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Substance P-induced lung inflammation in mice is mast cell dependent

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Author contribution

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Tao zhang: Data Curation. Shuanying Yang: investigation (clinical study).

.**Key words:** SP, Asthma, mast cell, Th2.

Key messages:

1. SP levels increased significantly in serum of asthmatic patients.
2. SP is an independent factor associated with the risk of asthma.
3. SP induced Th2 lung inflammation in mice, which was dependent on MCs.

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ABSTRACT

Background

Allergic asthma is a common inflammatory lung disease and a major health problem worldwide. Mast cells (MCs) play a key role in the early stage pathophysiology of allergic asthma. Substance P (SP) functions in neurogenic inflammation by activating MCs, and therefore it may to participate in the occurrence and development of asthma.

Objective

We examined the relationship between SP and lung inflammation, and also whether SP can directly trigger asthma.

Methods

We measured the number of peripheral blood eosinophils, neutrophils, and basophils and evaluated the levels of IgE and SP in blood samples of 86 individuals with allergic asthma. Serum IgE and SP levels were also determined in 29 healthy individuals. C57BL/6 mice were subjected to different doses of SP and bronchoalveolar lavage fluid (BALF) was collected to count the inflammatory cells. Lung tissues were analyzed using histopathological methods to evaluate lung peribronchial inflammation, fibrosis, and glycogen deposition. Levels of IgE, interleukin (IL)-1, IL-2, IL-4, IL-5, IL-13, IL-17 and IFN-γ were determined in mice serum.

Results

SP levels were increased in the serum samples of patients with asthma. SP induced mouse lung peribronchial inflammation, fibrosis, and glycogen deposition, with high levels of Th2-related cytokines such as IL-4, IL-5, and IL-13 observed in the BALF. Furthermore, low level of total IgE was noted in the serum, and SP had little effect on MC-deficient $kit^{W\text{-}sh/W\text{-}sh}$ mice.

Conclusions & Clinical Relevance

SP levels increased significantly in serum of asthmatic patients and independently associated with the risk of asthma. Furthermore, SP induced Th2 lung inflammation in mice, which was dependent on MCs.

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Introduction

Asthma is a common chronic respiratory disease. Epidemiological studies have shown that allergic asthma accounts for approximately 13% of all allergic diseases[1, 2] and can be caused by inhalation of environmental allergens, insect stings, or intake of allergens in food[3-6]. Asthma attacks cause excessive cough and can lead to shortness of breath, and chest tightness. Long-term consequences of asthma include chronic inflammation, airway hyperresponsiveness (AHR), and pulmonary fibrosis[9, 10]. Asthma pathophysiology is mediated by a variety of cells, including eosinophils, neutrophils, macrophages, and mast cells (MCs). This disease is broadly classified as Th2 type or non-Th2 type depending on the type of T helper cell differentiation[11]. Th2 type is characterized by increased eosinophil count in the serum and sputum and increased serum levels of IgE, and FeNO, whereas non-Th2 type asthma is characterized by a neutrophil-dominated inflammatory response[13, 14]. In most cases, allergic asthma is considered Th2 type, which promotes the continuous secretion of IL-4, IL-5, IL-13, and other Th2 type cytokines by increased count of Th2 cells[15, 16].

MCs play a key role in the early stages of allergic asthma by migrating toward inflamed tissues and stimulating the contraction of bronchial smooth muscle bundles, which are involved in the pathophysiology of allergic asthma[18-21]. Inhaled allergens can bind to and activate specific receptors on the plasma membranes of MCs, inducing MCs to release allergic mediators such as histamine, interleukins, and prostaglandins, thereby causing smooth muscle contraction, mucosal secretion, allergic inflammation, and inflammatory cell infiltration[22-25]. MCs can be activated by IgE binding to the high-affinity IgE receptor[26]; serum IgE is a biomarker of Th2 type asthma[27]. Although both IgE and its receptor are known to be potential therapeutic targets for allergic asthma, few drugs targeting MCs are currently available[28].

The neuropeptide substance P (SP) is a member of the tachykinin polypeptide family and acts as a neurotransmitter or regulator in the peripheral and central nervous system of mammals. SP is secreted by nerve fibers and is mainly related to the itch signal[29]. Its endogenous receptor is NK-1R. After stimulation by antigens, SP can increase bronchial stenosis and aggravate AHR[30]. SP can also aggravate asthma symptoms in obese mice. A recent study reported that SP is an endogenous agonist of MC Mas-related G protein-coupled receptor X2(MrgX2) to induce MC activation[11]. MrgX2 is a

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potential biomarker and therapeutic target in asthma and can be inhibited by imperatorin to ameliorates airway inflammation[31, 32]. However, whether SP can directly trigger asthma is unknown. In this study, we analyzed the role of SP in asthma patients and whether it is a direct inducer of lung inflammation in mice.

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Materials and Methods

Materials

Materials

Substance P (SP; purity ≥98%) was obtained from Aladdin Reagent Co. (Shanghai, China). Imperatorin (IMP, purity ≥98%) was obtained from Pufeide Biotech (Chengdu, China). Dexamethasone (purity ≥98%), acetylcholine chloride (Ach, purity ≥99%), and mass spectrometry-grade formic acid and ovalbumin (OVA; grade V, purity ≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from by Tiangen Biotech (Beijing, China). High-performance liquid chromatography-grade methanol and acetonitrile were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Human and Mouse Substance P ELISA kits were provided by Westang Biotech Co., Ltd. (Shanghai, China), Diff-quick Stain kit was provided by Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China), ELISA Kits were provided by CCL2 and TNF-α ELISA kits were purchased from Sino Biological (Beijing, China); IL-4, IL-5, IL-13, IL-17and IgE ELISA kits were from MultiSciences Biotech Co., Ltd. (Hangzhou, China)

Mice

Female C57BL/6 (wild-type, 6-8 weeks) mice were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). $Kit^{W\text{-}sh/W\text{-}sh}$ female (MC-deficient, C57BL/6 background, 6-8 weeks) mice were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). All animals were housed at the Experimental Animal Center of Xi'an Jiaotong University and provided with food and water *ad libitum*. All experiments requiring identical animal treatments were conducted by investigators blinded to the conditions.

Clinical study design and study population

Total 86 patients with asthma and 29 healthy individuals visiting the Second Affiliated Hospital of Xi'an Jiaotong University were enrolled in the study. Patients presented with wheezing, dyspnea, chest tightness, and cough, with exacerbations during the night or early morning, mean variability of adult diurnal PEF variation > 10 % is diagnosed as asthma. Patients were divided into two groups

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according to their total IgE levels. Asthma severity score was evaluated as follows: symptom frequency, peak expiratory flow/forced expiratory volume in 1 s (PEF/FEV1), and mutation rate, as indicated by the Chinese guidelines for the prevention and treatment of bronchial asthma: Intermittent (Grade 1), symptoms appear briefly (<once per week) combined with night-time asthma<twice per month (PEF = normal; PEF/FEV1 mutation rate <20%); Mild (Grade 2), symptoms may affect daily activities and appear > once per week but <once per day, with night-time asthma occurring >twice per month but < once per week (predicted PEF/FEV1 ≥ 80 %; mutation rate = 20-30%); Moderate (Grade 3), symptoms appear every day, with night-time asthma occurring more than once per week (PEF/FEV1 >60% but <80% predicted; mutation rate >30%); Severe (Grade 4), symptoms and night-time asthma appear continuously, with limited physical activity (predicted PEF/FEV1 <60%; mutation rate >30%).

Patient blood sample assessment

Blood samples were collected in vacuum tubes without any additives and allowed to clot for 30 min before centrifuging for 10 min at 1,000 ×g, Next, serum samples were transferred to new tubes and stored at -80 ℃. Serum SP and total IgE levels was detected in one week using an ELISA kit following the manufacturer's protocol

SP-induced lung allergic inflammation in mice

To verify whether SP can independently induce lung allergic inflammation and AHR, 8-week-old female C57BL/6 mice were challenged with 4 mL of vaporized SP (10, 30, or 90 μg/mL) or phosphate-buffered saline (PBS) on days 1, 3, 5, 7, 9, 11, 13, and 15. Lung inflammation and AHR were evaluated one day after SP challenge.

OVA induced lung allergic inflammation in mice

Briefly, 8-week-old female C57BL/6 mice in each group were intraperitoneally sensitized with 0.5 mg/kg OVA on Day 1, 3, 5 and 7, while vehicle group was sensitized with PBS. On Day 21, 23, 25, 27, and 29, mice were placed in sealed containers and given filtered 1% OVA inhalation for 30 min each time to induce asthma attacks. Vehicle group was atomized and inhaled with PBS. After the last challenge, mice were anesthetized and evaluated for AHR severity with flexiVent apparatus (SCIREQ, Quebec, Canada). Mice were sacrificed and lung tissues were subjected to H&E staining.

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Measurement of airway reactivity in mice

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium salt (1%) and ventilated. Baseline lung function was measured using a flexiVent apparatus (SCIREQ, Quebec, Canada), as described previously. Mice were exposed to acetylcholine, and Newtonian resistance (Rn), respiratory resistance (Rrs), tissue damping (G), and respiratory compliance (Crs) were determined using flexiVent.

Bronchoalveolar lavage fluid (BALF) collection

BALF was collected by lavage of the mouse lungs twice with saline (0.75 mL). Cells were collected by centrifugation (200×g) and were resuspended in 200 μL PBS. Total cell numbers were determined, cytospin specimens were prepared and stained with Diff-Quik Stain, and proportions of different cell types (eosinophils, neutrophils and total cell count) were quantified by counting at least 200 cells per cytospin preparation. The supernatants from BAL samples were collected by centrifugation (200×g). Mouse TNF-α, CCL-2, IL-4, IL-5, IL-13 and IL-17 levels in the supernatant were detected with ELISA kits according to manufacturer's instructions.

Lung histology

Mouse lungs were inflated via the trachea, removed, and fixed in 10% neutral-buffered formalin. Formalin-fixed tissues were embedded in paraffin, 5-μm-thick sections were cut, and subjected to hematoxylin and eosin, periodic acid–Schiff (PAS), or Masson's trichrome staining to quantify infiltrated cell counts, mucus production, and collagen deposition, respectively. Inflammatory cells, PAS-positive bronchial epithelial cells, and collagen deposition area were quantified as described previously. To identify MCs, paraffin-embedded lung sections were stained with fluorescein isothiocyanate labeled avidin. Non-degranulated MCs contained characteristic granules while degranulated MCs were characterized by the appearance of extracellular green fluorescence granules and lower levels of condensed intracellular granules.

Cytokine and chemokine measurements

ELISA kits were used to measure the levels of Mouse TNF-α, CCL-2, IL-4, IL-5, IL-13 and IL-17 concentrations in the BALF according to the manufacturer's instructions.

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Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the clinical data. Differences between the means of two samples were tested by the Mann–Whitney test. Kruskal–Wallis test was performed to evaluate the relationship between SP levels and asthma severity in groups with different IgE levels. In addition, to investigate the independent effect of SP on risk of asthma, we compared the SP levels in patients with asthma and controls in groups with different IgE level. Other in vitro and in vivo quantitative data were analyzed using the Student's t-test (to compare two groups) and analysis of variance (to compare three or more groups). Results with $P < 0.05$ were considered statistically significant.

Study approval

All animal experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mouse experimental protocols were approved by the Animal Ethics Committee at Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019-711). The clinical study was registered at the Chinese Clinical Trials Registry (ChiCTR-1900024672) and was approved by the ethics committee of the Second Affiliated Hospital of Xi'an Jiaotong University. Written informed consent was obtained from at least one guardian of each patient before enrollment. Patient data were analyzed anonymously.

Results

SP concentration increased in the serum of patients with allergic asthma

To study the association between SP levels and asthma, blood serum was collected from patients with asthma and normal subjects, SP and IgE levels were detected in the serum. As serum total IgE is a diagnostic marker of asthma, we devised the patients into two groups: IgE<100 IU/mL and IgE≥100 IU/mL. Serum SP level was increased obviously in the patients with asthma, especially when total IgE was below 100 IU/mL (Figure S1). We then evaluated the normality of the data in different groups using Shapiro-Wilk test. Data of either SP levels or IgE levels in asthma and control groups did not fit normal distributions (Table S1). So, we used non-parametric tests for further analysis. We performed Mann-Whitney test to determine the SP concentration in the serum of 86 patients with asthma whose clinical characteristics, including IgE and SP levels, are summarized in Table 1.

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Among the 86 patients, 47 had a total IgE content below 100 IU/mL, while 39 patients showed an IgE content of over 100 IU/mL; total IgE was 30.07 ±28.30 IU/mL in 29 healthy individuals. Disease severity and IgE levels did not differ between patients with different demographic or clinical characteristics. There was no significant difference in the numbers of white blood cells, eosinophil granulocytes, neutrophil granulocytes, or basophil granulocytes in each group. However, the serum SP level was 121.30 ± 131.10 pg/mL in patients with an IgE concentration below 100 IU/mL, and it was significantly higher in patients with serum IgE levels over 100 IU/mL. In contrast, the SP level was50.9±36.9 pg/mL in patients over 100 IU/mL serum IgE, but it was still significantly higher than in healthy individuals (33.93 ± 19.79 pg/mL, n = 29). These results demonstrate that SP levels are significantly increased in the serum of patients with asthma; the SP level in these patients increased with the decreasing IgE level.

We have investigated which groups are changing and the results were summarized in Table S2, none of the two by two comparisons showed significance after being adjusted for multiple comparison. We further evaluated the relation between SP and asthma status, and the results revealed that the results were not statistically significant (Figure 1B and C, Table 2).

In order to present the independent effect of SP levels on the risk of asthma, we have compared IgE and SP levels between asthma cases and healthy controls. As we know IgE levels have been considered as one of a main risk factor for asthma. Therefore, in single factor analysis, although SP level was significantly associated with risk of asthma (W=510, P=5.73×10^{-7}), it is still not clear whether this result has been confounded by IgE level. Therefore, we have adjusted the potential confounding effects of IgE level by stratifying this factor. The results in table 3 showed that SP level was still a significant factor on risk of asthma in different groups of IgE level. The above results indicated that the SP level is independently associated with the risk of asthma irrespective e of the IgE level.

Furthermore, we have assessed the correlation of SP levels with bronchial dilation using Mann-Whitney test. We have collected the bronchial dilation test of 86 asthmatic patients, but only 56 asthmatic patients underwent pulmonary function tests. Among the 56 patients, the serum SP level was 86.20±96.10 pg/mL in patients with negative bronchodilation test, the serum SP level was

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88.60±119.50 pg/mL in patients with positive bronchodilation test. There is no significant difference between negative and positive patients.

SP induced AHR in mice

In the murine model of OVA-mediated asthma, OVA challenge increased the levels of SP both in the blood serum and lung tissue (Figure S2). Therefore, we examined whether SP triggered inflammation and AHR, the major pathological changes observed in OVA-challenged mice. Mice were challenged with different doses of SP on days 4, 8, 12, and 16. After SP stimulation, AHR was detected every day, the treatment method and detection timepoints are shown in Figure 1A. Our results showed that AHR was induced by SP in a dose- and time-dependent manner. As shown in Figure 1B, after SP stimulation twice, levels of Rn, Rrs, and Gin creased significantly by 30 and 90 μg/mL SP, whereas that of Crs decreased; however, the differences compared with the vehicle group were not significant. Following stimulation with 10 μg/mL SP, no significant changes were observed in AHR indicators. After four stimulations, levels of Rn, Rrs, and G further increased in the 90 μg/mL SP group, along with a significant decrease in Crs level. After six SP stimulations, levels of Rn, Rrs, and G further increased, whereas that of Crs decreased gradually. After eight SP stimulations, a low concentration of SP led to maximum increase in Rn, Rrs and G levels, while levels of Crs decreased significantly. Collectively, these results indicated that SP induce AHR in mice in a dose- and time-dependent manner.

Furthermore, we also compared SP induced and OVA induced AHR in mice, our results showed that in mice treated with 30 μg/mL SP for 12 days, AHR severity was similar to that induced by OVA (Figure S3).

SP induced lung inflammation, mucus production collagen deposition and MC degranulation

We examined lung inflammation, mucus production, and collagen deposition by staining lung sections with hematoxylin and eosin, PAS, and Masson's trichome, respectively on days 4, 8, 12, and 16. As shown in Figure 2A, SP treatment induced significant inflammatory cell infiltration and bronchial wall thickening, which were accompanied by extensive infiltration of eosinophils and neutrophils around the airway (Figure 2B). Furthermore, PAS staining of the lung showed that SP challenge induced a notable increase in mucus production. On day 8 after challenge, mucus

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production increased significantly in treated group compared with the vehicle group (Figure 3). Masson trichome staining revealed an increased lung collagen content in SP-challenged mice, with the severity dependent on the dose and number of SP challenges (Figure S4), which was consistent with the results of AHR experiment.

SP activated MCs in mouse lung tissue

MCs are key mediators of allergy; therefore, we evaluated whether MC activation was involved in the SP-induced mouse lung inflammation. As shown in Figure 4, after treated with 10 μg/mL SP four times, the total MCs numbers and rate of MCs degranulation are significantly increased in the mice lung tissue (Figure 4B and C). In contrast, in mice treated with 30 μg/mL SP, more MCs were activated and the total numbers of MCs continued to increase. The same trend was observed in mice subjected to treatment with 90 μg/mL SP. Notably, the serum IgE levels did not increase in response to SP challenge (Figure S5).

SP increased inflammatory cell content and levels of cytokines, and chemokines in the *BALF*

Lung inflammation is caused by an increase in inflammatory mediators and cells. When animals were treated with 30 μg/mL SP for 12 days, lung inflammation and AHR severity were similar to those induced by OVA. Therefore, we measured the changes in counts for total cells, eosinophils, and basophils after challenge with 30 μg/mL SP on day 12. SP challenge induced infiltration of inflammatory cells in the BALF; moreover, the numbers of total cells, eosinophils, and basophils were elevated compared to those in the vehicle group (Figure 5A). Next, we measured the levels of several cytokines in the BALF. As shown in Figure 5B, levels of Th2-type cytokines IL-4, IL-5 and IL-13 increased significantly following SP challenge. Significantly, the pro-inflammatory factor TNF-αshowed the maximum increase in SP-challenged mice compared with vehicle group. Furthermore, expression of CCL-2, which is largely dependent on MCs, also increased in SP- challenged mice. We also measured the levels of IL-17, IL-2, and IFN-γ in BALF and the results are showed in Figure 5C and no significant differences were observed between the vehicle and the control groups. Taken together, these data demonstrated that SP induced significantly increase in Th2 type cytokines, while SP had no obvious effect on the content of Th17 and Th1 type factors.

SP-induced lung inflammation in mice depends on MCs

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To verify whether SP-induced lung inflammation in mice is dependent on MCs, we used MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. In wild type mice, AHR was induced notably after challenge with 30 μg/mL SP (Figure 6A, red line), whereas AHR did not occur in MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice (Figure 6A, green line). When MCs were functionally deficient, SP had no significant effect on lung inflammation, mucus production and collagen deposition (Figure 6B). Furthermore, compared to WT mice, little AHR or lung inflammation was observed in $Kit^{w\text{-}sh/w\text{-}sh}$ mice subjected to the same treatment.

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Discussion

In this study, we demonstrated that serum SP levels are much higher in asthmatic patients than it in healthy controls. SP levels in asthmatic patients with low total IgE levels are particularly higher than those in patients with high IgE level. Furthermore, SP was independently associated with the risk of asthma. SP induced lung inflammation in mice, which was solely dependent on MCs. Previous studies reported significantly elevated SP levels in the lungs, BALF, sputum, and plasma samples of patients with asthma; therefore, we analyzed blood serum samples from 86 patients with allergic asthma[33-36]. As our statistical analyses revealed a non-normal distribution, we adopted non-parametric methods to analyze our data. We observed that SP levels were much higher in patients with asthma than in healthy individuals. Additionally, a lower IgE concentration associated with a higher SP level in the serum of patients with asthma. We also analyzed the relationship between the SP level and the asthma status and observed that SP levels increased with the severity of asthma (assessed by grade 1, 2 and 3), although the result was not significant. However, these are interesting results and therefore, future studies based on larger sample sizes are necessary to confirm the relationship between SP levels and asthma severity. Based on our analyses of SP levels with different IgE levels, we inferred that SP levels increase in asthma patients, especially in patients with low IgE levels, and that SP level is independently associated with the risk of asthma regardless of the IgE level. Thus, SP may participate in asthma pathogenesis and could be a new biomarker of asthma. Future studies may validate SP level as a supplementary diagnostic index for asthma

Clinical data indicate that SP is involved in the asthma pathogenesis; however, it remains unclear whether elevated SP levels can cause asthma. We stimulated mice with different concentrations of SP to verify whether SP can directly cause lung inflammation in mice. Following SP inhalation at different doses, inflammatory cells such as eosinophils migrated into the lung tissue and BALF, and AHR was aggravated in treated mice. The severity of inflammation and AHR increased with increasing doses and frequency of SP administration. When animals were treated with 30 μg/mL SP for 12 d, AHR severity was similar to that induced by OVA. Therefore, we measured the changes in counts of total cells, eosinophils, and basophils after treatment with 30 μg/mL SP for 12 days. Interestingly, no changes were observed in mice serum IgE levels following SP treatment, whereas serum SP levels increased significantly in mice induced by OVA. Our clinical data showed that high

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IgE level in asthmatic patients is usually accompanied by the increased SP level. However, high SP level can work as an independent factor in asthma when IgE level is low. This finding provides a new avenue for the clinical diagnosis of patients with asthma. SP has a high affinity for NK-1R in airway smooth muscle cells and MCs, through which it promotes airway contraction [37-40], NK-1R antagonists have been shown to partially alleviate asthma in obese mice[41], however, their clinical effects are not significant[42, 43]. Potentially because SP activates human MCs via MrgX2, independently of NK-1R, and NK-1R antagonists did not inhibit SP-induced MC degranulation. Since drugs that can directly degrade SP directly are not yet available, further studies are required to determine whether reducing the serum SP content would be helpful for treating patients with asthma and whether SP can be a drug target. SP amplifies Th2 cell influx and polarization in the airways, thereby inducing Th2-type lung inflammation and AHR in mice[44], but its underlying mechanism is unclear. Although extensive migration of inflammatory cells and airway remodeling were observed following SP inhalation, serum IgE levels did not increase, suggesting that SP induces IgE-independent lung inflammation in mice. After SP stimulation, the number of lung MCs and degranulation of MCs increased significantly in mice. Thus, SP may cause lung inflammation in mice via MCs. TNF-α is released in allergic responses from mast cells as well as macrophages via IgE-dependent mechanisms and elevated TNF-α levels are frequently observed in bronchoalveolar fluid of asthmatic subjects subjected to undergoing allergen challenge[46]. Another study showed that TNF-α is released from MCs stimulated by SP[47]. We found that TNF-α levels increased along with SP induced mice lung inflammation which is in accordance with our in *vivo* study. CCL2, also called monocyte chemotactic protein 1 (MCP-1), is a potent chemotactic molecule that attracts lymphocytes, monocytes, mast cells, and memory T cells, but not neutrophils, and SP is an important neurotransmitter that can stimulate the CCL2 in mast cells[48]. In our study, CCL2 expression was elevated in SP stimulated mice lung inflammation. These results give us a hint that SP-induced asthma may be related to mast cells.

Asthma is characterized by airway inflammation, AHR, and reversible airflow obstruction and can be mediated by Th2 cells, which play a key role by producing cytokines such as IL-4, IL-5, and IL-13[49]. Recently, Th17 cells have received attention[50], since factors such as IL-17A and IL-17F can amplify several signal pathways related to asthma[51], particularly, IL-17A may contribute to

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neutrophilic airway inflammation by upregulating the chemokines CSF3 and CXCL expression, mucus gland hyperplasia, AHR, and corticosteroid resistance[52]. Th2 cells and Th17 cells represent Th2 and Th17 pathways, respectively, which engage in a complex crosstalk[53]. Macrophages account for more than 70 % of the total lymphocytes in the lungs. M0 to M1 are differentiated by IFN-γ and LPS, while M2 are differentiated by IL-4 and IL-13. Therefore, the polarization ratio of macrophages can reflect the direction of Th1-Th2 differentiations. In allergic asthma, the proportion of M2 increases. Therefore, the polarization was corresponding to the expression of Th2 cytokines[54]. We further evaluated Th1, Th2 and Th17 type indicators of lung inflammation in mice to determine the type of immune response triggered by SP. SP significantly increased IL-4, IL-5, and IL-13 in the BALF, whereas IL-17, IL-2 and IFN-γ levels did not increase significantly. The IgE concentration in the serum did not change significantly under stimulation of SP at different times. Thus, SP induced Th2 lung inflammation with low levels of IgE. In patients with low IgE levels in Th2 asthma, serum SP levels may have obvious advantages as a biomarker of asthma.

We further used MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice in which c-kit was knocked out to confirm this inference. After the loss of MC function, the AHR of SP-challenged mice mostly disappeared and lung inflammation did not appear, Deposition of collagen and glycogen was also significantly reduced. Based on these results, we confirmed that lung inflammation caused by SP in mice was dependent on MCs.

As we found out from SP induced mice lung inflammation model, serum IgE content did not significantly change, indicating that the asthma was Fc-receptor independent but mast cell dependent. Enriquez et al reported that house dust mite (HDM) induced contraction of sensitized mouse trachea occurred in a mast cell- and Fc-receptor dependent fashion, suggesting that HDM in this setting lacks effect mediated by other receptors[55]. Also, it is reported that HDM induced degranulation of MrgB2+ mast cells in skin, SP causes MCs degranulation obtained from BALF, and MrgX2 is upregulated in asthmatic lung MCs and severe asthma is dominated by the presence of MrgX2+ MCs in the airway[56]. Therefore, we assumed that SP-induced lung inflammation may associate with MrgX2.

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In conclusion, SP has an independent effect in asthma after being adjusted for IgE level based on our clinical data, and thus is a potential biomarker for diagnosing patients with asthma showing low IgE levels. Furthermore, the ability of SP to induce lung inflammation in mice depends on MCs.

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Conflicts of interests

The authors declare no competing financial interest.

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Tables

Table 1. Baseline demographic and clinical characteristics in asthma patients by IgE level (n=86)

Characteristic	IgE		Statistics	*P value*
	<100 IU/mL	**≥100 IU/mL**		
Age, years	50.0±14.9	51.8±15.9	*W = 900.50*	0.77
Sex				
Male	20	19		
Female	27	20	χ^2= 0.33	0.85
White blood cell count (1×10^9/L)	6.90±2.60	7.30±2.50	*W* = 702.00	0.27
Eosinophil granulocyte count (1×10^9/L)	0.20±0.30	0.30±1.20	*W* = 816.50	0.98
Neutrophil granulocytes (%)	65.30±17.40	63.2±19.1	*W* = 859.00	0.72
Eosinophil granulocytes (%)	2.80±4.30	2.20±3.50	*W* = 867.00	0.66
Basophil granulocytes (%)	0.40±0.40	0.60±1.20	*W* = 737.00	0.43
Asthma symptom score				
1	13	11		
2	20	14		
3	11	11		
4	3	3	Fisher's Exact Test	0.93

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Table 2. Kruskal–Wallis test of SP and asthma status (n=86)

Asthma symptom score	SP (pg/mL)	
	IgE<100 IU/mL	**IgE≥100 IU/mL**
1	102.30+126.30	50.0+12.90
2	125.50+126.10	46.60+22.10
3	135.20+165.60	66.70+65.00
4	117.20+119.70	39.50+8.70
Kruskal–Wallis *H* Statistic	1.54	2.41
P-Value	0.67	0.49

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Table 3. SP levels in patients with asthma and controls in groups with different IgE level (n=86)

	Stratified by IgE level		Single factor effects	
	IgE		IgE	SP
	<100 IU/mL	≥100 IU/mL		
W	350	300	744	510
P-Value	0.00014	0.012	0.00034	5.73×10^{-7}

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Figure legends

Figure 1. Substance P (SP) induces airway hyperresponsiveness (AHR) in mice. (A) SP challenge protocol. (B) Mice were challenged by inhalation of aerosol with 4 mL of phosphate buffered saline (PBS) containing SP at various concentrations (10, 30, and 90 μg/mL) or 4 mL of PBS alone on days 1, 3, 5, 7, 9, 11, 13, and 15. On days 4, 8, 12, and 16, airway responses to Ach were measured. Data are presented as the mean ± standard deviation (SD). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the vehicle group (*n* = 7).

Figure 2. SP induces mucus production in lung. Mice were treated as described in Figure 1. On days 4, 8, 12, and 16, lung sections were fixed and stained with hematoxylin and eosin (HE). (A) HE staining of lung sections. (B) Quantification of inflammatory cells was scored as described in the Material and Methods section. The figures were photographed under a light microscope at ×200 magnification (scale bar **=** 50 μm). Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the vehicle group (*n* = 7).

Figure 3. SP induces mucus production in lung. Mice were treated as described in Figure 1. On days 4, 8, 12, and 16, lung sections were fixed and stained with periodic acid–Schiff (PAS). (A) PAS staining of lung sections. (B) Quantification of PAS-positive bronchial epithelial cells was scored as described in the Materials and Methods section. The figures were photographed under a light microscope at ×200 magnification (scale bar **=** 50 μm). Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the vehicle group (*n* = 7).

Figure 4. SP induces mast cell (MC) degranulation in lung. Mice were treated as described in Figure 1. On days 4, 8, 12, and 16, lung sections were fixed and stained with avidin. (A) Avidin staining of lung sections. (B) Total number of MCs increasing in the lungs of the mice. (C) Degranulation rate of MCs in lung sections were determined as described in the Materials and Methods section. The figures were photographed under a light microscope at ×200 magnification (scale bar **=** 50 μm). Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the vehicle group (n = 7).

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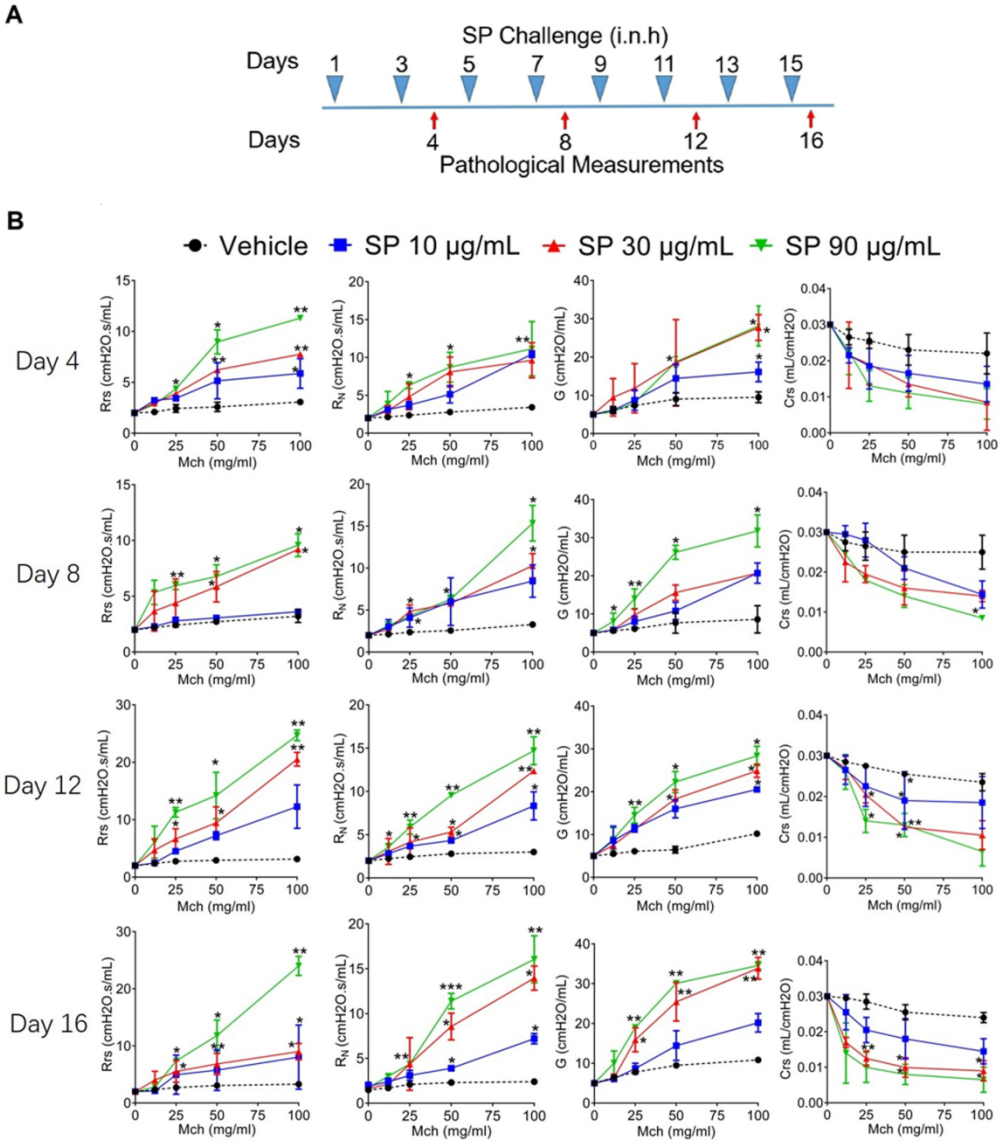
Figure 5. SP increases levels of inflammatory cells, cytokines, and chemokines in the bronchoalveolar lavage fluid (BALF). Mice were challenged with SP (30 μg/mL) by inhalation of aerosol with 4 mL PBS containing SP (30 μg/mL) or 4 mL of PBS alone on days 1, 3, 5, 7, 9, and 11. (A) 24 h after the last challenge, BALF was collected and the numbers of eosinophils, basophils, and total cells were determined. (B) At the same time point, CCL-2, IL-5, IL-4, IL-13, and TNF-α levels in the BALF. (C) IL-2, IFN-γ and IL-17 levels in the BALF. Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the vehicle group (n = 7).

Figure 6. SP-induced lung inflammation and AHR in mice depend on MCs. Airway responses to Ach were measured on day 12 and 24 h after the last SP or PBS challenge by assessing Rn, Rrs, G, and Crs. (A) Airway responses in wild-type and $Kit^{W\text{-}sh/W\text{-}sh}$ mice. (B) Lung sections were fixed and stained with hematoxylin and eosin, PAS, and Masson's trichome. The figures were photographed under a light microscope at ×200 magnification (scale bar **=** 50 μm). Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 *vs.* the vehicle group (n = 7).

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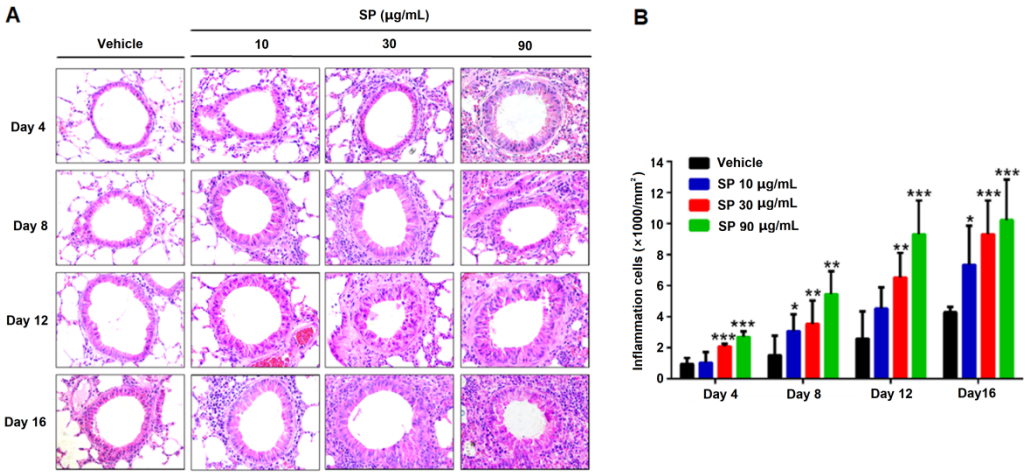
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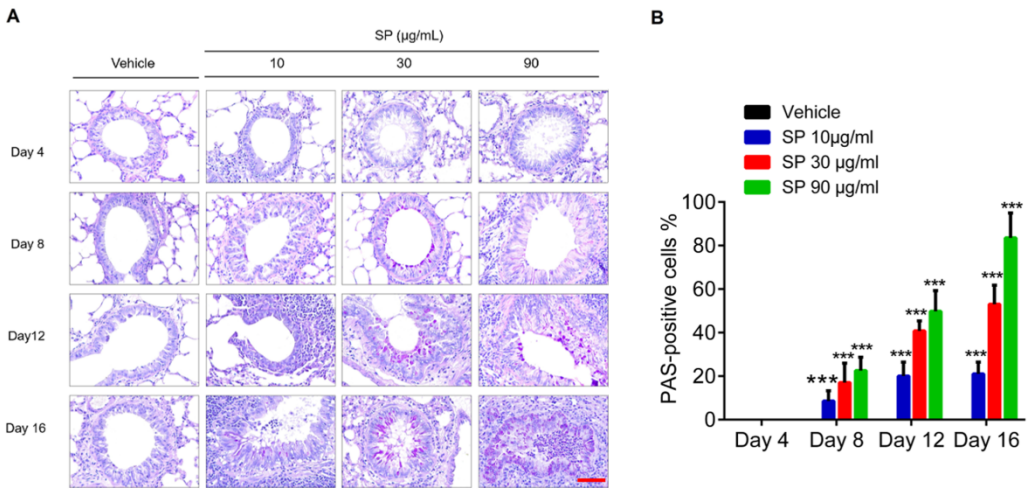
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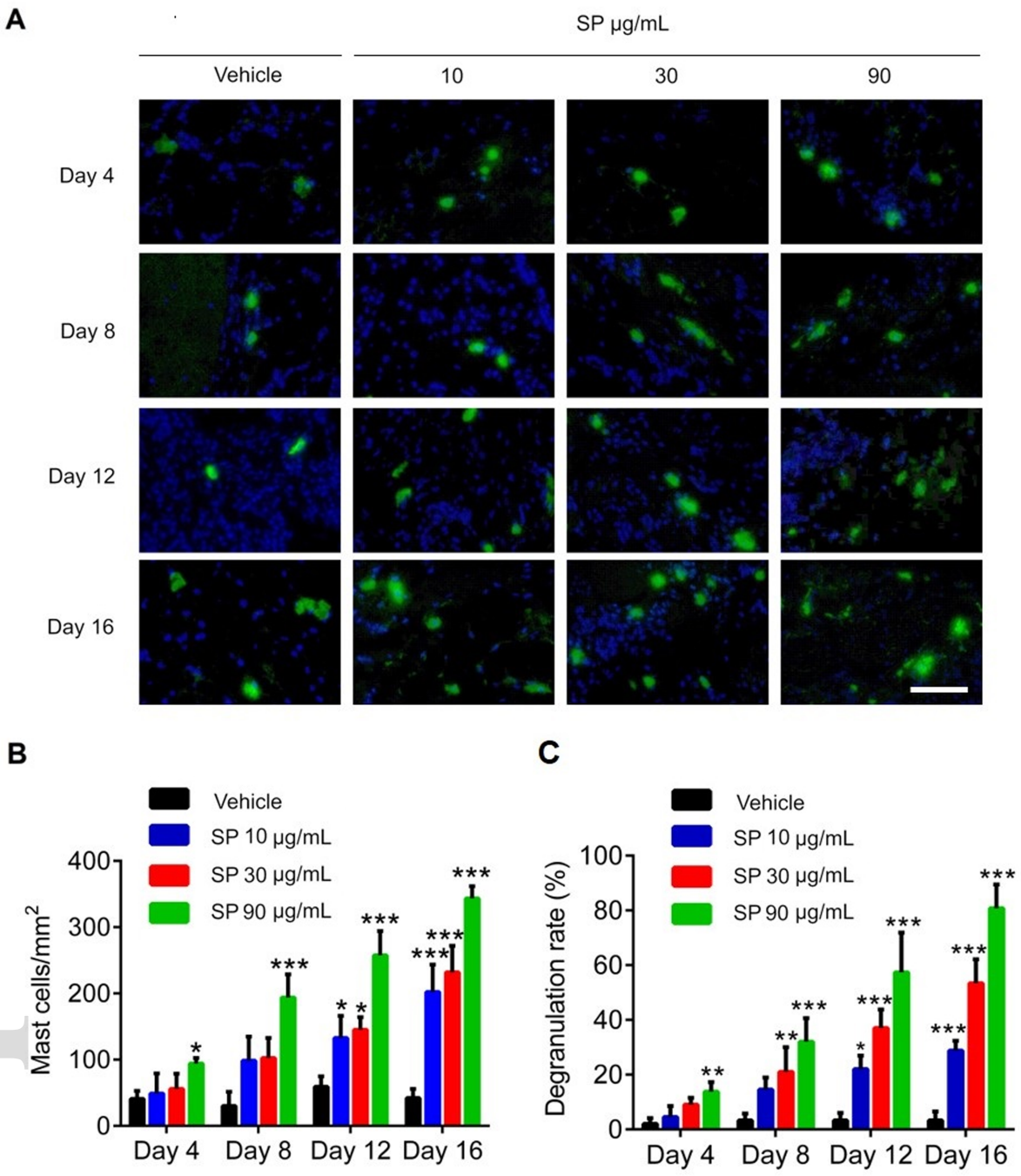
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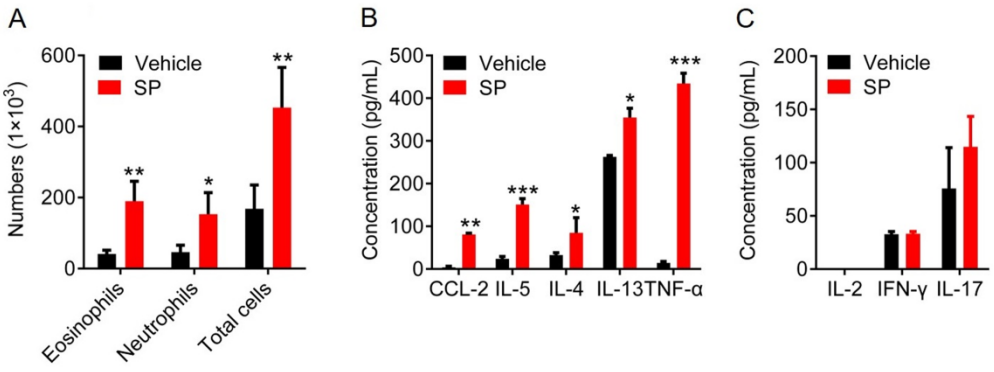
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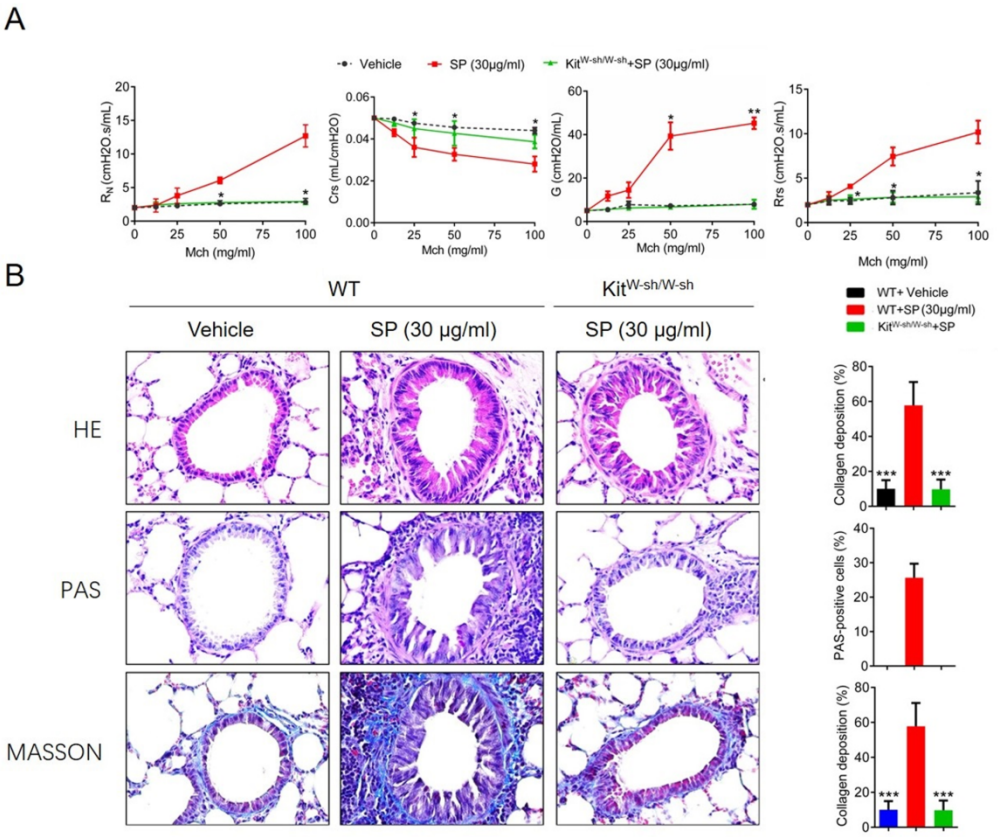
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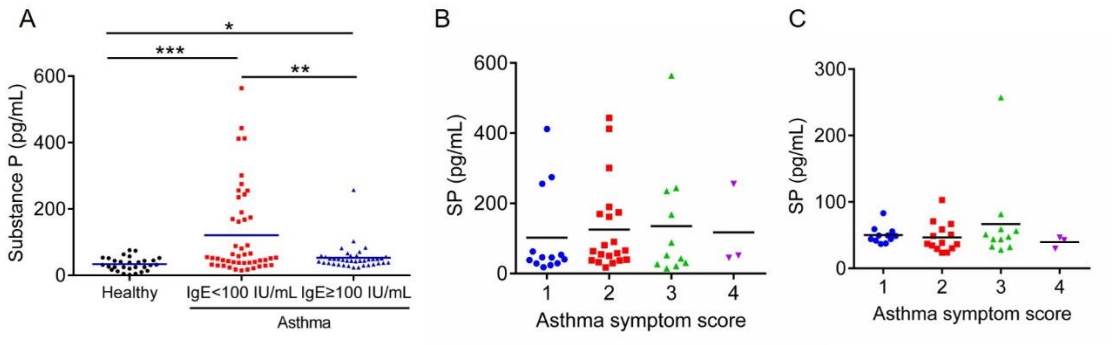
Supplement Table 1. Shapiro-Wilk normality test of different groups

Samples	SP		IgE	
	W	*P*	*W*	*P*
asthma	0.63	5.96×10^{-14}	0.66	2.57×10^{-13}
Controls	0.97	0.54	0.69	1.30×10^{-6}

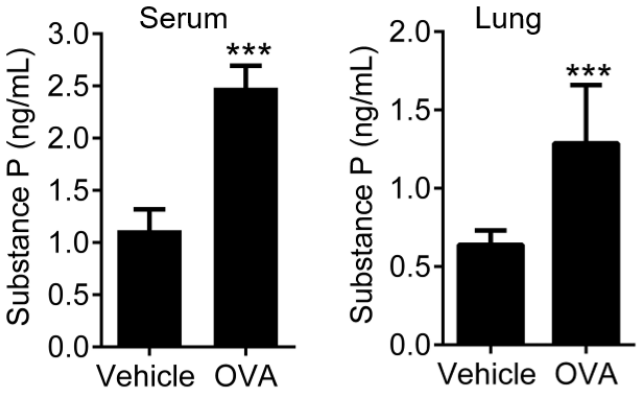
Supplement Table 2. Results of Dunn' Tests after Kruskal-Wallis tests of SP and asthma status (n=86)

Comparison	IgE<100 IU/mL		IgE≥100 IU/mL	
	Z	*Adjusted *P* Values	Z	*Adjusted *P* Values
1 - 2	-1.15	1.00	1.13	0.77
1 - 3	-0.34	0.87	0.20	0.84
2 - 3	0.71	1.00	-0.93	0.53
1 - 4	-0.67	1.00	1.19	1.00
2 - 4	-0.03	0.97	0.50	0.74
3 - 4	-0.44	0.99	1.06	0.58

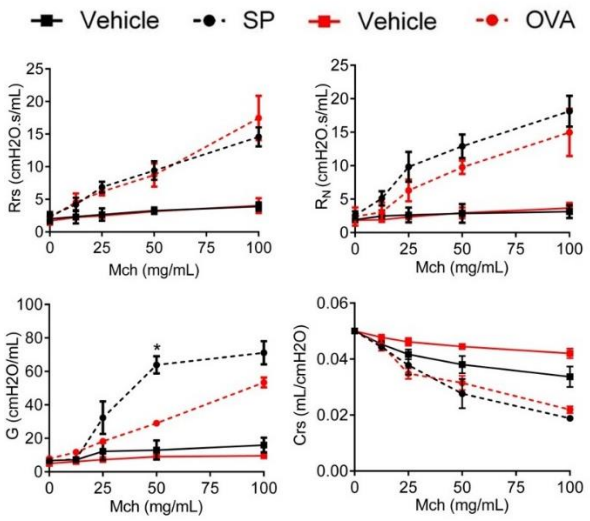
* *P* values were adjusted by Benjamini-Hochberg method.



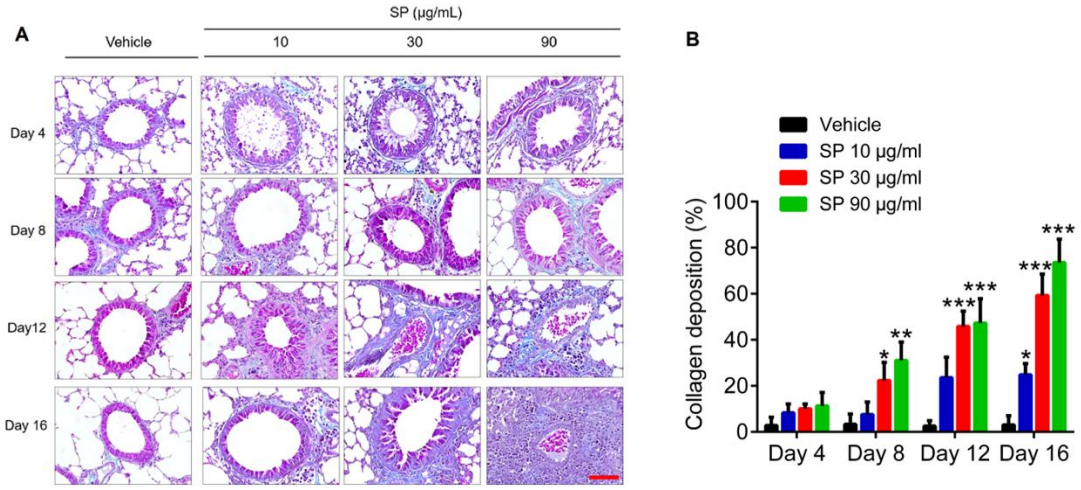
Supplement Figure 1. Substance P (SP) levels in the serum in healthy controls and patients with asthma. A. SP levels in the serum in healthy controls and patients with asthma. B. SP levels in patients with different asthma symptom score whose total IgE < 100 IU/mL. C. SP levels in patients with different asthma symptom score whose total IgE ≥100 IU/mL *P<0.05, **P<0.01, ***P<0.001.*vs.* the related groups.



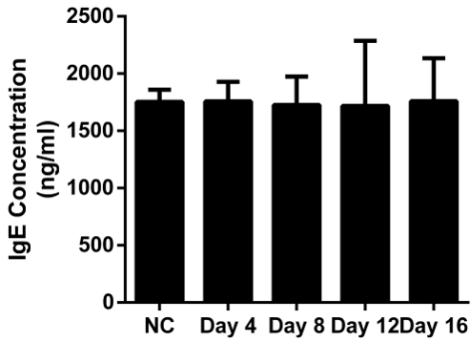
Supplement Figure 2. SP concentration in the serum and lung tissue after OVA challenge. ***P<0.001 compared with Vehicle group. (n=7)



Supplement Figure 3. SP-induced AHR in mice and OVA-induced AHR in mice were measured by assessing Rn, Rrs, G, and Crs. **P*<0.05 compared with OVA group. (n=7).



Supplement Figure 4. SP induces collagen deposition in lung. Mice were treated as described in Figure 1. On days 4, 8, 12, and 16, lung sections were fixed and stained with Masson's trichrome. A. Masson's trichrome staining of lung sections. B. Quantification of the collagen deposition area was scored as described in the Materials and Methods section. The figures were photographed under a light microscope at ×200 magnification (scale bar = 50 μm). Data are presented as the mean SD. **P* < 0.05, ***P* <0.01, ****P* <0.001 vs. Vehicle group (n = 7).



Supplement Figure 5. Total IgE concentration in mice serum after SP stimulation. Mice were challenged by inhalation of aerosol with 4 mL of phosphate buffered saline (PBS) containing SP at 30 μg/mL or 4 mL of PBS alone on days 1, 3, 5, 7, 9, 11, 13, and 15. On days 4, 8, 12, and 16, total IgE in the serum was detected. **P* < 0.05, ***P* <0.01, ****P* <0.001 *vs.* NC. (n=7)