The Journal of Immunology
RESEARCH ARTICLE | JULY 15 2004

Resistance to $CD4^+CD25^+$ Regulatory T Cells and TGF-β in Cbl-$b^{-/-}$ Mice FREE

Elizabeth A. Wohlfert; ... et. al

J Immunol (2004) 173 (2): 1059–1065.
https://doi.org/10.4049/jimmunol.173.2.1059

Resistance to $CD4^+CD25^+$ Regulatory T Cells and TGF-β in Cbl-$b^{-/-}$ Mice

Elizabeth A. Wohlfert,* Margaret K. Callahan,† and Robert B. Clark[1]*

Cbl-$b^{-/-}$ mice have signaling defects that result in CD28-independent T cell activation, increased IL-2 production, hyper-reactive T cells, and increased autoimmunity. Although the increased autoimmunity in these mice is believed to result from the hyper-reactive T cells, the mechanisms leading from T cell hyper-reactivity to autoimmunity remain unclear. Specifically, the function and interaction of $CD4^+CD25^+$ regulatory T cells (T_{reg}) and $CD4^+CD25^-$ effector T cells (T_{eff}) in Cbl-$b^{-/-}$ mice have not been examined. We now report that Cbl-$b^{-/-}$ $CD4^+CD25^+$ T_{reg} exhibit normal regulatory function in vitro. In contrast, the in vitro response of Cbl-$b^{-/-}$ $CD4^+CD25^-$ T_{eff} is abnormal, in that it is not inhibited by either Cbl-$b^{-/-}$ or wild-type T_{reg}. This resistance of Cbl-$b^{-/-}$ T_{eff} to in vitro regulation is seen at the levels of both DNA synthesis and cell division. In addition to this resistance to $CD4^+CD25^+$ T_{reg}, Cbl-$b^{-/-}$ T_{eff} demonstrate in vitro resistance to inhibition by TGF-β. This second form of resistance in Cbl-$b^{-/-}$ T_{eff} is seen despite the expression of normal levels of type II TGF-β receptors and normal levels of phosphorylated Smad3 after TGF-β stimulation. Coupled with recent reports of resistance to T_{reg} in T_{eff} exposed to LPS-treated dendritic cells, our present findings suggest that resistance to regulation may be a relevant mechanism in both normal immune function and autoimmunity. ***The Journal of Immunology,*** **2004, 173: 1059–1065.**

Optimal T cell activation requires signaling through both the TCR and the CD28 costimulatory receptor. CD28 costimulation is believed to set the threshold for T cell activation. Cbl-b, a member of the Cbl/Sli family of molecular adaptors and a ubiquitin ligase, has been shown to negatively regulate CD28-dependent T cell activation (1, 2). Recent studies using gene-targeted mice lacking Cbl-b (Cbl-$b^{-/-}$ mice) have demonstrated the importance of this molecule in both T cell activation and the development of autoimmunity (1, 2). Cbl-$b^{-/-}$ T cells display increased proliferation after TCR stimulation and produce increased amounts of IL-2, but not IFN-γ or TNF-α (1). Cbl-$b^{-/-}$ mice have T cells that are independent of CD28 costimulation, in that they do not require CD28 engagement for IL-2 production and proliferation (1, 2).

Bachmaier et al. (1) have demonstrated that Cbl-$b^{-/-}$ mice develop spontaneous autoimmunity characterized by autoantibody production, infiltration of activated T and B lymphocytes into multiple organs, and parenchymal damage. Chiang et al. (2) have demonstrated that Cbl-$b^{-/-}$ mice are highly susceptible to experimental autoimmune encephalomyelitis. The increased autoimmunity found in Cbl-$b^{-/-}$ mice is believed to be a product of the signaling abnormalities, the CD28 independence, and the resultant T cell hyper-reactivity. However, the mechanisms leading from T cell hyper-reactivity to autoimmunity remain unclear. In the present study we have asked whether the signaling abnormalities in Cbl-$b^{-/-}$ T cells could play a role in the increased autoimmunity in Cbl-$b^{-/-}$ mice by affecting the interactions of Cbl-$b^{-/-}$ regulatory T cells (T_{reg})[2] and Cbl-$b^{-/-}$ effector T cells (T_{eff}). Although $CD4^+CD25^+$ T_{reg} have been shown to suppress $CD4^+CD25^-$ T_{eff} and $CD8^+$ T cells in vitro and to play a role in the prevention of autoimmunity in vivo (3–9), the function and interaction of T_{reg} and T_{eff} have not been examined in Cbl-$b^{-/-}$mice.

Tang et al. (10) have recently demonstrated that CD28 controls both the survival and proliferation of T_{reg} in vivo. In addition, T_{reg} derived from TCR-transgenic mice have been found to be capable of proliferating in response to specific Ags, but this proliferation is dependent on dendritic cells and B7 costimulation (11). Thus, in addition to the fact that Cbl-$b^{-/-}$ mice are prone to autoimmunity, the relevance of CD28-B7 interactions in the normal physiology of T_{reg} suggested that $CD4^+$ T cell regulatory interactions could be abnormal in CD28-independent Cbl-$b^{-/-}$ mice.

In the present studies we have examined for the first time the status of $CD4^+$ T cell regulatory interactions in vitro in Cbl-$b^{-/-}$ mice. We now report that $CD4^+CD25^+$ T_{reg} from Cbl-$b^{-/-}$ mice function normally in vitro in suppressing wild-type (WT) $CD4^+CD25^-$ T_{eff}. However, we have found that $CD4^+CD25^-$ T_{eff} from Cbl-$b^{-/-}$ mice are resistant to regulation by both Cbl-$b^{-/-}$ and WT T_{reg}. In addition, Cbl-$b^{-/-}$ $CD4^+CD25^-$ T_{eff} are resistant to regulation by exogenously added TGF-β. This resistance of T_{eff} to inhibition by T_{reg} and TGF-β may help explain the increased autoimmunity seen in Cbl-$b^{-/-}$ mice. Furthermore, in light of similar findings in both aged NOD mice and T_{eff} exposed to LPS-treated dendritic cells, our present findings suggest that resistance to regulation may be a relevant mechanism in both normal immune function and autoimmunity (12, 13).

Materials and Methods

Mice

C57BL/6 (WT) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Cbl-$b^{-/-}$ mice on a C57BL/6 background (a gift from Dr. H. Gu (National Institutes of Health, Bethesda, MD) were bred in our facilities

*Department of Medicine, Division of Rheumatic Diseases, and †Center of Immunotherapy for Cancer and Infectious Diseases, University of Connecticut Health Center, Farmington, CT 06030

Received for publication January 20, 2004. Accepted for publication May 14, 2004.

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] Address correspondence and reprint requests to Dr. Robert B. Clark, Room L6032, Department of Medicine, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030. E-mail: rclark@nso2.uchc.edu

[2] Abbreviations used in this paper: T_{reg}, regulatory T cell; Tds, T cell-depleted splenocyte; T_{eff}, effector T cell; TGF-βRII, TGF-β receptor type II; WT, wild type.

0022-1767/04/$02.00

under specific pathogen-free conditions. All animals were used between the ages of 6 and 10 wk.

Reagents

The following Abs/reagents were used: from Miltenyi Biotec (Auburn, CA): anti-CD25-PE (clone 7D4) and anti-PE microbeads; and from BD Pharmingen (San Diego, CA): anti-CD25-PE (clone PC61), anti-CD4-FITC (clone GK1.5), anti-CD8α (clone 5H10-1), anti-CD24 (clone J11d), anti-CD3ϵ (clone 145-2C11), anti-CD4 PerCP (RM4-5), anti-CD45RB, and CD62L. Affinity-purified goat anti-rat IgG (Kierkegaard & Perry, Keene, NH) was used for panning. Human recombinant TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Cell purification

Splenocytes from WT and Cbl-b$^{-/-}$ mice were stained with anti-CD8α and anti-CD24 Abs and plated onto petri dishes (27×10^6/petri dish) coated with goat anti-rat IgG Ab (25 μg/ml). After 1.5 h at 4°C, CD8α^+ T cells and CD24$^+$ cells were depleted by panning, and the CD4$^+$-enriched population was separated on magnetic columns (MS MACS separation column; Miltenyi Biotec) after staining with anti-CD25-PE, followed by anti-PE-conjugated microbeads.

In vitro proliferation assays

CD4$^+$CD25$^-$ T_{eff} (5×10^4), 5×10^4 irradiated (2600 rad) WT splenocytes, and various numbers of CD4$^{+/+}$CD25$^{+/+}$ T_{reg} were plated in round-bottom, 96-well plates in RPMI 1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME. Cultures were stimulated with varying doses of soluble anti-CD3 Ab for 48 h, and [^{3}H]thymidine was added for the last 6 h. Cultures were harvested using a semiautomated cell harvester and assayed using a beta scintillation counter. To derive T cell-depleted splenocytes, Cbl-b$^{-/-}$ splenocytes were stained with anti-CD4 and anti-CD8 microbeads. This population was then negatively selected on magnetic columns, and the negatively selected cells were used as a source of T cell-depleted APCs. This population routinely consisted of <5% T cells (data not shown).

CFSE staining

Freshly isolated CD4$^+$CD25$^-$ WT and Cbl-b$^{-/-}$ T_{eff} (10×10^6cells/ml) were labeled with 2.5 μM CFSE for 10 min at 37°C and subsequently washed once in 20% FCS-PBS and twice in RPMI 1640 with 10% FCS. CFSE-labeled Cbl-b$^{-/-}$ T_{eff} and CFSE-labeled WT T_{eff} were cultured with irradiated T cell-depleted WT splenocytes (as APCs) and 0.5 μg/ml soluble anti-CD3 Ab with or without T_{reg} for the time periods indicated. After culture, cells were stained with PerCP-anti-CD4 (BD Pharmingen; RM4-5), washed, and fixed for subsequent FACS analysis.

Stimulation of T_{eff} with TGF-β

Freshly isolated CD4$^+$CD25$^-$ T_{eff} (5×10^6) were plated in 24-well plates in 1 ml of RPMI 1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME. Cultures were stimulated with or without TGF-β1 (5 ng/ml) for 30 min and harvested, and lysates were prepared for Western blots.

Western blots

Samples were prepared by lysing 5×10^6 purified cell populations in lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.5% octyl glucoside, and 4 mM Pefabloc SC (Sigma-Aldrich, St. Louis, MO); for the Smad3 studies, 1 mM NaF, 1 mM $NaVO_4$, and 50 mM β-glycerophosphate were also added), followed by centrifugation at 14,000 rpm to remove debris. The protein content of the lysates was quantified by the Bradford method using the Bio-Rad protein assay (Hercules, CA). The indicated quantities of lysates were denatured by boiling 5 min in sample buffer, resolved by reducing SDS-PAGE (10% (w/v) acrylamide), and electrophoretically transferred to an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad). Membranes were blocked for 1 h in blocking buffer (PBS with 5% dehydrated milk) and probed with anti-TGF-β receptor type II (anti-TGF-βRII) Ab (sc-17799; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1/500 in blocking buffer, followed by incubation with HRP-conjugated goat anti-mouse secondary Ab (170-6516; Bio-Rad) at a dilution of 1/2000 in blocking buffer. Smad3 was detected with anti-Smad3 Ab (sc-6202; Santa Cruz Biotechnology), followed by HRP-conjugated anti-goat secondary Ab. For p-Smad3, membranes were blocked for 1 h in TBS with Tween 20 with 5% dehydrated milk, then probed with anti-phospho-Smad3 Ab (gift from Dr. E. Loef, Mayo Clinic, Rochester, MN), followed by incubation with HRP-conjugated goat anti-rabbit secondary Ab (170-6515; Bio-Rad). Alternatively, membranes were stripped and probed with anti-β-actin Ab (A-2066; Sigma-Aldrich) at a dilution of 1/1000, followed by incubation with HRP-conjugated goat anti-rabbit secondary Ab (170-6515; Bio-Rad) at a dilution of 1/2000 in blocking buffer. Bands were visualized using ECL substrate (Amersham Pharmacia Biotech, Arlington Heights, IL) detected by BioMax LS film (Eastman Kodak, Rochester, NY) and developed on an M35A X-OMAT film processor (Eastman Kodak).

Results

T_{reg} function in Cbl-b$^{-/-}$ and WT mice

Our first goal in these studies was to characterize CD4$^+$CD25$^+$ T_{reg} cells derived from Cbl-b$^{-/-}$ mice. We isolated CD4$^+$CD25$^+$ T_{reg} and CD4$^+$CD25$^-$ T_{eff} from the spleens of WT C57BL/6 mice and Cbl-b$^{-/-}$ mice. The T_{reg} populations isolated were usually >95% pure, and the T_{eff} populations isolated were usually 88–95% pure, as assessed by FACS analysis. To more completely characterize the T_{eff} populations used, we analyzed the expression of CD45RB and CD62L as well as the expression of CD25 within the T_{eff} populations derived from WT and Cbl-b$^{-/-}$ mice. Our WT and Cbl-b$^{-/-}$ T_{eff} populations were similar not only in CD25 expression, but also in CD45RB and CD62L expression (Fig. 1, *A–C*). These results are consistent with those reported by Bachmaier et al. (1) and indicate that the T_{eff} populations derived from Cbl-b$^{-/-}$ mice did not consist of a greater proportion of activated or memory cells than the T_{eff} populations derived from WT mice. Also shown in Fig. 1*D* is the similarity of CD25 expression of the WT and Cbl-b$^{-/-}$-derived T_{reg} populations. The number of T_{reg} isolated from WT mice was ~0.35–0.40 $\times$ 10^6/spleen, and the number of T_{reg} isolated from Cbl-b$^{-/-}$ mice was ~0.40–0.55 $\times$ 10^6/spleen.

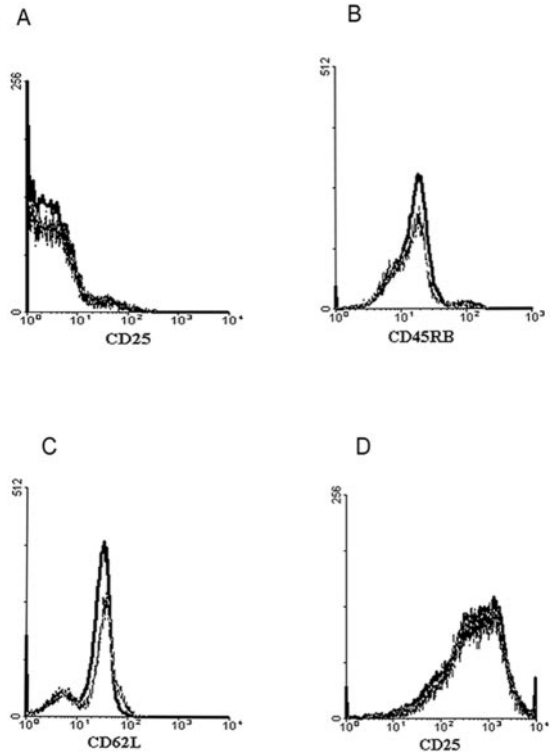

FIGURE 1. T_{eff} and T_{reg} were derived from WT or Cbl-b$^{-/-}$ splenocytes as described in *Materials and Methods*. Using FACS analysis gated on CD4$^+$ cells, T_{eff} derived from WT mice (solid line) or from Cbl-b$^{-/-}$ mice (broken line) were analyzed for the expression of CD25 (*A*), CD45RB (*B*), or CD62L (*C*). *D*, Also using FACS analysis gated on CD4$^+$ cells, T_{reg} derived from WT mice (solid line) or from Cbl-b $^{-/-}$ mice (broken line) were analyzed for the expression of CD25.

WT and Cbl-b$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} and CD4$^+$CD25$^-$ T_{eff} were tested for their ability to respond and/or suppress after stimulation in vitro with soluble anti-CD3 Ab. In confirmation of previous reports, WT T_{reg} showed no significant proliferation in response to anti-CD3 Ab using either 0.5 or 2 μg/ml anti-CD3 Ab. As expected, the WT T_{reg} were capable of suppressing the anti-CD3 Ab-stimulated response of WT T_{eff} using either 0.5 or 2 μg/ml anti-CD3 Ab (Fig. 2, *A* and *B*). Cbl-b$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} also showed no significant proliferation in response to anti-CD3 Ab using either 0.5 or 2 μg/ml anti-CD3 Ab. However, in contrast to WT cultures, we found that the Cbl-b$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} were incapable of suppressing the response of Cbl-b$^{-/-}$ CD4$^+$ CD25$^-$ T_{eff} at both 0.5 and 2 μg/ml anti-CD3 Ab stimulation (Fig. 2, *C* and *D*). On occasion, the responses of Cbl-b$^{-/-}$ T_{eff} were actually increased in cocultures with Cbl-b$^{-/-}$ CD4$^+$CD25$^+$ T_{reg}.

T_{reg} function in mixed WT/Cbl-b$^{-/-}$ cultures

To further assess the lack of regulation seen in cultures of Cbl-b$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} and CD4$^+$ CD25$^-$ T_{eff}, we next set up mixed cultures combining WT T_{eff} with Cbl-b$^{-/-}$ T_{reg} and Cbl-b$^{-/-}$ T_{eff} with WT T_{reg}. We found that Cbl-b$^{-/-}$ T_{reg} were fully capable of suppressing the anti-CD3 Ab-stimulated response of WT T_{eff}. This was true using either 0.5 or 2 μg/ml anti-CD3 Ab (Fig. 3, *A* and *B*). This suggests that Cbl-b$^{-/-}$ T_{reg} are fully capable of normal regulatory function. In contrast, WT T_{reg}, which were capable of suppressing WT T_{eff}, were not capable of suppressing the response of Cbl-b$^{-/-}$ T_{eff} using either 0.5 or 2 μg/ml anti-CD3 Ab (Fig. 3, *C* and *D*). Again, the Cbl-b$^{-/-}$ T_{eff} response was often slightly increased when cells were cocultured with WT

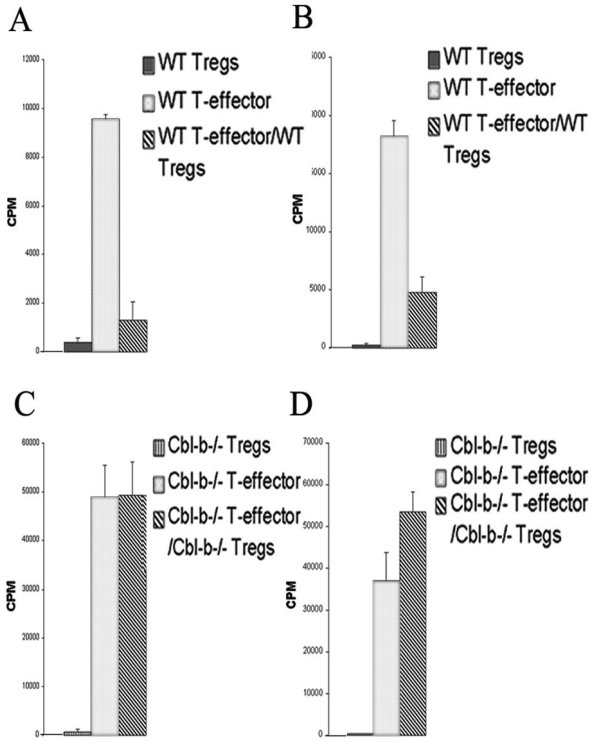

FIGURE 2. Regulation of T_{eff} responses using differing anti-CD3 Ab concentrations. WT T_{eff} with or without WT T_{reg} (*A* and *B*) or Cbl-b$^{-/-}$ T_{eff} with or without Cbl-b$^{-/-}$ T_{reg} (*C* and *D*), each at 5×10^4 /well, were cultured in duplicate or triplicate wells along with 5×10^4 /well irradiated WT splenocytes and were stimulated for 48 h with 0.5 μg/ml (*A* and *C*) or 2 μg/ml (*B* and *D*) of anti-CD3 Ab. [^{3}H]thymidine was added for the final 6 h of culture. Results are expressed as the mean cpm. The results of wells not stimulated with anti-CD3 Ab were all <500 cpm and are not shown.

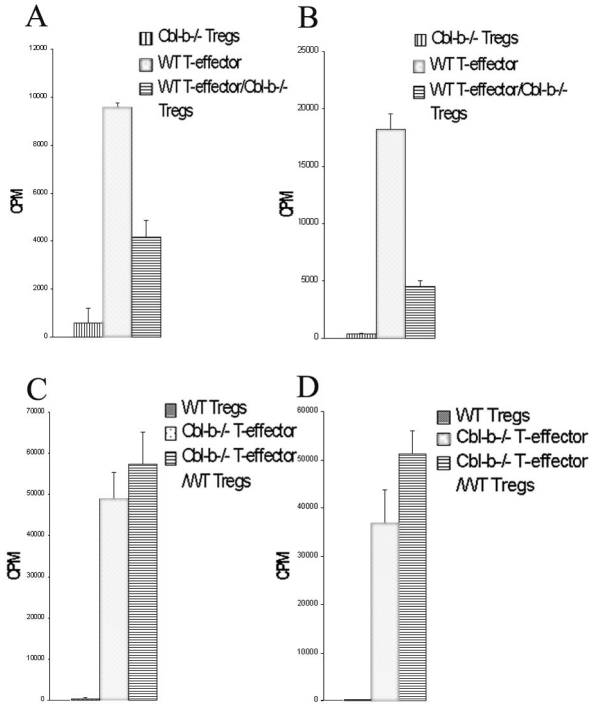

FIGURE 3. Regulation of T_{eff} responses using differing anti-CD3 Ab concentrations and cultured with mixed T_{reg}. WT T_{eff} with or without Cbl-b$^{-/-}$ T_{reg} (*A* and *B*) or Cbl-b$^{-/-}$ T_{eff} with or without WT T_{reg} (*C* and *D*), each at 5×10^4 /well, were cultured in duplicate or triplicate wells along with 5×10^4 /well irradiated WT splenocytes and stimulated for 48 h with 0.5 μg/ml (*A* and *C*) or 2 μg/ml (*B* and *D*) of anti-CD3 Ab. [^{3}H]thymidine was added for the final 6 h of culture. Results are expressed as the mean cpm. The results of wells not stimulated with anti-CD3 Ab were all <500 cpm and are not shown.

T_{reg}. These results suggest that although the in vitro regulatory function of Cbl-b$^{-/-}$ T_{reg} is normal, the ability of Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} to be suppressed by either WT or Cbl-b$^{-/-}$ T_{reg} is abnormal.

Response of Cbl-b$^{-/-}$ T_{eff} with varied T_{reg}:T_{eff} ratios

Having found an abnormality in the suppressibility of Cbl-b$^{-/-}$ T_{eff} at what is usually considered an optimal or supraoptimal T_{reg}:T_{eff} ratio of 1:1, we next tested the ability of WT and Cbl-b$^{-/-}$ T_{eff} to be suppressed at a lower and a higher ratio. At a ratio of 0.5:1, the response of WT T_{eff} was suppressed 62% by WT T_{reg}. In contrast, at this ratio, the response of Cbl-b$^{-/-}$ T_{eff} was not suppressed by Cbl-b$^{-/-}$ T_{reg} (Fig. 4). We then asked whether we could detect any suppression of the Cbl-b$^{-/-}$ T_{eff} response using the very high T_{reg}:T_{eff} ratio of 2:1. At a ratio of 2:1, the response of WT T_{eff} was suppressed 90% by WT T_{reg}. In contrast, at a 2:1 ratio, the response of Cbl-b$^{-/-}$ T_{eff} was only 34% suppressed by Cbl-b$^{-/-}$ T_{reg} (Fig. 4). Thus, even at the very high T_{reg}:T_{eff} ratio of 2:1, there was very little suppression of Cbl-b$^{-/-}$ T_{eff}.

Cbl-b$^{-/-}$ vs WT APCs

All of our prior studies were performed using WT-derived, irradiated, whole splenocytes as the APC population. We next wanted to examine the possibility that there might be functional differences between the WT and Cbl-b$^{-/-}$ APC populations that played a role in the resistance to regulation seen with Cbl-b$^{-/-}$ T_{eff}. In addition, we wanted to confirm our results using an APC population that was depleted of T cells. We, therefore, repeated our studies using irradiated, T cell-depleted (Tds), Cbl-b$^{-/-}$ splenocytes as

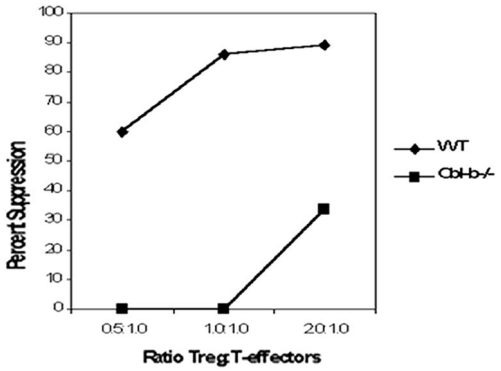

FIGURE 4. Regulation of WT and Cbl-b$^{-/-}$ T_{eff} responses at differing ratios of T_{reg}:T_{eff}. T_{eff}, at 5×10^4 /well, were cultured in duplicate or triplicate wells along with 5×10^4 /well irradiated WT splenocytes and were stimulated for 48 h with 0.5 μg/ml anti-CD3 Ab with or without varying numbers of T_{reg}. WT T_{eff} were cultured with or without WT T_{reg}, and Cbl-b$^{-/-}$ T_{eff} were cultured with or without Cbl-b$^{-/-}$ T_{reg}. [^{3}H]thymidine was added for the final 6 h of culture. % suppression = 100% − [response (mean cpm) T_{eff} cultured with T_{reg}/response of T_{eff} without T_{reg} × 100].

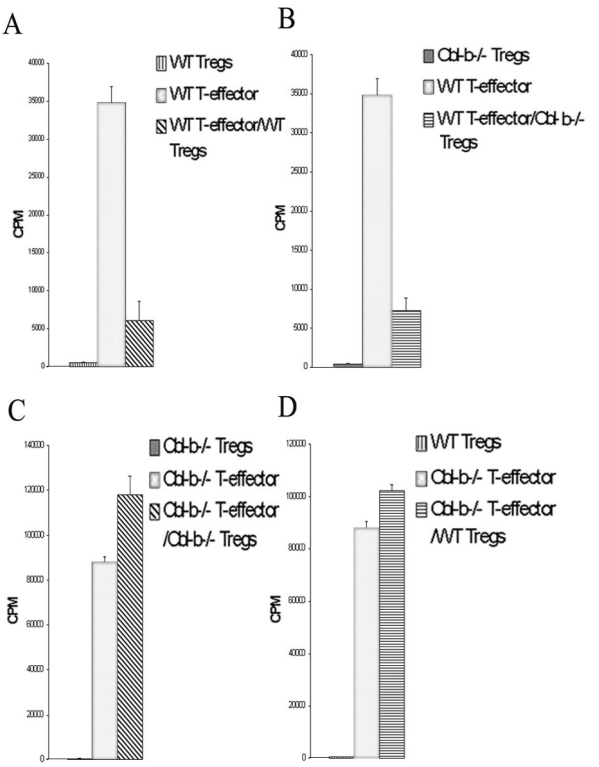

FIGURE 5. Regulation of WT and Cbl-b$^{-/-}$ T_{eff} responses using Cbl-b$^{-/-}$ Tds as APCs. Cbl-b$^{-/-}$ T_{reg}, WT T_{reg}, WT T_{eff}, Cbl-b$^{-/-}$ T_{eff} or combinations of these populations, each at 5×10^4 /well, were cultured in duplicate or triplicate wells along with 5×10^4 /well irradiated Cbl-b$^{-/-}$ Tds and stimulated for 48 h with 0.5 μg/ml anti-CD3 Ab. *A*, WT T_{eff} and WT T_{reg}; *B*, WT T_{eff} and Cbl-b$^{-/-}$ T_{reg}; *C*, Cbl-b$^{-/-}$ T_{eff} and Cbl-b$^{-/-}$ T_{reg}; *D*, Cbl-b$^{-/-}$ T_{eff} and WT T_{reg}. [^{3}H]thymidine was added for the final 6 h of culture. Results are expressed as the mean cpm. The results of wells not stimulated with anti-CD3 Ab were all <500 cpm and are not shown.

the APC population. Using these APCs, we found no significant difference in results from previous experiments. At a 1:1 T_{reg}:T_{eff} ratio and using 0.5 μg/ml anti-CD3 Ab for stimulation, the responses of WT T_{eff} were ~85% suppressed. This level of suppression was mediated by both WT T_{reg} and Cbl-b$^{-/-}$ T_{reg} (Fig. 5, *A* and *B*). However, the responses of Cbl-b$^{-/-}$ T_{eff} were unable to be suppressed by either WT T_{reg} or Cbl-b$^{-/-}$ T_{reg} (Fig. 5, *C* and *D*). These results suggest that the resistance to regulation of Cbl-b$^{-/-}$ T_{eff} is not related specifically to either WT or Cbl-b$^{-/-}$ APCs.

Evaluation of T_{reg} function using CFSE staining

To further evaluate the resistance to regulation seen in Cbl-b$^{-/-}$ T_{eff}, we next examined the T_{eff} responses at the level of cell division rather than at the level of DNA synthesis (i.e., [^{3}H]thymidine incorporation). WT and Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} were labeled with CFSE, stimulated with anti-CD3 Ab and APCs, and cultured with or without the addition of T_{reg}. In the FACS analyses used to evaluate these responses, we gated on CD4$^+$ T cells. As such, in those cultures to which T_{reg} were added, the T_{reg} were seen as a CSFE-negative population (Fig. 6, *B*, *D*, and *F*). The T_{eff} responses were examined at 48 and 60 h. In confirmation of our [^{3}H]thymidine incorporation studies, WT T_{reg} suppressed the anti-CD3 Ab-stimulated division of WT T_{eff}. This was seen as a decrease in the number of waves of division in the presence of T_{reg}. The results of a typical experiment for WT T_{eff}, harvested at 60 h, are shown in Fig. 6, *A* and *B* (data not shown for the 48 h WT T_{eff} responses). In contrast, Cbl-b$^{-/-}$ T_{reg} did not suppress anti-CD3 Ab-stimulated division of Cbl-b$^{-/-}$ T_{eff}. This was seen as either no decrease in the waves of division or even a slight increase in the progression through cell division of the CFSE-labeled Cbl-b$^{-/-}$ T_{eff}. The results of typical experiments for Cbl-b$^{-/-}$ T_{eff} are shown in Fig. 6, *C* and *D* (harvested at 48 h), and in Fig. 6, *E* and *F* (harvested at 60 h). These results suggest that the resistance to T_{reg} regulation of Cbl-b$^{-/-}$ T_{eff} occurs not only at the level of DNA synthesis, but also at the level of cell division.

Effect of exogenously added TGF-β on the response of T_{eff}

TGF-β has been demonstrated to be an important regulator of immune responses in vivo (14, 15). In addition, TGF-β has been postulated to be a mechanism of suppression used by T_{reg} (5, 16). To investigate the possibility that the resistance to regulation of Cbl-b$^{-/-}$ T_{eff} is a more general characteristic of these cells, we next examined the effect of exogenously added TGF-β on the anti-CD3 Ab-stimulated responses of WT and Cbl-b$^{-/-}$ T_{eff}. No T_{reg} were used in these assays. As shown in Fig. 7, *A* and *B*, 5 and 25 ng/ml TGF-β suppressed the anti-CD3 Ab response of WT T_{eff} by ~55–65%. In contrast, neither 5 nor 25 ng/ml TGF-β was able to significantly suppress the response of Cbl-b$^{-/-}$ T_{eff}. At 5 ng/ml TGF-β, the response of Cbl-b$^{-/-}$ T_{eff} actually increased (Fig. 7*C*). At 25 ng/ml TGF-β, the Cbl-b$^{-/-}$ T_{eff} response decreased, but only by ~11% (Fig. 7*D*). Ab to TGF-β reversed the effects of the added cytokine, confirming the specificity of the TGF-β effect (data not shown). Thus, Cbl-b$^{-/-}$ T_{eff} demonstrated resistance to regulation not only by both WT and Cbl-b$^{-/-}$ T_{reg}, but also by TGF-β. Together these findings of Cbl-b$^{-/-}$ T_{eff} resistance to both TGF-β-mediated suppression and regulation by T_{reg} suggest that the autoimmunity seen in Cbl-b$^{-/-}$ mice might be the result of numerous regulatory abnormalities associated with Cbl-b$^{-/-}$ T cells.

TGF-βRII expression by CD4$^+$CD25$^-$ T_{eff}

To further characterize the defect in suppression of Cbl-b$^{-/-}$ T_{eff} by TGF-β, we examined the expression of TGF-βRII by WT T_{eff} and Cbl-b$^{-/-}$ T_{eff}. Western blots were performed on lysates of freshly isolated, nonstimulated WT T_{eff} and Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff}. Using Ab specific for TGF-βRII, we examined two dilutions of lysates for both Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} (Fig.

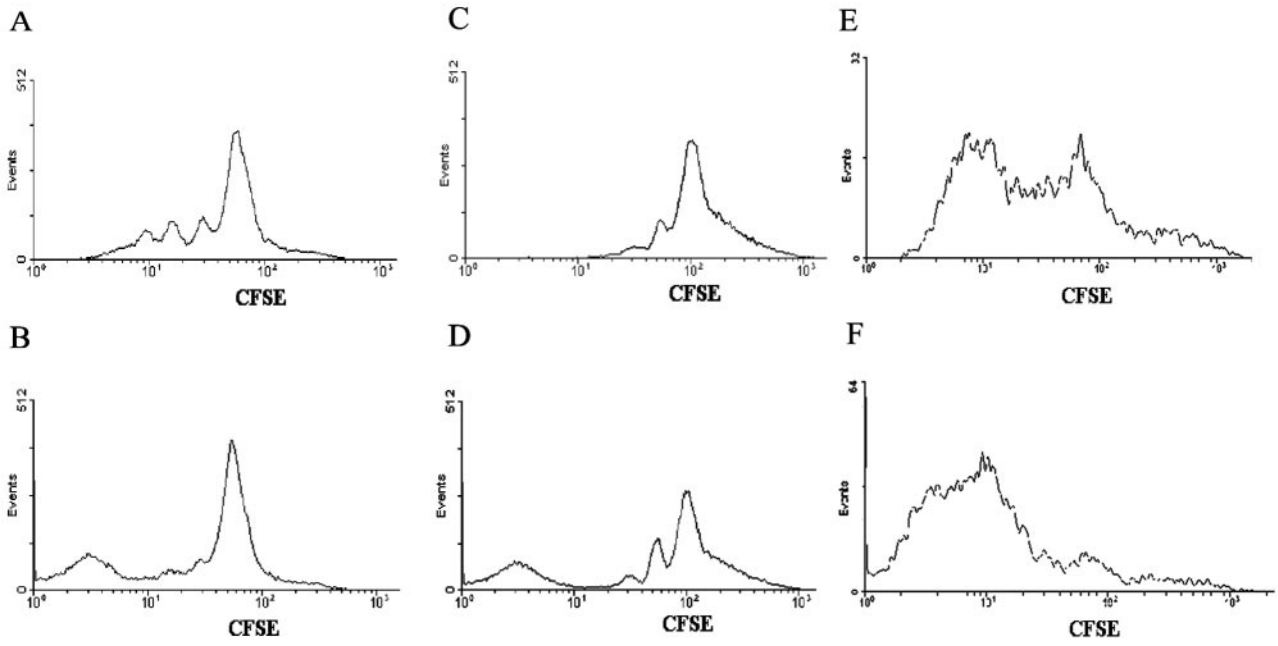

FIGURE 6. Response of CFSE-labeled T_{eff}. WT T_{eff} and Cbl-b$^{-/-}$ T_{eff} were labeled with CFSE and cultured (5×10^4 cells/well) with 5×10^4 cells/well WT Tds and 0.5 μg/ml anti-CD3 Ab with or without T_{reg} (2.5×10^4 cells/well). The FACS analyses, performed at the time points indicated, are gated on CD4$^+$ cells. *A*, WT T_{eff}, no T_{reg}, 60 h response; *B*, WT T_{eff} and WT T_{reg}, 60 h response; *C*, Cbl-b$^{-/-}$ T_{eff}, no T_{reg}, 48 h response; *D*, Cbl-b$^{-/-}$ T_{eff} and Cbl-b$^{-/-}$ T_{reg}, 48 h response; *E*, Cbl-b$^{-/-}$ T_{eff}, no T_{reg}, 60 h response; *F*, Cbl-b$^{-/-}$ T_{eff} and Cbl-b$^{-/-}$ T_{reg}, 60 h response.

8*A*, *lanes b* and *c*) and WT CD4$^+$CD25$^-$ T_{eff} (Fig. 8*A*, *lanes d* and *e*). We found that the levels of expression of TGF-βRII were not significantly different in Cbl-b$^{-/-}$ T_{eff} vs WT T_{eff} (Fig. 8*A*). These results suggest that the abnormality in TGF-β-mediated suppression of Cbl-b$^{-/-}$ T_{eff} is not the result of a decrease in expression of TGF-βRII.

Finally, we examined Smad3 phosphorylation in WT T_{eff} and Cbl-b$^{-/-}$ T_{eff} using Western blots. The ligation of TGF-βRII by TGF-β leads to the heterodimerization of TGF-βRII with TGF-βRI. This activated receptor complex then recruits Smad2 and Smad3, which are phosphorylated by TGF-βRI. The phosphorylated Smad2 and Smad3 moieties each then heterodimerize with Smad4, and these Smad complexes can translocate to the nucleus and activate transcriptional responses (17). We incubated WT and Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} with 5 ng/ml TGF-β for 30 min and then performed immunoblots on the cell lysates using Abs to both Smad3 and phosphorylated Smad3. We found that non-TGF-β-stimulated WT T_{eff} and Cbl-b$^{-/-}$ T_{eff} expressed equal levels of nonphosphorylated Smad3 (Fig. 8*B*). Furthermore, although TGF-β suppresses WT CD4$^+$CD25$^-$ T_{eff} proliferation, but does not suppress (and often enhances) Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} proliferation, we found that WT T_{eff} and Cbl-b$^{-/-}$ T_{eff} demonstrated an equal increase in phosphorylated Smad3 after incubation with TGF-β (Fig. 8*B*). Thus, our results suggest that the resistance of Cbl-b$^{-/-}$ T_{eff} to TGF-β-mediated suppression is the result of neither a decrease in expression of TGF-βRII nor an abnormality in the subsequent phosphorylation of Smad3, but may relate instead to abnormalities distal to these initial signaling events.

Discussion

Cbl-b, a ubiquitin ligase, acts as a type of gate-keeper in that it serves as a negative influence on T cell activation unless unleashed through CD28 stimulation (18). This unleashing occurs, at least in part, by CD28's ligation leading to Cbl-b ubiquitination and degradation (19). Two different laboratories have generated C57BL/6 mice that do not express Cbl-b (1, 2). In these mice the Cbl-b deficiency does not affect cellular development, but does lead to T cells that demonstrate increased proliferation and produce increased amounts of IL-2 after TCR stimulation (1, 2, 18). Chiang et al. (2) have shown that in Cbl-b$^{-/-}$ T cells, the main TCR signaling pathways are not affected, but Vav activation is significantly enhanced. Thus, mutation of Cbl-b uncouples T cell proliferation, IL-2 production, and phosphorylation of Vav1 from the requirement for CD28 costimulation, leading to CD28 independence of Cbl-b$^{-/-}$ T cell activation (1, 2).

Cbl-b$^{-/-}$ mice have been found to be prone to both spontaneous and induced autoimmune diseases (1, 2). The spontaneous autoimmunity involves the development of autoantibodies to dsDNA and massive infiltration in multiple organs (1). The induced autoimmunity involves an increased susceptibility to the induction of experimental autoimmune encephalomyelitis (2). In addition, a mutation in the *Cbl-b* gene has been shown to be associated with a spontaneous model of diabetes in the rat (20). Although the CD28 independence, the decreased threshold for stimulation, and the T cell hyper-reactivity are postulated to be responsible for the increased autoimmunity in Cbl-b$^{-/-}$ mice, the actual mechanisms underlying the increased autoimmunity are as yet unclear.

The mechanisms of suppression via CD4$^+$CD25$^+$ T_{reg} have yet to be definitively identified (9). Unresolved issues include questions about the cytokines and stimulatory factors required for the development and function of these cells and the nature of their Ag specificity. Shevach et al. (7, 9) have demonstrated that T_{reg} require stimulation through their TCR to mediate suppressive function and that T_{reg} suppress IL-2 production by T_{eff}. This suppression is noted at both the IL-2 mRNA and protein levels (7, 9). These CD4$^+$CD25$^+$ T_{reg} appear to have significant in vivo functional relevance, as evidenced by their ability to prevent certain autoimmune syndromes and their regulatory role in normal immune responses (3, 4, 9, 21, 22). The suppressive function of T_{reg} has also been demonstrated in vitro, where they have been found to suppress both anti-CD3 Ab-stimulated and Ag-specific responses of CD4$^+$ and CD8$^+$ T cells (8, 9).

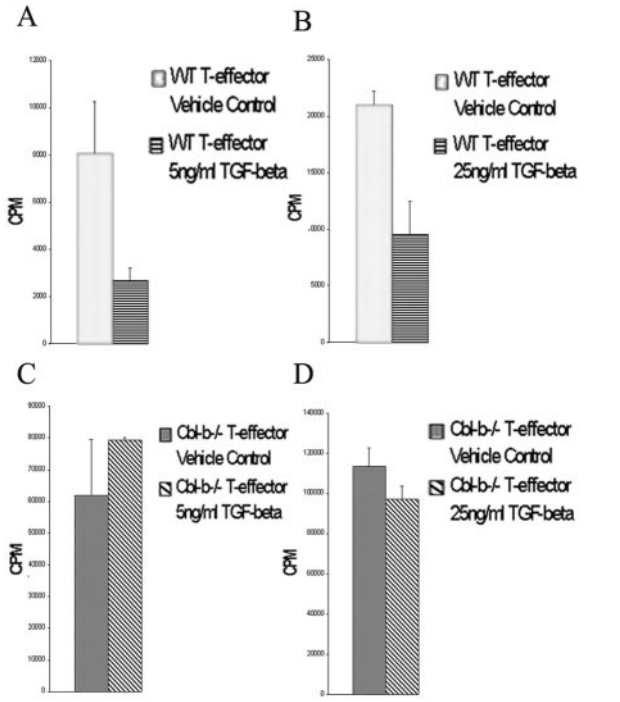

FIGURE 7. Effect of TGF-β on the responses of WT and Cbl-b$^{-/-}$ T_{eff}. WT T_{eff} (5×10^4 /well; *A* and *B)* and Cbl-b$^{-/-}$ T_{eff} (5×10^4 /well; *C* and *D)* were cultured in triplicate wells along with 5×10^4 /well irradiated WT splenocytes and stimulated for 48 h with 0.5 μg/ml anti-CD3 Ab in the absence of T_{reg}. 5 ng/ml (*A* and *C)* or 25 ng/ml (*B* and *D)* of human recombinant TGF-β1 or the appropriate concentration of vehicle control were added to the wells at the initiation of culture. [^{3}H]thymidine was added for the final 6 h of culture. Results are expressed as the mean cpm. The results of wells not stimulated with anti-CD3 Ab were all <500 cpm and are not shown.

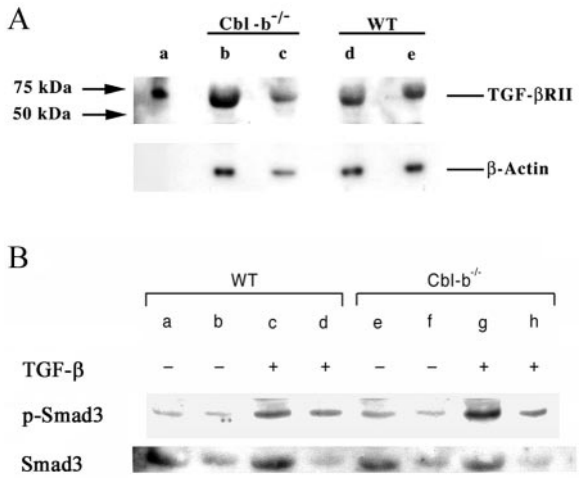

FIGURE 8. *A*, Western blots of TGF-βRII expression in T_{eff}. Lysates were prepared from purified WT and Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} populations directly ex vivo. These lysates, at two different protein concentrations, were then analyzed for TGF-βRII expression using immunoblotting. *Lane a*, Positive control: H-567 TGF-βRII; *lane b*, Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} lysate (20.7 μg of protein); *lane c*, Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} lysate (6.9 μg of protein); *lane d*, WT CD4$^+$CD25$^-$ T_{eff} lysate (20.7 μg of protein); *lane e*, WT CD4$^+$CD25$^-$ T_{eff} lysate (6.9 μg of protein). The expression of β-actin is shown as a loading control for *lane a* through *lane e*. *B*, Western blots of Smad3 and p-Smad3 expression in T_{eff}. Lysates were prepared from purified WT and Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} populations cultured ex vivo for 30 min in the presence or the absence of 5 ng/ml TGF-β. These lysates, at two different protein concentrations, were then analyzed for Smad3 (~55–60 kDa) and p-Smad3 (~52–56 kDa) expression using immunoblotting. *Lanes a* and *c*, WT CD4$^+$CD25$^-$ T_{eff} lysate (48 μg of protein); *lanes b* and *d*, WT CD4$^+$CD25- T_{eff} lysate (24 μg of protein); *lanes e* and *g*, Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} lysate (48 μg of protein); *lanes f* and *h*, Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} lysate (24 μg of protein).

CD4$^+$CD25$^+$ T_{reg} have been shown to depend on CD28 for their development, homeostatic proliferation, and Ag-driven proliferation (4, 10, 11). CD28$^{-/-}$ NOD mice have been demonstrated to develop exacerbated diabetes because of the lack of CD4$^+$CD25$^+$ T_{reg} development (4). Tang et al. (10) have recently shown that CD28 controls both the survival and the proliferation of T_{reg} in vivo. In addition, T_{reg} derived from TCR-transgenic mice have been found to be capable of proliferating in response to specific Ags, but this proliferation is dependent on dendritic cells and B7 costimulation (11). Thus, given the relevance of CD28-B7 interactions in the normal physiology of T_{reg}, the CD28 independence of Cbl-b$^{-/-}$ T cells and the increased proclivity of Cbl-b$^{-/-}$ mice to autoimmunity suggested that CD4$^+$ T cell regulatory interactions could be abnormal in these mice.

We now report that the function of Cbl-b$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} is essentially identical with that of T_{reg} derived from WT mice. However, the ability of Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T_{eff} to be regulated is significantly different from that of T_{eff} derived from WT mice. Specifically, in the in vitro response to soluble anti-CD3 Ab, Cbl-b$^{-/-}$ T_{eff}, compared with WT T_{eff}, are significantly less able to be suppressed by either Cbl-b$^{-/-}$ or WT T_{reg}. Using [^{3}H]thymidine incorporation proliferation assays, this resistance to regulation of Cbl-b$^{-/-}$ T_{eff} is seen over various ratios of T_{reg}:T_{eff}. In addition, this resistance is found when the strength of activating signal is varied by stimulation with either 0.5 or 2 μg/ml anti-CD3 Ab and when either WT or Cbl-b$^{-/-}$ APCs are used in the assays. Finally, using CFSE labeling, we confirmed that the resistance to T_{reg} regulation of Cbl-b$^{-/-}$ T_{eff} occurs not only at the level of DNA synthesis, but also at the level of cell division.

To date, almost all studies of CD4$^+$ regulatory function have focused on the T_{reg} populations rather than on the regulated T_{eff}. However, our present results of T_{eff} resistance to regulation are suggestive of two other recently described findings (12, 13). In the first, a decrease in suppressibility of murine CD4$^+$CD25$^-$ T_{eff} has been found in conjunction with LPS-activated dendritic cells (13). Such LPS-activated dendritic cells have been found to mediate a decrease in CD4$^+$CD25$^-$ T_{eff} suppressibility through the secretion of IL-6 and an as yet unidentified cytokine (13). At a 1:1 ratio, T_{reg}-mediated suppression has been shown to decrease from ~85% with normal dendritic cells to ~25% when LPS-treated dendritic cells were used (13). These results are similar to ours; at a 1:1 ratio we have found 85% suppression of the WT response and no suppression of the Cbl-b$^{-/-}$ T_{eff} response. In a second relevant study, Gregori et al. (12) have reported age-related changes in both CD4$^+$CD25$^+$ T_{reg} and CD4$^+$CD25$^-$ T_{eff} in NOD mice. These authors have found that as autoimmune diabetes progresses, the CD4$^+$CD25$^-$ pathogenic (effector) T cells become progressively less sensitive to immunoregulation by CD4$^+$CD25$^+$ T_{reg} (12). These results in NOD mice suggest that our present findings may be applicable to various autoimmune states in addition to that seen in Cbl-b$^{-/-}$ mice.

The mechanisms underlying our findings of Cbl-b$^{-/-}$ T_{eff} resistance to regulation are as yet unclear. There is evidence suggesting that the strength of the activating signal may play a role in determining the suppressibility of T_{eff} (23, 24). These studies have demonstrated that stronger activating signals are associated with a decrease in the suppressibility of CD4$^+$CD25$^-$ T_{eff}. Baecher-Allen et al. (23) have demonstrated a correlation between the in

vitro strength of signal of activation and the ability of human $CD4^+CD25^-$ T_{eff} to be suppressed by T_{reg} in vitro. In these studies the stronger activating signals are associated with a decrease in suppressibility of T_{eff} (23). The stronger activating signals are also associated with a decrease in the suppressor function of T_{reg} (23). Although we did not examine this issue of T_{reg} function specifically, in general, our findings indicate that WT T_{reg} and Cbl-$b^{-/-}$ T_{reg} function with equal efficiency. We believe that it is possible that the resistance to regulation described now for Cbl-$b^{-/-}$ T_{eff} results from the fact that the hyper-reactivity of Cbl-$b^{-/-}$ T cells is equivalent to a state of increased strength of activating signal for any given stimulus. However, the resistance to regulation of Cbl-$b^{-/-}$ T_{eff} is not simply related to differences in absolute levels of proliferation between Cbl-$b^{-/-}$ and WT T_{eff}. This can be seen in our results, where Cbl-$b^{-/-}$ T_{eff} are not suppressible when proliferating at a level of ~38,000 cpm of [^{3}H]thymidine incorporation (Fig. 3*D*), whereas WT T_{eff} are highly suppressible at a similar level of proliferation (Fig. 5, *A* and *B*).

Given the finding of resistance to T_{reg} regulation, we also examined whether Cbl-$b^{-/-}$ $CD4^+CD25^-$ T_{eff} are resistant to other forms of immunoregulation. It has previously been reported that Cbl-$b^{-/-}$ T cells are suppressed normally by CTLA-4 (2). Suppression by IL-10 has been reported to be mediated through down-regulation of B7 expression on APCs and also through alteration of the CD28 costimulation pathway in T cells (25, 26). The CD28 dependence of these mechanisms suggested that Cbl-$b^{-/-}$ T_{eff} are unlikely to be suppressible by IL-10. We therefore examined the suppressibility of Cbl-$b^{-/-}$ T_{eff} by TGF-β. TGF-β plays a role in immunoregulation, and there are reports suggesting that cell-bound TGF-β is important in the suppression mediated by T_{reg} (5, 14–16). TGF-β inhibits T cell proliferation and function at many different levels, including the production of IL-2 (27). We now report that Cbl-$b^{-/-}$ T_{eff} are also significantly less suppressed by exogenously added TGF-β than are WT T_{eff}. To further understand this TGF-β resistance, we first characterized the expression of TGF-βRII by freshly isolated T_{eff}. We found that the expression of TGF-βRII did not significantly differ between WT and Cbl-$b^{-/-}$ T_{eff}. We then examined Smad3 phosphorylation in WT T_{eff} and Cbl-$b^{-/-}$ T_{eff} after incubation with TGF-β. Although TGF-β suppresses WT $CD4^+CD25^-$ T_{eff} proliferation, but does not suppress (and often enhances) Cbl-$b^{-/-}$ $CD4^+CD25^-$ T_{eff} proliferation, we found that WT T_{eff} and Cbl-$b^{-/-}$ T_{eff} demonstrated an equal increase in phosphorylated Smad3 after incubation with TGF-β (Fig. 8*B*). This suggests that the resistance of Cbl-$b^{-/-}$ T_{eff} to TGF-β-mediated suppression is the result of neither a decrease in the expression of TGF-βRII nor an abnormality in the subsequent phosphorylation of Smad3, but may relate instead to abnormalities distal to these initial signaling events. Future studies in our laboratory will involve identifying these more distal TGF-β signaling alterations in Cbl-$b^{-/-}$ T cells.

The findings of resistance to TGF-β-mediated suppression together with the resistance to regulation by T_{reg} suggest that the autoimmunity seen in Cbl-$b^{-/-}$ mice might be the result of numerous regulatory abnormalities associated with Cbl-$b^{-/-}$ T cells. Given recent reports of similar resistance to T_{reg} regulation in aged NOD mice and in T_{eff} exposed to LPS-treated dendritic cells (12, 13), resistance to regulation may be a relevant mechanism in both normal immune function and autoimmunity. Future studies in our laboratory will attempt to confirm these immunoregulatory abnormalities in Cbl-$b^{-/-}$ mice using in vivo approaches.

Acknowledgments

We thank Dr. Ethan Shevach, National Institutes of Health, for his critical reading of this manuscript and his helpful comments.

References

1. Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y. Y. Kong, T. Sasaki, A. Oliveira-dos Santos, S. Marlathasan, D. Bouchard, A. Wakeham, A. Itie, et al. 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature 403:211.*
2. Chiang, Y. J., H. K. Kole, K. Brown, M. Naramua, S. Fukuhara, R.-J. Hu, I. K. Jang, J. S. Gutkind, E. M. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature 403:216.*
3. Malek, T. R., A. Yu, V. Vincek, P. Scibelli, and L. Kong. 2002. CD4 regulatory T cells prevent lethal autoimmunity in Il-2Rβ-deficient mice: implications for the nonredundant function of IL-2. *Immunity 17:167.*
4. 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.*
5. Green, E. D., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. $CD4^+CD25^+$ T regulatory cells control anti-islet $CD8^+$ T cells through TGF-β-TGF-β receptor interactions $CD4^+CD25^+$ in type 1 diabetes. *Proc. Natl. Acad. Sci. USA 100:10878.*
6. Mottet, C., H. H. Uhlig, and F. Powerie. 2003. Cure of colitis by $CD4^+CD25^+$ regulatory T cells. *J. Immunol. 170:3939.*
7. Thornton, A. M., and E. M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med. 188:287.*
8. Piccirillo, C. A., and E. M. Shevach. 2001. Control of $CD8^+$ T cell activation by $CD4^+CD25^+$ immunoregulatory cells. *J. Immunol. 167:1137.*
9. Shevach, E. M., R. S. McHugh, C. A. Piccirillo, and A. M. Thornton. 2001. Control of T-cell activation by $CD4^+CD25^+$ suppressor T cells. *Immunol. Rev. 182:58.*
10. Tang, Q., J. H. Kammi, E. K. Boden, A. J. Tooley, J. Ye, S. K. Subudhi, X. X. Zheng, T. B. Strom, and J. A. Bluestone. 2003. CD28 controls peripheral homeostasis of $CD4^+CD25^+$ regulatory T cells. *J. Immunol. 171:3348.*
11. Yamazaki, S., T. Iyoda, K. Tarbell, K. Olson, K. Velinzon, K. Inaba, and R. M. Steinman. 2003. Direct expansion of functional $CD4^+CD25^+$ regulatory T cells by antigen-processing dendritic cells. *J. Exp. Med. 198:235.*
12. Gregori, S., N. Giarratana, S. Smiroldo, and L. Adorini. 2003. Dynamics of pathogenic and suppressor T cells in autoimmune diabetes development. *J. Immunol. 171:4040.*
13. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of $CD4^+CD25^+$ T cell-mediated suppression by dendritic cells. *Science 299:1033.*
14. Leveen, P., J. Larsson, M. Ehinger, C. M. Cilio, M. Sundler, L. J. Sjostrand, R. Holmdahl, and S. Karlsson. 2002. Induced disruption of the transforming growth factor β type II receptor gene in mice causes a lethal inflammatory disorder that is transplantable. *Blood 100:560.*
15. Johns, L. D., K. C. Flanders, G. E. Ranges, and S. Sriram. 1991. Successful treatment of experimental allergic encephalomyelitis with transforming growth factor-β1. *J. Immunol. 147:1792.*
16. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J. Exp. Med. 194:629.*
17. Böttner, M., K. Kerstin Krieglstein, and K. Unsicker. 2000 The transforming growth factor-βs structure, signaling, and roles in nervous system Development and Functions. *J. Neurochem. 75:2227.*
18. Liu, Y. C., and H. Gu. 2002. Cbl and Cbl-b in T cell regulation. *Trends Immunol. 23:140.*
19. Zhang, J., T. Bardos, D. Li, I. Gal, C. Vermes, J. Xu, K. Mikecz, A. Finnegan, S. Lipowitz, and T. T. Glant. 2002. Regulation of T cell activation threshold by CD28 costimulation through targeting Cbl-b for ubiquitination. *J. Immunol. 169: 2236.*
20. Yokoi, N., K. Komeda, H. Y. Wang, H. Yano, K. Kitada, Y. Saitoh, Y. Seino, K. Yasuda, T. Serikawa, and S. Seino. 2002. Cbl-b is a major susceptibility gene for rat type 1 diabetes mellitus. *Nature 31:391.*
21. Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. 3:253.*
22. Maloy, K. J., L. Salaun, R. Cahill, G. Dougan, N. J. Saunders, and F. Powrie. 2003. $CD4^+CD25^+$ Tr cells suppress innate immune pathology through cytokine-dependent mechanisms. *J. Exp. Med. 197:111.*
23. Baecher-Allen, C., V. Viglietta, and D. A. Hafler. 2002. Inhibition of human $CD4^+CD25^{+high}$ regulatory T cell function. *J. Immunol. 169:6210.*
24. Bilsborough, G. T. C., J. L. Viney, and A. M. Norment. 2003. High Antigen dose and activated dendritic cells enable Th cells to escape regulatory T cell-mediated suppression in vitro. *Eur. J. Immunol. 33:502.*
25. de Waal Malefyt, R., J. Haanen, H. Spits, M. G. Roncarolo, A. te Velde, C. G. Figdor, K.E. Johnson, R. Kastelein, H. Yssel, and J. E. de Vries. 1991. Interleukin 10 and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. *J. Exp. Med. 174:915.*
26. Joss, A., M. Adkis, A. Faith, K. Blaser, and C. A. Adkis. 2000. IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway. *Eur. J. Immunol. 30:1683.*
27. Pearson-White S., and M. McDuffie. 2003. Defective T-cell activation is associated with augmented transforming growth factor β sensitivity in mice with mutations in the *Sno* gene. *Mol. Cell. Biol. 23:5446.*