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CTLA-4 Is Not Required for Induction of CD8⁺ T Cell Anergy In Vivo

Kenneth A. Frauwirth,* Maria-Luisa Alegre, † and Craig B. Thompson ²*

Recent studies of T cell anergy induction have produced conflicting conclusions as to the role of the negative regulatory receptor, CTLA-4. Several in vivo models of tolerance have implicated the interaction of CTLA-4 and its ligands, B7.1 and B7.2, as an essential step in induction of anergy, while results from a number of other systems have indicated that signals from the TCR/CD3 complex alone are sufficient to induce T cell unresponsiveness. One explanation for this disparity is that the requirements for anergy induction depend closely on the details of the system: in vivo vs in vitro, route of stimulus administration, naive vs memory cells, CD4⁺ vs CD8⁺ cells, etc. To test this possibility, we established an in vivo anergy model using mice transgenic for the 2C TCR on a recombination-activating gene-2-deficient background, that either express or lack the CTLA-4 molecule. This system provides us with a very homogeneous pool of naive Ag-specific CD8⁺ T cells, allowing us to control some of the conditions mentioned above. We found that T cells from CTLA-4-deficient mice were anergized by injections of soluble antigenic peptide as efficiently as were CTLA-4-expressing cells. These results indicate that CTLA-4 is not universally required for in vivo T cell anergy induction and may point to distinctions between regulation of peripheral tolerance in CD4⁺ and CD8⁺ T cells. The Journal of Immunology, 2001, 167: 4936–4941.

Although a model with CTLA-4 as the anergy initiator molecule is an attractive one, there is a significant body of conflicting data. Anergy is not blocked by anti-CTLA-4 Abs in some in vivo systems (24, 25), despite close parallels to those in which CTLA-4 appears critical. Furthermore, B7.1/2-blocking agents, which prevent interaction with both CD28 and CTLA-4, can be used to induce anergy in vitro (26–29) and long-term allograft tolerance in vivo (30, 31). Because interaction with B7 is required for CTLA-4 to signal, it is difficult to reconcile the model with these data. Furthermore, CTLA-4-deficient CD8⁺ T cells can be anergized in vitro (32). These apparent inconsistencies have led to the suggestion that the mechanisms of anergy induction may differ among systems, with details such as route of stimulus administration (in vitro/in vivo, i.p./i.v./oral/intranasal), differentiation state of the cells (naïve/memory, primary cells/clones), and T cell subset (CD4⁺/CD8⁺, Th1/Th2) influencing the requirements. We therefore undertook a direct examination of the role of CTLA-4 in the initiation of T cell anergy in vivo in TCR-transgenic (Tg) mice, providing a very homogeneous population of naïve T cells and allowing us to control over many of the variables that may influence tolerance induction. Using CD8⁺ TCR-Tg, recombination-activating gene (RAG)2-deficient, CTLA-4-deficient T cells, we found that CTLA-4 signaling is unnecessary for in vivo anergy induction of naïve CD8⁺ T cells.

Materials and Methods

Animals

C57BL/6 mice (4–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). 2C TCR-Tg (33/RAG2⁻/⁻/CTLA-4⁻/⁻) mice were kindly provided by Dr. T. Gajewski (University of Chicago, Chicago, IL). All mice were maintained in the University of Pennsylvania Animal Barrier Facilities (Philadelphia, PA). Animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (Bethesda, MD).

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Abbreviations used in this paper: Tg, transgenic; RAG, recombination-activating gene.
Abs and reagents

FITC-labeled hamster IgG, anti-Thy1.2, anti-CD3, anti-TCR, and anti-CD45RB, and PE-labeled hamster IgG, anti-Thy1.2, anti-CD44, anti-CD25, and anti-Fas were purchased from PharMingen (San Diego, CA). The H-2Kb-restricted 2C TCR-reactive peptide SIYRYYGL was purchased from Multiple Peptide Systems (San Diego, CA). Recombinant human IL-2 was purchased from Boehringer Mannheim (Mannheim, Germany).

Cell culture

All cells were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with glutamine, penicillin/streptomycin, HEPES buffer, MEM nonessential amino acids, 2-ME, and 10% FCS (Life Technologies) at 37°C in a 7% CO2 atmosphere.

T cell purification

Murine T cells were isolated from spleen using the StemSep negative-selection system (StemCell Technologies, Vancouver, British Columbia, Canada) following the manufacturer’s instructions, as previously described (32). The nonretained fraction typically contained >95% Thy1+ cells, as determined by flow cytometry. For separation of CD4high and CD4low cells, purified T cells were passed through the StemSep system a second time, using biotinylated anti-CD44 mAb (StemCell Technologies) as the negatively selecting agent, and both flow-through (CD44low) and retained (CD44high) fractions were collected.

In vivo anergy induction

2C TCR-Tg T cells were anergized in vivo by multiple peptide injections, modifying the protocol of Dubois et al. (8). Mice were injected i.p. one to three times, at 4-day intervals, with either 20 µg of aminobutyric acid (GABA) or 25 µg of the 2C agonist peptide (SIYRYYGL) in PBS alone. Spleens were harvested 7 days after the final injection. T cells were purified as described above and tested for proliferation and cytokine secretion upon stimulation in vitro with irradiated C57BL/6 splenocytes plus titrated doses of peptide.

Cytokine and proliferation assays

IL-2, IFN-γ, TGFβ1, and IL-10 levels in 1-day restimulation culture supernatants were measured by sandwich ELISA. Primary and biotinylated secondary anti-cytokine Abs and recombinant cytokine standards were purchased from BD PharMingen and used at the concentrations recommended by the manufacturer. Alkaline phosphatase-conjugated avidin was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and used at a 1/3000 dilution. Colorimetric alkaline phosphatase substrate was purchased from Sigma-Aldrich (St. Louis, MO) and used at 1 mg/ml in 10% diethanolamine buffer, and quantitation was performed on a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were analyzed using SoftMax Pro software (Molecular Devices) by comparison with a standard curve generated using recombinant cytokines at known concentrations. Proliferation after 3 days of restimulation was determined by [3H]thymidine incorporation. Cells were pulsed for 6–8 h with 1 µCi/well [3H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA), transferred to glass-fiber filters with a 96-well cell harvester (Tomtec, Hamden, CT), and analyzed by liquid scintillation using a 1450 Microbeta scintillation counter (Wallac, Turku, Finland). Data points for all analyses are presented as the mean of triplicate wells.

Flow cytometry

Levels of CD3, TCR, CD25, CD44, CD45RB, and Fas were determined on T cells by two-color flow cytometry. Following harvest, cells were washed once in flow cytometry buffer (1% BSA and 0.01% sodium azide in PBS, pH 7.4) and incubated for 30 min on ice in 100 µl of flow cytometry buffer containing FITC-conjugated (anti-CD3, anti-TCR, anti-CD45RB) or PE-conjugated (anti-CD25, anti-CD44, anti-Fas) mAb; FITC- and PE-conjugated hamster IgG were used as negative controls. Cells were simultaneously stained with PE- or FITC-conjugated anti-Thy1.2 to assess T cell purity. Cells were resuspended in flow cytometry buffer and analyzed on either a FACSCaliber or an LSR flow cytometer (BD Biosciences, Mountain View, CA). Forward and side scatter gates were used to exclude dead cells. Data from ~104 live cells were analyzed using CellQuest software (BD Biosciences). For cell cycle analysis, 5 × 105 cells were fixed in 25% PBS/75% ethanol overnight at 4°C. The fixed cells were resuspended in propidium iodide staining solution (3.8 mM sodium citrate, 0.125 mg/ml RNase A, and 0.01 mg/ml propidium iodide), incubated on ice for 30 min, and then analyzed by flow cytometry as described above.

Results

Induction of in vivo anergy in 2C TCR-Tg mice

To examine whether anergy could be induced in vivo in the absence of CTLA-4, we developed an anergy induction system using mice Tg for the 2C TCR on a RAG2-deficient background. Anergy was induced by multiple i.p. injections of the 2C agonist peptide.
SIYRYYGL. Injecting CTLA-4-positive, 2C TCR-Tg, RAG-deficient mice two or three times with 2C agonist peptide led to a significant depletion of splenic T cells, ranging from ~40 to 80% reduction in T cell recovery relative to saline-injected controls (data not shown). Although the peptide-treated T cells had a small (resting) size (Fig. 1A) and showed no increase in CD25 levels (Fig. 1B), they expressed increased CD44 (Fig. 1C) and decreased CD45RB (data not shown), a pattern generally associated with Ag-experienced T cells. As previously reported (8), TCR levels on T cells from peptide-injected mice were consistently at least 80% of those on control T cells (Fig. 1D).

T cells from control and peptide-injected mice were stimulated in vitro with antigenic peptide and irradiated splenic APC, and the responses were compared. Peptide injection caused a dramatic reduction in both proliferation (Fig. 2A) and IL-2 secretion (Fig. 2B). We consistently saw at least 90% inhibition of both proliferation and IL-2 production with this protocol. We were unable to detect IL-10 in any culture supernatants, and TGF-β production was comparable for T cells from control and peptide-injected mice (data not shown). Viable T cells were recovered after 2 days of in vitro stimulation and analyzed for cell cycle distribution. Peptide-treated cells were arrested in the G1 phase of the cell cycle, whereas the majority of control T cells were in S or G2/M phase at this point (Fig. 2C). Thus, injection of soluble peptide induces a state of proliferative unresponsiveness in 2C TCR-Tg T cells.

The high level of inhibition was somewhat surprising, as the fraction of cells with a CD44 high (Ag-exposed) phenotype was generally <70%, and in some experiments was <30% (Fig. 1C and data not shown). The presence of a sizable fraction of nonresponsive, but apparently naive, cells suggested that anergic cells might be suppressing the responses of CD44low T cells (34–37). To test this possibility, T cells from peptide-injected mice were separated into CD44high and CD44low fractions (Fig. 3A) before restimulation. Although proliferation of the CD44low T cells was somewhat higher than that of the CD44high T cells, responses in both fractions were strongly inhibited (Fig. 3B), indicating that the CD44low cells were independently anergic and were not being suppressed by CD44high cells. CD44low cells separated from control (saline-injected) T cells responded identically to unseparated cells, showing that the separation procedure itself did not inhibit subsequent proliferation. This suggests that a fraction of T cells may have encountered injected Ag and been rendered nonresponsive, but failed to up-regulate CD44. This is supported by an analysis of CD25 and CD44 levels at various times after Ag injection. T cells from saline-treated mice were CD25<~1% CD25> and CD44low (<10% CD44high). Within 24 h of peptide injection, ~92% of splenic T cells were found to be CD25+, indicating exposure to Ag, whereas the CD44high fraction was 33% at this time and reached a maximum of 62% at 48 h postinjection (data not shown).

**CTLA-4-deficient T cells can be rendered anergic in vivo**

We next compared anergy induction in 2C TCR-Tg/RAG2−/−/CTLA-4−/− and CTLA-4−/− mice. Mice were injected with peptide as described above, and then T cells were isolated and stimulated in vitro. Peptide-induced depletion of T cells from spleen was comparable in CTLA-4−/− and CTLA-4−/− mice (41% depletion in CTLA4−/− and 56% depletion in CTLA4−/− in one

**FIGURE 2.** Multiple injections of peptide induce T cell anergy in vivo. T cells purified from mice injected as in Fig. 1 were stimulated in vitro with APC plus peptide, and proliferation (A) and IL-2 production (B) were measured. Data are presented as the means of triplicate wells, and error bars represent SD from the mean. C. DNA content was analyzed in freshly isolated (left panels) and stimulated (right panels) cells.
FIGURE 3. CD44<sup>low</sup> T cells from peptide-injected mice are anergic. T cells were purified from mice injected as in Fig. 1 and further separated into CD44<sup>high</sup> and CD44<sup>low</sup> fractions. A. CD44 levels on total (dashed line), CD44<sup>low</sup> fraction (thick gray line), and CD44<sup>high</sup> fraction (thin black line) peptide-injected T cells. The portion of each that expresses high levels of CD44 (indicated by M1) is: total, 70%; CD44<sup>low</sup>, 9.5%; CD44<sup>high</sup>, 82.5%. B. Proliferation of total and fractionated T cells was measured after in vitro stimulation with APC plus peptide.

representative experiment). As shown in Fig. 4, proliferation and IL-2 secretion were inhibited to comparable levels in CTLA-<sup>+</sup> and CTLA-<sup>-</sup> T cells. As with CTLA-4-expressing cells, TCR levels on peptide-treated CTLA-4-deficient T cells were at least 80% of control TCR levels (Fig. 4C). CD25, CD44, and CD45RB expression levels on CTLA-<sup>-</sup> and CTLA-<sup>+</sup> cells were also comparable (data not shown).

Although the T cells from peptide-injected mice appeared to be resting when isolated, we were concerned that the multiple injections may have led to an increased sensitivity to death (such as activation-induced cell death or TNF-mediated killing) upon stimulation (8), and that this could account for the nonresponsiveness of the CTLA-4-deficient cells. However, addition of rIL-2 to cultures restored the proliferation of T cells from multiply injected mice (Fig. 4D), indicating that the cells were not being killed upon in vitro stimulation. We also tested for anergy induction after only one injection of peptide, as single-injection protocols were used in several of the models implicating CTLA-4 (18, 19, 23) and might

FIGURE 4. Anergy induction in CTLA-4-expressing vs CTLA-4-deficient T cells. CTLA-4 wild-type (+/+ ) or knockout (−/−) mice were injected with either PBS or peptide as indicated. Proliferation (A) and IL-2 secretion (B) of purified T cells were measured after in vitro stimulation with APC plus peptide. C. TCR levels from PBS-injected (thin black line) and peptide-injected (thick gray line) mice are comparable. Isotype control is indicated by the dashed line. Upper panel, CTLA-4-positive mice. Lower panel, CTLA-4-negative mice. D. Proliferation of T cells anergized by multiple injections is rescued by addition of IL-2.
Using the 2C Tg/RAG2-deficient mice, we compared in vivo anergy induction in CTLA-4-expressing and CTLA-4-deficient T cells. With both single-injection and multiple-injection protocols, CTLA-4-deficient T cells were anergized as efficiently as cells expressing CTLA-4. Thus, there appears to be no difference between in vitro and in vivo anergy induction for CD8+ TCR-Tg T cells with regard to CTLA-4 requirement. However, these in vitro and in vivo results conflict with the recent report of Greenwald et al. (23). Using a CD4+ TCR-Tg system, DO11.10, Greenwald et al. found that i.p. injection of soluble Ag tolerized CTLA-4-positive, but not CTLA-4-negative, naive T cells. Similar results were obtained by Perez et al. (18), who showed that a blocking anti-CTLA-4 Ab could prevent anergy induction in the same DO11.10 Tg system. Understanding the difference between the 2C and DO11.10 models will be important for determining the role that CTLA-4 plays in regulating peripheral tolerance.

One distinction between the two models involves the details of the anergy induction protocols. The DO11.10 system uses adoptive transfer of a relatively small number of Ag-specific T cells into non-Tg mice, whereas the 2C system involves Ag injection into intact Tg mice. We would predict that this would make it more difficult to induce anergy in the 2C system due to the higher number of Ag-specific T cells. However, it is possible that the monoclonality of the 2C system leads to a massive immune response to peptide injection, causing systemic effects that result in anergy as a secondary consequence independent of CTLA-4 expression. We do not favor this explanation, as the strong systemic response induced by bacterial superantigen does not bypass the CTLA-4 requirement for anergy induction by staphylococcal enterotoxin B (19); however, the spectrum of cytokines induced by soluble peptide may differ from that induced by superantigen.

A second distinction between the systems is that the DO11.10 TCR is MHC class II-restricted, giving rise to CD4+ T cells, while the 2C TCR is MHC class I-restricted, giving rise to CD8+ T cells. It has been found that there is a skewing toward preferential activation of CD4+ T cells in (non-TCR-Tg) CTLA-4-deficient mice, and that both the activated T cell phenotype and lymphoproliferative disease in these mice are dependent on CD4+, but not CD8+ T cells (40). This suggests that the inhibitory function of CTLA-4 plays a greater role in the regulation of CD4+ cells than that of CD8+ cells. Thus, induction of anergy in CD8+ T cells may depend on CTLA-4-independent signals, while CD4+ T cells require CTLA-4. Viewed in a larger context, this may reflect the fact that a wide variety of B7-negative cells in the body are potential class I MHC APC, allowing tolerization of CD8+ T cells without the need to cross-present tissue-specific Ags on “professional,” B7-expressing APC.

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