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The Cytoplasmic Domain of Fas Ligand Costimulates TCR Signals

Mingyi Sun, Kristina T. Ames, Ivy Suzuki, and Pamela J. Fink

Optimal T cell activation generally requires costimulation in addition to a signal delivered through the TCR. Although FasL is well-characterized for its capacity to deliver a death signal through Fas, this TNF family member can also transmit a reverse signal to enhance Ag-driven T cell proliferation. In this study, we describe this reverse signal through FasL as costimulation by showing it requires TCR engagement and is CD28 independent. We demonstrate that FasL-mediated costimulation drives FasL recruitment into lipid rafts and association with select Src homology 3 (SH3)-containing proteins. We further show that the proline-rich intracellular domain of FasL is sufficient to costimulate by enhancing the phosphorylation of Akt, ERK1/2, JNK, and FasL itself, by activating the transcription factors NFAT and AP-1, and by enhancing IFN-γ production. These results elucidate the pathway of costimulation through the death inducer FasL, and comprise the first mechanistic analysis of a newly emerging group of costimulators, the TNF family.

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3 Abbreviations used in this paper: TRAF, TNFR-associated factor; CKI, casein kinase I; SH3, Src homology 3; HuIG, human IgG1; KO, knockout; TM, transmembrane domain; WT, wild type; CID, chemical inducers of dimerization; CIP, calf intestinal phosphatase; HA, hemagglutinin; HPRT, hypoxanthine phosphoribosyltransferase.
surface upon activation, and the proline-rich region in the FasL cytoplasmic tail has been reported to be responsible for sorting FasL from the Golgi to secretory vesicles (42, 43). Moreover, recent studies suggest that the region between residues 23–33 in the cytoplasmic tail of FasL is both a negative regulator of FasL surface expression and a positive regulator of FasL-mediated cytotoxicity (44, 45).

In this report, we investigate the molecular basis of FasL-mediated costimulation through reverse signaling in T cells. We show that costimulation mediated by FasL requires TCR coengagement and is independent of costimulation through CD28. We also show that FasL costimulation correlates with the association of FasL with lipid rafts and select SH3-containing downstream adaptors, the phosphorylation of FasL on serine residues, the activation of Akt, ERK1/2, and JNK, the activation and nuclear translocation of transcription factors, and the induction of IFN-γ transcription and secretion. Thus, while FasL is a death-inducing ligand for Fas, it also transduces a costimulatory signal through its intracellular domain. This is the first comprehensive study of the mechanism of costimulation through a newly identified family of costimulators.

Materials and Methods

Mice

Wild-type C57BL/6 (B6.WT), B6.MRL-Fasl−/− (B6.lpr), B6 Smm.C3H-Fasl−/− (B6.gld), C57BL/6-scid/ScidJ (B6.SCID), and BALB/cJ mice were purchased from The Jackson Laboratory. C3H/HeJ and C57BL/6-Cd28−/−/−.MoAb mice (B6.CD28KO) were maintained in our animal facility using breeders obtained from The Jackson Laboratory. All mice were used at 6–9 wk of age, in accordance with the Institutional Animal Care Use Committee guidelines of University of Washington.

Reagents

Con A, cholera toxin, subunit B, and PMA were purchased from Sigma-Aldrich, and ERK1/2 inhibitor PD98059, PI3K inhibitor LY294002, ionomycin, and human IgG1 (HulGg) were obtained from Calbiochem. Anti-CD3 (145–2C11), anti-Thy-1 (G7), anti-FasL (Kay-10), and puritised mouse anti-Ly49a were purchased from BD Pharmingen. Santa Cruz Biotechnology provided anti-LAT, anti-ERK1/2, anti-phospho-ERK1/2, anti-phospho-JNK, and anti-phospho-Akt were obtained from Cell Signaling Technology, and Chemicon International supplied mouse anti-IKKβ, anti-Brg1, and anti-actin. Cappel Pharmaceuticals supplied alkaline phosphatase-conjugated goat anti-rabbit IgG. Affinity-purified goat anti-human IgG, anti-mouse IgG, and anti-hamster IgG were obtained from Rockland. FasIgG fusion protein, consisting of the extracellular domain of murine Fas joined to the hinge and constant regions of HuIgG, was purchased from R&D Systems, or used as diazylated sera from B6.SCID mice injected 5 wk previously with 1–2 × 106 NIH 3T3 transfectants. The transfectants were derived by Dr. B. Stanger and provided by Dr. A. Marshak-Rothstein (Boston University, Boston, MA).

Cell lines

Jurkat cells were cultured in RPMI 1640 with 10% FBS, 10 mM HEPES, 4 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5.5 × 10−3 M 2-ME, and 50 µg/ml gentamicin sulfate. H-2d-specific B6.WT, B6.lpr, and B6.gld CD8+ T cells were generated by coculturing responder spleen and lymph node cells with irradiated (3000 rad) C3H splenocytes every 8–10 days in RPMI 10% FBS, with the addition of 50 U/ml rIL-2 after the third stimulation. H-2d-reactive CD8+ L3 CTLs were stimulated every 8–9 days with irradiated BALB/c spleenocytes and 50 U/ml rIL-2. Stable transfectants of L3 CTL were generated by electroporation at 250 mV, 975 µF (Bio-Rad), 5 days after restimulation. Electroporated cells were selected for 2–3 wk in medium containing 0.75 mg/ml G418 (Invitrogen Life Technologies) and 25 U/ml rIL-2.

Cell stimulation

Responder T cells were seeded in plates coated overnight with 15 µg/ml goat anti-hamster IgG alone, goat anti-HuIgG, or goat anti-mouse IgG alone, or both, and after washing with PBS, for 4 h with 0.2–1.0 µg/ml suboptimal anti-CD3, and where indicated, with 5.0–10 µg/ml titered doses of anti-Ly49a. Proliferation assays

Proliferation assays

For proliferation of B6.lpr and B6.gld CTLs to mitogens in conjunction with FasL, costimulation, 2 × 104 responders were seeded in triplicate wells of 96-well plates to which appropriate mitogens were added at suboptimal concentrations. Con A was added at 0.75 µg/ml, PMA and ionomycin were used at 0.5 and 10 ng/ml, respectively, and azide-free anti-Thy-1 was added in soluble form at 5 µg/ml. Con A stimulation required the presence of filler cells that were provided by irradiated B6 splenocytes. For anti-CD3 activation with FasL, costimulation, 2 × 104 responders were seeded in triplicate wells of 96-well plates with 9.4 µg/ml plate-bound FasIgG plus 0.2 µg/ml plate-bound anti-CD3. For Thy-1 activation, 2 × 105 spleen and lymph node cells pretreated with anti-CD4 plus guinea pig complement (Invitrogen Life Technologies) were used as responders. For FasL-blocking assays, soluble FasIgG was added to cultures at 2.8–10 µg/ml on the day of Ag stimulation. HuIgG and/or B6.SCID preimmune serum were used as negative controls. For proliferation of transfected L3 CTLs, cells were added to triplicate wells with 0.2 µg/ml plate-bound anti-CD3 alone or with titered doses of plate-bound anti-Ly49a. Proliferation assays

FIGURE 1. The cytoplasmic tail of FasL is highly conserved and contains potential signaling domains and docking sites. Schematic diagram of the FasL intracellular, TM, and extracellular domains, including amino acid sequences of murine, rat, and human FasL cytoplasmic regions. Trimerization, glycosylation, and receptor-binding sites are denoted in the extracellular domain. Small dots represent spaces left to maximize sequence alignment. Proline residues of the FasL cytoplasmic tail are marked in bold, the consensus SH3-binding site is overlaid with a thick line, boxes mark the two CK1 binding motifs, and dots overlay serine, tyrosine, and threonine residues. There are no ITAM, ITIM, YXXM, or TRAF interacting motifs in the FasL tail.
was measured by \(^{[3]}\text{H}\)TdR incorporation 18 h after pulsing with 1 µCi/well on the indicated day.

**Recombinant DNA constructs**

For constructs in chemical inducers of dimerization (CID) assay, the \(\zeta\) construct was provided by Dr. D. Spencer (Baylor College of Medicine, Houston, TX). In this construct, the myristoylation-target domain (M) is linked to the murine \(\zeta\) cytoplasmic domain, three FK506-binding protein domains (FKBPs), and the HA epitope (HA), and is cloned into the eukaryotic expression vector pcDNA3 (Invitrogen Life Technologies). For CIP treatment of immunoprecipitates, samples were washed with three times lysis buffer, the phosphate group specificity of the anti-phosphoserine Ab. For CIP treatment of (CIP; New England Biolabs) treatment was included to address the phosphoserine Ab.

**Isolation of lipid rafts**

Equal numbers of L3 CTLs were lysed in 1 ml of ice-cold MBS (25 mM MES, 150 mM NaCl, 0.5% Triton X-100, protease inhibitor mixture (pH 6.5)). Lysates were homogenized 10 times through 22-gauge needles before being mixed with an equal volume of 85% sucrose (w/v) in lysis buffer, overlaid with 6 ml of 35% sucrose and 3 ml of 5% sucrose in lysis buffer with 1 mM NaCl, and centrifuged at 200,000 \(\times\) g for 16 h at 4°C. Eleven 1-ml fractions were then collected, starting at the top of the gradient. Immunoblotting confirmed that lipid rafts were recovered from the low-density gradient fractions 3–6, while cytosolic material and soluble membranes were recovered predominantly in fractions 9–11.

**ELISA**

Cells were left unstimulated or stimulated as indicated for 24 or 48 h. Cell supernatants were collected and assessed for IFN-\(\gamma\) protein levels by ELISA according to the manufacturer’s instructions (BD Pharmingen).

**Results**

**FasL costimulation requires concomitant TCR engagement**

Previous studies have demonstrated that FasL-mediated costimulation is required for CTLs to achieve maximal proliferation and expansion in vitro and in vivo (30, 32), and that the effects of costimulation are most apparent under conditions of suboptimal stimulation through the TCR (Ref. 30 and our unpublished data). Costimulation can be measured by the ability of soluble FasLG fusion protein to block proliferation, and by the ability of highly cross-linked FasLG to enhance proliferation of Ag-activated T cells. To further examine FasL costimulation in CD8\(^+\) T cells, we compared the influence of FasL cross-linking in the context of TCR-dependent stimulation by suboptimal levels of Con A and anti-CD3 (Fig. 2A), and TCR-independent stimulation by suboptimal levels of anti-Thy-1 and PMA plus ionomycin (Fig. 2B). Although both Con A and anti-Thy-1 stimulations require the expression of the TCR/CD3 complex by the responding T cells, Con A activates T cells in a TCR-dependent manner (47), whereas Thy-1 cross-linking operates via a distinct pathway (48). Both types of stimuli up-regulated FasL surface expression on CD8\(^+\) T cells (data not shown), demonstrating that TCR engagement is not an essential component of this process. However, the increase in proliferation of WT and lpr (Fas mutant) responders upon FasL cross-linking by plate-bound FasLG with a suboptimal dose of anti-CD3 was observed only with TCR-dependent stimulation (top panels, Fig. 2A), and not with TCR-independent stimulation (top panels, Fig. 2B). The heightened sensitivity to FasL cross-linking of lpr responders may be due to their enhanced FasL expression (49, 50). FasL cross-linking did not enhance proliferation of gld (FasL mutant) responders with either type of stimulus.

In line with these data, the addition of soluble FasLG to block FasL cross-linking inhibited FasL costimulation in cells activated by TCR-dependent (bottom panels, Fig. 2A), but not by TCR-independent stimuli (bottom panels, Fig. 2B). In addition, soluble FasLG blocked FasL costimulation when allotypic H-2\(^d\) splenocytes were used to stimulate naive CD8\(^+\) B6 T cells (Fig. 2C). Thus, assays of both plate-bound FasLG-mediated induction and soluble FasLG-mediated blocking of FasL costimulation demonstrate that TCR coengagement is required for the FasL-mediated 2- to 3-fold enhancement of T cell proliferation. Therefore, FasL cross-linking provides a signal that costimulates the TCR-induced proliferation of both naive and previously activated CD8\(^+\) T cells.

**Costimulation through FasL is independent of CD28 costimulation**

The relationship between costimulation through the FasL and CD28 molecules was explored by testing whether cross-linking...
FasL on CD28-deficient cells results in costimulation of Ag-driven T cell proliferation. Although CD8\(^+\) T cells from CD28 knockout (KO) mice proliferated poorly in response to allogeneic splenocytes compared with WT CD8\(^+\) T cells, this proliferative capacity was further reduced by blocking FasL costimulation with the addition of soluble FasIgG (left panel, Fig. 2C). Furthermore, the deficiency in CD28 costimulation, but not FasL costimulation, could be overcome by addition of exogenous IL-2 (right panel, Fig. 2C), consistent with previous results (51). These data demonstrate that FasL costimulation is independent of costimulation through CD28 and cannot be substituted for by the addition of exogenous IL-2.

The FasL cytoplasmic domain alone can mediate costimulation

To explore the mechanism of costimulatory signal transduction through FasL, pharmacological regulators of oligomerization were used to measure costimulation through the FasL cytoplasmic tail. In these experiments, the \(\xi\) and FasL cytoplasmic tails, lacking both TM and ligand-binding extracellular domains, were targeted to the inner leaflet of the plasma membrane of Jurkat cells through a myristoylation motif (Fig. 3A). These membrane-bound domains were then cross-linked by FK1012 or AP1510, chemical inducers that oligomerize fusion molecules containing FK506-binding domains (46, 52).
1.2–3.9 reporter to transfection control for cells incubated with medium alone was (46). Although cross-linking FasL on its own had no effect on 3
B, Jurkat cells cotransfected with combinations of CID constructs, firefly luciferase reporter (NFAT or AP-1) and Renilla luciferase reporter (pRL-TK) were subjected to CID analysis. The combinations were: empty vector alone, ε+empty vector, FasL+empty vector, and ε+FasL. Protein analysis by immunoblotting with anti-HA showed similar expression levels of constructs among all the transfectants (our unpublished data). Values are presented as the ratio of emission from the firefly reporter relative to the Renilla transfection control. The ratio of reporter to transfection control for cells incubated with medium alone was 1.2–3.9 × 10^2. Each signal was normalized to the medium control and represents the mean ± SD of three experiments. * Significant differences relative to the ε only control. Using the two-tailed Student’s t test on the data from the optimal dimerizer concentration, p = 0.0058 for NFAT and p = 0.002 for AP-1. Similar results (p = 0.00035) were obtained with an NF-κB reporter. C, Jurkat cells cotransfected with combinations of CID constructs, Renilla and IL-2 or IFN-γ firefly luciferase reporters were subjected to CID analysis. Data represent the mean ± SD of two experiments.

To assess the delivery of signals dependent on CID, Jurkat cells were cotransfected with the relevant ε and FasL CID constructs and firefly luciferase reporters of NFAT, AP-1, IFN-γ, IL-2, or NF-κB activity. Cotransfection with a Renilla luciferase reporter served as a transfection control. cross-linking ε fusion proteins generated a minor up-regulation of NFAT reporter activity (Fig. 3B), recapitulating earlier experiments establishing the CID system (46). Although cross-linking FasL on its own had no effect on reporter activity, co-cross-linking ε and FasL cytoplasmic tails generated a dimerizer-dependent synergistic up-regulation of NFAT, AP-1 (Fig. 3B), IFN-γ, IL-2 (Fig. 3C), and NF-κB (our unpublished data) activity. These results offer the first direct evidence at the molecular level that oligomerization of the FasL cytoplasmic domain is sufficient for FasL-mediated costimulation, and further emphasize the dependence of this signal on TCR cross-linking.

The FasL cytoplasmic domain mediates costimulation in nontransformed murine CD8+ T cells
To test further the capacity of the FasL cytoplasmic tail to costimulate TCR-derived signals in nontransformed murine CD8+ T cells in a less artificial system, an additional construct was generated to encode a fusion protein composed of the murine FasL cytoplasmic tail and the Ly49a TM and extracellular domains (Fig. 4A). Using an Ly49a-specific Ab, FasL cross-linking was induced in stably transfected L3 cells, an established H-2b-specific CTL line of B6 origin (53). Cross-linking the fusion protein boosted the anti-CD3-induced proliferative capacity of the transfectants in an Ab dose-dependent manner (Fig. 4B). Further increasing the dose of anti-Ly49a gradually decreased the proliferation of L3 transfectants, suggesting that the degree of cross-linking of the FasL chimeric molecules is crucial to its costimulatory capacity. Ly49a-specific Abs did not influence the anti-CD3-induced proliferation of L3 CTLs transfected with empty vector alone (Fig. 4B). These data clearly illustrate that anti-CD3 stimulated nontransformed CD8+ T cells are susceptible to costimulatory signals transduced through the FasL cytoplasmic tail of a chimeric molecule cross-linked by an extracellular ligand. Thus, we are now capable of measuring FasL-mediated costimulation through reverse signaling at both the molecular and cellular levels.

FasL-mediated costimulation recruits FasL into lipid rafts
To examine whether FasL localizes to lipid rafts upon FasL-mediated costimulation, sucrose gradient centrifugation was performed to separate lipid rafts and soluble membranes from L3 CTLs cultured with a suboptimal dose of plate-bound anti-CD3 alone or with FasIgG. Although endogenous FasL in L3 CTLs was recruited into lipid rafts upon TCR cross-linking, this recruitment from soluble to raft-associated fractions was more complete with TCR and FasL co-cross-linking (Fig. 5). Interestingly, the Src family tyrosine kinase Fyn has a similar redistribution pattern in rafts. In contrast, another Src family member, Lck, redistributed to rafts

FIGURE 3. The cytoplasmic domain of FasL is sufficient to mediate costimulation. A, Murine FasL and ε fusion constructs encode an N-terminal myristylation motif (M) that tethers the molecules to the membrane, the relevant cytoplasmic domains, FKBP3, and a C-terminal HA epitope. The addition of FK1012 or AP1510 induces hetero-oligomers of these membrane-bound proteins. B, Jurkat cells cotransfected with combinations of CID constructs, firefly luciferase reporter (NFAT or AP-1) and Renilla luciferase reporter (pRL-TK) were subjected to CID analysis. The combinations were: empty vector alone, ε+empty vector, FasL+empty vector, and ε+FasL. Protein analysis by immunoblotting with anti-HA showed similar expression levels of constructs among all the transfectants (our unpublished data). Values are presented as the ratio of emission from the firefly reporter relative to the Renilla transfection control. The ratio of reporter to transfection control for cells incubated with medium alone was 1.2–3.9 × 10^2. Each signal was normalized to the medium control and represents the mean ± SD of three experiments. * Significant differences relative to the ε only control. Using the two-tailed Student’s t test on the data from the optimal dimerizer concentration, p = 0.0058 for NFAT and p = 0.002 for AP-1. Similar results (p = 0.00035) were obtained with an NF-κB reporter. C, Jurkat cells cotransfected with combinations of CID constructs, Renilla and IL-2 or IFN-γ firefly luciferase reporters were subjected to CID analysis. Data represent the mean ± SD of two experiments.

FIGURE 4. The FasL intracellular domain costimulates TCR-induced CD8+ T cell proliferation. A, Schematic diagram of the FasL/Ly49a chimeric construct encoding murine FasL cytoplasmic and Ly49a TM and extracellular domains. B, L3 CTLs stably transfected with a construct encoding the chimeric FasL/Ly49a molecule or the empty EGFP C1 vector were cultured with 0.2 μg/ml plate-bound anti-CD3 alone or with the indicated doses of plate-bound anti-Ly49a. Proliferation was measured by [3H]TdR uptake in cultures pulsed on day 2.
were cultured with a suboptimal dose of plate-bound anti-CD3 IgG before being subjected to lipid raft isolation by sucrose gradient ultracentrifugation. Equal volumes of gradient fractions were separated by 12% SDS-PAGE and blotted with anti-FasL, anti-Fyn, anti-LAT, anti-Lck, and cholera toxin B subunit to detect respectively FasL, Fyn, LAT, Lck, and GM1 in lipid rafts. Blots with anti-LAT, anti-Lck, and cholera toxin B subunit confirmed that lipid rafts were recovered from low density gradient fractions 3–6, and cytosolic material and soluble membranes were recovered in fractions 9–11. The tabulated values represent the percentage of each protein in rafts, calculated as band densities in fractions 3–6/band densities in fractions 1–11. Data are representative of three experiments.

To investigate whether Akt is involved in FasL-mediated costimulation, Akt phosphorylation was measured after TCR and FasL co-cross-linking. Lipid rafts may thus provide a platform to which FasL is translocated to facilitate recruitment of downstream adaptors and induction of costimulatory signaling.

**FasL is phosphorylated upon FasL-mediated costimulation**

Although there is only a single tyrosine residue and no YXXM motif in the murine FasL cytoplasmic tail, this domain contains two potential CKI-binding motifs and three threonine and eight serine residues (Fig. 1). To determine whether these serine residues are phosphorylated upon FasL-mediated costimulation, the phosphoserine content was measured of FasL molecules immuno-precipitated from L3 and gld CTLs stimulated through the TCR in the presence or absence of plate-bound FasIgG. Although FasL expression levels in the two T cell populations were very similar under each condition, FasL costimulation of L3 but not gld T cells resulted in an almost 4-fold increase in phosphatase sensitive serine residues on FasL (Fig. 6A).

**Costimulation drives association of FasL with select SH3-containing proteins**

Given the SH3-binding domains found in the FasL cytoplasmic tail (Fig. 1), SH3-containing molecules were investigated to identify proteins that interact with the FasL intracellular domain to induce downstream signaling. Although FasL expression in both L3 and gld T cells was similar under each condition tested, TCR and FasL co-cross-linking led to enhanced association between WT but not gld T cells and Fyn, Grb2, and the p85 subunit of PI3K, with the p85 subunit of PI3K and activating Akt. Thus, FasL mediates costimulation may operate through the PI3K pathway, and would be expected to activate transcription factors such as AP-1 via the Ras/MAPK pathway.

To assess directly whether the Ras/MAPK pathway is involved in FasL-mediated costimulation, the phosphorylation of ERK1/2 upon TCR and FasL co-cross-linking was examined in cells cultured with a suboptimal dose of plate-bound anti-CD3 alone, plate-bound FasIgG alone, or both. The level of phosphorylated ERK1/2 was augmented 6-fold in L3 but not gld T cells stimulated with anti-CD3 plus FasIgG over that in cells stimulated with suboptimal anti-CD3 alone, after normalization against FasL (Fig. 7A). This enhancement was inhibited by LY294002. These data suggest that FasL transduces a costimulatory signal in CD8⁺ T cells by associating with the p85 subunit of PI3K and activating Akt. Thus, FasL-mediated costimulation may operate through the PI3K pathway, and would be expected to activate transcription factors such as AP-1 via the Ras/MAPK pathway.

**FasL-mediated costimulation enhances Akt, ERK1/2, and JNK phosphorylation**

To investigate whether Akt is involved in FasL-mediated costimulation, Akt phosphorylation was measured after TCR and FasL co-cross-linking. Lipid rafts may thus provide a platform to which FasL is translocated to facilitate recruitment of downstream adaptors and induction of costimulatory signaling.

**The FasL cytoplasmic domain is sufficient to activate the MAPK pathway**

To determine whether the FasL intracellular domain alone is sufficient to enhance ERK1/2 phosphorylation, we measured the effect of dimerizers on Jurkat transfectants of CID constructs. Although the level of total ERK1/2 expression in each transfectant was similar, cells transfected with constructs encoding the ζ and ζ FasL tails had 3- to 4-fold more ERK1/2 phosphorylation than cells transfected only with the construct encoding the ζ tail (Fig. 7C), indicating that FasL costimulation induces AP-1 activation indirectly through phosphorylating JNK. No p38 activation was observed (our unpublished data).
were cultured for 1 h with either medium alone, or 0.72 μg/ml plate-bound anti-CD3 alone or with 5 μg/ml plate-bound FasIgG. Lysates from 5 to 10 × 10^6 cells were immunoprecipitated overnight with anti-FasL (Kay-10). Cell lysates with or without CIP pretreatment were blotted with Ab specific for phosphoserine, and the blot was reprobed with anti-FasL (Q-10). Suppression by the ERK inhibitor PD98059 indicates that MAPK activation is essential for the induction of IFN-γ transcription and secretion upon TCR cross-linking alone or with FasL co-cross-linking (Fig. 9). Thus, costimulation through FasL augments IFN-γ production in CD8^+ T cells in a MAPK-dependent way.

**Discussion**

Our results indicate that coengagement of the TCR is necessary for FasL cross-linking to enhance proliferation, defining this reverse signaling as costimulation (Fig. 2, A and B). This requirement ensures that FasL costimulation enhances neither the proliferation of bystander T cells nor of Ag-specific T cells after Ag clearance. Because both TCR-dependent and TCR-independent means of activation can induce the expression of surface FasL (our unpublished data), this requirement for TCR coengagement by FasL costimulation is unrelated to FasL expression. Instead, after TCR and FasL co-cross-linking, downstream molecules such as Fyn, Grb2, and PI3K may be recruited to lipid rafts, along with the TCR and FasL, and thereby gain access to the FasL intracellular domain to form a scaffold for TCR and FasL cross-talk. The degree of FasL cross-linking appears to be important for delivery of a costimulatory signal, as suggested by the influence on costimulation of cross-linking Ab (Fig. 4) and dimerizer (Fig. 3) concentration. Also suggestive is the absence of positive FasL reverse signaling in the presence of soluble FasIgG (Fig. 2) or FasIgG bound directly to plates in the absence of a first stage Ab (our unpublished data and Ref. 54).

Although CD28 is widely considered the primary costimulatory receptor for initial expansion and survival of T cells, a number of costimulators can modulate the quantity, quality, or duration of immune responses independently of CD28 (55). Our data indicate that FasL is one of these molecules that induces CD28-independent costimulation (Fig. 2C). In addition, exogenous IL-2 compensates for the deficiency of CD28 but not of FasL costimulation (Fig. 2C). Similar to CD28 and ICOS, FasL can costimulate both naive and preactivated T cells, but enhances proliferation by a comparatively subtle, but consistent, 2- to 3-fold.

Using two separate approaches, we provide here the first direct evidence at the molecular level for FasL-mediated costimulation through reverse signaling. The CID system offers several advantages for assessing costimulation through FasL. It can generate higher order oligomers of the type we suspect generate the positive reverse signal through FasL. Furthermore, the absence of extracellular domains eliminates the death-inducing function of FasL and obviates the ensuing complications, thereby facilitating the direct demonstration of the synergy between TCR- and FasL-dependent signaling. Balancing these advantages is the artificiality of the system, both from cellular and molecular standpoints. In an alternative approach, the use of nontransformed murine transfectants of a construct encoding a chimeric FasL/Ly49a molecule...
solves this problem (Fig. 4). Here, the readout is cellular proliferation, presumably the sum of the numerous molecular readouts our CID assay measures. Both systems complement each other. The CID data indicate that the Fasl cytoplasmic domain is sufficient to activate the transcription factors NFAT, AP-1, and NF-κB, and to up-regulate IL-2 and IFN-γ promoter activity (Fig. 3). NFATc1 nuclear translocation and c-Jun phosphorylation upon TCR co-cross-linking with endogenous Fasl molecules in nontransformed T cells (Fig. 8) confirm these findings and link transcription factor activation to IFN-γ transcription and secretion in WT but not gld CD8+ T cells (Fig. 9 and our unpublished data). These data are consistent with a recent report that Fasl cross-linking can activate NF-κB and AP-1, although in this case, induction of IL-8 expression was the final readout (56).

Recent studies have shown that plasma membrane compartmentalization plays an essential role in T cell activation and costimulation (57–59). After cross-linking, the TCR translocates into lipid rafts that function as platforms to recruit downstream signaling molecules such as Src family kinases (60). It has been demonstrated that the related proteins TNFR1, Fas, and LIGHT localize into lipid rafts (23, 61, 62). Our data show that Fasl is similarly recruited into lipid rafts after translocation to the cell surface upon TCR and Fasl co-cross-linking (Fig. 5). Thus, rafts may help organize the downstream mediators of Fasl costimulation. Recent data also demonstrate that the localization of Fasl to lipid rafts regulates Fasl-induced killing activity (63).

One molecular signpost of reverse signaling through Fasl is the phosphorylation state of Fasl itself. Co-cross-linking Fasl and the TCR on WT but not gld T cells leads within an hour to the appearance of phosphoserine residues in Fasl, with no concomitant increase in Fasl surface expression (Fig. 6A and our unpublished data). Because the cytoplasmic domain of gld Fasl is identical with that of WT, these data also emphasize that Fas-mediated cross-linking of Fasl is the essential trigger for the phosphorylation of Fasl serine residues and the events that follow. It is unknown which specific serine residues in the Fasl cytoplasmic tail are phosphorylated, or which serine kinases phosphorylate them, although the presence of two conserved CKI sites in the cytoplasmic domain of Fasl is notable (Fig. 1). In contrast, costimulation through members of the Ig superfamily results in phosphorylation of tyrosine residues (4), suggesting that these two costimulatory pathways are distinct.

Our work identifies several potential downstream mediators of the Fasl costimulation pathway. Unlike CD28 and the TCR, Fasl does not associate with Lck, the Src family protein tyrosine kinase whose binding to the SH3 domain of CD28 leads to kinase activation and sustained signaling at the immunological synapse (64). An association between Lck and an SH3 domain-containing peptide of Fasl has been reported in PMA- and ionomycin-stimulated Jurkat cells (65), but our data are not consistent with this finding. In our hands, endogenous Fasl in normal murine CD8+ T cells interacts in an activation-dependent manner with another Src family protein tyrosine kinase, Fyn (Fig. 6B). Interestingly, Fyn but
FIGURE 8. FasL costimulation drives NFAT nuclear translocation and AP-1 activation. Cells were cultured for 6 h with either medium alone, or 0.72 μg/ml plate-bound anti-CD3 alone or with 5 μg/ml plate-bound FaslgG. The presence of NFATc1 and phospho-c-Jun in each cellular component was assessed by immunoblotting. Probing for the nuclear marker Brg1 and the cytoplasmic marker IKKβ showed no cross-contamination of nuclear and cytoplasmic extracts. The tabulated values represent the normalized ratios of band densities generated from nuclear NFATc1, phospho-c-Jun or Brg1 under each condition to that generated with medium alone. Data are representative of three experiments.

not Lck can also be recruited into rafts upon FasL costimulation (Fig. 5), suggesting that the association between FasL and Fyn may be responsible for their raft localization. Although it is unclear what role Fyn plays in FasL costimulation, we predict that it works at the interface of signals through the TCR and FasL, and likely functions to phosphorylate downstream proteins. FasL shares with CD28 an association with PI3K and Grb2 (Fig. 6A), although FasL interacts via the SH2 domain of this molecule and not, as does CD28, with the SH2 domain (4).

Akt is a known target of PI3K, and Akt activation in turn drives the activation of GSK3, further enhancing the production of IL-2 by prolonging the nuclear residency of NFAT (66). Recent studies show Akt phosphorylation is induced by cross-linking another TNF family member, CD27L (67). Our data indicate that FasL costimulation promotes the association of FasL with the p85 subunit of PI3K (Fig. 6B), and that Akt activity is enhanced by TCR and FasL co-cross-linking (Fig. 7A). How this is achieved is not clear, but we predict that FasL associates with and activates PI3K, which in turn activates Akt.

Our data identify Grb2 as an additional activation-dependent FasL-interacting protein (Fig. 6B). Grb2 is an adaptor that constitutively associates with Sos, thereby triggering the activation of Ras/MAPK pathway and the transcription of a number of proteins required for T cell activation and differentiation (68). We observe ERK1/2 activation upon FasL costimulation (Fig. 7B), similar to that observed through LIGHT costimulation in mouse T cells (23). The FasL cytoplasmic tail is sufficient for this activation (Fig. 7D), suggesting that signaling proteins such as Fyn, Grb2, and PI3K may interact with the FasL tail and indirectly activate ERK1/2. PI3K inhibition also blocks ERK1/2 activation induced by FasL costimulation (Fig. 7B), further confirming that the PI3K pathway is involved in ERK1/2 activation upon FasL costimulation. We do not observe p38 activation through FasL costimulation, although this protein apparently can be activated by CD40L, TRANCE, and TRAIL cross-linking (20, 27, 69, 70). JNK is activated through FasL costimulation (Fig. 7C), which phosphorylates c-Jun, a component of the transcriptionally active AP-1 complex. Another component of the AP-1 complex is Fos, which is activated by the MAPK pathway (71). Suppression of IFN-γ production through FasL costimulation by the ERK inhibitor suggests that the MAPK pathway plays an important role in FasL costimulation (Fig. 9). Taken together, these data suggest that FasL induces costimulation through the PI3K pathway, which leads to MAPK activation, transcription factor activation, cytokine production, and cell proliferation. However, it is still not clear what links Akt and ERK1/2 activation, and how the MAPKs mediate transcription factor activation.

The biological significance of TNF family member-mediated costimulation through reverse signaling is underexplored. Although FasL delivers a strong death signal to Fas-bearing cells, reverse signaling through FasL costimulates FasL-expressing T cells at a very modest level. Why does one single molecule play two opposite roles by sending and receiving bidirectional signals? The answer is unclear, but what we know is that a very subtle variation of immune behavior can make all the difference between health and illness (72). It is likely that bidirectional signaling through FasL-Fas interaction helps modulate the balance between life and death of the target cell and contributes to the fine tuning of the cellular and functional interactions in the immune system. The
differential control of FasL expression may regulate these distinct functions played by this complex molecule (31).

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Disclosures
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