

Nerve growth factor induces increased airway inflammation via a neuropeptide-dependent mechanism in a transgenic animal model of allergic airway inflammation

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Summary

Background Nerve growth factor (NGF) exerts an important functional impact on the pathogenesis of allergic diseases. Data obtained in animal models of allergic bronchial asthma indicate that NGF alters sensory nerve function and promotes allergic inflammation, bronchial hyper-reactivity, and airway obstruction.

Objective To further delineate the effects of NGF on airway inflammation, we employed a transgenic (tg) animal model of allergic inflammation and asthma.

Methods NGF-tg mice, which overexpress NGF in Clara cells of the airways, were compared with wild-type (wt) littermates regarding their ability to mount IgE-related airway inflammatory responses. Mice were sensitized intraperitoneally to ovalbumin (OVA) and locally challenged via the airways according to established protocols.

Results NGF-tg mice displayed enhanced levels of OVA-specific IgE antibody titres after repeated OVA aerosol exposure. In the airways, increased numbers of eosinophils were detected. These results were confirmed to be NGF specific, because similar results were obtained following local application of NGF into the airways of wt mice. The effect of NGF was partly mediated via neuropeptides, as treatment of OVA-sensitized NGF-tg mice with the dual neurokinin (NK) receptor NK-1/NK-2 antagonist partly prevented enhanced airway inflammation.

Conclusion The present data indicate an important functional role of NGF in allergic airway inflammation and point to an involvement of tachykinins as mediators of NGF effects.

Keywords airway hyper-reactivity, airway inflammation, allergy, mouse, nerve growth factor, neuroimmunology, neurotrophin

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Introduction

Initial evidence for the importance of nerve growth factor (NGF) in human allergic disease originated from a paper by Bonini et al. [1], demonstrating elevated serum NGF values in subjects with allergic diseases, such as bronchial asthma, keratoconjunctivitis and urticaria-angioedema. Levels of NGF in serum correlated with the severity of the allergic disease. To examine local allergic inflammation as a source of enhanced NGF production, segmental allergen provocation in mild asthmatics was performed. Unilateral allergen challenges resulted in increased NGF levels in bronchoalveolar lavage fluid (BALF) from the challenged segment after 18 h [2]. In addition, subjects with allergic rhinitis demonstrated elevated baseline NGF levels in nasal lavage fluid, which fur-

ther increased after allergen challenge [3]. In animal models of allergic airway inflammation, macrophages and lymphocytes have been identified as major sources of NGF in this condition [4].

The functional relevance of NGF in human allergic asthma is not completely defined. NGF exerts a central role in development, differentiation and function of neuronal and immune cells. *In vitro* data indicate a role of NGF as a powerful regulator of tachykinin production in sensory neurons [5, 6]. In the guinea-pig lung, nerve-tracing experiments demonstrated that allergen challenge in sensitized animals induced tachykinin production in sensory mechano-sensitive $\alpha\delta$ -nerve fibres, which normally do not express these neuropeptides [7]. This process could be mimicked by application of exogenous NGF, suggesting that the allergen-induced plasticity of sensory nerves is regulated by NGF [8, 9].

In asthmatic subjects, tachykinins such as substance P (SP) are elevated in the lung during asthma attacks [10]. As sensory nerves are critical for reflex-mediated bronchoconstriction, as well as for the development of neurogenic

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inflammation, an involvement in the occurrence of airway hyper-responsiveness (AHR) has been suggested. In fact, several *in vivo* experiments indicate a functional relevancy of these neuronal changes in asthma. In a mouse model of airway inflammation, treatment with anti-NGF reduced the AHR measured by electrical field stimulation. Furthermore, blockade of NGF in this model reduced IL-4 levels in the BALF of sensitized and challenged animals [4]. In a guinea-pig model, exogenously applied NGF significantly increased histamine-induced bronchoconstriction. This effect on airway reactivity was attenuated by a neurokinin (NK)-1 receptor antagonist, pointing to an involvement of tachykinins in mediating the actions of NGF *in vivo* [11].

In addition to AHR, airway inflammation is another crucial feature of bronchial asthma. Neurotrophin receptors are expressed on nerve cells as well as on a wide variety of immune cells, including monocytes, mast cells, and B and T lymphocytes [12–14]. *In vitro*, NGF exerts several effects on immune cells, including mast cell degranulation [15], eosinophilic activation [16], T cell proliferation [17] and antibody production [18]. However, its functional role *in vivo* is not completely characterized. To address this issue, we utilized a transgenic (tg) mouse model, in which NGF is overexpressed in the lung under control of a Clara cell-specific promoter [19]. These mice are characterized by increased NGF production in the lungs, which induces sensory and sympathetic hyper-innervation of the airways, resulting in elevated airway reactivity upon provocation with capsaicin or using electrical field stimulation [19, 20].

The aim of this study was to analyse the effect of enhanced NGF content in the airways on allergic inflammation and to characterize the underlying mechanisms of the pro-inflammatory activity of NGF.

Methods

Animals

Tg mice overexpressing human NGF in Clara cells of the airways had been generated on a mixed genetic background derived from the C57BL/6 and SJL inbred strains as described before [19]. In each experiment, both wild-type (wt) and tg animals derived from the same litter were analysed. Mice were housed under specific pathogen-free conditions. All experiments were approved by the animal care facility (Berlin Office for Occupational Safety, Protection of Health and Technical Safety – LAGetSi).

Genotyping of animals

Before each experiment, animals were tested for tg genotype using PCR with following primer sequences upstream 5'-CAT ACC CAC ACA TAC CCA CA and downstream 5'-ACA TTA CGC TAT GCA CCT GG. Phenotype was confirmed by measurement of NGF content in BALF of each animal after analysis.

Protocol of allergic sensitization

The animal groups were either sensitized to ovalbumin (OVA) (10 µg/injection) (Sigma, Deisenhofen, Germany)

adsorbed to 1.5 mg Al (OH)₃ (Pierce, Rockford, IL, USA), or received phosphate-buffered saline (PBS) vehicle and Al (OH)₃ alone by intraperitoneal injections on days 1, 14, and 21 (Fig. 1). Before analysis, animals received two consecutive local aerosol challenges of 1% OVA diluted in PBS or of PBS alone, applied by aerosolization for 20 min on days 26, 27, 33, 34, 40, 41, 47, and 48 (8 × challenge), as previously described [21]. Animals were analysed 24 h after the last challenge (day 49).

Application of NGF and NK-1/2 antagonist

Slightly anaesthetized mice received 2 × 25 µL saline-containing NGF (10 µg/mL), into the anterior nose. FK224 (1 mg dissolved in 0.1 mL 10% DMSO and 0.01% ethanol) or vehicle was administered i.v. Both substances were applied once a week 60 min before the weekly airway provocation (Fig. 1).

Determination of allergen-specific antibodies

Blood was sampled from the lateral tail veins. Blood was clotted at room temperature and centrifuged (1200 g, 10 min, room temperature). Total IgE and allergen-specific IgE, IgG1, and IgG2a antibody titres were measured by ELISA, as described previously [4].

Bronchoalveolar lavage

Twenty-four hours after the last airway provocation, animals were killed and the tracheae cannulated. Airways were lavaged twice with 0.8 mL ice-cold PBS, and cell numbers and cytokine contents were determined as described previously [4]. Cell-free BALF was frozen at –20 °C until further analysis.

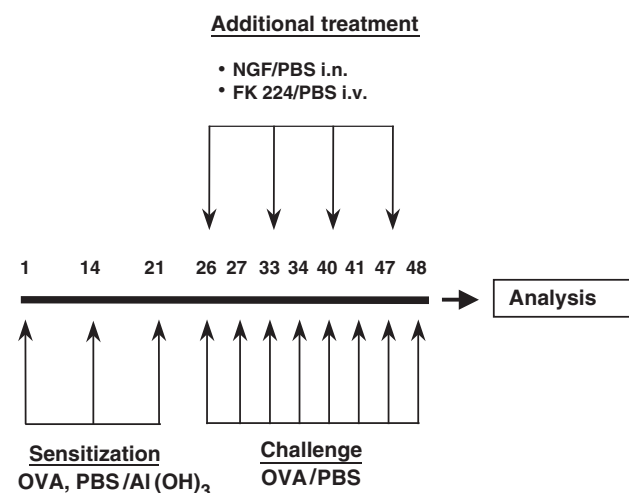


Fig. 1. Study protocol nerve growth factor (NGF) wild-type (wt) and transgenic (tg) animals were systemically sensitized with ovalbumin (OVA)/Al (OH)₃ (on days 1, 14 and 21) and repeatedly challenged via the airways with allergen (on days 26, 27, 33, 34, 40, 41, 47 and 48). In the experiments with the neurokinin antagonist, FK224 was given to NGF wt and tg animals once prior to weekly airway challenges (i.v., intravenous). To study the effect of external NGF application, wt animals were additionally treated either with NGF or vehicle (i.n., intranasally) once prior to weekly airway challenges. Analysis was performed on day 49.

Determination of cytokines and NGF

Ninety-six-well ELISA plates (Nunc, Roskilde, Denmark) were coated with either rat-anti-mouse IL-4 (1 mg/mL, clone 11B11), IL-5 (2 mg/mL, clone TRFK5) (Pharmingen, Hamburg, Germany), IFN- γ (3 mg/mL, clone 37801,11 and 37875,11), or anti-human β -NGF (2 μ g/mL, clone 25623,1) (R&D, Wiesbaden, Germany) diluted in 0.1 mL carbonate coating buffer, pH 8.2 (for Pharmingen products) or PBS (for R&D antibodies). After overnight incubation, plates were washed twice with 0.1% Tween-20/PBS, pH 7.2 and blocked for 1 h with 3% bovine serum albumin (BSA)/PBS buffer (w/v) (Sigma) at room temperature. After washing, cell-free BALF or supernatant was added in duplicates for 2 h at 37 °C. The following biotin-labelled monoclonal antibodies were incubated for 1 h at room temperature: rat-anti-mouse IL-4 (0.25 mg/mL, clone BVD6-24G2), IL-5 (1 mg/mL, clone TRFK4) (Pharmingen), IFN- γ (0.5 mg/mL, clone BAF485, R&D) and anti-human β -NGF (75 ng/mL, clone BAF256, R&D). After washing, plates were incubated with streptavidin-peroxidase (Calbiochem-Novabiochem, Bad Soden, Germany) diluted 1:1000 in 3% BSA/PBS for 30 min at room temperature. The substrate for the reaction was tetramethylbenzidine (TMB) (Fluka, Neu-Ulm, Germany) diluted 1:100 in Gallati buffer. The reaction was stopped with 2 N sulphuric acid and plates were measured at 450/490 nm. Sensitivities were 10 pg/mL for IL-4, 7 pg/mL for IL-5, 15 pg/mL for IFN- γ and 8 pg/mL for NGF.

Statistical analysis

Results are presented as mean values $\pm$ standard deviation. Means were compared by using Mann-Whitney *U*-test, SPSS, Version 7.5.2G (SPSS, Inc., Chicago, IL, USA). *P*-values <0.05 were regarded as significant differences.

Results

NGF-tg mice and antibody response

To determine the effect of local NGF overexpression, NGF content in BALF and serum of untreated mice was measured in wt and NGF-tg animals. An about 50-fold increase in NGF in the BALF was detected in naïve NGF-tg mice compared with wt controls (Fig. 2). In contrast, NGF was not detectable in sera from both groups (data not shown). In animals subjected to sham sensitization or sham provocation, no airway inflammation was measurable (characterized by enhanced infiltration of immune cells and elevated cytokine levels). OVA-specific Ig were found only in OVA-sensitized animals. Total IgE and OVA-specific Ig levels did not differ between wt and tg groups (data not shown). These data indicate that mice overexpressing NGF have no airway inflammation and normal antibody titres. Sensitization alone or allergen provocation of naïve mice did not alter this pattern.

Sensitization of NGF-tg mice compared with wt

It was then examined whether mice overexpressing NGF would show an altered Ig response after allergen sensitization

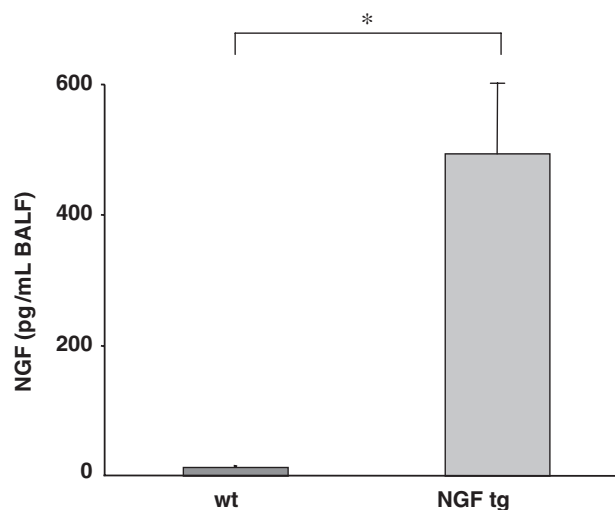


Fig. 2. Nerve growth factor (NGF) content in bronchoalveolar lavage fluid (BALF). The NGF content in BALF of naïve mice was determined by ELISA. Results are expressed as mean values with standard deviation, asterisks indicate statistically significant differences ($P < 0.05$). Six mice were analysed in each group.

Table 1. Ig levels in serum

Genotype	NGF wt	NGF tg
Anti-OVA IgE (LU/mL serum)	185 ($\pm$ 51)	*533 ($\pm$ 229)
Anti-OVA IgG1 (LU/mL serum)	709 ($\pm$ 65)	711 ($\pm$ 73)
Anti-OVA IgG2a (LU/mL serum)	2442 ($\pm$ 567)	2848 ($\pm$ 599)

Sensitized NGF wt and tg animals were repeatedly challenged with allergen via the airways, serum was sampled 24 h after the last airway challenge. Total IgE and OVA-specific IgE, IgG1 and IgG2a were measured by ELISA. Six mice were analysed in each group in two independent experiments. Average value and standard deviation are expressed, asterisk indicates statistically significant difference between NGF wt and tg ($P < 0.05$). NGF, nerve growth factor; OVA, ovalbumin; wt, wild-type; tg, transgenic; LU, lab units.

or after airway challenge as compared with wt mice. Allergen sensitization in both groups resulted in similar elevations of total and OVA-specific antibody levels (data not shown). After allergen challenge, NGF overexpression had an additional enhancing effect on development of anti-OVA IgE antibody level as compared with wt mice (Table 1).

Inflammation in the lung of NGF-tg mice as compared with wt

Local airway inflammation was evaluated by measurement of cytokine levels and determination of BALF cell counts after repeated allergen challenges. Enhanced IL-5 levels (Fig. 3) and increased numbers of inflammatory cells (189 ± 59 vs. $253 \pm 39 \times 10^3$ (cells/mL) total leucocytes $n = 10$, in two independent experiments, $P < 0.05$) were detected in the BALF of NGF-tg animals as compared with wt mice. The difference between the two groups was predominately based on an increase in eosinophils, whereas the numbers of other cell populations such as neutrophils remained similar between both groups (Fig. 4). The data indicate that the presence of NGF during elicitation and onset of inflammation was required for this augmenting effect. To further investigate the effect, NGF or PBS were applied nasally to the airways of sensitized wt

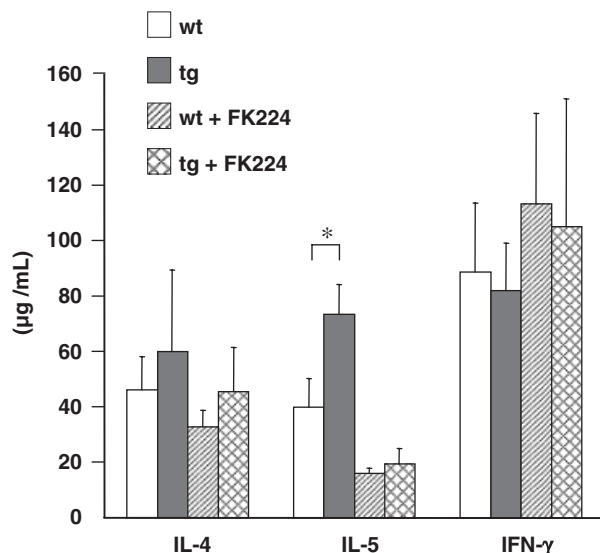


Fig. 3. Cytokine level in BALF sensitization, challenge and treatment protocols, as described in Fig. 1. Cytokines were measured in BALF from ovalbumin-sensitized mice 24 h after aerosol challenge by ELISA. Results are expressed as single values with medium bar, asterisks indicate statistically significant differences ($P < 0.05$) between NGF-tg and wt mice. Six mice were analysed in each group in two independent experiments. BALF, bronchoalveolar lavage fluid; NGF, nerve growth factor; tg, transgenic; wt, wild-type.

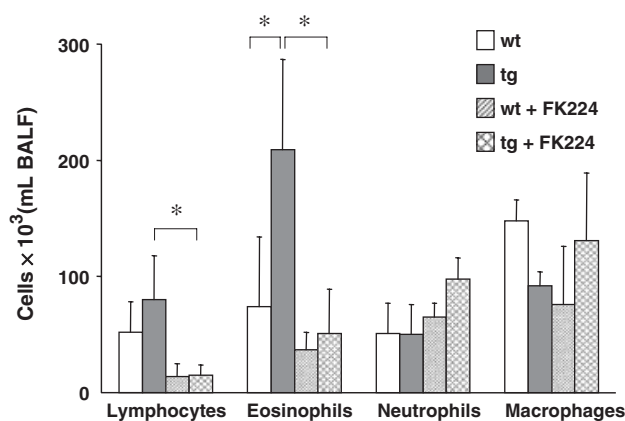


Fig. 4. Cell differentiation in bronchoalveolar lavage fluid (BALF) sensitization, challenge and treatment protocols as described in Fig. 1. Neutrophils, lymphocytes, and eosinophils were differentiated according to morphologic criteria in BALF 24 h after the last aerosol challenge. Results are expressed as average values with standard deviation, asterisks indicate statistically significant differences ($P < 0.05$). Ten mice were analysed in each group in two independent experiments.

mice prior to weekly OVA challenges (Fig. 1). In the OVA-sensitized animals, NGF treatment triggered a significant increase in total leucocytes (153 ± 63 vs. $372 \pm 125 \times 10^3$ (cells/mL) total leucocytes ($n = 6$, in two independent experiments), $P < 0.05$), which was again due to a 2-fold increase in eosinophil numbers, whereas neither all of the other cell populations (Fig. 5) nor cytokine or Ig levels differed significantly between the two groups (Table 2).

Effect of tachykinin antagonists on airway inflammation

To test whether tachykinins were involved in mediating NGF-induced airway inflammation, OVA-sensitized and

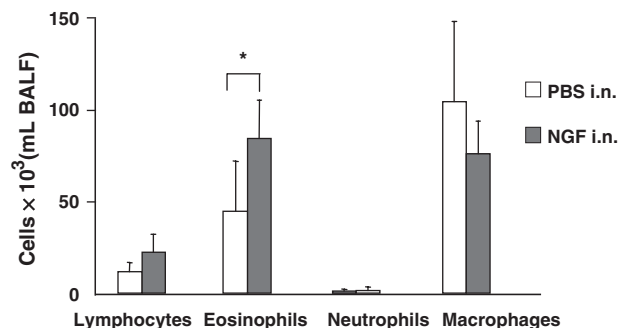


Fig. 5. Cell differentiation in bronchoalveolar lavage fluid (BALF) of externally nerve growth factor (NGF)-treated wild-type mice sensitization, challenge and treatment protocols as described in Fig. 1. Neutrophils, lymphocytes, and eosinophils were differentiated according to morphological criteria in BALF 24 h after the last aerosol challenge. Results are expressed as average values with standard deviation, asterisks indicate statistically significant differences ($P < 0.05$). Six mice were analysed in each group in two independent experiments.

Table 2. Serum Igs and cytokines in BALF of intranasally NGF-treated animals

Treatment	PBS intranasally	NGF intranasally
Anti-OVA IgE (LU/mL)	290 ($\pm$ 244)	745 ($\pm$ 384)
Anti-OVA IgG1 (LU/mL)	191 ($\pm$ 17)	186 ($\pm$ 104)
Anti-OVA IgG2a (LU/mL)	1077 ($\pm$ 279)	653 ($\pm$ 371)
IL-4 (pg/mL BALF)	28 ($\pm$ 18)	23 ($\pm$ 10)
IL-5 (pg/mL BALF)	28 ($\pm$ 27)	21 ($\pm$ 12)
IFN- γ (pg/mL BALF)	DL	DL

Sensitized wt animals were repeatedly OVA-challenged via the airways. Additionally, mice were treated intranasally with NGF. Total IgE and OVA-specific IgE, IgG1, IgG2a, and cytokines were measured by ELISA. Six mice were analysed in each group in two independent experiments. Average value and standard deviation are expressed. BALF, bronchoalveolar lavage fluid; NGF, nerve growth factor; OVA, ovalbumin; wt, wild-type; tg, transgenic; LU, lab units; DL, detection limit.

challenged wt and NGF-tg mice were treated with the dual neurokinin (NK-1, NK-2) receptor antagonist FK224 before weekly allergen provocation (Fig. 1). As depicted in Fig. 4, treatment with FK224 resulted in a significantly lower number of eosinophils in tg animals, but not in the wt group. Furthermore, treatment with FK224 significantly reduced IL-5 levels in the BALF of tg mice, but not in wt animals. The pre-existing difference between wt and NGF-tg animals was not evident anymore (Fig. 3).

Discussion

Local overexpression of NGF in the airways had the following effect on allergic immune responses in animal models of OVA sensitization: (i) augmentation of anti-OVA-specific IgE antibody production; (ii) augmentation of eosinophilic airway inflammation following allergen challenges; and (iii) augmentation of IL-5 production in BALF. These effects were at least partially mediated via tachykinins as treatment with the tachykinin receptor antagonist FK224 prevented these increases to a large extent. Furthermore, these effects were NGF specific as local treatment with NGF in wt mice gave similar results.

Augmentation of airway inflammation might be due to (i) a direct effect of NGF on inflammatory cells, (ii) neuropeptide release leading to intensified neurogenic inflammation, or (iii) anatomical lung differences between tg and wt mice as a consequence of developmental effects of NGF.

There is ample data suggesting that NGF enhances effector function of various immune cells. The mechanism of chemoattraction and degranulation of mast cells is partially understood on a molecular level [15, 22]. Hamada et al. [16] found that NGF treatment resulted in an increased cytotoxicity of eosinophils. *In vivo*, the NGF overexpression of allergic mice led to an augmented early-phase reaction [23]. In addition, these mice displayed an increased inflammatory response in the airways upon ozone provocation [24]. It has not been completely established whether this amplified effector function is solely due to a direct effect of NGF on immune cells.

NGF is known to be a strong regulator of tachykinin synthesis [25, 26]. This is of particular importance, because tachykinins have been found to be increased systemically and in the airways of asthmatic subjects after allergen challenges [10]. Interestingly, increased amounts of SP are present in the airways of NGF-tg mice [19]. Tachykinins have been shown to play an important role in mediating neuronal inflammation. They are capable of inducing bronchial constriction [27], microvascular leakage [28], activation of immune cells and mucous secretion [29] (for a review see [30]). NK-1 receptors are mainly responsible for mediating the inflammatory effects of tachykinins, while NK-2 receptors induce bronchial constriction [31]. As SP binds to the NK-1 receptor with high affinity, this may be the predominate mediator molecule of immunomodulatory effects among tachykinins [32]. SP exerts effects on various immune cells including neutrophils, eosinophils, mast cells, monocytes/macrophages, and lymphocytes [33].

To delineate whether NGF acts directly on immune cells or through neuropeptide-induced pathways, animals were treated with the neuropeptide antagonist FK224. Although the exact mechanism of action of the NK antagonists is not known yet, dual or selective NK-1 and NK-2 antagonists have been shown to inhibit several components of allergen-induced airway inflammation, both in asthmatic subjects and animal models [34, 35]. Antagonists differ in their strength to modulate airway pathology. In our model, FK224 did not significantly alter airway inflammation in sensitized and challenged wt animals, but prevented the increase in eosinophil numbers observed in NGF-tg mice (Fig. 4). This result suggests that the pro-inflammatory effect of NGF on cell influx into the lung is mediated in parts via tachykinins. We found a decrease in BALF IL-5 level together with a tendency to reduce cell influx into the airway in both wt and tg mice treated with the antagonist. Main source of IL-5 in the allergic airway inflammation are T cells and mast cells. The reduction or an additional inhibition of IL-5-producing cells in the airways might have caused the decreased IL-5 level in the BALF of the animals treated with the dual NK antagonists.

As NGF is constitutively overexpressed in this tg mouse line, anatomical lung differences between tg and wt mice as a consequence of development effects of NGF may have had a potential influence on our results. To further investigate this

possibility, wt mice were sensitized and treated intranasally with NGF before each allergen challenge. This protocol gave comparable results in terms of augmented airway inflammation as in the NGF-tg animals. These data indicate that the presence of NGF itself rather than an NGF-dependent developmental difference in airway architecture seems to be responsible for the demonstrated effects. However, induction of growth of sensory neurons in adult animals by NGF cannot be completely ruled out from these data.

In conclusion, this study provides further evidence for the concept that neurotrophins, particularly NGF, contribute to allergic responses in the lung. Previous studies by us and others have shown that neurotrophins are produced by several structural and immune cells in the airways and the lung including airway epithelium, alveolar and bronchial macrophages, T and B lymphocytes [4]. Increased production of NGF has been demonstrated in both, human and mice. To our knowledge, this is the first study showing that at least some effects of NGF-induced augmentation of airway inflammation can be prevented by treatment with tachykinin receptor antagonists. Further investigations are now required to find out whether similar effects can be reached also in human asthmatic patients.

Acknowledgements

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