CTLA-4 · FasL Induces Early Apoptosis of Activated T Cells by Interfering with Anti-Apoptotic Signals[1]

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The fusion protein CTLA-4 · FasL, a paradigmatic "*trans* signal converter protein", can attach to APC surfaces and in effect convert B7-activating costimulator signals into inhibitory Fas receptor-generated signals. The present study investigates CTLA-4 · FasL's mechanism of action. A combination of $p27^{kip}$ and proliferating cell nuclear Ag Western blot and propidium iodide flow cytometric analysis showed no CTLA-4 · FasL effect on cell cycle entry and progression, pointing away from the kind of classical anergy associated with CTLA-4 · Ig. Significantly, CTLA-4 · FasL elicited apoptosis (as detected by annexin-V/propidium iodide costaining) as early as 24 h after T cell activation, suggesting that some coordinate signaling might be capacitating the Fas receptor. Significantly, CTLA-4 · FasL, but not CTLA-4 · Ig, anti-Fas mAb, or the two in combination, abrogated the usual increase in expression of the anti-apototic protein, cFLIP. Furthermore, activation of caspases 8 and 3 were not affected by CTLA-4 · FasL. These findings suggest a model for CTLA-4 · FasL action wherein there is coordinate triggering of a death receptor and suppression of a proapoptotic protein. *The Journal of Immunology,* **2007, 179: 7287–7294.**

There is an expanding palette of fusion proteins with immunotherapeutic potential. Fusion protein derivatives have been designed for a variety of functional endpoints, including targeted delivery of functional moieties (e.g., ligands, toxins, Ags) (1–4), bridging cells and matrices (5), coordinate signaling (e.g., coupled cytokines) (6), and intercellular signal blockade (7–12). In some instances, the fusion protein derivatives achieve more than one of these endpoints, creating new functional possibilities.

One interesting class of fusion proteins are "*trans* signal converter proteins" (TSCP).[3] TSCPs substitute one intercellular signal for another, and in essence, combine the "intercellular signal blockade" and "targeted delivery of functional moiety" endpoints. The paradigmatic TSCP we first configured is CTLA-4 · FasL (7, 13, 14). This particular T cell-directed TSCP builds upon the costimulation blockade potential of soluble derivatives of CTLA-4 (which can bind and block APC-anchored B7 costimulators) (15) and the inhibitory signaling capacity of soluble derivatives of FasL (CD95L) (which can bind and trigger inhibitory Fas receptors (CD95) on activated T cells) (16). Not only is CTLA-4 · FasL effecting a B7-to-FasL intercellular signal conversion, but also, by anchoring to $B7^+$ APC surface membranes, it is in effect generating $FasL^+$ deletional APC, or "artificial veto cells" (17). Genetically engineered APC expressing FasL can clonally delete activated Fas-bearing T cells that recognize cognate Ags on these APC (18).

A series of studies, from our group (7, 13, 14) and others (19, 20), have by now documented CTLA-4 · FasL's effectiveness both in vitro and in vivo. This TSCP demonstrates remarkably higher potency than either CTLA-4 · Ig or soluble FasL, alone or in combination, in blocking T cell proliferation, and inducing death of proliferating T cells in vitro (7, 13). CTLA-4 · FasL inhibits primary MLR, induces alloantigen-specific hyporesponsiveness ex vivo (13), and modulates the in vivo response of adoptively transferred allogeneic splenocytes (14). There is now evidence that CTLA-4 · FasL delays rejection of cardiac grafts (19) and alleviates autoimmune diabetes (20).

As it turns out, each of CTLA-4 · FasL's component parts, CTLA-4 and FasL, are more functionally complex than originally appreciated. The most intensively studied soluble CTLA-4 derivative is CTLA-4 · Ig, a costimulator blocker that has even reached the clinic (21, 22). Yet, although it has been studied extensively in an array of in vitro and in vivo experimental systems, its mechanisms of action remain unclear, with a more complex picture of its functional potentials steadily emerging. Costimulation is required for complete T cell activation (23) and cell cycle progression postactivation (24, 25). CTLA-4 · Ig, via its capacity to block B7 costimulators, causes T cell anergy (24). However, this fusion protein also induces cell death (26), and at high concentrations, sensitizes reactive T cells to apoptosis by preventing bcl-X_L and bcl-2 anti-apoptotic signals (26, 27). In addition, there is now evidence that CTLA-4 · Ig has immunomodulatory activities that tie into the promotion of inhibitory APC and regulatory cells (28–30).

Functional complexity also extends to CTLA-4 · FasL's FasL component. Although Fas is mostly thought of as a death receptor, capable of inducing apoptosis of activated T cells via the caspase cascade (16), this receptor has also been implicated as an enabler of effective T cell activation (31–33). This type of functional pleiotropism must be accounted for in dynamic models of T cell

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[3] Abbreviations used in this paper: TSCP, *trans* signal converter proteins; PI, propidium iodide; PCNA, proliferating cell nuclear Ag; cFLIPs, cFLIP short.

activation and regulation, and must ultimately be factored into any complete description of FasL-containing fusion proteins.

With the diverse functional possibilities for the CTLA-4 and FasL components as a backdrop, the present study delves further into the mechanisms underlying CTLA-4 · FasL's inhibitory action. Most significantly, we uncover this fusion protein's capacity to promote early T cell apoptosis, and offer an explanation for this effect that is grounded in coordinate triggering of a death receptor (Fas) and abrogation of activation-driven induction of a pivotal anti-apoptotic protein (cFLIP). These findings position CTLA-4 · FasL as a unique immunoregulatory agent, fundamentally different from its related soluble CTLA-4 derivative, anergy-inducing CTLA-4 · Ig.

Materials and Methods

his$_6$CTLA-4 · FasL fusion protein

A hexahistidine-tagged derivative of CTLA-4 · FasL (his$_6$CTLA-4 · FasL), with the tag appended to the amino terminus, was prepared as described (7, 13).

Mice

Eight- to 10-wk-old female C57BL/6 mice were purchased from the Harlan Animals Farm. The animals were maintained in a pathogen-free animal facility at the Hadassah-Hebrew University Medical School, Jerusalem, Israel. Animals were treated according to the standards of the Animal Ethics Committee, Hebrew University Medical School Animal Care Facilities.

Abs and reagents

Goat anti-Syrian and anti-Armenian hamster IgG (H+L) were obtained from Jackson ImmunoResearch Laboratories. Anti-mouse CD28 mAb, anti-mouse CD3 mAb (OKT3), soluble anti-mouse CD3ε, and CTLA-4 · Ig (CTLA-4/Fc) were purchased from R&D Systems. Agonistic anti-mouse Fas receptor (Jo2) and its matching control were obtained from BD Pharmingen. For Western blot analysis, anti-β actin Ab and anti-mouse GAPDH Ab were purchased from Sigma-Aldrich and Chemicon International, respectively. Anti-PCNA mAb and anti p27^{kip1} mAb were purchased from Zymed Laboratories and Santa Cruz Biotechnology, respectively.

Proliferation assays

Splenocytes were prepared as described previously (14). All experiments were performed in mR-10, which consists of RPMI 1640, supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate (Biological Industries), and 14.3 mM 2-ME (Sigma-Aldrich). Two $\times$ 10^5 splenocytes were plated in 96-well round-bottom tissue culture plates in a total volume of 200 μl/well. Medium or his$_6$CTLA-4 · FasL at different concentrations was added 20 min before the addition of different concentrations of agonist anti-CD3 mAb. For proliferation assays, cells were pulsed with 25 μl of mR-10 containing 0.5 μCi of [^{3}H]thymidine (PerkinElmer) for 18 h and then harvested onto glass fiber filters for scintillation counting. Wells were prepared in triplicate.

For some experiments, T cells were enriched from splenocytes by negative selection using SpinSep enrichment mixture (StemCell Technologies), and activated using plate-bound anti-murine CD3 and CD28 mAb. These plates were prepared by incubating 2 μg/ml goat anti-Syrian hamster and 5 μg/ml anti-Armenian hamster IgG for 2 h in borate buffer in 96-well flat-bottom plates. Plates were washed three times with HBSS, and 2 μg/ml hamster anti-murine CD28 mAb and 5 μg/ml hamster anti-murine CD3 mAb were added. Plates were left overnight at 4°C. Before T cell addition, plates were washed three times with HBSS. Stimulation was performed in the presence or absence of his$_6$CTLA-4 · FasL. Assays were performed in triplicate, and wells were pulsed with 25 μl mR-10 containing 0.5 μCi of [^{3}H]thymidine for 18 h and then harvested onto glass fiber filters for scintillation counting. Where indicated, splenocytes or purified T cells were alternatively stimulated with 40 ng/ml PMA and 200 ng/ml ionomycin (Sigma-Aldrich).

Whole cell lysates and Western blotting analysis

Splenocytes (20 $\times$ 10^6) suspended in mR-10 were stimulated with 0.5 μg/ml anti-CD3 mAb, in the presence or absence of different concentrations of his$_6$CTLA-4 · FasL, in six-well plates. After 24 h, splenocytes were collected, washed twice with ice-cold PBS, and lysed in lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 20–30 min on ice. The protein concentration of whole cell lysates was determined using the Bio-Rad Protein Assay Kit (Bio-Rad), according to the manufacturer's protocol. The lysates were mixed 1/2 with Laemmli sample buffer (Bio-Rad), heated for 5 min at 95°C, and equal amounts of protein were loaded onto 8 or 12% SDS-PAGE. Following electrophoresis, gels were blotted onto nitrocellulose membranes (Schleicher & Schuell), blocked with 5% milk/PBS, and probed overnight with primary Ab. After extensive washing, blots were incubated with HRP-conjugated (Bio-Rad) matching secondary Ab, and developed with enhanced chemiluminescent substrate (Sigma-Aldrich) before exposure to x-ray film. Films were scanned and quantified by ImageMaster VDS-CL (Amersham Pharmacia Biotech). Either anti-β actin mAb or anti-GAPDH mAb were used as control.

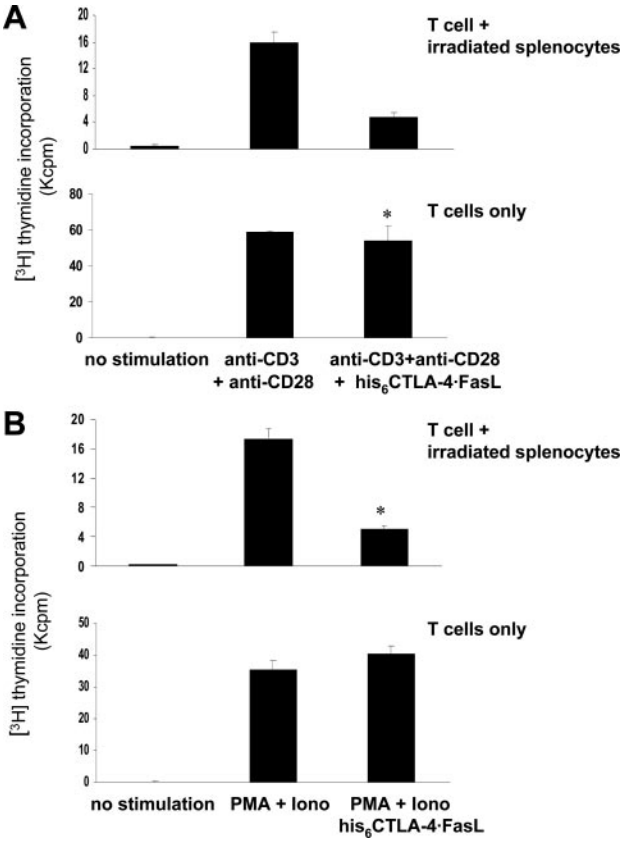

FIGURE 1. his$_6$CTLA-4 · FasL activity is APC-dependent. *A*, Purified T cells (2 $\times$ 10^5 cells/well), with (*upper panel*) or without (*lower panel*) APC (irradiated splenocytes, 2 $\times$ 10^5 cells/well), were stimulated with plate-bound anti-CD28 mAb (2 μg/ml) and anti-CD3 mAb (5 μg/ml), in the presence or absence of 10 ng/ml his$_6$CTLA-4 · FasL. *B*, Purified T cells (2 $\times$ 10^5 cells/well), with (*upper panel*) or without (*lower panel*) APC (irradiated splenocytes, 2 $\times$ 10^5 cells/well), were stimulated with 40 ng/ml PMA plus 200 ng/ml ionomycin, in the presence or absence of 10 ng/ml his$_6$CTLA-4 · FasL. In all assays, cultures were pulsed with [^{3}H]thymidine for 18 h before harvesting. Cells were harvested 72 h after stimulation and tested for [^{3}H]thymidine incorporation. Experiments were performed in triplicates. Error bars represent SE. *, $p < 0.01$ vs anti-CD3.

Flow cytometry

For cell cycle analysis, splenocytes (20 $\times$ 10^6) were stimulated with 1 μg/ml anti-CD3 mAb, in the presence or absence of 30 ng/ml his$_6$CTLA-4 · FasL in six-well plates for 24–96 h. The cells were then collected, washed with cold PBS, and fixed with 95% ethanol overnight at 20°C. On the following day, splenocytes were washed twice with FACS buffer (PBS supplemented with 0.5% BSA and 0.01% sodium azide), and incubated on ice for 30–45 min with allophycocyanin-conjugated anti-CD4 mAb, anti-CD8 mAb, or their matching controls. Before flow cytometry, splenocytes were resuspended in PBS containing 5 mg/ml propidium iodide (PI) and 10

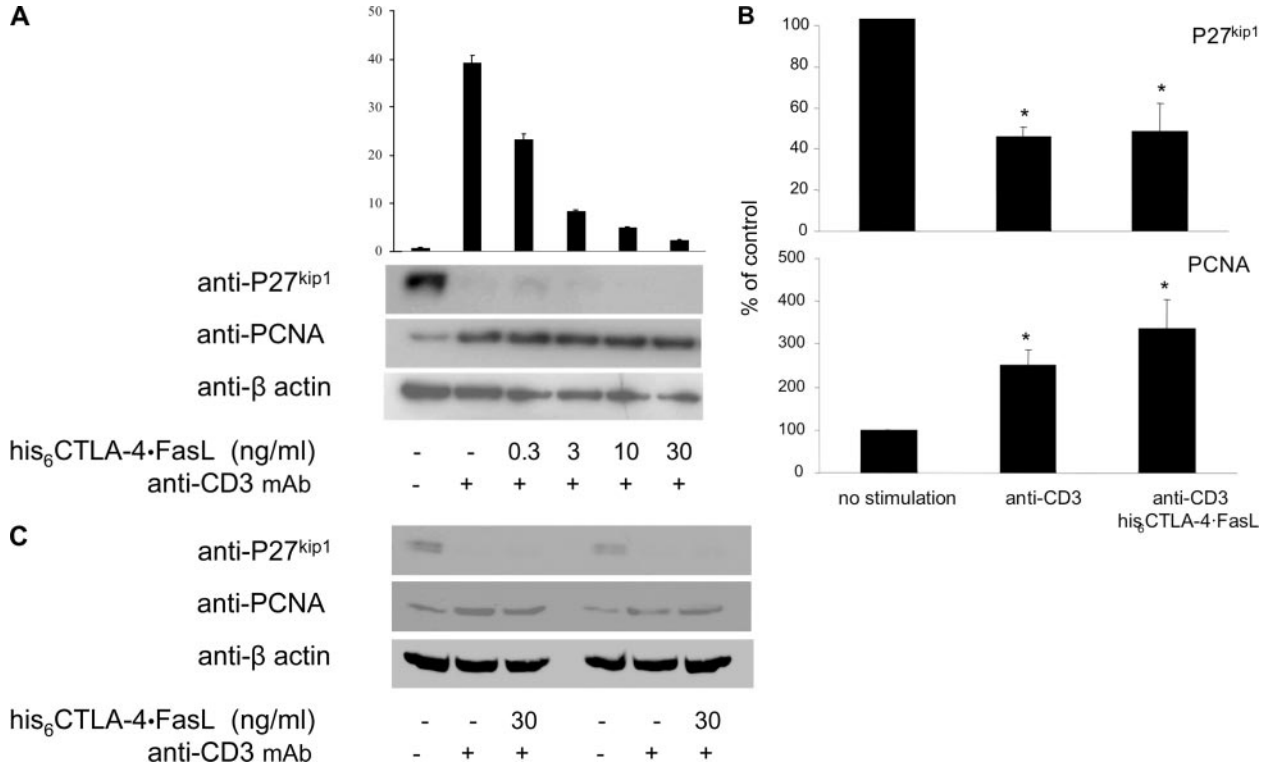

FIGURE 2. his$_6$CTLA-4 · FasL does not reduce cell cycle markers in activated T cells. *A*, Splenocytes were stimulated with anti-CD3 mAb (0.5 μg/ml), in the presence or absence of various doses of his$_6$CTLA-4 · FasL (as indicated), for 24 h. Samples were loaded on 12% SDS-PAGE, and immunoblots were incubated with the indicated Ab (*lower panel*). Proliferation, as detected by [^{3}H]thymidine incorporation, was determined in parallel to assure functionality of the fusion protein and establish dose-responsiveness (*upper panel*). *B*, Splenocytes were stimulated with anti-CD3 mAb (0.5 μg/ml), in the presence or absence of 30 ng/ml of his$_6$CTLA-4 · FasL for 24 h. Lysates were analyzed by Western blot analysis, using anti-p27^{kip1} mAb (*upper panel*) or anti-PCNA mAb (*lower panel*) as detecting Ab and then reblotted with anti-β actin mAb. In each experiment, bands were normalized against actin, and the value obtained from nonstimulated splenocytes (control) was designated as 100%, with values obtained from experimental groups calculated as percentage of control. These results summarize five independent experiments. Error bars represent SE. *, $p < 0.05$ vs nonstimulated. *C*, Splenocytes were stimulated with anti-CD3 mAb (1 μg/ml), in the presence or absence of 30 ng/ml of his$_6$CTLA-4 · FasL, for 24 h. Lysates were analyzed by Western blot analysis, using anti-p27^{kip1} mAb (*upper panel*) or anti-PCNA mAb (*lower panel*) as detecting Ab. This is a representative experiment from five independent experiments.

μg/ml RNase A (Sigma-Aldrich). Fluorescence-labeled cells were analyzed on a FACScan, using a CellQuest software package (Becton Dickinson).

To track T cells undergoing apoptosis, 2×10^7 splenocytes were stimulated with 1 μg/ml anti-CD3 mAb in the presence or absence of one of the following: his$_6$CTLA-4 · FasL, anti-Fas mAb, CTLA-4 · Ig, or a combination of the latter two. These cultures were incubated in 6-well plates for 24–96 h. At the indicated times, splenocytes were collected, washed twice with cold FACS buffer (1× PBS/0.5% BSA/0.02% sodium azide), and incubated on ice for 30–45 min with either APC-conjugated anti-CD4 mAb or anti-CD8 mAb, or their matching isotype Ab. FITC-conjugated anti-annexin-V mAb was used as a marker of early stages of apoptosis. To differentiate apoptotic cells from necrotic ones, splenocytes were costained with PI and annexin-V-FITC in a PI kit (MBL), according to the manufacturer's protocol. Flow cytometry was performed on a FACScan, and data were analyzed using CellQuest software (Becton Dickinson). A total of 5×10^5 events were counted for each sample.

Results

his$_6$CTLA-4 · FasL activity is APC dependent

CTLA-4 · FasL was designed as a trans signal converter protein that would block APC-resident B7 costimulators via its CTLA-4 domain and trigger T cell-resident Fas receptors via its FasL domain. The functionality of the FasL domain was established by demonstrating that splenocytes from lpr$^{-/-}$ mice, which are devoid of Fas receptors, are not affected by CTLA-4 · FasL (14) and that anti-Fas Ab can completely reverse CTLA-4 · FasL's action (7). The functionality of the CTLA-4 domain was demonstrated in vitro in two cellular contexts: B7-rich Daudi cells (for CTLA-4 · FasL membrane anchoring) combined with Fas$^+$ Jurkat T cells as targets, and stimulated PBMC (which offer both native B7$^+$ APC and Fas$^+$ T cell targets together) (7). Using these cellular combinations, we showed that CTLA-4 · FasL can induce Jurkat apoptosis and inhibit anti-CD3 mAb-stimulated proliferation of primary T cells, and it is significantly more potent than CTLA-4 · Ig and soluble FasL, alone or in combination (7). Although our data were consistent with the notion that CTLA-4 · FasL's enhanced activity stems from its ability to anchor to APC membranes, we did not formally show this via APC depletion.

To this end, and to delve further into CTLA-4 · FasL's mechanisms of action, we have proceeded to examine CTLA-4 · FasL's activity using enriched T cells, with or without irradiated splenocytes added as a source of APC. Specifically, purified murine T cells were stimulated with plate-bound anti-CD28 and anti-CD3 mAb, in the presence or absence of his$_6$CTLA-4 · FasL and added APC (irradiated splenocytes). In the presence of APC, his$_6$CTLA-4 · FasL effectively inhibited T cell proliferation, as measured by [^{3}H]thymidine incorporation (Fig. 1*A*, *upper panel*). In contrast, in the absence of APC, there was no observable inhibition of purified T cells by his$_6$CTLA-4 · FasL (Fig. 1*A*, *lower panel*).

We repeated this experiment substituting for anti-CD28/anti-CD3 mAb the combination of PMA plus ionomycin as a non-TCR T cell trigger that does not require costimulation. Again,

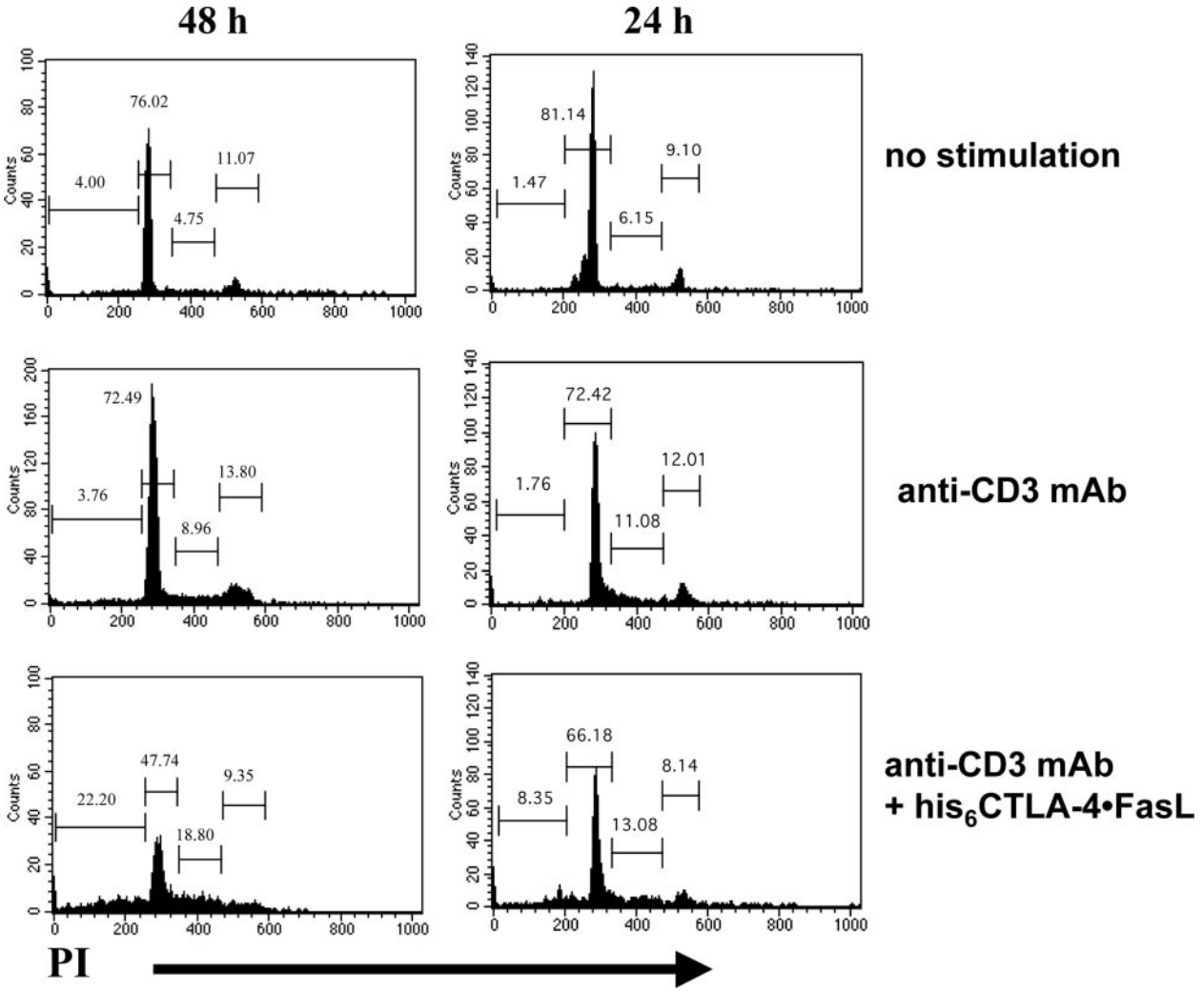

FIGURE 3. his_6CTLA-4 · FasL does not prevent cell cycle entry of $CD8^+$ T cells. Splenocytes were stimulated with anti-CD3 mAb (0.5 μg/ml) in the presence or absence of 30 ng/ml of his_6CTLA-4 · FasL for 24–96 h. Splenocytes were fixed and stained with APC-conjugated anti-CD8 mAb, along with PI for cell cycle analysis. The different cell cycle phases: hypodiploid (apoptotic), G_1, S, and G_2/M are presented in the histogram (left to right), respectively. Cell cycle analysis was preformed using flow cytometry. This is a representative of four independent experiments.

his_6CTLA-4 · FasL did not inhibit purified T cells (Fig. 1*B*, *lower panel*), but effectively inhibited T cell responses when APCs were present (Fig. 1*B*, *upper panel*). Taken together, these data indicate that his_6CTLA-4 · FasL's action is APC dependent, consistent with the notion that it binds to B7 molecules on APC surfaces.

his_6CTLA-4 · FasL does not interfere with cell cycle progression

In previous studies, we found that exogenous IL-2 can partially reverse his_6CTLA-4 · FasL's inhibitory activity (13). This suggested the possibility that his_6CTLA-4 · FasL might interfere with cell cycle progression, perhaps mirroring CTLA-4 · Ig's cell cycle effects (24). To test for a cell cycle effect, splenocytes were stimulated for 24 h with soluble anti-CD3 mAb, in the presence or absence of his_6CTLA-4 · FasL, CTLA-4 · Ig, anti-Fas mAb, or a combination of the latter two. Expression of $p27^{kip1}$, a marker for cell cycle entry, and proliferating cell nuclear Ag (PCNA), a marker up-regulated in dividing cells from late G_1 to early M phases, was assessed in Western blots. As expected, anti-CD3 mAb-mediated activation of splenocytes induced down-regulation of $p27^{kip1}$ and up-regulation of PCNA. Significantly, this was not affected by his_6CTLA-4 · FasL (Fig. 2), indicating that this agent does not interfere with cell cycle entry and progression at 24 h.

To solidify this observation, and to look directly at T cell subsets, we evaluated cell cycling via flow cytometry, using PI staining. Splenocytes were stimulated for 24–96 h with anti-CD3 mAb, in the presence or absence of his_6CTLA-4 · FasL (30 ng/ml). Cells were then collected, fixed with ethanol, immunostained with either APC-conjugated anti-CD4 or anti-CD8 mAb, counterstained with PI, and analyzed by flow cytometry. As shown in Fig. 3, more cells of the stimulated groups were in phases S and G_2 ($p < 0.05$ vs nonstimulated). No significant difference was found between cells stimulated in the presence or absence of his_6CTLA-4 · FasL at 24 h when data were collected from four independent experiments. This was true for $CD4^+$ cells as well (data not shown).

As expected, at 24 h relatively few cells entered the cell cycle upon anti-CD3 activation, and very few cells were apoptotic. Of note, in the presence of his_6CTLA-4 · FasL the cell cycle entry was not prevented, albeit more cells were apoptotic (for both $CD8^+$ and $CD4^+$ cells). At 48 h, more cells were in the S plus G_2 phases in the cells stimulated in the presence of his_6CTLA-4 · FasL. However, this cannot be attributed to a more effective cell cycle entry but probably reflects the increasing fraction of activated cells that underwent apoptosis. Interestingly, his_6CTLA-4 · FasL-promoted apoptosis was evident as early as 24 h, and it peaked at 48 h. Taken together, these findings

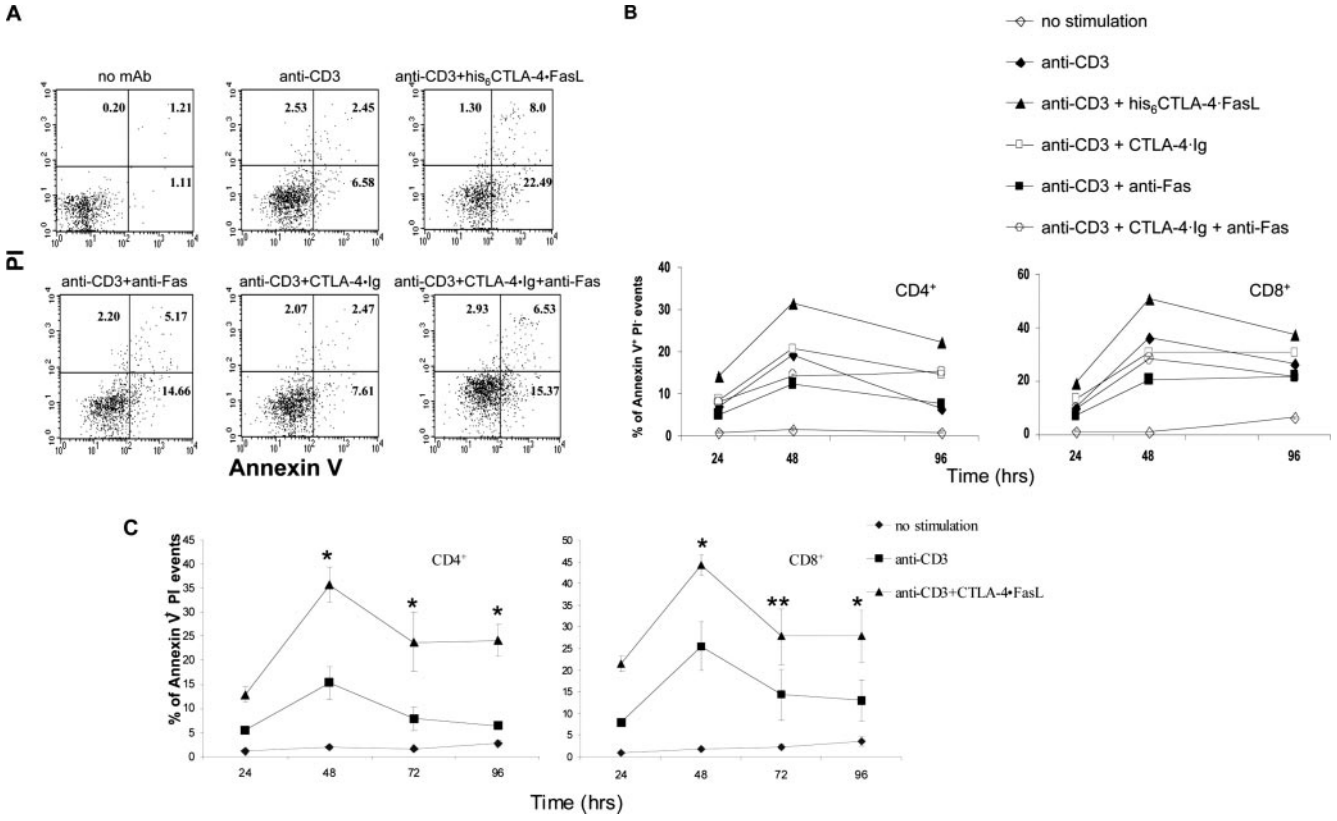

FIGURE 4. his_6CTLA-4 · FasL induces apoptosis in reactive T cells. Splenocytes were stimulated with anti-CD3 mAb (1.0 μg/ml), in the presence or absence of either his_6CTLA-4 · FasL (30 ng/ml), anti-Fas mAb (300 ng/ml), his_6CTLA-4 · Ig (100 ng/ml), or the latter two in combination (as indicated) for up to 96 h. Splenocytes were harvested and costained with annexin-V and PI, as set forth in *Materials and Methods*. *A*, Dot plot analysis of $CD8^+$ cells with the different treatment regimens after 96 h. *B*, Summary of FACS analysis data for $CD4^+$ (*left panel*) and $CD8^+$ (*right panel*) cells at 24, 48, and 96 h after treatment. Data acquisition and analysis were preformed on a FACScan, using three-color flow cytometry. This is a representative of four independent experiments. *C*, Summary of FACS analysis data for $CD4^+$ (*left panel*) and $CD8^+$ (*right panel*) cells at 24–96 h after treatment. This is the summary of four independent experiments. Data are presented as mean ± SE. *, $p < 0.02$ vs anti-CD3; **, $p = 0.05$ vs anti-CD3.

clearly demonstrate that his_6CTLA-4 · FasL does not lead to early cell cycle arrest, and in this respect differs from what is observed with classical anergy induction. However, his_6CTLA-4 · FasL does promote apoptosis of T cells, which interestingly is evident at a relatively early time point.

his_6CTLA-4 · FasL induces apoptosis of reactive T cells

We proceeded to evaluate the proapoptotic activity of his_6CTLA-4 · FasL in more depth. Splenocytes were stimulated for 24–96 h with anti-CD3 mAb, in the presence or absence of his_6CTLA-4 · FasL (30 ng/ml). Cells were collected, immunostained with APC-conjugated anti-CD4 or anti-CD8 mAb, counterstained with anti-annexin-V mAb and PI, and analyzed by flow cytometry. Dual staining with anti-annexin-V mAb plus PI enables one to distinguish apoptotic cells (annexin-V^+PI^-) from necrotic ones (annexin-V^+PI^+, annexin-V^-PI^+). The addition of his_6CTLA-4 · FasL resulted in a larger fraction of both apoptotic and necrotic cells at 24 h (Fig. 4, *B* and *C*), and this persisted for at least 96 h (Fig. 4, *A–C*). The necrotic and apoptotic fractions were also increased by anti-Fas mAb, or CTLA-4 · Ig, and anti-Fas mAb in combination, but only at 96 h (Fig. 4, *A* and *B*). These various data are consistent with the notion that his_6CTLA-4 · FasL primarily acts through apoptosis induction early after T cell activation.

Caspase inhibitor (zVAD-FMK) can block the effect of his_6CTLA-4 · FasL on proliferating T cells

To determine whether his_6CTLA-4 · FasL's proapoptotic activity is linked to the caspase pathway, as is the case for Fas receptor signaling triggered by other soluble FasL agents, we tested whether blocking the activity of caspases prevents the apoptosis of his_6CTLA-4 · FasL-treated cells. zVAD-FMK is an irreversible inhibitor of caspases. Splenocytes were stimulated with anti-CD3 mAb in the presence or absence of his_6CTLA-4 · FasL, anti-Fas

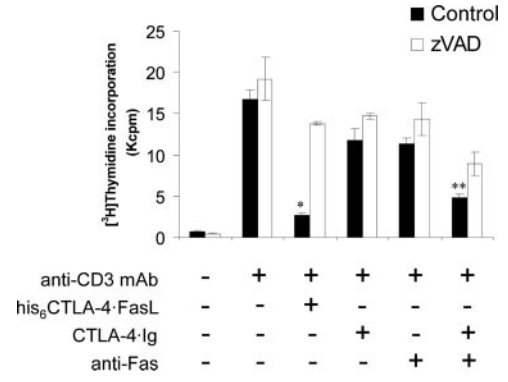

FIGURE 5. Caspase inhibitor (zVAD-FMK) blocks his_6CTLA-4 · FasL inhibition of T cell proliferation. Splenocytes were stimulated with anti-CD3 mAb (1 μg/ml), in the presence or absence of either his_6CTLA-4 · FasL (30 ng/ml), anti-Fas mAb (300 ng/ml), his_6CTLA-4 · Ig (100 ng/ml), or the latter two in combination (as indicated), in 96-well plates, with or without 20 μM zVAD-FMK. Cells were harvested after 72 h and tested for [^{3}H]thymidine incorporation. Data shown are an average of triplicates, and SEs are indicated. *, $p < 0.01$ vs zVAD; **, $p < 0.05$ vs zVAD. This is a representative of four independent experiments.

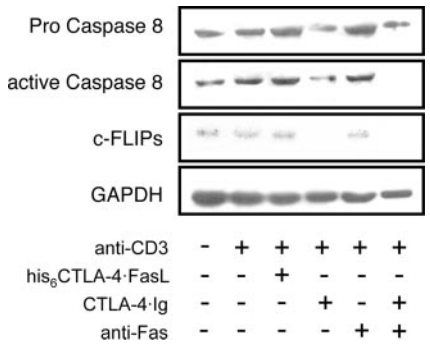

FIGURE 6. his_6CTLA-4 · FasL decreases anti-apoptotic markers. Splenocytes were stimulated with anti-CD3 mAb (1 μg/ml) in the presence or absence of either his_6CTLA-4 · FasL (30 ng/ml), anti-Fas mAb (300 ng/ml), his_6CTLA-4 · Ig (100 ng/ml), or the latter two in combination (as indicated) for 24 h. T cells were enriched by negative selection, and lysates were fractionated on 8% SDS-PAGE and immunoblotted overnight at 4°C with the indicated Ab. This is a representative experiment of four independent experiments.

mAb, CTLA-4 · Ig, or the latter two in combination, with or without 20 μM zVAD-FMK. As shown in Fig. 5, zVAD-FMK prevented his_6CTLA-4 · FasL-mediated inhibition of anti-CD3 mAb-driven T cell proliferation.

his_6CTLA-4 · FasL interferes with cFLIP up-regulation during T cell activation

Although Fas is up-regulated soon after T cell activation, there is a lag in the functional ability of this surface receptor to mediate apoptosis induction (34–36). Consequently, the relatively early apoptosis induced by his_6CTLA-4 · FasL in our system suggested that it might act to capacitate Fas-mediated apoptosis induction, perhaps by interfering with anti-apoptotic signals within the cell. One well-documented anti-apoptotic signal emanates from cFLIP, which inhibits the Fas signaling pathway by interfering with caspase 8 binding to the death complex, thereby preventing its activation and continuation of the caspase cascade (37). The level of cFLIP short (cFLIPs) levels is up-regulated during T cell activation and decreases as the cell cycle progresses, increasing the susceptibility of these cells to Fas receptor engagement (38).

To this end, we examined cFLIPs expression in T cells activated in the presence of his_6CTLA-4 · FasL. Specifically, splenocytes were stimulated for 24 h with anti-CD3 mAb, in the presence or absence of his_6CTLA-4 · FasL (30 ng/ml). cFLIPs expression was evaluated by immunobloting extracts from enriched T cells. As expected (38), cFLIPs expression was up-regulated after 24 h of anti-CD3 mAb stimulation. Significantly, addition of his_6CTLA-4 · FasL abrogated cFLIPs induction altogether (Fig. 6). In contrast, CTLA-4 · Ig, anti-Fas mAb, or the two in combination did not prevent cFLIPs induction. Of note, none of these immunomodulatory agents (his_6CTLA-4 · FasL, CTLA-4 · Ig, anti-Fas mAb, or the latter two in combination) interfered with the activation of caspase 8 in stimulated T cells (Fig. 6), nor did they affect caspase 3 activation (data not shown).

Discussion

In this study, we have further probed the mechanism of action of our paradigmatic TSCP, his_6CTLA-4 · FasL. In the process, we have uncovered unique features of this fusion protein, which distinguish it from the more extensively studied CTLA-4 · Ig, as well as from FasL. Key findings are that his_6CTLA-4 · FasL 1) does not interfere with cell cycle progression per se, and in this way differs from anergy induced by CTLA-4 · Ig; 2) induces early apoptosis of $CD8^+$ and $CD4^+$ T cells; 3) mediates its proapoptotic effect through caspases; and 4) prevents the postactivation induction of the anti-apoptotic protein cFLIP, and in this way, differs from CTLA-4 · Ig and anti-Fas mAb which do not affect cFLIP fluxes, alone or in combination.

CTLA-4 · Ig has been shown to induce classical anergy in stimulated T cells, preventing their entrance into the cell cycle, and its inhibitory effect is reversed by exogenous IL-2 (24, 25). A primary finding of this study is that his_6CTLA-4 · FasL, which can be used at substantially lower concentrations than CTLA-4 · Ig, does not interfere with cell cycle progression and entrance into M phase. This was established by two complementary approaches: monitoring effects on $p27^{kip1}$ and PCNA protein expression, and cell cycle analysis by PI staining. The cell cycle analysis not only pointed away from anergy induction for his_6CTLA-4 · FasL, but also pointed toward induction of apoptosis as a primary effect. The his_6CTLA-4 · FasL vs CTLA-4 · Ig difference in this setting may stem from the widely divergent concentrations at which they are optimally used.

Interestingly, by permitting entry into, and progression within, the cell cycle, his_6CTLA-4 · FasL (unlike CTLA-4 · Ig) may favor activation-induced cell death. Activation-induced cell death, which plays a central role in clonal deletion and tolerance induction, is known to be dependent on efficient T cell activation and cell cycle progression (39, 40). Indeed, we have previously shown that cells stimulated in the presence of his_6CTLA-4 · FasL do express activation markers such as CD25 and Fas (CD95) (13). Thus, the fact that his_6CTLA-4 · FasL permits cell cycle progression may be key to its effectiveness as an apoptosis-inducing agent, and may tie into its difference from CTLA-4 · Ig, which is instead linked to anergy induction.

A cornerstone observation of this study is that his_6CTLA-4 · FasL-driven apoptosis appears relatively early after T cell-activation (already at 24 h). Although activated T cells up-regulate Fas receptor (CD95) on their surfaces from the first day after activation, they become sensitive to proapoptotic triggering ligand (FasL) only at days 3–6 after stimulation (34–36). The initial resistance to FasL-induced apoptosis is believed to be related to anti-apoptotic signals that emerge in the course of T cell stimulation (35, 36). One critical anti-apoptotic protein appears to be the FLICE inhibitory protein, cFLIP (37, 38, 41–44). Our demonstration that his_6CTLA-4 · FasL blocks the usual increase in cFLIPs expression at 24 h poststimulation implicates this signal mediator as possibly being key here. That is, an inability to up-regulate cFLIPs expression could account for the apoptosis seen as early as 24 h after splenocytes are activated in the presence of his_6CTLA-4 · FasL. Furthermore, it is even possible that the his_6CTLA-4 · FasL-driven cFLIP perturbation could have a dual effect. In addition to enabling Fas-mediated apoptosis, cFLIP could also contribute to inhibition of T cell proliferation. cFLIP has been associated with T cell responses to TCR stimulation (45), and when cFLIP is over-expressed, Fas ligation induces proproliferative signals mediated by NF-κB (42–44, 46). Furthermore, inhibition of the NF-κB pathway effectively inhibits vFLIP-induced lymphocyte proliferation in Kaposi sarcoma virus-infected lymphomas (17). Thus, his_6CTLA-4 · FasL-driven down-regulation of cFLIPs at an early stage may both favor apoptosis and also coordinately prevent proliferation, the latter by inhibiting the NF-κB pathway.

What emerges from these findings is an intriguing model for his_6CTLA-4 · FasL action, wherein this fusion protein promotes apoptosis to occur early by both engaging the Fas receptor (via its FasL component) and simultaneously capacitating this receptor by

preventing cFLIP induction. It is tempting to speculate that this latter molecular effect hinges upon his_6CTLA-4 · FasL's CTLA-4 component. For example, it is possible that cFLIPs down-modulation ensues from exposure to a soluble CTLA-4-containing derivative (i.e., his_6CTLA-4 · FasL) at a relatively lower concentration (than is used for CTLA-4 · Ig) that preferentially blocks B7 engagement of the CD28 costimulator receptor on T cells, and yet still permits B7 binding to the higher affinity CTLA-4 inhibitory receptor on the same cells. If this were the case, a unique mechanism would be operative here, in which an immunomodulatory agent is capable of skewing from CD28 to CTLA-4 triggering. However, to date, there has been no direct linking of CTLA-4 signaling to modulation of FLIP expression. Thus, according this model, the his_6CTLA-4 · FasL TSCP can at the same time induce proapototic signals and inhibit the anti-apoptotic ones, with the latter reinforcing the former. Moreover, these pro- and anti-apoptotic signals merge at the junction of FLIP/caspase 8, with both signals sent by the protein favoring caspase 8 activation and apoptosis induction.

Although the focus of the this study is on CTLA-4 · FasL's direct effects on T cells, there is the additional possibility of indirect effects mediated through cellular intermediaries such as APC. One interesting possibility is that the fusion protein's CTLA-4 domain binds to, and "back-signals" through, surface B7 proteins on APC, and in so doing, elicits T cell inhibitory activity. CTLA-4 · Ig induces IDO expression in a specific subset of DC by back-signaling through B7-1 (47–49), and these DC are then able to inhibit T cell proliferation. However, our observation that irradiated splenocytes are as effective as nonirradiated ones in restoring the sensitivity of enriched T cells toCTLA-4 · FasL, argues against B7-mediated back-signaling as an predominant operative mechanism in our system. Nonetheless, the possibility that CTLA-4 · FasL has indirect effects on T cells merits further exploration.

The unfolding mechanistic insights into his_6CTLA-4 · FasL showcase how even a seemingly straightforward "two-component" fusion protein can feature a more complex array of functions, going beyond simple "bi-functionality." That is, a TSCP such as his_6CTLA-4 · FasL is more than just the sum of its two parts. Importantly, the his_6CTLA-4 · FasL fusion protein effect cannot be recapitulated by simply using its soluble CTLA-4 and FasL components in combination. The functional difference goes beyond dosing per se, in that even when concentrations of the components are matched on a molar basis with that of his_6CTLA-4 · FasL, one does not achieve the latter's proliferative inhibition (data not shown). Instead, the explanation may stem in some fashion from the way in which the functional domains are being presented to the responding cells. For instance, it is known that ligands can differ in their signaling properties when they are in membrane-bound vs soluble forms (50, 51). The exquisite APC-dependence of his_6CTLA-4 · FasL, as demonstrated in this study, is consistent with the notion that membrane anchorage is a critical driver of this fusion protein's functional profile.

The present study suggests that in the case of his_6CTLA-4 · FasL, early apoptosis can be elicited via the melding of the triggering of a proapoptotic receptor and the coordinate down-regulation of an anti-apoptotic protein. Moreover, there may be more subtle nuances to "costimulator blockade" than have been previously appreciated. As the repertoire of TSCP and other therapeutic fusion proteins expands, with capacities to target various cell subsets and even mediate autoinhibitory functions (8, 12), the functional richness of the protein fusion paradigm should multiply.

Disclosures

The authors have no financial conflict of interest.

References

1. Kreitman, R. J., W. H. Wilson, K. Bergeron, M. Raggio, M. Stetler-Stevenson, D. J. FitzGerald, and I. Pastan. 2001. Efficacy of the anti-CD22 recombinant immunotoxin BL22 in chemotherapy-resistant hairy-cell leukemia. *N. Engl. J. Med.* 345: 241–247.
2. Foss, F. M., P. Bacha, K. E. Osann, M. F. Demierre, T. Bell, and T. Kuzel. 2001. Biological correlates of acute hypersensitivity events with DAB(389)IL-2 (denileukin diftitox, ONTAK) in cutaneous T-cell lymphoma: decreased frequency and severity with steroid premedication. *Clin. Lymphoma* 1: 298–302.
3. Kreitman, R. J. 2006. Immunotoxins for targeted cancer therapy. *Aaps J.* 8: E532–E551.
4. Casares, S., C. S. Zong, D. L. Radu, A. Miller, C. A. Bona, and T. D. Brumeanu. 1999. Antigen-specific signaling by a soluble, dimeric peptide/major histocompatibility complex class II/Fc chimera leading to T helper cell type 2 differentiation. *J. Exp. Med.* 190: 543–553.
5. Schmoekel, H. G., F. E. Weber, J. C. Schense, K. W. Gratz, P. Schawalder, and J. A. Hubbell. 2005. Bone repair with a form of BMP-2 engineered for incorporation into fibrin cell ingrowth matrices. *Biotechnol. Bioeng.* 89: 253–262.
6. Bulfone-Paus, S., R. Ruckert, H. Krause, H. von Bernuth, M. Notter, T. Pohl, T. H. Tran, R. Paus, and U. Kunzendorf. 2000. An interleukin-2-IgG-Fas ligand fusion protein suppresses delayed-type hypersensitivity in mice by triggering apoptosis in activated T cells as a novel strategy for immunosuppression. *Transplantation* 69: 1386–1391.
7. Huang, J. H., and M. L. Tykocinski. 2001. CTLA-4-Fas ligand functions as a trans signal converter protein in bridging antigen-presenting cells and T cells. *Int. Immunol.* 13: 529–539.
8. Dranitzki Elhalel, M., J.-H. Huang, M. Sasoon, J. Rachmilewitz, M. Parnas, and M. L. Tykocinski. 2005. CD40 · FasL inhibits human T cells: evidence for an auto-inhibitory loop-back mechanism. *Am. J. Soc. Nephrol.* 16: 232A.
9. Assohou-Luty, C., J. Gerspach, D. Siegmund, N. Muller, B. Huard, G. Tiegs, K. Pfizenmaier, and H. Wajant. 2006. A CD40-CD95L fusion protein interferes with CD40L-induced prosurvival signaling and allows membrane CD40L-restricted activation of CD95. *J. Mol. Med.* 84: 785–797.
10. Gerspach, J., D. Muller, S. Munkel, O. Selchow, J. Nemeth, M. Noack, H. Petrul, A. Menrad, H. Wajant, and K. Pfizenmaier. 2006. Restoration of membrane TNF-like activity by cell surface targeting and matrix metalloproteinase-mediated processing of a TNF prodrug. *Cell Death Differ.* 13: 273–284.
11. Samel, D., D. Muller, J. Gerspach, C. Assohou-Luty, G. Sass, G. Tiegs, K. Pfizenmaier, and H. Wajant. 2003. Generation of a FasL-based proapoptotic fusion protein devoid of systemic toxicity due to cell-surface antigen-restricted Activation. *J. Biol. Chem.* 278: 32077–32082.
12. Dranitzki-Elhalel, M., J. Huang, M. Sasson, J. Rachmilewitz, M. Parnas, and M. Tykocinski. 2007. CD40 · FasL inhibits human T cells: evidence for an auto-inhibitory loop-back mechanism. *Int. Immunol*. 19: 355–363.
13. Elhalel, M. D., J. H. Huang, W. Schmidt, J. Rachmilewitz, and M. L. Tykocinski. 2003. CTLA-4. FasL induces alloantigen-specific hyporesponsiveness. *J. Immunol.* 170: 5842–5850.
14. Dranitzki-Elhalel, M., J. H. Huang, J. Rachmilewitz, O. Pappo, M. Parnas, W. Schmidt, and M. L. Tykocinski. 2006. CTLA-4.FasL inhibits allogeneic responses in vivo. *Cell. Immunol.* 239: 129–135.
15. Chen, L. 2004. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat. Rev. Immunol.* 4: 336–347.
16. Nagata, S. 1999. Fas ligand-induced apoptosis. *Annu. Rev. Genet.* 33: 29–55.
17. Keller, S. A., D. Hernandez-Hopkins, J. Vider, V. Ponomarev, E. Hyjek, E. J. Schattner, and E. Cesarman. 2006. NF-κB is essential for the progression of KSHV- and EBV-infected lymphomas in vivo. *Blood* 107: 3295–3302.
18. Zhang, H. G., X. Su, D. Liu, W. Liu, P. Yang, Z. Wang, C. K. Edwards, H. Bluethmann, J. D. Mountz, and T. Zhou. 1999. Induction of specific T cell tolerance by Fas ligand-expressing antigen-presenting cells. *J. Immunol.* 162: 1423–1430.
19. Feng, Y. G., Y. Z. Jin, Q. Y. Zhang, J. Hao, G. M. Wang, and S. S. Xie. 2005. CTLA4-Fas ligand gene transfer mediated by adenovirus induce long-time survival of murine cardiac allografts. *Transplant. Proc.* 37: 2379–2381.
20. Jin, Y., A. Qu, G. M. Wang, J. Hao, X. Gao, and S. Xie. 2004. Simultaneous stimulation of Fas-mediated apoptosis and blockade of costimulation prevent autoimmune diabetes in mice induced by multiple low-dose streptozotocin. *Gene Ther.* 11: 982–991.
21. Vincenti, F., C. Larsen, A. Durrbach, T. Wekerle, B. Nashan, G. Blancho, P. Lang, J. Grinyo, P. F. Halloran, K. Solez, D. Hagerty, et al. 2005. Costimulation blockade with belatacept in renal transplantation. *N. Engl. J. Med.* 353: 770–781.
22. Bluestone, J. A., E. W. St Clair, and L. A. Turka. 2006. CTLA4Ig: bridging the basic immunology with clinical application. *Immunity* 24: 233–238.
23. Janeway, C. A., Jr., and K. Bottomly. 1994. Signals and signs for lymphocyte responses. *Cell* 76: 275–285.
24. Greenwald, R. J., V. A. Boussiotis, R. B. Lorsbach, A. K. Abbas, and A. H. Sharpe. 2001. CTLA-4 regulates induction of anergy in vivo. *Immunity* 14: 145–155.
25. Greenwald, R. J., M. A. Oosterwegel, D. van der Woude, A. Kubal, D. A. Mandelbrot, V. A. Boussiotis, and A. H. Sharpe. 2002. CTLA-4 regulates cell cycle progression during a primary immune response. *Eur. J. Immunol.* 32: 366–373.
26. Wekerle, T., J. Kurtz, M. Sayegh, H. Ito, A. Wells, S. Bensinger, J. Shaffer, L. Turka, and M. Sykes. 2001. Peripheral deletion after bone marrow transplantation with costimulatory blockade has features of both activation-induced cell death and passive cell death. *J. Immunol.* 166: 2311–2316.

27. Lehnert, A. M., S. Yi, J. S. Burgess, and P. J. O'Connell. 2000. Pancreatic islet xenograft tolerance after short-term costimulation blockade is associated with increased $CD4^+$ T cell apoptosis but not immune deviation. *Transplantation* 69: 1176–1185.
28. Wei, L., L. Wei-Min, G. Cheng, and Z. Bao-Guo. 2006. Upregulation of $CD4^+CD25^+$ T lymphocyte by adenovirus-mediated gene transfer of CTLA4Ig fusion protein in experimental autoimmune myocarditis. *Autoimmunity* 39: 289–298.
29. Grohmann, U., F. Fallarino, and P. Puccetti. 2003. Tolerance, DCs and tryptophan: much ado about IDO. *Trends Immunol.* 24: 242–248.
30. Grohmann, U., C. Orabona, F. Fallarino, C. Vacca, F. Calcinaro, A. Falorni, P. Candeloro, M. L. Belladonna, R. Bianchi, M. C. Fioretti, and P. Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism in vivo. *Nat. Immunol.* 3: 1097–1101.
31. Alderson, M. R., R. J. Armitage, E. Maraskovsky, T. W. Tough, E. Roux, K. Schooley, F. Ramsdell, and D. H. Lynch. 1993. Fas transduces activation signals in normal human T lymphocytes. *J. Exp. Med.* 178: 2231–2235.
32. Kennedy, N. J., T. Kataoka, J. Tschopp, and R. C. Budd. 1999. Caspase activation is required for T cell proliferation. *J. Exp. Med.* 190: 1891–1896.
33. Maksimow, M., T. S. Soderstrom, S. Jalkanen, J. E. Eriksson, and A. Hanninen. 2006. Fas costimulation of naive CD4 T cells is controlled by NF-κB signaling and caspase activity. *J. Leukocyte Biol.* 79: 369–377.
34. Klas, C., K. M. Debatin, R. R. Jonker, and P. H. Krammer. 1993. Activation interferes with the APO-1 pathway in mature human T cells. *Int. Immunol.* 5: 625–630.
35. O'Flaherty, E., S. Ali, S. J. Pettit, and J. A. Kirby. 1998. Examination of the sensitivity of T cells to Fas ligation: induction of allospecific apoptosis. *Transplantation* 66: 1067–1073.
36. O'Flaherty, E., W. K. Wong, S. J. Pettit, K. Seymour, S. Ali, and J. A. Kirby. 2000. Regulation of T-cell apoptosis: a mixed lymphocyte reaction model. *Immunology* 100: 289–299.
37. Kirchhoff, S., W. W. Muller, A. Krueger, I. Schmitz, and P. H. Krammer. 2000. TCR-mediated up-regulation of c-FLIPshort correlates with resistance toward CD95-mediated apoptosis by blocking death-inducing signaling complex activity. *J. Immunol.* 165: 6293–6300.
38. Lee, S. W., Y. Park, J. K. Yoo, S. Y. Choi, and Y. C. Sung. 2003. Inhibition of TCR-induced CD8 T cell death by IL-12: regulation of Fas ligand and cellular FLIP expression and caspase activation by IL-12. *J. Immunol.* 170: 2456–2460.
39. Wells, A. D., X. C. Li, Y. Li, M. C. Walsh, X. X. Zheng, Z. Wu, G. Nunez, A. Tang, M. Sayegh, W. W. Hancock, et al. 1999. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat. Med.* 5: 1303–1307.
40. Wells, A. D., X. C. Li, T. B. Strom, and L. A. Turka. 2001. The role of peripheral T-cell deletion in transplantation tolerance. *Philos. Trans. R Soc. Lond B Biol. Sci.* 356: 617–623.
41. Kirchhoff, S., W. W. Muller, M. Li-Weber, and P. H. Krammer. 2000. Up-regulation of c-FLIPshort and reduction of activation-induced cell death in CD28-costimulated human T cells. *Eur. J. Immunol.* 30: 2765–2774.
42. Scaffidi, C., I. Schmitz, P. H. Krammer, and M. E. Peter. 1999. The role of c-FLIP in modulation of CD95-induced apoptosis. *J. Biol. Chem.* 274: 1541–1548.
43. Thome, M., P. Schneider, K. Hofmann, H. Fickenscher, E. Meinl, F. Neipel, C. Mattmann, K. Burns, J. L. Bodmer, M. Schroter, et al. 1997. Viral FLICE-inhibitory proteins (FLIPs) prevent apoptosis induced by death receptors. *Nature* 386: 517–521.
44. Maksimow, M., M. Santanen, S. Jalkanen, and A. Hanninen. 2003. Responding naive T cells differ in their sensitivity to Fas engagement: early death of many T cells is compensated by costimulation of surviving T cells. *Blood* 101: 4022–4028.
45. Chau, H., V. Wong, N. J. Chen, H. L. Huang, W. J. Lin, C. Mirtsos, A. R. Elford, M. Bonnard, A. Wakeham, A. I. You-Ten, et al. 2005. Cellular FLICE-inhibitory protein is required for T cell survival and cycling. *J. Exp. Med.* 202: 405–413.
46. Kataoka, T., R. C. Budd, N. Holler, M. Thome, F. Martinon, M. Irmler, K. Burns, M. Hahne, N. Kennedy, M. Kovacsovics, and J. Tschopp. 2000. The caspase-8 inhibitor FLIP promotes activation of NF-κB and Erk signaling pathways. *Curr. Biol.* 10: 640–648.
47. Mellor, A. L., B. Baban, P. Chandler, B. Marshall, K. Jhaver, A. Hansen, P. A. Koni, M. Iwashima, and D. H. Munn. 2003. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J. Immunol.* 171: 1652–1655.
48. Mellor, A. L., P. Chandler, B. Baban, A. M. Hansen, B. Marshall, J. Pihkala, H. Waldmann, S. Cobbold, E. Adams, and D. H. Munn. 2004. Specific subsets of murine dendritic cells acquire potent T cell regulatory functions following CTLA4-mediated induction of indoleamine 2,3 dioxygenase. *Int. Immunol.* 16: 1391–1401.
49. Baban, B., A. M. Hansen, P. R. Chandler, A. Manlapat, A. Bingaman, D. J. Kahler, D. H. Munn, and A. L. Mellor. 2005. A minor population of splenic dendritic cells expressing CD19 mediates IDO-dependent T cell suppression via type I IFN signaling following B7 ligation. *Int. Immunol.* 17: 909–919.
50. Schneider, P., N. Holler, J. L. Bodmer, M. Hahne, K. Frei, A. Fontana, and J. Tschopp. 1998. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J. Exp. Med.* 187: 1205–1213.
51. Knox, P. G., A. E. Milner, N. K. Green, A. G. Eliopoulos, and L. S. Young. 2003. Inhibition of metalloproteinase cleavage enhances the cytotoxicity of Fas ligand. *J. Immunol.* 170: 677–685.