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Roles of TNF-Related Apoptosis-Inducing Ligand in Experimental Autoimmune Encephalomyelitis

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TRAIL, the TNF-related apoptosis-inducing ligand, induces apoptosis of tumor cells, but not normal cells; the roles of TRAIL in nontransformed tissues are unknown. Using a soluble TRAIL receptor, we examined the consequences of TRAIL blockade in an animal model of multiple sclerosis. We found that chronic TRAIL blockade in mice exacerbated experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein. The exacerbation was evidenced primarily by increases in disease score and degree of inflammation in the CNS. Interestingly, the degree of apoptosis of inflammatory cells in the CNS was not affected by TRAIL blockade, suggesting that TRAIL may not regulate apoptosis of inflammatory cells in experimental autoimmune encephalomyelitis. By contrast, myelin oligodendrocyte glycoprotein-specific Th1 and Th2 cell responses were significantly enhanced in animals treated with the soluble TRAIL receptor. Based on these observations, we conclude that unlike TNF, which promotes autoimmune inflammation, TRAIL inhibits autoimmune encephalomyelitis and prevents activation of autoreactive T cells. *The Journal of Immunology*, 2001, 166: 1314–1319.

TRAIL, the TNF-related apoptosis-inducing ligand, is a newly identified member of the TNF superfamily (1). Unlike other members of the TNF superfamily that interact with one or two specific receptors, TRAIL can potentially interact with five different receptors. These include death receptor 4 (DR4, TRAIL-R1), death receptor 5 (DR5, TRAIL-R2), decoy receptor 1 (DcR1, TRAIL-R3, TRID), decoy receptor 2 (DcR2, TRAIL-R4, TRUNDD) (2–8), and a soluble receptor called osteoprotegerin (9). TRAIL and its receptors are constitutively expressed in a variety of cell types, including lymphocytes, NK cells, and neural cells (1, 2, 4, 10–15). The levels of TRAIL expression in lymphocytes can be markedly up-regulated following cell activation (5, 16, 17).

While the presence of multiple TRAIL receptors strongly suggests that TRAIL be involved in multiple processes, the precise roles of TRAIL in health and disease are unknown. In vitro studies suggest that TRAIL induces apoptosis of some, but not all, tumor cell lines (2, 6). This may be mediated through DR4 and DR5, which are capable of activating the caspase cascade. The presence of DcR1 and DcR2, which do not contain functional death domains, blocks TRAIL-induced apoptosis (2, 6). TRAIL may not induce apoptosis of most nontransformed cells (2, 6). In vivo administration of rTRAIL selectively kills tumor cells, but not normal cells, leaving the host organ systems unharmed (18, 19). To explore the roles of TRAIL in vivo, we have recently studied the consequences of chronic TRAIL blockade in an animal model of rheumatoid arthritis (20). We found that chronic TRAIL blockade exacerbated autoimmune arthritis, whereas intraarticular TRAIL gene transfer diminished it. Additionally, we found that unlike Fas ligand or TNF, TRAIL did not mediate activation-induced cell death of T lymphocytes. Instead, TRAIL prevented cell cycle progression of T cells and inhibited their differentiation into effector cells (20). In this study, we examine the roles of TRAIL in an animal model of multiple sclerosis. We propose that unlike many other members of the TNF family that promote autoimmune encephalomyelitis, TRAIL inhibits experimental autoimmune encephalomyelitis (EAE) and prevents activation of encephalitogenic T cells.

**Materials and Methods**

**Mice**

Female C57BL/6 mice, 4–6 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in the University of Pennsylvania (Philadelphia, PA) animal care facilities and were acclimated for 5–7 days before being used for experiments.

**Induction and clinical evaluation of EAE**

Mice were immunized by s.c. injection with 500 μg of myelin oligodendrocyte glycoprotein (MOG) 38–50 peptide emulsified in CFA containing 500 μg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, MI). Pertussis toxin, 250 ng per mouse, was injected i.v. on the day of immunization and 48 h later. Mice were evaluated daily and scored for EAE, as follows (21): 0, no disease; 1, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 4, hind limb paralysis plus forelimb paralysis; 5, moribund or dead.

**Production of soluble rTRAIL receptor**

Soluble DR5 (sDR5) was produced using the *Pichia pastoris* system, as follows (20). The cDNA that contains the full-length extracellular domain of the human DR5 (a gift from Dr. W. El-Deiry (University of Pennsylvania, Philadelphia, PA)) (22) was cloned into pGAPZa (Invitrogen, Carlsbad, CA), which contains a Poxyni promoter and a six-histidine tag as well as a zeocin resistance gene. Several recombinant *P. pastoris* clones with zeocin resistance were generated, which secreted up to 25 mg sDR5 per
FIGURE 1. sDR5 blocks TRAIL-induced apoptosis of mouse L929 cells. L929 cells were treated with different concentrations of rTRAIL (Bi- omol Research Laboratory, Plymouth Meeting, PA) with or without 5 μg/ml of sDR5. The percentage of dead cells was determined by MTT assay, as described in Materials and Methods.

Histopathological studies
Mice were perfused with PBS and 10% Formalin phosphate. Spinal cords were first embedded in paraffin, cut into five pieces, and then sectioned at 5 μm and stained with luxol fast blue and cresyl violet (23). The total area of tissue section and the area of inflammation were measured using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) in a blinded manner. The percentage of the spinal cord with inflammation was calculated as follows: (area of the spinal cord that is infiltrated by inflammatory cells/total area of the spinal cord sections measured) × 100. A total of 10 tissue sections from cervical, thoracic, lumbar, and sacral spinal cord was analyzed for each animal.

Apoptosis studies
TUNEL staining was performed on paraffin-embedded Formalin-fixed spinal cord sections, as described (24). Briefly, sections were dewaxed in xylene, hydrated in water/ethanol, and washed in PBS. Endogenous peroxidase activity was inactivated by incubating the tissue in 3% H2O2. Fragmented DNA in apoptotic cells was labeled with digoxigenin-conjugated dUTP (Roche Molecular Biochemicals, Indianapolis, IN) using TdT enzyme (Clontech, Palo Alto, CA). The labeled DNA was then detected by peroxidase-conjugated anti-digoxigenin Ab (Roche) using diaminobenzidine as substrate. Counterstain was performed with methyl green. The number of apoptotic cells in the lesions was determined by light microscopy.

Reagents and ELISA
Mouse MOG38-50 peptide was synthesized using Fmoc solid-phase methods and purified through HPLC by Research Genetics (Huntsville, AL). Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). The following reagents were purchased from PharMingen (San Diego, CA): rat anti-mouse IL-2 (clone JES6-1A12), IL-4 (BVD4-1D11), and IFN-γ (R4-6A2) mAb; biotin-labeled rat anti-mouse IL-2 (clone JES6-5H6), IL-4 (BVD6-24G2), and IFN-γ (XMG112D) mAb; recombinant mouse IL-2, IL-4, IL-10, and IFN-γ. Quantitative ELISA for IL-2, IL-4, and IFN-γ was performed as per manufacturer’s recommendations.

Cell culture
For cytokine assays, splenocytes were cultured at 1.5 × 106 cells/well in 0.2 ml of DMEM (Life Technologies, Grand Island, NY) containing 10% FBS and various amounts of MOG38-50 peptide. Culture supernatants were collected 48 h later, and cytokine concentrations were determined by ELISA. For proliferation assays, 0.5 × 105 cells/well were used. [3H]Thymidine was added to the culture at 48 h, and cells were harvested 16 h later. Radioactivity was determined using a flatbed beta counter (Wallac, Gaithersburg, MD).

MTT assay
L929 cells were first cultured in flat-bottom 96-well plate, at 2 × 104 cells/well, in 100 μl AIM-V medium (Life Technologies). Sixteen hours later, actinomycin D was added to the culture at 1 μg/ml and cells were cultured for another 2 h. rTRAIL was then added, and culture was continued for an additional 5 h. MTT was added only for the last hour of the culture. At the end of the culture, medium was removed and DMSO (100 μl/well) was added, and absorbance was determined at 595 nm. The percentage of dead cells was calculated using untreated cells as control (25).

Results
Generation of a soluble rTRAIL receptor that blocks TRAIL function
The rTRAIL receptor used in this study was the sDR5 produced in the yeast P. pastoris system (26). To assess the biological activities of sDR5, we studied TRAIL-induced apoptosis of mouse L929 cells. As shown in Fig. 1, TRAIL induces apoptosis of L929 cells in a dose-dependent manner. This was completely blocked by addition of sDR5. In parallel experiments, we also studied TRAIL-induced apoptosis of other tumor cell lines, including human Jurkat cells and K562 cells. The sDR5 selectively blocked TRAIL-induced apoptosis...
of these cells (20) (data not shown). Thus, sDR5 is biologically active and can be used to block TRAIL function in vitro.

**TRAIL blockade exacerbates MOG-induced autoimmune encephalomyelitis**

To investigate the roles of TRAIL in vivo, we examined the consequences of TRAIL blockade in an animal model of multiple sclerosis. C57BL/6 mice were immunized with MOG35-55 peptide to induce EAE. Eight days after disease onset (when ~90% of the mice had developed signs of EAE), mice were injected i.p. with either 200 μg of sDR5 or a control protein once every other day for a total of 17 days. As shown in Fig. 2A, C57BL/6 mice developed typical EAE starting ~18 days after immunization. Injection of sDR5 significantly exacerbated the disease. The mean maximal disease score in the control group was 2.6 ± 0.5. This was increased to 3.3 ± 0.4 in the sDR5-treated group. One of six mice died from EAE in the sDR5-treated group, whereas none died in the control group.

To determine whether the effect of TRAIL blockade is limited to the effector phase of EAE, we also investigated the consequences of TRAIL blockade during the inductive phase of the disease. Thus, MOG-immunized mice were treated with sDR5 or a control protein from the day of immunization until the day of disease onset (when 1 mouse of 12 developed signs of EAE). As shown in Fig. 2B, no significant differences between control and sDR5-treated groups were observed with respect to disease onset or severity. This suggests that TRAIL blockade during the inductive phase of EAE alone may not be sufficient to affect the disease course.

**TRAIL blockade enhances the formation of inflammatory lesions in the CNS**

To investigate the effect of TRAIL blockade on the formation of inflammatory lesions in the CNS, we performed quantitative histopathological studies of spinal cords. As shown in Fig. 3, A and B, FIGURE 3. Inflammation and apoptosis in the spinal cord. Mice were treated as in Fig. 2A and sacrificed 42 days after immunization. Spinal cord was treated and examined for histology and apoptosis, as described in Materials and Methods. A and B, Luxol fast blue staining of spinal cords from sDR5 (A)- and HSA (B)-treated mice (original magnification ×100). C and D, TUNEL staining of spinal cords from sDR5 (C)- and HSA (D)-treated mice (original magnification ×200). Arrows indicates apoptotic nuclei, which are shown in brown.

FIGURE 4. Quantitative analysis of the degree of inflammation in the CNS. C57BL/6 mice, six mice per group, were treated as in Fig. 2A and sacrificed 42 days after immunization. Spinal cord was treated and examined for histology, as described in Materials and Methods. Each data point represents a percentage of spinal cord section that is inflamed. The horizontal bars represent the means of respective groups. The differences between the two groups are statistically significant, as determined by Student t test (p < 0.05). ■, Mice treated with HSA. ○, Mice treated with sDR5.

FIGURE 5. Quantitative analysis of the degree of apoptosis in the CNS. C57BL/6 mice, four mice per group, were treated as in Fig. 2A and sacrificed 42 days after immunization. Spinal cord was treated and examined for apoptosis, as described in Materials and Methods. The numbers of apoptotic nuclei/mm² of inflamed tissue were counted and plotted against the percentages of the corresponding spinal cord sections that were inflamed. The differences between the two groups are not statistically significant, as determined by Mann-Whitney test (p > 0.05). ■, Mice treated with HSA. ○, Mice treated with sDR5.
inflammatory lesions were readily detectable in both control and sDR5-treated mice. The inflammatory lesions consisted mostly of lymphocytes and macrophages as well as granulocytes and microglial cells. To quantify the degree of inflammation, the area of spinal cord sections that showed signs of infiltration as well as the total area of each spinal cord sections were measured using the Image-Pro Plus software. The degree of inflammation was then evaluated based on the percentage of the spinal cord areas that show signs of inflammation. As shown in Fig. 4, mice treated with sDR5 had significantly more severe inflammation than control mice. The extent of demyelination correlated well with the degree of inflammation and clinical score. Mice with similar disease scores exhibited similar degrees of demyelination and inflammation regardless of treatment groups (data not shown).

TRAIL blockade does not affect apoptosis of inflammatory cells in the CNS

Some members of the TNF family are capable of inducing apoptosis of normal and/or tumor cells. TRAIL has been shown to induce apoptosis of some, but not all, tumor cell lines (2–8). The effect of sDR5 on EAE can be explained by its blockade of TRAIL-induced apoptosis of inflammatory cells. To test this theory, we examined the effect of TRAIL blockade on apoptosis of inflammatory cells and neural cells in the CNS. As shown in Fig. 3C/D, apoptotic cells were readily detectable in the spinal cords of both control and sDR5-treated mice.

The vast majority of apoptotic cells were localized within the inflammatory lesions. In sections that did not contain inflammatory lesions, few or no apoptotic cells were detected. To compare the degree of apoptosis between control and sDR5-treated groups, we first calculated the number of apoptotic cells/mm² of spinal cord that showed signs of inflammation. The number of apoptotic cells was then plotted against the degree of inflammation in the same section. As shown in Fig. 5, no statistically significant differences in the degree of apoptosis were observed between control and sDR5-treated groups. We then examined whether neural cell death was affected by TRAIL blockade in EAE. Apoptotic neural cells were identified based on their morphology and anatomic location in addition to TUNEL staining. We found that the number of apoptotic neural cells per cross section of spinal cord for control and sDR5-treated animals were 3.6 ± 2.9 and 4.3 ± 1.4, respectively (p > 0.05, as determined by ANOVA). In mice that did not develop EAE, few or no apoptotic neural cells were detected. Taken together, these results strongly suggest that TRAIL may not regulate apoptosis in the CNS during EAE.

TRAIL blockade enhances anti-MOG T cell responses

EAE is a T cell-mediated autoimmune disease. To determine whether TRAIL blockade affected the functions of encephalitogenic T cells, we studied anti-MOG T cell responses in mice following TRAIL blockade. Mice were immunized with MOG peptide to induce EAE and treated with either sDR5 or BSA for a total of 16 days as in Fig. 2B. Mice were sacrificed 10 days after the last injection of sDR5, and splenocytes were tested for anti-MOG proliferative and cytokine responses, as described in Materials and Methods. Data presented are representative of two experiments.
of 16 days. Anti-MOG T cell responses were determined ex vivo 10 days after the last injection of sDR5. As shown in Fig. 6, splenocytes of BSA-treated mice produced primarily Th1-type cytokines (i.e., IL-2 and IFN-γ) in response to MOG peptide. This was significantly increased in mice treated with sDR5. A small but significant amount of IL-4 was also detected in sDR5-treated group. Interestingly, lymphocyte proliferative responses were comparable between BSA- and sDR5-treated groups. These results suggest that TRAIL blockade enhances functions of both Th1- and Th2-type cells in vivo.

Discussion

The TNF family of proteins play crucial roles in a number of biological processes, including apoptosis, immunity, inflammation, and development. TRAIL is a newly identified member of the TNF family, which, unlike TNF, induces apoptosis of tumor cells, but not normal cells. However, both TRAIL and TRAIL receptors are expressed in normal nontransformed tissues; the roles of TRAIL in these tissues are unknown and are the subjects of this investigation. Results presented in this work strongly suggest that one of the functions of TRAIL in vivo is to inhibit autoimmune inflammation in the CNS. Blocking endogenous TRAIL with sDR5 eliminates this inhibition, and exacerbates autoimmune encephalomyelitis. Furthermore, our data also indicate that TRAIL may inhibit activation of autoreactive T cells that initiate autoimmune inflammation. To our knowledge, this is the first report examining the roles of TRAIL in animal models of multiple sclerosis.

Thus, unlike TNF, which initiates and exacerbates autoimmune diseases (27–30), TRAIL inhibits autoimmune inflammation. Other members of the TNF family that have been reported to inhibit autoimmune inflammation are CD95 ligand (CD95L, Fas) and CD30 ligand (CD30L). Mutations in CD95/CD95L genes lead to the development of systemic autoimmune diseases in both humans (31, 32) and mice (33–36), although paradoxically prevent several organ-specific autoimmune diseases (37–43). We and others have shown that up-regulating CD95 or CD95L function in synovial joints ameliorates autoimmune arthritis (44, 45). Similarly, CD30L plays crucial roles in regulating autoimmune inflammation (46). Autoreactive CD8+ T cells deficient in CD30L elicit more severe autoimmune insults in mice (46). Thus, unlike TNF, but similar to CD95L and CD30L, TRAIL may be a member of an inhibitor protein subgroup that prevents autoimmune diseases (20).

The precise mechanism(s) whereby TRAIL inhibits autoimmune inflammation in vivo is not clear. Our demonstration that autoreactive T cell activation was enhanced in mice treated with sDR5 suggests that TRAIL may inhibit functions of autoreactive T cells. Because EAE is a T cell-mediated autoimmune disease, inhibiting T cell function may diminish the disease. However, it should be pointed out that enhancing T cell function alone during the inductive phase of EAE may not be sufficient to exacerbate the disease, because treating mice with sDR5 before the onset of the disease failed to significantly affect EAE (Fig. 2B). Recently, we have observed a similar effect of TRAIL in another model of autoimmunity, i.e., collagen-induced arthritis in DBA/1 mice (20). We found that TRAIL blockade during the effector phase of the disease enhanced the arthritic inflammation (20).

An alternative mechanism whereby TRAIL may inhibit autoimmune inflammation is by inducing apoptosis of inflammatory cells. Although it has been shown that TRAIL does not induce apoptosis of most nontransformed cells (2, 6, 20), there is evidence to suggest that dendritic cells and some T cells may be susceptible to TRAIL-induced apoptosis in vitro (17, 47). Whether this is also true in vivo remains to be determined. Our demonstration that the degrees of apoptosis in the CNS and arthritic joints (20) were not affected by TRAIL blockade suggests that TRAIL may not regulate apoptosis of inflammatory cells in these systems. Experiments are underway to investigate whether different types of inflammatory cells respond differently to TRAIL signals in inflammation.

Thus, by in vivo TRAIL blockade, we have established that, unlike TNF, TRAIL inhibits autoimmune encephalomyelitis and prevents activation of autoreactive T cells. Because EAE is an animal model for human multiple sclerosis and because TRAIL and its receptors are also expressed by human cells, results reported in this study may be important not only for our understanding of the pathogenesis of EAE, but also for designing therapeutic strategies for the treatment of autoimmune diseases such as multiple sclerosis.

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