

Immunologic Self-Tolerance Maintained by CD25⁺CD4⁺ Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte-associated Antigen 4

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Abstract

This report shows that cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) plays a key role in T cell-mediated dominant immunologic self-tolerance. In vivo blockade of CTLA-4 for a limited period in normal mice leads to spontaneous development of chronic organ-specific autoimmune diseases, which are immunopathologically similar to human counterparts. In normal naive mice, CTLA-4 is constitutively expressed on CD25⁺CD4⁺ T cells, which constitute 5–10% of peripheral CD4⁺ T cells. When the CD25⁺CD4⁺ T cells are stimulated via the T cell receptor in vitro, they potently suppress antigen-specific and polyclonal activation and proliferation of other T cells, including CTLA-4-deficient T cells, and blockade of CTLA-4 abrogates the suppression. CD28-deficient CD25⁺CD4⁺ T cells can also suppress normal T cells, indicating that CD28 is dispensable for activation of the regulatory T cells. Thus, the CD25⁺CD4⁺ regulatory T cell population engaged in dominant self-tolerance may require CTLA-4 but not CD28 as a costimulatory molecule for its functional activation. Furthermore, interference with this role of CTLA-4 suffices to elicit autoimmune disease in otherwise normal animals, presumably through affecting CD25⁺CD4⁺ T cell-mediated control of self-reactive T cells. This unique function of CTLA-4 could be exploited to potentiate T cell-mediated immunoregulation, and thereby to induce immunologic tolerance or to control autoimmunity.

Key words: CTLA-4 • autoimmune disease • regulatory T cell • CD25 • self-tolerance

Introduction

CTL-associated antigen 4 (CTLA-4 [CD152]) is a CD28 homologue expressed on activated T cells and, upon interaction with CD80 or CD86 on APCs, exerts a downregulatory or attenuating effect on T cell-mediated immune responses (1, 2). Accumulating evidence indicates that it also plays an important role in immunologic self-tolerance in the periphery. CTLA-4-deficient mice, for example, develop a severe lymphoproliferative disorder and die from autoimmune-like disease within 1 mo after birth (3, 4).

Administration of anti-CTLA-4 Ab exacerbated autoimmune responses in a murine model of multiple sclerosis or insulin-dependent diabetes mellitus (IDDM [5–7]), enhanced tumor immunity (8), or prevented the induction of immunologic tolerance to concurrently administered non-self-antigens (9). However, it is uncertain whether these findings can be explained by the current notion that CTLA-4 transduces negative signals to activated effector T cells (1, 2, 10). In this report, we show another possible role of CTLA-4 in immunologic self-tolerance.

There is accumulating evidence that, in addition to clonal deletion or anergy, T cell-mediated dominant control of self-reactive T cells represents one mechanism for maintaining immunologic self-tolerance (11). For example, removal

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of a subpopulation of CD4⁺ T cells leads to spontaneous development of various T cell-mediated autoimmune diseases (e.g., thyroiditis, gastritis, and insulin-dependent diabetes mellitus) in otherwise normal rodents, and reconstitution of the removed population prevents the autoimmune development (12, 13). Elimination of CD25⁺CD4⁺ T cells (which constitute 5–10% of peripheral CD4⁺ T cells in normal naive mice), for example, elicits various autoimmune diseases in genetically susceptible strains of mice (14–20). The elimination also evokes potent tumor immunity in otherwise nonresponding animals (17). These *in vivo* and *in vitro* findings, especially similar effects of CTLA-4 blockade and CD25⁺ T cell elimination on autoimmunity and tumor immunity, have led us to examine possible involvement of CTLA-4 in T cell-mediated immunoregulation.

Here, we demonstrate that CTLA-4 is indeed a key costimulatory molecule for activating CD25⁺CD4⁺ regulatory T cells to exert suppression and thereby to control self-reactive T cells, and that *in vivo* blockade of CTLA-4 expressed on the regulatory T cells suffices to break natural self-tolerance and elicit pathological autoimmunity.

Materials and Methods

Mice. 8-wk-old BALB/c, BALB/c athymic nude, or C57BL/6 (B6) mice were purchased from SLC. DO11.10 transgenic mice expressing transgenic TCR specific for an OVA peptide were the gift of Dr. D.Y. Loh (Roche Japan, Kamakura, Japan). CTLA-4- or CD28-deficient mice on the B6 background were described previously (3, 21). All these mice were maintained in our animal facility and cared for in accordance with the institutional guidelines for animal welfare.

***In Vivo* Injection of mAb.** The hybridoma cells secreting hamster anti-CTLA-4 mAb (UC10-4F10-11) were a gift from Dr. J. Bluestone (University of Chicago, Chicago, IL [22]). The Ab was purified from ascites in SCID mice using protein A columns (22, 23). Rat anti-CD25 mAb (PC61) was purified from ascites in SCID mice by 40% ammonium sulfate precipitation twice as described previously (17).

Serologic Analysis. For flow cytometric analysis, 10⁶ cells were incubated with FITC- or PE-labeled or biotinylated mAbs, with

RPE-CyChrome 5-streptavidin (Dako) as the secondary reagent for biotinylated Abs, and analyzed by flow cytometry (Epics-XL; Beckman Coulter). FITC-labeled or biotinylated anti-CD25 mAb (7D4), FITC-anti-CD4 mAb (H129.19), PE-labeled anti-CTLA-4 mAb (UC10-4F10-11), and anti-FcR mAb (2.4G2) were purchased from BD PharMingen.

Cell Sorting. Spleen and lymph node cell suspensions prepared from 8-wk-old BALB/c, DO11.10, or CD28-deficient mice or 2–3-wk-old CTLA-4-deficient mice were stained with FITC-anti-CD25 mAb (7D4) and PE-anti-CD4 mAb (H129.19; BD PharMingen) and sorted by a FACS® (Epics-Elite; Beckman Coulter), as described previously (18, 19). Purity of the CD25⁺ or CD25⁻CD4⁺ population was >90% and ~99%, respectively.

***In Vitro* Proliferation Assay.** Lymph node and spleen T cells (2.0–2.5 × 10⁴), sorted as described above, and red blood cell-lysed, x-irradiated (20 Gy) BALB/c spleen cells (5 × 10⁴) as APCs were cultured for 3 d in 96-well round-bottomed plates (Costar) in RPMI 1640 medium supplemented with 10% FCS (GIBCO BRL), penicillin (100 U/ml), streptomycin (100 µg/ml), and 50 µM 2-ME. Anti-CD3 mAb (145-2C11; Cedarlane Laboratories) at a final concentration of 10 µg/ml or Con A at 1 µg/ml were added to the culture for stimulation (18, 19). Incorporation of ³H-TdR (1 µCi/well; Dupont) by proliferating lymphocytes during the last 6 h of the culture was measured (18, 19). Fab fragments of anti-CTLA-4 mAb or normal hamster IgG (Cortex Biochem, Inc.) were prepared by digesting these Abs with immobilized papain (Pierce Chemical Co.) according to the manufacturer's instruction.

Histology and Serology. Stomachs and other organs were fixed with 10% formalin and processed for hematoxylin and eosin staining. Serum titers of autoantibodies specific for the gastric parietal cells were assayed by ELISA (14, 15). Gastritis was graded 0–2+ depending on macroscopic and histological severity, as described previously (12, 14, 15).

Results and Discussion

Constitutive Expression of CTLA-4 on CD25⁺CD4⁺ T Cells in Normal Naive Mice. CTLA-4 is supposed to be expressed on T cells only after activation (1, 2). However, in the peripheral lymphoid organs of normal naive mice, flow cytometric analysis of cell surface as well as cytoplasmic

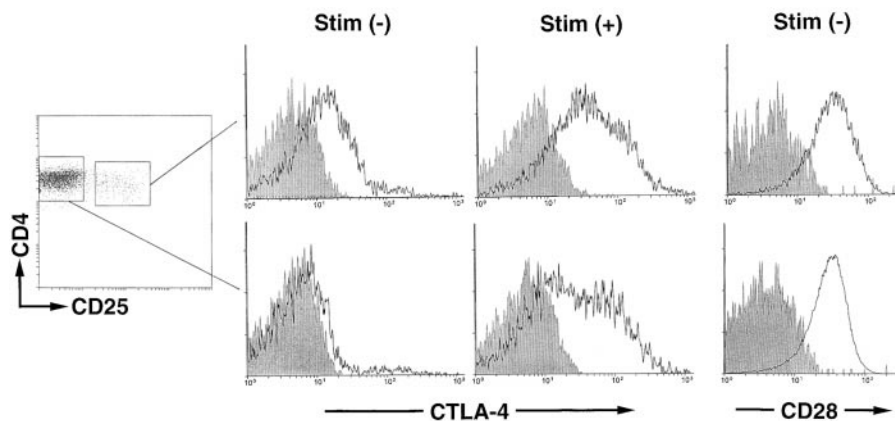


Figure 1. Constitutive expression of CTLA-4 on CD25⁺CD4⁺ T cells in normal naive mice. Lymph node and spleen cell suspensions prepared from a 2-mo-old BALB/c mouse were stained with FITC-anti-CD4 Ab and biotinylated anti-CD25 Ab with RPE-CyChrome 5-conjugated streptavidin as the secondary reagent. Cells enclosed with rectangles were sorted as CD25⁺ or CD25⁻CD4⁺ T cells. The sorted cells were stained for 2 h at 37°C with PE-conjugated anti-CTLA-4 Ab or PE-conjugated anti-human CD4 Ab as a staining control, and are shown as histograms (solid lines or shaded areas, respectively); the abscissa shows the staining intensity on a logarithmic scale and the ordinate shows the number of cells on an arbitrary scale (reference 28). The

sorted cells were also cultured with Con A (1 µg/ml) at 37°C for 24 h and incubated for the last 2 h with PE-anti-CTLA-4 Ab. CD28 expression on CD25⁺ or CD25⁻CD4⁺ T cells is shown as histograms by staining the cells with PE-anti-CD28 Ab. A representative result of five independent experiments is shown. Stim., stimulation.

mic expression of CTLA-4 revealed that the molecule was constitutively expressed on/in CD25⁺CD4⁺ T cells but not on/in CD25⁻CD4⁺ T cells (or CD8⁺ T cells; data not shown) whether the mice were raised in a specific pathogen-free condition or not (Fig. 1). Both populations expressed high levels of CTLA-4 upon *in vitro* T cell stimulation (for example, with Con A). The two populations showed no difference in their expression levels of CD28, which appears to be constitutively expressed on all murine T cells (24).

Induction of Autoimmune Disease in Normal Mice by Anti-CTLA-4 Ab Treatment. To determine the role of CTLA-4 expressed on CD25⁺CD4⁺ T cells in self-tolerance and autoimmunity, we administered purified anti-CTLA-4 mAb (0.8 or 0.1 mg), anti-CD25 mAb (1 mg), or the mixture of anti-CTLA-4 mAb (0.1 mg) and anti-CD25 mAb (1 mg) once a week three times to normal BALB/c mice

from the age of 10 d, and examined the mice at 3 mo of age (17, 25; Fig. 2). All the mice treated with high doses (0.8 mg/injection) of anti-CTLA-4 mAb spontaneously developed histologically and serologically evident autoimmune gastritis and some other autoimmune diseases (i.e., oophoritis or mild sialoadenitis, in one mouse each) (12, 14, 15; Fig. 2, A–C). Low doses of anti-CTLA-4 mAb or anti-CD25 mAb alone induced only low titers of autoantibody without histological evidence of autoimmune disease, whereas treatment with the mixture of the two mAbs produced autoimmune gastritis in the majority (~85%) of mice.

Administration of anti-CTLA-4 mAb did not significantly alter the number of CD25⁺CD4⁺ T cells or the ratio of CD25⁺ T cells among CD4⁺ T cells, whereas administration of anti-CD25 mAb or the mixed mAbs reduced CD25⁺CD4⁺ T cells to 10–30% of the controls

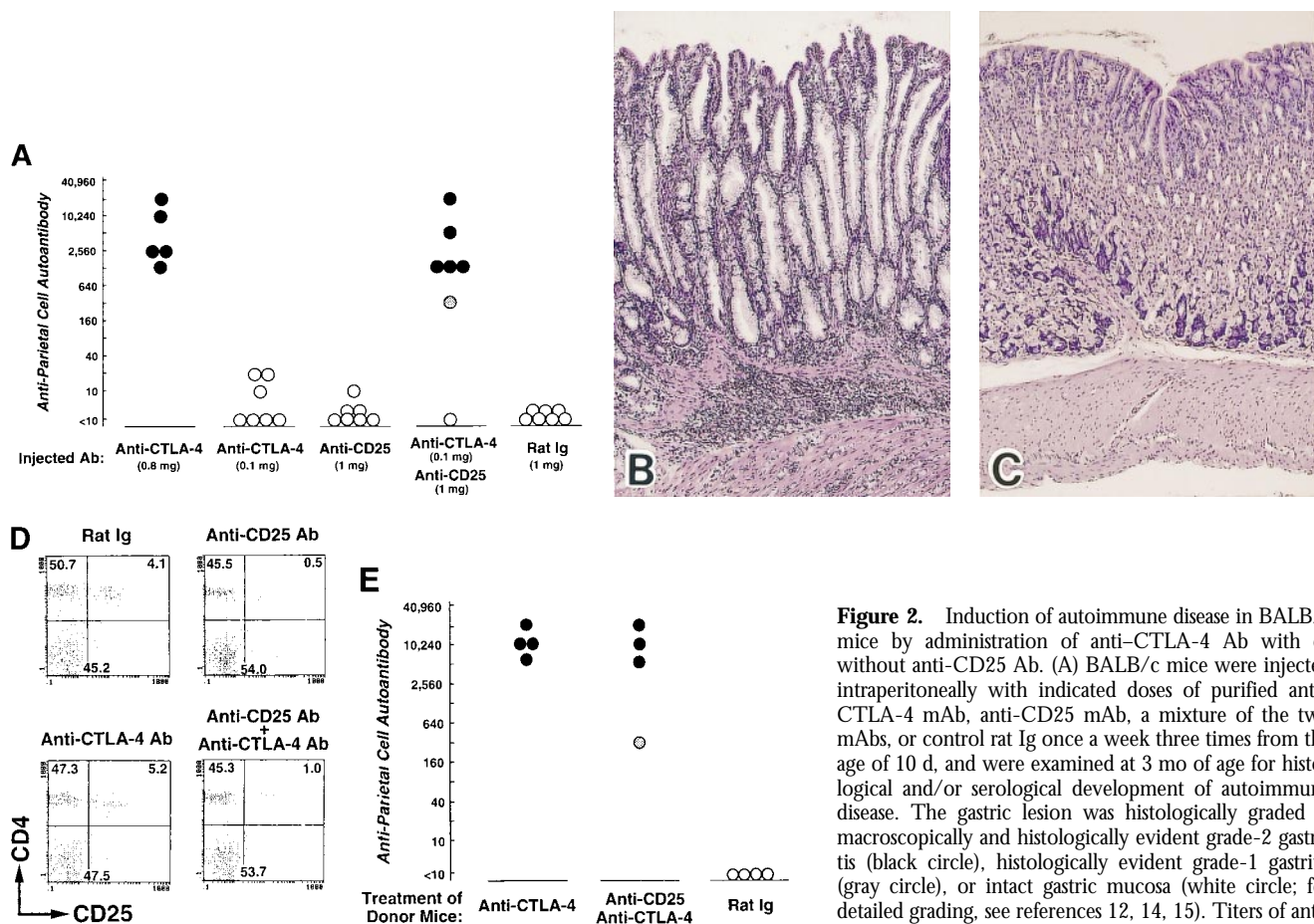


Figure 2. Induction of autoimmune disease in BALB/c mice by administration of anti-CTLA-4 Ab with or without anti-CD25 Ab. (A) BALB/c mice were injected intraperitoneally with indicated doses of purified anti-CTLA-4 mAb, anti-CD25 mAb, a mixture of the two mAbs, or control rat Ig once a week three times from the age of 10 d, and were examined at 3 mo of age for histological and/or serological development of autoimmune disease. The gastric lesion was histologically graded as macroscopically and histologically evident grade-2 gastritis (black circle), histologically evident grade-1 gastritis (gray circle), or intact gastric mucosa (white circle; for detailed grading, see references 12, 14, 15). Titers of anti-parietal cell autoantibodies were assessed by ELISA. The gastric mucosa of a grade-2 gastritis in a mouse treated with anti-CTLA-4 mAb is shown in B, and the intact gastric mucosa of a mouse treated with rat Ig is shown in C. Samples were stained with hematoxylin and eosin (original magnification: $\times 25$). (D) Composition of T cell subpopulations defined by the expression of CD4 and CD25 7 d after the last Ab treatment. Lymph node cells were stained with PE-anti-CD4 mAb and FITC-anti-CD25 mAb, 7D4, another anti-CD25 mAb with different epitope specificity, as described previously (reference 18). A representative staining of three independent experiments is shown. (E) Spleen cells (5×10^7) from anti-CTLA-4 mAb- or mixed mAb-treated mice with gastritis or from rat Ig-treated mice were transferred intravenously to BALB/c athymic nude mice, which were histologically and serologically examined 2 mo later. Black circle, macroscopically and histologically evident grade-2 gastritis; gray circle, histologically evident grade-1 gastritis; white circle, intact gastric mucosa. A representative result of more than three independent experiments is shown. The means of duplicate cultures are shown, and the SEM was within 10% of the mean for each experiment.

(Fig. 2 D). For example, 1 wk after the last treatment, percentages of CD25⁺ cells among the CD4⁺ cells in the lymph nodes were 7.2 ± 0.3% in the control mice, 10.1 ± 0.30% and 9.6 ± 0.26% in the high- or low-dose anti-CTLA-4 mAb-treated mice, respectively, 0.93 ± 0.15% in the anti-CD25 mAb-treated mice, and 2.1 ± 0.1% in the mixed mAb-treated mice (*n* = 3 for each).

The affected gastric mucosa was characterized by loss and damage of the parietal cells and the chief cells, compensatory hyperplasia of mucous-secreting cells, and a massive infiltration of mononuclear cells (Fig. 2, B and C). The lesion was immunopathologically similar to human autoimmune gastritis with pernicious anemia (26). Furthermore, the gastritis thus induced could be adoptively transferred to naive BALB/c athymic nude mice by spleen cells from gastritis-bearing BALB/c mice, indicating that the lesion was a result of a bona fide autoimmune disease (Fig. 2 E).

Thus, autoimmune diseases similar to those produced by elimination of CD25⁺CD4⁺ T cells could be elicited in normal mice by blockade of CTLA-4 with high-dose anti-CTLA-4 Ab, or reduction of CD25⁺CD4⁺ T cells accompanied by CTLA-4 blockade on the residual T cells with low-dose anti-CTLA-4 Ab (14, 15). In the latter treatment, it is plausible that reduction of CD25⁺CD4⁺ T cells might

have partially triggered the activation of autoimmune effector T cells, which might then be further activated by the blockade of CTLA-4 expressed on them. Nevertheless, successful autoimmune induction by CTLA-4 blockade alone without reduction of CD25⁺CD4⁺ T cells (Fig. 2 D), together with the constitutive expression of CTLA-4 on normal CD25⁺CD4⁺ T cells (Fig. 1), indicates that the blockade of CTLA-4 on the CD25⁺CD4⁺ T cells is most likely the triggering mechanism of this autoimmunity.

Role of CTLA-4 in CD25⁺CD4⁺ T Cell-mediated Suppression In Vitro. CD25⁺CD4⁺ T cells present in normal naive mice exhibited no proliferative response to TCR stimulation, whereas, upon TCR stimulation, they potently suppressed the activation and proliferation of other T cells, as reported previously (18–20, 27; Fig. 3). To examine the possible role of CTLA-4 in this natural anergy and suppression, we added anti-CTLA-4 Ab, its Fab fragments, or control hamster Ab to 3-d cultures of CD25⁺CD4⁺ T cells or CD25⁻CD4⁺ T cells (purified as shown in Fig. 1), or a mixture of an equal number of the two populations, and stimulated the cultures with Con A (Fig. 3 A) or soluble anti-CD3 mAb (Fig. 3 B) along with APCs. Anti-FcγII/III receptor (FcR [CD16/CD32]) mAb was also added to some cultures to inhibit anti-CTLA-4 mAbs from attaching to FcRs on APCs, thereby preventing the Abs

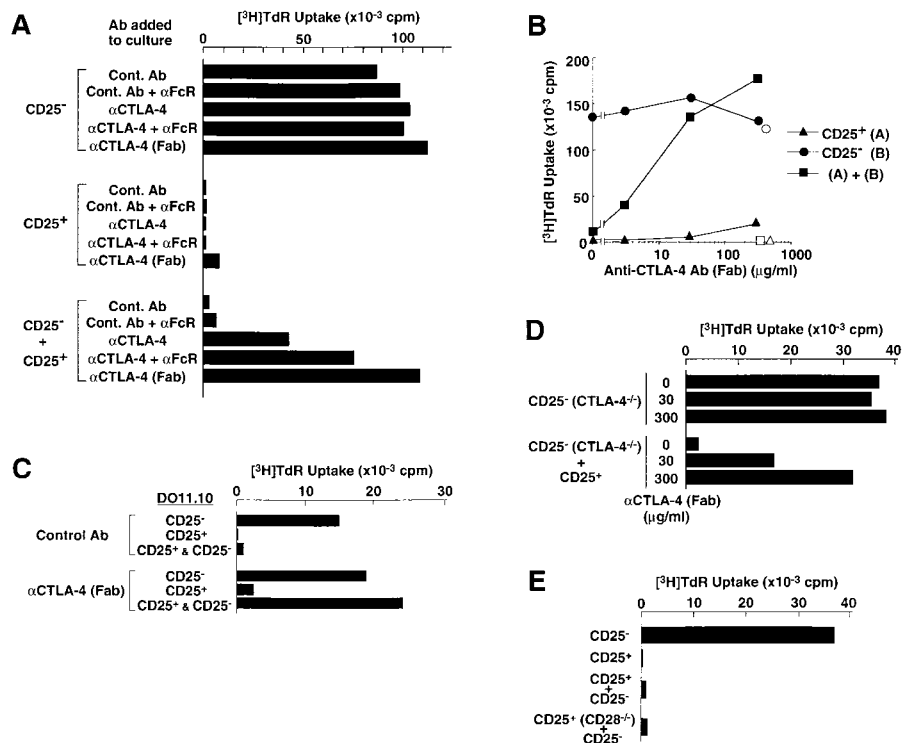


Figure 3. Key role of CTLA-4 expressed on CD25⁺CD4⁺ regulatory T cells in downregulating the activation and proliferation of other T cells. (A) Anti-CTLA-4 mAb (100 μg/ml) or control hamster Ab with or without anti-FcR mAb (10 μg/ml) or Fab-anti-CTLA-4 mAb (100 μg/ml) was added to the culture of CD25⁺ or CD25⁻CD4⁺ T cells purified from BALB/c spleen and lymph node cells (as shown in Fig. 1), or these two populations were mixed at a 1:1 ratio. The culture was stimulated for 3 d with Con A (1 μg/ml) along with irradiated BALB/c spleen cells as APCs. Incorporation of ³H-TdR during the last 6 h of the culture is shown as the degree of proliferation. Background counts in the wells with APCs alone were always <1,500 cpm in these experiments and those below. α, anti-. (B) CD25⁺ or CD25⁻CD4⁺ T cells or a 1:1 mixture of the two populations were stimulated with anti-CD3 mAb (10 μg/ml) in the presence of Fab-anti-CTLA-4 mAb at various concentrations (black symbols) or control Fab-hamster IgG at 300 μg/ml (white symbols). (C) CD25⁺ or CD25⁻CD4⁺ T cells or two populations mixed at an equal ratio, purified from the spleens of DO11.10 transgenic mice, were cultured for 3 d with 0.1 μM OVA-P(323–339) in the presence of Fab-anti-CTLA-4

mAb or control Fab-hamster IgG at 100 μg/ml. α, anti-. (D) CD25⁻CD4⁺ T cells prepared from 3-wk-old CTLA-4-deficient (CTLA-4^{-/-}) B6 mice or a 1:1 mixture of these cells with CD25⁺CD4⁺ T cells from 2-mo-old normal B6 mice was stimulated with anti-CD3 mAb for 3 d in the presence of Fab-anti-CTLA-4 mAb at indicated concentrations. The counts of CTLA-4-deficient CD25⁻CD4⁺ T cells cultured without stimulation were background levels (i.e., <2,000 cpm). α, anti-. (E) CD25⁺CD4⁺ T cells prepared from CD28-deficient (CD28^{-/-}) B6 mice or normal B6 mice were mixed with an equal number of CD25⁻CD4⁺ T cells from normal B6 mice and stimulated with anti-CD3 mAb for 3 d. A representative result of more than three independent experiments is shown. The means of duplicate cultures are shown, and the SEM was always with 10% of the mean for each experiment.

from cross-linking CTLA-4 and transducing signals (22, 23). In these experiments, intact anti-CTLA-4 mAb, especially in the presence of anti-FcR mAb, significantly neutralized CD25⁺CD4⁺ T cell-mediated suppression of the activation and proliferation of CD25⁻CD4⁺ T cells (Fig. 3 A). Although others reported no significant effect of anti-CTLA-4 mAb in a similar cell culture system (20), the discrepancy could be attributed to the concentration of anti-CTLA-4 mAb in the culture; i.e., with a high dose (100 µg/ml) of anti-CTLA-4 mAb, as shown here, the majority of CTLA-4 molecules could be blocked rather than cross-linked by the Ab attached to FcR on APCs. This blocking effect was further supported by the result that Fab-anti-CTLA-4 mAb, which is presumably able to block but unable to cross-link CTLA-4 (22, 23), more efficiently abrogated the suppression in a dose-dependent fashion (Fig. 3, A and B). In addition to these polyclonal T cell activations, the Fab fragments also neutralized CD25⁺CD4⁺ T cell-mediated control of antigen-specific activation and proliferation of OVA-specific T cells from DO11.10 transgenic mice (18, 19; Fig. 3 C). On the other hand, intact or Fab-anti-CTLA-4 mAb did not significantly affect the responses of CD25⁻CD4⁺ T cells or break the anergic state of CD25⁺CD4⁺ T cells (Fig. 3, A-C).

To determine more definitely the role of CTLA-4 on CD25⁺CD4⁺ T cells, we used CTLA-4-deficient mice. CD25⁺CD4⁺ T cells from normal mice efficiently suppressed CD25⁻CD4⁺ T cells from CTLA-4-deficient mice, and Fab-anti-CTLA-4 mAb abrogated this suppression in a dose-dependent fashion (Fig. 3 D). In the reciprocal combination, CTLA-4-deficient CD25⁺CD4⁺ T cells also exhibited weak suppressive activity (~50% in percentage suppression compared with >95% by normal CD25⁺CD4⁺ T cells at 1:1 mixing with CD25⁻CD4⁺ T cells). The CD25⁺ T cells constituted up to 30% of CD4⁺ T cells in the enlarged spleens of CTLA-4-deficient mice and appeared to be different from normal CD25⁺CD4⁺ T cells in including a large number of abnormally activated T cells (3). It remains to be determined whether this weak suppressive activity could be peculiar to CTLA-4-deficient CD25⁺CD4⁺ T cells (e.g., because of compensatory activation of other genes encoding molecules concerned with the suppression). Indeed, blockade of certain other cell surface molecules could abrogate the suppressive activity of CTLA-4-deficient as well as normal CD25⁺CD4⁺ T cells (our unpublished results). Despite this possibility, the results in Fig. 3, A-D, when taken together, indicate that CTLA-4 expressed on CD25⁺CD4⁺ regulatory T cells is critically involved in this *in vitro* T cell-mediated immunoregulation.

Activation of T cells usually requires a costimulatory signal through CD28 in addition to the TCR signal (24). However, CD25⁺CD4⁺ T cells from CD28-deficient mice exhibited a potent suppressive activity (Fig. 3 E). This indicates that CD28 on CD25⁺CD4⁺ T cells (Fig. 1) is dispensable for the CD25⁺CD4⁺ T cell-mediated suppression, even if CD28 deficiency might affect the development of regulatory T cells (e.g., the number of CD25⁺CD4⁺ T cells in CD28-deficient B6 mice reduced to

~20% of normal B6 mice; our unpublished data) or their level of CTLA-4 expression (28, 29). On the other hand, we have shown previously that ligation of the CD28 molecule with anti-CD28 Ab, in conjunction with TCR stimulation, can break the naturally anergic state of CD25⁺CD4⁺ T cells and simultaneously abolish their suppressive activity (18, 19). These findings collectively imply unique roles for CTLA-4 and CD28 in CD25⁺CD4⁺ regulatory T cells; i.e., costimulation via CTLA-4 may activate the regulatory T cells to exert suppression, whereas costimulation via CD28 may attenuate their suppressive activity. The balance between the two signals in a particular CD25⁺CD4⁺ regulatory T cell may be critical in determining its activation threshold and/or its suppressive activity. Furthermore, the balance between regulatory T cells activated through CTLA-4 and effector T cells activated through CD28 may contribute to determining the threshold or the magnitude of a particular immune response.

It remains to be determined how CD25⁺CD4⁺ regulatory T cells suppressively control other T cells upon stimulation via CTLA-4 and TCR. Although TGF-β possibly secreted by TCR- and CTLA-4-stimulated T cells may contribute to attenuating activated T cells, as shown by others (30), anergic/suppressive CD25⁺CD4⁺ T cells stimulated via CTLA-4 and TCR did not secrete detectable amounts of TGF-β, and the suppression was not abrogated by adding neutralizing anti-TGF-β Ab to the culture (18, 20; our unpublished data). As a possible mechanism of the suppression, CD25⁺CD4⁺ T cells stimulated via CTLA-4 and TCR may physically interfere with the interaction of other T cells with APCs by competing for the costimulatory molecules on APCs, as they are predominantly expressing CTLA-4 (which has a higher affinity for CD80/CD86 than CD28 [22]) and various adhesion molecules (such as intercellular adhesion molecule [ICAM]-1, LFA-1, and CD2) at high levels (18–20). Alternatively, the signals via TCR and CTLA-4 may activate CD25⁺CD4⁺ T cells to deliver to other T cells a negative signal for activation and proliferation. These possibilities are currently under investigation.

There are accumulating demonstrations that CTLA-4 deficiency or blockade induces or exacerbates autoimmunity, enhances tumor immunity, or prevents induction of immunologic tolerance (4–9). These effects of CTLA-4 deficiency or blockade have been largely explained as a hindrance of CTLA-4-transduced negative signals to activated T cells mediating immune responses (1, 2). However, our present *in vivo* and *in vitro* results indicate that the effects can also be attributed, at least in part, to the blockade of CTLA-4 molecules expressed on CD25⁺CD4⁺ regulatory T cells and consequent interference with T cell-mediated immunoregulation. CTLA-4 also appears to play a similar role in the function of regulatory T cells that control inflammatory bowel disease (31). Exploitation of this unique role of CTLA-4 in immunologic self-tolerance would help to develop novel strategies for treating or preventing autoimmune disease, enhancing immunity to autologous tumor cells, or inducing tolerance to allotransplants.

We thank Drs. K.S.K. Tung and T. Sakihama for critically reading the manuscript, Dr. J.A. Bluestone for providing us with the hybridoma-secreting anti-CTLA-4 Ab, Ms. E. Moriizumi for preparing histology, and Ms. Sue Plyte for taking care of sending mice abroad.

This work was supported by grants-in-aid from the Ministry of Human Welfare and the Organization for Pharmaceutical Safety and Research of Japan.

Submitted: 10 February 2000

Revised: 23 May 2000

Accepted: 8 June 2000

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