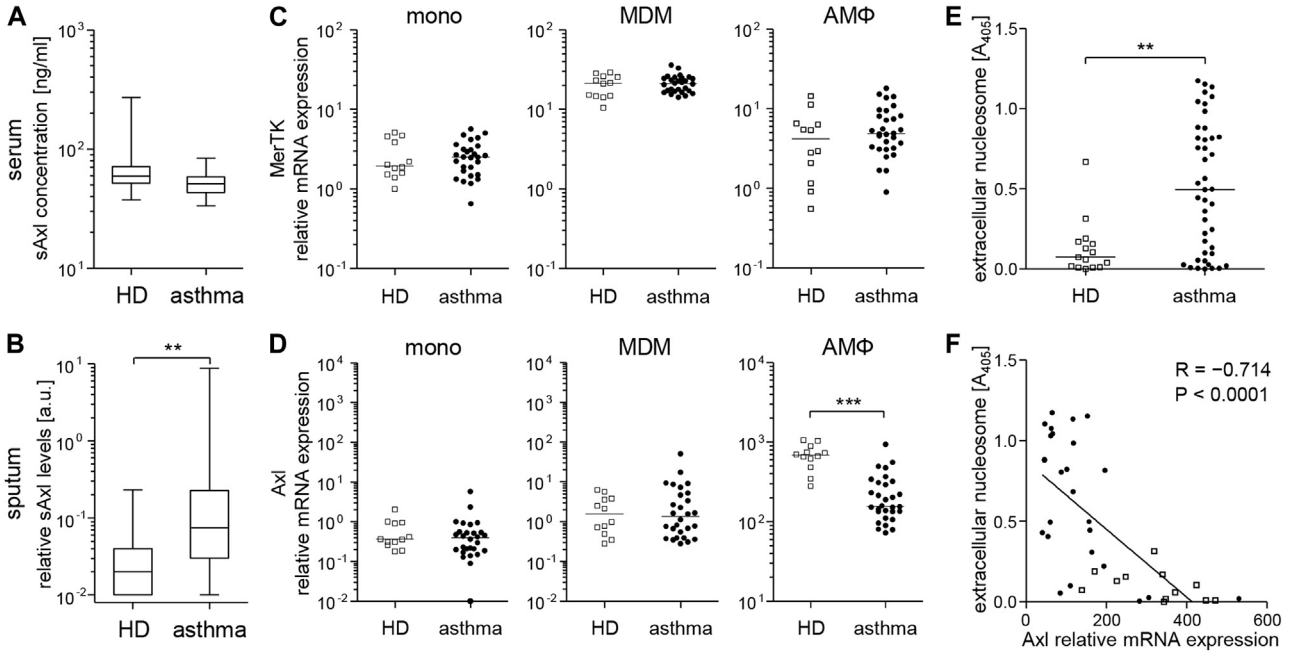

FIG 2. Airway macrophage Axl expression and shedding are altered in patients with moderate-to-severe asthma and are associated with accumulation of necrotic cell debris in the airways. **A,** Levels of sAxl in serum samples from HDs (n = 15) and patients with asthma (n = 43). **B,** Analysis of sAxl in sputum samples from HDs (n = 15) and patients with asthma (n = 40). **C** and **D,** MerTK and Axl relative mRNA expression in monocytes (mono), MDMs, and airway macrophages (AMΦ) from HDs (n = 12) and patients with asthma (n = 30). **E,** Amount of nucleosomes in sputum samples from HDs (n = 15) and patients with asthma (n = 43). **F,** Correlation analysis of AMΦ Axl mRNA expression and sputum nucleosome levels. Symbols represent values from individual HDs *(open squares)* and asthma patients *(closed circles)* and the *lines* denote the median. $^{**}P < .01$ and $^{***}P < .001$.

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

To the Editor:

Allergic rhinitis (AR), which affects approximately 10% to 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

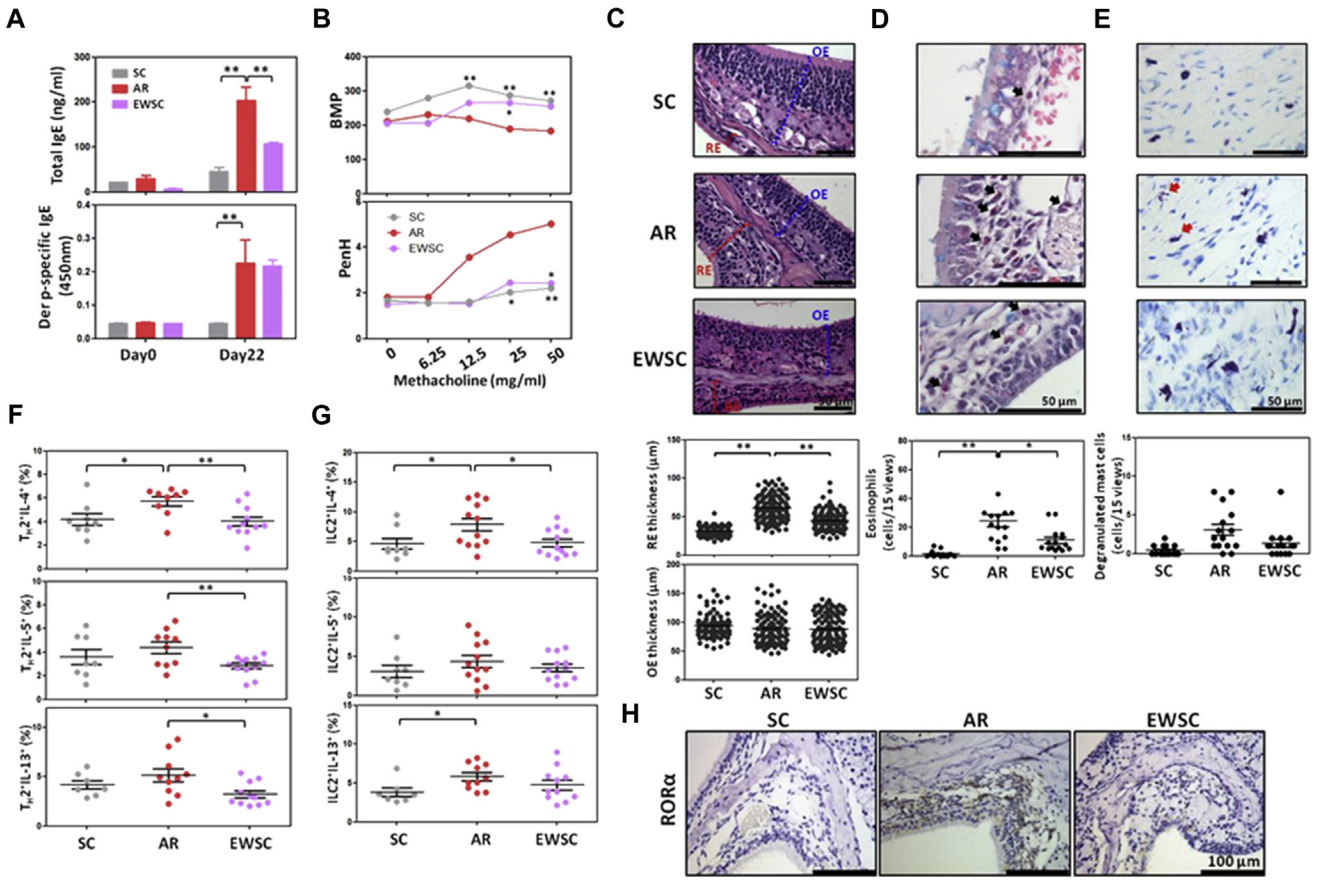

FIG 1. EWSC improves nasal pathological function and airway inflammationin Der p–induced AR. **A,** Total IgE and Der p–specific IgE concentrations of serum were measured through ELISA. **B,** Respiration rate and airway resistance were measured using the Buxco noninvasive airway-resistant measurement system. **C,** Thickness of the nasal RE (OE: olfactory epithelium). The positive-stained eosinophils *(black arrows)* **(D)** and degranulated mast cells *(red arrows)* **(E)** were counted in 400× 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 and $T_H2^+IL4^+$, $T_H2^+IL5^+$, 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 RORα antibodies and reacted with DAB (scale bars = 100 μm). Data are expressed as mean ± SEM (n ≥ 8). $*P < .05$; $**P < .01$ compared with the AR group, in Student *t* test.

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 tropomyosin receptor kinase A (TrkA), activates ion channels via the capsaicin receptor transient receptor potential cation channel subfamily V member 1 (TRPV-1) in sensory neurons and further modulates vasoactive intestinal polypeptide (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 (deacetylated chitin) could reduce house dust mite, *Dermatophagoides pteronyssinus* (Der p), allergen-induced airway inflammation, through thymic stromal lymphopoietin and arginase 1 inhibition in a mouse model of allergic asthma.[5]

To evaluate the effect of extensive water-soluble chitosan (EWSC) in AR, a Der p–sensitized and –challenged AR mouse model was established (see text and Fig E1, *A*, 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 1, *A*) and decreased respiratory rates (breath per minute) and AHR (as measured by PenH; Fig 1, *B*), which are the 2 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 breath per minute and lower AHR than did nontreated AR mice (Fig 1, *B*). Significantly lower levels of NGF and IL-6 and decreased levels of TNF-α, IL-4, IL-5, and IL-13 were observed in the nasal lavage fluids (NLFs) of EWSC mice (Fig E1, *B-E*). Brain-derived neurotrophic factor was not detectable in the NLFs of the experimental mice (Fig E1, *F*). In contrast, significantly higher NGF, IL-6, and VIP levels were noted in the NLFs of AR mice than of sensitization control group mice, and these levels were significantly decreased after EWSC treatment (Fig E1, *G-I*). In histology, EWSC treatment

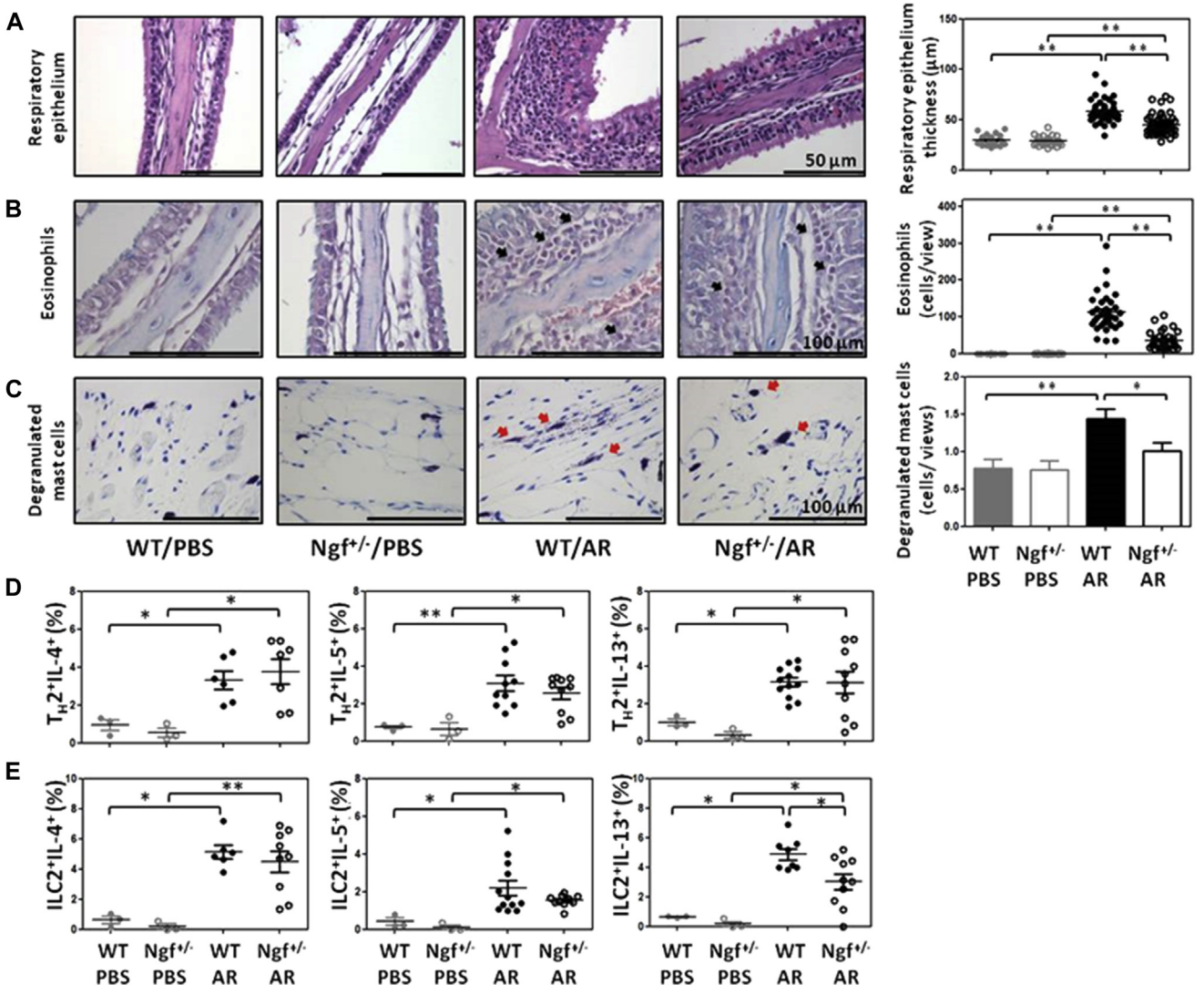

FIG 2. Der p fails to promote allergen-induced inflammation in NGF-deficient mice. **A,** Thickness of the nasal respiratory epithelium. The positive-stained eosinophils *(black arrows)* **(B)** and degranulated mast cells *(red arrows)* **(C)** were counted in 400× microscopic fields per group, and 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* < .05; ***P* < .05, compared with the AR group in Student *t* test.

significantly reduced Der p–induced nasal respiratory epithelium (RE) hyperplasia (Fig 1, *C*), and the infiltration of eosinophils (Fig 1, *D*) and degranulated mast cells (Fig 1, *E*) 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 (see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). NGF was also markedly expressed in the nasal epithelium, particularly in the RE, of AR mice but was decreased in EWSC and sensitization control mice (Fig E2, *C*). No significant differences between the degrees of staining for NGF receptors, such as p75 neurotrophin receptor (Fig E2, *D*) and TrkA (Fig E2, *E*), and TRPV-1 (Fig E2, *F*), were noted in the nasal epithelia of our experimental mice. In the AR group, the Der p allergen challenge significantly increased the levels of $IL\text{-}4^+T_H2$, $IL\text{-}4^+$ group 2 innate lymphoid cell $(ILC2)^+$, $IL\text{-}13^+ILC2^+$, and $ROR\alpha^+$ staining cells in the nasal draining lymph nodes and nasal mucosal, respectively (Fig 1, *F-H*). Intranasal administration of EWSC significantly decreased T_H2 cells expressing IL-4, IL-5, and IL-13 (Fig 1, *F*). Furthermore, EWSC treatment significantly reduced Der p allergen challenge–induced $IL\text{-}4^+ILC2^+$ (Fig 1, *G*), and $ROR\alpha^+$ staining cell infiltration (Fig 1, *H*) 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 (see Fig E3, *A* and *B*, in this article's Online Repository at www.jacionline.org). 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 (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org). The experimental mice exhibited a mild reduction in total serum IgE (Fig E4, *B*), and reduced NGF expression (Fig E4, *C* and *D*), RE thickness (Fig 2, *A*), eosinophil infiltration (Fig 2, *B*), and mast cell degranulation were noted in the nasal epithelium (Fig 2, *C*) 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 2, *D* and *E*), 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 ($P < .05$).

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 (see Fig E5, *B*, in this article's Online Repository at www.jacionline.org), NGF acts on the nociceptor TRPV-1 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, thymic stromal lymphopoietin, T_H2, and ILC2 cells, which amplify type 2 inflammation (see Fig E6 in this article's Online Repository at www.jacionline.org). 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 a natural product such as 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]

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Severe asthma: Differential chemokine response of airway epithelial cells

To the Editor:

Approximately 10% to 15% of asthmatic adults belong to a group with severe refractory asthma and have debilitating chronic symptoms despite optimal standard asthma treatment.[1] Unraveling the complex pathophysiology of severe asthma has proved to be a major research challenge.[1] There is growing interest in the role of the airway epithelium and its interactions with inhaled aeroallergens and pathogens in the pathogenesis of severe asthma.

In a study of patients with severe asthma and healthy control subjects, we have recently shown that profound ciliary dysfunction and marked ultrastructural abnormalities of the airway epithelium are features of severe asthma.[2] One potential consequence of these abnormalities is prolonged and more intense exposure of the airway epithelium to inhaled aeroallergens and pathogens. Moreover, given the marked epithelial disintegrity seen in patients with severe asthma and the ability of proteolytically active substances, such as *Dermatophagoides pteronyssinus* allergens, to cause disruption of the intercellular tight junctions, resulting in increased transepithelial permeability,[3] airway basal cells could also be exposed to inhaled allergens and pathogens. In this regard we studied the effect (in terms of cytokine and chemokine release) of a common respiratory pathogen *(Streptococcus pneumoniae)* on primary airway basal cells of patients with atopic severe asthma and compared this with the effect in healthy control subjects. As a positive control, cytokine and chemokine release in response to a common inhaled allergen (*D pteronyssinus* allergen 1 [Der p 1]) by primary airway basal cells was also studied.

Detailed methodology is provided in the Methods section in this article's Online Repository at www.jacionline.org. Briefly, we studied 8 patients with severe asthma and 6 healthy control subjects. Patients with severe asthma met the American Thoracic Society criteria for refractory asthma,[1] were current nonsmokers,

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 of the National Cheng Kung University (permit no. 103302) approved the study protocol. Mice were divided into 3 groups, sensitization control group (SC), AR group (AR), and EWSC pretreatment group (EWSC), and each group consisted of 6 mice. The procedures for allergen sensitization and treatment are summarized in Fig E1, *A*. Briefly, mice were sensitized with an intraperitoneal injection of 50 μg of Der p and 1 mg of aluminum hydroxide in 200 μL sterile PBS on days 0 and 7. After general sensitization, the mice were challenged with 50 μg of Der p in 20 μL sterile intranasal PBS without anesthesia from days 14 to 20. For the experimental control, sensitization control mice were sensitized and challenged with PBS. Along with sensitization and challenge, selected groups of mice were intranasally treated with 20 μL of 1% EWSC at 6 hours 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 pyogenic-free isotonic saline and filtered through a 0.22-μm filter; LPS contamination was removed through the column method and stored at −80°C before use. Water-soluble chitosan was purchased from BioChina Inc. It was prepared by using ultrasonic electrolysis and a multistep 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) 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, St Louis, Mo) at concentrations of 0, 6.25, 12.5, 25, and 50 mg/mL at 24 hours after the Der p challenge. Methacoline was aerosolized for ventilation by using an ultrasonic nebulizer for 3 minutes.

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 hour at room temperature, and then centrifugation at 13,000 rpm was performed for 30 minutes 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, Montgomery, Tex; 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 10 mM 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 staining. For the histological analysis of eosinophils, nasal cavity sections were stained using the Combined Eosinophil-Mast Cell Stain kit (ScyTek Laboratories, Inc, West Logan, Utah) 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) 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 minutes at 4°C to separate the cells and the supernatants, which were stored at −80°C until use.

Measurement of NGF, brain-derived neurotrophic factor, VIP, and cytokines in NLFs

ELISA kits were used to determine the concentrations of NGF (G7630, Promega, Madison, Wis and CY304, Merck Minipore, Temecula, Calif), brain-derived neurotrophic factor (E90011Muk, Wuhan USCN Business Co, Houston, Tex), and VIP (abx254505, Abbexa Ltd, Cambridge, United Kingdom) in the NLF, according to the manufacturers' instructions. The concentrations of TNF-α (DY410, R&D Systems, Inc, Minneapolis, Minn), IL-4 (DY404, R&D Systems, Inc), IL-5 (DY405, R&D Systems, Inc), IL-6 (DY406, R&D Systems, Inc), and IL-13 (DY413, R&D Systems, Inc) in the NLFs were measured using an ELISA kit, according to the manufacturer's instructions (R&D Systems, Inc).

Immunohistochemistry

Nasal cavity sections were heated at 65°C for 30 minutes, and then deparaffinized sections were prepared and hydrated with distilled water. For antigen retrieval, the sections were heat-induced with 1 mM 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, Santa Cruz, Calif), rabbit polyclonal anti-TrkA antibody (ab76291, Abcam, Cambridge, United Kingdom), rabbit polyclonal anti–T-bet antibody (bs-3599R, Bioss, Inc, Woburn, Mass), rabbit polyclonal anti-GATA3 antibody (bs-1452R, Bioss, Inc), or TRPV-1 (ab74855, Abcam), or RORα (ab60134, Abcam) at 4°C overnight. In addition, the sections were stained with rabbit polyclonal anti–p75 neurotrophin receptor antibody (ab8874, Abcam) at room temperature for 30 minutes. Immunoreactivity was visualized through the diaminobenzidine immunoperoxidase methodology by using the Novolink Polymer Detection Systems kit according to the manufacturer's instructions (Leica, Newcastle, United Kingdom).

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-μm cell strainers. After blood cell deletion, the cells were stimulated with a leukocyte activation cocktail plus GolgiPlug (550583, BD Bioscience, San Jose, Calif) at 37°C for 4 hours in a complete RPMI 1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μM β-mercaptoethanol, and 10% FBS (all from Life Technologies, Carlsbad, Calif). Thereafter, the isolated single cells were harvested and stained with fluorescein isothiocyanate–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, San Jose, Calif), and Alexa Fluor700–conjugated ST2 (FAB10041N, R&D Systems, Inc). TrkA antibodies (ab76291) were conjugated with phycoerythrin (PE) by using an R-phycoerythrin–conjugated kit (ab102918, Abcam) and were prepared for staining. Cells were stained for 30 minutes at 4°C and washed once with PBS. After incubation with CD16/CD32 mAb 2.4G2 (553142, BD Bioscience) to block Fc receptors, intracellular staining for allophycocyanin-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 Affymetrix eBioscience, San Diego, Calif) was performed using the Foxp3 intracellular staining kit (Affymetrix eBioscience). Cells were fixed with 200 μL of 2% paraformaldehyde. Flow cytometric analysis samples were analyzed through LSRFortessa flow cytometry (BD Bioscience), collecting 1 million events per

sample. These data were analyzed using FlowJo software v8.8.6 (Tree Star, Ashland, Ore). T_H2s were identified as $CD45^+CD4^+GATA3^+$ through the gating strategy shown in Fig E5, *A*. ILC2s were identified as $CD45^+Lineage^-GATA3^+CD127^+ST2^+$ through the gating strategy shown in Fig E5, *B*.

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 minutes at 37°C. The digestion media were composed of the RPMI 1640 medium (Life Technologies, Carlsbad, Calif) containing 100 U/mL penicillin (Life Technologies), 100 μg/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 μg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 μM β-mercaptoethanol, and 10% FBS (all from Life Technologies). Single cells from digested lung suspensions were obtained by passing through 70-μm cell strainers. After blood cell deletion, the cells were incubated for 10 to 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 hours with EWSC at concentrations of 0, 8, 40, and 200 μg/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, Temecula, Calif).

In vitro study with nasal septum epithelial cell line

RPMI-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). RPMI-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 hours 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 μg/mL Der p for 48 hours.

Western blot analysis of NGF expression in cell lines

Whole-cell lysates were prepared using the radioimmunoprecipitation assay buffer (R0278, Sigma-Aldrich) and then boiled in sample buffer (62.5 mM Tris–Cl [pH 6.8], 2% SDS, 20% glycerol, 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated through 10% SDS-PAGE and transferred to a PVDF membrane (Merck Minipore). The membrane was then blocked with 10% skim milk in TBST for 1 hour at room temperature and incubated with anti-NGF (ab52918, Abcam) and anti–α-tubulin (NB100-690, NOVUS, St Charles, Mo) antibodies at 4°C overnight. After washing in TBST 3 times, the blots were hybridized using horseradish peroxidase–conjugated goat anti-rabbit IgG (7074, Cell Signaling Technology Inc, Danvers, Mass) or horseradish peroxidase–conjugated rat anti-mouse IgG antibodies (7076, Cell Signaling Technology, Inc) for 2 hours, 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, Waltham, Mass). 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 $Ngf^{+/-}$ mice

C57BL/6-$Ngf^{tm1c(GEMMS)Narl}$ (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 ($Ngf^{+/-}$) were produced through cross-breeding C57BL/6-$Ngf^{tm1c(GEMMS)Narl}$ and C57BL/6-Tg(Pgk1-RFP, -cre/ERT2)3Narl mice. Six-week-old female $Ngf^{+/-}$ mice and C57BL/6 were divided into 4 groups: $Ngf^{+/-}$/PBS, WT/PBS, $Ngf^{+/-}$/AR, and WT/AR. The procedures for allergen sensitization and treatment are summarized in Fig E4, *A*. 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 ± SEM and analyzed through Student t test using Graph-Pad Prim 4.0 (GraphPad, San Diego, Calif). $P < .05$ was considered significant.

4-6 week-old	Sensitization	Pre-treatment	Challenge
Sensitization control (SC)	PBS+1mg alum	-	PBS
Allergic rhinitis (AR)	50μg Derp+1mg alum	PBS	50μg Derp
EWSC pretreated AR (EWSC)	50μg Derp+1mg alum	1% EWSC	50μg Derp

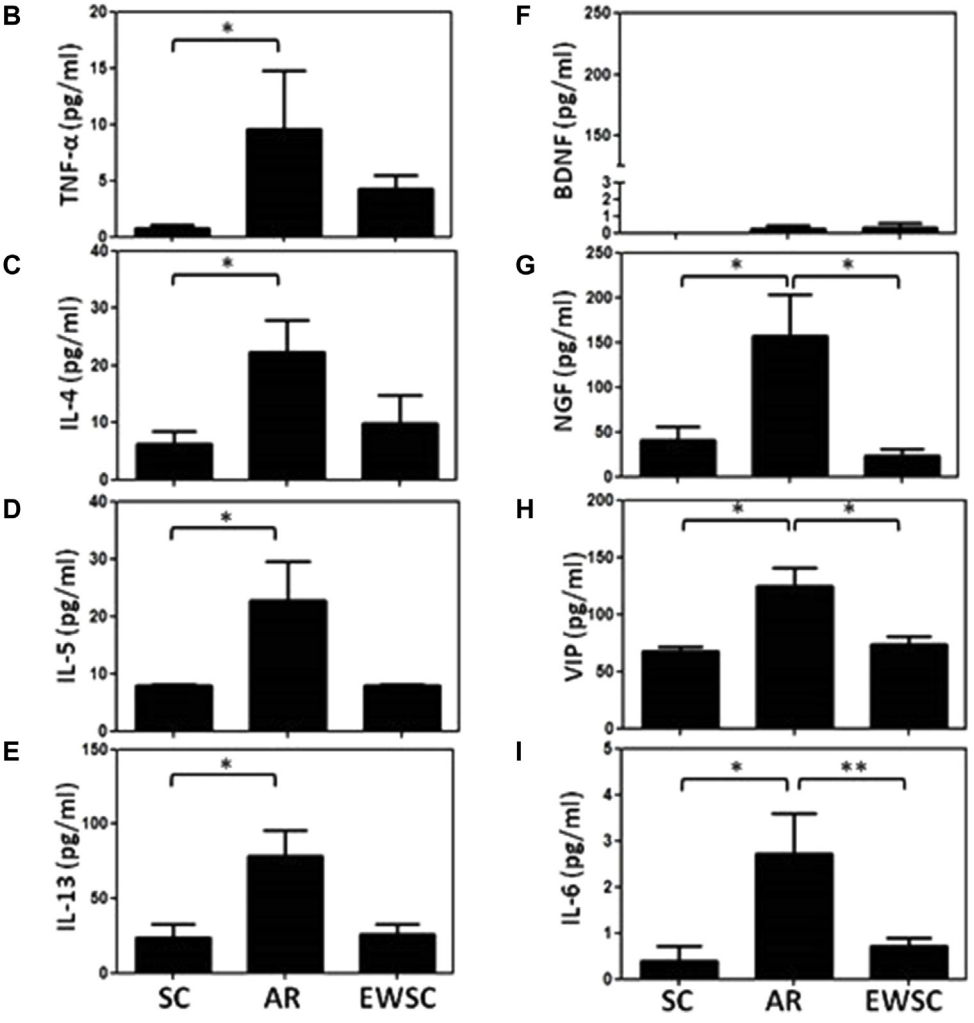

FIG E1. EWSC inhibits allergen-induced NGF production and airway inflammation. **A,** Experimental schema. Mice were sensitized with PBS or Der p on days 0 and 7. After the second sensitization, mice were challenged intranasally with PBS or Der p without anesthesia from days 14 to 20. Selected groups of mice were intranasally administered with 1% EWSC at 6 hours before the Der p challenge without anesthesia. TNF-α **(B)**, IL-4 **(C)**, IL-5 **(D)**, IL-13 **(E)**, BDNF **(F)**, NGF **(G)**, VIP **(H)**, and IL-6 **(I)** production in NLFs. The levels were determined through ELISA. Data are expressed as mean ± SEM ($n \geq 12$). *BDNF*, Brain-derived neurotrophic factor. $^{*}P < .05$; $^{**}P < .01$, compared with the AR group in Student *t* test.

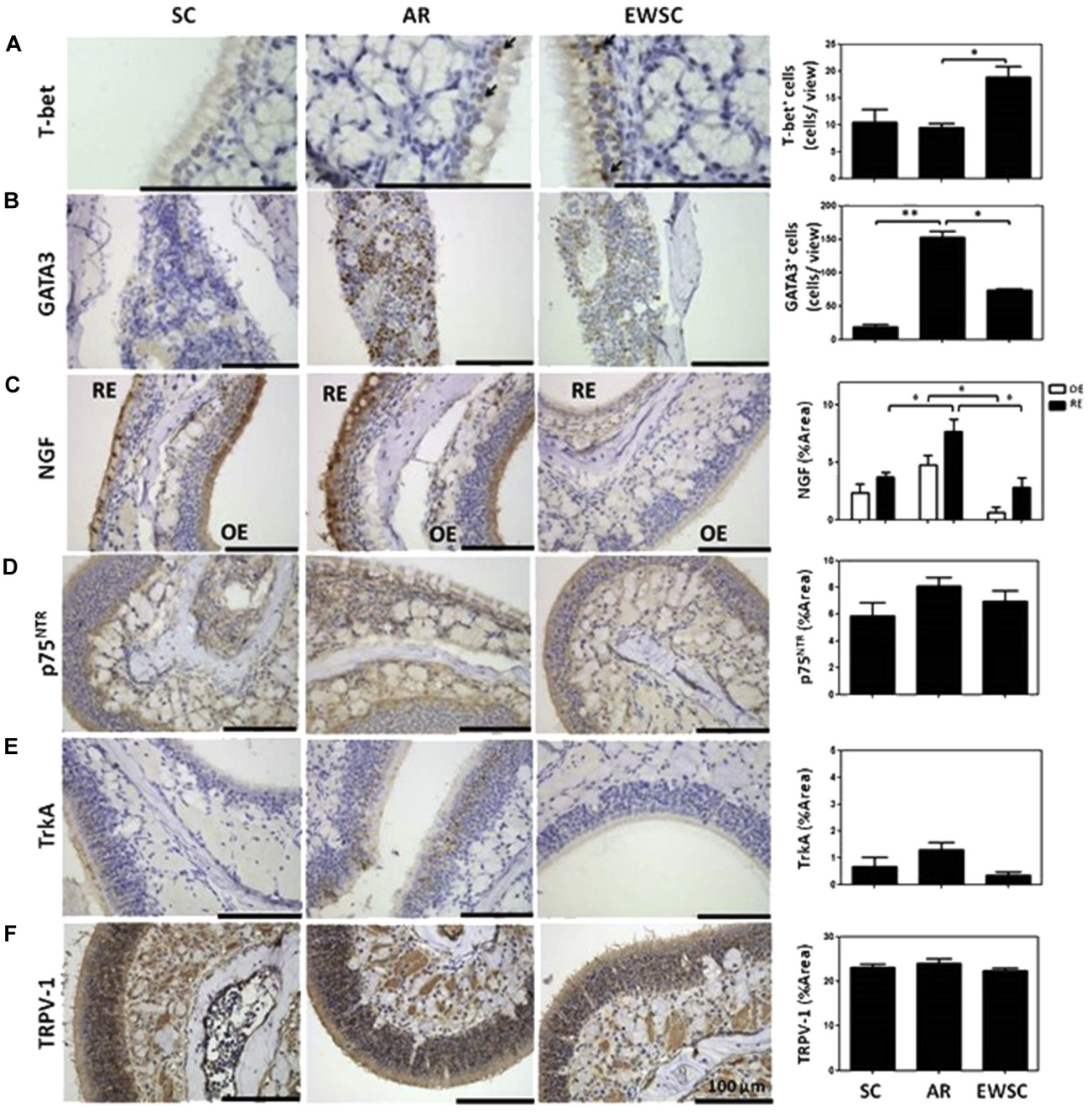

FIG E2. EWSC inhibits allergen-induced NGF production and airway inflammation. Sections of the nasal cavity were immunostained with T-bet **(A)**, GATA3 **(B)**, NGF **(C)**, $p75^{NTR}$ **(D)**, TrkA **(E)**, and TRPV-1 **(F)** antibodies and reacted with DAB (scale bars = 100 μm). The positive-stained T-bet and GATA3 cells were counted in 400× 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. *OE*, Olfactory epithelium *$p75^{NTR}$*, p75 neurotrophin receptor. Data are expressed as mean ± SEM ($n \geq 6$). **P* < .05; ***P* < .01 compared with the AR group in Student *t* test.

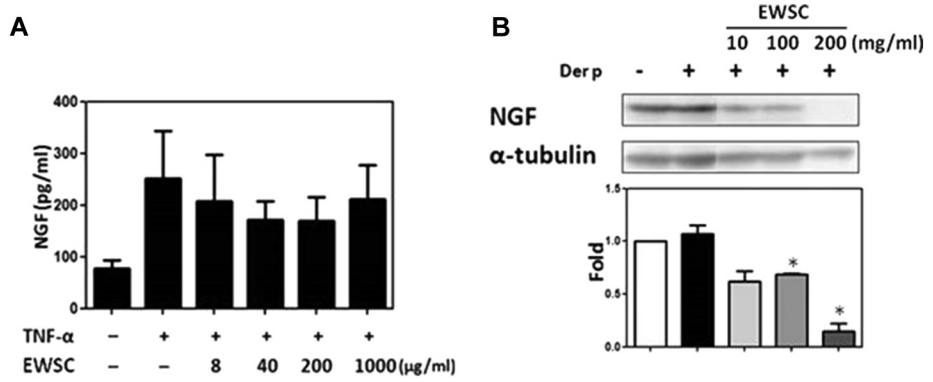

FIG E3. EWSC inhibits NGF expression *in vitro*. **A,** Primary lung cells were incubated for 6 hours with EWSC at different concentrations (0, 8, 40, 200 μg/mL) before TNF-α (20 ng/mL) stimulation. NGF levels in the culture supernatants were measured through ELISA. **B,** RPMI-2650 cells were incubated for 6 hours with EWSC at different concentrations (10, 100, 200 mg/mL) before Der p (100 μg/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 ± SEM ($n \geq 3$). *$P < .05$ compared with the nontreatment group in Student *t* test.

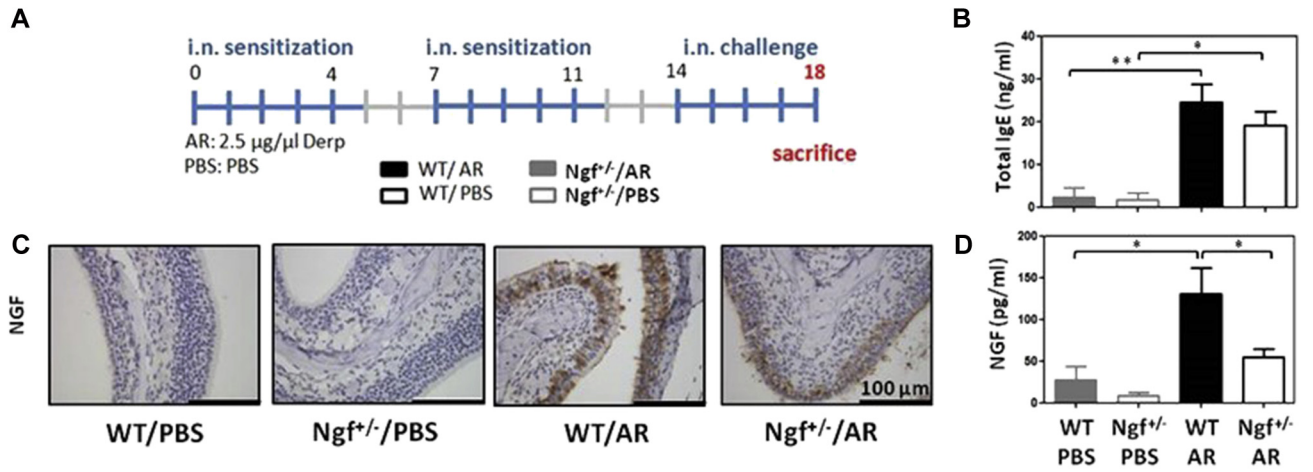

FIG E4. Der p fails to promote allergen-induced inflammation in NGF-deficient mice. **A,** Experimental schema. Mice were sensitized and challenged intranasally with Der p allergen daily for 5 days per week for 3 consecutive weeks. **B,** Total IgE concentrations of serum were measured through ELISA. **C,** Sections of the nasal cavity were immunostained with NGF antibodies and reacted with DAB. **D,** NGF production in NLFs was determined through ELISA. *i.n.*, Intranasal. Data are expressed as mean ± SEM ($n \geq 12$). $^{*}P < .05$; $^{**}P < .05$ compared with the AR group in Student *t* test (scale bars: 100 μm).

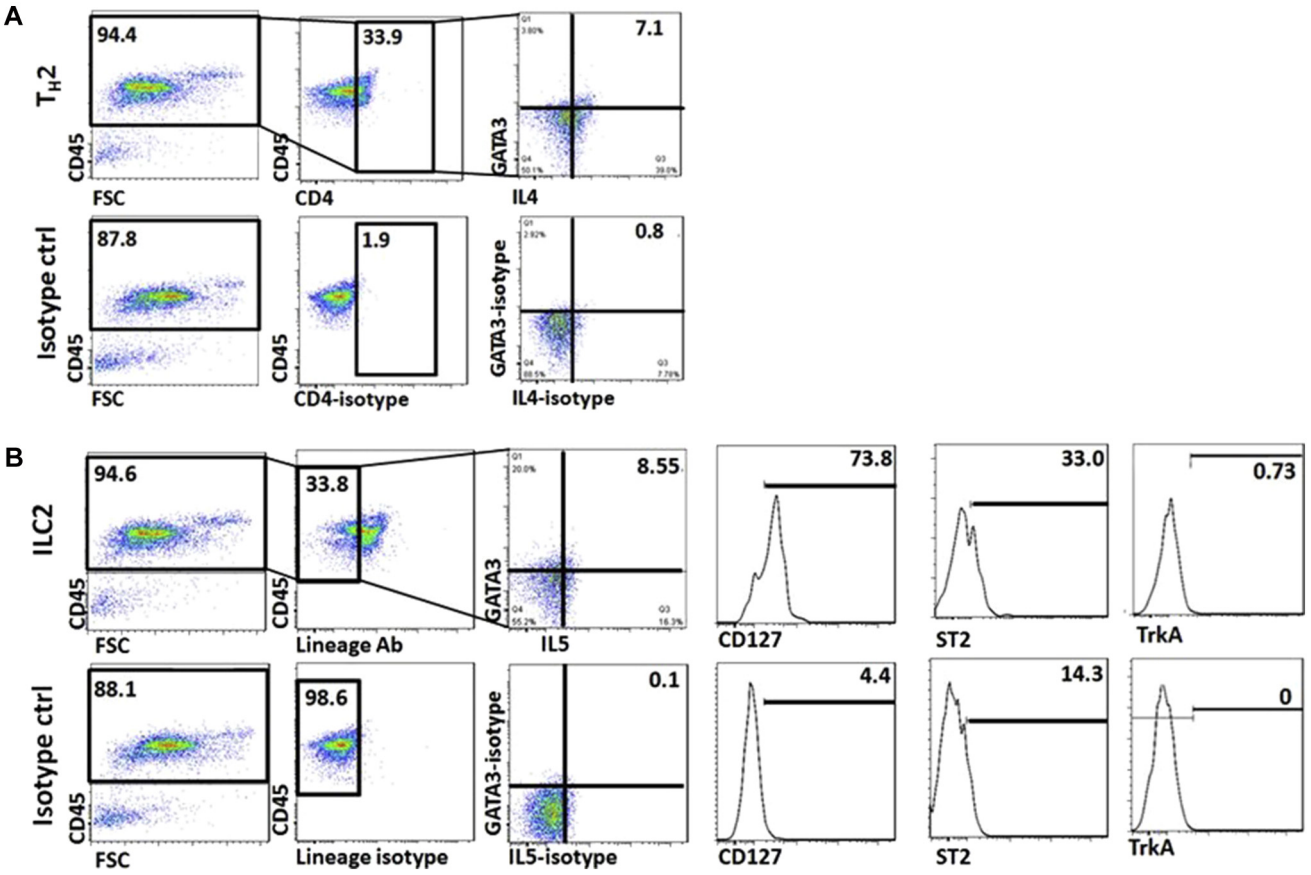

FIG E5. Gating strategy of isolated T_H2 and ILC2 cells in the draining lymph nodes of the nasal mucosa. **A,** Draining lymph nodes of the nasal mucosa were isolated, and T_H2 cells were analyzed using flow cytometry. T_H2s were identified as $CD45^+CD4^+GATA3^+$ through the gating strategy. **B,** 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.

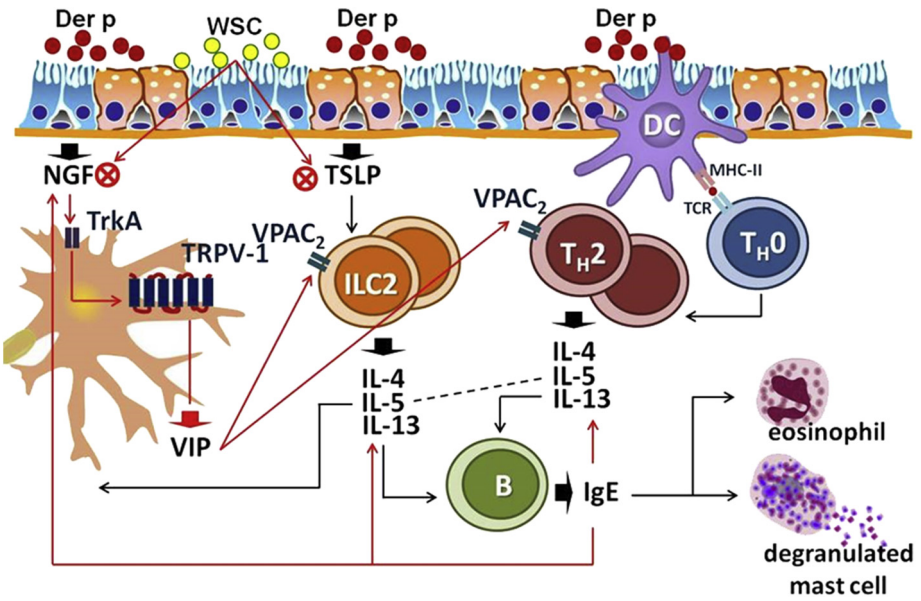

FIG E6. Model of NGF involvement in type 2 inflammation and the EWSC inhibition mechanism. During allergen exposure, the nasal mucosa was activated by dendritic cells and epithelial cells. Dendritic cells polarize precursor T_H0 cells into T_H2 cells, whereas epithelial cells produce NGF and thymic stromal lymphopoietin, 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, andIL-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.