

Activation of the specific neurotrophin receptors TrkA, TrkB and TrkC influences the function of eosinophils

O. Noga*, C. Englmann*, G. Hanf*, A. Grützkau†, S. Guhl† and G. Kunkel*

*Allergy and Asthma Clinic, and †Department of Dermatology, Charité, Campus Virchow, Humboldt University Berlin, Germany

Summary

Background Recent studies have shown that nerve growth factor (NGF) can act on several immune cells as well as residential cells. But little is known about their role in modulating eosinophil function via activation of high-affinity receptors.

Objectives The aim of this study was to assess whether eosinophils express functional receptors and if their function is influenced by NGF, brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3).

Methods Eosinophils were purified by negative immunoselection (purity > 96%). High-affinity neurotrophin receptors were demonstrated by reverse transcription polymerase chain reaction, western blotting and flow-cytometry analysis. Functionality of receptors was demonstrated by receptor phosphorylation after ligand binding. Eosinophils were incubated with NGF, BDNF and NT-3, and cells and supernatants were collected for measurement of the mediators IL-4, IL-5, IL-8, transforming growth factor (TGF)- β_1 , eosinophil cationic protein (ECP), eosinophil protein X (EPX) as well as eosinophil viability.

Results Eosinophils expressed mRNA for neurotrophin receptors. Proteins were detectable by western blot and fluorescent-activated cell sorter analysis. The receptors were phosphorylated after stimulation with neurotrophins. After NGF stimulation, a significant increase in IL-4 was detectable. BDNF and NT-3 stimulation led to a significant increase in EPX. Eosinophil viability was not influenced.

Conclusions Eosinophils express the functionally active receptors TrkA, TrkB and TrkC. Receptor activation stimulates eosinophils. This might be an additional pathway regulating inflammatory responses in allergic reactions.

Keywords allergy, eosinophils, neurotrophins, neurotrophin receptors, NGF

Submitted 22 November 2001; revised 14 January 2002; accepted 27 March 2002

Introduction

The neurotrophins nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) belong to a chemically well-described family of neurotrophic factors with high homology. Neurotrophins are expressed in the central and peripheral nervous system and they play an important role in the development, function and survival of neural cells [1] as well as the production of neuropeptides [2]. All actions of these neurotrophins are modulated via their specific high-affinity receptors TrkA, TrkB, TrkC and their low-affinity unspecific receptor p75 [3]. These are all membrane-bound single-strain receptors of the tyrosine kinase superfamily. After ligand-induced dimerization and initial activation of kinase activity with phosphorylation of tyrosines, various downstream cascades are activated. The Ras/raf/MEK/ERK and the phosphatidylinositol-3-kinase (PI3K)-AKT1

pathway which in neural cells regulate growth and survival; another downstream pathway via phospholipase C gamma (PLC γ) leads to calcium influx [4, 5].

Some recent studies have described effects of neurotrophins, especially NGF, on systems other than the nervous system. Neurotrophin receptors have been found on various immunologically active cells, e.g. lymphocytes [6] and mast cells. Some of these cells are known, or there is evidence that they produce, store and release NGF [7] but their proliferation and differentiation is also influenced by neurotrophins [8]. T and B cell proliferation and activation are influenced by NGF, leading to the release of cytokines and the production of immunoglobulins [9]. Basophiles can also be stimulated to release their mediators, and their survival is prolonged [10]. Besides neural cells and astrocytes, T and B cells, mast cells [7], fibroblasts and basophiles are known to produce NGF [11, 12].

In view of the influence on immunologically active cells, the role of neurotrophins in various immune diseases is discussed [13, 14]. Elevated plasma levels have been found in patients suffering from asthma, particularly in allergic asthma [15]. We were able to demonstrate increased plasma levels of neurotrophins in patients with untreated allergic asthma, while therapy

Correspondence: Gert Kunkel, Allergy and Asthma Clinic, Charité, Virchow Klinikum, Humboldt University, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail: oliver.noga@charite.de

with inhaled steroids was able to regulate circulating neurotrophin levels [16]. After segmental allergen provocation, an increase of the neurotrophins NGF and BDNF in the bronchoalveolar lavage fluid (BAL) has been described in the late allergic reaction (LAR) [17]. The tissue in LAR is characterized by the influx of eosinophils and activated T cells. Eosinophils are one of the main effector cells of allergic inflammation. They are found in BAL and characterize the cellular infiltrate in asthma. In animal experiments, NGF was able to induce bronchial hyper-responsiveness (BHR) [18, 19]. The production of NGF and the presence of TrkA receptor at the mRNA level in eosinophils has been described [20].

Our aim in the presented study was to clarify whether eosinophils express functional high-affinity receptors for neurotrophins and if their function can be influenced by neurotrophins.

Methods

Subjects

Blood was drawn from different volunteers. All subjects had mild blood eosinophilia (range 4–9%) and demonstrated signs and history of allergic disease: all suffered from allergic rhinitis and 50% also suffered from allergic asthma. No subject received any systemic or local treatment for at least 1 week before blood withdrawal. All subjects gave informed consent.

Isolation of eosinophils

Eosinophils were purified from the peripheral blood as previously described [21, 22]. Briefly, venous blood (120 mL) was collected and left to sediment in 6% dextran (Sigma Chemicals, St Louis, MO, USA). Leucocytes were centrifuged on Ficoll-Paque (density 1.077; Biochrome, Berlin, Germany) for 25 min at 700 *g* at 4 °C. Neutrophils in the granulocyte-enriched pellet were tagged with micromagnetic beads to anti-CD16 antibodies (Miltenyi, Biotech GmbH, Bergisch Gladbach, Germany). The tagged cells were then eliminated by passing them through a magnetic field (MACS). Eosinophils were collected at a purity of > 96% assessed by flow cytometry using anti-CD16 phycoerythrin (PE)-conjugated and anti-CD49d fluorescein isothiocyanate (FITC)-conjugated antibodies (Coulter Immunotech, Brea, CA, USA) and cytopins at a viability of more than 95% as assessed by flowcytometry with propidium iodide and trypan blue staining (Sigma).

Culture of isolated eosinophils

Isolated eosinophils were cultured for up to 72 h in RPMI-1640 medium containing 10% fetal calf serum (FCS); 5% penicillin/streptomycin on 6-well plates. Experiments were performed in medium and medium supplemented with the various neurotrophins. Cells were centrifuged and cell culture supernatants were collected.

Determination of cytokines in culture supernatant

To determine the release of mediators from eosinophils after stimulation with neurotrophins, cells were cultured for 24 h in the presence of either 100 ng/mL NGF, 20 ng/mL BDNF and NT-3 or medium. Samples were centrifuged and cell culture supernatants were collected. Mediators were detected in sandwich enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions for IL-4, IL-5,

IL-8, transforming growth factor (TGF)- β_1 (R & D Systems, Minneapolis, MN, USA), eosinophil cationic protein (ECP) and eosinophil protein X (EPX) (Pharmacia, Uppsala, Sweden). All assays were performed on 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish peroxidase. Wells were developed with tetramethylbenzidine and measured at 450 nm. Mediator content was quantified against a standard curve calibrated with known amounts of protein.

Eosinophil viability

Stimulated cells were collected by centrifugation. Approximately 10^5 cells were incubated with propidium iodide for 15 min, and then washed with phosphate-buffered saline (PBS). Fluorescence was determined by fluorescent-activated cell sorter (FACS) analysis. For control experiments, cells were also stained with trypan blue and viability was determined by light microscopy.

RNA isolation and RT-PCR amplification

Total RNA was extracted from approximately 10^6 freshly isolated eosinophils or from HMC-1 cells using the commercial RNeasy kit (Qiagen, Valencia, CA, USA). First-strand cDNA synthesis reaction was catalysed by AMV reverse transcriptase (RT) and oligo (dT) primer according to the manufacturer's instructions (Boehringer, Indianapolis, IN, USA). The generated complementary DNA was amplified in a total volume of 25 μ L with 1.25 U of Taq DNA polymerase and 0.2 mmol/L dNTP mixture, 0.4 μ mol/L of neurotrophin primers. Primers were: 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; TrkC upstream (5'-3') 5'CCA AAT ATA TTG CGT TTA CCC CC3', downstream (5'-3') 5'CAC ACC GTG GGG GAT AGT AGA CA3' with a product of 546 bp. Neurotrophin mRNA has been shown to be present in HMC-1 cells [23]. CDNA of this mast cell line was consequently used as a positive control. The thermocycler (MWG Biotech, Ebersberg, Germany) was used for the polymerase chain reaction (PCR) amplifications 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 photographed under UV light.

Protein electrophoresis and western immunoblotting

Briefly, 10^6 human peripheral eosinophils were homogenized on ice with 1.5% sodium dodecyl sulphate (SDS), 62.5 mM Tris-HCl buffer, pH 6.8, supplemented with 1% Nonidet P-40 (NP-40), 5% mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 10 μ g/mL of proteinase inhibitors (Sigma). Lysates were centrifuged at 20 000 *g* for 20 min and supernatants were collected as total lysate. Protein content was measured at 535 nm (BCA, Pierce, Rockford, IL, USA). Aliquots were stored at – 80 °C until analysis. SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot were performed as described previously [24–26]. Samples of 50 μ g protein were loaded onto a 10–20% polyacrylamide gel and electrophoresed in parallel with a molecular weight marker

(Bio-Rad, Hercules, CA, USA). Recombinant NGF, BDNF and NT-3 were used as standards. Proteins were blotted on supported nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA). Western immunoblotting was performed using 1:500 rabbit polyclonal 1° anti-human TrkA, TrkB or TrkC antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and 1:10000 horseradish peroxidase-linked donkey anti-rabbit 2° antibody (Dianova, Hamburg, Germany). The blot was analysed using a chemiluminescence system (Amersham Life Sciences, Uppsala, Sweden). For absorption control experiments, the membrane was stripped of bound antibody, and 1° antibody was incubated with blocking peptide overnight before reprobing.

Detection of the phosphorylation state of Trk receptors

The phosphorylation state of receptors was detected by stimulating freshly isolated eosinophils with NGF, BDNF or NT-3 (20 ng/mL) at baseline (0 min) and 10 min. Cells were lysed as described above. Phosphorylated receptors were immunoprecipitated. Twenty micrograms of anti-p-TrkA or anti-p-TrkB were supplemented and incubated for 1 h with constant rocking. Twenty microlitres of Protein A agarose beads (Bio-Rad, Hercules, CA, USA) were added and incubated for 1 h. After washing three times with lysis buffer, sample buffer was added and the precipitate was boiled for 5 min. The supernatant was sampled by centrifugation. Western blotting was performed as described.

Calcium assay

Calcium influx was detected by the FURA method [27,28]. Purified eosinophils, approximately 10^7 were suspended in PBS with Ca^{2+} , Mg^{2+} and 2% bovine serum albumin (BSA; Sigma). FURA-2 AM (Calbiochem, San Diego, CA, USA) was added to a final concentration of 2 µmol/L. After incubation for 30 min, excess dye was removed by centrifugation, and the cells were resuspended in a buffer containing 119 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl_2 , 1 mmol/L MgCl_2 , 0.03% human serum albumin and 25 mmol/L Pipes, pH 7.4. NGF, BDNF or NT-3 were added at concentrations of 20–100 ng/mL. Calcium influx was determined using excitation at 340 and 380 nm with a fluorescence spectrometer (Shimadzu, Kyoto, Japan) calibrated with 0.1% Triton-X for total Ca^{2+} release and 10 mmol/L EGTA to chelate free Ca^{2+} .

Intracellular staining and flow cytometry

Approximately 10^5 eosinophils were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.025% saponin. Cells were incubated for 1 h in 50 µL AB serum containing either 20 µL antibody to either TrkA, TrkB or TrkC (Santa Cruz Biotechnology) or antibodies pre-incubated overnight with specific blocking peptides (Santa Cruz Biotechnology) for 1 h. Cells were washed with PBS supplemented with 2% FCS and 0.1% saponin, followed by incubation with a secondary anti-rabbit FITC-conjugated antibody for 1 h. Isolated eosinophils were washed again with PBS containing 2% PFA. Fluorescence was analysed by flow cytometry (Epic XL, Coulter).

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) with SPSS, Version 10 (SPSS, Inc., Chicago, IL, USA). *P*-values below 0.05 were regarded as significant.

Results

RNA isolation and RT–PCR amplification

Each cDNA sample from isolated eosinophils or cultured HMC-1 cells demonstrated gluceraldehyde 3-phosphate dehydrogenase (GAPDH) amplification products of the expected size. There was no evidence of genomic amplification product, indicating the absence of contaminating genomic DNA. These results demonstrate the quality of the cDNA used for PCR detection of mRNA expression. During PCR amplification, control reactions were negative for amplification products, demonstrating that the PCR method and the reagents used yielded specific amplified products only when a cDNA source was included. Specific bands demonstrating the presence of RNA encoding for neurotrophin receptors was detectable in 50–75% of the samples after reverse transcription (Fig. 1).

Protein electrophoresis and western immunoblotting

To confirm the expression of the neurotrophin receptors at the protein level, soluble extracts from isolated eosinophils or HMC-1 cells as controls were immunoblotted using specific antibodies. As shown in Fig. 2, specific bands were detectable for TrkA, TrkB as well as TrkC. Next to full-length receptors, truncated receptors were also detectable for TrkC (Fig. 2). The specificity was confirmed by the fact that the staining was absorbed by pre-incubation of antibodies with excess amount of blocking peptides.

Intracellular staining and flow cytometry

To demonstrate the presence of neurotrophin receptors in intact cells, intracellular staining was performed with the antibody directed against an intracellular domain. FACS analysis showed positive results for TrkA and TrkB. TrkC receptor was not detectable (Fig. 3).

Detection of phosphorylation state of Trk receptors

After stimulation with specific ligands NGF, BDNF and NT-3, and immunoprecipitation of neurotrophin receptors with specific TrkA or anti-p-Trk antibodies, increasing band intensities on

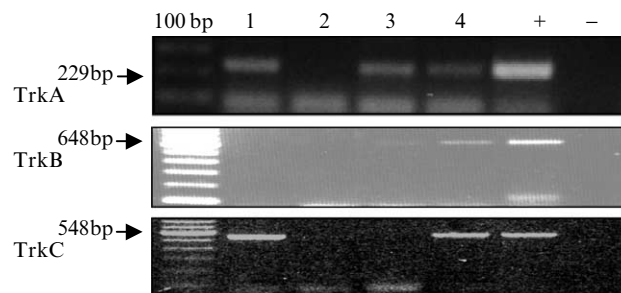


Fig. 1. RT–PCR for neurotrophin receptors TrkA, TrkB, TrkC. Lane 1: 100 bp ladder; lanes 2–5: samples of different donors; lane 6: positive control; lane 7: negative control. Positive control was performed with cDNA from HMC-1 cells. Negative control was performed by adding isolated RNA.

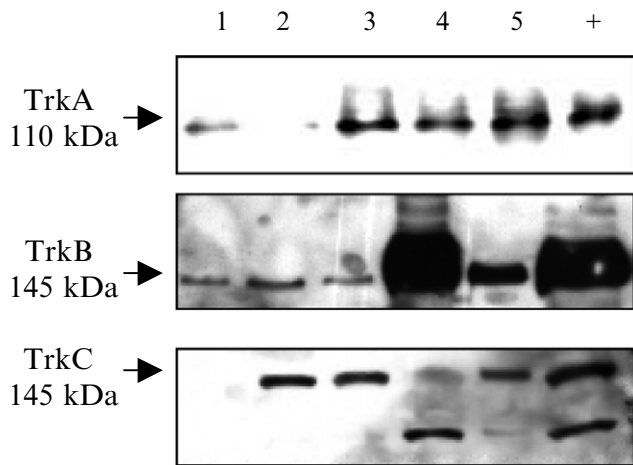


Fig. 2. Western blot analysis of TrkA, TrkB and TrkC protein. Lanes 1–5: samples of different subjects; lane 6: positive control. Cell lysates of HMC-1 cells were used as positive control.

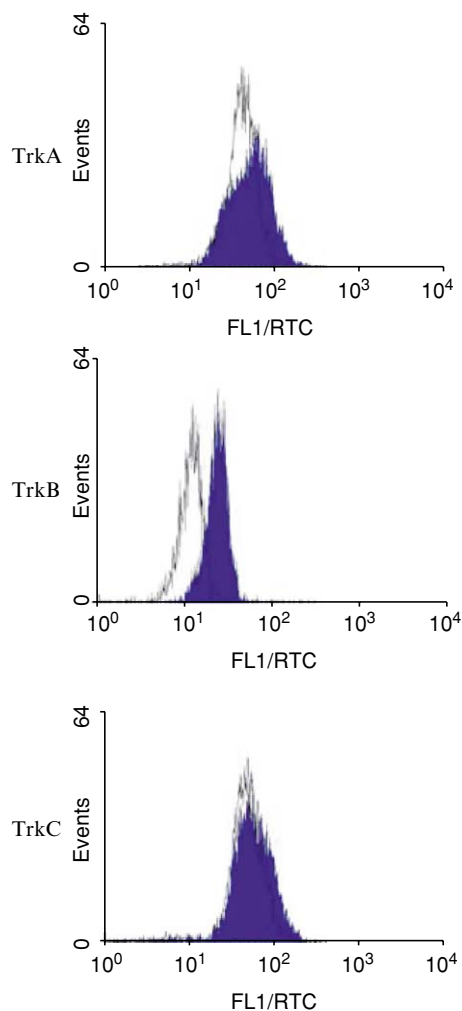


Fig. 3. FACS analysis of intracellular domain of TrkA, TrkB and TrkC with and without blocking of the antibodies with excess amount of blocking peptide. Controls are unfilled, samples are filled. Single experiment out of three.

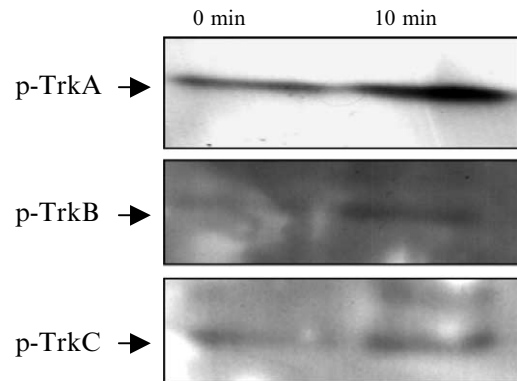


Fig. 4. Phosphorylation of neurotrophin receptors at baseline (0 min) and after ligand activation for 10 min.

western blots were detectable compared to baseline after 10 min of incubation (Fig. 4).

Calcium assay

Eosinophils did not undergo a measurable calcium influx after stimulation with the neurotrophins NGF, BDNF and NT-3, while control reactions with IL-8 showed positive signals (data not shown).

Determination of mediators in cell culture supernatant

IL-4 was significantly increased after stimulation with NGF compared to unstimulated samples. IL-5 also demonstrated a slight increase after NGF stimulation, though without reaching statistical significance. The same was also observed for $\text{TGF}\beta_1$. IL-8 and ECP did not demonstrate any difference between different stimulations, while EPX was significantly increased after stimulation with BDNF and NT-3 (Fig. 5).

Eosinophil viability

Eosinophil viability was not influenced by stimulation with either NGF, BDNF or NT-3. No difference was seen to cells cultivated with medium alone (Fig. 6).

Discussion

In this study we were able to prove the expression of TrkA receptor on eosinophils. Furthermore, we could demonstrate for the first time the expression of TrkB and TrkC receptors. These observations were made at RNA, protein and cellular levels. All the neurotrophin receptors detected are functionally active, which was demonstrated by phosphorylation after stimulation with their specific ligands. Surprisingly, in contrast to neural cells, receptor activation did not appear to lead to downstream calcium influx; probably $\text{PLC}\gamma$ activity in eosinophils is too weak to induce an influx measurable by the Fura assay. However, it might be possible that signalling in eosinophils is different to that in neural cells. This should be clarified, as it could lead to new therapeutical approaches. However, eosinophils were activated by stimulation with neurotrophins as shown by a significant increase of IL-4 after NGF stimulation and EPX after stimulation with BDNF or NT-3. These results might offer an explanation for increased Th2 cytokines

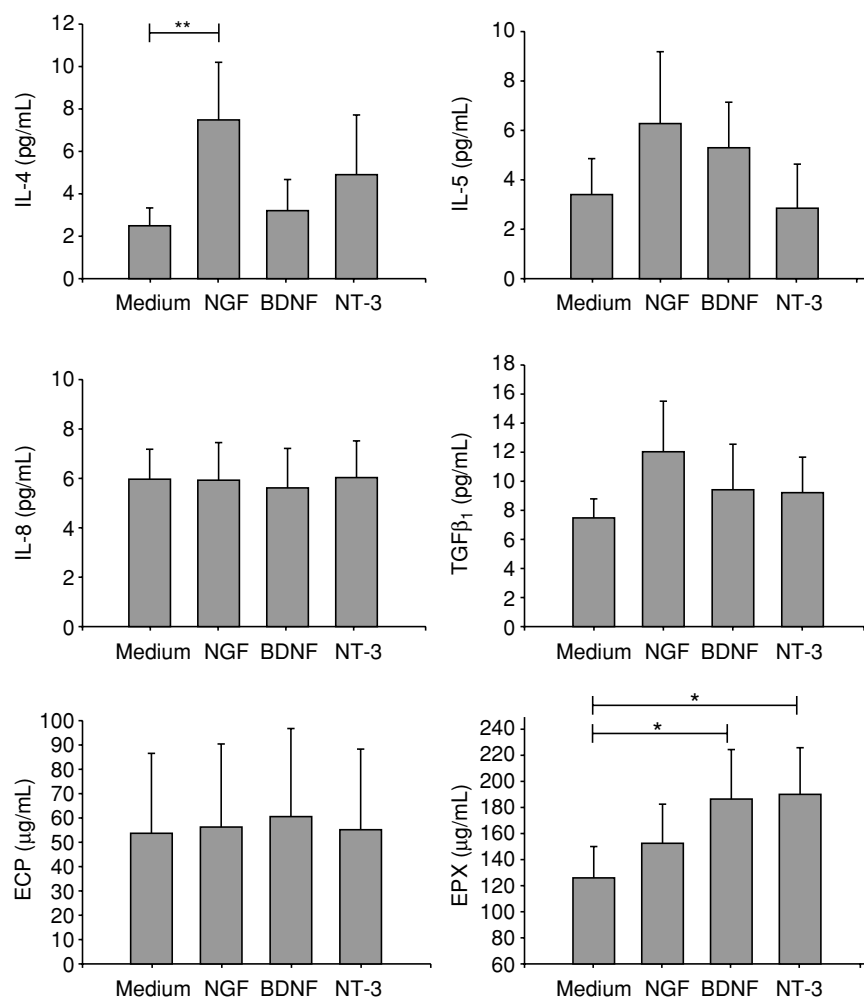


Fig. 5. Mediator measurements in cell culture supernatants after 24 h of stimulation with NGF, BDNF or NT-3 ($n = 18$). Mean values $\pm$ SEM. Calculated per 10^6 cells. * $P < 0.05$; ** $P < 0.01$.

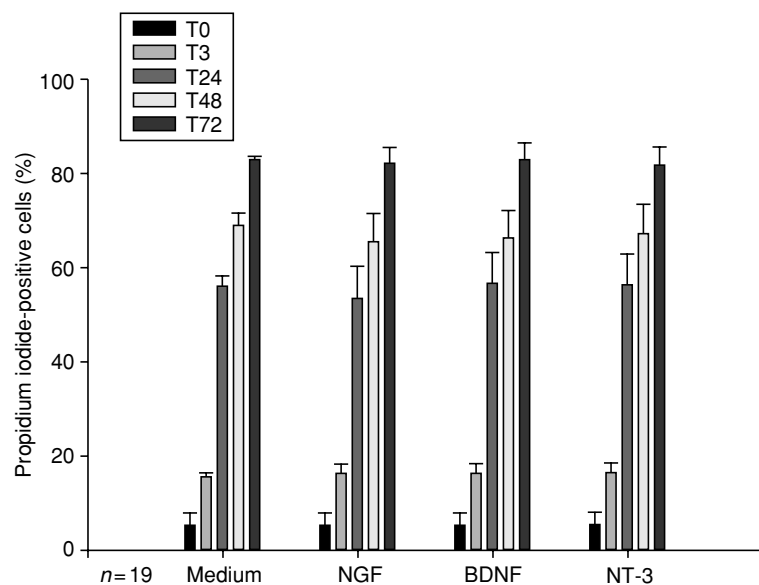


Fig. 6. Eosinophil viability determined by propidium iodide staining and FACS analysis of isolated cells of 19 different subjects ($n = 19$). Viability was determined immediately after isolation (T0); after 3 h of culture (T3); 24 h (T24); 48 h (T48) and 72 h of culture (T72).

IL-4 and IL-5 in splenic mononuclear cells of allergen-sensitized mice after exogenously added NGF, while nasal application of anti-NGF significantly reduced IL-4 [18]. Influence on eosinophil survival is still controversial [20, 29]. In our study we were unable to demonstrate any influence of neurotrophins on eosinophil survival.

Because eosinophils are a main player in allergic inflammation, our results provide an additional role for neurotrophins in modulating asthma. An important pathway triggering inflammation in the airways is neurogenic inflammation. Levels of substance P (SP) are elevated in lavage fluids of allergies [30]. Activated eosinophils increase the amount of SP while known activators, except major basic protein (MBP), fail to increase neuropeptides in dorsal root ganglion cells [31]. The expression of neurotrophins as activators of neuropeptide synthesis might be an explanation for this [32]. NGF as the best known neurotrophin not only increases SP expression in airway neurones but also changes the neuronal phenotype [33]. The increased levels of neuropeptides also influence eosinophil function because SP is chemoattractive for eosinophils. Neurotrophins influence neurogenic inflammation and, as we were able to demonstrate, neurotrophins also have a direct influence on eosinophils by increasing their release for IL-4 and EPX and possibly other inflammatory mediators. This can explain, to a certain extent, the correlation of IgE levels with neurotrophin concentrations [15]. Neurotrophins regulate the function of eosinophils and various other immune cells, e.g. mast cells, T cells and B cells. But residential cells like epithelial cells [34] and fibroblasts [35] are also influenced, providing a role for neurotrophins in modulating inflammation and remodelling in asthma, especially as neurotrophin receptors are expressed on various immune cells. The special influence of the neurotrophins NGF, BDNF and NT-3 on eosinophil function might also be triggered by an autocrine pathway as eosinophils are not only activated by neurotrophins but also produce neurotrophins themselves [20]. Modulation of eosinophil function by neurotrophins through activation of high specific neurotrophin receptors provides a further pathway for eosinophils in inflammation, repair and remodelling mechanisms present in allergic asthma.

References

- Chun LL, Patterson PH. Role of nerve growth factor in the development of rat sympathetic neurons *in vitro*. I. Survival, growth, and differentiation of catecholamine production. *J Cell Biol* 1977; 75:694–704.
- Amann R, Sirinathsinghji DJ, Donnerer J, Liebmann I, Schuligoi R. Stimulation by nerve growth factor of neuropeptide synthesis in the adult rat *in vivo*: bilateral response to unilateral intraplantar injections. *Neurosci Lett* 1996; 203:171–4.
- Johnson D, Lanahan A, Buck CR et al. Expression and structure of the human NGF receptor. *Cell* 1986; 47:545–54.
- Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* 2000; 10:381–91.
- Friedman WJ, Greene LA. Neurotrophin signaling via Trks and p75. *Exp Cell Res* 1999; 253:131–42.
- Santambrogio LBM, Chao MV. Nerve growth factor production by lymphocytes. *J Immunol* 1994; 153:4488–92.
- Leon A, Buriani A, Dal Toso R et al. Mast cells synthesize, store, and release nerve growth factor. *Proc Natl Acad Sci USA* 1994; 91:3739–43.
- Welker P, Grabbe J, Grutzkau A, Henz BM. Effects of nerve growth factor (NGF) and other fibroblast-derived growth factors on immature human mast cells (HMC-1). *Immunology* 1998; 94:310–7.
- Torcia M, Bracci-Laudero L, Lucibello M. Nerve growth factor is an autocrine survival factor for memory B lymphocytes. *Cell* 1996; 85:345–56.
- Bischoff SC, Dahinden CA. Effect of nerve growth factor on the release of inflammatory mediators by mature human basophils. *Blood* 1992; 79:2662–9.
- Lambiase A, Bracci-Laudiero L, Bonini S et al. Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. *J Allergy Clin Immunol* 1997; 100:408–14.
- Kerschensteiner M, Gallmeier E, Behrens L et al. Activated human T cells, B cells, and monocytes produce brain-derived neurotrophic factor *in vitro* and in inflammatory brain lesions: a neuroprotective role of inflammation? *J Exp Med* 1999; 189:865–70.
- Bracci-Laudiero L, Aloe L, Levi Montalcini R et al. Increased levels of NGF in sera of systemic lupus erythematosus patients. *Neuroreport* 1993; 4:563–5.
- Falcini F, Cerinic MM, Ermini M et al. Nerve growth factor circulating levels are increased in Kawasaki disease: correlation with disease activity and reduced angiotensin converting enzyme levels. *J Rheumatol* 1996; 23:1798–802.
- Bonini S, Lambiase A, Angelucci F, Magrini L, Manni L, Aloe L. Circulating nerve growth factor levels are increased in humans with allergic diseases and asthma. *Proc Natl Acad Sci USA* 1996; 93:10955–60.
- Noga O, Hanf G, Schäper C, O'Connor A, Kunkel G. The influence of inhalative corticosteroids on circulating NGF, BDNF and NT-3 in allergic asthmatics. *Clin Exp Allergy* 2001; 31:1906–12.
- Virchow JC, Julius P, Lommatzsch M, Luttmann W, Renz H, Braun A. Neurotrophins are increased in bronchoalveolar lavage fluid after segmental allergen provocation. *Am J Respir Crit Care Med* 1998; 158:2002–5.
- Braun A, Appel E, Baruch R et al. Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur J Immunol* 1998; 28:3240–51.
- de Vries A, Dessing M, Engels F, Henricks P, Nijkamp F. Nerve growth factor induces a neurokinin-1 receptor-mediated airway hyperresponsiveness in guinea pigs. *Am J Respir Crit Care Med* 1999; 159:1541–4.
- Solomon A, Aloe L, Pe'er J, Frucht Pery J, Bonini S, Levi-Schaffer F. Nerve growth factor is preformed in and activates human peripheral blood eosinophils. *J Allergy Clin Immunol* 1998; 102:454–60.
- Hansel TT, Pound JD, Pilling D et al. Purification of human blood eosinophils by negative selection using immunomagnetic beads. *J Immunol Methods* 1989; 122:97–103.
- Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. *Cytometry* 1990; 11:231–8.
- Tam SY, Tsai M, Yamaguchi M, Yano K, Butterfield JH, Galli SJ. Expression of functional TrkA receptor tyrosine kinase in the HMC-1 human mast cell line and in human mast cells. *Blood* 1997; 90:1807–20.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J Biol Chem* 1977; 252:1102–6.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979; 76:4350–4.
- Fujisawa T, Kato Y, Nagase H et al. Chemokines induce eosinophil degranulation through CCR-3. *J Allergy Clin Immunol* 2000; 106:507–13.

- 28 Oshiro T, Kakuta Y, Shimura S, Nara M, Shirato K. Characterization of platelet-activating factor-induced cytosolic calcium mobilization in human eosinophils. *Clin Exp Allergy* 2000; 30:699–705.
- 29 Hamada A, Watanabe N, Ohtomo H, Matsuda H. Nerve growth factor enhances survival and cytotoxic activity of human eosinophils. *Br J Haematol* 1996; 93:299–302.
- 30 Nieber K, Baumgarten C, Rathack R, Furkert J, Oehme P, Kunkel G. Substance P and β -endorphin-like immunoreactivity in lavage fluids of subjects with and without allergic rhinitis. *J Allergy Clin Immunol* 1992; 106:199–201.
- 31 Garland A, Necheles J, White SR et al. Activated eosinophils elicit substance P release from cultured dorsal root ganglion neurons. *Am J Physiol* 1997; 273:L1096–102.
- 32 Malcangio M, Garrett NE, Cruwys S, Tomlinson DR. Nerve growth factor- and neurotrophin-3-induced changes in nociceptive threshold and the release of substance P from the rat isolated spinal cord. *J Neurosci* 1997; 17:8459–67.
- 33 Hunter DD, Myers AC, Undem BJ. Nerve growth factor-induced phenotypic switch in guinea pig airway sensory neurons. *Am J Respir Crit Care Med* 2000; 161:1985–90.
- 34 Fox AJ, Patel HJ, Barnes PJ, Belvisi MG. Release of nerve growth factor by human pulmonary epithelial cells: role in airway inflammatory diseases. *Eur J Pharmacol* 2001; 424:159–62.
- 35 Olgart C, Frossard N. Human lung fibroblasts secrete nerve growth factor: effect of inflammatory cytokines and glucocorticoids. *Eur Respir J* 2001; 18:115–21.