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Inhibitor of γ-secretase alleviates middle ear inflammation by regulating Th2 response in OVA-mediated allergic OME in vivo

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ABSTRACT

Otitis media with effusion (OME) often occurs in infants and young children, which is related to allergic reactions. Notch signaling pathway plays an important role in allergic responses. In this study, we aimed to investigate the role of Notch signaling pathway in the ovalbumin (OVA)–mediated allergic OME in vivo. OVA-induced OME rats were treated with a control vehicle or a γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-*L*-*alanyl*]-*S*-phenylglycine t-butyl ester (DAPT) suppressing the Notch signaling. We studied the effect of Notch signaling pathway in OME model, including histopathological assessment, the expression of Th1 cytokines (IFN-γ), Th2 cytokines (IL-4, IL-5), key transcription factors (T-bet, GATA-3) by quantitative real-time polymerase chain reaction (qRT-PCR). In addition, the level of Notch ligand (Jagged1) and the downstream target gene Hes1 were also evaluated by qRT-PCR and immunofluorescent staining. We observed that the production of Th2 cytokines was increased, the level of Th1 cytokines was decreased in OME experimental model. Likewise, Th2-cytokine(IL-4)level was reduced, but the level of Th1 cytokines(IFN-γ) was no changes. Additionally, administration of DAPT induced a decrease in the expression of GATA-3 mRNA, however, no influence on T-bet mRNA production. These results suggest that there is an imbalance with Th1/Th2 in OVA-mediated allergic OME. DAPT treatment can block the Notch signaling pathway and relieve the middle ear inflammation through modulating the level of Th2 responses in OVA-induced allergic OME.

1. Introduction

Otitis media with effusion (OME) occurs in approximately 2/3 of infants and young children. Because of the anatomy and function of their Eustachian tube, enough ventilation and drainage of mucus in the middle ear are not acquired until about 5 years old(Pichichero, 2018). Conductive hearing loss caused by OME is the most common clinical symptom. It can also have an important impact on children's emotion, communication, attention, socialization, academic achievement and language development. OME in early childhood contributes to verbal intelligence quotient (IQ), behavior problem, and reading ability which can continue into late childhood and the early teens (Bennett et al., 2001),and it can affect quality of life (QoL) in children (van Brink and Gisselsson-Solen, 2019). Given the negative effects of OME on children development, it is necessary to find the causes and provide intervention appropriately for children with OME. Children with upper respiratory tract infection, and allergic diseases, such as eczema, asthma, and allergic rhinitis (AR), are even more susceptible to suffer from OME (Pichichero, 2018; Oh and Kim, 2016).

Recently, some studies on subjects and animal models of OME suggest that it is associated with allergic reactions. The middle ear mucosa originate from the upper respiratory epithelial system, causing them to have similar immune function (Nguyen et al., 2004). OME has a shift toward T helper (Th) 2 response, which Th2-cytokine levels are increased and Th1-cytokine levels are decreased in the impaired middle ear. Th2 cells and other inflammatory cells, such as eosinophils and mast cells, act in the pathological development and progression of OME. Therefore, there is also an imbalance in Th1/Th2 cells in OME just like asthma and AR(Oh and Kim, 2016; Kariya et al., 2006). Th2 cells mainly produce interleukin (IL)-4, IL-5, and IL-13, which play an essential role in allergies. These cytokines can promote the production of IgE from B cells, cause the proliferation and differentiation of eosinophils, and induce the hyperplasia and metaplasia of goblet cell. It is widely considered that the cytokine IL-4 is the major inducer for the

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initial differentiation of Th2 cell. IL-4 can boost expression of the crucial transcriptional factor GATA-3 for development of Th2 cell via IL-4 receptor(IL-4R) phosphorylation and activation of signal transducer and activator of transcription 6(STAT6). Thus, the initial differentiation of Th2 cells by the IL-4/STAT6 axis are suggestive of an autocrine loop which induces expansion of T cell produced by IL-4. However, it is still unclear to the elementary origin of IL-4, which promotes the Th2 response. And, GATA-3 promotes the Th2 responses but inhibits the Th1 pathway (Scheinman and Avni, 2009; Tindemans et al., 2017a). Th1 cells express the transcription factor T-bet, encoded by Tbx21, and secrete various cytokines, such as interferon gamma (IFN-γ), tumor necrosis factor(TNF).IL-12 is believed to the main inducer to drive the initial differentiation of Th1 cell, but there is also IL-12-independent Th1 cell responses(Amsen et al., 2015).

Many studies support that the Notch signaling pathway regulates cellular behavior of $CD4^+$ T cells (Amsen et al., 2015; Vijayaraghavan and Osborne, 2018; Tindemans et al., 2017a, 2017b). Notch signaling pathway is highly conserved during both evolution and homeostasis. It modulates cell differentiation, proliferation and fate decisions in many tissues. Notch family includes four receptors (Notch1-4) and five ligands comprising three Delta-like ligands (Dll1, Dll2 andDll4) and two Jagged ligands (Jag1-2) in mammals. The canonical Notch signaling pathways acts in cell–cell contacts manner. This pathway is activated when the transmembrane receptor and ligand are interacted, along with a serious of cleavages. The Notch intracellular domain (NICD) fragment in a signal-receiving cell is released under variety enzymes (furin, ADAM metalloproteases, γ-secretase complex) through sequential reactions. The NICD immediately reaches the nucleus stimulated by its nuclear localization signal. The NICD fragment in conjunction with CBF-1/Su(H)/LAG1 (CSL), which is also known as the DNA-binding protein RBPJκ (recombination signal–binding protein for the immunoglobulin Jκ), lead to the recruitment of the co-activator Mastermind-like (MAML) and the other transcriptional co-activator (Raphael and Ilagan, 2009). This is RBPJκ-dependent Notch signaling (canonical Notch signaling). It regulates the downstream target genes hairy/enhancer of split (Hes) and Hes-related proteins such as hairy/enhancer of split with YRPW motif(Hey) (Amsen et al., 2015; Vijayaraghavan and Osborne, 2018).

It is demonstrated that antigen-presenting cells (APC) can apply Jagged-mediated Notch signaling pathway to instruct Th2 cell differentiation, which is independent of IL-4/STAT6 axis. This process is implemented by inducing GATA-3 gene, as well as by directly regulating Il4 gene transcription through RBP-Jκ sites in a 3′enhancer (Tindemans et al., 2017a, Amsen et al., 2004). Other researches also suggest that differentiation of Th1 and Th2 cells is performed by Notch-like ligands and Jagged respectively (Amsen et al., 2015; Vijayaraghavan and Osborne, 2018). Jagged1 is considered to play an essential role in the Th2 response in a model of airway hyperresponsiveness (AHR) (Okamoto and Matsuda, 2009). However, another study shows that Jagged1 and Jagged 2 are dispensable for the Th2 cell differentiation in vivo. They find that expression of Jagged1, but not Jagged2, is increased on dendritic cells (DCs) after house dust mite (HDM) exposure, whereas Th2 cell differentiation has no difference in contract to the control group when DC-specific Jagged1 or Jagged2 single- or double-deficient mice is intranasal sensitized and challenged with HDM in vivo (Tindemans et al., 2017a). In addition, Minter et al. demonstrate that DAPT can effectively attenuated Th1 polarization by upregulating the level of Tbx21 (Minter et al., 2005). They point that Notch1 can directly regulate Tbx21 via the Notch1/CSL complex formed on the Tbx21 start site. In vitro and in vivo, DAPT reduce the secretion of IFN-γ and prevent the Th1 polarization. However, the polarization of Th2 is not affected, which seems to be proceed through Notch-independent pathway.

For Th2-mediated diseases, it is assessed by Kang et al. that the therapeutic potential of DAPT against allergic asthma. The result shows that DAPT directly lead to down-regulate Hes1 together with reduction of NICD and GATA-3 levels in an experimental model of asthma immunized by ovalbumin (OVA). Moreover, DAPT can result in decreasing Th2 cytokine production and increasing Th1 cytokine secretion, which is dependent on Notch signaling pathway (Kang et al., 2009). It is reported that in AR model sensitized by OVA, DAPT cause the reduction of Th2 cytokine levels, but have no impact on Th1 responses (Shi et al., 2017). These studies indicate that the inhibition of Notch signaling pathway can be a potential method for treating Th2-type and airway inflammatory disease. OME is associated with an imbalance with th1/th2 cells immune response. However, little is known regarding blocking Notch signaling by DAPT whether the Th1 and Th2 differentiation are affected in OME model and inflammation of middle ear are attenuated in vivo. To better understand the relationship between Notch signaling and OME, we investigated whether Notch signaling was associated with OME and observed the pathophysiologic changes of OME by blocking the notch signaling pathway used DAPT in OME model sensitized and challenged with OVA.

2. Materials and methods

2.1. Animals and treatments

Male Sprague-Dawley (SD) rats, weighing 250–300 g, were used in our experiment. The animal experiment was approved by the Institutional Animal Care and Use Committee of Capital Medical University (Permit Number: AEEI-2018-102). According to the method established as described previously, it was slightly improved to establish the model of OME (Hardy et al., 2001; Pollock et al., 2002; Labadie et al., 1999). In brief, experimental rats were sensitized on Days 1 and 8 by intraperitoneal(i.p.) injection 1.2 mg of ovalbumin (OVA, Sigma-Aldrich, USA), emulsified in 5.14 mg of aluminum hydroxide of 1.2 ml of phosphate-buffered saline (PBS). And rats were anesthetized with 10% chloral hydrate (0.03 mg/kg, i.p.) and 1% sodium pentobarbital (50 mg/kg i.p.). Bilateral tympanic membranes (TMs) were examined by a surgical microscope to exclude visible middle ear diseases (e.g. perforation, suppuration, inflammation, etc.). Then rats were challenged with 0.1 mg OVA in 35ul PBS, which were injected by the anterior and inferior quadrant of TM with a microsyringe once a day on days 15 and 16. The controls were sensitized with 5.14 mg aluminum hydroxide of 1.2 ml PBS and challenged with 35ul PBS. DAPT (Sigma-Aldrich, USA) was dissolved in the vehicle, which was 5% dimethylsulfoxide (DMSO, Sigma-Aldrich, USA, diluted in corn oil) (vol/vol). Rats were injected (i.p.) vehicle (DMSO) or different dose of DAPT for seven consecutive days from Day 12 to Day18. And DAPT or vehicle were administered at a half hour before each OVA challenge (Fig. 1A). These rats were randomly separate into five groups: (1) control group; (2) OME group; (3) OME + DMSO group;(4) OVA + DAPT (5 mg/kg) group;(5) OVA + DAPT (10 mg/kg) group. Rats were reanesthetized 4 h after last injection of DAPT or DMSO. The morphology of TM was evaluated once again under the surgical microscope and the tympanic picture was taken with 0°-angled endoscope (STORZ, German). Then rats were sacrificed and bullae were discreetly removed.

2.2. Histopathology

Bullae were fixed for 24 h at 4℃ in 4% paraformaldehyde (PFA) dissolved in PBS, and decalcified in 10% EDTA decalcifying solution (E1171, Solarbio) for 3 weeks. Then bullae were dehydrated and embedded with paraffin wax. Sections with a thickness of 5–6 μm were stained with hematoxylin and eosin (H&E) or Alcian Blue Periodic acid Schiff (AB-PAS, G1285, Solarbio), following the instructions. These sections were automatically scanned by Pannoramic SCAN. The severity of inflammation was evaluated by measuring the thickness of the epithelium and submucosa. The thickness of the epithelium and submucosa was measured in two areas, a portion of bulla near the cochlea apex and over the cochlea apex (Kim et al., 2016). The thickness of the

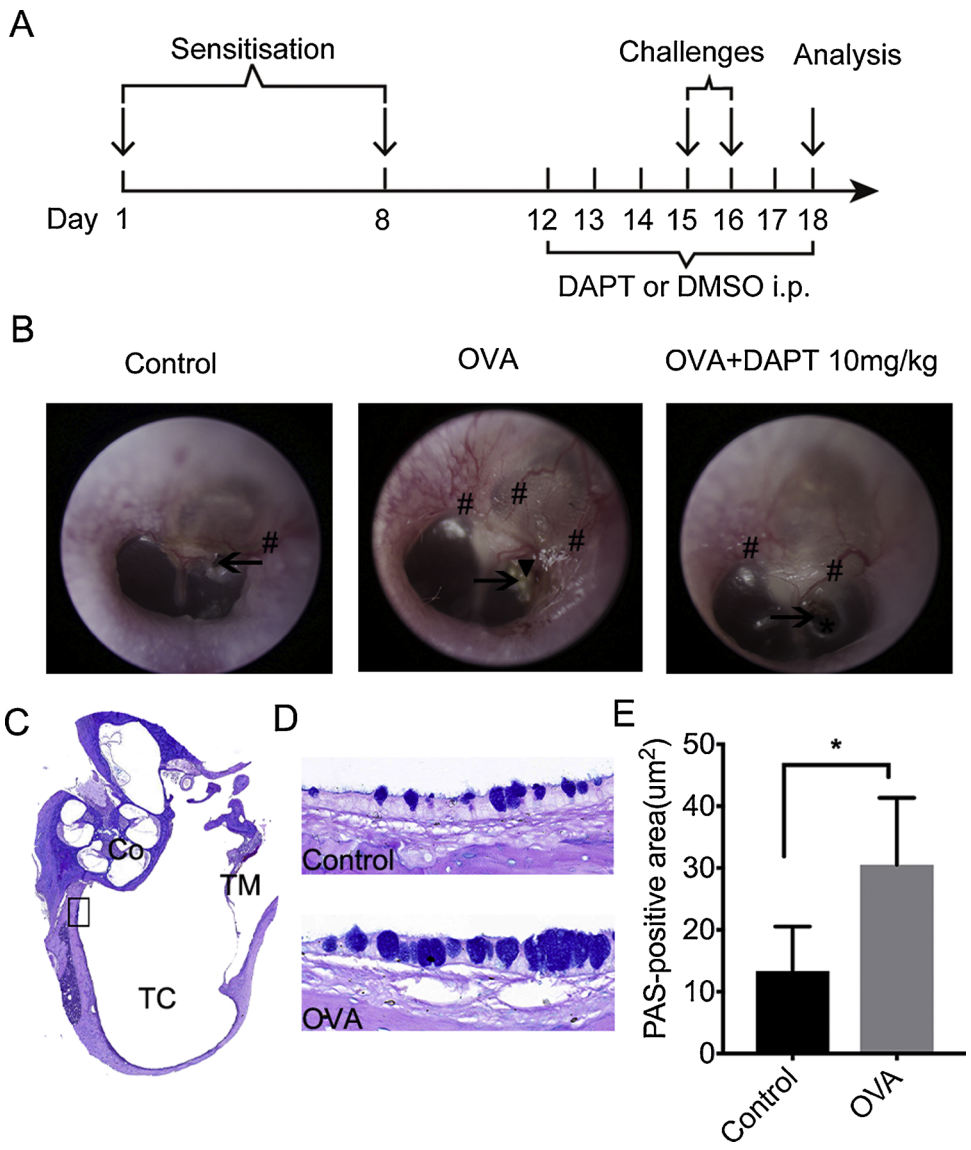

Fig. 1. Evaluated of OVA-induced OME model. A. Experimental OVA-mediated OME outline, showing that rats were sensitized on day1 and 8, and challenged with OVA or PBS as a control. And then they were treated with DAPT (5 mg/kg,10 mg/kg) or vehicle from day 12 to 18. Analyses were performed four hours after the last injection. i.p., intraperitoneal. B. The morphology of tympanic membrane. Radial vascularity (pound sign), The puncture site (arrow), epithelialization (asterisk), secretions (inverted triangle). C. We evaluated goblet cells near the cochlea apex. TM, tympanic membrane, cochlea, TC, tympanic cavity. Representative photos of PAS-stained middle ear sections different groups (n = 5 ears per group). The positivity of PAS-staining is dark blue. E. Quantification the PAS-positive area in the middle ear. Shown is mean ± s.d.. *P < 0.05.

epithelium was referred to the distance between the epithelial surface and basement membrane, and the thickness of the submucosa was defined as the distance between basement membrane and the bone surface (Hirokazu et al., 2002). The thickness was measured at the thickest and thinnest region in three contiguous sections by case view software (×400 magnification). Goblet cells, quantified using Image J software, were the area of PAS-positive staining area in same position in each section.

2.3. Quantitative real-time PCR

Total mRNA was extracted using the RNeasy Mini Kit (74104, Qiagen) following its instructions. The first-strand complementary DNA (cDNA) was synthesized by FastKing RT Kit (KR116, TIANGEN), and amplified with PowerUp™ SYBR™ Green Master Mix (A25742, Thermo Fisher Scientific), which was applied for quantitative real-time PCR (qRT-PCR) with Bio-Rad IQ5 system. All primers were as follows. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level of mRNA (Schmittgen and Livak, 2008). Primer sequences for qRT-PCR were as follows:

GAPDH: forward-(F): 5′-TCATTGACCTCAACTACATGG-3′, reverse-(R):5′-TCGCTCCTGGAAGATGGTG -3′, Jagged1: F: 5′-CGCCCTCTGAA AAACAGAAC-3′ R: 5′-ACCCAAGCCACTGTTAAGAC-3′, Hes1: F: 5′-AGAA AAATTCCTCGTCCCCG-3′ R: 5′-TTTCATTTATTCTTGCCCGGC-3′, IL-4: F: 5′-TGTCAGCGGTCT GAACTCAC-3′ R: 5′-TTCAGTGTTGTGAGC GTGGA-3′, IL-5: F: 5′-AGCACAGTG GTGAAAGAG ACC-3′ R: 5′-CCTCA TCGTCTCATTGCTCGT-3′, IFN-γ: F: 5′- TCTGGAGGAACTGGCAAAAG-3′ R: 5′-TCAAGACTTCAAAGAGTCTGAG-3′, GATA-3: F: 5′-CGATAGCATG AAGCTGGAGAC-3′R: 5′-TAGGGCGGATAGGTGGTAAT-3′, T-bet: F: 5′-AACAAGGGGGCTTCCAAC-3′ R: 5′-TGGTACT TATGGAGGGAC TGC-3′.

2.4. Immunofluorescence staining

Bullae were fixed for 2 h at room temperature in 4% PFA and washed 3 times in PBS (10 min/time). Middle ear mucosa was carefully separated under the microscope, then permeabilized with 0.3% Triton X-100 in 0.2 M PBS at room temperature for 1 h and subsequently blocked for 1 h with 10% goat serum (ASGB-BIO). Then it was incubated with primary antibodies over-night at 4 °C. The following primary antibodies were used: rabbit anti-Jagged 1 antibody (diluted 1:100, ab7771, Abcam), rabbit anti-Hes1 antibody (diluted 1:100, ab108937, Abcam), mouse anti-Hes1 antibody (diluted 1:200, sc166410, Santa Cruz). After washing with PBS 3 times(10 min/time) in next day, middle ear epithelium mucosa was incubated with the following second antibodies at room temperature for 2 h: anti-rabbit Alexa

Fluor™ 488(1:300, A11008, Invitrogen), Alexa Fluor™ 594 Phalloidin (1:300, A12381, Invitrogen). Samples were washed three times and spread on the glass slide with DAPI (ZSGB-BIO, ZLI-9557), used for staining nuclei. Slides were imaged with Leica TCS SP8 Laser Scanning Confocal Microscope. Images were edited with Adobe Photoshop CS6. The expression of Jagged1 was measured by Image J software.

2.5. Statistical analysis

Animals were males and randomly divided into five groups. We applied the Student's *t*-test to calculate statistical difference, using the SPSS statistics. The differences between the groups were analyzed by an independent-sample *t*-test. Diagrams were drawn with GraphPad Prism7.0 software. The results are showed as mean ± s.d. The statistical significant difference was described as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

3. Result

3.1. Establishment of OVA-sensitized and challenged OME model

To evaluate the model of OME, we assessed the pathologic features of middle ear. In control group, the TM was normal with a few radial vessels around it. There was no effusion in middle ear cavity or inflammatory changes. In OME group, the TM was muddy and thickened with different degrees of radial vascularity. The puncture site was epithelialized and partially blocked by secretions (Fig. 1B). In these animals, there was otopiesis, and bubbles were informed in tympanic cavity in some rats. It was in line with previous reports (Hardy et al., 2001; Labadie et al., 1999). In addition, all experimental TMs had OME pathological condition under the surgical microscope.

In addition, the thickness of epithelium and submucosa were analyzed in two position, over the cochlea apex (arrow) and near the cochlea apex (asterisk). The locations of two area were shown in Fig. 2A. In OME group, it was found that inflammatory cell infiltration, telangiectasia, mucosal edema in middle ear cavity. Compared to the control group, the thickness of the epithelium in OME group was significantly greater at two different areas. The thickness of the epithelium in OVA-induced group in two position was (1.63 ± 0.33)μm and (11.74 ± 1.78)μm respectively (Fig. 2B and C). In addition, the submucosa was much thicker than controls over the cochlea apex and near the cochlea apex ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 2B, D).

The mucus secretion is found in animals and human with OME (Labadie et al., 1999; Mills and Hathorn, 2016; Bhutta et al., 2017). Thus, we tested the expression of mucins in OVA-induced OME model. Mucins are secreted by goblet cells in mucosal epithelia (McCauley and Guasch, 2015). Notably, we found that the majority of goblet cells were found in OVA group, whereas only a small percentage were detected in control group (Fig. 1C–E).

3.2. Notch signaling inhibition relieved middle ear inflammation

To investigate the effect of DAPT on OME caused by allergy, we used the model induced by OVA. There was no striking difference between OME group and DMSO group. Simultaneously, two different dose of DAPT (5 mg/kg,10 mg/kg) were administrated, respectively. DAPT was injected intraperitoneally for consecutive seven days from day 12 (Fig. 1A). Contrast with OME rats, there was no change in epithelial and submucosal thickness after injected low-dose DAPT (Fig. 2B–D). However, after high-dose DAPT treatment, there was a small amount of blood vessels around the TM (Fig. 1B). The effect of high-dose DAPT was further assessed in the histological changes. Notably, the epitheliums of middle ear were even thinner than OME group at two position, over the cochlea apex ((1.44 ± 0.29)μm vs. (1.63 ± 0.33)μm) and near the cochlea apex((10.69 ± 1.88)μm vs. (11.74 ± 1.78)μm) (Fig. 2B and C). Similarly, we also observed a significant decrease in the submucosal thickness after Notch signaling blockade (Fig. 2B, D). So, DAPT can alleviate Th2-associated pathology in allergic OME, including the thickness of mucosa and submucosa and middle ear inflammation.

3.3. DAPT decreased the expression of Jagged1 and Hes1 in rats with OME

To study the localization and the expression of Notch signaling in middle ear epithelium, we surveyed middle ear mucosa from rats with OVA-induced OME, DMSO-treated OME, DAPT-treated OME compared to control group. Immunofluorescent staining was applied for recognizing the Jagged1 and the downstream target gene Hes1 and their localization. Quantitative RT-PCR was used for testing the level of Jagged1 mRNA and Hes1 mRNA. Previous study has shown that Notch receptors and their ligands were expressed in most of normal mouse middle ear epithelial cells (Liu et al., 2016). In accordance with previous study, our results showed that the Jagged1 staining was located on the cell membrane of middle ear mucosa (Fig. 3A). Analysis of immunofluorescence stained middle ear mucosa, Jagged1 expression was increased in OVA-induced OME, as compared with it in control middle ear($p < 0.05$). Furthermore, OME treated with high-dose DAPT showed decreased Jagged1 expression (Fig. 3B). When tested the expression of Jagged1 mRNA, it was found that Jagged1 expression in OME was upregulated in contrast with the normal group. There was no striking difference in OME group and in vehicle-treated group. Analysis of the influence of DAPT on Jagged1 in OME, we also observed that levels of Jagged1 mRNA were downregulated after administering DAPT (Fig. 3C). Likewise, we also detected that DAPT regulated the expression of Jagged1 in a dose-dependent manner in OVA-induced OME rats. To further assess the role of DAPT in OME, we measured the Notch target gene Hes1 in these rats. There was no nucleus staining in middle ear mucosa with anti-Hes1 antibody, but Hes1 immunoreactivity was appeared on the epithelial cell membrane (data not shown). Moreover, the outcomes found that the level of Hes1 in OME group, compared to that of control group, was dramatically increased. In addition, it was found that treatment with DAPT led to a decrease in the Hes1-expressing (Fig. 3D). These data suggest that Notch signaling is active in OVA-induced OME, and it is effectively blocked by DAPT in vivo.

3.4. DAPT alleviated the expression of Th2 cytokines but no effect on the level of Th1 cytokines

To confirm that the effect of Notch signaling on Th1 and Th2 cytokines in OME, we sensitized and challenged SD rats with OVA to establish experimental model because of the imbalance in Th1/Th2 cells in OME. Compared with normal rats, the levels of IL-4mRNA and IL-5 mRNA in middle ear mucosa with OME rats were increased, but IFN-γ mRNA-expressing was decreased (Fig. 4A–C). It was in line with previous study (Pollock et al., 2002). We also observed subsequent changes in Th1 and Th2 cytokines by blocking Notch signaling pathway with DAPT. When rats were treated with DAPT (5 mg/kg,10 mg/kg), the production of IL-4 mRNA was reduced (Fig. 4A), whereas the IL-5 expression was no change (Fig. 4B). The level of Th2 cytokines could be alleviated by DAPT in a dose-dependent approach. However, the expression of IFN-γ mRNA were not striking different between vehicle-treated group and DAPT-treated group (Fig. 4C). Although the effect of enhancing Th1 cytokine production has been reported with DAPT administration to asthma mice (Kang et al., 2009), we did not find same effect with DAPT treatment in OME rats. Whatever, inhibiting Notch signaling was possible to result in a reverse in Th1/Th2 balance of middle ear epithelium in OVA-induced OME. Furthermore, the expression of GATA-3 and T-bet, which were crucial transcription factors of Th1 and Th2 cells, were also evaluated, respectively. It was found a decrease in GATA-3 and increase in T-bet in OME rats contrast to normal rats, as tested by RT-PCR. In addition, it showed that there was no difference in the level of T-bet between vehicle-treated rats and DAPT-treated rats. And DAPT seemed no remarkable influence on the

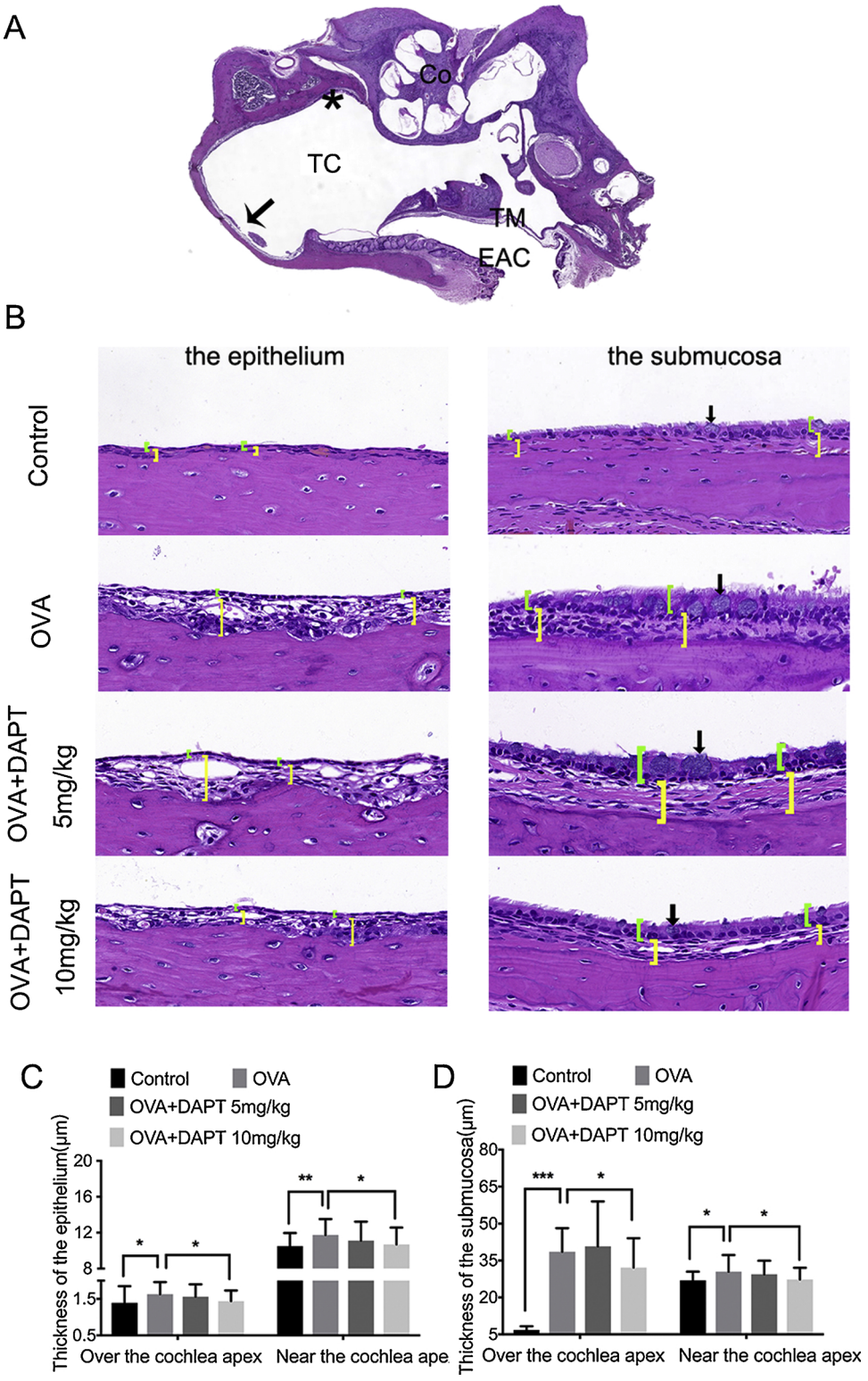

Fig. 2. Notch signaling inhibition(DAPT) relieved middle ear inflammation in OVA-driven OME rat model. A. We measured the thickness of the epithelium and submucosa in two different position, a portion of bulla over the cochlea apex(arrow) and near the cochlea apex (asterisk). EAC, external auditory canal, TM, tympanic membrane, Co, cochlea, TC, tympanic cavity. Scale bars = 1000 μm. B–D. The thickness of the epithelium(green) and submucosa(yellow) were measured to evaluated the severity of inflammation by H&E staining (n = 5 ears per group, ×400 magnification). The thickness of the epithelium and submucosa in DAPT-treated group were greater than vehicle-treated group. Goblet cell(arrow). Scale bars = 20 μm. Data are presented as mean ± s.d.. *P < 0.05, **P < 0.01, ***P < 0.01.

expression of T-bet (Fig. 4E). The results suggest that Notch signaling is related to the secretion of Th1 and Th2 type cytokines in OME, and DAPT seemingly suppresses the expression of Th2 cytokines but no impact on the production of Th1 cytokines.

4. Discussion

The Notch signaling pathway involves in the development of Th2 immunity response. It was demonstrated earlier in OVA or HDM-driven mouse models of asthma (Tindemans et al., 2017a, Kang et al., 2009) and in murine allergic rhinitis models using OVA (Shi et al., 2017). And Notch receptors and ligands were also expressed in the normal mouse middle ear epithelium (Liu et al., 2016). We provide several lines of evidence that Notch signaling plays an important role in OME induced by OVA.

In line with previous reports, OVA-sensitized and challenged OME model was successfully established by morphological characteristics of middle ear (Hardy et al., 2001; Labadie et al., 1999; Mills and Hathorn,

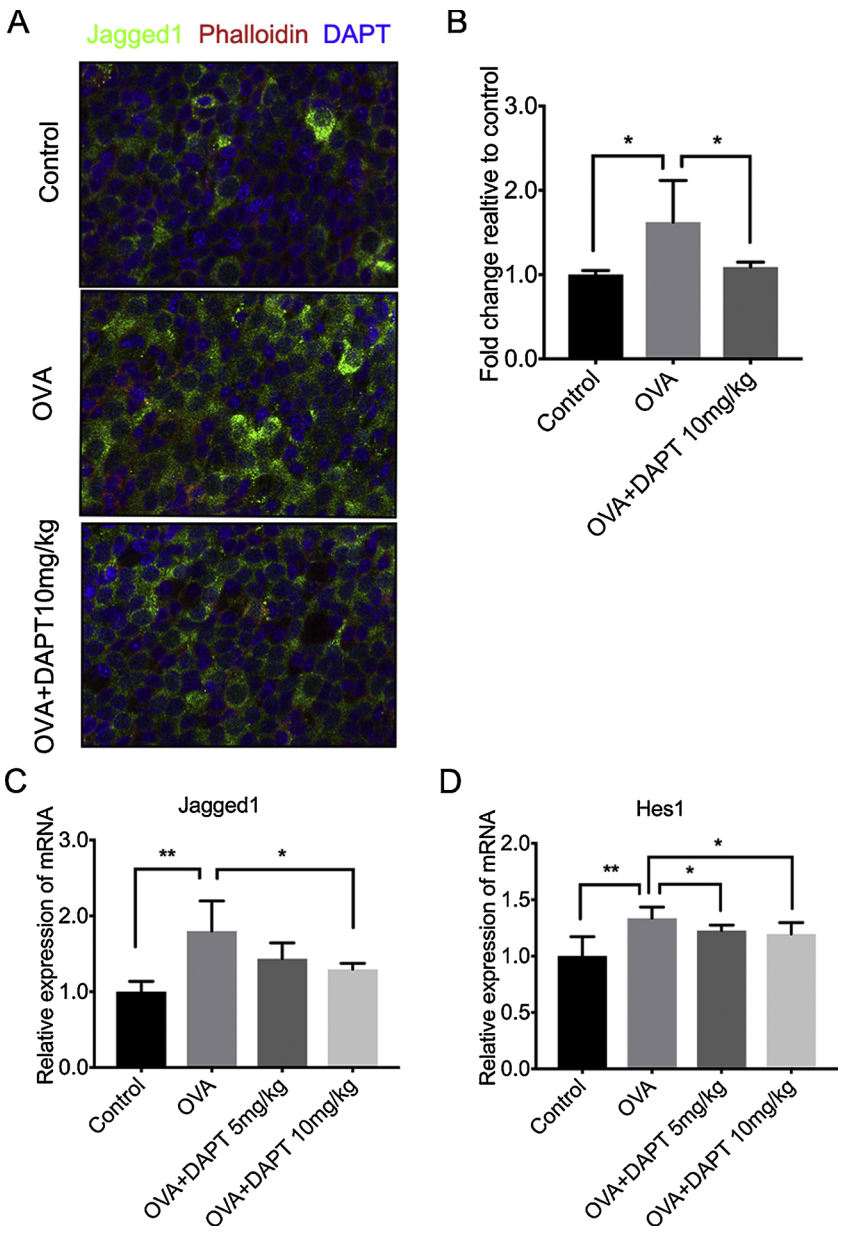

Fig. 3. The expression of Jagged1 and Hes1 in rats with OME after DAPT treatment. A. Immunofluorescent staining for the Jagged1 marker (green) and cytoskeleton marker (Phalloidin, red) in the middle ear epithelium. Nuclei was stained with DAPI(blue). Jagged1 immunoreactivity was located in the cell membrane. Scale bars = 50 μm. B. Quantification of the fluorescence intensity for Jagged1 marker (n = 5 ears per group). C–D. The middle ear mucosa was isolated from the bullae, and then total RNA was extracted to estimate the level of mRNA via qRT-PCR. The level of Jagged1 mRNA and Hes1 mRNA was showed (n = 6 rats per group). Data are expressed as mean ± s.d..*P < 0.05, **P < 0.01.

2016; Bhutta et al., 2017). In the present study, we found that inflammatory cells were increased, the middle ear mucosa and submucosal tissue became thick and capillary were proliferation. Numerous mucins secreted by goblet cells were observed as well. Hurst DS et al reported that the middle ear mucosa also caused an allergic response, like the other upper respiratory tract (Hurst and Venge, 2000).They pointed that the inflammatory response by eosinophils and mast cells, which were vital to a Th2 type immune response, were present in most ears with OME. Consistent with previous studies, we observed that Th2 cytokines were notably expressed in OVA-induced allergic OME (Pollock et al., 2002; Kim et al., 2016). It demonstrated the applicability of this modeling method for investigations of OME.

Our results indicated that the pathological characteristics of OME were alleviated by DAPT treatment. It has been demonstrated that the cytokine IL-4 played a key role in the sensitization of allergies, and IL-5 was essential for the proliferation and differentiation of eosinophils (Scheinman and Avni, 2009; Tindemans et al., 2017a). Furthermore, it is well-known that IL-4 plays a vital role in modulating Th2 inflammatory response. It promotes B cell maturation as well as converts IgG to IgE. Mast cells, stimulated by IgE, secrete mediators such as histamine, tumor necrosis factor (TNF). And IgE-activated mast cells also produce many cytokines, chemokines and growth factors. They are crucial for pathophysiological changes and tissue remodeling in allergic disease (Galli and Tsai, 2012). So, these inflammatory substances can cause edema of the middle ear epithelium and submucosa and vasodilation in OME rats. Meanwhile, the thickness of epithelium and submucosa was relieved after treatment with DAPT. It might be related to a decrease in IL-4 expression in DAPT-administrated group.

Although Notch has been demonstrated to be important in the regulation of most T helper cells differentiation, it remains controversial whether it undertakes a molecular switch or acts as delicate context-dependent role in helper T-cell responses (Vijayaraghavan and Osborne, 2018). Besides, there is dispute on the role of Notch signaling pathway in Th1 and Th2 differentiation (promoting or suppressing). Minter and colleagues reported that treatment by DAPT or deleted Notch1 to mice with aplastic anemia (AA), which is a th1-mediated diseases model, ameliorated symptoms of AA (Roderick et al., 2013). Moreover, it was in line with their earlier research that DAPT effectively attenuated the symptom of EAE, a th1-mediated classical autoimmune diseases model, and effectively blocking Th1 differentiation

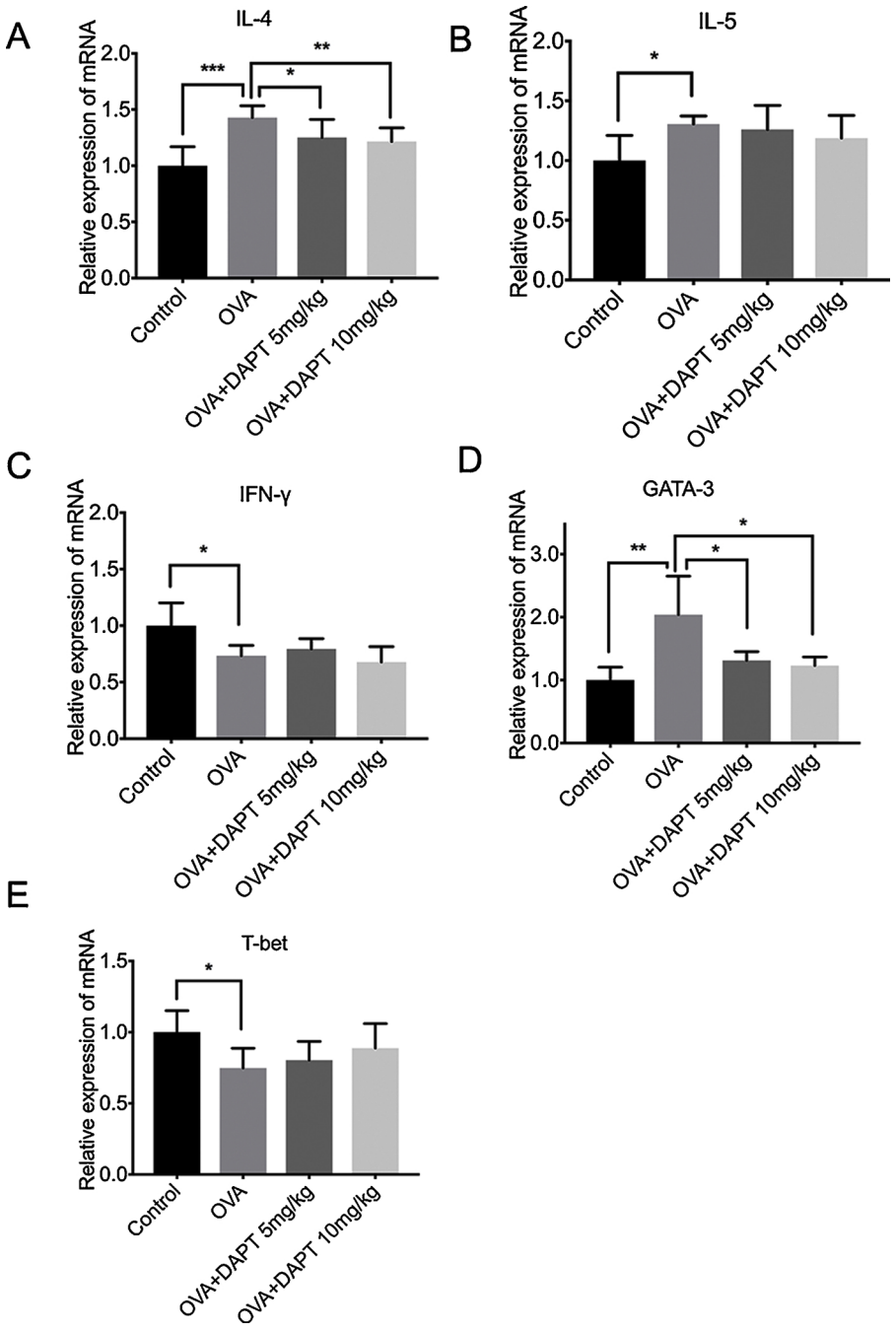

Fig. 4. DAPT treatment alleviated the expression of Th2 cytokines in middle ear mucosa. Quantitative RT-PCR was used for analyzing of Th1 and Th2 cytokines expression (n = 6 rats per group). In addition, transcription factor GATA-3 and T-bet were also measured. Data are showed as mean ± s.d..*P < 0.05, **P < 0.01, ***P < 0.001.

(Minter et al., 2005). They suggested that the Th1 polarization was weaken by inhibition of Notch signaling, which was achieved by Notch1 directly regulating the expression of T-bet (Minter et al., 2005; Roderick et al., 2013). Contrary to these results, it was demonstrated that suppression Notch signaling with DAPT contributed to a dramatic shift from Th2 to Th1 phenotypic change by GATA-3-mediated mechanism in allergic pulmonary inflammation (Kang et al., 2009). In our study, the level of Th2 cytokines (IL-4) was decreased in OME in a dose-dependent manner by DAPT treatment, and there was a significant increase of GATA-3 expression in OVA-mediated OME model in vivo. DAPT treatment could down-regulate GATA-3 production, but no effect on the level of T-bet. These data indicate that the GATA-3 production is regulated by Notch signaling. Similarly, Jiang and his colleagues reported that the blunting Notch signaling could inhibit of Th2 polarization and improve Th1 cell differentiation, so as to reverse the Th1/Th2 imbalance in the mice model of food allergy (Jiang et al., 2017). They found that the level of transcription factor GATA-3 and T-bet was decreased and increased separately when Notch signaling was blocked, and suggested that GATA-3 might be a direct target gene of Notch signaling pathway. In contrast, other study reported that the Dll4, one of Notch ligands, significantly decreased airway hyperreactivity and the production of Th2-type cytokines (IL-4) in the pathogenesis of allergic respiratory disease by virus infection (Mukherjee et al., 2014). We found that the ligand of Notch signaling (Jagged1) was increased significantly in OME rats, Jagged1 protein was located in the cell membrane and DAPT validly blocked the Notch signaling pathway.

Consistent with previous study, it was reported that Jagged1 production were significantly elevated in the lung of OVA-induced mice (Hu et al., 2018). Another study reported that allergic airway inflammation was alleviated by inhibiting Notch signaling pathway, by which treatment of $CD4^+$T cells with DAPT or Jagged1 expression through siRNA-Jagged1 silencing of bone marrow-derived dendritic cells (BMDCs) (Okamoto and Matsuda, 2009). They suggested that Jagged1 played an important role in Th2-type allergic inflammation.

In consonance with these results, Shi et al also reported that blocking Notch signaling could regulate Th2 immune response and alleviate upper airway inflammation in AR. They found that DAPT ameliorated the eosinophils infiltration, diminished the level of IL-4, but the level of IFN-γ was no change in AR model (Shi et al., 2017). We also found that DAPT can decrease IL-4 expression but has no impact on the production of IFN-γ. As mentioned earlier, there is controversial for the IFN-γ expression regulated by Notch cascade. Besides, our result was inconsistent with previous studies that DAPT effectively reduced the secretion of IL-5. The reason for these differences are unclear. The following factors might be the account for discrepancies. Firstly, it is related to different disease models in different experimental animal species. It was found that the level of IL-4 and IL-5 was decreased but secretion of IFN-γ was increased when Notch signaling was inhibited by DAPT in asthmatic mouse (Kang et al., 2009). However, DAPT efficiently suppressed T-bet and IFN-γ expression in EAE model (Minter et al., 2005). Secondly, the route of DAPT administration causes the difference. Rats in our experiment were injected with DAPT by intraperitoneal, whereas mouse were treated by intranasal (Kang et al., 2009; Shi et al., 2017). Thirdly, different observational indicators and experiments in vitro and in vivo may affect the results. So, the expression of these cytokines may be influenced by diverse experimental conditions. Furthermore, it may be related to other signal pathways, such as the nuclear factor (NF)-kB signaling pathway.

NF-kB, a nuclear transcription factor, is considered to play an important role in immunity process and regulating inflammation (Poynter et al., 2004; Moon et al., 2018; Shin et al., 2006; Espinosa et al., 2010). The airway inflammation and the expression of T cell cytokines were alleviated by repressing of NF-kB in OVA-induced allergic airway mice (Poynter et al., 2004). Blockage of NF-kB pathway repressed the level of GATA-3 and Th2 cytokines (Das et al., 2001). Similarly, the symptoms of rhinitis were reduced through blocking NF-kB activation, associated with decreased the infiltration of inflammatory cells in AR mice (Shin et al., 2006). Several significant evidences support the relationship between Notch signaling and NF-kB pathway. The intracellular domain of Notch1 directly mediated the level of IFN-γ through the complex formed with p50 and c-Rel, as well as augmented retention of NF-kB in the nucleus to promote NF-kB activity. In addition, other study suggested that Notch promoted the activation of NF-kB in T cell acute lymphoblastic leukemia (T-ALL) cells and in T-ALL animal model (Espinosa et al., 2010). They showed that blockage of Notch signaling by DAPT depressed the activation of NF-kB cascade. Kang et al. also found that the regulatory effect of cytokines mediated by DAPT was partly relied on its blockade of NF-kB activity, or acted through inhibition NF-kB-independent mechanism (Kang et al., 2009).

Our results suggested that there was the imbalance of Th1/Th2 response in OVA-induced allergic OME. DAPT entirely prevents the activation of Notch signaling pathway by blockage the proteolytic cleavage (Raphael and Ilagan, 2009).Blocking Notch signaling pathway can suppress Th2 polarization to regulate the balance of Th1/Th2 response and improve middle ear inflammation. Moreover, the upregulation of Jagged1 is accompanied by the increase of Th2-type cytokines (IL-4, IL-5), and expression of IL-4 and GATA-3 was downregulated after high-dose DAPT treatment. When allergic OME rats were administrated by low-dose DAPT, the secretion of IL-4 was decreased but the production of Jagged1 and IL-5 was no change. We suspect that other members of Notch signaling may participate in Th2 cytokines regulation. Besides, the relationship between Th2-type cytokines and Jagged1 also needs further investigation.

5. Conclusions

In summary, we demonstrated that Notch signaling pathway played an important role in OVA-induced allergic OME in vivo. In addition, our results showed that the middle ear inflammation was relieved via DAPT-mediated blocking Notch signaling pathway. The imbalance of Th1/Th2 was changed, regulating Th2-related cytokines (IL-4) secretion and key transcription factor (GATA-3) expression. It is indicated that DAPT may be a potential treatment for allergic OME.

Declaration of Competing Interest

The authors declare that there is no financial interest in this article.

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