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CD40·FasL inhibits human T cells: evidence for an auto-inhibitory loop-back mechanism

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Abstract

A chimeric CD40·FasL (CD40–CD95L) protein was designed with the combined capacities to bind to two surface receptors on activated T cells, CD40 ligand (CD40L; CD154) and Fas receptor (CD95). CD40·FasL, once tethered to the cell surface via one of its ends, can transmit a signal via its other end. In principle, simultaneous triggering from both ends is possible, and thus there is the intriguing potential for 'auto-inhibition' if such dual triggering occurs on the same cell itself. Several lines of evidence support this mechanism: (i) CD40·FasL is cytotoxic to Fas receptor-positive cell lines of different cell lineages, (ii) CD40·FasL's function is potentiated when there is enforced expression of CD40L on target cells, (iii) CD40·FasL inhibition does not require intercellular contact, as demonstrated by soft agar clone formation and cell dilution analysis and (iv) introduction of exogenous CD40 into the system interferes with CD40·FasL inhibition. Taken together, these data are consistent with a 'loop-back' inhibitory mechanism within individual activated (CD40L and Fas receptor expressing) T cells causing suicide of these T cells. Significantly, this type of fusion protein provides a unique way to confine immunoinhibition to activated T cells.

Introduction

T cell activation requires a primary antigenic stimulus, in the form of MHC–antigen complexes, along with a secondary co-stimulus (1). One strategy for attenuating T cell responses relies upon passive blockade of co-stimulatory signals, for example, by using soluble CTLA-4 derivatives to interfere with B7–CD28 co-stimulation (2). An alternative strategy invokes active T cell inhibition, via the enforced expression on antigen-presenting cell (APC) surfaces of ligands that can trigger T cell inhibitory receptors, such as Fas receptor and MHC class I (3–8). Such co-inhibitor-expressing 'deletional' APCs can induce hyporesponsiveness of T cells to antigenic stimulation and clonal deletion of antigen-specific T cells (7, 8).

Previously, we described a novel fusion protein, CTLA-4·FasL, that functionally integrates these alternative T cell inhibitory strategies (9). CTLA-4·FasL has the complementary capacities to block co-stimulation (via its CTLA-4 domain binding to the B7 co-stimulator on APC) and simultaneously provide a source of surface-anchored FasL that can deliver apoptotic signals through its Fas counter-receptor on T cells. In fact, this paradigmatic '*trans* signal converter protein (TSCP)' is converting a stimulatory intercellular (*trans*) signal into an inhibitory one, and has remarkably higher potency than either CTLA-4-Ig or soluble FasL, alone or in combination, in blocking *in vitro* alloresponses and inducing allo-antigen-specific hyporesponsiveness upon re-stimulation (10). CTLA-4·FasL expressed via an adenoviral vector prevents the development of autoimmune diabetes (11).

The efficacy of the CTLA-4·FasL TSCP has prompted us to devise other types of bi-functional fusion proteins with T cell inhibitory capacities. In the present study, we describe one such protein, CD40·FasL. CD40 ligand (CD40L; CD154) is neo-expressed on T cell surfaces upon activation, and is found on the T cells of acute inflammatory infiltrates (12). CD40L functions as an activating receptor on T cells, driven by its CD40 counter-receptor on APCs (13–15). However, signaling through CD40L also promotes apoptosis of activated T cells (16). Thus, a soluble CD40·FasL fusion protein would be expected to combine a number of interesting properties, such as blocking endogenous CD40–CD40L-mediated co-stimulation and at the same time rendering T cells more sensitive to activation-induced apoptosis. The latter would be achieved not only through artificial

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CD40L-mediated signals but also through Fas receptors that become active upon T cell activation (17, 18).

Interestingly, while CD40·FasL can bridge CD40L and Fas receptor proteins on neighboring cells, there is also the theoretical possibility that this fusion protein could bridge these two surface receptors when situated on the same cell. This creates the potential for auto-inhibition, since negative signals could be triggered via both ends of the chimeric CD40·FasL protein. The present study demonstrates the efficacy of the CD40·FasL protein and offers experimental support for this type of *cis* auto-inhibitory 'loop-back' mechanism.

Methods

Reagents, antibodies and cells

Cell culture media (RPMI-1640, DMEM and DCCM-1), FCS, glutamine and penicillin/streptomycin were purchased from Biological Industries, Beit Haemek, Israel. G418 was purchased from Calbiochem and protein A-agarose from Sigma–Aldrich, Israel. Antibody specific for FasL (NOK-1), antibody specific for human CD40 (5C3) and fluorescent antibody specific for CD40, CD40L, CD80, CD86 and CD95 were purchased from PharMingen (San Diego, CA, USA). Goat anti-CD40 was purchased from R&D Systems (Minneapolis, MN, USA). Agonistic Fas-specific antibody (CH-11) was purchased from MBL (Cambridge, MA, USA). A commercial soluble FasL ELISA was purchased from Bender BioSciences (Vienna, Austria). Daudi, Raji and 293 human kidney cells were originally obtained from the American Type Culture Collection (ATCC) (Bethesda, MD, USA). Two Jurkat sublines (J-CD40L$^+$ and J-CD40L$^-$) and NIH 3T3 transfectants were kindly provided by John Fayen (CWRU, Cleveland, OH, USA).

CD40·FasL production

cDNA sequence encoding the extracellular domain of human FasL (amino acids 127–281) was amplified by PCR using 5′-ATCAAGCTTGGAGAAGCAAATAGGC-3′ and 5′-TTTGGATCCTTAGAGCTTATATAAGCCGAA-3′ as 5′ and 3′ primers, respectively. Primers were purchased from Genosys (Woodlands, TX, USA). The PCR product was digested with *Hin*dIII and *Bam*HI and sub-cloned into the respective sites of our human EBV episomal expression vector pCEP9β (incorporating the cytomegalovirus promoter), generating pFasL-CEP9β. Next, cDNA sequence encoding the extracellular domain of human CD40 (amino acids 1–193) was amplified by PCR using 5′-CGGGGTACCATGGTTCGTCTGCCTCT-3′ and 5′-CCCCAAGCTTCTCAGCCGATCCTGCGGA-3′ as 5′ and 3′ primers, respectively. The PCR product was digested with *Hin*dIII and *Kpn*I and was sub-cloned into the respective sites of pFasL-CEP9β to generate pCD40-FasL/CEP9β.

pCD40-FasL/CEP9β was introduced into 293 cells using Lipofectin reagent (Life Technologies, Bethesda, MD, USA). Stable transfectants were selected using 0.4 mg dl^{-1} G418 in serum-supplemented DMEM. For protein production, expressors were grown in serum-free media (DCCM-1), and conditioned media were collected every 3–4 days. Immunoaffinity purification was performed with protein A agarose beads prepared by sequentially adding rabbit anti-mouse Ig (Dako, Glostrup, Denmark) and anti-human FasL mAb (NOK-1), followed by chemical cross-linking with dimethyl pimelimidate dihydrochloride (Sigma–Aldrich). Recombinant protein was eluted in 0.1 M sodium citrate, pH 2.5, and neutralized with 1 M NaH_2PO_4. Eluted fractions were concentrated using a Centicon-30 (Millipore, Bedford, MA, USA) into PBS. To verify protein size and multimerization, western blot analysis was performed (Fig. 1). In brief, samples for SDS–PAGE analysis were prepared in Laemmli sample buffer, with or without 2-mercaptoethanol, and were boiled prior to loading onto 10% SDS–PAGE. Following electrophoresis, gels were blotted onto Protran BA cellulosenitrate membranes (Schleicher & Schuell, Dassel, Germany), blocked with 5% milk/PBS and probed with rabbit anti-human FasL as primary antibody (Chemicon International). After extensive washing, blots were incubated with HRP-conjugated goat anti-rabbit antiserum (BioRad Laboratories, Hercules, CA, USA) and developed with enhanced chemiluminescent substrate (NEN, Boston, MA, USA) prior to exposure to X-ray film. A commercially available ELISA (Bender, Grünberg, Germany) was used to quantify CD40·FasL. Purified CD40·FasL was stored frozen at −80°C, and thawed immediately prior to use. The production of his_6CTLA-4·FasL has been described elsewhere (10).

Cytotoxicity assays

Cytotoxicity assays were performed in triplicate as described (19), with a few modifications. Jurkat, Daudi or Raji cell lines in exponential growth phase were labeled at 37°C for 4–5 h with 3 μCi ml^{-1} of [^{3}H]thymidine ([^{3}H]TdR) in RPMI-1640 medium supplemented with glutamine, streptomycin, penicillin and 10% FCS (R-10). Cells were washed

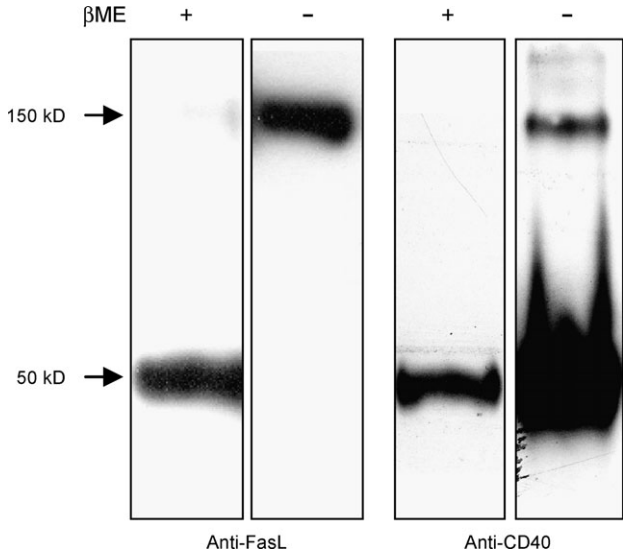

Fig. 1. CD40·FasL forms trimers. Western blot analysis was performed, in which samples of immunopurified protein were prepared in Laemmli sample buffer, with (left lane) or without (right lane) 2-mercaptoethanol as reducing agent, separated on 10% SDS–PAGE, and after transfer to Immobilon P membranes, probed with anti-human FasL mAb or anti-human CD40 as primary antibody and HRP-conjugated goat anti-rabbit antiserum as secondary antibody.

twice and re-suspended in the same medium at 2×10^6 ml^{-1}, and 0.1 ml of the cell suspension was added to each well of round-bottom 96-well tissue culture plates. Clarified CD40·FasL-containing supernatants, purified CD40·FasL or his_6CTLA-4·FasL were diluted into R-10, and 0.1 ml of each diluted sample was added to individual wells. Anti-CD40 mAb (5C3; PharMingen) was added at 0.1–10 μg ml^{-1} to indicated samples. Anti-Fas mAb (CH-11) was used as positive control. In each experiment, a medium-only control was used to determine the spontaneous loss of radiolabeled DNA. In some experiments, wells were pre-coated with CD40L- or CD8-expressing 3T3 NIH cells. For other experiments, flat-bottom plates were pre-coated with anti-CD40 antibodies (R&D Systems). Cultures were incubated for 17–20 h at 37°C, and radiolabeled DNA was then harvested onto glass filters for scintillation counting. 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.

Proliferation assays

Jurkat, Daudi or Raji cells in exponential growth phase were washed twice and re-suspended in R-10 at 1×10^6 cells ml^{-1}. A 50 μl of this cell suspension was added to individual wells of round-bottom 96-well tissue culture plates. CD40·FasL, CTLA-4·FasL, or anti-Fas antibody (CH-11) were added at different concentrations. In some experiments, irradiated CD40L-expressing or non-expressing Jurkat cells, CD40-expressing Daudi cells or 3T3 NIH cells expressing CD40L or CD8 were added at different ratios to the wells. Assays were performed in triplicate in round-bottom wells (or round- and flat-bottom wells for 3T3 NIH cell derivatives). Culture wells were pulsed with 25 μl of R-10 containing 0.5 μCi of [^{3}H]TdR for 18 h and then harvested onto glass fiber filters for scintillation counting.

PBMCs were isolated from healthy volunteers by Ficoll-Hypaque (Pharmacia Biotech, Israel) and re-suspended in R-10. PBMCs were plated at 1×10^5 in 96-well round-bottom tissue culture plates in a total volume of 200 μl per well. Medium or CD40·FasL at different concentrations were added 20 min prior to the addition of different concentrations of agonistic anti-CD3 antibody (OKT3), originally obtained from ATCC. Assays were performed in triplicate. Cultures were incubated at 37°C, 5% $C0_2$ and 95% humidity. At 54 h, culture wells were pulsed with 0.5 μCi of [^{3}H]TdR for 18 h and then harvested onto glass fiber filters for scintillation counting.

Soft agar colony formation

CD40L-expressing or non-expressing cells (5×10^2 cells ml^{-1}) were seeded in 2.5 ml 0.3% agarose containing 10% R-10, with or without varying concentrations of CD40·FasL or his_6CTLA-4·FasL, and this suspension was overlaid over 2.5 ml 0.6% agarose in 35-mm plates. After culturing for 3 weeks, numbers of colonies (>100 μM) per plate were counted.

Flow cytometry

Cells were washed twice with FACS buffer (1× PBS/0.5% FCS/0.01% sodium azide) and incubated on ice for 30–45 min with PE-conjugated anti-CD40L or anti-CD40 antibody, FITC-conjugated anti-CD95, -CD80 or -CD86 or their matching isotype controls (PharMingen). Fluorescence-labeled cells were analyzed on a FACScan, using a CellQuest software package (Becton Dickinson, Mountain View, CA, USA). For apoptosis detection, PBMCs were stained with annexin–propidium iodide (PI) kit (MBL) according to the manufacturer's instructions and analyzed by FACScan using a CellQuest software package.

Results

CD40·FasL induces cytotoxicity that is Fas dependent

The ability of CD40·FasL to induce cytotoxicity was first tested using as targets EBV-transformed B cell lines differing in Fas receptor expression. Fas receptor expression was verified on Raji B cells by immunofluorescence and flow cytometry, with Daudi cells demonstrating negativity (Fig. 2A). Both cell lines were negative for surface CD40L staining (data not shown). Dose-dependent killing of the Fas$^+$ Raji cells, ranging from 20 to 40% cell death, was observed after 16 h of incubation with CD40·FasL (Fig. 2B). In contrast, no cell death was observed under the same conditions for the Fas$^-$ Daudi cells. As an additional negative control, no

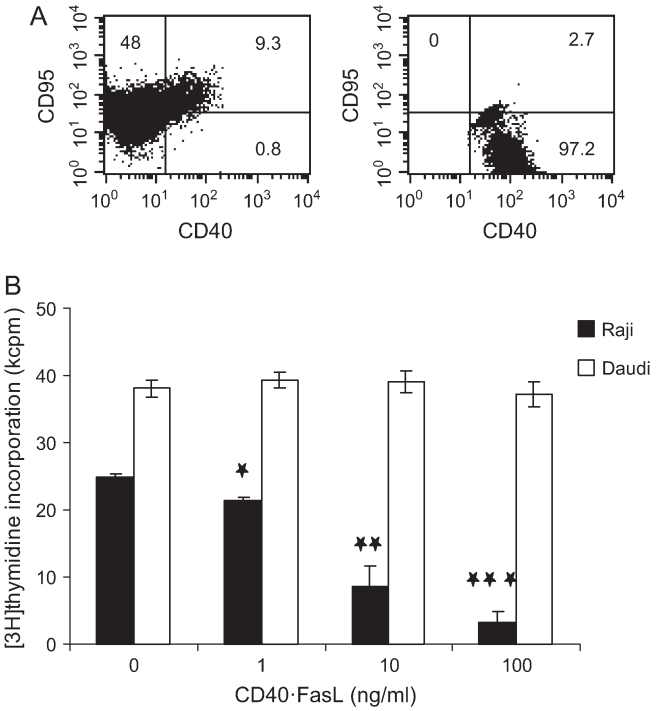

Fig. 2. Inhibition of proliferation by CD40·FasL is dependent on Fas receptor expression. (A) The EBV-transformed B cell lines Raji (left panel) and Daudi (right), differing in Fas receptor expression, were stained with PE-conjugated anti-CD40 and FITC-conjugated anti-CD95 (Fas receptor) mAb. (B) Raji or Daudi cells (10^5) were plated in round-bottom, 96-well plates in the presence or absence of the indicated concentrations of CD40·FasL. Assays were performed in triplicate. Cells were pulsed with [^{3}H]TdR, incubated at 37°C for 18 h and then evaluated for [^{3}H]TdR incorporation. Data are presented as mean ± SD. *$P < 0.01$ versus no CD40·FasL, **$P < 0.001$ versus no CD40·FasL and ***$P < 0.0005$ versus no CD40·FasL.

CD40·FasL-induced death was observed for 3T3 NIH cells, which also do not express Fas receptor (data not shown). These results established the functionality of the FasL component of the CD40·FasL fusion protein.

Efficacy of CD40·FasL is enhanced when CD40L and Fas are co-expressed on Jurkat targets

We next used variants of a Fas$^+$ human T cell line (Jurkat) differing in CD40L expression to assess the role of the CD40 component of CD40·FasL. Approximately 2% of wild-type Jurkat cells constitutively express CD40L (data not shown). Two Jurkat sublines, J-CD40L$^+$ and J-CD40L$^-$, comprise 96 and 0.6% CD40L-expressing cells, respectively (Fig. 3A, left and right panels, respectively). The wild-type Jurkat cells and the two Jurkat sublines do not express CD40 (data not shown), and expressed similar levels of Fas on a per-cell basis, with 96 and 98.3% of J-CD40L$^+$ and J-CD40L$^-$ cells, respectively, displaying surface Fas. Both sublines do not express either CD80 or CD86 (data not shown). While CD40·FasL induced cytotoxicity in all three Jurkat populations in a dose-dependent fashion, cell death was dramatically greater for the Jurkat subline enriched for CD40L expression (Fig. 3B). The increased CD40·FasL-mediated cell death observed for the J-CD40L$^+$ subline, as compared with the J-CD40L$^-$ subline and wild-type Jurkat cells, is all the more striking given that the former were less sensitive to anti-Fas antibody (CH-11)-induced cytotoxicity (data not shown). Furthermore, his$_6$CTLA-4·FasL, that acts as soluble FasL in this system as no B7 is present, was more efficient in death inducing in J-CD40L$^-$ subline than in the J-CD40L$^+$ subline. Thus, CD40L co-expression on Fas$^+$ T cell targets substantially augments the activity of CD40·FasL against them.

Antibody blocking was used to further verify that the CD40 domain of CD40·FasL contributes to its function. To this end, CD40·FasL-mediated cytotoxicity against the J-CD40L$^+$ subline was assessed in the presence or absence of an anti-CD40 mAb, 5C3. As shown in Fig. 4, cytotoxicity was substantially blocked with this anti-CD40 mAb, with 30% inhibition at the maximal concentration used.

One possible explanation for enhanced CD40·FasL killing when CD40L is present on the Jurkat T cells is that anchoring of CD40·FasL to cell-surface CD40L increases the valency of the former and its consequent ability to send inhibitory FasL–Fas signals intercellularly to neighboring cells. To test this possibility, we pre-adhered CD40L-expressing NIH 3T3 transfectants (Fig. 5A) to confluence on 96-well flat-bottom plates, and then asked whether CD40L presented in this way amplifies the inhibitory function of added CD40·FasL against J-CD40L$^-$ cells. Significantly, the presence of CD40L-expressing NIH 3T3 cells in the co-culture did not enhance CD40·FasL-induced killing of J-CD40L$^-$ cells, as compared with negative control CD8-expressing NIH 3T3 transfectants (Fig. 5B, upper panel, left). CD40L$^+$ (or CD8$^+$) NIH 3T3 transfectants also did not alter the killing of J-CD40L$^+$ cells as well (Fig. 5B, upper panel, right). As expected, CD40L- or CD8-expressing NIH 3T3 cells did not affect the killing of J-CD40L$^+$ or J-CD40L$^-$ cells by soluble his$_6$CTLA-4·FasL, which serves as a control since it is not attached to the NIH 3T3 cells (Fig. 5B, lower panel, left and right, respectively).

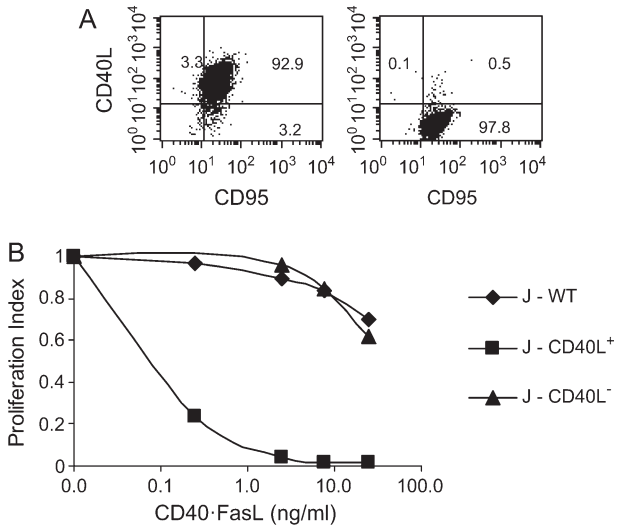

Fig. 3. Inhibition of proliferation by CD40·FasL is dependent on CD40L expression. (A) The Jurkat sublines J-CD40L$^+$ (left panel) and J-CD40L$^-$ (right panel) were immunostained with PE-conjugated anti-CD40L mAb and FITC-conjugated anti-CD95 mAb. (B) Wild-type, J-CD40L$^+$ and J-CD40L$^-$ cells (10^5) were plated in round-bottom, 96-well plates in the presence or absence of the indicated concentrations of CD40·FasL. Assays were performed in triplicate. Cells were pulsed with [^{3}H]TdR and incubated at 37°C for 18 h, and then evaluated for [^{3}H]TdR incorporation. Data are presented as percentage of maximal proliferation (proliferation index). The *x*-axis is on a logarithmic scale.

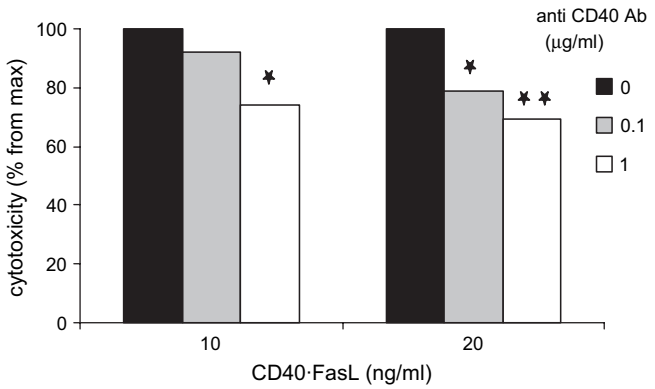

Fig. 4. CD40·FasL-induced cytotoxicity is blocked by anti-CD40 mAb. CD40·FasL was pre-incubated with different concentrations of anti-CD40 mAb for 1 h at 4°C. J-CD40L$^+$ cells were pre-labeled with [^{3}H]TdR for 4.5 h, washed, plated (10^5 cells per well) in the presence or absence of different combinations of CD40·FasL and anti-CD40 mAb, incubated for 18 h at 37°C and evaluated for cell killing. Data are presented as percentage of maximal cytotoxicity. Assays were performed in triplicate. *$P < 0.05$ versus no antibodies and **$P < 0.01$ versus no antibodies.

As an alternative approach, we performed cytotoxicity assays in which labeled J-CD40L$^-$ Jurkat targets were combined with varying concentrations of J-CD40L$^+$ cells. The question was whether addition of the latter would promote CD40·FasL-mediated killing of the former. Only marginal enhancement (~15–20%) was observed even at the highest

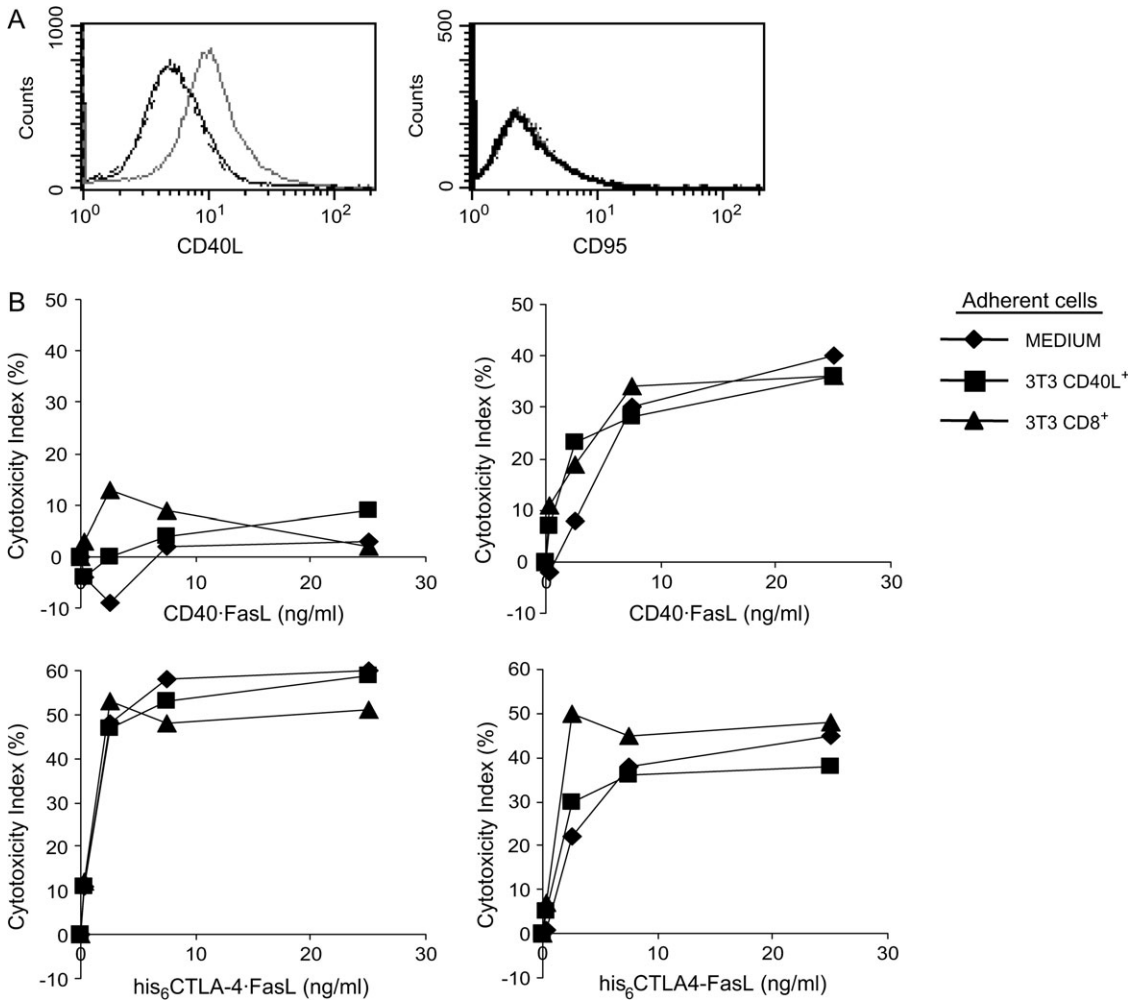

Fig. 5. CD40·FasL-induced cytotoxicity is not affected by the presence of CD40L-expressing cells. (A) 3T3 NIH transfectants expressing either CD40L or CD8 were immunostained with either PE-conjugated anti-CD40L mAb (left panel) or FITC-conjugated CD95 mAb (right panel), and analyzed by FACS. (B) 3T3 NIH transfectants expressing either CD40L or CD8 were pre-incubated in flat-bottom, 96-well plates for 24 h at 37°C. J-CD40L$^+$ (left panels) and J-CD40L$^-$ (right panels) cells were labeled with [^{3}H]TdR for 4.5 h, washed and then added to the 3T3 NIH pre-coated wells, in the presence or absence of the indicated concentrations of CD40·FasL (upper panels) or his$_6$CTLA-4·FasL (lower panels). After incubation for 18 h at 37°C, wells were evaluated for cell killing. A representative experiment from four independent experiments is shown. Assays were performed in triplicate.

ratio (data not shown). Taken together, these data argue that the role of cell-surface CD40L in this system is not principally to augment the capacity of CD40·FasL to transmit intercellular signals.

We also used plate-bound agonistic anti-CD40 antibodies in order to immobilize the protein to the plate. No enhancement of its activity on the J-CD40L$^-$ subline was noted. In fact, immobilizing CD40·FasL results in significant (50%) inhibition of its cytotoxic effect on J-CD40L$^+$ subline (data not shown).

CD40·FasL inhibits colony formation of CD40L-expressing Jurkats suspended in soft agar

Given data pointing away from a dominant contribution of '*trans*' intercellular signaling in CD40·FasL function, we entertained the alternative possibility that CD40L promotes CD40·FasL function by anchoring this fusion protein onto cell surfaces and enabling triggering of Fas receptor on the same cell. This would constitute a *cis* auto-inhibitory mechanism predicated on what can be termed a loop-back feature. This mechanism of action would be especially appealing in contexts where intercellular interactions among pathogenic T cells may be limited.

To test whether such an auto-inhibitory mechanism may be operative, we turned to an experimental system in which intercellular contacts are minimized. To this end, we determined whether CD40·FasL is capable of inhibiting colony formation of J-CD40L$^+$ cells in soft agar. As depicted in Fig. 6(A), CD40·FasL inhibited colony formation of J-CD40L$^+$ cells at low concentrations which did not affect J-CD40L$^-$ colony formation. This difference was all the more striking given that a soluble FasL-containing fusion protein, his$_6$CTLA-4·FasL, inhibited the colony formation of J-CD40L$^-$ to an even greater extent than J-CD40L$^+$ (Fig. 6B). his$_6$CTLA-4·FasL is considered soluble in this system since Jurkat cells

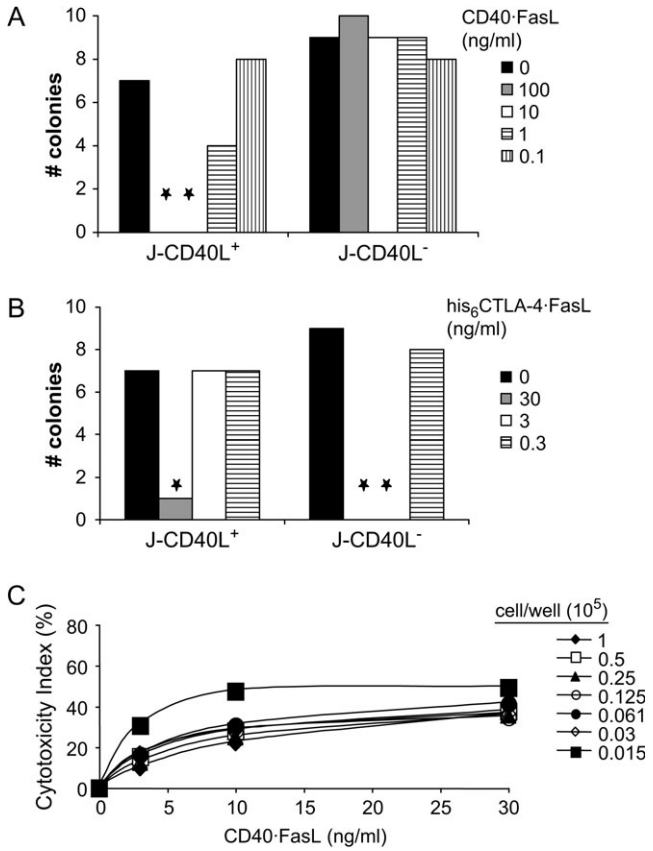

Fig. 6. CD40·FasL inhibits J-CD40L$^+$ colony formation in soft agar. J-CD40L$^+$ (left columns) or J-CD40L$^-$ (right columns) cells (0.5×10^3 cells ml^{-1}) were seeded in 2.5 ml 0.3% agarose, in the presence of the indicated concentrations of CD40·FasL (A) or his$_6$CTLA-4·FasL (B). This cell suspension was overlaid on 2.5 ml 0.6% agarose in 35-mm plates. After culturing for 3 weeks, colonies were counted. A representative experiment of three independent experiments is shown. (C) J-CD40L$^+$ cells were labeled with [^{3}H]TdR for 4.5 h, serially diluted from 10^5 to 10^3 cells per well in the presence or absence of the indicated concentrations of CD40·FasL, incubated at 37°C for 18 h and evaluated for cell killing. The cytotoxicity index was calculated as described in Methods. A representative experiment from four independent experiments is shown. Assays were performed in triplicate. *$P < 0.01$ versus control.

do not bear B7 on their surfaces, and therefore do not bind the his$_6$CTLA-4·FasL.

We queried the *cis* auto-inhibition model for CD40·FasL function from yet another experimental angle, invoking another set-up that minimizes intercellular contacts. [^{3}H]TdR-labeled J-CD40L$^+$ and J-CD40L$^-$ cells were serially diluted from 10^5 to 10^3 cells per well (in 96-well plates), and then incubated in the presence or absence of CD40·FasL (or his$_6$CTLA-4·FasL as positive control) at varying concentrations. Cytotoxicity was assessed after 18 h of incubation. Significantly, CD40·FasL killing of J-CD40L$^+$ cells was not diminished by successive cell dilution (Fig. 6C). Killing of J-CD40L$^-$ cells by CD40·FasL or his$_6$CTLA-4·FasL, functioning as soluble FasL derivatives triggering the Fas receptor, was also not affected by cell dilution, as one would expect (data not shown). Of note, at the lowest cell concentration used here (10^3), virtually no proliferation was observed, even in the presence of preconditioned medium, supporting the sufficiency of the cell dilution incorporated into this experiment (data not shown). This cell concentration independence of CD40·FasL inhibitory function reinforces the notion that cell-to-cell contact is not a prerequisite for this inhibition and that interaction with both CD40L and Fas receptor on the same cell is a key determinant.

Evidence that CD40·FasL acts via a loop-back mechanism

The soft agar suspension and cell dilution experiments constitute evidence that CD40·FasL can inhibit single cells independent of cell contact. There are two mechanistic models that could explain this observation. One model is that CD40·FasL anchors to CD40L on the cell surface and loops back to engage neighboring Fas receptors. According to this model, there is the potential for signaling not only via the Fas receptor but also through CD40L. Of interest in this regard, anti-CD40L antibody triggering induces early T cell proliferation, but later renders these cells more sensitive to Fas-mediated death (16). An alternative model is that CD40L and Fas are being triggered in parallel by distinct CD40·FasL molecules, with no molecular bridging *per se*. To distinguish between these molecular bridging versus non-bridging models, we asked whether addition of an exogenous source of CD40 interferes with or potentiates CD40·FasL inhibition. If the loop-back mechanism is operative, one would expect extraneous CD40 to interfere with CD40·FasL inhibition, whereas if there is simply parallel bimolecular signaling, extraneous CD40 might well promote inhibition.

To this end, irradiated EBV-transformed, CD40$^+$ Fas$^-$ Daudi B cells were co-cultured with J-CD40L$^+$ or J-CD40L$^-$ at varying cellular ratios. These Daudi cells provided an exogenous source of CD40 for triggering of the CD40L on the Jurkat cells. At all CD40·FasL concentrations tested, added Daudi cells interfered with inhibition of J-CD40L$^+$ proliferation in a dose-dependent fashion (Fig. 7, upper panel, gray bars). In contrast, addition of Daudi B cells which are B7-1 positive and hence can bind CTLA-4 potentiated negative signaling induced by CTLA-4·FasL, as expected for a fusion protein functioning intercellularly in *trans* (Fig. 7, upper panel, white bars; [9]). Adding Daudi cells did not change CD40·FasL's inability to inhibit significantly J-CD40L$^-$ proliferation, but as expected, it enhanced CTLA-4·FasL-mediated inhibition of these cells (Fig. 7, lower panel). Thus, these data favor the existence of a loop-back mechanism for CD40·FasL.

CD40·FasL inhibits activated T cells

Looking beyond Jurkat cell line variants, we determined whether CD40·FasL can inhibit the proliferation of anti-CD3 mAb-activated T cells within a PBMC pool. Significant dose-dependent inhibition of proliferation was observed (Fig. 8A), and at a concentration range (1–100 ng ml^{-1}) that pointed to its considerably greater potency than CD40–Fc, which requires cross-linking for efficacy, and is active at the microgram per milliliter range (20). This inhibition was observed only when CD40·FasL was added at the start of the reaction. If added 24 h prior to stimulation, no inhibition was observed (data not shown), suggesting that cells have to be activated in order to become sensitive to CD40·FasL.

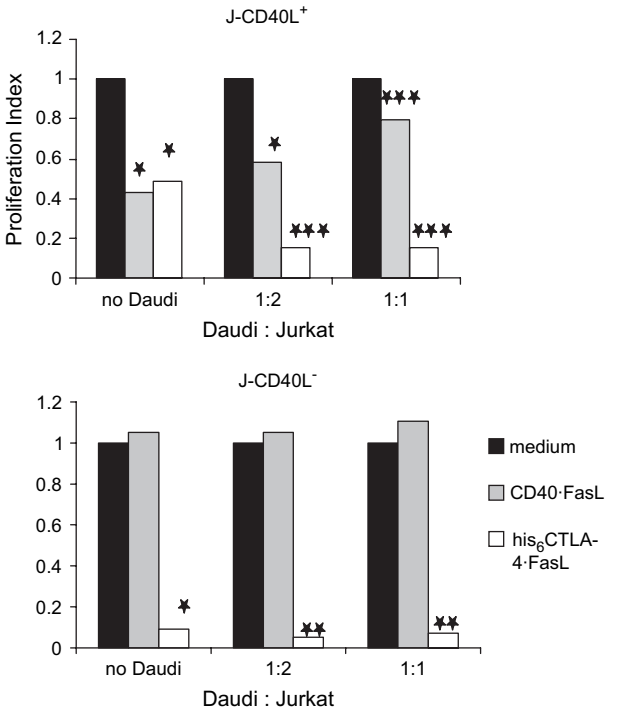

Fig. 7. CD40-expressing Daudi cells interfere with CD40·FasL-mediated inhibition of J-CD40L$^+$ proliferation. J-CD40L$^+$ (upper panel) and J-CD40L$^-$ (lower panel) cells (10^5) were mixed at varying ratios with irradiated (21 000 rads) Daudi cells, plated in the presence or absence of CD40·FasL (10 ng ml^{-1}) or his$_6$CTLA-4·FasL (10 ng ml^{-1}), pulsed with [^{3}H]TdR at 37°C for 18 h and evaluated for [^{3}H]TdR incorporation. Data are presented as percentage of maximal proliferation (proliferation index). A representative experiment from three independent experiments is shown. Assays were performed in triplicate. * $P < 0.005$ versus control, ** $P < 0.05$ versus no Daudi added and *** $P < 0.005$ versus no Daudi added.

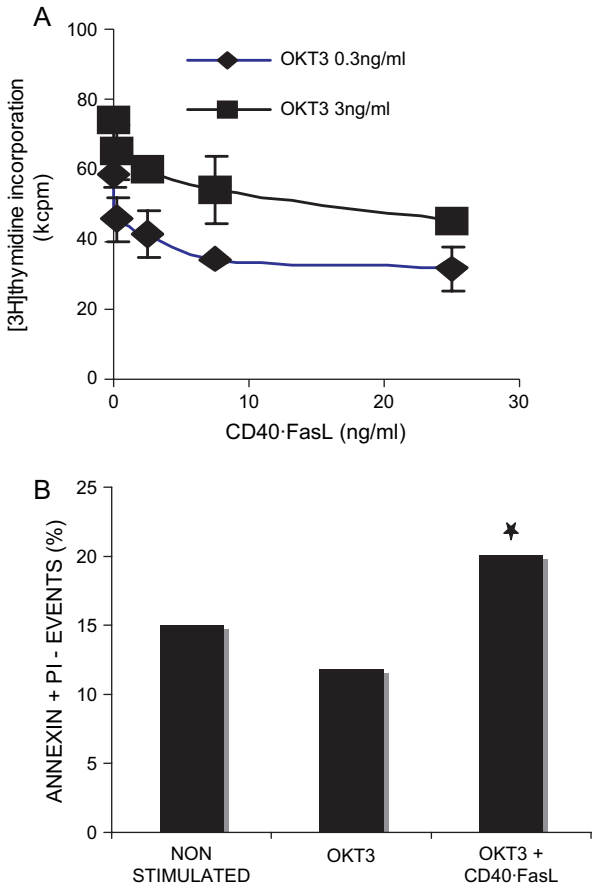

Fig. 8. CD40·FasL inhibits proliferation of anti-CD3 mAb-stimulated human PBMC. (A) CD40·FasL at the indicated concentrations was added to PBMC (1×10^5 per well) 20 min prior to stimulation with agonistic anti-CD3 mAb. At 54 h of culture, cells were pulsed with [^{3}H]TdR for 18 h and then evaluated for [^{3}H]TdR incorporation. Data are presented as mean ± SD. A representative experiment from five independent experiments is shown. Assays were performed in triplicate. (B) CD40·FasL at 30 ng ml^{-1} was added to PBMC (1×10^5 per well) 20 min prior to stimulation with agonistic anti-CD3 mAb (0.3 ng ml^{-1}). At 72 h of culture, cells were collected, stained with annexin and PI and analyzed by flow cytometry. Percentage of apoptotic, non-necrotic cells are presented. * $P < 0.05$ versus control.

Several reasons can account for the lack of T cell response in our system, one of which is induction of apoptosis. In order to verify that the activated cells undergo apoptosis in the presence of CD40·FasL, PBMCs were activated with anti-CD3 mAb in the presence or absence of CD40·FasL for 72 h and the percentage of apoptotic cells was evaluated by annexin–PI staining and flow cytometry analysis. The percentage of apoptotic cells (annexin$^+$, PI$^-$) increased while stimulation was done in the presence of CD40·FasL (Fig. 8B), supporting the likelihood that apoptosis mediates the protein's action.

Discussion

In this study, we describe CD40·FasL as a novel fusion protein designed for the preferential inhibition of activated T cells. Key findings include the following: (i) CD40·FasL is cytotoxic to cell lines of both T and B lineages, (ii) CD40·FasL's action is dependent on Fas receptor expression on target cells, (iii) CD40·FasL's inhibitory effect is potentiated by the presence of CD40L on target cells, (iv) soft agar clone formation and cell dilution analyses suggest that CD40·FasL inhibition, though potentiated by CD40L, does not require intercellular contact, (v) exogenous CD40 interferes with CD40·FasL inhibition, arguing for a novel loop-back mechanism leading to *cis* auto-inhibition and (vi) CD40·FasL can inhibit the proliferation of anti-CD3 mAb-activated primary human T cells. Thus, CD40·FasL emerges as an agent that has auto-inhibitory, suicide-inducing potential that is selective for activated T cells.

CD40·FasL inhibits CD40L-expressing Jurkat T cells with 1000-fold and 100-fold higher activity than it inhibits CD40L-non-expressing Jurkat T cells and Raji B cells, respectively. In principle, one possible explanation for this enhanced efficacy is that CD40L, which itself can exist in multimeric configurations, could serve to create higher order FasL signaling units. Indeed, it has been reported that FasL is most active when two trimers are conjoint (21), and it is thus possible that CD40L somehow brings together two or more CD40·FasL molecules into higher order units. However, the fact that neither CD40L-mediated tethering of CD40·FasL

nor the plate-bounded anti-CD40 binding of CD40·FasL augment its ability to inhibit in *trans* T cells lacking CD40L tends to argue against this higher order Fas arraying possibility. Also, arguing against it is our repeated demonstration that J-CD40L$^-$ cells were more sensitive than J-CD40L$^+$ cells to both an anti-Fas receptor antibody (CH-11) and the soluble his_6CTLA-4·FasL.The auto-inhibition mechanism, supported by various lines of evidence presented in the present study, provides an alternative explanation for the enhanced potency of CD40·FasL when CD40L is present on target cells. In this case, one argues that surface anchoring of the CD40·FasL (via CD40L engagement) forces it into proximity of neighboring Fas receptors on the same cells and achieves a higher functional affinity between them.

CD40·FasL features bi-functional potential in its dual capacities to signal not only through Fas receptor but also through CD40L. Agonistic anti-CD40L mAb can trigger intracellular T cell signaling pathways (22), and can co-stimulate T cells in conjunction with anti-CD3 mAb as first signal (16). Interestingly, CD40L signaling results in marked apoptosis of activated T cells, without inducing anti-apoptotic factors (Bcl-xL; Bcl-2) typically associated with CD28 co-stimulation. Thus, both ends of CD40·FasL have the inherent abilities to promote T cell apoptosis, with the intriguing possibility that CD40L triggering contributes to an 'apoptosis-prone' state which potentiates Fas receptor apoptotic signaling.

CD40·FasL's bi-functionality is expected to manifest only against activated T cell targets, sparing non-activated (naive and memory) T cells. In agreement with that, our results indicate that CD40·FasL function is dependent on simultaneous T cell activation. Activated T cells express both CD40L and Fas receptor. CD40L is expressed on T cells mostly after activation, and its expression on T cell surfaces is time limited (23). Fas receptor is up-regulated and becomes more functional after T cell activation (17, 18). An immunosuppressive agent that selectively targets activated T cells has special appeal for therapy of diverse allo- and autoimmune diseases. CD40L is over-expressed on T cells infiltrating kidney allografts during chronic rejection (24), as well as T cells of both F1 lupus-prone mice (25) and lupus patients (26). Thus, CD40·FasL offers potential therapeutic advantage in these and other clinical settings where CD40L is over-expressed on pathogenic, activated T cells. Since steroids can drive CD40L over-expression on T cells (27), there is also the possibility that steroid might be used to potentiate CD40·FasL's inhibition.

The concept of T cell suicide was suggested some time ago, prompted by the potential for FasL–Fas receptor signaling on individual activated T cells suspended in soft agar or serially diluted (28). The present study introduces a novel fusion protein with the capacity to artificially elicit this T cell suicide phenomenon, and provides a number of lines of evidence pointing to such 'auto-inhibition' as the operative mechanism for this agent. Using soft agar colony formation and serial cell dilution analyses, the findings here establish that intercellular contact is not a prerequisite for the enhanced potency of CD40·FasL inhibition. Furthermore, the fact that CD40 on a CD40-expressing, EBV-transformed B cell line competitively inhibits CD40·FasL-mediated inhibition of J-CD40L$^+$ cells, rather than providing a parallel trigger for CD40L bolsters the notion that CD40·FasL induces T cell suicide via a loop-back mechanism bridging neighboring ligands and receptors anchored to the same cell surface. This loop-back mechanism can mean that propagation of both intracellular signaling cascades is needed for optimal efficiency of CD40·FasL action. However, it is possible that by binding to CD40L on the target cells the protein induced clustering of the receptors and secondary to that clustering of the Fas receptors, thereby enhancing the protein potency in activating the death receptor.

Beyond a role in T cell auto-inhibition, CD40·FasL may have other therapeutic applications down the road. For example, triggering of CD40 promotes resistance to apoptosis in malignant B and plasma cells (29, 30). Thus, CD40·FasL could potentially function to deprive such cells of *trans* CD40L–CD40 generation of apoptotic resistance, while at the same time delivering apoptotic signals through Fas receptor on these cells.

One can envision other '*cis* loop-back proteins (CLBPs)' beyond CD40·FasL. For example, CLBPs might be designed that target other activation-induced surface molecules that are specific to other T cell activation stages or are associated with specific pathological states. Alternatively, CLBP might be configured to activate rather than inhibit T cells or other immune cells. Furthermore, other classes of fusion proteins, such as the TSCPs that we originally designed to convert *trans* intercellular signals, may under certain circumstances have *cis* loop-back signaling potential. For example, CTLA-4·FasL, a prototypical TSCP (9, 10), could potentially function in a loop-back mode when B7-1 is neo-expressed on T cells in the contexts of autoimmunity and HIV infection (31–33). Zhu *et al.* (34) described yet another approach to designing bi-functional fusion protein. Using the Fc portion of IgE and IgG, they successfully produced a fusion Fc protein to enhance the physiologically occurring co-aggregation of Fc_ϵ receptor 1 ($Fc_\epsilon R1$) with the inhibitory FcγRIIb on mast cells and basophils (34). The co-aggregation of both receptors is crucial for the inhibitory effect of FcγRIIb. CD40·FasL and the previously described CTLA-4·FasL fusion proteins differ from the Fc fusion protein as they are formed from membrane proteins (ligands), and are meant to ligate to receptors that are not previously known to co-aggregate, and this co-aggregation is not crucial, in their physiological state, for their functionality. In the case of CD40·FasL, data suggest that although not known to co-aggregate, CD40L and Fas receptor might co-ligate in a loop-back mechanism mediated by the novel protein CD40·FasL. However, both tactics highlight the wide spectrum of possibilities for designing immunomodulatory proteins. These and other spin-offs from the loop-back signaling concept can now be explored.

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Abbreviations

APC	antigen-presenting cell
ATCC	American Type Culture Collection

CD40L	CD40 ligand
CLBP	*cis* loop-back protein
[^{3}H]TdR	[^{3}H]thymidine
PI	propidium iodide
TSCP	*trans* signal converter protein

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