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Continued Administration of Ciliary Neurotrophic Factor Protects Mice from Inflammatory Pathology in Experimental Autoimmune Encephalomyelitis

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Multiple sclerosis is an inflammatory disease of the central nervous system that leads to loss of myelin and oligodendrocytes and damage to axons. We show that daily administration (days 8 to 24) of murine ciliary neurotrophic factor (CNTF), a neurotrophic factor that has been described as a survival and differentiation factor for neurons and oligodendrocytes, significantly ameliorates the clinical course of a mouse model of multiple sclerosis. In the acute phase of experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein peptide 35-55, treatment with CNTF did not change the peripheral immune response but did reduce the number of perivascular infiltrates and T cells and the level of diffuse microglial activation in spinal cord. Blood brain barrier permeability was significantly reduced in CNTF-treated animals. Beneficial effects of CNTF did not persist after it was withdrawn. After cessation of CNTF treatment, inflammation and symptoms returned to control levels. However, slight but significantly higher numbers of oligodendrocytes, NG2-positive cells, axons, and neurons were observed in mice that had been treated with high concentrations of CNTF. Our results show that CNTF inhibits inflammation in the spinal cord, resulting in amelioration of the clinical course of experimental autoimmune encephalomyelitis during time of treatment. ***(Am J Pathol 2006, 169:584–598; DOI: 10.2353/ajpath.2006.051086)***

Multiple sclerosis (MS), a chronic inflammatory disease of the central nervous system (CNS), is the most common cause of acquired disability in adulthood.[1,2] The pathological hallmarks of MS are demyelination, inflammation, gliosis, and axonal damage.[3,4] In recent years the significance of axonal damage for the permanent clinical deficit has been highlighted.[5–7] Immunomodulatory treatments such as interferon (IFN)-β and glatiramer acetate have only limited success in progressive MS disease.[8,9] Complementary neuroprotective approaches could therefore be beneficial.

Ciliary neurotrophic factor (CNTF) is a four α-helix bundle cytokine initially identified as a survival factor for chick ciliary neurons.[10–12] CNTF lacks a signal peptide, is localized in the cytoplasm, and is therefore considered a lesion factor that is released from cells after tissue damage.[12,13] CNTF binds and activates a tripartite receptor comprising a nonsignaling GPI-anchored α chain (CNTFRα) and two signal transducing subunits, gp130 and leukemia inhibitory factor receptor β (LIFRβ).[13] Phospholipase C-mediated cleavage of the CNTFRα chain GPI anchor leads to soluble derivatives that can combine with CNTF to trigger activation of cells expressing only the two signaling subunits of the receptor, gp130 and LIFRβ.[14] The expression of CNTF is restricted to Schwann cells in the periphery and astrocytes in the central nervous system (CNS), whereas CNTFRα can be

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detected mainly in neural tissues and skeletal muscle, with lower expression in the adrenal gland, skin, liver, kidney, and testis.[13,15,16] In the nervous system, prominent expression in brain cortex and facial nucleus, motor-related brain and spinal cord areas has been reported, as well as in sympathetic, sensory, and parasympathetic ganglia.[13,17]

CNTF is a potent survival and differentiation factor for a wide range of cell types of the nervous system *in vitro*, including neurons and oligodendrocytes.[10–12,18–22] *In vivo*, it prevents degeneration of axotomized neurons, attenuates motor deficits in different strains of mice with neuromuscular deficiencies,[23–27] and was tested successfully in animal models of Huntington's disease.[28–30] CNTF was administered systemically to patients with amyotrophic lateral sclerosis, but no beneficial effect was detected.[28,31,32] The lack of therapeutic efficiency could be linked to the poor ability of CNTF to pass the blood-brain barrier (BBB).[33] More promising preclinical or clinical results have been obtained with CNTF targeted to the CNS by intrathecal injection or implantation of encapsulated transfected cells.[34–37]

A mutation inactivating the CNTF gene has been identified in humans.[38] It does not appear to result in neurological abnormalities but was associated with early onset of amyotrophic lateral sclerosis (in patients with superoxide dismutase mutations).[39] Studies of the effect of CNTF gene mutation in MS patients led to conflicting results: some authors detected an association between CNTF inactivation and early MS initiation whereas others did not.[39,40] In mice deficient for CNTF, earlier onset of symptoms and increased disability were observed in experimental autoimmune encephalomyelitis (EAE) induced with myelin oligodendrocyte glycoprotein (MOG) (35-55) peptide.

To further investigate the potential of CNTF in inflammatory diseases, we have tested the effects of daily injection of recombinant mouse CNTF in the MOG35-55 EAE model. We observed a marked reduction of EAE symptoms associated with reduced numbers of inflammatory infiltrates in the acute disease phase and increased numbers of oligodendrocytes, neurons, and axons in mice treated with high doses of CNTF in the chronic disease phase. Our results complement recent reports on the beneficial effect of intravitreal administration of CNTF in acute optic neuritis induced by MOG immunization in rat, and of LIF injection in mouse EAE.[41,42]

Materials and Methods

Production of Recombinant Mouse CNTF

Mouse CNTF cDNA was cloned, expressed as carboxy-terminal six histidine-tagged protein, and purified by immobilized metal-ion affinity chromatography as described.[43,44] CNTF was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biological activity was assessed using a derivative of the Ba/F3 cell line expressing the three subunits of the CNTF receptor, CNTFRα, gp130, and LIFRβ.[44] Extract from bacteria transformed with empty vector were subjected to parallel immobilized metal-ion affinity chromatography, and the elution fractions corresponding to the one which would have contained CNTF were dialyzed and used as injection control.

EAE Induction

All animal procedures were performed in accordance with the guidelines set forth by the Canadian Council for Animal Care as approved by the McGill University animal care committee. Female C57BL/6 mice (6 to 8 weeks old) (Jackson Laboratories, Bar Harbor, ME) were immunized on each side of the base of the tail with a subcutaneous injection of 100 μg of MOG peptide 35-55 (Sheldon Biotechnology Centre, Montreal, QC, Canada) emulsified in complete Freund's adjuvant (Fisher, Montreal, QC, Canada) containing 500 μg of heat killed *Mycobacterium tuberculosis* (H37RA; Difco, Detroit, MI). Mice received intraperitoneal injections of 300 ng of pertussis toxin (Sigma-Aldrich, St. Louis, MO) in Hanks' balanced salt solution (Invitrogen, Burlington, CA) on the day of the immunization as well as 2 days later. Mice were assessed daily for symptoms and weighed. The grading scheme was as follows: grade 1 (partial floppy tail), grade 2 (floppy tail), grade 3 (floppy tail and slow to right), grade 4 (partial hind limb paralysis), grade 5 (complete hind limb paralysis), and grade 6 (moribund or dead). Mice received intraperitoneal injections of CNTF (60 μg/kg/day, 180 μg/kg/day, 1.2 mg/kg/day) in 200 μl of sterile saline from days 8 to 24 after immunization. The mice were treated daily and sacrificed either at the peak of disease or 30 days after immunization for histology. Control groups immunized at the same time as the CNTF-treated mice received 200 μl of sterile saline daily.

Proliferation Assay

Mice immunized with MOG in complete Freund's adjuvant and treated with either saline or CNTF were sacrificed 15 to 22 days after immunization, and lymph nodes were collected. Cells were plated with a density of 4×10^5 lymph node cells/well into a 96-well flat-bottom plate. Cells were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (Sigma), 100 μg/ml of streptomycin, and 100 U/ml of penicillin (Invitrogen), 2 mmol/L L-glutamine (Invitrogen), and 50 μmol/L β-mercaptoethanol (Sigma) and maintained at 37°C in a 5% CO_2 humidified atmosphere. For stimulation, 50 μg of MOG(35-55) peptide, purified protein derivative (Cedarlane, Hornby, Canada) or ovalbumin (257-264) peptide (Sheldon Biotechnology Center, Montreal, Canada) were added to wells in triplicate for each. At 54 hours before the addition of the [^{3}H] thymidine, 100 μl of the supernatants from each well were removed and replaced with 100 μl of fresh complete RPMI 1640 containing 0.5 μC of [^{3}H] thymidine (MP Biomedicals, Aurora, OH) and the cultures were harvested after 72 hours.

Multiplex Bead Immunoassay

Supernatants collected from the proliferation assay (see above) were examined for specific cytokine content using a multiplex bead immunoassay. A mouse TH1/TH2 six-plex Luminex kit (BioSource, Camarillo, CA) containing detection reagents for interleukin (IL)-2, IL-4, IL-5, IL-10, IL-12, and interferon (IFN)-γ was purchased from Medicorp (Montreal, Canada). Duplicate wells of proliferation assay supernatants from individual animals, stimulated with 50 μg of MOG, were run according to the manufacturer's instructions (BioSource). Data were collected using a Luminex-100 system (Luminex, Austin, TX). Data were analyzed using STarStation software version 2.0 (Applied Cytometry, Sacramento, CA) and a five-parameter curve analysis was applied to each standard curve to calculate the sample concentrations. The detection limit for each cytokine was set at the lowest standard.

Real-Time Polymerase Chain Reaction (PCR) Methods

Lymph nodes, spleens, and spinal cords were extracted from mice treated with CNTF or saline between days 15 to 17 after immunization. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions and reverse-transcribed into cDNA using myeloleukemia virus reverse transcriptase (Invitrogen). Real-time PCR was performed on cDNA from pooled lymph nodes, spleens, and spinal cord and normalized to 18S rRNA message (18S primers and probes from Applied Biosystems, Foster City, CA). Primers (Sigma) and probe (Applied Biosystems) sequence for IL-10 were taken from Overbergh and colleagues[45] and for IFN-γ from Shi and colleagues.[46] The cDNA for the 18S reaction was diluted 1/1000. Real-time PCR was performed using an ABI Prism 7000 as previously described.[47] Cycle conditions consisted of an initial period of 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C.

To determine the relative mRNA levels of IFN-γ and IL-10 within the samples, standard curves were prepared by serial dilutions. Standard curves for cycle threshold (CT: the cycle at which the detected signal becomes significantly different from the background signal) versus arbitrary levels of input RNA were prepared, and relative levels of mRNA in each sample were calculated. CT values were verified to be in the linear amplification range on the appropriate standard curves.

Immunohistochemistry and in Situ *Hybridization*

Mice were perfused under deep anesthesia through the left cardiac ventricle with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brain, spinal cord, spleen, and liver from EAE mice and five nonimmunized control mice were embedded in paraffin. Cervical, thoracic, and higher lumbar spinal cord was cut in 8 to 11 3-mm-thick transverse segments before embedding. Five-μm-thick sections were stained for hematoxylin and eosin (H&E), Luxol-fast blue (LFB), periodic acid-Schiff (PAS), and Bielschowsky's silver impregnation.

Immunohistochemical staining was performed with an avidin-biotin technique. After deparaffinization intrinsic peroxidase activity was blocked by incubation with 5% H_2O_2 in PBS for 20 minutes. Nonspecific antibody binding was inhibited with 10% fetal bovine serum in PBS for 25 minutes. The primary antibodies were anti-proteolipid protein (anti-PLP; Biozol, Diagnostica Vertrieb GmbH, Eching, Germany), anti-2′,3′-cyclic nucleotid 3′-phosphodiesterase (anti-CNPase; Sternberger Monoclonals, Baltimore, MD), anti-MAC-3 [similar or identical to CD107b[48] (PharMingen, San Diego, CA)], anti-CD3 (Serotec, Oxford, UK), anti-NG2 (PharMingen), anti-NeuN (Chemicon, Temecula, CA), anti-immunoglobulin (Ig) (Vector Laboratories, Burlingon, CA), anti-claudin 5 (Abcam, Cambridge, UK), and anti-amyloid precursor protein (Chemicon).

Nonradioactive *in situ* hybridization for PLP mRNA was performed as described in detail previously[49] with an automated immunostainer (Discovery; Ventana Medical Systems, Tucson, AZ). Hybridization was performed using digoxigenin-labeled riboprobes detected by alkaline phosphatase-conjugated anti-digoxigenin Fab fragments. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate were used as chromogens. For double staining after *in situ* hybridization, sections were incubated with anti-PLP antibody and processed as described above.

Terminnal dUTP Nick-End Labeling (TUNEL) Staining

Sections were deparaffinized and then incubated for 1 hour at 37°C with a reaction mix containing 10 μl of tailing buffer, 2 μl of cobalt chloride, 1 μl of digoxigenin labeling mixture, and 6 U of terminal deoxynucleotidyl transferase in a total volume of 50 μl. Sections were washed, followed by incubation with an alkaline phosphatase-labeled anti-digoxigenin antibody (dilution, 1:250) for 1 hour at room temperature. The color reaction was performed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate. All reagents, enzymes, and antibodies were purchased from Roche Applied Systems (Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany). The sections were counterstained with nuclear fast red.

Morphometric Analysis

To quantify the demyelinated area, all (8 to 11) transverse spinal cord cross-sections in each animal were analyzed. The demyelinated area was measured in sections stained for LFB-PAS and expressed as percentage of total white matter. For statistical analysis the mean per animal was calculated. The permeability of the BBB was assessed in sections stained with anti-mouse Ig. To determine the extent of Ig leakage, the area of Ig leakage was measured in all spinal cord cross-section of each animal and expressed as percentage of total spinal cord area. For

statistical comparison the mean per animal was calculated.

Axonal density was determined in sections stained with Bielschowsky's silver impregnation using a 24-point eyepiece. The number of points crossing axons was counted in lesions of three transverse sections. The number of neurons was determined in sections stained with NeuN. Neurons were counted in the anterior horns of thoracic spinal cord transverse sections; one to three transverse sections per animal were counted.

To analyze the number of oligodendrocytes, T cells, macrophages, and NG-2-positive cells within lesions, the three most severely affected cross-sections were selected, and the number of stained cells/mm^2 within the lesions was determined. The number of inflammatory cells in unaffected white matter was determined by counting T cells and macrophages/microglia in white matter areas without myelin pallor and perivascular infiltrates.

Statistical Analysis

Statistical analysis was performed by using Prism Software (Graphpad) or the Instat statistics program. To analyze the clinical course a two-side analysis of variance test with Bonferroni's correction was used. Statistical analysis of the number of inflammatory cells, axons, neurons, oligodendrocytes, and oligodendrocyte progenitor cells as well as the extent of Ig leakage was performed by using an unpaired *t*-test. Statistical analysis of maximal and cumulative disease scores was done by using the Mann-Whitney *U*-test.

Results

Effects of CNTF on the Clinical Course of EAE

We examined the effect of recombinant mouse CNTF on EAE induced by MOG(35-55) peptide in C57BL/6 mice. To allow the establishment of a peripheral immune response to MOG immunization and to compare our results with earlier studies in which LIF was used to ameliorate MOG-EAE,[41] we injected CNTF intraperitoneally from days 8 to 24. Daily administration (days 8 to 24) resulted in a significant amelioration of clinical symptoms. Most (87%, seven of eight) of the mice treated with saline reached a clinical score of 4 or more compared to only 57% (four of seven) or 37.5% (three of eight) of mice treated with 60 or 180 μg/kg/day CNTF (Figure 1, A and E). The therapeutic effects of 180 μg/kg/day CNTF were replicated in a second independent experiment in which mice were sacrificed at the peak of disease with similar results. In this experiment, 77.7% (seven of nine) of the mice treated with saline reached a clinical score of 3 or more. In contrast only 20% (2 of 10) of the mice treated with CNTF at 180 μg/kg/day achieved a clinical score of 2 or more by the time of sacrifice. The disease course in mice treated with 180 μg/kg/day CNTF was statistically significantly different during treatment period compared to saline-injected mice ($P = 0.0138$). Although these treatment experiments were repeated several times with different doses of CNTF, the delay of disease onset shown in Figure 1A was observed only in one experiment. Intraperitoneal administration of a higher dose (1.2 mg/kg/day) of CNTF led to a further improvement of clinical symptoms (Figure 1B). Only 3 of 11 mice in the group treated with 1.2 mg/kg/day CNTF reached a maximal clinical score of 4 or greater, whereas 12 of 13 of the saline-injected group achieved such scores (Figure 1E). The clinical course was significantly different compared to saline-injected mice ($P < 0.0001$). Treatment with 180 μg/kg/day and 1.2 mg/kg/day CNTF reduced significantly the cumulative clinical score during the treatment period compared to saline-injected mice (Figure 1F); treatment with 1.2 mg/kg/day CNTF led also to a significant reduction in maximal disease scores (Figure 1E). Termination of CNTF treatment after 16 days (equals day 24 after immunization with MOG) led to worsening of clinical symptoms resulting after a few days in clinical scores comparable to those of saline-injected mice (Figure 1, A and B).

CNTF has been reported to induce weight loss.[50] In our EAE experiments treatment with 60 μg/kg/day or 180 μg/kg/day CNTF did not result in a significant higher maximal weight loss compared to saline-injected mice (Figure 1C). Saline-injected mice had the lowest body weight on day 16 after immunization (95 $\pm$ 3.1% of initial body weight) whereas animals treated with 60 or 180 μg/kg/day reached their minimal body weight on day 18 (92 $\pm$ 2.3% of initial body weight) or day 19 after immunization (91 $\pm$ 2.9% of initial weight), respectively (Figure 1C). Animals treated with 1.2 mg/kg/day CNTF had a maximal weight loss of 18% (82 $\pm$ 3.5% of initial body weight; range, 64 to 102%) on day 23 that was not statistically significantly higher compared to the maximal weight loss observed in saline-injected mice (91 $\pm$ 5.1%; range, 79 to 104%) on day 17 (Figure 1D). However, 1 of 11 mice treated with 1.2 mg/kg/day CNTF died and 3 were sacrificed for ethical reasons due to severe weight loss on days 22 or 23. These four mice had only mild disease scores at time of death (Figure 1E). Three of the four mice were available for histological analysis. The extent of demyelination was determined by LFB-PAS staining and the number of perivascular infiltrates by MAC3 immunohistochemistry. These mice had very few infiltrates and no signs of demyelination in the spinal cords, and were excluded from further histological analysis. These experiments show that intraperitoneal administration of 180 μg/kg/day or 1.2 mg/kg/day CNTF before onset of symptoms has significant therapeutic effects on the clinical course of EAE and that cessation of treatment is associated with worsening of clinical symptoms. Treatment with 1.2 mg/kg/day leads to severe weight loss, which restricted use of higher dosages.

Effects on the Immune Parameters and Blood-Brain Barrier

Peripheral Immune Response

To investigate how CNTF exerts its therapeutic effect on the clinical course of EAE, we measured T-cell responsiveness to antigens by proliferation assays in the acute

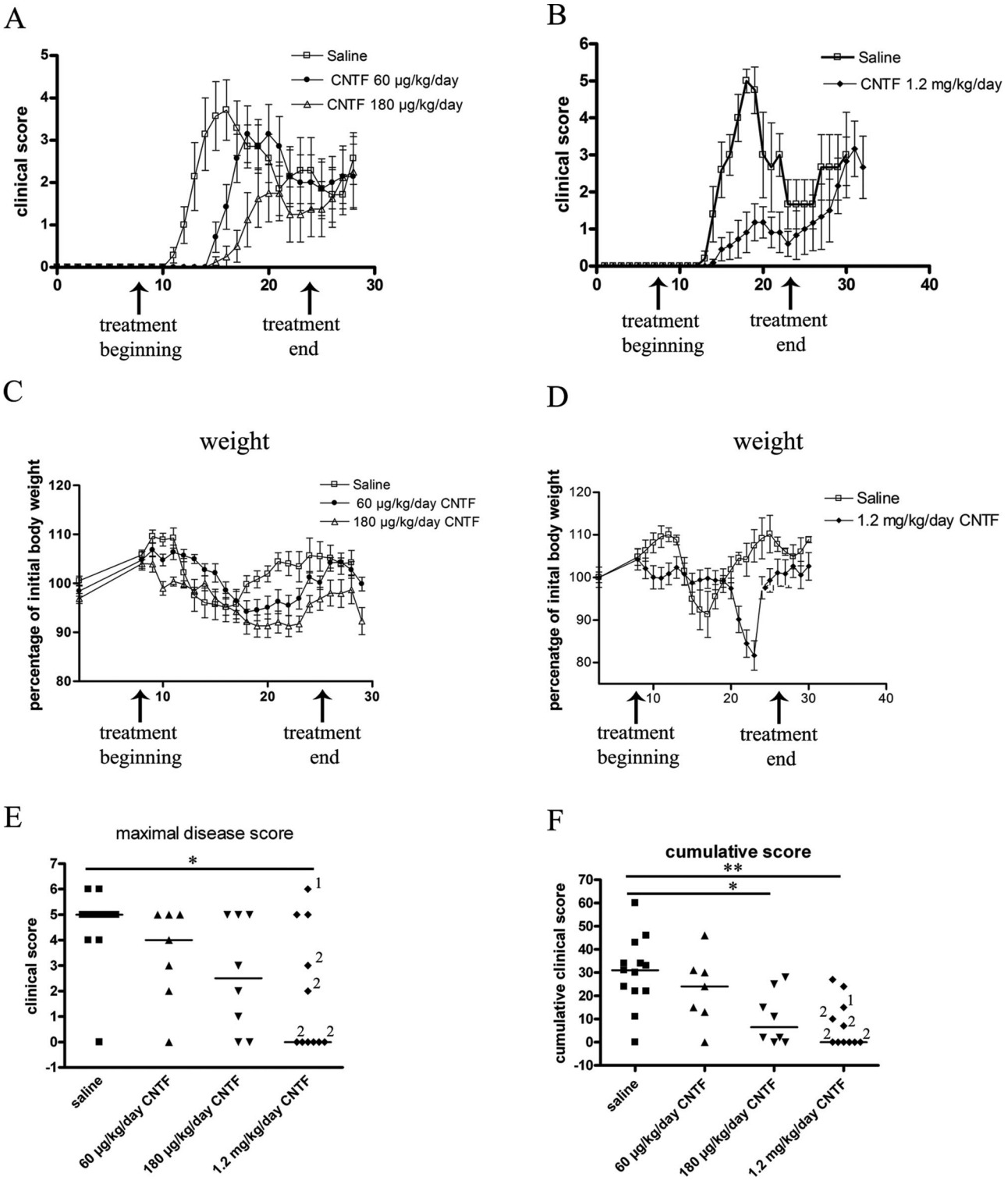


Figure 1. Daily intraperitoneal administration of CNTF reduces EAE symptoms in mice. **A:** Mice were immunized with MOG(35-55) peptide and complete Freund's adjuvant on day 0. From days 8 to 24 mice were either treated with 60 μg/kg/day CNTF (n = 7) (black circles), 180 μg/kg/day CNTF (n = 8) (white triangles), or saline (n = 8) (white squares). Treatment with 180 μg/kg/day significantly improved the clinical course during the time of treatment. **B:** Intraperitoneal injection of 1.2 mg/kg/day CNTF (n = 11) (black diamonds) led to a further improvement of the clinical symptoms compared to saline-treated mice (n = 5) (white squares). **C:** Intraperitoneal injection of 60 or 180 μg/kg/day CNTF modified the time course of weight loss in mice with EAE, but minimal body weight did not change significantly between CNTF- and saline-injected mice. **D:** Treatment with 1.2 mg/kg/day induced severe loss of body weight in some mice. These mice were sacrificed at day 23. Cessation of CNTF treatment at day 24 led to increase in body weight. **E:** Treatment with 1.2 mg/kg/day CNTF led to a significant improvement of the maximal clinical score during the treatment period compared to saline-injected mice (P = 0.02). **F:** Treatment with 180 μg/kg/day and 1.2 mg/kg/day significantly reduced the cumulative disease score during treatment period compared to saline-injected mice (180 μg/kg/day, P = 0.0112; 1.2 mg/kg/day, P = 0.0016). The graphs in **E** and **F** show the median scores. One sacrificed because of severe EAE and two sacrificed because of severe weight loss.

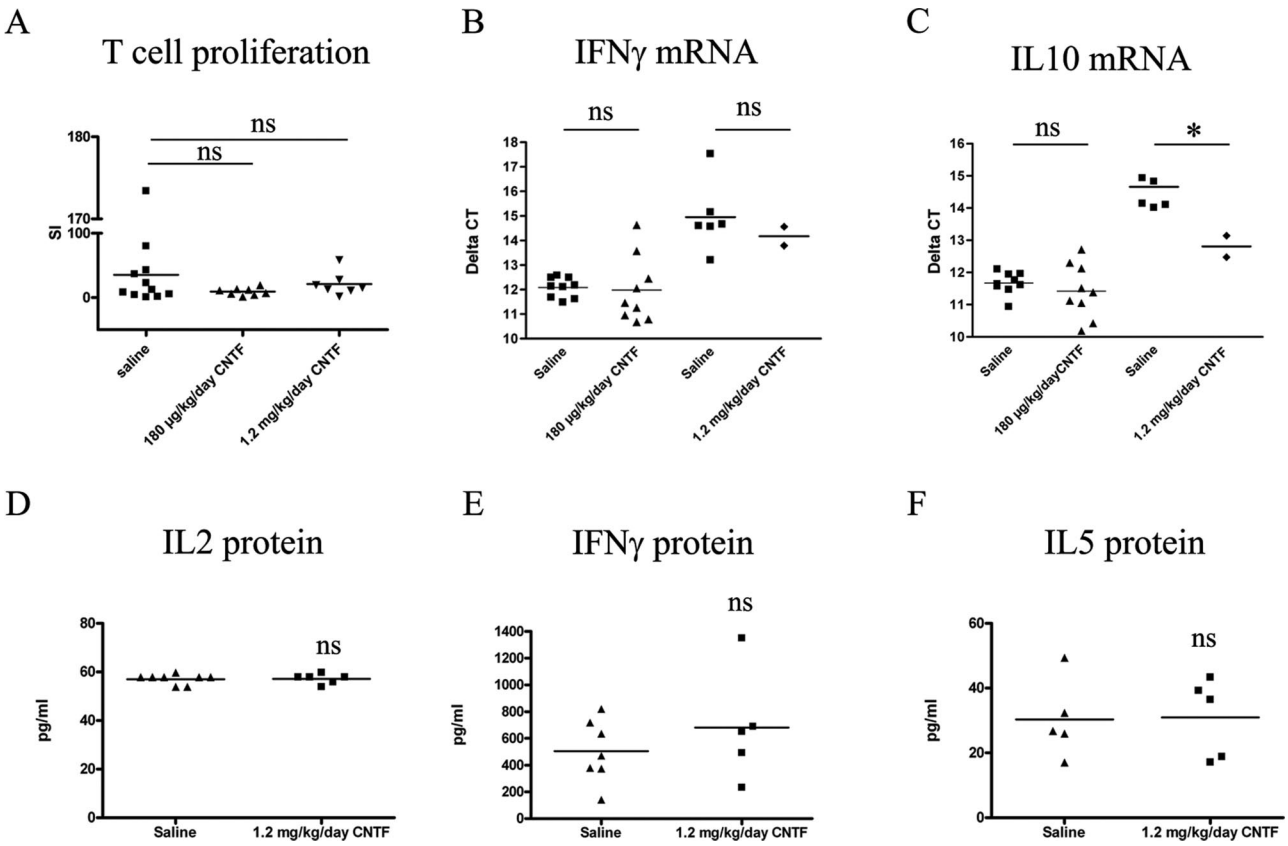
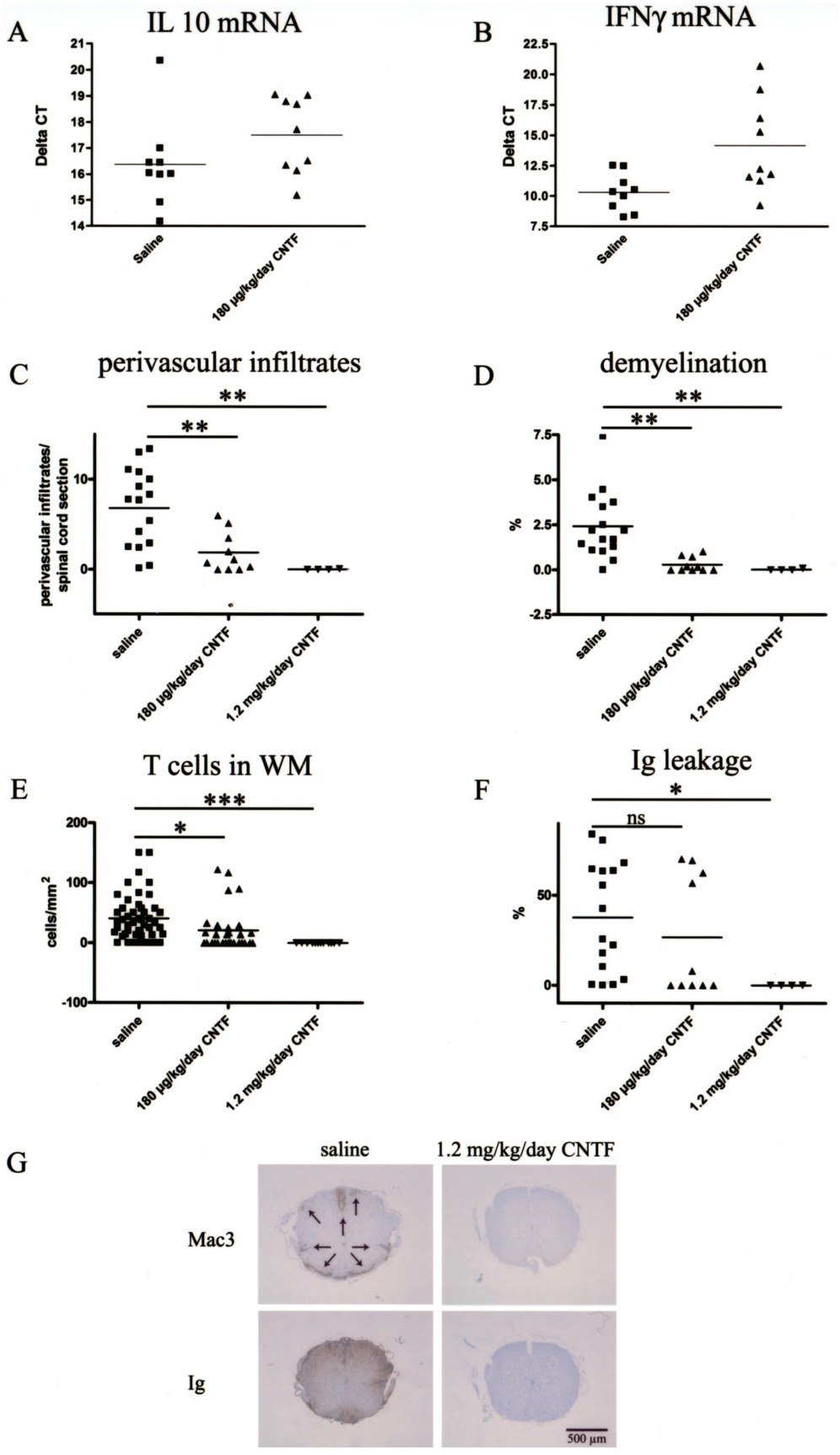


Figure 2. CNTF does not modify T-cell proliferation and cytokine mRNA levels in response to immunization with MOG peptide. **A:** Lymph nodes were collected from mice immunized with MOG plus complete Freund's adjuvant and treated with 180 μg/kg/day (n = 8), 1.2 mg/kg/day CNTF (n = 7), or saline (n = 11) at days 15 to 22 after immunization. Proliferative response of isolated LN cells was assayed *in vitro*. Stimulation with 50 μg of MOG(35-55) peptide led to a marked MOG-dependent proliferation that was not altered by CNTF treatment. **B** and **C:** Cytokine mRNA levels were assessed by real-time PCR analysis of RNA isolated from lymph nodes or spleen. The results for 180 μg/kg/day (n = 9) and 1.2 mg/kg/day CNTF (n = 2) were derived from two separate experiments. Results are shown within one graph with the appropriate saline control groups (n = 9 or 6, respectively). **B:** The levels of IFN-γ mRNA did not differ significantly between CNTF-treated and saline-injected mice. **C:** Mice treated with 1.2 mg/kg/day CNTF had significantly higher levels of IL-10 mRNA. **D–F:** Levels of IL-2, IFN-γ, and IL-5 protein in the supernatants of lymph node cell cultures from mice treated with saline (n = 8) or 1.2 mg/kg/day CNTF (n = 6) were measured by ELISA. No significant differences were detected.

disease phase. These experiments were performed with tissues from EAE mice either injected intraperitoneally with saline, 180 μg/kg/day CNTF, which ameliorated the clinical course of EAE without inducing significant weight loss, or 1.2 mg/kg/day CNTF. Lymph node cell cultures derived from MOG-immunized animals showed a marked MOG and purified protein derivative-dependent proliferation that was not significantly reduced by CNTF treatment compared to saline-injected mice that had severe EAE (SI = 35.4 $\pm$ 15.8 in control animals versus 9.5 $\pm$ 2.0 in 180 μg/kg/day and 21.1 $\pm$ 6.9 in 1.2 mg/kg/day CNTF-treated mice) (Figure 2A). EAE is considered to be a Th1-mediated disease.[51] To analyze whether CNTF treatment induces a change in the cytokine profile of EAE effector cells, we determined mRNA levels for IFN-γ and IL-10 from lymph nodes and spleen by real-time-PCR from mice that were sacrificed between days 15 to 17 after immunization. No significant differences were observed between CNTF and saline-injected mice in levels of the Th1 cytokine IFN-γ (Figure 2B). IL-10 mRNA levels did not differ significantly between mice injected with saline or 180 μg/kg/day CNTF. In mice treated with 1.2 mg/kg/day CNTF, slightly higher IL-10 mRNA levels were found compared to saline-injected mice, and these were significant (P = 0.0179) (Figure 2C). To further elucidate the effect of 1.2 mg/kg/day CNTF on T cells, we determined the protein levels of IL-2, IL-4, IL-5, IL-10, IL-12, and IFN-γ in the supernatants of lymph node cell cultures derived from MOG-immunized mice treated either with saline or 1.2 mg/kg/day CNTF. The protein levels of IL-2, IFN-γ, and IL-5 showed no significant differences as measured by enzyme-linked immunosorbent assay (ELISA) (Figure 2, D–F). The levels of IL-4, IL-10, and IL-12 were below the level of detection (between 5 to 30 pg/ml).

Immune Response in the CNS and BBB

To investigate the consequences of treatment with 180 μg/kg/day CNTF on the immune response within the CNS, we measured mRNA levels for IL-10 and IFN-γ in spinal cord tissue [days 15 to 17 after immunization with MOG(33-55) peptide] and performed a histological analysis of spinal cord and brain tissue sections from mice that were sacrificed during the acute phase of disease [days 13 or 18 after immunization with MOG(33-55) peptide]. The level of IFN-γ mRNA was significantly reduced in mice treated with 180 μg/kg/day CNTF compared to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B
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Ig
500 µm

Table 1. Number of NG2-Positive Cells, Oligodendrocytes, Axons and Thoracic Neurons in Healthy Animals and in EAE Mice Injected with Saline or Different Concentrations of CNTF

	No EAE mice (n = 5)	Saline (n = 12)	60 μg/kg/day CNTF (n = 7)	180 μg/kg/day CNTF (n = 8)	1.2 mg/kg/day CNTF (n = 6)	Significance (saline versus 1.2 mg/kg/day CNTF)
NG2-positive cells (cells/mm^2)	98 ± 6	173 ± 9	186 ± 26	177 ± 10	234 ± 13	P = 0.001
Oligodendrocytes (cells/mm^2, PLP-ISH)	956 ± 36	163 ± 30	n.d.	176 ± 24	303 ± 33	P = 0.012
CNPase-positive apoptotic figures	n.d.	33 ± 4	11 ± 3	21 ± 6	18 ± 7	P = 0.058
Axons (relative number, Bielschowsky's staining)	17.97 ± 0.51	2.87 ± 0.18	2.77 ± 0.20	3.0 ± 0.26	4.16 ± 0.21	P < 0.0001
Neurons (cells/mm^2, NeuN staining)	788 ± 25	632 ± 18	681 ± 38	641 ± 20	774 ± 24	P = 0.001
Perivascular infiltrates		4.9 ± 0.8	2.4 ± 1.0	2.8 ± 0.7	4.3 ± 0.7	n.s.
Macrophages in lesion		1893 ± 121	2332 ± 185	2259 ± 130	1796 ± 167	n.s.
T cells in lesion		769 ± 66	824 ± 78	795 ± 90	636 ± 86	n.s.

Animals without disease were sacrificed at 8 to 10 weeks, EAE mice were between 10 to 12 weeks of age when sacrificed.
All values are expressed as mean ± SEM.
n.d., not determined; n.s., not significant.

saline-injected mice (P = 0.0135) (Figure 3B). Levels of IL-10 mRNA also showed a trend to lower levels in CNTF-treated mice (P = 0.1513) (Figure 3A). These results are consistent with a reduced immune response in the spinal cord. This interpretation was confirmed by histological analysis. At the acute phase of disease significantly fewer perivascular infiltrates per spinal cord cross-section were found in tissue sections stained with Mac3 of mice treated with 180 μg/kg/day CNTF (6.8 ± 1.1 infiltrates/cross-section in saline-injected mice, 1.9 ± 0.7 infiltrates/cross-section in 180 μg/kg/day CNTF-treated mice; P < 0.01) (Figure 3, C and G). No perivascular infiltrates were observed in mice treated with 1.2 mg/kg/day CNTF (Figure 3C). The cellular composition of the infiltrates was comparable in the control and the 180 μg/kg/day group, consisting mostly of T cells (567 ± 67 in saline-injected mice versus 492 ± 37 cells/mm^2 in 180 μg/kg/day CNTF-treated mice) and macrophages/microglia (1856 ± 82 cells/mm^2 in saline-injected mice versus 1545 ± 137 cells/mm^2 in 180 μg/kg/day CNTF-treated mice) and few B cells. In addition to their presence in perivascular infiltrates, sparse infiltration of T cells and diffuse microglial activation were observed in otherwise normal-appearing white matter parenchyma, and this was significantly less prominent in CNTF-treated animals (microglia: 212 ± 18 cells/mm^2 in saline-injected mice, 88 ± 17 cells/mm^2 in 180 μg/kg/day CNTF-treated mice, P < 0.001, 25 ± 4 in 1.2 mg/kg/day CNTF-treated mice, P < 0.001; data not shown) (T cells: 41 ± 5 cells/mm^2 in saline-treated mice, 20 ± 6 cells/mm^2 in 180 μg/kg/day CNTF-treated mice, P < 0.05, 0 in 1.2 mg/kg/day CNTF-treated mice, P < 0.001; Figure 3E). The permeability of the BBB was assessed by measuring the spinal cord area that showed a positive staining for Ig (Figure 3G). In the saline group 3 of 16 mice showed no Ig staining, in contrast to 5 of 10 in the group treated with 180 μg/kg/day CNTF and 4 of 4 in the group treated with 1.2 mg/kg/day CNTF (Figure 3F). We also quantified the area of BBB leakage, expressed as percentage of total spinal cord cross-section area (Figure 3F). In the saline group on average 38 ± 7.7% of the spinal cord tissue area displayed a positive staining for Ig, whereas 26.7 ± 10.5% of tissue area showed a staining signal in mice treated with 180 μg/kg/day (ns, P = 0.4). In mice treated with 1.2 mg/kg/day CNTF no staining for Ig was detected (P = 0.028). To address further the effect of CNTF on the BBB, we investigated the expression of the tight junction protein claudin 5. However, no differences were observed between naïve mice and saline or CNTF-treated mice (data not shown). This is in line with recent studies demonstrating that BBB leakage can occur independent of tight junction protein expression.[52,53]

After cessation of CNTF treatment, disease returned to control levels. We analyzed inflammatory pathology ~10 days after cessation of CNTF treatment. The number of perivascular infiltrates per spinal cord cross-section, as well as the numbers of macrophages/microglia or T cells within lesions did not differ significantly between CNTF and saline-injected mice (Table 1). In summary, these results demonstrate that during the acute phase of EAE,

Figure 3. CNTF reduces the immune response in the spinal cord. Levels of mRNA for IL-10 and IFN-γ, number of perivascular infiltrates, extent of demyelination, number of T cells in unaffected white matter, and permeability of the BBB for Ig were analyzed in spinal cord during the acute phase of the disease. **A** and **B:** Mice were treated with 180 μg/kg/day CNTF (n = 9) or saline (n = 9) and levels of mRNA for IL-10 and IFN-γ were determined by real-time PCR. In mice treated with 180 μg/kg/day CNTF (n = 9) a trend to lower mRNA levels for IL-10 (P = 0.1513) (**A**) and significantly lower IFN-γ (P = 0.0135) (**B**) were observed in spinal cord tissue compared to saline-injected mice (n = 9). **C–F:** Tissue sections from mice injected with saline (n = 16), 180 μg/kg/day (n = 10) or 1.2 mg/kg/day CNTF (n = 4) were stained with the macrophage marker MAC3, the T-cell marker CD3, the myelin stain LFB-PAS, and anti-Ig for BBB leakage. Mice treated with CNTF showed significantly fewer perivascular infiltrates/cross-section (**C**), reduced loss of LFB-PAS staining (**D**), fewer T cells in the apparently unaffected white matter (**E**), and reduced Ig leakage (**F**) compared to saline-injected mice. **G:** Immunohistochemistry for MAC3 and Ig revealed a complete absence of perivascular macrophages/microglia cells and BBB leakage in mice treated with 1.2 mg/kg/day in contrast to saline-injected mice. **Arrows** indicate perivascular infiltrates [immunohistochemistry for Mac3 (brown) and mouse Ig (brown), counterstained with hematoxylin]. The graphs in **A–F** show means. In **A–D** and **F**, each point represents one animal, in **E** each point shows the result for a single spinal cord cross-section.

daily treatment from day 8 onwards with 180 μg/kg/day CNTF or higher inhibits spinal cord inflammation, seen as the recruitment or persistence of inflammatory cells into the CNS, the formation of perivascular infiltrates within the CNS, and activation to cytokine production, without affecting the T-cell response in the periphery.

Effects on Oligodendrocytes, Oligodendrocyte Precursor Cells, Neurons, and Axons

To analyze the neurobiological consequences of CNTF treatment (60 μg/kg/day, 180 μg/kg/day, and 1.2 mg/kg/day) on EAE, we studied its effects on oligodendrocytes, myelin, oligodendrocyte progenitor cells, and axons during acute and chronic disease stages [days 28 to 36 after immunization with MOG(35-55) peptide]. In the acute disease phase, mild demyelination was observed in the saline-injected mice (Figure 3D). However, demyelination in CNTF-treated mice was significantly reduced compared to saline-injected mice (Figure 3D). We determined the amount of axonal loss, acute axonal damage (immunohistochemistry for APP), and numbers of apoptotic oligodendrocytes in areas with inflammatory infiltrates in CNTF-treated mice which showed an inflammatory response in the spinal cord and compared them with saline-injected mice. No significant difference was observed for these parameters between the two groups (data not shown).

In the chronic disease phase marked infiltration of the CNS parenchyma by macrophages and T cells led to edema and loss of myelin staining. Although treatment with CNTF reduced the percentage of demyelinated white matter, this difference was not significant compared to saline-injected mice (9.8 ± 1.6% in saline-injected mice versus 5.0 ± 2.5%, 6.0 ± 2.4%, and 6.5 ± 1.9% in mice treated with 60 μg/kg/day, 180 μg/kg/day, and 1.2 mg/kg/day, respectively).

To determine the number of oligodendrocytes within lesions and normal-appearing white matter, *in situ* hybridization for PLP mRNA was performed. The number of PLP mRNA-positive oligodendrocytes within the lesions was significantly reduced in all treatment and saline groups compared to unaffected, normal-appearing white matter (Table 1, Figures 4B and 5A). A dose-response effect could be discerned, in that mice that had received higher dosages of CNTF (1.2 mg/kg/day) had a significantly higher number of oligodendrocytes within the lesion compared to saline-injected mice ($P = 0.012$) (Figure 4B; Table 1). To test whether this higher number of oligodendrocytes was caused by a reduction of oligodendrocyte cell death, we counted the number of total (Figure 5C) and CNPase-positive cells (Figure 5D) that displayed morphological characteristics of apoptosis, such as condensed and/or fragmented nuclei and apoptotic bodies. Administration of high doses of CNTF was associated with reduced numbers of cells with apoptotic morphology [67 ± 5 cells/mm^2 in saline-injected mice versus 43 ± 10 cells/mm^2 in mice treated with 1.2 mg/kg/day CNTF ($P = 0.022$) as well as reduced numbers of CNPase-positive apoptotic cells (33 ± 4 cells/mm^2 in saline-injected mice versus 18 ± 7 cells/mm^2 in mice treated with 1.2 mg/kg/day CNTF ($P = 0.058$) (Table 1)].

Oligodendrocyte precursor cells were identified by immunohistochemistry for the chondroitin sulfate proteoglycan NG2. NG2 is a single-pass transmembrane protein that carries a variable number of glycol- and glycosamine chains according to cell type and developmental stage and is expressed by oligodendrocyte progenitor cells in the mature CNS.[54,55] The number of NG2-positive cells was significantly increased within the lesions compared to normal-appearing white matter independent of treatment with CNTF or saline (Table 1; Figure 4A; Figure 5, E and F). This observation is in agreement with previous studies that showed proliferating and increasing numbers of NG2-positive cells in inflammatory and toxic demyelinating CNS lesions.[56,57] Treatment with 1.2 mg/kg/day CNTF was associated with a significant increase in the number of NG2-positive cells within the lesions compared to treatment with lower doses of CNTF or saline (173 ± 9 cells/mm^2 in saline-injected mice versus 234 ± 13 cells/mm^2 in mice treated with 1.2 mg/kg/day CNTF, $P = 0.0002$) (Table 1, Figure 4A).

CNTF is known to promote neuronal survival and axonal regeneration. Therefore we investigated the effect of CNTF administration on the loss of axons in EAE lesions and the number of neurons in the gray matter of spinal cord. Axonal density was determined in sections stained with Bielschowsky's silver impregnation using a 24-point eyepiece. The number of points crossing axons was counted. In normal-appearing spinal cord white matter, 18 points of the eye grid crossed axons whereas in EAE lesions this number was significantly reduced (Figures 4C and 5B, Table 1). A higher number of axons within lesions was observed in mice treated with CNTF, and this difference was significant in mice treated with 1.2 mg/kg/day compared to saline-injected mice (relative number of axons: 2.87 ± 0.18 in saline-injected mice versus 4.16 ± 0.21 in 1.2 mg/kg/day CNTF-treated mice, $P < 0.0001$) (Table 1, Figure 4C). The number of neurons was counted in the ventral horns of the thoracic spinal cord on sections stained with the neuronal marker NeuN from MOG-immunized animals treated with CNTF or saline as well as in mice without EAE (Figure 4D, Table 1). In saline-injected mice a significant reduction in the number of NeuN-positive neurons was observed compared to mice without EAE (788 ± 25 cells/mm^2 in mice without EAE compared to 632 ± 18 cells/mm^2 in saline-injected EAE mice; $P = 0.001$). No TUNEL-positive neurons or neurons with apoptotic morphology were observed (Figure 5G). Instead, we found swollen, weakly eosinophilic neurons that lacked Nissl bodies with either no or large vesicular nuclei similar to changes observed in neurons as a reaction to axonal damage (Figure 5H). Loss of NeuN-positive neurons was prevented completely by treatment with 1.2 mg/kg/day CNTF (774 ± 24 cells/mm^2, $P = 0.001$) as it is shown in Figure 4D and Table 1. In summary, these findings indicate that administration of high doses of CNTF reduces loss of NeuN-positive neurons and axonal damage, increases number of oligodendrocyte precursor cells, and protects mature oligodendrocytes against apoptotic cell death in EAE lesions.

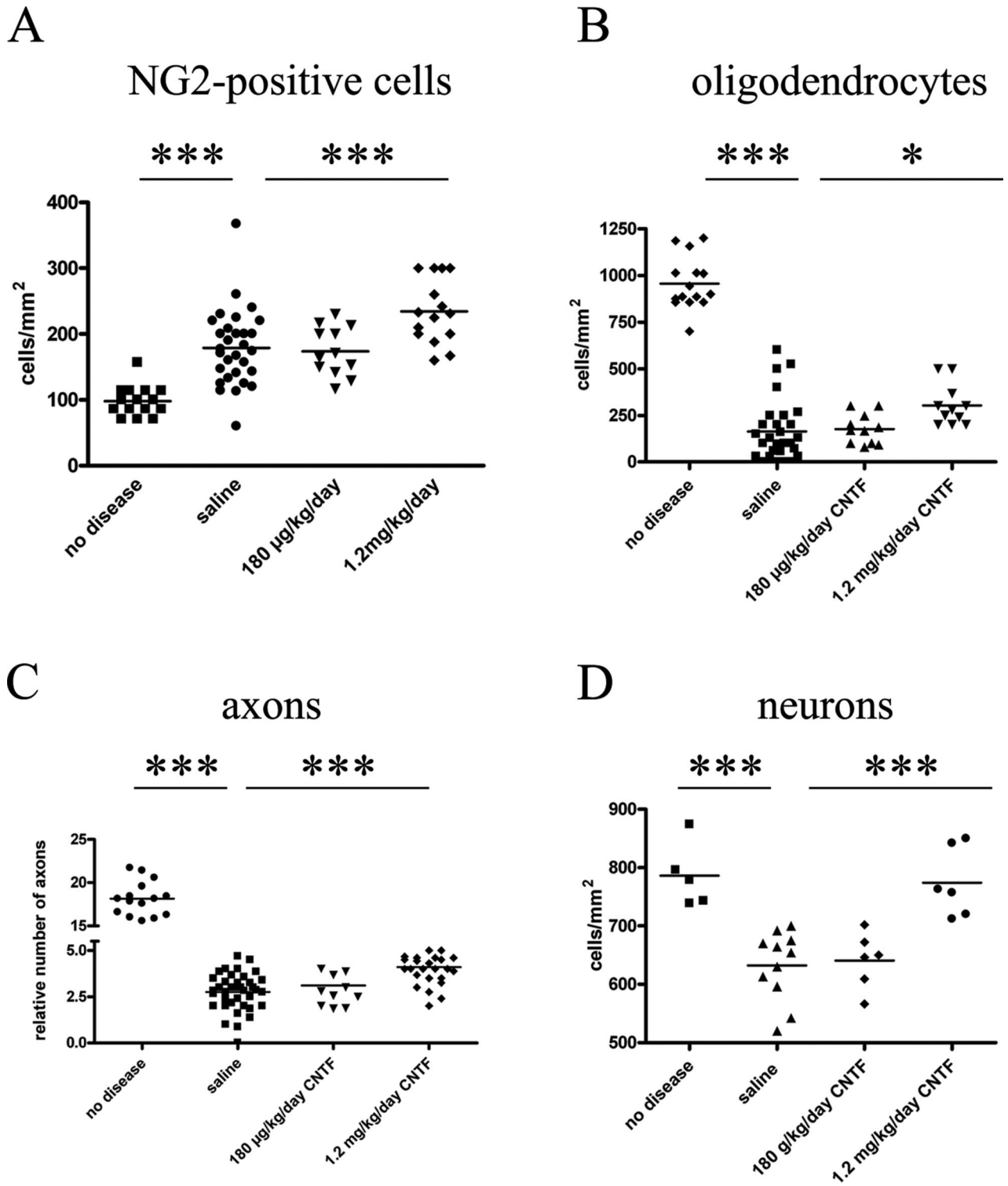
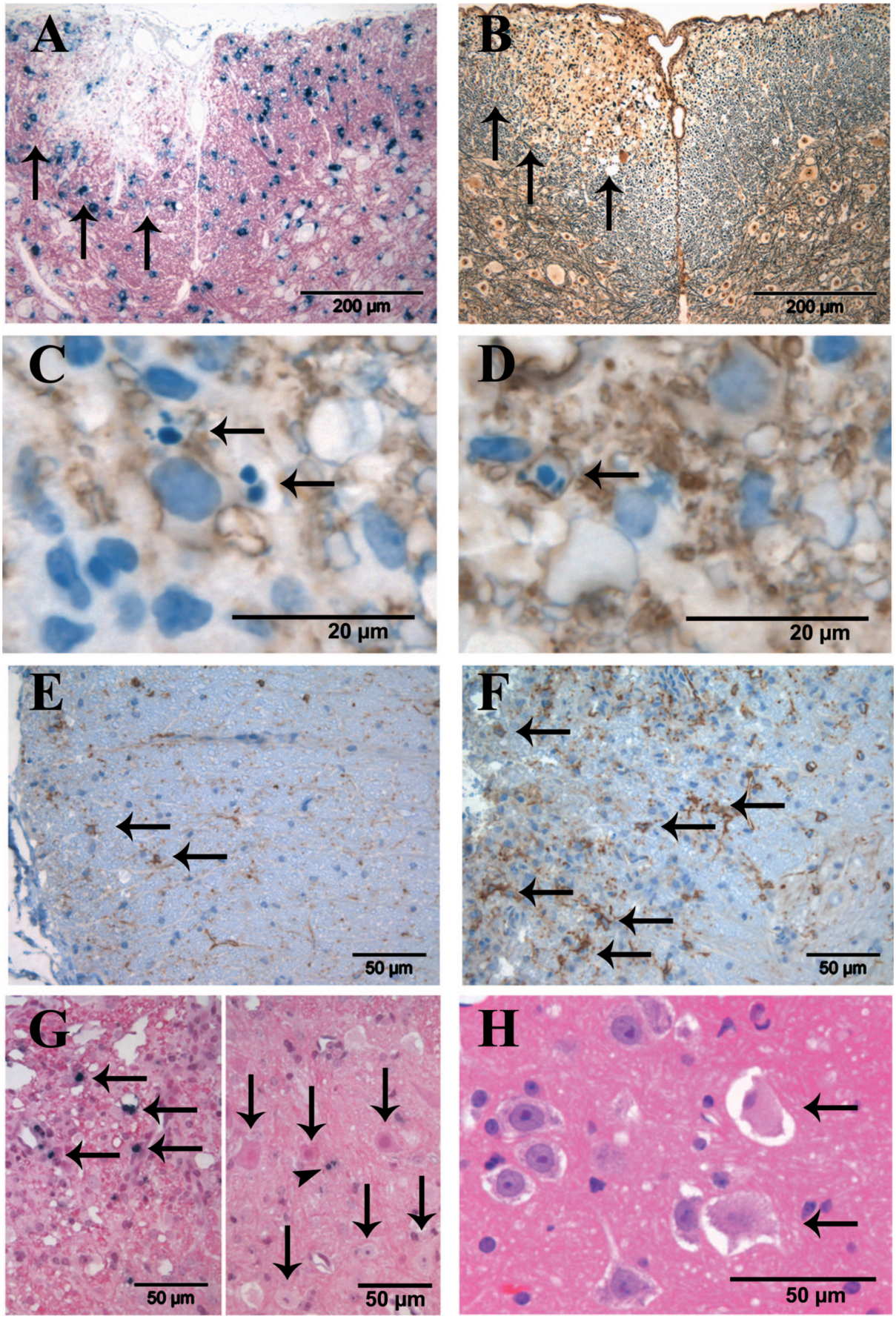


Figure 4. CNTF increases the number of oligodendrocytes and their precursors and decreases axonal and neuronal damage. **A–D:** To analyze the effects of CNTF treatment in the chronic disease phase, spinal cord tissues from mice in the chronic disease phase [days 28 to 36 after immunization with MOG(35-55) peptide] were studied. The numbers of NG-2-positive cells, oligodendrocytes (PLP-ISH), axons (Bielschowsky's staining), and neurons (NeuN) in healthy mice or mice treated either with different concentrations of CNTF or saline were determined. The animals from the two experiments shown in Figure 1, A and B, were pooled for this analysis; the saline-treated mice in these two experiments did not show significant differences in the clinical course or different histological parameters. **A–C:** In saline-treated EAE, the numbers of NG2-positive cells, PLP-positive oligodendrocytes, and axons within the lesions were significantly reduced compared to naive mice ($P < 0.001$ for all parameters). **D:** Also the number of NeuN-positive neurons in the ventral horn of the thoracic spinal cord of saline-injected mice with EAE was significantly lower compared to healthy animals without disease ($P < 0.001$). Treatment with 1.2 mg/kg/day CNTF led to significantly higher numbers of NG-2-positive cells ($P = 0.0002$) (**A**), PLP-positive oligodendrocytes ($P = 0.012$) (**B**), and axons ($P < 0.0001$) (**C**) within the lesions. **D:** Administration of 1.2 mg/kg/day CNTF prevented completely the reduction in NeuN-positive neurons observed in control EAE mice ($P = 0.0006$). Notice the different scale of the *y* axis in **D**. Each point in **A–C** represents the result of one spinal cord cross-section, in **D** each dot represents the result of one animal.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Discussion

Our data demonstrate that treatment with CNTF before onset of symptoms significantly ameliorates the clinical course of EAE in a dose-dependent manner. CNTF delivered at 180 μg/kg/day was sufficient to inhibit T-cell and macrophage/microglia recruitment into the CNS without affecting the peripheral immune response. Treatment with 1.2 mg/kg/day CNTF resulted in complete prevention of BBB leakage, formation of perivascular infiltrates, and migration of T cell into the CNS in the acute disease phase, as well as a slightly better preservation of oligodendrocytes, neurons, and axons and an increased number of oligodendrocyte progenitors in the chronic disease phase.

The role of CNTF as a survival and differentiation factor for neurons and oligodendrocytes is well established. *In vitro* CNTF promotes the survival of neurons[20,58] and reduces tumor necrosis factor-α and serum deprivation-induced cell death of oligodendrocytes.[19,59,60] *In vivo*, it prevents the degeneration of axotomized neurons.[23] In CNTF-deficient mice induction of EAE with MOG(35-55) peptide results in more severe axonal damage, increased number of apoptotic oligodendrocytes, and a reduction in proliferating oligodendrocyte progenitor cells compared to WT mice.[61]

Although these direct neuroprotective effects of CNTF are well known, our results argue for a different mode of action of CNTF in EAE. The underlying cause for the beneficial effect of CNTF during the treatment period appears to be because of reduced spinal cord inflammation. This conclusion is based on the observation of an almost complete absence of clinical symptoms that is associated with a complete prevention of BBB leakage and infiltration of T cells into the CNS in mice treated with 1.2 mg/kg/day CNTF. A similar but less pronounced tendency is observed in mice treated with 180 μg/kg/day CNTF. After cessation of CNTF treatment, disease returned to control levels, and inflammatory response measured ~10 days after CNTF withdrawal was equivalent in both groups. Mice treated with 1.2 mg/kg/day CNTF had higher numbers of axons, NG2-positive oligodendrocyte progenitor cells, and oligodendrocytes within the lesions, as well as no sign of significant neuronal loss. These relatively mild morphological changes observed in mice treated with 1.2 mg/kg/day CNTF in the chronic disease phase were not associated with a better clinical score. Whether these higher numbers of axons, oligodendrocytes, and neurons are a direct neuroprotective effect of CNTF or secondary to reduced inflammation in the acute disease phase remains open. The short half-life of CNTF and the complete absence of inflammation in mice treated with 1.2 mg/kg/day CNTF in the acute phase favor the second hypothesis.

The decreased CNS infiltration was not caused by suppression of peripheral T-cell proliferation because treatment with CNTF did not significantly reduce T-cell proliferation in response to immunization with MOG(35-55) peptide. We have no indication for a Th1/Th2 shift: treatment with 180 μg/kg/day CNTF did not change levels of mRNA levels for IFN-γ or IL-10 in peripheral immune tissues. We observed slightly increased IL-10 mRNA levels in mice treated with 1.2 mg/kg/day CNTF, but this did not result in detectably higher protein levels. Also other cytokines (IL-2, IL-4, IL-5, IL-12, IFN-γ) showed no detectable change in protein levels. These observations make it unlikely that the beneficial effect of CNTF is mediated by immunomodulation of the peripheral immune response. This conclusion is consistent with earlier studies showing that induction of EAE in CNTF-deficient mice or treatment of EAE with recombinant LIF (a member of the IL-6 family whose receptors comprises the two signaling subunits of CNTFR, gp130, and LIFRβ[62–64]) did not result in an altered peripheral immune response.[41,61] The effect of CNTF on spinal cord inflammation is unlikely to be linked to a direct effect of this cytokine on T cells because these cells are neither known to express CNTFRα nor LIFRβ.[13,65,66] It is conceivable that CNTF exerts its effects on the spinal cord immune response via immune modulating molecules produced by microglia, a cell population known to respond to CNTF[67,68] or a direct neuroprotective effect preventing initial myelin and axon damage and therefore blocking secondary inflammatory response.[69] The ability of CNTF to cross the BBB is prerequisite for a direct effect of CNTF on microglial cells or myelin and axons. Studies investigating the permeability of the intact BBB for CNTF led to contradictory results.[70–72] However, in EAE the BBB is disrupted early during the disease course,[73] so that transport of CNTF into the CNS may not be restricted by the BBB—preliminary data support this possibility (I.C., J.-F.G., data not shown).

An alternative interpretation compatible with the observation of an unchanged immune response in the peripheral immune system and reduced inflammation in the spinal cord is that CNTF prevents inflammatory cell recruitment into the CNS via effects on the BBB. This hypothesis is suggested by the observation of significantly reduced T-cell infiltration and diffuse microglia activation within the noninflamed spinal cord parenchyma and a significant reduction of Ig leakage in spinal cord tissue of

Figure 5. Morphological changes in EAE lesions. Immunohistological analysis of mice in the chronic disease phase (days 28 to 36 after immunization with MOG(35-55) peptide. **A:** Immunization with MOG(35-55) peptide leads to focal loss of oligodendrocytes and myelin [immunohistochemistry for PLP (red) combined with *in situ* hybridization for PLP mRNA (blue cytoplasmic signal)]. The **arrows** mark the border of the lesion. **B:** EAE lesions are characterized by a clear reduction in the number of axons (same lesion as in **A**, Bielschowsky's silver impregnation). **C:** In EAE lesions cells with morphological characteristics of apoptosis such as condensed and/or fragmented nuclei are found (**arrows**). **D:** A proportion of apoptotic cells are oligodendrocytes as identified by immunohistochemistry with CNPase (brown). The apoptotic cell expresses CNPase on the cell surface (**arrow**). **C** and **D:** Immunohistochemistry with anti-CNPase, counterstained with hematoxylin. **E** and **F:** Immunohistochemistry for NG2 (brown) reveals a marked up-regulation of NG2-positive cells (**arrows**) in EAE lesions (**F**) compared to unaffected white matter (**E**). **G:** Within white matter lesions numerous cells with fragmented DNA are observed that stain positive in the TUNEL reaction (**left**, **black arrows**). The **right** panel depicts the adjacent gray matter. No TUNEL-positive neurons are seen in the gray matter (**arrows**), but one small nonneuronal cell is TUNEL-positive (**arrowhead**) [TUNEL staining (black) combined with nuclear fast red]. **H:** In the anterior horns of spinal cord gray matter swollen, weakly eosinophilic neurons were found that lack Nissl bodies (**arrows**).

CNTF-treated animals. It has been shown that brain endothelial cells express mRNA coding for gp130 and LIFRβ.[74] Although expression of CNTFRα mRNA was not detected,[74] requirement for the nonsignaling chain can be bypassed by the formation of complexes between CNTF and soluble CNTFRα.[14]

The CNTF doses used in our EAE experiments are in same range as other studies in which CNTF or LIF were administered systemically.[26,41,75,76] However, we observed severe weight loss in mice treated with 1.2 mg/kg/day CNTF, to the extent that some mice had to be sacrificed because of ethical reasons. CNTF is known for its capability to induce weight loss and is currently under investigation as a potential treatment in obesity.[77] Induction of severe weight loss and death induced by CNTF in a dose-dependent manner has been described in a previous study in which the authors discussed cachexia-like effects of CNTF, although none of the cytokines typically involved in cachectic wasting such as IL-6, TNF-α, and LIF was up-regulated.[78] In contrast, in genetic animal models (ob/ob mice) and in diet-induced obesity restricted food-uptake appears to be the major reason for weight loss.[50,79] There are also indications that CNTF might induce weight loss by neurogenesis in areas of the hypothalamus relevant for energy-balance regulation[80] or by activation of hypothalamic leptin-like pathways and subsequent phosphorylation of STAT3, because of similarities in the structure of the CNTF and leptin receptor.[50] So far, side effects of CNTF such as fever, malaise, and weight loss as well as the most likely poor ability of CNTF to cross the intact BBB have limited the success of clinical studies in amyotrophic lateral sclerosis patients who were given CNTF because of its known neuroprotective effect.[28,31,32] More promising results were obtained when CNTF was administered to the CNS by intrathecal injections or implantation of encapsulated transfected cells into the ventricular system.[34,36,37,81,82] Recent progress on human CNTF biochemistry indicated that this cytokine can use soluble or membrane-bound IL-6Rα as a substitute to CNTFRα to activate the receptor signaling subunits, gp130 and LIFRβ.[83] This led to the suggestion that some of the side effects of CNTF in clinical trials might be linked to this species-specific property of the human cytokine, which has been mapped to Gln 63.[83] This amino acid is substituted by an arginine in rat and mouse CNTF, preventing interaction with IL-6Rα.[44,83] Modified forms of human CNTF with increased specificity such as axokine in which human Gln 63 is replaced by murine Arg 63[84] could therefore have an improved safety profile.

In summary, we show that the clinical benefit of CNTF in EAE is caused mainly by impaired inflammation in the CNS. The demonstration of beneficial effects of recombinant mouse CNTF in EAE together with the development of new applications to reduce side effects might sharpen the focus on CNTF as a potential therapeutic for MS.

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