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Induction of T Cell Anergy in the Absence of CTLA-4/B7 Interaction

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

Immunologic tolerance in T lymphocytes is maintained through both thymic and peripheral contributions. One peripheral tolerance mechanism is the induction of T cell anergy, a form of nonresponsiveness resulting from incomplete T cell activation, such as stimulation through the TCR in the absence of costimulation. Recent reports have suggested that engagement of the inhibitory receptor CTLA-4 by its B7 ligand is critical for the initiation of anergy. We tested the importance of CTLA-4 in anergy induction in primary T cells with an in vitro anergy system. Using both CTLA-4/B7-blocking agents and CTLA-4-deficient T cells, we found that T cell anergy can be established in the absence of CTLA-4 expression and/or function. Even in the absence of CTLA-4 signal transduction, T cells activated solely through TCR ligation lose the ability to proliferate as a result of autocrine IL-2 production upon subsequent receptor engagement. Thus, CTLA-4 signaling is not required for the development of T cell anergy. The Journal of Immunology, 2000, 164: 2987–2993.

The activation of resting T lymphocytes is a highly regulated process requiring two distinct signaling events. Upon interaction of the TCR with its cognate peptide/MHC complex on an APC, signal 1 is initiated by the TCR-associated CD3 complex. However, full activation does not occur unless a second, costimulatory signal (signal 2) is also received. An important costimulatory molecule on resting T cells appears to be CD28 (1–3), which initiates signaling after binding to members of the B7 family on the APC; however, other T cell surface molecules, such as CD2 (4), LFA-1 (5–7), and CD27 (8), have been reported to have costimulatory activity.

T cells that receive signal 1 in the absence of signal 2 enter a state of nonresponsiveness to subsequent stimulation (9–11). This nonresponsiveness, termed anergy, is characterized by a failure to proliferate or secrete IL-2 upon Ag receptor engagement even in the presence of costimulation. Anergic T cells have been reported to have defects in the Ras and mitogen-activated protein kinase signaling pathways (12–14), reduced Ca2+ flux (15), and altered tyrosine kinase activity (15, 16). These signaling deficiencies appear to lead to reduced NF-κB and AP-1 transcriptional activities (17–19). Anergy was originally described in CD4+ T cell clones, but it has since been shown to occur in primary CD4+ and CD8+ T cells, both in vitro and in vivo (20–24), and may be a significant component of peripheral tolerance.

Several groups have recently proposed that CTLA-4, a molecule expressed on activated T cells, acts as the trigger for anergy (20, 21). CTLA-4, like CD28, binds to members of the B7 family, but has an inhibitory effect on T cell activation (25, 26), making CTLA-4 a logical candidate for initiating anergy. Further, the defects in anergic cells appear to be similar to the acute effects of CTLA-4 cross-linking, namely blockade of proliferation and IL-2 production (25, 26), reduction of mitogen-activated protein kinase activation (27, 28), and inhibition of AP-1 and NF-κB activity (29). Indeed, administration of anti-CTLA-4 blocking Abs during anergy induction appears to inhibit the induction of T cell hyporesponsiveness in several systems (20, 21, 30–32), and these observations are the basis for models invoking CTLA-4 signal transduction in the induction of anergy.

Although the hypothesis that CTLA-4 is the anergy initiator molecule is an attractive one, many in vitro anergy systems exclude B7.1/2-expressing APC, and B7.1/2-blocking agents have been used to induce anergy when B7-expressing cells are present (33–38). Because interaction with B7 is required for CTLA-4 signaling, it is difficult to reconcile the model with these data. We therefore undertook a direct examination of the role of CTLA-4 in the initiation of T cell anergy. Using both reagents that block the CTLA-4/B7 interaction and CTLA-4-deficient T cells, we found that CTLA-4 signaling is not required for anergy induction in vitro.

Materials and Methods

Animals
Female C57BL/6 mice (4–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Lck-Bcl-xL transgenic mice have been described previously (39). F5 TCR transgenic (40)/recombination-activating gene 1 (RAG1−/−)/CTLA-4−/− mice were provided by Dr. Philip Ashton-Rickardt (University of Chicago, Chicago, IL). 2C TCR transgenic (41)/RAG2−/−/CTLA-4−/− mice were provided by Dr. Thomas Gajewski (University of Chicago). DO.11.10 TCR transgenic mice (42) were the gift of Dr. Kenneth Murphy (Washington University, St. Louis, MO). All mice were maintained in the University of Chicago Animal Barrier Facilities.

Abs and reagents
UC10-4F10-11 (4F10; hamster anti-CTLA-4) and 145-2C11 (2C11; hamster anti-CD3ε) were purified from hybridoma supernatants by passage over protein A-Sepharose columns. FITC-labeled hamster IgG, anti-Thy1.2, anti-CD3, anti-TCR, anti-CD25, PE-labeled hamster IgG, anti-Thy1.2, anti-CD69, anti-CD44, and anti-CD26L were purchased from PharMingen (San Diego, CA). Murine CTLA4-Ig was provided by Genetics Institute (Cambridge, MA). Influenza nucleoprotein 366–374 peptide ASNENMDAM (H-2Db restricted, F5 TCR reactive) was provided by Dr.

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3 Abbreviations used in this paper: RAG, recombination-activating gene; Tg, transgenic.
Philip Ashton-Rickardt (University of Chicago). The H-2K- restricted 2C TCR-reactive peptide SIYRYYGL and the OVA 223-239 peptide SQAVHAAEINAGR (I-A<sup>d</sup> restricted, DO.11.10 TCR reactive) were purchased from Multiple Peptide Systems (San Diego, CA).

Cell lines and culture

The OVA<sub>223-239</sub>I-A<sup>d</sup>-specific T<sub>1</sub>L<sub>1</sub> clone pGL10 was the gift of Dr. Thomas Gajewski (University of Chicago). All cells were maintained in DMEM (Life Technologies, Grand Island, NY) supplemented with 1-glutamine, penicillin, streptomycin, HEPES buffer, MEM nonessential amino acids, 2-ME, and 10% FBS (Life Technologies), at 37°C in a 7% CO<sub>2</sub> atmosphere.

T cell purification

Murine T cells were isolated from spleen and mesenteric lymph nodes using the StemSep negative selection system (StemCell Technologies, Vancouver, Canada) following the manufacturer’s instructions. Briefly, single-cell suspensions were prepared by manual disruption of spleen and lymph nodes and passage of cells through nylon mesh. Erythrocytes were depleted by hypotonic lysis. The cells were washed and resuspended in PBS supplemented with 2% FBS and 5% heat-inactivated normal rat serum (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were incubated with StemSep T cell enrichment Ab cocktail (a mixture of biotinylated anti-B cell, anti-erythrocyte, and anti-myectoid Abs) at 4°C, washed, and resuspended in PBS plus 2% FBS. The cells were then incubated with StemSep anti-biotin tetramer (a bifunctional, anti-biotin/anti-dextran tetramer), followed by incubation with dextran-linked magnetic beads and passage over magnetized columns to deplete non-T cells. The nonretained fraction typically contained >95% Thy1<sup>+</sup> cells, as determined by flow cytometry. To purify CD4<sup>+</sup> or CD8<sup>+</sup> T cells, the appropriate StemSep Ab cocktails (containing the components of the T cell enrichment Ab cocktail plus either anti-CD8 or anti-CD4 Abs, respectively) were used in place of StemSep T cell enrichment Ab cocktail.

In vitro anergy induction

Purified murine T cells were cultured on immobilized anti-CD3 mAb (2C11) for 2 days in the presence or absence of either 50 µg/ml soluble CTLA4-Ig or 25 µg/ml soluble anti-CTLA-4 mAb (4F10). Cells were harvested, resuspended in fresh medium, and cultured for 2 additional days in the absence of anti-CD3 mAb (“rest”). T cells were collected, counted, and tested for anergy by measuring cytokine secretion and proliferation upon restimulation with irradiated C57BL/6 splenocytes and titrated doses of anti-CD3 mAb. For proliferation assays, 5 × 10<sup>5</sup> T cells plus 2 × 10<sup>4</sup> splenocytes were cocultured in a final volume of 200 µl. For cytokine assays, 2.5 × 10<sup>4</sup> T cells plus 1 × 10<sup>6</sup> splenocytes were cocultured in a final volume of 1 ml. In some experiments with TCR transgenic T cells, the specific peptide was used instead of anti-CD3 mAb in the restimulation cultures.

Cytokine and proliferation assays

IL-2, IL-4, and IFN-γ levels in 1-day restimulation culture supernatants were measured by sandwich ELISA. Primary and biotinylated secondary anti-cytokine Abs were purchased from PharMingen and used at the concentrations recommended by the manufacturer. Alkaline phosphatase-conjugated avidin was purchased from Jackson ImmunoResearch Laboratories and used at a 1/3000 dilution. Colorimetric alkaline phosphatase substrate (Sigma, St. Louis, MO) was used at 1 mg/ml in 10% dithiothreitol buffer, and colorimetric readout 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 [<sup>3</sup>H]thymidine incorporation. Cells were pulsed for 7–8 h with 1 µCi of [<sup>3</sup>H]thymidine (ICN, Costa Mesa, CA) /well, transferred to glass-fiber filters with a 96-well cell harvester (Tomtec, Hamden, CT), and analyzed by liquid scintillation using a 1205 Betaplate 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, CD69, CD44, and CD62L were determined on freshly purified, anti-CD3-treated, and rested T cells by two-color flow cytometry. Following harvest, cells were washed once in FACS buffer (1% BSA and 0.01% sodium azide in PBS, pH 7.4) and incubated for 30 min on ice in 100 µl of FACS buffer containing FITC-conjugated (anti-CD3, anti-TCR, anti-CD25 mAbs) or PE-conjugated (anti-CD69, anti-CD44, anti-CD62L mAbs) Ab. FITC- and PE-conjugated hamster IgG were used as negative controls. Cells were simultaneously stained with PE- or FITC-conjugated anti-Thy1.2 mAb to assess T cell purity. After a final wash, cells were resuspended in FACS buffer and analyzed on a FACSSort flow cytometer (Becton Dickinson, Mountain View, CA). Forward and side scatter gates were used to exclude dead cells. Data from 10<sup>4</sup> live cells were analyzed using CellQuest software (Becton Dickinson). For cell cycle analysis, 10<sup>6</sup> cells were fixed in 25% PBS/75% ethanol for 1 h 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 anergy in primary T cells in vitro

Many of the studies used to define T cell anergy have been performed using T cell clones. To determine whether anergy could also be established in normal mouse T cells, an anergy induction system using immobilized anti-CD3 Ab (mAb 2C11) to stimulate primary T cells was developed, based on the protocol of Jenkins et al. (10). Culturing purified C57BL/6 T cells on anti-CD3-mAb coated dishes for 2 days followed by 2 days of rest (culture in the absence of Ab) resulted in a profound nonresponsiveness to restimulation through Ag receptor. The T cells failed to proliferate or secrete IL-2 when stimulated with splenic APC plus anti-CD3 mAb (Fig. 1), but were hyper-responsive to exogenously added IL-2 (data not shown), indicating that the lack of response was not due to T cell death during the restimulation. This was confirmed by manual cell count (data not shown).

A major fate of purified T cells was to undergo apoptotic death during the initial 4-day culture, however, limiting the yields of viable anergic cells. This difficulty in maintaining cell survival was not limited to the anergic cell population, as freshly isolated T cells cultured in the absence of stimulation also displayed low levels of viability after 4 days (data not shown). To avoid these obstacles, T cells isolated from animals bearing a transgene for the anti-apoptotic protein Bcl-x<sub>L</sub> under the control of the Lck proximal promoter (39) were used. The Bcl-x<sub>L</sub> transgenic (Tg) T cells maintained high viability in culture with or without anti-CD3 mAb stimulation (39) and, like their nontransgenic counterparts, became anergic when treated with immobilized anti-CD3 mAb (Fig. 1, b and c). Bcl-x<sub>L</sub> Tg T cells left untreated in culture for 4 days generally had proliferative and cytokine responses comparable to those of freshly isolated T cells, indicating that the culture conditions themselves did not contribute significantly to nonresponsiveness (data not shown).

During anergy induction, the T cells were induced to express several activation markers, including CD25 (Fig. 2a) and CD69 (data not shown), both of which dropped back to near initial levels during the rest period. Culture with anti-CD3 mAb caused a marked reduction in surface CD3 levels, but CD3 was re-expressed to nearly starting levels by the end of the rest period (Fig. 2b). T cells also went through blastogenesis during anergy induction (Fig. 3). Several reports have linked anergy induction with G<sub>1</sub> arrest (43–45). The DNA content of T cells was therefore analyzed over the course of anergy induction. Treatment with immobilized anti-CD3 mAb induced progression through S and G<sub>2</sub>/M phases (Fig. 3), and cells returned to G<sub>0</sub> phase during the rest period. Upon restimulation, however, the anergized T cells were unable to exit the G<sub>0</sub> phase of the cell cycle, unlike T cells that had been left untreated in culture for 4 days (Fig. 3). These findings are consistent with results from other models, in which anergy is preceded by an initial proliferative response (21, 46–48).
Blocking CTLA-4/B7 does not prevent anergy induction

The T cell inhibitory receptor CTLA-4 is thought to be important for the induction and/or maintenance of peripheral tolerance (20, 21, 30–32, 49–51). We therefore used the immobilized anti-CD3 mAb/Bcl-xL Tg T cell system to investigate the role of CTLA-4/B7 interactions in anergy induction. Freshly isolated T cells (>95% Thy1+ after purification) did not express detectable B7.1 or B7.2, but up-regulated both B7.2 (transiently) and B7.1 during anti-CD3 mAb treatment (data not shown), raising the possibility that B7 molecules on the T cells themselves could act as the ligands for CTLA-4. We therefore added either CTLA4-Ig, which binds B7 molecules with high affinity, or α-CTLA-4 mAb (mAb 4F10) to prevent any potential CTLA-4/B7 interactions. Addition of CTLA4-Ig to cultures was completely ineffective at preventing nonresponsiveness (Fig. 4, a and b), even at levels that totally abrogated CD28 costimulation in parallel cultures (Fig. 4c). Likewise, coculture with soluble α-CTLA-4 mAb consistently had no appreciable effect on anergy induction (Fig. 5).
Analysis of CD4+ /CD8+ subsets after anergy induction in some experiments revealed a skewing toward CD8+ cells, although the reasons for this are unknown. The reports linking CTLA-4 to anergy either evaluated only CD4+ cells (20) or concluded that α-CTLA-4 treatment most dramatically affected CD4+ cells (21), raising the concern that our failure to prevent anergy by CTLA-4 blockade might be due at least in part to this CD8+ skewing. We therefore compared anergy induction in purified CD4+ and CD8+ T cells. Culture on immobilized anti-CD3 mAb inhibited subsequent proliferative and IL-2 responses of both subsets, and treatment with α-CTLA-4 mAb was unable to prevent anergy in either CD4+ (Fig. 6) or CD8+ (data not shown) cells. Likewise, CTLA4-Ig did not block induction of anergy in T cells purified from DO.11.10 TCR (OVA/I-Ad-specific) transgenic mice, and neither CTLA4-Ig nor anti-CTLA-4 mAb inhibited anergy induction in the OVA/I-Ad-specific CD4+ clone pGL10 (data not shown).

CTLA-4-deficient T cells can be anergized

The inability of either CTLA4-Ig or α-CTLA-4 mAb to prevent T cell nonresponsiveness in this system strongly suggested that CTLA-4 is not required for anergy induction; however, it was possible that neither reagent completely blocked CTLA-4/B7 interactions, allowing a small, but sufficient, amount of CTLA-4 signaling to occur. We therefore made use of CTLA-4-deficient T cells to address this concern. CTLA-4 knockout mice develop a fatal lymphoproliferative disease within 3–4 wk of birth (49, 50). However, when the CTLA-4 deficiency was bred onto TCR transgenic, RAG2−/− mice, the exclusive expression of either the 2C (L2-alloreactive (41)) or F5 (D3/influenza nucleoprotein-reactive (40)) receptor led to T cell populations that avoided the lymphoproliferative disease (M. Alegre, unpublished observations).

To study anergy in the complete absence of CTLA-4, we purified 2C TCR Tg T cells from CTLA-4+ and CTLA-4− age-matched mice and cultured them with immobilized anti-CD3 mAb as described above. As shown in Fig. 7, both proliferation and IL-2 secretion were greatly reduced upon restimulation in CTLA-4+.
and CTLA-4+ T cells after culture on plate-bound anti-CD3 mAb. Although the CTLA-4+ cells consistently gave higher overall responses, the degree of inhibition by the anergizing treatment was comparable (IL-2 secretion: ~84% inhibition with CTLA-4+ cells, ~79% with CTLA-4− cells; proliferation: ~94% inhibition with CTLA-4+ cells, ~89% with CTLA-4− cells; averaged over the top three peptide concentrations). Treatment with immobilized anti-CD3 mAb also inhibited subsequent proliferative responses equally in CTLA-4+ and CTLA-4− F5 TCR Tg T cells (Fig. 7c).

Discussion

To study anergy in primary T cells, we established an in vitro system using immobilized anti-CD3 mAb as the anergizing agent. We found that induction of anergy follows an activation phase, which includes expression of CD25 and CD69, modulation of CD3, blastogenesis, and progression through the cell cycle. We further demonstrated that anergy can be induced in primary T cells in the absence of any CTLA-4/B7 interaction. Neither CTLA4-Ig nor α-CTLA-4 mAb was able to prevent anergy induced by immobilized anti-CD3 mAb, and CTLA-4− deficient T cells were as susceptible as CTLA-4+ expressing cells to anergy induction. Thus, CTLA-4 is not required for initiating T cell anergy in this system.

These results conflict with reports from the Abbas (20) and Bluestone (21) groups, which identified CTLA-4 as critical for anergy induction. One explanation for this discrepancy may be our use of an in vitro system. Perez et al. analyzed anergy induction in vivo by soluble Ag, and Walunas et al. studied induction of anergy by staphylococcal enterotoxin B, also in vivo. Although it is possible that the requirements for anergy in vivo differ from those in vitro, one must also interpret in vivo results carefully due to the increased complexity of in vivo systems. Injection of anti-CTLA-4 Ab may alter the basal responses of T cells other than those being assayed. Unimmunized CTLA-4− deficient mice develop a severe lymphoproliferative disease (49, 50), indicating that CTLA-4 regulates immune responses to environmental and self-Ags. An increase in IL-2 levels due to an enhancement of general T cell activity could prevent or reduce anergy induction (52, 53), independent of any cell-autonomous role for CTLA-4. Consistent with our in vitro results, a recent study found that blockade of CTLA-4 Ab may alter the basal responses of T cells other than those being assayed.

Earlier models of anergy have generally proposed that TCR/CD3 ligation leads to both positive and negative signals in T cells. The decision between activation and anergy would depend on the balance of those signals, with costimulatory pathways tipping the balance from anergy to activation. Kaufman et al. (54) have formulated such a model in mathematical terms, predicting anergy vs
activation based on the kinetics of ligand binding and signal transduction. Although this model does not exclude the possible contributions of inhibitory receptors such as CTLA-4, only Ag receptor and IL-2 signaling are required. The model predicts that anergy will follow an activation phase if dissociation of the activating ligand is slow, resulting in a prolonged signal, and this is consistent with our results using anti-CD3 mAb.

An important consideration in trying to build models to understand anergy induction is that the phenomena being studied as anergy are, in fact, a collection of responses to tolerizing stimuli, with different requirements and mechanisms. For example, the ability of anergized cells to differentiate into effector cells or to produce cytokines other than IL-2 (particularly IL-4, IL-10, and IFN-γ) depends on the anergy induction protocol used, as does the ability of CD28 costimulation and exogenous IL-2 to prevent anergy. Thus, CTLA-4 may be important for some pathways to anergy, but is not a universal trigger. Analysis of anergy induction is further complicated by the observation that T cells exposed to various tolerizing conditions can develop into regulatory cells able to suppress the responses of nontolerized cells (55–59). One mechanism of suppression, observed after injection of bacterial superantigen, involves the production of TGF-β (59). Induction of TGF-β secretion is also a result of CTLA-4 signaling (60), suggesting that some apparent effects of CTLA-4 on anergy may, in fact, represent contributions to suppressive mechanisms.

There are considerable data to support a role for CTLA-4 in the down-regulation of immune responses, and a number of strategies to restore or enhance in vivo immune responses by inhibiting CTLA-4 signal transduction have been proposed (61, 62). However, our findings indicate that T cell anergy can be established in the absence of CTLA-4 function and that studies to establish or reverse Ag-specific anergy should not focus exclusively on the absence of CTLA-4 function, and that studies to establish or reverse Ag-specific anergy should not focus exclusively on CTLA-4. Continued characterization of alternate mechanisms for anergy induction using either the TCR or other T cell-specific receptors is warranted.

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References


