



Notch ligand Delta-like1 enhances degranulation and cytokine production through a novel Notch/Dok-1/MAPKs pathway in vitro

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Abstract

Food allergy includes sensitization phase and effect phase, and effect cells degranulate and secrete cytokines in the effect phase, causing allergic clinical symptoms. We have demonstrated that Notch signaling plays an important role in the sensitization phase, but its role in effect phases still remains unclear. In this study, we investigated the role of Notch signaling in degranulation and cytokine production of the effect phase response. A RBL-2H3 cell model was used and Notch signaling was induced by priming with Notch ligands. Our results showed after priming with Notch ligand, Delta-like1 (Dll1)-Fc, β -hexosaminidase release, and cytokines production, including TGF- β , IL-1 β , IL-4, IL-6, and IL-13, were increased significantly, and the enhancement was abolished after DAPT treatment, a γ -secretase inhibitor, indicating that Dll1 Notch signaling enhanced RBL-2H3 cell degranulation and cytokine production. Western blot analysis showed that Dll1 Notch signaling augmented high-affinity IgE receptors-mediated phosphorylation of MAPKs through suppressing the expression of downstream tyrosine kinases 1 (Dok-1). Besides, a passive systemic anaphylaxis mouse model was used to confirm the role of Notch signaling. And our data showed that allergic clinical features of mice were alleviated, and the level of degranulation was decreased significantly after inhibiting Notch signaling in vivo. Therefore, we demonstrated Notch ligand Dll1 enhanced RBL-2H3 cell degranulation and cytokine production through a novel Notch/Dok-1/MAPKs pathway, suggesting Notch signaling played a key role in the effect phase of food allergy.

Keywords Notch signaling · Delta-like1 · Degranulation · Cytokine production · MAPKs · Dok-1

Introduction

Food allergy can cause a series of allergic disease complications, such as urticaria, asthma, and diarrhea, which could endanger human health [1, 2]. In recent years, the incidence of food allergy has increased, but the specific mechanism still remains unclear [3]. Many studies have demonstrated that most food allergies are immunoglobulin E (IgE)-mediated type 1 hypersensitivity, which depend on the antigen-

specific differentiation of Th2 cells in the sensitization phase and degranulation and cytokine production of mast cells, basophils in the effect phase [4–6]. In the effect phase, cross-linking of high-affinity IgE receptors (Fc ϵ RI) on effect cells by IgE/antigen complexes induces the release of preformed and newly synthesized proinflammatory mediators that give rise to allergic symptoms, varying in severity from mild to life-threatening, including histamine, β -hexosaminidase, mast cell protease (MCP), and a series of cytokines [7, 8].

Mast cells and basophils are key effector cells in the effect phase of IgE-mediated food allergy [8]. Rat basophilic leukemia (RBL) cells are of mucosal mast cell origin and have been used frequently as an established model for the study of IgE-mediated degranulation and cytokine production [9, 10]. RBL-2H3 cells express Fc ϵ RI on the cell-surface membrane [11]. Antigen-induced cross-linking of Fc ϵ RI initiates numerous early signaling events to promote degranulation and cytokine production. Mast cells are widely distributed in tissues, and their functions are regulated by the microenvironment of the body, such as local mediators, including cytokines, chemokines, and molecules on cell surface [12, 13]. Notch receptors and ligands are important pairs of costimulatory

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12026-017-8977-0>) contains supplementary material, which is available to authorized users.

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molecules on cell surface. Four Notch receptors (Notch 1, 2, 3, and 4) and five known ligands (Jagged [Jag] 1, 2, and Delta-like [Dll] 1, 3, and 4) have been found in mammals [14]. As a highly conserved signaling pathway, Notch signaling is induced when the Notch receptors encounter the ligands. This interaction finally initiates the release of Notch intracellular domain (NICD), which is affected by γ -secretase, and then NICD translocates to the nucleus to induce the transcription of genes [15]. We have illuminated that the Notch signaling pathway plays an important role in Th2 cell differentiation in the sensitization phase of IgE-mediated food allergy [16], which is consistent with many other findings [17–19]. However, the role of Notch signaling in the effect phase is still unclear.

In this study, we investigated the effect of Notch signaling on degranulation and cytokine production of effect cells. A RBL-2H3 cell model was used and Notch signaling was induced by priming with Notch ligands. Then, preformed and newly synthesized proinflammatory mediators were examined. Besides, a passive systemic anaphylaxis (PSA) mouse model was used to confirm the role of Notch signaling in effector phase response *in vivo*. Our results showed that Dll1 enhanced RBL-2H3 cell degranulation and cytokine production via a novel Notch/downstream of tyrosine kinases 1 (Dok-1)/MAPKs pathway, suggesting Notch signaling was a key mediator in the effect phase of food allergy. These findings provide scientific basis for Notch signaling as a potential target in suppressing food allergy processes.

Materials and methods

Cells and mice

RBL-2H3 cells were maintained in Eagle's Minimal Essential Medium supplemented with 15% defined fetal bovine serum as described previously [20].

Twenty-four 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, maintained at $23 \pm 3^\circ\text{C}$ and a relative humidity of 40–70% with a light/dark cycle of 12 h. All experimental studies were conducted under a protocol approved by the Animal Ethics Committee of China Agricultural University.

Coculture of RBL-2H3 cells with Jagged1-Fc and Delta1-Fc *in vitro*

RBL-2H3 cells were cultured with plate-bound Jag1-Fc (5 $\mu\text{g}/\text{mL}$) or Dll1-Fc (5 $\mu\text{g}/\text{mL}$) for 4 days. In some experiments, RBL-2H3 cells were pretreated with DAPT, a γ -secretase inhibitor for 24 h after coculture with Notch ligands.

Detection of RBL-2H3 proliferation after priming with Notch ligands by CCK-8

After priming with Notch ligands, RBL-2H3 cells were plated (10^5 cells/well) in 96-well round-bottom plates, and 200 μL of CCK-8 reagent was added into each well. The plates were centrifuged to remove supernatant after 4 h. Then, 125 μL of dimethyl sulfoxide was added into each well, and the absorbance at 450 nm was detected after 10 min shaking with an oscillator at room temperature in darkness.

Stable lentiviral transduction of RBL-2H3 cells with short hairpin RNAs

Stable knockdown of Dok-1 expression was achieved by transduction with the lentiviral vector pLKO.1-puro expressing a short hairpin RNA (shRNA) targeting rat Dok-1 (Dok-1 shRNA1, shRNA2, shRNA3), which were obtained from Hanbio (Shanghai, China). A vector was used as a control. The lentiviral transduction particles (2×10^6 transduction units) were incubated for 5 h at 37°C in RetroNectin-coated plates to attach the virus particles onto the RetroNectin. After removing the supernatants, RBL-2H3 cells (5×10^5 cells) were plated onto the virus-attached plates. The transduced cells were selected by additional culture in the presence of 1.6 mg/mL puromycin for 14 days.

Measurement of the β -hexosaminidase release

Degranulation was assessed by the enzymatic activity of the granule marker, β -hexosaminidase. RBL-2H3 cells were seeded in 96-well plates (10^5 cells/well) and cultured for 24 h at 37°C . Cells were sensitized with 1 $\mu\text{g}/\text{mL}$ anti-DNP IgE for 2 h at 37°C and washed and stimulated at 37°C with 100 ng/mL DNP-HSA in Tyrode's buffer for 45 min (135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 20 mM HEPES, 5.6 mM glucose, 0.05% BSA [pH 7.4]). In some experiments, sensitized cells were pretreated with MAPK inhibitors prior to the addition of DNP-HSA for 1 h. β -hexosaminidase in supernatants was measured as described previously [21].

Determination of cytokine production

RBL-2H3 cells were dispensed in 12-well plates (2×10^6 cells/well) and cultured for 24 h at 37°C . Cells were sensitized with 10 $\mu\text{g}/\text{mL}$ anti-DNP IgE for 2 h at 37°C , washed and stimulated at 37°C with 500 ng/mL DNP-HSA in Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 1.8 mM CaCl_2 , 20 mM HEPES, 5.6 mM glucose, 0.05% BSA [pH 7.4]), and incubated for 8 h at 37°C in 5% CO_2 . In the supernatants, the levels of IL-1 β , IL-4, IL-6, IL-13, and TNF- α were determined with Rat ELISA kits (eBioscience, Inc., San Diego, CA).

Real-time PCR

Total RNA was extracted from cultured cells directly by using the TRIzol reagent (Invitrogen, USA) and reverse transcribed to generate first-strand complementary DNA (cDNA) (SuperScript II reverse transcriptase, Invitrogen, USA). SYBR Green Master Mix kit (Takara) was used for real-time PCR with the Bio-Rad iCycler iQ system (Bio-Rad). The real-time PCR was carried out under the following conditions: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. As a control, each cDNA sample was simultaneously subjected to β -actin amplification. 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). Primer sequences are shown in supplementary Table 1.

Western blotting

RBL-2H3 cells were lysed after different treatments and subjected to SDS-PAGE, and then the separated proteins were transferred to PVDF membranes, which were blocked in Tris-buffered saline, 0.1% Tween 20, and 5% milk, and then incubated with primary antibodies overnight at 4 °C. After incubation, the membranes were incubated with specific HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the membranes were incubated in ECL reagents (Pierce) and exposed to film. For semi-quantification of the expression levels, the blots were scanned, and the density of each band was determined using image analysis software.

Passive systemic anaphylaxis protocol

Mice were grouped into negative group, positive group, and dual antiplatelet (DAPT) group ($n = 8$) randomly. Positive and DAPT groups were sensitized with 200 μ L of IgE solution (the mice will receive a total of 20 μ g of IgE anti-DNP). And the negative group were injected with 200 μ L of 0.9% NaCl. All mice were challenged with 100 μ L of DNP-HSA solution by intravenous injection after 24 h. And the DAPT group received DAPT (1 mg/kg) treatment by tail vein injection 1 h before challenge. Rectal temperature was measured at 10-min intervals for the first hour following the challenge. Besides, 100 μ L 0.5% Evans Blue was also injected through tail vein along with the DNP-HSA solution. The blue exudation of the soles of mice was also observed. For additional assessment of degranulation, blood samples were collected from orbital venous plexus of mice eyes 20 min after challenge and were collected in centrifugal tubes to obtain plasma, which contained heparin. Blood samples were collected again to obtain serum 45 min after challenge. Blood samples were centrifuged for 10 min at 3000g and 4 °C following coagulation for 2 h at room temperature to get the plasma and serum.

The plasma was stored at -80 °C before analyzing for plasma histamine levels by a commercial mouse ELISA kit (eBioscience, Inc., San Diego, CA), and the serum were stored at -80 °C before analyzing for mMCP-1 levels by commercial mouse ELISA kits (eBioscience, Inc., San Diego, CA).

Statistical analyses

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA). One-way analysis of variance was used to determined significant differences between groups. Significance was defined as $p < 0.05$.

Results

RBL-2H3 cells expressed Notch receptors on cell surface

As a highly conserved signaling pathway, Notch signaling is widespread in many types of cells [14]. To determine whether Notch signaling is functional in RBL-2H3 cells, we first examined the expression of Notch receptors on RBL-2H3 cells. Western blot analysis showed Notch 1, 2, 3, and 4 were all expressed, and Notch 1, 3, and 4 were expressed much more than Notch 2 ($p < 0.05$) (Fig. 1). These observations indicated that RBL-2H3 cells constitutively express Notch receptors on cell surface, suggesting Notch signaling might be an important mediator of its functions.

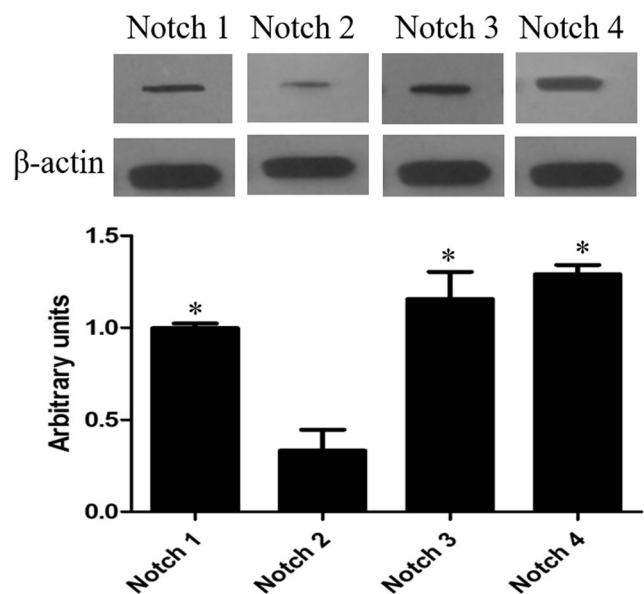


Fig. 1 Western blot analysis of Notch receptors on RBL-2H3 cell surface. * $p < 0.05$ vs. Notch 2. The results presented are from three independent experiments

Priming with Notch ligands induced *Hes1* expression in RBL-2H3 cells, but did not promote the proliferation

Since RBL-2H3 cells expressed Notch receptors, we then investigated the effect of Notch signaling in RBL-2H3 cells. *Hes1*, *Hes5*, and *Hey2* are the direct target genes of Notch signaling [22]. We detected the expression of these genes after priming with Notch ligands for 4 days. As shown in Fig. 2a, the expression of *Hes1* was significantly increased after priming with both Notch ligands, Jag1 and Dll1 ($p < 0.05$), while the expression of *Hes5* and *Hey2* did show a significant increase ($p > 0.05$), suggesting that the Notch signaling pathway was induced after priming with Notch ligands, and *Hes1* was the target gene.

In addition, many studies have proved that Notch signaling regulates cell proliferation and differentiation broadly [23–25]. Thus, we examined the effect of Notch signaling on the proliferation of RBL-2H3 cells. As shown in Fig. 2b, although the proliferation was slightly increased in the Notch ligand groups, there was no significant difference when compared with the control group ($p > 0.05$), indicating that the Notch signaling pathway did not significantly promote the proliferation of RBL-2H3 cells in the present study.

Dll1 priming augmented β -hexosaminidase release and cytokine production of RBL-2H3 cells

Next, we examined the effects of Notch signaling on IgE-mediated degranulation and cytokine production, the important effector phase response of allergic hypersensitivity. As shown in Fig. 3a, compared with the control group, Jag1-Fc enhanced the β -hexosaminidase release slightly, but it did not show a significant difference ($p > 0.05$), while the β -hexosaminidase release was significantly increased after priming with Dll1-Fc ($p < 0.05$). Furthermore, the enhancement of β -hexosaminidase release in the Dll1-Fc group was abolished after treatment of DAPT, a γ -secretase inhibitor, suggesting

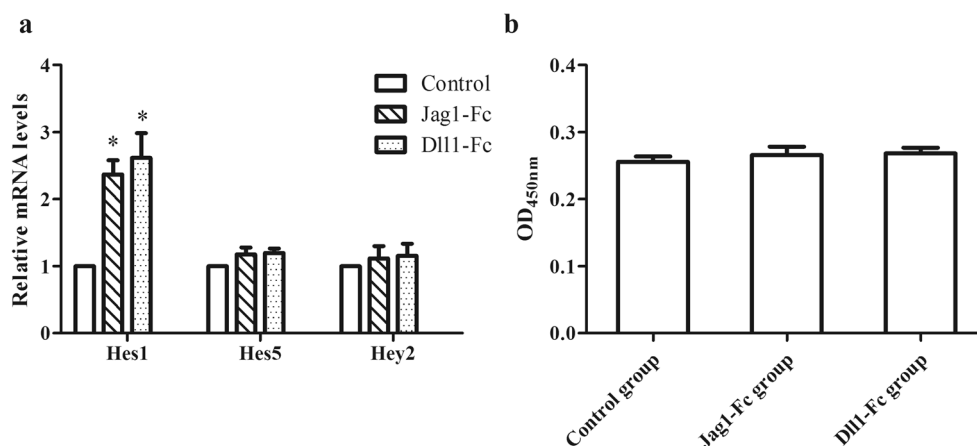
that Dll1 Notch signaling augmented the degranulation of RBL-2H3 cells.

Effect cells also secrete a series of cytokines, such as IL-4, IL-6, TNF- α , and IL-13, upon cross-linking of their Fc ϵ RI receptors. IL-1 β , IL-6, and TGF- α play a key role in inflammatory response, while IL-4 and IL-13 are important in Th2 cell differentiation [26–28]. We next investigated whether Notch signaling had an effect on cytokines secretion after cross-linking their Fc ϵ RI receptors with DNP-HAS and DNP-specific IgE antibodies. As shown in Fig. 3b, the levels of IL-1 β , IL-13, and TNF- α were significantly increased after priming with Jag1-Fc ($p < 0.05$), but it showed no significant difference on the secretion of IL-4 and IL-6 ($p > 0.05$). In contrast, Dll1 priming augmented the IL-1 β , IL-4, IL-6, IL-13, and TNF- α production significantly ($p < 0.05$) compared to the control group. To further confirm whether this enhancement of cytokine production was caused by Notch signaling, DAPT was used to blunt Notch signaling in Dll1-treated RBL-2H3 cells. As expected, the enhancement on cytokine secretion completely disappeared, suggesting that Dll1 Notch signaling pathway had an enhancement on the cytokine production in RBL-2H3 cells, which was consistent with the release of β -hexosaminidase.

Dll1-Fc priming enhanced Fc ϵ RI-mediated phosphorylation of MAPKs in RBL-2H3 cells

In Fc ϵ RI-mediated signaling events, MAPK activation plays a critical role in degranulation and cytokine production [29, 30]. To investigate the mechanism by which Dll1 Notch signaling augmented the degranulation and cytokine production in RBL-2H3 cells, we examined whether Notch signaling influenced the phosphorylation of MAPKs induced by Fc ϵ RI cross-linking. As shown in Fig. 4a, JNKs, p38 MAPK, and ERK1/2 were phosphorylated following Fc ϵ RI cross-linking. And the degree of phosphorylation was significantly augmented in Dll1-Fc-treated RBL-2H3 cells compared with the control group ($p < 0.05$). And the enhancement was almost

Fig. 2 Real-time PCR analysis of Notch target genes (a) and the effect of Notch signaling on proliferation of RBL-2H3 cells (b). * $p < 0.05$, relative to the control group. The results are from three independent experiments



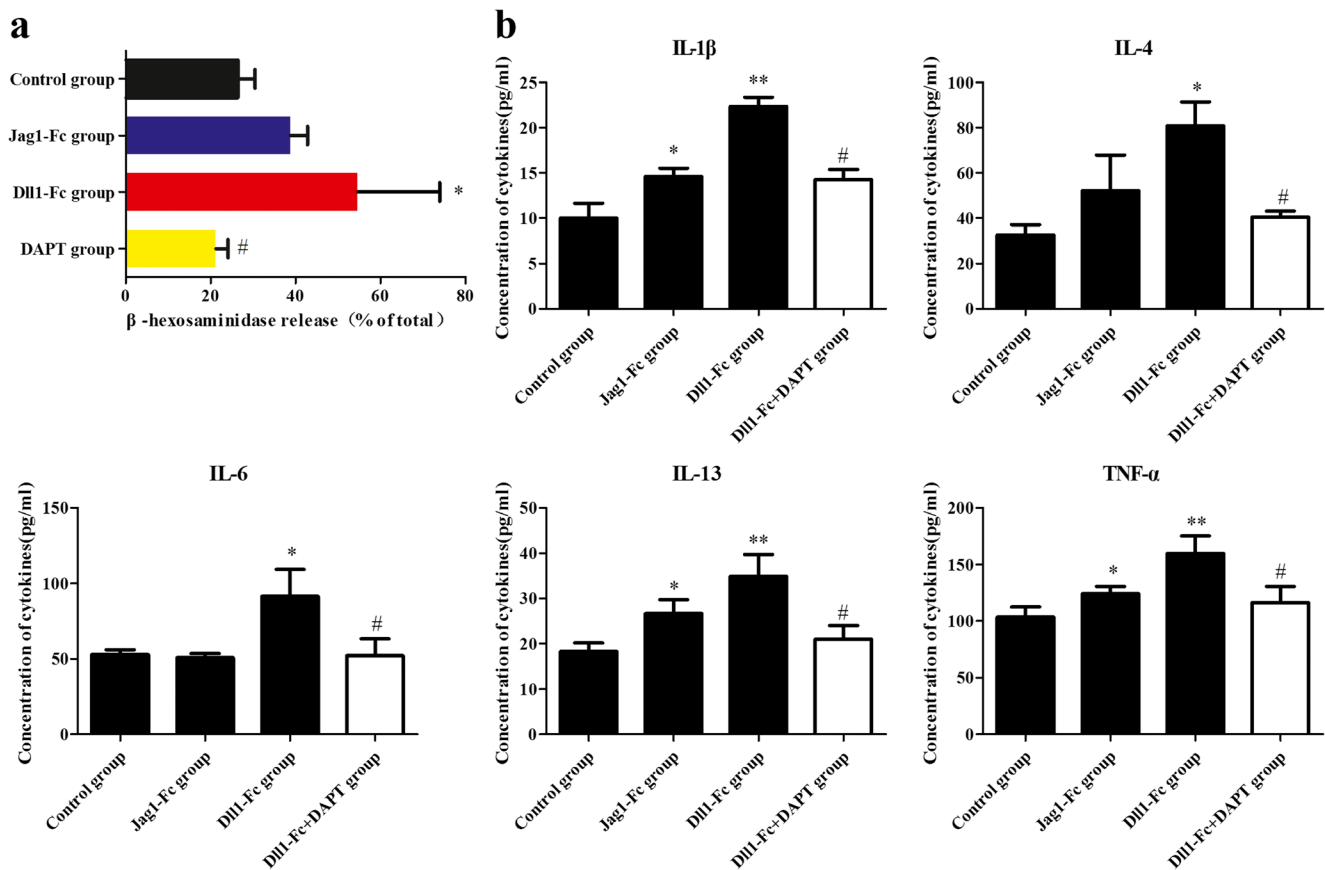


Fig. 3 The effect of Notch ligands on the IgE-mediated degranulation (a) and cytokine production (b) of RBL-2H3 cells. * $p < 0.05$, relative to the control group, ** $p < 0.05$, relative to the Jag1-Fc group, # $p < 0.05$, relative to the Dll1-Fc group

abolished after treatment with DAPT, suggesting that Dll1 Notch signaling augmented phosphorylation of MAPKs might contribute to the enhancement of FcεRI-mediated degranulation and cytokine production in RBL-2H3 cells. In addition, the release of β-hexosaminidase and cytokine production (IL-1β, IL-4, IL-6) was inhibited significantly by treatment with SP600125 (JNK inhibitor), SB203580 (p38 inhibitor), and U0126-EtOH (ERK1/2 inhibitor) ($p < 0.05$) in Dll1-Fc-treated RBL-2H3 cells (Supplementary Fig. 1). These results indicated that augmented phosphorylation of MAPKs was important to the enhancement of FcεRI-mediated degranulation and cytokine production in RBL-2H3 cells.

Downregulation of Dok-1 expression contributed to the enhancement of FcεRI-mediated degranulation and cytokine production by Dll1 Notch signaling

Many studies indicated that the phosphorylation of MAPKs might be related to an alteration in downstream signaling of FcεRI [31, 32]. Therefore, we focused on a negative regulator in FcεRI signaling. Dok-1 was originally identified as a tyrosyl-phosphorylated molecule of 62 kDa [33]. In the present study, western blot analysis showed that the expression of

Dok-1 was clearly reduced in Dll1-treated RBL-2H3 cells compared with control ($p < 0.05$), and the reduction of Dok-1 was prevented by DAPT (Fig. 4b). These results indicated that Dll1 Notch signaling suppressed the expression of Dok-1.

To ascertain whether the enhancement of FcεRI-mediated degranulation and cytokine production in Dll1-RBL-2H3 cells can be explained by the reduction in expression of Dok-1, we next determined the degranulation and cytokine production of Dok-1 knockdown RBL-2H3 cells. In this experiment, three shRNAs against Dok-1 were used to exclude the possibility of any off-target effects of shRNAs. As shown in Fig. 5, the expression of Dok-1 mRNA was decreased by 82% in the shRNA 3 group, and shRNA 3 also showed the best inhibition effect in protein level. So, we established a Dok-1 downregulated expression RBL-2H3 cell strain with shRNA 3. Then we used this strain to investigate the role of Dok-1 in degranulation and cytokine production. As shown in Fig. 5c, compared with the control group, the release of β-hexosaminidase was significantly increased in the Dok-1 knockdown group ($p < 0.05$). Besides, the production of IL-1β, IL-4, IL-6, IL-13, and TNF-α of Dok-1 knockdown RBL-2H3 cells upon FcεRI cross-linking was significantly enhanced ($p < 0.05$) (Fig. 5d). These data indicated that Dok-1 was a key mediator in degranulation and cytokine production of RBL-2H3 cells.

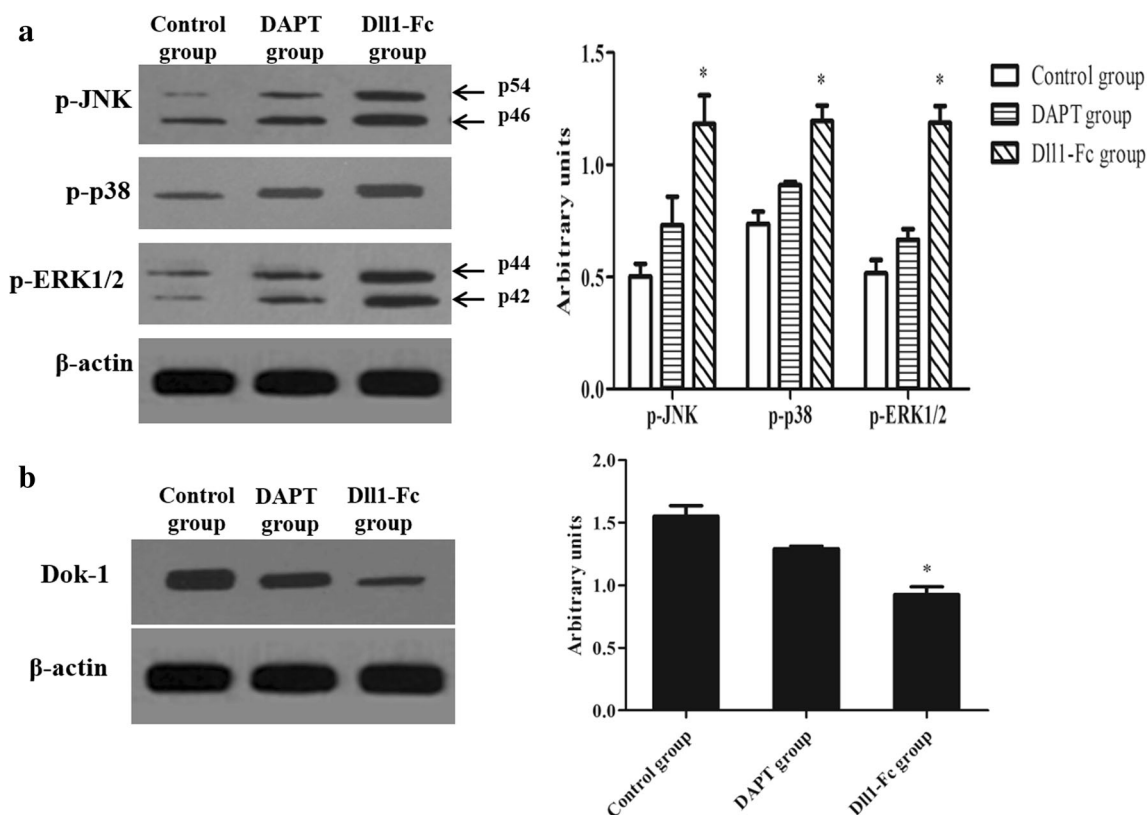


Fig. 4 The effect of Dll1-Fc priming on the phosphorylation levels of MAPKs (**a**) and the expression of Dok-1 (**b**) in RBL-2H3 cells. * $p < 0.05$, relative to the control group. The results are from three independent experiments

We next investigated the role of Dok-1 in phosphorylation of MAPKs. As shown in Fig. 5e, knockdown of Dok-1 resulted in marked augmentation of Fc ϵ RI-mediated phosphorylation of JNKs, p38 MAPK, and ERK1/2 ($p < 0.05$), which was similar to Dll1-Fc priming (Fig. 4a). These results indicated that the reduced expression of Dok-1 contributed to the enhancement of Fc ϵ RI-mediated degranulation and cytokine production through the augmentation phosphorylation of MAPKs in Dll1-Fc priming RBL-2H3 cells.

Notch signaling was important in the effect phase of food allergy in vivo

Systemic anaphylaxis mouse model is an important tool for the study of mast cells and basophil functions of food allergy [34]. Thus, we used a PSA mouse model to further confirm whether Notch signal was a key mediator of effect cell functions in vivo. As shown in Fig. 6a, mice in the positive group showed a marked drop in body temperature ($p < 0.05$) while mice in the DAPT group did not ($p > 0.05$). Besides, compared with the control group, the vascular permeability of mice in the positive group was significantly increased ($p < 0.05$), while the change vanished after treatment with DAPT (Fig. 6b), indicating that mice in the positive group showed some clinical symptoms, and the clinical symptoms

were alleviated after inhibiting Notch signaling in vivo. In addition, we detected the level of degranulation by detecting mMcp-1 and histamine in different groups. As shown in Fig. 6c, compared to the positive group, the levels of serum mMcp-1 and plasma histamine were significantly decreased after treatment with DAPT ($p < 0.05$). These results illustrated that Notch signaling was an important mediator in the effect phase in vivo, which was consistent with the results in vitro.

Discussion

Mast cells and basophils are important effector cells in IgE-mediated food allergy, and their functions are influenced by various microenvironmental factors [35]. Studies have shown that Notch receptors are expressed on mast cells, while Notch ligands are expressed on many types of cells, such as intestinal epithelial cells and vascular endothelial cells in various tissues [36, 37]. Therefore, we assumed that Notch signaling might be a key mediator factor of effector cells function in food allergy.

To verify this, we firstly detected the expression of Notch receptors on RBL-2H3 cells to illustrate the possibility that Notch signaling is functional in RBL-2H3 cells. We found that Notch 1, 2, 3, and 4 were expressed on the RBL-2H3 cell surface (Fig. 1), but Nakano et al. [38] found that only Notch 1

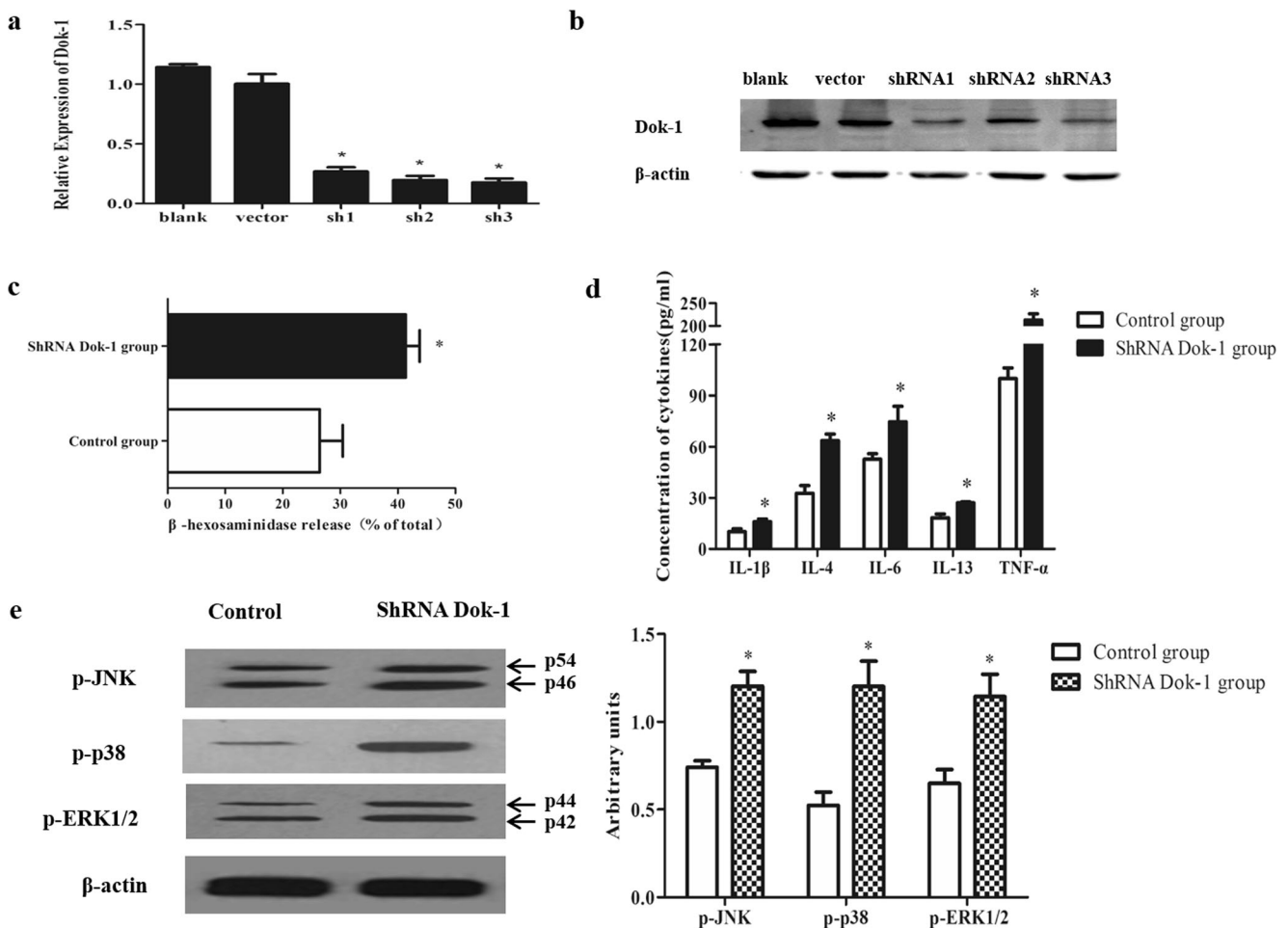


Fig. 5 The effect of Dok-1 on degranulation, cytokine production, and phosphorylation of MAPKs. Real-time PCR analysis (**a**) and western blot analysis (**b**) of Dok-1; the effect of knockdown of Dok-1 on FcεRI-

mediated degranulation (**c**) and cytokine production (**d**); the effect of knockdown of Dok-1 on phosphorylation of MAPKs (**e**). * $p < 0.05$, relative to the control group

and 2 were expressed on mouse mast cells; this difference might be due to the discrepancy of different cell types. Despite this, the expression of Notch receptors suggested that Notch signaling was a possible mediator in RBL-2H3 cells.

We further investigated the effect of Notch signaling on degranulation and cytokine production of RBL-2H3 cells. Jag1-Fc and Dll1-Fc enhanced the degranulation and cytokine production of RBL-2H3 cells, especially Dll1-Fc remarkably enhanced the FcεRI-mediated degranulation and cytokine production (Fig. 3). And the potentiation effect disappeared after treatment of DAPT, a γ-secretase inhibitor, which indicated that Dll1 Notch signaling pathways enhanced RBL-2H3 cell degranulation and cytokine production. Besides, the proliferation of RBL-2H3 cells was not affected by priming with Notch ligands at concentrations of 5 μg/mL (Fig. 2b), suggesting that the enhancement of degranulation and cytokine production was not due to its growth acceleration effects. In fact, many studies have demonstrated that Notch signaling pathways promote proliferation of many types of cells, such as T cells and endothelial cells [23, 39]. However, Notch

signaling showed no promotion effect in proliferation of RBL-2H3 cells in the present study, and this might be related to the concentrations of Notch ligands and priming time.

Interestingly, we found different Notch ligands showed different enhancement effects in RBL-2H3 cell degranulation and cytokine production. Dll1-Fc significantly enhanced FcεRI-mediated production of IL-1β, IL-4, IL-6, IL-13, and TNF-α, whereas Jag1-Fc only increased IL-1β, IL-13, and TNF-α production (Fig. 3b), and the enhancement of Jag1-Fc was not as remarkable as Dll1, the same tendency in degranulation (Fig. 3a). This difference might be the result of the different affinities of Notch receptors for different ligands. Shimizu et al. [40] have found that Jag1 and Jag2 are mainly combined with Notch 2 on a mouse pro-B cell line, but we found that Notch 2 receptor was expressed much less than Notch 1, Notch 3, and Notch 4 on RBL-2H3 cells (Fig. 1). Thus, we considered that the binding affinity of Jag1 for Notch receptors on RBL-2H3 cells was weaker compared with that of Dll1, and this might contribute to the different effects of different Notch ligands on degranulation and

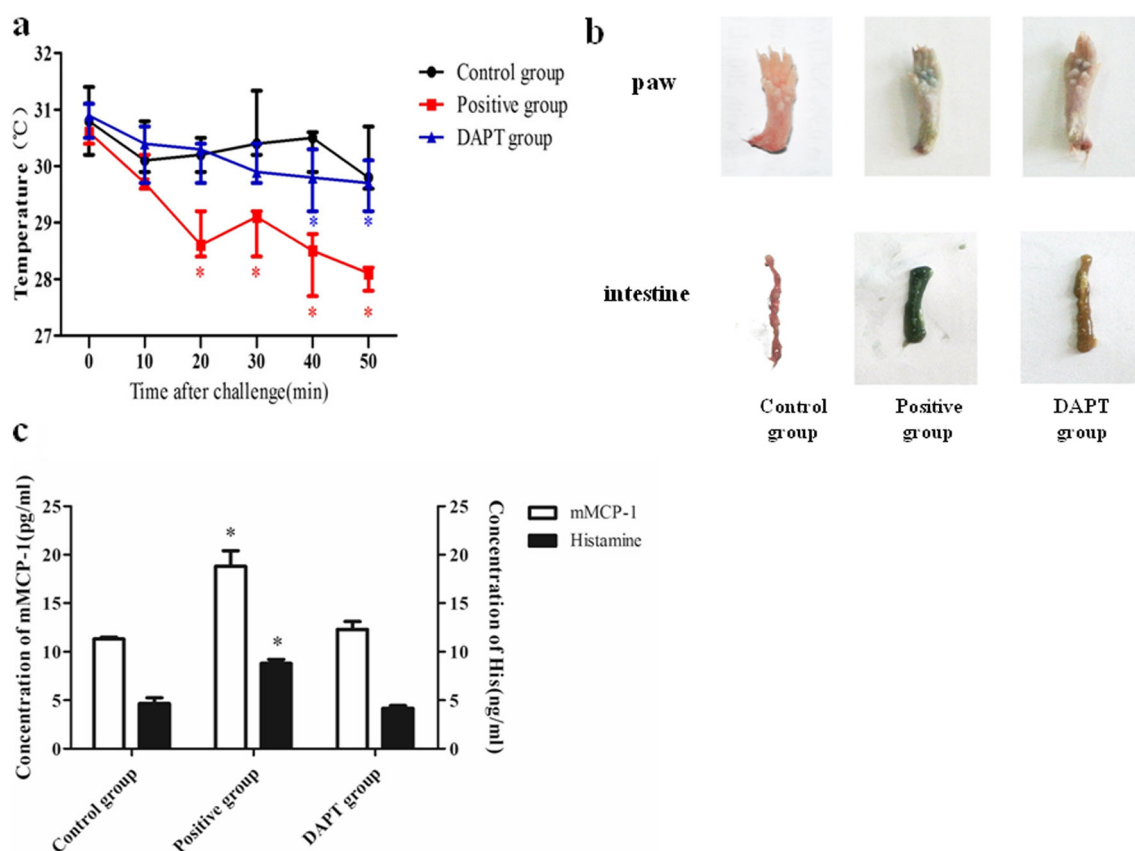


Fig. 6 The effect of blunting Notch signaling on passive systemic anaphylaxis in vivo. The effect of DAPT on body temperature changes (a), vascular permeability (b), and degranulation of effect cells (c). * $p < 0.05$, relative to the control group

cytokine production. However, which Notch receptor/receptors plays/play a key role needs further investigation.

How did Notch signaling enhance degranulation and cytokine production in RBL-2H3 cells? We found a novel indirect action through the modulation of activation signals. Our results indicated that Dll1-Fc priming significantly suppressed the expression of Dok-1, leading to augmentation of FcεRI-mediated phosphorylation of MAPKs, and this enhancement was almost abolished after treatment of DAPT (Fig. 4). Thus, we considered that Dok-1 played an important role in the reinforcement of degranulation and cytokine production of Dll1 priming.

To confirm this, we established a Dok-1 knockdown RBL-2H3 cell strain with shRNA. We found that knockdown of Dok-1 enhanced the RBL-2H3 cell degranulation and cytokine secretion (Fig. 5c, d), indicating that Dok-1 indeed played a negative regulatory role in FcεRI-mediated degranulation and cytokine production. Besides, downregulation of Dok-1 resulted in enhanced phosphorylation of MAPKs (Fig. 5e), indicating that knockdown of Dok-1 enhanced degranulation and cytokine secretion via enhancement of phosphorylation of MAPKs in Dll1-treated RBL-2H3 cells, or at least partly. Dok-1 is a member of a recently discovered family of docking proteins

phosphorylated by different PTKs and involved in the negative regulation of signaling downstream of cell surface receptors [41]. Carpino et al. [42] demonstrated that Dok-1 was a negative regulator of Ras/Erk signaling. Moreover, upon anti-CD3/TCR stimulation, Dok-1 associates with CD45 in a phosphotyrosine-dependent manner and negatively regulates JAK/STAT pathways [43]. Recent studies have demonstrated that Dok-1 is also a negative regulator of allergen-induced Th2 inflammation and airway hyperresponsiveness through selective inhibition and activation of STAT-6 and STAT-4 signaling pathways in airway epithelial cells [33]. In this study, we found that Dok-1 was a negative regulator of degranulation and cytokine production via negative regulation of phosphorylation of MAPKs. Together, these data suggested Dok-1 might have an important negative regulatory role in food allergy.

But, how did the downregulation of Dok-1 enhance phosphorylation of MAPKs? It remains unclear. Abramson et al. [44] have found that Dok-1 binds to CrkL, non-catalytic protein kinase (Nck), Lyn, SH2-domain-containing inositol polyphosphate 5'-phosphatase (SHIP), and RasGAP in FcεRI-stimulated RBL-2H3 cells in vitro. Thus, we supposed the regulatory mechanism might be indirect, through the interaction with SHIP, Nck, etc., and this needs to be further studied.

In conclusion, this study demonstrated that Dll1 Notch signaling significantly enhanced RBL-2H3 cell degranulation and cytokine production by enhancing FcεRI-mediated phosphorylation of MAPKs through downregulation of the expression of Dok-1, suggesting that Notch signaling might be an important mediator in the effect phase of food allergy. And this was confirmed in a PSA mouse model. In addition, we have indicated that Notch signaling is necessary in Th2 cell differentiation in the sensitization phase of food allergy [16]. So, our studies suggested that Notch signaling may be a therapeutic target of food allergy.

Acknowledgements We thank all colleagues who contributed to this research.

Funding information This work was supported by the National Natural Science Foundation of China (No. 81573158).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

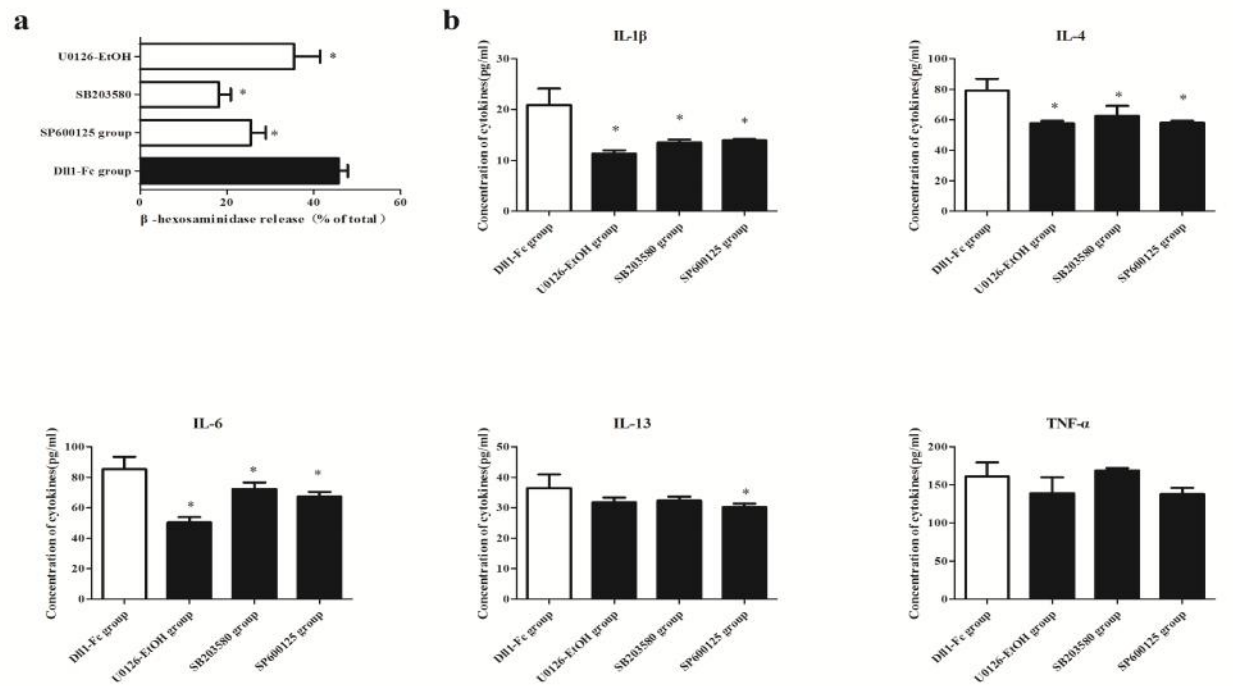
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Supplementary table 1. List of primer sequences used in RT-qPCR.

Gene Name	Forward primer sequences	Reverse primer sequences
Hes1	F: 5'-CGAAAATGCCAGCTGATATAA-3'	R: 5'- ACACGCTCGGGTCTGTGCTGA-3'
Hes5	F: 5'-CTCCGCTCCGCTCCGCTCGCTAAT-3'	R: 5'-GGGGCCGCTGGAAGTGGTAAAGCA-3'
Hey2	F: 5'-CAGTTTGCAAGAGTTTGGGTTAAT-3'	R: 5'- CTCACGTGCTCAATTTCAATGTAT-3'
Dok-1	F: 5'-ATACGCCTGGCTGAATGTGT-3'	R: 5'- GGTTCTCCGTCTGAGCCAAA-3'
GAPDH	F: 5'- ATGATTCTACCCACGGCAAG-3'	R: 5'- CTGGAAGATGGTGATGGGTT-3'



Supplementary figure 1. The effect of inhibitors of MAPKs on the IgE-mediated degranulation (a) and cytokine production (b) of RBL-2H3 cells. * $p < 0.05$, relative to the Dll1-Fc group.