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CTLA-4–Fas ligand functions as a *trans* signal converter protein in bridging antigen-presenting cells and T cells

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Abstract

Co-stimulator blockade and *trans* inhibitory signaling, using agents such as CTLA-4–Ig and Fas ligand (FasL) respectively have been invoked as alternative strategies for suppressing pathogenic T cells. This study describes a novel hetero-bifunctional fusion protein, CTLA-4–FasL, designed to combine within a single protein both co-stimulator blocking and *trans* inhibitory signaling potentials. A chimeric expression cassette, in which the ectodomain coding sequences for CTLA-4 and FasL were linked in-frame, was used to produce a CTLA-4–FasL fusion protein. CTLA-4–FasL binding to both B7-1/B7-2-expressing Daudi B cells and Fas-expressing Jurkat T cells was documented by immunofluorescence and flow cytometry. The capacity of CTLA-4–FasL to induce apoptosis in Jurkat targets was markedly enhanced by the addition of Daudi and other B7-1/B7-2$^+$ B cell lines, which provided a membrane platform for the otherwise soluble CTLA-4-fusion protein. Moreover, in dual-chamber experiments, Daudi cells pre-coated with CTLA-4–FasL demonstrated Jurkat inhibitory activity that was cell-contact dependent. Significantly, when used to inhibit *in vitro* cellular proliferation of peripheral blood mononuclear cells, CTLA-4–FasL was ~1000-fold more potent than the extensively characterized CTLA-4–Ig fusion protein. Furthermore, the degree of inhibition induced by CTLA-4–FasL substantially surpassed that observed for CTLA-4–Ig and a soluble FasL when used in combination. CTLA-4–FasL represents the first of a novel class of fusion proteins, designated here as '*trans* signal converter proteins', that combine *trans* signal masking and direct *trans* signaling functions.

Introduction

T cells integrate positive (1) and negative (2) inputs through a diverse set of surface receptors and associated signaling pathways. Co-stimulator receptors on T cells, when triggered *in trans* by antigen-presenting cell (APC) membrane-associated co-stimulators, enhance T cell receptor-dependent cellular proliferation and cytokine secretion. Alternatively, inhibitory receptors on T cells, when triggered *in trans* by their cognate inhibitors anchored on APC surfaces, induce T cell unresponsiveness and/or apoptosis. In devising strategies for inhibiting pathogenic T cells, investigators have targeted both co-stimulatory and inhibitory pathways, using soluble recombinant proteins directed at one or the other. However, the possibility now emerges for combining within a single recombinant protein the capacities to simultaneously interface with both pathways.

The Fas (CD95) receptor on T cell surfaces participates in activation-induced cell death (3). Fas ligand (FasL; CD95L), which binds to Fas, is a member of the tumor necrosis factor superfamily and is expressed by a variety of immune cells, including monocytes, NK cells, and activated B and T cells (4–6). FasL is a trimeric, type II membrane-associated protein (7,8) that is released as soluble FasL (sFasL) trimers from membranes by metalloproteinase cleavage (9–11).

Several observations have prompted us to devise FasL protein derivatives that can associate with cell membranes. Membrane-associated FasL induces apoptosis more effectively than does sFasL (12–16), possibly stemming from the higher functional valency expected for membrane-associated ligands, as compared to their soluble forms. Indeed, experiments with different sFasL proteins support the notion that FasL's functional valency dictates its potency. For example, whereas naturally processed, trimeric sFasL induces

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apoptosis relatively poorly, a longer recombinant sFasL variant incorporating the entire FasL ectodomain forms aggregates of trimers which display higher apoptotic activity (12,17). In fact, for non-aggregating recombinant sFasL trimers, antibody cross-linking is essential for the lysis of Fas-sensitive cells (13).

Additional potential benefits for membrane-associated FasL, as opposed to sFasL, relate to toxicity. When administered systemically, aggregated sFasL (18,19), as well as Fas-specific antibody (20), are toxic *in vivo*, causing rapid-onset liver damage. Hence, while *in vivo* loss of Fas–FasL manifests as lymphoproliferation and autoimmune disease syndromes in both mice and humans, exaggerated sFasL function is implicated in tissue destruction (21). Membrane association represents a reasonable strategy for bypassing the toxic effects of sFasL. This is supported by the finding that transfected FasL on APC surfaces is associated with less systemic toxicity, while maintaining desired FasL-mediated function (22,23).

CD28 is a co-stimulator receptor on T cells that is triggered by B7 surface proteins (B7-1/CD80 and B7-2/CD86) of APC. A second receptor for B7 proteins, cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), is a type I, disulfide-linked homodimeric glycoprotein that emerges on activated T cells and functions as an inhibitory receptor (24–26). CTLA-4–Ig, a soluble fusion protein in which the CTLA-4 ectodomain is linked to the Fc domain of IgG1, competitively blocks B7:CD28 interaction (27,28), and in so doing, suppresses T cell immunopathogenesis in animal models of alloimmune (29–33) and autoimmune (34,35) diseases. Given the soluble CTLA-4's well-documented immunoregulatory activity, it represents an attractive APC 'homing element' to be appended to sFasL derivatives.

Previously, we have demonstrated that glycosyl-phosphatidylinositol (GPI)-modified derivatives of proteins are amenable to exogenous re-incorporation into membranes and can be used for the engineering, or painting, of APC surfaces (2,36–38). However, since GPI anchors are appended to the C-termini of proteins, type II membrane proteins such as FasL, with their functional structures positioned near their C-termini, cannot be made as functional GPI derivatives. In this study, we report an alternative class of membrane-associating fusion proteins that is suited for type II membrane proteins and combines APC binding potential along with the additional capacity to send *trans* signals to interacting T cells. The model protein described here is CTLA-4–FasL (CD152–CD95L), which both associates with, and thereby masks, B7 co-stimulators on APC membranes, and simultaneously delivers FasL inhibitory signals in *trans* to T cells. The rationale for linking B7 blockade and FasL inhibition is made more compelling by findings that CTLA-4–Ig increases T cell susceptibility to Fas-dependent apoptosis and B7 counteracts FasL's apoptosis-inducing activity (39–42). The present data establish that CTLA-4–FasL's T cell inhibitory function is substantially augmented when this fusion protein is anchored on APC surfaces. Moreover, CTLA-4–FasL's inhibitory activity exceeds that of the extensively studied CTLA-4–Ig fusion protein, which has well-documented therapeutic potential.

Methods

Plasmid construction and transfection

A step-wise approach was employed to assemble an expression construct for CTLA-4–FasL. First, the cDNA sequence encoding amino acids 127–281 of human FasL (7) was amplified by PCR using 5′-ATCAAGCTTGGAGAAGCAAATAGGC-3′ and 5′-TTTTGGATCCTTAGAGCTTATATAAGCCGAA-3′ as 5′ and 3′ primers respectively. Primers were purchased from Genosys (The Woodlands, TX). The PCR product was digested with *Hin*dIII and *Bam*HI (which cut within the primers), and the resulting 480 bp DNA fragment was subcloned into the respective sites of our human Epstein–Barr virus (EBV) episomal expression vector pCEP9β (incorporating the cytomegalovirus promoter), to generate pXFasL/CEP9β. Next, the *Hin*dIII fragment of phCTLA-4:IgG1/REP7β (43), containing the coding sequences for the oncostatin M signal sequence and the ectodomain of human CTLA-4 (amino acids 1–127), was subcloned into the respective site in pXFasL/CEP9β, yielding pCTLA-4–FasL/CEP9β.

A β_2-microglobulin (β_2m)–FasL expression construct was produced in several steps. First, the human β_2m cDNA sequence was PCR-amplified from pβ_2m/REP12β. The primers used for this amplification (5′-TTGGGGTACCATGTCTCGCTCCGTGG-3′ and 5′-AAAGGATCCAAGCTTTCCATGTCTCGATCCCACTT-3′) were designed to replace the stop codon of human β_2m with a *Hin*dIII restriction site. Following DNA purification, the 360 bp β_2m PCR product was digested with *Kpn*I and *Hin*dIII, and subcloned into the respective sites of pCEP9β, yielding pβ_2mX/CEP9β. Human FasL sequence was mobilized from pXFasL/CEP9β using *Hin*dIII and *Bam*HI, and this 480 bp DNA fragment was subcloned into the respective sites of pβ_2mX/CEP9β to generate pβ_2m–FasL/CEP9β. Partially overlapping synthetic oligonucleotides (5′-AGCTTAGGTGGTGGTTCTGGTGGTGGTTCTGACTACAAGGACGACGA-3′ and 5′-AGCTACCTCCTCCAGATCCTCCTCCCTTGTCATCGTCGTCCTTGTAGT-3′), encoding the Flag epitope tag flanked by a linker sequence, $(GGGS)_2$DYKDDDDK$(GGGS)_2$, were subcloned into the *Hin*dIII site of pβ_2m–FasL/CEP9β, yielding pβ_2m–Flag–FasL/CEP9β. All constructs were confirmed by DNA sequencing.

Transfectant cell lines were generated by introducing the various EBV episomal expression constructs (within the pREP and pCEP vector backbones) into 293 cells, using Lipofectin reagent (Life Technologies, Bethesda, MD). Selection of stable transfectants was performed in D10 growth medium (*vide infra*) supplemented with 0.4 mg/ml G418 (Life Technologies). Multiple individual colonies were picked and screened for recombinant protein expression.

Cell culture

Human cell lines obtained from the ATCC (Rockville, MD) included Jurkat T cells, 293 embryonic kidney cells, and Raji and Daudi EBV-transformed B cell lines. The EBV-transformed B cell line, JY, was provided by Dr F. Chisari (Scripps Institute). All cells were maintained in humidified incubators providing 5% CO_2. RPMI 1640 and high glucose DMEM media, antibiotics, glutamine, and FBS were obtained from Biowhittaker (Bethesda, MD). Jurkat cells were cultured in RPMI 1640 supplemented with 100 μg/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine and 10% heat-inactivated FBS (together designated as R10 medium). The 293 cells were cultured in DMEM supplemented with 100 μg/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine and 10% heat-inactivated FBS (together designated as D10 medium). Conditioned media

were prepared by culturing cells for 5–6 days in Cellgro-FREE supplemented with penicillin/streptomycin and L-glutamine (Mediatech, Herndon, VA). Cellular debris was removed from cell culture supernatants by centrifugation (6 min at 400 *g*) followed by filtration through 0.2 μm sterile syringe filters. Clarified supernatants were stored at 4°C for 2 months without significant loss of activity. A commercially available solid-phase assay (MBL, Cambridge, MA) was used to quantitate sFasL in samples.

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). Proliferation assays with human PBMC were performed in 96-well U-bottom tissue culture plates. The assay medium consisted of R10 supplemented with 25 mM HEPES pH 7.4 (R10-H). Assays were performed in triplicate. Purified CTLA-4–FasL (*vide infra*), CTLA-4–Ig (43) or commercial sFasL (Alexis Biochemicals, San Diego, CA) were diluted in R10-H, and 50 μl of each diluted protein sample, along with 2×10^5 PBMC in 100 μl, was added per well. Cultures were then pre-incubated at 37°C for 30–60 min, prior to the addition of graded amounts of mitogenic anti-CD3 mAb (OKT3) in 50 μl R10-H. At 22 h and every 24 h thereafter, culture wells were pulsed with 25 μl of R10-H containing 0.5 μCi of [methyl-^{3}H]thymidine (ICN, Chicago, IL) for 18 h and then harvested onto glass fiber filters for scintillation counting.

Immunoaffinity purification

Immunoaffinity beads specific for human FasL were prepared according to a standard protocol. First, 0.1 ml of Protein A–agarose beads (Life Technologies, Bethesda, MD) was sequentially loaded with 100 μg of rabbit anti-mouse Ig and after washing, with 50 μg of the mouse anti-human FasL mAb NOK-1 (PharMingen, La Jolla, CA). After washing, antibodies were cross-linked to beads in 0.2 M sodium borate, pH 9.0, with 5 mg/ml of dimethyl pimelimidate (Pierce, Rockford, IL). Chemical cross-linking reactions were quenched with 0.2 M ethanolamine, pH 8.0, for 2 h, and the beads were washed with PBS containing 0.05% sodium azide and then stored at 4°C.

Immunoaffinity purification of CTLA-4–FasL was performed in batches. An aliquot of 0.1 ml of the FasL-specific beads was added to cell culture supernatants containing CTLA-4–FasL and rocked overnight at 4°C. Beads were collected by centrifugation and washed with 0.2 M sodium phosphate, pH 6.8. CTLA-4–FasL was batch-eluted from the beads with 0.1 M citrate, pH 2.5, and transferred into tubes containing 1 M phosphate, pH 8.0, for neutralization. Purified CTLA-4–FasL was quantitated by the commercial sFasL solid-phase assay cited above.

Gel filtration chromatography and Western blotting

For analyses of molecular size and stoichiometry, clarified cell culture supernatants containing CTLA-4–FasL were concentrated 15- to 30-fold with Centricon-10 ultrafiltration devices (Amicon, Beverly, MA). The retentate was applied to a pre-calibrated Superdex-200 (Pharmacia Biotech) column in 1×PBS, and 0.5 ml fractions were collected and analyzed using a sFasL ELISA. Fractions containing active CTLA-4–FasL were concentrated 50-fold and then co-incubated with the chemical cross-linker dithiobis(succinimidyl proprionate) (DSP; Pierce) at room temperature for 30 min, prior to quenching with 0.1 M Tris base/0.1M glycine. Samples for SDS–PAGE analysis were prepared in Laemmli sample buffer, with or without 2-mercaptoethanol (2-ME), and were boiled prior to loading onto 10% SDS–PAGE gels. Following electrophoresis, gels were blotted onto Immobilon P membranes (Millipore, Beverly, MA), blocked with 5% milk/PBS and probed with anti-human FasL antiserum (anti-C20; Santa Cruz Biologicals, Santa Cruz, CA). After extensive washing, blots were incubated with horseradish peroxidase-conjugated anti-rabbit antiserum (BioRad, Richmond, CA) and developed with enhanced chemiluminescent substrate (NEN, Boston, MA) prior to exposure to X-ray film.

Flow cytometry

Cells were pre-incubated at 37°C for 30–45 min with various dilutions of cell culture supernatants containing either β_2m–FasL or CTLA-4–FasL. These cells were then pelleted, washed twice with FACS buffer (1×PBS/0.5% BSA/0.02% sodium azide) and incubated on ice for 30–45 min with 10 μg/ml of primary anti-human FasL mAb (NOK-1; PharMingen) or anti-human CTLA-4 mAb (BNI3; PharMingen), with mouse IgG1 (Dako, Carpenteria, CA) serving as a negative control. Cells were then incubated on ice for 30–45 min with FITC-conjugated goat F(ab′)$_2$ anti-mouse IgG (Boerhinger Mannheim, Indianapolis, IN). Following washes, fluorescence-labeled cells were run on a FACScan and propidium iodide-excluding populations were gated for analysis with the CellQuest software package (Becton Dickinson, Mountain View, CA).

Cytotoxicity assays

Cytotoxicity assays were performed in triplicate as described (44), with a few modifications. Jurkat cells in exponential growth phase were labeled at 37°C for 4–5 h with 3 μCi/ml of [^{3}H]thymidine (ICN, Costa Mesa, CA) in R10 medium, pelleted and washed twice. Cells were resuspended in R10 at 2×10^5/ml, and 0.1 ml of the cell suspension was added to each well. Clarified supernatants containing CTLA-4–FasL or β_2m–FasL were diluted into R10 and 0.1 ml of each diluted sample was added to individual wells of round-bottom 96-well tissue culture plates. Anti-Flag mAb (Sigma) was added as cross-linker at 0.5 μg/ml to indicated samples. Pre-incubation of cells with defined quantities of CTLA-4–FasL or β_2m–FasL was performed on ice for 1 h, followed by centrifugation (6 min at 400 *g*) and two washes with R10. In each experiment, a medium-only control was used to determine the spontaneous loss of radiolabeled DNA. For cell–cell killing assays, 10^4 pre-treated effector cells were added per well. Cultures were incubated for 17–20 h at 37°C and radiolabeled DNA was then harvested onto glass filters for scintillation counting. In blocking experiments, Jurkat cells and supernatants to be blocked were pre-incubated with anti-Fas mAb (ZB4; Coulter Immunotech, Westbrook, ME) and anti-FasL mAb (NOK-1; PharMingen) respectively. Percent Fas-dependent specific lysis was calculated according to the equation $100\times(S - E)/S$, where S and E represent retained DNA in the absence of added protein and experimentally retained DNA in the presence of added protein respectively. Maximal killing ranged from 40 to 70%, depending on the assay.

Annexin V binding assays

Daudi cells (10^6) were pre-treated with 300 ng/ml of CTLA-4–FasL or β_2m–FasL on ice for 1 h, pelleted and washed. Pre-treated Daudi cells (5×10^5) were plated beneath semipermeable 3 μm membranes (Becton Dickinson) in 24-well tissue culture plates. Jurkat cells (10^6) were added either above or below the membrane, as indicated. After an 18 h incubation period, cells were pre-stained with CD3–FITC (Dako) and then tested for externalization of phosphatidylserine (used as a marker for early apoptosis), as detected by binding of Annexin V–phycoerythrin (PharMingen), according to the manufacturer's protocol. Propidium iodide-excluding populations of $CD3^+$ cells were gated for analysis, and Annexin V^{bright} cells were counted and plotted as percent of total $CD3^+$ cells.

Results

Expression of CTLA-4–FasL and β_2m–FasL fusion proteins

Two FasL-containing fusion proteins, CTLA-4–FasL and β_2m–FasL, were produced using chimeric coding sequences. The CTLA-4–FasL fusion protein, incorporating as its second component the ectodomain of human CTLA-4, was designed with dual functions in mind: (i) binding to, and thereby masking of, resident B7-1/B7-2 (CD80/CD86) on APC surfaces, and (ii) conferring pro-apoptotic *trans* signaling activity to APC (with the membrane-anchored FasL on APC signaling to the Fas receptor on responding T cells). β_2m–FasL was developed as a FasL-containing, control fusion protein lacking the capacity to engage B7 proteins. These two fusion proteins were produced by stably transfecting the respective chimeric coding sequences, carried in EBV episomal expression vectors, into 293 human embryonic kidney cells (see Methods). The 293 cells were chosen for producing these FasL-containing fusion proteins since this cell line has been used previously with success for the expression of functional sFasL trimers (13). Of note, a Flag epitope tag was incorporated into β_2m–FasL (in between the β_2m and FasL sequences) in order to facilitate molecular analyses and permit antibody cross-linking (with anti-Flag mAb) of this fusion protein in some experiments (*vide infra*). Secretion of the two fusion proteins from transfectant cell lines was quantitated using a FasL-specific ELISA, with levels in 5–6-day conditioned media ranging from 0.1 to 1.0 μg/ml (data not shown).

Both FasL and CTLA-4 proteins form multimers in their native forms, existing as non-covalently-associated homotrimers and disulfide-linked homodimers respectively. The multimerization status of the CTLA-4–FasL fusion protein was first evaluated via immunoblotting experiments. Conditioned media from two CTLA-4–FasL transfectant clones were processed for SDS–PAGE and immunoblotting with FasL-specific antiserum, in the presence or absence of reducing agent. In the absence of reducing agent, two prominent ~70–90 kDa molecular species, along with a weaker one at ~45 kDa, were detected (Fig. 1A). In contrast, in the presence of reducing agent, the larger bands disappeared, leaving only the ~45 kDa band. As expected, no significant FasL immunoreactivity was detected in conditioned media from a negative control, i.e. vector-only 293 cell transfectants. This finding was consistent with the presence of inter-chain disulfide bridges between ~45 kDa CTLA-4–FasL monomers.

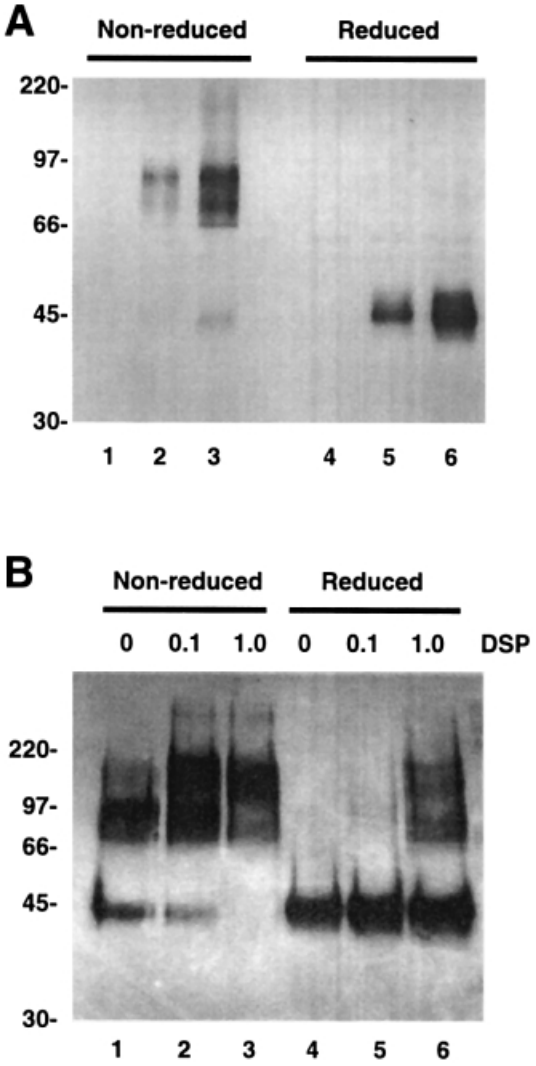

Fig. 1. CTLA-4–FasL forms trimeric complexes with interchain disulfide bridges. (A) Conditioned media from CTLA-4–FasL-expressing (lanes 2 and 3) or control (lane 1) 293 cellular transfectants were prepared for SDS–PAGE in the presence (reduced) or absence (non-reduced) of 2-ME. Immunoblots were incubated sequentially with anti-FasL antiserum (anti-C20) and goat anti-mouse IgG–horseradish peroxidase, and immunoreactivity was visualized by enhanced chemiluminescence and fluorography. Molecular weight markers (kDa) are indicated to the left of the gel. (B) CTLA-4–FasL-containing conditioned medium was pre-incubated at room temperature for 30 min with the homo-bifunctional reducible cross-linker DSP dissolved in DMSO (lanes 2, 3, 5 and 6) or DMSO alone (lanes 1 and 4). DSP concentrations in the cross-linking reactions are indicated in mM. Reactions were quenched and processed for SDS–PAGE (under non-reducing or reducing conditions as indicated) and immunoblotting as described in Methods.

To determine the molecular stoichiometry of CTLA-4–FasL complexes, proteins in transfectant cell conditioned media were cross-linked with increasing concentrations of the homo-bifunctional, reducible cross-linker DSP and analyzed by

immunoblotting using FasL-specific antiserum. A high-mol.-wt ~140–150 kDa CTLA-4–FasL molecular species appeared with DSP cross-linking, and its level increased significantly (with a concomitant reduction in the lower mol. wt species) with higher concentrations of cross-linker (in the absence of reducing agent; Fig. 1B). Partial reduction of the cross-linked (1 mM DSP) samples permitted the visualization of three molecular species of ~45, ~70–90 and ~140–150 kDa. The most straightforward interpretation for this pattern is that the CTLA-4–FasL monomers (~45 kDa) are progressively cross-linked into dimeric (~70–90 kDa) and then trimeric (~140–150 kDa) complexes (Fig. 1B). Of note, recombinant ~26–29 kDa sFasL monomers were reported to migrate as ~75 kDa trimeric complexes by gel filtration chromatography (18). These findings, together with those of Fig. 1(A), are consistent with the existence of CTLA-4–FasL trimers in which at least two of the polypeptide chains are disulfide linked. Of note, in similar chemical cross-linking experiments, β_2m–FasL monomers (~40 kDa) were found to form trimeric complexes of ~120 kDa (data not shown).

To confirm these gel-based CTLA-4–FasL multimerization findings, Superdex-200 gel filtration chromatography was performed. Peak column fractions containing FasL immunoreactivity, as determined by ELISA, eluted, as expected, at ~140–150 kDa. Chemical cross-linking of peak fractions followed by anti-FasL immunoblotting also revealed the ~70–90 and ~140–150 kDa CTLA-4–FasL complexes (data not shown). Hence, the chemical cross-linking and gel filtration analyses, taken together, suggest that CTLA-4–FasL complexes have a trimeric molecular stoichiometry, with the presence of intermolecular disulfide bridges between at least two of the component chains.

Apoptotic activity of CTLA-4–FasL versus β_2m–FasL

Functional differences between recombinant membrane-binding FasL and sFasL proteins have been observed (12,13). Apoptosis of Fas-sensitive target cells is readily detected after exposing these cells to 0.1 ng/ml of membrane-binding FasL on membrane microvesicles (13); in contrast, certain sFasL trimers have been shown to antagonize Fas receptor triggering (12). The relative capacities of CTLA-4–FasL versus β_2m–FasL to activate (or antagonize) apoptosis in the presence (or absence) of a 'membrane-anchoring' cell was determined. To this end, the transfectant supernatants were tested for their respective abilities to induce apoptosis of Fas-

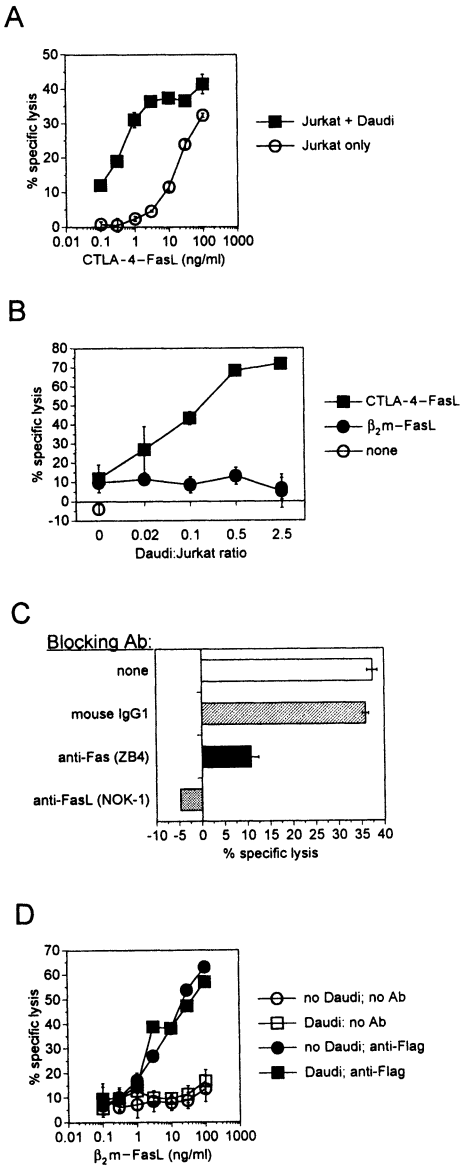

Fig. 2. CTLA-4–FasL's apoptotic activity is potentiated by the addition of B7-expressing cells. (A) Conditioned media containing defined amounts of CTLA-4–FasL, as indicated on the *x*-axis, were titrated into individual wells of 96-well U-bottom plates containing 2×10^4 [^{3}H]thymidine-labeled Jurkat cells (Jurkat only, ○). In some wells, 10^4 B7$^+$ Daudi cells were added (Jurkat + Daudi, ■). Plates were incubated for 17–20 h and harvested onto fiberglass filters for scintillation counting. Percent specific lysis is plotted on the *y*-axis. CTLA-4–FasL concentration, in ng/ml, is indicated on the *x*-axis. (B) 0.6 ng/ml CTLA-4–FasL (■), 1 ng/ml β_2m–FasL (●) or R10 medium alone (○) were added to assay wells containing ^{3}H-labeled Jurkat cells and varying numbers of Daudi cells. Plates were incubated for 17–20 h and harvested onto fiberglass filters for scintillation counting. Percent specific lysis is plotted on the *y*-axis. The ratio of Daudi to Jurkat cells is plotted on the *x*-axis. (C) For mAb blocking experiments, 10 μg/ml mAb were pre-incubated with 2 ng/ml CTLA-4–FasL proteins for 1 h at 37°C prior to the addition of radiolabeled Jurkat cells. For FasL blocking, anti-FasL (NOK-1) was used. For anti-Fas blocking, the Fas-specific blocking mAb, ZB4, was pre-incubated with [^{3}H]thymidine-labeled Jurkat cells for 1 h at 37°C and 2×10^4 pre-treated cells added per assay well. Daudi cells (10^4) were added to each assay well prior to 17–20 h culture. Cultures were harvested onto fiberglass filters for scintillation counting. For comparison, a mouse IgG1 control (Leu2a) was also included. Percent specific lysis is shown on the *x*-axis. (D) Graded amounts of β_2m–FasL, as indicated on the *x*-axis in ng/ml, were titrated into assay wells for Jurkat cell cytotoxicity assays. The presence or absence of 10^4 Daudi cells/well or 0.5 μg/ml anti-Flag antibody is indicated in the key as: cross-linking anti-Flag antibody with Daudi cells (Daudi, anti-flag; ■), anti-Flag (no Daudi, anti-Flag; ●), Daudi (Daudi, no antibody; □) and medium alone (no Daudi, no antibody; ○) respectively. Assays were performed as described in Methods. Percent specific lysis is displayed on the *y*-axis. Error bars represent SD. Similar results were obtained in at least three independent experiments.

sensitive Jurkat T cells, as measured by a radiometric DNA fragmentation assay. [^{3}H]Thymidine-labeled Jurkat cells were co-incubated with comparable amounts, as quantitated by a soluble sFasL ELISA, of CTLA-4–FasL or β_2m–FasL. CTLA-4–FasL demonstrated dose-dependent killing of Jurkat cells from 1–100 ng/ml (Fig. 2A). Importantly, the addition of Daudi cells (a Burkitt's lymphoma B cell line), which constitutively express moderate levels of B7-1 and high levels of B7-2, increased CTLA-4–FasL cytotoxic activity by ~60-fold and lowered the limit of detection of apoptosis to 0.1 ng/ml of CTLA-4–FasL. Two other EBV-transformed B cell lines, JY and Raji, both with B7 expression profiles similar to Daudi cells, also potentiated CTLA-4–FasL-mediated cytotoxicity (data not shown).

In addition, titrating the number of Daudi cells added to wells revealed a Daudi cell dose-dependent increase in Jurkat cell death (Fig. 2B). The dependence of the observed cytotoxicity upon the FasL component within the CTLA-4–FasL fusion protein was established by demonstrating that killing could be reversed with the addition of either anti-Fas or anti-FasL blocking mAb (Fig. 2C). No significant killing of Jurkat cells was detected with β_2m–FasL alone over the range of 0.1–100 ng/ml, except in the presence of a cross-linking, epitope tag (Flag)-specific mAb (Fig. 2D). Of note, similar results were obtained for both CTLA-4–FasL and β_2m–FasL when Annexin V binding assays, which measure the externalization of phosphatidylserine as an early marker of apoptosis (45), were used as a readout (data not shown). Hence, B7$^+$ cells potentiate the ability of CTLA-4–FasL to signal *in trans* through Fas receptors on Jurkat cell targets.

Dependence of CTLA-4–FasL trans effector activity upon intercellular contact

CTLA-4–Ig binds to cells expressing B7-1 and/or B7-2 (46). To test whether CTLA-4–FasL similarly binds to B7$^+$ cell surfaces, we incubated B7-1/B7-2$^+$ Daudi cells with increasing concentrations CTLA-4–FasL-containing cell supernatants and detected surface FasL expression by indirect immuno-

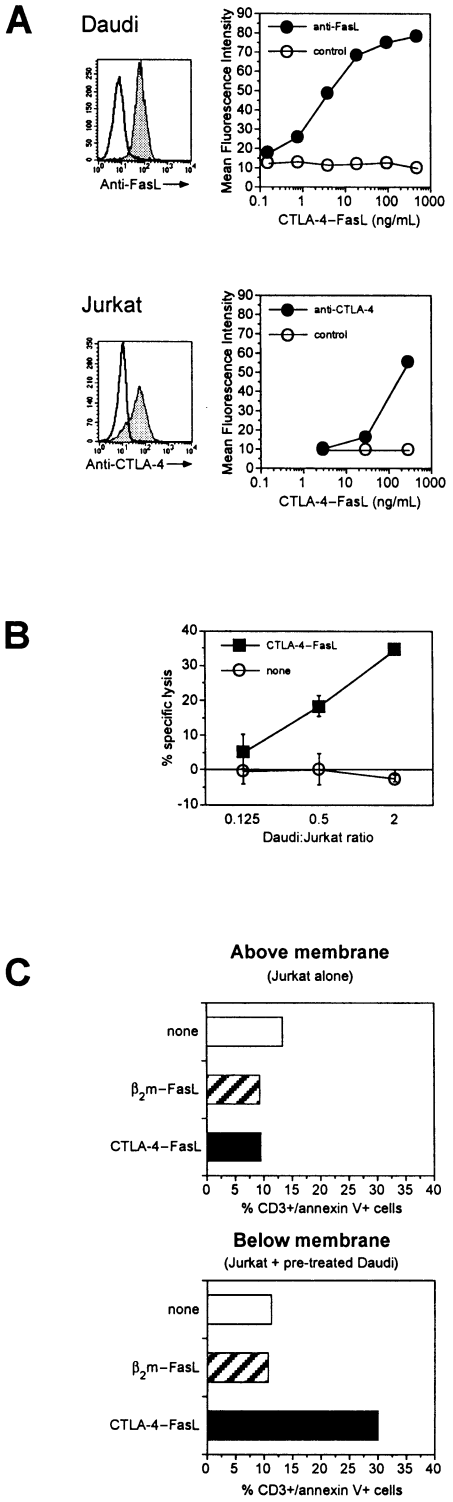

Fig. 3. CTLA-4–FasL-pre-coated cells trigger apoptosis of Fas-sensitive cells. (A) Daudi or Jurkat cells were pre-incubated with 300 ng/ml of CTLA-4–FasL at 37°C for 30–45 min, washed and immunostained for flow cytometry. In the upper panels, CTLA-4–FasL-coated, B7$^+$ Daudi cells were stained with anti-FasL mAb (●) or isotype-matched control mAb (control, ○) and FITC-conjugated goat anti-mouse IgG. In the lower panels, CTLA-4–FasL-coated Jurkat cells were immunostained with anti-CTLA-4 (●) or isotype-matched control mAb (control, ○) and FITC-conjugated goat anti-mouse IgG. The left panels display histogram plots of the fluorescence labeled cells. In the right panels, mean fluorescence intensity is plotted on the *y*-axes and the amount of CTLA-4–FasL plotted on the *x*-axes. Data acquisition and analysis were performed on a FACScan flow cytometer with CellQuest software. (B) Daudi cells were pre-coated with 300 μg/ml of CTLA-4–FasL (■) or R10 medium (○) at 37°C for 30–45 min. After washing, varying ratios of CTLA-4–FasL-coated Daudi cells (displayed on the *x*-axis as the ratio of Daudi to Jurkat cells) were plated into wells containing 2×10^4 ^{3}H-labeled Jurkat cells for apoptosis assays. After a 17–20 h co-incubation, wells were harvested onto fiberglass filters for scintillation counting. Percent specific lysis is plotted on the *y*-axis. (C) Daudi cells were pre-incubated with medium alone (open bars), 300 ng/ml of β_2m–FasL (hatched bars) or 300 ng/ml of CTLA-4–FasL (black bars), washed and 0.25×10^6 cells plated beneath 3 μm semipermeable membranes in 24-well plates. Jurkat cells (0.5×10^6) were added both above and below the membranes. After 20 h at 37°C, cells were harvested and processed for flow cytometry as described in Methods. Percentages of propidium iodide$^-$, CD3^{bright}, Annexin V^{bright} events are plotted on the *x*-axis. Data are representative of at least three independent experiments.

fluorescence and flow cytometry. A CTLA-4–FasL dose-dependent increase in the detection of cell surface-associated FasL epitopes was observed, with a plateau at ~100 ng/ml (Fig. 3A, upper panel). In parallel, we demonstrated CTLA-4–FasL also binds to Fas^+ ($B7^-$) Jurkat cells, with a dose-dependent increase in CTLA-4 epitopes upon adding 30–300 ng/ml of CTLA-4–FasL (Fig. 3A, lower panel). Hence, CTLA-4–FasL exhibits the expected binding potentials for cells expressing either B7 or Fas at their surfaces.

Building upon the capacity of CTLA-4–FasL to bind to Daudi cell surfaces, these cells were pre-coated with CTLA-4–FasL and their apoptosis-inducing potential was evaluated. As shown in Fig. 3(B), CTLA-4–FasL-coated, but not untreated, Daudi cells trigger dose-dependent Jurkat cell apoptosis. In contrast, Daudi cells pre-incubated with control β_2m–FasL did not induce apoptosis of Jurkat targets (data not shown).

Next, the cell-contact dependence of the apoptotic effector function of CTLA-4–FasL-coated Daudi cells was tested in a dual-chamber experiment. CTLA-4–FasL-coated Daudi cells were plated below semipermeable membranes in culture wells, and Jurkat target cells were then added to the same wells both above and below the semipermeable membranes. Following a 20 h co-incubation period, Jurkat cell apoptosis was monitored by flow cytometric detection of fluorochrome-conjugated Annexin V binding to apoptotic cell surfaces. Significant Jurkat cell apoptosis was observed only in the lower compartment where CTLA-4–FasL-coated Daudi effector cells and the Jurkat target cells were in direct contact on the same side of the semipermeable membrane (Fig. 3C, lower panel); the Jurkat cells present in isolation above the membrane did not demonstrate Annexin V positivity (Fig. 3C, upper panel). As expected, no Jurkat cell apoptosis was observed when either untreated or β_2m–FasL-treated Daudi cells were used as effectors. This experiment clearly shows that once bound to $B7^+$ cells, CTLA-4–FasL function is cell–cell contact dependent. Moreover, these dual-chamber experiments indicate that CTLA-4–FasL-coated cells do not shed sufficient CTLA-4–FasL protein, either through direct protein loss or microvesiculation, to induce apoptosis at a distance in Jurkat cell targets. Furthermore, the experiment rules out the possibility that the Daudi cells, once coated with CTLA-4–FasL, release some type of other soluble inhibitory factor that could act on its own.

Relative activity of purified CTLA-4–FasL versus CTLA-4–Ig against peripheral T cell responders

The immunomodulatory activity of CTLA-4–FasL was compared to that of CTLA-4–Ig and sFasL, the latter two proteins corresponding to CTLA-4–FasL's component parts. For this experiment, peripheral T cells (within PBMC) were used in place of the Jurkat T cell line, and the capacity of the various proteins in their purified forms to inhibit anti-CD3 mAb (OKT3)-induced proliferation of these T cells was assessed. As baseline data, graded doses of OKT3 mAb (from 0.1 to 1.0 ng/ml) yielded the expected increases in T cell proliferation (data not shown). CTLA-4–FasL potently inhibited this proliferative response at all time points (Fig. 4A), with complete inhibition observed at 30 ng/ml CTLA-4–FasL at an OKT3 concentration of 1 ng/ml (Fig. 4B). In contrast, the addition of equivalent amounts of CTLA-4–Ig or sFasL resulted in significantly less inhibition. At higher concentrations of sFasL, dose-dependent inhibition was detected, reaching ~43% at 1000 ng/ml (Fig. 4C). Likewise, antibody cross-linked sFasL was inhibitory at higher concentrations (~77% inhibition at 1000 ng/ml). Of note, in control assays, commercial sFasL that was maximally activated by antibody cross-linking demonstrated quantitatively comparable apoptosis-inducing activity to cell-anchored CTLA-4–FasL (data not shown).

The combination of sFasL (with and without cross-linking antibody) and CTLA-4–Ig was tested for inhibitory activity at the peak of cellular proliferation (Fig. 4C). Adding CTLA-4-4–Ig and sFasL in combination increased inhibition by up to 20% at each concentration tested, as compared to sFasL alone. Similar increases in inhibition, up to 20%, were observed for the combination of CTLA-4-4–Ig and antibody-cross-linked sFasL, as compared to antibody-cross-linked sFasL alone. These limited increases are consistent with additive effects when CTLA-4–Ig and sFasL are combined.

The 50% inhibitory concentration (IC_{50}) for CTLA-4–FasL was compared to that of CTLA-4–Ig, sFasL, antibody-cross-linked sFasL, and the combination of CTLA-4–Ig with sFasL (or antibody-cross-linked FasL). The IC_{50} versus proliferation for CTLA-4–FasL (~45 kDa) was ~6 ng/ml, which on a molar basis translates into ~130 pM. By contrast, the IC_{50}s for CTLA-4–Ig (40 kDa) or sFasL (35 kDa) were calculated as ~130 and ~29 nM respectively. Therefore, CTLA-4–FasL demonstrates potencies ~1000- and ~220-fold greater than CTLA-4–Ig and sFasL respectively. Antibody-cross-linking of sFasL increased inhibitory potency and reduced the IC_{50} of sFasL to ~5.7 nM (~5-fold).

Combining CTLA-4–Ig and sFasL lowered the doses required for inhibition, regardless of the presence of cross-linking antibodies. In calculating IC_{50}s for these combinations, the IC_{50} of sFasL was determined in the presence of a maximal dose of CTLA-4–Ig (10 μg/ml). The inhibition curves take into consideration the baseline offset observed in the presence of 10 μg/ml CTLA-4–Ig. IC_{50}s (molar calculations based on the mol. wt of sFasL) for CTLA-4–Ig plus sFasL and for CTLA-4–Ig plus antibody-cross-linked sFasL were ~26 and ~2.9 nM respectively. Taken together, findings indicate that CTLA-4–FasL's inhibitory capacity substantially exceeds that of CTLA-4–Ig and sFasL, whether used alone or in combination.

Discussion

In this report, we describe CTLA-4–FasL as a novel fusion protein that physically and functionally bridges APC and T cells. The CTLA-4 component targets this fusion protein to APC surfaces and endows it with CTLA-4–Ig's well-documented potential to block B7 co-stimulation. However, unlike CTLA-4–Ig, CTLA-4–FasL incorporates a second functional component, FasL, that confers to it *trans* signaling (apoptosis-inducing) potential. Thus, within CTLA-4–FasL, a solubilized co-stimulator receptor capable of blocking co-stimulation of T cells has been combined with a soluble derivative of a protein capable of inducing T cell apoptosis. Our data indicate that the CTLA-4–FasL fusion protein offers functional features that surpass those of its CTLA-4 and FasL components,

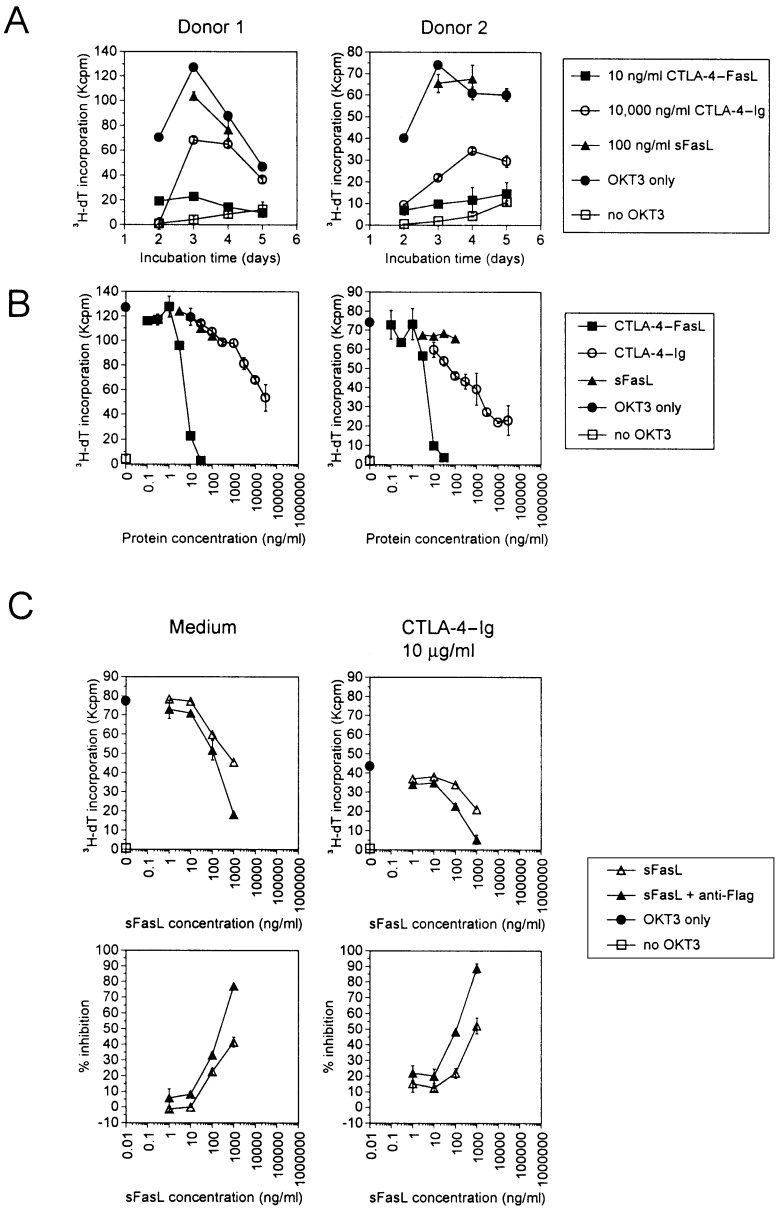

Fig. 4. CTLA-4–FasL inhibits proliferation of human peripheral blood T cells. (A) In 96-well U-bottom plates, varying amounts of proteins were titrated into assay wells. PBMC (2×10^5/well) were added and incubated for 30–60 min. Mitogenic OKT3 was added to a final concentration of 1 ng/ml, and the plates incubated for various times. At 22 h in culture, and subsequently, every 24 h up to 118 h total incubation time, a 22 h [^{3}H]thymidine pulse was performed prior to harvesting for scintillation counting. One concentration for each protein was plotted to simplify comparison. Proteins plotted include 10 ng/ml CTLA-4–FasL (■), 10 μg/ml CTLA-4–Ig (○) and 100 ng/ml sFasL (▲). Controls with or without OKT3 are represented in the figure by the filled circles and the open squares respectively. (B) Effects of CTLA-4–FasL (■), CTLA-4–Ig (○) and sFasL (▲) in ng/ml, at the peak of PBMC T cell proliferation to OKT3 stimulation (day 3), are shown for the same donors displayed in (A). OKT3 alone (□) and no OKT3 (●) are plotted on the *y*-axis. Plates were harvested for scintillation counting. (C) Effects of sFasL (△) and anti-Flag cross-linked sFasL (▲) alone (left panel) or in addition to 10 μg/ml of CTLA-4–Ig (right panel) at the peak of PBMC T cell proliferation on OKT3 stimulation (day 3) are shown. OKT3 alone (●) and no OKT3 (□) are plotted on the *y*-axis. The percent inhibition by sFasL of OKT3 stimulation (lower left) and the percent inhibition by sFasL of OKT3 stimulation in the presence of 10 μg/ml CTLA-4–Ig (lower right) are plotted. Incorporation of [^{3}H]thymidine and percent inhibition are shown on the *y*-axes of the upper and lower panels respectively. Results are representative of nine similar experiments with six individual donors.

whether used as immunomodulators in isolation or in combination.

The major findings of this study include the following: (i) Like native sFasL, CTLA-4–FasL forms functional trimers, with evidence in this case for disulfide bridging between at least two of the component polypeptide chains. This latter feature mirrors the disulfide bridging within native CTLA-4 homodimers. (ii) CTLA-4–FasL's capacity to induce apoptosis in

Jurkat T cell targets is substantially potentiated by the addition of $B7^+$ cells that serve to anchor the fusion protein to APC membranes. (iii) The apoptosis observed in this system is dependent upon the FasL:Fas molecular axis, as shown by antibody blocking. (iv) CTLA-4–FasL binding to both $B7^+$ APC and Fas^+ T cells can be readily demonstrated by immunostaining. (v) APC pre-coated with CTLA-4–FasL induce apoptosis of Jurkat T cell targets in a cell-contact-dependent fashion. (vi) Immunoaffinity-purified CTLA-4–FasL potently inhibits the proliferative response of native T cells (within PBMC pools) triggered via their TCR complexes, with the degree of inhibition substantially surpassing that for CTLA-4–Ig or sFasL when used alone or combinatorially.

Membrane anchorage potentiates CTLA-4–FasL's apoptosis-inducing activity. This was directly demonstrated in experiments where $B7^+$ anchoring cells were either titrated into the killing assay or were pre-coated with the fusion protein prior to the addition of target cells. The membrane anchorage effect likely reflects an augmentation of functional valency. Furthermore, the data and interpretation are consistent with the literature comparing the functional activities of membrane-associated versus sFasL. For example, whereas 0.1 ng of neuro2A cell membrane preparations containing membrane-associated FasL readily kill Jurkat cells (13), 10- to 100-fold more recombinant, sFasL molecular aggregates derived from COS cell transfectants are required to trigger significant Fas-dependent cell death of mouse cell lines (17,47). CTLA-4–FasL's activity level contrasts with that of β_2m–FasL, which does not detectably bind to APC surfaces and displays no significant cytocidal effects in the absence of cross-linking antibody. The enhancement of β_2m–FasL function with epitope tag-specific cross-linking antibody parallels that documented for other recombinant sFasL trimers (13).

As an immunomodulator, CTLA-4–FasL combines CD28 antagonism with Fas receptor agonism. This particular combination was motivated by data pointing to an antagonistic relationship between the CD28 and Fas pathways. CD28 signaling inhibits Fas-dependent apoptosis (48), possibly due to up-regulation of Bcl-x_L (48,49). Furthermore, CD28 co-stimulator blockade inhibits survival pathway signaling (49) and thereby promotes the Fas apoptosis pathway (39-42). One group has suggested that co-stimulator-induced cytoprotection from Fas-mediated apoptosis stems from FLIP/I-FLICE/FLAME-1/CASH/casper-mediated inhibition of death cascade signaling (50); however, this conclusion was not supported in another study (51). In recent experiments, we have demonstrated B7-1:FasL antagonism by combining B7-1 and FasL protein transfer (A. Chen and M. Tykocinski, unpublished observations). That said, the precise extent to which B7 blockade contributes to CTLA-4–FasL's observed activity remains unclear and will require more complicated experimental approaches.

The onset of Fas-mediated, activation-induced cell death in peripheral human T cells, *in vitro*, occurs ~4–5 days post-activation (52). Hence, the observation that CTLA-4–FasL significantly inhibits T cell proliferation as early as culture days 2–3 is unexpected. There would appear to be a previously unappreciated kinetic difference between Fas-mediated apoptosis induction versus proliferative inhibition. Since the Fas receptor is up-regulated within 24 h of T cell activation (52,53 and data not shown), it is certainly available to mediate CTLA-4–FasL-driven effects at the 2–3 day time period. There are a variety of downstream intracellular mediators linked to Fas receptor triggering that could potentially explain our observed early effect on T cell proliferation. Additionally, it should be pointed out that a blockade in cell division, regardless of the mechanisms, is likely to impact other T cell functions, such as cytokine secretion. Of note in this regard, secretion of some (e.g. IL-2) but not all (e.g. IFN-γ) cytokines requires the T cells to undergo multiple rounds of replication (54). Hence, it will be important to determine if CTLA-4–FasL differentially regulates the secretion of various cytokines during T cell activation.

FasL is a type II membrane protein, i.e. its functional C-terminus projects outward from the membrane (55). Previously, a variety of heterologous sequences have been appended to type II membrane proteins for purposes such as directing secretion, influencing multimerization and appending epitope tags (17,56–59). In the present study, we report a unique type of type II membrane protein derivative, in which the N-terminal intracellular and transmembrane domains of this protein have been replaced with a functional domain that anchors it to cell surfaces. In the case of the CTLA-4–FasL fusion protein developed here, the CTLA-4 anchoring domain, derived from a type I membrane protein, confers an added functional feature, i.e. *trans* signal masking. An elegant aspect of CTLA-4–FasL's design is that this chimeric soluble type I–type II protein positions the type II component at the C-terminus, projecting this business end of the molecule away from the membrane following membrane association.

The observation that CTLA-4–FasL results in apoptosis of $B7\text{-}1^-/B7\text{-}2^-$ Jurkat cells was unexpected, given that another trimeric sFasL derivative has been reported to function as an antagonist (11). One explanation for this finding revolves around the structure of CTLA-4–FasL. Based upon our gel analyses, each CTLA-4–FasL trimer likely consists of two disulfide-linked CTLA-4 domains and an unpaired, 'free', CTLA-4 domain. Hence, in principle, interactions between the free CTLA-4 domains of two CTLA-4–FasL trimers could occur. Our chemical cross-linking data indicate soluble CTLA-4–FasL predominates in trimeric complexes, it is unlikely that free CTLA-4 domains dimerize readily in solution. However, the fact that soluble CTLA-4–FasL kills Jurkat cells suggests the possible dimerization of unpaired CTLA-4 domain units within cell surface-bound CTLA-4–FasL trimers. In principle, this would yield CTLA-4–FasL 'hexamers' with proapoptotic activity analogous to that of antibody-cross-linked sFasL hexamers. In support of this hypothesis, both CTLA-4–FasL and antibody-cross-linked β_2m–FasL display similar dose–response curves for Jurkat cell apoptosis (Fig. 1). This CTLA-4–FasL hexamerization hypothesis will require further testing.

Targeting of pathogenic tissues with soluble, Fas-specific agents reveals a significant toxicity issue *in vivo*. Intraperitoneal or i.v. administration of either Fas-specific antibody (20) or recombinant sFasL (18,19) result in fulminant hepatitis, intrahepatic hemorrhage and rapid death. In principle, such *in vivo* toxicity might be reduced by directing the Fas triggering agents to the cell surfaces of cells within immune organs. However, it should be noted that Kupffer cells are $B7^+$ and,

hence, adjoining sinusoidal hepatocytes could, in principle, be susceptible to apoptosis induction by CTLA-4–FasL. However, if this proved to be a problem, therapeutic windows might be exploited, or alternatively, fusion proteins incorporating different co-stimulator receptors could be designed that bypass resident APC. Of note, our preliminary studies have shown no evidence for *in vivo* lethality or hepatotoxicity of CTLA-4–FasL (J. Huang and M. Tykocinski, unpublished observations).

In a mouse transplantation model, APC transduced with an adenovirus vector expressing recombinant FasL effected short-term elimination of alloreactive T cells with little hepatotoxicity. However, the alloreactive T cell depletion achieved by this *ex vivo* cell engineering method was incomplete, with the eventual repopulation of pathogenic T cells with time. The '*in vivo* cell engineering' made possible by CTLA-4–FasL could potentially yield more effective elimination of pathogenic T cells, while sharing in the lower toxicity of cell-based approaches.

By definition, hetero-bifunctional fusion proteins of the '*trans* signal converter protein (TSCP)' class serve to block one *trans* signal and simultaneously confer another. In the case of CTLA-4–FasL, APC are being targeted, and the B7-1 co-stimulatory signal is being replaced with a FasL apoptotic signal. However, multiple permutations can be envisioned for TSCP, with alternative designs drawing upon other molecular pairs and cellular effectors and targets. Thus, it should be possible not only to tap into other T cell co-stimulator:receptor combinations, but also to move beyond T cell inhibition altogether, targeting other regulatory endpoints of potential therapeutic benefit.

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Abbreviations

2-ME	2-mercaptoethanol
β_2m	β_2-microglobulin
APC	antigen-presenting cell
B7-1	CD80
B7-2	CD86
DSP	dithiobis(succinimidyl proprionate)
EBV	Epstein–Barr virus
FasL	Fas (CD95) ligand
GPI	glycosyl-phosphatidylinositol
PBMC	peripheral blood mononuclear cells
sFasL	soluble FasL
TSCP	*trans* signal converter protein

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