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Essential Role of the E3 Ubiquitin Ligase Cbl-b in T Cell Anergy Induction

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Summary

Antigen-specific immunotolerance limits the expansion of self-reactive T cells involved in autoimmune diseases. Here, we show that the E3 ubiquitin ligase Cbl-b is upregulated in T cells after tolerizing signals. Loss of Cbl-b in mice results in impaired induction of T cell tolerance both in vitro and in vivo. Importantly, rechallenge of Cbl-b mutant mice with the tolerizing antigen results in massive lethality. Moreover, ablation of Cbl-b resulted in exacerbated autoimmunity. Mechanistically, loss of Cbl-b rescues reduced calcium mobilization of anergic T cells, which was attributed to Cbl-b-mediated regulation of PLCγ-1 phosphorylation. Our results show a critical role for Cbl-b in the regulation of peripheral tolerance and anergy of T cells.

Introduction

Peripheral mature T cells play a critical role in mounting immune responses against invading pathogens through the engagement of the T cell antigen receptor (TCR) with antigenic peptides presented by the major histocompatibility complex molecules (MHCs) on antigen-presenting cells (APCs) and, at the same time, are tolerant to self-antigens. The induction of peripheral T cell tolerance involves a variety of mechanisms, including the deletion of self-reactive T cells, ignorance of self-antigen, generation of regulatory T cells, and T cell anergy (Kamradt and Mitchison, 2001; Walker and Abbas, 2002). Understanding the mechanisms of the peripheral tolerance is of paramount significance, since breakdown of self-tolerance can lead to disastrous consequences like autoimmune diseases. Indeed, recent gene targeting in mice has helped in linking intracellular biochemical signals with autoimmunity (Ohashi, 2002). How these signaling molecules are involved in the process of T cell tolerance induction remains largely unclear.

Cbl-b is a member of mammalian Cbl family proteins that consist of c-Cbl, Cbl-b, and Cbl-3 (Keane et al., 1995; Thien and Langdon, 2001). Proteins of this family contain a N-terminal tyrosine kinase binding domain, a RING finger, and C-terminal proline-rich sequences and function as adaptor proteins (Thien and Langdon, 2001). The RING finger recruits ubiquitin (Ub)-loaded Ub conjugation enzymes or E2 to help transfer Ub to target proteins (Joazeiro et al., 1999; Zheng et al., 2000). For example, Cbl-b was shown to promote Ub conjugation to the p85 regulatory subunit of phosphatidylinositol-3 (PI-3) kinase, which regulates the recruitment of p85 to upstream molecules in T cells (Fang and Liu, 2001). The importance of Cbl-b in T cells is underscored by the studies of Cbl-b-deficient mice: Cbl-b$^{-/-}$ T cells show effective activation in the absence of costimulation, which results in spontaneous autoimmunity or enhanced susceptibility to autoantigens (Bachmaier et al., 2000; Chiang et al., 2000). A more recent study suggested that Cbl-b is a major susceptibility gene product for rat type 1 diabetes (Yokoi et al., 2002).

Here, we report that Cbl-b is selectively induced during the early phase of T cell unresponsiveness. Cbl-b$^{-/-}$ T cells are largely resistant to T cell anergy induction both in vitro and in vivo. Importantly, in vivo, the presence of Cbl-b determines whether mice die or survive after challenge with the same antigen. The results identify an essential role of the E3 ligase Cbl-b in the regulation of clonal T cell anergy.

Results

Cbl-b Controls T Cell Unresponsiveness In Vitro

T cell clonal anergy is a growth arrest state in which cell proliferation is greatly impaired in a number of different ways (Schwartz, 2003). We first investigated whether Cbl-b is indeed involved in ionomycin-induced functional T cell anergy, a model system previously used to mimic early events of T cell tolerance (Macian et al., 2002). As reported previously, wild-type T cells did not proliferate after ionomycin-treatment as compared to control cells upon restimulation (Figure 1A). Surprisingly, Cbl-b$^{-/-}$ T cells displayed no or only a slight reduction in proliferation when low concentrations of ionomycin were used. Similarly, the levels of IL-2 were markedly reduced in ionomycin-treated wild-type T cells, whereas IL-2 production remained much higher in Cbl-b$^{-/-}$ T cells even when the concentration of ionomycin reached 400 ng per ml (Figure 1B). To test whether Cbl-b is a true

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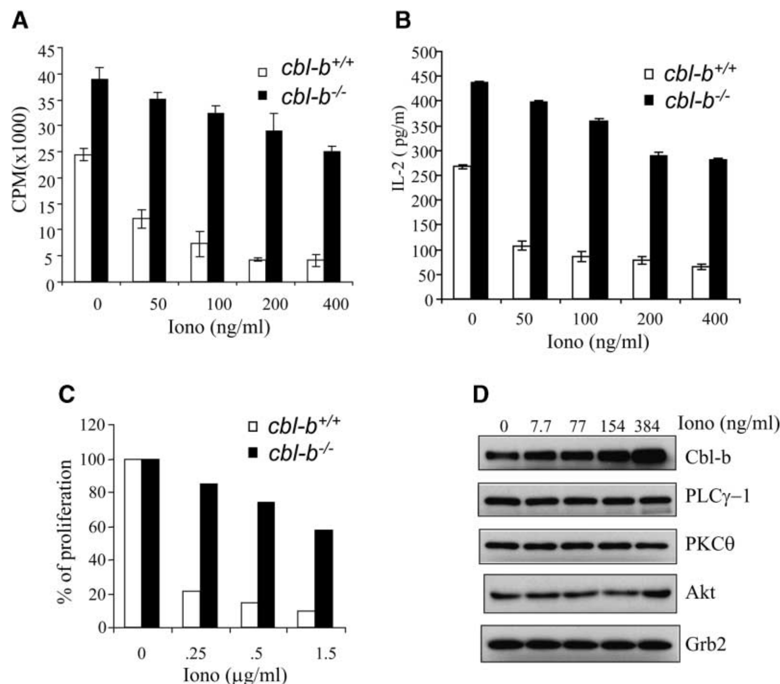


Figure 1. A Role of Cbl-b in T Cell Anergy In Vitro

(A) Activated $CD4^+$ T cells from the lymph nodes of $Cbl\text{-}b^{+/+}$ and $Cbl\text{-}b^{-/-}$ mice were cultured in the absence or the presence of different concentrations of ionomycin (Iono) for 16 hr. Cells were washed and restimulated with anti-CD3 and anti-CD28. Proliferation was measured after 48 hr by ^3H-thymidine incorporation.

(B) Supernatants from the cultured cells as in (A) were collected and measured for IL-2 production by ELISA.

(C) $CD4^+$ T cells as in (A) were pretreated with low to high doses of ionomycin and ^3H-thymidine incorporation was measured. The proliferative response is shown as percentage of proliferation. Values from cells without ionomycin treatment were considered as 100%. Data in (A) to (C) are representative of three repeated experiments.

(D) Cbl-b induction in ionomycin-treated T cells. Purified $CD4^+$ T cells from OT-II transgenic mice were cultured with OVA peptide and irradiated splenic cells for 5 days and then incubated with indicated doses of ionomycin (Iono) for 16 hr. Cell lysates were probed with antibodies as indicated. The intensity of PLCγ-1 band was quantified by Bio-Rad Quantity One software and normalized. 0 nM ionomycin treatment was considered to be basal level (1.0). The relative Cbl-b protein expression levels from lanes 1–5 are: 1.0, 1.4, 1.6, 2.2, and 3.0, respectively.

mediator of anergy induction, we next examined the proliferative responses by using very high concentrations of ionomycin. At the highest concentration of ionomycin (1.5 μg/ml), wild-type T cells showed 90% reduction in proliferation (Figure 1C), whereas the $Cbl\text{-}b^{-/-}$ T cells showed only 40% reduction; i.e., the proliferative response of $Cbl\text{-}b^{-/-}$ T cells remained strong even at the highest concentration of ionomycin. However, other factors must also be capable of inducing the anergic state.

In an antigen-induced anergy model in the absence of CD28 costimulation, we found that Cbl-b expression is induced both in the levels of mRNA and protein (data not shown). We then examined Cbl-b expression in T cells rendered unresponsive through ionomycin treatment. Treatment of T cells under anergizing culture conditions by using ionomycin alone triggered increased Cbl-b expression (Figure 1D). In addition, the ionomycin-induced Cbl-b expression was specific, since the protein levels of PLCγ-1, PKCθ, Akt, or the adaptor protein Grb2, did not show obvious alterations (Figure 1D). These data show that expression levels of the E3 ligase Cbl-b are selectively increased in anergized T cells in response to ionomycin treatment. Importantly, our data show that ablation of Cbl-b results in impaired induction of ionomycin-induced T cell tolerance.

Cbl-b Regulates PLCγ-1 Phosphorylation in Anergic T Cells

One of the characteristics of ionomycin- or antibody-induced T cell anergy is the reduced calcium mobilization upon restimulation with anti-CD3 (Heissmeyer et al., 2004; Wells et al., 2003). To gain molecular insights into Cbl-b-regulated T cell anergy, we examined whether Cbl-b affects calcium signaling. Consistent with recent findings (Heissmeyer et al., 2004; Wells et al., 2003), ionomycin-treated anergic wild-type T cells showed almost no Ca^{2+} mobilization upon TCR/CD3 crosslinking compared to untreated control T cells (Figure 2A). However, $Cbl\text{-}b^{-/-}$ T cells were largely resistant to ionomycin-induced effects on calcium mobilization. These results suggest that a Ca^{2+}-dependent pathway is at least one of the mechanisms by which Cbl-b mediates T cell anergy.

The reduced Ca^{2+} influx in anergic T cell prompted us to investigate whether TCR stimulation of ionomycin-treated T cells affects PLCγ-1, which controls Ca^{2+} mobilization in T cells. Anti-CD3 stimulation of ionomycin-pretreated T cells caused only a little or even no downmodulation of PLCγ-1 as compared to control cells (Figure 2B). Intriguingly, the amount of the phosphorylated form of PLCγ-1 markedly decreased upon anti-CD3 stimulation in ionomycin-treated wild-type T cells as compared to nonstimulated T cells. The reduced phosphorylation of PLCγ-1 in wild-type anergic T cells was not observed in $Cbl\text{-}b^{-/-}$ T cells under anergy-inducing conditions (Figure 2C). Notably, an increased phosphorylation of PLCγ-1 was observed in $Cbl\text{-}b^{-/-}$ T cells in ionomycin-untreated cells. In both wild-type and $Cbl\text{-}b^{-/-}$ T cells, anti-CD3-induced Akt phosphorylation was similar between untreated and ionomycin-treated cells. The phosphorylation of PLCγ-1 remained at increased levels in $Cbl\text{-}b^{-/-}$ T cells even when very high concentrations of ionomycin were used (Figure 2D). Since we used $Cbl\text{-}b^{-/-}$ T cells from two different mutant mouse lines (Bachmaier et al., 2000; Chiang et al., 2000) for the present study, it was important to compare PLCγ-1 phosphorylation in the anergic T cell from both types of mutant mouse lines by using higher concentrations of ionomycin. $Cbl\text{-}b^{-/-}$ T cells isolated from both

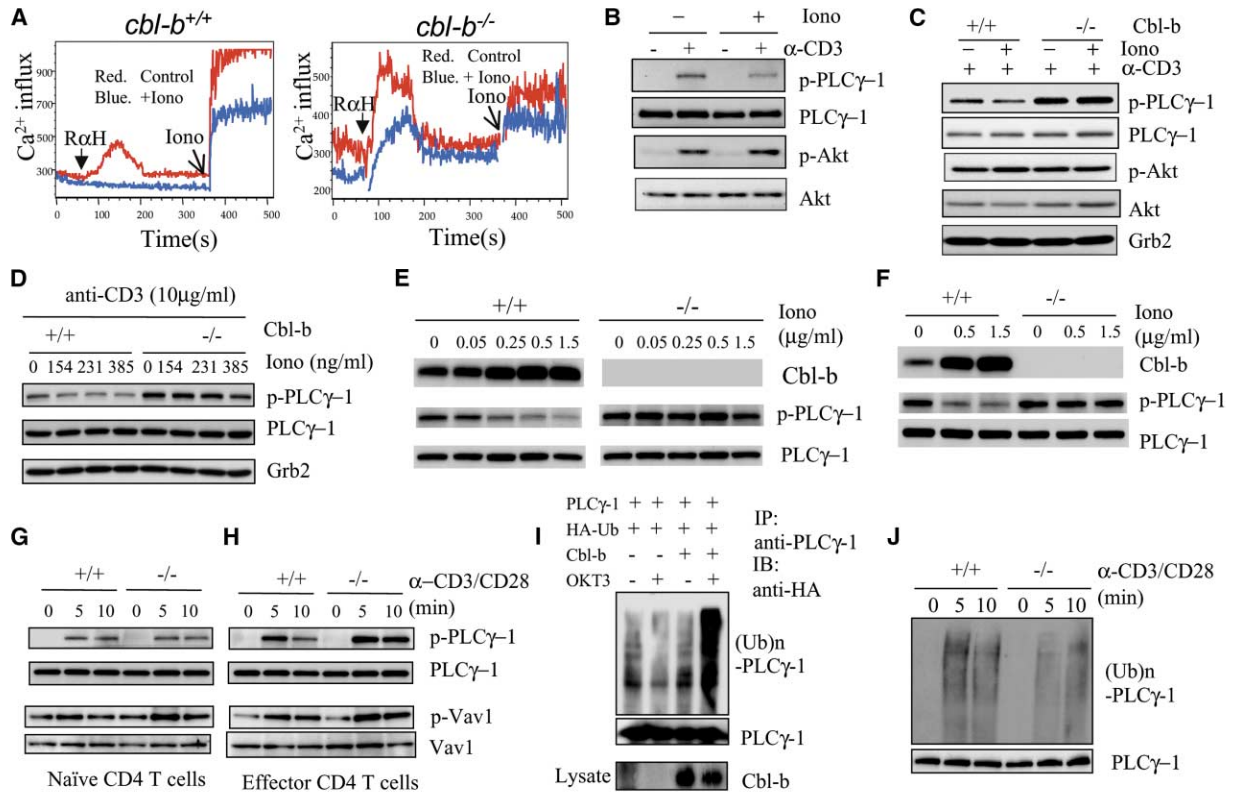


Figure 2. Cbl-b Controls PLCγ-1 Phosphorylation in Anergic T Cells

(A) Activated $CD4^+$ T cells from wild-type or Cbl-$b^{-/-}$ mice were left untreated (control) or treated with ionomycin (Iono) for 16 hr and loaded with Indo-1. Cells were incubated with anti-CD3, and Ca^{2+} release was initiated by crosslinking with secondary rabbit anti-hamster Ab (RαH, arrow). Ionomycin (open arrow) was added to control for viability.

(B) Activated $CD4^+$ T cells from wild-type mice were left untreated or treated with ionomycin as in (A). T cells were then stimulated with anti-CD3 for 3 min and cell lysates probed with phosphospecific antibodies against PLCγ-1 (p-PLCγ-1) or Akt (p-Akt). Membranes were reprobed with Abs to PLCγ-1 and Akt to control for loading.

(C) Purified $CD4^+$ T cells from wild-type (+/+) and Cbl-b deficient mice (−/−) were cultured without or with ionomycin (100 ng/ml). T cells were stimulated with anti-CD3 for 10 min and cell lysates probed with Abs as indicated. Grb2 was used as a loading control.

(D) $CD4^+$ T cells from control (+/+) and Cbl-$b^{-/-}$ (−/−) mice were treated with different concentrations of ionomycin, washed, and then stimulated with anti-CD3 for 10 min. Phosphorylation of PLCγ-1 was examined by using anti-phospho-PLCγ-1 Abs. Cell lysates were probed with anti-PLCγ-1 and anti-Grb2 Abs.

(E and F) Comparison of phosphorylation of PLCγ-1 in ionomycin-induced anergic Cbl-$b^{-/-}$ T cells isolated from two different Cbl-b mutant mouse lines ([E] = Cbl-$b^{-/-}$ mice generated by Chiang et al. 2000; [F] = Cbl-$b^{-/-}$ mice generated by Bachmaier et al. 2000). In both mouse lines we observed the same PLCγ-1 phosphorylation phenotypes. Blots were probed for Cbl-b to show Cbl-b induction and absence of Cbl-b protein in both mutant mouse lines.

(G) Purified naive $CD4^+$ T cells from wild-type (+/+) and Cbl-$b^{-/-}$ (−/−) mice were stimulated with anti-CD3 plus anti-CD28 and cell lysates blotted with Abs as indicated. To detect the phosphorylation of Vav1, lysates were subjected to anti-Vav1 immunoprecipitation and subsequent blotting with anti-phosphotyrosine Abs followed by reprobing with anti-Vav1. PLCγ-1 phosphorylation was determined as above.

(H) Purified $CD4^+$ T cells from wild-type (+/+) and Cbl-$b^{-/-}$ (−/−) mice were cultured for 2 days in the presence of anti-CD3 plus anti-CD28 (effector CD4 T cells). Cells were then rested, and restimulated with anti-CD3 and anti-CD28. Lysates were analyzed as in (G).

(I) Jurkat T cells were transfected with PLCγ-1, Xpress-Cbl-b and HA-Ub plasmids. Transfected cells were left untreated or stimulated with anti-CD3ε for 15 min. Cell lysates were immunoprecipitated with anti-PLCγ-1 and blotted with anti-HA. Membranes were then reprobed with PLCγ-1. Total cell lysates were blotted with anti-Xpress to reveal Xpress-Cbl-b. (Ub)n-PLCγ-1 indicates polyubiquitinated PLCγ-1.

(J) Purified $CD4^+$ T cells from Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ mice were stimulated and then pretreated with ionomycin as in (A). The cells were then stimulated with anti-CD3 plus anti-CD28 and subjected to immunoprecipitation with anti-PLCγ-1. Immunoprecipitates were blotted with anti-Ub antibody and membranes reprobed with anti-PLCγ-1.

lines displayed a rescue in the reduced PLCγ-1 phosphorylation induced by ionomycin treatment, even at a concentration of 1.5 μg/ml (Figures 2E and 2F). Thus, in two different genetically mutant lines, Cbl-b controls PLCγ−1 phosphorylation in ionomycin-induced T cell tolerance.

We and others have previously shown that in freshly isolated T cells Cbl-b deficiency does not affect tyrosine phosphorylation of PLCγ-1 but controls Vav1 phosphorylation (Bachmaier et al., 2000; Chiang et al., 2000). To test whether the increased phosphorylation of PLCγ-1 in Cbl-$b^{-/-}$ T cells is unique in previously stimulated T cells, we directly compared the phosphorylation of these two proteins in naïve versus activated T cells. Consistent with our previous observation, the phosphorylation of Vav1, but not PLCγ-1, was increased in naive Cbl-$b^{-/-}$ T cells (Figure 2G). Tyrosine phosphorylation of PLCγ-1 was augmented in activated Cbl-$b^{-/-}$ T cells

upon restimulation, whereas the tyrosine phosphorylation of Vav1 was not further altered in activated T cells as compared to naive T cells (Figure 2H). Thus, the regulation of PLCγ-1 and calcium mobilization by Cbl-b appears to be distinct in naive versus activated T cells.

We next examined whether Cbl-b can act as an E3 ligase for PLCγ-1. Transient Cbl-b overexpression in Jurkat cells increased PLCγ-1 ubiquitination that depended on TCR stimulation (Figure 2I). More importantly, after ionomycin-treatment, anti-CD3 stimulation induced Ub conjugation to PLCγ-1 in wild-type T cells, whereas the PLCγ-1 ubiquitination in Cbl-b$^{-/-}$ T cells was substantially reduced (Figure 2J). Taken together, these data indicate that induction of T cell tolerance results in increased Cbl-b expression and subsequently impaired PLCγ-1 activation and calcium mobilization. Loss of Cbl-b prevents this impaired PLCγ−1 activation and restores calcium influx. Moreover, Cbl-b can directly regulate ubiquitination of endogenous PLCγ-1 in T cells after antigen receptor engagement.

Adoptive Transfer of OVA-Tolerized CD4$^+$ T Cells

To examine the relevance of Cbl-b in the T cell tolerance, we employed an adoptive transfer experiment by using OT-II TCR transgenic mice (Bansal-Pakala et al., 2001; Kearney et al., 1994). OT-II transgenic mice recognize an OVA peptide in the context of MHC class II (Barnden et al., 1998). Loss of Cbl-b had no apparent effect on development and selection of OT-II TCR transgenic thymocytes or the numbers and surface receptor expression of mature OT-II T cells (not shown).

Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ OT-II transgenic mice were first injected with high dose of soluble OVA peptide to induce T cell tolerance or with PBS as control. CFSE-labeled CD4$^+$ T cells from these mice were then transferred into syngeneic recipients followed by immunization with OVA peptide in CFA (Figure 3A). Both wild-type and Cbl-b$^{-/-}$ T cells from PBS-treated mice proliferated in response to OVA peptide challenge. OVA-tolerized wild-type T cells failed to divide in the recipient. However, Cbl-b$^{-/-}$ T cells from OVA-tolerized mice displayed cell proliferation after antigen restimulation. Wild-type T cells from OVA-tolerized mice showed also a marked reduction in IL-2 concentration, accompanied by decreased in vitro proliferation (Figure 3B). Notably, the reduced cell proliferation and IL-2 production were again rescued in Cbl-b$^{-/-}$ OVA-tolerized T cells. These data show that Cbl-b controls the induction of antigen-specific CD4$^+$ T cell tolerance.

Cbl-b Controls T Cell Anergy Induction in CD8$^+$ P14 Transgenic Mice

To evaluate whether Cbl-b was also relevant for anergy induction of cytotoxic CD8$^+$ T cells in vivo, we crossed Cbl-b$^{-/-}$ mice (Bachmaier et al., 2000) with mice that carry the P14 TCRVα2Vβ8.2 transgene (Pircher et al., 1989). The P14 TCR recognizes the LCMV p33 peptide presented by MHC class I. P14 TCR expressing cells do not respond to the "wrong" peptide (AV), which also binds to the same MHC class I molecule. Loss of Cbl-b had no apparent effect on development and selection of P14 TCR transgenic thymocytes or the numbers and surface receptor expression of mature P14 T cells (not shown). Intravenous injection of the cognate p33 peptide results in expansion of antigen-specific P14 T cells followed by deletion of the activated cells (Figure 4A and Kyburz et al., 1993). Repeated injection of P14 TCR transgenic mice with the p33 antigen leads to peptide-specific T cell anergy defined by impaired proliferation and IL-2 production to rechallenge with the p33 peptide. The T cell tolerance phenotype can be first detected around day 4 (Figure 4B). Addition of IL-2 reverses the proliferative defect of in vivo tolerized P14 TCR transgenic cells (Figure 4B). Importantly, whereas Cbl-b expression was low in activated T cells on day 2 after the initial injection, Cbl-b protein expression was increased in anergized T cells after a second p33 challenge (Figure 4C).

Injection of p33 into Cbl-b$^{-/-}$ mice resulted in increased in vivo expansion of Cbl-b$^{-/-}$ P14 T cells after the first peptide injection (Figure 4D). This increased in vivo response paralleled increased in vitro responses of naive Cbl-b$^{-/-}$ P14 T cells to the cognate p33 antigen (Figure 4E). However, although Cbl-b$^{-/-}$ P14 T cells showed increased expansion on day 2 after the first p33 injection, rechallenge of P14 TCR expressing cells in a Cbl-b-deficient mouse background did not result in increased proliferation in vitro (Figure 4F). Importantly, the kinetics of deletion of activated T cells was comparable between Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ mice (Figure 4D). Intriguingly, whereas control T cells display peptide-specific anergy after the second p33 injection, in vivo antigen rechallenge of P14 TCR-expressing cells in a Cbl-b-deficient mouse background did not induce T cell anergy; Cbl-b$^{-/-}$ P14 T cells still proliferated ex vivo to the cognate antigen p33 but did not respond to the wrong peptide AV. The in vitro response of Cbl-b$^{-/-}$ P14 T cells also occurred at low antigen doses (range 0.1–5 μg/ml) and at different time points of analyses (days 4, 7, and 8) (Figures 4G–4I). Since Cbl-b$^{-/-}$ P14 T cells exhibited increased expansion to the initial p33 injection, it was possible that we had "hyperactivated" these cells; however, even three challenges (days 0, 3, and 6) of Cbl-b$^{-/-}$ P14 mice with low doses of p33 (0.1 μg per mouse per injection) could not induce anergy in Cbl-b$^{-/-}$ P14 T cells. Thus, loss of Cbl-b in CD8$^+$ P14 TCR transgenic T cells prevents the induction of T cell tolerance to their cognate peptide antigen in vivo.

Increased Mortality of Cbl-b$^{-/-}$ Mice upon p33 Antigen Rechallenge

Intriguingly, whereas all Cbl-b expressing P14 TCR transgenic mice survived a second challenge with the cognate p33 antigen on day 3, over 50% of Cbl-b$^{-/-}$ P14 TCR transgenic mice died within hours after a second p33 peptide challenge. Only four out of 41 Cbl-b$^{-/-}$ P14 mice survived a third challenge with p33 (Figure 5A). Even at low doses of in vivo restimulation, i.e., variation of the p33 dose at the third p33 injection, this lethal phenotype was frequently observed. Challenge of Cbl-b$^{-/-}$ P14 TCR transgenic mice with the wrong antigen AV or the low-affinity peptide A4Y, a moderate agonist relative to p33 (Sebzda et al., 1996) did not result in any detectable in vivo T cell responses or lethality (Figure 5A), indicating that the response was p33 peptide specific. Mutant mice developed massive edemas of multiple tissues including the liver, lung, and intestine and died most likely due to respiratory and cardiovascular failure

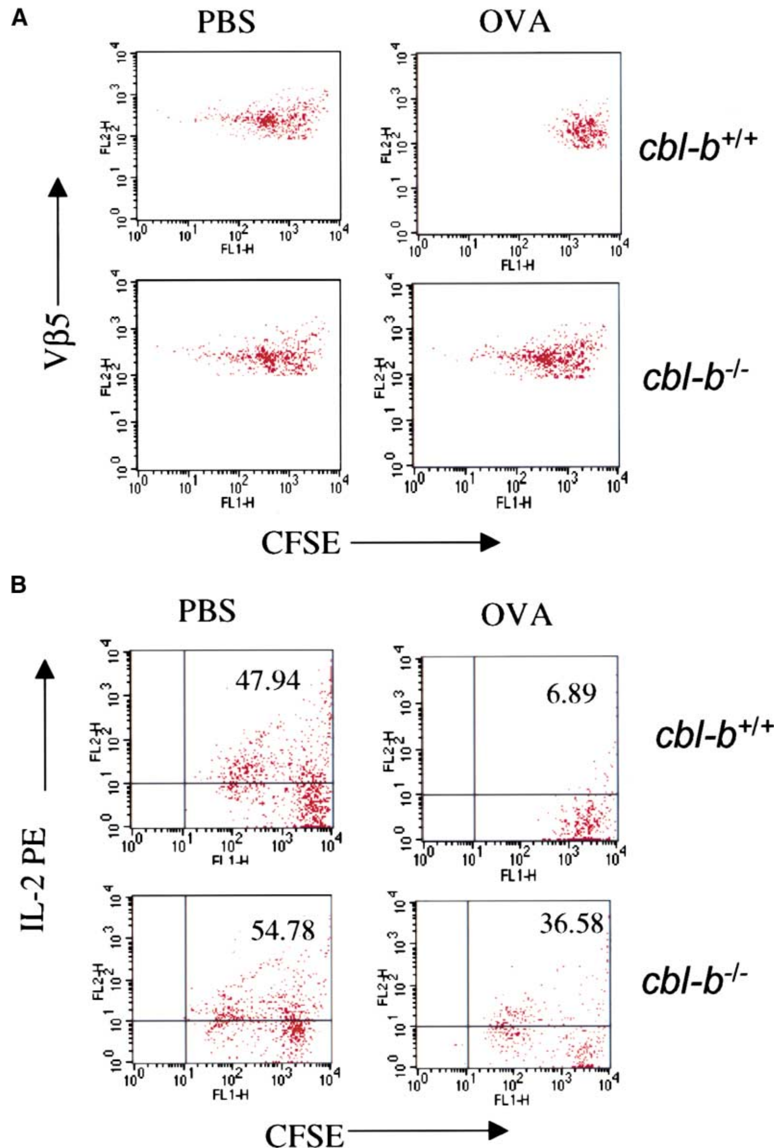


Figure 3. Cbl-b Controls Anergy Induction of $CD4^+$ OT-II T Cells

(A) Cbl-$b^{+/+}$ and Cbl-$b^{-/-}$ OT-II transgenic mice were tolerized with high doses of soluble OVA peptide or with PBS as control. $CD4^+$ T cells ($V\beta5^+$Thy1.2^+) purified from those mice were labeled with CFSE and adoptively transferred into syngeneic Thy1.1 mice. The recipients were immunized with peptide in CFA. Three days later, CFSE/$V\beta5^+$ T cells were analyzed by FACS.

(B) $CD4^+$ T cells from the recipients as in (A) were restimulated in vitro by incubating with OVA peptide plus irradiated APCs and the intracellular IL-2 concentration as well as proliferation (as determined by CFSE) were measured by FACS.

(Figure 5B). We also generated Cbl-$b^{-/-}$ P14 mice on a Rag$2^{-/-}$ background. In these mice, not a single Cbl-$b^{-/-}$ Rag$2^{-/-}$ P14 Tg mouse (n = 9) survived the second p33 challenge on day 3, presumably due to the larger numbers of P14 TCR Tg cells in such mice. Moreover, organ damage was also more severe in Cbl-$b^{-/-}$Rag$2^{-/-}$ P14 mice when compared to Cbl-$b^{-/-}$Rag$2^{+/+}$ P14 mice.

At the time of the in vivo rechallenge on day 3 or day 6 and at days 4, 5, and 8, we did not observe any apparent differences in antigen receptor levels or expression of surface molecules such as CD28, CD8, CD4, CD44, CD62L, or CD69 between Cbl-b-expressing and Cbl-b mutant P14 T cells. By contrast, transient induction of CD25 expression was markedly increased in Cbl-$b^{-/-}$ P14 T cells, suggesting that anergized T cells display selective activation defects in vivo. Since we observed a defect in CD25 upregulation, we analyzed cytokine expression levels in vivo following p33 immunization. As compared to Cbl-$b^{+/-}$ P14 mice, Cbl-$b^{-/-}$ P14 mice produced large amounts of serum IFNγ, TNFα, and IL-2 after in vivo rechallenge with p33 on day 3 (Figure 6A), suggesting that loss of Cbl-b releases "anergized" T cells from their defective cytokine production in vivo. Increased cytokine levels were also detectable after adjustment to $CD8^+$ P14 T cell numbers (Figures 6B–6D). These increased cytokine levels were only observed after the in vivo p33 rechallenge on day 3, indicating that this cytokine storm was triggered by the cognate antigen (Figures 6B–6D). Which one of these cytokines is the main mediator of death needs to be determined. Thus, expression of the E3 ligase Cbl-b is essential to induce in vivo tolerance of peptide-specific T cells, and expression of Cbl-b determines whether animals survive or die due to toxic T cell activation in response to repeated antigenic challenge.

Regulation of SEB-Induced Tolerance by Cbl-b

T cells recognize antigens as peptides in the groove of MHCs or as superantigens (Kotzin et al., 1993). To examine whether, in addition to peptide-specific $CD8^+$ and $CD4^+$ T cells, Cbl-b has also a role in superantigen-induced tolerance (Kawabe and Ochi, 1990; Rellahan

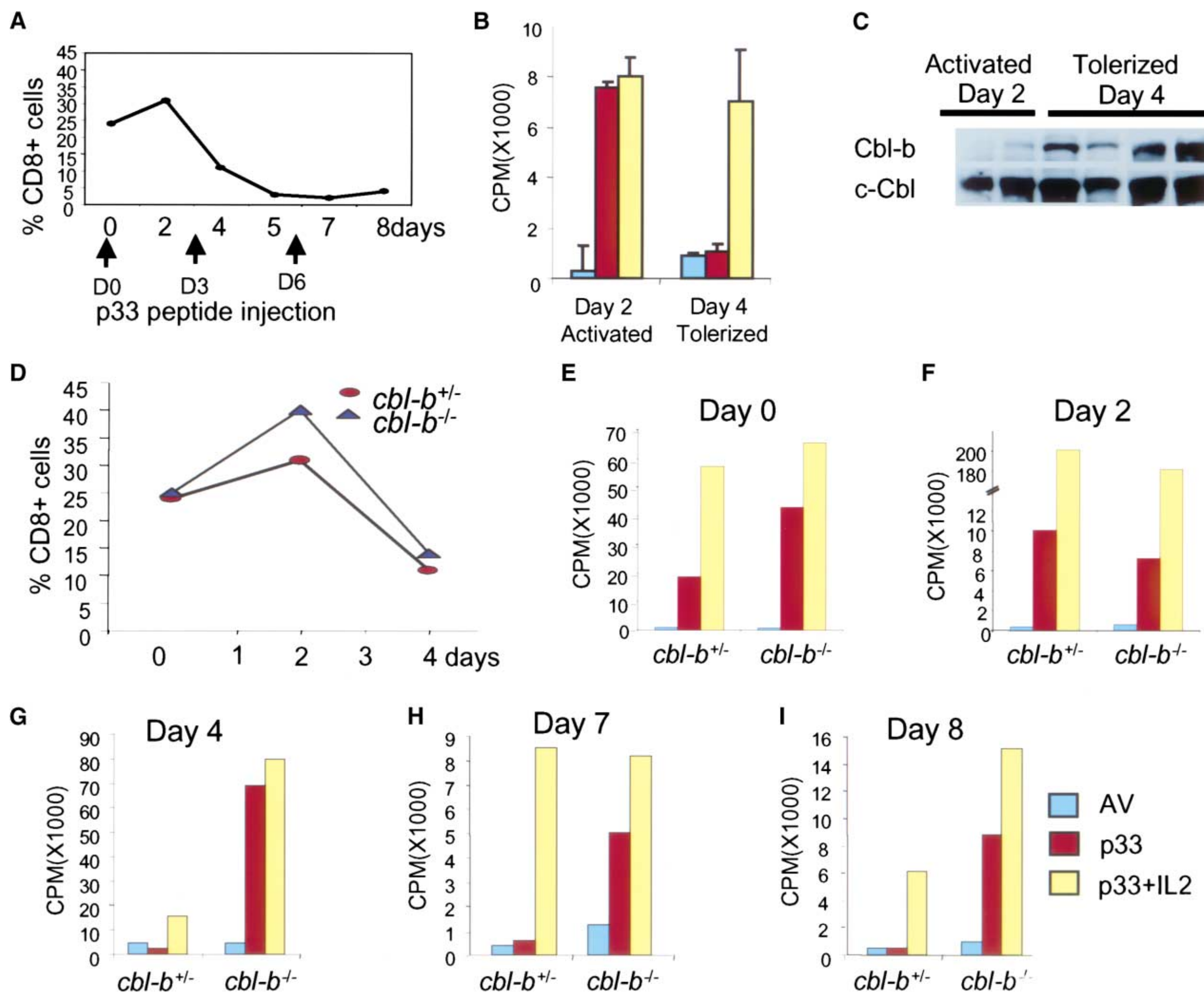


Figure 4. Loss of Cbl-b Prevents Induction of Peptide-Specific In Vivo Tolerance of CD8$^+$ T Cells

(A) Typical expansion and deletion curve of wild-type CD8$^+$ P14 Tg splenic T cells. The time points of intravenous p33 peptide injections are indicated. Mean values of four mice per group are shown.

(B) Splenic T cells were isolated from wild-type mice on day 2 and day 4 after p33 peptide injection (i.v., see scheme in [A]) and restimulated in vitro with APCs loaded with the wrong peptide AV or the high-affinity peptide p33 (10^{-6} M).

(C) Western blot for Cbl-b and c-Cbl expression in T cells isolated from wild-type mice on day 2 (activated) and day 4 (anergic) after p33 injection. Data from individual mice are shown.

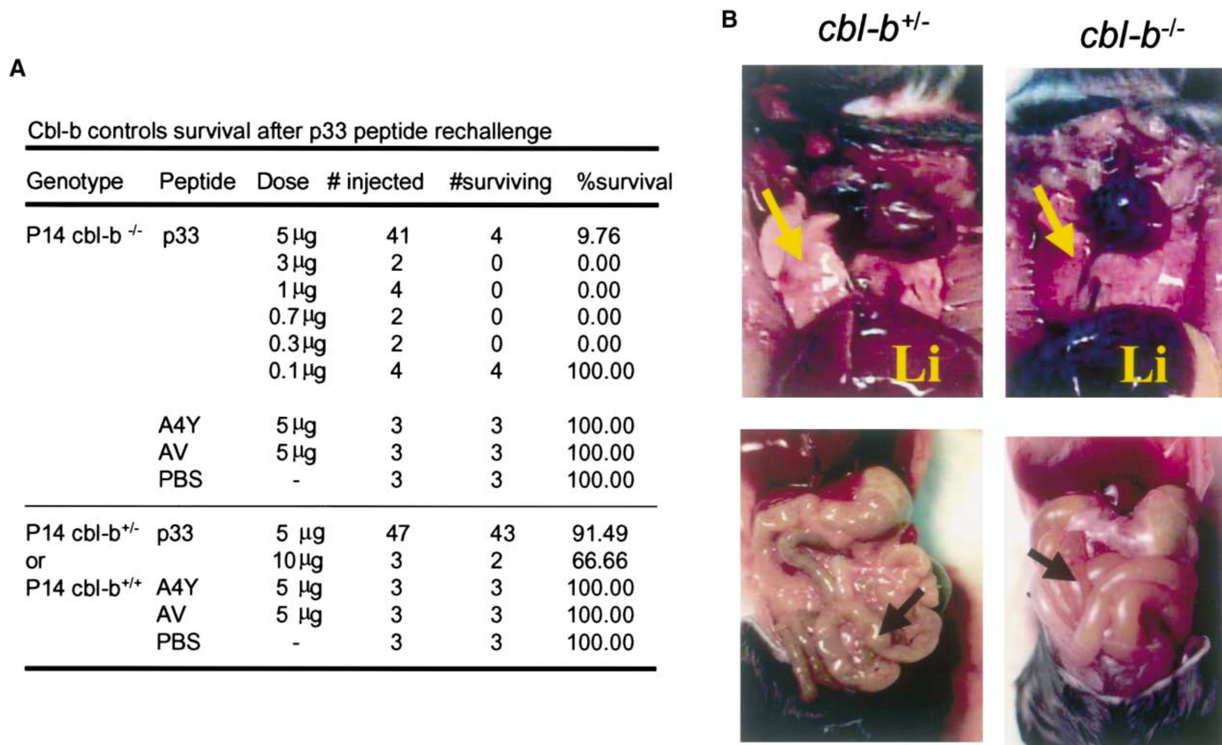
(D) Typical expansion and deletion curve of Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ CD8$^+$ P14 Tg splenic T cells after p33 peptide injections on days 0 and 3. Mean values of at least four mice per group are shown.

(E–I) Splenic T cells were isolated from P14 Cbl-b$^{+/-}$ and P14 Cbl-b$^{-/-}$ mice on day 0 (nonimmunized mice), and day 2 (activated), day 4, day 7, and day 8 after p33 peptide injection on day 0, day 3, and day 6 (i.v., see scheme in [A]). Cells were restimulated in vitro with APCs loaded with the wrong peptide AV or the high-affinity peptide p33 (10^{-6} M). Addition of murine rIL-2 (10 Units/ml) rescues the anergic phenotype in Cbl-b expressing T cells. Data are representative of at least five different experiments.

et al., 1990), we crossed Cbl-b$^{-/-}$ mice onto an H2^d background and challenged these mice with the bacterial superantigen staphylococcal enterotoxin B (SEB), a causative agent of food poisoning and toxic shock syndrome. Similar to the peptide challenge, the initial expansion of SEB reactive CD4$^+$ T cells was increased in Cbl-b$^{-/-}$ mice, but both Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ T cells displayed similar kinetics and extents of activation-induced T cell deletion (Figure 6E). Cbl-b$^{+/+}$ mice were tolerant to SEB and did not display apparent CD4$^+$ T cell responses after a second in vivo challenge with SEB. By contrast, CD4$^+$ T cells from Cbl-b$^{-/-}$ mice could be reactivated to expand in vivo 6 days after the primary injection (not shown) or 25 days after the primary injection (Figure 6E), and such SEB reactivated Cbl-b$^{-/-}$ T cells produced enhanced amounts of cytokines (Figure 6F). Importantly, whereas none of the Cbl-b expressing mice showed morbidity in response to a second SEB injection, five out of eight Cbl-b$^{-/-}$ mice died within hours after the SEB rechallenge on day 6. Thus, Cbl-b is a critical molecule involved in in vivo immunotolerance to peptide- as well as superantigens.

Increased Incidence of Autoimmune Arthritis

To extend our tolerance studies to in vivo autoimmunity, we backcrossed the Cbl-b mutation onto DBA/1 mice



A

Cbl-b controls survival after p33 peptide rechallenge

Genotype	Peptide	Dose	# injected	#surviving	%survival
P14 cbl-b -/-	p33	5 μg	41	4	9.76
		3 μg	2	0	0.00
		1 μg	4	0	0.00
		0.7 μg	2	0	0.00
		0.3 μg	2	0	0.00
		0.1 μg	4	4	100.00
	A4Y	5 μg	3	3	100.00
	AV	5 μg	3	3	100.00
	PBS	-	3	3	100.00
P14 cbl-b+/-	p33	5 μg	47	43	91.49
or		10 μg	3	2	66.66
P14 cbl-b+/+	A4Y	5 μg	3	3	100.00
	AV	5 μg	3	3	100.00
	PBS	-	3	3	100.00

Figure 5. Repeated Peptide Challenge Results in Death of P14 Cbl-b$^{-/-}$ Mice

(A) Lethality/survival table. Mouse genotypes, mouse numbers, and percentages of survival are indicated. All mice were injected with the indicated peptides or PBS as a vehicle control on day 0, day 3, and day 6. Lethality occurred in all instances within 24 hr after the in vivo rechallenge.

(B) Macroscopic images of lungs (top, arrows) and intestines (bottom, arrows) of P14 Cbl-b$^{+/-}$ and P14 Cbl-b$^{-/-}$ mice after a second p33 challenge on day 3. P14 Cbl-b$^{-/-}$ mice display labored breathing followed by irregular breathing near death. At necropsy lungs were filled with fluid. We observed massive capillary dilation and hemorrhaging. All morbid mice have spider veins in their skin and flushed skin. Arrows show lungs (top) and intestines (bottom). Li = livers.

for six to eight generations. Cbl-b$^{+/+}$ and Cbl-b$^{-/-}$ DBA/1 littermates were immunized with collagen emulsified in complete Freund's adjuvant (CFA). 21 days later, immunized mice were boosted with collagen in mineral oil only containing incomplete Freund's adjuvant (IFA). By day 32 after the first injection, about 50% of Cbl-b$^{+/+}$ DBA/1 mice developed arthritis, whereas almost 100% of Cbl-b$^{-/-}$ DBA/1 mice were arthritic, with a few mice showing sign of arthritis before the second booster (Figure 7A). Cbl-b$^{-/-}$ DBA/1 mice also displayed markedly more severe joint inflammation, with earlier appearance of paw swelling and concurrent occurrence of both forelimb and hindlimb thickening as compared to their Cbl-b$^{+/+}$ DBA/1 littermates.

The first injection of collagen with IFA does not induce arthritis development in DBA/1 mice (Germann et al., 1995). The increased incidence of arthritis in Cbl-b$^{-/-}$ DBA/1 mice prompted us to investigate whether Cbl-b deficiency can overcome the requirement of CFA coinjection. Intriguingly, immunization with collagen-IFA in the initial phase caused a late onset of arthritis in Cbl-b$^{-/-}$ DBA/1 mice, with 50% of the mice suffering from arthritis at day 40 (Figure 7B). By day 50, all Cbl-b$^{-/-}$ DBA/1 mice developed arthritis. In contrast, none of the Cbl-b$^{+/+}$ DBA/1 littermates showed any disease signs at any time point. At day 43 after immunization with collagen in IFA, joints from Cbl-b$^{-/-}$ DBA/1 mice exhibited synovial hyperplasia and erosion of cartilage and bone (Figure 7C). By contrast, the joints of Cbl-b$^{+/+}$ DBA/1 mice did not display any apparent changes in the morphology of the synovia, the cartilage, and the bone, findings that are consistent with the failure to develop arthritis in Cbl-b$^{+/+}$ DBA/1 mice. In addition, we observed marked increases in the collagen-specific IgG2a production at day 27 in Cbl-b$^{-/-}$ DBA/1 mice as compared with Cbl-b$^{+/+}$ DBA/1 mice after immunization with collagen in IFA (Figure 7D). Thus, ablation of Cbl-b exacerbates autoimmune arthritis even in the absence of mycobacterial adjuvant. These results provide novel experimental evidence that Cbl-b is a critical negative regulator of antigen-induced arthritis induction in vivo.

Discussion

Multiple mechanisms of T cell immunotolerance have been reported, such as deletion of autoreactive T cells in the thymus and peripheral lymphoid organs or the control of T cell activation by regulatory T cells (Schwartz, 2003). Moreover, it has been observed that antigen receptor stimulation in the absence of appropriate costimulation results in T cell anergy. However, the molecular mechanisms of stimulation-dependent T cell anergy are largely unknown. Cbl-b is of particular interest since in naive T cells loss of Cbl-b uncouples TCR stimulation

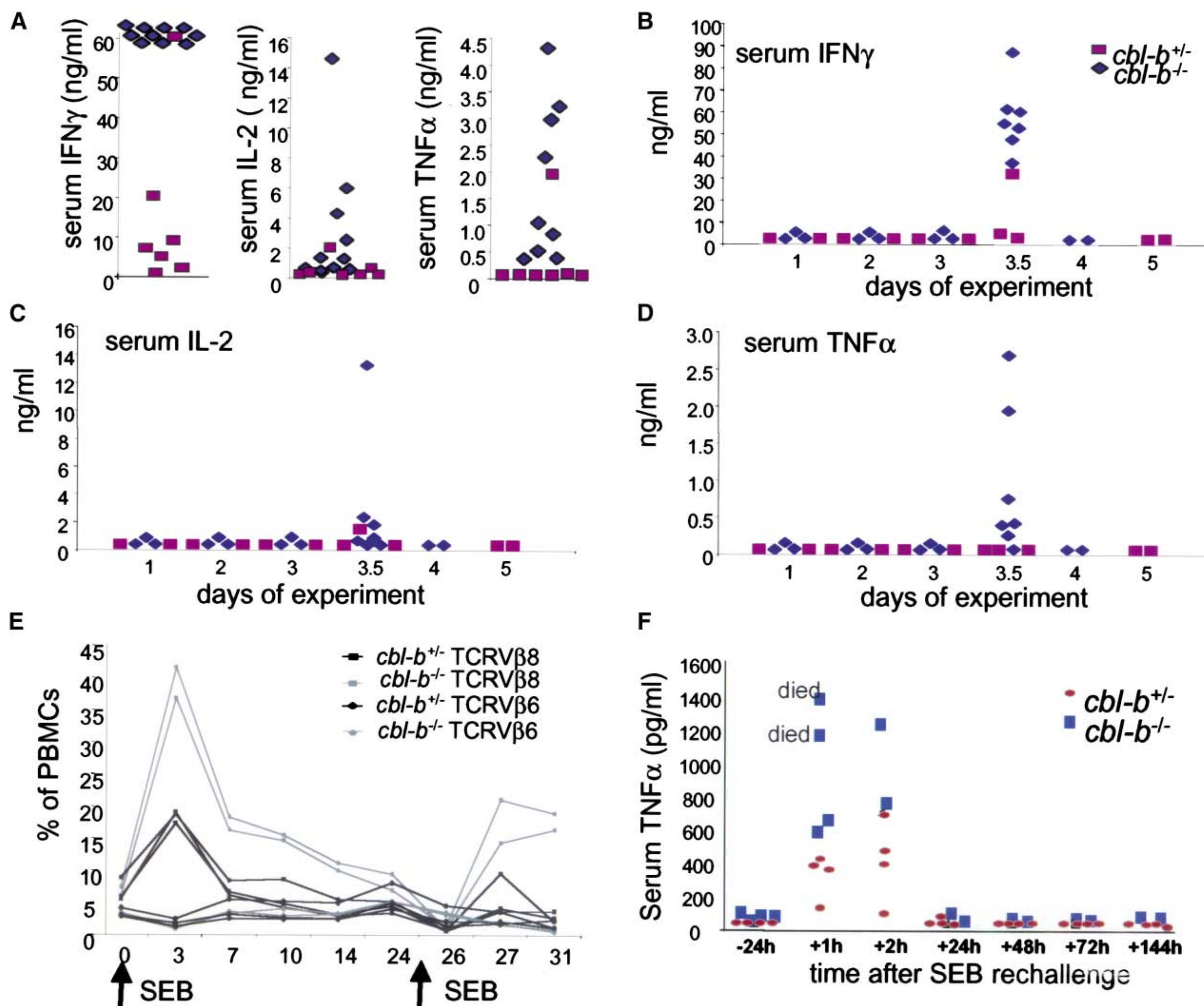


Figure 6. Cbl-b Controls In Vivo Tolerance

(A) In vivo serum cytokine levels in P14 Cbl-b$^{+/-}$ and P14 Cbl-b$^{-/-}$ mice measured at 8–16 hr after the second p33 (5 μg/ml) injection. TNFα, IFNγ, and IL-2 serum levels were determined by ELISA. For IFNγ it should be noted that the detected serum levels were at the upper detection limit of our ELISA assay indicating that the "real" values are much higher. Data from individual mice are shown.

(B–D) Kinetics of cytokine serum levels in P14 Cbl-b$^{+/-}$ and P14 Cbl-b$^{-/-}$ mice immunized with p33 (5 μg/ml) on day 0 and day 3. In these panels cytokine levels were adjusted to the relative numbers of CD8$^+$ P14 T cells in each mouse to exclude a bias in cell numbers. Cytokine serum levels immediately after the p33 injection were not determined, and cytokine levels in P14 Cbl-b$^{-/-}$ mice could only be followed until day 4 due to the massive lethality in response to the second p33 injection. TNFα, IFNγ, and IL-2 serum levels were determined by ELISA. Data from individual mice are shown.

(E) Cbl-b controls SEB-induced tolerance in vivo. Expansion and deletion curves of TCRVβ8$^+$ CD4$^+$ Cbl-b$^{+/-}$ and TCRVβ8$^+$ CD4$^+$ Cbl-b$^{-/-}$ T cells in response to SEB (20 μg). The time points of SEB injections on days 0 and 25 are indicated. Data from individual mice are shown. Data are representative of three different experiments.

(F) In vivo serum cytokine levels in Cbl-b$^{+/-}$ and Cbl-b$^{-/-}$ mice measured at 8–16 hr after a second SEB (20 μg) injection on day 6. TNFα levels were determined by ELISA. Data from individual mice are shown.

from the requirements of CD28 costimulation for effective proliferation and IL-2 production (Bachmaier et al., 2000; Chiang et al., 2000). Cbl-b$^{-/-}$ and c-Cbl$^{-/-}$Cbl-b$^{-/-}$ double mutant mice develop spontaneous multiorgan autoimmunity and display increased susceptibility to EAE, vasculitis, and arthritis induction (Bachmaier et al., 2000; Chiang et al., 2000; Krawczyk et al., 2000; Naramura et al., 2002; and the current study). Moreover, Cbl-b has been recently identified as a type I diabetes susceptibility gene in rats (Yokoi et al., 2002), indicating that Cbl-b is a critical autoimmunity gene. Our results provide a molecular framework for the role of Cbl-b in autoimmunity; that is, Cbl-b is selectively induced in tolerizing conditions and Cbl-b expression is essential for antigen-receptor specific induction of immunotolerance in vivo.

Based on our results we propose that Cbl-b regulates the autoimmune response via participating in the process of T cell unresponsiveness. Since cbl-b$^{-/-}$ T cells can be still anergized at high doses of ionomycin, albeit less efficiently, other factors must also be capable of inducing the anergic state. Earlier studies showed that anergic T cells display altered levels of protein tyrosine phosphorylation (Bhandoola et al., 1993; Cho et al.,

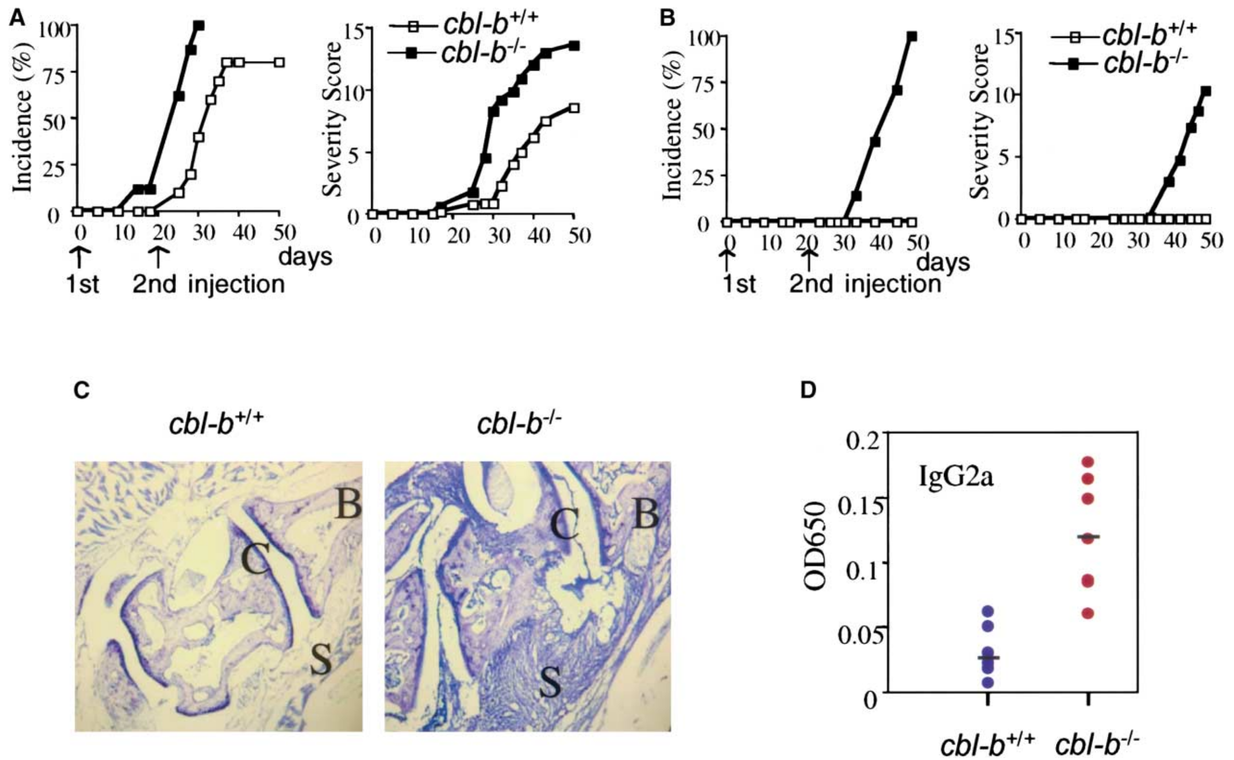


Figure 7. Collagen-Induced Arthritis in Cbl-b$^{-/-}$ DBA/1 Mice

(A) Cbl-b$^{+/+}$ or Cbl-b$^{-/-}$ DBA/1 mice were immunized with collagen in CFA on day 0 and boosted with collagen in IFA on day 21. Ten Cbl-b$^{+/+}$ and 8 Cbl-b$^{-/-}$ DBA/1 mice were used. Incidence of arthritis (left) and severity of joint inflammation (right) were scored.

(B) Cbl-b$^{+/+}$ (n = 8) and Cbl-b$^{-/-}$ (n = 7) DBA/1 mice were immunized with collagen in IFA in the first and the second injections and monitored for the incidence (left) and severity of arthritis (right).

(C) At day 43 after immunization with collagen in IFA (as in B), mice were sacrificed and joints removed for histological examination with crystal violet staining. Joints from Cbl-b$^{-/-}$ mice showed erosion of cartilage (C), bone (B), and synovial hyperplasia (S) (right), in comparison with joints from Cbl-b$^{+/+}$ mice (left).

(D) Sera from collagen-IFA immunized mice (n = 6) were collected at day 27 after immunization with collagen-IFA and collagen-specific IgG2a was measured by ELISA.

1993; Gajewski et al., 1994; Wells et al., 2003). T cell anergy can also result in a block in the downstream signaling events such as activation of the small GTPase Ras or the activation of Erk and JNK (Fields et al., 1996; Li et al., 1996). The recent discovery of anergy-associated genes suggested that intracellular signaling molecules are indeed involved in the T cell anergy process, and it was proposed that unbalanced NFAT signaling induces genes that differ from those induces by the combined activation of both NFAT and AP-1 (Macian et al., 2002). A recent study suggested that the E3 Ub ligase Grail, a transmembrane protein residing in the endosomes, is a mediator of T cell anergy induction. Grail is only expressed in anergized T cells and might control T cell unresponsiveness through targeting endocytosis of its potential substrates (Anandasabapathy et al., 2003). Our study is consistent with a recent study showing that E3 ligases such as Cbl-b, Itch, and Grail were upregulated during T cell anergy induction, which caused the downmodulation of signaling molecules and unstable synapse formation by using in vitro models (Heissmeyer et al., 2004). Whether Itch and Grail indeed control immunotolerance in vivo needs to be determined. Importantly, our genetic data on Cbl-b provide in vivo evidence that an E3 ubiquitin ligase; i.e., Cbl-b, is indeed critically involved in T cell anergy.

It should be noted that our study shows reduced phosphorylation of PLCγ-1 in contrast to enhanced PLCγ-1 degradation as reported previously (Heissmeyer et al., 2004). The discrepancy between these two studies may reflect the time frame for the examination of PLCγ-1: the change of phosphorylation is more obvious at the early stage of T cell restimulation, as revealed in our study. Indeed, the observed reduction of PLCγ-1 phosphorylation in anergic T cells occurs rapidly after subsequent TCR engagement (3–10 min) and coincides with the failed Ca^{2+} mobilization, in contrast to the protein degradation event that happens much later (30 min to 2 hr, a time frame much later than Ca^{2+} influx) (Heissmeyer et al., 2004). Thus, our data provide a more physiologically relevant model supporting ubiquitination-dependent modification of signaling molecules in the earlier phase of T cell anergy induction.

Our results suggest the following scenario for Cbl-b regulated immunotolerance: in response to tolerigenic signals, Cbl-b expression is selectively induced in anergic T cells. Cbl-b then participates in T cell tolerance via promoting ubiquitination of PLCγ-1 and regulation of antigen receptor-induced calcium mobilization. Significantly, we found that Cbl-b upregulation does not lead to obvious degradation of PLCγ-1. Instead, Cbl-b is largely involved in functional modifications (i.e., phos-

phorylation) of PLCγ-1, most likely via Cbl-b-mediated protein ubiquitination. It should be noted that Cbl-b plays a more important role for PLCγ-1 ubiquitination in activated and/or tolerized T cells, since Cbl-b does not seem to regulate the phosphorylation of PLCγ-1 in naive T cells. It therefore appears that increased Cbl-b expression resets the activation threshold for antigen receptor stimulation. However, this reset must be specific for at least some defined signaling pathways since we only observed PLCγ-1, but not Akt, hyperphosphorylation. Our data provide a novel molecular framework on the role of ubiquitination-dependent modification of signaling molecules in the earlier phase of T cell anergy induction. Importantly, our results show that the regulation of signaling molecules via E3 ligases such as Cbl-b is critical in setting up and maintaining immunotolerance in vitro and in vivo.

Great efforts have been made to understand the mechanisms of the peripheral tolerance, since loss of self-tolerance is closely related to the development of autoimmune diseases in animal models and humans. However, the pathways leading to tolerance induction are not well elucidated. Our data directly link the regulation of protein ubiquitination to T cell immunotolerance and clonal anergy in vivo. Intriguingly, in most instances the outcome of only a single challenge with a recall peptide- or superantigen in Cbl-b$^{-/-}$ mice is death of the animal. Thus, Cbl-b-regulated T cell tolerance appears to be essential for the survival of an animal after repeated encounters with the same antigen. These data also show that the molecular control of antigen receptor signaling is an essential component for the maintenance of T cell immunotolerance. Moreover, we show here that Cbl-b is an important negative regulator of autoimmune arthritis; ablation of Cbl-b exacerbates the autoimmune arthritis even in the absence of mycobacterial adjuvant. Thus, our results might have relevance for novel vaccine designs and therapeutic interventions in autoimmune diseases.

Experimental Procedures

Mice

Two different Cbl-b$^{-/-}$ mutant mouse lines (Bachmaier et al., 2000; Chiang et al., 2000) were used for the present studies. DBA/1 mice and B6.PL-Thy1a (Thy1.1) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). For the generation of TCR transgenic mice, $OVA_{323-329}$-specific TCR transgenic OT-II mice were crossed with Cbl-b$^{-/-}$ mice (Chiang et al., 2000). Moreover, P14 TCR Tg mice (Pircher et al., 1989) were bred onto the Cbl-b$^{-/-}$ (Bachmaier et al., 2000; Chiang et al., 2000) and Rag2$^{-/-}$ backgrounds. Genotyping was performed by DNA blotting, PCR, and FACS analyses. For Figures 1, 2, 3, and 7, mice generated by Chiang et al. (2000) were used. For the Figures 2F, 4, 5, and 6, mice generated by Bachmaier et al. (2000) were used. All mice were housed according to institutional guidelines.

Induction of Ionomycin-Induced T Cell Anergy In Vitro

For ionomycin-induced anergy, we used a protocol described previously (Macian et al., 2002) with slight modifications. Naive CD4 T cells were first stimulated with immobilized anti-CD3 (1 μg/ml) plus anti-CD28 (5 μg/ml) for two days. The cells were rested for 3 days and then incubated with ionomycin at the indicated concentrations (Calbiochem). After washing twice with medium, cells were restimulated with indicated concentrations of anti-CD3 and anti-CD28 for 24–48 hr. Proliferation and IL-2 production were measured by using ^{3}H-thymidine incorporation and ELISA.

In Vivo Tolerance Models

To study tolerance of OT-II transgenic CD4^{+} T cells, an adoptive transfer experiment was performed (Bansal-Pakala et al., 2001; Kearney et al., 1994). Briefly, Cbl-b$^{+/+}$ and Cbl$^{-/-}$ OT-II transgenic mice were injected intravenously with 500 μg of OVA peptide in PBS or PBS alone on day 0 and day 3. On day 10, CD4^{+} T cells were isolated and labeled with CFSE. Cells (Thy1.2^{+}Vβ5^{+}, 5×10^{5}) were injected into syngeneic B6.Thy1.1 recipients, followed by immunization with OVA peptide (50 μg) in CFA. On day 3, draining lymph node cells were collected, and cells were stained with Thy1.2-APC and Vβ5-PE. Thy1.2 cells were gated and CFSE profiles analyzed on transgenic Vβ5^{+} cells. Isolated lymph node cells were also stimulated in vitro with OVA peptide and irradiated antigen-presenting cells and cultured for 8 hr. Brefeldin A was added for the last 2 hr. Cells were washed and stained with Thy1.2 APC, followed by intracellular staining with anti-IL-2-PE.

P14 TCR transgenic mice were intravenously injected with different doses of the high-affinity peptide p33, the low-affinity peptide A4Y, and the wrong peptide AV on day 0, day 3, and in some instances on day 6 after the initial injection. For detection of proliferation, T cells were isolated from spleens, and CD8^{+} T cells stimulated in triplicate for different time periods with p33 and AV loaded antigen-presenting cells in the presence or absence of recombinant IL-2 and pulsed for the last 16–20 hr with 1 μCi/well ^{3}H-thymidine (Amersham). APCs were loaded with 10^{-6} M (unless otherwise indicated) of p33 or AV peptide for 1 hr at 37°C. ^{3}H-thymidine incorporation was measured by using a β-scintillation counter (Coulter). For induction of superantigen induced tolerance, Cbl-b$^{-/-}$ mice were crossed onto an H2^{d} background and mice injected with 20 μg/mouse of SEB (Toxin Technologies). To determine cytokine production, sera were harvested and assayed for the production of IL-2, TNFα, and IFNγ by using ELISA (R&D Systems, Minneapolis). Numbers of responding cells and phenotypes of these cells were followed by FACS analyses. Single cell suspensions of lymph nodes, blood ,and spleens were stained with FITC-, PE-, APC-, or biotin-conjugated Abs reactive to CD3ϵ, TCR$\alpha\beta$, TCRVβ6, TCRVβ5, TCRVβ8.1/8.2, TCRVα2, CD4, CD8, CD25, CD28, CD44, CD69, Thy1.2, and CD62L. Biotinylated Abs were visualized by using streptavidin-RED670 (Gibco BRL). Samples were analyzed by flow cytometry via a FACScan (Becton Dickinson).

Biochemistry

Antibodies to Cbl-b (H121), PLCγ-1 (1249), Ub (8017), and Grb2 (255) were purchased from Santa Cruz Biotechnology (CA). Anti-PKCθ, anti-phospho-Akt (Ser493), anti-Vav1 (Tyr174), and anti-Akt (9272) Abs were from Cell Signaling (Berverly, MA). The anti-phospho-PLCγ-1 (pY783) antibody was from Biosource (Camarillo, CA). Cell lysates were subjected to 8%–10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). Immunoblot and ubiquitination assays were performed as described (Fang and Liu, 2001). For in vitro Ca^{2+} mobilization, purified CD4^{+} T cells were first cultured with ionomycin to induce anergy. Cells were loaded with 1 mM indo-1 for 45 min at 37°C. Cells were then incubated with hamster anti-CD3 for 15 min at 4°C. The ratio of indo-1 violet/blue was analyzed by flow cytometry with a BD LSR (BD Biosciences, CA). Ca^{2+} mobilization was induced by crosslinking with rabbit anti-hamster IgG or addition of ionomycin (1 μg/ml). Data were analyzed by using FlowJo software (Tree Star, Inc., San Carlos, CA).

Collagen-Induced Arthritis

Cbl-b$^{-/-}$ mice (Chiang et al., 2000) were backcrossed onto a DBA/1 background to the sixth to eighth generations. Native bovine collagen II (Chondrex, Seattle, MA) was dissolved at 4 mg/ml in 10 mM acetic acid at 4°C overnight and emulsified with an equal volume of CFA or IFA (4 mg/ml each; Chondrex, Seattle, MA). Disease was induced by intradermal injection at the base of the tail with 50 μl of emulsion containing 100 μg collagen either in CFA or IFA. On day 21, the mice were boosted by intradermal injection with 100 μg collagen in IFA and monitored for signs of arthritis. Clinical arthritis was assessed by the following scoring system: grade 0, no swelling; grade 1, mild, but definite redness and swelling of the ankle or wrist or digits; grade 2, moderate redness and swelling of ankle and wrist;

grade 3, severe redness and swelling of entire paw including digits; grade 4, maximally inflamed limb with involvement of multiple joints. Each limb was graded, giving a maximum possible score of 16/mouse. During the development of disease, joints were prepared for histopathology examination of erosions and synovial infiltrates.

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