Inactivation of c-Cbl Reverses Neonatal Lethality and T Cell Developmental Arrest of SLP-76–deficient Mice

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Abstract

c-Cbl is an adaptor protein that negatively regulates signal transduction events involved in thymic-positive selection. To further characterize the function of c-Cbl in T cell development, we analyzed the effect of c-Cbl inactivation in mice deficient in the scaffolding molecule SLP-76. SLP-76–deficient mice show a high frequency of neonatal lethality; and in surviving mice, T cell development is blocked at the DN3 stage. Inactivation of *c-cbl* completely reversed the neonatal lethality seen in SLP-76–deficient mice and partially reversed the T cell development arrest in these mice. SLP-76$^{-/-}$ Cbl$^{-/-}$ mice exhibited marked expansion of polarized T helper type (Th)1 and Th2 cell peripheral CD4$^+$ T cells, lymphoid infiltrates of parenchymal organs, and premature death. This rescue of T cell development is T cell receptor dependent because it does not occur in recombination activating gene 2$^{-/-}$ SLP-76$^{-/-}$ Cbl$^{-/-}$ triple knockout mice. Analysis of the signal transduction properties of SLP-76$^{-/-}$ Cbl$^{-/-}$ T cells reveals a novel SLP-76– and linker for activation of T cells–independent pathway of extracellular signal–regulated kinase activation, which is normally down-regulated by c-Cbl.

Key words: c-Cbl • SLP-76 • LAT • T cell development • ERK

Introduction

T cell development and activation are regulated by signals (1) resulting from the interaction between TCR and peptide/MHCs on APCs. This signaling cascade is initiated by the phosphorylation of TCR subunits by Lck, which in turn creates binding sites for ZAP-70. Subsequently, the binding of ZAP-70 to phosphorylated TCR leads to autophosphorylation and activation of ZAP-70 kinase activity (2–4). Activated ZAP-70 phosphorylates downstream substrates, such as SLP-76 and linker for activation of T cells (LAT), which act as critical scaffolding molecules that bind and deliver signals to molecules including phospholipase Cγ-1, Itk, Vav, Grb2, extracellular signal–regulated kinases (ERKs), and Gads (2–5). These biochemical events trigger the activation of transcription factors that participate in thymic development and peripheral T cell responses. This model for signal transduction through a ZAP-70–LAT–SLP-76 pathway is supported by studies using knockout mouse models in which thymic development is blocked at the CD4$^-$ CD8$^-$ double negative (DN) stage in LAT- or SLP-76–deficient mice (6–10).

TCR-mediated signaling is also modulated by negative or inhibitory influences. Cbl family proteins have been proposed to be critical factors in mediating this negative regulation (11, 12). Three Cbl family members have been identified as c-Cbl (Cbl), Cbl-b, and Cbl-3 (Cbl-c) in mammalian cells. Cbl proteins consist of multiple functional domains, including an NH$_2$-terminal tyrosine kinase binding domain, a ring finger, a proline-rich region, and a COOH-terminal leucine-zipper/UBA domain (11). They interact with multiple protein molecules to affect signaling events via their protein–protein interaction domains. Recently, the ring finger domains of Cbl proteins were shown

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Abbreviations used in this paper: DN, double negative; DP, double positive; ERK, extracellular signal–regulated kinase; LAT, linker for activation of T cells; SP, single positive.

to function as E3 ubiquitin ligases that down-regulate the activities of signaling molecules associated with Cbl proteins through protein ubiquitination/degradation (13, 14).

c-Cbl appears to have multiple and complicated influences on T cell development. Thymocytes from c-Cbl knockout mice have enhanced cell surface expression of TCR and CD3 in comparison to control mice (15, 16). Studies of positive selection in a number of TCR transgenic mouse models have further found that MHC class II–restricted positive selection is markedly enhanced by c-Cbl inactivation (15). In addition, it has been observed that phosphorylation of ZAP-70, LAT, and SLP-76 is increased in c-Cbl$^{-/-}$ mouse thymocytes (15–17). These observations suggested that c-Cbl down-regulates a ZAP-70–dependent TCR–CD3 signaling pathway, thus inhibiting T cell–positive selection during thymic development. However, a recent report characterized a c-Cbl knock-in mouse with an amino acid substitution in the c-Cbl protein that results in loss of the ability to bind ZAP-70 (18). Unexpectedly, the effect of this mutation differed from that of c-Cbl deletion and did not result in any increase in phosphorylation of LAT and SLP-76. CD3 expression was normal in double positive (DP) thymocytes from mutant c-Cbl knock-in mice, again in contrast to the enhanced CD3 expression in c-Cbl knockout mice (18). These results suggest that c-Cbl may also down-regulate TCR signaling through a mechanism that is independent of a ZAP-70 pathway.

To further assess the possibility that c-Cbl proteins may inhibit T cell development through a pathway that is independent of ZAP-70–LAT–SLP-76, we examined the effect of c-Cbl inactivation in mice deficient in either SLP-76 or LAT. Strikingly, c-Cbl inactivation completely reversed the neonatal lethality observed in SLP-76 knockout mice and partially reversed their defect in thymic development. Moreover, mice deficient in both c-Cbl and SLP-76 exhibited a marked expansion of peripheral CD4$^+$ T cells as well as B cells and macrophages, leading to splenomegaly and lymphadenopathy as well as lymphoid infiltrates of multiple parenchymal organs, and premature death in these mice. These peripheral CD4$^+$ cells had decreased cell surface expression of CD3 and TCR in contrast to the significant increase in CD3 and TCR observed in single knockout c-Cbl$^{-/-}$ mice. The majority of peripheral CD4$^+$ cells in SLP-76/Cbl-deficient mice were polarized Th1 or Th2 cytokine-secreting cells. Interestingly, however, SLP-76/Cbl-deficient CD4$^+$ cells were unresponsive to TCR stimulation in vitro, but exhibited a constitutive phosphorylation of ERKs ex vivo. Thus, c-Cbl inactivation partially bypasses the defects seen in SLP-76–deficient mice and results in dysregulated and SLP-76–independent expansion of Th1/Th2 polarized CD4$^+$ T cells. LAT$^{-/-}$ Cbl$^{-/-}$ mice were found to express a phenotype similar to that of SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. In contrast, SLP-76$^{-/-}$ Cbl$^{-/-}$ RAG-2$^{-/-}$ triple knockout mice failed to show rescue of T cell development. These findings indicate that inactivation of c-Cbl results in partial reversal of the defects in T cell development seen in SLP-76$^{-/-}$ mice through a LAT- and SLP-76–independent but TCR–dependent signaling pathway.

Materials and Methods

Mice. C-Cbl knockout (Cbl$^{-/-}$), LAT knockout (LAT$^{-/-}$), RAG-2 knockout (RAG-2$^{-/-}$), and SLP-76 knockout (SLP-76$^{-/-}$) were mice generated as described previously (8, 10, 15). The c-Cbl knockout line was maintained by breeding homozygous knockout mice (c-Cbl$^{-/-}$), and SLP-76 knockouts were maintained as heterozygous mice (SLP-76$^{+/-}$) that were intercrossed to generate SLP-76$^{+/+}$, SLP-76$^{+/-}$, and SLP-76$^{-/-}$ offspring. The LAT knockout line was maintained by breeding homozygous knockout mice (LAT$^{-/-}$). All animals were housed at Bioqual.

Abs. Anti–mouse CD3 (2C11), anti-CD28 (4F10), anti–ZAP-70, anti-CD4 PE, anti–IL-4 PE, anti–IL-2 PE, anti–IFN-γ PE, anti-CD8 FITC, anti-CD25 biotin, anti–human CD3 (leu-4) biotin, anti-CD62L biotin, and anti–TCR-β (H57) biotin mAbs were purchased from BD Biosciences. Anti–phospho-tyrosine (4G10) mAb was purchased from Upstate Biotechnology. Anti-Lck, anti-ERK, and polyclonal Ab specific for serine- and tyrosine-phosphorylated ERK were purchased from Cell Signaling.

T Cell Proliferation and IL-2 Assays. T cells or CD4$^+$ T cells were purified from spleens or lymph nodes of 6–8-wk-old mice using T cell or CD4 cell separation columns (Accurate), respectively. For T cell proliferation assays, 100 μl purified CD4$^+$ cells (10^6/ml) were cultured in a 96-well plate precoated with 1 μg/ml CD3 Ab at 37°C for 48 h. 1 μl/well [^{3}H]thymidine was added and [^{3}H]thymidine incorporation was measured after an additional culture of 16 h at 37°C. For IL-2 assay, 10^5 cells (100 μl) were cultured in a 96-well plate with 10 μg/ml anti-CD3 Ab in the presence or absence of 1 μg/ml soluble anti-CD28 Ab (19). Supernatant was collected after 48 h of culture at 37°C and secreted IL-2 in the supernatant was detected using an ELISA kit (BD Biosciences).

Intracellular Cytokine Staining. Lymphoid cells were stimulated with 200 ng/ml PMA and 300 ng/ml ionomycin for 4 h. 2 μm monensin was added and cells were cultured for an additional 2 h (20). Cells were harvested and fixed with 4% paraformaldehyde for 20 min at room temperature, and fixed cells were stained with Abs as indicated and analyzed by flow cytometry using a FACScan™ (Becton Dickinson; reference 20).

Measurement of Intracellular Calcium Concentration. For intracellular calcium determination, 2 × 10^6 spleen cells were suspended in 1 ml of HBSS buffer containing 0.5% BSA and 1.8 mM Indo 1-AM (Molecular Probes) and incubated at 37°C for 30 min. The indo-1–loaded cells were then washed twice with HBSS buffer and incubated with biotin-conjugated anti-CD3 and anti-CD4 mAbs, followed by staining with anti-CD4 FITC Ab to allow detection and gating of CD4$^+$ cells. The stained cells were warmed to 37°C and analyzed on a FACStar™ (Becton Dickinson) for responses to 1 μg streptavidin or 2 μg ionomycin. For indo-1 fluorescence excitation, the Inova-305 laser was tuned to 200 mW power UV excitation. The emitted indo-1 fluorescence at 405 and 510 nm was collected (21). The 405:510 nm fluorescence ratio as a measurement of Ca^{2+} was calculated and kinetics were plotted with the FlowJo program (TreeStar, Inc.).

Western Blot Analysis T cells were enriched from spleen and lymph node by negative selection with anti-mIg Ab. Enriched T cells were incubated with 10 μg/ml biotin-conjugated anti-CD3 and anti-CD4 Abs for 30 min, and then stimulated with 20 μg/ml streptavidin for the indicated time (19). The stimulated cells were lysed in buffer containing 50 μm Tris, pH 7.4, 150 μm NaCl, 1 mM Na$_2$VO$_4$, 1% NP-40, and protease inhibitor cocktail. Protein lysates were used for biochemical analysis.

Online Supplemental Materials. Results of experiments, which complement the data presented in this study, are shown in Figs.

Table I. *The Perinatal Lethal Defect of SLP-76 Knockout Mice Is Rescued by c-Cbl Inactivation*

Total offspring		SLP-76 genotypes		
		+/+	+/−	−/−
SLP-76$^{+/−}$	Exp.	32	64	32
×	127			
SLP-76$^{+/−}$	Obs.	41	78	8
SLP-76$^{+/−}$ Cbl$^{−/−}$	Exp.	19	39	19
×	78			
SLP-76$^{+/−}$ Cbl$^{−/−}$	Obs.	19	38	21

Exp., expected; Obs., observed.

S1–S3. In Fig. S1, the expression of CD4 on peripheral CD4$^+$ T cells of WT, LAT$^{−/−}$ Cbl$^{−/−}$, and SLP-76$^{−/−}$ Cbl$^{−/−}$ mice is analyzed by flow cytometry. In Fig. S2, lymph nodes and spleens from WT and SLP-76$^{−/−}$ Cbl$^{−/−}$ mice at the age of 16 wk were isolated and photographed. In Fig. S3, DN cells were purified from thymocytes by depleting CD4$^+$ and CD8$^+$ cells with mouse CD4 and CD8 MicroBeads (Miltenyi Biotec). 3×10^6 DN cells from WT, c-Cbl$^{−/−}$, SLP-76$^{−/−}$, and SLP-76$^{−/−}$ Cbl$^{−/−}$ mice were lysed and immunoblotted with anti–Zap-70 and anti-actin Abs as described above. Figs. S1–S3 are available at http://www.jem.org/cgi/content/full/jem.20040262/DC1.

Results

Generation of SLP-76$^{−/−}$ Cbl$^{−/−}$ Double Knockout Mice. SLP-76$^{−/−}$ Cbl$^{−/−}$ double knockout mice were generated by intercrossing SLP-76$^{+/−}$ Cbl$^{−/−}$ mice. All mice were genotyped at 4 wk of age. As expected from previous reports (6, 9), by the time of weaning there was a selective loss of SLP-76$^{−/−}$ progeny (Table I) attributable to platelet dysfunction (8) and a more recently described failure of normal vascular development (22). Surprisingly, however, concomitant loss of c-Cbl and SLP-76 rescues the perinatal lethality, as the observed proportion of SLP-76$^{−/−}$ Cbl$^{−/−}$ mice was equivalent to that expected by Mendelian segregation (Table I). The mechanism by which c-Cbl mutation reverses the survival defect of SLP-76$^{−/−}$ mice is currently under investigation.
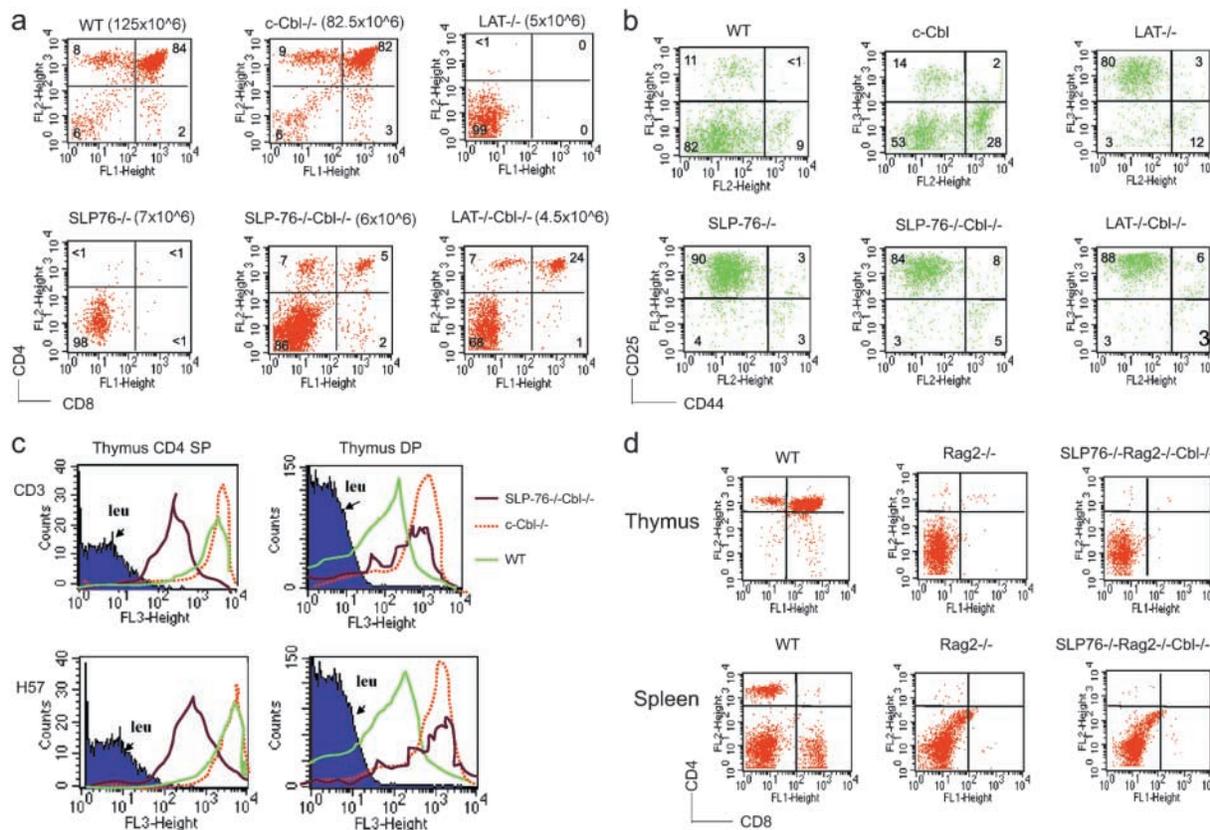
Figure 1. Inactivation of c-Cbl partially reverses the defect of thymic development of SLP-76$^{−/−}$ or LAT$^{−/−}$ mice but does not reverse the defect of thymic development of RAG-2$^{−/−}$ SLP-76$^{−/−}$ mice. (a) CD4/CD8 profiles and thymic cellularity of WT, Cbl$^{−/−}$ LAT$^{−/−}$, SLP-76$^{−/−}$, SLP-76$^{−/−}$ Cbl$^{−/−}$, and LAT$^{−/−}$Cbl$^{−/−}$ mice at 6 wk of age. (b) CD4/CD8/NK1.1/Mac-1/Gr1/CD3/B220$^-$ thymocytes from the indicated mice were analyzed for surface expression of CD44 and CD25 by flow cytometry. (c) CD4 SP cells and CD4/CD8 DP cells from the indicated mouse thymi were analyzed for the expression of CD3 and TCR-β (H57). The results presented are representative of more than five experiments. (d) CD4/CD8 profiles in WT, RAG-2$^{−/−}$, and RAG-2$^{−/−}$ SLP-76$^{−/−}$ Cbl$^{−/−}$ mouse thymi and spleens. The results presented are representative of three experiments.

c-Cbl Inactivation Allows Development of DP and CD4 Single Positive (SP) Thymocytes in SLP-76$^{-/-}$ or LAT$^{-/-}$ Mice. As previously reported, thymic development in SLP-76$^{-/-}$ mice is arrested at the CD4$^-$ CD8$^-$ (DN) stage with total number of thymocytes severely reduced in comparison to WT mice (8, 9). In SLP-76$^{-/-}$ Cbl$^{-/-}$ double knockout mice, total cellularity of thymus was not different from that of SLP-76$^{-/-}$ mice. However, in contrast to SLP-76$^{-/-}$ mice, SLP-76$^{-/-}$ Cbl$^{-/-}$ mice generated significant proportions of CD4$^+$ CD8$^+$ (DP) and CD4$^+$ CD8$^-$ (SP) cells, although in numbers substantially lower than those observed in WT or Cbl$^{-/-}$ thymi (Fig. 1 a). The proportion of CD4$^+$ and DP cells varied in thymi of individual mice, with 6-wk-old SLP-76$^{-/-}$ Cbl$^{-/-}$ mice expressing an average of 10% DP cells and 5% CD4$^+$ cells compared to the absence of detectable DP and CD4 SP cells in SLP-76$^{-/-}$ single knockouts. The majority of DN thymocytes were CD25$^+$ CD44$^-$ (DN3) in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice, as in SLP-76$^{-/-}$ mice (Fig. 1 b). Cell surface expression of CD3 or TCR-β was significantly decreased on SLP-76$^{-/-}$ Cbl$^{-/-}$ thymic CD4 SP cells relative to expression on WT thymic CD4 SP cells and to the increased expression observed in c-Cbl$^{-/-}$ mice. In contrast, SLP-76$^{-/-}$ Cbl$^{-/-}$ DP cells expressed CD3 and TCR-β at markedly enhanced levels that were similar to those observed in c-Cbl$^{-/-}$ DP cells (Fig. 1 c). Expression of CD5 and CD69 was increased on both DP and CD4 SP

SLP-76$^{-/-}$ Cbl$^{-/-}$ thymocytes relative to WT or c-Cbl single knockout mice (unpublished data).

The ability of c-Cbl inactivation to partially rescue T cell development in SLP-76–deficient mice suggested the existence of a developmental pathway distinct from that involving the conical TCR→ZAP70→LAT→SLP-76 pathway. To further test this possibility, the effect of c-Cbl inactivation on T cell development was studied in LAT-deficient mice. T cell development in LAT$^{-/-}$ mice is also arrested at the DN stage (10) with absence of DP and SP thymocytes, similar to the SLP-76$^{-/-}$ phenotype. c-Cbl inactivation induced partial rescue of thymic development in LAT$^{-/-}$ Cbl$^{-/-}$ mice, similar to the effect observed in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. Fig. 1 a shows that ~24% DP and 7% CD4 SP cells were detected in LAT$^{-/-}$ Cbl$^{-/-}$ thymi but not in LAT$^{-/-}$ thymi. As seen in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice, c-Cbl inactivation only partially reversed the defects of thymic development in LAT$^{-/-}$ mice. The cellularity of LAT$^{-/-}$ Cbl$^{-/-}$ thymi was <1/20 that of WT mice, and T cell development of LAT$^{-/-}$ Cbl$^{-/-}$ mice showed a block at DN3 (Fig. 1 b), similar to that in LAT$^{-/-}$ or SLP-76$^{-/-}$ mice. As was seen in SLP-76/c-Cbl double deficient mice, decreased expression of CD3 and TCR-β was observed in LAT$^{-/-}$ Cbl$^{-/-}$ CD4 SP cells, whereas expression of CD3 and TCR-β was enhanced in LAT$^{-/-}$ Cbl$^{-/-}$ DP cells (unpublished data).
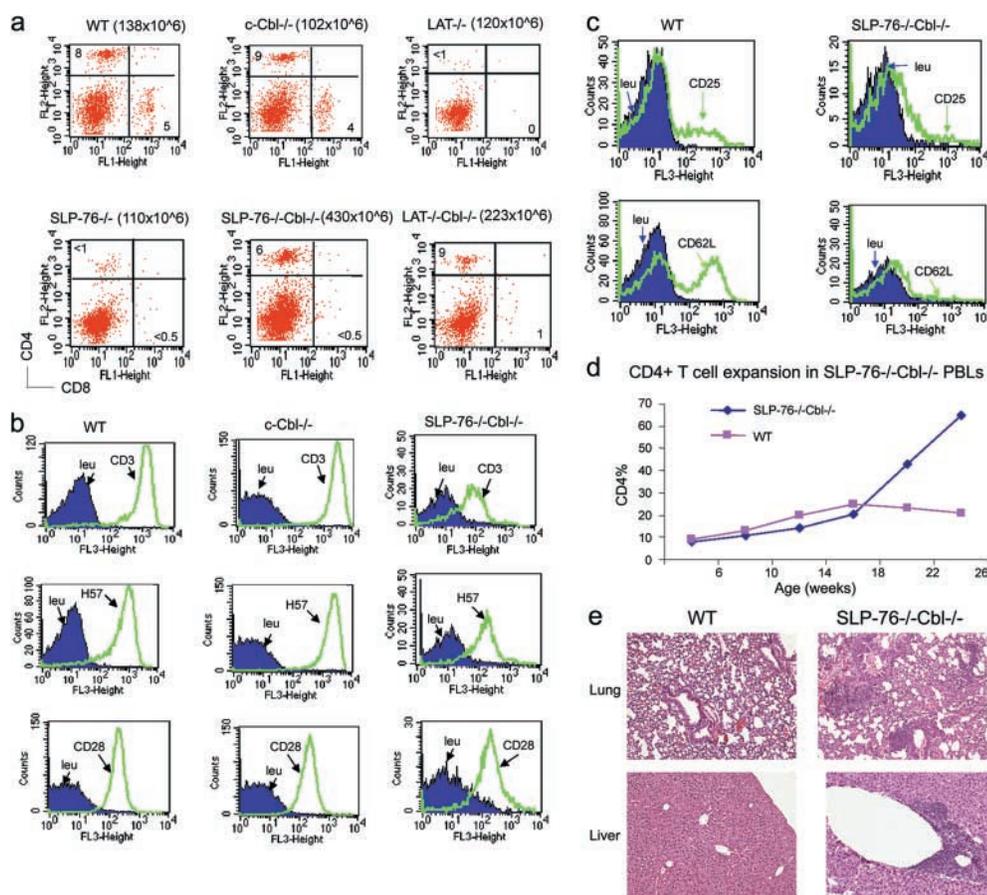

Figure 2. There is a marked expansion of peripheral CD4 T cells in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. (a) CD4/CD8 profiles of spleens of WT, Cbl$^{-/-}$, LAT$^{-/-}$, SLP-76$^{-/-}$, SLP-76$^{-/-}$ Cbl$^{-/-}$, and LAT$^{-/-}$ Cbl$^{-/-}$ mice at 6 wk of age. (b) Gated peripheral CD4$^+$ cells of WT, Cbl$^{-/-}$, and SLP-76$^{-/-}$ Cbl$^{-/-}$ mice were analyzed for the expression of CD3, TCR-β (H57), and CD28. (c) Gated peripheral CD4$^+$ cells of WT and SLP-76$^{-/-}$ Cbl$^{-/-}$ mice were analyzed for the expression of CD25 and CD62L. (d) Percentage of CD4$^+$ cells of WT and SLP-76$^{-/-}$ Cbl$^{-/-}$ PBLs was determined at the indicated ages. (e) Hematoxylin and eosin staining of sections from the lungs and livers of WT and SLP-76$^{-/-}$ Cbl$^{-/-}$ mice at ~24 wk of age. Magnification, 20. The flow cytometric results shown are representative of more than five experiments.

Inactivation of c-Cbl Does Not Rescue the T Cell Defects in RAG-2$^{-/-}$ SLP-76$^{-/-}$ Mice. c-Cbl inactivation rescued significant T cell development in the absence of SLP-76 or LAT, two scaffolding molecules that appear to be otherwise critical for TCR-mediated signal transduction. Therefore, it was of interest to determine whether T cell development in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice is in fact TCR independent. To test this possibility, RAG-2$^{-/-}$ SLP-76$^{-/-}$ Cbl$^{-/-}$ triple knockout mice were bred in which expression of rearranged TCR genes cannot occur. No DP or SP T cells were detected in RAG-2$^{-/-}$ mouse thymi or spleens, as expected. RAG-2$^{-/-}$ SLP-76$^{-/-}$ Cbl$^{-/-}$ triple knockout mice similarly had no DP or SP T cells in either the thymus or spleen (Fig. 1 d). This finding indicates that c-Cbl inactivation permits T cell development through an SLP-76– and LAT-independent, but nevertheless TCR-dependent, mechanism.

Massive Splenomegaly and Lymphadenopathy Develop in SLP-76$^{-/-}$ Cbl$^{-/-}$ and LAT$^{-/-}$ Cbl$^{-/-}$ Mice. In contrast to SLP-76$^{-/-}$ and LAT$^{-/-}$ mice that do not have peripheral T cells, SLP-76$^{-/-}$ Cbl$^{-/-}$ and LAT$^{-/-}$ Cbl$^{-/-}$ mice had substantial numbers of peripheral CD4$^+$ T cells (Fig. 2 a). Remarkably, the total number of CD4$^+$ T cells in the spleen and lymph nodes of 6-wk-old SLP-76$^{-/-}$ Cbl$^{-/-}$ or LAT$^{-/-}$ Cbl$^{-/-}$ mice was in fact markedly elevated above numbers in WT or c-Cbl$^{-/-}$ mice. In distinction to the observed increase in numbers of CD4$^+$ T cells, there were few if any peripheral CD8$^+$ T cells in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. SLP-76$^{-/-}$ Cbl$^{-/-}$ mice had ∼10 times the number of splenic and lymph node CD4$^+$ cells in comparison to either WT or c-Cbl$^{-/-}$ mice at the age of 24 wk.

Phenotypic analysis also demonstrated that the peripheral CD4$^+$ population in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice differs from that in WT controls or c-Cbl$^{-/-}$ mice. In comparison to WT or c-Cbl$^{-/-}$ mice, CD3 and TCR-β expression was markedly reduced and CD4 was modestly but consistently reduced in SLP-76$^{-/-}$ Cbl$^{-/-}$ peripheral CD4$^+$ cells (Fig. 2, a and b, and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20040262/DC1). CD28 expression on SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells was equivalent to that on WT cells (Fig. 2, a and b). The CD25$^+$ CD4$^+$ subpopulation of CD4$^+$ T cells that is observed in WT mice, and commonly associated with suppressor or regulatory function, is largely absent in SLP-76$^{-/-}$ Cbl$^{-/-}$ spleen and lymph nodes. SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells also have dramatically decreased expression of CD62L compared to WT CD4$^+$ cells (Fig. 2 c), suggesting that this is a population of previously activated cells. The expansion of SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells was polyclonal as analyzed by TCR Vβ usage (unpublished data). The proportion of Mac-1$^+$ cells in the SLP-76$^{-/-}$ Cbl$^{-/-}$ mice (∼20%) was substantially greater than that in controls (<5%), as were total numbers of Mac-1$^+$ cells (Table II). Total B cell numbers were also increased in the spleens and lymph nodes of SLP-76$^{-/-}$ Cbl$^{-/-}$ mice compared to control mice, although the proportion of B cells was not dramatically increased (Table II).

Table II. *The Distribution of Splenocytes Subpopulations*

	Cell number (×10⁶)				
	Total	CD4	CD8	B cell	Mac-1$^+$ cells
WT (6 wk)	138	11	6.9	11	5.8
WT (24 wk)	120	26.5	15.8	69	6.4
SLP-76$^{-/-}$ Cbl$^{-/-}$ (6 wk)	430	25.8	1	317	82
SLP-76$^{-/-}$ Cbl$^{-/-}$ (24 wk)	820	295	4.3	335	180

The results presented are for individual mice and are representative of three to five mice per group.

SLP-76$^{-/-}$ Cbl$^{-/-}$ mice developed progressive splenomegaly and lymphadenopathy with age and died at ∼6 mo. Older (4–6-mo-old) SLP-76$^{-/-}$ Cbl$^{-/-}$ mice had approximately 8–12 times the number of splenic CD4$^+$ cells observed in age-matched control WT or c-Cbl$^{-/-}$ mice and three to four times the number found in young SLP-76$^{-/-}$ Cbl$^{-/-}$ mice (Table II and Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20040262/DC1). Old SLP-76$^{-/-}$ Cbl$^{-/-}$ mice also had more B cells and macrophages in the spleen and lymph nodes than in control mice. The increase in peripheral CD4$^+$ T cells was also reflected in an increasing CD4$^+$ cell percentage in PBL with age of SLP-76$^{-/-}$ Cbl$^{-/-}$ mice (Fig. 2 d). To further analyze the state of other organ systems and potential contributors to the early death observed in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice, histological analysis was performed. Extensive lymphocyte infiltration was observed in the lungs and livers of SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. Lymphocytes were observed as clusters around pulmonary vessels, bronchioles, and hepatic parenchyma in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice but not in normal mice (Fig. 2 a). Occasionally, clusters of multinucleate giant cells were detected. Increased numbers of macrophages were also observed in the lungs of SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. Similar findings were observed in LAT$^{-/-}$ Cbl$^{-/-}$ mice (unpublished data).

SLP-76$^{-/-}$ Cbl$^{-/-}$ Peripheral CD4$^+$ Cells Are Polarized to Th1 and Th2 Cell Cytokine Secretion. In normal mice, the majority of peripheral T cells are naive CD62L^{hi} cells that have not encountered antigen and are not differentiated into specialized cells capable of secreting Th1 and Th2 cell cytokines. It was of interest to determine whether the peripheral CD4$^+$ T cells found in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice, the majority of which express a CD62L^{lo} memory cell phenotype, were functionally differentiated. More than 50% of SLP6$^{-/-}$ Cbl$^{-/-}$ CD4 cells were IL-4–producing cells when acutely stimulated with PMA and ionomycin, whereas IL-4–producing CD4$^+$ cells were essentially undetectable in WT or c-Cbl$^{-/-}$ mice (Fig. 3 a). The proportions of IL-2– and IFN-γ–producing cells were also increased in SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells. Multiparameter staining demonstrated that IL-4– and IFN-γ–producing cells represented mutually exclusive populations (Fig. 3 b).
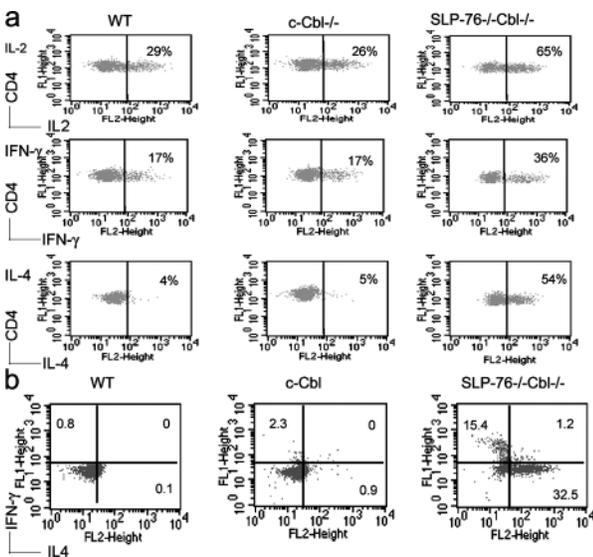
Figure 3. Peripheral CD4^{+} T cells in SLP-76^{−/−} Cbl^{−/−} mice are polarized to IL-4 or IFN-γ production. (a) IL-2, IL-4, and IFN-γ intracellular staining for PMA/ionomycin-stimulated CD4^{+} cells from indicated mice. (b) IFN-γ and IL-4 double intracellular staining for PMA/ionomycin-stimulated CD4^{+} cells from indicated mice. (c) Ig production in WT and SLP-76^{−/−} Cbl^{−/−} mice. The results are representative of three experiments.

The peripheral CD4^{+} T cells that are expanded in SLP-76^{−/−} Cbl^{−/−} mice thus appear to represent differentiated and polarized Th2 (IL-4) and Th1 (IFN-γ) cell populations. Consistent with the observed expansion of Th2 cytokine-secreting cells, a dramatic increase in Th2 cell–dependent IgE and IgG1 was detected in the serum of SLP-76^{−/−} Cbl^{−/−} mice with total IgE increased >10[5] times above levels in WT mice, and IgG1 10[2]–10[3] times higher than controls (Fig. 3 c). Despite these elevated levels of total Ig, immunization with TNP-KLH failed to induce antigen-specific Ig responses (unpublished data).

SLP-76^{−/−} Cbl^{−/−} CD4^{+} T Cells Are Unresponsive to TCR Stimulation. To test the function of SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells in vitro, enriched peripheral T cells were treated with titrated concentration of anti-CD3 Ab in the presence or absence of anti-CD28 Ab. Although WT CD4^{+} cells proliferated and produced IL-2 in response to TCR stimulation in vitro, SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells did not respond detectably to the same stimuli (Fig. 4). To measure early and proximal signaling events in response to

TCR stimulation, intracellular Ca^{2+} flux was analyzed. WT CD4^{+} cells generated Ca^{2+} responses to CD3/CD4 cross-linking, and c-Cbl^{−/−} CD4^{+} cells exhibited detectable but reduced Ca^{2+} responses, a finding not previously reported. In contrast, there was no detectable Ca^{2+} response in SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells (Fig. 5).

As noted above, SLP-76^{−/−} Cbl^{−/−} CD4^{+} T cells failed to respond to TCR stimulation in vitro as measured by proliferation, IL-2 production, or Ca^{2+} flux. Therefore, additional parameters were measured in further efforts to detect biochemical responses to TCR stimulation. Enriched CD4^{+} cells were stimulated with anti-CD3/CD4 Abs and cell lysates were analyzed for total tyrosine phosphorylation. Tyrosine phosphorylation of a 110-kD protein, probably c-Cbl, was observed in WT but not c-Cbl^{−/−} or SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells in response to TCR stimulation. Additional tyrosine phosphorylated proteins, including 37- (probably LAT) and 23-kD (probably TCR-ζ chain) species, were detected in WT and c-Cbl^{−/−} but not SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells. In fact, there was no detectable tyrosine phosphorylation induced in SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells in response to TCR stimulation (Fig. 6 a).

ERK Is Constitutively Phosphorylated in SLP-76^{−/−} Cbl^{−/−} CD4^{+} T Cells. Several bands representing proteins 65–80 kD were constitutively tyrosine phosphorylated in the absence of in vitro stimulation in WT and c-Cbl^{−/−} T cells, but not in SLP-76^{−/−} Cbl^{−/−} T cells. In contrast, one constitutively phosphorylated 45-kD protein showed the reciprocal pattern and was detected in SLP-76^{−/−} Cbl^{−/−} CD4^{+} cells but not in WT or c-Cbl^{−/−} CD4^{+} cells (Fig. 6 b).

To identify specific tyrosine phosphorylated proteins, ERK was first analyzed as a candidate for the 45-kD phosphoprotein detected constitutively in SLP-76^{−/−} Cbl^{−/−} T cells. Cell lysates were fractionated by gel electrophoresis and immunoblotted with Abs specific for total or phosphorylated protein. ERK was expressed at similar total protein levels in WT, c-Cbl^{−/−}, and SLP-76^{−/−} Cbl^{−/−} T cells. ERK phosphorylation was low to undetectable in un-
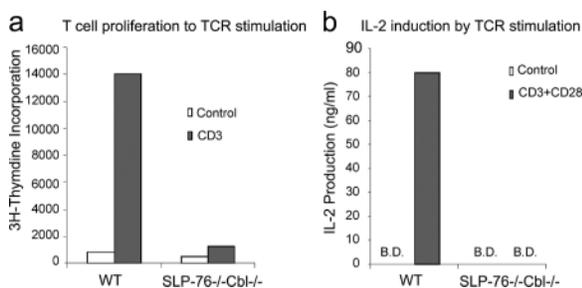

Figure 4. T cell proliferation and IL-2 production in response to TCR stimulation are deficient in peripheral CD4^{+} cells from SLP-76^{−/−} Cbl^{−/−} mice. (a) T cell proliferation assay: 10[5] cells were plated in 96-well plates precoated with anti-CD3 Abs. (b) IL-2 induction: 10[6] CD4 cells were plated in 24-well plates coated with 10 μg/ml anti-CD3 Ab in the presence of 1 μg/ml anti-CD28 Ab. B.D., below detection. The results are representative of three experiments.
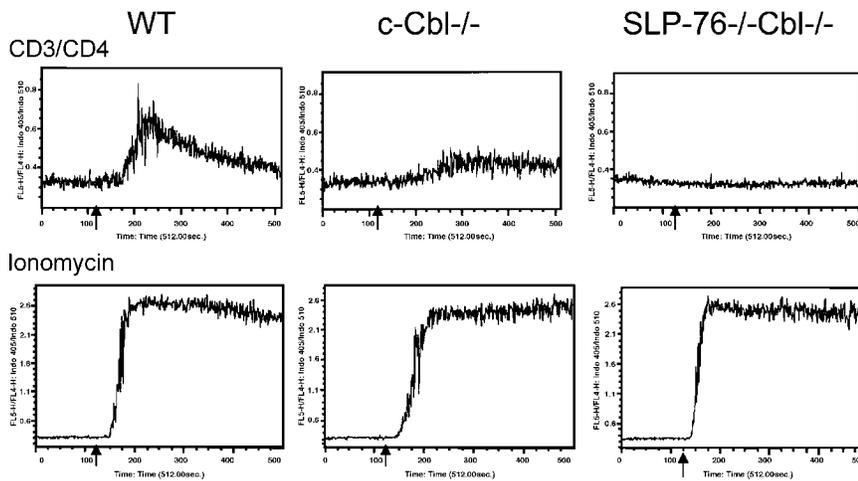
Figure 5. Ca²⁺ influx in response to anti–CD3/CD4 stimulation is deficient in peripheral CD4⁺ cells from SLP-76⁻/⁻ Cbl⁻/⁻ mice. CD4⁺ T cells were isolated and assayed for Ca²⁺ response as described in Materials and Methods. Arrows indicate the time at which streptavidin or ionomycin was added. The results presented are representative of three experiments.
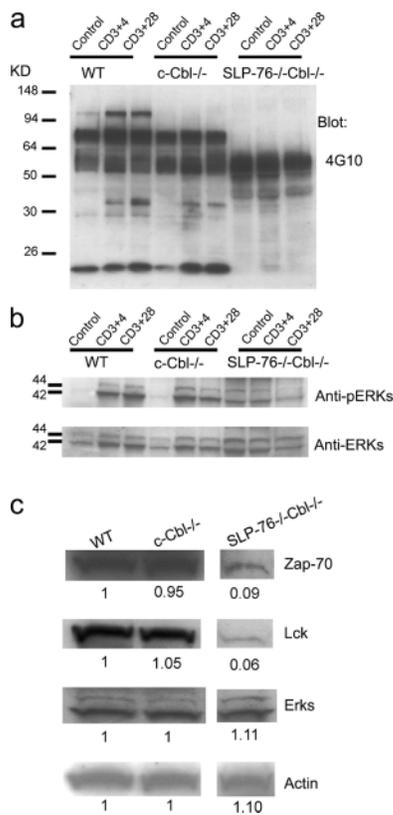
Figure 6. (a) SLP-76⁻/⁻ Cbl⁻/⁻ T cells fail to induce protein tyrosine phosphorylation in response to TCR engagement. Enriched T cells (75–85% T cells) from the indicated mouse lymph nodes and spleens were stimulated with anti-CD3/CD4 Abs or anti-CD3/CD28 Abs. The protein lysates were blotted with 4G10 anti-phosphotyrosine Ab. (b) ERKs of SLP-76⁻/⁻ Cbl⁻/⁻ T cells are constitutively phosphorylated. The blots from (a) were stripped and blotted with anti-ERK or anti-phospho-ERKs Abs. (c) The expression of ZAP-70 and Lck is reduced in SLP-76⁻/⁻ Cbl⁻/⁻ CD4⁺ cells. Purified CD4 cells from the indicated mouse lymph nodes and spleens were blotted with anti–ZAP-70, anti-Lck, anti-ERKs, and anti-actin Abs. The results presented are representative of three experiments.

stimulated WT or c-Cbl⁻/⁻ T cells, and increased in response to TCR stimulation. In contrast, both Erk1 and Erk2 were found to be constitutively phosphorylated in SLP-76⁻/⁻ Cbl⁻/⁻ CD4⁺ T cells, and ERK phosphorylation was not increased in response to TCR stimulation in these cells (Fig. 6 b).

ZAP-70 and Lck Are Expressed at Greatly Reduced Levels in SLP-76⁻/⁻ Cbl⁻/⁻ CD4⁺ Cells. Tyrosine phosphorylation of ZAP-70 is a critical event in normal T cell development and activation, and appears to be upstream of LAT and SLP-76 in TCR–initiated signal transduction pathways. Because no tyrosine phosphorylation of ZAP-70 was detected in SLP-76⁻/⁻ Cbl⁻/⁻ T cells, levels of total ZAP-70 expression were measured in SLP-76⁻/⁻ Cbl⁻/⁻ and control CD4⁺ cells by immunoblotting with two different anti–ZAP-70 Abs. Surprisingly, total ZAP-70 protein in SLP-76⁻/⁻ Cbl⁻/⁻ CD4⁺ cells was only one tenth the level detected in WT or c-Cbl⁻/⁻–deficient CD4⁺ T cells. Lck protein expression was also reduced >10-fold in SLP-76⁻/⁻ Cbl⁻/⁻ CD4⁺ cells (Fig. 6 c). In contrast, there was no difference in expression of ERK1 or ERK2 in the double knockout or control CD4⁺ cells (Fig. 6 c). RT-PCR analysis showed that equivalent steady state levels of both ZAP-70 and Lck mRNA were expressed in WT, c-Cbl⁻/⁻, and SLP-76⁻/⁻ Cbl⁻/⁻ CD4 T cells, indicating that the reduced expression of ZAP-70 and Lck protein in the double knockout CD4⁺ cells occurs at a translational or posttranslational level (unpublished data).

To determine whether expression of ZAP-70 protein was also altered in developing SLP-76⁻/⁻ Cbl⁻/⁻ thymocytes, lysates from predominantly DN SLP-76⁻/⁻ Cbl⁻/⁻ thymocytes were compared with purified WT DN thymocytes by immunoblotting. In contrast to the greatly reduced levels of ZAP-70 observed in peripheral CD4⁺ cells, ZAP-70 expression was not comparably decreased in SLP-76⁻/⁻ Cbl⁻/⁻ thymic lysates (Fig. S2). Therefore, reduction in ZAP-70 protein is most marked in peripheral CD4⁺ cells

and appears to reflect later events in development or peripheral differentiation.

Discussion

In this study, we have analyzed the effect of c-Cbl inactivation on the phenotype observed in SLP-76 knockout and LAT knockout mice. The inactivation of c-Cbl rescued completely the perinatal survival defect in SLP-76$^{-/-}$ mice and partially overcame the deficiency of T cell development in both SLP-76$^{-/-}$ and LAT$^{-/-}$ mice. SLP-76$^{-/-}$ Cbl$^{-/-}$ double knockout or LAT$^{-/-}$ Cbl$^{-/-}$ double knockout mice developed progressive splenomegaly and lymphadenopathy, and died by 6 mo of age with lymphoid infiltration of parenchymal organs. Hyperplasia was dominated by peripheral CD4$^+$ T cells that were polarized to differentiated Th1 or Th2 cytokine-producing cells. In contrast, RAG-2$^{-/-}$ SLP-76$^{-/-}$ Cbl$^{-/-}$ triple knockout mice did not generate DP or SP T cells. In vitro TCR stimulation of SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells failed to induce T cell proliferation, IL-2 production, Ca^{2+} influx, or protein tyrosine phosphorylation.

The majority of SLP-76 knockout mice die in the first week after birth (6, 9). This early lethality is completely rescued by c-Cbl inactivation with SLP-76$^{-/-}$ Cbl$^{-/-}$ double knockout mice surviving through early adulthood, but subsequently dying by ∼6 mo of age. The precise mechanism of the early mortality of SLP-76$^{-/-}$ mice is not yet completely understood, however, it is likely a consequence of a vascular development defect and platelet signaling abnormalities. The mechanism by which c-Cbl inactivation rescues early lethality has not yet been established, but preliminary results suggest that partial reversal of both platelet and vascular abnormalities occurs in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice (unpublished data).

The finding of splenomegaly and lymphadenopathy with massive expansion of CD4$^+$ cells was unexpected. It was notable that these mice are deficient in the CD4$^+$ CD25$^+$ subpopulation that in many settings functions as a regulatory population capable of suppressing T cell proliferation and IL-2 production (23, 24). The loss of T cell suppressor function could contribute to dysregulated proliferation in SLP-76$^{-/-}$ Cbl$^{-/-}$ or LAT$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells, a possibility that is currently being investigated. The observation that most SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells are differentiated into Th1 and Th2 cells suggests that these T cells have responded in vivo to unknown stimuli, despite their in vitro unresponsiveness to TCR stimulation. The decreased TCR density observed on the surface of SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells is in fact consistent with their in vivo activation, and the failure to rescue T cell development in RAG-2$^{-/-}$ SLP-76$^{-/-}$ Cbl$^{-/-}$ mice further reinforces the possibility that the phenotype of peripheral T cell expansion reflects chronic TCR-dependent stimulation. The relationship between peripheral CD4$^+$ T cells and the small number of thymocytes observed in SLP-76$^{-/-}$ Cbl$^{-/-}$ thymi is unclear, and it is possible that the thymic CD4 SP cells are at least in part derived from recirculating peripheral CD4$^+$ T cells.

SLP-76 is a central adaptor protein for T cell development. T cell development of SLP-76$^{-/-}$ mice is arrested at the DN3 stage (8, 9) presumably because in the absence of SLP-76, pre-TCR signals cannot be transmitted to downstream signaling molecules such as Vav, Grb2, Gads, and PLC-γ1, all of which interact with SLP-76 (2–5, 25). c-Cbl mutation partially overcame the defects in T cell development of SLP-76$^{-/-}$ mice. In studies of the TCR signaling capacity of SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ T cells, these cells failed to produce detectable tyrosine phosphorylation in response to in vitro TCR stimulation. However, constitutive tyrosine phosphorylation of ERKs was observed in SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4$^+$ cells, which was not seen in either WT or c-Cbl$^{-/-}$ T cells. c-Cbl binds to multiple molecules involved in signal transduction, and could in principle negatively regulate the function of these molecules by competitive binding or by inducing ubiquitin–dependent degradation through its E3 ligase activity. Based on the findings reported here, we hypothesize that SLP-76$^{-/-}$ Cbl$^{-/-}$ T cells use an alternative ERK signaling pathway to partially reverse the defects of T cell development in SLP-76$^{-/-}$ mice. Ca^{2+} signaling as well as ERK signaling are essential for normal T cell development. In WT T cells, c-Cbl constitutively binds to critical signal molecules such as Grb2 (26, 27), serving to maintain T cells in an inactivated state in the absence of TCR stimulation. SLP-76$^{-/-}$ mice cannot transmit either Ca^{2+} or ERK signals from the TCR or pre-TCR, resulting in an essentially complete block of T cell development at the DN3 stage. In contrast, SLP-76$^{-/-}$ Cbl$^{-/-}$ cells constitutively activate ERK, possibly through a Grb2–SOS pathway resulting from the release of Grb2 molecules in the absence of c-Cbl. The events that might contribute to this ERK activation are not fully defined, but appear to require TCR expression, despite the fact that SLP-76$^{-/-}$ Cbl$^{-/-}$ T cells did not produce detectable Ca^{2+} or ERK signals in response to in vitro TCR stimulation. LAT$^{-/-}$ mice exhibit a profound defect in T cell development that is similar to that observed in SLP-76$^{-/-}$ mice, and LAT$^{-/-}$ Cbl$^{-/-}$ mice show rescued development and an overall phenotype similar to that seen in SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. Together, these results suggest that c-Cbl may normally inhibit a LAT–SLP-76–independent pathway that is capable of mediating differentiation of a CD4$^+$ population that can undergo extensive peripheral expansion and functional polarization. It is notable that this pathway might be incapable of differentiating a population of CD25$^+$ CD4$^+$ regulatory cells, the absence of which could contribute to extensive CD4$^+$ T cell expansion in vivo. The dramatically decreased expression of both Lck and ZAP-70 protein in SLP-76$^{-/-}$ Cbl$^{-/-}$ CD4 T cells further suggests that inactivation of c-Cbl reverses the T cell defects of SLP-76$^{-/-}$ mice through a pathway that is independent of Lck and ZAP-70, as well as SLP-76 and LAT.

The phenotype of peripheral CD4$^+$ T cell expansion, as well as increased constitutive ERK phosphorylation, was found in LAT$^{-/-}$ Cbl$^{-/-}$ and SLP-76$^{-/-}$ Cbl$^{-/-}$ mice, but not in c-Cbl$^{-/-}$ single knockout mice. A number of mech-

anisms may underlie this observation. First, a molecule responsible for the unique phenotype of LAT$^{-/-}$ Cbl$^{-/-}$ and SLP-76$^{-/-}$ Cbl$^{-/-}$ mice may normally be ubiquinated and degraded via c-Cbl, and might be more stable in the absence of c-Cbl. The consequences of this stabilization might be regulated in the presence of a functional TCR pathway, but not if SLP-76 or LAT is missing. Alternatively, the presence of a normal SLP-76 and LAT-dependent T cell developmental pathway may competitively suppress the abnormal developmental pathway seen in SLP-76– or LAT-deficient mice. It is also possible that regulatory cells such as the CD25$^+$ CD4$^+$ T regulatory cell population are dependent upon an intact TCR–SLP-76–LAT pathway, and function to prevent the abnormal peripheral expansion of CD4$^+$ cells in c-Cbl$^{-/-}$ mice.

It is of interest that LAT knock-in mice, expressing a mutant LAT that has lost the ability to bind PLC-γ1, were recently reported to exhibit defects in T cell development and to develop splenomegaly (28, 29). The TCR-induced activation of PLC-γ1 and calcium influx is dramatically reduced in LAT knock-in CD4$^+$ cells, but ERK activation remains intact. The T cell phenotype of SLP-76$^{-/-}$ Cbl$^{-/-}$ mice is thus similar to that described for mice expressing a mutated form of LAT. In another LAT knock-in mouse model, expression of a different mutation in intracellular tyrosines was recently reported to block αβ T cell development and to partially impair γδ T cell development (30). These mice also accumulated large numbers of peripheral T cells, in this case γδ T cells, with a Th2 cell phenotype. It will be of interest to further compare the functional status of LAT knock-in with LAT$^{-/-}$ Cbl$^{-/-}$ or SLP-76$^{-/-}$ Cbl$^{-/-}$ mice. Collectively, the patterns of T cell development and peripheral dysregulation observed in these genetically altered mice indicate the presence of previously unrecognized alternative pathways for critical TCR-dependent signaling in vivo.

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