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Neurturin Influences Inflammatory Responses and Airway Remodeling in Different Mouse Asthma Models

Marion Mauffray,* Olivia Domingues,* François Hentges,* Jacques Zimmer,* Daniel Hanau,[†] and Tatiana Michel*

Neurturin (NTN) was previously described for its neuronal activities, but recently, we have shown that this factor is also involved in asthma physiopathology. However, the underlying mechanisms of NTN are unclear. The aim of this study was to investigate NTN involvement in acute bronchial Th2 responses, to analyze its interaction with airway structural cells, and to study its implication in remodeling during acute and chronic bronchial inflammation in C57BL/6 mice. We analyzed the features of allergic airway inflammation in wild-type and NTN^{-/-} mice after sensitization with two different allergens, OVA and house dust mite. We showed that NTN^{-/-} dendritic cells and T cells had a stronger tendency to activate the Th2 pathway in vitro than similar wild-type cells. Furthermore, NTN^{-/-} mice had significantly increased markers of airway remodeling like collagen deposition. NTN^{-/-} lung tissues showed higher levels of neutrophils, cytokine-induced neutrophil chemoattractant, matrix metalloproteinase 9, TNF- α , and IL-6. Finally, NTN had the capacity to decrease IL-6 and TNF- α production by immune and epithelial cells, showing a direct anti-inflammatory activity on these cells. Our findings support the hypothesis that NTN could modulate the allergic inflammation in different mouse asthma models. *The Journal of Immunology*, 2015, 194: 000–000.

Allergic asthma is a chronic inflammatory disease characterized by a Th2-geared immune response composed of the production of IgE, the secretion of Th2 cytokines and an accumulation of eosinophils in response to specific environmental allergen. The Th2-mediated inflammation in allergic airways proceeds from lung dendritic cells (DCs) through the capture and internalization of allergens leading to their migration into the draining lymph nodes (LN) where they can prime T cells and thereby generate a Th2 cytokine IL-4, IL-5, and IL-13 response (1, 2). This pathway is an optimal target for drug development by targeting Th2 inflammation and the development of the humoral response (3). In individuals with chronic asthma, there is in addition a structural remodeling of the airways, consisting of an increase in smooth muscle thickening, collagen deposition, accumulation of extracellular matrix components, and changes in the airway epithelium (4, 5). Important interactions take place between epithelial cells and DCs including the release of thymic

stromal lymphopoietin, which promotes a Th2 polarizing trend in DC precursors (6). The secretion of the Th2 cytokines IL-4 and IL-13 will raise the production of chemokines like IL-8/cytokine-induced neutrophil chemoattractant (KC) by the airway epithelial cells, which attract neutrophils (5). These cells will then release matrix metalloproteinase-9 (MMP-9) involved in tissue remodeling (7). Many studies have explored therapeutic agents for asthma, nevertheless the variation in efficacy and possible side effects of these therapies determine a need for new treatments (8).

The neurotrophic factor neurturin (NTN) was originally identified for its role on the development and the maintenance of neuronal cells (9). In contrast to its neuronal activity, only a limited amount of information is available regarding its involvement in inflammatory or immune response. NTN belongs to the glial cell line–derived neurotrophic factor (GDNF) family ligands (GFLs), which are distant members of the TGF- β superfamily. Anti-inflammatory activities and upregulation in serum of asthmatic patients were described in the case of TGF- β 1 (10). Indeed, TGF- β 1 produced by CD4⁺ T cells inhibited the Th2-driven airway hyperresponsiveness and inflammation in a murine model of asthma (11). However, the effect of TGF- β 1 in asthma diseases is diverse. Thus, this cytokine can have promoting effects, because when expressed by airway epithelial cells, it participates to the remodeling and fibrosis in asthmatic patients (12–14). On its side, the secreted NTN preferentially activates a pathway via a GPI-linked receptor termed GDNF family receptor α -2 (GFR α -2) and the tyrosine kinase RET (15). In addition to this interaction, NTN might cross-talk weakly with the receptor GFR α -1 (16). Expression of NTN, RET, and GFR α -2 mRNAs was observed during the development of the respiratory system (17). In adult mice, NTN was detected at a moderate level in the lung alveoli and in the thymus (18). Jugular trachea and lung transient receptor potential vanilloid type 1–positive neurons expressed GFR α -2 and RET at the mRNA level in guinea pig (19). Moreover, tracheal sections have revealed a positive staining for other neurotrophic factors like GDNF in the OVA-induced asthma model (20). It was also shown that NTN and GDNF were important for the differentiation

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Abbreviations used in this article: ATF3, activating transcription factor 3; BALF, bronchoalveolar lavage fluid; BMDC, bone marrow–derived DC; DC, dendritic cell; GDNF, glial cell line–derived neurotrophic factor; GFR α 2, GDNF family receptor α 2; HDM, house dust mite; KC, cytokine-induced neutrophil chemoattractant; LN, lymph node; MHCII, MHC class II; MMP-9, matrix metalloproteinase-9; NTN, neurturin; WT, wild-type.

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and survival of pulmonary neurons (21, 22). NTN^{-/-} mice develop a dry-eye disease, characterized by a high level of MMP-9 and TNF- α mRNA expressed by the corneal epithelium (23). In a previous study, using a murine OVA-induced asthma model, we observed that the absence of NTN increased the peribronchial inflammatory cell infiltration. Thus, NTN^{-/-} mice showed a stronger bronchial inflammation associated with higher amounts of Th2 cytokines and eosinophils as well as increased airway hyperresponsiveness. Furthermore, administration of NTN before challenge with OVA partially rescued the phenotype (24). How the anti-inflammatory effect of NTN could impact on immune and structural cells remains unclear.

In the current study, we investigated whether NTN was able to modulate the function of Th2 responses and examined the relationships between NTN and the structural airway remodeling by studying NTN^{-/-} mice in models of acute and chronic allergic airway inflammation induced by the model allergen OVA and the natural allergen source house dust mite (HDM) extract. To further determine a direct anti-inflammatory activity of NTN on immune and epithelial cells, NTN was added with OVA to these cells in vitro and tested for its capacity to decrease proinflammatory cytokine production.

Materials and Methods

Mice

C57BL/6 mice were obtained from Harlan (Horst, the Netherlands). NTN^{-/-} mice on C57BL/6 background were provided by Prof. J. Milbrandt (Washington University School of Medicine, St. Louis, MO). Mice were housed under a constant light/dark cycle and specific pathogen-free conditions. They were used at 6–8 wk of age. All procedures respected current European regulations and were approved by the National Animal Research Authority.

Acute airway inflammation protocol

Mice were sensitized with OVA (20 μ g) emulsified in 2.25 mg aluminum hydroxide (Sigma-Aldrich, Bornem, Belgium) in PBS (a total volume of 0.2 ml) as described previously (24). Briefly, mice were sensitized by i.p. injection on days 0, 14, and 21 and challenged intranasally on days 27, 28, and 29. Control mice were sensitized and challenged with PBS/alum and PBS. On day 30, mice were sacrificed, and samples were collected for further analysis. OVA (grade V; Sigma-Aldrich) contained endotoxin: 250 endotoxin units/mg protein OVA as measured by Pyrogen Plus Gel Clot LAL assays (Lonza, Belgium).

Mice were sensitized intranasally, according to the protocol described by Willart et al. (25), with 1 μ g HDM (*Dermatophagoides pteronyssinus*) in 50 μ l PBS on day 0 after a short anesthesia with isofluran (CP-pharma, Burgdorf, Germany) and challenged 7 d later with 10 μ g HDM in 50 μ l PBS for 5 consecutive days. Control mice were sensitized and challenged with PBS. On day 14, mice were sacrificed, and samples were collected for further analysis. HDM (Greer, Lenoir, NC) contained endotoxin: 3.86 endotoxin units/mg protein.

Chronic airway inflammation protocol

Mice were treated with OVA as described previously (24) with some modifications. Briefly mice were sensitized on days 0, 7, 14, and 21 and challenged intranasally on days 27, 29, 30, 34, 37, 41, 44, 48, 51, 55 and 57. Control mice were sensitized and challenged with PBS. On day 58, mice were sacrificed, and samples were collected for further analysis.

Sample acquisition and preparation

Mice were anesthetized and sacrificed as described previously (24). Blood was collected by cardiac puncture for measurement of allergen-specific Ig. Bronchoalveolar lavage (BAL) was performed using PBS/5% BSA/mixture protease inhibitor (700 μ l) (Roche, Mannheim, Germany), followed by PBS (2 $\times$ 500 μ l). Lungs were excised from mice and directly flash-frozen for RNA extraction or fixed in 4% paraformaldehyde for histology analyses.

Cell cultures

Cells from spleen and lung draining LNs of OVA-induced mice were isolated and adjusted to 5 $\times$ 10⁶ cells/ml and put in culture for 4 d in DMEM supplemented with IL-2 (1000 U/ml) and restimulated with OVA (200 μ g/ml).

LN cells from HDM-induced mice were adjusted to 2 $\times$ 10⁶ cells/ml with IL-2 and restimulated with HDM (15 μ g/ml), BAL fluid (BALF), spleen, and LN supernatants were frozen at -80°C for later cytokine analysis.

Bone marrow from naive mice was flushed from tibiae and femurs and passed through a 40- μ m cell strainer. Bone marrow-derived DCs (BMDCs) were generated from culture of 1 $\times$ 10⁷ cells per 3 ml in 6-well plates for 6 d in medium supplemented with GM-CSF (20 ng/ml) and IL-4 (20 ng/ml), and half of the medium was replaced two times with fresh medium. At day 7, cells were replated with GM-CSF (20 ng/ml) for 24 h, then harvested and used for RNA extraction, DC-T cell coculture or stimulated for 2 d with LPS (100 ng/ml) or OVA (200 μ g/ml) and stained with specific Abs for FACS analysis.

Spleen DCs were generated from splenocyte cultures of 1 $\times$ 10⁷ cells per 3 ml for 14 d with GM-CSF (10 ng/ml). Half the medium was replaced three times per week with fresh medium. At day 14, cells were harvested for RNA extraction or stimulated for 2 d with LPS (100 ng/ml) or OVA (200 μ g/ml) and stained with specific Abs for FACS analysis.

LA-4 murine bronchial epithelial cells (European Collection of Cell Cultures, ECACC cell lines, reference number 90040512) were cultured at 2 $\times$ 10⁵ cells in collagen-precoated Transwells in culture medium (DMEM 1:2, Ham's F12 1:2, 5% FBS, 1% penicillin/streptomycin, 0.1% insulin). After 4 d, medium in the apical side was removed to set up air-liquid interface culture. Cells were cultured for 14 d in USG medium (DMEM 1:2, Ham's F12 1:2, 2% Ultrosor G, and 1% penicillin/streptomycin) and medium was changed 2 times per week. Cells were stimulated with NTN (15 ng/ml) and/or OVA (200 μ g/ml) on the apical side for 3 d and supernatants were stored at -80°C before cytokine analysis.

DC and T cell coculture

BMDCs were harvested at day 8, cultured at 3.10⁴ cells in medium (100 μ l) in 96 well plates and stimulated for 48 h with TNF- α (10 ng/ml) and OVA (200 μ g/ml) or medium alone as negative control in presence of different doses of NTN. In parallel, OVA-induced spleen and LN CD4⁺ T cells were isolated by negative selection using the CD4⁺ T cell isolation kit (Miltenyi Biotec). The purity of CD4⁺ T cells was $\geq$ 90%. Then, 1 $\times$ 10⁵ CD4⁺ T cells were added to the culture of BMDCs. Supernatants were collected after 4 d and stored at -80°C before cytokine analysis.

Lung histology and morphometric analysis

After BAL, lungs were collected, fixed in 4% paraformaldehyde, and embedded in paraffin. Lung tissue sections (6 μ m) were stained to visualize collagen with a Trichrome stain Masson Kit (H15-1KTI; Sigma-Aldrich). Pictures were taken with a Leica microscope mounted with a camera (model DMI 6000B). Morphometric analysis was performed using a Leica application Suite version S3.8 software (model DMI 6000B; Leica microscope). The peribronchial collagen thickness was measured (in square micrometers) in five bronchioles per mouse. The mean depth of collagen was quantified by the measurement of the differences between the area of the bronchiole and the area of the bronchiole extended with collagen deposition. Results are expressed as mean of the collagen depth for each animal.

Western blot

Protein homogenates of lung tissue samples were prepared in lysis buffer (50 mM Tris, 200 mM NaCl, 10 mM CaCl₂, and 10 μ M ZnCl₂). Proteins (50 μ g) were loaded on a 15% SDS-PAGE, transferred onto a nitrocellulose membrane, and blocked in TBS/Tween 0.05%/BSA 3% before incubation overnight with anti-activating transcription factor 3 (ATF3) Ab (1/100; Santa Cruz SC188) or anti-actin Ab (1/6000; Millipore MAB1501). Secondary Abs were added for 2 h before revelation using West Femto chemiluminescent Substrate kit (Fisher Scientific PI: 34094). The resulting bands were analyzed using ImageQuant (GE HealthCare) and normalized to those of actin.

Zymography

Protein homogenates were prepared from each lung tissue as described in Western blot. MMP-9 activity was obtained by analysis of the resulting bands on a gelatin gel zymogram (Bio-Rad, 10% Ready Gel Zymogram: 161-1167) by densitometry using ImageQuant (GE HealthCare). The mean $\pm$ SE density of MMP-9 activity was plotted in a graph and expressed as the relative ratio of the values in the control group, which were expressed as one.

RNA extraction

Total RNA was extracted with RNeasy mini kit for cell lysates and RNeasy midi kit for lung samples (Qiagen, Venlo, the Netherlands). RNA quality assessment was tested using Agilent RNA 6000 Nano Kit (Agilent). One microgram of RNA was used to synthesize cDNA using the PrimeScript Reverse Transcriptase kit (Takara).

Real-time PCR

Primers designed for real-time PCR assay were GFR α -2 (5'-AGGACA-AAGAGAAGCCCAAT-3', 3'-CCTGTTGATGGTCCGTACAG-5'), RET (5'-GGTCAACAACAACCTCCGTTTC-3', 3'-CAGGAGGTAAAGTTGCA-CAG-5'), and GAPDH (5'-GTGGAGTCTACTGGTGTCTTC-3', 3'-CT-GGTGCTCTTTACTGTTGA-5') as endogenous control. The reaction mix was as follows: 10 μ l Fast Sybr green Master Mix (Life Technologies), 25–50 ng cDNA, 2 μ M primers in a final volume of 20 μ l. The amplification conditions were: 20s at 95°C (hold stage) and then 40 cycles of 1 s at 95°C and 20 s at 58°C. All samples were analyzed in triplicates using an Applied Biosystems ViiA 7 Real-Time PCR System.

Measure of cytokines in culture supernatant

Levels of IL-4, IL-5, IL-6, IL-13, TNF- α , and KC were measured by Cytometric Bead Array (CBA; BD Biosciences), according to the manufacturer's protocol.

ELISA

TGF β -1 levels were measured by ELISA (eBioscience), according to the manufacturer's protocol. Specific IgE, IgG1 and IgG2a were measured in the serum as described previously (24).

Flow cytometry

Abs used for flow cytometry were CD80-FITC (BD Biosciences), CD3-FITC, CD11c-PerCP-Cy5.5, CD86-PE-Cy7 (BioLegend), Gr1-PE, MHC class II (MHCII)-allophycocyanin (eBioscience), CD4-allophycocyanin (Immunotools, Friesoythe, Germany), IgG-allophycocyanin, IgG-PE (Jackson ImmunoResearch Laboratories), RET (R&D Systems, Abingdon, U.K.). Viable cells were identified with live/dead fixable near-IR dead cells stain (Life Technologies, Europe B.V.). Cells were acquired on a FACSCanto flow cytometer and analyzed with FlowJo software (Tree Star).

Statistical analysis

Data were analyzed with Graph Pad Prism 5.0 software. One-way ANOVA-Newman-Keuls multiple comparison and *t* tests were used to determine the statistical significance of the results. The values were expressed as means $\pm$ SEM from independent experiments. Any difference with a *p* value <0.05 was considered significant.

Results

NTN^{-/-} splenocytes promote Th2 cytokine production

To investigate at which step the inflammation is overinduced in NTN^{-/-} mice, we tested the presence of OVA-specific Th2 cells in lymphoid tissues. As we observed previously a higher Th2 response in NTN^{-/-} lung draining LN cells (24), we performed in vitro experiments to analyze the capacity of NTN^{-/-} spleen cells to induce the Th2 pathway. After sensitization and challenge with the allergen OVA (Fig. 1A), wild-type (WT) and NTN^{-/-} spleen cells were put in culture and restimulated with OVA to activate the Th2 cytokine secretion. We found that spleen NTN^{-/-} cells were able to produce more IL-4 compared with WT and that the same tendency was observed for the secretion of IL-5 and IL-13 (Fig. 1B).

Spleen NTN^{-/-} DCs have a distinct phenotype

To characterize the nature of the cells at the origin of the NTN^{-/-} phenotype, spleen DCs were put in culture and analyzed for their activation marker expression. NTN^{-/-} DCs exhibited a more mature cell surface phenotype than WT cells, characterized by higher expression of the costimulatory molecules CD80 and CD86 and the MHCII molecules (Fig. 1C). The same differences were observed upon stimulation with LPS or OVA (Fig. 1C). Unlike spleen DCs, the NTN^{-/-} BMDCs showed no phenotypic differences with WT (Fig. 1D). To determine the NTN^{-/-} DC capacity to uptake allergen, FITC-dextran beads were added in vitro in the presence of spleen DCs or BMDCs. NTN^{-/-} spleen and BMDCs showed the same phagocytosis capacities than WT DCs (data not shown).

NTN^{-/-} DCs elicit stronger Th2 cell responses

To further investigate the ability of DCs to modulate the immune response of NTN^{-/-} mice, purified spleen OVA-specific CD4⁺T cells were restimulated in vitro with OVA-pulsed BMDCs, which resulted in production of Th2 cytokines (Fig. 2). NTN^{-/-}

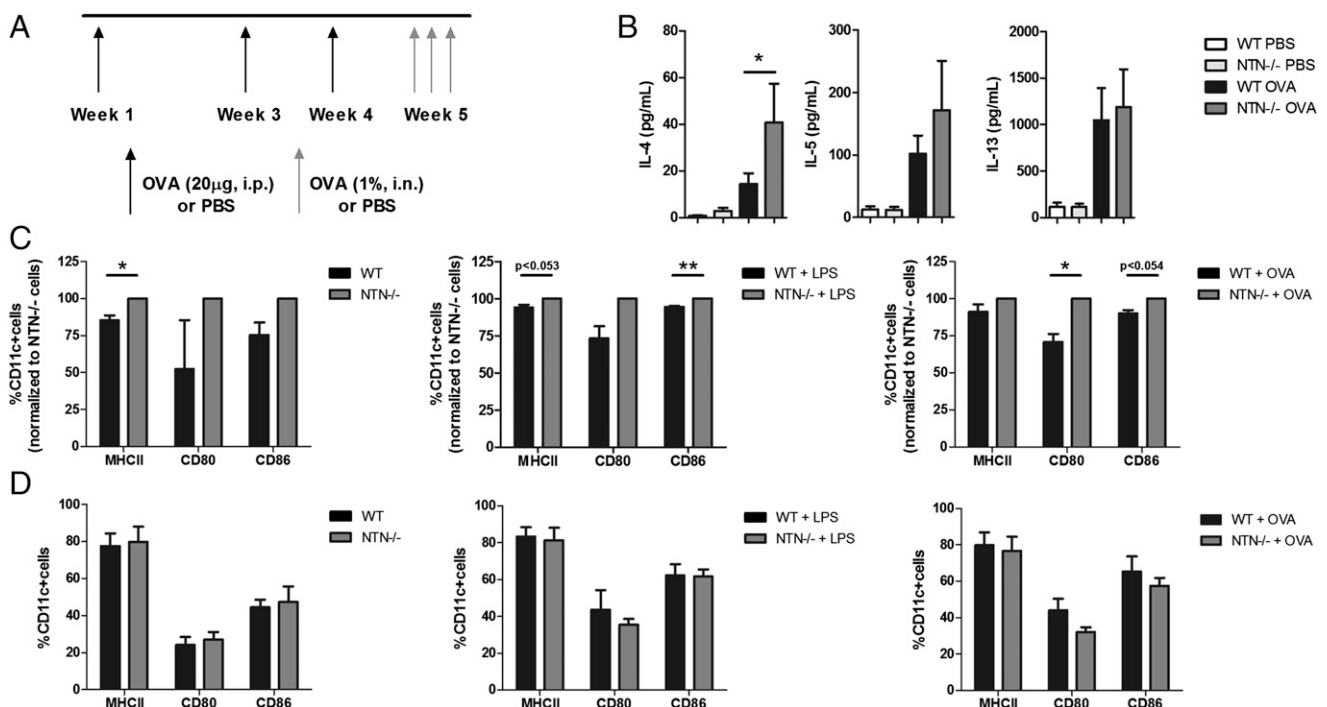


FIGURE 1. NTN^{-/-} splenocytes produce more Th2 cytokines and DCs show different phenotypes. **(A)** Schematic of sensitization and challenge protocol. **(B)** Cytokine levels from spleens of sensitized and challenged mice. Levels of IL-4, IL-5, and IL-13 were measured in supernatants of splenocytes restimulated in vitro with OVA for 4 d. Data are derived from three mice per group of four independent experiments (*n* = 12). **(C)** Expression level of MHCII, CD80, and CD86 among CD11c⁺ spleen DCs stimulated with LPS or OVA from naive mice (normalized to NTN^{-/-} DC condition). **(D)** Expression level of MHCII, CD80, and CD86 among CD11c⁺ BMDCs stimulated with LPS or OVA. Results are derived from three independent experiments. Values are expressed as means $\pm$ SEM (**p* < 0.05, ***p* < 0.01). i.n., intranasal.

T cells in contact with NTN^{-/-} BMDCs produced more IL-5 and IL-13 compared with WT T cells (Fig. 2A). To determine whether DCs or T cells were responsible for this phenotype, we next assessed whether differences in Ag-induced Th2 cytokine production were attributable to the ability of NTN^{-/-} DCs to present Ag. WT CD4⁺ T cells had more capacity to secrete IL-5 and IL-13 in coculture with NTN^{-/-} BMDCs in comparison with WT BMDCs (Fig. 2B). Thus, stimulation of T cells by NTN^{-/-} BMDCs led to a more robust proinflammatory response. We next compared the ability of NTN^{-/-} and WT CD4⁺ T cells to secrete the Th2 cytokines. So, WT or NTN^{-/-} CD4⁺ T cells were put in contact with WT BMDCs. Results showed that Th2 responses were better activated by NTN^{-/-} T cells (Fig. 2C). Furthermore, NTN^{-/-} OVA-specific CD4⁺ T cells purified from lung draining LN restimulated in vitro with OVA-pulsed DCs showed the same profile than NTN^{-/-} spleen CD4⁺ T cells (Supplemental Fig. 1A–

C). Thus, in NTN^{-/-} mice, both BMDCs and CD4⁺ T cells played a role in the stronger activation of the Th2 responses.

Higher Th2 secretion level in HDM-inflamed NTN^{-/-} mice

To obtain a better relevance for human disease, the acute airway inflammation experiments were reproduced with HDM as a natural allergen source for sensitization and airway challenge (Fig. 3A). After the induction of acute airway inflammation with the allergen, HDM-specific IgG1, IgG2a, and IgE were increased in WT and NTN^{-/-} sera and lung tissues showed immune cell infiltration around bronchi (Fig. 3B). At this stage, no significant differences were found between NTN^{-/-} and WT HDM-specific IgG1, IgG2a, and IgE levels. A strong elevation of total cell numbers, in particular the eosinophils, DCs, and lymphocytes, was observed in the BALF of NTN^{-/-} mice compared with WT (Fig. 3C). The analysis of Th2 cytokine levels in the BALF showed a tendency to

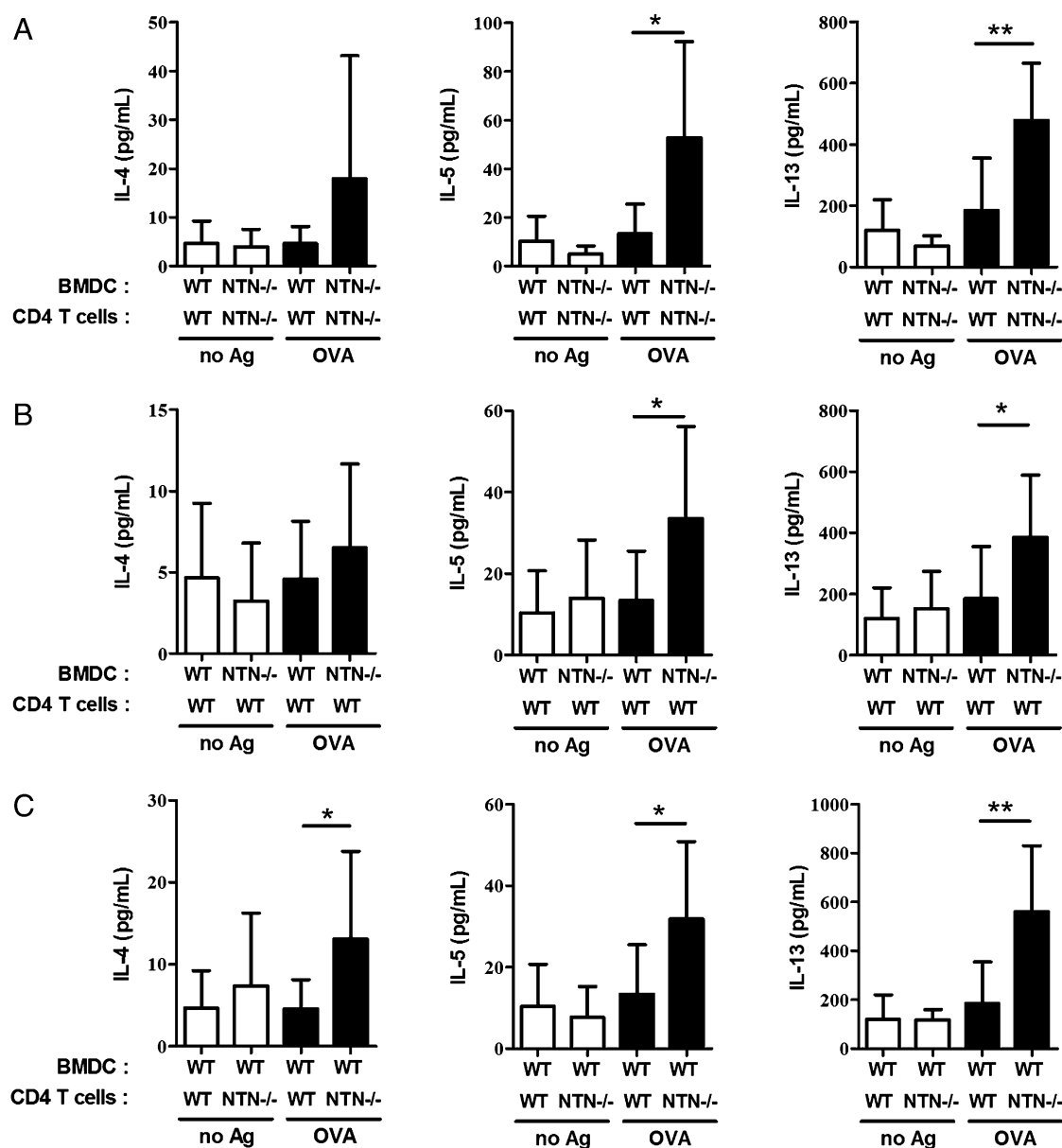


FIGURE 2. NTN^{-/-} BMDCs and T cells induce higher Th2 cytokine secretion. Purified OVA-induced spleen CD4⁺ T cells were cocultured with BMDCs in the presence of OVA (■) or no Ag (□). (A) Levels of IL-4, IL-5, and IL-13 in culture supernatants of WT and NTN^{-/-} BMDCs and T cells. (B) Levels of IL-4, IL-5, and IL-13 in culture supernatants of WT or NTN^{-/-} BMDCs in the presence of WT T cells. (C) Levels of IL-4, IL-5, and IL-13 in culture supernatants of WT BMDCs in the presence of WT or NTN^{-/-} T cells. Data are derived from three mice per group of three independent experiments ($n = 8/9$). Results of each group are expressed as means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$).

higher IL-4, IL-5, and IL-13 release in $NTN^{-/-}$ mice (Fig. 3D). The Th2 cytokine production was also analyzed after expansion of the lung draining LN cells in the presence of HDM for 4 d. As previously shown with the allergen OVA (24), the level of IL-4, IL-5, and IL-13 was strongly increased in $NTN^{-/-}$ LN in the presence of HDM (Fig. 3E). These results confirmed in another model of inflammation that $NTN^{-/-}$ mice have higher capacities to secrete Th2 cytokines.

Stronger effects of chronic allergic airway inflammation and remodeling in $NTN^{-/-}$ mice

The amount of TGF β -1 was significantly increased after acute airway inflammation in WT and $NTN^{-/-}$ mice. Furthermore, TGF β -1 expression was raised to higher levels in $NTN^{-/-}$ BALF compared with WT (Supplemental Fig. 2). Because this molecule has been identified as an anti-inflammatory and profibrotic cytokine playing a role in tissue remodeling (11, 26), we used a murine model of chronic allergic airway inflammation to assess the involvement of NTN in OVA-induced airway remodeling and chronic inflammation (Fig. 4A). IgE, IgG1, and IgG2a were upregulated in WT and $NTN^{-/-}$ sera after inflammation; in particular the IgE level was higher in the $NTN^{-/-}$ strain (Fig. 4B). BALF cell counts were increased in OVA-challenged mice. Thus, the level of eosinophils and the Th2 responses were elevated after inflammation. However, no difference was found between $NTN^{-/-}$ and WT mice (Fig. 4C, 4D). The same results were observed after analysis of the Th2 cytokine production of the lung

draining LN cells put in culture with OVA for 4 d (data not shown). In contrast, BALF composition showed an increase of neutrophils in $NTN^{-/-}$ and WT mice after a chronic airway inflammation, and the number was significantly higher in $NTN^{-/-}$ mice compared with WT mice (Fig. 4C). Because $NTN^{-/-}$ mice showed a higher level of neutrophils, we examined whether the lack of NTN affected the level of the proinflammatory markers IL-6, KC, and TNF- α , in BALF and the activity of the metalloproteinase MMP-9 in lung tissues (Fig. 4D, 4E). $NTN^{-/-}$ mice sensitized and challenged with OVA had a higher level of KC (Fig. 4D). TNF- α found in the BALF was also higher in $NTN^{-/-}$ compared with WT mice and MMP-9 activity followed the same way after inflammation in the lung (Fig. 4D, 4E). In addition, the proinflammatory cytokine IL-6 was significantly more secreted in $NTN^{-/-}$ BALF compared with WT (Fig. 4D).

Chronic allergen exposure is associated with peribronchial collagen deposition (27). We assessed the thickness of airway walls with Masson's trichrome stain. After repeated exposure to OVA, WT and $NTN^{-/-}$ lung sections showed an increase of peribronchial collagen deposition (Fig. 4F). In the airways of OVA-sensitized and -challenged $NTN^{-/-}$ mice, the area of subepithelial collagen staining appeared more intense and the quantitative image analysis showed significant differences compared with WT (Fig. 4G).

NTN treatment downregulates IL-6 secretion by immune and lung epithelial cells

The presence of OVA in the coculture of WT BMDCs and CD4 $^{+}$ T cells triggered the release of IL-6 (Fig. 5A). The simultaneous

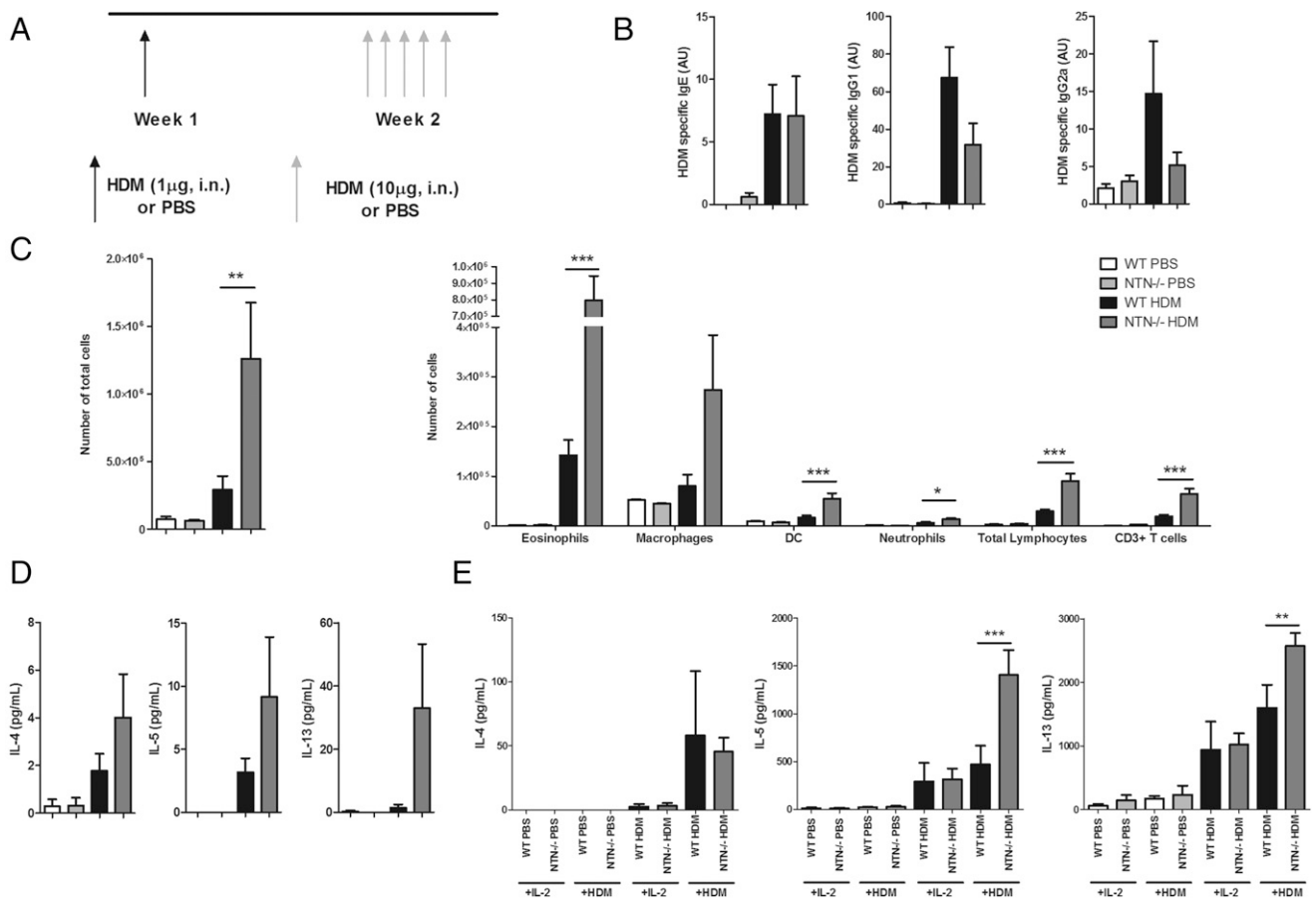


FIGURE 3. $NTN^{-/-}$ mice develop a strong airway inflammation after exposure to HDM. (A) Schematic of HDM disease protocol. (B) HDM-specific IgE, IgG1, and IgG2a levels in the serum ($n = 12$). (C) Numbers of total cells and cellular composition in BALF ($n = 9$). (D) Levels of IL-4, IL-5, and IL-13 in BALF ($n = 9$). (E) Levels of IL-4, IL-5 and IL-13 in culture supernatant of LN restimulated in vitro with IL-2 $\pm$ HDM for 4 d ($n = 9$). Data are derived from three mice per group of three or four independent experiments. Results of each group are expressed as means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). AU, arbitrary unit; i.n., intranasal.

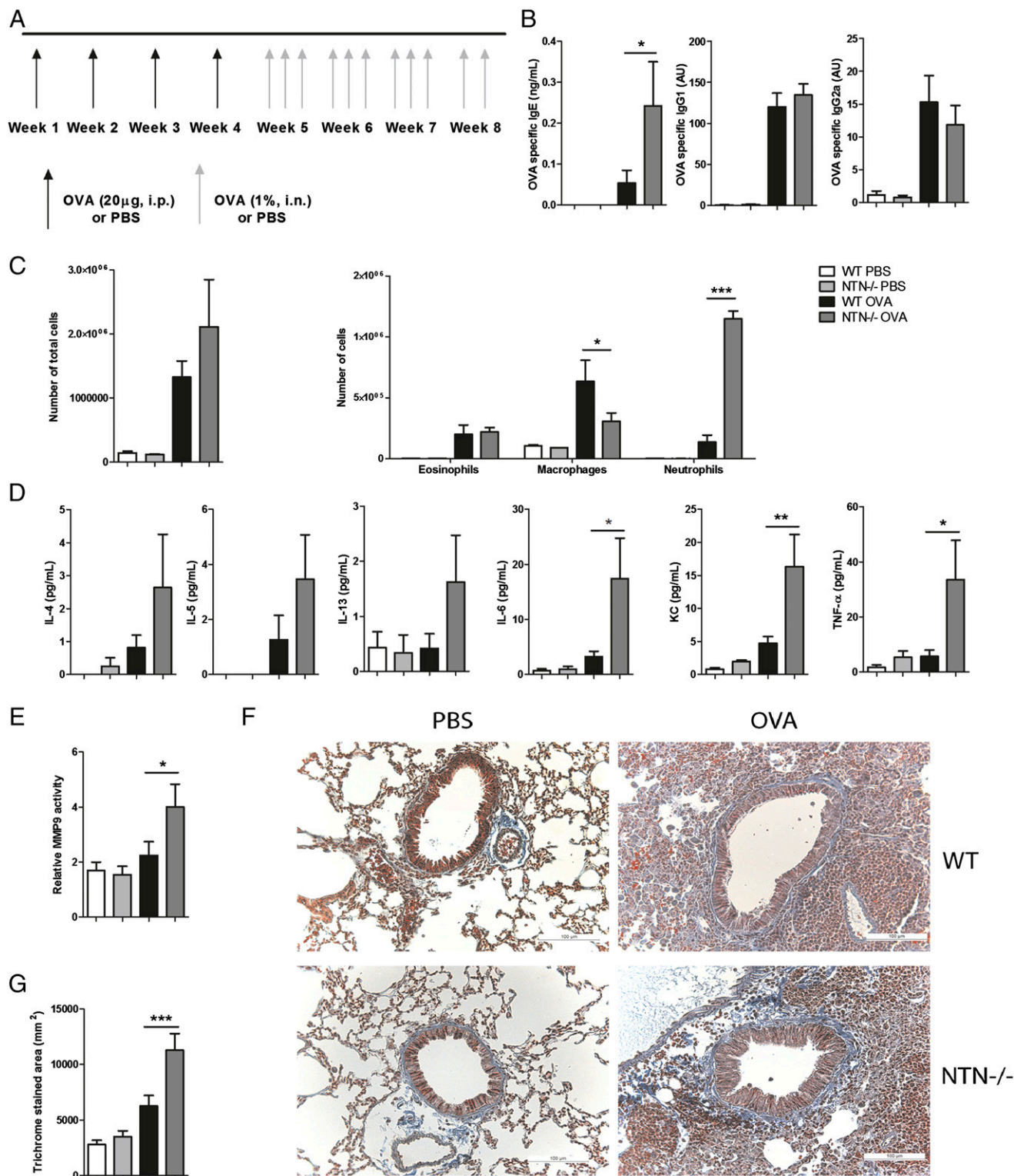


FIGURE 4. NTN^{-/-} mice develop stronger features of chronic airway inflammation and remodeling. **(A)** Schematic of chronic OVA disease protocol. **(B)** OVA-specific IgE, IgG1, and IgG2a levels in serum (AU, arbitrary unit) ($n = 9$). **(C)** Total cell numbers and eosinophils, macrophages and neutrophils in BALF ($n = 6$). **(D)** Levels of IL-4, IL-5, IL-13, IL-6, KC, and TNF- α in BALF ($n = 6/9$). **(E)** MMP-9 activity quantified in lung tissues from gelatin-zymogram ($n = 9$). **(F)** Lung histology. Representative sections of bronchi of WT or NTN^{-/-} mice. Sections were stained with Trichrome. Scale bars, 100 μ m. **(G)** Peribronchial collagen thickness was measured as described in *Materials and Methods* ($n = 15$). Data are derived from three mice per group of two or three independent experiments. Results of each group are expressed as means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). i.n., intranasal.

addition of NTN and OVA to WT BMDCs significantly decreased the IL-6 production. The NTN treatment generated also a reduction of the TNF- α level (Fig. 5B). Nevertheless, the secretion of the Th2 cytokines was not influenced as the level of IL-4, IL-5,

and IL-13 was comparable in the presence or absence of NTN (data not shown).

IL-6 is secreted by lung epithelial cells in asthmatic patients, and may promote Th2 differentiation of CD4⁺ T cells (28). To test the

capacity of NTN to influence the secretion of IL-6 by mouse lung epithelial cells, the LA-4 cell line was put in air-liquid interface culture. Then, OVA or OVA and NTN were added on the apical side, and the levels of the IL-6 production were quantified. The consequence of adding NTN was a significant reduction of the IL-6 production (Fig. 5C).

NTN receptors are expressed in lung tissues, DCs, and lung epithelial cells

To determine whether the effects of NTN on immune and lung epithelial cells could be the result of a binding to the NTN receptor complex, we investigated the expression of RET and GFR α -2 in lung tissues, DCs, and the LA-4 cell line by quantitative RT-PCR analysis. Previous reports have shown the expression of NTN, RET, and GFR α -2 in the airways (17, 18, 29). Our results demonstrated the mRNA expression of GFR α -2 and RET in the LA-4 cell line, WT BMDCs, and spleen DCs. Furthermore, the absence of NTN had no influence on the basal expression of these receptors (Fig. 6A). WT and NTN^{-/-} lung tissues also expressed GFR α -2 and RET, and the transcript levels measured in control mice and after inflammation were similar (Fig. 6A). RET expression at protein level was also detected in lung tissues of WT and NTN^{-/-} mice by flow cytometry (Fig. 6B). Thus, we confirmed that the molecules required for active NTN signaling are expressed in lung tissues, DCs, and lung epithelial cells, suggesting a role for this neurotrophic factor in airway lung inflammation.

Lower expression of ATF3 in NTN^{-/-} lung tissue

Activating transcription factor 3 (ATF3) is activated in response to asthmatic inflammation in the lung and negatively regulates the gene expression of many cytokines like IL-4, IL-5, IL-13, and the proinflammatory IL-6 (30, 31). Thereby, in the absence of ATF3, the Th2 cytokine production is enhanced (32). We tested the hypothesis that NTN modulates the immune response by inducing expression of ATF3. Therefore, the ATF3 protein expression level was quantified in WT and NTN^{-/-} lung tissues after a chronic airway inflammation protocol. The data showed that ATF3 expression was significantly increased in the lungs of WT mice after OVA sensitization (Fig. 6C), confirming results from Gilchrist et al. (32). On the contrary, NTN^{-/-} mice didn't show any rise of the ATF3 protein in their lung tissues following the overinflammatory phenotype observed in these mice (Fig. 6C).

Discussion

The implication of NTN in the regulation of asthmatic airway inflammation had not yet been further worked out. Our study of

NTN^{-/-} mice in airway inflammation demonstrates an essential role of NTN in the control of epithelial and immune cells like DC and CD4⁺ T cells through the downregulation of the Th2 response. Thus, we tested the Ag-specific Th2 cell responses in lymphoid tissue and locally in the respiratory tract. As previously shown for NTN^{-/-} lung draining LN cells (24), NTN^{-/-} spleen cells better induce Th2 responses. Analyses of NTN^{-/-} BMDCs and NTN^{-/-} lymphoid CD4⁺ T cells, demonstrated a higher capacity of these cells to activate the secretion of the Th2 cytokines compared with WT cells. Furthermore, the allergic airway inflammation in a HDM instead of OVA asthma model showed similar results by exhibiting more immune cells in the BALF of NTN^{-/-} mice, in particular eosinophils, DCs, T cells, and neutrophils, and in addition, Th2 responses were increased. The same phenomenon was observed when NTN^{-/-} mice had undergone chronic inflammation challenge using OVA. In this situation, NTN^{-/-} lung tissues also exhibited increased markers of airway remodeling as shown by collagen deposition, neutrophils, KC, MMP-9, and the proinflammatory cytokines TNF- α and IL-6. In addition, we were able to demonstrate that NTN, added to OVA on DCs or LA-4 cell line, has the capacity to decrease in vitro the level of IL-6 and TNF- α and thus plays a direct role as an anti-inflammatory molecule. Finally, we showed the presence of the receptors GFR α -2 and RET on DCs and epithelial cells and a possible influence of NTN on the transcription factor ATF3 expression, because its level is lower in NTN^{-/-} mice after OVA sensitization and challenge compared with WT mice.

To confirm our previous studies using OVA in acute airway asthma model inflammation (24), we tested another allergen model that is considered more relevant for human asthma (33). HDM extract contains many allergenic components to which 50–85% of asthmatics are allergic (34). In our model, HDM exposure induces a strong increase of airway inflammation markers in NTN^{-/-} mice. Thus, NTN seems to play a role in the control of this pathology in both HDM- and OVA-induced acute asthma models. Nevertheless, the global Th2 response in the HDM model of airway inflammation is weaker than in the OVA model as reflected by a lower level of serum HDM-specific Ig.

DCs, as professional APCs, have an important function in the airways to initiate and modulate immune responses by influencing differentiation of T cells. It was shown that DCs use different signals to activate the T cell responses; one is delivered by MHCII molecules to the TCR and another one from costimulatory molecules such as CD80 and CD86 (35). Despite similar MHCII, CD80, and CD86 expression levels as in WT,

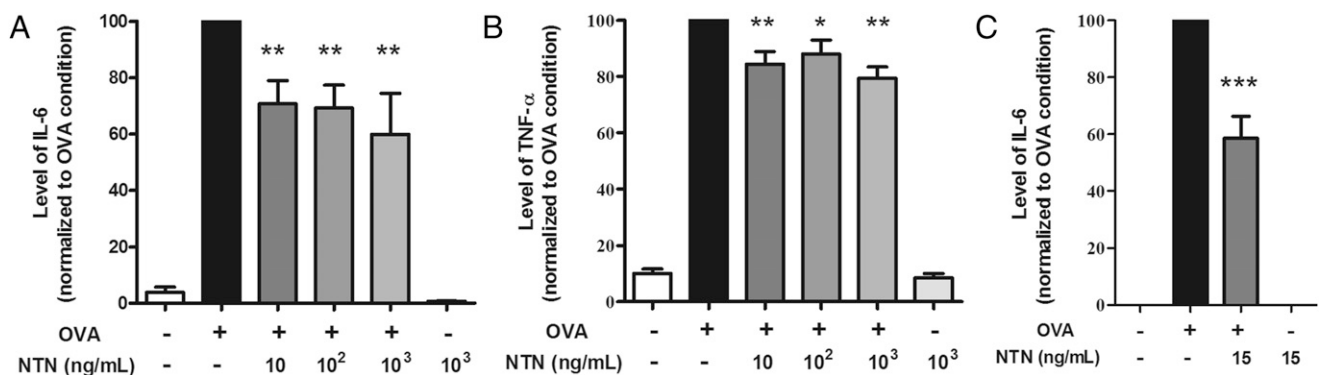


FIGURE 5. NTN has a potential anti-inflammatory role on immune and epithelial cells. Levels of IL-6 (**A**) and TNF- α in supernatants (**B**) of purified OVA-induced WT spleen CD4⁺ T cells cocultured with WT BMDCs in the presence of OVA and/or 10 ng/ml NTN, 10² ng/ml, and 10³ ng/ml. Data are derived from three mice per group of two or three independent experiments ($n = 9$). (**C**) Levels of IL-6 in supernatants of LA-4 cells stimulated with OVA (200 μ g/ml) and/or NTN (15 ng/ml). Data are derived from seven independent experiments. Results of each group are expressed as means $\pm$ SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

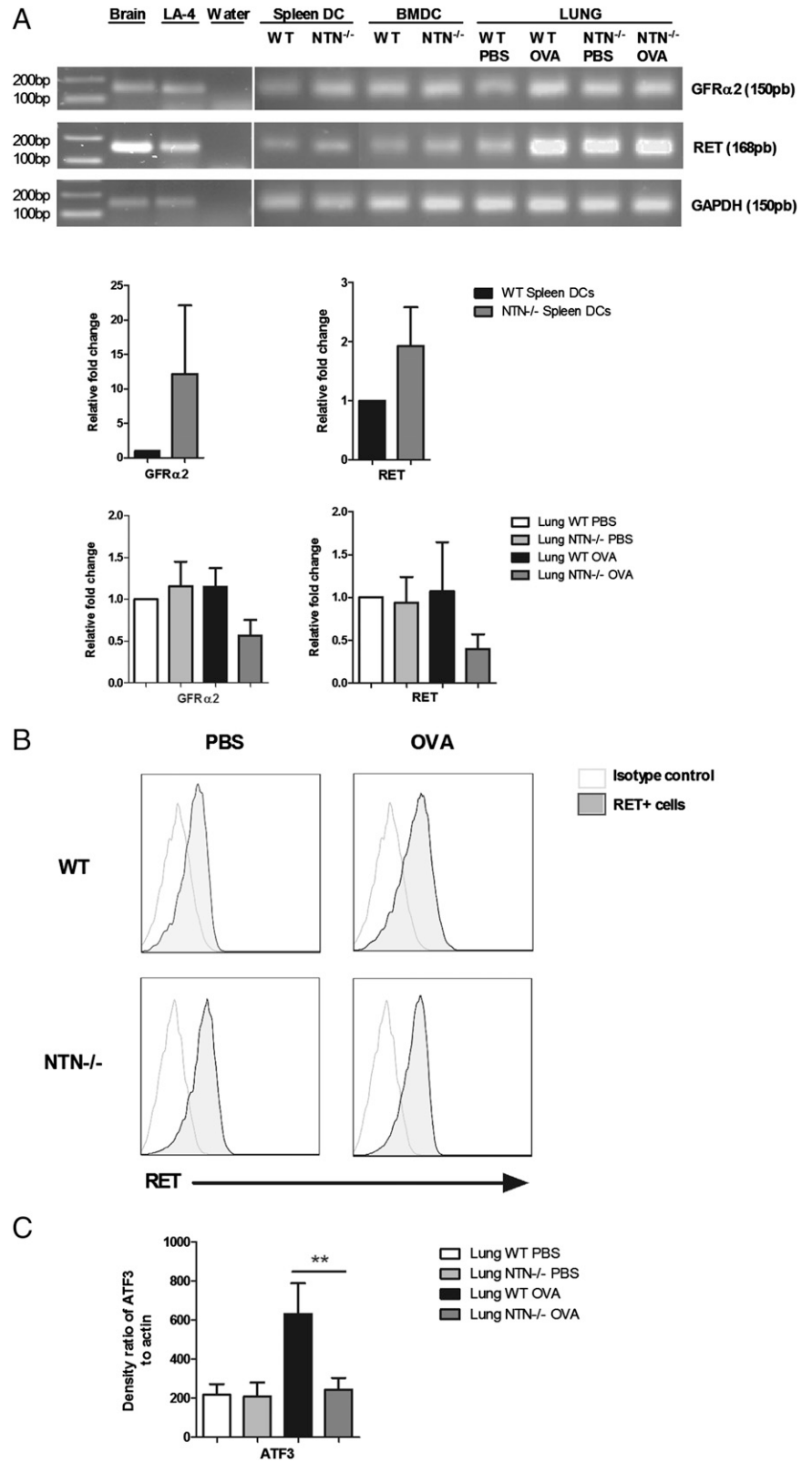


FIGURE 6. WT and NTN^{-/-} mice express GFRα-2 and RET in lung tissues, DCs, and in LA-4 cell line. **(A)** GFRα-2 and RET mRNA expressions in LA-4 cell line ($n = 3$), in BMDCs and in spleen DCs from naive mice ($n = 3$), in lung tissues from OVA-induced mice ($n = 5$) and in brain tissue as positive control. For each sample, GAPDH was used as endogenous control. **(B)** RET expression was examined by flow cytometry in OVA-induced WT and NTN^{-/-} lung cells (RET⁺CD19⁻IgG⁻) compared with isotype control ($n = 3$). Data are derived from three independent experiments. **(C)** ATF3 protein expression in OVA-induced WT and NTN^{-/-} lung tissues were examined by Western blot. Data are derived from three mice per group of three independent experiments ($n = 7/9$). Results of each group are expressed as means $\pm$ SEM (** $p < 0.01$).

NTN^{-/-} BMDCs have higher T cell stimulatory capacities in vitro. Moreover, stimulated NTN^{-/-}CD4⁺ T cells induce a more robust activation compared with WT. These findings suggest that Th2 cell responses might at least in part be negatively regulated by NTN but that control might be dependent on several other cosignaling pathways, through adhesion or costimulatory molecules like TIM family or members of the TNF superfamily:

CD70, OX40 ligand and LIGHT (36). LIGHT could be an interesting candidate as it was described to be involved in T cell activation and in the promotion of MMP-9 and IL-13 expression in airway remodeling in OVA-induced animal models of chronic asthma (4, 37). This hypothesis needs to be tested to detail the potential anti-inflammatory effect of NTN demonstrated previously (24).

Because we observed the expression of GFR α -2 and RET mRNA in lung tissues, in immune and epithelial cells, this suggests a direct action of NTN on DCs and T cells. Our results are in accordance with previous reports, which described that mice and human lung, LN, and immune cell subsets like CD4⁺ T cells and monocytes are able to produce NTN and express its receptors GFR α -2 and RET (29, 38–41). Furthermore, after a stimulation with OVA and NTN in a culture of DCs or the LA-4 cell line, the IL-6 levels were lower in comparison with the condition with OVA alone. IL-6 was shown to be produced by macrophages or DCs, by epithelial, endothelial, and some tumor cells. A potential role of IL-6R in controlling Th2 function was suggested (42). Thus, IL-6 might be released by DCs and then influence T cells by promoting a Th2 response in the airways. We can make the hypothesis that NTN, through the binding of its receptors GFR α -2 and RET on immune cells, may inhibit the secretion of IL-6, leading to a decrease of the inflammation as illustrated in Fig. 7. Accordingly, in absence of NTN, IL-6 is overexpressed resulting in an increase of the inflammation observed in NTN^{-/-} mice.

Airway epithelial cells play an important role in asthma pathophysiology via the interaction with immune cells like DCs and the secretion of proinflammatory cytokines such as IL-6 and IL-8/KC.

These cytokines have been associated not only with inflammation but also with airway remodeling and hyperresponsiveness (43). Direct interactions have been shown between the RET pathway and immune response as the RET mRNA level is correlated with the transcript of IL-8 in monocytes and macrophages (40). This could be a link to our results showing a strong increase of IL-8/KC secretion in lung in the absence of NTN. Furthermore, the secretion of TNF- α and KC inducing a recruitment of neutrophils, is associated with airway remodeling of chronic bronchial inflammation (44). In our experiments, TNF- α levels were higher in NTN^{-/-} mice, as were the levels of neutrophils, which also differed between NTN^{-/-} and WT mice. This could correlate with findings reported previously by Vargas-Leal et al. (39) who showed that addition of NTN to immune cells reduced the amount of TNF in the supernatants of PBMC. Taken together with our results, this study suggests that NTN might regulate the expression of TNF- α during chronic asthma.

Following stimulation by TNF- α and IL-8/KC, neutrophils will release granules whose major component is MMP-9 (45). MMP-9 is found in the BALF of asthmatic patients, and the increase of MMP-9 activity in the lung tissues is related to the increase of collagen deposition in the airway walls (46). We have shown that

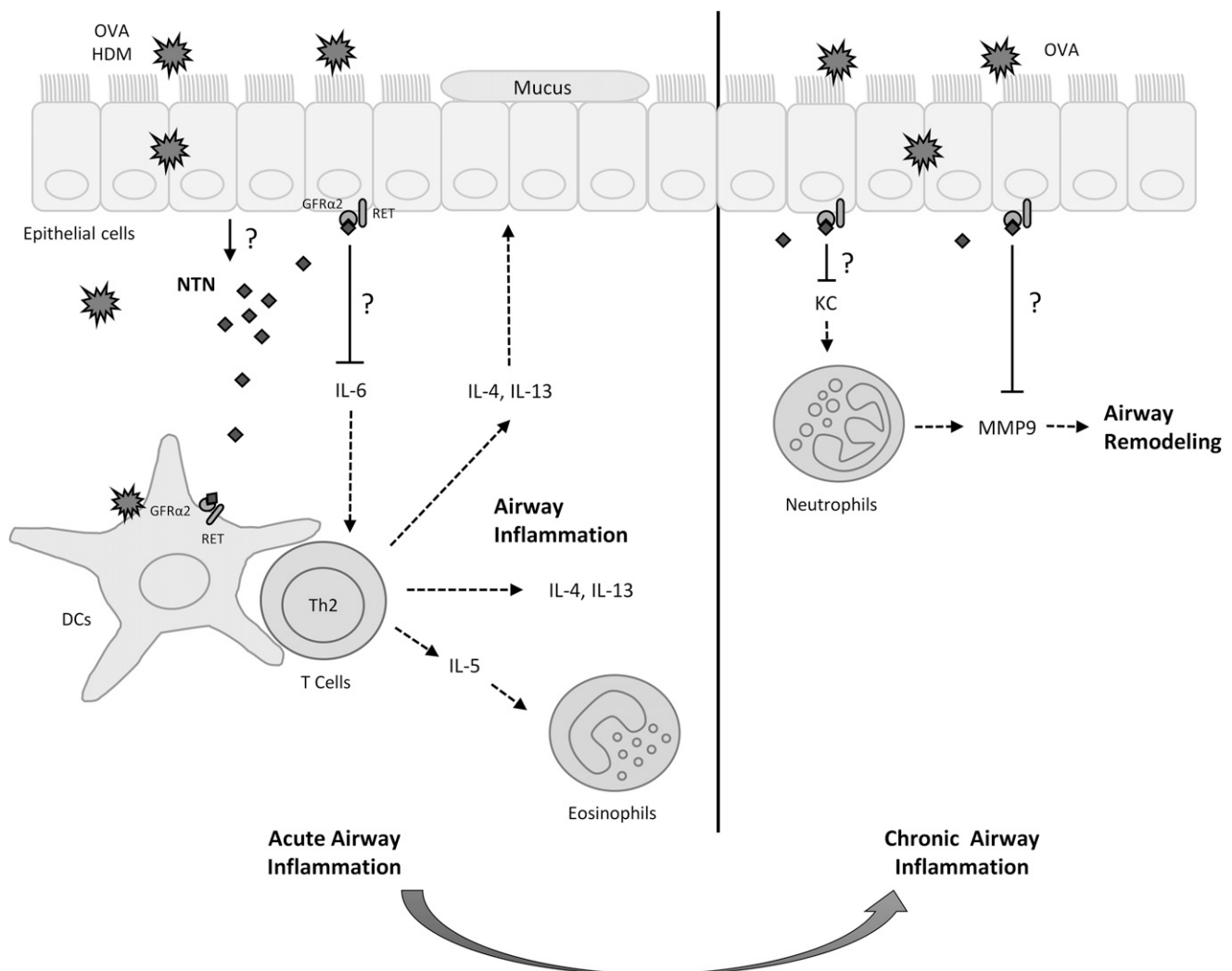


FIGURE 7. Model of mechanism of action of NTN during airway inflammatory responses. As a response to stimulation from the allergen, NTN might be produced by airway epithelial cells. This molecule could be able to modulate epithelial and immune cells like DCs through the regulation of the acute airway inflammation. During chronic airway inflammation, this effect would lead to a partial control of the airway remodeling via various cytokines like, KC, MMP-9, TNF- α , and collagen deposition.

NTN^{-/-} mice presented a higher MMP-9 activity than WT mice resulting in a higher level of peribronchial collagen deposition. As previously shown for the influence of the neurotrophin BDNF on eosinophils (47), one hypothesis could be that NTN produced by airway epithelial cells could be able to modulate immune cells like DCs, T cells, or neutrophils by the way of various cytokines like IL-6, KC, MMP-9, and TNF- α and collagen deposition (Fig. 7).

ATF3 regulates proinflammatory cytokine production like IL-4, IL-5, IL-6, and IL-13 in immune cells through a negative feedback mechanism to prevent acute inflammatory syndromes (48, 49). In response to LPS, ATF3 is also able to downregulate TNF- α (30). The fact that ATF3 is expressed at a lower level in NTN^{-/-} mice after OVA sensitization and challenge compared with WT mice could be linked with the cytokine level observed in these mice. By which mechanism NTN is able to influence the ATF3 level expression remains to be determined. However, through the activation of GFR α -2 and RET pathway, it has been shown that NTN could modulate the ATF3 expression in neurons (50).

In conclusion, treatment with NTN can reduce the secretion of IL-6 by immune cells. The absence of this neurotrophic factor increases cellular infiltration by eosinophils and neutrophils in the airways, activates the synthesis and secretion of cytokines, and increases the IgE synthesis by B cells, related to acute and chronic airway inflammation and remodeling. Further studies should be performed using NTN in vitro with different partners like airway epithelial cells, DCs and T cells or neutrophils to define exactly the anti-inflammatory potential role of NTN. It now remains to be established whether and how this pathway is also operative in humans, as it was recently shown that RET is likewise present in human peripheral T cells (39, 40). In addition, NTN was identified in the transcriptome of the normal nonsmoker healthy human small airway epithelium (51) and was able to reduce TNF expression in human PBMC (39).

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Disclosures

The authors have no financial conflicts of interest.

References

- Plantinga, M., H. Hammad, and B. N. Lambrecht. 2010. Origin and functional specializations of DC subsets in the lung. *Eur. J. Immunol.* 40: 2112–2118.
- Liu, Y. J. 2006. Thymic stromal lymphopoietin: master switch for allergic inflammation. *J. Exp. Med.* 203: 269–273.
- Kaur, D., and C. Brightling. 2012. OX40/OX40 ligand interactions in T-cell regulation and asthma. *Chest* 141: 494–499.
- Doherty, T. A., P. Soroosh, N. Khorram, S. Fukuyama, P. Rosenthal, J. Y. Cho, P. S. Norris, H. Choi, S. Scheu, K. Pfeffer, et al. 2011. The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. *Nat. Med.* 17: 596–603.
- Lambrecht, B. N., and H. Hammad. 2012. The airway epithelium in asthma. *Nat. Med.* 18: 684–692.
- Barnes, P. J. 2011. Pathophysiology of allergic inflammation. *Immunol. Rev.* 242: 31–50.
- Page, K., J. R. Ledford, P. Zhou, and M. Wills-Karp. 2009. A TLR2 agonist in German cockroach frass activates MMP-9 release and is protective against allergic inflammation in mice. *J. Immunol.* 183: 3400–3408.
- Kim, M. S., K. A. Cho, Y. J. Cho, and S. Y. Woo. 2013. Effects of interleukin-9 blockade on chronic airway inflammation in murine asthma models. *Allergy Asthma Immunol Res* 5: 197–206.
- Heuckeroth, R. O., H. Enomoto, J. R. Grider, J. P. Golden, J. A. Hanke, A. Jackman, D. C. Molliver, M. E. Bardgett, W. D. Snider, E. M. Johnson, Jr., and J. Milbrandt. 1999. Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron* 22: 253–263.
- Pulleyn, L. J., R. Newton, I. M. Adcock, and P. J. Barnes. 2001. TGF β 1 allele association with asthma severity. *Hum. Genet.* 109: 623–627.

- Hansen, G., J. J. McIntire, V. P. Yeung, G. Berry, G. J. Thorbecke, L. Chen, R. H. DeKruyff, and D. T. Umetsu. 2000. CD4⁺ T helper cells engineered to produce latent TGF- β 1 reverse allergen-induced airway hyperreactivity and inflammation. *J. Clin. Invest.* 105: 61–70.
- Joseph, J., S. Benedict, P. Badrinath, S. Wassef, M. Joseph, S. Abdulkhalik, and M. G. Nicholls. 2003. Elevation of plasma transforming growth factor β 1 levels in stable nonatopic asthma. *Ann. Allergy Asthma Immunol.* 91: 472–476.
- Redington, A. E., J. Madden, A. J. Frew, R. Djukanovic, W. R. Roche, S. T. Holgate, and P. H. Howarth. 1997. Transforming growth factor- β 1 in asthma: measurement in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med.* 156: 642–647.
- Palomares, O., M. Martín-Fontecha, R. Lauener, C. Traidl-Hoffmann, O. Cavkaytar, M. Akdis, and C. A. Akdis. 2014. Regulatory T cells and immune regulation of allergic diseases: roles of IL-10 and TGF- β . *Genes Immun.* 15: 511–520.
- Buj-Bello, A., J. Adu, L. G. Piñón, A. Horton, J. Thompson, A. Rosenthal, M. Chinchetru, V. L. Buchman, and A. M. Davies. 1997. Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. *Nature* 387: 721–724.
- Airaksinen, M. S., and M. Saarma. 2002. The GDNF family: signalling, biological functions and therapeutic value. *Nat. Rev. Neurosci.* 3: 383–394.
- Widenfalk, J., C. Nosrat, A. Tomac, H. Westphal, B. Hoffer, and L. Olson. 1997. Neurturin and glial cell line-derived neurotrophic factor receptor-beta (GDNFR- β), novel proteins related to GDNF and GDNFR- α with specific cellular patterns of expression suggesting roles in the developing and adult nervous system and in peripheral organs. *J. Neurosci.* 17: 8506–8519.
- Golden, J. P., J. A. DeMaro, P. A. Osborne, J. Milbrandt, and E. M. Johnson, Jr. 1999. Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. *Exp. Neurol.* 158: 504–528.
- Lieu, T., M. Kollarik, A. C. Myers, and B. J. Udem. 2011. Neurotrophin and GDNF family ligand receptor expression in vagal sensory nerve subtypes innervating the adult guinea pig respiratory tract. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 300: L790–L798.
- Lieu, T. M., A. C. Myers, S. Meeker, and B. J. Udem. 2012. TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 302: L941–L948.
- Langsdorf, A., K. Radzikinas, A. Kroten, S. Jain, and X. Ai. 2011. Neural crest cell origin and signals for intrinsic neurogenesis in the mammalian respiratory tract. *Am. J. Respir. Cell Mol. Biol.* 44: 293–301.
- Tollet, J., A. W. Everett, and M. P. Sparrow. 2002. Development of neural tissue and airway smooth muscle in fetal mouse lung explants: a role for glial-derived neurotrophic factor in lung innervation. *Am. J. Respir. Cell Mol. Biol.* 26: 420–429.
- Song, X. J., D. Q. Li, W. Farley, L. H. Luo, R. O. Heuckeroth, J. Milbrandt, and S. C. Pflugfelder. 2003. Neurturin-deficient mice develop dry eye and keratoconjunctivitis sicca. *Invest. Ophthalmol. Vis. Sci.* 44: 4223–4229.
- Michel, T., M. Thérésine, A. Poli, O. Domingues, W. Ammerlaan, N. H. Brons, F. Hentges, and J. Zimmer. 2011. Increased Th2 cytokine secretion, eosinophilic airway inflammation, and airway hyperresponsiveness in neurturin-deficient mice. *J. Immunol.* 186: 6497–6504.
- Willart, M. A., K. Deswarte, P. Pouliot, H. Braun, R. Beyaert, B. N. Lambrecht, and H. Hammad. 2012. Interleukin-1 α controls allergic sensitization to inhaled house dust mite via the epithelial release of GM-CSF and IL-33. *J. Exp. Med.* 209: 1505–1517.
- Border, W. A., and N. A. Noble. 1994. Transforming growth factor β in tissue fibrosis. *N. Engl. J. Med.* 331: 1286–1292.
- Kudo, M., Y. Ishigatsubo, and I. Aoki. 2013. Pathology of asthma. *Frontiers Microbiol.* 4: 263.
- Neveu, W. A., J. L. Allard, D. M. Raymond, L. M. Bourassa, S. M. Burns, J. Y. Bunn, C. G. Irvin, D. A. Kaminsky, and M. Rincon. 2010. Elevation of IL-6 in the allergic asthmatic airway is independent of inflammation but associates with loss of central airway function. *Respir. Res.* 11: 28.
- Almeida, A. R., S. Arroz-Madeira, D. Fonseca-Pereira, H. Ribeiro, R. Lasrado, V. Pachnis, and H. Veiga-Fernandes. 2012. RET/GFR α signals are dispensable for thymic T cell development in vivo. *PLoS One* 7: e52949.
- Gilchrist, M., V. Thorsson, B. Li, A. G. Rust, M. Korb, J. C. Roach, K. Kennedy, T. Hai, H. Bolouri, and A. Aderem. 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. *Nature* 441: 173–178.
- Calton, C. M., L. K. Wade, and M. So. 2013. Upregulation of ATF3 inhibits expression of the pro-inflammatory cytokine IL-6 during *Neisseria gonorrhoeae* infection. *Cell. Microbiol.* 15: 1837–1850.
- Gilchrist, M., W. R. Henderson, Jr., A. E. Clark, R. M. Simmons, X. Ye, K. D. Smith, and A. Aderem. 2008. Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation. *J. Exp. Med.* 205: 2349–2357.
- Chapman, D. G., J. E. Tully, J. D. Nolin, Y. M. Janssen-Heininger, and C. G. Irvin. 2014. Animal models of allergic airways disease: where are we and where to next? *J. Cell. Biochem.* 115: 2055–2064.
- Nelson, R. P., Jr., R. DiNicolo, E. Fernández-Caldas, M. J. Seleznick, R. F. Lockey, and R. A. Good. 1996. Allergen-specific IgE levels and mite allergen exposure in children with acute asthma first seen in an emergency department and in nonasthmatic control subjects. *J. Allergy Clin. Immunol.* 98: 258–263.
- Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 14: 233–258.
- Bakdash, G., S. P. Sittig, T. van Dijk, C. G. Figdor, and I. J. de Vries. 2013. The nature of activatory and tolerogenic dendritic cell-derived signal II. *Front. Immunol.* 4: 53.

37. Tamada, K., K. Shimozaiki, A. I. Chapoval, Y. Zhai, J. Su, S. F. Chen, S. L. Hsieh, S. Nagata, J. Ni, and L. Chen. 2000. LIGHT, a TNF-like molecule, costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell response. *J. Immunol.* 164: 4105–4110.
38. Yang, C., D. Hutto, and D. W. Sah. 2006. Distribution of GDNF family receptor $\alpha 3$ and RET in rat and human non-neural tissues. *J. Mol. Histol.* 37: 69–77.
39. Vargas-Leal, V., R. Bruno, T. Derfuss, M. Krumbholz, R. Hohlfeld, and E. Meinel. 2005. Expression and function of glial cell line-derived neurotrophic factor family ligands and their receptors on human immune cells. *J. Immunol.* 175: 2301–2308.
40. Rusmini, M., P. Griseri, I. Matera, E. Pontarini, R. Ravazzolo, D. Mavilio, and I. Ceccherini. 2014. Expression variability and function of the RET gene in adult peripheral blood mononuclear cells. *J. Cell. Physiol.* 229: 2027–2037.
41. Almeida, A. R., D. Fonseca-Pereira, S. Arroiz-Madeira, H. Ribeiro, C. Labão-Almeida, and H. Veiga-Fernandes. 2014. The neurotrophic factor receptor RET regulates IL-10 production by in vitro polarised T helper 2 cells. *Eur. J. Immunol.* 44: 3605–3613.
42. Doganci, A., K. Sauer, R. Karwot, and S. Finotto. 2005. Pathological role of IL-6 in the experimental allergic bronchial asthma in mice. *Clin. Rev. Allergy Immunol.* 28: 257–270.
43. Vieira, R. P., T. Müller, M. Grimm, V. von Gernler, B. Vetter, T. Dürk, S. Cicko, C. K. Ayata, S. Soricther, B. Robaye, et al. 2011. Purinergic receptor type 6 contributes to airway inflammation and remodeling in experimental allergic airway inflammation. *Am. J. Respir. Crit. Care Med.* 184: 215–223.
44. Thomas, P. S., D. H. Yates, and P. J. Barnes. 1995. Tumor necrosis factor- α increases airway responsiveness and sputum neutrophilia in normal human subjects. *Am. J. Respir. Crit. Care Med.* 152: 76–80.
45. Khokha, R., A. Murthy, and A. Weiss. 2013. Metalloproteinases and their natural inhibitors in inflammation and immunity. *Nat. Rev. Immunol.* 13: 649–665.
46. Atkinson, J. J., and R. M. Senior. 2003. Matrix metalloproteinase-9 in lung remodeling. *Am. J. Respir. Cell Mol. Biol.* 28: 12–24.
47. Hahn, C., A. P. Islamian, H. Renz, and W. A. Nockher. 2006. Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation. *J. Allergy Clin. Immunol.* 117: 787–794.
48. Thompson, M. R., D. Xu, and B. R. Williams. 2009. ATF3 transcription factor and its emerging roles in immunity and cancer. *J. Mol. Med.* 87: 1053–1060.
49. Roussel, L., S. Robins, A. Schachter, J. Bérubé, Q. Hamid, and S. Rousseau. 2011. Steroids and extracellular signal-regulated kinase 1/2 activity suppress activating transcription factor 3 expression in patients with severe asthma. *J. Allergy Clin. Immunol.* 127: 1632–1634.
50. Young, B. A., B. M. Girard, and R. L. Parsons. 2008. Neurturin suppresses injury-induced neuronal activating transcription factor 3 expression in cultured guinea pig cardiac ganglia. *J. Comp. Neurol.* 508: 795–805.
51. Hackett, N. R., M. W. Butler, R. Shaykhiev, J. Salit, L. Omberg, J. L. Rodriguez-Flores, J. G. Mezey, Y. Strulovici-Barel, G. Wang, L. Didon, and R. G. Crystal. 2012. RNA-Seq quantification of the human small airway epithelium transcriptome. *BMC Genomics* 13: 82.

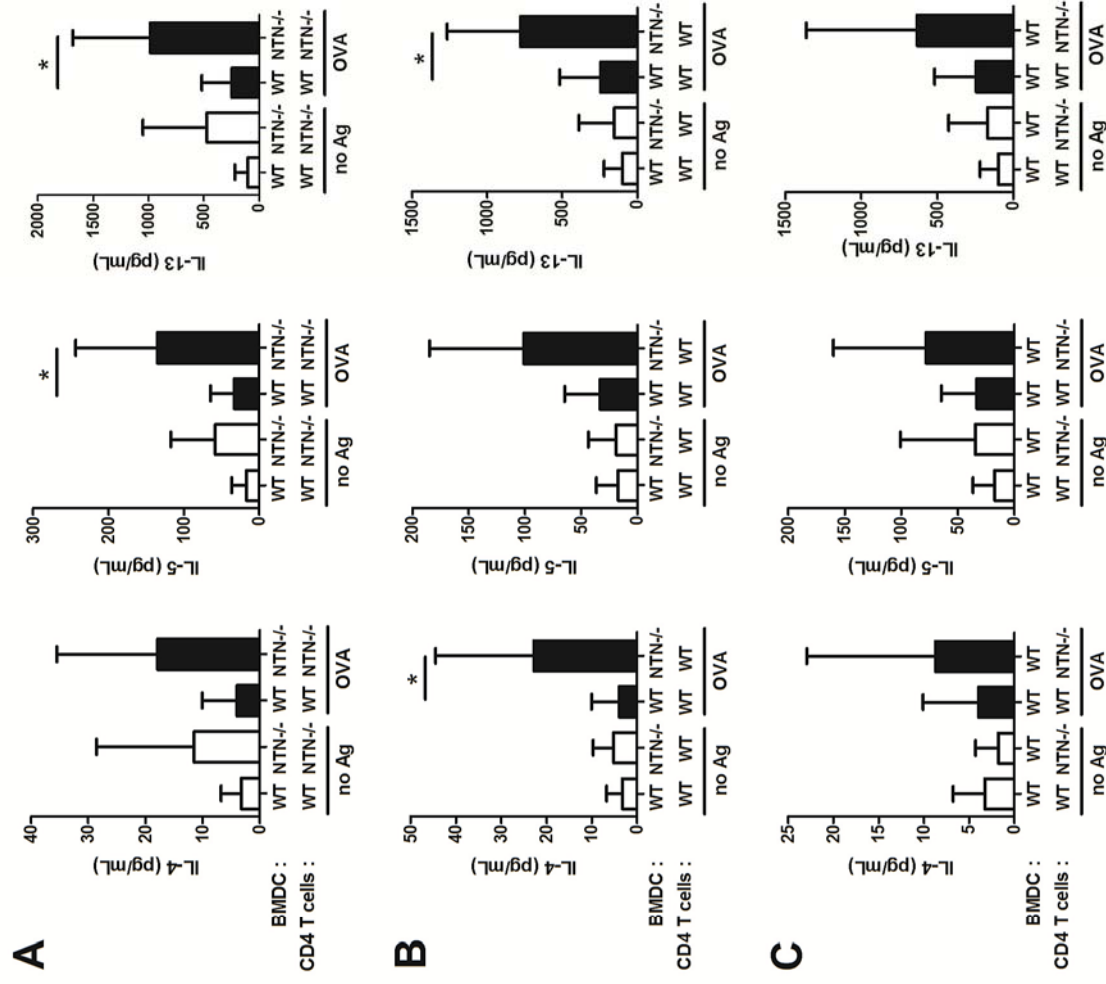


Figure S1: NTN^{-/-} lung draining LN T cells and DCs induce higher Th2 cytokine secretion. Purified OVA-induced LN CD4⁺ T cells were co-cultured with BMDCs in the presence of OVA (black bars) or no Ag (white bars). (A) Levels of IL-4, IL-5 and IL-13 in culture supernatants of WT and NTN^{-/-} BMDCs and T cells. (B) Levels of IL-4, IL-5 and IL-13 in culture supernatants of WT or NTN^{-/-} BMDCs in the presence of WT T cells. (C) Levels of IL-4, IL-5 and IL-13 in culture supernatants of WT BMDCs in the presence of WT or NTN^{-/-} T cells. Data are derived from two or three mice per group of three independent experiments (n=7/8). Results of each group are expressed as means $\pm$ SEM (* p < 0.05).

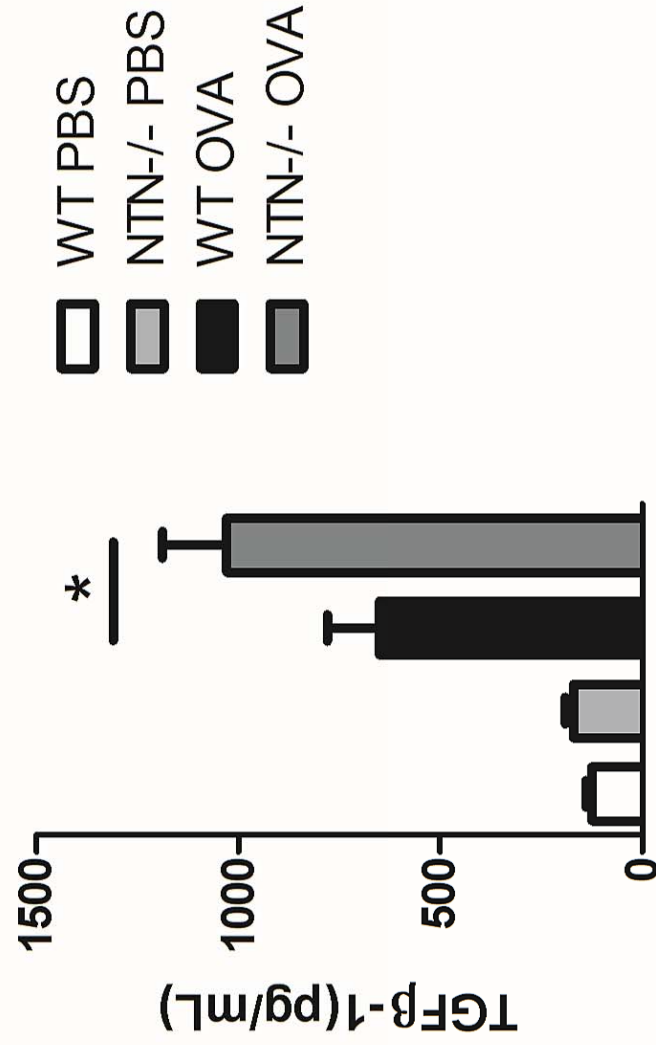


Figure S2: NTN^{-/-} mice produce higher levels of TGFβ-1 after an acute airway inflammation. Mice were treated with OVA or PBS. Levels of TGFβ-1 in BALF are shown. Data are means ± SEM (* p < 0.05).