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E3 Ubiquitin Ligase Cbl-b Regulates Thymic-Derived CD4⁺CD25⁺ Regulatory T Cell Development by Targeting Foxp3 for Ubiquitination

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CD28 costimulation is essential for the development of thymic-derived CD4⁺CD25⁺Foxp3⁺ regulatory T cells (“tTregs”). E3 ubiquitin ligase Cbl-b has been shown to regulate CD28 dependence of T cell activation. In this paper, we report that the loss of Cbl-b partially but significantly rescues the defective development of tTregs in Cd28−/− mice. This partial rescue is independent of IL-2. Mechanistically, Cbl-b binds to Foxp3 upon TCR stimulation and, together with Stub1, targets Foxp3 for ubiquitination and subsequently degradation in the proteasome. As Cbl-b self-ubiquitination and proteasomal degradation is impaired in Cd28−/− T cells, the defective development of tTregs in Cd28−/− mice may in part be due to increased Foxp3 ubiquitination and degradation targeted by Stub1 and Cbl-b. Treating Cd28−/− mice with a proteasome inhibitor completely rescues defective tTreg development in these mice. Therefore, Cbl-b, together with Stub1, ubiquitinate Foxp3, and regulate tTreg development. The Journal of Immunology, 2015, 194: 1639–1645.

Thymic-derived CD4⁺CD25⁺ regulatory T cells (tTregs) have an important role in the mechanisms of peripheral immune tolerance and in the prevention of pathogenic autoimmunity through the suppression of proliferation and production of proinflammatory cytokines in effector immune cells. Similar to other T cells, tTregs develop in the thymus and are defined by expression of the forkhead family transcription factor Foxp3. Expression of Foxp3 is required for tTreg development and appears to control a genetic program specifying this cell fate (1, 2). Mice deficient for Foxp3, or carrying a loss-of-function mutation in Foxp3 (Scurfy mice), present with fatal autoimmune-like disease caused by hyperresponsive CD4⁺ T cells (2–4). CD28 is required for tTreg development and peripheral homeostasis because mice deficient for CD28 have very few tTregs in both thymuses and spleens, and this defect cannot be rescued by IL-2 (5, 6). In addition to CD28, it has been documented that IL-2 is essential for tTreg expansion and maintenance, whereas IL-2Rγ is also required for tTreg development (5, 7, 9).

Casitas-B-lineage lymphoma protein-b (Cbl-b), an E3 ubiquitin ligase, plays an important role in regulating T cell signaling threshold and T cell differentiation (10–12). Gene targeting in mice has shown that Cbl-b functions as a gatekeeper involved in the maintenance of a balance between immunity and tolerance (13, 14). Indeed, we demonstrated that CD28 costimulation potentiates TCR-induced Cbl-b ubiquitination and degradation, whereas CTLA-4–B7 interaction induces Cbl-b expression (15, 16). These observations indicate that CD28 and CTLA-4 tightly regulate Cbl-b expression, which is critical for establishing the threshold for T cell activation and tolerance. In strong support of this notion, Cblb−/− T cells are resistant to anergy induction in vitro and in vivo (17, 18), and Cblb−/− mice are highly susceptible to autoimmune/inflammatory diseases (12–14, 19).

T cells from Cblb−/− mice show enhanced proliferation and IL-2 production in response to TCR stimulation (13, 14) as well as uncoupling the requirement of CD28 costimulation to promote T cell proliferation and IL-2 production (13, 14, 20). On the basis of these observations, we reasoned that Cbl-b deficiency might rescue the defective development of tTregs in Cd28−/− mice. In this study, we found that Cbl-b deficiency partially rescues impaired tTreg development in Cd28−/− mice, and this rescue is independent of increased IL-2 production. Further analysis showed that Cbl-b binds to ubiquitinated Foxp3 upon TCR stimulation via its ubiquitin-associated (UBA) domain and, together with Stub1, targets Foxp3 for ubiquitination and subsequent degradation in the proteasome. Treating Cd28−/− mice with PS-341, a proteasome inhibitor, completely rescues defective tTregs indicating that Foxp3 ubiquitination and degradation are major mechanisms regulating Foxp3 expression.

Materials and Methods

Mice

Wild-type (WT) BALB/c, C57BL/6, and Cd28−/− mice were purchased from The Jackson Laboratory. Cblb−/− mice were obtained from Dr. J. M. Penninger (University of Toronto, Toronto, ON, Canada) and have been backcrossed onto a BALB/c background for at least 14 generations. CblbC373A mice were described previously (21). Cblb−/− mice were crossed to Cd28−/− mice to generate Cblb−/−Cd28−/− mice. Foxp3gfp mice were obtained from Dr. A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY). Cd28−/− mice were crossed onto...
**Plasmids and transfection**

Cbl-b cDNAs encoding full-length or different mutant Cbl-b with an HA epitope in pCEFL described previously (22) were obtained from Dr. Stanley Lipkowitz (National Cancer Institute, Bethesda, MD). His6-tagged ubiquitin plasmid was a gift from Dr. D. Bohmann (University of Rochester, Rochester, NY). GFP-tagged Stub1 plasmid was obtained from OriGene (Rockville, MD). HEK293T cells were transfected with HA-tagged Cbl-b, Cbl-b N1/3, C2/3, and UBA together with Flag-tagged Foxp3 and His-tagged ubiquitin were lysed in radioimmunoprecipitation assay (RIPA) buffer containing 1% SDS.

**Immunoprecipitation and Western blot**

The isolation of Cbl-b and Cbl-b was assessed by immunoblotting. The transfected cells were stimulated with anti-CD3 and anti-CD28 for 15 min and lysed in RIPA buffer containing 1% SDS.

**Isolation of Tregs**

The isolation of C4*CD25* Tregs was performed using mouse Treg isolation kits from Miltenyi Biotec as described previously (19, 23). For some experiments, C4*CD25*GFP* cells were sorted from Foxp3*GFP* mice using a FACS Aria II cell sorter (BD Biosciences).

**Subcellular fractionation**

The cytosolic and nuclear fractionations were isolated using a membrane/cytosolic and nuclear protein isolation kit (Thermo Scientific, Rockford, IL).

**Knockdown of Stub1 and Cbl-b**

For Stub1 and Cbl-b knockdown experiments, naive C4*CD25* T cells from BALB/c mice were nucleofected with Stub1 or Cbl-b small interfering RNAs (siRNAs) or scrambled siRNA, according to the manufacturer’s instruction (Lonza, Allendale, NJ). The efficiency of knockdown was assessed by immunoblotting. The transfected cells were stimulated with anti-CD3 and anti-CD28 for 15 min and lysed in RIPA buffer containing 1% SDS.

**FIGURE 1.** Loss of Cbl-b partially rescues defective development of C4*CD25*Foxp3* Tregs in C28* mice. (A) Thymocytes and splenocytes from WT, Cblb*−/−, Cd28*−/−, and Cblb*−/−Cd28*−/− mice (n = 5/group; 8 wk of age) were surface stained with anti-CD4, anti-CD8, and anti-CD25 and intracellularly stained with anti-Foxp3. The percentage of CD4* CD8* CD25*Foxp3* cells in thymi and CD4* CD25*Foxp3* cells in spleens of different groups of mice were measured. Statistical significance was calculated using the Student t test. *p < 0.05 compared with WT, **p < 0.01 compared with WT, ***p < 0.001 compared with C28−/−. (B) WT CD4*CD25* T cells were incubated with CD4*CD25* T cells isolated from WT and Cblb*−/−Cd28*−/− mice at different ratios in the presence of anti-CD3 and T-depleted APCs for 72 h. T cell proliferation was determined by [3H]thymidine incorporation. WT and CD28*−/− mice received daily i.v. injections of a combination of mouse IL-2 (1.5 μg/day; eBioscience) and anti-mouse IL-2 mAb (clone JES6-1A12) (50 μg/day; Bio-XCell) for 7–9 d. Thymi and spleens were analyzed for Tregs on days 8–10 by flow cytometry as above. (D) CD28*−/− mice were treated with anti–IL-2 daily by i.v. for 7–9 d, and Tregs in thymi and spleens were analyzed by flow cytometry. The data shown are one representative of three independent experiments. *p < 0.05 compared with PBS-treated controls.
arbitrary densitometric units were determined by the ImageJ 1.48 (National Institutes of Health, Bethesda, MD).

In vivo PS-341 treatment

Cd28<sup>−/−</sup> Foxp3<sup>gfp</sup> mice (6 wk of age) were i.p. injected with PS-341 (Bortezomib) or vehicle at 0.25 mg/kg twice per week for 2 wk. On day 14, the mice were sacrificed, and the expression of GFP<sup>+</sup> cells within CD4<sup>+</sup> CD25<sup>+</sup> population in thymus and spleens was determined by flow cytometry.

Statistical analysis

A two-tailed Student t test was used for calculation of statistical significance unless otherwise stated.

Results

Cbl-b deficiency partially rescues defective development of CD4<sup>+</sup> Foxp3<sup>+</sup> tTregs in Cd28<sup>−/−</sup> mice

To test whether Cbl-b deficiency rescues the defective development and/or expansion of tTregs in Cd28<sup>−/−</sup> mice, we crossed Cblb<sup>−/−</sup> mice onto a Cd28<sup>−/−</sup> background and generated Cblb<sup>−/−</sup> Cd28<sup>−/−</sup> mice. Although very few CD4<sup>+</sup> Foxp3<sup>+</sup> tTregs were found in Cd28<sup>−/−</sup> thymi and spleens, Cblb<sup>−/−</sup> mice had increased tTregs in their thymi and spleens compared with their WT controls (Fig. 1A). Loss of Cbl-b partially but significantly rescued this defect in both thymuses and spleens of Cd28<sup>−/−</sup> mice (Fig. 1A; p < 0.001 compared Cd28<sup>−/−</sup> tTregs with Cblb<sup>−/−</sup> Cd28<sup>−/−</sup> tTregs in both thymuses and spleens). Therefore, Cbl-b deficiency rescues at least in part tTreg development in Cd28<sup>−/−</sup> mice. Functionally, Cblb<sup>−/−</sup> Cd28<sup>−/−</sup> CD4<sup>+</sup>CD25<sup>+</sup> tTregs suppressed TCR-induced WT effector T cell proliferation comparable to WT CD4<sup>+</sup>CD25<sup>+</sup> tTregs (Fig. 1B). This partial rescue is likely to be independent of IL-2 because treating Cd28<sup>−/−</sup> mice with IL-2/anti–IL-2 complex, which has been shown to selectively stimulate tTregs (8), failed to rescue defective development of CD4<sup>+</sup>CD8<sup>+</sup> Foxp3<sup>+</sup> tTregs in thymuses of Cd28<sup>−/−</sup> mice, although this treatment indeed increased splenic tTregs (Fig. 1C). Furthermore, treating Cblb<sup>−/−</sup> Cd28<sup>−/−</sup> mice with a neutralizing anti–IL-2 Ab failed to abrogate the increased tTregs in thymuses and spleens of Cblb<sup>−/−</sup> Cd28<sup>−/−</sup> mice (Fig. 1D).

Cbl-b associates with Foxp3 upon TCR stimulation via its UBA domain

To address how Cbl-b inhibits CD28-dependent tTreg development, we investigated whether Cbl-b and Foxp3 form a physical association. We found by coimmunoprecipitation that Cbl-b, but not c-Cbl, forms an inducible association with Foxp3 at 30 min after TCR/CD28 stimulation (Fig. 2A). To assess whether Foxp3 associates with other E3 ubiquitin ligases, we also blotted Foxp3 immunoprecipitates with Abs against Cul2 and Stub1. It has been shown that Stub1 ubiquitinates Foxp3 under an inflammatory condition (24), whereas Cul2 may directly target Foxp3 under hypoxic conditions (25). Stub1 was found to constitutively associate with Foxp3, and this increased upon TCR/CD28 stimulation, but we failed to detect Cul2–Foxp3 interaction (Fig. 2A).

To map the structural requirements for Cbl-b’s association with Foxp3, we generated serial Cbl-b truncated mutants as shown in Fig. 2B. Cbl-b–Foxp3 interaction only occurred when the cells...
were cotransfected with WT Cbl-b and a Cbl-b C2/3 fragment, but not with Cbl-b constructs lacking the UBA domain (Fig. 2C). These data indicate that the Cbl-b UBA domain is critical for the binding of Cbl-b to Foxp3. These findings also raise the possibility that another E3 ubiquitin ligase initiates Foxp3 ubiquitination, which results in the recruitment of Cbl-b via the interaction between ubiquitin chains on Foxp3 and the UBA domain of Cbl-b, thus enhancing Foxp3 ubiquitination. Indeed, knocking down Stub1 by siRNA in WT T cells disrupted Cbl-b–Foxp3 interaction (Fig. 2D).

**Stub1 and Cbl-b sequentially induce Foxp3 for ubiquitination and degradation**

Although Foxp3 undergoes ubiquitination mediated by Stub1 upon inflammatory stimulation (24, 26), it is unknown whether TCR engagement also induces Foxp3 ubiquitination and degradation, which may provide a mechanism of maintaining Foxp3 expression at a steady state. Indeed, Foxp3 underwent ubiquitination upon TCR/CD28 stimulation (Fig. 3A). To test whether Cbl-b is involved in the ubiquitination of Foxp3, we transfected 293T cells

**FIGURE 3.** Stub1 and Cbl-b sequentially ubiquitinate Foxp3 upon TCR/CD28 stimulation. (A) CD4+CD25+ T cells from WT mice were stimulated with anti-CD3 and anti-CD28, or left unstimulated, and lysed in RIPA buffer. The cell lysates were immunoprecipitated with anti-Foxp3 and blotted with anti-ubiquitin. (B) HEK293T cells were transfected with HA-tagged Cbl-b or Cbl-b C373A, Flag-tagged Foxp3, and His-tagged ubiquitin. The cell lysates were immunoprecipitated with anti-Flag and blotted with anti-HA. (C and D) CD4+CD25+ T cells from WT, Cblb<sup>−/−</sup>, or Cblb<sup>C373A</sup> mice and stimulated with anti-CD3 and anti-CD28 and lysed. The Foxp3 ubiquitination was determined. (E) Cytoplasmic and nuclear fractions of WT CD4+CD25+ T cells stimulated with anti-CD3 and anti-CD28 were separated and immunoprecipitated with anti-Foxp3 and blotted with anti-ubiquitin. (F) WT CD4+CD25+ T cells were stimulated with anti-CD3 and anti-CD28 for 1, 2, and 4 h in the presence or absence of MG-132. Foxp3 expression was determined. (G) BALB/c CD4+CD25+ T cells were transfected with siRNAs specific for Stub1, Cbl-b, or a scrambled siRNA and stimulated with anti-CD3 and anti-CD28 and lysed in RIPA buffer. The cell lysates were immunoprecipitated with anti-Foxp3 and blotted with anti-ubiquitin. The fold changes of Foxp3 ubiquitination bands in arbitrary densitometric units were determined by the ImageJ 1.48. The data shown are one representative of three independent experiments.
respectively. The data shown are one representative of three independent
lysates (WCLs) were blotted with anti-Foxp3, anti-Cbl-b, and anti-actin,
membrane was stripped and reprobed with anti-Foxp3. The whole-cell
immunoprecipitated with anti-Foxp3 and blotted with anti-ubiquitin. The
hamster IgG for 15 min, and lysed in RIPA buffer. The cell lysates were
anti-CD3 and anti-CD28, followed by cross-linking with rabbit anti-
mouse IL-2 (R&D Systems) for 72 h. The cells were restimulated with
initiates Foxp3 ubiquitination, which in turn allows the recruit-
ubiquitination (Fig. 3G). These data strongly indicate that Stub1
whether Stub1 is the initial E3 ubiquitin ligase for Foxp3, we
Foxp3 undergoes proteasome-mediated degradation. To determine
was significantly decreased at 1 h after TCR/CD28 stimulation,
cyttoplasm (Fig. 3E). In keeping with these data, Foxp3 expression
with HA-tagged Cbl-b, Flag-tagged Foxp3, and His-tagged ubiq-
We have shown that Cbl-b facilitates Foxp3 expression by naive CD4+CD25+ T cells, but once Foxp3 is expressed, Cbl-b targets it for ubiquitination and degradation, thus maintaining Foxp3 expression at a steady state.

**Proteasome inhibitor PS-341 potentiates tTreg development in CD28−/− mice**

Our previous studies showed that Cbl-b self-ubiquitination and degradation is impaired in CD28−/− T cells upon TCR stimulation (15). Indeed, Cbl-b degradation was impaired in thymic Treg precursors lacking CD28 (Fig. 5A). These observations indicate that higher levels of Cbl-b in CD28−/− T cells may display higher E3 Ubligase activity, which results in a significantly higher degradation of Foxp3 in these T cells. In support of this notion, treating Cd28−/−Foxp3gfp mice with PS-341 (bortezomib), a clinically approved drug for cancer therapy, completely rescued defective tTregs in thymuses and spleens of Cd28−/− mice to WT levels (Fig. 5B), and these newly generated tTregs were able to suppress effector T cell proliferation in vitro (Fig. 5C).

**Discussion**

In this study, we demonstrate that Cbl-b regulates CD28-dependent tTreg development via targeting Foxp3 for ubiquitination. Interestingly, we found that Foxp3 is sequentially ubiquitinated by Stub1 and Cbl-b. Stub1 initiates Foxp3 ubiquitination, which results in the recruitment of Cbl-b to the ubiquitin chains that are attached to Foxp3 via the Cbl-b UBA domain. This mechanism is important to maintain Foxp3 at a steady state. Therefore, our study provides a novel molecular mechanism for the regulation of Foxp3 expression in Tregs.

It has been documented that CD28 costimulation is required for tTreg development because Cd28−/− mice have very few tTregs (5, 6). The defective tTreg development in Cd28−/− mice appears to be independent of IL-2 (5), and the molecular mechanism for CD28 in controlling tTreg development remains poorly defined. Because we and others have previously shown that Cbl-b regulates CD28-dependent T cell activation (13–16), we explored the possibility that Cbl-b regulates CD28-dependent Foxp3 expression. By introducing Cbl-b deficiency into Cd28−/− mice, we found that loss of Cbl-b partially but significantly increases tTregs and that this partial rescue of tTreg development is independent of IL-2 (Fig. 1), a finding consistent with a previous report (5). Further analysis revealed that Foxp3 undergoes polyubiquitination and proteasome-mediated degradation and that Foxp3 ubiquitination is significantly reduced in tTregs lacking Cbl-b or expressing an E3 ubiquitin ligase death mutation (Fig. 3). These data suggest that Cbl-b is an E3 ubiquitin ligase that promotes Foxp3 ubiquitination. Interestingly, our analysis of the molecular interaction between Cbl-b and Foxp3 reveals that Cbl-b binds to Foxp3 via its UBA domain (Fig. 2), suggesting that Cbl-b interacts with ubiquitinated Foxp3 that is initiated by another E3 ubiquitin ligase. Indeed, Stub1, which was reported to induce Foxp3 under the inflammatory condition (24), is the E3 ubiquitin ligase that initiates Foxp3 ubiquitination that allows Cbl-b to be recruited to the complex, thus synergistically enhancing Foxp3 ubiquitination. As we have shown that Cbl-b self-ubiquitination is impaired in Cd28−/− mice (15), our data suggest that the defective tTreg development in Cd28−/− mice may at least in part be ascribed to heightened expression of Cbl-b. In support of this notion, treating Cd28−/− mice with PS-341 (bortezomib), a clinically approved drug for cancer therapy, completely rescued defective tTregs in the thymus and spleens of Cd28−/− mice to WT levels (Fig. 5B). Therefore, we have unveiled a novel molecular mechanism for CD28-dependent tTreg development. Our data also suggest that administration of bortezomib may provide a useful approach to treat certain autoimmune diseases.
In summary, we show that loss of Cbl-b partially uncouples the requirement for tTreg development from CD28 costimulation, and this effect is independent of IL-2. At the molecular level, Stub1 initially induces Foxp3 ubiquitination upon TCR/CD28 stimulation. This ubiquitination event leads to the recruitment of Cbl-b to Foxp3 via the Cbl-b UBA domain and ubiquitin chains attached to Foxp3, thus enhancing Foxp3 ubiquitination. Because Cbl-b facilitates Foxp3 expression by naive CD4+CD25+ T cells via inactivating Akt-2 (19), our data reveal a previously unappreciated role for Cbl-b in controlling the development of tTregs and maintaining Foxp3 expression at a steady state.

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Disclosures
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