

ORIGINAL PAPER

Selective induction of nerve growth factor and brain-derived neurotrophic factor by LPS and allergen in dendritic cells

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Summary

Background Neurotrophins are produced by various cells upon different stimuli and participate in the initiation and regulation of inflammation in various diseases including allergy and asthma, but little is known about the production and control of neurotrophins by dendritic cells (DCs). The aim of this study was to assess whether DCs produce the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), and whether inflammatory stimuli or allergens are able to induce the production of neurotrophic factors.

Methods Monocyte-derived dendritic cells (MoDCs) were generated from different donors. The neurotrophins NGF and BDNF were demonstrated by RT-PCR, Western blotting, flow cytometry analysis and fluorescence microscopy. MoDCs were cultured and stimulated with lipopolysaccharide (LPS) or allergen for 24 h. The supernatants and cells were collected. Measurement for NGF and BDNF was performed by ELISA.

Results DCs express mRNA for the neurotrophins NGF and BDNF. Proteins were detectable by Western blot, FACS analysis and fluorescence microscopy. LPS led to an up-regulation of BDNF, while NGF was unaffected. Cell lysates demonstrated an increased amount of BDNF after stimulation with LPS or allergen, while NGF was not affected significantly.

Conclusions DCs are a source of neurotrophins. LPS selectively regulates the production of BDNF. Allergen stimulation leads to an LPS-independent regulation. This contributes to a complex involvement of neurotrophins in allergic diseases.

Keywords allergy, asthma, BDNF, dendritic cells, inflammation, neurotrophins, NGF

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Introduction

The neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) belong to a well-described family of neurotrophic factors sharing high homology. Neurotrophins play an essential role in the development, function and survival of neural cells [1]. The involvement of the neurotrophins NGF and BDNF in inflammatory and allergic diseases has become evident in recent years [2, 3]. Elevated plasma levels of NGF were found in patients suffering from allergic diseases, and the highest levels were found in those with allergic asthma [4]. We were able to demonstrate increased plasma levels of neurotrophins in patients with untreated allergic asthma, while therapy with inhaled steroids was able to normalize circulating neurotrophin levels [5]. The effect

of steroids on the production of neurotrophins was proved by *in vitro* experiments for eosinophils and peripheral blood mononuclear cells (PBMC) [6, 7]. Segmental allergen provocation led to an increase of the neurotrophins NGF and BDNF in broncho-alveolar lavage fluid in the late allergic reaction [8]. Important sources of neurotrophins in allergic reactions include various immune cells including key cells of allergic inflammation like mast cells and eosinophils [9–12] but also structural pulmonary cells [13].

Neurotrophins are strong inducers of neuropeptide synthesis [14, 15], which contribute to neural inflammation. Furthermore, as inducers of nerve growth and differentiation, the observed change of pulmonary innervation with a shift towards c-fibres might be explained by locally released neurotrophins [16, 17].

Recently, their role in childhood wheezing during episodes of respiratory syncytial virus (RSV) infections has dependently led to remodelling changes of the airways [18]. Further factors leading to neurotrophin release of immune cells or structural cells are rarely described and differ depending on the cell type. They include platelet-activating factor for eosinophils, IgE cross-bridging for mast cells, and lipopolysaccharide (LPS) leading to neurotrophin release in monocytes but also stress induced by parachute jumping was able to increase circulating NGF or BDNF [11, 19].

In animal models, neurotrophins are able to induce bronchial hyper-responsiveness and airway inflammation [20, 21]. Cellular effects leading to these outcomes have also been examined recently in humans. Eosinophils stimulated with neurotrophins secrete a significantly increased amount of IL-4 and eosinophil protein X (EPX) [22]. Furthermore, eosinophils that migrated in lung tissue demonstrated a prolonged survival, dependent on the presence of neurotrophins [23]. Neurotrophins are proliferation and differentiation factors for mast cells and inducers of immune globulin synthesis by B cells [24]. T cells demonstrated a T-helper type 1 Th1/Th2-polarized expression of neurotrophin receptors [25].

The role of DCs in the regulation of inflammation and allergy has become evident in recent years as techniques were developed to purify and generate these cells *in vitro*. DCs provide a high capacity for antigen uptake and presentation. After migration to a draining lymph node and contact with a T cell with a cognate T cell receptor (TCR), they induce T cell differentiation and proliferation. This is the key mechanism resulting in inflammatory responses characterized by Th1 or Th2 cells or tolerance characterized by T_{Reg} cells [26]. Antigen presentation, environmental factors for DCs and probably genetic factors define costimuli resulting in different T cell responses [27].

The aim in the present study was to assess whether DCs express neurotrophins and whether they are regulated by LPS or allergens.

Methods

Subjects

Blood was drawn from different allergic or non-allergic subjects. An allergy diagnosis was made on the basis of the medical history and a skin prick test to common inhalation allergens, including birch and grass pollen, house dust mites and animals. All allergic subjects suffered from allergic rhinitis and some also had mild asthma. No subject received any systemic or local treatment for at least 1 week before blood withdrawal. All subjects gave informed consent.

Generation of monocyte-derived dendritic cells (MoDCs)

MoDCs were generated after negative immunomagnetic monocyte isolation from peripheral blood. For this purpose, 50 mL of heparinized venous blood was collected. Cells were washed and centrifuged on Ficoll-Paque (density 1.077 g/mL) (Biochrome, Berlin, Germany) for 45 min at 900 g. The washed pellet of PBMC was incubated with paramagnetic microbeads (Monocyte Isolation Kit II; Miltenyi Biotech, Germany) for negative selection. The tagged cells were then passed through a magnetic field (MACS, Miltenyi Biotech, Germany), and the negative fraction was collected. Monocytes were concentrated at a high purity of >95% assessed by flow cytometry using anti-CD14 IgG- and fluorescein isothiocyanate (FITC)-conjugated antibodies (Jackson Immuno Research, Suffolk, UK). Isolated monocytes demonstrated a viability of more than 95% as evaluated with propidium iodide and trypan blue staining (Sigma Chemicals, St Louis, MO, USA). Monocytes were cultured in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 for 6 days to generate MoDCs. Experiments were performed after 6 days of culture in the presence of GM-CSF and IL-4. MoDCs were recovered from the plates, and a shift from CD14- to human leukocyte antigen-DR (HLA-DR)-positive cells was controlled by flow cytometry analysis.

Stimulation of MoDCs

MoDCs, 10^6 /mL were cultured in RPMI 1640 medium containing 10% fetal calf serum, and 2% penicillin/streptomycin on six-well plates. Experiments were performed in medium and medium supplemented with LPS (1 µg/mL) or birch pollen extract (1 µL/mL) (Allergopharma, Rheinbeck, Germany) supplemented with polymyxin-B (10 µg/mL) (Calbiochem, Darmstadt, Germany) to exclude LPS-dependent effects of allergen extract. For stimulation with the CD40 ligand, MoDCs were cocultured with hCD40L-expressing murine L cells (CD40L⁺ cells). Cells were centrifuged and the cell culture supernatants were collected.

Intracellular staining and immunofluorescence

MoDCs were recovered from the plates after 6 days, checked for a shift from CD14- to HLA-DR-positive cells by fluorescence activated cell sorting (FACS) and stained for expression of neurotrophins. After blocking Fc receptors in phosphate-buffered saline 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 rabbit anti-human antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min on ice. After washing, the

primary antibody was stained with 5 µL FITC-labelled donkey anti-rabbit IgG (Jackson, Soham, UK) for 20 min on ice. After extensive washing, cells were analysed by flow cytometry (FACSCalibur, BD Biosciences) and microscopy. We used a Leica DMRBE fluorescence microscope (Bensheim, Germany), equipped with a mercury lamp, a Leica × 40 water-immersion objective, filter sets from Omega Optical (Seefeld, Germany) and a Hamamatsu digital camera C4742-95 (Hersching, Germany). Images were taken using Openlab software (Improvision, Heidelberg, Germany).

RNA isolation and reverse transcription PCR amplification

Total RNA was extracted from MoDCs using a commercial kit (RNeasy; Qiagen, Hilden, Germany). First-strand cDNA synthesis reaction was catalysed by Avian myeloblastosis virus (AMV) reverse transcriptase and oligo (dT) primer (Boehringer, Indianapolis, IN, USA). The cDNA generated was amplified in a total volume of 25 µL with 1.25 U of Taq DNA polymerase and 0.2 mmol/L dNTP mixture, and 0.4 µmol/L of neurotrophin primers. The primers were NGF Upstream (5′–3′) 5′-TAA AAA GCG GCG ACT CCG TT-3′, Downstream (5′–3′) 5′-ATT CGC CCC TGT GGA AGA TG-3′ with a product of 167 bp; BDNF Upstream (5′–3′) 5′-AGC CTC CTC TGC TCT TTC TGC TGG A-3′, Downstream (5′–3′) 5′-CTT TTG TCT ATG CCC CTG CAG CCT T-3′ with a product of 298 bp. The PCR amplifications were performed with the following settings: 36 cycles at 94 °C for 1 min, 60 °C for 1 min and 74 °C for 1 min. Amplified products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and detected under UV light.

Western immunoblotting

Approximately 10⁶ MoDCs were homogenized on ice with 1.5% SDS, 62.5 mM Tris-HCl buffer, pH 6.8, supplemented with 1% nonidet P-40 (NP-40), 5% mercaptoethanol, 2 mM phenylmethylsulfonylfluoride and 10 µg/mL of proteinase inhibitors (Sigma Chemicals). Lysates were centrifuged at 15 000 g. for 20 min and the supernatants were collected as total lysates. Protein content was measured at 535 nm (BCA, Pierce, Rockford, IL, USA). Fifty micrograms of protein was loaded on a 10% polyacrylamide gel and electrophoresed with a molecular weight marker (Bio Rad, Hercules, CA, USA). Recombinant NGF or BDNF was used as control (R&D Systems, Minneapolis, MN, USA). Proteins were blotted on an activated polyvinylidene difluoride membrane (Pharmacia, Uppsala, Sweden). Detection of NGF and BDNF proteins was performed using 1 : 500 rabbit polyclonal anti-human NGF or BDNF antibody (Santa Cruz Biotechnology). Proteins were detected by incubation with a secondary cyanin 5.5 (Cy5.5)-labelled anti-rabbit antibody (Jackson). Proteins were

visualized using the Odyssey infrared imaging system (LI-COR, Lincoln, NE, USA).

Determination of NGF and BDNF in cell culture supernatants and lysates

In order to determine neurotrophins in the lysates and supernatants from MoDCs, cells were cultured for 24 h and upto 48 h in the presence of LPS (1, 10 µg/mL) or birch allergen extract (1 µL/mL) in combination with polymyxin B (10 µg/mL) to avoid LPS-dependent effects. Samples were centrifuged and the cell culture supernatants were collected. Cells were lysated by ultrasound disruption. Neurotrophins were detected in sandwich ELISAs according to the manufacturer's instructions (R&D Systems) on 96-well plates (Nunc, Wiesbaden, Germany) in duplicate. Wells were developed with tetramethylbenzidine and measured at 450 nm. The neurotrophin content was quantified against a calibrated standard curve. The detection limit was <4 pg/mL for NGF and BDNF.

Statistical analysis

Results are presented as mean values + SEM. Values were compared by using the non-parametric test for independent values (Mann-Whitney *U*-test). *P* values below 0.05 were considered to be significant.

Results

Detection of neurotrophins in MoDCs

We analysed MoDCs for intracellular expression of the neurotrophins NGF, BDNF and neurotrophin-3 (NT-3) by fluorescence microscopy and flow cytometry. In the microscopic analysis, we found bright signals for NT-3, while NGF and BDNF showed weaker fluorescence values, but emission was found in all analysed cells. Bright spots indicating protein accumulation could be found in the Golgi area. FACS analysis confirmed this by positive fluorescence signals for the neurotrophins detected (Fig. 1).

Transcripts of NGF and BDNF in PCR analysis of MoDCs

Each cDNA sample from DCs or control cells (keratinocytes) demonstrated actin amplification products of the expected size and comparable intensity. There was no evidence of genomic amplification product, indicating the absence of contaminating genomic DNA. Specific bands demonstrating the presence of RNA encoding for neurotrophins were detectable. Stimulation with LPS or the CD40 ligand led to increased band intensity for BDNF expression while NGF was unaffected (Fig. 2).

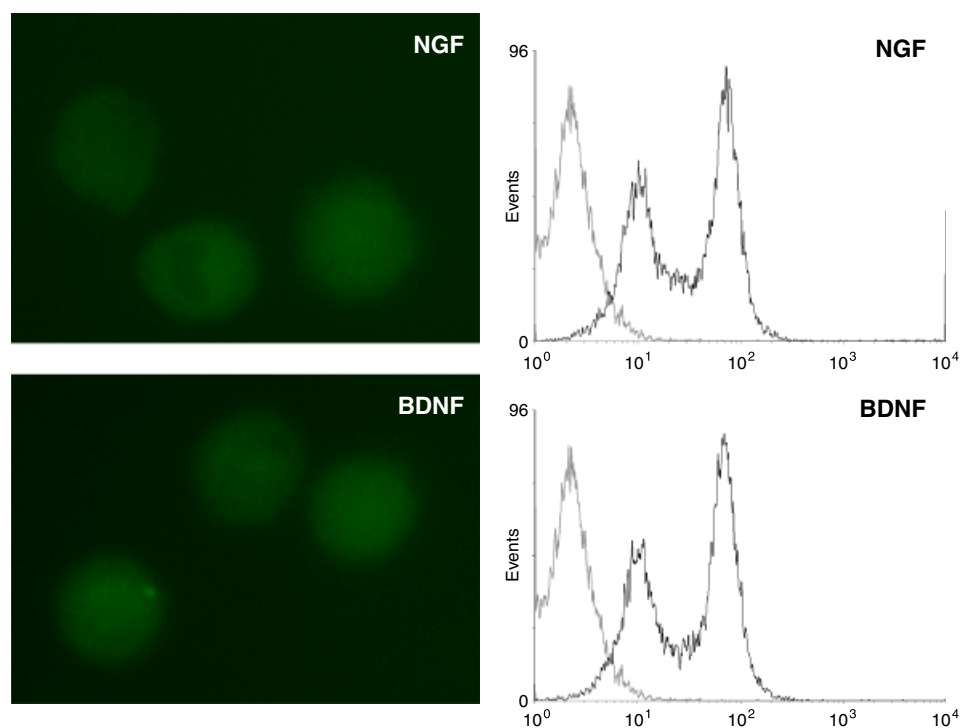


Fig. 1. Analysis of fluorescein isothiocyanate (FITC)-labelled neurotrophins. Left: fluorescence microscopy. Fluorescence immersion-microscopy detected the expression of nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neutrophin-3 (NT-3). Images show fluorescence values for a neurotrophins that represent emission found in most of the cells analysed. Right: fluorescence activated cell sorting (FACS) analysis of intracellular NGF, BDNF and NT-3. Isotype controls are light grey, and samples are dark grey.

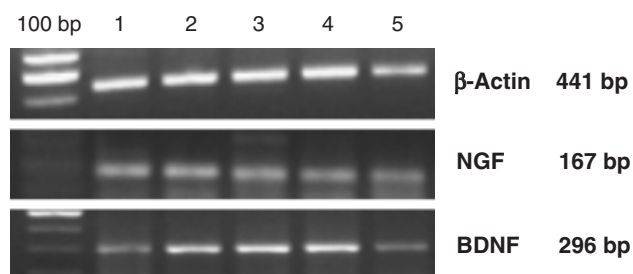


Fig. 2. RT-PCR for neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF). Lane 1: 100 bp ladder, lane 2: unstimulated control. Lane 3: stimulated with CD40 ligand. Lane 4: stimulated with lipopolysaccharide (LPS) and CD40 ligand. Lane 5: positive control. Positive control was performed with cDNA from keratinocytes.

Regulation of NGF and BDNF protein in allergic and non-allergic individuals

Western blot analysis of isolated protein extracts from untreated and stimulated MoDCs demonstrated the presence of NGF and BDNF proteins in MoDCs. As shown in Fig. 3, specific bands were detectable for NGF and BDNF. Band intensities varied individually, also between allergic and non-allergic subjects, but were found in all preparations. LPS stimulation led to increased band intensities in BDNF while NGF was unchanged (Fig. 3).

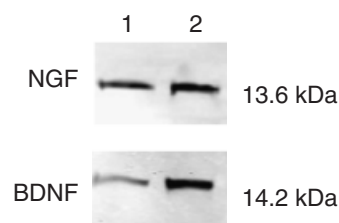


Fig. 3. Western blot analysis of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) control (lane 1) and after stimulation with lipopolysaccharide (lane 2).

Determination of NGF and BDNF in cell culture supernatants and lysates

The lysates of MoDCs after 24 h of culture demonstrated a significant ($P < 0.05$) increase of the cellular content of BDNF in response to LPS stimulation. Allergen was also able to induce the production of BDNF significantly ($P < 0.05$) (Fig. 4). Stimulation of allergen-treated cells by LPS could be excluded by the use of polymyxin B in all allergen experiments. The production of NGF was not affected by LPS or by allergen stimulation. LPS and allergen led to an increased release of BDNF. Determining the allergy state of cell donors a trend towards a higher neurotrophin release was observed in allergic subjects.

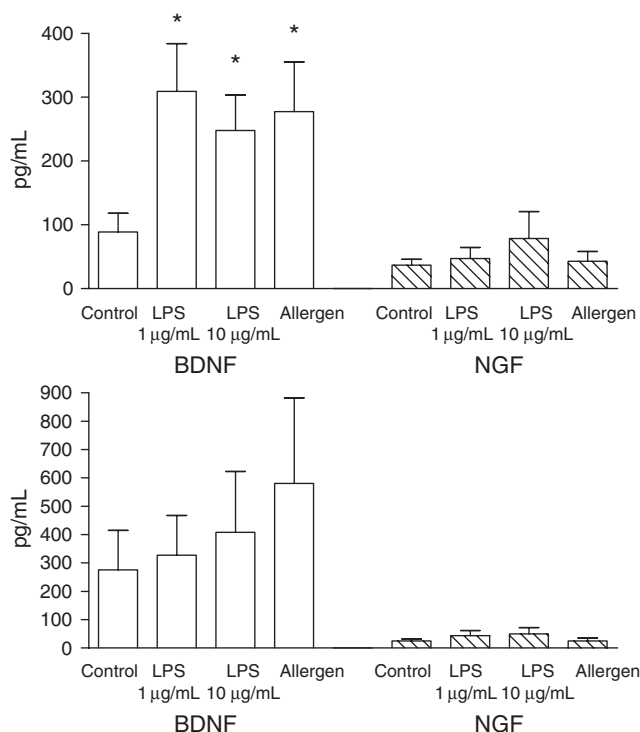


Fig. 4. Mediator measurements in cell lysates (up) and cell culture supernatants (down) for the neurotrophins nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) after 24 h of stimulation with lipopolysaccharide (LPS) or allergen ($n = 15$). Mean values $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

Discussion

In this study, we were able to demonstrate the production of NGF, BDNF and NT-3 by DCs. Stimulation with LPS as an inflammatory stimulus led to a selective induction of BDNF production. A similar effect could also be achieved by incubation with allergen. The expression of NGF was not affected significantly by LPS or birch pollen extract. The stimuli used in this experimental systems up-regulated neurotrophins but failed to demonstrate a significant release of NGF and BDNF by DCs. There is possibly a need for additional factors to achieve a significant release of neurotrophins.

DCs are immune cells with the highest capacity for antigen presentation in human tissues, and their immense regulatory potential for inflammatory responses has become evident in the recent years. They play an essential role in the induction of tolerance or inflammation. DCs are the main players in the polarization of T cells towards a Th1 or Th2 response, although the mechanisms are not understood in detail [28]. Neurotrophins might provide an additional factor contributing to T cell polarization in inflammatory diseases and asthma.

LPS is a strong inducer of DC cell maturation with a shift of DC cell physiology from an antigen-uptaking and -processing cell towards a T-cell activating cell after

migrating to the draining lymph node [29]. The induction of BDNF therefore indicates the maturation of DCs and represents a further indicator. There is also evidence that many allergens can induce maturation of DCs directly in the absence of microbial stimuli and might therefore be independent of toll-like receptor (TLR) and nucleotide binding oligomerization domain (NOD) signalling. Many allergens, such as Der p 1, directly induce DC maturation through their enzymatic activity [30]. The selective induction of BDNF in contrast to NGF points to a divergent role of neurotrophins in the maturation of DCs and the initiation of inflammatory processes. Platelets after sheer stress were able to induce mature DCs [31]. Platelets are a main source of BDNF *in vivo* as they are able to store high amounts of this neurotrophin [7]. Therefore, BDNF might also play a role in the maturation and differentiation of DCs with different potential of activating inflammatory T cells or regulatory T cells. Recently, the induction of different effects on cytokine production was described for LPS and soluble extracts of pollen [32]. The selective induction of neurotrophin production by these factors provides a further hint of the role of neurotrophins as an essential mediator in inflammatory processes.

The location of DCs in the airways is associated with nerves. The production of neurotrophins by DCs towards different stimuli present in various bacterial infections or allergen exposure has the potential to modulate neuronal growth and differentiation, accompanied by a cascade of neurogenic changes initiated by sprouting of nerves and an increase of c-fibres. The lung mucosa is innervated by sensory, sympathetic and parasympathetic nerve fibres that lead to a co-transmission of transmitters and of neuropeptides (NPY, VIP, CGRP and SP) after stimulation [33]. Neurotrophins are known physiological inducers of neuropeptide synthesis. The change of sensory airway innervation has recently been demonstrated to participate in airway inflammatory changes [15, 16]. Neurotrophins have the potential to change the neural phenotype and increase the amount of inflammatory transmitters leading to neuronal inflammation, with known consequences contributing to airway hyper-responsiveness in asthma [34].

In conclusion, the production of neurotrophins by DCs and their selective regulation by inflammatory stimuli or allergens provides an additional role in the initiation of auto- or paracrine BDNF-dependent pathways. Focusing on the pivotal role of BDNF in allergic diseases, elevated levels of BDNF in DCs might contribute to a Th2 response to allergens in allergic subjects, accompanied by the initiation of neurogenic inflammation.

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