



Ambient particulate matter-associated autophagy alleviates pulmonary inflammation induced by *Platanus* pollen protein 3 (Pla3)

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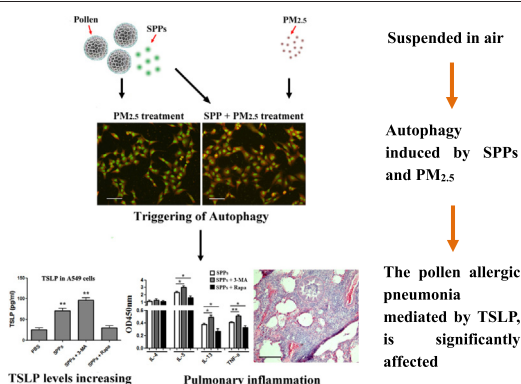
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HIGHLIGHTS

- Subpollen particles (SPPs) promote ROS generation and increase TSLP levels.
- Autophagy induced by PM_{2.5}, suppressed ROS and TSLP levels, leading to the alleviation of inflammation.

GRAPHICAL ABSTRACT



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ABSTRACT

Subpollen particles (SPPs) with diameter less than 1 mm released from allergenic pollen grains contain allergens could trigger asthma and lung inflammation after being inhaled. In the meaning time, ambient fine particles attached on the pollen grains could have further effects on the inflammation. However, the mechanisms underlying these phenomena have not been fully elucidated. In this study, the effects of autophagy triggered by PM_{2.5} and *Platanus* SPPs were evaluated by using the A549 cell lines and a pollen sensitized rat model. First, autophagy in A549 cells was analyzed after exposure to PM_{2.5} using acridine orange staining, real-time quantitative PCR (qRT-PCR), and western blot (WB) assays. The increased levels of ROS, superoxide dismutase, and malonaldehyde in the lung homogenates of rats exposed to SPPs indicated that inflammatory response was triggered in the lungs. Treatment with autophagy-inhibiting drugs showed that autophagy suppressed ROS formation and decreased the production of thymic stromal lymphopoietin (TSLP), a critical pathway altering the inflammatory response. Although the effect was indirect, autophagy appeared to negatively regulate TSLP levels, resulting in a compromised immune response. These results suggested that SPPs promote ROS generation and increase TSLP levels, triggering downstream inflammation reactions. However, ambient PM_{2.5} could aggravate autophagy, which in turn effectively suppressed ROS and TSLP levels, leading to the alleviation of the immune response and pulmonary inflammation.

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Abbreviations: AL, avoiding light; AO, acridine orange; DPI, diphenyleneiodonium; FBS, fetal bovine serum; 3-MA, 3-methyladenine; MDA, malonaldehyde; NAC, N-acetyl-L-cysteine; NBT, nitroblue tetrazolium; PNC, penicillin; Rapa, rapamycin; ROS, reactive oxygen species; SM, streptomycin; SOD, superoxide dismutase; SPPs, subpollen particles; TMB, tetramethylbenzidine; TSLP, thymic stromal lymphopoietin.

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1. Introduction

Ambient particulate matter (PM) has become a major pollutant in the urban atmosphere. Pollen allergen, one of the biological component of PMs, contributes to several health problems, such as asthma, pollinosis, and allergic rhinitis (Butres et al., 2015; Humbal et al., 2018; Fernandez-Gonzalez et al., 2019). There are tree pollen in spring, herbaceous pollen in summer, and ragweed pollen in autumn. Previous studies have identified and isolated abundant pollen allergens, including Birch allergen Bet v 1, Japanese cedar allergen Cry j1 and Crj2, and *Humulus japonicus* allergen Hum j1. *Platanus acerifolia* is a common tree species in urban areas in cities of the world (Asturias et al., 2002; Lu et al., 2014), and its airborne pollen has become a major allergy factor during bloom in spring. *Platanus* pollen contains three main allergen proteins, Pla a1, Pla a2, and Pla a3 (Liu et al., 2015; Ni et al., 2017a; Wang et al., 2018), which induce allergic reactions in humans. The purified allergens can also be used to prepare polyclonal and monoclonal antibodies by immunizing animals (Ni et al., 2017b; Zhou et al., 2019). Notably, the pollen allergens, found in subpollen particles (SPPs) (0.1–1 μm) can be released under conditions of relatively high humidity, and the SPPs are transported to the deep lung through the respiratory tract (Yan et al., 2019). We previously reported that the mass concentration of Pla 3 in the Shanghai atmosphere increased during the flowering season, especially after heavy rainfall, which correlated with the high prevalence of atopic individuals admitted to hospitals (Zhou et al., 2019). The SPPs released from pollen not only contain allergens but also NAD(P)H oxidase and NADH dehydrogenases (Bacsi et al., 2006; Wang et al., 2009; Smiljanic et al., 2017), which can stimulate reactive oxygen species (ROS) generation in cells. Furthermore, some studies have reported that the ROS produced by NAD(P)H oxidase induce complete allergic inflammation in mice (Bacsi et al., 2006; Dharajiya et al., 2008). ROS, as signaling molecules, regulate the expression of apoptotic genes by activating the MAPK signaling pathway, and cause organelle damage via oxidative stress (Zakki et al., 2018; Rashmi et al., 2019). ROS initiate cell defense mechanisms against foreign body invasion when cells are subjected to oxidative stress. Conversely, cell death is accelerated by severe oxidative damage (Hu et al., 2015; Redza-Dutordoir and Averill-Bates, 2016; Rendra et al., 2018). Furthermore, investigation of the oxidative stress signaling pathway has revealed that ROS modulation directly affects the level of thymic stromal lymphopoietin (TSLP), a critical pathway transition hub, altering the inflammatory response (Tang et al., 2010).

TSLP is a cytokine produced and secreted by epithelial cells, fibroblasts, and mast cells. It can stimulate dendritic cells (DCs) to promote the differentiation, maturation, and migration of DCs, as well as the proliferation and maturation of Th2 cells, regulating the body's immune balance (Pandey et al., 2000). The increase in TSLP levels could promote the production and secretion of inflammatory factors, and the transformation of B2 cells into IgE-secreting plasmacytes, activating type I hypersensitivity (Huang et al., 2019). Pollen, acting as a functional Toll-like receptor 4 (TLR4) agonist, initiates TSLP signaling, which triggers Th2-dominant allergic inflammation (Li et al., 2011). Thus, TSLP is also considered a potential molecular marker for allergic disease diagnosis and assessment (Zhu et al., 2009).

Generally, cells need to regulate endogenous ROS levels to achieve physiological balance, of which autophagy is a universal regulator (Hensley and Harris-Whit, 2015; Rezabakhsh et al., 2017). Autophagy is a process involving the lysosomal degradation and recycling of cellular proteins and cytoplasmic organelles. Autophagy is initiated at membrane structures called phagophores, which engulf cellular and cytoplasmic components, followed by the elongation and recruitment of microtubule-associated protein light chain 3 (LC3) to form the characteristic double membrane autophagosome. Endogenous LC3 is detected as two bands following SDS-PAGE and immunoblotting: one represents LC3-I, which is cytosolic, and the other LC3-II, which is conjugated with phosphatidylethanolamine and is present on isolation

membranes and autophagosomes. The amount of LC3-II or the LC3-II/LC3-I ratio can be used to estimate the degree of autophagosome formation. High levels of autophagy can effectively eliminate excessive ROS in cells, while autophagy inhibition leads to ROS accumulation (Sotthibundhu et al., 2018; Tu et al., 2019). The autophagy pathway occurs in many distinct stages, which are tightly regulated by several autophagy-related (Atg) proteins, including the unc-like kinase 1 (Ulk1)/Atg 1 complex, ATG3, ATG5, and ATG7.

Ambient particles could induce autophagy in cells (Park et al., 2018; Miao et al., 2019), and cellular autophagy has a dual effect, promoting apoptosis and protecting cells. Considering that air pollutants can alter the physicochemical characterization of pollen allergens and increase their allergenicity (Sousa et al., 2012; Hong et al., 2018), ambient particles might have affected allergenicity of pollen protein, which may manifest through the autophagy. We found that allergenicity of pollen protein was weaker in highly polluted area than in an area with low degree of pollution, suggesting that the biological effect of air pollutants on pollen allergenicity was not positively correlated with pollutant mass concentration. Therefore, we designed an experiment and examined the relationship between autophagy and pollen allergenicity using animal model and A549 cells. Our results could help in the elucidation of pollen allergy mechanisms and in finding effective ways to prevent and treat pollen allergy disease.

2. Materials and methods

2.1. PM_{2.5} collection and preparation PM_{2.5} solution

The PM_{2.5} sampling was carried out on the rooftop of a building which was about 15 m above the ground level (31°19'00.01"N, 121°23'23.25"E) (Fig. S1) during spring season of 2017. The ambient particles were collected on pre-baked 8" × 10" quartz filters (Pallflex Tissuquartz, Pall Life Sciences) using a high-volume sampler (TE-6001-2.5 I, Tisch Environmental Inc., Ohio 45,002 USA; nominal flow rate 1.13 m³/min, PM_{2.5} impactor). After collecting the samples, the filters were immediately wrapped with clean aluminum foil in sealed plastic bags and kept frozen (at −4 °C) until further analysis. Detailed protocol for obtaining PM_{2.5} water soluble fraction is described in Lu et al. (2019). Briefly, five punches (12.56 cm²/punch) of quartz filter (20.3 × 25.4 cm) were extracted with ultrapure water (resistivity >18 M Ω cm^{−1}, 3 cm² per 1 ml) in an ultrasonic bath for 30 min; the extraction was repeated twice. The aqueous extracts were filtered through a syringe PTFE filter with a 0.22 μm pore size (Millex GV 13 mm) to avoid any interference from residual quartz filters.

2.2. Pollen collection and sub-pollen particle extraction

Platanus pollen was collected from mature inflorescence from three sampling sites in Shanghai (Songjiang, Shangda Road and Wu Song) in the spring season of 2017 and filtered with 40 μm mesh before storage at 4 °C, as previously described (Zhou et al., 2019). The pollen material used in the cell- and animal-based experiments was washed 5–6 times with ddH₂O to remove the contaminants adsorbed on the pollen surface.

0.2 g *Platanus* pollen was soaked in 5 ml of ddH₂O. Six hours later, the pollen suspension was centrifuged at 1600g for 5 min. Then, the supernatant was centrifuged at 13,000g for 15 min at 4 °C. Finally, the precipitate was dissolved, and the amount of SPP protein was quantified using the Pierce™ BCA Protein Assay kit (Thermo Fisher, America).

2.3. Pla a3 protein extracted from *Platanus* pollen grains

Pollen sample (0.2 g) was crushed by grinding. Then the total protein was extracted using ProteoPrep® Total Extraction Sample Kit (Sigma-Aldrich). Pla a3 protein concentration was quantified according to the standard curve of the protein concentration by ELISA using the

anti-Pla a3 antibody (NB biolab. China). Moreover, after sufficient lysis of the SPPs extracted from 0.2 g pollen sample, the total protein was extracted and Pla a3 concentration was quantified as explained above.

2.4. A549 cell culture

Human pulmonary epithelial cell line A549 was obtained from Cell Bank of Chinese Academy of Sciences and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/ml penicillin (NC) and 0.1 mg/ml streptomycin (SM) in a humidified atmosphere of 5% CO₂ at 37 °C.

2.5. Establishment of pollen-sensitized animal model

Pollen-sensitized animal model was established following the procedure shown in Fig. S2. Three independent experiments were performed. SD rats were divided into different treatment groups, with 5 rats in each group. The Pla a3 CDS was cloned from *Platanus* pollen, and Pla a3 protein was expressed and purified using a prokaryotic expression system, as previously described (Zhou et al., 2019). Two milligrams of Pla a3 protein was dissolved in 5 ml of Freund's complete adjuvant and injected into SD rats from tail and limbs (once a week). Subsequent reinforcing inoculation was performed with Pla a3 protein dissolved in Freund's incomplete adjuvant, once a week. After 2 weeks, rats received an atomization treatment challenge with SPPs every day for a week (each time for 30 min), according to the procedure described previously (Hong et al., 2018). PBS (pH 7.0) was used as the blank control and 0.5% NaCl as the negative control. Finally, the rats were deeply anesthetized and sacrificed, and the blood and lungs were obtained for subsequent detection and analysis.

Serum was isolated and was placed at room temperature for 5 min and then in a waterbath at 37 °C for 15 min. Serum was then centrifuged at 3000 rpm for 15 min. Then, an equal volume of PBS (with glycerol, v: v = 1:1) was added to the supernatant and the sample was stored at -20 °C. The tip of the left lung was cut off for pathological examination, and the rest of the lung was preserved for the detection of physiological parameters.

2.6. In vitro fluorescence labeling and ROS quantification caused by PM_{2.5}

Cells were loaded with 50 μM 2',7'-dihydro-dichlorofluorescein diacetate (H₂DCF-DA, Molecular Probes, 0.5 mM) and incubated for 15 min at 37 °C AL. After removing any excess probes, the cells were exposed to different treatments for 2 h and washed twice with PBS (pH 7.0). Then, the images were obtained under a fluorescence microscope (Leica DM2500, Germany). For semi-quantification of the fluorescence intensity, five images were randomly selected, and the intensity was measured and normalized by the SMART software.

2.7. Detection of autophagy induced by PM_{2.5} and SPPs by using of acridine orange staining assay

After the cells were exposed to different treatments (PM_{2.5}, SPPs) for 2 h at 37 °C AL. The cells were washed with 1× PBS twice and then incubated for 15 min at room temperature for 1 μg/ml AO (A6014, Sigma) staining. Then, the cells were washed with 1× PBS twice, observed, and photographed under a fluorescence microscope (Leica DM2500, German).

2.8. Detection of ATG transcription by quantitative real-time PCR

To quantify the transcription of the autophagy-associated genes, A549 cells were collected after exposure to different samples, and the total RNA was extracted according to a method described by Zhou et al. (2014). Then, reverse transcription was performed according to instructions of the Prime Script 1st Strand cDNA Synthesis Kit (Treasure

Table 1

Primers used to detect transcription of autophagy-associated genes by qRT-PCR.

Name	Forward primer	Reverse primer
ATG5	AGCAACTCTGGATGGGATTG	AGGTCCTTCAGTCGTTGTCTG
ATG7	GCTACTTCTGCAATGATGTGG	AGATACCATCAATTCACGGC
ULK	AATGGCTAATCTGTCTACCTGG	GAAGAGCCTGATGGTGTC
Bac1in	GGACAATCAATAACTTCAGGC	TTATTGGCCAGAGCATGGAG
Actin-β	ACCTTCTACAATGAGCTGCG	CCTGGATAGCAACGTACATGG

Bioengineering). qRT-PCR was performed using the SYBR Green PCR Master Mix Kit (TaKaRa Bio Co. Ltd, China). Actin-β from A549 cells was used as an internal control for calculating the relative expression levels of the target gene. The primers used in the reactions are listed in Table 1.

2.9. Detection of autophagy marker protein (LC3II) in A549 cells

The proteins extracted from A549 cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), before transferring onto the NC membrane. After sealing with 1% BSA for 1 h, the membrane was incubated with 1:200 diluted rat sera at 37 °C for 1 h. Then, the membrane was washed with TBST buffer (pH 7.4) 3 times and 1:5000 diluted HRP-labeled anti-IgG/IgE antibody was added (YESEN Biotechnology Co. Ltd, China), followed by incubation at 37 °C for 1 h AL. Finally, the washed membrane and TMB were co-incubated at 37 °C for 30 min. The gray value of the protein bands was analyzed by Image J software and the histogram drawn using GraphPad Prism5 software.

2.10. Detection of serum antibody level in rats serum by ELISA

Two hundred microliters of serum samples were incubated in 96-well plates for 12 h at 4 °C, avoiding light (AL). After rinsing 3 times with PBS, the immunological reaction was obtained by adding 100 μl horseradish peroxidase (HRP)-labeled rabbit anti-IgG/IgE antibody (1:10,000 dilution) into each coated well at 37 °C for 2 h AL. After PBS washing, the color reaction was carried out with 100 μl freshly concocted tetramethylbenzidine (TMB) substrate solution and terminated with 50 μl 2 M sulfate. Then, OD was detected at 450 nm, and the data were statistically analyzed using SARS software.

2.11. Inflammation factors detection in rat lung tissue

0.2 mg lung tissue was fully ground in 2 ml extraction reagent, and centrifuged at 12,000g for 5 min at 4 °C. Then, the supernatant was collected and the inflammatory factors and TSLP levels were detected using corresponding detection kits, e.g., INF-α, IL-4, IL-5, IL-13 and TSLP assay kits (Nanjing Jiancheng Bioengineering Institute, China). The experiment was performed according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute., Inc.).

2.12. Statistical analysis

Data from at least three independent experiments were analyzed and expressed as mean ± standard error (SD). Comparisons between the experimental groups were performed using the paired Student t-test and ANOVA with SPSS version 11.5. A value of *p < 0.05 and **p < 0.01 were considered statistically significant.

3. Results

3.1. Mass level of air pollutants in the atmosphere of Shanghai different areas

Mass level of PM_{2.5} in SD, SJ and in WS was 45.45 ± 24.60, 46.07 ± 25.60 and 52.04 ± 26.38 μg/m³ respectively during the sampling time

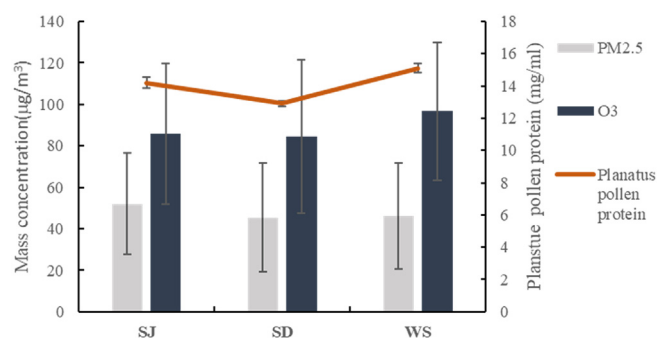


Fig. 1. Mass concentration of PM_{2.5} and total protein in *Platanus* pollen grains collected in different areas.

(Fig. 1). The mass concentrations of the PM₁₀, NO₂ had no significant difference at the three different sampling sites. However, mass level of O₃ and SO₂ in WS sampling site was higher than that at the other two sampling sites (Table 2). The total protein extracted from *Platanus* pollen grains collected in WS (15,100 ± 300 µg/mL) was higher than that in the other two sampling sites (SD, 12900 ± 360 µg/mL; SJ 14200 ± 173 µg/mL). It might suggest that an increase in environmental pollutants might lead to an increase in pollen total protein levels.

3.2. Pla a3 distribution in the *Platanus* pollen grains collected from different areas

To further clarify the characteristics of allergens in pollen, western blot assay was used to detect the Pla a3 allergens in the *Platanus* pollen. Our data clearly showed that the mass level of the Pla a3 protein in the pollen sampled from WS was the highest (Fig. 2a), and followed by SD, SJ samples. It was noted that the Pla3 protein in the SPPs released from different pollen grains collected from different areas followed a similar trend (Fig. 2b). These results indicated that the amount of Pla a3 allergen protein in the pollen grains and the amount of SPPs increased with the mass level of air pollutants. Pla a3 protein is a non-specific lipid transfer protein, so considering its biological function, the changing feature of Pla a3 protein might be closely related to the functions in plant defense (Schmitt et al., 2018).

Table 2
Analysis of air pollutants from different sampling points in Shanghai during spring season of 2017.

Place	Mass conc. (µg/m ³)					Protein (µg/mL)
	PM _{2.5}	PM ₁₀	O ₃	SO ₂	NO ₂	
SJ	52.04 ± 26.38	77.71 ± 34.48	85.96 ± 37.01	16.83 ± 5.90	47.21 ± 23.48	14,200 ± 173
WS	46.07 ± 25.60	84.82 ± 43.80	96.60 ± 33.20	22.65 ± 12.60	45.81 ± 25.50	15,100 ± 300
SD	45.45 ± 24.60	78.75 ± 33.96	84.29 ± 33.87	11.75 ± 4.39	46.54 ± 25.47	12,900 ± 360

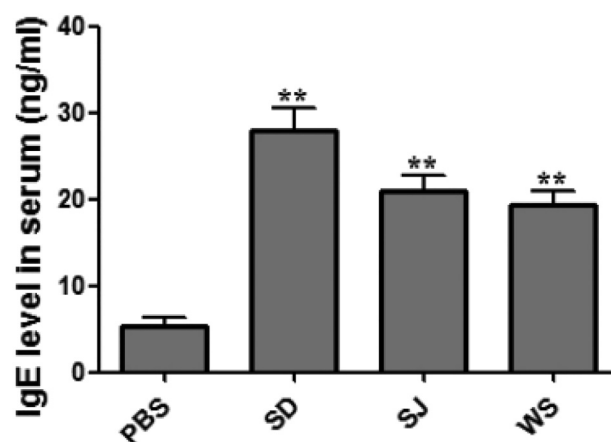


Fig. 3. Quantitative detection of IgE in serum of immunized rats by ELISA, **p < 0.01.

3.3. IgE levels in rat serum after exposure to *Platanus* SPPs

Our previous studies have shown that pollen-releasing SPPs can be inhaled into the body and deep into the lungs causing IgE increase (Hong et al., 2018; Yan et al., 2019). The IgE level variety induced by the treatment of extracted SPPs in pollen from different regions was analyzed. Our data showed that the level of IgE protein in the serum of rats immunized by *Platanus* pollen extracts decreased following order: SJ > SD > WS (Fig. 3). This phenomenon might imply that allergic reactions in the mice associated other physiological reactions besides immunity reaction. The multiple pathways might lead to the weakest allergic reaction even if the pollen and SPPs in WS samples with high allergen content.

3.4. Autophagy in A549 cells caused by PM_{2.5} and SPPs

Autophagy is a protective physiological response of organisms at the cellular level. Our data showed that autophagy could be found after A549 cells exposed to the SPP, PM_{2.5}, SPP + PM_{2.5} (Fig. 4a). The expression levels of autophagy related genes were detected by qRT-PCR. The results showed that expression of ATG5, ATG7, BACLIN and ULK1 varied with the different samples. The transcription levels of ATG5 and ATG7

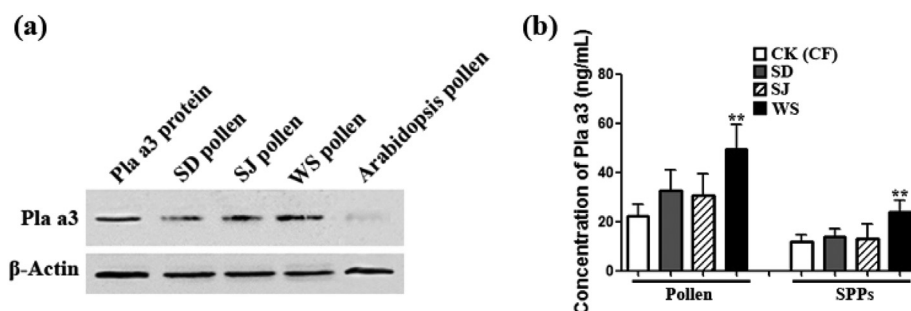


Fig. 2. Analysis of Pla a3 protein concentration in pollen collected from different sampling sites. (a) Pla a3 allergenic protein in pollen grains collected from different areas; (b) quantitative detection of Pla a3 allergen in *Platanus* pollen collected from different areas. CK = Arabidopsis pollen-control, **p < 0.01.

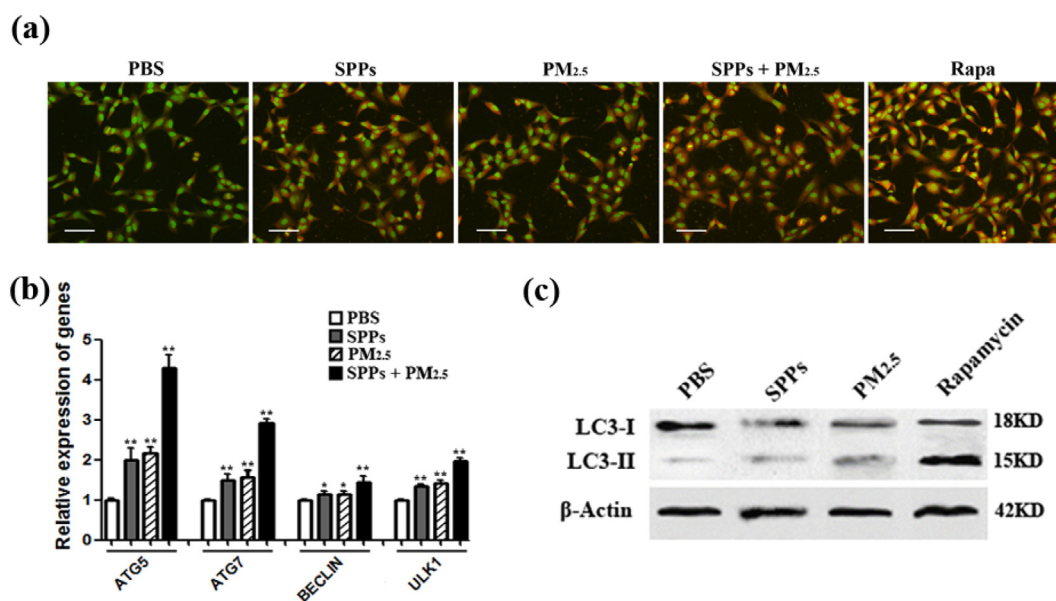


Fig. 4. Autophagy induced by PM_{2.5} in A549 cells. (a) Autophagy in the A549 cells induced by different samples, (b) transcription levels of different genes in the A549 cell; (c) distribution of autophagy marker protein LC3-II/LC3-I after the A549 cell treated with PM_{2.5}, SPPs and autophagy modulator (Rapamycin) (*p < 0.05, **p < 0.01).

in the cells treated with WS samples were significantly higher than that of the other samples (Fig. 4b). In addition, the results of the western blot indicated increased expression of the autophagy marker protein LC3-II and a significant increase in the ratio of LC3-II/I (Fig. 4c) after the cells were exposed to PM_{2.5}, suggesting that the ambient particles and SPPs could induce the increase of autophagy in A549 cells.

Autophagy induced by the SPPs was confirmed by using of autophagy modulator, 3-MA and Rapa (Fig. 5a, b). When the autophagy was inhibited by 3-MA or activated by Rapamycin, variety of autophagy induced by the SPPs could be found. The value of LC3-II/LC3-I increased significantly when the autophagy was activated by the Rapamycin (Fig. 5b). Synthesizing the above results, it shows that in addition to PM_{2.5} triggering autophagy, SPPs triggers the body's allergic reaction as well as autophagy.

3.5. Autophagy regulated the level of ROS and TSLP and further alleviated pulmonary inflammation

After incubation with SPPs for 20 min, ROS could be detected in the A549 cells. The fluorescent staining of ROS showed that the SPP-treated group cells showed green fluorescence, and the fluorescence intensity was between the positive and negative control group (Fig. S3a and b). The ROS level in A549 cells increased rapidly in a short time in both Rosup- (a stimulator triggering the production of ROS) and SPP-treated groups. After 12 min, ROS levels gradually stabilized, and

20 min later, ROS levels decreased (Fig. S3c). In addition, both *in vivo* and *in vitro* tests showed that SPPs stimulation resulted in the increase of TSLP and IgG/IgE in sensitized rats and A549 cells (Fig. S3d and e). These results suggest that SPPs could stimulate cells to produce ROS, TSLP and induce significant immune responses in the organism.

When DPI/NAC (ROS inhibitors) was used to inhibit ROS, the levels of TSLP and inflammatory factors were analyzed. The results of the fluorescence labeling analysis showed that the SPPs could induce high levels of ROS, but upon co-treatment with DPI, the ROS levels decreased (Fig. 6A and B). Simultaneously, the results of ELISA showed that the TSLP levels in A549 cells exhibit the same pattern (Fig. 6C). Results of the *in vivo* experiments also demonstrated that the levels of SOD and MDA increased in lungs treated with SPPs; however, upon co-treatment with NAC, the levels of SOD and MDA decreased markedly (Fig. 6D). In addition, the levels of TSLP and inflammatory factors in the lavage fluid of the rat lung increased, and resulted in significant inflammatory reactions (Fig. 6E, F and G-a, -b). When NAC was added to the treatment regimen—due to its synergistic effect—inflammation was greatly relieved (Fig. 6E, F and G-c, -d). The above results suggest that the intracellular ROS is a key upstream signal for inducing TSLP levels and inflammation.

When the autophagy inhibitor, 3-MA, was used to treat A549 cells, the green fluorescence associated with ROS was enhanced, but upon co-treatment with SPPs and rapamycin, the fluorescence signal associated with ROS decreased (Fig. S4A and B). However, both SOD and

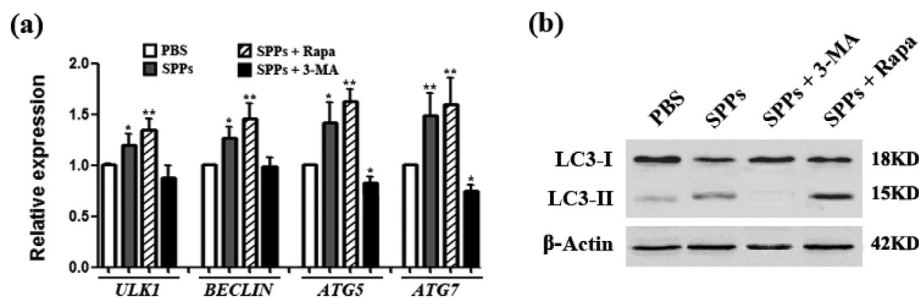
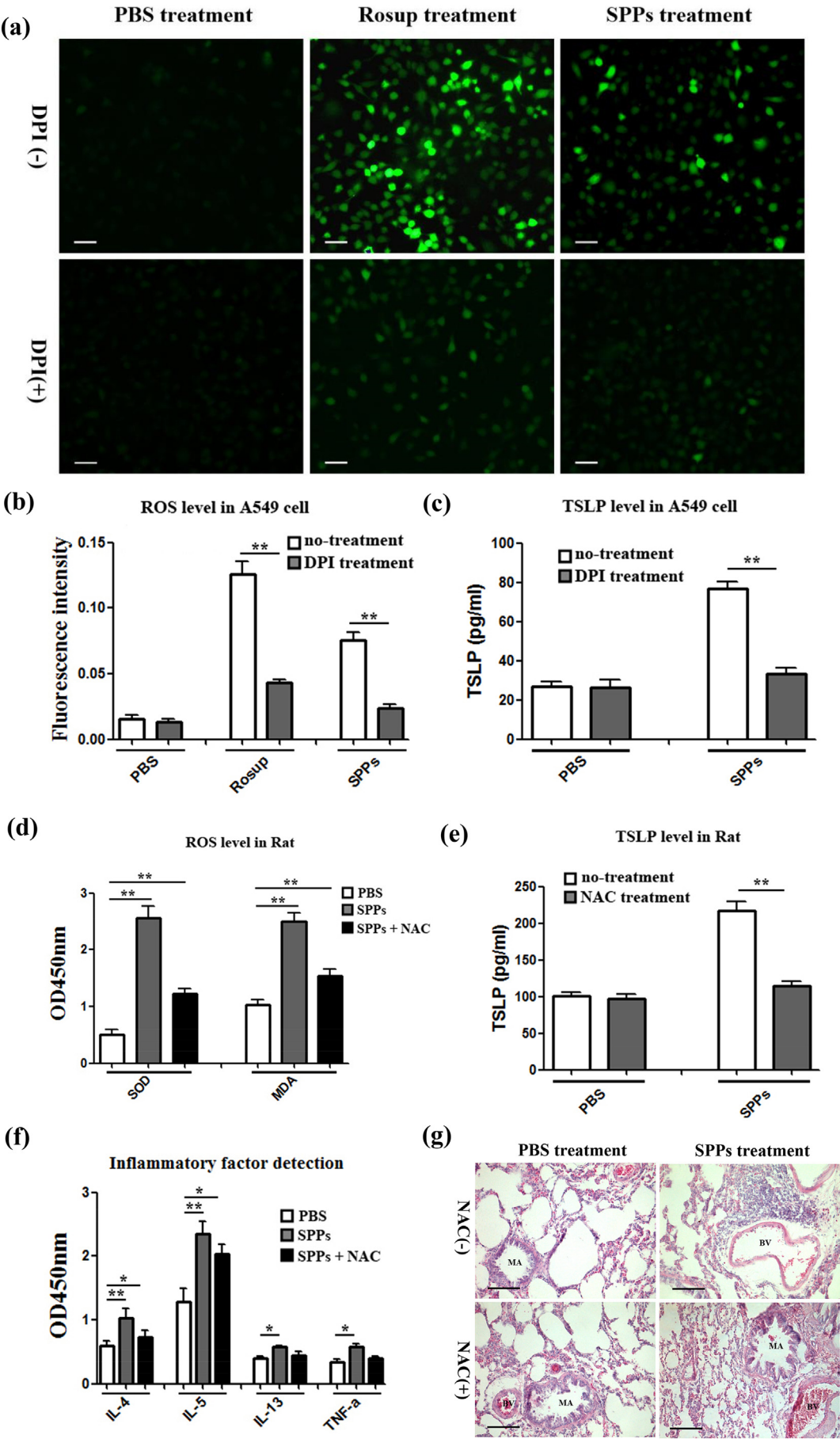


Fig. 5. (a) Autophagy production induced by SPPs in A549 cells. Transcription levels of different genes in the cell; (b) autophagy marker protein LC3-II/LC3-I after the A549 cell treated with SPPs and modulating autophagy modulator (3-MA and Rapa).



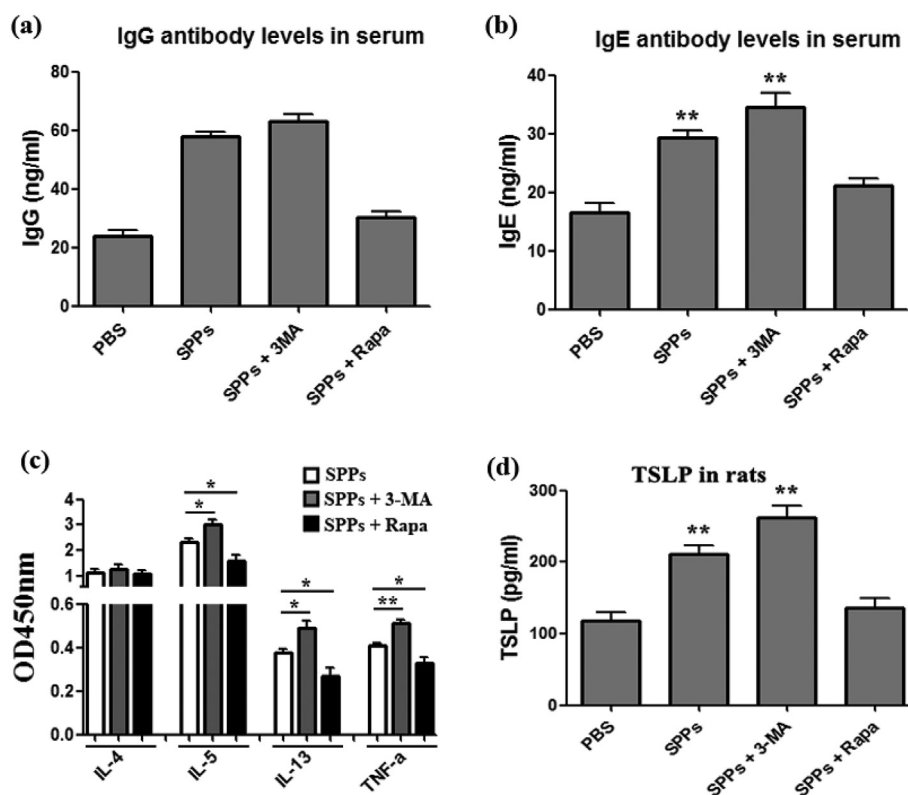


Fig. 7. Autophagy alleviates inflammation reaction. (a) Serum IgG levels in rats from different treatment groups. (b) Serum IgE levels in rats from different treatment groups. (c) variety of inflammation factors in rats when autophagy inhibited and stimulated. (d) Levels of TSLP produced in rats when autophagy inhibited and stimulated. The data were obtained from five independent samples of each group and repeated three times (* $p < 0.05$, ** $p < 0.01$).

MDA levels in the lung increased after the autophagy was inhibited. Furthermore, when autophagy was pre-stimulated, the levels of SOD and MDA in the rat lung were significantly decreased (Fig. 4c). These results suggest that autophagy could play a role in the elimination of excess ROS and the maintenance of the normal function of cells.

After modulating autophagy using 3-MA and Rapa, the TSLP, IgG and IgE antibody levels in the serum were analyzed. The results showed that when autophagy was inhibited by 3-MA, the levels of IgG and IgE were significantly higher than that in the no treatment group. However, after high levels of autophagy were induced by Rapa, the IgG and IgE levels in sensitized rats were significantly lower than in those in the without Rapa group (Fig. 7a and b). The levels of inflammatory cytokines, IL-5, IL-13, and TNF- α , in the lung tissue fluid of rats treated with Rapa decreased to varying degrees. However, the 3-MA treatment group showed increased level of these cytokines (Fig. 7c). However, various treatments showed no effect on the level of IL-4 cytokines.

As TSLP is known to have a positive effect on the inflammatory response (Li et al., 2011), the levels of TSLP in sensitized rats were analyzed. The results showed the TSLP level in the SPP-treated group increased substantially compared to that in the control group. When

autophagy was inhibited by treatment with 3-MA, the levels of TSLP increased further. However, when autophagy was pre-stimulated using rapamycin, the TSLP levels decreased significantly and were similar to those in the control group (Fig. 7d).

Further analysis of the inflammatory reaction occurring in pathological sections of the rat lung showed aggregation of some inflammatory cells in the pulmonary microairways and around the blood vessels in the SPP-alone treated group (Fig. S5A-b). When 3-MA was used to inhibit autophagy, the number of inflammatory cells increased dramatically and extensively infiltrated into the rat lung tissue (Fig. S5A-c). However, inflammatory cells were hardly found in the lung tissue of rats treated by Rapa combined with SPPs (Fig. S5A-d). The observations of pathological sections were similar to those for the control group (Fig. S5A-a). At the same time, compared with the control group, the wet weight/dry weight ratio of the lung was increased in groups with SPPs treatment alone and 3-MA/SPPs co-treatment, whereas Rapa combined with SPPs could decrease the W/D weight ratio (Fig. S5B). These results indicated that autophagy could not only affect the production of IgG and IgE in sensitized rats, but also had a significant effect on TSLP. Therefore, lung inflammation could be relieved and the occurrence of pulmonary edema could be prevented by lowering the level of TSLP.

Fig. 6. Analysis of the correlation between ROS, TSLP and inflammatory reaction in A549 cells or rat lungs. (A) Effects of DPI treatment on intracellular ROS level in A549 cells, the cells were treated as follows: PBS treatment, Rosup (ROS inducer) treatment, SPPs treatment, joint treatment of PBS and diphenyleneiodonium, joint treatment of Rosup and diphenyleneiodonium, joint treatment of SPPs and diphenyleneiodonium. (B) Semi-quantitative analysis of the effect of DPI treatment on intracellular ROS level in A549 cells, randomly 5 images were selected and the intensity was measured and normalized by the SMART software. Statistics analysis of at least 3 independent samples per treatment group was carried. (C) Semi-quantitative analysis of the effect of DPI treatment on the level of TSLP in A549 cells. (D) Semi-quantitative analysis of the effect of DPI treatment on the level of ROS in lung tissue of rats, each group tested 5 samples and repeated 3 times independently. (E) Semi-quantitative analysis of the effect of DPI treatment on the level of TSLP in lung of rats. (F) Detection of inflammatory factors in lung lavage fluid. 3 rats were treated in each group, and the experiment was repeated 3 times independently. (G) Observation of inflammatory cells in rat lung tissue with different treatment groups, including PBS treatment, SPPs treatment, co-treatment of PBS and NAC and co-treatment of SPPs and NAC, the black arrows pointed the inflammatory cells. Bar = 50 μ m, * $p < 0.05$, ** $p < 0.01$.

4. Discussion

4.1. Autophagy triggered by air pollutants might cause suppression of pollen allergy

Our data showed that allergenicity of *Platanus* pollen protein was weaker in high polluted area than in low polluted area (Fig. 3). We hypothesized that the biological effect of air pollutants might affect the pathogenicity of pollen allergen; therefore, we then carried out experiments to study the relationship between autophagy and pollen allergenicity by using animal model and A549 cells. We found that the ROS played a key role in subsequent inflammatory response induced by SPPs (Fig. 6). Once the generation or accumulation of ROS was inhibited, inflammatory response in the rats decreased significantly. Autophagy could effectively remove the intracellular ROS (Sotthibundhu et al., 2018; Tu et al., 2019). When autophagy increased, the level of ROS in the cell would decrease, further suppressing the downstream inflammatory response mediated by the ROS signals. Therefore, improvement of autophagy properly within a certain range could effectively reduce the symptoms of the pollen allergy. Drugs, such as Rapamycin, could modify intracellular autophagy and play a role similar to that of the air pollutants (Fig. 4). Considering that the ambient particles could trigger autophagy (Park et al., 2018; Miao et al., 2019) when they were inhaled together with pollen allergen into the human body, these particles could strengthen intracellular autophagy; therefore, autophagy could decrease the allergenic inflammatory responses induced by the SPPs. Moreover, our previously studied air pollutants, such as O₃, could significantly affect the protein structure causing an increase in allergenicity of the pollen protein (Hong et al., 2018); therefore, the effect of air pollutants on pollen allergy should be regarded as the superposition of positive and negative effects. Taken together, it could be revealed that, on one hand, the allergen content and release of the *Platanus* pollen collected from SD, SJ, and WS areas had been enhanced because of the efficacy of air pollution (Fig. 2). On the other hand, air pollutants (such as PM_{2.5}, O₃) in atmosphere could derive the pollens to evoke the highest ability of autophagy, so the pollen allergic reaction caused by WS pollen samples was the lightest among the three groups (Fig. 3).

4.2. Autophagy may be a potential method for cure and prevention of pollen allergic inflammation

Exposure to allergens can result in skin pruritus, asthma, rhinitis, inflammation, and many other symptoms, which has a tremendous negative impact on human health and quality of life (Zhu et al., 2009; Werfel et al., 2015; Saunders et al., 2016; Huang et al., 2019). Therefore, it is a major challenge to find an effective method for prevention and treatment of allergic diseases. In this study, we demonstrated that SPPs could not only induce oxidative stress response (Figs. S4, 4) but also increase the level of TSLP in A549 cells (Figs. 5 and 6) and rats (Fig. 7). Although high levels of autophagy induce apoptosis, appropriate levels of autophagy could help to degrade damaged organelles in cells, such as mitochondria and peroxisomes (Seino et al., 2013), and played a negative role in the release of interleukins from airway epithelial cells exposed to allergens, thus the autophagy reduced Th2-type responses (Shi et al., 2012; Murai et al., 2015). In autophagy-deficient cells, damaged organelles, misfolded proteins and ROS would accumulate and resulted in inflammatory activation (Rao and Eissa, 2019). When autophagy was stimulated and inhibited by rapamycin and 3-MA (Figs. 7 and S5), respectively, the results revealed that the levels of ROS and TSLP decreased as autophagy increases, and when autophagy was inhibited, both ROS and TSLP levels increased. Similar results were confirmed from the results of *in vivo* experiments too (Fig. 6). Taking the above data together, our findings suggested that autophagy might be highly effective at relieving symptoms of allergic diseases. Moreover, the substances that regulate autophagy could be potential therapeutic targets for the prevention and treatment of allergic diseases.

CRediT authorship contribution statement

S.Z., W.Z. and S.L. designed the study, instructed all experiments. S.Z. drafted the manuscript. J.P. carried out animal experiment, L.Y. was responsible for cell culture and related experiment. Z.L. analyzed the data of air pollutants. X.T. and R.L. collected and prepared pollen samples. L.S. provided instruction on allergenic reaction. W.W. and W.Q. revised the manuscript.

Declaration of competing interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2020.143696>.

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