



Production of Neurotrophins by Activated T Cells: Implications for Neuroprotective Autoimmunity

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Neurotrophins (NTs) promote neuronal survival and maintenance during development and after injury. However, their role in the communication between the nervous system and the immune system is not yet clear. We observed recently that passively transferred activated T cells of various antigen specificities home to the injured central nervous system (CNS), yet only autoimmune T cells specific to a CNS antigen, myelin basic protein (MBP), protect neurons from secondary degeneration after crush injury of the rat optic nerve. Here we examined the involvement of NTs in T-cell-mediated neuroprotection, and the possible significance of the antigen specificity of the T cells in this activity. Analysis of cytokine and NT expression in various rat T cell lines showed that the T cells express mRNA for cytokines of Th1, Th2, and Th3 phenotypes. In addition, the T cells express mRNA and protein specific to nerve growth factor, brain-derived neurotrophic factor, NT-3, and NT-4/5. Antigen activation significantly increased NT secretion. Thus, reactivation of CNS autoimmune T cells by locally presented antigens to which they are specific can lead to enhanced secretion of NTs and possibly also of other factors in injured optic nerves. mRNA for TrkA, TrkB and p75 receptors was expressed in the injured nerve, suggesting that these specific receptors can mediate the effects of the T-cell-derived NTs. The neuroprotective effect of the passively transferred autoimmune anti-MBP T cells in injured optic nerves was significantly decreased after local application of a tyrosine kinase inhibitor known to be associated with NT-receptor activity. These results suggest that the neuroprotective effect of autoimmune T cells involves the secretion of factors such as NTs by the T cells reactivated by their specific antigen in the injured CNS. T cell intervention in the injured CNS might prove to be a useful means of promoting post-injury CNS maintenance and recovery, possibly via supply of NTs and other factors.

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Introduction

We recently demonstrated that although the central nervous system (CNS) is a site of immune privilege, in which immune responses are normally limited, it can nevertheless benefit from immune activity for its protection and recovery after injury. More specifically, we showed that the T cell response to axonal injury is much more limited in the CNS than in the peripheral nervous system (PNS); significantly more endogenous T cells were found to accumulate in injured PNS axons than in injured CNS axons [1]. Moreover, elimination of T cells via apoptosis occurred extensively in the injured CNS, but only to a very small extent in the injured PNS [1]. Systemic injection of activated T cells

of different antigen specificities immediately after CNS injury resulted in an increased accumulation of T cells at the injury site [2, 3]. Notably, injection of activated T cells specific to a CNS self-antigen myelin basic protein (MBP), but not to non-CNS antigens, reduced the secondary degeneration of neurons after a primary crush injury of CNS axons [3]. This neuroprotective effect induced by the autoimmune T cells was found to be similar whether the T cells are weakly or strongly encephalitogenic [3].

The mechanism underlying the neuroprotective effect of autoimmune anti-MBP T cells in the injured CNS has not yet been established. However, several lines of evidence implicate cytokines and neurotrophic factors as mediators in the reciprocal relationship between the immune and nervous systems. The therapeutic application of neurotrophins (NTs) prevents neuronal degeneration after axotomy and other forms of neuronal injury [4]. In addition, beneficial

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effects of NTs have been reported in animal models of neurodegenerative disease [5]. We were therefore interested in determining whether the T cells that confer neuroprotection after optic nerve crush injury (a model for CNS axonal injury) do so by providing a natural source of imported NTs.

Neurotrophins are biologically active proteins that promote neuronal survival through receptor-mediated processes and are thought to participate in nervous system development, maintenance, and response to trauma. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 have all been shown to enhance neuronal survival *in vitro* and *in vivo*. For example, NGF is required for the survival of sympathetic and some sensory and cholinergic neuronal populations [6], BDNF prevents the death of motoneurons in newborn rats after nerve transection [7] and rescues spinal cord motoneurons from axotomy-induced cell death [8], and NT-4/5 increases adult rat retinal ganglion cell (RGC) survival and neurite outgrowth [9, 10]. The responsiveness of target cells to a given NT is governed by the expression of two classes of NT receptors: the low-affinity receptor p75, which binds all NTs with similar affinity [11], and the high-affinity tyrosine kinase receptors of the Trk family, which interact with NTs in a specific manner. TrkA is the receptor for NGF, TrkB is the receptor for BDNF and NT-4/5, and TrkC is the main receptor for NT-3 [12, 13]. Specific binding of the NT to its receptor causes receptor dimerization and autophosphorylation. These processes can activate multiple signal transduction pathways depending on the cell type [14], leading to different biological functions.

Accumulating evidence suggests that the NTs, in addition to their neurotrophic effects, participate in the inflammatory response. Recently, NGF and the NGF receptor TrkA were shown to be expressed in activated CD4-positive T cell clones [15, 16]. NGF is also expressed in B cells [17], in macrophages and microglia [18, 19], in mast cells [20], in eosinophils [21], and in basophils [22]. NT mRNA is expressed in spleen and thymus [23] and in inflamed tissue [24]. Recent studies have shown that activated human T cells, B cells, and monocytes produce BDNF *in vitro* and in inflammatory brain lesions [25], and that human stimulated peripheral blood mononuclear cells express TrkB and TrkC receptors [26]. The production of NTs by immune cells provides a novel example of the commonality of the nervous and the immune systems. Some NTs have been shown to modulate certain functions of the immune system. For example, NGF was recently shown to influence lymphocyte proliferation and differentiation [27, 28], stimulate immunoglobulin production [28], and induce mast cell degranulation [29]. Both NT-3 and BDNF induce proliferation and phagocytic activity of microglia [18].

In the present study we show that rat T cells expressing cytokines of Th1, Th2, and Th3 phenotypes produce various NTs and secrete them upon antigen activation. The optic nerve was found to express receptors for TrkA, TrkB, and p75. The neuroprotective effect mediated by autoimmune T cells in the

injured optic nerve appears to involve T cell-secreted factors such as NTs, as shown by the fact that local application of K252a, a tyrosine kinase-associated inhibitor of signal transduction [30–33], partially prevented this neuroprotective effect. These findings suggest that T-cell autoimmunity specific to CNS myelin antigens can, under certain circumstances, be beneficial in CNS trauma via neurotrophic factor supply.

Materials and Methods

Animals

Inbred female adult Lewis rats (8–12 weeks old) were supplied by the Animal Breeding Center of The Weizmann Institute of Science. The rats were housed in a light- and temperature-controlled room and matched for age in each experiment. Animals were handled according to the regulations formulated by IACUC (Institutional Animal Care and Use Committee).

Antigens

MBP from the spinal cords of guinea-pigs was prepared as described [34]. Ovalbumin (OVA) was purchased from Sigma (St Louis, MO, USA). Peptide 277 (p277) of the human 60-kDa heat shock protein (hsp60) (sequence VLGGGCALLRCPALDSLTPA NED) [35] was synthesized by the 9-fluorenylmethoxycarbonyl technique using an automatic multiple peptide synthesizer (AMS 422, ABIMED, Langenfeld, Germany). The purity of the peptide was analysed by HPLC and amino acid composition.

Antibodies (Abs)

Rabbit polyclonal Abs raised against amino acids 1–20 of rat NGF, amino acids 128–147 of rat BDNF, and amino acids 139–158 of rat NT-3 were purchased from Santa Cruz (Santa Cruz, CA, USA). Mouse monoclonal anti-rat T cell receptor (TCR) Ab [36] was provided by Dr Boris Reizis. Rhodamine goat anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (with minimal cross-reaction to rat, human, bovine and horse serum proteins), and phycoerythrin (PE) donkey anti rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

T cell lines

T cell lines were generated from draining lymph node cells obtained from Lewis rats immunized with the above antigens [37]. The antigen was dissolved in phosphate-buffered saline (PBS) (1 mg/ml) and emulsified with an equal volume of incomplete

Freund's adjuvant (IFA) (Difco Laboratories, Detroit, MI, USA) supplemented with 4 mg/ml *Mycobacterium tuberculosis* (Difco). Ten days after the antigen was injected into the rats' hind footpads in 0.1 ml of the emulsion, the rats were killed and the draining lymph nodes were surgically removed and dissociated. The cells were washed and activated with the antigen (10 µg/ml) in stimulation medium containing Dulbecco's modified Eagle's medium (DMEM) supplemented with l-glutamine (2 mM), 2-mercaptoethanol (5×10^{-5} M), sodium pyruvate (1 mM), penicillin (100 IU/ml), streptomycin (100 µg/ml), non-essential amino acids (1 ml/100 ml), and autologous serum 1% (volume/volume). After incubation for 72 h at 37°C, 90% relative humidity and 7% CO₂, the cells were transferred to propagation medium consisting of DMEM, l-glutamine, 2-mercaptoethanol, sodium pyruvate, non-essential amino acids, and antibiotics in the same concentrations as above, with the addition of 10% fetal calf serum (FCS) (volume/volume) and 10% T-cell growth factor derived from the supernatant of concanavalin A (ConA)-stimulated spleen cells [38]. Cells were grown in propagation medium for 4–10 days before being restimulated with their antigen (10 µg/ml) in the presence of irradiated (2000 rad) thymus cells (10^7 cells/ml) in stimulation medium. The T-cell lines were expanded by repeated stimulation and propagation [39].

Crush injury of optic nerve

Crush injury of the optic nerve was performed as previously described [40]. Briefly, rats were deeply anaesthetized by intraperitoneal (ip) injection of Rompun (xylazine, 10 mg/kg; Vitamed, Israel) and Vetalar (ketamine, 50 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA, USA). Using a binocular operating microscope, lateral canthotomy was performed in the right eye, and the conjunctiva was incised lateral to the cornea. After separation of the retractor bulbi muscles, the optic nerve was exposed intraorbitally by blunt dissection. Using calibrated cross-action forceps, the optic nerve was subjected to a moderate crush injury 1–2 mm from the eye. The uninjured contralateral nerve was left undisturbed.

Immunocytochemistry

Activated T cells were separated from irradiated thymus cells using a Ficoll gradient (Pharmacia Biotech, Uppsala, Sweden), followed by centrifugation for 20 min at 2000 rpm. The interphase was collected and washed three times in PBS. The blood-derived T cells were purified using a Uni-Sep_{Maxi+} kit (Novamed, Jerusalem, Israel), which is based on density separation of lymphocytes. This was followed by passage through a nylon wool column (Novamed), exploiting the property of B cells, but not T cells, to adhere to nylon wool. The cells were fixed with ethanol for 10 min, washed three times with PBS, permeabilized with acetone for 3 min, washed twice

with PBS, and resuspended in PBS containing 3% FCS and 2% bovine serum albumin (BSA). The cells were then incubated for 1.5 h at 37°C with the primary Ab. For the neutralization assay, the antigen-blocking peptide (Santa Cruz) was added to the sample at a peptide:Ab rate of 5:1 (by weight), according to the manufacturer's instructions. The cells were washed three times in PBS containing 0.05% polyoxyethylene sorbitan–monolaurate (Tween-20), then incubated with the second Ab for 1 h at room temperature. The cells were again washed three times with PBS containing 0.05% Tween-20, and then analysed by fluorescence-activated cell sorter (FACS) analysis or viewed with a Zeiss Universal fluorescence microscope. In the latter case, the cells were treated with glycerol containing 1,4-diazobicyclo-(2,2,2)octane to inhibit quenching of fluorescence.

FACS analysis

The immunostained cells were resuspended in 0.4 ml of PBS and analysed by FACScan, with at least 1700 events scored. In single-colour analysis, positive cells were defined as cells with greater immunofluorescence, on a logarithmic scale, than that of control cells incubated with second Ab only, or with second Ab in the presence of the non-corresponding first Ab as a cross test. The cells were scored from a region defined according to physical parameters that indicate the size (forward scatter) and granularity (side scatter) of lymphocytes.

Reverse transcriptase–polymerase chain reaction (RT–PCR)

To examine the expression of NTs and cytokines, activated rat T cells were separated from irradiated thymus cells as described above, and total RNA was extracted from the activated T cells in each cell line using the commercial TRI reagent (Molecular Research Center, Cincinnati, OH, USA). To examine NT receptor expression, total RNA was isolated from rat optic nerves 7 days after injury, as well as from normal nerves (using the TRI reagent, Molecular Research Center), according to the manufacturer's instructions. First-strand cDNA synthesis reaction was carried out as follows: 1 µg of RNA and 3.5 mM oligo (dT) primer were incubated at 65°C for 5 min and then chilled on ice, after which 0.5 mM dNTP mixture, AMV–RT (Promega, Madison, WI, USA), and reverse transcriptase buffer were added, according to the manufacturer's instructions. The mixture was incubated at 42°C for 1 h, then at 94°C for 2 min, and placed on ice. From the generated complementary DNA, a volume of 2–4 µl was amplified in a total volume of 50 µl, with 2.5 U of taq-zol (Tal-Ron, Rehovot, Israel), 0.2 mM dNTP mixture, and 50 pmoles of each primer. The primer sequences are listed in Table 1. For the PCR amplification, we used the thermocycler Cetus with the following settings: 94°C for 3 min for RNA denaturation, followed by

Table 1. Details of primers used in the study

Gene	Product size (bp)	Direction	Sequence
IL-4	294	Forward (F) Reverse (R)	TGCTTTTCATATGTACCGGG TGAGTTCAGACCGCTGACAC
IL-6	371	F R	ACTGCCTTCCCTACTTCAC GTATTGCTCTGAATGACTCTG
IL-10	329	F R	GAGTGAAGACCAGCAAAGGC TCGCAGCTGTATCCAGAGG
TGF- β	411	F R	AAGGAGACGGAATACAGG TGTGTTGGTTGTAGAGGG
IFN- γ	405	F R	ATGAGTGCTACACGCCGCGTCTTGG GAGTTCATTGACTTTGTGTCTGG
NGF	271	F R	TGCTGAACCAATAGCTGCC ATCTCCAACCCACACACTGAC
BDNF	574	F R	GCTGACACTTTTGAGCAC AAATCCACTATCTTCCCC
NT-3	510	F R	CTCTCTCAATTCCCTCATTATC GCTTCTTTACACCTCGTTTC
NT-4/5	580	F R	CCCTGCGTCAGTACTTCTTC TTTCTCGTCTCTCTTGCC
TrkA	571	F R	CACTAACAGCACATCAAGAGAC GAAGACCATGAGCAATGGG
TrkB	188	F R	TCATTGGGATGACCAAGATCC GCAGAGGTTATAGCACTCC
TrkC	503	F R	CTAATATTGAGTCGCTGTTGTGG TGATCCTTGTGGATGGACAGCCAC
P75	447	F R	TGCAGTGTGCAGATGTGCCTATGGC AGGAATGAGCTTGTCGGTGGTGCCG

35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 60°C, and elongation for 2 min at 72°C. The amplified products were electrophoresed on 1.5% agarose gel, stained with ethidium bromide and photographed under UV light. A band of each NT and Trk receptor was purified from the gel using the GenElute[®] kit (Suplenco, Bellefonte, PA, USA), and sequenced to confirm its specificity.

Enzyme-linked immunosorbent assay (ELISA)

Anti-MBP T cells were grown for a week in a propagation medium, then washed with PBS and resuspended in stimulation medium. The T cells (0.5×10^6 cells/ml) were incubated in the presence of irradiated thymocytes (10^7 cells/ml), with ConA (1.25 μ g/ml), or with MBP antigen (10 μ g/ml), or with OVA antigen (10 μ g/ml), or with no antigen, in stimulation medium at 37°C, 90% relative humidity and 7% CO₂. In addition, irradiated thymocytes (10^7 cells/ml) alone were incubated in stimulation medium. After 48 h the cells were centrifuged and their supernatants were collected and sampled. Concentrations of NT-3, NGF, and NT-4/5 in the samples were determined by the use of sandwich ELISA kits (Promega) and comparison with an NT standard (absorbance measurement at 450 nm using an ELISA reader). BDNF concentrations in the samples were determined with a sensitive sandwich ELISA. In brief, 96-well flat-bottomed plates were coated with a chicken anti-

human BDNF Ab (Promega) in 0.025 M NaHCO₃ and 0.025 M Na₂CO₃ (pH 8.2). Recombinant human BDNF (used as standard; Research Diagnostics, Flanders, NJ, USA) was used in serial dilutions in blocking solution containing 3% BSA, 0.05% Tween-20, and 1% FCS in PBS (pH 8.2). Bound BDNF was detected by incubating the plates with a mouse anti-human BDNF Ab (Research Diagnostics) followed by peroxidase-conjugated goat anti-mouse IgG (Jackson Immuno-Research) in blocking solution. The plates were developed using a 3,3',5,5'-tetramethylbenzidine liquid substrate system (Sigma). The reaction was stopped by adding 1 M H₃PO₄, and the optical density was determined at 450 nm. Results for each experiment were calculated as the amount of secreted NT per 1 ml of sample, after subtraction of the background levels of the irradiated thymocytes incubated with the stimulation medium.

Measurement of secondary degeneration by retrograde labelling of retinal ganglion cells

Secondary degeneration of the optic nerve axons and their attached RGCs was measured after post-injury application of the fluorescent lipophilic dye, 4-(4-(didecylamino)styryl)-N-methylpyridinium iodide (4-Di-10-Asp) (Molecular Probes Europe BV, Netherlands), distally to the site of injury, 2 weeks after the primary injury. Only axons that are intact are capable of transporting the dye back to their cell

bodies. Therefore, application of the neurotracer dye distally to the site of the primary crush after 2 weeks ensures that only axons that survived both the primary damage and the secondary degeneration will be counted. This approach makes it possible to differentiate between neurons that are still functionally intact and neurons in which the axons are injured but the cell bodies are still viable, because only those neurons whose fibres are morphologically intact can take up dye applied distally to the site of injury and transport it to their cell bodies. Using this method, the number of labelled TGCs reliably reflects the number of still-functioning neurons. Labelling and measurement were carried out as follows: the right optic nerve was exposed for the second time, again without damaging the retinal blood supply. Complete axotomy was performed 1–2 mm from the distal border of the injury site and solid crystals (0.2–0.4 mm diameter) of 4-Di-10-Asp were deposited at the site of the newly formed axotomy. Five days after dye application the rats were killed. The retina was detached from the eye, prepared as a flattened whole mount in 4% paraformaldehyde solution, and examined for labelled RGCs by fluorescence microscopy.

Administration of K252 compounds

Sterile stock solutions (10 mM) of K252a and K252b were prepared in dimethyl sulfoxide, stored at -20°C , and diluted in PBS before use. One day after right optic nerve injury and injection of T cells or PBS, each rat received one drop of $1\ \mu\text{M}$ K252a, K252b, or vehicle in the right eye, three times a day for 13 days. On day 14, the neurotracer dye 4-Di-10-Asp was applied to the optic nerves distally to the site of injury, and 5 days later the retinas were examined for labelled RGCs (reflecting still-viable axons).

Clinical evaluation of experimental autoimmune encephalomyelitis (EAE)

To examine for the presence and severity of EAE, rats injected with activated anti-MBP T cells were scored every 1–2 days according to the following neurological scale: 0, no abnormality; 1, tail atony; 2, hind limb paralysis; 3, paralysis extending to thoracic spine; 4, front limb paralysis; 5, moribund state.

Results

mRNA for cytokines of Th1, Th2, and Th3 phenotypes, and for the NTs NGF, BDNF, NT-3, and NT-4/5, is expressed in rat T-cell lines

Because passive transfer of T cells specific to a CNS self-antigen MBP, but not of T cells specific to the non-CNS antigens p277 or OVA, exerts a neuroprotective effect [3], we examined whether these T-cell lines differ in their cytokine mRNA profiles and whether

they transcribe NT mRNA. The anti-MBP, anti-p277 and anti-OVA T-cell lines were activated with their respective antigens for 2 days and total RNA was then extracted from the lymphoblasts of each line. Qualitative RT-PCR was then carried out, using primers specific for the various cytokines and NTs. The three tested T-cell lines, anti-MBP (T_{MBP}) (Figure 1A), anti-p277 (T_{p277}) (Figure 1B), and anti-OVA (T_{OVA}) (Figure 1C), all expressed cytokines of Th₁ [interferon- γ (IFN γ), and interleukin-2 (IL-2) which is not shown], Th₂ (IL-10 and IL-6), and Th₃ [transforming growth factor- β (TGF β)] subsets. In addition, all three T-cell lines expressed NT-3, NGF, NT-4/5 and BDNF mRNA. The PCR product of each NT was sequenced and its identity confirmed. A PCR control reaction for each NT and cytokine, containing all the components except the complementary DNA, was negative.

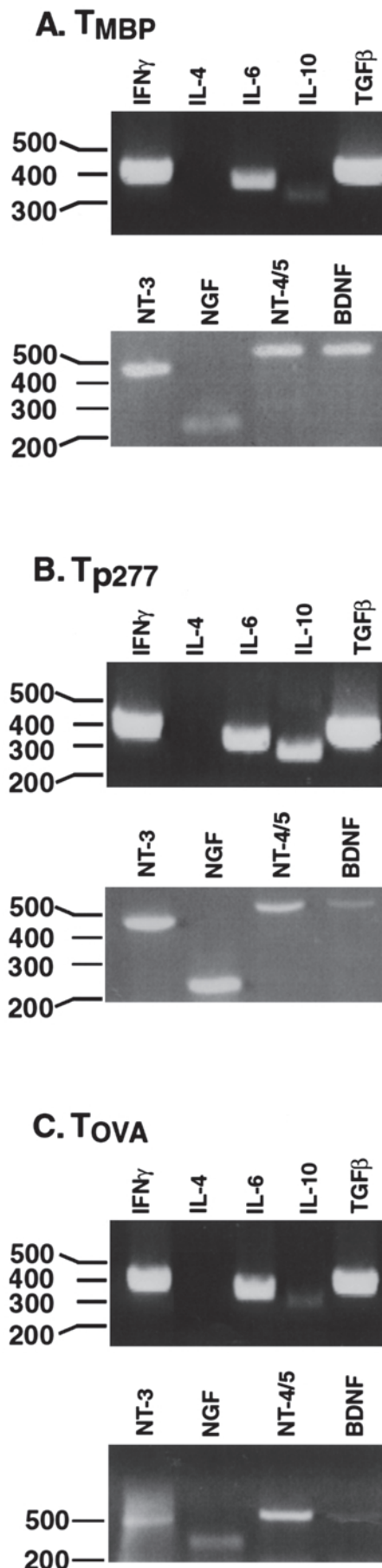
Activated rat T cells produce NT proteins

To determine whether the NT mRNA is translated into a corresponding protein in the activated T cells, we performed immunocytochemistry for NTs. Activated rat anti-MBP, anti-p277, and anti-OVA T cells were immunostained with rabbit Abs directed against rat NGF, BDNF, or NT-3, detected by rhodamine goat anti-rabbit IgG. As shown in Figure 2, the T cells expressed NGF, BDNF, and NT-3 immunoreactive proteins. The cells were viewed and photographed with a fluorescence microscope using a filter that detects rhodamine (Figure 2, left), and using phase contrast (Figure 2, right). Staining in the absence of the first Ab was negative (–) (Figure 2). Binding of the first Ab was neutralized with the corresponding blocking peptide, yielding negative staining.

To examine the incidence of NT-producing cells in the T-cell lines, we used FACS analysis. Activated anti-MBP, anti-p277, and anti-OVA T cells were immunostained for BDNF, NT-3, or NGF, and in each case also for TCR. The FACS analysis is presented in Figure 3A and the definition of quadrants in Figure 3B. FL₁ indicates FITC staining for TCR and FL₂ indicates PE staining for the specific NT. Regardless of antigen specificity, in each T cell line almost all of the cells that were stained for TCR (right side of each plot) were also stained for NT (upper right of each plot) (Figure 3A). These results indicate that the three tested T cell lines produce NTs, and that the incidence of T cells expressing NTs is high and is similar in all three lines.

Blood-derived T cells express intracellular NTs

The T cell lines were cultured by repeated stimulations and propagations *in vitro*. To determine whether non-manipulated T cells also express NTs, we isolated T cells from the blood of naive Lewis rats. One day later we subjected the cells to immunostaining for BDNF, NT-3, or NGF and in each case also for TCR, and then subjected them to analysis by FACS. As



shown in Figure 4, naïve blood-derived T cells were stained for NGF, BDNF, and NT-3. The percentage of T cells that were stained for NTs (upper right of each plot in double staining) was higher than 80%, suggesting that the high prevalence of intracellular NT expression in T cells is not exclusive to particular T cell lines. To examine the specificity of the staining, binding of the first Abs to their corresponding blocking peptides was performed. Neutralization of the anti-NT Abs by addition of the blocking peptides led to a marked decrease in staining (Figure 4), indicating that the observed staining is specific.

Secretion of NTs by T cells is significantly increased upon activation

Next we examined whether the T cells also secrete NTs, and if so, whether the NT secretion depends on antigen activation. Since autoimmune anti-MBP cells, unlike anti-p277 or anti-OVA T cells, are the only ones capable of recognizing their antigen (MBP) and hence becoming reactivated in the injured optic nerve, we used the anti-MBP T cell line in this experiment. T cells can be activated by polyclonal mitogens or by their specific antigen. Therefore, supernatants were collected after 48 h from activated anti-MBP T cells that were incubated with the polyclonal mitogen ConA or with their specific antigen MBP, and from non-activated anti-MBP T cells that were incubated with a non-specific OVA antigen or with no antigen. The supernatants were then subjected to sensitive sandwich ELISA. The NTs are known to function physiologically at extremely low (picomolar) concentrations. The activated T cells secreted NGF, NT-3, NT-4/5, and BDNF proteins (Figure 5). However, significantly higher levels of each of these proteins were secreted by the activated anti-MBP cells than by the non-activated anti-MBP T cells, indicating that NT secretion by T cells is induced by activation. The non-specific antigen (OVA) induced no more NT secretion than that obtained by the anti-MBP T cells alone. This finding demonstrates that secretion of NTs by T cells is significantly increased by activation, and suggests that autoimmune anti-MBP T cells—unlike T cells with other antigen specificities—upon recognizing their specific antigen at the site of the damaged optic nerve, can be reactivated to secrete substantial amounts of NTs.

Figure 1. Expression of mRNA for cytokines and NTs in activated rat T cell lines. Anti-MBP T cells (T_{MBP}), anti-p277 T cells (T_{p277}), and anti-OVA T cells (T_{OVA}) were activated with their respective antigens for 2 days. Total RNA was extracted from the lymphoblasts, and qualitative RT-PCR was performed using primers specific for various cytokines and NTs. T_{MBP} (A), T_{p277} (B) and T_{OVA} (C) cells expressed mRNA for cytokines of Th1 (IFN γ), Th2 (IL-10 and IL-6), and Th3 (TGF β) subsets, as well as for the NTs NT-3, NGF, NT-4/5, and BDNF.

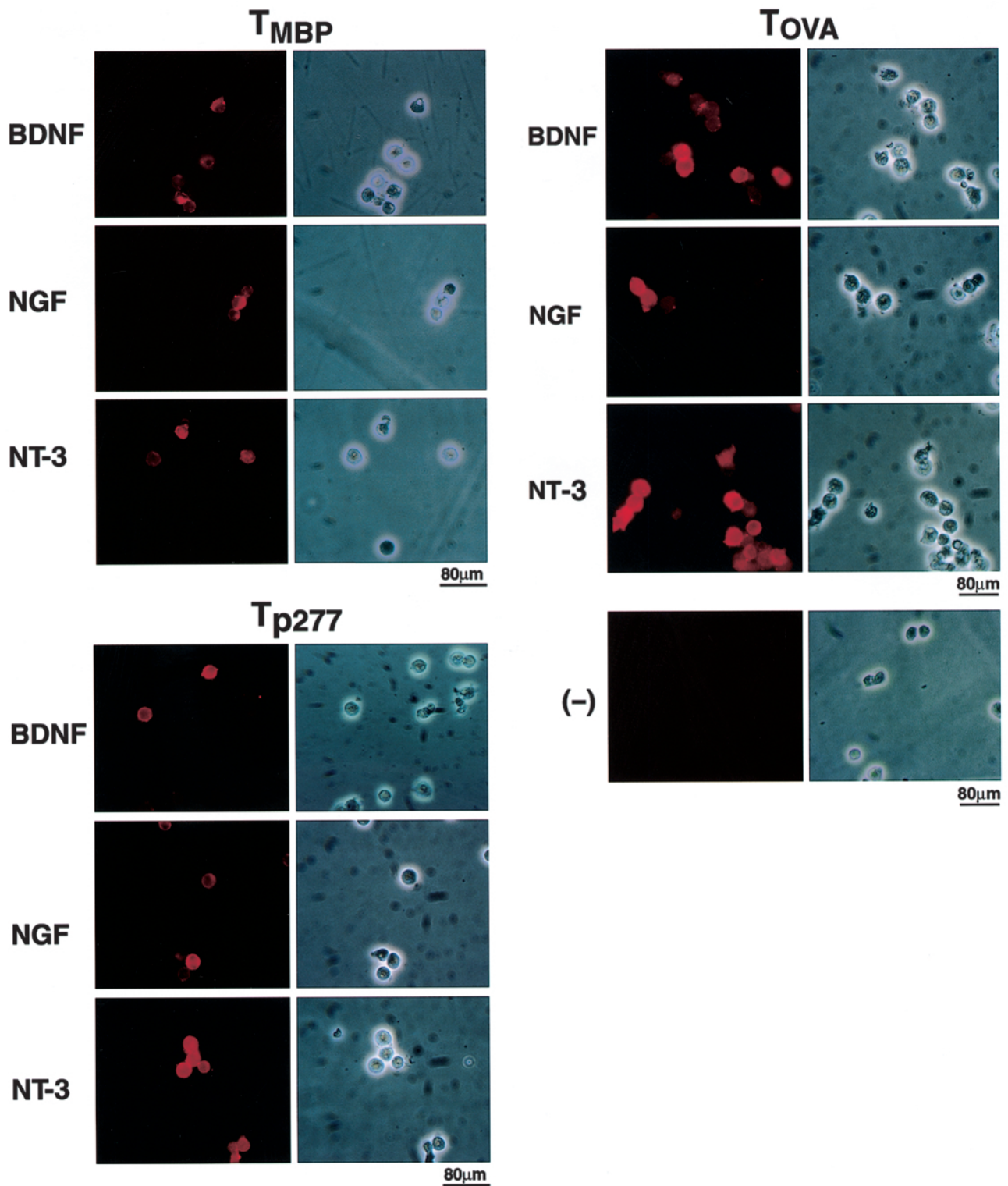


Figure 2. Detection of NTs in the T cells by immunocytochemistry. The rat T cell lines T_{MBP} , T_{p277} , and T_{OVA} were activated with their respective antigens for 3 days, and then immunostained with rabbit Abs directed against rat NGF, BDNF, or NT-3, detected by rhodamine goat anti rabbit IgG. The cells were viewed and photographed with a fluorescence microscope, using a filter that detects rhodamine (left), and using phase contrast (right). Staining in the absence of the first Ab (-) was negative.

mRNA for TrkA, TrkB, and p75 receptors is expressed in the optic nerve

To express their physiological effects, the NTs should recognize their specific tyrosine kinase receptors of the Trk family and/or the low-affinity p75 receptor [41].

In a previous study we demonstrated that auto-immune T cells exert a neuroprotective effect in rat injured optic nerves [3]. We were therefore interested in determining whether mRNA for Trk or p75 receptors is expressed in the optic nerve, a potential site of

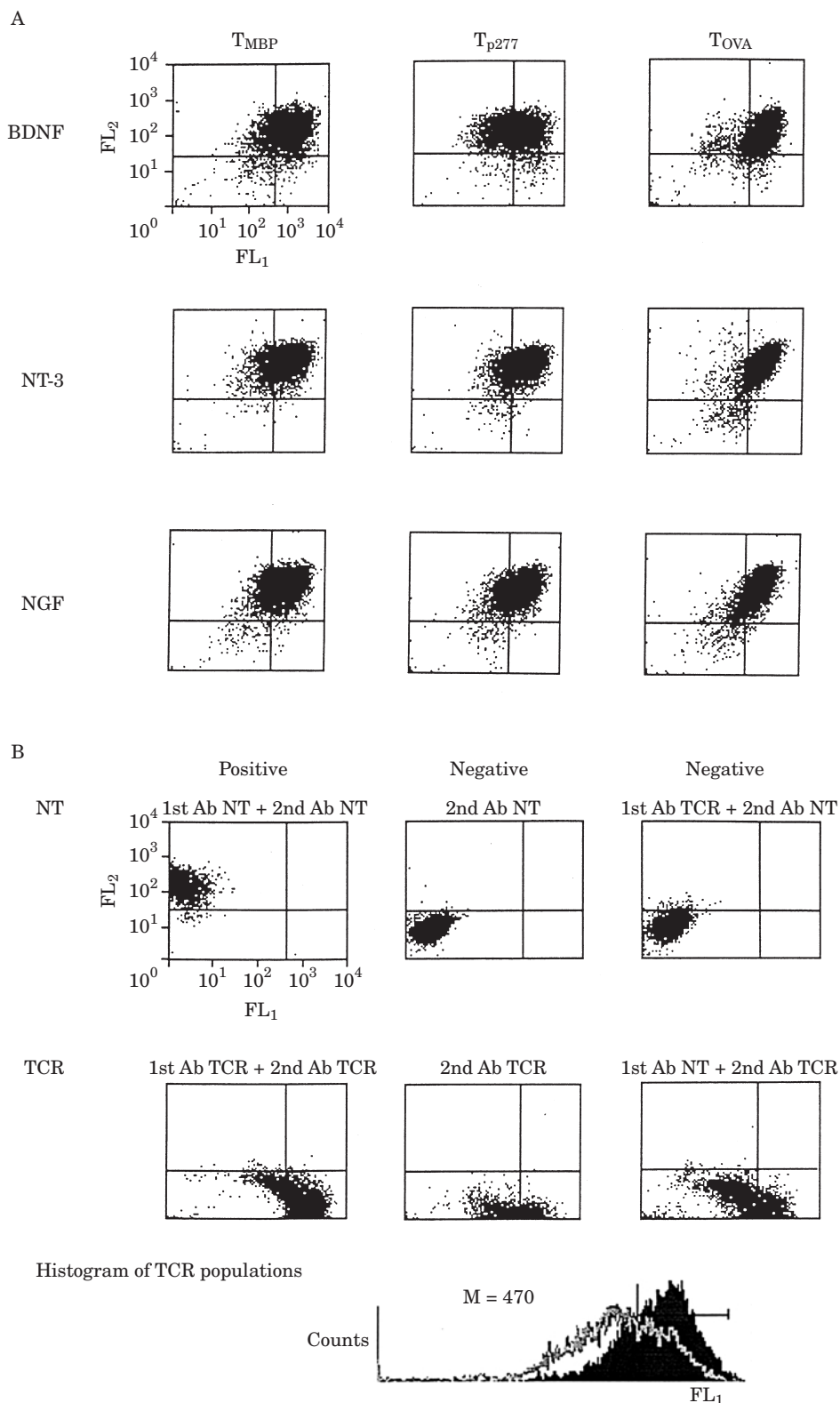


Figure 3. Flow cytometric analysis for the expression of NTs by activated T cells. A. Activated rat T_{MBP} , T_{p277} , and T_{OVA} cells were immunostained for BDNF, NT-3, or NGF, and in each case also for TCR, and then analysed by FACS. FL_1 indicates FITC staining for TCR, and FL_2 indicates PE staining for the specific NT. About 70% of the cells were double-stained. Irrespective of their antigen specificity, almost all cells that showed staining for TCR (right side of each plot) also showed staining for NT (upper right of each plot). B. Definitions of quadrants. To define the cell populations that showed staining for NT or for TCR, a single staining was done for each of them. The populations were then defined according to the positive and negative controls. For each single staining the positive control, with the corresponding first and second Abs, is shown on the left. The negative controls were stained with second Ab only (middle), and as a cross test, cells were stained with the corresponding second Ab, in the presence of the non-corresponding first Ab (right). Because of overlapping of the TCR-stained and TCR-unstained populations, they were distinguished using the histogram (bottom).

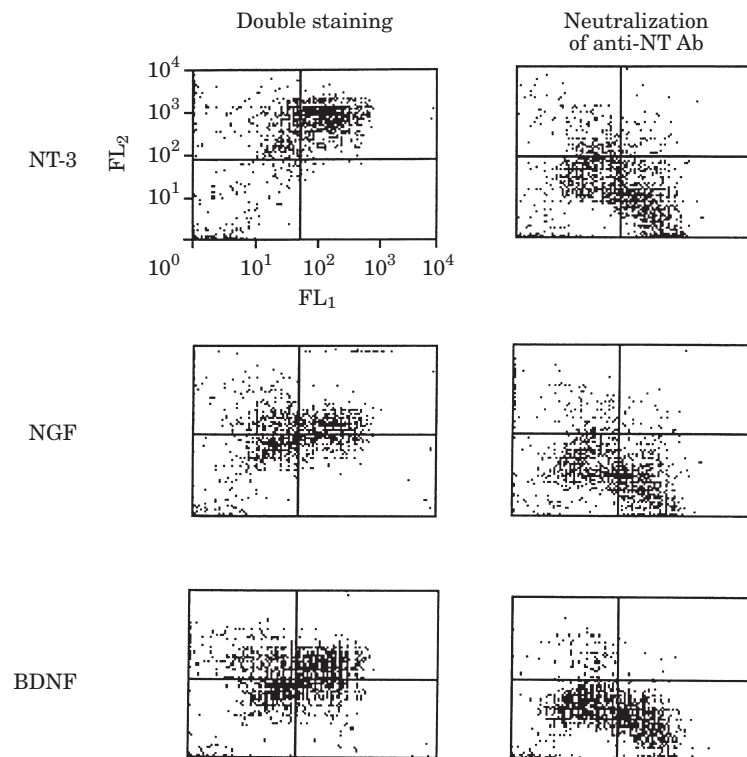


Figure 4. Flow cytometric analysis of blood-derived T cells for expression of NTs. T cells were derived from rat blood by passage through a Percoll gradient and then through a nylon wool column. The cells were immunostained for NTs and TCR and then analysed by FACS. FL₁ indicates FITC staining for TCR and FL₂ indicates PE staining for the specific NT. About 50% of the cells were double-stained. A high proportion of the cells that were stained for TCR (right side of each plot) also showed staining for NT (upper right of each plot). For each NT a neutralization assay was carried out, using the commercial blocking peptides of the first Abs, to confirm their specificity. The results can be seen by comparing the plots on the left side (double staining) with those on the right (neutralization of anti-NT Ab).

interaction with T cell-derived NTs. Total RNA was extracted from normal optic nerves and from injured optic nerves 7 days after moderate crush injury. The RNA was then subjected to qualitative RT-PCR, using primers specific for TrkA, TrkB, TrkC, and the p75 receptor. Both the normal and the injured optic nerves were found to express mRNA for TrkA, TrkB, and p75 receptors, but not for the TrkC receptor (Figure 6). A positive control PCR for TrkC receptor was performed on total RNA isolated from rat brain (data not shown). For each NT receptor we performed a negative control PCR, in which all the components except the complementary DNA were present. The PCR product of TrkA and TrkB receptors was sequenced and its identity confirmed. The finding that mRNA for TrkA, TrkB, and p75 receptors is expressed in the optic nerve points to the ability of T cell NGF, BDNF, NT-4/5, and possibly also NT-3 to interact with their receptors and mediate their biological effects on neuronal survival in the injured optic nerve.

Blockade of tyrosine kinase signalling diminishes the neuroprotective effect of autoimmune anti-MBP T cells in the injured optic nerve

K252a and K252b are microbial alkaloids that block signal transduction by preventing autophosphoryl-

ation of the tyrosine kinase domain of several receptors, including the NT receptors [30–33]. Both K252a and K252b are hydrophobic, but K252a, being irreversibly concentrated in intracellular membranes, is more potent than K252b [42]. To determine whether blocking signal transduction of tyrosine kinase receptors (e.g., NT receptors) influences the neuroprotective effect of autoimmune anti-MBP T cells at and near the optic nerve lesion site, we performed the following experiment. Immediately after undergoing moderate unilateral optic nerve crush injury (right side), rats were injected ip with 10^7 activated anti-MBP T cells or with PBS, and a drop of $1\ \mu\text{M}$ K252a, K252b, or vehicle was instilled in the right eye, three times a day, from day 1 after injury until day 14. On day 14, RGCs were retrogradely labelled and the mean numbers of surviving RGCs with intact axons were calculated. In addition, the rats were scored every 1–2 days for clinical EAE. Neither the course of EAE nor the disease severity (Figure 7A) was affected by the daily eye-drop treatment with K252a or K252b, eliminating the possibility of any systemic influence of K252 on T-cell infiltration into the CNS. After daily treatment with vehicle eye drops, the mean numbers of surviving RGCs were significantly greater in the retinas of rats injected with anti-MBP T cells than in rats injected with PBS, demonstrating the neuroprotective effect of the autoimmune T cells. However, the mean numbers

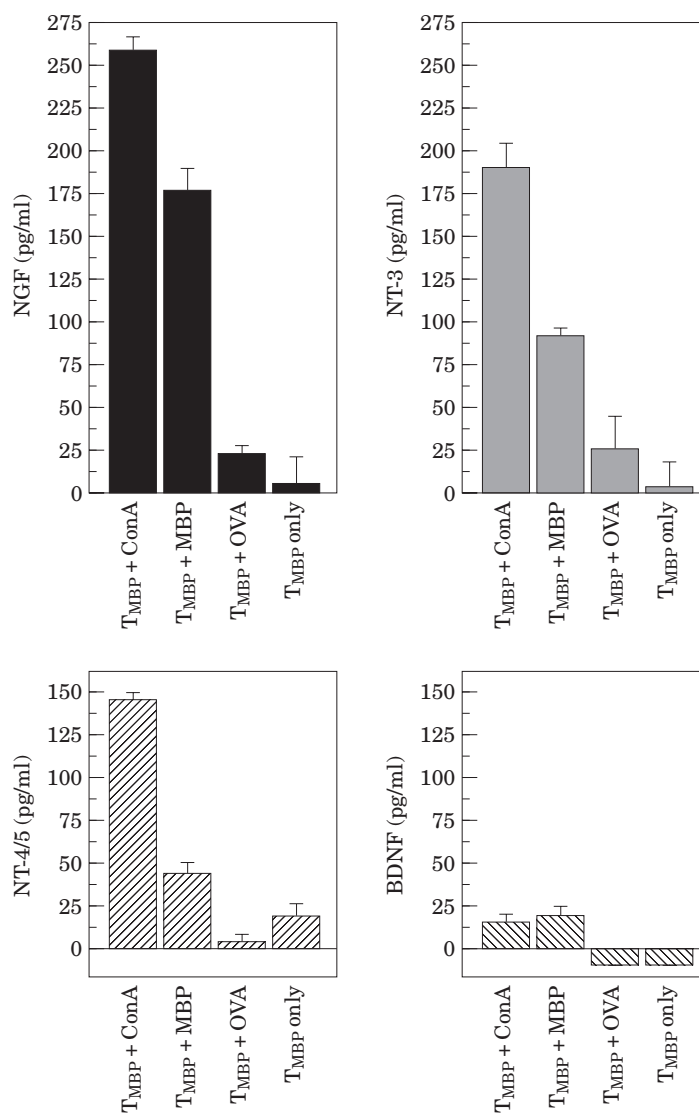


Figure 5. ELISA of secreted NTs. Rat anti-MBP T cells were cultured for 48 h in stimulation medium with ConA, MBP, OVA, or no antigen. The supernatants of the T cells were collected and subjected to sandwich ELISA. The histogram shows the concentration of secreted NTs \pm SD in each sample. The amount of secreted NTs was significantly higher in the supernatants of activated anti-MBP T cells that were stimulated with ConA or with their specific antigen MBP, than in non-activated anti-MBP T cells that were incubated with the non-specific antigen OVA or with no antigen ($P < 0.01$ or $P < 0.001$ for all NTs, one-way ANOVA followed by Bonferroni's multiple comparison *t*-test). There were no significant differences in amounts of secreted NTs between the anti-MBP T cells that were incubated with OVA and with no antigen.

of surviving RGCs were significantly lower in the retinas of rats injected with anti-MBP T cells and treated daily with K252a eye drops than in those injected with anti-MBP T cells and treated daily with the vehicle eye drops (Figure 7B). No significant effect of K252b eye drops on the mean numbers of surviving RGCs was observed in the rats injected with anti-MBP T cells. Likewise, no effect of K252a or K252b eye drops on the mean numbers of surviving RGCs was observed in PBS-injected rats (Figure 7B), suggesting that the inhibitory effect of K252a in the rats injected with anti-MBP T cells is associated with T cell-derived factors. This finding shows that local blockade of tyrosine kinase receptor signalling reduces RGC survival in the injured optic nerves of rats injected with anti-MBP T cells, and supports the contention

that secreted factors such as NTs mediate, at least in part, the neuroprotective effect of the autoimmune T cells.

Discussion

Neurotrophins are essential for neuronal survival and maintenance during the processes of development and maturation [6, 43] and for the regulation of neurotransmitter release and dendritic growth [44–46]. Several studies have shown that administered NTs can rescue injured or degenerating neurons and induce axonal outgrowth and regeneration [7, 8, 47–49]. While NTs have been studied intensively in

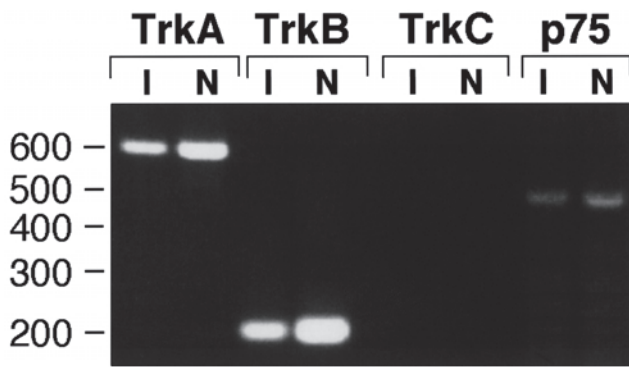


Figure 6. Expression of mRNA for TrkA, TrkB and p75 receptors in the rat optic nerve. Total RNA was extracted from normal and injured optic nerves, and qualitative RT-PCR was performed using primers specific to the various NT receptors. Normal (N) and injured (I) optic nerves expressed mRNA for TrkA, TrkB, and p75 receptors, but not for TrkC receptor.

the nervous system, little is known about their role in the interaction between the nervous and the immune systems. We showed here that rat T cells express mRNA and protein of NGF, BDNF, NT-3, and NT-4/5 and that secretion of the NT proteins by the T cells is significantly increased by antigen activation. We also demonstrated that the neuroprotective effect of the autoimmune T cells is significantly decreased after local application of K252a in injured optic nerves. These results suggest that the neuroprotection mediated by autoimmune T cells might be attributable, at least in part, to their ability to supply the injured optic nerve with various factors such as NTs. Several studies have suggested that some of the NTs are protective only in the short-term [50–51] and that some (NGF and NT-3) do not promote survival [9] in the optic nerve. These studies cannot be compared to studies demonstrating the physiological effect of the autoimmune anti-MBP T cells, since the latter are present at the injury site for a prolonged period [1, 2] and are able to continuously secrete a variety of factors, such as cytokines and NTs, which are locally regulated by signalling molecules presented to them by the damaged tissue. It is conceivable that these factors work in synchrony, in accordance with the dialogue between the immune cells and the tissue, and in a way that cannot be mimicked by the artificial delivery of a single NT or by cells genetically modified to overexpress NT.

Neurotrophins bind to and activate specific receptor tyrosine kinases of the Trk family [12, 13, 41]. The interaction of NGF with TrkA receptor, and of BDNF and NT-4/5 with TrkB receptor, is rather specific. However, although NT-3 primarily activates TrkC receptor [52], it can induce moderate activation of TrkA [53] and TrkB receptors [54–56], at least in certain cell culture systems [57]. In addition to binding a specific Trk, each of these four NTs (NGF, BDNF, NT-3, and NT-4/5) interacts with a common receptor, the low-affinity p75 receptor [58, 59]. This receptor is primarily expressed on NT-responsive cells and has been shown to play a role in apoptosis [60, 61] and cell

migration [62]. Despite its ability to interact specifically with each of the NTs, p75 appears to be neither necessary nor sufficient for many aspects of NT signalling. Nevertheless, the presence of p75 may modulate the cellular response to NTs [63]. For example, the presence of p75 appears to enhance the sensitivity of the response of TrkA to NGF [64, 65]. Our finding that TrkA, TrkB, and p75 receptors are expressed in the injured optic nerve supports the possibility that T cell-derived NGF, NT-4/5, BDNF, and perhaps NT-3 can mediate a neuroprotective action at the lesion site. Local blockade of tyrosine kinase-receptor signalling by K252a indeed caused a marked attenuation of the neuroprotective effect of the autoimmune anti-MBP T cells in injured optic nerves. These findings suggest that secretion by the autoimmune T cells of factors whose activity is mediated by signal transduction involving tyrosine kinase receptors, such as NTs, is responsible—at least in part—for their neuroprotective effect.

Although K252a is a non-selective inhibitor of tyrosine kinase activity, at low concentrations it specifically inhibits Trk receptors and blocks TrkA, TrkB, and TrkC with approximately equal efficacy, while leaving intact other tyrosine kinase signalling pathways, as well as the protein kinase C pathway [30–33]. Since the Trk receptors can be expressed in axons and/or glial cells of the optic nerve [66], the T cell-derived NTs may act directly on the RGCs or on glial cells that can indirectly affect neuronal survival. For example, T cell-derived NTs may locally induce microglia to release a second NT [67]. Several studies have indeed shown that NTs induce secretion of NTs [68, 69], and that inflammatory cytokines such as IL-1 β and tumour necrosis factor- α stimulate microglial NGF transcription and protein release [70]. Thus, NTs and/or cytokines released by T cells can stimulate microglial cells to secrete NTs. Regardless of whether the NT is released directly by the T cells or indirectly by the glial cells, after the NT binds its receptors the NT-receptor complex is then internalized and transported retrogradely in the axon to the cell body, where it initiates signal transduction [67]. Thus, it is conceivable that K252a, applied locally as eye drops in this study, acted at the RGCs by blockade of tyrosine kinase-associated receptor-mediated activities.

We have demonstrated previously that autoimmune anti-MBP T cells, but not anti-p277 or anti-OVA T cells, exert a neuroprotective effect in rat injured optic nerves [3]. Regardless of their different antigen specificities, these T cell lines did not differ in the expression of their NT profiles *in vitro*. The *in vivo* neuroprotection mediated by the autoimmune T cells and not by the other tested T cell lines can be partly explained, however, in terms of NT secretion from the CNS-specific T cells following interaction of the T cells with their target self antigen presented at the lesion site. We showed here that secretion of NTs by the T cells depends on antigen activation. Thus, the exposure of myelin at the site of injury might activate MBP-specific T cells to secrete NTs that increase neuronal survival, whereas T cells specific to other

antigens might not be activated because of inadequate antigen recognition. Signalling via the TCR might therefore be required for NT secretion, allowing only CNS-specific T cells to exert a neuroprotective effect after axonal injury.

Intensive research over the last few decades has been aimed at uncovering the reasons why the CNS fails to accomplish processes of protection, repair, and regeneration. A growing body of evidence indicates that under normal physiological conditions the interaction between the immune system and the CNS is restricted [71], and that this restriction strongly influences the events which follow CNS injury [1, 72]. Effective dialogue between any injured tissue and the immune system is, however, a prerequisite for healing. Circumvention of the restricted communication

between the CNS and macrophages by transplantation of activated macrophages into the CNS lesion site in the rat optic nerve or spinal cord results in regrowth and partial recovery [73, 74]. Passive transfer of activated T cells specific to a self antigen, MBP, is beneficial in reducing the spread of damage following CNS axotomy in rats [3]. We have suggested that autoimmunity, unlike autoimmune disease, is a benign immune response that protects the CNS against pathogen-free damage. The fact that the spontaneous T cell response does not exert enough protection to cause significant improvement after CNS injury might be attributable to the restricted and/or inefficient communication between the CNS and the immune system. T cell accumulation at a site of CNS axonal insult is significantly increased after injury and passive transfer of activated autoimmune T cells [2, 3], but the phenotype of the neuroprotective T cells is not yet known. We showed here that the injected anti-MBP T cells express cytokines of Th1, Th2 and Th3 subtypes as well as various NTs *in vitro*. It is not yet possible, however, to determine whether the neuroprotective effect seen *in vivo* is produced by all of the injected cells or only by a certain subpopulation of

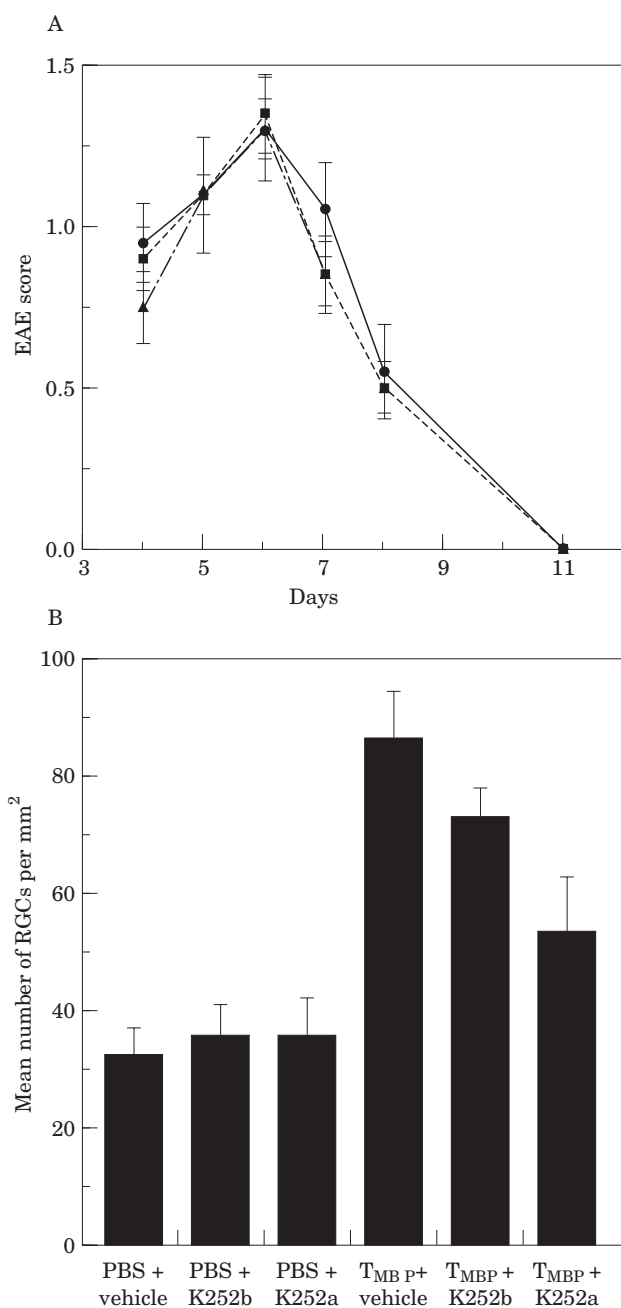


Figure 7. K252a inhibits the neuroprotective effect of anti-MBP T cells. **A.** The course and severity of EAE are not influenced by local treatment with K252 eye drops. Immediately after moderate optic nerve injury Lewis rats were injected ip with 10^7 activated anti-MBP T cells, and then treated three times a day, from day 1 to day 14, with $1 \mu\text{M}$ of K252a (---▲---), K252b (---■---), or vehicle (—●—) eye drops. EAE was evaluated according to a neurological paralysis scale. Data points represent means \pm SE. The figure shows the result of a representative experiment, in which each group contained five rats. No difference in the course of EAE or in disease severity was observed among the groups. **B.** The mean number of surviving RGCs is significantly diminished by treatment with K252a eye drops. Immediately after moderate optic nerve injury, Lewis rats were injected ip with 10^7 activated anti-MBP T cells or with PBS and then treated three times a day, from day 1 to day 14, with $1 \mu\text{M}$ of K252a, K252b, or vehicle eye drops. On day 14, 4-Di-10-Asp was applied to the optic nerves. After 5 days the retinas were excised and flat-mounted. Labelled RGCs from five randomly selected fields in each retina (all located at approximately the same distance from the optic disk) were counted by fluorescence microscopy, and their average numbers per mm^2 were calculated. The histogram shows the mean number of RGCs \pm SE. Each group contained five to ten rats. The neuroprotective effect of the anti-MBP T cells compared with that of PBS in the rats treated with vehicle eye drops was significant ($P < 0.001$, one-way ANOVA followed by Bonferroni's multiple comparison *t*-test). The neuroprotective effect of the anti-MBP T cells was significantly decreased in rats treated with K252a eye drops relative to rats treated with vehicle eye drops ($P < 0.05$, one-way ANOVA followed by Bonferroni's multiple comparison *t*-test). The neuroprotective effect of the anti-MBP T cells in rats treated with K252b eye drops did not differ significantly from that in rats treated with vehicle eye drops. No effect of K252a or K252b eye drops on RGC survival was observed in the control PBS-injected rats.

them, and what factors and in which combination are responsible for it. We are currently investigating the phenotype of the T cells isolated from the injury site.

In conclusion, we demonstrate that rat T cells produce NTs and secrete them upon activation. We suggest that the beneficial effect of passively transferred CNS autoimmune T cells on injured CNS axons is exerted via local secretion of various factors such as cytokines and NTs by the T cells in response to reactivation by their specific antigens. Withdrawal of growth factors is one of the mechanisms leading to death of neurons in CNS injuries and neurodegenerative diseases [4]. It might be possible to prevent this neuronal cell loss by a direct delivery of neurotrophic factors. An obstacle to the development of effective therapy, however, is delivery of NTs to the CNS, because these proteins are not able to cross the blood-brain barrier.

Systemic injection of activated autoimmune T cells specific to a CNS antigen appears to be a feasible cell therapy that offers some advantages: first, these T cells can cross the blood-brain barrier [75] and specifically accumulate at the site of CNS lesions [2], and secondly, the T cells are capable of continuously releasing various cytokines and NTs at the CNS injury site, and the timing and dynamics of such factor release might be in accordance with the needs of the tissue. This procedure may therefore potentially offer a physiological form of CNS maintenance that might be worth developing as a therapeutic modality for CNS injuries if the autoimmune responses are properly controlled. In other words, neuroprotection—without risk of autoimmune disease—might be achieved by the use of autoimmune T cells specific to CNS antigens that do not induce an autoimmune disease but do accumulate at the site of CNS injury, and provide neuroprotective factors like NTs.

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