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Expression and Function of Glial Cell Line-Derived Neurotrophic Factor Family Ligands and Their Receptors on Human Immune Cells[1]

Vivian Vargas-Leal,* Roxana Bruno,* Tobias Derfuss,*† Markus Krumbholz,*† Reinhard Hohlfeld,*† and Edgar Meinl[2]*†

There is increasing evidence that factors originally identified due to their neurotrophic activity also function within the immune system. This study focused on the related molecules glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) as well as their receptors. GDNF and NTN signaling is mediated by a two-component receptor: a signal-transducing component, RET, which is shared by both ligands, and a ligand-specific binding component, GFRα-1 (higher GDNF affinity) or GFRα-2 (higher NTN affinity). We report that human T cells, B cells, and monocytes produce NTN but not GDNF, as seen by RT-PCR and immunocytochemistry. *RET* was expressed by B cells, T cells, and monocytes. Exons 2–5 of *RET* encoding the cadherin-like domains 1–3 in the extracellular part and exons 16–19 encoding a section of the second tyrosine kinase domain were transcribed in CD4$^+$ T cells, CD8$^+$ T cells, B cells, and monocytes. Different splice variants encoding the C-terminal intracellular part (exons 19–21) of *RET* were detected. The ligand-binding receptors GFRα-1 and GFRα-2 were transcribed in all immune cell subsets. Quantitative PCR showed that GFRα-2 is by far the dominant ligand binding chain in T cells, B cells, and monocytes. Addition of GDNF or NTN to activated PBMCs reduced the amount of detectable TNF protein without altering its transcription. Together, this suggests that immune cells communicate with each other via NTN. Production of NTN by immune cells might also contribute to the neuroprotective immunity in the CNS observed in different model systems. ***The Journal of Immunology,*** **2005, 175: 2301–2308.**

Neurotrophic factors were originally identified because of their effects on the development and maintenance of neuronal cells. There is growing evidence, however, that some neurotrophic factors can also be produced by immune cells and regulate immune functions (reviewed in Refs. 1 and 2). Factors with a neurotrophic activity can be divided into at least three structurally different families: the neurotrophins (nerve growth factor, brain-derived neurotrophic factor, NT3, and NT4/5), the CNTF/IL-6 family, and the glial cell line-derived neurotrophic factor (GDNF)[3] family ligands (GFLs). The GFLs, which are distant members of the TGF-β superfamily (3, 4), include GDNF, neurturin (NTN), artemin, and persephin.

Signaling of the GFLs is mediated by two-component receptors. The common signal-transducing component is RET, a receptor protein kinase. *RET* was originally identified as a protooncogene with 21 exons and multiple splice variants. RET is activated by a complex of a GFL and a GDNF family α receptor (GFRα). Four GFRαs have been identified, which determine the ligand specificity. GFRα-1 binds preferentially to GDNF, GFRα-2 to NTN, GFRα-3 to artemin, and GFRα-4 to persephin. In addition to these high-affinity interactions, more promiscuous bindings between GFLs and their receptors have been described, e.g., binding of NTN to GFRα-1 (reviewed in Refs. 3 and 4).

GFRαs are usually bound to the plasma membrane via glycosylphosphatidylinositol, but cleavage by an unknown phospholipase or protease can produce soluble forms of these coreceptors (4). Soluble forms of cleaved GFRαs can bind GFLs, and the complex of soluble GFRαs and GFLs can also activate RET (5, 6).

In addition to its neurotrophic activity, GDNF has important roles outside of the nervous system. Among other functions, GDNF acts as a morphogen in kidney development, as a migration factor for neural crest cells, and as a regulator of the differentiation of spermatogonia (4). Similar to GDNF, NTN is a potent neurotrophic factor. Its mRNA has been strongly detected in peripheral blood, heart, brain, and other tissues (7, 8).

Mutations of *RET* have been linked to different human diseases: activating mutations of *RET* cause cancer (papillary thyroid carcinoma and multiple endocrine neoplasia syndrome), whereas inactivating mutations of *RET* lead to Hirschsprung's disease (aganglionic megacolon) (3). A putative role of RET in the functional regulation of hemopoietic cells has been suggested by the presence of RET, GFRα-1, and GFRα-2 in lymphohemopoietic tissues, including fetal liver, thymus, spleen, and lymph nodes as well as in human leukemia cell lines (9–13). Bone marrow stroma cells expressed GFRα-1 and GFRα-2 but not RET (12, 13). In the thymus, GDNF is produced by stroma cells and supports the survival of

*Department of Neuroimmunology, Max-Planck-Institute of Neurobiology, Martinsried, Germany; and †Institute for Clinical Neuroimmunology, Ludwig-Maximilians-University, Munich, Germany

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[2] Address correspondence and reprint requests to Dr. Edgar Meinl, Department of Neuroimmunology, Max-Planck-Institute of Neurobiology, Am Klopferspitz 18, D-82152 Martinsried, Germany. E-mail address: meinl@neuro.mpg.de

[3] Abbreviations used in this paper: GDNF, glial cell line-derived neurotrophic factor; GFL, GDNF family ligand; GFRα, GDNF family receptor α; NTN, neurturin; SAC, *Staphyloccocus aureus* Cowan I; RT, reverse transcriptase; DAPI, 4′,6′-diamidino-2-phenylindole; CLD, cadherin-like domain; NCAM, neural cell adhesion molecule; BDNF, brain-derived neurotrophic factor.

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immature $CD4^-CD8^-$ thymocytes, which express GFRα-1 and RET (14).

In the present study, we investigated the expression and potential function of the GFLs GDNF and NTN in mature human immune cells. We asked whether T cells, B cells, and monocytes can produce GDNF and NTN, quantified the expression of the corresponding ligand-binding receptors, and identified the isoforms of *RET* expressed in different resting and activated immune cell subsets. Finally, we attempted to identify functional effects of these GFLs on immune cells.

Materials and Methods

Cell lines

The following human cell lines were cultured in RPMI 1640 (Invitrogen Life Technologies) with 10% FCS (Invitrogen Life Technologies) and 1% penicillin-streptomycin and served as positive or negative controls for GFLs or their receptors: the neuroblastoma cell lines TGW (kindly provided by Dr. M. Takahashi, Department of Pathology, Nagoya University, Nagoya, Japan) and SHSY-5Y, the promyelocytic leukemia cell line HL-60, and the histiocytic lymphoma cell line U937. 3T3 GDNF-transfected fibroblasts were provided by Dr. A. Flügel (Max-Planck-Institute of Neurobiology, Martinsried, Germany).

Purification and activation of immune cell subsets

$CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes were isolated from PBMCs by immunomagnetic selection using Dynabeads (Dynal Biotech). The separated cells were analyzed by flow cytometry, and the purity for each subset was >93%.

PBMCs and purified immune cells were cultured in RPMI 1640 (Invitrogen Life Technologies) containing 5% FCS (Invitrogen Life Technologies) and 1% penicillin-streptomycin. The following activators were applied: Con A (10 μg/ml), PHA (10 μg/ml), PMA (3 ng/ml), ionomycin (300 ng/ml), LPS (300 ng/ml), and PWM (5 μg/ml) (all from Sigma-Aldrich); IFN-γ (100 U/ml; Roche); anti-CD40 mAb (10 μg/ml; BD Pharmingen); and *Staphyloccocus aureus* Cowan I (SAC; 1:7500; Calbiochem).

The functional experiments that showed an effect of GDNF and NTN on the TNF metabolism were done as follows. PBMCs (2×10^6 cells/ml) were seeded in 48-well plates (Nunc) and activated with either Con A or (LPS + IFN-γ). After 5 or 6 days without any medium exchange, human recombinant GDNF (100 ng/ml; R&D Systems) or human recombinant NTN (100 ng/ml; R&D Systems) was added for another 24 h. Controls (adding PBS) were always included. In another set of experiments, the activated PBMCs were washed after 5 or 6 days, and exogenous human recombinant TNF (2,000 pg/ml; R&D Systems) in fresh medium was added to the washed cells together with either GDNF or NTN at the indicated concentrations. Supernatants were collected 24 h later and stored until the TNF ELISA was done.

ELISA

Supernatants from resting and activated immune cells were spun down to eliminate cell debris and then stored at −20°C. The TNF ELISA (BD Pharmingen) was performed as described in the manufacturer's instructions. The GDNF ELISA kit was purchased from Promega. Supernatants from 3T3 GDNF-transfected fibroblasts were used as the positive control.

RT-PCR

Isolation of total RNA was performed using RNAzol (WAK-Chemie Medical). The RNA was treated with DNase I (Promega). For reverse transcription, 1–2 μg RNA was converted into a ssDNA by a standard 20-μl reverse transcriptase (RT) reaction using oligo(dT) primers (Invitrogen Life Technologies) and Superscript II RT (Life Technologies). One-tenth of the total cDNA was amplified in a 50-μl reaction mixture using 0.2 μM each of forward and reverse primers and 1.25 U *Taq* polymerase (Qiagen). Expression of the β-actin messenger RNA was determined after 28 PCR cycles and used as control for RNA integrity. Because of the high GC content of the NTN amplicon, 5% DMSO was added to the NTN PCR. The PCR products were separated by electrophoresis through 1.7% agarose gels, stained with ethidium bromide (0.5 μg/ml; Sigma-Aldrich), and visualized by UV illumination.

NTN forward: 5′-CCT CAG TGC TCT GCA GCT C-3′ (exon 1: bp 29–47), and NTN reverse was 5′-TCG TGC ACC GTG TGG TAG-3′ (exon 2: bp 546–563). RET forward was 5′-TGT CCG CAA CCG CGG CTT TC-3′ (exons 2 and 3: bp 525–544), and RET reverse was 5′-TAC GGT CGC CCG CAC GAA GC-3′ (exon 5: bp 1229–1248). Human β-actin forward was 5′-CGG GAA ATC GTG CGT GAC AT-3′ (bp 689–708), and β-actin reverse was 5′-GAA CTT TGG GGG ATG CTC GC-3′ (bp 1381–1400). The primers for GDNF (15); RET-9, RET-43, and RET-51 (16, 17); RET-B (exons 16–19) (13); and GFRα-2 (18) were used as described. To detect GFRα-1, three different sets of primers were used: primers HRAF1 and HRAR2 spanning exon 1–2 from Ref. 19 and the primers used in Refs. 12 and 15.

Real-time PCR

Quantitative PCR was performed with ABI 5700 (Applied Biosystems) using the qPCR Core kit and uracyl *N*-glycosylase for carryover prevention (both from Eurogentec). Primers for the housekeeping genes cyclophilin A (peptidyl-prolyl isomerase A) and GAPDH were from Applied Biosystems. The primers and probes for TNF were used as previously described (20). Primer and TaqMan probes for GFRα1–3 were designed with Primer Express software. GFRα-1 forward (exon 6) was 5′-CAG GCA TCC TGC AAG ACG AAT TA-3′, with reverse (exon 7) being 5′-AGC TGC TGA CAG ACC TTG ACT CT-3′ and probe (exons 6/7) being 5′-TCT GCA GAT CTC GCC TTG CGG ATT T-3′.

GFRα-2 forward (exon 5) was 5′-CCG ACT TCC ATG CCA ATT GT-3′, with reverse (exons 5/6) being 5′-ATG TCA AAC CCA ATC ATG CCA-3′ and probe (exon 5) being 5′-CCT GCG GAC AAT TAC CAG GCG TGT C-3′.

GFRα-3 forward (exon 4) was 5′-GCG CCT CTG CTT CTC CGA-3′, with reverse (exon 5) being 5′-ATC TGG ACT GCT CTG TTG CAC A-3′ and probe (exons 4/5) being 5′-CGC TTT GCA GAT CAC GCC TGG TG-3′.

Flow cytometry

To determine the purity of the isolated cells, directly labeled Abs recognizing CD3, CD4, CD8, or CD19 (BD Biosciences) and corresponding isotype controls (BD Biosciences and DakoCytomation) were used. A goat anti-human GFRα-2 Ab (BD Transduction Laboratories) was applied at 2 μg/ml, followed by a donkey anti-goat Ig PE-labeled Ab (1/100; Jackson ImmunoResearch Laboratories). To stain RET transmembrane receptor, a nonlabeled mouse IgG1 anti-RET mAb (R&D Systems) was used at 10 μg/ml, followed by goat anti-mouse Ig-FITC (1/150; DakoCytomation). Dead cells were excluded with the Via Probe (10 μl/sample; BD Biosciences). Cells were analyzed immediately after they were stained on a FACScan using CellQuest software (BD Biosciences).

Immunocytochemistry

Immunostaining was used to detect intracellular NTN in immune cells. Cytospins were performed with nonactivated or activated PBMC onto slides (Superfrost*/Plus; Menzel). Cells were fixed with 4% paraformaldehyde, washed with PBS, and blocked with 2% goat serum in PBS. Subsequently, a mouse IgG2b anti-human NTN (R&D Systems) was added for 1 h at room temperature at 5 μg/ml, and then the slides were incubated for 45 min with a secondary labeled goat anti-mouse Cy3 Ab (Jackson ImmunoResearch Laboratories). Background labeling was determined by incubation with primary purified isotype control mouse IgG2b (1 mg/ml; BD Biosciences) diluted 1/200 in the blocking solution. The nuclei were counterstained with 1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich).

Double staining was performed to identify immune cell subsets producing NTN. For that purpose, after slides were stained with anti-NTN and secondary Ab, slides were blocked with mouse serum (1:100) for 30 min at RT and then incubated with the following mAbs: anti-CD4-FITC, anti-CD8-FITC, anti-CD14-FITC, and anti-CD19-FITC (BD Biosciences). Isotype controls (mouse IgG1-FITC and mouse IgG2a; BD Biosciences) were applied in parallel.

Statistical analysis

TNF ELISA data were expressed as the weighted means ± weighted SD. Differences in cytokine expression between two groups (GDNF- or NTN-treated and nontreated samples) were examined for statistical significance using Student's *t* test.

Results

Differential expression of GDNF and NTN in human immune cells

To analyze whether immune cells express GDNF or NTN, we separated $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and $CD14^+$

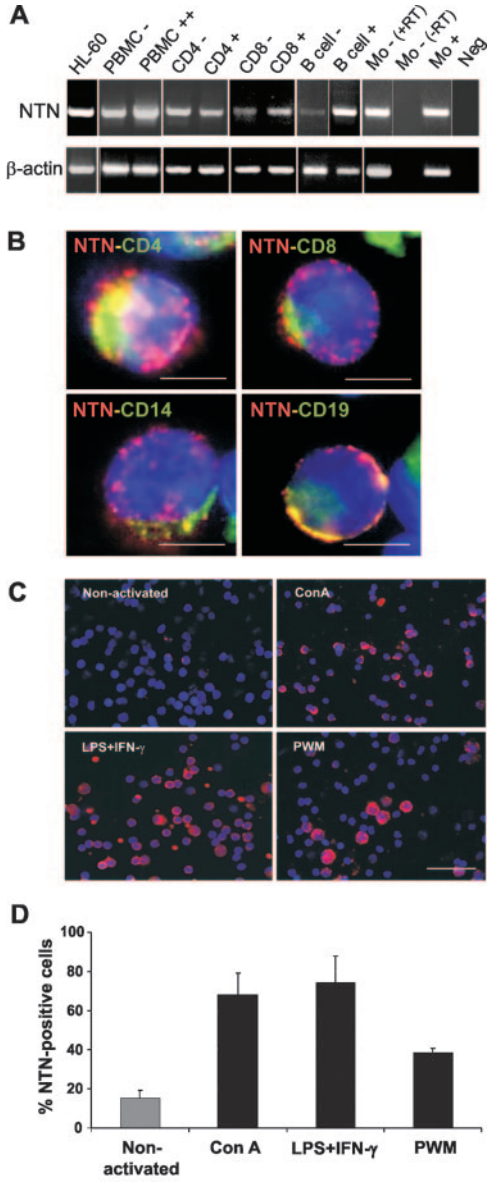

FIGURE 1. Neurturin is produced by different immune cell subsets. *A*, NTN transcripts are present in immune cell subsets as seen by RT-PCR. Immune cell subsets were purified and cultured for 24 h in the absence of activation (−) or stimulated (+) as follows: $PBMC^{++}$, (LPS + IFN-γ)-activated PBMC; $CD4^+$, PHA-activated $CD4^+$ T cells; $CD8^+$, PHA-activated $CD8^+$ T cells; B $cell^+$, SAC-activated B cells; Mo^+, LPS-activated monocytes. HL-60 cell line was used as positive control. Neg, water control. *B*, NTN is detected in immune cell subsets by immunocytochemistry. PBMCs, activated with Con A for 5 days ($CD4^+$, $CD8^+$) or LPS + IFN-γ ($CD14^+$, $CD19^+$), were double stained for NTN (red) and the indicated immune cell markers (green). Colocalization of NTN with $CD4^+$, $CD8^+$, $CD19^+$, and $CD14^+$ cell markers resulted in a yellow color. Nuclei were counterstained with DAPI (in blue). Scale bar, 10 μm. *C*, NTN expression was demonstrated by immunocytochemistry, resulting in a red staining. PBMCs were analyzed in a resting state and after activation for 72 h with Con A, LPS + IFN-γ, or PWM. The applied activating reagents induced a higher percentage of NTN-expressing cells and a more intense staining in the individual NTN-positive cells. Nuclei were counterstained with DAPI. Scale bar, 50 μm. *D*, Quantitative analysis of the staining shown in *C* and two additional experiments showed that polyclonal activation enhances NTN expression; 100 cells per activation condition were counted, and the number of NTN-positive cells was calculated. Each column represents the mean of three independent experiments ± SD.

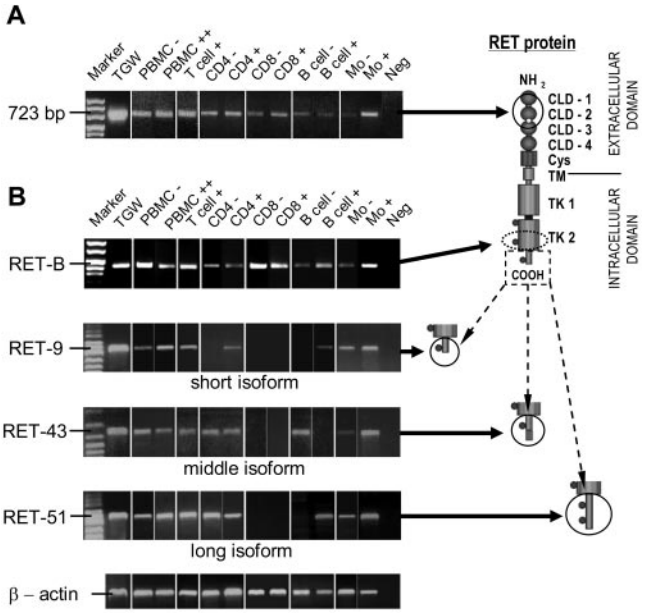

FIGURE 2. Transcription of different isoforms of *RET* in immune cells. Immune cell subsets were purified and cultured for 24 h in the absence of activation (−) or stimulated (+) as follows: $CD4^+$, PHA-activated $CD4^+$ T cells; $CD8^+$, PHA-activated $CD8^+$ T cells; B $cell^+$, SAC-activated B cells; Mo^+, LPS-activated monocytes; $PBMC^{++}$, 5-day (LPS + IFN-γ)-activated PBMC; T $cell^+$, 5-day Con A-activated PBMC. The neuroblastoma cell line TGW was used as a positive control. Neg, water control. Subsequently, RT-PCR was performed. *A*, *RET* transcripts coding for the extracellular part were recognized by RT-PCR with a primer pair spanning exons 2–5. A full-length transcript of this region was amplified in all subsets of immune cells, either resting or activated. *Right*, structure of the translated RET protein, which includes four CLDs (CLD-1/4) below the N-terminal region (NH_2), the cysteine-rich domain (Cys), the transmembrane domain (TM), the tyrosine kinase domains (TK1 and TK2), and the C terminus (COOH). The PCR product obtained represents CLD-1, CLD-2, and part of CLD-3, as well as the Ca^{2+} binding motif located between CLD-2 and CLD-3. This part of RET is shown on the right inside of the closed ellipse. *B*, *RET* transcripts coding for the intracellular part. All immune cell subsets contain a transcript spanning exons 16–19 (RET-B). This codes for a part of the second tyrosine kinase domain (TK2), which is encircled on *right* with a dashed ellipse. Different splice variants were detected at the 3′ end of *RET*. These splice variants were amplified using one common forward primer and three different reverse primers: RET-9 (short isoform), RET-43 (middle isoform), and RET-51 (long isoform). Resting $CD4^+$ T cells expressed RET-43 and RET-51; they also expressed RET-9 after activation. $CD8^+$ T cells expressed none of these three splice variants. Con A-activated T cell blasts and resting or activated monocytes expressed RET-9, RET-43, and RET-51. B cells displayed a complex pattern that was changed after activation: resting B cells expressed only RET-43, whereas SAC-activated B cells also expressed RET-9 and RET-51. *Right*, C-terminal part of RET protein is marked with a dashed square, indicating the region codified between exons 19 and 21. Differences in the C terminus (closed circles) of the predicted proteins translated from each isoform are shown beside each of the corresponding PCRs. RET-9 (short isoform) contains 9 amino acids at the C terminus and only 1 tyrosine residue (small circle on the left side of the protein), RET-43 (middle isoform) generates 43 different amino acids instead of the 9 aforementioned, and RET-51 (long isoform) translates 51 unrelated amino acids at the C terminus and another tyrosine residue.

monocytes by magnetic beads. RT-PCR indicated that $CD4^+$ and $CD8^+$ T cells, B cells, and monocytes expressed NTN transcripts constitutively and after activation (Fig. 1*A*). The cell line HL-60 served as a positive control for NTN (21). Furthermore, we investigated the expression of NTN by different immune cell subsets by immunofluorescence microscopy. In Con A and (LPS + IFN-γ)-activated PBMC, NTN could be detected in $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes (Fig. 1*B*).

As shown in Fig. 1*C*, in nonactivated PBMC preparations, only very few cells were weakly labeled with the anti-NTN mAb. After activation with Con A, (LPS + IFN-γ), or PWM, the percentage of NTN-containing immune cells and the amount of NTN per cell increased (Fig. 1*C*). Quantitative analyses showed that in nonactivated PBMCs <20% of cells expressed NTN, after stimulation with PWM ~40% of PBMC expressed NTN, and after activation with Con A or (LPS + IFN-γ) ~80% of PBMCs contained NTN (Fig. 1*D*).

In contrast to NTN, GDNF was undetectable by RT-PCR, Western blot, and ELISA in all analyzed immune cell subsets (data not shown).

Differential expression of different RET *isoforms by immune cells*

We next asked whether immune cells express *RET* and aimed to identify the transcribed *RET* splice variants, using different primer pairs. Primers were located at the 5′-end region (coding for the extracellular domain of RET) and at the 3′-end region of the *RET* gene (coding for the intracellular part of the protein).

To identify transcripts of the extracellular domain of *RET* in immune cells, we applied a primer pair spanning exons 2–5. These exons coded for the cadherin-like domains (CLD)-1, -2, and part of -3, as well as the Ca^{2+} binding motif located between CLD-2 and CLD-3 (22). We found that $CD4^+$ T cells, $CD8^+$ T cells, B cells, and monocytes expressed this part of the 5′-end region of *RET* (Fig. 2*A*).

We applied a set of primers spanning exons 16–19 (previously designated RET-B) (13), which encode a part of the intracellular region and contribute to formation of the second tyrosine kinase domain (TK2; Fig. 2*B*). This part was expressed in $CD4^+$ and $CD8^+$ T cells, monocytes, and B cells (Fig. 2*B*).

Next, we analyzed the expression of the three 3′-end region splice variants, namely, RET-9, RET-43, and RET-51, using one common forward primer and three different reverse primers. Immune cell subsets expressed different splice variants of this region of the gene (Fig. 2*B*). T cell blasts at 5–6 days after activation and resting or activated monocytes expressed RET-9, RET-43, and RET-51. Resting $CD4^+$ T cells expressed RET-43 and RET-51 and coexpressed RET-9 at 24 h after PHA activation. In contrast, $CD8^+$ T cells expressed none of the three splice variants, neither in the resting state nor at 24 h after activation with PHA or PMA + ionomycin. B cells displayed a particularly complex pattern of *RET* expression: resting B cells expressed only RET-43, whereas

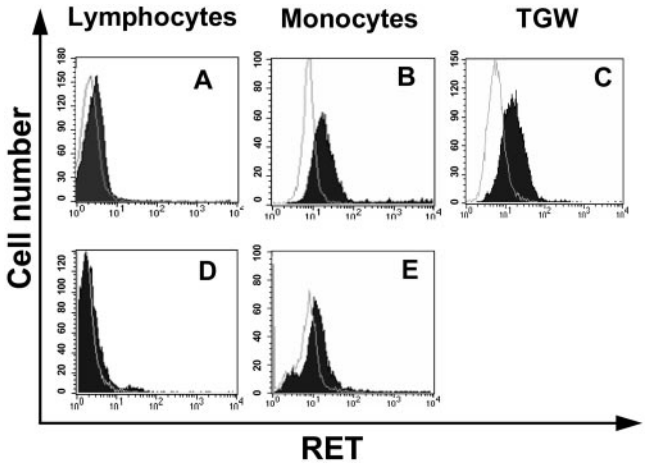

FIGURE 3. Surface expression of RET. Freshly isolated PBMC were stained with a mAb recognizing the extracellular part of RET and analyzed by FACS. Based on SSC and forward light scatter, gates were set on the lymphocyte and monocyte fractions. The filled graph illustrates the specific staining; the open graph illustrates the isotype control. RET staining was far more intense on monocytes (*B* and *E*) compared with lymphocytes (*A* and *D*). The neuroblastoma cell line TGW (*C*) served as a positive control. In *A* and *B*, freshly isolated immune cells were analyzed. *D* and *E*, PBMC 24 h after activation with LPS+IFN-γ.

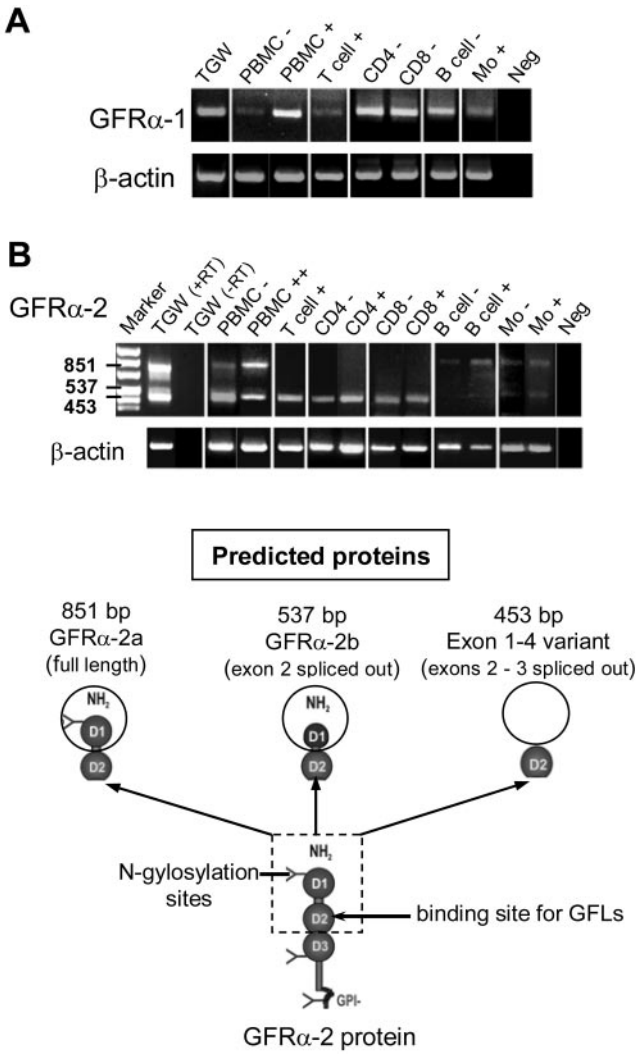

FIGURE 4. Transcription of GFRα-1 and GFRα-2. Immune cell subsets were purified and cultured for 24 h in the absence of activation (−) or stimulated (+) as follows: $PBMC^+$, PMA + ionomycin-activated PBMC; $CD4^+$, PHA-activated $CD4^+$ T cells; $CD8^+$, PHA-activated $CD8^+$ T cells; B $cell^+$, SAC-activated B cells; Mo^+, LPS-activated monocytes; T $cell^+$, 5-day Con A-activated PBMC; $PBMC^{++}$, 5-day (LPS+IFN-γ-activated) PBMC. The neuroblastoma cell line TGW was used as a positive control. Neg, water control. Subsequently, RT-PCR was performed. *A*, GFRα-1 was detected in $CD4^+$ and $CD8^+$ T cells and in B cells, monocytes, and PBMC. In this PCR, 40 cycles were necessary to see an amplicon. β-Actin is shown as housekeeping gene. *B*, Different isoforms of GFRα-2 gene were detected in human immune cells using primers spanning exons 1–5. The full length of 851 bp (GFRα-2a) was expressed by TGW and resting and activated monocytes and by (LPS + IFN-γ)-activated PBMC. The GFRα-2b isoform of 537 bp (lacking exon 2) was detected at low abundance in TGW, (LPS + IFN-γ)-activated PBMC ($PBMC^{++}$), and resting monocytes (Mo^-). The exon 1–4 mRNA variant (453 bp) was seen in $CD4^+$ T cells, $CD8^+$ T cells, and monocytes. Predicted protein structures translated from each isoform are depicted below the PCR picture and marked with an open circle. The smallest isoform (lacking exons 2 and 3) does not translate the first Cys-rich domain (D1), a region not critical for NTN and RET binding (24).

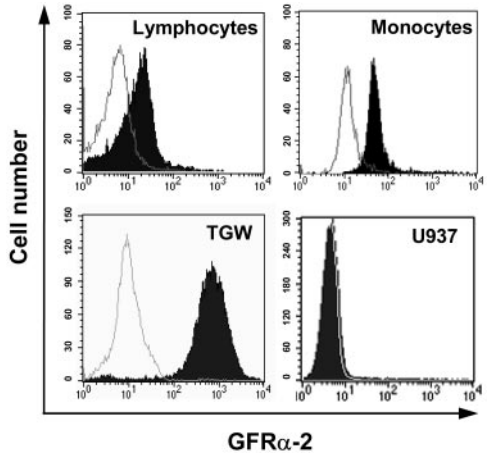

FIGURE 5. Surface expression of GFRα-2. Freshly isolated PBMCs were stained with a GFRα-2-specific polyclonal Ab and analyzed by FACS. Based on SSC and forward light scatter, gates were set on the lymphocyte and monocyte fractions. The filled graph illustrates the specific staining; the open graph illustrates the isotype control. The neuroblastoma cell line TGW served as a positive control, and the cell line U937 served as a negative control.

SAC-activated B cells transcribed only RET-9 and RET-51. Figure 2 displays the differences in the C terminus of RET encoded by the different splice variants.

We used flow cytometry for demonstration of RET protein, using a mAb directed against the extracellular domain of RET. FACS analysis of freshly isolated PBMCs showed weak expression of RET on lymphocytes and stronger expression on monocytes (Fig. 3). PBMCs, which had been activated with LPS+IFN-γ, displayed a similar staining pattern of RET (Fig. 3). The neuroblastoma cell line TGW served as positive control.

Preferential expression of the GFRα by immune cells

Using primers spanning exon 1–2 and RT-PCR, we detected the same full-length isoform of GFRα-1 in $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, and neuroblastoma cells (Fig. 4*A*). During our analyses, we noted a variable detection of GFRα-1, presumably due to its low expression level (see below). A total of 15 RT-PCR experiments (comprising a total of 23 amplifications) for GFRα-1 with immune cells was performed using three different sets of primers (12, 15, 19). In two RT-PCRs, all cDNAs from immune cells amplified the corresponding product; two other RT-PCR experiments were negative, and 11 reactions showed variable results, being sometimes positive and sometimes negative for the same subtype of cells. Figure 4*A* shows the results obtained with one set of primers (19). With these primers, two RT-PCR experiments showed all samples as positive, and four other RT-PCRs had variable results.

To detect splice variants of GFRα-2, we applied primers spanning exons 2–5, which amplify all known isoforms (23, 24). Three different isoforms of GFRα-2 were transcribed in immune cells (Fig. 4*B*), namely, the full-length mRNA (GFRα-2a), an isoform lacking exons 2 and 3 (exon 1–4 variant), and low levels of an isoform lacking exon 2 (GFRα-2b). The full-length mRNA was seen in PBMCs (resting and activated with (LPS + IFN-γ)), in monocytes (resting and activated with LPS), and in B cells (especially after activation with SAC). The GFRα-2 isoform lacking exons 2 and 3 (exon 1–4 variant) was clearly seen in $CD4^+$ T cells, $CD8^+$ T cells, and monocytes. The predicted protein from this variant lacks the N-terminal domain, a domain that is not critical for binding of GFRα-2 to either RET or NTN (see scheme of GFRα-2 protein in Fig. 4*B*, *bottom*). The central domain (D2) of GFRα-2 protein is critical for ligand binding (24). The GFRα-2 isoform lacking exon 2 (GFRα-2b) was the least abundant isoform; it was seen at low levels in PBMCs activated with (LPS + IFN-γ) and weakly transcribed in the neuroblastoma cells.

FACS analysis showed that GFRα-2 protein was expressed by the lymphocytic fraction of PBMCs and even stronger by the monocytes (Fig. 5). Again, the neuroblastoma cell line TGW served as a positive control and the U937 cell line as negative control.

To quantitatively compare the expression of GFRα-1, GFRα-2, and GFRα-3 in PBMCs, we applied real time-PCR. The primers for GFRα-2 (exons 5–6) were located such that they detect all three isoforms expressed in immune cells (see above). The expression of GFRα-1, GFRα-2, and GFRα-3 was determined in PBMCs and purified immune cell subsets. GFRα-2 was clearly the predominantly expressed type of GFRα in PBMCs and $CD3^+$, $CD14^+$, and $CD19^+$ cells. Experiments with immune cells from two different donors gave essentially the same results; a representative experiment is shown in Fig. 6. We also compared the expression levels of the GFRαs detected in immune cells with those of the neuroblastoma cell line SHSY5Y, which is used as a model system to study biological effects of GFLs. Although the expression of GFRα-1 and GFRα-3 was several hundredfold lower in immune cells compared with the neuroblastoma cells, GFRα-2 expression reached the expression level detected in the studied neuroblastoma cells (Fig. 6). Six days after treatment with Con A or (LPS + IFN-γ), the activated immune cells displayed the same hierarchy of GFRα expression (GFRα-2 > GFRα-3, with GFRα-1 barely detectable) as resting PBMCs (data not shown).

Search for functional effects of GDNF and NTN on immune cells

Based on our findings suggesting that immune cell subsets express the GFL ligand NTN, as well as different isoforms of RET and GFR-α, we searched for potential functional effects of GFLs on immune cells. In a series of experiments, we added GDNF or NTN to resting PBMC or to PBMC activated with PHA, Con A, or (LPS + IFN-γ). We assessed proliferation, production of IL-4 or IFN-γ, and expression of the activation markers HLA-DR, CD25, CD40, CD69, and CD80. GDNF and NTN had no detectable effects on any of these responses or markers (data not shown).

There was, however, one functional effect detected during these experiments. We found that GDNF and NTN reduced the amount

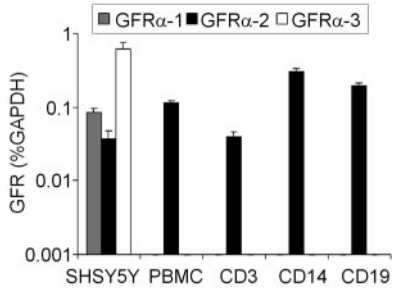

FIGURE 6. Quantification of GFRα expression. The expression of GFRα-1, GFRα-2, and GFRα-3 in PBMCs, purified $CD3^+$ T cells, $CD14^+$ monocytes, $CD19^+$ B cells, and the neuroblastoma cell line SHSY5Y was quantified by real-time PCR. The applied GFRα-2 primers could detect all three isoforms expressed in immune cells. Expression of GFRα-1, GFRα-2, and GFRα-3 of the indicated cell types is shown as percentage of the housekeeping gene GAPDH. Error bars represent the SD of triplicates. The expression of GFR-α1 and GFR-α3 was below 0.001% GAPDH.

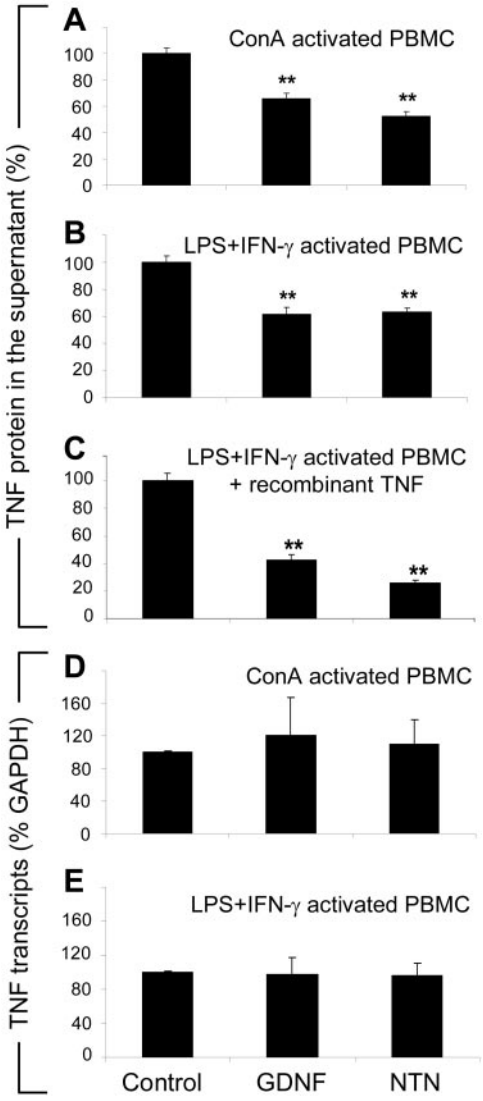

FIGURE 7. GDNF and NTN reduce TNF in the supernatant of activated PBMC without altering its transcriptional level. PBMC (2×10^6 cells/ml) were activated during 5 or 6 days with Con A or with (LPS + IFN-γ). GDNF or NTN was applied at a concentration of 100 ng/ml as indicated. *A* and *B*, GDNF or NTN was added to cells, which had been activated with Con A (*A*) or LPS + IFN-γ (*B*); 24 h later, supernatants were collected, and the amount of TNF was measured by ELISA. The TNF content in the medium/PBS-treated cells was set as 100%, and the relative TNF content in the other cultures was calculated. *A*, GDNF column represents the weighted mean of 19 different experiments ± weighted SD, and NTN column represents the weighted mean of 16 different experiments ± weighted SD. *B*, GDNF column represents the weighted mean of 17 different experiments ± weighted SD, and NTN column represents the weighted mean of 15 different experiments ± weighted SD. Both GDNF and NTN significantly reduced the TNF content in the culture medium (**, $p < 0.01$; Student's *t* test). *C*, PBMCs, activated with (LPS + IFN-γ) for 5 or 6 days, were washed, and either PBS (control), GDNF, or NTN was added together with fresh medium containing 2,000 pg/ml human recombinant TNF; 24 h later, the concentration of TNF was determined by ELISA. The TNF concentration in the control wells was set as 100%, and the relative TNF content in the GDNF- and NTN-treated wells was calculated. The weighted mean ± weighted SD of five different experiments is shown. Both GDNF and NTN significantly reduced the amount of exogenously added TNF in the cultures (**, $p < 0.01$, Student's *t* test). *D* and *E*, After 6 days of activation with Con A (*D*) or (LPS + IFN-γ) (*E*), GDNF or NTN was added; 24 h later, cDNA was prepared, and the TNF content in the supernatant was determined by ELISA. The level of TNF transcripts in relation to the housekeeping gene peptidyl-prolyl isomerase A was determined by real-time PCR. The TNF transcript level in control samples was set as 100% and that in the GDNF- or NTN-treated samples was calculated. Each column represents mean ± SEM of TNF transcripts of three (*D*) or four (*E*) different experiments. Although the TNF concentration in the supernatant of treated cells was reduced 1 day later in all these experiments (data included in *A* and *B*), amount of TNF transcripts measured in the corresponding cells did not show a significant difference.

of TNF in the supernatants of cultured T cell blasts. Specifically, when we added GDNF or NTN for 24 h to PBMCs that had been activated with Con A 5–6 days previously, the concentration of TNF in the supernatants was reduced ($p < 0.01$; Fig. 7*A*). This effect was seen in 17 of 21 experiments. Figure 7*A* shows the weighted mean of all these experiments. However, if we added GDNF or NTN at day 1 of culture, i.e., together with the activator Con A, then the TNF content of the supernatants was not detectably changed (data not shown).

To further explore this effect, we stimulated monocytes by adding (LPS + IFN-γ) to PBMC cultures. Again, when GDNF or NTN was added at day 5 or 6, the TNF content of these cultures was reduced after 24 h ($p < 0.01$; Fig. 7*B*). This reduction was seen in 18 of 22 separate experiments. Figure 7*B* shows the weighted mean of all of these experiments. Similar to the observations with activated T cells, GDNF and NTN did not reduce the TNF content when cells were treated at day 1 together with the activators LPS and IFN-γ (data not shown).

To learn whether the observed reduction in TNF content was due to a reduced production or enhanced uptake and/or consumption of TNF, we performed two types of experiments. First, PBMCs, which had been activated with (LPS + IFN-γ), were washed at day 6, and exogenous human recombinant TNF was added in the absence or presence of GDNF or NTN. Under these conditions, GDNF and NTN reduced significantly ($p < 0.01$) the concentrations of TNF measured 24 h later. The weighted mean of five such experiments is shown in Fig. 7*C*. Second, we examined the transcript levels of TNF by quantitative PCR. To this end, we added GDNF or NTN to PBMC at day 6 after stimulation with Con A or (LPS + IFN-γ). In four experiments with Con A-activated PBMC and in three experiments with (LPS+IFN-γ)-activated PBMC, the transcript levels of TNF were unchanged, although the TNF protein content in the supernatants was significantly reduced (Fig. 7, *D* and *E*).

Discussion

We demonstrate that different immune cell subsets, namely, $CD4^+$ T cells, $CD8^+$ T cells, B cells, and monocytes, are all capable of producing NTN, a member of the GFL. We could also detect the ligand-binding and signal-transducing components of the GFL receptors in immune cells. Notably, immune cells preferentially expressed GFRα-2, the receptor with the highest affinity for NTN. Finally, we found that both GDNF and NTN reduced TNF levels in supernatants of activated PBMCs at the posttranscriptional level.

NTN was present in resting PBMCs and was readily up-regulated after T cell activation with Con A or monocyte activation with (LPS + IFN-γ). This result is in harmony with previous reports, which described strong transcription of NTN mRNA in fetal peripheral blood and liver and in adult blood cells from rodents and human subjects (7). Using double-staining and fluorescence microscopy, we could localize NTN in $CD4^+$ and $CD8^+$ T cells, $CD19^+$ B cells, and $CD14^+$ monocytes. In contrast to NTN, GDNF, which is the prototype of the GFLs, was not expressed by any of these blood-derived immune cells. Although we did not

detect GDNF production by monocytes, other investigators described microglia as producers of GDNF (25, 26), suggesting the possibility that GDNF production is differentially regulated in microglia and blood monocytes.

Immune cells not only produced NTN but also expressed the corresponding receptors, indicating that immune cells might communicate via NTN. T cells, B cells, and monocytes expressed receptors for GFLs, namely, both the signal-transducing chain RET and the ligand-binding GFRα components. Quantitative PCR showed that T cells, B cells, and monocytes preferentially expressed GFRα-2, the receptor with the highest binding affinity for NTN. The expression level of GFRα-2 in immune cells reached about the same magnitude as in neuroblastoma cells, which are commonly used as a model system to study GFL effects.

RET is the common signal-transducing component of the GFL receptors. In all studied immune cell subsets, transcripts encoding a component of the extracellular part (cadherin-like domains 1–3) and a component of the intracellular part (tyrosine kinase domain 2) of RET were present. In addition, we observed a differential transcription of different isoforms of the 3′ end of the gene. Interestingly, $CD4^+$ and $CD8^+$ T cells expressed a different pattern of *RET* isoforms. Furthermore, in B cells and $CD4^+$ T cells, the transcribed isoforms of *RET* were regulated by activation. Previous studies had detected *RET* in monocytes, macrophages, granulocytes (12), and bone marrow hemopoietic cells (13). In T cells and B cells, *RET* transcripts had not been previously detected with a primer pair spanning exons 7–12 (12). With primers spanning exons 2–5 and 16–19, we found *RET* expression in all studied immune cell subsets. With primer combinations of the 3′ end of the gene (exons 19–21), we detected different *RET* isoforms in $CD4^+$ T cells, B cells, and monocytes. Further studies are needed to clarify whether splice variants lacking exons 7 or 12 in T cells and B cells might explain the different results. The functional relevance of the different isoforms of *RET* at the 3′ region for GFL-mediated effects on immune cells has to be elaborated in future studies. In sympathetic neurons, different signaling complexes were linked to RET-9 and RET-51 (27).

Regarding possible functional effects of GFLs on immune cells, we found that GDNF and NTN consistently reduced the concentrations of TNF protein in supernatants of PBMCs activated with Con A or (LPS + IFN-γ). In contrast, there was no significant reduction of TNF transcripts as measured by quantitative PCR. Furthermore, when we added exogenous TNF along with GDNF and NTN to (LPS + IFN-γ)-stimulated cells, the amount of TNF in the supernatants was again reduced, indicating that GDNF and NTN enhance the uptake or consumption of TNF. Together, these findings raise the possibility that GDNF and NTN influence TNF turnover at the posttranscriptional level.

The effect of GDNF and NTN on the TNF content of the supernatant was observed only when GDNF and NTN were added to activated but not to resting cells, although both expressed GFL receptors. Previous studies in neurons had identified cofactors that synergize with GFLs. For example, GDNF may cooperate with TGF-β (28, 29) and BDNF (30) as cofactors for survival of neuronal cells. Indeed, as shown previously, activated T cells and monocytes produce not only TGF-β but also BDNF (31). Moreover, in neuronal cells, cAMP elevation promotes GDNF-induced formation of lamellipodia (32). cAMP-elevating PGs are produced by activated macrophages. Thus, TGF-β, BDNF, and PGs are all candidate factors that might synergize with GFLs to regulate immune cell function; future studies are necessary to clarify the relative importance of these and other potential cofactors.

Recently, the neural cell adhesion molecule (NCAM; CD56) was identified as an alternative, RET-independent, signal-transducing receptor for GFLs (33). NCAM is not only expressed on neuronal cells but is known to be expressed also on NK cells and a subset of CTLs (34). We found NCAM expression after Con A or (LPS + IFN-γ) stimulation on $CD16^+$ NK cells and a subset of $CD8^+$ T cells, with a lower percentage also on $CD4^+$ T cells (data not shown). The relative contribution of RET and NCAM to GFL-mediated effects on immune cells is currently unknown.

Previous studies of the role of GFL in hematopoiesis (13, 21) and thymocyte development (14) did not describe GFL production by developing immune cells but rather by stroma cells. Bone marrow stroma cells expressed GDNF, GFRα-1, and GFRα-2. It was suggested that bone marrow stroma cells provide GDNF and soluble GFRαs, which might signal in RET-expressing hemopoietic cells (13, 21); however, the precise role of GFLs during hematopoiesis in the bone marrow has not been identified. The thymic stroma provides GDNF, which signals via GFRα-1 and RET expressed by $CD4^-CD8^-$ immature thymocytes (14). Expression of GFRα-1 and RET had been observed on rat microglia (35), and GDNF supported microglia survival and reduced NO production by microglia (36).

Immune cell infiltrates are found in the CNS of patients with inflammatory diseases such as multiple sclerosis. Furthermore, inflammatory cells may be found after trauma and stroke and, in small numbers, also in neurodegenerative diseases. Our findings demonstrating that activated human immune cells produce the neurotrophic factor NTN have several implications for the mutual interaction of immune cells and nerve cells. First, CNS-derived GDNF and NTN might influence immune cells in many other ways, which have yet to be discovered. Second, immune cell-derived production of NTN might confer neuroprotective effects on CNS cells, as have been observed in several previous studies (1, 2). In addition to NTN, GFRα-2 produced by immune cells also might exert neurotrophic functions because secreted GFRαs can function in *trans* and synergize with GFLs to mediate neuroprotection (5).

In conclusion, our data demonstrate that different immune cell subsets express the GDNF family ligand NTN and different isoforms of the GFL receptors RET and GFRα. Furthermore, NTN and GDNF modulate TNF turnover in immune cells at the post-transcriptional level. Within the nervous system, production of NTN and GFRα-2 by immune cells would be expected to exert certain neuroprotective functions, as have indeed been observed in different model systems.

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Disclosures

The authors have no financial conflict of interest.

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