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J Immunol 2011; 186:2138-2147; Prepublished online 19 January 2011;
doi: 10.4049/jimmunol.1003390
http://www.jimmunol.org/content/186/4/2138

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/01/19/jimmunol.1003390.DC1

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The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Essential Role of E3 Ubiquitin Ligase Activity in Cbl-b–Regulated T Cell Functions

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E3 ubiquitin ligases have been placed among the essential molecules involved in the regulation of T cell functions and T cell tolerance. However, it has never been experimentally proven in vivo whether these functions indeed depend on the catalytic E3 ligase activity. The Casitas B-cell lymphoma (Cbl) family protein Cbl-b was the first E3 ubiquitin ligase directly implicated in the activation and tolerance of the peripheral T cell. In this study, we report that selective genetic inactivation of Cbl-b E3 ligase activity phenocopies the T cell responses observed when total Cbl-b is ablated, resulting in T cell hyperactivation, spontaneous autoimmunity, and impaired induction of T cell anergy in vivo. Moreover, mice carrying a Cbl-b E3 ligase-defective mutation spontaneously reject tumor cells that express human papilloma virus Ags. These data demonstrate for the first time, to our knowledge, that the catalytic function of an E3 ligase, Cbl-b, is essential for negative regulation of T cells in vivo. Thus, modulation of the E3 ligase activity of Cbl-b might be a novel modality to control T cell immunity in vaccination, cancer biology, or autoimmunity.

The Journal of Immunology, 2011, 186: 2138–2147.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1003390
rejection. Our results demonstrate an essential role of the E3 ligase activity for Cbl-b–mediated T cell functions.

Materials and Methods

Mice

Cbl-b E3 ligase-defective (C373Aklb/klb) knock-in mice were generated by homologous recombination as previously described (30). In brief, a targeting vector was constructed to introduce a cytceine (TGC) to alanine (GCC) substitution at amino acid position 373 encoded within the RING finger domain (at exon 8) of Cbl-b. The pGKNeo resistance cassette was removed by crossing C373A mice to β-actin–Cre transgenic mice. Cbl-b knockout mice were generated in our laboratory and have been previously described (6). Rag2−/− and P14 TCR transgenic mice were obtained from our in-house breeding stock. For in vivo T cell tolerance experiments, P14 TCR mice were bred onto the C373A and Cbl-b deficient backgrounds to generate P14/Cbl-b−/−, P14/C373Aklb/klb and the respective control mice. All experimental procedures were performed on mice backcrossed for at least six generations onto the C57BL/6J strain. Except for autoimmune experiments and if not otherwise stated, only 6–12-wk-old littermate mice were used in all experiments. All animal experiments were carried out according to an ethical animal license protocol complying with the current Austrian law.

Western blot and in vitro ubiquitylation assays

For Cbl-b and c-Cbl protein expression analysis, proteins were extracted from thymocytes and total lymph node cells using a protein lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 50 mM HEPES pH 7.8, 1 mM EDTA pH 8) containing protease inhibitors (Thermo Scientific). After clearance of the homogenates by centrifugation, 35 μg tissue lysate was resolved by 4–12% SDS-PAGE, electro-transferred to polyvinylidene difluoride membranes, and probed with anti-Cbl-b (Santa Cruz Biotechnology) and anti-c-Cbl (Pharmingen) Abs according to standard procedures. For in vitro ubiquitylation assays, N-terminal regions (aa 29–483) containing the high-affinity peptide p33 on day 0, day 3, and day 6. Non-TCR transgenic C373Aklb/klb and P14/Cbl-b−/− were used as controls. Mice were observed for the following 6–12 h, and those mice found dead or moribund mice (labored breathing, restricted movement, and spike veins in the ears) were dissected and examined for signs of toxic T cell activation, namely inflammation, edema formation, hemorrhages, and/or vascular dilatation. Blood was collected, and serum was prepared for the detection of IL-2 and IFN-γ levels using Bioplex systems (Bio-Rad).

Detection of spontaneous autoimmune phenotypes

For detection of tissue infiltrates, the submandibular salivary gland, kidney, pancreas, lung, and liver were harvested from 6- to 8-wk-old mice and fixed overnight at 4°C in 4% formalin. Tissues were then embedded in paraffin and sectioned (3.5 μm) at three depth levels (interval 200–300 μm). Tissue sections were stained with H&E for detection of mononuclear cell infiltration and with anti-CD3ε Abs (clone SP7; Neo Markers) for detection of T cells. Two independent investigators scored the presence of lymphocyte infiltrates in a blind fashion. For the detection of serum autoantibodies, indirect immunofluorescence on Rag2−/− tissue sections was performed as described (31). Briefly, 5-μm cryostat sections from several organs of Rag2 knockout mice were incubated with 1/40 dilutions of sera obtained from 6- to 8-wk-old mice, followed by detection with Alexa Fluor-555 labeled anti-mouse IgG secondary Abs (Invitrogen). DAPI was used for nuclear counterstaining. Slides were examined in a blind fashion, and representative photographs were taken using a fluorescence microscope (Axioplan2; Zeiss). Serum IgG1 levels and anti-nuclear Ag (ANA) and anti-dsDNA autoantibodies were detected by ELISA according to the manufacturer’s protocol (Bethyl Lab for IgG1; α Diagnostic for anti-nuclear and ds-DNA autoantibodies). Serum glucose was measured using a glucose meter (One Touch Ultra Easy; LifeScan), and serum insulin levels were detected by ELISA according to the manufacturer’s protocol (Merckodia AB).

TC1 tumor rejection

TC1 cells are primary C57BL/6 mouse lung fibroblasts co-transformed with an activated c-Ha-ras oncogene and the HPV-16 E6 and E7 oncoproteins (32). Culture conditions and TC1 inoculation protocols have been described elsewhere (18). In brief, 2.5 × 105 TC1 tumor cells were s.c. injected into the shaved left flank of young (8–12 wk old) mice (day 0) and tumor growth was monitored twice per week with a caliper. Because TC1 cells are derived from female mice, only female animals were used for the described experiments. Mice were euthanized when tumor volume reached 1 cm3 in accordance with animal license protocols. For CD8+ cell depletion studies, mice were i.p. injected with 50 μg anti-CD8 depletion Abs (clone 24.3) at day −4 and day −2 before TC1 inoculation. Efficient and specific depletion of CD8+ cells was confirmed by FACS analysis at day −1 (see Supplemental Fig. 6). CD8+ depletion was repeated every week throughout the whole experimental observation period. For immunohistochemistry analysis, TC1 tumors were harvested at day 14
after tumor inoculation, fixed overnight at 4˚C in 4% formalin, and embedded in paraffin. Several 3.5-μm-thick sections (at two different tumor depths, interval 200–300 μm) were stained for each mouse with Abs against cleaved (activated) caspase 3 (Asp175; Cell Signaling), Ki67 (Ncl-Ki67p; Novacastra), and CD3ε (SP7; Neo Markers) using the automated Ventana Discovery System (Ventana). Slides were scanned on a Mirax Scanner (Zeiss), and Definiens Tissue Map software (Definiens AG) was used for the automated determination of positively stained cells. All cells in the section (6–12 sections per mouse) were counted.

Tumor-infiltrating lymphocytes

Analysis of tumor-infiltrating lymphocytes was performed in tumors isolated on day 14 after inoculation, a time point when all experimental mice have similar tumor volume. For immunofluorescence staining of CD8+ intra-tumor cells, 5-μm tumor cryosections were stained with anti-CD8 Abs (clone KT15; Chemicon) and detected with Alexa Fluor-555 labeled anti-rat IgG secondary Abs (Invitrogen). DAPI was used for nuclear counterstaining. Sections were examined and photographed in a fluorescence microscope (Axioplan2; Zeiss). For staining quantifications, slides were scanned on a Mirax Scanner (Zeiss), and positively stained cells at five different image fields were quantified per mouse using the Metamorph Imaging Software (Molecular Devices). For flow cytometry analysis, tumor single-cell suspensions were stained with anti-CD45, anti-CD3ε, anti-CD4, and anti-CD8 Abs (all Pharmingen). FACS data were acquired using a six-color flow cytometer (FACSCanto; BD) equipped with the FACSDiva software (BD). Data analysis was performed using the FlowJo software (Tree Star).

Statistical analyses

Unless otherwise stated, data are shown as mean values ± SEM. Illustrations and statistical analyses were generated using GraphPad Prism 4 (GraphPad Software). Data were analyzed using the two-tailed Student t test for single comparisons or ANOVA for multiple analyses. Ordinal and normally distributed data were analyzed using the Mann–Whitney U test. For analyzing the presence of autoantibodies, we set a cutoff at p#0.05 based on a Z-statistic for the serum concentration measured in each individual mouse related to the mean and SD of the wild-type population (X2Xwt/SDwt). Kaplan–Meier survival curves were analyzed using a log-rank test. A p value <0.05 was considered to indicate statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001).

Results

Inactivation of Cbl-b E3 ligase activity does not alter T cell populations

To dissect genetically the enzymatic function of Cbl-b in vivo, we analyzed mice that carry a RING finger inactivating point mutation (C373AKI/KI) (30). These mice are viable, fertile, and grossly normal. The C373A mutation substitutes the N-terminal consensus cysteine residue in the RING finger domain of Cbl-b for alanine. Nucleotide sequence analysis on total RNA extracted from...
T cells of C373AKI/KI mice confirmed a single cysteine/alanine substitution at the right 373 position within the Cbl-b RING finger domain (Supplemental Fig. 1). To verify the functional inactivation of Cbl-b by this particular point mutation, we performed in vitro ubiquitiyation assays using wild-type Cbl-b, C373A mutant Cbl-b, and Cbl-b mutants containing a tryptophan to alanine substitution at position 400 (W400A), a second residue required for E3 ligase activity (13, 25). Whereas Cbl-b auto-ubiquitiyation was clearly detected using wild-type Cbl-b protein, the capacity to auto-ubiquitiyation was virtually abolished in both C373A and W400A Cbl-b mutant proteins (Fig. 1A). We next examined whether this loss-of-function mutation affects Cbl-b protein expression. Western blot analysis on whole thymus and lymph node lysates revealed similar Cbl-b protein levels between C373A+/+, C373AKI/KI, and C373AKI/KI mice (Fig. 1B).

Genetic ablation of Cbl-b neither alters T cell populations nor TCR expression in naive mice (6, 7). Nonetheless, as an important control for our experiments and because an equivalent loss-of-function mutation in the c-Clip RING finger domain led to additional and more severe phenotypic changes than those observed in whole-body c-Clip knockout mice (33), we assessed whether the Cbl-b C373A mutation might affect thymocyte development or peripheral T cell subpopulations. Flow cytometry analysis on total thymus showed that C373AKI/KI mice exhibit normal thymocyte development (Fig. 1C, Supplemental Fig. 2A). Moreover, similar to total Cbl-b mutant mice, C373AKI/KI animals exhibited normal mature CD4+ and CD8+ peripheral T cell populations (Fig. 1D). Of note, TCR and CD3 surface expression levels on developing thymocytes as well as gated on CD4+ and CD8+ splenocytes (Supplemental Fig. 2B) and lymph node cells (not shown) were also comparable between C373A+/+, C373AKI/KI, and Cbl-b−/− mice. In addition, these animals contain comparable numbers of regulatory CD4+CD25+Foxp3+ T cells (Fig. 1E). Naive/memory T cell populations as defined by the CD25, CD69, CD44 surface markers were also normally represented in C373AKI/KI mice compared with those in E3 ligase-sufficient C373A+/+ and C373A+/+ control mice or Cbl-b−/− mice (Supplemental Fig. 2C). In addition, splenic B cell populations were unaffected by the C373A mutation (Supplemental Fig. 3). Thus, genetic inactivation of the Cbl-b RING finger domain has no overt effect on the development and subpopulations of T and B cells.

C373AKI/KI T cells hyperrespond to TCR stimulation

It has previously been shown that Cbl-b-deficient T cells hyperproliferate and can be activated even without CD28 costimulation (6, 7). To determine whether negative regulation of T cell activation is dependent on the E3 ligase activity of Cbl-b, purified peripheral CD3+ T cells from C373A+/+, C373AKI/KI, and Cbl-b−/− mice were stimulated in vitro with different concentrations of anti-CD3e Abs alone or together with anti-CD28 Abs. A significant, stronger proliferation of T cells from C373AKI/KI mice was observed under these stimulatory conditions compared with that of C373A+/+ (not shown) or C373A+/+ T cells (Fig. 2A). Importantly, peripheral T cells lacking Cbl-b enzymatic function were able to proliferate at suboptimal concentrations of anti-CD3e Abs and even in the absence of CD28 costimulation (Fig. 2A). In all stimulatory conditions tested, the hyperproliferative responses of C373AKI/KI T cells were similar to that observed in T cells lacking Cbl-b (Fig. 2A). TCR/CD28 independent T cell stimulation with PMA and the calcium ionophore ionomycin was comparable between all genotypes (Fig. 2A).

TCR stimulation of C373AKI/KI mutant T cells not only resulted in hyperproliferation but also in a marked increase in the secretion of IL-2, IL-17, and IFN-γ (Fig. 2B). Strikingly, stimulation of C373AKI/KI T cells with anti-CD3e Abs alone triggered equal cytokine levels as the stimulation of control C373A+/+ T cells with anti-CD3e plus anti-CD28 costimulation. As in the case of proliferation, the elevated levels of IL-2, IL-17, and IFN-γ produced by C373AKI/KI T cells were comparable with those generated by Cbl-b−/− deficient T cells (Fig. 2B). Taken together, these in vitro data show that inactivation of Cbl-b RING finger domain
is functionally equivalent to total Cbl-b ablation, rendering T cells hyperresponsive to TCR stimulation, even in the absence of CD28 costimulation. Thus, Cbl-b E3 ligase activity is essentially required in the negative regulation of TCR/CD28-induced T cell proliferation and cytokine production.

**Cbl-b E3 ligase activity is critically required for inducing T cell tolerance in vivo**

To investigate whether Cbl-b functions as an E3 ligase in the induction of T cell tolerance in vivo, we decided to challenge Cbl-b E3 ligase mutant mice in a mouse model for Ag-specific CD8\(^+\) T cell tolerance. For this purpose, we crossed the C373A mutation onto a P14 TCR transgenic background, where the P14\(^+\) TCR specifically recognizes with high affinity the cognate Ag p33 (derived from the lymphocytic choriomeningitis virus) presented in the MHC class I complex (34). Non-TCR-transgenic C373AKI/KI, as well as P14 Cbl-b\(^{-/-}\) mice were included as controls.

For T cell anergy induction, mice received i.v. injections of either high (5 \(\mu\)g) or low (0.5 \(\mu\)g) doses of p33 peptides at day 0, day 3, and day 6 (Fig. 3A). Whereas all P14 C373A\(^{K/K}\) survived and showed no overt phenotype in response to repetitive p33 injections (Supplemental Fig. 4), P14 C373A\(^{K/K}\) mice died upon Ag rechallenge (Fig. 3B). Approximately 50% of the P14 C373A\(^{K/K}\) mice died after the second injection of p33 at day 3, and with the exception of only one mouse injected with a low dose of p33, no P14 C373A\(^{K/K}\) mouse survived a third Ag encounter on day 6. In all cases, lethality occurred within 6–12 h after the last p33 administration. Moribund C373A\(^{K/K}\) mice displayed restricted movements, severely impaired breathing, and vascular dilation (Fig. 3C). Dissection of diseased P14 C373A\(^{K/K}\) mice further revealed signs of severe edema formation, capillary dilation, and hemorrhagic areas in several organs (Fig. 3C). After p33 rechallenge, serum levels of IL-2 and IFN-\(\gamma\) were up to several hundred-fold elevated in P14 C373A\(^{K/K}\) mice compared with those in P14 C373A\(^{K/K}\) mice (Fig. 3D). Challenge of P14 Cbl-b knockout mice either at low or high doses of p33 resulted in 100% lethality (Fig. 3B). The inflammatory features as well as the massive increase in serum IL-2 and IFN-\(\gamma\) were comparable among P14 C373A\(^{K/K}\) and P14 Cbl-b\(^{-/-}\) mice (data not shown). These results show that the functional inactivation of the Cbl-b RING
fingervdomain leads to a massive activation of P14+ T cells, a severe cytokine storm, and death due to a T cell “shock.” Thus, Cbl-b E3 ligase activity is essential for the induction of immunotolerance of CD8+ P14+ T cells in vivo.

Cbl-b E3 ligase-defective mutants develop systemic autoimmune responses

The observations that Cbl-b RING finger mutant mice present hyperproliferative T cells that cannot be tolerized in vivo prompted us to test whether, similar to Cbl-b knockout mice (6), Cbl-b E3 ligase-defective mutants develop autoimmunity. To this end, C373A+/+, C373A+/KI, C373AKI/KI, and Cbl-b−/− mice were analyzed for the presence of spontaneous pathological tissue infiltrates and autoantibodies. Exhaustive histological analyses revealed a high incidence of mononuclear inflammatory tissue infiltrates in several organs of C373AKI/KI as well as age-matched Cbl-b−/− mice (Fig. 4A, Supplemental Fig. 5A). Lung, submandibular salivary glands, and kidney were the most affected organs, followed by liver and pancreas (Fig. 4A, Supplemental Fig. 5B, and data not shown). For most tissues, cellular infiltrates were mostly located adjacent to tissue vasculature. In the particular case of pancreas, virtually all infiltrates were located within islets of Langerhans. However, the presence of cellular infiltrates in C373AKI/KI and Cbl-b−/− mice did not result in pancreatic malfunction, as determined by physiological levels of glucose and insulin in serum (Supplemental Fig. 5E, 5F). Staining with anti-CD3ε Abs revealed significant numbers of T cells among the infiltrating cells (Fig. 4B). In tissue cross-sections of age-matched C373A+/+ and C373A+/KI littermate mice, lymphocytic infiltrates were also observed, albeit at significantly reduced incidence and severity (Fig. 4A, Supplemental Fig. 5A, 5B, and not shown).

In addition to cellular infiltrates in multiple organs, immunofluorescence staining revealed the presence of autoantibodies against several tissue Ags in the serum of C373AKI/KI mice (Fig. 4C). We could also detect a higher prevalence for anti-nuclear (ANA) (Fig. 4D) and anti-dsDNA autoantibodies (Supplemental Fig. 5C) in C373AKI/KI E3 ligase-defective mice compared with that for C373A+/+ and C373A+/KI littermates. Additionally, C373AKI/KI mice present augmented serum titers of total IgG1 (Supplemental Fig. 5D). In accordance with previously published data (6), 6- to 8-mo-old Cbl-b−/− mice exhibited highly elevated serum levels of autoantibodies (Fig. 4D, Supplemental Fig. 5C). These data show that mice carrying a loss-of-function mutation in Cbl-b RING finger domain develop a spontaneous autoimmune phenotype.

Cbl-b E3 ligase-defective mice can spontaneously reject TC1 tumors

Recently, our group and others have demonstrated that genetic ablation of Cbl-b renders mice able to reject several different types of tumors (17, 18). Based on these observations, we set out to investigate whether inactivation of Cbl-b E3 ligase function is sufficient to mediate tumor rejection in vivo. For this purpose, we challenged C373A ligase-defective mice with TC1 tumor cells expressing the human papilloma virus oncoproteins (32). We used this tumor because we have previously shown that Cbl-b−/− mice

**FIGURE 4.** Cbl-b E3 ligase-defective mice develop spontaneous autoimmunity. A, H&E staining on lung, submandibular salivary gland, and kidney from 6- to 8-mo-old C373AKI/KI, C373AKI/KI, and Cbl-b−/− mice. Cellular infiltrates are marked with an asterisk or arrows. Representative images for a total of n = 10 (C373A+/KI, C373AKI/KI) and n = 5 (Cbl-b−/−) mice are shown. Original magnification ×20. B, Anti-CD3ε immunostaining showing T cells among the cellular infiltrates in lung, pancreas, and submandibular salivary gland of C373AKI/KI and Cbl-b−/− mice. Representative images are shown. Original magnification ×20. Scale bars, 200 m. C, Detection of autoantibodies (red) by indirect immunofluorescence staining on tissue sections of Rag2−/− mice using serum obtained from 6- to 8-mo-old C373A+/KI and C373AKI/KI littermates. Autoantibodies were visualised using Alexa Fluor-555 labeled anti-mouse IgG secondary Abs. Representative images are shown. Nuclei were counterstained using DAPI (blue). Original magnifications ×63 and ×40 (for liver). D, Quantification of serum autoantibodies reactive against nuclear Ags (ANA) in the serum of 6- to 8-mo-old C373A+/+ (n = 6), C373A+/KI (n = 10), C373AKI/KI (n = 11), and Cbl-b−/− (n = 5) mice, determined by ELISA. OD values for individual mice are shown. Red dots represent significantly increased ANA autoantibodies serum concentration based on a Z-scored cutoff of 1.65 (dashed line, p ≤ 0.05) related to the serum concentration measured in wild-type mice.
can spontaneously reject TC1 cells (18). Subcutaneous injections of $2.5 \times 10^5$ TC1 cells in the flanks of C373A+/+ and C373A+/KI mice lead to a fast and progressive growth of palpable tumors. For both cohorts, tumors reached 1 cm$^3$ (the end point of the experiment) within the first 3–4 wk after inoculation (Fig. 5A).

C373A/KI/KI mice were able to efficiently and spontaneously reject TC1 tumor (Fig. 5A). It should be noted that we injected a tumor cell number ($2.5 \times 10^5$) that is 10 times higher than the dose that is lethal for wild-type mice (35). Tumor rejection by Cbl-b ligase-defective mutant mice started between 2 and 3 wk...
after tumor inoculation, when tumor mass was clearly visible, and this rejection progressed extremely fast for \(\sim 10\) d (Fig. 5A, 5B). The kinetics of tumor rejection in C373A\(^{+/+}\) mice were very similar to that observed in Cbl-\(b\)-deficient mice (Fig. 5A). On average, whereas half of the C373A\(^{+/+}\) and Cbl-\(b^{-/-}\) mice could reject TC1 cells for up to 100 d, the median survival time for C373A\(^{+/+}\) and C373A\(^{+/+}\) mice was \(\sim 30\) d. Remarkably, whereas not a single C373A\(^{+/+}\) or C373A\(^{+/+}\) mouse was able to survive

**FIGURE 6.** CD8\(^+\) T cells are required for tumor rejection in Cbl-\(b\)-E3 ligase-defective mice. A, Flow cytometry analysis of lymphocytes infiltrating TC1 tumors isolated from C373A\(^{+/+}\), C373A\(^{+/+}\), C373A\(^{+/+}\), and Cbl-\(b^{-/-}\) mice at day 14 post-tumor inoculation. Representative FACS blots of CD4\(^+\) and CD8\(^+\) intratumoral T cells and their percentages per group are shown (top panels). Bottom panel, Quantification of intratumoral CD4\(^+\) and CD8\(^+\) T cells (mean \(\pm\) SEM). \(n=4\) to 7 animals per group. \(*p<0.05\) versus C373A\(^{+/+}\) or Cbl-\(b^{-/-}\) (t test). B, Representative images of individual mice showing tumor-infiltrating CD8\(^+\) cells at day 14 after TC1 inoculation (top panel). Bottom panel, Quantification. Five optical fields were counted for each mouse (\(n=4\) to 7 mice per group); that is, data from 20–35 optical fields were counted per genotype. Tumor infiltrating CD8\(^+\) cells were detected with anti-CD8 Abs followed by Alexa Fluor-555 labeled anti-rat IgG secondary Abs. Data are shown as mean numbers of intratumoral CD8\(^+\) T cells/mm\(^2\) \(\pm\) SEM. ***\(p<0.001\) versus C373A\(^{+/+}\). C, Kinetics of TC1 tumor cell growth in untreated (left panels, control) or CD8\(^+\) cell-depleted (right panels) C373A\(^{+/+}\) and C373A\(^{+/+}\) mice. Data are pooled from two different experiments. D, Kaplan–Meier survival curves for untreated C373A\(^{+/+}\) (\(n=7\)) and C373A\(^{+/+}\) (\(n=8\)) mice and C373A\(^{+/+}\) (\(n=6\)) and C373A\(^{+/+}\) (\(n=9\)) CD8\(^+\) cell-depleted mice. **\(p<0.01\) (C373A\(^{+/+}\) control versus C373A\(^{+/+}\) CD8\(^+\) depleted mice), ***p < 0.01 (C373A\(^{+/+}\) untreated versus C373A\(^{+/+}\) CD8\(^+\) depleted mice).
beyond 48 d, ~30% of C373AKI/KI and Cbl-b–/– animals remained tumor-free their whole lives, in some cases for up to 2 y (Fig. 5C).

To understand better the mechanism involved in tumor rejection by Cbl-b E3 ligase inactivation, we performed histological analyses on tumors isolated at day 14 post-TC1 inoculation. Of note, this time point immediately preceded tumor rejection, and thus tumor volume was still comparable between all experimental mice groups. Staining for apoptosis markers revealed markedly increased cell death in the tumors from C373AKI/KI and Cbl-b–/– mice compared with that in E3 ligase-sufficient control mice (Fig. 5D). We also observed slightly reduced proliferation of TC1 cells as detected by Ki67 immunostaining. Moreover, whereas tumors from C373A+/+ or C373A+/KI mice contained few CD3+ cells, tumors isolated from C373A/KI and Cbl-b–/– mice exhibited a marked increase in tumor-infiltrating CD3ε+ T cells (Fig. 5D). Thus, inactivation of the Cbl-b RING finger catalytic domain results in slightly reduced tumor proliferation, increased tumor cell death, and higher numbers of intratumoral T cells. Most importantly, these data using TC1 tumor cells show that inactivation of Cbl-b enzymatic function is sufficient to confer antitumor activity in vivo.

**CD8* T cells control tumor rejection in Cbl-b E3 ligase mutant mice**

As greater numbers of CD3+ T cells were present in the tumors of C373A+/KI animals, we speculated that, as in the case of Cbl-b–/– deficient mice (17, 18), T cells might be relevant mediators of tumor rejection in these mutant mice. Whereas flow cytometry analysis of tumor tissue revealed similar numbers of intratumoral CD4+ T cells in all animal cohorts, the percentages of CD8+ T lymphocytes present in the tumors of C373A+/KI and Cbl-b–/– mice were significantly elevated compared with those observed in C373A+/KI and C373A+/KI mice (Fig. 6A). Further immunofluorescence analyses on tumor cross-sections confirmed the increased numbers of CD8+ cells within the tumors of C373A+/KI and Cbl-b–/– mice (Fig. 6B).

To examine whether CD8+ T cells are indeed required for the spontaneous rejection of TC1 tumor cells in Cbl-b E3 ligase-defective mice, we depleted mice of CD8+ cells prior to tumor inoculation by i.p. injections of specific depleting Abs (Supplemental Fig. 6). As expected, C373A+/KI could not reject tumors, and absence of CD8+ cells in these mice led to an even faster tumor growth (Fig. 6C, 6D). Importantly, whereas C373A+/KI untreated control mice exhibited efficient and spontaneous tumor rejection, C373A+/KI mice depleted of CD8+ T cells were unable to reject TC1 cells, displaying instead a progressive and lethal tumor growth (Fig. 6C, 6D). Thus, Cbl-b E3 ligase-defective CD8+ T cells mediate spontaneous tumor rejection.

**Discussion**

The characterization of Cbl-b knockout mice during the past decade has established the essential role of Cbl-b in the regulation of T cell activation, immunotolerance, and autoimmunity. The remarkably evolutionary conservation of the RING finger domain of Cbl proteins (8), together with the confirmation that this domain is directly responsible for protein ubiquitylation (13, 14), suggested that Cbl-b physiological functions are mediated by the catalytic E3 ligase activity. However, Cbl proteins contain also highly evolutionarily conserved protein-interacting domains (8), and Cbl-b binds to multiple key molecules of the TCR signalosome (6, 7) that, so far known, are not being ubiquitylated. Similarly, it has been shown that other E3 ubiquitin ligases, such as GRAIL and Itch, play critical roles in T cell activation and immunological tolerance (19, 20, 22–24). However, it was entirely unknown whether the in vivo functions of any of these key E3 ligases, and in particular Cbl-b, are indeed dependent on the E3 ligase catalytic activity or whether these molecules function in vivo as multivalent adapters (9, 10, 36). The generation of point mutant Cbl-b E3 ligase-defective mice provided us with the unique possibility to ultimately dissect the relative contribution of Cbl-b enzymatic and adapter functions in vivo.

Our data show that a loss-of-function mutation in the Cbl-b RING finger domain renders peripheral T cells hyperproliferative and able to be fully activated in the absence of CD28 costimulation. Hence, a functional RING finger catalytic domain is required for Cbl-b to repress T cell activation. More strikingly, by crossing the C373A point mutation into a mouse model of T cell tolerance, we could show that Cbl-b controls T cell anergy to specific Ags and determines the survival/lethal outcome after repetitive encounter with the same Ag in an E3 ligase-dependent manner. Additionally, Cbl-b enzymatic function is essential in the control of autoimmune responses, as inactivation of Cbl-b catalytic activity phenocopies the spontaneous autoimmunity observed in Cbl-b knockout mice. Moreover, inactivation of Cbl-b E3 ligase activity in mice resulted in a spontaneous capacity to reject TC1 tumors. Although in our model CD8* T cells are a major population contributing to tumor rejection, additional studies are needed to understand better the cellular mechanisms implicated in the remarkable tumor rejection capacity of Cbl-b E3 ligase-defective mice. Nonetheless, our study importantly suggests that Cbl-b ligase inactivation could be a novel modality to confer antitumor activity in vivo.

Cbl-b is ubiquitously expressed and participates in the modulation of other cellular immune responses beyond T cells, for example in B cells (37, 38), mast cells (39), and macrophages (40). Recent studies have confirmed that Cbl-b functions as an E3 ligase in many but interestingly not all cellular contexts. By analyzing the same Cbl-b C373A ligase-defective mice, very recent publications have demonstrated that Cbl-b enzymatic function is required to induce anergy in NKT cells (41) as well as for Foxp3 expression in TGF-β–induced regulatory T cells (42). In contrast, Cbl-b ligase activity has been reported to be differentially required for IgE receptor-mediated mast cell functions. Whereas loss of Cbl-b enzymatic activity altered some signaling pathways downstream of the FceRI receptor, Cbl-b negative regulation of the signaling pathway leading to the production of inflammatory cytokines was found to be largely independent of the RING finger domain (30). Thus, Cbl-b may biochemically function as both E3 ligase and multivalent adapter protein depending on the cellular context or even in different signaling pathways within each cell type. Our results establish the essential in vivo role of Cbl-b E3 ligase activity in the regulation of T cell tolerance, autoimmunity, and tumor immunosurveillance. A more complete understanding of the cellular and molecular mechanism involved in Cbl-b ubiquitylation-dependent immune regulation could offer a unique possibility for the development of future therapies to modulate Cbl-b E3 ligase activity to control T cell immunity in vaccination, cancer biology, or autoimmunity.

**Acknowledgments**

We thank Gabri Stengl, Mihaela Zeba, Karin Aumayr, Pawel Pasierbek, Harold Scheuch, Maria Novatchkova, and Ricardo de Matos Simoes for technical support, Cornelia J.M. Mielief for providing TC1 cells, and Stefanie Loeser and all other current and former members of the Penninger laboratory for helpful discussions.

**Disclosures**

J.M.P. holds shares in Apeiron Biologics.
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