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Death Ligand TRAIL Induces No Apoptosis but Inhibits Activation of Human (Auto)antigen-Specific T Cells

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TRAIL can interact with five different receptors. Of these, only TRAIL receptor 1 (also referred to as DR4) and TRAIL receptor 2 (DR5) are capable of transmitting a death signal, whereas the transmembrane TRAIL receptor 3 (DcR1) and TRAIL receptor 4 (DcR2) do not contain a functional death domain. The latter two are considered to act as decoy receptors because their overexpression was shown to inhibit TRAIL-induced apoptosis. The soluble receptor osteoprotegerin is reported to block TRAIL-mediated apoptosis by competitive inhibition of TRAIL binding to TRAIL receptors 1 and 2. Although TRAIL and its receptors were shown to be constitutively expressed by a variety of cell types including human (auto)antigen-specific T cells, the physiologic relevance of the TRAIL system and its potential role in inflammatory processes are not completely understood. Previous studies emphasized the selectivity of TRAIL to induce apoptotic cell death in tumor cells. However, this view has been challenged because TRAIL was also reported to induce massive apoptosis in normal human hepatocytes and human brain cells, indicating a species difference between rodents and man.

Materials and Methods

Materials and cell lines

The human recombinant soluble TRAIL used together with an enhancer Ab for multimerization, and the human recombinant protein consisting of the extracellular domain of TRAIL receptor 2 fused to the Fc portion of human IgG1 (rhTRAIL-R2:Fc fusion protein; Alexis, San Diego, CA) were used for the functional analysis of the TRAIL system. T cells were activated, independent of Ag, by anti-CD3 Ab (OKT3; American Type Culture Collection, Manassas, VA) and anti-CD28 Ab (Ancell, Bayport, MN). FITC-conjugated rat anti-human IFN-γ (XMG1.2; BD PharMingen), and FITC-PE-conjugated rat anti-mouse IL-4 (11B11; BD PharMingen) were used for multimerization, and the human recombinant protein consisting of the extracellular domain of TRAIL receptor 2 fused to the Fc portion of human IgG1 (rhTRAIL-R2:Fc fusion protein; Alexis, San Diego, CA) were used for the functional analysis of the TRAIL system. T cells were activated, independent of Ag, by anti-CD3 Ab (OKT3; American Type Culture Collection, Manassas, VA) and anti-CD28 Ab (Ancell, Bayport, MN). FITC-conjugated rat anti-human IFN-γ (XMG1.2; BD PharMingen), and FITC-PE-conjugated rat anti-mouse IL-4 (11B11; BD PharMingen) were used for multimerization.
cells were incubated together with anti-CD28 (10 μg/ml H9262) using a modified "split-well" protocol (15). Briefly, 2 × 10^5 PBMC were seeded in 200 μl medium (RPMI supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS). Human Ag-specific T cell lines

Ag-specific activation. Ag-specific CD4^+ T cell lines were established using a modified "split-well" protocol (15). Briefly, 2 × 10^5 PBMC were seeded in 200 μl medium (RPMI supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% pooled human AB serum) in the presence of 20 μg/ml myelin basic protein (MBP), 5 μg/ml histone (BP), or 4 μg/ml tetanus toxoid (TT) in 96-well round-bottom microtiter plates. After 7 days, 10 U/ml recombinant IL-2 (human—proleukin; Eurocetus, Frankfurt, Germany) was added to the cultures. Five to 7 days thereafter, 10 μl were taken from each original well and split into two wells on a new plate. Irradiated (3000 rad) autologous PBMC (1 × 10^5) were added to each well of the master plate and to the split plate in the presence or absence of Ag. Specificity was tested by a standard proliferation assay.

Proliferation. [3H]Thymidine (0.5 μCi; Amersham, Braunschweig, Germany) was added to each well of the split plate. Incorporation of radioactivity was measured after 18 h with a beta counter (Microbeta. Wallac, Turku, Finland). Specifically responding populations with stimulation indices >3 were selected for further expansion.

Antigens. Human MBP was purified as described in established protocols (16). TT was purchased as a vaccine concentrate (Chiron Behring, Marburg, Germany), and BP was purchased from a crude extract of Betula verrucosa (Iantirug, Pharmacia & Upjohn, Angelholm, Sweden).

Th differentiation. The Th differentiation was based on the ratio of IFN-γ and IL-4 production. Predominant IFN-γ staining by intracellular flow cytometry (17) determined a Th1-like cell line, double staining determined a Th0 cell line, and predominant IL-4 staining determined a Th2-like cell line. Briefly, 10^5 resting T cells were stimulated with 0.1 μg/ml PMA and 1 μg/ml ionomycin in the presence of 2 μM monensin. After 6 h, cells were permeabilized and stained by incubating with 1.5 μg/ml FITC-conjugated rat anti-human IFN-γ, 0.5 μg/ml PE-conjugated rat anti-mouse IL-4, and equivalent concentrations of FITC/PE-labeled rat IgG1 isotype control Abs diluted in 0.1% saponin permeabilization solution. Analysis was performed with a FACSscan flow cytometer (BD Biosciences, Mountain View, CA) equipped with CellQuest software (BD Biosciences), and 10,000 events were acquired.

Unspecific activation. For analysis of activated T cells, a protocol using Abs to CD3 and CD28 was used (18). Cell culture plates were coated with anti-CD3 Abs overnight at 10 μg/ml in PBS. After washing the plates, T cells were incubated together with anti-CD28 (10 μg/ml) under the respective experimental conditions.

Anergy. T cells (5 × 10^5/well) and irradiated matched PBMC (1.5 × 10^5/well) were incubated with or without Ag for 7 days using different concentrations of TRAIL. Surviving T cells were washed and seeded at 5 × 10^4 cells/well together with irradiated matched PBMC (1.5 × 10^5/well) and Ag. Proliferation was measured by [3H]thymidine uptake. Parallel to this, IL-2 secretion 24 h after the initial and second stimulation was measured using the IL-2-dependent T cell line CTLL-2.

DNA fragmentation

For quantitative analysis of DNA fragmentation, 10^5 cells were treated according to the protocol described by Nicoletti et al. (19). Briefly, a hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate and 0.1% Triton X-100) was added to 10^5 cells followed by incubation overnight at 4°C. The level of the hypodiploid DNA peak was determined as the percentage of total events by flow cytometry. Data are presented as specific DNA fragmentation (with stimulus/control).

Cytokines

IFN-γ and IL-4 production upon T cell activation with PMA/ionomycin was measured by using intracellular cytokine staining as described above. Secreted IFN-γ (Biomedical Laboratories, New Brunswick, NJ) and IL-4 (R&D Systems, Minneapolis, MN) were further quantified using sandwich ELISAs. For the latter analysis, 2 × 10^3 cells/well were stimulated with anti-CD3/CD28 and cultured for 48 h in the absence or presence of TRAIL.

Soluble TRAIL was quantified in the supernatants of Ag-activated T cells after 24, 48, and 72 h of culture and in sera of 20 healthy volunteers using a sandwich ELISA (Trinova Biochem, Gielnien, Germany).

IL-2 levels were measured 48 h after Ag stimulation according to a standard bioassay protocol using the IL-2-dependent T cell line CTLL-2.

Measurement of [Ca^{2+}]_i

Intracellular free calcium concentrations of fura 2-AM-loaded T cells were monitored with a Hitachi F4500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). T cell suspensions (10 × 10^5 cells/ml) were loaded with 1 μl of the acetoxymethyl ester precursor of fura 2 (Sigma-Aldrich) for 30 min at 37°C. A dual mirror chopping mechanism within the spectrophotometer permitted rapid alternating (30 Hz) excitation of fura 2 at 2 wave-lengths (340 and 380 nm). Unloaded dye was removed by centrifugation, and cells were resuspended in PBS without calcium. Typical measurements involved 2–3 × 10^5 cells in 1 ml of cell medium in a temperature-controlled plastic cuvette at 37°C with constant stirring. Fura 2-loaded cells were incubated with 0.5 mM EGTA for 5 min to bind any external calcium. During the last minute of incubation, the measurement was started and 2 μM thapsigargin (Tg; Calbiochem, Schwalbach, Germany) was subsequently added to fully deplete intracellular Ca^{2+} stores. Following a 5-min incubation with Tg, 1.2 mM calcium was added to the cell suspension to monitor the extent of Ca^{2+} influx. Each measurement was terminated by a calibration for maximum (R_max) and minimum (R_min) calcium release with Triton X-100 and 3 mM MnCl_2, respectively. The results obtained (intracellular calcium concentration expressed in nanomoles) were calculated using the standard equation: intracellular Ca^{2+} concentration ([Ca^{2+}]_i) = K_d × (R - R_{min})/(R_{max} - R) as described (20), using 224 nm as the apparent K_d for Ca^{2+} and fura 2.

Western blot

Immunoblot studies were performed according to standard procedures. T cell lines were harvested and washed in PBS. Cell pellets (minimum of 3 × 10^6 cells) were lyzed by resuspending in lysis buffer (0.15 M NaCl, 0.01 M Tris-HCl, 0.005 M EDTA, 1% Triton X-100, 2 μg/ml aprotinin, and 100 μg/ml PMSF) with a Hamilton syringe. After a 15-min incubation on ice, cell lysates were centrifuged for 15 min in a microfuge at 13,000 rpm. Cellular lysates equivalent to 10 μg protein, as determined by the bicinchoninic acid method (Pierce, Rockford, IL), were separated using 12% PAGE and blotted onto nitrocellulose membranes using standard procedures. Following a 2-h incubation at 37°C in a blocking mixture (TBS containing 2% BSA and 0.05% Tween 20), membranes were incubated overnight at 4°C with specific primary Abs: monoclonal mouse anti-p27^Kip1, monoclonal mouse anti-p27^Kip2, and monoclonal mouse anti-p27^Kip3. Following a series of washing steps, the membranes were incubated for 1 h with 1.25 μg/ml secondary Ab coupled to HRP (Dako, Carpinteria, CA). Specific bands were detected using the ECL-plus system (Amersham Pharma Biotech, Uppsala, Sweden) and densitometrically quantified using TINA Version 2.09g (Santa Cruz Biotechnology, Heidelberg, Germany). CDK4 and p27^Kip1 immunobots were sequentially incubated with anti-β-actin as control.

Statistics

Statistics were performed using SPSS 10.0 software for Windows (SPSS, Chicago, IL). A p value <0.05 was regarded as significant.

Results

TRAIL inhibits proliferation of human Ag-specific T cells independent of APC

Recently, we reported that human (auto)antigen-specific T cells are resistant to apoptosis mediated by functionally active TRAIL, which does induce apoptosis in human Jurkat T lymphoma cells (6). Because there are lines of evidence indicating that different recombinant TRAIL versions vary in their biochemical properties
Human recombinant soluble TRAIL induces apoptosis in tumor cells but not in human (auto)antigen-specific T cell lines (n = 16). Cells were treated with various concentrations of TRAIL (30, 100, and 300 ng/ml) together with an enhancer Ab for multimerization (see Materials and Methods). Apoptosis was determined by specific DNA fragmentation as measured by flow cytometry at 24 h and is demonstrated as mean ± SD.

and cytotoxic potential (21), we first assessed the biological activity of the TRAIL preparation used in this study by investigating its apoptosis-inducing potency in susceptible cells. Fig. 1 illustrates that the TRAIL preparation used in this study is capable of inducing apoptosis in tumor cells in a dose-dependent manner. Incubation of human T cell lines with different concentrations of this TRAIL preparation did not reveal any susceptibility to apoptotic cell death as determined by DNA fragmentation. Furthermore, human unstimulated CD3+ T cells that were separated from freshly isolated PBMC were also fully resistant to TRAIL-induced apoptosis (specific DNA fragmentation index: 1.035 (100 ng/ml TRAIL) and 1.026 (300 ng/ml TRAIL)). Thus, the biologically active TRAIL used throughout the whole study is not capable of killing nontransformed human T cells in vitro.

As outlined in Table I, TRAIL inhibited the Ag-induced proliferation of 22 well-characterized T cell lines, in eight of them by >40%. This effect was also observed in freshly isolated CD3/CD28-stimulated T cell lines (maximal inhibition 22.94% after 5 days of culture), but not as marked as in the long-term Ag-specific T cell lines. The inhibitory effect was independent of the Ag specificity and Th1/Th2 differentiation of these T cells. Furthermore, no differences in the proliferative response of the T cell lines derived from MS patients or healthy individuals were observed. TRAIL at concentrations ranging from 30 to 300 ng/ml did not affect proliferation of the T cell lines cultured with APC in the absence of the nominal Ag (data not shown).

To investigate whether the TRAIL-induced hypoproliferation is due to interference with Ag processing or presentation, 12 representative T cell lines were stimulated with plate-bound anti-CD3 and soluble anti-CD28 Abs. As demonstrated in Fig. 2, TRAIL substantially inhibited T cell proliferation in the absence of APC, indicating that this cytokine influences T cell function directly rather than via APC-mediated signals.

### Table I. Human Ag-specific T cell lines

<table>
<thead>
<tr>
<th>T Cell Line</th>
<th>Disease</th>
<th>Specificity</th>
<th>Tb Status</th>
<th>Inhibition$_{max}$ (%)</th>
<th>Concentration at Maximal Inhibition (ng/ml)</th>
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$^a$ All T cell lines showed a stimulation index $>3$ and the effect of TRAIL on Ag-stimulated proliferation was investigated in each particular cell line more than three times. Different initials indicate different individuals.

$^b$ Ratio: (cpm in the presence of Ag and the maximal inhibitory TRAIL concentration/cpm in the presence of Ag) $\times 100$.

$^c$ Lowest TRAIL concentration at which maximal inhibition is observed.

$^d$ NA, Not available.
effect could be confirmed in freshly isolated T cells (data not shown). The blockade of calcium influx was further accompanied by an inhibition of proliferation (Fig. 3B). Thus, interaction of TRAIL with its receptors negatively regulates human T cell calcium channels resulting in reduced T cell activation.

No induction of anergy by TRAIL

Because failure to produce IL-2 and proliferate after reencountering Ag is a hallmark of clonal T cell anergy, we used four representative cell lines (FZ2, LS4, MA1, and GU3) to investigate whether TRAIL can anergize Ag-specific T cells. T cells were stimulated with Ag and APC in the presence of different TRAIL concentrations, allowed to rest for 7 days, and then rechallenged with Ag and APC. The capacity of the T cells to proliferate and to produce IL-2 was studied after both the initial and second stimulations.

Although we found a trend toward an inhibition of proliferation after the rechallenge in some T cell lines, these effects were not reflected by an inhibition of IL-2 production (Fig. 4).

TRAIL inhibits G1/S transition

T cell proliferation upon TCR engagement is tightly controlled by a large number of positive regulators, such as cyclins and CDK, and negative regulators, such as CDK inhibitors (25, 26). Because DNA synthesis and [3H]thymidine incorporation occur during the S phase of the cell cycle, TRAIL could block cell cycle progression during the G1 to S phase transition. CDK4 allows transit through the G1 phase of the cell. Therefore, we examined whether TRAIL is capable of regulating the expression of this kinase. Furthermore, we investigated the expression of the CDK inhibitor, p27Kip1, which has recently been found to be responsible for the blockade of clonal expansion of anergic T cells (27, 28). The expression levels of CDK4 and the kinase inhibitor p27Kip1 were measured following both antigenic stimulation and anti-CD3/CD28 stimulation in the presence or absence of TRAIL in seven T cell lines. Fig. 5 depicts data from a representative T cell line showing an up-regulation of CDK4 upon T cell stimulation as expected (25, 26). Coincubation of TRAIL with either of both stimuli, MBP and anti-CD3/CD28, resulted in decreased levels of CDK4 expression compared with stimulation in the absence of TRAIL. This indicates an inhibition of cell cycle progression at the G1/S transition level. However, p27Kip1 expression was unaltered in the presence of TRAIL. These data further argue against the induction of clonal anergy by TRAIL as an underlying mechanism of the observed inhibitory properties on T cell activation.

FIGURE 2. Soluble TRAIL inhibits proliferation of human (auto)antigen-specific T cell lines independent of APC. T cell lines were cultured in the absence and presence of different TRAIL concentrations for 96 h. Proliferation was assessed by [3H]thymidine uptake in triplicate, and the presented results are expressed as means ± SEM. The diagram represents four exemplary experiments. A, Ag-stimulated MBP-specific T cell line FZ2 (background 198 cpm). B, Anti-CD3/CD28-stimulated MBP-specific T cell line FZ2 (background 227 cpm). C, Ag-stimulated BP-specific T cell line MB7 (background 121 cpm). D, Anti-CD3/CD28-stimulated BP-specific T cell line MB7 (background 276 cpm).
TRAIL by human T cells does not affect their Ag-induced proliferation.

**TRAIL inhibits IFN-γ and IL-4 production**

To investigate whether TRAIL is capable of inhibiting cytokine secretion upon TCR stimulation, we quantified IFN-γ and IL-4 levels in the supernatants of six Th1/Th0 and five Th2/Th0 T cell lines, respectively. As shown in Fig. 7, treatment with TRAIL led to a significant down-regulation of both cytokines as compared with anti-CD3/CD28 stimulation in the absence of the death ligand. The IFN-γ response was also inhibited in freshly isolated CD3/CD28-stimulated T cells (data not shown). Thus, reduced production of typical Th1 and Th2 cytokines might indicate an inhibition of T cell effector functions due to diminished activation rather than a specific cytokine regulation by TRAIL.
Discussion

In this study, we demonstrate that soluble multimerized TRAIL, serving as a model of selective TRAIL influence on T cells, inhibits proliferation and IFN-γ/H9253 production of human T cell lines independent of their differentiation phenotype. The reduction of T cell proliferation was not dependent on the Ag specificity. Both foreign (TT/BP-specific) and autoreactive (MBP-specific) T cell lines and T cells derived from patients with multiple sclerosis as well as healthy individuals were inhibited. Recent studies on murine models of autoimmune diseases suggested that, unlike TNF, TRAIL might be a member of an inhibitor protein subfamily due to negative regulation of the T cell cycle progression (13, 14). However, the mechanisms of the observed TRAIL effects were not clarified. Because TRAIL had primarily aroused tremendous interest due to its ostensible selectivity of killing tumor cells as observed in rodents (10) and was later reported to induce apoptosis in human hepatocytes and brain cells (11, 12), different effector functions among different species have to be assumed. In the human immune system, we have now also found TRAIL-mediated inhibition of T cell proliferation, albeit to varying degrees (Table I). Whereas marked dose-dependent inhibition was apparent in one-third of the T cell lines, the other T cell lines exhibited only slight effects.

Our finding of increasing TRAIL concentrations in the T cell supernatants upon Ag stimulation is compatible with the idea that the ligand also exerts its effects in a soluble form. This is further supported by data from Martinez-Lorenzo et al. (29), who reported that the cytotoxicity induced by supernatants of PHA-stimulated...
human PBMC on Jurkat cells could partially be blocked by a neutralizing anti-TRAIL mAb. However, blocking endogenously produced TRAIL by Ag-specific T cells through application of a soluble TRAIL receptor did not result in an enhanced proliferation. Because the inhibitory effect on T cell proliferation by exogenously applied TRAIL was shown to be dose dependent, we conclude that lower TRAIL concentrations in supernatants of activated T cells, which are comparable to serum levels of healthy volunteers, are apparently ineffective in modulating T cell growth. However, this does not inevitably exclude a possible impact of soluble TRAIL on T cell activation and growth at inflammatory sites in vivo.

Concerning the mechanism of the apoptosis-independent properties of the death ligand TRAIL, we found an independence of APC because the inhibitory effect was also apparent in CD3/CD28-stimulated T cells (Fig. 2). Thus, TRAIL directly influences T cell function.

T cell proliferation is tightly regulated by positive factors such as cyclins and CDK, and negative regulators such as CDK inhibitors. Cells entering the G1 phase after TCR engagement are characterized by an up-regulation of cyclin D and CDK4/6 (30, 31). We found the TRAIL-induced hypoproliferation to be accompanied by an down-regulation of CDK4 (Fig. 5), indicating an inhibition of cell cycle progression at the G1/S transition. Apart from their known function in cell cycle progression, cell cycle regulators have also been shown to control mechanisms implicated in T cell tolerance, such as anergy (32). The decision between activation and anergy upon TCR occupancy is generally considered to be dependent on the balance between positive and negative signals in T cells, with costimulatory pathways tipping this balance from anergy to activation (33). Investigating the relationship between cell cycle progression and tolerance, Gilbert and Weigle (34) demonstrated that the G1 blocker n-butylate, but not drugs that block cell cycle progression in G0 or S/G2 phases, is capable of inducing anergy as determined by the loss of T cell proliferation and IL-2 secretion upon subsequent Ag rechallenge. Consistent with this paradigm, the cycling inhibitor p27Kip1, which contributes to the association and activation of cyclin D with their complementary CDK, was recently demonstrated to be important for the induction and maintenance of T cell anergy (27, 28). We did not find any evidence that TRAIL collaborates in peripheral T cell tolerance as determined by p27Kip1 expression as well as proliferation and IL-2 production upon secondary Ag challenge (Fig. 4). Therefore, other mechanisms must be responsible for the observed inhibition of T cell proliferation.

Because calcium influx is crucial to lymphocyte activation, including cytokine generation and cell proliferation (35), and the inhibition of calcium-dependent signaling pathways was shown to completely suppress T cell activation (36), we investigated whether the inhibitory effect of TRAIL was related to alterations in calcium signaling. Following TRAIL stimulation, we observed an inhibition of store-operated calcium entry in human T cells. Of note, inhibition of the CRACs has been described for other TNF superfamily members. For example, applying a model of Tg-evoked calcium entry, Tormoquist et al. (37) found that TNF-α inhibits store-operated calcium influx in a rat thyroid cell line. Moreover, Lepple-Wienhues et al. (38) reported that CD95 stimulation inhibited activation of calcium channels and subsequent IL-2 synthesis in apoptosis-resistant Jurkat T cells. The latter observation indicates that the CD95 system might play a role in anergy induction before or in the absence of apoptosis. However, TRAIL reduced calcium influx to a level just within the lower concentration limit required for subsequent IL-2 production and proliferation (23), and thus showed no anergy-inducing properties in the T cell lines. Having defined a novel mechanism of action for TRAIL, which has been previously reported for TNF and CD95 stimulation, these observations indicate that the suppression of calcium-dependent lymphocyte activation signals might represent a primary mechanism responsible for the immunomodulatory properties of TNF/nerve growth factor superfamily members.

These molecules are known to be critically involved in the regulation of immune responses and are currently being targeted for therapeutic modulation in a multitude of recalcitrant autoimmune and malignant diseases. Our data indicate that TRAIL can act as a negative regulator of human T cell activation via mechanisms different from the induction of anergy or T cell clonal deletion by apoptosis.

In combination with the well-documented cytotoxic activities of TRAIL (11, 12), our data strongly suggest a two-edged role for TRAIL in human T cell effector functions, promoting both destructive and anti-inflammatory properties. Given that recent reports demonstrated that different recombinant TRAIL preparations differ widely in their capacity to induce apoptosis (21), attempts to exploit the anti-inflammatory features of TRAIL might be promising in future therapeutic strategies targeting autoimmune inflammation.

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References


