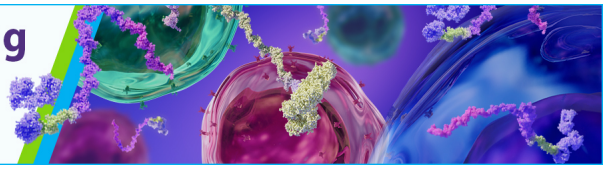

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CUTTING EDGE

Cutting Edge: Deficiency in the E3 Ubiquitin Ligase Cbl-b Results in a Multifunctional Defect in T Cell TGF-β Sensitivity In Vitro and In Vivo

Elizabeth A. Wohlfert, Leonid Gorelik,§ Robert Mittler,† Richard A. Flavell,‡ and Robert B. Clark[1]**

Mice deficient in the E3 ubiquitin ligase Cbl-b have CD28-independent T cells and develop autoimmunity. We previously reported that Cbl-b$^{-/-}$ CD4$^+$CD25$^-$ T effector cells are resistant in vitro to the antiproliferative effects of CD4$^+$CD25$^+$ regulatory T cells and TGF-β. We have now asked whether the resistance noted in Cbl-b$^{-/-}$ T cells is restricted solely to TGF-β's antiproliferative effects, whether the TGF-β resistance has in vivo relevance, and whether a defect can be identified in the TGF-β signaling pathway. We now demonstrate the following: 1) in vitro, Cbl-b deficiency prevents the TGF-β-mediated induction of Foxp3$^+$ functional regulatory T cells; 2) in vivo, Cbl-b$^{-/-}$ mice show a significantly enhanced response to a tumor that is strictly TGF-β regulated; and 3) Cbl-b$^{-/-}$ T effector cells have defective TGF-β-mediated Smad2 phosphorylation. These studies are the first to document that the E3 ubiquitin ligase Cbl-b plays an integral role in T cell TGF-β signaling, and that its absence results in multifunctional TGF-β-related defects that have important disease-related implications. The Journal of Immunology,* 2006, 176: 1316–1320.

The E3 ubiquitin ligase Cbl-b plays an important role in regulating T cell signaling, particularly through the CD28 pathway (1–3). Cbl-b-deficient (Cbl-b$^{-/-}$) mice represent an important model for studying the complex relationship among cell signaling, costimulation, and autoimmunity, because they demonstrate CD28-independent T cell activation, T cell hyperreactivity, and spontaneous autoimmunity (4, 5). Although the mechanisms underlying the development of autoimmunity in Cbl-b$^{-/-}$ mice remain unclear, Cbl-b deficiency has been associated with a T cell anergy defect, which has been suggested to underlie the autoimmunity in Cbl-b$^{-/-}$ mice (6, 7). However, in initial studies, we recently described a novel concept, "resistance to regulation," which we believe may represent an important alternative mechanism underlying the autoimmunity in Cbl-b$^{-/-}$ mice (8).

In those studies, we reported that CD4$^+$CD25$^-$ T effector cells (Teff)[2] from Cbl-b$^{-/-}$ mice are resistant in vitro to the antiproliferative effects of CD4$^+$CD25$^+$ regulatory T cells (Tregs) and TGF-β (8). To further characterize our initial finding of TGF-β resistance, we have now asked whether the resistance noted in Cbl-b-deficient T cells is restricted solely to TGF-β's antiproliferative effects, whether the TGF-β resistance has in vivo relevance, and whether a defect can be identified in the TGF-β signaling pathway.

We now report that Cbl-b deficiency prevents the TGF-β-mediated in vitro induction of Foxp3$^+$, functional Tregs, and, in vivo, allows Cbl-b$^{-/-}$ mice to mount an immune response to the tumor EL-4 comparable to that of mice with T cells engineered to be TGF-β resistant. Most importantly, we have identified a defect in T cell TGF-β-mediated Smad2 phosphorylation that is associated with Cbl-b deficiency. E3 ubiquitin ligases are proving crucial in many immunologically relevant mechanisms (6, 7, 9–11). Our studies are the first to document that such an E3 ubiquitin ligase, Cbl-b, plays an integral role in T cell TGF-β signaling and that its absence results in multifunctional TGF-β-related defects that have important disease-related implications.

Methods

Mice

C57BL/6 wild-type (WT) mice (Ly5.1$^+$) and B6.129S2-Cd28<tm1Mak>/J (CD28$^{-/-}$) mice were obtained from The Jackson Laboratory. Ly5.2$^+$ C57BL/6 mice were obtained from the National Institutes of Health. Cbl-b$^{-/-}$ mice, on a C57BL/6 background, were obtained as a gift from Dr. H. Gu (Columbia University, New York, NY). Dominant-negative TGF-β receptor II (dnTGF-RII) mice, on a C57BL/6 background, were obtained from Dr. R.

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[2] Abbreviations used in this paper: Teff, T effector cell; Treg, regulatory T cell; WT, wild type; rhTGF-β1, human recombinant TGF-β1; HPRT, hypoxanthine phosphoribosyltransferase; dnTGF-RII, dominant-negative TGF-β receptor II; Tds, T-depleted spleen; qPCR, quantitative PCR.

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Flavell (Yale University, New Haven, CT) and bred in our facilities under specific pathogen-free conditions in accordance with the guidelines and regulations of the Center for Laboratory Animal Care at the University of Connecticut Health Center.

Reagents, cell isolation, and purification

$CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated using a murine $CD4^+CD25^+$ Treg isolation kit (Miltenyi Biotec). T-depleted spleen (Tds) were obtained by depleting splenocytes with anti-CD4 and anti-CD8 microbeads (Miltenyi Biotec). The following Abs/reagents were used: anti-CD25-PE (PC61), anti-CD4-FITC (GK1.5), anti-CD3 Ab (145-2C11), all purchased from BD Pharmingen; and anti-Foxp3 (FJK-16s), obtained from eBioscience. Human rTGF-β1 (rhTGF-β1) was purchased from R&D Systems.

Cell culture for conversion to Foxp3 positivity

WT and Cbl-$b^{-/-}$ Teff were isolated as described above and then stimulated on plate-bound anti-CD3 Ab in the presence of soluble anti-CD28 Ab and, where indicated, with rhTGF-β1. Cells were cultured in RPMI 1640 supplemented with 10% FCS and 5×10^{-5} M 2-ME. Cells were harvested, and RNA was extracted for quantitative PCR (qPCR) or stained for intracellular Foxp3 as per the manufacturer's protocol after 3 days.

RNA isolation and quantitative RT-PCR

Total RNA was extracted using an RNeasy Mini kit (Qiagen). Before cDNA synthesis, 1 μg of RNA was cleaned with Amplification Grade DNase I (Invitrogen Life Technologies) and then subjected to cDNA synthesis using the iScript cDNA Synthesis kit (Bio-Rad). Foxp3 mRNA was quantified by real-time qPCR using the Quantitect SYBR Green PCR kit (Qiagen) with the iCycler iQ Real-Time PCR Detection System (Bio-Rad). Relative Foxp3 mRNA expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT).

In vitro functional assay

After 3 days of culture as above, WT and Cbl-$b^{-/-}$ cells were removed from the plate-bound anti-CD3 Ab wells, and medium was replaced (with fresh TGF-β for the TGF-β cells) for 4 additional days, yielding control and TGF-β cells.

Anergy. Cells were harvested and cocultured with irradiated (2600 rad) Tds and with or without anti-CD3 Ab in 96-well plates. Cultures were labeled with [^{3}H]thymidine at 48 h and harvested 18 h later; proliferation was assessed using a liquid scintillation counter.

Regulatory function. Naive $Ly5.2^+$ $CD4^+CD25^-$ "responder cells" were isolated as described above and labeled with 2.5 μM CFSE. CFSE-labeled $Ly5.2^+$ responder cells were plated in round-bottom, 96-well plates with irradiated Tds in the absence or presence of soluble anti-CD3 Ab. Control and TGF-β cells were harvested after 7 days as above and cocultured (5×10^4/well) where indicated with responder cells. At the end of 60 h, cultures were harvested and labeled with anti-CD45.1-biotin-conjugated Ab, followed by streptavidin-conjugated APC. $Ly5.2^+$ cells were gated, and CFSE dilution was assessed on a FACSCalibur (BD Biosciences).

In vivo EL-4 survival assay

Groups consisting of three to six mice each of WT, $CD28^{-/-}$, dnTGF-RII, and Cbl-$b^{-/-}$ mice were challenged i.p. with 5×10^5 live EL-4 tumor cells and observed daily for survival.

Western blots

Lysates were prepared from WT and Cbl-$b^{-/-}$ Teff that were cultured on plate-bound anti-CD3 Ab in the presence of soluble anti-CD28 Ab and in the absence or presence of TGF-β. After 30 min or 2 h, cell lysates were prepared (from 5×10^6 cells) and analyzed by Western blot as described previously (8). For pSmad2 and pSmad3, blots were probed with anti-phospho-Smad2 Ab (no. 3101S; Cell Signaling Technology) or pSmad3, (gift from Dr. E. Loef, Mayo Clinic, Rochester, MN), followed by incubation with HRP-conjugated goat anti-rabbit Ab (170-6515; Bio-Rad). Blots were stripped and probed with an anti-Smad2 or anti-Smad3 (51-1300, 51-1500; Zymed Laboratories), followed by HRP-conjugated goat anti-rabbit and subsequently stripped and probed with anti-β-actin Ab (A2228; Sigma-Aldrich), followed by incubation with HRP-conjugated goat anti-rabbit Ab. Bands were visualized using ECL substrate (Amersham Biosciences) detected by BioMax LS film (Eastman Kodak) and developed on an M35A X-OMAT film processor (Eastman Kodak). Densitometry was assessed on a Kodak Image Station 440CF, using Kodak 1D Image Analysis software.

Results and Discussion

Cbl-$b^{-/-}$ $CD4^+$ $CD25^-$ Teff are defective in conversion to $Foxp3^+$ T cells

We previously reported that Cbl-$b^{-/-}$ Teff are resistant in vitro to the antiproliferative effects of $CD4^+CD25^+$ Tregs and TGF-β (8). To further define the scope of Cbl-$b^{-/-}$ Teff resistance, we began by examining the in vitro TGF-β-mediated conversion of $CD4^+CD25^-Foxp3^-$ Teff to $CD4^+CD25^+Foxp3^+$ Tregs using both WT C57BL/6 and Cbl-$b^{-/-}$ Teff (12–14). Teff were purified from WT and Cbl-$b^{-/-}$ spleens and cultured on plate-bound anti-CD3 Ab with soluble anti-CD28 Ab, in either the absence (control) or presence (TGF-β) of rhTGF-β1. After 3 days of culture, cells were analyzed for conversion to Foxp3 positivity using qPCR and intracellular staining for Foxp3.

In the qPCR assays, Foxp3 mRNA expression was normalized to HPRT mRNA expression, and then the fold-induction of Foxp3 expression was normalized using the preculture Teff values set as 1. The results of a typical experiment comparing WT Teff vs Cbl-$b^{-/-}$ Teff conversion are shown in Fig. 1, *a* and *b*. WT control demonstrated a consistent decrease in Foxp3 mRNA expression from preculture levels. This likely reflects either a dilution of Foxp3-expressing cells by a preferential expansion of cells that express little or no Foxp3 or, alternatively, a decrease in Foxp3 mRNA expression by all the cells within the stimulated population. However, WT TGF-β demonstrated a consistent increase in Foxp3 mRNA expression compared with preculture Teff levels with an average increase of 3.85-fold over numerous experiments (Fig. 1*a*).

After 3 days of culture, the level of Foxp3 mRNA expression in Cbl-$b^{-/-}$ control decreased in a similar fashion to that seen with WT control. However, in contrast to the increase seen with WT TGF-β, Cbl-$b^{-/-}$ TGF-β demonstrated a decrease in Foxp3 mRNA expression (compare Fig. 1, *a* and *b*). Over numerous experiments, Cbl-$b^{-/-}$ TGF-β consistently demonstrated Foxp3 mRNA levels that were essentially similar to that seen in cultures of Cbl-$b^{-/-}$ controls (Fig. 1*b*).

We next used intracellular staining and FACS analysis to determine the expression of Foxp3 protein after 3 days of culture. Results of a typical experiment are shown in Fig. 1*c*. Despite equal purity (≥90–95%) of the WT and Cbl-$b^{-/-}$ Teff populations, the preculture levels of Foxp3 expression in Cbl-$b^{-/-}$ Teff were usually slightly higher than those in WT Teff. After 3 days, WT control and Cbl-$b^{-/-}$ control populations both showed a significant decrease in the percentage of $Foxp3^+$ cells (Fig. 1*c*, *middle two panels*). As expected, the WT TGF-β population demonstrated a significant increase in the percentage of Foxp3-expressing cells at the end of the culture period (Fig. 1*c*, *top right panel*). In contrast, Cbl-$b^{-/-}$ TGF-β cells demonstrated only a small increase in Foxp3-expressing cells from preculture levels (Fig. 1*c*, *bottom right panel*). To determine whether Cbl-$b^{-/-}$ mice have a more general defect in expression of the Foxp3 protein, we compared the Foxp3 expression of $CD4^+CD25^+$ Tregs purified directly from WT and Cbl-$b^{-/-}$ mice. We found the expression of Foxp3 in WT and Cbl-$b^{-/-}$ Tregs to be essentially identical (Fig. 1*d*). Thus, both by qPCR and protein expression, Cbl-$b^{-/-}$ Teff demonstrate a severe defect in the ability to undergo in vitro, TGF-β-mediated conversion of Foxp3- Teff to $Foxp3^+$ Tregs.

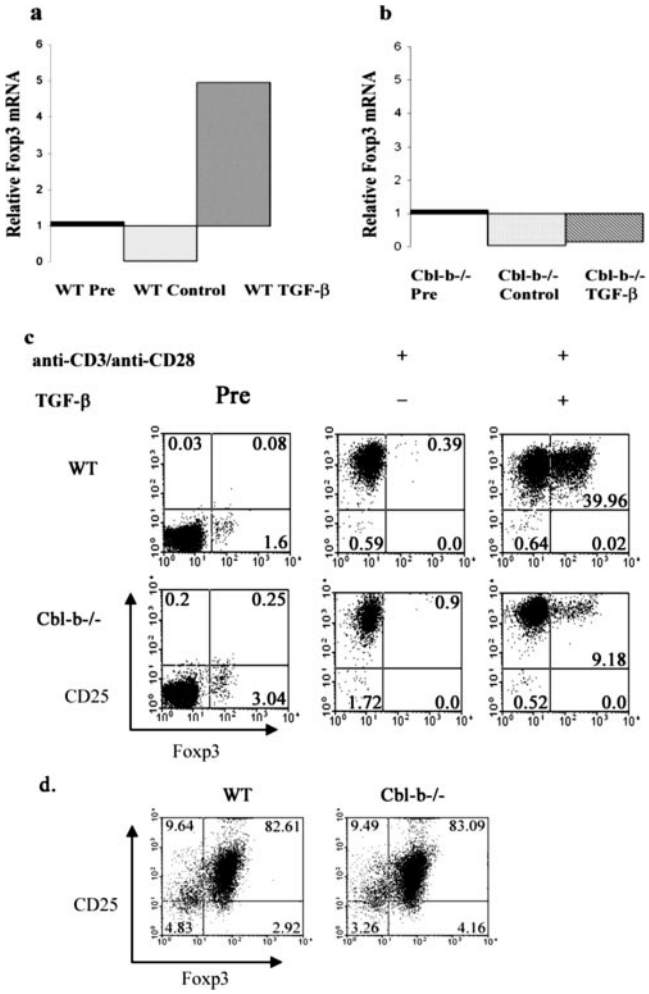

FIGURE 1. Cbl-b$^{-/-}$ Teff do not convert to Foxp3 positivity. qPCR: WT Teff (*a*) and Cbl-b$^{-/-}$ Teff (*b*) (1×10^6/well) were stimulated on plate-bound anti-CD3 Ab (5 μg/ml) with 2 μg/ml anti-CD28 Ab in either the absence or presence of 2 ng/ml rhTGF-β1. At the end of 3 days, RNA was extracted for qPCR. Foxp3 mRNA expression was normalized to HPRT mRNA expression. Then the fold-induction of Foxp3 expression for both WT and Cbl-b$^{-/-}$ was normalized using the respective preculture Teff values set as 1. The results of one of four typical experiments comparing WT Teff vs Cbl-b$^{-/-}$ Teff conversion are shown. Foxp3 staining: *c*, WT Teff (*top panels*) and Cbl-b$^{-/-}$ Teff (*bottom panels*) were stimulated as above, and Foxp3 protein expression and CD25$^+$ expression were analyzed via flow cytometry gated on CD4$^+$ T cells. *d*, CD4$^+$CD25$^+$ Tregs were purified from WT and Cbl-b$^{-/-}$ spleen and analyzed directly ex vivo as in *c*.

In vitro function of WT and Cbl-b$^{-/-}$ TGF-β cells

We next assessed the in vitro regulatory function of WT and Cbl-b$^{-/-}$ control and TGF-β cells. WT and Cbl-b$^{-/-}$ Teff were cultured with anti-CD3 Ab and anti-CD28 Ab in the absence or presence of TGF-β for 7 days. The four groups of cells were then tested for anergy and for regulatory function.

In the anergy studies, WT control demonstrated a normal proliferative response to anti-CD3 Ab stimulation. In contrast, WT TGF-β were anergic to anti-CD3 Ab stimulation, proliferating only to ~20% of the WT control level (Fig. 2*a*). As expected, Cbl-b$^{-/-}$ control proliferated vigorously to anti-CD3 Ab stimulation. However, in contrast to the WT TGF-β, the Cbl-b$^{-/-}$ TGF-β did not show an anergic response, proliferating almost equally (85%) to that of Cbl-b$^{-/-}$ control (Fig. 2*a*).

In the regulatory experiments, the responder cells, when cultured alone, proliferated as expected in the presence of anti-CD3 Ab (compare Fig. 2, *b* and *c*). Responder cells cocultured with WT control demonstrated slightly enhanced proliferation, which was likely a result of the IL-2 production by the WT control (compare Fig. 2, *c* and *d*). In contrast to WT control, WT TGF-β demonstrated regulatory function, completely inhibiting the proliferation of the responder cells (compare Fig. 2, *c* and *e*). Responder cells cocultured with Cbl-b$^{-/-}$ control demonstrated slightly enhanced proliferation (compare Fig. 2, *c* and *f*). However, in contrast to WT TGF-β, Cbl-b$^{-/-}$ TGF-β did not demonstrate regulatory function, showing no inhibition of the proliferation of the responder cells. Instead, Cbl-b$^{-/-}$ TGF-β actually enhanced the proliferation of the responders (compare Fig. 2, *c* and *g*).

Thus, unlike WT TGF-β, Cbl-b$^{-/-}$ TGF-β were not anergic and did not suppress the proliferative response of naive responder cells. This inability to be converted to functional Tregs further confirms the resistance of Cbl-b$^{-/-}$ Teff to the TGF-β-mediated effects required in the Foxp3$^-$ Teff to Foxp3$^+$ Treg conversion. Our findings not only document an important additional function of TGF-β to which Cbl-b$^{-/-}$ T cells are resistant but, given the potential for "resistance to regulation" being a more common mechanism underlying autoimmunity, also raise a potential roadblock for using in vitro-generated Tregs as immunotherapy in autoimmune diseases.

In vivo resistance of Cbl-b$^{-/-}$ T cells to TGF-β

To investigate the in vivo resistance of Cbl-b$^{-/-}$ T cells to TGF-β, we used a model of TGF-β-regulated tumor rejection. Gorelik and Flavell (15) demonstrated that the tumor EL-4 secretes TGF-β at levels that can inhibit the antitumor response of normal T cells. They further showed that C57BL/6 mice were fully capable of rejecting the TGF-β secreting syngeneic mouse thymoma EL-4 if the mice transgenically expressed a dnTGF-RII specifically in T cells. Using this approach to test for T cell TGF-β-resistance, we assessed the survival of WT, CD28$^{-/-}$, Cbl-b$^{-/-}$, and dnTGF-RII mice after an i.p. challenge with live EL-4 cells.

In the present studies, groups of mice (three to six mice per group) were injected i.p. with 5×10^3 live EL-4 cells and evaluated daily for survival. Fig. 3 shows the results of a typical challenge study with EL-4. In agreement with Gorelik and Flavell (15), we found that WT mice succumbed to the tumor in an average of 20 days. CD28$^{-/-}$ mice were equally as vulnerable and succumbed to the tumor challenge in an average of 21 days. In contrast to WT mice, dnTGF-RII mice had a significantly delayed mortality and increased survival after EL-4 challenge, with a small proportion of the mice surviving indefinitely. Most interestingly, the survival of Cbl-b$^{-/-}$ mice after challenge with EL-4 was comparable to the survival of dnTGF-RII mice, with delayed mortality and a small proportion of mice surviving indefinitely (Fig. 3).

This indicates that Cbl-b$^{-/-}$ mice are able to mount an in vivo immune response to EL-4 comparable to that of mice with T cells engineered to be TGF-β resistant. Although Cbl-b deficiency could hypothetically alter a number of aspects of the T cell response to EL-4, ultimately, Cbl-b$^{-/-}$ T cells must be resistant to the TGF-β secreted by the EL-4 to mount a successful antitumor response. These results are the first to document that Cbl-b deficiency leads to T cell resistance to TGF-β in vivo and

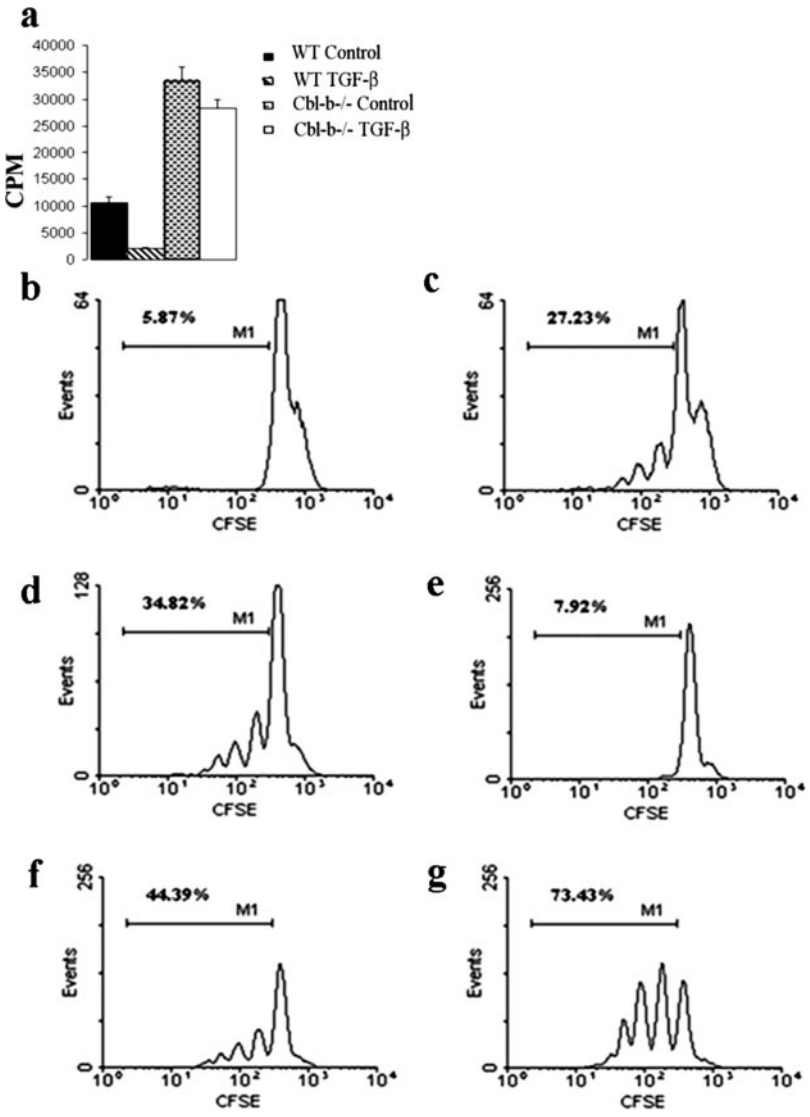

FIGURE 2. In vitro function of TGF-β cells. WT and Cbl-b$^{-/-}$ control and TGF-β cells were cultured for 7 days as described in *Methods* and then assessed. *a*, Anergy: WT and Cbl-b$^{-/-}$ control and TGF-β cells (5×10^4/well) were cocultured with irradiated Tds (5×10^4/well) and with or without 0.5 μg/ml anti-CD3 Ab in 96-well plates. Cultures were labeled with [^{3}H]thymidine at 48 h and harvested 18 h later. Results are expressed as cpm. Background counts of nonstimulated cultures were always <700 cpm. *b–g*, Regulatory function: CFSE-labeled Ly5.2$^+$ CD4$^+$CD25$^-$ responder cells (1.5×10^4/well) and irradiated Tds (5×10^4/well) were cocultured in 96-well plates along with no anti-CD3 Ab or additional populations (*b*), anti-CD3 Ab (0.5 μg/ml) (*c*), anti-CD3 Ab and WT control cells (*d*), anti-CD3 Ab and WT TGF-β cells (*e*), anti-CD3 Ab and Cbl-b$^{-/-}$ control cells (*f*), and anti-CD3 Ab and Cbl-b$^{-/-}$ TGF-β cells (*g*). CFSE dilution was assessed 60 h later using FACS analysis gated on Ly5.2$^+$ cells.

further support the concept that this resistance may play a role in the development of autoimmunity.

Reduced TGF-β-mediated Smad2 phosphorylation in Cbl-b$^{-/-}$ Teff

In our initial studies, we found that Cbl-b$^{-/-}$ Teff express levels of TGF-βRII that are equivalent to WT Teff levels, and that phosphorylation of Smad3 is equivalent in Cbl-b$^{-/-}$ and WT Teff 30 min after TGF-β-stimulation (8). In the present studies, we examined both Smad2 and Smad3 phosphorylation in WT and Cbl-b$^{-/-}$ Teff at 30 min and 2 h after TGF-β stimulation. WT and Cbl-b$^{-/-}$ Teff were established in conversion cultures as described above and 30-min and 2-h cellular lysates were analyzed by Western blot. The results of a typical Western blot are shown in Fig. 4. We found that the 2-h lysates consistently revealed a difference between WT and Cbl-b$^{-/-}$ Teff in TGF-β signaling. Two hours after TGF-β stimulation, WT TGF-β showed an increase in pSmad2 compared with baseline levels. In contrast, Cbl-b$^{-/-}$ TGF-β showed no increase in pSmad2 over baseline levels (Fig. 4*a*). Although the 30-min lysates most often revealed no relative decrease in Cbl-b$^{-/-}$ TGF-β Smad2 phosphorylation, we occasionally noted a small relative decrease in Cbl-b$^{-/-}$ TGF-β Smad2 phosphorylation beginning at this time point (Fig. 4*a*). Interestingly, a difference was never seen between WT TGF-β and Cbl-b$^{-/-}$ TGF-β Smad3 phosphorylation either at 30 min or 2 h (Fig. 4*b*). These results demonstrate for the first time that a deficiency of the E3 ubiquitin ligase Cbl-b is associated with a defect in T cell Smad2 phosphorylation.

Our findings are similar to those described for the E3 ubiquitin ligase Itch in fibroblast TGF-β-mediated signaling. Bai et al. (10) found that mouse embryonic fibroblasts deficient in Itch (Itch$^{-/-}$) were resistant to the growth-inhibitory effect of TGF-β. Itch deficiency was associated with a reduction in

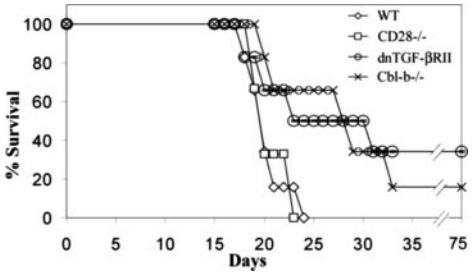

FIGURE 3. In vivo resistance to TGF-β. In groups of three to six mice, WT, CD28$^{-/-}$, dnTGF-RII, and Cbl-b$^{-/-}$ mice were challenged i.p. with 5×10^3 live EL-4 tumor cells and observed daily for survival.

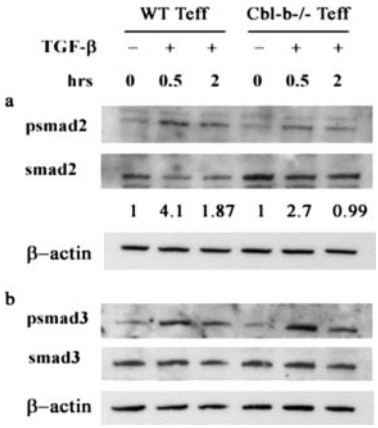

FIGURE 4. Reduced TGF-β-mediated Smad2 phosphorylation in Cbl-b$^{-/-}$ Teff. WT and Cbl-b$^{-/-}$ Teff were stimulated on plate-bound anti-CD3 Ab (5 μg/ml) with 2 μg/ml soluble anti-CD28 Ab in the presence or the absence of 2 ng/ml TGF-β. Before culture and after 30 min or 2 h of culture, the cells were harvested and lysates prepared and analyzed for levels of pSmad2, Smad2 (*a*); and pSmad3, Smad3, and β-actin (*b*). A total of 1.75×10^6 cell-equivalents per lane of WT Teff or Cbl-b$^{-/-}$ Teff lysate was loaded. The ratio of pSmad2:Smad2 (shown below the pSmad2 blot) was determined for each sample by densitometry and normalized by assigning the pSmad2:Smad2 ratio for nonstimulated WT Teff and Cbl-b$^{-/-}$ Teff cells a value of 1.

Smad2 phosphorylation, and Itch was reported to normally augment Smad2 phosphorylation directly via proteolysis-independent ubiquitination (10). Interestingly, the reduction in Smad2 phosphorylation in Itch$^{-/-}$ fibroblasts was apparent at 2 h but not at 30 min after the initiation of TGF-β stimulation.

Overall, our in vitro, in vivo, and signaling results represent the first documentation that the E3 ubiquitin ligase Cbl-b plays a critical role in T cell TGF-β signaling. Recently, there has been great interest in the E3 ubiquitin ligases that play a role in regulating immune responses, and our results now suggest that at least one of these, Cbl-b, also can regulate T cell TGF-β signaling (6, 7, 9–11). Finally, our studies document that the absence of Cbl-b results in multifunctional in vitro and in vivo defects in TGF-β sensitivity that may have important disease-related implications.

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Disclosures

The authors have no financial conflict of interest.

References

1. Fang, D., and Y. C. Liu. 2001. Proteolysis-independent regulation of PI3K by Cbl-b-mediated ubiquitination in T cells. *Nat. Immunol.* 2: 870–875.
2. Fang, D., H. Y. Wang, N. Fang, Y. Altman, C. Elly, and Y. C. Liu. 2001. Cbl-b, a RING-type E3 ubiquitin ligase, targets phosphatidylinositol 3-kinase for ubiquitination in T cells. *J. Biol. Chem.* 276: 4872–4878.
3. Li, D., I. Gal, C. Vermes, M. L. Alegre, A. S. Chong, L. Chen, Q. Shao, V. Adarichev, X. Xu, T. Koreny, et al. 2004. Cutting edge: Cbl-b: one of the key molecules tuning CD28- and CTLA-4-mediated T cell costimulation. *J. Immunol.* 173: 7135–7139.
4. Chiang, Y. J., H. K. Kole, K. Brown, M. Naramura, S. Fukuhara, R. J. Hu, I. K. Jang, J. S. Gutkind, E. Shevach, and H. Gu. 2000. Cbl-b regulates the CD28 dependence of T-cell activation. *Nature* 403: 216–220.
5. Bachmaier, K., C. Krawczyk, I. Kozieradzki, Y. Y. Kong, T. Sasaki, A. Oliveira-dos-Santos, S. Mariathasan, D. Bouchard, A. Wakeham, A. Itie, et al. 2000. Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. *Nature* 403: 211–216.
6. Jeon, M. S., A. Atfield, K. Venuprasad, C. Krawczyk, R. Sarao, C. Elly, C. Yang, S. Arya, K. Bachmaier, L. Su, et al. 2004. Essential role of the E3 ubiquitin ligase Cbl-b in T cell anergy induction. *Immunity* 21: 167–177.
7. Heissmeyer, V., F. Macian, S. H. Im, R. Varma, S. Feske, K. Venuprasad, H. Gu, Y. C. Liu, M. L. Dustin, and A. Rao. 2004. Calcineurin imposes T cell unresponsiveness through targeted proteolysis of signaling proteins. *Nat. Immunol.* 5: 255–265.
8. Wohlfert, E. A., M. K. Callahan, and R. B. Clark. 2004. Resistance to CD4$^+$CD25$^+$ regulatory T cells and TGF-β in Cbl-b$^{-/-}$ mice. *J. Immunol.* 173: 1059–1064.
9. Uchida, D., S. Hatakeyama, A. Matsushima, H. Han, S. Ishido, H. Hotta, J. Kudoh, N. Shimizu, V. Doucas, K. I. Nakayama, et al. 2004. AIRE functions as an E3 ubiquitin ligase. *J. Exp. Med.* 199: 167–172.
10. Bai, Y., C. Yang, K. Hu, C. Elly, and Y. C. Liu. 2004. Itch E3 ligase-mediated regulation of TGF-β signaling by modulating smad2 phosphorylation. *Mol. Cell* 15: 825–831.
11. Zhao, H., C. C. Li, J. Pardo, P. C. Chu, C. X. Liao, J. Huang, J. G. Dong, X. Zhou, Q. Huang, B. Huang, et al. 2005. A novel E3 ubiquitin ligase TRAC-1 positively regulates T cell activation. *J. Immunol.* 174: 5288–5297.
12. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4$^+$CD25$^-$ naive T cells to CD4$^+$CD25$^+$ regulatory T cells by TGF-β induction of transcription factor Foxp3. *J. Exp. Med.* 198: 1875–1886.
13. Fu, S., N. Zhang, A. C. Yopp, D. Chen, M. Mao, H. Zhang, Y. Ding, and J. S. Bromberg. 2004. TGF-β induces Foxp3$^+$ T-regulatory cells from CD4 + CD25 - precursors. *Am. J. Transplant.* 4: 1614–1627.
14. Park, H. B., D. J. Paik, E. Jang, S. Hong, and J. Youn. 2004. Acquisition of anergic and suppressive activities in transforming growth factor-β-costimulated CD4$^+$CD25$^-$ T cells. *Int. Immunol.* 16: 1203–1213.
15. Gorelik, L., and R. A. Flavell. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor-β signaling in T cells. *Nat. Med.* 7: 1118–1122.