups was significantly higher than that in the control group ($P < 0.05$). Comparison between experimental groups showed that the serum IL-4 concentration gradually increased with the increase of administration times, and the IL-4 concentration in day 24 and day 27 group was comparable ($P > 0.05$) (Figure 4).

Expression of HDM-sIgE in Serum of Mice in Each Group

The serum HDM-sIgE concentration in day 0 group was 26.31 ± 1.46 ng/mL, day 7 group was 22.92 ± 1.54 ng/mL, day 14 group was 26.93 ± 3.40 ng/mL, and day 21 group was 27.54 ± 3.26 ng/mL. The concentration in day 24 group was 31.29 ± 3.09 ng/mL, the concentration in day 27 group was 32.99 ± 3.11 ng/mL, and the concentration of control group was 24.33 ± 1.33 ng/mL. Compared with the control group, the concentration of IL-4 in day 21, day 24, and day 27 group was significantly higher than that in the control group ($P < 0.05$). Compared between both experimental groups, serum HDM-sIgE concentration gradually increased with the increase of administration times, and the IL-4 concentration in day 24 and day 27 group was comparable ($P > 0.05$) (Figure 5).

The Expression of LC3 in Nasal Mucosa Was Observed by Histochemical Staining

The nasal mucosa of mice in both the experimental groups and the control group was stained with immunohistochemistry and observed under the light microscope. LC3 was expressed in the epithelial layer of the mucosa and the glandular tissue, showing a brown color. Image Pro Plus 6.0 software was used to calculate and analyzed the average optical density value of each group. The results were as follows: In the control group, the brown color of the epithelial layer was lighter, representing the level of basal autophagy, and the average optical density value was 0.036 ± 0.011. The average optical density of day 0 group was 0.045 ± 0.031, the average optical density of day 7 group was 0.034 ± 0.024, the average optical density of day 14 group was 0.125 ± 0.010, the average optical density of day 21 group was 0.108 ± 0.012, the average optical density of day 24 group was 0.059 ±

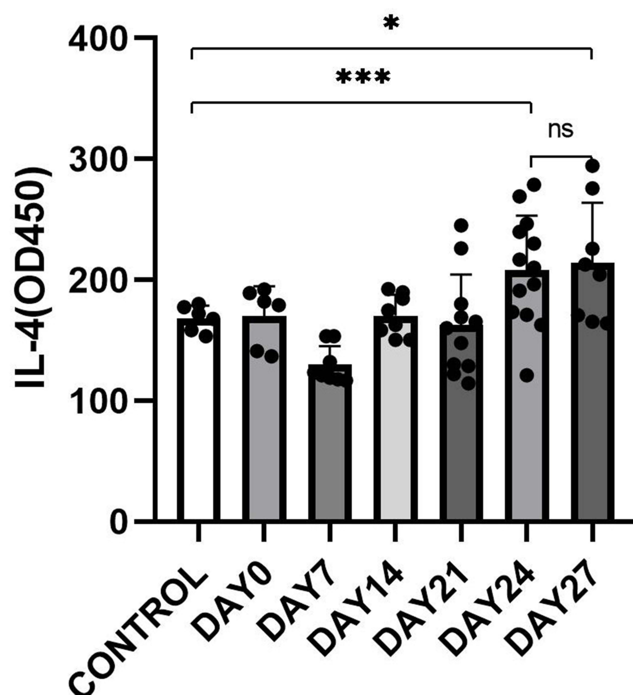

Figure 4 Comparison of serum IL-4 expression levels between experimental mice and control mice.*P<0.05, ***P<0.001.

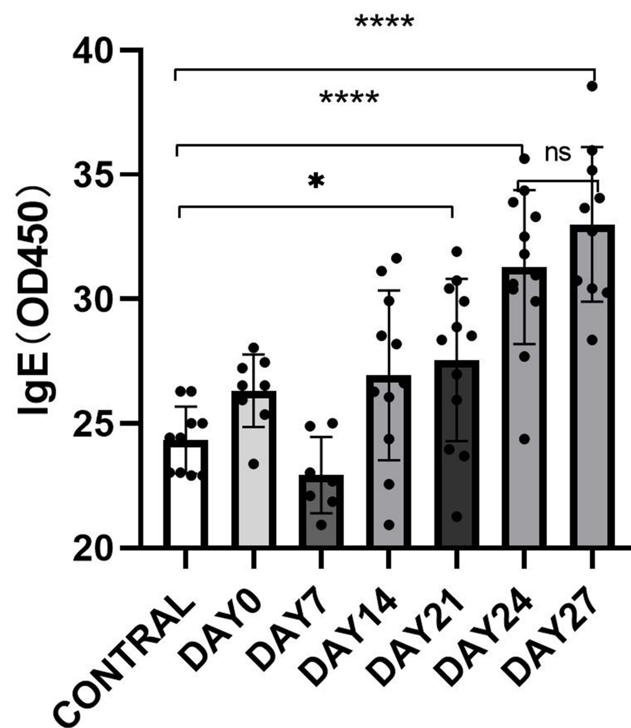

Figure 5 Comparison of serum house dust mite specific IgE antibody levels between experimental mice and control mice *P<0.05, **** P< 0.0001.

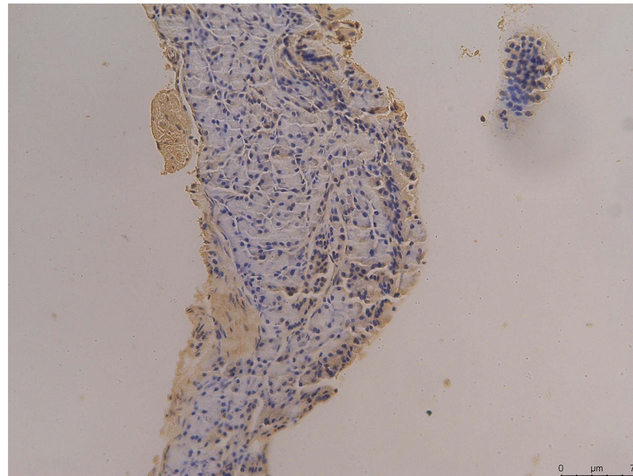

Figure 6 The immunohistochemical staining results of the control group were observed under 200X microscope.

0.025. The average optical density of day 27 group was 0.041 ± 0.006. Compared with the control group, the average optical density value of the control group represented the basal autophagy level; the average optical density value of the experimental group increased first and then decreased with the increase of administration times, indicating that the autophagy level changed from low to high (higher than the basal level), and then decreased to the basal autophagy level (Figures 6–12).

Correlation Analysis of LC3 Expression in Nasal Mucosa and Serum IL-5 Concentration

The expression of LC3 in nasal mucosa was positively correlated with serum IL-5 concentration during intraperitoneal administration (r = 0.549, $P < 0.05$).

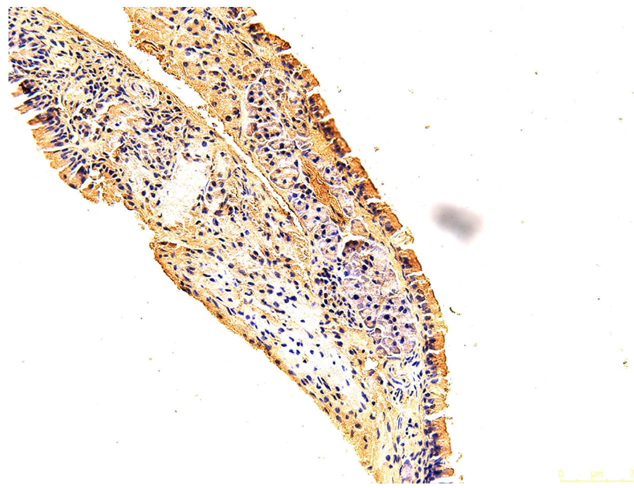

Figure 7 The immunohistochemical staining results of the day 0 group were observed under 200X microscope.

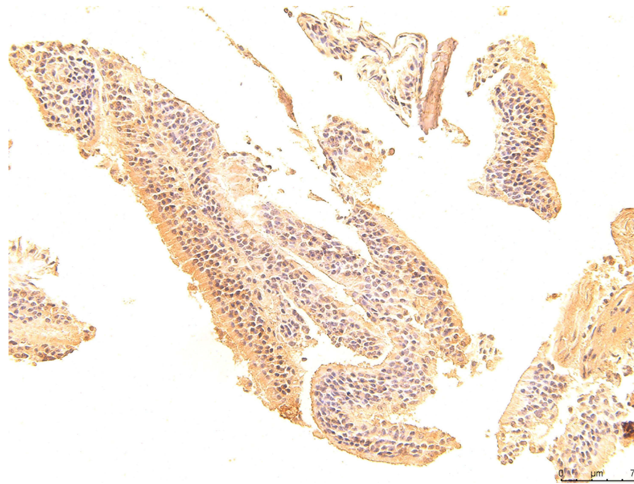

Figure 8 The immunohistochemical staining results of the day 7 group were observed under 200X microscope.

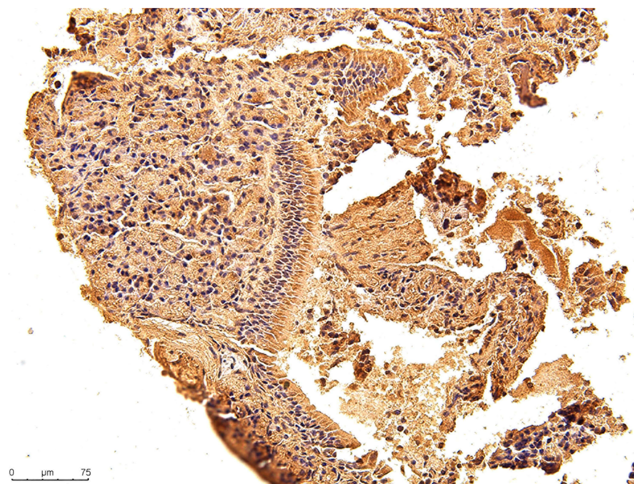

Figure 9 The immunohistochemical staining results of the day 14 group were observed under 200X microscope.

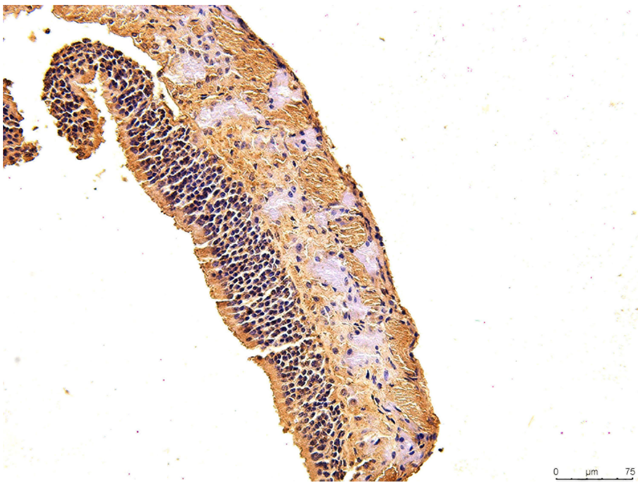

Figure 10 The immunohistochemical staining results of the day 21 group were observed under 200X microscope.

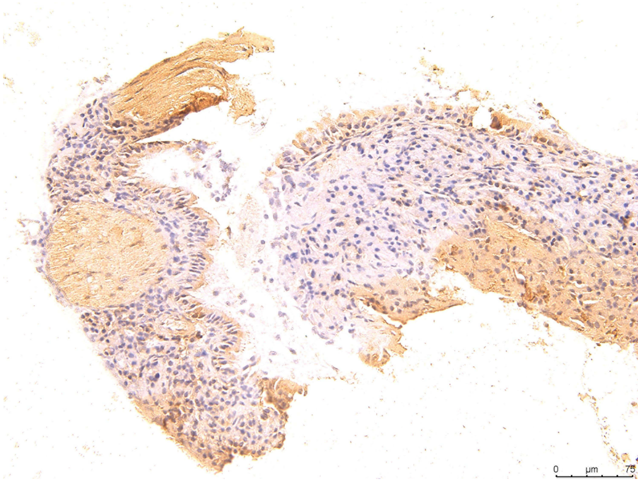

Figure 11 The immunohistochemical staining results of the day 24 group were observed under 200X microscope.

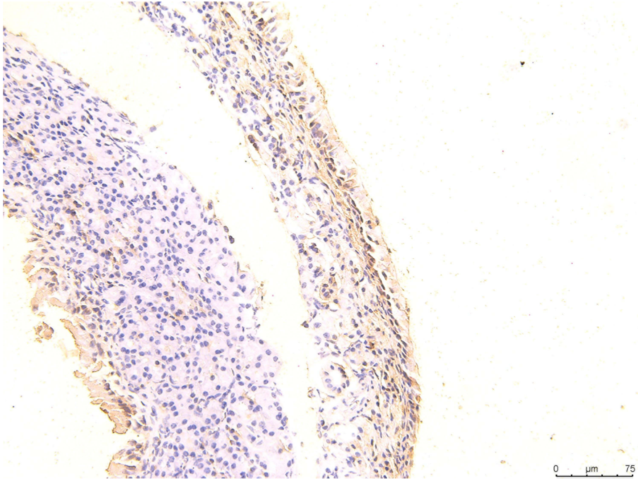

Figure 12 The immunohistochemical staining results of the day 27 group were observed under 200X microscope.

Discussion

The successful establishment of animal models is an important part of the study of the mechanisms of AR disease. In this experiment, the experimental group and control group mice were observed after all the administration of the drug and the experimental group mice showed obvious symptoms of sneezing, runny nose, and nasal itching, which were statistically different from the control group mice after the behavioral score. The nasal mucosa of mice in the experimental group was swollen, and the turbinate was hypertrophy, while the nasal mucosa of mice in the control group was light red, without swelling, and the turbinate was not hypertrophy. It was we observed by the H&E staining that the epithelial layer of the nasal mucosa of mice in the experimental group was significantly thickened, with disorderly arrangement of epithelial cells, thickened basement membrane, increased glands, and obvious lymphocyte infiltration, showing an obvious evident inflammatory infiltration. The nasal mucosa of the control mice was normal. These results indicate that intraperitoneal injection of Der f and aluminum hydroxide gel combined with nasal stimulation of Der f can effectively establish AR animal models. Many scholars have successfully established AR animal models by using ovalbumin[15] or dust mite[14] combined with aluminum hydroxide adjuvant. Intraperitoneal injection of sensitizing agent added with aluminum hydroxide adjuvant can effectively enhance TH1-type immune response.[16] Gel aluminum hydroxide has better affinity for ovalbumin than powder aluminum hydroxide and has better sensitization effect.[17] Different mouse species have different emphasis on the type of immune response, and Balb/C mice have more significant Th2 type immune response than C57BL/6 mice.[14] The choice of adjuvant, the different dosage forms of adjuvant, the different species of mice, the choice of sensitizer and the dose difference,[14] all have different effects on AR animal models. The animal model established in this study can simulate the process of human AR, which is helpful to understand the changes of cytokines and nasal mucosa function in each stage of AR and provide ideas for the early diagnosis of the disease.

In this experiment, samples were collected at each stage of drug administration, and the serum IL-4 and IL-5 curves of AR animal models were drawn. It was found that IL-4 and IL-5 did not change synchronously in the progression of AR disease. There was a significant peak of IL-5 at the stage of intraperitoneal administration, which was not significantly different from the level at the end of modeling, but IL-4 increased gradually throughout the progression of the disease. Although IL-4 and IL-5 belong to the same type 2 cytokine, which is mainly regulated by the expression of transcription factor GATA3 and plays an important role in maintaining the growth and survival of B cells in acquired immune response, the sources of IL-4 and IL-5 are not completely the same. IL-5 can also be produced by ILC2s, mast cells, eosinophils, NK and NKT cells, and epithelial cells.[18] IL-4 can also be derived from NKT cells, mast cells, basophils, eosinophils, and follicular helper Th cells.[19] The concentration of IL-5 increased gradually while the concentration of IL-4 did not change significantly during intraperitoneal administration, which may be due to the different sources of IL-4 and IL-5 in the early stages of AR disease. However, this study did not confirm the exact source of IL-5, so it cannot be used as direct evidence of different sources of IL-4 and IL-5 in the early stages of AR.

Autophagy, a hot topic in the research of respiratory allergic diseases in recent years, and its mechanism in AR is also being gradually explored. In this study, the AR mouse model was established with Der f as a sensitizer. The LC3 expression level in nasal mucosa of the tested mice gradually increased in the early stage of modeling, but there was no significant difference between the LC3 expression level in nasal mucosa of the tested mice and the control group at the end of modeling. This is different from previous studies on LC3 expression in nasal mucosa of AR patients and mice. LC3 expression was not uniformly elevated in animal models of asthma using ovalbumin as a sensitizer. The expression of LC3 was significantly increased in the asthma animal model established with ovalbumin as sensitizer, and the number of eosinophils in the alveolar lavage fluid was significantly decreased after the administration of autophagy inhibitor 3-MA.[20] In an animal study of asthma using ovalbumin as sensitizer, the expression level of autophagy-related gene LC3 mRNA was decreased.[21] We found that the change in mean optical density values in the AR experimental group compared with the control group increased and then decreased with increasing administration, indicating that the change in autophagy level from low to high (above basal level) and then decreased to basal autophagy level, indicating that the autophagy level in the nasal mucosa of mice with allergic rhinitis increased with repeated exposure to allergens and returned to basal autophagy level in the later stages of the disease. We suggest that nasal mucosal autophagy may also play a role in promoting the development of inflammation in the early stages of AR disease, and its elevated level

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correlates with the elevated level of IL-5, but the specific mechanism of nasal mucosal autophagy in AR needs to be further investigated.

Conclusion

In this study, a dynamic change of LC3 expression level in nasal mucosa was highlighted, and the changes of IL-4 and IL-5 concentrations of type 2 cytokines were detected during the same period. Although we found a positive correlation between LC3 and IL-5 expression in the early stage of AR, the pathway which LC3 leads to increased IL-5 secretion need to be explored in further studies.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon request.

Ethics Approval and Consent to Participate

This study was conducted with approval from the Ethics Committee of Yangtze University Health Science Center. Principles of Laboratory Animal Care (NIH Publication Vol 25, No. 28 revised 1996; http://grants.nih.gov/grants/guide/notice-files/not96-208.html) were followed, as well as specific national laws (eg the current version of the German Law on the Protection of Animals) where applicable.

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Disclosure

The authors report no conflicts of interest in this work.

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