Substance P Increases STAT6-Mediated Transcription Activation of Lymphocyte Cytosolic Protein 2 to Sustain M2 Macrophage Predominance in Pediatric Asthma

Miao Li, Xiao Zhong, and Wenting Xu

From the Department of Pediatrics, Shengjing Hospital of China Medical University, Liaoning, China

Asthma, a chronic inflammatory disease of the airways, is the most common disease in children, adolescents, and young adults worldwide that severely affects patients’ quality of life, strains health systems, and reduces productivity. This heterogeneous disease is marked by variable bronchial hyperresponsiveness, airflow obstruction, and chronic airway inflammation, which result in similar clinical symptoms, such as episodic wheeze, shortness of breath, chest tightness, and cough. Allergic asthma is usually induced by sensitization of the airways to environmental allergens, such as house dust mite (HDM), pet dander, pollens, cockroaches, and mold. Most asthmatic patients have evidence of type 2 inflammation, characterized by T-helper type 2 (Th2) inflammatory cytokines, such as IL-4, IL-13, and IL-5, secreted by innate or adaptive immune system. Targeting these key Th2 cytokines has therefore been proposed as a promising strategy to achieve therapeutic benefit in allergic asthma.

Macrophages are the most abundant immune cells (approximately 70%) in the lung and play crucial roles in innate immunity and homeostasis. They are extremely heterogeneous cells, generally classified into the classically activated (M1) and the alternatively activated (M2) phenotypes, which mirror the M1-M2 polarization of T cells. The M2 macrophages can be further allocated into three subtypes (M2a, M2b, and M2c) according to the nature of

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inducing agent and the expressed markers.\textsuperscript{10} Not surprisingly, previous publications have reported significantly increased population as well as pathogenesis-promoting roles of M2 macrophages in patients or animal models with allergic asthma.\textsuperscript{11,12}

Substance P (SP) is a neuropeptide primarily secreted by sensory nerves and immune cells and participates in a multitude of biological processes, such as nociception and inflammation.\textsuperscript{13,14} SP acts by G-protein–coupled neurokinin receptors, including NK-1R, NK-2R, and NK-3R, among which the NK-1R has the highest affinity for SP, and the SP-NK-1R interaction has widely reported regulatory roles in the immunity of immune cells.\textsuperscript{15} SP exerts proinflammatory effects in immune and epithelial cells and is involved in a variety of inflammatory diseases of the musculoskeletal, gastrointestinal tract, and respiratory tract systems, including asthma.\textsuperscript{16} In prior research, it was discovered that SP induced inflammation and pyroptosis of bronchial epithelial cells by activating the phosphatidylinositol 3-kinase/Akt/NF-κB signaling pathway.\textsuperscript{17} In addition, SP has been reported to play various roles in immune cells, including macrophages, such as promoting cell survival, activation, and phagocytosis, and the expression of SP receptors on macrophages is reportedly markedly increased on infection.\textsuperscript{14} Moreover, SP has recently been documented to induce M2 skewing of macrophages in a rat model of ischemic brain injury.\textsuperscript{18} This piqued interest in unraveling SP’s role in macrophage phenotype in allergic asthma and the implicated molecules.

Bioinformatics analyses were conducted (details are provided later in the text), revealing STAT6 and lymphocyte cytotoxic protein 2 (LCP2) as a candidate downstream cascade of SP. STAT6 is activated following either IL-4 or IL-13 exposure and is essential for the function of cytokines and for Th2 development.\textsuperscript{19} Indeed, STAT6 has been demonstrated to activate M2a macrophages and increase type 2 inflammation in asthmatic lungs.\textsuperscript{20} Similarly, LCP2 has also been reported to be positively correlated with the M2 polarization of macrophages.\textsuperscript{21} However, whether SP can regulate STAT6 activity and LCP2 in the context of macrophage polarization remains largely unknown. The hypothesis posited is that SP might induce M2 polarization of macrophages through the STAT6/LCP2 axis and therefore affect the progression of allergic asthma.

Materials and Methods

Clinical Sample Collection

Eighteen asthmatic children treated at Shengjing Hospital of China Medical University (Liaoning, China) from November 2019 to April 2021 and 13 children who underwent a bronchoscopy for treatment of bronchial foreign body during the same period were included into this research. The two cohorts of respondents had no significant difference in age and sex. The peripheral blood samples and bronchoalveolar lavage fluid (BALF) were collected. The proportions of M1 (CD68<sup>+</sup>CCR7<sup>+</sup>) and M2 (CD68<sup>+</sup>CD206<sup>+</sup>) macrophages in BALF were analyzed by flow cytometry. The study protocol was approved by the Institutional Review Board of Shengjing Hospital of China Medical University. All procedures involving human samples were performed in line with the tenets of the Declaration of Helsinki. Informed consent was obtained from their statutory guardians.

Isolation of Bone Marrow—Derived Macrophages

All animal experiments were approved by the Animal Ethics Committee of Shengjing Hospital of China Medical University and performed in line with the Guide for the Care and Use of Laboratory Animals. C57BL/6j mice were procured from Charles River Laboratory Animal Technology Co, Ltd (Beijing, China). The mice were euthanized by i.p. injection of excessive pentobarbital sodium,\textsuperscript{22,23} and then the femur was detached and placed into a culture dish containing sterile phosphate-buffered saline (PBS). After the removal of the excess muscle tissue from the femur, the leg bone near the joint’s proximal end was cut using sterilized scissors. The leg bone was inserted into the femur marrow cavity with cold PBS to rinse the marrow. The rinsing medium was collected in a centrifuge tube and centrifuged at 500 × g for 10 minutes at room temperature. Following this, the culture medium was removed. Nonadherent cells were washed with PBS. A nonenzymatic cell dissociation solution was added, and the detached cells were collected. They were then centrifuged at 400 × g for 10 minutes at 4°C. Finally, the precipitated bone marrow–derived macrophages (BMDMs) were resuspended in Dulbecco’s modified Eagle’s medium/F12-10 medium and cultured in a 37°C, 5% CO₂ incubator.

Cell Culture and Treatment

THP-1 monocyes (TIB-202) were procured from ATCC (Manassas, VA). The THP-1 cells were cultured in Dulbecco’s modified Eagle’s medium and RPMI-1640 medium, along with 10% fetal bovine serum, at 37°C with 5% CO₂. The THP-1 cells were incubated with 50 ng/mL 12-O-tetradecanoylphorbol 13-acetate (TPA; catalog number 79346; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) to induce differentiation. After that, the TPA-stimulated THP-1 cells were treated with 100 nmol/L SP (catalog number S6272; Sigma-Aldrich) for 48 hours. For STAT6 inhibitor, 10 nmol/L STAT6-specific inhibitor YM-341619 (HY-134771; MedChemExpress, Monmouth Junction, NJ) was added to the culture solution before SP treatment, with equal volume of dimethyl sulfoxide used as negative control. To analyze the effect of SP on BMDMs, the isolated mouse BMDMs were incubated with 50 ng/mL SP for 48 hours.
Cell Transfection

shRNA of STAT6 (1#, 2#, and 3#), overexpression plasmid of LCP2 (oe-LCP2), and the negative control (NC) plasmids (sh-NC and oe-NC) were procured from VectorBuilder Inc. (Guangzhou, China). For cell transfection, approximately 2 × 10⁶ THP-1 cells were seeded into culture flasks and transfected with the above-mentioned plasmids using Lipofectamine 3000 (L3000015; Invitrogen, Thermo Fisher Scientific, Rockford, IL). After 48 hours, the cells were collected for subsequent analyses.

Animal Grouping and Treatment

Pregnant C57BL/6J mice (aged 4 months; weight, 24 ± 2 g) were procured from Charles River Laboratory Animal Technology. The offspring mice (aged 3 to 5 days) were randomly allocated into the following eight groups: saline group, HDM group, HDM + saline group, HDM + anti-SP group, HDM + saline group, HDM + LV-YM-341619 group (LV refers to lentivirus), HDM + YM-341619 + LV-oe-NC group, and HDM + YM-341619 + LV-oe-LCP2 group (n = 5 in each).

HDM was used to induce allergic asthma in the neonate mice. In short, each mouse was intranasally administrated with 10 μg HDM diluted in 20 μL saline. The induction was performed three times per week for 3 consecutive weeks. Mice in the saline group were given 20 μL saline each. After the final HDM or saline administration, the mice were euthanized.

The neutralizing antibody of SP (customized by GeneScript Biotech Corp., Jiangsu, China) or YM-341619 was administrated at 1 week after the first HDM treatment. The neutralizing antibody of SP (150 μg) was intraperitoneally injected into mice twice a week for 2 weeks, and YM-341619 (0.02 mg/kg) was administrated orally once a day for 2 weeks. The i.p. injection or oral administration of saline was set as control.

The lentivirus vectors for plasmid injection were procured from VectorBuilder as well. The lentivirus vector–carried plasmids were injected into mice through retro-orbital venous sinus at 1 week after the first HDM treatment.

BALF Preparation

Pulmonary bronchus of mice was washed with 500 μL PBS to obtain BALF, which was centrifuged at 400 × g at 4°C for 8 minutes to collect supernatant and cells in precipitates. The cell number was calculated by a Countess 3 automatic cell counter (AMQAX2000; Invitrogen, Thermo Fisher Scientific). The eosinophils (SSChighSiglecF⁺CD11c⁻), lymphocytes (SSClowCD45⁺), macrophages (SSChighSiglecF⁺CD11c⁺), and neutrophils (SSChighGr-1⁺) were sorted by flow cytometry.²⁴,²⁵

Flow Cytometry

Proportions of M1 and M2 macrophages in BALF from asthmatic or nonasthmatic children were analyzed by flow cytometry. In short, the BALF was centrifuged at 400 × g for 10 minutes to discard supernatant. The sediment was filtered through a 100-mesh filter, followed by centrifugation at 400 × g for 5 minutes. The cells were collected and resuspended in RPMI-1640 medium containing 10% fetal bovine serum. These cells were cultured in plates for 2 hours, after which the nonadherent cells were washed away. The adherent cells were incubated with CD68 and CCR7/CD206 antibodies in the dark for 30 minutes, followed by PBS washes and flow cytometric analysis. The immune cells in mouse BALF were stained by SglecF, CD11c, CD45, or Gr-1 antibody, and then the staining of each channel was analyzed. Compensation was adjusted to rule out the interference from autofluorescence. The antibodies used were as follows: CD68-PE (1:50; bs-0649R-PE; Bioss Biotechnology, Co, Ltd, Beijing, China), CCR7-fluorescein isothiocyanate (FITC; 1:100; 561271; BD Biosciences, Franklin Lakes, NJ), CD206-FITC (1:50; bs-4727R-FITC; Bioss), SiglecF-PerCP-eFluor 710 (1:50; 46-1702-82; Invitrogen), CD11c-APC (1:100; 550261; BD Biosciences), CD45-APC (1:100; 103112; Biolegend, San Diego, CA), and Gr-1–FITC (1:50; 11-5931-82; Invitrogen).

Histologic Staining

After mouse euthanasia, the lung tissues were harvested and fixed with paraformaldehyde at 4°C overnight. On the next day, the tissue samples were dehydrated in graded ethanol, cleared in xylene, embedded in paraffin, and cut into sections. After that, the sections were deparaffinized and rehydrated in ethanol for hematoxylin–eosin (G1120; Solarbio Science & Technology Co, Ltd, Beijing, China) and periodic acid–Schiff (PAS; G1281; Solarbio) staining. The tissue samples were washed with PBS washes and treated with Triton X-100 for 15 minutes, and blocked with 5% bovine serum for 30 minutes. After that, the sections were incubated with 150 μg/ml neutralizing antibody of SP (customized by GenScript Biotech Corp.) or YM-341619 for 1 hour. After staining, the sections were washed with PBS for 3 times, blocked with normal goat serum for 30 minutes, and incubated with primary antibody for 1 hour. After that, the sections were incubated with goat anti-primary antibody (1:50; 11-5931-82; Biolegend, San Diego, CA) for 1 hour, and then the sections were incubated with secondary antibody for 1 hour. After that, the sections were incubated with streptavidin–biotin–horseradish peroxidase (HRP) complex (1:50; bs-0649R-PE; Bioss Biotechnology, Co, Ltd, Beijing, China) for 1 hour, and then the sections were washed with PBS for 3 times, and incubated with 3,3′-diaminobenzidine (DAB) and hematoxylin for 10 minutes. After that, the sections were sealed with neutral resin and captured under a microscope.

Immunofluorescence Staining

Prepared mouse lung tissue sections were washed with PBS, penetrated with Triton X-100 for 15 minutes, and blocked with 5% bovine serum albumin for 30 minutes. After that, the sections were incubated with F4/80–FITC (1:100; LS-C447914; LSBio) and Arg-1–FITC (1:100; LS-C447914; LSBio) at 4°C overnight. The ratio of Arg-1⁺ cells/F4/80⁺ cells was then calculated.

Enzyme-Linked Immunosorbent Assay

Contents of SP and IgE in the collected human serum samples, and the contents of SP, IgE, HDM-IgE in collected
mouse BALF, were examined following the instructions of the enzyme-linked immunosorbent assay kits. In short, the reagents were added, and the OD (450 nm) of samples and standards of different concentration gradients was detected using the microplate reader. A standard curve was produced, by which the contents of the factors of interest were calculated. The enzyme-linked immunosorbent assay kits used included the following: substance P (KGE0107; R&D Systems, Minneapolis, MN), IgE (human; BMS2097; Invitrogen), IgE (mouse; EMIGHX10; Invitrogen), and HDM-IgE (mouse; customized by GenScript).

**Western Blot Analysis**

Tissues or cells were lysed in radioimmunoprecipitation assay lysis buffer (R0278; Sigma-Aldrich) to collect total protein, whose concentration was examined by the bicinchoninic acid method. Equal amounts of prepared protein samples were separated by gel electrophoresis and wet transferred onto polyvinylidene fluoride membranes. The membranes were blocked by 5% nonfat milk for 45 minutes and then incubated with anti-STAT6 (1:1000; 5397; Cell Signaling Technology, Beverly, MA), anti- phosphorylated STAT6 (1:1000; AP1390; ABclonal Technology Co, Ltd, Wuhan, China), or anti-γ-glutamyl transferase (GAPDH; 1:2000; ab9485; Abcam Inc., Cambridge, MA) at 4°C overnight, followed by incubation with horseradish peroxidase–conjugated secondary antibody (1:5000; ab6721; Abcam) at ambient temperature for 40 minutes. The blots were developed by enhanced chemiluminescence reagent (WBULS0500; Sigma-Aldrich). Expression of target protein relative to GAPDH was analyzed by ImageJ software (NIH, Bethesda, MD).

**RNA Extraction and Quantification**

The TRI reagent (T9424; Sigma-Aldrich) was used for total RNA extraction. After examination of the concentration and purity, the extracted RNA was reverse transcribed to cDNA using the PrimeScript RT Master Mix (RR036Q; Takara Holdings Inc., Kyoto, Japan). After that, quantitative PCR (qPCR) was performed using TB Green Premix Ex Taq II (qPCR) was performed using TB Green Premix Ex Taq II (Tli RNaseH Plus; TaKaRa). Gene expression relative to GAPDH was determined by the 2−ΔΔCT method. The following primers were used: LCP2, 5′-GGAGGAAAGCCACCCGTGGCCAAA-3′ (forward) and 5′-GCTCAATAGGAAGTGGTGGG-3′ (reverse); IL-10, 5′-TCTCCAGAATGCTGCTTTG-3′ (forward) and 5′-TCGACAAGGCTTGCCACCA-3′ (reverse); CD204, 5′-TGGCAACAGGTCCTGTCGTC-3′ (forward) and 5′-GTGCAAGGTAGCCTTCCAGGCATTC-3′ (reverse); CD163, 5′-CAGAGAAGAATCTTGAGCACAG-3′ (forward) and 5′-CAGGCACCAAGGCTTTTGAGCT-3′ (reverse); and GAPDH, 5′-GCTCTCCCTGGCTTGAGC-3′ (forward) and 5′-ACCACCTGTGCTGTAGC-3′ (reverse).

**ChIP-qPCR**

Binding between STAT6 and the LCP2 promoter was examined using the EZ-Magna chromatin immunoprecipitation (ChIP) kit (17-408; Sigma-Aldrich). In short, TPA-induced THP-1 cells were treated with methanol for protein-DNA cross-linking, which was terminated by the addition of glycine. The cells were then lysed, followed by ultrasonication for DNA truncation. Thereafter, the lysates were reacted with anti-STAT6 (1:50; 5397; Cell Signaling Technology) or the isotype control IgG to precipitate the DNA-protein complexes. The complexes were de-cross-linked by proteinase K. The DNA was collected and purified for qPCR analysis.

**Statistical Analysis**

Data were analyzed by Prism 8.0.2 (GraphPad, La Jolla, CA) and expressed by the means ± SD. Intergroup differences were compared by the unpaired t test. When over two groups were involved, one- or two-way analysis of variance was applied, followed by the Tukey post hoc test. Differences in clinical characteristics between asthmatic and control children were analyzed by the Fisher exact test and the unpaired t test. Correlation between SP and M2 polarization of macrophages was determined by the Pearson correlation analysis. P < 0.05 was considered significant.

**Results**

**SP Is Up-Regulated in Asthmatic Children and Linked to M2 Polarization of Macrophages**

First, increased eosinophil population and IgE level were detected in peripheral blood samples from asthmatic children compared with those from nonasthmatic controls (Supplemental Table S1). According to flow cytometry, the asthmatic children had a significantly elevated proportion of M2 macrophages (CD68+CD204+) in their BALF compared with the control children, although the proportion of M1 macrophages (CD68+CD86+) was not significantly elevated (Figure 1A). Meanwhile, the asthmatic children showed a significantly elevated proportion of M2 macrophages (CD68+CD204+). However, the M1/M2 ratios did not differ between asthmatic and control children (Figure 1B). This suggested that increased SP levels in asthmatic children were associated with the differentiation of M2 macrophages (Figure 1C). To validate the effect of SP on macrophage polarization, THP-1 cells were treated with TPA to induce monocyte-to-macrophage differentiation, followed by SP treatment. The flow cytometry showed that the SP treatment significantly increased the proportion of M2 macrophages, and similar results were found in the isolated BMDMs (Figure 1D). In addition, positive correlation of NK-1R was detected in both TPA-treated THP-1 cells and the BMDMs (Figure 1E), indicating a possible interaction between SP and macrophages.
Figure 1  Substance P (SP) is up-regulated in asthmatic children and linked to M2 polarization of macrophages. A: Proportions of M1 (CD68⁺ CCR7⁻) and M2 (CD68⁺ CCR7⁺) macrophages in bronchoalveolar lavage fluid (BALF) of control and asthmatic children determined by flow cytometry. B: Content of SP in the serum and BALF of control and asthmatic children determined using enzyme-linked immunosorbent assay kits. C: Positive correlations between SP contents and proportion of M2 macrophages in BALF in asthmatic children. D: Proportion of M2-skewed 12-O-tetradecanoylphorbol 13-acetate (TPA)—induced THP-1 cells (macrophages) and the isolated mouse bone marrow—derived macrophages (BMDMs) analyzed by flow cytometry. E: Expression of neurokinin receptor NK-1R in TPA-induced THP-1 cells and in mouse BMDMs determined using quantitative PCR analysis. A, B, and D: Differences were compared by the unpaired t test. C: Pearson correlation analysis was applied. n = 13 (A and B, control children); n = 18 (A–C, asthmatic children); n = 3 (D and E). *P < 0.05. FITC, fluorescein isothiocyanate; NS, no significance; PBS, phosphate-buffered saline.

Neutralizing Antibody of SP Alleviates Inflammation in HDM-Challenged Mice

Asthmatic models were induced on neonatal mice by HDM challenge, followed by treatment with the neutralizing antibody of SP (anti-SP). Enzyme-linked immunosorbent assay results showed that the HDM-challenged mouse pups had significantly elevated SP content in their BALF, which was suppressed by the anti-SP treatment (Figure 2A). \(^\text{32}\) Compared with saline treatment, the HDM challenge led to significant inflammatory cell infiltration in mouse bronchus, bronchial congestion, and shedding of epithelial cells, which were alleviated by anti-SP as well (Figure 2B). PAS results showed that the population of goblet cells (PAS⁺) in mouse
Neutralizing antibody of substance P (SP; anti-SP) alleviates inflammation in house dust mite (HDM)—challenged mice. A murine model of pediatric asthma was induced by intranasal administration of HDM, followed by treatment of saline or anti-SP. A: SP contents in mouse bronchoalveolar lavage fluid (BALF) examined by enzyme-linked immunosorbent assay (ELISA). B: Pathologic changes in mouse lung tissues examined by hematoxylin-eosin staining (specific locations of pathologic injuries are indicated by arrows). C: Number of goblet cells in mouse lung tissues detected by periodic acid-Schiff staining (specific locations of goblet cells are indicated by arrows). D and E: Serum contents of total IgE (D) and HDM-specific IgE (E) in mice determined using ELISA kits. F: Populations of total cells and immune cells (eosinophils, lymphocytes, macrophages, and neutrophils) in BALF of mice determined by flow cytometry (gating strategy: after the exclusion of doublets and debris, SSC<sup>high</sup> and SSC<sup>low</sup> cells were first identified; lymphocytes within the SSC<sup>low</sup> cells were sorted using the pan-hematopoietic marker CD45; eosinophils and macrophages within the SSC<sup>high</sup> cells were sorted as Siglec F<sup>+</sup>CD11c<sup>+</sup>; and neutrophils within the SSC<sup>high</sup> cells were sorted using Gr-1). G: Proportion of M2 macrophages in mouse lung tissues examined by immunofluorescence staining (green fluorescence: Arg-1; red fluorescence: F4/80). A and D–G: Differences were compared by one-way analysis of variance, followed by the Tukey post hoc test. n = 5 (B–G). *P < 0.05. Scale bars = 100 μm (B, C, and G).
**Figure 3**  Substance P (SP) promotes M2 polarization of macrophages by up-regulating STAT6. **A:** Volcano plots for differentially expressed genes between M1 and M2 macrophages in the GSE162698 data set [dashed vertical lines denote the criterion of log2 (fold change) > 1, and dashed horizontal line denotes the criterion of P < 0.05]. **B:** A Venn diagram for the screened differentially expressed genes, downstream targets of SP predicted from the SEA database, and the human transcription factors predicted from the human TFDB system. **C:** Phosphorylation of STAT6 (p-STAT6) in macrophages after dimethyl sulfoxide (DMSO) or YM-341619 treatment, examined by WB analysis. **D:** Phosphorylation of STAT6 in macrophages after SP induction and YM-34169 treatment, examined by WB analysis. **E:** Proportion of M2 macrophages after SP induction and YM-341619 treatment, examined by flow cytometry. **F:** Relative protein expression of STAT6 in macrophages after dimethyl sulfoxide (DMSO) or YM-341619 treatment, examined by WB analysis. **G:** Proportion of M2 macrophages after SP induction and YM-341619 treatment, examined by flow cytometry.
lungs tissues was increased by HDM challenge but reduced by anti-SP (Figure 2C). The concentrations of total IgE and HDM-specific IgE in mouse serum were increased by HDM treatment but decreased by anti-SP treatment as well (Figure 2, D and E). Cells in the BALF of mice were harvested. A significant increase in the population of immune cells (eosinophils, lymphocytes, macrophages, and neutrophils) was found in the HDM group, but this increase was significantly decreased after further anti-SP treatment (Figure 2F). Immunofluorescence staining on mouse lung tissues identified a significantly elevated proportion of M2 macrophages (F4/80^Arg-1^') in HDM-treated mice. The elevation was reduced by anti-SP as well (Figure 2G).

SP Promotes M2 Polarization of Macrophages by Up-Regulating STAT6

To unravel the target(s) of SP participating in M2 polarization of macrophages, differentially expressed genes between M1 and M2 macrophages were screened using the Gene Expression Omnibus data set GSE162698 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162698, last accessed April 19, 2021) (Figure 3A). Meanwhile, the targets of SP were queried in the SEA database (https://sea.bkslab.org/jobs/search_16828fad-4d23-4a54-a930-3785addb6cd8, last accessed April 21, 2021), which were intersected with human transcription factors predicted in the human TFDDB system (http://bioinfo.life.hust.edu.cn/HumanTFDB#!/download, last accessed May 7, 2021), resulting in three shared outcomes: MLX, STAT3, and STAT6 (Figure 3B). Among the three candidates, STAT6 has recently been reported to activate M2a macrophages to exacerbate allergic inflammation in asthmatic lungs.20

Thereafter, the STAT6 activity in the TPA-induced THP-1 cells (macrophages) was determined. It was found that the phosphorylation of STAT6 was significantly enhanced after SP treatment (Figure 3C). Moreover, the macrophages were treated with the STAT6 inhibitor, YM-341619, which successfully suppressed phosphorylation of STAT6 (Figure 3D). In this context, the proportion of M2 macrophages was significantly reduced following SP treatment (Figure 3E). In addition, STAT6 knockdown was induced in the macrophages by using three shRNAs. The shRNA of STAT6 1# with the greatest interfering efficiency (Figure 3F) was selected for subsequent use. Following this, the flow cytometric analysis showed that the SP-induced M2 polarization of macrophages was decreased (Figure 3G). This evidence indicates that the SP promotes M2 polarization of macrophages due, at least in part, to the up-regulation of STAT6.

STAT6 Inhibition Suppresses Inflammation in HDM-Challenged Mice

The function of STAT6 in macrophages was further examined in vivo. The HDM-challenged mice were treated with YM-341619 as well. The STAT6 suppression was then detected in mouse lung tissues (Figure 4A). In this context, the inflammatory cell infiltration, bronchial congestion, and shedding of epithelial cells (Figure 4B) and the number of goblet cells (Figure 4C) in mouse lungs were reduced according to hematoxylin-eosin and PAS staining results. Meanwhile, the YM-341619 treatment led to a decline in the concentrations of total IgE as well as HDM-specific IgE in mouse serum (Figure 4, D and E). Immunofluorescence staining showed that the proportion of M2 macrophages in mouse lung tissues was decreased by YM-341619 as well (Figure 4F).

YM-341619 Suppresses Transcription of LCP2

The downstream targets of STAT6, as a transcription factor, were predicted in the hTFTarget system (http://bioinfo.life.hust.edu.cn/hTFtarget#!/targets/chipseq_tf?tf=STAT6, last accessed March 1, 2022). The predicted targets were input to the bioinformatics system STRING (https://cn.string-db.org/cgi/input?sessionId=b8v4gX6PG&input_page_show_search=off, last accessed March 10, 2022) to construct a protein-protein interaction network, in which SH2B3, LCP2, and SOCS1 were found as the core proteins (Figure 5A). Among them, only SH2B3 and LCP2 showed differential expression between M1 and M2 macrophages in the GSE162698 data set. Meanwhile, LCP2 has been reported as an immune-related factor showing a positive correlation with the M2 macrophage marker CD206 in cancer.21 Following this, significant expression of LCP2 was detected in both the TPA-treated THP-1 cells and the BMDMs, the according to qPCR analysis (Figure 5B). In addition, the LCP2 expression was up-regulated in SP-treated macrophages but reduced after YM-341619 treatment (Figure 5C). The binding sites between STAT6 and LCP2 promoter were predicted from the hTFTarget system (Figure 5D). The direct binding of STAT6 with LCP2 promoter fragments was validated by the ChIP-qPCR assay (Figure 5E). The THP-1 cells were transfected with oe-LCP2. The successful up-regulation of LCP2 was detected by qPCR analysis (Figure 5F), which significantly increased the proportion of M2 macrophages (Figure 5G). Complementing this evidence, the qPCR analysis further revealed that the LCP2 overexpression significantly increased the expression of several M2

by flow cytometry. F: Protein level of STAT6 in macrophages after shRNA of STAT6 (sh-STAT6) 1-3# transfection, determined by WB analysis. G: Proportion of M2 macrophages after STAT6 silencing, determined by flow cytometry. C–G: Differences were compared by the unpaired t test (C–E and G) or one-way analysis of variance, followed by the Tukey post hoc test (F). n = 3 (C–E and G). *P < 0.05. FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sh-NC, shRNA of negative control.
Figure 4  STAT6 inhibition suppresses inflammation in house dust mite (HDM)—challenged mice. The HDM-challenged mice were given YM-341619 orally. A: Phosphorylation of STAT6 (p-STAT6) in mouse lung tissues, detected by Western blot analysis. B: Pathologic changes in mouse lung tissues examined by hematoxylin-eosin staining (specific locations of pathologic injuries are indicated by arrows). C: Number of goblet cells in mouse lung tissues detected by periodic acid-Schiff staining (specific locations of goblet cells are indicated by arrows). D and E: Serum contents of total IgE (D) and HDM-specific IgE (E) in mice, determined using enzyme-linked immunosorbent assay kits. F: Proportion of M2 macrophages in mouse lung tissues examined by immunofluorescence staining (green fluorescence: Arg-1; red fluorescence: F4/80). A and D–F: Differences were compared by the unpaired t test. n = 5 (A–F). *P < 0.05. Scale bars = 100 μm (B, C, and F). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 5  STAT6 inhibition suppresses transcription of lymphocyte cytosolic protein 2 (LCP2). A: A protein–protein interaction network of the downstream targets of STAT6 predicted in hTFtarget. B: LCP2 mRNA expression in 12-O-tetradecanoylphorbol 13-acetate (TPA)–treated THP-1 cells and in the bone marrow–derived macrophages (BMDMs), determined using quantitative PCR (qPCR) analysis. C: LCP2 mRNA expression in the TPA-treated THP-1 cells after substance P (SP) or YM-341619 treatment, examined by qPCR analysis. D: Binding relationship and the detailed binding sites between STAT6 and LCP2 promoter predicted in hTFtarget. E: Binding between STAT6 with LCP2 promoter, examined by chromatin immunoprecipitation–qPCR assay. F: LCP2 expression in THP-1 cells after overexpression of LCP2 (oe-LCP2) transfection determined by qPCR analysis. G: Proportion of M2 macrophages after SP and YM-341619 treatment.
markers, including IL-10, CD204, and CD163, in the THP-1 cells (Figure 5H).

LCP2 Overexpression Restores Lung Inflammation in Mice by Elevating the Proportion of M2 Macrophages

To examine the function of LCP2 in vivo, the HDM-challenged mice were injected with lentivirus vector–carried and oe-LCP2 given YM-341619 orally. Successful up-regulation of LCP2 mRNA was detected in mouse lung tissues (Figure 6A). The LCP2 up-regulation restored the infiltration of inflammatory cells, bronchial congestion, and shedding of epithelial cells (Figure 6B), as well as the abundance of goblet cells (Figure 6C), in the lung tissues of mice. In the meantime, the serum concentrations of total IgE and HDM-specific IgE in mouse were rescued as well (Figure 6, D and E). In the collected lung tissues, increased intensity of immunofluorescence staining of F4/80 “Arg-1” was detected (Figure 6F). In the BALF of mice, elevated contents of IL-4 and IL-13 were detected as well.

Discussion

The inability of the existing treatments in controlling the symptoms of allergic asthma without adverse effects indicates that more effective and noninvasive therapeutic options are being expected. Directly treating and alleviating the acute symptoms induced by the immune system over-reaction to accelerate discomfort relief as much as possible has become a promising therapeutic approach for this allergic disease, which, however, requires more intensive understanding of the underpinning molecular mechanisms involved in immune hyperactivation. In the present work, it is reported that an SP-STAT6-LCP2 axis potentially plays a role in sustaining the predominance of M2 macrophages in allergic asthma and aggravating inflammation. These findings offer new insights for asthma management.

The first findings of the present study were elevated SP concentrations in the serum samples of asthmatic children, along with increased population of M2 macrophages in their BALF. Indeed, up-regulation of SP and its major receptor NK-1R has been detected in patients with allergic asthma or with allergic rhinitis. Similarly, the SP up-regulation, along with elevated infiltration of inflammatory cells as well as increased expression of immune cytokines, has been detected in asthmatic animal models, which was crucial for the airway hyperresponsiveness and disease severity. In fact, many substances in the lung, including allergen, histamine, prostaglandins, and leukotrienes, can induce SP secretion from sensory nerves, and SP receptors on macrophages could be elevated on infection. As for the direct impact of SP on macrophages, it was found that the SP treatment increased M2 polarization of TPA-induced THP-1 cells in vitro. In a previous work by Lim et al., SP induced an M1-to-M2 phenotype shift of macrophages by activating the phosphorylated inositol 3-kinase/Akt/mammalian target of rapamycin cascade, as manifested by increased M2 markers, including Arg-1, CD163, and CD206. Similarly, SP treatment elevated the M2 markers, including IL-4, IL-10, and Arg-1, in lipopolysaccharide-stimulated RAW 264.7 macrophages by elevating hemeoxygenase-1. Similar promoting effects of SP on M2 skewing of macrophages have also been witnessed in several disease model of tissue injuries. It was observed that treatment of anti-SP alleviated serum IgE level, inflammatory cell infiltration, and goblet cell hyperplasia, and reduced the M2 macrophage population, in mice induced by HDM, which are all typical type 2 inflammatory responses. This ample evidence suggests that SP is linked to increased population of M2 macrophages and inflammation aggravation in asthmatic lungs.

When it comes to the downstream targets of SP responsible for M2 skewing, the GSE162698 data set and the SEA and human TFBDB bioinformatics systems were analyzed, with STAT6 identified as a key target among the three candidates (MLX, STAT3, and STAT6). STAT6 stood out as an interesting study subject as it has been recognized as a driver for macrophage M2 polarization. In general, STAT6 is primarily activated in response to IL-4 and IL-13 stimulations, which induce M2a polarization of macrophages. STAT6 has been reported to activate the transcription of M2 macrophage-related genes, such as Arg-1, mannose receptor, and chitinase-like protein 3, and several chemokines, such as C-C motif chemokine ligand (CCL) 17 and CCL24. In allergic asthma, STAT6 has been found to activate M2a macrophages and lead to allergic inflammation in the lungs. Another work by Williams et al. demonstrates that STAT6 mediates the type 2 inflammation induced by CCL17 only but not that induced by CCL22 in asthma. However, in the study by Lim et al., SP was demonstrated to induce M2c polarization of macrophages without affecting STAT6 activity. Conversely, it was found that the SP treatment did increase STAT6 phosphorylation in the TPA-treated THP-1 cells. This was partly aligned with the findings by Miyazaki et al. that SP increased STAT3 activity in human dermal microvascular endothelial cells, like IL-4 and IL-13 did. This discrepancy might be attributed to specific cellular and pathologic contexts.

In addition, SP itself has been demonstrated to function upstream of IL-4, potentially inducing IL-4 release. As mentioned earlier, SP has been found to increase the production of IL-4, IL-10, and Arg-1 in lipopolysaccharide-
Figure 6  Lymphocyte cytosolic protein 2 (LCP2) overexpression restores lung inflammation in mice by elevating the proportion of M2 macrophages. The house dust mite (HDM)—challenged mice were infected with lentivirus (LV)—carried overexpression of LCP2 (oe-LCP2) and treated with YM-341619 orally.  

A: mRNA expression of LCP2 in mouse lung tissues detected by quantitative PCR analysis.  

B: Pathologic changes in mouse lung tissues examined by hematoxylin–eosin staining (specific locations of pathologic injuries are indicated by arrows).  

C: Number of goblet cells in mouse lung tissues detected by periodic acid–Schiff staining (specific locations of goblet cells are indicated by arrows).  

D and E: Serum contents of total IgE (D) and HDM-specific IgE (E) in mice, determined using enzyme-linked immunosorbent assay kits.  

F: Proportion of M2 macrophages in mouse lung tissues examined by immunofluorescence staining.
stimulated macrophages. Therefore, SP possibly activates STAT3, at least in part, through the IL-4 signaling. Not surprisingly, in the constructed mouse model of asthma, it was found that the specific inhibition of STAT6 reduced M2 polarization of macrophages. Moreover, by constructing a protein-protein interaction network established on the basis of the transcription targets of STAT6 predicted in the hTFtarget system, LCP2 was identified as a critical target of STAT6, with the direct transcriptional regulation validated by ChIP assay. Although there has been no direct evidence concerning the role of LCP2 in asthmatic inflammation, a positive correlation between LCP2 and M2 macrophages has been reported in several types of human cancers, indicating the potential association of LCP2 with type 2 inflammation in allergic asthma. Indeed, the artificial up-regulation of LCP2 rescued M2 polarization of macrophages and aggravated type 2 inflammation cytokines in mice.

Collectively, this study demonstrates that SP sustains M2 macrophage predominance and allergic inflammation in pediatric asthma by increasing STAT6-dependent transcription activation of LCP2 (Figure 7). However, there are several limitations of the present study that should be acknowledged. First, the GSE162698 data set, which contains gene expression profiles of M2 macrophages induced by IL-4 or IL-10 stimulations, was applied as a reference to probe downstream targets of SP. This was not the optimal strategy, and an RNA-sequencing analysis would be better to identify the targets most relevant to SP effect. Second, SP has been summarized to be expressed by an array of cell types, including neurons, astrocytes, microglia, epithelial cells, endothelial cells, immune cells, and stem cells. The primary cell types that secret SP in the lung tissue microenvironment were not investigated, primarily because of the time and economic constraints. In addition, the authors performed abundant in vitro experiments. However, conducting additional experiments, such as cell-specific gene knockouts or conditional knockout models targeting macrophages specifically, would provide more intensive insight into the specific roles of STAT6 and LCP2 in macrophage...

**Figure 7**

A: Structure diagram of substance P (SP). B: During the course of asthma, SP is upregulated, which promotes STAT6 expression and the consequent transcriptional activity of lymphocyte cytosolic protein 2 (LCP2). This leads to an elevated proportion of M2 macrophages (MΦ), ultimately exacerbating allergic inflammation in mice.

(green fluorescence: Arg-1; red fluorescence: F4/80). A and D–F: Differences were compared by the unpaired t test. n = 5 (A–F). *P < 0.05. Scale bars = 100 μm (B, C, and F).
polarization in the asthma context. Such experiments were not performed because of the time, economic, and technique constraints. These gaps need to be addressed in future research endeavors. Nevertheless, this study provides direct evidence that specific suppression of any member(s) of the SP/STAT6/LCP2 axis may reduce the population of M2 macrophages to alleviate the symptoms of allergic asthma. In addition, although both STAT6 and LCP2 have been found to be closely correlated with the M2 macrophage population, they may also regulate other types of immune cells. This may represent an interesting and worth-investigating direction for future research.

Author Contributions
M.L. conceived and designed this study, analyzed the data, drafted the manuscript, and participated in funding acquisition; X.Z. performed the experiments, helped to analyze the data, and drafted the manuscript; and W.X. participated in experiment design, edited the manuscript, and performed the experiments. All the authors read and approved the final manuscript.

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None declared.

Supplemental Data
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