

Inhibition effect of blunting Notch signaling on food allergy through improving T_H1/T_H2 balance in mice

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ABSTRACT

Background: Notch signaling regulates proliferation, differentiation, and function of dendritic cells, T cells, and mast cells, as well as many other immune cells, which act as important parts in food allergy. Notch signaling may play an important role in food allergy.

Objective: To investigate the role of Notch signaling in IgE-mediated food allergy.

Methods: An ovalbumin-induced food allergy mouse model was built (cholera toxin as adjuvant) and Notch signaling was blunted by FLI-06 and MW167, which inhibited Notch receptor-expressing phase and the γ -secretase-affecting phase, respectively. Then food allergy indicators, including levels of serum antibodies, cytokines, and degranulation, were examined. Meanwhile, clinical features, such as vascular permeability changes, intestinal permeability changes, body temperature changes, and symptoms, were also observed.

Results: After blunting Notch signaling, the levels of serum ovalbumin specific IgE and IgG₁ were decreased significantly, suggesting that blunting Notch signaling inhibited antibody responses. The levels of T_H1 cytokines (interferon- γ) were increased significantly, whereas the levels of T_H2 cytokines (interleukin-4, -5, and -13) were decreased significantly, suggesting T_H2 polarization was suppressed after blunting Notch signaling. The expression of T-bet was significantly increased, whereas the expression of Gata-3 was significantly reduced in both messenger RNA and protein levels, indicating T_H2 polarization was inhibited and T_H1 polarization was enhanced after blunting Notch signaling. Moreover, allergic clinical features of mice were alleviated after blunting Notch signaling.

Conclusion: Food allergy was inhibited by blunting Notch signaling through suppressing T_H2 polarization, enhancing T_H1 cell differentiation and promoting T_H1/T_H2 balance in mice. Notch signaling plays a key role in IgE-mediated food allergy.

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Introduction

Food allergy often induces severe allergic diseases complications and seriously affects quality of life. A total of 3% to 4% of adults and 5% of infants have food allergies every year in developed countries.¹ The underlying mechanism of this immune disorder currently remains a mystery. Most food allergies are IgE-mediated type 1 immediate hypersensitivity. CD4⁺ T cells activation and differentiation are key steps in IgE-mediated food allergy.² The naive CD4⁺ T cells selectively differentiate into different subtypes, including interferon- γ (IFN- γ)-producing T_H1; interleukin (IL)-4, IL-5, IL-13 secreting T_H2, IL-17A producing T_H17; and inducible regulatory T cell lineages. T_H2 polarization and T_H2-dominant

immune response are major contributors to food allergy.^{3,4} Unfortunately, the mechanism of T_H2 polarization induction is still unclear. The differentiation of naive T cells depends on various factors; the recognition of allergen presented to CD4⁺ T cells by antigen-presenting cells (APCs) is a major effector axis. Many molecules expressed on the surface of T cells and APCs have capacities to drive T_H1 or T_H2 polarization, such as Toll-like receptors on dendritic cells inducing T_H1 polarization⁵ and the costimulatory molecules OX40 on T cells and OX40L on APCs driving T_H2 polarization.⁶

Recently, the expression of Notch ligands on APCs in concert with Notch receptors on T cells has been found to play a key role in T_H1- or T_H2-promoting stimuli.^{7–10} Four Notch receptors (Notch1, 2, 3, and 4) and 5 known ligands (Jagged 1 and 2 and Delta-like 1, 3, and 4) have been found in mammals.¹¹ As a highly conserved signaling pathway, Notch signaling is induced when the Notch receptor encounters the ligand. This interaction finally initiates the release of Notch intracellular domain (NICD), which is affected by γ -secretase. Then the NICD translocates to the nucleus to induce the transcription of genes.¹² Data from several laboratories revealed

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that Notch signaling is required in T_H2 -cell differentiation.^{13–15} However, the role of Notch signaling in T_H1 differentiation is still controversial. Many studies have found that Delta-like proteins indeed promoted the differentiation of naive $CD4^+$ T cells into the T_H1 -cell lineage,^{16–18} but some researchers found that it was not essential to T_H1 cell differentiation by using Notch knockout mice.^{13,19} A number of studies have found that Notch signaling plays an important role in a variety of diseases, such as cancer,²⁰ diabetes,²¹ and asthma,²² but its role in food allergy was not explored.

In this study, the role of Notch signaling in IgE-mediated food allergy was explored. We speculated that Notch signaling was necessary in IgE-mediated food allergy. An ovalbumin-induced IgE-mediated food allergy mouse model was built, and Notch signaling was blunted by FLI-06 and MW167, which inhibited Notch receptor-expressing phase and the γ -secretase-affecting phase, respectively. Then, related indicators of food allergy were examined. The study will define the role of Notch signaling in IgE-mediated food allergy and provide evidence of potential therapy of food allergy.

Methods

Mice

Forty-eight female specific pathogen free BALB/c mice (14–18 g) with 4 weeks of age were obtained from Weitong Lihua Inc (Beijing, China) and left to acclimatize for 7 days. These mice were provided with fresh water and diet without ovalbumin ad libitum, maintained at a mean (SD) temperature of 23°C (3°C) and a relative humidity of 40% to 70% with a light/dark cycle of 12 hours. All experimental studies were conducted under a protocol approved by the Animal Ethics Committee of China Agricultural University.

Experimental Protocol

Mice were grouped into the ovalbumin group, dimethyl sulfoxide (DMSO) group, MW167 group, or FLI-06 group ($n = 12$) randomly. All groups were sensitized intragastrically with 1 mg of ovalbumin (Sigma, St Louis, Missouri) plus 10 μ g of cholera toxin (CT) (Sigma) per mouse on days 0, 7, 14, 21, and 28. Mice in the FLI-06 and MW167 groups received FLI-06 (1 mg/kg) and MW167 (1 mg/kg) treatment by tail vein injection 1 hour before gavage each time, respectively, and mice in the DMSO group received DMSO treatment (0.1% concentration). All sensitized mice were orally challenged with 10 mg of ovalbumin on day 42.

Antigen Specific IgE, IgG₁, and IgG_{2a} Analysis

Blood samples were collected from the orbital venous plexus of mice eyes at on day 35 and were centrifuged for 10 minutes at 3000g and 4°C after coagulation for 2 hours at room temperature to obtain the serum. The serum were stored at –80°C before being analyzed for antigen specific IgE, IgG₁, and IgG_{2a} by enzyme-linked immunosorbent assay (ELISA).

Assessment of Hypersensitivity Responses

Symptoms of systemic anaphylaxis were assessed within 45 minutes after the challenge on day 42. Symptoms were evaluated by using a scoring system modified slightly from previous reports²³ and scored as follows: 0, no symptoms; 1, scratching and rubbing around the nose and head; 2, puffiness around the eyes and mouth, pilar erection, reduced activity, and/or decreased activity with increased respiratory rate; 3, wheezing, labored respiration, and cyanosis around the mouth and the tail; 4, no activity after prodding or tremor and convulsion; and 5, death. Body temperatures were recorded before and after challenge using a WI88375 probe (Beijing Science and Technology, Beijing, China).

Measurement of Plasma Histamine, Serum Mouse Mast Cell Protease 1, and Serum Diamine Oxidase

Blood samples were collected from orbital venous plexus of mice eyes 30 minutes after challenge on day 42 and were collected in centrifugal tubes to obtain plasma, which contained heparin. Blood samples were collected again to obtain serum 45 minutes after challenge. Blood samples were centrifuged for 10 minutes at 3000g and 4°C after coagulation for 2 hours at room temperature to get the plasma and serum. The plasma samples were stored at –80°C before being analyzed for plasma histamine levels by a commercial mouse ELISA kit (eBioscience Inc, San Diego, California), and the serum were stored at –80°C before being analyzed for diamine oxidase (DAO) levels and mouse mast cell protease 1 (mMCP-1) levels by commercial mouse ELISA kits (eBioscience Inc).

Determination of Cytokine Levels

Mesenteric lymph nodes (MLNs) and spleens were isolated from mice in the ovalbumin group. Single-cell suspensions were prepared as described previously.²⁴ MLN and spleen single suspensions were separately suspended in complete RPMI 1640 medium, which contained 10% fetal calf serum and 1% antibiotics (penicillin and streptomycin) (Zhejiang Tianhang Biotechnology, Shanghai, China) at 10^6 cells/mL. For the culture steps, aliquots of each cell population were loaded into wells of 96-well plates (2×10^5 cells per well) and were separated into the ovalbumin group (control), DMSO group, FLI-06 group, and MW167 group, which were incubated only with DMSO (0.1%), MW167 (20 μ M), and FLI-06 (20 μ M), respectively. Then, ovalbumin was add in the well to the final concentration of 100 μ g/mL. The plates were cultured for 3 days at 37°C with 5% carbon dioxide. In the supernatants, the levels of IFN- γ , IL-4, IL-5, IL-13, and IL-17A were determined with mouse ELISA kits (eBioscience Inc).

Real-Time Polymerase Chain Reaction

Total RNA was extracted from cultured cells and from MLNs and spleens directly by using the TRIzol reagent (Invitrogen, Waltham, Massachusetts) and reverse transcribed to generate first-strand complementary DNA (SuperScript II reverse transcriptase, Invitrogen). SYBR Green Master Mix kit (Takara, Tokyo, Japan) was used for real-time polymerase chain reaction (PCR) with the Bio-Rad iCycler iQ system (Bio-Rad). The real-time PCR was performed under the following conditions: 95°C for 30 seconds followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. As a control, each complementary DNA sample was simultaneously subjected to β -actin amplification. The real-time PCR primer sequences used were as follows: IFN- γ , 5'-CGCTCTTGGTTTTCAGCTC-3' (sense) and 5'-ACCGTCTTTTGCAGTCC-3' (antisense); IL-4, 5'-CACCTTGCTGT-CACCTGTT-3' (sense) and 5'-CGTTCCTCGTGTTCCTT-3' (antisense); IL-17A, 5'-CACTTCACAAGTCGGAGGCT-3' (sense) and 5'-TCTGACAGTGCATCATCGCT-3' (antisense); and β -actin, 5'-TGTGTCGCTCGTGGATCTGA-3' (sense) and 5'-TTGCTGTGAAGTCGAGGAG-3' (antisense); NICD, 5'-CCAACTGAGGACAGACGGAC-3' (sense) and 5'-GGGATCAGAGGCCACATAGC-3' (antisense); Hes-1, 5'-CGAGTGCATGAACGAGGTGA-3' (sense) and 5'-ATCTGGGTCATGCAGTTGGC-3' (antisense); Gata-3, 5'-AAGCTAGTATCCGCTGACG-3' (sense) and 5'-GATACCTCTGCACCGTAGCC-3' (antisense); T-bet, 5'-CGTTTCTACCCGACCTTCC-3' (sense) and 5'-ATGCTCACAGCTCGGAATC-3' (antisense); The threshold cycle (Ct) for each sample was determined according to the manufacturer's instructions, and the relative level of each transcript ($2^{\Delta Ct}$) was calculated from ΔCt (test Ct – β -actin Ct).

Western Blotting

Proteins were exacted from MLNs and spleens and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and

then the separated proteins were transferred to polyvinylidene fluoride membranes, which were blocked in Tris-buffered saline, 0.1% Tween 20, and 5% milk and incubated with primary antibodies overnight at 4°C. After incubation, the membranes were incubated with specific horseradish peroxidase–conjugated secondary antibodies for 2 hours at room temperature. Finally, the membranes were incubated in ECL reagents (Pierce Biotechnology, Waltham, Massachusetts) and exposed to film. For semiquantification of the expression levels, the blots were scanned, and the density of each band was determined using ImageJ Software (Bethesda, Maryland).

Histologic Analysis

The liver, kidney, and small intestine of 3 mice per group were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. Hematoxylin-eosin (HE) staining was then performed to permit examination of inflammation and damage in each organ. Mast cells degranulation in small intestine were detected using toluidine blue staining.

Statistical Analysis

Statistical analysis was performed using SPSS statistical software, version 13.0 (SPSS Inc, Chicago, Illinois), and GraphPad Prism 5 (GraphPad Software, San Diego, California). One-way analysis of variance was used to determine significant differences between groups. Significance was defined as $P < .05$.

Results

The expressions of NICD and *Hes-1* were decreased significantly after being treated with FLI-06 and MW167

Notch signaling works, depending on the release of NICD and the transcription of target genes, such as *Hes-1*.¹² In this study, we detected the expressions of NICD and *Hes-1* in MLNs and spleen by quantitative reverse-transcription PCR and Western blotting, respectively. The results indicate that the expressions of NICD and

Hes-1 were significantly decreased in both messenger RNA (mRNA) and protein levels in MLNs and spleen after treatment with FLI-06 and MW167 ($P < .05$). However, there was no significant difference in expression between the FLI-06 and MW167 groups (Fig 1). These results indicated that FLI-06 and MW167, which inhibited the expression of Notch receptors and the effect of γ -secretase, respectively, blunted Notch signaling effectively and had a similar inhibitory effect.

The levels of ovalbumin specific IgE and IgG₁ were reduced significantly after blunting Notch signaling

Sun Na et al²⁵ found that the levels of serum antibodies reached their maximum on day 35 during the establishment process of food allergy mouse model. Therefore, the levels of serum ovalbumin specific IgE, IgG₁, and IgG_{2a} were measured by ELISA on day 35. As shown in Figure 2, the levels of serum antibodies of mice in the DMSO group had no difference with those in the ovalbumin group, suggesting that DMSO did not affect the antibody responses. The level of ovalbumin specific IgE and IgG₁ was significantly reduced after treatment with FLI-06 and MW167 ($P < .05$). Although the level of specific IgG_{2a} was slightly decreased, there was no significant difference when compared with the ovalbumin group ($P > .05$) (Fig 2). In addition, there was no significant difference in antibody levels between the FLI-06 and MW167 groups. Our results indicated that the IgE and T_H2-antibody response were significantly inhibited after blunting Notch signaling in mice.

The levels of T_H2 cytokines and T_H17 cytokines were reduced significantly after blunting Notch signaling

CD4⁺ T cells and many other immune cells maintain the immune balance through secreting cytokines after differentiation and maturation.^{26,27} In this study, MLN and spleen single-cell suspensions from the mice in the ovalbumin group were obtained and cultured with ovalbumin for 72 hours under different conditions in vitro. There was no significant difference in any

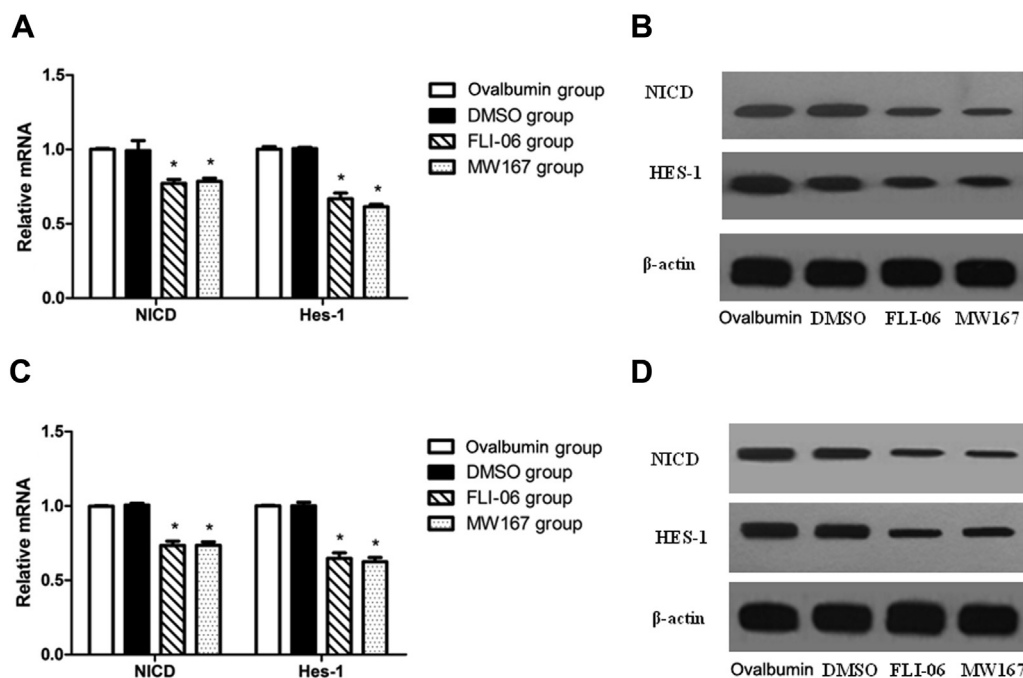


Figure 1. The messenger RNA (mRNA) and protein levels of Notch intracellular domain (NICD) and *Hes-1* of different groups. A and B, The mRNA and protein levels of NICD and *Hes-1* in mesenteric lymph nodes. C and D, The mRNA and protein levels of NICD and *Hes-1* in spleens. * $P < .05$ vs the ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 3$). Error bars indicate SDs.

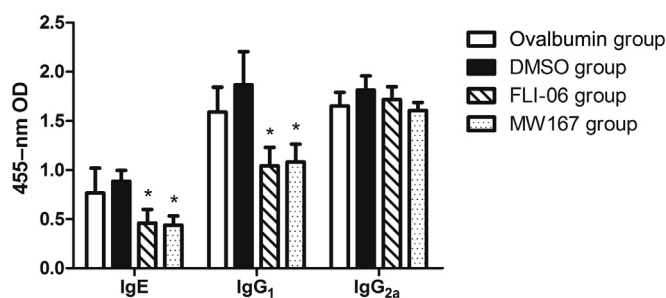


Figure 2. The levels of serum ovalbumin specific IgE, IgG₁, and IgG_{2a}. * $P < .05$ relative to the ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 12$). Error bars indicate SDs.

cytokine levels between the DMSO and OVA groups, which illuminated that DMSO did not affect the cytokine secretion. However, the levels of IL-4, IL-5, and IL-13 were significantly reduced after being treated with FLI-06 and MW167 ($P < .05$), whereas the level of IFN- γ was significantly increased ($P < .05$). In addition, the levels of IL-17A and T_H2 cytokines were also significantly decreased ($P < .05$) (Fig 3). There were no significant differences in any cytokine levels between the FLI-06 and MW167 groups ($P > .05$). These results indicate that T_H2 cytokine secretion was significantly decreased, whereas T_H1 cytokines were significantly increased after blunting Notch signaling, which promoted the T_H1/T_H2 balance in mice. In addition, the results also indicated that T_H17 immune response might also be suppressed after blunting Notch signaling.

The level of degranulation was decreased significantly after blunting Notch signaling

The levels of degranulation in mice were analyzed by detecting the levels of serum mMCP-1 and plasma histamine. As shown in

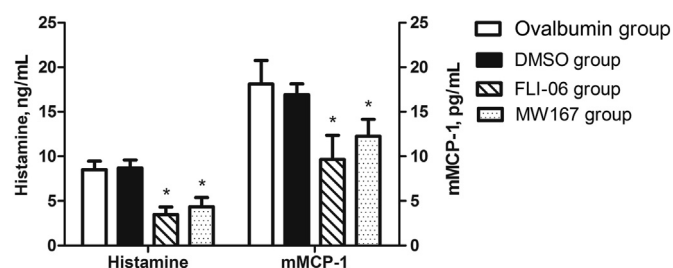


Figure 4. Quantity of plasma histamine and serum mouse mast cell protease 1 (mMCP-1) of mice of all groups. * $P < .05$ vs ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 12$). Error bars indicate SDs.

Figure 4, the levels of serum mMCP-1 and plasma histamine were significantly decreased ($P < .05$) after treatment with FLI-06 or MW167, and there was no difference between the FLI-06 and MW167 groups ($P > .05$) (Fig 4), which indicated that FLI-06 and MW167 had a similar inhibition effect on degranulation. In addition, there was no difference between the DMSO and OVA groups ($P > .05$) (Fig 4). These results suggested that the degranulation of mast cells and basophils in mice in the ovalbumin and DMSO groups occurred after the challenge, which released a large number of inflammatory mediators, and the degranulation was suppressed after blunting Notch signaling.

Intestinal and vascular permeability was decreased after blunting Notch signaling

Many studies found that intestinal permeability increases after food allergy.²⁸ Therefore, the changes of intestinal permeability in mice were evaluated after the challenge by detecting the content of DAO in serum. Compared with the ovalbumin and DMSO groups, both the FLI-06 and MW167 groups exhibited significant decrease in concentration of serum DAO ($P < .05$) (Fig 5A), indicating that the

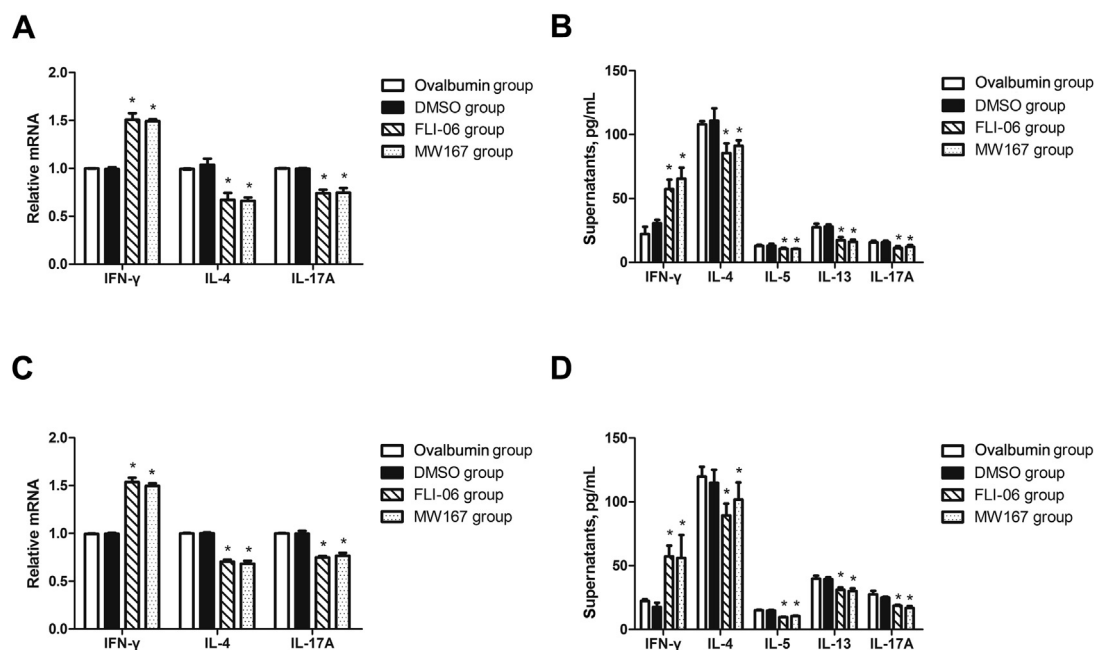


Figure 3. Real-time polymerase chain reaction (PCR) analysis and quantity of cytokines after different treatments. Real-time PCR analysis of cytokines in mesenteric lymph nodes (MLN) cells (A) and spleen cells (C) of allergic mice after different treatments. Quantity of interferon- γ (IFN- γ), interleukin (IL)-4, IL-5, IL-13, and IL-17A in the supernatants of cultured MLN cells (B) and spleen cells (D) after different treatments. * $P < .05$ vs the ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 3$). Error bars indicate SDs.

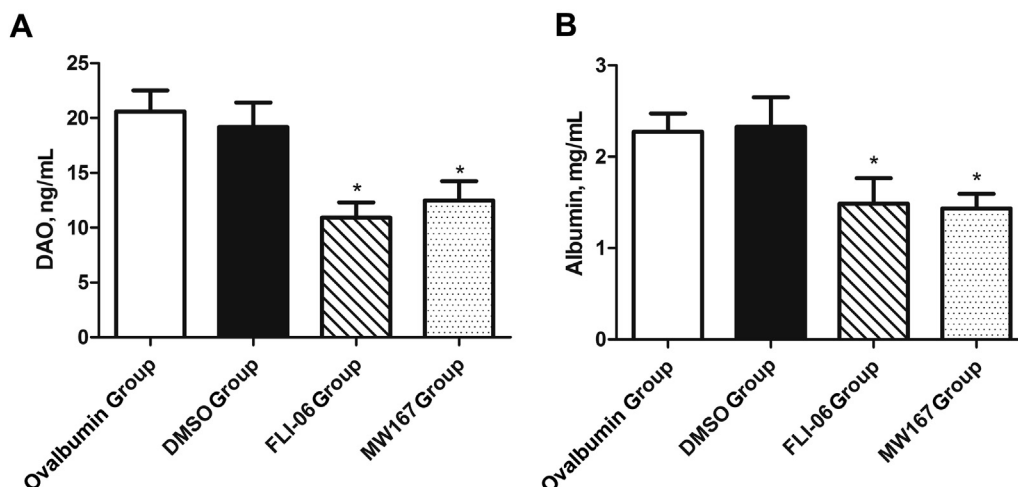


Figure 5. Quantity of albumin in peritoneal fluid and serum diamine oxidase (DAO) after challenge. * $P < .05$ vs the ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 9$).

intestinal permeability of mice was significantly reduced after blunting Notch signaling.

Because systemic allergy symptoms were related to the increase of vascular permeability closely,²⁹ we detected the concentration of albumin in the peritoneal fluid to evaluate changes of vascular permeability. As shown in Figure 5B, the concentration of albumin was significantly decreased after treatment with FLI-06 and MW167 ($P < .05$), indicating that the vascular permeability was decreased after blunting Notch signaling. In addition, the concentration of albumin revealed no significant difference between the DMSO and ovalbumin groups ($P > .05$), suggesting that DMSO did not affect the changes of vascular permeability.

The clinical symptoms of allergy were alleviated after blunting Notch signaling

Mice with food allergies usually had by a decrease in body temperature and a range of clinical symptoms, such as scratching ears and asthma.³⁰ After the challenge, the body temperature was measured and the symptoms were observed in each group. As shown in Figure 6A, mice in the ovalbumin and DMSO groups had a decrease in body temperature, whereas mice in the FLI-06 and

MW167 groups did not (Fig 6A). In addition, mice in the ovalbumin and DMSO groups had more severe clinical symptoms, whereas mice in the MW167 and FLI-06 groups did not have obvious symptoms or had very weak allergic symptoms (Fig 6B), suggesting that the clinical symptoms of allergy were alleviated after blunting Notch signaling in mice.

The expression of Gata-3 was significantly decreased, whereas the expression of T-bet was significantly increased after blunting Notch signaling

On the basis of the above results, we considered that food allergy was inhibited by blunting Notch signaling. To explore the mechanism, the key transcription factor *T-bet* of T_H1 -type immune response and *Gata-3* of T_H2 -type immune response were examined in both mRNA and protein levels. The results indicate that the expression of *Gata-3* was significantly decreased, whereas the expression of *T-bet* was significantly increased ($P < .05$) in both mRNA and protein levels after treatment with FLI-06 and MW167 (Fig 7), and there was no difference between the FLI-06 and MW167 groups, indicating that inhibition of the Notch receptor-expressing phase and the γ -secretase-affecting phase had a similar effect.

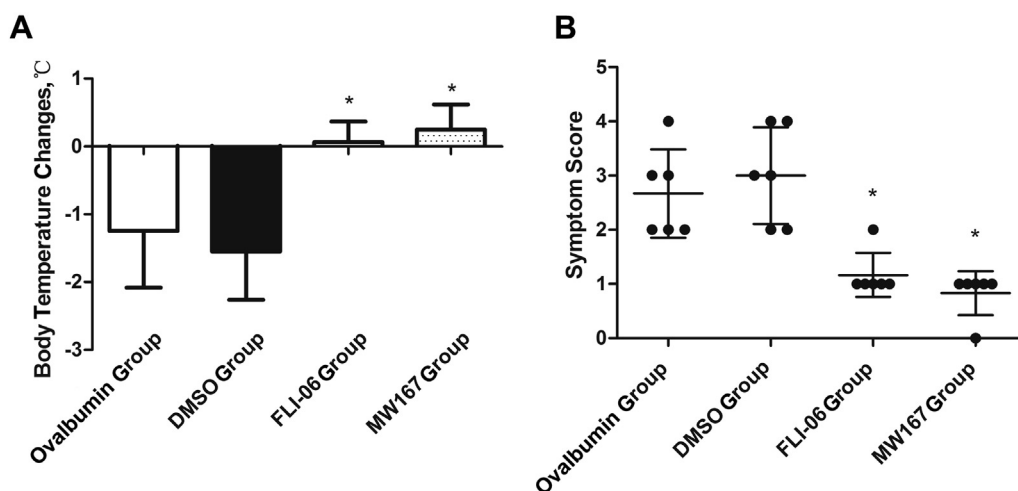


Figure 6. Clinical symptoms after challenge of different groups. A, Body temperature changes; B, symptom scores of different groups. * $P < .05$ vs the ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 6$). Error bars indicate SDs.

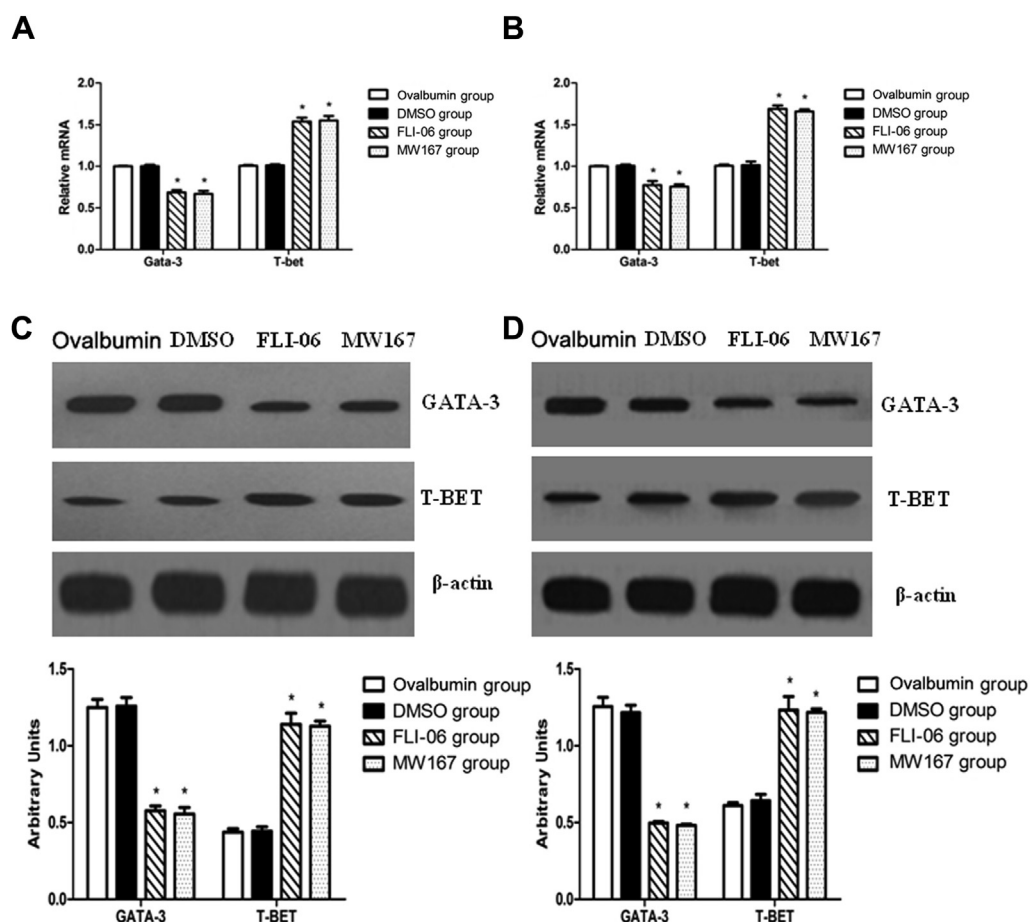


Figure 7. The messenger RNA (mRNA) and protein levels of Gata-3 and T-bet of different groups. Real-time polymerase chain reaction (PCR) analysis (A) and Western blotting analysis (C) of Gata-3 and T-bet in mesenteric lymph nodes. Real-time PCR analysis (B) and the Western blotting analysis (D) of Gata-3 and T-bet in spleens. * $P < .05$ vs the ovalbumin group and dimethyl sulfoxide (DMSO) group ($n = 3$). Error bars indicate SDs.

These results suggest that blunting Notch signaling inhibited IgE-mediated food allergy in mice through suppressing T_H2 -type immune response, enhancing T_H1 -type immune response, and promoting T_H1/T_H2 balance or at least partly.

Pathologic Analysis

To determine the toxicity of DMSO to mice, HE staining in liver and kidney of mice was observed. The structure of liver and kidneys in the DMSO, MW167, and FLI-06 groups was normal, and no obvious pathologic changes were apparent compared with the ovalbumin group (Fig 8A), suggesting that this dose of DMSO had no toxic effects on mice. HE staining results revealed that the small intestine of mice in the ovalbumin and DMSO groups appeared to have infiltration of lymphocytes and distribution of inflammatory cells locally (Fig 8A), whereas the morphologic findings and structure of the small intestine in mice in the FLI-06 and MW167 groups were normal, and there was no obvious inflammation phenomenon. These results indicate that blunting Notch signaling inhibited the local inflammation response caused by IgE-mediated food allergy in mice. In addition, we also observed mast cell degranulation visually by toluidine blue staining of small intestine mast cells. Most mast cell membrane ruptured and revealed an indistinct outline, and degranulation occurred in the ovalbumin and DMSO groups (Fig 8B and C). In the FLI-06 and MW167 groups, most mast cell membrane maintained integrity, their outline was clear, and degranulation hardly occurred (Fig 8D and E). These results indicate that

degranulation was inhibited by blunting Notch signaling in mice, which was consistent with histamine and mMCP-1 results.

Discussion

In this study, we blunted Notch signaling in mice to explore the role of Notch signaling in IgE-mediated food allergy. We used 2 kinds of inhibitors, FLI-06 and MW167, to suppress Notch signaling in 2 phases of receptor expression and γ -secretase, respectively, and found both significantly inhibited the expression of NICD and *Hes-1* similarly (Fig 1). This finding indicated Notch signaling could be inhibited effectively by suppressing the Notch receptor-expressing phase or the γ -secretase-affecting phase. In addition, our results indicate that inhibiting Notch receptor-expressing phase and the γ -secretase-affecting phase had a same effect on secretion changes of cytokines, allergic indexes (vascular permeability, intestinal permeability, body temperature, symptoms), and the expression of key nuclear transcription factor (*T-bet*, *Gata-3*), which further confirmed that Notch signaling was a highly conservative signaling; this finding is in line with many other research results.^{7,12,13}

The process of IgE-mediated food allergy includes 2 phases: the sensitization phase and the effector phase. First, after food allergens intake, T cells in gut associated lymphoid tissue, spleen, and many other immune tissues are activated and differentiated into different T_H cell lineages, which is affected by many factors, such as the antigen-presenting process and the microenvironment.^{31,32} In this study, we found that the levels of T_H2 cytokines (IL-4, IL-5, IL-13) in

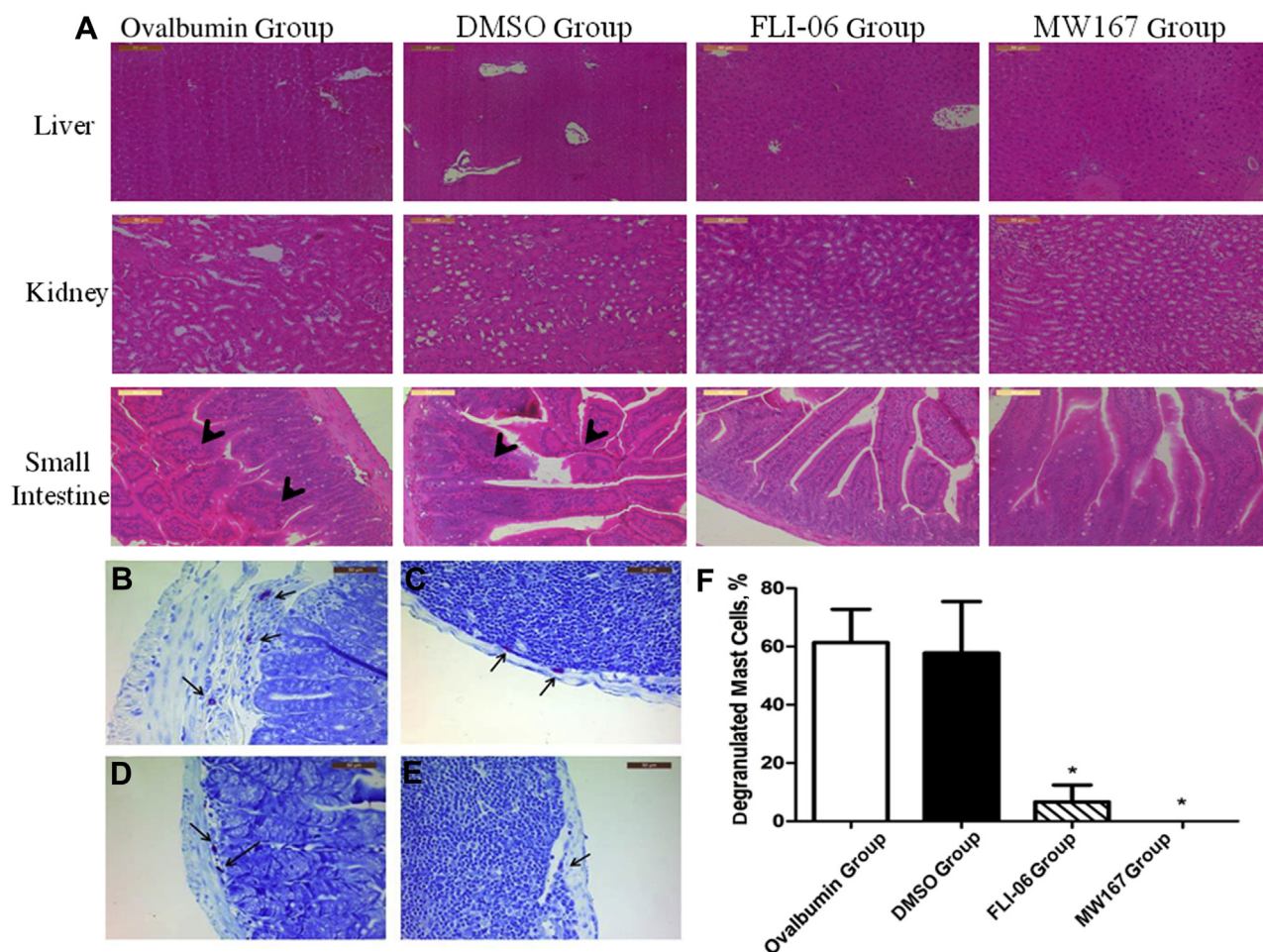


Figure 8. Histologic results of different groups. A, Hematoxylin-eosin staining of liver, kidney, and small intestine. Toluidine blue staining of small intestine of the ovalbumin group (B), dimethyl sulfoxide (DMSO) group (C), FLI-06 group (D), and MW167 group (E). F, Percentage of degranulated mast cells of different groups. * $P < .05$ vs the ovalbumin group and DMSO group ($n = 3$). Error bars indicate SDs.

MLNs and the spleen were reduced significantly, whereas the levels of T_H1 cytokine (IFN- γ) were remarkably elevated after blunting Notch signaling in ovalbumin allergic mice (Fig 3). Thus, we supposed that blunting Notch signaling could inhibit T_H2 polarization and enhance the T_H1 cell differentiation to improve T_H1/T_H2 response balance. Sauma et al³³ found that Notch signaling regulated the levels of IL-10 and IFN- γ , and the regulation ability was abolished when mice were exposed to γ -secretase inhibitor treatment in mice. Meanwhile, Liotta et al³⁴ also reported that blocking the Notch signaling pathway reduced the level of IL-4 significantly but increased the level of IFN- γ in T cells, cocultured with dendritic cells, which was consistent with our results.

Interestingly, the level of IL-17A was decreased significantly after blunting Notch signaling (Fig 3), suggesting that Notch signaling played an important role in T_H17 cell differentiation. Shilpa et al³⁵ also confirmed that Notch signaling was required for T_H17 cell differentiation. Some other studies pointed out that T_H17 cells play a key role in food allergy through interaction with T_H1 cells and T_H2 cells, but the mechanism of the interaction is still unclear.^{36,37} Weaver et al³⁸ reported that T_H17 and T_H2 are types of inflammatory cell that promote allergic reactions. Therefore, suppression of T_H17 cell differentiation might be one of the reasons for blunting Notch signaling to inhibit food allergy in mice.

In the sensitization phase, B cells synthesize and secrete antibodies, such as IgE, which is a key step in food allergy. In this study, the levels of serum ovalbumin specific IgE and IgG₁ were

significantly reduced after blunting Notch signaling in mice. It is known that IgE plays a key role in IgE-mediated food allergy and is regulated by T_H cells and cytokines, whereas IL-4 is a key factor in inducing differentiation of plasma B cells.^{39–41} Therefore, the significant decrease of ovalbumin specific IgE and IgG₁ antibody levels might be related to the decrease of IL-4 after blunting Notch signaling.

In the effector phase of food allergy, mast cells and basophils are key effector cells.^{42,43} The effector cell activation induced by food antigen- and IgE-dependent crossing of Fc ϵ RI leads to the degranulation and release of inflammatory cytokines, such as histamine and mMCP-1, which induces a series of clinical symptoms.⁴⁴ This study found that blunting Notch signaling significantly inhibited degranulation and alleviated allergic clinical symptoms in allergic mice (Fig 4). This inhibition of degranulation in vivo was likely caused partly by the reduced production of IgE antibodies. However, whether Notch signaling is able to regulate mast cells and basophils degranulation directly still remains uncertain. Nobuhiro et al⁴⁵ reported that Notch signaling enhanced the production of inflammatory cytokines and aggravated degranulation in mast cells. Therefore, blunting Notch signaling might affect the function of effector cells, such as mast cells, and inhibit the degranulation directly.

Food allergy indicator analysis revealed that blunting Notch signaling inhibited IgE-mediated food allergy in mice (Figs 4–8). To explore the mechanism, we detected the levels of T_H1 -type

response key transcription factor *T-bet* and T_H2 -type response key transcription factor *Gata-3*, and results revealed that the expression of *Gata-3* was significantly decreased, whereas the expression of *T-bet* was significantly increased after blunting Notch signaling (Fig 8). Moreover, T_H2 cytokines, such as IL-4, were decreased, whereas T_H1 cytokines, such as IFN- γ , were increased after blunting Notch signaling (Fig 3). On the basis of these results, it could be concluded that blunting Notch signaling promoted T_H1/T_H2 balance through inhibiting T_H2 -type immune response and enhancing T_H1 -type immune response, which could relieve food allergy in mice. In addition, these results also suggested that *Gata-3* might be a direct target gene of Notch signaling and *Hes-1*. In fact, Notch-mediated regulation of *Gata-3* was found in many other studies.^{7,46,47}

In addition, CT was used as adjuvant to avoid tolerance in this study. CT is considered to be one of the most effective oral immunization adjuvants and can enhance the immune response to antigens when administrated with other antigens simultaneously. Krawczyk et al.⁴⁸ found that CT enhanced the expression of Jagged 2 significantly, suggesting that CT might exert the immune adjuvant activity through Notch signaling. In this study, it was likely that the immune activity of CT adjuvant was suppressed by blunting Notch signaling, and it might also be a measure for inhibition of food allergy by blunting Notch signaling in mice.

Because Notch receptor and ligands exist in humans and mice,¹¹ we supposed that inhibition of Notch signaling might be an effective treatment of IgE-mediated food allergy in humans. However, regarding this inhibition effect as a therapy needs to consider many more factors, such as safety and adverse effects. In this article, we found that it was safe for mice treated with the 2 inhibitors by daily observation and histologic analysis (Fig 8A). T_H1 lymphocytes are potent inducers of some chronic inflammation, such as inflammatory bowel disease⁴⁹ and rheumatoid arthritis.⁵⁰ Our findings indicate that blunting Notch signaling inhibited the food allergy through suppressing T_H2 polarization and enhancing T_H1 cell differentiation, which might cause chronic inflammation. However, we did not find any inflammation in the small intestine after treatments (Fig 8A), although it was not clear whether there was any other inflammation, which needed to be further studied. Currently, our findings indicated that Notch signaling might be a potential therapeutic target and provided new ideas for effective inhibition of food allergy.

In summary, this study confirmed that Notch signaling played a key role in IgE-mediated food allergy, and blunting Notch signaling inhibited the food allergy by suppressing T_H2 polarization and enhancing the T_H1 cell differentiation, which improved T_H1/T_H2 balance in mice. However, the specific role of Notch signaling in food allergy still remains unclear because of the diversity of Notch receptors and ligands⁴⁶ and needs to be further studied.

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