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Control of the B Cell-Intrinsic Tolerance Programs by Ubiquitin Ligases Cbl and Cbl-b

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SUMMARY

B cell receptor (BCR) signaling plays a critical role in B cell tolerance and activation. Here, we show that mice with B cell-specific ablation of both Cbl and Cbl-b ($Cbl^{-/-}Cblb^{-/-}$) manifested systemic lupus erythematosus (SLE)-like autoimmune disease. The Cbl double deficiency resulted in a substantial increase in marginal zone (MZ) and B1 B cells. The mutant B cells were not hyperresponsive in terms of proliferation and antibody production upon BCR stimulation; however, B cell anergy to protein antigen appeared to be impaired. Concomitantly, BCR-proximal signaling, including tyrosine phosphorylation of Syk tyrosine kinase, Phospholipase C-γ2 (PLC-γ2), and Rho-family GTP-GDP exchange factor Vav, and Ca^{2+} mobilization were enhanced, whereas tyrosine phosphorylation of adaptor protein BLNK was substantially attenuated in the mutant B cells. These results suggested that the loss of coordination between these pathways was responsible for the impaired B cell tolerance induction. Thus, Cbl proteins control B cell-intrinsic checkpoint of immune tolerance, possibly through coordinating multiple BCR-proximal signaling pathways during anergy induction.

INTRODUCTION

B cell development, activation, and tolerance are interconnected processes controlled by signals delivered by the B cell receptor (BCR) (Healy and Goodnow, 1998; Rajewsky, 1996; Reth and Wienands, 1997). Paradoxically, the same BCR can either signal immunogenically, stimulating the proliferation and differentiation of B cells specific for for-eign antigens, or signal tolerogenically to eliminate or silence cells that bind to self-antigens. Although divergent hypotheses exist as to how precisely BCR signaling is triggered by antigen and how this signaling is quantitatively and differentially altered in tolerized B cells (Healy et al., 1997; Vilen et al., 2002), the developmental timing when B cells encounter antigens may determine the final outcomes (Cancro, 2004; Chung et al., 2003). In particular, evidence indicate that triggering of the antigen receptors on bone-marrow (BM) immature and peripheral transitional (T1 or T2) B cells leads to B cell tolerance in the absence of T cell help (Allman et al., 1992; Carsetti et al., 1995; Fulcher and Basten, 1994). These findings thus support the idea that the immature stages of B cell development may represent a time window during which B cell tolerance is established. After these stages, binding of antigens to the BCR on mature B cells results in B cell activation.

The BCR complex is composed of antigen-binding immunoglobulin Ig molecules and noncovalently associated signal-transduction molecules, Ig-α- and Ig-β-containing cytoplasmic domain immunoreceptor tyrosine-based activation motifs (ITAMs) (Cambier, 1995b; Campbell, 1999; Reth, 1989, 1992). Crosslinking of the BCR results in tyrosine phosphorylation of the ITAMs by Src-family tyrosine kinase Lyn followed by recruitment and activation of Syk tyrosine kinase (Cambier, 1995a; Reth and Wienands, 1997). Recruitment and activation of Syk by the phosphorylated BCR is a key event in the assembly of the BCR signalosome composed of the adaptor protein BLNK (B cell linker protein) and downstream signaling components phospholipase C-γ2 (PLC-γ2), Bruton's tyrosine kinase (Btk), and Rho-family GTP-GDP exchange factor Vav (Kurosaki, 2002; Pierce, 2002). These components coordinately induce Ca^{2+} influx and activate nuclear-transcription factors including NF-AT, AP-1, and NF-κB that are essential for B cell development and activation (Campbell, 1999; Kurosaki, 2000).

Casitas B lineage lymphoma (Cbl) proteins were recently identified as E3 ubiquitin ligase (Joazeiro et al., 1999). They interact with E2-ubiquitin-conjugating

enzyme (Ubc) through their RING finger (RF) domain and regulate the signaling of a broad range of receptors by promoting ubiquitination of the components involved in this receptor signaling (Duan et al., 2004; Liu and Gu, 2002; Thien and Langdon, 2005). In mammals, the Cbl family of proteins has three members, Cbl, Cbl-b, and Cbl-3, among which Cbl and Cbl-b are expressed in hematopoietic cells (Duan et al., 2004). Recent genetic studies from our and several other laboratories have revealed a critical role of Cbl proteins in T cell development and activation (Bachmaier et al., 2000; Chiang et al., 2000; Murphy et al., 1998; Naramura et al., 1998, 2002). The role of Cbl in B cell development and function requires further investigation. The involvement of Cbl proteins in BCR signaling has been reported in several papers, in which Cbl and Cbl-b were shown to regulate PLC-γ2 activation and Ca^{2+} response (Sohn et al., 2003; Yasuda et al., 2000, 2002). Cbl proteins associate with Syk and BLNK upon BCR stimulation, suggesting that they are part of the BCR signalosome. Cbl-b deficiency leads to an enhanced tyrosine phosphorylation of Syk and Ca^{2+} response in mouse B cells, despite normal BCR-induced proliferation of $Cblb^{-/-}$ B cells (Sohn et al., 2003). However, the precise signaling and physiological function of Cbl proteins in B cell biology has not yet been fully addressed, to some extent as a result of functional redundancy between Cbl and Cbl-b.

In order to understand the biochemical and physiological functions of Cbl proteins in B cells, we have generated a mouse model in which Cbl and Cbl-b are simultaneously inactivated in B lineage cells. Our study revealed that these mice manifested systemic lupus erythematosus (SLE)-like disease. The mutation substantially increased the rate of B cell maturation and impaired B cell anergy. These results thus indicate that Cbl proteins control a checkpoint of B cell tolerance, possibly by extending the duration of B cell maturation, providing sufficient time for the induction of B cell tolerance.

RESULTS

Generation of B Cell-Specific Cbl and Cbl-b Double-Deficient Mice

Our previous data demonstrate that inactivation of the germline Cbl or $Cblb$ gene alone results in a negligible impact on the development and function of B cells; however, the simultaneous ablation of both Cbl and $Cblb$ genes in germline leads to embryonic lethality (Naramura et al., 2002), suggesting that Cbl and Cbl-b may have a redundant role in intracellular signaling. To assess whether Cbl and Cbl-b have a redundant function in B cells, we generated mutant mice in which the Cbl and $Cblb$ genes were simultaneously inactivated only in B cells. These mice carried the homozygous $Cbl^{f/f}$ (Cbl gene flanked by $loxP$ sequences) alleles and $Cblb^{-/-}$ ($Cblb$ null) alleles and a $Cd19$-cre transgene (Tg). Deletion of the $Cbl^{f/f}$ alleles in a given cell by the Cre recombinase resulted in ablation of both genes, so we expected that the double deficiency

in these mice would occur only in B cells, because $Cd19$-cre transgene was expressed specifically in B lineage cells (Rickert et al., 1997). Indeed, we found that in these mice, the $Cbl^{f/f}$ alleles were deleted efficiently in B but not T cells (Figures S1A–S1C in the Supplemental Data available online). Hereafter we will refer to $Cbl^{f/f}Cblb^{-/-}Cd19$-cre Tg mice as $Cbl^{-/-}Cblb^{-/-}$ mice.

Altered B Cell Development in $Cbl^{-/-}Cblb^{-/-}$ Mice

$Cbl^{-/-}Cblb^{-/-}$ mice were born normal and fertile and exhibited no gross abnormality in major organs (data not shown). To determine whether the $Cbl^{-/-}Cblb^{-/-}$ mutation altered B cell development, we analyzed B cell compartments of the bone marrow (BM), spleen, lymph nodes, and peritoneal cavity from the mutant mice by flow cytometry (Figure 1A). $Cbl^{-/-}Cblb^{-/-}$ and WT control mice possessed comparable numbers of BM B (B220$^+$) cells, as well as similar representation of BM B cell subsets, including pro- and pre- (B220^{lo} IgM$^-$), immature (B220^{lo} IgM$^+$), and mature recirculating (B220^{hi} IgM$^+$) B cells. These observations were expected because Cd19-Cre-mediated deletion occurred in less than 40% of pro- and pre-B cells, whereas almost complete deletion was found only in mature B cells (Figure S1D). On the contrary, we found that the mutant mice possessed approximately 30% more B (B220$^+$) cells than did the WT mice and altered representations of B cell subsets in spleen and peritoneal cavity (Figures 1A and 1C), suggesting that the $Cbl^{-/-}Cblb^{-/-}$ mutation affected peripheral B cell development. To determine which subsets of peripheral B cells were affected by the $Cbl^{-/-}Cblb^{-/-}$ mutation, we analyzed the cellularity of splenic B cell subsets by flow cytometry. We found that $Cbl^{-/-}Cblb^{-/-}$ mice possessed approximately 2-fold more splenic T1 (B220$^+$ AA4.1^{hi} HSA^{hi} CD21^{lo} CD23^{lo}), B1 (B220$^+$ AA4.1^{lo} HSA^{lo} CD21$^-$ CD23$^-$), and marginal zone (MZ) (B220$^+$ AA4.1^{lo} HSA^{lo} CD21^{hi} CD23$^-$) B cells as compared to WT mice; however, the total numbers of follicular (FO) (B220$^+$ AA4.1^{lo} HSA^{lo} CD21$^+$ CD23$^+$) and T2 (B220$^+$ AA4.1^{hi} HSA^{hi} CD21^{hi} CD23^{hi}) B cells were comparable between the mutant and WT mice (Figures 1B and 1C). $Cbl^{-/-}Cblb^{-/-}$ mice also had an increased number (up to 20%–40% more) of B1 B cells in the peritoneal cavity than did the WT mice (Figure 1A and data not shown). Based on these results, we conclude that the $Cbl^{-/-}Cblb^{-/-}$ mutation alters the development of multiple B cell subsets in the periphery.

$Cbl^{-/-}Cblb^{-/-}$ Mice Spontaneously Manifest SLE-like Disease

Our inspection revealed that while the WT control mice (10/10) remained normal beyond 10 months of age, 50% (6/11) of $Cbl^{-/-}Cblb^{-/-}$ mice became moribund during the same period. The mutant mice also possessed a substantially (3- to 5-fold) higher amount of serum IgM antibodies as compared to WT, $Cbl^{-/-}$, and $Cblb^{-/-}$ mice; however, the serum amounts of other Ig isotypes in the mutant mice appeared to be similar to that in control mice (Figure 2A). To determine whether immune tolerance was impaired in the mutant mice, we first examined the
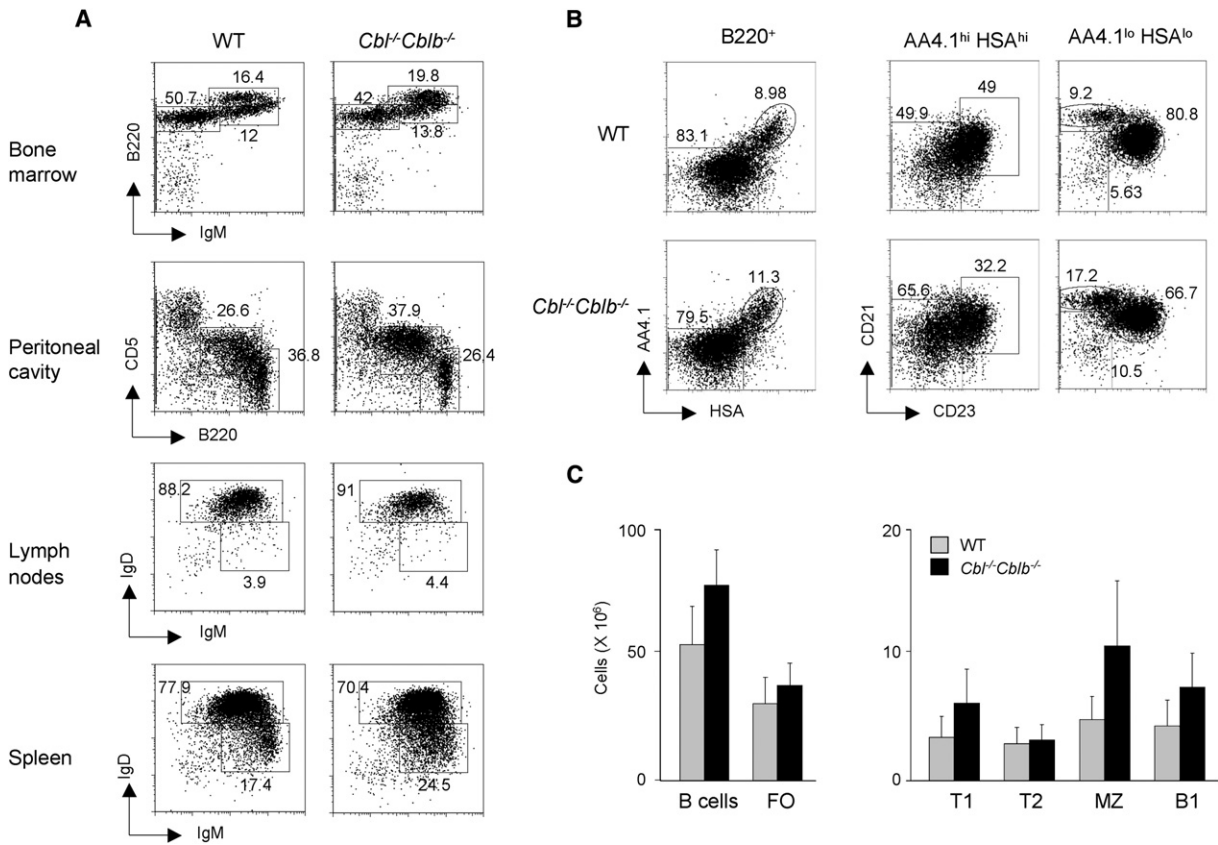
Figure 1. Flow Cytometric and Statistic Analysis of B Cell Subsets in $Cbl^{-/-}Cblb^{-/-}$ Mice

(A) B cell subsets in bone marrow, spleen, lymph node, and peritoneal cavity. Pro- and pre- (B220^{lo} IgM$^-$), immature (B220^{lo} IgM$^+$), and recirculating mature B (B220^{hi} IgM$^+$) cells were gated as shown for analysis of B220 and IgM expression on bone-marrow cells. Percentages of bone-marrow cells in those B cell subsets are indicated. Total splenic and lymph node cells were stained with anti-B220, anti-IgM, and anti-IgD. Percentage of B220$^+$ B cells that are IgD^{hi} IgM$^+$ and IgD^{lo} IgM^{hi} are indicated. Peritoneal cavity cells were stained with anti-CD5, anti-B220, and anti-IgM. Shown in the gates are the percentage of peritoneal cavity cells in B1 (CD5^{lo} B220^{lo}) and B2 (CD5$^-$ B220^{hi}) B cell subsets.

(B) Spenic B cell subsets. Splenic B cells were stained with anti-B220, anti-AA4.1, anti-CD24 (HSA), anti-CD21, and anti-CD23. Shown are AA4.1 and HSA expression of the gated B220$^+$ B cells (left), CD21 and CD23 expression on the gated B220$^+$ AA4.1^{hi} HSA^{hi} immature B cells (middle), and B220$^+$ AA4.1^{lo} HSA^{lo} mature B cells (right). Percentages of B cell subsets within the indicated gates are given. Phenotypes of T1, T2, MZ, B1, and FO B cells are described in text.

(C) The numbers of total splenic B cells and B cell subsets. Data were collected from five 2-month-old WT and $Cbl^{-/-}Cblb^{-/-}$ mice.

titers of serum autoantibodies of anti-double-stranded DNA (anti-dsDNA) and presence of anti-nuclear antigen (ANA) by ELISA and immunofluorescent staining, respectively. We found that a high percentage of $Cbl^{-/-}Cblb^{-/-}$ mice but not WT littermates possessed anti-dsDNA and ANA of both IgG (Figures 2B and 2C) and IgM (data not shown) isotypes in the sera, suggesting that the mutant mice developed autoimmune diseases. To directly assess whether $Cbl^{-/-}Cblb^{-/-}$ mice manifested autoimmune diseases, we performed histopathological analysis on various tissues from the mutant and control mice. Compared to the WT or $Cblb^{-/-}$ (data not shown) mice in which the tissue infiltration of leukocytes were absent, we found massive infiltrations of leukocytes in liver, lung, kidney, and salivary grand of $Cbl^{-/-}Cblb^{-/-}$ mice that developed the diseases (Figure 2D). Immunohistological analysis on kidney sections of the mutant mice revealed greatly enlarged glomeruli (average diameter, 76.02 ± 12.72 versus

49.12 ± 8.28 μM [n = 40 glomeruli], p < 0.001) and severe glomerular deposits of IgG and IgM antibodies when compared to controls (Figure 2E). These results thus suggest that the $Cbl^{-/-}Cblb^{-/-}$ mutant mice developed SLE-like autoimmune disorders. Because our double-deficient mice carried the $Cbl^{-/-}Cblb^{-/-}$ mutation only in B cells and $Cblb^{-/-}$ mice did not develop spontaneous autoimmune diseases (Chiang et al., 2000), we propose that the simultaneous ablation of Cbl and Cbl-b in B cells disrupts the B cell-intrinsic program for immune-tolerance induction.

$Cbl^{-/-}Cblb^{-/-}$ B Cells Are Not Hyperactive in Response to Antigen Stimulation

Impaired B cell tolerance is frequently associated with B cell hyperactivity or resistance to apoptosis. To determine whether the $Cbl^{-/-}Cblb^{-/-}$ mutation affected B cell activation in vivo, we examined T-dependent (TD) and
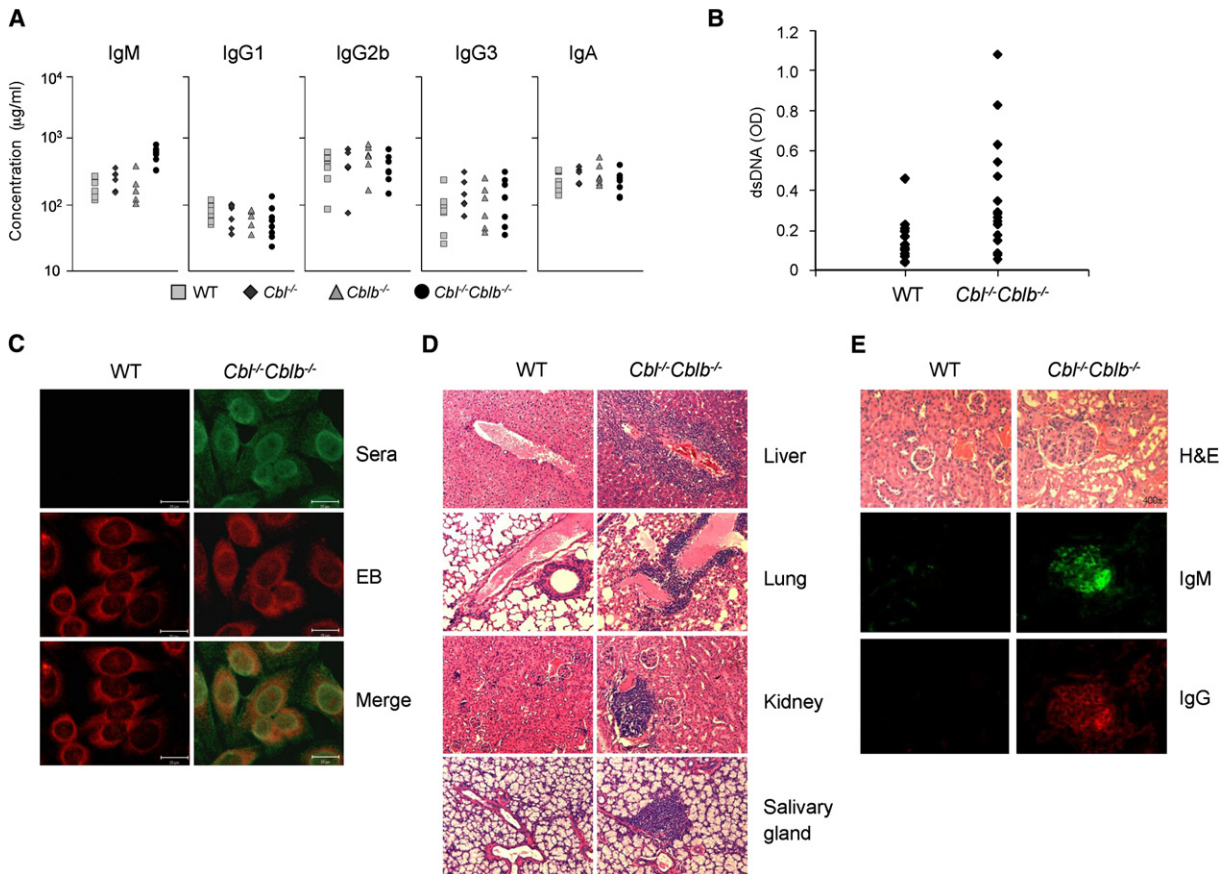
Figure 2. Serum Antibody Titers and Pathological Analyses of $Cbl^{-/-}Cblb^{-/-}$ Mice

(A) Serum concentrations of immunoglobulins (Ig) of different Ig isotypes. Data were collected from 8- to 10-week-old WT, $Cbl^{-/-}$, $Cblb^{-/-}$, and $Cbl^{-/-}Cblb^{-/-}$ mice. Each symbol represents the data from one individual mouse.

(B) Increased titers of dsDNA antibodies in $Cbl^{-/-}Cblb^{-/-}$ mice. Sera were obtained from 8- to 10-month-old WT and $Cbl^{-/-}Cblb^{-/-}$ mice. Shown are the titers of anti-dsDNA IgG in sera (1:1000 dilution) of WT and $Cbl^{-/-}Cblb^{-/-}$ mice.

(C) Serum anti-nuclear antibodies (ANA) of $Cbl^{-/-}Cblb^{-/-}$ mice. Sera samples (1:100 dilution) from $Cbl^{-/-}Cblb^{-/-}$ mice and WT littermates were used for the ANA analysis. Shown are Hep2 cells stained with sera from WT and $Cbl^{-/-}Cblb^{-/-}$ mice. Bound IgG (green) was detected with FITC-conjugated anti-mouse IgG. Cells are counterstained with Evans blue (EB) to visualize cytoplasmic region (red).

(D) Perivascular infiltration of leukocytes in $Cbl^{-/-}Cblb^{-/-}$ mice. Liver, lung, kidney, and salivary gland sections from WT and $Cbl^{-/-}Cblb^{-/-}$ mice were stained with haematoxylin and eosin (H&E).

(E) Glomerulonephritis and immunoglobulin deposits in kidney of $Cbl^{-/-}Cblb^{-/-}$ mice. Kidney sections from $Cbl^{-/-}Cblb^{-/-}$ and WT mice were stained with H&E (top) or immunofluorescently stained with anti-mouse IgM (IgM) (middle) or anti-mouse IgG (IgG) (bottom). Shown are glomeruli with IgM (green) or IgG (red) antibody deposits, respectively. Results are representative of 4 $Cbl^{-/-}Cblb^{-/-}$ and 4 WT mice.

T-independent (TI) antibody responses in these mice. We immunized WT and $Cbl^{-/-}Cblb^{-/-}$ mice either with hapten nitrophenyl-acetyl (NP)-conjugated keyhole limpet hemocyanin (KLH) for TD antibody responses or with NP-Ficoll or NP-lipopolysaccharide (LPS) for type I or type II TI responses. In NP-KLH-immunized $Cbl^{-/-}Cblb^{-/-}$ mice, the amounts of NP-specific IgM responses were comparable to that produced by WT, $Cbl^{-/-}$, and $Cblb^{-/-}$ mice; however, the production of NP-specific IgG1 and IgG2b in the mutant mice were moderately lower than that in control mice (Figure 3A). Similarly, while $Cbl^{-/-}Cblb^{-/-}$ mice had a comparable titer of anti-NP IgM to that produced by WT, $Cbl^{-/-}$, and $Cblb^{-/-}$ mice after NP-Ficoll immunization, they produced substantially lower amounts of anti-NP IgG3 than did the control

mice (Figure 3B). The titers of NP-specific IgM and IgG3 in NP-LPS-immunized mutant and WT mice were comparable (Figure 3C), indicating that the type II TI antibody response was not affected by the $Cbl^{-/-}Cblb^{-/-}$ mutation.

To determine whether the $Cbl^{-/-}Cblb^{-/-}$ mutation influenced B cell activation and survival in vitro, we assessed the proliferation and apoptosis of purified splenic B cells upon anti-IgM stimulation. The proliferative response of $Cbl^{-/-}Cblb^{-/-}$ B cells to anti-IgM stimulation alone was severely impaired as compared to that of WT B cells; however, the proliferation of the mutant B cells was restored to normal in the presence of IL-4 or anti-CD40 (Figure 3D). The defective proliferation of mutant B cells in response to anti-IgM stimulation was not likely caused by a different
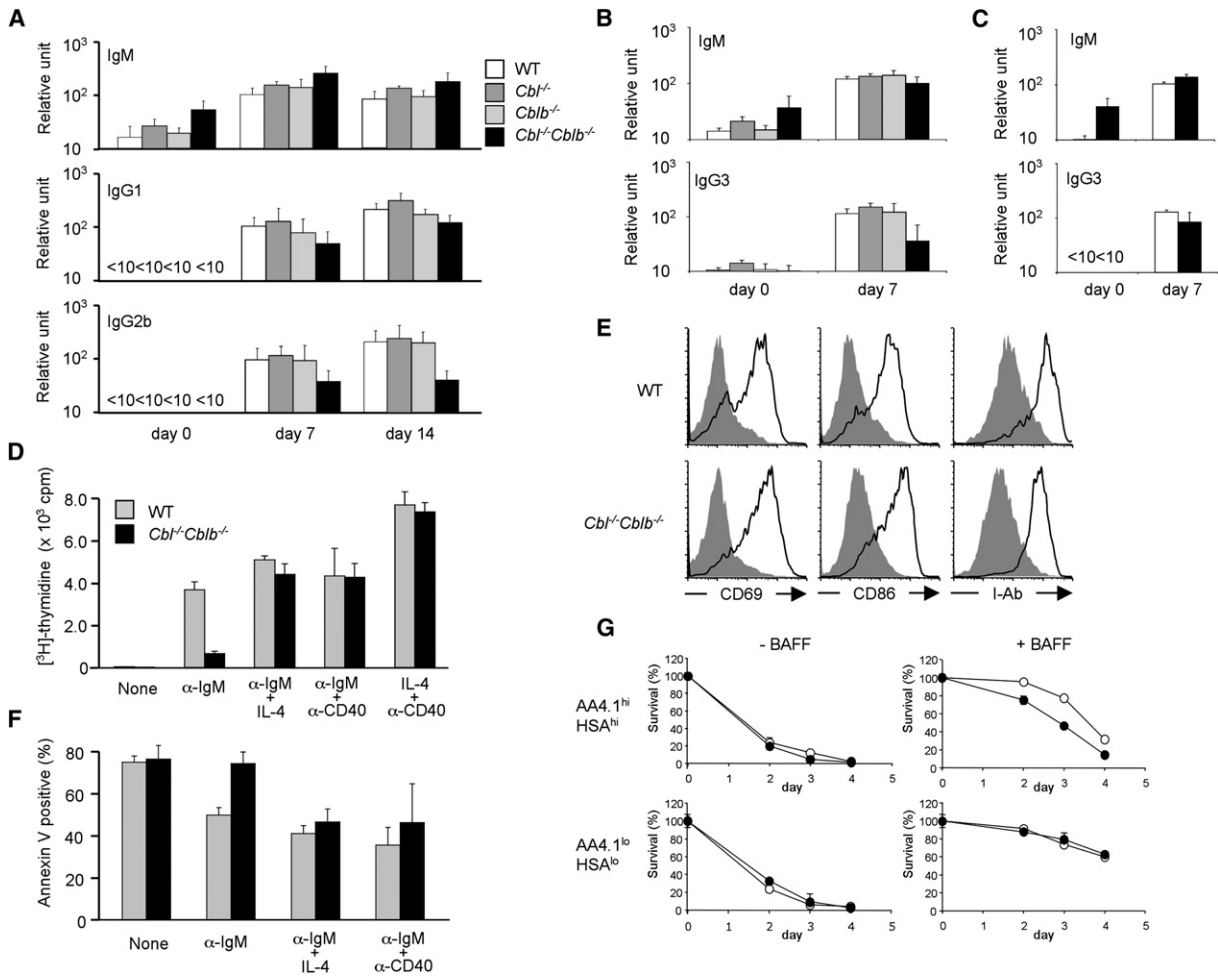
Figure 3. In Vivo and In Vitro B Cell Responses to Antigen Stimulation

(A) T-dependent antibody responses to NP-KLH in $Cbl^{-/-}Cblb^{-/-}$ mice. Sera were collected from WT, $Cbl^{-/-}$, $Cblb^{-/-}$, and $Cbl^{-/-}Cblb^{-/-}$ mice at days 7 and 14 after immunization. Shown are the titers of NP-specific antibodies in different Ig isotypes. Antibody titers in day 7 WT immunized mice are arbitrarily defined as 100 (relative unit).

(B and C) Type I and type II T-independent antibody responses. Sera were collected from WT, $Cbl^{-/-}$, $Cblb^{-/-}$, and $Cbl^{-/-}Cblb^{-/-}$ mice at day 7 after immunization with NP-Ficoll (B) or NP-LPS (C). Results in (A), (B), and (C) are representatives of at least three independent experiments, each containing at least 5 mice of each genotypes.

(D) BCR-induced B cell proliferation. Purified splenic B cells were stimulated with anti-IgM alone, with anti-IgM together with anti-CD40 or IL-4, or with anti-CD40 and IL-4. The rate of cell proliferation was determined based on [3H]thymidine incorporation.

(E) BCR-induced upregulation of cell-surface activation markers. Purified B cells from WT and $Cbl^{-/-}Cblb^{-/-}$ mice were stimulated with anti-IgM for 24 hr. The expression of cell-surface CD69, CD86, and I-A^b were determined by flow cytometry. Shadowed areas, nonstimulated B cells; solid lines, anti-IgM-stimulated B cells.

(F) BCR-induced apoptosis of $Cbl^{-/-}Cblb^{-/-}$ B cells. Purified B cells were stimulated with either anti-IgM alone or with anti-IgM plus anti-CD40 or IL-4 for 3 days. Cell death was determined by Annexin V staining. Shown are the percentages of Annexin V-positive cells.

(G) BAFF-dependent cell-survival assay. Purified splenic T1 and T2 (AA4.1^hi HSA^hi) and mature (AA4.1^lo HSA^lo) B cells from WT (open circles) and $Cbl^{-/-}Cblb^{-/-}$ (solid dots) mice were cultured for 4 days in the presence or absence of 100 ng/ml BAFF. Apoptotic cells were analyzed by Annexin V staining and propidium iodide (PI) double staining, and percentages of viable (Annexin V^- and PI^-) cells are plotted against time in days. Results are representative of three independent experiments.

ratio of immature versus mature B cells between WT and $Cbl^{-/-}Cblb^{-/-}$ B cell compartment, because a similar defect was also found in purified mature (B220^+ AA4.1^lo HSA^lo) B cells (Figure S2). It was unlikely a result of impaired B cell activation either, because the mutant B cells upregulated cell-surface markers CD69, CD86, and MHC II as efficiently as did the WT B cells upon anti-IgM stimula-

tion (Figure 3E). To determine whether the $Cbl^{-/-}Cblb^{-/-}$ mutation promoted BCR activation-induced cell death, we analyzed apoptosis of $Cbl^{-/-}Cblb^{-/-}$ B cells after BCR stimulation in the presence or absence of anti-CD40 or IL-4 (Figure 3F). $Cbl^{-/-}Cblb^{-/-}$ B cells exhibited a much higher rate of apoptosis than did the WT cells upon stimulation with IgM antibody alone; however, the
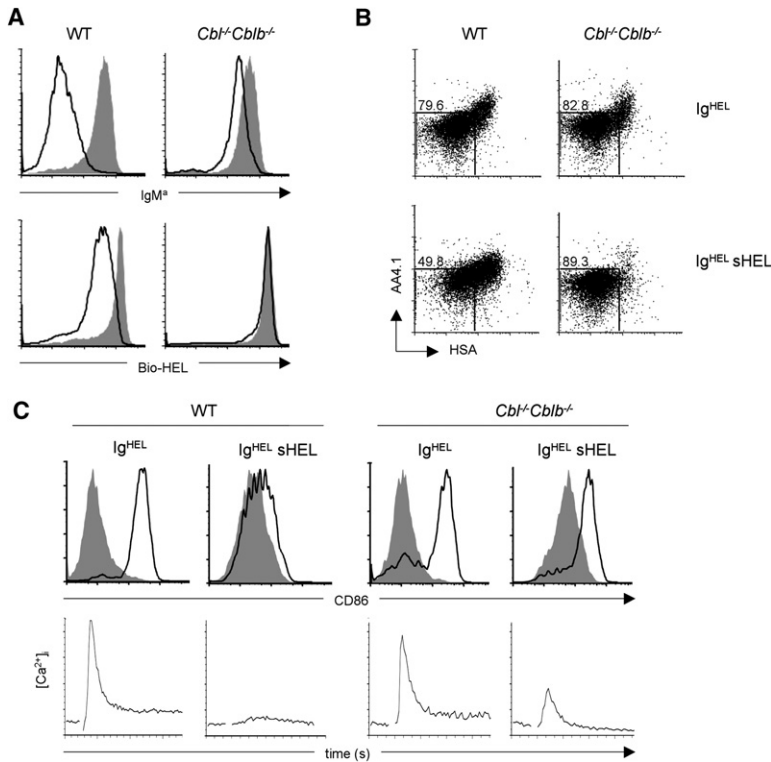
Figure 4. Impaired B Cell Anergy to sHEL Antigen in $Cbl^{-/-}Cblb^{-/-}$ Mice

(A) Cell-surface BCR expression on $Cbl^{-/-}Cblb^{-/-}$ and WT B cells. Shown are histograms of HEL-specific BCR (IgM[a]) expression (top) and sHEL-binding activity (bottom) on WT (Ig[HEL] sHEL double transgenic) and $Cbl^{-/-}Cblb^{-/-}$ ($Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] sHEL double transgenic) B cells (solid lines). Shadowed areas represent Ig[HEL] Tg mice. The IgM[a] expression and sHEL-binding activity on WT B cells are lower than that on $Cbl^{-/-}Cblb^{-/-}$ B cells.

(B) Normal maturation of $Cbl^{-/-}Cblb^{-/-}$, Ig[HEL] B cells against HEL antigen. Total splenic cells were stained with anti-B220, anti-AA4.1, and anti-HSA. Shown are dot plot profiles of anti-AA4.1 and anti-HSA staining of the gated B cells from Ig[HEL] sHEL double transgenic and $Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] sHEL double transgenic mice. The numbers indicate the percentage of B220^{+} B cells that are mature (AA4.1[lo] HSA[lo]).

(C) BCR-induced activation of Ig[HEL] sHEL double transgenic B cells. B cells from Ig[HEL] transgenic and Ig[HEL] sHEL double transgenic and $Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] and $Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] sHEL double transgenic mice were stimulated with anti-IgM (Fab')$_2$. Shown at the top are histograms of cell-surface CD86 expression on stimulated (solid line) and unstimulated (shadowed area) B cells. Ca^{2+} mobilization was determined by measuring the intracellular Ca^{2+} concentration [Ca^{2+}]$_i$ by flow cytometry (bottom). Results are representatives of at least three independent experiments.

difference of cell death between the mutant and WT B cells diminished substantially in the presence of anti-CD40 or IL-4.

BAFF (B cell activation factor of the tumor necrosis factor family) signaling plays a critical role in B cell survival, and enhanced BAFF signaling has been linked to B cell-mediated autoimmune diseases (Lesley et al., 2004; Thien et al., 2004). To test whether the $Cbl^{-/-}Cblb^{-/-}$ mutation affected BAFF signaling, we cultured $Cbl^{-/-}Cblb^{-/-}$ and WT B cells for 4 days in the presence or absence of BAFF and then analyzed the rates of cell apoptosis by flow cytometry. We found that the addition of BAFF rescued both mutant and WT B cells from apoptosis as compared to the cultured cells without BAFF. In the immature B cell compartment, WT B cells exhibited a slightly better survival rate than did $Cbl^{-/-}Cblb^{-/-}$ B cells. These results thus suggest that BAFF signaling is not enhanced in $Cbl^{-/-}Cblb^{-/-}$ B cells (Figure 3G).

Taken together, we conclude that $Cbl^{-/-}Cblb^{-/-}$ B cells are neither hyperresponsive to BCR stimulation (in vivo or in vitro), nor are they resistant to anti-IgM-induced apoptosis or exhibiting an enhanced BAFF signaling. These results also suggest that manifestation of the SLE-like disease in $Cbl^{-/-}Cblb^{-/-}$ mice is unlikely a consequence of generalized B cell hyperactivation or improved survival mediated by BAFF signals.

Impaired B Cell Anergy to Self-Antigen in $Cbl^{-/-}Cblb^{-/-}$ Mice

Whereas B cell tolerance can be achieved through different mechanisms such as clonal deletion, BCR editing, and anergy of autoreactive B cells (Chen et al., 1995; Goodnow et al., 1988, 1995; Nemazee and Buerki, 1989), induction of anergy to self-antigen is a final safeguard to prevent autoreactivity (Rajewsky, 1996). To determine whether the $Cbl^{-/-}Cblb^{-/-}$ mutation affected B cell anergy, we crossed $Cbl^{-/-}Cblb^{-/-}$ mice to BCR transgenic mice that expressed membrane IgM specifically recognizing hen egg lysozyme (HEL) (Ig[HEL]) and soluble HEL (sHEL) transgenic mice. We found that approximately 50% (3/6) of $Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] sHEL mice became sick and eventually died by 3 months of age. Further analyses revealed that $Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] B cells in $Cbl^{-/-}Cblb^{-/-}$ Ig[HEL] sHEL mice were not anergic to the HEL antigen, as shown by the fact that they showed a higher expression of IgM[a] (encoded by Ig[HEL] transgene), exhibited a higher sHEL binding activity, and entered the stage of mature (AA4.1[lo] HSA^{lo}) B cells (Figures 4A and 4B). Additionally, upon anti-IgM stimulation, the mutant B cells efficiently upregulated CD86 and MHC II and elicited Ca^{2+} influx albeit at an amount slightly lower than that in WT cells (Figure 4C). On the contrary, the WT Ig[HEL] B cells from Ig[HEL] sHEL double-transgenic mice exhibited typical
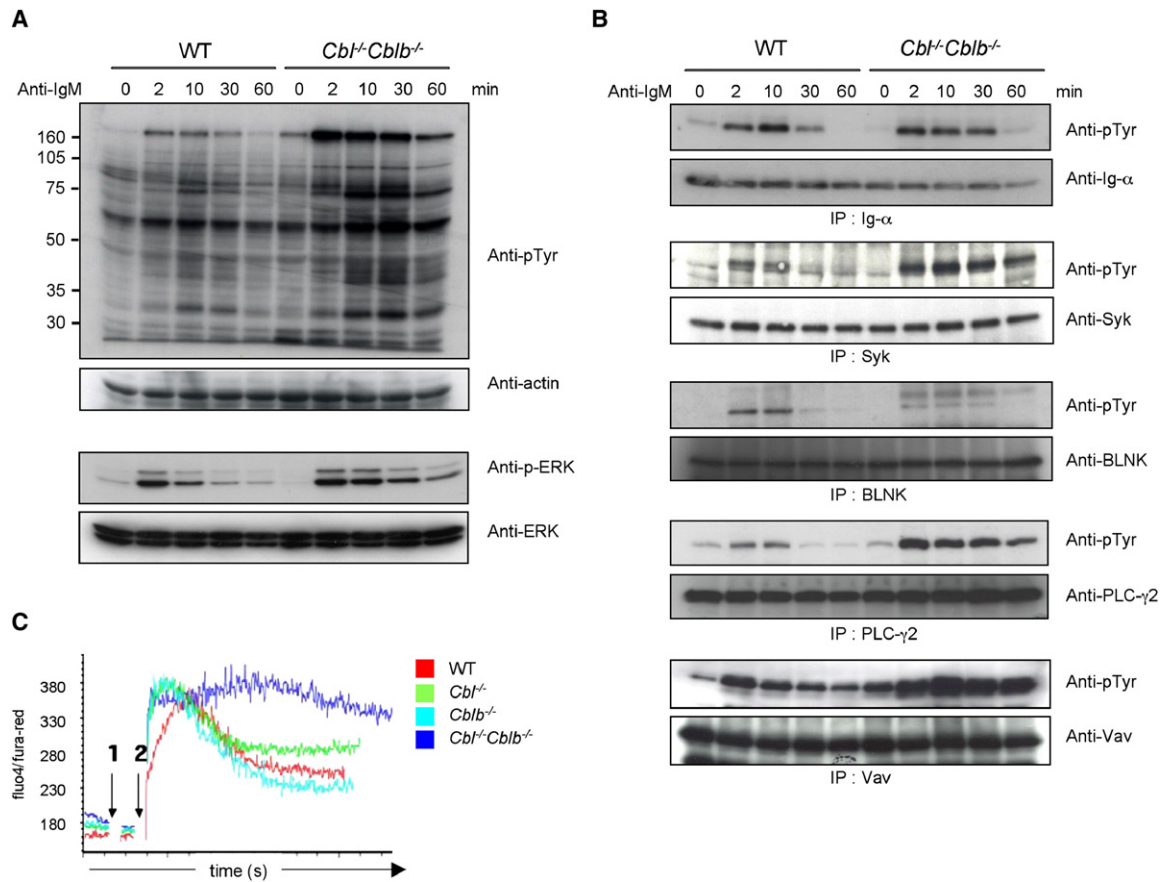
Figure 5. Differential Dysregulation of BCR Downstream Signaling Pathways in $Cbl^{-/-}Cblb^{-/-}$ B Cells

Purified B cells were stimulated with anti-IgM F(ab')$_2$ for various periods of time as indicated. The amounts of tyrosine phospho-proteins in the cell lysates were determined by immunoprecipitation and immunoblot analyses by an anti-phosphotyrosine. Protein loadings were quantified with the corresponding antibodies.

(A) Tyrosine phosphorylation of total cellular proteins in $Cbl^{-/-}Cblb^{-/-}$ and WT B cells.

(B) Tyrosine phosphorylation of individual BCR downstream signaling components in $Cbl^{-/-}Cblb^{-/-}$ and WT B cells.

(C) BCR-induced Ca^{2+} mobilization in WT, $Cbl^{-/-}$, $Cblb^{-/-}$, and $Cbl^{-/-}Cblb^{-/-}$ B cells.

phenotypes of anergic B cells, because they substantially downmodulated cell-surface IgM^a and HEL-binding ability and failed to develop into follicular B cells (Figures 4A and 4B). Anti-IgM stimulation could not induce CD86 and MHC II expression, nor did the Ca^{2+} response in these anergic B cells (Figure 4C). Based on these data, we conclude that B cell anergy is at least one of the major reasons that contribute to the manifestation of the SLE-like autoimmune disease in these mice.

Differential Alterations of BCR-Proximal Signaling in $Cbl^{-/-}Cblb^{-/-}$ B Cells

The strength of BCR-proximal signaling plays a critical role in peripheral B cell tolerance and activation (Monroe, 2004; Rajewsky, 1996). It may also dictate the development and differentiation of immature B cells into follicular, B1, and MZ B cells: it has been shown that strong BCR signaling facilitates the development of MZ and B1 B cells whereas weak BCR signaling favors the differentiation of follicular B cells (Casola et al., 2004). The $Cbl^{-/-}Cblb^{-/-}$

mutation altered the ratios of follicular B cells to B1 and MZ B cells (Figure 1C), so we decided to assess whether BCR-proximal signaling was enhanced in $Cbl^{-/-}Cblb^{-/-}$ B cells after anti-IgM stimulation. We found that stimulation of $Cbl^{-/-}Cblb^{-/-}$ B cells elicited markedly enhanced and prolonged tyrosine phosphorylation of total cellular proteins as compared to that of WT B cells (Figure 5A). In particular, the mutant B cells exhibited substantially protracted higher amounts of tyrosine phosphorylation of Ig-α, Syk, PLC-γ2, and Vav, as well as Erk MAP kinase activities, than did the control cells (Figure 5B). Additionally, a prolonged and elevated amount of Ca^{2+} mobilization was also found in $Cbl^{-/-}Cblb^{-/-}$ B cells as compared to WT cells (Figure 5C). Surprisingly, tyrosine phosphorylation of BLNK was dramatically decreased in the mutant B cells as compared to that in WT B cells (Figure 5B), indicating that the $Cbl^{-/-}Cblb^{-/-}$ mutation exerted a differential effect on BCR-proximal signaling pathways. A strong phosphorylated band of approximate 70–75 kDa was reproducibly coimmunoprecipitated with BLNK, although

the identity of this protein remained unclear (Figure 5B). Taken together, we conclude that Cbl proteins differentially control both the strength and duration of multiple BCR-proximal signaling pathways. Additionally, since most BCR-proximal signaling pathways are enhanced whereas BLNK phosphorylation is attenuated in $Cbl^{-/-}$ $Cblb^{-/-}$ B cells, we propose that the loss of coordination between these BCR-proximal signaling pathways is likely responsible for the impaired B cell tolerance in $Cbl^{-/-}$ $Cblb^{-/-}$ mice.

Blockage of BCR Downmodulation and Ig-α and Syk Ubiquitination in $Cbl^{-/-}Cblb^{-/-}$ B Cells

Cbl proteins negatively regulate TCR signaling by promoting downmodulation of the TCR complex and ubiquitination of intracellular signaling components such as Lck tyrosine kinase and phosphoinositide-3 kinase p85 subunit (PI-3 kinase [p85]) (Fang and Liu, 2001; Naramura et al., 2002; Rao et al., 2002b). The BCR delivers signals in a manner similar to the TCR, so we investigated whether the $Cbl^{-/-}Cblb^{-/-}$ mutation affected BCR downmodulation and ubiquitination of BCR-downstream signaling components. To determine whether BCR downmodulation was blocked in the absence of Cbl proteins, we crosslinked cell-surface IgM of $Cbl^{-/-}Cblb^{-/-}$ mutant and WT B cells with biotinylated anti-IgM (Fab')₂ for various periods of time and then monitored the remaining cell-surface IgM by staining the cells with fluorescent streptavidin (Figure 6A). In the absence of crosslinking, the mutant B cells expressed a slightly higher amount of cell-surface IgM than did the WT cells. Although BCR crosslinking for 5 min already resulted in a substantial loss of cell-surface IgM on the WT B cells, the same treatment induced little change with respect to the amount of cell-surface IgM on the mutant B cells for at least 20 min, indicating that Cbl proteins indeed play a critical role in BCR downmodulation.

Cbl proteins form complexes with BCR-downstream signaling components, including Ig-α, Syk, PLC-γ2, BLNK, PI-3 kinase (p85), and Vav. To explore whether Cbl protein-mediated ubiquitination was involved in BCR downmodulation and BCR signaling, we examined ubiquitination of these signaling components in WT and $Cbl^{-/-}$ $Cblb^{-/-}$ B cells after anti-IgM stimulation. We found that Ig-α and Syk were heavily ubiquitinated in WT but not $Cbl^{-/-}Cblb^{-/-}$ B cells (Figure 6B). By contrast, we could not detect any meaningful ubiquitination of PLC-γ2, BLNK, PI-3 kinase (p85), and Vav in either WT or $Cbl^{-/-}$ $Cblb^{-/-}$ B cells (data not shown). Interestingly, despite their ubiquitinations, the amounts of Ig-α and Syk in WT B cells did not seem to be affected even at 60 min after the stimulation (Figure 6B and data not shown), suggesting that Cbl proteins might regulate the signaling of these molecules through a nondegradation mechanism.

Taken together, our results indicate that Cbl proteins selectively promote ubiquitination of the BCR-downstream signaling components including Ig-α and Syk during BCR activation. Ig-α is constitutively associated with membrane IgM and its ubiquitination state is closely
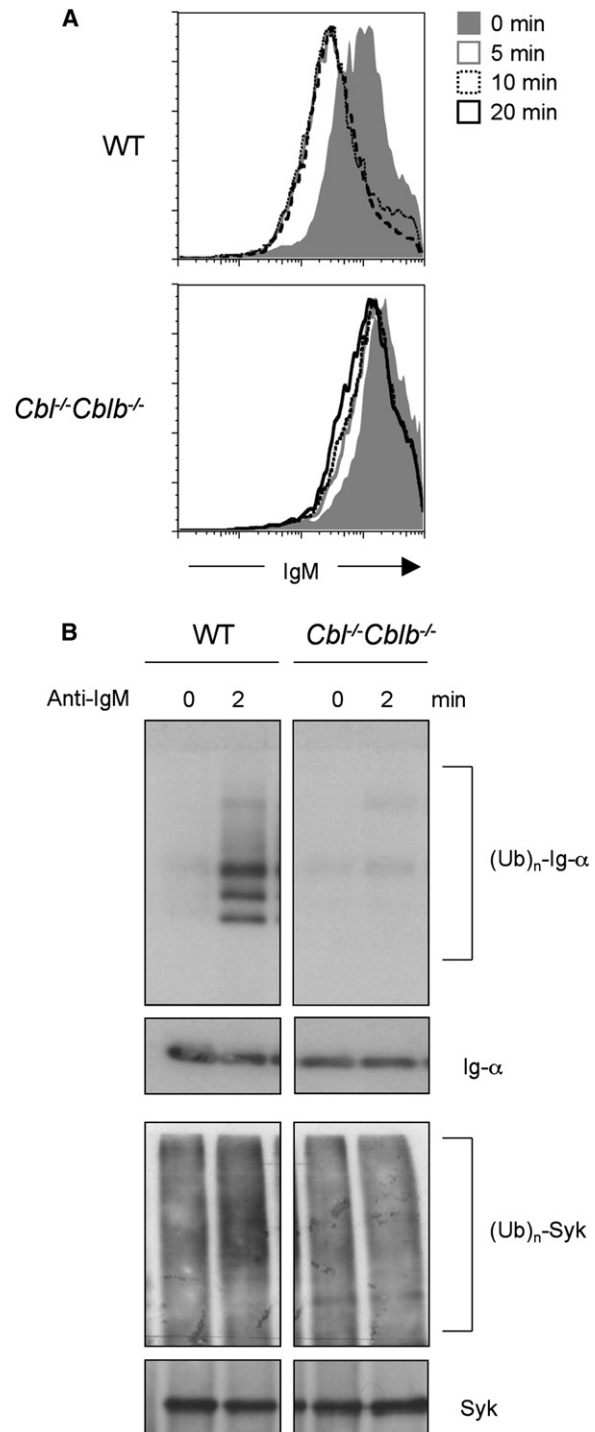

Figure 6. Impaired BCR Downmodulation and Ubiquitination in $Cbl^{-/-}Cblb^{-/-}$ B Cells
(A) BCR downmodulation. Shown are histograms of cell-surface IgM expression on $Cbl^{-/-}Cblb^{-/-}$ and WT B cells after BCR crosslinking for 5, 10, and 20 min.
(B) Ubiquitination of BCR-proximal signaling components. $Cbl^{-/-}$ $Cblb^{-/-}$ and WT B cells were stimulated with anti-IgM (Fab')₂ for 2 min. Ig-α and Syk in the cell lysates were immunoprecipitated and immunoblotted to a membrane. The amounts of Ig-α and Syk ubiquitination were determined by an ubiquitin antibody.

correlated with the BCR downmodulation, so it is therefore likely that Cbl proteins downmodulate the activated BCR, hence the BCR signaling by promoting Ig-α ubiquitination.

DISCUSSION

In this study, we show that the simultaneous ablation of E3 ubiquitin ligases Cbl and Cbl-b in B cells results in manifestation of SLE-like autoimmune disease, as evidenced by high production of serum autoantibodies against dsDNA and ANA, as well as pathological alterations in kidney and other major organs. $Cbl^{-/-}Cblb^{-/-}$ B cells were not generally hyperresponsive to antigen stimulation in terms of antibody production and proliferation. However, BCR anergy to soluble protein antigen (sHEL) was impaired. Because in this mouse model the Cbl and Cbl-b are simultaneously inactivated only in B cells, we conclude that Cbl proteins control B cell-intrinsic checkpoint of tolerance induction.

B cell-mediated autoimmunity is frequently linked to the hyperactivation of B cells. In this regard, mice deficient in tyrosine kinase Lyn, tyrosine phosphatase SHP-1, or membrane receptor CD22 exhibit B cell hyperresponsiveness upon BCR stimulation and manifest systemic autoimmune diseases (Cyster and Goodnow, 1995; Hibbs et al., 1995; O'Keefe et al., 1996). In addition to this mechanism, mutations that influence B cell apoptosis and survival may also affect immune tolerance, because the germline deletion of protein kinase C-δ (PKC-δ) or overproduction of BAFF in mice, both of which promote the survival of B cells, cause severe autoimmune diseases (Lesley et al., 2004; Saijo et al., 2003; Thien et al., 2004). In contrast to these two mechanisms, we found that $Cbl^{-/-}Cblb^{-/-}$ B cells were not hyperactive to antigen stimulation either in vitro or in vivo. They were not resistant to BCR-induced apoptosis, nor did they exhibit any enhancement in BAFF signaling. These findings therefore suggest that Cbl proteins control B cell tolerance through a different mechanism. It is generally believed that B cell tolerance may occur at immature B cell stage. In contrast to mature B cells that are activated when encountering an antigen, immature B cells usually become tolerized upon BCR triggering (Allman et al., 2001; Carsetti et al., 1995; Loder et al., 1999; Monroe, 2004; Rajewsky, 1996). Under physiological condition, immature B cells may take 3–4 days to become immune-competent mature B cells (Allman et al., 1993; Rolink et al., 1998). This period of B cell maturation naturally constitutes a time window when autoreactive B cells can be checked by various tolerance mechanisms such as clonal deletion, BCR editing, and anergy (Chen et al., 1995; Goodnow et al., 1995; Nemazee and Buerki, 1989). Our preliminary data suggest that the $Cbl^{-/-}Cblb^{-/-}$ mutation may expedite B cell maturation (data not shown). We therefore propose that this alteration could dramatically shorten the susceptible period of immune tolerance against autoreactive B cells, consequently breaking down the immune tolerance. This hypothetic model of B cell tolerance is also supported by

a recent observation that administration of female hormone prolactin may concomitantly facilitate B cell maturation and development of SLE-like disease in anti-DNA antibody transgenic mice (Peeva et al., 2003). Our results revealed that B cell anergy to soluble HEL antigen was impaired in $Cbl^{-/-}Cblb^{-/-}$ mice, so more experiments are needed to assess whether a shortened duration of B cell maturation is directly responsible for the impaired B cell anergy to autoantigen in $Cbl^{-/-}Cblb^{-/-}$ mice. It should be noted that we also found that while the mutant B cells still underwent BCR editing, the efficiency of the secondary rearrangement of the κ chains in the absence of Cbl proteins seemed to be less efficient (data not shown). It is therefore necessary to further investigate whether BCR editing is partially impaired by $Cbl^{-/-}Cblb^{-/-}$ mutation. Finally, we found that $Cbl^{-/-}Cblb^{-/-}$ mice possessed more B1 B cells, and it remains to be determined whether the observed autoimmune symptom is linked to the abnormal development and function of B1 B cells.

Molecular mechanisms by which Cbl proteins regulate BCR signaling remain unclear. Previous experiments show that both Cbl and Cbl-b may function as E3 ubiquitin ligases in T cells (Joazeiro et al., 1999). Cbl and Cbl-b directly or indirectly form complexes with the Ig-α, Syk, PLC-γ2, PI-3 kinase (p85), Vav, and BLNK (Bachmaier et al., 2000; Fang and Liu, 2001; Rao et al., 2002a; Sohn et al., 2003; and data not shown). However, we noted that in B cells, the $Cbl^{-/-}Cblb^{-/-}$ mutation abolished BCR-induced Ig-α and Syk ubiquitination but did not affect the ubiquitination states of PI-3 kinase, PLC-γ2, Vav, and BLNK, despite the fact that tyrosine phosphorylation of these molecules was markedly altered. Ig-α and Syk function at the top of the BCR-signaling cascade, so our results thus support the idea that Cbl proteins may negatively regulate BCR-signaling cascade at the top of BCR-induced tyrosine phosphorylation cascade by promoting Ig-α and Syk ubiquitination. We could not detect any meaningful degradation of Ig-α and Syk even after 1 hr of BCR stimulation, so we believe that the ubiquitination of Ig-α and Syk by Cbl proteins might not direct them for degradation, but rather alter their transportation and/or association with other molecules during BCR signaling. A similar observation that PI-3 kinase (p85) is ubiquitinated but not degraded by Cbl-b has been reported in T cells (Fang and Liu, 2001). This conclusion of course cannot exclude the possibility that only a small fraction of Ig-α and Syk are ubiquitinated by Cbl proteins so that the degradation of these molecules is below the detectable level in our assay system.

It should be mentioned that T cells in our $Cbl^{-/-}Cblb^{-/-}$ mice were deficient in Cbl-b. Because our $Cblb^{-/-}$ mice are susceptible to autoimmune diseases (Chiang et al., 2000), it is possible that in $Cbl^{-/-}Cblb^{-/-}$ mice, the $Cblb^{-/-}$ T cells also contribute to the development of SLE-like disease. However, because we did not find a similar autoimmune symptom in the littermate $Cblb^{-/-}$ ($Cbl^{f/f}Cblb^{-/-}$) mice, we believe that B cell-intrinsic ablation of Cbl and Cbl-b are necessary for the development of the SLE-like diseases in our animal model. In support of this

idea, our results showed that $Cbl^{-/-}Cblb^{-/-}$ B cells indeed exhibited B cell-intrinsic alterations in terms of maturation and BCR signaling. Additionally, anergy of Ig^{HEL} B cells to sHEL was abolished, indicating that a B cell-intrinsic defect has developed in the absence of Cbl proteins. Further experiments will be helpful to assess to what extent B cell-intrinsic $Cbl^{-/-}Cblb^{-/-}$ mutation affects B cell tolerance induction and whether $Cbl^{-/-}Cblb^{-/-}$ B cells are sufficient for the manifestation of the SLE-like diseases in the absence of T cell help.

EXPERIMENTAL PROCEDURES

Mice

Cbl-floxed, $Cblb$-deficient, and $Cd19$-Cre transgenic mice were described previously (Naramura et al., 2002). To generate $Cbl^{-/-}Cblb^{-/-}$ mice, Cbl-floxed mice were crossed to $Cblb^{-/-}$ mice and then to $Cd19$-Cre transgenic mice kindly provided by A. Tarakhovsky and K. Rajewsky (Rickert et al., 1997). Mice used in this study were of a mixed C57BL/6 and 129 background. Ig^{HEL} and sHEL transgenic mice were originally generated by Goodnow et al. (1988) and were kindly provided by A. Tarakhovsky. All mice used in this study were maintained and used at The Twinbrook II Facility of NIAID and the Columbia University Hammer Health Science Center mouse facility under specific pathogen-free conditions according to institutional guidelines and animal study proposals approved by the institutional animal care and use committees.

Antibodies and Reagents

CD5, CD21, CD23, CD24 (HSA), B220, IgM, IgD, CD69, CD86, I-A^b, and AA4.1 antibodies were from BD PharMingen. The purified and biotinylated goat anti-mouse IgM F(ab')$_2$ antibody used for BCR crosslinking was from Jackson Immunoresearch Laboratory. Ig-α antibody was from S.K. Pierce. Anti-BLNK was obtained from A. Chan and D. Kitamura. Anti-Syk, PLC-γ2, Vav, ubiquitin, phospho-ERK, and ERK1/2 were from Santa Cruz Biotechnology, Inc. Anti-phosphotyrosine (4G10) was from Upstate. BAFF was from Apotech Biochemicals. Anti-CD40 and IL-4 were from BD PharMingen.

T-Dependent and T-Independent Antibody Responses and ELISA

6- to 10-week-old mice were immunized i.p. with 50 μg of NP-KLH for TD immune response, NP-Ficoll for type I TI immune response, or NP-LPS for type II TI immune response. The antigens were precipitated in 100 μl of Imject-Alum adjuvant (Pierce). Immunized mice were bled from the tail vein on day 7 and 14 after primary immunization. The titers of NP-specific antibodies of different Ig isotypes were determined by ELISA as described previously (Chiang et al., 2000).

In Vitro B Cell Proliferation and Apoptosis Assays

B cells were purified with MACS column according to a B cell enrichment protocol (Miltanyi Biotek) and were more than 95% pure based on cell-surface CD19 staining. FACS purification of T1 and T2 (AA4.1^{hi} HSA^{hi}) and follicular (AA4.1^{lo} HSA^{lo} CD21^{hi} CD23^{hi}) B cells was performed after staining cells with anti-AA4.1, HSA, CD23, and CD21. Purified B cells (1 × 10^5 cells/well) were stimulated with 10 μg/ml anti-IgM F(ab')$_2$ or 10 μg/ml of LPS for 2 days in a 96-well plate. For the anti-CD40 and IL-4 culture, 50 μg/ml anti-CD40 or 20 U/ml IL-4 were included in the culture. To determine the rates of cell proliferation, cultured cells were pulsed with [^{3}H]thymidine for 16 hr. Cells were then harvested on a cell harvester and [^{3}H]thymidine incorporation was measured on a β-counter. To determine the dependence of cell survival on BAFF, purified immature or mature B cells were cultured for up to 4 days in the presence of recombinant BAFF (100 ng/ml). Apoptotic cells were quantified by staining the cells with FITC-conjugated

Annexin V and propidium iodide (PI). Upregulation of cell-surface CD69, CD86, and I-A^b was determined by flow cytometry.

Ca^{2+} Mobilization

Freshly purified splenic B cells were loaded with Fura-red and Fluo-4 (Molecular Probe) in HBSS buffer containing 1% FBS at 37°C for 30 min. After washing once with HBSS buffer, cells were stimulated with 5 μg/ml anti-IgM (Fab')$_2$ fragment. Increase of intracellular Ca^{2+} concentration in B220$^+$ B cells was recorded in real time by flow cytometry for 300 s.

BCR Downmodulation

Purified B cells were stained with biotinylated anti-IgM (Fab')2 at 4°C for 15 min. After washing with PBS, cells were incubated at 37°C prewarmed HBSS buffer containing 1% FBS to allow internalization of BCR-anti-IgM (Fab')$_2$ complexes to occur. After various periods of incubation, cells were immediately transferred into cold HBSS buffer containing 0.1% sodium azide to stop further internalization. Cell-surface-remaining anti-IgM (Fab')$_2$ were stained with streptavidin-PE and quantified on an LSR II.

Immunoprecipitation and Immunoblot Analyses

Immunoprecipitation and immunoblot analyses were performed as previously described (Naramura et al., 2002). In brief, purified B cells were stimulated with anti-IgM (Fab')$_2$ for various periods and lysed in RIPA buffer containing a mixture of proteinase and phosphatase inhibitors (0.1 μg/ml Aprotinin, 0.01 μg/ml Leupeptin, 0.2 mM PMSF, 1 mM NaF, and 1 mM NaVO$_4$). Ig-α, Syk, BLNK, Vav, and PLC-γ2 in the cell lysates were immunoprecipitated with the corresponding antibodies and subjected to electrophoresis and immunoblotting. The amounts of tyrosine phosphorylation and protein ubiquitination were determined with anti-phosphotyrosine and anti-ubiquitin, respectively (Santa Cruz Biotechnology).

Histochemical and Immunofluorescent Staining

Mouse tissues were harvested, snap frozen in liquid nitrogen, and embedded in OCT-embedding medium (Sakura Finetek). 8 μM sections were air-dried and fixed with acetone. H&E staining was performed according to a standard protocol (Miyamoto et al., 2002). Immunofluorescent staining was performed with the following reagents: anti-IgM-FITC, anti-IgG-biotin (BD Biosciences), and streptavidin-Alexa 568 (Molecular Probes). Anti-nuclear antibodies (ANA) were detected by intracellular staining of Hep2 cells with mouse serum (1:100 dilution), followed by FITC-conjugated anti-mouse IgG. Evans Blue staining was used to visualize cytoplasm.

Supplemental Data

Two figures are available at http://www.immunity.com/cgi/content/full/26/5/567/DC1/.

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