

ORIGINAL PAPER

Differential activation of dendritic cells by nerve growth factor and brain-derived neurotrophic factor

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Summary

Background Neurotrophins are involved in inflammatory reactions influencing several cells in health and disease including allergy and asthma. Dendritic cells (DCs) play a major role in the induction of inflammatory processes with an increasing role in allergic diseases as well.

Objective The aim of this study was to investigate the influence of neurotrophins on DC function.

Methods Monocyte-derived dendritic cells were generated from allergic and non-allergic donors. Neurotrophin receptors were demonstrated by western blotting, flow cytometry and fluorescence microscopy. Activation of small GTPases was evaluated by pull-down assays. DCs were incubated with nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) and supernatants were collected for measurement of IL-4, IL-6, IL-10, IL-12p70, TNF- α and TGF- β .

Results Receptor proteins were detectable by western blot, fluorescence activated cell sorting analysis and fluorescence microscopy. Signalling after neurotrophin stimulation occurred in a ligand-specific pattern. NGF led to decreased RhoA and increased Rac activation, while BDNF affected RhoA and Rac activity in a reciprocal fashion. Cells of allergics released a significantly increased amount of IL-6, while for healthy subjects a significantly higher amount of IL-10 was found.

Conclusion These data indicate that DCs are activated by the neurotrophins NGF and BDNF by different pathways in a receptor-dependant manner. These cells then may initiate inflammatory responses based on allergic sensitization releasing preferred cytokines inducing tolerance or a T-helper type 2 response.

Keywords allergy, asthma, BDNF, dendritic cells, neurotrophins, NGF, TrkA, TrkB

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Introduction

The neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) play an extended role in inflammatory and allergic diseases. In 1996, Bonini et al. [1] were able to demonstrate elevated plasma levels of NGF in patients suffering from allergic diseases with the highest levels in allergic asthma. We observed increased circulating levels of neurotrophins in patients with untreated allergic asthma, while therapy with inhaled steroids was able to normalize neurotrophin levels [2]. After segmental allergen provocation, an increase of

the neurotrophins NGF and BDNF in bronchoalveolar lavage fluid was shown in the late allergic reaction [3]. The neurotrophins NGF and BDNF belong to a well-described family of neurotrophic factors sharing high sequence homology. They play an essential role in the development, function and survival of neural cells [4]. The effects of neurotrophins are mediated by ligand-dependent activation of their specific high-affinity receptors TrkA, TrkB and TrkC, which are transmembrane receptors of the tyrosine kinase superfamily and their unspecific low-affinity receptor p75 [5]. These various downstream cascades are described to become activated after ligand-induced receptor dimerization and subsequent activation of their kinase activity [6]. Important sources of

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neurotrophins in allergic reactions include not only immune cells but also structural pulmonal cells [7, 8]. Mast cells are demonstrated to produce neurotrophins and release NGF [9]. Recently, we also identified eosinophils as a source of neurotrophins present in the late allergic response [10].

Additional cells like monocytes [11] and T and B cells also contribute to peripheral neurotrophin release [12, 13].

In animal models, neurotrophins were able to mimic bronchial hyperresponsiveness and airway inflammation as characteristic features in asthma [14, 15]. Furthermore, they were able to influence the neuronal phenotype of pulmonary innervation with a shift towards c-fibres [16].

Underlying cellular mechanisms have also been examined recently in humans. Eosinophils stimulated with NGF or BDNF secreted a significantly increased amount of IL-4 or EPX [17]. Eosinophils migrated in the lungs demonstrated prolonged survival in the presence of neurotrophins [18]. It was demonstrated that neurotrophins act as chemoattractants for mast cells and as proliferation factors for B cells [19, 20]. The amount of platelet BDNF correlated inversely with lung function parameters in asthmatics [21].

In recent years, the role of dendritic cells (DCs) in the regulation of inflammation and allergy has become evident. Their critical role in the induction of tolerance or in a shift towards a T-helper type (Th1) or 2 response has been investigated extensively. Numerous important functions of DCs, such as Ag presentation, migration, chemotaxis and endocytosis, spine outgrowth and adhesion, are dependent on the activation of the small GTPases Rho and Rac, as these are essential features in the biology of DCs. Furthermore, it has been demonstrated that DCs are crucial not only in the process of sensitization to allergens but also in the regulation of allergic inflammation [22].

The aim of the present study was to assess whether DCs express functional high-affinity neurotrophin receptors and whether ligand binding induces DC signalling and altered function.

Methods

Subjects

Blood was drawn from different allergic and non-allergic subjects. Allergy diagnosis was made on the basis of medical history and skin prick tests to common inhalative allergens. None of the subjects received any systemic or local treatment for at least 1 week before blood withdrawal. All subjects gave informed consent.

Generation and culture of monocyte-derived dendritic cells

Human monocytes were isolated by Ficoll-gradient and negative selection with paramagnetic microbeads

(Monocyte Isolation Kit II; Miltenyi Biotech, Bergisch Gladbach, Germany). The tagged cells were passed through a magnetic field (MACS, Miltenyi Biotech) and the negative fraction was collected. Cells were harvested and cultured in RPMI 1640 medium containing 10% fetal calf serum, 5% penicillin/streptomycin on six-well plates (5×10^6 cells/3 mL/well) supplemented with 100 ng/mL granulocyte macrophage-colony forming factor (GM-CSF) and 50 ng/mL IL-4 (both R&D Systems, Minneapolis, MN, USA) for 6 days. On days 2 and 4, two-thirds of the culture medium were replaced by fresh medium containing GM-CSF and IL-4 as described [23]. Monocyte-derived dendritic cells (MoDCs) were recovered from the plates after 6 days, and checked for shifting from CD14 to HLA-DR-positive cells by fluorescence activated cell sorting (FACS). Cells without CD14 expression were regarded as MoDCs and used in further assays. Experiments were performed in medium and medium supplemented with NGF (100 ng/mL) or BDNF (20 ng/mL). MoDCs were stimulated by lipopolysaccharide (LPS) (1 µg/mL, from *Escherichia coli* 0128:B12, Sigma, Deisenhofen, Germany), Poly I : C (100 µg/mL, Apotech, Epalingen, Switzerland) and soluble CD40 ligand trimer (1 µg/mL, Bender, Vienna, Austria). Cells were centrifuged and cell culture supernatants were collected.

Detection of neurotrophin receptors in human monocyte-derived dendritic cells

Fluorescence microscopy. MoDCs were recovered from the plates and stained for expression of neurotrophins. After blocking Fc receptors in phosphate-buffered saline (PBS) containing 10% HuS for 20 min at 4 °C, intracellular staining was performed using Cytofix/Cytoperm (BD Biosciences, San Diego, CA, USA). MoDCs were incubated with 10 µL neurotrophin-receptor rabbit anti-human antibody [Santa Cruz Biotechnology, Santa Cruz, CA, USA; TrkA (763): sc-118, TrkB (H-181): sc-8316] for 20 min on ice. After washing, primary antibody was stained with 5 µL fluorescein isothiocyanate (FITC)-labelled donkey anti-rabbit IgG (Jackson, Soham, UK) for 20 min on ice. After extensive washing, cells were analysed by microscopy. We used a Leica DMRBE fluorescence microscope (Bensheim, Germany), equipped with an Hg lamp, a Leica $\times 40$ water-immersion objective, filtre sets from Omega Optical (Seefeld, Germany) and a Hamamatsu digital camera C4742-95 (Herrsching, Germany). Images were taken using Openlab software (Improvision, Heidelberg, Germany).

Flow cytometry. Approximately 10^5 DCs were treated with Cytofix/Cytoperm (BD Biosciences). Cells were incubated for 30 min on ice in 50 µL AB serum containing either 10 µL polyclonal antibody to high-affinity neurotrophin receptors TrkA and TrkB (Santa Cruz Biotechnology) or isotype

control for 30 min. HLA-DR or costimulatory molecules CD80 and CD86 (TÜ36, L307.4, FUN-1, respectively, all BD Biosciences, Heidelberg, Germany) or isotype control were also stained for 30 min. Cells were washed, followed by incubation with a secondary anti-rabbit FITC-conjugated antibody for 30 min. Isolated cells were washed again with PBS containing 2% paraformaldehyde. Samples were analyzed using a FACSCalibur cytometer (BD Biosciences, Oxnard, CA, USA).

Protein electrophoresis and western immunoblotting

MoDCs were homogenized on ice with lysis buffer containing 1.5% SDS, 62.5 mM Tris-HCl buffer, pH 6.8, supplemented with 1% non-idet P-40 (NP-40), 5% mercaptoethanol, 2 mM phenylmethylsulfonylfluoride and 10 µg/mL of proteinase inhibitors (Sigma, St Louis, MO). The lysates were centrifuged at 10 000 g for 20 min and supernatants were collected as total lysate. The protein content was measured with a modified Lowry assay (BCA, Pierce, Rockford, IL, USA). Samples of 50 µg protein were loaded onto 10% polyacrylamide gel and electrophoresed in parallel with a molecular weight marker (Bio-Rad, Hercules, CA, USA). Lysates of eosinophils were used as controls. Proteins were blotted on pre-activated polyvinylidene difluoride membrane (Pharmacia, Upsala, Sweden). Western blotting was performed using 1:500 rabbit polyclonal 1° anti-human TrkA or TrkB antibody (Santa Cruz Biotechnology). Proteins were visualized by incubation with secondary cyanin 5.5 (Cy5.5) labelled anti-rabbit antibody. Proteins were visualized using the Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA).

RNA isolation and reverse transcription polymerase chain reaction amplification

Total RNA was extracted from 10^6 isolated DCs or cells stimulated with LPS 1 µg/mL from *E. coli* 0128:B12 (Sigma), CD40 ligand 1 µg/mL (Bender) and poly (I:C) 50 µg/mL (Axxora, Grunberg, Germany) using the RNeasy kit (Qiagen, Hilden, Germany). First-strand cDNA synthesis reaction was catalysed by AMV reverse transcriptase and oligo(dT) primer (Boehringer, Indianapolis, IN, USA). The generated complementary DNA was amplified in a total volume of 25 µL with 1.25 U of Taq DNA polymerase and 0.2 mmol/L dNTP mixture, 0.4 µmol/L of neurotrophin primers. Primers were as follows: TrkA upstream (5'-3') 5'TCT TCA CTG AGT TCC TGG AG3', downstream (5'-3') 5'AGA CCT CTG GGC CAC CTC TT3' with a product of 229 bp. TrkB upstream (5'-3') 5'CTT TTG GTA ATG CTG TTT CTG3', downstream (5'-3') 5'CGC GGC GAT CTG CTG GGC TAT3' with a product of 648 bp. PCR amplifications were performed with the following settings: 36 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Amplified products were electro-

phoresed on 1.5% agarose gel, stained with ethidium bromide and detected under UV light.

Detection of cytokines

To determine the release of mediators from DCs after stimulation with neurotrophins, cells were cultured for 24 h in the presence of either 100 ng/mL NGF, 20 ng/mL BDNF or medium. Samples were centrifuged and cell culture supernatants were collected. Mediators were detected in sandwich ELISAs according to the manufacturer's instructions for IL-4, IL-6, IL-10, IL-12p70, TNF-α and TGF-β (R&D Systems). The assays were performed on 96-well plates (Nunc, Wiesbaden, Germany). Streptavidin was conjugated to horseradish peroxidase. Wells were developed with tetramethylbenzidine and measured at 450 nm.

Rho and Rac GTPase activity assays

Lysates from untreated and stimulated DCs were subjected to GTPase pull-down assays. GTP-bound Rho was precipitated as previously described using immobilized GST-rhotekin-RBD as bait [24]. Rho and Rac activity assays were performed simultaneously by adding 1 µg/mL of purified biotinylated Cdc42/Rac interactive binding (CRIB) peptide to the lysis reaction. CRIB peptide-bound GTP-Rac was precipitated after addition of streptavidin-agarose (Sigma-Aldrich) as described [25]. Proteins were eluted with SDS sample buffer and analysed by SDS-PAGE.

Mixed leucocyte reaction

To determine the potential of neurotrophin-stimulated MoDCs to induce proliferation in allogeneic CD4⁺ T cells, a carboxyfluorescein succinimidyl (CFSE)-based assay was used. T cells were isolated from peripheral blood mononuclear cells by cell sorting (CD4⁺ T Cell Isolation Kit II, Miltenyi Biotech) according to the manufacturer's recommendation. CD4⁺ T cells were stained with CFSE (Molecular Probes, Eugene, OR, USA) at a final concentration of 50 nM for 10 min at RT. 10^5 stained T cells were co-cultured in 96-well plates with MoDCs at a ratio of 10:1. As a control, proliferation was induced in T cells by 10^4 anti-CD3/anti-CD28-coated Dynabeads (Dynal Biotech, Oslo, Norway) in each well. Proliferation of the CFSE-stained responder T cells was determined by flow cytometry using a FACSCalibur (BD Biosciences).

Statistical analysis

Results are presented as mean values ± SEM. Values were compared using the non-parametric test for independent values (Mann-Whitney *U*-Test). *P* values below 0.05 were regarded as significant.

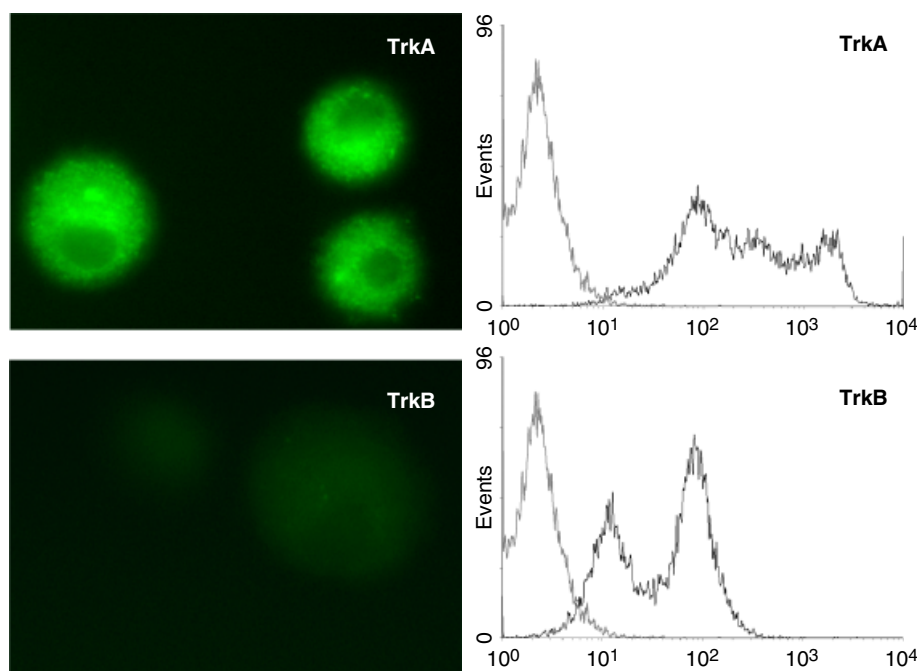


Fig. 1. Neurotrophin receptors are expressed in monocyte-derived dendritic cells. Left: fluorescence microscopy analysis of anti-neurotrophin receptor labelled dendritic cells generated from monocytes by cytokine culture. Fluorescence immersion-microscopy detected expression of TrkA and TrkB. Images show fluorescence values for a neurotrophin receptor that represents emission found in most of the cells analysed. Right: flow cytometric detection of TrkA and TrkB. Isotype controls (light grey), samples (dark grey).

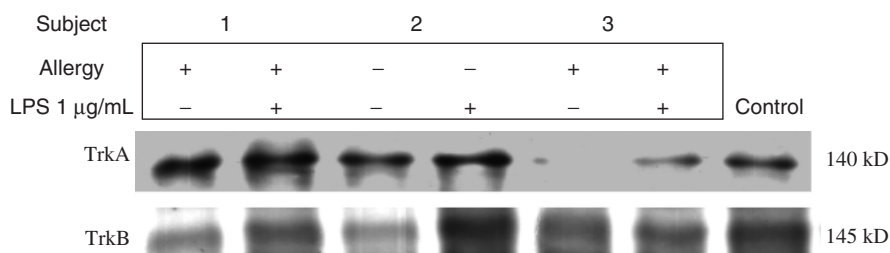


Fig. 2. Western blot analysis of TrkA and TrkB protein of three different subjects. Cells were cultured in medium or stimulated with lipopolysaccharide 1 µg/mL. Positive control was performed with lysates of eosinophils.

Results

Human monocyte-derived dendritic cells express the neurotrophin receptors TrkA and TrkB

We analysed MoDCs after a 6-day culture in GM-CSF and IL-4 for expression of neurotrophins receptors by fluorescence microscopy. We found a bright signal for TrkA. TrkB showed a weaker fluorescence value, but emission was found in all MoDCs. Bright spots indicating protein accumulation in the Golgi area could be found. Dim fluorescence was detected for TrkB (Fig. 1).

In order to prove the results of fluorescence microscopy, flow cytometry was performed. FACS analysis showed positive results for TrkA and TrkB with comparable fluorescence intensities as found in microscopic analysis (Fig. 1).

TrkA and TrkB proteins in allergics and non-allergics

The expression of the neurotrophin receptors TrkA and TrkB protein, from MoDCs or eosinophils, as controls were immunoblotted using specific antibodies. As shown in Fig. 2, specific bands were detectable for TrkA and TrkB. While no difference in disease state was detectable, LPS stimulation led to an increase of the band intensity of TrkB (Fig. 2).

Lipopolysaccharide and CD40 ligand selectively induce TrkB mRNA expression

The high-affinity neurotrophin receptors TrkA and TrkB were not expressed constitutively in MoDCs. Stimulation with LPS or the CD40 ligand led to a selective induction of TrkB expression. The Co-stimulatory CD40 ligand strongly up-regulated in synergy with LPS transcripts of TrkB. Poly (I:C), mimicking double-stranded RNA and triggering TLR3, could not induce Trk receptors. TrkA was not expressed upon application of any of the used stimuli (Fig. 3).

Neurotrophins enhance inflammatory interleukin-6 and anti-inflammatory interleukin-10 upon disease state

A significantly increased amount of IL-6 was detectable after stimulation with NGF ($P=0.0001$) and BDNF ($P=0.0003$) compared with unstimulated controls after 24 h. IL-10 was also significantly increased in NGF ($P=0.012$) and BDNF ($P=0.008$) stimulated cell culture supernatants. IL-4, TNF- α and TGF- β did not differ between the neurotrophin and unstimulated samples (Table 1). IL-12p70 was below the detection limit in the

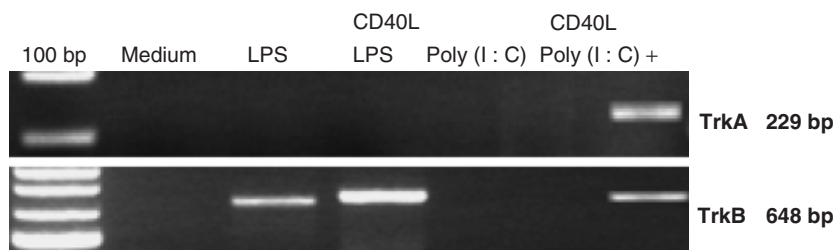


Fig. 3. RT-PCR amplification for TrkA and TrkB. Cells were stimulated with lipopolysaccharide, CD40 ligand or poly (I : C) for 6 h.

Table 1. Cell culture supernatants after 24 h of stimulation with NGF (100 ng/mL) or BDNF (20 ng/mL)

	Control			NGF			BDNF		
	All subjects N = 14	Allergics N = 9	Non-allergics N = 5	All subjects N = 14	Allergics N = 9	Non-allergics N = 5	All subjects N = 14	Allergics N = 9	Non-allergics N = 5
IL-6	217.7 ± 66.1	261 ± 88.6	140 ± 95.	490 ± 60.9**	620 ± 14.9***	231 ± 84	544 ± 55.5**	642 ± 15.9***	327 ± 107
IL-10	137.7 ± 38.6	198 ± 48.9	29 ± 18.4	377.9 ± 39.9**	296 ± 36.3	524.5 ± 38***	329 ± 31.5**	271 ± 33.5	435 ± 25***
IL-4	4247 ± 1357	4942 ± 1661	2857 ± 1580	5175 ± 250	5152 ± 279	3692 ± 691	4653 ± 339	4681 ± 377	2188 ± 187.2
TNF α	80 ± 16.4	93 ± 25.8	62 ± 17	102 ± 10.5	126 ± 5.3	69.7 ± 15.7	98 ± 10.5	123 ± 8.7	65.1 ± 16
TGF β	1727 ± 201.6	2042 ± 244.1	1225 ± 217	1754 ± 188.8	2462 ± 58.3	1371 ± 218.2	2042 ± 175.5	2143 ± 143.6	1133 ± 251

Mean values (pg/mL) ± SEM. ** $P < 0.01$ (compared with control); # $P < 0.05$; ## $P < 0.01$ (allergics vs. non-allergics). NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor.

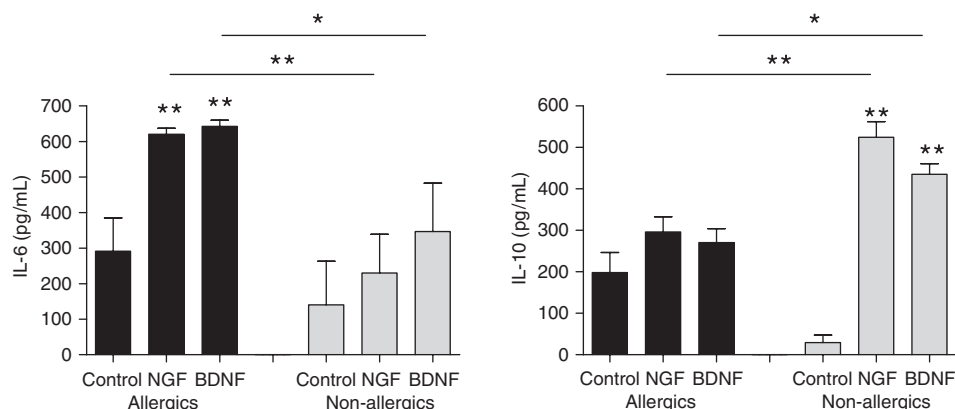


Fig. 4. Cytokine detection in cell culture supernatants after 24 h of stimulation with nerve growth factor 100 ng/mL or brain-derived neurotrophic factor 20 ng/mL ($n = 14$, nine allergics, five non-allergics). Allergics (unfilled bars), non-allergics (striped bars). Mean values ± SEM. * $P < 0.05$; ** $P < 0.01$.

majority of the samples (data not shown). When comparing allergic to healthy donors, a significant increase of IL-6 was detected after stimulation with NGF ($P = 0.002$) or BDNF ($P = 0.004$) in allergics. In contrast, the cells of healthy donors demonstrated a significant increase in IL-10 compared with those of allergics (Fig. 4).

Nerve growth factor and brain-derived neurotrophic factor activate Rho and Rac GTPase diversely

Cell stimulation with NGF or BDNF led to opposite effects towards RhoA and Rac activity. While NGF stimulation resulted in a decreased RhoA activity and an increased Rac activity, BDNF was able to activate RhoA while down-regulating Rac activity (Fig. 5).

Neurotrophins do not influence dendritic cell markers or T cell proliferation

After stimulation with neurotrophins, we analysed the expression of costimulatory molecules on DCs. With the exception of NT3, no significant up-regulation of CD80 and CD86 could be observed. NT3 slightly increased CD86 (Fig. 6a) but in allogeneic leucocyte reaction, even NT-stimulated cells were not able to induce massive T-cell proliferation (Fig. 6b).

Discussion

In this study we were able to demonstrate the expression of the high-affinity neurotrophin receptors TrkA and TrkB on dendritic cells and could demonstrate an NGF- and BDNF-

related downstream signalling in DCs with a ligand-dependent activation pattern. RhoA was down-regulated by NGF and up-regulated by BDNF. Conversely, Rac

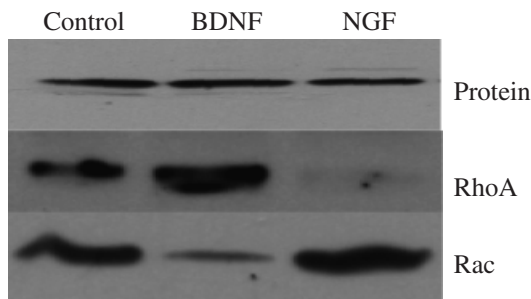


Fig. 5. Pull-down assay for the analysis of RhoA and Rac activation after cell stimulation with the neurotrophins nerve growth factor or brain-derived neurotrophic factor. Controls were performed without neurotrophin stimulation. Controls indicate similar activation without stimulation.

activity was up-regulated by NGF and down-regulated by BDNF. Stimulation with the neurotrophins NGF and BDNF resulted in an increased release of IL-6 and IL-10, while IL-4, TNF- α and TGF- β remained unaffected. In light of the allergy state of the donors, it is remarkable that cells of allergics release a significantly increased amount of IL-6, while cells of healthy subjects respond to stimulation with neurotrophins by an increased release of IL-10. The general amount of cytokines released from the cells of allergic donors was increased compared with those of healthy donors. This confirms the results of former reports [26].

The neurotrophins NGF and BDNF are produced by various residential and immune cells when in contact with inflammatory stimuli and might be additional factors in the regulation of T-cell activation by DCs. The process of T-cell activation in allergic diseases is characterized by a Th2-type reaction. Neurotrophins are factors that are increased in allergic diseases and asthma and might tip

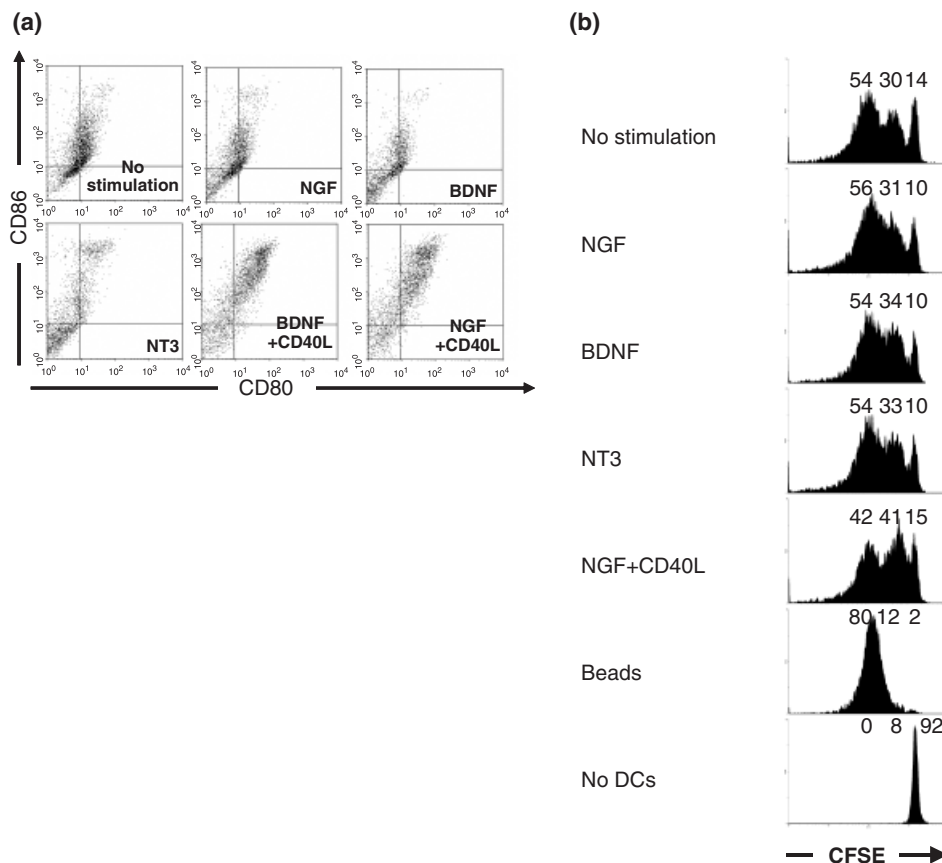


Fig. 6. (a) Expression of costimulatory molecules on dendritic cells (DCs) stimulated with neurotrophins. Monocyte-derived dendritic cells (MoDCs) were stimulated by nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) (500 ng/mL), NT3 (100 ng/mL), soluble CD40L (1 μ g/mL) for 2 days. DR-positive cells were measured by fluorescence activated cell sorting, quadrants indicate isotype control. (b) Induction of CD4⁺ T cell proliferation after stimulation of DCs with neurotrophins. MoDCs were stimulated by NGF, BDNF (500 ng/mL), NT3 (100 ng/mL), soluble CD40L (1 μ g/mL) or Dynalbeads. Allogeneic carboxyfluorescein succinimidyl-labelled T cells were measured by fluorescence-activated cell sorting after 5 days of co-culture with DCs.

the balance towards a Th2-dominant T-cell activation. IL-10 secreting DCs induce the formation of regulatory T cells [27].

Hallmarking the participation of neurotrophins in allergic diseases, it seems to be of importance that the amount of released inflammatory IL-6 was increased in allergics and the opposite is true for IL-10 in non-allergics after neurotrophin stimulation. This fact indicates a selective induction of pro- and anti-inflammatory cytokine release dependent on the disease state. IL-6 production by DCs leads to a strong induction of c-Maf as inducer of IL-4 production by T cells [28, 29]. A down-regulation of Th1 inducing IL-12 by IL-6 in pulmonary DCs was reported. Furthermore, in cocultures of DCs with T cells supplementing IL-6 was able to induce Th2 polarization [30].

The selective induction of cytokine production by NGF and BDNF is part of the growing role of neurotrophins in the regulation of inflammatory processes.

Recently, the important role of small GTPases has been demonstrated in regulating DC adhesion, Ag presentation, migration, chemotaxis and endocytosis [31]. They regulate the highly characteristic dendrite morphology, spine outgrowth and adhesion [32]. Here we report for the first time about the selective ability of BDNF and NFG to induce respectively to reduce RhoA and Rac activity in non-neural cells. This effect is most probably related to the activation of specific neurotrophin receptors because p75 binds all neurotrophins unspecifically.

Diverse effects on characteristic DC functions are likely. RhoA inhibition led to a decreased DC : T cell interaction as a result of reduced spine formation [33]. Opposite effects mediated by neurotrophins were described for Schwann cells by the regulation of different pathways with the consequence of inhibiting migration [34]. The influx of DCs was identified as a crucial factor in the initiation of allergic inflammation after allergen challenge [35]. The inhibition of RhoA-dependent migration therefore contributes to the diverse and probably also anti-inflammatory effects of BDNF contrary to those of NGF. An additionally important factor might be the recently observed differences between the cellular amount of NGF and BDNF in different cells of allergics and non-allergics. We demonstrated a decreased amount of cellular BDNF in eosinophils and an increased cellular NGF in monocytes of allergics compared with non-allergics as well as a decreased cellular content of BDNF in allergics [11, 12]. Therefore the amount of released neurotrophins is different compared with allergics and non-allergics.

Neurotrophins provide mediators in the regulation of inflammation by regulating key mechanisms of trafficking and T-cell activation by DCs. Regulating the neurotrophin-induced effects on DCs might provide a tool for the modulation of allergic inflammation in asthma.

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