Enhanced Proliferation and Increased IFN-\(\gamma\) Production in T Cells by Signal Transduced Through TNF-Related Apoptosis-Inducing Ligand

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Enhanced Proliferation and Increased IFN-γ Production in T Cells by Signal Transduced Through TNF-Related Apoptosis-Inducing Ligand

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TNF-related apoptosis-inducing ligand (TRAIL, also called Apo2L), a novel member of TNF superfamily, induces apoptosis in transformed cell lines of diverse origin. TRAIL is expressed in most of the cells, and the expression is up-regulated in activated T cells. Four receptors for TRAIL have been identified, and there is complex interplay between TRAIL and TRAIL receptors in vivo. The actual biological function of TRAIL/TRAIL receptor is still not clear. Growing evidence has demonstrated that members of TNF superfamily transduce signals after engagement with their receptors. Cross-linking of TRAIL by plate-bound rTRAIL receptor, death receptor 4-Fc fusion protein enhanced T cell proliferation and increased IFN-γ production in conjunction with immobilized suboptimal anti-CD3 stimulation in mouse splenocytes. The increase of T cell proliferation by death receptor 4-Fc was dose dependent, and this effect could be blocked by soluble rTRAIL proteins, indicating the occurrence of reverse signaling through TRAIL on T cell. The enhanced secretion of IFN-γ mediated via TRAIL could be blocked by SB203580, a p38 mitogen-activated protein kinase-specific inhibitor. Thus, in addition to its role in inducing apoptosis by binding to the death receptors, TRAIL itself can enhance T cell proliferation after TCR engagement and signal the augmentation of IFN-γ secretion via a p38-dependent pathway. This provides another example of reverse signaling by a member of TNF superfamily. In conclusion, our data suggest that TRAIL can itself transduce a reverse signal, and this may shed light on the biological function of TRAIL. The Journal of Immunology, 2001, 167: 1347–1352.

The TNF family of cytokines includes physiological death factors and influences a variety of immunological functions, such as cell activation and death (1). TNF and Fas ligand (L)4 have received the most intense study, and were shown to participate in activation-induced cell death, immune privilege, autoimmune disorders, and tumor evasion from the immune system (2–5). TNF-related apoptosis-inducing ligand (TRAIL; APO-2L) is another family member of TNF superfamily that is capable of inducing apoptosis (6). Four receptors for TRAIL have been identified. The ability to transduce death signals is restricted to death receptor (DR)4/TRAIL-R1 and DR5/TRAIL-R2 (7–10). In contrast, TRAIL receptor without an intracellular domain/decoy receptor 1 (DcR1)/TRAIL-R3 and TRAIL receptor with a truncated death domain DcR2/TRAIL-R4 lack functional death domains and are unable to activate apoptosis (7, 9, 11–13). Furthermore, it has been shown that these two inhibitory receptors could inhibit TRAIL-mediated apoptosis, and TRAIL-R3 and TRAIL-R4 were suggested to act as decoy receptors to protect normal tissues from cell death (11–13), based on the selective expression of TRAIL-R3 on normal tissue, but not in transformed cell lines, suggesting that TRAIL may be involved in tumor killing in vivo (7, 9). There is complex interplay between TRAIL and TRAIL receptors in TRAIL-induced apoptosis in vivo. So far, the actual biological function of TRAIL/TRAIL receptor is still not clear.

TRAIL exists mainly in membrane-bound form, and its expression on T cells is induced after T cell activation by anti-CD3 or type I IFN (14). TRAIL and members of this ligand superfamily primarily interact with their receptors by direct cell-cell contact (15). This observation, coupled with the cross-species sequence conservation of the cytoplasmic domains of these ligands, has led to the suggestion that signaling occurs in both directions for this family of ligand-receptor pairs (15). Recently, there is growing evidence that ligands of the TNF superfamily, such as CD40L (CD154) (16–18), CD30L (19), CD27L (CD70) (20), FasL (21, 22), CD137L (23), OX40L (24), and TNF-related activation-induced cytokine (TRANCE) (25), also transduce signals after engagement with their receptors. It has been shown that reverse signaling via CD40L is involved in a range of different immune processes, such as cytokine production, costimulation of T cell activation, and proper formation of germinal centers (17). Blair et al. (18) also demonstrated that CD40L could trigger short-term CD4 T cell activation as well as mediating the secretion of immunomodulatory cytokines and apoptosis. Cross-linking of OX40L on CD40L-stimulated B cells results in a significantly enhanced...
proliferative response of B cells and the down-regulation of the transcription factor B cell lineage-specific activator protein (24). In addition, cross-linking of CD30L by a mAb or by CD30-Fc fusion protein induced the production of IL-8 by freshly isolated neutrophils (19). Recently, it has been further demonstrated that maximal proliferation of CTL requires reverse signaling through FasL (21, 22). Moreover, reverse signaling via CD27L/CD70 has been shown to induce a subset of leukemic B cells to proliferate vigorously, an effect that is synergistically enhanced by ligation of CD40, but inhibited by the presence of IL-4 (20). Meanwhile, addition of CD137-Fc fusion protein induces a substantial degree of proliferation in human peripheral monocytes (23). In a recent report, Chen further demonstrated that TRANCE enhanced IFN-γ secretion in activated Th1 cells (25). These studies provide evidence to demonstrate the importance of reverse signaling in activation of the immune system. It is interesting to know whether bidirectional signaling might also occur in other members of TNF receptor superfamily. Therefore, we investigated the possible signal transduction via TRAIL after engagement with its receptor on T cells.

In this study, we report that cross-linking of TRAIL by plate-bound DR4-Fc fusion protein enhanced T cell proliferation and increased IFN-γ production in conjunction with immobilized suboptimal anti-CD3 stimulation in activated T cells in a dose-dependent manner. The effect of increased IFN-γ production could be blocked by SB203580, a p38 mitogen-activated protein kinase (MAPK)-specific inhibitor. Thus, it appears that reverse signaling is also occurring following the interaction of TRAIL and DR4. This provides yet another example of reverse signaling by a member of TNF superfamily.

Materials and Methods
Expression and purification of soluble DR4-Fc and TRAIL
To generate soluble rDR4-Fc fusion molecule, the coding sequence for the extracellular domain of human DR4 was isolated by RT-PCR using the forward primer, CGGATTCTCGAGGGCCCCACCAACCA, and the reverse primer, GAAGATCTATTATGTCATCTGCCC. The amplified product was ligated in-frame into BamHI-cut pUC19-IgG1-Fc vector containing the human IgG1 Fc coding sequence. The fusion gene was then subcloned into pBacPAK9 vector (Clontech, Palo Alto, CA). DR4-Fc fusion protein was recovered from the filtered supernatants of the recombinant virus-infected Sf21 cells using protein G-Sepharose beads (Pharmacia, Piscataway, NJ). The bound DR4-Fc protein was eluted with glycine buffer (pH 3) and dialyzed into PBS.

The extracellular portion of the TRAIL molecule was subcloned into pRSET(B) His vector (Invitrogen, Groningen, The Netherlands) and expressed in Escherichia coli. The purification of rHis-TRAIL fusion protein was performed by metal chelate column chromatography using Ni-NTA resin, according to the manufacturer’s recommendations (Qiagen, Hilden, Germany).

Immunoblotting
For immunoblotting, proteins were boiled for 5 min in SDS sampling buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol), separated by 12% SDS-PAGE, and transferred to nitrocellulose membrane. The membrane was blocked with 5% milk in TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl), washed with TBST (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.2% Tween 20), and incubated with the indicated Ab for 2 h at room temperature. The mouse anti-human IgG1 Fc (Chemicon, Temecula, CA) was used as first Ab. Bound Ab was revealed with HRP-conjugated anti-mouse IgG (Pharmacia) using ECL (Amersham, Arlington Heights, IL).

In vitro binding assay
For the in vitro binding assay, 10 µg soluble rTRAIL with or without DR4-Fc was incubated for 1 h at agitation at 4°C. Protein A-Sepharose beads (30 µl; Pharmacia, Piscataway, NJ), swollen and washed, were added and incubated for 4 h at agitation at 4°C. The beads were washed five times in cold buffer (50 mM HEPES, pH 7, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), and proteins were eluted by boiling for 5 min in SDS sampling buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol), separated by 12% SDS-PAGE. The gel was then stained with Coomassie blue staining buffer (0.25% Coomassie blue, 25% methanol, 10% acetic acid).

Mouse T cell isolation
The BALB/c mice were maintained in the animal center at the National Taiwan University Medical Center and were used between 8 and 12 wk of age. All experiments were performed in accordance with our institutional guidelines.

Mice were sacrificed by cervical dislocation, and total splenocytes were isolated and treated with RBC lysis solution (Sigma, St. Louis, MO), and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. Adherent cells were removed by incubation with nylon wool, and the enriched T cells were isolated by passing through a nylon wool column. The purity of T cells isolated was near 90% after checking with anti-CD3 staining in flow cytometry.

T cell proliferation assay
For assaying T cell proliferation with DR4 costimulation, isolated T cells (2 × 10^6 cells/well) were cultured for 72 h in 96-well flat-bottom microtiter plates precoated with anti-murine CD3 (500 ng/ml, 2C11 clone) and DR4-Fc recombinant protein (10 µg/ml). The cultures were pulsed with [3H]thymidine (1 µCi/well) 18 h before harvesting the cells, and [3H]thymidine incorporation was measured in a Microbeta Plus liquid scintillation counter (Wallac, Gaithersburg, MD). Cultures were run in triplicate, and each experiment was repeated at least three times.

Cytokine assays
To trigger the activation of T cells via TRAIL, purified T cells (2 × 10^6 cells/well) were stimulated with suboptimal concentration of plate-bound anti-CD3 mAb (500 ng/ml, 2C11) and DR4-Fc fusion protein (10 µg/ml) or human IgG1 (10 µg/ml; Sigma) for 72 h in 96-well flat-bottom microtiter plates in the presence or absence of p38 MAPK inhibitor, SB203580. Cell culture supernatants were collected, and levels of IFN-γ and IL-4 were quantified using commercial ELISA kits (Endogen, Woburn, MA), according to the vendor’s instructions. For some experiments, after stimulation with plate-bound anti-CD3 and DR4-Fc or human IgG1 for 72 h, the cells were rested on noncoated plate for 24 h, and the T cells were then restimulated with plate-bound anti-CD3 mAb (500 ng/ml, 2C11), in conjunction with immobilized DR4-Fc fusion protein (10 µg/ml) or human IgG1 (10 µg/ml; Sigma). Supernatants were separated from cells by centrifugation, and cytokine content was determined by ELISA.

Results
Expression of TRAIL in mouse-activated T cells
To study the expression of TRAIL on T cells, we constructed a soluble fusion protein containing the extracellular domain of human DR4 and the Fc domain of human IgG1 and a soluble recombinant protein of TRAIL containing the extracellular domain of human TRAIL. The rDR4-Fc fusion protein was recovered from the filtered supernatants of the recombinant baculovirus-infected Sf21 cells using protein G-Sepharose beads. The cultured supernatant of recombinant baculovirus was purified via the protein G column, and analyzed in SDS-PAGE electrophoresis. As shown in Fig. 1A, the purified rDR4-Fc protein was demonstrated by immunoblotting using anti-human IgG1Fc as the primary Ab. To determine that the rDR4-Fc protein is able to interact with TRAIL, an in vitro binding assay was used to demonstrate the binding between DR4-Fc and TRAIL. The results of the in vitro binding of TRAIL to DR4-Fc were shown in Fig. 1B. The soluble DR4-Fc protein was incubated with or without soluble rTRAIL, and was then subjected for immunoprecipitation with protein A-Sepharose beads. rTRAIL was immunoprecipitated with DR4-Fc, as shown in Fig. 1B, indicating that the purified DR4-Fc could bind to rTRAIL protein in vitro. We also tested the apoptosis-inducing ability of rTRAIL on an in vitro apoptosis system. As shown in Fig. 1C, the rTRAIL protein induced apoptosis in TRAIL-susceptible target cells, Jurkat cells (6) in a dose-dependent manner (Fig. 1C). Moreover, the apoptosis induced in Jurkat
cells by rTRAIL could be specifically blocked by DR4-Fc fusion protein (Fig. 1C), indicating that both recombinant TRAIL and DR4-Fc fusion protein are with function.

To determine the expression of TRAIL on mouse-activated T cells, T cells isolated from mouse spleen were cultured in vitro, activated with anti-CD3 mAb for 48 h, and stained with DR4-Fc and detected by FITC-labeled anti-human IgG1Fc Ab in flow cytometry. The results in Fig. 1D demonstrated that rDR4-Fc fusion proteins bound to surface of activated mouse splenic T cells. A significant shift in TRAIL-associated fluorescence was observed at 48 h after stimulation in mouse T cells, but not in isotype control (Fig. 1D). The surface expression of TRAIL was in accordance with the expression of TRAIL mRNA in mouse-activated T cells in RT-PCR analysis (data not shown). The results indicated that mouse-activated T cells expressed TRAIL on their surface.

Cross-linking of TRAIL by plate-bound DR4-Fc enhanced proliferation of murine T cells activated by suboptimal anti-CD3

Proliferation assays using purified T cells from mouse splenocytes revealed that cross-linking of TRAIL by plate-bound DR4-Fc induced proliferation of murine T cells activated by immobilized suboptimal anti-CD3 (Fig. 2). The plates precoated with human IgG1 were used as controls. As shown in Fig. 2A, the proliferation of T cells was significantly enhanced by immobilized DR4-Fc compared with immobilized human IgG1. This effect is dependent on anti-CD3, because cell proliferation was not detected in the absence of anti-CD3 (Fig. 2A). This proliferation effect by plate-bound DR4-Fc was dose dependent, and higher concentration of the plate-bound DR4-Fc induced increased proliferation of preactivated murine T cells (Fig. 2B). We found that cross-linking of TRAIL alone had no effect on the T cell proliferation. In contrast, when both TCR and TRAIL were cross-linked by anti-CD3 mAb (500 ng/ml) and DR4-Fc (10 μg/ml), respectively, the proliferation of T cells was enhanced dramatically (Fig. 2A). To pinpoint TRAIL as the source of the proliferative signal, soluble rTRAIL protein was added to block cell surface TRAIL/DR4 interactions. A significant decrease in the proliferation of T cells to the background level was observed upon the addition to the culture of soluble TRAIL (Fig. 2A). Soluble TRAIL alone did not affect the proliferation response on murine splenic T cells. To further exclude the possibility that the neutralizing effect of TRAIL could be due to its cytotoxic effect on T cells, thereby suppressing their proliferation directly, and to ensure that the proliferation effect is via interaction between DR4 and TRAIL, we used anti-DR4-specific Ab (polyclonal antiserum to DR4; Alexis Biochemicals, San Diego, CA) to block the interaction between immobilized DR4-Fc and TRAIL on T cell surface. The results in Fig. 2C demonstrated that anti-DR4 Ab, like that of TRAIL, could neutralize the stimulatory effect of immobilized DR4-Fc. The anti-DR4 Ab alone did not affect the proliferation response on mouse T cells. These results indicated that cross-linking of TRAIL on T cell surface by plate-bound DR4-Fc induced maximal proliferation of murine T cells in conjunction with suboptimal anti-CD3. Similar results were also observed when purified human T cells were used (data not shown).

TRAIL engagement increased production of IFN-γ in murine-activated T cells

We then investigated the role of TRAIL in IFN-γ secretion during T cell activation. To address this question, T cells were stimulated with plate-bound suboptimal concentration of anti-CD3 mAb, 2C11 (500 ng/ml), in the presence of either immobilized soluble DR4-Fc fusion protein or control human IgG1. The supernatant
FIGURE 2. TRAIL engagement enhances T cell proliferation in conjunction with suboptimal concentration of immobilized anti-CD3. A, The mouse T cells were cultured in plates precoated with anti-mouse CD3 mAb (500 ng/ml) and human IgG1 Fc (10 μg/ml) or DR4-Fc (10 μg/ml) in the presence or absence of soluble TRAIL protein (250 μg/ml), as indicated in the figure. The cultures were pulsed with \[^{3}H\]thymidine (1 μCi/well) 18 h before harvesting the cells, and \[^{3}H\]thymidine incorporation was measured. Statistical analysis by two-tailed Student’s t test revealed significant differences between immobilized human IgG1- or DR4-Fc-treated samples (\(p < 0.05\)). B, Purified mouse T cells from splenocytes were cultured for 72 h in 96-well flat-bottom microtiter plates precoated with anti-mouse CD3 mAb (500 ng/ml) and DR4-Fc recombinant protein (1–10 μg/ml) or human IgG1 (10 μg/ml). The cultures were pulsed with \[^{3}H\]thymidine (1 μCi/well) 18 h before harvesting the cells, and \[^{3}H\]thymidine incorporation was measured (\(p < 0.05\)). The results shown are representative of three independent experiments. C, The mouse T cells were cultured in plates precoated with anti-mouse CD3 mAb (500 ng/ml) and human IgG1 Fc (10 μg/ml) or DR4-Fc (10 μg/ml) in the presence or absence of anti-DR4 Ab (15 μg/ml), as indicated in the figure. The cultures were pulsed with \[^{3}H\]thymidine (1 μCi/well) 18 h before harvesting the cells, and \[^{3}H\]thymidine incorporation was measured (\(p < 0.05\), when compared with immobilized human IgG1-treated samples). The results shown are representative of three independent experiments.

We found that cross-linking of TRAIL alone had no effect on the production of IFN-γ. In contrast, when both TCR and TRAIL were cross-linked by anti-CD3 mAb (500 ng/ml) and DR4-Fc (10 μg/ml), respectively, the secretion of IFN-γ was enhanced dramatically (Fig. 3A). We also tested whether the engagement of TRAIL by DR4-Fc was cumulative. To address this question, T cells were preactivated by plate-bound anti-CD3 mAb with or without plate-bound DR4-Fc in both the priming and restimulation stages. Among the four groups tested, we found that the highest level of IFN-γ production was produced when T cells were cross-linked by anti-CD3 mAb and DR4-Fc fusion proteins in both the priming and restimulation stages. The levels of IFN-γ produced, from highest to lowest, were DR4-Fc/DR4-Fc > DR4-Fc/IgG1 > IgG1/DR4-Fc > IgG1/IgG1 (Fig. 3C). This observation suggested that engagement of TRAIL could transduce a costimulatory signal to enhance IFN-γ secretion during both priming and restimulation.

To understand the signaling pathway transduced by TRAIL, mouse T cells activated by plate-bound anti-CD3 mAb and DR4-Fc were incubated with SB203580, a p38 MAPK inhibitor. As shown in Fig. 4, the increased IFN-γ secretion by immobilized DR4-Fc could be significantly suppressed by SB203580 in a dose-dependent manner (Fig. 4). The results indicated that the p38 MAPK inhibitor SB203580 blocked the up-regulation of IFN-γ secretion via TRAIL on activated T cells. This suggested that the engagement of TRAIL enhances the secretion of IFN-γ and which was dependent on the activation of p38 MAPK.

Discussion

Our study has demonstrated that triggering of TRAIL by immobilized DR4-Fc, in conjunction with immobilized suboptimal anti-CD3 mAb, induced maximal proliferation response and enhanced IFN-γ secretion by activated T cells. In our results, the T cell costimulation effects induced by immobilized DR4-Fc were dose dependent and could be specifically blocked by soluble TRAIL and anti-DR4 Ab. In contrast, these effects were not observed in immobilized human IgG1; therefore, our data indicated that these effects might result from immobilized DR4-Fc acting directly on preactivated T cells’ surface, instead of acting indirectly via FcRs on surface of macrophages or other APCs. Thus, the T cell proliferation response and enhanced IFN-γ production induced by immobilized DR4-Fc in our assay system might result from the reverse signaling by TRAIL on T cells. When plate-bound DR4-Fc fusion protein was used in conjunction with suboptimal amounts of anti-CD3, a costimulatory signal was delivered for proliferation by TRAIL to T cells. These data demonstrated that the source of this positive signal is the TRAIL expressed on the T cells.

The role of TCR engagement in conjunction with the TRAIL signal remains unclear. Other molecules known for their positive signaling capabilities have recently been implicated in the death of cells in the absence of a concomitant Ag receptor signal. For example, signaling through CD40 without concurrent engagement of the B cell receptor leads to Fas-mediated cell death (26, 27), and may serve an immunoregulatory role by removing nonspecific B
It will be interesting to determine whether TRAIL can still signal without engagement of the CD3/TCR complex, and to analyze the consequences of such uncoupled signaling. In light of the discovery that CD40 signals can direct germinal center B cells to become memory B cells (28), one could speculate on the role of TRAIL in the clonal expansion of Ag-specific T cells and the generation of memory T cells.

Although it is important to note that the molecules mediating these signals have yet to be identified, due to the short cytoplasmic domain of TRAIL, it has not been noticed that TRAIL might have the capability to transduce signal by itself. This implied that there might be other important intracellular molecules associated with TRAIL to transduce the signal. Even though the phenomenon of reverse signaling has been observed in several members of TNF superfamily, including CD40L/CD154, CD30L, CD27L/CD70, FasL, CD137L, OX40L, and TRANCE (16–25), the downstream signaling pathways after cross-linking of TNF and other members of TNF family have not been elucidated until recently. It has been reported that a casein kinase I (CKI) consensus sequence is conserved in the cytoplasmic domain of 6 of 15 members of the type II integral membrane TNF ligand family (29). Therefore, Watts et al. (29) speculated that the CKI motif might be also phosphorylated in other TNF ligand family member. This represents a new insight into the mechanism of reverse signaling in this cytokine family. However, there is no CKI motif in the cytoplasmic region of TRAIL, and our study provides evidence that p38 MAPK is involved in reverse signaling via TRAIL. This raises the question as to whether MAPK signaling pathways are also initiated via other members of TNF superfamily. In a recent report, Chen et al. (25) also demonstrated that p38 MAPK was involved in reverse signal through TRANCE. The presence of reverse signaling further increases the complexity to our current understanding of TNF/TNF superfamily.

In recent studies, results obtained using soluble rTRAIL receptor DR5-Fc in mice exacerbated autoimmune arthritis and led to profound hyperproliferation of synovial cells and arthritogenic (30). Furthermore, Hilliard et al. (31) found that chronic TRAIL blockade in mice with soluble DR5 exacerbated experimental autoimmune encephalomyelitis induced by myelin oligodendrocyte glycoprotein. These effects might not only result from the blockage of TRAIL/TRAIL receptor interaction in vivo, but it also raised the possibility that these effects might result from the DR5/TRAIL engagement to transduce a reverse signal to preactivated T cells. Our study has clearly demonstrated that triggering of TRAIL by
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