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Water-soluble chitosan inhibits nerve growth factor and attenuates allergic inflammation in mite allergen-induced allergic rhinitis

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Capsule summary (35 words)

Nerve growth factor induces nasal inflammation by augmenting the existing T_H2 immune response, and this can be suppressed by water-soluble chitosan, a natural, nontoxic product that provides new therapy for allergic rhinitis.

Keywords: house dust mite, allergic rhinitis, nerve growth factor, innate lymphoid cells, water-soluble chitosan

Abbreviations:

AR: allergic rhinitis

AHR: airway hyperresponsiveness

BPM: breath per minute

CGRP: calcitonin gene-related peptides

Der p: Dermatophagoides pteronyssinus

ILC2: group 2 innate lymphoid cells

NGF: nerve growth factor

NLF: nasal cavity lavage fluid

p75^{NTR}: p75 neurotrophin receptor

ROS: reactive oxygen species

SP: substance P (SP)

T_H2s: type 2 helper T lymphocytes

TLR: toll-like receptor

TrkA: tropomyosin receptor kinase A

TRPV-1: transient receptor potential cation channel subfamily V member 1

TSLP: thymic stromal lymphopoietin

VIP: vasoactive intestinal polypeptide

EWSC: extensive water-soluble chitosan.

WT: wild type

ACCEPTED MANUSCRIPT

To the editor,

Allergic rhinitis (AR), which affects approximately 10%–15% of the population worldwide, is a chronic inflammatory disease of the nasal mucosa involving nasal airway hyperresponsiveness (AHR), an inappropriate activity of the nervous system and neuropeptides released.¹ Nerve growth factor (NGF), one of the neurotrophins produced by the nasal epithelium, eosinophils, and mast cells, plays an important role in neuroimmune interactions by augmenting the existing T_H2 immune response.² NGF, via its high affinity receptor TrkA, activates ion channels via the capsaicin receptor TRPV1 in sensory neurons and further modulates VIP production, which activates the T_H2 immune response.³ This bidirectional communication between nerves and immune cells forms feedback loops contributing to AHR and mucosal inflammation in AR. Although blocking the NGF–TrkA axis can alleviate allergic disease,⁴ an appropriate inhibitor for interrupting NGF function remains undiscovered. The intranasal application of nontoxic water-soluble chitosan (WSC, deacetylated chitin) could reduce house dust mite, *Dermatophagoides pteronyssinus* (Der p), allergen-induced airway inflammation, through TSLP and arginase 1 inhibition in a mouse model of allergic asthma.⁵

To evaluate the effect of extensive WSC (EWSC) in AR, a Der p-sensitized and -challenged AR mouse model was established (see Online Repository text and Fig E1A

in this article's Online Repository at www.jacionline.org). These mice developed the hallmarks of AR, with significantly higher levels of serum total and Der p-specific IgE (Fig. 1A) and decreased respiratory rates (BPM) and AHR (as measured by PenH; Fig. 1B), which are the two cardinal signs of AR. To evaluate the effect of EWSC in AR, Der p-challenged mice were intranasally administrated with EWSC, and they showed significantly higher BPM and lower AHR than did non-treated AR mice (Fig. 1B). Significantly lower levels of NGF and interleukin (IL)-6 and decreased levels of tumor necrosis factor (TNF) α , IL-4, IL-5, and IL-13 were observed in the nasal lavage fluids (NLFs) of EWSC mice (Fig. E1B-E). Brain-derived neurotrophic factor (BDNF) was not detectable in the NLFs of the experimental mice (Fig. E1F). By contrast, significantly higher NGF, IL-6, and VIP levels were noted in the NLFs of AR mice than of sensitization control group (SC) mice, and these levels were significantly decreased after EWSC treatment (Fig. E1G-I). In histology, EWSC treatment significantly reduced Der p-induced nasal respiratory epithelium (RE) hyperplasia (Fig. 1C), and the infiltration of eosinophils (Fig. 1D) and degranulated mast cells (Fig. 1E) in the nasal mucosa. Compared with AR mice, EWSC mice exhibited increased expression of T-bet-positive and decreased expression of GATA3-positive cells in their nasal epithelium (Fig. E2A and E2B). NGF was also markedly expressed in the nasal epithelium, particularly in the RE, of AR mice but was decreased in EWSC and SC

mice (Fig. E2C). No significant differences between the degrees of staining for NGF receptors, such as p75^{NTR} (Fig.E2D) and TrkA (Fig.E2E), and TRPV-1 (Fig.E2F) was noted in the nasal epithelia of our experimental mice. In the AR group, the Der p allergen challenge significantly increased the levels of IL-4⁺T_H2, IL-4⁺ILC2⁺, IL-13⁺ILC2⁺, and ROR α ⁺ staining cells in the nasal draining lymph nodes (Fig. 1F-H). Intranasal administration of EWSC significantly decreased T_H2 cells expressing IL-4, IL-5, and IL-13 (Fig. 1F). Furthermore, EWSC treatment significantly reduced Der p allergen challenge-induced IL-4⁺ILC2⁺ (Fig. 1G), and ROR α ⁺ staining cell infiltration (Fig. 1H) in the nasal mucosal of AR mice. EWSC inhibited TNF- α and Der p-induced NGF production in primary lung cells and the human nasal epithelial cell line (RPMI-2650), respectively (Fig. E3A, E3B). These results suggest that NGF- and T_H2-related cytokines created a positive feedback loop amplifying allergic inflammation in the upper-airway epithelial cells, and EWSC attenuated allergic inflammation and epithelial cell damage, by inhibiting NGF biosynthesis during allergic T_H2 immune responses. To confirm the role of NGF in allergen-induced inflammation of AR, NGF heterozygote-deficient (Ngf^{+/-}) mice were raised in the C57BL/6 background and sensitized using the same protocol as wild-type AR mice (Fig. E4A). The experimental mice exhibited a mild reduction in total serum IgE (Fig. E4B) and reduced NGF expression (Fig. E4C and E4D), RE thickness (Fig. 2A), eosinophil infiltration (Fig. 2B),

and mast cell degranulation were noted in the nasal epithelium (Fig. 2C) compared with the wild-type AR (WT/AR) mice. Although no significant baseline differences existed in the number of T_H2 and ILC2 cells in the draining lymph nodes between NGF heterozygote-deficient (Ngf^{+/-}) and WT mice (Fig. 2D and 2E), after the Der p allergen sensitization and challenge, only the IL-13⁺ ILC2⁺ cell numbers were significantly lower in Ngf^{+/-}/AR mice than in WT/AR mice ($p < 0.05$).

The nose and lung airway epithelium is densely innervated by sensory fibers, most of which express markers of nociceptors, including TRPV-1, and TRPA-1.⁶ T_H2 cytokine IL-5 activated lung nociceptive neurons, which in turn secreted VIP to activate innate lymphoid cells and lymphocytes associated with the development of airway allergic responses. These cells secrete cytokines, such as IL-5, recruit effector immune cells integral to reaching the disease state and further activate the sensory neurons.⁷ After EWSC treatment, the NGF and VIP levels were decreased in NLFs examined in this study. We speculate that although no NGF receptor, TrkA, is present on the cell surface of ILC2 (Fig. E5B), NGF acts on the nociceptor TRPV1 to increase VIP release from sensory neurons, stimulating lung-resident ILC2 and T_H2 cells via the VPAC2 receptor in allergic inflammation.⁸ Therefore, the nasal administration of EWSC not only reduced NGF production by inflamed tissues but also inhibited the NGF-induced positive feedback loop involving nociceptors, VIP, TSLP, T_H2, and ILC2

cells, which amplify type 2 inflammation (Fig.E6). In summary, our results have demonstrated that the nasal administration of EWSC attenuated allergen-induced nasal AHR and inflammation via the inhibition of NGF and its amplification loop of type 2 immune response. This strategy of using natural product like EWSC focused on interrupting NGF sensory neuron immunosignaling and innate immune protective actions, thereby constituting a possible novel therapeutic approach to allergic airway inflammation such as in asthma and AR.⁹

Sincerely

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Figure Legends

Figure 1. EWSC improves nasal pathological function and airway inflammation in Der p-induced AR. (A) Total IgE and Der p-specific IgE concentrations of serum were measured through ELISA. (B) Respiration rate and airway resistance were measured using the Buxco[®] noninvasive airway resistant measurement system. (C) Thickness of the nasal respiratory epithelium (RE: respiratory epithelium; OE: Olfactory epithelium). (D) The positive-stained eosinophils (black arrows) and (E) degranulated mast cells (red arrows) were counted in 400 $\times$ microscopic fields per group, and the average numbers were calculated. (F) Draining lymph nodes of the nasal mucosa were isolated, and T_H2 and ILC2 cells were analyzed using flow cytometry. T_H2⁺IL4⁺, T_H2⁺IL5⁺, and T_H2⁺IL13⁺ cell percentage; (G) ILC2⁺IL4⁺, ILC2⁺IL5⁺, and ILC2⁺IL13⁺ cell percentages in the draining lymph nodes of the nasal mucosa. (H) Sections of the nasal cavity were immunostained with ROR α antibodies and reacted with DAB (scale bars = 100 μ m). Data are expressed as mean $\pm$ SEM (n $\geq$ 8). *: p < 0.05; **: p < 0.01 compared with the AR group, in Student's t-test.

Figure 2. Der p fails to promote allergen-induced inflammation in NGF-deficient

mice. (A) Thickness of the nasal respiratory epithelium. (B) The positive-stained eosinophils (black arrows) and (C) degranulated mast cells (red arrows) were counted in 400× microscopic fields per group, and the average numbers were calculated (scale bars: 50 µm or 100 µm). (D) Draining lymph nodes of the nasal mucosa were isolated, and T_H2 and ILC2 cells were analyzed using flow cytometry and T_H2⁺IL4⁺, T_H2⁺IL5⁺, and T_H2⁺IL13⁺ cell percentages; (E) ILC2⁺IL4⁺, ILC2⁺IL5⁺, and ILC2⁺IL13⁺ cell percentages in the draining lymph nodes of the nasal mucosa. Data are expressed as mean ± SEM (n ≥ 8). *: p < 0.05; **: p < 0.05, compared with the AR group in Student's t-test.

Fig. 1

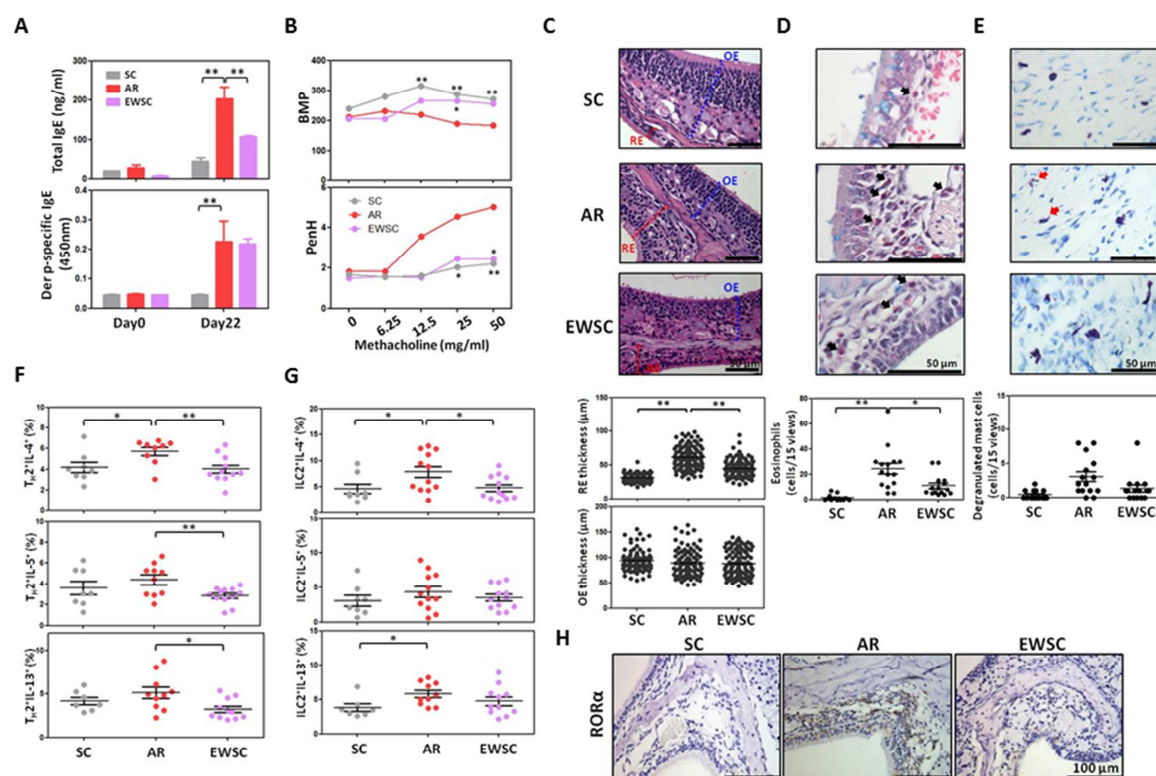
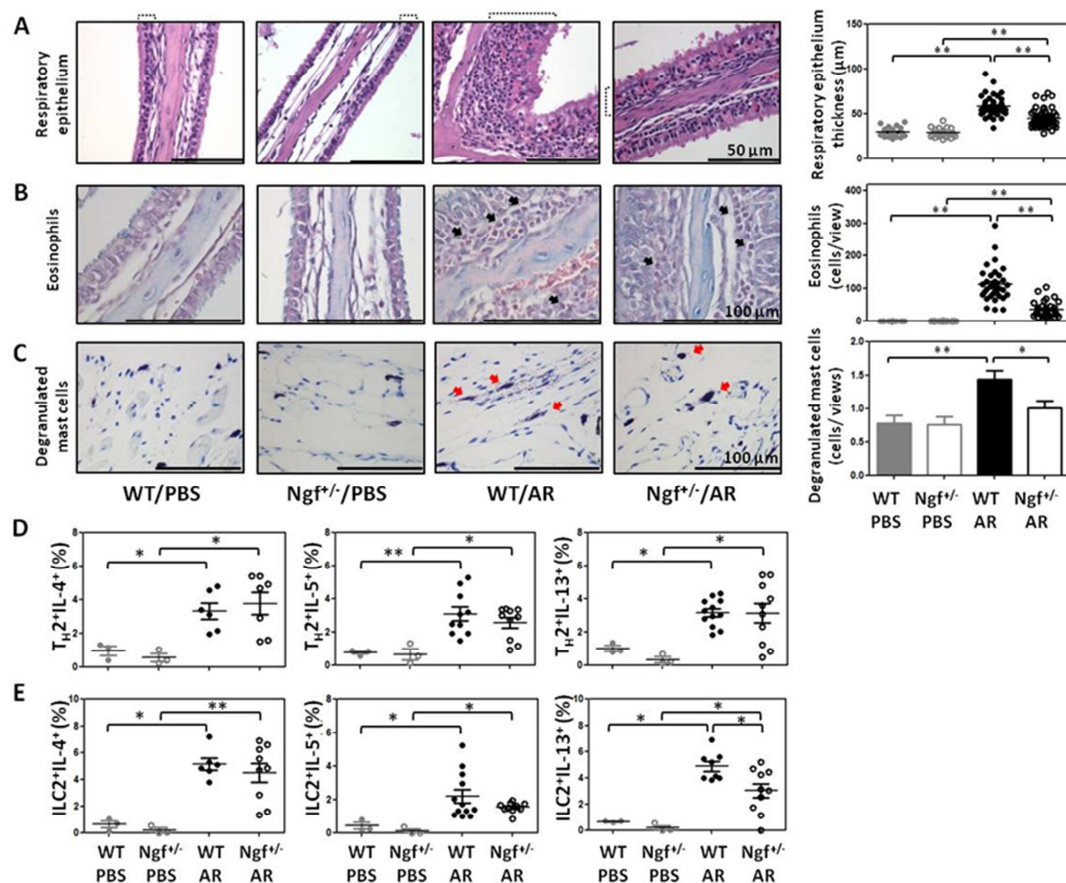


Fig. 2 A



Supplemental text file

Material and Methods

Murine allergic rhinitis model and treatment

Six-week-old female BALB/c mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). These mice were kept in specific-pathogen-free conditions and provided with sterile food and water at National Cheng Kung University, Taiwan, in accordance with animal experimentation guidelines. The Institutional Animal Care and Use Committee (IACUC) of the National Cheng Kung University (Permit Number: 103302) approved the study protocol. Mice were divided into three groups, and each group consisted of six mice: sensitization control group (SC), AR group (AR) and EWSC pretreatment group (EWSC). The procedures for allergen sensitization and treatment are summarized in Fig. E1A. Briefly, mice were sensitized with an intraperitoneal injection of 50 μ g of Der p and 1 mg of aluminum hydroxide in 200 μ L sterile phosphate-buffered saline (PBS) on days 0 and 7. After general sensitization, the mice were challenged with 50 μ g of Der p in 20 μ L sterile intranasal PBS without anesthesia from days 14 to 20. For the experimental control, SC mice were sensitized and challenged with PBS. Along with sensitization and challenge, selected groups of mice were intranasally treated with 20 μ L of 1%EWSC at 6 h before the intranasal Der p challenge without anesthesia (EWSC). Allergic nasal responses in mice were measured using a noninvasive Enhanced Pause System on day 21, and the mice were sacrificed on day 22.

Reagents

House dust mite, *Dermatophagoides pteronyssinus* (Der p, 1 g lyophilized whole body extract in ether; Allergon, Engelholm, Sweden) was dissolved in pyrogenic-free isotonic saline and filtered through a 0.22 μ m filter; Lipopolysaccharides (LPS)

contamination was removed through the column method and stored at -80°C before use. WSC was purchased from BioChina Inc. It was prepared by using ultrasonic electrolysis and a multi-step membrane separation process. The EWSC (50–100 kDa) was dissolved in PBS (pH 7.4) and then sterilized. The EWSC stock solution (1 %) was stored at 4°C .

Measuring of the nasal response by using a noninvasive Enhanced Pause System

The nasal response was measured in unrestrained, spontaneously breathing mice through barometric whole-body plethysmography by using the Biosystem XA[®] (Buxco, Troy, NY, USA) whole-body plethysmography system. Mouse respiration rates and airway resistance were used to evaluate the nasal responses of the airway to acetyl- β -methylcholine chloride (A2251, Sigma-Aldrich Co. LLC. MO, USA) at concentrations of 0, 6.25, 12.5, 25, and 50 mg/mL at 24 h after the Der p challenge. Methacoline was aerosolized for ventilation by using an ultrasonic nebulizer for 3 min.

Measurement of total and Der p-specific IgE in the serum

Blood was collected from the mice on days 0 and 22. The collected blood was left to stand and clot for 1 h at room temperature, and then centrifugation at 13,000 rpm was performed for 30 min to remove the clotted matter and obtain the serum. Serum levels of total and Der p-specific IgE were measured using an ELISA kit (Mouse IgE ELISA Quantitation Set, Bethyl Laboratories, Inc. E90-115).

Histopathology analysis

For examining the RE in the nasal cavity, the heads were fixed in 10% neutral buffered formalin (pH 7.4) for 3 days and decalcified in 20 % EDTA in 10mM Tris buffer (pH 7.4) for 2 weeks at room temperature. The nasal areas were sliced at the anterior margin of the orbit transversely. The specimens were then embedded in paraffin and sliced at a thickness of 4 μm for hematoxylin and eosin (H&E) staining. For the histological analysis of eosinophils, nasal cavity sections were stained using

the Combined Eosinophil-Mast Cell Stain kit® (ScyTek Laboratories, Inc. UT, USA) for the differential counting of eosinophils through microscopy. For the histological analysis of mast cells, the sections were stained with toluidine blue (T3260, Sigma-Aldrich Co. LLC. MO, USA) to identify and count mast cells and degranulated mast cells through microscopy.

Collection of NLFs

After euthanasia, the mouse trachea was incised at the upper level, and then a catheter was guided into the nasopharynx. Subsequently, 1 mL of 3% NaCl in PBS (pH 7.4) was injected slowly into the nasal cavity and collected in an Eppendorf tube. The NLFs were centrifuged at 300×g for 5 min at 4°C to separate the cells and the supernatants, which were stored at -80°C until use.

Measurement of NGF, BDNF, VIP, and cytokines in NLFs

ELISA kits were used to determine the concentrations of NGF (G7630, Promega, WI, USA and CY304, Merck Minipore, Germany), BDNF (E90011Muk, Uscn, Germany), and VIP (abx254505, Abbexa Ltd, UK) in the NLF, according to the manufacturers' instructions. The concentrations of TNF- α (R&D Systems, Inc. DuoSet ELISA DY410), IL-4 (R&D Systems, Inc. DuoSet ELISA DY404), IL-5 (R&D Systems, Inc. DuoSet ELISA DY405), IL-6 (R&D Systems, Inc. DuoSet ELISA DY406), and IL-13 (R&D Systems, Inc. DuoSet ELISA DY413) in the NLFs were measured using an ELISA kit, according to the manufacturer's instructions (R&D Systems, MN, USA).

Immunohistochemistry

Nasal cavity sections were heated at 65°C for 30 min, and then deparaffinized sections were prepared and hydrated with distilled water. For antigen retrieval, the sections were heat-induced with 1mM EDTA (pH 8.0) in the presence of 0.05% Tween 20, by using a pressure cooker. The sections were immunostained with either rabbit polyclonal NGF antibody (sc-549, Santa Cruz Biotechnology, Inc., Europe), rabbit

polyclonal anti-TrkA antibody (ab76291, Abcam, Cambridge, UK), rabbit polyclonal anti-T-bet antibody (bs-3599R, Bioss Inc. MA, USA), rabbit polyclonal anti-GATA3 antibody (bs-1452R, Bioss Inc. MA, USA), or TRPV-1 (ab74855, Abcam, Cambridge, UK), or ROR α (ab60134, Abcam, Cambridge, UK) at 4°C overnight. In addition, the sections were stained with rabbit polyclonal anti-p75NTR antibody (ab8874, Abcam, Cambridge, UK) at room temperature for 30 min. Immunoreactivity was visualized through the diaminobenzidine immunoperoxidase methodology by using the Novolink™ Polymer Detection Systems kit according to the manufacturer's instructions (Leica, Newcastle, UK).

Flow analysis of T_H2s and ILC2s

Draining lymph nodes of the nasal mucosa were isolated from Der p-challenged mice. Single cells were obtained by passing through 70- μ m cell strainers. After blood cell deletion, the cells were stimulated with a leukocyte activation cocktail plus GolgiPlug (550583, BD Bioscience, NJ, USA) at 37°C for 4 h in a complete RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μ M β -mercaptoethanol (β -ME), and 10% fetal bovine serum (FBS; all from Life Technologies, CA, USA). Thereafter, the isolated single cells were harvested and stained with FITC-conjugated CD45 (553079), PerCP-Cy5.5-conjugated CD4 (553052), PerCP-Cy5.5-conjugated lineage marker mAbs (CD3e, B220, CD11b (Mac-1), Gr-1 (Ly-6C & Ly-6G, and Ter119; 561317), BV421-conjugated CD127 (562959, all from BD Bioscience, NJ, USA), and Alexa Fluor®700-conjugated ST2 (FAB10041N, R&D, Systems, MN, USA). TrkA antibodies (ab76291) were conjugated with PE by using an R-phycoerythrin conjugated kit (ab102918, Abcam, Cambridge, UK) and were prepared for staining. Cells were stained for 30 min at 4°C and washed once with PBS. After incubation with CD16/CD32 mAb 2.4G2 (553142, BD Bioscience, NJ, USA)

to block Fc receptors, intracellular staining for APC-conjugated GATA3 (50-9966-42) as well as PE-conjugated IL-4 (12-7041-82), IL-5 (12-7052-82), and IL-13 (12-7133-82, all from eBioscience, CA, USA) was performed using the Foxp3 intracellular staining kit (eBioscience). Cells were fixed with 200 μ L of 2% paraformaldehyde. Flow cytometric analysis samples were analyzed through LSRI Fortessa flow cytometry (BD Bioscience), collecting 1 million events per sample. These data were analyzed using FlowJo software v8.8.6 (Tree Star, Ashland, OR, USA). T_H2s were identified as CD45⁺CD4⁺GATA3⁺ through the gating strategy shown in Fig. E5A. ILC2s were identified as CD45⁺Lineage⁻GATA3⁺CD127⁺ST2⁺ through the gating strategy shown in Fig. E5B.

Preparation of primary cultures and protocol of NGF induction

Lung cells were isolated from BABL/c mice. The lung was first perfused with 20 mL of sterile ice-cold isotonic saline through the right ventricle of the heart, dissected from the trachea, cut into small pieces, and incubated in digestion media for 90 min at 37°C. The digestion media were composed of the RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) containing 100 U/mL penicillin (Life Technologies), 100 μ g/mL streptomycin (Life Technologies), 0.7 mg/mL collagenase (Sigma-Aldrich), and 0.03 mg/mL deoxyribonuclease (DNase) I (Sigma-Aldrich). The enzymatic digestion was stopped by adding complete RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μ M β -ME, and 10% FBS (all from Life Technologies). Single cells from digested lung suspensions were obtained by passing through 70- μ m cell strainers. After blood cell deletion, the cells were incubated for 10–14 days. The primary cells were harvested and prepared for assay. Primary cells were seeded into 24-well plates (5×10^4 /well) for the cytokine assay. Isolated cells were incubated for 6 h with EWSC at concentrations of 0, 8, 40, and 200 μ g/mL in serum-free medium,

and then the EWSC was washed out. After being washed twice with PBS, the cells were stimulated with 20 ng/mL of TNF- α . After TNF- α stimulation, NGF levels in the culture medium were harvested and determined through ELISA (CY304, Merck Minipore, Germany).

***In vitro* study with nasal septum epithelial cell line**

RPMP-2650 cells (ATCC[®] CCL-30[™]) were cultured in MEM complete medium (Life Technologies), which was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/L sodium bicarbonate, and 10% FBS (all from Life Technologies). RPMP-2650 cells were seeded at a concentration of 1×10^6 cells/mL and cultured until they adhered and filled the well. Cells were incubated for 6 h with EWSC at concentrations of 10, 100, and 200 mg/mL in the serum-free medium, and then the EWSC was washed out. After being washed twice with PBS, the cells were stimulated with 100 μ g/mL Der p for 48 h.

Western blot analysis of NGF expression in cell lines

Whole-cell lysates were prepared using the radioimmunoprecipitation assay buffer (R0278, Sigma-Aldrich Co. LLC., MO, USA) and then boiled in sample buffer (62.5 mM Tris-Cl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated through 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Millipore Corporation, MA, USA). The membrane was then blocked with 10% skim milk in TBST for 1 h at room temperature and incubated with anti-NGF (ab52918, Abcam, Cambridge, UK) and anti- α -tubulin (NB100-690, NOVUS, MO, USA) antibodies at 4°C overnight. After washing in TBST three times, the blots were hybridized using HRP-conjugated goat anti-rabbit IgG (7074, Cell Signaling Technology Inc., MA, USA) or HRP-conjugated rat anti-mouse IgG antibodies (7076, Cell Signaling Technology, Inc. MA, USA) for 2 h, and the antibody-specific proteins were visualized

using the enhanced chemiluminescence detection system, according to the recommended procedure (Western Lightning Chemiluminescence Reagent PLUS; PerkinElmer Life Sciences Inc., MA, USA). The densities of NGF were calculated and compared with the density of α -tubulin, and the results are shown as a proportion.

Allergic rhinitis model and treatment of $\text{Ngf}^{+/-}$ mice

C57BL/6- $\text{Ngf}^{tm1c(GEMMS)Narl}$ (RMRC13175) and C57BL/6-Tg(Pgk1-RFP, -cre/ERT2)3Narl (RMRC13179) mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). NGF heterozygote-deficient mice ($\text{Ngf}^{+/-}$) were produced through cross-breeding C57BL/6- $\text{Ngf}^{tm1c(GEMMS)Narl}$ and C57BL/6-Tg(Pgk1-RFP, -cre/ERT2)3Narl mice. Six-week-old female $\text{Ngf}^{+/-}$ mice and C57BL/6 were divided into four groups: $\text{Ngf}^{+/-}$ /PBS, WT/PBS, $\text{Ngf}^{+/-}$ /AR, and WT/AR. The procedures for allergen sensitization and treatment are summarized in Fig. E4A. Mice were intranasally sensitized and challenged with 50 μg of Der p in 20 μL sterile PBS without anesthesia daily for 5 days per week for 3 consecutive weeks. For the experimental control, the selected groups were sensitized and challenged with 20 μL sterile PBS.

Statistical analysis

The data are presented as mean $\pm$ SEM and analyzed through Student's t-test using Graph-Pad Prim 4.0 (GraphPad, San Diego, CA, USA). $P < 0.05$ was considered significant.

Fig. E1

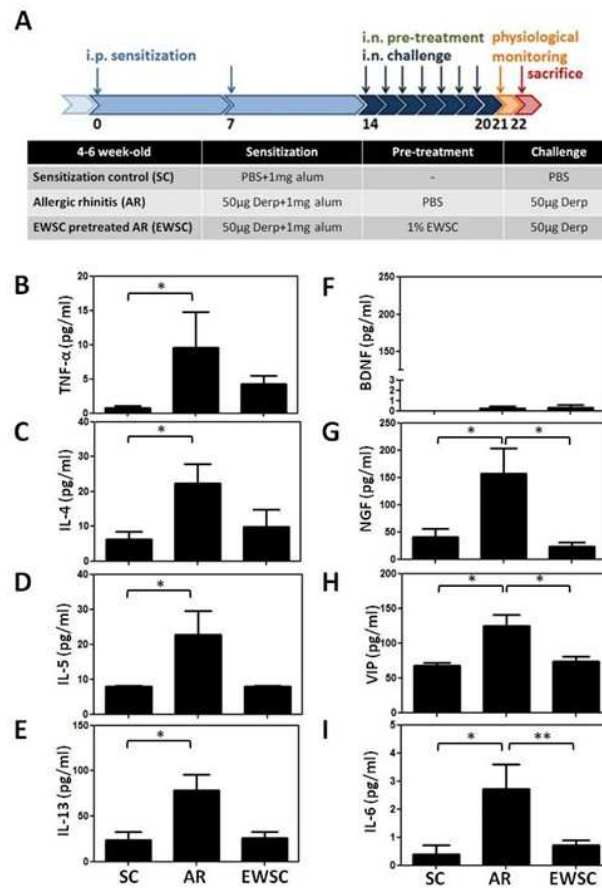


Fig. E2

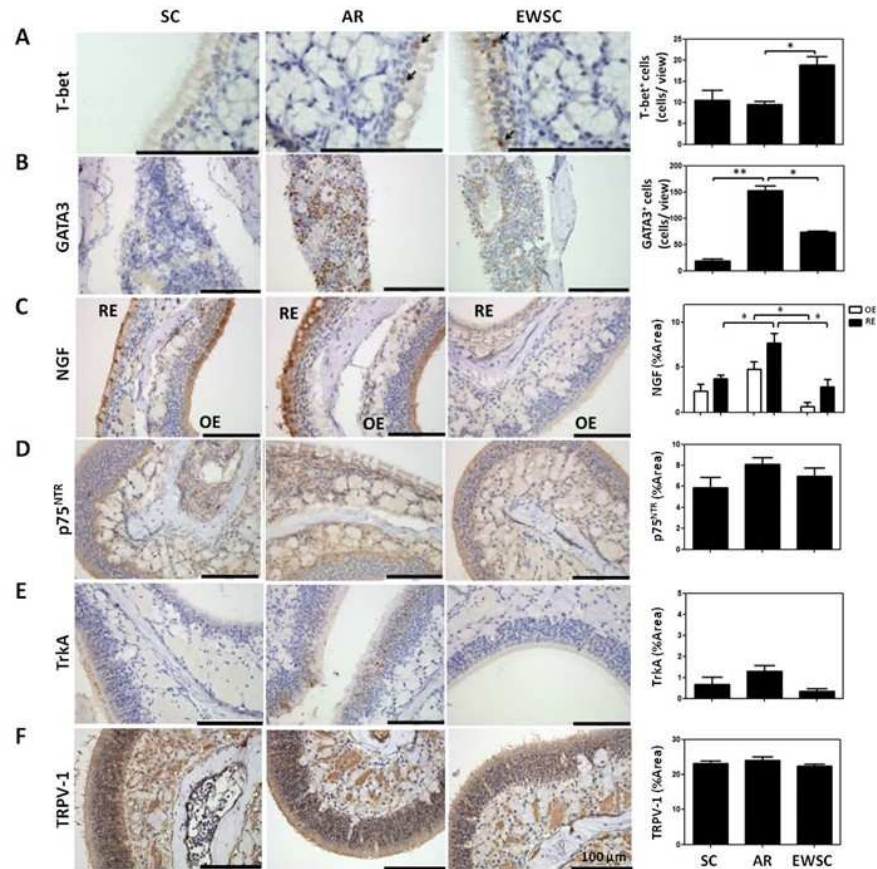


Fig. E3

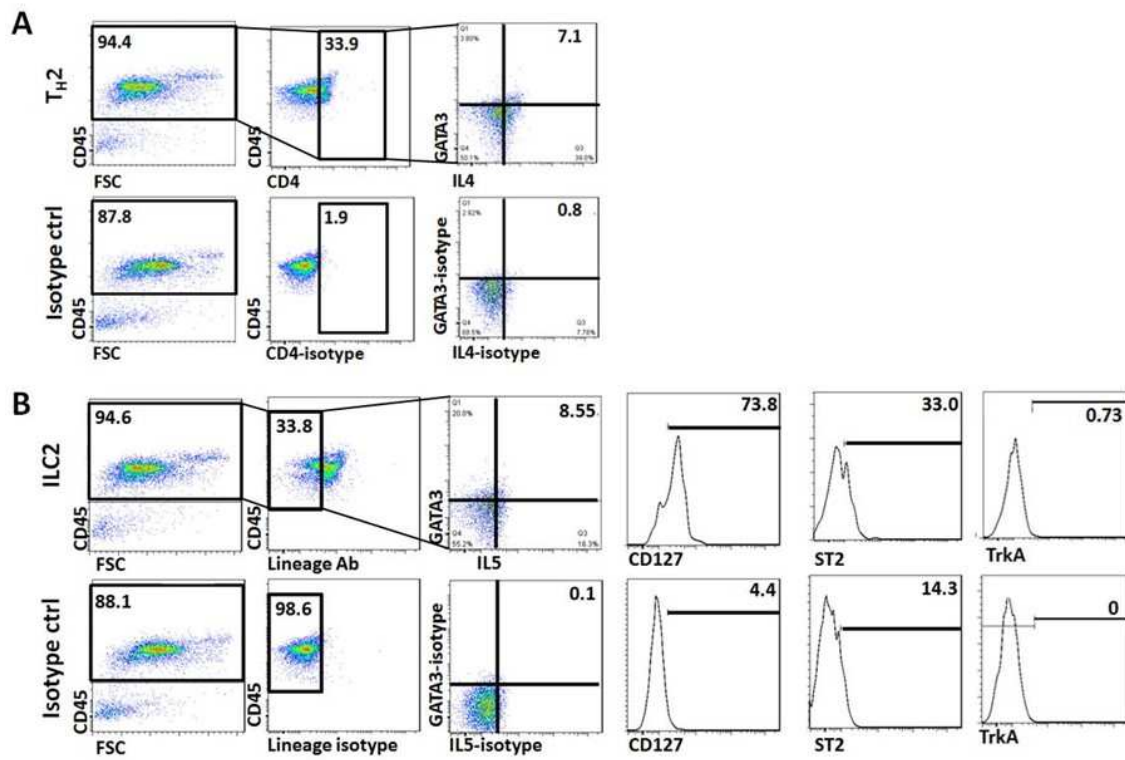


Fig. E4

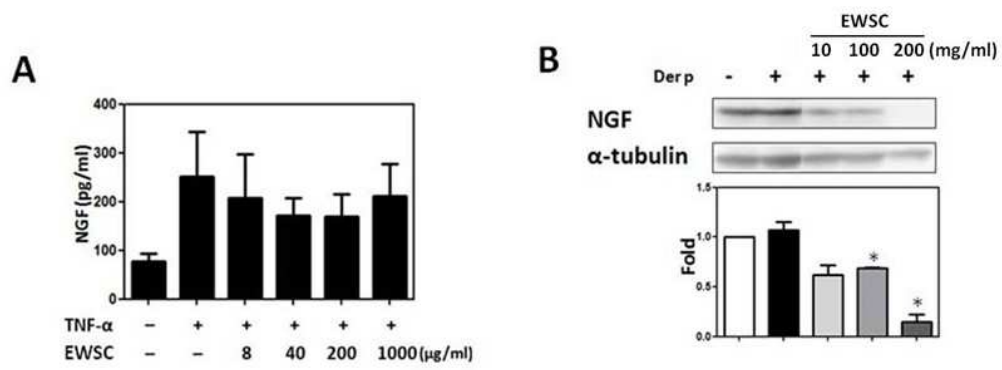


Fig. E5

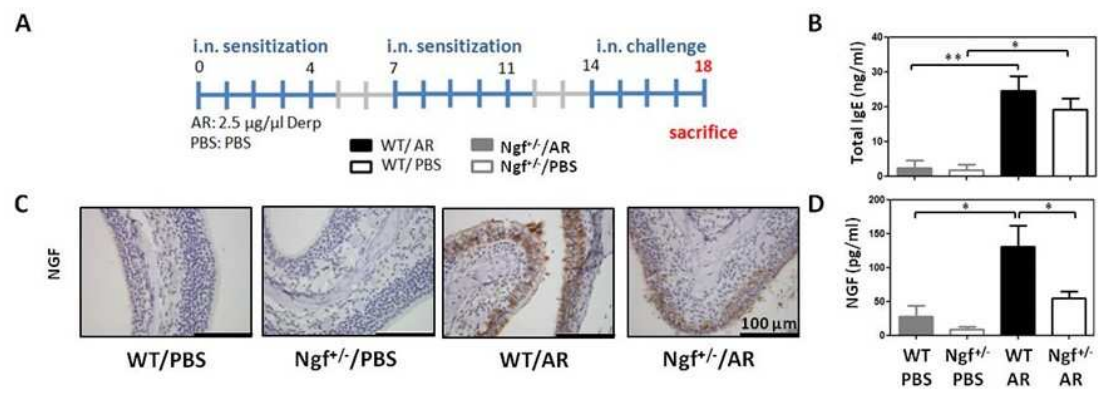
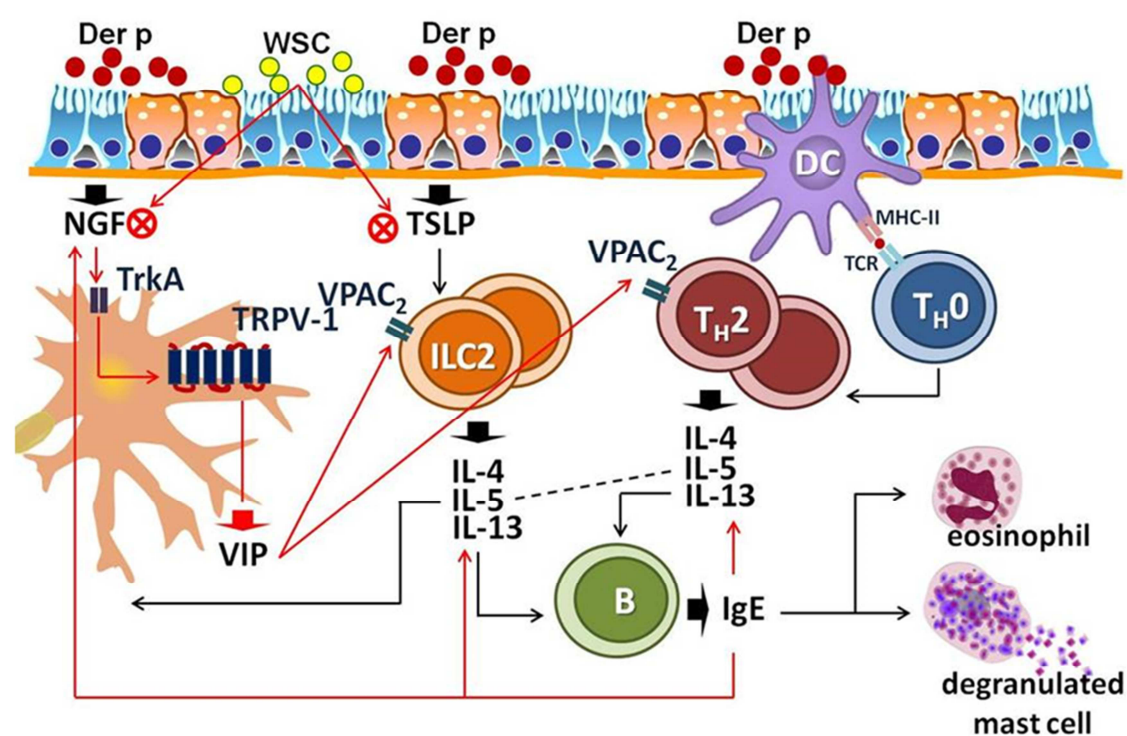


Fig. E6



Supplementary Figure legends

Supplementary Fig. E1. EWSC inhibits allergen-induced NGF production and airway inflammation. (A) Experimental schema. Mice were sensitized with PBS or Der p on days 0 and 7. After the second sensitization, mice were challenged intranasally with PBS or Der p without anesthesia from days 14 to 20. Selected groups of mice were intranasally administered with 1% EWSC at 6 h before the Der p challenge without anesthesia. (B) TNF- α , (C) IL-4, (D) IL-5, (E) IL13, (F) BDNF, (G) NGF, (H) VIP, and (I) IL-6 production in NLFs. The levels were determined through ELISA. Data are expressed as mean $\pm$ SEM ($n \geq 12$). *: $p < 0.05$; **: $p < 0.01$, compared with the AR group in Student's t-test.

Supplementary Fig. E2. EWSC inhibits allergen-induced NGF production and airway inflammation. Sections of the nasal cavity were immunostained with (A) T-bet, (B) GATA3, (C) NGF, (D) p75^{NTR}, (E) TrkA, and (F) TRPV-1 antibodies and reacted with DAB (Scale bars = 100 μ m) (RE: respiratory epithelium; OE: olfactory epithelium). The positive-stained T-bet and GATA3 cells were counted in 400 $\times$ microscopic fields per group, and the average numbers were calculated. The NGF, TrkA, p75^{NTR}, and TRPV-1 in the immunohistochemically stained nasal sections were quantified by Image J. Data are expressed as mean $\pm$ SEM ($n \geq 6$). *: $p < 0.05$; **: $p < 0.01$ compared with the AR group in Student's t-test.

Supplementary Fig. E3. EWSC inhibits NGF expression *in vitro*. (A) Primary lung cells were incubated for 6 h with EWSC at different concentrations (0, 8, 40, 200 μ g/mL) before TNF- α (20 ng/mL) stimulation. NGF levels in the culture supernatants were measured through ELISA. (B) RPMI-2650 cells were incubated for 6 h with WSC at different concentrations (10, 100, 200 mg/ml) before Der p (100 μ g/mL). Thereafter, whole-cell extracts were used for Western blotting with anti-NGF. Protein expression levels were detected using densitometry and were depicted as proportional lines and bar graphs. Data are expressed as mean $\pm$ SEM ($n \geq 3$). *: $p < 0.05$ compared with the non-treatment group in Student's t-test.

Supplementary Fig. E4. Der p fails to promote allergen-induced inflammation in NGF-deficient mice. (A) Experimental schema. Mice were sensitized and challenged intranasally with Der p allergen daily for 5 days per week for 3 consecutive weeks. (B) Total IgE concentrations of serum were measured through ELISA. (C) Sections of the nasal cavity were immunostained with NGF antibodies and reacted with DAB. (D) NGF production in NLFs was determined through ELISA. Data are expressed as mean

± SEM (n ≥ 12). *: p < 0.05; **: p < 0.05 compared with the AR group in Student's t-test (scale bars: 100µm).

Supplementary Fig. E5. Gating strategy of isolated T_H2 and ILC2 cells in the draining lymph nodes of the nasal mucosa.

(A) Draining lymph nodes of the nasal mucosa were isolated, and T_H2 cells were analyzed using flow cytometry. T_H2s were identified as CD45⁺CD4⁺GATA3⁺ through the gating strategy. (B) Draining lymph nodes of the nasal mucosa were isolated, and ILC2 cells were analyzed using flow cytometry. ILC2s were identified as CD45⁺Lineage⁻GATA3⁺CD127⁺ST2⁺ through the gating strategy. ILC2s were negative for TrkA expression.

Supplementary Fig. E6. Model of NGF involvement in type 2 inflammation and the EWSC inhibition mechanism.

During allergen exposure, the nasal mucosa was activated by dendritic cells and epithelial cells. Dendritic cells polarize precursor T_H0 cells into T_H2 cells, whereas epithelial cells produce NGF and TSLP, which activates nociceptors to release VIP and stimulates ILC2 cell activation, respectively. Furthermore, VIP also stimulates ILC2 and newly differentiated T_H2 cells via the VPAC2 receptor. Type 2 cytokines, including IL-4, IL-5, and IL-13, are released by ILC2 and T_H2 cells and promote eosinophilia and degranulated mast cell infiltration in the nasal mucosa, IgE secretion by B cells, mucus production by goblet cells, and nasal hyperresponsiveness. Furthermore, NGF- and T_H2-related cytokines create an amplification loop resulting in broader allergic inflammation in the upper-airway epithelium. EWSC attenuates allergen-induced nasal AHR and inflammation through the inhibition of NGF and its amplification loop of type 2 immune response.