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CTLA-4·FasL inhibits allogeneic responses *in vivo* ☆

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Abstract

CTLA-4·Fas ligand (CTLA-4·FasL), a paradigmatic '*trans* signal converter protein (TSCP)', can attach to APC (via CTLA-4 binding to B7) and direct intercellular inhibitory signals to responding T cells (via FasL binding to Fas receptor), converting an activating APC-to-T cell signal into an inhibitory one. Our previous studies established that CTLA-4·FasL inhibits human primary mixed lymphocyte reactions (MLR) and induces alloantigen-specific hyporesponsiveness *ex vivo*. The present study extends this to an *in vivo* context. Using splenocytes from MHC-mismatched C57BL/6 and Balb/c mice, we demonstrated that his_6CTLA-4·FasL, effectively inhibits murine MLR. Moving *in vivo*, we demonstrated that subcutaneously administered his_6CTLA-4·FasL modulates the *in vivo* response of infused allogeneic splenocytes. his_6CTLA-4·FasL reduces the number of cells in each cell division, and increases the percentage of dead cells in each division. These findings are consistent with an antigen-induced cell death of the alloreactive cells, and bolsters recombinant TCSP promise as a therapeutic for transplantation diseases.
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1. Introduction

Alloreactive T cells are principal mediators of transplant rejection [1] and graft-versus-host disease [2]. Consequently, promoting activation-induced cell death in such cells is considered key for effecting transplantation tolerance [3–5]. There remains a pressing need for agents that are capable of inducing effective tolerance in alloreactive T cells. While costimulation blockade with agents such as CTLA-4·Ig (a blocker of antigen-presenting cell-driven B7 costimulation) induces hyporesponsiveness to alloantigens [6] and prolongs graft survival [7–9], it fails to prevent graft rejection [10–12].

Triggering of inhibitory receptors on T cells offers an alternative therapeutic tactic. For example, triggering of the inhibitory Fas receptor can be exploited for deleting antigen-specific T cells [13], and interference with this signaling prevents specific tolerance induction [14]. Deletional APC (artificial veto cells), defined as APC bearing on their surfaces T cell inhibitory ligands (coinhibitors), can inhibit T cell proliferative responses and induce alloreactive T cell apoptosis *ex vivo* [15–17] and alloantigen-specific T cell clonal deletion *in vivo* [13,18].

We have previously described a novel bi-functional fusion protein, CTLA-4·FasL, which combines the complementary capacities to bind (and block) B7 and deliver FasL inhibitory signals, in effect converting a stimulatory *trans* signal into an inhibitory one [19].

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This paradigmatic '*trans* signal converter protein (TSCP)' demonstrated significantly higher potency than either CTLA-4·Ig or soluble FasL, alone or the combination, for blocking alloresponses *ex vivo* [20]. Moreover, this TSCP was shown to induce alloantigen-specific hyporesponsiveness in restimulation assays, with the likelihood that this hyporesponsiveness is mediated by both anergy and clonal deletion [20].

In the present study, we have further explored CTLA-4·FasL's ability to inhibit alloresponses, moving to an *in vivo* context. Using adoptive transfer of allogeneic splenocytes as a straightforward *in vivo* model system, we now demonstrate that CTLA-4·FasL, unlike soluble FasL [21,22], is well-tolerated by mice when administered subcutaneously, and effectively inhibits both $CD4^+$ and $CD8^+$ T cells responses *in vivo*. This now extends recombinant TSCP efficacy to the *in vivo* level for alloresponses.

2. Materials and methods

2.1. Production of his_6CTLA-4·FasL

Production of recombinant his_6CTLA-4·FasL, a CTLA-4·FasL derivative with a hexahistidine tag at its amino terminus, was as described, with minor modifications [19,20]. In particular, stable G418-resistant 293 cell transfectants were selected in serum-supplemented DMEM as before, but in this case, they were subsequently grown in DCCM-1 serum-free medium (Biological Industries, Beit Haemek, Israel). his_6CTLA-4·FasL, purified from conditioned media by metal chelate chromatography as previously described [20], was quantified here using a FasL ELISA kit (Bender BioSciences, Vienna, Austria). A single ~46 kDa band was observed on reducing SDS–PAGE gels upon staining with Coomassie blue, and activity of the purified protein was confirmed by a radiometric DNA fragmentation assay, using Jurkat cells as targets [23], as well as in a proliferation assay, using human PBMC stimulated with an anti-CD3 mAb (OKT3; prepared from a hybridoma obtained from American Type Culture Collection, Bethesda, MD).

2.2. Mice

Male BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were purchased from the Harlan, Israel. Wild-type CBA, CBA-LPR/cg, and B6.MRL-*Faslpr*/J LPR mice were purchased from Jackson laboratories (Bar Harbor, Maine). All mice were housed in the pathogen-free Hebrew University Medical School animal facilities and were used at age 8–12 weeks. Animals were treated according to the standards of the Animal Ethics Committee, Hebrew University Medical School Animal Care Facilities.

2.3. Ab, CFSE labeling, and flow cytometry

Spleens from donor mice (C57BL/6) were harvested and splenocytes were isolated by density-gradient centrifugation using Ficoll–Hypaque. 10^7 cells/ml in PBS were labeled with 5 μM CFSE (Sigma–Aldrich, Israel) for 10 min at RT and immediately washed three times with ice-cold RPMI medium supplemented with 10% fetal calf serum (Biological Industries, Beit Hemek, Israel). APC-conjugated anti-murine CD4 and CD8 mAb and their matching controls were purchased from Biotest (Kfar Saba, Israel), PE-conjugated anti-murine MHC H-$2K^b$ mAb and its matching control was purchased from Serotec (Oxford, UK), and 7-AAD for live/dead discrimination was purchased from Sigma–Aldrich (Israel).

2.4. In vitro MLR and anti-CD3 mAb stimulation assay

Splenocytes were resuspended in RPMI medium supplemented with penicillin, streptomycin, 4 mM L-glutamine, 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, and 10 mM Hepes. 2×10^5 BALB/c splenocytes (H-2^d) were incubated with 2×10^5 gamma-irradiated (1700 rads) C57BL/6 (H-2^b) splenocytes in round-bottommed 96-well plates, in a total volume of 0.2 ml, in the presence or absence of his_6CTLA-4·FasL that was added at the start of the MLR. Triplicate cultures were incubated at 37 °C with 5% CO_2, and were pulsed with 0.5 μCi [^{3}H]thymidine per well 16–18 h before harvesting and scintillation counting. In some assays, splenocytes were activated with anti-CD3 mAb or Staphylococcal endotoxin B (SEB) in the presence or absence of his_6CTLA-4·FasL. After 54 h, cultures were pulsed with 0.5 μCi [^{3}H]thymidine per well, and harvested after 16–18 h. [^{3}H]Thymidine incorporation was measured in a β-counter.

2.5. In vivo MLR (adoptive transfer of allogeneic splenocytes)

To assess allogeneic cell division *in vivo*, a total of $3–5 \times 10^7$ CFSE-labeled splenocytes from C57BL/6 (H-2^b) in a total volume of 0.25 ml PBS were injected into the tail veins of sub-lethally irradiated (750 rads) BALB/c mice (H-2^d). Recipient mice were treated with subcutaneously administered his_6CTLA-4·FasL, divided into three doses daily, and were then sacrificed at 72 h. Spleens and livers were harvested, and the latter were fixed in formalin, stained with haematoxylin–eosin, and examined for histopathological changes in a blinded-fashion. Splenocytes were isolated as described above and subjected to flow-cytometric analysis. Unlabeled mAb with specificity for CD16/CD32 (anti-FcRγIII/FcRγII, Serotec, Oxford, UK) were used to block Fc receptor binding. PE-conjugated anti-H-$2K^b$ mAb was used to distinguish between donor and recipient cells, and 7-AAD staining was used to differentiate live cells from dead ones. Flow cytometry was performed on a FACScan and analyzed using CellQuest software. A total of $1–2 \times 10^6$ events were counted for each sample. Gating on lymphocyte-characteristic forward light scatter, cell divisions were demarcated according to CFSE-staining brightness. By gating on each division, the proportion of $CD4^+$ or $CD8^+$ dead cells was calculated.

3. Results

3.1. Human CTLA-4·FasL effectively inhibits murine MLR

Previously, we demonstrated that human his$_6$CTLA-4·FasL inhibits human MLR *ex vivo* and induces specific hyporesponsiveness to restimulation. As a first step towards evaluating this fusion protein's function *in vivo*, we asked whether human his$_6$CTLA-4·FasL can function cross-species to inhibit *in vitro* murine MLR. To this end, BALB/c splenocytes were co-cultured with irradiated, MHC-mismatched C57BL/6 splenocytes, and *vice versa*, in the presence or absence of human his$_6$CTLA-4·FasL. As shown in Fig. 1, human his$_6$CTLA-4·FasL inhibits murine and human allogeneic responses to similar extents. This functional equivalence obviated the need to craft and separately validate a murine his$_6$CTLA-4·FasL homologue.

3.2. CTLA-4·FasL inhibition is Fas-dependent

To establish the Fas receptor-dependence of his$_6$ CTLA-4·FasL's inhibitory action, we compared the his$_6$CTLA-4·FasL-sensitivity of lpr-cg splenocytes (with defective Fas receptor signaling) to that of Fas receptor-positive controls. Splenocytes from lpr-cg mice, but not controls with intact Fas receptor signaling, proliferated normally when stimulated with anti-CD3 mAb or SEB in the presence of his$_6$CTLA-4·FasL (Fig. 2). We repeated the experiments using lpr mice (that lack Fas receptor) as responders to confirm his$_6$CTLA-4·FasL's inability to inhibit anti-CD3-induced proliferation. We also used lpr mice as responders in *in vitro* MLR and found no inhibition (data not shown). These findings, along with our demonstration elsewhere that Ab-blocking of FasL:Fas receptor interaction overcomes his$_6$CTLA-4·FasL-induced inhibition of T cell triggering by anti-CD3 mAb [19], together indicate that functional Fas receptor is essential for his$_6$CTLA-4·FasL action. Of note, we have also shown previously that his$_6$CTLA-4·FasL activity is not strictly dependent on the CTLA-4 domain, and thus, in the absence of B7 for anchoring, his$_6$CTLA-4·FasL can still act to some extent as a soluble FasL [20].

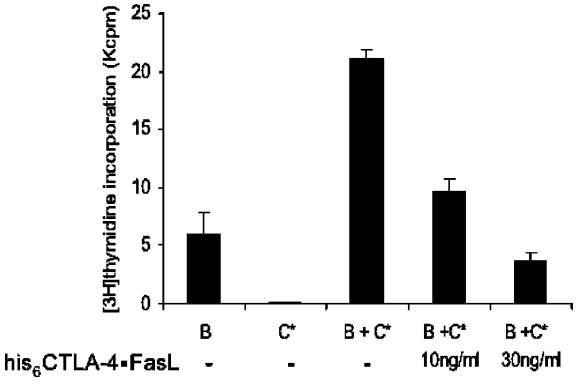

Fig. 1. his$_6$CTLA-4·FasL inhibits murine in vitro MLR. 2×10^5 BALB/c splenocytes (H-2^d) were incubated with 2×10^5 gamma-irradiated C57BL/6 (H-2^b) splenocytes in round-bottommed 96-well plates. his$_6$CTLA-4·FasL was added at the start of the MLR. Four-day cultures were performed in triplicate. Cells were pulsed with [^{3}H]thymidine for the last 18 h, and then evaluated for [^{3}H]thymidine incorporation. This is a representative experiment from eight independent experiments performed. Data are presented as means ± SD. (*B* = splenocytes from BALB/c, C* = irradiated C57BL/6 splenocytes).

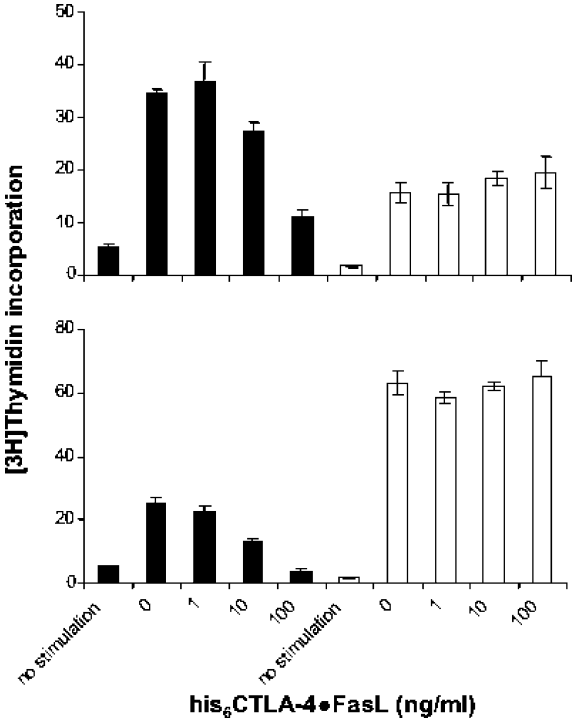

Fig. 2. his$_6$CTLA-4·FasL inhibition is dependent on functional Fas receptor on responders. Wild-type CBA (black bars) or (white bars) CBA-LPR/cg splenocytes were activated with either anti-CD3 mAb (upper panel) or SEB (lower panel), in the presence or absence of his$_6$CTLA-4·FasL. After 54 h, cultures were pulsed with [^{3}H]thymidine for 18 h, and then evaluated for [^{3}H]thymidine incorporation. Data are presented as means ± SD.

3.3. CTLA-4·FasL induces death of proliferating alloantigen-driven, adoptively transferred splenocytes

We next transitioned to *in vivo* analysis of his$_6$CTLA-4·FasL allo-inhibition, using *in vivo* murine MLR. $3–5 \times 10^7$ CFSE-labeled C57BL/6 splenocytes were adoptively transferred to sub-lethally irradiated BALB/c mice. his$_6$CTLA-4·FasL was injected subcutaneously three times a day to a total dose of 30 μg/day or 100 μg/day. Control mice were injected with vehicle (PBS). After 72, spleens from recipient mice were recovered. Spleens from irradiated mice that did not receive any transferred cells weighed approximately half that of age-matched, non-irradiated mice. Spleens from irradiated mice injected with splenocytes and treated with either vehicle ($n = 11$) or 30 μg/day his$_6$CTLA-4·FasL ($n = 7$) weighed 80–90% and 60–70%, respectively, of age-matched, non-irradiated mice. Of note, to factor for potential inter-experiment differences attributable to age and irradiation variability, all experimental groups were replicated in each experiment.

Harvested splenocytes were immunostained with PE-conjugated anti-murine H-2k^b mAb (to detect donor cells) and 7-AAD (to detect dead cells). As seen in the representative experiment of Fig. 3A, at least 5–6 cell divisions can be detected at 72 h. Significantly, his$_6$CTLA-4·FasL, at a dose of 100 μg/day decreased the number of proliferating T cells. When less his$_6$CTLA-4·FasL was administrated to the mice (30 μg/day), his$_6$CTLA-4·FasL had no significant effect on the percentage of cells that undergo division (Fig. 3B). However, even at this lower his$_6$CTLA-4·FasL dose, there was a significantly greater percentage of dead splenocytes among proliferating T cells (Figs. 3B and C). This striking effect was seen in both 30 μg/day and 100 μg/day his$_6$CTLA-4·FasL treatment groups, and was more prominent for cells that had undergone more than one cell division (Figs. 3B and C). Even though some death was seen among non-dividing cells, this was substantially less extensive than for dividing cells.

Next, since both CD4$^+$ and CD8$^+$ T cells play a major role in allogeneic responses, we asked whether his$_6$CTLA-4·FasL induces death of alloreactive cells of both subsets. The harvested spleen cells were co-immunostained with APC-conjugated anti-CD4 or anti-CD8 mAb, in addition to PE-conjugated anti-H-2K^b mAb and 7-AAD, and four color analysis was performed. his$_6$CTLA-4·FasL markedly increased the percentage of dead cells among both CD4$^+$ and on CD8$^+$ proliferating T cells (Fig. 4).

3.4. his$_6$CTLA-4·FasL is not hepatotoxic

We asked whether his$_6$CTLA-4·FasL is hepatotoxic, as is the case for soluble FasL and anti-Fas Ab. Livers of all irradiated mice, analyzed in a blinded-fashion, demonstrated some degree of hepatocyte swelling and mononuclear infiltration. Additionally, mild perivascular inflammation could be detected after 72 h in irradiated animals transfused with splenocytes. his$_6$CTLA-4·FasL did not significantly impact the histological appearance of livers in these animals, whether at 30 μg/day or 100 μg/day doses (not shown). No hepatotoxic damage was observed when healthy, non-irradiated mice were injected with his$_6$CTLA-4·FasL at concentrations up to 100 μg/day (not shown).

4. Discussion

The present study extends his$_6$CTLA-4·FasL's allo-inhibitory capacity from the *ex vivo* to *in vivo* settings. Key findings are: (1) human his$_6$CTLA-4·FasL effectively inhibits murine T cells *ex vivo* cross-species; (2) his$_6$CTLA-4·FasL does not inhibit lpr-cg T cells that are defective in Fas receptor signaling, documenting the importance of the FasL:Fas receptor axis for his$_6$CTLA-4·FasL function; (3) his$_6$CTLA-4·FasL induces death of both CD4$^+$ and CD8$^+$ allo-responsive T cells *in vivo*; and (4) subcutaneously

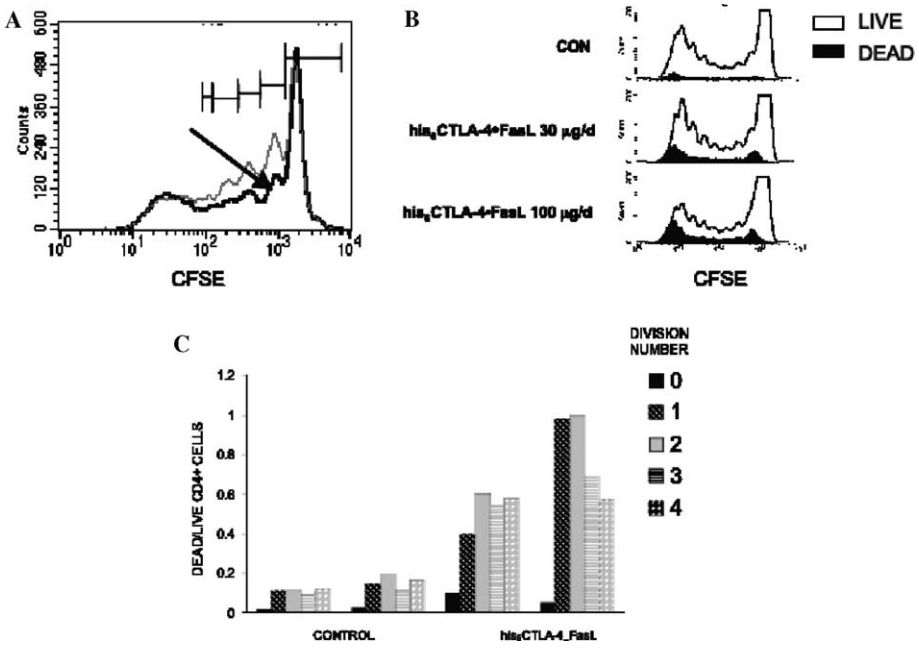

Fig. 3. his$_6$CTLA-4·FasL partially inhibits allogeneic proliferation *in vivo*. 3–5 × 10^7 CFSE-labeled splenocytes from C57BL/6 (H-2^b) were injected into the tail veins of sub-lethally irradiated BALB/c mice (H-2^d). Recipient mice were treated with subcutaneously administered his$_6$CTLA-4·FasL or PBS and sacrificed at 72 h. Splenocytes were isolated and analyzed by flow-cytometric analysis. (A) Recipient mice treated with 100 μg/day his$_6$CTLA-4·FasL versus PBS (no arrow) were compared. Gating on lymphocyte-characteristic forward light scatter, cell divisions were demarcated according to CFSE-staining brightness. Unlabeled Ab specific for CD16/CD32 were used to block Fc receptor binding. PE-conjugated anti-H-2K^b Ab was used to differentiate between donor and recipient cells. (B) Recipient mice treated with indicated amounts of his$_6$CTLA-4·FasL versus PBS were compared. Gating and Ab blocking were as described under (A), and in this case, 7-AAD staining was used to differentiate live cells from dead ones. (C) Histogram presenting the ratio of dead to live cells detected in splenocytes from two representatives his$_6$CTLA-4·FasL-treated mice (his$_6$CTLA-4·FasL) versus two representatives non-treated mice (control), at the various cell divisions shown. Treated mice were injected with 30 μg/day (left group of columns) or 100 μg/day (right group of columns) of his$_6$CTLA-4·FasL.

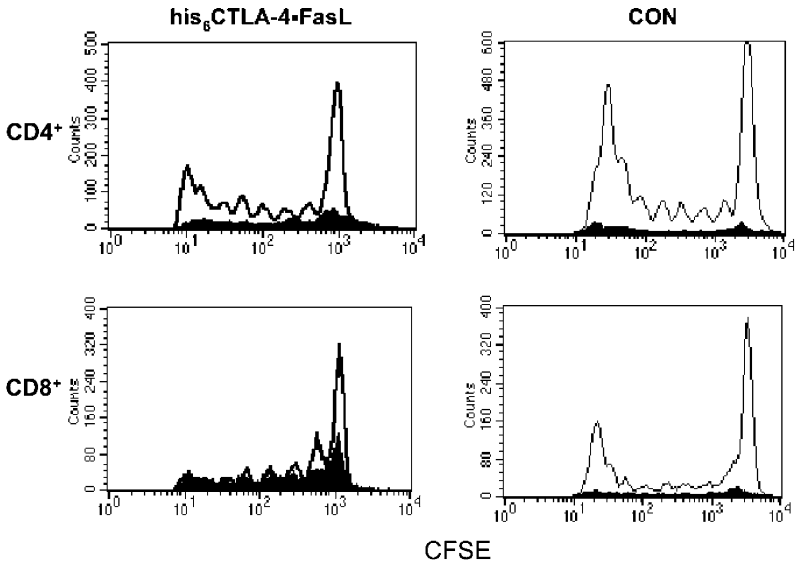

Fig. 4. his_6CTLA-4·FasL induces death of $CD4^+$ and $CD8^+$ alloreactive T cells. $3–5 \times 10^7$ CFSE-labeled splenocytes from C57BL/6 (H-2^b) mice were injected into the tail veins of sub-lethally irradiated BALB/c mice (H-2^d). Recipient mice were injected with 30 μg/day his_6CTLA-4·FasL versus PBS and sacrificed at 72 h. Splenocytes were immunostained with a combination of PE-conjugated anti-H-$2K^b$ mAb, APC-conjugated anti-CD4 or anti-CD8 mAb, and 7-AAD. Unlabeled Ab with specificity for CD16/CD32 were used to block Fc receptor binding. CFSE staining of events gated for H-$2K^b$ and CD4 (or CD8) is displayed. A representative of three independent experiments is shown.

administered his_6CTLA-4·FasL is not overtly hepatotoxic in this murine model system.

The *in vivo* analyses of this study solidify the notion that his_6CTLA-4·FasL's primary inhibitory effect, at least in the MLR model, occurs post-activation. We have previously shown that his_6CTLA-4·FasL does not interfere with the appearance of activation markers on stimulated T cells [19]. Consistent with this earlier observation, we now demonstrate that adoptively transferred T cells can still proliferate in his_6CTLA-4·FasL-treated mice, albeit at a reduced level. Thus, his_6CTLA-4·FasL does not prevent progression to a proliferative response *per se*, but instead, functions primarily to induce death of proliferating T cells. This post-activation inhibitory mechanism mirrors the activation-induced cell death (AICD) previously associated with FasL:Fas receptor signaling [4,5]. In a similar vein, administration of a combination of CTLA-4·Ig/anti-CD40L/rapamycin in an adoptive transfer model has been shown by others to spare alloreactive cell proliferation, but induce apoptosis of the reacting cells [5]. Furthermore, this 'proliferation-sparing' immunosuppressive regimen was found to evoke better tolerance than one that prevents alloreactive cell proliferation altogether.

Presumably, the persistence of proliferation in the face of our CTLA-4·FasL regimen reflects incomplete blockade of B7 costimulation (due to the lower doses of therapeutic fusion protein being used, as compared to standard CTLA-4·Ig treatment) and/or second signals provided by other non-B7 costimulators. It is likely that generation of FasL-coated APC accounts in large part for his_6CTLA-4·FasL's T cell inhibitory activity. It is now well-established that APC genetically engineered to express FasL on their surfaces can delete alloreactive clones and induce allo-tolerance [13]. Also, since it is now known that there can be 'backward' signaling through B7 into APC [24], it is conceivable that his_6CTLA-4·FasL, even at the relatively low doses being used, signals through B7 resident on APC and thereby impacts their functional activity.

Both $CD4^+$ and $CD8^+$ T cells contribute significantly to allo-immune responses [25–30], and there has been an increasing focus on the latter. Immunosuppressive protocols that spare $CD8^+$ cells are ineffective in some transplantation models [31–33], whereas protocols that deplete alloreactive $CD8^+$ T cells *in vivo* prolong skin graft survival [29]. Fetal FasL expression is crucial for the development of maternal-fetal tolerance, and this tolerance is mediated by clonal deletion of $CD8^+$ T cells [34]. Such findings imply that preferred therapeutic agents will be those that are cytotoxic not just to $CD4^+$, but also $CD8^+$ T cells. Our present *in vivo* findings indicate that his_6CTLA-4·FasL fits this mold, affecting both of these T cell subsets.

Another group has recently reported *in vivo* efficacy of gene-transferred CTLA-4·FasL in murine models for diabetes mellitus [35] and cardiac allograft rejection [36]. Our present study represents the first demonstration of *in vivo* efficacy of recombinant CTLA-4·FasL protein, and adds mechanistic insights into this TSCP's allo-immunity effect, using a straightforward *in vivo* MLR approach. Taken together, these studies substantiate the therapeutic promise of TSCP-based therapeutics, with possible future extensions to the treatment of other allo-immune and autoimmune disorders. Significantly, neither gene-transferred CTLA-4·FasL [35,36] nor subcutaneously administered CTLA-4·FasL appear to cause the kind of hepatotoxicity and mortality previously associated with soluble FasL or anti-Fas Ab [21,22].

his_6CTLA-4·FasL is a paradigmatic TSCP, and its *in vivo* efficacy provides motivation for designing more TSCP with optimal safety and target cell selectivity profiles. In terms of the latter, it should be possible to develop TSCP that preferentially modulate distinct T cell subsets, for example, naïve versus memory T cells, or effector versus regulatory T cells. This would substantially expand potential therapeutic applications for this new class of immunomodulators.

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