Title

House dust mite-induced endoplasmic reticulum stress mediates mucus MUC5AC hyper secretion via TBK1-STAT6/NF-κB in asthmatic mice

Short title: HDM mediates MUC5AC hyper secretion via TBK1-STAT6/NF-κB

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Conceived and designed the study: X.W., J.D., L.G. Performed the experiments: Y.Z., H.T., X.Y., N.M., H.H., X.Y.W., C.L., G.X., Y.L., S.W. Wrote the paper: L.G., X.W., Y.Z. Analyzed the data: Y.Z., H.T., X.W. All authors read and approved the final manuscript.

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There were no potential conflicts of interest to be disclosed.

ABSTRACT

Background Exposed to house dust mite (HDM) is known to be associated with allergic asthma. Endoplasmic reticulum (ER) stress is involved in the regulation of mucus hyper secretion. However, the mechanism remains unclear. Our main goal was to investigate the relationship of TBK1 pathway regulates the expression of MUC5AC under the HDM induced ER stress promote allergic asthma.

Methods Using HDM induced allergic mice and stimulated over expression of MUC5AC in human airway epithelial cells. We examined the mucus and the expression of ER stress markers both in vivo and in vitro. Additionally, we investigated whether TBK1, NF-κB, STAT6 play an important role in the HDM induced ER stress promote airway mucus hyper secretion.

Results Mice exposed to HDM were identified ER stress, hyper secretion of mucus, and activated TBK1-NF-κB/STAT6 signaling pathway in airway epithelial cells of asthmatic mice. Similarly results had also been observed in the human airway epithelial cells after exposed to HDM. Both in vivo and in vitro study not only revealed that an anti-allergy drug, Amlexanox, reduced super response of mucus and weaken TBK1-NF-κB/STAT6 signal of induced asthma. But also indicated HDM induced ER stress result in over production of MUC5AC, which can be decreased by the inhibition of TBK1, NF-κB, STAT6, or even by using ER stress inhibitor 4-PBA, respectively.

Conclusions Our results show that TBK1-NF-κB/STAT6 plays a pivotal role in the HDM induced ER stress result in over production of mucus protein MUC5AC in the asthmatic airway.

Keywords: Asthma; endoplasmic reticulum stress; house dust mite; mucus protein MUC5AC;

INTRODUCTION

Over millions of people suffered allergic asthma worldwide. Accumulated evidence revealed that long time exposure to environmental allergens can cause allergic airway inflammation result in asthma which is a common chronic disease affect our life and has become a public health problem [1]. Airway remodeling, airflow limitation and mucus hyper secretion etc. are known pathological symptoms of asthma [2]. Over 20 members of proteins in airway mucus family like Muc1, Muc2, MUC5AC and Muc5b etc. MUC5AC has been reported as one of the important biomarkers of goblet cells in allergic airway lung disease [3]. Clinical research showed MUC5AC is the major mucus of serious asthmatic patients [4].

Endoplasmic reticulum (ER) is the largest intercellular membrane system of eukaryotic cells, which conduct protein synthesis, folding and transportation etc. It plays a central role in maintaining cell function and calcium homeostasis. Any malfunction of ER will cause unfavorable effects or even life threatening issues. ER stress is associated with varies of human diseases, cancer, diabetes and lung diseases etc. [5]. Cigarette smoke, LPS, ovalbumin (OVA), mites or other pathogens can induce ER stress to develop chronic lung function disorder result in COPD and asthma [6]. Home dust mite (HDM) is one of the major indoor components cause allergic asthma. Continuously expose to HDM would impact healthy problem especially those sensitive to HDM and result in chronic allergic lung disease [7]. HDM is known to induce airway epithelial cell apoptosis and fibrosis through ER stress [8]. And ER stress might promote MUC5AC expression via JNK signaling pathway [9]. However, the relationship between ER stress and hyper secretion of mucus does not well documented, this need to be further clarified.

It reported cyclic GMP-AMP synthase (cGAS) mediates HDM and OVA to effect experimental allergic airway inflammation of asthmatic mice models and human bronchial epithelia cells [10]. And cGAS-STING can recruit and activate TBK1 [11]. STING is located in ER, which plays an important role in dsDNA induced native immune response. IRF3 response early in the first stage of alcoholic liver disease induced by ER stress and then binds with p-TBK1 and STING. P-TBK1 further enhance phosphorylation of IRF3 to promote cytokines and cell apoptosis related genes expression [12]. IRF7 and NF-κB activated by dsDNA via STING-TBK1 pathway [13]. All those indicate ER stress might activate downstream factors via non-classical STING-TBK1 signal to promote gene expression. Activated STING recruit STAT6 and TBK1 to ER to form a complex, TBK1 promotes phosphorylation of STAT6 to promote inflammation factor and cytokines expression [14]. Additionally, LPS does not only promote STAT6 phosphorylation, but also activates IRF3 via activating TBK1 [15]. In allergic lung diseases, ER stress accelerates p-NF-κB to up-regulate asthmatic related cytokines expression, like IL5 and IL13 etc. [16]. However, it's still unclear whether ER stress promotes MUC5AC expression via STING-TBK1. Evidence showed STING-TBK1-IRF3 signaling is required for cGAMP induced lung allergic inflammation [17].

MAPK, ERK1/2, STAT6 pathways involved in regulation of asthmatic mucus over expression [18]. Our study indicated NF-κB mediates IL-13 to regulate over production of MUC5AC [19]. ER stress up-regulate expression of inflammation response factor NF-κB [20]. OVA induced mice airway inflammation model showed TLR4 and NF-κB modulated this pathogenesis [21]. TBK1 phosphorylates NF-κB to regulate cell proliferation, immune response and apoptosis [22]. Some reports pointed STAT6 plays a role in experimental chronic asthma [23] and STAT6 deficient mice avoid from OVA induced development of experimental asthma [24, 25]. Lyn kinase as a negative

control factor regulates STAT6 to MUC5AC promoter binding to promote its expression [26]. And activated NF-κB binds to MUC5AC promoter region to active gene expression [27]. These indicated ER stress might affect MUC5AC secretion through STAT6, NF-κB pathway.

Few reports discussed relationship between ER stress and mucus hyper response. Current research will focus on ER stress induced mucus over production of asthma, and the contribution of TBK1 in ER stress and mucus over expression. Our results give a clear understanding of TBK1 in HDM induced ER stress result in asthmatic mucus hyper secretion. TBK1 is activated in HDM induced ER stress. P-TBK1 promotes phosphorylation of NF-κB and STAT6 to elevate over formation of mucus in vivo and in vitro. NF-κB and STAT6 as downstream factors of TBK1 in the regulation of HDM induced ER stress result in MUC5AC hyper-expression of asthma.

METHODS

A detailed description of materials, methods and additional data figures are available in online supporting files.

RESULTS

Asthmatic mucus MUC5AC over secretion correlated with activated TBK1

The endoplasmic reticulum plays an important role in protein synthesis, folding and transportation in cells. ER homeostasis disorder can cause healthy problem. ER stress has already been well studied in affected stroke, inflammation, cancer and heart disease etc. [5]. It indicated HDM can induce ER stress and promote airway epithelial inflammation and fibrosis [8]. MUC5AC is one of the gel formation proteins involved in asthmatic airway epithelial remodeling [28]. And TBK1 has been reported to regulate the inflammation response as an upstream factor of NF-κB [29]. In this study, an established asthmatic mouse model was used to investigate whether TBK1 pathway and ER stress are involved in asthmatic mice pathogenesis.

To obtain asthmatic mice, C57BL/6J mice were sensitized twice by intraperitoneal (i.p.) injection of HDM (20 μg) and $Al(OH)_3$ (1 mg) on day 1 and day 8. Then the mice were challenged with HDM (20 μg) daily through intratrachel instillation (i.n.) to induce airway allergy from day 15 to day 21 (**figure 1A**). Asthmatic mice airway inflammation has been tested by HE histology staining of lungs' tissues **(see online supplementary figure S1A)**. And airway inflammation quantification index indicated HDM induced asthmatic pathology lung **(see online supplementary figure S1B).** Goblet cells and mucus secretion had been tested increase by exposed to HDM. The total periodic acid-shiff (PAS) staining (**figure 1B**) areas were measured as percentage (**figure 1C**) of airway epithelium cells in lung tissue sections. The mice exposed to HDM represent significantly higher percent of goblet cells in airway lung tissue than PBS control mice (**figure 1B-C**). Moreover, mucus secretion had been elevated by explosion of HDM as well. This pointed to the hyper expression of Muc5ac was detected in model mice lung tissues by immunofluorescence, which showed significant higher level of Muc5ac in HDM induced asthmatic mice compared to PBS control (**figure 1D-E**). And this has been further proved by western blotting, which released dramatically higher expression of Muc5ac in asthmatic mice (**figure 1F-G**).

Western blotting also indicated Tbk1 had been activated in model mice result from exposing to HDM. As expected, the significantly increased p-Tbk1 in lung tissues of asthmatic mice than PBS control mice has been tested versus with total Tbk1 (**figure 1F-H**). Interestingly and most importantly, a significant positive correlation between Muc5ac and p-Tbk1 expression in asthmatic mice has been noticed ($p<0.05$, **figure 1I**).

These results confirmed our hypothesis, HDM induced allergic and established asthmatic mice models, which presented over secretion of mucus in airway through Tbk1 signal. Activated p-Tbk1 has close relation with Muc5ac expression. Further experiments need to be carried out to reveal this mechanism.

NF-κB and SATA6 are involved in TBK1 signal to regulate MUC5AC secretion of BEAS-2B cells

Beside of NF-κB, STAT6 had also been found regulated by TBK1. STAT6 is one of the important genes involved in regulation of asthmatic and allergic diseases pathogenesis [30]. In order to confirm in vivo results, a human bronchial epithelial cell line, BEAS-2B was used for further investment. We found HDM (200 μg/ml) was able to

induce MUC5AC overexpression in BEAS-2B cells (**figure 2A-B**). TBK1 was knocked down by using TBK1 specific siRNA lentivirus to explore the linkage between p-TBK1 and MUC5AC expression. BEAS-2B cells were treated with TBK1 siRNA lentivirus for 24 h while si-NC as control. Expression of MUC5AC remarkably decreased in TBK1 knocked down group than control (**figure 2A-B**).

The activities of NF-κB and STAT6 have been inhibited after knocked TBK-1 down, which have been confirmed by western blotting and immunofluorescence (**figure 2C-G**). These results indicated NF-κB and STAT6 are downstream factors of TBK1 in HDM stimulated BEAS-2B cells. Knock down of TBK1 would inhibit NF-κB and STAT6 activity to decrease MUC5AC expression. For future exploring NF-κB and STAT6 are downstream factors of TBK1 to mediate hyper secretion of MUC5AC, NF-κB or SATA6 specific siRNA lentivirus were used to inhibit NF-κB or STAT6 expression, respectively. Similar results showed, after NF-κB (**figure 2H**) and STAT6 (**figure 2K**) have been blocked, MUC5AC expression dropped to basal level which had not been detected in scramble siRNA treated group (**figure 2I-J and figure 2L-M**).

Consistent with asthmatic mice, these data indicated TBK1, NF-κB and STAT6 are required to regulate MUC5AC expression. TBK1-NF-κB/STAT6 pathway regulates MUC5AC expression of human airway epithelial cells. Inhibition of TBK1 would de-phosphorylation of p-NF-κB and p-STAT6, down regulated NF-κB and STAT6 result in relative lower expression of MUC5AC induced by HDM in BEAS-2B cells.

Amlexanox restore HDM induced MUC5AC overexpression of BEAS-2B cells

For further investigation the development of allergic asthma, a commercial anti-inflammation and anti-allergy drug Amlexanox (Aphthasol) was used to against induced allergic injury. Amlexanox is a clinical small molecule used to treat allergic rhinitis, ulcer and asthma. It has the ability to inhibit TBK1 signal [31, 32]. BEAS-2B cells were treated with Amlexanox at 25 μM for 24 h. As expected, the activity of TBK1 had been inhibited which proved by the decreased p-NF-κB and p-STAT6 in western blotting density of Amlexanox groups versus with positive control which enhanced by exposure of HDM (**figure 3A**).

To determine the effects of Amlexanox on HDM induced MUC5AC of BEAS-2B cells. We tested NF-κB, STAT6 and MUC5AC expression by immunofluorescence. **Figure 3B-C** and **figure 3D-E** representative the translocation of NF-κB and STAT6 had been reduced by Amlexanox, respectively. And the secretion of MUC5AC had been restored by Amlexanox compared with HDM group (**figure 3F-G**).

All together, these results revealed NF-κB and STAT6 are the downstream factors of TBK1 mediate hyper secretion of MUC5AC in HDM induced allergic response of BEAS-2B cells.

NF-κB/STAT6 mediate Amlexanox attenuate MUC5AC hyper secrete in asthmatic mice

Asthmatic mice were induced by HDM as usual, while the mice were i.p. injected with Amlexanox (100 mg/kg) daily from day 15 up to day 21 (**figure 4A**). Western blotting results indicated p-NF-κB and p-STAT6 were elevated in asthmatic mice and both factors were dropped back by treating with Amlexanox (**figure 4B**).

The mice exposed to HDM result in greater goblet cells in airway epithelia cells than control mice. However, the percentage of goblet cells falling off when asthmatic mice had been treated with Amlexanox, which detected by

PAS staining (**figure 4C-D**). From airway inflammation quantification index results (**see online supplementary figure S2A-B),** we can see the asthmatic mice with severe airway inflammation and Amlexanox can restore this proved by HE staining. Due to upstream signal p-NF-κB and p-STAT6 had been blocked by Amlexanox, the over expressed MUC5AC in lung tissues of asthmatic mice had also been attenuated in Amlexanox group (**figure 4E-F**) compared with HDM group.

Collectively, these data showed Amlexanox was able to inactive p-NF-κB and p-STAT6 induced by HDM, and resulted in further decreasing hyper secretion of MUC5AC in asthmatic mice.

HDM induced ER stress elevated mucus MUC5AC expression in BEAS-2B cells

As allergy resource, HDM continually impaired people's respiration system. Lung tissue ER homeostasis has been destroyed by exposure of HDM. The elevated BIP and CHOP indicated the ER stress had been induced by HDM (**figure 5A-C**). And HDM induced ER stress leads to the hyper secretion of MUC5AC (**figure 5D-E**).

4-PBA is known can improve protein maturation and alleviate ER stress [33]. ER molecular chaperone BIP and transcription factor CHOP take part in the regulation of ER stress. Their expression levels were used as an indicator of ER homeostasis [34]. As one of the known ER stress inhibitor, 4-PBA attenuated this process after treated BEAS-2B cells 24 h at 10 mM, and result in dramatically decrease expression of MUC5AC (**figure 5D-E**). These indicated HDM induced ER stress of BEAS-2B cells lead to the over production of MUC5AC, and 4-PBA can reverse ER stress to decrease elevated MUC5AC to basal level.

4-PBA reduced NF-κB and STAT6 transcriptional activity in BEAS-2B cells

For further understand the mechanism which factor mediates ER stress to promote HDM induce airway gel formation, we examined NF-κB and STAT6, well known allergic airway remodeling pathogenesis factors.

The levels of p-NF-κB and p-STAT6 were increased after 24 h treated with HDM (100 μg/ml) compared with PBS group. These induced over expression of p-NF-κB and p-STAT6 had been reversed by 4-PBA in the human epithelial cells, tested by western blotting (**figure 5F**) and Immunofluorescence (**figure 5J**). Results suggest that exposed to HDM can induce ER stress and further promote downstream factors NF-κB and STAT6 activation. Inhibiting ER stress by 4-PBA, p-NF-κB and p-STAT6 were reduced.

Taken together, stimulate BEAS-2B cells with HDM can induce ER stress, and this can significantly activated TBK1-STAT6/NF-κB pathway result in MUC5AC over expression, inhibition of ER stress can reverse this.

ER stress enhance expression of mucus MUC5AC of asthmatic mice via TBK1-NF-κb/STAT6

For further confirm ER stress involved in chronic asthmatic disease we investigated effect of 4-PBA on the level of ER stress markers, Bip and Chop in lung tissues of asthmatic mice. Another set of asthmatic mice were used to treat with or without 4-PBA (**figure 6A**) to detect the therapeutic efficiency. As similar as in vitro results, 4-PBA decreased ER stress by remarkable lower the expression of Bip and Chop in 4-PBA treated group (**figure 6B-C**) versus with mice exposed to HDM alone.

HE staining (**see online supplementary figure S3A**) showed the inflammation of mice asthmatic lung tissues.

Inflammation index indicated 4-PBA could significantly rescue the inflammation of asthmatic mice airway inflammation (**see online supplementary figure S3B**). Moreover, goblet cells and MUC5AC secretion had been reversed by 4-PBA. Total periodic acid-shiff (PAS) staining (**figure 6D-E**) areas were measured as percentage of airway epithelium cells in lung tissue sections indicated the mice exposed to HDM represent greater goblet cells in airway than the combination treatment with 4-PBA and PBS control mice. And we can see 4-PBA significantly reverse the Muc5ac expression in asthmatic mice lung tissues (**figure 6F-G**). What's more, asthmatic mice treated with 4-PBA showed lower expression of Bip and Chop, and inactivity of p-Tbk1, p-NF-κb, p-Stat6 as well (**figure 6H**).

In all, these results suggest that HDM can induce ER stress lead to activation of Tbk1, p-Tbk1 is elevated. P-Tbk1 activated NF-κb and Stat6 to promote over production of Muc5ac result in airway asthmatic remodeling of mucus. Inhibiting ER functional disorder by 4-PBA can reverse asthmatic pathogenesis. Decrease p-Tbk1, p-NF-κb and p-Stat6 can attenuate airway mucus hyper secretion. TBK1-NF-κB/STAT6 pathway plays an important role in regulation of MUC5AC expression in HDM induced ER stress to trigger allergic asthmatic response both in mice and human epithelial cells (**figure 6I**). Well investigated the mechanism of ER stress mediate mucus MUC5AC over expression via TBK1-NF-κB/STAT6 pathway is important in the therapeutic of asthma.

DISCUSSION

Arising allergic asthmatic population in the past decades indicates it is a worldwide public health problem which warns us have to pay attention to. Super sensitivity to mites is known as one of the important resource result in allergic airway inflammation [35]. HDM is a majority of indoor component cause asthma. Allergic people sensitive to HDM develop chronic asthma always associated with airway remodeling and over production of mucus [36]. The over expressed mucus result in airway blockage, lung function disorder are major problems of therapeutic of seriously asthmatic patients. Traditional asthma medications are reported with following side effects, like cough, headache and would cause drug tolerance as well. However, the therapeutic efficiency with anti-inflammation medication is only temporary or even useless. It said the basic function of airway super sensitive reflection might be mucus hyper secretion [37]. In this case, inhibiting over production of mucus probably play a pivotal role in the treatment of severe asthma condition or other lung diseases. In the present study we showed some new relations between well-known signaling pathway and gene transcription factors which might be raise some new ideas for allergic asthma therapy.

ER stress via classical UPR activation model effects the development of asthmatic airway super reflection. Under normal condition, BIP bind with PERK, AFT6, and IRE1 to make them maintain no activation single form. Over stocked unfolding protein on ER result in BIP released from UPR sensor factor, activate PERK, ATF6 and IRE1 three classical signaling pathways to interrupt protein biosynthesis and secretion, then promote inflammation cytokines secretion and degradation of related proteins [38].

In present study, ER stress was found imbalance in HDM induced asthma mice and human epithelial cells. Mucus protein MUC5AC level was also elevated in the airway tissues from asthma mice and BEAS-2B cells compared with PBS controls. These findings indicate ER stress might be as parts of the signaling pathway regulate the pathogenesis of HDM induced allergic asthma in vivo and in vitro. Additionally, we detected TBK1 as a downstream molecular factor of ER stress via NF-κB/STAT6 nuclear transcriptional factors regulate MUC5AC

hyper secretion in HDM induced airway epithelial disorders. What's more, inhibition of TBK1 can remarkably rescue HDM induced asthmatic responses in vivo and in vitro. Collectively, these results indicate TBK1 plays an important role in the regulation of HDM induced excess production of MUC5AC.

Our previous study showed ER stress mediate asthma development which induced by IL-13 [19]. Our recently study indicated HDM induced oxidative stress cause DNA damage result in allergic asthma. In current research, mice exposed to HDM result in ER stress markers Bip and Chop highly increased in asthmatic lung tissues. Similar results have also been detected in the human bronchial epithelial cells under the same stimulate condition. Additionally, inhibitory of ER stress by using 4-PBA can turn over excess production of MUC5AC which impaired by exposed to HDM. The detectable significantly decreased Bip and Chop, and less mucus formation are the support evidences.

Activated NF-κB plays a key role in the development of inflammation diseases [29]. What's more, NF-κB also show the ability in the regulation of ER stress induced lung disease [39]. In this study, NF-κB remarkably increased in HDM induced ER stress groups compared with PBS control, and NF-κB dropped to basal level after treated with ER stress inhibitor 4-PBA. However, NF-κB nuclear translocation also had been blocked when ER stress downstream TBK1 had been silenced by using si-TBK1. Knock down NF-κB itself also ameliorated allergic asthma induced by HDM and no significant mucus over production had been detected versus with HDM group and PBS control, respectively. All these indicated NF-κB plays an important role in the HDM induced ER stress to promote the development of allergic asthma disease.

Aside of NF-κB, STAT6 is also involved in the pathogenesis of asthma. Previous study showed STAT6 bind to MUC5AC promoter to enhance gene expression in the induced asthmatic mice [26]. In the present study we found STAT6 transcription factor cooperate with NF-κB mediate TBK1 pathway to regulate MUC5AC expression. As expect and similar to NF-κB, no detectable increase level of STAT6 had been found after ER stress or upstream factor TBK1 had been blocked. And silencing STAT6 can decrease HDM induced allergic response as blocking NF-κB did. These indicate STAT6 plays the same important role in the pathogenesis of allergic asthma as NF-κB.

All together, we show 4-PBA dramatically attenuated HDM induced airway remodeling of hyper production of mucus MAC5AC both in vivo and in vitro. Our data also suggest this process accompanied with the imbalance of ER homeostasis and activated TBK1 to trigger the activation of NF-kB and STAT6 to promote MUC5AC over expression of asthmatic mice's airway lung tissues and human epithelial cells. NF-κB and STAT6 are involved in the regulation of mucus remodeling. Additionally, after exposed human epithelial cells to HDM combined with Amelxanox the decreased phosphorylation forms of TBK1, NF-κB, STAT6 and MUC5AC had been detected compared with exposed to HDM alone. Moreover, the asthmatic mice with the treatment of Amelxanox showed less airway goblet cells and inflammation and with lower MUC5AC expression as well. These evidences indicated TBK1, NF-κB and STAT6 all together to regulate the secretion of MUC5AC in the HDM induced allergic asthmatic pathogenesis.

In conclusion, our study demonstrated HDM induced endoplasmic reticulum stress resulted in hyper secretion of MUC5AC of asthmatic mice and BEAS-2B cells, and the activated TBK1-NF-κB/STAT6 signals are involved in the pathogenesis. These results suggest that blockage of TBK1 pathway or attenuate endoplasmic reticulum stress is sufficient enough to down regulate mucus secretion of allergic asthmatic mice and human airway epithelial cells

(**Fig.6I**). Both of NF-κB and STAT6 are the downstream signaling factors of TBK1. And both are required to mediate the expression of MUC5AC. This is a novel way to develop effective target specific medications to cure this worldwide chronic allergic healthy problem to benefit more asthmatic patients.

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FIGURE LEGENDS

Figure 1. Unregulated Muc5ac and p-Tbk1 represented in the asthma mice lung tissue. (A) Schematic of HDM induced asthma mice model protocol (n = 6 mice for each group). (B-C) 6 random fields were selected to quantification the percentage of goblet cells, which representative in the images from PAS-stained lung tissue sections of asthma model mice and control mice. (D-E) Immunofluorescence staining images showed the Muc5ac protein expression in lung tissues of asthma mice and control mice. 6 random fields were selected to quantification the fluorescence intensity of Muc5ac. (F-H) Relative changes in density of Muc5ac v.s. Gapdh, phosphor-Tbk1 and Tbk1 as detected by western blotting. (I) Association between the expression of Muc5ac protein and phosphor elated of Tbk1. Each point is an individual mouse. All data are presented as mean ± *s.d.* **p<0.01 or ***p<0.001 was determined by t-test; (I) Linear regression analysis was performed by using SPSS, $p < 0.05$.

Figure 2. NF-κB and STAT6 are activated by TBK1 regulate the expression of MUC5AC in the HDM induced human bronchial epithelial cells. (A-B) Immunofluorescence staining images represented the MUC5AC protein expression in human bronchial epithelial cells (BEAS-2B cells) treated with or without si-TBK1 exposed to HDM or PBS. (C) The expression of p-NF-κB and NF-κB, p-STAT6 and STAT6, p-TBK1 and TBK1 were detected by western blotting with or without si-TBK1 treated BEAS-2B cells exposed to HDM or PBS. (D-E and F-G) Immunofluorescence staining images represented the transcription factor NF-κB (D-E) and STAT6 (F-G) expression in BEAS-2B cells treated with or without si-TBK1 exposed to HDM or PBS. (H) The expression of p-NF-κB and NF-κB were detected by western blotting with or without si-NF-κB treated BEAS-2B cells exposed to HDM or PBS. (I-J) Images represented immunofluorescence staining of MUC5AC protein expression in BEAS-2B cells treated with or without si-NF-κB exposed to HDM or PBS. (K) The expression of p-STAT6 and STAT6 were detected by western blotting with or without si-STAT6 treated BEAS-2B cells exposed to HDM or PBS. (L-M) Immunofluorescence staining images represented the MUC5AC protein expression in BEAS-2B cells treated with or without si-STAT6 exposed to HDM or PBS. All values are presented as means ± s.d. 10 random fields were selected to quantification the fluorescence intensity. At least three independent experiments were performed. Si-NC was used as negative control of siRNA induced gene silencing. GAPDH was used as an internal control of western blotting. *p<0.05, **p<0.01 or ***p<0.001 was determined by one way ANOVA followed Tukey-Kramer posttest.

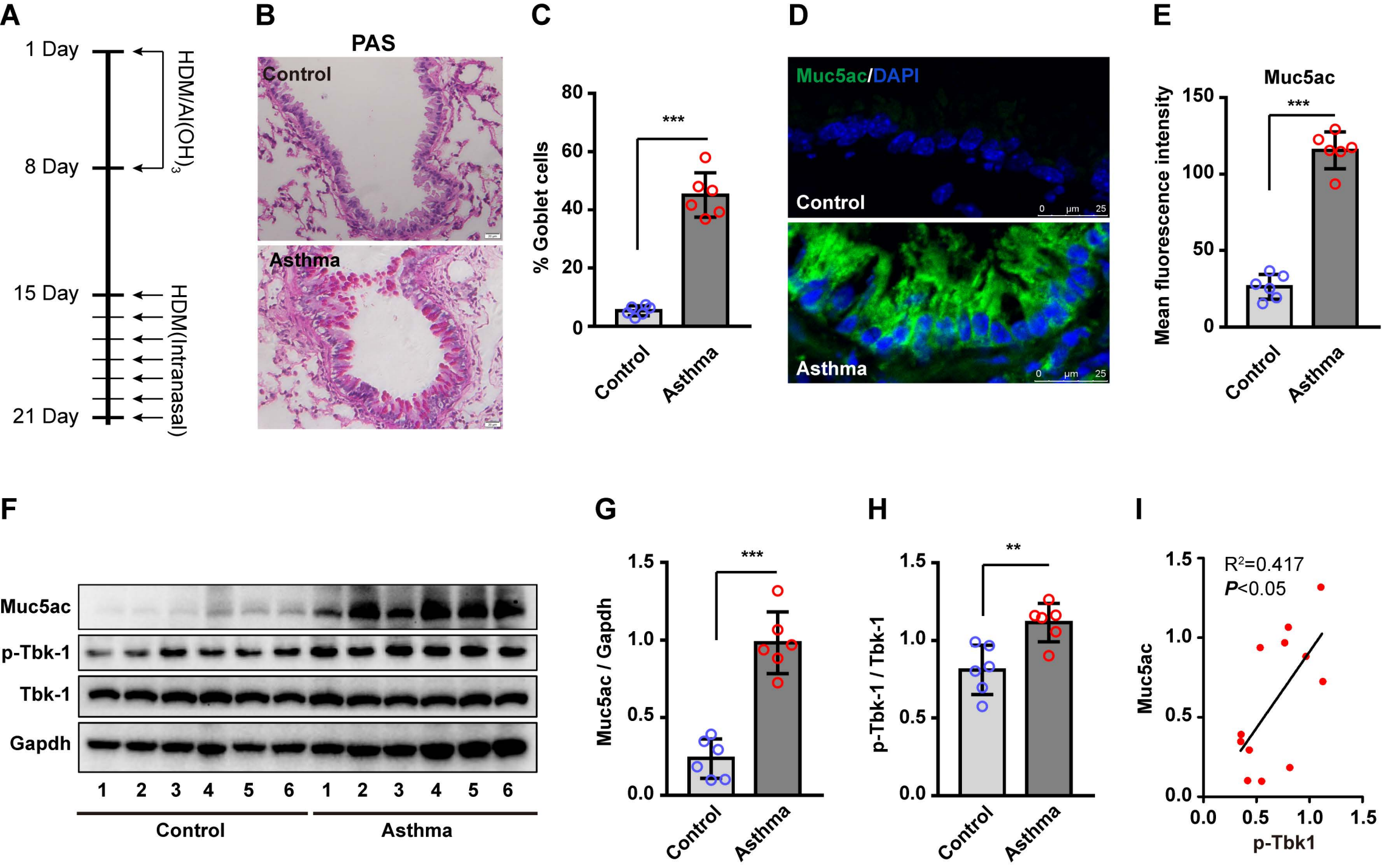
Figure 3. NF-κB/STAT6 is necessary for the HDM induced allergic response of human airway epithelial cells (A) The expression of p-TBK1 and TBK1, p-NF-κB and NF-κB, p-STAT6 and STAT6 were detected by western blotting with or without Amlexanox treated BEAS-2B cells exposed to HDM or PBS. GAPDH was used as an internal control. (B-G) Immunofluorescence staining images represented the NF-κB (A), STAT6 (D) or MUC5AC (F) expression in BEAS-2B cells treated with or without Amlexanox exposed to HDM or PBS, respectively. 10 random fields were selected to quantification the fluorescence intensity of NF-κB (C), STAT6 (E) or MUC5AC (G). At least three independent experiments were performed. All values are presented as means ± s.d. **p<0.01 or ***p<0.001 was determined by one way ANOVA followed Tukey-Kramer posttest.

Figure 4. Amlexanox rescue Asthma by down regulate the expression of MUC5AC through NF-κB/STAT6 signaling pathway (A) Schematic of HDM induced asthma mice and therapeutic with Amlexanox procedure (n = 6

mice for each group). (B) Western blotting was used to detect total and phosphor forms of NF-κB/STAT6 signal factors expression change in density v.s. Gapdh; (C-D) 6 random fields were selected to quantification the percentage of goblet cells, which representative in the images from PAS-stained lung tissue sections of control mice group, Amlexanox treated mice group, and asthma mice model without or with Amlexanox treatment group; (E-F) Immunofluorescence staining images showed the Muc5ac protein expression in lung tissues of four groups as shown in the images. 6 random fields were selected to quantification the fluorescence intensity of Muc5ac. Each point is an individual mouse. All values are presented as means ± s.d. **p<0.01 or ***p<0.001 was determined by one way ANOVA followed Tukey-Kramer posttest.

Figure 5. HDM induced ER stress elevate MUC5AC expression via TBK1-NF-κB/STAT6 signaling pathway (A-C and D-E and G-J) Immunofluorescence staining images represented the BIP, CHOP, MUC5AC, NF-κB or STAT6 expression in BEAS-2B cells treated with PBS, HDM, 4-PBA and HDM+4-PBA in different groups. 10 random fields were selected to quantification the fluorescence intensity of BIP and CHOP (C), MUC5AC (E), NF-κB (H) or STAT6 (G), respectively. (F) The expression of BIP, CHOP, p-TBK1 and TBK1, p-NF-κB and NF-κB, p-STAT6 and STAT6 were detected by western blotting in BEAS-2B cells treated with PBS, HDM, 4-PBA and HDM+4-PBA as designed. GAPDH was used as an internal control. At least three independent experiments were performed. All values are presented as means ± s.d. *p<0.05, **p<0.01 or ***p<0.001 was determined by one way ANOVA followed Tukey-Kramer posttest.

Figure 6. ER stress enhance hyper expression of mucus in allergic asthma mice through Tbk1-NF-κb/Stat6 (A) Schematic of HDM induced asthma mice and therapeutic with 4-PBA procedure (n = 6 mice for each group). (B-C) Immunohistology staining images showed the Bip and Chop protein level in lung tissues of control mice, asthma mice and asthma mice treated with 4-PBA. 3 random fields were selected to quantification the fluorescence intensity of Muc5ac. (D-E) 3 random fields were selected to quantification the percentage of goblet cells, which representative in the images from PAS-stained lung tissue sections of control mice, asthma mice and asthma mice treated with 4-PBA. (F-G) Immunohistology staining images showed the Muc5ac protein expression in lung tissues of control mice, asthma mice and asthma mice treated with 4-PBA. 3 random fields were selected to quantification the fluorescence intensity of Muc5ac. (H) The expression of Bip, Chop, p-Tbk1 and Tbk1, p-NF-κb and NF-κb, p-Stat6 and Stat6 were detected by western blotting in lung tissues of control mice, asthma mice and asthma mice treated with 4-PBA. Gapdh was used as an internal control. (I) Schematic diagram of the mechanisms of HDM induced ER stress which triggers MUC5AC overexpression via TBK1-NF-κB/STAT6 signaling pathway. Each point is an individual mouse. All data are presented as mean ± s.d. *p<0.05, **p<0.01 or ***p<0.001 was determined by one way ANOVA followed Tukey-Kramer posttest.


A
1 Day
8 Day
15 Day
21 Day
HDM/Al(OH)3
HDM(Intranasal)
B
PAS
Control
Asthma
C
% Goblet cells
***
Control
Asthma
D
Muc5ac/DAPI
Control
Asthma
0 μm 25
E
Muc5ac
Mean fluorescence intensity
***
Control
Asthma
F
Muc5ac
p-Tbk-1
Tbk-1
Gapdh
1 2 3 4 5 6 1 2 3 4 5 6
Control
Asthma
G
Muc5ac / Gapdh
***
Control
Asthma
H
p-Tbk-1 / Tbk-1
**
Control
Asthma
I
R²=0.417
P<0.05
Muc5ac
p-Tbk1

A

si-NC

PBS

MUC5AC

MUC5AC/DAPI

HDM

si-TBK1

PBS

HDM

B

MUC5AC

Mean fluorescence intensity

200

150

100

50

0

**

**

HDM - + - +

si-NC

si-TBK1

C

si-NC

si-TBK1

HDM - + - +

p-NF-κB

NF-κB

p-STAT6

STAT6

p-TBK1

TBK1

GAPDH

D

si-NC

si-TBK1

PBS

HDM

PBS

HDM

NF-κB

NF-κB/DAPI

E

NF-κB nuclear translocation (%)

50

40

30

20

10

0

***

***

HDM - + - +

si-NC

si-TBK1

F

si-NC

si-TBK1

PBS

HDM

PBS

HDM

STAT6

STAT6/DAPI

G

STAT6 nuclear translocation (%)

50

40

30

20

10

0

***

*

HDM - + - +

si-NC

si-TBK1

H

si-NC

si-NF-κB

HDM - + - +

p-NF-κB

NF-κB

GAPDH

I

si-NC

si-NF-κB

PBS

HDM

PBS

HDM

MUC5AC

MUC5AC/DAPI

J

MUC5AC

Mean fluorescence intensity

200

150

100

50

0

**

*

HDM - + - +

si-NC

si-NF-κB

K

si-NC

si-STAT6

HDM - + - +

p-STAT6

STAT6

GAPDH

L

si-NC

si-STAT6

PBS

HDM

PBS

HDM

MUC5AC

MUC5AC/DAPI

M

MUC5AC

Mean fluorescence intensity

300

200

100

0

*

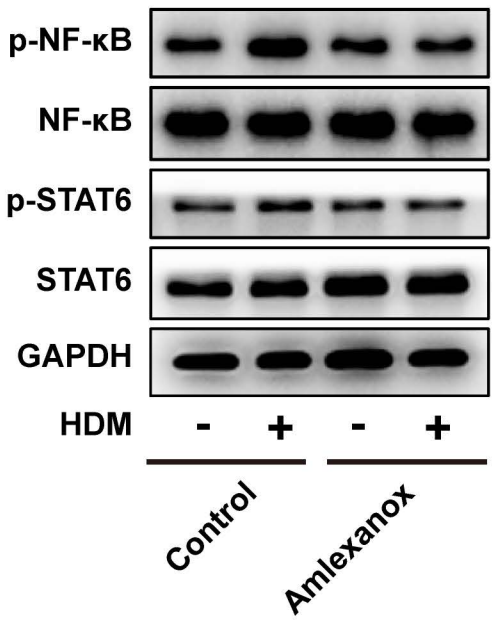
*

HDM - + - +

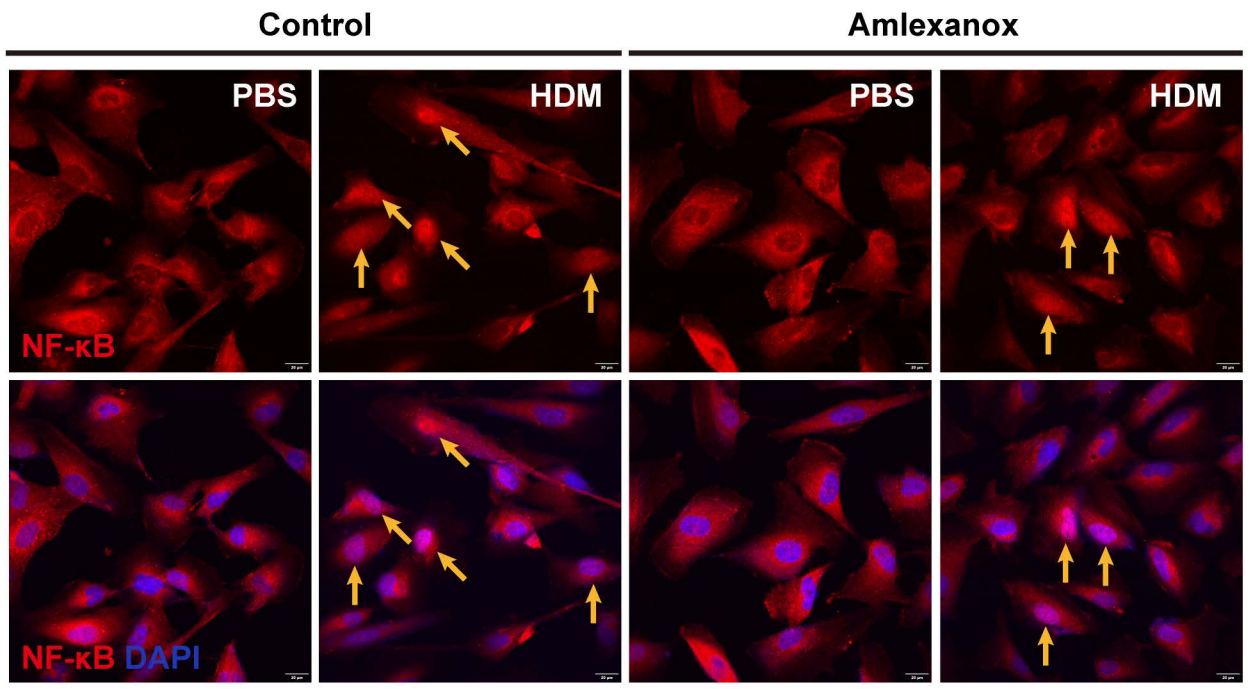
si-NC

si-STAT6

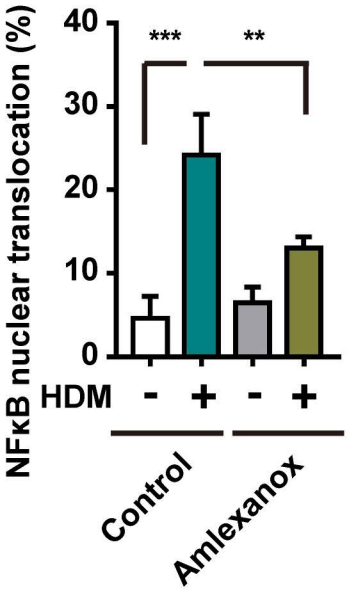
A


p-NF-κB
NF-κB
p-STAT6
STAT6
GAPDH
HDM
-
+
-
+
Control
Amlexanox

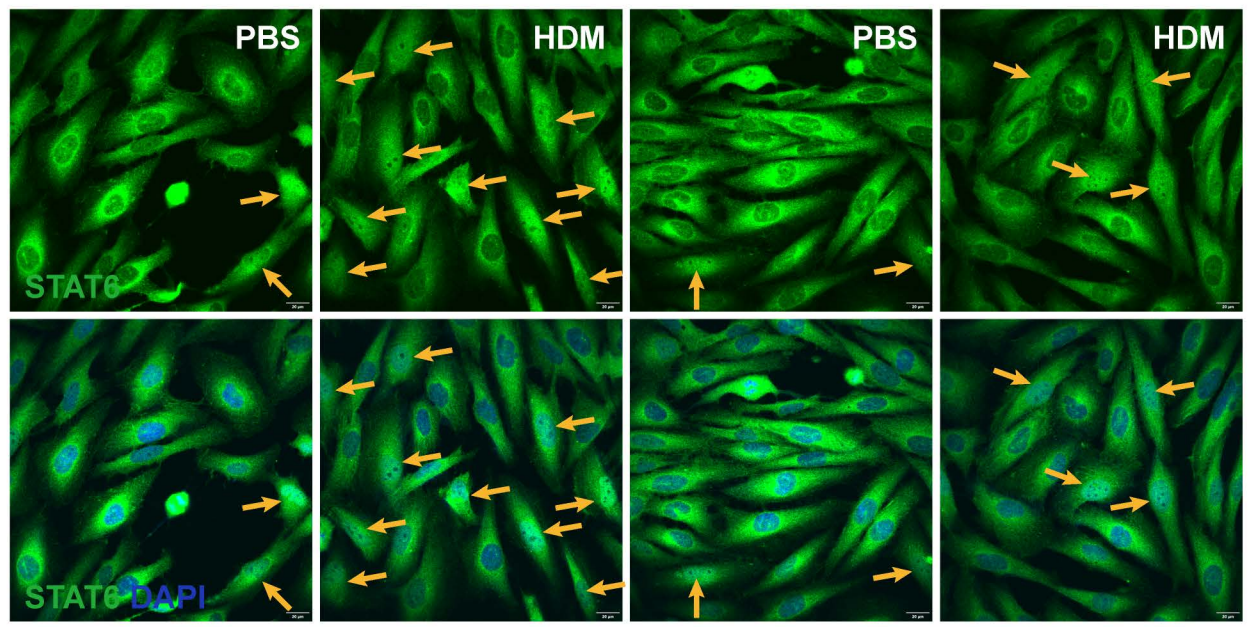
B


Control
Amlexanox
PBS
HDM
PBS
HDM
NF-κB
NF-κB DAPI

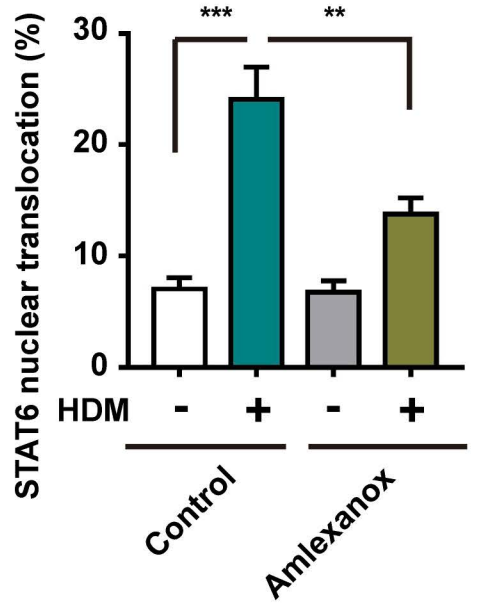
C


NFκB nuclear translocation (%)
40
30
20
10
0
***
**
HDM
-
+
-
+
Control
Amlexanox

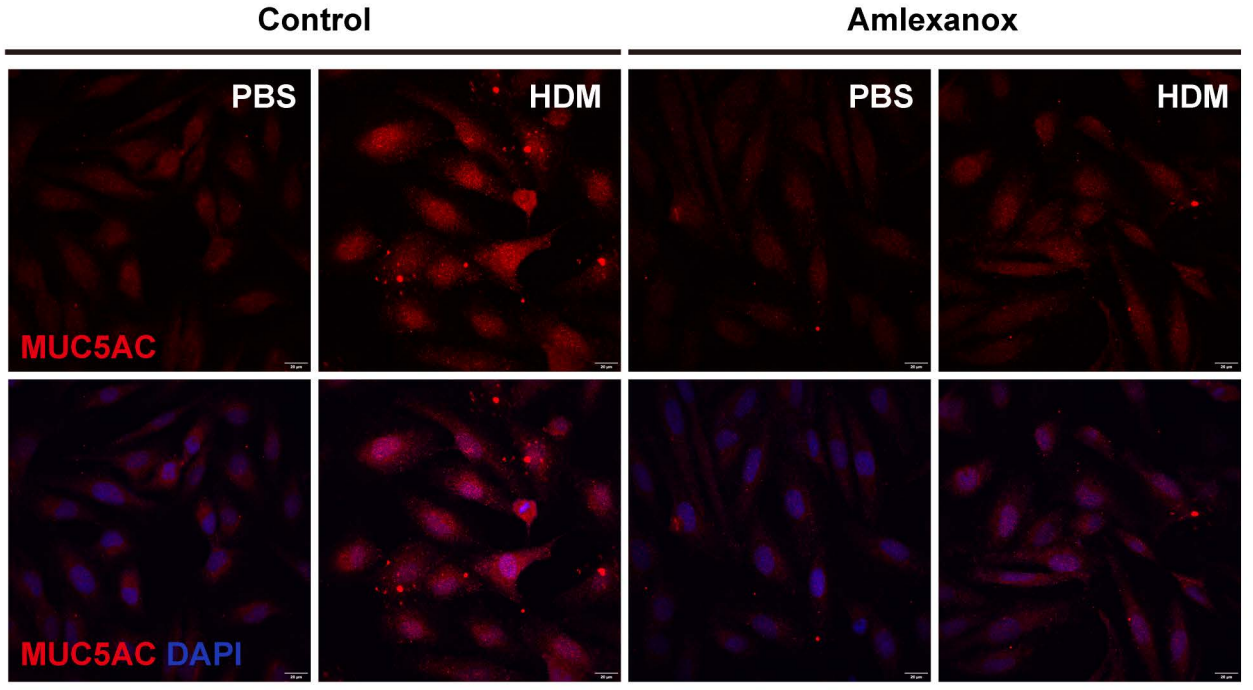
D


Control
Amlexanox
PBS
HDM
PBS
HDM
STAT6
STAT6 DAPI

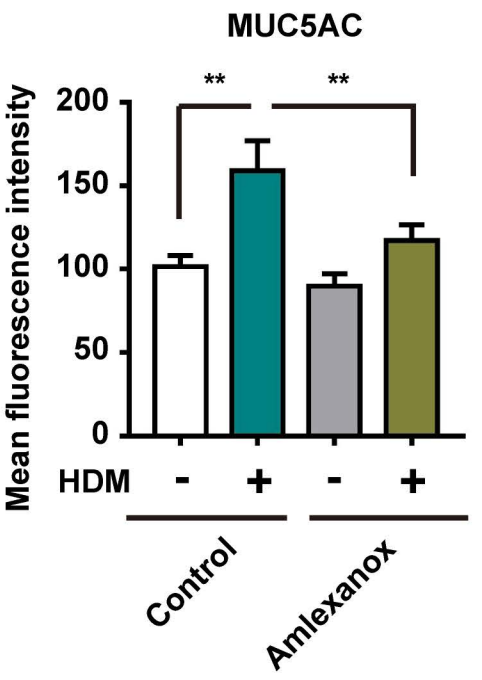
E

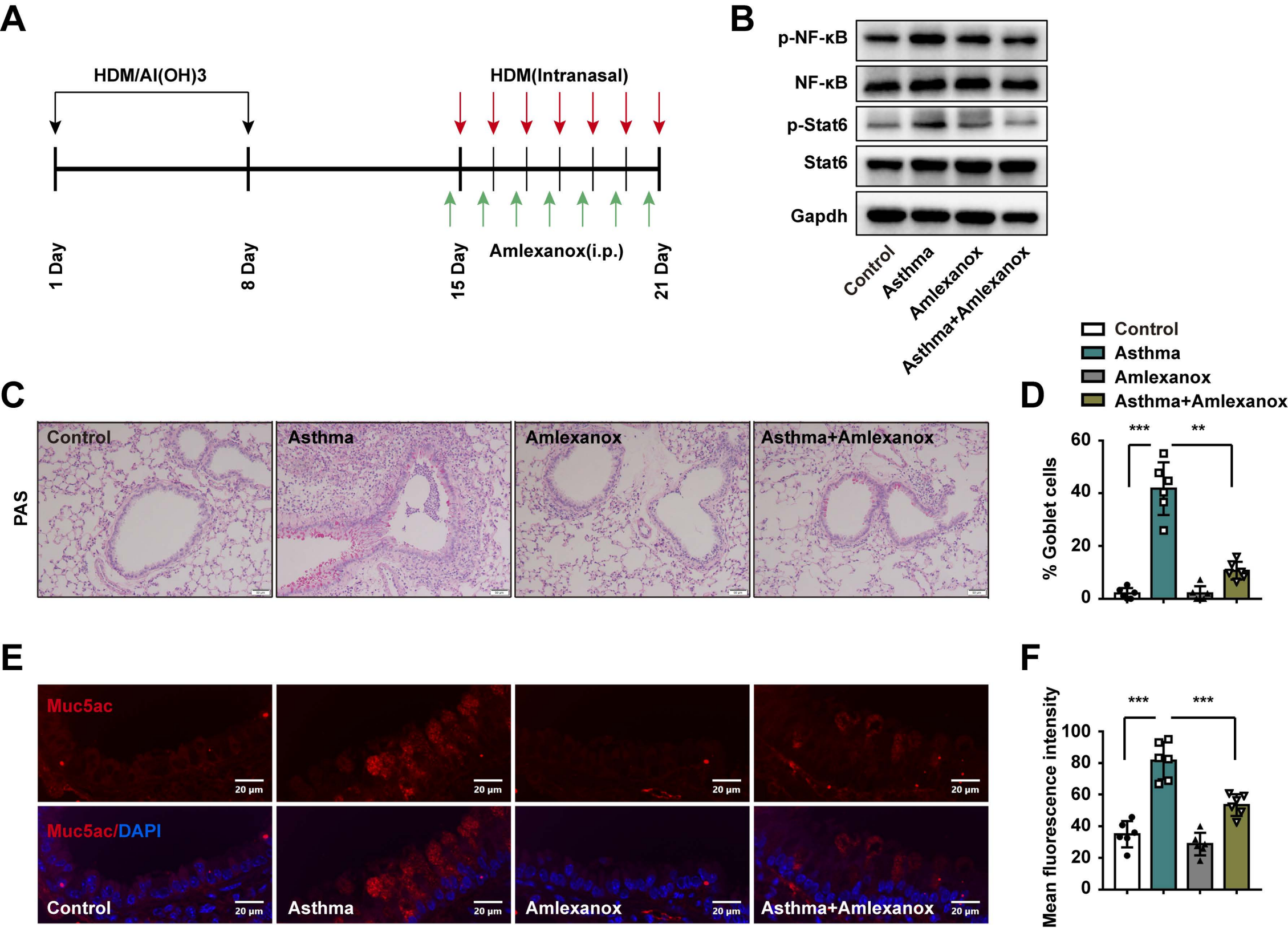

STAT6 nuclear translocation (%)
30
20
10
0
***
**
HDM
-
+
-
+
Control
Amlexanox

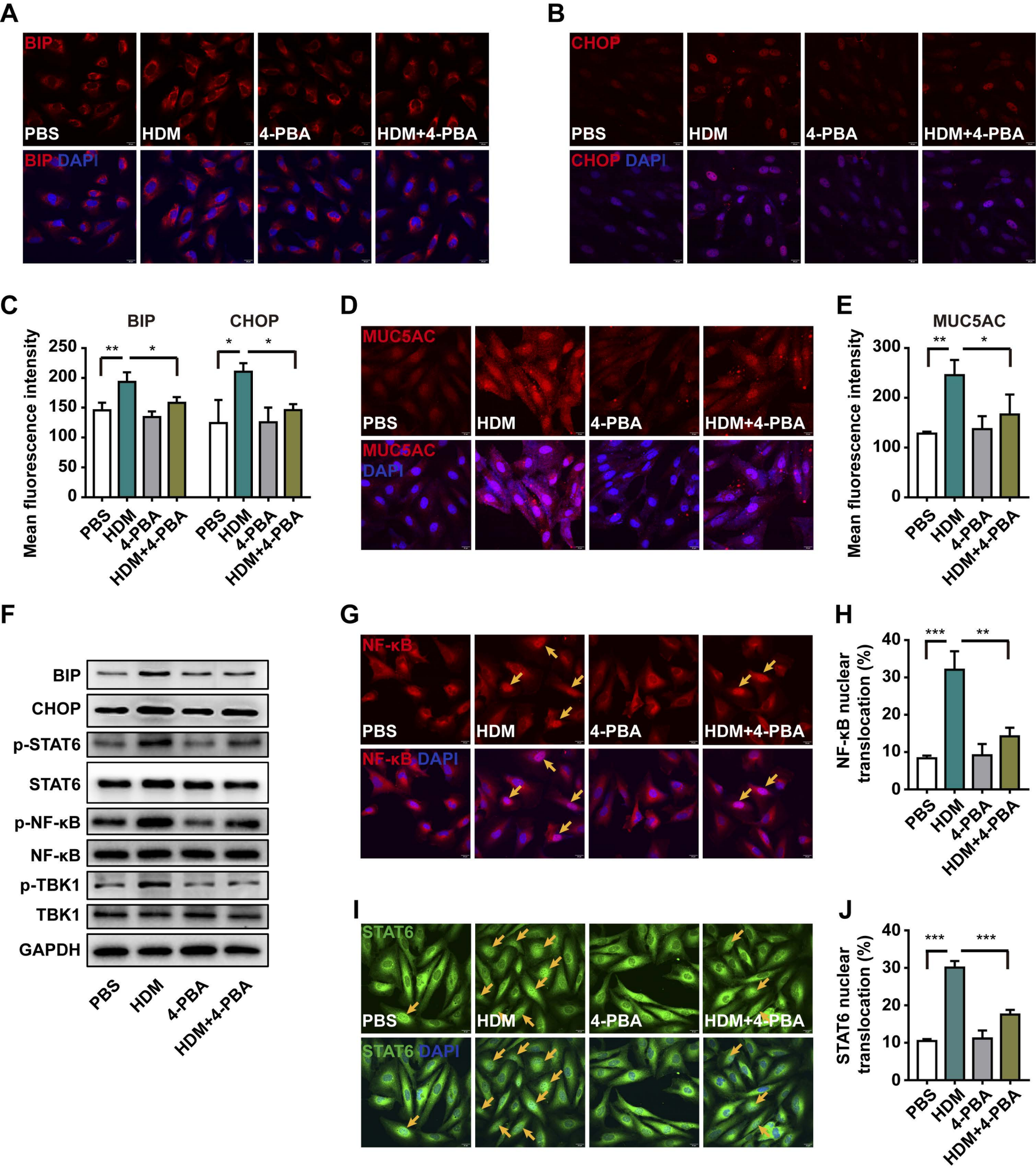
F


Control
Amlexanox
PBS
HDM
PBS
HDM
MUC5AC
MUC5AC DAPI

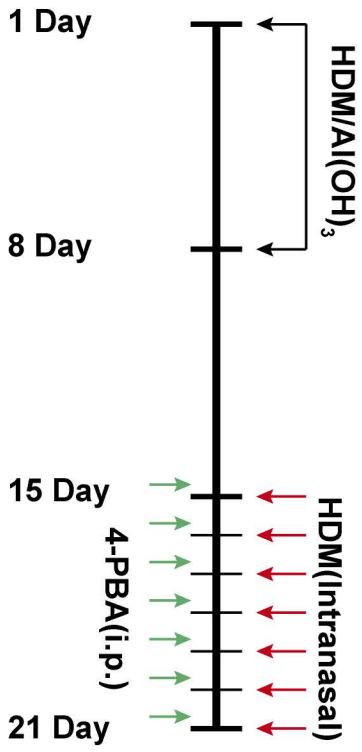
G


MUC5AC
Mean fluorescence intensity
200
150
100
50
0
**
**
HDM
-
+
-
+
Control
Amlexanox

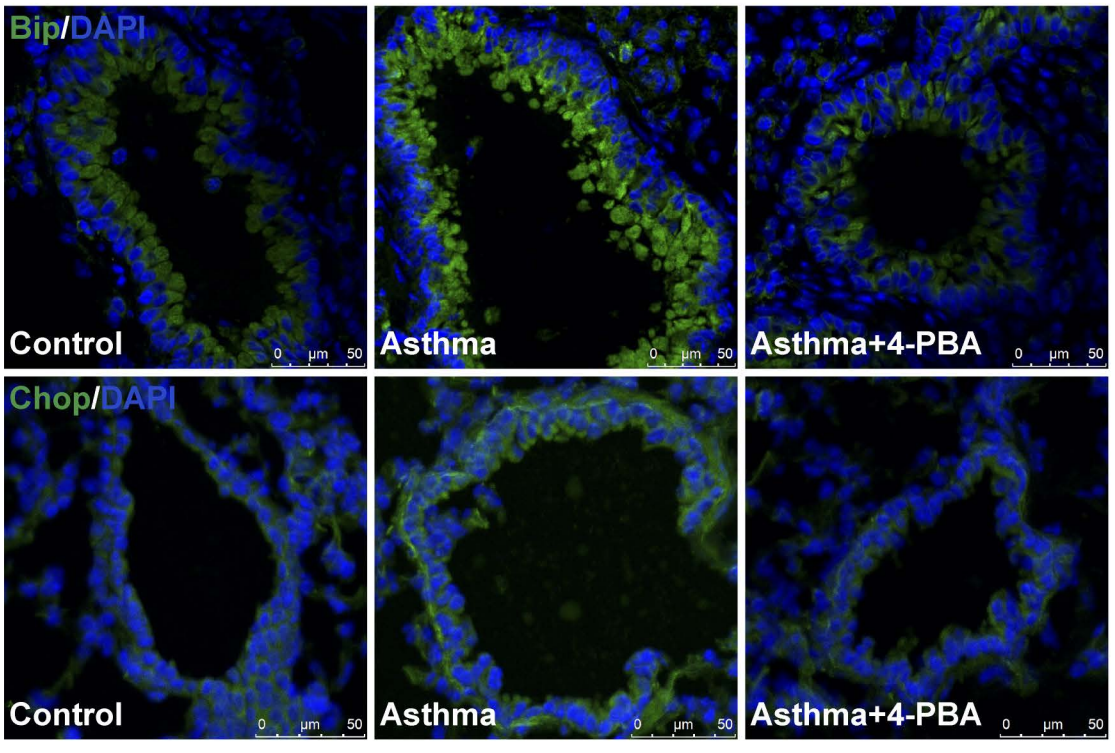

A
HDM/Al(OH)3
HDM(Intranasal)
1 Day
8 Day
15 Day
21 Day
Amlexanox(i.p.)
B
p-NF-κB
NF-κB
p-Stat6
Stat6
Gapdh
Control
Asthma
Amlexanox
Asthma+Amlexanox
C
PAS
Control
Asthma
Amlexanox
Asthma+Amlexanox
D
Control
Asthma
Amlexanox
Asthma+Amlexanox
% Goblet cells
***
**
E
Muc5ac
Muc5ac/DAPI
20 μm
F
Mean fluorescence intensity
***
***


A
BIP
PBS
HDM
4-PBA
HDM+4-PBA
BIP DAPI
B
CHOP
PBS
HDM
4-PBA
HDM+4-PBA
CHOP DAPI
C
BIP
CHOP
Mean fluorescence intensity
250
200
150
100
50
0
**
*
*
*
PBS
HDM
4-PBA
HDM+4-PBA
PBS
HDM
4-PBA
HDM+4-PBA
D
MUC5AC
PBS
HDM
4-PBA
HDM+4-PBA
MUC5AC
DAPI
E
MUC5AC
Mean fluorescence intensity
300
200
100
0
**
*
PBS
HDM
4-PBA
HDM+4-PBA
F
BIP
CHOP
p-STAT6
STAT6
p-NF-κB
NF-κB
p-TBK1
TBK1
GAPDH
PBS
HDM
4-PBA
HDM+4-PBA
G
NF-κB
PBS
HDM
4-PBA
HDM+4-PBA
NF-κB DAPI
H
NF-κB nuclear translocation (%)
40
30
20
10
0
***
**
PBS
HDM
4-PBA
HDM+4-PBA
I
STAT6
PBS
HDM
4-PBA
HDM+4-PBA
STAT6 DAPI
J
STAT6 nuclear translocation (%)
40
30
20
10
0
***
***
PBS
HDM
4-PBA
HDM+4-PBA

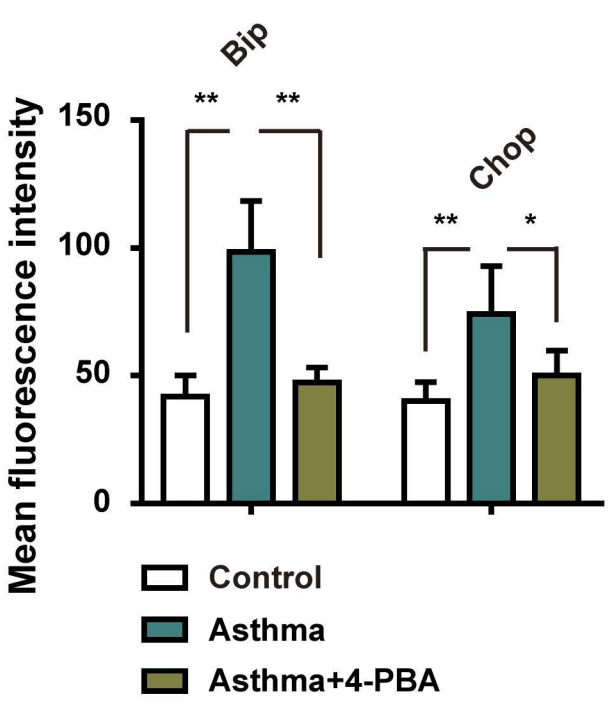
A


1 Day
8 Day
15 Day
21 Day
HDM/Al(OH)3
4-PBA(i.p.)
HDM(Intranasal)

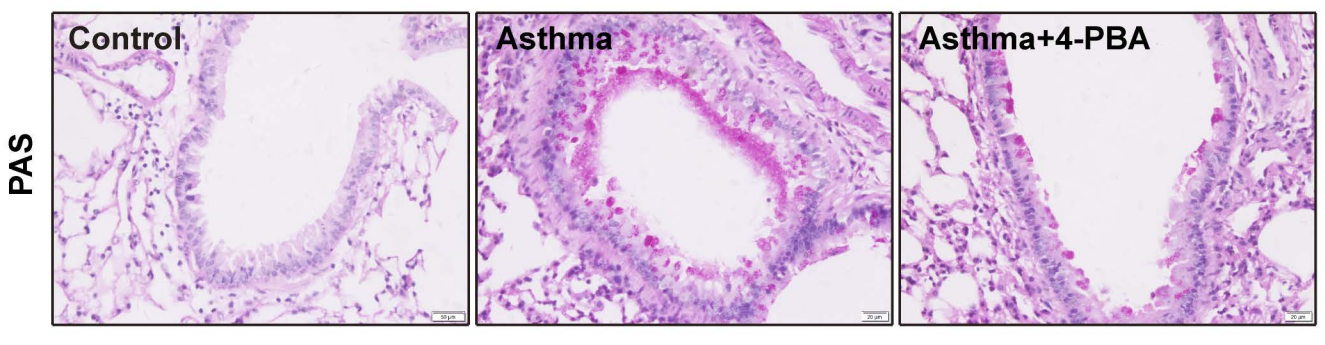
B


Bip/DAPI
Control
Asthma
Asthma+4-PBA
Chop/DAPI
Control
Asthma
Asthma+4-PBA

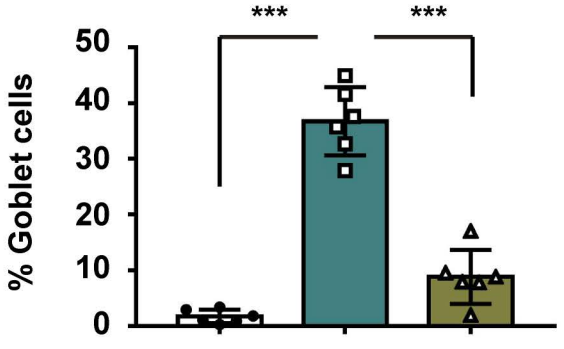
C


Bip
Chop
**
**
**
*
Mean fluorescence intensity
150
100
50
0
Control
Asthma
Asthma+4-PBA

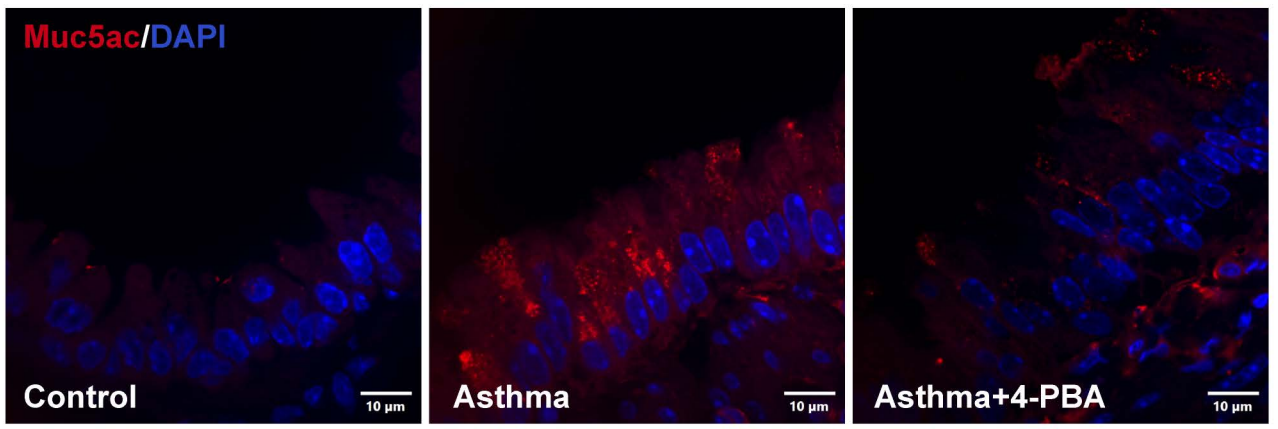
D


PAS
Control
Asthma
Asthma+4-PBA

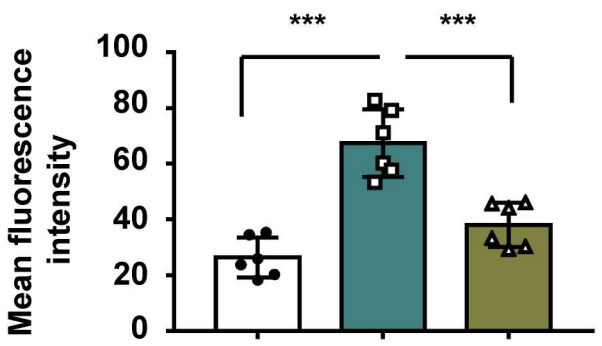
E


***
***
% Goblet cells
50
40
30
20
10
0

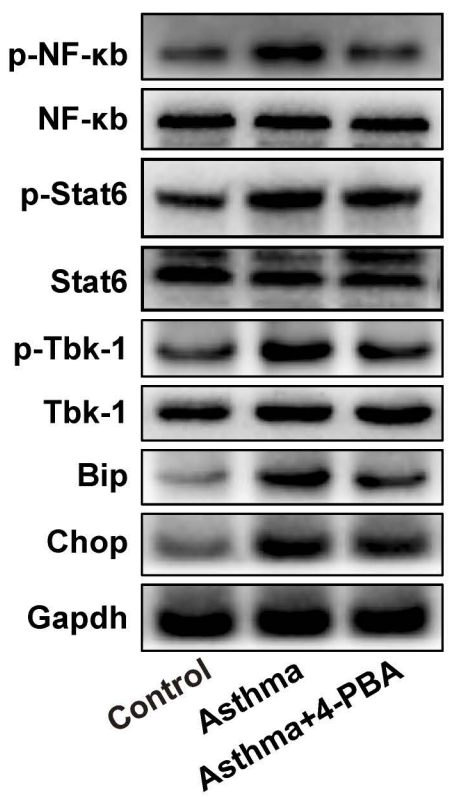
F


Muc5ac/DAPI
Control
Asthma
Asthma+4-PBA
10 μm

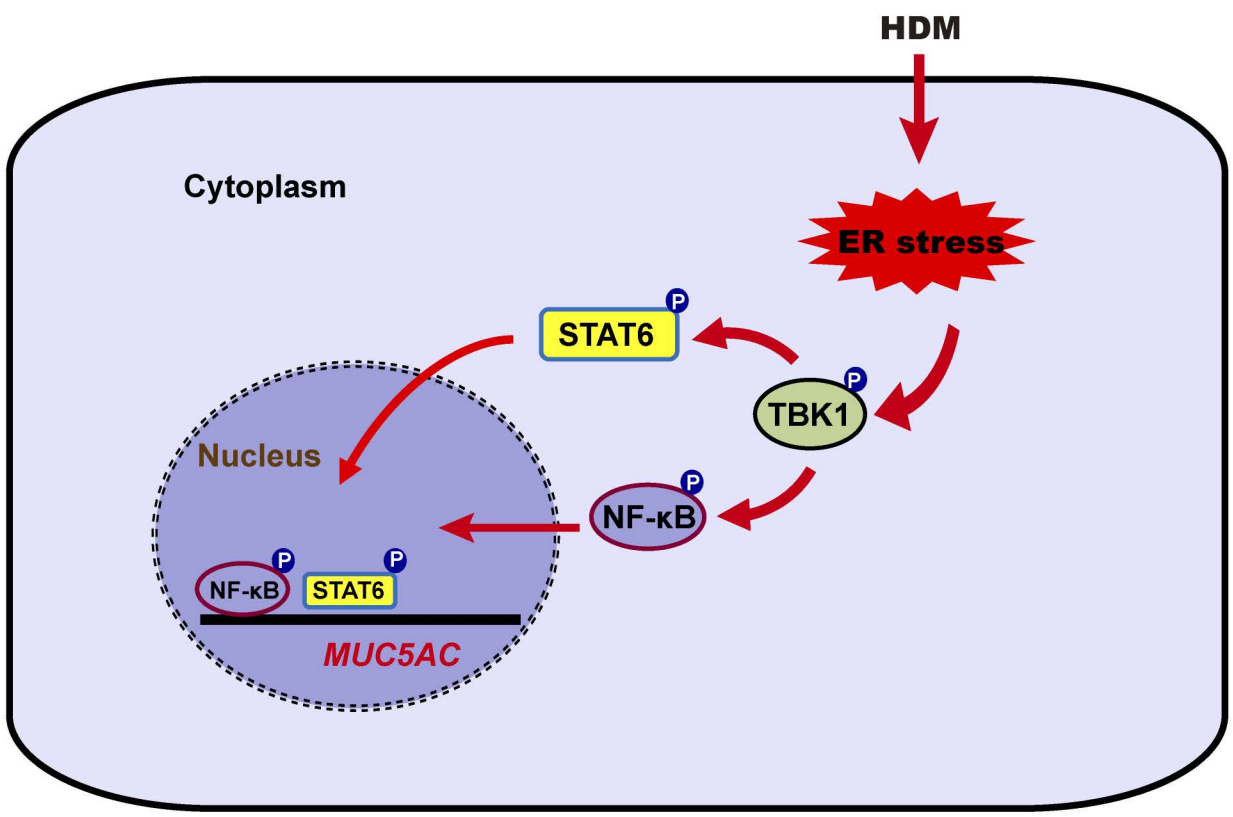
G


***
***
Mean fluorescence intensity
100
80
60
40
20
0

H


p-NF-κb
NF-κb
p-Stat6
Stat6
p-Tbk-1
Tbk-1
Bip
Chop
Gapdh
Control
Asthma
Asthma+4-PBA

I


HDM
Cytoplasm
ER stress
STAT6
TBK1
NF-κB
Nucleus
NF-κB
STAT6
MUC5AC

Supplemental material, method and figure legends

Mice asthma model protocol

C57BL/6J mice asthma model was established as previous study [1]. All mice were maintained under specific pathogen-free conditions in the Animal Experimental Center of Southwest Medical University. All animal experiments in this study were approved by and performed in accordance with the guidelines of the Committee of Animal Experiments Center of Southwest Medical University and the National Institute of Health guidelines on the care and use of animals.

PAS Tissue histology staining

Periodic acid-Schiff (PAS) staining was performed as previously description [1].

HE Histological analysis

Mice's lung tissue samples were fixed in 10% neutral-buffered formalin for 24 h at room temperature and then embedded in paraffin. The tissues were then cut into 5 μm sections and subjected to standard hematoxylin-eosin staining was performed as lab route protocol. The DM4000 light microscope (Leica, Germany) was used to analyze the stained slides. The severity of peribronchial and perivascular inflammation was scored by using previously described methods. Each bronchus observed was scored from 0 to 3, with approximately 10 areas scored in total [2, 3] .

Cell culture

BEAS-2B human bronchial epithelial cell line was maintained as previously description [1]. In brief, BEAS-2B cells were cultured in DMEM culture medium with 10% fetal calf serum at 37 °C with 5% CO_2. After BEAS-2B cells reached 50% confluence in 6-well plates, the medium was replaced with serum-free culture medium. The cells were then transduced with si-NC, si-TBK1, si-NF-κB and si-STAT6 lentivirus vectors (with the MOI=20) with or without HDM (100 μg/ml). Or BEAS-2B cells were treated with Amlexanox (25 μM) or 4-PBA (10 mM) with or without HDM.

Western Blotting

All tissue samples or cell samples from different treatment groups were collected and western blotting was performed to analysis protein expression based on lab standard protocol, which previously described in detail [1]. While specific primary antibodies in this study are: p-TBK1 (1:1,000; CST, 5483S), TBK1 (1:1,000; CST, 38066S), p-NF-κB (1:1,000; 3033S), NF-κB (1:1,000; CST, 8242S), p-STAT6 (1:1,000; CST, 56554S), STAT6 (1:1,000; CST, 5397S), BIP (1:2,000; Abcam, ab21685), CHOP (1:1,000; CST, 2895S), and GAPDH (1:1,000; Beyotime Institute of Biotechnology, AF0006).

Immunofluorescence staining

BEAS-2B cells were treated as designed strategies in this study. Immunofluorescence staining was performed as

lab standard protocol [1]. Specific primary antibodies in this study are: MUC5AC (1:50; Abcam, ab24070), NF-κB (1:200; CST, 8242S), STAT6 (1:200; CST, 5397S), BIP (1:200; Abcam, ab21685), CHOP (1:100; CST, 2895S), and followed by stained with Alexa Fluor 555 or Alexa Fluor 488 conjugated secondary antibody (1:500; Invitrogen, cat. no. A32727, A32732, A28175) was used to against primary antibody. DAPI was used to stain nuclei.

Statistics

All data are presented as the means ± standard deviation. Statistical analysis was performed by SPSS 17.0 (SPSS, Inc.). Student's t-test or one way ANOVA followed by Tukey-Kramer post-test was used to compare data between two groups or multiple groups, respectively. SPSS was used to analysis the linear regression. P value less than 0.05 was considered statistically significant differences.

Figure S1

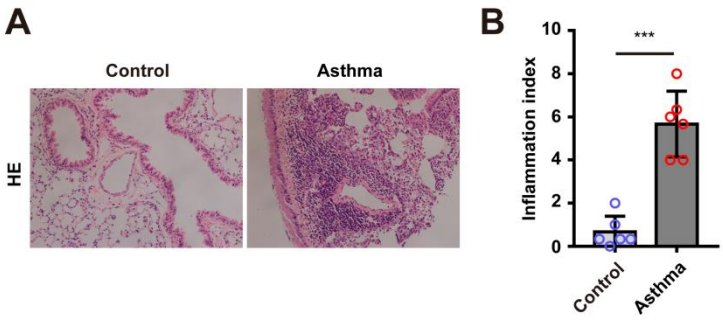


Figure S1. Pathology of asthmatic mice's lung tissues, related to figure 1.

To obtain asthmatic mice, C57BL/6J mice were sensitized twice by intraperitoneal (i.p.) injection of HDM and $Al(OH)_3$ on day 1 and day 8. Then the mice were challenged with HDM through intratrachel instillation (i.n.) to induce airway allergic asthma from day 15 to day 21, scheme as in figure 1a. (A) Representative images of hematoxylin and eosin-stained lung tissue sections; (B) Quantification of inflammatory cell infiltration in lungs. Data are presented as mean ± s.d. ***$p<0.001$ was determined by t-test.

Figure S2

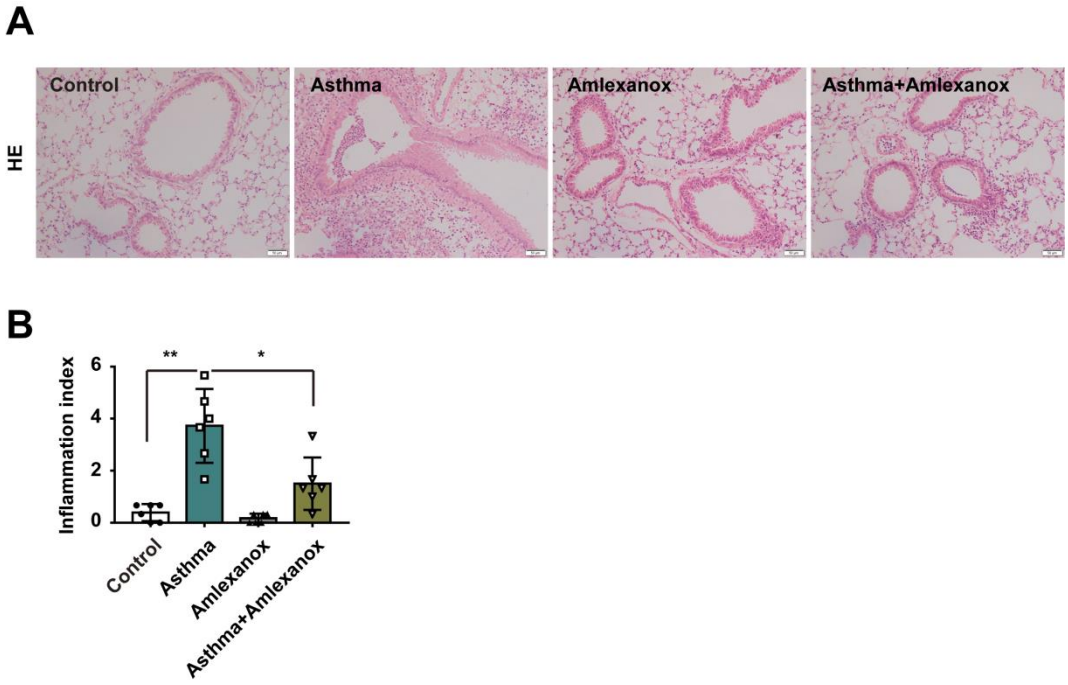


Figure S2. Pathology of asthmatic mice's lung tissues, the effect of Amlexanox, related to figure 5.

Induced asthmatic mice were generated as usual, while the mice were i.p. injected with Amlexanox (100 mg/kg) daily from day15 up to day21 as showed in figure 5a. (A) Representative images of hematoxylin and eosin-stained lung tissue sections. (B) Quantification of inflammatory cell infiltration in lungs. Data are presented as mean ± *s.d.* *p<0.05 or **p<0.01 was determined by t-test for two groups comparison or one-way ANOVA for three groups comparison.

Figure S3

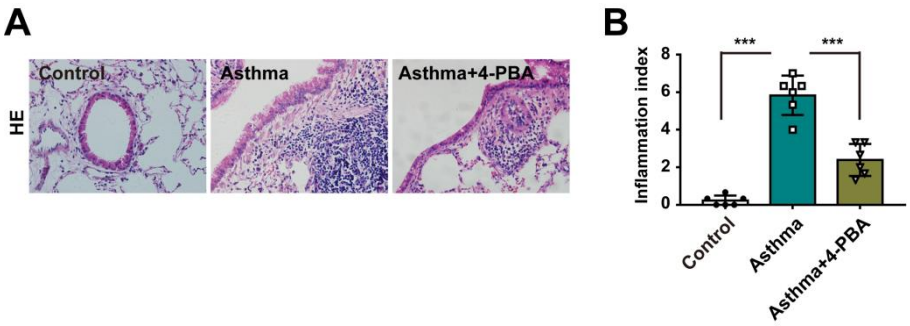


Figure S3. Pathology of asthmatic mice's lung tissues, the effect of 4-PBA, related to figure 7.

As shown in figure 7a, mice were treated with HDM/Al(OH)$_3$, followed by HDM (i.n.) with or without 4-PBA to investigate the therapeutic efficiency of 4-PBA. (A) Representative images of hematoxylin and eosin-stained lung tissue sections. (B) Quantification of inflammatory cell infiltration in lungs. Data are presented as mean ± *s.d.* ***p<0.001 was determined by one way ANOVA followed Tukey-Kramer posttest.

References

1. Zhang Y, Tang H, Yuan X, Ran Q, Wang X, Song Q, Zhang L, Qiu Y: **TGF-β3 Promotes MUC5AC Hyper-Expression by Modulating Autophagy Pathway in Airway Epithelium**. *EBioMedicine* 2018, **33**:242-252.
2. Wang X, Yang X, Li Y, Wang X, Zhang Y, Dai X, Niu B, Wu J, Yuan X, Xiong A *et al*: **Lyn kinase represses mucus hypersecretion by regulating IL-13-induced endoplasmic reticulum stress in asthma**. *EBioMedicine* 2017, **15**:137-149.
3. Han Y, Chen L, Liu H, Jin Z, Wu Y, Wu Y, Li W, Ying S, Chen Z, Shen H *et al*: **Airway Epithelial cGAS Is Critical for Induction of Experimental Allergic Airway Inflammation**. *The Journal of Immunology* 2020, **204**(6):1437-1447.