Original article

Nerve growth factor induces type III collagen production in chronic allergic airway inflammation

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Background: Excessive extracellular matrix deposition occurs as a result of repetitive injury-repair cycles and plays a central role in the pathogenesis of chronic inflammatory diseases, such as allergic asthma. The molecular mechanism leading to aberrant collagen deposition is not fully understood.
Objective: We sought to test the hypothesis that increased nerve growth factor (NGF) production contributes to collagen deposition in the airways during chronic allergic airway inflammation.
Methods: Antibody-blocking experiments were performed in an *in vivo* model for chronic allergic airway inflammation (allergic asthma), which is accompanied by matrix deposition in the subepithelial compartment of the airways, to study the profibrotic effect of NGF. The signaling pathways were delineated with *in vivo* and i*n vitro* studies in primary lung fibroblasts.
Results: Functional blocking of NGF in chronically affected mice markedly prevented subepithelial fibrosis. Transgenic overexpression of NGF in murine airways resulted in altered airway wall morphology with increased peribronchial collagen deposition and impaired lung physiology in the absence of inflammation. NGF exerted a direct effect on collagen expression in murine lung fibroblasts, which was mainly mediated through the activation of the receptor tropomyosin-related kinase A. NGF-induced collagen expression was dependent on downstream activation of p38 mitogen-activated protein kinase independent of the TGF-β1/mothers against decapentaplegic homolog (SMAD) pathway.
Conclusion: The results of this study demonstrate that NGF exerts profibrotic activities in the airways by inducing type III collagen production in fibroblasts independently of TGF-β1.
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Key words: *Allergic asthma, airway remodeling, type III collagen, nerve growth factor*

Excessive collagen deposition by fibroblasts is a prominent characteristic in a variety of chronic inflammatory diseases and is associated with loss of organ function.[1] It is considered the consequence of exaggerated wound repair after repetitive or chronic insult to the tissue.[1] During inflammation and tissue injury, growth factors are secreted by the injured epithelium and infiltrating immune cells and drive the subsequent wound-healing process.[2] The production and deposition of type III collagen by fibroblasts as part of the provisional matrix for epithelial cell migration and proliferation is immediately initiated on injury.[1] Allergic asthma represents a chronic inflammatory condition in which tissue remodeling and restructuring is of disease-limiting importance. In patients with chronic asthma, excessive matrix deposition manifests as subepithelial fibrosis in the airways and contributes to lung function decline in the progression of the disease.[3,4] TGF-β1 has been identified as a major profibrotic growth factor[5,6]; however, there is strong evidence that antagonizing TGF-β1 might not be sufficient to prevent disease pathology. Furthermore, profibrotic pathways acting independently of TGF-β1/mothers against decapentaplegic homolog (SMAD) signaling have been recently identified.[7,8]

Nerve growth factor (NGF), a prototypic member of the neurotrophin family, was initially described in promoting the survival and differentiation of neuronal cells.[9] In addition to this neuroprotective activity, there is increasing evidence supporting the role of NGF in repair mechanisms. NGF is actively secreted on injury and during inflammation from various epithelial and inflammatory cells. Its ability to accelerate wound-healing processes has been shown in several pathological situations[10] and rodent models of tissue repair.[11] In this context NGF promotes epithelial cell proliferation[12] and migration, as well as contraction of lung fibroblasts.[13] These events represent an important step in the repair process and link this growth factor to profibrotic mechanism. Therefore we hypothesized that as a consequence of repetitive induction of lung damage, NGF levels are strongly upregulated and participate in deregulated repair mechanisms that result in subepithelial fibrosis in chronic inflamed airways. To investigate this concept and explore the mechanisms of how NGF might promote collagen deposition in the airways, we used a well-established and well-characterized murine model of chronic allergic airway inflammation that demonstrates the pathological process of subepithelial fibrosis.[14]

METHODS

The methods used are described in detail in the Methods section in this article's Online Repository at www.jacionline.org.

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Abbreviations used
BALF: Bronchoalveolar lavage fluid
col3(α1): Type III collagen (α1) chain
ERK: Extracellular signal-regulated kinase
JNK: c-Jun terminal kinase
MAPK: Mitogen-activated protein kinase
MLF: Murine lung fibroblast
NF-κB: Nuclear factor κB
NGF: Nerve growth factor
OVA: Ovalbumin
siRNA: Small interfering RNA
SMAD: Mothers against decapentaplegic homolog
SURS: Systematic uniform random sampling
TrkA: Tropomyosin-related kinase A
WT: Wild-type

Animals

Female C57BL/6 (wild-type [WT]) mice obtained from Harlan Winkelmann (Borchen, Germany), $Ngfr^{tm1Jae}$ ($p75^{NTR\ exonIII-/-}$), mice on a C57BL/6 background purchased from Jackson Laboratory (Bar Harbor, Me), and transgenic mice encoding NGF (NGF-Tg) under the control of the lung-specific Clara cell secretory protein (CCSP) promoter[15,16] at 6 to 8 weeks of age were used for the experiments. NGF-Tg mice were mated with $p75^{NTR\ exonIII-/-}$ ($p75^{-/-}$) mice.[17] Mice were genotyped for the presence of the NGF construct or deletion of exon III of the p75 gene. All mice used for the experiments were maintained under pathogen-free conditions in a 12/12-hour light/dark cycle with food and water available *ad libitum*. Experimental procedures were approved by the local animal ethics committee.

Ovalbumin sensitization and challenge

Mice were sensitized by 3 intraperitoneal injections of 10 μg of ovalbumin (OVA; grade VI; Sigma-Aldrich, Hamburg, Germany) adsorbed to 1.5 mg of aluminum hydroxide (Pierce, Rockford, Ill) dissolved in 200 μL of PBS at days 1, 14, and 21. OVA aerosol challenges (1% OVA, grade V, Sigma) were performed for 1 or 4 weeks, with 2 consecutive challenges per week (see Fig E1 in this article's Online Repository at www.jacionline.org).[14] Experimental groups were either sham immunized and sham treated (PBS/PBS), OVA immunized and sham treated (OVA/PBS), or OVA immunized and either treated with a control IgG (OVA/IgG) or anti-NGF (OVA/anti-NGF). Antibody dose and route are described in detail in the Methods section of this article's Online Repository.

Measurement of lung mechanics

Lung mechanics were assessed by using the invasive flexiVent-System (SCIREQ, Montreal, Quebec, Canada). Measurements are described in detail in the Methods section of this article's Online Repository.

Assessment of leukocyte distribution in bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously described.[18]

Measurements of cytokines in bronchoalveolar lavage fluid

Measurement of cytokines is described in the Methods section in this article's Online Repository.

Lung histology and morphometric analysis

After bronchoalveolar lavage fluid (BALF) collection, the right lung was fixed in paraformaldehyde (6% wt/vol). Systematic uniform random sampling (SURS) was performed to obtain a representative collection of lung tissue samples.[19] After paraffin embedment, 3-μm sections were stained with hematoxylin and eosin. Collagen fibrils were detected by means of staining with Sirius Red (Sigma-Aldrich)/Fast Green (Rowley Biochemicals, Danvers, Mass). See the Methods section in this article's Online Repository for additional details on the methods used for collagen quantification.

Isolation of murine lung fibroblasts, RNA isolation, and analysis of mRNA expression

Murine lung fibroblasts of WT and $p75^{-/-}$ mice were isolated by enzymatic digestion and magnetic bead isolation. For signaling pathway analysis, cells were pretreated with specific inhibitors for ERK1/2 (20 μmol/L U0126; Calbiochem, Nottingham, United Kingdom) and p38 MAPK (20 μmol/L SB202190, Calbiochem) followed by stimulation with 100 ng/mL rhNGF (PeproTech, Hamburg, Germany). Col3(α1) mRNA expression was analyzed by means of real-time PCR. Cell isolation, RNA isolation, and mRNA expression analysis are described in detail in the Methods section in this article's Online Repository.

Construction of luciferase reporter plasmid

Genomic fibroblast DNA was used to generate a col3(α1) promoter reporter construct containing the flanking sequence 5′ to the transcriptional start ATG (+1) cloned into pGL4.10 (firefly luciferase) mammalian expression vector (Promega, Madison, Wis). Construction of luciferase reporter is described in detail in the Methods section in this article's Online Repository.

Transient transfection and reporter gene analysis

HEK293 cells were transfected with pcDNA-ratTrkA (WT and mutant K538R) using FuGene HD (Roche, Penzberg, Germany). After 24 hours, the pCol3(α1)-luc and pGL4.73 were co-transfected. Cell extracts were analyzed using the dual-luciferase reporter assay system (Promega). Transient transfection and reporter gene analysis are described in detail in the Methods section in this article's Online Repository.

RNA interference

RNAi knockdown experiments were performed using specific siRNAs for TrkA and SMAD4, both puchased from Qiagen. RNA interference is described in detail in the Methods section in this article's Online Repository.

Immunoprecipitation and immunoblot analysis

Cells were lysed in 50 mM Tris/HCl pH7.5, 150 mM NaCl, 1% NP-40, 1 × protease- and phosphatase- inhibitor mix (both Roche) and used for immunoprecipitation and immunoblot analyses. Immunoprecipitation and immunoblot analysis are described in the Methods section in this article's Online Repository.

Statistical analysis

Data analyses were performed with the Prism 4 Software package (GraphPad Software, Inc, San Diego, Calif). Bar graph data were expressed as means ± SEMs. The box and whisker plots represent the 10th, 25th, 50th (median), 75th, and 90th percentiles. Where appropriate, a 2-tailed Student *t* test was done. For multiple-group comparisons, 1-way ANOVA followed by the Tukey posttest was applied. *P* values of less than .05 were considered statistically significant.

RESULTS

Development of subepithelial fibrosis in experimental chronic asthma is NGF dependent

Well-established murine models of acute and chronic allergic airway inflammation were used to assess the involvement of NGF in the production and deposition of peribronchial collagen.[14] We observed that NGF levels were augmented during disease

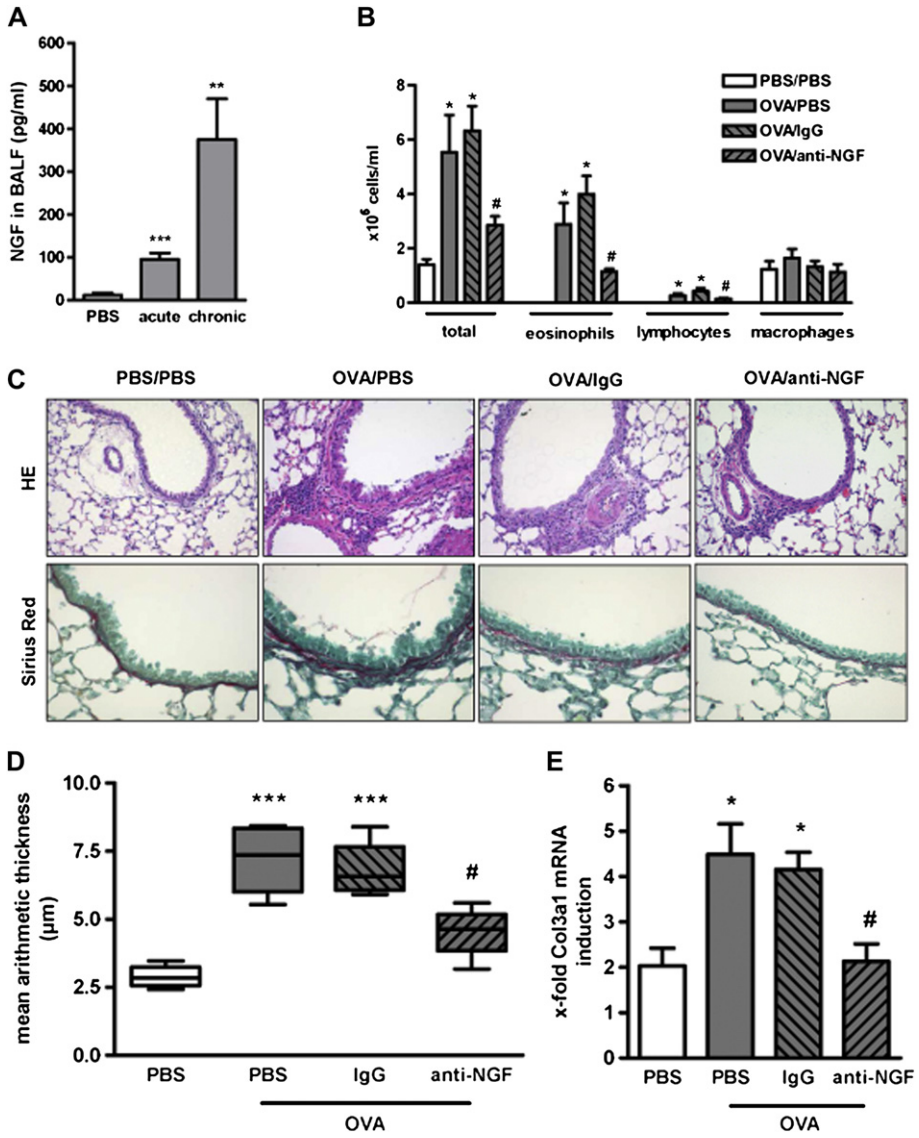

FIG 1. Neutralization of NGF in OVA-induced experimental chronic asthma suppresses airway inflammation and subepithelial collagen deposition. **A**, NGF levels in BALF after acute and chronic allergen exposure. **B**, Total and differential cell counts in BAL. **C**, Lung sections stained for hematoxylin and eosin (*HE*; magnification ×200) and Sirius Red (magnification ×400). **D**, Stereological quantification of subepithelial collagen layer thickness. **E**, Col3(α1) mRNA levels in total lung RNA. N = 4 to 6 per group. **P* < .05, ***P* < .01, and ****P* < .001 versus PBS/PBS; #*P* < .05 versus OVA/PBS and OVA/IgG.

progression (Fig 1, *A*), and the highest levels of NGF were detected in the chronic phase, which was accompanied by peribronchial fibrosis (Fig 1, *C*, Sirius Red, and Fig 1, *D*, OVA/PBS). NGF was neutralized with a function-blocking polyclonal antibody to examine whether collagen deposition in the airways during asthma is dependent on NGF. Assessment of subepithelial collagen deposition in these mice revealed that anti-NGF treatment markedly prevented OVA allergen–induced collagen deposition (Fig 1, *C*, OVA/anti-NGF), as confirmed by means of quantification of peribronchial collagen layer thickness (Fig 1, *D*, OVA/anti-NGF), as well as type III collagen (α1) chain (col3[α1]) mRNA expression in lung tissues (Fig 1, *E*), the major component of reticular connective tissue. In addition, administration of anti-NGF prevented the OVA-induced influx of inflammatory cells into the lung tissue, as revealed by hematoxylin and eosin staining on lung sections (Fig 1, *B* and 1, *C*), together with markedly reduced inflammatory cell numbers in the BALF of these mice (Fig 1, *B*). In contrast, the administration of a control IgG antibody (OVA/IgG) affected neither collagen deposition nor tissue and airway inflammation.

Constitutive overexpression of NGF in the airways causes subepithelial collagen deposition and altered lung mechanics

Mice were examined that constitutively overexpress NGF under the control of the Clara cell secretory protein promoter in the airways (NGF-Tg) to distinguish between the indirect effects of NGF through augmentation of inflammatory cell activity and a direct effect of NGF on structural cells.[16] The

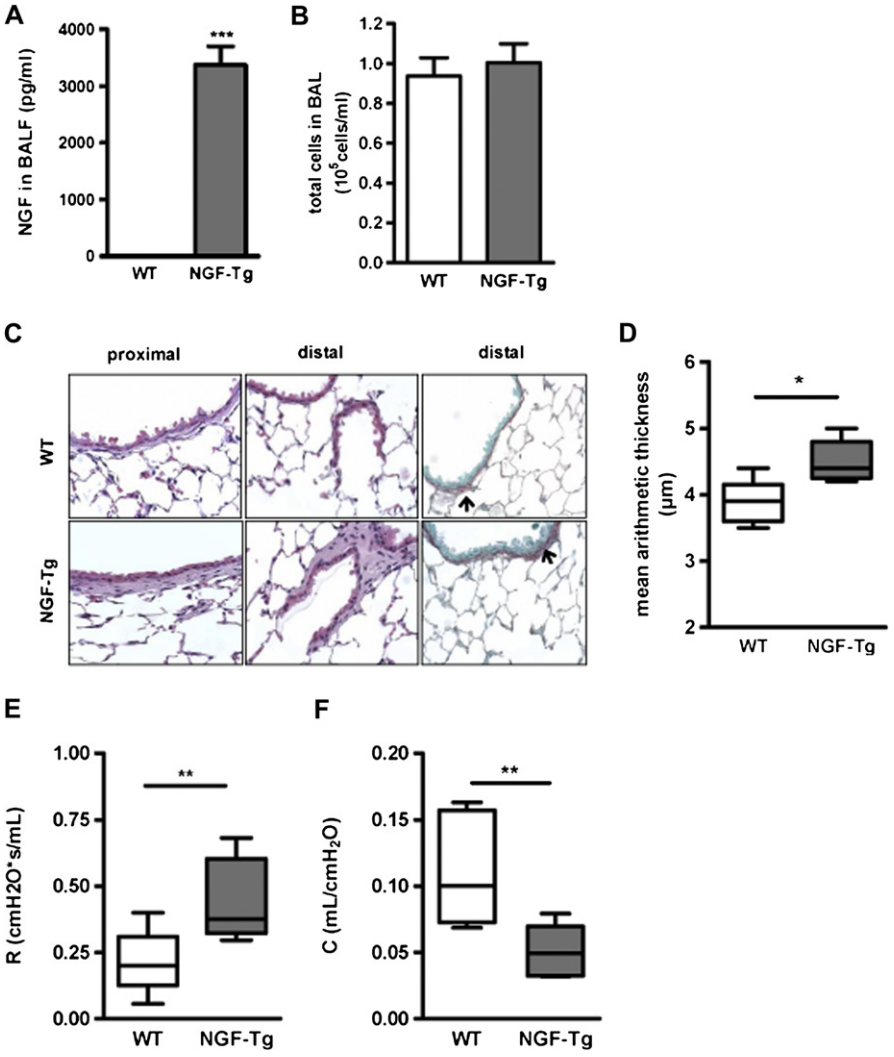

FIG 2. Increased subepithelial collagen deposition in NGF-Tg mice. **A**, NGF levels in BALF of naive WT and NGF-Tg mice. **B**, Cell counts in BAL. **C**, Hematoxylin and eosin (HE)– and Sirius Red–stained proximal and distal lung sections (magnification ×200). *Arrows* indicate collagen fibrils. **D**, Quantification of subepithelial collagen deposition in the airways. **E** and **F**, Baseline airway resistance (Fig 2, *E*) and lung compliance (Fig 2, *F*) were measured. N = 5. $*P < .05$, $**P < .01$, and $***P < .001$ versus WT mice.

Clara cell promoter becomes active before birth,[20] and high levels of NGF are readily detectable in BALF at a young age. Despite increased NGF levels in the airways (Fig 2, *A*), naive mice did not exhibit any sign of airway inflammation (Fig 2, *B*). In lung sections of naive 6- to 8-week-old NGF-Tg mice, thickening of the proximal and distal airway wall was observed (Fig 2, *C*), which was mainly due to an enlargement of the peribronchial collagen layer (Fig 2, *D*).

Invasive lung function measurements were performed to assess whether the airway wall thickness in NGF-Tg mice affects lung physiology. These revealed a significantly higher baseline level of airway resistance (Fig 2, *E*) and a significantly lower lung compliance in NGF-Tg mice compared with that seen in WT littermates (Fig 2, *F*).

NGF-Tg mice were sensitized and chronically challenged to OVA to examine whether an additional inflammatory response in the airways would further augment the effect of NGF. A marked influx of inflammatory cells into the airways was observed (Fig 3, *A* and *B*), which was accompanied by thickening of the peribronchial collagen layer (Fig 3, *C*).

Collagen deposition in NGF-Tg mice occurs independently of the NGF receptor p75^{NTR}

Important signaling pathways for collagen production comprise the TGF-β1–activated SMAD complex, as well as the p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, and c-Jun terminal kinase (JNK).[21-23] NGF triggers these signaling pathways through 2 structurally unrelated receptor systems: the tyrosine kinase tropomyosin-related kinase A (TrkA) and TNF receptor type I p75^{NTR}. The binding of NGF to TrkA leads to activation of p38 MAPK and ERK1/2, whereas signaling through p75^{NTR} results in activation of JNK. Murine lung fibroblasts (MLFs), representing the major cellular source for matrix protein production, were isolated from WT and p75$^{NTR-/-}$ mice to delineate the pathway NGF uses to

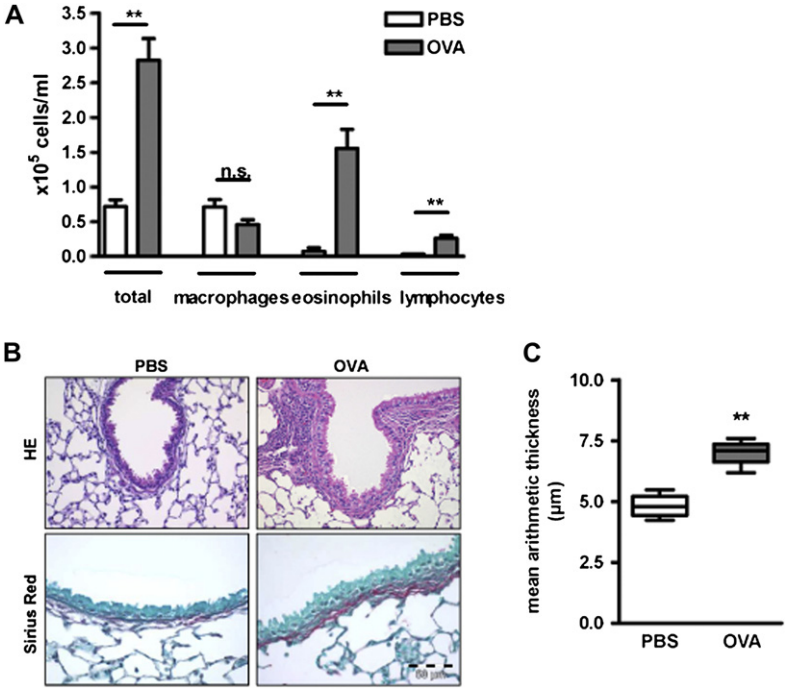

FIG 3. NGF-Tg mice develop experimental chronic asthma. **A**, Total and differential cell counts in PBS- and OVA-treated mice. **B**, Lung sections of PBS- and OVA-treated mice stained with hematoxylin and eosin (*HE*; magnification ×200) and Sirius Red (magnification ×400). **C**, Quantification of subepithelial collagen deposition. Data were obtained from 2 independent experiments and depict the results of 4 mice. *n.s.*, Not significant. $^{**}P < .01$ versus PBS.

regulate collagen deposition in the lung. In $p75^{NTR-/-}$ mice exon III in the $p75^{NTR}$ gene is deleted, which codes for the neurotrophin-binding domain. As a consequence of this deletion, $p75^{NTR}$ protein is not expressed. Because of alternative splicing of exon III in the $p75^{NTR}$ gene, a functional inactive protein lacking the ligand-binding domain is transcribed and expressed.[24] Activation of the downstream target nuclear factor κB (NF-κB) was investigated to confirm functional knockout in $p75^{NTR}$ signaling. Although activation of the NF-κB subunit p65 was detected in WT MLFs after stimulation with NGF and pro-NGF, it was absent in $p75^{NTR-/-}$ MLFs (Fig 4, *A*). Stimulation of both WT and $p75^{-/-}$ MLFs with NGF resulted in increased col3(α1) mRNA expression in both groups (Fig 4, *B*).

NGF-Tg mice were crossed to mice lacking functional $p75^{NTR}$ to further investigate the role of $p75^{NTR}$ in NGF-induced collagen expression *in vivo*. Loss of functional $p75^{NTR}$ (NGF-WT/$p75^{NTR-/-}$) *per se* did not induce a pathological phenotype in the lung compared with NGF-WT/$p75^{NTR+/+}$, and deletion of $p75^{NTR}$ in the NGF-Tg background (NGF-Tg/$p75^{NTR+/-}$ and NGF-Tg/$p75^{NTR-/-}$) did not change lung morphology compared with NGF-Tg/$p75^{NTR+/+}$ (Fig 4, *C*). Furthermore, quantification of subepithelial collagen in lung sections of these mice confirmed that the deletion of $p75^{NTR}$ was without an effect (Fig 4, *D*).

NGF induces type III collagen expression by activating p38 MAPK independent of TGF-β1

After confirming the activation of the TrkA-signaling pathways p38MAPK and ERK1/2 on NGF stimulation in MLFs (Fig 5, *A*), the contribution of both pathways regarding col3(α1) mRNA expression was investigated. MLFs were pretreated with specific inhibitors for 30 minutes before NGF stimulation (Fig 5, *B*), and col3(α1) mRNA expression was assessed by means of RT-PCR. A strong induction of col3(α1) mRNA was observed after NGF stimulation, which was not affected by blockade of the ERK1/2 pathway. In contrast, pretreatment with the p38 inhibitor resulted in a marked reduction in col3(α1) mRNA expression (Fig 5, *C*). These results are strengthened by studies performed in HEK293 cells in which the expression of an active TrkA receptor led to increased col3(α1) promoter activation. The activation of the col3(α1) promoter was decreased when p38 MAPK activation was inhibited (see Fig E2 in this article's Online Repository at www.jacionline.org).

Small interfering RNA (siRNA) knockdown experiments were performed in MLFs to investigate a possible relationship between NGF/p38 and the TGF-β1/SMAD pathway with regard to collagen production. siRNAs directed against either the NGF receptor TrkA or SMAD4 mRNA, which is the central molecule in TGF-β1/SMAD signaling, were used. Scrambled siRNA was used as the negative control (Fig 5, *D*).

After the efficient knockdown of TrkA and SMAD4, MLFs were cultured in serum-reduced medium to exclude the effect of excess TGF-β1 present in FCS. Stimulation of MLFs with NGF or TGF-β1 strongly induced col3(α1) expression. The effect of NGF was abrogated when the NGF-signaling pathway was blocked by TrkA knockdown. In contrast, disruption of the TGF-β1 pathway by means of knockdown of SMAD4 did not affect col3(α1) mRNA levels when cells were stimulated with NGF. In turn, TGF-β1–induced expression of col3(α1) was exclusively dependent on SMAD signaling because knockdown of SMAD4 but not TrkA caused a reduction in collagen expression (Fig 5, *E*).

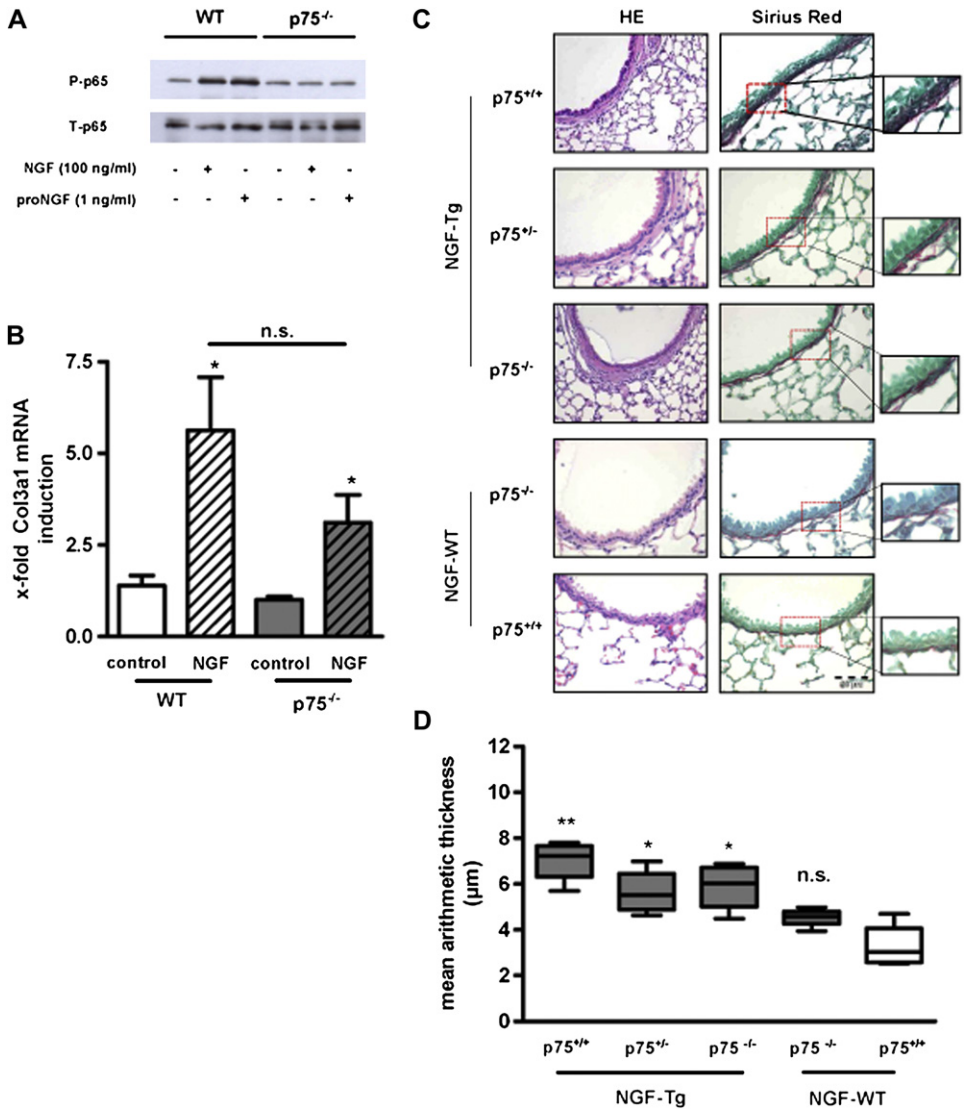

FIG 4. Collagen deposition seen in NGF-Tg mice occurs independently of functional $p75^{NTR}$. **A**, Functional knockout of $p75^{NTR}$ confirmed by testing activation of the NF-κB subunit p65 in WT and $p75^{-/-}$ lung fibroblasts. **B**, NGF-induced col3(α1) mRNA in WT and $p75^{-/-}$ MLFs. **C**, Lung sections of the presented genotypes stained with hematoxylin and eosin and Sirius Red (both magnification ×200). **D**, Quantification of subepithelial collagen. N = 4 per group. *n.s.*, Not significant; *P*, phospho; *T*, total. $*P < .05$ versus control and NGF-WT/$p75^{+/+}$. $**P < .01$ versus NGF-WT/$p75^{+/+}$.

DISCUSSION

This study describes a novel function and pathway of NGF in chronic allergic airway inflammation contributing significantly to subepithelial fibrosis. Experimental chronic asthma involves structural changes that readily occur in the airways, and this is associated with increased NGF levels. Treatment with anti-NGF in this model inhibited the deposition of collagen and, subsequently, the development of subepithelial fibrosis. Increased pulmonary NGF levels (NGF-Tg) lead to subepithelial collagen deposition independent of airway inflammation. The structural changes observed in NGF-Tg mice were linked to altered lung physiology. A direct effect of NGF on col3(α1) expression in lung fibroblasts was demonstrated. This was mediated by the activation of TrkA/p38 kinase by NGF and not $p75^{NTR}$. Finally, these effects occur independently of TGF-β1/SMAD signaling.

The presence of subepithelial fibrosis has been reported in all severities of asthma, and the increased deposition of collagens in the reticular basement membrane has been associated with severity of the disease.[3,4] During inflammation and tissue injury, locally increased growth factor levels drive the subsequent wound-healing process.[2] Therefore a close interaction of epithelial cells and adjacent fibroblasts is required for proper wound healing. Yet a misbalance in this physiological process can result in an exaggerated pathological process, leading to remodeling.

In allergic patients NGF concentrations are increased, and this has been related to disease severity.[25] Serum and BALF concentrations of NGF are higher in asthmatic subjects compared with those seen in healthy control subjects.[25,26] A further increase in NGF levels was reported after both segmental allergen provocation[27] and bronchial allergen provocation.[28] The upregulation of NGF and its receptors has been described in several injured tissues

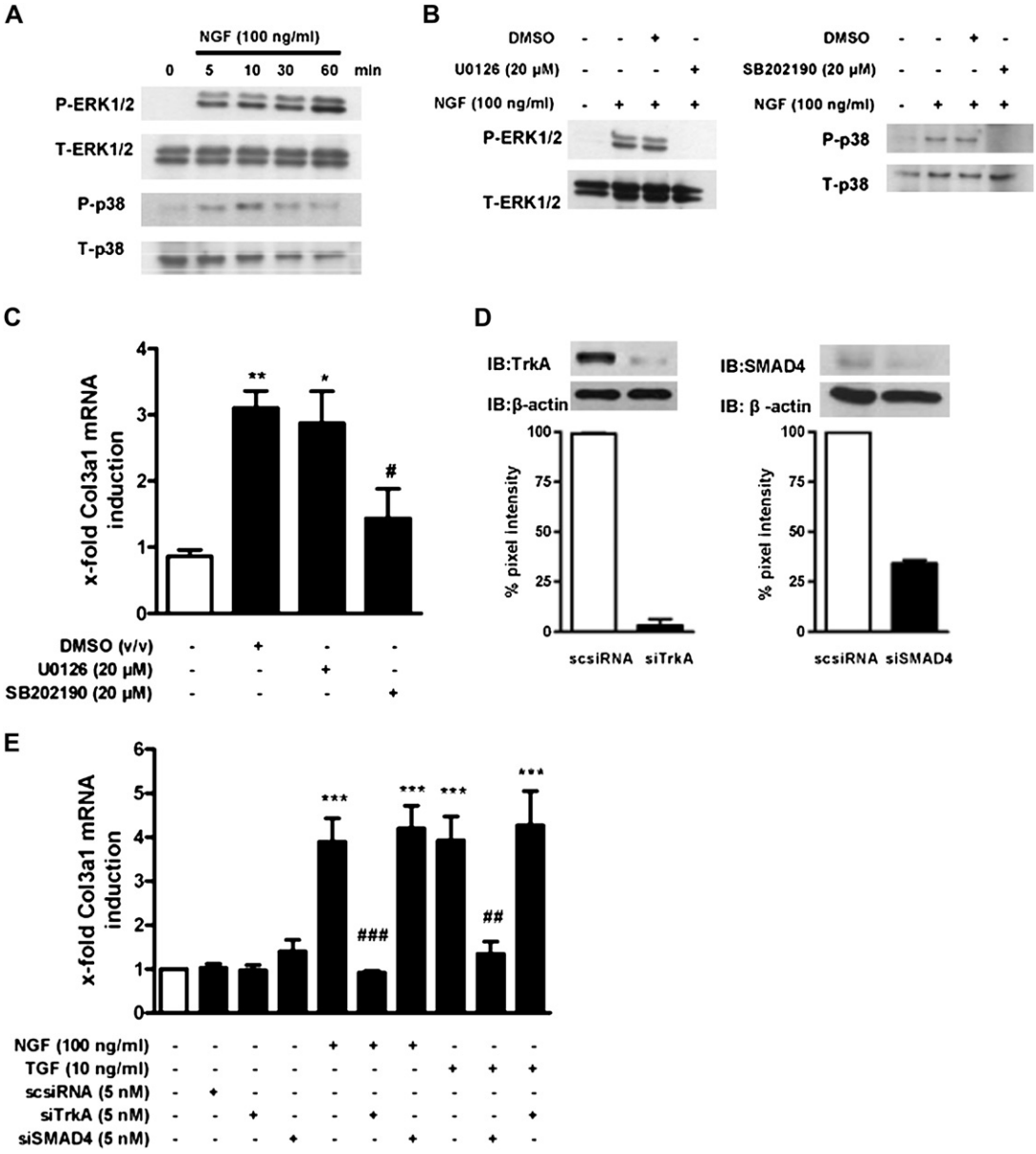

FIG 5. NGF induces type III collagen expression in MLFs independently of TGF-β1. **A**, NGF-induced p38 and ERK1/2 activation in MLFs. **B**, Inhibitors for p38 and ERK1/2 were tested. **C**, NGF-induced col3(α1) mRNA expression after p38 and ERK1/2 inhibition. **D**, Protein knockdown of TrkA and SMAD4 by RNAi. Scrambled *(sc)* siRNA was used as a negative control. **E**, Col3(α1) mRNA expression analyzed after RNAi knockdown of TrkA and SMAD4 and stimulation with NGF and TGF-β1. Data are representative of 1 of 3 experiments (n = 4). *DMSO,* Dimethyl sulfoxide; *IB,* immunoblot; *P,* phospho, *T,* total. **P* < .05, ***P* < .01, and ****P* < .001 versus control. #*P* < .05 versus NGF. ##*P* < .01 versus TGF. ###*P* < .001 versus NGF.

and was shown to initiate epithelial wound-healing processes.[10,11] A previous study identified the activated airway epithelium as a major source for NGF during allergic airway inflammation.[29] Infiltrating immune cells, including mast cells, T cells, and eosinophils,[30-32] together with airway smooth muscle cells, have also been identified as producers of NGF.[33] Lung fibroblasts, which express the NGF receptors TrkA and $p75^{NTR}$,[13] have been reported to be targets of NGF at healing sites.[34]

In vitro and *in vivo* experiments were performed to investigate the role of NGF in subepithelial fibrosis. *In vitro* results demonstrate that NGF induced col3(α1) expression by activating TrkA and downstream p38 kinase. These data are supported by *in vivo* findings in the NGF-Tg mice, which display increased amounts of collagen deposition in the bronchial wall, as well as in a murine model of chronic allergic airway inflammation. In this model, which is accompanied by structural changes, NGF levels were found to be increased at a disease stage in which remodeling occurs, and treatment with anti-NGF antibodies markedly prevented the development of allergen-induced subepithelial collagen deposition. However, the inhibition of tissue remodeling in experimental chronic asthma was accompanied by decreased infiltration of the airways and lung tissue with inflammatory cells, including eosinophils and lymphocytes.

Fibroblasts, which express the NGF receptors TrkA and $p75^{NTR}$, have been shown to migrate, contract, and differentiate into myofibroblasts on *in vitro* NGF stimulation.[13] Smooth muscle cells were reported to proliferate after NGF stimulation.[35] Neutralization of NGF might affect these important events during inflammation and remodeling. NGF is also an important factor for maturation of granulocyte colonies and provision of a survival signal for eosinophils during allergic airway inflammation.[36]

Remodeling is considered imperfect wound healing of the tissue after repetitive injury and frustrating repair cycles. In this phase production and release of growth factors at wound sites occur in a more and more uncontrolled fashion, and increased levels of profibrotic factors, such as IL-13 and TGF-β1, induce collagen production by structural cells. NGF has been increasingly reported to mediate processes, such as proliferation of epithelial and smooth muscle cells and migration of lung fibroblasts *in vitro*, which link this molecule to potential profibrotic mechanisms. To uncover this, we tested whether high amounts of pulmonary NGF can induce collagen expression *in vivo*. NGF-Tg mice were examined, which have been reported to be hyperresponsive to cholinergic and sensory stimuli[15,16] because of hyperinnervation of the airways. These mice already have a thickened airway wall in the naive state in the absence of allergic asthma. In contrast, several studies that use conditional overexpression of remodeling- associated factors, including IL-13 and IL-11, were able to produce a situation in which subepithelial fibrosis occurs. Yet an increased level of each factor in these studies was accompanied by airway and tissue inflammation in naive mice.[37,38]

The structural changes in naive NGF-Tg mice were associated with an alteration of lung mechanics. A higher baseline airway resistance was detected in these mice, which is likely the consequence of the thickened airway wall in the distal compartment of the lung. The decrease in lung compliance could either have been caused by airflow heterogeneity during perturbations or because of matrix deposition in the alveolar compartment of the lung resulting in stiffening of the alveolar walls.[39] These data suggest that locally increased NGF levels drive tissue remodeling and eventually lead to altered lung function and lung physiology.

The chronic allergen exposure model was performed with NGF-Tg mice to confirm whether increased NGF production further deteriorates the phenotype in experimental chronic asthma. The baseline levels of collagen in the airways of NGF-Tg mice were already increased compared with those seen in WT animals. After chronic allergen challenge, a further increase was detected in the OVA–NGF-Tg group. However, this did not exceed the levels observed in OVA-WT mice, suggesting that a plateau of airway wall thickening might have been reached in this model that could not be further augmented.

To delineate the NGF pathways that could mediate the observed *in vivo* effects, we used *in vivo* and *in vitro* experiments. Known signaling pathways, which have been linked to fibrosis, include activation of stress-activated JNK, p38 kinase, and ERK1/2. Because NGF signals through 2 structurally unrelated receptors, the tyrosine kinase TrkA and $p75^{NTR}$, which belongs to the TNF receptor family, the role of both signaling systems in terms of tissue fibrosis was investigated. Both receptors have been shown to either signal independently from each other, counteract each other, or act synergistically because $p75^{NTR}$ was reported to support TrkA signaling.[40] While TrkA was reported to activate p38 and ERK1/2, $p75^{NTR}$ signaling activates JNK. The obtained data suggest that remodeling induced by NGF is strongly dependent on TrkA and downstream p38 activation because inhibition of ERK1/2 did not affect col3(α1) mRNA expression and promoter activation. Furthermore, NGF-induced collagen expression can occur in the absence of functional $p75^{NTR}$ *in vivo* and *in vitro*. However, a role for $p75^{NTR}$ in NGF-induced collagen expression cannot be excluded completely because in the absence of functional $p75^{NTR}$, the amount of collagen deposition was reduced *in vivo*, although not in a statistically significant manner. This suggests that signaling pathways activated by $p75^{NTR}$, such as JNK or NF-κB, could augment the NGF/TrkA/p38 effect.

Several studies have shown the importance of TGF-β1,[41] which is secreted by epithelial cells and eosinophils during allergic airway inflammation, and SMAD signaling as a key pathway in matrix deposition by fibroblasts. Remodeling-associated factors, including IL-13, were shown to induce airway fibrosis by activating TGF-β1.[42] For MAPK-signaling pathways, including JNK,[21] p38, and ERK1/2, the direct activation of SMAD-signaling molecules has been described.[21-23] Also, for NGF, an activation of the SMAD pathway has been previously reported.[43] By using the cell-culture model of primary MLFs, it was demonstrated that NGF-activated collagen deposition occurs independently of TGF-β1/SMAD signaling. The NGF-activated p38 kinase did not depend on SMAD activation because knockdown of SMAD4 in parallel to NGF stimulation still induced collagen expression. These results are further strengthened by findings in NGF-Tg mice, which display increased amounts of subepithelial collagen deposition in the absence of increased TGF-β1 levels in either BALF or lung tissue (data not shown). Taken together, these results indicate that NGF, in terms of collagen expression, signals through a TGF-β1/SMAD–independent signaling pathway.

In conclusion, we demonstrate for the first time a novel function of NGF in airway remodeling. High levels of NGF produced in chronic allergic airway disease not only contribute to the pathogenesis of chronic asthma through augmenting allergic airway inflammation and triggering airway reactivity. NGF also activates collagen production and consecutively participates in airway remodeling by inducing peribronchial fibrosis. This then contributes to the altered lung morphology and lung mechanics in the disease. These data provide evidence for a novel profibrotic pathway that might be of therapeutic importance in human disease.

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Clinical implications: The identification of a TGF-β1–independent profibrotic pathway activated by NGF might serve as a new therapeutic target for chronic allergic airway disease.

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METHODS

OVA sensitization and challenge

Anti-NGF (polyclonal rabbit anti-human β NGF 500-P85; PeproTech) and control rabbit IgG antibodies (Sigma-Aldrich) at a concentration of 10 μg/200 μL in PBS were applied intraperitoneally 24 hours before each challenge episode. Control animals were treated with intraperitoneal application of PBS. All analyses were performed 24 hours after the last OVA or PBS aerosol challenge.

Measurement of lung mechanics

Baseline lung mechanics of airway resistance and lung compliance were assessed with flexiVent, an animal mechanical ventilator system (SCIREQ).[E1] Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine (Rompun) solution, tracheostomized, and connected to the flexiVent. Mechanical ventilation was set at 120 breaths/minute with a tidal volume of 0.16 mL/kg and a positive end-expiratory pressure of 3 cm H_2O.

Measurements of cytokine levels in BALF

NGF (NGF ImmunoAssay System; Promega, Mannheim, Germany) levels were measured in cell-free BALF, according to the manufacturer's instructions. Plates were read in a microplate autoreader (Tecan, Salzburg, Austria) at 405 nm. The detection limit for NGF was 7.8 pg/mL.

Lung histology and morphometric analysis

Collagen quantification was performed in SURS samples with either a computer-based CastGrid System (Fig 2) or light microscopy and CellF-Software (both Olympus, Center Valley, Pa; Figs 1, 3, and 4), according to design-based stereology.[E2] SURS-sampled pictures of at least 10 airways were taken at a magnification of ×400. The arithmetic mean thickness (Tcomp) was measured as the volume of the respective component, which was determined by counting all points hitting Sirius Red components.[E3] Results were referred to the reference surface assessed by counting all intersections with the airway epithelial basement membrane. The arithmetic mean thickness was calculated according to the following formula:

$$T_{comp} = L(P) \cdot \frac{\sum P_{comp}}{2 \cdot \sum I_{bl}},$$

where *L(P)* represents the test line length per test point, *Pcomp* represents the number of points hitting the respective component, and *Ibl* represents the number of intersections of the test lines with the epithelial basal lamina. Selection of sections and morphometric analysis were performed by a blinded investigator.

Isolation of MLFs, RNA isolation, and analysis of mRNA expression

Lungs were perfused through the right-heart ventricle with PBS, excised, and homogenized with GentleMACS (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by collagenase D (2 mg/mL; Roche) treatment for 1 hour at 37°C. The cell suspension was depleted of immune and endothelial cells by means of negative selection for CD45 (clone 30F11.1, Miltenyi Biotec) and CD31 (clone MEC13.3; BD Biosciences, San Diego, Calif) with magnetic cell sorting. The resulting cells were cultured in Dulbecco modified Eagle medium (low glucose) supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, 1% L-glutamine, 1% sodium pyruvate, and 1% nonessential amino acids (all reagents purchased from PAA, Cölbe, Germany). Cells in passages 2 to 4 were used for the experiments.

Activation of signaling pathways for col3(α1) mRNA expression in MLFs was studied by using either control (dimethyl sulfoxide) or pretreatment with specific inhibitors for ERK1/2 (20 μmol/L U0126; Calbiochem, Nottingham, United Kingdom) and p38 MAPK (20 μmol/L SB202190, Calbiochem) for 30 minutes, followed by stimulation with 100 ng/mL rhNGF (Peprotech) for 8 hours. Stimulation with rhTGF-β1 (PeproTech) at a concentration of 10 ng/mL was performed for 8 hours.

Total RNA from MLFs or lung tissue was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Genomic DNA was removed by means of DNase treatment (DNaseI; Invitrogen, Karlsruhe, Germany), and first-strand cDNA synthesis was performed with Superscript reverse transcriptase II (Invitrogen). Quantitative real-time PCR was performed with the QuantiTect SYBR Green PCR Kit (Qiagen) and a RotorGene3000 System (Corbett, Basel, Switzerland). After an initial denaturation step for 15 minutes at 95°C, 40 PCR cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 15 seconds were performed.

Primers specific for col3(α1) (5′-GCA GGA CCC AGA GGA GTA G-3′/5′-TTC CAT CAT TGC CTG GTC-3′) and L32 (5′-AAG CGA AAC TGG CGG AAA CC-3′/5′-CTG GCG TTG GGA TTG GTG AC-3′) were used to test the mRNA expression of col3(α1) mRNA expression. PCR quantification was done with the $2^{-\Delta\Delta Ct}$ method, with normalization to murine ribosomal L32 as the housekeeping gene. All standard procedures were performed according to the manufacturer's instructions.

Construction of luciferase reporter plasmid

The flanking sequence 5′ to the transcriptional start site of the col3(α1) gene was PCR amplified with HotStart-Polymerase (Invitrogen) by using genomic fibroblast DNA as the template. The primers were designed according to the published col3(α1) promoter sequence (ENSMUSG00000026043). The construct was designed with the sense (5′) primer 5′-AATGCACATGGC CATGATTAGGAT-3′ located at position −1000 relative to the transcriptional start ATG (+1). The antisense primer 5′-ACCGGGCCCGTCATAAAACT CAG-3′ was located at +92 relative to ATG. The PCR product was analyzed on a 1% agarose gel, excised, and subcloned into the TOPOpCR2.1 Vector (Invitrogen) for sequencing. The insert was cut out by using *Kpn*I and *XhoI* restriction sites and cloned into pGL4.10 (firefly Luciferase) mammalian expression vector (Promega, Madison, Wis). The constructed plasmid was termed pCol3α1-luc.

Transient transfection and reporter gene analysis

HEK293 cells were seeded in 48-well plates and grown until 60% to 70% confluence. Cells were transfected with 0.5 μg of pcDNA3-ratTrkA (WT and mutant K538R) plasmid DNA (both kindly provided by Moses V. Chao, Skirball Institute, New York University, New York, NY) by using FuGene HD (Roche). After 24 hours, the cells were serum starved for 2 hours and transfected with 1 μg of pCol3α1-luc and 0.04 μg of pGL4.73 (Renilla-Luc-SV40) vector (Promega) to determine transfection efficiency. For signaling pathway analysis, cells were treated with inhibitors for p38 kinase (20 μmol/L SB202190) and ERK1/2 (20 μmol/L U0126), both purchased from Calbiochem. Cells were either control treated or stimulated with 100 ng/mL rhNGF (PeproTech) for 8 hours. Cell extracts were prepared and assayed with the dual-luciferase reporter assay system (Promega). Luciferase activity was measured for 10 seconds by using a microplate autoreader (Tecan). Relative luciferase activity was calculated by normalizing the relative light units of the firefly construct to those of the Renilla construct.

RNA interference

MLFs were seeded in 6-well plates and grown to 70% to 80% confluence. Specific siRNAs for TrkA (r[AGU GGA GAA GAA AGA UGA A]dTdT/r[UUCAUC UUU CUU CUC CAC U]dGdG)[E4] and SMAD4 (r[GCA CAA GGU UAG UUA UUU A]dTdT/r[UAA AUA ACU AAC CUU GUG C]dCdT) were purchased from Qiagen and transfected with HiPerfect transfection reagent (Qiagen) at a final concentration of 5 nmol. After 24 hours, protein knockdown was determined by using SDS-PAGE and immunoblotting.

Immunoprecipitation and immunoblot analysis

MLFs were serum starved for 2 hours and subsequently stimulated with NGF (100 ng/mL) or pro-NGF (1 ng/mL). Cells were lysed in 50 mmol/L Tris/HCl (pH 7.5), 150 mmol/L NaCl, 1% NP-40, and 1× protease and phosphatase inhibitor mix (both Roche) for 15 minutes on ice. Lysates were clarified, and total protein concentrations were measured with BCA (Pierce). Lysates were either used for immunoprecipitation or direct immunoblotting.

For immunoprecipitation, 100 μg of total protein was used. Briefly, protein solution was incubated with 2 μg of anti-TrkA (Santa Cruz, Cambridge, United Kingdom) for 3 hours at 4°C on a rotator. Protein A–Agarose was added and incubated overnight at 4°C. immunoprecipitates were washed 3 times and boiled in Laemmli sample buffer and analyzed by means of SDS-PAGE/immunoblotting.

For direct immunoblotting, 20 μg of total protein was separated by means of SDS–10% PAGE and transferred onto polyvinylidene difluoride membranes. The blots were incubated overnight at 4°C with primary antibodies. Rabbit polyclonal anti-TrkA (1:1000), anti-SMAD4 (1:1000), anti–phospho tyrosine (1:500), and p65 (1:1000) were purchased from Santa Cruz. Anti–phospho-p65 (1:1000), anti–phospho-ERK1/2 (1:1000), anti-ERK1/2 (1:1000), anti–phospho-p38 (1:500), and anti-p38 (1:500) were purchased from Cell Signaling Technology (Danvers, Mass), and murine monoclonal anti–β-actin (1:40,000, Sigma-Aldrich) was diluted in TBS/0.1% Tween supplemented with 3% milk powder. Incubation with secondary antibody anti-rabbitpox or anti-mousepox (1:2000 in TBS/0.1% Tween, Sigma-Aldrich) was performed for 1 hour at room temperature. Immunoreactive proteins were detected by using Luminol Reagent (Santa Cruz) on x-ray films (GE Healthcare, Freiburg, Germany).

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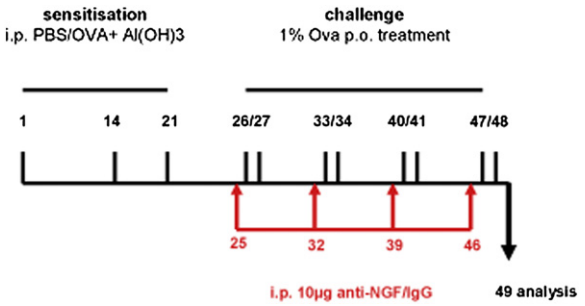

FIG E1. Neutralization of NGF in experimental chronic asthma. Mice were immunized with 3 intraperitoneal *(i.p.)* injections of OVA/Al(OH)3 on days 1, 14, and 21. OVA challenge was performed on 2 consecutive times per week at the indicated time points. Antibodies were applied 24 hours before each challenge period by means of intraperitoneal injection. Animals were analyzed 24 hours after the final challenge.

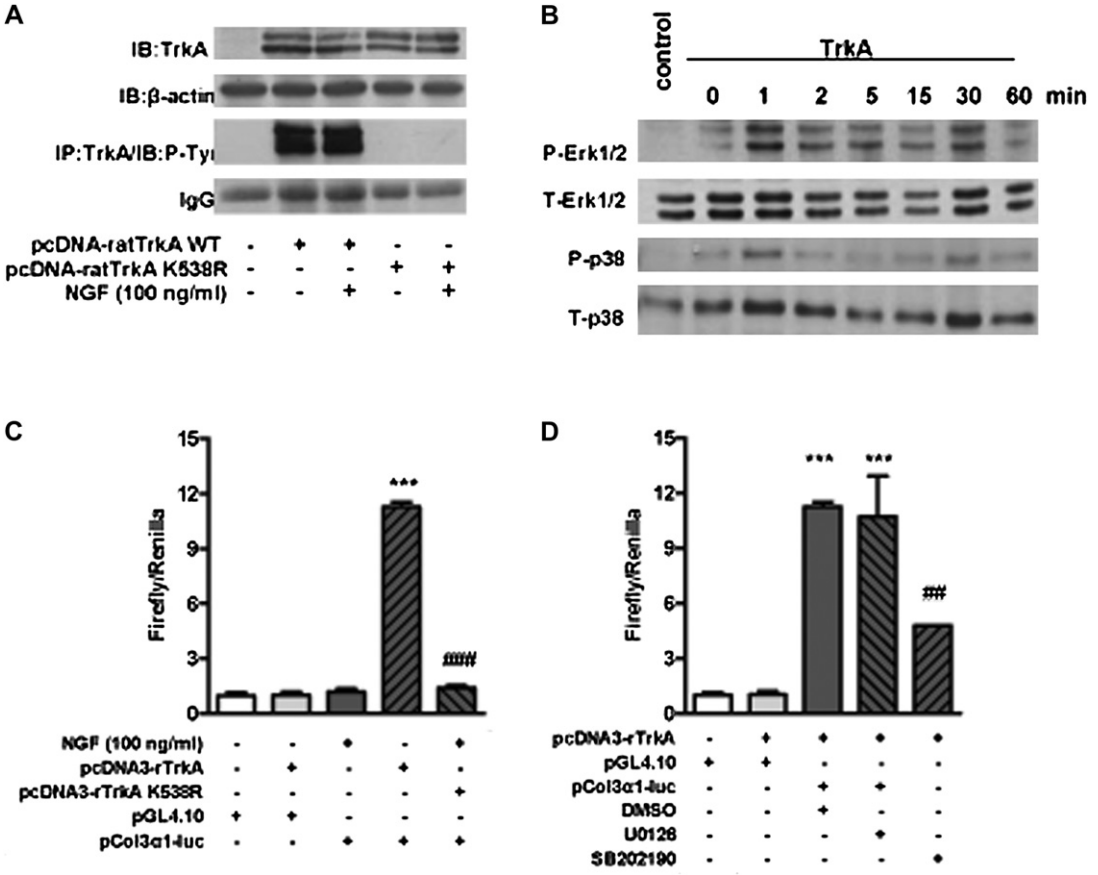

FIG E2. NGF induces type III collagen transcription through TrkA. **A**, TrkA and activated TrkA expression in HEK293 cells transfected with pcDNA3-ratTrkA (WT) and K538R. **B**, Kinetics of p38 and ERK1/2 activation. **C**, Relative luciferase activities of transfected pCol3α1-luc in HEK293 cells expressing TrkA (WT) or K538R. **D**, Relative luciferase activity after inhibition of p38 kinase (SB202190) and ERK1/2 (U126). Data are presented as means ± SEMs and are representative for 1 of 3 independent experiments (n = 4-5 per group). *DMSO*, Dimethyl sulfoxide; *IB*, immunoblot; *IP*, immunoprecipitation; *P*, phospho; *T*, total; *Tyr*, tyrosine. ***P < .001 versus control. ##P < .01 and ###P < .001 vs TrkA (WT).