CTLA-4 · FasL Induces Alloantigen-Specific Hyporesponsiveness[1]

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The APC:T cell interface can be effectively targeted with immunotherapeutic proteins. We previously described a unique *trans* signal converter protein, CTLA-4 · Fas ligand (FasL), that has the inherent capacities to tether the T cell inhibitor FasL (CD95 ligand) to the surfaces of B7 (CD80 and CD86)-positive APC (via CTLA-4:B7 interaction), and in so doing, to simultaneously interfere with B7-to-CD28 T cell activation signals. Given the continuing need for agents capable of inducing allograft tolerance without generalized immunosuppression, we have explored in depth the functional activity of CTLA-4 · FasL in human allogeneic MLR. CTLA-4 · FasL inhibits 1° MLR and induces specific hyporesponsiveness in 2° MLR, with both effects only partially reversible with exogenous IL-2. Moreover, the presence of exogenous IL-2 during the 1° MLR does not affect the induction of hyporesponsiveness upon restimulation. Furthermore, CTLA-4 · FasL enables partial activation of allostimulated T cells, reduces the fraction of actively dividing cells, and increases the percentage of dead cells among dividing T cells. Taken together, these findings suggest that CTLA-4 · FasL-mediated inhibition of secondary alloantigenic responses involves both anergy induction and clonal deletion. Thus, CTLA-4 · FasL, a paradigmatic *trans* signal converter protein, manifests unique functional properties and emerges as a potentially useful immunotherapeutic for modulating alloresponsiveness. ***The Journal of Immunology*, 2003, 170: 5842–5850.**

Controlling alloreactive T cells is a major goal of transplant immunotherapy. Although nonspecific immunosuppressive drugs dominate the current therapeutic landscape, the search continues for more directed therapies that target alloreactive T cells in a specific fashion. By inducing T cell hyporesponsiveness, or even tolerance, there is the prospect of engendering long-lasting graft survival without the need for chronic therapeutic intervention.

One T cell-directed therapeutic strategy that has been explored in depth is costimulator blockade. This strategy invokes agents that competitively block the interaction between APC-anchored costimulators and their cognate receptors on T cells, in this way inhibiting T cell activation (1). CTLA-4 · Ig, which blocks B7 costimulation, has received the most attention in this regard, with its documented capacities to induce anergic hyporesponsiveness to alloantigens and prolong allograft survival (2–5). However, CTLA-4 · Ig fails to prevent graft rejection in some transplantation models (6–8), highlighting the continuing need for other more effective therapeutic approaches.

As an alternative to passive costimulator blockade, it is possible to actively inhibit T cells by triggering inhibitory receptors on their surfaces. One strategy for achieving this end involves the use of deletional APC, also referred to as artificial veto cells (9), that have inhibitory ligands (termed coinhibitors), such as CD8 and Fas ligand (FasL),[4] anchored to their surfaces. We (10) and others (11, 12) have demonstrated that APC engineered in this way can inhibit proliferative responses and induce alloreactive T cell apoptosis in vitro. Moreover, APC with enforced FasL expression can induce alloantigen-specific T cell tolerance (12, 13) and clonal deletion in vivo (14). These effects of $FasL^+$ APC have largely been attributed to the capacity of FasL to induce the apoptotic deletion of T cells.

The concept of combining passive costimulator blockade and active inhibition has emerged. Increased costimulation via the B7:CD28 axis interferes with Fas-mediated apoptosis (15, 16), and conversely, blockade of this costimulatory pathway via CTLA-4 · Ig increases susceptibility to Fas-mediated apoptosis (17, 18). Prompted by these observations, we recently produced CTLA-4 · FasL, integrating within a single fusion protein the complementary capacities to bind B7 and deliver FasL inhibitory signals and thereby converting a stimulatory *trans* signal into an inhibitory one (19). This first *trans* signal converter protein (TSCP) demonstrated remarkably higher potency than either CTLA-4 · Ig or soluble FasL, alone or even in combination, in blocking mitogen-induced T cell proliferation and inducing apoptosis of Fas^+-susceptible T cells ex vivo.

In the present study, we further explore CTLA-4 · FasL-mediated inhibition, moving from nonspecific to specific stimuli. We ask whether CTLA-4 · FasL can induce alloantigen-specific hyporesponsiveness, using human MLR as an in vitro model, and if so, by what mechanism. Importantly, given the functional capacities of its component elements (CTLA-4 and FasL), CTLA-4 · FasL could potentially induce specific hyporesponsiveness by promoting both anergy and apoptosis of alloresponsive clones.

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[4] Abbreviations used in this paper: FasL, Fas ligand; 7-AAD, 7-amino actinomycin D; sFasL, soluble FasL; TSCP, *trans* signal converter protein.

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Materials and Methods

Primary MLR

Peripheral blood was obtained from unrelated, healthy laboratory volunteers, according to a protocol approved by our Institutional Review Board. Responders and stimulators were unrelated individuals chosen so that there was at least one HLA class I and one HLA-DR Ag mismatched within each pair. HLA typing was performed by the Histocompatibility Laboratory at the Hospital of the University of Pennsylvania. PBMC were isolated by density-gradient centrifugation using Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and were resuspended in AIM-V medium (Life Technologies, Bethesda, MD). MLR were performed in round-bottom 96-well plates using 10^5 cells responder and 10^5 gamma-irradiated (3000 rad) stimulator cells in a total volume of 0.2 ml. Immunoinhibitory proteins (CTLA-4 · FasL, CTLA-4 · Ig, or soluble FasL (sFasL)) were diluted in AIM-V and added to the culture wells at the start of the MLR. Cultures were performed in triplicate and incubated at 37°C with 5% CO_2 for up to 10 days. Cultures were pulsed with 0.5 μCi [^{3}H]thymidine per well 16–18 h before harvesting and scintillation counting.

Restimulation assays

Bulk culture 1° MLR were prepared in 25-cm^2 tissue culture flasks by coculturing 10^7 responder and 10^7 gamma-irradiated (3000 rad) stimulator PBMC in 5 ml AIM-V. Cultures were incubated for 7 days before washing and resting for 3 days. On day 10, the cells were collected, viability was tested using trypan blue exclusion, and restimulation was performed in round-bottom 96-well plates, using 10^5 viable cells and 10^5 stimulator cells. Of note, all stimulator cells were dead. In addition, cells collected on day 10 were stained with PE-conjugated anti-CD83 or PE-conjugated anti-CD14, to confirm the presence of APC in both CTLA-4 · FasL-treated and nontreated cultures. Cell viability was assessed using the exclusion of 7-amino actinomycin D (7-AAD). Freshly prepared, gamma-irradiated stimulator cells from the original donor or an HLA-mismatched, third-party donor were used in the restimulation experiments. In some experiments, gamma-irradiated (15,000 rad), EBV-transformed Raji B cells were also used as third-party stimulator cells. Assays were performed in triplicate. Restimulation assays were cultured for up to 7 days before pulsing and harvesting for scintillation counting, as described above for the 1° MLR.

CTLA-4 · FasL fusion proteins

Immunoaffinity-purified CTLA-4 · FasL was prepared as described (19). To facilitate purification, we additionally produced a hexahistidine-tagged derivative of CTLA-4 · FasL (his$_6$CTLA-4 · FasL), with the tag at the amino terminus. An expression vector for his$_6$CTLA-4 · FasL was generated in sequential subcloning steps. First, partially overlapping long oligonucleotides (leaving single-stranded gaps) were used to amplify a DNA sequence encoding (from 5′ to 3′) a *Kpn*I site, the human Ig secretion signal, six histidines, and a *Hin*dIII site. Following digestion with *Kpn*I and *Hin*dIII, the DNA fragment was isolated by gel electrophoresis and ligated into the respective sites of pFasL/CEP9, yielding phis$_6$FasL/CEP9. The extracellular domain sequence of human CTLA-4 was amplified by PCR, digested with *Hin*dIII, and subcloned into the *Hin*dIII site of phis$_6$FasL/CEP9, generating phis$_6$CTLA-4 · FasL/CEP9. All plasmids were sequence confirmed.

The 293 cells were lipofected with phis$_6$CTLA-4 · FasL/CEP9, and stable transfectants were obtained by selection in 0.2 mg/ml G418. Expressing cell lines were identified by radiometric DNA fragmentation assays, using Jurkat cells as targets (20). His$_6$CTLA-4 · FasL was purified by metal chelate chromatography from conditioned CellGro-Free medium (Mediatech, Herndon, VA) and quantitated using a sFasL ELISA (MBL, Cambridge, MA). A single band at ~46 kDa was seen on reducing SDS-PAGE. CTLA-4 · FasL and his$_6$CTLA-4 · FasL activities were shown to be comparable in MLR assays as well as in their ability to bind to the CD80/CD86-bearing, human B cell line, Daudi cells, and the two were therefore used interchangeably. CTLA-4 · FasL, his$_6$CTLA-4 · FasL, and CTLA-4 · Ig displayed similar binding to cell surface CD80. However, on a molar basis, the affinity of CTLA-4 · Ig for CD86 was ~1.5 log more efficient than either CTLA-4 · FasL protein.

Reagents, Abs, and cytokines

CFSE and 7-AAD were purchased from Molecular Probes (Eugene, OR). Neutralizing chicken anti-human TGF-β1 mAb, control chicken Ab, neutralizing goat anti-human IL-10 mAb, and normal goat IgG were purchased from R&D Systems (Minneapolis, MN). Fluorochrome-conjugated mAb specific for human CD3, CD4, CD25, CD69, and CD95 (Fas), along with isotype control Ab, were obtained from BD PharMingen (La Jolla, CA). Anti-human CD3 mAb was prepared from the OKT3 hybridoma (American Type Culture Collection, Manassas, VA). Human IL-2 and IL-10 were purchased from Chiron (Emeryville, CA) and BD PharMingen, respectively. Recombinant human CTLA-4 · Ig and sFasL were purchased from R&D Systems and Alexis Biochemicals (San Diego, CA), respectively.

Flow cytometry

Bulk culture primary MLR were prepared by combining 0.5×10^7 responder and 0.5×10^7 gamma-irradiated (3000 rad) stimulator PBMC in 2.5 ml AIM-V in 12-well tissue culture plates. Duplicate cultures were prepared in the presence or absence of 30 ng/ml his$_6$CTLA-4 · FasL. Beginning on day 0, cells were collected for immunostaining with FITC-conjugated anti-human CD3 mAb and PE-conjugated mAb specific for the activation markers CD25, CD69, CD40L, and Fas receptor (CD95). A total of 0.5 μg/sample of 7-AAD was used for live/dead discrimination. Three-color flow cytometry was performed to monitor FITC- and PE-conjugated Ab and 7-AAD. Flow cytometry was performed on a FACScan, using CellQuest software (BD Biosciences, Mountain View, CA); 25,000 events were collected per sample. Gating for live cells was performed on 7-AAD-negative populations demonstrating lymphocyte-characteristic forward light scatter.

To enable tracking of cell divisions during MLR, responding PBMC were labeled with CFSE. A total of 10^7 PBMC/ml was labeled with 5 μM CFSE for 10 min at room temperature and immediately washed three times with ice-cold AIM-V medium. A total of 10^7 CFSE-labeled PBMC was coincubated with 0.2×10^7 gamma-irradiated Raji cells in 5 ml AIM-V in six-well plates. Duplicate cultures were performed in the presence or absence of varying concentrations of his$_6$CTLA-4 · FasL. In parallel, CFSE-labeled responder cells were stimulated with 0.3 ng/ml anti-human CD3 mAb. After 4 days, cells were collected for immunostaining with PE-conjugated anti-CD4 mAb and 7-AAD. Flow cytometric data were acquired on a FACScan and analyzed using CellQuest software. A total of 10^6 events was counted from each sample. Cell divisions were demarcated according to CFSE-staining brightness. By gating on each cell division, the proportion of dead (7-AAD-positive) CD4-positive cells was calculated.

Results

CTLA-4 · FasL inhibits 1° MLR

Previously, we demonstrated that CTLA-4 · FasL inhibits the proliferation of mitogen-activated human peripheral T cells. To test whether a TSCP incorporating CTLA-4 and FasL inhibits one-way MLR, standard in vitro MLR were performed with human PBMC and cultured in the presence or absence of varying concentrations of a hexahistidine-tagged CTLA-4 · FasL fusion protein (his$_6$CTLA-4 · FasL). In the absence of his$_6$CTLA-4 · FasL, typical 1° MLR proliferative kinetics were observed, peaking at days 5–7 and returning to baseline by days 9–13 (Fig. 1*A*). When his$_6$CTLA-4 · FasL was added at the start of the MLR, inhibition was observed at the earliest point of detectable proliferation and persisted throughout the culture. This his$_6$CTLA-4 · FasL-mediated proliferative inhibition was dose dependent (Fig. 1*B*), and at the highest concentration of his$_6$CTLA-4 · FasL (100 ng/ml), proliferation was reduced to background. Similar results were observed in 15 independent experiments with 9 different PBMC donors in variable combinations, in which PBMC were completely or partially HLA mismatched. Of note, his$_6$CTLA-4 · FasL and CTLA-4 · FasL consistently demonstrated comparable inhibition in these experiments (data not shown). In addition, CTLA-4 · FasL blocked the proliferation of PBMC stimulated with HLA-mismatched, EBV-transformed B cell lines, as well as two-way MLR (data not shown). Taken together, these findings clearly demonstrate that CTLA-4 · FasL is highly effective in inhibiting 1° MLR.

We next compared the inhibitory potency of CTLA-4 · FasL to CTLA-4 · Ig and sFasL, alone or in combination. All agents were added only at the start of the MLR. CTLA-4 · Ig, at a concentration of 2.5 μg/ml, reportedly yields only partial inhibition (50–85%) of 1° MLR (3). Whereas 100 ng/ml CTLA-4 · FasL inhibited 1° MLR by 91%, the same concentration of either CTLA-4 · Ig or sFasL inhibited proliferation by 33 and 12%, respectively (Fig. 1*C*).

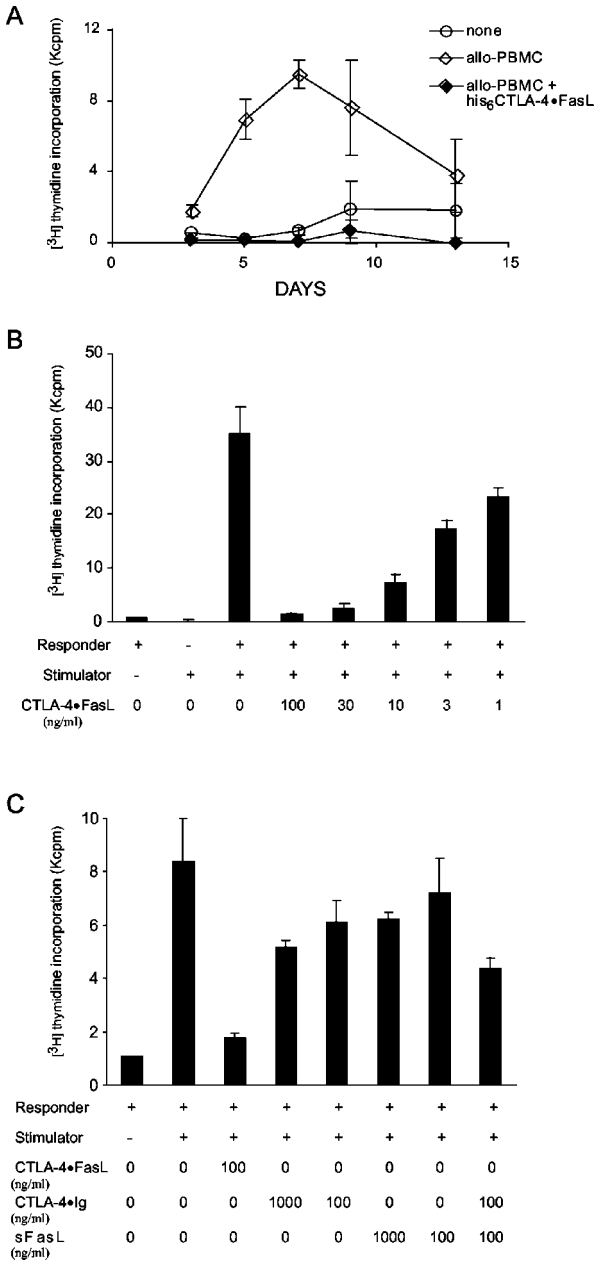

FIGURE 1. CTLA-4 · FasL inhibits proliferation of one-way 1° MLR. *A*, 1° MLR, containing a 1:1 ratio of responder and gamma-irradiated, HLA-mismatched, stimulator PBMC, were treated with 100 ng/ml his_6CTLA-4 · FasL (♦), medium alone (◇), or no stimulation (○), respectively. [^{3}H]Thymidine incorporation was determined, as described in *Material and Methods*, on the indicated days, as shown. *B*, Dose-dependent his_6CTLA-4 · FasL inhibition of [^{3}H]thymidine incorporation in day 5 MLR was analyzed. *C*, The inhibition of one-way 1° MLR by CTLA-4 · FasL was compared with that by CTLA-4 · Ig and sFasL, alone or in combination, at the various doses indicated. The MLR cells were harvested on day 5. Error bars represent SD.

Moreover, when CTLA-4 · Ig and sFasL were added in combination, each at a concentration of 100 ng/ml, there was 55% inhibition. Of note, when used at the same concentration, CTLA-4 · Ig and FasL are in 1.1- and 1.5-fold molar excess, respectively, over CTLA-4 · FasL. Even with a 10-fold higher concentration of CTLA-4 · Ig or sFasL (1 μg/ml), neither protein blocked proliferation by >60%. Thus, CTLA-4 · FasL is more potent than its individual components used in combination.

CTLA-4 · FasL induces alloantigen-specific hyporesponsiveness

CTLA-4 · Ig- and FasL-expressing APC have been used in recent studies to induce alloantigen-specific hyporesponsiveness (3, 4, 12, 14). To assess whether CTLA-4 · FasL similarly induces alloantigen-specific hyporesponsiveness, the effect of its presence during a 1° MLR on subsequent alloantigen restimulation was assessed. To this end, bulk 1° MLR cultures were incubated for 7 days in the presence or absence of CTLA-4 · FasL, and recovered cells were washed, rested for 3 days, and then restimulated with gamma-irradiated PBMC from the same donor in the absence of CTLA-4 · FasL. As expected, the proliferation response during restimulation was stronger compared with the primary stimulation and appeared earlier, peaking at days 2–4. Significantly, the presence of CTLA-4 · FasL during the 1° MLR reduced the 2° proliferative response to background levels (Fig. 2*A*). In contrast, when CTLA-4 · FasL was omitted from the 1° MLR, the expected 2° MLR proliferative response was observed.

Next, the specificity of the CTLA-4 · FasL-induced hyporesponsiveness was examined. PBMC treated with CTLA-4 · FasL during 1° stimulation were restimulated with either specific alloantigen (from the same donor) or completely HLA-mismatched PBMC from another donor (Fig. 2*B*) or EBV-transformed Raji B cells (Fig. 2*C*) as a third-party stimulator. Significantly, the response to third-party stimulators remained fully intact, despite the observed hyporesponsiveness to specific alloantigenic rechallenge (Fig. 2, *B* and *C*). Thus, CTLA-4 · FasL induced alloantigen-specific hyporesponsiveness.

The capacity of CTLA-4 · FasL to inhibit restimulation was also tested. CTLA-4 · FasL, when added to the 2° MLR, completely blocked proliferative responses, regardless of whether it was also present in the 1° MLR (Fig. 2*D*).

CTLA-4 · FasL-mediated inhibition of 1° MLR is partially reversed by IL-2

CTLA-4 · FasL induced hyporesponsiveness during both primary and secondary stimulations. CTLA-4 · Ig also can induce Ag-specific hyporesponsiveness when present during 1° MLR (3). Notably, coincubation of CTLA-4 · Ig with 20 IU/ml IL-2 prevents the induction of hyporesponsiveness. In addition, CTLA-4 · Ig-induced hyporesponsiveness is reversed by the presence of IL-2 during restimulation. We determined whether IL-2 has similar effects on hyporesponsiveness induced by CTLA-4 · FasL. Addition of IL-2 (20 IU/ml) to 1° MLR (in the absence of CTLA-4 · FasL) resulted in an earlier peak of proliferation (day 4) and modestly increased the extent of proliferation (Fig. 3*A*). Significantly, IL-2 partially reversed the proliferative inhibition mediated by CTLA-4 · FasL, to ~50% of the peak proliferation detected in the absence of CTLA-4 · FasL (but in the presence of IL-2). Peak proliferation in the presence of both CTLA-4 · FasL and IL-2 occurred on day 5, midway between the peak proliferation observed with 1° MLR in the absence (day 6) or presence of IL-2 (day 4).

The effect of coincubating IL-2 and CTLA-4 · FasL during 1° MLR on allospecific restimulation responses was tested. Bulk 1° MLR cultures were cultured in the presence of CTLA-4 · FasL alone, IL-2 alone, or the combination of CTLA-4 · FasL and IL-2. After 10 days, alloantigen restimulation responses were measured. Importantly, the presence of IL-2 during the 1° MLR phase did not interfere with the capacity of CTLA-4 · FasL to induce hyporesponsiveness during the subsequent 2° MLR (Fig. 3*B*). Of note, the addition of IL-2 to the 1° MLR did enhance the 2° response when CTLA-4 · FasL was absent.

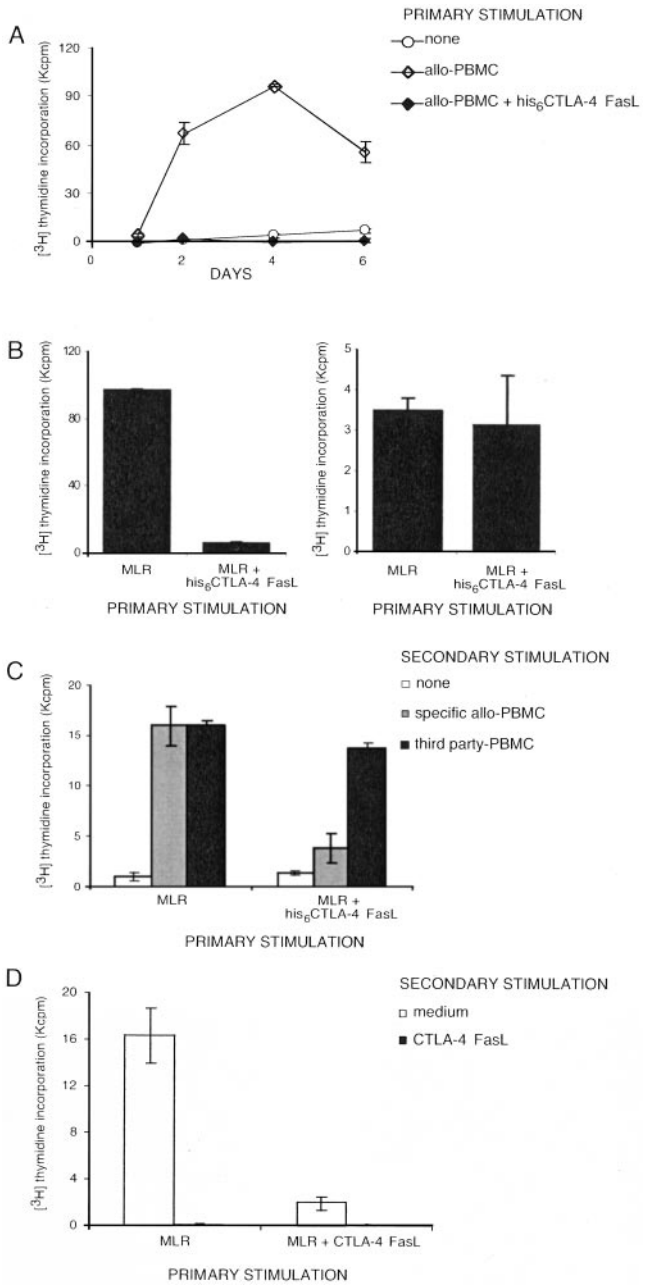

FIGURE 2. his_6CTLA-4 · FasL induces hyporesponsiveness to alloantigen restimulation. *A*, 1° MLR cultures were incubated for 7 days in the presence or absence of 30 ng/ml his_6CTLA-4 · FasL, washed and rested for 3 days, and then restimulated with freshly prepared, gamma-irradiated allogeneic stimulator cells. [^{3}H]Thymidine incorporation was determined at the days indicated. *B*, Following primary stimulation and resting as in *A*, cells were restimulated either with freshly prepared, gamma-irradiated stimulator PBMC purified from the original donor (*left panel*) or with HLA-mismatched PBMC from an unrelated donor as third-party stimulators (*right panel*). The data shown represent the maximal responses in each stimulation. Similar results were obtained in nine independent experiments, using responder and stimulator PBMC from different donors. *C*, Following primary stimulation and resting as in *A*, cells were restimulated either with freshly prepared, gamma-irradiated stimulator PBMC purified from the original donor or with HLA-mismatched EBV-transformed Raji B cells as third-party stimulators. Plates were harvested on day 5. *D*, Following primary stimulation and resting as in *A*, restimulation was performed in the presence of CTLA-4 · FasL (30 ng/ml), using freshly prepared gamma-irradiated PBMC from the original donor as stimulators. Plates were harvested on day 5. Error bars represent SD.

Hyporesponsiveness to antigenic restimulation could be due to multiple mechanisms, including anergy, suppressor cells, or deletion. To determine whether CTLA-4 · FasL induces anergy, restimulations were performed in the presence of exogenous IL-2 (Fig. 3*C*). The addition of IL-2 to 2° MLR reversed CTLA-4 · FasL-induced hyporesponsiveness, with proliferation ~30% of that observed when CTLA-4 · FasL is absent (during the 1° MLR) and IL-2 is present (during the 2° MLR); this points to an anergic component in CTLA-4 · FasL-mediated hyporesponsiveness. However, the capacity of IL-2 to reverse hyporesponsiveness depended on the concentration of his_6CTLA-4 · FasL present in the 1° MLR, with hyporesponsiveness being less reversible as the dose of his_6CTLA-4 · FasL during 1° MLR was increased. This suggested that mechanisms other than anergy might also contribute to CTLA-4 · FasL-induced hyporesponsiveness.

The effect of IL-10 and TGF-β1 blockade on CTLA-4 · FasL action

The inhibition of MLR responses caused by costimulation blockade has been linked to the inhibitory cytokines IL-10 and TGF-β1 (21–23). To determine whether the inhibitory effect of CTLA-4 · FasL is similarly associated with these inhibitory cytokines, neutralizing mAb directed against them were added at the outset of 1° MLR cultures. As shown in Fig. 4*A*, adding blocking mAb against IL-10 did not reverse CTLA-4 · FasL-mediated inhibition of the 1° MLR. In parallel experiments, no effect of neutralizing TGF-β1 mAb was observed (data not shown). The activity of these blocking activities was verified by demonstrating that they could block the inhibitory activity of exogenously added human rIL-10 (10 ng/ml) or human TGF-β (10 ng/ml), respectively, on 1° MLR (data not shown).

We also tested the effects of blocking IL-10 or TGF-β on 2° MLR responses. The addition of anti-IL-10 mAb during secondary MLR had no significant effect on the proliferative response (Fig. 4*B*). Although the addition of anti-TGF-β mAb during restimulation partially reversed CTLA-4 · FasL-induced hyporesponsiveness (which would be consistent with a TGF-β-dependent suppressor cell mechanism), the implications of this change are unclear, given that TGF-β blockade more generally increased the proliferation of 2° MLR (in the absence of CTLA-4 · FasL during the primary stimulation; Fig. 4*B*).

To look more directly for a suppressor cell mechanism, 1° MLR cells, cultured in the presence or absence of CTLA-4 · FasL, were tested for their ability to inhibit allostimulation of freshly isolated PBMC. As much as a 5-fold excess of CTLA-4 · FasL-treated, 1° MLR cells failed to inhibit the 1° MLR response of freshly isolated PBMC that are syngeneic with the 1° MLR cells (data not shown).

Taken together, these data suggest that CTLA-4 · FasL lacks the putative connections of CTLA-4 · Ig to the inhibitory cytokines IL-10 and TGF-β and to suppressor cell activity.

CTLA-4 · FasL delays the expression of CD25 on stimulated T cells

Activation of T cells renders them susceptible to activation-induced cell death and correlates with the induction of tolerance in vivo (24, 25). To assess whether his_6CTLA-4 · FasL perturbs T cell activation, its effects on the expression of T cell surface activation markers (CD25 and CD95) in 1° MLR through day 11 were assessed. At day 3, there was a decrease in the percentage of $CD3^+CD25^+$, but not $CD3^+CD95^+$, events within the 1° MLR (Fig. 5*A*). As expected, the percentage of $CD3^+CD25^+$ events on stimulated cells within 1° MLR was already elevated by day 1, and peaked at ~20% on day 8–9 (Fig. 5*B*). The addition of his_6CTLA-4 · FasL delayed the expression of CD25 on stimulated cells up to

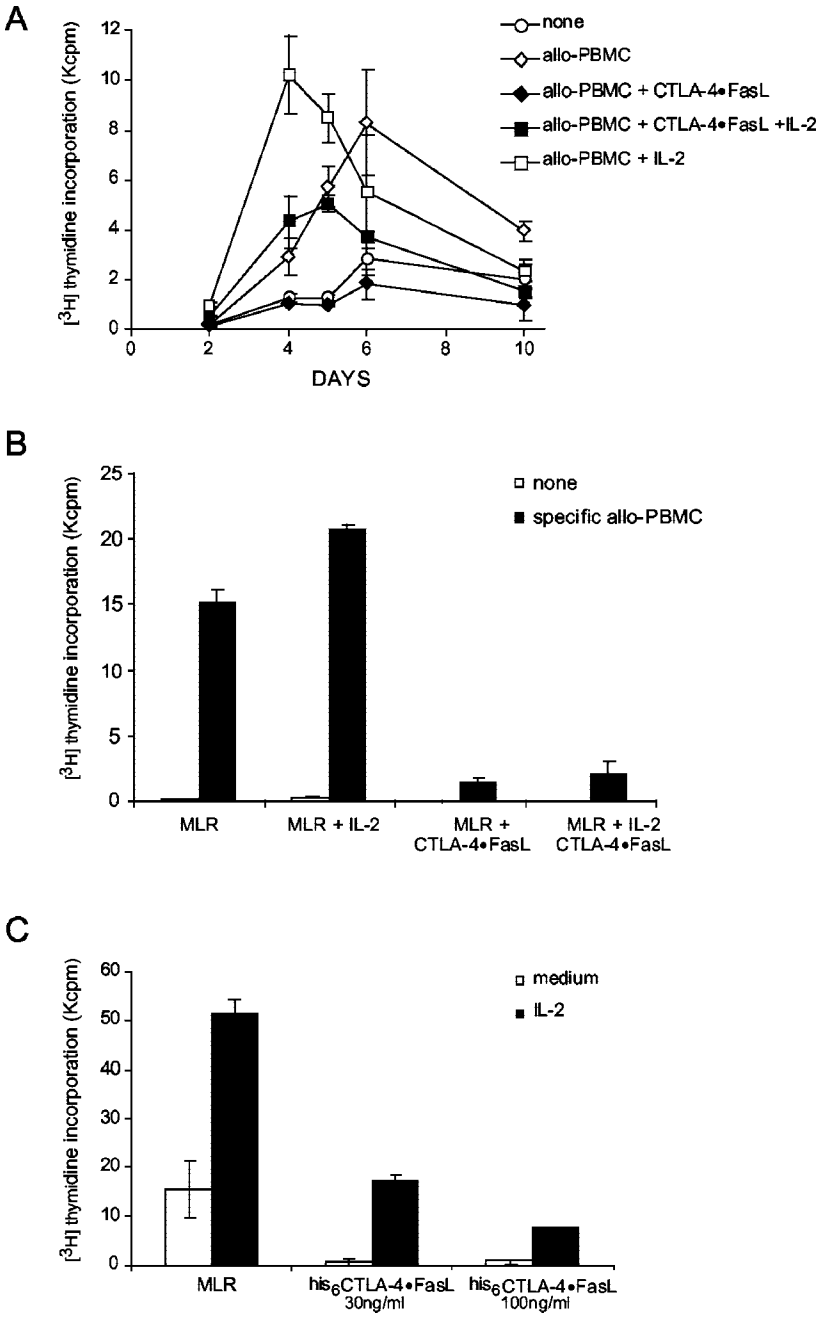

FIGURE 3. IL-2 partially overcomes CTLA-4 · FasL-mediated inhibition. *A*, The effect of IL-2 on CTLA-4 · FasL-mediated inhibition of 1° MLR was evaluated. Assays were performed in the presence of either 20 IU/ml IL-2 (□), 30 ng/ml CTLA-4 · FasL (♦), 20 IU/ml IL-2 plus 30 ng/ml CTLA-4 · FasL (■), medium without IL-2/CTLA-4 · FasL (◇), or no allostimulating cells (□). Cells were harvested at the indicated time points and tested for [^{3}H]thymidine incorporation. *B*, 1° MLR bulk cultures were incubated in the presence of either 30 ng/ml CTLA-4 · FasL, 30 ng/ml CTLA-4 · FasL plus 20 IU/ml IL-2, 20 IU/ml IL-2, or medium without CTLA-4 · FasL or IL-2 for 7 days, and then washed and rested for 3 days. Cells were collected and restimulated with freshly prepared, gamma-irradiated stimulator PBMC. Plates were harvested on day 14. *C*, 1° MLR were performed in the presence or absence of his$_6$CTLA-4 · FasL (30 or 100 ng/ml) for 7 days, and then washed and rested for 3 days. Cells were collected and restimulated with freshly prepared, gamma-irradiated stimulator PBMC in the presence or absence of 20 IU/ml of IL-2. Plates were harvested on day 14. Similar results were obtained in four independent experiments. Error bars represent SD.

day 5. However, starting on day 6, CD25 expression was equivalent on CTLA-4 · FasL-treated and nontreated cells (Fig. 5*B*). Moreover, his$_6$CTLA-4 · FasL treatment had no effect on the expression of CD95 (Fig. 5*B*), CD69, and CD40L (data not shown). Thus, notwithstanding a delaying effect on one surface activation marker (CD25), his$_6$CTLA-4 · FasL does not introduce a major block in early T cell activation processes.

CTLA-4 · FasL increases the fraction of dead cells among dividing T cells

Two outcomes of T cell activation are proliferation and death. CTLA-4 · FasL clearly inhibited proliferation, and given the well-characterized proapoptotic activity of FasL, CTLA-4 · FasL has the potential to induce T cell death. Because Fas receptor (CD95) expression is up-regulated on activated T cells in 1° MLR, even in the presence of CTLA-4 · FasL, we tested whether CTLA-4 · FasL can induce T cell death.

PBMC were labeled with CFSE to enable the simultaneous tracking of cell division and death. CFSE-labeled PBMC were stimulated with gamma-irradiated, allogenic EBV-transformed Raji B cells or anti-CD3 mAb for 4 days, before harvesting the cells for flow cytometric analysis. CD4$^+$ T cells were detected with PE-conjugated anti-CD4 mAb, and dead cells were identified by 7-AAD staining. In a typical experiment, three to four divisions of CD4$^+$ T cells were readily visible on day 4 within both anti-CD3 mAb-stimulated and MLR cultures (Fig. 6*A*). Fewer dividing cells were detected when CTLA-4 · FasL was present, confirming the results of the previous [^{3}H]thymidine incorporation experiments (Figs. 1 and 2). By gating on the individual cell divisions, the fractions of live and dead CD4$^+$ events were calculated. CTLA-4 · FasL increased the percentage of dead cells in each division (Fig. 6*D*). Thus, CTLA-4 · FasL inhibited the division of most, but not all T cells, and the small minority of T cells that divided were more prone to death.

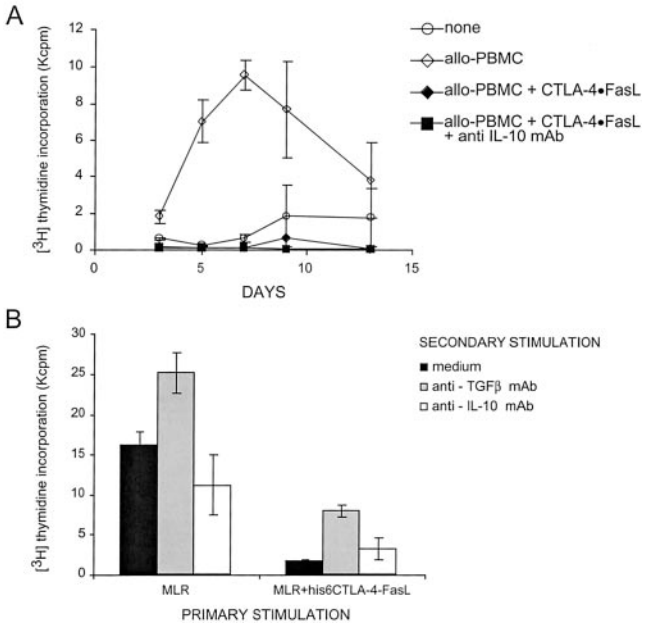

FIGURE 4. his_6CTLA-4 · FasL-mediated inhibition is not dependent upon IL-10 and TGF-β. *A*, 1° MLR were incubated in the presence of 5 μg/ml anti-IL-10 Ab, 30 ng/ml his_6CTLA-4 · FasL, or 5 μg/ml anti-IL-10 Ab plus 30 ng/ml CTLA-4 · FasL. Similar results were obtained in three independent experiments. Cells were harvested at the indicated time points and tested for [^{3}H]thymidine incorporation. *B*, 1° MLR bulk cultures were prepared in the presence or absence of 30 ng/ml his_6CTLA-4 · FasL for 7 days, and then washed and rested for 3 days. Cells were collected and restimulated with freshly prepared, gamma-irradiated stimulator PBMC from the same donor used in the 1° MLR. Restimulation assays were performed in the presence of 5 μg/ml anti-IL-10 Ab, 2 μg/ml anti-TGF-β mAb, or medium alone. Assays were harvested on day 4. Similar results were obtained in two independent experiments. Error bars represent SD.

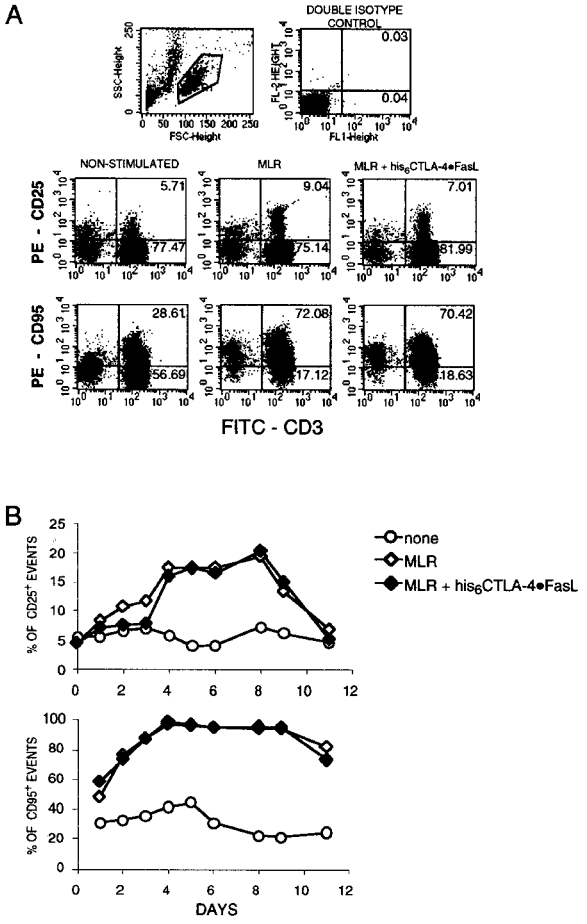

FIGURE 5. his_6CTLA-4 · FasL delays the early expression of CD25, but not CD95, during 1° MLR. *A*, 1° MLR cultures, using CD3-negative EBV-transformed Raji B cells, were incubated in the absence or presence of 30 ng/ml his_6CTLA-4 · FasL. On day 2, harvested cells were immunostained with FITC-conjugated anti-CD3 Ab, and either PE-conjugated anti-CD25 or anti-CD95 Ab. The percentage of cells in each quadrant is specified. Similar results were obtained in two independent experiments. The difference was statistically significant ($p < 0.01$ by χ^2 analysis). *B*, 1° MLR bulk cultures, using CD3-negative EBV-transformed Raji B cells, were incubated in the presence or absence of 30 ng/ml his_6CTLA-4 · FasL. Beginning on day 0, cells were collected for immunostaining with FITC-conjugated anti-CD3 Ab and PE-conjugated anti-CD25 Ab (*upper panel*) or anti-CD95 Ab (*lower panel*). A total of 0.5 μg 7-AAD/sample was used for live/dead discrimination. Three-color flow cytometry was performed to monitor FITC- and PE-conjugated Abs, and 7-AAD. Flow cytometry was performed on a FACScan using CellQuest software (BD Biosciences). A total of 25,000 events was collected per sample. Live cell gates were drawn around 7-AAD$^-$ populations demonstrating lymphocyte-characteristic forward light scatter.

Discussion

CTLA-4 · FasL integrates two distinct functional elements within a single protein: one, CTLA-4, binds to the B7 surface costimulator on APC, and another, FasL (CD95L), triggers the inhibitory Fas receptor (CD95) on T cells. Previously, we demonstrated that this unique TSCP potently inhibits mitogenic stimulation of T cells (19). The present study extends this early observation, with several key findings: 1) CTLA-4 · FasL inhibits allogeneic stimulation in 1° MLR, providing the first evidence that this new class of fusion protein can inhibit a physiological T cell stimulus; 2) CTLA-4 · FasL induces alloantigen-specific hyporesponsiveness, as demonstrated by decreased restimulation in 2° MLR; and 3) the hyporesponsiveness induced by CTLA-4 · FasL results from a combination of apoptotic and anergic mechanisms.

The hyporesponsiveness induced by CTLA-4 · FasL differs from that induced by CTLA-4 · Ig, both quantitatively and qualitatively. On the quantitative side, this study has confirmed that CTLA-4 · FasL has substantially higher efficacy as a T cell inhibitor than CTLA-4 · Ig and sFasL, even in combination. Just as significant are the qualitative differences that have emerged from the present studies. First, CTLA-4 · FasL, in contrast to CTLA-4 · Ig (3, 26), is capable of inducing hyporesponsiveness to restimulation, even when the primary stimulation is potentiated with exogenous IL-2. Second, CTLA-4 · FasL-induced hyporesponsiveness, unlike that associated with CTLA-4 · Ig (3, 26), is only partially reversed when exogenous IL-2 is added during the restimulation phase. Thus, there is a more pronounced hyporesponsiveness associated with CTLA-4 · FasL, which most likely stems from its additional proapoptotic activity.

The capacity of CTLA-4 · FasL to induce alloantigen-specific hyporesponsiveness, notwithstanding the presence of IL-2 during the 1° MLR, has implications for its potential use in preventing transplant rejection. Circulating IL-2 levels become elevated in response to inflammatory insults, including transplant rejection and infection. This would tend to limit the therapeutic utility of CTLA-4 · Ig for treating these conditions, given the susceptibility of its immunoinhibitory activity to IL-2 override. In contrast, based upon the present findings, CTLA-4 · FasL would be expected to be more effective as an immunoinhibitor in the face of a systemic IL-2 challenge. Moreover, whereas CTLA-4 · Ig is most

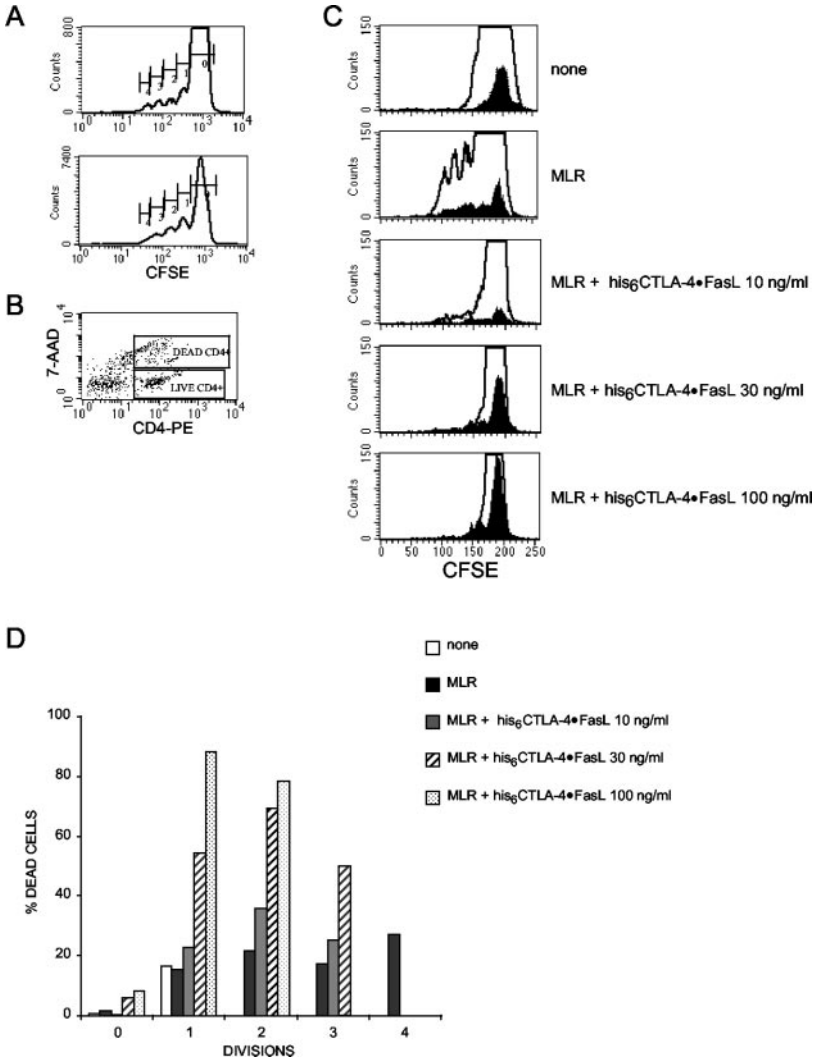

FIGURE 6. his_6CTLA-4 · FasL increases the percentage of dead cells among dividing T cells during 1° MLR. A total of 10^7 CFSE-labeled PBMC was stimulated with 0.2×10^7 allogeneic Raji cells in 5 ml AIM-V in six-well plates. Duplicate cultures were performed in the presence or absence of varying concentrations of his_6CTLA-4 · FasL (10–100 ng/ml). On day 4, cells were collected and immunostained with PE-conjugated anti-CD4 Ab and 7-AAD. A total of 10^6 flow cytometric events was collected from each sample. Cell divisions were demarcated according to CFSE-staining brightness. For each cell division, gates were drawn to enable the calculation of the percentage of dead (7-AAD$^+$), CD4$^+$ cells/total CD4$^+$ cells. *A*, The histogram shows the CFSE fluorescence of 1° MLR cells (*upper panel*) and anti-CD3 stimulation of PBMC (*lower panel*) collected on day 4. Peaks representing cell divisions are indicated by the markers. *B*, Dot plot shows gating on CD4$^+$/7-AAD$^+$ or CD4$^+$/7-AAD$^-$ events. *C*, 7-AAD$^-$ (live) and 7-AAD$^+$ (dead) cells are plotted in the open and filled histograms, respectively. *D*, Percentage of dead/total CD4$^+$ cells in each division.

effective in inhibiting primary responses of naive T cells (3, 26), CTLA-4 · FasL is as effective in inhibiting recall responses by memory T cells as it is in inhibiting primary ones. This suggests yet another potential advantage for CTLA-4 · FasL over CTLA-4 · Ig as a tolerogenic agent that could handle various stages of transplant rejection.

CTLA-4 · FasL was designed with both inhibitory FasL signaling and B7 blockade in mind. Combining the two seemed especially appropriate, given evidence that costimulator blockade can increase the sensitivity of T cells to Fas-mediated apoptosis B7 (15, 17). However, in reality, it is difficult to decipher the exact extent to which CTLA-4-mediated blockade is actually potentiating FasL-mediated inhibition, and it remains possible that the primary role of CTLA-4:B7 binding is merely to anchor FasL to the membrane. Several observations bear upon this issue, and the question of whether FasL *trans* signaling is the dominant factor here. First, CTLA-4 · FasL shows efficacy at molar concentrations substantially below (10- to 300-fold lower) those required for effective CTLA-4 · Ig costimulator blockade (generally 2.5 μg/ml). Yet, this does not in and of itself preclude the possibility of costimulator blockade occurring in this setting, because CTLA-4 · FasL could in principle be more effective as a costimulator blocker, due to any number of reasons (e.g., more favorable cell surface distribution or conformational properties, somehow shifting the balance of low affinity CD28 vs high affinity CTLA-4 triggering). Second, when added during primary stimulation, exogenous IL-2, which is expected to overcome any costimulation blockade, does not interfere with CTLA-4 · FasL-induced hyporesponsiveness to restimulation.

Membrane association is apparently a critical functional feature of CTLA-4 · FasL. CTLA-4 · Ig and sFasL in combination did not yield the same level of 1° MLR inhibition as did CTLA-4 · FasL, even when the former two were used at a 10-fold higher molar concentration. Similar results were obtained previously when CTLA-4 · FasL was compared with CTLA-4 · Ig plus sFasL for blocking anti-CD3 mAb stimulation of human T cells (19). Evidence offered in that earlier study (e.g., potentiation by B7-1-bearing cells) attributed the enhanced activity of CTLA-4 · FasL to its membrane-associating ability. Moreover, other findings in the literature suggest that membrane-anchored FasL is more effective than its soluble counterpart (12, 14, 17).

Systemic administration of anti-Fas mAb and sFasL has been associated with severe hepatotoxicity (27, 28). However, cell membrane-associated FasL, as for example on FasL-APC, is well tolerated and has no liver toxicity. Due to its membrane-binding property, CTLA-4 · FasL offers the possibility of avoiding toxicities associated with other soluble FasL derivatives. Indeed, in preliminary experiments, the s.c. and i.v. injection of up to 120 μg/mouse his_6CTLA-4 · FasL was found to be well tolerated (data not shown). More extensive studies will be required to fully evaluate the in vivo potential and toxicities of this multifaceted fusion protein.

Several mechanisms (anergy, clonal deletion, cell-mediated immunoregulation) have been invoked to explain alloantigen-specific hyporesponsiveness in different systems. In the case of CTLA-4 · FasL, more than one mechanism is apparently operative. The finding that partial responsiveness is restored when exogenous IL-2 is added during the secondary MLR is consistent with an anergic mechanism of action for CTLA-4 · FasL. The anergic component of CTLA-4 · FasL-induced hyporesponsiveness is most prominent at lower doses (30 ng/ml) of CTLA-4 · FasL, because IL-2 was less effective at reversing hyporesponsiveness at higher doses (>100 ng/ml).

The irreversible component of the observed CTLA-4 · FasL-induced hyporesponsiveness is likely to reflect clonal deletion, although proving this will require comprehensive in vivo systems. The present findings are consistent with a recent model for peripheral tolerance induction that entails the early activation and subsequent apoptotic death of alloreactive T cells (18, 24). The deletion of allospecific T cells in this way leaves significantly reduced numbers of potentially graft-reactive T cells in the recipient, which can then be more readily controlled by other mechanisms (e.g., suppressor/regulatory cells). Significantly, CTLA-4 · FasL did not interfere with early T cell activation events per se, given normal expression kinetics for the activation markers CD69 and CD95, albeit with delayed expression kinetics for CD25. Moreover, multicolor flow cytometric analyses showed that most of those cells undergoing division died, consistent with deletion of alloreactive clones.

There was no evidence in the present study for CTLA-4 · FasL-inducing regulatory/suppressor cells. By secreting immunoregulatory cytokines, including IL-10 and TGF-β, immune-deviated Th2 cells have been shown to effectively suppress alloactivation (29). In addition, Fas-dependent immunoinhibition has been linked to the secretion of IL-10 (30, 31). However, our findings did not point to a a direct involvement of either IL-10 or TGF-β in CTLA-4 · FasL-mediated inhibition of 1° MLR. Furthermore, treatment with CTLA-4 · FasL inhibited the accumulation of intracellular IL-10 in anti-CD3-stimulated human peripheral T cells (W. Schmidt and J. Huang, unpublished observations).

Of note, in the case of 2° MLR, TGF-β-neutralizing Ab (but not IL-10-neutralizing Ab) did partially restore 2° MLR responses of cultures preincubated with CTLA-4 · FasL. However, this effect of TGF-β-neutralizing Ab in 2° MLR cultures was not specifically tied to CTLA-4 · FasL, with anti-TGF-β Ab enhancing observed responses regardless of the presence or absence of CTLA-4 · FasL. This enhancement suggests the emergence of TGF-β-secreting suppressor/regulatory cells after 1° MLR stimulation, which are apparently not negatively impacted by the addition of CTLA-4 · FasL. Additional experiments will be required to determine whether immunoregulatory cells contribute to the TGF-β-dependent immunomodulation observed in restimulation assays.

There are some intriguing aspects to CTLA-4 · FasL-mediated alloinhibition. First, as discussed above, both anergy induction and clonal deletion are operative. Second, the kinetics of inhibition are notable. T cells responding to allogeneic stimulation up-regulate Fas receptor within 24–48 h (32, 33) (Fig. 6*C*), but resist Fas-mediated apoptosis until day 5. Yet, we observed CTLA-4 · FasL-mediated inhibition as early as [^{3}H]thymidine incorporation could be detected (usually the second day of the 1° MLR). We now have evidence that this earlier-than-expected inhibition results from proliferative inhibition that is dependent upon the FasL component (J.-H. Huang et al., data not shown). Future experiments will seek to decipher the functional web connecting the antiproliferative and apoptosis-inducing activities (which are possibly temporally dissociated) of CTLA-4 · FasL, its FasL-signaling and B7-blocking potentials, and its dual capacity to effect anergy and clonal deletion. The mechanistic possibilities point to the functional richness that may arise within TSCPs that integrate more than one immunoregulatory function into a single fusion protein. Moreover, such TSCP, and CTLA-4 · FasL as a paradigmatic TSCP, can now be evaluated for their immunotherapeutic potential in alloimmune disease models.

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