Immunity 25, 571–581, October 2006 ©2006 Elsevier Inc. DOI 10.1016/j.immuni.2006.08.021

Establishment of the Major Compatibility Complex-Dependent Development of CD4$^+$ and CD8$^+$ T Cells by the Cbl Family Proteins

Fang Huang,[1,4,5] Yasuyuki Kitaura,[1,4,6] IhnKyung Jang,[1,4] Mayumi Naramura,[2,7] Hemanta H. Kole,[2] Liping Liu,[1,8] Haiyan Qin,[1] Mark S. Schlissel,[3] and Hua Gu[1,*]

[1] Department of Microbiology
Columbia University College of Physicians and Surgeons
701 West 168th Street
New York, New York 10032

[2] Laboratory of Immunogenetics
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Rockville, Maryland 20852

[3] Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

Casitas B cell lymphoma (Cbl) proteins are negative regulators for T cell antigen receptor (TCR) signaling. Their role in thymocyte development remains unclear. Here we show that simultaneous inactivation of c-Cbl and Cbl-b in thymocytes enhanced thymic negative selection and altered the ratio of CD4$^+$ and CD8$^+$ T cells. Strikingly, the mutant thymocytes developed into CD4$^+$- and CD8$^+$-lineage T cells independent of the major histocompatibility complex (MHC), indicating that the CD4$^+$- and CD8$^+$-lineage development programs are constitutively active in the absence of c-Cbl and Cbl-b. The mutant double-positive (DP) thymocytes exhibited spontaneous hyperactivation of nuclear factor-kappa B (NF-κB). Additionally, they failed to downregulate the pre-TCR and pre-TCR signaling. Thus, our data indicate that Cbl proteins play a critical role in establishing the MHC-dependent CD4$^+$ and CD8$^+$ T cell development programs. They likely do so by suppressing MHC-independent NF-κB activation, possibly through downmodulating pre-TCR signaling in DP thymocytes.

Introduction

T lymphopoiesis is a sequential developmental process regulated by thymic environmental signals (Fowlkes and Pardoll, 1989; Love and Chan, 2003; von Boehmer, 1988). The first step of T cell development involves successive rearrangements of the V-(D)-J genes in CD4 and CD8 double-negative (DN) T precursors, and only those cells with a successfully rearranged TCRβ gene may develop further into CD4 and CD8 DP stage of thymocytes. This step in T cell development is controlled by signaling of growth factor receptors such as interleukine-7 receptor (IL-7R) and pre-TCR and occurs independently from the MHC because pre-TCR signaling is constitutively active in the absence of the MHC (Hayday et al., 1999; von Boehmer and Fehling, 1997). At the DP stage, thymocytes may decide to become either CD4$^+$ helper-T (Th) or CD8$^+$ cytotoxic-T (Tc) cells and are positively and negatively selected based on the interaction between the TCR and the MHC expressed by thymic accessory cells (Kaye et al., 1989; Sha et al., 1988; Teh et al., 1988). While thymocytes expressing a TCR that recognize MHC-I choose CD8$^+$ T cell fate and develop into Tc cells, cells that express a TCR recognizing MHC-II commit to CD4$^+$ lineage and become Th cells (Cosgrove et al., 1991; Grusby et al., 1991; Zijlstra et al., 1990).

Development of CD4$^+$ and CD8$^+$ T cells is regulated by signals delivered by the TCR; however, it remains unclear how the incoming TCR signals are connected to the CD4$^+$ and CD8$^+$ lineage-development program. Current opinion indicates that engagement of the TCR with MHC initiates a TCR-downstream signaling cascade, starting with tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex by the Src-family tyrosine kinase Lck (lymphocyte-specific protein tyrosine kinase) (Weiss and Littman, 1994). The phosphorylated ITAMs then recruit tyrosine kinase Zap70 (zeta-chain associated protein 70 kDa) to the engaged TCR complexes, leading to Zap70 activation. Upon activation, Zap70 phosphorylates membrane protein LAT (linker for activation of T cells), which then assembles a complex of multisignaling molecules, including Grb2 (growth-factor receptor binding protein 2), Gads (Grb2-related adaptor protein), SLP-76 (the SH2-domain-containing leukocyte phosphoprotein of 76 kDa), Vav, PLCγ-1 (phospholipase Cgamma-1), and Itk (interleukine-2 tyrosine kinase) (Koretzky and Myung, 2001; Lucas et al., 2003). Coordination between these signaling components leads to multiple downstream cellular responses, including Ca^{2+} mobilization, cytoskeleton reorganization, and activation of various nuclear factors that eventually determine the various T cell development programs.

The Cbl family of proteins are signaling adaptors that associate with many different receptors and intracellular signaling components (Rao et al., 2002; Thien et al., 2001). c-Cbl and Cbl-b, two highly homologous members of the Cbl family, are coexpressed in hematopoietic-lineage cells and function as E3-ubiquitin ligases (Joazeiro et al., 1999). Biochemical analyses reveal that Cbl proteins promote the ubiquitination of several Cbl-associated proteins, thereby regulating the degradation or intracellular association of these molecules (Liu, 2004). In the immune system, protein ubiquitination has been implicated in the regulation of T-lymphocyte

*Correspondence: hg2065@columbia.edu
[4] These authors contributed equally to this work.
[5] Present address: Fu Dan University Medical School, Shanghai 200032, P. R. China.
[6] Present address: RIKEN BioSource Center, Tsukuba-Shi, Ibaraki 305-0074, Japan.
[7] Present address: Department of Medicine, Evanston Northwestern Healthcare Research Institute, Northwestern University, Illinois 60201.
[8] Present address: Neuroinflammation Research Center, Department of Neurosciences, Lerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195.

differentiation into Th2 cells, as well as in the induction of immune tolerance (Fang et al., 2002; Naramura et al., 2002). However, it remains unclear whether Cbl proteins also control the development of early T cells. c-Cbl and Cbl-b are differentially expressed in thymocytes and mature T cells (Naramura et al., 2002). Such developmentally regulated pattern of expression is consistent with their predominant role either in thymocyte development or in peripheral T cell activation (Bachmaier et al., 2000; Chiang et al., 2000; Naramura et al., 1998, 2002). At the molecular level, c-Cbl and Cbl-b share a high sequence homology and are coexpressed in developing thymocytes (Naramura et al., 2002), thus suggesting that they may have a redundant function in early T cell development and differentiation. In this report, we show that simultaneous ablation of c-Cbl and Cbl-b in thymocytes resulted in spontaneous development of $CD4^+$ and $CD8^+$ T cells in the absence of the MHC. The mutant DP thymocytes failed to downmodulate pre-TCR and exhibited constitutive pre-TCR signaling. These results together indicate that Cbl proteins are the key players in switching pre-TCR signaling to TCR signaling, a process critical for establishing the MHC-restricted repertoire of $CD4^+$ and $CD8^+$ T cells.

Results

Generation of c-Cbl and Cbl-b Double-Deficient Mice

Homozygous germline c-Cbl and Cbl-b double-deficient mice died before day 10.5 of embryogenesis (E10.5) (Naramura et al., 2002). To study the function of Cbl proteins in T cell development, we generated T cell-specific c-Cbl and Cbl-b double-deficient mice with the Cre-loxP system (Naramura et al., 2002) (see also Figure S1A in the Supplemental Data available online). These mice were homozygous for the Cbl-b null ($Cblb^{-/-}$) and c-Cbl floxed ($Cbl^{f/f}$) mutations and carried an *Lck-cre* transgene that specifically expressed the Cre recombinase in thymocytes (Takahama et al., 1998). Our results indicated that the Lck-Cre transgenic mice mediated deletion of floxed gene in as early as DN3 stage of thymocytes (Figure S1B). We confirmed the absence of c-Cbl and Cbl-b proteins in the purified DP thymocytes of the resulting c-Cbl and Cbl-b double-deficient mice by immunoblot hybridization (Figure S1C). Hereafter, we refer to these T cell-specific c-Cbl and Cbl-b double-deficient ($Cbl^{f/f}$, $Cblb^{-/-}$, *Lck-cre*) mice as Cbl double-deficient ($Cbl^{-/-}$, $Cblb^{-/-}$) mice.

The Cbl Double Deficiency Enhances Thymic Negative Selection

It has been shown previously that both c-Cbl-deficient ($Cbl^{-/-}$) and Cbl-b-deficient ($Cblb^{-/-}$) mice have normal numbers of thymocytes (Bachmaier et al., 2000; Chiang et al., 2000; Naramura et al., 1998). In contrast, we found that $Cbl^{-/-}$, $Cblb^{-/-}$ mice had significantly lower numbers of thymocytes than did wild-type controls (Figure 1A). Analysis of DN thymocytes revealed that the total numbers of DN1-4 subsets were mostly comparable between $Cbl^{-/-}$, $Cblb^{-/-}$ and wild-type mice, except a slight increase in DN3 thymocytes in the mutant mice ($p < 0.001$) (Figures 1A and 1B). However, the number of DP thymocytes in $Cbl^{-/-}$, $Cblb^{-/-}$ mice was reduced to about 30%–40% of that in wild-type controls ($p < 0.001$) (Figure 1A). The impaired DP T cell development could not be explained by the deficiency in TCRβ gene rearrangement, as shown by the fact that we detected comparable levels of dsDNA breaks at TCRβ recombination signal sequences (RSSs) in purified mutant DN2-4 cells (Figure S2A), nor could it be explained by lack of DN3-DN4 expansion (β-selection), as shown by the fact that the mutant DN3 and DN4 cells proliferated as efficiently as did the wild-type cells (Figure S2B). These results thus suggest that the reduction of thymocyte numbers happens after the DN stage.

The total number of DP thymocytes could be affected by thymic positive and negative selection, so we next determined whether thymic selection was altered in the absence of c-Cbl and Cbl-b. To this end, we crossed $Cbl^{-/-}$, $Cblb^{-/-}$ mice to H-Y and 5C.C7 TCR transgenic mice, respectively, and examined thymic positive and negative selection in the resulting $Cbl^{-/-}$, $Cblb^{-/-}$, H-Y and $Cbl^{-/-}$, $Cblb^{-/-}$, 5C.C7 TCR transgenic mice. We found that in the absence of Cbl proteins, $CD8^+$ T cells were negatively selected in male H-Y TCR transgenic mice as efficiently as that in wild-type H-Y TCR transgenic mice (Figure 1C); however, in female H-Y TCR transgenic mice, the ablation of Cbl proteins appeared to convert thymic positive selection of H-Y TCR-expressing $CD8^+$ T cells into negative selection; DP and $CD8^+$ thymocytes were markedly diminished in thymi of $Cbl^{-/-}$, $Cblb^{-/-}$ mice (Figure 1D). Conversion of positive selection into negative selection was also observed in the $CD4^+$ T cell compartment: both DP and $CD4^+$ thymocytes were deleted in $Cbl^{-/-}$, $Cblb^{-/-}$ but not in wild-type 5C.C7 TCR transgenic mice (Figure 1D). Based on these results, we conclude that the Cbl double deficiency enhances thymic negative selection, leading to deletion of T cells that are usually positively selected in wild-type mice. Moreover, we propose that the enhanced negative selection is at least one of the reasons responsible for the reduced number of DP thymocytes in the mutant mice.

Altered Ratio of $CD8^+$ to $CD4^+$ Thymocytes in Cbl Double-Deficient Mice

In addition to the reduction in the numbers of DP cells, the Cbl double deficiency markedly altered the ratio of $CD8^+$ versus $CD4^+$ thymocytes (Figures 2A and 2B). While the wild-type, $Cbl^{-/-}$, and $Cblb^{-/-}$ mice each had approximately 2–3 times more $CD4^+$ than $CD8^+$ thymocytes, $Cbl^{-/-}$, $Cblb^{-/-}$ mice possessed 20%–50% more $CD8^+$ than $CD4^+$ T cells ($p < 0.001$). This result suggests that the Cbl double deficiency alters the $CD4^+$ and $CD8^+$ lineage-development program.

We have previously demonstrated that $Cbl^{-/-}$, $Cblb^{-/-}$ mice develop severe vasculitis in adulthood and become moribund by the age of 4–6 months (Naramura et al., 2002). To exclude the possibility that the inflammation is responsible for the reduced mutant DP cells and biased ratio of $CD4^+$ versus $CD8^+$ T cells, we transferred mixed bone marrow (BM) stem cells (at a 1:1 ratio) from $Cbl^{-/-}$, $Cblb^{-/-}$ ($H\text{-}2K^{d/b}$) and wild-type ($H\text{-}2K^{b/b}$) mice into $Rag2^{-/-}$ ($H\text{-}2K^{b/b}$) mice and analyzed the donor-derived DP, $CD4^+$, and $CD8^+$

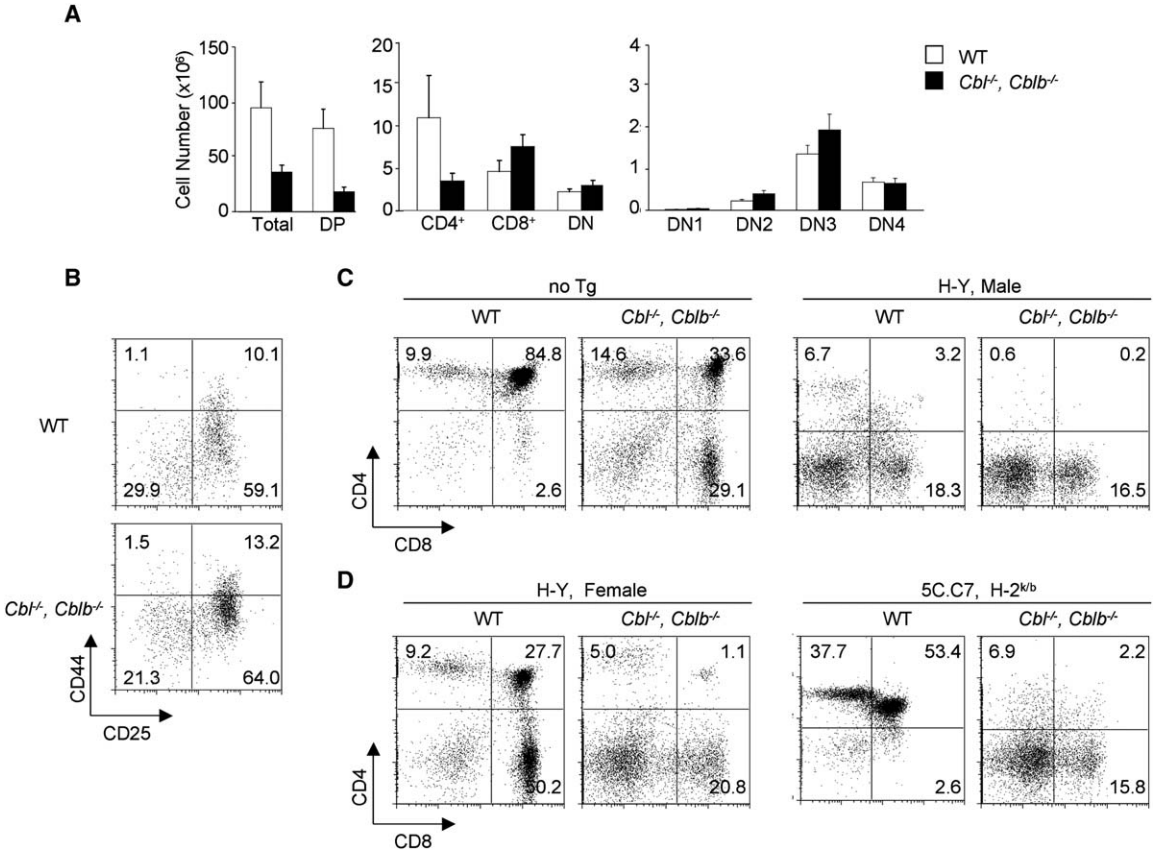

Figure 1. Flow Cytometric Analyses of the Thymocyte Subsets and Thymic Positive and Negative Selection

(A) Comparison of the thymocyte subsets in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ and wild-type mice. Shown are the mean values of cell numbers of each thymocyte subset. DP, CD4CD8 double-positive thymocytes; DN, CD4CD8 double-negative thymocytes; DN1-4, CD4CD8 double-negative 1 to 4 subpopulations of thymocytes as defined by cell-surface markers c-Kit, CD25, and CD44 (Godfrey et al., 1993); DN1 cells represent c-Kit^{+} cells in gated CD44^{+} CD25^{-} population. The results were summarized from 4- to 5-week-old *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ (n = 5) and wild-type or *Cblb*$^{-/-}$ mice (n = 5).

(B) Flow cytometric analysis of DN thymocyte subsets from wild-type and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice. Cells were stained with anti-CD25-FITC, anti-CD44-APC, anti-c-Kit-PE, and biotinylated anti-CD4, CD8, CD3, Mac-1, Gr-1, NK1.1, TCR$\gamma\delta$, and B220, followed by straptavidin-PE-Cy7 staining. DN cells are gated DN (CD4^{-} CD8^{-} CD3^{-} Mac-1^{-} Gr-1^{-} NK1.1^{-} TCR$\gamma\delta^{-}$ B220^{-}) thymocytes. Shown are the profiles of anti-CD44 and CD25 staining of the c-Kit^{+} CD44$^{+/-}$ and c-Kit^{-} CD44^{-} populations among the gated DN cells.

(C) Thymic negative selection. Shown are dot plot analyses of anti-CD4 and anti-CD8 staining of thymocytes from wild-type and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ non-TCR transgenic mice (left) and male wild-type and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ H-Y TCR transgenic mice (right).

(D) Thymic positive selection. Dot plots represent anti-CD4 and anti-CD8 staining profiles of thymocytes from female wild-type and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ H-Y TCR transgenic mice (left) or wild-type and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ 5C.C7 TCR transgenic mice (right). 5C.C7 TCR transgenic mice were of H-2$^{k/b}$ background.

thymocytes in the recipient *Rag2*$^{-/-}$ mice (Figure 2C). *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ and wild-type thymocytes could be distinguished by the cell-surface marker H-2K^{d}. We found that in the recipient mice, the total number of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes was approximately one-third of the wild-type donor thymocytes ($p < 0.01$), consistent with our finding that the Cbl double deficiency reduced the total number of thymocytes (Figure 1). In addition, the ratio of CD8^{+} to CD4^{+} T cells derived from *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ donors was also higher than that from wild-type controls. These results thus refute the notion that the reduced number of DP T cells and altered ratio of CD8^{+} versus CD4^{+} T cells in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice are caused by inflammatory cytokines. Taken together, we conclude that c-Cbl and Cbl-b regulate the T cell-intrinsic programs that control the total number of thymocytes and define the ratio of CD4^{+} and CD8^{+} T cells during thymic development.

The Cbl Double Deficiency Leads to MHC-Independent CD4^{+} and CD8^{+} T Cell Development

The increase in CD8^{+} thymocytes in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice suggested that the Cbl double deficiency might enhance thymic positive selection or attenuate negative selection of CD8^{+}-lineage cells. To investigate this possibility, we transferred respectively fetal liver stem cells from *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ and *Cblb*$^{-/-}$ mice into lethally irradiated MHC-I-deficient mice and then examined development of donor-derived thymocytes in the recipient mice. Donor-derived cells can be distinguished from the recipients based on the cell-surface marker Ly9.1. If the increase of CD8^{+} T cells in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice was a result of the altered thymic selection for CD8^{+}-lineage cells, we expect that the development of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD8^{+} T cells was blocked in this experimental setting because thymic selection of CD8^{+} T cells

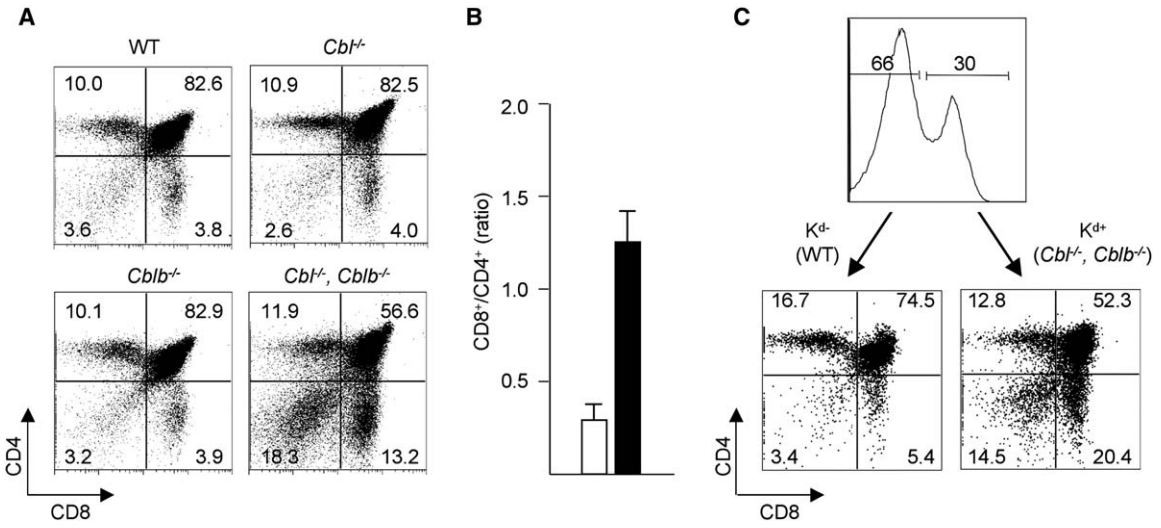

Figure 2. Flow Cytometric Analysis of the Ratio of CD8$^+$ to CD4$^+$ Thymocytes

(A) Anti-CD4 and CD8 staining profiles of thymocytes from wild-type, *Cbl*$^{-/-}$, *Cblb*$^{-/-}$, and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice.

(B) Ratios of CD8$^+$ to CD4$^+$ thymocytes in wild-type (open bar) and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ (solid bar) mice. The results represent data collected from 3-week-old mice (n = 5) in each group.

(C) Flow cytometric analysis of CD4$^+$ and CD8$^+$ thymocytes from *Rag2*$^{-/-}$ bone marrow (BM) chimera receiving mixed (1:1 ratio) BM cells from the *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ (H-2k$^{d/b}$) and wild-type (H-2K$^{b/b}$) mice. Shown at the bottom are the CD4 and CD8 staining profiles of the gated *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ (Kd$^+$) or wild-type (Kd$^-$) thymocytes. Results represent more than three independent experiments.

requires TCR-MHC-I interaction. As shown in Figure 3A, we found that CD8$^+$ T cells were still generated from *Cbl*$^{-/-}$, *Cblb*$^{-/-}$, but not MHC-I-deficient nor *Cblb*$^{-/-}$ donor stem cells in these chimeras, thus indicating that the increase in CD8$^+$ T cells in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice is unlikely resulted from the enhanced positive or attenuated negative selection for this lineage.

Alternatively, increase in CD8$^+$ T cells in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice could be a consequence of an altered CD4$^+$-and CD8$^+$-lineage commitment program so that, in addition to MHC-I-restricted thymocytes, some mutant thymocytes that recognize MHC-II developed into CD8$^+$ cells. To examine this possibility, we transferred *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ fetal liver stem cells into irradiated MHC-I and II double mutant mice and examined donor-derived thymocyte development in the recipients. If the Cbl double deficiency switches the fate of MHC-II-restricted thymocytes to the CD8$^+$ lineage, we expect that the development of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD8$^+$ T cells should be blocked in MHC-I and II double-deficient mice because in this scenario CD4$^+$- and CD8$^+$-lineage determination requires interaction between the TCR and MHC-I or MHC-II. Surprisingly, we found that in the recipient thymi, while CD4$^+$ and CD8$^+$ T cells from MHC-I and II double mutant or *Cblb*$^{-/-}$ donor stem cells were completely absent, they were efficiently generated from *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ T cell precursors (Figure 3B). Interestingly, *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ T cells were not significantly increased in the spleens and lymph nodes of recipient MHC-I and II double mutant mice as compared to wild-type cells (data not shown), suggesting that build-up of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ T cell populations in the periphery still requires the MHC.

Recent experiments demonstrate that enforced expression of MHC-II in BM stem cell-derived thymocytes may promote positive selection of CD4$^+$ and CD8$^+$ T cells in MHC-II-deficient recipient mice (Choi et al., 2005; Li et al., 2005). To exclude the possibility that development of T cells in MHC-deficient recipients involved donor-derived MHC-expressing cells, we bred *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice to MHC-I and II double-deficient mice and analyzed T cell development in the resulting c-Cbl, Cbl-b, MHC-I, and MHC-II (MHC-Cbl) quadruple-deficient mice (Figure 3C). We found that the total numbers of thymocytes in Cbl-MHC quadruple-deficient mice were comparable to that in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice. Strikingly, while few CD4$^+$ and CD8$^+$ thymocytes were generated in MHC-I and II double-deficient mice, both CD4$^+$ and CD8$^+$ T cells were as efficiently generated in Cbl-MHC quadruple-deficient mice as that in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice. Additionally, a biased ratio of CD8$^+$ versus CD4$^+$ T cells was also observed in Cbl-MHC quadruple-deficient mice (Figure 3C). These Cbl-MHC quadruple-deficient CD4$^+$ and CD8$^+$ T cells resembled positively selected *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ thymocytes, as shown by the fact that the majority of them downmodulated HSA and expressed the TCR at the amounts comparable to that expressed by *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ T cells (Figure 3D). Additionally, the quadruple-deficient CD4$^+$ and CD8$^+$ T cells appeared to be functionally normal T cells rather than immature CD4$^+$ and CD8$^+$ single-positive T cells: they proliferated vigorously and secreted a high amount of IL-2 upon TCR stimulation (Figure 3E). Taken together, the above results demonstrate that Cbl proteins play an essential role in establishing the MHC-dependent CD4$^+$ and CD8$^+$ T cell development programs. Since *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes develop into CD4$^+$ and CD8$^+$ T cells in the absence of MHC-I and II, we propose that Cbl proteins function as a barrier to prevent "unauthorized" (through TCR-MHC interaction) CD4$^+$ and CD8$^+$ T cell development under physiological conditions.

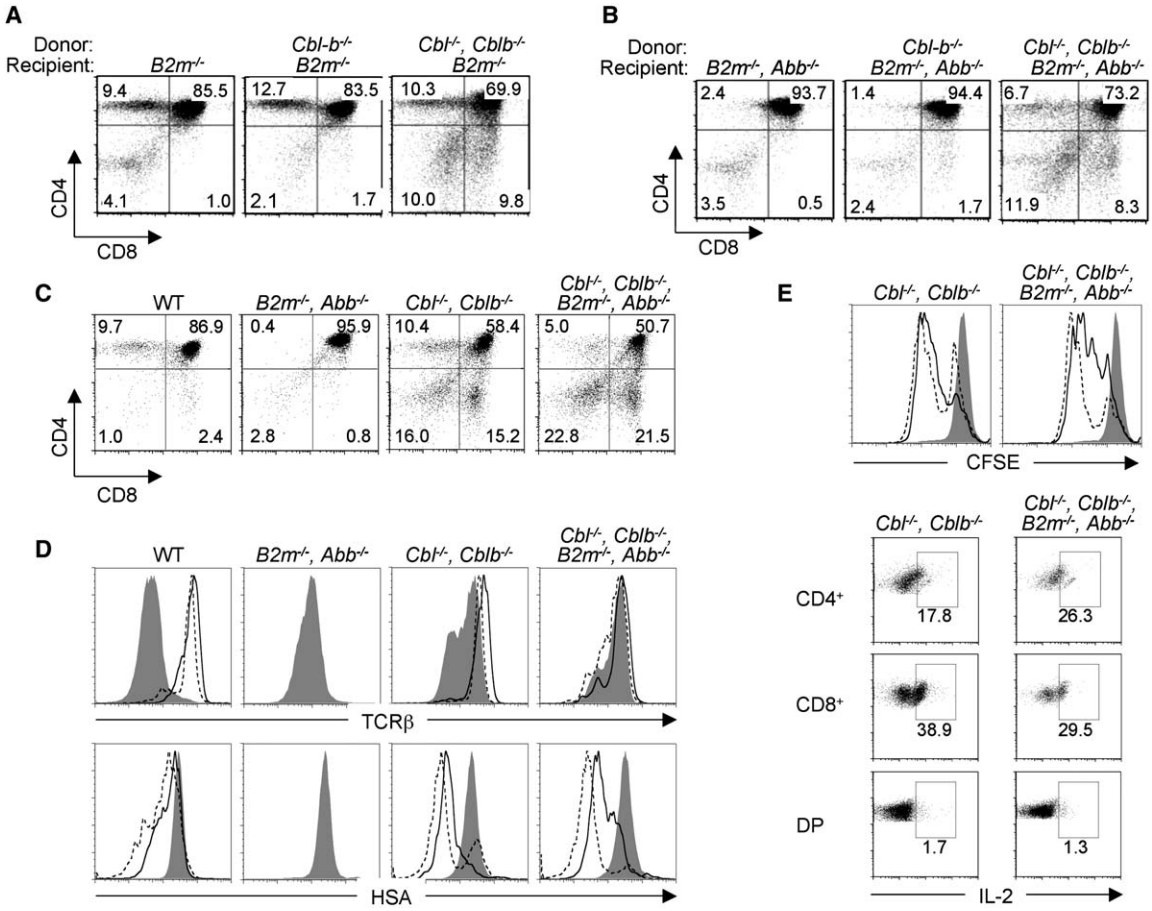

Figure 3. Development of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ Thymocytes in MHC-I Deficient, MHC-I and II Double-Deficient BM Chimeric, and Cbl-MHC Quadruple-Deficient Mice

(A and B) Fetal liver cells (Ly9.1$^+$) were collected from 14-day-old *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ and wild-type embryos and transferred into irradiated-MHC-I deficient or MHC-I and II double-deficient (Ly9.1$^-$) mice by i.v. injection. One month later, mice were sacrificed, and thymocytes were analyzed by flow cytometry. Shown are anti-CD4 and CD8 staining profiles of gated recipient (Ly9.1$^-$) or donor (Ly9.1$^+$) derived cells from MHC-I-deficient (*B2m*$^{-/-}$) (A) or MHC-I and II double-deficient (B) recipient (*B2m*$^{-/-}$, *Abb*$^{-/-}$) mice.

(C) Flow cytometric analysis of thymocyte subsets in Cbl-MHC quadruple-deficient mice. Shown are anti-CD4 and anti-CD8 staining profiles of thymocytes from wild-type, MHC-I and II double-deficient (*B2m*$^{-/-}$, *Abb*$^{-/-}$), Cbl double-deficient (*Cbl*$^{-/-}$, *Cblb*$^{-/-}$), and Cbl-MHC quadruple-deficient (*Cbl*$^{-/-}$, *Cblb*$^{-/-}$, *B2m*$^{-/-}$, *Abb*$^{-/-}$) mice.

(D) Expression of HSA and TCRβ on *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ DP, CD4$^+$, and CD8$^+$ thymocytes. Thymocytes from wild-type, MHC-I and II double-deficient, Cbl double-deficient, and Cbl-MHC quadruple-deficient mice were stained with anti-CD4 and CD8 in combination with either anti-HSA or anti-TCRβ chain. Shown are histograms of HSA and TCRβ expression on the gated DP (shadow), CD4$^+$ (solid line), CD8$^+$ (dashed line) thymocytes. Results are representatives of three independent experiments.

(E) Proliferation and IL-2 production of Cbl-MHC quadruple-deficient thymocytes. Total thymocytes were either unstimulated or stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 3 days for CFSE-labeling experiment, or for 18 hr followed by 6 hr of incubation with brefeldin A for IL-2 production assay. Top, histograms show the CFSE-fluorescent intensity of unstimulated thymocytes (shadow), CD4$^+$ (solid line), or CD8$^+$ (dashed line) after stimulation with anti-CD3 and anti-CD28. Bottom, dot plots show intracellular staining of IL-2 in gated CD4$^+$, CD8$^+$, and DP thymocytes. For DP cells, only CD8 staining is shown in the y axis.

Most TCR-Proximal Signaling Pathways Were Not Spontaneously Activated in Cbl Double-Deficient Thymocytes

Development of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ T cells in the absence of the MHC suggests that the Cbl double deficiency results in spontaneous activation of the CD4$^+$ and CD8$^+$ lineage-development program. To assess this possibility, we examined various TCR-proximal signaling pathways in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ DP thymocytes in the presence or absence of anti-CD3 stimulation. To eliminate background signaling elicited by intrathymic stimuli in vivo, we cultured thymocytes at 37°C for 10–12 hr in vitro before biochemical analysis. This treatment has been shown to eliminate the basal TCR signaling activated by the MHC molecules that are expressed on thymic accessory cells (Nakayama et al., 1991). In the absence of anti-CD3 stimulation, we could not find substantial amounts of spontaneous tyrosine phosphorylation of the total cellular proteins or individual signaling components such as LAT, Zap70, Vav, PLCγ-1, and Erk1 and 2 (Figures 4A and 4B), suggesting that the TCR-proximal signaling events examined above are not spontaneously activated in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes in the absence of TCR stimulation. After anti-CD3 stimulation, the magnitude of tyrosine phosphorylation of total cellular proteins was not measurably

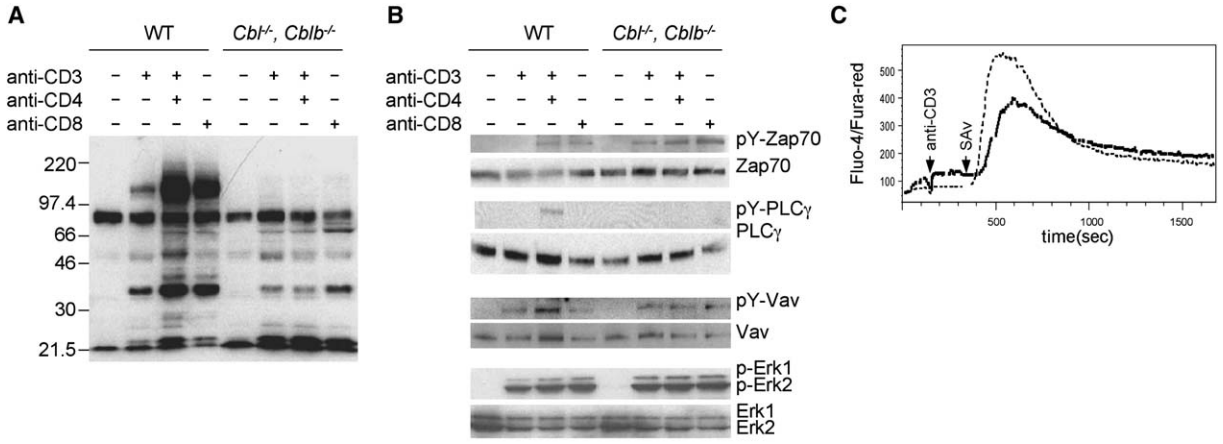

Figure 4. Intracellular Signaling of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ and Wild-Type Thymocytes

(A and B) FACS-purified DP thymocytes or total thymocytes were stimulated with anti-CD3, anti-CD3 + anti-CD4, or anti-CD3 + anti-CD8 for 2 min. Shown are tyrosine phosphorylation of total cellular proteins (A) or individual signaling proteins (B). Erk1 and 2 activities were determined with an antibody against the active forms of Erk1 and 2.

(C) TCR-induced Ca^{2+} mobilization. Shown are the histograms of Ca^{2+} mobilization in wild-type (dashed line) and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ (solid line) DP thymocytes. $CD4^+CD8^+$ thymocytes are gated and displayed as a ratio of fluo 4 versus fura-red intensity. Arrows indicate the time points when anti-CD3ε-biotin and straptavidin (SAV) were added.

enhanced, and in some cases, for example p90 and p36-p38, was even weakened in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes as compared to that in wild-type cells (Figures 4A and 4B). The strong phosphorylated 120 kDa protein band in wild-type cells, which was absent in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ cells, might represent tyrosine phosphorylated c-Cbl, as indicated by the fact that preclearing of c-Cbl from the lysates via anti-c-Cbl depleted this band (Figure S3). Tyrosine phosphorylation of CD3ζ chain and Zap70 was augmented. However, tyrosine phosphorylation of p36-p38 (possibly LAT) and PLCγ-1 was markedly reduced in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes, suggesting that the enhanced CD3ζ chain and Zap70 signals could not be efficiently transduced to downstream targets in the absence of c-Cbl and Cbl-b. Consistent with the weaker tyrosine phosphorylation of PLCγ-1, TCR-induced Ca^{2+} mobilization was also attenuated in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ DP thymocytes (Figure 4C). These results collectively indicate that the TCR-proximal signaling pathways studied above are neither spontaneously activated nor generally hyperactive in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes.

Constitutive Hyperactivation of NF-κB in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ Thymocytes

Our finding that in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes most TCR-proximal signaling pathways are not spontaneously activated suggests that the Cbl double deficiency might affect the TCR-distal signaling cascade. Since development of $CD4^+$ and $CD8^+$ T cells is controlled by a series of transcription factors, we next examined the activities of several transcription factors that are known to be essential for $CD4^+$- and $CD8^+$-lineage development.

NF-κB is a transcription factor that can be activated by the TCR (Kane et al., 2002). Enforced activation of NF-κB may substitute pre-TCR and TCR signals to support the development and survival of DP and $CD4^+$ and $CD8^+$ thymocytes (Voll et al., 2000). We found that, in contrast to wild-type thymocytes in which NF-κB binding activity was moderately enhanced only after TCR stimulation, *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes exhibited a markedly higher level of NF-κB binding activities even in the absence of TCR stimulation (Figure 5). The constitutive activation appeared to be selective for NF-κB because the activity of AP-1, another transcription factor whose activation depends on TCR-induced MAP kinases, was not spontaneously active in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ cells (Figure 5). Based on these results, we conclude that the signaling cascade upstream of the NF-κB pathway is constitutively active in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes. Since NF-κB activation may substitute TCR signaling in promoting T cell development (Voll et al.,

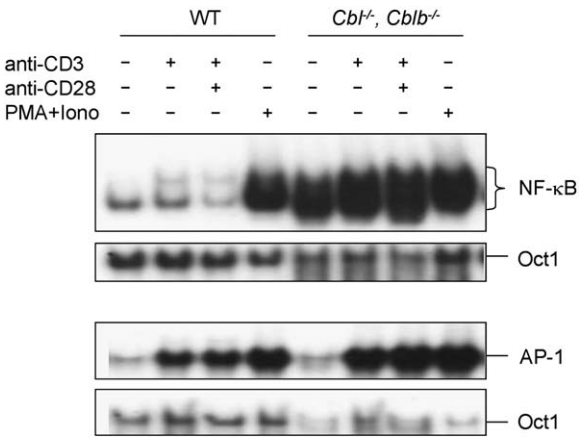

Figure 5. Constitutive Activation of NF-κB in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ Thymocytes

DP thymocytes were purified by FACS sorting. The purified cells were either left unstimulated or stimulated with plate-bound anti-CD3, anti-CD3 + anti-CD28, or PMA and Ionomycin. NF-κB and AP-1 activities were determined by EMSA with oligonucleotides of NF-κB and AP-1 binding sites as probes, respectively. Oct 1 binding activities were used as protein quantity controls.

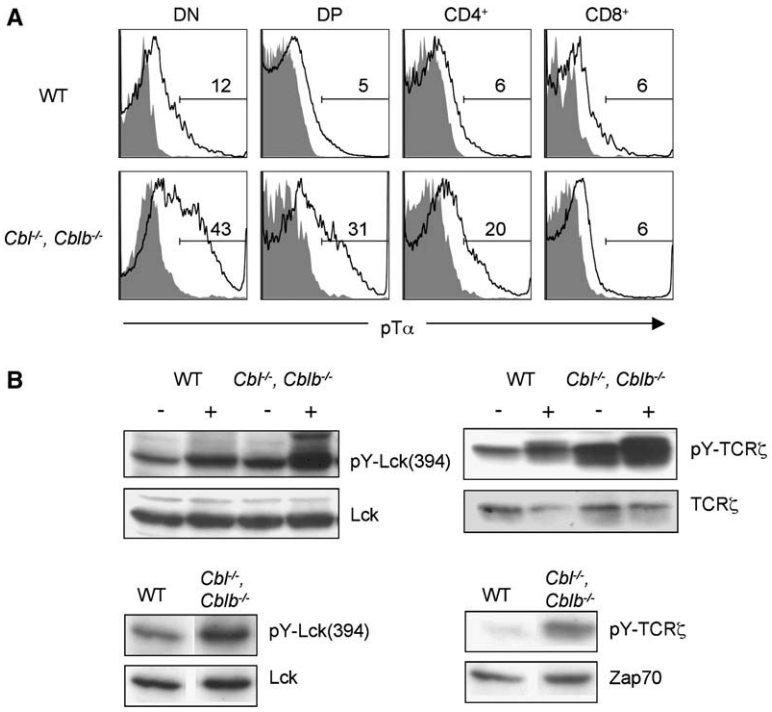

Figure 6. Enhanced Pre-TCR Expression and Signaling in $Cbl^{-/-}$, $Cblb^{-/-}$ DP Thymocytes

(A) Shown are histograms of pTα expression on DN, DP, $CD4^+$, and $CD8^+$ thymocytes from wild-type (top) and $Cbl^{-/-}$, $Cblb^{-/-}$ mice (bottom). Shadow, thymocyte subsets stained with isotype-matched control antibody. Solid line, thymocytes stained with anti-pTα.

(B) Lck (Y394) phosphorylation and tyrosine phosphorylation of CD3ζ chain. Left, tyrosine phosphorylation of Lck (Y394) in total thymocytes either with or without anti-CD3 + anti-CD4 stimulation (top left) or in purified DP thymocytes without stimulation (bottom left). Phosphorylation of Lck (Y394) was determined with an anti-Lck (Y394). Right, tyrosine phosphorylation of CD3ζ chain in total thymocytes either with or without anti-CD3 + anti-CD4 stimulation (top right) or in purified DP thymocytes without stimulation (bottom right). In the latter, the amounts of Zap70 were used as protein loading controls.

2000), our data also suggest that the constitutive activation of NF-κB is one of the reasons contributing to the loss of MHC-dependent T cell development in the mutant mice.

Impaired Pre-TCR Downmodulation and Constitutive Pre-TCR Signaling in $Cbl^{-/-}$, $Cblb^{-/-}$ DP Thymocytes

It is generally believed that the TCR and the pre-TCR deliver signals through the same CD3 complexes; however, unlike the TCR, the pre-TCR is constitutively active even in the absence of MHC engagement (Panigada et al., 2002; Yamasaki et al., 2006). Cbl proteins may attenuate TCR signaling through promotion of TCR downmodulation, so we speculated that the spontaneous activation of the $CD4^+$ and $CD8^+$ T cell development programs in $Cbl^{-/-}$, $Cblb^{-/-}$ thymocytes might be triggered by enhanced pre-TCR signaling, resulting from impaired pre-TCR downmodulation. We found that indeed pre-TCR expression was much higher on $Cbl^{-/-}$, $Cblb^{-/-}$ DN and DP thymocytes than on wild-type controls; however, the expression of pre-TCR was only minimally increased on the mutant $CD4^+$ thymocytes but not detectable on the mutant $CD8^+$ thymocytes nor on the peripheral $CD4^+$ and $CD8^+$ T cells (Figure 6A and data not shown). To determine whether pre-TCR signaling is constitutively enhanced in $Cbl^{-/-}$, $Cblb^{-/-}$ DP thymocytes, we examined pre-TCR downstream signaling including Lck activation and tyrosine phosphorylation of CD3ζ chain in $Cbl^{-/-}$, $Cblb^{-/-}$ DP thymocytes. We found that even in the absence of TCR stimulation, the mutant total thymocytes as well as purified DP T cells possessed significantly higher levels of Lck (Y394) phosphorylation, which represents the active form of Lck, and of tyrosine phosphorylated CD3ζ chain (p21) than did the wild-type controls (Figure 6B). These data indicate that pre-TCR signaling is indeed enhanced in $Cbl^{-/-}$, $Cblb^{-/-}$ DP thymocytes and support our hypothesis that the enhanced pre-TCR signaling is at least one of the reasons responsible for constitutive NF-κB activation and MHC-independent development of $CD4^+$ and $CD8^+$ T cells.

Discussion

MHC-dependent recognition is established during T cell development in thymus in which only those T cells expressing a TCR that recognizes either MHC-I or MHC-II are permitted to develop further into $CD4^+$ or $CD8^+$ T cells (Fink and Bevan, 1978; Fowlkes and Pardoll, 1989; von Boehmer, 1988). Our study reveals that Cbl proteins play a critical role in T cell development at multiple stages, particularly in controlling the program responsible for MHC-dependent $CD4^+$ and $CD8^+$ T cell genesis. In the absence of c-Cbl and Cbl-b, both $CD4^+$- and $CD8^+$-lineage T cells are efficiently generated in thymi even in the absence of MHC-I and II, indicating that Cbl proteins function as a barrier to prevent "unauthorized" (MHC-independent) $CD4^+$ and $CD8^+$ T cell development under physiological conditions. Interestingly, $Cbl^{-/-}$, $Cblb^{-/-}$ $CD4^+$ and $CD8^+$ T cells generated in Cbl-MHC quadruple-deficient mice do not accumulate in the peripheral lymphoid organs. Since the survival of peripheral T cells depends on TCR-MHC interaction (Kirberg et al., 1997; Takeda et al., 1996; Tanchot et al., 1997; Viret et al., 1999; Witherden et al., 2000), our observation may suggest that the survival signals in $Cbl^{-/-}$, $Cblb^{-/-}$ $CD4^+$ and $CD8^+$ T cells are not constitutively active. Alternatively, it is possible that $Cbl^{-/-}$, $Cblb^{-/-}$ $CD4^+$ and $CD8^+$ T cells cannot exit the thymus in the absence of the MHC. It has been shown that thymocyte egress from thymi depends on sphingosine-1 phosphate (S1P) signaling that is upregulated in newly generated mature thymocytes (Matloubian et al., 2004). In this

regard, it is of interest to examine whether the responsiveness to S1P is diminished in Cbl-MHC quadruple-deficient CD4$^+$ and CD8$^+$ thymocytes.

The multiple effects on thymocyte development by the Cbl double deficiency may involve different mechanisms. *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymi contain a significantly low number of thymocytes as compared to that in wild-type mice. This defect is not likely a result of reduced DN cell proliferation, but rather can be attributed to the enhanced thymic negative selection, resulting in depletion of DP thymocytes that usually are positively selected and develop into CD4$^+$ or CD8$^+$ thymocytes in wild-type mice. As for the altered ratio of CD4$^+$ and CD8$^+$ lineages and MHC-independent development of CD4$^+$ and CD8$^+$ T cells in the mutant mice, we propose that the Cbl double deficiency regulates these development programs through a different mechanism. It is generally believed that the development program for the CD4$^+$ and CD8$^+$ lineages is controlled by TCR signals that may influence CD4$^+$ and CD8$^+$ lineage choice through different strength and duration (Germain, 2002). Our finding that *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ CD4$^+$ and CD8$^+$ T cells may develop in the absence of the MHC thus suggests that TCR signaling is constitutively active in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes. Although our experiments show that the majority of TCR-proximal signaling pathways are not spontaneously activated in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes, we indeed find that NF-κB but not AP-1 transcription factors are constitutively hyperactive in the mutant cells even in the absence of TCR stimulation. NF-κB controls cell development, death, and survival (Ghosh and Karin, 2002). Under physiological conditions, however, constitutive NF-κB activity is restricted to DN3 and DN4 populations and is likely to be induced by pre-TCR signaling (Aifantis et al., 2001; Voll et al., 2000). It has been reported that enforced expression in thymocytes of a "superinhibitor" of NF-κB, which inhibits endogenous NF-κB activation by 3- to 4-fold, suppresses DN4 thymocyte development. In contrast, expression of a constitutively active form of IκB kinase β in *Rag2*$^{-/-}$ thymocytes, which results in a 2- to 3-fold increase in NF-κB activity, promotes *Rag2*$^{-/-}$ thymocyte differentiation into DP T cells (Voll et al., 2000). Interestingly, in thymi of the IκB kinase β transgenic mice, a small number of *Rag2*$^{-/-}$ CD4$^+$ and CD8$^+$ thymocytes emerge and there are more CD8$^+$ T cells than CD4$^+$ cells, thus suggesting that the hyperactivation of NF-κB alone may be sufficient to substitute TCR signaling, leading to a biased ratio and MHC-independent development of CD4$^+$ and CD8$^+$ cells (Voll et al., 2000). Finally, it should be mentioned that in addition to the TCR, development of CD4$^+$- and CD8$^+$-lineage T cells is also regulated by Notch. At the present time, we cannot exclude the possibility that Notch signaling is altered in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes. Notch 1 has been shown to be ubiquitinated by c-Cbl in myoblasts in an overexpression experiment (Jehn et al., 2002). Therefore, it will be interesting to examine whether Notch ubiquitination and expression is affected in thymocytes in the absence of Cbl proteins.

How can c-Cbl and Cbl-b selectively regulate NF-κB but not AP-1 activation in developing thymocytes? At present, it is not well understood how TCR signaling activates NF-κB in thymocytes. Because Cbl proteins function as E3 ubiquitin ligases, they may control NF-κB activation by selectively promoting ubiquitination and degradation of signaling components that act between the TCR and NF-κB, but not AP-1. Bcl-10, Carma-1, PKCθ, and MALT-1 are candidate components that meet this criterion (Li and Verma, 2002; Ruland et al., 2001; Thome and Tschopp, 2003; Wang et al., 2004). Therefore, it is worthwhile to examine whether ubiquitination and degradation of these proteins are altered in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes. Alternatively, we favor an idea that constitutive NF-κB activation in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes results from impaired pre-TCR downmodulation and degradation, since the pre-TCR signals constitutively through the CD3 complex in the absence of ligand stimulation (Aifantis et al., 2001). It has been shown that pre-TCR downmodulation and degradation depend on c-Cbl (Panigada et al., 2002). We found that pre-TCR expression was markedly upregulated on *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ DN and DP thymocytes that correlated with constitutive Lck activation and CD3ζ chain phosphorylation in the mutant DP cells (Figure S5), thus indicating that the pre-TCR signaling is enhanced in these cells. However, since pre-TCR signaling activates multiple TCR signaling pathways in addition to NF-κB, other mechanisms must be involved in order to explain how the Cbl double deficiency selectively affects activation of NF-κB, but not that of AP-1. One possible explanation is that the Cbl double deficiency might also elicit other signaling pathways that selectively suppress pre-TCR-induced branch signaling leading to AP-1, but not NF-κB activation. One candidate molecule along this line is CD148, a transmembrane phosphatase that may selectively dephosphorylate LAT and PLC-γ in Jurkat T cells (Baker et al., 2001). This possibility is supported by our preliminary data showing that CD148 is markedly upregulated on *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ DP thymocytes (Figure S4). Additionally, it is also consistent with our observation that despite an enhanced tyrosine phopshorylation of CD3ζ chain, tyrosine phosphorylation of PLC-γ and p36-38 (possibly LAT) appears to be attenuated in *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes. Taken together, current data from our laboratory and others are consistent with our hypothesis that Cbl proteins switch MHC-independent pre-TCR signaling to MHC-dependent TCR signaling in DP thymocytes, and failure to do so may result in constitutive activation of pre-TCR signaling pathways, such as NF-κB, consequently promoting T cell differentiation into CD4$^+$ and CD8$^+$ T cells in the absence of the MHC. Further genetic studies, with mice in which pre-TCR signaling is either attenuated or ablated in DP T cells, may help to determine whether the impaired pre-TCR downmodulation is the sole contributor to constitutive NF-κB activation and whether this is sufficient to drive the MHC-independent development of CD4$^+$ and CD8$^+$ T cells.

Stimulation through the TCR activates a series of coordinated intracellular signaling events that control the CD4$^+$ and CD8$^+$ lineage-development program. However, it is worthwhile to notice that these individual signaling events can also be activated by receptors other than the TCR (Dikic and Blaukat, 1999; Ghosh and Karin, 2002; Jorissen et al., 2003; Sanchez-Martin et al., 2004; Zell et al., 1999). In particular, the pre-TCR, which also signals through the CD3 complex, activates a signaling

cascade similar to that activated by the TCR (Aifantis et al., 2001). In this regard, it is conceivable that activation of these signals by receptors other than the TCR in the thymic environment may generate basal level signaling in the TCR signaling cascade and that loss of control over these "tonic" signals may result in accumulation of the transcription activities required for T cell development. Thus, by eradicating this "tonic" signaling, Cbl proteins may enforce use of the TCR to activate the $CD4^+$ and $CD8^+$ T cell development program under physiological conditions, thus establishing an MHC-dependent mechanism for T cell development and differentiation.

One may envisage that loss of MHC dependence during T cell development might lead to the generation of T cells that recognize antigens outside of the context of MHC presentation. These cells may exist only in small numbers and may receive stimulation from native autoantigens in the periphery. The survival and activation of these cells would be dangerous for animals because they could potentially cause autoimmune diseases. In this regard, *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice indeed developed severe systemic autoimmune vasculitis (Naramura et al., 2002). Given that *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice exhibit enhanced thymic negative seletion, which should eliminate autoreactive cells more efficiently, it will be of great interest to determine whether the development of the autoimmune disease involves non-MHC-restricted autoreactive T cells. Finally, our preliminary data suggest that *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ T cells are resistant to TGF-β suppression (data not shown). It is feasible that *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ T cells may escape negative regulation of TGF-β secreted by regulatory T cells. In conclusion, our data demonstrate that Cbl proteins play multiple functions in T cell development, function, and autoimmunity. Further dissection of signaling controlled by Cbl proteins may be useful for immunomodulation.

Experimental Procedures

Mice

Generation of c-Cbl floxed (*Cbl*$^{f/f}$) and *Cblb*$^{-/-}$ mice has been described previously (Chiang et al., 2000; Naramura et al., 2002). The *Lck-cre* transgenic (Tg) mice were kindly provided by J. Takeda (Takahama et al., 1998). To generate *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice (*Cbl*$^{-/-}$, *Cblb*$^{-/-}$), we crossed *Cbl*$^{f/f}$ mice to *Lck-cre* Tg mice and then crossed their offspring with *Cblb*$^{-/-}$ mice to obtain *Cbl*$^{f/f}$, *Cblb*$^{-/-}$, *Lck-cre* Tg mice. The genotypes of these mice were determined by Southern blot hybridization according to a previous protocol (Naramura et al., 2002). Cbl-MHC quadruple-deficient mice were obtained by crossing *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice to MHC-I and II double-deficient mice (*B2m*$^{-/-}$, *Abb*$^{-/-}$). C57BL/6 *Rag2*$^{-/-}$, MHC-I-deficient (*B2m*$^{-/-}$), and MHC-I and II double-deficient mice were from Taconic (Germantown, NY). These mice were kept in specific pathogen-free facilities at the NIH and Columbia University. Animal experiments were approved by the Institutional Animal Care and Use Committees of the National Institute of Allergy and Infectious Diseases, NIH, and Columbia University.

Flow Cytometric Analysis

To study thymocyte cellularities, we collected total thymocytes from wild-type and mutant mice and prepared them as single-cell suspensions. For fluorescent staining, we stained 1×10^6 cells in PBS buffer containing 0.5% BSA and 0.05% azide with fluorescent-coupled antibodies on ice for 30 min. After staining, we washed cells twice with PBS buffer and analyzed the staining profiles on a FACS LSR II (BD Biosciences, San Diego, CA). To examine the development of the $CD4^+$ and $CD8^+$ lineage T cells, we stained total thymocytes with different fluorescent-coupled CD4, CD8, TCRβ, and HSA antibodies. To examine the development of DN thymocytes, we stained total ($CD3^-$ $CD4^-$ $CD8^-$ $B220^-$ $TCR\gamma\delta^-$ $NK1.1^-$ $Gr\text{-}1^-$ $Mac\text{-}1^-$) thymocytes with CD44, CD25, and CD117/c-Kit antibodies. The subpopulations of DN1-4 thymocytes were defined as DN cells that express $CD117^+$ $CD44^+CD25^-$, $CD117^+$ $CD44^+CD25^+$, $CD117^-$ $CD44^-CD25^+$, and $CD117^-$ $CD44^-CD25^-$ markers, respectively (Godfrey et al., 1993). Antibodies used are the following: anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-B220 (RA3-6B2), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8c5), anti-NK1.1 (PK136), anti-TCRβ (H57-597), anti-TCR$\gamma\delta$ (GL3), anti-CD44 (1M7), anti-CD117 (2B8), anti-CD25 (7D4), anti-HSA (M1/69), and anti-pTα (Pharmingen, San Diego, CA). The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Cell Transfer and Bone Marrow Chimera Studies

We excluded the possible influence of inflammation on the development of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes in mixed stem-cell bone marrow chimeras. To generate these chimeras, we intraperitoneally injected wild-type ($H\text{-}2K^{b/b}$) and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ ($H\text{-}2K^{d/b}$) mice (4–5 weeks old) with 5 mg of 5-fluorouracil (5-FU) to deplete the actively dividing BM cells. Five days later, BM cells were recovered from the mice, mixed in a 1:1 ratio (1×10^6 from each donor), and then transferred into lethally irradiated (900 Rads) *Rag2*$^{-/-}$ recipient ($H\text{-}2K^{b/b}$) mice by i.v. injection. One month later, thymocytes were recovered from these BM chimeras and subjected to flow cytometric analysis. *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ thymocytes developed in the recipient mice were distinguished from wild-type cells by flow cytometry with antibody against $H\text{-}2K^d$ (34-2-12, Pharmingen). To examine the dependence of T cell development on MHC-I alone or on both MHC-I and II, we generated BM chimeras with 5′-FU-treated *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ and Cbl-b$^{-/-}$ mice as the donors and lethally irradiated MHC-I-deficient (*B2m*$^{-/-}$) or MHC-I and II double-deficient (*B2m*$^{-/-}$, *Abb*$^{-/-}$) mice as the recipients. One month later, we analyzed the development of $CD4^+$ and $CD8^+$ lineage cells on a FACS LSR II after staining the cells with anti-Ly9.1 (30C7, Pharmingen), anti-CD4, and anti-CD8. Donor cells were $Ly9.1^+$ so they could be distinguished from the recipient cells ($Ly9.1^-$) based on Ly9.1 expression.

Biochemical Studies

We used total thymocytes or purified DP thymocytes for biochemical analyses. DP thymocytes were purified by FACS sorting. Activation of individual signaling events in thymocytes induced by the TCR was examined according to previously published protocols (Naramura et al., 1998). In brief, to determine tyrosine phosphorylation of total cellular proteins, we stained 1×10^7 total or purified DP thymocytes with biotinylated anti-CD3ε (145-2C11) (5 μg/ml) alone or together with biotinylated anti-CD4 or CD8 (each 5 μg/ml) followed by straptavidin (10 μg/ml) at 37°C for 2 min. Cells were then lysed in RIPA buffer supplemented with proteinase and phosphatase inhibitors. Tyrosine phosphorylation of total proteins or activation of Erk1/2 in the lysates was determined by immunoblot with antibodies against phosphotyrosine (4G10, Upstate Biotech, Charlottesville, VA) or the anti-active forms of Erk1 and 2 (E10, New England Biolabs, Beverly, MA). To determine the tyrosine phosphorylation of Zap70, Vav, CD3ζ chain, and PLCγ-1, we immunoprecipitated the corresponding proteins from the cell lysates and examined the levels of tyrosine phosphorylation of these proteins by western blot hybridization with 4G10 antibody. The antibodies used for immunoprecipitation were: anti-Zap70 (sc 574 AC), anti-Vav (H-21), anti-CD3ζ (C-20), and anti-PLCγ-1 (sc-426) (Santa Cruz Biotechnologies, Inc, Santa Cruz, CA). To determine Ca^{2+} mobilization, total thymocytes were loaded with Fluo-4 and Fura-red (Molecular Probes, Eugene, OR), surface stained with anti-CD4 and anti-CD8, and then stimulated with biotinylated anti-CD3ε (1 μg/ml) and straptavidin (5 μg/ml). Ca^{2+} influx were determined by flow cytometry according to a previously published protocol (Naramura et al., 2002). To determine the amount of Lck (Y394) phosphorylation, membrane was directly hybridized with rabbit anti-phosphorylated Lck (Y394) antibody (a gift from Andrey Shaw).

Thymocyte Proliferation and IL-2 Production Assay

To measure thymocyte proliferation, CFSE-labeled total thymocytes (10^5 cells/well) were stimulated in 96-well plate with plate-bound

anti-CD3 (5 μg/ml) and soluble anti-CD28 (5 μg/ml) for 3 days. After the stimulation, cells were stained with anti-CD4 and anti-CD8 and then analyzed on an LSR II. For IL-2 production assay, cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (5 μg/ml) for 18 hr followed by incubation with 10 μg/ml Brefeldin A for 6 hr. After stimulation, cells were surface stained with anti-CD4 and anti-CD8, permeabilized, and intracellularly stained with anti-IL-2 before being subjected to FACS analysis.

Electrophoresis Mobility Gel Shift Assay

We stimulated DP thymocytes with plate-bound anti-CD3ε (5 μg/ml) alone or together with soluble anti-CD28 (5 μg/ml) or PMA (10 ng/ml) + Ionomycin (10 ng/ml) for 8–10 hr at 37°C. For unstimulated cells, we cultured DP thymocytes in the normal medium in the absence of stimuli antibodies. Nuclear extracts were prepared and EMSAs conducted according to a previous protocol (Sun et al., 2000). To assess the binding activities of the corresponding nuclear factors, we used the following oligonucleotides: NF-κB: 5′-ACCAA GAGGGATTTCACCTAAATC-3′; AP-1: 5′-CGCTTGATGACTCAGCC GGAA-3′.

Supplemental Data

Five Supplemental Figures can be found with this article online at http://www.immunity.com/cgi/content/full/25/4/571/DC1/.

Acknowledgments

We thank Drs. Y. Zou for advice and comments, J. Takeda for *Lck-cre* transgenic mice, F. Costantini for R26R-EYFP mice, and A. Shaw for phospho-Lck (Y394) antibody. Y.K. was supported by postdoctoral fellowships from The Uehara Memorial Foundation and from The Charles H. Revson Foundation. M.S.S. is supported by a grant from the NIH (R01 HL48702). H.G. is supported by the Irene Diamond Fund. This work was partly supported by the NIH intramural research program.

Received: December 14, 2004
Revised: January 24, 2006
Accepted: August 1, 2006
Published online: October 12, 2006

References

Aifantis, I., Gounari, F., Scorrano, L., Borowski, C., and von Boehmer, H. (2001). Constitutive pre-TCR signaling promotes differentiation through Ca2+ mobilization and activation of NF-kappaB and NFAT. Nat. Immunol. *2*, 403–409.

Bachmaier, K., Krawczyk, C., Kozieradzki, I., Kong, Y.Y., Sasaki, T., Oliveira-dos-Santos, A., Mariathasan, S., Bouchard, D., Wakeham, A., Itie, A., et al. (2000). Negative regulation of lymphocyte activation and autoimmunity by the molecular adaptor Cbl-b. Nature *403*, 211–216.

Baker, J.E., Majeti, R., Tangye, S.G., and Weiss, A. (2001). Protein tyrosine phosphatase CD148-mediated inhibition of T-cell receptor signal transduction is associated with reduced LAT and phospholipase Cgamma1 phosphorylation. Mol. Cell. Biol. *21*, 2393–2403.

Chiang, Y.J., Kole, H.K., Brown, K., Naramura, M., Fukuhara, S., Hu, R.J., Jang, I.K., Gutkind, J.S., Shevach, E., and Gu, H. (2000). Cbl-b regulates the CD28 dependence of T-cell activation. Nature *403*, 216–220.

Choi, E.Y., Jung, K.C., Park, H.J., Chung, D.H., Song, J.S., Yang, S.D., Simpson, E., and Park, S.H. (2005). Thymocyte-thymocyte interaction for efficient positive selection and maturation of CD4 T cells. Immunity *23*, 387–396.

Cosgrove, D., Gray, D., Dierich, A., Kaufman, J., Lemeur, M., Benoist, C., and Mathis, D. (1991). Mice lacking MHC class II molecules. Cell *66*, 1051–1066.

Dikic, I., and Blaukat, A. (1999). Protein tyrosine kinase-mediated pathways in G protein-coupled receptor signaling. Cell Biochem. Biophys. *30*, 369–387.

Fang, D., Elly, C., Gao, B., Fang, N., Altman, Y., Joazeiro, C., Hunter, T., Copeland, N., Jenkins, N., and Liu, Y.C. (2002). Dysregulation of T lymphocyte function in itchy mice: a role for Itch in TH2 differentiation. Nat. Immunol. *3*, 281–287.

Fink, P.J., and Bevan, M.J. (1978). H-2 antigens of the thymus determine lymphocyte specificity. J. Exp. Med. *148*, 766–775.

Fowlkes, B.J., and Pardoll, D.M. (1989). Molecular and cellular events of T cell development. Adv. Immunol. *44*, 207–264.

Germain, R.N. (2002). T-cell development and the CD4-CD8 lineage decision. Nat. Rev. Immunol. *2*, 309–322.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-kappaB puzzle. Cell (*Suppl*) *109*, S81–S96.

Godfrey, D.I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J. Immunol. *150*, 4244–4252.

Grusby, M.J., Johnson, R.S., Papaioannou, V.E., and Glimcher, L.H. (1991). Depletion of CD4+ T cells in major histocompatibility complex class II-deficient mice. Science *253*, 1417–1420.

Hayday, A.C., Barber, D.F., Douglas, N., and Hoffman, E.S. (1999). Signals involved in gamma/delta T cell versus alpha/beta T cell lineage commitment. Semin. Immunol. *11*, 239–249.

Jehn, B.M., Dittert, I., Beyer, S., von der Mark, K., and Bielke, W. (2002). c-Cbl binding and ubiquitin-dependent lysosomal degradation of membrane-associated Notch1. J. Biol. Chem. *277*, 8033–8040.

Joazeiro, C.A., Wing, S.S., Huang, H., Leverson, J.D., Hunter, T., and Liu, Y.C. (1999). The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. Science *286*, 309–312.

Jorissen, R.N., Walker, F., Pouliot, N., Garrett, T.P., Ward, C.W., and Burgess, A.W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. Exp. Cell Res. *284*, 31–53.

Kane, L.P., Lin, J., and Weiss, A. (2002). It's all Rel-ative: NF-kappaB and CD28 costimulation of T-cell activation. Trends Immunol. *23*, 413–420.

Kaye, J., Hsu, M.L., Sauron, M.E., Jameson, S.C., Gascoigne, N.R., and Hedrick, S.M. (1989). Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. Nature *341*, 746–749.

Kirberg, J., Berns, A., and von Boehmer, H. (1997). Peripheral T cell survival requires continual ligation of the T cell receptor to major histocompatibility complex-encoded molecules. J. Exp. Med. *186*, 1269–1275.

Koretzky, G.A., and Myung, P.S. (2001). Positive and negative regulation of T-cell activation by adaptor proteins. Nat. Rev. Immunol. *1*, 95–107.

Li, Q., and Verma, I.M. (2002). NF-kappaB regulation in the immune system. Nat. Rev. Immunol. *2*, 725–734.

Li, W., Kim, M.G., Gourley, T.S., McCarthy, B.P., Sant'Angelo, D.B., and Chang, C.H. (2005). An alternate pathway for CD4 T cell development: thymocyte-expressed MHC class II selects a distinct T cell population. Immunity *23*, 375–386.

Liu, Y.C. (2004). Ubiquitin ligases and the immune response. Annu. Rev. Immunol. *22*, 81–127.

Love, P.E., and Chan, A.C. (2003). Regulation of thymocyte development: only the meek survive. Curr. Opin. Immunol. *15*, 199–203.

Lucas, J.A., Miller, A.T., Atherly, L.O., and Berg, L.J. (2003). The role of Tec family kinases in T cell development and function. Immunol. Rev. *191*, 119–138.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355–360.

Nakayama, T., Samelson, L.E., Nakayama, Y., Munitz, T.I., Sheard, M., June, C.H., and Singer, A. (1991). Ligand-stimulated signaling events in immature CD4+CD8+ thymocytes expressing competent T-cell receptor complexes. Proc. Natl. Acad. Sci. USA *88*, 9949–9953.

Naramura, M., Kole, H.K., Hu, R.J., and Gu, H. (1998). Altered thymic positive selection and intracellular signals in Cbl-deficient mice. Proc. Natl. Acad. Sci. USA *95*, 15547–15552.

Naramura, M., Jang, I.K., Kole, H., Huang, F., Haines, D., and Gu, H. (2002). c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. Nat. Immunol. *3*, 1192–1199.

Panigada, M., Porcellini, S., Barbier, E., Hoeflinger, S., Cazenave, P.A., Gu, H., Band, H., von Boehmer, H., and Grassi, F. (2002). Constitutive endocytosis and degradation of the pre-T cell receptor. J. Exp. Med. *195*, 1585–1597.

Rao, N., Dodge, I., and Band, H. (2002). The Cbl family of ubiquitin ligases: critical negative regulators of tyrosine kinase signaling in the immune system. J. Leukoc. Biol. *71*, 753–763.

Ruland, J., Duncan, G.S., Elia, A., del Barco Barrantes, I., Nguyen, L., Plyte, S., Millar, D.G., Bouchard, D., Wakeham, A., Ohashi, P.S., and Mak, T.W. (2001). Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell *104*, 33–42.

Sanchez-Martin, L., Sanchez-Sanchez, N., Gutierrez-Lopez, M.D., Rojo, A.I., Vicente-Manzanares, M., Perez-Alvarez, M.J., Sanchez-Mateos, P., Bustelo, X.R., Cuadrado, A., Sanchez-Madrid, F., et al. (2004). Signaling through the leukocyte integrin LFA-1 in T cells induces a transient activation of Rac-1 that is regulated by Vav and PI3K/Akt-1. J. Biol. Chem. *279*, 16194–16205.

Sha, W.C., Nelson, C.A., Newberry, R.D., Kranz, D.M., Russell, J.H., and Loh, D.Y. (1988). Positive and negative selection of an antigen receptor on T cells in transgenic mice. Nature *336*, 73–76.

Sun, Z., Arendt, C.W., Ellmeier, W., Schaeffer, E.M., Sunshine, M.J., Gandhi, L., Annes, J., Petrzilka, D., Kupfer, A., Schwartzberg, P.L., and Littman, D.R. (2000). PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. Nature *404*, 402–407.

Takahama, Y., Ohishi, K., Tokoro, Y., Sugawara, T., Yoshimura, Y., Okabe, M., Kinoshita, T., and Takeda, J. (1998). Functional competence of T cells in the absence of glycosylphosphatidylinositol-anchored proteins caused by T cell-specific disruption of the Pig-a gene. Eur. J. Immunol. *28*, 2159–2166.

Takeda, S., Rodewald, H.R., Arakawa, H., Bluethmann, H., and Shimizu, T. (1996). MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. Immunity *5*, 217–228.

Tanchot, C., Lemonnier, F.A., Perarnau, B., Freitas, A.A., and Rocha, B. (1997). Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science *276*, 2057–2062.

Teh, H.S., Kisielow, P., Scott, B., Kishi, H., Uematsu, Y., Bluthmann, H., and von Boehmer, H. (1988). Thymic major histocompatibility complex antigens and the alpha beta T-cell receptor determine the CD4/CD8 phenotype of T cells. Nature *335*, 229–233.

Thien, C.B., Walker, F., and Langdon, W.Y. (2001). RING finger mutations that abolish c-Cbl-directed polyubiquitination and down-regulation of the EGF receptor are insufficient for cell transformation. Mol. Cell *7*, 355–365.

Thome, M., and Tschopp, J. (2003). TCR-induced NF-kappaB activation: a crucial role for Carma1, Bcl10 and MALT1. Trends Immunol. *24*, 419–424.

Viret, C., Wong, F.S., and Janeway, C.A., Jr. (1999). Designing and maintaining the mature TCR repertoire: the continuum of self-peptide:self-MHC complex recognition. Immunity *10*, 559–568.

Voll, R.E., Jimi, E., Phillips, R.J., Barber, D.F., Rincon, M., Hayday, A.C., Flavell, R.A., and Ghosh, S. (2000). NF-kappa B activation by the pre-T cell receptor serves as a selective survival signal in T lymphocyte development. Immunity *13*, 677–689.

von Boehmer, H. (1988). The developmental biology of T lymphocytes. Annu. Rev. Immunol. *6*, 309–326.

von Boehmer, H., and Fehling, H.J. (1997). Structure and function of the pre-T cell receptor. Annu. Rev. Immunol. *15*, 433–452.

Wang, D., Matsumoto, R., You, Y., Che, T., Lin, X.Y., Gaffen, S.L., and Lin, X. (2004). CD3/CD28 costimulation-induced NF-kappaB activation is mediated by recruitment of protein kinase C-theta, Bcl10, and IkappaB kinase beta to the immunological synapse through CARMA1. Mol. Cell. Biol. *24*, 164–171.

Weiss, A., and Littman, D.R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263–274.

Witherden, D., van Oers, N., Waltzinger, C., Weiss, A., Benoist, C., and Mathis, D. (2000). Tetracycline-controllable selection of CD4(+) T cells: half-life and survival signals in the absence of major histocompatibility complex class II molecules. J. Exp. Med. *191*, 355–364.

Yamasaki, S., Ishikawa, E., Sakuma, M., Ogata, K., Sakata-Sogawa, K., Hiroshima, M., Wiest, D.L., Tokunaga, M., and Saito, T. (2006). Mechanistic basis of pre-T cell receptor-mediated autonomous signaling critical for thymocyte development. Nat. Immunol. *7*, 67–75.

Zell, T., Kivens, W.J., Kellermann, S.A., and Shimizu, Y. (1999). Regulation of integrin function by T cell activation: points of convergence and divergence. Immunol. Res. *20*, 127–145.

Zijlstra, M., Bix, M., Simister, N.E., Loring, J.M., Raulet, D.H., and Jaenisch, R. (1990). Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. Nature *344*, 742–746.

Supplemental Data

Establishment of the Major Compatibility Complex-Dependent Development of $CD4^+$ and $CD8^+$ T Cells by the Cbl Family Proteins

Fang Huang, Yasuyuki Kitaura, IhnKyung Jang, Mayumi Naramura, Hemanta H. Kole, Liping Liu, Haiyan Qin, Mark S. Schlissel, and Hua Gu

Supplemental References

Hempel, W.M., Stanhope-Baker, P., Mathieu, N., Huang, F., Schlissel, M.S., and Ferrier, P. (1998). Enhancer control of V(D)J recombination at the TCRbeta locus: differential effects on DNA cleavage and joining. Genes Dev. *12*, 2305–2317.

Schlissel, M.S., Durum, S.D., and Muegge, K. (2000). The interleukin 7 receptor is required for T cell receptor gamma locus accessibility to the V(D)J recombinase. J. Exp. Med. *191*, 1045–1050.

Srinivas, S., Watanabe, T., Lin, C.S., William, C.M., Tanabe, Y., Jessell, T.M., and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev. Biol. *1*, 4.

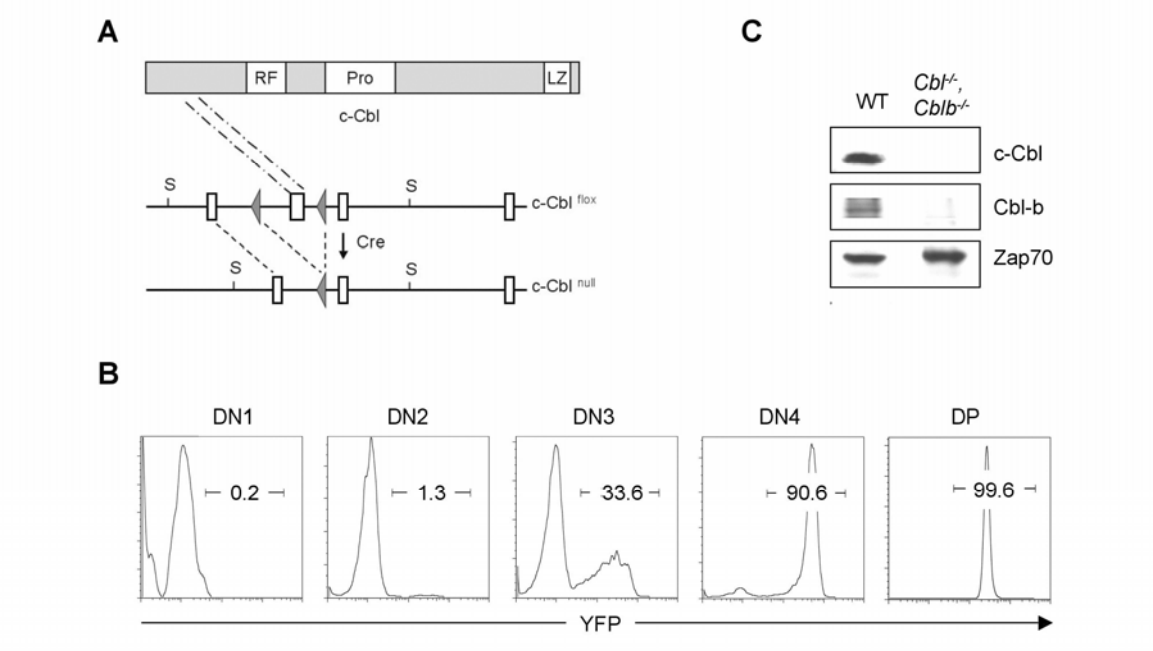

Figure S1. *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ Mice

A) Strategy to generate c-Cbl floxed (*Cbl*$^{f/f}$) mice. Two loxP sites (triangles) were inserted into the *Cbl* locus to flank the exon encoding a part of the phosphotyrosine-binding (PTB) domain of the *Cbl* gene by gene targeting. Deletion of this exon creates a reading-frame-shift in the coding region of *Cbl* gene down stream of this exon. *Cbl*$^{f/f}$ mice were bred to *Cblb*$^{-/-}$ mice and then to *Lck-cre* Tg to obtain *Cbl*$^{f/f}$, *Cblb*$^{-/-}$, *Lck-cre* Tg (*Cbl*$^{-/-}$, *Cblb*$^{-/-}$) mice. Because the *Cre* gene is expressed only in thymocytes, the Cbl double-deficiency occurs only in T-lineage cells in these mice. **B)** YFP expression in DN1-4 and DP thymocytes from *Lck-cre* Tg R26R-EYFP mice (Srinivas et al., 2001). We tested the efficiency of *Lck-cre*-mediated deletion in thymocyte subsets using R26R-EYFP reporter mice. In these mice, cells with Cre-mediated deletion of the transcription stopper in R26R-EYFP gene express EYFP. Shown are the histograms of YFP expression on the gated DN1, 2, 3, and 4, and DP thymocytes, respectively. Percentages of YFP positive cells are indicated in each histogram. **C**) Immunoblot analysis of c-Cbl and Cbl-b expression in DP thymocytes from wildtype (WT) and Cbl double-deficient (*Cbl*$^{-/-}$, *Cblb*$^{-/-}$) mice.

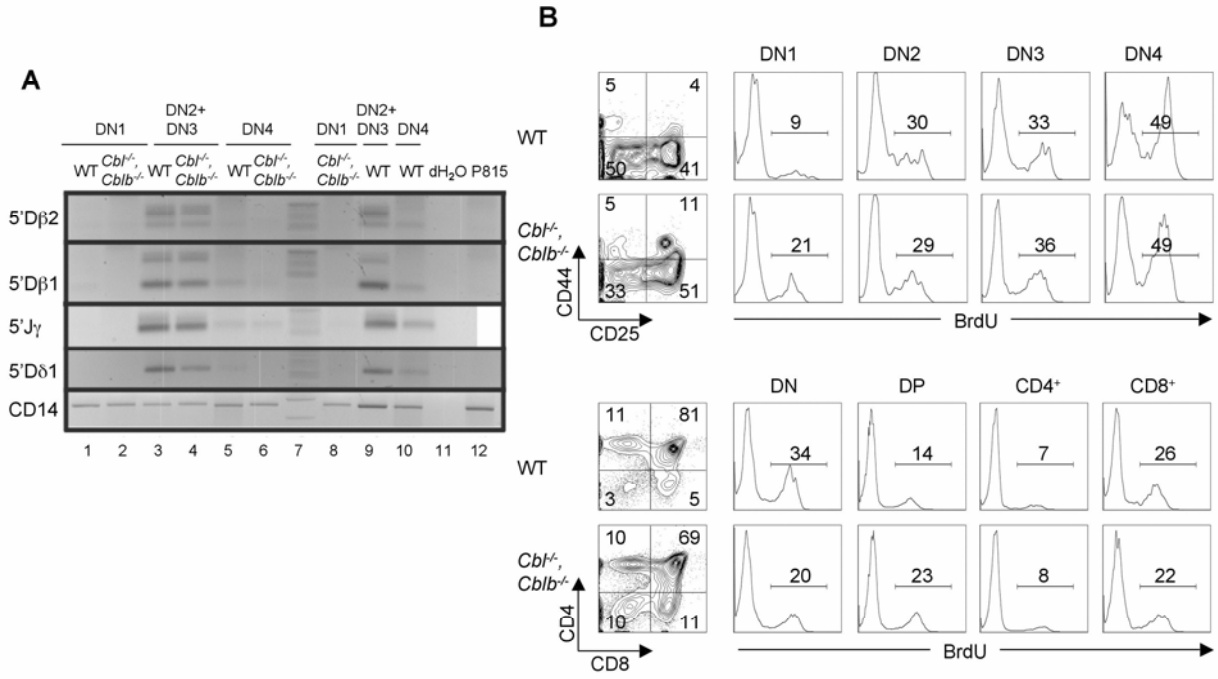

Figure S2. TCRβ and TCRγ Gene Rearrangements in DN Thymocytes.

A) Equivalent amounts of genomic DNA purified from the indicated sorted cell populations were subjected to LM-PCR analysis for dsDNA breaks associated with V(D)J recombination at the indicated TCR gene segments. CD14 consists of a series of control amplifications of a non-rearranging locus demonstrating equivalence of DNA samples. P815 is a myeloid tumor line and dH_20 indicates reactions where water was substituted for linker-ligated DNA template. Negative images of ethidium bromide stained gel photographs are shown. These assays were performed as described previously (Hempel et al., 1998; Schlissel et al., 2000). **B)** Normal proliferation of *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ DN3 and DN4 thymocytes. Wildtype and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ mice were injected with BrdU (1mg/mouse) by *i.v.* injection. Three hours later, mice were sacrificed and BrdU positive cells in each thymocyte subsets were analyzed by flow cytometry. Shown are histograms of BrdU positive cells among each subsets of gated DN, DP and SP thymocytes. The results are representatives of three independent experiments.

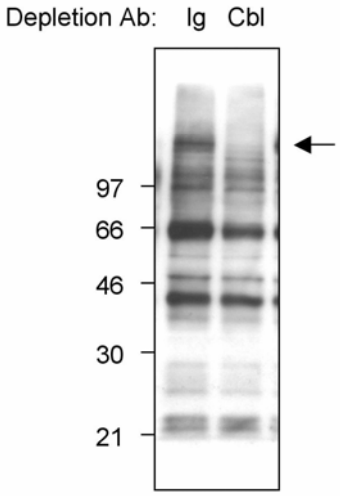

Figure S3. Identification of 120 kD Phosphoprotein in Total Thymocyte Lysate

Total thymocytes were stimulated with anti-CD3 for 2 minutes. To determine whether the strong phosphoprotein (120 kD band) represented Cbl protein, we pre-depleted Cbl protein from the cell lysate using anti-Cbl. Tyrosine phosphorylation of total cellular proteins pre-cleared Cbl was determined by immunoblot using 4G10 antibody. Ig: cell lysate treated with isotype matched rabbit Ig. Cbl: cell lysate pre-cleared with anti-Cbl. 120 kD band representing Cbl is indicated by an arrow.

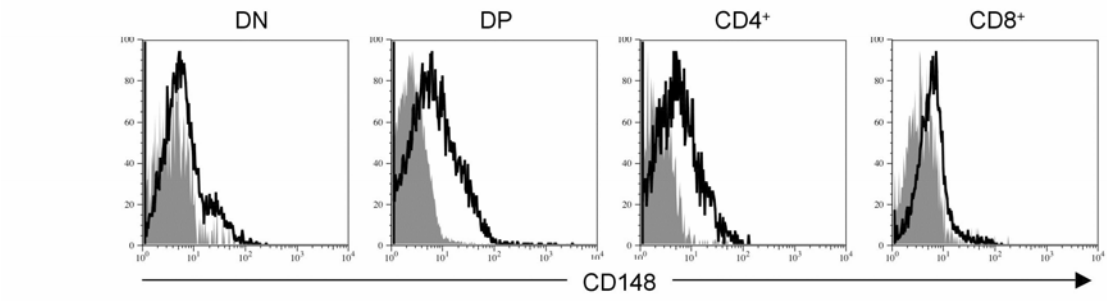

Figure S4. Flow Cytometric Analysis of CD148 Expression on Thymocytes

Shown are histograms of CD148 expression on wildtype (shadow) and *Cbl*$^{-/-}$, *Cblb*$^{-/-}$ (solid line) DN, DP, CD4^{+}, and CD8^{+} thymocytes.

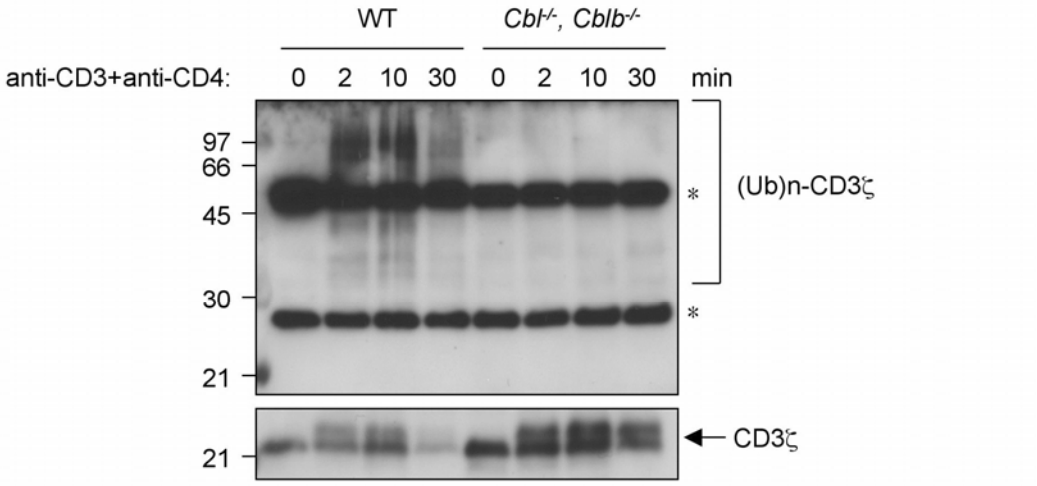

Figure S5. Ubiquitination of CD3ζ Chain by Cbl Proteins

Purified T cells were stimulated with anti-CD3 for 2 minutes. Cells were lysed in RIPA buffer, and CD3ζ chain in the lysate was immunoprecipitated and immunoblotted with an anti-body against ubiquitin.