HDM-induced Atg5 Mediates TSLP Expressing to disrupt epithelial barrier

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Abstract

Background: Asthma is a complex and heterogeneous disease. Autophagy, process of self-protection in cells, is an intracellular process when cells are being attacked by certain stress. Our team focused upon the disruption of airways epithelial barrier in asthma, and we interested in whether autophagy played a key role in asthma.

Methods: 400U HDM was used to treat HBECs and established asthmatic mice model. Western blotting, RT-PCR and immunofluorescence were mainly used to detect autophagy process in vivo and in vitro. One way ANOVA and Mann Whitney test were used for statistic.

Results: After treated with HDM, expression of LC3ab increased in vivo and in vitro. Using Rapamycin, 3-MA and Chloroquine to treat HBECs, then we surprisingly found that HDM disrupts epithelial barrier through incomplete autophagy. To find out the connection between asthma and autophagy, we chose known autophagy related genes to determine the association between autophagy and disruption of airway epithelial barrier. Atg5 and atg12 were chosen because these two genes varied upon the time dependent manner. Knocked down the expression of atg5 or atg12 by siRNA, the expression of TSLP, which can induce the disruption of airway epithelial barrier, remarkably reduced.

Conclusions: These results demonstrated that HDM induced inflammatory in airway epithelium through autophagy, and then knocked down autophagy related genes alleviated the inflammatory in HBECs.
Keywords: Asthma, HDM, autophagy, TSLP, Atg5, epithelial barrier.

Abbreviation

Human bronchial epithelial cells, HBECs
Background

Asthma is a complex chronic inflammatory heterogeneous lung disease[1]. Chronic inflammation may finally lead to hyperresponsiveness to common allergens, microorganisms, oxidants, pollutants, and It is also associated with immune system activation, airway hyperresponsiveness (AHR), epithelial cell activation, airway epithelial barrier disruption, mucus overproduction and airway remodeling[2, 3]. Under normal circumstances, airway epithelial cells connected by tight junctions secrete mucus, airway surface lining fluid, host defense peptides, and antioxidants and express innate immune pattern recognition receptors to respond to inhaled foreign substances and pathogens[4]. Airway epithelium has been thought to function mainly as the first defensive barrier to protect lung from allergens. The integrity of airway epithelial barrier depends on apical tight junctions composed of zonula occludens 1-3, occludin, and claudin 1-5, and on adherens junctions, which consist of E-cadherin, β-catenin, and junctional adhesion molecule, that keep bronchial epithelial cells together and maintain their apicobasal polarity. When asthma patients were exposed to allergens, such as House Dust Mites (HDM), airway epithelial cells activated and released cytokines, such as IL-33, IL-25 and TSLP, induced Th2 inflammation that play a critical role in asthma[3].

Autophagy, connect to a large number of human diseases, is a process that intracellular components and dysfunctional organelles are delivered to the lysosome for degradation and recycling, and it is essential for cell survival, bioenergetic homeostasis, organism development, and cell death regulation[5]. Recent studies showed that dysfunction in autophagy have been associated with environmental pollutant and allergen-induced oxidative stress, mitochondrial dysfunction, secretion of multiple inflammatory proteins, and subsequently development of asthma[6]. During the past two decades, some evidences have revealed that autophagy upregulation or activation may help to reduce levels of toxic protein species and apoptosis, thereby alleviate disease[7-9]. On the other hands, researchers also found that inhibition of autophagy can enhance the action of anti-cancer drugs by modulating the apoptotic response (Regulation of Apoptosis by Autophagy to Enhance Cancer Therapy.). Whether autophagy plays a positive or negative role in certain diseases is still under argument.

Our team focus on the airway epithelial barrier disruption induced by HDM. We hypothesized autophagy process plays critical roles in HDM-induced asthma, and in this study we evaluated that different stage of autophagic process played distinct roles in HDM-induced asthma.

Methods and Materials

Animals and experimental protocol
Specific Pathogen Free (SPF) C57BL/6 mice (female, 6 weeks old) were purchased from...
Southern Medical University (Guangzhou, China). The mice were housed in the SPF environment (room temperature 24°C, humidity range 40-70%, 12 h light/dark cycle). Providing sterilized water and food. All experiments were conducted under the guidelines outlined by the committee of Southern Medical University on the Use and Care of Laboratory Animals. Standard guidelines for laboratory animal care followed the Guide for the Care and Use of Laboratory Animals. HDM was purchased from ALK-Abello A/S (Denmark). C57BL/6 mice were randomly assigned into each of following 2 groups: (1) control group, phosphate-buffered saline (PBS; Gibco, Life Technology); (2) HDM group, treated intranasally with HDM (400 U/mouse each irritation, dissolved in sterilized PBS). The protocol of establishing the mouse model of HDM-induced asthma was as follows: The mice were injected 100 µl HDM solution intraperitoneally once a week in total two weeks. Three days after the last injection, mice were exposed to 25 ul of all treatments twice (50ul in total) for each irritation under inhaled isoflurane-anesthesia two times every week, for a total of 8 weeks.

Airway Hyper-responsiveness Measurement
The mice were anesthetized by 2,2,2-Tribromoethanol intraperitoneally, being performed tracheal intubation then baseline response was determined. After that, aerosol inhaled increasing concentrations (3.125, 6.25, 12.5, 25 and 50 mg/ml) of methacholine (Sigma Aldrich) respectively, the measuring interval was set to 5 min following next aerosol inhalation.

Cell culture and treatment
The human bronchial epithelial cell line, HBE-135 (ATCC, USA) was raised in Keratinocyte Medium (Sciencell) and placed in a humidified incubator at 37 °C with an atmosphere of 5% CO2. When the cells reached 70–80% confluence, the cells were treated with trypsin and seeded into culture plates at a density of 106 cells per cm for experiments. When the cells reached 90% confluence, the cells were rinsed with PBS twice and then stimulated with Rapamycin (Solarbio, China), 3-MA (Selleck, USA) and Chloroquine (Sigma, USA) and with or without HDM (ALK-Abello A/S, Denmark) for the indicated times and doses.

Pulmonary histologic examination
Left lungs were gently infused with 4% neutral formalin to fully inflate all lobes (inflation was judged visually) and immersed in formalin for at least 48h, then fixed, paraffin-embedded, cut in 5µm sections, and stained with hematoxylin and eosin (H&E) for blinded histopathologic assessment. For immunohistochemistry of LC3a/b, lung sections (5µm) were prepared with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany), and then submerged in citrate buffer (pH 6.0) for antigen retrieval. Samples were treated with PBS contained 5%BSA for 10 min at room temperature to block endogenous peroxidases, and then incubated overnight at 4 °C in recommended dilutions of anti-LC3ab (Cell Signaling Technology). After washing with PBS three times, slices were incubated with a secondary antibody for 30 min at room temperature. Signals were visualized with a DAB peroxidase substrate kit (ZhongShanJinQiao, Beijing).

Evaluation of epithelial barrier function
Epithelial barrier integrity was assessed by measuring TEER and FITC-Dx flux across the
monolayers of cultured epithelial cells. Briefly, confluent monolayers of HBECs, polarized at an air-liquid interface, were cultured in 12-well Transwell inserts (Corning Costar). TEER was measured using a Millicell ERS-2 Epithelial Volt-Ohm meter with an STX01 electrode (Millipore Corp, Billerica, MA, USA). Then, the apical medium (luminal side) was replaced with 200 μL of phenol red-free RPMI 1640 containing 0.5 mg/ml FITC-Dx (Sigma, USA), and the basal medium (non-luminal side) was replaced with 800 μL of phenol red-free RPMI 1640 without FITC-Dx, and the cells were incubated at 37 °C for 90 min. Samples were analyzed by fluorimetry (excitation 492nm; emission 530nm). Epithelial permeability was expressed as the percent leakage of FITC-Dx from apical to basolateral compartments.

**Western blotting analysis**

Cells were lysed in the RIPA lysis buffer (KeyGEN Biotech) containing PMSF (KeyGEN Biotech) at 4°C, 15min. After ultrasonicated 10s, the samples were centrifuged at 12000 rpm for 10min, and the supernatants were collected and boiled with standard SDS sample buffer. The samples were resolved by SDS-PAGE and western blotting analysis was performed for detection of the following antigens: TSLP (Abcam), p-ERK1/2 (Cell signaling technology), ERK1/2 (Cell signaling technology), LC3a/b (Cell signaling technology), p62 (Proteintech), E-cadherin (Cell signaling technology), and β -catenin (Santa Cruz), ATG5 (Santa Cruz), ATG12 (ABclonal), ATG7 (ABclonal), ATG16 (ABclonal), β-actin (Proteintech). After incubation with a secondary antibody, signal intensities were analyzed by using the Odyssey infrared Image System (Licor, USA).

**Quantitative real-time PCR (RT-qPCR)**

Total RNA was extracted from the treated cells by using Cell Total RNA Isolation Kit (Foregene, China). RNA samples were then reverse transcribed into first-strand cDNA using the PrimeScriptTM RT reagent kit (Takara). SYBR Green (Yeasen, China) was used to perform Quantitative RT-PCR by Real-Time PCR instrument (Bio-Rad, USA). Primers for amplifying glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5’-ATCAGCAATGCCTCCTGCAC-3’ (forward) and 5’-TGGCATGGACCTGTCGATTGCTG-3’ (reverse), autophagy related 5 (ATG5) were 5’-AAAGATGTGCTTCGCCAGATGTT-3’ (forward) and 5’-CCTTGTTGAGGAGTACAGGGTT-3’ (reverse), autophagy related 7 (ATG7) were 5’-CTGCCAGCTCGTAAACATTG-3’ (forward) and 5’-CTTGTGAGAGTCAGGTTTT-3’ (reverse), autophagy related 10 (ATG10) were 5’-ATAAGATGCGACTGCTACAGG-3’ (forward) and 5’-CAATGCCAGCCATGATGCT-3’ (reverse), autophagy related 12 (ATG12) were 5’-GTGCCGACACAAAGAA3’ (forward) and 5’-GTCCTGCGTCTGACTGCCC-3’ (reverse), autophagy related 16-like 1 (ATG16L1) were 5’-CTGGGACATCCAGGAGAG-3’ (forward) and 5’-CCTTTCTGGTTTAAGTCAGG-3’ (reverse). The amplification protocol was set as follows: denaturation at 95 °C for 10 min; 40 or 50 cycles denaturation at 95 °C for 15 s; followed by 1 min of annealing/extension at 60 °C. The levels of mRNA were normalized to those of GAPDH mRNA (the internal control; Δ Ct method) and were calculated and displayed as 2−ΔCt values.

**Adenovirus mRFP-GFP-LC3 infected**

HBECs were seeded in confocal plates at a density of 5x103 cells per well, cultured in a
humidified incubator at 37 °C with an atmosphere of 5% CO2 overnight, then infected with adenovirus mRFP-GFP-LC3 at indicated MOI for 8 hours. Changed fresh medium to culture 40 hours after adenovirus infected, then stimulated with HDM and Chloroquine for indicated time, the labeled sections were viewed and images captured using a confocal microscope (FV1000, Olympus) at 100× objective magnification.

**Immunofluorescence**
HBECS were seeded in confocal plates at a density of 5x103 cells per well cultured in a humidified incubator at 37 °C with an atmosphere of 5% CO2 overnight. When the cells reached 90% confluence, treated with reagents mentioned above for indicated times. At the end of the treatment period, the cells were fixed with 4% paraformaldehyde at room temperature for 10 min, washed with PBS for 3 times, 10 min per time, incubated with 0.2% Triton X-100 in PBS for 10 min, and rinsed again with PBS. Cells were blocked with 3–5% BSA in PBS at room temperature for 2h. The cell monolayers were then incubated overnight with a primary antibody at 4 °C in PBS containing 3–5% BSA, primary rabbit anti-E-cadherin (Santa Cruz, USA) and anti-β-catenin (Santa Cruz, USA) antibodies. Following PBS washes, the cell monolayers were incubated with a secondary antibody, Alexa Fluor 488 (R37118) or Alexa Fluor 594 (R37119) (1:200 diluted in PBS) (Invitrogen, USA), for 1 h at room temperature protect from light. The cell nuclei were stained with DAPI (Sigma Aldrich) for 10min at room temperature. The images captured using a confocal microscope (FV1000, Olympus) at 100× objective magnification.

**Statistical analysis**
Statistical analysis was performed using the GraphPad Prism 5 software package. Data are means ± SD and assessed for significance by one way ANOVA accompanied by Bonferonni’s post hoc test or by Mann-Whitney U test as specified on figure legends. Values of P < 0.05 were considered significant.

**Results**
**The expression of LC3ab could be induced by treatment of HDM both in vivo and in vitro.**
Autophagy is a common intracellular process induced by varied stimulation, so we hypothesized that allergen, House Dust Mites (HDM), induced autophagy in airway epithelium in vivo and in vitro. We successfully established the allergic asthma model mice initially to determine that autophagy induced by HDM in asthma mice, the airway epithelial structure of the mice stimulated by HDM were disrupted versus control group, and the inflammation in HDM group was severer than control group (Fig1 A), and also the mucous secretion more than control group (Fig1 B), while the level of airway resistance increased, and the level of pulmonary dynamic compliance decreased (Fig1 C). These data suggested that the structure of the lung in mice disrupted, mucus secretion increased and the lung dysfunction after treated with HDM. LC3ab, the marker of autophagy, was used to determine that autophagy upregulated significantly in mice airway epithelium, the expression of LC3ab increased significantly in HDM group (Fig1 D, left panel). We were interested in whether the expression
of LC3ab was increase in vitro, then we examined the expression of LC3ab in Human Bronchial Epithelial Cells (HBECs) after treated with HDM in indicated concentration for 24h. As we thought, the expression of LC3ab upregulated in HBECs (Fig2 A). These data suggested that HDM induced autophagy in both mice and HBECs.

The autophagy process was induced by treating with HDM in HBECs. According to our team researched before, HDM disrupted airway epithelial barrier in HBECs[10]. As autophagy was induced by starvation, virus infection and other stress, we hypothesized that airway epithelial barrier disruption associated with autophagy. To confirm that the association between asthma and autophagy in HBECs, we used three reagents respectively to verify our hypothesis. Rapamycin (Rap) induces autophagy through disrupted mTOR pathway. 3-MA, suppresses autophagy through PI3K pathway. Chloroquine (CQ) suppresses autophagolysosome formation to block autophagy flux[11-13]. CCK8 assay was used to choose the suitable concentration of those reagents for our experiments (Fig2 B). Under the treatment of three reagents respectively for 24h, western blotting was first used to detect the expression of LC3ab, and the LC3ab was increase under HDM, Rap and CQ treating, as CQ suppressed autophagolysosome formation, the expression of P62 increased under treating with CQ in HBECs (Fig2 C). Adenovirus mRFP-GFP-LC3 infected assay could be used to detect autophagic flux, then the result showed that the autophagy was induced in HBECs when treating with HDM and Rap, and the autophagy process was suppressed by 3-MA and CQ treatments (Fig2 D). These data demonstrated that the autophagy was induced by the treatment of HDM in HBECs.

HDM disrupted airway epithelial barrier through autophagy. The FITC-Dx permeability and Transepithelial electrical resistance (TEER) were measured to assess the monolayer cells integrity, we found that induced autophagy by Rap and treated with HDM in HBECs both disrupted monolayer cells integrity, but blocked autophagic flux by CQ could not rescue the integrity of monolayer cells, on the other hand, suppressed autophagy process initially by 3-MA exacerbated the disruption of monolayer cells (Fig3 A and B). These data showed that the disruption of airway epithelial barrier maybe an irreversible process. We next used immunofluorescence assay to detect the integrity of epithelial barrier, and found that the integrity of epithelial barrier was disrupted by treating with all stimuli (Fig3 C). The western blotting was used to detect the expressions of E-cadherin and β-catenin, the markers of epithelium, both in total protein and cytomembrane protein in HBECs. The expressions of E-cadherin and β-catenin were not change under treating with HDM in total protein level, which means HDM was not affected epithelial proteins expression in total protein level (Fig3 D). Interestingly, induced autophagy by Rap and suppressed autophagic flux by CQ both decreased the expressions of E-cadherin and β-catenin, and suppressed autophagy by 3-MA decreased β-catenin expressing though 3-MA not affected the expression of E-cadherin in total protein level (Fig3 D). To further investigate the disruption of epithelial barrier, we detected the expression of E-cadherin and β-catenin in cytomembrane. The expressions of E-cadherin and β-catenin decreased in cytomembrane under treating with HDM, and we surprisingly found that induced autophagy by Rap and suppressed autophagic flux by CQ showed different result compared with stimulated by HDM (Fig3 E), but the result of being suppressed autophagy by
3-MA as same as stimulated by HDM both in total and cytomembrane protein level (Fig3 D, E). Intriguingly, the expression of P62 didn’t change under the treatment with HDM in vitro and in vivo (Fig1 D, Fig2 A), treating with CQ, suppressing the formation of autophagolysosome for blocking autophagic flux, increased the expression of P62 (Fig2 C), we hypothesized that HDM induced autophagy process initially but the autophagic flux did not complete. These data suggested that initiating autophagy process plays important role in HDM-induced epithelial barrier disruption but complete autophagic flux not affect the disruption.

**HDM induced autophagy through Atg5 & Atg12 in HBECs.**

Our results mentioned above demonstrated that HDM induced autophagy in HBECs in initial stage, we hypothesized that block autophagy initially may be useful for alleviating the epithelial barrier disruption. To clarify how HDM induced autophagy to disrupted epithelial barrier, we used HDM to stimulate HBECs for indicated times, we found that autophagy was induced from 12 to 24h (Fig4 A). Autophagy related genes (ATGs) associated with both autophagy and human disease pathogenesis, especially neurodegenerative, inflammatory disorders and cancer[14], so we screened the autophagy related genes, including atg5, atg7, atg10, atg16L1 and atg12, for finding out the most important gene in autophagic process in HBECs. Under the treatment with HDM for indicated times, the expressions of Atg5 and Atg12 upregulated most remarkable in HBECs from 12 to 24h(Fig4 B), these two proteins were also a known complex in the autophagy process[15], on the other hand, the levels of ATGs mRNA were not change under the treatment (Fig4 C). These results suggested that HDM induced autophagy in HBECs through Atg5/Atg12, and the two proteins expression upregulated in 12h.

**TSLP plays critical role in HDM-induced allergic inflammation.**

And the immunochemistry was used to detected the alarmins, including TSLP, IL-25 and IL-33 which are the alarmins that is released in the lung mainly by epithelial cells[17, 18], for our next investigation. Our team had confirmed that the airway epithelial barrier was disrupted by HDM in vivo and in vitro, and the TSLP played essential role in the epithelial barrier disruption, and the expression of TSLP also upregulated in 12h under the treatment with HDM in HBECs[10, 16]. As our expected, only the expression of TSLP increased in mice lung sections in HDM group (Fig5 A). The expressions of Atg5 and Atg12 also increased in 12h, so that we hypothesized Atg5 and Atg12 associated with TSLP expressing. As we targeted the two proteins for our further investigation, we examined the most efficient siRNA for interfering the mRNA of Atg5 and of Atg12 (Fig5 B). The mRNA level of TSLP decreased significantly after interfering Atg5 and Atg12, but the result of IL-25 and IL-33 mRNA level showed no difference under interfering Atg5 and Atg12 compared with the control group (Fig5 C). These data showed that TSLP, but no IL-25 and IL-33, plays critical role in HDM-induced allergic inflammation through upregulating Atg5 and Atg12 expressing.

**The expression of TSLP was blocked after silencing Atg5.**

We hypothesized that the increased expression of TSLP was associated with Atg5 and Atg12 upregulating. Then we interfered protein levels of ATG5 and of ATG12 respectively (Fig6 A), for further investigating the association between Atg5/Atg12 and TSLP in HBECs under HDM-treated, western blotting was used to assess the protein expression of TSLP while Atg5
or Atg12 silenced respectively with or without HDM. Expectably, the expression of E-cadherin and β-catenin didn’t change whatever silenced Atg5 or Atg12 (Fig6 B, C). And the protein expression of TSLP was downregulated remarkably after interfering Atg5 under the treatment with HDM (Fig6 B), but we can’t get the same results after Atg12 interfering treatment (Fig6 C). These results demonstrated that HDM induced Atg5 to initiate autophagic process, knock down the expression of Atg5 decreased the expression of TSLP in HBECs after treated with HDM.

**Discussion**

Autophagy is a conserved process that substrates are endocytosed by the double membrane vesicles and are transported to the lysosomes for degradation[5, 19]. The process plays an important role in many diseases, such as Crohn disease, a type of inflammatory bowel disease. A recent research had demonstrated that ATG16L1, one of the initial genes of autophagy, affects the respond of autophagy in Salmonella typhimurium, which means host cells and autophagy response to stimulation in the pathogenesis of Crohn disease[20]. Another research found that the autophagy receptor p62 are able to mediate the anti-inflammation in LPS-induced macrophages, it has revealed a novel mechanism of macrophage inflammation[21]. As to asthma, plenty of evidence showed that autophagy plays an important role in it, such as environmental particulate matter (PM) induced autophagy in bronchial epithelial cells, while airway inflammation reduced after blocked autophagy in asthmatic mice[22]. More and more evidence showed that autophagy is associated with asthma, but the mechanisms of autophagy affecting airway epithelial inflammation in allergic asthma are not really understood. We attempted to make clear the underlying mechanisms of the function of autophagy in allergic asthma.

In this study, we have demonstrated that autophagy plays different roles in its different stage of process in HDM-induced asthma. Our team have investigated the disruption of airway epithelial barrier for years, and we found that the 400U HDM destroyed the barrier by inducing TSLP expression, then redistributed E-cadherin and β-catenin[10, 16]. Blocking the autophagy process induced by HDM at the early stage, like knocked down the autophagy related gene 5(Atg5), we surprisingly found the expression of TSLP, which damaged the airway epithelial barrier, was downregulated in HBECs. On the other hand, complete autophagy process and blocked autophagic flux both disrupted epithelial barrier in our study. These data suggested initial autophagy process is a matter of TSLP expressing resulting in epithelial barrier disruption.

Interestingly, our research has found that the expression of P62 in HBECs wasn’t affected by HDM. Only the CQ, a reagent could induce P62 accumulation due to inhibit the autophagolysosome formation, increased the expression of P62 in vitro (Fig2 C), it was a pity that we couldn’t explorer further reasons of this phenomenon as our research limited.

There are more and more evidences showed that autophagy highly associated with inflammation in asthma[9, 23-25], but to our knowledge, researchers studied autophagy, considered it plays either good or bad role in certain disease[26-29]. Hence, our findings demonstrated that HDM induced Atg5 to mediate TSLP expressing resulted in disruption of airway epithelial barrier.
Legends

Fig 1. Autophagy could be induced by treatment of HDM both in vivo. A. H&E staining mice lung sections with HDM stimulation (400 × magnification). B. PAS staining mice lung sections with HDM stimulation (400 × magnification). C. Airway hyper-responsiveness was measured by PFT Pulmonary Maneuvers. D. Immunohistochemistry (IHC) staining image of LC3ab and P62 in mice lung sections. Data are presented as the mean±SD; n≥3. *P < 0.05, **P < 0.01, ***P < 0.0001.

Fig 2. HDM induced autophagy in vitro. A. Western blotting analysis of LC3ab and P62 in HBECs which were stimulated with HDM (400U) for 24h. B. CCK8 analysis of HBECs in 24h stimulated with Rapamycin, 3-MA and CQ respectively. C. Western blotting analysis of P62 and LC3ab in HBECs stimulated with or without HDM for 24h and treated with Rapamycin, 3-MA and CQ respectively. D. HBECs that expressed mRFP-EGFP-LC3 fusion protein were stimulated with HDM (400U). Confocal microscopic analysis is shown (1000 × magnification). Data are presented as the mean±SD; n≥3. *P < 0.05, **P < 0.01, ***P < 0.0001 vs to control, #P < 0.05, ##P < 0.01, ###P < 0.0001 vs to HDM.

Fig 3. HDM disrupted airway epithelial barrier through autophagy. A. The FITC-Dx permeability was measured to determine epithelial barrier integrity after the HBECs treated with or without HDM (400U) and with Rapamycin, 3-MA and CQ relatively for the indicated times. B. Transepithelial electrical resistance (TEER) was measured to determine epithelial barrier integrity after the HBECs treated with or without HDM (400U) and with Rapamycin, 3-MA and CQ relatively for the indicated times. C. The distribution of E-cadherin and β-catenin was monitored using immunofluorescence. Green represents E-cadherin, red represents β-catenin, blue represents the nucleus. Confocal microscopic analysis is shown (1000 × magnification). D. Western blotting analysis of E-cadherin and β-catenin in HBECs stimulated with or without HDM for 24h and treated with Rapamycin, 3-MA and CQ respectively. E. Western blotting detected the expressions of E-cadherin and β-catenin in membrane in HBECs stimulated with or without HDM for 24h and treated with Rapamycin, 3-MA and CQ respectively (ATP1A1 for membrane, β-tublin for cytoplasm, Lamin B for nucleus). Data are presented as the mean±SD; n≥3. *P < 0.05, **P < 0.01, ***P < 0.0001 vs to control, #P < 0.05, ##P < 0.01, ###P < 0.0001 vs to HDM.

Fig 4. HDM induced autophagy through Atg5 & Atg12 in HBECs. A. Western blotting analysis of LC3ab in HBECs stimulated with HDM(400U) for indicated times. B. Western blotting analysis of Atg7, Atg16L1, Atg12 and Atg5 in HBECs stimulated with HDM (400U) for indicated times. C. qPCR assay of Atg7, Atg16L1, Atg10, Atg12 and Atg5 mRNA in HBECs stimulated with HDM (400U) for indicated times. Data are presented as the mean±SD; n≥3. *P < 0.05, **P < 0.01, ***P < 0.0001.

Fig 5. The mRNA level of TSLP was mediated by Atg5 or Atg12.
A. Immunohistochemistry (IHC) staining image of TSLP, IL-25 and IL-33 in mice lung sections. B. qPCR assay of Atg5 and Atg12 mRNA in HBECs treated with siATG5 & siATG12 respectively. C. qPCR assay of TSLP, IL-33 and IL-25 in HBECs with or without siATG5 and siATG12. Data are presented as the mean±SD; n≥3. *P < 0.05, **P < 0.01, ***P < 0.0001 vs to control, #P < 0.05, ##P < 0.01, ###P < 0.0001 vs HDM.

Fig 6. Expression of TSLP was blocked after interfering Atg5 but not Atg12. A. Western blotting analysis of Atg5 or Atg12 in HBECs treated with siATG5 and siATG12 respectively. B. Western blotting analysis of HBECs treated with siATG5 and with or without HDM (400U). C. Western blotting analysis of HBECs treated with siATG12 and with or without HDM (400U). Data are presented as the mean±SD; n≥3. *P < 0.05, **P < 0.01, ***P < 0.0001 vs to control, #P < 0.05, ##P < 0.01, ###P < 0.0001 vs HDM.

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Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors declare that they are consent for the publication.

Competing interests
The authors declare that they have no competing interests.

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