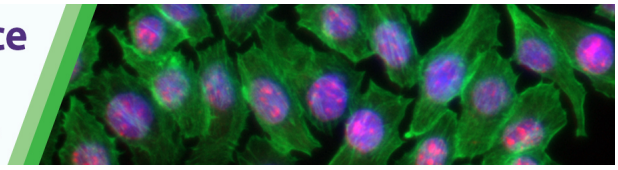

The Journal of Immunology

Cutting Edge: Clonally Restricted Production of the Neurotrophins Brain-Derived Neurotrophic Factor and Neurotrophin-3 mRNA by Human Immune Cells and Th1/Th2-Polarized Expression of Their Receptors

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Cutting Edge: Clonally Restricted Production of the Neurotrophins Brain-Derived Neurotrophic Factor and Neurotrophin-3 mRNA by Human Immune Cells and Th1/Th2-Polarized Expression of Their Receptors[1]

Michal Besser and Rudolf Wank[2]

Neurotrophins, such as neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF), are potent regulators of neuronal functions. Here we show that human immune cells also produce NT-3 mRNA, secrete BDNF, and express their specific receptors trkB and trkC. The truncated trkB receptor, usually expressed in sensory neurons of the central nervous system, was also constitutively expressed in unstimulated Th cells. Full-length trkB was detectable in stimulated PBMC, B cell lines, and Th1, but not in Th2 and Th0 cell clones. Clonally restricted expression was also observed for trkC, until now not detected on blood cells. The Th1 cytokine IL-2 stimulated production of trkB mRNA but not of trkC, whereas the Th2 cytokine IL-4 enhanced NT-3 but not BDNF mRNA expression. Microbial Ags, which influence the Th1/Th2 balance, could therefore modulate the neurotrophic system and thereby affect neuronal synaptic activity of the central nervous system. ***The Journal of Immunology,*** **1999, 162: 6303–6306.**

Cytokines function as intracellular messenger molecules. ILs are defined mainly through regulatory effects on immune cells, whereas neurotrophins are capable of regulating survival, differentiation, and maintenance of specific neuronal populations (1–5).

It has been demonstrated that the tyrosine kinase receptors trkA, trkB, and trkC mediate functions of several neurotrophin family members including nerve growth factor (NGF),[3] brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (6–8). Furthermore, the presence of NGF receptor trkA on activated T cells (9) and basophils (10) and the contribution of NGF to inflammatory processes (11) have been proved.

With the exception of trkB expression during T cell development (12), the information about the influence of the neurotrophins BDNF and NT-3 and their receptors on innate or acquired immunity is scarce. We therefore investigated whether PBMC could receive neurotrophin messages by expressing the specific receptors trkB and trkC and particularly which immune cell subpopulations, cell lines, or T cell clones were responsible for the receptor production. We were, furthermore, most interested in whether cytokines or neurotrophins could regulate trkB or trkC expression in bulk PBMC. This could indicate whether microbial Ags, stimulating production of Th1 immune cytokines, e.g., IL-2 and IFN-γ, or Th2 immune cytokines (IL-4) could affect receptor expression. Finally, we tested whether those cytokines could stimulate immune cells to produce BDNF and NT-3 mRNA and thereby influence neuronal adaptive responses.

Materials and Methods

Cell cultures

Human PBMC were purified from whole blood by Ficoll gradient (Amersham Pharmacia Biotech, Freiburg, Germany). Th cells were isolated by using CD4 mAbs coupled to magnetic beads (Dynal, Hamburg, Germany). T cells or PBMC were cultured at $1–2 \times 10^6$ cells/ml either in the presence of 1% PHA (Difco, Hamburg, Germany), 20 μg of anti-CD3 (OKT-3, Cilag, Sulzbach, Germany)-precoated plates in combination with IL-2 (20 U/ml) or IL-4 (100 U/ml), or with 100 ng/ml neurotrophins (Prepro, London, England), 20 U/ml IL-2 (Chiron, Ratingen, Germany), 100 U/ml IL-4 (Genzyme, Ruesselsheim, Germany) or 500 U/ml IFN-γ (Thomae, Bicherach an der Riss, Germany) alone. RPMI 1640 culture medium (Life Technologies, Eggenstein, Germany) was supplemented with 10% FCS (Life Technologies).

Cloning of T lymphocytes

Alloprimed PBMC were seeded at 0.4 cell/well on allogeneic specific feeder layer. Clonality was confirmed by FACS analysis; T cell clones 234 and 305 and EM-17 were also recloned at a cell concentration of 0.1 cell/well. Constitutive expression in immune cell bulk cultures was assessed immediately after cell separation, in cloned T lymphocytes after 3 days without further addition of IL-2.

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[3] Abbreviations used in this paper: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; B-LCL, EBV-transformed lymphoblastoid B cell line; NT-3 (-4), neurotrophin-3 (-4).

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ELISA

For cytokine-ELISA assays, 2.5×10^5 $CD4^+$ clone cells were cultured together with 2.5×10^5, 80-Gy-irradiated, specific stimulating EBV-transformed lymphoblastoid B cell lines (B-LCL)[3] in 2 ml of RPMI 1640 containing 10% FCS. Cell culture supernatants were collected after 24 h. IL-4 and IFN-γ ELISA (PharMingen, Hamburg, Germany) was performed according to the manufacturer's protocols. For BDNF ELISA, cells were irradiated with different doses (0, 5, 10, 20, 30, 40, 60 Gy) and cultured at a density of 5×10^5 per 2 ml of RPMI 1640 containing 10% FCS. Cell culture supernatants were collected after 1 and 3 days. BDNF ELISA kit was purchased from Promega (Mannheim, Germany).

Reverse transcription and amplification of cDNA

Total RNA from cultured cells was prepared following RNAzol B (Biozol, Eching, Germany) protocol. After treating RNA with DNase I (Boehringer Mannheim, Mannheim, Germany) for 30 min of repeated precipitation, first-strand cDNA was synthesized from 7 μg of total RNA in a 50-μl final incubation volume by using RAV-2 reverse transcriptase (Pharmacia Amersham Biotech) and oligo$(dT)_{12-18}$ primer (Life Technologies). PCR was conducted in a 50-μl reaction mixture containing 3 μl of the above first-strand cDNA, 5 μl of 10× PCR buffer (Amersham Pharmacia Biotech), 3 μl of 10 mM dNTP mix, 2 μl of each primer (10 pmol/μl), 2 μl of standard primers (1 pmol/μl), and 2 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech). The sequence of the primers (5′–3′) and length of the product were as follows. trkB gp145: upstream primer, CAACCCGCCCACGGAACTGA; downstream primer, CTCATGTGGGGCTCTCGCTG, 464 bp; trkB gp95: upstream primer, GTTTCATAAGATCCCACTGGATGG; downstream primer, TGCTGCTTAGCTGCCTGAGAGTTA, 260 bp; trkC: upstream primer, GGAGTCCAAGATCATCCATGTGGA; downstream primer, CATTCCAAATTTGGACCGTCGACC; 336, 363 bp; BDNF: upstream primer, TACTTTGGTTGCATGAAGGCTGCC; downstream primer, ACTTGACTACTGAGCATCACCCTG; 266 bp; NT-3: upstream primer, GTATCTCATGGAGGATTACGTGGG; downstream primer, TGTTCTCTGAAGTCAGTGCTCGGA; 343 bp; GAPDH: upstream primer, AATTCCATGGCACCGTCAAG; downstream primer, GCCTGCTTCACCACCTTCTT, 631 bp.

Conditions for the PCR were 94°C for 1 min, 65°C for 30 s, and 72°C for 30 s (35 cycles) using a PTC-200 Thermocycler (Biozym, Oldendorf, Germany).

Sequencing

DNA/cDNA sequencing was performed with an automated sequencer (ALF, Amersham Pharmacia Biotech) to confirm the identity of the PCR products trkB gp145, trkC, BDNF, and NT-3.

FACS analysis

Cells were incubated for 30 min with 2 μg/ml CD4-Tri-color-labeled Ab (Medac, Hamburg, Germany) in PBS containing 1% FCS and 0.1% sodium azide. After the cells were washed with PBS, they were fixed for 1 h in 4% paraformaldehyde, washed 4 times in PBS containing 0.1% saponin (Sigma, Deisenhofen, Germany), and incubated with 10 μg/ml anti-trkB gp95 (Santa Cruz, Ismaning, Germany) polyclonal rabbit Ab, or alternatively with 10 μg/ml rabbit IgG (Sigma) at 4°C for 30 min. After four washings with 0.1% saponin/PBS, the cells were incubated with 8.8 μg/ml fluorescein-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) for 30 min. All Abs were suspended in PBS with 0,1% saponin. After fixation with 1% paraformaldehyde, cells were analyzed in a flow cytometer (Becton Dickinson, Heidelberg, Germany).

Results

Unstimulated Th cells express constitutively the truncated trkB receptor

Our experiments with human bulk immune cells using unstimulated and stimulated PBMC showed that unstimulated PBMC expressed only the tyrosine kinase-lacking form (gp95) of the BDNF high affinity receptor trkB. The full length (gp145) trkB receptor was not expressed (not shown). FACS analysis for gp95 trkB revealed that 12% of all unstimulated PBL were positive (Fig. 1*a*). This gp95 trkB-stained cell fraction was at the same time positive for CD4 (Fig. 1*a*). To confirm the gp95 trkB expression in $CD4^+$ Th cells, PBMC subpopulations were separated by using Abs against cluster-defined molecules coupled to magnetic beads. The isolated CD4-positive (+) Th cells, $CD8^+$ cytotoxic cells, $CD14^+$ monocytes, and $CD19^+$ B lymphocytes were subsequently analyzed by RT-PCR, and the resulting PCR products were sequenced. Among these unstimulated subpopulations, only $CD4^+$ T cells constitutively expressed gp95 trkB mRNA (Fig. 1*b*). This picture changed dramatically when PBMC were stimulated with PHA or anti-CD3 Abs; PBMC, even if depleted from $CD4^+$ T cells, expressed now the truncated trkB receptor (Fig. 1*b*). After activation with anti-CD3 or PHA, separated $CD8^+$, $CD14^+$, as well as $CD19^+$ cells were able to produce the gp95 trkB mRNA (data not shown).

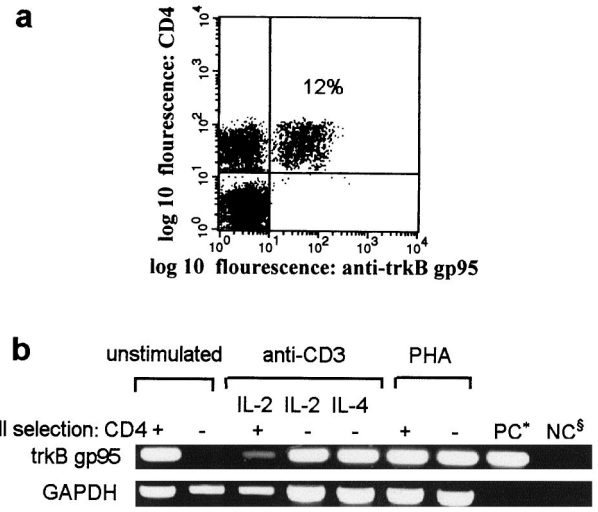

FIGURE 1. Expression of trkB gp95 on PBMC. *a*, FACS analysis of unstimulated lymphocytes. PBL were stained with anti-trkB gp95 polyclonal rabbit Ab and CD4 Ab. Twelve percent of the Th cells carried the receptor. *b*, Expression of trkB gp95 mRNA in CD4-positive (+) and CD4-depleted (−) PBMC. Cells were either unstimulated or stimulated with the following: PHA (1%).or anti-CD3 (20 μg) in combination with 20 U/ml IL-2 or 100 U/ml IL-4. Only unstimulated $CD4^+$ cells expressed the receptor mRNA, whereas after stimulation $CD4^+$ as well as $CD4^-$ cells were positive for trkB gp95 mRNA. PC*, positive control (cDNA of neuroblastoma cell line SMS-KCN); NC§, negative control (no cDNA added). The constitutively expressed GAPDH served as a PCR and gel-loading control.

$CD4^+$ T cell clones expressed the truncated trkB receptor independently from IL profiles corresponding to the Th0 type (represented by clones 234 and 403), Th1 type (represented by clones 200, 305, 407, 411, and 425), and Th2 type (represented by clone 401) (Fig. 2*a*). Expression of the truncated trkB receptor was also observed in the $\gamma\delta^+$ T cell clone EM-17 and in B-LCL (Fig. 2*a*). Among blastoid cell lines, the monocyte line MM1 (13) was the notable exception not expressing gp95 trkB (Fig. 2*a*).

Influence of cytokines on the gp145 trkB expression

Induction of the full-length trkB receptor in bulk cultures required stimulation of immune cells with PHA or anti-CD3 Abs in combination with IL-2. To investigate whether IL-2 alone, other cytokines, or neurotrophins could regulate full-length trkB expression in PBMC, immune cells were incubated with IL-2, IL-4, IFN-γ, BDNF or NT-4 for 1 day. Only IL-2, but not IL-4, IFN-γ, BDNF, or NT-4, could up-regulate the full-length trkB receptor in PBMC (Fig. 3).

Th1/Th2 polarized trkB gp145 receptor expression

Following this observation, we examined whether gp145 trkB receptor expression differed between Th1 and Th2 T cell clones. Interestingly, only five of eight T cell clones showed constitutive trkB gp145 mRNA expression. These five clones (200, 305, 407, 411, and 425) were identified as Th1 clones, producing very little or no IL-4 but high levels of IFN-γ (Fig. 4). The remaining three

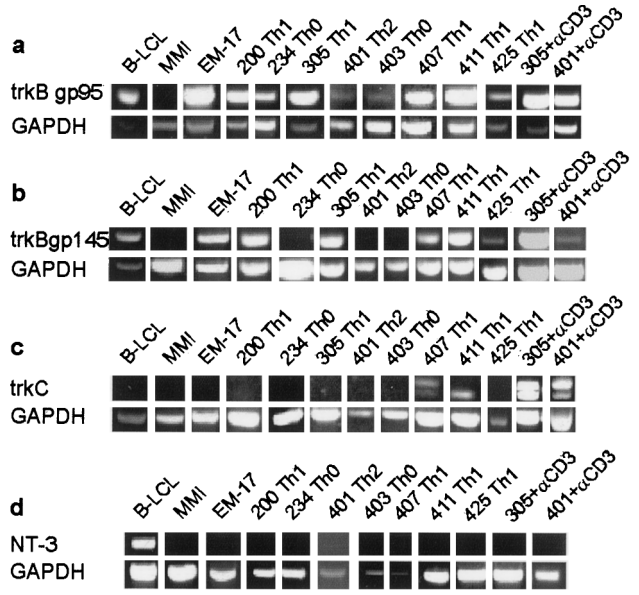

FIGURE 2. Amplification of trkB, trkC, and NT-3 mRNA in various cell lines and clones by RT-PCR. trkB gp95 mRNA (*a*),trkB gp145 mRNA (*b*), trkC mRNA (*c*), and NT-3 mRNA (*d*) expression in B-LCL, the monocyte cell line MM1, the $\gamma\delta^-$ clone EM-17, and $CD4^+$ T cell clones 200, 234, 305, 401, 403, 407, 411, and 425. The $CD4^+$ clones 305 and 401 were also stimulated with anti-CD3 (20 μg) and 20 U/ml IL-2 for 3 days. The constitutively expressed GAPDH served as a PCR and gel-loading control.

trkB gp145-negative Th clones exhibited Th2-type (401) or Th0-type (234, 403) cytokine profiles (Fig. 4). The full-length trkB receptor was furthermore expressed by the $\gamma\delta$T cell clone EM-17 and the B-LCL but not by the monocyte line MM1 (Fig. 2*b*).

Expression of trkC requires activation of immune cells

According to current documentation, the NT-3 receptor trkC is found to be in almost all tissues of the body except in PBMC (14). However, in our findings, trkC was discovered in immune cells. We analyzed the presence of trkC by RT-PCR using a primer pair, with one primer binding to the extracellular and the other to the intracellular domain of trkC. On sequencing the PCR products, we detected both forms of trkC that have been described within this region, one with an additional insert of 24 amino acids (14). Neither form was detectable in unstimulated PBMC, and addition of IL-2 or IL-4 alone had no effect on trkC up-regulation (not shown). However, stimulation of PBMC with PHA or with anti-CD3 Abs induced trkC mRNA levels (not shown). Stimulation of the CD3 coreceptor up-regulated trkC expression in all T cell clones tested

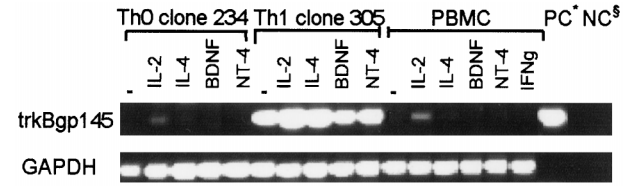

FIGURE 3. Influence of different cytokines on trkB gp145 expression. trkB gp145 mRNA was amplified by RT-PCR. Th0 clone 234, Th2 clone 305, and PBMC were treated with IL-2 (20 U/ml), IL-4 (100 U/ml), BDNF (100 ng/ml), NT-4 (100 ng/ml), IFN-γ (500 U/ml), or with medium alone (-) for 24 h. PC*, positive control (cDNA of the neuroblastoma cell line SMS-KCN was added), NC§, negative control (cDNA of PC-12 cells, not expressing the trkB mRNA, was added). The constitutively expressed GAPDH served as a PCR and gel-loading control.

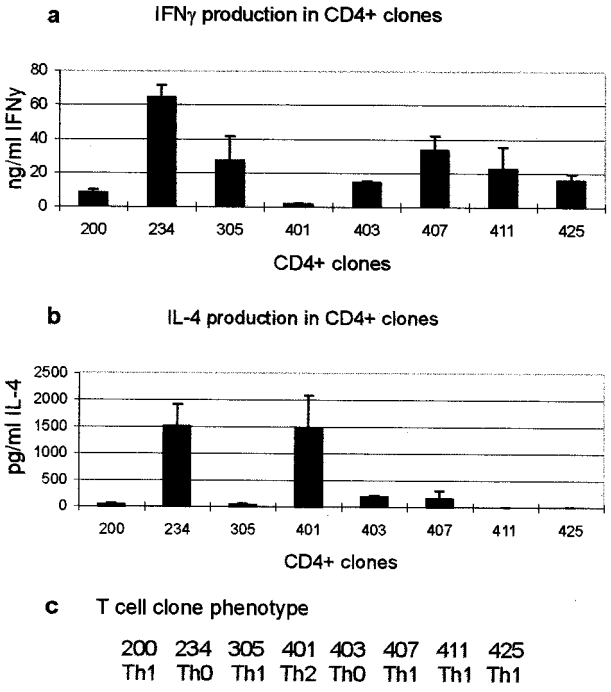

FIGURE 4. Phenotyping of $CD4^+$ Th clones according to their secretion of IL-4 and IFN-γ determined by ELISA of cell culture supernatants after 24 h. *a*, IFN-γ production as measured in nanograms per ml. *b*, IL-4 production in pg/ml. Data represent the mean $\pm$ SEM of eight experiments. *c*, Clones producing mainly IL-4 were assigned a Th2 phenotype; clones producing mainly IFN-γ as Th1 and clones secreting both cytokines in higher amounts were assigned a Th0 phenotype.

(Fig. 2*c*). Only two of the $CD4^+$ T cell clones, 407 and 411, showed constitutively weak expression of trkC (Fig. 2*c*). T cell clone 411 expressed the trkC-splicing variant that lacks the 24-amino acid insert (Fig. 2*c*). The $\gamma\delta^+$ T cell clone EM-17, B-LCLs, and the monocyte line MM1 did not express trkC (Fig. 2*c*).

BDNF and NT-3 production in immune cell subpopulations

We found NT-3 mRNA expression in PHA-stimulated and IL-4 treated PBMC, but not in anti-CD3-stimulated, IL-2-treated, or unstimulated immune cells (not shown). NT-3 mRNA expression was detectable neither in T cell clones nor in the monocyte cell line MM1 (Fig. 2*d*). As indicated by the NT-3 mRNA production in PBMC after IL-4 treatment, NT-3 mRNA was expressed in B-LCL (Fig. 2*d*).

From the strong expression of BDNF mRNA in immune cells, we hoped to find sufficient BDNF concentrations in cell culture supernatants to be recorded by ELISA. We had to consider, however, autocrine consumption of BDNF by cells constitutively expressing truncated trkB, e.g., $CD4^+$ T cell clones and B-LCL. Production of cytokines coupled with the prevention of autocrine consumption has been reported by using low dose irradiation of immune cells (15). We therefore irradiated immune cells at the beginning of various culture periods using different irradiation doses (only 3-day culture period and 0 and 20 Gy shown (Fig. 5)). In most T cell clones, irradiation with 20 Gy did not change cell culture supernatant BDNF levels (Fig. 5), indicating that in these T cell clones BDNF was probably not only produced for autocrine but also for paracrine consumption. Conversely, BDNF was first detectable in B-LCL R.E. and in T cell clone 411 following irradiation, indicating that autocrine consumption of low BDNF concentrations was prevented by irradiation. Similarly, higher concentrations of BDNF were detectable in 3-day cell culture supernatants of MM1 cells after irradiation (Fig. 5).

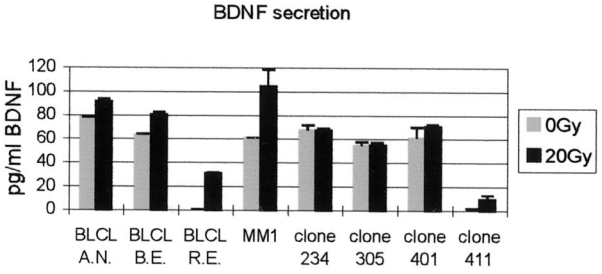

FIGURE 5. Effect of irradiation on BDNF secretion. BDNF levels in pg/ml were determined by ELISA for three untreated and 20-Gy-irradiated B-LCL (A.N., B.E., R.E.), the monocyte cell line MM1, and four Th clones (234, 305, 401, 411). The cell culture supernatants were collected 3 days after irradiation. Data represent the mean ± SEM of three experiments.

Discussion

In our results, we could demonstrate that specific PBMC subpopulations express mRNA for the neurotrophins BDNF and NT-3 and their receptors trkB and trkC. Whether PBMC-bound neurotrophins affect PBMC function or decrease the supply of neurotrophins for neurons and thereby affect the neurotrophin balance in the nervous system, still needs to be examined. Especially CD4-positive lymphocytes constitutively expressing truncated trkB receptor could function as a BDNF buffer/supply system. The truncated trkB receptor may also use novel signaling pathways (16, 17).

Since immune cells could secrete BDNF or NT-3, they could possibly communicate back to neurons expressing trkB or trkC. Neurotrophin-secreting immune cells might show autocrine and paracrine effects in the local microenvironment in which there are produced; e.g., it has been observed that BDNF plays a role in the repair of peripheral nerve injury (18). Therefore, neurotrophin-secreting immune cells that can be recruited after nerve injury might effect nerve repair.

Furthermore, it has been demonstrated that environmental factors, such as microbial Ags, can dictate on one hand development and expansion of distinct T helper subsets and on the other hand induce secretion of distinct ILs (19) and influence adaptive neuronal responses (20–21). Our finding that only Th1 but not Th0/Th2 clones constitutively expressed full-length trkB, and the divergent influence of the Th1 cytokine IL-2 and the Th2 cytokine IL-4 on neurotrophin and receptor expression, might be one possibility to explain this phenomenon.

In addition, we could show that expression of BDNF and NT-3 mRNA and of their receptors can be influenced by ILs: IL-2 stimulates production of trkB gp145 mRNA but not of trkC mRNA, whereas IL-4 stimulates expression of NT-3 mRNA but not of BDNF. Furthermore, it has been demonstrated that BDNF and NT-3 enhance the release of transmitters from neurons (22, 23) and that their influence on synaptic efficacy and neuronal plasticity affects behavior in rats (24, 25). This might shed new light on the importance of elevated IL-2 concentrations in cerebrospinal fluid of schizophrenic patients (26), the predictive value for relapses of such elevated IL-2 concentrations after neuroleptic treatment (27), and the increased IL-4 concentrations in pediatric neuropsychiatric cases (28). Symptoms of paranoia and dementia in AIDS could be influenced by the depletion of CD4 T lymphocytes, constitutively expressing the gp95 trkB receptor.

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