

Nerve growth factor derived from bronchial epithelium after chronic mite antigen exposure contributes to airway hyperresponsiveness by inducing hyperinnervation, and is inhibited by *in vivo* siRNA

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Abstract

Background Bronchial asthma is a chronic allergic airway inflammatory disease. Neurotrophins, including nerve growth factor (NGF), play an important role in the pathogenesis of asthma. However, the effects of NGF derived from epithelium on airway hyperresponsiveness (AHR) after antigen sensitization/exposure remain uncertain.

Objective In this study, we examined the role of NGF on AHR after chronic antigen exposure and the effect of inhibiting NGF by *in vivo* siRNA on AHR exacerbation.

Methods We generated chronic mouse models of bronchial asthma using house-dust mite antigen (*Dermatophagoides pteronyssinus*; Dp). NGF concentrations in bronchoalveolar lavage fluid (BALF), lung histopathology, hyperresponsiveness, and related neuronal peptides and cytokines in supernatants of lung homogenates were determined.

Results NGF in BALF was increased in a dose- and time-dependent manner, and was expressed primarily in bronchial epithelium. Nerve fibres and substance P-positive fibres were detected in subepithelium of Dp-sensitized and challenged mice over 4 weeks of mite antigen exposure. AHR was positively correlated with NGF concentration and nerve fibre innervation. AHR, modulation of innervation, and increased substance P were inhibited by *in vivo* administration of siRNA that targeted NGF, although the inhibition of NGF did not affect allergic inflammation and subepithelial fibrosis.

Conclusion and clinical relevance These findings suggest that NGF derived from bronchial and alveolar epithelium plays an important role in AHR after chronic exposure to mite antigen. NGF inhibition could potentially manage bronchial asthma, including AHR.

Keywords airway hyperresponsiveness, bronchial epithelium, hyperinnervation, nerve growth factor, substance P

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Introduction

Bronchial asthma is characterized by allergic airway inflammation, airway obstruction and airway hyperresponsiveness (AHR). These features are related to various inflammatory cells and cytokines, including Th2 cytokines, chemokines and growth factors. Recently, neuropeptides and neurotrophins were found to be important in immune responses during the pathogenesis of bronchial asthma. The neurotrophins include brain-derived neurotrophic factor, nerve growth factor (NGF), neurotrophin-3(NT3) and neurotrophin-4(NT4). Neurotrophins bound to the tyrosine kinase (trk) receptor on nerve fibres promoted the development and survival of

sensory neurons [1]. Regarding NGF for immune response in asthma, NGF levels are increased in the serum [2] and in bronchoalveolar lavage fluid (BALF) [3] of asthmatic patients. Neurotrophins also promoted the survival and activation of eosinophils in the airways [4] and induced allergic inflammation and AHR via neurotrophin- p75NTR signalling in immune cells [5]. In NGF Tg mice, AHR was accompanied by over-expression of NGF in the airways [6]. NGF administration also induced AHR in animal models in both *in vivo* and *in vitro* studies [7, 8]. In contrast, administering an anti-NGF antibody inhibited the progression of AHR in mice [9]. Thus, these previous reports demonstrated that NGF has been characterized in terms of specific gene

modulations in mice and other defined conditions and that NGF contributed to AHR. NGF was produced by mast cells [10], macrophages [9] and T cells [11] in allergic inflammation. Moreover, structural cells such as lung epithelial cells [12] and fibroblasts [13] also produce NGF and play an important role in the asthmatic response. NGF produced by epithelial cells promoted the survival of eosinophils [14] and plasma cells [15]. However, little is known regarding whether NGF derived from the bronchial epithelium contributes to AHR following chronic antigen exposure.

To clarify this question, a similar allergic disease, atopic dermatitis, can serve as a reference because the role of NGF after antigen exposure has been investigated in atopic dermatitis. Keratinocytes synthesize and secrete biologically active NGF [16]. In atopic dermatitis, neurogenic inflammation and pro-inflammatory cytokine production increased NGF expression by keratinocytes [17]. Augmented NGF expression may have been responsible for an increased number of nerve fibres and increased SP-positive nerve fibres in the skin [17]. In NC/Nga mice, NGF derived from skin keratinocytes led to itching via increased PGP9.5 positive nerve fibres [18, 19]. On the basis of these findings, we hypothesized that similar mechanisms associated with NGF also occurs in bronchial asthma after antigen exposure and that these mechanisms could result in AHR.

To confirm the effects of NGF derived from bronchial and alveolar epithelium, we used *in vivo* transfection of short interfering RNA (siRNA) that targeted NGF into Dp-sensitized and challenged mice and evaluated its possible inhibitory effects on AHR. RNA interference was originally determined to be an ancient endogenous defence mechanism against viral infections [20, 21]. RNA interference is a cellular mechanism in which siRNA can induce the sequence specific degradation of a complementary mRNA target. Recently, siRNA has been shown to be a powerful new tool for effective and specific gene silencing. In particular, because direct intranasal application of siRNA has shown promise as a gene delivery method for treating respiratory disease [22–25], we chose this method to knock-down NGF gene expression in bronchial and alveolar epithelium.

In this report, we demonstrate the functional role of NGF derived from bronchial and alveolar epithelium on AHR using *in vivo* siRNA that targeted NGF (NGF-siRNA) in a mite allergen-induced chronic model of asthma.

Materials and methods

Preparation of antigen

House-dust mite antigen (*Dermatophagoides pteronyssinus*, Dp) was purchased from LSL (Tokyo, Japan). Endotoxin levels were reduced using endotoxin removal

solution (Sigma-Aldrich Japan, Tokyo, Japan). Dp endotoxin was reduced to < 0.3 IU/mg [26].

Mouse experimental protocols

Six-week-old female BALB/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained in the animal facility of the University of Tokushima under specific pathogen-free conditions, according to the guidelines of and approval by the ethics committee of our university. Each experiment used 3–5 mice per group.

All mice were sensitized on days 0 and 7 by intraperitoneal injections of 10 µg of Dp dissolved in 500 µl saline and mixed with 1 mg of Alum (Sigma-Aldrich). As an acute model, mice were challenged intranasally with 80 µl of solution mixed with 5, 10 or 20 µg of antigen on days 14 and 16 (Fig. 1a) [27]. As a chronic model, we prepared subgroups for 1, 2, 4 and 8 weeks of antigen challenge [26]. Antigen challenges (10 µg) were performed from days 14 to 18 (1-week model), 14 to 25 (2-week model), 14 to 39 (4-week model) or 14 to 67 (8-week model), every other day, 3 days per week (Fig. 1b). Mice were killed 3 days after their final antigen challenge. Bronchoalveolar lavage (BAL) was performed [27]. The lungs were harvested for histopathology and to obtain supernatants after lung homogenization [27].

Preparation and administration of *in vivo* siRNA

NGFsiRNA and negative control siRNA were purchased from Invitrogen (Carlsbad, CA, USA). The siRNA sequences were the following; NGF: MSS276008 (si08);

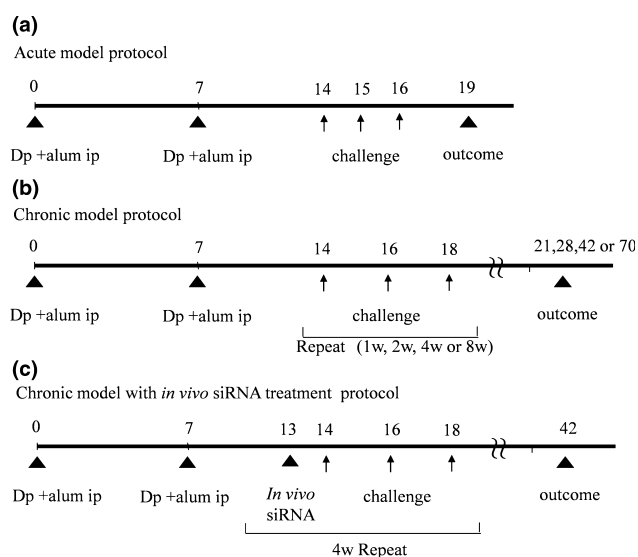


Fig. 1. Mouse experimental protocols. (a) Acute model (3-day challenge). (b) Chronic model (4-week challenge). (c) Chronic model with *in vivo* siRNA treatment (4-week challenge).

sense: CAGUGCUGGCCGAGGUGAACAUUAA; antisense: UUA AUGUUCACCUCGGC CAGCACUG. MSS207044 (si44): sense: GGCAGAACCGUACACAGAUAGCAAU, antisense: AUUGCUAUCUGUGUACGGUUCUGCC.

siRNA was dissolved in a solution of 5% glucose and *in vivo* jetPEI (Polyplus Transfection, New York, NY, USA) to an N/P ratio of 5 (number of nitrogen residues of jetPEI per RNA phosphate), according to the manufacturer's instructions [24]. A total of 50 µl of siRNA-jetPEI complex was administrated intranasally to Dp-sensitized/challenged mice on days 13, 21, 28 and 35 (Fig. 1c).

To examine the effects of transfection, Alexa 488 labelled si08 was purchased from Invitrogen. This was administrated to wild type mice. After 24 hr, lungs were harvested and frozen sections were prepared. Fluorescence signals were detected using an OLYMPUS BX61 microscope.

Measurement of airway resistance

Lung resistance (R_L) was measured by restrained whole-body plethysmography (Buxco Electronics, Troy, NY, USA) [26]. R_L values were expressed as a percentage of the baseline R_L . As an index of AHR, the provocative concentration of methacholine (Mch) that caused a 200% increase in R_L (PC200) was calculated by linear interpolation of the dose-response curves [28].

Histopathology

Lung tissue was fixed in 10% formalin and embedded in paraffin. Tissue sections (3 µm thickness) were prepared using a microtome. Eosinophils were detected using Luna modified staining. Briefly, a section was stained with 1% Biebrich Scarlet solution for 10 min followed by Haematoxylin counter stain. Azan stain was used to detect subepithelial fibrosis.

Immunohistochemistry and immunofluorescent staining

For immunohistochemistry, a Dako CSAII immunohistochemistry kit (Dako Japan, Tokyo, Japan) was used according to the manufacturer's instructions. The primary antibody was anti-NGFβ antibody diluted 1:100 (Epitomics, 1895-1, rabbit monoclonal IgG, Burlingame, CA, USA).

To evaluate nerve innervation, double staining immunofluorescence methods were used. Briefly, sections were prepared in boiling water at 100°C for 30 min for antigen retrieval. Sections were incubated with an anti-mouse Substance P (SP) antibody diluted 1:100 (Millipore, AB1566, rabbit polyclonal IgG, Billerica, MA, USA) overnight at 4°C. Sections were incubated with a secondary antibody, Alexa 594 anti-rabbit IgG antibody (Invitrogen) for 1 h, and blocked with 5% rabbit serum

and 20% anti-rabbit IgG Fab (Jackson ImmunoResearch Laboratory, West Grove, PA, USA). Then, the sections were incubated with anti-mouse protein gene product (PGP) 9.5 antibody diluted 1:300 (Enzo Life Sciences, rabbit polyclonal IgG, Plymouth Meeting, PA, USA) overnight at 4°C and were incubated with Alexa 488 anti-rabbit IgG antibody (Invitrogen) for 1 h. Fluorescent signals were analysed using an OLYMPUS BX61 microscope.

The methods used to count the numbers of eosinophils and to measure the thickness of subepithelial fibrosis were described previously [26, 27]. Numbers of nerve fibres around a bronchus were counted in 15–20 fields per lung lobe at a magnification of 200 × .

Measurements of total protein and cytokine concentrations

Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). NGF, SP and cytokines were determined using commercial ELISA kits. ELISA kits and their sensitivities were the following: NGF (Promega, Madison, WI, USA) with a sensitivity of 7.8 pg/ml; SP (Assay Designs Inc. Ann Arbor, MI, USA) with a sensitivity of 8.04 pg/ml; IL-4, IL-5, IL-13 and TGF-β (R&D Systems, Minneapolis, MN, USA), with sensitivities of, 2, 7, 1.5 and 1.7 pg/mL respectively.

Statistical analysis

Results are given as means ± SEs. Experimental groups were compared using one-way analysis of variance (ANOVA). If statistical significance was identified by ANOVA, a Tukey-Kramer post-hoc test was used to correct for multiple comparisons. Stat-View 5.0 (Abacus Concept, Inc., Berkeley, CA, USA) was used for data analysis. *P*-values < 0.05 were considered significant.

Results

Nerve growth factor concentrations in bronchoalveolar lavage fluid and nerve growth factor -producing cells

To determine if NGF levels increased upon exposure to Dp, NGF concentrations in BALF were measured by ELISA. As shown in Fig. 2a, NGF concentrations in BALF increased in response to Dp exposure for 3 days in a dose-dependent manner. During chronic antigen exposure, NGF concentrations increased in a manner that depended on the period of exposure. The degree of NGF production was higher with chronic exposure than with short-term exposure.

NGF-producing cells were identified by immunohistochemistry. NGF was expressed primarily in bronchial epithelial cells in controls (Fig. 2b). Although the NGF

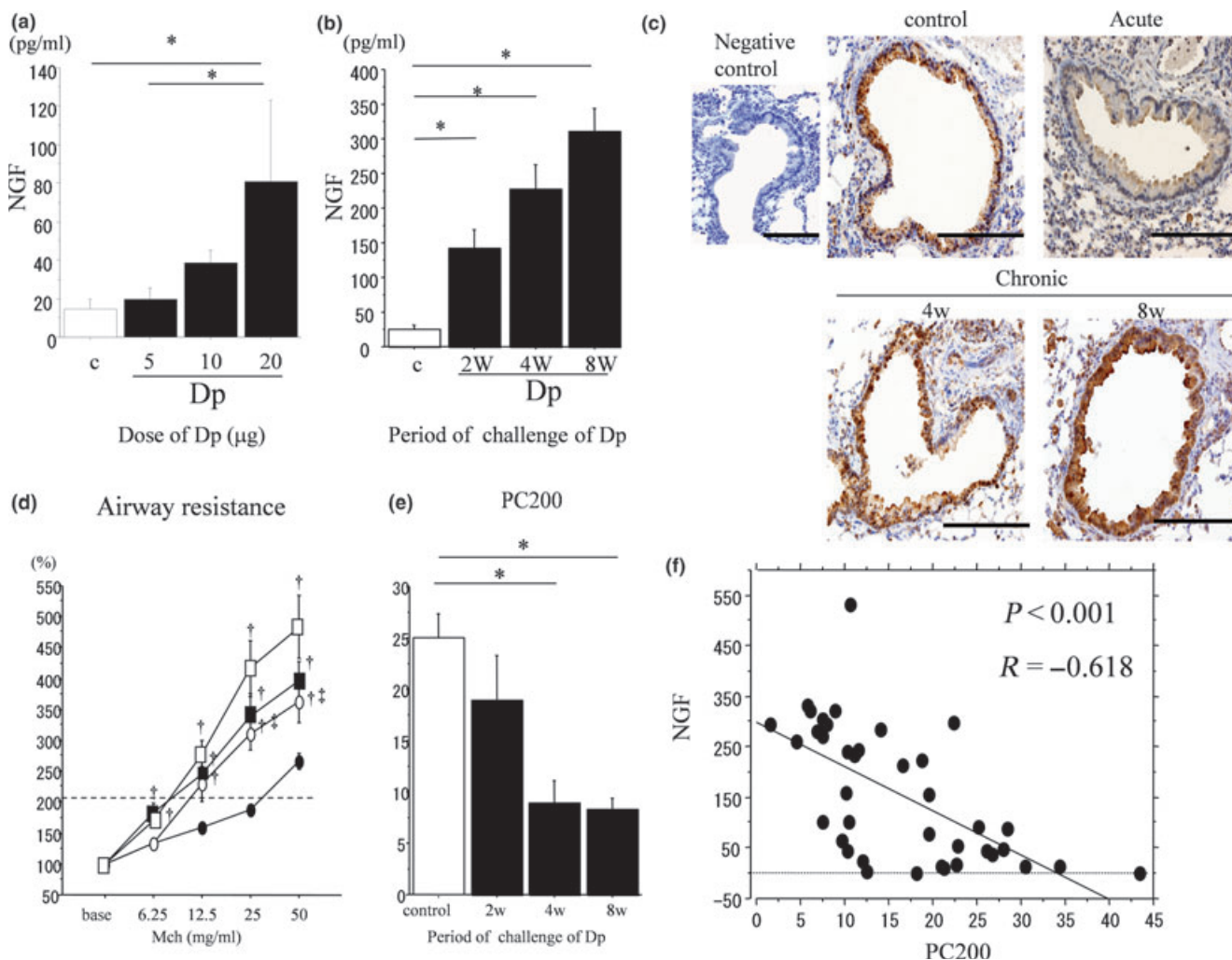


Fig. 2. NGF production in the lungs and correlations between NGF and AHR. (a) Acute model using different doses of Dp (3-day challenge). (b) Chronic model with different periods of Dp challenge (2-, 4- and 8-week challenges). Results are means $\pm$ SE from three different experiments (3–5 mice/group). The number for statistical analysis was 10. (c) Immunohistochemistry for NGF in lungs. Original magnification was 200 $\times$. Scale bar = 200 μ m. (d) Airway resistance. (e) PC200 values after Dp challenge. Results are means $\pm$ SEs from three different experiments (3–5 mice/group). The numbers of mice (n) used for statistical analysis were: control mice: $n = 10$; Dp 2-week challenge model: $n = 8$; Dp 4-week challenge model: $n = 8$; Dp 8-week challenge model: $n = 10$. (f) Correlation between NGF(Y) and AHR(X); $n = 36$. White bar: control mice; Black bar: Dp sensitized/challenged mice; Closed circles: control mice; Open circles: Dp 2-week challenge; Closed squares: Dp 4-week challenge; Open square: Dp 8-week challenge. * $P < 0.05$. † $P < 0.05$ compared with control mice. ‡ $P < 0.05$ compared with Dp 8-week challenge.

expression in epithelial cells was lower in acute model compared with control mice, it was detected in chronic exposure models at the same degree as control mice (Fig. 2b). Some alveolar cells around the bronchus and alveolar macrophages also expressed NGF (Fig. 2b). These results suggested that structural cells of the respiratory tract could produce NGF and the levels increased depending on the period of Dp exposure.

Correlations between nerve growth factor and airway hyperresponsiveness

We evaluated the correlations between NGF and AHR (PC200 values) in the chronic exposure mouse models.

As shown in Fig. 2c, there were significant differences among the curves for all groups ($F(3, 32) = 8.93$; $P = 0.002$). ANOVA also showed an interaction between treatment and the Mch dose ($F(4, 128) = 69.86$; $P < 0.001$). R_L values for all Dp-challenged groups increased at a dose of 12.5 mg/ml and at all higher Mch doses. Increased airway resistance was dependent on the period of Dp exposure (Fig. 2c). Similarly, PC200 values decreased in a manner that was dependent on the period of Dp exposure (Fig. 2c). PC200 values for the 4 and 8 week challenge models were significantly lower than for the control mice (Fig. 2c). NGF concentration in BALF were significantly correlated with PC200 values (Fig. 2d). These results suggest

that NGF derived from the bronchial epithelium after chronic Dp exposure was related to an increase in AHR.

Nerve fibres around the bronchus

As NGF can maintain and prolong nerve fibre production, [29] lung sample histopathology was analysed by immunofluorescence to demonstrate pathological changes in the lungs via continuous NGF production. Nerve bundles around large bronchi were detected in all groups (online supplementary Figure S1). In addition, PGP9.5-positive thin fibres were detected around the bronchioles in the 4 w and 8 w Dp exposure models (Figs 3c and 3d), although these nerve fibres were not detected in controls and the acute Dp exposure group (Figs 3a, 3b, 3e, 3f, 3i and 3j). Some of these fibres in chronic model expressed PGP9.5 and SP (Figs 3g, 3h, 3k and 3l). These results suggest that hyperinnervation may have occurred in mice after chronic Dp antigen

exposure. The numbers of PGP9.5-positive fibres also increased as a function of the time of Dp exposure (Fig. 3m). The numbers of SP-positive fibres were also similar to the results for PGP9.5, although the results were statistically significant only for the 4 w challenge model (Fig. 3m). NGF levels were significantly correlated with the numbers of nerve fibres under the epithelium (Fig. 3n). The numbers of nerve fibres were also significantly correlated with PC 200 values (Fig. 3n). These results suggest that hyperinnervation derived by NGF may be associated with AHR.

In vivo transfection and inhibitory-effects of siRNA targeting nerve growth factor

To confirm that NGF derived from the bronchial and alveolar epithelium played a role in AHR, we evaluated AHR in mice after knocking down NGF mRNA expression by *in vivo* siRNA administration. First, we

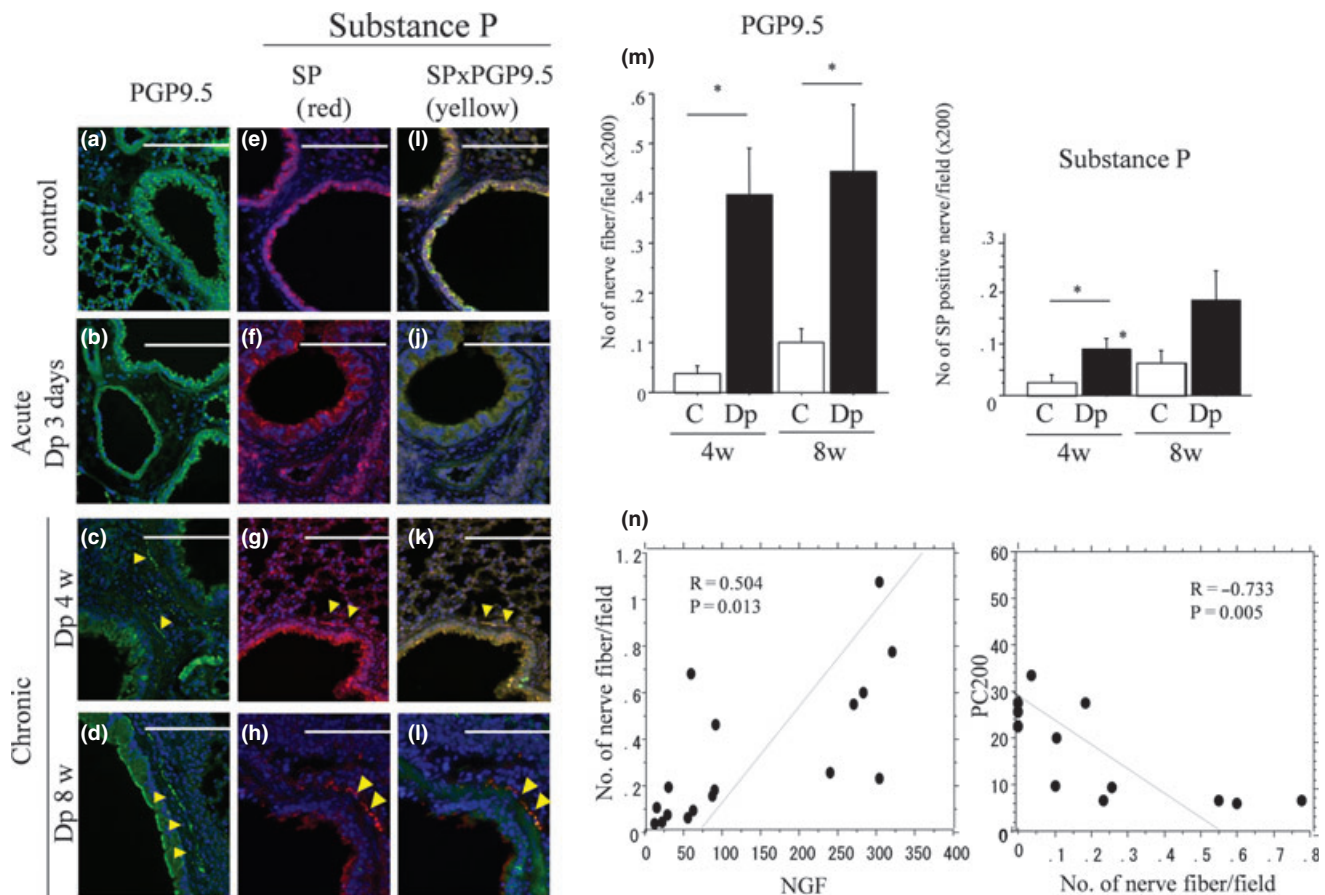


Fig. 3. (a–l) Immunofluorescence of nerve fibres. Photomicrographs are representative of 3 different experiments. (a–d) PGP9.5 (green), (e–h): SP (red), (i–l): merge of PGP9.5 and SP (yellow). Original magnification was 200 $\times$. Scale bar = 200 μ m. (m) Number of PGP9.5 positive and SP positive nerve fibres per field (magnification was 200 $\times$). Results are means $\pm$ SEs from three different experiments (3 mice/group). Numbers of mice (*n*) used for statistical analysis were: White bars: control mice (4 week: *n* = 7; 8 week: *n* = 6); Black bars: Dp-sensitized/challenged mice (4 week: *n* = 7; 8 week: *n* = 8). (n) Associations between NGF, and numbers of nerve fibres and AHR (*n* = 24). Left panel: correlation between NGF in BALF (X) and the number of nerve fibres (Y). Right panel: correlation between the number of nerve fibres (X) and PC200 values (Y). **P* < 0.05.

addressed the effect of *in vivo* transfection of siRNA using fluorescence-labelled siRNA. As shown in Figs 4b and d, fluorescent signals were detected in bronchial and lung epithelial cells in transfected mice. In contrast, there were no signals detected in untreated mice (Figs 4a and c), and in BALF cells (Figs 4e and 4c). To examine the inhibitory effects of NGF-siRNA, siRNA was administered to the acute and 1 w chronic models, and NGF concentrations in BALF were determined. In the acute model, the NGF concentrations increased in Dp-challenged mice (Dp/control siRNA) compared with control mice and control mice with NGF-siRNA (control/NGF-siRNA), and decreased in response to siRNA with two different sequences: Si08 and Si44 (Figs 4g). These results suggest that NGF-siRNA could be transfected into the epithelium and inhibit NGF production without off-target effects. In the 1 w model, the

inhibitory effect of siRNA was retained after 1 w of siRNA administration (Fig. 4g). On the basis of these results, we decided upon the treatment protocol shown in Fig. 1c.

Effect of in vivo siRNA targeting nerve growth factor on airway hyperresponsiveness

In the chronic model generated with this treatment protocol, NGF levels in BALF were significantly decreased in Dp- sensitized/challenged mice with NGF-siRNA (Dp/NGF-siRNA) compared with those in Dp/control siRNA mice (Fig. 5). In contrast, *in vivo* NGF-siRNA did not affect the production of TGF- β and Th2 cytokines, including IL-4, IL-13 and IL-5 (Fig. 5). As shown in Fig. 6a, there were significant differences between the curves for all the groups ($F(3, 30) = 8.96$; $P = 0.002$).

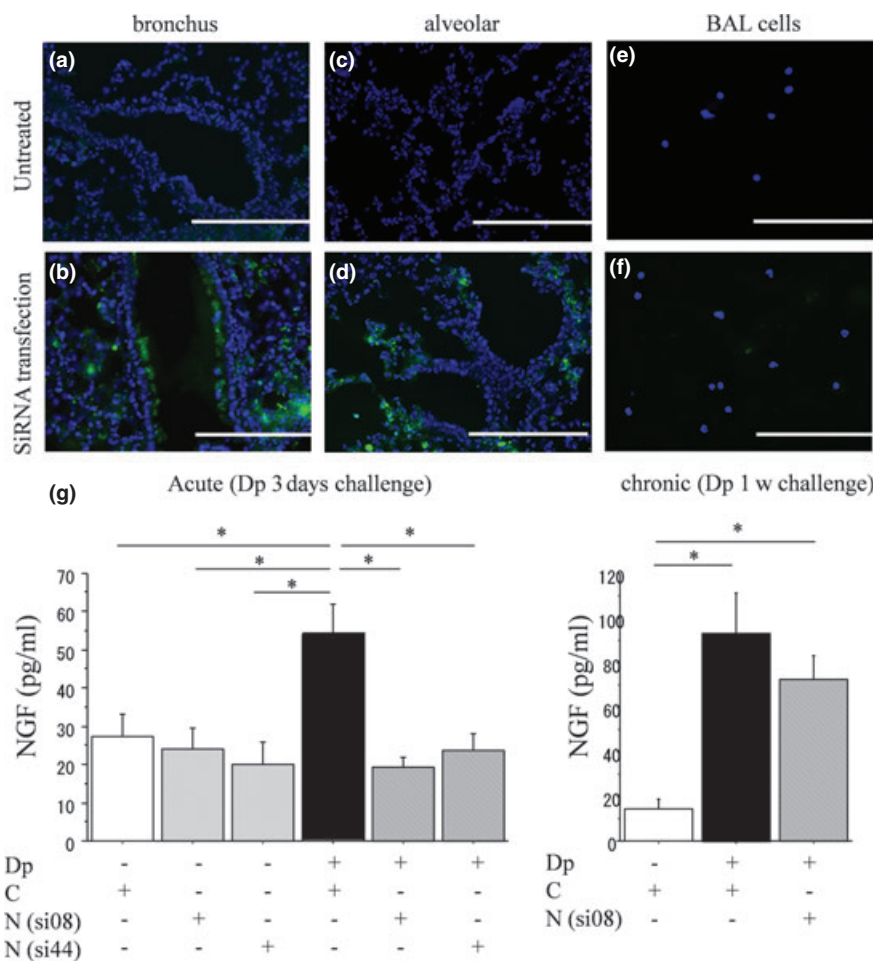


Fig. 4. *In vivo* transfection and inhibitory effects of siRNA targeting NGF. (a–f) Photomicrographs of lungs transfected with fluorescence-labelled siRNA. (a and c) Lung tissue of mice not transfected with siRNA. (b and d): Lung tissue of mice transfected with fluorescence-labelled siRNA. (e) BAL cells of mice not transfected with siRNA. (f) BAL cells of mice transfected with fluorescence-labelled siRNA. Photomicrographs are representative of three experiments. Original magnification was 200 $\times$. Scale bar = 200 μ m. (g) NGF concentrations in BALF in acute model (3-day challenge) and chronic model (1-week challenge). Results are means $\pm$ SEs from two different experiments (5 mice/group). The number of mice in each group used for statistical analysis was eight. White bar: control/control siRNA mice; Grey bar: control/NGF-siRNA mice; Black bar: Dp/control siRNA mice; Shaded bar: Dp/NGF-siRNA mice. * $P < 0.05$.

ANOVA also showed an interaction between the treatment and the Mch dose ($F(4, 120) = 57.21$; $P < 0.001$). Airway resistance decreased upon siRNA treatment at Mch concentrations of 6, 12.5, and 50 mg/ml compared with the Dp/control siRNA mice group. PC200 values were significantly decreased in Dp/control siRNA mice compared with controls and tended to be restored by siRNA treatment, but this was not statistically significant (Fig. 6a).

Although it is known that airway inflammation and airway remodelling can affect airway hyperresponsiveness [30], *in vivo* administration of NGF-siRNA did not alter the infiltration of eosinophils and the thickness of subepithelial fibrosis (Figs 6b and c). These results suggest that NGF derived from the bronchial epithelium directly contributes to the increased AHR in the chronic Dp exposure model.

Effect of in vivo siRNA targeting nerve growth factor on nerve fibre innervation

Although nerve fibres under the subepithelium were detected in each of the Dp/control siRNA mice groups and the Dp/NGF-siRNA treatment group (Figs 7a–d), the number of fibres in the Dp/NGF-siRNA group was lower compared with Dp/control siRNA mice (Fig. 7e). In addition, we determined the SP concentration in whole lung, because these nerve fibres were not detected by histology in the siRNA treatment group. SP

concentrations in whole lung were significantly lowered by treatment with NGF-siRNA compared with those in Dp/control siRNA mice (Fig. 7f).

Discussion

In this study, we demonstrated that repeated Dp exposures in a chronic model of asthma induced the production of NGF derived from bronchial and alveolar epithelium, and that NGF production correlated with the development of AHR, which may have been secondary to hyperinnervation in the airway epithelium.

The mechanisms underlying AHR remain unclear and are controversial. Cockcroft et al. suggested that there are two components to AHR, reflected by variable AHR and persistent AHR [30]. Persistent AHR is related to airway remodelling and airway inflammation that leads to chronic, irreversible AHR [30], for which only partially reversible changes can be achieved by anti-inflammatory therapy. Chronic prolonged airway inflammation induces airway pathological changes, including subepithelial collagen deposition, airway smooth muscle cell hypertrophy/hyperplasia, increased vascularity and changes in the extracellular matrix composition [30]. The present findings demonstrate one of the mechanisms involved in persistent AHR and add to our understanding of these mechanisms by showing that prolonged antigen exposure results in increased AHR.

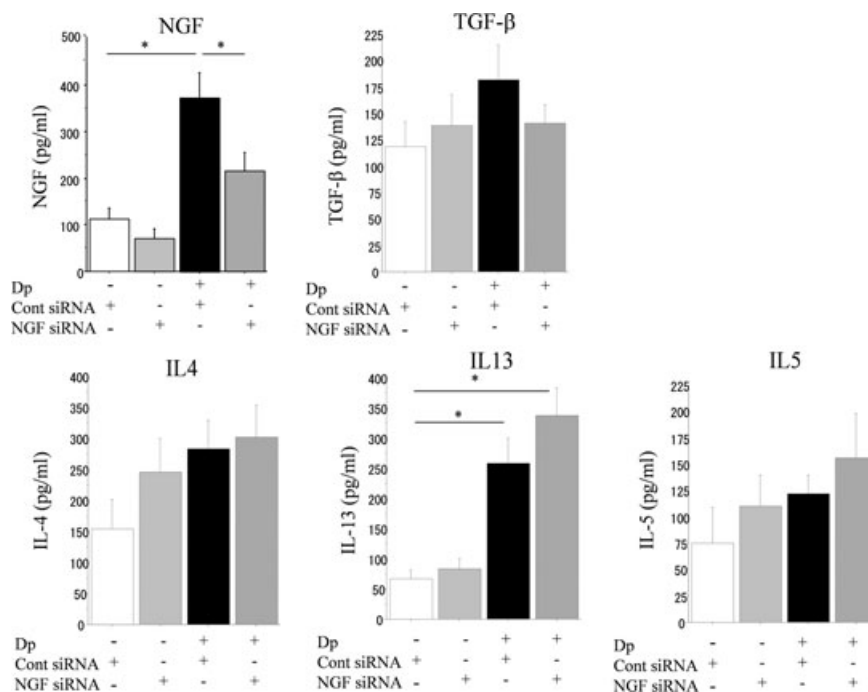


Fig. 5. NGF, TGF- β and Th2 cytokine concentrations after *in vivo* treatment with siRNA targeting NGF. Results are means $\pm$ SEs from three different experiments (3–4 mice/group). The numbers of mice (n) used for statistical analysis were: White bar: control/control siRNA mice ($n = 8$); Grey bar: control/NGF-siRNA mice ($n = 8$); Black bar: Dp/control siRNA mice ($n = 10$); Shaded bar: Dp/NGF-siRNA mice ($n = 10$). * $P < 0.05$.

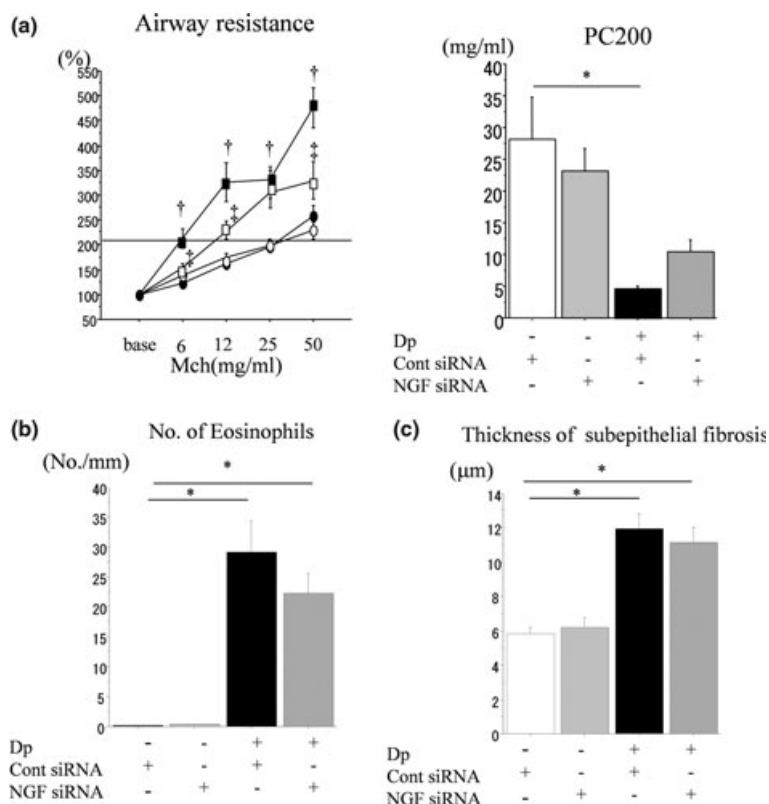


Fig. 6. AHR and morphological analysis after *in vivo* treatment with siRNA targeting NGF. (a) Airway resistance and PC200 after Dp challenge. (b) Numbers of eosinophils in the subepithelium. (c) Thickness of subepithelial fibrosis. Results are means $\pm$ SEs from three different experiments (3–4 mice/group). Numbers of mice (*n*) used for statistical analysis were: Open circles and white bar: control/control siRNA mice (*n* = 8); Closed circles and grey bar: control/NGF-siRNA mice (*n* = 8); Closed squares and black bar: Dp/control siRNA mice (*n* = 8); Open squares and shaded bar: Dp/NGFsiRNA mice (*n* = 10). **P* < 0.05. †*P* < 0.05 compared with control/control siRNA mice. ‡*P* < 0.05 compared with Dp/control siRNA mice.

In this study, we also demonstrated that AHR could be inhibited by knock-down of the NGF gene in bronchial and alveolar epithelium. siRNA elicits sequence-specific degradation of complementary mRNA. Recently, *in vivo* siRNA delivery was used to leverage its effects *in vivo* after developing the appropriate reagents and dispensing methods. siRNA that targeted heme oxygenase-1 reduced ischaemic reperfusion injury. [22] Suppressing the discoidin domain receptor 1 by siRNA attenuated lung inflammation in a bleomycin-induced pulmonary fibrosis model [23]. In bronchial asthma, AHR and airway inflammation were inhibited by siRNA that targeted IL-13 [24] or STAT6 [25]. Similarly, our present findings showed that nasal administration of NGF-siRNA resulted in its transfection into bronchial cells, bronchiole cells or alveolar cells and resulted in reduced NGF concentrations in BALF. Attenuation of NGF also resulted in inhibiting hyperinnervation and reducing AHR. SP concentrations were also significantly reduced after *in vivo* siRNA administration. Moreover, we found that eosinophil infiltration and the thickness of subepithelial fibrosis were not affected by this treatment. These results suggest that inhibiting NGF-derived hyperinnervation by *in vivo* siRNA plays a

critical role in reducing AHR, rather than the inhibition of airway inflammation or airway remodelling.

Based on these findings, the following mechanism may be postulated. NGF released by bronchial and alveolar epithelia resulting from chronic mite antigen exposure can bind either to high-affinity tyrosine kinase A (trkA) receptors or to low-affinity p75 receptors expressed on the surfaces of surrounding nerve fibres, and thereby engender hyperinnervation [1, 31]. NGF can also enhance the production of SP and another tachykinins, Neurokinin A (NKA) by hyperinnervated sensory nerve fibres via the binding to these receptors including trkA and p75 [32, 33]. SP is a neuropeptide involved in contractility in the airways and induces AHR [34], although it induced Prostaglandin E production by airway epithelium, which is a bronchodilator [35]. In addition, SP and Neurokinin (NK)1 receptors, which are receptors for SP, are also implicated in the extravasation caused by antigen challenge [36]. NKA could induce AHR by activating mast cells and their release of serotonin, which is also a bronchoconstrictor [37]. Thus, the effect of tachykinins, including SP, may lead to a readily contractile state that we term AHR [34]. In previous reports, blocking NK1 receptors with

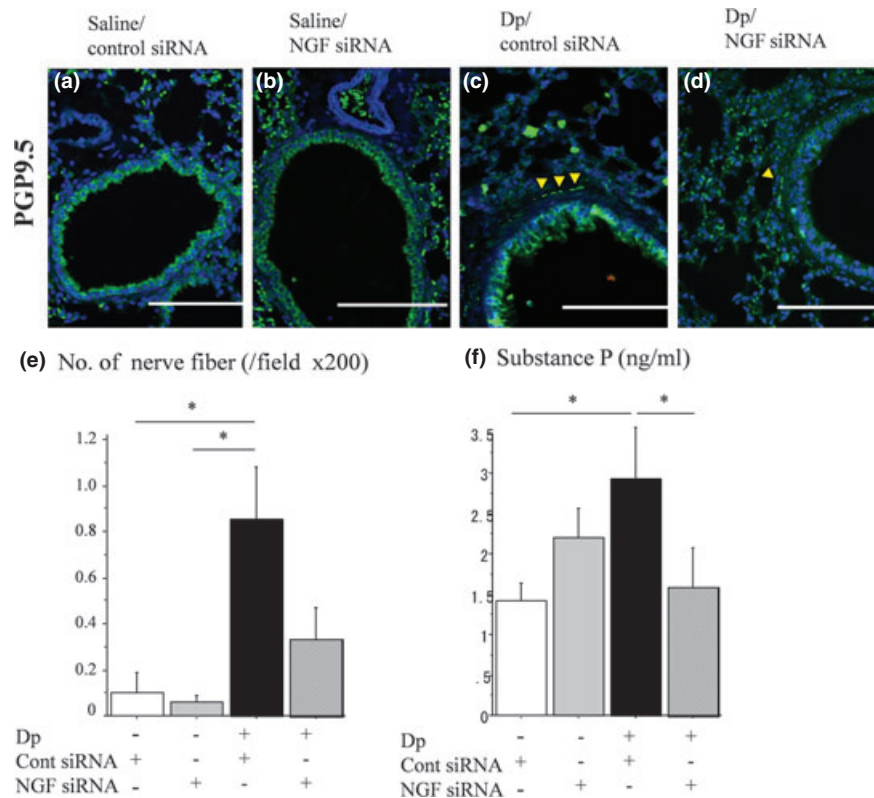


Fig. 7. Nerve innervation and SP concentrations after *in vivo* treatment with siRNA targeting NGF. (a) Control/control siRNA mice. (b) Control/NGF-siRNA mice. (c) Dp/control siRNA mice. (d) Dp/NGF-siRNA mice. Photomicrographs are representative of three different experiments. Original magnification was 200 $\times$. Scale bar = 200 μ m. (e) Number of PGP9.5 positive nerve fibres per field (magnification was 200 $\times$). (f) SP concentrations in lung homogenates. Results are means $\pm$ SEs from three different experiments (3–4 mice/group). The numbers of mice (*n*) used for statistical analysis were: White bar: control/control siRNA mice (*n* = 8); Grey bar: control mice/NGF-siRNA (*n* = 8); Black bar: Dp/control siRNA mice (*n* = 8); Shaded bar: Dp/NGF-siRNA mice (*n* = 10). **P* < 0.05.

an antagonist reduced hyperresponsiveness, suggesting that NGF induced SP-NK1-mediated hyperresponsiveness [8, 38]. NGF induced a change in the neuronal phenotype, which increased SP-positive nerve fibres [39]. The present findings and those of previous reports support our hypothesis that NGF-related mechanisms similar to those seen in atopic dermatitis occur in bronchial asthma after antigen exposure and that these processes may lead to AHR. Alternatively, in atopic dermatitis, these mechanisms involved itch-scratch cycles that eventually resulted in chronic inflammation [40]. When AHR becomes exacerbated, allergic inflammatory cells and cytokines are increased in the airways [41]. Increased NGF due to allergic inflammation induces Th2 cells to increase their production of Th2 cytokines [9] and mast cells to release histamine [10]. These mediators can induce SP production by nerve fibres [34]. Based on this evidence, it appears that NGF-induced nerve hyperinnervation and the resulting AHR of the airways contribute to a cycle of exacerbation similar to the itch-scratch cycle in atopic dermatitis.

With regard to NGF production by epithelial cells, the findings of this study showed that NGF secretion was increased in a time-dependent manner (Fig. 2A), and that its production and storage in epithelial cells also increased with time (Fig. 2B). Moreover, significant hyperinnervation was increased in chronic Dp exposure model compared with our acute exposure model, although both models exhibited NGF production (Fig. 3). Based on these findings, it is likely that there may be difference in the mechanisms between the production and secretion of NGF by epithelial cells. In addition, due to these mechanism, the NGF production and secretion may be gradual and accumulate around the bronchus by chronic Dp exposure, and chronic allergen exposure is critically important for inducing the hyperinnervation derived from the accumulative effects of NGF production by the epithelium. Fox et al. showed that antigen exposure increased the concentrations of IL-1 β and TNF- α , which led to an increase in NGF production by the A549, airway epithelial cell line [12]. Some allergens, such as Dp, can potentially stimulate directly the bronchial epithelium, leading to increased production of

inflammatory cytokines [42]. Der p2 regulates the release of NGF by epithelial cells via the reactive oxygen stress-MAP kinase pathway [43]. NGF bound to macrophages can induce the production of IL-1 β and TNF- α [44]. These responses described in previous reports may be associated with the unique NGF production by the bronchial epithelium upon allergen exposure; however, the detailed mechanism involved remains unknown. Additional studies will be necessary to clarify this.

In summary, the present findings demonstrate that NGF derived from bronchial and lung epithelial cells plays an important role in nerve hyperinnervation-induced AHR. Our results suggest that NGF derived from bronchial and alveolar epithelia may be an attractive target for the management of chronic AHR.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Immunofluorescence of nerve bundles around large bronchi. A: control mice; B: Acute model; C: Chronic model 4 weeks; D: Chronic model 8 weeks. Photomicrographs are representative of three different

experiments. Original magnification was 100×. Scale bar = 50 μ m. Arrow head: nerve bundles.

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