

Combined Treatment With H1 and H4 Receptor Antagonists Improves Th2 Inflammatory Responses in the Nasal Mucosa of Allergic Rhinitis Rats

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Abstract

Background: Histamine H1 receptor (H1R) antagonists are the first-line drugs for the treatment of allergic rhinitis (AR) at present. Emerging evidence supports an important role of histamine H4 receptor (H4R) in allergic diseases. However, information regarding the effects of combined treatment with H1 and H4 receptor antagonists in AR is limited.

Objectives: We aimed to assess the effects of combined treatment with H1R and H4R antagonists on Th2 inflammatory responses in the nasal mucosa of AR rats.

Methods: Sprague Dawley rats were sensitized with ovalbumin and treated with H1R antagonist desloratadine or/and H4R antagonist JNJ7777120. Western blotting was used to assay the phenotypic markers of mature dendritic cells in the nasal mucosa, including major histocompatibility complex class II (MHC-II) and co-stimulatory molecules CD80, CD86 and OX40 ligand (OX40L). Th2 inflammatory cytokines including interleukin-4, 5 and 13 in nasal lavage fluids were determined by using enzyme-linked immunoassay.

Results: The treatment with desloratadine alone down-regulated the CD86 expression, and decreased the production of Th2 cytokines, but had no impact on the expression of MHC-II, CD80 and OX40L. The administration of JNJ7777120 alone reduced the levels of CD86, OX40L and Th2 cytokines, whereas MHC-II and CD80 expression was unaffected. The combination of desloratadine and JNJ7777120 showed more significant synergistic therapeutic effects than monotherapy.

Conclusion: H4R antagonist acted synergistically with H1R antagonist to reduce Th2 inflammatory responses by down-regulating CD86 and OX40L expression in the nasal mucosa of AR rats. The combination with H1R and H4R antagonists might be a new strategy for AR treatment.

Keywords

allergic rhinitis, histamine H4 receptor, histamine H1 receptor, major histocompatibility complex class II, co-stimulatory molecules, Th2 inflammatory responses

Allergic rhinitis (AR) results from type I hypersensitivity reactions associated with nasal inflammation of variable intensity.¹ Over the past 20 years, the prevalence of AR has been increasing, affecting 10–40% of the worldwide population.² AR seriously affects the patients' quality of life, and becomes a global problem with considerable socioeconomic and healthcare burden.³

The imbalance between Th1 and Th2 cells is identified as the main effector mechanism for AR. During this process, naïve CD4⁺ T cells preferentially differentiate into Th2 cells that release Th2 cytokines including

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interleukin (IL)-4, 5 and 13, leading to aberrant Th2 inflammatory responses.⁴⁻⁶ Accumulated evidence has revealed a critical role of mature dendritic cells (mDCs) in the induction of Th2 polarization.⁷ The mDCs capture and present antigens and thus activate naïve CD4⁺ T cells by expressing high levels of major histocompatibility complex class II (MHC-II) and co-stimulatory molecules including CD80, CD86 and OX40 ligand (OX40L), triggering the Th2 pathway.⁸⁻¹⁰ Understanding the pathways that regulate the expression of MHC II and co-stimulatory molecules might provide more specific strategies for AR therapy.

Histamine is a critical mediator in allergic diseases, and exerts its effects by binding to four receptors including histamine H1, H2, H3 and H4 receptors.¹¹ AR has long been considered to be mainly mediated by histamine H1 receptor (H1R), and H1R antagonists are recommended as the first-line drugs for AR therapy.¹² But H1R antagonists are not entirely effective in atopic dermatitis (AD), asthma and other allergic diseases.¹³ Histamine H4 receptor (H4R) is the most recently identified histamine receptor, and is expressed on numerous immune and inflammatory cells intimately associated with the pathology of allergic diseases.¹⁴ Proof for the effective results of H4R antagonists in models of AR, asthma and AD highlights H4R in allergic diseases.¹⁵⁻¹⁷

Recently, H1R and H4R antagonists have shown a synergistic anti-inflammatory effect in a mouse model of AD.¹⁷ However, it remains unknown whether this effect can be transferred into the *in vivo* situation of experimental AR. In this study, we hypothesized that H4R acted synergistically with H1R to promote Th2 inflammatory responses by regulating the expression of (MHC-II) and co-stimulatory molecules, and the combination of H1R and H4R antihistamines was an optimal therapeutic option for AR. To test this hypothesis, rats were sensitized with ovalbumin (OVA), and were treated with H1R antagonist desloratadine or/and H4R antagonist JNJ7777120. We investigated the effect and mechanism of the combination of H1 and H4 antagonists on Th2 inflammatory responses in AR compared with either antagonist alone.

Materials AND Methods

Animals

This study was approved by the Institutional Review Board of Huzhou University, and was performed in accordance with the accepted policy on the use of animals. Seventy Sprague Dawley rats, 200 to 250 g (Slaccas Co, Ltd, Shanghai, China), were randomized into 5 groups: group control (group A, 14 rats), group model (group B, 14 rats), group treated with desloratadine (group C, 14 rats), group treated with JNJ7777120

(Group D, 14 rats) and group treated with JNJ7777120 and desloratadine (Group E, 14 rats).

The Protocol for AR Model

The AR model was established according to literature-based standard protocols.¹⁸ Rats were immunized with an intraperitoneal injection of 1 ml normal saline (NS) containing 0.3 mg ovalbumin (OVA; Sigma, St Louis, Missouri) and 300 mg aluminum hydroxide (Alum; Sigma) once every other day. From day 14, 0.1 ml of NS containing 10 mg OVA was instilled into both nasal cavities of rat once a day for 7 days.

The Sensitization, Treatment and Sample Collection

Groups B, C, D, and E were sensitized according to the protocol above, whereas group A were treated with NS without OVA. After the establishment of AR, group C were intraperitoneally injected with desloratadine (10 mg/kg/day; Sigma) for 3 days, group D were intraperitoneally injected with JNJ7777120 (30 mg/kg/day; Tocris Bioscience, Bristol, United Kingdom) for 3 days, group E were intraperitoneally injected with desloratadine (100 mg/kg/day) and JNJ7777120 (30 mg/kg/day) for 3 days. Groups A and B were treated by intraperitoneal injection of NS for 3 days. Based on the previous studies, the dosages of desloratadine and JNJ7777120 were chosen.^{19,20}

Rats were sacrificed with an intraperitoneal overdose of sodium pentobarbital (100 mg/kg, Sigma) on three hours after the treatment. Before the nasal mucosa tissues were collected, an epidural anesthesia tube was placed and fixed into the nasal cavity at 2 cm. The rats were placed upside down, and the bilateral nasal cavities were lavaged with 1 ml NS. The nasal lavage fluids (NLF) were centrifuged at 3000 rpm/min for 15 minutes, and the supernatants were collected.

Western Blotting

The protein from nasal mucosa was extracted using radio-immunoprecipitation assay buffer (Beyotime, Haimen, Jiangsu, China). After denaturation, equal amounts of protein from each sample were prepared for 12% sodium dodecyl sulfate gel electrophoresis (Beyotime). Protein was transferred to PVDF membranes after electrophoresis. The membranes were blocked with blocking buffer, and then were incubated with a 1:200 dilution of mouse anti-MHC-II antibody (Abcam, Cambridge, MA), a 1:300 dilution of rabbit anti-CD80 (Abcam), a 1:300 dilution of mouse anti-CD86 (Abcam), a 1:200 dilution of rabbit anti-OX40L (4 A Biotech Co, Ltd, Beijing, China), or a 1:800 dilution of mouse anti- β -actin antibody (Beyotime). The membranes were then incubated with secondary antibody

(Beyotime). Antibody binding was detected with Beyond ECL Plus (Beyotime). The density of the bands was analyzed using Quantity One software (Silk Scientific Corporation, Orem, UT).

Enzyme-Linked Immunoassay

The concentrations of IL-4, 5 and 13 in NLF were analyzed using an enzyme-linked immunoassay (ELISA) kit (4 A Biotech). The procedures were performed according to the manufacturer's instructions. Optical density values were measured at 450 nm by an ELISA plate reader (Bio-Rad, Hercules, California).

Statistical Methods

Each data point was expressed as mean $\pm$ standard deviation (SD). SPSS 13.0 software (SPSS Inc, Chicago, IL) was used for statistical analysis. Each experiment was repeated seven times for each group. Statistical significance was evaluated by using analysis of variance, followed by a Dunnett's test. A P value <0.05 was considered to indicate statistical significance.

Results

To investigate the effects of H1R and H4R antagonists on antigen-presentation capability of DCs, the expression of MHC-II protein in the nasal mucosa was determined using Western blotting. As seen from Figure 1, the level of MHC-II protein in group B was 3.04-fold

higher than that in group A ($P < 0.05$). Although the expression in groups C, D and E was respectively decreased by 5.79%, 7.27% and 10.35% compared with group B ($P > 0.05$), no difference was observed. These results showed that H1R and H4R antagonists could not affect antigen-presentation capability of DCs.

To further identify the effect of H1R and H4R antagonists on the expression of co-stimulatory molecules, we detected the expression of CD80 and CD86 protein using Western blotting. As shown in Figure 2, a 7.59-fold increase of CD80 protein level was seen in group B compared with group A ($P < 0.05$), whereas the expression in groups C, D and E showed no difference compared with group B ($P > 0.05$). Figure 3 showed that a 4.34-fold increase of CD86 expression was observed in group B, when compared with group A ($P < 0.05$), but the expression was reduced by 36.50%, 63.26% and 80.44% in groups C, D and E compared with group B ($P < 0.05$). The level of CD86 protein in groups D and E was 42.14% and 69.19% lower than that in group C ($P < 0.05$), and was decreased by 46.75% in group E compared with group D ($P < 0.05$). These present results showed that H1R and H4R antagonists inhibited DC maturation in the nasal mucosa by down-regulating CD86 expression.

We then assayed the level of co-stimulatory molecule OX40L using Western blotting (Figure 4), founding the expression of OX40L protein in group B was 6.69 times higher than that in group A ($P < 0.05$). The level of OX40L protein in group C was decreased by 5.39% compared with group B, but this did not reach

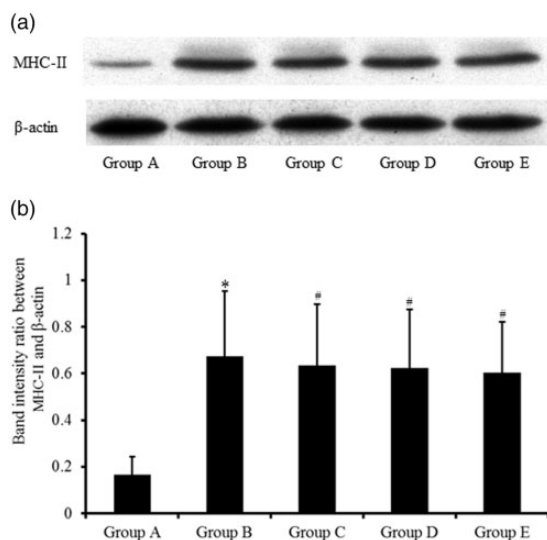


Figure 1. Western-blotting for MHC-II protein in the nasal mucosa. β -actin was used as loading control. The intensity of MHC-II bands was normalized to the intensity of β -actin bands. The band intensity ratio between MHC-II and β -actin was calculated, and the data are presented as mean $\pm$ SD ($n = 7$). * $P < 0.05$ vs group A, and # $P > 0.05$ vs group B.

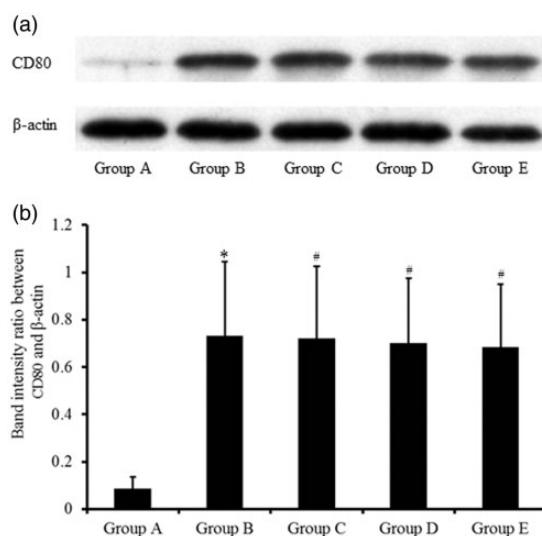


Figure 2. Western-blotting for CD80 protein in the nasal mucosa. β -actin was used as loading control. The intensity of CD80 bands was normalized to the intensity of β -actin bands. The band intensity ratio between CD80 and β -actin was calculated, and the data are presented as mean $\pm$ SD ($n = 7$). * $P < 0.05$ vs group A, and # $P > 0.05$ vs group B.

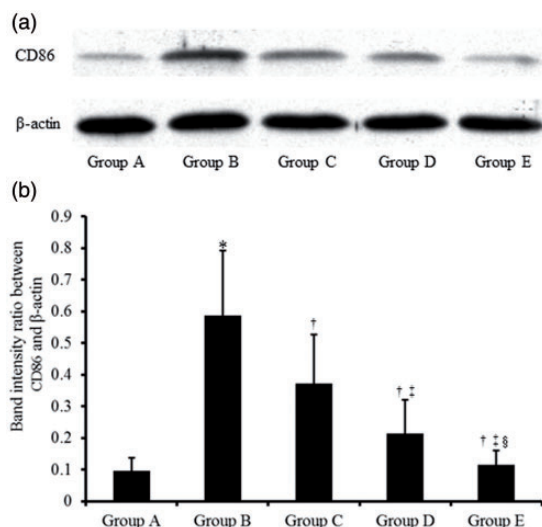


Figure 3. Western-blotting for CD86 protein in the nasal mucosa. β -actin was used as loading control. The intensity of CD86 bands was normalized to the intensity of β -actin bands. The band intensity ratio between CD86 and β -actin was calculated, and the data are presented as mean $\pm$ SD ($n = 7$). * $P < 0.05$ vs group A, † $P < 0.05$ vs group B, ‡ $P < 0.05$ vs group C, and § $P < 0.05$ vs group D.

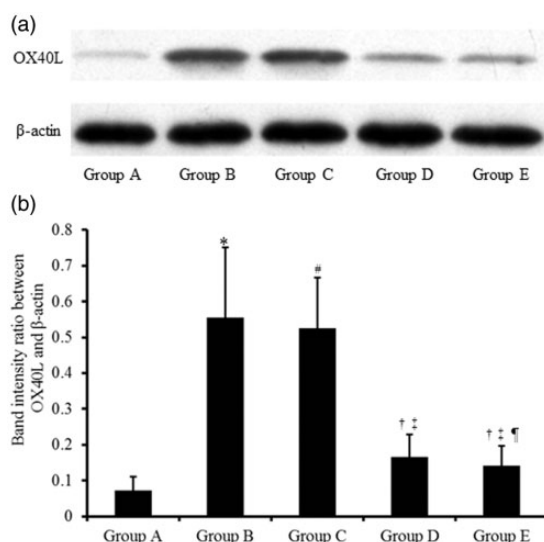


Figure 4. Western-blotting for OX40L protein in the nasal mucosa. β -actin was used as loading control. The intensity of OX40L bands was normalized to the intensity of β -actin bands. The band intensity ratio between OX40L and β -actin was calculated, and the data are presented as mean $\pm$ SD ($n = 7$). * $P < 0.05$ vs group A, # $P > 0.05$ vs group B, † $P < 0.05$ vs group B, ‡ $P < 0.05$ vs group C, and ¶ $P > 0.05$ vs group D.

significance ($P > 0.05$). The expression in groups D and E was 70.07% and 74.63% lower than that in group B ($P < 0.05$), and was decreased by 68.37% and 73.18% in groups D and E compared with group C ($P < 0.05$), but

there was no difference though the level of OX40L protein was decreased by 15.22% in group E compared with group D ($P > 0.05$). Our data indicated that H1R and H4R antagonists inhibited DC maturation in the nasal mucosa by down-regulating OX40L expression.

To investigate the effects of H1R and H4R antagonists on Th2 inflammatory responses, we assayed the concentrations of Th2 inflammatory cytokines in NLFs. First, we determined IL-4 level by using ELISA. As seen in Figure 5(A), a 4.35-fold increase of IL-4 level was observed in group B compared with group A ($P < 0.05$). The level in groups C, D, and E was respectively reduced by 26.33%, 49.78% and 68.27% compared with group B ($P < 0.05$), and expression in groups D and E was 31.83% and 56.94% lower than that in group C ($P < 0.05$). IL-4 level in group E was decreased by 36.83% compared with group D ($P < 0.05$). These results showed that the combination of H1R and H4R antagonists showed more effective in inhibiting IL-4 production than monotherapy.

Next, we determined IL-5 level in NLFs by using ELISA. Figure 5(B) showed that IL-5 level was increased by 3.20-fold in group B compared with group A ($P < 0.05$), whereas the expression was reduced by 23.92%, 45.19% and 62.57% in groups C, D and E compared with group B ($P < 0.05$). IL-5 expression in groups D and E was 27.96% and 50.80% lower than that in group C ($P < 0.05$), and was decreased by 31.70% in group E compared with group D ($P < 0.05$). Therefore, the combined treatment with H1R and H4R antagonists had a more significant inhibitory effect on IL-5 production than monotherapy.

Then, IL-13 concentration in NLFs was detected by using ELISA. From Figure 5(C), we observed that IL-13 level in group B was 2.99-fold higher than that in group A ($P < 0.05$). The level in groups C, D and E was 22.14%, 41.88% and 59.94% lower than in group B ($P < 0.05$), and the expression in groups D and E was decreased by 25.35% and 48.55% compared with group C ($P < 0.05$). IL-13 concentration in group E was significantly decreased by 31.08% compared with group D ($P < 0.05$). These results indicated that the treatment with either antagonist was effective in inhibiting IL-13 production, but the combination of both drugs enhanced this efficacy.

Discussion

Nasal DCs play an essential role in AR.²¹ In the nasal mucosa of AR patients, increased DCs are observed, and aberrant DC function conditions Th2 polarization.²² The capability of DCs to initiate Th2 immunity requires high levels of expression of MHC II and co-stimulatory molecules.²³ DCs express H1R, H2R and H4R, and are the major target of histamine.²⁴ H1R and H4R were

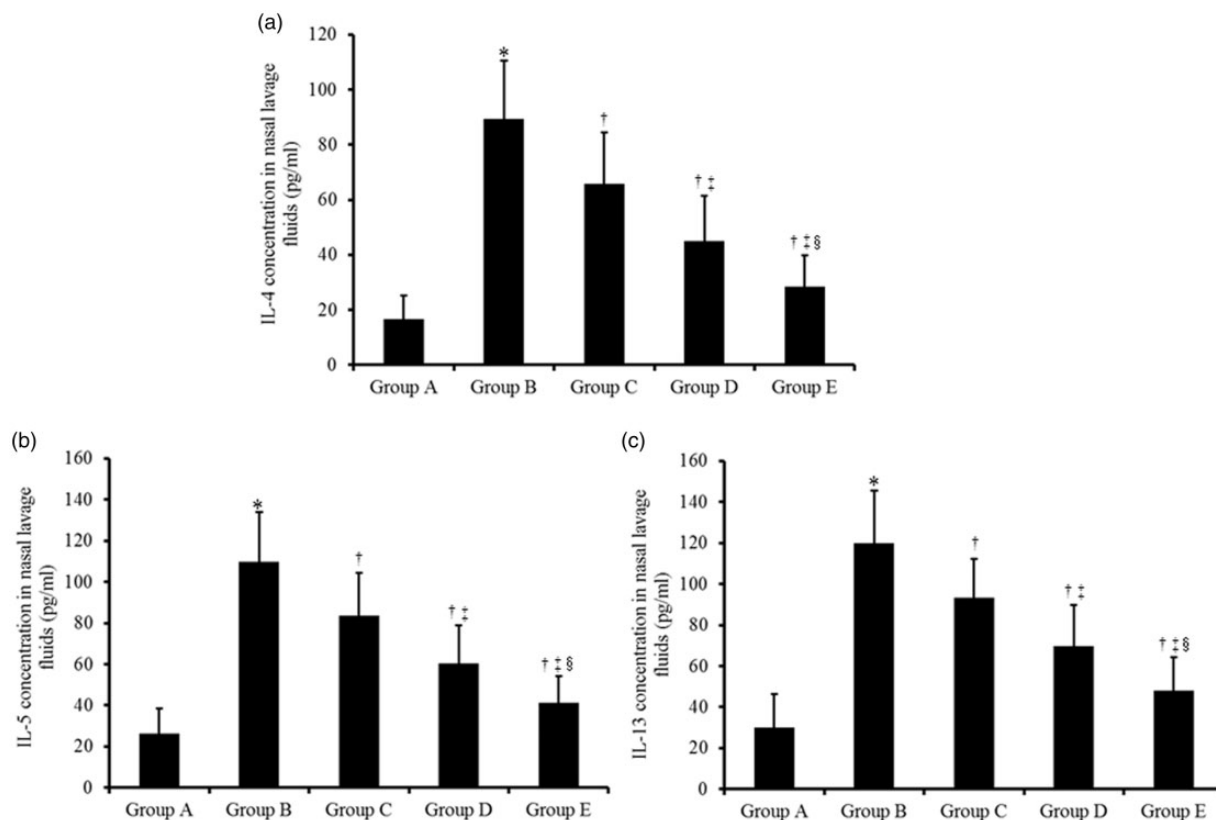


Figure 5. ELISA for the concentrations of Th2 cytokines in NLF. The data are presented as mean $\pm$ SD ($n = 7$). * $P < 0.05$ vs group A, † $P < 0.05$ vs group B, ‡ $P < 0.05$ vs group C, and § $P < 0.05$ vs group D.

shown to contribute to nasal allergic inflammation in AR.²⁵ In this study, we investigated whether H4R and H1R antagonists reduced Th2 responses by regulating MHC II and co-stimulatory molecules in AR.

A pivotal step to initiate Th2 immunity is the presentation of antigens by DCs. In the absence of stimulation signals, DCs remain in an immature state. Their function is regulated by a maturational process in which immature DCs capture and process antigens and are thus activated into mDCs, subsequently expressing with high level of MHC II.²⁶ MHC II is necessary for the presentation of antigens to T cells and the subsequent initiation of Th2 immunity.²⁷ In this study, the levels of MHC II protein were up-regulated in the nasal mucosa of AR rats, whereas the treatment with H1R antagonist desloratadine or/and H4R antagonist JNJ777120 had no impact on MHC II production. These data proved consistent with the previous results showing that H1R and H4R antagonist did not alter MHC II expression in histamine-stimulated DCs.²⁸ Hence, H1R and H4R antagonist could not affect the capacity of DCs to present antigens in AR.

The interaction between DCs and T cells requires signals mediated by co-stimulatory molecules. The mDCs highly express co-stimulatory molecules, providing the

signals for T-cell activation, expansion and differentiation.²⁹ Co-stimulatory molecules CD80/CD86 on mDCs that interact with CD28 on T cells are necessary for the difference of CD4⁺ T cells into Th2 cells.³⁰ Inhibition of CD80/CD86 expression reduces Th2 immune responses in asthma mice.³¹ Our data showed that the expression of CD80/CD86 protein was increased in the nasal mucosa of AR rats. The treatment with desloratadine or JNJ777120 alone reduced CD86 production, but had no effect on CD80 production. Previous study showed that H1R or H4R antagonist reduced histamine-induced CD86 expression on DCs in vitro, while CD80 expression was unaffected.²⁸ Our results were consistent with this report. We also found that the combination of desloratadine and JNJ777120 was more effective in inhibiting CD86 expression than monotherapy.

Co-stimulatory molecule OX40L is also a well-known phenotypic marker of mDCs, and interacts with OX40 on T cells to induce Th2 polarization.³² Inhibition of OX40L expression reduces Th2 responses in AR mice.³³ We found that the levels of OX40L protein were increased in the nasal mucosa of AR rats. The treatment with JNJ777120 alone or in combination with desloratadine reduced OX40L production, whereas

desloratadine alone had no impact on OX40L production. Thymic stromal lymphopoietin (TSLP) is a key player by induction of OX40L on DCs.³⁴ TSLP is mainly produced by epithelial cells, and its expression is increased in the nasal mucosa of AR patients.^{35,36} Recently, H4R activation is shown to induce TSLP production by epithelial cells in nasal mucosa and skin.^{15,37} We concluded that H4R antagonist reduced OX40L expression in the nasal mucosa by inhibiting TSLP production. H1R was found to be not involved in TSLP production by nasal epithelial cells.¹⁵ Therefore, H1R antagonist had no effect on TSLP expression in this study. This is also the possible reason why OX40L level showed no difference between JNJ777120 monotherapy and the combined treatment with JNJ777120 and desloratadine in our results.

AR is characterized by Th2 inflammatory responses in the nasal mucosa.³⁸ Th2 cells are crucial for the initiation and maintenance of allergic immune responses, with overproduction of Th2 inflammatory cytokines at nasal mucosal sites, including IL-4, 5 and 13.³⁹ Th2 inflammatory cytokines induce the activation of immunological effector system assembled by B cells, eosinophils and mast cells, leading to immunoglobulin E production, mast-cell degranulation, mucus hypersecretion, and eosinophilic inflammation.⁴⁰ The levels of Th2 cytokines are responsible for Th2 inflammatory responses in allergic diseases.⁴¹ In this study, the concentrations of IL-4, 5 and 13 in NLF were increased in AR rats. JNJ777120 alone significantly inhibited the levels of Th2 cytokines. Previous report showed that H4R antagonists reduced Th2 responses in AR rats with decreased Th2 cytokines.¹⁵ Desloratadine alone also decreased the levels of Th2 cytokines, but this effect was weaker than JNJ777120 alone. We also found that the combination of desloratadine and JNJ777120 showed more significant inhibitory effects on Th2 cytokines than monotherapy.

Increasing evidence has shown that a combination of H1 and H4 antagonists is more effective than H1R antagonists alone in allergic diseases, which counteracts the disadvantages of monotherapy.⁴² Our present results indicated that individual treatment with either antagonist was effective in inhibiting Th2 inflammatory responses, but the combination of both drugs maximized efficacy. Our preliminary evidence obtained in this study showed that the combined treatment had more significant therapeutic effects, and elucidated the mechanism by which H4R and H1R antagonists regulated Th2 inflammatory responses. Based on these experimental data, there may be benefit in treating AR patients with the combination of H1R and H4R antagonists when the H4R antagonists become clinically available.

However, there are some limitations in this study. The interaction between costimulatory molecules on DCs

and their receptors on T cells triggers the differentiation of naive CD4⁺ T cells. We only determined the levels of costimulatory molecules CD80, CD86 and OX40L in the nasal mucosa, and did not assay the receptors of these costimulatory molecules such as CD28 and OX40. We plan to investigate the effect of H4R and H1R antagonists on CD28 and OX40 expression. In the present study, we investigated the effect of H4R and H1R antagonists on the markers of mDCs at nasal mucosal sites, but it remains unknown whether this effect can be transferred into DCs in peripheral blood. In the future, we will evaluate how H4R and H1R antagonists affects DCs in peripheral blood. Furthermore, a reliance on pharmacological inhibitors is one weakness of this study. We get the results on nasal samples and NLF by using sensitized rat model. However, behavior changes of animals are also important in predicting the clinical effect of H4R antagonist. In the next experiment, we will evaluate the changes of allergy signs such as sneezing, runny nose and rubbing nostrils.

Conclusion

H4R antagonist reduced Th2 inflammatory responses by inhibiting CD86 and OX40L expression in the nasal mucosa of AR rats, and H1R antagonist exerted this effect by down-regulating CD86 production. The combined treatment with H4R and H1R antagonists had synergistic effects, and was more effective than monotherapy. Therefore, the combination of H1R and H4R antagonists might be an optimal strategy for AR therapy.

Author Contributions

W. W.: Final approval of the version to be published. H. Y.: Data analysis and final approval. Y. P.: Data analysis and final approval.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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