Inhibiting ATG5 mediated autophagy to regulate endoplasmic reticulum stress and CD4$^+$ T lymphocyte differentiation: Mechanisms of acupuncture's effects on asthma

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A B S T R A C T

Objective: Asthma is characterized by airway hyperresponsiveness(AHR), inflammation and remodeling. Autophagy and endoplasmic reticulum stress(ERS) are dysregulated in asthma, and ATG5 has attracted wide attentions a representative gene of autophagy. Previous evidence shows that acupuncture may treat asthma by regulating the immune environment.However,the precise mechanism involved in acupuncture's effects on asthma is unclear. Thus, we investigated the inner-relationships of acupuncture and ATG5-mediated autophagy, ERS and CD4$^+$ T lymphocyte differentiation in asthma.

Methods: Ovalbumin (OVA)-sensitized and challenged ATG5$^{+/-}$ and ATG5$^{-/-}$mice with asthma were treated by acupuncture at Dazhui(GV14),Feishu(BL13) and Zusanli(ST36),and sacrificed the next day.Then blood and bronchoalveolar lavage fluid (BALF)samples were collected to determine inflammatory cell counts and cytokine levels. Lung tissue samples were obtained for histological examination, and the spleen was harvested for flow cytometry.

Results: Compared with the untreated group, acupuncture decreased BALF inflammatory cell counts and AHR in OVA-induced mice.Acupuncture decreased autophagy-related protein and mRNA (ATG5,Beclin-1,p62 and LC3B) amounts and ERS-related protein (p-PERK, p-IRE-1,Grp78, and ATF6)levels as well as autophagosome formation in lung tissue, concomitant with increased IFN-γ and decreased IL-4, IL-17 and TGF-β amounts in BALF. Consistently, the imbalance of CD4$^+$ T lymphocyte subsets(Th1/Th2 and Treg/Th17) was also corrected by acupuncture.Meanwhile, AHR and inflammation were decreased in ATG5$^{-/-}$ mice compared with ATG$^{+/-}$animals, without affecting the therapeutic effect of acupuncture.

Conclusion: Acupuncture reduces airway inflammation and AHR in asthma by inhibiting ATG5-mediated autophagy to regulate endoplasmic reticulum stress and CD4$^+$T lymphocyte differentiation.

1. Introduction

Asthma is a heterogeneous disease that has different underlying disease processes, with a morbidity of about 4.2% in China and as high as 18% in some parts of the world[1]. Although pharmacological therapy recommended by evidence-based asthma management strategies is updated every year, uncontrolled severe asthma still accounts for 5–10% of all patients[2]. Expiratory airflow limitation triggered by asthma exacerbation threatens the life of patients and imposes a significant burden on both the affected individuals and the social healthcare system.

Asthma is a serious chronic respiratory disease characterized by airway inflammation, hyperresponsiveness(AHR) and remodeling[3]. Based on different pathological features of the airway, diverse phenotypes of asthma show different therapeutic responses to inhaled corticosteroid (ICS) treatment. Allergic asthma is the most widely recognized asthma phenotype,with an IgE related Th2-induced background[3–5]. The inflammatory response is currently widely considered an important

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part of the pathophysiology of allergic asthma.Moreover,the chemotaxis of inflammatory cells is closely related to T lymphocyte-mediated immune responses.A complex relationship exists between molecular signals released from epithelial cells and type 2 cytokines that are mainly secreted from type 2 immune cells[6]. A number of studies have shown that T lymphocytes, particularly CD4$^+$ T lymphocytes, and secreted cytokines, are targeted in allergic asthma[7–9]. Due to the heterogeneity of asthma, exploring its mechanism is imperative.

Autophagy, a conservative degradation process in eukaryotic cells, plays an important role in maintaining cellular homeostasis and adapting to adverse environments.However, abnormal autophagy has a dual function in pathological processes[10]. Disturbed intracellular environment from insufficient autophagy may result in neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease. Meanwhile,excessive activation of autophagy could lead to tissue damage[11]. Studies have shown that autophagy is related to airway inflammation response in asthma[12]and plays an essential role in the development and function of lymphocytes[13]. Silencing of the key autophagy gene ATG5decreases cytokine secretion and airway hyperresponsiveness in varying degrees, indicating that ATG5 participates in the pathogenesis of asthma.

The endoplasmic reticulum (ER) is important in maintaining the stability of cell function and structure.In this organelle,proteins are folded and then transported to the extracellular surface or to different intracellular organelles[14]. However, inflammatory cells in the airway that generate reactive oxygen species (ROS)could disrupt ER homeostasis, triggering ER stress(ERS),eventually leading to apoptotic cell death. As a consequence,the cell activates the unfolded protein response (UPR),an adaptive signaling pathway, enhancing the folding capability to relieve ERS.In recent years, endoplasmic reticulum stress and the UPR have been shown to play important roles in chronic lung diseases[15]. Wu et al.[16] found that chronic ERS and UPR activation leads to apoptotic T cell death in the Sting$^{N153S/+}$ mouse as well as in human T cells. These changes were reversed by ERS inhibitors.However, evidence that links ERS and T lymphocytes in asthma is lacking.

The ER also contributes to the production of autophagosomes and peroxisomes[17,18]. Several studies have demonstrated that ER stress and autophagy are mechanistically interconnected.Autophagy is stimulated by the UPR,the key ER stress pathway,in a variety of diseases such as kidney diseases,bone loss and neurode generative disorders[19–21]. In addition, evidence indicates that ERS and autophagy are interconnected for inflammation in bowel epithelial cells.Knocking out the autophagy-related gene ATG7 further exacerbates intestinal inflammation[22]. However, the mechanism governing the interaction of ERS and autophagy in asthmatic airway inflammation and hyperresponsiveness remains unclear.

Chinese clinicians have used traditional Chinese medicine and acupuncture for ages to relieve acute exacerbation symptoms in asthma patients.Several animal experiments and clinical trials have validated the effect of acupuncture for asthma treatment [23–26]. We previously found that acupuncture reduces stress response and β-endorphin release in surgery patients, potentially providing novel mechanisms for the development of new therapeutic strategies [27]. Here, we further hypothesized that the effect of acupuncture on asthma might be explained by inhibited ATG5-mediated autophagy to regulate endoplasmic reticulum stress and CD4$^+$ T lymphocyte differentiation.

2. Materials and methods

2.1. Experimental animals

Female C57BL/6 mice(6–8 week old) were purchased from Shanghai Medel Organisms Center,Inc.Then, mice were housed in standard cages in a temperature-controlled environment under a 12 h light/dark cycle and 60 ± 10% relative humidity.All mice were provided with standard food and tap water daily. All animal experiments were approved by the Animal Care and Use Committee on the Ethics of Animal Experiments of Guangzhou University of Chinese Medicine.

2.2. Asthma model establishment and acupuncture treatment

Thirty-six female mice were divided into six groups randomly(n = 6 per group). ATG5$^{+/-}$(ATG5$^{flox/flox}$CD4^{Cre-}) mice were divided into three groups,including the sham group(Sham(-)),untreated OVA-induced asthma group(AHR(-)),and OVA-induced asthma group treated with manual acupuncture at Dazhui(GV14),Feishu(BL13) and Zusanli(ST36) (AAHR(-)). Similarly,ATG5-/-(ATG5$^{flox/flox}$CD4^{Cre+}) mice were divided into the sham group(Sham(+)),untreated OVA-induced asthma group (AHR(+)),and OVA-induced asthma group treated with manual acupuncture(AAHR(+)). The asthma model was induced by multiple OVA(Sigma-Aldrich,US) sensitization and challenge rounds (Fig. 1, A). On Days 0,7 and 14,mice were sensitized with 0.2 mL 0.9% saline containing 10 μg OVA and 1 mg AL(OH)$_3$(ThermoFisher, US). From Day 15, mice in the AAHR group were administered the acupuncture treatment with a fixation device while awake every 2 days for 7 times by a same acupuncturist.The acupoints were selected according to Experimental Acupuncture Science(Sixth Edition). GV14 is located in the subspinous depression of cervical vertebra 7. BL13 is located in the depression outside the lower edge of the spinous process of the bilateral third thoracic spine.ST36 is located approximately 5 mm inferolaterally to the bilateral anterior tibial tubercle. Disposable stainless steel acupuncture needles measuring 0.3 × 13 mm (Zhongyan,China) were bilaterally inserted to a depth of 3 mm and maintained for 20 minManual manipulations(20 cycles) were performed every 5 minFrom Day28 to Day30, mice were placed in plastic cages by group and challenged with 1% OVA for 20 min every day using ultrasonic nebulizers(402AI, Yuyue Medical Equipment Company, Jiangsu, China).

2.3. AHR measurement

Airway hyperresponsiveness was measured the second day after the final challenge.Mice were treated by inhalation with increasing doses of methacholine(3.125, 6.25 12.5, 25 and 50 mg/mL, respectively) after anesthesia with 0.2 mL of 1% pentobarbital sodium.Pulmonary resistance(RI) was assessed on a Buxco Pulmonary function measurement in Resistance/Compliance System(RC, Buxco Electronics, USA). Results were expressed as percentage change of the difference between a given dose and baseline values.

2.4. Histopathological assessment

The right lung was collected after bronchoalveolar lavage fluid (BALF) collection,fixed with 4% paraformaldehyde,embedded in paraffin and cut into 4 μm sections.The sections were stained with hematoxylin and eosin(H&E)(Beyotime,Shanghai,China) and observed under an optical microscope(BX51,OLYMPUS,Japan).

2.5. Inflammatory Cell Counts in BALF

BALF was collected from the left lung through the tracheal cannula using 0.5 mL of sterile PBS for three times(1.5 mL in total) and centrifuged at 1500 rpm for 5 min. The cell pellet was resuspended with 100 μL of PBS for incubation with Giemsa Stain(419015,Baso,Zhuhai, China) to count inflammatory cells.

2.6. Cell Sample Preparation and Flow Cytometric Analysis

The spleen was harvested from each mouse and washed with ice-cold PBS. Then, single cells were separated through a 100 μm mesh.Red blood cells in the suspension were removed with the ACK Lysis Buffer (0.4% NH$_4$Cl). CD4$^+$ T cells were then isolated from mononuclear cells by positive selection using MicroBeads and a MiniMACS™ Separator
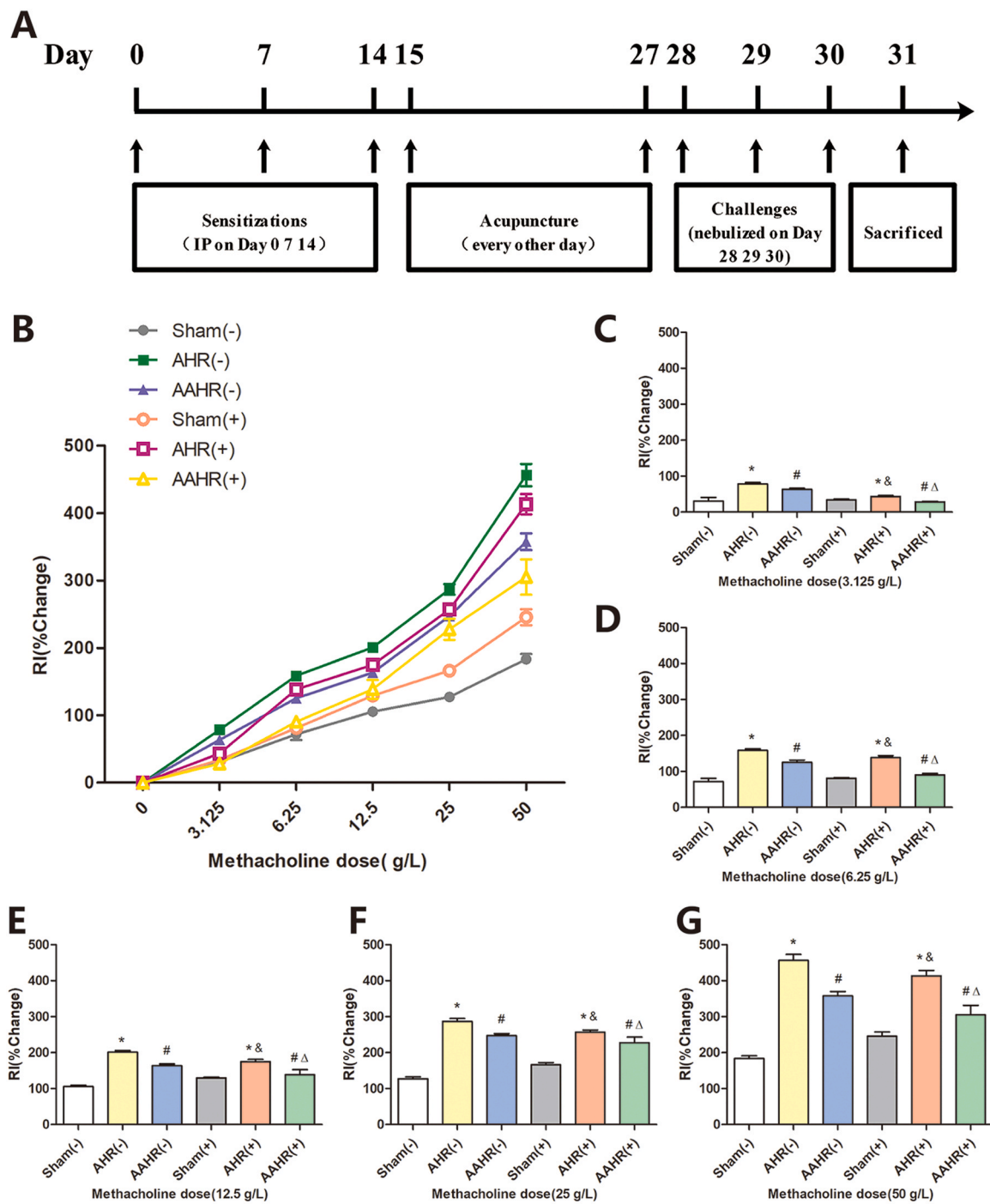
Fig. 1. ATG5 deficiency and acupuncture treatment protect against methacholine-induced AHR in mice. Flow chart of acupuncture treatment of the airway of OVA-induced asthma in mice (A).Acupuncture suppressed airway hyperresponsiveness(AHR) in mice with OVA-induced asthma and affected the autophagy gene ATG5. AHR developed with OVA dose-dependent increase (3.125, 6.25, 12.5, 25 and 50 g/L)(B). Degrees of RI (%change) significantly increased with methacholine concentration in the AHR groups and decreased in the AAHR groups(C-G). Data are mean±SD, n = 6 per group.*P < 0.05 vs Sham group.#P < 0.05 vs AHR group.&P < 0.05 vs AHR(-) group. ΔP < 0.05 vs AAHR(-) group.RI, airway resistance.

(Miltenyi Biotec, Bergisch Gladbach, Germany)according to the manufacturer's instructions.The isolated CD4$^+$ T cells were incubated in RPMI 1640 containing 10% FCS and 0.2%PMA(Sigma-Aldrich,US)at 37 °C in 5% CO_2 for 5 h. Antibodies used for flow cytometry were purchased from BD(Becton Dickinson,US). Anti-mouse CD16/32 antibody was used to block non-specific binding to Fc receptors before staining.FITC-linked anti-mouse CD4 and APC-linked anti-mouse CD25 antibodies were used for intracellular staining, PE-conjugated anti-mouse Foxp3 and PE-Cyanine7-linked anti-mouse IL-17A antibodies were used for

extracellular staining. Flow cytometry data were collected on the BD FACS Calibur (Becton Dickinson,US) and analyzed by the Flow Jo x64 software.

2.7. Cytokine measurement in serum and BALF

Blood samples were collected after AHR detection and centrifuged for serum collection.IL-4,IL-17,IFN-γ and TGF-β amounts in serum and BALF specimens were detected by ELISA according to the

manufacturer's instructions.

2.8. Western blot analysis

Lung samples were lysed in ice-cold RIPA buffer(Beyotime,Shanghai, China) for 30 min Protein concentrations were determined with the Protein BCA Assay Kit(Beyotime,Shanghai,China)and proteins were boiled at 95 °C for 5 min Sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE) was performed with 30 µg protein per samples followed by transfer on to PVDF membranes and blocking with 5% skimmed milk.Target proteins, including p-PERK(S3179),ATF6 (S65880),Grp78(S3177),ATG5(S12994),LC3I/II(S12741),Beclin-1 (S3495),p62(S23214) and GAPDH(S2118),were detected by antibodies obtained from Cell Signaling Technology (CST, USA; 1:1000).p-IRE-1 (ab48187)was obtained from Abcam(Abcam,England , 1:1000). Membranes were further incubated with HRP-conjugated secondary antibodies (Abcam,ab6721,England) at 1:5000 on the next day. Quantification was performed by densitometry using the Image J software.

2.9. Real-time Quantitative PCR(qPCR)

Total RNA in lung samples was collected for PCR assay with a MiniBest Universal RNA Extraction Kit(Takara,Ostu,Japan) according to the manufacturer's instructions. qPCR was performed on a CFX Connect Real-Time PCR System with iQ SYBR Green Supermix (Bio-Rad).β-actin was used as an endogenous control.The primer sequences are listed in Table 1.

2.10. Immunofluorescence

The sections were dewaxed, rehydrated and blocked with 3% bovine serum albumin for 1 h at room temperature.Slides were successively incubated with anti-LC3(CST;1:100, S12741) antibodies overnight at 4 °C and the corresponding secondary antibody(Abcam,ab150074; 1:500) for 1 h at room temperature.After PBS washes, the sections were counterstained with antifade Mounting Medium contained DAPI and stored at 4 °C.

3. Transmission electron microscopy

Lung samples were fixed with 2.5% glutaraldehyde and 2%

Table1
Primer sequences used for q-PCR analysis in this study.

Gene	Primers (5'−3')
ATG5	Forward: CACCCCTGAAATGAGTTTTCCA
	Reverse: AAAGTGAGCCTCAACCGCAT
LC3II	Forward: TACATGGTCTACGCCTCCCA
	Reverse: GCCTAATCCACTGGGGACTG
PERK	Forward: GTCTCTGCCCTCGAAAACCA
	Reverse: TGATGCGCTTGTTTGGGTTG
IRE-1	Forward: TCGCCACTTGGTGAATGTGT
	Reverse: CAAAGTCTGCACGGTGCTTC
ATF6	Forward: CCTAAAGAGGACCTGTGGCTT
	Reverse: TTGGTCCATCGTGGGAGGA
Grp78	Forward: GTGTGTGAGACCACAACCGT
	Reverse: GCAGTCAGGCAGGAGTCTTA
IL-4	Forward: TACCAGGAGCCATATCCACGGATG
	Reverse: TGTGGTGTTCTTCGTTGCTGTGAG
IL-17a	Forward: GAGCTTCATCTGTGTCTCTGAT
	Reverse: GCCAAGGGAGTTAAAGACTTTG
IFN-γ	Forward: CTTGAAAGACAATCAGGCCATC
	Reverse: CTTGGCAATACTCATGAATGCA
TGF-β	Forward: CCAGATCCTGTCCAAACTAAGG
	Reverse: CTCTTTAGCATAGTAGTCCGCT
β-actin	Forward: GTGTGTGAGACCACAACCGT
	Reverse: GCAGTCAGGCAGGAGTCTTA

paraformaldehyde for 2 h and washed with PBS three times. Then, the samples were dehydrated with 50%, 70%, 80%, 90%, and 100% ethanol and 100% acetone.A mixture of acetone and Epon812(1:1) was used to infiltrate the samples overnight.Finally,the samples were embedded in Epon812 and stained.Images were acquired under a JEM-1200EX transmission electron microscope.

4. Statistical analysis

Data are mean ±standard deviation (SD),and were assessed with SPSS (version 20.0). Data with normal distribution were compared by one-way ANOVA with Bonferroni post-test.Data with a non-normal distribution were compared by the Kruskal-Wallis *H* test for differences among groups, and further analyzed by the Nemenyi test for pairwise comparisons.ATG5 and acupuncture were analyzed by two-way classification ANOVA.$P < 0.05$ was considered statistically significant.

5. Results

1. Acupuncture suppresses AHR in OVA-induced asthma mice,and these effects enhanced by the autophagy gene ATG5.

   Airway hyperresponsiveness was assessed by measuring airway resistance(RI),which is significantly increased in asthma.AHR was increased with methacholine dose(Fig. 1 B). Mice in the AHR groups showed overtly increased RI compared with the Sham groups regardless of the ATG5 gene status,with increasing dose of methacholine($P < 0.05$). After acupuncture treatment,RI was decreased in the AHR groups compared with model mice ($P < 0.05$). Meanwhile, ATG5 deficiency expanded the therapeutic effect of acupuncture in both the AHR and AAHR groups ($P < 0.05$)(Fig. 1 C-G).

2. Airway inflammation and lung injury are attenuated by acupuncture in OVA-induced asthma model

   We next investigated the effects of acupuncture and ATG5 on inflammation by measuring BALF inflammatory cell and cytokine levels and serum cytokine levels.Eosinophil(EOS)and neutrophil (NEU)counts were significantly higher in the OVA-induced groups than the sham groups($P < 0.05$), and both were decreased after acupuncture treatment($P < 0.05$)(Fig. 2 A-B). In contrast,macrophagocyte(MAC) counts tended to change in the opposite direction (Fig. 2 C). Furthermore, there was no significant change in lymphocyte(LYM) amounts between groups without ATG5 deficiency, but lymphocyte counts were increased in the AHR(+) and AAHR(+) groups compared with the AHR(-) and AAHR(-) groups (Fig. 2 D).

   OVA-induced lung injury was assessed by H&E staining. The micrographs showed large amounts of inflammatory cells infiltrating the airway, with congestive edema of the bronchial mucosa and partial exfoliation of epithelial cells in OVA-induced mice. Acupuncture treatment resulted in significantly decreased infiltration of inflammatory cells compared with the AHR groups. As expected, the factors reflecting lung injury decreased more profoundly in mice with ATG5 deficiency,including less inflammatory cells infiltrating the airway(Fig. 2 E).

   To further address the level of inflammation, we examined representative cytokines in BALF and serum,including TGF-β,IL-4, IFN-γ and IL-17. The results showed markedly increased TGF-β(Fig. 3 A,B), IL-4(Fig. 3 G,H) and IL-17(Fig. 3 E,F) and decreased of IFN-γ(Fig. 3 C,D)amounts in the AHR groups compared with sham animals ($P < 0.05$),confirming the establishment of an inflammatory environment in the asthma model. Meanwhile,the levels of these cytokines decreased after acupuncture treatment (AHR group vs. AAHR group; $P < 0.05$). Some differences were observed in the amounts of cytokines in BALF and serum with ATG5 deficiency.We found that IFN-γ and IL-4 expression changes had similar trends,both in BALF and serum from mice with ATG5 deficiency($P < 0.05$),but
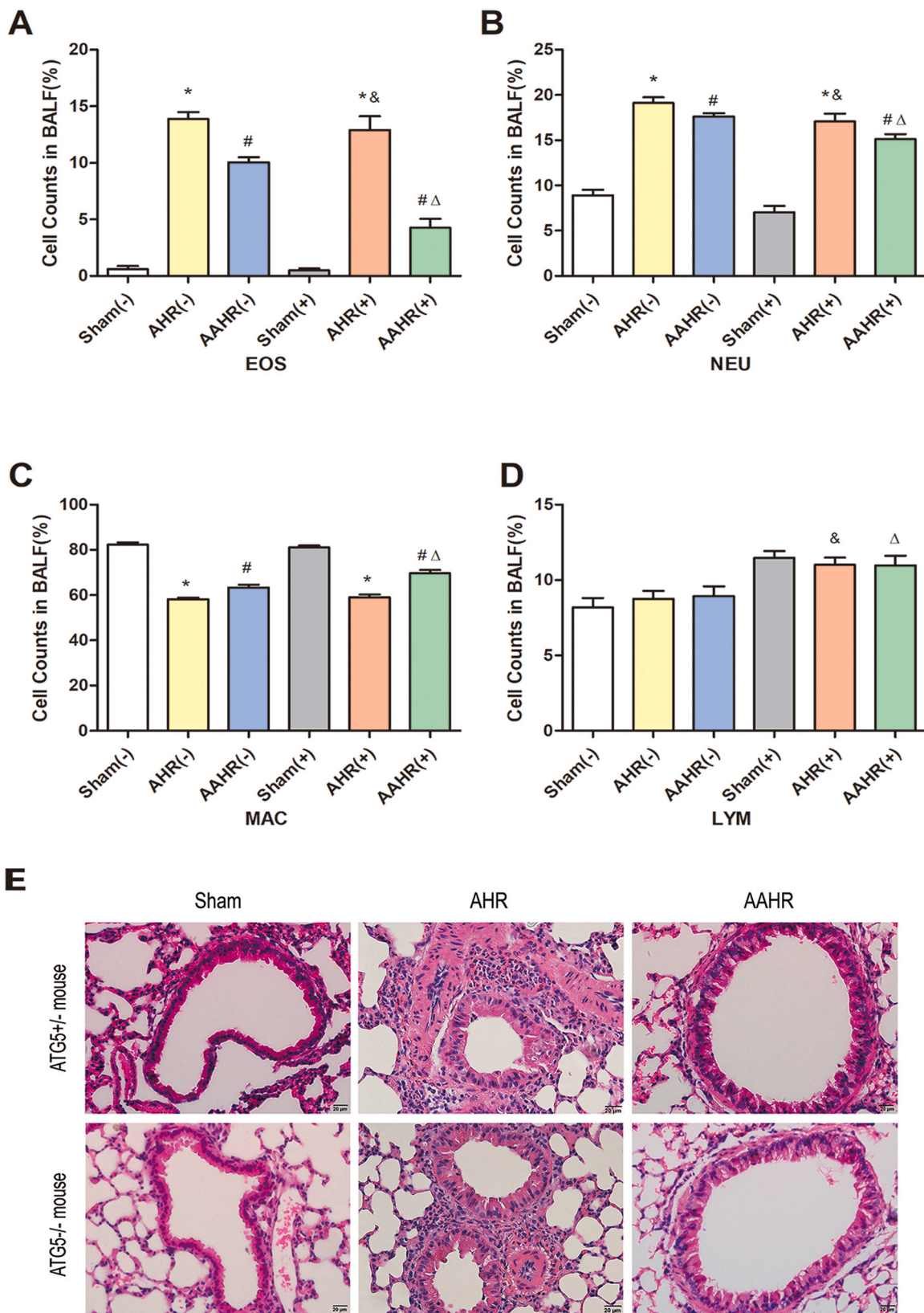
Fig. 2. Effects of acupuncture and ATG5 gene knockout on inflammatory cells in BALF of OVA-induced asthma models.Counts of eosinophils (EOS)(A), neutrophils (NEU)(B),macrophagocyte(MAC)(C),lymphocytes (LYM)(D), expressed as percentages.Data are mean±SD; n = 6 per group.*P < 0.05 vs Sham group.#P < 0.05 vs AHR group.&P < 0.05 vs AHR(-) group. ΔP < 0.05 vs AAHR(-)group. Representative images of H&E staining of the lung tissue(E). Scale bar: 20 μm.
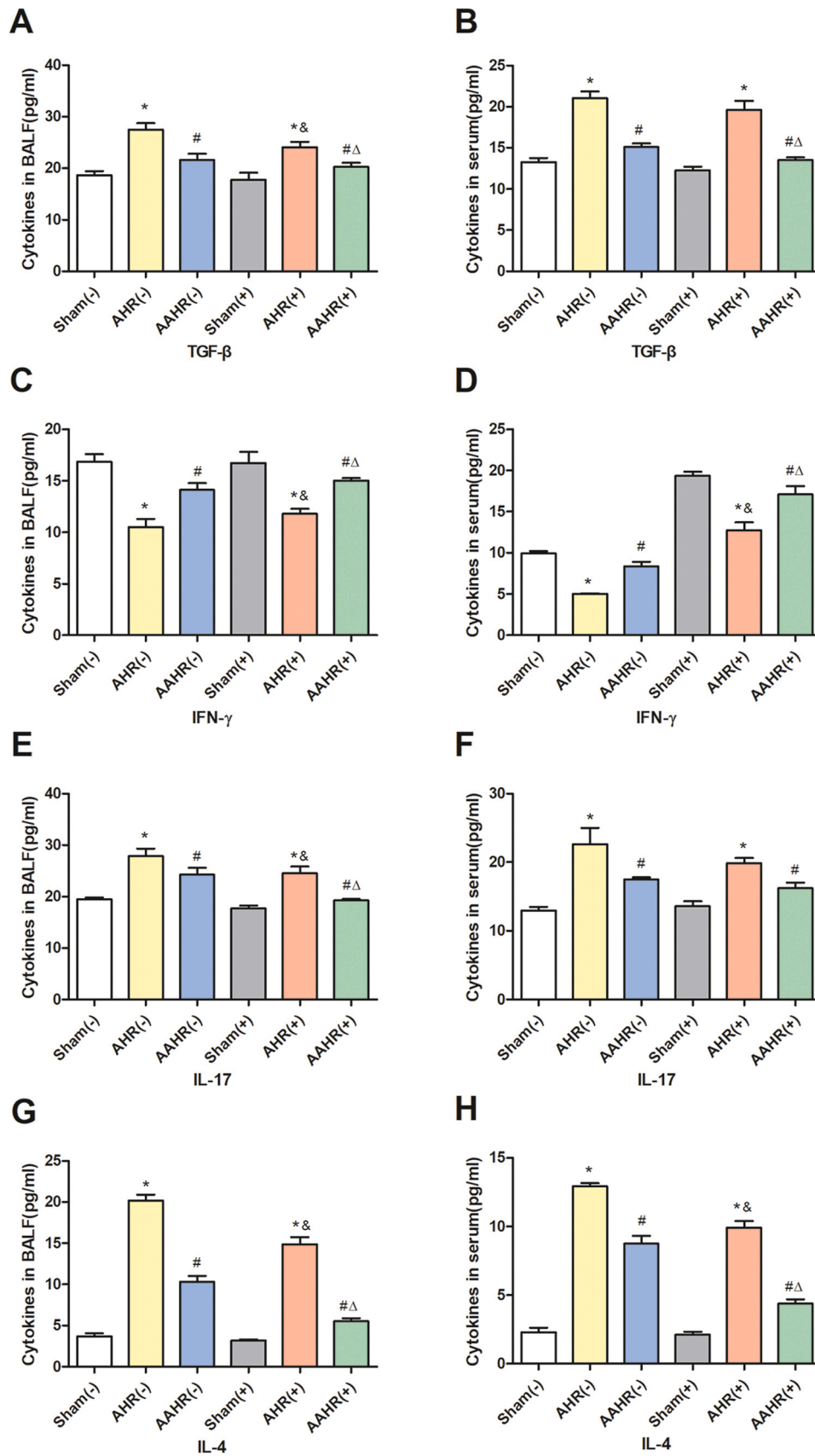
Fig. 3. Effects of acupuncture and ATG5 on cytokine levels. TGF-β(A and B),IFN-γ (C and D), IL-17(E and F) and IL-4(G and H) were assessed in BALF and serum samples from mice with OVA-induced asthma. Data are mean±SD, n = 6 per group.*P < 0.05 vs Sham group.#P < 0.05 vs AHR group.&P < 0.05 vs AHR(-) group. ΔP < 0.05 vs AAHR(-) group.TGF,transforming growth factor;IL, interleukin; IFN, interferon.

acupuncture treatment had no significant effect on serum TGF-β and IL-17 amounts.We also measured changes of inflammatory cytokines (IL-4,IL-17a,TGF-β and IFN-γ) in lung tissue samples by qPCR(Fig. 4 A-D). As the mRNA levels of cytokines had no significant differences except IL-4 and IL-17a in asthmatic mice(vs sham group,P < 0.05),we presumed that the gene levels of inflammatory cytokines were not regulated by acupuncture therapy.

3. Acupuncture treatment suppresses autophagy and ER stress

We next investigated the mRNA and protein expression levels of ERS and autophagy-related factors. The ERS-related genes *PERK, IRE-1,ATF6 and Grp78*(Fig. 4 E-H)were significantly downregulated in the acupuncture treatment group compared with the AHR groups (P < 0.05). Similarly, the amounts of the autophagy-related genes*LC3B*(Fig. 4 J) and ATG5(Fig. 4 I)were reduced upon acupuncture treatment(P < 0.05). Meanwhile,consistent changes of protein amounts were detected by Western blot analysis.The expression levels of related proteins were increased in the AHR groups and decreased after acupuncture treatment.*ATG5* gene deficiency reduced protein elevation in the AHR groups(Fig. 5A-D). Comparing the ATG5$^{-/-}$and ATG5$^{+/-}$groups,we found that ATG5 had different effects on mRNA and protein.There were significantly decreased amounts of related proteins(P < 0.05),while the changes of ERS-related mRNAs were limited(P > 0.05). In contrast,ATG5 deficiency was associated with significantly downregulated autophagy protein (LC3,ATG5,p62 and Beclin-1),and mRNA (*ATG5* and *LC3B*) amounts were further reduced compared with the non-knockdown group.LC3-

positive punct a were increased in the AHR groups, and acupuncture treatment decreased the expression of LC3 in the AAHR group (Fig. 6),consistent with the trend of protein expression.Moreover,in two-factor crossover analysis, the interaction between acupuncture and ATG5 was significantly different at the protein level(Fig. 9 E), which may help identify a target in future studies.

4. Typical autophagosomes are observed in the OVA-induced asthma model, but not in groups administered acupuncture or with ATG5 deficiency.

To further examine the effects of ATG5 on autophagy and acupuncture treatment, we observed the formation of autophagosomes in the lung by electron microscopy.There were many typical autophagosomes in the lung tissue of AHR mice, most of which showed closed spherical bilayer membrane formed by organelles, proteins and other components to be degraded in cells(Fig. 7). The diameters of autophagosomes were about 0.5–1.5 μm, and a few autophagosomes were monolayer structures containing degraded cytoplasmic components. Meanwhile,no typical structure was found in the AAHR groups. Concomitantly, the degree of autophagy in mice with ATG5 deficiency was attenuated compared with the non-knockdown groups in both the model and acupuncture groups.

5. Acupuncture helps restore the balance of Th1/Th2 and Treg/Th17 in the mouse spleen.

To determine whether the ATG5 gene participates in the pathophysiologic processes of OVA-induced asthma in mice by regulating
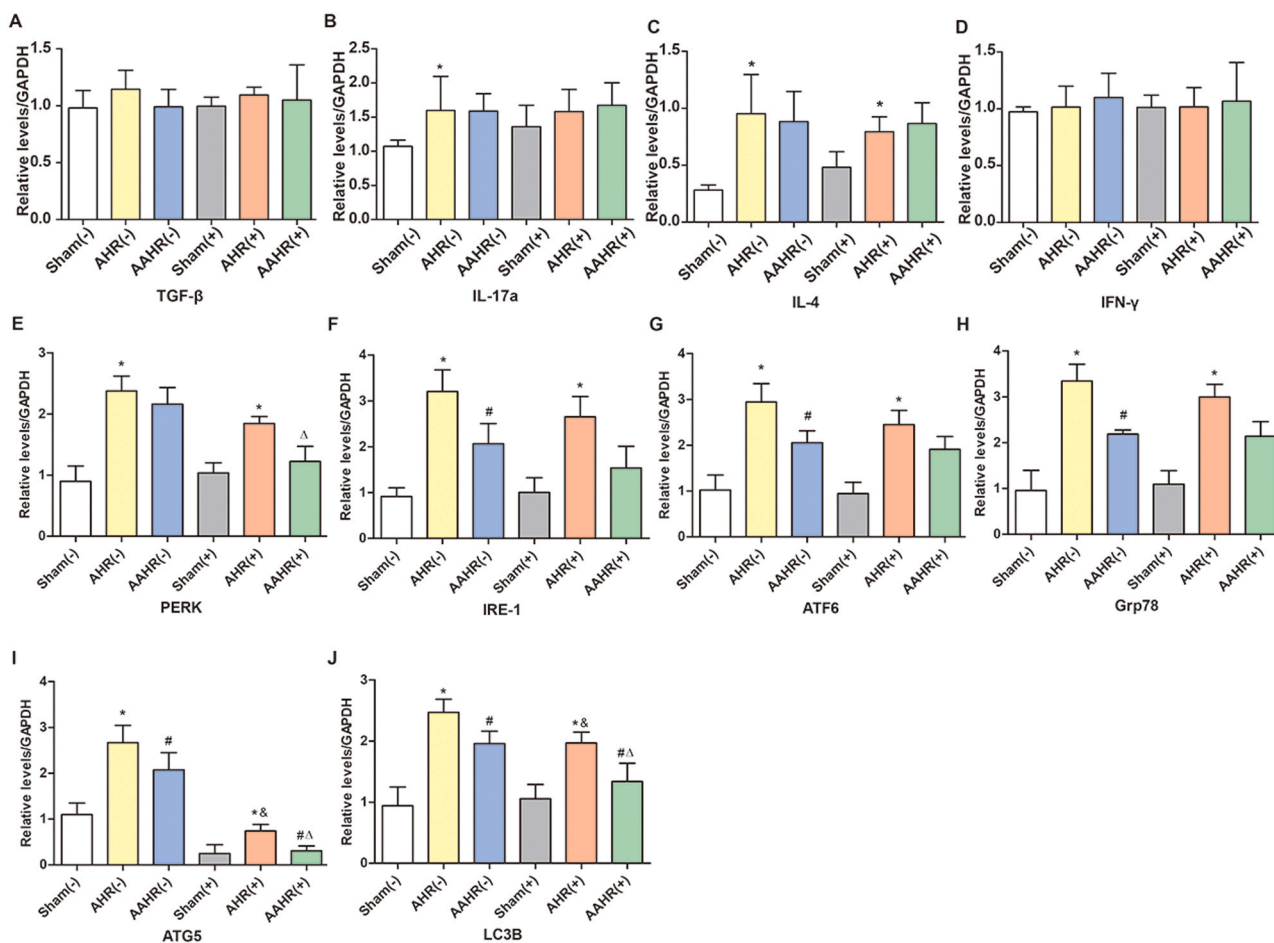

Fig. 4. Effects of acupuncture and ATG5 knockout on inflammation-, ERS- and autophagy-associated genes in the lung tissue of OVA-induced asthma mice. The inflammatory cytokines TGF-β(A),IL-17a(B),IL-4(C) and IFN-γ(D),the ERS-related factors PERK(E), IRE-1(F),ATF6(G) and Grp78(H), and the autophagy-related factorsATG5(I) and LC3B (J) were examined at the mRNA level. Data are mean±SD, n = 6 per group.*P < 0.05 vs. Sham group.#P < 0.05 vs AHR group.&P < 0.05 vs AHR(-) group. ΔP < 0.05 vs AAHR(-) group.
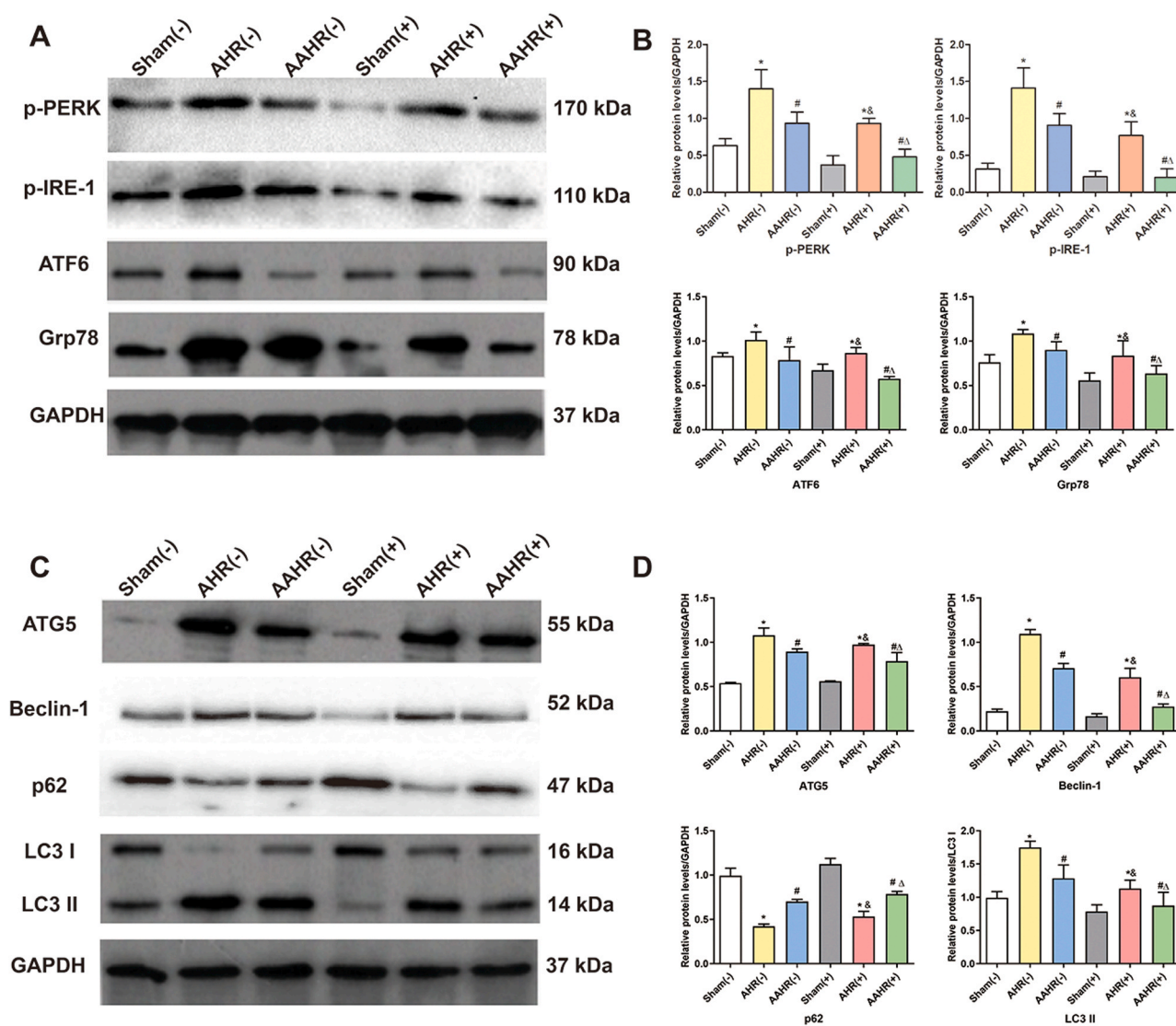
Fig. 5. Effects of acupuncture and ATG5 gene knockout on ERS- and autophagy-associated proteins in the lung tissue of OVA-induced asthma mice. The ERS-related proteins p-PERK, p-IRE-1,ATF6 and Grp78 (B) and the autophagy-related proteins ATG5,Beclin-1,p62 and LC3(D) were examined at the protein level. Representative images are also shows in the figure above(A and C).Data are mean±SD, n = 3 per group.*P < 0.05 vs Sham group.#P < 0.05 vs AHR group.&P < 0.05 vs AHR(-) group. ΔP < 0.05 vs AAHR(-) group.

CD4$^+$ T cell-associated immune responses, subsets of CD4$^+$ T cells(Th1, Th2, Th17 and Treg cells) in lung tissue samples were tested by flow cytometry. The results showed that the percentages of Th2 and Th17 cells were significantly increased while those of Th1 and Treg cells were remarkably decreased in OVA-induced mice compared with control animals (P < 0.05). Meanwhile,acupuncture treatment could reverse these adverse changes in asthmatic mice. Besides, ATG5 deficiency further increased the amounts of Th17 cells but reduced those of Th1 and Treg cells in mice with OVA-induced asthma(P < 0.05). Interestingly, there was no significant difference in the improvement degree of Th2 ratio between the ATG5 knockout and non-knockout groups after acupuncture treatment(Fig. 8).

6. Discussion

Asthma is a typical chronic inflammatory disease with high morbidity worldwide,in which the majority of cases are characterized by an allergic response such as AHR or airway inflammation [28].Given the features of recurrent chronic disease, asthma imposes a serious burden on patients and the society. In the present study, we investigated the role

of ATG5-mediated autophagy in mice with OVA-induced asthma as well as the effect of acupuncture treatment.The results demonstrated that acupuncture was effective in alleviating AHR and autophagy by inhibiting ATG5 to regulate endoplasmic reticulum stress and CD4$^+$ T lymphocyte differentiation in the OVA-induced asthma model Fig. 9.

Autophagy plays a crucial role of cell adaptation and survival under extreme conditions.In recent years,researchers have shown that autophagy is involved in multiple aspects of cell physiology and pathology.In addition to maintaining homeostasis,autophagy participates in tissue remodeling, growth control, cell differentiation and defense[11,29]. Thus, autophagy activation may play diverse roles in different diseases. Determining whether autophagy promotes or inhibits cell damage is critical to analyzing its role in disease. Poon et al.[30,31] found that autophagosomes are more likely to be detected in bronchial epithelial cells and fibroblasts in patients with moderate or severe asthma; in addition, ATG5 gene expression is negatively correlated with airway collagen fiber content, suggesting that autophagy may be involved in the process of airway sub-epithelial fibrosis in patients with refractory asthma.A report showed that autophagy is a potentially new therapeutic tool to target ILC2-dependent inflammation in asthma[32].However,
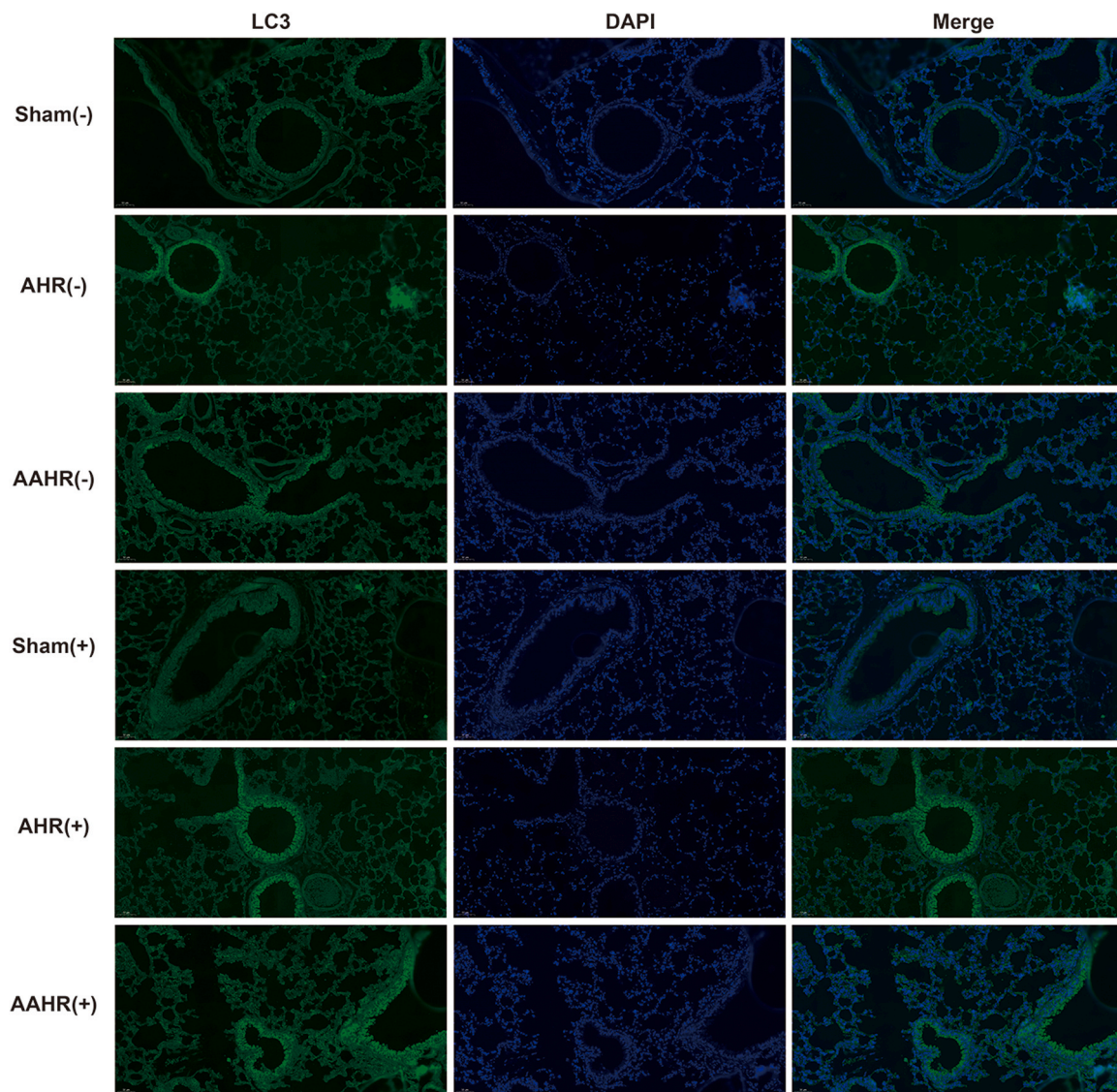
Fig. 6. Immunofluorescent detection of the autophagy protein(LC3) in the lung tissue of OVA-induced asthma mice. Representative images are also shows in the figure above.

whether autophagy is a pivotal part in airway hyperresponsiveness of asthma is still not fully elucidated.

To date, at least thirty-seven autophagy-related genes(ATG) have been identified in mammalian cells, including ATG8-PE and ATG12-ATG5-ATG16L ubiquitination pathways that play key roles. ATG5 is regulated by various stress-induced transcription factors, and represents a key protein involved in the formation of autophagosomes and LC3-II [33].A tight relationship exists between the single nucleotide polymorphism (SNP) rs12212740G>A of the ATG5 gene and asthma[30]. Additional SNPs in Atg5 (rs12201458 and rs510432) were also shown to be associated with childhood asthma by increasing promoter activity [34].The present study demonstrated that the expression levels of autophagy-related proteins and genes,e.g., LC3 and ATG5,were increased in asthmatic mice, accompanied by elevated amounts of autophagosomes in the lung.Autophagy and AHR levels in mice with ATG5 deficiency were lower than those of counterparts without ATG5 knockdown, suggesting that ATG5-mediated autophagy can negatively regulate airway hyperresponsiveness in asthmatic mice.

The ER is involved in the production of secretory and membrane proteins in the cell.Protein homeostasis, or proteostas is, is easily disrupted by internal or external stressors, altering the complex

biochemical environment required for proper protein folding, leading to the unfolded protein response (UPR)[35].Prolonged UPR activation could lead to pathological reprogramming of the cell or the initiation of proapoptotic pathways leading to cell death. Evidence suggests that the UPR in endoplasmic reticulum stress is involved in autophagy induction in some diseases[15,17,36],but such findings have not been reported for asthma.

GRP78, also referred to as BiP, is a central regulator of ER stress due to its anti-apoptotic properties and ability to monitor the activation of transmembrane ER stress sensors (IRE1, PERK, and ATF6) through a binding-release mechanism[37].When the UPR occurs, BIP is isolated by a large number of unfolded proteins and releases transmembrane proteins, activating the three above mentioned signaling pathways[37–39]. In acute and chronic respiratory diseases, ERS affects inflammatory signaling through a variety of pathways.IRE-1 promotes airway mucus synthesis and secretion by regulating its downstream transcription factor XBP-1[40].PERK promotes metabolite expression and antioxidant activity through the downstream signaling molecule HO-1[41].Previous studies have reported that autophagy and ERS are connected in neuro degenerative disorders through the PERK-eIF2α signaling pathway[42], and Bcl-2 blocks endoplasmic reticulum stress by inhibiting autophagy
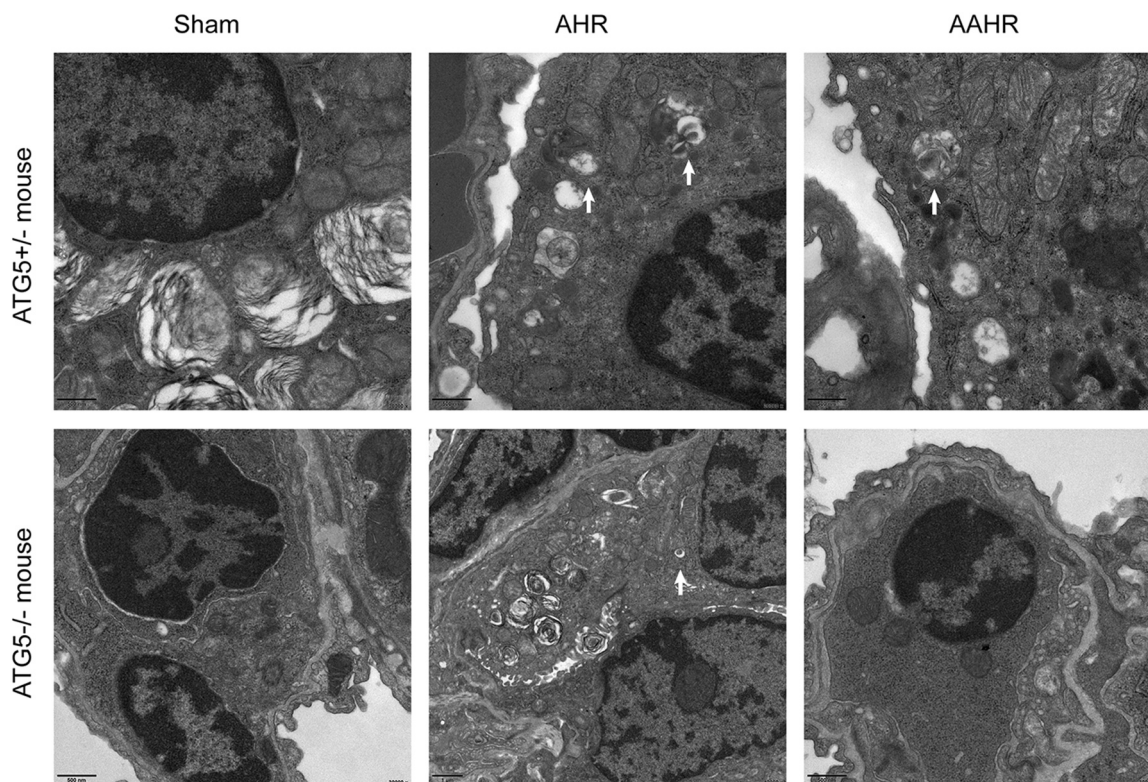
	Sham	AHR	AAHR

Fig. 7. Representative autophagosomes(white arrows)detected by transmission electron microscopy. Scale bar,500 nm.

and promoting the formation of protein complexes[43].Similarly, we found that ERS markers were highly expressed in OVA-induced mice and decreased after acupuncture treatment, and these changes were positively correlated with autophagy markers.

To further examine the effects of autophagy on ERS,we compared the protein and mRNA levels in mice with ATG5 deficiency. The results showed that ERS marker proteins (p-PERK,p-IRE-1,ATF6 and Grp78) were significantly reduced in ATG5^{-/-} mice compared with ATG5^{+/-} mice, while the corresponding mRNAs showed no significant differences, suggesting that acupuncture further inhibits the expression of ER stress proteins by suppressing ATG5-mediated autophagy.It is worth noting that total ERS marker protein changes cannot precisely reflect ERS levels,as UPR is mediated by three different signaling pathways that start with the activation of protein sensors on the membrane.Further analysis of proteins in different locations is necessary in future research.

Immune responses in asthma have attracted increasing attention in the last decades. Research suggests that T cell responses are crucial factors in the outcomes of AHR and inflammation in asthma as they establish a balance between immunopathology and allergenicity [44]. The change of T lymphocyte subsets, represented by the balance of Th1/Th2 and Th17/Treg, plays a critical role.Promoting the restoration of Th1/Th2 balance by enhancing Th1 cells and inhibiting Th2 cell differentiation can contribute to the prevention and treatment of asthma [45,46].In this study,there were decreased Th1 and increased Th2 rates in spleen cells from model animals compared with the sham group, which could be partially alleviated by acupuncture treatment. Similarly, the imbalance of Treg/Th17 could also lead to airway hyperresponsiveness in asthma. Tregs enhance immune tolerance, regulating abnormal inflammatory response by stimulating neutrophil chemotaxis to establish a protective CD4^{+}T cell immune response [47].We also detected an imbalance of Treg/Th17 in asthmatic mice that could be alleviated by acupuncture,demonstrating that the balance of CD4^{+}T cell subgroups is involved in the treatment process of acupuncture treatment for airway hyperresponsiveness in asthma.However, cell type distributions within the lung or other immune organs,such as the lymph gland

and thymus,indifferent mouse groups were not analyzed in the present research for comprehensively assessing the impact of acupuncture on asthma development.The potential mechanism still requires a great deal of follow-up work.

The degree of inflammatory cell infiltration in the airway and the levels of inflammatory cytokines can reflect the extent of inflammation in asthma.According to H&E staining results for the lung tissue in this study, inflammatory cell infiltration in the airway was obvious in the model group, which was relieved after acupuncture treatment.Correspondingly, the inflammatory cytokines IL-4,IL-17 and TGF-βwere elevated in the model group.TGF-βis a representative factor secreted by Treg cells.Previous evidence shows that smooth muscle cells promote fibroproliferative airway remodeling in asthma, and TGF-β1 is a key inductive signal[48]. TGF-βalso plays an important role in structural changes that promote airway remodeling in asthma[49].Th17 cells mainly play a role by secreting the effector cytokine IL-17. Studies have shown that IL-17 is positively correlated with asthma severity in clinical and animal studies, and involved in the activation of Th2 cells[50,51]. Similarly,IFN-γ and IL-4 are representative factors secreted by Th1 and Th2 cells, reflecting inflammation levels in asthma. Taken together, the corresponding inflammatory factor levels were also consistent with the change of CD4^{+} T lymphocyte subset balance.

As one of the key genes of autophagy, ATG5 is functionally associated with asthma and improves promoter activity.Evidence shows that ATG5 deficiency results in progressive liver inflammation, fibrosis, and tumorigenesis of liver cancer[52].A recent study showed that miR-30a suppresses airway fibrosis and autophagy by targeting ATG5 in asthma.However, the function of ATG5 in asthma is not fully understood [53].The above data suggest that acupuncture may play a therapeutic role by regulating ATG5-mediated autophagy to reduce asthma-related inflammation and airway hyperresponsiveness, as well as endoplasmic reticulum stress and T lymphocyte homeostasis.These results may help further explore and evaluate ATG5 targeting agents or acupuncture for asthma therapy.

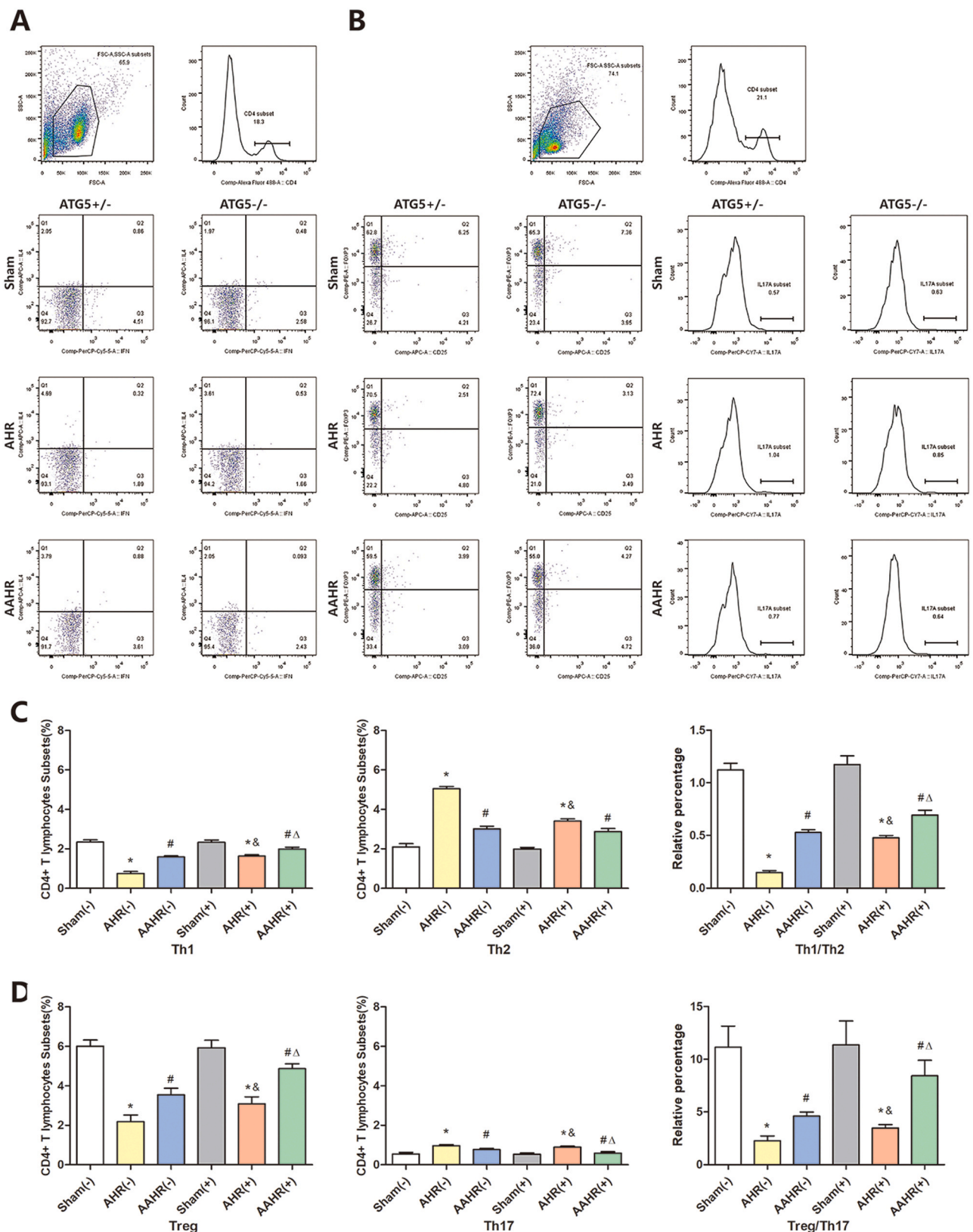Recent studies have suggested that acupuncture plays a therapeutic

Fig. 8. Effects of acupuncture and ATG5 gene knockout on the Treg, Th17, Th1 and Th2 subsets of CD4 + T lymphocytes. Dot plot analyses of anti-CD4,anti-IL-4 and anti-IFN-γ staining forTh1 and Th2 subsets(A). Dot plot analyses of anti-CD4,anti-CD25,anti-Foxp3 and anti-IL-17 staining for Treg and Th17 subsets(B).Percentages of Th1 and Th2 subgroups and Th1/Th2 ratios(C). Percentages of Treg and Th17 subgroups and Treg/Th17ratios(D).Data are mean±SD, n = 6 per group.*P < 0.05 vs Sham group.#P < 0.05 vs AHR group.&P < 0.05 vs AHR(-) group. ΔP < 0.05 vs AAHR(-) group.
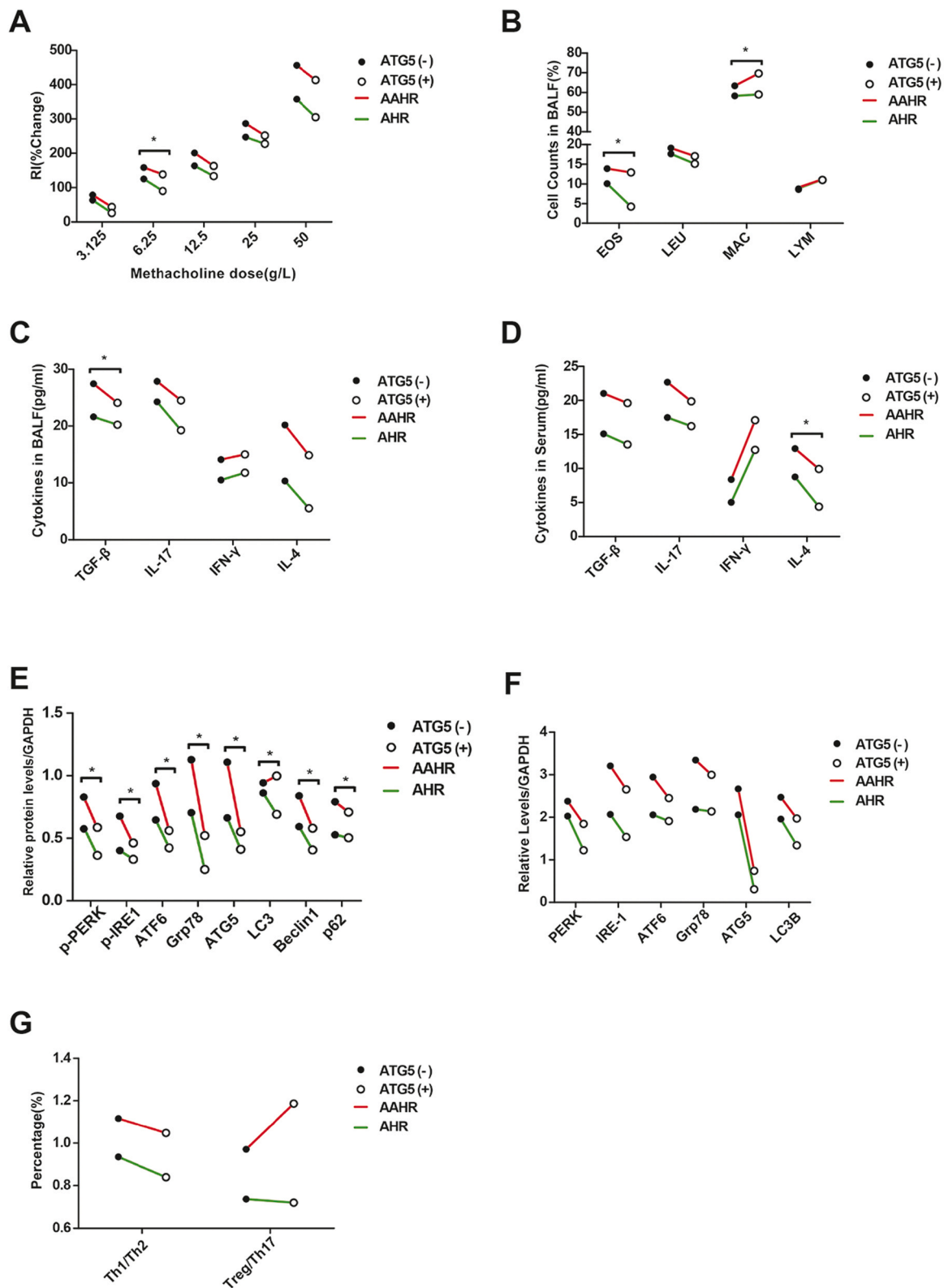
Fig. 9. Two-factor analysis of the effects of ATG5 and acupuncture on various factors.
AHR(A), cell counts in BALF(B),inflammatory cytokines in BALF(C), inflammatory cytokines in serum(D),protein levels(E), mRNA levels(F) and proportions of CD4 + T lymphocyte subsets(G) were examined. Data are mean ± SD, n = 6 per group.*P < 0.05 Acupuncture vs. ATG5.

role through the complex "neuro-endocrine-immunological" network [54],and improves the quality of life of asthmatic patients. However, the mechanism of acupuncture has not been clarified.Our data corroborated a previous research suggesting that Th1/Th2 and Treg/Th17 balance can be adjusted by acupuncture treatment.In addition,acupuncture treatment significantly decreased inflammation and AHR in OVA-induced mice as shown above.

With advances in novel biological therapies, biologic medications,

including anti-IgE, anti-IL-5 and anti-IL4/IL13, can help relieve asthma symptoms in patients with failed conventional therapy. Further assessment of the cellular and functional relevance of autophagy in asthma would facilitate clinical translation of laboratory findings. Studies have shown that acupuncture regulates Beclin-1 to inhibit autophagy[55] and controls ERS by downregulating GRP78 to interfere with PERK and IRE-1 signaling[56,57]. Here, we also found that acupuncture affected endoplasmic reticulum stress by regulating ATG5-mediated autophagy, and regulated the balance of T lymphocyte subsets, with a positive correlation. These data indicate that autophagy regulation via ATG5 is crucial for inflammatory and immune environments in asthma. It should be noted that our results did not clearly disclose the ERS or autophagy-related molecular pathways involved in acupuncture treatment of asthma. Due to the complex interactions between immune cells, further evidences for a molecular link between acupuncture and autophagy is needed in future in vitro studies.

In summary, this study firstly demonstrated that acupuncture inhibits ATG5-mediated autophagy to regulate endoplasmic reticulum stress and CD4$^+$ T lymphocyte differentiation,thereby reducing asthma airway inflammation and AHR.

CRediT authorship contribution statement

Wuhua Ma: Conceptualization, Methodology. **Yong Wang:** Verification, Methodology. **Huanyi Zhao:** Provision of study materials, Investigation, Data curation, Writing – original draft. **Fang Dong:** Provision of study materials, Investigation. **Yuhui Li:** Supervision. **Xiaojie Ren:** Provision of study samples. **Zhengyuan Xia:** Writing – review & editing.

Conflict of interest statement

None of the material related to this manuscript has been published or is under consideration for publication elsewhere, including the internet. The authors declare that they have no conflicts of interest.

Acknowledgements

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112045.

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