Role of CTLA-4 in the Activation of Single- and Double-Positive Thymocytes

Hyokjoon Kwon, Hee-Sook Jun, Lee-Yong Khil and Ji-Won Yoon

*J Immunol* 2004; 173:6645-6653; doi: 10.4049/jimmunol.173.11.6645

http://www.jimmunol.org/content/173/11/6645

**References**

This article cites 43 articles, 21 of which you can access for free at:

http://www.jimmunol.org/content/173/11/6645.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Role of CTLA-4 in the Activation of Single- and Double-Positive Thymocytes

Hyokjoon Kwon,* Hee-Sook Jun,2*† Lee-Yong Khil,* and Ji-Won Yoon2*†

CTLA-4, a homologue of CD28, is a negative regulator of T cell activation in the periphery and is transiently expressed on the cell surface after T cell activation. However, the role of CTLA-4 in T cell activation in the thymus is not clear. This investigation was initiated to determine the role of CTLA-4 in the activation of CD4+CD8+ double-positive (DP) and CD4+CD8- and CD4-CD8+ single-positive (SP) thymocytes using fetal thymic organ cultures (FTOC) of MHC class II-restricted, OVA323–339-restricted TCR transgenic mice (DO11.10). We found that treatment of the FTOC with anti-CTLA-4-blocking Ab during activation with OVA323–339 increased the proportion and number of DP thymocytes, but decreased the proportion and number of SP thymocytes compared with OVA323–339-stimulated FTOC without anti-CTLA-4 Ab treatment. In addition, anti-CTLA-4 Ab treatment inhibited OVA323–339-induced expression of the early activation marker, CD69, in DP thymocytes, but increased CD69 in SP thymocytes. Similarly, CTLA-4 blockage decreased phosphorylation of ERK in DP thymocytes by Ag-specific TCR engagement, but increased phosphorylation of ERK in SP thymocytes. CTLA-4 blockage inhibited deletion of DP thymocytes treated with a high dose of OVA323–339, whereas CTLA-4 blockage did not inhibit deletion of DP thymocytes treated with a low dose of OVA323–339. We conclude that CTLA-4 positively regulates the activation of DP thymocytes, resulting in their deletion, whereas blocking CTLA-4 suppresses the activation of DP thymocytes, leading to inhibition of DP thymocyte deletion. In contrast, CTLA-4 negatively regulates the activation of SP thymocytes. The Journal of Immunology, 2004, 173: 6645–6653.

T cells in the thymus differentiate through positive and negative selection upon ligation of the TCR with self-peptide/MHC complexes (1–5). Low affinity/avidity interactions elicit positive selection, whereas high affinity/avidity interactions elicit negative selection. T cell stimulation during this process requires signaling not only through the TCR, but also through co-stimulatory molecules (6), including CD28 (7, 8), CD5/CD43 (9), CD80/86 (10), CD40L (11, 12), and CTLA-4 (13). The CD28/CTLA-4:B7-1/B7-2 costimulatory pathway is essential for the control of T cell responses. CD28-mediated signaling leads to the enhancement and maintenance of T cell activation. CTLA-4, a homologue of CD28, is known to be a negative regulator in T cell activation (14–17). CTLA-4 and CD28 differ in their expression pattern and in avidity to their common ligands, B7-1 (CD80) and B7-2 (CD86). CD28, which binds B7 molecules with relatively low avidity, is constitutively expressed on the cell surface of resting and activated T cells. In contrast, CTLA-4 is transiently expressed on the cell surface only after T cell activation (18–20), and its avidity for B7 molecules is 10- to 2000-fold higher than the avidity of CD28 (21, 22). It is clear that CTLA-4 functions as a regulator of T cell activity and plays a major role in the establishment of self-tolerance in the periphery (18, 23–25). However, the role of CTLA-4 in the development of T cells in the thymus is still unclear.

Some studies using CTLA-4-deficient mice showed that thymocyte development is not altered in the absence of CTLA-4 (26, 27), suggesting that CTLA-4 is not involved in thymocyte development. Other studies showed that the administration of CTLA-4-naturalizing Abs inhibited negative selection in vivo (13), suggesting that CTLA-4 is necessary for negative selection. This investigation was initiated to determine the role of CTLA-4 in T cell development in the thymus by analyzing the proportion and number of CD4+CD8+ double-positive (DP) thymocytes, the expression of the activation marker, CD69, and the activation of ERK after blocking CTLA-4, using Ag-specific stimulation and a fetal thymic organ culture (FTOC) system. We found that the expression of CTLA-4 was significantly increased in CD4+CD8+ DP T cells after TCR engagement, and blockade of CTLA-4 inhibited TCR-mediated activation of DP thymocytes and inhibited ERK phosphorylation, indicating that CTLA-4 positively regulates the activation of DP thymocytes after TCR engagement, whereas it negatively regulates the activation of SP thymocytes.

Materials and Methods

**Animals**

C57BL/6 and DO11.10 TCR transgenic mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CTLA-4-deficient C57BL/6 mice were provided by T. W. Mak (University of Toronto, Toronto, Canada). The animals were bred and maintained under specific pathogen-free conditions and were provided with sterile food and water ad libitum at University of Calgary. Female mice were used for all experiments. The use and care of the animals in this study were approved by the animal care committee of University of Calgary.

**Abs and chemicals**

Purified anti-CTLA-4 (UC10-4F10-11), anti-CD16/CD32 (FcRIII/II; 2.4G2), and control hamster isotype IgG; FITC- or PE-labeled anti-CD4, anti-CD8, anti-CD16/CD32 (FcRIII/II; 2.4G2), and control hamster isotype IgG; FITC- or PE-labeled anti-CD4,
anti-CD8, and anti-CTLA-4; and biotinylated anti-CD3, anti-CD4, and anti-CD69 Abs were purchased from BD Pharmingen (San Diego, CA). Streptavidin-PerCP and -PE were purchased from BD Biosciences (Mountain View, CA). OVA peptide (OVA323–339: ISQAVHAAHAEINEAGR) and T cell unstimulating mimic of OVA peptide (TUM) (OVA 324 –334: SQAVHAAHAEL) were synthesized by TANA Laboratories (Houston, TX). Anti-ERK and anti-phospho-ERK Abs were purchased from New England Biolabs (Beverly, MA).

**FTOC**

Fetal thymic lobes were prepared from C57BL/6, DO11.10 TCR transgenic and CTLA-4-deficient C57BL/6 mice on embryonic day 17.5. To prepare CTLA-4-deficient C57BL/6 FTOC, heterozygous CTLA-4/H11001/H11002 mice were crossed, and the genotype of the offspring was determined by PCR of fetal tail DNA as previously described (28). Fetal thymic lobes were placed on 0.8-μm pore size polycarbonate filters (Costar, Cambridge, MA), which were floated on IMDM supplemented with 12% FCS, 2 mM glutamine, 5×10^-5 M 2-ME, 100 U/ml penicillin, and 100 g/ml streptomycin, and incubated for 4 d at 37°C. The thymic lobes were then treated with Abs, OVA peptide, and/or chemical inhibitors. The lobes were teased apart, and the thymocytes were counted and stained for FACS analysis.

**Flow cytometric analysis**

Abs (anti-CD3, anti-CTLA-4, isotype IgG, anti-CD4, anti-CD8, and anti-CD69) were added to 5×10^5 cells in FACS buffer (PBS containing 1% FCS and 0.1% sodium azide, pH 7.2), and the cells were incubated for 20 min at 4°C. For detection of cells stained with biotinylated Abs, the cells were incubated with streptavidin-PE or -PerCP for 20 min at 4°C and analyzed by FACSscan. Data files were analyzed using FlowJo software (TreeStar, San Carlos, CA). For CTLA-4 intracellular staining, cells were first stained for surface markers after FcR blocking, fixed in 2% paraformaldehyde, washed twice in saponin buffer (0.03% saponin in PBS), and permeabilized with 0.3% saponin in PBS for 5 min on ice. Cells were then stained with PE-labeled anti-CTLA-4 Ab in the presence of 0.3% saponin for 30 min, washed thoroughly in saponin buffer and once in staining buffer, and resuspended in PBS for FACS.

![Figure 1](http://www.jimmunol.org/Downloadedfrom)
Western blot

Cell lysates from total thymocytes or sorted DP thymocytes were prepared in cold lysis buffer (20 mM HEPES (pH 7.6), 20% glycerol, 150 mM NaCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, a mixture of protease inhibitors, and a mixture of phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO)), and proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Hybond-P, Amersham Bioscience, Arlington Heights, IL). The membranes were incubated with anti-phospho-ERK Ab to determine the phosphorylation of ERK protein, then stripped with Re-Blot Plus (Chemicon International, Temecula, CA), and total ERK protein levels were determined by incubating the membranes with anti-ERK Ab. Proteins were detected by ECL (ECL Plus; Amersham Bioscience).

Statistical analyses

The significance of differences between groups was analyzed by Student’s t test. A level of $p < 0.05$ was accepted as significant. Data are expressed as the mean ± SEM.

Results

CTLA-4 signal acts as a positive regulator in the activation of DP thymocytes, but a negative regulator in the activation of SP thymocytes

To determine whether blocking CTLA-4 inhibits the anti-CD3 Ab-induced deletion of DP thymocytes in C57BL/6 FTOC, we treated the FTOC with anti-CTLA-4 Ab during activation with anti-CD3 Ab and examined the proportion of DP thymocytes. We found that stimulation of C57BL/6 FTOC with anti-CD3 Ab resulted in a significant decrease in the proportion of DP thymocytes compared with isotype IgG-treated C57BL/6 FTOC (Fig. 1, A and B). Treatment of FTOC with anti-CTLA-4 during stimulation with anti-CD3 Ab for 24 h resulted in the recovery of DP thymocyte proportion, indicating that blocking CTLA-4 inhibits the anti-CD3 Ab-induced deletion of DP thymocytes (Fig. 1C). However, treatment of C57BL/6 FTOC with anti-CTLA-4 Ab without stimulation with anti-CD3 Ab did not change the proportion of DP thymocytes compared with isotype IgG-treated FTOC (Fig. 1D). To determine whether DP thymocyte numbers, in addition to proportions, are increased when CTLA-4 was blocked during stimulation with anti-CD3 Ab, we examined the number of CD4⁺CD8⁺ DP, CD4⁺ SP, and CD8⁺ SP thymocytes in anti-CTLA-4 Ab-treated FTOC. We found that the total number of thymocytes and DP thymocytes in anti-CD3 Ab-stimulated C56BL/6 FTOC was significantly decreased compared with that in isotype Ab- or anti-CTLA-4 Ab-treated FTOC (Fig. 1E, F). Treatment with anti-CTLA-4 Ab significantly inhibited anti-CD3 Ab-induced DP thymocyte deletion, as evidenced by the increase in the actual number of DP thymocytes (Fig. 1F), even though the total number of thymocytes was similar in anti-CD3 Ab-treated FTOC and FTOC treated with a combination of anti-CTLA-4 and anti-CD3 Abs (Fig. 1E). In contrast, the number of CD4⁺ and CD8⁺ SP thymocytes was decreased by anti-CTLA-4 Ab treatment compared with anti-CD3 Ab-stimulated FTOC without anti-CTLA-4 Ab treatment (Fig. 1, G and H). These results suggest that CTLA-4 plays a critical role in the activation of DP thymocytes that is opposite its functional role in SP thymocytes and peripheral T cells.

To confirm that CTLA-4 is involved in the activation of DP thymocytes, we activated C57BL/6 FTOC with anti-CD3 Ab and examined the expression of CTLA-4 and CD69, an early activation marker in DP thymocytes. We found that the expression of CD69

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Blockage of CTLA-4 inhibits the expression of CD69 in DP thymocytes. C57BL/6 FTOC was treated with isotype IgG (A), anti-CTLA-4 (B), anti-CD3 (C), or a combination of anti-CD3 and anti-CTLA-4 Abs (D). Thymocytes were isolated 24 h later and triple-stained. CD4⁺ SP, CD8⁺ SP, and CD4⁺CD8⁺ DP thymocytes were gated and further analyzed for the expression of CD69 and CTLA-4 by three-color FACS. Representative results from four independent experiments are shown.
and CTLA-4 was significantly increased in DP and SP thymocytes (Fig. 2C) compared with either isotype IgG- or anti-CTLA-4 Ab-treated FTOC (Fig. 2, A and B). When we treated the cultures with anti-CTLA-4 Ab to block CTLA-4 during activation with anti-CD3 Ab, we found that the anti-CD3 Ab-induced CD69 expression was significantly inhibited in DP thymocytes, but was increased in CD4^+ and CD8^+ SP thymocytes (Fig. 2D). These results clearly suggest that the CTLA-4 signal acts as a positive regulator in the activation of DP thymocytes that is opposite its functional role in SP thymocytes, and thus, blockage of CTLA-4 results in a decrease in the induction of CD69 expression.

**CTLA-4 plays a positive role in Ag-specific, activation-induced DP thymocyte deletion**

Because anti-CD3-Ab-induced activation may result in nonspecific T cell activation (2), we used MHC class II-restricted, OVA-restricted TCR-transgenic mice, DO11.10, to induce Ag-specific T cell activation. To determine whether CTLA-4 affects the deletion of DP thymocytes after TCR engagement in DO11.10 FTOC, we examined the deletion profile of DP thymocytes by flow cytometric analysis after treatment with 6 μg/ml anti-CTLA-4 Ab during stimulation with various doses of OVA_{323-339} (0.5, 1, 5, 10, 20, or 40 μg/ml). We found that stimulation with OVA_{323-339} induced a dose-dependent deletion of DP thymocytes that reached a plateau at 10 μg/ml in DO11.10 FTOC (Fig. 3A, A and B), whereas stimulation with a control peptide, TUM, did not result in DP thymocyte deletion (Fig. 3A). Blockage of CTLA-4 by treatment with anti-CTLA-4 Ab significantly inhibited OVA_{323-339}-induced deletion of DP thymocytes (Fig. 3A, A and B).

To determine the dose- and time-dependent effect of anti-CTLA-4 Ab treatment on the OVA_{323-339}-induced deletion of DP thymocytes, we treated DO11.10 FTOC with various doses of anti-CTLA-4 Ab for different lengths of time and examined the deletion profile of DP thymocytes. We found that 6 μg/ml anti-CTLA-4 Ab was the most effective dose for inhibiting the deletion of DP thymocytes at 24 h after TCR engagement (Fig. 3, C and D). A lower dose of anti-CTLA-4 Ab (3 μg/ml) did not inhibit OVA_{323-339}-induced deletion of DP thymocytes; the number of DP thymocytes was slightly increased (data not shown).

To determine whether blocking CTLA-4 inhibits the OVA_{323-339} induced deletion of DP thymocytes, resulting in an increase in the number of DP thymocytes, we treated DO11.10 FTOC with

**FIGURE 3.** Blockage of CTLA-4 inhibits TCR-specific peptide-induced deletion of DP thymocytes after TCR engagement. DO11.10 FTOC was treated with various amounts of OVA_{378-387} (OVA peptide) or TUM peptide and CTLA-4-blocking Ab. Thymocytes were isolated at different times thereafter, stained with anti-CD4 and anti-CD8 Abs, and analyzed by FACS. 

A. FACS profile of thymocytes 24 h after treatment with isotype IgG or anti-CTLA-4 Ab (6 μg/ml) combined with either TUM peptide or OVA peptide (20 μg/ml). Representative results of five independent experiments are shown.

B. Proportion of DP thymocytes at various times after treatment with TUM peptide, anti-CTLA-4 Ab, or OVA peptide in combination with either isotype IgG or anti-CTLA-4 Ab. The number of CD4^+CD8^- DP thymocytes after 24 h of treatment with TUM or OVA peptide (20 μg/ml) in combination with isotype IgG or anti-CTLA-4 Ab. Numbers were calculated from the total number of thymocytes and the proportion of CD4^+SP and CD4^+CD8^- DP thymocytes, as determined by FACS. Values are the mean ± SEM of more than five independent experiments (n ≥ 5/group). *p < 0.02; **p < 0.003; ***p < 0.0004 (compared with anti-CTLA4 Ab-treated DO11.10 FTOC).
anti-CTLA-4 Ab during stimulation with OVA_{323-339} and examined the number of CD4^{+}CD8^{+} DP and SP (CD4^{+}) thymocytes. We found that anti-CTLA-4 Ab treatment resulted in a significantly higher number of DP thymocytes compared with the control (isotype IgG-treated FTOC; Fig. 3E). In contrast, anti-CTLA-4 Ab treatment further decreased the number of CD4^{+} SP thymocytes compared with control Ab-treated FTOC (Fig. 3F). Stimulation with a control peptide, TUM, did not change cell numbers (Fig. 3, E and F). These results indicate that CTLA-4 plays a positive role in deletion of DP thymocytes by Ag-specific activation, which naturally occurs during T cell selection in the thymus.

**FIGURE 4.** Cross-linkage of the anti-CTLA-4 Ab is not involved in the CTLA-4 blockage-mediated prevention of DP thymocyte deletion after TCR engagement. DO11.10 FTOC was treated with anti-FcR-blocking Ab (10 μg/ml) for 1 h before treatment with anti-CTLA-4-blocking Ab (6 μg/ml) and OVA_{378-387} (OVA peptide) or TUM peptide (20 μg/ml). Thymocytes were isolated 24 h later, stained with anti-CD4 and anti-CD8 Abs, and analyzed by FACS. Representative results of three independent experiments are shown.

**FIGURE 5.** Blockage of CTLA-4 inhibits the OVA peptide-induced activation of DP thymocytes after TCR engagement. DO11.10 FTOC was treated with isotype IgG or anti-CTLA-4 Ab (6 μg/ml) in combination with TUM or OVA peptide (20 μg/ml). Thymocytes were isolated 12 or 24 h later and triple-stained. CD4^{+} SP and CD4^{+}CD8^{+} DP thymocytes were gated and further analyzed for the expression of CD69 (A) and CTLA-4 (B) by three-color FACS. Representative results from four independent experiments are shown. C, Splenocytes from DO11.10 mice were treated with OVA peptide (1 μg/ml) and/or anti-CTLA-4 Ab (6 μg/ml). Splenocytes (1 × 10^{6}) were prepared 40 h later and triple-stained. CD4^{+} splenocytes were gated and further analyzed for the expression of CD69 and CTLA-4 by three-color FACS. Representative results from two independent experiments are shown.
To determine whether the effect of anti-CTLA-4 Ab on the deletion profile of DP thymocytes depends on cross-linking of the anti-CTLA-4 Ab, we treated DO11.10 FTOC with anti-FcR III/II-blocking Ab (anti-FcR) before treatment with anti-CTLA-4 Ab and OVA323–339 and examined the deletion profile of DP thymocytes. We found that there was no significant difference in the deletion profile of DP thymocytes in FTOC treated with anti-FcR-blocking Ab compared with FTOC without anti-FcR Ab treatment (Fig. 4), suggesting that cross-linking of the anti-CTLA-4 Ab is not involved in the inhibition of DP thymocyte deletion.

**CTLA-4 positively regulates the activation of DP thymocytes by Ag-specific TCR engagement, but negatively regulates the activation of SP thymocytes**

We found that blocking CTLA-4 inhibited OVA323–339-induced deletion of DP thymocytes in DO11.10 FTOC. Deletion of DP thymocytes is mediated by activation-induced cell death through apoptosis. To determine whether blocking CTLA-4 inhibits the TCR-mediated activation of DP thymocytes, we treated DO11.10 FTOC with anti-CTLA-4 Ab during stimulation with OVA323–339 and examined the activation-induced expression of CD69 and CTLA-4 at 12 and 24 h after stimulation. We found that the expression of CD69 (Fig. 5A) and CTLA-4 (Fig. 5B) was significantly increased in CD4^+CD8^- DP thymocytes as well as in CD4^-CD8^+ SP thymocytes 24 h after stimulation with OVA323–339, whereas stimulation with TUM peptide had no effect. Anti-CTLA-4 Ab treatment of the FTOC resulted in the inhibition of OVA323–335-induced CD69 expression in DP thymocytes, but an increase in CD69 expression in CD4^-SP thymocytes (Fig. 5, A and B).

To determine the function of CTLA-4 in peripheral T cells, we examined the expression of CD69 and CTLA-4 in splenocytes from DO11.10 transgenic mice after treatment with OVA323–339. We found that the expression of CD69 and CTLA-4 was significantly increased in splenic CD4^+ T cells 40 h after TCR engagement (Fig. 5C), and blocking CTLA-4 with anti-CTLA-4 Ab enhanced the expression of CD69 and CTLA-4 during stimulation with OVA323–339 compared with isotype IgG control Ab-treated CD4^+ T cells. These results indicate that CTLA-4 positively regulates the activation of DP thymocytes by Ag-specific TCR engagement, but negatively regulates the activation of both CD4^+ SP thymocytes and CD4^- peripheral T cells.

**Signaling through CTLA-4 linkage activates ERK in DP thymocytes, but suppresses ERK in SP thymocytes**

Because we found that CTLA-4 ligation plays a critical role in the TCR-mediated activation of DP thymocytes, as evidenced by the inhibition of CD69 expression after blocking CTLA-4, we examined the effect of anti-CTLA-4 Ab on the activation of signaling molecules involved in thymocyte activation. It is known that MAPKs, including ERK, are phosphorylated after TCR-mediated T cell activation (29) and are involved in positive (29–33) and negative (34, 35) selection. We treated DO11.10 FTOC with anti-CTLA-4 Ab during stimulation with OVA323–339 and examined the phosphorylation of ERK 1, 3, 6, and 12 h after stimulation. We found that phosphorylation of ERK was significantly increased 3, 6, and 12 h after stimulation with OVA323–339 and anti-CTLA-4 Ab treatment inhibited this increase in total thymocytes 6 and 12 h after Ab treatment (Fig. 6A). When we examined the effects of anti-CTLA-4 Ab on TCR-mediated phosphorylation of ERK in sorted CD4^-CD8^- DP and CD4^-SP thymocytes, we found that anti-CTLA-4 Ab treatment inhibited the OVA323–339-induced increase of ERK phosphorylation in DP thymocytes, but increased ERK phosphorylation in SP thymocytes (Fig. 6B). We then examined the effects of anti-CTLA-4 Ab on the phosphorylation of ERK in splenocytes of DO11.10 mice after stimulation with OVA peptide. We found that CTLA-4 blockage significantly increased

![Figure 6](http://www.jimmunol.org/Downloaded-from.)

**FIGURE 6.** Blockage of CTLA-4 inhibits the activation of ERK in DP thymocytes after TCR engagement. DO11.10 FTOC was treated with OVA peptide (20 µg/ml) and/or anti-CTLA-4 Ab (6 µg/ml). A. Total thymocytes were isolated at the indicated times thereafter. B, CD4^-CD8^- DP and CD4^- SP thymocytes were sorted at 10 h after TCR engagement. C, DO11.10 splenocytes were treated with OVA peptide (1 µg/ml) and/or anti-CTLA-4 Ab (6 µg/ml) and prepared 20 h after TCR engagement. Western blots were performed using phospho-specific Abs against ERK (P-ERK) or total ERK. Numbers below bands indicate the ratio of phosphorylated ERK to total ERK, as determined by densitometric analysis. Lane 1, isotype IgG and TUM peptide; lane 2, anti-CTLA-4 Ab and TUM peptide; lane 3, isotype IgG and OVA peptide; lane 4, anti-CTLA-4 Ab and OVA peptide. Representative results from three independent experiments are shown.
OVA323–339-induced phosphorylation of ERK in splenic T cells compared with isotype IgG control Ab-treated splenic T cells (Fig. 6C). These results indicate that signaling through CTLA-4 linking induces the activation of ERK, which results in the activation of DP thymocytes, whereas the same signaling suppresses the activation of ERK in CD4+/CD8− SP thymocytes and splenic T cells.

**Effect of anti-CTLA-4 Ab on TCR-mediated deletion of DP thymocytes is correlated with the level of CTLA-4 expression**

To determine whether there is any difference in the deletion of DP thymocytes in DO11.10 FTOC between low and high dose stimulation with OVA323–339, we treated DO11.10 FTOC with 0.5 μg/ml (low dose) or 20 μg/ml (high dose) OVA323–339 and examined the deletion profile of DP thymocytes. We found that there was no significant difference in the proportion of DP thymocytes between these two groups (Fig. 7A). However, anti-CTLA-4 Ab treatment significantly inhibited the deletion of DP thymocytes when stimulated with a high dose, but not a low dose, of OVA323–339. We then examined whether there is any difference in the expression of CD69 and CTLA-4 between FTOCs stimulated with low and high doses of OVA323–339. We found that strong TCR stimulation with a high dose of OVA323–339 significantly increased the expression of CD69 and CTLA-4, whereas weak TCR stimulation with a low dose of OVA323–339 induced only a little expression of CD69 and CTLA-4 in DP thymocytes (Fig. 7B). There was no significant difference in the expression of CD69 and CTLA-4 between FTOCs stimulated with a low or a high dose of TUM peptide (Fig. 7C). To determine whether there is any difference in the degree of ERK phosphorylation after stimulation with a low or a high dose of OVA323–339, we treated DO11.1 FTOC with 0.5 or 20 μg/ml OVA323–339, and examined the level of ERK phosphorylation. We found that the phosphorylation of ERK was slower and milder after weak TCR stimulation (Fig. 6A). These results suggest that impaired inhibition of DP thymocyte deletion after treatment with anti-CTLA-4 Ab is not due to a lack of ERK activation in DP thymocytes but rather to a slower and milder ERK activation after weak TCR stimulation.

**FIGURE 7.** CTLA-4 blockage efficiently inhibits the deletion of DP thymocytes after strong TCR stimulation. DO11.10 FTOC was treated with a low (0.5 μg/ml) or a high (20 μg/ml) dose of OVA or TUM peptide and/or anti-CTLA-4 Ab (6 μg/ml). A, Thymocytes were isolated 24 h after TCR engagement, stained with anti-CD4 and anti-CD8 Abs, and analyzed by FACS. Representative results of five independent experiments are shown. B and C, Thymocytes were isolated after 24 h of treatment with OVA or TUM and triple-stained. CD4+CD8− DP thymocytes were gated and further analyzed for the expression of CD69 and CTLA-4 by three-color FACS. Representative results from four independent experiments are shown. D, DO11.10 FTOC was treated with a low dose of OVA peptide (0.5 μg/ml) and/or anti-CTLA-4 Ab (6 μg/ml). Total thymocytes were isolated at the indicated times, and Western blots were performed using phospho-specific Abs against ERK (p-ERK) or total ERK (ERK). Numbers indicate the ratio of phosphorylated ERK to total ERK determined by densitometric analysis. Lane 1, Isotype IgG and TUM peptide; lane 2, anti-CTLA-4 Ab and TUM peptide; lane 3, isotype IgG and OVA peptide; lane 4, anti-CTLA-4 Ab and OVA peptide. Representative results from three independent experiments are shown.

**FIGURE 8.** The number of DP thymocytes is increased in CTLA-4-deficient mice. Fetal thymic lobes were removed on embryonic day 17.5 and incubated in FTOC for 4 days. Thymocytes were isolated after an additional 24 or 48 h of incubation, stained with anti-CD4 and anti-CD8 Abs, and analyzed by FACS. Numbers of CD4+CD8− DP thymocytes were calculated from the total number of thymocytes and the proportion of DP thymocytes, as determined by FACS. Values are the mean ± SEM of more than three independent experiments (n ≥ 3/group). *, p < 0.04; **, p < 0.015 (compared with wildtype (+/+)) or heterozygous (+/-) littermate control FTOC.)
anti-CTLA-4 Ab in low dose OVA\textsubscript{323–339}-stimulated FTOC may be correlated with the low expression of CTLA-4.

**Number of DP thymocytes is increased in CTLA-4-deficient mice**

To determine whether the absence of CTLA-4 affects the DP thymocyte population, we examined the proportion and number of DP thymocytes in CTLA-4\textsuperscript{−/−} C57BL/6 FTOC. We found that the number of DP thymocytes in CTLA-4\textsuperscript{−/−} C57BL/6 FTOC was significantly higher than that in wild-type and heterozygous littermate control FTOCs (Fig. 8). However, there were no differences in the proportion of DP thymocytes, as determined by FACS analysis (data not shown). These results indicate that CTLA-4 deficiency may inhibit the deletion of DP thymocytes in the thymus, resulting in an increase in the number of DP thymocytes.

**Discussion**

CTLA-4 is generally known as a negative regulator in peripheral T cell activation (14–17), because CTLA-4-deficient mice show lymphoproliferative disease (28, 36), and anti-CTLA-4 mAbs enhance T cell responses (18, 37, 38). In contrast, CTLA-4 has also been suggested to play a positive role in T cell activation (39). T cells from CD28-deficient mice showed a significant clonal expansion, which was mediated by CTLA-4, and a mutant B7-1 molecule that does not bind to CD28, but does bind to CTLA-4, promoted T cell clonal expansion (40), suggesting a positive role of CTLA-4 in T cell activation. In addition, lymphocyte infiltration was found in many organs in CTLA-4-deficient mice, but lymphoproliferative disease did not develop if all CTLA-4-deficient T cells were specific for a foreign Ag (27), suggesting an alternative interpretation of the function of CTLA-4 as a positive regulator. Autoreactive T cells might escape negative selection in the absence of CTLA-4 signaling, because of the decrease in avidity of the interaction between thymocytes and APCs. In this scenario, CTLA-4-mediated positive signaling may enhance the activation and deletion of DP thymocytes after TCR engagement during negative selection.

It was previously suggested that CTLA-4 may be involved in negative selection during thymocyte development (39). However, there are conflicting reports on the role of CTLA-4 in negative selection in the thymus. Anti-CTLA-4-blocking Ab was shown to inhibit anti-CD3 Ab-induced negative selection of DP thymocytes in vivo (13), and the proportion of DP thymocytes was found to decrease in CTLA-4-deficient mice (28, 36). In contrast, later studies showed normal development of thymocytes in CTLA-4-deficient mice (26) and H-Y TCR transgenic CTLA-4-deficient mice (27).

In the present study we used FTOC to exclude any effects of activated peripheral T cells on the deletion of DP thymocytes and to maintain the thymic microenvironment, including an intact cortex, medulla, and endogenous stromal cells in vivo (41). Because CTLA-4 Ab (UC10-4F10-11) was shown to be an antagonist of CTLA-4-mediated signaling (37, 38, 42), we used this Ab to examine the effect of CTLA-4 blockage on the deletion of DP thymocytes in C57BL/6 FTOC activated with anti-CD3 Ab and found that the number of DP thymocytes was significantly increased compared with that in FTOC without anti-CTLA-4 Ab treatment. This finding is consistent with previous results of the effect of CTLA-4 blockage on DP thymocyte deletion in mice treated with anti-CD3 Ab in vivo (13). Anti-CD3 Abs can induce TCR-mediated signaling, resulting in nonspecific activation of T cells with out TCR engagement, which is distinct from activation by peptide/MHC ligation (2). To exclude this possibility, we used OVA\textsubscript{323–339}-specific TCR transgenic mice, DO11.10, to induce thymocyte selection by specific ligation between the TCR and the peptide/MHC complex. We found that CTLA-4 blockage significantly inhibited OVA\textsubscript{323–339}-induced deletion of DP thymocytes. These results indicate that CTLA-4 is a positive regulator of the deletion of DP thymocytes.

Because DP thymocytes in the thymus are deleted by activation-induced cell death to eliminate autoreactive T cells, we examined whether blockage of CTLA-4 inhibits activation of DP thymocytes, resulting in a decrease in activation-induced deletion of DP thymocytes. We found that blockage of CTLA-4 further increased OVA\textsubscript{323–339}-induced CD69 expression in CD4\textsuperscript{+} CD8\textsuperscript{−} and CD4\textsuperscript{−} CD8\textsuperscript{+} SP thymocytes, suggesting that CTLA-4 is a negative regulator of T cell activation, as previously reported (14–17). In contrast, blockage of CTLA-4 inhibited OVA\textsubscript{323–339}-induced CD69 expression in DP thymocytes. Anti-FcR-blocking Ab treatment did not influence the effect of anti-CTLA-4 on the activation and deletion profile of SP and DP thymocytes, suggesting that the differential function of CTLA-4 on SP and DP thymocytes is not due to FcR-mediated cross-linking. In addition, we found that blockage of CTLA-4 inhibited TCR-stimulated activation of ERK in DP thymocytes, whereas blockage of CTLA-4 further increased the activation of ERK in CD4\textsuperscript{+} SP thymocytes as well as in peripheral T cells. These results clearly indicate that CTLA-4 ligation delivers a positive signal to activate DP thymocytes, which is opposite its function in SP thymocytes and peripheral T cells.

There is an apparent discrepancy between the relatively small proportion of cells that express CTLA-4 after anti-CD3 Ab treatment and the strong inhibitory effect of anti-CTLA-4 blocking Ab on anti-CD3 Ab-induced deletion of DP thymocytes. Although the surface expression of CTLA-4 in thymocytes was readily detected about 24–48 h after TCR engagement in our studies and others, CTLA-4 mRNA is detectable within 1 h (19), and CTLA-4/B7 interaction affects IL-2 mRNA accumulation about 4 h after T cell activation (20), suggesting that CTLA-4 might be functionally available before it can be detected on the cell surface. In addition, CTLA-4 has a short half-life (∼2 h) in activated T cells (43). In the present study DP thymocytes might express CTLA-4 before TCR engagement to receive signals, but the presence of CTLA-4 would be transient because of its short half-life. Therefore, a low proportion of CTLA-4-expressing DP thymocytes would be detected at any sampling time.

We further examined whether the CTLA-4-dependent deletion of DP thymocytes in DO11.10 FTOC depends on the dose of OVA\textsubscript{323–339}. We found that CTLA-4 dependence was less evident in the deletion of DP thymocytes when a low dose of OVA\textsubscript{323–339} was used. This was probably because weak TCR stimulation may be insufficient to induce the full expression and trafficking of CTLA-4 to activation-induced, scattered immunological synapses of DP thymocytes after TCR engagement (43). Thus, anti-CTLA-4 Ab could not efficiently block CTLA-4-mediated signaling, resulting in impaired inhibition of DP thymocyte deletion after weak TCR engagement. Under conditions of weak TCR stimulation, ERK activation could also be slower, resulting in less efficient expression of activation-induced CTLA-4. Therefore, TCR engagement with high affinity/avidity self-peptide/MHC complexes may significantly induce the expression of CTLA-4 on the surface of DP thymocytes, and CTLA-4-mediated costimulatory positive signals induce overactivation, resulting in apoptosis of DP thymocytes. The complete deletion of these DP thymocytes during T cell development may contribute to the induction of tolerance.

We carefully checked the proportion and number of DP thymocytes in CTLA-4-deficient C57BL/6 FTOC without T cell activation. We found that there was no difference in the proportion of DP thymocytes between CTLA-4-deficient and control FTOC. However, the number of DP thymocytes in CTLA-4-deficient FTOC was significantly higher than that of control FTOC (Fig. 8). This result supports the hypothesis that CTLA-4 is involved in negative...
selection as a positive regulator, which contrasts with previous studies showing normal development of DP thymocytes in CTLA-4-deficient mice and CTLA-deficient H-Y TCR transgenic mice (26, 27). Our results consistently showed that CTLA-4 plays a positive role in the activation and deletion of DP thymocytes, which contrasts with previous studies. There may be several reasons for this discrepancy. First, the absence of CTLA-4-mediated negative signals in CTLA-4-deficient mice may have allowed nonspecific activation of peripheral T cells in vivo, resulting in the production of soluble mediators that may have resulted in DP thymocyte deletion (28, 36). To exclude possible nonspecific activation of peripheral T cells, we used FTOC from OVA253–339-specific TCR transgenic mice to blockage of CTLA-4 suppressed the activated C57BL/6 mice, the excellent technical assistance of L. Robertson and the editorial assistance of Dr. A. L. Kyle.

Acknowledgments

We gratefully acknowledge Dr. Tak W. Mak for providing CTLA-4-deficient C57BL/6 mice, the excellent technical assistance of L. Robertson with FACS analysis, and the editorial assistance of Dr. A. L. Kyle.

References