Differential Regulation of the B Cell Receptor-mediated Signaling by the E3 Ubiquitin Ligase Cbl*

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The E3 ubiquitin ligase Cbl has been implicated in intracellular signaling pathways induced by the engagement of the B cell antigen receptor (BCR) as a negative regulator. Here we showed that Cbl deficiency results in a reduction of B cell proliferation. Cbl−/− B cells show impaired tyrosine phosphorylation, reduced Erk activation, and attenuated calcium mobilization in response to BCR engagement. The phosphorylation of Syk and Btk is also down-modulated. Interestingly, Cbl−/− B cells display enhanced BCR-induced phosphorylation of CD19 and its association with phosphatidylinositol 3-kinase. Importantly, Lyn kinase activity is up-regulated in Cbl−/− B cells, which correlates inversely with the Cbl-mediated ubiquitination of Lyn. Because Lyn has both negative and positive roles in B cells, our results suggested that Cbl differentially modulates the BCR-mediated signaling pathways by targeting Lyn ubiquitination, which affects B cell development and activation.

B lymphocyte development, selection, and activation are critically dependent on the surface expression of the B cell antigen receptor (BCR) and the BCR-mediated signal transduction (1). The BCR is composed of membrane-bound immunoglobulin and an immunoreceptor tyrosine-based activation motif-containing αβ heterodimer (2). Upon the engagement of the BCR, tyrosine residues within immunoreceptor tyrosine-based activation motifs are phosphorylated by Src family protein-tyrosine kinases, Lyn, Fyn, or Btk followed by the recruitment and activation of various protein-tyrosine kinases such as Syk or Btk. These tyrosine kinases activate additional signaling molecules including phospholipase C (PLC)-γ2 and Vav and ultimately lead to the activation of transcription factors such as NFAT, AP-1, and NFκB, which can achieve a multitude of cellular responses, such as proliferation, activation, differentiation, or cell death (3). Adapter proteins also play an essential role in regulating the interactions and recruitments of effector enzymes with the BCR and protein-tyrosine kinases (4). Among this group of adapter proteins, there are increasing evidences for the role of Cbl and another member of its family, Cbl-b, in the BCR signaling pathway (5, 6).

The Cbl family proteins are composed of an N-terminal tyrosine kinase binding domain, followed by a RING finger domain, and C-terminal multiple proline-rich regions (7). Cbl and Cbl-b function as adaptor proteins by interacting with protein-tyrosine kinases and other crucial signaling molecules and function as E3 ubiquitin (Ub) ligases, in which the RING finger recruits Ub-bound E2 enabling the transfer of Ub to the substrate, resulting in its ubiquitination and subsequent down-modulation (7). Earlier studies identified Cbl as one of the rapidly tyrosine phosphorylated substrates upon the BCR engagement (5). Overexpression of Cbl leads to an inhibition of Syk kinase activity (8) but has no effect on the Lyn function (9). A later study showed that Cbl acts as an E3 Ub ligase to promote Syk ubiquitination (10). Experiments with Cbl-deficient chicken DT40 cells demonstrated that Cbl inhibits the BCR-mediated PLC-γ2 activation by regulating PLC-γ2 recruitment to B cell linker protein (BLNK) and Vav (11). However, a physiological function of Cbl in primary murine B cells remains unclear.

To understand the role of Cbl in B cells, we examined B cells from Cbl-deficient mice and investigated the mechanism by which Cbl regulates BCR signaling. It was found that a loss of Cbl resulted in reduced B cell proliferation and diminished phosphorylation of BCR proximal signaling molecules. However, the tyrosine phosphorylation of CD19 and CD19-mediated downstream signaling were increased. We provided evidence that Cbl differentially regulates the BCR signaling via targeting the ubiquitination of Lyn.

MATERIALS AND METHODS

Mice—Cbl−/− mice (12) were provided by M. Naramura and H. Gu. Cbl−/− mice on a mixed 129 and C57BL/6 background were further back-crossed with C57BL/6 mice for six more generations to obtain Cbl−/−, Cbl+/−, and Cbl−/− B6 mice. The genotyping of the mutant mice was done by PCR using tail DNA. Mice between 6 and 8 weeks after birth were mostly used for the experiments. All the animal experiments were performed according to the institutional guidelines.

B Cell Proliferation—B cells from splenocytes were negatively selected with a B cells isolation kit following the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). Purified B cells (5 × 10⁶ cells/ml) were stimulated with increasing amount of LPS or anti-IgM F(ab)₂ (Jackson Immunoresearch Laboratories, West Grove, PA) or in the presence of recombinant mouse IL-4 (100 units/ml; Calbiochem, San Diego, CA) either alone or with anti-CD40 monoclonal antibody (BD Biosciences). Proliferation was measured by pulsing the cells with 1 μCi of [3H]thymidine for the final 16 h of the 2-day culture and then by counting the radioactivity uptake.

Calcium Influx Measurement—Spleenic B cells were resuspended at 1 × 10⁶/ml in Hanks’ buffer (Invitrogen) containing 1% (w/v) bovine serum album and loaded with 1 μM Indo-1-AM (Sigma-Aldrich) at 37 °C for 45 min. The cells were washed twice at room temperature and resuspended at 2 × 10⁶/ml. Continuous monitoring of the fluorescence ratio (525/405 nm) was performed using BD-LSR (BD Biosciences). Base-line fluorescence ratios were collected for 1 min before anti-IgM F(ab)₂ was added. The fluorescence ratios were collected at real time for 5 min following addition of antibodies.

Immunoprecipitation and Immunoblotting—Spleen B cells (1 × 10⁷)

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were stimulated with 10 μg/ml goat anti-mouse IgM F(ab)2 fragment for the indicated time intervals. Lysates were prepared in 1% Nonidet P-40 lysis buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, and 10 μg/ml of both aprotinin and leupeptin at 4 °C for 30 min. Lysates were cleared by centrifugation at 15,000 × g at 4 °C for 15 min. For immunoprecipitation, cleared lysates were incubated with indicated antibodies and protein G-Sepharose at 4 °C, washed three times in lysis buffer, and boiled with Laemmli buffer. Samples were analyzed by SDS-PAGE using 10% gels followed by transfer to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were probed with antibodies and developed using the ECL detection system (Amersham Biosciences). The following antibodies used for immunoprecipitation or immunoblotting: polyclonal antibodies specific to Lyn (44), Syk (N-19), Btk (M138), BLNK (H80), p85, ERK1/2, and PLC-γ2 (Q-20) (Santa Cruz Biotechnology, Santa Cruz, CA); CD19, Akt, Bcl-xL, and phospho-Akt, JNK, phospho-ERK, and phospho-JNK from Cell Signaling Technology (Beverly, MA); and anti-Bcl-2 and monoclonal anti-Lyn antibody from BD Transduction Laboratory (Lexington, KY).

In Vitro Kinase Assay—Lysates were prepared from splenic B cells in radioimmuno precipitation assay buffer. The anti-Lyn precipitates were washed four times with the radioimmune precipitation assay buffer followed by washing two times with 20 mM HEPES, pH 8.0, containing 150 mM NaCl, 10 mM magnesium acetate, and 20 mM MnCl2 (kinase assay buffer). The Lyn kinase activity was measured by the in vitro kinase assay system according to the manufacturer’s instructions (Chemicon, Temecula, CA). Briefly, the immunoprecipitates were incubated in kinase assay buffer including biotin-conjugated 5′rC kinase-specific peptide substrates for 30 min at 30 °C and then subjected to 96-well enzyme-linked immunosorbent assay-based colorimetric assay. All in vitro kinase activity experiments were repeated at least three times, and representative results are shown.

Plasmids and Cell Transfection—Plasmids containing wild-type Cbl or Cbl RING finger Cys to Ala mutant (Cbl-CA); Myc-Ub have been described previously (13). The pME plasmids containing Lyn or Lyn kinase-dead mutant (Lyn KR) cDNA were kindly provided by Dr. T. Kawakami and the cDNAs were subcloned into pCMV-FLAG mammalian expression vector (Sigma). Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Irvine Scientific, Santa Ana, CA) containing 10% fetal calf serum, 100 units/ml of penicillin, and 100 units/ml of streptomycin. Cells were transfected with appropriate plasmids (usually 5–10 μg in total) using FuGENE 6 transfection reagent (Roche Applied Science). After 48 h, cells were collected and resuspended in 0.5 ml of Dulbecco’s modified Eagle’s medium. Cells were either untreated or treated with pervanadate for 30 min at 37 °C.

Cells were then pelleted and resuspended in 1% Nonidet P-40 lysis buffer. For the detection of ubiquitinated proteins, 0.1% SDS and 5 mM N-ethylmaleimide (Sigma-Aldrich) were added to the lysis buffer to disrupt nonspecific protein interactions (14). For the transfection of DT40 cells (ATCC number CRL-2111), cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 100 units/ml of penicillin, and 100 units/ml of streptomycin. Cells were transfected with plasmids (usually 15–20 μg each, and 45–50 μg in total) by electroporation (260 V, 975 microfarad, Bio-Rad).

Flow Cytometry—Single-cell suspension from various tissues was stained with conjugated monoclonal antibodies for 45 min at 4 °C. Cell-associated fluorescence was analyzed using a FACSCalibur instrument (BD Biosciences) and the equipped FlowJo software. Analysis of surface marker expression on lymphocytes was done using monoclonal antibodies against CD3ε (2C11), B220 (RA3-2B2), IgD (1.3-5), IgM (R3-24.12), CD5 (53-7.3), CD19 (1D9), CD21 (B-Ly4), CD23 (B2B4), or CD43 (24.12) purchased from BD Biosciences.

Pulse-chase Experiment—Purified B cells (1 × 10⁷) from Cbl+/+ or Cbl−/− mice were cultured in methionine-free Dulbecco’s modified Eagle’s medium containing 5% dialyzed fetal calf serum for 1 h at 37 °C. The cells were stimulated with 10 μg/ml anti-IgM F(ab)2 and then labeled for 1 h with 100 μCi/ml Trans[35S] (ICN Biomedicals, Costa Mesa, CA). Cells were then washed three times with cold phosphate-buffered saline and cultured for different time intervals in complete Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. At each time point, the cells were collected and lysed by Nonidet P-40 lysis buffer. The cell lysates were preclarified with protein G-Sepharose for 30 min and then immunoprecipitated with anti-Lyn. The immune complexes were resolved by 12% SDS-PAGE and were subjected to autoradiography. The radiolabeled protein bands were quantified by using NIH Image 1.61 software.

RESULTS

Reduced Proliferation of Cbl−/− B Cells—To examine the effects of the Cbl deficiency on the biological responses of B cells, we assessed the proliferation of purified splenic B cells following anti-IgM, anti-CD40, anti-CD40 plus IL-4, or LPS stimulation. As shown in Fig. 1A, the proliferative response of Cbl−/− B cells to anti-IgM stimulation was severely impaired. However, no defect in proliferation was observed after stimulation with LPS in Cbl−/− B cells compared with wild-type B cells (Fig. 1B). The B cell proliferation was reduced after stimulation with anti-CD40 or anti-CD40 plus IL-4 (Fig. 1, C and
D). The data suggested that the proliferative responses induced by the engagement of the BCR, CD40, and IL-4, but not LPS, are affected by Cbl deficiency.

**Impaired BCR Signaling in Cbl−/− B Cells**—Although several biochemical studies have previously provided evidence about Cbl in BCR signaling (5), it is still unclear whether Cbl regulates BCR signaling in murine primary B cells in a similar manner as in cultured cell lines. To study Cbl-mediated downstream signaling events following the BCR engagement, we assessed the BCR-induced tyrosine phosphorylation patterns of cellular proteins in purified wild-type and Cbl−/− splenic B cells. In the absence of Cbl, the overall tyrosine phosphorylation in the whole cell lysates was reduced as compared with wild-type B cells during a period of 60-min stimulation (Fig. 2A). We then examined the activation of downstream mitogen-activated protein kinases, Erk and JNK in those cells by using phospho-specific antibodies. The Erk phosphorylation in Cbl−/− B cells was obviously reduced after a 5-min stimulation and remained lower than in wild-type B cells even after a 60-min stimulation (Fig. 2B). However, the amount of phosphorylated JNK in Cbl−/− B cells was comparable with wild-type B cells under the same stimulation conditions (Fig. 2B). We next examined the BCR-triggered Ca2+ influx and found that Cbl−/− B cells showed slightly reduced Ca2+ mobilization in comparison with wild-type B cells (Fig. 2C).

**Reduced Phosphorylation of Proximal Signaling Molecules in Cbl−/− B Cells**—To further understand the molecular mechanisms that account for the BCR signaling defects in Cbl−/− B cells, we compared the BCR-mediated tyrosine-phosphorylation status of Syk, Btk, and PLC−γ2, the critical proximal signaling molecules for the initial of BCR signaling. It was observed that the phosphorylation of Syk and Btk was decreased in Cbl−/− B cells (Fig. 3, A and B). It has been shown previously that a Cbl deficiency in the DT40 cell line can affect the association of PLC−γ2 with BLNK and also PLC−γ2 phosphorylation (11). Surprisingly, there seemed to be no significant difference in the tyrosine phosphorylation of PLC−γ2 and the association of PLC−γ2 with BLNK between wild-type and Cbl−/− B cells (Fig. 3C). Thus our data from Cbl−/− mice suggested that Cbl may not directly play a negative role in the BCR-mediated phosphorylation of Syk or PLC−γ2 as previously proposed (8, 11).

**Up-regulation of CD19 Signaling Pathway**—We next examined the potential contribution of Cbl in BCR-induced CD19 signaling pathway. Surprisingly, we found that CD19 phosphorylation was enhanced in Cbl−/− B cells upon BCR engagement (Fig. 4A). Because it has been reported that engagement of CD19 leads to the activation of Akt (15), we next examined the Akt phosphorylation by blotting the cell lysates with phospho-specific anti-Akt antibody. It was found that Akt phosphorylation was augmented in Cbl−/− B cells compared with wild-type B cells upon BCR stimulation (Fig. 4A). The increased CD19 phosphorylation was not because of the change of cell surface expression of CD19, which was equivalent between wild-type and Cbl−/− B cells (Fig. 4B). BCR stimulation did not further alter the expression levels of CD19 in those cells (data not shown). We also examined other CD19 downstream effector molecules such as the Bcl family proteins (16). We detected increased levels of Bcl-XL and, to a lesser extent, Bcl-2 in Cbl−/− B cells after anti-IgM stimulation for 6–48 h (Fig. 4C).

It was shown previously that engagement of BCR, but not CD19 itself, induces an association between CD19 and the p85 subunit of PI3-kinase (17). The increased Akt phosphorylation in Cbl−/− B cells, a downstream target of PI3-kinase, could result from an altered association between CD19 and the p85. To test this hypothesis, we immunoprecipitated CD19 and examined the associated p85 in anti-IgM stimulated wild-type and Cbl−/− B cells. Consistent with a previous observation (17), anti-IgM stimulation caused a rapid association between CD19 and p85 and gradual dissociation 5 min after stimulation (Fig. 4D). The association of CD19 with p85 was markedly increased in Cbl−/− B cells under the same stimulation conditions. Because Lyn also forms a complex with CD19 (18), we blotted the same membrane with anti-Lyn antibody and found that more Lyn protein was coimmunoprecipitated with anti-CD19 antibody.
in Cbl−/− B cells (Fig. 4D). Thus, in contrast to the positive role of Cbl in BCR-induced phosphorylation of Syk or Btk as described earlier, the results described here suggest that Cbl is a negative regulator of BCR-mediated CD19 signaling pathways.

**Cbl Regulates Lyn Activation through Ubiquitination**—Genetic and biochemical studies have shown that the tyrosine kinase Lyn plays both a negative and a positive role in BCR signaling (19). The reduced proximal BCR signaling, the up-regulation of CD19 phosphorylation, and its association with Lyn in Cbl−/− B cells led us to speculate that Cbl may negatively regulate Lyn, which differentially affects the downstream signaling. To test such model, we examined the in vitro kinase activity of immunoprecipitated Lyn from wild-type and Cbl−/− B cells. Although the kinase activity was quite similar between wild-type and Cbl−/− B cells 2 min after BCR stimulation, higher kinase activity was observed after a 5-min stimulation (Fig. 5A). Cbl−/− B cells still showed higher kinase activity after a 15-min stimulation, whereas at the same time point the kinase activity in wild-type B cells decreased dramatically. Thus, the loss of Cbl caused a slower but stronger Lyn activation upon BCR engagement.

We next investigated whether Lyn is a target for Cbl E3 ligase activity and performed an in vitro ubiquitination assay in transiently transfected human embryonic kidney 293 cells by overexpressing wild-type Lyn, or kinase-dead Lyn, together with Myc-tagged Ub and Cbl. The overexpression of Cbl promoted Ub conjugation to wild-type Lyn (Fig. 5B). However, the kinase-dead Lyn KR mutant was not ubiquitinated by Cbl. Stimulation of the transfected cells with pervanadate, a phosphatase inhibitor, induced a stronger ubiquitination of wild-type Lyn. In addition, the ubiquitination of Lyn by Cbl requires an intact RING finger, because a mutation at a critical cysteine residue in the RING finger (Cbl CA) disrupted the Ub conjugation to Lyn (Fig. 5C). Interestingly, we observed that the immunoprecipitated Lyn from samples transfected with the Cbl CA mutant showed higher levels of tyrosine phosphorylation than from cells transfected with wild-type Cbl (Fig. 5C, bottom panel), suggesting that Lyn ubiquitination is inversely related to its phosphorylation. We further examined Cbl-promoted Lyn ubiquitination in a chicken DT40 B cell line and found that only wild-type Lyn, but not the Lyn KR mutant, was ubiquitinated by the expression of Cbl (Fig. 5D).

To further confirm the physiological relevance of Cbl-mediated Lyn ubiquitination, we examined the protein levels of Lyn in wild-type and Cbl−/− B cells following anti-IgM stimulation for different time periods. It was found that the protein amounts of Lyn in wild-type B cells decreased rapidly after BCR stimulation, becoming obvious 10 min after stimulation (Fig. 5E). However, the rate of reduction in Cbl−/− B cells was attenuated, and the Lyn protein was still detectable 3 h after BCR stimulation. We further performed a pulse-chase experiment to examine the protein stability of Lyn in wild-type and Cbl−/− B cells. Lyn in Cbl−/− B cells was more stable than in wild-type B cells (Fig. 5F). Thus, Lyn is a physiological target for Cbl-induced ubiquitination and subsequent degradation.

**Altered B Cell Development in Cbl−/− Mice**—To understand a functional implication of Cbl in the differential regulation of BCR signaling, we investigated the B cell development in wild-type and Cbl−/− mice, the B cell subpopulations in the spleen and peritoneal cavity were analyzed in wild-type and Cbl−/− littermates by flow cytometry. The analysis of splenocytes showed a normal ratio of B220− B cells versus CD3ε+ T cells in Cbl−/− mice, and the total cell numbers were comparable between wild-type and Cbl−/− mice (data not shown). As shown in Fig. 6A, double staining with anti-IgM and IgD of splenocytes showed an apparent increase in the population of IgM+/IgDlow immature B cells, but a substantial decrease in the mature IgMhighIgDhi B cells. We further examined the cell surface expression of CD21 and CD23 and found a marked reduction of CD21highCD23low (mostly follicular B cells) in Cbl−/− mice (Fig. 6B). But the CD21hiCD23m (enriched in the marginal zone) and CD21mCD23hi (mostly newly formed B cells) populations were increased in Cbl−/− mice compared with wild-type mice.

We next examined the population of B1 B cells in the peritoneal cavities. B1 B cells share many phenotypic and functional properties with marginal zone B cells and are characterized by the cell surface expression of CD5 and IgM (20). We found that the population of IgM+CD5+ B1 B cells was markedly increased in Cbl−/− mice (Fig. 6C). These data collectively
suggest that a Cbl deficiency blocks the development of follicular B cells but promotes the development of the marginal zone and B1 B cells.

**DISCUSSION**

Although the Cbl<sup>−/−</sup> mice have been available for awhile, a vigorous investigation on Cbl in B cell development and activation has been lacking. Based on previous studies, it could have been predicated that a Cbl deficiency in B cells results in an up-regulation of BCR signaling. However, our data suggested that Cbl plays differential roles in the signal transduction pathways initiated by the engagement of the BCR. The reduced proximal BCR signaling and increased CD19 signaling in Cbl<sup>−/−</sup> B cells prompted us to speculate that Cbl regulates a further upstream molecule or molecules that act as switches between CD19 and BCR signaling pathways. The present study leads us to conclude that Lyn is such a candidate as the physiological target for Cbl in B cells, which is supported by the increased Lyn kinase activity in Cbl<sup>−/−</sup> B cells, Cbl-mediated ubiquitination of Lyn in transfected cells, and augmented Lyn stability upon BCR stimulation. An early study showed that Lyn associates with Cbl and induces its phosphorylation in B cells (9). Another recent study showed that Lyn is ubiquitinated by Cbl and/or Cbl-b in mast cell studies upon stimulation of the high affinity IgE receptor (21). It was previously reported that Lyn<sup>−/−</sup> B cells are hyperproliferative and show enhanced Erk activation (22, 23). In the present study, we observed that Cbl deficiency results in increased Lyn kinase activation and reduced Erk activation, which is the opposite of Lyn<sup>−/−</sup> B cells. Thus, our data showing reduced proliferation in Cbl<sup>−/−</sup> B cells are consistent with the hypothesis that Cbl regulates B cell proliferation via modulating Lyn activity.

The finding that Lyn is a target of Cbl in B cells is further supported by another interesting observation of the increased CD19 phosphorylation in Cbl<sup>−/−</sup> B cells upon BCR stimulation. It was previously shown that Lyn plays a positive role in phosphorylating CD19 tyrosine residues and the phosphorylated CD19 in turn positively regulates Lyn activation (18). To be consistent with this observation, we indeed observed an increased complex formation between CD19 and Lyn in anti-IgM-stimulated B cells from Cbl<sup>−/−</sup> mice. However, opposite observations have also been reported that Lyn and CD19 independently transduce downstream signaling (24). It is possible that in addition to Lyn, other Src kinases may compensate for the function of Lyn for the CD19 phosphorylation. The observation of increased CD19 signaling in Cbl<sup>−/−</sup> B cells is further supported by the augmented association of CD19 with p85 of PI3-kinase, which then induces the Akt phosphorylation and probably the expression of Bcl proteins.

Although previous biochemical studies have clearly established a negative role of Cbl in regulating Syk either through inhibiting its kinase activity (8) or inducing Syk ubiquitination (10), it is surprising to find that Syk activation is not up-regulated, but rather down-modulated in Cbl<sup>−/−</sup> B cells. The results shown here may imply that Syk is not a primary target for Cbl under physiological conditions. The reduced Syk activation in Cbl<sup>−/−</sup> B cells correlated with the attenuated B cell proliferation and decreased Erk phosphorylation. A possible explanation for the reduced Syk activation is that Cbl functions as an adaptor to facilitate the phosphorylation of Syk by Src family kinases, which becomes defective in the absence of Cbl. This explanation is consistent with previous biochemical studies that Syk interacts with Cbl via the N-terminal tyrosine kinase binding domain, whereas Src family kinases associate with Cbl via its C-terminal proline-rich sequences (7). Similarly, we could not detect a change of tyrosine phosphorylation of PLC-γ2 or its association with BLNK, as observed in DT40 cells (11). The discrepancy could be the difference of B cell origin. To support this notion, it was observed that Cbl-b<sup>−/−</sup>...
murine B cells display enhanced BCR signaling through the ubiquitination of Syk (6), whereas a decreased PLC-β2 activation was observed in Cbl-b−/− DT40 B cells (25). In any case, the present genetic study using Cbl−/− primary B cells allows us to revisit the issue of Cbl in the regulation of BCR signaling.

In this study, we also showed that Cbl−/− mice display an expanded subset of marginal zone B cells and a reduction of follicular B cells in the spleen, together with a marked increase in the population of B1 cells in the peritoneal cavity. Thus, Cbl regulates the cell fate decision during peripheral B cell development. Several mechanisms have been proposed for the lineage commitment of follicular versus marginal zone B cells,
which includes the strength of BCR signal, chemokine-mediated cell migration, or Notch-regulated differentiation (26, 27).

Our results partially supported the idea that BCR signaling strength is involved in follicular B cell development, because we detected decreased total tyrosine phosphorylation and Erk activation in Cbl−/− splenic B cells in response to BCR engagement. Intriguingly, we also observed an increase in marginal zone and B1 B cells in Cbl−/− mice, which does not fit with the signal strength model. Interestingly, both CD19 and PI3-kinase signaling are important in marginal zone and B1 B cell development (28–30). To be consistent with these observations, it was found that in Cbl−/− splenic B cells, CD19 phosphorylation and the downstream PI3-kinase activation were up-regulated. Thus, we propose that Cbl plays distinct roles in regulating BCR signaling pathways, which differentially affects B cell development.

In summary, we presented evidence that Cbl plays relatively complicated roles in the signaling transduction emanated from the BCR engagement under physiological conditions. The present study focused on the analysis of splenic B cell development and activation and revealed unexpected findings different from previous biochemical studies. It is obvious that Cbl is ubiquitously expressed in other cells or tissues such as T cells or dendritic cells, which might also influence the B cell phenotype in Cbl−/− mice, although our adoptive transfer study showed that Cbl has an intrinsic role in B cell development (data not shown). Further dissection of BCR signaling in Cbl−/− B cells using biochemical and genetic approaches is needed to fully understand the diverse roles of Cbl in B cell signaling and B cell-mediated immune responses.

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