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Expression of TRAIL receptors in human autoreactive and foreign antigen-specific T cells

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Abstract

Deletion of T cells due to apoptosis induction is a regulatory mechanism in the human immune system that may be impaired in autoimmune diseases such as multiple sclerosis (MS). Involvement of the apoptosis-mediating CD95/CD95 ligand system in MS has been demonstrated. Here, we report that (auto)antigen-specific human T cells are not killed *in vitro* by soluble TNF-related apoptosis-inducing ligand (TRAIL) although expressing death-inducing receptors, TRAIL receptor 1 (TRAIL-R1) and TRAIL-R2. Apoptosis was assessed by caspase activation and DNA fragmentation, receptor expression was detected by RT–PCR and flow cytometry. The (auto)antigen-specific T cells were also resistant to specific TRAIL-R1/TRAIL-R2-directed induction of apoptosis, indicating that coexpression of the truncated TRAIL-R3 and TRAIL-R4 in these T cells is not responsible for the observed resistance. Upon stimulation, levels of death-inducing TRAIL receptors decreased whereas TRAIL was up-regulated on the cell surface. In contrast to CD95, the role of TRAIL receptors in MS might not involve regulation of T cell vulnerability. *Cell Death and Differentiation* (2000) **7,** 637–644.

Keywords: TRAIL; apoptosis; T lymphocytes; multiple sclerosis

Abbreviations: TRAIL, TNF-related apoptosis-inducing ligand; Trail-R, TRAIL receptor; MS, Multiple Sclerosis; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; TT, tetanus toxoid

Introduction

Apoptotic death of lymphocytes is involved in the maintenance of tolerance to self antigens.[1,2] The mechanism of T cell control, namely activation-induced T cell death, critically depends on the CD95 (APO-1/Fas) pathway.[3–5] In particular, there is evidence for the pathogenic and therapeutic role of CD95-mediated apoptosis in autoimmune diseases such as Multiple Sclerosis (MS).[6–8] In the course of this disease, myelin-specific T cells invade the central nervous system (CNS) and promote demyelination. Induction of T cell death, especially via CD95-mediated apoptosis, would decrease T cell numbers, reduce invading T cells and thus autoimmune-mediated tissue damage. This regulatory process is effective during recovery[9] and experimental treatment of the MS model disease, experimental autoimmune encephalomyelitis (EAE).[10–12] It has, however, been difficult to reconcile the protection from active EAE in mice lacking functional CD95 (*lpr*) or CD95 ligand (*gld*)[13–15] with the increasing evidence for impaired CD95-dependent T cell elimination in MS.[16–20] Thus, other death receptor/ligand systems might be involved in the pathogenesis. Recently, it was demonstrated that *gld* mice with passive EAE, induced by transfer of autoreactive T cells, developed prolonged clinical signs when immunized with wild-type T cells.[21] Thus, in the passive EAE model, inhibiting the CD95 ligand results in reduced disease remission. The latter clearly indicates the role of CD95-mediated apoptosis in the regulation of T cells in EAE. However, it is still not fully understood under which conditions T cells are rendered susceptible to apoptosis.[8,22,23] Over the past few years it has become evident that there are several other apoptosis-associated receptor/ligand interactions besides the CD95/CD95 ligand system involved in the fate of a cell, in particular the recently described TNF-related apoptosis-inducing ligand (TRAIL) receptor/TRAIL system, mainly including TRAIL;[24,25] the death-inducing receptors TRAIL receptor 1 (TRAIL-R1) (also referred to as DR4)[26] and TRAIL-R2 (also referred to as DR5);[27,28] and the truncated TRAIL receptors TRAIL-R3 (also referred to as DcR1,[27] TRID[29] or LIT)[30,31] and TRAIL-R4 (also referred to as DcR2).[32,33] Interactions between TRAIL and truncated TRAIL receptors are considered to physiologically block apoptosis[27,29–32] which might thus contribute to the pathogenesis of autoimmunity. However, it is not known whether and, if so, how the TRAIL/TRAIL receptor system participates in the pathogenesis of MS. So far, contradictory reports have been published concerning the susceptibility of non-specifically stimulated T cells to soluble recombinant TRAIL.[34,35]

Here, we studied the expression pattern and modulation of the TRAIL receptor/TRAIL system in human myelin- and foreign antigen-specific T cells of MS patients and healthy donors. Upon stimulation, death-inducing TRAIL receptors were down-regulated whereas TRAIL itself was up-regulated. The T cells were not susceptible to apoptosis induced by three different soluble TRAIL preparations although we found expression of both apoptosis-mediating receptors, TRAIL-R1 and TRAIL-R2. Moreover, these T cells were also resistant to antibody-mediated TRAIL-R1/TRAIL-R2-specific apoptosis. This argues against the coexpression of the truncated TRAIL receptors TRAIL-R3

and -R4 being responsible for the protection of the (auto)antigen-specific T cells.

Results

No induction of apoptosis in human antigen-specific T cells by soluble TRAIL

First, we examined whether human T cells are susceptible to TRAIL-induced apoptosis. Antigen-specific T cell lines, recognizing the myelin antigen, myelin basic protein (MBP) or the foreign antigen tetanus toxoid (TT) (see Table 1), were resistant to three different preparations of soluble recombinant TRAIL, whereas all of these TRAIL preparations induced apoptosis in Jurkat T lymphoma cells. This is shown in Figure 1 for the functionally active leucine zipper(LZ)-TRAIL measured by caspase activation (Figure 1a) and DNA fragmentation (Figure 1b). No differences in TRAIL-mediated apoptosis susceptibility between MBP- and TT-specific T cell lines and between those derived from patients and healthy individuals were observed. Caspase activation and DNA fragmentation in Jurkat T lymphoma cells induced by soluble LZ-TRAIL confirmed the functional activity of this TRAIL preparation (see Figure 1a,b). Even in the presence of cycloheximide (10 ng/ml) no induction of apoptosis by soluble LZ-TRAIL was found (data not shown).

Human antigen-specific T cells express death-inducing TRAIL receptors

We asked whether the unresponsiveness of the human antigen-specific T cells to TRAIL-induced apoptosis was due to a lack of TRAIL receptor expression. Therefore, antigen-specific T cell lines were analyzed for presence of death-inducing TRAIL receptors. We isolated cDNA from different antigen-specific T cell lines and tested for presence of message for TRAIL-R1 and -R2 (Figure 2). All T cell lines tested were found positive for both death-signaling TRAIL receptors, although the expression level revealed a great

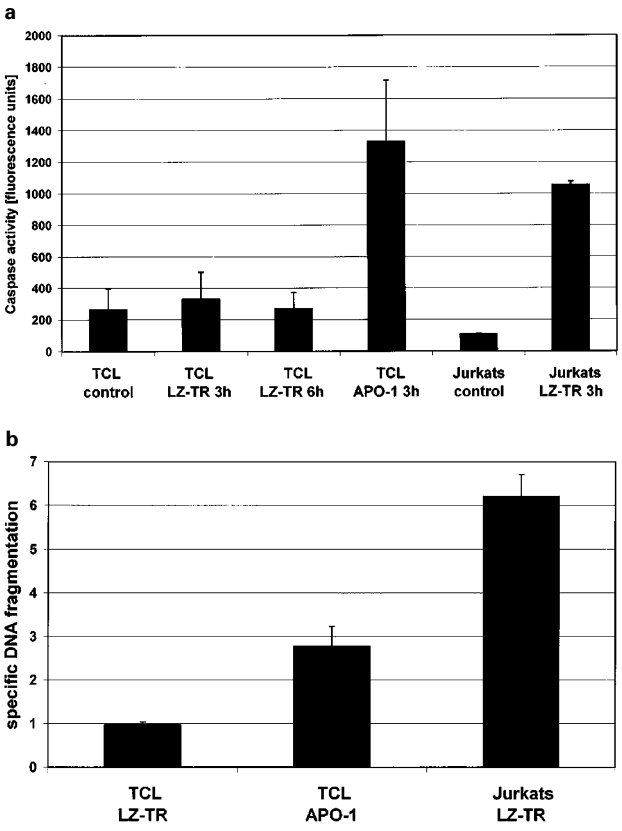

Figure 1 No induction of apoptosis in human antigen-specific T cells by soluble TRAIL. Antigen-specific T cell lines (TCL) AV4, W2, AH2, MB3, MB5, MB7 and MB15 and as positive control Jurkat T lymphoma cell line were treated with soluble functionally active trimerized TRAIL (LZ-TR) (2 μg/ml) and as positive control for TCL with APO-1 (1 μg/ml) plus protein A (10 ng/ml). (**a**) Caspase activity was measured at 3 h exposure to the apoptosis stimuli (at 3 h maximum activity was found for most apoptosis-inducing stimuli), the exposure time for LZ-TR was extended to 6 h in order to detect a delayed peak. Data are expressed as mean fluorescence units and standard error of the mean (S.E.M.), each assay was performed in triplicates (see Materials and Methods). (**b**) Specific DNA fragmentation was measured by flow cytometry at 24 h and is demonstrated as mean and S.E.M. (see Materials and Methods) with control values of 17.0% apoptotic cells (S.E.M. 3.0) for TCL and 13.8% for Jurkat cells (S.E.M. 1.4)

Table 1 Human antigen-specific T cell lines

T cell line	Antigen specificity	Origin
AH2	MBP	MS
DE1	MBP	MS
DE3	MBP	MS
SC1	MBP	MS
AV2	MBP	healthy individual
AV4	MBP	healthy individual
W2	MBP	healthy individual
U5	MBP	healthy individual
FZ18	TT	healthy individual
FZ20	TT	healthy individual
MB3	TT	healthy individual
MB5	TT	healthy individual
MB7	TT	healthy individual
MB15	TT	healthy individual

All T cell lines showed a stimulation index >3 (AH, DE, SC, AV, W, U, FZ, MB are different individuals). The MBP-specific T cell lines are T-Helper-1- or T-helper-0-like, whereas all TT-specific T cell lines display the T-helper-0 status

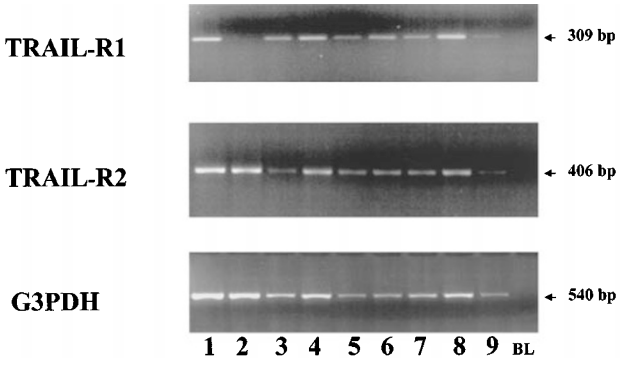

Figure 2 Transcription of death-inducing TRAIL receptors in human antigen-specific T cells assessed by RT–PCR. Expression of TRAIL receptors was analyzed by RT–PCR in seven human T cell lines (lanes 3–9; AV4, U5, W2, FZ18, FZ20, MB5, MB15) and two human glioma lines (lane 1: LN18, lane 2: T98G). Bands represent the product of RT–PCR for TRAIL-R1, 2, and reference gene G3PDH (for details, see Materials and Methods)

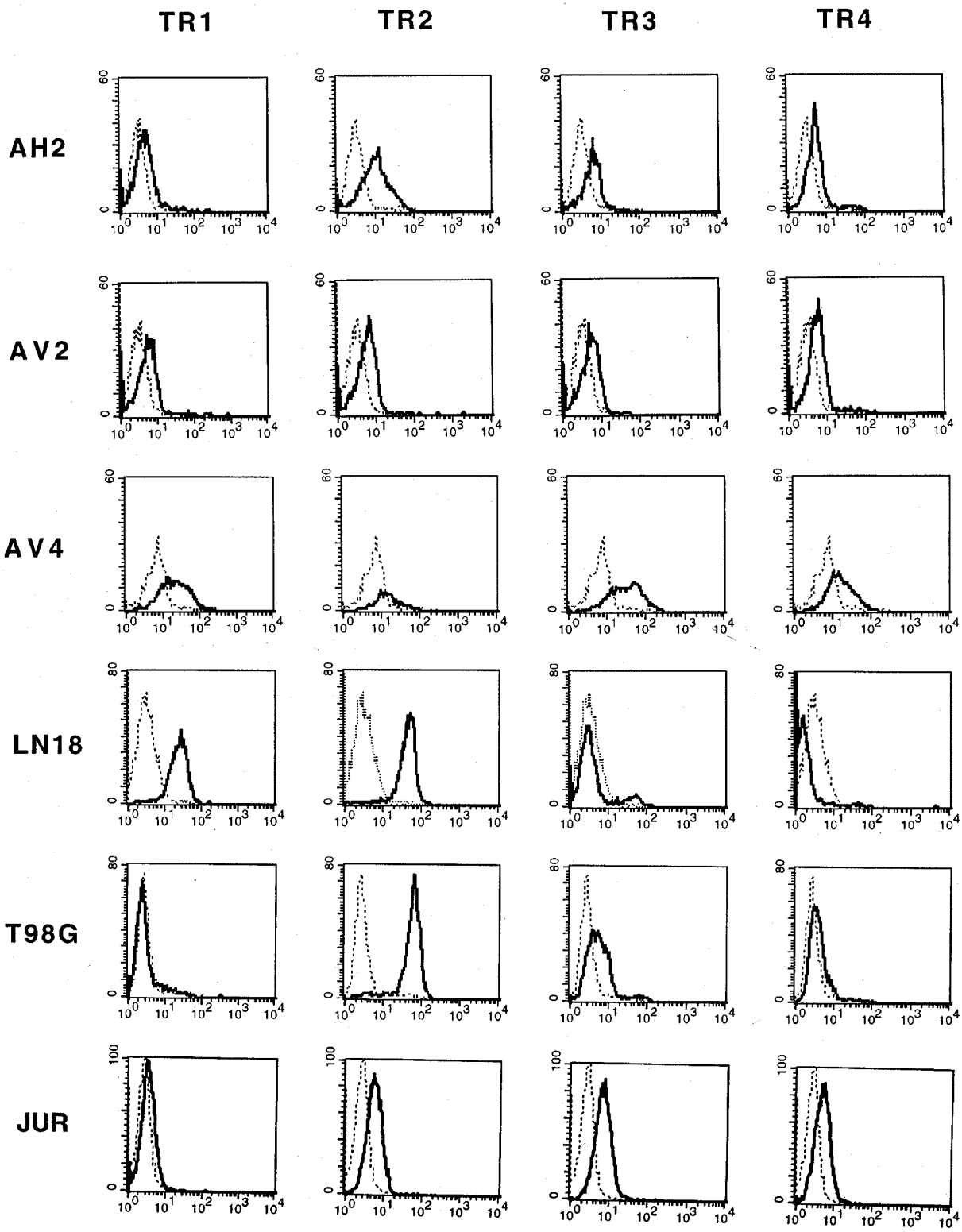

Figure 3 Human antigen-specific T cells express all TRAIL receptors. Cell surface expression of TRAIL receptors in three representative human MBP-specific T cell lines (AH2, AV2, AV4), in comparison TRAIL-susceptible human glioma cell lines (LN-18, T98G) and a human T cell lymphoma line (Jurkat; JUR) was performed by flow cytometry (see Materials and Methods). Data are expressed in a fluorescence histogram overlay depicting TRAIL receptor staining (solid line) *versus* negative control staining (dotted line)

variability of expression between the different T cell lines. No significant difference of receptor RNA levels was observed between MBP- and TT-specific T cell lines.

Co-expression of death-inducing and death-blocking TRAIL receptors in human antigen-specific T cells

In order to find out whether the resistance of antigen-specific T cells is due to coexpression of inducible and blocking TRAIL receptors we also investigated the distribution of these receptors. We found expression of all TRAIL receptors in antigen-specific T cells on the RNA (shown for TRAIL-R1 and TRAIL-R2 in Figure 2, data for TRAIL-R3 and TRAIL-R4 are not shown) and the protein level (Figure 3). In Figure 3 the surface expression pattern of three T cell lines is shown as representative examples. The TRAIL receptor expression on the cell surface was tested with monoclonal antibodies specific for the four TRAIL receptors 1-4. Glioma cell lines LN18 and T98G, previously investigated regarding RNA levels of the TRAIL receptors[36] and the Jurkat T cell lymphoma line are demonstrated in comparison. We found a stronger surface expression of at least one death-inducing TRAIL receptor in these glioma cells than in the antigen-specific T cell lines, whereas Jurkat cells displayed a receptor pattern similar to the T cell lines. As shown for the RNA level (Figure 2) TRAIL-R1 is not expressed on the cell surface of T98G. The cell surface expression of the truncated TRAIL-R3 and -R4 was, however, markedly higher in the antigen-specific T cells than in T98G and was not present in LN18.

All T cell lines tested displayed the four TRAIL receptors. The level of surface expression varied between T cell lines. Yet, there was no consistent difference between MBP- and TT-specific T cell lines. The expression of the death-inducing TRAIL receptors was similar in T cell lines derived from MS patients as in those derived from healthy donors. However, we observed a higher expression of TRAIL-R3 in a panel of T cell lines derived from patients (specific fluorescence intensity (SFI) $\pm$ S.E.M.=31.5% $\pm$ 3.9) compared to those from healthy donors (SFI $\pm$ S.E.M.=14.1% $\pm$ 7.0).

Agonistic TRAIL-R1 and TRAIL-R2 antibodies induce apoptosis in Jurkat but not in human antigen-specific T cells

In order to address the question whether the truncated TRAIL-R3 and TRAIL-R4 are capable of protecting the antigen-specific T cells from TRAIL-induced apoptosis, we compared the effect of LZ-TRAIL to the effect of immobilized agonistic TRAIL-R1/TRAIL-R2 antibodies. Therefore, antigen-specific T cells and Jurkat T lymphoma cells as positive control were incubated with LZ-TRAIL, immobilized agonistic TRAIL-R1/TRAIL-R2 antibodies and the combination of these TRAIL-mediated apoptotic stimuli (Figure 4). Whereas TRAIL-R1/TRAIL-R2-specific apoptosis was comparable to LZ-TRAIL-triggered apoptosis in Jurkat cells, the myelin and foreign antigen-specific T cells were resistant to both apoptosis stimuli. Further, the combination of antibody-and ligand-mediated apoptosis did not act synergistically in Jurkat T cells. As expected, the antigen-specific T cells also stayed resistant when incubated with the combination of apoptosis stimuli.

TRAIL and death-inducing, but not truncated TRAIL receptors are regulated upon activation of antigen-specific T cells

The regulation of TRAIL and its receptors in the human antigen-specific T cells upon activation was tested by RT–

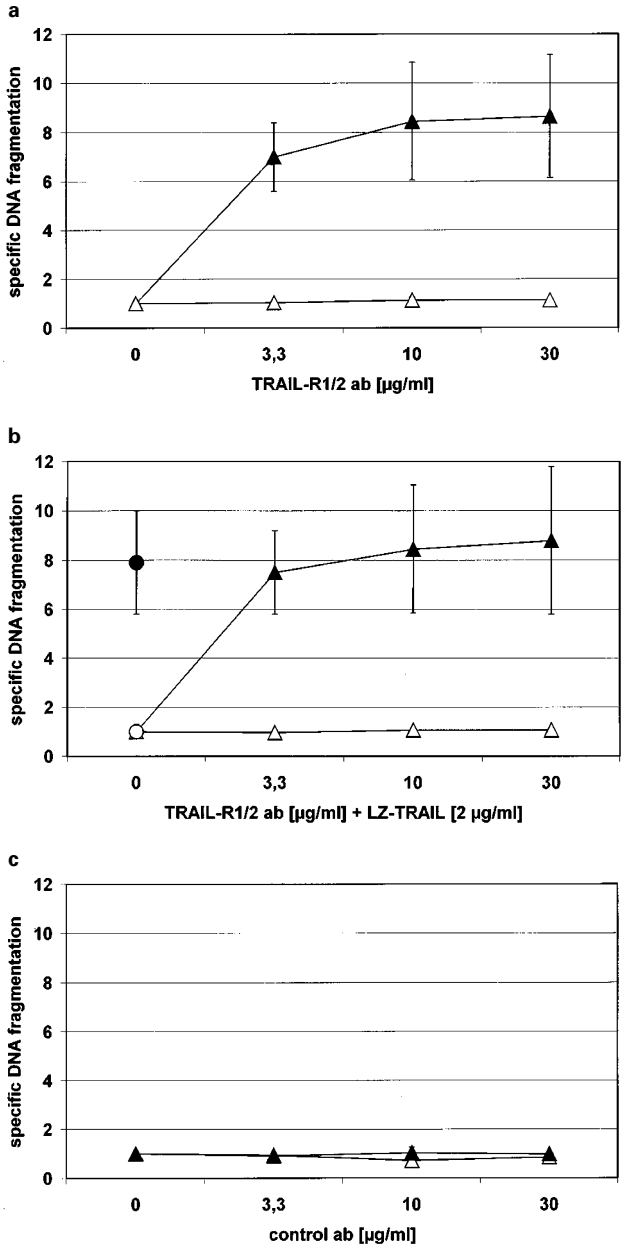

Figure 4 No induction of apoptosis by TRAIL-R1- and TRAIL-R2 mediated signal. Human antigen-specific T cell lines (open symbols) or Jurkat T lymphoma cells (closed symbols) were treated with (**a**) immobilized agonistic TRAIL-R1/TRAIL-R2, (**b**) either with LZ-TRAIL (2 μg/ml) alone (circles) or the combination of ligand and antibodies (triangles), or (**c**) an irrelevant control mouse IgG1 antibody alone. After 24 h specific DNA fragmentation was measured (for details, see Materials and Methods). Control values for Jurkat cells were 15.9% (S.E.M. 0.9) and 16.3% (S.E.M. 0.4) for TCL

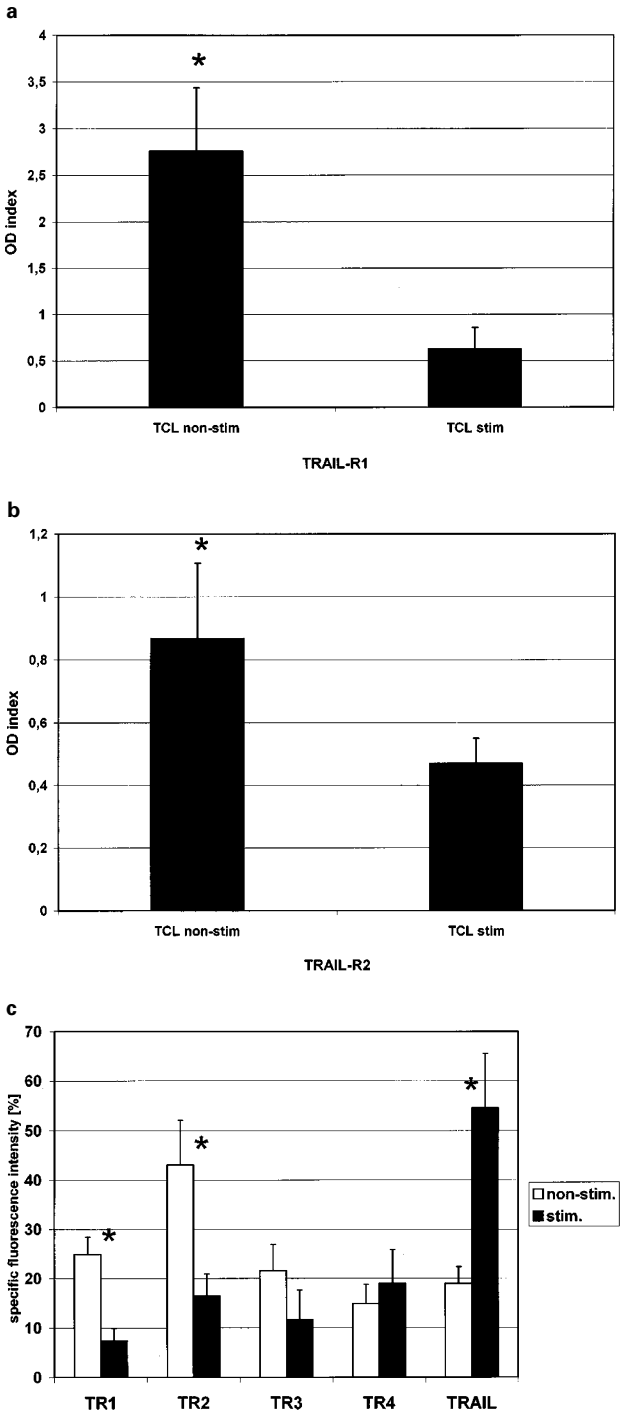

Figure 5 TRAIL-receptors 1 and 2 are down-regulated on various T cell lines upon T cell receptor stimulation whereas TRAIL is up-regulated. TRAIL-R1-4 and TRAIL were analyzed in human antigen-specific T cell lines from healthy donors (AV2, AV4, FZ18, MB15) and MS patients (AH2, DE1, DE3, SC1) on the RNA and protein level prior and after activation of T cells via immobilized anti-CD3 and anti-CD28. (**a**,**b**) Transcripts were detected by PCR using primers specific for TRAIL-R1-4 and G3PDH as control. Semiquantitative analysis of TRAIL-R1 (**a**) and -R2 (**b**) RT–PCR product levels is expressed as mean OD index and S.E.M. (for details see Materials and Methods). (*$P<0.05$, Mann-Whitney *U*-test. (**c**) Cell surface expression was detected by flow cytometry (see Materials and Methods). Data is expressed as specific fluorescence intensity and represents the mean and S.E.M. of five MBP-specific and 2 TT-specific T cell lines. (*$P<0.05$, Mann-Whitney *U*-test)

PCR and flow cytometry employing antigen-specific T cells that were activated by immobilized anti-CD3 and anti-CD28 antibodies to avoid contamination by antigen-presenting cells. In Figure 5 mean levels of TRAIL-R1 (Figure 5a) and -R2 (Figure 5b) measured by RT–PCR before and 24 h after stimulation are depicted for seven T cell lines (three from MS patients and four from healthy donors). These death-signaling TRAIL-R1 and -R2 are down-regulated on the RNA level when T cells are activated. Figure 5c demonstrates the cell surface expression of the entire TRAIL receptor/TRAIL system as measured by flow cytometry. Expression levels of (cell surface) accessible death-inducing TRAIL receptors 1 and 2 decreased upon T cell stimulation – at the same time TRAIL surface expression was up-regulated. No significant regulation was observed for TRAIL receptors 3 and 4. Further, there was no consistent difference between MBP- and TT-specific T cell lines and between T cell lines derived from MS patients and those from healthy donors in the regulation of the TRAIL receptors in response to activation.

Discussion

In recent years it became evident that one major regulatory mechanism of autoimmune diseases such as multiple sclerosis is apoptosis. Natural mouse mutants carrying mutations in the CD95 (*lpr*) or CD95 ligand (*gld*) genes in which activation-induced cell death is impaired exhibit lymphoproliferative and autoimmune syndromes.[37] Further, children with CD95-gene defects suffer from similar autoimmune disorders.[38–41] Autoreactive myelin-specific T cells considered to initiate and perpetuate the immune processes in MS, are found both in patients and healthy individuals.[42,43] Thus, dysregulation of T cell apoptosis as a control mechanism might be crucial for the pathogenesis and heterogeneous course of MS. Apart from CD95-mediated apoptosis currently being investigated with respect to its exact role in autoimmune diseases[8] other death receptor/death ligand systems might also contribute to pathophysiologic processes.

Here, we studied the role of the TRAIL receptor/TRAIL system containing receptors which mediate apoptosis[26–28] and those which block apoptosis.[30–32,44] We investigated the latter in autoreactive and foreign antigen-specific T cells of MS patients and healthy individuals. In contrast to transformed Jurkat T cells, (auto)antigen-specific T cells were resistant to soluble TRAIL (Figure 1). Such a difference was also demonstrated in our previous work on monomeric soluble CD95 ligand.[45] Notably, even trimerized soluble LZ-TRAIL[46] was not capable of inducing apoptosis in the (auto)antigen-specific T cells (Figure 1). The resistance towards the ligand was apparent, although we found expression of both death-mediating TRAIL receptors, TRAIL-R1 and TRAIL-R2, on the RNA and protein level (Figures 2 and 3). As overexpression of truncated TRAIL receptors was shown to inhibit TRAIL-induced apoptosis,[27,29,31] we then investigated the distribution of death-blocking truncated TRAIL receptors in the (auto)antigen-specific T cells derived from MS patients and healthy individuals. Our data demonstrate coexpression of death-

inducing and death-blocking TRAIL receptors (Figures 3 and 5c).

Furthermore, we studied the apoptotic cell death mediated by specific TRAIL-R1 and TRAIL-R2 antibodies in comparison to TRAIL. These agonistic antibodies were employed in order to assess a potential functional influence of the truncated on the death-inducing TRAIL receptors with respect to the apoptosis properties of TRAIL which binds all receptors. The auto(antigen)-specific T cells were neither sensitive to TRAIL nor to direct apoptosis induction by agonistic TRAIL-R1 and TRAIL-R2 antibodies, whereas Jurkat T cells readily underwent apoptosis when incubated with TRAIL or these antibodies (Figure 4). The amount of apoptosis in Jurkat T cells induced by the agonistic antibodies was comparable to the ligand-induced apoptosis rate. These data indicate that there is no inhibition of apoptosis by the truncated TRAIL receptors, and that the resistance of the (auto)antigen-specific T cells is not conferred by these receptors.

So far, we found RNA and surface expression of TRAIL receptors 1–4 in both the autoreactive myelin-specific and foreign antigen-specific T cell lines, and in both T cell lines from MS patients and healthy individuals (Figures 3 and 5). Interestingly, a difference between MS patients and healthy donors was only present for the surface expression of TRAIL-R3, namely a higher expression of TRAIL-R3 in patients. Recently, it was postulated that several death ligands, including TRAIL, are involved in activation-induced cell death.[47] In line with previous results in murine T cells, we found a marked up-regulation of TRAIL surface expression in human antigen-specific T cells when these were activated (Figure 5).[48] Expression of death-inducing TRAIL-R1 and TRAIL-R2 was even downregulated in the antigen-specific T cells upon activation (Figure 5).

In conclusion, we were interested whether, and to which extent, the TRAIL receptor/TRAIL system contributes to T cell homeostasis and is involved in autoimmunity, since evidence has been provided that the general mechanism of apoptosis and, in particular, failure of T cell apoptosis might be part of the pathogenesis of autoimmune diseases such as MS, a T cell-mediated disorder of the central nervous system.[8] Up to now, mainly the CD95/CD95 ligand system, known to be important for activation-induced T cell death, has been studied in this context. For the first time, we report on the expression of agonistic and truncated TRAIL receptors in human (auto)antigen-specific T cells. Although autoreactive and foreign antigen-specific T cells of MS patients and healthy individuals express death-inducing TRAIL receptors, soluble active TRAIL does not induce death in these cells independently of their antigen reactivity and the corresponding disease status. Our data provide no evidence for a protective role of the truncated TRAIL receptors in (auto)antigen-specific T cells.[27,29,31] This suggests that the role of TRAIL receptors in MS does not refer to regulation of T cell vulnerability. Future studies should elucidate whether local elimination of autoreactive T cells in the effector organ, the immune privileged central nervous system, might involve TRAIL-mediated apoptosis in MS.

Materials and Methods

Materials and cell lines

Human recombinant soluble TRAIL,[48] a soluble TRAIL-kit from Alexis Corporation (San Diego, CA, USA; employed together with an enhancer antibody for multimerization) and trimerized soluble leucine zipper TRAIL (LZ-TRAIL),[46] kindly provided by Immunex Corporation (Seattle, WA, USA), were used. The anti-APO-1 antibody[49] was used in the presence of the crosslinker protein A. Cycloheximide (CHX) was purchased from Sigma (Deisenhofen, Germany). Monoclonal antibodies specific for TRAIL and TRAIL receptors 1, 2, 3, 4 (M180, M270, M413, M430 and M444, respectively) were of the mouse IgG1 subclass; additionally, an agonistic antibody specific for TRAIL receptor 1 (M271) was employed for induction of apoptosis. Antibody specificity was determined by transient expression of the different receptors in CV-1 cells. These antibodies were obtained from Immunex Corporation. As isotype-matched control antibody a mouse IgG1 antibody was used (Sigma, Deisenhofen, Germany). Anti-CD3 antibody (OKT3) was obtained from the American Type Culture Collection (Manassas, VA, USA) and anti-CD28 antibody from Ancell (Bayport, MN, USA). Jurkat T lymphoma, LN18 and T98G glioma cell lines,[36] cultured in RPMI supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum, were used as human transformed cell lines in comparison to human antigen-specific T cell lines.

Human antigen-specific T cell lines

Antigen-specific CD4+ T cell lines were established using a modified 'split-well' protocol.[45] Briefly, 2×10^5 peripheral blood mononuclear cells (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) or 4 μg/ml tetanus toxoid (TT) in 96-well round bottom microtiter plates. After 7 days, 10 IU/ml recombinant human interleukin-2 (IL-2) (Proleukin, Eurocetus, Frankfurt, Germany) was added to the cultures. Five to 7 days thereafter, 100 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 antigen. Specificity was tested by a standard proliferation assay. [^{3}H]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 indexes greater than three, were selected for further expansion.

For RT–PCR and flow cytometric analysis of activated T cells a protocol employing antibodies to CD3 and CD28 was used.[50] Cell culture plates were coated with anti-CD28 and anti-CD3 antibodies overnight at 10 μg/ml in PBS. After washing of the plates T cells were incubated for 24 h prior to assays.

RT–PCR

Isolation of RNA was performed using a total RNA purification kit (Peqlab, Erlangen, Germany). Possibly contaminating genomic DNA was removed by DNAse-digestion (Boehringer Mannheim, Mannheim, Germany). From 1 μg of RNA, cDNA was synthesized using a first-strand cDNA synthesis kit (Pharmacia Biotech, Freiburg, Germany). The PCR conditions were as follows: TRAIL-R1 29 cycles, 45 s/95°C, 45 s/60°C, 60 s/72°C, primer sequences ACTCGCTGTC-CACTTTCGTCTCTGA (nucleotides 911–935) and

CATCCCCTGGGCCTGCTGCTGTA (nucleotides 1200–1219); TRAIL-R2 28 cycles, 45 s/95°C, 45 s/60°C, 60 s/72°C, primer sequences GGGAGCCGCTCATGAGGAAGTT (nucleotides 1083–1104) and CTGGGTGATGTTGGATGGGAGAGT (nucleotides 1465–1488); TRAIL-R3 33 cycles, 45 s/95°C, 45 s/72°C, 45 s/72°C, primer sequences GAAGAATTTGGTGCCAATGCCACT (nucleotides 600–623) and CTCTTGGACTTGGCTGGGAGATGT (nucleotides 1188–1224); TRAIL-R4 32 cycles, 45 s/95°C, 45 s/64°C, 60 s/72°C, primer sequences CAACTGGTGGGCTCCGAAAAG (nucleotides 1253–1273) and ACCGCATGTGGCCTAAAACGAC (nucleotides 1578–1599); Glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) 26 cycles, 45 s/95°C, 45 s/54°C, 60 s/72°C, primer sequences GTCAACGGATTTGGTCGTATT (nucleotides 82–102) and AGTCTTCTGGGTGGCAGTGAT (nucleotides 601–621).

The PCR fragments were separated in 3% agarose gels and visualized by ethidium bromide. A cDNA fragment of the housekeeping gene G3PDH was amplified as reference for the relative expression of the other genes. For all genes, PCR protocols were standardized so that the cycle number ensured PCR-amplification was in its exponential phase. To quantify the expression of the TRAIL receptors, optical density (OD) was determined using BioDocII (Biometra, Göttingen, Germany) documentation system and Scanalytics ONE-Dscan software (Scanalytics Inc, Fairfax, VA, USA). Relative TRAIL receptor expression is presented as arbitrary value OD index, calculated as ratio of the integrated OD of the TRAIL receptor to the appropriate OD of G3PDH.

Induction of cell death by recombinant human TRAIL or TRAIL-R1- and -R2-specific monoclonal antibodies

Recombinant LZ-TRAIL was used at 2 μg/ml. For induction of apoptosis with TRAIL-R1 (M271) and -R2 (M413)-specific monoclonal antibodies, 96 well plates were coated overnight at 4°C with the indicated concentrations of the monoclonal antibodies. 1.5×10^5 T cells were subsequently added to 3 × PBS-washed wells.

DNA fragmentation

For quantitative analysis of DNA fragmentation, 10^5 cells were treated according to the protocol described by Nicoletti.[51] Briefly, a hypotonic fluorochrome solution (propidium iodide 50 μg/ml 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 percentage of total events by flow cytometry. Data are presented as specific DNA fragmentation (with stimulus/control).

DEVD-amc-cleaving caspase activity

To measure DEVD-amc-cleaving caspase activity, 10^5 T cells were plated in 96-well flat bottom plates, incubated with various stimuli for 3 h, or 6 h in serum-free medium (AIM-V), and subsequently lysed in lysis buffer (60 mM NaCl, 5 mM Tris-HCl, 2.5 mM EDTA, 0.25% NP40) for 10 min. The fluorogenic caspase substrate DEVD-amc (20 μM) (BIOMOL, Hamburg, Germany) was added to the lysates and fluorescence was measured after 30 and 60 min at 360 nm excitation and 480 nm emission wavelengths using a CytoFluor 2400 cytofluorometer (Millipore Corp., Eschborn, Germany). The amount of caspase activity is given as fluorescence units.

Flow cytometry

T cells (10^5 per sample) were stained with 10 μg/ml of monoclonal mouse IgG1 antibodies for 30 min at 4°C in PBS containing 0.01% Na-azide and 0.25% bovine serum albumin. Washed cells were incubated with phycoerythrin (PE)-labeled secondary $F(ab')_2$ fragments of a goat-anti-mouse antiserum (DAKO, Hamburg, Germany) for 30 min. Samples were measured using a FACScan flow cytometer and analyzed using the Cellquest software (Becton-Dickinson, Mountain View, Ca, USA). Values are expressed as specific fluorescence intensity, calculated by histogram statistics and defined by the percentage of fluorescence intensity with a certain antibody excluding the fluorescence intensity with the appropriate control antibody. A maximal background staining of 5% was tolerated within the region of specific fluorescence intensity.

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