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Corinna Lehnert, Maxi Weiswange, Irmela Jeremias, Carina Bayer, Michaela Grunert, Klaus-Michael Debatin and Gudrun Strauss

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TRAIL–Receptor Costimulation Inhibits Proximal TCR Signaling and Suppresses Human T Cell Activation and Proliferation

Corinna Lehnert,* Maxi Weiswange,* Irmela Jeremias,[†] Carina Bayer,[‡] Michaela Grunert,[†] Klaus-Michael Debatin,* and Gudrun Strauss*

The TRAIL–receptor/TRAIL system originally described to induce apoptosis preferentially in malignant cells is also known to be involved in T cell homeostasis and the response to viral infections and autoimmune diseases. Whereas the expression of TRAIL on activated NK and T cells increases their cytotoxicity, induction of TRAIL on APCs can turn them into apoptosis inducers but might also change their immunostimulatory capacity. Therefore, we analyzed how TRAIL–receptor (TRAIL–R) costimulation is modulating TCR-mediated activation of human T cells. T cells triggered by rTRAIL in combination with anti-CD3 and -CD28 Abs exhibited a strong decrease in the expression of activation markers and Th1 and Th2 cytokines compared with CD3/CD28-activated T cells. Most importantly, proliferation of TRAIL–R costimulated T cells was strongly impaired, but no apoptosis was induced. Addition of exogenous IL-2 could not rescue T cells silenced by TRAIL–R costimulation, and TRAIL-mediated inhibition of T cell proliferation only prevented TCR-triggered proliferation but was ineffective if T cells were activated downstream of the TCR. Inhibition of T cell proliferation was associated with abrogation of proximal TCR signaling by inhibiting recruitment of TCR-associated signaling molecules to lipid rafts, followed by abrogation of protein tyrosine phosphorylation of ZAP70, phospholipase C- γ 1, and protein kinase C- θ , and impaired nuclear translocation of NFAT, AP-1, and NF- κ B. Most importantly, TRAIL–R costimulation efficiently inhibited alloantigen-induced T cell proliferation and CD3/28-induced activation and proliferation of autoreactive T cells derived from patients with Omenn syndrome, indicating that coactivation of TRAIL–R and TCR represents a mechanism to downmodulate T cell immune responses. *The Journal of Immunology*, 2014, 193: 4021–4031.

A member of the TNF superfamily, TRAIL was originally described as a death-inducing ligand activating the extrinsic apoptotic cell death pathway. In human cells, it activates apoptosis by binding to TRAIL receptors 1 (TR1) and 2 (TR2), whereas interaction with the decoy receptors TR3 and TR4 does not transmit an apoptotic signal. Upon binding of the trimeric TRAIL to TRAIL–receptor (TRAIL–R), it induces clustering of the receptors, followed by recruitment of the adapter protein Fas-associated death domain and caspase-8. Autocatalytic caspase-8 cleavage initiates activation of the effector caspase-3, -6, and -7, leading to DNA fragmentation and cleavage of cellular substrates (1). Among the proapoptotic ligands, TRAIL has emerged as a prominent candidate in tumor therapy for its ability to mediate apoptosis in various cancer cells while causing virtually no

damage to normal cells, although clinical trials showed only small therapeutic effects using TRAIL agonists as a monotherapy (2).

TRAIL is preferentially expressed on cells of the innate and adaptive immune system depending on their activation status; for example, TRAIL expression is low in naive human T cells but upregulated after TCR stimulation, and expression is augmented in combination with type I IFNs (3, 4). In cells of myeloid origin, TRAIL expression is detected after infections with HIV, SARS-coronavirus, and measles virus (5–7), indicating that TRAIL might exhibit immunoregulatory functions apart from apoptosis induction. This is in accordance with the fact that the course of several experimental-induced autoimmune diseases can be modulated by interfering with the TRAIL–R/TRAIL system. Type 1 diabetes can be prevented by treating NOD mice with TRAIL-expressing adenovirus (8), whereas TRAIL-deficient mice are more susceptible for streptozotocin-induced diabetes (9), and blockade of TRAIL exacerbates the onset of the disease (10). In autoimmune arthritis, general blockade of TRAIL increases autoimmune inflammation by hyperproliferation of synovial cells and arthritogenic lymphocytes (11). The role of TRAIL in experimental autoimmune encephalomyelitis (EAE) is controversially discussed. Clinical severity and neuronal apoptosis are reduced upon brain-specific blockade of TRAIL after EAE induction, and intracerebral delivery of TRAIL into EAE-animals increased clinical deficits (12). However, chronic TRAIL blockade exacerbates EAE (13), and treatment of EAE developing mice with genetically modified dendritic cells expressing TRAIL prevented the development of EAE (14) by increasing cell death of CD4⁺ T cells and simultaneously preventing proliferation of Ag-specific T cells, indicating that TRAIL–R activation on nonmalignant cells can exert apoptosis but also modulate Ag-specific T cell activation and proliferation.

*Department of Pediatrics and Adolescent Medicine, University Medical Center Ulm, 89075 Ulm, Germany; [†]Helmholtz Zentrum München, German Research Center for Environmental Health, 81377 Munich, Germany; and [‡]University Medical Center Ulm, Institute of Virology, 89081 Ulm, Germany

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Address correspondence and reprint requests to Dr. Gudrun Strauss, Department of Pediatrics and Adolescent Medicine, University Medical Center Ulm, Eythstrasse 24, 89075 Ulm, Germany. E-mail address: gudrun.strauss@uniklinik-ulm.de

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Abbreviations used in this article: 7-AAD, 7-amino-actinomycin-D; EAE, experimental autoimmune encephalomyelitis; LAT, linker of activated T cells; OS, Omenn syndrome; PKC- θ , protein kinase C- θ ; PLC- γ 1, phospholipase C- γ 1; TR1, TRAIL–R 1; TRAIL–R, TRAIL–receptor.

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The execution of additional functions of death receptors apart from cell death induction was first described for CD95. The CD95/CD95L system plays a key role in T cell apoptosis and immunohomeostasis because patients with mutations in the CD95 or CD95L gene suffer from autoimmunity and lymphoproliferation (15). However, CD95 serves as a coactivator if T cells are triggered with suboptimal doses of anti-CD3 Abs (16). We could recently show that CD95 silences immune responses in naive human T cells when costimulated during T cell activation by inhibiting proximal TCR signaling events (17). T cell activation and proliferation require TCR-mediated reorganization of signaling molecules in lipid rafts, cholesterol- and glycosphingolipid-enriched signaling platforms of the plasma membrane, which provide close proximity for molecules involved in T cell activation (18, 19). Subsequently, ITAMs of the CD3 chains are phosphorylated, followed by a cascade of protein tyrosine phosphorylations of TCR-associated signaling molecules such as ZAP70, linker of activated T cells (LAT), cytosolic adaptor protein SLP-76, and phospholipase C- γ 1 (PLC- γ 1). PLC- γ 1 hydrolyzes phosphatidylinositol 3,4-bisphosphate into the secondary messengers inositol 1,4,5-triphosphate and diacylglycerol, initiating Ca^{2+} influx and activation of protein kinase C- θ (PKC- θ) and MAPK, and finally activation of transcription factors NF- κ B, NFAT, and AP-1 (20).

Considering the multifunctional roles of death ligand/death receptor systems, we analyzed in this study whether and how TRAIL-R stimulation influences activation, proliferation, and signaling of primary human T cells. TRAIL-R triggering in combination with anti-CD3/28 stimulation inhibits T cell activation by blocking early proximal TCR signaling events showing that TRAIL-R can function as silencer of T cell activation in human T cells.

Materials and Methods

Cell culture

Human T cells were cultured in complete RPMI 1640 medium (Life Technologies) supplemented with 2% FCS (Lonza), 2 mM L-glutamine, and 1 mM sodium pyruvate at 37°C in a humidified atmosphere containing 7.5% CO_2 . Proliferation experiments were carried out in complete medium containing 5% FCS.

T cells were isolated from buffy coats of healthy donors by Rosette Sep (Stemcell Technologies) with a purity >90%. For proliferation and cytokine assays, plates were coated with 0.2 $\mu\text{g/ml}$ anti-CD3 (cl. OKT3) and 0.2 $\mu\text{g/ml}$ anti-CD28 (cl. 15E8) unless indicated differently. For Western blot analyses, EMSAs, and lipid raft isolations, 0.25 $\mu\text{g/ml}$ of each Ab was used.

MLR

PBLs were isolated from buffy coats of healthy donors by density centrifugation on Ficoll, and subsequently labeled with CFSE and stimulated with isolated, irradiated (30 Gy) PBLs of healthy, unrelated, non-HLA-matched donors in a ratio of 1:1 in the presence or absence of TRAIL for 7 d. Proliferation was determined on live cells by CFSE dilution.

Patients and proliferation assay

PBLs from two patients with Omenn syndrome (OS) were analyzed. Patients were between 1 and 3 mo old and had a RAG-1 mutation. PBLs consist of >75% of activated T cells, no B cells, and between 3% and 25% of NK cells. A total of 2×10^5 PBLs was incubated on plates coated with anti-CD3/28 Abs (0.2 $\mu\text{g/ml}$) in either the absence or the presence of rTRAIL (50 $\mu\text{g/ml}$). After 6 d, cells were pulsed with 1 $\mu\text{Ci/well}$ [methyl- ^3H]thymidine (GE Healthcare Life Sciences) for 18 h before being harvested on an Inotech harvester (Wallac, Freiburg, Germany), and thymidine incorporation was determined on a Microbeta Trilux Counter (PerkinElmer). Institutional ethics committee approved collection of blood from patients. Blood samples were obtained from patients of the Department of Pediatrics and Adolescent Medicine, University Medical Center Ulm.

Purification of rTRAIL

TRAIL was either purchased from R&D Systems and used with concentrations of 5 $\mu\text{g/ml}$ for proliferation assays or purified as described pre-

viously (21). In brief, TRAIL was produced in *Escherichia coli*, inclusion bodies were isolated and lysed, and rTRAIL was refolded and dialyzed. Protein concentrations were determined by SDS gels and subsequent Coomassie blue staining. Activity of the self-made TRAIL was compared with purchased TRAIL in apoptosis assays by treating Jurkat cells with both types of TRAIL. Activity of self-produced TRAIL was 10 times lower than the activity of TRAIL purchased from R&D Systems. Therefore, self-produced TRAIL was used in 10-fold higher concentrations than purchased TRAIL. Self-produced TRAIL was used in concentrations of 50 $\mu\text{g/ml}$ for proliferation assays and 62.5 $\mu\text{g/ml}$ for Western blot analyses, EMSAs, and lipid raft isolations.

Flow cytometry

A total of 2×10^5 cells was stained with the following Abs: CD2-allophycocyanin, CD8-eFluor605, HLA-DR-allophycocyanin-eFluor780 (eBioscience), CD4-Pacific Blue, CD8-PE-Cy7, CD25-PE-Cy7, CD69-PE, and CD71-FITC (BD Biosciences). Dead cells were visualized using 7-amino-actinomycin-D (7-AAD; Sigma-Aldrich), and analysis was done on LSR II flow cytometer (BD Biosciences).

CFSE labeling

A total of 2×10^6 T cells was labeled with 100 μl 50 μM CFSE (eBioscience) at 37°C for 10 min, immediately washed five times with ice-cold PBS-5% FCS, and subsequently used for proliferation assays.

Determination of cytokine concentration in cell culture supernatants

Purified T cells or PBLs from patients with OS were cultured in medium alone or with CD3/28 in the absence or presence of TRAIL for different days. Supernatants were collected, immediately stored at -80°C, and analyzed simultaneously by Immumed GmbH (Munich, Germany) using Bioplex technology.

Nuclear protein extraction and EMSA

Purified T cells were stimulated with CD3/28 in the absence or presence of rTRAIL or with 10 ng/ml PMA and 0.5 μM ionomycin for 1 or 4 h. Nuclear protein extraction and EMSAs were performed as described previously (17). In brief, cells were harvested and resuspended in low-salt buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 10 mM KCl) and incubated for 14 min on ice. Ten percent Nonidet P-40 (10% of volume of low-salt buffer) was added, and nuclei were collected by centrifugation and resuspended in high-salt buffer (25% of volume of low-salt buffer; 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 25% glycerol). After incubating nuclei 19 min on ice, nuclear extracts were isolated by centrifugation and protein concentrations were determined.

Single-strand oligonucleotides were labeled with γ -[^{32}P]-ATP by T4 polynucleotide kinase (Thermo Scientific). A 2-fold molar excess of unlabeled complementary oligonucleotides was annealed, and double-stranded oligonucleotides were purified on spin columns (Micro Bio Spin P30; Bio-Rad). On ice binding, reactions between 5 μg nuclear extract protein and 1 μg poly(dI:dC) (Sigma) and 10,000 cpm labeled oligonucleotide were performed for 30 min in 20 μl buffer (1 mM MgCl_2 , 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM TRIS-HCl pH 7.5, 4% glycerol). Binding complexes were resolved in 6% nondenaturing polyacrylamide gels, and gels were dried and autoradiography films detected radioactive signals. The following oligonucleotides were used: NF- κ B, 5'-AGTTGAGGGGACTTTCCAGGC-3' (sense); NFAT, 5'-TCTAAGAGGAAA-ATTTTCATG-3' (sense); AP-1, 5'-CGCTTGATGAGTCAGCCGAA-3' (sense).

Analysis of protein tyrosine phosphorylation

Purified T cells were serum starved overnight in complete medium containing 0.5% FCS, incubated for the indicated time points on plates coated with CD3/28 in the absence or presence of TRAIL, and subsequently lysed in 50 mM Hepes, 100 mM NaCl, 1% IGEPAL CA-630, 1% laurylmaltoside, 1 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4 , 50 mM NaF, and 10 mM $\text{Na}_4\text{O}_7\text{P}_2 \times 10 \text{ H}_2\text{O}$ for 15 min, and protein lysates were subjected to Western blot analysis.

Isolation of lipid rafts

Purified T cells were stimulated on CD3/28-coated wells in the absence or presence of rTRAIL for 48 h and subsequently lysed in 50 mM Hepes, 100 mM NaCl, 3% Brij 58, 1 mM PMSF, 5 mM EDTA, 1 mM Na_3VO_4 , 50 mM NaF, and 10 mM $\text{Na}_4\text{O}_7\text{P}_2 \times 10 \text{ H}_2\text{O}$ for 10 min on ice. The lysate was 1:1 diluted in 80% ice-cold sucrose in 25 mM MES, 5 mM EDTA, and

150 mM NaCl, pestled on ice, transferred to Ultra-Clear centrifuge tubes (Beckman Coulter), and overlaid with ice-cold 30% sucrose, followed by ice-cold 5% sucrose. After centrifugation for 20 h at 4°C and 40,000 rpm in a Beckman Coulter SW41 rotor, eleven 1-ml fractions were collected and lipid raft fractions were determined by CD59 positivity in dot-blot analysis. Subsequently, fractions were subjected to TCA and acetone precipitation, and raft-containing fractions were pooled.

Western blot analysis

Western blot analysis was carried out as described previously (22). In brief, 20 µg cell lysates was separated on 8.5 or 10% SDS-PAGES and electroblotted onto Hybond ECL nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked in PBS, 5% milk powder, 0.1% Tween 20 for 1 h, and stained overnight with the first Ab, followed by incubation with HRP-conjugated second-step Ab. Signals were detected by enhanced chemoluminescence membrane (GE Healthcare Life Sciences). The following Abs were used: anti-phosphotyrosine (clone 4G10), LAT (clone 2E9), TRAIL-R2 (Merck Millipore), phospho-PLC-γ1(Tyr⁷⁸³), phospho-LAT(Tyr¹⁹¹), phospho-Zap-70(Tyr³¹⁹)/Syk(Tyr³⁵²), PLC-γ1 (Cell Signaling Technology), ZAP70 (cl. 29), PKC-θ (cl. 27), JNK1/JNK2

(cl. G151-666; BD Biosciences), β-actin (cl.AC-15), ERK-1/ERK-2 (Sigma-Aldrich), caspase-8 (cl.12F5; Alexis Biochemicals), ERK1/2 (phospho-Thr²⁰²/Tyr²⁰⁴), p38 MAPK (phospho-Thr¹⁸⁰/Tyr¹⁸²; Assay Designs), anti-active JNK pAb (Promega), p38 (Stressgen), TRAIL-R1 (Upstate Biotechnology), SH-PTP1 and SH-PTP2 (cl. B-1) goat anti-mouse IgG-HRP, and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology).

Statistical analysis

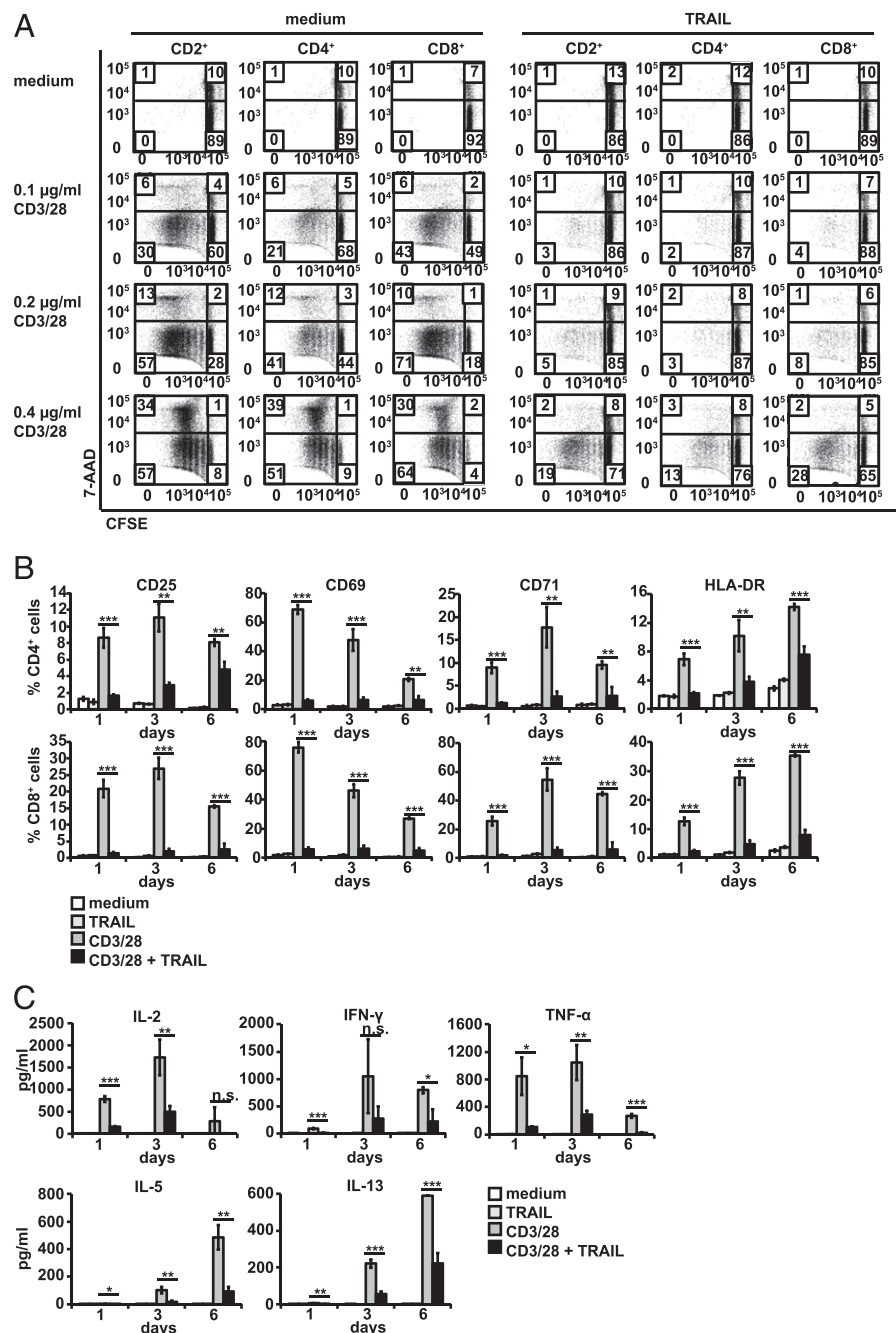
Data were analyzed using GraphPad Prism and are presented as mean ± SD. A two-way ANOVA or a Student *t* test was used for the analysis; *p* < 0.05 was considered significant.

Results

TRAIL-R costimulation blocks CD3/28-induced T cell activation and proliferation

To define the role of TRAIL-R costimulation in TCR-mediated proliferation, we stimulated CFSE-labeled T cells with increasing

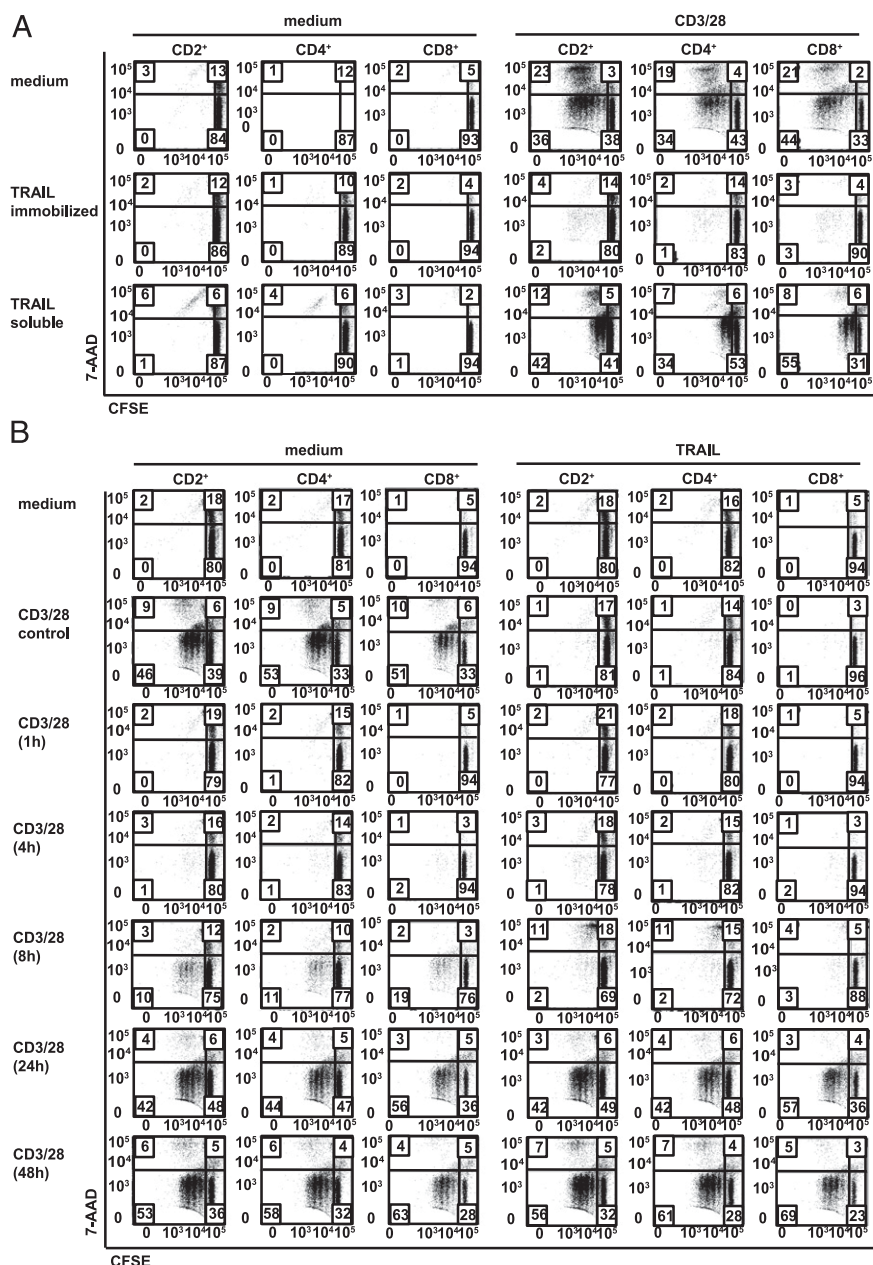
FIGURE 1. TRAIL-R triggering during TCR stimulation inhibits T cell activation and proliferation. **(A)** Purified CFSE-labeled human T cells were activated with different concentrations of anti-CD3/28 Abs in the absence or presence of TRAIL. After 6 d, the cells were simultaneously stained for CD2, CD4, CD8, and 7-AAD. CD2⁺, CD4⁺, and CD8⁺ T cells were gated by using dot blots showing SSC versus CD2, CD4, or CD8 expression. Proliferation (CFSE) and cell death (7-AAD) were then analyzed in CD2⁺, CD4⁺, and CD8⁺ T cells. **(B and C)** Purified T cells were activated with anti-CD3/28 Abs in the absence or presence of TRAIL. After 1, 3, or 6 d, cells were stained for CD4, CD8, and CD25, CD69, CD71, or HLA-DR. By gating on the different T cell subpopulations, the expression of activation markers was determined in CD4⁺ and CD8⁺ T cells by flow cytometry (B) or supernatants were analyzed for cytokine secretion (C). All experiments are representative of one experiment out of three. Values represent the mean of triplicates ± SD. **p* ≤ 0.05, ***p* ≤ 0.01, ****p* ≤ 0.001. n.s., not significant.



concentrations of anti-CD3 and -CD28 Abs in the presence or absence of rTRAIL. TRAIL-R activation strongly decreased CD3/28-mediated proliferation in CD4⁺ and CD8⁺ T cells, whereas no significant induction of apoptosis was observed. Effectiveness of TRAIL-mediated inhibition of T cell proliferation was dependent on the strength of the TCR-mediated activation signal because T cell proliferation was not completely blocked at high CD3/28 concentrations (Fig. 1A). Accordingly, inhibition of CD3/28-mediated T cell proliferation was dependent on the TRAIL concentration used (Supplemental Fig. 1). Agonistic TRAIL-R1 and -R2 Abs partially prevented anti-CD3/28-induced apoptosis, although triggering both TRAIL-Rs by rTRAIL was more efficient, showing that both functional TRAIL-Rs are involved in TRAIL-mediated inhibition of T cell proliferation (data not shown). Because CD95 triggering induces T cell proliferation in combination with suboptimal doses of anti-CD3 Ab (16), we analyzed whether costimulation of TRAIL-R with plate-bound TRAIL would also induce proliferation in anti-CD3-activated T cells.

However, independent of the anti-CD3 Ab concentrations used, TRAIL-R costimulation prevented T cell proliferation and had no costimulatory effect (Supplemental Fig. 2A). Plate-bound TRAIL was also not able to induce significant apoptosis in nonactivated and CD3/28 preactivated T cells, whereas Jurkat cells underwent apoptosis at all TRAIL concentrations tested (Supplemental Fig. 2B). TRAIL-induced inhibition of T cell proliferation was also reflected by a decreased expression of activation markers and reduced cytokine production. Stimulation of T cells with CD3/28 increased the expression of activation markers CD25, CD69, CD71, and HLA-DR in CD4⁺ and CD8⁺ T cells. Triggering TRAIL-R in the presence of CD3/28 activation reduced the expression of all activation markers, whereas TRAIL-R stimulation alone had no effect (Fig. 1B). Accordingly, the presence of TRAIL during T cell activation prevents secretion of Th1- and Th2-specific cytokines (Fig. 1C). Taken together, these results clearly show that T cell activation and proliferation can be efficiently suppressed by TRAIL-R costimulation.

FIGURE 2. Immobilized, but not soluble, TRAIL blocks TCR-mediated T cell proliferation, and inhibition of proliferation requires coactivation of TCR and TRAIL-R. **(A)** CFSE-labeled purified human T cells were activated with anti-CD3/28 Abs in the absence or presence of immobilized or soluble TRAIL. After 6 d, cells were harvested and stained for CD2, CD4, CD8, and 7-AAD. CD2⁺, CD4⁺, and CD8⁺ T cells were gated by using dot blots showing SSC versus CD2, CD4, or CD8 expression. By gating on CD2, CD4, and CD8 T cells, proliferation (CFSE) and cell death (7-AAD) were defined in the different cell populations by flow cytometry. **(B)** Purified, CFSE-labeled T cells were transferred to wells coated with rTRAIL or with medium after they have been activated for different time points (1, 4, 8, 24, 48 h) with anti-CD3/28 Abs. Control represents cells that were simultaneously activated with anti-CD3/28 and rTRAIL. After 6 d, cells were harvested and stained for CD2, CD4, CD8, and 7-AAD. CD2⁺, CD4⁺, and CD8⁺ T cells were gated by using dot blots showing SSC versus CD2, CD4, or CD8 expression. Proliferation (CFSE) and cell death (7-AAD) were then defined in the different T cell populations. Experiments were performed in triplicates, and results are representative for one of three experiments done.



Immobilized, but not soluble, TRAIL blocks TCR-mediated T cell proliferation, and inhibition of proliferation requires simultaneous activation of TCR and TRAIL-R

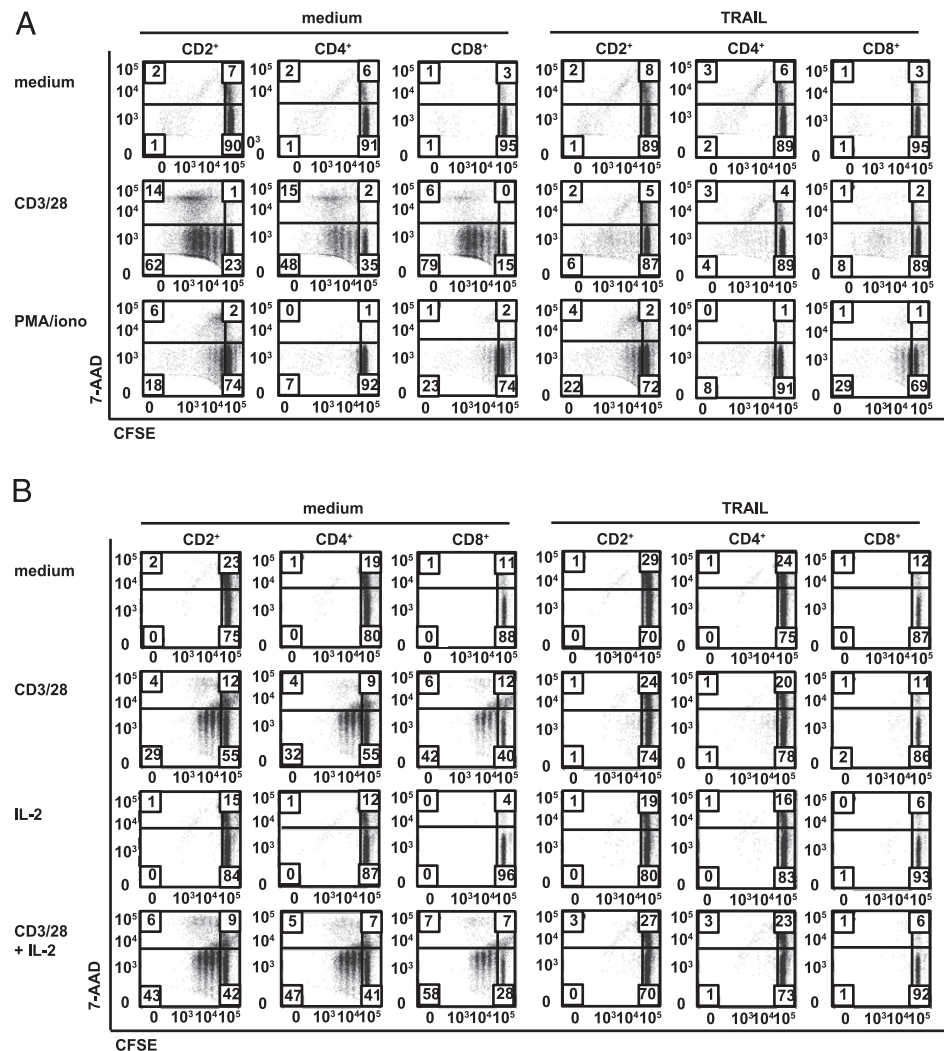
To analyze whether immobilized and soluble TRAIL inhibit T cell proliferation, we stimulated CFSE-labeled T cells with anti-CD3 and -CD28 Abs in the presence or absence of TRAIL coated on multiwell plates or added TRAIL soluble to the stimulation culture. Immobilized TRAIL completely inhibited CD3/28-induced T cell proliferation in CD4⁺ and CD8⁺ cells. Soluble TRAIL did not reduce the number of proliferating cells, although fewer cell-cycle divisions were detected compared with cells stimulated with CD3/28, indicating that soluble TRAIL cannot prevent initial T cell priming but blocks further T cell divisions (Fig. 2A). To analyze whether TRAIL-R triggering prevents T cell proliferation only when simultaneously activated together with the TCR, we stimulated CFSE-labeled T cells with TRAIL and CD3/CD28 simultaneously (CD3/28 control) as described before or activated the T cells for different time points (1, 4, 8, 24, or 48 h) with CD3/CD28 before they were transferred to plates coated with rTRAIL. Proliferation of all T cells was measured independent of the way they were activated on day 6 (Fig. 2B). Short CD3/28 activation for 1 or 4 h was not sufficient to induce T cell proliferation, whereas activation for 8 h induced proliferation of 10% CD2⁺ T cells, and activation for 24 or 48 h induced proliferation rates between 42 and 53% of CD2⁺ T cells similar to proliferation rates induced by the continuous presence of CD3 and CD28 Abs during

the whole experiment (46% proliferation of CD2⁺ T cells). Most importantly, however, TRAIL-R costimulation was unable to prevent T cell proliferation in fully activated T cells, which were preactivated for 24 or 48 h with CD3/28. Inhibition of preactivated T cell proliferation by TRAIL was observed only when most T cells have not yet received an efficient activation signal by CD3/28 Abs (8 h). These data show that TRAIL-R costimulation is mostly efficient in blocking T cell proliferation in the phase of T cell priming, and efficient inhibition of proliferation requires the simultaneous activation of TCR and TRAIL-R.

TRAIL-R activation prevents only TCR-triggered proliferation, and exogenous IL-2 cannot restore TRAIL-R-mediated T cell unresponsiveness

To analyze whether TRAIL-R triggering only interferes with TCR-mediated proliferation, we activated purified T cells with PMA and ionomycin known to directly activate PKC and Ca²⁺ mobilization independent of TCR stimulation. Activation by PMA/ionomycin induced T cell proliferation predominantly in CD8⁺ T cells and to a lesser extent than CD3/28 activation. Although CD3/28-mediated T cell proliferation was efficiently blocked by TRAIL-R triggering, PMA/ionomycin-induced proliferation was not inhibited (Fig. 3A), clearly showing that TRAIL-R stimulation only interferes with proximal T cell activation events. Because IL-2 is known to rescue anergic T cells from their unresponsive state, we also tested whether the addition

FIGURE 3. TRAIL-R triggering inhibits only TCR-induced proliferation, and exogenous IL-2 cannot rescue TRAIL-mediated inhibition of T cell proliferation. CFSE-labeled purified T cells were activated with anti-CD3/28 Abs or PMA/ionomycin in the absence or presence of TRAIL (**A**), or with anti-CD3/28, anti-CD3/28 + IL-2, or IL-2 alone in the absence or presence of TRAIL (**B**). After 6 d, cells were harvested and stained for CD2, CD4, CD8, and 7-AAD. CD2⁺, CD4⁺, and CD8⁺ T cells were gated by using dot blots showing SSC versus CD2, CD4, or CD8 expression. Proliferation (CFSE) and cell death (7-AAD) were then defined in the different T cell populations. All data shown are representative of three independent experiments performed in triplicates.



of exogenous IL-2 could abrogate TRAIL-mediated inhibition of T cell proliferation in the presence of TCR triggering. Exogenous IL-2 further increased proliferation of CD4⁺ and CD8⁺ T cells activated in the presence of CD3/28, but most importantly IL-2 could not revert the inhibition of T cell proliferation induced by TRAIL-R costimulation. Neither TRAIL nor IL-2 alone affected T cell proliferation (Fig. 3B).

TRAIL-R costimulation inhibits proximal TCR signaling

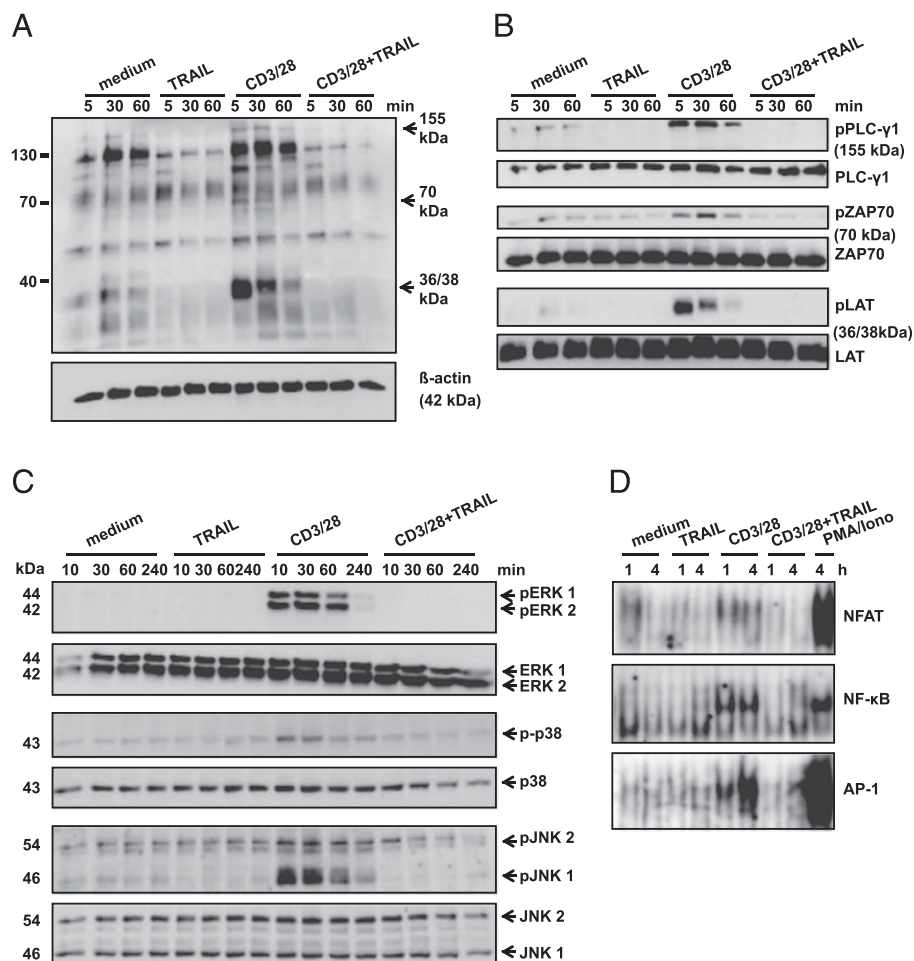
To elucidate the effects of TRAIL-R activation on proximal TCR signaling, we first addressed the influence on protein tyrosine phosphorylation, an early event in TCR signaling. Five minutes of CD3/28 stimulation induced phosphorylation of tyrosine residues in proteins corresponding to a size of ~150, 70, and 40 kDa (Fig. 4A). At later time points, protein tyrosine phosphorylation decreased but was still detectable. Importantly, costimulation of TRAIL-R totally blocked protein tyrosine phosphorylation, whereas treatment with TRAIL alone had no effect. Using phosphospecific Abs, we identified phosphorylated proteins as PLC- γ 1, ZAP70, and LAT (Fig. 4B). Also, phosphorylation of MAPK known to be activated after TCR triggering (23) was totally prevented in T cells stimulated with TRAIL and CD3/28 (Fig. 4C). Activation by CD3/28 significantly induced phosphorylation of ERK1/2, p38, and JNK1 after 10 min of stimulation. Significant protein phosphorylation was detected until 60 min after stimulation and was nearly absent after 240 min. JNK2 was constitutively phosphorylated independent of the stimulus. TRAIL treatment alone exhibited no effect on MAPK phosphorylation. Inhibition of protein phosphorylation of TCR-associated signaling molecules

and MAPKs was reflected by impaired nuclear translocation of transcription factors NF- κ B, NFAT, and AP-1 in T cells stimulated with TRAIL and CD3/28 for 1 or 4 h compared with T cells activated with CD3/28 (Fig. 4D). PMA/ionomycin activation served as a positive control. TRAIL treatment alone did not influence nuclear translocation of any transcription factor analyzed. In summary, we could show that proximal TCR signaling is strongly impaired by TRAIL-R costimulation of CD3/28 activated T cells.

TRAIL-R engagement during T cell activation prevents the recruitment of TCR-associated signaling molecules to lipid rafts

Because TCR signaling requires the recruitment of TCR-associated molecules into lipid rafts, we analyzed whether TRAIL-R costimulation influences their composition. Lipid rafts were isolated by discontinuous sucrose gradient from CD3/28-activated T cells in the presence or absence of TRAIL treatment after 48 h. Raft-containing fractions were identified by CD59 expression (Supplemental Fig. 3) and pooled for further analysis, whereas nonraft fractions were negative for CD59. CD3/28 activation induced recruitment of TCR-associated signaling molecules PLC- γ 1, PKC- θ , and ZAP70 into lipid rafts. Importantly, costimulation with TRAIL during T cell activation totally abrogated the expression of PLC- γ , PKC- θ , and ZAP70 in lipid rafts. Expression levels of LAT, which is known to be constitutively expressed in lipid rafts (24), were similarly independent of the activation signal. Untreated T cells and TRAIL-treated T cells showed no difference in the recruitment of TCR-associated signaling molecules. Nonraft fractions contained all TCR-associated molecules in similar

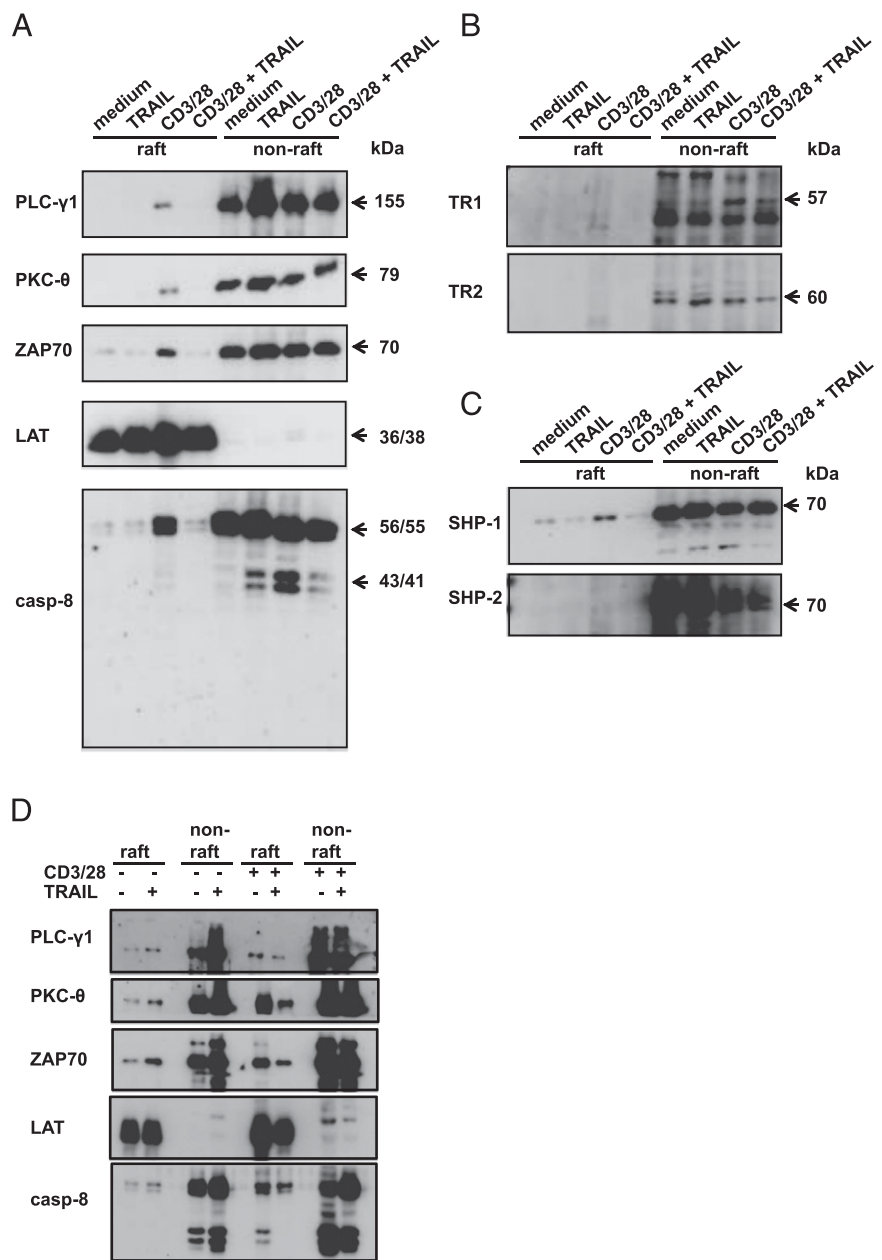
FIGURE 4. Proximal TCR signaling events are inhibited in T cells simultaneously activated by TCR and TRAIL-R. (A–C) Purified T cells were stimulated with CD3/28 Abs in the absence or presence of TRAIL for the indicated time points. Expression of total protein tyrosine phosphorylation (A) (arrows indicate differences in protein tyrosine phosphorylation), specific phosphorylated TCR-associated proteins (B), or phosphorylated and nonphosphorylated MAPKs (C) was determined by Western blot analysis at different time points. (D) Purified T cells were activated with anti-CD3/28 Abs in the presence or absence of TRAIL. After 1 and 4 h, nuclear extracts were prepared and analyzed by EMSAs for DNA binding activity of NFAT, NF- κ B, or AP-1. PMA/ionomycin stimulation served as a positive control. Experiments were performed three times with similar results.



expression levels independent of the stimulation signal (Fig. 5A). Because T cell proliferation requires the activation of caspase-8 (25), lipid rafts of CD3/28-stimulated T cells exhibited high expression of the caspase-8 proforms and also the intermediate cleavage products (p43/41), which were not detectable in T cells coactivated by TRAIL or in T cells treated with medium or TRAIL alone. Although TRAIL treatment does not induce apoptosis in T cells (Fig. 1A and Supplemental Fig. 2B), we observed an increase of the intermediate p43/41 caspase-8 cleavage product in the nonraft fraction of T cells stimulated with TRAIL, CD3/28, or CD3/28/TRAIL, which, however, was not further processed to the active p18/10 fragment (Fig. 5A). TRAIL-induced apoptosis was reported to be dependent on recruitment of TRAIL-R to lipid rafts in which the death-inducing signaling complex is formed to initiate the extrinsic apoptosis pathway (26, 27). Because TRAIL-R triggering in T cells does not induce apoptosis, no recruitment of TR1 or TR2 into lipid rafts was observed, although the receptors were expressed in the nonraft fractions (Fig. 5B). Whereas TR2 was expressed with comparable expression levels

independent of the activation signal, TR1 expression was slightly increased after CD3/28 activation. Although SHP-1 is known to function as a negative regulator of TCR-mediated signaling and is predominantly expressed in hematopoietic cells (28), no increased recruitment of SHP-1 was detected in the raft fractions of CD3/28/TRAIL-treated T cells (Fig. 5C), indicating that SHP-1 is apparently not responsible for the impaired protein tyrosine phosphorylation of TCR-associated molecules. Although SHP-1 acts as a negative regulator of T cell activation, we observed a slight increase of SHP-1 expression in CD3/28-activated T cells compared with the medium control. SHP-2 was not detectable in the raft fraction of T cells independent of the activation signal (Fig. 5C). Because we have shown in Fig. 2B that proliferation of preactivated, CD3/28-stimulated T cells cannot be inhibited by subsequent TRAIL-R triggering, we analyzed the raft composition of preactivated T cells, which were triggered by TRAIL-R 4 d after continuous CD3/28 activation. CD3/28-activated T cells recruited PLC- γ 1, PKC- θ , ZAP70, and caspase-8 into lipid rafts, and TRAIL-R costimulation did not abrogate their recruitment

FIGURE 5. TRAIL-R stimulation during T cell activation prevents translocation of TCR-associated molecules into lipid rafts. **(A–C)** Purified human T cells were simultaneously activated with anti-CD3/28 Abs in the presence or absence of TRAIL, or stimulated with TRAIL or medium alone. After 48 h, cells were harvested and lysed, and lysates were separated on a discontinuous sucrose gradient. Raft fractions were identified by CD59 expression, pooled and subsequently analyzed by Western blot analysis, and compared with nonraft fractions. Blots shown are representative of three independent experiments. **(D)** Purified T cells were activated with anti-CD3/CD28 Abs (0.2 μ g/ml). After 4 d, preactivated T cells were directly incubated in the presence or absence of TRAIL, or preactivated T cells were restimulated on plates coated with anti-CD3/28 Abs in the absence or presence of TRAIL. After 48 h, raft and nonraft composition was analyzed as described in (A)–(C). Blots are representative of two independent experiments performed.



but rather slightly increased their expression, further supporting that TRAIL-R triggering in the absence of CD3/28 stimulation does not substantially interfere with activation and proliferation of preactivated T cells (Fig. 5D). However, if CD3/28-activated T cells were restimulated again with anti-CD3/28 in combination with TRAIL-R triggering, recruitment of PLC- γ 1, PKC- θ , ZAP70, and caspase-8 was reduced compared with T cells reactivated with anti-CD3/CD28 alone (Fig. 5D). These results clearly indicate that TRAIL-R triggering in the presence of TCR activation prevents the recruitment of TCR-associated signaling molecules into signaling platforms in T cells.

TRAIL-R costimulation inhibits alloantigen-specific T cell proliferation and prevents activation and proliferation of autoreactive T cells

Because rTRAIL efficiently inhibited CD3/28-induced T cell activation, we analyzed whether TRAIL-R triggering could also prevent alloantigen-induced T cell proliferation. We isolated PBLs from buffy coats of different donors and activated these PBLs with irradiated, unrelated, non-HLA-matched PBLs in the presence or absence of plate-bound, rTRAIL. Seven days later, proliferation of CD2⁺ T cells was determined by CFSE dilution. For all MLRs performed, costimulation of TRAIL-R significantly decreased T cell proliferation, although the efficiency of inhibition varied between 26 and 59% depending on the responding T cells used (Fig. 6). Incompleteness of T cell inhibition might be due to the fact that TRAIL and Ag are not expressed on the same APC.

To analyze whether activation and proliferation of autoreactive T cells can also be inhibited by TRAIL-R costimulation, we isolated PBLs from two patients suffering from OS (OS1, OS2), which is a form of SCID. PBLs of these patients preferentially consist of activated oligoclonal T cells (OS1: 75% CD3⁺; OS2: 95% CD3⁺), which are nearly 100% CD45RO⁺CD45RA⁻ (Fig. 7A) and are

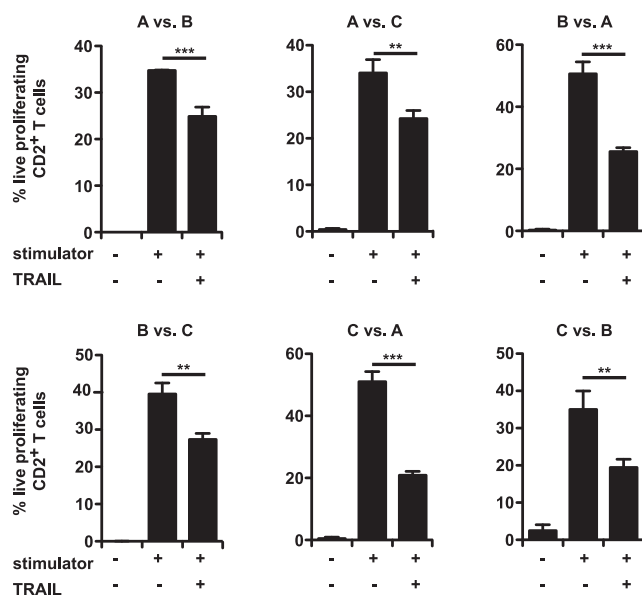


FIGURE 6. TRAIL-R costimulation inhibits alloantigen-specific T cell proliferation. CFSE-labeled PBLs isolated from buffy coats of healthy, unrelated, non-HLA-matched donors (buffy A, B, and C) were stimulated with irradiated PBLs from buffy coats A, B, and C in the absence or presence of immobilized TRAIL. After 7 d, cells were stained for CD2 and 7-AAD. Live cells were identified by FSC/SSC and 7-AAD negativity. CD2⁺ T cells were identified in live cell population using dot blots showing SSC versus CD2 expression, and proliferation was determined in CD2⁺ live cells by CFSE dilution. ** $p \leq 0.01$, *** $p \leq 0.001$.

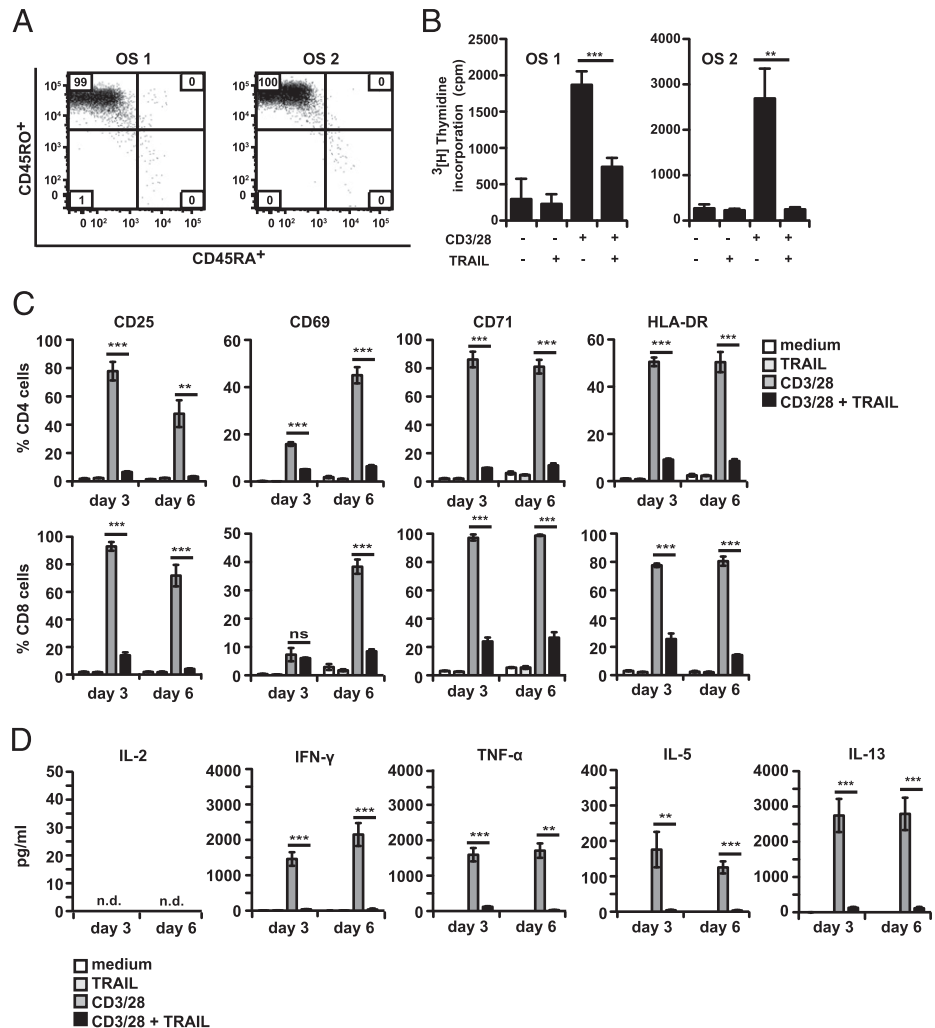
present in peripheral blood and infiltrate the skin, intestine, spleen, and liver, causing graft-versus-host-like disease (29, 30). PBLs from OS patients were stimulated with immobilized anti-CD3/28 Abs in the absence or presence of TRAIL. CD3/28 activation induced proliferation of OS T cells, which was efficiently blocked by simultaneous TRAIL-R triggering (Fig. 7B). [³H]Thymidine incorporation was used because numbers of PBLs obtained from OS patients was too little for CFSE labeling. TRAIL-induced inhibition of T cell proliferation was also reflected by decreased expression of activation markers on CD4⁺ and CD8⁺ T cells after TRAIL-R costimulation (Fig. 7C). In addition, secretion of Th1- and Th2-specific cytokines was nearly totally prevented when patient PBLs were simultaneously activated by anti-CD3/28 Abs and TRAIL compared with CD3/28 stimulation alone. Interestingly, IL-2 was not secreted in detectable amounts from CD3/28-activated PBLs of OS2 (Fig. 7D). Stimulation with TRAIL alone did not induce expression of activation markers or the secretion of cytokines. TRAIL-mediated inhibition of T cell activation and proliferation in OS T cells was comparable with the inhibition detected in PBLs, which were preactivated with anti-CD3/28 Abs for 5 d. These preactivated T cells preferentially expressed CD45RO, and TRAIL-R costimulation also prevented CD3/28-induced T cell proliferation (Supplemental Fig. 4A, 4B). Expression of activation markers in preactivated T cells was higher compared with patient material; however, stimulation by CD3/28 further increased their expression, which was prevented after TRAIL-R costimulation (Supplemental Fig. 4C). TRAIL-R costimulation inhibited CD3/28-induced secretion of Th1 and Th2 cytokines (Supplemental Fig. 4D) showing that TRAIL-R costimulation has essentially the same inhibitory effects on autoreactive T cells from OS patients compared with CD3/28 preactivated T cells from healthy donors.

Discussion

TRAIL, a cytokine of the TNF superfamily described to specifically induce apoptosis in tumor cells, is now increasingly recognized to regulate various immune cell functions. In this article, we describe for the first time, to our knowledge, that TRAIL-R triggering during Ag-independent T cell activation prevents proliferation of primary human T cells by inhibiting proximal TCR signaling. Also, alloantigen-specific T cell proliferation and activation and proliferation of CD3/28-activated autoreactive T cells was significantly inhibited by TRAIL-R costimulation, indicating that TRAIL-R can function as suppressor of T cell activation if simultaneously triggered with the TCR.

The TRAIL-R/TRAIL system was previously described to contribute to T cell homeostasis because blocking Abs against TRAIL reduced the efficiency of activation-induced cell death (31), and mice deficient for CD95L and TRAIL developed a more severe lymphoproliferative disease than mice deficient for CD95L alone (32). Also, "helpless" CD8⁺ Ag-activated T cells generated in the absence of CD4⁺ T cells die by TRAIL-mediated, activation-induced cell death upon restimulation (33). In contrast, nonactivated T cells and activated PHA/IL-2-stimulated T cells and autoantigen-specific T cells from multiple sclerosis patients were described to be TRAIL resistant, which was correlated with high expression levels of c-FLIP (34–36). Using nonactivated T cells isolated from buffy coats or CD3/28-activated T cells, we also did not detect significant T cell apoptosis by using immobilized TRAIL. TRAIL-induced apoptosis by TRAIL-R1 requires palmitoylation of the receptor, which is a prerequisite for its raft localization and its ability to oligomerize, two features essential for TRAIL-mediated death induction (26). Resistance of tumor cells toward TRAIL correlates with impaired translocation of DR4 and

FIGURE 7. TRAIL-R costimulation prevents activation and proliferation of autoreactive T cells. **(A)** PBLs from patients with OS (OS1, OS2) were stained for CD3, CD45RA, and CD45RO (% of CD3⁺ T cells: OS1: 75%, OS2: 95%). By gating on CD3⁺ cells, expression of CD45RA and CD45RO was determined on T cells. **(B)** PBLs from OS1 and OS2 were stimulated with anti-CD3/28 Abs in the absence or presence of TRAIL or with TRAIL alone. After 7 d, proliferation was determined by [³H]thymidine incorporation. **(C)** and **(D)** PBLs from OS2 were activated by anti-CD3/28 Abs in the absence or presence of TRAIL or TRAIL alone. After 3 or 6 d, cells were stained for CD4, CD8, and CD25, CD69, CD71, or HLA-DR. By gating on the different T cell subpopulations, the expression of activation markers was determined in CD4⁺ and CD8⁺ T cells by flow cytometry (C), or supernatants were analyzed for cytokine secretion (D). Values represent the mean of triplicates \pm SD. ** p < 0.01, *** p < 0.001. n.d., not detectable.



DR5 into lipid rafts (27, 37). Concordant with the reported results, TR1 and TR2 were not detected in lipid rafts of nonactivated and anti-CD3/28-stimulated human primary T cells.

TRAIL-R triggering in combination with TCR activation, however, efficiently prevented T cell proliferation in nonactivated T cells and CD3/28-stimulated T cells. Inhibition of proliferation was associated with a decrease in the expression of activation markers and reduced Th1 and Th2 cytokine secretion. Importantly, inhibition of proliferation is only achieved if TRAIL-R stimulation and TCR activation are simultaneously provided because proliferation of CD3/28-activated T cells cannot be prevented if these T cells are subsequently incubated with immobilized TRAIL in the absence of further TCR activation. Furthermore, only immobilized TRAIL prevented activation and proliferation of TCR-stimulated T cells, although soluble ligand reduced the numbers of cell divisions while not changing the amount of dividing cells. The role of soluble and membrane-bound TRAIL in apoptosis induction and immunomodulation is not well characterized. Membrane-bound TRAIL expressed on NK cells supplements NK cell cytotoxicity against neuroblastoma cells (38), whereas recombinant soluble TRAIL increases neuroblastoma cell numbers (39), indicating that probably dependent on the cellular microenvironment, TRAIL can act either proapoptotic or anti-apoptotic. Interestingly, also adding exogenously rIL-2 known to rescue anergic cells from their unresponsive state could not revert the inhibitory effects of TRAIL-R triggering on T cell activation.

In several models of experimentally induced autoimmune diseases, the interference with the TRAIL-R/TRAIL system was reported to influence the course of disease. rTRAIL suppressed type 1 diabetes (8), and intra-articular injection of TRAIL-expressing virus in mice suffering from collagen-induced arthritis 6 d after the onset of disease alleviated signs of autoimmunity (11). Likewise, inhibition of TRAIL by recombinant TR2 exacerbated autoimmune arthritis reflected by increase in lymphocyte proliferation associated with increased IL-2 and IFN- γ secretion and an enhanced development of autoantibodies (11), and a blockade of TRAIL in EAE enhanced autoantigen-specific T cell responses (13). These *in vivo* studies strongly point to an important role of the TRAIL-R/TRAIL system in modulating Ag-specific T cell activation and expansion. TRAIL-R costimulation significantly decreased alloantigen-specific T cell proliferation in MLR. Although Ag-independent T cell proliferation induced by anti-CD3/28 activation was inhibited by TRAIL to nearly 100%, inhibition of alloantigen-specific proliferation ranged between 25% and 60%. This might be due to the fact that Ag and TRAIL were not expressed on the same APC, because we could show earlier in the CD95/CD95L system that development of alloantigen-specific CTLs is prevented only if APCs coexpressed the alloantigen together with the death ligand CD95L (40). In addition, costimulation of TRAIL-R in T cells derived from patients with OS prevented CD3/28-induced T cell activation, proliferation, and cytokine secretion, suggesting that also proliferation of

autoreactive T cells can be prevented by simultaneous activation of TCR, CD28, and TRAIL-R.

TRAIL-induced interference with T cell proliferation can be either indirect, by inducing immunosuppressive cells, or direct, by modulating T cell signaling. Although increase of regulatory T cell numbers after TRAIL treatment in mice suffering from experimental autoimmune thyroiditis or EAE (41, 42) was observed, direct influence of TRAIL on T cell activation could also be demonstrated by TRAIL-expressing DCs interfering with T cell proliferation and cytokine secretion in vitro (14, 43). T cell activation is initiated by the recognition of Ag-MHC complexes through TCRs, followed by reorganization of signaling molecules in lipid rafts. Signaling molecules are subsequently tyrosine phosphorylated, finally leading to activation of MAPK and transcription factors. We could show that triggering TRAIL-R in combination with TCR activation prevents the initial event of recruiting signaling molecules into lipid rafts. Inefficient recruitment of signaling molecules into lipid rafts abrogated further downstream events such as protein tyrosine phosphorylation of ZAP70, PLC- γ 1, PKC- Θ , and nuclear translocation of transcription factors AP-1, NF- κ B, and NFAT. TRAIL-R costimulation did not induce a global change in raft composition because molecules constitutively associated to lipid rafts such as LAT were still recruited. The underlying mechanism by which TRAIL-R costimulation prevents the formation of the signaling platform remains unclear. Steric hindrance of TCR signaling by TRAIL-R is unlikely because TRAIL-Rs were not detected in lipid rafts. Also, caspase-8 and MAPK activation, which are needed for efficient T cell proliferation (23, 44), were impaired by TRAIL-R costimulation. In Ag-specific human T cell lines, rTRAIL was reported to inhibit calcium influx and cell-cycle progression (36), further supporting our findings that TRAIL-R can function as a suppressor of T cell activation. Suppressing functions were also described for CD95 in primary human T cells. We have recently shown that rCD95L or CD95L expressed on APCs prevents T cell activation and proliferation by inhibiting proximal TCR signaling, and that upregulation of CD95L on APCs might serve as a mechanism of immune evasion (17).

In summary, we propose that TRAIL-R can function as silencer of T cell activation by inhibiting early T cell signaling events when coactivated together with the TCR, and that rTRAIL or TRAIL-expressing APCs might possibly be therapeutically applicable to prevent T cell activation and proliferation.

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Disclosures

The authors have no financial conflicts of interest.

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