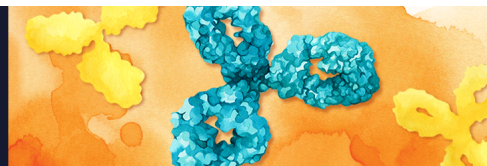


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## Cutting Edge: Targeted Ligation of CTLA-4 In Vivo by Membrane-Bound Anti-CTLA-4 Antibody Prevents Rejection of Allogeneic Cells

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## Cutting Edge: Targeted Ligation of CTLA-4 In Vivo by Membrane-Bound Anti-CTLA-4 Antibody Prevents Rejection of Allogeneic Cells<sup>1</sup>

Kwang Woo Hwang,<sup>2\*‡</sup> William B. Sweatt,<sup>2\*‡</sup> Ian E. Brown,<sup>†‡</sup> Christian Blank,<sup>†‡</sup> Thomas F. Gajewski,<sup>†‡</sup> Jeffrey A. Bluestone,<sup>3,4§</sup> and Maria-Luisa Alegre<sup>3\*‡</sup>

**Natural engagement of CTLA-4 on host B7 limits T cell activation. We hypothesized that therapeutic cross-linking of CTLA-4 in vivo may further inhibit T cell function and prevent allograft rejection. However, none of the currently available CTLA-4-binding reagents have ligating properties when injected in vivo. The observation that surface-immobilized anti-CTLA-4 mAb inhibits T cell activation in vitro prompted us to develop a membrane-bound single-chain anti-CTLA-4 Ab (7M). To model whether tissue expression of 7M could suppress allograft rejection, we examined the ability of H-2L<sup>d</sup>-specific TCR-transgenic T cells to reject 7M-expressing allogeneic tumor cells injected s.c. Expression of 7M significantly inhibited allogeneic rejection in mice that received CTLA-4<sup>+/+</sup> but not CTLA-4<sup>-/-</sup> T cells. Furthermore, CTLA-4<sup>+/+</sup> T cells that had encountered 7M-expressing tumors in vivo acquired defects in cytokine production and cytotoxicity. Thus, deliberate ligation of CTLA-4 in vivo potentially inhibits allogeneic T cell responses. *The Journal of Immunology*, 2002, 169: 633–637.**

**T** cell receptor-dependent activation of naive T cells is aided by CD28-mediated costimulatory signals delivered upon engagement of B7-1 (CD80) and B7-2 (CD86) on APCs. However, B7 ligands can also bind the inducible CD28-related receptor CTLA-4 (CD152), resulting in attenuation of T cell responses. Initial support for an inhibitory role mediated by CTLA-4 engagement was derived from in vitro experiments in which soluble anti-CTLA-4 mAb was shown to enhance T cell proliferation while surface-immobilized CTLA-4 mAb inhibited

IL-2 production and cell cycle progression (1, 2). In addition, CTLA-4-deficient mice developed a lymphoproliferative disorder (3, 4) secondary to uncontrolled polyclonal CD4<sup>+</sup> T cell expansion (5). Furthermore, CTLA-4 blockade during i.v. administration of soluble Ag prevented tolerance induction in CD4<sup>+</sup> T cells (6) and anti-CTLA-4 therapy reversed tolerance in vivo (7), supporting a role for CTLA-4 in regulating both the initiating and the maintenance phase of tolerance.

Because CTLA-4 is expressed on activated T cells, a strategy for deliberate ligation of CTLA-4 could theoretically suppress immune responses in vivo, thus having applications toward the prevention of allograft rejection. However, the anti-CTLA-4 mAbs that have been described to date block CTLA-4 ligation in vivo, resulting in augmentation of T cell responses. The observation that surface-immobilized anti-CTLA-4 mAb inhibits T cell function prompted the development of genetically engineered cells expressing membrane-bound single-chain anti-CTLA-4 Ab (4F10scFv). Cells transiently transfected to express anti-CD3scFv and 4F10Fv were previously shown to inhibit production of cytokines and proliferation by T cells in vitro (8). However, the low affinity and low level of expression of 4F10scFv could potentially limit the application of this system to in vivo models. Thus, a yeast display strategy was used (9) to engineer a mutant of 4F10scFv with increased CTLA-4 binding capacity (9, 10). In this work we report that expression of 4F10scFv mutant, 7M, on allogeneic tumor cells resulted in suppression of T cell activation in vivo, leading to a failure to reject the allogeneic tumor. This is the first approach resulting in successful deliberate ligation of CTLA-4 in an allogeneic model in vivo, a strategy with potential applications for clinical transplantation.

### Materials and Methods

#### Mice

2C/recombination-activating gene (RAG)<sup>52-/-</sup>CTLA-4<sup>+/+</sup> or 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> mice (H-2<sup>b</sup>) have been described previously (11). RAG1<sup>-/-</sup> mice (H-2<sup>b</sup>) were obtained from The Jackson Laboratory (Bar Harbor, ME). Animals were kept in specific pathogen-free facilities.

#### Reagents

CTLA-4-Ig was a gift from M. Collins (Wyeth Laboratories, Cambridge, MA) and was FITC-coupled by standard laboratory procedures. The mastocytoma P815 and its highly transfectable variant PIHTR (H-2<sup>d</sup>) were grown in complete DMEM (Life Technologies, Grand Island, NY) (11).

<sup>5</sup> Abbreviation used in this paper: RAG, recombination-activating gene.

Departments of \*Medicine and †Pathology, and ‡Committee in Immunology, University of Chicago, Chicago, IL 60637; and §Diabetes Center, University of California, San Francisco, CA 94143

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<sup>2</sup> K.W.H. and W.B.S. contributed equally to this project and should be considered co-first authors.

<sup>3</sup> J.A.B. and M.-L.A. should be considered co-senior authors for this study.

<sup>4</sup> Address correspondence and reprint requests to Dr. Jeffrey A. Bluestone, Diabetes Center, University of California, 513 Parnassus Avenue, Box 0540, San Francisco, CA 94118. E-mail address: jbluest@diabetes.ucsf.edu

The P815-B7-1 cell line was previously generated (12) and grown in complete medium in the presence of geneticin (1 mg/ml), PMA (50 ng/ml) and ionomycin (0.5  $\mu$ g/ml) were purchased from Cellgro (Herndon, VA). Abs for flow cytometry were purchased from BD PharMingen (San Diego, CA).

#### Generation of 7M mutant

Generation of mutants of 4F10scFv using yeast display technology was described previously (10). One mutant, 7M, had the greatest binding capacity of the screened library for CTLA-4-Ig and was selected for transfection into PIHTR cells. The cDNA for 7M was cloned into a mammalian expression vector as previously described for the gene encoding the parental 4F10scFv (10), except that pEF-puro.PL3 (pEF) vector, a variant of the pEF-BOS vector (13), was used in place of the LZRSpBMN vector. PIHTR cells were transfected by calcium phosphate precipitation with either pEF vector control or 7M-encoding vector and were selected in the presence of puromycin (2  $\mu$ g/ml). Stable transfectants were further subcloned using limiting dilution techniques and were screened for comparable expression of L<sup>d</sup>, B7-1, and B7-2, as well as for similar growth properties and morphologic features *in vitro* and *in vivo*.

#### T cell purification

CD8<sup>+</sup> T cells were purified by negative selection (StemCell Technologies, Vancouver, Canada) from spleens from 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup>, 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup>, or reconstituted RAG1<sup>-/-</sup> mice. An aliquot of purified cells was stained with the clonotypic Ab 1B2-FITC and analyzed by flow cytometry. T cell purity was routinely >95%.

#### Detection of cytokines and proliferation

Purified T cells were incubated with mitomycin C-treated (50  $\mu$ g/ml for 90 min at 37°C) PIHTR-PEF, PIHTR-7M, or P815-B7-1 at a 1:3 ratio. In some cases, T cells were primed for 4 days in the presence of mitomycin C-treated PIHTR and human IL-2 (20 U/ml) added at 24 h. Live cells were isolated after Ficoll density centrifugation, were rested for 8 h, and were restimulated with the indicated cell lines. Supernatants were collected at 24 h and concentration of cytokines was detected by ELISA using Ab pairs (BD PharMingen). Parallel plates were cultured for 72 h and 1  $\mu$ Ci of [<sup>3</sup>H]thymidine was added to each well for the last 8 h of the culture (14).

#### Tumor experiments

Indicated numbers of purified 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> or 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells were resuspended in 100  $\mu$ l of PBS and injected *i.v.* into the retro-orbital plexus of syngeneic RAG1<sup>-/-</sup> recipients. One to 7 days after adoptive transfer, recipient mice received a *s.c.* injection of P815, PIHTR-PEF, or PIHTR-7M tumor cells (1  $\times$  10<sup>6</sup>) in PBS. Tumor growth was monitored biweekly using graduated calipers. The product of the longest and shortest diameter of each tumor was recorded at each measurement. Animals were sacrificed when the tumor area exceeded 1200 mm<sup>2</sup> for ethical reasons related to the morbidity associated with a large tumor mass.

#### Statistical analysis

Differences in tumor size were determined using the unpaired *t* test as calculated with InStat 3.0 software (GraphPad, San Diego, CA).

## Results

#### Generation of control and 7M-expressing tumor lines with similar growth characteristics *in vivo*

To obtain surface-immobilized anti-CTLA-4 Ab, a membrane-bound single chain anti-CTLA-4 Ab (4F10scFv) was generated (8) and subjected to random mutagenesis in a yeast display assay to increase its binding affinity to CTLA-4 (10). One such mutated clone, 7M, displayed the highest affinity for CTLA-4-Ig by flow cytometry (10) and was shown to have one mutation in the framework of the variable light domain (L89M) and one just outside the L2 complementarity-determining region (L49Q) (10).

To introduce expression of membrane-bound 7M in an allogeneic tissue, L<sup>d</sup>-expressing PIHTR tumor cells were stably transfected. Subclones of control transfectants (PIHTR-pEF) and 7M-transfectants (PIHTR-7M) were generated and selected for similar expression of H-2L<sup>d</sup> and lack of expression of B7-1 and B7-2 (Fig. 1A). Expression of 7M in PIHTR-7M but not PIHTR-pEF was

verified by staining with CTLA-4-Ig-FITC (Fig. 1A). To ensure that these subclones had comparable growth kinetics *in vivo*, tumor growth was compared over time following *s.c.* injection of PIHTR-pEF and PIHTR-7M cells into RAG1-deficient mice. As shown in Fig. 1B, *left panel*, both tumors grew with similar kinetics in these lymphocyte-deficient mice. Finally, it was important to ensure that both tumors elicited similar T cell responses *in vivo* when CTLA-4 was not ligated. To verify comparable immunogenicity, RAG1-deficient mice were transfused with graded numbers of L<sup>d</sup>-specific, 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells before the *s.c.* injection of PIHTR-pEF or PIHTR-7M cells. Growth of both tumors was comparably controlled by 1  $\times$  10<sup>5</sup> CTLA-4<sup>-/-</sup> T cells (Fig. 1B, *right panel*) and was completely prevented by 1  $\times$  10<sup>6</sup> CTLA-4<sup>-/-</sup> T cells (data not shown), indicating that the immunogenicity of the two tumors was comparable *in vivo* in the absence of CTLA-4 ligation.

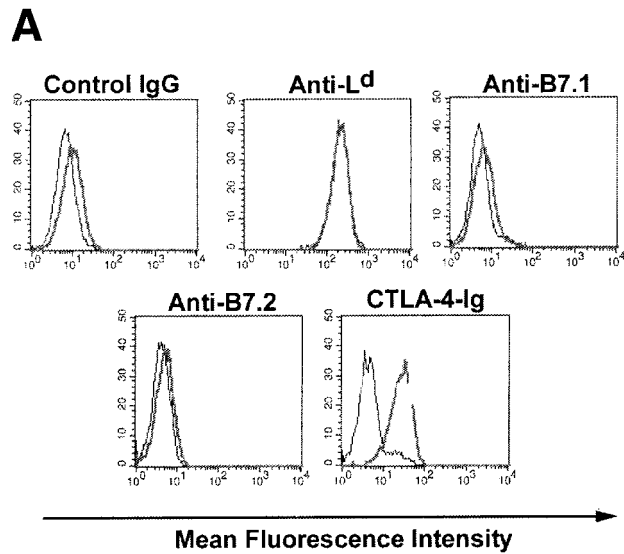
#### Expression of 7M resulted in reduced cytokine production and proliferation by CTLA-4-expressing allogeneic T cells *in vitro*

To determine whether ligation of CTLA-4 by membrane-bound 7M inhibited T cell activation *in vitro*, primed 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> T cells were stimulated with PIHTR-PEF or PIHTR-7M, and supernatants were analyzed for cytokine content. IL-2 and IFN- $\gamma$  production by T cells was dramatically reduced in the presence of PIHTR-7M when compared with stimulation with PIHTR-pEF (Fig. 2). Similarly, incorporation of [<sup>3</sup>H]thymidine was severely decreased in T cells cultured with PIHTR-7M (Fig. 2). These results indicate that engagement of this highly stable anti-CTLA-4 scFv effectively inhibited T cell responses *in vitro*.

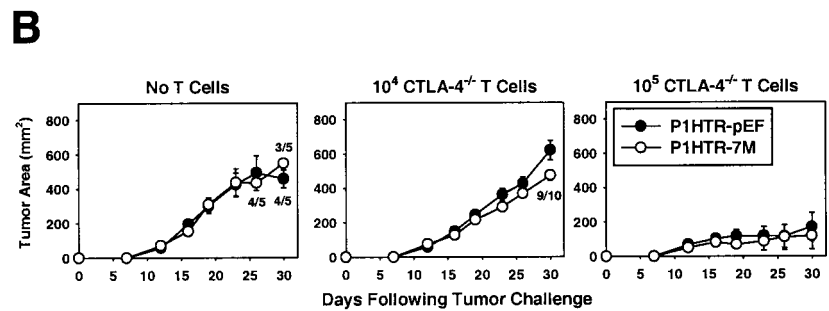
#### Engagement of CTLA-4 inhibits allogeneic responses *in vivo*

Before testing the effect of deliberate ligation of CTLA-4 by 7M *in vivo*, it was important to assess whether engagement of CTLA-4 by host B7 molecules was already limiting T cell responses against tumors lacking a CTLA-4 ligand. To this end, the growth of parental PIHTR tumors was compared in RAG1<sup>-/-</sup> mice that received 2C/RAG2<sup>-/-</sup>CTLA4<sup>+/+</sup> vs 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells. As shown in Fig. 3A, CTLA-4<sup>-/-</sup> T cells were quantitatively superior to CTLA-4<sup>+/+</sup> T cells at controlling allogeneic tumor growth. Transfer of 1  $\times$  10<sup>6</sup> CTLA-4<sup>+/+</sup> or CTLA-4<sup>-/-</sup> T cells completely prevented tumor growth in all recipients (data not shown). These results indicate that engagement of CTLA-4 by host B7 ligands normally attenuates T cell responses to alloantigens *in vivo*.

To address whether deliberate ligation of CTLA-4 by the tissue allograft would further suppress T cell activation and prevent rejection, growth of PIHTR-pEF and PIHTR-7M tumors was compared in CTLA-4-competent animals. RAG1<sup>-/-</sup> mice received 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> T cells before the *s.c.* inoculation of PIHTR-PEF or PIHTR-7M cells. As a control, some mice received only tumor cells. As shown in Fig. 3B, a dramatic difference in tumor growth rate was observed between PIHTR-pEF- and PIHTR-7M-injected mice that received 1  $\times$  10<sup>4</sup> T cells. PIHTR-7M tumors grew almost as aggressively as did tumors in mice that did not receive any T cells, and 9 of 10 mice died by wk 6 in the PIHTR-7M tumor-bearing group. In contrast, growth of control PIHTR-pEF tumors was effectively controlled with 8 of 10 animals completely rejecting their allogeneic tumors. The inability to reject the PIHTR-7M tumors could be overcome by increasing the number of T cells transferred, as injection of 1  $\times$  10<sup>5</sup> T cells supported rejection of both PIHTR-pEF and PIHTR-7M tumors (Fig. 3B). Growth rates of both tumors were similar in the absence of transferred T cells (Fig. 3B) as well as in mice adoptively transferred with 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells (Fig. 1 and data not



**FIGURE 1.** Generation of P1HTR-pEF and P1HTR-7M subclones with similar properties. *A*, P1HTR-pEF (thin line) and P1HTR-7M (thick line) subclones were analyzed by flow cytometry. *B*, P1HTR-pEF and P1HTR-7M cells were injected s.c. in the flank of RAG1-deficient mice either left untreated (*left panel*, five mice per group) or transfused with 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells ( $1 \times 10^4$  (*middle panel*) vs  $1 \times 10^5$  (*right panel*), 10 mice per group). The numbers on the plots refer to the remaining live animals per group used for each measurement. Results are representative of three independent experiments and indicate the mean and SE of the tumor area.



shown), confirming that lack of rejection of P1HTR-7M was due to engagement of CTLA-4 *in vivo*. Together, these data demonstrate that deliberate engagement of CTLA-4 by a tumor allograft can prevent rejection *in vivo*.

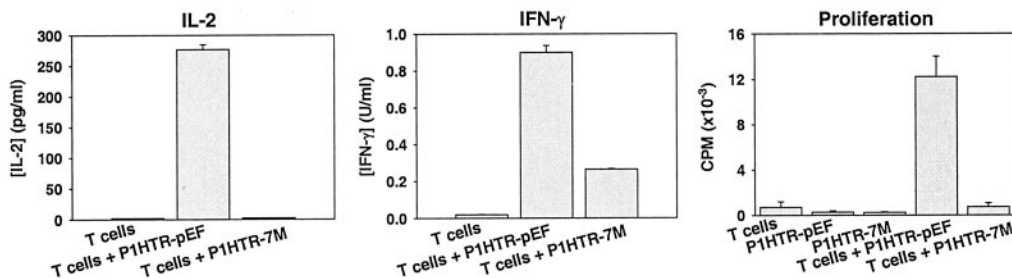
*Encounter of P1HTR-7M tumor in vivo promotes hyporesponsiveness of CTLA-4-competent allogeneic T cells*

Engagement of CTLA-4 has been shown to play a role in the induction and maintenance of tolerance in several systems (6, 7). To determine whether prior engagement of CTLA-4 *in vivo* down-regulated T cell effector functions, T cells were analyzed *ex vivo* 6 wk after tumor inoculation. Splens from each group were pooled and T cells were purified and restimulated *in vitro* with P815-B7-1 cells. CTLA-4<sup>+/+</sup> T cells that had encountered P1HTR-7M *in vivo* failed to produce detectable levels of IL-2 and produced markedly reduced amounts of IFN- $\gamma$  upon restimulation when compared with those exposed *in vivo* to P1HTR-pEF (Fig.

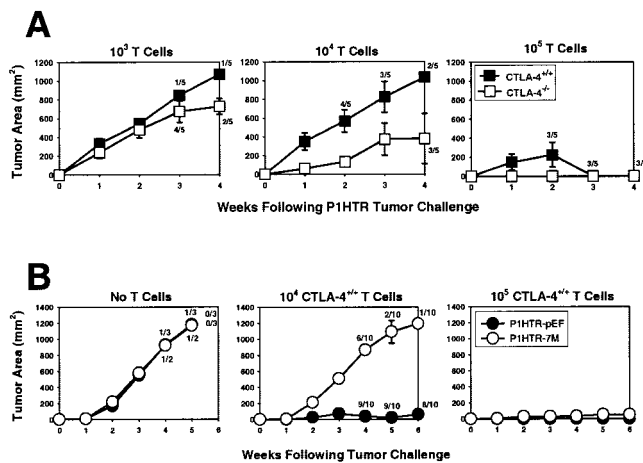
4). Interestingly, CTLA-4<sup>-/-</sup> T cells produced greater amounts of IL-2 and IFN- $\gamma$  when compared with CTLA-4-expressing T cells, and exposure to the 7M-expressing tumor *in vivo* had no effect on this effector function. Stimulation with PMA and ionomycin resulted in similar cytokine production by all T cells, indicating a comparable intrinsic cytokine-producing capability and implying that hyporesponsiveness of 7M-exposed T cells was specific to TCR engagement.

**Discussion**

Although it has been demonstrated in numerous systems that engagement of CTLA-4 by B7 down-regulates T cell responses, lack of appropriate reagents has precluded attempts at deliberately ligating CTLA-4 *in vivo* to inhibit the function of allogeneic T cells. In this study, we show that exposure to allogeneic tumor cells expressing membrane-bound anti-CTLA-4 scFv results in T cell hyporesponsiveness and failure to reject tumor allografts.

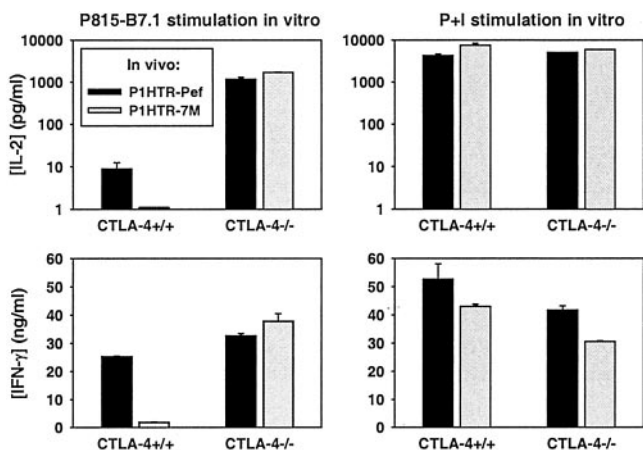


**FIGURE 2.** 7M expression inhibits allogeneic T cell responses by 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> T cells *in vitro*. Primed 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> T cells were restimulated with P1HTR-pEF or P1HTR-7M. Supernatants were analyzed for cytokine content by ELISA. Parallel plates were assayed for [<sup>3</sup>H]thymidine incorporation. Results represent the mean and SD of triplicate wells.



**FIGURE 3.** CTLA-4 engagement inhibits allogeneic T cell responses in vivo. *A*, Purified 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> and 2C/RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells were transfused into syngeneic RAG1<sup>-/-</sup> recipient mice before the injection of P1HTR cells (five mice per group). Symbols represent the mean and SE in each group. *B*, Purified 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> T cells were adoptively transferred into syngeneic RAG1<sup>-/-</sup> recipients before the inoculation of P1HTR-pEF or P1HTR-7M tumor cells (10 mice per group). Results are representative of two independent experiments.

T cells that had encountered anti-CTLA-4-bearing tumor cells in vivo had reduced cytokine production and specific lytic activity (data not shown) upon restimulation with Ag in vitro, suggesting that targeted CTLA-4 ligation in vivo had led to T cell hyporesponsiveness. Because tumor burden was greater in P1HTR-7M-bearing mice than in P1HTR-pEF-bearing mice, it was possible that reduced T cell function ex vivo was the result of the immunosuppressive effects of remaining tumor cells. This is unlikely, because CTLA-4<sup>-/-</sup> T cells had greater cytolytic activity than CTLA-4<sup>+/+</sup> T cells, even in recipient mice that rejected their tumors. This was not due to intrinsically greater function of CTLA-4-deficient T cells, as lack of CTLA-4 does not affect T cell thymic development (15) and naive 2C/RAG2<sup>-/-</sup>CTLA-4<sup>+/+</sup> and 2C/



**FIGURE 4.** CTLA-4 engagement in vivo resulted in reduced T cell function ex vivo. RAG1<sup>-/-</sup> mice having received T cells and tumors and remaining alive at 6 wk were sacrificed. Spleens were harvested and pooled (five mice with CTLA-4<sup>+/+</sup> T cells and P1HTR-pEF, four mice with CTLA-4<sup>+/+</sup> and P1HTR-7M, eight mice with CTLA-4<sup>-/-</sup> T cells and P1HTR-pEF, and eight mice with CTLA-4<sup>-/-</sup> T cells and P1HTR-7M). T cells were purified and restimulated with P815-B7-1 (*left panels*) or with PMA and ionomycin (P+I, *right panels*). Supernatants were assayed for cytokine content by ELISA.

RAG2<sup>-/-</sup>CTLA-4<sup>-/-</sup> T cells display comparable cytokine production and proliferation in vitro (14). Furthermore, cytokine production by CTLA-4-competent and -deficient T cells was similar following PMA and ionomycin stimulation. Our results are consistent with CTLA-4 engagement by 7M in vivo resulting in TCR-specific T cell hyporesponsiveness or anergy.

The role of CTLA-4 in inducing anergy in different T cell subsets has been controversial. CTLA-4 engagement contributed to tolerance induction in adoptively transferred CD4<sup>+</sup> DO11.10 TCR-transgenic T cells following systemic injection of OVA Ag (6, 16). However, CTLA-4 engagement was not required to induce anergy of CD8<sup>+</sup>2C/RAG2<sup>-/-</sup> TCR-transgenic mice challenged with soluble peptide (17). Whether these disparate results are due to differences between CD4<sup>+</sup> and CD8<sup>+</sup> T cells is under investigation. Nevertheless, our current results indicate that direct engagement of CTLA-4 on CD8<sup>+</sup>2C/RAG2<sup>-/-</sup> T cells by a target tissue can result in T cell hyporesponsiveness, suggesting that, whereas CTLA-4 ligation is not necessary for tolerization of 2C CD8<sup>+</sup> T cells (17), persistent ligation of CTLA-4 in effector cells can lead to their hyporesponsiveness.

Inhibition of T cell function in vivo by CTLA-4/7M ligation can theoretically occur during the initial recognition of Ag by the 2C TCR or during secondary engagement of effector T cells that have migrated into the tumor. Naive CD8<sup>+</sup> T cells are less susceptible to the inhibitory effect of CTLA-4 engagement than previously activated T cells (14, 18), suggesting that CTLA-4 may preferentially regulate effector CD8<sup>+</sup> T cells. In support of this hypothesis, Luhder et al. (19) have demonstrated in a diabetes model that CTLA-4 inhibits T cells upon re-encounter of target cells in the pancreas rather than during initial priming in pancreatic lymph nodes, supporting an inhibitory role of CTLA-4 at the effector stage of the immune response.

P1HTR tumor cells lacked B7 expression, as detected by flow cytometry. Therefore, greater growth of wild-type P1HTR tumors in recipients of CTLA-4<sup>+/+</sup> than in recipients of CTLA-4<sup>-/-</sup> T cells suggests that engagement of CTLA-4 by host B7 ligands limits T cell activation in vivo. Previous studies using P1HTR tumor cells have revealed that positive costimulation can be provided by host B7 molecules (20), supporting the importance of host B7 for rejection of B7-negative tumors. Our observations imply that host B7 molecules also ligate the negative regulator CTLA-4, suggesting that CTLA-4 may be engaged in *trans* with the TCR during recognition of 7M-negative tumors. However, in vitro, inhibition of T cell function occurs only when CTLA-4 is ligated in *cis* with the TCR (8). It is unlikely that CTLA-4 engagement occurs in *cis* in vivo following challenge with wild-type P1HTR, as the 2C TCR recognizes the p2Ca peptide with a 1000-fold lesser affinity when bound to K<sup>b</sup> vs L<sup>d</sup> (21), suggesting that CTLA-4 may be ligated effectively in *trans* in vivo.

Cross-linking of CTLA-4 is achieved in vitro upon its binding to dimeric Abs. How monomeric membrane-bound 7M triggers CTLA-4-mediated signals is unclear but may be similar to how monomeric B7-1, CTLA-4's natural ligand, functions. The costimulatory function of B7-1 was reported to depend on the presence of its cytoplasmic tail, which may interact with the actin-based cytoskeleton and localize B7-1 to complexes required for effective T cell costimulation (22). 7M comprises the transmembrane and cytoplasmic portion of B7-1 and could thus ensure clustering of CTLA-4 via a similar mechanism, as its level of expression on P1HTR cells is similar to that of B7-1 on APCs.

Prud'homme et al. (23) have recently described another approach to ligate CTLA-4 in vivo. The cDNA encoding for a membrane-bound form of B7-1 mutated to reduce its affinity for CD28 but retain CTLA-4 binding was coinjected in vivo with cDNA

encoding preproinsulin in nonobese diabetic mice. Such an approach prevented generation of insulin-reactive T cells. However, low levels of CD28 binding to mutated B7-1 could account for such results, because CD28 ligation can prevent autoimmune diabetes in nonobese diabetic mice (24). Expression of a membrane-bound form of an Ab specific for CTLA-4 in our study circumvents these caveats. Therefore, our data demonstrate a novel mechanism by which to effectively down-regulate allogeneic T cell responses through deliberate ligation of CTLA-4 *in vivo*. Allogeneic organs engineered to express membrane-bound anti-CTLA-4 Ab may represent a novel strategy to induce Ag-specific immunosuppression in clinical transplantation settings.

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