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Neuronal Damage in Autoimmune Neuroinflammation Mediated by the Death Ligand TRAIL

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Summary

Here, we provide evidence for a detrimental role of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in neural death in T cell-induced experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). Clinical severity and neuronal apoptosis in brainstem motor areas were substantially reduced upon brain-specific blockade of TRAIL after induction of EAE through adoptive transfer of encephalitogenic T cells. Furthermore, TRAIL-deficient myelin-specific lymphocytes showed reduced encephalitogenicity when transferred to wild-type mice. Conversely, intracerebral delivery of TRAIL to animals with EAE increased clinical deficits, while naive mice were not susceptible to TRAIL. Using organotypic slice cultures as a model for living brain tissue, we found that neurons were susceptible to TRAIL-mediated injury induced by encephalitogenic T cells. Thus, in addition to its known immunoregulatory effects, the death ligand TRAIL contributes to neural damage in the inflamed brain.

Introduction

Multiple sclerosis (MS) is a multiphasic autoimmune disease of the CNS (Steinman, 2001) in which myelin-specific CD4+ Th1 cells are thought to orchestrate the effector processes resulting in the destruction of the myelin sheath (Brosnan and Raine, 1996). Recent studies in MS and its animal model, experimental autoimmune encephalomyelitis (EAE), suggest, however, that already during the early phase of inflammation, neuronal pathology involving axonal transection plays a pivotal role in disease pathology (Ferguson et al., 1997; Kornek et al., 2000; Meyer et al., 2001; Trapp et al., 1998). It has become evident that long-term disability in MS correlates better with axonal damage than with the degree of demyelination (Bjartmar et al., 2003). In the EAE model, we have recently reported the occurrence of apoptotic neurons in the brainstem of affected animals, indicating irreversible neuronal cell loss (Diestel et al., 2003). However, the mechanisms of this neuronal damage have to be elucidated, as they constitute one of the main targets in the prevention of persisting clinical deficits during autoimmune neuroinflammation (Zamvil and Steinman, 2003).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also known as Apo2 ligand, is a member of the TNF/nerve growth factor (NGF) superfamily (Pitti et al., 1996; Wiley et al., 1995). In humans, four membrane bound receptors for TRAIL have so far been identified. Of these, only TRAIL receptor 1 (TRAIL-R1 or death receptor 4 [DR4]) (Pan et al., 1997) and TRAIL receptor 2 (TRAIL-R2, TRICK2, KILLER, DR5) (Walczak et al., 1997) have the capacity to induce caspase-dependent apoptotic cell death, whereas TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) lack a functional death domain and are considered to act as decoy receptors (Degli-Esposti et al., 1997b; Degli-Esposti et al., 1997a). In the murine system, two decoy receptors have been reported recently (Schneider et al., 2003), whereas only one death-mediating TRAIL receptor is known, which has the highest homology with the human TRAIL receptor 2 (Wu et al., 1999).

Initial reports indicated a specific and selective action of TRAIL on transformed cells (Ashkenazi et al., 1999; Walczak et al., 1999). They also provided evidence for the role of TRAIL in the immunosurveillance of tumor cells (Cretney et al., 2002; Smyth et al., 2001). Expression of TRAIL has been shown for a variety of immune cells, and T cells were found to upregulate TRAIL upon antigen-specific stimulation (Mariani and Krammer, 1998; Wendling et al., 2000). Systemic (intraperitoneal) injection of soluble neutralizing TRAIL-R2 (DR5) leads to exacerbation of murine collagen-induced arthritis (Song et al., 2000), autoimmune diabetes (Lamhamedi-Cherradi et al., 2003a), and myelin oligodendrocyte glycoprotein (MOG)-mediated EAE in C57BL/6 mice (Hilliard et al., 2001). Moreover, recent reports have discussed the possible contribution of TRAIL to selection processes within the thymus (Cretney et al., 2003; Lamhamedi-Cherradi et al., 2003b). We have even shown that TRAIL is capable of inhibiting T cell activity and is upregulated in MS patients responding to interferon-β therapy (Lünemann et al., 2002; Wandinger et al., 2003).

Further research has revealed that TRAIL is also able to induce apoptosis in nontransformed human cells, as certain recombinant TRAIL preparations were cytotoxic

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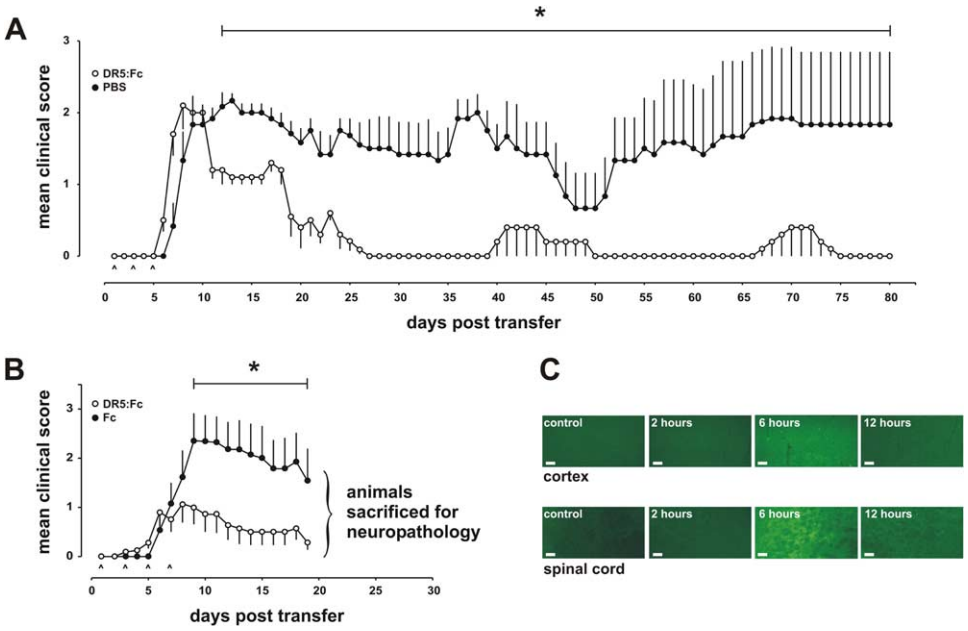

Figure 1. Autoimmune Encephalomyelitis Is Ameliorated by Intracerebral Injection of DR5:Fc

Adoptive transfer EAE in SJL mice was induced by intravenous injection of 3 × 10^7 encephalitogenic PLP-specific lymphocytes, and mean disease scores were significantly reduced by blockade of TRAIL in the CNS. (A) From the time of T cell transfer, mice were narcotized intravenously at the indicated intervals (arrowheads), received intracisternal injections of DR5:Fc (n = 5; open circles) or PBS (n = 6; filled circles), and were monitored for up to 80 days. Mean clinical disease scores were significantly reduced in the DR5:Fc-treated group during the indicated period [*$F_{12–80}(1, 68) = 7.6$; $p < 0.05$, ANOVA]. (B) In order to rule out an unspecific Fc-mediated effect, mice were treated in a similar way but received either DR5:Fc or Fc fragment only as a control (n = 8 for both groups). Disease was significantly attenuated in DR5:Fc-treated animals [*$F_{11–20}(1, 10) = 5.0$; $p < 0.05$, ANOVA]. For (A) and (B), mean clinical disease scores with SEM are given. (C) Distribution of DR5:Fc throughout the brain and spinal cord was assessed 2, 6, and 12 hr following injection into the cisterna cerebello-medullaris. A dispersed signal was found at 6 hr in the cortex, and a strong diffuse signal with the peak at 6 hr was found throughout the spinal cord. Scale bars, 20 μm.

to normal human (but not murine) hepatocytes (Jo et al., 2000); human endothelial cells (Li et al., 2003); and human erythropoietic cells in vitro (Zamai et al., 2000). In the brain, isolated human oligodendrocytes and human brain cells preserved in slice cultures were shown to be susceptible to TRAIL (Matysiak et al., 2002; Nitsch et al., 2000). These findings suggest that TRAIL has an ambivalent role in inflammation by promoting anti-inflammatory mechanisms on the one hand, and by a destructive effector function on the other hand. We therefore hypothesized that TRAIL acts as a mediator of brain cell death during autoimmune inflammatory CNS damage. Our experiments involving targeted inhibition of TRAIL in the brain and TRAIL-deficient mice show that the death ligand TRAIL indeed contributes to T cell-induced neuronal pathology in autoimmune neuroinflammation.

Results

Targeted Modulation of TRAIL in the CNS

In view of the existing literature, we hypothesized that TRAIL, apart from its reported anti-inflammatory properties, contributes to the damage mechanisms in EAE. In order to modulate TRAIL function selectively within the brain, we injected human TRAIL receptor 2 (TRAIL-R2; DR5) fused to human Fc (DR5:Fc, 1 μg in 10 μl) intracisternally (i.c.) at three time points after the adoptive transfer of encephalitogenic lymphocytes inducing EAE. This DR5:Fc fusion protein has been shown to recognize mouse TRAIL (Schmaltz et al., 2002), and it protects glioma cells (Dörr et al., 2002b) and murine fibroblasts from TRAIL-mediated apoptosis in vitro (O.A. and F.Z., unpublished data). Indeed, DR5:Fc-treated mice showed markedly decreased clinical signs, as compared to PBS-injected control animals (Figure 1A), over a prolonged observation period [$F_{12–80}(1, 68) = 7.6$; $p < 0.05$, ANOVA; n = 5 for DR5:Fc; n = 6 for PBS control group]. However, we were concerned about a possible nonspecific effect of the Fc portion of the DR5:Fc molecule used in this experiment. We therefore repeated this experiment using the corresponding human Fc protein alone as a control for DR5:Fc and were able to confirm our findings, as DR5:Fc-treated animals showed reduced disease scores (Figure 1B) [*$F_{11–20}(1, 10) = 5.0$; $p < 0.05$, ANOVA; n = 8 for both groups]. A time course analysis (2, 6, and 12 hr) showed distribution of intracisternally injected DR5:Fc throughout the brain and spinal cord (Figure 1C).

Effects of Intracerebral DR5:Fc Injections on Immune Cells in the Brain and outside the Brain

We realized that animals receiving DR5:Fc showed a slightly earlier disease onset, suggesting a possible

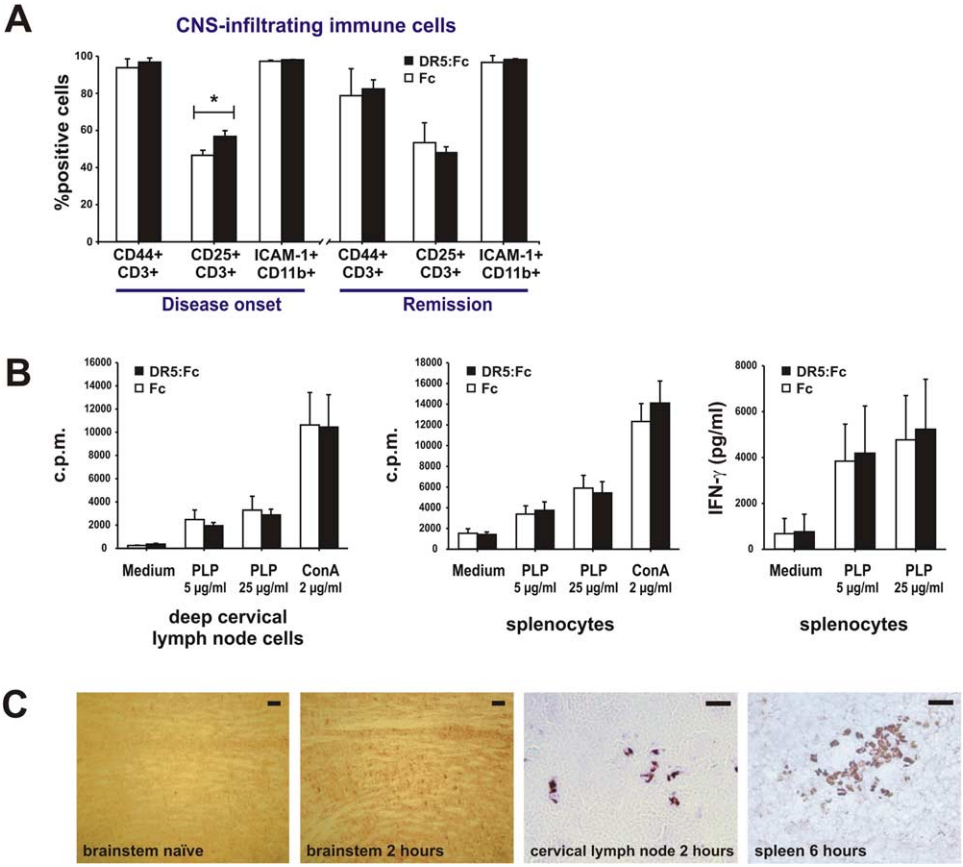

Figure 2. Immunomodulatory Effect and Systemic Distribution of Intracisternal DR5:Fc

(A) T cells and macrophages/microglia cells were isolated from the brain at the onset of the disease (day 7) (mean disability score ± SEM for DR5:Fc-treated group, 2.63 ± 1.31; for Fc-treated control group, 0.75 ± 0.38; n = 4 for both groups) and at the time of remission (day 14) (mean disability score ± SEM at the disease peak for DR5:Fc-treated group, 1.95 ± 0.81 [n = 5]; for Fc-treated control group, 2.92 ± 0.60 [n = 6]). Activation markers determined by FACS analysis are given as means with SEM (open bars, treatment with Fc fragment only; filled bars, treatment with DR5:Fc; *$p < 0.05$, Mann-Whitney U test).

(B) Spleens and draining cervical lymph nodes were harvested from animals with passive EAE at day 11 after cell transfer. Animals had been treated with intracisternal DR5:Fc or Fc fragment only, as shown in Figure 1B. Proliferation (given as counts per minute [c.p.m.]) and cytokine synthesis were measured as described in the Experimental Procedures. Data are presented as means with SD, with each bar corresponding to four animals treated with Fc fragment only (open bars) or DR5:Fc (filled bars).

(C) In order to check the distribution of the soluble receptor, naive SJL/J animals were intracisternally injected with DR5:Fc and sacrificed 2 and 6 hr later. CNS-draining deep cervical lymph nodes and spleens were isolated and stained for DR5:Fc by immunohistochemistry for the human Fc fragment of this molecule. Scale bars, 30 μm.

systemic immunoregulatory impact of the intracisternally applied DR5:Fc, as previously shown by others (Hilliard et al., 2001). Therefore, we recovered T cells and macrophages/microglia cells from the brains of SJL animals transferred with encephalitogenic T cells and treated with three intracisternal injections of DR5:Fc or Fc alone. We found that, at the very onset of the disease (day 7 posttransfer), in the DR5:Fc-treated animals even more T cells expressed the activation marker CD25 (Figure 2A). After the peak of the disease, no differences in the activity of the T cells were further observed. Absolute numbers and proportion of T cells and macrophages/microglia cells were comparable at both time points: at disease onset, the mean total number of infiltrating cells per mouse (± SEM) was $3.2 \times 10^6 \pm 1.6$ for Fc-treated animals and $2.7 \times 10^6 \pm 1.4$ for DR5:Fc-treated animals (n = 4 for both groups; $p > 0.05$, Mann-Whitney U test). After disease peak, the mean total number of infiltrating cells per mouse (±SEM) was $2.0 \times 10^6 \pm 0.8$ for Fc-treated animals (n = 6) and $2.5 \times 10^6 \pm 1.1$ for DR5:Fc-treated animals (n = 5; $p > 0.05$, Mann-Whitney U test). In a similar experiment, CNS-draining deep cervical lymph node cells, as well as splenocytes, were isolated at day 11 posttransfer and checked for antigen-specific proliferation and cytokine profiles (IFN-γ and IL-4). However, analysis of these parameters revealed neither a systemic effect of DR5:Fc treatment on lymphocyte proliferation nor cytokine synthesis in lymphocytes from deep lymph nodes or in splenocytes (Figure 2B). We next investigated the body distribution of intracisternally applied DR5:Fc. This was possible by detection of the human Fc fragment contained in this recombinant molecule. We found this fusion protein diffusely distributed in the

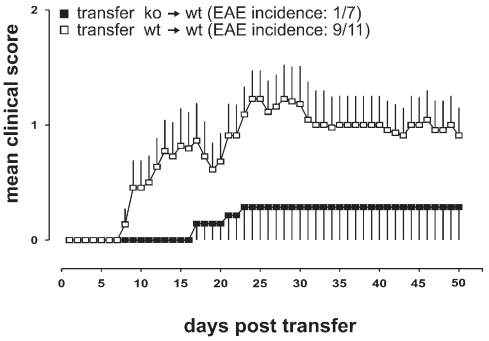

Figure 3. Reduced Encephalitogenicity of TRAIL-Deficient Myelin-Reactive Lymphocytes

MOG-specific lymphocytes from either TRAIL-deficient (ko) or wild-type (wt) donors were transferred into wild-type recipients. Disease was significantly milder in mice receiving TRAIL-deficient cells (filled squares; n = 7), compared to recipients of wild-type cells [open squares; n = 11; comparison of mean clinical scores, $F_{10-49}(1, 40) = 4.9$; $p < 0.05$]. Moreover, disease incidence was significantly lower in mice receiving TRAIL-deficient lymphocytes (EAE manifestation, i.e., a score of at least 1, was found in one out of seven mice), as compared to recipients of wild-type lymphocytes (EAE in 9 out of 11). Mean clinical disease scores with SEM are shown.

brainstem up to a maximum of 2 hr after intracisternal application. At that time point, we detected DR5:Fc confined to macrophage-like cells in deep cervical lymph nodes, but not diffusely distributed. After no sooner than 6 hr, we found DR5:Fc-containing macrophage-like cells prominently in the spleen (Figure 2C). A similar distribution was found with control Fc injection alone indicating unspecific uptake of DR5:Fc into phagocytes, which would rule out a systemic distribution of soluble DR5:Fc.

Attenuated Passive EAE upon Transfer of TRAIL-Deficient T Cells

Our data so far suggested that TRAIL plays a detrimental role in effector mechanisms occurring during neuroinflammation within the CNS. In view of the pivotal role of encephalitogenic T lymphocytes in both initiation of disease and tissue damage (Flugel et al., 2001), we considered that infiltrating myelin-specific T lymphocytes might employ TRAIL as an effector molecule. We therefore immunized TRAIL-deficient mice (Sedger et al., 2002) or wild-type C57BL/6 littermates with MOG peptide 35-55 (MOG35-55) and obtained MOG-reactive lymphocytes. In MOG-immunized TRAIL-deficient mice, we found an increased number of CD25+ T cells in draining lymph nodes on day 13 as compared to wild-type (mean percentage ± SEM was 64.8% ± 1.6 for TRAIL-deficient animals versus 51.5% ± 1.4 for wild-type littermates; $p < 0.05$, Mann-Whitney U test; n = 5 per group), whereas no difference in CD44 expression and proliferation was observed. Thus, there are indications of an increased activation of T cells in TRAIL-deficient animals. Subsequently, we transferred these

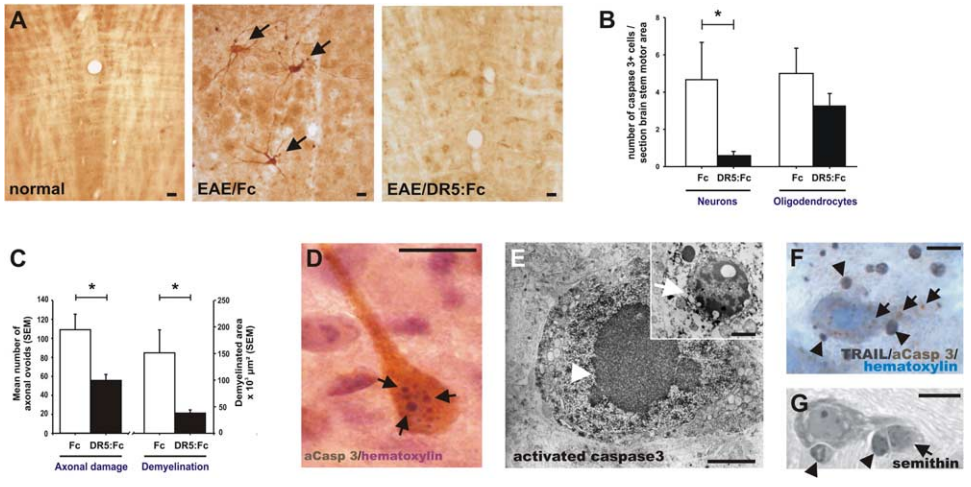

Figure 4. TRAIL Inhibition Is Associated with Decreased Brain Cell Death in EAE

(A) Brain cells expressing activated caspase 3 were identified by immunohistochemistry (amplified with ABC and visualized with HRP/DAB) in transverse brainstem sections from animals shown in Figure 1B. Scale bars, 20 μm. Sections are from normal (left panel), Fc-treated (middle panel), or DR5:Fc-treated (right panel) mice, respectively. Arrows point to active caspase 3-positive cells. (B) Average number of oligodendrocytes and neurons positive for activated caspase 3 in brainstem sections. Each bar represents three mice from the vehicle (Fc)-treated and DR5:Fc-treated groups, with four sections analyzed per mouse (*$p < 0.05$, vehicle control-treated compared with DR5:Fc-treated animals, Mann-Whitney U test). Data show means with SEM. (C) Axonal pathology and areas of demyelination with bars representing five mice per group (means with SEM; *$p < 0.05$, Mann-Whitney U test). (D) Neuron from a control (Fc)-treated mouse exhibiting immunolabeling for activated caspase 3 (aCasp 3) throughout its cytoplasm, which was counterstained with hematoxylin. Arrows point to chromatin condensation indicating the final phase of apoptosis. (E) Ultrathin section and electron micrograph of apoptotic neuron exhibiting caspase 3 immunolabeling (DAB; diffuse black precipitates) and morphologic characteristics of apoptosis, i.e., nuclear condensation (white arrowhead) and membrane blebbing (white arrow; insert). Scale bars, 2.5 μm. (F) Light micrograph of TRAIL-expressing cells (dark blue/black signal reflecting DAB/Ni chromogen) in the vicinity of and in direct contact (arrowheads) with a brainstem neuron in the early phase of apoptosis exhibiting activated caspase 3 (aCasp 3) staining (brown signal reflecting DAB chromogen; arrows) in its cytoplasm. (G) Semithin section revealing that invading TRAIL-expressing cells show classical morphological characteristics of lymphocytes (arrowheads). The arrow points to a neighboring cell exhibiting morphological characteristics of macrophages. Scale bars (D, F, and G), 20 μm.

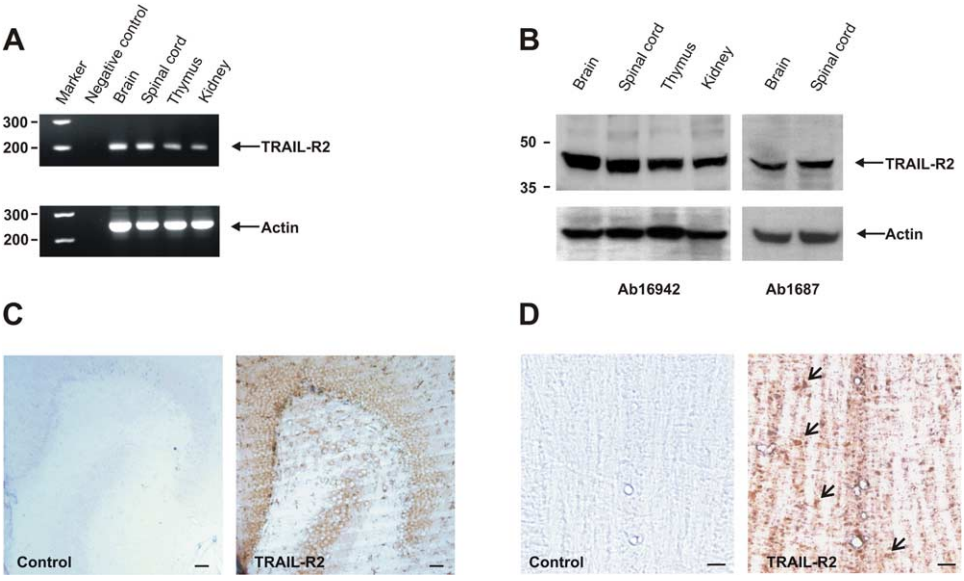

Figure 5. Expression of TRAIL Receptor 2 in the CNS

Brains from naive SJL mice were removed after perfusion with PBS (for RT-PCR and Western blot) or with 4% PFA (for immunohistochemistry) and tested for the expression of the mouse TRAIL-R2. (A) TRAIL-R2 mRNA expression in selected tissue samples assessed by RT-PCR. (B) TRAIL-R2 protein expression assessed by Western blot analysis using two different antibodies. (C and D) TRAIL-R2 expression analyzed by immunohistochemistry in the hippocampus (C) or brainstem (D) of the murine CNS. Arrows point to cells in the brainstem that are clearly positive. Scale bars, 50 μm.

cells upon antigen-specific restimulation into wild-type C57BL/6 littermates. In this setting, we were able to closely investigate the roles of TRAIL outside and within the CNS, as the immunomodulatory function of TRAIL was preserved in the wild-type recipients. In fact, these TRAIL-deficient lymphocytes showed a significantly reduced capacity to induce EAE: disease manifestation and mean clinical scores (Figure 3) were significantly milder in mice receiving TRAIL-deficient cells (EAE incidence: 1 out of 7), compared to recipients of wild-type cells [EAE incidence, 9 out of 11; comparison of mean clinical scores, $F_{10-49}(1, 40) = 4.9$; $p < 0.05$].

Reduced CNS Damage upon Intracisternal DR5:Fc Injection

The clinical and immunological data so far suggested a striking effect of intracisternal DR5:Fc application: although the peripheral antigen response was not altered, DR5:Fc-receiving animals showed a favorable outcome after the disease peak. We then addressed the question of whether intracisternal DR5:Fc modulates the inflammatory infiltration within the CNS. However, we were unable to find a difference, as we observed a comparable number of inflammatory foci/section corrected for focus size (2.4 ± 0.4 in the DR5:Fc-treated versus 2.5 ± 0.6 in the Fc-treated control animals, counted and calculated in four sections per mouse of three mice from each group in a blinded manner; $p > 0.05$, Mann-Whitney U test) (Aktas et al., 2004). Thus, we assumed that intracisternal DR5:Fc may have an impact on the tissue damage pattern. Indeed, immunohistochemical staining for activated caspase 3, a central effector enzyme involved in the execution of apoptotic cell death, revealed positive neurons with transected neurites (Figure 4A). Quantitative assessment of caspase-positive neurons indicated a significant difference between DR5:Fc- or vehicle-treated animals (Figure 4B). Further, we quantified the caspase-positive oligodendrocytes in a blinded fashion in the same way as the neurons. We also found a reduced number of caspase-positive oligodendrocytes in DR5:Fc-treated animals, although this difference showed a statistical trend only (Figure 4B). Moreover, both axonal pathology determined by APP staining (four longitudinal sections per mouse with five mice per group assessed in a blinded manner) and demyelination measured with Luxol fast blue staining (areas were assessed using a MicroBrightField setup; n = 5 mice per group) were reduced in the DR5:Fc-treated animals (Figure 4C).

Direct Contact between TRAIL-Expressing Immune Cells and Apoptotic Neurons during EAE

Our findings so far suggest a role of TRAIL in contributing to tissue damage during autoimmune neuroinflammation. The comparable immune response in the periphery and inflammatory infiltration of the brain argued against a possible indirect, i.e., immunomodulatory, effect of intracerebral DR5:Fc. We therefore speculated that TRAIL, released by or localized to infiltrating myelin-specific immune cells, may be responsible for death in TRAIL receptor-bearing target cells during EAE. We observed that activation of caspase 3 throughout the cytoplasm of dying brain cells was indeed regularly accompanied by (1) chromatin condensation, as detected by hematoxylin-eosin counterstaining (Figure 4D), and (2) nuclear condensation and membrane blebbing, as detected by electron microscopy (Figure 4E).

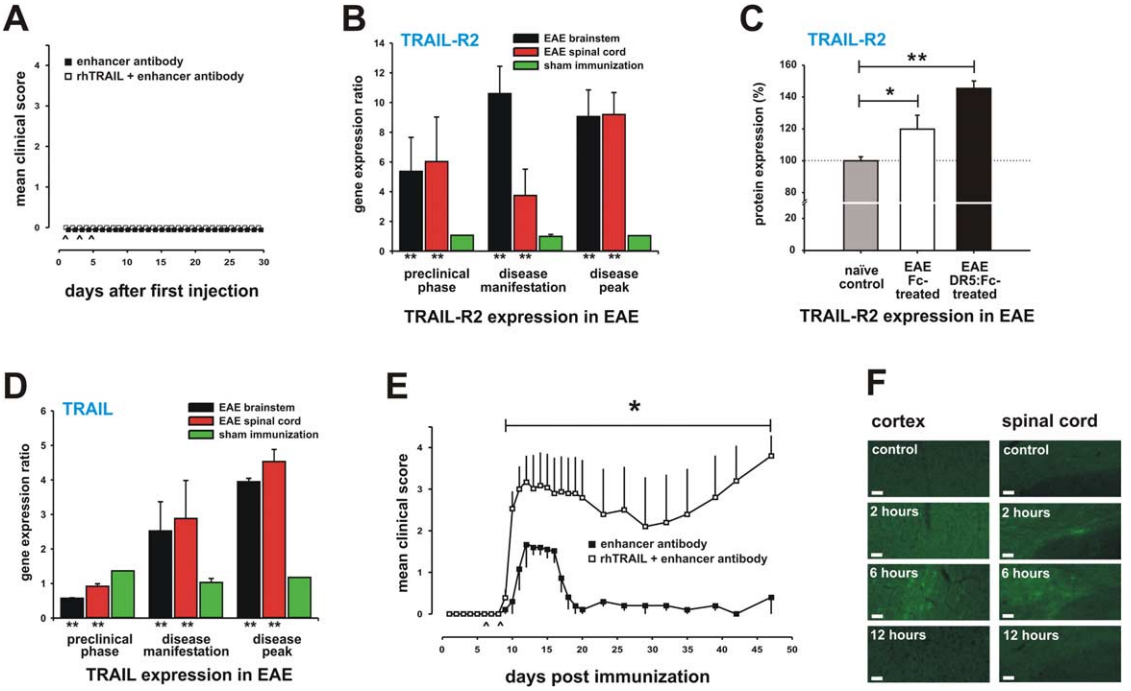

Figure 6. Selective TRAIL Susceptibility of the Inflamed CNS

(A) Recombinant human TRAIL (rhTRAIL together with an enhancer antibody for multimerization; open squares) or enhancer antibody alone (filled squares) were injected intracisternally into healthy mice (arrowheads; n = 5). No clinical deficit was observed. (B and D) Results of quantitative gene expression analysis for TRAIL receptor 2 (B) and TRAIL (D) performed in different regions of the CNS (brainstem, spinal cord) from animals with active EAE (induced by immunization with PLP139-151; see Experimental Procedures for details) or from control animals (sham immunized, i.e., the same immunization protocol as used for active EAE, including pertussis toxin injections, but without PLP139-151). Animals were analyzed in the course of EAE, i.e., at day 3 (preclinical phase), at day 9 (disease manifestation), or at day 13 (disease peak) after immunization, respectively. Mean relative changes of gene expression assessed with real-time PCR are given (with SEM) and compared to sham immunized controls (**p < 0.01, Mann-Whitney U test). (C) Quantitative assessment of TRAIL-R2 protein expression on dentate granule cells in naive controls, Fc-treated passive EAE, and DR5:Fc-treated passive EAE (disease peak for EAE animals). Four sections from four animals per group were analyzed. Results (mean + SEM) indicate TRAIL-R2 protein expression in relation to naive control animals (control = 100%) (*p < 0.05, **p < 0.01 for comparison with naive control group, respectively, Mann-Whitney U test). (E) Mild active EAE was significantly deteriorated by delivery of recombinant human TRAIL into the CNS prior to and at the onset of disease. SJL mice were immunized and treated with active rhTRAIL (400 ng per mouse) together with enhancer antibody for multimerization (n = 5; open squares; 2 μg per mouse) or enhancer antibody alone (n = 5; filled squares) intracisternally at days 6 and 8 after immunization [arrowheads; *F_{9-47}(1, 20) = 6.7; p < 0.05 for comparison of mean clinical scores, ANOVA]. Mean clinical disease scores with SEM are given. (F) Distribution of recombinant human TRAIL throughout the brain and spinal cord was assessed 2, 6, and 12 hr following injection into the cisterna cerebellomedullaris. TRAIL was found distributed as far as the cortex and the spinal cord with a peak 6 hr following injection. Scale bars, 20 μm.

In contrast, apoptotic neurons in normal control mice were entirely absent. Our immunohistochemical analysis of normal mouse brain showed expression of the death-mediating TRAIL receptor 2 predominantly on neurons and, to some extent, on oligodendrocytes (Figures 5A–5D). Importantly, in the course of EAE, we detected TRAIL-positive lymphocytes that had invaded the brain in close vicinity and direct contact with neurons showing the appearance of caspase 3 activation in their cytoplasm (Figures 4F and 4G). These data suggest that brain-infiltrating and TRAIL-expressing lymphocytes are able to deliver an apoptosis-inducing signal to receptor-bearing neurons in the course of neuroinflammation.

Regulation of Intracerebral Expression of TRAIL Receptor 2 and TRAIL in the Course of EAE

In order to confirm the role of TRAIL in the induction of neuronal apoptosis in vivo, we injected recombinant TRAIL into the compartment containing the cerebrospinal fluid. However, even repeated intracisternal injections of recombinant preparations of multimerized human TRAIL induced neither clinical disability nor apoptosis of neurons and oligodendrocytes, respectively, in healthy SJL mice (Figure 6A). In contrast, the same TRAIL preparations were effective in killing mouse fibroblasts (O.A. and F.Z., unpublished data). We therefore assumed that susceptibility to TRAIL-mediated cell death is differentially regulated in normal and inflamed murine brain tissue. Supporting this idea, quantitative gene expression analysis showed that both the death-mediating TRAIL receptor 2 and TRAIL itself are significantly upregulated in the CNS during EAE (Figures 6B and 6D). Further, analysis of protein expression on neurons (Figures 5A–5D) revealed a significant increase of TRAIL-R2 in passive EAE (both in Fc-injected animals and animals treated with DR5:Fc in order to block TRAIL signaling) (Figure 6C). Thus, we decided to test intracisternal TRAIL in the course of the inflammatory EAE paradigm. A recombinant TRAIL preparation containing a FLAG tag was injected together with an enhancer anti-FLAG antibody for multimerization (Figure 6E). Indeed, as

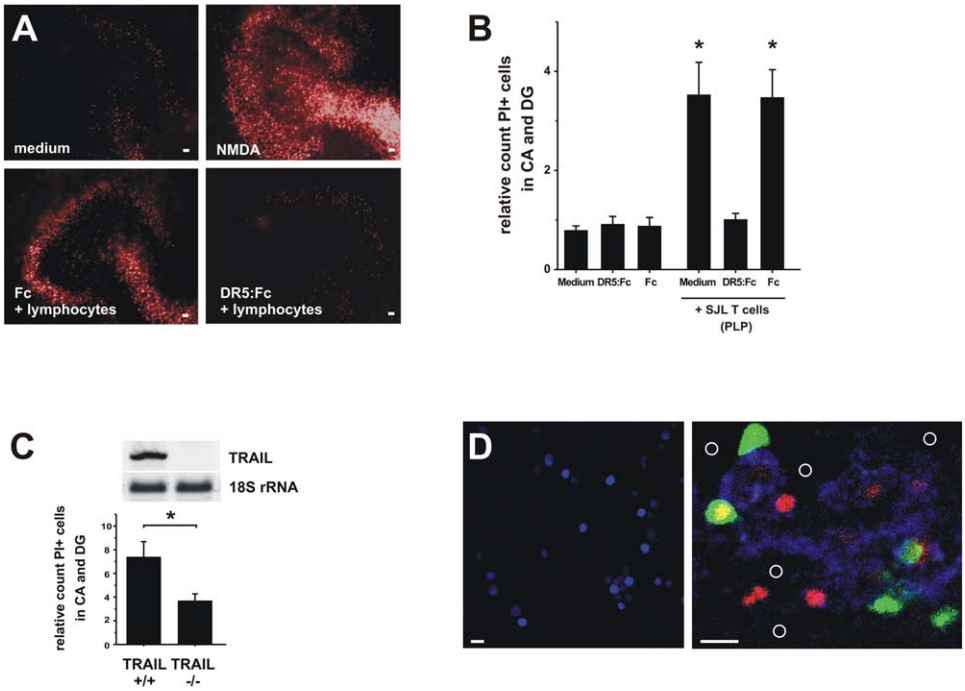

Figure 7. DR5:Fc Protects from Encephalitogenic T Cell-Induced Brain Injury In Vitro

(A) Propidium iodide-fluorescence microscopy of the SJL mouse living hippocampal brain slices without (left upper quadrant) or with the addition of PLP-specific encephalitogenic T cells (left lower quadrant, with Fc control). As a positive control, the hippocampus is shown in response to application of the neurotoxic agent NMDA (right upper quadrant). Cell death induced by encephalitogenic lymphocytes was reduced by soluble DR5:Fc (right lower quadrant). Scale bars, 100 μm.

(B) The number of PI-positive cells was assessed in the neuronal cell layer of the slice culture incubated with culture medium alone, and with DR5:Fc or Fc, and with or without encephalitogenic T cells obtained from PLP-immunized SJL mice. An average number of 26 slices was analyzed per treatment group. Results (means + SEM) indicate cell counts in relation to nondamaged brain tissue (control = 1). The number of PI+ cells was significantly reduced upon incubation with soluble DR5:Fc (1 μg/ml), compared to Fc alone (1 μg/ml) or medium control (*$p < 0.05$, Mann-Whitney U test).

(C) TRAIL mRNA expression was detected in MOG-specific T cells from wild-type animals but not in TRAIL-deficient T cells. Number of PI-positive cells (means with SEM) is demonstrated in the neuronal cell layer of CA and DG after invasion of wild-type or TRAIL-deficient MOG-reactive lymphocytes (*$p < 0.05$, Mann-Whitney U test).

(D) With confocal laser scanning microscopy, TRAIL expression (blue) is demonstrated by fluorescence labeling of fixed and permeabilized encephalitogenic T cells using a wide pinhole and aperture for detection (left panel). After invasion into brain tissue (right panel), CFDA-labeled T cells (green) were found adjacent to dead PI-stained neuronal nuclei (red) surrounded and contacted by secreted TRAIL (blue). Areas without T cells and neurons do not contain any labeling for TRAIL (circles). Scale bars, 10 μm.

compared to injection of the enhancer alone, application of TRAIL prior to and at the onset of EAE significantly increased EAE severity [Figures 6E and 6F; $F_{9-47}(1, 20) = 6.7$; $p < 0.05$ for comparison of clinical scores, ANOVA; n = 5 for both groups]. Moreover, considering reports on diverging cytotoxic capacities of different TRAIL preparations (Lawrence et al., 2001; Qin et al., 2001), we tested and confirmed this finding (O.A., R. Lauster, and F.Z., unpublished data) using a recombinant TRAIL preparation that is active without any enhancing antibodies, i.e., N-terminal truncated human TRAIL (residues 95–281) containing an N-terminal 6-His tag (Aktas et al., 2004). These results provide evidence that, during EAE, TRAIL mediates cell death in the CNS via its specific receptors, which are upregulated under these inflammatory conditions.

TRAIL-Mediated Neurotoxicity of Encephalitogenic T Cells In Vitro

In a final set of experiments, we assessed the role of TRAIL as a mediator of neuronal cell death by encephalitogenic lymphocytes in living organotypic brain slices. PLP-specific activated lymphocytes used to induce transfer EAE were stained with carboxyfluorescein diacetate (CFDA) and applied to the surface of these brain slices (Gimsa et al., 2000). Cell death was visualized by propidium iodide (PI) staining (Figure 7A). N-methyl-D-aspartate (NMDA)-induced damage in the pyramidal cell layer of the cornu ammonis (CA) and granule cell layer of the dentate gyrus (DG) was performed as a positive control. The number of PI+ cells in the neuronal layer of the CA and DG (for quantification, see Ullrich et al., 2001) induced by PLP-restimulated lymphocytes was significantly reduced in the presence of DR5:Fc(1 μg/ml), as compared to their number in the presence of Fc alone (Figures 7A and 7B). In addition, we employed TRAIL-deficient T cells and observed significantly reduced neuronal cell death when compared to wild-type T cells (Figure 7C). In further confocal studies, we identified invading CFDA-labeled T cells in the vicinity of dead neurons whose DNA was stained by PI (Figure 7D). Importantly, immu-

nolabeling of TRAIL detected by PerCP-Cy5.5 fluorescence was confined to sites surrounding these invaded T cells and dying neurons (Figure 7D). These results suggest that both soluble and lymphocyte bound TRAIL may contribute to neuronal death in the brain.

Discussion

Following a series of recent findings (Diestel et al., 2003; Ferguson et al., 1997; Kornek et al., 2000; Meyer et al., 2001; Peterson et al., 2001; Trapp et al., 1998), neuronal pathology is now considered to be a key feature of autoimmune neuroinflammation, namely EAE and MS. However, the precise mechanisms leading to the destruction of neurons during the myelin-specific immune attack have yet to be elucidated.

The death ligand TRAIL has primarily aroused tremendous interest due to its ostensible selectivity in killing tumor cells, as observed in various animal models (Ashkenazi et al., 1999; Cretney et al., 2002; Smyth et al., 2001; Walczak et al., 1999). Later, several studies including our own work (Lünemann et al., 2002; Wandinger et al., 2003; Wendling et al., 2000) have demonstrated the immunomodulatory capacity of TRAIL in the immune system. Importantly, the blockade of TRAIL action outside the CNS results in the exacerbation of various autoimmune disease models (Lamhamedi-Cherradi et al., 2003a; Song et al., 2000) including EAE (Hilliard et al., 2001). These studies propose the potentially therapeutic use of TRAIL as a protective agent for autoimmunity. Surprisingly, it became clear that, apart from these immunomodulatory functions, TRAIL may act as a death-inducing effector molecule on nontransformed human cells: certain TRAIL preparations were found to have toxic effects on hepatocytes as well as brain cells (Jo et al., 2000; Matysiak et al., 2002; Nitsch et al., 2000). Since we could show that TRAIL is upregulated by activated human lymphocytes (Dörr et al., 2002b; Wendling et al., 2000), we speculated about a potential role of TRAIL in the course of the immune cell attack to the brain. With these aspects of TRAIL biology in mind, we employed strategies for a selective blockade of TRAIL within the CNS, which is an anatomically and immunologically separate compartment. In fact, in contrast to systemic application of soluble TRAIL receptor molecules outside the brain (Hilliard et al., 2001), DR5:Fc injection selectively into this compartment resulted in an amelioration of EAE. We were able to show that our intracisternal route of injection did not influence the peripheral immune response including T cell proliferation and cytokine release. Furthermore, transfer of myelin-specific TRAIL-deficient T cells into wild-type recipients yielded a significantly attenuated disease score. In this experimental setting, the peripheral immunomodulatory role of TRAIL was preserved, as recipient wild-type animals were still able to regulate the transferred T cells via TRAIL. These findings indicate that brain-invading myelin-specific T cells (Flugel et al., 2001) that lack the important effector molecule TRAIL are significantly reduced in their damaging capacity. An initial increase in T cell activity in the brain following CNS-specific blockade of TRAIL signaling is in line with the anti-inflammatory capacity of TRAIL as earlier reported for the peripheral immune system (Hilliard et al., 2001; Lünemann et al., 2002; Wandinger et al., 2003). We conclude that TRAIL plays an ambivalent role during EAE: extracerebrally, i.e., within the peripheral immune system, TRAIL has an anti-inflammatory (and thus protective) effect, whereas in the central nervous system this anti-inflammatory effect is less important for EAE outcome than the apoptosis-inducing effector function targeting neural cells.

Our analysis of healthy CNS tissue revealed that main targets during neuroinflammation, i.e., neurons and oligodendrocytes, express the death-mediating TRAIL receptor 2 (Dörr et al., 2002a). In contrast, TRAIL itself is not expressed within the brain (Dörr et al., 2002a). Indeed, soluble TRAIL, when applied to living CNS tissue (Nitsch et al., 2000) or oligodendrocytes (Matysiak et al., 2002), can induce caspase-dependent brain cell death. Thus, it may well be the case that TRAIL-expressing activated T cells that invade the brain during neuroinflammation (Flugel et al., 2001) mediate brain damage using TRAIL as an effector molecule. In fact, we observed that caspase-dependent neuronal cell death in the course of EAE (Diestel et al., 2003) was significantly reduced by blocking TRAIL within the CNS as shown by our present data. In our study, caspase-positive neurons were frequently contacted by small, TRAIL-expressing lymphocytes, favoring T cells as the cellular source of TRAIL.

We and other investigators have previously shown that (1) TRAIL is upregulated on the surface of T cells upon antigen-specific stimulation (Mariani and Krammer, 1998; Wendling et al., 2000), (2) TRAIL is secreted by activated T cells (Martinez-Lorenzo et al., 1999), and (3) TRAIL plays a significant role in T cellular cytotoxicity (Dörr et al., 2002b). Our in vitro studies using organotypic slices show that myelin-specific T cells invading organotypic slice cultures release TRAIL in the vicinity of dying neurons. In fact, these encephalitogenic T cells are able to induce substantial neuronal damage. This is in line with recent reports by Giuliani et al. reporting a marked toxicity of human T cells toward human neurons in vitro (Giuliani et al., 2003). In the present study, activated TRAIL-deficient lymphocytes from knockout animals showed a significant reduction in their killing capacity. Using 2-photon laser scanning microscopy for live imaging in slices (Nitsch et al., 2004), we recently observed that TRAIL-deficient T cells directly contact neurons but show a reduced neurotoxic capacity (O.A., E.P., R.N., and F.Z., unpublished data). Moreover, functional blockade of TRAIL using DR5:Fc was effective in preventing this neuronal cell death, indicating a pivotal role of TRAIL in damage processes. However, it is possible that TRAIL secreted by infiltrating macrophages may contribute to TRAIL-mediated neural damage in EAE, as we recently identified activated macrophages as a significant source of soluble TRAIL (Ehrlich et al., 2003). This is supported by Miura et al., who have demonstrated the involvement of the TRAIL system in macrophage-mediated neuronal cell death occurring in a humanized mouse model of HIV-associated encephalopathy in vivo (Miura et al., 2003).

Interestingly, we could not discern any cytotoxic effects in connection with either of two different TRAIL preparations applied intracisternally in healthy animals.

This observation is in line with a previous report demonstrating that intracerebral injection of TRAIL in a xenograft brain tumor model only affects the tumor cells and does not show significant neurotoxicity to the normal CNS tissue (Roth et al., 1999). However, a recent report showed that TRAIL, which lacks any hepatotoxicity in healthy rodents, critically contributes to hepatocyte cell death in different models of murine hepatitis (Zheng et al., 2004). We therefore performed quantitative expression analysis for TRAIL receptor 2 and found a significant upregulation prior to and during the peak phase of EAE. Accordingly, we observed that intracisternally applied soluble TRAIL enhanced disease severity in animals with ongoing EAE, while healthy animals were not affected. This indicates that receptor levels under normal conditions are not sufficient to mediate a significant death signal, which is only achieved in the course of inflammation. Indeed, inflammation itself was not modulated by intracerebral TRAIL blockade, and this further corroborates the idea that TRAIL is a specific death-inducing effector molecule in the brain. This finding might also explain why the use of TRAIL for tumor therapy did not result in toxic effects to healthy neurons.

Taken together, our study reveals that the death ligand TRAIL critically contributes to irreversible CNS damage during autoimmune neuroinflammation. Evidence is provided for an important role of TRAIL expressed on and secreted by CNS-invading lymphocytes in the effector phase of a multiple sclerosis animal model. One has to bear in mind, however, that the immunosuppressive properties of TRAIL may be useful to enhance the effects of TRAIL outside the CNS. This complex action of TRAIL resembles a double-edged sword, as known for other members of the TNF family such as the CD95 system (Zipp et al., 1999). However, targeted modulation of TRAIL receptor-TRAIL interactions within the inflamed brain may represent a possible therapeutic strategy for the prevention of neurological deficits in MS patients.

Experimental Procedures

PCR

Reverse Transcriptase PCR

Total RNA was isolated from PBS-perfused frozen tissue followed by digestion with DNase I. PCR conditions were as follows: 30 cycles, 30 s/95°C, 30 s/65°C, 45 s/72°C; TRAIL-R2 primer sequences (GenBank AF176833) 5′-CACAAATACGGTGTGTCGATG-3′ (nucleotides 372–392) and 5′-CTGGAACCAGGAGTCCTATCC-3′ (nucleotides 551–571); TRAIL primer sequences (Genbank NM009425) 5′-TGAGGATTTCTGGGACTCCACTGA-3′ (nucleotides 241–264) and 5′-CTTCAGCTTCCTGAAATCGG-3′ (nucleotides 628–647); β-actin primer sequences (GenBank J04181) 5′-GTGGGCCGCCCTAGG CACCA-3′ (nucleotides 165–184) and 5′-CGGTTGGCCTTAGGGTT CAGGGGG-3′ (nucleotides 386–409). Isolated PCR fragments were reamplified and checked with an ABI 377 DNA sequencing system (Applied Biosystems, Weiterstadt, Germany).

Quantitative Real-Time PCR

For quantitative real-time PCR, reverse transcription and Taqman real-time PCR analysis was done in duplicate using the 2-$\Delta\Delta C_T$ relative quantification method (ABI-Prism 7700, Applied Biosystems). PCR conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles, 15 s/95°C, 60 s/60°C. The following PCR primers and fluorogenic probes (Eurogentec, Seraing, Belgium; designed with Primer Express Software, Applied Biosystems) were used: TRAIL forward, 5′-GATCACTCGGAGAAGCAACTCA-3′; TRAIL reverse, 5′-GAGAGGACTCCCAGGATT-CAATC-3′; TRAIL probe, 5′-FAM-CAA TCTCCAAGGATGGAAAGACCTTAGGCCA-TAMRA-3′; TRAIL-R2 forward, 5′-TGCAAACCAGGCACCTTTG-3′; TRAIL-R2 reverse, 5′-TCT-TCCCCGTCAGTGCAGTT-3′; TRAIL-R2 probe, 5′-FAM-AGACT CCCCTGAGATC-TGCCAGTCATGCT-TAMRA-3′; GAPDH forward, 5′-CTCAACTACATGGTCTACAT-GTTCCA-3′; GAPDH reverse, 5′-CCATTCTCGGCCTTGACTGT-TAMRA-3′; GAPDH probe, 5′-FAM-TGACTCCACTCA CGGCAAATTCAACGT-TAMRA-3′.

Western Blot

PBS-perfused frozen tissue or lymphocytes were homogenized in extraction buffer containing 1% Triton X-100 and protease inhibitors (0.2 mM PMSF, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin). Samples were separated by 12% SDS-PAGE, membranes were incubated with anti-TRAIL-R2 antibodies (dilution 1:1000; AB1687 or AB16942; Chemicon, Temecula, CA). Antibodies coupled to horseradish peroxidase (Dako Diagnostica, Hamburg, Germany) were used as secondary antibodies, and an ECL-plus system (Amersham Pharmacia, Freiburg, Germany) was used for development. Membranes were sequentially incubated with an antibody against β-actin (Sigma, Deisenhofen, Germany).

Histology and Immunohistochemistry

Mice euthanized with narcotics were transcardially perfused with 4% paraformaldehyde (PFA). Horizontal 50 μm thick vibratome sections (Shandon, Pittsburgh, PA) or 10 μm thick paraffin sections were immersed with 3% H_2O_2 to quench unspecific endogenous peroxidase activity, then washed and blocked (10% normal serum and 0.5% Triton X-100) prior to overnight incubation with anti-activated caspase 3 (1:100; R&D, Wiesbaden, Germany), anti-TRAIL-R2 antibody (Ab16942; 1:50; Chemicon), or anti-β-APP (1:1000; Zymed Laboratories, San Francisco, CA). Sections were immunostained using FITC-labeled secondary antibodies or the avidin:biotinylated enzyme complex technique (ABC-Elite, Vector Laboratories, Burlingame, CA) before development with diaminobenzidine ([DAB]; brown signal) as a substrate. Ig-control experiments were performed for all primary antibodies, and no staining was observed under these conditions. Axonal ovoids indicating transection (Aktas et al., 2004) were counted in longitudinal spinal cord sections (cervical to lumbosacral level; five sections per mouse). Areas of demyelination were quantitatively evaluated using Luxol fast blue staining (American Histolabs, Gaithersburg, MD). Cell-specific expression of TRAIL-R2 was assessed as immunofluorescence signal intensity on vibratome sections using a MetaMorph analysis setup. For counterstaining of nuclei, brainstem sections stained for active caspase 3 were incubated with Harris hematoxylin for 1 min at room temperature. For the staining of TRAIL, these sections were labeled with mouse DR5:human Fc chimera (1:25; R&D) and visualized using DAB/Ni as a chromogen (dark blue/black signal), as described elsewhere (Leranth and Nitsch, 1994). Electron microscopy was performed on a Zeiss EM 900 (Jena, Germany).

Induction of Active and Passive EAE, Treatment Procedures, and Distribution Analysis

For adoptive transfer (passive) EAE in SJL mice, female donors (6–8 weeks; Harlan, Rehovot, Israel; Charles River, Sulzfeld, Germany) were immunized with murine PLP139-151 (purity > 95%; Pepceuticals, Leicester, UK) as previously described (Steinbrecher et al., 2001). Primary lymph node cells were prepared 10 days later, restimulated with PLP139-151 (10 μg/ml) for 4 days, and injected intravenously (i.v.) into syngenic recipients (3 × 10^7 cells per mouse). Antigen specificity of injected cells was routinely checked with a standard ^{3}[H]-thymidine proliferation assay. For active EAE in SJL mice, animals were immunized as previously described (Aktas et al., 2003; Aktas et al., 2004) with the following modifications: each mouse received 100 μg PLP139-151 in complete Freund adjuvant (CFA; supplemented with *M. tuberculosis* H37Ra, 2 mg/ml), and pertussis toxin (200 ng per mouse; List) was injected intraperitoneally at days 0 and 2 after immunization. The control group for serial assessment of gene expression in the course of active EAE (Figures 6B and 6D) was sham immunized, i.e., using the same immunization protocol as for active EAE, including pertussis toxin injections, but without PLP139-151. For passive EAE in TRAIL-defi-

cient animals, C57BL/6 *TRAIL* knockout mice (kindly provided by Amgen, Seattle, WA) (Cretney et al., 2002; Sedger et al., 2002) and wild-type littermates were immunized with MOG35-55 (purity >95%; Pepceuticals; in CFA supplemented with *M. tuberculosis* H37Ra, 4 mg/ml). Twelve days later, animals were sacrificed, whereupon draining lymph node cells were isolated, restimulated with MOG35-55 (30 μg/ml) in the presence of IL-12 (20 ng/ml, R&D), harvested after 72 hr, and injected into wild-type recipients (20×10^6 blasts per mouse). Pertussis toxin (400 ng per mouse) was administered at day 0 and 2 after immunization. For all EAE procedures, neurological deficits were graded as previously described (Aktas et al., 2003). For intracisternal treatment procedures, mice were intravenously anesthetized with ketamine (50 mg/kg; Curamed, Karlsruhe, Germany) and xylazine (10 mg/kg; Bayer, Leverkusen, Germany) before puncture of the cisterna magna with a 26G syringe (Hamilton, Bonaduz, Switzerland). Human recombinant DR5:Fc (1 μg per mouse) or vehicle control (1 μg human Fc for Figure 1B or PBS alone for Figure 1A; see text and figures for explanation) was injected in a volume of 10 μl (Okuda et al., 1998; Reijneveld et al., 1999; Saeki et al., 1992). Human Fc fragment was obtained from Calbiochem (San Diego, CA) and dialyzed against sterile PBS before use. Similarly, recombinant human TRAIL (400 ng with 2 μg enhancer antibody for multimerization per mouse; Alexis, San Diego, CA) or vehicle control (enhancer antibody alone) was injected intracisternally in a total volume of 10 μl. Distribution of DR5:Fc or Fc was assessed in sections of brain, lymph nodes, and spleen using anti-human Fc fragment antibody (Sigma) and FITC or ABC/DAB for visualization. TRAIL was visualized using a DR5:Fc-FITC detection system (Alexis). All procedures were conducted according to protocols approved by the local animal welfare committees.

Isolation of CNS Mononuclear Cells and FACS Analysis

Isolation of CNS mononuclear cells was performed basically as previously described (Ford et al., 1995; Mack et al., 2003), with minor adaptations: tissue digestion was performed in DMEM containing 363 U/ml clostridiopeptidase A (Sigma) and 200 U/ml DNase I (Roche, Mannheim, Germany) for 30 min at 37°C. Homogenate was resuspended in 23% Percoll (Amersham Pharmacia) and underlain with 73% Percoll. The gradients were centrifuged for 30 min. Phenotypic analysis of the infiltrate was performed using a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Activation of T cells was monitored as described previously (Nitsch et al., 2004) using antibodies against CD3, CD25, and CD44. Activation of CNS-derived macrophages was analyzed by monitoring the expression of ICAM-1 on nonlymphocytic CD11b-positive cells (all antibodies from BD Pharmingen, Heidelberg, Germany).

Ex Vivo Proliferation

Lymph node cells and splenocytes were isolated as previously described (Brocke et al., 1996). After 72 hr culture with PLP (5 or 25 μg/ml) or concanavalin A (ConA; 2 μg/ml), 0.5 μCi ^{3}[H]-thymidine was added to each well, and 18 hr later incorporation was measured in counts per minute (c.p.m.) with a Microbeta counter (Wallac, Freiburg, Germany).

Cytokines

For detection of IFN-γ or IL-4, sandwich ELISAs of supernatants were performed according to the manufacturer's instructions (Becton Dickinson).

Coculture of Slices and T Cells

Organotypic entorhinal-hippocampal slice cultures (350 μm thick) from 10-day-old SJL or C57BL/6 mice were prepared on a tissue chopper (Bachhofer, Reutlingen, Germany) and cultured as described (Ullrich et al., 2001). For coculture, 5×10^4 encephalitogenic lymphocytes (as used for EAE induction) were prelabeled with CFDA (Gimsa et al., 2000) and transferred to the surface of slices (day 7 in vitro) preincubated with DR5:Fc or Fc alone (both 1 μg/ml) for 3 hr. NMDA (50 μM) was applied as a positive control. Encephalitogenicity of PLP-specific lymphocytes was routinely checked by successful induction of adoptive transfer EAE. Damage in neuronal cell layers of the CA and DG was detected 12–24 hr later by adding 5 μg/ml PI (30 min at 36°C) (Ullrich et al., 2001) using a rhodamine filter (488 nm/515 nm) with a dark-field inverse fluorescence microscope (Olympus, Tokyo, Japan). Dead lymphocytes were distinguished by double-fluorescence microscopy and subtracted from the counts of PI+ cells within the neuronal layers of the slices. For resectioning, slices were immersed prior to the preparation of 20 μm thin horizontal cryostat sections. Brain slices were fixed using the same fixative and were treated as whole mounts in the same way as with vibratome sections. For confocal laser scanning microscopy, CFDA and PI were excited at 488 nm, emission of CFDA was detected between 510 and 560 nm, and emission of PI was detected between 570 nm and 625 nm. TRAIL was immunostained with the M19 antibody (Santa Cruz, CA) (Dörr et al., 2002a) and labeled with PerCP-Cy5.5 fluorescence dye (excitation at 633 nm, detected between 680 and 710 nm) using a Leica TCS SP2 confocal microscope (Leica, Heidelberg, Germany). Corresponding Ig-control experiments were performed, and no staining was observed under these conditions.

Statistics

For group comparisons, the Mann-Whitney U test was applied. To compare the EAE courses, statistical analysis of repeated measurements was performed by means of variance using the Fisher's pairwise least significant difference test (Aktas et al., 2003).

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References

Aktas, O., Waiczies, S., Smorodchenko, A., Dorr, J., Seeger, B., Prozorovski, T., Sallach, S., Endres, M., Brocke, S., Nitsch, R., et al. (2003). Treatment of relapsing paralysis in experimental encephalomyelitis by targeting Th1 cells through atorvastatin. J. Exp. Med. *197*, 725–733.

Aktas, O., Prozorovski, T., Smorodchenko, A., Savaskan, N.E., Lauster, R., Kloetzel, P.-M., Infante-Duarte, C., Brocke, S., and Zipp, F. (2004). Green tea epigallocatechin-3-gallate mediates T cellular NF-κB inhibition and exerts neuroprotection in autoimmune encephalomyelitis. J. Immunol. *173*, 5794–5800.

Ashkenazi, A., Pai, R.C., Fong, S., Leung, S., Lawrence, D.A., Marsters, S.A., Blackie, C., Chang, L., McMurtrey, A.E., Hebert, A., et al. (1999). Safety and antitumor activity of recombinant soluble Apo2 ligand. J. Clin. Invest. *104*, 155–162.

Bjartmar, C., Wujek, J.R., and Trapp, B.D. (2003). Axonal loss in the pathology of MS: consequences for understanding the progressive phase of the disease. J. Neurol. Sci. *206*, 165–171.

Brocke, S., Quigley, L., McFarland, H.F., and Steinman, L. (1996). Isolation and characterization of autoreactive T cells in experimental autoimmune encephalomyelitis of the mouse. Methods Enzymol. *9*, 458–462.

Brosnan, C.F., and Raine, C.S. (1996). Mechanisms of immune injury in multiple sclerosis. Brain Pathol. *6*, 243–257.

Cretney, E., Takeda, K., Yagita, H., Glaccum, M.B., Peschon, J.J., and Smyth, M.J. (2002). Increased susceptibility to tumor initiation and metastasis in TNF-related apoptosis-inducing ligand-deficient mice. J. Immunol. *168*, 1356–1361.

Cretney, E., Uldrich, A.P., Berzins, S.P., Strasser, A., Godfrey, D.I., and Smyth, M.J. (2003). Normal thymocyte negative selection in TRAIL-deficient mice. J. Exp. Med. *198*, 491–496.

Degli-Esposti, M.A., Dougall, W.C., Smolak, P.J., Waugh, J.Y., Smith, C.A., and Goodwin, R. (1997a). The novel receptor TRAIL-R4 induces NF-κB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. Immunity *7*, 813–820.

Degli-Esposti, M.A., Smolak, P.J., Walczak, H., Waugh, J.Y., Huang, C.P., DuBose, R.F., Goodwin, R., and Smith, C.A. (1997b). Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. J. Exp. Med. *186*, 1165–1170.

Diestel, A., Aktas, O., Hackel, D., Hake, I., Meier, S., Raine, C.S., Nitsch, R., Zipp, F., and Ullrich, O. (2003). Activation of microglial poly(ADP-ribose)-polymerase-1 by cholesterol breakdown products during neuroinflammation: a link between demyelination and neuronal damage. J. Exp. Med. *198*, 1729–1740.

Dörr, J., Bechmann, I., Waiczies, S., Aktas, O., Walczak, H., Krammer, P.H., Nitsch, R., and Zipp, F. (2002a). Lack of tumor necrosis factor-related apoptosis-inducing ligand but presence of its receptors in the human brain. J. Neurosci. *22*, RC209.

Dörr, J., Waiczies, S., Wendling, U., Seeger, B., and Zipp, F. (2002b). Induction of TRAIL-mediated glioma cell death by human T cells. J. Neuroimmunol. *122*, 117–124.

Ehrlich, S., Infante-Duarte, C., Seeger, B., and Zipp, F. (2003). Regulation of soluble and surface-bound TRAIL in human T cells, B cells, and monocytes. Cytokine *24*, 244–253.

Ferguson, B., Matyszak, M.K., Esiri, M.M., and Perry, V.H. (1997). Axonal damage in acute multiple sclerosis lesions. Brain *120*, 393–399.

Flugel, A., Berkowicz, T., Ritter, T., Labeur, M., Jenne, D.E., Li, Z., Ellwart, J.W., Willem, M., Lassmann, H., and Wekerle, H. (2001). Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. Immunity *14*, 547–560.

Ford, A.L., Goodsall, A.L., Hickey, W.F., and Sedgwick, J.D. (1995). Normal adult ramified microglia separated from other central nervous system macrophages by flow cytometric sorting. Phenotypic differences defined and direct ex vivo antigen presentation to myelin basic protein-reactive CD4+ T cells compared. J. Immunol. *154*, 4309–4321.

Gimsa, U., Peter, S.V.A., Lehmann, K., Bechmann, I., and Nitsch, R. (2000). Axonal damage induced by invading T cells in organotypic central nervous system tissue in vitro: Involvement of microglial cells. Brain Pathol. *10*, 365–377.

Giuliani, F., Goodyer, C.G., Antel, J.P., and Yong, V.W. (2003). Vulnerability of human neurons to T cell-mediated cytotoxicity. J. Immunol. *171*, 368–379.

Hilliard, B., Wilmen, A., Seidel, C., Liu, T.-S.L., Göke, R., and Chen, Y. (2001). Roles of TNF-related apoptosis-inducing ligand in experimental autoimmune encephalomyelitis. J. Immunol. *166*, 1314–1319.

Jo, M., Kim, T.-H., Seol, D.-W., Esplen, J.E., Dorko, K., Billiar, T.R., and Strom, S.C. (2000). Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat. Med. *45*, 564–567.

Kornek, B., Storch, M.K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M., and Lassmann, H. (2000). Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am. J. Pathol. *157*, 267–276.

Lamhamedi-Cherradi, S.E., Zheng, S., Tisch, R.M., and Chen, Y.H. (2003a). Critical roles of tumor necrosis factor-related apoptosis-inducing ligand in type 1 diabetes. Diabetes *52*, 2274–2278.

Lamhamedi-Cherradi, S.E., Zheng, S.J., Maguschak, K.A., Peschon, J., and Chen, Y.H. (2003b). Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL$^{-/-}$ mice. Nat. Immunol. *4*, 255–260.

Lawrence, D., Shahrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P., et al. (2001). Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat. Med. *7*, 383–385.

Leranth, C., and Nitsch, R. (1994). Morphological evidence that hypothalamic substance P-containing afferents are capable of filtering the signal flow in the monkey hippocampal formation. J. Neurosci. *14*, 4079–4094.

Li, J.H., Kirkiles-Smith, N.C., McNiff, J.M., and Pober, J.S. (2003). TRAIL induces apoptosis and inflammatory gene expression in human endothelial cells. J. Immunol. *171*, 1526–1533.

Lünemann, J.D., Waiczies, S., Ehrlich, S., Wendling, U., Seeger, B., Kamradt, T., and Zipp, F. (2002). Death ligand TRAIL induces no apoptosis but inhibits activation of human (auto)antigen-specific T cells. J. Immunol. *168*, 4881–4888.

Mack, C.L., Vanderlugt-Castaneda, C.L., Neville, K.L., and Miller, S.D. (2003). Microglia are activated to become competent antigen presenting and effector cells in the inflammatory environment of the Theiler's virus model of multiple sclerosis. J. Neuroimmunol. *144*, 68–79.

Mariani, S.M., and Krammer, P.H. (1998). Surface expression of TRAIL/Apo-2 ligand in activated mouse T and B cells. Eur. J. Immunol. *28*, 1492–1498.

Martinez-Lorenzo, M.J., Anel, A., Gamen, S., Monleon, I., Lasierra, P., Larrad, L., Pineiro, A., Alava, M.A., and Naval, J. (1999). Activated human T cells release bioactive Fas ligand and APO2 ligand in microvesicles. J. Immunol. *163*, 1274–1281.

Matysiak, M., Jurewicz, A., Jaskolski, D., and Selmaj, K. (2002). TRAIL induces death of human oligodendrocytes isolated from adult brain. Brain *125*, 2469–2480.

Meyer, R., Weissert, R., Diem, R., Storck, M.K., de Graaf, K.L., Kramer, B., and Bähr, M. (2001). Acute neuronal apoptosis in a rat model of multiple sclerosis. J. Neurosci. *21*, 6214–6220.

Miura, Y., Misawa, N., Kawano, Y., Okada, H., Inagaki, Y., Yamamoto, N., Ito, M., Yagita, H., Okumura, K., Mizusawa, H., et al. (2003). Tumor necrosis factor-related apoptosis-inducing ligand induces neuronal death in a murine model of HIV central nervous system infection. Proc. Natl. Acad. Sci. USA *100*, 2777–2782.

Nitsch, R., Bechmann, I., Deisz, R.A., Haas, D., Lehmann, T.N., Wendling, U., and Zipp, F. (2000). Human brain-cell death induced by tumour-necrosis-factor-related apoptosis-inducing ligand (TRAIL). Lancet *356*, 827–828.

Nitsch, R., Pohl, E.E., Smorodchenko, A., Infante-Duarte, C., Aktas, O., and Zipp, F. (2004). Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue. J. Neurosci. *24*, 2458–2464.

Okuda, Y., Sakoda, S., Fujimura, H., and Yanagihara, T. (1998). Aminoguanidine, a selective inhibitor of the inducible nitric oxide synthase, has different effects on experimental allergic encephalomyelitis in the induction and progression phase. J. Neuroimmunol. *81*, 201–210.

Pan, G., O'Rourke, K., Chinnaiyan, A.M., Gentz, R., Ebner, R., Ni, J., and Dixit, V.M. (1997). The receptor for the cytotoxic ligand TRAIL. Science *276*, 111–113.

Peterson, J.W., Bo, L., Mork, S., Chang, A., and Trapp, B.D. (2001). Transected neurites, apoptotic neurons, and reduced inflammation in cortical multiple sclerosis lesions. Ann. Neurol. *50*, 389–400.

Pitti, R.M., Marsters, S.A., Ruppert, S., Donahue, C.J., Moore, A., and Ashkenazi, A. (1996). Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family. J. Biol. Chem. *271*, 12687–12690.

Qin, J., Chaturvedi, V., Bonish, B., and Nickoloff, B.J. (2001). Avoiding premature apoptosis of normal epidermal cells. Nat. Med. *7*, 385–386.

Reijneveld, J.C., Taphoorn, M.J., and Voest, E.E. (1999). A simple mouse model for leptomeningeal metastases and repeated intrathecal therapy. J. Neurooncol. *42*, 137–142.

Roth, W., Isenmann, S., Naumann, U., Kugler, S., Bahr, M., Dichgans, J., Ashkenazi, A., and Weller, M. (1999). Locoregional Apo2L/TRAIL eradicates intracranial human malignant glioma xenografts

in athymic mice in the absence of neurotoxicity. Biochem. Biophys. Res. Commun. *265*, 479–483.

Saeki, Y., Mima, T., Sakoda, S., Fujimura, H., Arita, N., Nomura, T., and Kishimoto, T. (1992). Transfer of multiple sclerosis into severe combined immunodeficiency mice by mononuclear cells from cerebrospinal fluid of the patients. Proc. Natl. Acad. Sci. USA *89*, 6157–6161.

Schmaltz, C., Alpdogan, O., Kappel, B.J., Muriglan, S.J., Rotolo, J.A., Ongchin, J., Willis, L.M., Greenberg, A.S., Eng, J.M., Crawford, J.M., et al. (2002). T cells require TRAIL for optimal graft-versus-tumor activity. Nat. Med. *8*, 1433–1437.

Schneider, P., Olson, D., Tardivel, A., Browning, B., Lugovskoy, A., Gong, D., Dobles, M., Hertig, S., Hofmann, K., Van Vlijmen, H., et al. (2003). Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J. Biol. Chem. *278*, 5444–5454.

Sedger, L.M., Glaccum, M.B., Schuh, J.C., Kanaly, S.T., Williamson, E., Kayagaki, N., Yun, T., Smolak, P., Le, T., Goodwin, R., et al. (2002). Characterization of the in vivo function of TNF-alpha-related apoptosis-inducing ligand, TRAIL/Apo2L, using TRAIL/Apo2L gene-deficient mice. Eur. J. Immunol. *32*, 2246–2254.

Smyth, M.J., Cretney, E., Takeda, K., Wiltrout, R.H., Sedger, L.M., Kayagaki, N., Yagita, H., and Okumura, K. (2001). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. J. Exp. Med. *193*, 661–670.

Song, K., Chen, Y., Göke, R., Wilmen, A., Seidel, C., Göke, A., and Hilliard, B. (2000). Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression. J. Exp. Med. *191*, 1095–1103.

Steinbrecher, A., Reinhold, D., Quigley, L., Gado, A., Tresser, N., Izikson, L., Born, I., Faust, J., Neubert, K., Martin, R., et al. (2001). Targeting dipeptidyl peptidase IV (CD26) suppresses autoimmune encephalomyelitis and up-regulates TGF-beta 1 secretion in vivo. J. Immunol. *166*, 2041–2048.

Steinman, L. (2001). Multiple sclerosis: a two-stage disease. Nat. Immunol. *2*, 762–764.

Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R.A., Mork, S., and Bo, L. (1998). Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. *338*, 278–285.

Ullrich, O., Diestel, A., Eyupoglu, I., and Nitsch, R. (2001). Regulation of microglial expression of integrins by poly(ADP-ribose) polymerase-1. Nat. Cell Biol. *3*, 1035–1042.

Walczak, H., Degli-Esposti, M.A., Johnson, R.S., Smolak, P.J., Waugh, J.Y., Boiani, N., Timour, M.S., Gerhart, M.J., Schooley, K.A., Smith, C.A., et al. (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. EMBO J. *16*, 5386–5397.

Walczak, H., Miller, R.E., Ariail, K., Gliniak, B., Griffith, T.S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., et al. (1999). Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. Nat. Med. *5*, 157–163.

Wandinger, K.P., Lunemann, J.D., Wengert, O., Bellmann-Strobl, J., Aktas, O., Weber, A., Grundstrom, E., Ehrlich, S., Wernecke, K.D., Volk, H.D., et al. (2003). TNF-related apoptosis inducing ligand (TRAIL) as a potential response marker for interferon-beta treatment in multiple sclerosis. Lancet *361*, 2036–2043.

Wendling, U., Walczak, H., Dörr, J., Jacobi, C., Weller, M., Krammer, P.H., and Zipp, F. (2000). Expression of TRAIL receptors in human autoreactive and foreign antigen-specific T cells. Cell Death Differ. *7*, 637–644.

Wiley, S.R., Schooley, K., Smolak, P.J., Din, W.S., Huang, C.P., Nicholl, J.K., Sutherland, G.R., Smith, T.D., Rauch, C., Smith, C.A., et al. (1995). Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity *3*, 673–682.

Wu, G.S., Burns, T.F., Zhan, Y., Alnemri, E.S., and El-Deiry, W.S. (1999). Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor. Cancer Res. *59*, 2770–2775.

Zamai, L., Secchiero, P., Pierpaoli, S., Bassini, A., Papa, S., Alnemri, E.S., Guidotti, L., Vitale, M., and Zauli, G. (2000). TNF-related apoptosis-inducing ligand (TRAIL) as a negative regulator of normal human erythropoiesis. Blood *95*, 3716–3724.

Zamvil, S.S., and Steinman, L. (2003). Diverse targets for intervention during inflammatory and neurodegenerative phases of multiple sclerosis. Neuron *38*, 685–688.

Zheng, S.J., Wang, P., Tsabary, G., and Chen, Y.H. (2004). Critical roles of TRAIL in hepatic cell death and hepatic inflammation. J. Clin. Invest. *113*, 58–64.

Zipp, F., Krammer, P.H., and Weller, M. (1999). Immune (dys)regulation in multiple sclerosis: Role of the CD95/CD95 ligand system. Immunol. Today *20*, 550–554.