

CTLA4 Expression Is an Indicator and Regulator of Steady-State CD4⁺FoxP3⁺ T Cell Homeostasis¹

Anita L. Tang,* John R. Teijaro,* Modesta N. Njau,* Smita S. Chandran,* Agnes Azimzadeh,* Steven G. Nadler,[†] David M. Rothstein,[‡] and Donna L. Farber^{2*}

The presence of FoxP3⁺ regulatory T cells (Tregs) is necessary for control of deleterious immune responses in the steady state; however, mechanisms for maintaining the frequency and quality of endogenous Tregs are not well defined. In this study, we used *in vivo* modulators of the CD28 and CTLA4 pathways administered to intact mice to reveal mechanisms controlling the homeostasis and phenotype of endogenous Tregs. We demonstrate that expression of the negative costimulatory regulator CTLA4 on FoxP3⁺ Tregs *in vivo* is a direct consequence of their rapid, perpetual homeostasis. Up-regulation of CTLA4 expression occurs only on FoxP3⁺ Tregs undergoing extensive proliferation and can be abrogated by inhibiting the CD28 pathway, coinciding with a reduction in FoxP3⁺ Treg proliferation and frequency. We further demonstrate that CTLA4 negatively regulates steady-state Treg homeostasis, given that inhibiting CTLA4 signaling with an anti-CTLA4 blocking Ab greatly enhances Treg proliferation and overall Treg frequency. Our findings provide new insight into the origin and role of CTLA4 expression on natural FoxP3⁺ Tregs and reveal opposing effects of costimulation modulators on the steady-state level and quality of Tregs, with implications regarding their effects on endogenous Tregs in patients receiving immunotherapy. *The Journal of Immunology*, 2008, 181: 1806–1813.

Regulatory T cells (Tregs)³ constitute 6–12% of peripheral CD4 T cells and maintain self-tolerance and control Ag-specific responses (1). The FoxP3 transcription factor delineates Treg development and functional capacity (2–4), with genetic mutations in FoxP3 manifesting as a fatal autoimmune lymphoproliferative disease in both humans and mice (5, 6). Tregs have been shown to suppress immune responses to infectious pathogens (7), tumors (8, 9), and transplants (10–12) and also ameliorate inflammatory bowel disease (13–15) and autoimmune diabetes (16–18). Moreover, Tregs have an active role in maintaining immune homeostasis during healthy conditions in the absence of exogenous Ags, because *in vivo* ablation of FoxP3⁺ Tregs in immunocompetent adult mice results in a wasting, lymphoproliferative scurfy-like disease with multiorgan immune infiltration (19, 20). At present, it is not known how Tregs maintain control of deleterious immune responses in steady-state conditions and what in turn controls the maintenance and quality of Tregs during healthy conditions.

The identification of FoxP3⁺ Tregs as crucial cellular controllers of immunity suggests that direct manipulation of Tregs *in vivo* can result in the enhancement or suppression of specific immune responses. Two potential targets for Treg manipulation are the co-

stimulatory molecules CD28 and CTLA4, which promote and inhibit naive T cell activation, respectively (21), and play distinct roles in Treg generation, function, and homeostasis (22, 23). CD28 is constitutively expressed on all T cells and binds the B7-1 (CD80) and B7-2 (CD86) ligands, providing the necessary second signal for TCR-mediated activation, differentiation, and survival of naive T cells (24–26). CD28 signaling in Tregs has been shown to promote FoxP3⁺ Treg generation from developing thymocytes (27) as well as peripheral Treg homeostasis and expansion (28, 29). However, other studies have shown that FoxP3⁺ Tregs can be generated in CD28^{-/-} and B7.1/7.2^{-/-} mice (28–30), suggesting the existence of CD28-independent pathways for Treg generation and/or homeostasis.

CTLA4 (CD152), a homolog of CD28, is up-regulated on naive T cells after stimulation and suppresses T cell activation upon binding its B7 ligands (31). CTLA4 is also expressed constitutively on a subset of Tregs (32), although the origin of CTLA4 expression on Tregs is not known. CTLA4 expression on Tregs does not appear to be a direct consequence of activation by the FoxP3 transcription factor (33, 34), suggesting that additional factors drive the up-regulation of CTLA4 expression on Tregs. Whether CTLA4 expression is required for Treg generation or intrinsic regulatory capacity likewise remains unresolved (23). CD4⁺FoxP3⁺ Tregs are generated in CTLA4-deficient mice, and these CTLA4-deficient Tregs can suppress T cell responses *in vitro* and *in vivo* (15, 35). However, CTLA4 up-regulation is also associated with enhanced Treg activity in inflammatory bowel disease models (15, 32) and is required in Ab-mediated therapies for promoting allograft survival (36). In addition, the factors or pathways that induce or maintain CTLA4 expression on Tregs are not well defined.

Both the CD28 and CTLA4 pathways are targets for current immunomodulatory therapies: the B7-binding fusion protein CTLA4Ig (37) is an approved immunosuppressant for treatment of rheumatoid arthritis (38); and an anti-CTLA4 blocking Ab is in trials for immune enhancement in cancer (39). Although these treatments have specific effects on Ag-driven activation of

*Department of Surgery, University of Maryland School of Medicine, Baltimore, MD 21201; [†]Immunology and Inflammation Drug Discovery, Bristol-Myers Squibb Research and Development, Princeton, NJ 08543; and [‡]Department of Medicine, Yale University School of Medicine, New Haven, CT 06510

Received for publication March 20, 2008. Accepted for publication May 26, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by a grant from Bristol-Myers Squibb and by National Institutes of Health Grant AI050632 (to D.L.F.).

² Address correspondence and reprint requests to Dr. Donna L. Farber, Department of Surgery, University of Maryland School of Medicine, 685 West Baltimore Street, Baltimore, MD 21201. E-mail address: dfarber@smail.umaryland.edu

³ Abbreviations used in this paper: Treg, regulatory T cell; GITR, glucocorticoid-induced tumor necrosis factor receptor; MFI, mean fluorescence intensity.

conventional naive and memory T cells (40–42), systemic administration has the potential to affect nontargeted T cell responses, such as those mediated by Tregs which are required to maintain immune homeostasis in healthy individuals.

In this study, we examined steady-state Treg turnover and used *in vivo* modulators of the CD28 and CTLA4 pathways administered to intact mice to reveal new mechanisms controlling CTLA4 expression and steady-state homeostasis of endogenous Tregs. We demonstrate that constitutive CTLA4 expression on peripheral FoxP3⁺ Tregs is a consequence of their rapid, steady-state homeostasis that is driven by CD28 signaling. Inhibiting CD28 costimulation with the B7-binding reagent CTLA4Ig blocked endogenous Treg proliferation and decreased CTLA4 expression on the remaining FoxP3⁺ Tregs. Conversely, inhibition of CTLA4 signaling with an anti-CTLA4 blocking Ab greatly enhanced Treg homeostasis and overall frequency, revealing a novel role for CTLA4 in the control of Treg turnover. Our findings provide new insight into the origin and role of CTLA4 expression on natural FoxP3⁺ Tregs and reveal that costimulation modulators can alter the steady-state level and quality of Tregs, with implications regarding the role of endogenous Tregs in immunotherapies.

Materials and Methods

Mice

BALB/c (Thy1.2⁺) mice were purchased from the National Cancer Institute, and BALB/c (Thy1.1⁺) congenic mice (University of North Carolina, Chapel Hill, NC) were bred and maintained under specific pathogen-free conditions and used at 6–10 wk of age. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore.

Antibodies

The following Abs were purchased from Bio X Cell: anti-CD8 (TIB105); anti-CD4 (GK1.5); anti-I-A^d (212.A1); anti-Thy1 (TIB238); murine IgG2a (C1.18.4); anti-FcγR (2.4G2); and anti-CTLA4 (UC10-4F10-11). FITC-conjugated anti-BrdU and anti-IgG1; PE-conjugated anti-CD25, anti-CTLA4, and anti-IgG1; PerCP-conjugated anti-CD4; and allophycocyanin-conjugated anti-CD90.2 and anti-IgG2a Abs were purchased from BD Pharmingen. PE-conjugated anti-FoxP3; PE-Cy-7-conjugated anti-CD4 and anti-CD90.2; allophycocyanin-conjugated anti-FoxP3; allophycocyanin-Cy-7-conjugated anti-CD25; and allophycocyanin-Alexa Fluor 750-conjugated anti-CD25 Abs were purchased from eBiosciences. Polyclonal hamster IgG Ab was purchased from Jackson ImmunoResearch. Goat anti-rat IgG, anti-rat IgM, and anti-mouse IgG conjugated to magnetic beads were purchased from Polysciences.

Treatment of mice with costimulation blockade and thymectomy

BALB/c mice were treated with murine CTLA4Ig (250 μg/dose *i.p.*; Bristol-Myers Squibb) or anti-CTLA4 (250 μg/dose, *i.p.*) every other day for a total of 3 doses, with murine IgG2a, IgG, and PBS as controls. For CD28 inhibition, mice were treated with a single-chain anti-CD28 blocking Ab (Wyeth Pharmaceuticals) in doses of 100 μg twice daily for 7 days.

In some experiments, mice were thymectomized before treatment with CTLA4Ig. Briefly, mice were anesthetized *i.p.* with a 2% solution of 2,2,2-tribromoethanol (Sigma-Aldrich), and the thymus was removed with negative suction. Animals were allowed to recover for at least 7 days before further manipulation.

Isolation of Treg and non-Treg fractions

To isolate CD4⁺CD25⁺ Tregs, CD4 T cells were purified from the spleen by density centrifugation (Lymphocyte Separation Medium; Mediatech) and immunomagnetic depletion as described previously (43), stained with PE- or allophycocyanin-Alexa Fluor 750-conjugated anti-CD25 Ab and anti-PE or anti-Cy-7 magnetic microbeads (Miltenyi Biotec), and positively selected by separation over a ferromagnetic column using AutoMACS (Miltenyi Biotec). Non-Tregs (CD4⁺CD25⁻) were isolated from splenic CD4 T cells after staining and conjugation to magnetic microbeads as above and depleted of CD4⁺CD25⁺ Tregs using AutoMACS. Isolated CD4⁺CD25⁺ fractions were >90% pure and CD4⁺CD25⁻ were >95% pure.

In vivo BrdU labeling and flow cytometry

To assess the turnover of Tregs in comparison with non-Treg populations, CTLA4Ig-, IgG2a, and PBS-treated mice were injected with BrdU (1 mg, *i.p.*) for 3 consecutive days before sacrifice. Splenic CD4 T cells were harvested at various time points (3–28 days), purified as above, and resuspended in stain buffer. Cells were surface stained with fluorescently coupled Abs specific for CD4, Thy1.2, and CD25; fixed; and permeabilized (Cytofix/Cytoperm, Perm/Wash; BD Biosciences) for subsequent intracellular staining. For analysis of intracellular BrdU content, permeabilized cells were incubated with DNase (Sigma-Aldrich) for 60 min at 37°C, washed with Perm-Wash, incubated with Fc-block (Miltenyi) to block non-specific binding, followed by fluorescently conjugated anti-BrdU, anti-FoxP3, and/or anti-CTLA4 Ab at 4°C. Cells were further washed and analyzed on LSRII using FACSDiva software (BD Biosciences).

In vivo proliferation assays

To monitor the proliferation of T cell subsets *in vivo*, CD4⁺CD25⁺ Tregs or CD4⁺CD25⁻ non-Tregs were isolated from BALB/c (Thy1.2⁺) mice, labeled with 2.5 μM CFSE (Invitrogen), and transferred via tail vein injection (1 × 10⁶ cells/mouse) into congenic BALB/c (Thy1.1⁺) hosts undergoing treatment with CTLA4Ig, anti-CTLA4, anti-CD28, IgG2a, IgG or PBS. Splenic CD4 T cells were harvested and purified 6 days after adoptive transfer, stained for intracellular FoxP3 in accordance with the manufacturer's protocol, and analyzed by flow cytometry. Treg proliferation was determined by gating on CD4⁺FoxP3⁺Thy1.2⁺ cells isolated from mice transferred with CD4⁺CD25⁺Thy1.2⁺ cells. Non-Treg proliferation was assessed by gating on CD4⁺FoxP3⁻Thy1.2⁺ cells from mice receiving CD4⁺CD25⁻Thy1.2⁺ cells.

Statistical methods

Student's two-tailed *t* test was used to evaluate differences between treatment groups. A *p* value of <0.05 was considered significant.

Results

Rapid steady-state turnover of Tregs *in vivo* is associated with up-regulated CTLA4 expression

We initially analyzed the steady-state turnover of endogenous FoxP3⁺ Tregs compared with the FoxP3⁻ non-Treg population using short-term BrdU incorporation. We injected intact, healthy BALB/c mice with BrdU for 3 days before harvest and analyzed BrdU uptake as a function of FoxP3 expression on splenic CD4 T cells. We found that a higher proportion of FoxP3⁺ Tregs incorporated BrdU than did FoxP3⁻ non-Tregs. Of the FoxP3⁺ CD4 T cells, 19% incorporated BrdU compared with only 7% of FoxP3⁻ cells (Fig. 1A, *right*). Overall, FoxP3⁺ Tregs exhibited 3-fold greater proliferation compared with FoxP3⁻ non-Tregs, based on the extent of BrdU incorporation (Fig. 1B). These results demonstrate rapid and enhanced turnover of FoxP3⁺ CD4 T cells (Tregs) relative to their FoxP3⁻ (non-Treg) counterparts.

To more closely analyze the dynamics of steady-state Treg homeostasis, we examined the ability of Treg and non-Treg populations to proliferate *in vivo* when transferred into intact hosts. For these experiments, we isolated Treg and non-Treg populations from intact BALB/c (Thy1.2⁺) mice by sorting on CD25 expression, yielding CD4⁺CD25⁺ and CD4⁺CD25⁻ populations, respectively. These subsets were CFSE-labeled, transferred into intact BALB/c (Thy1.1⁺) congenic hosts, and harvested after 6 days *in vivo* to assess spontaneous turnover of the transferred populations. In our analysis of the transferred populations (Fig. 2A), we further gated on FoxP3⁺ cells within the CD25⁺ transferred population, and FoxP3⁻ cells within the CD25⁻ transferred population to ensure that true Treg and non-Treg populations were being evaluated. We found extensive *in vivo* proliferation of transferred FoxP3⁺ Tregs (53% dividing) compared with non-Tregs (6%) after only 6 days *in vivo* in intact mice (Fig. 2A). These results demonstrate that the natural population of polyclonal FoxP3⁺ Tregs contains a subset capable of rapid and extensive proliferation in steady-state conditions.

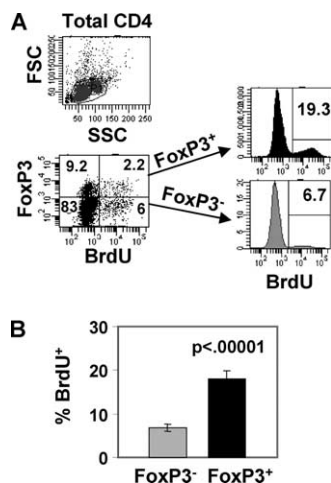


FIGURE 1. Enhanced steady-state turnover of FoxP3⁺ Tregs in healthy mice. BALB/c mice were given BrdU (1 mg/dose i.p.) for 3 consecutive days, and splenic CD4 T cells were harvested the following day, stained intracellularly for FoxP3 and BrdU, and analyzed by flow cytometry. *A, Top*, Forward scatter (FSC) and side scatter (SSC) plots of total CD4 T cells with gating indicated. *Bottom*, FoxP3 vs BrdU staining of total live CD4 T cells, with percentages indicated in each quadrant. To the right of the dot plot are histograms showing BrdU incorporation gated on FoxP3⁺ and FoxP3⁻ CD4 T cells. *B*, Mean BrdU incorporation \pm SD by FoxP3⁺ and FoxP3⁻ CD4 T cells from five mice per group (representative of three experiments).

Given this heterogeneity in the ability of FoxP3⁺ Tregs to undergo rapid homeostasis, we asked whether FoxP3⁺ Tregs that underwent maximal division (CFSE^{low}) differed in the expression of Treg functional markers compared with the undivided (CFSE^{high}) population. Indeed, we found that FoxP3⁺ CFSE^{low} Tregs exhibiting extensive division expressed higher levels of FoxP3, CTLA4, and glucocorticoid-induced tumor necrosis factor receptor (GITR) and lower levels of CD25 compared with non-proliferating Tregs (Fig. 2*B*). The most striking phenotypic difference between proliferating and nonproliferating Tregs was in CTLA4 expression, with maximally dividing Tregs expressing high levels of CTLA4, and undivided Tregs exhibiting low CTLA4 expression (Fig. 2*B*, boxed area). These results demonstrate a correlation between the level of CTLA4 expression on FoxP3⁺ Tregs and the extent of peripheral homeostasis, and also show that FoxP3 and GITR expression varies between actively dividing and quiescent Tregs.

Costimulatory inhibition with CTLA4Ig decreases Treg turnover, frequency, and CTLA4 expression in vivo

The association of CTLA4 expression with rapidly dividing Tregs suggests that CTLA4 up-regulation could either occur as a direct result of *in vivo* turnover, or that CTLA4 directly signals for rapid turnover. To address the first possibility, we investigated whether inhibiting steady-state homeostasis would reduce CTLA4 expression on endogenous Tregs. As the CD28 pathway is important for peripheral turnover of CD4⁺CD25⁺ Tregs *in vivo* (28, 29), we used the B7-binding reagent CTLA4Ig (37) to down-modulate Treg homeostasis and determine potential effects on CTLA4 expression. We first examined the extent of CTLA4Ig-mediated inhibition of steady-state FoxP3⁺ Treg homeostasis in intact BALB/c hosts, as earlier studies had not addressed direct effects on *in vivo* proliferation of transferred or endogenous FoxP3⁺ Tregs (29). To analyze the effects of CTLA4Ig treatment on transferred populations of Tregs and non-Tregs, we treated BALB/c

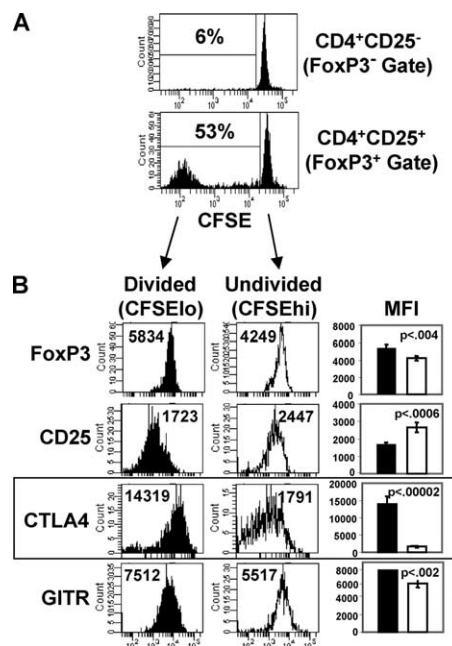


FIGURE 2. Rapid steady-state homeostasis of FoxP3⁺ Tregs is associated with increased CTLA4 expression. CFSE-labeled CD4⁺CD25⁺ (Tregs) and CD4⁺CD25⁻ (non-Tregs) from BALB/c (Thy1.2⁺) mice were transferred into BALB/c (Thy1.1⁺) congenic hosts (1×10^6 cells/mouse), harvested after 6 days, stained intracellularly for FoxP3 and additional Treg markers, and analyzed by flow cytometry. *A*, CFSE dilution of transferred Thy1.2⁺ populations, gating on FoxP3⁺ cells for analysis of Tregs within the CD4⁺CD25⁺ transferred population, and gating on FoxP3⁻ cells for analysis of non-Tregs within the CD4⁺CD25⁻ transferred population. Mean percentage \pm SD of divided (CFSE^{lo}) Tregs (CD4⁺FoxP3⁺Thy1.2⁺) and non-Tregs (CD4⁺FoxP3⁻Thy1.2⁺) are $53 \pm 1\%$ and $6 \pm 1\%$, respectively ($n = 10$ mice per group). *B*, Expression of FoxP3, CD25, CTLA4, and GITR on maximally divided (CFSE^{lo}) and undivided (CFSE^{hi}) FoxP3⁺ cells with mean fluorescence intensity (MFI) of each marker indicated. Bar graphs show mean MFI \pm SD for each marker expressed by maximally-divided (black) and undivided (white) Tregs from four mice per group. Box highlights histogram plots of CTLA4 expression, with the most striking differences in expression between dividing and non-dividing Tregs.

(Thy1.1⁺) mice with murine CTLA4Ig or IgG2a isotype control Ab according to the schematic in Fig. 3*A*, transferred CFSE-labeled Thy1.2⁺ CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells into treated mice on day 1 (Fig. 3*A*), and assessed spontaneous *in vivo* turnover of the transferred populations after 6 days. Whereas FoxP3⁺ CD4 T cells proliferated extensively when transferred into control-treated hosts as in Fig. 2*A*, CTLA4Ig substantially inhibited this steady-state homeostasis by 75% (Fig. 3*B*). These results demonstrate a near-complete inhibition of FoxP3⁺ Treg proliferation *in vivo* by CTLA4Ig.

To assess whether CTLA4Ig similarly inhibited endogenous FoxP3⁺ Treg turnover, we pulsed IgG2a- and CTLA4Ig-treated BALB/c mice with BrdU for 3 days before harvest and analyzed FoxP3 expression in conjunction with BrdU incorporation by splenic CD4 T cells. In control-treated mice, as in intact mice, FoxP3⁺ Tregs divided more extensively than FoxP3⁻ non-Tregs (16% vs 6% incorporating BrdU, respectively; Fig. 3*C*). However, in CTLA4Ig-treated mice, BrdU incorporation by FoxP3⁺ Tregs was almost completely inhibited, compared with the slight inhibition of BrdU incorporation by FoxP3⁻ non-Tregs (Fig. 3*C*). Interestingly, CTLA4Ig treatment substantially altered the Treg/non-Treg proliferation ratio, from a 3-fold higher proliferation by

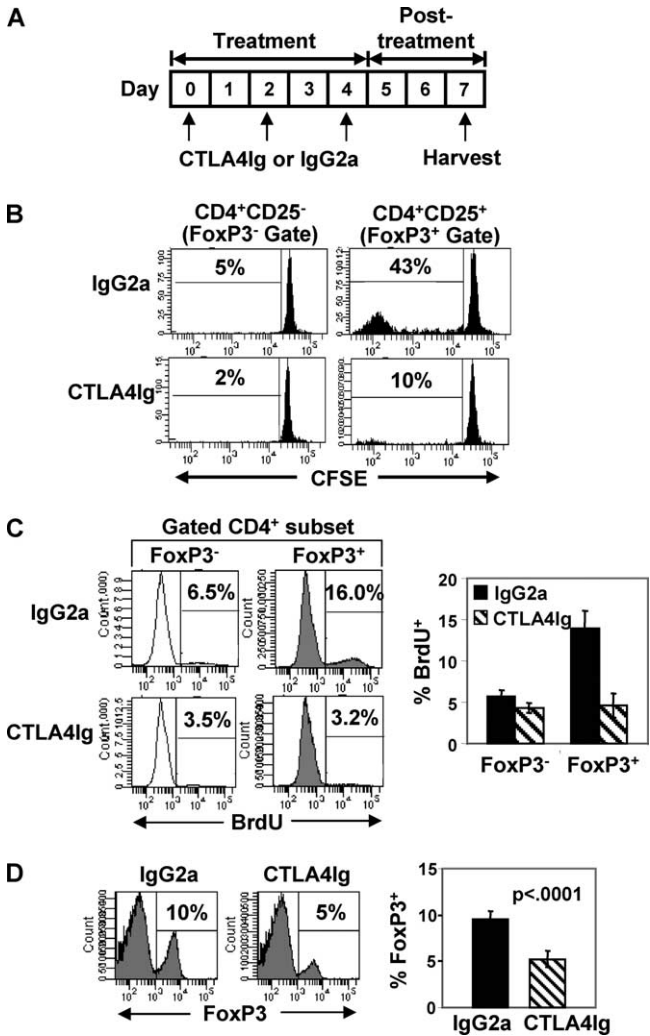


FIGURE 3. Steady-state homeostasis of FoxP3⁺ Tregs is blocked by CTLA4Ig-mediated inhibition of CD28 costimulation. **A**, Experimental scheme for measuring effects of CTLA4Ig on steady-state homeostasis of transferred and endogenous Treg populations. BALB/c (Thy1.1⁺) congenic hosts were treated with CTLA4Ig or IgG2a on days 0, 2, and 4. For Treg transfer experiments in **B**, CFSE-labeled CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from untreated BALB/c (Thy1.2⁺) mice were transferred on day 1 into Thy1.1⁺ mice undergoing treatment, harvested at day 7, and stained intracellularly for FoxP3 expression. To examine the effects on endogenous FoxP3⁺ and FoxP3⁻ populations, BrdU was administered to treated mice on days 4–6, and mice were harvested at day 7 in **C** and **D**. **B**, Representative CFSE histograms show percentage of divided Thy1.1⁺FoxP3⁺ Tregs and Thy1.1⁺FoxP3⁻ non-Tregs gated as in Fig. 2A. Mean percentage ± SD of divided Thy1.2⁺FoxP3⁺ and Thy1.2⁺FoxP3⁻ CD4 T cells are as follows: IgG2a 45 ± 4% and 5 ± 1%, and CTLA4Ig 10 ± 2% and 2 ± 1%, respectively (*n* = 3–7 mice/group). **C**, CTLA4Ig inhibits endogenous Treg homeostasis. *Left*, Representative histograms show BrdU incorporation by FoxP3⁺ and FoxP3⁻ CD4 T cells in IgG2a and CTLA4Ig-treated mice, with the percentage of BrdU⁺ cells indicated. *Right*, Mean proportion ± SD of FoxP3⁺ and FoxP3⁻ CD4 T cells incorporating BrdU from five mice per group. **D**, Reduced frequency of CD4⁺FoxP3⁺ T cells in CTLA4Ig-treated mice. *Left*, Representative histograms indicate the percentage of FoxP3⁺ Tregs among total CD4 T cells. *Right*, Mean percentage ± SD of FoxP3⁺ Tregs of total CD4 T cells from five mice per group. Results are representative of four experiments.

FoxP3⁺ compared with FoxP3⁻ cells in control-treated mice to equivalent low-level proliferation of both FoxP3⁺ and FoxP3⁻ cells in CTLA4Ig-treated hosts (Fig. 3C). This decrease in endogenous Treg turnover resulted in a net 50% decrease in overall

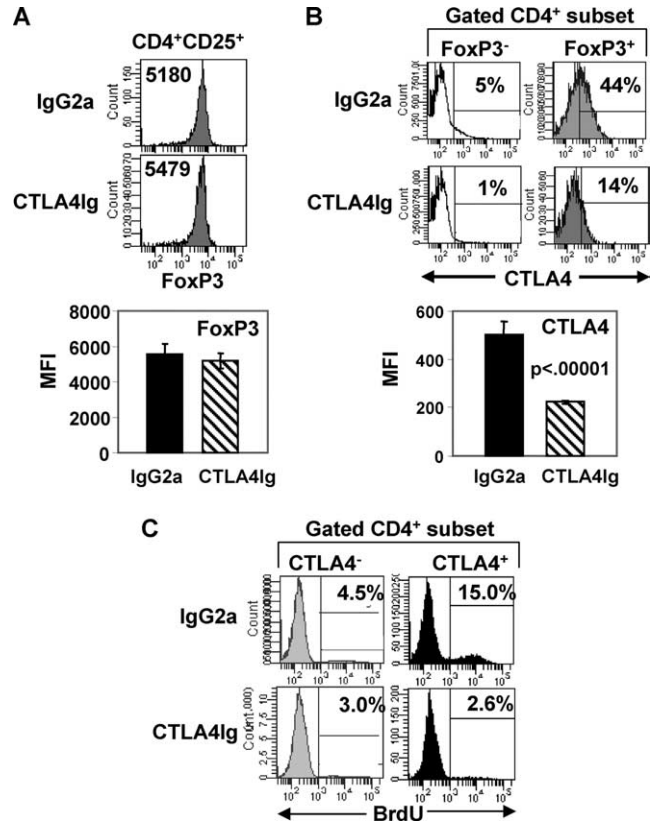


FIGURE 4. Inhibition of steady-state homeostasis selectively reduces CTLA4 expression on Tregs while FoxP3 expression is maintained. BALB/c mice were treated with IgG2a or CTLA4Ig as in Fig. 3A, administered BrdU on days 4–6, and splenic CD4 T cells were harvested at day 7, and analyzed for FoxP3 and CTLA4 expression. **A**, Comparable level of FoxP3 expression in IgG2a- and CTLA4Ig-treated mice. *Top*, Histograms show the level of FoxP3 expression of CD4⁺CD25⁺ T cells from IgG2a- and CTLA4Ig-treated mice, with MFI of FoxP3 indicated in each histogram. *Bottom*, Average MFI of FoxP3 expression from five mice per group. Results are representative of four experiments. **B**, CTLA4Ig treatment reduces CTLA4 expression on Tregs. *Top*, Histograms show CTLA4 expression gated on FoxP3⁺ and FoxP3⁻ CD4 T cells from IgG2a- and CTLA4Ig-treated mice with the percentage CTLA4⁺ shown based on isotype control staining indicated by marker. *Bottom*, Average MFI of CTLA4 expression by FoxP3⁺ Tregs from IgG2a- and CTLA4Ig-treated mice (*n* = 5 mice per group). Results are representative of six experiments. **C**, Steady-state homeostasis of CTLA4⁺ and CTLA4⁻ CD4 T cells in IgG2a- and CTLA4Ig-treated mice. Histograms show percent BrdU incorporation by CD4⁺CTLA4⁺ and CD4⁺CTLA4⁻ T cells from total CD4 T cells in IgG2a- and CTLA4Ig-treated mice (*n* = 5 mice per group).

FoxP3⁺ Treg frequency compared with control mice (Fig. 3D). These results indicate that the rapid steady-state proliferation of FoxP3⁺ Tregs is driven by CD28 engagement whereas the low-level turnover of the non-Treg population appears to be independent of CD28 costimulation.

We asked whether the expression of FoxP3 or CTLA4 was altered within the endogenous Treg population in CTLA4Ig-treated mice, given their reduced frequency and *in vivo* turnover. We found that endogenous FoxP3⁺ Tregs from CTLA4Ig-treated mice had equivalent levels of FoxP3 expression as Tregs from IgG2a-treated mice (Fig. 4A). However, FoxP3⁺ Tregs from CTLA4Ig-treated mice exhibit greatly reduced CTLA4 expression compared with the high levels of CTLA4 expression in Tregs from IgG2a-treated hosts (Fig. 4B). These results indicate that CD28 engagement is required for CTLA4 up-regulation on FoxP3⁺ Tregs in steady-state conditions.

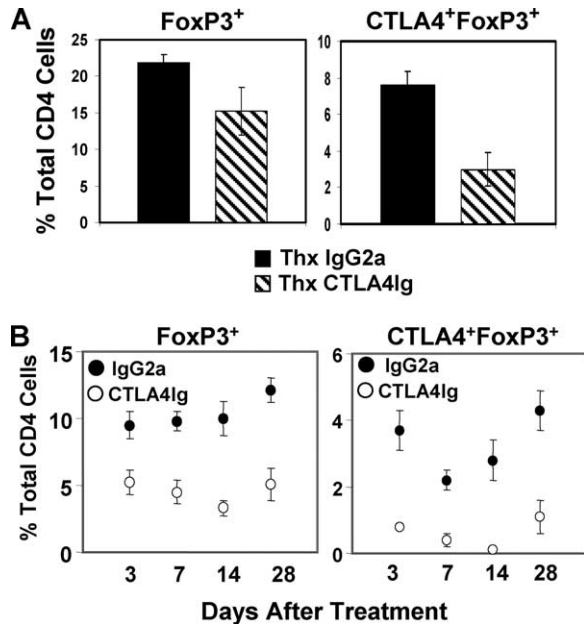
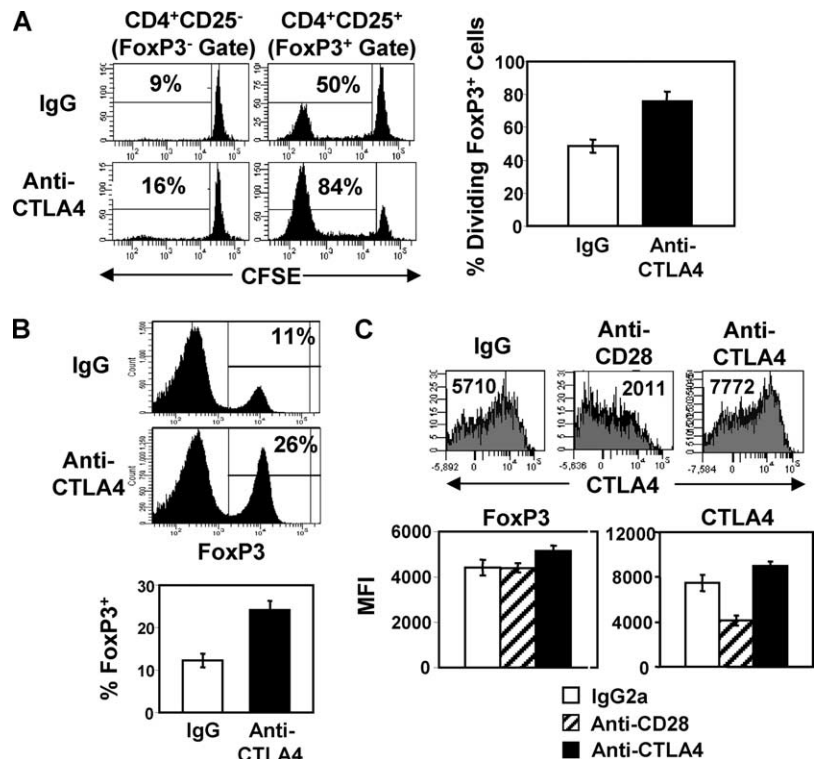


FIGURE 5. Abrogation of CTLA4 expression on Tregs by CTLA4Ig treatment is due to decreased peripheral homeostasis. *A*, Decreased Treg frequency and CTLA4 expression in thymectomized (Thx) mice treated with CTLA4Ig. BALB/c mice were thymectomized and treated with CTLA4Ig or IgG2a as in Fig. 3*A*, and splenic CD4 T cells were harvested 3 days posttreatment (day 7). Graphs show percentage of FoxP3⁺ (*left*) and CTLA4⁺FoxP3⁺ CD4 T cells (*right*) among total CD4 T cells from thymectomized IgG2a- and CTLA4Ig-treated mice. Results are an average of four mice per group and are representative of two experiments. The proportion of Tregs in IgG2a- and CTLA4Ig-treated sham-thymectomized controls were indistinguishable from intact mice similarly treated (data not shown). *B*, Long-term reduction of FoxP3⁺ frequencies and CTLA4 expression on FoxP3⁺ Tregs following CTLA4Ig treatment. BALB/c mice were treated with IgG2a or CTLA4Ig as in Fig. 3*A*, and the proportions of FoxP3⁺ (*left*) and CTLA4⁺FoxP3⁺ CD4 T cells (*right*) of total CD4 T cells were determined 3–28 days posttreatment. Results are expressed as mean percentage \pm SD from four to five mice per group.

FIGURE 6. CTLA4 negatively regulates Treg homeostasis. *A*, Effect of anti-CTLA4 treatment on in vivo proliferation of Tregs. CFSE-labeled CD4⁺CD25⁺ or CD4⁺CD25⁻ T cells from BALB/c (Thy1.2⁺) mice were transferred (1×10^6 cells/mouse) on day 1 into BALB/c (Thy1.1⁺) congenic mice undergoing treatment with anti-CTLA4 or IgG control using a similar treatment schedule as with CTLA4Ig (days 0, 2 and 4; Fig. 3*A*) and harvested after 6 days in vivo (day 7). *Left*, Representative CFSE histograms showing the division of transferred FoxP3⁺ Tregs and FoxP3⁻ non-Tregs gated as in Fig. 2*A*. *Right*, Mean percentage \pm SD of divided CD4⁺FoxP3⁺Thy1.2⁺ Tregs in IgG- and anti-CTLA4-treated mice ($n = 6-7$ mice per group). *B*, FoxP3 expression of endogenous CD4 T cells isolated from IgG- and anti-CTLA4-treated mice. Top two plots are representative histograms showing FoxP3⁺ expression of total CD4 T cells from IgG- and anti-CTLA4-treated BALB/c mice, with the percentage of CD4⁺FoxP3⁺ cells indicated. Bottom plot shows the mean percentage \pm SD of CD4⁺FoxP3⁺ T cells of 10 mice per group, compiled from 3 independent experiments. *C*, FoxP3 and CTLA4 expression of endogenous CD4⁺FoxP3⁺ cells from mice treated with anti-CD28, anti-CTLA4, or IgG2a depicted as representative histograms and average MFI \pm SD of $n = 3-4$ mice per group. Results are representative of three experiments.



To determine whether CTLA4 expression directly correlated with in vivo homeostasis, we performed a similar BrdU incorporation analysis as in Fig. 1*A*, examining BrdU uptake in CD4 T cells as a function of CTLA4 expression. Thus, we injected intact BALB/c mice with BrdU for 3 consecutive days and harvested and analyzed splenic CD4 T cells as in Fig. 1. This analysis revealed that CTLA4 expression marks the population of cells undergoing rapid endogenous turnover in intact mice (Fig. 4*C*, *top row*) and that homeostasis of CTLA4⁺ T cells in CTLA4Ig-treated mice is inhibited by CTLA4Ig (Fig. 4*C*, *bottom row*). These results suggest that homeostasis drives CTLA4 expression, rather than CTLA4 signaling directing homeostasis.

The reduced proportion of CTLA4⁺FoxP3⁺ Tregs seen after CTLA4Ig treatment was also observed in thymectomized mice (Fig. 5*A*), indicating that CTLA4Ig-mediated effects on Treg homeostasis acted on peripheral Tregs. Furthermore, the decrease in FoxP3⁺ and CTLA4⁺FoxP3⁺ cells in CTLA4Ig-treated mice, as described in Figs. 2 and 3, persisted between 14 and 28 days posttreatment (Fig. 5*B*). These results indicate that CD28-driven peripheral homeostasis is required for CTLA4 expression on Tregs and the accumulation of CTLA4⁺ Tregs in the periphery, although maintenance of FoxP3 expression is CD28 independent. We also found that CTLA4Ig-treated Tregs maintained in vitro suppressive capacity (data not shown), indicating that CTLA4⁺ Tregs maintain suppressive function, presumably due to the maintenance of FoxP3 levels.

CTLA4 expression on Tregs controls their steady-state homeostasis

The above results strongly suggest that the lack of CTLA4 expression on Tregs is due to the lack of CD28-driven Treg homeostasis; however, it is important to consider that CTLA4Ig binds the B7 ligands, potentially inhibiting signaling through both CD28 and CTLA4. To test whether the association of CTLA4 expression with homeostasis was affected by CTLA4 engagement by Tregs, we treated mice with an anti-CTLA4 blocking Ab and assessed its

effects on steady-state proliferation of adoptively transferred and endogenous Tregs. Remarkably, spontaneous turnover of adoptively transferred FoxP3⁺ Tregs was substantially increased in anti-CTLA4-treated mice with 80–90% of Tregs undergoing maximal division after only 6 days *in vivo* (Fig. 6A). In addition, steady-state homeostasis of FoxP3⁻ non-Tregs was also increased, although remained significantly lower than that of Tregs (Fig. 6A). Consistent with the increased level of spontaneous Treg turnover *in vivo*, there was a substantial 2- to 3-fold increase in the proportion of endogenous FoxP3⁺ cells in anti-CTLA4-treated mice (24–28%) compared with controls (10–13%; Fig. 6B). These results demonstrate that engagement of the CTLA4 pathway on Tregs negatively regulates steady-state homeostasis and limits Treg frequency *in vivo*, in contrast to the CD28 pathway which positively regulates Treg homeostasis and frequency. These results further establish that homeostasis drives CTLA4 expression and not the converse mechanism.

To directly compare the effect of the CTLA4 vs CD28 pathway on CTLA4 expression by Tregs, we examined CTLA4 and FoxP3 expression by endogenous Tregs isolated from intact mice treated with anti-CTLA4 or anti-CD28 blocking Abs. Anti-CD28 treatment resulted in significant down-regulation of CTLA4 expression on Tregs and comparable levels of FoxP3 compared with control mice (Fig. 6C), similar to effects of CTLA4Ig on these markers (Fig. 4, A and B). Additionally, anti-CD28 treatment resulted in a reduced frequency of endogenous CD4⁺FoxP3⁺ T cells, but not to the degree observed with CTLA4Ig due to the limited stability and partial receptor coverage of the single-chain anti-CD28 Ab (data not shown). By contrast, anti-CTLA4 treatment resulted in higher levels of CTLA4 expression and slight increases in FoxP3 expression on endogenous Tregs (Fig. 6C). These results demonstrate opposing effects of the CD28 and CTLA4 pathways on the steady-state homeostasis, frequency, and expression of CTLA4 on FoxP3⁺ Tregs.

Discussion

We demonstrate here that CD4⁺FoxP3⁺ T cells (Tregs) undergo rapid and dynamic proliferation during steady-state conditions which can be reciprocally modulated by targeting costimulatory pathways. We show a striking bias in the steady-state proliferation of FoxP3⁺CD4 T cells and an association between CTLA4 expression and the subset of FoxP3⁺ Tregs undergoing rapid turnover. In addition, both Treg steady-state homeostasis and CTLA4 up-regulation were substantially inhibited by blocking the CD28 pathway. Conversely, Treg homeostasis and endogenous frequency were significantly enhanced when the CTLA4 pathway was blocked by *in vivo* administration of anti-CTLA4. Our findings demonstrate that Treg frequency and CTLA4 expression are determined by the extent of homeostasis during steady-state conditions and can be altered by immunotherapies in the absence of an antigenic stimulus. These findings have implications for the effects of costimulation-based immunotherapies on the endogenous host regulatory environment.

Our findings of biased incorporation of BrdU by FoxP3⁺ CD4 T cells *in vivo* over several days are consistent with reports of homeostatic turnover of CD4⁺CD25⁺ populations in lymphopenic or intact hosts (44, 45). However, we show here that steady-state turnover of endogenous FoxP3⁺ Tregs in intact hosts is rapid and continuous. We also demonstrate a direct and novel association between CTLA4 expression and the peripheral homeostasis of FoxP3⁺ Tregs, with the expression of FoxP3 and GITR also varying between actively dividing and quiescent Tregs. Although CD28 signaling has been recognized as promoting Treg homeostasis (22, 29), our results indicate that CD28 signaling is required for

both rapid steady-state Treg homeostasis and CTLA4 up-regulation. Conventional (FoxP3⁻) T cells likewise require CD28 signaling for optimal CTLA4 up-regulation during antigenic stimulation (46); however, homeostasis of FoxP3⁻ cells during steady-state conditions was only slightly dependent on CD28 signaling, and CTLA4 up-regulation did not occur in the dividing FoxP3⁻ population. These results indicate that homeostasis and CTLA4 expression on FoxP3⁺ and FoxP3⁻ T cells is controlled by distinct mechanisms during steady-state conditions.

One mechanism to account for the difference in steady-state behavior of Tregs and conventional T cells is the TCR specificity. It has been shown that self-reactive TCRs are disproportionately represented within the FoxP3⁺ Treg population (47). Therefore, reaction with self Ags and CD28 may account for the rapidly dividing Treg population in healthy, normal conditions, as was also suggested by a study in which peripheral turnover of Tregs expressing a fixed, transgene-encoded TCR required endogenous expression of the peptide Ag (48). Up-regulation of CTLA4 expression on conventional FoxP3⁻ T cells requires both TCR triggering and CD28 costimulation (46), suggesting that a similar mechanism of CD28 triggering and TCR ligation by endogenous Ags functions to up-regulate CTLA4 expression by Tregs.

The role of CTLA4 expression of Tregs has been actively investigated. Whereas CTLA4-deficient mice possess FoxP3⁺ Tregs (35) and CTLA4-deficient T cells exhibit regulatory activity *in vivo* (15), blockade of CTLA4 can abrogate Treg function (13, 15). Our finding that Treg homeostasis is enhanced following anti-CTLA4 blockade suggests that CTLA4 signaling down-modulates steady-state Treg turnover. A role for CTLA4 in controlling peripheral Treg frequency is also implied by a previous report showing an association between a single nucleotide polymorphism in CTLA4 and an increase in Treg frequency (49). Our results suggest that impaired CTLA4 signaling may lead to an increase in Treg proliferation and an augmented Treg frequency, with potential consequences on immune regulation.

We demonstrate here that the a subset of FoxP3⁺ Tregs exhibits rapid steady-state proliferation that greatly exceeds that of FoxP3⁻ non-Tregs and propose that this rapid and extensive turnover comprises the homeostatic noise associated with a healthy, regulated state (Fig. 7). We show here that immunotherapies targeting costimulation can affect the homeostatic turnover, frequency, and quality of FoxP3⁺ Tregs, raising the question whether their effect on steady-state Treg homeostasis can predict their effect on immune responses. Inhibiting the CD28 pathway with CTLA4Ig reduces steady-state proliferation of FoxP3⁺ Tregs more extensively than FoxP3⁻ T cells, resulting in an equal frequency of proliferating cells in both FoxP3⁺ and FoxP3⁻ populations (Fig. 7). However, these treated mice do not develop autoimmunity up to 2 mo posttreatment (data not shown), and clinically, CTLA4Ig treatment results in dampening of autoimmune-induced inflammation in rheumatoid arthritis (38) and in suppressing allogeneic responses in transplantation (50, 51), indicating that the net effect of CTLA4Ig treatment is immunosuppressive. Conversely, although anti-CTLA4 promotes Treg homeostasis and increases Treg frequency, it also increases non-Treg turnover under healthy conditions (Fig. 7). However, in tumor models, the net result of anti-CTLA4 treatment is an increase in tumor-specific immune responses (52, 53), although an increase in both effector and Treg numbers has been observed (54). Thus, CTLA4Ig decreases and anti-CTLA4 increases Treg frequency and homeostasis, which appear to be in opposition to their therapeutic outcomes in disease models.

We propose that the predominant outcome of immunomodulation of the CD28/CTLA4 costimulatory pathways in a disease state

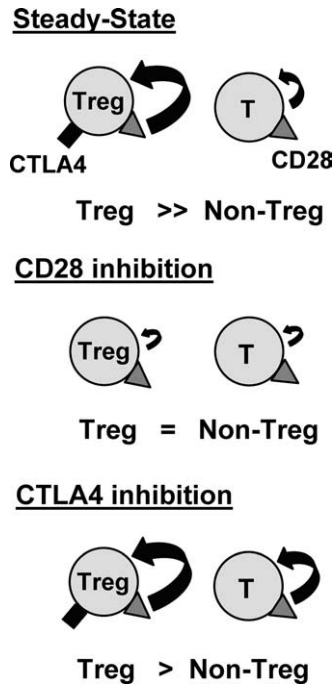


FIGURE 7. Treg and non-Treg turnover during steady-state conditions and during modulation of the CD28 or CTLA4 costimulation pathways. Regulatory T cells (Tregs) and conventional T cells (non-Tregs) undergo steady-state homeostasis, with the proliferation of Tregs exceeding that of non-Tregs in healthy conditions. CD28 (triangle) is expressed by both subsets and CTLA4 (rectangle) is only expressed by proliferating Tregs in the steady state. Treatment with CD28 pathway inhibitors reduces Treg proliferation to the low level seen with non-Tregs, thereby inhibiting CTLA4 up-regulation. Conversely, inhibition of the CTLA4 pathway increases both Treg and non-Treg homeostasis, with an increased proportion of CTLA4⁺FoxP3⁺ T cells.

with an active Ag response is determined primarily by the effects on the non-Treg population. Moreover, our results suggest that the immune system demonstrates plasticity with regard to the frequency and steady-state levels of Tregs. However, long-term use of these costimulation modulators in the clinic, particularly once the disease state is ameliorated, may result in their effects on Tregs dominating over those on the non-Treg population. Further studies are warranted to establish whether spontaneous Treg proliferation is a mechanism for maintaining proper immune homeostasis in the steady state or is a by-product of the CD28 and/or TCR signals encountered in the periphery.

Acknowledgments

We thank Drs. Stephen T. Bartlett and David Scott for critical reading of the manuscript, Emily Welty for anti-CD28 coverage studies and Wendy Lai for mouse colony maintenance.

Disclosures

The authors have no financial conflict of interest.

References

- Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25⁺ CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
- Fontenot, J. D., M. A. Gavin, and A. Y. Rudensky. 2003. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat. Immunol.* 4: 330–336.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
- Ziegler, S. F. 2006. FOXP3: of mice and men. *Annu. Rev. Immunol.* 24: 209–226.
- Brunkow, M. E., E. W. Jeffery, K. A. Hjerrild, B. Paepfer, L. B. Clark, S. A. Yasayko, J. E. Wilkinson, D. Galas, S. F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat. Genet.* 27: 68–73.
- Bennett, C. L., J. Christie, F. Ramsdell, M. E. Brunkow, P. J. Ferguson, L. Whitesell, T. E. Kelly, F. T. Saulsbury, P. F. Chance, and H. D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat. Genet.* 27: 20–21.
- Belkaid, Y., and B. T. Rouse. 2005. Natural regulatory T cells in infectious disease. *Nat. Immunol.* 6: 353–360.
- Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, et al. 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat. Med.* 10: 942–949.
- Zhou, G., and H. I. Levisky. 2007. Natural regulatory T cells and de novo-induced regulatory T cells contribute independently to tumor-specific tolerance. *J. Immunol.* 178: 2155–2162.
- Edinger, M., P. Hoffmann, J. Ermann, K. Drago, C. G. Fathman, S. Strober, and R. S. Negrin. 2003. CD4⁺CD25⁺ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat. Med.* 9: 1144–1150.
- Graca, L., S. Thompson, C. Y. Lin, E. Adams, S. P. Cobbold, and H. Waldmann. 2002. Both CD4⁺CD25⁺ and CD4⁺CD25⁻ regulatory cells mediate dominant transplantation tolerance. *J. Immunol.* 168: 5558–5565.
- Ochando, J. C., C. Homma, Y. Yang, A. Hidalgo, A. Garin, F. Tacke, V. Angeli, Y. Li, P. Boros, Y. Ding, et al. 2006. Alloantigen-presenting plasmacytoid dendritic cells mediate tolerance to vascularized grafts. *Nat. Immunol.* 7: 652–662.
- Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25⁺CD4⁺ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192: 295–302.
- Singh, B., S. Read, C. Asseman, V. Malmstrom, C. Mottet, L. A. Stephens, R. Stepankova, H. Tlaskalova, and F. Powrie. 2001. Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* 182: 190–200.
- Read, S., R. Greenwald, A. Izcue, N. Robinson, D. Mandelbrot, L. Francisco, A. H. Sharpe, and F. Powrie. 2006. Blockade of CTLA-4 on CD4⁺CD25⁺ regulatory T cells abrogates their function in vivo. *J. Immunol.* 177: 4376–4383.
- Tang, Q., K. J. Henriksen, M. Bi, E. B. Finger, G. Szot, J. Ye, E. L. Masteller, H. McDevitt, M. Bonyhadi, and J. A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J. Exp. Med.* 199: 1455–1465.
- Bresson, D., L. Togher, E. Rodrigo, Y. Chen, J. A. Bluestone, K. C. Herold, and M. von Herrath. 2006. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J. Clin. Invest.* 116: 1371–1381.
- Lepault, F., and M. C. Gagnerault. 2000. Characterization of peripheral regulatory CD4⁺ T cells that prevent diabetes onset in nonobese diabetic mice. *J. Immunol.* 164: 240–247.
- Lahl, K., C. Lodenkemper, C. Drouin, J. Freyer, J. Arnason, G. Eberl, A. Hamann, H. Wagner, J. Huehn, and T. Sparwasser. 2007. Selective depletion of Foxp3⁺ regulatory T cells induces a scurfy-like disease. *J. Exp. Med.* 204: 57–63.
- Kim, J., J. Rasmussen, and A. Rudensky. 2007. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat. Immunol.* 8: 191–197.
- Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 14: 233–258.
- Boden, E., Q. Tang, H. Bour-Jordan, and J. A. Bluestone. 2003. The role of CD28 and CTLA4 in the function and homeostasis of CD4⁺CD25⁺ regulatory T cells. *Novartis Found. Symp.* 252: 55–63.
- Sansom, D. M., and L. S. Walker. 2006. The role of CD28 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) in regulatory T-cell biology. *Immunol. Rev.* 212: 131–148.
- Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147: 2461–2466.
- Salomon, B., and J. A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol.* 19: 225–252.
- Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-x_L. *Immunity* 3: 87–98.
- Tai, X., M. Cowan, L. Feigenbaum, and A. Singer. 2005. CD28 costimulation of developing thymocytes induces Foxp3 expression and regulatory T cell differentiation independently of interleukin 2. *Nat. Immunol.* 6: 152–162.
- Salomon, B., D. J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J. A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4⁺CD25⁺ immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12: 431–440.
- Tang, Q., K. J. Henriksen, E. K. Boden, A. J. Tooley, J. Ye, S. K. Subudhi, X. X. Zheng, T. B. Strom, and J. A. Bluestone. 2003. Cutting edge: CD28 controls peripheral homeostasis of CD4⁺CD25⁺ regulatory T cells. *J. Immunol.* 171: 3348–3352.
- Lohr, J., B. Knoechel, S. Jiang, A. H. Sharpe, and A. K. Abbas. 2003. The inhibitory function of B7 costimulators in T cell responses to foreign and self-antigens. *Nat. Immunol.* 4: 664–669.

31. Carreno, B. M., and M. Collins. 2002. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu. Rev. Immunol.* 20: 29–53.
32. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T. W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by CD25⁺CD4⁺ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J. Exp. Med.* 192: 303–310.
33. Khattri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for scurf in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 4: 337–342.
34. Lin, W., D. Haribhai, L. M. Relland, N. Truong, M. R. Carlson, C. B. Williams, and T. A. Chatila. 2007. Regulatory T cell development in the absence of functional Foxp3. *Nat. Immunol.* 8: 359–368.
35. Tang, Q., E. K. Boden, K. J. Henriksen, H. Bour-Jordan, M. Bi, and J. A. Bluestone. 2004. Distinct roles of CTLA-4 and TGF- β in CD4⁺CD25⁺ regulatory T cell function. *Eur. J. Immunol.* 34: 2996–3005.
36. Ariyan, C., P. Salvalaggio, S. Fecteau, S. Deng, L. Rogozinski, D. Mandelbrot, A. Sharpe, M. H. Sayegh, G. P. Basadonna, and D. M. Rothstein. 2003. Cutting edge: transplantation tolerance through enhanced CTLA-4 expression. *J. Immunol.* 171: 5673–5677.
37. Linsley, P. S., P. M. Wallace, J. Johnson, M. G. Gibson, J. L. Greene, J. A. Ledbetter, C. Singh, and M. A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science* 257: 792–795.
38. Kremer, J. M., R. Westhovens, M. Leon, E. Di Giorgio, R. Alten, S. Steinfield, A. Russell, M. Dougados, P. Emery, I. F. Nuamah, et al. 2003. Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N. Engl. J. Med.* 349: 1907–1915.
39. Ribas, A., L. H. Camacho, G. Lopez-Berestein, D. Pavlov, C. A. Bulanagui, R. Millham, B. Comin-Anduix, J. M. Reuben, E. Seja, C. A. Parker, et al. 2005. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J. Clin. Oncol.* 23: 8968–8977.
40. Bluestone, J. A., E. W. St Clair, and L. A. Turka. 2006. CTLA4Ig: bridging the basic immunology with clinical application. *Immunity* 24: 233–238.
41. Ndejemi, M. P., J. R. Teijaro, D. S. Patke, A. W. Bingaman, M. R. Chandok, A. Azimzadeh, S. G. Nadler, and D. L. Farber. 2006. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. *J. Immunol.* 177: 7698–7706.
42. Peggs, K. S., S. A. Quezada, A. J. Korman, and J. P. Allison. 2006. Principles and use of anti-CTLA4 antibody in human cancer immunotherapy. *Curr. Opin. Immunol.* 18: 206–213.
43. Ahmadszadeh, M., S. F. Hussain, and D. L. Farber. 1999. Effector CD4 T cells are biochemically distinct from the memory subset: evidence for long-term persistence of effectors in vivo. *J. Immunol.* 163: 3053–3063.
44. Fisson, S., G. Darrasse-Jeze, E. Litvinova, F. Septier, D. Klatzmann, R. Liblau, and B. L. Salomon. 2003. Continuous activation of autoreactive CD4⁺CD25⁺ regulatory T cells in the steady state. *J. Exp. Med.* 198: 737–746.
45. Gavin, M. A., S. R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of CD4⁺CD25⁺ suppressor T cells in vivo. *Nat. Immunol.* 3: 33–41.
46. Alegre, M. L., P. J. Noel, B. J. Eisfelder, E. Chuang, M. R. Clark, S. L. Reiner, and C. B. Thompson. 1996. Regulation of surface and intracellular expression of CTLA4 on mouse T cells. *J. Immunol.* 157: 4762–4770.
47. Hsieh, C. S., Y. Liang, A. J. Tyznik, S. G. Self, D. Liggitt, and A. Y. Rudensky. 2004. Recognition of the peripheral self by naturally arising CD25⁺ CD4⁺ T cell receptors. *Immunity* 21: 267–277.
48. Cozzo, C., J. Larkin, 3rd, and A. J. Caton. 2003. Cutting edge: self-peptides drive the peripheral expansion of CD4⁺CD25⁺ regulatory T cells. *J. Immunol.* 171: 5678–5682.
49. Atabani, S. F., C. L. Thio, S. Divanovic, A. Trompette, Y. Belkaid, D. L. Thomas, and C. L. Karp. 2005. Association of CTLA4 polymorphism with regulatory T cell frequency. *Eur. J. Immunol.* 35: 2157–2162.
50. Baliga, P., K. D. Chavin, L. Qin, J. Woodward, J. Lin, P. S. Linsley, and J. S. Bromberg. 1994. CTLA4Ig prolongs allograft survival while suppressing cell-mediated immunity. *Transplantation* 58: 1082–1090.
51. Levisetti, M. G., P. A. Padrid, G. L. Szot, N. Mittal, S. M. Meehan, C. L. Wardrip, G. S. Gray, D. S. Bruce, J. R. Thistlethwaite, Jr., and J. A. Bluestone. 1997. Immunosuppressive effects of human CTLA4Ig in a non-human primate model of allogeneic pancreatic islet transplantation. *J. Immunol.* 159: 5187–5191.
52. Leach, D. R., M. F. Krummel, and J. P. Allison. 1996. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271: 1734–1736.
53. Sotomayor, E. M., I. Borrello, E. Tubb, J. P. Allison, and H. I. Levitsky. 1999. In vivo blockade of CTLA-4 enhances the priming of responsive T cells but fails to prevent the induction of tumor antigen-specific tolerance. *Proc. Natl. Acad. Sci. USA* 96: 11476–11481.
54. Quezada, S. A., K. S. Peggs, M. A. Curran, and J. P. Allison. 2006. CTLA4 blockade and GM-CSF combination immunotherapy alters the intratumor balance of effector and regulatory T cells. *J. Clin. Invest.* 116: 1935–1945.