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*J Immunol* 2011; 186:931-939; Prepublished online 15 December 2010; doi: 10.4049/jimmunol.1001092

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Lipid Raft Assembly and Lck Recruitment in TRAIL Costimulation Mediates NF-κB Activation and T Cell Proliferation

Shih-Chia Huang,* Hwei-Fang Tsai,†‡ Horng-Tay Tzeng,* Hsiu-Jung Liao,* and Ping-Ning Hsu*§

The TNF-related apoptosis-inducing ligand was shown to provide a costimulatory signal that cooperates with the TCR/CD3 complex to induce T cell proliferation and cytokine production. Although a number of signaling pathways were linked to the TCR/CD3 complex, it is not known how these two receptors cooperate to induce T cell activation. In this study, we show that TRAIL-induced costimulation of T cells depends on activation of the NF-κB pathway. TRAIL induced the NF-κB pathway by phosphorylation of inhibitor of κB factor kinase and protein kinase C0 in conjunction with anti-CD3. Furthermore, we demonstrated that TRAIL costimulation induced phosphorylation of the upstream TCR-proximal tyrosine kinases, Lck and ZAP70. Ligation of the TRAIL by its soluble receptor, DR4-Fc, alone was able to induce the phosphorylation of Lck and ZAP70 and to activate the NF-κB pathway; however, it was insufficient to fully activate T cells to support T cell proliferation. In contrast, TRAIL engagement in conjunction with anti-CD3, but not TRAIL ligation alone, induced lipid raft assembly and recruitment of Lck and PKC0. These results demonstrate that TRAIL costimulation mediates NF-κB activation and T cell proliferation by lipid raft assembly and recruitment of Lck. Our results suggest that in TRAIL costimulation, lipid raft recruitment of Lck integrates mitogenic NF-κB–dependent signals from the TCR and TRAIL in T lymphocytes. The Journal of Immunology, 2011, 186: 931–939.

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Received for publication April 7, 2010. Accepted for publication November 14, 2010.

This work was supported by grants from the National Health Research Institute (NHRI-EX95-9532SI), the National Science Council, Taiwan (NSC96-2811-B-002-043, NSC98-3112-B-002-047, and NSC98-2320-B-002-049-MY3), and National Taiwan University Hospital (VN-9801). Address correspondence and reprint requests to Dr. Ping-Ning Hsu, Graduate Institute of Immunology, College of Medicine, National Taiwan University, 1 Jen-Ai Road, Sec. 1, Taipei 100, Taiwan. E-mail address: phsu8635@ntu.edu.tw

Abbreviations used in this article: HDAC, histone deacetylase; IKK, inhibitor of κB factor kinase; LAT, linker for activation of T cells; MIICD, methyl-β-cyclodextrin; PDK1, phosphoinositide-dependent kinase-1; PKC, protein kinase C; siRNA, small interfering RNA.

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membrane adaptor linker for activation of T cells (LAT) and the cytosolic adaptor protein, SLP-76, constitute docking proteins (e.g., for phospholipase Cγ) to initiate Ca2+ influx and PKCθ and the MAPK cascade, ultimately resulting in activation of transcription through NFAT, NF-kB, and AP-1 (20).

In the studies reported below, we show that lipid raft assembly and the recruitment of Lck is required for cell proliferation and IL-2 production in TRAIL-induced costimulation of T lymphocytes. Ligation of the TRAIL by its soluble receptor, DR4-Fc, alone induced phosphorylation of Lck and ZAP70, resulting in activation of the downstream NF-kB pathway. However, TRAIL ligation alone was insufficient to activate T cells to produce IL-2. These results demonstrate that TRAIL costimulation mediates NF-kB activation and T cell proliferation by lipid raft assembly and recruitment of Lck.

Materials and Methods

Human T lymphocyte isolation

Heparinized peripheral venous blood was obtained from study subjects. T cells were separated by Rosette separation ( Stem Cell Technologies, Vancouver, BC, Canada) as previously described (12, 21). Briefly, non-T cells were selected by a tetramer Ab mixture against CD14, CD16, CD19, CD56, and glycA and bound to erythrocytes. These complexes were separated from the T cells by a Ficoll-Paque gradient. The purity of human T cells isolated was >97% as tested with an anti-CD3 mAb by flow cytometry.

T cell proliferation assay

To assay human primary T cell proliferation with TRAIL-mediated costimulation, 96-well flat-bottom microtiter plates were coated with an anti-CD3 mAb (OKT3; 0.5 μg/ml) at 4˚C overnight, followed by coating with the DR4-Fc recombinant protein (10 μg/ml) at 37˚C for 2 h. Fresh purified human T cells were labeled with CFSE dye for 5 min. Then, the NF-kB inhibitor, quinazoline (Merck, San Diego, CA)-pretreated T cells were seeded from the T cells by a Ficoll-Paque gradient. The purity of human T cells isolated was >97% as tested with an anti-CD3 mAb by flow cytometry.

IL-2 reporter assay

To assay T cell activation in a human T cell line, Jurkat cells (106 cells) were electroporated at 975 microfarads and 260 V using a Bio-Rad Gene Pulser (Bio-Rad) with 20 μg gpl2-ELAM-κB-Luc plasmid and 2 μg pRL-TK. Cells were allowed to sit for 2 h and then left unstimulated or stimulated for 8 h with an anti-TCR mAb alone (C305; 1.50 dilution of ascites), an anti-TCR mAb plus DR4-Fc, or DR4-Fc alone at the indicated dose. After harvesting, cells were washed and lysed. Cell lysates were subjected to a dual luciferase reporter assay (Promega, Madison, WI) according to the manufacturer’s instructions. The relative luciferase values were calculated by dual luciferase assays as described in the manual.

Cytosolic and nuclear extract separation

Nuclear and cytosolic proteins were separated upon resuspension of the pelleted cells (106) in 30 μl hypotonic lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl2, and 0.5% Nonidet P-40) by gentle pipetting, then incubation on ice for 5 min. The homogenate was centrifuged at 6000 rpm for 5 min at 4˚C. The supernatant representing the cytosolic fraction was collected then stored at −80˚C, and the pellet containing cell nuclei was lysed in 30 μl RIPA lysis buffer. The nuclear extract (supernatant) was retained after centrifugation at 13,000 × g for 5 min at 4˚C for subsequent Western blotting for NF-kB p65 (Santa Cruz Biotechnology, Santa Cruz, CA) and an EMSA.

Expression and purification of the recombinant soluble TRAIL receptor DR4-Fc

To generate the soluble rDR4-Fc fusion molecule, the coding sequence for the extracellular domain of human DR4 was isolated by RT-PCR, and the amplified product was ligated in-frame into the pUC19-IgG1-Fc vector containing the human IgG1 Fc coding sequence as previously described (9, 12). The fusion gene was then subcloned into the pBacPAK9 vector (Clontech, Palo Alto, CA). DR4-Fc fusion proteins were recovered from the filtered supernatants of recombinant virus-infected 293 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.

Preparation of lipid raft fractions

Jurkat cells (5 × 105) were treated with 375 μl cold lysis buffer (0.2% Triton X-100 in TNE buffer: 25 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% protease inhibitor, 1 mM Na3VO4, and 1 mM NaF) and incubated for 1 h on ice. The lysates were then mixed with 375 μl 85% sucrose in TNE buffer. The solution was overlaid with 2.25 ml 30% sucrose in TNE buffer followed by 1.5 ml 5% sucrose in TNE buffer. The sucrose gradients were centrifuged at 200,000 × g in an SW55Ti rotor (Beckman Coulter, Fullerton, CA) for 22 h at 4˚C. SDS-PAGE sample buffer was added to the proteins in the 12 harvested fractions. Samples at 100 μl were resolved by 10% SDS-PAGE and Western blot analysis.

Immunoblotting

For immunoblotting, proteins were boiled for 10 min in SDS sample buffer, separated by 10% SDS-PAGE, then transferred to nitrocellulose membranes. The membranes were blocked with 5% milk in TBST for 30 min. After washing with TBS-T three times, the membrane was incubated with the indicated primary Abs at room temperature for 1 h. Unbound Abs were washed with TBS-T three times. Then, the membrane was incubated with secondary Abs at room temperature for 1 h. After washing with TBS-T, the membrane was developed in an ECL system (Amersham Biosciences, Arlington Heights, IL). For the quantitative analysis, the anti–NF-kB and anti–PKCθ Abs were from Santa Cruz Biotechnology. The anti-phospho-PKCθ and anti–flotillin-1 Abs were from BD Biosciences (Franklin Lakes, NJ). The anti-actin Ab was from Millipore (Billerica, MA). The anti–TRAIL Ab (clone 55B709.3) was from Imgenex (San Diego, CA). The anti–phospho-IκBα, anti-IκBα, anti–phospho–IκBα kinase (IκK), anti-histone deacetylase (HDAC), Ab to Lck phosphorylated at Tyr506, anti-Lck, Ab to ZAP70 phosphorylated at Tyr319, and anti–ZAP70 were from Cell Signaling Technology (Danvers, MA). Ab to LAT phosphorylated at Tyr216 was from Epitomics (Burlingame, CA).

Lipid raft disruption and cytokine assays

Jurkat cells (106 cells/well) were pretreated with 10 mM methyl-β-cyclodextrin (MβCD) for 30 min and then stimulated with plate-bound anti-CD3 mAb and DR4-Fc recombinant protein (10 μg/ml) for 24 h in 96-well flat-bottom microtiter plates. Cell culture supernatants were collected, and cytokine levels of IL-2 were quantified using commercial ELISA kits (Endogen, Woburn, MA), according to the manufacturer’s instructions.

Immunofluorescence staining and confocal microscopy

Jurkat cells were stimulated with an anti-CD3 mAb (OKT3; 1.0 μg/ml) or with the combination of anti-CD3 and DR4-Fc (10 μg/ml), followed by cross-linking with 1 μg protein G for 15 min at 37˚C. Cells were fixed with 2% paraformaldehyde and first labeled with cholera toxin B-Alexa Fluor 647 (Invitrogen, Carlsbad, CA), anti-Lck polyclonal Ab (Cell Signaling Technology), and anti–PKCθ mAb (BD Biosciences). After 30 min, cells were incubated with goat-anti-rabbit-Ab–PE or goat-anti-mouse-Ab–FITC (Biolegend, San Diego, CA). Cells were then incubated on coverslips coated with poly-L-lysine for 5 min at 37˚C at room temperature. Before the confocal microscopic analysis, the coverslips were stained with Hoechst 33258. The confocal images were acquired using a Zeiss Axiocam 100TV microscope (Carl Zeiss).

Statistical analysis

The results of T cell proliferation and cytokine production were evaluated by Student t test. A p value of < 0.05 was regarded as statistically significant. The experiments were performed more than three times with T cells derived from a single donor, and a representative set of data are presented. A similar result in each figure was obtained with T cells purified from a second independent donor.

Results

TRAIL costimulation induced NF-kB activation and cell proliferation in human T lymphocytes

The critical role of costimulation in cell proliferation and cytokine production in T cells suggests that the NF-kB pathway may have an active role in TCR/CD3 plus TRAIL-mediated T cell activation. To demonstrate that TRAIL-induced costimulation of human T cells is dependent on NF-kB activation, we purified T cells from
human PBMCs and enriched them with a RosetteSep Ab mixture kit (StemCell Technologies). The purity of T cells after purification reached up to 97% according to CD3 staining by a FACScan analysis. The results in Fig. 1 demonstrated that cross-linking of the TRAIL by plate-bound DR4-Fc induced human T cell proliferation in conjunction with a suboptimal concentration of anti-CD3. This proliferative effect by DR4-Fc was neutralized by an inhibitor of NF-κB in a dose-dependent manner, indicating that these effects resulted from activation of an NF-κB pathway.

To further demonstrate activation of the NF-κB pathway in TRAIL-mediated costimulation, we further showed activation of NF-κB after TRAIL engagement by DR4-Fc in conjunction with anti-CD3 in an IL-2 reporter assay and EMSA (Fig. 1D, 1E). Moreover, translocation of NF-κB p65 into nuclei after stimulation with DR4-Fc and anti-CD3 was also demonstrated by Western blotting and a confocal image study (Fig. 1B, 1C). All of these results indicate that TRAIL costimulation induced activation of the NF-κB pathway in human T lymphocytes.

**FIGURE 1.** TRAIL costimulation induced NF-κB activation and cell proliferation in human T lymphocytes. A, CFSE-labeled purified primary human T lymphocytes were cultured for 5 d in 96-well flat-bottom plates precoated with an anti-CD3 mAb (0.5 μg/ml) and DR4-Fc (10 μg/ml) in the presence or absence of the NK-κB inhibitor, quinazoline (QNZ; 1.1, 5.5, and 11 pM). T cell proliferation was assessed by CFSE dye dilution in an FACS analysis. Numbers indicate the percentages of divided cells among total T cells. Mean values ± SD of the CFSE analysis from triplicate samples are shown. The experiments were performed more than three times, and a representative set of data is presented. Statistical analysis by Student t test revealed significant differences between quinazoline-untreated or quinazoline-treated samples. A similar result was obtained with T cells purified from two other independent donors. B, TRAIL costimulation induced nuclear translocation of NF-κB p65. Jurkat cells were stimulated with Abs as indicated, followed by cross-linking with protein G for the indicated time periods at 37 °C. Lysates of the nuclear fraction were prepared and immunoblotted with anti-NF-κB p65 and anti-HDAC Abs. C, Jurkat cells were stimulated as described in B for 2 h at 37 °C. Cells were fixed and processed for immunofluorescence staining of NF-κB p65 Ab (green) and Hoechst33258 (blue). Scale bars, 5 μm. D, The NF-κB luciferase reporter plasmid (20 μg) and Renilla-CMV (5 μg) plasmid as an internal control were transiently transfected into Jurkat cells. Transfected Jurkat cells were stimulated with Abs as indicated, followed by cross-linking with protein G for 12 h at 37 °C, and then dual luciferase assays were performed. The relative luciferase values were calculated by dual luciferase assays as described by the manufacturer (Promega). Mean ± SD of the luciferase assays from triplicate samples are shown. E, The DNA binding activity of Jurkat lysates of the nuclear fraction were analyzed for NF-κB by EMSA. Specificity of the DNA binding was determined in competition reactions using unlabeled oligonucleotides. *p < 0.05; **p < 0.01.
TRAIL costimulation-induced NF-κB activation is via activation of the upstream TCR-proximal tyrosine kinase, ZAP70, and Lck in human T lymphocytes

To study the upstream signaling pathways induced to activate NF-κB in TRAIL costimulation, we further investigated the phosphorylation of IkB, IKK, and PKCθ in TRAIL costimulation. The results in Fig. 2 demonstrate that TRAIL costimulation induced phosphorylation of IKK and degradation of IkB after stimulation with DR4-Fc and anti-CD3. Moreover, TRAIL engagement in conjunction of anti-CD3 also induced phosphorylation of PKCθ. These results indicated that TRAIL costimulation induces activation of IkB, IKK, and PKCθ in human T lymphocytes. We have performed extensive screening of all downstream pathways including NF-AT, JNK, and ERK to determine whether these pathways are affected in the presence of TRAIL costimulation. Our results demonstrated that in addition to NF-κB pathway, the NFAT pathway is also activated in TRAIL-induced costimulation (Fig. 2D). Moreover, in previous study, we have demonstrated that TRAIL costimulation induced activation of p38MAPK pathway (9, 12). However, both ERK and JNK pathways are not affected in the presence of TRAIL costimulation (Fig. 2E).

We then investigated the role of the upstream TCR-proximal tyrosine kinase signaling pathway in induction of activation of the IKK pathway and NF-κB activation in TRAIL costimulation. The results in Fig. 3 demonstrate that TRAIL engagement by DR4-Fc in conjunction with anti-CD3 induced phosphorylation of the TCR-proximal tyrosine kinases, Lck, ZAP70, and LAT. In contrast, stimulation with anti-CD3 alone was unable to induce phosphorylation of Lck, ZAP70, and LAT, indicating that TRAIL costimulation induces activation of TCR-proximal tyrosine kinase Lck, ZAP70, and LAT in human T lymphocytes.

TRAIL costimulation induced an association of Lck with TRAIL to mediate NF-κB activation

To study the signaling molecules associated with TRAIL’s activation of NF-κB in TRAIL costimulation, we further investigated the molecules associated with TRAIL in the presence of anti-CD3 and DR4-Fc stimulation. The results of communoprecipitation experiments in Fig. 4 demonstrate that Lck was associated with the TRAIL when T cells were stimulated with both anti-CD3 and DR4-Fc, but not DR4-Fc or anti-CD3 alone (Fig. 4A), indicating that Lck is associated with the TRAIL in mediating signaling upon TRAIL costimulation. Moreover, when JCaM1.6 cells, the Lck-deficient Jurkat T cells (22, 23), were stimulated with anti-CD3 and DR4-Fc, no NF-κB translocation was induced (Fig. 4B). These results indicate that Lck is associated with the TRAIL and mediates NF-κB activation upon TRAIL costimulation.

Engagement of the TRAIL by DR4-Fc alone induces activation of NF-κB and an upstream signaling pathway, but is insufficient to induce T cell proliferation in the absence of anti-CD3/TCR

To understand the signaling pathway transduced by the TRAIL, human T cells were activated by plate-bound DR4-Fc in the absence of anti-CD3/TCR. The results in Fig. 5 demonstrate that cross-linking of the TRAIL by DR4-Fc alone induced activation of NF-κB in an IL-2 reporter assay and EMSA (Fig. 5A, 5B). Furthermore, translocation of NF-κB p65 into nuclei after stimulation with DR4-Fc alone was also demonstrated by Western blots and a confocal image study (Fig. 5C, 5D). These results indicate that TRAIL engagement by DR4-Fc alone induced activation of the NF-κB pathway. We then investigated the upstream signaling pathway in induction of NF-κB activation induced by the TRAIL in the absence of anti-CD3/TCR. The results in Fig. 5E demonstrate that TRAIL engagement by DR4-Fc alone induced degradation of IkB and phosphorylation of IkB in the absence of anti-CD3. Moreover, TRAIL engagement by DR4-Fc alone in the absence of anti-CD3/TCR induced weak phosphorylation of the TCR-proximal tyrosine kinases Lck, ZAP70, and LAT (Fig. 5F). In contrast to activation by DR4-Fc in conjunction with anti-CD3/TCR, the phosphorylation of Lck, ZAP70, and LAT was weaker and transient when activated by DR4-Fc alone in the absence of anti-CD3/TCR (Fig. 5F).

We further investigated whether stimulation with DR4-Fc alone induces T cell proliferation. The results in Fig. 6 demonstrate that TRAIL engagement by DR4-Fc alone induced activation of the NF-κB pathway, but was insufficient to induce proliferation of

FIGURE 2. TRAIL costimulation induced phosphorylation of IkB, IKK, PKCθ, and NF-AT. Jurkat cells were stimulated with an anti-CD3 mAb or anti-CD3 with DR4-Fc, followed by cross-linking with protein G for the indicated time periods at 37°C. Cell lysates were prepared and immunoblotted with anti-phospho-IkB, anti-IkB, anti-actin (A), anti–phospho-IKKα (Ser180), anti-actin (B), anti–phospho-PKCθ (Thr538), and anti–PKCθ (C). Abs. D. TRAIL costimulation induced nuclear translocation of NF-AT. Jurkat cells were stimulated with same condition and lysates of the nuclear fraction were prepared and immunoblotted with anti–NF-AT and anti-HDAC Abs. E. Phosphorylation of ERK and JNK MAPK in TRAIL costimulation. Jurkat cells were stimulated with same condition, and cell lysates were prepared and immunoblotted with anti–phospho-ERK, anti-ERK, anti–phospho-JNK, and anti-JNK Abs.

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Recent studies indicated that membrane compartmentalization is required for efficient T cell activation, and lipid raft integrity is a prerequisite for efficient TCR signal transduction (18–20). To understand the roles of lipid raft organization and its associated signaling complex in signaling mediated by TRAIL-induced costimulation of human T lymphocytes, we investigated lipid raft assemblies and Lck recruitment in Jurkat cells activated by anti-CD3 mAb and DR4-Fc. As shown in Fig. 7, engagement of the TRAIL by DR4-Fc was in conjunction with anti-CD3–induced lipid raft assembly and recruitment of Lck and PKC0 (Fig. 7A–C). To directly demonstrate lipid raft reorganization induced by TRAIL-mediated costimulation, we analyzed the distribution of Lck and PKC0 on the surface of T cells by staining with DR4-Fc and anti-CD3 in the confocal image analysis. Results in Fig. 7 demonstrate that stimulation of T cells by DR4-Fc and anti-CD3 induced lipid raft assembly and recruitment of Lck and PKC0, whereas TRAIL ligation alone was insufficient to induce lipid raft recruitment of Lck and PKC0. Moreover, conditions that disrupted raft structure by dispersing their contents with MB with anti-CD3 and DR4-Fc (Fig. 7F). Thus, raft integrity is a prerequisite for efficient TRAIL-mediated costimulation of T cells. The results indicated that engagement of the TRAIL in conjunction with anti-TCR/CD3 enhanced lipid raft assembly and recruitment of Lck in human T cells, resulting in activation of the mitogenic NF-κB pathway.

**Discussion**

In this study, we demonstrated that TRAIL costimulation induced phosphorylation of the upstream TCR-proximal tyrosine kinases Lck and ZAP70, resulting in activation of the downstream NF-κB pathway. However, TRAIL ligation alone was unable to activate the proliferation of T cells, even though TRAIL ligation alone was sufficient to induce activation of NF-κB. Moreover, the TRAIL-induced costimulation of T cells that induced lipid raft assembly and recruitment of Lck and PKC0, whereas TRAIL ligation alone was insufficient to induce lipid raft recruitment of Lck and PKC0. These results demonstrate that TRAIL costimulation mediates NF-κB activation and T cell proliferation by lipid raft assembly and recruitment of Lck. Our results suggest that lipid raft recruitment of Lck integrates mitogenic NF-κB–dependent signals from the TCR and TRAIL in T lymphocytes. Moreover, we also showed that Embc was associated with the TRAIL in TRAIL costimulation in the coimmunoprecipitation assay (Fig. 4), indicating that Lck is associated with the TRAIL and mediates NF-κB activation upon TRAIL costimulation.
TRAIL costimulation. Meanwhile, in this study our results demonstrated that there is an apparent increase in the pTyr blots upon TRAIL costimulation, suggesting costimulation via TRAIL is a major inducer of the tyrosine phosphorylation of Lck. However, in our results, a change in the migration pattern of Lck is also seen in immunoblots of total Lck (Figs. 3, 7), which is commonly explained that it results from phosphorylation of Ser^59, presumably induced by Erk-1 or 2 (24–26). Therefore, although our results indicate there is an increase in the pTyr blots upon TRAIL costimulation, the possibility that other modification of Lck is dominant or more relevant is still not excluded.

CD28 was suggested to function in membrane translocation and lipid raft accumulation at immune synapses and support T cell responses through the recruitment of raft-localizing molecules (27–31). Shaw and colleagues (32) demonstrated that the accessory molecules, CD4 and CD28, simultaneously accumulated at immune synapses upon CD3/CD28 co-crosslinking, and phosphorylation of Lck was strongly enhanced upon this co-crosslinking.

**FIGURE 5.** TRAIL engagement with DR4 alone induced activation of NF-κB and proximal tyrosine kinases. A, An NF-κB luciferase reporter plasmid and Renilla-CMV plasmid, as an internal control, were transiently transfected into Jurkat cells. The transfected Jurkat cells were stimulated with DR4-Fc, followed by cross-linking with protein G for 12 h at 37˚C, and then the dual luciferase assays were performed. The relative luciferase values were calculated by dual luciferase assays as described by the manufacturer (Promega). Mean values ± SD of the luciferase assays from triplicate samples are shown. B, Jurkat cells were stimulated with DR4-Fc, followed by cross-linking with protein G for the indicated time periods at 37˚C. Lysates of the nuclear fraction were prepared and immunoblotted with anti–NF-κB p65 and anti-HDAC Abs. C, Jurkat cells were stimulated as described in B for 2 h at 37˚C. Cells were fixed and processed for immunofluorescence staining of an anti–NF-κB p65 Ab (green) and Hoechst33258 (blue). D, The DNA binding activity of Jurkat lysates of the nuclear fraction was analyzed using NF-κB by an EMSA. The specificity of DNA binding was determined in competition reactions using unlabeled oligonucleotides. E, Jurkat cells were stimulated with DR4-Fc, followed by cross-linking with protein G for the indicated time periods at 37˚C. Cell lysates were prepared and immunoblotted with anti–phospho-IκB, anti-IκB, and anti-actin. F, Jurkat cells were stimulated as described above, and the cell lysates were immunoblotted with anti–phospho-Lck (Tyr394), anti-Lck, anti–phospho-ZAP70 (Tyr319), anti-ZAP70, anti–phospho-LAT (Tyr200), and anti-actin Abs. *p < 0.05.
synapses and that CD28 enhanced the T cell response in cooperation with CD4 by recruiting Lck. Moreover, the accumulation of CD28-enhanced intracellular calcium flux and TCR signaling resulted in augmentation of IL-2 production and the proliferation of T cells (32). It was shown that CD28 organized the localization of PKCζ, which was translocated to the T cell–APC interface and was segregated from the TCR core within the c-SMAC for at least 4 h (33). Consistent with those results, CD28 costimulation was shown to sustain Lck activity in immune synapses by an interaction involving the C-terminal PxxP motif in the cytoplasmic domain of CD28. In CD4+ T cells, Lck is recruited to immune synapses by CD4; however, the association of CD4 with the immune synapse is transient, and in the absence of CD28, Lck activity is not sustained (32, 34). The capacity of CD28 to sustain Lck activity is a plausible mechanism for how CD28 maintains PI3K signaling and enhances TCR signaling. All of these results suggest that similar to CD28, TRAIL-induced costimulation may function in membrane translocation and lipid raft accumulation at immune synapses and supports T cell responses through recruiting raft-localizing molecules and Lck.

The integration of costimulatory receptor signaling with TCR signaling is still not clear. Although data already show that

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**FIGURE 6.** TRAIL engagement with DR4 alone was insufficient to induce T cell proliferation. A, CFSE-labeled purified primary human T cells were cultured for 5 d in 96-well flat-bottom plates precoated with immobilized Abs as indicated. CFSE dye dilution due to T cell proliferation was assessed by FACS analysis. Numbers indicate the percentage of divided cells among total T cells. Mean values ± SD of the CFSE analysis from triplicate samples are shown. Similar results were obtained with T cells purified from two other independent donors. Jurkat T cells were transfected with human TRAIL siRNA (B) or human CD28 siRNA (C). After 2 d, the knockdown expression of TRAIL or CD28 on Jurkat T cells were confirmed by immunoblotting (B) or flow cytometry (C), and then were stimulated with anti-CD3 mAb (0.5 μg/ml) with DR4-Fc (10 μg/ml) or anti-CD3 mAb (0.5 μg/ml) with anti-CD28 (5 μg/ml). Supernatants were harvested and assayed for IL-2 production by ELISA. Data are presented as the means ± SD of triplicate samples. *p < 0.05.
costimulatory signals are necessary for T cells to obtain optimal activation, cytokine production, survival, and memory generation, how these signals function still needs to be unraveled. Elucidating molecular targets of costimulation will provide new insight toward understanding the importance of these molecules in Ag-reactive T cells and may define novel targets for augmenting T cell immunity against diseases.

Our results suggest that lipid raft recruitment of Lck integrates mitogenic NF-κB-dependent signals from the TCR and TRAIL in T lymphocytes. T cell activation is initiated by binding of the TCR to the appropriate Ag presented by MHC molecules, followed by translocation of the TCR and its associated signaling molecules into lipid rafts. By inducing signaling molecules to come in close proximity, rafts serve as signaling platforms (18, 19). The Src family protein tyrosine kinase, Lck, subsequently phosphorylates the ITAMs of CD3 chains, followed by recruitment and activation of ZAP70. ZAP70 subsequently phosphorylates the transmembrane adapter protein LAT (35). When phosphorylated, LAT binds directly to the Src homology 2 domains of phospholipase Cγ, Grb2, and Gads (36), which then initiate Ca²⁺ influx and activation of PKCθ and the MAPK cascade, finally resulting in activation of transcription through NFAT, NF-κB, and AP-1 (36).

Therefore, our results support the notion that integration of the TRAIL with TCR signaling is via enhanced lipid raft recruitment of Lck, which integrates mitogenic NF-κB-dependent signals from the TCR and TRAIL in T lymphocytes. Moreover, when lipid rafts were disrupted by MβCD, T cell activation with the production of IL-2 in response to TRAIL costimulation was totally abolished (Fig. 7), indicating that intact lipid rafts are critical for TRAIL costimulation of T cells.

It remains unclear whether costimulatory receptors activate signaling pathways distinct from those triggered by the TCR complex or whether costimulatory receptors function by augmenting TCR-induced signaling events. Our present studies indicated that the TCR/CD3 complex and TRAIL had distinct roles in regulating NF-κB-dependent mitogenesis in T lymphocytes. Our results
demonstrated that TRAIL costimulation induced phosphorylation of the upstream TCR-proximal tyrosine kinases Lck and ZAP70, resulting in activation of the downstream NF-κB pathway. However, TRAIL ligation alone was unable to activate the proliferation of T cells, even though it was sufficient to induce activation of NF-κB. Furthermore, TRAIL-induced costimulation of T cells induced lipid raft assembly and recruitment of Lck and PKC0, whereas TRAIL ligation alone was insufficient to induce lipid raft recruitment of Lck and PKC0. These results demonstrate that TRAIL costimulation mediates NF-κB activation and T cell proliferation by lipid raft assembly and recruitment of Lck. Our results suggest that lipid raft recruitment of Lck integrates mitogenic NF-κB–dependent signals from the TCR and TRAIL in T lymphocytes.

In conclusion, in this study, we show that lipid raft assembly and the recruitment of Lck is required for cell proliferation and IL-2 production in T lymphocytes. Ligation of the TRAIL by its soluble receptor, DR4-Fc, alone was unable to recruit Lck into lipid rafts and sustain Lck activity and was insufficient to fully activate T cells to support T cell proliferation. These results demonstrate that TRAIL costimulation mediates NF-κB activation and T cell proliferation by lipid raft assemblies and recruitment of Lck. Targeting TRAIL-induced costimulation of T cells represents a new modality to reverse T cell hyperactivity and treatment of human autoimmune diseases.

Acknowledgments
We thank Dr. Shie-Liang Hsieh for critically reviewing the manuscript and W.-L. Wang and Y.-L. Chen for assistance. We also thank the Department of Medical Research and core laboratory of National Taiwan University Hospital for helping with confocal image analysis.

Disclosures
The authors have no financial conflicts of interest.

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